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[biodiv] Editor Decision

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Thank you for reminding me to submit our revised manuscript.
We have revised our manuscript according to your comments, please kindly check the attached file.
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The potential of indigenous bacteria from oil sludge for biosurfactant production using ~~hydrolysis product~~ ~~hydrolisate~~hydrolysate of agricultural waste

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Abstract. Biosurfactants are amphiphatic compounds which are useful in various fields of health, industry, and remediation. Biosurfactants are produced by bacteria that grow in hydrocarbon or sugar substrates. Hydrolysis product of agricultural waste can be used as a biosurfactant production medium. This research aims to obtain biosurfactant producing bacteria from Balongan oil sludge, Indonesia. The ability to grow and produce biosurfactant by indigenous bacteria was tested using a medium of Synthetic Mineral Water (SMW) added by 209.3 ppm of rice straw hydrolysis product (RSHP). The growth of bacteria was evaluated through Total Plate Count (TPC) and biosurfactant production was evaluated through measurement of emulsification activity and surface tension. Six indigenous bacteria capable to produce biosurfactants in the RSHP. Emulsification activity was not detected, but surface tension reduction was founded. The best biosurfactant was indicated by surface tension value of 53.56 mN/m with TPC value of 20.07 CFU/mL at the 5th day of incubation by BP(1) 5. The indigenous bacteria were identified as *Propionibacterium* BP(1) 1, *Propionibacterium* BP(1) 3, *Bacillus* BP(1) 4, *Corynebacterium* BP(1) 5, *Corynebacterium* BP(1) 8, and *Rothia* BP(1) 6. Utilization of sugar as hydrolysis product of agricultural waste is an innovation of raw materials for biosurfactant production.

Keywords: Agricultural waste, biosurfactant, indigenous bacteria, oil sludge, rice straw

Running title: Biosurfactant from rice straw hydrolyzate

INTRODUCTION

Biosurfactants are amphiphatic compounds which are useful in various fields of health, industry and remediation. Biosurfactants have low toxicity, easy manufacture, and wide application (Elazzazy et al. 2015). Biosurfactants are produced by bacteria that grow in hydrocarbon environments and can be secreted on the surface or outside cells in growth media with noticeable by surface tension reduction and emulsifying activities. (Kosaric 1993). Biosurfactant contains hydrophobic and hydrophilic molecules. Biosurfactant production by bacteria requires carbon nutrients, one of which is a sugar substrate.

The use of agricultural waste as a supplier of carbon sources in the form of sugar substrates as a result of hydrolysis has not been further exposed. The results of hydrolysis of agricultural waste can be used as a medium for biosurfactant production. Organic material in organic waste is a source of micronutrients (Wahyono 2011). The use of agricultural waste has been groundbreaking as an alternative material for biosurfactant production.Utilization of agricultural wastes (barley bran, trimming vine shoots, corn cobs, and *Eucalyptus globulus* chips) for biosurfactant production by bacteria have been done to reduce production costs (Moldes et al. 2007). Rice straw decomposing also have been successfully used for biosurfactant production by *Pseudomonas aeruginosa* BSZ-07 (Quizhuo et al. 2008).

One of agricultural waste that can produces hydrolyzing sugar is rice straw. According to the Indonesian Center for Rice Research, in the East Java region for March 2018, rice yields reached 2,026,739 tons (BB Padi 2018) and produced 12-15 tons per hectare of rice straw. Rice straw contains polysaccharides in lignocellulose. Lignocellulose is an abundant material, renewable resources, and less attention (Peralta et al. 2012). In rice straw, lignocellulose contains 32% cellulose, 24% hemicellulose, and 14% lignin (Chandel et al. 2007 in Novalina, 2014). Rice straw also produces 40-43% of carbons. The high cellulose and hemicellulose content can be used as a result of hydrolysis of rice straw and carbon for biosurfactant-producing bacteria. In this study, *Penicillium* sp. H9 was used to hydrolysis rice straw and itshydrolysis product called rice straw hydrolysis product (RSHP) was used as substrate for biosurfactant production.

