Drying Technology

An International Journal

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/ldrt20

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To cite this article: K. R. Karthika Parvathy , M. Ajanth Praveen , P. Balasubramanian & Bibekanand Mallick (2020): Impact of advanced extraction technologies and characterization of freeze-dried brown seaweed polysaccharides, Drying Technology

To link to this article: https://doi.org/10.1080/07373937.2020.1842440

Published online: 10 Nov 2020.

DRYING TECHNOLOGY
https://doi.org/10.1080/07373937.2020.1842440

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Impact of advanced extraction technologies and characterization of freeze-dried brown seaweed polysaccharides

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ABSTRACT

The main objective of the study is to evaluate the extraction efficiency methods followed by freeze-drying of bioactive brown seaweed polysaccharides (BSPs). Brown seaweeds such as *Sargassum wightii, Spatoglossum asperum, Colpomenia sinuosa, Padina tetrastromatica* were considered in this study. Conventional solvent extraction (CSE), and advanced extraction techniques such as microwave-aided (MAE), ultrasound-aided (UAE), and enzyme-aided extractions (EAE) were attempted. MAE (2.5 GHz, 15 min) and UAE (50–60 kHz, 30 min) require minimum time and found to be thermal effective compared to the CSE method. EAE exhibited the maximal extraction efficiency of BSPs and *S. wightii* shown high polysaccharide content (54.99%). The antioxidant activity of all the extracted polysaccharides was evaluated and found to be maximum with *P. tetrastromatica*. Prebiotic activity assays were attempted on all EAE polysaccharides. Based on antioxidant and prebiotic activity, enzyme-assisted *P. tetrastromatica* extract was chosen to study for its characteristics using sophisticated analytical techniques. This study signifies the intertwined characteristics of antioxidant properties and shelf life along with its prebiotic activity of freeze-dried bioactive compounds for its desired purpose as a functional ingredient.

ARTICLE HISTORY
Received 31 March 2020
Revised 8 October 2020
Accepted 11 October 2020

KEYWORDS
Brown seaweeds; dietary fiber; prebiotics; freeze-dried; polysaccharides

1. Introduction

Marine macroalgae are untapped prebiotic resources rich in carbohydrates, proteins, vitamins, and minerals that gained an attraction to be explored for its therapeutic properties on functional food development.[1,2] Prebiotics are non-digestible oligosaccharides, which enhances the growth of beneficial bacteria (*Lactobacilllus* and *Bifidobacterium* species) that act as food to them.[3] Prebiotics improves bone development by increasing the availability of minerals like calcium, hydroxyapatite for improved bone growth.[4] Macroalgae can be classified based on pigmentation and chemical composition as brown, red, and green seaweeds, which are enriched with carbohydrates (polysaccharides) that are species-specific. There are sulfated and non-sulfated polysaccharides that are characterized by its sugar residues and the nature of bonds between them. Dietary fibers are edible carbohydrate polymers that present naturally in consumed food. Brown seaweeds contain total dietary fiber of 25–70%, in which soluble dietary fiber constitutes 50–80%.[5] Brown seaweeds are predominantly abundant in sulfated polysaccharides such as laminarin, fucoidan, and alginates which had shown antibacterial, antioxidant, and immune-modulating activity.[6] Fucoidans stimulate resistance against Alzheimer's disease, disorders of the kidney and urinary system.[7]

The extraction technique influences the effectiveness in bioactivity of seaweed polysaccharides while time, cost, and yield are other important parameters to be considered for choosing the appropriate method. Consequently, the evolution of emerging technologies like microwave-aided extraction (MAE), ultrasound-aided extraction (UAE), and enzyme-aided extraction (EAE) were developed. In a conventional solvent extraction method (CSE), a stepwise methodology that includes the utilization of chloroform, t-butanol, formaldehyde for removing proteins and other contaminants was adopted.[8] MAE influenced by microwave heating which is generated by ionic conduction due to dissolved ions and dipole rotation of polar solvent.[9] The efficiency of UAE is based on the quantum of sound waves produced and creating cavitation under pressure and heat results in the extraction of polysaccharides.[10] EAE helps the substances to hydrolyze

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enzymatically that digests the bonds in polymer bonds and release out the bioactive components.[11] The structural complexity and cell wall rigidity hinders the efficient extraction of intracellular bioactive compounds.[12] The CSE method is not suitable for functional food development because of its toxicity, time consumption, poor selectivity, efficiency yield, high solvent necessity, and presence of remaining residues.[13] MAE is desirable because of less solvent utilization, enhanced yield, automated process, and minimum time requirement with a limitation of loss in bioactive property due to intense heating. UAE has a huge possibility for large-scale extraction of bioactive compounds because of its high efficiency and selectivity.[14] Although the enzyme cost is high, EAE is mostly preferred in a large scale for its high catalytic efficiency, non-toxicity, eco-friendly, and desired food-grade level.[10,15]

Freeze-drying (FD) is a technique that involves a low-temperature dehydration process by freezing the product. Research suggested that the dehydration process alters the nutritional properties in a sample but several influencing factors like drying conditions and pretreatment method could retain the nutritional and functional properties of the desired product.[16] This technology transforms the unstable, heat-sensitive, and value-added bioactive compounds into stable ingredients in food industries to increase the storage stability of BSPs. *Sargassam wightii, Spataglossum asperum, Colpomenia sinousa*, and *Padina tetrastromatica* are the most abundant brown seaweed species in the southern coastal region of India, which were not studied much earlier and investigated as a source of bioactive components for functional food development. Only certain studies have reported the utilization of microwave, ultrasound, and enzymes for polysaccharide extraction.[10,13] The purpose of the study is to assess the yield efficiency of various advanced extraction techniques such as CSE, MAE, UAE, and EAE of brown seaweed polysaccharides (BSPs). Also, the extracted polysaccharides were subjected to partial purification followed by freeze-drying techniques. The partially purified polysaccharides from all the studied BSPs were subjected to evaluation of its antioxidant activities, prebiotic assays along with detailed structural and functional characterization. Such studies would aid in comprehending the potential ingredients from BSPs on functional foods.

