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Thermoplasticization of euglenoid β-1,3-glucans by mixed esterification

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ABSTRACT

We experimentally demonstrated that paramylon, a storage polysaccharide of *Euglena gracilis*, is efficiently thermoplasticized by adding acyl groups that differ in alkyl chain length. Glass transition temperature of mixed paramylon esters was higher than those of plant-based polylactic acid (PLA), poly 11-aminoundecanoic acid (PA11), and petroleum-based acrylonitrile-butadiene-styrene (ABS) resin and was comparable to that of cellulose acetate stearate (CAS). Their thermoplasticity was equivalent to or higher than those of these reference plastics. The bending strength and bending elastic modulus of injection molded test specimens made from mixed paramylon esters were comparable to those of the reference plastics. While their impact strength was lower than that of specimens made from ABS resin and CAS, it was comparable to those of PLA and PA11. Euglenoid β-1,3-glucans are thus a potential component of thermoplastic materials.

1. Introduction

The use of microalgae to produce useful chemicals has recently attracted attention because they can transform carbon dioxide and water into carbohydrates by photosynthesis (Chisti, 2007). Attention has particularly focused on production of biofuels as part of the effort to reduce dependence on carbon-based fuels (Banerjee, Sharma, Chisti, & Banerjee, 2002; Kaya et al., 2011; Mata, Martins, & Caetano, 2010; Tucci, Vacula, Krajcovic, Proksch, & Martin, 2010; Yamane et al., 2013). By contrast, less attention has been directed at the use of microalgae to create other useful difficult-to-synthesize carbohydrates. Our efforts in this area have focused on *Euglena*-derived storage polysaccharide (β-1,3-glucan), which is generally referred to as paramylon (Shibakami, Sohma, & Hayashi, 2012; Shibakami, Tsubouchi, Nakamura, & Hayashi, 2013).

Since paramylon has unique helical structures due to its β-1,3-bonds, it is likely that materials made from this polysaccharide will exhibit intriguing thermal and mechanical properties that differ from those of materials made from other polysaccharides. One physical property that is useful for preparing materials of various shapes is thermoplasticity. Paramylon, however, does not inherently have this property. It has been reported that mixed cellulose esters having at least two types of substituents exhibit thermoplasticity (Peydecastaing, Vaca-Garcia, & Borredon, 2011; Teramoto, Yoshioka, Shiraishi, & Nishio, 2002). Wax esters, i.e., esters of long-chain fatty acid and higher alcohol, are another major euglenoid product in addition to paramylon. Of particular interest is that hydrolysis of wax esters releases the fatty acid and higher alcohol. We hypothesized that if wax ester-derived fatty acids are introduced into paramylon, we will likely obtain thermoplastic mixed paramylon esters with a *high euglenoid constituent ratio*. Toward this goal, we have started a program aimed at synthesizing mixed paramylon esters containing long-chain fatty acids.

Here we report the creation of thermoplastic paramylon derivatives. To the best of our knowledge, this is the first creation of *Euglena*-based thermoplastics despite the long study of *Euglena* since the establishment of genus *Euglena* by Ehrenberg in 1830 (Gojdics, 1953). The primary objective of the work described here was twofold: (i) to establish a mix-esterification method that provides thermoplastic paramylon derivatives and (ii) to investigate the relationship between the chemical structures of the derivatives and their thermal and mechanical properties.

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2. Experimental

2.1. General methods

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE 500 spectrometer. Quantitative ^{13}C NMR spectra were obtained by means of the inverse gated decoupling method. Fourier transform infrared (FT-IR) spectra were recorded using a JASCO FT/IR-480ST spectrophotometer equipped with an attenuated total reflectance accessory (ATR Pro 400-S, ZnSe prism, JASCO) with a resolution of 4 cm^{-1}. Melting behavior was observed using a Yanako MP-500D melting-point apparatus. Elemental analyses were performed using a CE Instruments EA1110 elemental analyzer.

All chemicals and reagents except for the paramylon used for synthesizing the paramylon derivatives are commercially available and were used without further purification. N,N-dimethylacetamide (DMAc), lithium chloride (LiCl), and triethylamine (NEt$_3$) were purchased from Sigma–Aldrich, Kishida Chemical, and Junsei Chemical, respectively. Myristoyl, palmitoyl, and stearoyl chloride were purchased from Wako Pure Chemical Industries, Tokyo Chemical Industry, and Kanto Chemical, respectively. Methanol and chloroform were obtained from Kishida Chemical and Junsei Chemical, respectively. Acetic anhydride and pyridine were obtained from Tokyo Chemical Industry and Sigma–Aldrich, respectively. Polylactic acid (PLA, TE-4000), poly-11-aminoundecanoic acid (PA11, Rilsan BMFO), and acrylonitrile-butadiene-styrene (ABS) resin (GA-701) were purchased from Unitika, Arkema Japan, and Nippon A&L Co, respectively. Paramylon particles were obtained from *Euglena gracilis* in accordance with a previously reported method (Shibakami et al., 2012). The degree of substitution (DS) values, which are the average number of functional groups attached to a glucose unit, were determined by comparing the integral values of the methyl protons of the long alkyl chains and acetyl groups with those of the glucosidic protons in the ^{1}H NMR spectrum; DS$_{lac}$ and DS$_{ace}$ in the text represent the DS of the long alkyl chains (myristoyl, palmitoyl, and stearoyl) and acetyl groups, respectively.

2.2. Synthesis of mixed paramylon esters

2.2.1. Paramylon acetate myristate (DS$_{lac}$ 0.28/DS$_{ace}$ 2.49) (***1a***)

To a homogeneous solution of paramylon (10.0 g, 61.7 mmol) in DMAc (0.5 L)/LiCl (8.0 g, 188.7 mmol) prepared by heating at ~110 °C for 0.5 h were added dropwise 7.8 mL (56.0 mmol) of NEt$_3$ and 0.5 L of a DMAc solution containing myristoyl chloride (8.4 mL, 30.9 mmol) at room temperature. This mixture was heated at 110 °C under a nitrogen atmosphere for 3 h. Methanol (2.0 L) was then added to precipitate a solid. This solid was washed with a mixture of methanol and chloroform (0.9 L, 1/2 (v/v)). Air drying overnight and subsequent vacuum drying at 70 °C for 2 h and 105 °C for 4 h produced paramylon myristate as a solid (13.0 g, 58.8 mmol).

