Journal of Environmental Biology
p-ISSN: 0254-8704 • e-ISSN: 2394-0379 • CODEN: JEBIDP

JEB®

Original Research Paper
Volume 47 Issue 2 March 2026 pp. 237-246
DOI: http://doi.org/10.22438/jeb/47/2/MRN-5804

J. Environ. Biol., 2026
© Triveni Enterprises, Lucknow (India)
Journal website : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Enzyme-assisted protein extraction from *Sargassum muticum* using response surface methodology

P. Niharika[1], P.P. Mahendran[1]*, P.M. Sundaram[2], J. Prabhaharan[3], A. Gurusamy[3], K. Chandrakumar[4], T.A. Naziba[4] M. Prasanthrajan[5], and M.M.R.A. Firnass[6]

[1]*Department of Soils & Environment, Agricultural College and Research Institute, Madurai 625 104, India*
[2]*Department of Biotechnology, Agricultural College and Research Institute, Madurai – 625 104, India*
[3]*Department of Agronomy, Agricultural College and Research Institute, Madurai-625 104, India*
[4]*Department of Renewable Energy and Engineering, Agricultural Engineering College and Research Institute, Coimbatore-641 003, India*
[5]*Department of Environmental Sciences, Agricultural College and Research Institute, Coimbatore-641 003, India*
[6]*Department of Soils Science and Agricultural Chemistry, Agricultural College and Research Institute, Coimbatore–641 003, India*

Received: 12 June 2025 **Revised:** 26 August 2025 **Accepted:** 25 September 2025

***Corresponding Author Email:** ppmsoil96@tnau.ac.in ***ORCiD:** https://orcid.org/0000-0003-0244-0504

Abstract

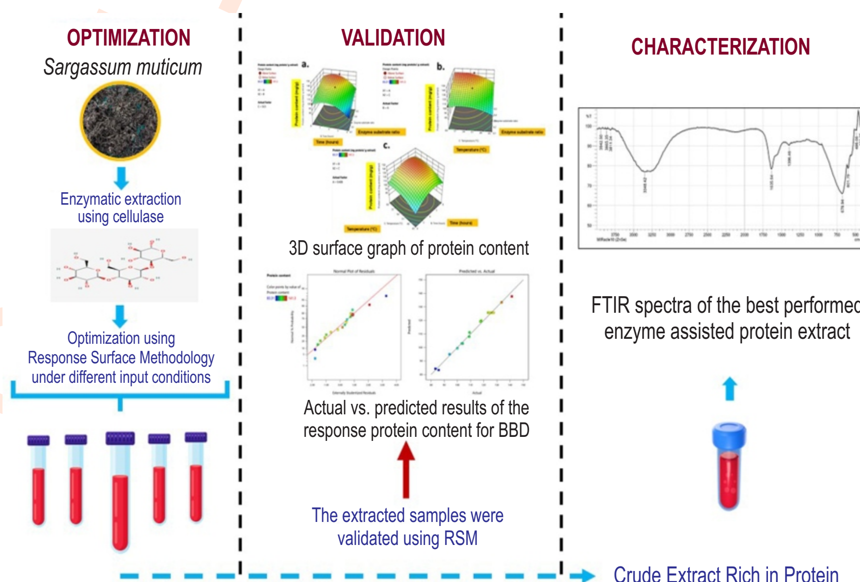
Aim: *Sargassum muticum,* a brown seaweed is emerging as a sustainable protein source for agriculture, aquaculture and food industry. This study aimed to enhance the efficacy of protein extraction through enzymatic extraction approach.

Methodology: An enzymatic extraction was performed using the input parameters such as enzyme-to-substrate (E/S) ratio (0.10–0.50), incubation time (4–8 hrs) and temperature (45–60°C). They were optimized using a Box-Behnken design-based Response Surface Methodology to achieve the maximum protein content.

Results: The results revealed that enzymatic extraction under E/S=0.50, 6 hrs, 52.5°C resulted in a maximum protein and phenol content of 141.3 mg g^{-1}, 35.5 mg GAE g^{-1}, respectively. The model validated through ANOVA showed a strong agreement between experimental and predicted values, with high R^2, adjusted R^2, predicted R^2 and F-values confirming the model's significance.

Interpretation: These findings emphasize the potential of response surface methodology as an effective tool for optimizing conditions to maximize protein extraction from *Sargassum muticum*. The resulting protein-rich extracts could serve as promising biostimulants for the agroindustry, enhancing crop productivity and nutritional security.

Key words: Biostimulant, Cellulase, Marine protein, Optimization, Seaweed extract



Triveni Enterprises (Educational Services Set-up)

How to cite: Niharika, P., P.P. Mahendran, P.M. Sundaram, J. Prabhaharan, A. Gurusamy, K. Chandrakumar, T.A. Naziba M. Prasanthrajan and M.M.R.A. Firnass: Enzyme-assisted protein extraction from *Sargassum muticum* using response surface methodology. *J. Environ. Biol.,* **47,** 237-246 (2026).

Introduction

Seaweeds are diverse and dominant group of photosynthetic macroalgae that play a crucial role in the aquatic ecosystems. They have been utilized as a dietary staple in many Asian countries for centuries and are highly valued for their biochemical properties across agriculture, pharmaceuticals, and food industries (Kadam *et al.,* 2017). Beyond agriculture, seaweeds are increasingly recognized as a sustainable protein source due to their balanced amino acid composition, which offer advantages over traditional plant-derived proteins and supports their use in food and feed industries (Kaur *et al.,* 2024). The growing demand for seaweed-based products has spurred expansion in the agricultural biostimulant market, accounting for more than one-third of the global share (Ma *et al.,* 2025). Seaweed extracts are widely utilized as biostimulants in modern agriculture as they contain antioxidant compounds that enhance the crop tolerance to biotic and abiotic stress. It offers a sustainable alternative to chemical fertilizers and pesticides for improving crop yields (Shukla *et al.,* 2016; Prajapati *et al.,* 2023).