Indonesia has a diversity of microorganisms that have the potential to be explored, one of which is a group of bacteria. Hydrocarbonoclastic bacteria in oil sludge are known to produce biosurfactants. Biosurfactant production by indigenous or soil bacteria has been carried out (Amani et al. 2011; Ni'matuzahroh et al. 2017; Wanet al. 2018). This study reveals the ability of indigenous bacteria to grow and produce biosurfactant and knowing the prospect of utilizing RSHP for alternative substrate for biosurfactant production.

MATERIALS AND METHODS

Procedures

Sample collection and bacteria isolation ~~*Microorganism handling and isolation*~~

Oil sludge was obtained from PT. Pertamina, Balongan, West Java, Indonesia.~~Microorganisms that used in this study are *Penicillium* sp. H9 obtained from Alas Purwo National Park and indigenous bacteria from PT. Pertamina, Balongan, West Java.~~ Ten grams of the oil sludge ~~samples collected from oil sludge~~ were added to 250 mL Erlenmeyer flasks containing 90 mL distilled water and homogenized with a shaker for 15 minutes. One mL of supernatants were put into a sterile Petri dish and then added 15 mL of Nutrient Agar. This Petri dish was incubated at 30°C for 24 hours on incubator. The bacteria isolates that have been obtained then characterized and purified.

Morphological and physiological characterization of bacteria

All isolated bacteria were characterized macroscopically, microscopically, and physiologically. Macroscopic characterization was observed by the morphological colony of form, color, elevation, edges, and consistency. Microscopic observation of the isolates were performed before 18 hours and after 24 hours to make sure the shape and the presence of Gram. Bacteria biochemical test was using Microbact Kit GNB 12A and 12B according to the protocol. Almost bacteria identified were included in the Gram-positive bacteria groups so that the species name was compared to the Bergey's Manual of Determinative Bacteriology 9th and Cowan and Steel's Manual for Identification of Medical Bacteria Third Edition.

Hydrolysis of rice straw by Penicillium sp. H9

The hydrolysis process was carried out enzymatically by *Penicillium* sp. H9, a microbial collection of Microbiology Laboratory of Universitas Airlangga which was obtained from Alas Purwo National Park. Organic agriculture waste, rice straw, was dried for two days to reduce the water content then delignified mechanically by the grinding process. Rice straw powder and dilute NaOH were mixed in a solid-liquid (1:10) for 60 minutes at 100ºC. The mixture was cooled to room temperature and washed to pH 7.0 with distilled water then dried to remove the water. Two gram of pretreatment rice straw was added to 100 mL media Mendel-Sternberg's and autoclaved at 121°C for 15 minutes. The media was cooled to room temperature and added 4% (v/v) spores suspension of *Penicillium* sp. H9 then incubated at shaker incubator for 6 days. Sample was filtrated through sterile filter unit (Minisart NML syringe filter 0.2 µm). The final filtrate was served as RSHP in the following experiments. The reducing sugar content was measured by the Somogyi-Nelson method.

Biosurfactant production

Media Synthetic mineral water used for biosurfactant production in this study was modified from Pruthi and Cameotra (1997). The composition of medium was the following (g/L): $(NH_4)_2SO_4$ (3.0 g/L), NaCl (10 g/L), $MgSO_4.7H_2O$ (0.2g/L), $CaCl_2$ (0.01g/L), $MnSO_4.H_2O$ (0.001 g/L), H_3BO_3 (0.001 g/L), $ZnSO_4.7H_2O$ (0.001 g/L), $CuSO_4.5H_2O$ (0.001 g/L), $CoCl_2.6H_2O$ (0.005g/L) dan $NaMoO_4.2H_2O$ (0,001 g/L). AMS buffer consists of (g/50mL): KH_2PO_4 (5 g), K_2HPO_4 (2.62047 g) and Fe (g/50mL) Fe_3O_4 (0.0006 g). The total culture volume was 20 mL and added 1.5 mLof rice straw sugar hydrolysis with the concentration 20~~2~~9.3 ppm. 2% (v/v) bacteria suspension was used for the culture. Microbial culture was incubated for 0, 1, 3, and 5 days in a rotary shaker 120 rpm at room temperature.

