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Polysaccharide from *Sargassum Oligocystum* Algae: Isolation, Antioxidant and Antibacterial Activities

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Abstract

Natural polysaccharides from algae have gained increasing attention for their biological activities and potential for applications in food, pharmacology, medicine, and biology fields. The study aimed to investigate the effects of the Viscozyme enzyme on the polysaccharide extraction from Sargassum oligocystum algae. Then, the obtained polysaccharides were purified by using the Sevag method and Sephadex G-75 gel filtration chromatography before evaluating the antioxidant and antibacterial activities. The results show that the obtained polysaccharide was 2.06 ± 0.027 mg/g based on dry mass after extraction and the obtained purified polysaccharide with a purity of 76.28%, which was determined via the UV-Vis and Fourier Transform Infrared Spectroscopy (FT-IR) spectra with characteristic peaks. Antioxidant capacity of polysaccharides from S. oligocystum algae by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical scavenging tests with IC_{50} values of 4.90 ± 0.09 ppm and 4.01 ± 0.03 ppm, respectively. The antioxidant capacity of the obtained polysaccharides by Ferric Reducing Antioxidant Power (FRAP) and Reducing Powder (RP) with $OD_{0.5}$ values were 3.52 ± 0.10 ppm and 0.27 ± 0.01 ppm, respectively. The antibacterial ability of the obtained polysaccharide was the concentration-dependent manner in the surveyed range of 200-1000 ppm via the antibacterial diameter of Escherichia coli (7.02± 1.01 mm to 13.33 ± 2.08 mm) is greater than Bacillus subtilis (4.67 ± 1.15 mm to 12.00 ± 1.73 mm).

Keywords: Antioxidant, antibacterial, polysaccharide, *Sargassum oligocystum.*

1. Introduction

S. oligocystum belongs to the *Sargassum* genus - a large natural reserve in the brown algae (*Sargassaceae*). About 250 genera have been discovered in the world, and there are over 1,500 species. In Vietnam, about 150 species have been discovered in the North of the Gulf of Tonkin, in the Central region and the Southern coast [1]. The growing season for most *Sargassum* lasts from November to June of the following year. The best time to harvest *Sargassum* is from May to June. The brown seaweed species lives deep and grows all year round. Algae species are very popular in traditional medicine in Asia. *Sargassum* species is also used in Vietnam as food additives or tea which has beneficial effects on health [2].

Algae-derived polysaccharides attract widespread attention for their nutritional benefits as well as their biological potential. They are found mainly in the form of fucoidan, alginate and laminarin. The polysaccharide in green algae is mainly starch, while polysaccharides in brown algae are laminaran composed of (1,3)-β-D-glucan with β-(1,6) linkage creating branches, laminaran is known to be an antioxidant, anti-cancer agent, an anticoagulant... Polysaccharide is a group of substances with many biological functions. Fibre is a polysaccharide that is a structural component of seaweed cells, including water-soluble fibre and water-insoluble fibre. Water-soluble fibre components include wax, gum, pectin, xyloglucan, galactomannan, hemicellulose, and water-insoluble fibre components such as cellulose, arabinoxylan, lignin [3]. Castro *et al.* reported that the fucans found in sulfated polysaccharides have strong antioxidant properties, anti-inflammatory activity, and cell inhibition of the HT-29 human colon cancer cell line [4]. It is noteworthy that polysaccharides from *Sargassum* exhibited high antioxidant capacity. For example, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals scavenging was 51.99% at 80 µg/mL (fucoidan isolated from *Sargassum cinereum*) [5] or 95.23% at 0.4 mg/mL (polysaccharide sulfate in *Sargassum elasticbergii*) [6].

