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ORIGINAL ARTICLE

Production of massoia lactone by *Aureobasidium pullulans* YTP6-14 isolated from the Gulf of Thailand and its fragrant biosurfactant properties

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Keywords
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Abstract

Aims: In order to add to the existing knowledge about structural diversity of biosurfactants, marine environment was chosen to discover a new type of biosurfactant-producing fungus.
Methods and Results: A number of fungi were collected from the Gulf of Thailand and examined for biosurfactant productivities. A dimorphic fungus, *Aureobasidium pullulans* YTP6-14, produced several different biosurfactants in both heavy oil and aqueous layers of the culture. Surface tension of the aqueous layer was decreased to 31·4 mN m^{-1} and oil displacement area reached 53 cm^2/10 μl after 7 days of cultivation. Critical micelle concentration and minimum surface tension values of the crude biosurfactants prepared from the aqueous layer were 39 mg l^{-1} and 31·6 mN m^{-1} respectively. Surface tension values remained unchanged over a wide range of pH and NaCl concentrations, suggesting their nonionic feature. LC/MS and NMR analyses revealed that one of the main active compounds in the aqueous layer was 5-hydroxy-2-decenoic acid delta-lactone, known as massoia lactone. Massoia lactone indeed showed significant surface tension reduction capacity of 43·3 mN m^{-1} at 1 mg ml^{-1}.
Significance and Impact of the Study: This is the first report for the production of a fragrant biosurfactant, massoia lactone by a fungus *A. pullulans*. Massoia lactone has been industrially prepared from aromatic bark of an endangered tree species, *Cryptocarya massoy*, growing in rainforests. This report expands the diversity of biosurfactants produced by *A. pullulans* and also points to its possibility in contributing to the green sustainable chemistry, and ultimately rainforest conservation.

Introduction

Biosurfactants are surface-active compounds produced by various micro-organisms including bacteria, yeasts and filamentous fungi (Plaza *et al.* 2006; Satpute *et al.* 2010), and are capable of reducing interfacial tension and surface tension. They are amphiphilic molecules containing both polar and nonpolar moieties, and are generally classified into fatty acids, phospholipids, glycolipids, lipopeptides, lipopolysaccharides and polymeric biosurfactants based on their chemical structure (Makkar *et al.* 2011; Roongsawang *et al.* 2011; Marchant and Banat 2012a). Some biosurfactants are also biologically active compounds with antifungal, antitumour and anticancer proliferative activities (Rodrigues *et al.* 2006). Biosurfactants are natural chemicals potentially useful in the pharmaceutical, cosmetic and food industries (Nitschkea and Costab 2007; Marchant and Banat 2012b). The utilization of microbial products meets with the recent trends in the nonpetroleum-based materials industry, but further technological innovation is necessary to use expensive biosurfactants in future industries. It is highly expected that

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uncovering useful hidden properties of biosurfactants, such as fragrance, flavour, moisture retention and texture will greatly expand the chances and field of their practical application. Moreover, from the viewpoint of protecting animals and plants, the trend, especially to regulate the use of animal-derived cosmetic ingredients, is also increasing. For these reasons, it is necessary to search for new biosurfactants beyond the existing concepts and structural categories.

Aureobasidium pullulans is a dimorphic fungus that grows in both single-celled yeast-like and multicellular filamentous forms, depending on the environmental conditions. *Aureobasidium pullulans* is distributed to extremely diverse environments such as tropical fruits, leaves, marine environments, timber, concrete and painted walls (Stratilova *et al.* 2005; Manitchotpisit *et al.* 2009). It is widely known as “black yeast” for abundant melanin pigment production (Campbell *et al.* 2004). *Aureobasidium pullulans* also produces a number of industrially useful extracellular enzymes including alkaline proteases, lipases, α-amylases, β-1,3-glucanases, endo- and exo-chitinases, and an exopolysaccharide, pullulan (Chi *et al.* 2009).

Manitchotpisit *et al.* (2011) reported that *A. pullulans* CU-43 produced sparingly soluble heavy oils with surface-active properties. The aqueous solution saturated with the heavy oils from strain CU 43 was able to lower the surface tension from 67 to 27 mN m^{-1}, suggesting that heavy oils are biosurfactant. Thereafter, it was found that the heavy oils from *A. pullulans* NRRL 50380 is a mixture of unique mannitol lipid conjugate, liamocins and nonconjugated exophillins (Price *et al.* 2013). Recently, a novel liamocin-related biosurfactant, glycerol-liamocin, was also reported from *A. pullulans* L3-GPY. Glycerol-liamocin was able to reduce the surface tension to 31·5 mN m^{-1} at 1·5 mg l^{-1} (Kim *et al.* 2015). Here, we report that one of the main biosurfactant compounds in aqueous layer of *A. pullulans* YTP6-14 culture is massoia lactone (ML). Massoia lactone is a coconut and creamy fragrant compound used in food, beverage and cosmetic industries. Massoia lactone has been largely prepared from an endangered tree species, *Cryptocarya massoy*, growing in rainforests (Sangat and Larashati 2002). This report describes the diversity of biosurfactants produced by *A. pullulans* and its potential future prospect for rainforest conservation.

