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Fungal Analysis of Aspergillus niger as an Alternative Biosurfactant for Microbial Injection-Enhanced Oil Recovery

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ABSTRACT - This study aims to evaluate the ability of Aspergillus niger to produce biosurfactants as a cost-effective and environmentally friendly alternative for the microbial-enhanced oil recovery (MEOR) process. Biosurfactants were produced using different carbon sources: waste cooking oil, liquid paraffin, and tapioca flour in Stone Mineral Salt Solution media. The growth and production of biosurfactants from these sources were analyzed through oil displacement tests, emulsification activity, and surface tension measurements. Tapioca flour emerged as the best carbon source, achieving the highest oil displacement area of 6 cm and an emulsification index of 51.3%. These findings suggest that biosurfactants obtained from Aspergillus niger have significant potential for MEOR applications, providing an eco-friendly solution for enhanced oil recovery.

Keywords: *aspergillus niger*, biosurfactants, liquid paraffin, MEOR, SMSS, carbon sources.

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INTRODUCTION

Microbial-enhanced oil recovery (MEOR) is a method of increasing oil recovery from old wells using microorganisms (Alpentri et al. 2001). It can produce several compounds that have the potential to increase oil recovery, such as gases, acids, polymers, and biosurfactants (Laini et al. 2014). One of the potential microorganisms is *Aspergillus niger*, a fungus that is capable of producing biosurfactants. This study focuses on the production of biosurfactants using *Aspergillus niger* fungi with the addition of various carbon sources, such as used cooking oil, liquid paraffin, and tapioca flour, to find environmentally friendly and economical alternatives to the MEOR process ((Septihanny et al. 2020; Nugroho 2006;Banat et al. 2000).

Silva et al. (2019) showed that *Aspergillus niger* fungus isolated from *Piper hispidum* plants can produce biosurfactants by adding soybean oil to its growth medium. Their study further revealed that the Aspergillus niger fungus isolates could produce biosurfactants that were quite significant in reducing

surface tension. The initial and final surface tensions were 68.4 dyne/cm and 44 dyne/cm, respectively. The objectives of this study are as follows: 1). To determine the growth of *Aspergillus niger* fungal isolates as an alternative MEOR material with the addition of carbon sources to the growth medium; 2). To determine the effect of biosurfactants produced by *Aspergillus niger* fungal isolates on the oil displacement zone through an oil displacement test; 3). To determine the emulsification ability of biosurfactants produced by *Aspergillus niger* fungal isolates; and 4). To determine the decrease in the surface tension value of biosurfactants produced by *Aspergillus niger* fungal isolates (Kadarwati & Herlina 2008).

METHODOLOGY

Carbon sources: Waste cooking oil, liquid paraffin, and tapioca flour.

Media composition: Stone Mineral Salt Solution (SMSS) media containing ammonium nitrate, manganese dichloride tetrahydrate, calcium chloride, sodium phosphate dibasic heptahydrate, magnesium sulfate heptahydrate, and potassium dihydrogen phosphate.

Analysis tools: Du-Nouy Tensiometer, centrifuge, and incubator (Gozan et al. 2014); (Afiati et al. 2014).

For the job analysis process, can be seen the flow Figure 1 below.

RESULT AND DISCUSSION

Aspergillus niger fungus growth media

In this study, pure isolates of *Aspergillus niger* fungus were grown in two media: solid media and liquid media. The solid and liquid media each have the same composition: 0.5 gr NH4NO3, 0.1 gr MnCl24H2O, 1 gr CaCl2, 0.2 gr Na2HPO47H2O, 0.1 gr MgSO47H2O, 0.1 gr KH2PO4, and 200 ml Aquadest (L. Welan & Mauboy 2019). The only difference between the two is the addition of 2% agar to the solid media as a solidifying agent. The fungal growth media used in this study is SMSS. As mentioned in the preceding section, the SMSS media contains water and mineral salts, which are required for the growth and development of mushrooms.

SMSS media is classified as a synthetic growth media in which the composition of chemical compounds is obtained with a formulation of known dosage and type (Subekti et al. 2009). SMSS media for the growth of *Aspergillus niger* fungi can be made in two forms: solid SMSS media and liquid SMSS media.

Factors affecting fungal growth

Substrat

(Junaini et al. 2019)*Aspergillus niger* fungus can produce amylolytic enzymes whose functions are similar to those of the α-amylase and glucoamylase enzymes (Junaini et al. 2019).

Humidity

Aspergillus niger fungus can grow in a lower humidity environment compared to rhizopus or mucor fungus, which requires an environment with 90% relative humidity. *Aspergillus niger* fungus can live at 80% relative humidity (Abdullah et al. 2018).

Temperature

Aspergillus niger is classified as a mesophilic fungus because it can grow at optimum (35ºC–37ºC), minimum (6ºC–8ºC), and maximum (45ºC–47ºC) temperatures (Yunasfi et al. 2020).

Environmental acidity degree (pH)

Fungi generally prefer environments with a pH below 7, although certain fungi can grow in environments with a pH of 45.5. The degree of acidity of the environment needs to be known, because some enzymes can only decompose substrates at a certain pH (Abdullah et al. 2018). In this study, the pH of the liquid medium for the growth of *Aspergillus niger* fungi was 4.

Solid SMSS Media

The growth of *Aspergillus niger* fungus on solid SMSS media with each carbon source occurred at different times. With used cooking oil as the carbon source, fungal growth began on the second day, while with tapioca flour and liquid paraffin as the carbon sources, fungal growth became noticeable on the third day.

