BIODIVERSITAS
Volume 23, Number 6, June 2022
Pages: 3108-3114

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d230638

Biosurfactant activity of phylloplane bacteria from an ornamental plant, *Colocasia esculenta* L.

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Manuscript received: 30 April 2022. Revision accepted: 27 May 2022.

Abstract. *Bukhori A, Suryanto D, Nurtjahja K. 2022. Biosurfactant activity of phylloplane bacteria from an ornamental plant,* Colocasia esculenta *L. Biodiversitas 23: 3108-3114.* Biosurfactants are surface-active molecules produced by living organisms predominantly by microorganisms with amphiphilic properties. Exploration of biosurfactant-producing bacteria has been promoted to find the suitable agent for mass production in the laboratory following its biochemical and genetic modification. Leaf-colonizing bacteria or phyllosphere bacteria are of great interest, including those colonizing the surface of ornamental plants which are still understudied. This study aimed to isolate the biosurfactant-producing bacteria from an ornamental plant, *Colocasia esculenta* L and to determine their physical characteristics. Four bacterial isolates coded as IC1, IC3, IC4 and IC5 were recovered from the surface of *C. esculenta* and were tested positive for their growth under Bushnell-Haas agar + 1% olive oil (v/v) as the sole carbon source. Two isolates, namely IC3 and IC5, later molecularly identified as *Bacillus cereus* and *Alcaligenes faecalis* produced the highest biosurfactant concentration (IC3 = 157 ppm, IC5 = 106 ppm) on 10th day incubation based on a colorimetric test using rhamnolipid as a standard solution. Crude biosurfactants produced by *A. faecalis* IC5 showed better physical attributes than *B. cereus* IC3 in terms of surface tension, emulsification index, and oil spreading capability on four different hydrophobic compounds i.e., kerosene, solar fuel, octane fuel (Pertalite, Pertamax). The results of this study confirmed the existence of biosurfactant-producing bacteria in *C. esculenta* and the possibility of developing prominent strains for the treatment of hydrocarbon pollution in the environment.

Keywords: *Alcaligenes*, *Bacillus*, biosurfactant, *Colocasia esculenta*, phyllosphere bacteria

INTRODUCTION

Petroleum is currently the most extensively used form of energy by mankind. The increased use of petroleum hydrocarbon compounds undoubtedly pose some negative effects to pollution and public or human health. Environmental pollution can occur on land and in the water, and if not addressed, it will be hazardous to the environment as well as humans, plants, and animals (Wang et al. 2017). Under perspective of negative ramifications, some efforts must be made to eliminate contamination from hydrocarbon pollution. Biodegradation using microorganisms is one of the methods that can be applied to rehabilitate the polluted site with cost-effective approach and is regarded as safe for the environment (Das and Chandran 2011).

Surfactants are surface-active chemicals that can lower surface tension and make insoluble compounds more soluble in water. Surfactants are widely employed in a variety of sectors, including soap, paint, paper, pharmaceutical, cosmetic, food and beverage bioremediation, and pharmaceutical, cosmetic, food and beverage bioremediation (Akbari et al. 2018; Singh et al. 2018). Surfactants are commonly made from petroleum-derived chemicals such as linear alkylbenzene sulfonate (LAS) and alkyl sulfonates (AS), but the surfactants made from these two compounds are not environmentally beneficial because they can become waste and are difficult to decompose. Microorganisms have been shown to synthesize biosurfactant molecules with similar properties to chemically synthesized surfactants. Biosurfactants are currently attracting more attention and interest due to the benefits they provide not only in the remediation of environment but also in the agriculture sector (Hussain et al. 2020a; Hussain et al. 2020b; Hussain et al. 2021). Biosurfactants have several advantages, high bioactivities against competing microbes, including being biodegradable, having minimal toxicity, having low production costs because they can be produced from inexpensive raw materials, and being stable under extreme pH and temperature settings (Hussain et al. 2020c; Gayathiri et al. 2022).

