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Biosurfactant Characterization of Bacterial Consortium from Soil Contaminated Hydrocarbon in Cepu Area, Central Java, Indonesia

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Abstract- Biosurfactant have been recognized as an important microbial products which was applicable in a number of industries and bioprocesses. In these study, characterization of biosurfactant producing bacterial consortium isolated from soil a hydrocarbon-contaminated area in Cepu Area, Central Java, Indonesia were conducted by measuring the growth of bacterial consortium, surface tension, interfacial tension, emulsification activity and its chemical composition. The results revealed that growth of bacteria exponential phase was reached at 24 hours no different growth pattern. The biosurfactant was a complex compound which consist of carbohydrate, protein and lipid. The emulsification used E24 of 93.75% unit was for bacterial consortium. The surface tension was reduced to below 51 dynes/cm from 72.00 dynes/cm (water surface tension) of bacterial consortium and the lowest interfacial tension values were obtained 10.00 dynes/cm.

Index Terms- Biosurfactant, characterization, bacterial consortium, surface tension, interfacial tension, emulsification activities.

I. INTRODUCTION

Biosurfactant was an important product of biotechnology today, especially for industrial and medical applications processes. It was also reported to have the ability as an antibacterial, antifungal, antitumor, antimycoplasmic and antiviral properties [1,2]. The mechanisms of biosurfactant has the ability to reduce surface tension and interfacial tension between two liquid phases are mixed. In nature in a wide range of chemical structures including glycolipids, lipopeptide and lipoproteins, fatty acids, neutral fats, phospholipids, lipid and lipid particulate polymer [1]. The chemical has the ability to stabilize emulsions, form and produce foam and non-toxic and can degrade other compounds in biological, naturally friendly to the environment, the ability of large-scale production, selectivity ability, the ability to exist in extreme conditions and potential applications for environmental protection [3,4]. In the biological compound produced in the extracellular amphiphilic or as part of various cell membranes of microorganisms such as bacteria, yeasts, and filamentous fungi [5,6]. The product was a secondary metabolite produced by microorganisms of various material substances include sugar, oil and waste [7].

Biosurfactant can provide a wider contact surface between the hydrocarbons and microorganisms through micelle formation, dissolution and emulsification of hydrocarbons and the release of droplets of a solution of non water from the pores of the solid particles due to surface tension reduction [8]. The properties are similar to synthetic surfactants and can be decomposed in the form of physicochemical properties such as critical micelle concentration (CMC), surface tension, interfacial tension and emulsion types that produced. Some of the biosurfactant showed a low CMC values and the reduction of surface tension in liquid fermentation approximately 30 mN/m.

Some advantages biosurfactant by [9,10] was to be biodegradable, low toxicity, biologically compatible and can be described as the application of cosmetics, pharmaceuticals and function as a food additive, available in rough material and production may be advantage economically, can also be used as a control environment and specific properties. A number of factors can affect the production of biosurfactant in nature including pH, salinity and temperature [11,12], agitation and oxygen availability [13], the availability of carbon substrate [14], the availability of nitrogen substrates [15], the source of carbon and nitrogen ratio (C/N) [16,17].

The purpose of this research is to characterize the biosurfactant produced isolates and bacterial consortium of the isolation of hydrocarbon-contaminated soil in the region of Cepu oil exploration, Central Java, Indonesia.

II. MATERIALS AND METHODS

A. Growth of Biosurfactant-Producing Bacteria

Four soil isolates (33, 34, 35, and 38 isolate) also a bacterial consortium (a combination of the isolates) obtained from the isolation in a hydrocarbon-contaminated area in Cepu Area, Central Java, Indonesia. Cultures were then stored at the Laboratory of Bioprocess Research Center of Biotechnology, Indonesia Institute of Science (LIPI), Cibinong Science Center. Study the growth of biosurfactant-producing bacteria started by growing colonies of bacteria on media nutrient agar (NA) at 37^0C for 48 hours. After incubation, morphologically different colonies were isolated to obtain the pure isolates culture. Each of these isolate were then grown in nutrient broth (NB) medium

with was added by Arabic Liquid Crude Oil (ALCO) as a carbon source at 37^0C and incubated for 72 hours.