Waste cooking oil as a carbon source

Used cooking oil is a good source of carbon for the growth of *Aspergillus niger* fungus. Figure 2 shows the growth of fungi on solid SMSS media with waste cooking oil as the carbon source**.**

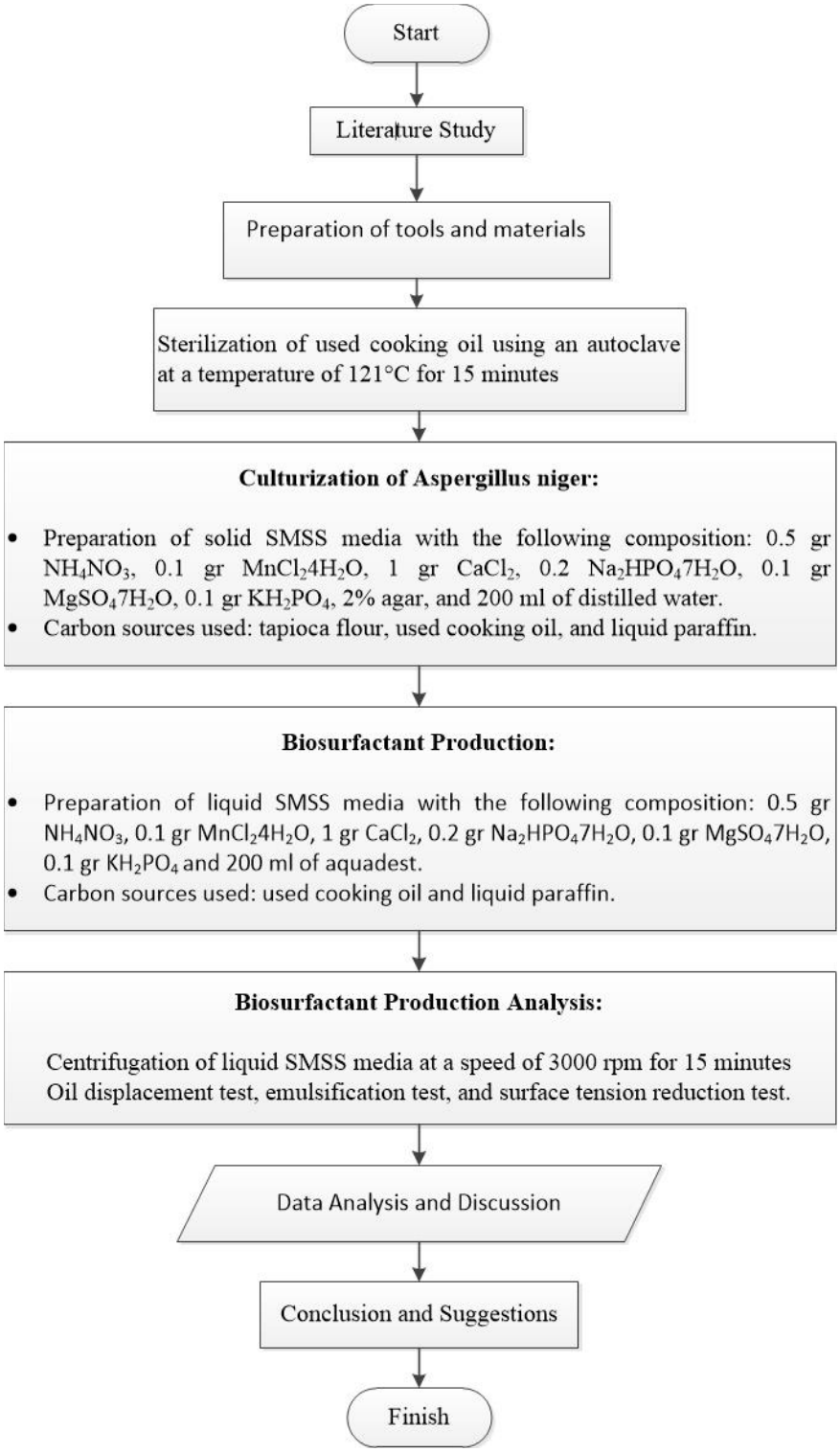

Figure 1
Flow chart

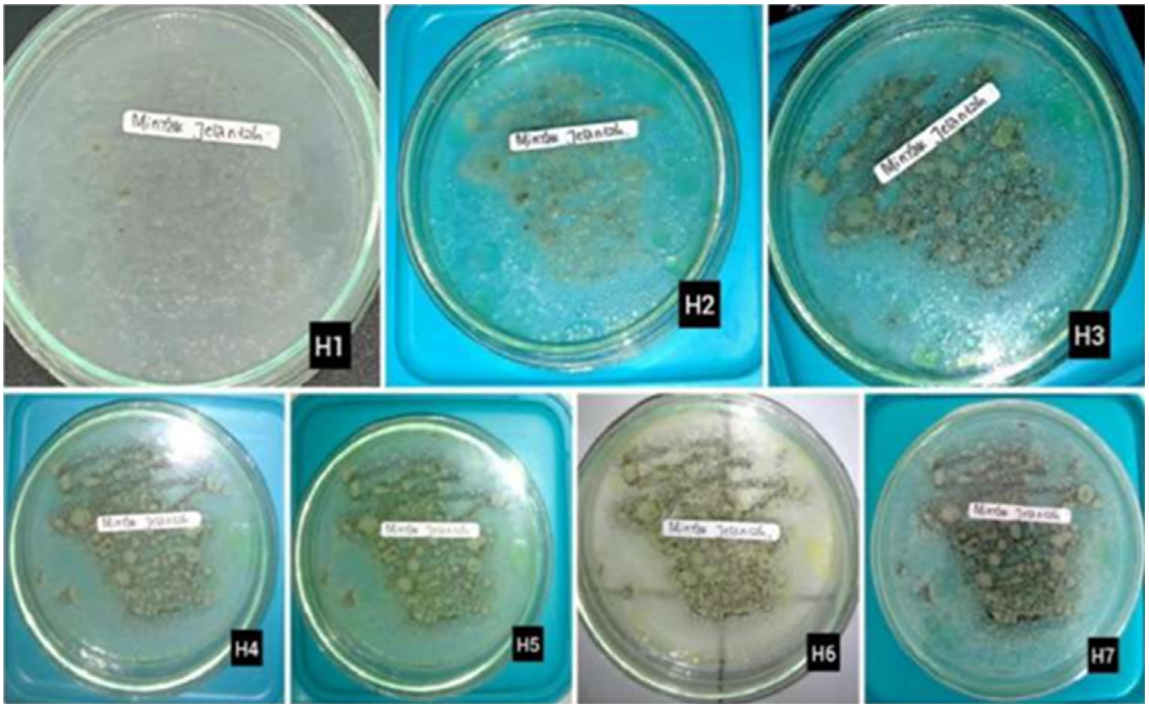

Figure 2
Growth of *Aspergillus niger* fungus on solid SMSS media with waste cooking oil as the carbon source over 7 days

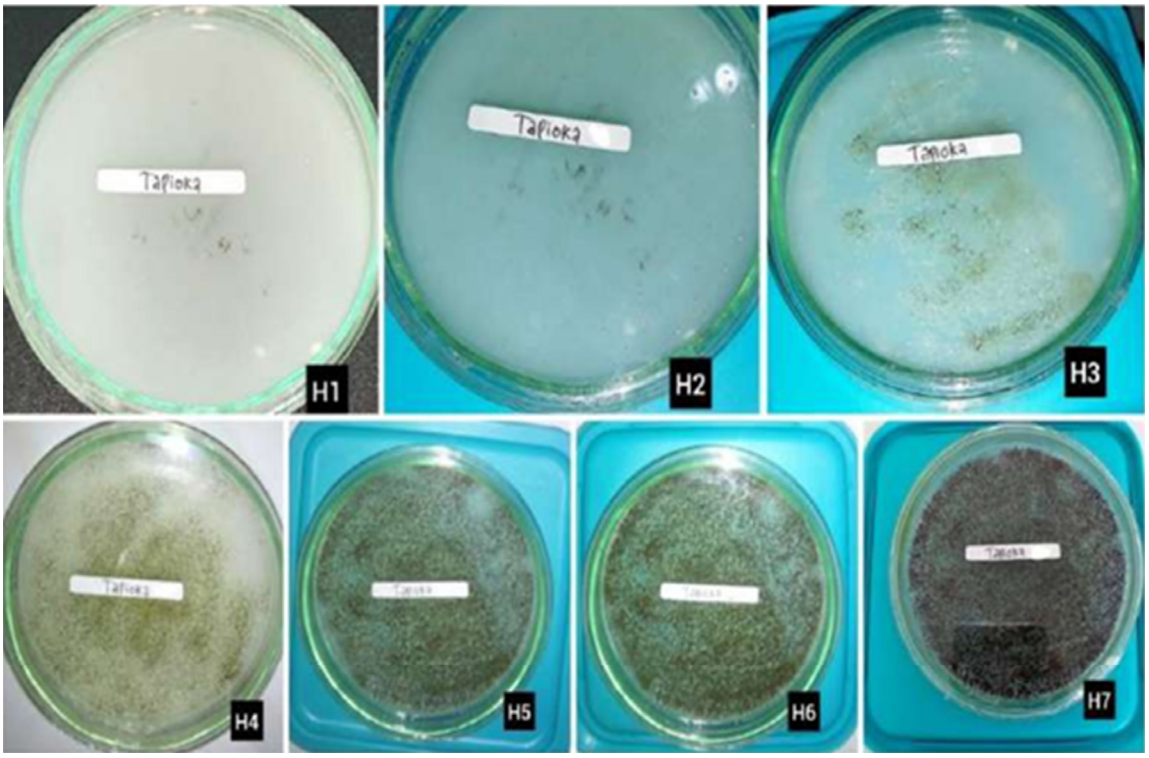

Figure 3
Growth of *Aspergillus niger* fungus on solid SMSS media with tapioca flour as the carbon source over 7 days

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The free fatty acids contained in the used cooking oil are long-chain saturated fatty acids (Sopianti et al. 2017). Saturated fatty acids contain 8–22 carbon atoms (Marlina & Ramdan 2017). Therefore, the number of fungal growths in solid media with used cooking oil as a carbon source is second only to that with tapioca flour.

Tapioca flour as a carbon source

Tapioca flour or cassava flour is a good source of nutrition for the growth of mushrooms (Askari, 2018). Figure 3 shows the growth of fungi on solid SMSS media with tapioca flour as the carbon source over seven days**.**

Tapioca flour, as a major source of starch, also has other contents, such as protein, vitamins (especially vitamin B), and minerals (Aswardi et al. 2020). Tapioca flour is the carbon source that produces the most *Aspergillus niger* fungus on solid media, followed by used cooking oil and then liquid paraffin.

Liquid paraffin as the carbon source

Figure 4 shows the growth of *Aspergillus niger* fungus on solid SMSS media with liquid paraffin as the carbon source.

