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Research article

Optimization of one-step enzymatic hydrolysis conditions for two large brown algae and analysis of the antioxidant activity of the hydrolysis products ☆

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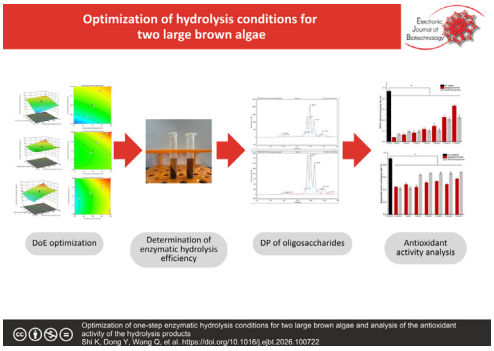
GRAPHICAL ABSTRACT

Optimization of one-step enzymatic hydrolysis conditions for two large brown algae and analysis of the antioxidant activity of the hydrolysis products



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ABSTRACT

Background: The industrial production of alginate lyase provides high-quality tool enzymes for the enzymatic hydrolysis of large brown algae. Establishing a seaweed enzymatic hydrolysis process that is simple to operate while offering both high enzymatic hydrolysis efficiency and a high yield of oligosaccharides contributes to the high-value utilization of large brown algae.
Results: A one-step enzymatic hydrolysis process combining alginate lyase, pectinase and cellulase was adopted to conduct enzymatic hydrolysis experiments on *Sargassum horneri* and *Saccharina japonica*, respectively. The Box-Behnken experimental design was applied to optimize the dosage of the three enzymes, as well as the temperature, pH and reaction time of enzymatic hydrolysis. The results showed that the optimal enzymatic hydrolysis conditions for *S. horneri* were as follows: alginate lyase 490 U/g, pectinase 4660 U/g, cellulase 380 U/g, hydrolysis temperature 47°C, hydrolysis pH 5.5 and hydrolysis time 8 h. For *S. japonica*, the optimal enzymatic hydrolysis conditions were alginate lyase 360 U/g, pectinase 5000 U/g, cellulase 284 U/g, hydrolysis temperature 49.5°C, hydrolysis pH 5.2 and hydrolysis time 8.8 h. After process optimization, the solid hydrolysis rates of *S. horneri* powder and *S. japonica* powder

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Saccharina japonica
Sargassum horneri
Seaweed oligosaccharides

were increased to 71.4% and 66.3%, respectively, and the proportion of low-molecular-weight alginate oligosaccharides (DP < 6) in the hydrolysates reached approximately 70%; both enzymatic hydrolysates exhibited favorable antioxidant activity according to the detection.
Conclusions: The findings of this study greatly simplify the technological process for preparing alginate oligosaccharides via brown algae enzymatic hydrolysis and significantly improve the production efficiency.
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1. Introduction

Brown algae, characterized by rapid growth, high photosynthetic efficiency, and non-dependence on arable land, serve as important raw materials in industries such as food, feed, health products, pharmaceuticals, and chemicals. *Sargassum horneri* and *Saccharina japonica* are both economically significant large brown algae species with wide global distribution. Particularly for *S. japonica*, extensive cultivation areas exist along the coastal regions of China, and its annual production has been steadily increasing year by year [1,2]. Against the backdrop of a global research boom in the development and utilization of marine biological resources, *S. horneri* and *S. japonica*, as abundant large brown algae, have become a research hotspot in the field of marine biotechnology due to the potential for in-depth exploration of their functional components. In China, *S. horneri and S. japonica* are also used as a major feed ingredient in the cultivation of valuable marine delicacies such as sea cucumbers and abalones.

S. horneri and *S. japonica* are rich in compounds including glyceroglycolipids, phytosterols, mixed terpenoids, phlorotannins, and nitrogen-containing compounds. They exhibit various biological activities such as anti-tumor, antioxidant, lipid-lowering, and antiviral effects [3,4], and have potential applications in industries including agriculture, medicine, and food. Currently, the development and application of brown algal polysaccharides and oligosaccharides have become major research hotspots.

Oligosaccharides are short-chain carbohydrates with low molecular weight. Generally, the degree of polymerization (DP) of the sugar groups is between 3 and 10, which is between monomeric monosaccharides and highly polymerized polysaccharides [5]. Brown algal oligosaccharides refer to sugar chains composed of 2–20 monosaccharide units. They are characterized by low molecular weight, good water solubility, safety, non-toxicity, and possess functions such as antioxidant activity, immune regulation, bacteriostasis, anti-inflammation, and anti-tumor effects [6]. They are attracting increasing attention in agricultural and medical fields. When added to aquatic feed, brown algal oligosaccharides can act as prebiotics that modulate intestinal flora, thereby improving feed utilization efficiency. They can also enhance metabolism and nutrient absorption by upregulating related gene expression, leading to improved growth performance in aquatic animals [7,8]. Treatment of plants with brown algal oligosaccharides can significantly enhance their stress and disease resistance [8,9]. Additionally, these oligosaccharides and their derivatives show potential for the prevention and treatment of major human diseases such as cardiovascular diseases, Alzheimer's disease, and tumors [10,11,12].

At present, the main processes for preparing brown algal oligosaccharides from large brown algae include chemical degradation, enzymatic degradation, physical degradation, and microbial fermentation [13]. Currently, the chemical preparation method typically employs acid hydrolysis, which directly cleaves the glycosidic bond between C1 and C4 in alginate to yield low-molecular-weight products without generating new structures. However, chemical degradation has drawbacks such as an uncontrolled reaction process, difficulty in obtaining products with specific molecular weights, as well as environmental pollution and energy waste resulting from the use of acidic substances and high-temperature operations [14]. In contrast, enzymatically produced brown algal oligosaccharides are unsaturated oligomers with low molecular weight and stable structure, effectively retaining the biological activity of oligosaccharides. The enzymatic reaction consumes less energy, can be conducted under mild conditions, offers high efficiency, and is more environmentally friendly [15]. Nevertheless, due to the lack of efficient enzymatic tools and optimized enzymatic process conditions for brown algal hydrolysis, most current methods for preparing brown algal oligosaccharides still rely primarily on chemical approaches.

S. horneri and *S. japonica* contain significant amounts of indigestible components, such as alginate, fucoidan, and cellulose. These constituents form a complex macromolecular network structure through tight glycosidic bonds, which hinders the full release and absorption of nutrients and bioactive substances [16]. In the compound enzyme system, pectinase first breaks down the pectin binding layer to provide action sites for other enzymes. Alginate lyases degrade alginate polymers into oligosaccharides or monosaccharides. Cellulase hydrolyzes the 1,4-β-D-glycosidic linkages in cellulose. Enzymatic hydrolysis of *S. horneri* and *S. japonica* using composite enzymes under suitable conditions can break down indigestible alginate into oligosaccharides and monosaccharides, thereby enhancing its nutritional value [17]. With the industrial production of enzyme preparations such as alginate lyase, efficient enzymatic tools are now available for the preparation of brown algal oligosaccharides from large brown algae.

Response Surface Methodology (RSM) is an effective statistical optimization method that can detect the impact of interactions between two or more variables on response values [18,19]. It uses statistical methods to design experiments with the minimum number of trials and also enables optimization of factor levels and response outcomes [20]. Therefore, RSM can reduce reagent consumption and significantly decrease experimental workload [21]. In this study, the optimal one-step enzymatic hydrolysis process for *S. horneri* and *S. japonica* was determined using RSM. The hydrolysis efficiency and the composition of brown algal oligosaccharides in the hydrolysate were analyzed. The results will provide a reference for the enzymatic production and preparation of oligosaccharides from *S. horneri* and *S. japonica*.

2. Materials and methods

2.1. Materials

2.1.1. Enzymes

Alginate lyase (10,000 U/g, Vland Biotech Inc., Qingdao, China); pectinase (100,000 U/g, Beijing Psaitong Biotechnology Co., Ltd.,

Beijing, China); cellulase (8000 U/g, Vland Biotech Inc., Qingdao, China).

2.1.2. S. horneri and S. japonica powders

S. horneri and *S. japonica* were both purchased from Rizhao, Shandong Province, China. The algae were rinsed thoroughly with flowing fresh water three times, 5 min per rinse, to remove surface sediments, salt residues, and planktonic attachments. Subsequently, the algae were air-dried in a well-ventilated area, cut into 2 cm segments, and transferred to an electric blast drying oven. The drying process was conducted at a temperature of 60°C and an air velocity of 1.5 m/s for 12 h until a constant weight was achieved (the mass difference was ≤ 0.1% for two consecutive hours of weighing). The dried algae were subjected to ultrafine pulverization, sieved through a 300-mesh sieve, and stored in sealed bags at 25°C until subsequent use.

2.2. Methods

2.2.1. Enzymatic hydrolysis procedure

S. horneri as well as *S. japonica* were processed in the same manner was as follows. 0.5 g of dried algal ultrafine powder (300 mesh), alginate lyase, pectinase, and cellulase were weighed and placed in a 10-mL centrifuge tube and mixed evenly. Five mL of buffer solution (citric acid-disodium hydrogen phosphate buffer) was added, mixed evenly, and placed in a metal bath at the preset temperature for enzymatic hydrolysis. During hydrolysis, shake the tube every 30 min to ensure complete enzymatic hydrolysis of the algal powder and sufficient release of reducing sugars. After enzymatic hydrolysis, immediately inactivate the enzymes by heating the tube at 100°C for 10 min in the metal bath. Allow the tube to cool to room temperature naturally. Centrifuge the hydrolysate at 4000 × g for 15 min and repeat the centrifugation twice. The reducing sugar content in the supernatant was determined using a plant total and reducing sugar assay kit (3,5-dinitrosalicylic acid colorimetric method, Yuanye Biotechnology Co., Ltd., Shanghai, China) to evaluate the enzymatic hydrolysis efficiency [22]. In this study, the enzyme dosages are expressed in U/g, representing the enzyme activity added per gram of algal powder substrate. Here, "U" denotes the International Unit of enzyme activity. For the three commercial enzyme preparations utilized, the activity values were those specified by the respective manufacturers.

2.2.2. Optimization of multi-enzyme dosage using response surface experimental design

For experiments on both *S. horneri* and *S. japonica*, a Box–Behnken design (BBD) was employed, with the reducing sugar content in the supernatant after enzymatic hydrolysis as the response value to evaluate the enzymatic hydrolysis efficiency. The addition amounts of alginate lyase, pectinase, and cellulase were set as independent variables in a three-factor, three-level experimental design. The following conditions were maintained during the experiments: pH = 7, enzymatic hydrolysis time = 8 h, and temperature = 50°C. The Box-Behnken response surface design was generated, and data were analyzed using Design-Expert software (version 13). The factors and levels used in the Box-Behnken design are presented in Table 1. Prior to the enzyme dosage optimization experiments corresponding to Table 1, the optimal addition ranges of 1–5% recommended by the commercial enzyme suppliers were taken into consideration. After comprehensively evaluating both enzymatic hydrolysis efficiency and performance, the final dose gradients for the three enzymes were set as follows: alginate lyase at 100, 300, and 500 U/g; pectinase at 1000, 3000, and 5000 U/g; and cellulase at 80, 240, and 400 U/g.

2.2.3. Optimization of enzymatic hydrolysis conditions using response surface experimental design

A BBD was applied with the reducing sugar content in the supernatant after enzymatic hydrolysis as the response value. Three factors, enzymatic hydrolysis temperature, time, and pH were selected as independent variables to design a three-factor, three-level experiment. Based on the results from the previous response surface experiment on the compound enzyme ratio, the following enzyme addition amounts were used for *S. horneri*: alginate lyase = 4.90%, pectinase = 4.66%, and cellulase = 4.75%. The enzyme addition amounts used for *S. japonica* are alginate lyase = 3.60%, pectinase = 5.00%, and cellulase = 2.84%. The Box-Behnken experimental design was generated, and data analysis was performed using Design-Expert software (version 13). The factors and levels of the Box-Behnken design are presented in Table 2.

2.2.4. Enzymatic degradation rate of S. horneri and S. japonica powders

The enzymatic degradation rate represents the proportion of solid material degraded through enzymatic hydrolysis. The procedure was carried out as follows: First, 0.5 g of dried *S. horneri* and *S. japonica* powders was dried in an oven at 80°C until constant weight was achieved. This weight was recorded as the initial dry weight of the *S. horneri* and *S. japonica* powders. After enzymatic hydrolysis, the precipitate from the centrifuged hydrolysate was washed and centrifuged twice again, then dried at 80°C to constant weight. This weight was recorded as the dry weight of the residual solid material after enzymatic hydrolysis.

The enzymatic degradation rate was calculated using **[Equation 1]**:

$$\text{Enzymatic degradation rate}\ (\%) = \frac{\text{WI} - \text{WR}}{\text{WI}} \times 100\% \quad (1)$$

In the formula: WI: Initial dry weight of *S. horneri* and *S. japonica* powders, WR: Dry weight of residual solids after enzymatic hydrolysis.

2.2.5. Determination of basic nutrient components in S. horneri and S. japonica powders and their enzymatic hydrolysate

The proximate composition of *S. horneri* and *S. japonica*, including protein, fat, crude ash, moisture, carbohydrate, and crude fiber content, was determined according to the Official Methods of Analysis of AOAC International [23]. Specifically, protein content was quantified using the Kjeldahl method; fat content was determined by acid hydrolysis; moisture content was obtained by drying samples at 105°C to constant weight; carbohydrate content was calculated by difference from the total mass after accounting for other components; and crude fiber content was analyzed via the acid-base digestion method.

