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Ultrasound-Assisted Extraction of Native Agar from *Gracilaria corticata*: Optimization by Response Surface Methodology and Evaluation of Its Physicochemical, Rheological, and Cytotoxic Properties

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Abstract

Gracilaria corticata, an abundant red seaweed rich in agar, is a valuable resource for sustainable hydrocolloid production. High-yield native crude agar with preserved structural and rheological properties was extracted using ultrasound pre-treatment followed by hot-water extraction (95 °C, 2 h) without alkali pre-treatment. Response surface methodology with central composite design predicted 32.87 min and 61.58% amplitude as optimum conditions, giving a predicted yield of 28.66% (w/w). Acoustic cavitation-induced cell wall disruption significantly enhanced agar yield (validated: 29.31 ± 0.70%) versus conventional hot-water extraction (14.01 ± 0.43%). Commercial agar (C-AP) had higher gel strength. Ultrasound-assisted (US-AP) and conventionally extracted agar (CE-AP) had higher sulfate and lower 3,6-anhydrogalactose contents ($p < 0.05$). C-AG showed higher gel strength. FTIR confirmed agar functional groups in all samples, indicating preserved functional groups. Syneresis remained below 2%. Microstructure showed a more porous network in CE-AG and US-AG than in C-AG. Rheology demonstrated thermo-reversible gel behaviour, with gelation onset at 38–46 °C. Extracted agars were cytocompatible with BJ fibroblasts after 48 h, supporting soft-gel applications without chemical modification. Low gel-strength seaweeds such as *G. corticata* can be utilized for high-yield agar extraction via ultrasonication.

Keywords: *Gracilaria corticata*; agar; ultrasonication; RSM; rheology; cytotoxicity

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1. Introduction

Red seaweeds are of considerable commercial importance due to the presence of valuable galactose-rich structural polysaccharide agar, widely used in the food, biotechnological and pharmaceutical industries due to its unique gelling and physicochemical properties [1]. This highly valued hydrocolloid consists of two main components, agarose and agaropectin. Agarose is a linear polysaccharide composed of alternating β-D-galactopyranose and 3,6-anhydro-α-L-galactopyranose units and is mainly responsible for the gelling properties

of agar. Agaropectin is a heterogeneous mixture of smaller molecules that possesses a structure similar to agarose but may also contain sulfate ester groups and other substituents, including methoxyl groups and pyruvic acid, functioning analogously to hemicellulose in terrestrial plants, acting as a matrix polysaccharide [2,3].

Agar is primarily extracted from red seaweeds of the genera *Gracilaria, Gelidium, Pterocladia, and Hydropuntia*, with food-grade agar from *Gracilaria* accounting for nearly 80% of the global supply [4]. Agars extracted from *Gracilaria* species generally exhibit lower gel strength attributed to the higher sulfate content [5]. Agar yield and gelling properties are determined by extraction methods, storage conditions, environmental factors, nutrient availability, biotic interactions, and the physiological state of seaweeds [6]. *G. corticata,* belonging to the phylum Rhodophyta, is one of the common macroalgae along the Indian coast, found predominantly in the intertidal rock pools and lower littoral zone as a submerged population [7]. *G. corticata* is rich in agar compared to the other *Gracilaria* species, and this species can even resist extreme environmental conditions such as varying salinity and ecological changes, and withstand high temperatures; therefore, it is considered for the commercial production of agar and other valuable components in the coastal regions [8].

Despite the natural abundance and industrial potential of *Gracilaria* species for agar production, a prevailing approach in the agar production industry is the use of gel strength as the sole evaluation criterion. This has led to heavy dependence on high gel strength-producing red seaweed species like *Gelidium*, as well as the enhancement of gel strength in inherently low gel strength species such as *Gracilaria* through intensive alkali pre-treatment [9]. Although alkaline pre-treatment improves agar gel strength by eliminating the sulfate groups, it involves heavy chemical usage and can alter polysaccharide structure through desulfation and structural rearrangement, ultimately affecting agar yield [9]. This prevailing practice of determining agar quality based on high gel strength has underscored the need for extraction methods that provide higher yield with diverse functional properties. Softer gel texture, reduced syneresis, and improved processability make agar particularly suitable [10].

So, there is a need for extraction strategies that improve agar yield for food-grade applications by employing physical assistance techniques while reducing reliance on harsh chemical treatments. In this study, the main objective is to produce native agar from *G. corticata* using the conventional hot water extraction method and compare it with ultrasonically pre-treated seaweed to evaluate improvements in extraction efficiency. Ultrasound-assisted extraction (UAE) is employed for hydrocolloids because acoustic cavitation disrupts algal cell walls, enhancing solvent penetration and accelerating mass transfer, thereby improving yield and efficiency while reducing time and energy consumption [11]. The optimization of ultrasound pre-treatment is done using Response Surface Methodology (RSM). RSM is a statistical tool used to evaluate the effects of independent variables on response parameters and to identify optimal conditions through systematic experimental design, modelling, and analysis, commonly employing central composite design (CCD) to reduce experimental error and the number of trials required [12].

While UAE and RSM provide the methodological framework for this optimization, the scientific significance of this contribution lies primarily in the material characterized and the properties it yields. *G. corticata* from the Someshwara coast has not previously been assessed for agar content or quality, and given that polysaccharide composition in agarophytes is known to vary with local environmental conditions [13], this population represents a distinct, uncharacterized biochemical resource. The agar in this study was extracted without alkali pre-treatment, and characterizing this native form therefore provides a compositional baseline for the species and supports lower-input extraction approaches. To the best of our knowledge, no prior studies report the extraction, optimization and char-

acterization of native agar from this population, and research on seaweeds from this region more broadly remains limited despite its documented diversity. Hence, the present study aimed to optimize ultrasonication time and amplitude via Response Surface Methodology (RSM) for the yield of crude native agar and the physicochemical, rheological and cytotoxic properties of the resulting agar were then characterized separately and compared with those of conventionally extracted and commercial agar.

2. Materials and Methods

2.1. Raw Materials and Chemicals

G. corticata was collected from the coast of Someshwara (12.7949° N, 74.8469° E), Karnataka, India, during September 2025. The collected seaweed was taxonomically identified and authenticated by a taxonomist and a voucher specimen RH005 was deposited at the Laboratory of Applied Biology, St Aloysius (Deemed to be University), Mangaluru, for future reference. The collected seaweed was manually washed with running tap water within 1 h of collection to remove sand and other impurities. The cleaned seaweed was spread evenly on aluminium trays and dried in a hot air oven at 60 °C for 24 h. The dried samples were then ground into fine powder and stored in vacuum-sealed bags at 4 °C until further use. Commercial agar powder was obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India).

All chemicals used were of analytical grade. Barium chloride was procured from Ajax Finechem Pty. Ltd. (Taren Point, New South Wales, Australia), and potassium sulfate was obtained from KemAus (Cherrybrook, New South Wales, Australia). All other chemicals were acquired from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

2.2. Proximate Analysis of G. corticata

The proximate composition of *G. corticata* was determined according to standard methods of AOAC [14], following analytical methods 928.08, 960.39, 920.153, and 950.46 for moisture, ash, fat, and protein, respectively. Total carbohydrate content was calculated by difference, subtracting the sum of moisture, ash, crude fat, and crude protein from 100. All analyses were performed in triplicate, and results were expressed on a wet weight basis.

2.3. Agar Extraction

2.3.1. Conventional Agar Extraction Method

Hot water agar extraction was carried out with slight modifications to the procedure described by Deepika et al. [12]. The dried seaweed was subjected to hot-water extraction at a solid–liquid ratio of 1:30 (w/v) in a water bath maintained at 95 °C for 2 h. The hot extract was filtered through muslin cloth, and the filtrate was allowed to cool and gel at room temperature for 12 h. After gel formation, the agar gel was cut into strips and frozen at −20 °C for 48 h. The frozen gel was subsequently thawed in tap water to obtain a hydrogel, which was manually pressed to remove excess water. The resulting gel was dried in a hot air oven at 60 °C to obtain the final dried agar extract, which was used for further analyses.

The dried agar samples were weighed, and the crude agar yield was calculated using the following Equation (1):

$$\text{Crude agar yield (\% w/w)} = \frac{\text{Weight of dried agar (g)}}{\text{Weight of initial dried seaweed (g)}} \times 100 \quad (1)$$

2.3.2. Ultrasound-Assisted Agar Extraction Method

A suspension of dried seaweed was prepared in distilled water at a solid–liquid ratio of 1:30 (w/v) and subjected to ultrasonication using a probe-type ultrasonic processor (Sonics,

Model VC750, Sonic and Materials, Inc., Newtown, CT, USA) operating at a frequency of 20 kHz ± 50 Hz with a maximum power output of 750 W and equipped with a 13 mm diameter probe. Different extraction times and ultrasound probe powers were evaluated as specified by the RSM design. Ultrasonication was performed at 20%, 50%, and 80% amplitudes for 15, 30, and 45 min using a fixed pulse mode of 5 s ON and 5 s OFF. An ice bath was used during sonication to maintain the sample temperature below 10 °C. After ultrasonication pre-treatment, agar extraction was carried out using the previously described conventional method.

