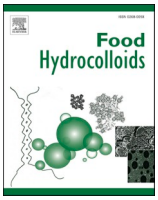
Food Hydrocolloids 129 (2022) 107664

Contents lists available at ScienceDirect

Food Hydrocolloids

journal homepage: www.elsevier.com/locate/foodhyd



Pressurized hot water-assisted recovery of crude residual agar from a never-dried algae industry waste stream: A Box-Behnken design approach

Cherif Ibrahima Khalil Diop [a,*], Ester Trigueros [a], Maria-Teresa Sanz [a], Sagrario Beltran [a], Javier García–Tojal [b]

[a] University of Burgos, Biotechnology and Food Science Dept. (Chemical Engineering Section), Pza. Misael Bañuelos s/n, 09001, Burgos, Spain
[b] University of Burgos, Department of Chemistry, Pza. Misael Bañuelos s/n, 09001, Burgos, Spain

ARTICLE INFO

Keywords:
Residual crude agar valorization
Pressurized hot water extraction
Never-dried algae industry waste stream
Physicochemical properties
Response surface methodology
Box-Behnken design

ABSTRACT

The potential of using pressurized hot water extraction to valorize the remaining crude agar in *Gelidium sesquipedale* waste stream after an initial industrial extraction was investigated. In this process, a four-factor Box-Behnken design was coupled with a response surface methodology. The impact of the operating temperature (°C), the internal pressure (bar), the extraction time (min), and the algae concentration (% w: v), as well as their quadratic effects and two-way interactions, on the physicochemical properties of the residual agar, was analyzed. The yield (%), gel strength (g/cm^2), gelling temperature (°C), melting temperature (°C), 3,6-anhydrogalactose content (%), and the sulfate content (%) were all considered in the evaluation. A multiple regression statistical model was used to fit all the experimental responses to a second-order polynomial equation that confirmed the suitability of the approach. Temperature of 120 °C, low pressure of 3.28 bar, and an extended extraction time of 150 min along with a 3% (w: v) algae concentration were projected to be optimum conditions for a high extraction yield of 17.03%. The strength of the recovered agar hydrogel oscillated between a minimum of 25 g/cm^2 and a maximum of 350 g/cm^2. The key parameters impacting the fluctuation of the sulfate content in the recovered agar (2% $\leq$ sulfate content $\leq$10%, with R^2 = 79.8%) appeared to be the temperature and the algae concentration, in addition to the quadratic effect of the solid concentration. By adjusting the parameters, the process can accommodate the physicochemical properties of agar for wider range of applications.

1. Introduction

A variety of seaweeds produce various forms of phycocolloids in their cell wall and intercellular spaces. Carrageenan and agar synthesized by red algae (Rhodophyta) are mostly made up of galactans, while alginates containing urinates residues are mainly present in brown algae (Ochrophyta and Phaeophycae). These two molecules are the primary categories of marine phycocolloids of interest.

High-quality agar characterized by a strong gel (strength greater than 700 g/cm^2 in a 1.5 wt% solution) is largely preferred for industrial purposes (Murakami, Yamaguchi, Sugawa-Katayama, & Katayama, 2016). The major genera of red algae producing agar are Gelidium and Gracilaria. However, when compared to Gracilaria agar, the agar isolated from Gelidium exhibits a superior quality given by its substantially higher gel strength.

Techno-chemically, agar refers to a thermoreversible marine hydrocolloid made of a mixture of agarose and agaropectin. Agarose is a homogeneous linear polysaccharide made up by the alternate of (1-4)-linked 3,6-anhydro-α-L-galactopyrannose and (1–3)-linked-β-D-galactose units (Tako & Nakamura, 1988). Fig. 1 illustrates the molecular structure of the agarose backbone in agar molecules. Agaropectin has the same backbone, but it contains heterogeneous anionic groups such as sulfate, pyruvate, and glycuronate.

The agarose exists as a disordered random coil structure at temperatures around 85 °C, which pseudoequilibrates upon cooling via hydrogen bonding and electrostatic interactions. It generates a 3D double helix structure or gel capable of immobilizing water molecules (García-González, Alnaief, & Smirnova, 2011; Zarrintaj et al., 2018).

New considerations have emerged, as a result of the multiple physical states of agar (dried, dissolved, or gelled). They are oriented toward non-food applications in the area of pharmaceutical, biomedical, bioengineering, biomaterials, and molecular biology, among other domains (Kondaveeti, Prasad, & Siddhanta, 2013; Meena et al., 2007). The thermoreversible 3D structure of agar has found key applications in

* Corresponding author.
E-mail address: cidiop@ubu.es (C.I.K. Diop).

https://doi.org/10.1016/j.foodhyd.2022.107664
Received 28 October 2021; Received in revised form 21 February 2022; Accepted 13 March 2022
Available online 16 March 2022

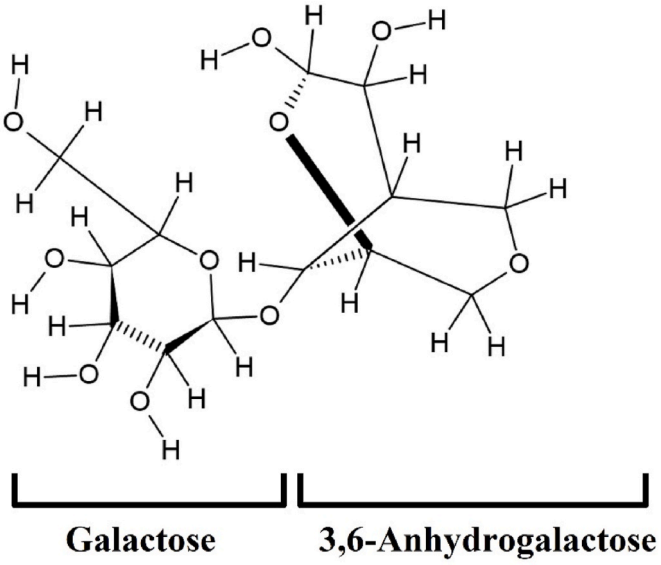


Fig. 1. Molecular structure of D-Galactose and the 3,6-anhydrogalactose unit in the agarose backbone of agar.

tissue engineering, as protein immobilizer, for denture molding (Madhavan & Abirami, 2015), in the reproduction of archaeological remains, and in security fingerprinting (Martínez-Sanz et al., 2019).

Agar, also known as vegetable gelatin in the food industry (Mariod & Adam, 2013; Matos et al., 2021), is one of the most common polysaccharides used as a thickening agent as well as texture and viscosity controller. Furthermore, its high soluble gross fiber content (≥94%) confers it suitable prebiotic properties for the nutraceutical industry (Armisen & Gaiatas, 2009).

In 2016, the global agar market was worth USD 268.58 million and was predicted to worth USD 338.17 million by 2022. Agar for the food industry accounted for more than 57% of the total amount in 2016. In terms of revenues, the market of seaweed polysaccharide will expand at a 3.9% compound annual growth rate (CAGR) to reach a total market size of US$ 350 million by 2024, from US$ 280 million in 2019 (Fiormarkets, 2019). However, a shortage in bacteriological and technical agar has recently been reported due to a decrease in resources (Sanchez-Cardozo, Quintanilla-Carvajal, Ruiz-Pardo, & Acosta-González, 2019). The overall amount of agar in *Gelidium sesquipedale* collected on the Moroccan coast varied according to the harvesting period. It oscillated around 40% and can reach a maximum of 44.5% of the algae dry weight when collected in November (Mouradi-Givernaud, Hassani, Givernaud, Lemoine, & Benharbet, 1999). Matsuoka patented the freezing method as the primary industrial production technology of agar as early as 1921 and 1922 in California. However, modern industrial extraction processes typically incorporate an initial alkaline pretreatment of the seaweed to improve the gelling properties of the hydrocolloid, particularly for Gracilaria agar.

A multitude of green extraction technologies has been reported since then. These methods use microwave equipment (Sousa, Alves, Morais, Delerue-Matos, & Gonçalves, 2010), autoclaves, ultrasounds coupled with hot water extraction (Öğretmen & Duyar, 2018) as well as an enzyme assisted process. Rhein-Knudsen, Ale, & Meyer, 2015). Matos et al. (2021) described a correlation between the severity of the treatment and the hydrolysis and degradation of the agar. Trigueros et al. (2021) has found that a certain amount of agar still remains in the discarded red algae industry waste stream when analyzing the protein fraction from this industrial end of line by-product using a semi-continuous pressurized liquid extraction also known as accelerated solvent extraction.

In this regard, recovering the residual agar in the waste stream as the first step to a cascade biorefining approach could be a sustainable and economical strategy.

When water is pressurized at temperatures over its typical boiling point, its viscosity is reduced. As a result, when compared to extraction under ambient or Soxhlet conditions, the pressurized hot water provides improved access to the analytes. Temperatures between 100 and 374 °C promote the rupture of the hydrogen bonds, lowering the dielectric constant (ϵ) of water to levels comparable to typical organic solvents. Consequently, a wide range of solvation power can be obtained, which can be exploited to selectively extract molecules of interest.

This current work aims to evaluate the potential of using pressurized hot water extraction technology for the recovery and physicochemical properties of the residual agar remaining in the discarded algae industry waste stream. The individual and combined influences of the time (min), pressure (bar), temperature (°C), and algae to water ratio (% w: v) were investigated using a Box-Behnken design associated with a multifactorial response surface methodology (RSM).

2. Material and methods

2.1. Material

To recover the residual agar, pressurized double distilled water subjected to high temperatures was used as a green solvent. The standard reagents used for the characterization of the agar and derivatives were of high purity. K_2SO_4 (99.5%) was supplied by Probus S.A. (Badalona, Barcelona, Spain). Alfa Aesar provided the Resorcinol (benzenediol 99%). Anhydrous GC-grade acetaldehyde was purchased from Sigma, and D-galactose (>98%) was obtained from the Tokyo Chemical Industry (TCI). Fructose (>99%, HPLC grade) was acquired from Fluka Analytical. HCl (37%) was provided by ACROS Organics (USA). Barium Chloride (99%) was purchased from Merck, and trichloroacetic acid (99.7%) was supplied by VWR (Germany). All other reagents were of analytical grade and were used as received without further purification.

Hispanagar (Burgos, Spain, https://www.hispanagar.com) generously donated the residual waste stream of *Gelidium sesquipedale* discarded after a primary industrial phycocolloid-centric extraction process. The waste biomass was washed thoroughly with tap water to remove the sand and contaminants, then hand-pressed, wrapped in sealed zippered plastic bags, and stored at −20 °C until utilization. The frozen algae waste was thawed at room temperature prior to the PHWE process, to obtain never-dried algae with a water content of approximately 75 ± 3%.

2.2. Methods

2.2.1. Characterization of the algae materials

The National Renewable Energy Laboratory (NREL) protocols (https://www.nrel.gov/bioenergy/biomass-compositional-analysis.html) were used to characterize the physicochemical properties of discarded algae industry waste stream.

The moisture content was determined gravimetrically after oven-drying at 105 °C for 24 h. The resulting moisture content was used to express results per gram of dried material. The ash content was determined after burning the dried biomass in a muffle at 575 ± 25 °C for 12 ± 3 h.

The carbohydrate content was quantified using high-performance liquid chromatography (HPLC) equipped with a Biorad Aminex-HPX-87 H column, a variable wavelength detector (VWD), and a refractive index detector (RID) was used for the characterization, using 0.005 M sulfuric acid as the mobile phase. The Bligh and Dyer method was used to determine total lipids (Bligh & Dyer, 1959).

The elemental composition (C, H, N, S, O) of the raw material was determined using an organic elemental microanalyzer (Thermo Scientific Model Flash 2000), with the oxygen content deducted from a mass

balance. A diamond micro-ATR crystal (Jasco FT-IR 4200) was used to perform FT-IR analysis of the algae and the recovered crude agar. 64 scans were used to capture IR spectra in the wavenumber range of 400–4000 cm^{-1}, which were then processed using Jasco Spectra Manager software (*Spectra Manager*, versión 2; JASCO Corporation: Easton, Maryland (EE.UU.), 2002–2012.).