2. Materials and methods

2.1. Materials

Brown seaweeds of interest (*S. wightii, S. asperum, C. sinousa, P. tetrastromatica*) were collected from the coastal regions of Tuticorin, Tamilnadu (8.7642°N, 78.1348°E) of India. Seaweeds were washed with distilled water thrice to eliminate the salt, epiphytes, and other debris. Subsequently, it was shade dried, milled, and stored in an airtight container until further use. Chemicals were procured from Sigma Aldrich (USA), and all other reagents were of analytical grade. Enzymes (protease-300 tyrosine units/mL, α-amylase-3000 units/mL, amyloglucosidase-3300 units/mL) were purchased from Megazyme kit (K-TDFR- 100A, Ireland).

2.2. Proximate composition

The total carbohydrate and protein content was estimated using Anthrone and Bradford methods, respectively.[17] The total dietary fiber was calculated by enzyme gravimetric method (AOAC – 991.43) as described by Lee et al.[18] The fat (AOAC – 2003.05) and crude fiber were assessed by acid and alkaline hydrolysis through soxhlet extraction method[19]; moisture (AOAC – 925.10), and ash or mineral content (AOAC – 923.03) were evaluated by the combustion process.

2.3. Extraction methods

The powdered seaweed samples were added with cold water and subjected to continuous stirring for three hours to remove impurities and then defatted by treating with petroleum ether followed by air drying. The protein content was removed by adding with Sevag reagent (Chloroform: t-butanol – 4:1) and air-dried. Henceforth, these samples were subjected to various extraction techniques like CSE, MAE, UAE, and EAE.

2.3.1. Conventional solvent extraction

Conventional solvent extraction was carried out by following the method outlined in Moumita et al.[20] The defatted and protein removed solid biomass was mixed with deionized water (1:6) and then incubated in a water bath at 50 °C for ten hours. The mixture was centrifuged (5000 rpm, 20 min) and the supernatant was collected and precipitated with three volumes of ethanol. Thus, the partially purified carbohydrate pellet was obtained by centrifuging at 5000 rpm for 10 min and stored after freeze-drying. The BSP extract of concentration 1 mg/mL was prepared and used for further analysis. The total carbohydrate in the extract was evaluated by the Anthrone method.[17]

2.3.2. Microwave-aided extraction

Microwave-aided extraction was done in a microwave oven [Electrolux- 2.4 GHz] with the defatted and protein removed sample mixing with deionized water (1:6) for 15 min to hydrolyze through the volumetric distribution of heat energy. The mixture was centrifuged, and carbohydrate pellets were obtained as similar to CSE.

2.3.3. Ultrasound-aided extraction

Ultrasound-aided extraction was carried out as per the method outlined in Kadam et al.,[6] (2015) with minor modification of utilizing defatted and deproteinized sample instead of crude ones. The sample with deionized water (1:6) was subjected to ultrasonication using lab sonicator probe (Sartorius, India) (15 mm diameter) with 50–60 kHz frequency for 30 min at an amplitude of 80%. A break time gap of 2 min was maintained for every 10 min cycle. The mixture was centrifuged, and carbohydrate pellets were obtained as similar to CSE.

2.3.4. Enzyme-aided extraction

Enzyme-aided extraction was carried out based on methods outlined in Lee et al.[18] with minor modifications. The crude powdered sample of weight 1.00 g was taken in 40 mL of 0.05 M MES-TRIS buffer of pH 8.2. The mixture was kept in an orbital shaker for 10 min to maintain homogeneity. α-amylase (50 μL) was added and kept in a boiling water bath (100 °C) for 30 min with continuous stirring. The mixture was cooled to 60 °C, and the beaker wall was scraped and rinsed with 10 mL of distilled water. Protease solution (100 μL) was added and incubated in a water bath at 60 °C for 30 min. Five milliliters of 0.561 N HCl was added, and the pH was adjusted to 4.5 using 1 N HCl and 1 N NaOH. Amyloglucosidase solution (200 μL) was added and kept in a water bath at 60 °C for 30 min. The mixture was centrifuged, and the collected supernatant was precipitated by adding three volumes of preheated (50 °C) 95% ethanol and left undisturbed overnight to collect the precipitate through vacuum filtration.

2.4. Antioxidant activities

The extracted brown seaweed polysaccharides were subjected to freeze-drying for future laboratory analysis. One mg/mL of partially purified, freeze-dried BSPs were prepared and used throughout the study. Radical scavenging activities of DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) were determined according to methods outlined in Fan et al.[21] Ferric reducing power assay (FRAP) was assessed using the method of Yıldırım et al.[22] Total phenolic content (TPC) and total flavonoid content (TFC) were estimated according to the protocol followed by Waterhouse[23] and Chang et al.,[24] respectively.

2.5. Prebiotic activity assay

A quantitative approach for the selectivity of prebiotics was carried out based on the fact that the enumeration of lactic acid bacteria (LAB) will be directly dependent on the increase in prebiotic activity and vice-versa. The stock cultures of probiotic LAB strains (*Lactobacillus plantaram* NCIM 2083 and *Bifidobacterium longum* NCIM 5684) were grown in MRS broth (de Man, Rogosa, and Sharpe) and enteric pathogen (*Escherichia coli* DH5α) were grown in Nutrient broth, respectively. Then their growth in glucose, inulin (commercial prebiotic) as a positive control[25] and samples (Enzyme-assisted polysaccharides of *S. wightii, S. asperum, C. sinuosa,* and *P. tetrastromatica*) were evaluated as outlined by Anprung and Sangthawan.[26] An overnight culture 1% (v/v) of LAB strains and enteric strain were inoculated in MRS and Nutrient broth with 1% (w/v) glucose or 1% (w/v) commercial prebiotic or 1% (w/v) extracted BSP samples. The cell load was counted (CFU/mL) for both the LAB strains and pathogen at 0 and 24 h in MRS and EMB (Eosin Methylene Blue) agar plates, respectively. The prebiotic score was calculated as given in Eq. (1).

$$\text{Prebiotic score} = \left[\frac{\log P_X^{24} - log\ P_X^0}{\log P_G^{24} - log\ P_G^0}\right] - \left[\frac{\log E_X^{24} - log\ E_X^0}{\log E_G^{24} - log\ E_G^0}\right] \quad (1)$$

where P_X, E_X are CFU/mL of LAB and enteric strain against prebiotics; P_G, E_G are CFU/mL of LAB and enteric strain against glucose, respectively.