These findings showed that algal polysaccharides have strong antioxidant activity. Biologically active polysaccharides exhibited antibacterial activity by interfering with cell walls and cell membranes or

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changing cell membrane permeability. Changing permeability can prevent the penetration of nutrients introduced into microorganisms. The disk diffusion method is performed by placing a sheet of antibiotic-absorbent paper on the surface of an agar plate that has been inoculated with bacteria. The antibacterial agent then diffuses into the agar, where it can inhibit bacterial growth in the area surrounding the plate. The antibacterial ability of polysaccharides is also influenced by their structure and composition. The diffusivity of different polysaccharides can directly affect the bacterial inhibition zone [7]. Vo Thi Tuyet Hoa *et al.* extracted and purified fucoidan from *Ceratophyllum submersum* algae and obtained fucoidan with a purity of over 60% [8]. Huynh Truong Giang *et al.* was isolated polysaccharides from brown algae *Sargassum mcclurei* in different solvents such as distilled water, hydrochloric acid (HCl) 0,1N, and ethanol 90% [9]. The chemical composition and antioxidant activities of polysaccharides extracted from *Sargassum microcystum* were also evaluated. Nguyen Duy Nhut *et al.* isolated and compared the content of fucoidans and their structure characteristics from five Sargassum brown seaweed species in the south provinces of Vietnam [10]. However, there are no available reports on the isolation and characterization of polysaccharides from *Sargassum oligocystum* in Ninh Thuan province. This study investigated the conditions for obtaining polysaccharides from *S. oligocystum*, enhanced the purity by using the Saveg method and gel filtration chromatography, and evaluated the antioxidant and antibacterial activities of the obtained extract. This study provides platform information related to polysaccharides from *S. oligocystum* algae in particular and *Sargassaceae* algae in general.

2. Materials and Methods

2.1. Materials

S. oligocystum algae were collected in Son Hai 1 village, Phuoc Dinh commune, Thuan Nam district, Ninh Thuan province. After harvesting, the algae was pre-washed, drained and transported to the laboratory, where they were washed with tap water to remove impurities such as sand, shells, snails... then dried at 50 °C until the moisture was under 10%, ground and sieved to collect powder less then 0.3 mm in a zip bag and stored at 5 °C for all experiments.

Chemicals: Chloroform (Merck), n-butanol (Merck), enzyme Viscozyme L (Novozyme, Denmark), phenol (Merck), H_2SO_4 (Merck), Mueller Hilton Agar (MHA, Sigma), LSB medium, DPPH (Merck), ABTS (Merck), $K_2S_2O_8$ (Merck), $K_3Fe(CN)_6$ (Merck), CCl_3COOH (Merck), Ampicillin (Sigma). Other chemicals were at an analytical level.

Bacterial strains included *Bacillus subtilis* ATCC®6633 and *Escherichia coli* ATCC®25922, which were selected for antibacterial activity assay. The bacteria cultures were maintained in their appropriate agar slants at 4 °C throughout the study and used as stock cultures.

2.2. Methods

2.2.1. Effects of enzymes on polysaccharide extraction

Polysacharide was isolated via enzyme-assisted extraction with Viscozyme, which helps destroy the cell wall and release polysacharide from the material. Briefly, 1 g of raw algae powder (calculated by dry matter) was put into different 100 mL glass beakers processed by the Soxhlet method, and then the solvent was added to the beakers. The material/solvent ratios were investigated (1/15, 1/20, 1/25, 1/30, 1/35 (w/v)), stirred, fixed enzyme concentration 1% compared to raw material weight dry, placed the mixtures in a thermostatic bath at the investigated temperatures (40 °C, 50 °C, 60 °C, 70 °C, 80 °C) for the investigation time (60, 90, 120, 150, 180 minutes) [11]. At the end, the enzymatic hydrolysis reaction was inactivated by keeping at 90 - 100 °C for 10 minutes and then immediately cooled to cool samples. The samples were centrifuged at 4800 rpm for 10 minutes to collect the supernatant containing polysaccharides and determine the polysaccharide content using the spectroscopic method.

