Purification and Characterization of a Novel Polysaccharide Involved in the Pellicle Produced by a Thermotolerant Acetobacter Strain†

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Acetobacter strains able to produce a thick pellicle at 37°C were screened among many thermotolerant strains isolated from fruits in Thailand. As a result, Acetobacter sp. SKU 1100 was selected as the producer of a relatively thick pellicle even when cultured at higher temperatures such as 37°C or 40°C. This strain could produce a pellicle polysaccharide in a shaking submerged culture as well as under static culture conditions. The polysaccharide was found to be attached to the bacterial cells. Although the polysaccharide production was higher at 30°C than at 37°C in shaking submerged culture, the productivity in static culture was not decreased even at higher temperatures. The membrane-attached polysaccharide was purified from the SKU 1100 strain by cell disruptions using either ultrasonic treatment or lysozyme treatment, followed by ultracentrifugation, enzyme treatments, dialysis against SDS, DEAE-cellulose column chromatography, alcohol precipitation, and gel filtration chromatography. The polysaccharide purified by the sonic treatment and also by the mild conditions using lysozyme treatment had the same average molecular mass of 120 kDa. The purified polysaccharide was composed of three different monosaccharides; glucose, galactose, and rhamnose, in an approximately equimolar ratio of 1:1:1.

Key words: thermotolerant acetic acid bacteria; Acetobacter species; pellicle polysaccharide formation; novel polysaccharide

Many bacterial species among both Gram-positive and Gram-negative bacteria have been known to produce extracellular hetero- or homopolysaccharides, which are attached to the bacterial cell or secreted into the environment.1) Acetobacter species, one genus of acetic acid bacteria, are also able to produce such extracellular polysaccharides, which have been isolated and characterized from Acetobacter xylinum,2–7) Acetobacter sp.,8,9) Acetobacter sp. NBI 1022,10) and Acetobacter methanolicus MB 129.11) Acetobacter species is a food-grade microorganism, strongly oxidizing ethanol to acetic acid and thus being used as a vinegar producer. The bacterial species is also well known to have an ability to grow floating on static culture by producing a pellicle on the top of the medium surface. Since A. xylinum is well known to produce a pellicle polysaccharide consisting of bacterial cellulose, it is one of the most famous model organisms for studying cellulose biogenesis.12,13) Thus, pellicles produced by other Acetobacter species have also been believed to be composed of cellulose or cellulosic material. However, we have discovered that Acetobacter acetii produces a pellicle consisting of a cell-attached heteropolysaccharide, composed of glucose and rhamnose.14) The pellicle formation of Acetobacter species is an important phenomenon for vinegar fermentation in static culture. However, the pellicle formation is not stable enough, and the ability to produce such a pellicle can be sometimes lost by a subtle change of culture conditions, especially a relatively small temperature change of 2–3°C. Thus, Acetobacter strains able to produce a stable pellicle would be very useful for such a vinegar fermentation. Furthermore, it would be even more useful if a thermostolerant strain able to produce a pellicle even at higher temperature could be obtained.

Thus, in this work, we screened Acetobacter strains able to produce a thick pellicle among many thermotolerant strains isolated from fruits in Thailand, and Acetobacter sp. SKU 1100 was selected as the best strain capable of producing a pellicle at relatively high temperatures. We also report the

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methods for the pellicle polysaccharide purification from the intact cells and cell membrane by using ultrasonic disruption and mild conditions using lysozyme treatment, and showed it is a novel pellicle heteropolysaccharide composed of three different sugars; galactose, glucose, and rhamnose.