The wax layers namely cuticles on the surface of leaves or phyllospheres are home to a wide variety of bacteria including the lipolytic and biosurfactant-producing bacteria (Oso et al. 2021). Phyllosphere refers to the aerial part or above-ground surface of a plant such as flowers, fruits, leaves and stems while phylloplane specifically refers to the surface area of leaves. The phylloplane region provides a suitable habitat for the colonization of various microorganisms such as algae, bacteria, fungi, nematodes, and yeasts. The number of bacterial populations on the leaf surface ranges between 10^5 to 10^7 CFU/g which may differ across plant species, communities, and ecosystems (Lindow and Brandl 2003; Baldotto and Olivares 2008; Wiraswati et al. 2020). This study therefore selected a common ornamental plant in the urban environment, *Colocasia esculenta* L, as a potential source of isolating

biosurfactant-producing bacteria from the phylloplane region (Pereira et al. 2005). To date, the isolation of phylloplane bacteria from ornamental plants is mostly exclusive to monitoring the concentration of polycyclic aromatic hydrocarbons (PAH) or indoor pollution in the urban environments (Siriratruengsuk et al. 2017). The finding from this study may be used to increase our knowledge on the existence of biosurfactant-producing bacteria on the surface of typical ornamental plants following its possibility to be developed for field application in the polluted sites.

MATERIALS AND METHODS

Isolation of phylloplane bacteria

Samples of *C. esculenta* L were collected from Percut Sei Tuan District, Medan City, North Sumatra (3°35'07.0"N, 98°44'10.9"E). Isolation of phylloplane bacteria was based on the method by Muliani et al. (2004). Briefly, the leaf sample was cut into smaller size, approximately 2-to-5-cm fragments then placed on top of Nutrient Agar (NA) medium, each for the upper and lower surface of leaves. The medium was incubated for 48 hr at 27°C. Any visible bacterial colonies showing distinguishable characteristics based on biochemical tests and gram staining were purified into single colony and stored for further experimentation.

Screening of biosurfactant-producing bacteria

All bacterial isolates were grown in the Bushnell-Hass Agar (BHA) containing (g/L): KH_2PO_4 1 g, K_2HPO_4 1 g, NH_4NO_3 1 g, $MgSO_4$ 0.2 g, $CaCl_2$ 0.02 g, $FeCl_3$ 0.05 g, agar 20 g, supplemented with 1% (v/v) olive oil and incubated at 27°C for 24 hr. The presence of visible colonies indicated the traits of biosurfactant-producing bacteria and subjected for production in the next experimentation.

Production and quantification of biosurfactant

The fermentation procedure followed the method by Naibaho et al. (2020). One mL of bacterial inoculum with a density of 10^8 cells/mL was inoculated into 48 mL of Bushnell-Haas Broth (BHB) + 2% olive oil aseptically. The medium was incubated at room temperature and agitated at 100 rpm for 15 d. The concentration of crude biosurfactant was quantified using a modified orcinol method. The liquid medium was centrifuged at 6000 rpm for 10 min and the supernatant was sampled for quantification and characterization in the next experimentation. Four mL of the supernatant was extracted with 2 mL of diethyl ether for 5 min. The ether layer was decanted, dried and redissolved in 2 mL of 0.05 M sodium bicarbonate ($NaHCO_3$) solution. The sample solution was homogenized and added with 3.6 mL of orcinol solution, heated to boiling, cooled to room temperature for 15 min and read with a spectrophotometer with a wavelength of 421 nm. A standard curve using pure rhamnose as a standard biosurfactant was treated in the similar step as sample reading in different concentrations (10, 50, 100, and 200 ppm). The concentration of biosurfactant in samples was plotted against the standard solution absorbance using a linear regression analysis (Figure 1).