To a homogeneous solution containing DMAc (1.5 L), LiCl (6.6 g, 155.7 mmol), and paramylon myristate (12.5 g, 56.5 mmol), which was prepared by heating at ~110 °C for 0.5 h, were added pyridine (168 mL, 2.09 mol) and acetic anhydride (240 mL, 4.89 mol) at 70 °C. The mixture was heated at 70 °C for 6 h and then left at ambient temperature for 17 h under a nitrogen atmosphere. Addition of water (3.0 L) to the mixture produced a solid. This solid was washed with water (1.6 L) and methanol (0.3 L). Air drying overnight and subsequent vacuum drying at 80 °C for 1 h and 105 °C for 4 h produced paramylon acetate myristate (**1a**) as a solid (15.1 g, 46.4 mmol, yield 75.2%). Successful preparation was confirmed by FT-IR and ^{1}H and ^{13}C NMR measurements. ^{1}H NMR (CDCl$_3$) δ 4.99–4.75 (m), 4.47–4.20 (m), 4.08–3.96 (m), 3.79–3.57 (m), 2.37–2.21 (m), 2.12 (s), 2.06 (s), 2.00 (s), 1.57 (m), 1.26 (s), 0.88 (t, J = 6.9). ^{13}C NMR (CDCl$_3$) δ 173.4, 170.5, 169.0, 168.8, 100.7, 78.4, 72.7, 71.8, 68.1, 62.0, 33.9, 31.9, 29.6, 29.5, 29.3, 29.2, 29.1, 24.6, 22.6, 20.8, 20.7, 20.4, 14.1. FT-IR (cm^{-1}) 2875, 2807, 1740, 1365, 1212, 1033, 886.

2.2.2. Paramylon acetate myristate (DS$_{lac}$ 0.61/DS$_{ace}$ 2.23) (***1b***)

By using procedures similar to those described for the preparation of **1a**, a 52.7% yield of **1b** (12.5 g, 32.5 mmol) was obtained; ^{1}H NMR (CDCl$_3$) δ 4.99–4.75 (m), 4.47–4.20 (m), 4.08–3.96 (m), 3.79–3.57 (m), 2.37–2.21 (m), 2.11 (s), 2.07 (s), 1.99 (s), 1.58 (m), 1.25 (s), 0.87 (t, J = 6.9). ^{13}C NMR (CDCl$_3$) δ 173.4, 170.5, 169.0, 168.8, 100.7, 78.4, 72.7, 71.8, 68.1, 62.0, 33.9, 31.9, 29.6, 29.5, 29.3, 29.2, 29.1, 24.6, 22.6, 20.9, 20.7, 20.5, 14.1. FT-IR (cm^{-1}) 2918, 2849, 1740, 1364, 1210, 1029, 888.

2.2.3. Paramylon acetate palmitate (DS$_{lac}$ 0.29/DS$_{ace}$ 2.41) (***1c***)

By using procedures similar to those described for the preparation of **1a**, a 71.6% yield of **1c** (14.7 g, 44.2 mmol) was obtained; ^{1}H NMR (CDCl$_3$) δ 4.99–4.75 (m), 4.47–4.20 (m), 4.08–3.96 (m), 3.79–3.57 (m), 2.37–2.21 (m), 2.11 (s), 2.06 (s), 2.00 (s), 1.56 (m), 1.26 (s), 0.88 (t, J = 6.9). ^{13}C NMR (CDCl$_3$) δ 173.4, 170.5, 169.0, 100.7, 78.4, 72.7, 71.8, 68.1, 62.0, 33.9, 31.9, 29.7, 29.5, 29.3, 29.1, 24.6, 22.6, 20.9, 20.7, 20.4, 14.1. FT-IR (cm^{-1}) 2911, 2846, 1741, 1365, 1213, 1032, 887.

2.2.4. Paramylon acetate palmitate (DS$_{lac}$ 0.50/DS$_{ace}$ 2.02) (***1d***)

By using procedures similar to those described for the preparation of **1a**, a 59.8% yield of **1d** (13.5 g, 36.9 mmol) was obtained; ^{1}H NMR (CDCl$_3$) δ 4.99–4.75(m), 4.47–4.20(m), 4.08–3.96(m), 3.79–3.57 (m), 2.37–2.21 (m), 2.11 (s), 2.05 (s), 1.98 (s), 1.58 (m), 1.25 (s), 0.87 (t, J = 6.9). ^{13}C NMR (CDCl$_3$) δ 173.4, 170.5, 169.0, 168.8, 100.7, 78.4, 72.7, 71.8, 68.2, 62.0, 33.9, 31.9, 29.7, 29.5, 29.3, 29.2, 29.1, 24.7, 22.7, 20.8, 20.7, 20.5, 20.4, 14.1. FT-IR (cm^{-1}) 2914, 2849, 1747, 1363, 1213, 1032, 890.

2.2.5. Paramylon acetate stearate (DS$_{lac}$ 0.31/DS$_{ace}$ 2.33) (***1e***)

By using procedures similar to those described for the preparation of **1a**, a 82.8% yield of **1e** (17.5 g, 51.1 mmol) was obtained; ^{1}H NMR (CDCl$_3$) δ 4.99–4.75 (m), 4.47–4.20 (m), 4.08–3.96 (m), 3.79–3.57 (m), 2.37–2.21 (m), 2.12 (s), 2.06 (s), 2.00 (s), 1.55 (m), 1.26 (s), 0.88 (t, J = 6.9). ^{13}C NMR (CDCl$_3$) δ 173.4, 170.5, 169.0, 100.7, 78.4, 72.6, 71.8, 68.1, 62.1, 33.9, 31.9, 29.7, 29.5, 29.3, 29.1, 24.6, 22.6, 20.8, 20.7, 20.4, 14.1. FT-IR (cm^{-1}) 2920, 2847, 1740, 1365, 1212, 1031, 889.