2.2.6. Determination of sugar molecular weight distribution and analysis of the degree of polymerization in oligosaccharides from S. horneri and S. japonica hydrolysates

The molecular weight of oligosaccharides in the supernatant was determined using Gel Permeation Chromatography (GPC). The method for determining polysaccharide molecular weight was as follows: both standards and samples were dissolved in the mobile phase, which consisted of a 0.1 mol/L sodium nitrate solution. The GPC column specifications were 7.8 mm × 30 cm, 10 μm; a differential refractive index detector was used; the column temperature was set to 40°C; the flow rate of the mobile phase was 0.5 mL/min; and the total analysis time per injection was 35 min.

The molecular weight and content were calculated using the following GPC procedure: the sample solution was analyzed under the specified chromatographic conditions alongside

Table 1
Factors and levels of Box-Behnken experimental design for enzyme dosage optimization experiment.

Items	*S. horneri*			*S. japonica*		
Level	**Dosage of Alginate Lyase (U/g)**	**Dosage of Pectinase (U/g)**	**Dosage of Cellulase (U/g)**	**Dosage of Alginate Lyase (U/g)**	**Dosage of Pectinase (U/g)**	**Dosage of Cellulase (U/g)**
−1	100	100	80	100	100	80
0	300	300	240	300	300	240
1	500	500	400	500	500	400

Table 2
Factors and levels of Box-Behnken experimental design for hydrolysis conditions optimization.

Items	*S. horneri*			*S. japonica*		
Level	**Temperature/°C**	**Time/h**	**pH**	**Temperature/°C**	**Time/h**	**pH**
−1	40	8	5	40	8	5
0	50	16	6	50	16	6
1	60	24	7	60	24	7

standard substances of known molecular weights. A calibration curve was constructed using retention time as the abscissa and the logarithm of molecular weight (log Mw) as the ordinate. The molecular weight of the sample was then calculated based on its peak retention time. Using GPC data processing software, the chromatographic data of the sample were substituted into the calibration curve equation to determine the relative molecular weight of the oligosaccharides and their distribution range. The oligosaccharide content was quantified by summing the relative percentages of the peak areas corresponding to oligosaccharides within different molecular weight ranges, using the peak area normalization method.

2.2.7. Determination of oligosaccharide DP composition

The oligosaccharide composition in the enzymatic hydrolysate was determined using high-performance liquid chromatography (HPLC) [24]. The oligosaccharide sample was prepared as a 10 mg/mL solution and analyzed on a Superdex™ Peptide column (G10/300). The mobile phase consisted of 0.1 mol/L ammonium bicarbonate, delivered at a flow rate of 0.4 mL/min. The injection volume was 25 μL, and detection was carried out using an ultraviolet detector set at a wavelength of 235 nm. The relative content of oligosaccharides was calculated by the peak area normalization method. Oligosaccharide standards and samples were analyzed under the same HPLC conditions. By comparing the retention times of peaks in the sample with those of the oligosaccharide standards, the DP corresponding to each peak was identified. The area of each peak was integrated by the chromatographic workstation, and the relative percentage was calculated using **[Equation 2]**:

$$w = \frac{Ai}{\sum Ai} \times 100\% \quad (2)$$

In the formula: w is the relative content of the oligosaccharide component (%), Ai is the peak area of the detected oligosaccharide component (mAU*min), $\sum Ai$ is the total peak area of all detected oligosaccharide components (mAU*min).

2.2.8. Determination of monosaccharide composition of the enzymatic hydrolysate

The monosaccharide composition of the enzymatic hydrolysate was analyzed using liquid chromatography. The specific procedure was as follows: A sample was treated with trifluoroacetic acid solution and hydrolyzed at 110°C for 2 h to achieve complete release of monosaccharides. The pH was adjusted to 7 using sodium hydroxide, and the solution was diluted to a final volume of 25 mL with water. Then, 1 mL of this solution was mixed with 1 mL of 0.3 mol/L sodium hydroxide and 1 mL of 0.5 mol/L 1-phenyl-3-methyl-5-pyrazolone methanol solution, followed by incubation in a water bath at 70°C for 70 min. After cooling, 1 mL of 0.3 mol/L acetic acid solution was added, and the mixture was diluted to 10 mL with water. Subsequently, 2 mL of the derivatized solution was transferred to a 10-mL sealed tube and extracted with chloroform. The upper layer was passed through an organic filter membrane for subsequent detection [25,26]. The standard curve working solution was derivatized, purified, filtered through a membrane, and injected following the same procedure as the sample.

The chromatographic conditions were as follows: Chromatographic column: C18 column (4.6 × 250 mm, 5 μm); Column temperature: 40°C; Detector: UV detector (SPD-20A); Wavelength: 254 nm; Flow rate: 1.0 mL/min; Mobile phase A: 50 mmol/L KH_2PO_4 solution; Mobile phase B: acetonitrile; Injection volume: 25 μL. The elution gradient is shown in Table 3.

The monosaccharide content in the two kinds of algae enzymatic hydrolysate was calculated by substituting the measured values into the regression equation of the standard curve. The calculation formula is as **[Equation 3]**:

$$x = a \times \frac{c \times v}{m \times 10000} \times f \quad (3)$$

In the formula: x is the content of the target component in the sample (%), c is the concentration of the analyte in the sample solution obtained from the standard curve (mg/L), v is the constant volume of the sample solution (mL), m is the weighed mass of the sample (g), a is the standard correction factor, defined as the actual concentration of the standard divided by its calibrated concentration, and f is the dilution factor.

2.2.9. Determination of antioxidant capacity in the enzymatic hydrolysates of S. horneri and S. japonica

To evaluate the antioxidant capacity of the enzymatic hydrolysates derived from *S. horneri* and *S. japonica*, the 2,2-diphenyl-1-picrylhydrazyl DPPH radical scavenging rate and superoxide anion scavenging ability were measured. The hydrolysates of both brown algae were diluted with water to various concentrations and then assessed using a DPPH radical scavenging assay kit and a superoxide anion scavenging assay kit (both from Beijing Solarbio Science & Technology Co., Ltd.), following the procedures specified in the manufacturer's instructions. The DPPH radical scavenging rate was determined by incubating the algal enzymatic hydrolysates with DPPH and measuring the extent of DPPH radical scavenging. The superoxide anion scavenging rate was determined using the

Table 3
Elution gradient for monosaccharide composition detection.

Time (min)	A	B
2	85	15
8	83	17
20	82	18
36	60	40
40	60	40
42	85	15
47	Stop	

hydroxylamine-azo colorimetric method. The ammonium persulfate – TEMED system generates superoxide anions, which react with hydroxylamine hydrochloride to produce NO_2^-. NO_2^- then reacts with sulfanilamide and α-naphthylamine to form a red azo compound with a characteristic absorption peak at 530 nm. The scavenging ability of the sample toward superoxide anions is negatively correlated with the absorbance value at 530 nm.

2.3. Data analysis

All experiments were performed in triplicate, and the results are expressed as mean ± standard deviation. Origin software (version 9.0) was used for plotting the experimental data, and the response surface test results were analyzed using Design-Expert software (version 13).

3. Results and analysis

3.1. Optimal dosage of enzyme via response surface methodology

A Box-Behnken design (BBD) was employed using the reducing sugar content in the supernatant after enzymatic hydrolysis as the response value. A total of 17 experiments were designed, including 12 factorial points and 5 center point replicates, with three factors: alginate lyase amount, pectinase amount, and cellulase amount. Table 4a and Table 5a show the BBD experimental design for the enzymatic hydrolysis of *S. horneri* and *S. japonica* with the three enzyme amounts as independent variables, along with the corresponding reducing sugar content measured after hydrolysis.

The experimental results were analyzed using response surface methodology. The model was found to be significant, while the lack of fit was not significant, indicating the validity of the model and the reliability of both the experimental design and results (Table 4b and Table 5b). "Prob > F" < 0.05 indicates a significant factor effect. Specifically, for the two brown algae used in the experiment, the addition amount of alginate lyase showed a significant effect on enzymatic hydrolysis efficiency, while the addition amount of pectinase exhibited a highly significant effect. The effect of cellulase addition amount was relatively minor. The order of factor influence on enzymatic hydrolysis efficiency was: pectinase addition amount > alginate lyase addition amount > cellulase addition amount. In addition, C.V. (Coefficient of Variation) = 6.98% for *S. horneri* and C.V. = 3.80% for *S. japonica*, which proves that the experiment has high credibility and accuracy; *R*-Squared (Coefficient of Determination) = 0.8557 for *S. horneri* and *R*-Squared = 0.9623 for *S. japonica*, indicating a high correlation. For *S. horneri*, the R^2, adjusted R^2, and predicted R^2 are 0.8557, 0.6701, and −0.7710, respectively, indicating a good fit but with local overfitting. The lower adjusted R^2 suggests the presence of non-significant terms. However, as shown in the predicted vs. actual scatter plot Fig. 1a, all data points cluster tightly around the diagonal line, confirming that the model accurately captures the relationship between the factors and the response. The negative predicted R^2 implies limited global generalizability, but not complete failure. Given the high R^2 and adjusted R^2, along with the strong fit observed in the scatter plot, the model can still be used for analysis within the experimental range, providing valuable reference for process optimization. For *S. japonica*, the R^2, adjusted R^2, and predicted R^2 are 0.9623, 0.9138, and 0.6414, respectively, demonstrating an excellent fit and strong predictive ability. The small difference between the adjusted R^2 and R^2 indicates that all included independent variables are effective predictors. As shown in the predicted vs. actual scatter plot Fig. 1b, the data points closely follow the diagonal line with no systematic deviation, reflecting excellent agreement between the predicted and measured values.

Based on the model optimization, the optimal enzyme dosage for *S. horneri* was obtained as follows: Addition amount of alginate lyase (A) = 490 U/g; addition amount of pectinase (B) = 4660 U/g; addition amount of cellulase (C) = 380 U/g. The predicted maximum reducing sugar content was 22.79 mg/mL, with a desirability of 1.00. The optimal enzyme dosage for *S. japonica* was obtained as follows: Addition amount of alginate lyase (A) = 360 U/g; addition amount of pectinase (B) = 5000 U/g; addition amount of cellulase (C) = 284 U/g. The predicted maximum reducing sugar content was 19.22 mg/mL, with a desirability of 0.9.

Using Design-Expert 13, the three-dimensional response surface plot and contour plot for *S. horneri* were generated as shown in Fig. 2. The three-dimensional response surface plot is a 3D spatial graph of the response value against the independent variables, which intuitively reflects the degree of influence of the independent variables on the response value. The effects of different enzyme addition levels (alginate lyase, cellulase, and pectinase) on the reducing sugar content were investigated. The results indicate that the interactions among the three factors are weak and not significant, as evidenced by the sparse and flattened contour lines. The interactive effects of the various factors on the reducing sugar content are insignificant, which is consistent with the variance analysis results of the regression equation. The three-dimensional response surface plot and contour plot for *S. japonica* were shown in Fig. 3. The contour plots showed some curvature but did not exhibit a distinct elliptical shape, while the three-dimensional surfaces were moderately inclined. These graphical characteristics suggested that the interactions among the three enzymes, namely alginate lyase, cellulase, and pectinase, were weak. This conclusion was further supported by the sparse and gentle distribution of the contour lines, which indicated non-significant interactive effects. The findings from the response surface graphics are consistent with the results of the analysis of variance for the regression model, confirming that the pairwise interactions did not significantly influence the yield of reducing sugars.

3.2. Optimal enzymatic hydrolysis conditions via response surface methodology

A BBD was employed using the reducing sugar content in the supernatant after enzymatic hydrolysis as the response value. The design consisted of 17 experiments, including 12 factorial points and 5 center point replicates, with three factors: enzymatic hydrolysis temperature, time, and pH. Based on the results from the previous response surface experiment on enzyme addition amounts, the following amounts were used for *S. horneri*: alginate lyase = 490 U/g; pectinase = 4660 U/g; and cellulase = 475 U/g. The enzyme addition amounts used for *S. japonica* were alginate lyase = 360 U/g; pectinase = 5000 U/g; and cellulase = 284 U/g. The experimental design and corresponding results for *S. horneri* and *S. japonica* were presented in Table 6a and Table 7a, respectively.