2.2.2. Pressurized hot water extraction (PHWE) of the residual agar

To recover the residual agar, a 500 mL laboratory-designed pressurized stainless-steel extractor vessel, equipped with a temperature-controlled heating jacket and a pressure gauge was used. Fig. 2 depicted a schematic illustration of the designed vessel used for the pressurized hot water extraction of the crude agar that remained in the discarded algae industry waste stream.

To achieve the appropriate solid to liquid ratio (from 3 to 10% w: v), a precise dry weight of the never-dried algae waste stream was placed in the extractor and the corresponding volume of double distilled water was added. A flux of N_2 gas was injected to pressurize the tightly sealed vessel in a static mode (while the outlet valves are closed) under the control of a pressure gauge. Using an attached heating jacket (230 V, 4000 W, ø95 cm, 160 mm height) controlled by a PID system, the reaction system was then heated at a rate of 3 ± 1 °C/min. Throughout the extraction process, a platinum resistance thermometer (Pt100 sensor) immersed in the extraction medium recorded any temperature variations. The residual agar recovery process was carried out at set temperatures (ranging from 80 to 130 °C) and pressures (ranging from 1 to 70 bar).

At the end of the process, the extractor was depressurized and unsealed when the temperature dropped to 80 ± 5 °C. A double-layered muslin cloth was used to filter the hot mixture, which separated the residual algae from the solution. The liquid fraction containing the recovered crude agar was allowed to gel entirely at room temperature. It was then subjected to a two-cycle freeze-thawing (freezing at -20 ± 2 °C for 16 h and then thawed at ambient temperature) to ensure that the agar and the liquid fraction were separated efficiently. The extracted agar was oven-dried at 45 ± 2 °C for 72 ± 5 h or until a constant weight was reached. It was then tightly sealed in a plastic bag and placed in a desiccator at room temperature until further characterizations.

2.2.3. Experimental design

A Box-Behnken design combined with a response surface methodology (RSM) analysis was used to assess the impact of the pressurized hot water extraction (PHWE) conditions on the recovery and the physicochemical properties of the crude residual agar. Statgraphics Centurion XVIII, version 18, software (2017, StatPoint, Inc., Warrenton, VA, USA) was used for the design and data analysis.

The statistical model allowed the use of a reduced number of experimental runs to evaluate the impacts of the key operational parameters on the defined responses. The parameters were chosen based on existing information on agar extraction methods and the operating limits of the designed PHWE extractor. The temperature (Factor A, °C), the pressure (Factor B, bar), the time (Factor C, minutes), and the algae to water percent ratio (Factor D, % w: v) were the four key factors chosen in the RSM design. Each of these variables was rated at a lower (−1), centered (0), and higher (1) level (Table 1). Two center points were added to confirm the curvature and the reproducibility of the extraction process based on the experimental runs. Table 2 summarized the results of a total of 27 experimental runs based on the specified Box-Behnken design.

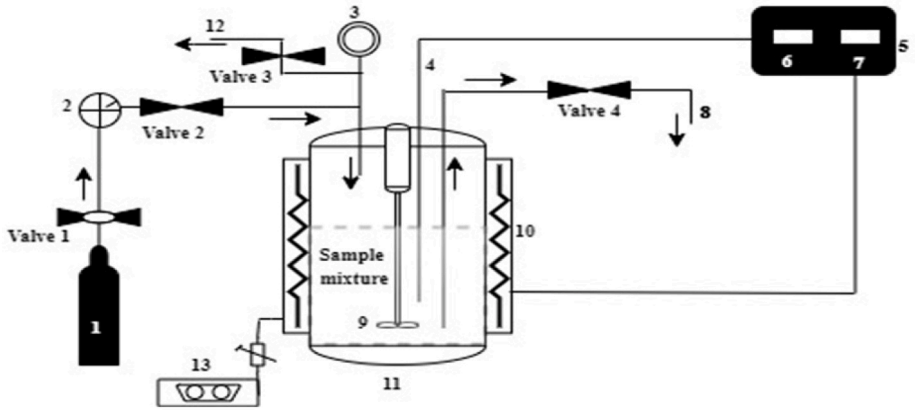
Because of all the assessed factors were measurable, the response surface can theoretically be described by the following equation:

$$y = f(x_1, x_2, x_3, \ldots\ldots, x_k) \quad (1)$$

where y is the specific experimental response and x_i the experimental factors.

Table 1
Experimental factors and their associated levels used during the development of the Box-Behnken design for the extraction of crude residual agar from the *Gelidium sesquipedale* waste-stream using Pressurized Hot Water.

Factors	Symbols	Levels		
		−1	0	+1
Reaction temperature (°C)	**A**	80	105	130
Reactor pressure (bar)	**B**	1	35.5	70
Extraction time (min)	**C**	40	97.5	150
Solid content (%)	**D**	3	6.5	10



1- N_2 gas tank
2- Pressure source regulator
3- Extractor pressure controller
4- Thermocouple temperature recorder
5- Centralized temperature control board
6- Extracor temperature controller
7- Heating jacket temperature controller
8- Sample collector
9- Propeller
10- Heating jacket
11- Pressurized liquid extractor vessel
12- Pressure release system
13- Resistor linked to a PID controller

Fig. 2. Schematic representation of the Pressurized Vessel used for Pressurized Hot Water Extraction of the remaining crude agar from the algae industry waste-stream.

Table 2
Experimental pattern of the Box-Behnken design reporting the different process factors and responses of the residual agar recovered via Pressurized Hot Water Extraction Process of the never-dried algae industry waste-stream.

	Factor A	*Factor B*	*Factor C*	*Factor D*	*Response 1*	*Response 2*	*Response 3*	*Response 4*	*Response 5*	*Response 6*
Run	Temperature (°C)	Pressure (bar)	Time (min)	Solid Content (% w:v)	Yield (%)	Gel Strength (g/cm^2)	Melting Temp. (°C)	Gelling Temp. (°C)	3,6-ANG (%)	Sulfate content (%)
1	105	37.5	45	3	14.9	25	84.2	35	21.1	2.3
2	80	70	97.5	6.5	14.1	198	83.5	31	34.8	2.8
3	105	35.5	45	10	12.4	286	84.5	35	22.7	4
4	105	1	97.5	10	11.8	235	84.5	35.1	27.4	4.3
5	130	35.5	97.5	3	16.7	30	75	30	22	2.7
6	105[a]	35.5[a]	97.5[a]	6.5[a]	13.3[a]	216.1[a]	80.0[a]	34.0[a]	26.3[a]	4.7[a]
7	105	70	97.5	3	15.1	202	81.5	32.3	36.6	1.7
8	130	35.5	97.5	10	12.5	12.7	73	28.5	37	4.8
9	105	70	97.5	10	10.7	174.1	82.9	33.5	37.1	4
10	80	35.5	97.5	10	11	320.2	83	34	22.5	3.6
11	130	70	97.5	6.5	12	8	68	29.2	32.6	7
12	130	1	97.5	6.5	14.9	50.8	73.6	29.8	25.3	8.4
13	105	70	45	6.5	13.9	112	84.6	34.7	27.5	2.7
14	130	35.5	45	6.5	13.8	189	77.1	33	32.2	10
15	105	1	45	6.5	13.7	229	85.5	34	22.4	4.2
16	105	1	150	6.5	13.3	289.3	83.7	35	29.4	4.9
17	80	1	97.5	6.5	11.1	277.7	82.5	35	21.3	3.5
18	105[a]	35.5[a]	97.5[a]	6.5[a]	13.1[a]	218.0[a]	83.6[a]	34.0[a]	25.6[a]	4.8[a]
19	105	35.5	150	3	16.1	210	81.5	33.5	29.9	2.8
20	80	35.5	97.5	3	14.2	226.2	80.6	32.5	19.2	1.5
21	105	1	97.5	3	14.1	307.3	82	33.5	25.6	2.6
22	130[b]	37.5[b]	150[b]	6.5[b]	12.6	–	–	–	39.8	6.0
23	105	70	150	6.5	11.5	28.7	69	28.7	38.6	3.9
24	80	37.5	45	6.5	12.8	35	86	35	21.2	3.2
25	105	35.5	150	10	10	29.4	73.9	29.4	25.3	6
26	105[a]	35.5[a]	97.5[a]	6.5[a]	13.1[a]	34.0[a]	82.0[a]	34.0[a]	25.8[a]	4.7[a]
27	80	35.5	150	6.5	12.5	34	85	34	24.8	3.2

a) Represent the factors and responses obtained at the center points in the Box-Behnken design and allowed experimental replications.
b) Responses 2, 3, and 4 were not obtained due to the non-gelling property of the recover solid extracts at high A factor value (temperature = 130 °C) and high C factor value (extraction time = 150 min) (the pressure was set to 37.5 bar and the solid concentration to 6.5 wt%).

On the other hand, the PHWE process factors being continuous and experimentally controllable with negligible errors, the second-order model was used to find the suitable approximation for the true functional relationship between independent variables. The model can be described mathematically as follows:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

where, $x_1, x_2, x_3, \ldots, x_k$ are the input factor that influence the response y, β_0, β_i, β_{ii}, β_{ij} ($i = 1, 2, 3, \ldots k$; and $j = 1, 2, 3, \ldots k$) are the unknown parameters and ϵ being the random error. The β coefficients, which should be determined in the second-order model, are acquired by the least square method (Aslan & Cebeci, 2007).

The linear relationship between variables was determined using Pearson product moment correlation carried out using Statgraphics Centurion XVIII, version 18, software (2017, StatPoint, Inc., Warrenton, VA, USA). The severity factor (SF) and the physicochemical properties of the residual crude agar were included among these variables. The following equation was used to calculate the severity factor also labeled log (R_0):

$$\log(R_0) = log\left\{t \times e^{[(T-100)/14.75]}\right\} \quad (3)$$

where T is the temperature (°C) and t, the time (min). The severity factor ranged between 1.06 (run 24: T = 80 °C and time = 45 min) and 3.06 (run 22: T = 130 °C and time = 150 min).

2.2.4. Physicochemical characterization of the recovered crude agar

2.2.4.1. Agar yield calculation. The yield of the recovered crude agar was determined based on the following equation and reported as the percentage of dry mass.

$$Agar\ Yield = \left(\frac{Dry\ weigth\ of\ agar\ (g)}{Dry\ weight\ of\ the\ never\ dried\ algae\ waste}\right) \times 100 \quad (4)$$

2.2.4.2. Determination of the agar gel strength. A 1.5% (w: v) colloidal gel was used to determine the gel strength of the recovered crude agar. The gel was molded in a cylindrical tube with 3 cm × 2.5 cm (diameter x height), stabilized at 4 °C for 12 h, and then equilibrated at room temperature for 2 h before analysis. To evaluate the gel strength (expressed in g/cm^2), a penetration test was performed at room temperature (25 ± 5 °C) using a texture analyzer (Stable Micro Systems model TA-XT2, Surrey, England). A flat-faced cylindrical Teflon plunger with a diameter of 10 mm was allowed to penetrate the gel at a constant velocity of 0.2 mm/s. Weight was added progressively until the plunger broke the surface and penetrated 5 mm into the agar gel (Lee, Namasivayam, & Ho, 2014).

2.2.4.3. Gelling and melting temperatures. The melting temperature was determined from a gel containing 1.5% (w: v) agar following the method described by (Freile-Pelegrín & Robledo, 1997) with slight modifications. 10 mL of the dispersed colloid solution was placed in a 50 mL screw-capped tube and allowed to gel at room temperature before being stabilized at 4 °C overnight. The gel surface was covered with glass beads (3 mm diameter), and the tube was heated gradually in a water bath. The melting point was recorded when the glass beads sank to the bottom of the tube.

The gelling temperature was determined by inserting a thermometer in the 50 mL screw-capped tube containing 10 mL of a fresh 1.5% (w: v) hot agar solution (temperature ≈ 95 °C). The tube was inclined to form a 45° angle and allowed to cool gradually. The gelling temperature (°C) was recorded when the gelling solution ceased to flow in the tube.