Liquid paraffin contains 10–16 carbon atoms (Nugroho 2006). It is a good carbon source, but not better than tapioca flour and used cooking oil because both carbon sources contain more carbon atoms than liquid paraffin. In this study, *Aspergillus niger* fungus grown on solid media can grow well at room temperature, so it does not need to be placed in an incubator.

Liquid SMSS media

The liquid SMSS media has the same composition as the solid SMSS, as well as the carbon source used, except that agar is not added as a solidifying agent for the liquid SMSS media. The pH value of the liquid SMSS media was also the same as that of the solid SMSS media, which was four. The fungi used in this second stage are the same as those grown in the first stage. This is done to prove whether fungi that grow on solid SMSS media can grow on liquid SMSS media with the same composition of chemicals and carbon sources. The growth of fungi in liquid SMSS media is characterized by the appearance of objects such as cotton or small white fibers floating in the media, not only white but also blackish brown. The cotton-like objects are single conidia or spores that have grown into the mycelium. Figure 4 shows the growth of *Aspergillus niger* on the liquid SMSS media with liquid paraffin as the carbon source and fungi from the solid media with the same carbon source over 14 days. On the 14th day, the solution becamee cloudier than the previous days, no color change was observed, the solution remained white, fungi grew and floated in the media in the form of very small black and white cotton, and undissolved sediment from the chemicals used at the beginning of making the liquid media was observed at the bottom of the solution.

Figure 6 shows the growth of *Aspergillus niger* fungi on liquid SMSS media containing liquid paraffin as the carbon source and fungi taken from the solid media with tapioca flour as the carbon source. On the 14th day, the solution became increasingly cloudy and brownish yellow, fungi shaped like brown and black cotton grew and floated in the media, and fungi also settled at the bottom of the solution.

Figure 7 shows the observations made on the liquid SMSS media with used cooking oil as the carbon source and fungi from the solid media with tapioca flour as the carbon source. On the 14th day, the solution became yellowish and slightly cloudier than the initial solution, which looked clearer, and lumps and foam of used cooking oil were mixed with dark brown fungi on the top of the liquid media and the walls of the Erlenmeyer flask.

Figure 8 shows changes in the solution in the liquid media with used cooking oil as the carbon source and fungi from the solid media with the same carbon source. On the 14th day, the solution was bright yellow and became cloudier than the previous days, and lumps and bubbles of used cooking oil in golden yellow were observed on the top of the solution and on the left and right sides of the Erlenmeyer flask.

The growth of microorganisms in liquid and solid media affects the production of biosurfactants. The adaptation phase in this study occurred on days 1–2. After the adaptation phase, the cells entered the exponential phase, in which they continued to grow at maximum speed. The exponential phase in this study occurred on days 3–10, and after the cells reached the maximum growth rate, the growth of fungal cells entered the stationary phase, in which the number of fungal cells remained constant. The stationary phase occurred on days 11–13. After the stationary stage, the number of cells decreased; this phase is called the death phase. The death phase occurred on day 14.

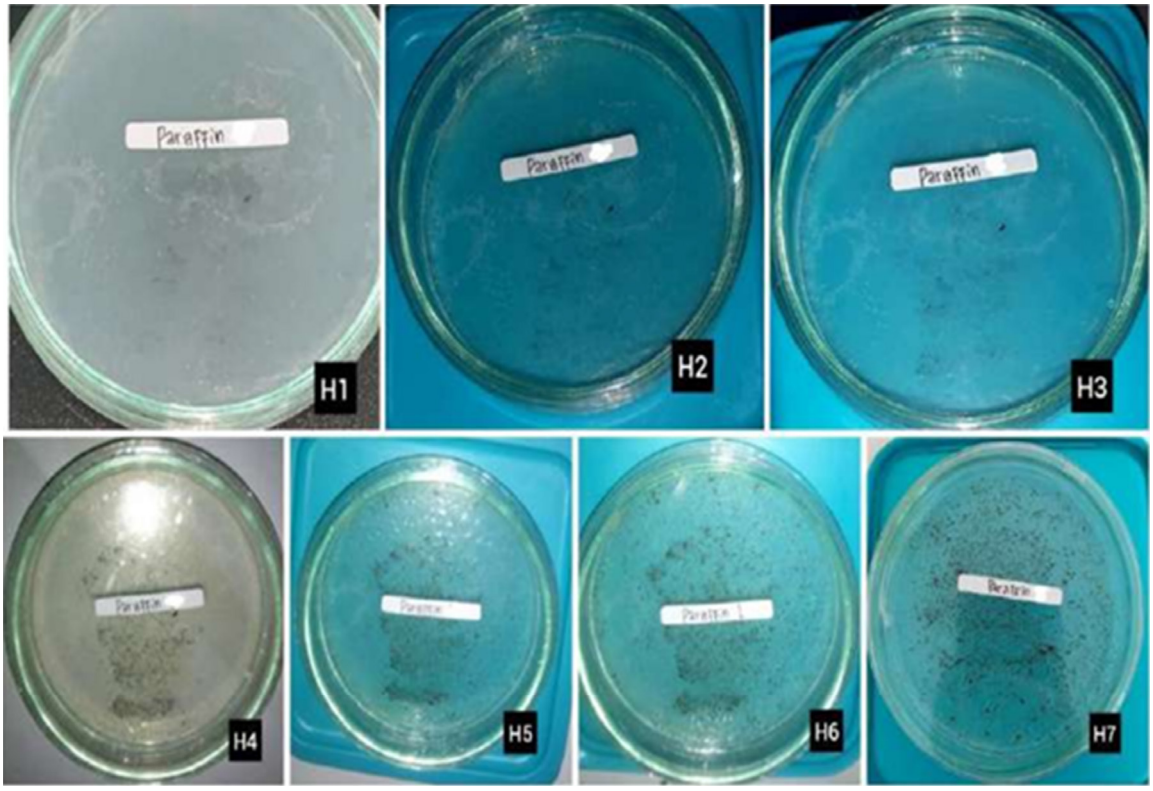

Figure 4
Growth of *Aspergillus niger* fungus on solid SMSS media with liquid paraffin as the carbon source over 7 days