Table 4a
Response surface design scheme for enzyme dosage experiment of *S. horneri.*

Run no./ Column no.	Dosage of alginate lyase (U/g) (A)	Dosage of pectinase (U/g) (B)	Dosage of cellulase (U/g) (C)	Concentration of reducing sugar in enzymatic hydrolysate (mg/mL)
1	100	1000	240	12.918
2	500	1000	240	16.973
3	100	5000	240	19.716
4	500	5000	240	21.902
5	100	3000	80	19.502
6	500	3000	80	19.103
7	100	3000	400	18.433
8	500	3000	400	20.656
9	300	1000	80	15.392
10	300	5000	80	19.075
11	300	1000	400	18.247
12	300	5000	400	22.367
13	300	3000	240	17.503
14	300	3000	240	18.405
15	300	3000	240	17.010
16	300	3000	240	19.177
17	300	3000	240	18.889

Table 4b
Analysis of variance results (BBD) for enzyme dosage experiment of *S. horneri.*

Source	Sum of squares	df	Mean square	F value	Prob > F	
Model	69.62	9	7.74	4.61	0.0282	Significant
A-Addition amount of alginate lyase	8.13	1	8.13	5.27	0.0390	
B-Addition amount of pectinase	47.68	1	47.68	30.89	0.0001	
C-Addition amount of cellulose	5.50	1	5.50	3.56	0.0817	
AB	0.8733	1	0.8733	0.5205	0.4940	
AC	1.72	1	1.72	1.02	0.3452	
BC	0.0477	1	0.0477	0.0285	0.8708	
A^2	0.1172	1	0.1172	0.0699	0.7992	
B^2	0.9961	1	0.9961	0.5937	0.4662	
C^2	4.73	1	4.73	2.82	0.1371	
Residual	11.05	7	1.68			
Lack of fit	8.37	3	2.79	3.31	0.1389	Not significant
Pure error	3.37	4	0.84			
Cor total	81.37	16				

Note: "Not significant" indicates that the effect on the results is not significant ($p > 0.05$); "Significant" indicates that the effect on the results is significant ($p < 0.05$).

Table 5a
Optimization response surface design scheme for enzyme dosage experiment of *Saccharina japonica.*

Run no./ Column no.	Dosage of alginate lyase (U/g) (A)	Dosage of pectinase (U/g) (B)	Dosage of cellulase (U/g) (C)	Concentration of reducing sugar in enzymatic hydrolysate (mg/mL)
1	100	1000	240	11.774
2	500	1000	240	15.383
3	100	5000	240	16.257
4	500	5000	240	18.824
5	100	3000	80	12.407
6	500	3000	80	15.039
7	100	3000	400	12.946
8	500	3000	400	14.518
9	300	1000	80	13.458
10	300	5000	80	17.308
11	300	1000	400	15.215
12	300	5000	400	17.484
13	300	3000	240	16.647
14	300	3000	240	17.633
15	300	3000	240	16.406
16	300	3000	240	16.573
17	300	3000	240	17.289

The results showed that the model was significant, while the lack of fit was not significant, indicating model validity and the reliability of the experimental design and results (Table 6b and Table 7b). "Prob > F" < 0.05 was considered statistically significant. Specifically, enzymatic hydrolysis time for these two brown algae showed a nearly significant effect on hydrolysis efficiency, pH had a significant effect, and temperature exhibited a relatively minor effect. The order of factor influence on enzymatic hydrolysis efficiency was: pH > enzymatic hydrolysis time > temperature. For *S. horneri*, a significant interaction was observed between factors A

Table 5b
Analysis of variance results (BBD) for enzyme dosage experiment of *S. japonica*.

Source	Sum of squares	df	Mean square	F value	Prob > F	
Model	62.66	9	6.96	19.85	0.0003	Significant
A-Addition amount of Alginate Lyase	13.47	1	13.47	38.40	0.0004	
B-Addition amount of Pectinase	24.65	1	24.65	70.29	<0.0001	
C-Addition amount of Cellulase	0.4758	1	0.4758	1.36	0.2823	
AB	0.4758	1	0.4758	0.7740	0.4082	
AC	0.2809	1	0.2809	0.8010	0.4005	
BC	0.6249	1	0.6249	1.78	0.2237	
A^2	12.81	1	12.81	36.54	0.0005	
B^2	0.6547	1	0.6547	1.87	0.2141	
C^2	8.70	1	8.70	24.82	0.0016	
Residual	2.45	7	0.3507			
Lack of Fit	1.35	3	0.4506	1.63	0.3160	Not significant
Pure Error	1.10	4	0.2758			
Cor Total	65.12	16				

Note: "Not significant" indicates that the effect on the results is not significant ($P > 0.05$); "Significant" indicates that the effect on the results is significant ($p < 0.05$).

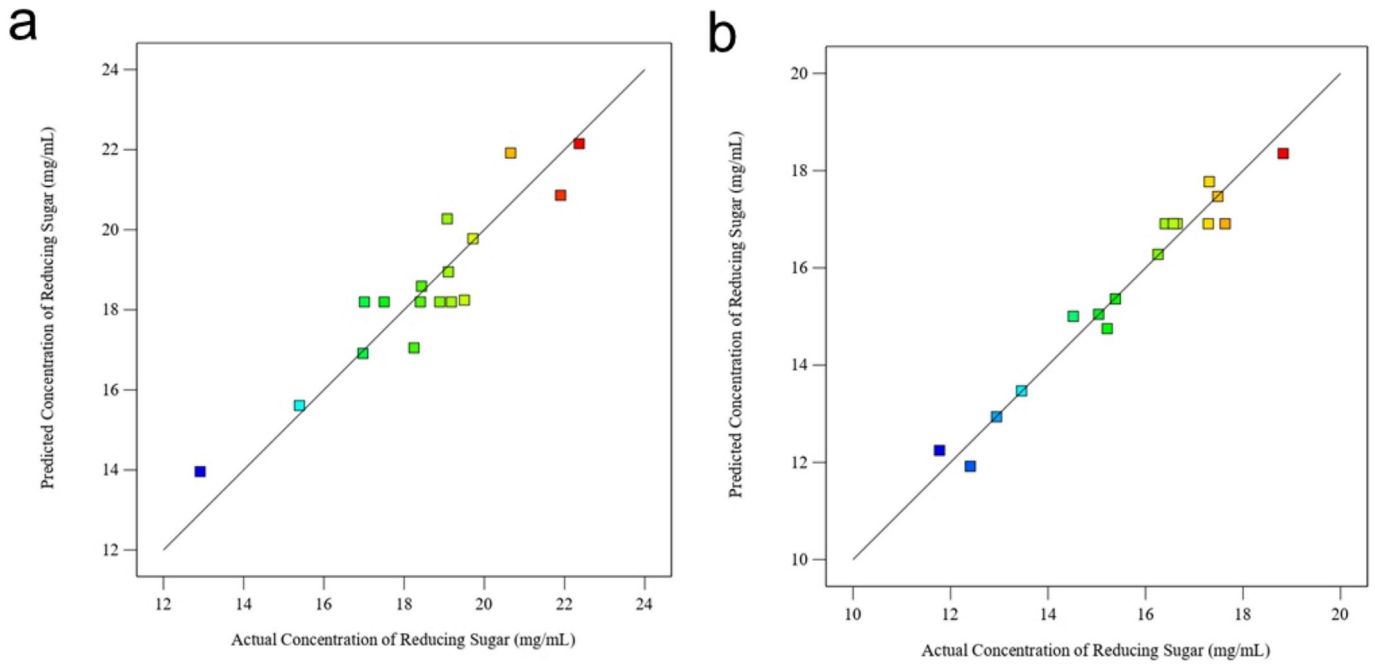

Fig. 1. Scatter plots of predicted vs. actual reducing sugar content under different enzyme addition amounts: (a) *S. horneri*, (b) *S. japonica*.

and C (i.e., temperature and pH). The C.V. = 5.17%, which proves high experimental credibility and accuracy; the *R*-Squared = 0.9045, indicating a high correlation. However, the interactions between factors A and B, and between factors B and C are weak and not significant. For *S. japonica*, the C.V. = 6.61%, which proves high experimental credibility and accuracy; the *R*-Squared = 0.8835, indicating a high correlation. However, the interactions between factors A and B, A and C, and between factors B and C are weak and not significant. For *S. horneri* and *S. japonica*, the R^2 values were 0.9045 and 0.8835, and the adjusted R^2 values were 0.7817 and 0.7336, respectively. Both models exhibited a good fit, although the gap between the two metrics in each case suggests the presence of a few non-significant independent variable terms; nevertheless, the models remain relatively reliable. The scatter plots for the two seaweeds (Fig. 4a, b) show that the experimental data points are densely distributed along the fitted diagonal line without systematic deviation, indicating that the predicted values of the models are in good agreement with the actual measured values, demonstrating good predictive performance. The predicted R^2 for the *S. horneri* model is –0.2501, indicating slight overfitting, while the predicted R^2 for the *S. japonica* model is 0.1079, suggesting moderate predictive ability. It should be clarified that an abnormal predicted R^2 does not imply complete invalidation of the model. The R^2 and adjusted R^2 values, together with the scatter plots, confirm that within the respective experimental condition ranges covered by the two models, they can still be effectively used to analyze process conditions, interactions among different factors, and the degree of influence of each factor.

Using Design-Expert 13, the 3D response surface plot and contour plot for *S. horneri* and *S. japonica* were generated as shown in Fig. 3 and Fig. 4. For *S. horneri,* the downward-opening curvature of the response surface indicates the existence of a maximum value for the response variable (reducing sugar content). There is a strong and significant interaction between temperature and pH, evidenced by the dense and elliptical contour lines. In contrast, the interactions between hydrolysis time and temperature, as well as between hydrolysis time and pH, are weak and have an insignificant impact on the reducing sugar content (Fig. 5). These findings are consistent with the variance analysis of the regression equation. For *S. japonica*, the contour plots for the interactions between temperature and time, as well as between time and pH, exhibited distinct curved shapes, accompanied by noticeable slopes in their

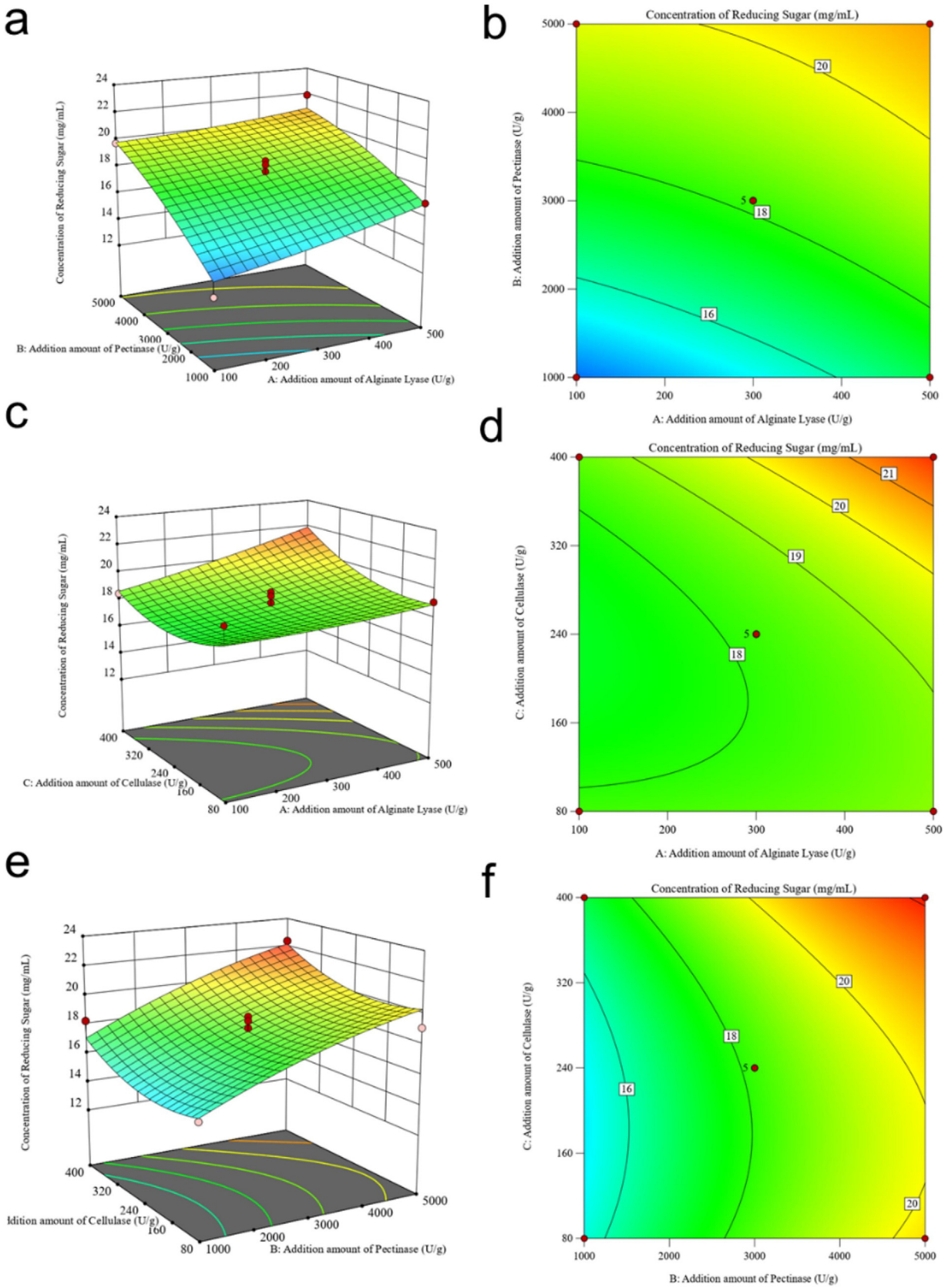

Fig. 2. Three-dimensional response surface plot (a, c, e) and contour plot of enzyme addition amount (b, d, f) for *S. horneri*.

corresponding three-dimensional surfaces. This indicates strong and significant interactive effects between these factors. In contrast, the contour plot for the interaction between temperature and pH was close to a perfect circle, suggesting a weak interaction. This factor pair did not exhibit a statistically significant influence on the reducing sugar yield (Fig. 6).