2.2.2. Polysaccharide purification

Protein was removed from the polysaccharide extract by reacting with Sevag solution (chloroform: n-butanol = 4:1 v/v) with the ratio of 1:1 v/v, vortexing for 20 minutes, and allowing it to settle. The mixture was separated into three phases, and most of the polysaccharides were concentrated in the supernatant. This process was repeated three times. Then, the precipitation was continued with 96% ethanol at 4 °C overnight in a ratio of 1:4 v/v. Then, the mixture was centrifuged at 5500 rpm for 20 minutes to obtain the crude polysaccharide for further purification stage. Next, 1 g of crude polysaccharide was mixed with 10 mL of distilled water, centrifuged to get the solution and put into a prepared Sephadex G75 gel filtration chromatography column, waiting for 30 minutes, then eluted with 0.2 M NaCl to collect fractions of 4 mL for each. The polysaccharide content was determined via the spectroscopic method.

2.2.3. Antioxidant activity

DPPH assay: The antioxidant activity via DPPH assay was performed according to the description of K. Mishra *et al* [12]. Polysaccharide samples and positive control acorbic acid concentrations were diluted with methanol in a 10 mL volumetric flask. 2 mL test solution was put into the test tube, followed by 2 mL of 0.1 mM DPPH solution. For the control sample, the test solution was replaced with MeOH. The blank sample only contains MeOH. The test tubes were incubated in the dark at room temperature for 30 minutes, and then the absorbance spectrum was measured at 517 nm.

ABTS assay: The antioxidant activity via ABTS assay was conducted as described by R. Re *et al.* [13]. $ABTS^{\bullet+}$ solution was from 2 mL of 2 mM ABTS solution and 2 mL of 2.45 mM $K_2S_2O_8$ solution in a 100 mL volumetric flask and incubated the solution in the dark for 16 hours, then diluted it with methanol and adjusted the absorbance of the solution at a wavelength of 734 nm with an optical density of 0.7 ± 0.05. 4 mL of $ABTS^+$ and 1 mL of the samples in test tubes at various concentrations. The reaction mixture was incubated for 6 minutes and then measured at 734 nm.

Reducing Powder (RP) assay: The Fe reduction capacity of polysaccharides was determined according to Rahate K. *et al.* [14]. The reaction mixtures consisted of 0.5 mL of polysaccharide samples at different concentrations, 0.5 mL of phosphate buffer (0.2 M, pH= 6.6), 0.5 mL of phosphate buffer (0.2 M, pH= 6.6) and 1% $K_3Fe(CN)_6$ in test tubes, the mixtures were incubated at 50 ℃ for 20 minutes and added 0.5 mL of 10% CCl_3COOH and then centrifuged at 3000 rpm for 10 minutes. After centrifugation, 0.5 mL of the upper layer, 0.5 mL of water and 0.1 mL of 0.1% $FeCl_3$ were mixed in a test tube, shaken well in the test tube and then measured at 700 nm. The positive control was ascorbic acid.

Ferric Reducing Antioxidant Power (FRAP) assay: The principle of determining the antioxidant activity of this method is based on the ability to reduce the Fe^{3+}-TPTZ complex [2, 4, 6-tripyridyl-s-triazine (TPTZ)] to the Fe^{2+}-TPTZ complex in an acidic environment. The Fe^{3+}-TPTZ complex is in an environment containing antioxidants, and the antioxidants donate electrons to this complex and form Fe^{2+}-TPTZ. In particular, the blue intensity is proportional to the antioxidant content in the samples, measured at a wavelength of 593 nm [15].

2.2.4. Antibacterial activity

Antibacterial activity of polysaccharide obtained from *S. oligocystum* was performed as described by A. W. Bauer *et al*. Mueller Hilton Agar (MHA) medium was to inoculate the strains at 37 ℃/24 hours for use [16]. Lauryl Tryptose Broth (LSB) medium was also mixed to create an enrichment medium for *E. coli* and *B. subtilis*. Then, the typical colony inoculated into a liquid LSB medium and then incubated at 37 ℃/24 hours. 100 µL of bacterial solution with a cell density of about 10^6-10^8 CFU/mL from the liquid medium put into the MHA medium plate, spread evenly, let dry for 15-30 minutes, then added 20 µL of the samples of different concentrations on 6 mm – diameter filter paper on the surface of the agar plate, incubated the plate upside down at 37 ℃/24 hours then measured the antibacterial diameter. The solvent was chosen as the negative control, and the positive control was ampicillin.