2.6. Characterization of extracted polysaccharide

Seaweeds are ample sources of bioactive compounds that are rich in anionic sulfated polysaccharides. The exploration for natural antioxidants replacing the synthetic antioxidants is gaining attention at present for developing a functional food. Therefore, the best extraction method with good antioxidant and prebiotic properties was chosen for further chemical characterization. Hence, detailed characterization was

carried out with the polysaccharide of *P. tetrastromatica* by EAE method in this study.

2.6.1. Environmental scanning electron microscopy

Environmental scanning electron microscope (Nova Nanosem 450, USA) was used to examine the morphological features of seaweed polysaccharide (freeze-dried) samples.[27] Freeze-dried EAE polysaccharide sample (*P. tetrastromatica*) was fixed over a black carbon tape and observed for its structural characteristics.

2.6.2. Ultraviolet–visible spectral analysis

The EAE-based polysaccharide from *P. tetrastromatica* was examined using UV–Vis Spectrophotometer (Shimadzu, Japan). The polysaccharides extract was subjected to a spectral scan of 200–800 nm.[28]

2.6.3. Fourier transform infrared spectroscopy

Freeze-dried EAE polysaccharide from *P. tetrastromatica* extract (1 mg/mL) was investigated using Fourier Transform Infrared (FTIR) Spectrophotometer (Shimadzu 8201PC, Japan). FTIR spectroscopy was carried out in the frequency range of 4000 cm^{-1} to 400 cm^{-1} for the identification of functional groups.[29]

2.6.4. X-Ray diffraction analysis

Freeze-dried EAE polysaccharide (*P. tetrastromatica*) was subjected to Ultima IV diffractometer (Japan) for its X-ray diffraction (XRD) pattern, which was noted at 2-theta range of 5–80° with a step size of 0.05° and a counting time of 20°/min.[30] The particle size was calculated from the graph obtained by plotting 2-theta value against intensity using Scherrer equation as given in Eq. (2).

$$D_p = \frac{0.94 \times \lambda}{\beta \times \cos\theta} \quad (2)$$

where D_p is the average crystallite size in nm, β is the line broadening in radians, θ is the Bragg angle in degree, and λ is the wavelength of X-ray spectrum in nm.

2.6.5. Nuclear magnetic resonance spectroscopy

Freeze-dried EAE polysaccharide (3 mg) of *P. tetrastromatica* was dissolved in 0.5 mL of 99% deuterium oxide (D_2O). The spectra were recorded in a frequency of 400 MHz, an acquisition time of 5.29 s and pulse duration of 11 ms, using Ultrashield Bruker 300 spectrometer (Germany).[30] Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy was performed to detect the hydrogen bonding and shift in the chemical structure of polysaccharides present in the sample.

2.6.6. Sugar composition and short-chain fatty acid profiling

Freeze-dried EAE polysaccharide of *P. tetrastromatica* was prefiltered (0.2 μm) and the filtrate (20 μL) was analyzed by High-Performance Liquid Chromatography (HPLC), (Shimadzu, Japan) with Hiplex H column (Agilent, USA) and refractive index detector. Sulfuric acid (mobile phase, 5 mM), with a flow rate of 0.7 mL/min and temperature of 60 °C were maintained.[27] The peaks obtained were compared with the standards retention time of mono and disaccharide sugars such as glucose, sucrose, galactose, fructose, ribose, maltose, xylose, and arabinose. The bacterial culture broth (*B. longum* NCIM 5684 and *L. plantarum* NCIM 2089) was added with 1% v/v extracted polysaccharide (EAE *P. tetrastromatica*) and incubated for 24 h followed by centrifugation for the removal of bacterial pellets. The collected supernatant was prefiltered and subjected to HPLC analysis following above-indicated conditions for evaluating the presence of short-chain fatty acids (SCFA) like acetic acid, propionic acid, butyric acid, and ethanol.

2.7. Statistical analysis

All experiments were carried out in triplicates. Mean and standard deviations were calculated, and data were reported in standard format with error bars. Statistical calculations were made using the OriginPro 8.5 and differences were evaluated.

3. Results and discussion

3.1. Proximate composition

Four species of Indian brown seaweeds such as *S. wightii, S. asperum, C. sinousa,* and *P. tetrastromatica* of Phaeophyceae family were collected for comprehending the proximal composition of polysaccharides and evaluating the extraction efficiency of dietary fibers. In the present study, the maximum dietary fiber was found in *S. wightii* (54.99%) followed by *S. asperum* (50.92%), *C. sinousa* (45.7%), and *P. tetrastromatica* (44.36%) which was shown in Figure 1a. The maximum protein content was noted in *S. wightii* (13.13%) followed by *S. asperum* (12.29%), *P. tetrastromatica* (11.55%), and *C. sinousa* (10.68%). The fat content of the brown seaweeds varied across 1 to 2.84%. The maximum fat content was found in *S. wightii* (2.84%). It was followed by *P. tetrastromatica* (2.4%), *S. asperum* (2.18%), and *C. sinousa* (1%). The ash or mineral content ranges across 28.15% to 34.35%. *P. tetrastromatica* holds the highest ash content (34.35%) followed by *C. sinousa* (33.25%),