Oil (hydrocarbon) spreading test

The hydrocarbon displacement activity of bacterial biosurfactants on top of aqueous environment was tested against four different hydrocarbon compounds namely kerosene, solar fuel, octane fuel 90 (Pertalite), and octane fuel 92 (Pertamax). A total of 40 mL of sterile distilled water was poured into a petri dish followed by the addition of 20 µL of crude hydrocarbon solution to form a thin layer on the surface of the distilled water. The supernatant from the bacterial culture was then added as much as 10 µL to the surface of the hydrocarbon. If the supernatant contains biosurfactants, the oil will separate and form a clear zone (Alyousif et al. 2020).

Emulsifying activity (Emulsification index/ EI_{24})

The emulsifying activity of bacterial biosurfactants was tested against five different hydrocarbon compounds namely kerosene, solar fuel, octane fuel 90 (Pertalite), octane fuel 92 (Pertamax), and hexane in a 1:1 ratio (v/v). The mixture of hydrocarbon compounds and bacterial biosurfactant was vortexed for 2 min and stored for 24 hr at room temperature. The emulsifying activity is expressed as an emulsification index (EI_{24}) calculated using the following formula (Santos et al. 2018):

$$Emulsification\ index\ (EI_{24}) = \frac{The\ height\ of\ emulsion\ layer}{The\ height\ of\ total\ solution}\ x100\%$$

Surface tension analysis

The surface tensions of air/water and oil/water interfaces were measured using a Du Noüy ring method in a tensiometer (Desai and Banat 1997). The interaction between bacterial surfactants with oil interface will reduce the surface tension to match the aqueous solution (72 nM/m) and be observed up to a critical level or known as the critical micelle concentration (CMC).

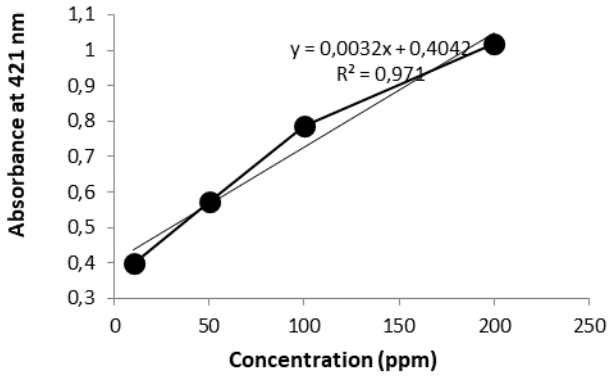

Figure 1. Calibration curve for standard rhamnose for determination of total crude biosurfactant produced by bacterial isolates at 421 nm using a modified orcinol method

Molecular identification of potential isolates based on 16S rRNA region

Two potential bacterial isolates, IC3 and IC5 were identified to the species level based on their sequence similarity with online database provided in BLAST NCBI in the 16S-rRNA region. The molecular identification was commercially employed by Macrogen, Inc (Singapore) resulting in raw forward and reverse sequence using a pair of primers (785F, 907R). Phylogenetic construction of genetic similarity among accessions was visualized using MEGA-11 (Tamura et al. 2021).

RESULTS AND DISCUSSION

Screening results and yield of biosurfactants produced by phylloplane bacteria of *Colocasia esculenta*

A total of 25 bacterial isolates were recovered from the phylloplane of *C. esculenta*. Only four bacterial isolates, namely IC1, IC3, IC4, IC5 showed a considerable growth performance on 1% olive oil-supplemented BHA medium with the biochemical and gram stain traits as presented in Table 1. The biosurfactant production was monitored until 15th days of incubation with the profile as presented in Figure 2. The maximum production of biosurfactant was observed at 10th day of incubation with the highest yield by IC3 (157 ppm) followed by IC5 (106 ppm) and IC4 (72 ppm) and drastically decreased until the end of incubation period. In contrary, IC1 only produced biosurfactant in the early fermentation stage (19 ppm).

