Utilization of Rice Straw Hydrolysis Product of Penicillium sp. H9 as A Substrate of Biosurfactant Production by LII61 Hydrocarbonoclastic Bacteria

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Submission date: 27-Apr-2021 10:47AM (UTC+0800)
Submission ID: 1570960650
File name: 3._Utilization_of_Rice_Straw_Hydrolysis-PS.pdf (1.02M)
Word count: 3956
Character count: 21301

IOP Conference Series: Earth and Environmental Science

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To cite this article: Ni'matuzahroh *et al* 2018 *IOP Conf. Ser.: Earth Environ. Sci.* **217** 012028

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Abstract. This study aims to reveal the prospect of rice straw as a biosurfactant production substrate by hydrocarbonoclastic bacteria. Rice straw can be hydrolysed enzymatically into simple sugar by *Penicillium* sp. H9. Hydrolysis products are used as growth medium and biosurfactant production by LII61 bacteria. The concentration sugar from hydrolysis product was analyzed by Nelson method. In this study, molasses were used as a comparison substrate in biosurfactant production. The growth response of LII61 bacteria was observed by measuring the turbidity of the culture. Biosurfactant products were evaluated by measuring the emulsification activity (%) and surface tension (mN/m). Acquisition of sugar from rice straw hydrolysis product (RSHP) was 209.25 µg/mL. The optimum growth both of RSHP and molasses substrates were obtained on the 5th day of incubation with a culture turbidity value of $OD_{\lambda 650}$ 0.201 and $OD_{\lambda 650}$ 0.157 respectively. The lowest surface tension obtained in culture of RSHP was 48.85 mN/m. It was better than biosurfactant product on the molasses substrate on the 3rd day incubation. However, during the incubation time both substrates did not show emulsification activity. Biosurfactants produced have certain characteristics on variations in pH and temperature.

Keyword: Rice Straw, *Penicillium* sp. H9, Biosurfactan, LII61 Hydrocarbonoclastic Bacteria

1. Introduction

Indonesia is a tropical country with most of its terrain is the agricultural area. Agricultural yields in Indonesia always leave agricultural wastes that are accumulating in the environment. Rice straw is one of the agricultural wastes in Indonesia. According to the Central Statistics Agency of Indonesia [1], rice production in 2015 amounted to 75.40 million tons with rice straw production reaching 12-15 tons per hectare per harvest. Rice straw contains polysaccharides in the form of cellulose, hemicellulose, lignin, and pectin [2]. Lignocellulose content in rice straw consists of cellulose by 32%, hemicellulose 24%, and lignin 14% [3]. The high content of cellulose in rice straw has the potential to be transformed by microorganisms into materials that can be reused.

The use of residual agricultural products is a breakthrough in handling environmental organic waste. Organic material in the agricultural waste can be a source of nutrition for microorganisms [4]. The enzymatic specifications possessed by microbes will determine the conversion of ingredients into desired products [5]. Hydrolysis is the first step in utilizing agricultural waste. Hydrolysis product is obtained from enzymatic alteration of organic waste, in which enzymatic hydrolysis have the advantage such as no toxic products [6], no corrosion and higher sugar production than acid hydrolysis [7]. According to Pratama [8], *Penicillium* sp. H9 has a cellulolytic activity which is able to hydrolyze cellulose. *Penicillium* sp. H9 has the potential as an agent to hydrolyze rice straw into sugar as a result of hydrolysis (reducing sugars), these products can be used as carbon sources and substrates for biosurfactant production.

Biosurfactants are amphipathic compounds produced by microorganisms. These compounds can reduce surface tension and emulsify hydrocarbons. Biosurfactants have the potential to be applied in

the oil industry, such as oil tank washing and processing of oil sludge waste [9] and for the health aspect which can be used as an antimicrobial compound [10]. Previous research shows that LII61 bacteria are able to grow and produce biosurfactants in sugarcane (molasses) waste substrates [11]. Molasses contain simple sugars like glucose, sucrose, and fructose which support bacterial growth. However, the presence of molasses is increasingly scarce as demand in the food industry increases. Rice straw hydrolysis product (RSHP) is one of the alternative substrates for biosurfactant production by bacteria.