2.4. Optimization of Ultrasound-Assisted Agar Extraction

Response surface methodology (RSM) was employed to evaluate the combined effects of ultrasonication time and ultrasound amplitude on agar yield during ultrasound-assisted extraction. The statistical software Design-Expert (version 13.0), Stat-Ease Inc., Minneapolis, MN, USA, was used to set up the experimental design and analyse data. The analysis aimed to determine the influence and interaction of process variables on agar recovery and to develop a predictive model describing the extraction behaviour. Two independent variables were selected for experimental design: ultrasonication time (X_1, min) and ultrasound amplitude (X_2, %). Experimental runs were designed using a central composite design (CCD) with a face-centred configuration. A face-centred CCD ($\alpha = 1$) was selected to keep all runs within the experimentally feasible ranges (15–45 min; 20–80% amplitude) using three levels per factor and 13 runs, sufficient to fit the six-term quadratic model (7 residual degrees of freedom). Pure error was estimated from five centre-point replicates. All experiments were conducted in randomized order to minimize systematic bias and improve data reliability. Experimental data were fitted to a second-order polynomial model using multiple regression analysis. The quadratic model used to describe the agar yield is expressed by the following Equation (2):

$$Y = \beta_0 + \beta_1 X^1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 \quad (2)$$

where Y represents agar yield (%), β_0 is the intercept, β_1 and β_2 are linear coefficients, β_{12} is the interaction coefficient, and β_{11} and β_{22} are quadratic coefficients.

The statistical significance of the model and regression coefficients was evaluated using analysis of variance (ANOVA) at a confidence level of $p < 0.05$. Model adequacy was assessed through determination coefficients (R^2 and adjusted R^2), coefficient of variation, and lack-of-fit testing. Three-dimensional response surface and contour plots were generated to visualize relationships between process variables and agar yield. Numerical and graphical optimization procedures were applied to determine the optimum level of independent variables. The optimum level of the response variables was determined by desirability with a scale ranging from 0 to 1.0. Different desirability functions can be obtained by keeping the dependent variables to be maximized, minimized or assigned a target value. The overall desirability was calculated as the geometric average of the partial desirability functions of individual components. Finally, the optimized levels of factors were validated experimentally.

2.5. Characterization of Extracted Agar

Samples are coded by origin as C (commercial agar), CE (conventionally extracted agar) and US (ultrasound-assisted extracted agar), and by the form analysed as AP (powder), AG (gel) and AS (solution).

2.5.1. Chemical Composition of Extracted Agar

Ash and moisture contents were determined following AOAC methods 960.39 and 928.08, respectively [14]. Protein content was determined by the Lowry method [15], with absorbance measured at 750 nm against a BSA standard curve, and expressed as mg BSA equivalents/g dry weight, to quantify co-extracted proteinaceous impurities.

3,6-Anhydrogalactose (3,6-AG) content was determined by the resorcinol-acetal method [16], with absorbance measured at 555 nm against a fructose standard curve and expressed as % wet weight. Sulfate content was determined turbidimetrically using the $BaCl_2$-gelatin method [17], with absorbance measured at 360 nm against a K_2SO_4 standard curve and expressed as % wet weight.

2.5.2. Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)

ATR-FTIR of dried agar samples was carried out using an FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with an ATR module. The spectra were recorded in the range of 4000–500 cm^{-1}. All spectra were normalized before interpretation.

2.5.3. Determination of Gel Properties

Determination of pH

The pH of the agar samples was determined according to AOAC International method 943.02 [14]. Briefly, 1 g of agar from the prepared 1.5% gel was dispersed in 10 mL of distilled water and mixed thoroughly to obtain a homogeneous solution. The pH was measured using a digital pH meter (Model pH 700, Eutech Instruments, Singapore). All measurements were performed in triplicate, and the results were expressed as mean pH values [18].

Determination of Colour

The colour characteristics of agar samples were analysed using a colourimeter (ColourFlex, HunterLab, Reston, VA, USA) following the CIE Lab* colour space. In this system, L* indicates lightness, while a* and b* correspond to red-green and yellow-blue colour components, respectively. To assess colour variation relative to the reference, total colour difference (ΔE*) and chroma difference (ΔC*) values were determined. The total colour difference (ΔE*) was calculated using the following Equation (3),

$$\Delta E^* = \sqrt{(L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (3)$$

where ΔL*, Δa*, and Δb* represent the differences between the colour coordinates of the sample and those of the white reference standard [5].

The chroma difference (ΔC*) was calculated using the following Equation (4):

$$\Delta C^* = C^* \text{ sample} - C^* \text{standard} \quad (4)$$

where $C^* = \sqrt{(a^*)^2 + (b^*)^2}$.

Gel Strength and Syneresis

Gel strength of 1.5% (w/v) agar gels was determined following the method described by Suzuki et al. [19] with minor modifications. Measurements were performed at 25–30 °C using a texture analyser (TA-XTplus C, Stable Micro Systems, Surrey, UK) equipped with a 5 kg load cell. A cylindrical probe (1/2″ diameter; Stable Micro Systems) was used, and the maximum force (g) required for the probe to penetrate 5 mm into the agar gel was recorded. Gel strength was calculated and expressed as g/cm^2.

Syneresis was determined following Banerjee et al. [20] with slight modifications. Agar solutions (1.5%, w/v; 30 mL) were poured into 50 mL graduated centrifuge tubes, and the initial weight of the tube with the sample was recorded (m_1). Gels were allowed to set at ambient temperature, stored at 4 °C for 12 h, and equilibrated at room temperature for 2 h. The samples were then centrifuged (2150× g, 10 min, 25 °C), the expelled liquid was decanted, and the tubes were reweighed (m_2). Syneresis was calculated as the percentage of liquid released relative to the initial gel weight using Equation (5).

$$\text{Syneresis (\%)} = \frac{m_1 - m_2}{m_1} \times 100 \quad (5)$$

Melting and Gelling Temperatures

Melting and gelling temperatures of agar gel were determined by the tube inversion method with slight modifications [21]. Agar gel (1.5%) was prepared in test tubes and allowed to set at room temperature, and a glass bead (3 mm) was placed on the surface. For melting temperature, the tubes were heated in a boiling water bath, and the temperature at which the bead passed through the gel was recorded. For gelling temperature, the samples were cooled, and the tubes were tilted at intervals; the temperature at which the bead stopped moving was noted using a thermometer.

Texture Profile Analysis

Texture profile analysis (TPA) of 1.5% (w/v) agar gel was performed using a texture analyser (Model TA-XT plus, Stable Micro Systems, Surrey, UK) with a load cell of 50 kg and a cylindrical aluminium probe (diameter 40 mm), and tests were run with two compression cycles. Gel samples (2.5 cm height × 2.5 cm diameter) were equilibrated at room temperature (26–28 °C) and placed on the flat platform of the instrument. The test parameters were set as probe type P/40, pre-test, test, and post-test speeds of 5.0 mm/s, deformation of 30%, and a residence time of 5 s. Textural parameters including hardness, springiness, cohesiveness, and chewiness were calculated from the force-time curves [22].

The microstructure of the selected agar gels (1.5%) was examined using scanning electron microscopy (SEM; SU3900, Hitachi, Tokyo, Japan). Agar gels prepared at 1.5% (w/v) were fixed in 2.5% (v/v) glutaraldehyde prepared in 0.2 mol/L phosphate buffer (pH 7.2) for 3 h at room temperature (25–27 °C). The samples were then rinsed with distilled water and dehydrated through a graded ethanol series (25, 50, 70, 80, 90, and 100%, v/v). Following dehydration, samples were critical-point dried using CO_2 as the transition fluid, mounted on aluminium stubs, and sputter-coated with gold prior to imaging. Cross-sectional microstructures were subsequently observed using SEM operated at an accelerating voltage of 10.0 kV.

2.5.4. Oscillatory Rheology

Agar solutions (1.5%, w/v) were prepared in Milli-Q water by heating at 95 °C without the addition of ions. Rheological measurements were performed using a rotational rheometer (Thermo Fisher Scientific HAAKE MARS 40, Karlsruhe, Germany) equipped with a serrated parallel-plate geometry (35 mm diameter, 1.0 mm gap). Prior to measurements, strain sweep tests were conducted to determine the linear viscoelastic region. Frequency sweep analysis was performed following the method of Norziah et al. [23] with slight modifications. The hot agar solution was loaded onto the preheated plate (95 °C) and cooled to 25 °C to allow gel formation. After equilibration for 3 min, measurements were carried out at 25 °C over a frequency range of 0.01–10 Hz. Temperature sweep tests were conducted at a constant frequency of 1 Hz to evaluate viscoelastic behaviour during gelation. Samples were cooled from 95 to 20 °C at a rate of 3 °C/min, followed by heating from 25

to 95 °C at the same rate. To prevent dehydration, the sample edges were sealed with paraffin oil. The storage modulus (G′) and loss modulus (G″) were recorded as a function of temperature [22].