2.2.4.4. Determination of the 3,6-anhydrogalactose content. The content of 3,6-anhydrogalactose expressed in percent of the agar dry weight was determined colorimetrically using a modified resorcinol method (Xie, Zhang, Liu, Chen, & Cheong, 2020). 0.03 mL of the agar solution (1mg/mL) was mixed with 0.2 mL of distilled water in a test tube, then was placed in an ice bath for 5 min. After adding 1 mL of resorcinol reagent to the solution, the tube was allowed to stand at room temperature for 2 min before being incubated at 80 °C during 10 min. The absorbance was measured at 555 nm using a spectrophotometer after cooling in an ice bath for 5 min. The concentration of 3,6-anhydrogalactose was calculated from the calibration curve using fructose as standard (0.06 mg/mL to 0.6 mg/mL). The colorimetric response of 3,6-anhydrogalactose was found to correspond at 92% to those obtained from fructose.

2.2.4.5. Determination of the sulfate content. The sulfate content in the recovered residual crude agar was determined using a modified barium chloride-gelatin ($BaCl_2$-Gelatin) turbidity method (Martínez-Sanz et al., 2019). 5 mg of the agar was hydrolyzed for 5 h at 100 °C in 1 mL of 1 mol/L HCl (37%) solution. The hydrolyzate was then vortexed and filtered with a Whatman N°1 filter paper (0.22 μm). 0.2 mL of the solution was blended with 3.8 mL of trichloroacetic acid (3%) and 1 mL of the $BaCl_2$-Gelatin reagent in a 10 mL test tube. The solution was mixed vigorously and allowed to stand at room temperature for 15 min. The absorbance was measured at 360 nm against a blank of distilled water. The calibration curve was prepared using K_2SO_4 at concentrations ranging from 0.06 to 0.6 mg/mL (~0.0533–0.533 mg SO_4^{2-}/mL). The sulfate content was calculated and expressed as a percentage on a dry weight basis.

3. Results and discussion

3.1. Characteristics of the materials

Table S1 (as supplementary material) illustrated the proximate analysis of the fresh algae (*Gelidium sesquipedale*) and the residual waste stream discarded following its primary agar industrial extraction (Ester, 2021). When looking at the galactan concentration in the algae, a considerable reduction was noticed in the waste stream following the industrial process. When compared to the unprocessed fresh seaweed (21.3 ± 0.5%), the amount of galactan in the waste algae (10.9 ± 0.5%) was roughly 51% lower. This observation could help to corroborate the presence of an exploitable amount of residual agar as a sulfated galactan in the industrially processed algae. It is worth noting, however, that the acid hydrolysis stage involved in the galactan content analysis process, can significantly degrade compounds such as 3,6-anhydrogalactose and its 2-O-methyl ether (Usov, 1998), making a precise estimation more challenging.

The CHNSO elemental analysis, on the other hand, revealed the modest difference in sulfate proportions between the fresh algae (0.26 ± 0.07%) and its residual industrial waste counterpart (0.21 ± 0.05%). Inversely, the industrial agar extraction process appeared to favor an increase of the ash content in the algae by-product. The higher ash content might demonstrate coherent preservation of the weight ratio of sulfate to galactans between the respective algae. In the same trend, the moisture content of the never-dried algae waste was found to be 75 ± 3% with an ash content of 22 ± 2%, while the moisture content in the recovered crude agar fluctuated between 8 ± 2 and 11 ± 2%, with an ash content ranging between 15 ± 3 and 19 ± 1% (dry basis).

The FT-IR spectra of the residual algae industry waste stream and the PHWE recovered crude agar were shown in Fig. S2 (as supplementary material). Most of the tested samples showed characteristic peaks appearing at comparable wavenumbers. The broad band about 3300 cm^{-1} was attributed to the combined stretching vibrations of NH and OH groups in both the algae and the residual agar (Selvalakshmi, Mathavan, Selvasekarapandian, & Premalatha, 2017), with a relevant contribution from the water content in the samples (Shahnaz, Shehnaz, & Haider, 2019). The peak emerging at 2920 cm^{-1} could be attributed to asymmetric ν(CH) stretching modes, whereas the band appearing at 1634 cm^{-1} could mostly involve the moisture water bending δ(HOH) vibrations, but also bending δ(NH_2) and stretching ν(C═O) modes could contribute in this region (Kumar, Boro, Ray, Mukherjee, & Dutta, 2019).

The most representative peaks for the agar phycocolloid, on the other hand, are generally detected in the region below 1600 cm^{-1}. The spectra bands of the algae waste were compared to commercial agar and selected crude agars recovered from a 6% (w: v) algae to water ratio in the 400 cm^{-1} to 1600 cm^{-1} region in Fig. 3.

Wagging ρ(CH) vibrational modes (Trivedi, Rao, & Kumar, 2014) have been assigned to the band at 1370 cm^{-1} for both sulfated and unsulfated agarose (Rochas, Lahaye, & Yaphe, 1986). The weak band observed at 1239 cm^{-1} could be due to the overlapped contributions of peaks at around 1250 cm^{-1} assigned to pyranose ring δ(CH_2) bending vibrations in agarose (Trivedi et al., 2014) together with an absorption arisen at about 1220 cm^{-1} in the recovered crude agar samples (Fig. S3, supplementary material). This latter could be ascribed to the ester-sulfate groups (Rajasulochana & Gunasekaran, 2009). In this regard, the comparison of the spectra of commercial pure agarose with negligible sulfate content (less than 0.1%) and the crude agar with the highest sulfate content (10%), recovered at a high temperature of 130 °C, with a pressure of 35.5 bar, a PHWE recovery time of 40 min with 6.5% (w: v) algae concentration (Table 2) could shed light on this assignment (see Fig. S3). In addition, an increase of the intensity of this band depending on the sulfate content could be observed. Unfortunately, the small magnitude of the changes together with the low

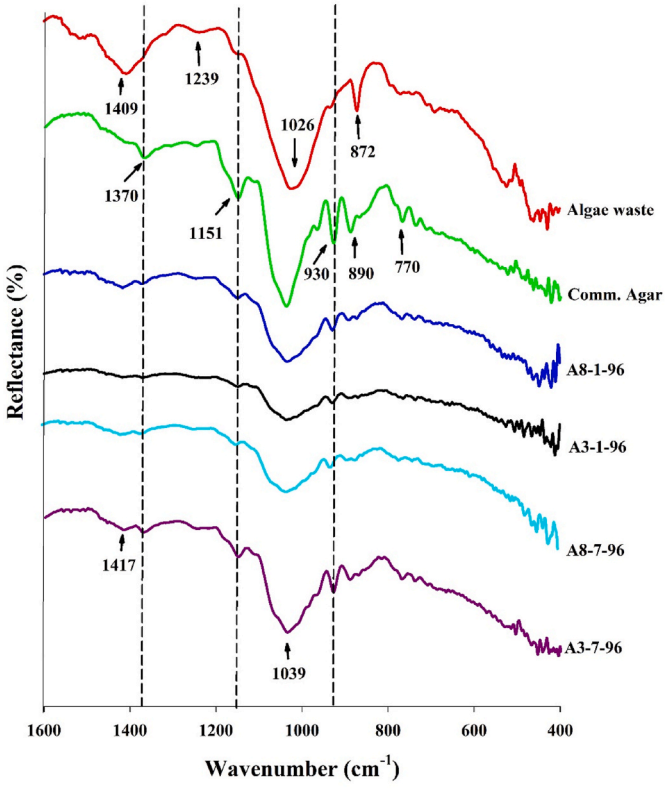


Fig. 3. FT-IR spectra showing the bands below the wavenumber of 1600 cm^{-1} of the residual algae industry waste stream, the commercial agar, and the recovered crude agar. A8-1-96 (Temperature (A) = 80 °C; Pressure (B) = 1 bar; Time (C) = 97.5 min and Algae to water ratio (D) of 6% (w: v); A3-1-96 (A = 130 °C; B = 1 bar; C = 97.5 min and D = 6% (w: v); A8-7-96 (A = 80 °C; B = 70 bar; C = 97.5 min and D = 6% (w: v); A3-7-96 (A = 130 °C; B = 70 bar; C = 97.5 min and D = 6% (w: v).

resolution of the spectra in this region precluded any quantification of the observed trend (Fig. S4). The appearance of an absorption between 1409 and 1417 cm^{-1} in the algae waste and recovered crude agar samples was observed in Fig. 3, which could be attributable to the $\delta(CH_2)$ modes (Trivedi et al., 2014). On the other hand, the peak at 1039 cm^{-1} was attributed to the C–O and C–O–C vibrations of the 3,6-anhydrogalactose bridge (Zhang, Fu, Duan & Gao, 2019) as well as the glycosidic linkage (Trivedi et al., 2014). This latter peak appeared nonetheless, to be located at a lower wavenumber of 1026 cm^{-1} for the algae waste. Peaks emerging between 1000 and 1100 cm^{-1} were considered to be typical of the C–O and O–CH vibrations of polysaccharides such agarose (Balkan, Coban, & Güven, 2005). Agarose characteristic bands were also observed at 930, 872 and 770 cm^{-1} (Fig. 3), which were attributed to 3,6-anhydro-β-galactose skeletal bending modes (Singh, Trivedi, & Kumar, 2010). On the other hand, the peak emerging at 890 cm^{-1} was assigned to both the C–C and C–O vibration in the 1,3-linked β-galactopyranosyl units (Balkan et al., 2005).

In this study, the Box-Behnken experimental matrix coupled with a response surface methodology (RSM) analysis, allowed to assess the theoretical impact of the pressurized hot water extraction (PHWE) process parameters on the recovery and some physicochemical properties of the crude agar.

Using the RSM, the Box-Behnken design was best suited to generate a polynomial quadratic model able to integrate the individual extraction process parameters. The significance of the impact of the PHWE parameters and their 2-way interactions on the agar recovery responses was examined using an ANOVA test presented in Table 3. The use of three replicates at the center point allowed for the evaluation of the process stability and of any key variability inclusion. The recovery and physicochemical properties of the agar were largely impacted by the interrelation between the pressurized hot water operating parameters, namely the temperature, the extraction time, the internal pressure, and the ratio of algae biomass to water (Table 3). Therefore, the variation induced on the yield along with the gel strength, the gelling and melting temperature as well as the content of 3,6-anhydrogalactose and sulfate groups will be discussed.

Fig. S1 (as supplementary material) showed the Pareto chart depicting the standardized effect of the pressurized hot water extraction parameters and their interactions on the yield and properties of the crude agar.

According to Alvarez-Rivera, Bueno, Ballesteros-Vivas, Mendiola, and Ibañez (2019), variables such as biomass particle size and initial water content can also influence the pressurized hot water extraction process. However, in the current study, the option to implement a cost and energy-efficient PHWE recovery process dictated the use of a pre-cleaned never-dried (75 ± 2% water content) and unground algae waste. Consequently, the potential influences of particle size change and initial moisture of the waste stream were not considered in this study. The Pearson moment correlation was found to effectively supplement the RSM analysis in elucidating the linear correlations between the severity factors of the PHWE and the physicochemical properties of the crude agar, as well as between the different properties.

3.2. Effects of PHWE parameters on the recovered crude agar yield

The PHWE technology allowed to recover agar yields ranging from 10 ± 2% to 17 ± 2% (Table 2) depending on the operating conditions. The F-test, the p-value ($p < 5\%$ tolerance level at a 95% confidence level), the R-square (R^2) as well as the adjusted R^2 (adj-R^2) (Table 3), assessed the compatibility of the mathematical model and the significance of factors' effects.

With p-values below the 5% tolerance level, the analysis of variance

Table 3
Analysis of variance ANOVA results of the effect of the pressurized hot water parameters and their 2-ways interaction on the yield and some physicochemical properties of the recovered crude agar at a 95% confidence level ($p < 5\%$) as well as R^2 and adjusted-R^2 values. (Insignificant interactions were omitted from the table).