2.2.5. Analysis method

Polysaccharide content determination:

Polysaccharide content was determined by using the phenol-sulfuric acid method. Based on the hydrolysis reaction of polysaccharide into monosaccharide, monosaccharide creates colour with phenol in an acidic environment. Briefly, 2 mL of the sample solution was put into lidded test tubes, 1 mL of 4% phenol solution was added, and 5 mL of concentrated H_2SO_4 solution was added to cover the test tubes tightly and vortex gently so that the solutions were uniform. First, heat the test tubes in a water bath at 40 ℃ for 30 minutes, then put them in ice water for 5 minutes. Absorbance was measured at 490 nm [17].

The polysaccharide purity of fractions was determined via the percentage of polysaccharide content calculated by phenol-sulfuric acid (*B*) and the mass of dry matter *(A*) [18].

$$\text{Purity (\%)} = \frac{B \times 100\%}{A}$$

FI-IR spectroscopic analysis:

The infrared (IR) spectra of the obtained polysaccharide were obtained using a Fourier transform infrared spectrophotometer, recorded at the absorbance mode from 4000 to 400 cm^{-1} (mid-infrared region) [17]. In this study, the fractions with the highest purity from the purification stage would be determined the FT-IR spectrum.

Nuclear magnetic resonance (NMR) analysis:

The spectra ^{1}H-NMR were recorded using the Brucker Advance DPX - 500 NMR spectrometer (Bruker, Berlin, Germany). The samples were sonicated at 75.5 MHz and 27 ℃ and before dissolving in D_2O with 20 µg/mL for measuring ^{1}H-NMR spectrum.

2.3. Data Analysis

The experiments were performed 3 times, and the results were presented as mean plus/minus SD. IBM SPSS Statistics 20.0 software was used to analyze experimental data, evaluate the difference between samples, and optimize the extract conditions. Microsoft Excel 2019 software was used to draw charts.

3. Results and Discussion

3.1. Effects of Enzyme on Polysaccharide Extraction from S. Oligocystum Algae

The enzyme will disrupt the cell wall, thereby releasing inside components like polysaccharides into the solvent and resulting in polysaccharide content. The effects of material and solvent ratio, temperature and extraction time in enzyme treatment on polysaccharide content are shown in Fig. 1.

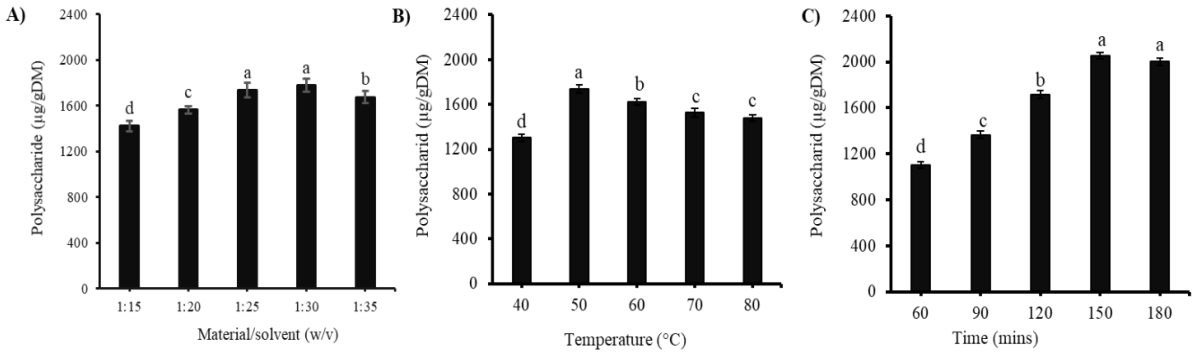

Fig. 1. Effects of material/solvent ratio (A), temperatures (B), and time (C) on polysaccharide extraction

Note: In each graph, different characters on the bar indicate different statistical significance at 5%.