2.2.6. Paramylon acetate stearate (DS$_{lac}$ 0.46/DS$_{ace}$ 2.07) (***1f***)

By using procedures similar to those described for the preparation of **1a**, a 48.8% yield of **1f** (11.2 g, 30.1 mmol) was obtained; ^{1}H NMR (CDCl$_3$) δ 4.99–4.75 (m), 4.47–4.20 (m), 4.08–3.96 (m), 3.79–3.57 (m), 2.37–2.21 (m), 2.12 (s), 2.08 (s), 2.00 (s), 1.58 (m), 1.25 (s), 0.87 (t, J = 6.9). ^{13}C NMR (CDCl$_3$) δ 173.4, 170.6, 169.1, 100.7, 78.5, 72.7, 71.8, 68.2, 62.0, 33.9, 31.9, 29.7, 29.5, 29.4, 29.2, 24.7, 22.7, 20.9, 20.7, 20.5, 14.1. FT-IR (cm^{-1}) 2920, 2849, 1740, 1366, 1209, 1030, 890.

2.2.7. Paramylon dimyristate (DS$_{lac}$ 2.08) (***2***)

To a homogeneous solution of paramylon (100 mg, 0.62 mmol) in DMAc (5.0 mL)/LiCl (79 mg, 1.86 mmol) prepared by heating at ~110 °C for 0.5 h were added dropwise 1.3 mL (9.34 mmol) of NEt$_3$ and 15 mL of a DMAc solution containing myristoyl chloride (1.3 mL, 4.79 mmol) at room temperature. This mixture was heated at 120 °C under a nitrogen atmosphere for 3 h. Methanol (40 mL) was then added to precipitate a solid. This solid was washed with a mixture of methanol and chloroform (30 mL, 2/1 (v/v)). Air drying overnight and subsequent vacuum drying at 90 °C for 2 h produced paramylon dimyristate (**2**) as a solid (390 mg, 0.65 mmol, yield nearly 100% (estimated from DS_{lac} of 2.09 tentatively calculated based on elemental analysis instead of ^{1}H NMR)). Since the product was not dissolved in NMR solvents, successful preparation was confirmed by FT-IR and elemental analysis. FT-IR (cm^{-1}) 3457, 2918, 2850, 1743, 1454, 1363, 1147, 1077, 718, 688. Anal. Found: C, 70.404; H, 10.746.

2.2.8. *Paramylon acetate (DS_{ace} 2.68) (**3**)*

To a homogeneous solution containing DMAc (250 mL), LiCl (4.0 g, 94.4 mmol), and paramylon (5.2 g, 32.1 mmol), which was prepared by heating at ~110 °C for 0.5 h, were added pyridine (84 mL, 1.04 mmol), acetic anhydride (120 mL, 1.27 mol), and DMAc (500 mL) at 70 °C. The mixture was heated at 70 °C for 5 h and then left at ambient temperature for 17 h under a nitrogen atmosphere. Addition of water (1.5 L) to the mixture produced a solid. This solid was washed with methanol (300 mL). Air drying overnight and subsequent vacuum drying at 90 °C for 4 h produced paramylon acetate (**3**) as a gumlike solid (8.73 g, 31.8 mmol, yield 99.1%). Successful preparation was confirmed by FT-IR and ^{1}H and ^{13}C NMR measurements. ^{1}H NMR ($CDCl_3$) δ 5.01–4.74 (m), 4.48–4.20 (m), 4.10–3.96 (m), 3.80–3.51 (m), 2.11 (s), 2.05 (s), 2.00 (s). ^{13}C NMR ($CDCl_3$) δ 170.5, 169.1, 168.9, 100.6, 78.4, 72.7, 71.8, 68.1, 62.0, 20.9, 20.7, 20.4. FT-IR (cm^{-1}) 3445, 1732, 1623, 1366, 1212, 1024, 885.

2.2.9. *Paramylon acetate myristate (DS_{lac} 0.07/DS_{ace} 2.58) (**4**)*

To a homogeneous solution of **3** (1.02 g, 3.71 mmol) in DMAc (50 mL)/LiCl (587 mg, 13.85 mmol) prepared by heating at ~110 °C for 0.5 h were added dropwise 0.85 mL (6.08 mmol) of NEt_3 and 50 mL of a DMAc solution containing myristoyl chloride (1.1 mL, 4.06 mmol) at room temperature. This mixture was heated at 110 °C under a nitrogen atmosphere for 3 h. Methanol (900 mL) was then added to precipitate a solid. This solid was washed with methanol (300 mL). Air drying overnight and subsequent vacuum drying at 90 °C for 4 h produced paramylon acetate myristate (**4**) as a solid (963 mg, 3.37 mmol, yield 90.8%). Successful preparation was confirmed by FT-IR and ^{1}H and ^{13}C NMR measurements. ^{1}H NMR ($CDCl_3$) δ 5.04–4.74 (m), 4.52–4.18 (m), 4.10–3.96 (m), 3.81–3.53 (m), 2.37–2.21 (m), 2.12 (s), 2.05 (s), 2.00 (s), 1.57 (m), 1.25 (s), 0.88 (t, J = 6.6). ^{13}C NMR ($CDCl_3$) δ 170.5, 169.1, 168.8, 100.6, 78.4, 72.7, 71.8, 68.1, 62.0, 31.9, 29.6, 29.5, 29.3, 22.7, 20.8, 20.7, 20.4, 14.1. FT-IR (cm^{-1}) 3426, 2919, 2823, 1735, 1626, 1361, 1214, 1018, 889.

2.3. *Size exclusion chromatography*

The weight-average molecular weights (M_w) of the mixed paramylon esters were determined by using size exclusion chromatography with multiangle laser light scattering (SEC-MALLS). The SEC-MALLS measurements were carried out on a DAWN HELEOS II multiangle laser photometer (Wyatt Technology) and an Optilab T-rEX refractive index detector (Wyatt Technology) equipped with two gel permeation chromatography columns (KD-805 plus K-802, Shodex) (eluent chloroform, 1.0 mL/min, 40 °C). All of the polysaccharide solutions were purified using a 1.0-μm filter. The injection volume was 200 μL with a concentration of approximately 1.0 mg/mL. The dn/dc values were 0.0405, 0.0367, 0.0472, 0.0384, 0.0408, and 0.0303 for **1a**–**1f**, respectively.

2.4. *Melt flow rate measurements*

The melt flow rates (MFR) of the mixed paramylon esters were determined using a CFT-500D capillary rheometer (Shimadzu) by weighing a melted sample heated at 200 °C that was extruded with a constant force (500 kgf/cm^2) using a piston through a die orifice for 10 min. The samples were heated at 105 °C for 5 h prior to the MFR measurements.