Yield (%)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Temperature	4.10875	1	4.10875	17.31	0.0008
B:Pressure	0.213333	1	0.213333	0.90	0.3582
C:Time	1.59835	1	1.59835	6.73	0.0203
D:Solid concentration	42.7141	1	42.7141	179.93	0.0000
AB	8.7025	1	8.7025	36.66	0.0000
BD	1.1025	1	1.1025	4.64	0.0478
CD	3.24	1	3.24	13.65	0.0022
R-squared (R^2) %	94.71				
Adjusted-R^2 (%)	91.20				

Gelling Temperature (°C)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Temperature	40.5687	1	38.5711	40.61	0.0001
B:Pressure	14.0833	1	14.0833	14.1	0.0032
C:Time	25.787	1	25.787	25.81	0.0004
D:Solid concentration	0.140833	1	0.140833	0.14	0.7145
AA	21.2925	1	21.2925	21.31	0.0007
BC	12.25	1	12.25	12.26	0.0050
R-squared (R^2) %	91.10				
Adjusted-R^2 (%)	79.77				

Melting Temperature (°C)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Temperature	297.476	1	297.476	49.79	0.0000
B:Pressure	13.6906	1	13.6906	2.29	0.1610
C:Time	56.8535	1	56.8535	9.52	0.0115
D:Solid concentration	0.75	1	0.75	0.13	0.7305
AA	81.2379	1	81.2379	13.6	0.0042
R-squared (R^2) %	88.76				
Adjusted-R^2 (%)	73.03				

Gel Strength (g/cm^2)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Temperature	112245	1	112245	35.08	0.0001
B:Pressure	19602.1	1	19602.1	6.13	0.0308
C:Time	19.021	1	19.021	0.01	0.9399
D:Solid concentration	1692.19	1	1692.19	0.53	0.4823
AA	18170.6	1	18170.6	5.68	0.0363
CD	31684	1	31684	9.9	0.0093
R-squared (R^2) %	84.27				
Adjusted-R^2 (%)	64.26				

3,6-Anhydrogalactose (%)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Temperature	169.501	1	169.501	8.71	0.0094
B:Pressure	259.47	1	259.47	13.33	0.0022
C:Time	138.041	1	138.041	7.09	0.0170
D:Solid concentration	25.8133	1	25.8133	1.33	0.2665
R-squared (R^2) %	67.77				
Adjusted-R^2 (%)	47.62				

Sulfate content (%)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Temperature	37.1008	1	37.1008	22.38	0.0005
B:Pressure	2.80333	1	2.80333	1.69	0.2178
C:Time	0.0133333	1	0.0133333	0.01	0.9300
D:Solid concentration	14.3008	1	14.3008	8.63	0.0124
DD	12	1	12	7.24	0.0196
R-squared (R^2) %	79.80				
Adjusted-R^2 (%)	56.22				

(ANOVA) revealed that three of the four investigated parameters, as well as several of their interactions (2-way), had significant impacts on the recovered yield. In other words, with the exception of the pressure, each process factor had a distinct impact on the extraction yield. As evidenced by the high F-value of 179.93 linked with a p-value less than 0.0001, the solid to water ratio in the reactor appeared to be the single factor having the most perceptible impact on the recovery yield.

Apart from the influence of the solid content, Table 3 revealed a tangible significance of the individual effects of the temperature and the extraction time, with p-values of 0.0008 (F = 17.31) and 0.0203 (F = 6.73) respectively. The 2-way-interaction between temperature and pressure ($p < 0.0001$; F = 36.66) has been found to have a clear effect on the extraction process and yield, as expected. In a similar trend, the effects of the two-way interaction between pressure and solid content (p = 0.0478; F = 4.64) as well as time and solid content (p = 0.0022; F = 13.65) were significantly related to the variability in recovered agar yield. The fitted quadratic model explained at 94.7% the variability, as evidenced by the R-squared value. With a standard error of estimate equal to 0.48 and a mean absolute error of 0.28, the adjusted R-squared statistic (adj-R^2 = 91.2%) included in the assessment of the model's appropriateness revealed that the suggested model was highly justified by the investigated variables.

Therefore, the mathematical relationships of the polynomial regression equation for the agar yield (%) using the combined extraction factors were given as follows:

$$\begin{aligned} \textit{Agar yield} = {} & 4.42075 + 0.0859443 \times A + 0.203961 \times B + 0.0244244 \times C \\ & + 0.0928512 \times D - 0.00171014 \times AB - 0.00434783 \times BD \\ & - 0.00489796 \times CD \end{aligned} \tag{5}$$

where A is the temperature (°C), B is the Pressure (bar), C is the time (min), and D is the algae to water ratio (% w: v).

At the stationary point, the fitted model projected a high recovered agar yield value of 17.03%. This value was calculated for a never-dried and unground industrial algae waste stream, indicating that an optimized model would be appropriate. Based on the combined influences of the PHWE parameters, the optimization tests retrieved from the mathematical model proposed a balance of the varied optimum process conditions for each response. The results of the balanced optimization tests in relation to the interrelated effect of the process factors on each response were presented in Table S2 (supplementary material). The balanced conditions suggested a temperature of 120.03 °C, with an internal pressure of 3.28 bar, a reaction time of 150 min at a 3% (w: v) algae concentration in the PHWE extractor to estimate this forecasted optimum yield value.

Overall, in comparison to earlier investigations, the yield values achieved for a secondary agar extraction via the Box-Behnken matrix were high. Authors such as Trigueros et al. (2021) have recovered a residual agar yield of 5% from dried *Gelidium sesquipedale* algae-industry waste stream after a semi-continuous subcritical water extraction at high temperature (200 °C) and an extraction time of 240 min (flow rate of 6 mL/min). The higher yield could be explained by the lower to moderate process temperatures ($80 \leq T\,°C \leq 130$ °C) used in this present study, as well as the shorter extraction times ($45 \leq t \leq 150$ min). It is commonly known that the physical properties of water change as the temperature vary. As a result, the combination of these two physical parameters fosters a decrease of the dielectric constant and water density. Furthermore, when exposed to high temperature and pressure, the generation of hydronium ions ($H_2O \rightleftharpoons H_3O^+ + OH^-$) that occurs from self-ionization of water molecules tended to acidify the reaction medium (Plaza & Turner, 2015). Therefore, the autohydrolysis action of the pressurized hot water combined with a lower dielectric constant impart an autocatalytic power to water. When operating under these conditions, the most amorphous and lower molecular weight fractions of biopolymers (Chandler, Deng, Dillow, Liotta, & Eckert, 1997; Gomes-Dias, Romaní, Teixeira, & Rocha, 2020) such as the agarose and the agaropectin backbones of the residual agar, could be hydrolyzed. However, when considering the PHWE process' severity factor (based on the synergistic effect of temperature and extraction time), the Pearson moment correlation analysis revealed a very limited positive linear relationship between the severity factor and the recovered agar yield. The Pearson moment correlations between the various factors and responses investigated, as well as their statistical significances, are represented in Table S3 (as supplementary material). A Pearson coefficient (r) of 0.19 was found to exist between the severity factor and the recovered agar yield a 95% confidence level, with an insignificant p-value ($p > 5\%$).

The use of wet never-dried algae waste, on the other hand, could have an impact on the PHWE recovery yield. According to Yousefi, Islami, and Filizadeh (2013), soaking *Gracilaria corticata* in water before agar extraction lowered the extracted agar yield. Wet plant biomass trap water in their interstitial tissues. Similar to the PHWE, steam pressurization at high temperature during a steam-explosion process causes hydrolysis inside the biomass matrix due to the formation of H_3O^+ (Diop, Lavoie, & Huneault, 2015). In the same trend, Trigueros (2021) also found a significant galactose concentration, possibly due to galactan hydrolysis under subcritical water conditions. As a result, harsher extraction conditions such as long extraction times at high temperatures might increase the hydrolysis rate, while also decreasing the agar yield. These phenomena could support the theory that the higher agar retrieved in this study could be related to the milder process conditions used. Furthermore, it can also confirm the effect of the 2-way interaction between the temperature and the pressure as well as the considerable influence of solid concentration on agar yield. The reduction of the solid to liquid ratio in the extractor improved the algae dispersal in the pressurized hot water. In accordance with the fundamental of mass transfer (Elboughdiri, 2018), it provided improved accessibility of the free solvent and a higher diffusion rate in a shorter time.

Fig. 4 showed the 3D response (Fig. 4 a-c-d) and contour plots (Fig. 4 b) illustrating the variation of the crude agar yield as a function of the concurrent influences of the four selected process factors and their interactions. The quadratic polynomial regression equation (Eq. (5)) was used to generate the graphs. Two continuous variables were used to determine the variation in agar yield, while the other two were kept constant.

The plots clearly supported the decrease in extraction yield as the concentration of the solid algae waste stream in the medium increased, as previously discussed. The lesser the yield of recovered agar, the higher the solid concentration in the reactor was. However, highest yields were obtained at a temperature above 110 °C and extended extraction time (150 min), while maintaining a reduced algae concentration of 3% (w: v), as indicated in the 3D response surface and contoured plots (Fig. 4 a-b). It was, however, necessary to operate at a low to medium pressure (<45 bar). Sousa et al. (2010) associated an increase in agar extraction rate at high temperatures with a drop in the solvent viscosity and the surface tension, which improved its solvation and diffusion abilities. But overall, because of the long recovery time and low algae concentration, it was necessary to operate at high pressure and low temperature to improve the recovery yield. The strong significance of the 2-way interaction between temperature and pressure ($p < 0001$) on the obtained yield, as shown by the ANOVA test, could support this statement. It could also support the idea that in addition to the subcritical state of water, which is generally reached at temperatures above 100 °C and pressure high enough to keep water in a liquid phase, combining temperatures below the water boiling point with high pressure could offer appealing extraction potentials to a lesser extent.

3.3. Effect of PHWE factors on the gel strength of the residual agar

One of the most widely used quality indicators for agar is the ability of the dispersed residual phycocolloid to form and maintain a strong gel

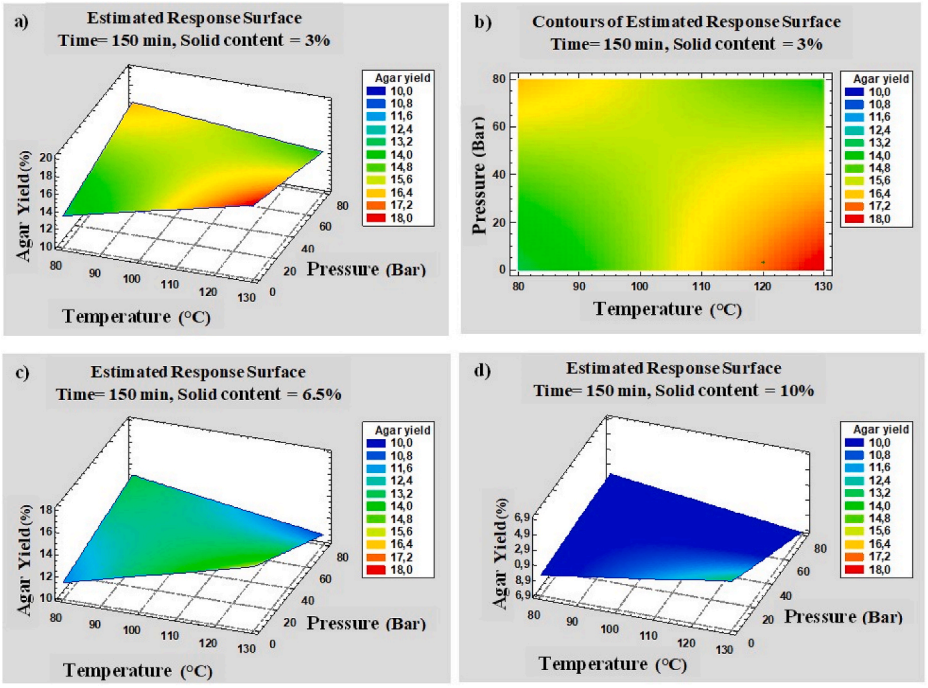


Fig. 4. 3D-response surface (a, c, d) and contour plots (b) showing the variation of the agar yields in function of the extraction parameters.

structure. ANOVA studies demonstrated that the two key individual process parameters, temperature and pressure, had substantial impacts on the variation in the agar gel strength. The temperature, with a very low p-value of 0.0001 and high F-value of 35.08 within a confidence level of 95%, was the dominating factor having a significant influence on the agar gel strength. The low p-value ($p = 0.0363$) found for the quadratic effect of the temperature with an F-value of 5.68 confirmed the preponderance of the temperature effect. Pressure ($p = 0.0308$ and $F = 6.13$), as well as the 2-way interaction between the recovery time and the solid concentration ($p = 0.0093$ and $F = 9.90$), influenced the fluctuation of the recovered agar gel strength in a similar way to the temperature.