Detection of growth and biosurfactant product

Bacteria cultures that incubated during the incubation time were measured for TPC, surface tension (ST), and emulsification activity (EA). Samples of culture medium were centrifuged for 15 minutes at 6000 rpm. 10 ml supernatants were taken for surface tension analysis using Tensiometer Du-Nuoy, Surface tension value was stated in mN/m or dyne/cm. The emulsification activity was obtained from 1 ml of supernatant which added by 1 ml of kerosene, the mixture was homogenized with vortex for 2 minute, and the emulsification activity can be observed after 1 hour and 24 hours.

RESULTS AND DISCUSSION

Morphological and microscopical characteristics of bacteria

Six morphologically distinct bacteria colonies were isolated from Balongan's oil sludge. Isolates were identified macroscopically, microscopically, and physiologically. Macroscopic and microscopic characters are presented in table 1.

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Every colony displayed different characters (Figure 1), but microscopic characters of some bacteria showed the similarity of characters. Only one bacterium has rod-shaped and Gram-negative, BP (1) 6 isolates, and others bacteria were identified with rod-shaped and Gram-positive (Figure 2). Isolate BP (1) 4 have a longer rod-shaped compared with the others.

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Table 1. Macroscopic and microscopic characters of oil sludge indigenous bacteria from Balongan

Isolates	Macroscopic characters of colony					Microscopic characters of cell	
	Colour	Elevation	Edge	Shape	Consistency	Shape	Gram
BP (1) 1	White	Raised	Entire	Circular	Opaque	Rod	Positive
BP (1) 3	White yellowish	Raised	Entire	Circular	Opaque	Rod	Positive
BP (1) 4	White	Flat	Undulate	Irregular	Transluents	Rod	Positive
BP (1) 5	White	Flat	Entire	Circular	Transparant	Rod	Positive
BP (1) 6	White Bone	Flat	Undulate	Irregular	Transluent	Rod	Negative
BP (1) 8	Orange	Flat	Entire	Circular	Transparant	Rod	Positive

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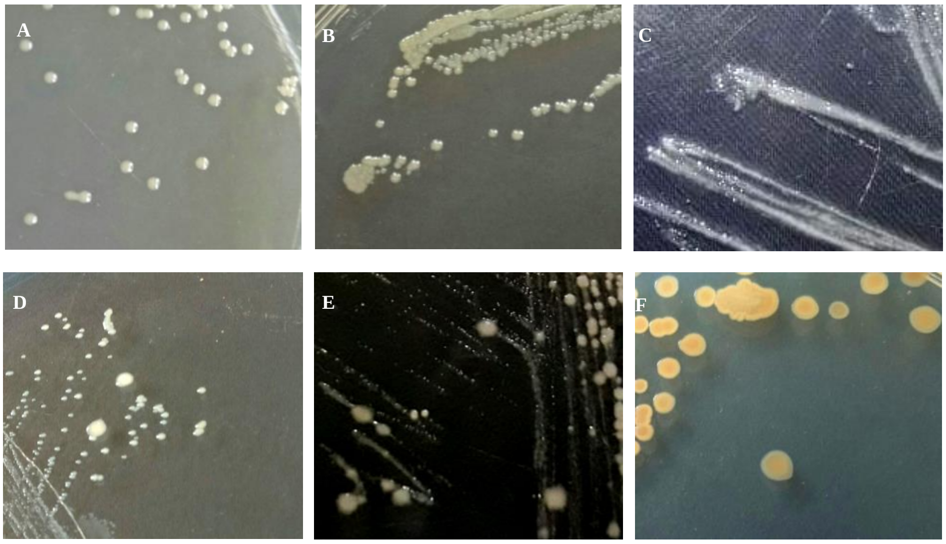
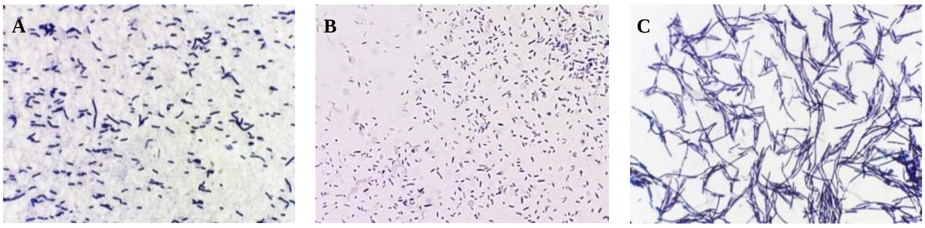