Materials and methods

Micro-organism

Aureobasidium pullulans YTP6-14 was isolated from seawater obtained from a coast near Koh Sichang, Chonburi Province, Gulf of Thailand on YM agar (w/v, yeast extract 0·3%, malt extract 0·3%, peptone 0·5%, glucose 1% and 2% agar (pH 4·5)) supplemented with 0·025% sodium propionate and 200 mg l^{-1} chloramphenicol (Thaniyavarn *et al.* 2013). The strain was maintained in YM broth supplemented with 10% (w/v) glycerol and stored at −80°C.

Optimization of carbon source for growth and biosurfactant production

The strain YTP6-14 was cultured in a production medium containing 0·06% peptone, 0·04% yeast extract, 0·5% K_2HPO_4, 0·04% $MgSO_4{\cdot}7H_2O$ and 0·1% NaCl (Manitchotpisit *et al.* 2011) supplemented with 5% of various carbon sources including glucose (w/v), sucrose (w/v), glycerol (v/v), palm oil (v/v) or soybean oil (v/v), and mixed carbon sources which were also tested. Cells were first precultured in YM broth for 18 h at 30°C with shaking at 200 rpm and inoculated at 10% to 50 ml production medium in 250-ml Erlenmeyer flask for further 7 days of cultivation.

Time course of the growth and biosurfactant production by *Aureobasidium pullulans* YTP6-14

Triplicate culture samples after 1, 3, 5 and 7 days were subjected to the following analyses; cell growth, pH, biosurfactant activities by oil displacement test and surface tension, and the amount of crude biosurfactant. Cell growth was measured by dry cell weight (DCW) after cell filtration by glass fibre filters (GF/A, Sigma-Aldrich, St. Louis, MO, USA), this was followed by drying overnight at 80°C until constant value (Albiol *et al.* 1993). An aqueous layer was prepared by centrifugation at 10 000 × ***g*** for 20 min to remove heavy oils and cells from the culture. Biosurfactants contained in the aqueous layer was quantified either by surface tension (K6 Krüss force tensiometer, Hamburg, Germany), oil displacement (O-D) area (Morikawa *et al.* 2000; Youssef *et al.* 2004) or dry weight of ethyl acetate extracts. O-D area value was measured by spotting 10 μl sample on the 10 μl mineral oil membrane previously formed on the 20 ml extra-pure water in a 20-cm diameter Petri dish. O-D activity is defined as the O-D area formed by 10 μl sample.

Preparation of crude biosurfactants

Culture supernatant was recovered by centrifugation at 10 000 × ***g*** for 20 min to remove heavy oil and cells. Biosurfactants were extracted three times with an equal volume of ethyl acetate. The bottom solvent layer

containing biosurfactants was recovered by centrifugation at 10 000 × ***g*** for 5 min, and dried by evaporation at 40°C under vacuum. The resultant pellet was used as “crude biosurfactants” for further analyses and purification.

Measurement of critical micelle concentration (CMC) of biosurfactant

Crude biosurfactants were dissolved in 50 mM Tris-HCl (pH 8) at 1 mg l^{-1} and serially diluted to concentrations in the range of 0·01–10 000 mg l^{-1}. The CMC was determined by plotting the surface tension values against the log of the biosurfactant concentration.

Purification and chemical structure analysis of the main biosurfactant compound

The crude biosurfactants were submitted to purification by preparative thin layer chromatography (TLC). Samples were dissolved in methanol, spotted onto silica gel 60 plates (#113895, 1 mm thickness, Merck, Darmstadt, Germany), and developed for separation using chloroform/methanol/water (CMW 65:25:4) as mobile phase. The compounds were visualized by iodine vapour. Each dense coloured area was collected by scraping off the plates, eluted by the CMW solvent and tested for O-D activity after dissolving in 50 mM Tris-HCl (pH 8). Further purification was performed by high-performance liquid chromatography (HPLC) using a reversed-phase column (Cosmosil 5C18–AR, 4·6 × 150 mm, NacalaiTesque, Kyoto, Japan) at a flow rate of 0·5 ml min^{-1}, and monitored at 210 nm by UV detector.