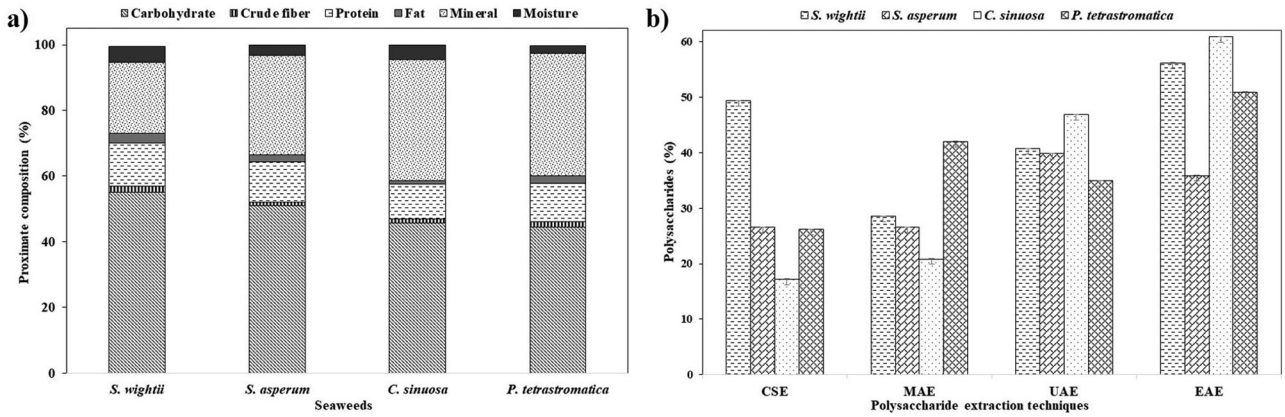

Figure 1. (a) Proximate composition analysis of selected brown seaweeds and (b) Comparison of total polysaccharide yield of brown seaweeds by different extraction methods [CSE: conventional solvent extraction; EAE: enzyme-aided extraction; MAE: microwave-aided extraction; UAE: ultrasound-aided extraction].

S. asperum (30.65%), and *S. wightii* (28.15%). The moisture content was found to be in the range from 2.3% (*P. tetrastromatica*) to 4.78% (*S. wightii*).

The proximate composition of the seaweeds is mainly influenced by seasonal changes and species. The protein content is usually high in green and red seaweeds when compared to the brown seaweeds. The range of protein content in brown seaweeds is low as 3–15% compared to the green and red seaweeds with 10–47%.[31] In the current study, the protein content of brown seaweeds was found to be in the range of 10–13% which was quite similar to reports of Zubia et al.,[32] Manivannan et al.,[33] and Murakami et al.[34] Fat content is generally low in seaweeds compared to the terrestrial plants. Protein, fat, and ash content in *C. sinousa* stated by Manivannan et al.[35] and Tabarsa et al.[36] were found to be in the same range that supports the present investigation. In general, the dietary fiber content of the seaweeds ranged between 33 and 62%, which is greater than the scale found in terrestrial plants.[37] The total dietary fiber in *S. wightii* was found to be 54.99% which is in similar to the results of 44–53% reported by Kumar.[31] The total dietary fiber content in other brown seaweeds were as follows: *S. horneri* (42-55%), *S. hemiphyllum* (61.3%), *S. patens* (54.8%), and *S. polycytum* (39.67%).[32,34] Ash content is usually high in seaweeds, which contain the mineral and trace elements that enhance human nutrition, and it varies from species, seasonal changes, and environmental origin. *Sargassum* species contain ash content that ranges from 24.37% (*S. echinocarpum*) to 42.4% (*S. polycystum*).[37]

3.2. Extraction methods

An extraction technique plays a major role in recovering the desired product in a shorter extraction time with yield improvement. In the present study, EAE was found to be the desirable method for extracting BSPs due to its extraction efficacy and specificity by targeting the desired end product. The extraction efficiency was calculated based on total carbohydrate percentage (Figure 1b) as estimated by the Anthrone method.[17] It was found that total carbohydrate in the yield to be 15–45% for CSE, 20–40% for MAE, 30–50% for UAE, and 50–60% for EAE methods. The total carbohydrate in the crude aqueous extract was observed to be $12.05 \pm 0.001\%$ for *S. wightii*, $16.47 \pm 0.09\%$ for *S. asperum*, $18.04 \pm 0.03\%$ for *C. sinuosa*, and $12.98 \pm 0.02\%$ for *P. tetrastromatica*. In CSE, maximum carbohydrate yield was obtained in *S. wightii* ($49.44 \pm 0.029\%$) and the minimum was noted in *C. sinousa* ($17.2 \pm 0.0115\%$). In MAE, maximum carbohydrate yield was obtained in *P. tetrastromatica* ($42.05 \pm 0.038\%$), and the minimum was noted in *C. sinousa* ($20.89 \pm 0.037\%$).

In UAE, maximum carbohydrate yield was obtained in *C. sinousa* ($46.91 \pm 0.003\%$) and the minimum was noted in *P. tetrastromatica* ($35 \pm 0.026\%$). In EAE, maximum carbohydrate yield was obtained in *C. sinousa* ($60.86 \pm 0.017\%$) and the minimum was noted in *S. asperum* ($35.92 \pm 0.042\%$). UAE (50–60 kHz, 30 min) and MAE (2.5 GHz, 15 min) require minimum time and found to be effective with improved efficiency of 5–25% compared to the CSE method that demands the operation time of 24 h. The inclusion of particular enzymes α-amylase, protease, and amyloglucosidase improved extraction efficiency and, at the same time, not much influence on sugar yield when compared with the corresponding solvent extraction. The polysaccharides yield as of reported literature includes *A. nodosum* 56–90%,[38] *L. digitata* 38–52%,[39] and *S. longicruris* 20.0%.[40] Okolie[38]