Figure 5
Growth of *Aspergillus niger* fungus on liquid SMSS media with liquid paraffin as the carbon source + fungus from solid SMSS media with liquid paraffin as the carbon source over 14 days

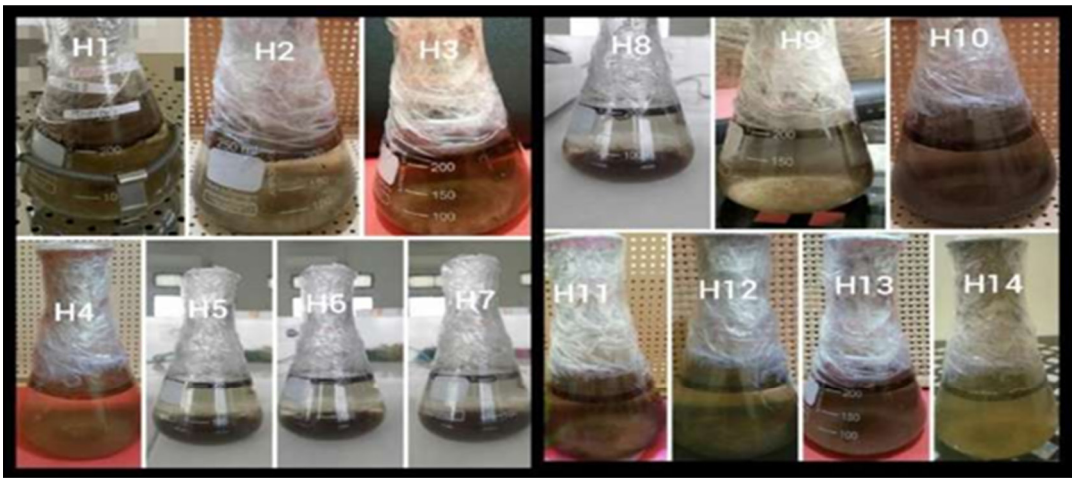

Figure 6
Growth of *Aspergillus niger* fungus on liquid SMSS media with liquid paraffin as the carbon source + fungus from solid SMSS media with tapioca flour as the carbon source over 14 days

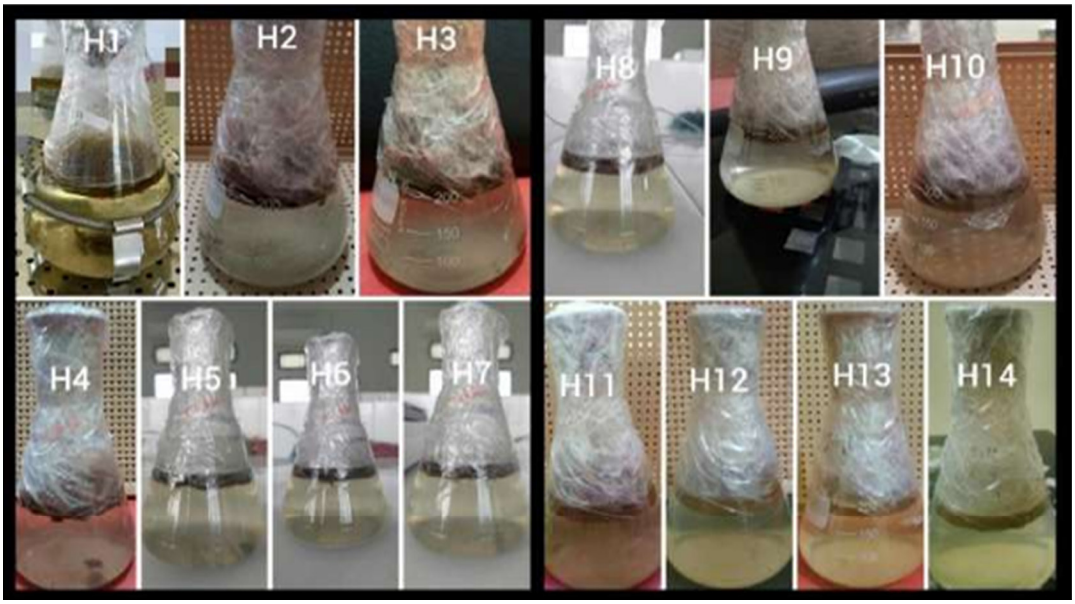

Figure 7
Growth of *Aspergillus niger* fungus on liquid SMSS media with waste cooking oil as the carbon source + fungus from solid SMSS media with tapioca flour as the carbon source over 14 days