Figure 1. Macroscopic morphology of indigenous bacteria from Balongan's oil sludge. A is isolate of BP (1) 1; B is isolate of BP (1) 3; C is isolate of BP (1) 4; D is isolate of BP (1) 5; E is isolate of BP (1) 6; and F is isolate of BP (1) 8



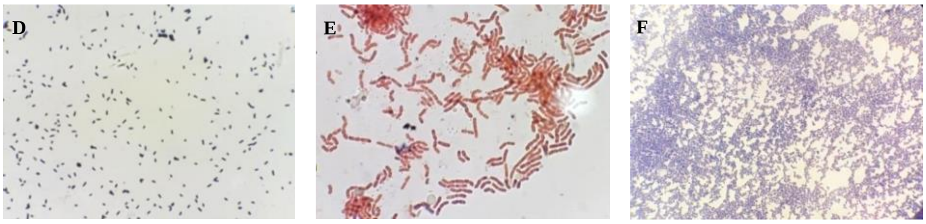


Figure 2: Cell of indigenous bacteria from Balongan's oil sludge. A is isolate of BP (1) 1; B is isolate of BP (1) 3; C is isolate of BP (1) 4; D is isolate of BP (1) 5; E is isolate of BP (1) 6; and F is isolate of BP (1) 8.

Physiological characteristic of bacteria

Based on differences in microscopic and macroscopic character, physiological characteristics were carried out to obtain the genus name. The physiological test of the bacteria is shown in a table 2.

Table 2. The biochemical test of bacteria using microbact GNB 12A and 12B

Type of test	Bacteria isolate code					
	1	3	4	5	6	8
Oxidase	+	+	+	+	+	+
Motility	-	-	-	-	-	-
Nitrate	+	+	-	+	-	-
Lysine	-	+	+	+	+	-
Ornithine	-	-	-	-	-	-
H2S	-	-	-	-	-	-
Glucose	-	-	+	-	-	-
Mannitol	-	-	-	-	-	-
Xylose	-	-	+	-	-	-
ONPG	-	-	-	-	-	-
Indole	-	-	-	-	-	-
Urease	+	-	-	-	-	-
VP	-	-	-	-	-	-
Citrate	-	+	-	+	+	-
TDA	-	-	-	-	-	-
Gelatin	-	-	-	-	-	-
Malonate	-	-	-	-	+	-
Inositol	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-
Lactose	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-
Salicin	-	-	-	-	-	-
Catalase	+	+	+	+	+	+
Arginine						
after 24 hours	-	-	-	-	-	-
after 48 hours	-	-	-	-	-	-

According to Jaccard's index (Real et al. 1996), the isolates were identified using similarity index (J) values. The isolates were identified as *Propionobacterium* sp. (isolate BP (1) 1) (J = 60%), *Propionobacterium* sp. (isolate BP (1) 3) (J = 60%), *Bacillus* sp. (isolate BP (1) 4) (J = 80%), *Corynebacterium* sp. (isolate BP(1) 5) (J = 75%), *Rothia* sp. (isolate BP (1) 6) (J = 75%), and *Corynebacterium* sp. (isolate BP (1) 8) (J = 51%).

Biosurfactant production

All isolated bacteria were able to grow and produce biosurfactants when grown on synthetic mineral water containing RSHP as a main carbon. This is evidenced by the growth response shown by the TPC are and the decrease in ST of the supernatant culture which indicates the production of biosurfactant. Value of TPC and ST are displayed in figure 2.

Growth response of bacteria had formed after first day incubation and increased continuously until the end of the incubation.This data shows that the addition of RSHP as a carbon source can be used by indigenous bacteria to grow.