**Materials and Methods**

**Chemicals.** All chemicals used in this study were commercial products of guaranteed grade.

**Bacterial strains, culture medium, and cultivation conditions.** The bacterial strains, thermotolerant acetate acid bacteria belonging to SKU strains, used in this study were isolated and kept by Prof. Napha Lotong and her colleagues at Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok, Thailand. The bacterial strains were maintained on a potato-glycerol-CaCO₃ agar slant, which was prepared by adding 2% agar and 0.5% CaCO₃ to a potato medium consisting of 5 g of glucose, 10 g of yeast extract, 10 g of polypeptone, 20 g of glycerol, and 100 ml of potato extract in 1 liter of tap water. Alternatively, all bacterial strains were preserved by mixing its fresh culture (24-h cultivation) with sterilized glycerol (ratio 1:1, v/v) in a glass vial and storing in a deep freezer at −80°C as stock culture. The test strains maintained on agar slants were inoculated in 1 ml of the potato liquid culture medium and cultivated with shaking (200 rpm) for 24 h as a seed culture.

**Screening for the pellicle-producing Acetobacter strains.** The culture static was formed by inoculating 5% of the seed culture into 5 ml of the same medium in a test tube (61.2 × 10.5 cm), and cultivating without shaking at 37°C for 3 days. Then, the cells able to produce a pellicle were harvested by centrifugation at 9,000 × g at 4°C for 10 min, and washed twice with 50 mM potassium-phosphate buffer (KPB), pH 6.5. After they were resuspended with the same buffer, sugar and protein contents of the intact cells (pellicle) were measured by the phenol-sulfuric acid method using glucose as the standard and by a modified Lowry method using bovine serum albumin as the standard, respectively. Thermotolerant Acetobacter strains selected as stable pellicle producers at 37°C in static culture were further screened at 30°C, 37°C, and 40°C. For the second screening, shaking and static cultures were done by inoculating 0.1% of the seed culture into 100 ml of the potato medium in a 500-ml Erlenmeyer flask and incubating with or without shaking at 30°C, 37°C, and 40°C for 3 days.

**Growth characterization of isolated Acetobacter sp.** Acetate overoxidization, acetate resistance, and ethanol oxidation of thermotolerant Acetobacter strains isolated were examined as described previously by Saeki et al.

**Isolation and purification of the pellicle polysaccharide from Acetobacter sp. SKU 1100 using ultrasonic disruption.** Shaking submerged culture was done by inoculating the seed culture into 100 ml of the same medium in a 500-ml Erlenmeyer flask and shaking (200 rpm) with a rotary shaker at 30°C for 24 h. Then, the culture was transferred to 1 liter of the same medium in a 3 liter Erlenmeyer flask and incubated with vigorous shaking for 30 h. Cells were harvested from the late exponential to the early stationary phase by centrifugation at 9,000 × g at 4°C for 10 min. Collected cells were washed twice with 50 mM KPB, pH 6.5, and then resuspended in the same buffer (about 1 g of the washed cells per 10 ml of buffer, w/v). Then, the cell suspension (40 ml) was ultrasonically disrupted in a 50-ml plastic serum tube (φ2.5 × 11.5 cm) maintained (0–4°C) on an ice bath by using an ultrasonic disruptor UD-201 (TOMY TECH, INC.) with a tapered microprobe (TP-040; 0.3 mm diameter) at the maximum condition for 20 min. After centrifugation at 9,000 × g at 4°C for 10 min to remove the debris and unbroken cells, the resultant clear supernatant was further centrifuged at 150,000 × g at 4°C for 1.5 h to collect the supernatant fraction (cell-free extract). To the cell-free extract fraction, DNase was added to the final concentration of 50 µg/ml, and then the solution was incubated at 37°C for 24 h in a water-bath shaker. Proteinase K was added to the suspension to the final concentration of 100 µg/ml, and then incubation was done under the same conditions. The supernatant was thoroughly dialyzed for 1–2 days against 25 mM Tris-HCl buffer, pH 8.5, containing 0.1% SDS with a cellulose membrane tube (molecular weight cutoff, 12,000–14,000). After removal of the precipitate by centrifugation at 9,000 × g at 4°C for 10 min, the dialyzed solution was put onto a Superdex S-200 column (φ3.5 × 12 cm) which had been equilibrated with 25 mM Tris-HCl buffer, pH 8.5, containing 0.1% SDS. The column was eluted with 300 ml of the same buffer, and then with 300 ml of 1 M NaCl at a flow rate of 1 ml/min. Polysaccharides were passed through the column, and the fractions containing polysaccharide were combined and centrifuged at 150,000 × g at 4°C for 1.5 h to remove insoluble materials. The resultant supernatant was precipitated by slowly adding 2 volumes of isopropyl alcohol, vigorously mixing for 1–2 min, and leaving at 4°C overnight. The precipitate was collected by centrifugation at 8,000 × g for 30 min, air-dried, and dissolved in 0.1 M NaCl containing 0.1% SDS. The crude polysaccharide was put onto a Superdex S-200 column (φ1.6 × 90 cm) equilibrated with the same solution, and eluted at a flow rate of 60 ml/h. The fractions containing...
polysaccharide were combined and precipitated by isopropyl alcohol as described above. Protein content was measured by a modified Lowry method and also to the amido black method, using bovine serum albumin as the standard.