The R^2 value indicated that the mathematical model as fitted explained 84.27% of the variance in the gel strength observed. The model provided a proficient mathematical tool that could forecast the expected gel strength in terms of values based on the combined impact of the PHWE process parameters.

The following equation gave the corresponding mathematical pattern for this specific response using the polynomial regression.

$$\textit{Gel strength} = -514.48 + 11.6774 \times A - 1.1715 \times B + 3.35271 \times C + 50.6173 \times D - 0.0740056 \times A^2 - 0.484354 \times CD \quad (6)$$

where A is the temperature (°C), B is the Pressure (bar), C is the time (min), and D is the algae to water ratio (% w: v).

The model predicted the best conditions for recovering a residual agar with a maximum gel strength of 394.38 g/cm^2 (Table S2). This value was expected at a low temperature of 87.9 °C, at atmospheric pressure ($p \approx 1$ bar), at a reaction time (150 min), and using the lowest ratio of solid to water (3% w: v).

The Pearson correlation (Table S3) revealed a moderate negative linear relationship between the severity factor and the gel strength ($r = -0.3548$, $p > 5\%$), but a very low negative linear correlation between the recovery yield and the gel strength ($r = -0.0332$, $p > 5\%$). This corroborates the theory that increasing the severity of the PHWE process, particularly the temperature and the extraction time, inexorably reduced the agar gel's strength.

The measured gel strength values for a 1.5% (w: v) agar hydrogel were in the range of 25–350 g/cm^2 (Table 2), based on the Box-Behnken design. These results were consistent with those obtained by Martínez-Sanz et al. (2019) for unpurified agar derived from non-pretreated algae. For agar extracted using hot water and combination of heating and sonication, this author obtained values of 245 ± 10 g/cm^2 and 275 ± 10 g/cm^2, respectively. Ahmad et al. (2011) and Arvizu-Higuera, Rodríguez-Montesinos, Murillo-Álvarez, Muñoz-Ochoa, and Hernández-Carmona (2008) noticed a significant decrease in gel strength when the extraction time was prolonged at high temperature. Martínez-Sanz et al. (2019) made a similar observation, confirming the decline of the agar strength as well as agar yield at elevated temperatures and prolonged extraction times. Suzuki, Sawai, and Takada (2001) established a linear correlation between the agar's molecular weight and its gel strength. The hydrolytic ability of the pressurized hot water solvent could cause depolymerization of the agar biopolymer at high temperatures and extended recovery times. Depolymerization reduces the molecular weight of the polymer, which weakens the gel strength. The stability of the molecular structure of the gel, as well as its strength, is related to the helical structure of the agarose fraction.

Fig. S5 (as supplementary material) showed the 3D response surface plots depicting the variability of the recovered residual agar gel strength in relation to the variation of the temperature and the pressure at a fixed reaction time and solid content. At a 5% tolerance level, the ANOVA revealed no statistically significant effect of the solid concentration on the resulting gel strength. It's worth noting, nevertheless, that using a 3% (w: v) algae concentration seemed to promote the recovery of agar with acceptable gel strength. This increased gel strength, however, necessitated a longer recovery time and temperatures below 100 °C, as seen in Fig. S5 (c-d). In line with the effect of subcritical water state on the agar yield stated above, it was found that harshening of the PHWE recovery conditions resulted in a visible reduction in gel strength. The strength of the agar gel, on the other hand, was known to be strongly influenced by the charge of sulfate groups in the molecule. During the formation of the double helix, sulfate groups can interfere with the intermolecular hydrogen bonding (Yarnpakdee, Benjakul, & Kingwascharapong, 2015). When the seaweed was desulfated with 10% NaOH prior to the extraction process, Matsuhiro and Urzúa (1990) demonstrated a subsequent rise of the gel strength of agar isolate from

Gelidium rex from 590 g/cm^2 to 1272 g/cm^2. The maximum force applied per 1 cm^2 of agar gel for a standard powdered industrial agar should be $\geq$ 400 g while this force range between 250 and 400 g for squared or agar strips.

Nonetheless, Pearson correlation revealed a statistically insignificant and weak negative linear relationship between the gel strength and the sulfate content ($r = -0.18$, $p > 5\%$), as well as between the gel strength and the 3,6-anhydrogalactose content ($r = -0.15$, $p > 5\%$) in the crude residual agar (Table S3).

The variability of gel strength values noticed when using PHWE could lead to a wide range of potential applications. In fact, for technical use as in microbiology, agar with a higher gel strength is preferred (Armisen, 1995). Lower gel strength agar, on the other hand, has been in high demand in the food, pharmaceutical, and cosmetic industries (Pereira-Pacheco, Robledo, Rodríguez-Carvajal, & Freile-Pelegrín, 2007).

Properties like the gelling and melting temperatures are other important properties to consider when assessing the quality of agar gel.

3.4. Effect of the PHWE extraction parameters on gelling and melting behaviors of the recovered agar

The ability of agar solution to form a compact hydrogel at low temperatures that liquefies when exposed to high temperatures, and vice versa, is a typical characteristic. It confers to agar polysaccharide exceptional properties with various benefits when compared to other natural gelling agent analogs such as gum, starch, sodium alginate or gelatin, etc. (Banerjee & Bhattacharya, 2012).

The response surface methodology analysis of the Box-Behnken experimental design revealed a clear dependency of the gelling temperature of the recovered crude agar gel on certain of the PHWE process parameters. The extraction temperature ($p = 0.0001$, $F = 40.61$), the applied pressure ($p = 0.0032$, $F = 14.1$), and the extraction time ($p = 0.0004$, $F = 25.81$) had significant influence on the temperature of gelation. At a 95% confidence level, the quadratic effect of the temperature ($p = 0.0007$, $F = 21.31$) and the 2-way interaction between the pressure and the extraction time ($p = 0.0050$, $F = 12.26$) were both highly significant. The ANOVA test's R-squared value ($R^2 = 91.1\%$) and adj-R^2 of 79.77% (Table 3) well validated a proper relationship between the observed variation in gelling and the fitted mathematical model.

Fig. 5, illustrates the linear correlation between the severity factor on the one hand, and the agar gel strength on the other hand, with some physicochemical properties of the residual crude agar. It was observed that when the severity factor of the PHWE process increased, both melting and gelling temperatures tended to drop (Fig. 5 a). In contrast, there appears to be a positive relationship between the strength of the crude agar gel and its melting and gelling temperatures (Fig. 5 c).

Furthermore, the analyzed RSM's second-order polynomial regression equation was mathematically fitted. The optimum extraction conditions for recovering residual agar with a high gelling temperature of 36.24 °C were predicted using the fitted equation (Eq. (7)) and included a temperature set to 98.46 °C, a pressure of 48.19 bar, a recovery time of 45 min and never-dried algae to liquid ratio of 9.9% (w: v) (Table S2).

$$\textit{Gelling Temperature} = 11.1814 + 0.513465 \times A + 0.0628019 \times B + 0.00608082 \times C - 0.0309524 \times D - 0.00280504 \times A^2 - 0.000966184 \times BC \quad (7)$$

where A is the temperature (°C), B is the Pressure (bar), C is the time (min), and D is the algae to water ratio (% w: v).

The 3D response surface plots obtained from the 2nd order polynomial regression equation are shown in Fig. S6 (as supplementary material). It showed how the gelling temperature of the crude residual agar varied as a function of the PHWE operating conditions. To recover agar with a high gelling temperature, shorter extraction times associated with low to medium temperatures were required. As expected, the central effect of the temperature on the gelling point was visible in a similar way to the agar gel strength. Extending the reaction time, on the other hand, was found to have a detrimental impact on the gelling temperature, marked by a decrease in their values. The lower gelling temperatures observed when harsher conditions were used could be related to a decrease in the molecular weight of the biopolymer, which was also evident in the gel strength. The acidification of the medium and the hydrolytic ability of the pressurized hot water have the aptitude to weaken the recombination capacity of the helical structure mostly implicated in the gel formation, thus lowering its molecular cohesion (Suzuki et al., 2001). Fig. S6. (a-b-c) depicted, however, that with a shorter time of 45 min, the recovery of agar with high gelling temperatures was dependent on an increase of the algae to liquid concentration from 3 to 10% (w: v). This favorable effect of the solid concentration was, nonetheless, restrained to the application of a short recovery time,

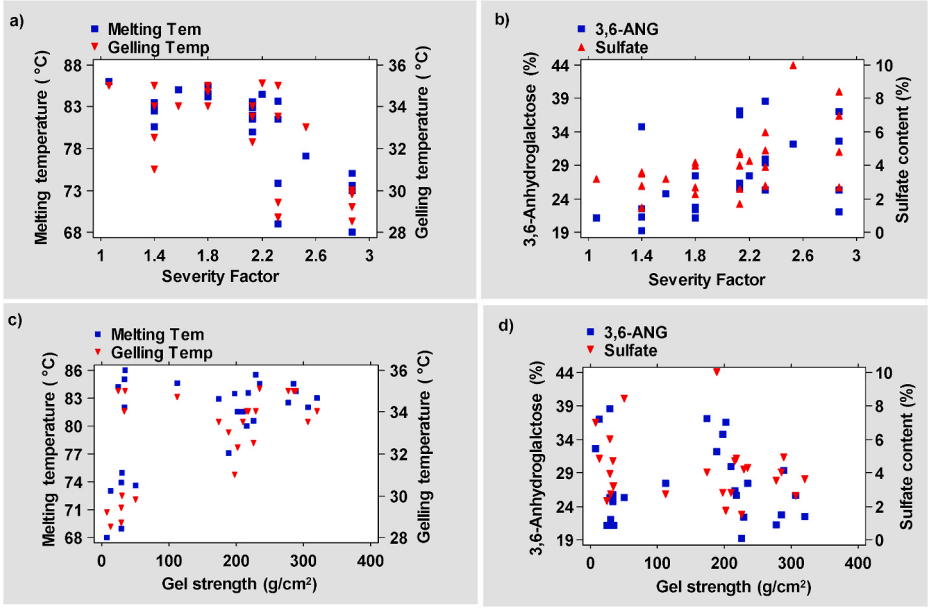


Fig. 5. **a**) Correlation between the severity factor of the PHWE process, the melting temperature (°C) and the gelling temperatures (°C) measured from gels containing 1.5% (w: v) agar. **b**) Correlation between the severity factor, the 3,6-Anhydrogalactose content (%) and the sulfate content (%). **c**) Correlation between the gel strength (g/cm^2), the melting temperature (°C) and the gelling temperature (°C) obtained from gels containing 1.5 wt% agar **d**) Correlation between the gel strength, the 3,6-anhydrogalactose content and the sulfate content in the recover crude agar.

whereas a longer extraction process had the opposite inversed impact (Fig. S6 d-e-f).

