J Appl Phycol (2015) 27:755–761
DOI 10.1007/s10811-014-0363-x

The effect of the water-soluble polymer released from *Botryococcus braunii* Showa strain on solvent extraction of hydrocarbon

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Received: 8 February 2014 / Revised and accepted: 9 June 2014 / Published online: 29 June 2014
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Abstract Hydrocarbons are easily extracted by organic solvents such as *n*-decane from wet samples of *Botryococcus braunii* by thermal pretreatment at 90 °C even after being cooled to room temperature. However, hydrocarbon recoveries are not as readily achieved at room temperature from samples pretreated at temperatures lower than 80 °C. This suggests that there is the point of no return for pretreatment temperature that enables effective solvent extraction of hydrocarbons at room temperature from wet algal samples of *B. braunii*. To elucidate the mechanism of hydrocarbon recovery from *B. braunii* following thermal pretreatments, we investigated the thermophysical properties of the water phase separated from heated algal slurry. Differential scanning calorimetry (DSC) measurements revealed sol–gel transitions in the water phase of algal slurry after protein denaturation at 64 °C in samples that was pretreated at 70 or 80 °C but not in those pretreated at 90 °C. Furthermore, the pretreated >70 °C water-soluble polymers revealed polysaccharides composed of galactose, arabinose, and uronic acid. These results suggest that the transition from sol state to gel state of water-soluble polysaccharides in algal slurry prevented hydrocarbon recovery with organic solvents since hydrocarbons were easily recovered from sol state samples pretreated at 70 or 80 °C when the extraction temperature was kept the same as the pretreatment temperature. These results reveal that the presence of water-soluble polymers with gelation ability in the water phase and removal of these polymers in sol state enable effective recovery of hydrocarbons at room temperature after thermal pretreatments.

Keywords *Botryococcus braunii* · Chlorophyta · Hydrocarbon · Water-soluble polymer · Sol–gel transition · Differential scanning calorimetry (DSC)

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Introduction

The microalga *Botryococcus braunii* accumulates a large amount of hydrocarbons and is therefore expected to be a suitable biofuel resource (Brown et al. 1969; Largeau et al. 1980; Yamaguchi et al. 1987). *B. braunii* is distributed worldwide and sometimes blooms so vigorously that it completely covers the surface of freshwater lakes or ponds with green, red, or yellow blooms (Swale et al. 1968; Wake et al. 1980; 1981). Many other lipid-producing microalgae produce fatty acids and triacylglycerol (TAG), whereas *B. braunii* produces hydrocarbons that contain no oxygen atoms in the molecular skeleton. Therefore, *B. braunii* is an extremely attractive option as a potential fuel source.

B. braunii is classified into three chemical races (A, B, and L) according to the structure of the hydrocarbons produced (Metzger et al. 1985). Among them, the B race seems to be the most promising fuel resource because it produces large amounts of branched unsaturated triterpenes with the formula C_nH_{2n-10} (n=30–37). The most striking feature of this microalga is that it forms colonies by connecting cells together with extracellular matrices composed of mucopolysaccharides and hydrocarbon-related compounds and it accumulates most of the hydrocarbons produced in the extracellular matrices instead of inside the cells (Chisti et al. 2007; Frenz et al. 1989).

