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Characteristics and function of an extracellular polysaccharide from a green alga *Parachlorella*

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ABSTRACT

The green alga *Parachlorella* sp. BX1.5 is a new strain that can significantly coproduce lipids and polysaccharides, inside and outside the cells, respectively. This study aimed to investigate the characteristics and functional properties of the extracellular polysaccharide (EPS). Ethanol-extracted EPS from the cells grown in N-starved BG11 medium was found to be a novel acidic rhamnan of straight-chain type, with a high molecular mass of $> 1.75 \times 10^6$ daltons. The water-soluble EPS exhibited high viscosity as sol, even at low concentrations, with 0.5 % EPS showing almost constant viscosity at 10–40 °C. Its addition to mayonnaise resulted in improved oil-off, suggesting its possible application as a thickener or food additive. Cells with added EPS were found to exhibit resistance to environmental stress conditions, such as heat, dryness, and decompression, and ultraviolet rays. Based on these rheological and biological effects, its unique properties with respect to biorefinery have been discussed here.

1. Introduction

Microalgae, considered a model microorganism, have strong photosynthetic capabilities and are used as a resource in biorefinery (Brennan & Owende, 2010; Caporgno & Mathys, 2018). For example, the green alga *Chlorella* cell contains 45 % protein, 20 % lipid, 20 % carbohydrate, and 10 % ash, with vitamins and minerals as other minor components. The high protein content of *Chlorella* reflects its promise as a food resource. Recent reports have indicated microalgal extracts to possibly show protective effects against antiviral infections and cancer metastasis, immunostimulatory effects, as well as prevention of diabetes. Microalgal materials have been known not only as nutrients but also as oil, polysaccharides, photosynthetic pigments, *etc*.

Polysaccharides from microalgae are mainly known as extracellular polysaccharides (EPS), their molecular structure and function summarized in Table S1. Sulfated EPS, a branched structure produced from the cyanobacterium *Aphanothece sacrum*, is known as sacran and consists of glucose and other sugars, having molecular weight, and is expressed as $>1.6 \times 10^7$ daltons (Da) (Okajima et al., 2008). Glycosides of sacran, modified with lipids or organic acids, have been reported to possess properties that make them applicable as food thickener or as antiviral agents (Kaneko & Kaneko, 2008). Anti-inflammatory effects of sacran on allergic dermatitis *in vivo* had been reported previously (Ngatu et al., 2012). Acidic EPS, a branched structure derived from the cyanobacterium *Arthrospira* (Spirulina) sp., is called spirulan and consists of rhamnose and other sugars; its molecular weight is 2.6×10^4 Da. The spirulina EPS has been shown to confer protective properties against tumor metastasis (Hayashi et al., 1997).

The branched-structure EPS from green alga *Scenedesmus* sp. consists of galactose and other sugars, with molecular weight of 4.0×10^4 Da. This EPS contains proteins as glycoproteins, and can function against tumor metastasis (Ando et al., 1994). The EPS from green alga *Monostroma nitidum* mainly consists of rhamnose (78 % w/w) and abundant sulfate esters, with molecular weight of 8.7×10^5 Da (Karnjanapratum & You, 2011; Lee, Koizumi, Hayashi, & Hayashi, 2010; Mao et al., 2008). The EPS from green alga *Parachlorella kessleri* consists of abundant galactose (67.1 % w/w) and other sugars like mannose (22.5 %), arabinose (4.7 %), rhamnose (4 %), and xylose (1.7 %), with molecular weight of 6.5×10^4 Da (Sato & Ri, 2014). This EPS can inhibit colon carcinoma in mice (Ishiguro et al., 2017). On the other hand, the linear EPS from non-photosynthesizing microorganism *Alcaligenes* sp., is known as curdlan and consists of glucose (approximate 100 %, β-1,

Abbreviations: FAMEs, fatty acyl methyl esters; FID, flame ionization detector; TEM, transmission electron microscopy.

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3-bond), with molecular weight $> 2.0 \times 10^5$ Da (Dobashi et al., 2006). This EPS has been utilized as food additive and hairdressing fee.

Therefore, microalgal polysaccharides, with molecular mass between 10^4 and 10^5 daltons, exhibit branched carbon chains in most cases. EPS, for application as food additive (as thickening stabilizer/gelling agent) or cosmetic moisturizer/emulsifying dispersant, may also be extracted from plant seed, fruit, sap, and seaweed, representatively referred to as guar gum, locust bean gum, pectin, Arabic gum, agar, and carrageenan. Their characteristics of thickening and gelation/solation, however, greatly depend on the degree of branching. Until date, reports of polysaccharides with a linear structure are limited. We have successfully isolated a novel green alga *Parachlorella* sp. BX1.5 that can significantly coproduce lipids (food oils) and acidic polysaccharides, inside and outside the cells, respectively (Sasaki et al., 2020). In this study, the unique BX1.5 EPS has been characterized and its effective impacts discussed in terms of algal biorefinery.

2. Material and methods

2.1. Cultivation conditions

The *Parachlorella* sp. BX1.5 strain was isolated from an outdoor culture consisting of natural water collected from a mountainous area in Hiroshima, Japan (Sasaki et al., 2020). The BX1.5 cells were pre-cultivated in 50 mL of BG11 in 100-mL Erlenmeyer flasks for 5 days. The flasks were placed in a cultivation chamber CF-415 (TOMY Company Ltd., Tokyo, Japan), supplied with 2 % CO_2, and subjected to reciprocating-shaking at 40 rpm under continuous white-light exposure at 100 μmol photons m^{-2} s^{-1}. Approximately 5 mL of the cell-culture was collected therefrom, and the cells suspended into 30 mL or 50 mL of new BG11 (Rippka, 1989), BG11-N (= $BG11_0$ as nitrogen-deficient condition without $NaNO_3$), BG11 supplied with 2.65 % (w/v) NaCl (= BG11 + NaCl), artificial sea water (= ASW) containing 3.6 % (w/v) of the powder from a package (MARIN MERIT; Matsuda Co. Ltd., Osaka, Japan), and BG11-P (as a phosphorous-deficient condition without K_2HPO_4) media in 300-mL Erlenmeyer flasks. These flasks were placed in the same cultivation chamber for the main cultivation. After 6 days, the cells were finally collected. Cells were also cultured under static (standing) conditions, with 0.04 % CO_2-air, for 13 days, and used after collection, as control.

2.2. Cell staining and microscopic observation

To detect the extracellular polysaccharide, a 100-μL aliquot of cell culture, harvested from the main culture, was suspended in 20 μL of India ink (Daiso Co. Ltd., Tokyo, Japan), and observed under an optical microscope (BX53; Olympus, Tokyo, Japan) with differential interference contrast (DIC) in half the amount of light and shutter speed of 0.48 s (Sasaki et al., 2020). Based on this observation, inner (Φ*ic*) and outer (Φ*ec*) diameters of the cells were measured.

2.3. Ethanol precipitation of polysaccharides

After pre-culture, a 5-mL aliquot of culture was collected and the cells suspended into 30 mL of new BG11, BG11-N, BG11 + NaCl, or ASW in 300-mL Erlenmeyer flasks. These flasks were put in the same cultivation chamber as used for pre-cultivation. After 6 days, 10 mL of culture was collected and transferred to a new tube. To the sample, 40 mL of 99.5 % (v/v) ethanol was added, mixed vigorously, and centrifuged at 6000×*g* for 10 min to obtain a precipitate fraction. Thereafter, 10 mL of ethanol was added to the pellets. This sample was again mixed, ultrasonically disrupted (UD-100; TOMY, Tokyo, Japan: set at an output of 50 for 5 min), and 40 mL of 99.5 % ethanol added. After centrifugation at 6000×*g* for 10 min, the pellet was collected, 5 mL of 99.5 % ethanol added, vigorously mixed, and 40 mL of 99.5 % ethanol added again. After another centrifugation at 6000×*g* for 10 min, the pellet was finally collected. To the pellet, 5 mL of 99.5 % ethanol was added once again, vortexed, and 2 mL of the sample subsequently transferred to a 2.5-mL syringe. Two days later, height of the bed volume of polysaccharide deposited in the syringe, was measured with a ruler.