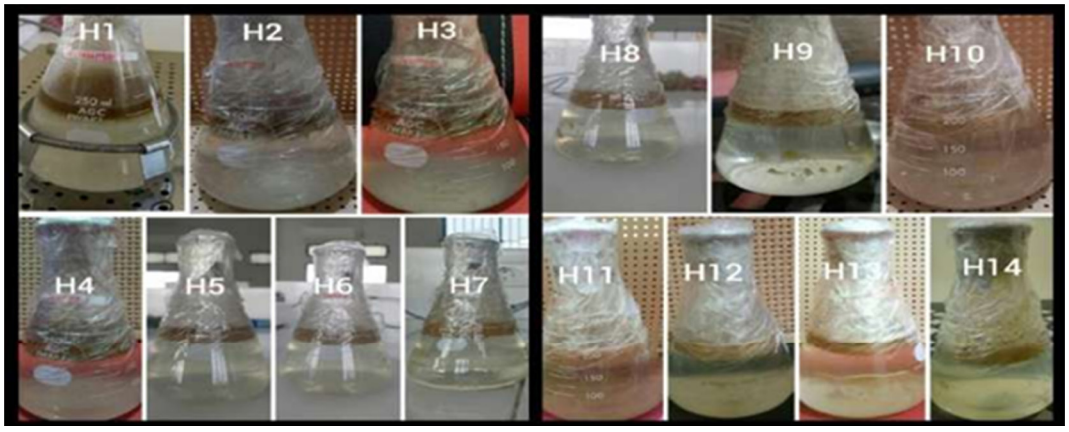

Figure 8
Growth of *Aspergillus niger* fungus on liquid SMSS media with waste cooking oil as the carbon source + fungus from solid SMSS media with waste cooking oil as the carbon source over 14 days

Figure 9 schematically shows the relationship between substrate utilization, cell growth, and biosurfactant formation (Ruzniza, 2005; Gozan et al. 2014).

Microscopic structure of *aspergillus niger* fungus

The characteristics of *Aspergillus niger* fungus are a yellow or white base color; a thick layer of conidiospores; round and large conidial heads; purple-brown, black, or black-brown in color; brown, green, or black conidial chains; branched mycelium; and septate hyphae (Wuryanti 2008).

The structures that are clearly visible in *Aspergillus niger* fungus with used cooking oil and tapioca flour as carbon sources are round black conidia and long white flower stalk-like conidiophores, respectively. The structure of *Aspergillus niger* fungus seen in a digital microscope is in accordance with the characteristics mentioned in (Wuryanti 2008).

Biosurfactant production analysis

In this study, biosurfactant production was carried out at room temperature (25°C–30°C), based on the statement of Kitamoto et al. (2001) in (Rengga et al., n.d.), which states that most biosurfactant production has been carried out at temperature variations of 25°C–30°C. The biosurfactant was produced by centrifuging the liquid SMSS media that contains fungi from the solid SMSS media and carbon sources. Centrifugation was carried out for 15 min with a centrifuge and at a rotation speed of 3,000 rpm at room temperature. The supernatant produced from the centrifugation process was put into a vial bottle to make it safer and more secure for emulsification activity tests, oil displacement tests, and surface tension reduction tests. Figure 11 shows the centrifugation results of the four samples.

The quantity and quality of the biosurfactants are influenced by the ability of the microorganisms to use carbon sources from their growth substrates. This can be seen in the different emulsification activities, the size of the clear zone value formed during the oil displacement test, and the ability of the biosurfactants to reduce the surface tension of the culture (Hamida 2010).

Biosurfactant production in a liquid medium by *Aspergillus niger* fungus with the addition of used cooking oil and liquid paraffin as carbon sources can be said to be successful even though it does not produce biosurfactants of very good quality. The quality of the biosurfactant produced by *Aspergillus niger* fungus with the addition of used cooking oil and liquid paraffin as carbon sources in its liquid growth medium is proven by the results of the emulsification, surface tension, and oil displacement tests conducted in this study.

Oil displacement test

Figure 12 **s**hows the results of the oil displacement test conducted in this study. The formation of a clear zone indicates that each supernatant is capable of producing a biosurfactant (Rahayu & Prasetyo 2020). In this study, the best biosurfactant was produced from the liquid SMSS medium with liquid paraffin as the carbon source and the addition of fungi from the solid SMSS medium with tapioca flour as the carbon source. This is evidenced by the oil displacement area (ODA) value formed from the supernatant produced from the media. The ODA value is one of the criteria for determining the best microorganisms and carbon sources to be used as biosurfactant production agents (Rahayu & Prasetyo 2020).

The oil displacement test in this study showed positive results with the formation of a clear oil zone or oil displacement zone. The presence of a clear oil zone is directly proportional to the presence of active surface compounds of the biosurfactants in the solution, as shown in Figure 12. Table 1 shows the ODA values from the oil displacement test results. The highest ODA value was given by the biosurfactant in sample 4, which was 6 cm, followed by the biosurfactant in sample 1 (4.1 cm), sample 2 (3.6 cm), and sample 3 (2.4 cm). In a study conducted by (Praharyawan et al., (2013), the biosurfactant produced by *Bacillus sp.* with used cooking oil as the carbon source showed good oil displacement test results with an ODA value of 5.95 cm. This shows that the good or bad quality of a biosurfactant is also related to the microorganisms used, even with the same carbon source.