2.5.5. Cytotoxicity

The cytotoxicity of the agar samples was evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay against BJ human dermal fibroblast cells (BJ-CRL2522, ATCC, Manassas, VA, USA). The cells were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution at 37 °C in a humidified 5% CO_2 incubator. BJ cells were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h prior to treatment. The cells were then exposed to different concentrations of the sample (31.25–500 µg/mL) for 24 and 48 h. After incubation, 100 µL of MTT reagent was added to each well and incubated for an additional 4 h. The formed formazan crystals were dissolved using solubilization solution, and absorbance was measured at 570 nm using a FLUOstar® Omega microplate reader (BMG Labtech, GmbH, Ortenberg, Germany). Cell viability was calculated relative to untreated control cells to determine cytotoxicity [24].

2.6. Statistical Analysis

All experiments and analyses were performed in triplicate, and the results are presented as mean ± standard deviation (SD) using descriptive statistics. Statistical analyses, including one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) for post-hoc mean comparisons, were carried out using SPSS software (Version 27.0, IBM Corp., Armonk, NY, USA). Statistical significance was determined at $p < 0.05$.

3. Results

3.1. Proximate Analysis of G. corticata

Proximate analysis of *G. corticata* was conducted as a preliminary assessment of its chemical composition to support the subsequent extraction studies (Table 1). The moisture content was 7.06%, indicating an appropriate drying process. The protein content was 15.97% and the lipid content was low (0.86%). The ash content was 12.83%, indicating the presence of inorganic matter in the seaweed. Carbohydrate was the major component, accounting for 63.28% (w.b).

Table 1. Proximate composition of *G. corticata*.

Components	Content (%, Wet Weight Basis)
Moisture	7.06 ± 0.79 *
Protein	15.97 ± 0.44
Lipid	0.86 ± 0.05
Ash	12.83 ± 0.08
Carbohydrates	63.28 ± 0.65

* Data are mean ± SD ($n = 3$).

3.2. Agar Yield by Conventional Extraction and Ultrasound-Assisted Extraction

The agar yield obtained by conventional hot water extraction was 14.01 ± 0.43%, which served as the reference for evaluating the effectiveness of ultrasound-assisted extraction. Alkaline treatment was excluded in the present study in order to evaluate the sole effect of the extraction process without any chemical pre-treatment.

3.3. Effect of Ultrasound Pre-Treatment on Agar Extraction Yield

3.3.1. Agar Yield and Regression Model

The agar yield obtained under the different ultrasound-assisted conditions of the central composite design (CCD) ranged from 24.15% to 29.05% (Table 2). A significant difference ($p < 0.05$) was observed between the ultrasound-assisted extracted agar and the conventionally extracted agar, showing that ultrasound pre-treatment significantly enhanced agar recovery. The experimental data were fitted to a second-order polynomial model by multiple regression analysis, and the regression coefficients were estimated by the least squares method. The resulting quadratic model relating the independent variables to agar yield is:

$$Y = 15.69686 + 0.431372\,X_1 + 0.190812\,X_2 - 0.002644\,X_1X_2 - 0.004086\,X_1^2 - 0.000844\,X_2^2$$

where X_1 represents ultrasonication time (min) and X_2 represents ultrasound amplitude (%).

Table 2. Central composite design (CCD) with coded and actual values and experimental results for ultrasound-assisted extraction of agar.

Run	X_1 (Coded)	X_2 (Coded)	X_1 (Extraction Time, min)	X_2 (Ultrasonic Amplitude, %)	Y (Yield, %)
1	0	0	30	50	28.29
2	0	0	30	50	28.13
3	0	0	30	50	28.80
4	+1	0	45	50	28.58
5	−1	−1	15	20	24.15
6	−1	0	15	50	26.70
7	+1	−1	45	20	28.02
8	+1	+1	45	80	26.83
9	0	0	30	50	28.50
10	0	+1	30	80	29.05
11	0	−1	30	20	26.55
12	0	0	30	50	28.14
13	−1	+1	15	80	27.72

X_1 and X_2 denote coded variables for extraction time and ultrasonic amplitude, respectively; Y represents agar yield (%). Central points were replicated to estimate experimental error.

3.3.2. Analysis of Variance

The analysis of variance (ANOVA) of the fitted model is summarized in Table 3. The model was highly significant ($p = 0.0002$; F-value = 26.63), and the lack-of-fit test was not significant ($p = 0.1554$), indicating no evidence of significant lack of fit. The adjusted coefficient of determination (Adjusted $R^2 = 0.9144$) showed that the model explained a substantial proportion of the variability in agar yield. Both linear terms, ultrasonication time (X1) and ultrasound amplitude (X2), had significant effects on agar yield ($p = 0.0013$). The quadratic terms X12 ($p = 0.0055$) and X22 ($p = 0.0138$) were also significant, indicating a non-linear relationship between the ultrasound parameters and extraction yield. The interaction term X1X2 was also significant ($p = 0.0005$). The pure-error standard deviation (0.28%) was small relative to the range of the response (24.15–29.05%), and adequate precision was 17.3. The coefficients of the fitted model (coded factors, ± standard error) were 28.43 ± 0.16 (intercept), 0.81 ± 0.16 (X_1), 0.81 ± 0.16 (X_2), −1.19 ± 0.19 (X_1X_2), −0.92 ± 0.23 (X_1^2) and −0.76 ± 0.23 (X_2^2), 95% confidence intervals excluded zero for all terms. Residual diagnostics showed approximately normal residuals (Shapiro–Wilk W = 0.979, $p = 0.97$),

no pattern against predicted values or run order, and no outliers (all |externally studentized residuals were less than 3.2). One corner point (run 8) at 45 min, 80% amplitude had high influence (Cook's distance 2.46), but refitting without it left all model terms significant and changed the predicted yield at the selected optimum by only 0.17 percentage points (28.66 to 28.83%).

Table 3. Analysis of variance (ANOVA) for the quadratic model of agar yield.

Source	Sum of Squares	df	Mean Square	*F*-Value	*p*-Value *
Model	19.88	5	3.98	26.63	0.0002
Main regressors					
X_1 (Time)	3.94	1	3.94	26.36	0.0013
X_2 (Amplitude)	3.97	1	3.97	26.58	0.0013
Interaction regressors					
X_1X_2	5.66	1	5.66	37.93	0.0005
Quadratic regressors					
X_1^2	2.33	1	2.33	15.63	0.0055
X_2^2	1.59	1	1.59	10.66	0.0138
Residual	1.05	7	0.1493	—	—
Lack of Fit	0.7266	3	0.2422	3.04	0.1554
Pure Error	0.3187	4	0.0797	—	—
Cor Total	20.93	12			
R^2	0.9501				
Adj. R^2	0.9144				
C.V. (%)	1.40				

* Significant ($p < 0.05$), Not significant ($p > 0.05$).

3.4. Interaction Effects of Ultrasound Parameters on Agar Yield

The combined effects of ultrasonication time and ultrasound amplitude on agar yield were visualized using two-dimensional contour plots and three-dimensional (3D) response surface plots generated with Design-Expert software (Figure 1). Agar yield increased with increasing ultrasonication time up to an optimum level, beyond which a decline was evident. Similarly, increasing ultrasound amplitude initially increased yield, and further increases led to a reduction. The curvature of the 3D response surface reflects the significant quadratic effects of both factors, and the elliptical contour lines indicate a strong interaction between time and amplitude.

The lowest yield (24.15%) was obtained at 15 min and 20% amplitude. Increasing amplitude from 20% to 50% at 15 min raised the yield from 24.15% to 26.70%. The maximum yield (29.05%) was achieved at 30 min and 80% amplitude, whereas a further increase in severity (45 min, 80%) reduced the yield to 26.83%. The centre-point values were consistent (28.13–28.80%), demonstrating good reproducibility. The effect of time was amplitude-dependent: at low amplitude (20%), increasing time improved yield, whereas at high amplitude (80%), prolonged sonication reduced yield.

3.5. Process Optimization and Model Validation

Numerical optimization using the desirability function approach was performed to maximize agar yield, keeping ultrasonication time (15–45 min) and amplitude (20–80%) within the experimental range. The predicted optimum was an ultrasonication time of 32.87 min and an amplitude of 61.58%, giving a predicted agar yield of 28.66% with an overall desirability of 0.92. Validation experiments were carried out in triplicate under these conditions. The experimental yield (29.31 ± 0.70%) was close to the predicted value (28.66%); the difference (0.65 percentage points; 95% CI −1.09 to 2.39) was not significant,

and the observed value fell within the model's 95% prediction interval (27.67–29.65%), supporting the model's predictive adequacy within the studied range. The optimized ultrasound-assisted extraction also gave a significantly higher agar yield than conventional hot water extraction (14.01 ± 0.43%). The yield at the optimum was not statistically distinguishable from the design centre points (28.13–28.80%) or from the highest observed run (29.05% at 30 min, 80% amplitude), indicating a broad plateau of high yield around these conditions.