B. Extraction of Biosurfactant

Biosurfactant extraction was done by pouring each of 250 ml of NB medium which contains the isolates (33, 34, 35, and 38 isolate) also the bacterial consortium aseptically into sterile Erlenmeyer, incubation for 5 days at 30°C, then centrifuged at 6000 rpm for 20 minutes at room temperatures to separate the bacterial cell. The supernatant was acidified up to pH 2 using HCl. The extraction from the supernatant using a solution of chloroform and ethanol with a ratio of 2:1. The bottom layer was extracted biosurfactant separated using a rotary evaporator at 40°C. The volume of each isolates and bacterial consortium are recorded and stored at a temperature -10°C to for further characterization.

C. Analysis of Carbohydrate Content

The presence of carbohydrate compounds was tested using a modified method of destruction rhamnose phenol-sulfuric acid [18]. A number of 0.5 ml of the supernatant was extracted with 0.5 ml of phenol solution and 2.5 ml sulfuric acid 5%, then incubated for 15 minutes, the absorbance was measured at 490 nm.

D. Analysis of Protein Content

The presence of protein compound was assayed using modified method of Bradford [19]. A number of 0.1 ml supernatant was mixed with 0.9 ml Bradford solution and then incubated for 15 minutes before the absorbance was measured at 595 nm.

E. Analysis of Lipid Content

The presence of lipid compounds was analyzed using modified method of blight and dyer [20]. A number of 1 ml of the supernatant solution was extracted with 20 ml of chloroform and then shaked using a vortex mixer for 1 hour. Chloroform evaporated and discharged while lipid content measured by analytical balance after drying in a hot plate at 50°C.

F. Emulsification Index (E24)

The ability of emulsification process was measured using the emulsification index (E24). Emulsification index of each isolate and a bacterial consortium was determined by adding 2 ml of oil Liquids Arabic Crude Oil (ALCO) and 2 ml of cell-free in nutrient broth medium in a test tube, then shaked using a vortex mixer at 6000 rpm for 2 min and allowed for 24 hours overnight. Emulsification index value (E24) was a high percentage of the emulsion layer (cm) divided by the total height of the solution (cm).

Percentage of emulsification index was calculated using the formula:

$$E24 = \frac{\text{High of emulsion formed} \times 100}{\text{High of total solution}}$$

[21,22].

G. Surface Tension Measurements

Supernatant which produced from the centrifugation process of isolates and bacterial consortium were measured the surface tension by Surface Tensiomat (Model 21, catalog no. 59951-14, Cole-Parmer), du Nouy ring method at 6000 rpm for 25 minutes. Supernatant which will be measured to be transfered to a clean beaker glass and placed on the sample table. Sample table was moved up to the position under ring of platinum-iridium exactly. Then sample table was carried up to the ring immersed in the supernatant. The ring must be wetted by supernatant approximately 1/8 inch below the surface. The scale will be read at the breaking point of the supernatant which was the value of surface tension.

H. Interfacial Tension Measurements

Interfacial tension measurement procedure the same as the surface tension measurements; an equal volume of ALCO and the supernatant was poured into a beaker glass with a diameter of 4 cm and the resulting mixture was used for the measurement of interfacial tension. Balancing analysis of Surface tensiomat readings done during rings of platinum-iridium was completely absorbed in the phase of the ALCO and not the surface or interface of ALCO-supernatant. Ring of platinum-iridium was absorbed then put into the surfactant phase till rings platinum-iridium is separated from ALCO-supernatant interface [23]. Scale will be read at the breaking point between the ALCO and the supernatant.