Emulsification activity test

Table 1 shows the results of the emulsification test conducted in this study. Sample 4, a biosurfactant obtained from a liquid medium with liquid paraffin as the carbon source and fungi added from a solid medium with tapioca flour as the carbon source, has the largest emulsification index value of 51.3%. The second largest emulsification index was that of sample 3, with a value of 28%. Sample 3 is a biosurfactant produced in a liquid medium with liquid paraffin as the carbon source and the addition of fungi from a solid medium with the

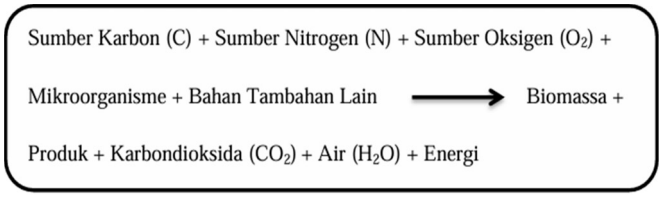

Figure 9
Schematic of the relationship between substrate utilization, cell growth, and biosurfactant formation
(An Awaludin & Sari 2017)

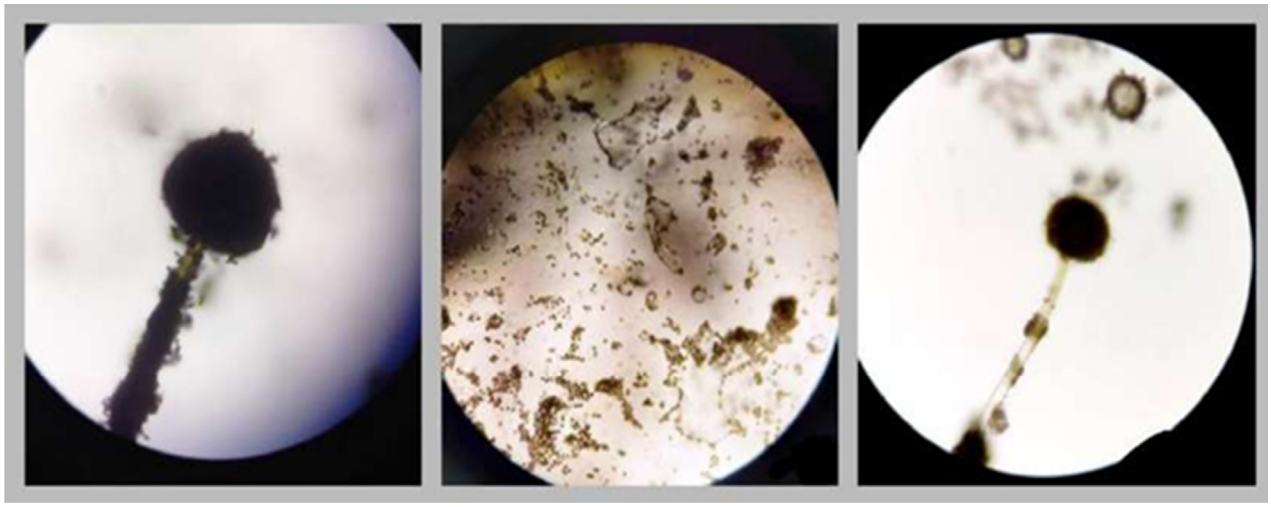

Figure 10
Microscopic structure of *Aspergillus niger* fungus with different carbon sources

Figure 11
Secondary metabolite products from the liquid SMSS medium after centrifugation

same carbon source. Samples 1 and 2 had the same emulsification index value of 13%. Samples 1 and 2 are the biosurfactants produced in the liquid medium with waste cooking oil as the carbon source and the addition of fungi from the solid medium with waste cooking oil as the carbon source (sample 1) and tapioca flour (sample 2).

Description:

(-) = No emulsification occurs
(+/-) = Emulsification occurs slightly
(+) = Emulsification occurs moderately
(++) = Emulsification occurs often

The best biosurfactant was produced by sample 4, which is evident from its ability to produce emulsions in the emulsification test with kerosene. Previously, an oil displacement test was carried out, and the results showed that sample 4 produced biosurfactants with better capabilities than samples 1, 2, and 3. Biosurfactant production was more optimal in sample 4 because the fungus used came from a solid medium with a carbon source of tapioca flour, which has a high carbon content (Askari 2018), so that it is used optimally by *Aspergillus niger* for cell growth. In the liquid medium, liquid paraffin was added as the carbon source and was used more for biosurfactant production (Zajic et al. 1977; Sheehan1997; Nugroho 2006).

The low emulsification index produced indicates the poor quality of the biosurfactant. Figure 13 shows the results of the biosurfactant emulsification test with kerosene.

Surface tension test

The method used for the surface tension testing in this study is the Du-Nouy Tensiometer method. The working principle of the Du-Nouy Tensiometer is to release a ring immersed in a liquid with a force that is proportional to the surface tension and interfacial tension. The force required to release the ring, in this case, is given by a torsion wire (Juliyanto et al. n.dn.d.).

Table 1 shows the results of the surface tension measurements using the Du-Nouy ring method.

The results of the surface tension test in Table 1 show a decrease in the surface tension in sample 1 from 63.5 dyne/cm to 63.3 dyne/cm, sample 2 from 63.5 dyne/cm to 63.2 dyne/cm, sample 3 from 65.8 dyne/cm to 61.6 dyne/cm, and sample 4 from 53.5 dyne/cm to 52 dyne/cm. The decrease in surface tension in each sample occurred because the *Aspergillus niger* fungus culture medium produced biosurfactants that had surface-active properties. Biosurfactants can reduce the surface tension between two phases, because their molecules tend to be toward both phases, so they can mix with both phases. The two phases cannot dissolve in each other because they have different polarities (Najiyah et al. 2013).