**Purification of polysaccharide from the spheroplast membrane of Acetobacter sp. SKU 1100.** Spheroplast membrane was prepared by the method described in another paper, except for the lysozyme concentration (0.5 mg/ml instead of 0.25 mg/ml) and the incubation time (2 h instead of 1 h). The spheroplast membrane was solubilized with 1% octyl β-D-glucoside (β-OG) and treated with 100 μg/ml proteinase K at 37°C for 24 h. The resulting solution was thoroughly dialyzed against 25 mM Tris-HCl, pH 8.5, containing 0.1% SDS. The following steps of purification were the same as described above.

**Measurement of molecular mass of the purified polysaccharide.** The average molecular mass of the polysaccharides was estimated by size exclusion chromatography on a Superdex S-200 column (φ1.6 cm × 90 cm). Elution was done at a flow rate of 60 ml/h with 0.1 M NaCl containing 0.1% SDS, and the sugar content was measured colorimetrically at 490 nm by the phenol-sulfuric acid method. A calibration curve was obtained with the following standard pullulans (Showa Denko K.K., Tokyo, Japan.): P200 (20 kDa), respectively.

**Analytical ultracentrifugation.** Homogeneity of the purified polysaccharide was estimated by an analytical ultracentrifugation, which was done on a Hitachi model SCP85H by using a Schlieren optical system at 20°C.

**Identification of monosaccharide in the purified polysaccharide by gas chromatography mass spectrometer (GC-MS).** The monosaccharide composition of the purified pellicle polysaccharide from thermotolerant *Acetobacter* sp. SKU 1100 was identified by GC-MS of their alditol acetate derivatives. After hydrolysis with 2N (final concentration) trifluoroacetic acid at 121°C for 1 h, the hydrolysate was reduced and acetylated with NaBD₄ and acetic anhydride/pyridine, respectively. After it was evaporated with toluene, it was extracted with dichloromethane. The alditol acetates were analyzed with a Shimadzu GC-MS QP5050A, equipped with a DB-WAX capillary column (φ0.25 mm × 60 m). Its temperature program was 190°C for 4 min and then 1°C/min to 220°C and holding for 20 min at 220°C.