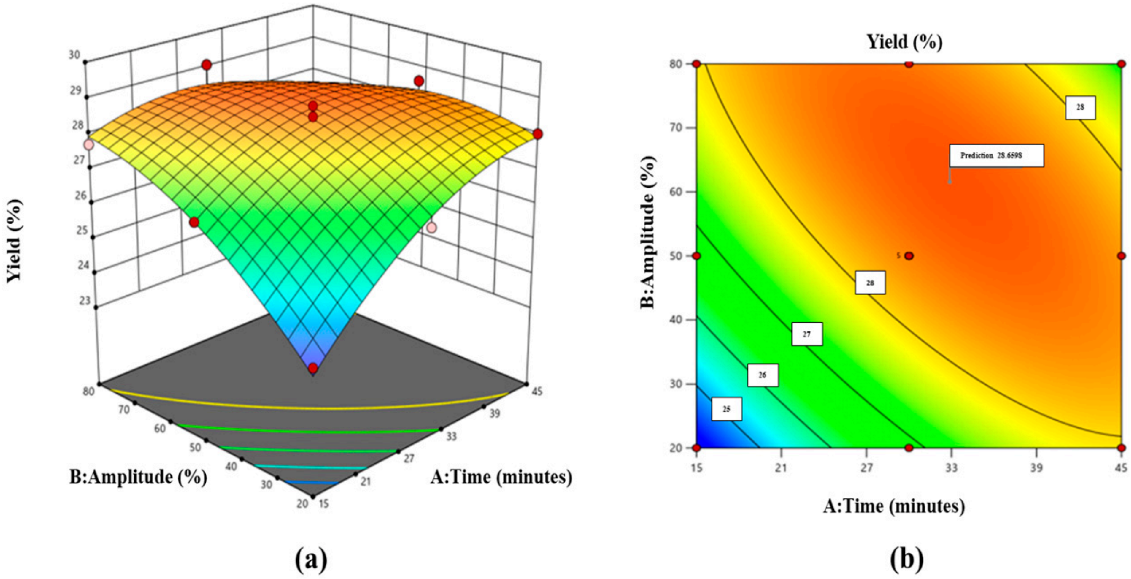

Figure 1. Response surface plots showing the interactive effects of ultrasonication time and amplitude on agar yield: 3D response surface plot (**a**) and contour plot (**b**).

3.6. Chemical Composition of Agar Powders

The ash, moisture and protein contents of the three agar powders (Table 4) differed significantly ($p < 0.05$). The ash contents of CE-AP (3.57%) and US-AP (3.81%) were higher than that of C-AP (1.96%), and US-AP was slightly higher than CE-AP. C-AP recorded the highest moisture content (8.19%), followed by US-AP (7.42%) and CE-AP (6.63%), with no significant difference ($p > 0.05$) between the two extracted preparations. Protein content was markedly lower in C-AP (0.35%) than in CE-AP (3.33%) and US-AP (3.48%), with US-AP marginally higher than CE-AP.

Table 4. Chemical composition of different agar powders.

Samples	Ash Content (%, Wet Weight Basis)	Moisture Content (%, Wet Weight Basis)	Protein Content (%, Wet Weight Basis)	Sulfate Content (%, Wet Weight Basis)	3,6-Anhydrogalactose (%, Wet Weight Basis)
C-AP	1.96 ± 0.17 ^{c}	8.19 ± 0.41 ^{a}	0.35 ± 0.03 ^{c}	1.03 ± 0.03 ^{c}	52.65 ± 2.99 ^{a}
CE-AP	3.57 ± 0.11 ^{b}	6.63 ± 0.48 ^{b}	3.33 ± 0.04 ^{b}	5.97 ± 0.39 ^{b}	25.94 ± 0.77 ^{b}
US-AP	3.81 ± 0.06 ^{a}	7.42 ± 0.18 ^{b}	3.48 ± 0.04 ^{a}	6.10 ± 0.07 ^{a}	22.43 ± 1.34 ^{b}

Values are expressed as mean ± standard deviation (SD) ($n = 3$). Different superscript letters within the same column indicate significant differences ($p < 0.05$). C-AP: Commercial agar powder; CE-AP: Conventionally extracted agar powder; US-AP: Ultrasound-assisted extracted agar powder.

Samples with higher sulfate content showed correspondingly lower 3,6-anhydrogalac tose (3,6-AG) content and lower gel strength (Table 4). US-AP had a slightly higher sulfate content (6.10%) and a lower 3,6-AG content (22.43%) than CE-AP (5.97% sulfate; 25.94%

3,6-AG). C-AP showed the lowest sulfate content (1.03%) and the highest 3,6-AG content (52.65%).

3.7. FTIR Analysis

The FTIR spectra of the extracted agars (CE-AP and US-AP) and commercial agar (C-AP) in the range 4000–500 cm^{-1} are shown in Figure 2. Characteristic absorption bands of agar were observed at 1367–1374, 1240–1250, 1037–1042, 928–929, 842–850 and 820–823 cm^{-1}. CE-AP and US-AP showed peak positions similar to those of commercial agar, with slight differences in intensity. A broad band at 3364–3278 cm^{-1} was assigned to O-H and N-H stretching vibrations. A weak to moderate band at 2917–2921 cm^{-1} was attributed to C-H stretching, and the band at 1632–1638 cm^{-1} to C = O stretching. The band at 1400–1450 cm^{-1} corresponds to symmetric stretching of carboxylate groups, and the band at 1140–1152 cm^{-1} to glycosidic C-O-C linkages. The band at 1037–1042 cm^{-1} indicates pyranose ring structures, and the band at 1240–1250 cm^{-1} corresponds to S=O stretching of sulfate esters. The band near 928–929 cm^{-1} corresponds to the C-O-C vibration of 3,6-anhydro-α-L-galactopyranose, and the bands at 820–850 cm^{-1} correspond to sulfate groups attached to the C-4 and C-6 positions of galactose. The intensity of the sulfate-associated band varied between CE-AP and US-AP. Overall, the FTIR profiles showed the characteristic agar structure in all samples, with minor variations depending on the extraction method.

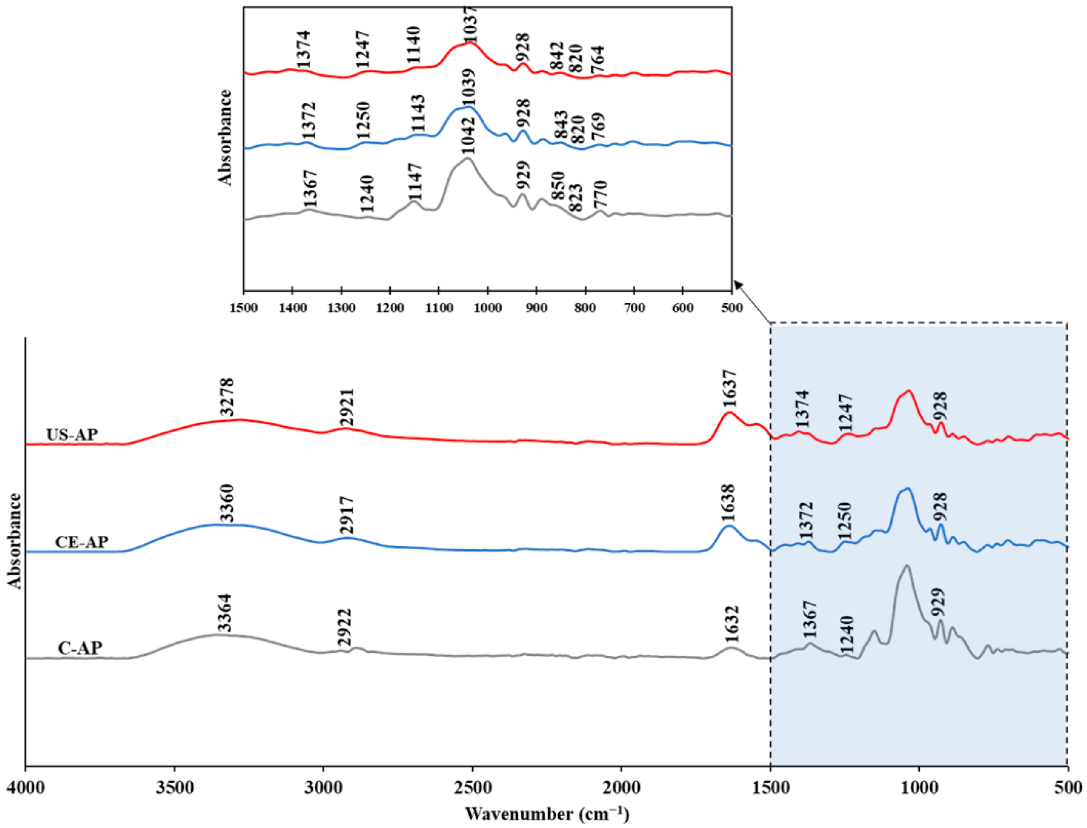

Figure 2. FTIR spectra of different agar powders. C-AP: Commercial agar powder; CE-AP: Conventionally extracted agar powder; US-AP: Ultrasound-assisted extracted agar powder.