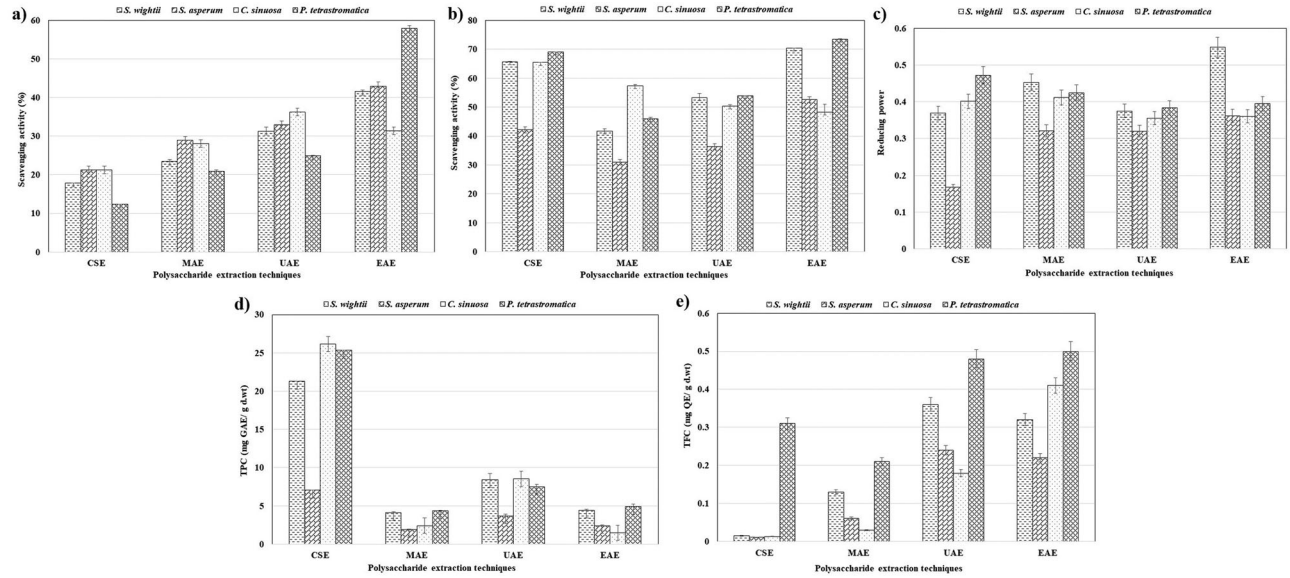

Figure 2. (a) DPPH radical scavenging activity, (b) ABTS radical scavenging activity, (c) Ferric reducing power activity, (d) Total phenolic content and (e) Total flavonoid content of various brown seaweed polysaccharides by different extraction methods [CSE: conventional solvent extraction; EAE: enzyme-aided extraction; MAE: microwave-aided extraction; UAE: ultrasound-aided extraction].

reported that polysaccharide extract such as alginate by UAE (70.15%) and EAE (90.32%) was significantly higher than CSE and MAE (56.35%). Charoensiddhi et al.[13,15] reported EAE enhances yield by 5–20% than the CSE, and higher yield of almost 70% was obtained with EAE (protease – alkalase and flavourzyme). The enzyme functions by attacking the cell wall, linkages, and bonds of the crude sample which intends to release the desired product like polysaccharides from it. The extracted algal/seaweed polysaccharides were partially purified with ethanol precipitation for removing the low molecular weight impurities from BSPs. These partially purified polysaccharides were utilized further for the studies of antioxidant, prebiotic properties, and structural characterization.

3.3. Antioxidant activities

Antioxidants are characterized to be oxygen scavengers and chelators intricate in the formation of free radicals. Seaweeds naturally possess potent free radical scavenging activity that might be related to the presence of phenolic and flavonoid compounds in it. The antioxidant activities of the polysaccharide extract obtained by different extraction methods were evaluated by performing DPPH, ABTS, and FRAP assays. The components (total phenolic and flavonoid content) responsible for antioxidant activity were also determined. Higher antioxidant activities were noted in the enzyme-assisted extraction as shown in Figure 2a. Percentage of DPPH antioxidant activity in the CSE was found to be maximum for *C. sinousa* and *S. asperum* (21.3 ± 0.003%) with the minimum for *P. tetrastromatica* (12.44 ± 0.002%). In MAE, *C. sinousa* (28.12 ± 0.28%) and *S. asperum* (28.93 ± 2.31%) revealed high DPPH scavenging activity and *P. tetrastromatica* with less scavenging activity (20.90 ± 2.39%). In UAE, the results were quite similar to that of *C. sinousa* (36.24 ± 0.28%) and *S. asperum* (32.99 ± 1.01%), which exhibits high antioxidant activity and *P. tetrastromatica* (24.91 ± 0.08%) with less scavenging property. In EAE, the maximum DPPH scavenging property was noticed in *P. tetrastromatica* (57.99%) and *S. asperum* (43.01 ± 2.59%), which may be due to several factors such as effective lysis of cell wall, external heat, pressure, and other extraction conditions.

The ABTS radical scavenging activity was identified to be high for the enzymatic method of extraction followed by CSE, UAE, and MAE (Figure 2b). In EAE, *P. tetrastromatica* (73.46 ± 1.49%) and *S. wightii* (70.38 ± 0.13%) exhibited higher ABTS radical scavenging activity. In CSE, *P. tetrastromatica* (69.03 ± 0.038%) exhibited higher antioxidant activity. In MAE, *C. sinousa* (53.88%) and *P. tetrastromatica* (45.93%) found to have higher ABTS scavenging activity.

FRAP activity was evaluated based on its absorbance value, where the higher absorbance indicates greater FRAP activity.[22] The reducing activity was found to be maximum in *P. tetrastromatica* followed by *S. wightii* and *C. sinousa* among the seaweeds (Figure 2c). The reducing power activity of *S. asperum* was found to be low in all BSP extracts by various extraction methods.

Table 1. Prebiotic activity score of polysaccharides obtained through enzyme-assisted extraction of brown seaweeds and commercial prebiotic.

	Prebiotic score				
Lactic acid bacterial strain	Inulin	*S. wightii*	*S. asperum*	*C. sinuosa*	*P. tetrastromatica*
L. plantaram NCIM 2083	0.77 ± 0.05	0.93 ± 0.09	0.87 ± 0.06	0.49 ± 0.09	1.05 ± 0.04
B. longum NCIM 5684	0.63 ± 0.03	0.76 ± 0.01	0.56 ± 0.03	0.44 ± 0.01	0.89 ± 0.02

Values represent mean ± standard deviation, $n = 3$.

The phenolic content was found to be higher in BSP extracts of CSE followed by UAE, EAE, and MAE (Figure 2d), which is based upon high polar nature. Mostly in all methods, *P. tetrastromatica* revealed high phenolic content that helps to exhibit higher antioxidant properties by ABTS assays. The phenolic content of *P. tetrastromatica* in terms of gallic acid equivalents (GAE) per gram dry weight was found to be higher in CSE (116.65 ± 0.007 mg GAE), UAE (37.55 ± 0.22 mg GAE), EAE (24.5 ± 0.22 mg GAE), and MAE (21.95 ± 0.063 mg GAE). It was reported that the presence of phenolic compound promotes antioxidant activity and Fe^{2+} chelating activity.[41]

The flavonoid content was observed to be high in EAE because of the complete breakdown of cell wall. *P. tetrastromatica* was found to exhibit more flavonoid content out of all the different extraction methods as shown in Figure 2e. The flavonoid content in terms of quercetin equivalents (QE) per gram dry weight was found to be higher in *P. tetrastromatica* [EAE (0.50 ± 0.065 mg QE), UAE (0.48 ± 0.014 mg QE), MAE (0.21 ± 0.036 mg QE), and CSE (0.031 ± 0.003 mg QE)]. The flavonoid and phenolic components are responsible for the free radical scavenging activity. The phenol content was found to be higher in CSE rather than EAE method. The polarity of the CSE plays a major role in releasing the phenolic compounds because of the presence of functional group –OH. The enzymes decompose the cell wall effectively and digest the internal material that stores the flavonoid content.