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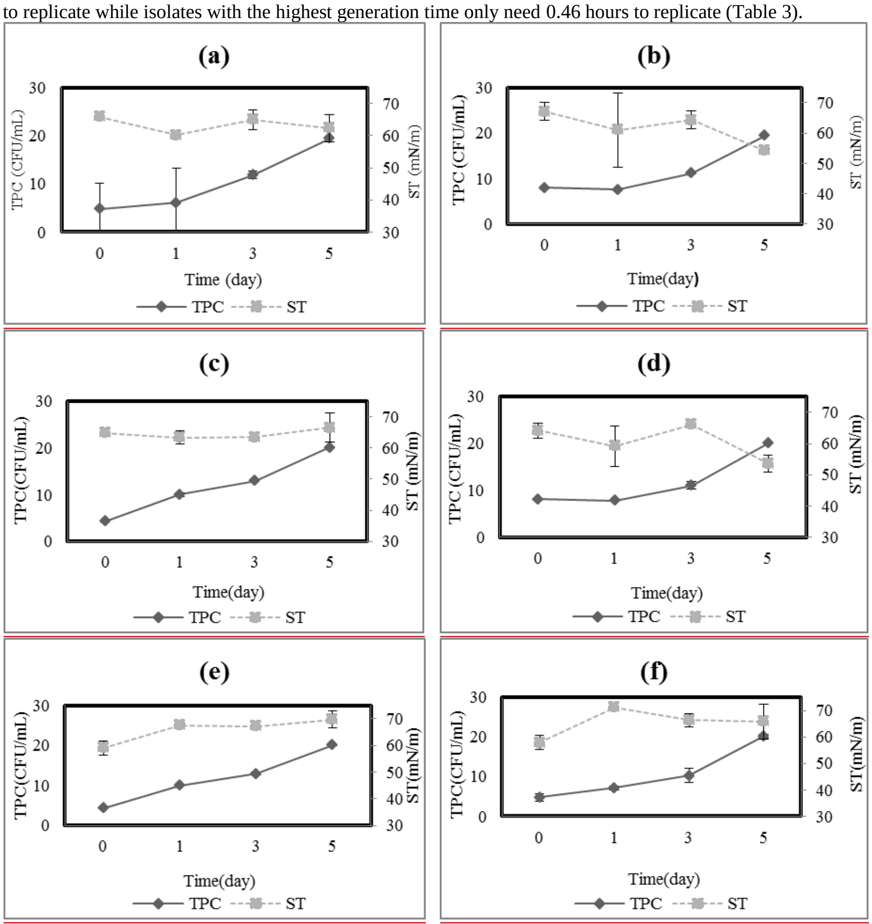
Biosurfactant production of isolates BP (1) 1, BP (1) 3, and BP (1) 5 tends increased from the first day of incubation to the end of incubation where all three bacteria are in the exponential phase.

The highest biosurfactant production was obtained on the fifth day incubation by isolate BP (1) 5 with a surface tension value of 53.56 ± 2.66 mN/m and 54.15±0.60 mN/m. The pattern of biosurfactant production by BP (1) 5 and BP (1) 3 shows that bacterial growth is in line with the quantity of biosurfactant product.The high value of surface tension is in line with the result of TPC values, but inversely proportional to the generation time of bacteria. Isolate BP (1) 5 takes 0.61 hours to replicate while isolates with the highest generation time only need 0.46 hours to replicate (Table 3).