3.8. pH and Color of Agar Gels

The pH values of the 1.5% agar gels are presented in Table 5. A significant difference ($p < 0.05$) was observed among the samples, with C-AG showing the highest pH (6.95), followed by US-AG (6.59) and CE-AG (6.45).

Table 5. Physicochemical and gel properties of gels prepared from different agars.

Samples	pH	Gel Strength (g/cm^2)	Melting Temperature (°C)	Gelling Temperature (°C)	Syneresis (%)	L^*	a^*	b^*	ΔE^*	ΔC^*
C-AG	6.95 ± 0.72^{a}	822.69 ± 11.96^{a}	86.70 ± 0.45^{a}	42.97 ± 0.76^{b}	1.02 ± 0.03^{b}	36.50 ± 0.14^{a}	-2.75 ± 0.01^{c}	-6.65 ± 0.02^{c}	58.88 ± 0.15^{c}	5.76 ± 0.16^{c}
CE-AG	6.45 ± 0.04^{c}	72.05 ± 1.97^{b}	79.40 ± 0.26^{b}	45.70 ± 0.20^{a}	1.77 ± 0.01^{a}	25.86 ± 0.03^{b}	-0.74 ± 0.04^{b}	14.64 ± 0.17^{a}	70.39 ± 0.01^{b}	13.22 ± 0.17^{a}
US-AG	6.59 ± 0.02^{b}	66.50 ± 0.73^{b}	77.37 ± 0.37^{c}	45.00 ± 0.45^{a}	1.79 ± 0.01^{a}	21.87 ± 0.17^{c}	-0.58 ± 0.02^{a}	11.77 ± 0.20^{b}	73.82 ± 0.14^{a}	10.34 ± 0.20^{b}

Values are expressed as mean ± standard deviation (SD) ($n = 3$). Different superscript letters within the same column indicate significant differences ($p < 0.05$). C-AG: Commercial agar gel; CE-AG: Conventionally extracted agar gel; US-AG: Ultrasound-assisted extracted agar gel.

The colour parameters of the gels are shown in Table 5, where L* represents lightness (0 = black, 100 = white), a* the redness (+a*) to greenness (−a*) axis, and b* the yellowness (+b*) to blueness (−b*) axis. Significant differences ($p < 0.05$) were observed among all three samples for all parameters. C-AG showed the highest lightness (L* = 36.50), whereas CE-AG (25.86) and US-AG (21.87) had significantly lower L* values. CE-AG and US-AG exhibited higher positive b* values (14.64 and 11.77, respectively) than C-AG (−6.65). The total colour difference (ΔE*) relative to the white reference standard (L* = 94.88, a* = −1.16, b* = 0.85) was highest in US-AG (73.82), followed by CE-AG (70.39) and C-AG (58.88). In contrast, the colour saturation difference (ΔC*) was higher in CE-AG (13.22) than in US-AG (10.34). Overall, the extraction method significantly influenced the colour characteristics of the gels.

3.9. Gel Strength and Syneresis

The gel strengths of CE-AG, US-AG and commercial agar gel (C-AG) are presented in Table 5. Both extracted agars showed markedly lower gel strength (72.05 and 66.50 g/cm^2, respectively) than C-AG (822.69 g/cm^2). The gel strength of CE-AG (72.05 g/cm^2) was slightly higher than that of US-AG (66.50 g/cm^2), although this difference was not significant; both were far below C-AG (822.69 g/cm^2). Syneresis values differed significantly among the gels ($p < 0.05$; Table 5). C-AG exhibited the lowest syneresis (1.02%), whereas CE-AG (1.77%) and US-AG (1.79%) showed significantly higher values, with no significant difference between the two extraction methods. The syneresis of all samples remained below 2%.

3.10. Melting and Gelling Temperatures

The melting and gelling temperatures of the agar gels differed significantly among the samples ($p < 0.05$; Table 5). C-AG exhibited the highest melting temperature (86.70 °C), followed by CE-AG (79.40 °C) and US-AG (77.37 °C). In contrast, CE-AG and US-AG had slightly higher gelling temperatures (45.70 and 45.00 °C, respectively) than C-AG (42.97 °C).

3.11. Texture Profile Analysis

The textural properties of the gels are presented in Table 6. C-AG exhibited the highest hardness (33.83 N), followed by CE-AG (4.11 N) and US-AG (2.81 N). Springiness showed the opposite trend: CE-AG (0.93 cm) and US-AG (0.91 cm) were significantly higher than C-AG (0.74 cm). Cohesiveness and resilience did not differ significantly ($p > 0.05$) among the samples. Gumminess and chewiness followed a trend similar to hardness, with C-AG showing the highest values and CE-AG and US-AG significantly lower values. Overall, the extracted agars formed softer but more elastic gels than the commercial agar.

Table 6. Texture profile analysis (TPA) of gels prepared from different agars.

Samples	Hardness (N)	Springiness (cm)	Cohesiveness	Gumminess (N)	Chewiness (N)	Resilience
C-AG	33.83 ± 0.86 ^{a}	0.74 ± 0.01 ^{b}	0.66 ± 0.01 ^{a}	22.53 ± 0.40 ^{a}	16.85 ± 0.65 ^{a}	0.45 ± 0.01 ^{a}
CE-AG	4.11 ± 0.37 ^{b}	0.93 ± 0.06 ^{a}	0.66 ± 0.08 ^{a}	2.73 ± 0.49 ^{b}	2.5 ± 0.59 ^{b}	0.48 ± 0.07 ^{a}
US-AG	2.81 ± 0.49 ^{c}	0.91 ± 0.02 ^{a}	0.51 ± 0.05 ^{a}	1.45 ± 0.38 ^{c}	1.33 ± 0.38 ^{c}	0.38 ± 0.08 ^{a}

Values are expressed as mean ± standard deviation (SD) ($n = 3$). Different superscript letters within the same column indicate significant differences ($p < 0.05$). C-AG: Commercial agar gel; CE-AG: Conventionally extracted agar gel; US-AG: Ultrasound-assisted extracted agar gel.

3.12. Microstructure Analysis

The microstructures of the gels (1.5%, w/v) prepared from C-AG, CE-AG and US-AG at magnifications of 250× and 500× are shown in Figure 3. C-AG exhibited a dense and continuous three-dimensional network of compact fibrous and sheet-like structures with limited pore spaces. In contrast, CE- AG and US-AG showed a less compact microstructure with noticeable cavities and a less uniform fibrous network. The US-AG gel showed a more open and interconnected porous structure with thinner network walls and larger voids.

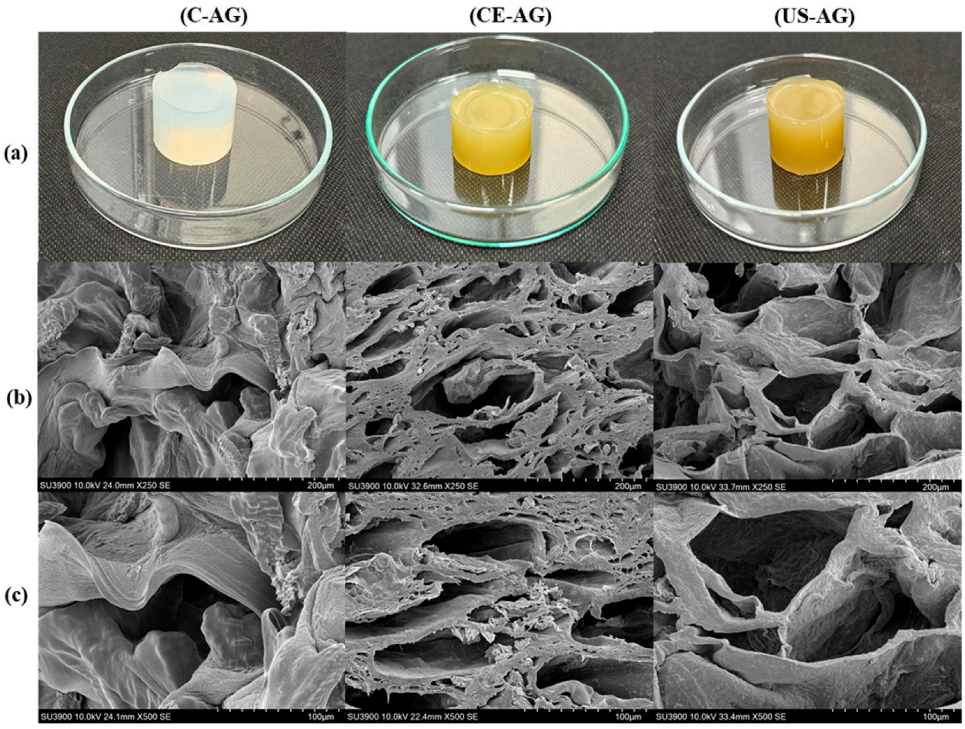

Figure 3. Photographs (**a**) and SEM micrographs of gels (1.5%, w/v) prepared from commercial agar (C-AG), conventionally extracted agar (CE-AG), and ultrasound-assisted extracted agar (US-AG) at magnification of 250× (**b**) and 500× (**c**).