3.4. Prebiotic activity assay

The efficient prebiotics is chosen based on their ability to selectively ferment by the LAB strains present in the gut.[42] Prebiotics are rich in polysaccharides which rapidly metabolize the probiotics and adhere in the epithelial membrane of the gut. Therefore, remaining enteric pathogens which are not able to consume prebiotics are flushed out from the body. The positive value represents that the given substrates (glucose, prebiotic and seaweed polysaccharides) are metabolized by the LAB strains and not by other gut microbes. *L. plantaram* NCIM 2083 and *B. longum* NCIM 5684 had positive prebiotic score when grown on all prebiotics. *P. tetrastromatica* shows a considerably higher prebiotic score of 1.05 ± 0.04 and 0.89 ± 0.02 for *L. plantaram* NCIM 2083 and *B. longum* NCIM 5684, respectively. The order of prebiotic score for *L. plantaram* was *P. tetrastromatica* > *S. wightii* > *S. asperum* > Inulin > *C. sinuosa* and in the case of *B. longum* NCIM 5684 was *P. tetrastromatica* > *S. wightii* > Inulin > *S. asperum* > *C. sinuosa*. The utilization of various prebiotics by gut microbes (LAB and other bacteria) are occurring through the presence of specific metabolic transport and hydrolysis systems.[43] The prebiotic score for various brown seaweeds subjected to EAE and commercial prebiotic were enlisted in Table 1.

3.5. Characterization of extracted polysaccharide

Amongst the various extraction methods carried out in the present study, EAE showed better results in terms of antioxidant and prebiotic properties. Moreover, enzyme-assisted *P. tetrastromatica* extract showed comparatively improved antioxidant activity than other extraction techniques and other seaweeds. Therefore, the detailed characterization of this particular seaweed, *P. tetrastromatica* has been carried out. The good antioxidant material could enhance additional shelf-life property which is an essential criterion for developing functional food in future studies.

3.5.1. Environmental scanning electron microscopy

The microscopical images depicted that freeze-dried polysaccharide (*P. tetrastromatica*) were aggregates of irregular shape rough surface like clouds with wrinkles as compared to the crude raw powdered sample (Figure 3a,b). The surface morphology of the polysaccharides might be affected by the extraction and purification process.[27] The texture could be improved by using other techniques like spray drying or spray-freeze drying techniques and also by making a combination with encapsulation agents like maltodextrin and gum arabic. The freeze-drying technique influences the retention ability of the material inside matrix, which might help the structure of extracted

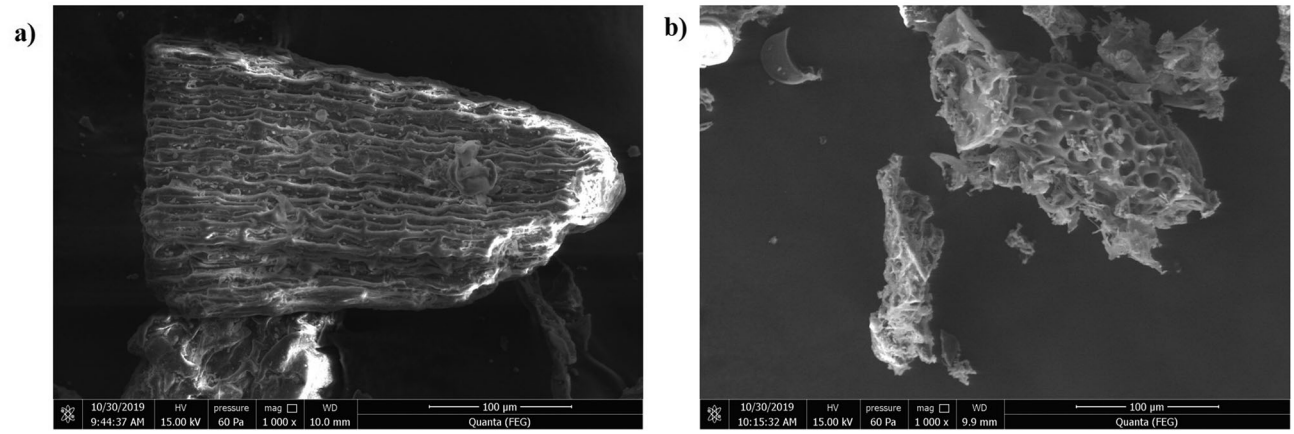

Figure 3. Environmental scanning electron microscopy for structural analysis of *P. tetrastromatica,* (a) Raw/crude powdered sample and (b) Polysaccharide obtained through enzyme-assisted extraction after freeze-drying.