Ni'matuzahroh et al, [12] has succeeded in isolating potential bacteria which decompose hydrocarbons known as hydrocarbonoclastic bacteria. These bacteria also have the potential to produce biosurfactants [11]. One strain of hydrocarbonoclastic bacteria that has activities to produce biosurfactants is LII61. According to Pratiwi [13], LII61 produces lipase as well as biosurfactant enzymes so as to reduce the surface tension of the growth medium. This study aims to determine the use of RSHP of *Penicillium* sp. H9 as a substrate of biosurfactant production by hydrocarbonoclastic bacteria LII61. Optimization and characteristics of biosurfactants were also carried out in this study. LII61 biosurfactant products on RSHP compared with biosurfactant products on molasses substrate. Utilization of RSHP for biosurfactant substrate is an effort to reduce agricultural waste.

2. Experimental Method

2.1 Microorganism

The bacteria used in this study was *Micrococcus* sp. L II (61) obtained from Pegirian slaughterhouse (RPH) Surabaya East Java which is a collection from the Laboratory of Microbiology, Faculty Science and Technology, Airlangga University, Surabaya. Bacterial isolates were grown in Broth Nutrient media and incubated using a shaker incubator at room temperature for 24 hours. The bacteria used was 2% (v/v) of the total culture volume of 0.4 mL at OD_{650} 0.5.

2.2 Hydrolysis of rice straw by Penicillium sp. H9

Organic agricultural waste, rice straw, obtained from local farmers. The rice straw is mechanically delignified by the grinding process and chemically delignified using 1% NaOH for 1 hour at 100 ºC. The pretreatment rice straw was enzymatically hydrolyzed by *Penicillium* sp. H9 for 6 days of incubation time. The results of hydrolysis was mechanically sterilized using a filter syringe and the sugar content was measured by the Somogy-Nelson method.

2.3 Screening of biosurfactant production by LII61 on RSHP substrate

Screening of biosurfactant production was carried out on Synthetic Mineral Water media (SMW) with the addition of 2% (v/v) RSHP. Media Synthetic mineral water used in this study is a modification from Pruthi and Cameotra (1997). The composition of synthetic mineral water are (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) and $NaMoO_4.2H_2O$ (0.001 g/L). SMWbuffer 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, microbial culture was incubated for 0, 1, 3, and 5 days in a rotary shaker 120 rpm at room temperature.

2.4 Optimization biosurfactant production by LII61

Optimization of biosurfactant production was executed with a variation of incubation time 0, 1, 3 and 5 days, incubation was carried out in a rotatory shaker 120 rpm at room temperature. The media used are synthetic mineral water (SMW) with the addition of RSHP substrate as much as 1.5 mL of rice straw hydrolysis with the concentration 229 ppm. The total culture volume used was 20 mL.

2.5 Detection of biosurfactant product from LII61

Bacterial cultures that were incubated during the incubation time measured for the final pH value. Bacterial cultures were centrifuged at 9000 rpm for 15 minutes and the supernatant that contains biosurfactant measured surface tension and emulsification activity.

Surface tension was measured with tensiometer Du-Nuoy, Surface tension value was stated in mN/m or dyne/cm and distilled water was used for control in this measurement. Surface tension was measured with this formula.

$$r = ro\frac{\theta}{\theta o} \quad (1)$$

Which are r = surface tension of sample, ro = surface tension of distilled water in t°C, θ = surface tension of surface sample that reads on the tool, and θo = surface tension of surface distilled water that reads on the tool

Emulsification activity was measured with 1 ml of supernatant from centrifugation. The centrifugation was carried out for 5 minutes with 3000 rpm. 1 ml of supernatant was added with 1 ml of kerosene. The mixture was compound with vortex for 2 minute, and the emulsification activity can be observed after 1 hour and 24 hours. Emulsification activity was observed by measuring the high of emulsy (cm) toward high liquid total (cm) multiplied 100% [14].