Characteristics of biosurfactant produced by phylloplane bacteria of *Colocasia esculenta*

The physical attributes of crude biosurfactants produced by phylloplane bacteria were analyzed based on several parameters. Surface tension measurements on four isolates of phyllosphere bacteria produced surface tension values ranging from 35 to 39 mN/m and were declared positive for biosurfactant producers (Figure 3). In the oil dispersion or displacing test, the four isolates could produce clear zones on the hydrocarbon compounds, except for IC4 which was unable to produce clear zones in octane fuel (Pertamax). The clear zone ranges from the four isolates ranged from 9-38 mm (Figure 4, Figure 5). The emulsifying activities were varied among bacterial isolates. Only IC1 that was able to emulsify diesel oil and hexane, IC3 was only able to emulsify kerosene, IC4 on solar fuel, and IC5 could emulsify four hydrocarbon compounds, namely diesel oil, octane fuels and hexane. The range of emulsifying activity (IE_{24}) produced was 2-7% (Figure 6).

Identity of potential phylloplane bacterial strains based on 16S-rRNA

Two potential strains, IC3 and IC5 were subjected to molecular identification based on 16S-rRNA region through BLAST search results and phylogenetic analysis. Based on the phylogenetic construction, the potential strains were identified as a member of Bacillaceae namely *Bacillus cereus* for IC3 (ON606231.1) and a member of Alcaligenaceae namely *Alcaligenes faecalis* for IC5 (ON606232.1) as presented in Figure 7.

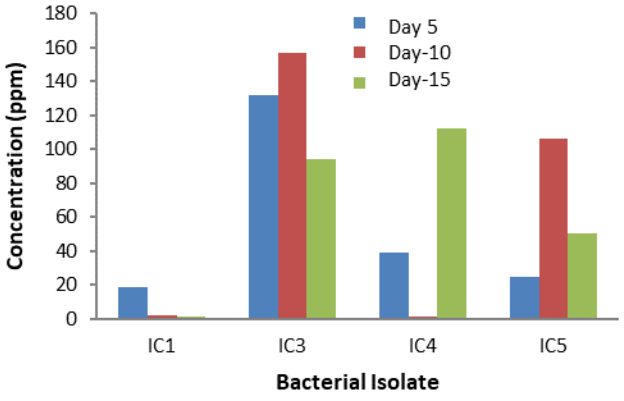

Figure 2. Biosurfactant concentration (ppm) in the crude supernatant of phylloplane bacteria was monitored until 15th day of incubation

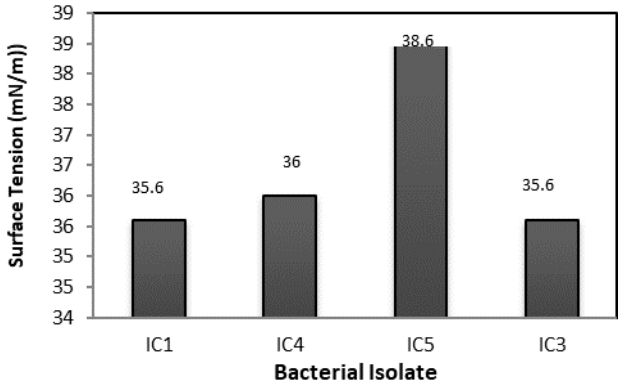

Figure 3. Surface tension (nM/m) of hydrocarbon tested with bacterial biosurfactants at 25^{o}C

Table 1. Morphological characteristics and biochemical properties of phyllosphere bacterial isolates

Isolate	Colony form	Edge of the colony	Elevation	Colony color	Gram staining	Motility	Catalase	Sugar utilization (TSIA)	Citric	Starch hydrolysis	Gelatin hydrolysis
IC1	Circular	Entire	Raised	Cream	-	+	+	Red	+	-	+
IC3	Irregular	Lobate	Raised	White	+	-	+	Yellow	-	+	+
IC4	Irregular	Undulate	Raised	White	+	+	+	Yellow	+	+	+
IC5	Filamentous	Filamentous	Flat	White	-	-	+	Red	+	+	+