The results indicate that the more used solvent resulted in higher polysaccharide concentration in the extract. In fact, the polysaccharide content increased from 1322.35 µg/gDM (1:10 w/v) to 1728.65 µg/gDM (1:25 w/v) (Fig. 1A). When this ratio was increased, the raw materials were in full contact with the enzymes, leading to the release of many polysaccharides. As the ratio of materials/solvents increases, the extraction rate of polysaccharides decreases because the excess solvents completely dissolve them.

The effectiveness of polysaccharide extraction showed an upward trend with the increase in temperature extraction. At 50 °C, the polysaccharide content was the highest with 1736.81 µg/gDM (Fig. 1B); this is because the Viscozyme works well at this temperature [19]. Enzyme activity is significantly influenced by the fact that different enzymes have different optimum temperature conditions for their best hydrolysis efficiency. Furthermore, a much higher temperature could result in the thermal breakdown of polysaccharides, increase energy expenditure, speed up solvent evaporation, and release impurities in the extraction process [11]. Besides, 150 mins were cited as the highest polysaccharide content with 2055.49 µg/gDM (Fig. 1C). The amount of polysaccharides decreases slightly as extraction time increases past this point. Enzymes hydrolyzing the polysaccharide at a specific temperature and extraction time may be a factor in this phenomenon.

In short, Fig. 1 shows that the highest polysaccharide content was the material/solvent 1/25 (w/v), temperature 50 °C, and extraction time was 150 minutes were the most appropriate conditions.

3.2. Polysaccharide Purification from S. Oligocystum Algae

Proteins and polysaccharides are biopolymers that have a diverse structure. The separation of proteins from crude polysaccharides is an important step in the process of splitting and purifying polysaccharides. The Sevag method is a simple method based on the principle that reagents denature and precipitate proteins instead of polysaccharides [20]. After the extraction, the polysaccharide extract was precipitated with 96% ethanol, then mixed with water, centrifuged to collect the supernatant and added the Sevag reagent to deproteinize for 20 minutes oscillation. Next, samples were left to stand for 30 minutes so that the mixtures split into 3 phases; polysaccharides were in the phase on the top. After three times of this stage, the obtained polysaccharide content was 1659.34 µg/gDM. The obtained polysaccharide was then fractioned via Gel filtration chromatography of Sephadex G75, eluted by 0.2M NaCl with the rate of 2 mL/min and collected 5 fractions (Table 1).

Based on Table 1, the highest polysaccharide content was fraction 2 with 2101.28 µg/gDM with a purity of 66.28%. The FT-IR spectrum of this fraction is shown in Fig. 2.

Table 1. Polysaccharide content and purity during the purification stage

Fractions	Polysaccharide content (µg/g DM)	Purity (%)
1	1156.23 ± 38.59	8.04 ± 0.45
2	2101.28 ± 30.26	66.28 ± 7.41
3	1560.99 ± 32.97	24.47 ± 1.88
4	297.62 ± 33.12	1.84 ± 0.37
5	53.66 ± 35.75	0.15 ± 0.10

The infrared spectrum (Fig. 2) showed a strong absorption range at 3500-3200 cm^{-1} with observed peaks of 3381.7 cm^{-1}, 3419 cm^{-1}, and 3445.9 cm^{-1} characterizing the elongated oscillations of O-H. The peak near 2936.0 cm^{-1} was due to prolonged oscillations of C-H, peaks appearing at 1730 cm^{-1} demonstrated that polysaccharides

contained carboxylic groups and peaks near 1610.3 cm^{-1} indicated the presence of O-C-O [21]. The peak at 1246.9 cm^{-1} was in the extended band at about 1120-1270 cm^{-1}, indicating an elongated S=O sulfate group branching from the amount of fucoidan or alginic acid [22]. The peak at 1090.6 cm^{-1} was attributed to prolonged oscillations of C-O-C. The peak at 821.1 cm^{-1} was considered the signal for the presence of α-mannopyranose [21]. In addition, ^{1}H-NMR spectrum expressed peak of 5.28 ppm (Fig. 3), which was respond the bond of α -1,2-linked mannopyranose unit [23].