2.6 Extraction of biosurfactant from LII61

Crude biosurfactant was obtained with acid deposition. 1 Litre of supernatant from bacteria culture was incubated until 72 hours and centrifugation for 15 minutes with 6000 rpm. The supernatant from centrifugation was deposited with HCl 6N until the final pH was 2,0. Then, supernatant was incubated in the refrigerate for overnight and was centrifugated again to obtained the sediment of crude biosurfactant.

2.7 Characterization of crude biosurfactant extract from LII61 towards temperature and pH

Crude biosurfactant extract was characterized with CMC (Critical Micelle Concentration). CMC measurement was carried out by making various concentration from crude biosurfactant. The surface tension of various concentration of crude biosurfactant were measured with Tensiometer Du-Nuoy. The measurements were stopped if the critical point was reached, which is the value of surface tension was remained. The critical point was the CMC value of crude biosurfactant extract.

The measurement of stability from crude biosurfactant extract towards temperature change was obtained by making a various concentrations of crude biosurfactant extract at CMC value, and then the solutions were incubated to a various temperature (30 ºC, 50 ºC, and 70 ºC) for 1 hour. The solutions were measurement for surface tension and emulsification activity with kerosene. The measurement of stability of crude biosurfactant extract towards pH change was obtained by making a various concentrations of crude biosurfactant extract at CMC value, and then the solutions were incubated to a various pH (4, 7, and 10) for 1 hour. The solutions were measurements for surface tension and emulsification activity with kerosene.

3. Results and Discussion

3.1 Hydrolysis of rice straw by Penicillium sp. H9

The lignocellulose content in organic waste has the potential to be hydrolyzed into simple sugar using *Penicillium* sp. H9 . The cellulose, hemicellulose, and lignin contents of some organic wastes are shown in Table 1. The delignification process was needed to break the bonds between lignin and cellulose. The results showed the success of delignification was shown by an increase of cellulose, hemiselulose, and lignin levels in rice straw, 52.8%, 30.4% and 4.3% respectively. Rice straw hydrolized acquisition from the hydrolysis of rice straw by *Penicillium* sp. H9 was 199 µg / mL for 6 days of incubation. The rice straw hydrolized obtained that used as a growth substrate and biosurfactant production by LII61.

Tabel 1. Content of cellulose, hemiselulose and lignin in organic wastes

Organic wastes	Content (%)			References
	Cellulose	Hemicellulose	Lignin	
Palm oil	71.5	9.9	19.9	[15]
Wheat straw	38.2	21.2	23.4	[16], [17]
Bagasse	38.2	27.1	20.2	[16], [18]
Sorghum Biomass	22.2	19.4	21.4	[19]
Molasses	25.0	17.0	12.0	[16]
Rice straw	35.0	18.0	20.9	[20]
Rice straw	42.2	21.3	4.4	This work

Hydrolysis enzymatically will break the polymer chain specifically on certain branch while breaking the polymer chain with chemical and physical hydrolysis occur randomly [21]. *Penicillium* sp. H9 produces cellulase that play a role in the hydrolysis [22]. The mechanism of cellulose hydrolysis is through enzymatic (1) endo-β-1,4-glucanase breaks the β-1,4-glucoside bond randomly, especially amorf part of polysaccharide chains to produces cellobiose (2) ekso-β-1,4-cellobiohydrolase split of cellobiose unit from non-reduce polysaccharide chain terminal (3) β-glucosidase hydrolyze cellobiose and small chain of cello-oligosaccharide for producing glucose [23]. Concentration of rice straw, microbial enzyme, and environment condition influenced the hydrolysis product.

3.2 Screening of biosurfactant production by LII61 on RSHP substrate

Based on Fig. 1, enhancement of OD value and TPC were showed by LII61 during 5 days incubation (0.20 and 19.05 CFU/mL). The growth of LII61 suggested that RSHP can be a carbon source converted to energy for growth process. LII61 also showed existence of biosurfactant which was showed by lowering the surface tension value. The optimum lowering of surface tension was obtained in three days of incubation time amount 48,85 mN/m. Bioemulsifier from LII61 in the RSHP not detected because the concentration of biosurfactant is low.