2.5. *Differential scanning calorimeter measurements*

Differential scanning calorimeter (DSC) measurements were carried out with a DSC6200/Exstar6000 differential scanning calorimeter (Seiko Instruments) on heating scans from −100 to 230 °C at a scan rate of 10.0 °C/min. Two DSC runs were performed for each sample, and grass transition temperature (T_g) was determined on the second run.

2.6. *Mechanical strength measurements*

Injection molded specimens (80 (length) × 12.4 (width) × 2.4 (depth) mm) were prepared from the mixed paramylon esters using a HAAKE MiniJet II injection molder (Thermo Fisher Scientific Co.) at 210 °C. Izod impact tests were carried out on these specimens using a Universal Impact Tester C1 (Toyo Seiki). The bending strength and bending elastic modulus were measured for specimens with a size of 40 (length) × 12.4 (width) × 2.4 (depth) mm using an Instron 5567 universal testing machine. The PLA test pieces were annealed at 100 °C for 4 h.

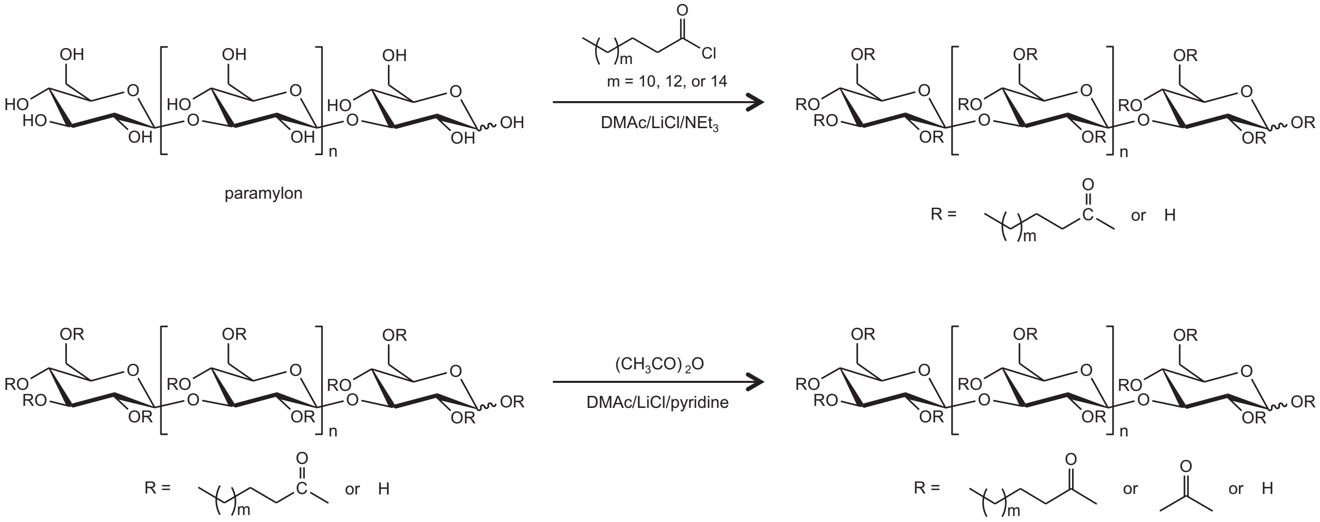
3. Results and discussion

3.1. *Synthesis of mixed paramylon esters*

There have been three main strategies for preparing thermoplastic polysaccharides: addition of external plasticizers, formation of polymer blends, and chemical modification or grafting of saccharide backbones (Warth, Mulhaupt, & Schatzle, 1997). In the work reported here, we examined the feasibility of a modification method that utilizes long-chain alkyl groups as an “internal plasticizer” for paramylon. This modification is advantageous in terms of obtaining a high euglenoid constituent ratio (theoretically as high as ~70% if euglenoid wax ester-derived fatty acids are used). For quantitative evaluation of the effect of alkyl chain length on the properties of the mixed paramylon esters, we used commercially available, structurally defined fatty acid chlorides.

The scheme for synthesizing the mixed paramylon esters (**1a**–**1f**) is depicted in Scheme 1. A primary benefit of using paramylon as a raw material is its ease of handling in chemical reactions. In brief, mechanical stirring of a heterogeneous mixture composed of paramylon, DMAc, and LiCl at around ~110 °C yields a homogeneous solution within 30 min while strict temperature and time control are required for preparing homogeneous solutions of cellulose (Edgar, Arnold, Blount, Lawniczak, & Lowman, 1995).

Long alkyl chains were introduced into glucose in the first step. Since the final goal of this study was to prepare thermoplastic paramylon derivatives with a higher euglenoid constituent ratio, our first choice for the long alkyl chains was myristoyl because the main component fatty acid of the *Euglena*’s wax esters is myristic acid (Inui, Miyatake, Nakano, & Kitaoka, 1983). Palmitoyl and stearoyl groups were chosen as references for examining how alkyl chain length affects the product properties. We prepared two types of long alkyl chain-introduced paramylons with different DS_{lac} values by adding different amounts of acid chlorides (i.e., 0.5 and 1.0 equiv. per glucose unit). Next, acetyl groups were introduced to cap the remaining hydroxyl groups of glucose and thereby weaken the interfibrillar hydrogen bonds. As shown in Table 1, the lower and higher DS_{lac} values ranged from 0.28 to 0.31 and from 0.46 to 0.61, respectively. The M_w values determined by SEC-MALLS ranged from 3.86 to 29.39 × 10^4. The degree of polymerization (DP) values ranged from 88 to 633. While the reason for the difference in M_w and DP values among the products remains to be unknown, we suppose that both values depend on the amount of fatty acid that can hydrolyze the polymer chain. An alternative synthetic route is



1a, m = 10, lower DSlac
1b, m = 10, higher DSlac
1c, m = 12, lower DSlac
1d, m = 12, higher DSlac
1e, m = 14, lower DSlac
1f, m = 14, higher DSlac

Scheme 1. Scheme for synthesizing mixed paramylon esters. Molar ratios of paramylon/acid chlorides are 0.5 and 1.0 for lower and higher DS_{lac}.

Table 1
Degree of substitution, weight-average molecular weight, and degree of polymerization of mixed paramylon esters.