Samples were dissolved in acetonitrile and injected to the column. The mobile phase was a linear gradient of acetonitrile from 10 to 100% in 0·1% trifluoroacetic acid. Major peaks were collected and tested for oil displacement activity. A main active peak, F2-3, was analysed for the chemical structure using NMR at GC-MS & NMR Laboratory, Research Faculty of Agriculture, Hokkaido University, Japan. Compound F2-3 was dissolved in deuterated methanol (CD_3OD). ^1H NMR, ^{13}C NMR and heteronuclear single quantum coherence (HSQC) analyses were carried out using Bruker AMX500 spectrometer (Bruker Biospin Corp., Billerica, MA, USA); ^1H spectra were recorded at 500 MHz. Chemical shifts (δ) were described by ppm scales relative to solvent peaks. Field ionization mass spectrometry (FI-MS) and electron impact ionization mass spectrometry (EI-MS) analyses were performed using a JMS-T100GCV mass spectrometer (JEOL, Tokyo, Japan) at the Global Facility Center, Hokkaido University.

Results

Isolation of biosurfactant-producing micro-organisms

A total of nine morphologically different colonies out of 246 isolates were surrounded by clear O-D area on YM agar plate covered with 20 μl of crude oil membrane. Formation of O-D area is a sensitive and useful indicator of biosurfactant production (Morikawa *et al.* 1993). The most significant biosurfactant-producing fungal strain, YTP6-14, was selected for further experiments. Its culture supernatant showed the largest O-D area among the strains. A partial DNA fragment of *c.* 600 bp encoding the D1/D2 domain of the large subunit 26S rRNA was PCR amplified and used to determine the nucleotide sequence. The 579 nucleotide sequence showed 100% identity to a number of *A. pullulans* strains and 97% identity to the strain *A. pullulans* var. *pullulans* CBS 100524^T. The strain YTP6-14 was thus identified as *A. pullulans*.

Time course profiles of cell growth and biosurfactant production

Growth of cells and production of biosurfactants were observed for 7 days at 30°C in the optimized production medium containing 2·5% (w/v) glucose and 2·5% (v/v) glycerol as the carbon source and initial pH of 6·5 (Fig. 1). When the culture was centrifuged, three layers were formed in the centrifuge tube, which were clear aqueous layer, cell pellet and bottom heavy oil layer. Surface tension and O-D area were measured for the aqueous layer of the culture. The maximum O-D area and minimum surface tension were 53·3 cm^2/10 μl and 32·1 mN m^{-1} respectively. The production of crude biosurfactants reached a maximum yield of 1·26 g l^{-1} after 7 days of cultivation. These values are comparable to those reported for biosurfactant-producing fungi such as *A. pullulans* L3-GPY (Kim *et al.* 2015), *Cyberlindnera samutprakarnensis* (Poomtien *et al.* 2013) and *Candida* sp. (Kim *et al.* 2006). The surface tension of the aqueous layer had dropped rapidly in 1 day and then gradually decreased to 32·1 mN m^{-1}. In contrast, its O-D activity was not evident for 1 day but then dramatically increased to 53 cm^2/10 μl after 7 days. These results suggest that *A. pullulans* YTP6-14 produced surface tension-reducing compounds and O-D active compounds in growth-associated and growth-nonassociated manners respectively. This discrepancy motivated the further analysis of biosurfactant compounds from the strain.

Critical micelle concentration of the crude biosurfactants

In order to determine the critical micelle concentration of crude biosurfactants produced by *A. pullulans* YTP6-14, the relationship between the concentration of

biosurfactants and surface tension was examined. Crude biosurfactants showed excellent surface tension reduction capacity, from 72·8 to 31·6 mN m^{-1}. The critical micelle concentration was determined as 39 mg l^{-1} (Fig. 2).

Effects of pH, salinity and temperature on the biosurfactant activity

The surface tension of crude biosurfactants remained unchanged around 32 mN m^{-1} over the range of pH 2–12 conditions. There was no significant but slight decrease in the minimum surface tension 31·0 mN m^{-1} in increasing amount of NaCl up to 12% (w/v). These results suggest that the major biosurfactant compounds are nonionic compounds. Reduction of pH to 4·1 in the culture medium (Fig. 1) suggests the production of some organic acids by strain YTP6-14. The surface tension also remained unchanged at temperatures from 4 to 100°C. Excellent thermostability of the crude biosurfactants was confirmed by no significant reduction in the activity after autoclaving at 121°C for 15 min.