2.4. Preparation of BX1.5 EPS extracts

The BX1.5 strain was pre-cultivated in BG11 medium under 2 % CO_2-air supply in the CO_2 cultivation chamber (see the section of Cultivation conditions). Thereafter, a 5-mL aliquot of culture was collected and suspended in 50 mL of new BG11-N medium in a 300-mL Erlenmeyer flask. This culture was kept in the cultivation chamber, supplied with 2% CO_2-air, for 6 days. Subsequently, the cells (from 495-mL culture) were harvested and transferred into a new tube. This sample was added to three volumes of ethanol (1485 mL), resulting in 75 % (v/v) concentration. It was vigorously mixed, allowed to stand at room temperature (25 °C) for 30 min, and centrifuged at 6000×*g* for 10 min. The resultant pellets were collected and mixed with 495 mL of ethanol. The ethanol-based extractions were performed four times, and finally the extracts added to 100 mL of acetone and dehydrated overnight. The dried samples were ground in a mortar.

2.5. Analysis of sugar composition of BX1.5 extracts

Proteins in the BX1.5 extract were assayed by the method of Lowry et al. (Lowry, Rosebrough, Farr, & Randall, 1951) using bovine serum albumin as the standard. The total carbohydrate content of the BX1.5 extract was measured by phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) using rhamnose as a reference. To measure the neutral sugar composition of BX1.5 extracts, the ground samples (0.05 *g*) were dissolved in 1 mL of 72 % (v/v) sulfuric acid, and allowed to stand for 2 h at room temperature. The resultants were added to 8 mL of water and hydrolyzed at 105 °C for 20 h to obtain neutral monosaccharides. After the hydrolysate was neutralized by adding barium carbonate, the aqueous phase collected was centrifuged at 10,000×*g* for 10 min, leading to precipitates. The supernatant was filtered through a USY-1 filter (Advantec Co. Ltd., Tokyo, Japan), and freeze-dried. Neutral sugar content was measured by HPLC, using the Shimadzu 10Avp chromatography system (Shimadzu Co., Ltd., Kyoto, Japan), consisting of an NH2P-50 4E column (Φ4.6 mm × 250 mm). The eluent was 80 % acetonitrile (CH_3CN) with 250 mM phosphate, at a flow rate of 0.8 mL/min. Thirty microliters of the sample, which had been dissolved in ultrapure water and filtered through a cellulose acetate filter (0.45 μm; Advantec Co. Ltd.), was injected into the column. Relative monosaccharide content (mol%, w/w) was calculated referring to total monosaccharides as 100 %. Uronic acid content of the sample was measured by Blumenkrantz method (Blumenkrantz & Asboe-Hansen, 1973) using galacturonic acid as the standard. Uronic acid composition was measured by HPLC using the Shimadzu 10Avp chromatography system (Shimadzu Co. Ltd., Kyoto, Japan), consisting of a Shodex SUGAR SH-1821 column (Φ7.6 mm × 300 mm). The eluent was 5/1,000 N H_2SO_4 with a flow rate of 1.0 mL/min. Standards of arabinose (Ara), fucose (Fuc), galactose (Gal), glucose (Glc), rhamnose (Rha), and xylose (Xyl) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan).

2.6. Analysis of FT-IR spectrum

The organic functional groups of BX1.5 EPS extracts, involving acidic rhamnans, were identified by Fourier-transform infrared (FT-IR) spectroscopy in the range of 4,000–400 cm^{-1} (Lee et al., 2010; Mao et al., 2008). Polysaccharides (1 mg) were adequately dried and ground with spectroscopic grade KBr powder (10 mg), and pressed into a pellet for FT-IR measurement using a FT/IR-4200 Fourier-transform infrared spectrometer (Jasco Co. Ltd., Tokyo, Japan).

2.7. Measurement of molecular weight and distribution

Analytical gel-filtration chromatography of BX1.5 acidic rhamnan was performed in an HPLC system (880-PU; Jasco Co. Ltd.), employing a TSK guard column PWXL (Φ6.0 mm × 40 mm; Tosoh Co. Ltd., Tokyo, Japan) and TSKgel G6000PWXL column (Φ7.8 mm × 300 mm, possible separation range being MW 50–7,000 × 10^3 Da in a case using dextrans; Tosoh Co. Ltd.) in 50 mM sodium acetate buffer, pH 5. Aliquots (100 μL) of 1.0 % (w/v) BX1.5 acidic rhamnan solution, which had been filtered through a cellulose acetate filter (0.80 μm; Advantec Co. Ltd.), was injected into the G6000PWXL column, and separation carried out at a flow rate of 1.0 mL/min. The BX1.5 EPS sample was detected using a refractive index detector (Shodex RI-101; Showa Denko Kogyo Co. Ltd., Tokyo, Japan) and a multi-angle laser light scattering detector with a He-Ne laser (632.8 nm) as the light source (DAWN EOS; Wyatt Technology Co. Ltd., Santa Barbara, CA, USA). The absolute molecular weight and radius of gyration were calculated using a multi-angle light scattering (MALS) detection system. Toluene was used for calibration of the apparatus and a value of 1.406 × 10^{-5} was used as the Rayleigh ratio. A value of 0.15 mL/g was used as the refractive index increment (dn/dc) for molecular weight calibration (Nakamura, Fujii, Tobe, Adachi, & Hirotsuka, 2012).

2.8. Atomic force microscopy

BX1.5 EPS extracts were dispersed into ultrapure water to final concentrations of approximately 1 mg/mL, using a magnetic stirrer at room temperature, and then heated at 70 °C for 1 h. They were filtered through cellulose acetate filters (0.80 μm; Advantec Co. Ltd.), and then diluted up to 10 μg/mL with a 10 mM aqueous solution of non-ionic surfactant Tween-20 (Wako Chemical Co. Ltd., Tokyo, Japan). A 0.5-μL aliquot of the sample was immediately deposited onto mica surfaces, and dried at room temperature for 20 min. Atomic force microscope (AFM) imaging was performed in air at 20 °C using an alternating current cyclic contact mode of a multi-mode imaging unit (SPI4000; Seiko Instruments Inc., Chiba, Japan) controlled by a probe station (SPI3800 N; Seiko Instruments Inc.). Beam-shaped Si cantilevers (spring constant 20 N/m) were excited at a frequency proximate to a resonant frequency of 136 kHz. The sample surface was scanned with the probe at a scanning frequency of 0.5–1 Hz (Ikeda, Funami, & Zhang, 2005).

2.9. Measurement of viscosity

The viscosity of BX1.5 EPS extracts, as 0.5 % (w/v) hydrates, was measured using a corn-plate type viscometer, LV-DV2T, with a spindle CPA-52Z (AMETEK Brookfield Inc., Middleborough, MA, USA), in the temperature range of 10–40 °C. Samples of ι- or κ-carrageenans were also prepared by the same procedure as for standard controls.

2.10. Emulsion stability test

Mixtures were prepared with 13.9 g of water (14 g, if no stabilizer is added), 4 g of emulsifier (gum arabic), 0.2 g of granulated sugar, 1.4 g of 10 % (w/v) acetic acid, 0.4 g of sodium chloride, and stabilizer [none, 0.12 g of xanthan gum (XG), or 0.12 g of BX1.5 EPS extract]. After stirring overnight, 20 g of canola oil (J-oil Mills Inc., Tokyo, Japan) were added and homogenized using a homogenizer at 15,000 × *g* for 3 min to prepare egg-free mayonnaise [oil, 50 % (w/w)] (Chivero, Gohtani, Yoshii, & Nakamura, 2016). After the egg-free mayonnaise had been stored in the refrigerator (at 4 °C) for 3 weeks, 1-mL drops were spotted on a filter paper (No.1; Advantec Co. Ltd) and heated at 210 °C in an oven (MRO-TS8; Hitachi Co. Ltd., Tokyo, Japan) for 3 min. After heating, area of the oil stain from the egg-free mayonnaise was measured with a digital microscope (VHX-950F; Keyence Co. Ltd., Osaka, Japan). The average oil-stained area of egg-less mayonnaise, without any added stabilizer (none), was considered 100 %; the oil-stained area was also calculated for XG and BX1.5 EPS extract (bxEPS) ($n = 3$).