III. RESULTS AND DISCUSSION

A. The growth of bacteria producing biological surfactant

Isolate 33, 34, 35, and 38 also bacterial consortium had indicated that no different time grown pattern. The results of measurements of optical density (OD) each isolate and bacterial consortium were in Figure 1:

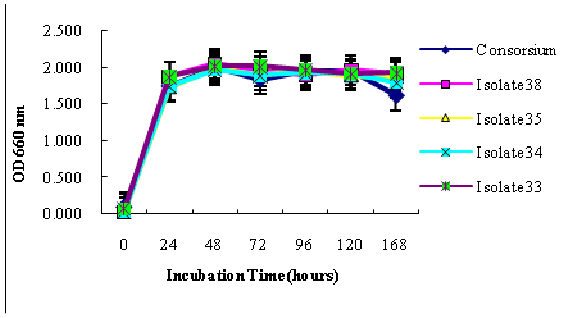

Figure 1: The growth pattern of isolates and bacterial consortium during incubation time

The results of measurement of OD on bacterial growth patterns showed that isolates and bacterial consortium achieve an exponential phase after incubation for 24 hours. After that, isolate 33, 34, 35, 38 and bacterial consortium enter the stationary phase up to 120 hours and finally to decline phase at 168 hours.

During the lag phase, increasing the cell size slowly. This phase is characterized by increasing macromolecular components, metabolic activity, and susceptibility to chemical and physical factors. Phase lag is a very important adaptation period to add metabolites in the cell group to achieve level of maximum cell synthesis [24].

In the exponential or logarithmic phase, cells were in a state of balanced growth. During this phase, mass and cell volume increases by the same factors in terms the average of cell composition and concentrations of metabolites are constant relatively. This phase was also characterized by the highness of cell proliferation rate that can be expressed by an exponential function naturally. Cell proliferation was determined by the intrinsic properties of bacteria and environmental conditions. In this case, there was a diversity of growth rate of isolates and bacterial consortium [25].

Based on figure 1, the stationary phase was a phase that resulting decrease in growth rate. It was begun by the accumulation of waste products, lack of nutrients, pH changes, and other factors which affect the growth culture,. During this phase, the number of living cells decline for different periods, culture cell population did not grow up, it began to occure swollen cell abnormally, or an aberration, a manifestation of unequal growth.

In the phase of population decline or death phase, nutrients of microorganisms growth is empty critically, occurred bacterial population decreased in number. In this phase, the current number of died cells are more than a life cell [26].

B. Analysis of carbohydrate

The result of carbohydrate in the biosurfactant shown in Figure 2:

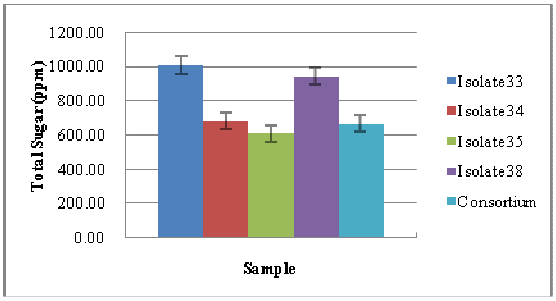

Figure 2: Average of total sugar content for each isolate and bacterial consortium

Analysis result of the average of total sugar content showed that four isolates (33, 34, 35, and 38 isolate) also a bacterial consortium have the ability to produce biosurfactant containing carbohydrate (total sugars) for 7 days of observation. The lowest total sugar content was found in the sample with treatment by isolate 35 (608.18 ppm) then followed by a bacterial consortium (666.36 ppm), isolate 34 (680.00 ppm), isolate 38 (940.30 ppm) and the highest is isolate 33 (1006.67 ppm). The use of ALCO compound as a carbon source for biosurfactant production by four isolates and a bacterial consortium are varies. This condition is related with emulsification activity during the incubation process [27].