3.13. Oscillatory Rheology

The frequency sweep results are shown in Figure 4a(i). The storage modulus (G′) remained higher than the loss modulus (G″) over the entire frequency range for all samples, indicating predominantly elastic behaviour. C-AG exhibited the highest G′ values, whereas CE-AG and US-AG showed lower G′ values. The frequency dependence of both moduli was weak for all samples, with a slight increase in both moduli with frequency for CE-AG and US-AG. US-AG exhibited slightly higher G′ values than CE-AG across most frequencies.

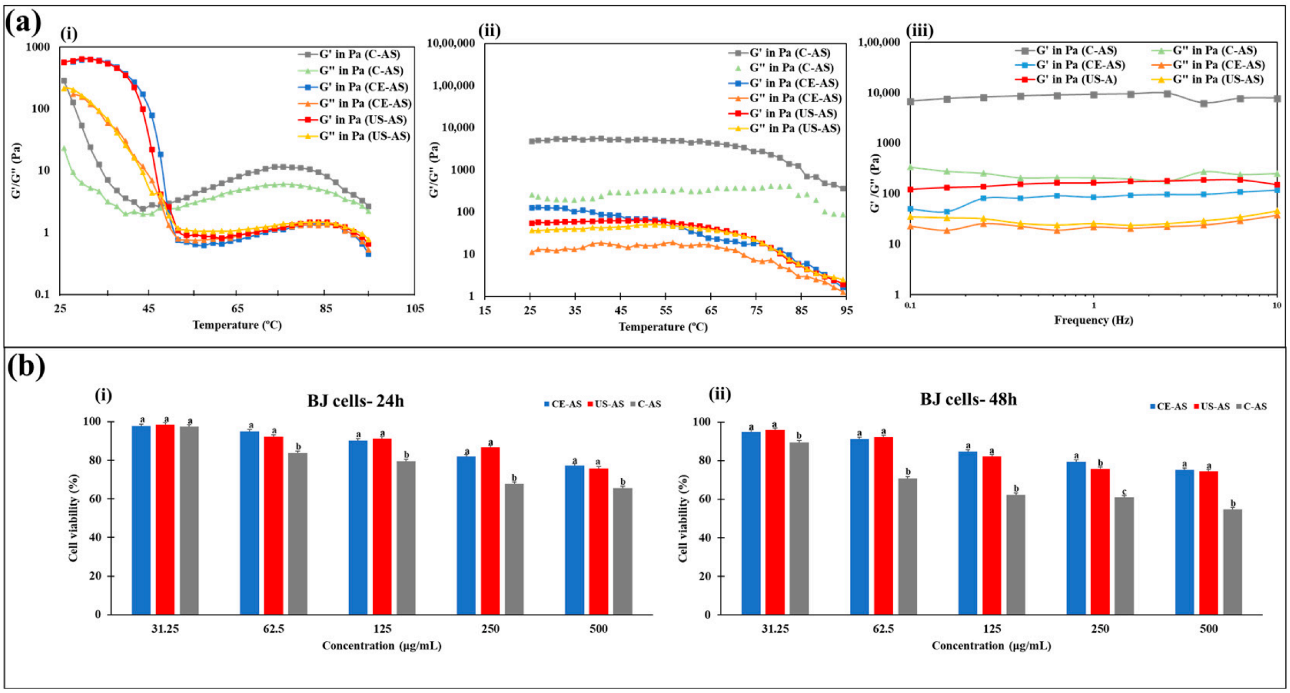

Figure 4. Rheological properties of agar solutions, including (**a**(i)) frequency-dependent viscoelastic behaviour evaluated over a frequency range of 0.1–10 Hz, (**a**(ii)) temperature-dependent behaviour during the cooling cycle from 95 °C to 25 °C, and (**a**(iii)) temperature-dependent behaviour during the heating cycle from 25 °C to 95 °C. Cell viability of BJ fibroblast cells treated with CE-AS, US-AS, and C-AS at different concentrations for 24 (**b**(i)) and 48 h (**b**(ii)). Bars represent standard deviation ($n = 3$); different lowercase letters in the same concentration indicate significant differences ($p < 0.05$). C-AS: commercial agar solution; CE-AS: conventionally extracted agar solution; US-AS: ultrasound-assisted extracted agar solution.

During the cooling sweep (Figure 4a(ii)), the agar solutions at 95 °C showed low G′ and G″ values, and both moduli increased gradually as the temperature decreased. G′ of US-AG increased rapidly at 43.65 °C and that of CE-AG at 45.76 °C, after which G′ exceeded G″. For C-AG, G′ was slightly higher than G″ at higher temperatures and no distinct crossover point was observed; a sharp increase in G′ occurred at 38.74 °C. At 25 °C, C-AG exhibited the highest storage modulus, followed by US-AG and CE-AG. During the heating sweep (25–95 °C; Figure 4a(iii)), both G′ and G″ decreased with increasing temperature; G′ remained higher than G″ at lower temperatures, and C-AG showed higher thermal stability than CE-AG and US-AG.

3.14. Cytotoxicity

The cytotoxicity of the extracted agar samples (CE-AS and US-AS) and commercial agar (C-AS) was assessed by MTT assay using human BJ fibroblast cells at concentrations of 31.25, 62.5, 125, 250 and 500 µg/mL (Figure 4b). After 24 h, CE-AS and US-AS showed cell viability above 90% at 31.25–125 µg/mL (Figure 4b(i)), which decreased gradually with increasing concentration to 77.21% and 75.78% at 500 µg/mL, respectively. C-AS showed lower cell viability at all concentrations, decreasing to 65.53% at 500 µg/mL. After 48 h, viability ranged from 94.86% to 75.19% for CE-AS and from 95.83% to 74.35% for US-AS, whereas C-AS decreased significantly to 54.82% at 500 µg/mL (Figure 4b(ii)). Cell viability of all samples exceeded 80% at the lower concentrations, and CE-AS and US-AS maintained viability above 70% at both 24 and 48 h. No additional cytotoxicity was observed for US-AS relative to CE-AS.

4. Discussion

4.1. Proximate Composition of G. corticata

The moisture content of the seaweed was low, as expected for dried *G. corticata* [25], and low moisture levels are generally considered suitable for maintaining raw material quality [26]. The protein content is typical of red seaweeds, which are known to contain moderate levels of protein that vary with species and growth conditions [27]. The low lipid content is a common feature of *Gracilaria* species [28] and is advantageous in hydrocolloid processing, as lipids can interfere with extraction efficiency. The ash content is characteristic of the *Gracilaria* genus and varies with growth conditions, reflecting the accumulation of minerals from the marine environment [29]. Carbohydrate, calculated by difference, was the dominant fraction, as also reported for other *Gracilaria* species [30]. This value represents total carbohydrate, including agar together with other polysaccharides such as fibre, and therefore does not represent the agar content. The dominance of carbohydrates is important because agar, the target hydrocolloid, consists mainly of galactan polysaccharides [8].

4.2. Conventional Extraction and Ultrasound-Assisted Extraction

The relatively low yield of the conventional method may be attributed to limited disruption of the seaweed cell wall matrix and reduced mass transfer efficiency, resulting in incomplete release of agar polysaccharides. Conventional extraction relies primarily on thermal diffusion, which may not be sufficient to solubilize agar entrapped within the rigid cell wall of red seaweeds. Orduña-Rojas et al. [31] reported native agar yields of 34.4% and 29.7% for *G. longissima* and *G. vermiculophylla*, respectively, and noted that yield is influenced by the month of collection, which may explain the variation in yield observed in the present study. Tatary et al. [8] reported a higher extraction yield of 22.91% for agar from *G. corticata*, which may be attributed to alkaline treatment improving disruption of the cell wall matrix; alkaline treatment was deliberately excluded here to evaluate the effect of the extraction process alone.

The significantly higher yield obtained with ultrasound pre-treatment indicates the effectiveness of ultrasonic energy in facilitating the release of agar from the seaweed matrix. The increase in agar yield under ultrasound treatment can be attributed to acoustic cavitation phenomena, including microbubble formation, collapse, and microjet generation, which disrupt the cell wall structure and enhance solvent penetration into the intracellular matrix [12,32,33]. The significance of the linear, quadratic and interaction terms of the model indicates that the combined influence of time and amplitude plays an important role in optimizing agar extraction.