2.11. Spot assay for stress resistance

The assay was performed as per a previously described method (Sasaki et al., 2020), with slight modifications as follows. After the pre-cultivation (see the section Cultivation conditions), a 5-mL aliquot of culture was harvested and suspended into 30 mL of fresh BG11 medium in a 300-mL Erlenmeyer flask. This was placed in the chamber CF-415 for 2 days for main cultivation; EPS was not secreted much on the cell surface. A 100-μL aliquot of cell-culture was harvested and centrifuged at 10,000 × *g* for 5 min. Thereafter, the cell pellet was collected and 0.5 % (w/v) BX1.5 EPS aqueous solution (100 μL) was either added (+EPS) or not added (control) to the pellet. They were subjected to heat drying stress (HD), freeze-thaw stress (FT), freeze-dry stress (FD), and ultraviolet stress (UV), separately. For heat drying stress, it was left overnight at 42 °C. Sterilized and deionized water (100 μL), as controls, were added to the cell pellet when the stress was applied by ultraviolet irradiation. To these samples, 100 μL of fresh BG11 medium were added and mixed well. For freeze-thaw stress, it was naturally thawed after freezing at −80 °C. This sample was added to 100 μL of fresh BG11 medium and mixed well. For freeze-dry stress, the sample was vacuum freeze-dried using V-850 (BUCHI) for 90 min under a 0.002 mBar setting after freezing at −80 °C. To the dried cell-pellet, 100 μL of fresh BG11 medium was added and mixed well. For ultraviolet stress, the sample was transferred to a 96-well plate, and irradiated by UV (260 nm) light at 100 μmol photons m^{-2} s^{-1} for 5 min at a distance of 4 cm from the UV-light source. After each stress treatment, a 5-μL aliquot of the sample was spotted onto the BG11 agar plate. These cells were incubated under a 0.04 % CO_2-air condition with 30 μmol photons m^{-2} s^{-1} of white fluorescent-light at 30 °C for 15 days. Cells were observed on days 5, 10, and 15, sequentially.

3. Results

3.1. Condition for EPS production

The new strain *Parachlorella* sp. BX1.5 could grow in BG11 liquid medium under conditions of 0.04 % CO_2-air for 13 days, producing a small amount of extracellular polysaccharide (EPS), as observed in cells stained with India ink (Fig. 1a). The EPS production was, however, significant under air conditions of 2% CO_2, even if the incubation time was only one day (Fig. S1), thus suggesting high concentration of CO_2 to be effective for EPS overproduction (Fig. 1b). EPS production was most notable under conditions of 2 % CO_2 and nitrogen-starved BG11 ($BG11_0$, BG11-N) medium (Fig. 1c). The overproduction of EPS could also be observed under conditions of 2% CO_2 and phosphate-starved BG11 (BG11-P) medium (Fig. 1d). No overproduction was observed when another strain *Parachlorella* sp. 11h was cultured under the same conditions (data not shown). This suggested the ability of EPS overproduction to be specific for the BX1.5 stock. We investigated the BX1.5 EPS-production status based on values indicated by the ratio of EPS diameter (Φ*ei*) to cell diameter (Φ*ci*) under various culture conditions (Fig. 1 bottom, Fig. S1). Results showed the most effective value of Φ*ei*/Φ*ci* under BG11-N condition, although other culture conditions were also tested for EPS production (Fig. S2A). EPS extracts could be recovered using artificial sea water (ASW) while there was almost no accumulation when BG11+NaCl medium was used (Fig. S2B). Therefore, we used the BG11-N condition for overproduction and extraction of EPS in the subsequent experiments.

3.2. EPS extraction

After EPS overproduction, BX1.5 cells were harvested from the BG11-N medium (Fig. S3a), and EPS samples prepared with several rounds of ethanol extraction (Fig. S3b, c). The resultant was added to

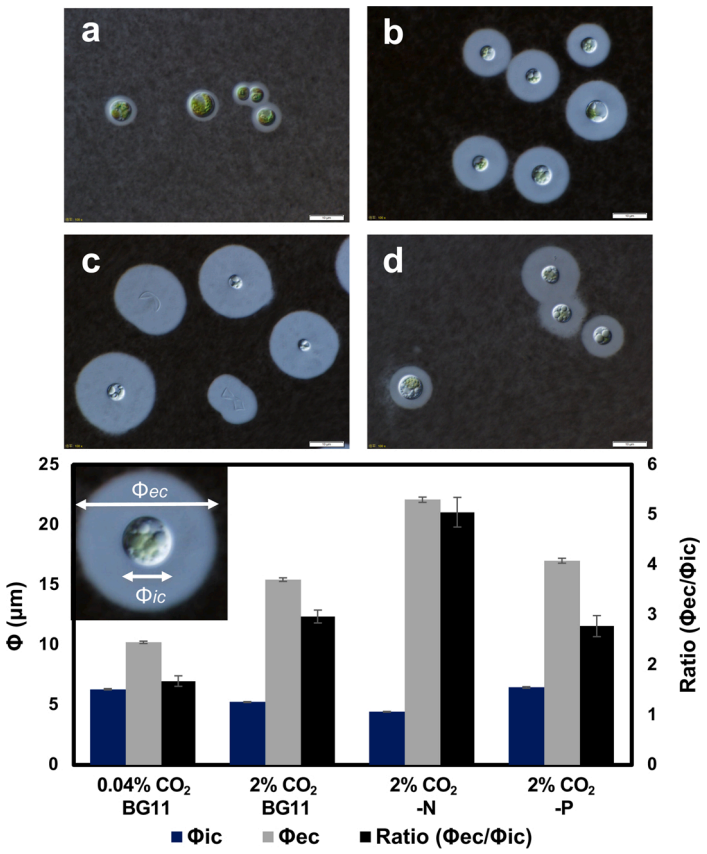

Fig. 1. Sizes of the cells producing exopolysaccharide. Diameters of the cells inside (Φ*ic*) and outside (Φ*ec*), and the ratio (Φ*ic*/Φ*ec*) were determined along with standard deviations from the photographs shown in the top panels. The bar indicates 10 μm. Sample numbers: CO_2 0.04 % BG11 **(a)**, $n = 17$; CO_2 2 % BG11 **(b)**, $n = 28$; CO_2 2 % -N **(c)**, $n = 13$, and CO_2 2 % -P **(d)**, $n = 19$, respectively.

acetone and dried for a day (Fig. S3d). The dried EPS extract was crushed in a mortar and eventually ground (Fig. S3e). It was stored as EPS extract at room temperature until further use. The extracts were dissolved in water to prepare 0.5 % (w/v) solutions of each (Fig. S3f), and used for investigating their characteristics and functional properties. It is noteworthy that the EPS extract was obtained with high yield (approximately 1.6 g/L of BG11-N culture). It, however, contained small amounts of cells that could not be completely removed despite trying several extraction methods. This implied that cells are tightly wrapped and protected by EPS. Therefore, we selected this grade of EPS extract for further experiments.

3.3. Composition of EPS extract

The EPS extract contained proteins at 9.62 wt% and total sugar at 73.8 wt%, with sugar monosaccharide composition being 49 mol% rhamnose, 17 mol% galactose, 13 mol% glucose, 13 mol% xylose, and 9.0 mol% galacturonic acid (Table 1). Arabinose, fructose, or glucuronic acid was not detected. Since the content profile was apparently different from what had been known until date, BX1.5 EPS was considered an acidic rhamnan and a novel glycan. Since acidic EPS from the green alga *Monostroma nitidum* is referred to as rhamnan sulfate (Karnjanapratum & You, 2011; Lee et al., 2010; Mao et al., 2008), we investigated whether acidic BX1.5 EPS also possesses sulfate group. The organic functional groups of BX1.5 acidic rhamnan were identified by Fourier-transform infrared (FT-IR) spectroscopy and the results are shown in Fig. 2. When pullulan was used as a control polysaccharide polymer without sulfate group, consisting of maltotriose units, also known as α-1, 4- (and α-1, 6-) glucan, a profile with specific peaks was obtained. When κ-carrageenan was used as a control polymer, with one sulfate group per disaccharide, made up of repeating galactose units joined by alternating α-1, 3- and β-1, 4-glycosidic linkages, specific peaks referring to S═O and C—O—S bonds in the sulfate group were identified. BX1.5 EPS did not contain a sulfate group, and the glycosyl bond pattern (area marked with a dashed circle) was found to be simpler than in pullulan. Therefore, the results reconfirmed BX1.5 EPS as a new type of acidic glycan.

3.4. Molecular mass and structure

Molecular mass of BX1.5 EPS was determined by gel-filtration

Table 1
BX1.5 EPS information.

Protein (weight %)	Carbohydrate (weight %)	Monosaccharide composition (mol%) *							Absolute molecular weight (kDa)	Radius of Gyration (nm)	CPS [log (nm)/log (g/mol)] †	Length (μm) ‡
		Rha	Gal	Glc	Xyl	Ara	Fuc	Gal A				
9.62	73.8	48.5	16.7	13.2	13.1	ND	ND	8.52	1.75×10^3	194.2	0.89	0.85

* Rha, rhamnose; Gal, galactose; Ara, arabinose; Fuc, fucose; Xyl, xylose; Glc, glucose; Gal A, galacturonic acid. ND, not detected.
† CPS, conformation plot slope.
‡ An average value per a linear molecule.