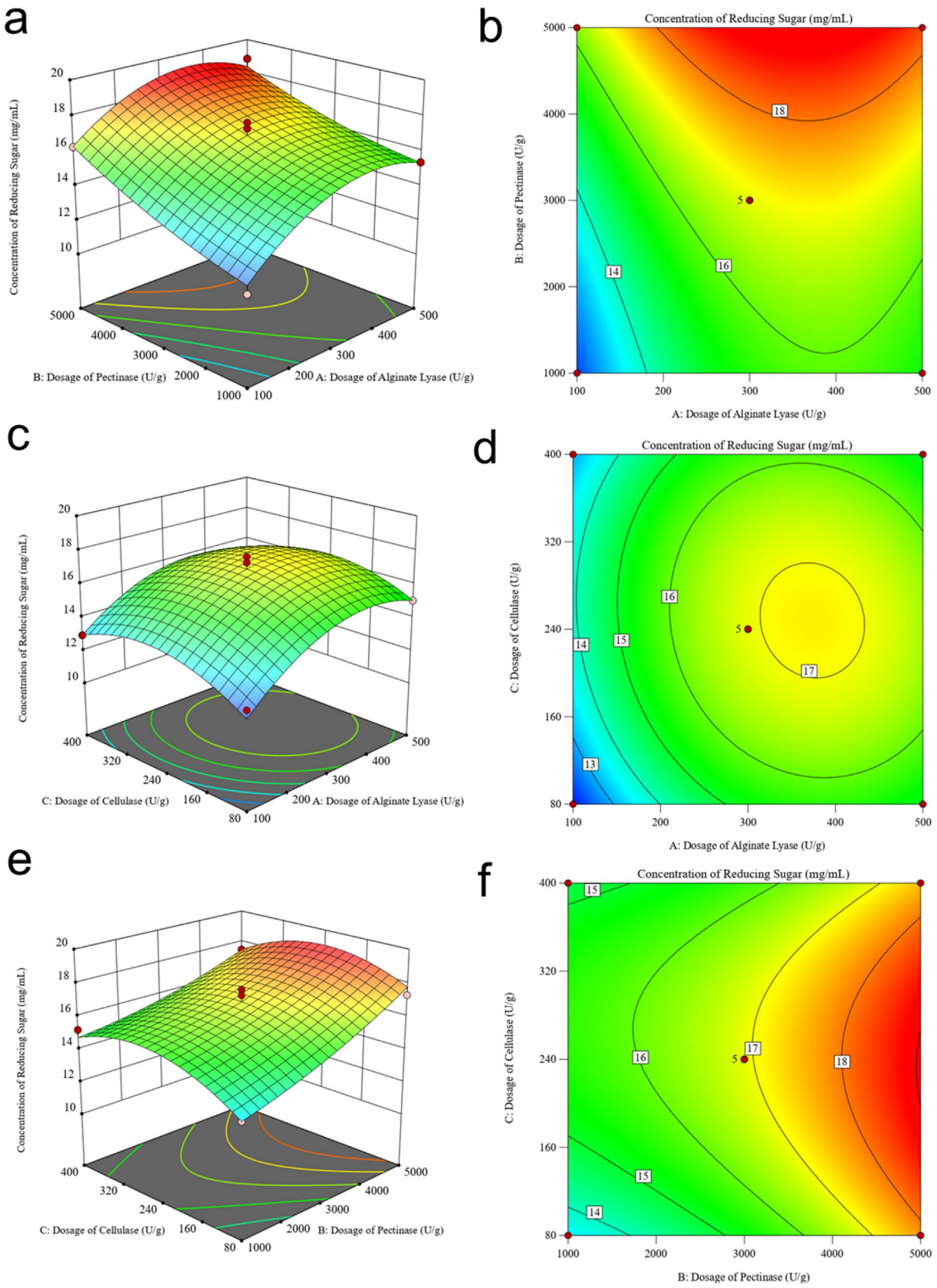

Fig. 3. Three-dimensional response surface plot (a, c, e) and contour plot of enzyme addition amount (b, d, f) for *S. japonica*.

Table 6a
Response surface design scheme for enzymatic hydrolysis conditions experiment of *Sargassum horneri.*

Run no./Column no.	Temperature/°C (A)	Time/h (B)	pH (C)	Concentration of reducing sugar in enzymatic hydrolysate (mg/mL)
1	40	8	6	23.111
2	40	16	5	24.431
3	40	16	7	16.136
4	40	24	6	18.517
5	50	8	5	23.353
6	50	8	7	21.930
7	50	16	6	24.301
8	50	16	6	23.287
9	50	16	6	23.585
10	50	16	6	23.669
11	50	16	6	24.989
12	50	24	5	23.827
13	50	24	7	19.809
14	60	8	6	23.539
15	60	16	5	20.535
16	60	16	7	19.372
17	60	24	6	22.860

Table 6b
Analysis of variance results (BBD) for enzymatic hydrolysis conditions experiment of *Sargassum horneri.*

Source	Sum of squares	df	Mean square	F value	Prob > F	
Model	87.16	9	9.68	7.37	0.0077	Significant
A-Temperature	2.11	1	2.11	1.61	0.2454	
B-Time	5.99	1	5.99	4.55	0.0703	
C-pH	27.75	1	27.75	21.11	0.0025	
AB	3.83	1	3.83	2.92	0.1315	
AC	12.72	1	12.72	9.67	0.0171	
BC	1.68	1	1.68	1.28	0.2950	
A^2	17.44	1	17.44	13.27	0.0083	
B^2	0.024	1	0.024	0.018	0.8958	
C^2	13.83	1	13.83	10.52	0.0142	
Residual	9.20	7	1.31			
Lack of Fit	7.35	3	2.45	5.29	0.0707	Not significant
Pure Error	1.85	4	0.46			
Cor Total	96.36	16				

Note: "Not significant" indicates that the effect on the results is not significant ($p > 0.05$); "Significant" indicates that the effect on the results is significant ($p < 0.05$).

Table 7a
Response surface design scheme for enzymatic hydrolysis condition experiment of *Saccharina japonica.*

Run No./Column No.	Temperature/°C (A)	Time/h (B)	pH (C)	Concentration of reducing sugar in enzymatic hydrolysate (mg/mL)
1	40	8	6	23.027
2	40	16	5	23.938
3	40	16	7	17.494
4	40	24	6	20.265
5	50	8	5	25.445
6	50	8	7	19.437
7	50	16	6	24.562
8	50	16	6	23.287
9	50	16	6	25.12
10	50	16	6	22.581
11	50	16	6	21.558
12	50	24	5	22.85
13	50	24	7	18.87
14	60	8	6	24.003
15	60	16	5	21.865
16	60	16	7	15.364
17	60	24	6	20.749

3.3. Verification of optimal reaction conditions

The reducing sugar contents in the enzymatic hydrolysates of *S. horneri* and *S. japonica* under the optimal enzymatic hydrolysis conditions are shown in Table 8.

Based on the optimization experiments for *S. horneri*, the optimal enzymatic hydrolysis conditions were determined as follows: alginate lyase = 490 U/g; pectinase = 4660 U/g, cellulase = 475 U/g; temperature = 46.67°C, time = 8 h, and pH = 5.5. A verification experiment was conducted using *S. horneri* powder under adjusted conditions close to the optimal values: 0.5 g of *S. horneri* ultrafine powder, with enzyme addition amounts set at alginate lyase = 50 0 U/g, pectinase = 5000 U/g, and cellulase = 400 U/g. Hydrolysis was carried out under the optimized conditions (temperature = 4

Table 7b
Analysis of variance results (BBD) for enzymatic hydrolysis conditions experiment of *Saccharina japonica.*

Source	Sum of squares	df	Mean square	F value	Prob > F	
Model	109.94	9	12.22	5.90	0.0145	Significant
A-Temperature	0.9405	1	0.9405	0.4540	0.5221	
B-Time	10.53	1	10.53	5.08	0.0588	
C-pH	65.74	1	65.74	31.73	0.0008	
AB	0.0605	1	0.0605	0.0292	0.8691	
AC	0.0008	1	0.0008	0.0004	0.9848	
BC	1.03	1	1.03	0.4963	0.5039	
A^2	12.14	1	12.14	5.86	0.0461	
B^2	0.3476	1	0.3476	0.1678	0.6943	
C^2	17.84	1	17.84	8.61	0.0219	
Residual	14.50	7	2.07			
Lack of Fit	6.12	3	2.04	0.9734	0.4882	Not significant
Pure Error	8.38	4	2.10			
Cor Total	124.44	16				

Note: "Not significant" indicates that the effect on the results is not significant ($p > 0.05$); "Significant" indicates that the effect on the results is significant ($p < 0.05$).

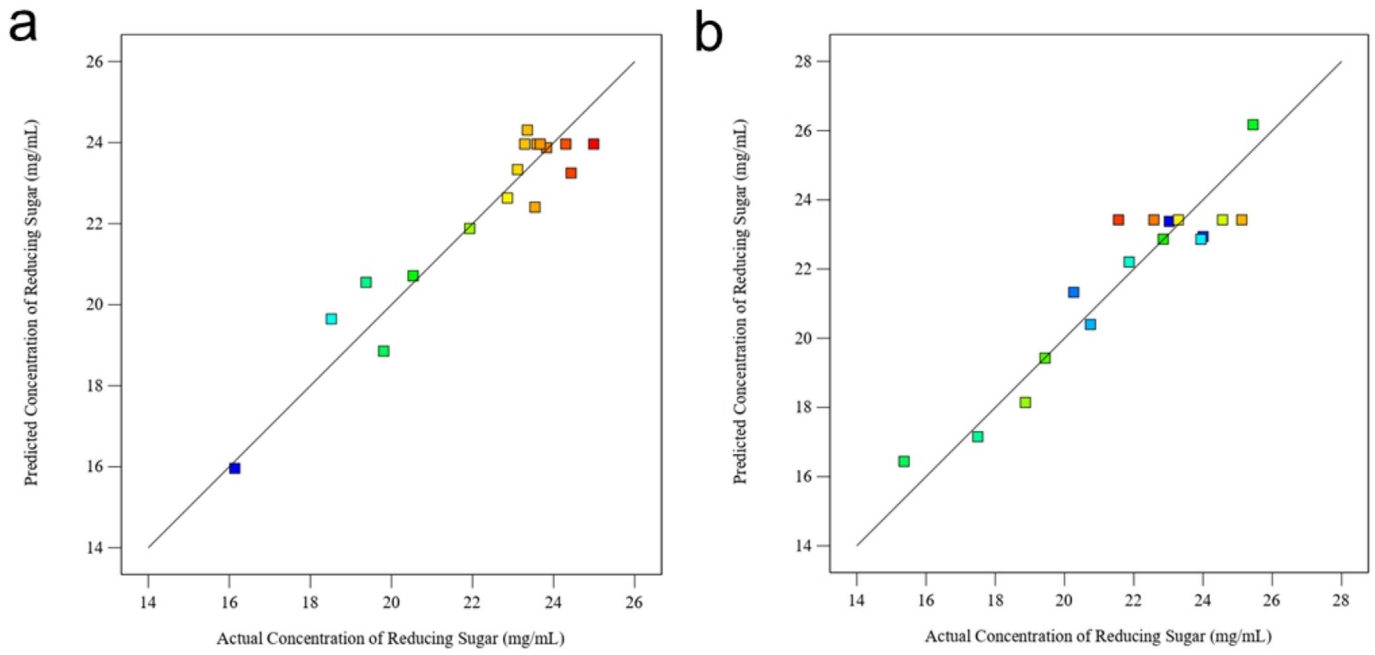

Fig. 4. Scatter plots of predicted vs. actual reducing sugar content under enzymatic hydrolysis conditions: (a) *S. horneri*, (b) *S. japonica.*

7°C, pH = 5.5, time = 8 h) in triplicate. The average reducing sugar content obtained was 25.47 mg/mL (relative to the extraction rate at the center point), which was consistent with the model-predicted value of 25.29 mg/mL, showing only minor deviation. These results confirm the accuracy of the response surface optimization and demonstrate the feasibility of the enzymatic hydrolysis conditions determined by response surface methodology.

Based on the optimization experiments for *S. japonica*, the optimal enzymatic hydrolysis conditions were determined as follows: Alginate lyase = 360 U/g, pectinase = 5000 U/g, cellulase = 284 U/g, temperature = 49.51°C, time = 8.8 h, and pH = 5.15. A verification experiment was conducted using *S. japonica* powder under adjusted conditions close to the optimal values: 0.5 g of *S. japonica* ultrafine powder, with enzyme addition amounts set at alginate lyase = 360 U/g, pectinase = 5000 U/g, and cellulase = 284 U/g. Hydrolysis was carried out under the optimized conditions (temperature = 49.5°C, pH = 5.1, time = 8.8 h) in triplicate. The average reducing sugar content obtained was 25.27 mg/mL (relative to the extraction rate at the center point), which was consistent with the model-predicted value of 26.03 mg/mL, showing only minor deviation. These results confirm the accuracy of the response surface optimization and demonstrate the feasibility of the enzymatic hydrolysis conditions determined by response surface methodology.

