Preliminary Studies on Isolation of Lipid-Degrading Bacteria from Contaminated Water

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Preliminary Studies on Isolation of Lipid-Degrading Bacteria from Contaminated Water

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Abstract. The increase in human activities has caused water pollution, where more pollutants are released into the water. Lipid is one of the common substances that can be found in contaminated water. Lipid-degrading bacteria refer to bacterial strains that can produce lipase and break down the lipid. This study was conducted to isolate and screen lipid-degrading bacteria from contaminated water samples. Four potential lipid-degrading bacteria were isolated from contaminated seawater and pond water. The ability of bacteria strains in degrading lipids was tested by growing the bacteria on Rhodamine B agar. The colony that emits orange fluorescent indicates the presence of lipase activity. The rate of lipid degradation by the bacterial strain on olive oil and motorcycles oil was carried out using the liquid-liquid extraction method with chloroform and methanol as solvents. Strain T1 isolated from Teluk Batik, Malaysia seawater showed lipid-degradation activity and capable of degrading commercial olive oil and motorcycles oil at 75.59% and 85.43%, respectively.

1. Introduction

Lipid is a substance that is non-soluble in polar solvents and consists of hydrocarbon. It can be found in water sources and land due to the pollution that appears nowadays. The revolution of industry and tourism has caused environmental pollution and pollution, whether air, water or land are still considered one type of pollution since most pollutants will end up in the ocean [1]. The industry's revolution mostly has caused river pollution since most of the factory will release the waste into the river. For tourism industry especially for the island, the increasing number of tourists contributing to the increase of waste products in that area. Irresponsible tourists contribute to this pollution as they dump the waste as they please without thinking about their activities to the environment. Most of the famous beach has an issue regarding garbage where there are garbage everywhere on the beach that later will end up in the ocean. Human has practiced to dispose waste into waterways and most people will use an open waterway to dump every type of waste that produced. The pollution of aquatic environment is very serious and some of the pollution is more critical in area that located near human settlements.

Oil pollution can be considered the most severe pollution for marine pollution, where approximately 700 million gallons of oil are released on ocean worldwide every year [2]. There are six oil pollution sources: natural seeps, offshore drilling, smoke, big spills, routine maintenance, and the drain. Surprisingly the main contributor to oil pollution came from the drain, which is around 350 million gallons. Oil spills occurred worldwide, and it cause a serious problem as it lead to large-scale fish kills. The oils at the water surface also pollute marine organisms as the crude oil remains in the

culture site for prolonged periods [3]. Lipid-degrading bacteria are bacteria that can produce lipase and break down lipid. Previous research shows various types of bacteria can be isolated and contain a high level of lipase activity. Different type of bacteria has a different kind of optimum temperature for growth. The optimum temperature for lipolytic bacteria is at 30 °C, but some species such as *Pseudomonas aeruginosa* have an optimum temperature between 37 °C up to 42 °C [4]. Lipolytic bacteria are bacteria that release energy when decomposing vegetable and animal fat. The most common type of lipid-degrading bacteria belongs to Bacillus sp. and Pseudomonas sp. The urbanization process causing the amount of lipid-rich wastewater to increase every year, making lipid-degrading bacteria potentially be used in the wastewater treatment [5]. Wastewater treatment is a process to remove the suspended solids from wastewater before releasing the water back to the environment. Clean and safe water is essential for humans as it is a fundamental need. Wastewater treatment is a very important process, and with the help of lipid-degrading bacteria, the process could be done quickly. This study was conducted to isolate and screen the lipid-degrading bacteria from the beach and pond's contaminated water sample. Different types of bacteria were isolated from both sampling locations with one strain shows the ability to degrade lipid.

2. Experimental

2.1. Sampling of polluted water

The seawater samples were collected from Teluk Batik beach, Lumut, Perak, Malaysia. Whereas pond water samples were collected from fishponds at the Universiti Malaysia Kelantan (UMK), Jeli Campus, Kelantan, Malaysia. Each water sample (300 mL) was collected in aseptic condition, inside sterile bottles by dipping the bottle into the water without disturbing the surface and sediment. Samples were kept at 30 °C until further use.

2.2. Isolation and screening of lipid-degrading bacteria

The procedure was conducted based on Phong, Duyen & Diep [6]. Ten mL of each water sample was added into 200 mL screening medium (0.5% $MgSO_4$, 0.05% $(NH_4)_2$ SO_4, 0.1% KH_2PO_4 and 1% of cooking oil) and incubated at 30 °C, 140 rpm for 72 hours. 0.5 mL of the mixture was then spread on Luria-Bertani (LB) plate and incubated for 48 hours at 30 °C. LB plate consists of 1% of peptone, 0.5% of yeast extract, 1% of NaCl, 1.5% of agar and supplemented with 100 µg/mL nystatin. Experiment was carried out in duplicates. Bacterial growths were observed daily and morphological characteristics of the isolated strains were recorded.