Furthermore, the RSM analysis, and ANOVA test revealed significant influences of the temperature ($p < 0.0001$, $F = 49.79$), the time ($p = 0.0115$, $F = 9.52$), and the quadratic effect of the temperature ($p = 0.0042$, $F = 13.60$) on the melting temperature of the recovered agar hydrogel. In fact, when exposed to high temperatures, the intermolecular interaction in the agar gel is altered, causing dissociation and a change from a gel structure to a liquid phase. The corresponding R^2 of 88.76% with an adj-R^2 of 73.03% (Table 3), indicated a good relationship between the proposed model and the variability of the melting temperature. Moreover, the fitted mathematical model described a minor effect of the other PHWE parameters on the change in the melting temperature observed for the crude agar hydrogel, principally the pressure and algae to water ratio. The statistical approach suggested using an optimal combinatorial effect of extraction temperature set at 86.5 °C, at a high pressure fixed to 69.75 bar, with a short reaction time of 45 min and a high algae concentration of 10% (w: v) to maintain the melting temperature of the residual agar gel to a predicted higher value of 89.06 °C (Table S2). As a result, the 2nd order polynomial regression of the fitted quadratic model was given in the following equation:

$$\textit{Melting temperature} = 38.6693 + 1.15411 \times A - 0.0302068 \times B - 0.0452656 \times C - 0.0714286 \times D - 0.0065023 \times A^2 \quad (8)$$

where A is the temperature (°C), B is the Pressure (bar), C is the time (min), and D is the algae to water ratio (% w: v).

When PHWE was operated at mild temperatures and shorter processing times associated with a higher algae concentration, agar with higher melting temperature were recovered, similarly to the gelling temperature. Applying more severe conditions, in contrast, resulted in a reversal of this tendency. The negative impact of the severity factor on both the gelling temperature and melting temperature, on the other hand, was found to be strong and statistically significant, as fortified by the Pearson coefficients of −0.72 ($p < 0.0001$) and −0.61 ($p = 0.0009$) respectively. Furthermore, the Pearson linear relationships (Table S3) between the gel strength and respectively the gelling ($r = 0.55$, $p = 0.0034$) and melting temperatures ($r = 0.53$, $p = 0.0057$) were strongly positive as expected. The strongest Pearson coefficient of 0.89 ($p < 0.0001$) was evidently related to the linear relationship between the gelling and the melting temperatures.

On the one hand, the presence of agarose as the main molecule in charge of the agar's gelling behaviors is directly connected to both its gelling and melting properties. The stability of the helical colloids forming the agar gel being controlled by galactose and 3,6-anhydrogalactose. Furthermore, the sulfate groups that affect the agar's gelling capabilities are predominantly found in the agaropectin fraction. Prior investigations confirmed the negative correlation between the agar sulfate content and gelling temperatures (Yarnpakdee et al., 2015). In contrast to agarose, the presence of sulfate-charged molecules may generate electrostatic repulsions that inhibit polysaccharide chain interactions. As with the gel strength, alkaline desulfation generally was found to improve hysteresis performance (temperature gap between the melting temperature and the gelling temperature). Yarnpakdee et al. (2015) have demonstrated that the enhancement of gelling properties of agar from Gracilaria by alkaline pretreatment was related to the conversion of L-galactose-6-sulfate to 3,6-anhydro-L-galactose.

The industrial algae waste stream used in this study was employed as received with no extra chemical pretreatments applied. In order to conform with the principles of green chemistry and green engineering, the agar was neither purified nor bleached after the recovery process. Therefore, the PHWE responses obtained showing gelling temperatures ranging from 69 to 85 °C and melting temperatures detected between 29 °C and 35 °C were consistent with those obtained by authors such as Martínez-Sanz et al. (2019) and Pereira-Pacheco et al. (2007) for native unpurified hot water extracted agar from the Rhodophyta *Gelidium sesquipedale* and *Hydropuntia cornea* respectively.

Overall, the relationship between the gelling properties of agar with its chemical structure has been well established at a molecular level. As a result, the effects of hot water pressurization on the 3,6-anhydrogalactose and sulfate content in the crude agar were discussed further in this work.

3.5. Assessment of the 3,6-anhydrogalactose content in function of the PHWE process factors

Three of the four key PHWE process factors studied showed substantial effects, according to the analysis of variance. Within a 95% confidence level, the effect of the temperature, the pressure and the reaction time were found to influence the content of 3,6-anhydrogalactose with respective p-values of 0.0094 ($F = 8.71$), 0.0022 ($F = 13.33$), and 0.0170 ($F = 7.09$). Nonetheless, while the influence of the algae concentration on the variation of the 3,6-anhydrogalactose content was not statistically significant ($p = 0.2665$; $F = 1.33$), it did not appear to be completely inconsequential. Fig. 6 showed the 3,6-anhydrogalactose variability in the extracted residual agar as a function of reaction temperature and the extraction time using a 3D response surface (Fig. 6 a-e) and contour plots (Fig. 6 f). As a first remark, a slight increase of the 3,6-Anhydrogalactose content was observed when the solid to algae ratio in the medium increased from 3 to 10% (w: v), especially at high pressure ($P = 70$ bar) and elevated extraction temperatures. The fitted model explained 67.77% of the variation in 3,6-anhydrogalactose observed, as evidenced by the R squared value ($R^2 = 67.77\%$) and the adj-R^2 of 47.2%. The regression equation for the resulting second order quadratic model obtained was as follows:

$$3,6-\textit{Anhydrogalactose} = -1.66648 + 0.150333 \times A + 0.134783 \times B + 0.0646032 \times C + 0.419048 \times D \quad (9)$$

where A is the temperature (°C), B is the pressure (bar), C is the time (min), and D is the algae to water ratio (% w: v).

The regression model predicted in the crude agar a high value of 41.016% of 3,6-anhydrogalactose. To obtain this result, the projected optimal operating conditions (Table S2) were a temperature of 130 °C, high pressure of 70 bar, an extended reaction time of 149.84 min, and a solid concentration of 7.65% (w: v).

A positive correlation existed between the severity factor and the content of 3,6-anhydrogalactose in the agar, as shown in Fig. 6-b. Conversely, when the strength of the PHWE recovered crude agar gel increased, its content decreased (Fig. 6-c). The relationship between the severity factor and the content of 3,6-anhydrogalactose in the crude agar was moderately positive ($r = 0.44$, $p = 0.0242$), according to the Pearson linear correlation. It corroborates the observation that the detection of 3,6-anhydrogalactose in the residual agar was favored by higher temperatures and longer extraction times. On the other hand, the gel strength ($r = -0.15$, $p > 5\%$) revealed a weak negative and statistically insignificant relationship with the 3,6-anhydrogalactose content, similar to the recovery yield ($r = -0.12$, $p > 5\%$).

Furthermore, the Pearson correlation between respectively the gelling ($r = -0.48$, $p = 0.0138$) and melting ($r = -0.42$, $p = 0.0342$) temperatures and the level of 3,6-anhydrogalactose in the pressurized hot water extracted agar was found to be moderately negative (Table S3). Under the acidic conditions caused by water's self-ionization, the molecule of 3,6-anhydrogalactose is generally unstable and can be easily converted to 5-hydroxymethyl-furfural (5-HMF). Moreover, an incomplete release of sulfates from the galactose 2-sulfate has been reported under similar mild acidic conditions (Quemener & Lahaye, 1998). Consequently, it has been thoroughly established that sulfation can occur at the only free hydroxyl group of the 3,6-anhydrogalactose molecule (2-OH) in the presence of sulfates. The stability of

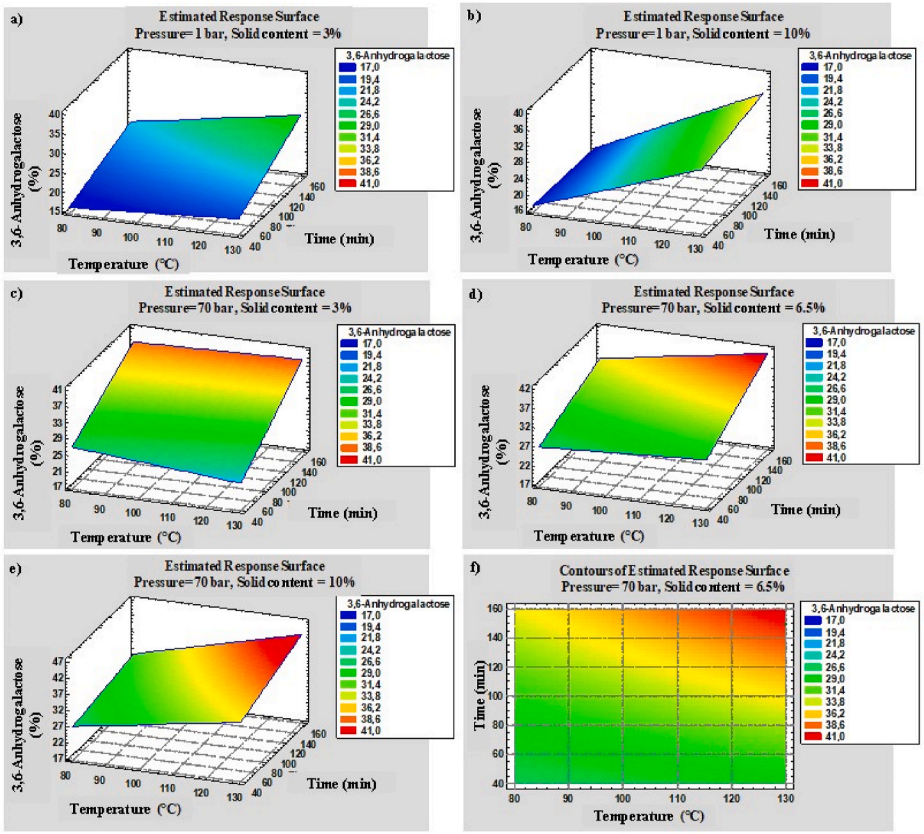


Fig. 6. 3D-response surface plots showing the variation of the 3,6-Anhydrogalactose content in function of the pressurized hot water extraction parameters.

the glycosidic bond at both the reducing and non-reducing ends is favored by the formation of 2-O sulfated 3,6-anhydrogalactose. The hydrolysis and the degradation of the 3,6-anhydrogalactose to 5-HMF become particularly difficult due to this chemical phenomenon (Ducatti, Colodi, Gonçalves, Duarte, & Noseda, 2011; Stevenson & Furneaux, 1991; Yang et al., 2009). This sulfation was also reported by Usov (1998), who used enzymatic hydrolysis to analyze the structure of red seaweed galactans. The formation of agarose blocks separated by short sulfated sections and by longer blocks tended to preserve the molecule from enzymatic hydrolysis, according to the author. This fact was confirmed to occur with the sulfation at position 6 of β-D-galacto-pyranose residues and to the presence of α-L-galactopyranose 6-sulfate instead of the initial 3,6-anhydrogalactose residues. Apart from the creation of more stable sulfated molecules, agaropectin, which has a more complex structure and a higher molecular weight (MW) than agarose (Mitsuiki, Mizuno, & Motoki, 1999), appeared to be less impacted by the autohydrolysis power of the pressurized hot water. As a result, a possible more premature fractionation of agarose in contrast to the higher MW agaropectin, as well as the presence of a stabilized 2-O sulfated 3,6-anhydrogalactose could be a logical explanation of the high 3,6-anhydrogalactose detected under more severe conditions.

3.6. *Effects of the PHWE parameters on the sulfate content of the residual agar*

The influence of the different factors on the content of sulfate groups in the extracted crude agar was illustrated using the quadratic model of the response surface methodology. The amount of sulfate was shown to have a substantial dependency on the reaction temperature ($p = 0.0005$ and $F = 22.38$) and the algae to water ratio ($p = 0.0124$ and $F = 8.63$) among the evaluated individual operating factors based on the analysis of variance. The quadratic effect of the algae to water ratio ($p = 0.0196$ and $F = 7.24$) was subsequently shown to have a significant impact on the charge of sulfate groups at a 5% tolerance level. This claim proved that the optimal points for the sulfate content response to change in relation to the variation of the algae to water ratio are located inside the experimental region. Therefore, the fact that the optimal zone was located remotely from the boundaries validated the suitability of the statistical approach. This assertion could be corroborated by the fact that the fitted model explained 76.9% (R^2) of the variation in sulfate charge observed, with an adj-R^2 of 56.22%.