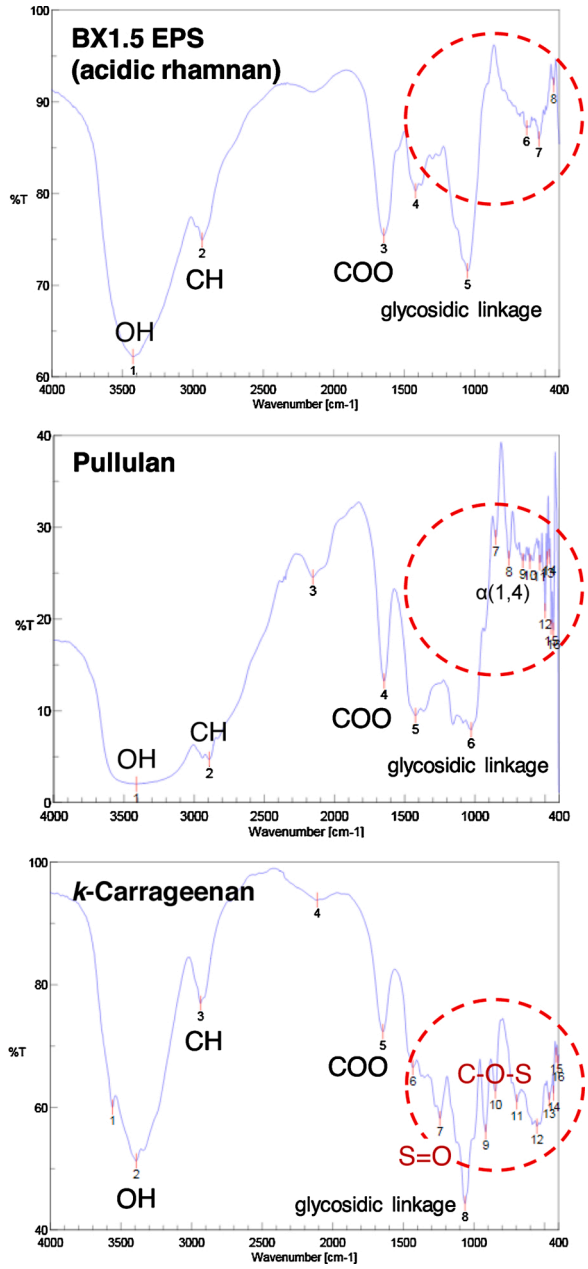

Fig. 2. FT-IR spectrum of BX1.5 acidic rhamnan. Respective peaks referring to the specific bonds or glycosidic linkage are shown. The BX1.5 acidic rhamnan does not contain sulfate group, as shown by the dashed circle. As controls, pullulan (with no sulfate group) and κ-carrageenan (with a sulfate group) were used and also shown with the area focusing by dashed circles.

chromatography using an HPLC system (Fig. 3A). Analysis revealed a high molecular mass of 1.75 (up to 2.11) × 10^6 daltons (Da), distributed uniformly, and a radius of gyration of 194.2 nm in water (Table 1). A conformation plot showed a slope of 0.89 ± 0.02 log (nm) / log (g/mol), the value calculated using the radius of gyration and absolute molecular weight, thus indicating the BX1.5 EPS structure to be almost linear in water-soluble form. The shape of EPS was further confirmed to be linear, with average chain length of one molecule estimated as 0.5–1.2 μm by atomic force microscope (AFM) analysis (Fig. 3B, *X*-, or *Y*-axis). The high molecular weight and linear structure of BX1.5 EPS was reconfirmed by comparing with the AFM results of HM pectin, whose structure resembles bifurcated glucan and has smaller molecular size than BX1.5 EPS (Nakamura, Furuta, Kato, Maeda, & Nagamatsu, 2003), as shown in Fig. S4. There has not been any report of linear structure of acidic rhamnans until date, suggesting a novel aspect of BX1.5 EPS. This discovery inspired further characterization of BX1.5 EPS properties.

3.5. Viscosity characteristics

Polysaccharides are widely used in food and other industries as thickening and stabilizing agents; therefore, it was considered important to characterize the viscosity of BX1.5 EPS. We prepared BX1.5 EPS extracts at various concentrations, in water, and even 1% (w/v) sample was found to show a sol state (Fig. 4a). ι- (loose gel) or κ- (gel) carrageenan was used as control material for food additive (Fig. 4b, c, Funami et al., 2007).

The viscosity characteristics are summarized in Table S2, with respect to other representative food additives. Although polysaccharides having a straight-chain structure generally tend to have high viscosity, the viscosity of aqueous BX1.5 EPS solution was found to be the lowest among all linear polysaccharides, and no gel-formation was observed in the 0.5–10 % aqueous solutions. Although BX1.5 acidic rhamnan did not form a gel at high concentrations (5–10 %), it did form a highly viscous sol. On the other hand, polysaccharides having multi-branched structure, with bifurcation or polytomy, have low viscosity that remained at 11.5 mPa × s (Arabic gum) or 110 mPa × s (soybean polysaccharides) even in 10 % aqueous solutions. BX1.5 EPS, as straight-chain rhamnan, had the same high viscosity of 25,000 mPa × s in 1% aqueous solution, thus clarifying BX1.5 acidic rhamnan to be a polysaccharide with unique properties, showing high viscosity at low concentrations while not forming gels. As a preliminary experiment, rhamnan of the present context had been seen to be soluble in water up to at least 20 % (data not shown).

Temperature dependence of viscosity of 0.5 % (w/v) aqueous solutions was also investigated using a corn-plate type viscometer having a spindle CPA-52Z. The viscosity of 0.5 % aqueous solution of BX1.5 was 9–10 Pa in shear stress (Fig. 5A), thus indicating a non-Newtonian fluid state, and a plastic-flow, like mayonnaise (Fig. 5A). Viscosity of polysaccharides is generally temperature-dependent; iota (ι)-carrageenan and kappa (κ)-carrageenan, commonly used as food additives, show large changes in viscosity depending on temperature. However, viscosity of BX1.5 EPS hardly changed in the temperature range of 10–40 °C, and remained stable over a wide range of temperature (Fig. 5B). Its resistance to change in viscosity over a general temperature range makes it an excellent candidate as food or cosmetic additive.

3.6. Property for food additives

For the purpose of stabilizing dispersion and improving fluidity of emulsified oil droplets, mayonnaise-like food were prepared as emulsions with polysaccharide extracts of BX1.5 or xanthan gum, generally used as stabilizer for food additives. When the sample with 0.3 % (w/w) BX1.5 EPS was prepared, separation of oil (oil-off) and aggregation of oil droplets were not observed under conditions of 50 % (w/w) oil content (Fig. 6A). Little change was observed in median particle size of oil droplets and little increase in viscosity was seen, over time, indicating the mayonnaise-like food with BX1.5 EPS to be stable. On the other hand, the sample containing 0.3 % (w/w) xanthan gum showed little change in the median particle size of the oil droplets and little increase in viscosity over time. However, aggregation of oil droplets was observed (Fig. 6A). Based on these results, BX1.5 acidic rhamnan was concluded to have an emulsion stabilization function superior to that of xanthan gum.