Notes: (+) = Positive Reaction; (-) = Negative Reaction

Discussion

The occurrence of biosurfactant-producing bacteria from the leaf surface or phylloplane of an ornamental plant species, *C. esculenta* is validated and reported for the first time in our study. Initial finding of bacterial colonization on the phylloplane of *Colocasia* has been reported from *Colocasia antiquorum*, revealing the existence of bacterial microcolonies, solitary bacterial cells and biofilm based on electron microscopy (Baldotto and Olivares 2008). Additional information included the existence of oxalotrophic bacteria as endophytes in *C. esculenta* capable of degrading oxalate (Ghate et al. 2021). Hence, the exploration of biosurfactant-producing bacteria is possible from a variety of substrates and ecosystems, including the phyllosphere region. Bacterial isolates recovered from a polluted environment, on the other hand, may produce more biosurfactants than isolates obtained in an unpolluted environment. Biosurfactants are secreted by microorganisms in order to survive in hostile environments. Biosurfactants are synthesized to aid in the attachment of microorganisms to the substrate and the adherence of cells to their natural substrate (Hussain and Khan 2018; Alyousif et al. 2020).

According to Viramontes-Ramos et al. (2010), bacteria that produce biosurfactants have two methods of secreting the biosurfactants either directly into their surroundings (extracellular biosurfactants) or on the cell surface (intracellular biosurfactants). Intracellular biosurfactants are lipid-based compounds and aid in the movement of water-insoluble substrates, whereas extracellular biosurfactants are built of complex lipids, proteins, and carbohydrates which aid in the solubilization of hydrocarbon substrates. Saharan et al. (2011) stated that environmental or culture conditions such as aeration, temperature, nutrients, and pH have a significant impact on estimating the biosurfactant capacity of an isolate in the laboratory.

The biosurfactant activity observed in this study was the oil spreading test, emulsification index and surface tension measurement. Measurement of surface tension activity is a common type of measurement that is commonly used to see the surfactant activity of a substance. Syahriansyah and Hamzah (2016) suggested that the surface tension value >45 mN/m is considered as negative in producing biosurfactants, while the surface tension value <40 mN/m is considered as potential. The emulsification index (*EI*) is one of the methods used to select potential microorganisms to produce biosurfactants. A low *EI* value indicates that the biosurfactant produced by microorganisms is in low quantity (Alyousif et al. 2020). Emulsification index is related to the concentration of surfactant produced, the smaller the concentration of surfactant produced, the lower its ability to emulsify oil. The emulsification index is a test used to see the ability of biosurfactants to emulsify liquids with different polarity levels.

Based on the biosurfactant activity of the four isolates of phyllosphere bacteria that have been tested, the selected isolates were IC3 which was identified as *B. cereus* and IC5 identified as *A. faecalis* bacteria. *Bacillus cereus* is a group of bacteria that can be found in agricultural products, food, soil, and water (Aradillas et al. 2011). Some strains of the *B. cereus* group are known to be pathogenic bacteria, but some may be classified as non-pathogenic and known as producers of biosurfactants especially from the environment. Some strains of *B. cereus* are known as a beneficial group in the agriculture and food industry sectors along with its close relatives such as *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus cytotoxicus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, and *Bacillus weihenstephanensis* (Santos et al. 2019).

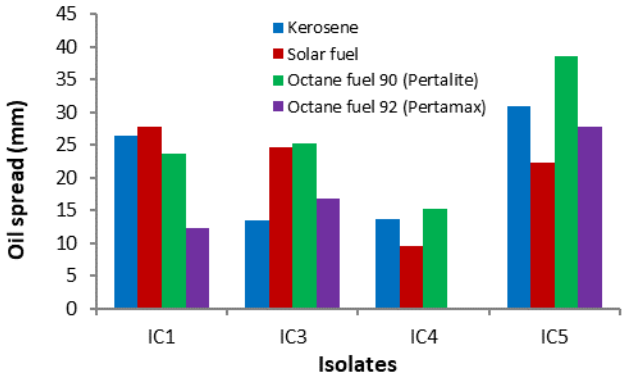

Figure 4. Oil or hydrocarbon displacement activity of biosurfactants produced by phylloplane bacteria on four different hydrocarbon compounds