Sulfate groups are commonly linked to the C-4 of D-Galactose and C-2 of L-Galactose on a molecular level (Andriamanantoanina, Chambat, & Rinaudo, 2007). Sulfated derivatives confer various nutraceutical and health benefits to marine polysaccharides, including but not limited to anticoagulant, antiviral, antioxidant, anticancer, and anti-immunomodulating effects (Jiménez-Escrig, Gómez-Ordóñez, & Rupérez, 2011). It was reported that the inclusion of sulfate groups in sulfated polysaccharides increased the reducing power and scavenging activity potentials of radicals (Ma et al., 2017). In contrast, several studies had reported a negative relationship between the sulfate content and the gelling behaviors for a specific agar from an industrial standpoint (Singh, Meena, & Kumar, 2009; Xiao et al., 2019). A high sulfate charge reduces the gel strength and corollary, has an unfavorable impact on gelling and melting temperatures, as discussed above.

The 3D response surface plots displaying the variation of the sulfate content in the recovered crude agar based on the PHWE parameters were shown in Fig. S7 (as supplementary material). It was clearly proven that increasing the solid concentration resulted in an increase in the sulfate content value in the extracted agar at a fixed reaction time (Fig. S7 a-e). Lower sulfate contents, on the other hand, were detected (Fig. S7 b-d-f) with a fixing of the algae percent concentration and a longer recovery time.

The best conditions for recovering highly sulfated crude agar seemed

to be an increase in solid consistency to a value of 6.5% (w: v) combined with a very short extraction time of 45 min. However, high process temperatures (T ≥ 120 °C) and pressures below 60 bar (Fig. S7 a) were required for this purpose.

The proposed second-order polynomial equation (Eq. (10)) was obtained based on the model integrating the different PHWE operating factors.

$$\textit{Sulfate content} = -9.47575 + 0.0703333 \times A - 0.0140097 \times B + 0.000634921 \times C + 1.98333 \times D - 0.128571 \times D^2 \quad (10)$$

where A is the temperature (°C), B is the Pressure (bar), C is the time (min), and D is the algae to water ratio (% w: v).

Globally, the mathematical model projecting the optimum conditions to recover crude agar with low sulfate derivatives as follows i.e. a-3% (w: v) algae concentration, a temperature set to 82.3 °C, an internal pressure of 70 bar, and an extraction time of 49 min (Table S2). As previously stated for the influence of the solid concentration on agar yield, a higher solid load would likely not allow a sufficient decrease of the surface tension around the algae biomass, resulting in a lower diffusivity of the pressurized hot water. Consequently, there may be less interactions between the algae waste and the water solvent. This phenomenon will tend to slow the desorption process of grafted anionic groups from the polysaccharide, including sulfate ions, and may result in a partial sulfate release from the galactose 2-sulfate.

In any case, adjusting the PHWE conditions could allow the recovery of agar with a wide range of sulfate charges (0–8%), according to the model. Therefore, the PHWE settings can be tailored based on the planned sulfate content and applications of the recovered residual crude agar.

Additionally, the sulfate content was found to be positively correlated with the severity of the treatment (Fig. 5-b), similarly to the content of 3,6-Anhydrogalactose.

A strong positive relationship ($r = 0.56$, $p = 0.0033$) between the severity factor and the sulfate content was also proven using Pearson linear correlation (Table S3), indicating that a high operating temperature has a noticeable impact on this molecule. Correlatively, the 3,6-anhydrogalactose ($r = 0.195$, $p > 5\%$) content exhibited a weak, but positive linear relationship with the sulfate content detected in the recovered agar.

On the other hand, Fig. S8 (as supplementary material) compared the 3D-response surface plots obtained for the gel strength and the sulfate content in the crude residual agar at varied extraction times ((t = 45 min; Fig. S8 a-b), (t = 150 min; Fig. S8 c-d)) and solid concentrations. A slight visual relationship was established between the lower agar gel strength value and the higher sulfate content detected at high temperature and prolonged extraction time, as expected. In the same trend, a negative correlation of the sulfate content appeared with increasing agar gel strength (Fig. 5 d). On one hand, these observations visibly corroborated the negative effect of the sulfate groups on the gel strength. On the other hand, it supported the hypothesis that the variations in sulfate content were not the primary cause of the crude agar hydrogel's weakening observed under more severe PHWE conditions. It might confirm the implication of the premature fractionation of the agarose compound, which is assumed to be caused by the autohydrolysis power of water under subcritical conditions.

Furthermore, the Pearson correlation demonstrated a negative linear relationship between the gelling behaviors and the recovered agar sulfate concentration. This relationship was weak with the gel strength ($r = -0.18$, $p > 5\%$) and the gelling temperature ($r = -0.29$, $p > 5\%$), but statistically significant and moderate ($r = -0.48$, $p = 0.0126$) with the melting temperature.

Nonetheless, the conversion of L-galactose-6-sulfate into 3,6-anhydrogalatose subsequent to an alkaline desulfation of algae might result in a beneficial increase of the content of the anhydrogalactose molecule. In this work, a clear correlation between low sulfate charges with higher content of 3,6-anhydrogalactose was not clearly observed, and may be plausibly explained by the molecular phenomena discussed above.

4. Conclusion

This study demonstrated that pressurized hot water extraction (PHWE) was a convenient tool for sustainably recovering and adding value to the remaining residual agar fraction. It was a need to develop an efficient and sustainable valorization method for the waste stream of *Gelidium sesquipedale* discarded by the algae industry. Coupled a Box-Behnken design with a response surface methodology was a suitable statistical approach. The impact of four key extraction parameters, namely the temperature, the extraction time, the concentration of algae, and the pressure on the yield, as well as various physicochemical aspects of the recovered agar was allowed to be assessed. It can be deducted that by varying the keys PHWE operating variables, agar with a wide range of specific properties and applications might be recovered. The focus of this study was on crude agar as a marine hydrocolloid with fascinating technological features for the food, nutraceutical, and pharmaceutical industry as well as the bioplastic and packaging sector. To optimally extract or degrade the non-sulfated carbohydrates (example of cellulose) and bioactive compounds i.e. protein, antioxidants, polyphenols, minerals, etc., remaining in the algae industry waste stream, more severe PHWE conditions were generally required. The mild conditions used, enabled the recovery of an exploitable amount of agar without markedly compromising the other functional molecules in the algae residue (unpublished data). Aside from the added value potential, the faster recovery process demonstrated in this study using mild conditions of temperature and pressure was the first step in a subsequent integral cascade valorization of the discarded algae waste stream. It will reduce the high gelation commonly encountered when processing marine macroalgae waste, which makes its biorefining technically challenging. Overall, the PHWE process offered a variety of technological and long-term benefits over traditional extraction methods.

Author agreement statement

We the undersigned declare that this manuscript entitled "**Pressurized Hot Water-Assisted Recovery of Crude Residual Agar From a Never-Dried Algae Industry Waste stream: A Box-Behnken Design Approach"** is original, has not been published before and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed.

We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We understand that the Corresponding Author is the sole contact for the Editorial process. He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs Signed by all authors as follows: Cherif Ibrahima Khalil Diop, Ester Trigueros, Maria-Teresa Sanz, Sagrario Beltran, Javier García–Tojal.

Declaration of conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by the European Commission, Horizon 2020 program through the Marie-Curie Individual Fellowship (H2020-MSCA-IF-2019), with regards to the ALGWAS-BIOR project (Grant agreement number 898804). This work was also supported by the Junta de Castilla y León (JCyL) and the European Regional Development Fund

(ERDF) [grant numbers BU301P18 and BU050P20]; as well as the Agencia Estatal de Investigación [grant number AEI /10.13039/501100011033].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2022.107664.

References

Ahmad, R., Surif, M., Ramli, N., Yahya, N., Nor, A. R. M., & Bekbayeva, L. (2011). A preliminary study on the agar content and agar gel strength of gracilaria manilaensis using different agar extraction processes. *World Applied Sciences Journal, 15*(2), 184–188.

Alvarez-Rivera, G., Bueno, M., Ballesteros-Vivas, D., Mendiola, J. A., & Ibañez, E. (2019). Pressurized liquid extraction. *Liquid-Phase Extraction*, 375–398. https://doi.org/10.1016/B978-0-12-816911-7.00013-X

Andriamanantoanina, H., Chambat, G., & Rinaudo, M. (2007). Fractionation of extracted Madagascan Gracilaria corticata polysaccharides: Structure and properties. *Carbohydrate Polymers, 68*(1), 77–88. https://doi.org/10.1016/j.carbpol.2006.07.023

Armisen, R. (1995). World-wide use and importance of Gracilaria. *Journal of Applied Phycology, 7*(3), 231–243. https://doi.org/10.1007/BF00003998

Armisen, R., & Gaiatas, F. (2009). Agar. In *Handbook of hydrocolloids* (pp. 82–107). Woodhead Publishing.

Arvizu-Higuera, D. L., Rodríguez-Montesinos, Y. E., Murillo-Álvarez, J. I., Muñoz-Ochoa, M., & Hernández-Carmona, G. (2008). Effect of alkali treatment time and extraction time on agar from Gracilaria vermiculophylla. *Journal of Applied Phycology, 20*(5), 515–519. https://doi.org/10.1007/s10811-007-9258-4

Aslan, N., & Cebeci, Y. (2007). Application of Box-Behnken design and response surface methodology for modeling of some Turkish coals. *Fuel, 86*(1–2), 90–97. https://doi.org/10.1016/j.fuel.2006.06.010

Balkan, G., Coban, B., & Güven, K. C. (2005). Fractionation of agarose and Gracilaria verrucosa agar and comparison of their IR spectra with different agar. *ACTA Pharmaceutica Sciencia, 47*(2).

Banerjee, S., & Bhattacharya, S. (2012). Food gels: Gelling process and new applications. *Critical Reviews in Food Science and Nutrition, 52*(4), 334–346. https://doi.org/10.1080/10408398.2010.500234

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology, 37*(8), 911–917.

Chandler, K., Deng, F., Dillow, A. K., Liotta, C. L., & Eckert, C. A. (1997). Alkylation reactions in near-critical water in the absence of acid catalysts. *Industrial & Engineering Chemistry Research, 36*(12), 5175–5179. https://doi.org/10.1021/ie9702688

Diop, C. I. K., Lavoie, J. M., & Huneault, M. A. (2015). Structural changes of Salix miyabeana cellulose fibres during dilute-acid steam explosion: Impact of reaction temperature and retention time. *Carbohydrate Polymers, 119*, 8–17. https://doi.org/10.1016/j.carbpol.2014.11.031

Ducatti, D. R. B., Colodi, F. G., Gonçalves, A. G., Duarte, M. E. R., & Noseda, M. D. (2011). Production of agaro- and- carraoligosaccharides by partial acid hydrolysis of galactans. *Revista Brasileira de Farmacognosia, 21*(2), 296–304. https://doi.org/10.1590/S0102-695X2011005000080

Elboughdiri, N. (2018). Effect of time, solvent-solid ratio, ethanol concentration and temperature on extraction yield of phenolic compounds from olive leaves. *Engineering, Technology & Applied Science Research, 8*(2), 2805–2808. https://doi.org/10.48084/etasr.1983

Fiormarkets. (2019). *Global agar market growth 2019-2024*. Retrieved from https://www.fiormarkets.com/report/global-agar-market-growth-2019-2024-368665.html. (Accessed 14 September 2021).