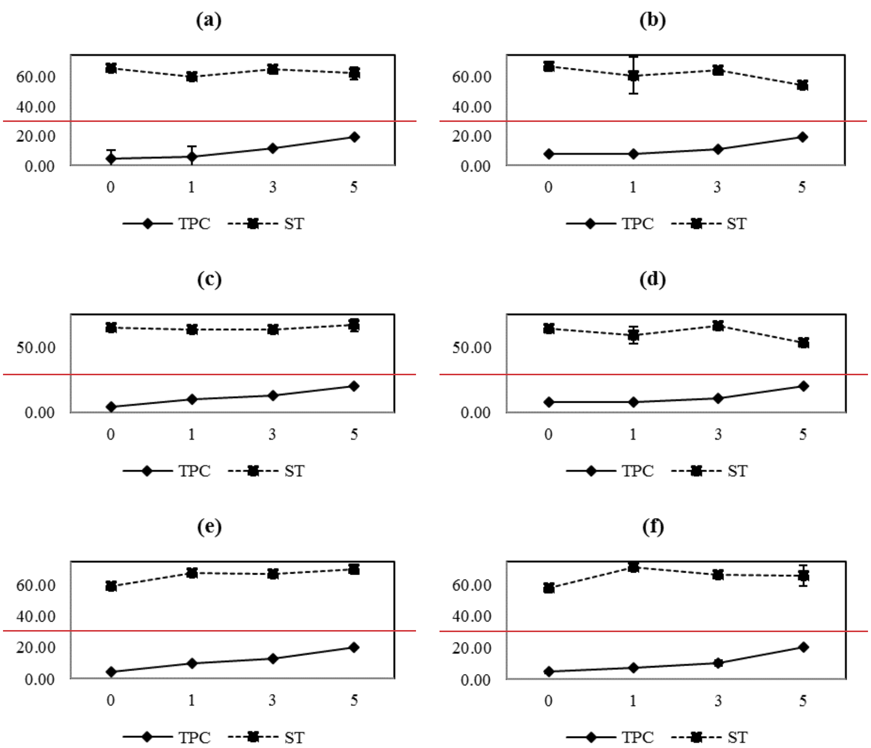


Figure 2. Total Plate Count and Surface Tension result of culture supernatant during 0, 1, 3 and 5 days incubation by **(a)** *Propionibacterium* sp. BP (1) 1, **(b)** *Propionibacterium* sp. BP (1) 3, **(c)** *Bacillus* sp. BP (1) 4, **(d)** *Corynebacterium* sp. BP (1) 5, **(e)** *Rothia* BP (1) 6, and **(f)** *Corynebacterium* sp. BP (1) 8 was using RSHP as carbon source

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Table 3. Growth rate, generation time, TPC and ST result in fifth day incubation time

Isolates	Growth rate (cell/hour)	Generation time (hour)	TPC (CFU/mL)	ST (mN/m)
BP (1) 1	0.41	2.47	19.42±0.02	62.30±4.31
BP (1) 3	0.32	3.15	19.48±0.02	54.15±0.60
BP (1) 4	0.44	2.28	20.12±0.05	66.52±4.61
BP (1) 5	0.33	3.03	20.07±0.04	53.56±2.66
BP (1) 6	0.44	2.30	20.08±0.02	69.72±3.09
BP (1) 8	0.43	2.34	20.22±0.46	65.80±6.62

Discussion

All isolated bacteria from Balongan's oil sludge were identified into four genera as *Propionobacterium* sp., *Bacillus* sp., *Corynebacterium* sp., and *Rothia* sp.based on the results of physiological tests using microbact 12A and 12B. Four genera were able to grow and produced biosurfactants when grown on sugar hydrolysis of rice straw. Generally, the type of biosurfactant that produced by *Propionobacterium* sp., *Bacillus* sp., *Corynebacterium* sp., and *Rothia* sp have potential in the process of hydrocarbon degradation. Several studies have reported similar results where the bacterial isolated from hydrocarbon contaminated areas was obtained *Bacillus*, *Corynebacterium*, *Propionibacterium*, and *Rothia* (Calvo et al. 2004; Sathishkumar et al. 2008; Sette et al. 2007; Yang et al. 2017). *Bacillus* produced surfactin, the type of biosurfactant which capable to emulsify the hydrocarbon. In addition, biosurfactant also have the potential as anti-microbial agents and inhibitors biofilm formation. The lipopeptide, biosurfactant types produced by *Bacillus* sp. strain SW9 that was capable of inhibiting biofilm formation in a wide range of bacteria.While, lipopeptide produced by *Propionibacterium freudenreichii* was known to inhibit *Pseudomonas aeruginosa* biofilm production (Wu et al. 2013; Hajfarajollah et al. 2014). The ability to inhibit biofilm formation is owned by Corixyn, the type of biosurfactant, produced by *Corynebacterium xerosis* strain NS. This biosurfactant disrupted biofilm preform of *S. aureus*, *S. mutans*, *P. aeruginosa*, and *E. coli* beside of that, it is also reported this ability as an anti-microbial agent (Dalili et al. 2015). Meanwhile, the type of biosurfactant from the genus *Rothia* has not been reported.