Purification of the main compound, F2-3, from crude biosurfactants

Separation of crude biosurfactants on TLC exhibited three dense coloured areas, named F1, F2 and F3, with R*f* values of 0·90, 0·77 and 0·66 respectively (Fig. 3). Their O-D activities were measured after dissolving at 20 mg ml^{-1} in 50 mM Tris-HCl (pH 8). Compounds F2 and F3 showed significantly high O-D activities, of 125 and 177 cm^2/10 μl (equivalent to 0·2 mg), respectively, while compound F1 showed much less activity, 10 cm^2/10 μl. Production yield of compounds F2 and F3 were calculated as 170·1 and 132·3 mg l^{-1} culture after TLC purification respectively. Further purification of F2 was performed by reverse-phase HPLC. The main peak F2-3, eluted at a retention time of 11·21 min, exhibited the highest O-D area value of 11 cm^2/10 μl (equivalent

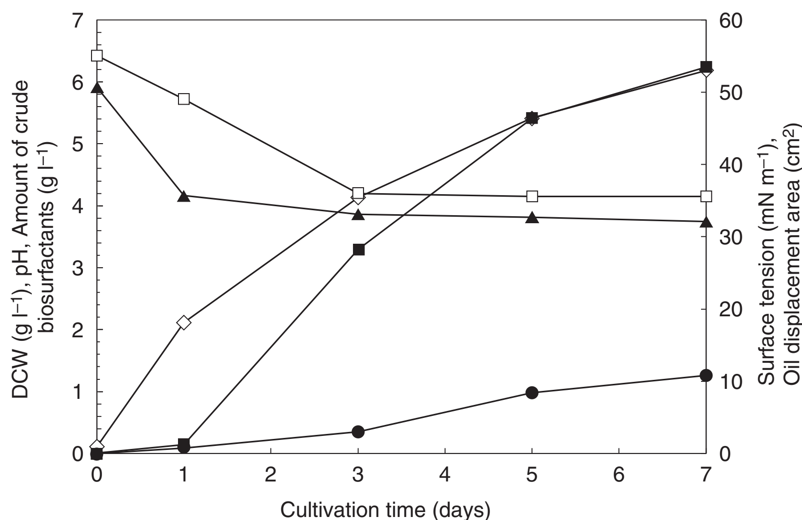

Figure 1 Time course profiles of cell growth, biosurfactant activity and amount of crude biosurfactants produced by *Aureobasidium pullulans* YTP6-14. Symbols are (◇) DCW (g l^{-1}); (□) pH; (●) Amount of crude biosurfactants (g l^{-1}); (▲) Surface tension (mN m^{-1}); (■) Oil displacement area (cm^2).

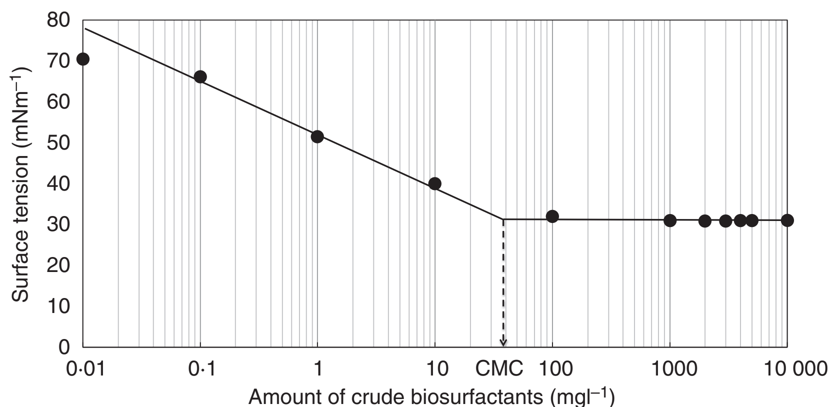

Figure 2 Relationship between the concentration of crude biosurfactants and surface tension. The CMC was determined to 39 mg l^{-1} by plotting the surface tension values against the log of biosurfactant concentration (Rosen 1989). CMC indicates the critical micelle concentration.

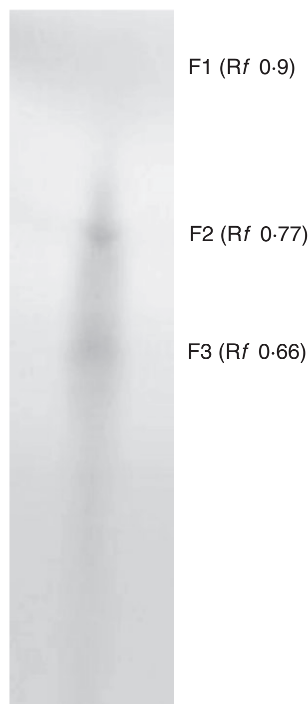

Figure 3 TLC separation of the crude biosurfactants produced by *Aureobasidium pullulans* YTP6-14. The crude biosurfactants were dissolved in methanol, spotted onto silica gel 60 plates, and developed using chloroform/methanol/water (65:25:4) as mobile phase. Spots or dense coloured area were visualized by exposing with iodine vapour in a sealed box. Rf value for each area (F1–F3) is shown in parenthesis.