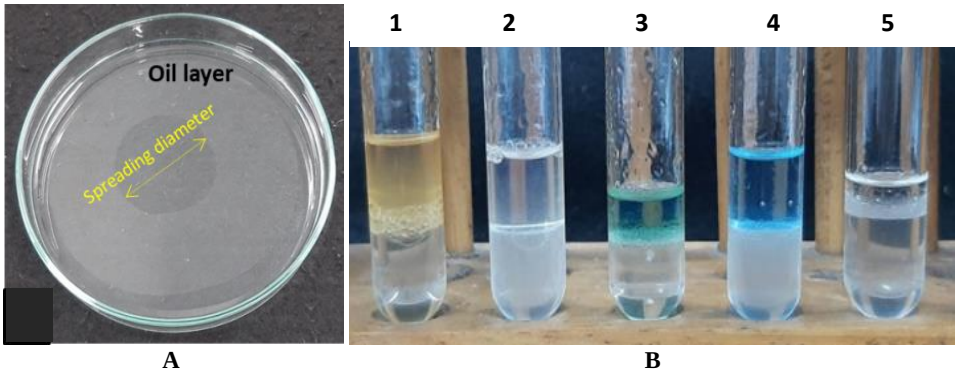

Figure 5. Oil displacement activity by IC5 (A). Emulsifying activity (IE_{24}) by IC5 on aqueous solution with kerosene (1), solar fuel (2), octane fuel 90 (Pertalite) (3), octane fuel 92 (Pertamax) (4), hexane (5)

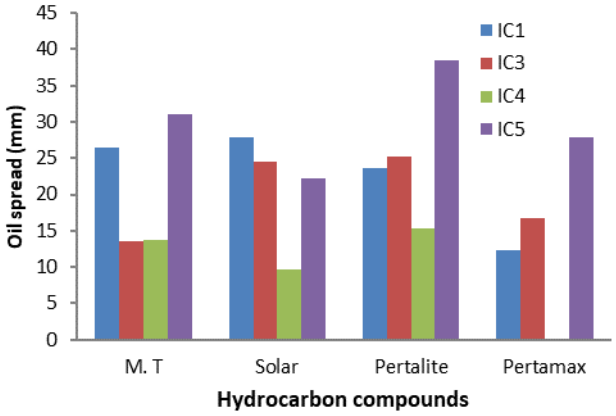

Figure 6. Emulsification index (EI_{24}) of different hydrocarbon compounds by bacterial biosurfactants.

The genus *Bacillus* has previously been reported as one of the genera of bacteria capable of producing biosurfactants such as *Bacillus siamensis* (Hussain and Khan 2022), and *Bacillus firmus* (Hussain et al. 2022), *Bacillus subtilis* (Hussain and Khan 2020d), etc. This is because the genus *Bacillus* is a group that is commonly found in environments contaminated with hydrocarbon compounds and, thus is highly tolerant under high levels of hydrocarbon compounds. The feature is due to the fact that members of *Bacillus* generate resistant endospores under stressful conditions (Alyousif et al. 2020). Santos et al. (2019) added that the genus *Bacillus* has a high level of resistance to various physical disturbances: such as wet and dry heat, UV and gamma radiation, and extreme environmental conditions.

Members of *Bacillus* generally produce lipopeptide-type of biosurfactants. The structure of lipopeptides is generally made up of heptapeptides and hydroxy fatty acid chains. The main classes of lipopeptides are surfactin, iturin and fengycin (Basit et al. 2018; Santos et al. 2019). Structurally, biosurfactants have a more complex structure than synthetic surfactants because they are composed of a combination of biomolecules such as proteins, carbohydrates and lipids. Biosurfactants produced by microorganisms are classified into several types based on the chemical structure that builds them, such as glycolipids, phospholipids, lipopeptides, neutral lipids, fatty acids, and polymeric biosurfactants (Alyousif et al. 2020; Sanches et al. 2021).