Protein content in seaweeds varies across taxa, with red and green species generally containing 4–50% (w/w), while brown algae typically range from 1–29% (w/w) (Harnedy and FitzGerald, 2013). Despite this relatively lower protein content, *Sargassum muticum* was selected for the present study owing to its unique biochemical characteristics, abundant, low-cost availability and as a potential source of bioactive compounds. *S. muticumis* a member of Phaeophyceae family, which is widely distributed in tropical and temperate marine regions. It is rich in polysaccharides, carotenoids, dietary fiber and essential minerals (Mouga *et al.,* 2025). However, the protein potential of *S. muticum* remains limited. Moreover, protein extraction from seaweed is inherently difficult due to their rigid cell walls matrix composed mainly of alginates and carrageenans, which limit access to intracellular proteins (Gandhi *et al.,* 2024).

Traditional approaches such as alkali hydrolysis may alter the amino acid side chains and unfold proteins, reduce sits solubility and functional properties. Similarly, physical methods such as ultrasound and pulsed electric fields often require high energy inputs and can cause heat or shear-induced degradation of sensitive biomolecules (Vasquez *et al.,* 2019; Kadam *et al.,* 2017). To overcome these limitations, enzyme assisted hydrolysis has emerged as an alternative method, as it targets to break the polysaccharide matrices and facilitates the release of intact proteins without changing the functional properties. Moreover, this process can enhance the recovery of phenolic compounds, thereby improving the overall valorisation of algal biomass (Marshall and Oyekola, 2025).

Recently, enzyme-assisted seaweed extraction technologies have largely focused either on seaweed with higher protein content or on the recovery of individual classes of bioactive compounds. For instance, Phuong *et al.* (2022) demonstrated enhanced protein release from *Gracilaria gracilis* using enzyme-assisted extraction, while Nguyen *et al.* (2024) optimized the co-extraction of phenolics and polysaccharides from *Padina gymnospora* through enzyme hydrolysis coupled with Response Surface Methodology (RSM). Similarly, da Silva *et al.* (2025) also emphasized the eco-friendly strategies for recovering bioactive metabolites from brown seaweed (*Saccharina latissimi*). While several advances have been achieved in enzyme-assisted extraction from seaweeds, *S. muticum* has received little attention and its simultaneous recovery of proteins and phenolics through optimized enzymatic hydrolysis conditions has yet to be explored.

The present study addresses the existing limitations in the valorisation of *S. muticum* by integrating enzyme-assisted hydrolysis with RSM-based optimization to enhance the simultaneous recovery of proteins and phenolic compounds. Unlike earlier investigations that relied on conventional extraction methods or examined isolated parameters such as enzyme concentration for the recovery of single bioactive metabolites (Dinc *et al.,* 2024), this work adopts a comprehensive, multi-variable optimization strategy that explicitly incorporates time and temperature as critical process factors. Beyond maximizing extraction efficiency, the study advances its novelty by systematically characterizing the recovered proteins with the presence of functional groups, thereby linking extraction outcomes with potential applications. Therefore, the objective of this study was to optimize enzyme-assisted extraction conditions for the simultaneous recovery of proteins and phenolic compounds from *S. muticum* and to characterize the functional properties of the recovered extract.

Materials and Methods

Sargassum muticum was identified based on its morphological features following the standard taxonomic descriptions given by Yoshida (1998).It was collected from Mandapam (9°22'54.06"N; 78°77'40.08"E), Keelakarai, Ramanathapuram district, Tamil Nadu, India. The algal samples were thoroughly rinsed with seawater to remove impurities, sand particles, epiphytes and other surface contaminants and shade-dried for one week. Once completely dried, the samples were ground using a commercial grinder and the powder was stored under appropriate conditions for subsequent analysis.

The proximate composition of dried *Sargassum muticum* was analyzed using various standard procedures. The moisture content was determined by drying the samples in a hot air oven at 70°C for 72 hr (Horwitz, 1975), while the ash content was estimated by incinerating the samples in a muffle furnace at 550°C for 3 hr (Atwater, 1892). Crude protein content was quantified by the Kjeldahl method, with a nitrogen-to-protein conversion factor of 6.25 (Kjeldahl, 1883). The crude fibre content of the algal sample was estimated through sequential acid and alkaline hydrolysis, each carried out under reflux for 30 min followed by filtration of the insoluble residues (Henneberg and Stohmann, 1862). Total carbohydrate content was calculated as

the difference between 100 and the sum of the percentages of moisture, protein, lipidand ash (Maynard, 1970). All analyses were performed in triplicate and the results were expressed as percentages on a dry weight basis. The mineral content was determined by digesting the dried seaweed samples (0.5 g) in a HNO_3:H_2SO_4 acid mixture (9:2:1 v/v) as described by Jackson (1958). The digested solutions were diluted and analyzed for macro- and micronutrients, except for nitrogen. Nitrogen was estimated separately by the Kjeldahl method following H_2SO_4: $HClO_3$ acid digestion (5:2 v/v) (Kirk, 1950).

Enzymatic hydrolysis of *S. muticum* for protein extraction: Proteins were extracted from *Sargassum muticum* using enzyme cellulase. The seaweed powder was incubated with cellulase under varying enzyme-to-substrate (E/S) ratios, reaction time and temperature as defined by the experimental design. After hydrolysis, the mixtures were centrifuged at 9,000 rpm for 20 min at 4 °C and the supernatants were collected and stored at −20 °C for subsequent analysis. Optimization of hydrolysis conditions was performed using Response Surface Methodology (RSM), which enables systematic evaluation of process variables and identification of optimum conditions while reducing the number of experimental trials required (Yuan *et al.,* 2015; Wani *et al.,* 2017). Among the available RSM designs, the Box–Behnken Design (BBD) was chosen as it avoids extreme factor levels, reduces the number of experimental runs and provides robust estimates of curvature effects (Myers *et al.,* 2016). Three independent variables were considered: E/S ratio (A: 0.10–0.50), incubation time (B: 4–8 h), and temperature (C: 45–60 °C). The actual and coded levels of independent variables for Box–Behnken design was given in Table 1. Protein content (Y_1, mg protein/g dry weight) and phenolic content (Y_2, mg GAE/g dry weight) were selected as response variables. The BBD consisted of 17 experimental runs, including five replicates at the center point to ensure model adequacy and estimate pure error. The experimental data were fitted to a second-order polynomial regression model (Tekin *et al.,* 2015):

$$Y_i = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ij} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ii} x_i + \varepsilon \quad \text{– Equation 1}$$

Where, Y_1 represents the response variable; x denotes the independent variables; β_o is the intercept; β_i, β_{ii}, and β_{ij} represent the coefficients for the linear, quadratic, and interaction terms, respectively; k is the number of variables.