to 0·2 mg) (Fig. 4). Production yield of F2-3 after HPLC purification was calculated as 8·6 mg l^{-1} culture. The specific O-D activity of F2-3 was determined as 57 cm^2 mg^{-1}. Compound F2-3 was subjected to chemical structure analyses by MS, ^1H and ^{13}C NMR spectroscopy. Compound F3 was also recovered from TLC plate and subjected to HPLC. The main peak F3-3 was unexpectedly eluted at exactly the same position as F2-3 in HPLC and no further structural analysis was performed (data not shown).

Chemical structure analyses of F2-3

A molecular ion peak $[M+H]^+$ was detected at 169·12 by FI-MS (Fig. 5). The molecular formula of this compound was estimated as $C_{10}H_{16}O_2$ by high-resolution MS (measured $(M+H)^+$ 169·12184, calculated 169·12285). EI-MS measurement showed fragment ion peaks at 97 $(C_5H_5O_2)^+$ and 68 $(C_4H_4O)^+$ (Fig. 6). ^1H NMR spectrum of F2-3 in CD_3OD is shown in Fig. 7a. HSQC analysis enabled the assigning of major proton peaks at δ 0·92 ppm (t, 7·0 Hz, 3H; **10**), 1·25–1·39 ppm (m, 4H; **8,9**), 1·42 ppm (m, 1H; **7′**), 1·52 ppm (m, 1H; **7**),

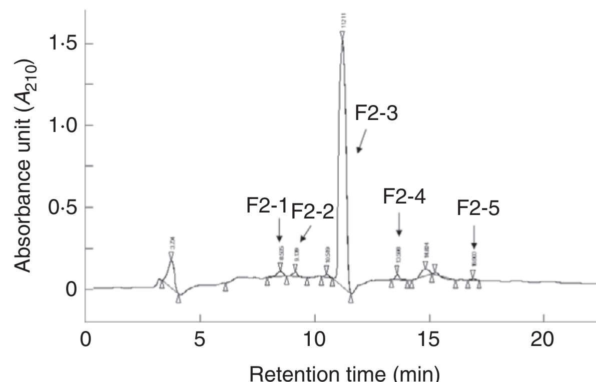

Figure 4 HPLC separation profile of F2 fraction.

1·66 ppm (m, 1H; **6′**), 1·75 ppm (m, 1H; **6**), 2·31 ppm (ddt 18·6/11·8/2·6 Hz, 1H; **4′**), 2·44 ppm (ddd 18·6/6·0/4·3 Hz, 1H; **4**), 4·45 ppm (dddd 12·0/8·6/4·3/4·3 Hz, 1H; **5**), 5·96 ppm (dd 9·7/2·5 Hz, 1H; **2**) and 7·02 ppm (ddd 9·9/6·3/2·1 Hz, 1H; **3**). ^{13}C NMR peaks were assigned as follows: 14·30 ppm (**10**), 23·58 ppm (**9**), 25·61 ppm (**7**), 30·32 ppm (**4**), 32·72 ppm (**8**), 35·85 ppm (**6**), 79·90 ppm (**5**), 121·30 ppm (**2**), 148·42 ppm (**3**) and 167·21 ppm (**1**) (Fig. 7b). These MS analyses and ^1H and ^{13}C NMR spectra data indicated that compounds F2-3 and probably also F3-3 produced by *A. pullulans* YTP6-14 were 5-hydroxy-2-decenoic acid delta-lactone, known as massoia lactone (ML). Similar coconut fragrance specific for ML was confirmed for compounds F2-3 and F3-3. Total production yield of ML by strain YTP6-14 was finally estimated as 19·7 mg l^{-1} culture.

Massoia lactone as a fragrant biosurfactant

The O-D activity of standard ML (>95% purity, Sigma-Aldrich W374400) was also determined. A 2% (w/v) solution in 50% methanol showed activity of 11 cm^2/10 μl (0·2 mg), whose value is comparable to the compound F2-3. Surface tension value of ML at 1 mg ml^{-1} in water was determined as 43·3 mN m^{-1}.