Biosurfactant which produced by microorganisms has different of total sugar content, this is because of differences of the level of water solubility and availability of substrates for microorganisms. ALCO compound as a main substrate, in a particular concentration may be the effect of inhibiting the growth of microorganisms and biosurfactant production. The inhibitory effect was related with ALCO compound solubility and the difficulty level of four isolates and bacterial consortium to gain access to nutrients in the culture media. The types, qualities and quantities of biosurfactant production are affected by the form and the availability of carbon substrates as an energy sources and the growth of microorganisms [28].

Based on the description above, the linkage of esterification in ALCO compound on isolate 33 and isolate 38 were enables hydrolysis reaction to release the total sugar in the production of biosurfactant rapidly. Thus, it is known that isolate 33 and isolate 38 are more capable to oxidize components of ALCO compound in the production of biosurfactant to produce carbohydrate.

C. Protein analysis

Characterization of biosurfactant which produced by isolates (33, 34, 35, and 38 isolate) also bacterial consortium in the form of protein content using modified method of Bradford [18], shown in Figure 3:

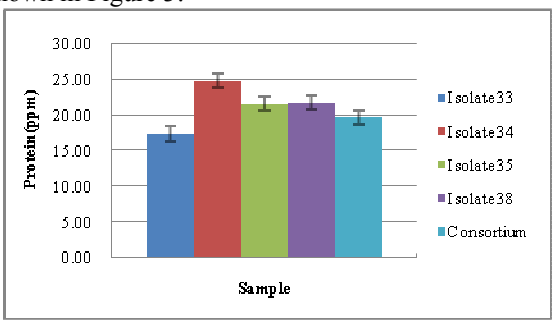

Figure 3: Average of protein content for each isolate and bacterial consortium

Analysis results of average of protein content showed that four isolates (33, 34, 35 and 38 isolate) also bacterial consortium are able to use the NH_3 compounds or nitrate or urea as a source of nitrogen for protein synthesis for cell growth.The availability of nitrogen sources as a macronutrient substrate in the growth of microorganisms was very important to obtain a high concentration of biosurfactant although every nitrogen sources was different in level of the effectiveness of protein synthesis [13].

Protein content in Figure 3, showed that the four isolates (33, 34, 35, and 38 isolate) and a bacterial consortium have the ability to produce biosurfactant. This was obtained from the production of biosurfactant by isolates and bacterial consortium which were distributed uniform containing protein. The lowest protein content was found in the sample with treatment by isolate 33 (17.44 ppm) then followed by a bacterial consortium (19.69 ppm), isolate 35 (21.57 ppm), isolate 38 (21.77 ppm) and the highest was isolate 34 (24.81 ppm).

The various of protein content on biosurfactant was caused by capability levels of nitrogen source conversion of each isolate and bacterial which are different in the transformation of one compound into another compound that can be utilized by isolates and bacterial consortium. This suitabiliy with reported by [13], that the source of nitrogen may be an important key for the regulation of biosurfactant synthesis.

It can be seen in Figure 3, that isolate 34 and isolate 38 are more able to transform nitrogen source into a protein compound in the biosurfactant synthesis compared with isolate 33, isolate 35 and a bacterial consortium. This condition was appropriate

with the statement of [28], that the types, qualities and quantities of biosurfactant produced was influenced by culture conditions such as pH, temperature, agitation, and the dilution rate as well as the culture itself is also influenced by the properties of the carbon substrate, concentrations of nitrogen, phosphorus, magnesium, iron, and manganese ions in the medium.

Relating to the availability of nitrogen as a constituent of protein compound in the biosurfactant synthesis, the high concentration of nitrogen (N) in a growth medium with hydrocarbons as the main source of carbon can cause toxic to hydrocarbonoclastic bacteria [29].