3.3. Antioxidant Activity of Polysaccharide from S. Oligocystum Algae

The antioxidant activity of the obtained polysaccharides was determined via four select assays of DPPH, ABTS, RP and FRAP. The antioxidant activity via four selected assays was concentration-dependent. Regarding antioxidant activity, the IC_{50} values were 4900 ppm (DPPH assay) and 4010 ppm (ABTS assay). In addition, higher polysaccharide concentrations resulted in higher absorbance values, which revealed better antioxidant properties in both FRAP and RP assays (Fig. 4).

DPPH free radicals have the ability to absorb hydrogen molecules of antioxidants. Therefore, DPPH is widely used to test the antioxidant capacity of various compounds [22]. As polysaccharide concentrations increased, antioxidant activity also increased markedly. The results were similar to those in the literature [21]. The antioxidant capacity of polysaccharides of the test sample increased from 1000 ppm to 5000 ppm; the free radical scavenging increased from 22.38% to 50.96% (Fig. 4A). The ABTS free radical scavenging increased from 22.66% (1000 ppm) to 58.23% (5000 ppm) (Fig. 4B). The ability of polysaccharides from *S. oligocystum* algae to scavenge ABTS free radicals was higher than that of DPPH.

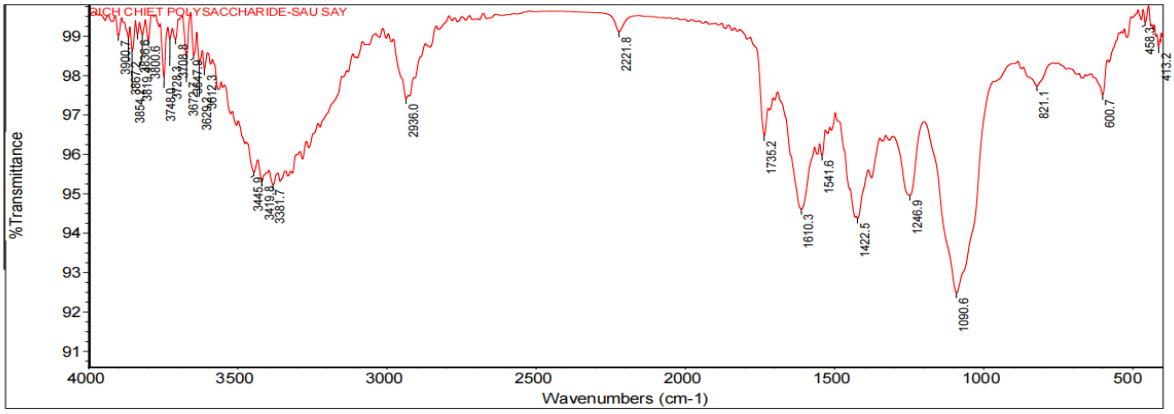

Fig. 2. FT-IR spectrum of polysaccharides

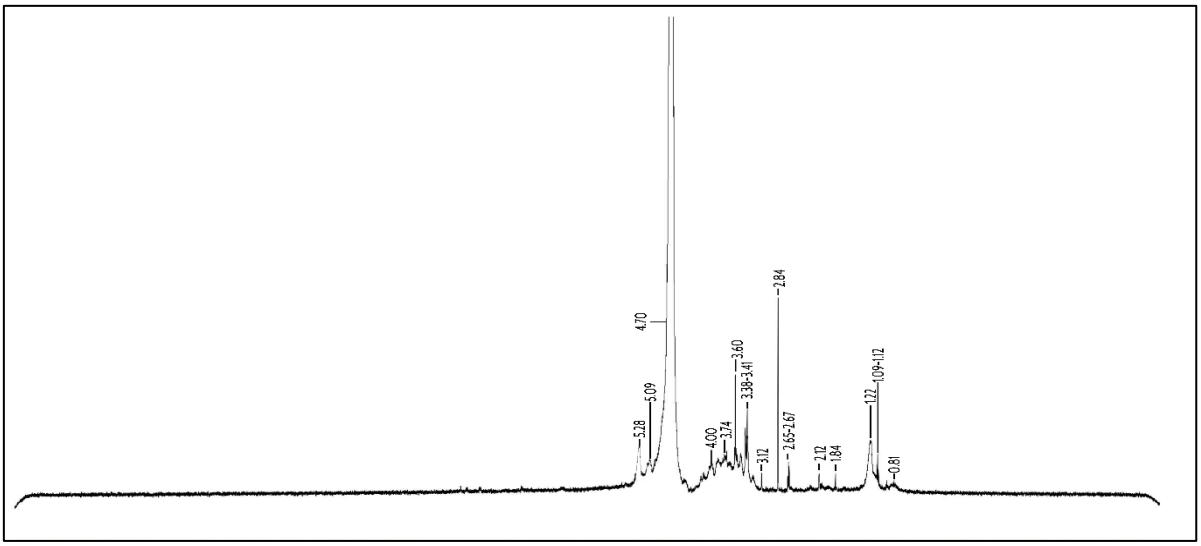

Fig. 3. ^{1}H-NMR spectrum of the isolated polysaccharides

This finding was in line with the report of J. Chen *et al.