Discussion

Carbon sources for biosurfactant production

Production yield of the crude biosurfactants by *A. pullulans* YTP6-14 was eventually increased from 0·2 g l^{-1} in the initial culture condition where 5% sucrose acts as a single carbon source to 1·3 g l^{-1} in the optimized condition with 2·5% each of glucose (w/v) and glycerol (v/v) as mixed carbon sources. Glycerol is a cheap and useful carbon source for biosurfactant production (Hamzah *et al.* 2013). Moreover, crude glycerol is the waste

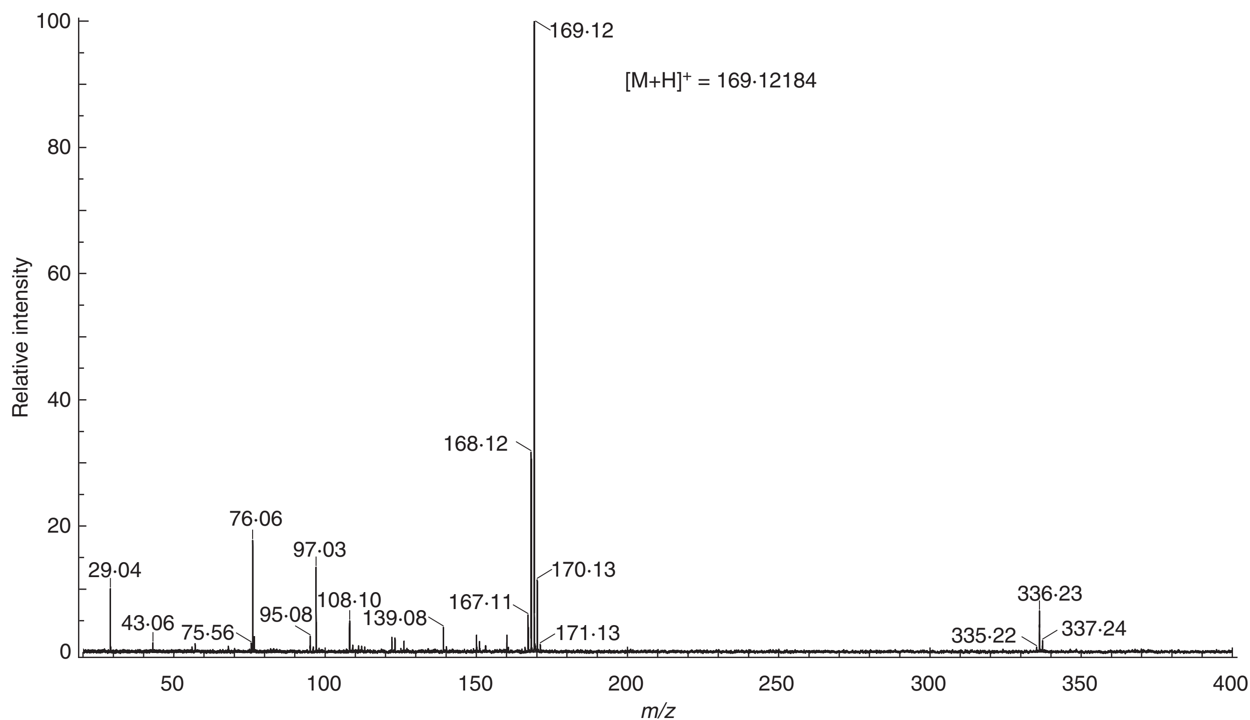

Figure 5 FI-MS analysis of F2-3.