In general, the hydrocarbons accumulated in *B. braunii* can be extracted by soaking dried alga in low-polar solvents such as *n*-hexane (Brown et al. 1969). However, the drying process requires an enormous amount of energy. Several studies have therefore been carried out to recover hydrocarbons from wet *B. braunii* without using drying processes. For example, Frenz et al. (1989) attempted to recover hydrocarbons by directly mixing the algal slurry with a low-polar organic solvent, but the recovery rate was relatively low. Dote et al. (1994) treated *B. braunii* suspended in water at 300 °C under a high pressure of 10 MPa with liquefaction and found that liquid fuel could be obtained with a high yield. However, the liquid fuel fraction obtained by the hydrothermal liquefaction contained contaminants other than the original hydrocarbons. By contrast, Kita et al. (2010) succeeded in recovering hydrocarbons with a high recovery rate of more than 97 % by thermally pretreating a low concentration of the microalgal slurry (corresponding to 1.5-g dry weight L^{-1}) at a relatively low temperature (90 °C) against above liquefaction before extraction with *n*-hexane. This thermal pretreatment method is advantageous in terms of energy costs compared with other methods involving drying processes because little energy is needed to heat wet algae to ~100 °C. Magota et al. (2012) also tried to recover hydrocarbons from three strains of *B. braunii* using a similar method and found that the lowest temperature necessary for efficient hydrocarbon recovery differed between the strains. We attempted the same method to recover hydrocarbons from slurry of the Showa strain with a much higher concentration than that used by Kita et al. (2010). The slurry at a concentration of 80 g L^{-1} was heated to 90 °C and then centrifuged after addition of *n*-hexane; however, this resulted in the formation of an emulsion that prevented hydrocarbon recovery. To avoid emulsion formation after the slurry was heated, the water phase had to be separated from the solid phase (composed of algae) by centrifugation. After that, hydrocarbons could be efficiently recovered from the solid phase using solvent extraction (Saga et al. 2013). The authors suggested that removing the water phase from the slurry enabled direct contact between the solid phase (containing hydrocarbons) and the extraction solvent and that the water phase of the thermally pretreated slurry contained some substance that inhibited hydrocarbon recovery.

In this study, we investigated the thermophysical properties of water-soluble polymers in the water phase from the *B. braunii* Showa strain. The samples were thermally pretreated using differential scanning calorimetry (DSC) measurements to elucidate the mechanism of hydrocarbon recovery from the alga following thermal pretreatments.

Materials and methods

The *Botryococcus braunii* Showa strain (Nonomura 1988) used in this study belongs to the B race. The microalga was grown in a modified Chu 13 medium at 25 °C at 100 μmol photons m^{-2} s^{-1} with a 12-h light/dark cycle in a 1,200-mL Roux culture bottle aerated with air containing 1.0 vol% of CO_2 (Okada et al. 1995). The algal culture was harvested 30 days after inoculation into the modified Chu 13 medium. The culture was suction-filtered through a nylon plankton net with a 20-μm mesh size, and a wet algal sample was obtained.

DSC measurement of the water phase of the microalgal slurry

DSC measurement was performed according to the following procedure. The 50 g L^{-1} of microalgal slurry (10 mL) with 95 % moisture content was placed into 50-mL plastic centrifuge tubes. The samples were heated at 60, 70, 80, or 90 °C for 10 min in a water bath. For DSC measurement, the thermally pretreated slurry was centrifuged (3,650×*g*, 10 min), and the separated water phase was transferred into closed batch cells made of Hastelloy. The closed batch cells were sealed tightly and placed into a DSC-7 calorimeter (Setarum), and the temperature was raised from 25 to 70 °C at a rate of 1 °C min^{-1}.

In addition, to investigate the thermophysical properties of the wet microalgal slurry without thermal pretreatment, DSC measurement was also performed for unheated microalgal slurry. Microalgal slurry without thermal pretreatment was prepared at the same concentration, and its DSC was measured by raising the temperature from 25 to 100 °C at a rate of 1 °C min^{-1}.

Hydrocarbon recoveries with *n*-decane at the same temperature as that used for thermal pretreatment

As we had recovered hydrocarbons from wet samples of *B. braunii* with *n*-hexane (C_6H_{14}, boiling point 69.0 °C) at room temperature after thermal pretreatment, it can be recovered almost 100 % from Showa strain (Magota et al. 2012). Then, we tried to extract the hydrocarbons using a solvent kept at the same temperature as that used for the thermal pretreatment (60, 70, 80, or 90 °C), so we selected *n*-decane ($C_{10}H_{22}$, boiling point 174.2 °C) as the extraction solvent because it is apolar (like *n*-hexane) but nonvolatile (unlike *n*-hexane). Figure 1 shows the GC chromatogram of hydrocarbons extracted from algal samples using both solvents. We confirmed that the hydrocarbon recovery rate using *n*-decane was equal to that obtained with *n*-hexane.

Details regarding hydrocarbon recovery with a solvent at the same temperature as that used for thermal preteatment are as follows. Microalgal slurry at a concentration corresponding to 5-g dry weight L^{-1} was placed into glass vials and pretreated for 10 min at 60, 70, 80, or 90 °C in a water bath.