Mixed paramylon ester	DS_{lac}/DS_{ace}	M_w ($\times 10^4$)	DP
1a	0.28/2.49	15.31	352
1b	0.61/2.23	3.86	88
1c	0.29/2.41	29.39	633
1d	0.50/2.02	5.02	116
1e	0.31/2.33	26.31	568
1f	0.46/2.07	7.53	176

one-step reaction in which two acid chlorides differing in alkyl chain length simultaneously react to paramylon. We did not take this route in spite of the procedure's simpleness because regioselective control of the acyl addition reaction is difficult.

3.2. Thermoplasticity

3.2.1. Melting behavior

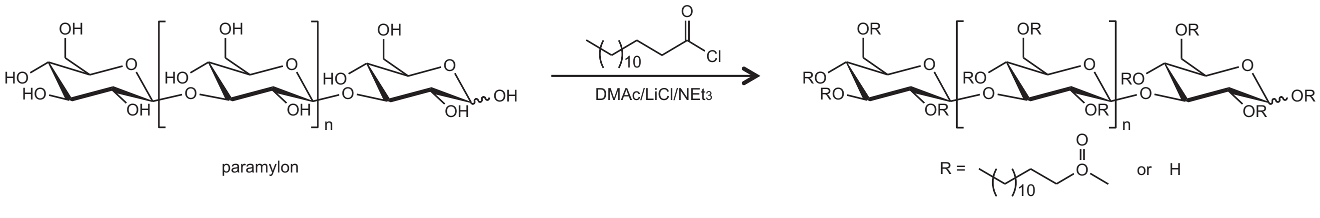
To simply determine whether the mixed paramylon esters (**1a**–**1f**) had thermoplasticity, we observed their melting behavior on the heating block of the melting-point apparatus. We found that the state of all the esters changed from powder to fluidic without charring upon heating up to 250 °C, indicating that these derivatives had a thermoplastic characteristic. The melting point was not evident in the DSC thermograms since the mixed paramylon esters were amorphous (see supplementary data).

3.2.2. Role of mix-esterification

To determine whether mix-esterification is necessary for obtaining thermoplasticity, we explored the melting behavior of granular paramylon dimyristate (**2**) prepared in accordance with the synthesis scheme shown in Scheme 2 and granular paramylon acetate (DS_{ace} 2.62) prepared according to a method previously reported (M. Shibakami et al., 2012). We found that these derivatives did not melt but charred upon heating to ~300 °C on the heating block. Although the detailed mechanism for the inability of dimyristoylation to induce thermoplasticity remains to be elucidated, close packing of the long-chain alkyl groups plausibly hinders thermoplasticization (Sealey, Samaranayake, Todd, & Glasser, 1996; Takada, Fujii, Watanabe, Fukuda, & Miyamoto, 1994; Teramoto, Miyata, & Nishio, 2006). It is likely that the acetyl group is too short to weaken the polymer chain interaction. Taken together, these results indicate that both long-chain alkyl and acetyl groups must be introduced to achieve thermoplasticity.

3.2.3. Synthesis procedure to induce thermoplasticity

The synthesis scheme used for preparing **1a**–**1f** is composed of successive introductions of long-chain alkyl and acetyl groups. A simple question occurred to us: does changing the order of introduction change the results? To answer this question, we



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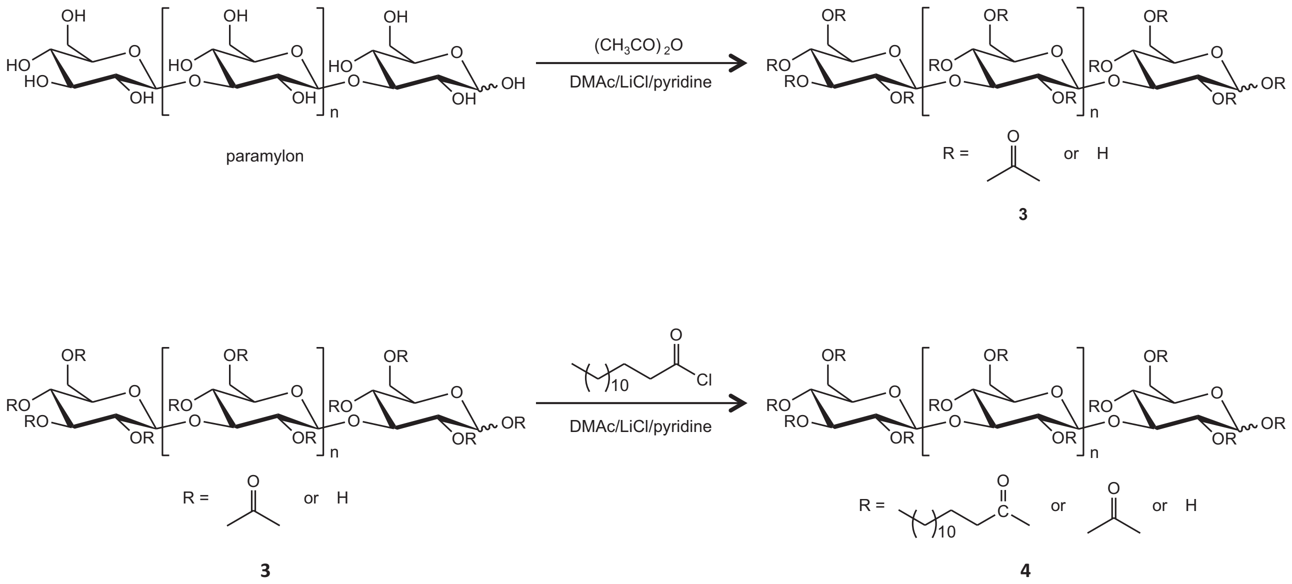
Scheme 2. Scheme for synthesizing paramylon dimyristate. Molar ratio of paramylon/myristoyl chloride is 7.7.



Scheme 3. Scheme for synthesizing paramylon acetate myristate in which acyl groups are introduced in reverse order.

examined the feasibility of a synthesis scheme in which myristoyl introduction is followed by acetyl introduction (Scheme 3). In brief, paramylon acetate (**3**) that had been prepared by partial acetylation with acetic anhydride was myristoylated with myristoyl chloride to obtain paramylon acetate myristate (**4**). ^{1}H NMR confirmed that the DS_{lac} and DS_{ace} of **4** were 0.07 and 2.58, respectively. Comparison of these values with those of **1b** indicates that introduction of myristoyl groups was substantially hindered. It is likely that preoccupation of the reactive site of glucose by acetyl groups resulted in the insufficient introduction of myristoyl groups. To determine whether **4** had a thermoplastic characteristic, we observed the morphological change in **4** on heating. As is apparent in Fig. 1, a rectangular transparent cast film made from **4** shrank and charred with retaining the original rectangular form upon heating to 300 °C, indicating that **4** did not have thermoplasticity. The insufficient thermoplasticity of **4** is plausibly ascribed to a lower number of substituted myristoyl groups. These results indicate that preparation of thermoplastic paramylon derivatives requires that the long-chain alkyl groups be introduced first.