3.4. Enzymatic degradation rate of the S. horneri and S. japonica powders

Triplicate enzymatic hydrolysis experiments were conducted on *S. horneri* and *S. japonica* powders using the optimized compound enzyme ratio and enzymatic hydrolysis reaction conditions. The average enzymatic degradation rate of *S. horneri* was 71.4%, and the average enzymatic degradation rate of *S. japonica* was 66.3%. In that case, compared with *S. japonica*, the enzymatic degradation rate of *S. horneri* increased by approximately 7.7%, which means that the structural components of *S. horneri* were more susceptible to enzymatic attack. This result also indicated that the enzyme efficiently hydrolyzed the refractory macromolecular components in *S. horneri* and *S. japonica* (such as cell wall polysaccharides and structural proteins) into soluble substances,

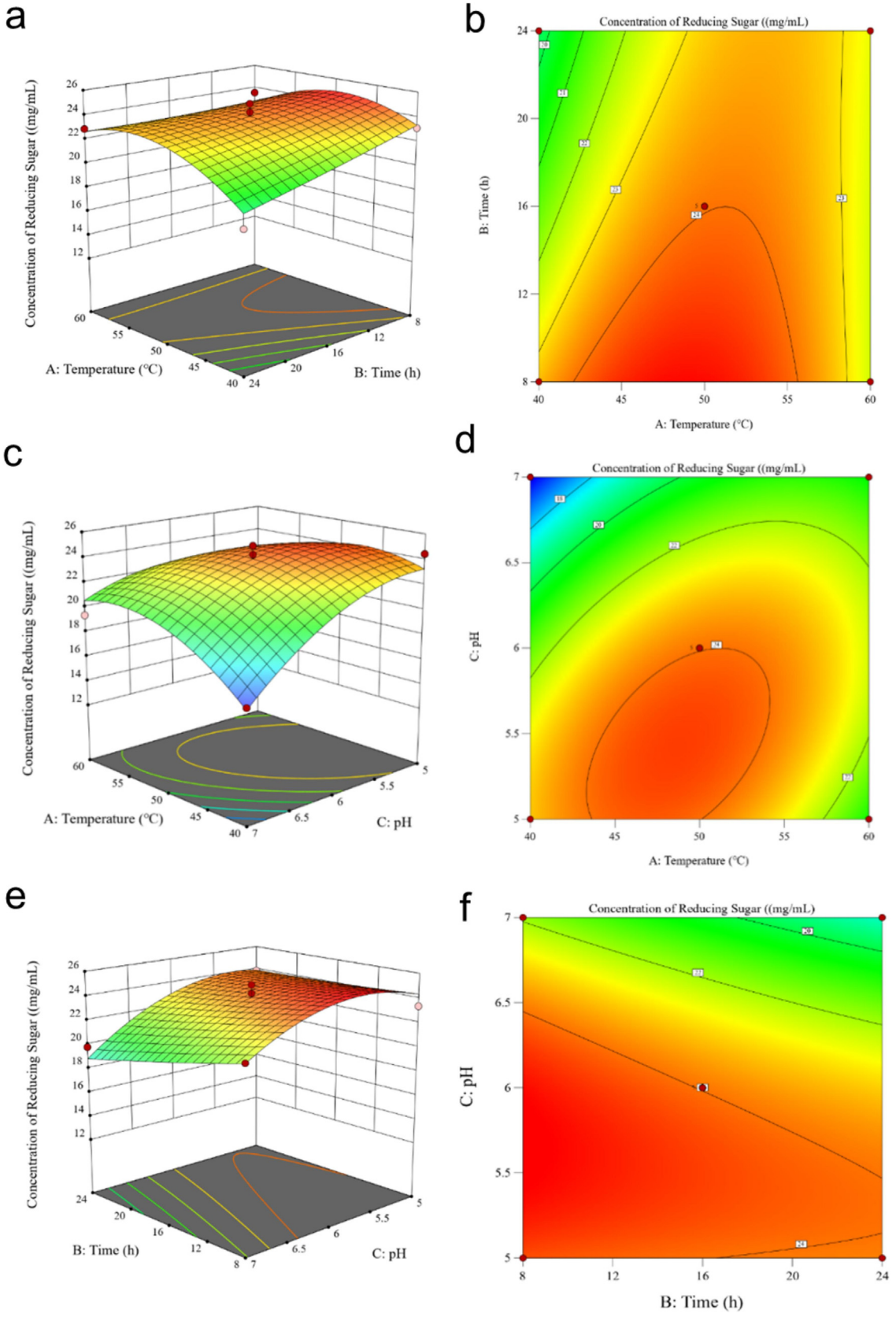

Fig. 5. Three-dimensional response surface plot (a, c, e) and contour plot of the enzymatic hydrolysis conditions (b, d, f) for *S. horneri*.

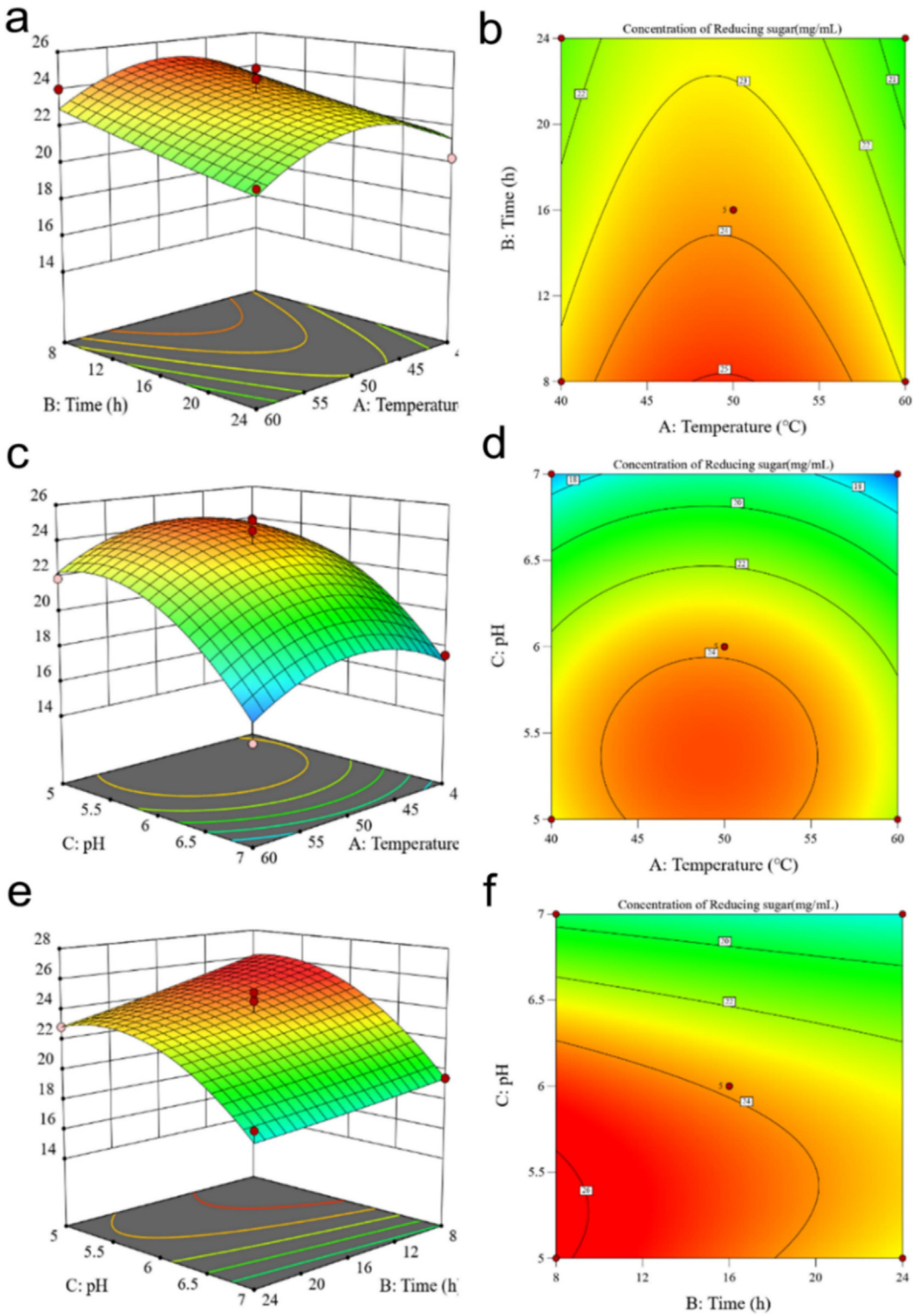

Fig. 6. Three-dimensional response surface (a, c, e) and contour plot of the enzymatic hydrolysis conditions (b, d, f) for *S. japonica*.

and most structural components were effectively converted. This is beneficial for the subsequent separation and purification of products, and the experimental results are shown in Table 9 and Fig. 7.

3.5. Basic nutrient composition of the S. horneri and S. japonica powders and its enzymatic hydrolysate

The basic nutrient composition of the *S. horneri* and *S. japonica* powders and their enzymatic hydrolysate is shown in Table 10. The hydrolysate of *S. horneri* contains high levels of carbohydrates, accounting for approximately 55.95% of its dry weight, which is higher than that in *S. horneri* powder. In contrast, the dry weight contents of protein and fat were 4.52% and 1.19%, which are lower than those in *S. horneri* powder. In terms of the hydrolysate of *S. japonica*, it also contains high levels of carbohydrates, accounting for approximately 53.09% of its dry weight, which is higher than that in *S. japonica* powder. Moreover, the dry weight contents of protein and fat were 4.82% and 1.24%, which are lower than that

Table 8
Validation experiment results under optimal reaction conditions.

Items	Addition of alginate lyase (U/g)	Addition of pectinase (U/g)	Addition of cellulase (U/g)	Temperature/°C	pH	Time/h	Concentration of reducing sugar (mg/mL)
S. horneri	500	5000	400	47	5.5	8	25.47
S. japonica	360	5000	284	49.5	5.1	8.8	25.27

in *S. japonica* powder. Additionally, no matter in hydrolysate or dry algae powder, *S. horneri* always contains more carbohydrate than *S. japonica.*

3.6. Oligosaccharide polymerization degree of S. horneri and S. japonica enzymatic hydrolysate

The molecular weight distribution of saccharide components in the enzymatic hydrolysates of *S. horneri* and *S. japonica*, as well as in the raw material of these two algae, was analyzed by Gel Permeation Chromatography, with the results presented in Table 11. The data indicate that in *S. horneri* and *S. japonica*, the percentages of oligosaccharides and monosaccharides with a molecular weight less than 1000 Daltons were 69.57% and 66.95%, respectively, corresponding to an approximate degree of polymerization of 2–6. The proportions of low-molecular-weight oligosaccharides in the range of 1000–2000 Daltons were 12.06% and 11.74%, respectively, with a corresponding degree of polymerization of approximately 6 to 12. The percentages of lower-molecular-weight oligosaccharides in the range of 2000–3000 Daltons were 8.01% and 8.10%, respectively, corresponding to a degree of polymerization of about 13 to 19. In summary, approximately 89.64% and 78.69% of the native seaweed polysaccharides in *S. horneri* and *S. japonica*, respectively, were degraded into oligosaccharides with a degree of polymerization of less than 19.

The degree of polymerization of oligosaccharides in the enzymatic hydrolysate was also determined by HPLC and integral analysis. For *S. horneri,* the peaks were identified as disaccharide (37 min; 22.66% of peak area), trisaccharide (39 min; 38.59%), and tetrasaccharide (42 min; 18.51%). Minor peaks eluting after 45 min, corresponding to pentasaccharides and other oligosaccharides with a higher degree of polymerization, accounted for less than 10% collectively (Fig. 7, Table 11). For *S. japonica*, the peaks were identified as disaccharide (37 min; 26.97%), trisaccharide (39 min; 32.00%), and tetrasaccharide (42 min; 20.97%). Minor low-intensity peaks eluting after 45 min, corresponding to other oligosaccharides such as pentasaccharides, accounted for less than 15% collectively (Fig. 8, Table 12). These results demonstrate that the hydrolysate is predominantly composed of low-molecular-weight oligosaccharides (disaccharides, trisaccharides, and tetrasaccharides), which are consistent with the molecular weight distribution data.

3.7. Monosaccharide composition of the S. horneri and S. japonica enzymatic hydrolysate

The monosaccharide composition is a critical determinant of polysaccharide structure and is closely associated with bioactivity [27]. This study analyzed the monosaccharide profiles of enzymatic hydrolysates from *S. horneri* and *S. japonica* in Table 13 and Fig. 9. Among the 12 monosaccharides assayed, the primary components in *S. horneri* were glucose, guluronic acid (G), and mannuronic acid (M), with minor amounts of galactose, xylose, fucose, and mannose also detected. Rhamnose, arabinose, ribose, and galacturonic acid were not found. The combined content of guluronic acid and mannuronic acid accounted for 38% of the total identified monosaccharides. Similarly, in *S. japonica*, glucose, guluronic acid, and mannuronic acid were the predominant monosaccharides, accompanied by small quantities of galactose, xylose, fucose, and mannose. Rhamnose, arabinose, ribose, and galacturonic acid were also undetectable. Notably, the sum of G and M constituted 44% of the identified monosaccharides, highlighting them as characteristic components of seaweed polysaccharides. Comparative analysis revealed that *S. horneri* possessed a higher content of guluronic acid and a significantly higher G/M ratio than *S. japonica.* In contrast, the fucose content was comparable between the two species.

