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CRUDE OIL DEGRADATION BY LACCASE *PSEUDOMONAS AERUGINOSA* NR. 22. A DRIVING FORCE OF BIOREMEDIATION

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Abstract

Pseudomonas aeruginosa NR. 22 (Ps.NR.22) was able to produce bio-surfactant and laccase enzyme to degrade complex hydrocarbon from crude oil. The bio-surfactant was used to increase bioavailability of the hydrophobic crude oil while laccase enzyme acts as an agent to initiate the breaking of hydrocarbon bond. Ps. NR. 22 were fermented for 21 days in nutrient broth containing 2, 5 and 7% (v/v) crude oils. Cell dry weight (CDW) representing cell growth, laccase, biosurfactant production and crude oil residual analysis were recorded throughout the fermentation. The growth was excellent in 5% (v/v) crude oil with 94.1% degradation; when the production of laccase was the lowest. Ideally, as the cells growth, enzyme and biosurfactant production will increase but it was different with Ps.NR.22. This finding suggested the laccase enzyme was not the main tool for the crude oil degradation to take place but the integration of both has made the degradation worked.

Keywords*: Pseudomonas aeruginosa, Biodegradation, Crude oil, Laccase, Biosurfactant*

Introduction

Pseudomonas species recently received a lot of attention since it can be easily found and isolated from many ecosystems including water, soil, and plants. It has been reported to be having great potential for bioremediation process and likely to be adapted for special environmental treatment. According to Nik Raikhan and De Valda [1], Pseudomonas has high capability to produce very high laccase concentration for degradation of many kinds of extreme substrates such as BPA, long chain hydrocarbon, crude oil and heavy metals. *Pseudomonas aeruginosa* NR. 22 (Ps.NR.22) has been proven to be a player in enhancing balance and sustainability of different kinds of extreme environments.

Apart from that, exposing the species under specific environmental conditions will enhance the bacterium to produce rhamnose compound containing glycolipid bio-surfactant [2]. Having capability producing surfactant to emulsify the oil surface and secreting enzyme to break the hydrocarbon chain are among of two most important trait needs to be possessed by any hydrocarbon degrading bacteria to ensure bioremediation can occur and can be run go continuously.

Crude oil is a naturally occurring complex mixture of hydrocarbon and non-hydrocarbon compounds which at certain concentration, possesses a measurable toxicity towards living system. The toxicity of crude oil or petroleum products varies widely, depending on their composition, concentration, environmental factors and on the biological state of organisms at

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the time of the contamination. Crude oil can cause harmed to the environmental when the oil accidentally spills from tankers or pipelines to the water surface. Tides will help in moving the oils to the beaches and sooner contaminates living and nonliving organisms. Microorganism on the other hand will be less infected as they have higher tolerance to the toxicity of the hydrocarbons due to their physiology and have the mechanism to eliminate oil spilled from the environment [1]. This microorganism is known as hydrocarbon degrader, which through similar processes has degraded some oil sources through a process named bioremediation [3].

Bioremediation is a process where organic waste such as crude oil or waste oil being biologically degraded by the bacteria to an established level below the concentration proven safe by the regulatory authorities. Besides bacteria, fungi and certain type of plants are also able to detoxify organic hazardous substances into harmless end products. The efficiency of the microorganism in degrading organic matter will depend on the environmental condition. Thus, it is crucial to ensure that the biodegradation process can take place in an optimum condition of the relevant species involve. One of the reasons why the biological approach is being a favored over the physical-chemical treatment is because this method can offer in situ biodegradation of oil fractions without needing high operation and process cost as it only requisite less complex equipment and doesn't need any addition chemical usage or support [4].

Bacteria are the most active agents in degrading petroleum in fact they work as primary degraders of oil spilled in environment. According to Das and Chandran [3], Acinetobacter sp. was found to be capable of utilizing n-alkanes of chain length C10-C40 as a sole source of carbon. Besides *Acinetobacter sp.*, there are humongous numbers of other species isolated from contaminated soils proved to have the potential to degrade crude oil such as *Gordonia, Brevibacterium, Aeromicrobium, Dietzia, Burkhoderia, Mycobacterium*. In this research paper, the kinetic of crude oil degradation by *Pseudomonas aeruginosa* NR. 22 (Ps.NR.22) will be discussed [5].

Most of the compounds in crude oil have very low solubility in water which led to low biodegradation. For the biodegradation to happen, the bacteria need to be in contact with the hydrocarbon substrates. One of the biological strategies that can improve contact between bacteria and water-insoluble hydrocarbons is emulsification of thehydrocarbon [6]. These surfactants help to disperse the oil, increase the surface area for growth by reducing the surface tension. Apart from that, surfactant also able to help to detach the bacteria from the oil droplets after utilizable hydrocarbon has been depleted.