D. Lipid analysis

Biosurfactant production has been demonstrated successfully with the availability of water-soluble substrates, hydrocarbons and oils. The results of the analysis of lipid compounds in the characterization of biosurfactant of four isolates (33, 34, 35 and 38 isolate) and bacterial consortium shown in Figure 4:

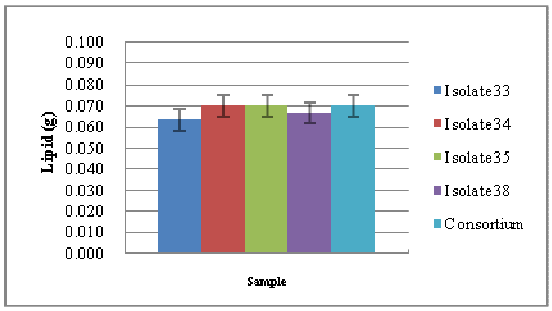

Figure 4: Average lipid content (g) for each isolate and bacterial consortium

Microorganisms utilize various organic compounds as carbon and energy source for their growth. When the source of carbon is not soluble substrates such as hydrocarbons (CxHy), microorganisms facilitate their diffusion into the cell by producing biosurfactant [13].

The results of the average lipid content analysis showed that four isolates (33, 34, 35, and 38 isolate) also a bacterial consortium have the ability to produce biosurfactant-containing lipids. The lowest lipid content was found in isolate 33 (0.070 g) then followed by isolate 34 and isolate 35 with similar lipid content (0.080 g) and the last isolate 38 (0.090 g). The highest lipid content was found on the bacterial consortium (0.120 g).

High and low lipid content is closely associated with the production of biosurfactant produced by isolates and bacterial consortium. Lipid content of the biosurfactant whose varies value is usually directly proportional to the ability of emulsification activity and surface tension reduction. The higher levels of lipid contained in the biosurfactant produced by microorganisms, usually is also higher the ability of emulsification activity and surface tension reduction [30].

Based on Figure 4, a bacterial consortium contain the highest lipid in its biosurfactant than isolates, it was believed to have strong relationship with its ability as an emulsifier and reduce surface tension. It also reported by [1], that types of biosurfactant are formed from the growth of microorganisms, greatly influenced by the ability of the microorganism itself to form emulsion and to reduce surface tension.

Bacterial consortium and isolate 38 have a higher lipid content than 33, 34 and 35 isolates although the source of carbon for their growth come from the same substrate (ALCO). Bacterial consortium were more able to adapt the growth process. This was apropriate with the reported by [31], that biosurfactant production was closely related with metabolism increase of microorganisms on hydrocarbon substrates (e.g. benzene, hexadecane, etc.). As a carbon source, hydrocarbons are non-polar substrates. Therefore, biosurfactant production may increase the bioavailability of non-polar substrates with emulsification properties.

E. Emulsification activity (E24)

The analysis result of emulsification activity of biosurfactant on four isolates (33, 34, 35 and 38 isolate) also bacterial consortium shown in Figure 5:

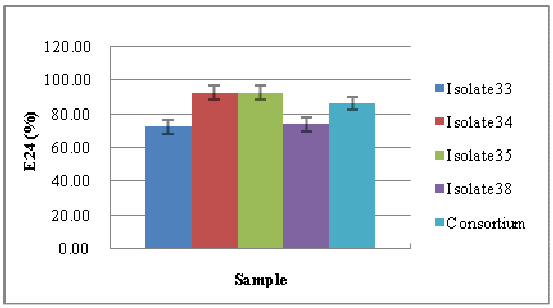

Figure 5: Percentage of emulsification activity for each isolate and bacterial consortium

The analysis result of percentage of emulsification activity showed that biosurfactant produced by bacterial consortium have the highest emulsification activity with emulsification index of 93.75% followed by isolate 34 and isolate 35 with emulsification index of 92.36%, isolate 38 with emulsification index of 73.61% and the last isolate 33 with emulsification index of 72.22%. In general, four isolates and bacterial consortium have the ability to optimize the absorption of hydrocarbons to produce biosurfactant through the mechanism of substrate emulsification until the contact between oil phase and water phase is to be larger.