4.3. Interaction Effects of Ultrasonication Time and Amplitude

Moderate ultrasonication time enhances cell wall disruption and improves mass transfer; however, prolonged exposure may lead to partial degradation of agar polysaccharide chains owing to excessive cavitation energy. Similarly, increasing amplitude initially improved extraction by intensifying cavitation effects and promoting solvent penetration into the algal matrix, whereas further increases reduced yield, possibly owing to structural degradation of agar molecules under high ultrasonic energy. The lowest yield (24.15%) at 15 min and 20% amplitude suggests that insufficient sonication energy is inadequate for effective cell wall disruption and agar release, and amplitude appeared to play a dominant role in promoting cavitation and mass transfer. The maximum yield (29.05%) at 30 min and 80% amplitude identifies this as the optimal region, in which sufficient acoustic energy disrupts the algal matrix without degrading the agar polymers. The reduced yield at 45 min and 80% amplitude indicates possible depolymerization under excessive ultrasonication and confirms a non-linear response. The consistent centre-point values support the repro-

ducibility and adequacy of the model. Overall, balanced ultrasound conditions appear essential to achieve maximum agar recovery without compromising molecular integrity.

4.4. Optimization and Comparison with Previous Studies

The optimized ultrasound-assisted extraction demonstrates the effectiveness of ultrasound as an efficient technique for agar recovery. The face-centred design is not rotatable, and replication was limited to the centre point, so the lack-of-fit test alone does not establish adequacy across the whole region. The model nevertheless identified the optimum within the studied range, which the independent validation confirmed. The optimisation therefore identified an operating window of high, stable yield, and the practical benefit lies in the large increase over conventional extraction and the avoidance of low-intensity conditions, and not in a significantly higher yield at the exact optimum. Several studies have explored the effect of ultrasound parameters, most notably treatment time and amplitude, on agar yield from seaweeds [34–36]. However, the use of statistical optimization techniques such as RSM to determine optimal extraction conditions has been relatively limited. Deepika et al. [12] optimized ultrasound-assisted agar extraction from *Hypnea pannosa* using a CCD to study ultrasound amplitude (40–80%) and treatment time (15–45 min). Both linear and quadratic terms of time and amplitude significantly ($p < 0.05$) affected agar yield, and 33.5 min and 62% amplitude were found to be optimal, yielding 32.99% agar, which is similar to the optimum identified in the present study. A similar study on *Gelidium sesquipedale* optimized extraction time (30–60 min) and ultrasound power (50–100%) using a CCD [32]. Unlike our study, the quadratic terms in that study were not statistically significant and a linear model best related yield to time and power; the predicted maximum yield (6.7%) at 60 min and 100% power was validated experimentally at 8.55 ± 0.16% agar. Besides agar, other seaweed polysaccharides have been extracted using ultrasound pre-treatment. For instance, alginate extraction from *Fucus vesiculosus* was optimized at 69% amplitude and 30 min using a CCD, yielding 6.71% alginate, and, as in the present study, a quadratic model best fitted the experimental data [37]. Although energy consumption was not measured, the near-optimal yields obtained under lower-intensity conditions (26.55–26.70% versus 29.31% at the optimum and 14.01% conventionally) suggest favorable energy efficiency at moderate settings. Ultrasound was an added step before the same 2 h hot-water extraction and gelling, freezing, drying and cooling were not assessed. The study used a laboratory-scale 20 kHz probe, and pilot-scale reproducibility was not tested. Quantifying delivered power, energy per gram of agar, pilot-scale performance and economic feasibility remains necessary before practical recommendations can be made.

4.5. Chemical Composition of Agar Powders

The higher ash contents of US-AP and CE-AP compared with C-AP can be attributed to the extensive purification employed in industrial agar processing, including repeated washing, bleaching and freeze–thaw cycles, which reduce inorganic mineral residues co-extracted with the polysaccharide [38]. The marginally higher ash content of US-AP relative to CE-AP may reflect the enhanced cell wall disruption induced by acoustic cavitation, facilitating greater mobilisation of mineral-associated polysaccharide fractions. All values remained within the internationally accepted food-grade specification of ≤ 6.5% ash, confirming the compositional suitability of both extracted agars for food applications [39]. The elevated moisture retention in C-AP is likely associated with the hygroscopic character of its highly purified, low-sulfate agarose chains, which retain water through hydrogen bonding with exposed hydroxyl groups. Agars from *Gracilaria* species collected from Java Island, Indonesia, show ash and moisture contents in a similar range to those observed in the present study [40].

The low-to-moderate protein content of the extracted agars was of similar order to that of crude sulfated polysaccharide extracts from *G. fisheri* [41], indicating the predominantly carbohydrate-rich nature of the extracts. The lower protein content of C-AP is attributable to the alkaline pre-treatment and extensive purification steps of commercial processing, which remove co-extracted proteinaceous residues, pigments and other non-polysaccharide impurities [9]. The protein content of CE-AP and US-AP falls within a plausible range typical of native agar, reflecting the natural retention of matrix-associated proteins rather than any processing deficiency. The marginally higher protein content of US-AP relative to CE-AP may similarly be attributed to enhanced cell wall disruption during ultrasound treatment, facilitating co-extraction with the polysaccharide matrix, consistent with the trend for ash.

In general, agar extracted from *Gracilaria* species has a relatively high sulfate content, which is often associated with lower 3,6-AG content, weaker gel networks and reduced gel strength [42]. The higher sulfate and lower 3,6-AG contents of US-AP compared with CE-AP may be attributed to enhanced extraction of sulfated agaropectin fractions due to cavitation-induced cell disruption. The lower sulfate and higher 3,6-AG contents of C-AP are consistent with its greater purity and gel strength, and reflect the effect of alkaline pre-treatment, which converts the L-galactose-6-sulfate precursor into the 3,6-AG bridge characteristic of agarose [43]. Agar from *G. corticata* extracted with alkaline pre-treatment is reported to have a lower sulfate content than the native agars obtained here, which may be attributed primarily to the alkaline pre-treatment and to differences in regional origin [8]. In contrast, sulfated polysaccharide from *G. birdiae* extracted without alkaline pre-treatment retains a higher sulfate content [44]. *Gracilaria* species from different geographical locations show varying sulfate and 3,6-AG contents, which may be influenced by environmental factors and growth conditions [5]. Overall, the elevated sulfate and reduced 3,6-AG contents observed here can be explained by the natural sulfation of *Gracilaria* species, the absence of alkaline pre-treatment and the enhanced release of sulfated agaropectin fractions during ultrasound treatment, which collectively influenced the gel strength and structural properties of the extracted agar.

4.6. FTIR Analysis

The characteristic bands confirm the presence of typical agar functional groups [5,8,45]. The similar peak positions of CE-AP and US-AP relative to commercial agar, with only slight intensity variations, indicate that ultrasound-assisted extraction did not significantly alter the fundamental chemical structure of agar. The broad band at 3364–3278 cm^{-1} indicates extensive hydrogen bonding within the polymeric network, which contributes to strong intermolecular bonding, gel formation and water-binding capacity [8]. The weak to moderate C-H band indicates a very low degree of methylation (O-CH3) [45]. The band at 1632–1638 cm^{-1} may be associated with ester or amide functionalities [46], and the carboxylate band supports the presence of acidic polysaccharide components. The bands at 1037–1042 cm^{-1} and 1140–1152 cm^{-1} confirm the galactan skeleton [5]; the bands at 1240–1250 cm^{-1} indicate sulfate ester groups, and the bands at 820–850 cm^{-1} and near 928–929 cm^{-1} are consistent with sulfate substitution and 3,6-AG, respectively [47]. The variation in the intensity of the sulfate-associated band between CE-AP and US-AP indicates a possible alteration in sulfate content during extraction [12]. Reduced sulfate-related peak intensity may indicate partial desulfation during processing, whereas sharper and more defined peaks suggest better preservation of the native polysaccharide structure.

4.7. pH and Colour

The higher pH of C-AG may be attributed to industrial purification and neutralization steps during commercial processing, which remove acidic impurities and improve product stability. The lower pH of CE-AG and US-AG may be associated with residual organic acids and naturally occurring compounds retained during extraction. Similar pH values (6.09–6.23) have been reported for 1.5% agar gels extracted from *G. verrucosa* [17].