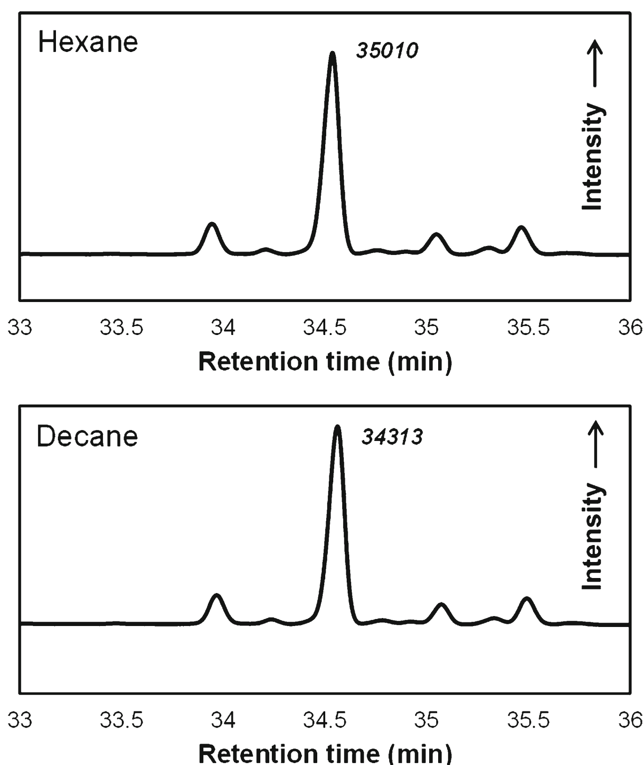

Fig. 1 GC chromatogram of hydrocarbons extracted from algal samples using both solvents

Sample temperature was measured directly using a thermometer inserted into the glass vials. After the pretreatment, 1 mL of the slurry was transferred into a glass test tube with a cap and combined with 5 mL of *n*-decane that had been prewarmed to the same temperature as that used for the thermal pretreatment. The test tube was shaken for 30 s and rewarmed to the pretreatment temperature in a water bath. This warming and shaking process was repeated three times. The mixture of slurry and *n*-decane was then centrifuged (3,650×*g*, 10 min) to separate the *n*-decane phase.

In order to determine the effect of extraction solvent temperature on hydrocarbon recovery, microalgal slurry was heated at 60, 70, 80, or 90 °C and extracted with *n*-decane at room temperature (25 °C). Hydrocarbon compositions in the *n*-decane phases were analyzed using gas chromatography (GC) (GC-2014; Shimadzu Corporation) with a capillary column (Rtx-1; 30 m). The column temperature was programmed as follows: 50 °C for 1 min, increase from 50 to 220 °C at 10 °C min^{-1}, 220 °C for 3 min, increase from 220 to 260 °C at 2 °C min^{-1}, and 260 °C for 3 min. The temperature of the flame ionization detector (FID) was set to 280 °C. Helium was used as the carrier gas.

In our study, the hydrocarbon recovery efficiency was calculated based on the peak area of the recovered hydrocarbon GC chromatograms. The first, the peak area of the most abundant on GC chromatograms that is considered the C34 botryococcene (Atobe et al. 2014) identified to the hydrocarbon recovery efficiency (Fig. 1). It can be recovered almost 100 % of the hydrocarbon recovery from Showa strain at room temperature extraction from the slurry thermally pretreated at 90 °C in our study (Magota et al. 2012). The peak area of that time was identified to 100 % of the hydrocarbon recovery efficiency. After that, the GC peak area of the samples was converted into the hydrocarbon recovery efficiencies respectively.

Composition analysis of water-soluble polymers in the water phase

The amount of water-soluble polymers was determined using the following method. Microalgal slurry (10 mL) with a moisture content of 95 % was placed in 50-mL plastic centrifuge tubes. The samples were heated at 90 °C for 10 min in an autoclave. For the composition analysis, thermally pretreated slurry samples were centrifuged (3,650×*g*, 10 min). The water phases were separated and filtered to purify foreign substances. The resultant solutions were considered to contain the water-soluble polymers of the water phase. They were freeze-dried, and the amounts of water-soluble polymers were determined on the basis of the weight of the dried samples.