**Results**

**Screening of pellicle-producing *Acetobacter* strains**

Some *Acetobacter* species have an ability to grow floating on static culture by forming a pellicle on the medium surface. In this study, thermotolerant *Acetobacter* species were screened for their ability to produce a stable and thick pellicle even at high temperatures. One hundred and fourteen *Acetobacter* strains isolated from fruits in Thailand were screened; 22 strains of the screened strains showed an ability to produce a stable pellicle, in static culture at 37°C, when compared with mesophilic *Acetobacter* strains that formed a very weak or no pellicle at 37°C or 40°C, respectively (data not shown). The 22 strains were listed in Table 1, together with their abilities for growth at 40°C, acetic acid resistance, ethanol oxidation, acetate overoxidation, pellicle formation, colony morphologies, and sugar content. As shown in the Table, of the 22 strains, 9 strains had high pellicle-producing ability at 37°C and even at 40°C while 13 strains had only weak productivity at these temperatures. Of the 9 strains, three strains, SKU 1013, SKU 1072, and SKU 1100, produced pellicles at 37°C at almost the same level as at 30°C. Especially, SKU 1100 showed the same pellicle productivity even at 40°C. Sugar content of the selected 9 thermotolerant *Acetobacter* strains were further examined when grown with shaking at 37°C. *Acetobacter* sp. SKU 1100, with the highest polysaccharide production was finally selected as a producer of thick pellicle in static culture and used for the further study. The thermotolerant *Acetobacter* sp. SKU 1100 strain showed a rough type-colony surface (Fig. 1) and grew well under shaking submerged culture as well as static culture conditions.

**Growth behavior and the polysaccharide production of thermotolerant *Acetobacter* sp. SKU 1100**

Growth of *Acetobacter* sp. SKU 1100 strain was done with shaking and static culture conditions at various temperatures (Fig. 2). *Acetobacter* sp. SKU 1100 strain could grow very well even at 40°C, comitant with some cell aggregation which occurred during cultivation for 18–36 h (data not shown). Despite of the good growth at the high temperature, the pellicle formation was decreased by increasing the culture temperature (Fig. 2(A)), although under static culture conditions, the thermotolerant strain grew very well (Fig. 2(B)) and also produced a thick pellicle even at 40°C (data not shown). Especially, the thermotolerant strain rapidly grew and produced a higher level of the pellicle polysaccharide in the first day of cultivation at 37°C or 40°C, when compared with 30°C (Fig. 2(B)). In the shaking culture of *Acetobacter* sp. SKU 1100, the polysaccharide production was increased up to 72 h of cultivation, and the maximum yielding was 2.51 g/l at 30°C, 1.22 g/l at 37°C and 0.44 g/l at 40°C, respectively (Fig. 2(A)). Whereas the production under static cul-
ture condition reached to its maximum of 1.52 g/l, 1.23 g/l, and 1.34 g/l at 30°C, 37°C, and 40°C, respectively after 3 days of cultivation (Fig. 2(B)).

Table 1. Pellicle Polysaccharide Formation and Their Characteristics of Thermotolerant Acetobacter Strains

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Acetobacter rancens SKU 1102

Acetobacter sp. SKU 1100 were done by both using sonication or using lysozyme treatment, as described in Materials and Methods. This is because the sonication method has been shown to cause severe fragmentation in the case of the pellicle heteropolysaccharide of A. aceti, where the lysozyme method was suitable for preparation of the full length polymer of such fragile polysaccharide.14

In the purification of the pellicle polysaccharide, the recovery was almost the same, 38% with the sonication method and 33% with the lysozyme treatment from the starting materials, the intact cells, or the spheroplast membranes respectively, (data not shown). The pellicle polysaccharides purified by both procedures were eluted at almost the same position in a gel filtration chromatography (Fig. 3(A)) indicating both have an average size of 120 kDa. However, the ultrasonic treatment seemed to cause some fragmentation of the polysaccharide as can be seen as a shoulder in the gel filtration (Fig. 3(A); upper panel), which is in contrast to the case of the lysozyme treatment where almost no shoulder was observed (Fig. 3(A); lower panel).