Colour is an important quality attribute of gels that primarily influences sensory perception without affecting functional properties [48]. The highest lightness of C-AG is expected because industrial purification removes most pigments. The significantly lower L* values of CE-AG and US-AG indicate that native residual seaweed pigments, such as chlorophyll, carotenoids and phycoerythrobilin, likely contributed to their darker appearance [49]. The slightly lower L* value of US-AG suggests that ultrasound may have enhanced cell disruption and thus pigment release. Residual chlorophyll a could contribute negative a* values (green tone), and the higher b* values of the extracted agars are primarily attributed to the release of carotenoids and degradation products of phycoerythrin [50]. The highest ΔE* of US-AG indicates that ultrasound pre-treatment produced the greatest overall colour deviation from the white reference, while the higher ΔC* of CE-AG indicates that ultrasound primarily darkens the agar (lowering L*) rather than making its colour more intense.

4.8. Gel Properties: Gel Strength, Syneresis, Melting and Gelling Temperatures

The markedly higher gel strength of C-AG is likely due to its higher purity and lower sulfate content. Gel formation in agar depends on the aggregation of polymer chains into hydrogen-bond-stabilized double helices, which can be disrupted by charged groups such as sulfates [5]. The lower gel strength of the extracted agars can therefore be attributed mainly to the higher sulfate content of the *G. corticata* variety used, and the small difference between CE-AG and US-AG may be attributed to minor structural modifications of the polysaccharides during ultrasonication. Luhan et al. [51] reported a gel strength of 43 g/cm^2 for agar from *G. heteroclada* collected in September, suggesting that seasonal variation and harvesting month strongly influence gel strength. Yarnpakdee et al. [5] reported 95 g/cm^2 for native agar from *G. tenuistipitata*, and Ganesan et al. [52] reported a maximum of 96.5 g/cm^2 for untreated *G. edulis*. The gel strength obtained here therefore lies within the range reported for non-alkaline-extracted *Gracilaria* agar, which supports the understanding that agar extracted without alkali pre-treatment generally shows lower gel strength owing to the absence of alkali-induced structural modification and inadequate removal of sulfate groups that interfere with gel network formation. Although this gel strength does not meet conventional industrial standards for agar grading, it need not be viewed as a limitation, as such agar can be used in food systems requiring soft or low-strength gels.

Syneresis is a critical factor in gel stability during storage, occurring when the network fails to retain bound water, resulting in phase separation and deterioration of texture and quality [48]. The lower syneresis of C-AG suggests a more stable and compact three-dimensional network capable of efficiently entrapping water. The higher syneresis of CE-AG and US-AG may be associated with their higher sulfate content and lower gel strength; elevated sulfate substitution can disrupt intermolecular hydrogen bonding, weakening junction zones and reducing water-holding capacity. Menaka et al. [18] reported syneresis of 0.6–7.98% for 1.5% native agar from *G. longissima* over 50–90 °C, with the lowest value at 90 °C. Although statistically significant differences were observed here, all samples remained below 2%, demonstrating good gel stability. The syneresis of CE-AG (1.77%) and US-AG (1.79%) differed by only 0.02 percentage points, and both remained below 2%, suggesting that ultrasound did not markedly affect water retention under these conditions.

The higher melting temperature of C-AG indicates a stronger and more stable gel network, which may be associated with the higher purity and agarose content of commercial agar, promoting more effective junction zones. The lower melting temperatures of CE-AG and US-AG may be associated with structural variations in the polysaccharide chains and natural components retained from the seaweed matrix. Their slightly higher gelling temperatures may be related to residual pigments, minerals or sulfate groups that influence the sol–gel transition. Villanueva et al. [53] reported a melting temperature of 92.3 °C and a gelling temperature of 46.2 °C for native agar from *L. flexilis*, which are similar to the present values.

4.9. Texture and Microstructure

Texture refers to the mechanical, structural and acoustic properties perceived by humans that define the physical characteristics of a food [54]. The higher hardness of C-AG is likely because commercial agar undergoes purification steps that improve gel-forming ability by removing components that weaken the gel structure. Gels of lower hardness can be beneficial for foods requiring a softer texture, such as low-fat or soft-textured products [10] and are often preferred by consumers such as children, the elderly or those requiring easily chewable foods. The higher springiness of CE-AG and US-AG suggests superior elastic recovery and flexibility of the gel network, which is important in foods where an elastic texture is desirable. Cohesiveness and resilience did not differ significantly among the samples, which suggests, but does not prove, comparable internal bonding. The similar resilience suggests that immediate elastic recovery after compression was relatively unaffected by the extraction method.

The compact morphology of C-AG, with limited pore spaces, may result from a higher degree of desulfation during commercial purification and is consistent with its higher gel strength and water retention. The larger pores in CE-AG and US-AG indicate reduced structural compactness, which may be influenced by seaweed species, polysaccharide composition and extraction method, contributing to the lower gel strength and reduced water retention observed. Yarnpakdee et al. [5] similarly reported that native agar showed a looser network, whereas alkaline-treated agar formed a more ordered and denser gel matrix with finer strands. The more open porous structure of US-AG is likely due to cavitation-induced shear forces during ultrasound treatment that enhance polysaccharide release from algal cell walls [55]. These observations indicate that extraction conditions play a crucial role in the microstructural organization and functional properties of agar gels.

4.10. Rheological Behaviour

The predominance of G′ over G″ for all samples is consistent with the behaviour reported by Norziah et al. [23] for 1.5% pure agar, is characteristic of thermoreversible polysaccharide gels, and confirms solid-like behaviour with a well-developed three-dimensional network [56]. The highest G′ of C-AG reflects its stronger gel network and higher rigidity, and the lower G′ of CE-AG and US-AG is consistent with their lower gel strength; it may be attributed to their higher sulfate content, which interferes with the formation of strong double-helix junction zones. The weak frequency dependence indicates stable gel networks, and the slight increase in moduli for CE-AG and US-AG suggests a moderately flexible network compared with the commercial sample. The higher G′ of US-AG relative to CE-AG indicates that ultrasonication did not adversely affect the viscoelastic properties of the gel.

The temperature-dependent behaviour agrees with the concept that agar molecules exist as random coils at elevated temperatures and associate on cooling into double helices that aggregate into higher-order structures, ultimately forming the gel network [56]. The higher moduli of C-AG may be attributed to its greater agarose purity and the presence of

higher molecular weight chains. The sharp rise in G′ at 38.74 °C can be used to extrapolate the curve and estimate complete gelation in the absence of a distinct crossover point [57]. During heating, the decrease in moduli reflects disruption of intermolecular interactions and melting of the gel structure.

4.11. Cytocompatibility

The concentration- and time-dependent decrease in viability may be attributed to residual components and bioactive compounds in the agar samples, particularly in the crude extracts. Notably, ultrasound treatment did not induce additional cytotoxicity, indicating that the extraction method did not adversely affect cytocompatibility. Sulfated seaweed polysaccharides show sulfate- and molecular-weight-dependent bioactivities in vitro [58–60]. The higher sulphate content of the extracted agars (Table 4) may therefore have contributed to their concentration-dependent response. The lower cell viability of C-AS may be attributed to residual impurities, particularly phenolic compounds such as 3-hydroxyphenylacetic acid and catechol, which are known to influence biological performance [61]. Lesgourgues et al. [62] reported that dialysis fractions gave significantly higher proliferation of human dermal fibroblasts than non-dialyzed carrageenan-rich fractions from *Solieria chordalis* at elevated concentrations, while no cytotoxic effects were observed for the red seaweed-derived polysaccharide extract. Overall, all samples showed good cytocompatibility at lower concentrations (viability > 80%), supporting their potential biological suitability under the tested conditions, and CE-AS and US-AS showed better cytocompatibility than C-AS, maintaining viability above 70% at both 24 and 48 h, which confirms no marked reduction in cell viability under the tested conditions [63].

4.12. Implications and Future Directions

Ultrasound-assisted extraction, optimized by RSM, roughly doubled agar recovery relative to conventional hot water extraction without altering the fundamental chemical nature of the agar or introducing additional cytotoxicity. The low gel strength suggests possible use in soft-gel food applications, which remains to be evaluated and energy consumption, cost, scalability and pilot-scale reproducibility were not assessed and remain to be evaluated. Further optimization, including additional replicates at the factorial and axial points of the RSM-CCD model to improve model reliability, additional purification of the crude agar, molecular-level characterization, and the incorporation of a mild alkali treatment, could enhance extraction efficiency, economic viability, and industrial applicability.

5. Conclusions

Ultrasound pre-treatment significantly increased crude agar yield from *G. corticata* (29.31% vs. 14.01% for conventional hot water extraction). The RSM model described the response within the studied region and identified a candidate optimum (32.87 min, 61.58% amplitude), supported by a triplicate validation run. The extracted agar was characterised and compared with a single commercial agar sample. The extracted agar showed the characteristic agar FTIR bands and thermoreversible gel behaviour, but its (3,6-AG) content, gel strength, hardness and melting temperature were lower, and its sulfate content higher than those of commercial agar. FTIR confirmed the characteristic agar functional groups in the extracted samples, indicating that the agar-type backbone was retained. Furthermore, CE-AS and US-AS maintained BJ fibroblast viability above 70% at 24 and 48 h in vitro, supporting their favorable cytocompatibility under the tested conditions.

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