Next, the composition of the water-soluble polymers was determined using the following method. Carbohydrates in the water-soluble polymer solutions were analyzed by the method recommended by National Renewable Energy Laboratory (NREL) (Sluiter et al. 2008). The yields of the natural sugars glucose, galactose, arabinose, and mannose were determined by high-performance liquid chromatography using a Shimadzu LC-20 system equipped with a SP0810 column (Showa Denko K. K.) and a refractive index detector (RID-10A; Shimadzu Co., Ltd.). The concentration of uronic acids, i.e., acidic sugars, was determined using the carbazole-sulfuric acid method. The total uronic acid content, calculated as glucuronic acid, was determined by measuring the absorbance at 530 nm using the U-2001 spectrophotometer (Hitachi Ltd.). The amount of nitrogen was determined by elemental analysis on an automatic nitrogen analyzer (Model 2400; Perkin Elmer Co., Ltd.). Furthermore, the total ash content was determined using the CHN corder (MT-6; Yanaco New Science Inc.).

Results and discussion

Thermophysical properties of the water phase of the microalgal slurry

First, we examined the thermophysical properties of the water phase of microalgae slurry without thermal pretreatment. Figure 2 shows an endothermic peak in the DSC curve for the microalgae slurry (95 % moisture content) before thermal

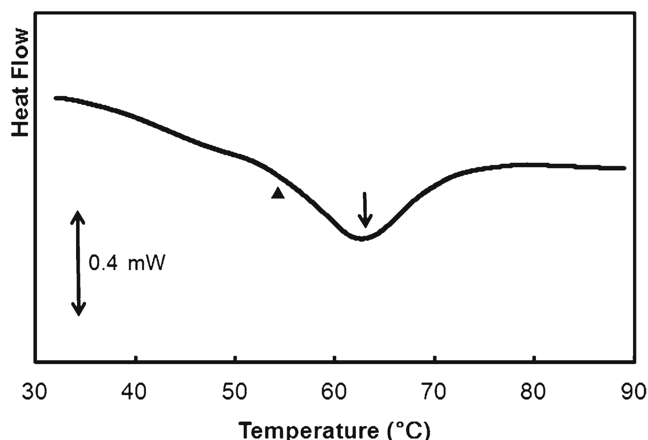

Fig. 2 Endothermic peak in the DSC curve for the microalgae slurry (95 % moisture content) before thermal pretreatment

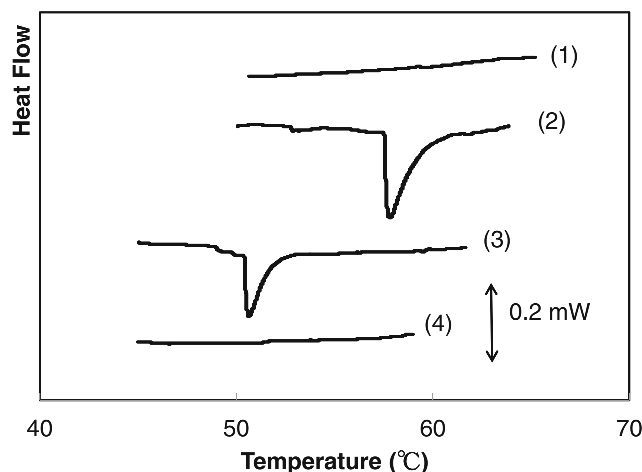

Fig. 3 DSC curves of water phases separated from the microalgal slurry that was thermally pretreated at different temperatures (1) 90 °C; (2) 80 °C; (3) 70 °C; and (4) 60 °C

pretreatment. In general, microalgae have been reported to contain 40–60 % protein in their dry biomass (Aaronson and Dubinsky 1982; Thomas et al. 1984); the protein content of *B. braunii* is lower at almost 15 % (Ben-Amotz and Tornabene 1985; Vaquez-Duhalt and Arredondo-Vega 1991).

In general, a protein has a three-dimensional structure involving helix and sheet structures. However, protein structures unfold upon heating, thereby increasing the random coil contents and ultimately denaturing the protein. When a protein denatures, the heat capacity to break the rigid helix and sheet structures increases and, consequently, an endothermic peak is observed in its DSC curve. Therefore, endothermic peaks can be readily used to determine whether proteins have been denatured. On this basis, we inferred that the endothermic peaks found in the microalga slurry DSC curves before thermal pretreatments indicate denaturation of the microalgal proteins. When the same slurry was reused to measure a DSC curve (second run) before thermal pretreatment, no endothermic peaks were detected. The endothermic peak observed in the first run could therefore be attributed to the denaturation of proteins. The denaturation temperature of proteins in the microalgae slurry was 63.5±0.6 °C (indicated by an arrow in Fig. 1), and the temperature at which the proteins started to denature was 56.3±4.9 °C (indicated by an arrow head in Fig. 1).