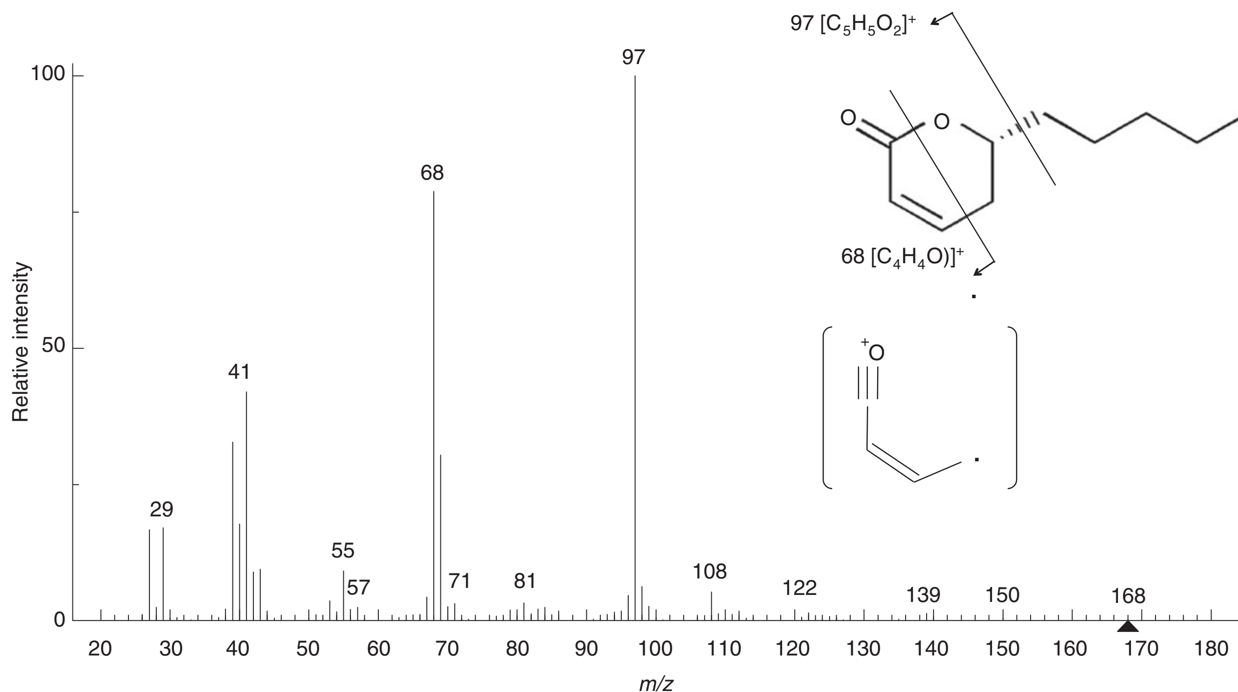

Figure 6 EI-MS analysis of F2-3.

obtained in biofuel production processes, and the development of value-added utilization methods for the crude glycerol is of a great demand not only for reducing the biodiesel production costs but also for constructing sustainable industries in the future (Garlapati *et al.* 2016). The culture condition of 5% glycerol as a sole carbon