3.8. Determination of antioxidant capacity of S. horneri and S. japonica

As the enzymatic hydrolysates of *S. horneri* and *S. japonica* were diluted, their DPPH radical scavenging activity and superoxide anion scavenging capacity continuously decreased. When the carbohydrate concentration in both algal hydrolysates reached 45 mg/mL (close to the concentration of the original hydrolysate), the DPPH radical scavenging activity was the highest (Fig. 10). At higher concentrations (15–45 mg/mL), the DPPH radical scavenging activity of *S. horneri* hydrolysate gradually surpassed that of *S. japonica*. At a carbohydrate concentration of 45 mg/mL, the DPPH radical scavenging activity of *S. horneri* hydrolysate was 1.21 times higher than that of *S. japonica*. In contrast, the superoxide anion scavenging capacity of *S. japonica* hydrolysate was generally superior to that of *S. horneri*, and it tended to stabilize at concentrations above 15 mg/mL. Although the DPPH radical scavenging activity and superoxide anion scavenging capacity of both hydrolysates showed some gaps compared to VC at concentrations of 1 mg/mL and 0.5 mg/mL, respectively, they overall exhibited favorable antioxidant properties.

4. Discussion

Prior to the industrial production of dedicated algae-degrading enzymes, cellulases and proteases were commonly used for seaweed hydrolysis. However, these enzymes lack the ability to degrade the specific components and structures of seaweed, and the enzymatic hydrolysis time is relatively long to increase the yield of extracts [28]. Some studies have reported that combining physical or chemical treatments with enzymatic hydrolysis of seaweed can enhance the degradation of polysaccharides by enzymes. For instance, organic solvent treatment, acid hydrolysis, or alkali hydrolysis is added to assist in enzymatic hydrolysis [28,29]. Nevertheless, reactions under strong acid or alkali conditions are violent, which damages the active components in seaweed and is neither energy-efficient nor environmentally friendly [30,31]. With the industrial production of seaweed-degrading enzymes, some studies have achieved deep enzymatic hydrolysis of large seaweeds. For instance, agarases and cellulases are used for the enzymatic hydrolysis of red algae. Kwon et al. [32] converted polysaccharides from the red alga *Gracilaria lemaneiformis* into utilizable monosaccharides through a combined process of citric acid pretreatment followed by agarase and cellulase hydrolysis. Using the *G. lemaneiformis* residue after citric acid pretreatment as the substrate, the highest and optimized monosaccharide extraction rate reached 57.8%, which was 3.1 times higher than that achieved without citric acid pretreatment. However, large brown algae are

Table 9
Enzymatic degradation rate of *S. horneri* and *S. japonica* powders.

Items	*S. horneri*			*S. japonica*		
	D1	D2	D3	D1	D2	D3
Original Weight (g)	0.50	0.50	0.50	0.50	0.50	0.50
Dry Original Weight (g)	0.47	0.47	0.46	0.47	0.47	0.47
Dry Weight of Hydrolysate (g)	0.12	0.15	0.13	0.13	0.18	0.16
Enzymatic Degradation Rate (%)	0.74	0.68	0.72	0.72	0.61	0.66

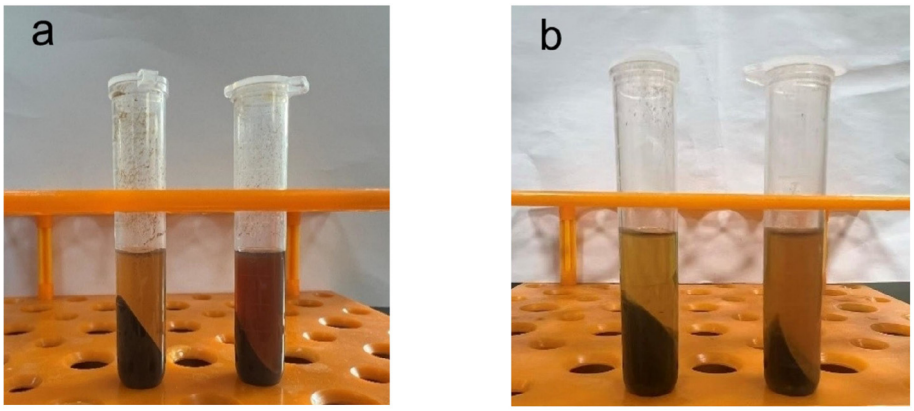

Fig. 7. *S. horneri* (a) and *S. japonica* (b) samples were centrifuged after being treated with and without enzymes in each panel; two test tubes containing brown algae were subjected to the optimal temperature, pH and reaction time determined by the experiment. The right test tube contained the enzyme, while the left test tube did not.

Table 10
Dry weight content of protein, lipid, ash and carbohydrate in the *S. horneri* and *S. japonica* powders and their enzymatic hydrolysate.

Items	*S. horneri*		*S. japonica*	
	Enzymatic hydrolysate (g/100 g)	Seaweed powder (g/100 g)	Enzymatic hydrolysate (g/100 g)	Seaweed powder (g/100 g)
Crude protein	4.52 ± 0.001[a]	10.94 ± 0.077[b]	4.82 ± 0.003[a]	9.95 ± 0.050[b]
Crude lipid	1.19 ± 0.002[a]	1.76 ± 0.005[b]	1.24 ± 0.011[a]	2.65 ± 0.020[b]
Crude ash	44.05 ± 0.005	38.52 ± 0.084	46.91 ± 0.409	42.25 ± 0.150
Carbohydrate	55.95 ± 0.208	48.76 ± 0.213	53.09 ± 0.229	44.96 ± 0.189

Values are presented as the mean of three replicates ± standard error (SE). Values with different superscript letters in the same row indicate significant differences ($p < 0.05$).

Table 11
Sugar molecular weight distribution of marine *S. horneri* and *S. japonica* enzymatic hydrolysate.

Items	*S. horneri*			*S. japonica*		
Molecular weight range (Da)	Content (%)	Number-average molecular weight (Mn)	Weight-average molecular weight (Mw)	Content (%)	Number-average molecular weight (Mn)	Weight-average molecular weight (Mw)
>50,000	0.00	/	/	1.00	88,658	156,946
40,000–50,000	0.00	/	/	0.37	44,355	44,542
30,000–40,000	0.00	/	/	0.56	34,469	34,701
20,000–30,000	0.00	/	/	0.53	25,118	25,433
15,000–20,000	0.02	16,274	16,347	0.15	17,574	17,695
10,000–15,000	0.11	11,741	11,892	0.13	12,134	12,307
8000–10,000	0.21	8761	8797	0.17	8741	8776
6000–8000	0.95	6707	6750	0.96	6688	6729
5000–6000	1.35	5431	5445	1.40	5432	5446
4000–5000	2.72	4441	4459	2.80	4442	4460
3000–4000	5.00	3444	3468	5.14	3444	3467
2000–3000	8.01	2436	2470	8.10	2440	2473
1000–2000	12.06	1414	1470	11.74	1414	1470
<1000	69.57	/	/	66.95	/	/

difficult to deeply hydrolyze using agarase and cellulase due to their high content of alginates. Before the industrial production of highly active alginate lyase, alginate-degrading bacteria were studied for their use in the degradation of large brown algae. Zhang et al. [33] employed a combination of *Vibrio* sp. B1Z05 (alginate-degrading bacteria) cell-free supernatant and cellulase to hydrolyze brown algae such as kelp. In recent years, high-activity alginate lyase has been successfully produced on a large industrial scale. This study explored the feasibility of achieving one-step deep enzymatic hydrolysis of *S. horneri* and *S. japonica* using a compound enzyme system consisting of cellulase, pectinase, and alginate lyase. The cell wall of *S. horneri and S. japonica* is mainly

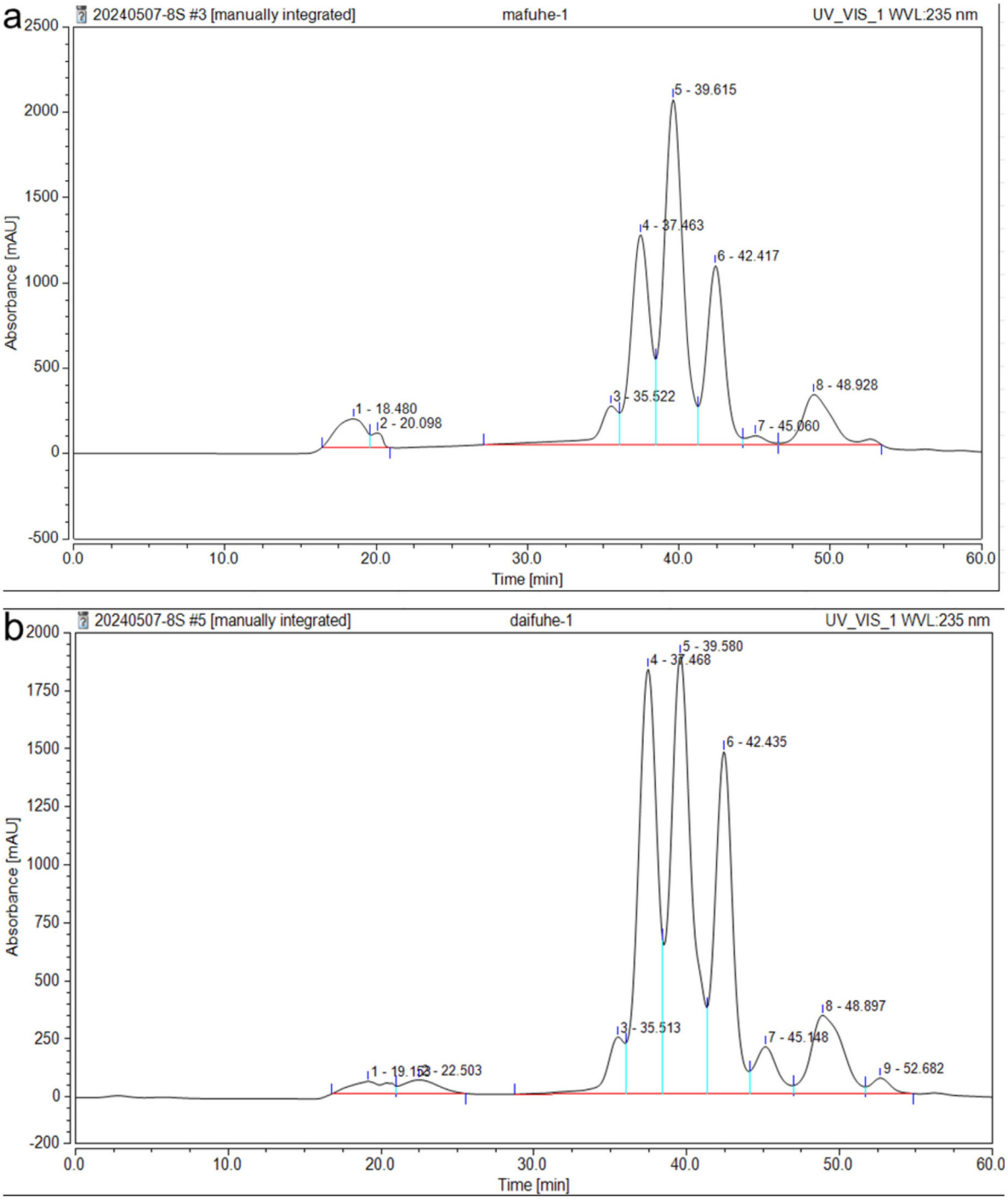

Fig. 8. Liquid chromatography profile of oligosaccharides obtained from the enzymatic digestion of *S. horneri* (a) and *S. japonica* (b) fusiform.

Table 12
Relative composition of oligosaccharides in the enzymatic hydrolysate of *S horneri* and *S. japonica.*

Items	*S. horneri*		*S. japonica*	
	Peak area (mAU*min)	Relative content (%)	Peak area (mAU*min)	Relative content (%)
Disaccharide	1758.99	22.66	2579.535	26.97
Trisaccharide	2995.28	38.59	3060.729	32.00
Tetrasaccharide	1437.06	18.51	2005.719	20.97
Pentasaccharides and others	755.71	9.73	1251.039	13.08

Table 13
Monosaccharide components of *S. horneri* and *S. japonica* enzymatic hydrolysate.