It is also obtained that bacterial consortium, isolate 34 and isolate 35 showed a greater ability to produce an emulsion (oil droplet size is to be smaller) respectively. The ability of bacterial consortium and each isolate to form emulsion on the biosurfactant production process is influenced by many factors, as suggested [9]. Factors that affect the ability of bacteria in the emulsification process consists of : 1). Factors affecting the bacterial cell surface include : a) Species-specific genetic potential for cell wall biochemistry (hydrophobic, hydrophilic), b) Metabolic state of cell influencing expression of genetic potential involve of cell cycle (averaged in nonsynchronized cultures); availability of C, N, 0; Substrate form (e.g. sugars vs alkanes), c) Physical organization of cell wall biochemicals at the cell surface, d) Binding of extracellular surface-active molecules of biological origin to the cell surface, involve of secreted metabolic byproducts; products of cell autolysis; lipids extracted from cells by organic substrates; 2) Factors of nonbiological

origin influencing interfacial and rheological properties of the system include organic phase composition, aqueous phase composition (salts and pH), nature of emulsion-stabilizing surfactants, presence of fine particulates (e.g. clays) and temperature.

Emulsification properties of the surfactant was measured in terms of emulsification activity (EA) and emulsification stability (ES) [32]. EA was the ability of a surfactant to form emulsions under certain conditions and directly related to the oil droplet size, the smaller of the size was the greater of the activity. Naturally, the emulsions were thermodynamic unstability and the separation of a solution into the oil phase and the water phase [33].

Emulsion was formed when one of liquid phase is dispersed as microscopic droplets in another liquid phase. Emulsion activity is tested by the ability of biosurfactant to produce hydrocarbons suspension such as n-hexadecane [34] or kerosene [15] or a mixture of n-hexadecane and 2 - methylnaphthalene [35] and others in an aqueous test systems. Therefore, emilsificationi stability (ES) is a measure of slow/fast of an emulsion separate into oil phase and water phase rapidly.

F. Surface tension measurements

Analysis results of surface tension measurements on biosurfactant characterization produced by four isolates (33, 34, 35, and 38 isolate) also bacterial consortium shown in Figure 6:

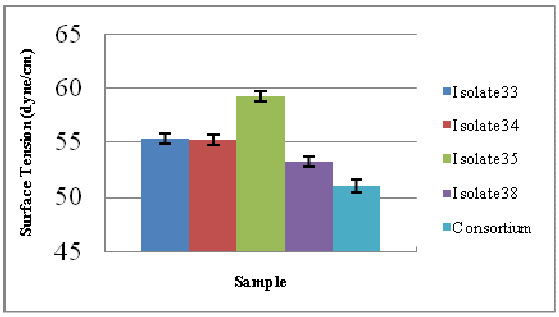

Figure 6: Surface tension value for each isolate and bacterial consortium

The highest of surface tension values was isolates number 35 (59.20 dyne/cm), the lowest surface tension values obtained in the sample with treatment by bacterial consortium of bacteria (51.00 dyne/cm), then followed by isolate 38 (53.20 dyne/cm). The low value of surface tension on bacterial consortium and isolate 38, indicating rapidly hydrolysis reaction to produce biosurfactant through the release of fatty acids and glycerol. Bacterial consortium and isolate 38 have more ability to adapt and oxidize hydrocarbons rapidly in the oil (ALCO) until resulting reduction of surface tension. [36] reported that one of the most important characteristics of the biosurfactant was the ability of a microorganism to reduce surface tension and interfacial tension between oil and water which can be measured by the method of du-Nouy ring classic. Surface tension values also showed that the biosurfactant produced was hydrophilic and can be used as a solvent agent, detergent and as an emulsifier between oil with water (O/W).