2.3. Lipase assay

The isolated bacteria strains were streaked on lipase assay agar (0.8% trypticase soy broth, 0.4% of yeast extract, 0.3% NaCl, 3% of agar powder, 3% of commercial olive oil and rhodamine B) and incubated at 30 °C for 48 hours. Presence of oranges fluorescent surrounding the bacterial strain that indicates the lipase activity were observed by naked eyes and recorded.

2.4. Lipid extraction by chloroform-methanol extraction

The procedure was conducted based on method by Matsumiya et al. [7]. The isolated bacterial strains were inoculated in LB broth and incubated at 30 °C, 140 rpm for 48 hours. 1% of bacteria culture was then inoculated into 100 mL seawater in a 250 mL flask. Two types of oils were used; commercial olive oil and motorcycles oil to test the ability of bacteria strains in degrading lipids. 1% of oil were added into the mixture and the samples were cultivated in shaker incubator at 30 °C, 140 rpm for 48 hours. Next, 30 mL of chloroform-methanol mixture (ratio 3:1, v/v) was added to the 100 mL culture and mixed for 5 minutes in a separating funnel. Chloroform layer was collected and 5 mL was transferred into pre-weighted 50 mL beaker and placed at 25 °C for 24 hours for drying process. The percentage of lipid degradations was calculated using the following formula [8]:

$$\text{Percentage of lipid degradation (\%)}: \frac{\text{1 - dry weight of oil after cultivation}}{\text{Dry weight of oil before cultivation}} \times 100$$

3. Results and Discussions

3.1. Isolated strains and morphological characteristics

Teluk Batik, Perak was selected as one of the sampling locations due to pollution in that area. It is one of the famous tourist attractions in Lumut, Perak, and has a higher number of tourists who visited the beach, especially during school holidays. Other than tourist attractions, Teluk Batik is also located near the iron ore terminal and power station where the shipping activity occurred. The activities at Teluk Batik have contributed to the seawater pollution. The pond in UMK was used as another sampling location. The water is stagnant and only used for recreational activities. The contaminants are brought to the pond by the runoff water. Screening of lipid-degrading bacteria was carried out using media containing 1% of cooking oil that acts as a source of carbon and energy for the lipid-degrading bacteria. Previous research used cooking oil [6] and salad oil [9]. The oil promotes the growth of lipid-degrading bacteria during cultivation process.

The isolated bacterial strains were observed and selected based on the morphological characteristic. Four out of 20 isolated strains were morphologically different (Table 1). Bacterial growth isolated from pond water can be observed after 24 hours incubation. However, bacterial from seawater samples need more than 24 hours incubation for growth. The morphological characteristics of Strain T1 show yellow colour while the other 3 strains were in white colour. The colony of Strain U3 was denser compared to the others.

Table 1. Bacterial strains isolated from polluted water samples.

Morphology of isolated strain				
Strain	T1	T2	U3	U4
Water Source	Seawater	Seawater	Pond water	Pond water

Lipid-degrading bacteria came from different sources. Phong et al. [10] and Sutrisno, Wardani, & Ratnawati [11] have isolated bacteria strains from wastewater sites. Percentage of successful isolation of lipid-degrading bacteria varies; Sixty-one bacterial were isolated and only 11 identified as potential lipid-degrading bacteria [6], whereas Sutrisno et al [9] had isolated 12 bacterial strains but only 7 strains showed lipolytic activity.

The morphological characteristics differed between bacteria strains isolated from seawater and pond water. Bacteria from seawater have a smaller colony size while pond water has a larger colony size. The microbial diversity between seawater and fresh water is very contrasted due to many factors such as salinity, average temperature, depth, and nutrient content. The microbial populations are larger in ocean estuaries than shoreline waters due to higher nutrient levels in that place [10]. In seawater, the microbial diversity is enormous where the microbial at the surface water is very different from the microbial at the seafloor. Bacteria richness for seawater is higher in the water column than in the sediment area [11] and phylum Proteobacteria abundant in seawater than freshwater [12].