Items	*S. horneri*	*S. japonica*
	Average content (g/100 g)	Average content (g/100 g)
Glucose	0.87	0.93
Guluronic Acid	0.42	0.38
Mannuronic Acid	0.25	0.51
Galactose	0.07	0.05
Ribose	0.00	0.00
Arabinose	0.00	0.00
Mannose	0.04	0.03
Xylose	0.02	0.02
Rhamnose	0.00	0.00
Fucose	0.06	0.07
Glucuronic Acid	0.03	0.03
Galacturonic Acid	0.00	0.00

composed of alginate and sulfated polysaccharides (FCSPs, including sulfated fucose), which are bonded with cellulose microfibrils to form bundles [34]. In traditional processes, hydrochloric acid or sulfuric acid is generally used for preliminary acid hydrolysis of the substrate to catalyze the hydrolysis and cleavage of glycosidic bonds, destroy the cell wall structure of seaweed, and improve the contact rate of enzymes [35,36]. However, H^+ in acid hydrolysis randomly attacks the glycosidic bonds of the oligosaccharide backbone, leading to backbone cleavage and a decrease in the degree of polymerization. Eventually, inactive small-molecule fragments (disaccharides and monosaccharides with DP = 1–2) are generated [37]. In this experiment, pectinase was used to replace the acid hydrolysis step and was compounded with other enzymes. This approach also achieved the goal of destroying the seaweed cell wall structure. In contrast, acid hydrolysis requires neutralizing the strongly acidic environment to the opti-

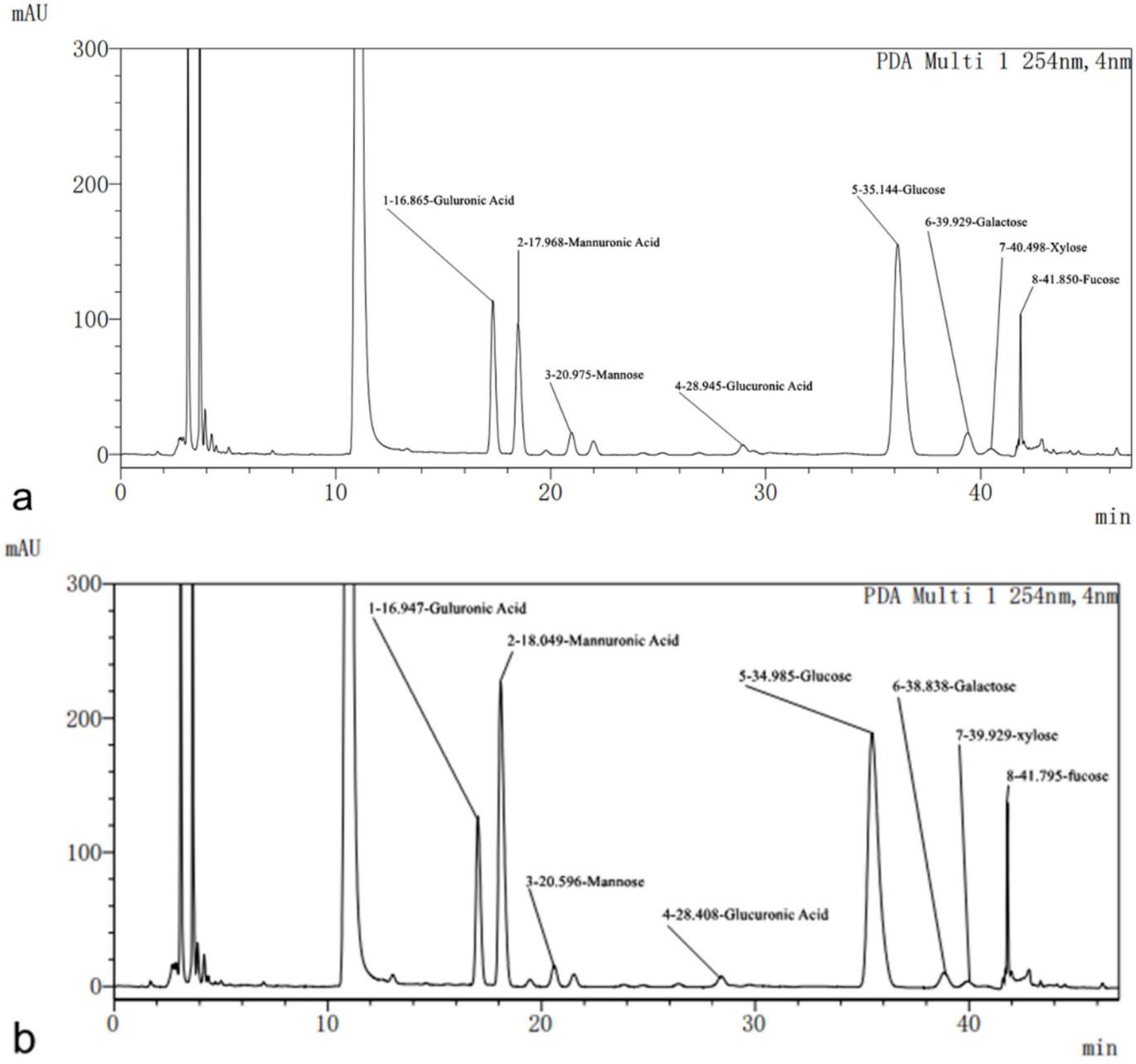

Fig. 9. Liquid chromatogram of monosaccharide composition analysis for *S. horneri* enzymatic hydrolysate (a) and *S. japonica* enzymatic hydrolysate (b).

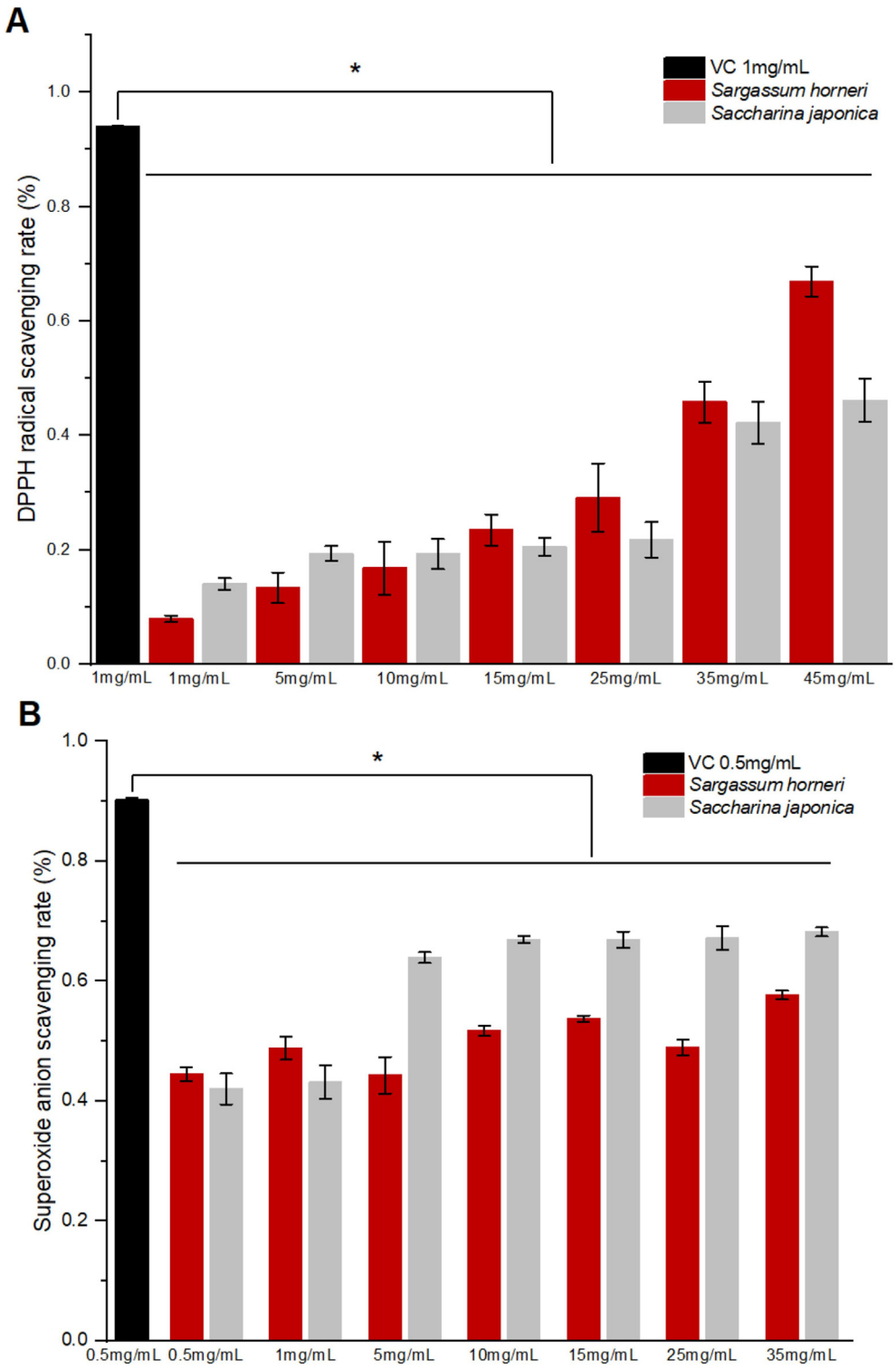

Fig. 10. Effects of enzymatic hydrolysates of *S. horneri* and *S. japonica* at different concentrations on DPPH radical scavenging activity (A) and superoxide anion scavenging capacity (B). *indicates significant difference between vitamin C and different concentrations of hydrolysates ($p < 0.05$). The oligosaccharide concentration in the enzymatic hydrolysate is expressed as carbohydrate content.

mal pH for enzymes, which involves cumbersome steps and easily introduces salts. The three enzymes work synergistically to achieve deep degradation of the cell wall and maximize the yield of active components. Moreover, the compounding of the three enzymes only requires one adjustment to the co-optimal conditions for all three enzymes. These conditions also fall within the stability range of active components in most seaweeds. The entire enzymatic hydrolysis process is mild without violent fluctuations, which can well maintain the activity of the products. Additionally, the results of this experiment showed that the addition amount of

pectinase is an extremely significant factor affecting enzymatic hydrolysis efficiency. It is evident that adding pectinase can significantly improve the enzymatic hydrolysis efficiency in the multi-enzyme compound hydrolysis of *S. horneri* and *S. japonica*.

Malvis Romero et al. [38] employed commercial cellulase and protease for enzyme-assisted extraction of the seaweed *Fucus vesiculosus*. They used response surface methodology to investigate the effects of enzyme type, enzyme activity, and extraction time on the yield, molecular weight distribution, functional groups, and purity of sodium alginate. After 15 h of extraction, when the amount of composite enzyme used was 2.4 L and the enzyme activity was 824.5 U, the highest yield of alginate reached 9.60% of the dry weight. In this experiment, industrially produced seaweed-degrading enzymes were used, and through the optimization of composite enzymatic hydrolysis conditions, one-step enzymatic hydrolysis was achieved to release a large number of oligosaccharides from *S. horneri* and *S. japonica*. In the enzymatic hydrolysate of *S. horneri*, oligosaccharides with a degree of DP < 19 accounted for 89.4%, with those in the DP 2–6 range comprising 69.4%. In contrast, in the enzymatic hydrolysate of *S. japonica*, oligosaccharides with DP < 19 accounted for 78.7%, and oligosaccharides within the DP 2 – 6 range accounted for 67.0%. This process exhibited better seaweed polysaccharide cleavage efficiency compared to traditional chemical processes and enzymatic hydrolysis processes. According to the results of response surface analysis, four factors, including alginate lyase addition amount, pectinase addition amount, enzymatic hydrolysis time, and pH had significant effects on enzymatic hydrolysis efficiency and are the key conditions that should be focused on in practical operations. In this study, the Box-Behnken response surface analysis was used to optimize the enzymatic hydrolysis process. The optimal enzyme dosages and process parameters for the enzymatic preparation of functional oligosaccharides from *S. horneri* and *S. japonica* were ultimately determined as follows: For *S. horneri*, the addition levels of alginate lyase, pectinase, and cellulase were 490 U/g, 4660 U/g, and 380 U/g, respectively. The optimal enzymatic hydrolysis conditions for the synergistic action of the three enzymes were temperature 47°C, pH 5.5, and hydrolysis time 8 h. For *S. japonica*, the addition levels of alginate lyase, pectinase, and cellulase were 360 U/g, 5000 U/g, and 284 U/g, respectively. The optimal enzymatic hydrolysis conditions for the synergistic action of the three enzymes were temperature 49.5°C, pH 5.2, and hydrolysis time 8.8 h. Under these optimized conditions, the enzymatic degradation rates of the solid substrates from the two seaweed species reached 71.4% and 66.3%, respectively, demonstrating the highly efficient hydrolysis of seaweed biomass achieved by the established process. Compared with the fermentation and enzymatic hydrolysis process in which Sun et al. [39] used the *Pseudoalteromonas agarivorans* A3 strain for the low-cost degradation of kelp root to produce alginate oligosaccharides (AOS), achieving a degradation efficiency of 54.58% and an AOS yield of 33.11%, the results of this study are more efficient, with a higher yield of AOS. With the discovery of different types of seaweed-degrading enzymes and their genetic engineering modification, the enzymatic hydrolysis process conditions can be further optimized to achieve energy savings. For example, Jiang et al. [40] identified a gene encoding a cold-adapted alginate lyase, which has an optimal operating temperature of 30°C and can achieve deep enzymatic hydrolysis of *Undaria pinnatifida*.