Next, we examined the thermophysical properties of the water phase of the microalgal slurry after thermal pretreatments. Figure 3 shows the DSC curves of water phases separated from the microalgal slurry that was thermally pretreated at different temperatures. The water phase from the 60 °C pretreatment showed no endothermic peak. Because the denaturation temperature of the *B. braunii* proteins was 64 °C, as mentioned above, the proteins in the microalgae slurry were not completely denatured by pretreatment at 60 °C. Consequently, the structural components of the water-soluble polymers in the microalga maintained the original conditions, were not released into the water phase, and did not show any endothermic peak. In contrast, clear endothermic peaks of released water-soluble polymers were observed in the DSC curves of the water phases of the slurry thermally pretreated at 70 and 80 °C. These endothermic peaks did not seem to originate from the denaturation of proteinaceous components released from the microalga because those would have been irreversibly denatured at 64 °C.

It has long been known that algal extracellular matrices are filled with mucopolysaccharides (Dodgson 1961; Hang et al. 1967). Polysaccharides extracted from seaweed are widely used in the food and pharmaceutical industries because of their gelling, thickening, stabilizing, and dispersing effects (Rinaudo 1994; Nishinari 2000). In particular, representative polysaccharides, such as agar, carrageenan,

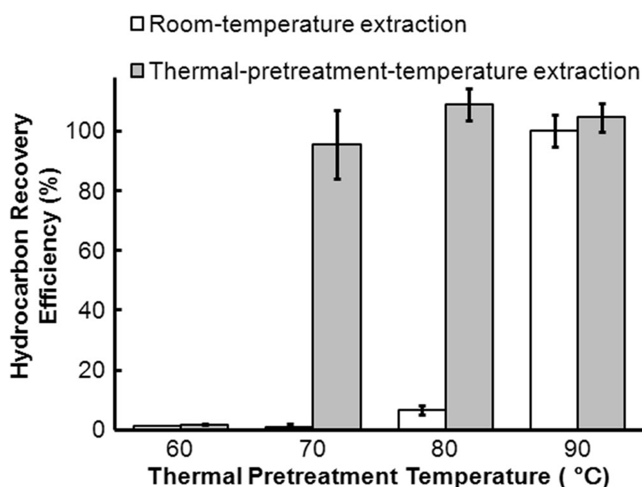

Fig. 4 Hydrocarbon recovery efficiencies from thermally preheated microalgae slurry using either *n*-decane at the thermal pretreatment temperature or an extraction solvent at room temperature. *White bars* indicate hydrocarbon recovery from the thermally pretreated slurry on which extractions were performed at room temperature. *Gray bars* indicate hydrocarbon recovery for extractions that were performed at the same temperature as that used for the thermal pretreatments

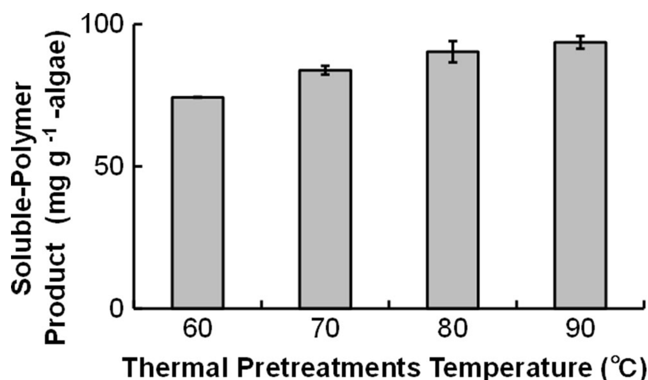

Fig. 5 Amount of water-soluble polymers extracted from the *B. braunii* Showa strain after thermal pretreatment at 60, 70, 80, or 90 °C