Since no protein was detected in the final preparation even when measured by the highly sensitive amido black method, the purified pellicle polysaccharides were obtained as a protein-free material after Superdex S-200 column chromatography in both

![Fig. 1. Colony Morphology of Thermotolerant Acetobacter sp. SKU 1100 Strain.](image-url)
Novel Pellicle Polysaccharide from Thermotolerant Acetobacter Strain

Fig. 2. Growth and Pellicle Productivity of Thermotolerant Acetobacter sp. SKU 1100 under Shaking and Static Culture Conditions at Various Temperatures. (A) The growth (Klett units: ○; 30°C, □; 37°C, and ▲; 40°C) and the pellicle productivity measured by sugar content (white bar; 30°C, gray bar; 37°C, and black bar; 40°C) in shaking culture were shown. (B) The growth (wet cell weight: ○; 30°C, □; 37°C, and ▲; 40°C) and the pellicle polysaccharide production under static culture (white bar; 30°C, gray bar; 37, and black bar; 40°C) after 1, 2, and 3 days cultivation were shown.

Fig. 3. Homogeneity of the Purified Pellicle Polysaccharide of Thermotolerant Acetobacter sp. SKU 1100. (A) Elution profile of crude polysaccharide of thermotolerant Acetobacter sp. SKU 1100 purified by the sonication method and the mild method using lysozyme treatment. Crude polysaccharides of thermotolerant Acetobacter sp. SKU 1100 prepared by sonic treatment and by lysozyme treatment, as described in Materials and Methods, were put onto a Superdex S-200 chromatography column (1.6 × 90 cm) equilibrated with 0.1 M NaCl containing 0.1% SDS, and eluted at a flow rate of 60 ml/h. Elution was detected by absorbance at 214 nm (for polysaccharide), 240 nm (for nucleic acid) and 280 nm (for protein). (B) Sedimentation pattern of the purified pellicle polysaccharides from thermotolerant Acetobacter sp. SKU 1100. The purified pellicle polysaccharides at a concentration of 10 mg/ml in 0.1 M NaCl were centrifuged at 60,000 rpm at 20°C. Photographs were taken 40 min after reaching the maximum speed.

Sugar composition of the purified pellicle polysaccharide

In order to examine the sugar composition of the purified pellicle polysaccharides from the thermotolerant Acetobacter strain, it was analyzed by GC-MS after their alditol acetate formation. The purified polysaccharides were composed of three different monosaccharides, rhamnose, glucose, and galactose (Fig. 4). The results clearly showed that this polysaccharide was composed of galactose, glucose, and rhamnose in the molar ratio of 1.00:1.03:1.04, respectively.

Discussion

Many Acetobacter strains are able to grow floating in static culture, in which their pellicle formation enables the cells to float on the medium surface. We have found that A. aceti has a novel pellicle polysaccharide different from bacterial cellulose produced by A. xylinum, an Acetobacter species. The pellicle polysaccharide of A. aceti has also shown to be attached to the outer membrane, and to consist of glu-
1100 formed a thick pellicle even at 40°C under static culture, when compared with mesophilic Acetobacter polysaccharide under both shaking and static culture strain could produce a higher level of the pellicle at a lower temperature with shaking culture, the thermotolerant polysaccharide productivity was decreased at higher temperatures. Although the productivity of the polysaccharide was not much changed at the higher temperatures. Although it produced a higher level of the pellicle at 30°C under static culture even at high temperatures. Although some strain seemed to be novel and to be specific for the thermotolerant strain was not as much fragmented by the sonication method, the pellicle polysaccharide prepared by the sonication method was not different from that of the polysaccharide prepared by the mild method. Thus, although the lysozyme method was suitable for preparation of the full length polymer of fragile pellicles such as the polysaccharide of A. acetii, the sonication method seems to be practically more useful for isolation and purification of the cell-attached polysaccharide from several Acetobacter species. Our study also suggested that the sonication method is useful to prepare a full-length polymer only in the case of native polysaccharide so as to be smaller than 200 kDa, and thus may be useful for the preparation of homogeneous, low molecular-mass polysaccharides without alteration of the chemical structure of the repeating units. In this study, the pellicle polysaccharide purified from the membrane of Acetobacter sp. SKU 1100 was shown to be a heteropolysaccharide, composed of galactose, glucose, and rhamnose in an approximately equimolar ratio of 1:1:1, and to have an average molecular mass of 120 kDa. Such characteristics of SKU 1100 pellicle polysaccharide can be distinguished from other extracellular heteropolysaccharides being found among another Acetobacter species; ones composed of glucose, mannose, rhamnose, and glucuronic acid in A. xylinum, and in Acetobacter sp. NBI 1022, one composed of glucose, mannose, galactose, and glucuronic acid in Acetobacter sp., one composed of glucose, galactose, rhamnose, and glyceric acid in A. methanolicus MB 129, and one composed of glucose, galactose, and mannose in Acetobacter sp. Thus, the polysaccharide of thermotolerant Acetobacter sp. SKU 1100 strain seemed to be novel and to be specific for the pellicle formation.