The results showed that most bacteria have an optimal capacity to degrade dissolved hydrocarbons compound through the mechanism of substrate emulsification and or dissolution process and or removal cell hidropobicity, so that, the cell can directly contact with the oil phase to produce biosurfactant [27]. Surface tension measurement would be the choice of method to quantify biosurfactant production. However, it gave an indirect relationship to the concentration of biosurfactant available in the liquid [1]. Reduction of surface tension was indicative to the production of biosurfactant by the microbe. The higher reduction of surface tension would lead to the high concentration of biosurfactant available [37].

However, biosurfactant were also occurred in the form of micelles at which point reduction of surface tension could not be determined anymore thus, the presence of increasing concentration of biosurfactant during prolonged incubation could not be measured [38].

G. Interfacial tension measurements

The results of interfacial tension measurements of the four isolates and bacterial consortium shown in Figure 7:

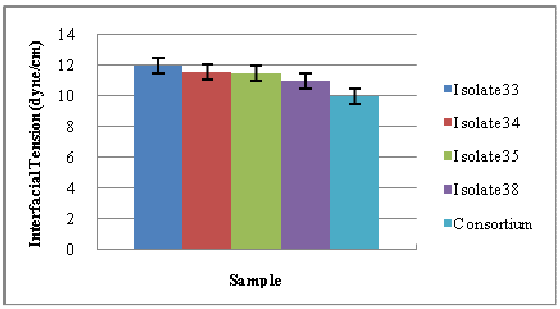

Figure 7: Interfacial tension value for each isolate and bacterial consortium

The result of analysis of interfacial tension showed that the lower value of interfacial tension was found in the samples with was treated by bacterial consortium (10.00 dyne/cm), then followed by isolate 38 (11.00 dyne/cm), isolate 35 (11.50 dyne/cm), isolate 34 (11.60 dyne/cm) and the highest is isolate 33 (12.00 dyne/cm). Reduction interfacial tension value indicates the ability of biosurfactant to enhance the contact between oil (ALCO) with supernatant by reducing the capillary tension between oil (ALCO) with supernatant until resulting mobilization of oil (ALCO) between the supernatant. In this case, a bacterial consortium have more ability to make contact between the oil with the supernatant.

Kaczorek et.al [39] reported that the values of surface tension and the interfacial tension on the characterization of biosurfactant depend on type factors: concentration of emulsifier, bioemulsifier, quantity and types of strain of bacteria, the complexity of hydrocarbons also products of emulsifier biodegradation. The influence of its resultant on the water can be expressed as a change in the surface tension and interfacial tension.

Analysis results of biological surfactant characterization of bacterial and consortium from the soil contaminated hydrocarbons in Cepu Area, Central Java, Indonesia can be summarized in the Table 1:

Table 1: Biosurfactant characterization of bacterial consortium from soil contaminated hydrocarbons in Cepu Area, Central Java, Indonesia.

Isolates	OD (48 hours)	Carbo hydrate (ppm)	Protein (ppm)	Lipid (gr)	Index of Emulsification (%)	Surface Tension (dyne/cm)	Interfasial Tension (dyne/cm)
33	2.004	1006.70	17.44	0.070	72.22	55.30	12.00
34	1.965	680.00	24.81	0.080	92.36	55.20	11.60
35	2.006	608.18	21.57	0.080	92.36	59.20	11.50
38	2.058	940.30	21.77	0.090	73.61	53.20	11.00
Consortium	2.075	666.36	19.69	0.120	93.75	51.00	10.00

IV. CONCLUSION

The results revealed that growth of bacteria exponential phase was reached at 24 hours. The biosurfactant was a complex compound which consist of carbohydrate, protein and lipid. The emulsification used E24 of 93.75% unit was observed for bacterial consortium. The surface tension was reduced to below 51 dynes/cm from 72.00 dynes/cm (water surface tension) of bacterial consortium and the lowest interfacial tension values were obtained 10.00 dynes/cm.

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