* [24]. The FRAP assay is also commonly used to determine the antioxidants and antioxidant activity of plant extracts. The antioxidant potential of the various samples was estimated from their ability to reduce TPTZ-Fe(III) complexes to TPTZ-Fe(II) complexes. The absorbance ranged from 0.36 (1000 ppm) to 0.48 (5000 ppm) (Fig. 4C). Regarding the reducing capacity (RP), the higher sample concentration resulted in higher reducing capacity. This may be because polysaccharide had functional groups linked to reducing agents and hydroxides that can act as electron donors and can react with free radicals to convert them into more stable products and thus stop radical chain reaction. The results in Fig. 4D were in agreement with the study of P. Vijaya Baskar *et al.* [25].

3.4. Antimicrobial Activity of Polysaccharide from S. Oligocystum Algae

Polysaccharides with antibacterial effects must provide surface-active properties, absorption capacity, and high binding affinity for bacterial cells [7]. The obtained polysaccharide was determined for antimicrobial activity via two selected strains of *E. coli* (gram-negative) and *B. subtilis* (gram-positive). The results are shown in Table 2 and Fig. 5.

The polysaccharide extract (200 ppm) inhibited the growth of bacteria with antimicrobial diameters of 7.00 ± 1.00 mm (*E. coli*) and 4.67 ± 1.15 mm (*B. subtilis*). In addition, the inhibitory zone becomes larger with increasing concentration and the maximum inhibition was recorded at 1000 ppm, with diameters of 13.33 mm (*E. coli*) and 12.00 mm (*B. subtilis*), with ampicillin as positive control, DMSO 10% as negative control. The samples showed not strong enough antimicrobial activity; the antimicrobial diameter ranged from less than 10 mm to 15 mm but did not exceed 20 mm [25]. The antimicrobial activity of polysaccharides from *S. oligocystum* algae is less effective than that of ampicillin (Fig. 5). The results in this experiment were in accordance with W. Violando *et al.* [26].