When using mayonnaise as a seasoning for the production of food, like pizza, it is desirable to have no oil separation upon heating, that is, the emulsified state remains maintained. Therefore, firing resistance reducing oil-off, for stability during baking, was investigated for the mayonnaise-like samples. The mayonnaise-like food with BX1.5 EPS was seen to maintain an emulsified state even when heated, since there was little oil-off (oil-exuded parts shown with arrow heads) caused by

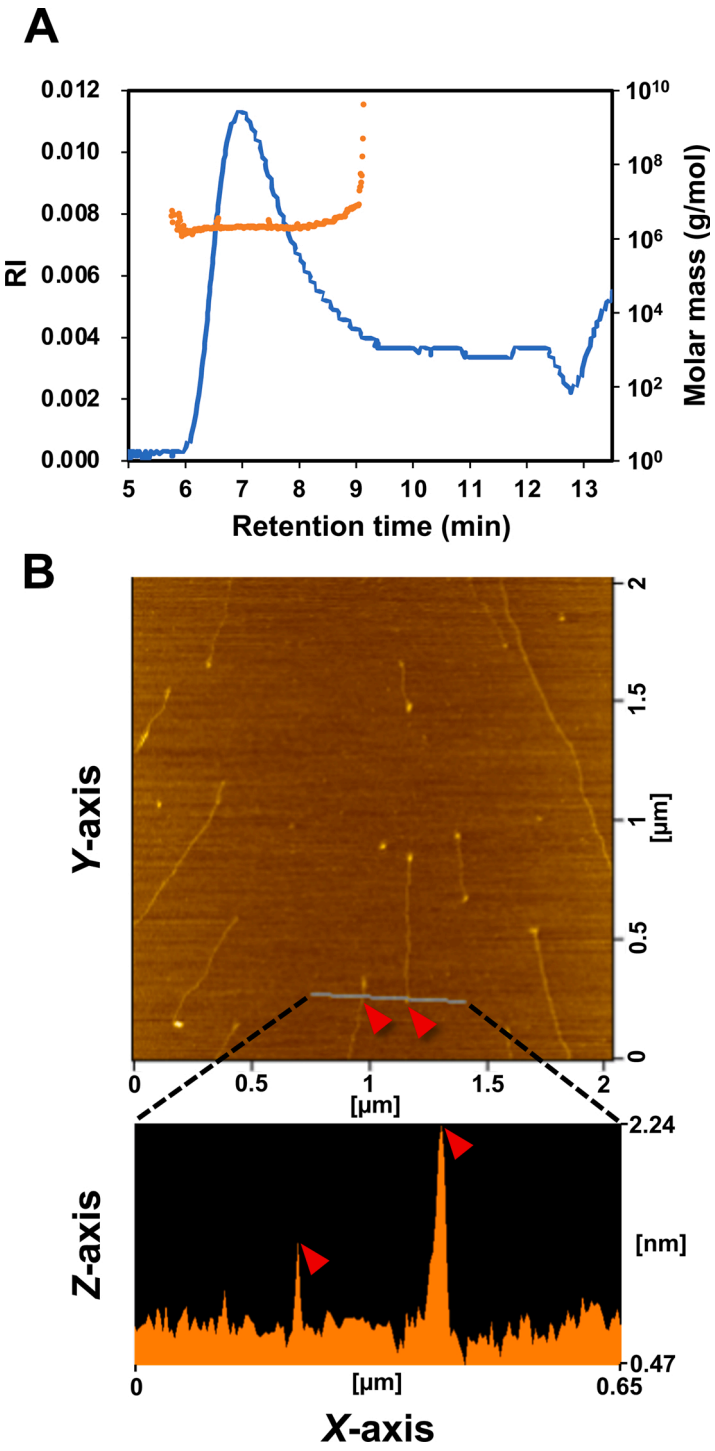

Fig. 3. Molecular weight and structure of BX1.5 EPS. **(A)** Analytical gel-filtration chromatography of the BX1.5 acidic rhamnan was performed using an HPLC system. Profiles of the refractive index (blue dots) and the absolute molecular mass (orange dots) for the effluent are shown. **(B)** AFM images. The *X-Y* (upper panel) and *X-Z* (lower panel with arrowheads) plots accord to a flat surface and height of the samples on a mica plane, respectively. The section is shown by a line with arrowheads.

heating (Fig. 6B). The results suggested BX1.5 EPS extract to be easy to mix with fats/oils, and to remain stable against heat treatment, thus implying its feasibility of application as a food additive.

3.7. Biological significance in stress tolerance

A spot assay was conducted to elucidate some of the biological functions of BX1.5 EPS. In this assay, 0.5 % (w/v) BX1.5 EPS extract was added to the BX1.5 cells that did not produce much EPS (Fig. 1a) and the latter were subjected to various stress conditions. The treated cells (5 µL) were spotted on a BG11 plate and growth was sequentially observed for 15 days (Fig. 7). Results confirmed that the cells could grow despite freeze and thaw (FT), with or without EPS, hence indicating the possibility of long-term cryopreservation for the BX1.5 strain. The test group with EPS showed better growth than the control group without EPS under any stress of freeze (vacuum) drying (FD), heat drying (HD), or

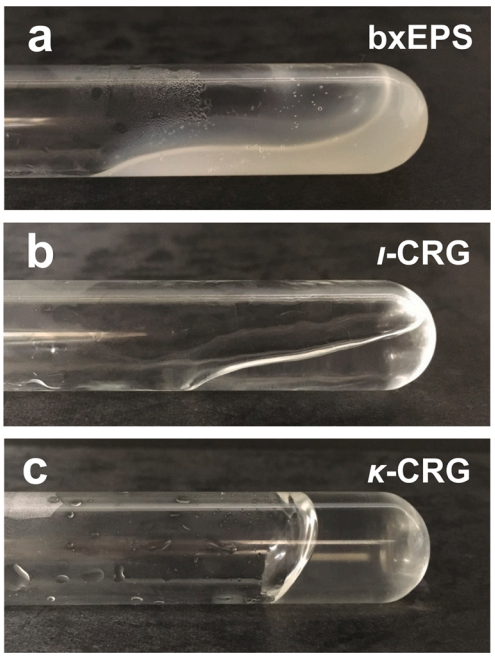

Fig. 4. Aqueous solution of EPS. Samples were dissolved in water as 1 % (w/v) solution and treated at 80 °C for 60 min; 2.5 mL of the resultant was left in a glass tube at 25 °C. **(a)** BX1.5 EPS (bxEPS) as a sol, **(b)** ι-carrageenan as loose gel, and **(c)** κ-carrageenan as a gel.

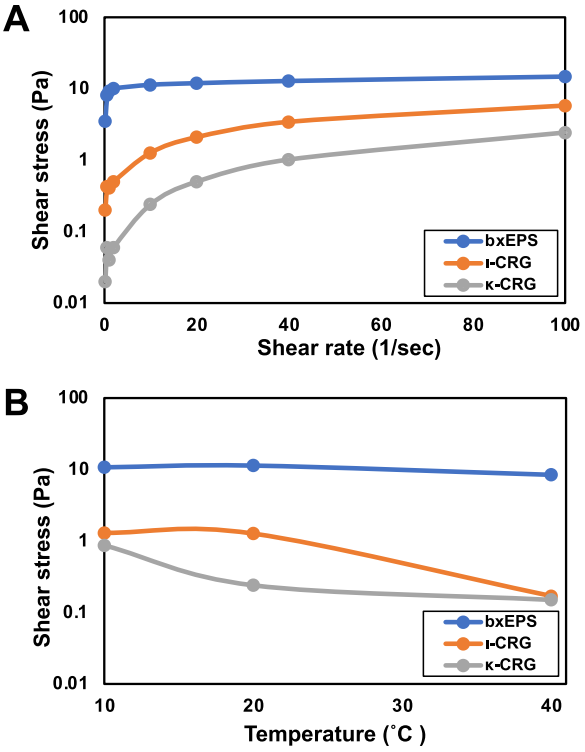

Fig. 5. Viscosity of aqueous BX1.5 EPS solution. A 0.5 % (w/v) bxEPS solution was prepared and subjected to analysis using a corn-plate type viscometer at temperatures 10–40 °C. Samples of ι-carrageenan or κ-carrageenan were used as controls. **(A)** Effect of shear rate at 20 °C; **(B)** Effect of temperature at a shear rate of 1/sec.