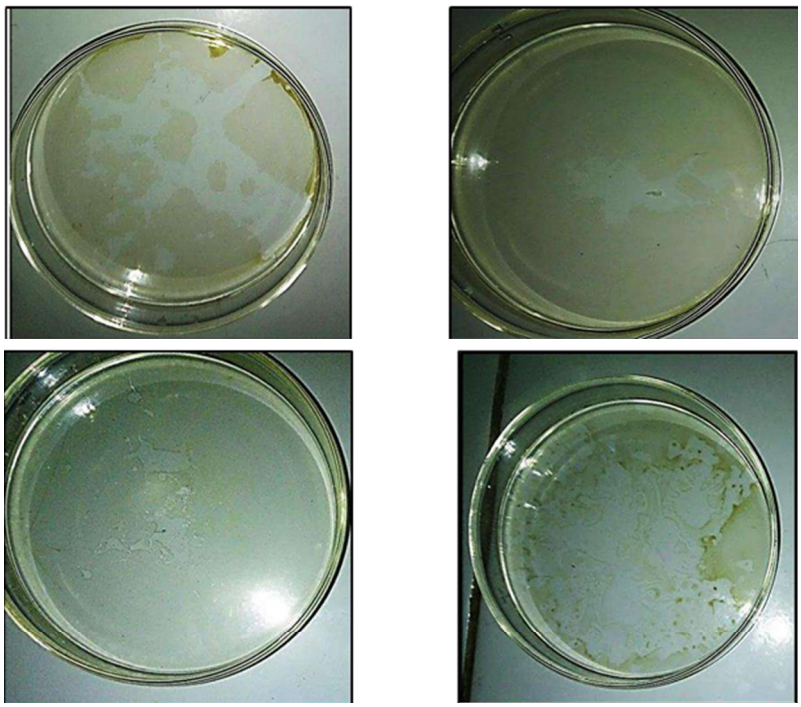

Figure 12
Results of the oil displacement test with the supernatant from the centrifuged liquid SMSS media

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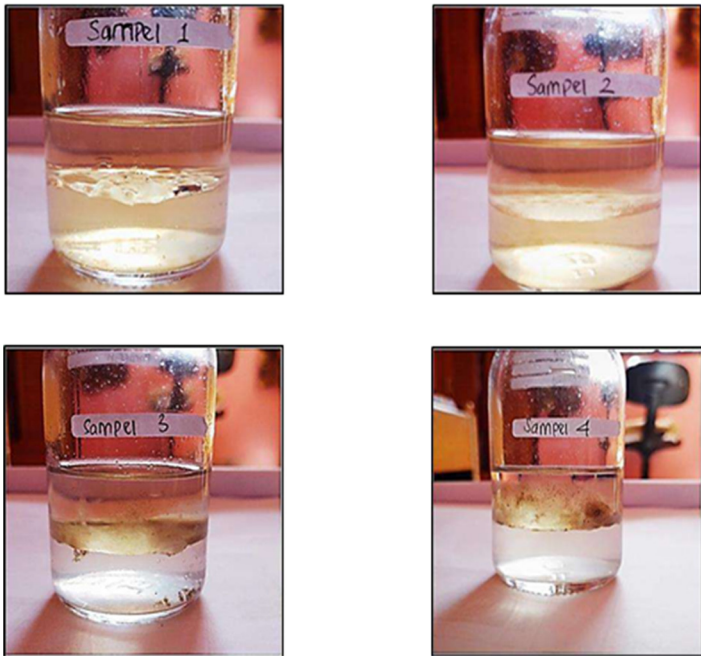

Figure 13
Emulsification test with supernatant from liquid SMSS media with liquid paraffin and waste cooking oil as carbon sources

Table 1
Oil displacement area (ODA) value, emulsification index value of biosurfactant + kerosene, and surface tension test results using the Du Nuoy Tensiometer method

No	Superstition	Oil Displacement Area (ODA) Value (cm)	Emulsification Index Value (%)		Surface Tension (dyne/cm) with three repetitions		
					Time 1	Time 2	Time 3
1	Liquid SMSS media with used cooking oil carbon source + fungi from solid SMSS media with used cooking oil carbon source (Sample 1).	4.1	(+/-)	13%	63.5	63.5	63.3
2	Liquid SMSS media with waste cooking oil carbon source + fungi from solid SMSS media with tapioca flour carbon source (Sample 2).	3.6	(+/-)	13+	63.5	63.5	62.3
3	Liquid SMSS media with a liquid paraffin oil carbon source + fungi from solid SMSS media with a liquid paraffin carbon source (Sample 3).	2.4	(+/-)	28%	65.8	63.2	61.6
4	Liquid SMSS media with a liquid paraffin carbon source + fungi from solid SMSS media with a tapioca flour carbon source (Sample 4)	6	(+)	51.3 %	53.5	53.2	51

CONCLUSION

This study shows that *Aspergillus niger* can produce effective biosurfactants in the MEOR process, especially with the addition of tapioca flour as a carbon source. This indicates that biosurfactants from *Aspergillus niger* can be an environmentally friendly and economical alternative for increasing petroleum recovery.

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GLOSSARY OF TERMS

Symbol	Definition	Unit
MEOR	Microbial Enhanced Oil Rec	
SMSS	Stone Mineral Salt Solution	
NH4NO3	Amonium nitrat	
ODA	Oil Displacement Area	Centi meter
E24	Emulsification Index	%
$MNCL_2$, $4H_2O$	Manganese dichloride tetrahydrate	
$CACL_2$	Calcium chloride	
NA_2HPO_4, $7H_2O$	Sodium phosphate dibasic heptahydrate	
KH_2PO_4	Potassium dihydrogen phosphate	
PH	Potential hydrogen	
mL	Volume	Mili liter
Gr	Massa	Gram

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