and alginic acid, are commonly extracted from seaweed with hot water. Because these polysaccharides show a reversible sol–gel transition, DSC was used for their identification (Miyoshi et al. 1995). Thus, the endothermic peaks found in the water phase of the *B. braunii* slurry thermally pretreated at 70 or 80 °C seemed to originate from the sol–gel transition of some water-soluble polymers (probably polysaccharides) that had been released rather than from the denaturation of proteins. The water phase of the slurry thermally pretreated at 80 °C was reanalyzed using DSC and produced a peak in the same temperature range. This result revealed the presence of some water-soluble polymers released into the water phase that showed a sol–gel transition during the heating process. As the sol–gel transition was reversible, there is a possibility that the water-soluble polymers form gels when thermally pretreated microalgae slurry is cooled to room temperature and that this affects the recovery of hydrocarbons by extraction with organic solvents. In contrast, the water phase of the slurry thermally pretreated at 90 °C no longer showed a sol–gel transition, possibly because the water-soluble polymers were completely denatured or degraded at 90 °C.

Effects of temperature of extraction on hydrocarbon recovery

Figure 4 shows the hydrocarbon recovery efficiencies from thermally preheated microalgae slurry using either *n*-decane at the thermal pretreatment temperature or an extraction solvent at room temperature. White bars indicate hydrocarbon recovery from the thermally pretreated slurry on which extractions were performed at room temperature. Gray bars indicate hydrocarbon recovery for extractions that were performed at the same temperature as that used for the thermal pretreatments. Hydrocarbons could not be extracted with either extraction process for the slurry thermally pretreated at 60 °C. For the microalgal slurry pretreated at 70 or 80 °C, however, hydrocarbons could be recovered with high efficiency (>80 %) by maintaining the extraction temperature similar to that of the thermal pretreatment but not by extraction at room temperature. The microalgae slurry pretreated at 90 °C showed high hydrocarbon recovery for extractions carried out both at 90 °C and at room temperature.

From these results, we suggest that the proteins in the microalgae slurry did not completely denature at 60 °C. Subsequently, water-soluble polymers were not removed from the microalgae, and consequently, hydrocarbons could not be extracted because the extraction solvent could not make contact with them. However, once the proteins were treated at 70 or 80 °C, which is higher than the protein denaturation temperature of 64 °C, water-soluble polymers in the slurry were released into the water phase. These were in a gel state during the room temperature extraction process, adhered to the surface of the alga colonies, and prevented contact between the accumulated hydrocarbons and a low-polar organic solvent, such as *n*-hexane.

In contrast, for extractions at the same temperature as that of thermal pretreatments at 70 or 80 °C, the water-soluble polymers remained in the sol state. We suggest that solation of the water-soluble polymers allowed the hydrocarbons in the solid phase to contact the organic solvent and consequently improved the hydrocarbon recovery efficiency. Furthermore, in the slurry thermally pretreated at 90 °C, the water-soluble polymers in the water phase were also in the sol state, but never returned to the gel state, even if cooled to room temperature. Thus, the extraction solvent could readily contact the hydrocarbons, and recovery improved. These results reveal that water-soluble polymers with gelation ability present in the water phase prevent hydrocarbon recovery by solvent extraction.

Table 1 Chemical composition of soluble-polymer by hydrothermal extraction from *Botryococcus braunii* Showa strain

Thermal treatment temperature (°C)	Carbohydrate		N (%)	S (%)	Ash (%)
	Neutral sugar (%)	Acidic sugar (%)			
60	51.1	10.2	2.3	0.26	17.5
70	53.0	12.8	1.8	0.22	16.4
80	62.2	13.5	1.8	0.23	15.2
90	60.9	13.4	1.8	0.2	15.4