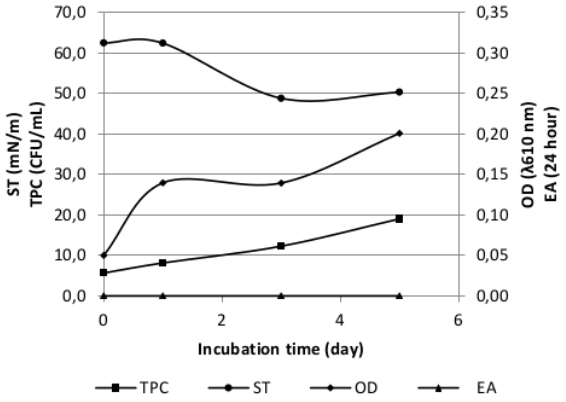

Figure 1. Screening of biosurfactant product by LII61 in rice straw substrate

3.3 Optimization of biosurfactant production by LII61

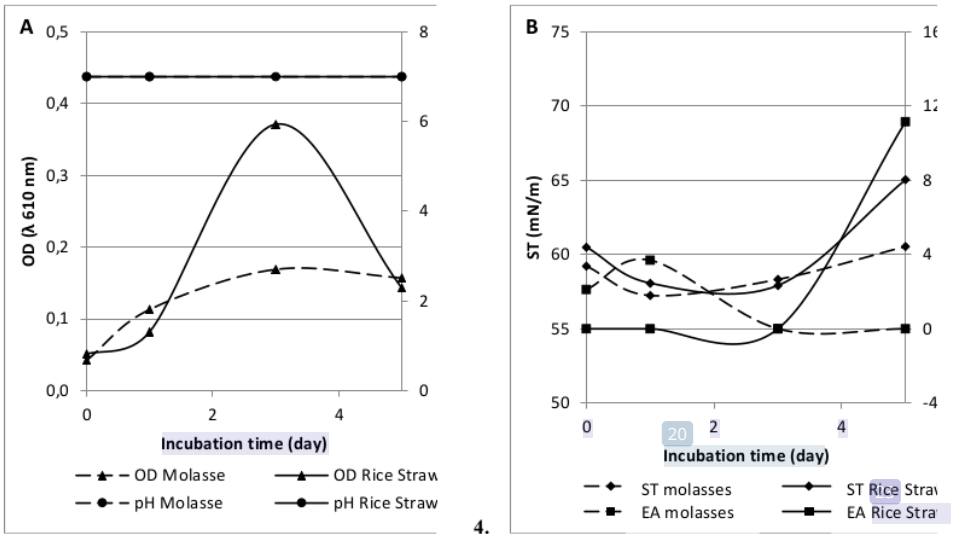

Figure 2. Optimalization of the biosurfactant production from LII61 in the RSHP substrate with molasses as control. A) growth and pH during incubation B) the activity of biosurfactant product

The results show that LII61 entered the log phase from the first day of incubation. In the molasses substrate and RSHP, LII61 showed a different response. The growth rate of LII61 on the RSHP was twice as fast as the molasses substrate at the same concentration (Fig. 2A). At the end of incubation, LII61 growth on RSHP substrates experienced a significant decrease. This shows the presence of cell death in culture. Whereas, in molasses culture, the decrease in cell counts did not differ significantly from the 3rd day of incubation. In this case pH changes were not found. Emulsification activity was detected in both substrates with the highest yield on RSHP substrate on the 5th day of incubation by 10.35% (Fig. 2B). Surface tension reduction was also detected on both substrates with significant differences. The optimum time for biosurfactant production of LII61 in the RSHP substrate is 3rd day incubation supported by the highest of number cell and the lowest of surface tention of culture supernatant. Similar with other bacteria, biosurfactant from *Corynebacterium lepus* are produced during exponential growth phase [24]. In this case, the increasing of emulsification activity in the 5th day incubation was affected by the lysis of cells. Utilization of RSHP as substrate have succesfull be a triger of LII61 to produce biosurfactant. This showed that at the same concentration, RSHP can be an alternative substrate to substitute molasses.