3.2.4. Regioselectivity of acyl addition reaction

We supposed that the preoccupation of the reactive site of glucose by acetyl groups may result in insufficient thermoplasticity of **4**. This is accounted for by that acetylation and myristoylation have the same regioselectivity. To confirm this, we explored the regioselectivity of acetylation and myristoylation by measuring the quantitative ^{13}C NMR of paramylon acetate (**3**) and paramylon acetate myristate (DS_{lac} 0.48/DS_{ace} 2.24) prepared according to a method described in Section 2.2.2. As shown in Fig. 2(a), **3** exhibited two large signals and one small one at 170.6, 169.1, and 168.9 ppm, respectively. If we consider the ^{13}C NMR spectrum of cellulose triacetate previously reported (Tezuka & Tsuchiya, 1995) and the higher steric hindrance at C4 than at C6 and C2 as suggested

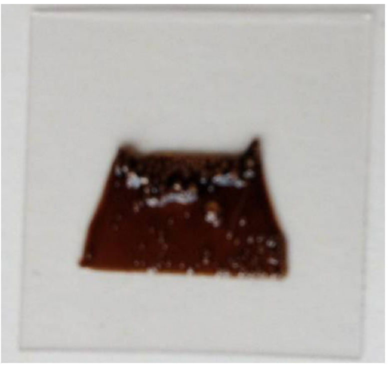


Fig. 1. Photograph of cast film made from paramylon acetate myristate (DS_{lac} 0.07/DS_{ace} 2.24) placed on cover glass (18 mm × 18 mm) upon heating to 300 °C.

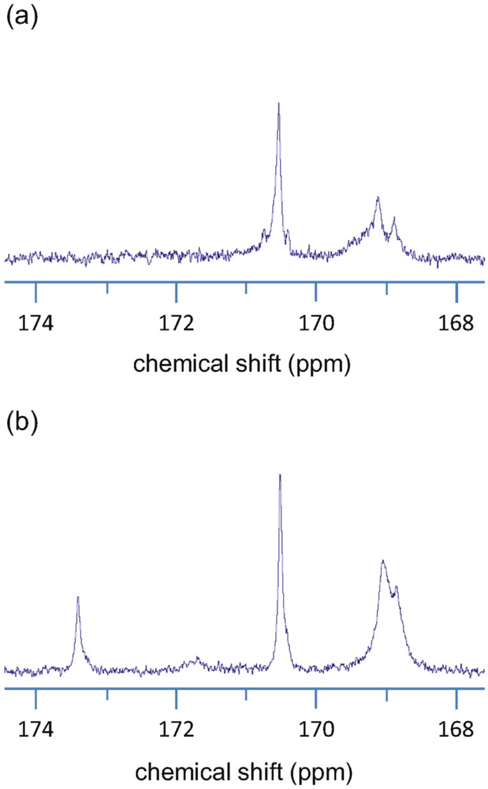


Fig. 2. Quantitative ^{13}C NMR spectra of (a) paramylon acetate (**3**) and (b) paramylon acetate myristate (DS_{lac} 0.48/DS_{ace} 2.24).

Table 2
Thermal properties of mixed paramylon esters and reference polymers.

Polymer	MFR (g/10 min)	T_g (°C)
Mixed paramylon ester		
1a	1080	133
1b	1354	94
1c	734	131
1d	1721	110
1e	948	127
1f	1289	113
Reference polymer		
PLA	1320	60
PA11	1340	45
ABS	1040	102
CAS ($\mathrm{DS}_{\mathrm{lac}}/\mathrm{DS}_{\mathrm{ace}}$)		
0.31/2.1[a]	160[a]	155[a]
0.51/2.1[a]	990[a]	145[a]

[a] Iji, Toyama, and Tanaka (2013).

by molecular modeling, it is reasonable that the signals at 170.6, 169.1, and 168.9 ppm can be assigned to C6, C2, and C4, respectively. The integral values of these signals are 1.00, 1.02, and 0.09, respectively. The overlapping peaks were deconvolved using the Bruker Topspin software in this study. These values quantitatively show the accessibility of an acetyl group to the hydroxyl groups of paramylon. Further, if we consider that C6 is less hindered than C2, it is likely that acetylation starts at C6.

Next, we examined the spectrum of paramylon acetate myristate ($\mathrm{DS}_{\mathrm{lac}}$ 0.48/$\mathrm{DS}_{\mathrm{ace}}$ 2.24). We measured this compound instead of paramylon myristate because the latter is insoluble in NMR solvents. As is apparent in Fig. 2(b), a sharp signal and a broad one appear at 173.4 and 171.8, respectively. Since three acetyl carbonyl-derived signals are observed in the higher magnetic field region, it is apparent that these two signals are due to the carbonyl carbon of myristoyl. The integral values of the myristoyl carbonyl carbon signals at 173.4 and 171.8 ppm were calculated to be 0.34 and 0.14, respectively. Since C6 is sterically less hindered than C2, the signal at 173.4 ppm is reasonably ascribed to myristoyl carbonyl carbon attached to position C6. Since molecular modeling suggested that C2 is less hindered than C4, the signal at 171.8 ppm is due to that at C2. The validity of this assignment is supported by the integration values due to acetyl carbonyl carbon at C6 (0.70, 170.5 ppm) and C2 (0.90, 169.0 ppm); that is, the total integration value of the acyl groups at C6 (1.04) is the same as that at C2 (1.04). Thus, the present myristoylation has the same regioselectivity as acetylation using acetic anhydride. Taken together, these NMR results support the assertion made in Section 3.2.3: successful preparation of thermoplastic mixed paramylon esters hinges on first introducing long-chain alkyl groups. Although plausible requirements for thermoplasticity deduced by the results are introduction at C6 and/or suitable introduction amount of the long-chain alkyl groups, the detailed thermoplasticizing mechanism remains to be elucidated.