Unlike members from *Bacillus* and *Pseudomonas* which are known as the main producers of biosurfactants, the genus *Alcaligenes* is not a common genus although several studies have uncovered its potential in laboratory studies. *Alcaligenes faecalis* strains being a producer of biosurfactants have been reported by Powthong and Suntornthiticharoen (2018) isolated from soil and water in agricultural areas of Thailand. Salehizadeh and Mohammadizad (2009) reported that some native bacterial strains isolated from oil and mud samples in Iranian oil fields were also identified as *A. faecalis*. *Alcaligenes faecalis* was first discovered from stool samples, but now the genus *Alcaligenes* can be found in soil, water, insects, the environment contaminated with hydrocarbon compounds and human clinical samples. The genus *Alcaligenes* has now been widely used as a bioremediation and biocontrol agent (Duran et al. 2019).

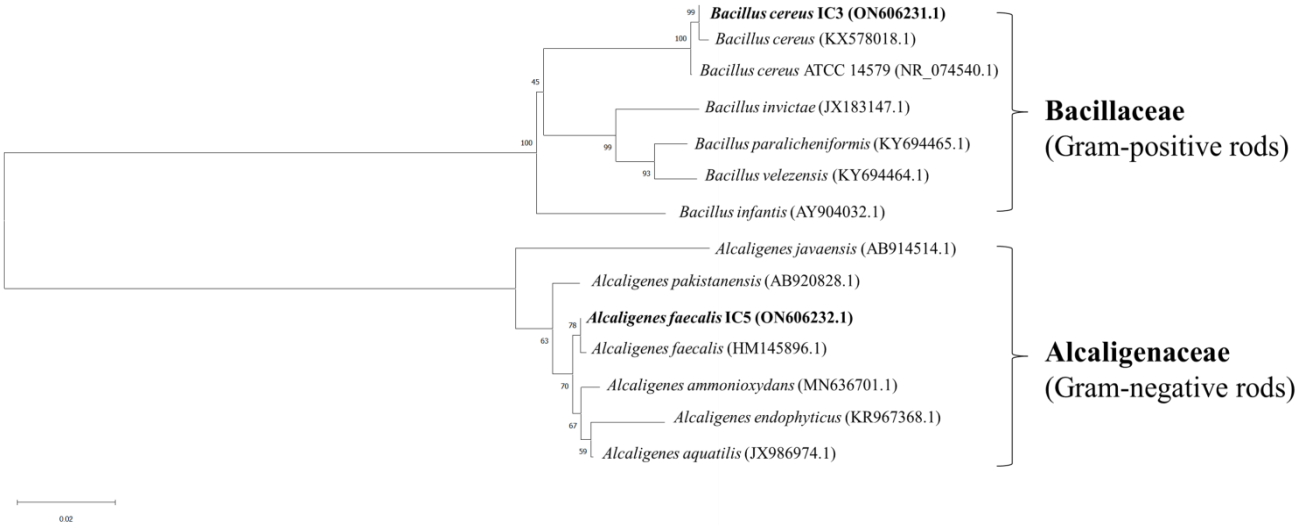

Figure 7. The neighbor-joining phylogenetic tree shows the genetic resemblance of potential phylloplane bacterial isolates (shown in bold) among other accessions retrieved from GenBank based on 16S-rRNA region. The tree was constructed using Kimura-2 parameter model with bootstrapping 1000×

In conclusion, an ornamental plant species, *C. esculenta* L, is studied for the presence of biosurfactant-producing bacteria in the phylloplane region. Screening of 25 bacterial isolates based on their colony growth on Bushnell-Hass Agar (BHA) supplemented with 1% olive oil as sole carbon source revealed four potential isolates namely IC1, IC3, IC4 and IC5. Two potential isolates later identified as *B. cereus* IC3 and *A. faecalis* IC5 were designated as the most prominent strains based on the characteristics of biosurfactant and concentration.

ACKNOWLEDGEMENTS

The authors wish to express their sincere thankfulness to the Laboratory of Microbiology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan, Indonesia in providing the analytical instruments and reagents for our study.

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