Bioactive compound analysis: Protein concentration in the enzymatic extracts was estimated by colorimetric method of Lowry *et al.* (1951) and the results were expressed as mg g^{-1} of sample. Total phenolic content was assessed by the Folin–Ciocalteu assay following the procedure outlined by Turkmen *et al.* (2005), with the values reported as milligrams of gallic acid equivalents (mg GAE) per gram. The experiments carried out in triplicate to ensure accuracy.

Characterization of protein extract: Fourier transform infrared spectroscopy (FT-IR) analysis was initiated to identify the functional groups present in both the dried seaweed and its extracted protein fractions. The spectra were recorded using a Perkin-Elmer Spectrum UATR Two FTIR spectrometer (PerkinElmer Inc., USA), equipped with a universal attenuated total reflectance (ATR).

Statistical analysis: Standard deviations were calculated where applicable, representing technical variation in the measurements rather than natural variability.The model's performance was assessed using statistical criteria, including coefficients of determination (R^2, adjusted R^2, predicted R^2), lack-of-fit tests and analysis of variance (ANOVA). Graphical visualization of the relationships between factors and responses was obtained from three-dimensional response surface plots generated using Design Expert® 13.0 software (Stat-Ease Inc., Minneapolis, USA). The model was validated experimentally by conducting hydrolysis under the given optimum conditions and observed values were compared with model predictions to confirm reliability.

Results and Discussion

The proximate composition of *Sargassum muticum* (Table 2) showed low moisture (10.3 ± 0.3%) and crude fat (2.7 ± 0.7%) contents, which are typical of dried brown seaweeds. In contrast, the ash content (28.0 ± 1.5%), crude protein (16.8 ± 1.2%), fibre (9.4 ± 0.2%) and carbohydrate (38.3 ± 1.0%) contents were comparatively higher, reflecting the mineral-rich and polysaccharide dominated nature of the algae. Similar compositional patterns have been reported for other *Sargassum* species, such as *S. wightii* and *S. thunbergii* with ash contents, crude protein, crude fat of 12.5 and 16.35%, 10.5± 1.0% and 12.3 ± 1.0%, 3.0 ± 0.7% and 0.8 ± 1.0%, respectively (Chatterjee and Mathew, 2023). Earlier studies have emphasized that brown algae usually contain less protein than red and green counterparts (Olsson *et al.,* 2020), yet the value obtained in the current investigation (~17%) remains substantial and emphasize *S. muticum* as a promising protein source among brown macroalgae. Mineral profiling of *S. muticum* revealed potassium

Table 1: Actual and coded levels of independent variables for Box–Behnken design

Input variables for extraction of proteins from *S. muticum*	Coded levels		
	-1	0	+1
A: Enzyme substrate ratio	0.10	0.30	0.50
B: Time (hours)	4	6	8
C: Temperature (°C)	45	52.5	60

Online Copy

Table 2: Proximal composition and nutrient profiling of *Sargassum muticum*

Proximal composition	Content (%)
Moisture	10.3 ± 0.3
Crude protein	16.8 ± 1.2
Crude fat	2.7 ± 0.7
Crude fibre	9.4 ± 0.2
Ash	28.0± 1.5
Carbohydrates	38.3 ± 1.0
Nutrients (g 100 g^{-1})	
Nitrogen	2.63 ±0.45
Phosphorous	0.44 ±0.02
Potassium	2.89 ±0.88
Calcium	2.33 ±0.32
Magnesium	1.18 ±0.11
Sulphur	0.23 ±0.67
Iron	0.09 ±0.02
Zinc	0.31±0.01
Copper	0.04 ±0.05
Manganese	0.01 ±0.07

Values are mean± S.D. and reflects technical error rather than biological variation.

(2.89 ± 0.88 g 100 g^{-1}) and nitrogen (2.63 ± 0.45 g 100 g^{-1}) as the dominant macronutrients, with zinc (0.31 ± 0.01 g 100 g^{-1}) being the most abundant micronutrient. These values are higher than those reported for *S. wightii* (K: 1.14 g 100 g^{-1}; Zn: 0.18 g 100 g^{-1}) and *S. thunbergii* (K: 0.87 g 100 g^{-1}; Zn: 0.14 g 100 g^{-1}) (Paul *et al.,* 2023), suggesting that *S. muticum* accumulates greater levels of essential minerals under the studied conditions. Although trace elements such as Fe, Cu, Zn and Mn occur in small quantities, their roles as enzyme cofactors and regulators of metabolic processes enhance the nutritional significance of *S. muticum*. Together, these findings establish *S. muticum* as a mineral-rich, polysaccharide-dominated species with moderate protein content reinforcing its suitability as a source for enzyme-assisted extraction of proteins and phenolics.

Enzyme-assisted extraction of *S. muticum* optimized using BBD was significantly influenced by the input factors, with temperature and time emerging as the most critical parameters (Table 3). In the present study, *S. muticum* achieved the maximum protein content of 141.3 mg g^{-1} or 14.1% dry weight under optimized conditions of enzyme substrate ratio of 0.50, an incubation time of 6 hrs and a temperature of 52.5°C. Beyond this range, the protein yield increase with increased in hydrolysis time and increased enzyme dosage (133.4 mg g^{-1} at E/S 0.5, 6 hours and 60°C), which could be due to the progressive breakdown of protein structures under higher concentration of enzymes. However, the protein yield got eventually stabilized after a certain point of time due to substrate saturation or partial enzyme inactivation (He *et al.,* 2013). On the other hand, the temperature exerted a dual effect: moderate conditions (45–60 °C) enhanced enzymatic activity and extraction efficiency, while excessive heating (>65 °C) reduced yield because of enzyme denaturation (Dinc *et al.,* 2024). The lowest protein content (83.1 mg g^{-1}) was recorded under the conditions of 0.1 E/S ratio, 4 hrs, and 52.5 °C. This finding suggests that enzyme concentration, even under favourable time and temperature, plays a decisive role in determining protein extraction efficiency. Although cellulase is crucial for disrupting the complex cell wall matrix and facilitating protein release, the unfavourable conditions like time and temperature also limits the extent of hydrolysis, resulting in reduced protein recovery. Thus, it is evident that effective extraction not solely depends on the enzyme concentration but also on its synergistic interaction with hydrolysis time and

Table 3: BBD with natural and coded extraction conditions and experimentally obtained values of output responses