Table 2. Antimicrobial results of polysaccharides from *S. oligocystum*

Concentration (ppm)	Antimicrobial diameter (mm)	
	E. coli	***B. subtilis***
200	7.02 ± 1.01	4.67 ± 1.15
400	8.33 ± 0.58	6.33 ± 1.53
600	10.33 ± 1.15	8.67 ± 0.58
800	11.67 ± 1.53	10.00 ± 1.00
1000	13.33 ± 2.08	12.00 ± 1.73
Ampicillin	25.83	30.16

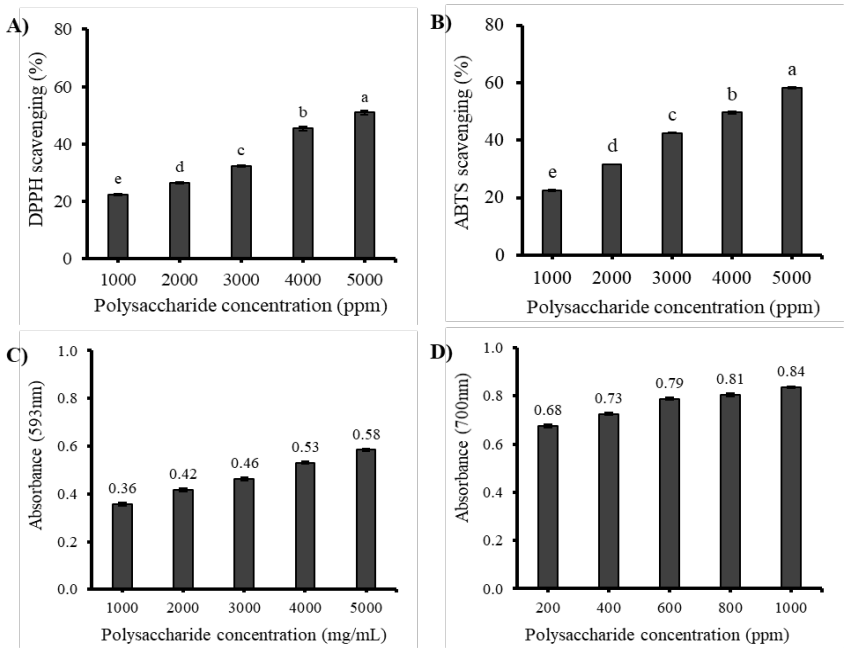

Fig. 4. Antioxidant activity of polysaccharide via assays of DPPH (A), ABTS (B), FRAP(C) and RP (D)

Note: In each graph, different characters (a, b, c…) on the bar indicate different statistical significance at 5%.

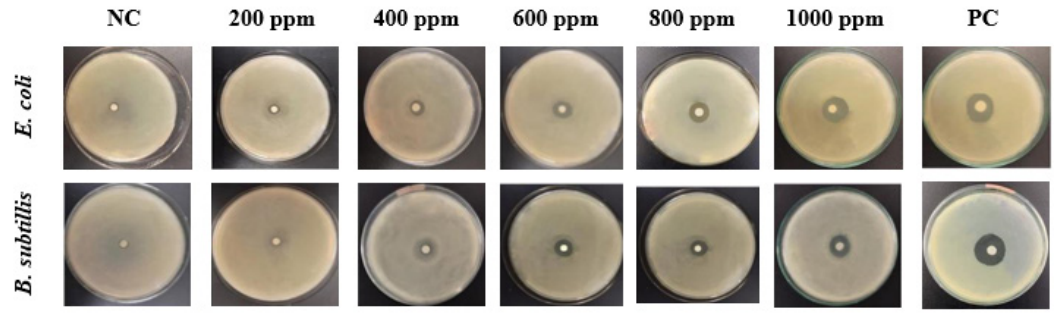

Fig. 5. Antibacterial capacity of *E. coli* and *B. subtilis,* NC: negative control, PC: positive control

4. Conclusion

This finding indicates that polysaccharides can be extracted from *S. oligocystum* algae with the support of the enzyme Viscozyme. The Sevag method and Sephadex G-75 gel filtration chromatography help to enhance the purity of polysaccharides to 76.28%. The obtained polysaccharide showed strong antioxidant properties via different assays, such as DPPH, ABTS, FRAP, and RP, but the antibacterial capacity was not highlighted. The study offers essential information for further investigations related to bioactive properties and the potential of polysaccharides from *S. oligocystum* algae before practical applications.

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