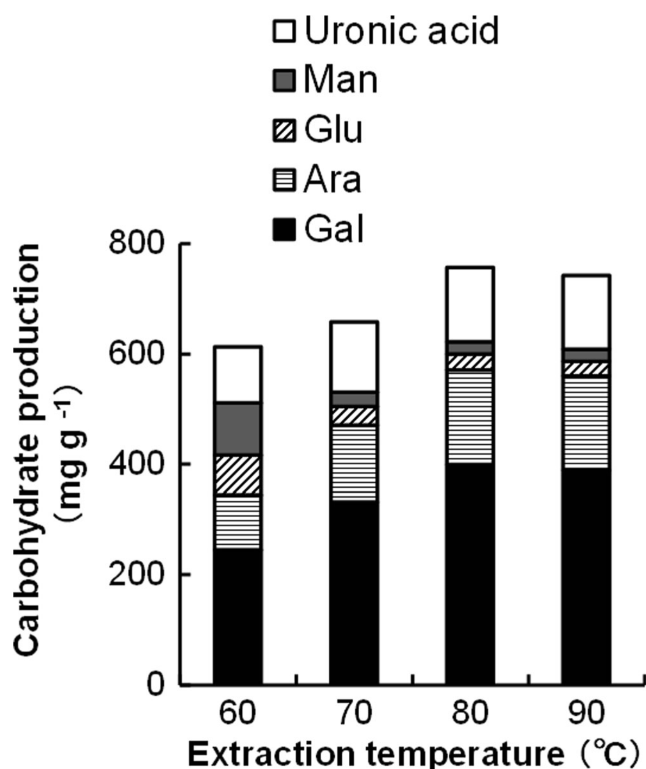

Fig. 6 Constituent sugars in water-soluble polymers from *Botryococcus braunii* Showa strain after thermal pretreatment. The values are means ± SD (n= 3). The amount of galactose and arabinose of water-soluble polymers were increased with more than 70 ºC pretreated. However, the amount of glucose and mannose were decreased with more than 70 ºC pretreated. Polysaccharides composition changed after protein denaturation of algal slurries

Production and composition of water-soluble polymers

Figure 5 shows the amount of water-soluble polymers extracted from the *B. braunii* Showa strain after thermal pretreatment at 60, 70, 80, or 90 °C. The amount of water-soluble polymers was 74.2±0.1 mg g^{-1} dry algae at 60 °C samples and 93.6± 2.2 mg g^{-1} dry algae at 90 °C samples. The amount of water-soluble polymers extracted from the 90 °C extraction samples was almost 25 % larger than that from the 60 °C extraction samples. Table 1 shows the compositions of water-soluble polymers extracted from the Showa strain by thermal pretreatments. These results revealed that the water-soluble polymers were polysaccharides that composed primarily of glucose, galactose, arabinose, mannose and uronic acid. It was suggesting that the polysaccharides contained almost no sulfuric acid. Furthermore, it was revealed that the amount of galactose and arabinose of water-soluble polymers was increased with more than 70 °C pretreated. However, the amount of glucose and mannose was decreased with more than 70 °C pretreated. These results suggested that polysaccharides composition changed after protein denaturation of algal slurry (Fig. 6).

The typical polysaccharides in seaweed are carrageenan, alginic acid, and fucoidan, which are mucilaginous polysaccharides that can be obtained through heated extraction from the ECM of any seaweed. Alginic acid has a copolymer polysaccharide consisting of the uronic acid of β-mannuronic acid and α-L-guluronic acid (Chanda et al. 1939; Nelson and Cretcher 1929, 1932). The mucilaginous polysaccharides of seaweed have the characteristics of gelling and thickening and are thus widely used as thickeners or gelators.

Based on this background, it is suggested that the water-soluble polymers, in particular galactose and arabinose and uronic acid which eluted at more than 70 °C after denaturation of the proteins of the algae slurry, are involved in the sol–gel transition of the liquid phase and as a result inhibit the hydrocarbon extraction at room temperature. It is therefore necessary to remove the water-soluble polymers to improve hydrocarbon recovery from slurry of *B. braunii*. Furthermore, it may be utilized as an alternative for the polysaccharides from seaweeds that are commercially used as thickeners or gelators. On the other hand, it may also be possible to improve hydrocarbon recovery from algal cells by preventing the production of the water-soluble polymer itself. In recent studies, hydrocarbon recovery was improved by changing the composition of the medium (Furuhashi et al. 2013). It is interesting that carbohydrate metabolism seems to contribute to the recovery of hydrocarbons.

In the future, it will be necessary to identify the water-soluble polymer. In addition, it is necessary to consider breeding, selection, and the construction of the culture method. It is believed that the water-soluble polymers extracted from *B. braunii* are particularly useful in the hydrocarbon recovery process.

Acknowledgments This study was performed as part of a project of the Strategic Development of Next-generation Bioenergy Utilization Technology, "Efficient Production of Hydrocarbon from Microalgae," supported by the New Energy and Industrial Technology Development Organization (NEDO). We would like to thank all the people involved.

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