Among the basic nutrients in the *S. horneri* and *S. japonica* enzymatic hydrolysate, the content of carbohydrates is approximately 55.95 g/100 g and 53.09 g/100 g on a dry weight basis. Compared to the raw seaweed powders, the enzymatic hydrolysates exhibited a higher carbohydrate content. Furthermore, *S. horneri* exhibited a higher carbohydrate content than *S. japonica*, indicating a greater abundance of polysaccharides. This suggests that *S. horneri* is a superior raw material for the production of functional oligosaccharides compared to *S. japonica*. This also explains why the enzymatic degradation rate of *S. horneri* solid matter is higher when using polysaccharide-degrading enzymes to hydrolyze the two types of seaweed. In contrast, their protein and fat contents on a dry weight basis were lower, measuring 4.52 g/100 g and 1.19 g/100 g for *S. horneri*, and 4.82 g/100 g and 1.24 g/100 g for *S. japonica*, respectively. All these values were lower than the corresponding contents found in the raw powders. This indicates that the enzymatic hydrolysis process established in this study mainly achieves the cleavage of seaweed polysaccharides, while some proteins and lipids fail to be completely released. During enzymatic hydrolysis, the complex polysaccharide structure inside *S. horneri* and *S. japonica* is gradually decomposed under the action of enzymes, releasing a large amount of carbohydrates and thus increasing the carbohydrate content in the enzymatic hydrolysates of the two seaweeds. If protease is added in future experiments, it will contribute to the further enzymatic hydrolysis of seaweed and may produce bioactive peptides, which represents another direction for the high-value processing of seaweed.

From the perspective of monosaccharide composition, a large amount of guluronic acid and mannuronic acid were detected in the enzymatic hydrolysates of both seaweed species. Among them, uronic acid is known to be widely present in the cell walls of algae. Polysaccharides or oligosaccharides containing uronic acid exhibit various physiological functions, such as lowering blood glucose and lipid levels, and possess strong antioxidant capacity. The hydroxyl radical scavenging capacity and DPPH radical scavenging capacity increase with the rise in the ratio of guluronic acid to mannuronic acid [41]. The monosaccharide composition of the *S. horneri* and *S. japonica* enzymatic hydrolysate is almost identical to that of their corresponding raw seaweed powders. This indicates that the combined use of alginate lyase, pectinase, and cellulase can effectively degrade a majority of polysaccharides in both *S. horneri* and *S. japonica*, rather than selectively targeting specific polysaccharide components. Glucose was the monosaccharide detected in the highest content; as cellulose is a macromolecular polysaccharide composed of glucose, this is consistent with the characteristic of brown algae having cellulose as the skeletal polysaccharide [42]. The cellulase used in this study can degrade cellulose present in the algal powder. Therefore, the glucose in the enzymatic hydrolysate primarily originates from the enzymatic degradation of these glucans by cellulase. Brown algae are the main source of alginate, a linear acidic polysaccharide composed of β-D-mannuronic acid linked by 1–4 glycosidic bonds and its C5-epimer α-L-guluronic acid [43,44]. Guluronic acid and mannuronic acid are exclusive sugar units of alginate and serve as the monosaccharide markers that distinguish brown algae from other phyla of seaweeds. The monosaccharide composition of both enzymatic hydrolysates was consistent with the basic profile of brown algae, characterized by substantial amounts of mannuronic and guluronic acids, along with a minor presence of fucose. Compared with *S. japonica, S. horneri* possesses a higher content of guluronic acid and a significantly higher guluronic acid to mannuronic acid ratio. Its alginate exhibits a guluronic acid-rich structure, also known as the G-block type; in contrast, *S. japonica* is characterized by a higher content of mannuronic acid, and its alginate predominantly features a mannuronic acid-rich structure, referred to as the M-block type [45]. The deep enzymatic hydrolysis of *S. horneri* and *S. japonica* lays a foundation for the further purification and preparation of functional oligosaccharides.

Seaweed oligosaccharides have unique biological activities and health benefits, and seaweed oligosaccharides with different DP exhibit distinct biological activities [46]. In terms of sugar molecular weight distribution, components with a molecular weight < 1000 Da (DP = 2–6) constituted 69.57% of the *S. horneri*

enzymatic hydrolysate. Similarly, such low molecular weight components reached 66.95% in the *S. japonica* hydrolysate. These results demonstrate a high level of oligosaccharification in the enzymatic hydrolysates. The molecular weight distribution determined by GPC and the DP distribution of oligosaccharides determined by HPLC largely corroborate each other. In this study, the molecular weight distribution results for the enzymatic hydrolysates of *S. horneri* and *S. japonica* were smaller than the measured oligosaccharide DP distribution. The reasons for this discrepancy are, on the one hand, that the detected product is not purified alginate oligosaccharides, and on the other hand, that the two detection methods are based on different principles. GPC separates analytes solely based on molecular size and hydrodynamic volume, independent of structural features, polarity, or charge. The enzymatic hydrolysates analyzed in this study contained impurities such as proteins and fatty acids. Given that the enzymatic system did not include proteases or lipases, the relative abundance of proteins and other macromolecules remained comparatively high. Consequently, the calculated proportion of substances with a molecular weight of <1000 Da appeared artificially low, because it was intended to represent the relative abundance of small molecules (primarily small-chain oligosaccharides) within the total sample matrix. In contrast, HPLC separates analytes based on polarity, hydrophobicity, monosaccharide composition, and glycosidic bond type. By utilizing an ultraviolet detector set at a wavelength of 235 nm, this method significantly mitigates interference from impurities such as proteins. As a result, HPLC provides a more accurate representation of the relative proportions of oligosaccharides with varying degrees of polymerization within the total carbohydrate fraction. In the natural components of *S. horneri* and *S. japonica*, carbohydrates mostly exist in the form of high-molecular-weight polysaccharides such as alginate and fucoidan. These components have complex structures and large molecular weights, which greatly limit the exertion of their nutritional and functional values. The enzymatic hydrolysis process successfully breaks down macromolecular polysaccharide chains into short-chain oligosaccharides and monosaccharides. Small-molecule sugars have higher bioavailability, and the optimized enzymatic hydrolysis conditions achieved excellent degradation efficiency of the *S. horneri* and *S. japonica* powders.

Antioxidant capacity is also a commonly assessed indicator of the biological functions of alginate oligosaccharides. Numerous studies have demonstrated that the molecular weight of alginate oligosaccharides determines their antioxidant activity. By comparing the antioxidant capacity of alginate oligosaccharides with different molecular weights, it has been found that low-molecular-weight oligosaccharides exhibit more pronounced DPPH radical scavenging effects and relatively stronger superoxide anion scavenging ability [47,48]. For example, Şen [49] investigated the DPPH radical scavenging effects of alginate oligosaccharides from the perspectives of molecular weight and G/M ratio. In this study, the antioxidant capacity of oligosaccharides in the enzymatic hydrolysates of *S. horneri* and *S. japonica* was evaluated by measuring DPPH radical scavenging rates and superoxide anion scavenging ability. The DPPH radical scavenging rates of the hydrolysates from *S. horneri* and *S. japonica* reached up to 66.94% and 46.13%, respectively, while the superoxide anion scavenging rates reached up to 57.67% and 68.23%, respectively. Compared with previous studies, Wu et al. [50] obtained low-molecular-weight ultrasonic *L. japonica* polysaccharides through ultrasonic degradation of *Laminaria* polysaccharides, and their experimental results showed a DPPH scavenging rate of 35.85%, which was significantly lower than the antioxidant activity of the enzymatic hydrolysates of *S. horneri* and *S. japonica* obtained in this study. Cheong et al. [51] prepared AOS with varying degrees of polymerization (DP 2–6) from *Laminaria digitata* via partial acid hydrolysis. The obtained AOS exhibited good DPPH radical scavenging activity, which was lower than that of the AOS produced by enzymatic degradation in this study. AOS generated by enzymatic methods typically possess an unsaturated non-reducing end (DEH), whereas those obtained by chemical or physical methods usually undergo ring opening at the reducing end, forming carboxyl groups and resulting in saturated terminal structures. The presence of a conjugated enolic acid structure in unsaturated AOS generally contributes to their enhanced antioxidant capacity [52]. However, compared with the study by Falkeborg et al. [53], who reported a superoxide anion scavenging rate of up to 87% for alginate oligosaccharides obtained by enzymatic hydrolysis of alginate using β-alginate lyase, the antioxidant activity of the algal enzymatic hydrolysates obtained in this study was slightly lower. This discrepancy may be attributed to the fact that Falkeborg et al. [53] used alginate as the substrate, yielding alginate oligosaccharides with higher purity. In contrast, the present study employed ultra-fine algal powder, which contains substantial amounts of cellulose, other polysaccharides, and proteins. Consequently, the enzymatic hydrolysate contained not only oligosaccharides but also incompletely degraded macromolecular carbohydrates, polypeptides, inorganic salts, and other impurities, resulting in a relatively lower concentration of active components and thus reducing the antioxidant capacity of the hydrolysate. Nevertheless, for the industrial application of algae, the one-step direct enzymatic hydrolysis approach can significantly simplify the process and reduce costs.

In the field of medicine, low-molecular-weight alginate oligosaccharides exhibit antioxidant, antiviral, antitumor, and immunomodulatory effects [54,55]. A study from Sun et al. [56] showed that alginate oligosaccharides such as guluronic acid and mannuronic acid can enhance the activity of immune cells, improve the body's resistance, participate in tissue repair, and exert a certain protective effect on tissue damage. The active functions of some polysaccharides may also be related to the molecular weight and ratio of mannuronic acid to guluronic acid; at a lower ratio, some polysaccharides show more significant antioxidant activity [57]. Fucose is a water-soluble monosaccharide unique to brown algae. Fucoidan sulfate is a specific fucose-containing polysaccharide with an α-L-fucose backbone linked by glycosidic bonds, combined with sulfate groups (-OSO3H) and trace side-chain sugars. It has various biological activities such as anticoagulation, antitumor, and prebiotic effects [58]. In animal husbandry, low-molecular-weight alginate oligosaccharides play an important role in animal growth, antioxidant defense, and immunity [59,60]. When added to feed, they can significantly reduce the feed conversion ratio, improve feed quality, enhance growth performance, boost the liver's antioxidant capacity, and strengthen non-specific immunity [61,62,63]. In agriculture, alginate oligosaccharides can promote plant growth, mainly manifested in stimulating root growth, enhancing chlorophyll synthesis, promoting photosynthesis, and increasing seed germination rate [64,65]. Moreover, alginate oligosaccharides can alleviate high-salinity and heavy-metal stresses in various plants and improve their drought and cold resistance [66,67]. In this study, the *S. horneri* and *S. japonica* enzymatic hydrolysate released a large amount of low-molecular-weight alginate oligosaccharides after enzymatic hydrolysis, laying a foundation for the further development and utilization of functional oligosaccharide products. Using the *S. horneri* and *S. japonica* enzymatic hydrolysis process established in this experiment, a deep seaweed enzymatic hydrolysate with high oligosaccharide content can be prepared, which simplifies the enzymatic hydrolysis steps and improves the enzymatic hydrolysis efficiency. The seaweed oligosaccharides prepared by enzymatic hydrolysis have high safety: the crude product is suitable as a

functional feed additive, while the refined product can be used to develop functional foods and pharmaceuticals, showing promising application prospects.

5. Conclusions

This study investigated the optimal enzymatic hydrolysis process for the preparation of functional oligosaccharides from *S. horneri* and *S. japonica* via enzymatic hydrolysis. Using the reducing sugar content in the enzymatic hydrolysate as the indicator, the Box-Behnken response surface analysis was employed to optimize the addition amounts of alginate lyase, pectinase, and cellulase, as well as the enzymatic hydrolysis temperature, pH, and time. The experimental results showed that pectinase, alginate lyase, hydrolysis time, and pH had significant effects on enzymatic hydrolysis efficiency. Finally, a one-step multi-enzyme complex hydrolysis process was successfully established for both *S. horneri* and *S. japonica*. The optimal parameters for deep hydrolysis were determined. For *S. horneri*, the enzyme dosages were 490 U/g alginate lyase, 4660 U/g pectinase, and 380 U/g cellulase, with the process conducted at 47°C, pH 5.5 for 8 h. For *S. japonica*, the optimal dosages were 360 U/g alginate lyase, 5000 U/g pectinase, and 284 U/g cellulase, under conditions of 49.5°C, pH 5.2 for 8.8 h. Under these conditions, the enzymatic degradation rate of the *S. horneri* and *S. japonica* solids reached over 65%. Furthermore, the enzymatic hydrolysates of both seaweeds exhibited good antioxidant activity. Meanwhile, oligosaccharides/monosaccharides with a molecular weight < 1000 Da (DP = 2–6) accounted for 69.57% and 66.95% of the *S. horneri* and *S. japonica* enzymatic hydrolysates, which effectively improved the yield of low-molecular-weight alginate oligosaccharides. This study can provide a scientific reference for the further processing and utilization of *S. horneri* and *S. japonica*.

CRediT authorship contribution statement

Keqing Shi: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Yitong Dong:** Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Qi Wang:** Validation, Methodology, Formal analysis, Data curation. **Ling Qin:** Writing – review & editing, Data curation, Conceptualization. **Chen Zhong:** Validation, Methodology, Data curation. **Kefeng Xu:** Validation, Resources, Formal analysis, Data curation. **Mei Liu:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Jie Sun:** Writing – review & editing, Validation, Supervision, Conceptualization.

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Data availability

Data will be made available on request.

Declaration of competing interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Appendix A. Supplementary material

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