3.2. Lipase activity

The morphologically different isolated strains were further tested for lipase activity with Rhodamine B as a dye in the growth media. Rhodamine B dye for lipase assay was introduced by Kouker and Jaegar [13] where the basic principle involves an interaction of hydrolyzed substrates with Rhodamine B resulting in the formation of orange fluorescent halos around microbial colonies which can be visible upon ultraviolet (UV) irradiation. Other methods that can be used are gel diffusion assays using various lipid substrates incorporated in to the media. This method tests microbes' ability to form a clear zone of lipolysis by breaking down the lipids that are fused inside the solid media [14]. Lipid substrates commonly used in gel diffusion assays are Tween 20, Tween 80, tributyrin, triolein, and olive oil. Indicator dyes such as phenol red, methylene blue, and nile blue were also used in the screening media where the dye's color changes can be observed directly. Out of 4 strains that were tested, only Strain T1 showed a positive result for lipase activity (Figure 1). Strain T1 shows oranges fluorescent around the colony while Strain T2 has a bright pink colour and pale pink for Strain U1. The lipase producing strains reflect the oranges fluorescent and non-lipase producing strains has pink colour around the colonies [9]. The color changes were due to the complex bond formation between Rhodamine B cation with uranyl ion from the hydrolysis of triglycerides by lipase [13].

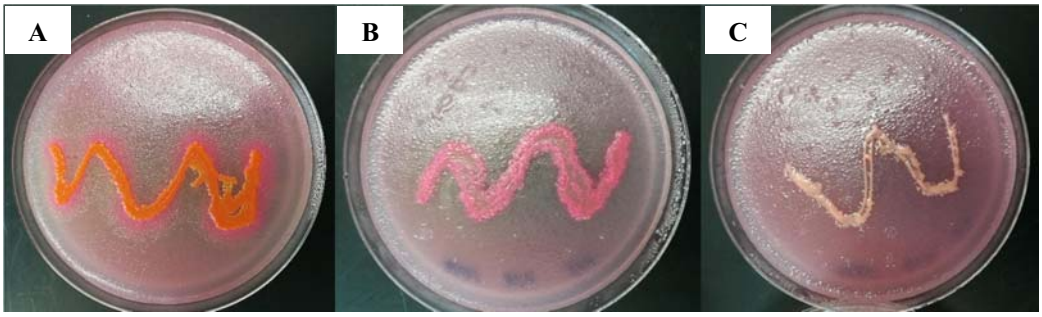

Figure 1. Assay plates after 48 hours incubation for lipid degradation activity on the isolated bacterial strains; Strain T1 (A), Strain T2 (B) and Strain U1 (C). Strain T1 shows orange fluorescence indicating lipase activity.

3.3. Lipid extraction

The percentage of lipid degradation was quantified by growing the Strain T1 in LB broth containing olive oil and motorcycles oil. Chloroform layer were formed after the mixing (Figure 2). Two different types of oil were used to study the effectiveness of Strain T1 to degrade lipids. Strain T1 efficiently degrades commercial olive oil and motorcycles oil at 75.59% and 85.43%, respectively. These suggest that Strain T1 capable of degrading various types of lipids. Lipid degradation ability is generally enhanced by emulsification with biosurfactant, which increases the interaction between microbial enzymes and lipids [7]. Rhodococcus sp. isolated by Koma et. al [8] able to degrade 24% of base oil and 15% of c-alkane fraction after 48 hours while Gordonia sp. degrade 14% of base oil and 8% of c-alkane fraction. Matsumiya et. al. [7] has isolated Burkholderia sp. and tested the lipase activity using 4 different types of lipids; beef tallow, olive oil, salad oil and sesame oil. The rate of degradation for each type of lipids is 77.4%, 92.3%, 96.7% and 90.1%. These show that different types of bacteria strains have different rates for lipid degradation and types of lipid use affect the degradation rate. Lipid degrading bacteria can be found widely in many various sources. Oil-contaminated sites such as wastewater and soils are rich with lipid-degrading bacteria due to the presence of lipid that promotes the growth of bacteria [15].

Figure 2. Broth cultures mixed with olive oil (left) and motorcycles oil (right) for lipid extraction using chloroform and methanol as solvent. Red arrow shows the chloroform layer.

4. Conclusions

Marine pollution occurs in every part of the globe and the rate of pollution is increasing every year. This is disturbing, as the pollution will affect the ocean's ecosystem, marine life and human itself. Results obtained suggested that Strain T1 capable of degrading lipid when tested on olive oil and motorcycles oil. Not all bacteria are capable of utilizing every lipid as it is highly dependent on their metabolic activities. The findings from this study can be further use for the development of natural wastewater treatment methods. The bacteria strains can be used for oil spilling cleaning in the sea, and treating polluted rivers in Malaysia.

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