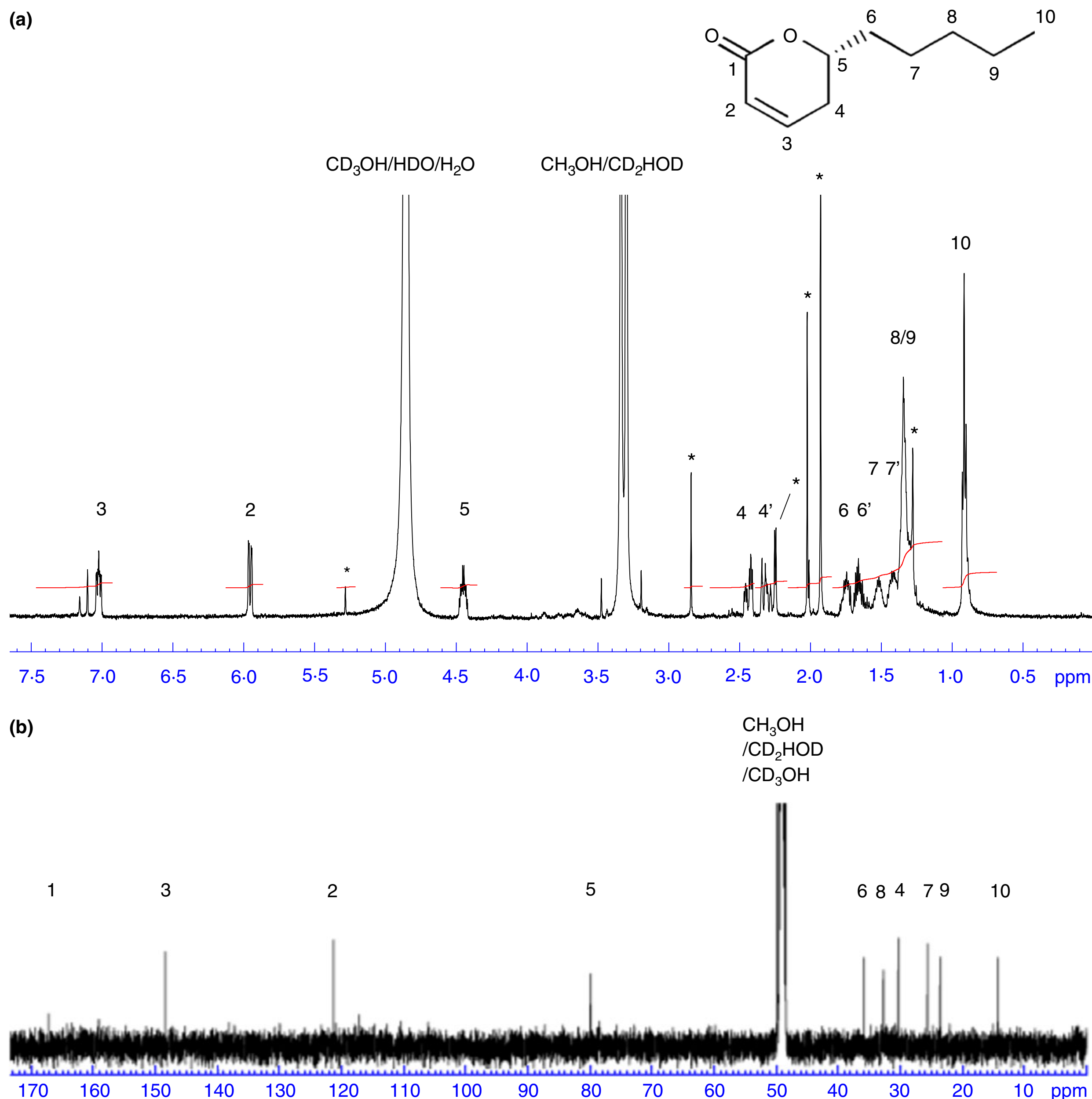

Figure 7 ^{1}H and ^{13}C NMR spectra of F2-3 biosurfactant. (a) ^{1}H NMR. (b) ^{13}C NMR. Asterisks indicate noise peaks. Chemical shifts (δ) were described by ppm scale relative to the solvent peak. [Colour figure can be viewed at wileyonlinelibrary.com]

source was tested; however, production yield of biosurfactants was very low, 20·4% of the maximum value.

Production of massoia lactone by *Aureobasidium pullulans* YTP6-14

Price *et al.* (2013) detected ML after methanolysis treatment of heavy oils containing glycolipid liamocins, produced by *A. pullulans* NRRL 50380. O-D activities of heavy oil layer from strain YTP6-14 were determined as 17 cm^2/10 μl (1 mg ml^{-1}). Although there has not been any analysis of the structure of heavy oil fraction produced by strain YTP6-14, it is probable that this fraction contains similar glycolipids produced by *A. pullulans* NRRL 50380 or *Aureobasidium* sp. A-2. Kurosawa *et al.* (1994) reported that 5-hydroxy-2-decenoic acid and 3,5-dihydroxydecanoic acid esters of arabitol or mannitol lipids were produced by *Aureobasidium* sp. A-2. They obtained ML after saponification of the lipids with 2N NaOH overnight followed by acidification with 5N

H_2SO_4 to pH 3. Vesonder *et al.* (1972) also reported that a fungus *Cephalosporium recifei* strain produced 3,5-dihydroxydecanoic acid delta-lactone. This compound was further converted to ML after dehydration in boiling benzene with *p*-toluene-sulphonic acid. Although the structure of F3 was not analysed, however, F2 should not be a dehydrated product of F3, which was formed in our preparation method.

Massoia lactone as a fragrant biosurfactant

Here, we characterized for the first time ML as a new type biosurfactant that could not be grouped into existing categories. The hydrophilic–lipophilic balance (HLB) value of ML was calculated as 4·85 and 4·71, respectively, by Davies' method and Griffin's method (Rosen 1989), indicating that it could function as a moderate w/o emulsifying agent. ML was first discovered in 1937 from the aromatic bark of *Cryptocarya massoy* and has been commercially used as flavouring agent in food industries (Pratiwi *et al.* 2015), however, its surfactant properties have never been described before. The genus *Cryptocarya* is of great ecological importance (Sangat and Larashati 2002). The natural habitats of *Cryptocarya* species are mostly in the rainforest in tropical and subtropical regions that face threats of destruction by human deforestation, and some species are on the verge of extinction due to loss of habitat. Our observation demonstrates that *A. pullulans* YTP6-14 produces ML in the culture. Thus, production yield is yet very low, however, production of ML by a fungus is noteworthy from the viewpoint of potential rainforest conservation.

On the other hand, extensive efforts have been made for biotransformation and metabolic engineering in order to produce various fragrance compounds, including ML (Romero-Guido *et al.* 2011). It has been reported that several fungi are able to transform fatty acids into lactones, and peroxisomal acyl coenzyme A oxidases such as POX1-5 are critical enzymes in the transformation process (Endrizzi *et al.* 1996; Waché *et al.* 2000). Gene cloning and expression of *POX* homologues from *A. pullulans* YTP6-14, followed by comparing activities and functions of the recombinant enzymes with those from *Yarrowia lipolytica* (García *et al.* 2007), *Saccharomyces cerevisiae* (Dmochowska *et al.* 1990) and *C. recifei* (unpublished), would shed light on the different molecular mechanisms of lactone formation and contribute to enhanced microbial production of ML in the future.

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Compliance with ethical standard

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author contributions

S.L., J.T. and M.M. designed the work. S.L. conducted the experiments. S.L., J.T. and M.M. analysed the data and wrote the manuscript.

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