As being mentioned earlier, referring to Ron and Rosenberg [7], type of surfactant produced by Pseudomonas sp. is rhamnolipid which consists of two moles of rhamnose and two moles of beta–hydroxydecanoic acid. Apart from emulsification, another driving force for petroleum biodegradation is the ability of the microorganism to utilize hydrocarbons [8] to satisfy their cell growth and energy needs. This only can happen if the microorganism managed to break the bonds. This is where laccase enzyme being needed. This enzyme excreted by Pseudomonas aeruginosa when it detects the presence of petroleum hydrocarbon as this enzyme will initiate the breaking of the complex hydrocarbon bond.

By referring to the metabolite pathway, this process is shown in Fig. 1. The first step of alkane degradation (Fig. 1a) is the oxidation process of the methyl group to the alcohol by the alkane hydroxylase system, which involve monooxygenase enzyme while the catabolic pathways of aromatic monoaromatics and polynuclear (Fig. 1b and 1c) including two oxygen dependent enzymes which are hydroxylase and dioxygenase. Monoxygenases, dioxygenase and tyrosinases are some of the members of laccase enzymes family [8]. Further breaking of the bond will not be able to take place prior this process.

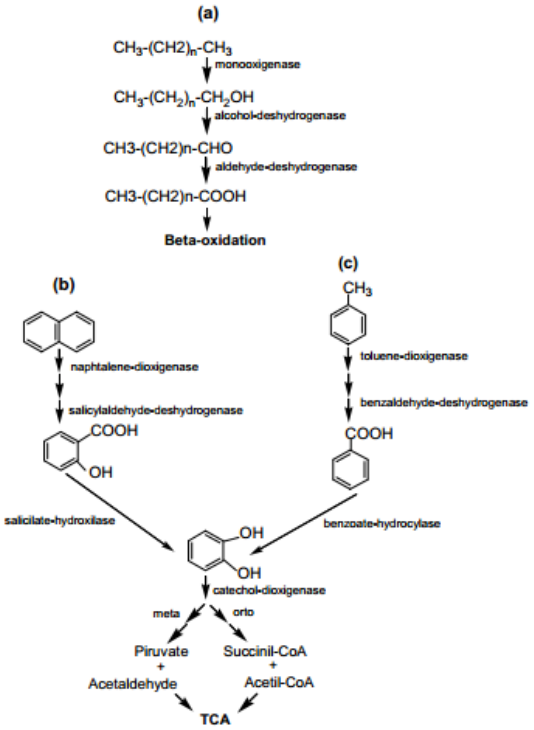

Fig. 1. Suggested metabolic pathway degradation of the main components in the crude oil by Pseudomonas aeruginosa (a) n-alkanes (b) naphthalene (c) toluene [6]

Experimental

Reagent, chemicals and pH

All material and chemicals were collected from various sources and quality as listed. Pseudomonas agar (Microbiology), nutrient agar (Merck), nutrient broth (Merck), potassium dihydrogen phosphate (KH_2PO_4, V90004, Vetec™ reagent grade, 99%, Vetec), sodium hydroxide (NaOH, Fluka) and hexane ($CH_3(CH_2)_4CH_3$, 95%, Sigma-Aldrich).

Bacteria isolation and degradation of crude oil

The method has been modified from Nik. Raikhan and Khairul Izwan [5]. Pseudomonas aeruginosa was isolated from one of the polluted lakes in Shah Alam, Selangor, Malaysia. It is a Gram negative-rod shaped bacterium (Fig. 2).

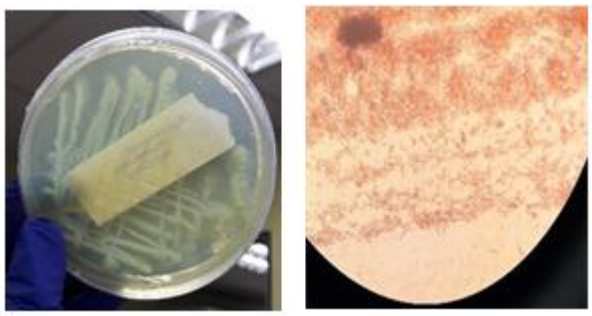

Fig. 2. Gram negative staining of *Pseudomonas aeruginosa* NR. 22 (Ps.NR.22)

The strain was cultured on Cetrimide agar (Fig. 2). The inoculum was prepared using nutrient broth (NB) at 150rpm at 37°C for 24 hours. Volume of the inoculum that will be added in the main fermentation was 10% (v/v) from the working volume used. Then, 2% (v/v), 5% (v/v) and 7% (v/v) of crude oil also added into the flask.

Optical density and Cell Dry Weight (CDW)

Optical density was determined spectrophotometrically at 560nm. 3.0mL of sample collected regularly within the fermentation period to measure the bacteria growth in the medium containing crude oil. The average from three readings of absorbance value will be recorded and from the data obtained, bacteria growth curve can be plotted. OD versus cell dry weight graph was plotted for growth performance [9].

pH

A volume of 3.0mL of fermentation medium was collected regularly during the fermentation period. Then, the pH of the medium was measured using pH meter.