Factor 1 A:Enzyme substrate ratio	Factor 2 B:Time (hours)	Factor 3 C: Temperature(°C)	Protein content (mg g^{-1})	Total phenol content (mg GAE g^{-1})
0.5	6	52.5	141.3	35.5
0.3	4	45	85.78	22.1
0.3	4	60	100	25.8
0.3	6	52.5	123.2	32.1
0.3	6	52.5	127.5	31.95
0.3	6	52.5	124.1	32.5
0.1	6	45	94	20.9
0.3	8	60	117.5	34.6
0.5	8	60	133.54	34.8
0.5	6	45	118.1	30.1
0.3	6	52.5	126.8	32.4
0.1	6	60	109	28.2
0.3	8	45	103.4	27.3
0.3	6	52.5	125.3	32.6
0.1	8	52.5	104	29.5
0.1	4	52.5	83.31	23.6
0.5	4	52.5	109.2	28.8

Online

Table 4: ANOVA for quadratic response surface model for protein and total phenol content

Source	Protein content (mg g^{-1})		Total phenol content (mg GAE g^{-1})	
	F-value	p-value	F-value	p-value
Model	73.08	< 0.0001*	315.23	< 0.0001*
A	229.11	< 0.0001	751.09	< 0.0001*
B	102.58	< 0.0001	769.97	< 0.0001*
C	68.79	< 0.0001	412.84	< 0.0001*
AB	0.1536	0.7068	7.66	0.0278
AC	1.13	0.3232	47.27	0.0002
BC	0.0002	0.9894	27.06	0.0013
A^2	2.33	0.1704	41.83	0.0003
B^2	129.65	< 0.0001	73.20	< 0.0001*
C^2	33.78	0.0007	340.09	< 0.0001*

temperature (Nguyen *et al.,* 2022). Recent studies further support the role of enzyme in effectively disturbing the cell wall to improve the protein recovery from seaweed. These are in line with other similar findings, for instance, Dinc *et al.* (2024) demonstrated that ultrasound-assisted enzymatic hydrolysis, optimized through RSM, enhanced protein extraction from *Sargassum vulgare* to yield a maximum protein content of 25.9%. Similar improvements were reported by Dhaouafi *et al.* (2024), where ultrasonication pretreatment increased protein recovery in the red seaweeds *Sphaerococcus coronopifolius* and *Gelidium spinosum*. Among the tested approaches, ultrasonication for 60 min proved effective, yielding protein content of 3.46 ± 0.06 mg g^{-1} and 9.73 ± 0.41 mg g^{-1} d.wt. in both the species, respectively.

In *Palmaria palmata*, the incorporation of cellulase enzyme increased the protein yield by 17% and improved the extraction efficiency from 9 to 37% (Suwal *et al.,* 2019). Collectively, these findings emphasize that protein recovery efficiency in macroalgae is strongly influenced by the choice of extraction strategy, particularly the application of enzyme-assisted or combined physical-enzymatic approaches. In line with these reports, the present study achieved protein yields of 8.3–14.1% dry weight from *S. muticum,* confirming that enzyme-assisted hydrolysis is an effective strategy for enhancing protein release from *S. muticum.* The regression model fitted for protein yield followed the second-order polynomial equation (Eq. 1). In the present study, the strength of the model predicted a second-order polynomial equation (Eq. 2, 3) in coded terms, effectively identifying the optimal conditions for maximizing the target responses.

$$\text{Protein content (mg g}^{-1}\text{)} = 125.72+13.98A+9.85B+8.06C \quad \text{(Eq. 2)}$$

$$+0.57AB+1.55AC+0.02BC$$

$$-1.92A^2-16.19B^2-8.26C^2$$

where, A is enzyme substrate ratio; B is the hydrolysis time in hrs and C is the temperature in °C.

Similar results were also achieved under the similar experimental condition for maximum phenol extraction (35.5 mg GAE g^{-1}). Total phenol content increased only upon a certain time and temperature, as phenolic compounds bound to proteins are released, while moderate temperature supports their stability and extraction (Tian *et al.,* 2025). Beyond this range, *i.e.,* at E/S ratio was 0.5, time at 8 hr and temperature at 60°C, the phenol content got slightly decreased to 34.80 mg GAE g^{-1} which could be due to degradation of the phenolic compounds due to thermal sensitivity. These results are in line with Paiva *et al.* (2016) who found that enzymatic hydrolysis of *Ulva rigida* using various carbohydrase and protease facilitated the release of phenolic compounds with strong antioxidant, antibacterial, and enzyme-inhibitory activities. Among the enzymes tested, viscozyme treatment yielded the highest total phenolic content, accounting for 6.4% of dry weight. Whereas, Gisbert *et al.* (2022) reported that ultrasound-assisted extraction, optimized using RSM, significantly enhanced the recovery of total phenolics, fucose and uronic acid from *Ascophyllum nodosum,* achieving yields of 14.3, 8.70 and 12.85%, respectively. This underscores the importance of optimized conditions for maximizing compound recovery.

This enhanced recovery is primarily due to the virulence of enzyme to cleave ester and glycosidic bonds more efficiently and release bound phenolic compounds into the extraction solution (Ara *et al.,* 2013). These findings reinforce the role of enzymatic extraction as a promising approach for efficiently releasing bioactive compounds, particularly phenolics and proteins from brown seaweeds. The lowest phenol content of 22.1 mg GAE g^{-1} was extracted under the conditions of 0.3 E/S ratio, 4 hr and 45 °C. This clearly explained the role of optimized time, temperature as an important parameter in extracting phenols efficiently. The regression model fitted for total phenol content followed the second-order polynomial equation (Eq. 1).