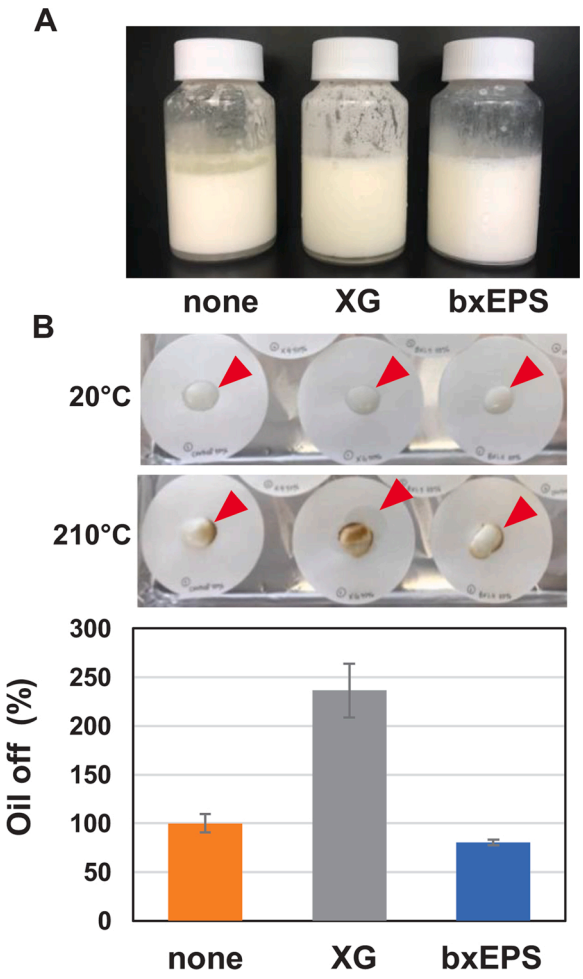

Fig. 6. Emulsion stability test using BX1.5 EPS. **(A)** Egg-free mayonnaise was added to BX1.5 EPS [bxEPS, 0.3 % (w/w)] as a stabilizer. Samples without bxEPS (none) or with xanthan gum (XG) were used as controls. A photograph of the sample after standing for 6 days is shown. **(B)** Egg-free mayonnaise [1 mL from panel A, oil content as 50 % (w/w)] was spotted on a filter paper, without or with a baking treatment in an oven at 210 °C for 3 min (upper panel). Area of the oil-off from egg-free mayonnaise on the filter (arrow heads) was measured with a digital microscope. The values (n = 3) were evaluated and shown with respect to the control using "no added stabilizer" as the condition for 100 % (lower panel).

ultraviolet (UV) treatment. Therefore, BX1.5 EPS was found to impart improved resistance (environmental stress tolerance) to living cells. Such enhancement of environmental stress tolerance suggested the usefulness of BX1.5 EPS as a cosmetic additive, culture additive, or a pharmaceutical additive.

4. Discussion

The green alga *Parachlorella* sp. BX1.5 was found to coproduce lipids and polysaccharides abundantly (Figs. 1 and S1). The lipids produced by BX1.5 were significantly enhanced under nitrogen-starved culture conditions ($BG11_0$, BG11-N). Those produced in a BX1.5 cell were approximately 30–70 % (w/w), and were composed of palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2, ω6 oil), and α–linolenic acid (C18:3, ω3 oil) as useful food oil (Sasaki et al., 2020). Dual production of useful EPS and food oil inside the cells recommend the use of this alga for economic production. Since the BX1.5 cells are coated with acidic EPS, they may be cultured in extensive alkaline conditions (pH 10–11) (Sasaki et al., 2020), for which carbon dioxide is effectively dissolved in liquid culture. It is appropriate for large-scale outdoor

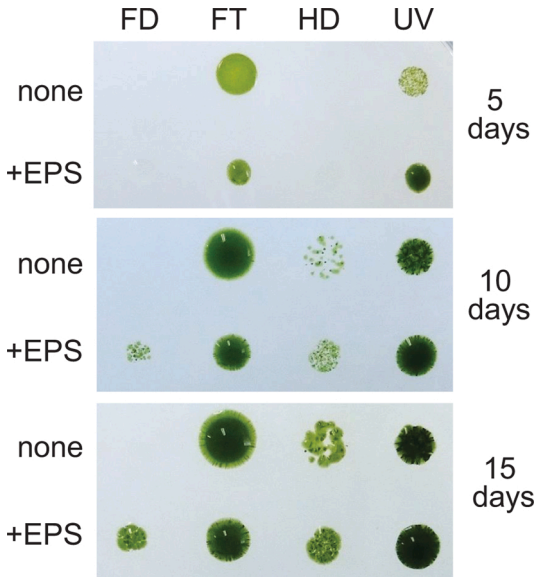

Fig. 7. Spot assay for stress tolerance by BX1.5 EPS. BX1.5 cells were prepared and either mixed with 0.5 % (w/v) BX1.5 EPS solution (+EPS) or not (none). The samples were exposed to stress conditions. An aliquot of 5 μL from the samples was spotted onto a BG11 plate, incubated for 5, 10, and 15 days to observe stress resistance. FD, freeze dry; FT, freeze-thaw; HD, heat dry; UV, ultraviolet-rays irradiation.

cultivation without contamination. In fact, we succeeded in cultivating BX1.5 cells in 10-ton scale in the outdoors. Since BG11 is a medium rich in sodium nitrate, we found nitrogen-deficient condition to be one of the best for EPS-overproduction (Sasaki et al., 2020), promising low cost of cultivation.

The molecular weight of BX1.5 EPS was determined to be 1.75 (up to 2.11) × 10^6 Da, which makes it the second largest natural polysaccharide known until date. The ultra-high molecular weight of BX1.5 EPS was found to be the highest among the natural glucans known until date, except for sacran (>1.6 × 10^7 Da) extracted from the cyanobacterium *Aphanothece sacrum* (Kaneko & Kaneko, 2008). Interestingly, the ends of each BX1.5 EPS molecule may be considered to be wound into a sphere and have a specific thickness (Fig. 3B, *Z*-axis). Although *Parachlorella kessleri* can also produce an EPS with branched-type molecular structure, its molecular weight (6.5 × 10^4 Da) and sugar composition (Table S1) (Ishiguro et al., 2017; Sato & Ri, 2014) were totally distinct from those of BX1.5 EPS (Table 1). Notably, the BX1.5 EPS has a linear structure (0.5–1.2 μm, Fig. 3B), is water soluble, and shows quite a unique property of solation with high viscosity (7,500 to 24,000 mPa × s) even at low concentrations (0.5–1 %) in water (Table S2, Fig. 4). Viscosity of the BX1.5 EPS remained constant from 10 to 40 °C (Fig. 5B). This is the first case of linear-chain EPS reported from microalgae. Another linear-type EPS, called curdlan, from the non-photosynthetic microorganism *Alcaligenes* sp., is well known, containing glucose (approximate 100 %, β1→3 bond), having a molecular weight > 2.0 × 10^5 Da (which is much less than that of BX1.5 EPS), and showing "gel" formation (Table S2, Dobashi et al., 2006). BX1.5 EPS was evaluated as an acidic rhamnan containing galacturonic acids (uronic acids, Table 1). Rhamnan sulfate, an acidic EPS from green alga *Monostroma nitidum*, is mainly composed of rhamnose [78 wt% (w/w)], and is reported to have a molecular weight of 8.7 × 10^5 Da (Karnjanapratum & You, 2011; Lee et al., 2010; Mao et al., 2008). Although curdlan and rhamnan sulfate are soluble in water, resulting in gelation, their properties are apparently different from those of BX1.5 EPS, thus suggesting the novelty of BX1.5 EPS. Of note, since BX1.5 EPS extract contains proteins (Table 1), this may influence the unique property of solation with constant high viscosity. EPS suppresses the coalescence of oil droplets by adding viscosity to the aqueous phase of emulsion. This function is considered similar to that of xanthan gum. On the other hand, protein in the EPS extract may work as an emulsifier, similar to soy polysaccharides (Chivero, Gohtani, Yoshii, & Nakamura, 2014). EPS is superior to xanthan gum in terms of its emulsification activity and emulsion stability (Chivero, Gohtani, Yoshii, & Nakamura, 2015).

The results shown in Table S2 and Figs. 4 and 5 suggest the viscosity characteristics of BX1.5 EPS to be appropriate for it to be a food additive and a thickener. Moreover, the results shown in Fig. 6 implied appreciable stabilization of the emulsion even when exposed to high temperatures (210 °C), thus reducing oil-off. This ability could not be complemented by xanthan gum under the same conditions, indicating BX1.5 EPS as appropriate for emulsion stabilization, as well as for water retention as a food additive. Further analyses of properties of BX1.5 EPS in terms of the shape and composition might explain how the EPS extract influences its stabilizing capacity. Results shown in Fig. 7 demonstrated the performance of BX1.5 EPS as environmental stress tolerance enhancer against freeze (vacuum) drying, heat drying, and ultraviolet-rays treatment. Therefore, the EPS could be suitable as a culture additive and/or cosmetic materials.

Taking the above findings into account, BX1.5 EPS might provide new insights for polysaccharides and be potent to contribute to algal biorefinery, not only for food and feed but also for cosmetic and medical considerations. Structural analysis of the sugar-chain-binding mode and further functional analysis of BX1.5 EPS are awaited in future.