3.3. Quantitative evaluation of thermal properties of mixed paramylon esters

3.3.1. Melt flow rate

As described in Section 3.2.1, we found by using a melting-point apparatus that the mixed paramylon esters (**1a**–**1f**) exhibited thermoplasticity. For quantitative evaluation, we measured their MFRs using a capillary rheometer. As shown in Table 2, the MFRs ranged from 734 to 1721 g/10 min. These rates are comparable to those of plant-based PLA, PA11, and petroleum-based ABS resin and are higher than that of CAS. Note that comparison of each reference with the mixed paramylon esters does not unambiguously clarify which is higher in terms of each parameter in this study because the thermal and mechanical properties of each reference plastic depend on its polymer characteristics including molecular weight and graft ratio. Comparison of the rates between mixed paramylon esters with alkyl chains of the same length revealed that the derivatives with a higher $\mathrm{DS}_{\mathrm{lac}}$ had a higher MFR, indicating that their plasticizing effect depended on $\mathrm{DS}_{\mathrm{lac}}$. Given that the mixed paramylon esters with a higher $\mathrm{DS}_{\mathrm{lac}}$ had a lower M_w, it is likely that the molecular weight of the polymer chains also affects thermoplasticity. Which factor primarily affects MFR remains to be elucidated.

3.3.2. Glass transition temperature

Next, we measured T_g using a differential scanning calorimeter. As shown in Table 2, the highest value was 133 °C for **1a**, higher than the 60 °C for PLA, 45 °C for PA11, and 102 °C for ABS resin and a bit lower than the 145 and 155 °C for CAS. Comparison of T_g among the mixed paramylon esters with alkyl chains of the same length revealed that the derivatives with a smaller $\mathrm{DS}_{\mathrm{lac}}$ exhibited a higher T_g. Comparison of T_g among the derivatives with a lower $\mathrm{DS}_{\mathrm{lac}}$ (i.e., **1a**, **1c**, and **1e**) revealed that they had a similar T_g. Comparison among the higher $\mathrm{DS}_{\mathrm{lac}}$ derivatives showed that **1d** and **1f** had a similar T_g and that **1b** had a lower T_g. This difference was likely due to the higher $\mathrm{DS}_{\mathrm{lac}}$ (i.e., 0.61) of **1b** than those of **1d** and **1f** (0.50 and 0.46, respectively). These results indicate that $\mathrm{DS}_{\mathrm{lac}}$ is an essential factor affecting T_g and that the alkyl chain length does not significantly affect it.

3.4. Mechanical properties

An additional property that is generally needed for many thermoplastic materials to be practically useful is mechanical strength. We prepared mechanical strength test specimens from the mixed paramylon esters by injection molding (Fig. 3). Since an injection molded specimen made from **1b** was too fragile for mechanical testing, a series of mechanical tests were not carried out.

Table 3 shows the bending strength, bending elastic modulus, and impact strength of the test specimens made from the mixed paramylon esters and the reference plastics. The highest bending strength (75.7 MPa) among the test specimens was that of **1a**. This is comparable to those of PLA, PA11, ABS, and CAS. Comparison of the bending strength among the test specimens with alkyl chains of the same length showed that it increased as the $\mathrm{DS}_{\mathrm{lac}}$ decreased. Comparison among the test specimens with a similar $\mathrm{DS}_{\mathrm{lac}}$ value clearly showed that it increased with a decrease in the alkyl chain length. A similar tendency was observed for the bending elastic modulus. The highest modulus was 2.11 GPa for **1a**, which is comparable to those of the reference plastics. Thus, **1a** had the highest bending strength and elastic modulus among the test specimens. Given that the M_w of **1a** was lower than those of **1c** and **1e**, the present results indicate that these mechanical strengths are not significantly affected by molecular weight but primarily by the alkyl chain length as well as by the $\mathrm{DS}_{\mathrm{lac}}$ value.

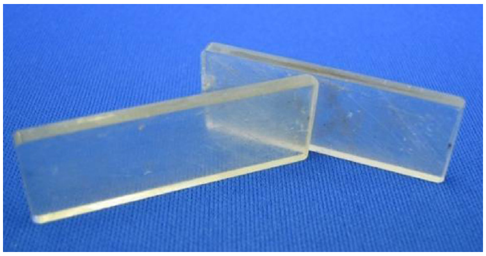


Fig. 3. Photograph of injection molded test specimens made from paramylon acetate myristate (**1a**) by injection molding (40 mm × 12.4 mm × 2.4 mm).

Table 3
Mechanical properties of injection molded test specimens made from mixed paramylon esters and of reference polymers.

Injection molded specimen	Bending strength (MPa)	Bending elastic modulus (GPa)	Impact strength (kJ/m^2)
Mixed paramylon ester			
1a	75.7	2.11	3.9
1b	–[a]	–[a]	–[a]
1c	65.6	1.74	2.7
1d	39.2	1.15	2.0
1e	58.3	1.60	2.3
1f	32.7	0.92	4.0
Reference polymer			
PLA	97	4.9	4.8
PA11	62	1.2	3.9
ABS	78	2.7	21.9
CAS (DS_{lac}/DS_{ace})			
0.31/2.1[b]	80[b]	2.3[b]	7.8[b]
0.51/2.1[b]	60[b]	1.9[b]	10.9[b]

[a] Mechanical tests could not be conducted because of fragility.
[b] Iji et al. (2013).

We also measured the impact strength. No clear dependence of the impact strength on any structural parameters was observed. The present results did show that the impact strengths of the mixed paramylon esters were comparable to those of PLA and PA11 while they were a bit lower than that of CAS and substantially lower than that of ABS.

4. Conclusion

We have demonstrated the synthesis and clarified the properties of mixed paramylon esters that exhibit thermoplasticity. The results fundamentally validated the idea of introducing long alkyl chains and acetyl groups into the glucose to weaken the polymer chain interaction, resulting in sufficient thermoplasticity. An additional benefit of using paramylon as a starting material for functional materials is carbon neutrality. Other benefits include the ease of extracting paramylon from *Euglena* cells and purifying it for use: since this polysaccharide is stored in the form of pure β-1,3-glucan particles inside thin cell membranes that can be easily disrupted, obtaining pure paramylon is potentially easy (Brandes, Buetow, Bertini, & Malkoff, 1964). Thermal and mechanical measurements of injection molded specimens revealed the potential of using mixed paramylon esters as thermoplastic materials for practical use. Further improvements in their properties can be obtained by fine tuning the alkyl chain structures. Studies currently in progress are aimed at developing high-value-added materials based on thermoplastics. We are also developing a mass cultivation method for *Euglena* that will enable inexpensive procurement of paramylon. The results will be reported in due course.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.01.053.