$$\text{Total phenol content (mg GAEg}^{-1}\text{)} = 32.3549+3.23A+3.48B+2.55C \quad \text{(Eq. 3)}$$

$$+0.52AB-1.29AC+0.84BC$$

Online Copy

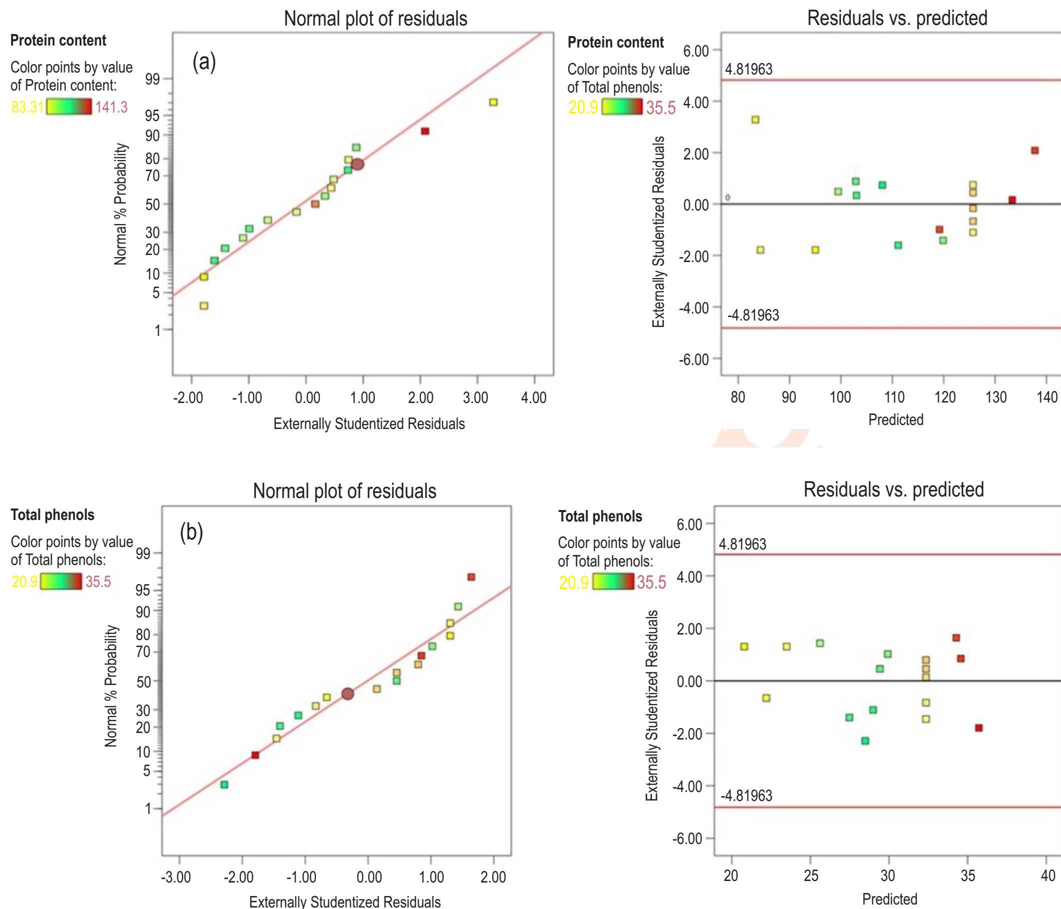


Fig. 1: Residual plotted graph graphs for (a) protein content and (b) total phenol content.

$$-1.055A^2-1.57B^2-3.38C^2$$

where, A is enzyme substrate ratio, B is the hydrolysis time in hr, C is the temperature in °C.

The ANOVA results also confirmed that the quadratic models were highly significant with large F-values of 73.08 and 315.23 for both protein and phenolic content, respectively (Table 4). The lack-of-fit tests were not significant, confirming that the model's adequately described the experimental data. Strong regression coefficients (R^2=0.98 for protein and 0.99 for phenolics; adjusted R^2= 0.97 and 0.99; predicted R^2=0.80 and 0.95) further validated the reliability and predictive power of the model. A strong agreement between the predicted and experimental values, further supported the model's reliability (Fig. 1). Three-dimensional response surface graphs showed the relationship of three variables at their best values. The plots had a distinct colour range within the design boundary, meaning that the highest protein content was achievable in that specified range (Fig. 2,3 for proteins and phenols). In addition, the coefficients of variation were low (2.27% for protein and 1.12% for phenolics), reflecting high experimental precision. Adequate precision values exceeded the standard threshold of 4, confirming that the models possessed sufficient signal-to-noise ratios to navigate the design space effectively. Such robustness is consistent with the earlier observations that high adequate precision is a strong indicator of reliable process optimization models (Demirel and Kayan, 2012).

FTIR-ATR analysis of the enzymatic extract with the highest protein yield (Fig. 4) confirmed the presence of characteristic functional groups associated with proteins and polysaccharides. A distinct absorption peak at 1635 cm^{-1} corresponded to the amide I band, which arises from C=O stretching vibrations in the peptide backbone and serves as a reliable marker of protein secondary structure. Another prominent band was observed at 3343 cm^{-1}, which was attributed to O–H stretching from polysaccharides and polyphenols, as well as N–H stretching from protein amide groups. These features are consistent with earlier studies on enzymatically hydrolyzed proteins from *Macrocystis pyrifera* and *Chondracanthus*