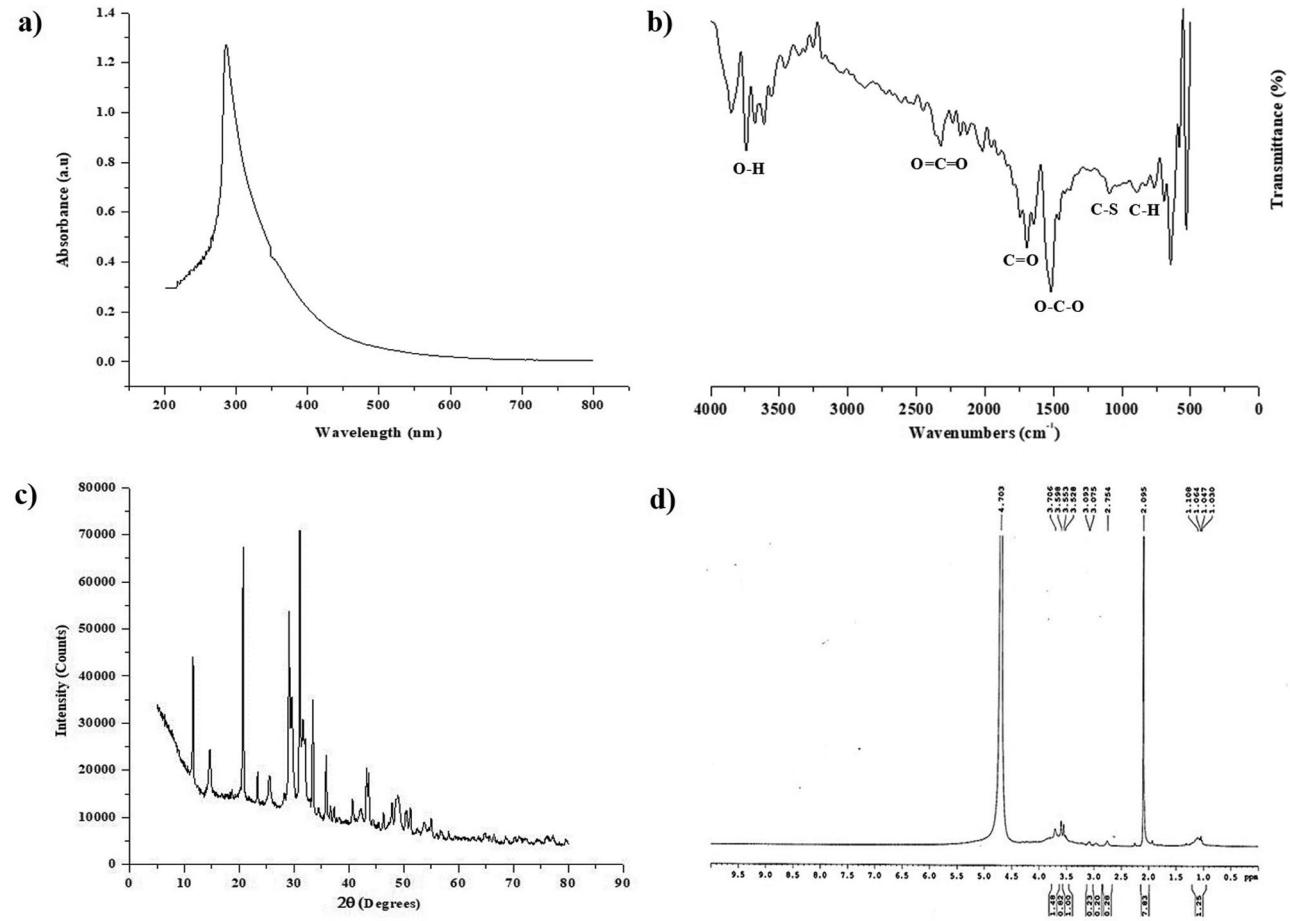

Figure 4. (a) Ultraviolet–Visible spectrum, (b) Fourier Transform Infrared spectrum, (c) X-ray Diffraction spectrum and (d) Nuclear Magnetic Resonance (^{1}HNMR) spectrum of polysaccharide obtained through enzyme-assisted extraction from *P. tetrastromatica* sample.

polysaccharide molecules to be smooth and regular in shape.[44]

3.5.2. Ultraviolet–visible spectral analysis

The enzyme-assisted polysaccharide (*P. tetrastromatica*) extract subjected to UV–Vis spectral scan from 200 to 800 nm had shown maximal absorption at 285 nm, which was due to be the presence of flavonoid components that are responsible for antioxidant activity.[45] The peak at 285 nm is observed at a longer wavelength and occurs when the chromophore group is attached to an aromatic ring[46] as represented in Figure 4a.

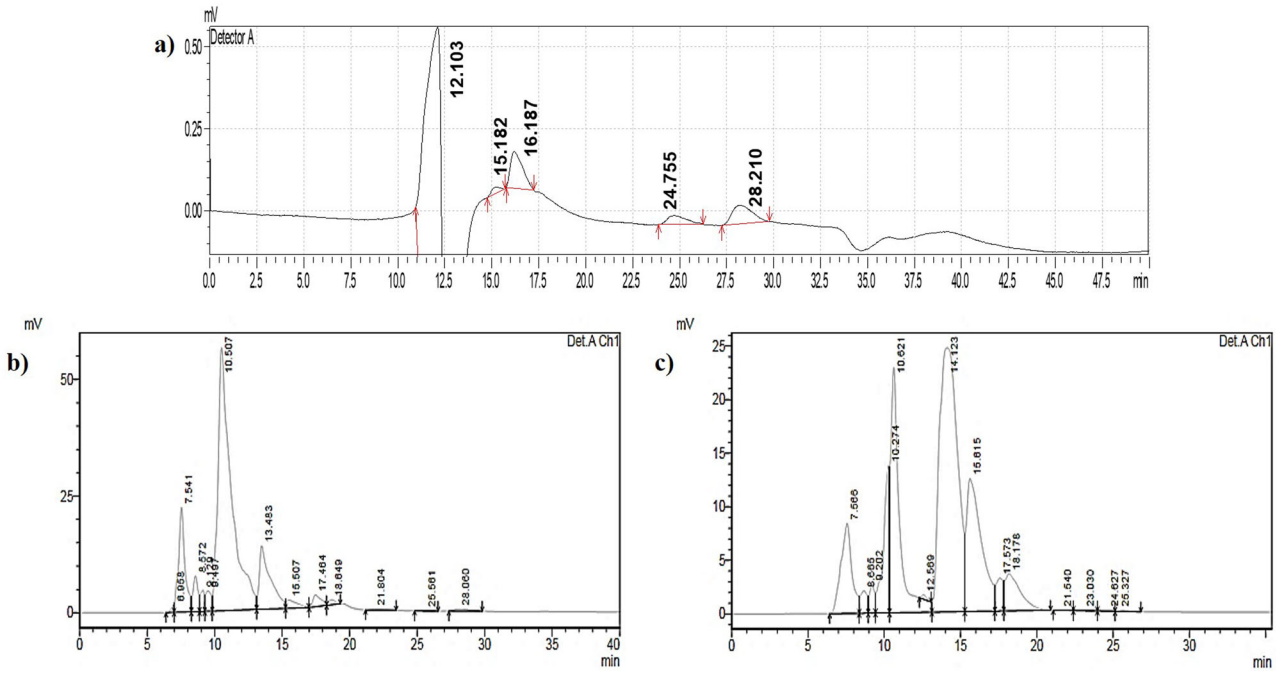

Figure 5. (a) Sugar composition profiling, (b) Short-chain fatty acids profiling for *Bifidobacterium longum* NCIM 5684 and (c) Short-chain fatty acids profiling for *Lactobacillus plantarum* NCIM 2083 of polysaccharide obtained through enzyme-assisted extraction from *P. tetrastromatica* sample.