All of indigenous bacteria from oil sludge were able to grow and produce biosurfactant in RSHP. Reducing sugar in hydrolysate of rice straw has the following composition of 41-43% glucose, 14.8-20.2% xylose, 2.7-4.5% arabinose, 1.8% mannose, and 0.4% galactose (Maiorella 1985; Roberto et al. 2003). Biosurfactan production asssosiated with growth respon has been reported for isolate AB-Cr1, where the bacterial growth showed a parallel relationship with biosurfactant production and correlated with the glucose utilization (Ruzniza 2005). The result of this study is similar with Mulligan & Gibbs (1993), biosurfactant compound was a bacterial bioproduct that produced during exponential and stationer phases. Biosurfactant production was found paralleled to the highest reduction of surface tension which observed during the exponential growth of bacteria (Ruzniza 2005).

Six indigenous bacteria can grow and produce biosurfactants in the RSHP. The most potential isolate was *Corynebacterium* BP (1) 5 with TPC value of 20.07 CFU/mL and ST value of 53.56 mN/m. RSHP have the prospect to be an alternative substrate for biosurfactant production. Moreover, utilization of RSHP can reduce organic waste in the environment.

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Commented [Office9]: Composition of RSHP from this result?

ACKNOWLEDGEMENTS

Authors ~~Researcher~~ would like to thank Science and Technology Faculty of Universitas Airlangga who has funded the research through the Penelitian Unggulan Fakultas grant scheme in 2018 and Mr. Suwarni, S.Sos. for his aid during this research.

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[biodiv] Editor Decision

2 messages

Smujo Editors <smujo.id@gmail.com> Mon, Apr 15, 2019 at 7:57 AM
Reply-To: Smujo Editors <editors@smujo.id>
To: NI’MATUZAHROH NI’MATUZAHROH <nimatuzahroh@fst.unair.ac.id>

NI’MATUZAHROH NI’MATUZAHROH:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "The potential of indigenous bacteria from oil sludge for biosurfactant production using hydrolysis product of agricultural waste".

Our decision is: Revisions Required

Smujo Editors
editors@smujo.id

Reviewer _:
Recommendation: Revisions Required

Biodiversitas Journal of Biological Diversity

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nimatuzahroh nimatuzahroh <nimatuzahroh@fst.unair.ac.id> Mon, Apr 15, 2019 at 5:36 PM
To: Smujo Editors <editors@smujo.id>

Dear Smujo editorial team,

Thank you for reminding me to submit our revised manuscript.
We have revised our manuscript according to your comments, please kindly check the attached file.
Thank you very much.

Best regards,
Dr. Ni'matuzahroh and team
[Quoted text hidden]

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nimatuzahroh nimatuzahroh <nimatuzahroh@fst.unair.ac.id>

[biodiv] Editor Decision

2 messages

Smujo Editors <smujo.id@gmail.com> Sat, Apr 27, 2019 at 4:00 PM
Reply-To: Smujo Editors <editors@smujo.id>
To: NI’MATUZAHROH NI’MATUZAHROH <nimatuzahroh@fst.unair.ac.id>

NI’MATUZAHROH NI’MATUZAHROH:

The editing of your submission, "The potential of indigenous bacteria from oil sludge for biosurfactant production using hydrolysis product of agricultural waste," is complete. We are now sending it to production.

Submission URL: https://smujo.id/biodiv/authorDashboard/submission/3498

Smujo Editors
editors@smujo.id

Biodiversitas Journal of Biological Diversity

nimatuzahroh nimatuzahroh <nimatuzahroh@fst.unair.ac.id> Sun, May 5, 2019 at 7:02 AM
To: Smujo Editors <editors@smujo.id>

Thank you for your information

Best regards

Dr. Ni'matuzahroh and team
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