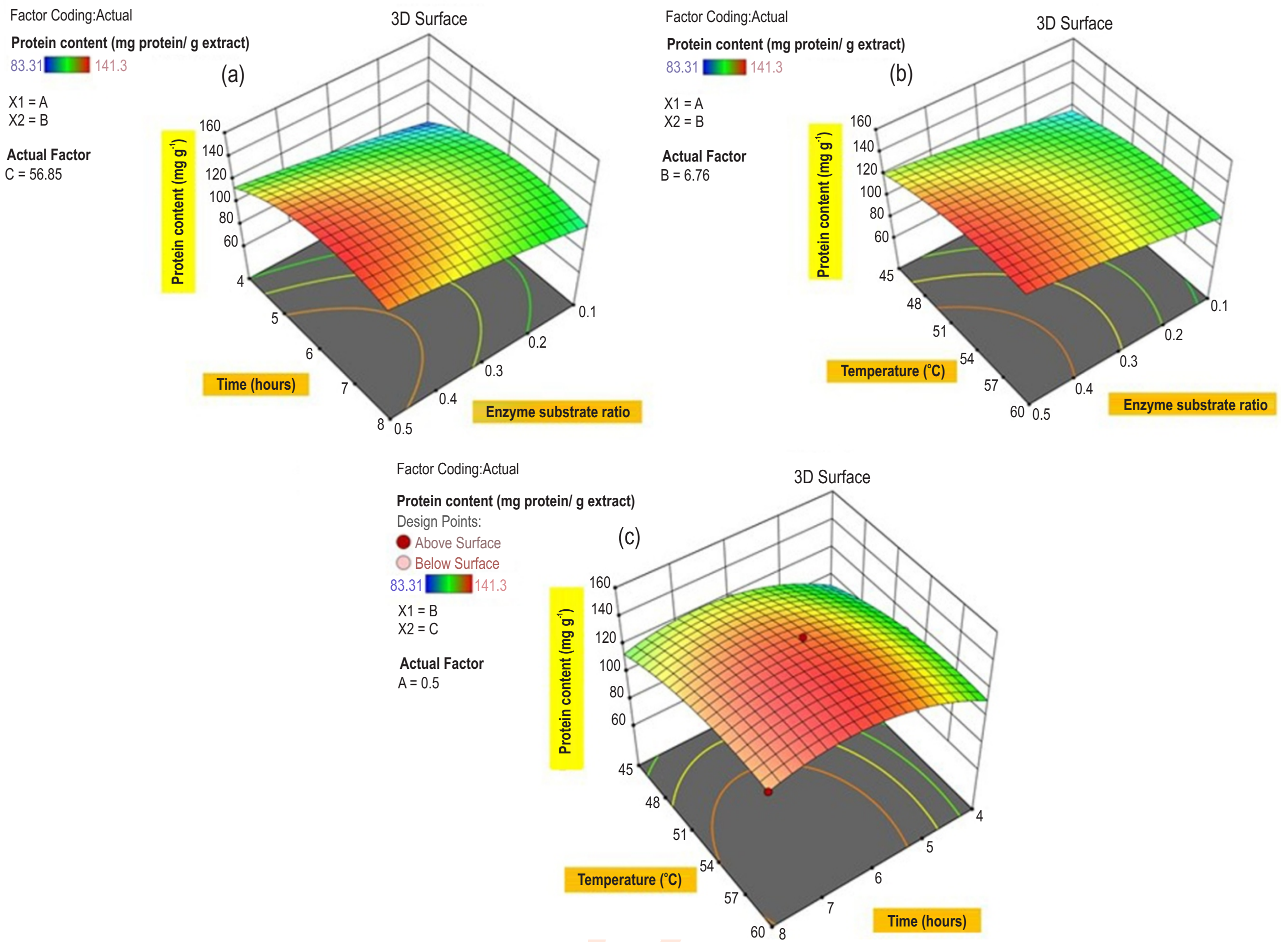


Fig. 2: 3D graphical representation of influence of input parameters on protein as response variable3D surface plots of protein content A. Effect of E/S ratio vs. time (hr) B. Effect of E/S ratio vs. temperature (°C) C. Effect of time (hr) vs. temperature (°C). The interactive effect is represented with color ranging from blue to red (blue, green, red); blue is least significant, green is moderately significant, and red is highly significant.

chamissoi, which showed broad peaks around 3380 cm^{-1} and amide- related bands at 1637 and 1544 cm^{-1} (Vasquez *et al.,* 2019).

Additional peaks were observed at 1396 cm^{-1}, corresponding to O–H bending of carboxylic acids whereas the peaks observed in 1350–1400 cm^{-1} range represent the sulphate ester groups (S=O). The latter are typically associated with sulphated polysaccharides, a characteristic feature of brown algae cell walls (Synytsya *et al.,* 2010). Similar spectral features have also been reported in methanol extracts of *Sargassum wightii,* where peaks between 1200 and 1390 cm^{-1} were attributed to C–H deformation and C–C stretching of carbohydrates (Rajeswari and Jeyaprakash, 2019). Furthermore, glycosidic bond vibrations detected near 658–890 cm^{-1} supported the presence of polysaccharides, as previously observed in alkali-treated *Melanothamnus somalensis* (Alawi *et al.,* 2018). Combined, these FT-IR observations confirm the successful recovery of proteinaceous compounds alongside polysaccharides in *S. muticum* extracts obtained under optimized enzymatic conditions. This study demonstrated that Response Surface Methodology effectively optimized enzyme-assisted extraction of *S. muticum.* The identified conditions enzyme-to-substrate ratio of 0.50, hydrolysis time of 6 hr and temperature of 52.5°C yielded maximum protein (141.3 mg g^{-1}) and phenolic content (35.5 mg GAE g^{-1}). The models displayed excellent statistical performance (R^2 = 0.98–0.99), confirming their predictive reliability. The protein and phenolic rich extract hold promise as a sustainable biostimulant and as a functional ingredient for food and nutraceutical industries. The enzymatic protein extraction of *Sargassum muticum* in this study was limited to a single enzyme system, which may not fully hydrolyze all protein fractions. Enzymatic extraction also has inherent limitations, including high enzyme costs, time-consuming processing, incomplete cell wall disruption and variability in protein solubility depending on pH and enzyme type. Hence, future research should focus on using alternative enzymes or

Fig. 3: 3D graphical representation of influence of input parameters on total phenol as response variableA. Effect of E/S ratio vs. time (hr) B. Effect of E/S ratio vs. temperature (°C) C. Effect of time (hr) vs. temperature (°C). The interactive effect is represented with color ranging from blue to red (blue, green, red); blue is least significant, green is moderately significant, and red is highly significant.

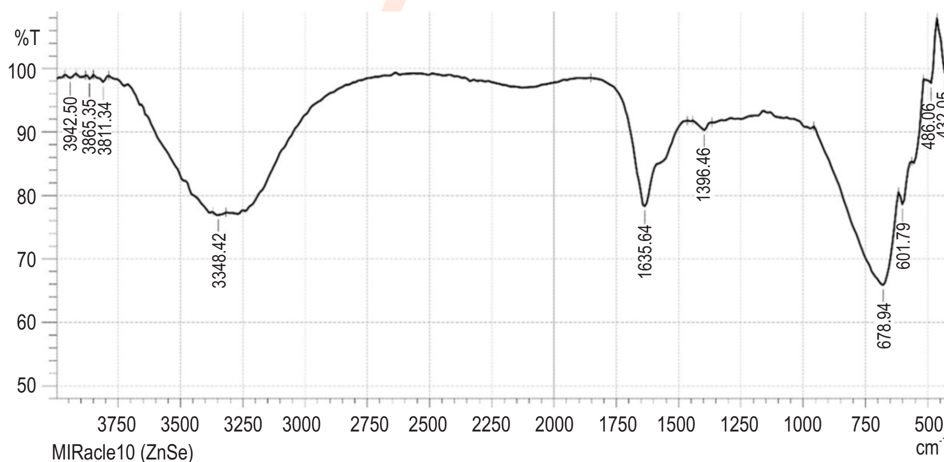


Fig. 4: FTIR spectra of the best performed protein extract.

optimized enzyme combinations. In addition, purification and functional characterization of protein fractions to be carried out and their efficacy as biostimulants should be assessed to enhance the applicability of these extracts in sustainable agriculture and related industries.