CRediT authorship contribution statement

Mitsuki Sasaki: Data curation, Formal analysis, Investigation. **Akari Takagi:** Data curation, Formal analysis, Investigation. **Daisaku Sasaki:** Data curation, Formal analysis, Investigation. **Akihiro Nakamura:** Data curation, Formal analysis, Investigation. **Munehiko Asayama:** Data curation, Formal analysis, Investigation.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2020.117252.

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Table S1. Polysaccharides.

PS name (bacteria)	Sugar composition * (weight % or mol %)	Molecular weight (Da)	References
BX1.5 extract *Parachlorella* sp. BX1.5 (Green alga)	Rha : Gal : Glc : Xyl : Gal A = 49 : 17 : 13 : 13 : 9 Uronic acid = 9	1.75 (～2.11) x 10^6	This study Sasaki et al., 2020
Kessleri extract *Parachlorella kessleri* (Green alga)	Gal : Man : Ara : Rha : Xyl = 67.1 : 22.5 : 4.7 : 4 : 1.7 (Gal = 50～79)	5～8 x 10^4	Ishiguro et al., 2017 Sato & Ri, 2014
Rhamnan sulfate *Monostroma nitidum* (Green alga)	Rha : Gal : Glc : Xyl = 79 : 5.3 : 10 : 5.2 Uronic acid = 7.62 Sulfate ester = 28.2	8.7 x 10^5	Karnjanapratum & You, 2011 Lee et al., 2010 Mao et al., 2008
Glycoprotein *Scenedesmus* sp. (Green alga)	Gal : Glc : Man : Rha : Fuc: Glcs : Xly = 29 : 18 : 16 : 16: 14 : 4 : 3	4.0 x 10^5	Ando et al., 1994
Sacran *Aphanothece sacrum* (Cyanobacterium)	Ara : Fuc : Rha : Xly : Man : Gal : Gal A : Glc A : Glc : Gals = 1 : 4 : 15 : 17 : 10.5 : 12 : 5 : 5 : 29 : 2	2.0 x 10^6 ～1.6 x 10^7	Kaneko & Kaneko, 2008
Spirulan *Arthrospira platensis* (Cyanobacterium)	Rha : Rib : Man : Fru : Gal : Xyl : Glc : Glc A : Gal A = 60 : 3 : 1 : 46 : 3 : 0.8 : 6 : 10 : 5	2.6 x 10^5	Hayashi et al., 1997
κ-Carrageenan *Kappaphycus cottonii* (Red alga)	Gal = 100	7.1 x 10^5	Funami et al., 2007
Curdlan *Alcaligenes* (Non photosynthesizing bacterium)	Glc = 100	>2.0 x 10^5	Dobashi et al., 2006

* Rha, rhamnose; Gal, galactose; Glc, glucose; Xyl, xylose; Man, mannose; Fuc, fucose; Ara, arabinose; Gal A, galacturonic acid; Glc A, glucuronic acid; Gals, galactosamine; Glcs, glucosamine; Fru, fructose.

Table S2. Polysaccharide property.

PS name	Structure	Shear rate (1/sec)	Viscosity (mPa・sec)*						Gelation†
			0.1%	0.2%	0.5%	1%	5%	10%	
BX1.5 extract	Linear	0.1	15	1,800	7,500	25,000	UM	UM	−
Curdlan	Linear	0.1	320	38,400	UM	UM	UM	UM	+
κ-Carrageenan	Linear	0.1	20	4,600	UM	UM	UM	UM	+
Guar gum	Bifurcation	0.1	120	10,500	UM	UM	UM	UM	−
Pectin	Bifurcation	0.1	8	80	750	6,500	UM	UM	+
Xanthan gum	Bifurcation	0.4	140	744	2,325	5,907	UM	UM	−
Soybean PS	Polytomy	100	1.2	2.5	5.4	12	64.5	110	−
Pullulan	Polytomy	100	0.7	0.9	1.1	1.9	12.8	92.5	−
Arabian gum	Polytomy	100	1.1	1.9	2.8	5	9.5	11.5	−

***** water soluble of polysaccharides [% (w/v)]; UM, unmeasurable cases under high viscosity. † +, gelation ; -, non gelation.

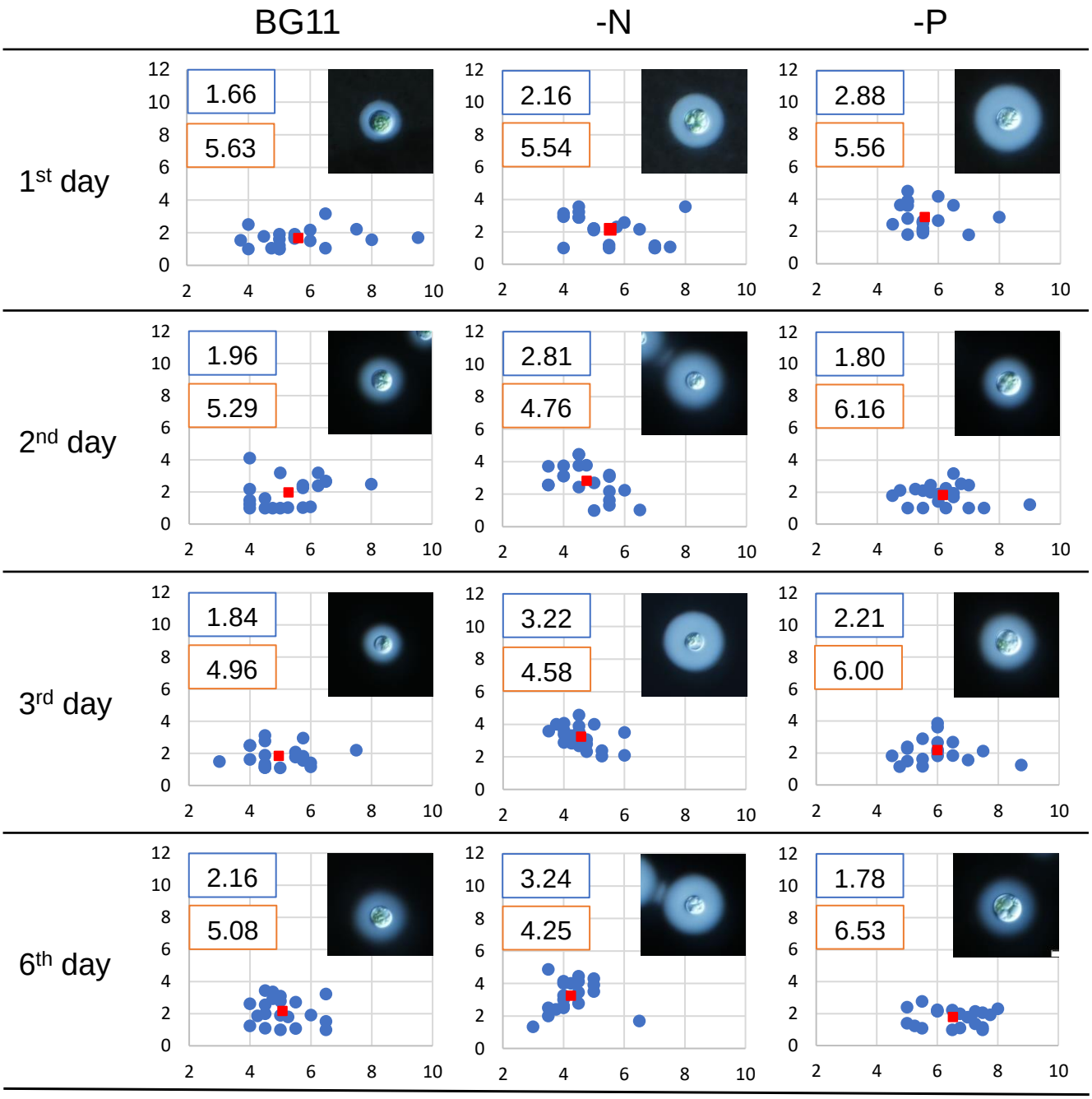

Figure S1. Sizes of the cells producing exopolysaccharide. After pre-culture, each culture was collected on the 1st, 2nd, 3rd, and 6th days of the main culture, and diameters inside (Φ*ic*) and outside (Φ*ec*) of the cells were measured. *X*-axis of the graph indicates the diameter inside (Φ*ic*, μm), and *Y*-axis indicates the ratio (Φ*ic*/Φ*ec*). The blue dots show values measured from 20 randomly chosen cells, and the red square shows the average value corresponding to Φ*ic* (red frame) and Φ*ic*/Φ*ec* (blue frame). This experiment was performed as independent session, as shown in Fig. 1.