3.5.3. Fourier transform infrared spectroscopy

The FTIR spectrum reveals the functional groups and chemical bonds present in EAE polysaccharides of freeze-dried *P. tetrastromatica* as shown in Figure 4b. The pattern of the recorded signals at 1456.1 cm^{-1} was attributed to scissoring vibration of CH_2 (galactose and xylose), asymmetric bending vibration of CH_3 (fucose and O-acetyls) and 1372.4 cm^{-1} was bending vibration of a methyl group which are the characteristic wavenumbers of a polysaccharide.[47] The peaks at 1519.3 cm^{-1} and 1698.7 cm^{-1} indicates the O–C–O asymmetric stretching and C = O asymmetric bending vibrations of uronic acids.[48] Also, the signal at 887.1 cm^{-1} attributes C–H deformation of β-mannuronic acid. The spectra at 1715.1 cm^{-1} correspond to the alginate of brown seaweed.[29] The spectral region of 2000 to 1700 cm^{-1} indicates the aromatic overtones of the benzene ring vibrational mode with stretching frequencies. The peaks after 3600 cm^{-1} are called as background peaks that are commonly noticed in brown seaweed species of *Sargassum* and *Padina*[49] and *Colpomenia peregrine*.[50]

3.5.4. X-Ray diffraction analysis

The crystallite size (D) of the freeze-dried seaweed polysaccharide (*P. tetrastromatica*) was determined from XRD plot (Figure 4c). The crystallite size was found to be 18.65 nm for *P. tetrastromatica* regarding the Scherrer equation and found to be semi-crystalline. The prominent 2θ values were at 11.612°, 20.712°, 29.063°, 31.045°, and 33.398°, respectively. The 2θ value of around 31° for lyophilized seaweed polysaccharide with a degree of crystallinity to be 35% was reported earlier.[27]

3.5.5. Nuclear magnetic resonance spectroscopy

The obtained ^{1}H NMR peaks (Figure 4d) are correlated with previously reported data. The spectra at 4.703 ppm and chemical shift at 3.5 to 3.7 ppm revealed the peaks correspond to laminarin and signal enhancement derived from mannitol, which is a characteristic of brown algae. Polysaccharides of brown seaweeds majorly constitute laminarin, mannitol and uronic composition of alginic acid.[51,52] Ulvan (Sulfated glucuronorhamnoxylan) composed of 4-linked L-rhamnose-3-sulfate and D-xylose residues (ulvobiose) with monomeric D-glucuronic acid or D-glucuronic acid-3-sulfate on O-2 of some L-rhamnose-3-sulfate units as the side chains alternatively.[53]

3.5.6. Sugar composition and short-chain fatty acids profiling

Sugar components present in enzyme-aided polysaccharide (*P. tetrastromatica*) extract was determined and compared the retention time with their standards (as mentioned in section 2.6.6) using HPLC. The chromatogram peaks were noted at a retention time of 15.182, 16.187, 24.755, 28.210 min as shown in

Figure 5a. The peaks obtained were found at 15.182 and 16.187 min were galactose and sucrose, respectively. The other two peaks might be corresponding to fucose, glucuronic acid, or mannuronic acid which was reported by Okolie et al.[10] who were using the same Hiplex H column and mobile phase. The fermented broth of both prebiotic (*P. tetrastromatica*) and probiotics (*B. longum* NCIM 5684 and *L. plantaram* NCIM 2083) were observed after 24 h for essential short-chain fatty acids (SCFA) like propionic acid, acetic acid, and butyric acid (Figure 5b,c). Here, *B. longum* was able to utilize *P. tetrastromatica* and produce acetic acid and propionic acid, whereas, *L. plantaram* produces all the three essential SCFA of acetic acid (15.5 min), propionic acid (18.1 min), and butyric acid (23.03 min) as compared with its standards, respectively.

4. Conclusion

The extraction efficiency, antioxidant activities, and prebiotic score for four brown seaweed polysaccharides with various advanced extraction techniques were evaluated in this study. The polysaccharides extracted by enzyme-assisted extraction techniques after partial purification showed maximum yield up to 50–70% of total dietary fiber content in *S. wightii*. Also, *P. tetrastromatica* exhibited potent antioxidant activity, which had good scavenging ability amongst the other brown seaweeds. The order of prebiotic score for *L. plantaram* was *P. tetrastromatica* > *S. wightii* > *S. asperum* > Inulin > *C. sinuosa* and for *B. longum* NCIM 5684 was *P. tetrastromatica* > *S. wightii* > Inulin > *S. asperum* > *C. sinuosa*. Furthermore, EAE polysaccharides of *P. tetrastromatica* were studied for its structural characteristics and FTIR confirmed the presence of sulfate groups and glycosidic linkages in the polysaccharides. NMR characterization revealed the existence of laminarin by chemical shift and hydrogen bonding in its structure. In conclusion, *P. tetrastromatica* had an essential ingredient for developing a functional food to improve health benefits.

Acknowledgement

The authors thank the Department of Life Science and the Department of Biotechnology and Medical Engineering of National Institute of Technology Rourkela for extending the required research facilities. The authors express their sincere thanks to the (late) Dr R. Jayabalan for his support and guidance.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The authors acknowledge the Department of Science and Technology (DST) of Government of India for funding the research under DST-WOS-B (Women Scientist Scheme – B category) [SR/WOS-B/282/2016(G)] awarded to the first author.

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