Acknowledgments

The authors would like to express their gratitude to the Centre of Innovation, AC&RI, Madurai, Tamil Nadu Agricultural University for providing laboratory facilities to complete the research successfully. The present work was financially supported by the Innovation in Science Pursuit for Inspired Research (INSPIRE) (grant no IF230027) awarded by the Department of Science and Technology, Govt of India.

Authors' contribution: P. Niharika: Conducted laboratory experiments, data interpretation and drafted the manuscript; **P. P. Mahendran:** Provided technical guidance, conceptualized the study and supervised the experimental design; **P.M. Sundaram:** Provided technical guidance and supervised the experiment; **J. Prabhaharan:** Assisted in data collection and preparation of figures; **A. Gurusamy:** Contributed to manuscript drafting and revision; **K. Chandrakumar:** Assisted with statistical analysis and ensured data accuracy; **M. Prasanthrajan:** Reviewed the manuscript and provided critical inputs for improvement; **T.A. Naziba:** Assisted with statistical analysis; **M.M.R.A. Firnass:** Assisted with preparation of figures and revised the manuscript.

Funding: DST INSPIRE grant no IF230027 awarded by the Department of Science and Technology, Govt of India.

Research content: The research content of this manuscript is original and has not been published elsewhere.

Ethical approval: Not applicable.

Conflict of interest: The authors declare no conflict of interest.

Data availability: All data analyzed in this study are included in the manuscript. Additional data, if required, can be obtained from the Corresponding author upon reasonable request.

Consent to publish: All authors agree to publish this paper in *Journal of Environmental Biology.*

References

Al-Alawi., P.C. Ahmed, A.M. Abdullah, A.M. Insaaf and S.R. Mohammad: Characterization of red seaweed extracts treated by water, acid and alkaline solutions. *Int. J. Food Eng.,* **14**, 20170353 (2018).

Ara, K.Z.G., S. Khan, S. Tejas, S. Kulkarni, P. Tania and N.K. Eva: Glycoside hydrolases for extraction and modification of polyphenolic antioxidants. In: Advances in enzyme biotechnology (P. Shukla and B. Pletschke), pp. 9-21 (2013).

Atwater, W.O.: The Science of Nutrition: Treatise upon the Science of Nutrition. Damrell & Upham, Boston, pp. 1-348 (1892).

Chatterjee, N.S. and S. Mathew: Evaluation of the proximate composition, minerals, and heavy metals of brown seaweeds: *Sargassum wightii* and *Sargassum thunbergii* collected from the mandapam coastal regions, Tamil Nadu. *Int. J. Zool. Appl. Biosci.,* **8**, 106-112 (2023).

da Silva, J., L.C. dos Santos, E. Ibañez and S.R.S. Ferreira: Green extraction methods applied to the brown macroalga *Saccharina latissima:* Assessing yield, total phenolics, phlorotannins and antioxidant capacity. *Foods,* **14**, 1017 (2025).

Demirel, M. and B. Kayan: Application of response surface methodology and central composite design for the optimization of textile dye degradation by wet air oxidation. *Int. J. Ind. Chem.,* **3**,1-10 (2012).

Dhaouafi, J., N. Nedjar, M. Jridi, M. Romdhani and R. Balti: Extraction of protein and bioactive compounds from Mediterranean red algae (*Sphaerococcus coronopifolius* and *Gelidium spinosum*) using various innovative pretreatment strategies. *Foods.*, **13**, 1362 (2024).

Dinc, H., E. Şencu, U. Altuntaş, E.S. Okudan, B. Özçelik and A. Yücetepe: Ultrasound-assisted enzymatic extraction of antioxidative protein extracts from *Sargassum vulgare*: optimization of extraction parameters using RSM. *Gıda.,* **49**, 439-452 (2024).

Gandhi, G., K. Biswas, P. Vaghela, J. Nayak, A. Nair, K. Moradiya, V.A.K. Gopalakrishnan, V. Veeragurunathan and A. Ghosh: In-depth metabolite characterization of seaweed-based plant biostimulants: Insights into bioactive components. *Algal Res.,* **81**, 103574 (2024).

Gisbert, M., J. Sineiro and R. Moreira: Polyphenols extraction kinetics from *Ascophyllum nodosum* seaweed employing water and saltwater- Effect of ultrasound sonication. *Algal Res.,* **66**, 102773 (2022).

Harnedy, P.A. and R.J. FitzGerald: Extraction of protein from the macroalga *Palmaria palmata.* LWT-Food Science and Technology., **51**, 375-382 (2013).

He, S., C. Franco and W. Zhang: Functions, applications and production of protein hydrolysates from fish processing co-products (FPCP). *Food Res. Int.,* **50**, 289–297 (2013).

Henneberg, W., F. Stohmann and F. Rautenberg: Ueber die Bestimmung von Hippursäure, Harnstoff und Kochsalzim Harn der Pflanzenfresser, und über die Zusammensetzung desselben bei verschiedenem Futter. *Justus Liebigs Ann. Chem.,* **124**, 181-203 (1862).

Horwitz, W.: Official Methods of Analysis of the Association of Official Analytical Chemists. 12th Edn., AOAC, Washington, D.C., pp. 1-1094 (1975).

Jackson, M.L.: Soil Chemical Analysis. Prentice-Hall Inc., Englewood Cliffs, New York, pp. 1-498 (1958).

Kadam, S.U., C. Álvarez, B.K. Tiwari and C.P. O'Donnell: Extraction and characterization of protein from Irish brown seaweed *Ascophyllum nodosum*. *Food Res. Int.,* **99**, 1021-1027 (2017).

Kaur, M., T. Shitanaka, K.C. Surendra and S.K. Khanal: Macroalgae-derived bioactive compounds for functional food and pharmaceutical applications—a critical review. *Crit. Rev. Food Sci. Nutr.,* **65**, 4172–4194 (2024).