3.4 Extraction of biosurfactant from LII61

The amount of biosurfactant produced by LII61 in RSHP was 80 mg/L during 3 days incubation. The crude biosurfactant can reduce the surface tension from 72 mN/m to 53.3 mN/m (Fig. 3). The CMC value of extracted biosurfactant was 1000 mg/L. Based on the previous study, the CMC value of crude biosurfactant produced by LII61 in the molasses substrate was 6000 mg/L with the value of surface tension was 51.5 mN/m [25]. This discovery is better than molasses as substrate on biosurfactant production. At CMC, surfactant molecules aggregate and form micelles in polar or aqueous environment [26]. The value of surface tension unable to significally decrease at CMC.

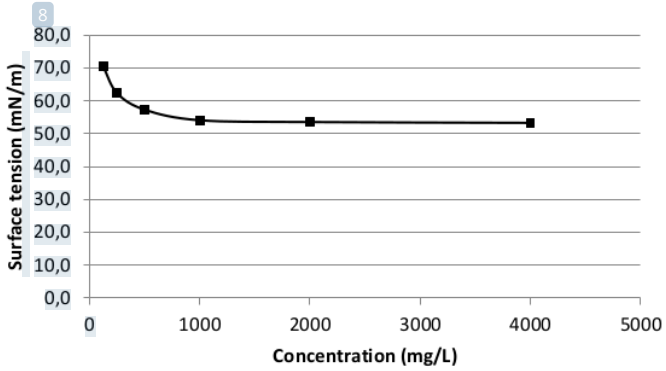

Figure 3. Critical Micelle Concentration (CMC) of crude biosurfactant in RSHP

Characterization of crude biosurfactant from LII61 towards temperature and pH

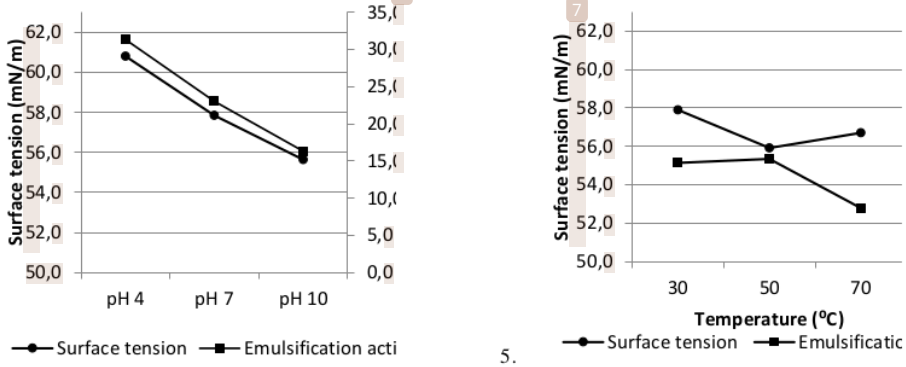

Figure 4. Characterization of crude biosurfactant from LII61 at various of condition A) pH B) temperature

Crude biosurfactant was tested at various pH and temperature. The surface tension value is showed at figure 4. The value of surface tension and emulsification activity at CMC of crude biosurfactant unstable in the various of pH. The surface tension decreased along with increased of pH (alkali) and the emulsification activity increased along with decreased of pH (acid). The optimum lowering of surface tension of LII 61 crude biosurfactant in the pH of 10 up to 55.6 mN/m approaching surface tension of CMC. This could be caused by a better stability of fatty acids surfactant micelles in the presence of sodium hydroxide and the precipitation of secondary metabolites at higher pH values [27]. The crude biosurfactant in the range of temperature 30°C - 70°C still show biosurfactant activies through emulsification activity and lowering surface tension. The crude biosurfactant capable to decrease the surface tension and show the emulsification activity in 30°C until 70 °C. The RSHP has the potential as substrate to produce biosurfactant from LII61.

4. Conclusions

The level of RSHP is 229 μg/mL in 6 days incubation time. RSHP can be used by LII61 to grow and produce biosurfactant as indicated by increasing of total plate count and optical density until 5 days. The best result of obtaining biosurfactant on RSHP are indicated by the surface tension 48.85 mN/m. Biosurfactants produced by LII61 have CMC value at 1000 mg/L and have the certain characteristic at

various pH and temperature. The use of rice straw hydrolysis product as a substitute for molasses in the manufacture of biosurfactant by LII61 shows good results and has favorable prospects.

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

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