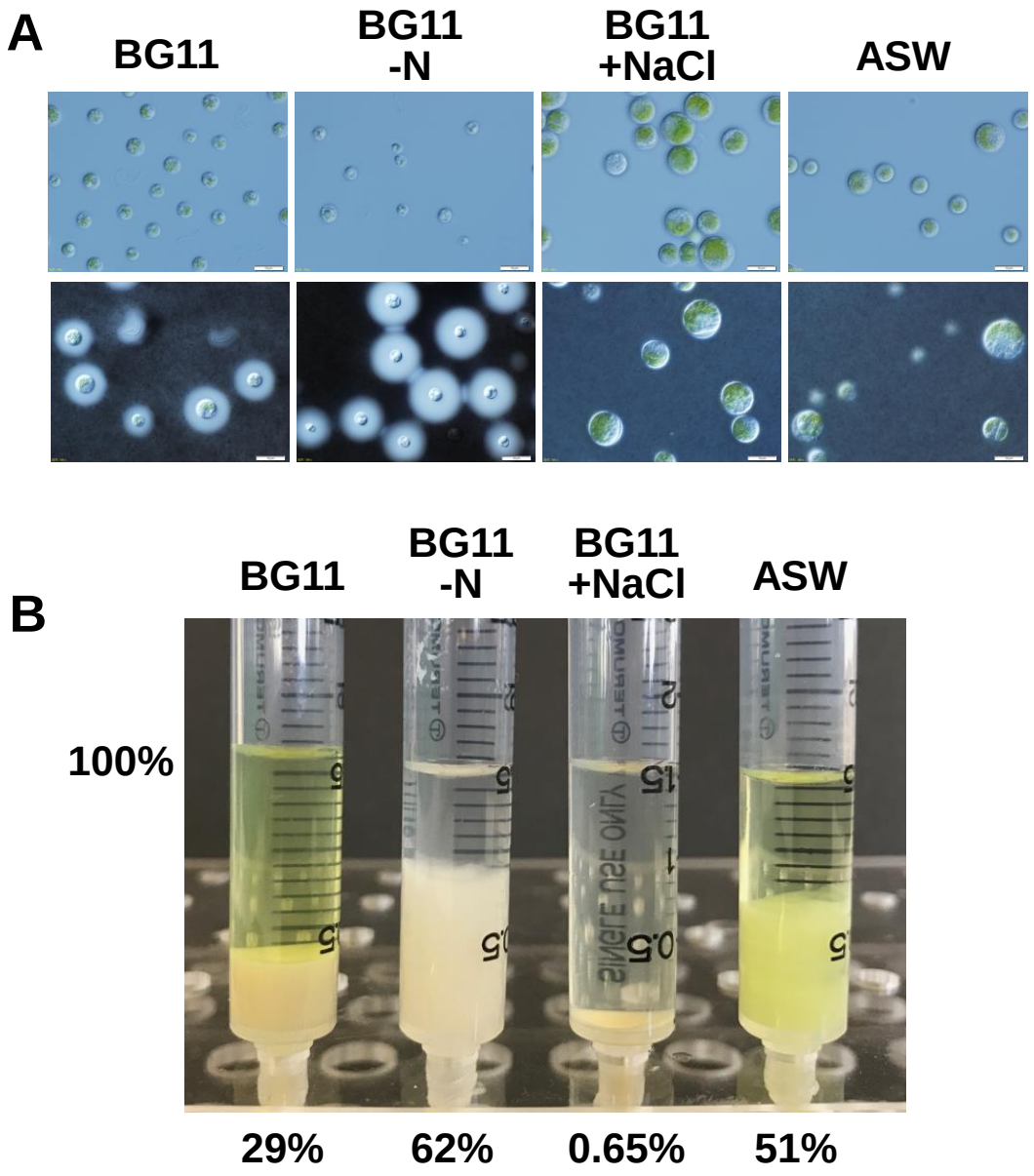

Figure S2. BX1.5 cells and ethanol precipitation. (**A**) Microscopic observation of the BX1.5 cells, stained without (upper) or with (lower) India ink, under different culture conditions. (**B**) Ethanol precipitation shown with relative bed volume (%, v/v); white-color pellet contains EPS.

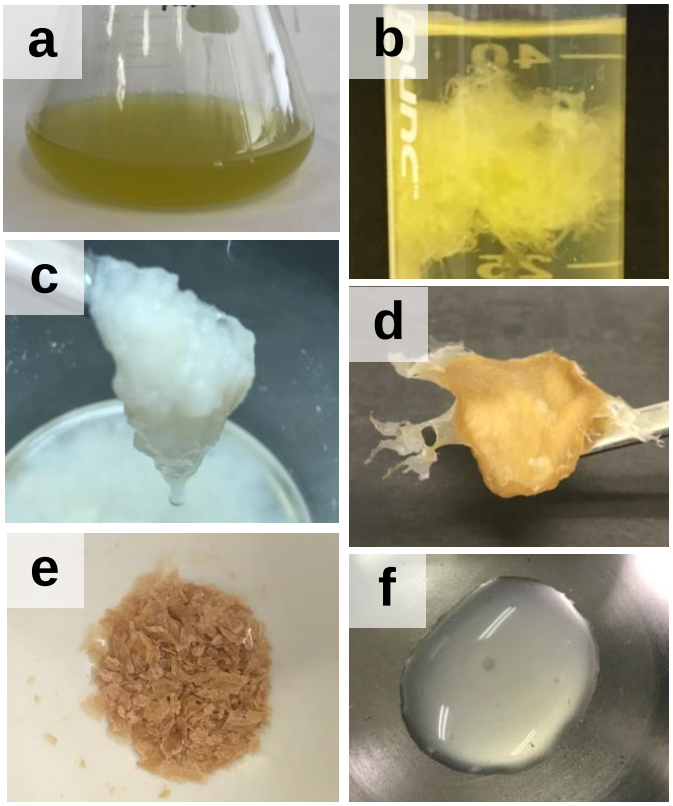

Figure S3. Preparation of BX1.5 EPS extracts. A 55-mL cell culture of BX1.5, grown in the BG11-N medium in the incubation chamber (supplied with 2% CO_2 gas) for 6 days (**a**), was harvested and transferred into a new tube. The sample was added to ethanol at 70% (v/v) concentration, vigorously mixed, allowed to stand at room temperature for 30 min (**b**), and centrifuged at 6,000 $\times$ *g* for 10 min. After this, the pellet fraction was collected and ethanol (50 mL) was added. This sample was again mixed, and centrifuged; the ethanol extractions were performed four times. Thereafter, 10 mL of acetone was added to the pellets (**c**) and dehydrated overnight (**d**). The dried samples were ground in a mortar (**e**). An aliquot of the sample from (**e**) was dissolved in water at 0.5% (w/v), treated at 95 ˚C for 10 min, and cooled back to room temperature (**f**). See more details in the text.

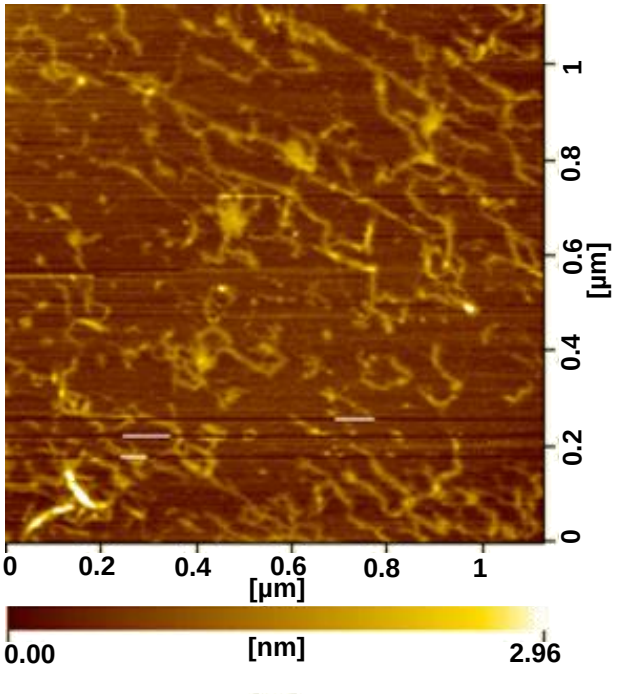

Figure S4. AFM image of HM pectin. The bifurcation glucan, of average molecular mass 3.8 x 10^5 Da, is composed of α-1,4-bonds of polygalacturonic acid. A color gradation of the bar indicates height of the polysaccharide on the mica.