Freile-Pelegrín, Y., & Robledo, D. (1997). Effects of season on the agar content and chemical characteristics of Gracilaria cornea from Yucatan, Mexico. *Botanica Marina, 40*(4), 285–290. https://doi.org/10.1515/botm.1997.40.1-6.285

García-González, C. A., Alnaief, M., & Smirnova, I. (2011). Polysaccharide-based aerogels - promising biodegradable carriers for drug delivery systems. *Carbohydrate Polymers, 86*(4), 1425–1438. https://doi.org/10.1016/j.carbpol.2011.06.066

Gomes-Dias, J. S., Romaní, A., Teixeira, J. A., & Rocha, C. M. R. (2020). Valorization of seaweed carbohydrates: Autohydrolysis as a selective and sustainable pretreatment. *ACS Sustainable Chemistry & Engineering, 8*(46), 17143–17153. https://doi.org/10.1021/acssuschemeng.0c05396

Jiménez-Escrig, A., Gómez-Ordóñez, E., & Rupérez, P. (2011). Seaweed as a source of novel nutraceuticals: Sulfated polysaccharides and peptides. *Advances in Food & Nutrition Research, 64*, 325–337. https://doi.org/10.1016/B978-0-12-387669-0.00026-0

Kondaveeti, S., Prasad, K., & Siddhanta, A. K. (2013). Functional modification of agarose: A facile synthesis of a fluorescent agarose-tryptophan based hydrogel. *Carbohydrate Polymers, 97*(1), 165–171. https://doi.org/10.1016/j.carbpol.2013.04.034

Kumar, S., Boro, J. C., Ray, D., Mukherjee, A., & Dutta, J. (2019). Bionanocomposite films of agar incorporated with ZnO nanoparticles as an active packaging material for shelf life extension of green grape. *Heliyon, 5*(6), Article e01867.

Lee, W. K., Namasivayam, P., & Ho, C. L. (2014). Effects of sulfate starvation on agar polysaccharides of Gracilaria species (Gracilariaceae, Rhodophyta) from Morib, Malaysia. *Journal of Applied Phycology, 26*(4), 1791–1799. https://doi.org/10.1007/s10811-013-0231-0

Madhavan, S., & Abirami. (2015). A review on hydrocolloids-agar and alginate. *Journal of Pharmaceutical Sciences and Research, 7*(10), 909–912.

Mariod, A. A., & Adam, H. F. (2013). Review: Gelatin, source, extraction and industrial applications. *Acta Scientiarum Polonorum, Technologia Alimentaria, 12*(2), 135–147.

Martínez-Sanz, M., Gómez-Mascaraque, L. G., Ballester, A. R., Martínez-Abad, A., Brodkorb, A., & López-Rubio, A. (2019). Production of unpurified agar-based extracts from red seaweed Gelidium sesquipedale by means of simplified extraction protocols. *Algal Research, 38*(January), 101420. https://doi.org/10.1016/j.algal.2019.101420

Ma, X. T., Sun, X. Y., Yu, K., Gui, B. S., Gui, Q., & Ouyang, J. M. (2017). Effect of content of sulfate groups in seaweed polysaccharides on antioxidant activity and repair effect of subcellular organelles in injured HK-2 cells. *Oxidative Medicine and Cellular Longevity*. https://doi.org/10.1155/2017/2542950, 2017.

Matos, G. S., Pereira, S. G., Genisheva, Z. A., Gomes, A. M., Teixeira, J. A., & Rocha, C. M. R. (2021). Advances in extraction methods to recover added-value compounds from seaweeds: Sustainability and functionality. *Foods, 10*(3). https://doi.org/10.3390/foods10030516

Matsuhiro, B., & Urzúa, C. C. (1990). Agars from gracilaria chilensis (Gracilariales). *Journal of Applied Phycology, 2*(3), 273–279. https://doi.org/10.1007/BF02179785

Meena, R., Siddhanta, A. K., Prasad, K., Ramavat, B. K., Eswaran, K., Thiruppathi, S., et al. (2007). Preparation, characterization and benchmarking of agarose from Gracilaria dura of Indian waters. *Carbohydrate Polymers, 69*(1), 179–188. https://doi.org/10.1016/j.carbpol.2006.09.020

Mitsuiki, M., Mizuno, A., & Motoki, M. (1999). Determination of molecular weight of agars and effect of the molecular weight on the glass transition. *Journal of Agricultural and Food Chemistry, 47*(2), 473–478. https://doi.org/10.1021/jf980713p

Mouradi-Givernaud, A., Hassani, L. A., Givernaud, T., Lemoine, Y., & Benharbet, O. (1999). Biology and agar composition of Gelidium sesquipedale harvested along the Atlantic coast of Morocco. *Hydrobiologia, 398–399*, 391–395. https://doi.org/10.1007/978-94-011-4449-0_46

Murakami, K., Yamaguchi, Y., Sugawa-Katayama, Y., & Katayama, M. (2016). Effect of water depth on seasonal variation in the chemical composition of Akamoku, *Sargassum horneri* (Turner) C. Agardh. *Natural Resources, 7*, 147–156. https://doi.org/10.4236/nr.2016.74015, 04.

Öğretmen, Ö. Y., & Duyar, H. A. (2018). The effect of different extraction methods and pre-treatments on agar yield and physico-chemical properties of Gelidium latifolium (Gelidiaceae, Rhodophyta) from Sinop Peninsula Coast of Black Sea, Turkey. *Journal of Applied Phycology, 30*(2), 1355–1360. https://doi.org/10.1007/s10811-017-1292-2

Pereira-Pacheco, F., Robledo, D., Rodríguez-Carvajal, L., & Freile-Pelegrín, Y. (2007). Optimization of native agar extraction from Hydropuntia cornea from Yucatán, México. *Bioresource Technology, 98*(6), 1278–1284. https://doi.org/10.1016/j.biortech.2006.05.016

Retrieved from Plaza, M., & Turner, C. (2015). Pressurized hot water extraction of bioactives. *TrAC - Trends in Analytical Chemistry, 71*, 39–54 https://doi.org/10.1016/j.trac.2015.02.022 http://linkinghub.elsevier.com/retrieve/pii/S0960077902004344.

Quemener, B., & Lahaye, M. (1998). Comparative analysis of sulfated galactans from red algae by reductive hydrolysis and mild methanolysis coupled to two different HPLC techniques. *Journal of Applied Phycology, 10*(1), 75–81.

Rajasulochana, N., & Gunasekaran, S. (2009). Fourier transform infrared spectral analysis of agars of Gelidium micropterum and Gracilaria fergusonii. *Asian Journal of Chemistry, 21*(6), 4543.

Rhein-Knudsen, N., Ale, M. T., & Meyer, A. S. (2015). Seaweed hydrocolloid production: An update on enzyme assisted extraction and modification technologies. *Marine Drugs, 13*(6), 3340–3359. https://doi.org/10.3390/md13063340

Rochas, C., Lahaye, M., & Yaphe, W. (1986). Sulfate content of carrageenan and agar determined by infrared spectroscopy. *Botanica Marina*, 335–340.

Sanchez-Cardozo, J., Quintanilla-Carvajal, M. X., Ruiz-Pardo, R., & Acosta-González, A. (2019). Evaluating gelling-agent mixtures as potential substitutes for bacteriological agar: An approach by mixture design. *DYNA (Colombia), 86*(208), 171–176. https://doi.org/10.15446/dyna.v86n208.72964

Selvalakshmi, S., Mathavan, T., Selvasekarapandian, S., & Premalatha, M. (2017). Study on NH4I composition effect in agar–agar-based biopolymer electrolyte. *Ionics, 23*(10), 2791–2797.

Shahnaz, L., Shehnaz, H., & Haider, A. (2019). Fourier transform infrared (FT-IR) spectroscopic investigations of four agarophytes from northern Arabian sea. *Bangladesh Journal of Botany, 48*(4), 925–932.

Singh, T., Meena, R., & Kumar, A. (2009). Effect of sodium sulfate on the gelling behavior of agarose and water structure inside the gel networks. *Journal of Physical Chemistry B, 113*(8), 2519–2525. https://doi.org/10.1021/jp809294p

Singh, T., Trivedi, T. J., & Kumar, A. (2010). Dissolution, regeneration and ion-gel formation of agarose in room-temperature ionic liquids. *Green Chemistry, 12*(6), 1029–1035.

Sousa, A. M. M., Alves, V. D., Morais, S., Delerue-Matos, C., & Gonçalves, M. P. (2010). Agar extraction from integrated multitrophic aquacultured Gracilaria vermiculophylla: Evaluation of a microwave-assisted process using response surface methodology. *Bioresource Technology, 101*(9), 3258–3267. https://doi.org/10.1016/j.biortech.2009.12.061

Stevenson, T. T., & Furneaux, R. H. (1991). Chemical methods for the analysis of sulphated galactans from red algae. *Carbohydrate Research, 210*(C), 277–298. https://doi.org/10.1016/0008-6215(91)80129-B

Suzuki, H., Sawai, Y., & Takada, M. (2001). The effect of Apparent molecular weight and components of agar on gel formation. *Food Science and Technology Research, 7*(4), 280–284. https://doi.org/10.3136/fstr.7.280

Tako, M., & Nakamura, S. (1988). Gelation mechanism of agarose. *Carbohydrate Research, 180*(2), 277–284.

Trigueros, E. (2021). *Valorization of industrial solid residue from macroalgae by emerging technologies*.

Trigueros, E., Sanz, M. T., Alonso-Riaño, P., Beltrán, S., Ramos, C., & Melgosa, R. (2021). Recovery of the protein fraction with high antioxidant activity from red seaweed industrial solid residue after agar extraction by subcritical water treatment. *Journal of Applied Phycology, 33*(2), 1181–1194. https://doi.org/10.1007/s10811-020-02349-0

Trivedi, T. J., Rao, K. S., & Kumar, A. (2014). Facile preparation of agarose–chitosan hybrid materials and nanocomposite ionogels using an ionic liquid via dissolution, regeneration and sol–gel transition. *Green Chemistry, 16*(1), 320–330.

Usov, A. I. (1998). Structural analysis of red seaweed galactans of agar and carrageenan groups. *Food Hydrocolloids, 12*(3), 301–308.

Xiao, Q., Weng, H., Ni, H., Hong, Q., Lin, K., & Xiao, A. (2019). Physicochemical and gel properties of agar extracted by enzyme and enzyme-assisted methods. *Food Hydrocolloids, 87*(April 2018), 530–540. https://doi.org/10.1016/j.foodhyd.2018.08.041

Xie, X. T., Zhang, X., Liu, Y., Chen, X. Q., & Cheong, K. L. (2020). Quantification of 3,6-anhydro-galactose in red seaweed polysaccharides and their potential skin-whitening activity. *3 Biotech, 10*(4), 1–9. https://doi.org/10.1007/s13205-020-02175-8

Yang, B., Yu, G., Zhao, X., Jiao, G., Ren, S., & Chai, W. (2009). Mechanism of mild acid hydrolysis of galactan polysaccharides with highly ordered disaccharide repeats leading to a complete series of exclusively odd-numbered oligosaccharides. *FEBS Journal, 276*(7), 2125–2137. https://doi.org/10.1111/j.1742-4658.2009.06947.x

Yarnpakdee, S., Benjakul, S., & Kingwascharapong, P. (2015). Physico-chemical and gel properties of agar from Gracilaria tenuistipitata from the lake of Songkhla, Thailand. *Food Hydrocolloids, 51*, 217–226. https://doi.org/10.1016/j.foodhyd.2015.05.004

Yousefi, M. K., Islami, H. R., & Filizadeh, Y. (2013). Effect of extraction process on agar properties of gracilaria corticata (rhodophyta) collected from the Persian gulf. *Phycologia, 52*(6), 481–487. https://doi.org/10.2216/13-165.1

Zarrintaj, P., Manouchehri, S., Ahmadi, Z., Saeb, M. R., Urbanska, A. M., Kaplan, D. L., et al. (2018). Agarose-based biomaterials for tissue engineering. *Carbohydrate Polymers, 187*, 66–84. https://doi.org/10.1016/j.carbpol.2018.01.060. October 2017.

Zhang, Y., Fu, X., Duan, D., Xu, J., & Gao, X. (2019). Preparation and characterization of agar, agarose, and agaropectin from the red alga Ahnfeltia plicata. *Journal of Oceanology and Limnology, 37*(3), 815–824.