Kirk, P.L.: Kjeldahl method for total nitrogen. *Anal. Chem.,* **22**, 354-358 (1950).

Kjeldahl, J.: New method for the determination of nitrogen. *Chem. News,* **48**, 101-102 (1883).

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **193**, 265-275(1951).

Ma, C., P. Wang, W. Tao, B. Zhou, Q. Wang and M. Deng: Seaweed biostimulant supplementation improves maize resilience to saline-

alkaline stress. *Plant Stress*, **17**, 100933 (2025).

Marshall, A. and O. Oyekola: Effects of the chemical and mechanical pre-treatment of brown seaweed on biomethane yields in a batch configuration. *Biomass,* **5**, 7 (2025).

Maynard, A.J.: Methods in Food Analysis. Academic Press, New York, 176 pages (1970).

Mouga, T., M.M. Almeida, F.I. Pitacas, A.M. Rodrigues, C. Vitória and O. Anjos: Elemental and nutritional characterisation with vibrational spectroscopy analysis of *Ulva sp., Gracilaria multipartita,* and *Sargassum muticum. Appl. Sci.,* **15**, 4212 (2025)

Myers, R.H., D.C. Montgomery and C.M. Anderson-Cook: Response Surface Methodology: Process and Product Optimization Using Designed Experiments. 4th Edn., John Wiley & Sons, Hoboken, NJ, pp. 1-857 (2016).

Nguyen, H.C., K.N. Ngo, H.K. Tran and C.J. Barrow: Enzyme-assisted co-extraction of phenolics and polysaccharides from *Padina gymnospora. Mar. Drugs,* **22**, 42 (2024).

Nguyen, D.L.D., L.T.T. Hoa, N.T.X. Quynh and N.D. Nguyen: Protein extraction from *Spirulina platensis* with the cellulase enzyme assistance. *J. Tech. Educ. Sci.,* **17**, 25–32 (2022).

Olsson, J., G.B. Toth and E. Albers: Biochemical composition of red, green and brown seaweeds on the Swedish west coast. *J. Appl. Phycol.,* **32**, 3305-3317(2020)

Paiva, L., E. Lima, A.I. Neto and J. Baptista: Isolation and characterization of angiotensin I-converting enzyme (ACE) inhibitory peptides from *Ulva rigida* C. Agardh protein hydrolysate. *J. Funct. Foods.,* **26**, 65-76 (2016).

Paul, P., J. Joy, F.T. Salim, A. Pradeep, N. Chatterjee and S. Mathew: Evaluation of the proximate composition, minerals, and heavy metals of brown seaweeds *Sargassum wightii* and *Sargassum thunbergii* collected from the Mandapam coastal regions, Tamil Nadu. *Int. J. Zool. Appl. Biosci.,* **8**, 106-112 (2023).

Phuong, H., A. Massé, J. Dumay, L. Vandanjon, H. Mith, J. Legrand and A. Arhaliass: Enhanced liberation of soluble sugar, protein, and R-phycoerythrin under enzyme-assisted extraction on dried and fresh *Gracilaria gracilis* biomass. *Front. Chem. Eng.,* **4**, 718857 (2022).

Prajapati, S.K., P. Dayal, V. Kumar and M. Kumari: The plant bio-stimulant properties of seaweed extracts: Potential to mitigate climate change for sustainable agriculture. *Food Sci. Rep.,* **4**, 70-75 (2023).

Rajeswari, R. and K. Jeyaprakash: Bioactive potential analysis of brown seaweed *Sargassum wightii* using UV-VIS and FT-IR. *J. Drug Deliv. Ther.,* **9**, 150-153 (2019).

Shukla, P.S., T. Borza, A.T. Critchley and B. Prithiviraj: Carrageenans from red seaweeds as promoters of growth and elicitors of defense response in plants. *Front. Mar. Sci.,* **3**, 81 (2016).

Suwal, S., V. Perreault, A. Marciniak, E. Tamigneaux, E. Deslandes, L. Bazinet, H. Jacques, L. Beaulieu and A. Doyen: Effects of high hydrostatic pressure and polysaccharidases on the extraction of antioxidant compounds from red macroalgae, *Palmaria palmata* and *Solieria chordalis. J. Food Eng.,* **252**, 53-59 (2019).

Synytsya, A., W.J. Kim, S.M. Kim, R. Pohl, A. Synytsya, F. Kvasnička, J. Čopíková and Y.I. Park: Structure and antitumour activity of fucoidan isolated from sporophyll of Korean brown seaweed *Undaria pinnatifida. Carbohydr. Polym.,* **81**, 41-48 (2010)

Tekin, K., M.K. Akalın and M.G. Şeker: Ultrasound bath-assisted extraction of essential oils from clove using central composite design. *Ind. Crops Prod.,* **77**, 954-960 (2015).

Tian, Y., D. Deng, H. Luo, M. He and S. Wang: Ultrasonic assisted enzymatic extraction, structural characterization and tyrosinase inhibition mechanism of phlorotannins from *Sargassum horneri. Algal Res.,* **88**,104038(2025).

Turkmen, N., F. Sari and Y.S. Velioglu: The effect of cooking methods on total phenolics and antioxidant activity of selected green vegetables. *Food Chem.,* **93**,713-718 (2005).

Vasquez, V., R. Martínez and C. Bernal: Enzyme-assisted extraction of proteins from the seaweeds *Macrocystis pyrifera* and *Chondracanthus chamissoi*: Characterization of the extracts and their bioactive potential. *J. Appl. Phycol.,* **31**, 1999-2010 (2019).

Wani, S.M., N. Jan, T.A. Wani, M. Ahmad, F.A. Masoodi and A. Gani: Optimization of antioxidant activity and total polyphenols of dried apricot fruit extracts (*Prunus armeniaca* L.) using response surface methodology. *J. Saudi Soc. Agric. Sci.,* **16**, 119-126 (2017).

Yoshida, T.: Marine Algae of Japan. Uchida Rokakuho Publishing Co., Ltd., Tokyo, pp. 1-1222 (1998).

Yuan, J., J. Huang, G. Wu, J. Tong, G. Xie, J.A. Duan and M. Qin: Multiple responses optimization of ultrasonic-assisted extraction by response surface methodology (RSM) for rapid analysis of bioactive compounds in the flower head of *Chrysanthemum morifolium* Ramat. *Ind. Crops Prod.,* **74**, 192-199 (2015).

online copy