References

Banerjee, A., Sharma, R., Chisti, Y., & Banerjee, U. C. (2002). *Botryococcus braunii*: A renewable source of hydrocarbons and other chemicals. *Critical Reviews in Biotechnology*, *22*(3), 245–279. http://dx.doi.org/10.1080/07388550290789513

Brandes, D., Buetow, D. E., Bertini, F., & Malkoff, D. B. (1964). Role of lysosomes in cellular lytic processes. I. Effect of carbon starvation in *Euglena gracilis*. *Experimental and Molecular Pathology*, *3*(6), 583. http://dx.doi.org/10.1016/0014-4800(64)90036-X

Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology Advances*, *25*(3), 294–306. http://dx.doi.org/10.1016/j.biotechadv.2007.02.001

Edgar, K. J., Arnold, K. M., Blount, W. W., Lawniczak, J. E., & Lowman, D. W. (1995). Synthesis and properties of cellulose acetoacetates. *Macromolecules*, *28*(12), 4122–4128. http://dx.doi.org/10.1021/Ma00116a011

Gojdics, M. (1953). *The genus Euglena*. Madison: University of Wisconsin Press.

Iji, M., Toyama, K., & Tanaka, S. (2013). Mechanical and other characteristics of cellulose ester bonded with modified cardanol from cashew nut shells and additional aliphatic and aromatic components. *Cellulose*, *20*(1), 559–569. http://dx.doi.org/10.1007/s10570-012-9832-z

Inui, H., Miyatake, K., Nakano, Y., & Kitaoka, S. (1983). Production and composition of wax esters by fermentation of *Euglena gracilis*. *Agricultural and Biological Chemistry*, *47*(11), 2669–2671.

Kaya, K., Nakazawa, A., Matsuura, H., Honda, D., Inouye, I., & Watanabe, M. M. (2011). *Thraustochytrid aurantiochytrium* sp. 18W-13a accummulates high amounts of squalene. *Bioscience Biotechnology and Biochemistry*, *75*(11), 2246–2248. http://dx.doi.org/10.1271/Bbb.110430

Mata, T. M., Martins, A. A., & Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: A review. *Renewable & Sustainable Energy Reviews*, *14*(1), 217–232. http://dx.doi.org/10.1016/j.rser.2009.07.020 (Review)

Peydecastaing, J., Vaca-Garcia, C., & Borredon, E. (2011). Bi-acylation of cellulose: Determining the relative reactivities of the acetyl and fatty-acyl moieties. *Cellulose*, *18*(4), 1015–1021. http://dx.doi.org/10.1007/s10570-011-9528-9

Sealey, J. E., Samaranayake, G., Todd, J. G., & Glasser, W. G. (1996). Novel cellulose derivatives. 4. Preparation and thermal analysis of waxy esters of cellulose. *Journal of Polymer Science Part B: Polymer Physics*, *34*(9), 1613–1620. http://dx.doi.org/10.1002/(Sici)1099-0488(19960715)34:9<1613::Aid-Polb10>3.0.Co;2-A

Shibakami, M., Sohma, M., & Hayashi, M. (2012). Fabrication of doughnut-shaped particles from spheroidal paramylon granules of Euglena gracilis using acetylation reaction. *Carbohydrate Polymers*, *87*(1), 452–456. http://dx.doi.org/10.1016/j.carbpol.2011.08.012

Shibakami, M., Tsubouchi, G., Nakamura, M., & Hayashi, M. (2013). Polysaccharide nanofiber made from euglenoid alga. *Carbohydrate Polymers*, *93*(2), 499–505. http://dx.doi.org/10.1016/j.carbpol.2012.12.040

Takada, A., Fujii, K., Watanabe, J., Fukuda, T., & Miyamoto, T. (1994). Chain-length dependence of the mesomorphic properties of fully decanoated cellulose and cellooligosaccharides. *Macromolecules*, *27*(6), 1651–1653. http://dx.doi.org/10.1021/Ma00084a057

Teramoto, Y., Miyata, T., & Nishio, Y. (2006). Mesomorphic assemblage of chitin normal acylates and rapid enthalpy relaxation of their side chains. *Biomacromolecules*, *7*(1), 190–198. http://dx.doi.org/10.1021/Bm050580y

Teramoto, Y., Yoshioka, M., Shiraishi, N., & Nishio, Y. (2002). Plasticization of cellulose diacetate by graft copolymerization of epsilon-caprolactone and lactic acid. *Journal of Applied Polymer Science*, *84*(14), 2621–2628. http://dx.doi.org/10.1002/app.10430

Tezuka, Y., & Tsuchiya, Y. (1995). Determination of substituent distribution in cellulose-acetate by means of a C-13 NMR-study on its propanoated derivative. *Carbohydrate Research*, *273*(1), 83–91. http://dx.doi.org/10.1016/0008-6215(95)00107-5

Tucci, S., Vacula, R., Krajcovic, J., Proksch, P., & Martin, W. (2010). Variability of wax ester fermentation in natural and bleached *Euglena gracilis* strains in response to oxygen and the elongase inhibitor flufenacet. *Journal of Eukaryotic Microbiology*, *57*(1), 63–69. http://dx.doi.org/10.1111/j.1550-7408.2009.00452.x

Warth, H., Mulhaupt, R., & Schatzle, J. (1997). Thermoplastic cellulose acetate and cellulose acetate compounds prepared by reactive processing. *Journal of Applied Polymer Science*, *64*(2), 231–242. http://dx.doi.org/10.1002/(Sici)1097-4628(19970411)64:2<231::Aid-App4>3.0.Co;2-S

Yamane, K., Matsuyama, S., Igarashi, K., Utsumi, M., Shiraiwa, Y., & Kuwabara, T. (2013). Anaerobic coculture of microalgae with thermosipho globiformans and methanocaldococcus jannaschii at 68 °C enhances generation of n-alkane-rich biofuels after pyrolysis. *Applied and Environmental Microbiology*, *79*(3), 924–930. http://dx.doi.org/10.1128/AEM.01685-12