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**Fig. 4.** Gas Chromatograms of the Alditol Acetate Derivatives Derived from the Purified Pellicle Polysaccharides of Thermotolerant Acetobacter sp. SKU 1100.

The purified polysaccharide (1 mg) and each authentic sugar (1 mg) were converted to the alditol acetate derivatives, and dissolved in chloroform. Then, the sample (1 μg) was injected (upper line), while each of the authentic samples (1 μg) was mixed, then injected (lower line).

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Cose and rhamnose. In addition, it has been shown to be a large fragile polymer of about 700-kDa when prepared under mild conditions.

In this study, we searched for other Acetobacter species able to produce such a pellicle polysaccharide, since some novel polysaccharides may be involved in the pellicle formation, and also some more efficient strains may be present to produce a high level of polysaccharide. Recently, we have shown that some thermotolerant acetic acid bacteria have more advantage than mesophilic strains as new microbial resources for development of oxidative fermentations such as vinegar fermentation and sugar alcohol oxidation. Thus, we searched for thermotolerant Acetobacter strains, which may be useful to produce a high level of polysaccharide even at high temperature, in this study. As the result, we found that Acetobacter sp. SKU 1100 was the most efficient strain, of 114 thermotolerant strains examined, as a producer of relatively thick pellicle polysaccharide under static culture even at high temperatures. Although it produced a higher level of the polysaccharide at 30°C than at 37°C or 40°C under shaking submerged culture, Acetobacter sp. SKU 1100 formed a thick pellicle even at 40°C at almost the same level at 30°C under static culture, where the productivity of the polysaccharide was not much changed at the higher temperatures. Although the polysaccharide productivity was decreased at higher temperature with shaking culture, the thermotolerant strain could produce a higher level of the pellicle polysaccharide under both shaking and static culture conditions, when compared with mesophilic Acetobacter strains. Thus, such a thermotolerant strain could be used in industrial production of the pellicle polysaccharide even at relatively high temperatures, implying it can be cultivated without any control of temperature.

These results clearly indicate that the pellicle polysaccharide produced by thermotolerant Acetobacter sp. SKU 1100 is tightly attached to the cell, more specifically to the spheroplast membrane, because the polysaccharide was retained in the spheroplast membrane when prepared by lysozyme treatment. For isolation and purification of the pellicle polysaccharide from the thermotolerant strain, both sonication and lysozyme treatment were used. Since, although some fragmentation was observed, the polysaccharide of the thermotolerant strain was not as much fragmented by the sonication method, the pellicle polysaccharide of the thermotolerant strain seems to be more tight physically than that of A. aceti. Also, the properties and quality of the pellicle polysaccharide prepared by the sonication method were not different from that of the polysaccharide prepared by the mild method. Thus, although the lysozyme method was suitable for preparation of the full length polymer of fragile pellicles such as the polysaccharide of A. acetii, the sonication method seems to be practically more useful for isolation and purification of the cell-attached polysaccharide from several Acetobacter species.
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