Analysis of laccase enzyme production

Laccase enzyme production was measured spectrophotometrically at 436 nm by using modified method from Niku-Paavola et al, [9]. The 21 days fermentation medium (21DFM) contained 7% (v/v) crude oil was used as crude laccase source. The calibration curve was prepared after 21DFM was centrifuge at 5000rpm for 30 minutes at 40°C. A mixture of 1.0mL crude laccase and 1.0mL crude oil was left for 2 hours to allow the reaction to take place followed by addition of 1.0mL of mixture of acetone and alcohol of ratio 1:1 to stop the reaction. The calibration curve was plotted by using Equation 1:

$$y = 0.00030x - 0.06298 \quad (1)$$

By using the equation 1, concentration of the laccase enzyme at certain relevant time was determined. The percentage of laccase enzyme produced was obtained by modifying the equation in Latha and Kalaivani [10]. All spectrophotometric measurements were carried out using a UV–Vis spectrophotometer in triplicate.

Analysis Percentage of Crude Oil Degradation by Using Residual Oil Analysis

Method to determine the percentage of crude oil residual was modified from Latha and Kalaivani [10]. An equal volume of fermentation medium (23FM) and hexane was poured into a 500mL separation funnel. The funnel was shake vigorously for few seconds to increase the contact rate between medium and hexane. The mixture was left for an hour to let the separation process to occur. The amount of residual oil was measured at 650°C (1atm). The weight of extracted oil was deducted from the previously weighed beaker and the percentage of residual calculated by subtracting the weighed of extracted oil with the weighed of the oil at the beginning of the experiment.

Kinetic models

All experiments were carried out with Ps.NR.22 strain at 37°C in 250mL Erlenmeyer flasks operating at 150 rpm to elucidate the optimum conditions (pH, contact time and initial crude oil concentration). The effect of pH on biodegradation was studied by adjusting pH values (4.5–8.0) with the addition of 0.1M HCl or NaOH. The experimental part of the crude oil degradation was accomplished by using 10–100mg/L crude oil concentrations. The amount of crude oil degraded by the strain was calculated by using modification of method of Othman et al. [11] with the below mass balance equation:

$$q_e = (C_0 - C_e)V/W, \quad (2)$$

where: C_0 is the initial of crude oil concentration, C_e is equilibrium of crude oil concentrations in the solution (mg/g), V is the volume of the Nutrient Broth (NB) in liter (L) and W is the mass (g) of bacterial cells mass.

Results and discussion

Ps.NR.22 growth in crude Oil

Potential of Ps.NR.22 as bio-degrader and as an oil degradation indicator for the crude oil has been studied. Figure 3 shows the growth profile of the Ps.NR.22 with the first 6 hours of laq phase and steady growth from 54-92 hours of incubation.

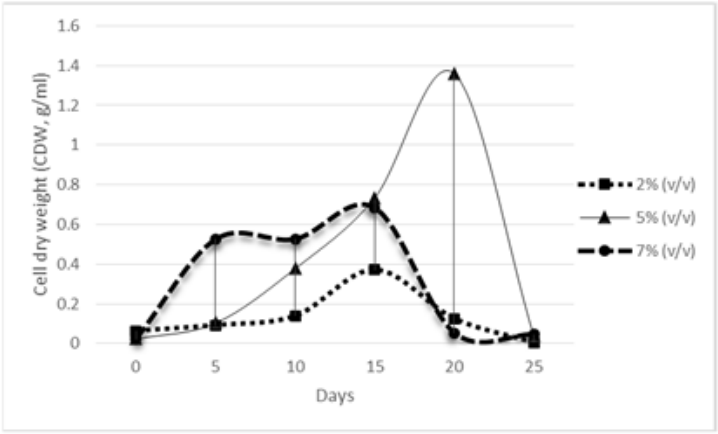

Fig. 3. Cell dry weight (g/ml) of the cells in different concentration of crude oil

The results revealed that Ps.NR.22 was showing the same significant growth curve with better growth stability in the crude oil. It can be clearly seen that; highest number of cells was recorded with 5% (v/v) concentration of crude oil was used. The range of growth was recorded between 0.0226g/mL to 1.3598g/mL before the cells enter death phase and cells dropped to 0.0397g/mL. The lowest growth was recorded with 2% (v/v) crude oil where the highest amount of cell mass was only 0.3714g/ml. But, the most stable pattern of growth was in beaker contained 7% (v/v) concentration crude oil since every phase of bacterial growth clearly shown from the lag phase until the death phase. The growth of the cells will depend on how well the cells are degrading the crude oil. This is related to the efficiency of the cells in getting carbon source which is commonly one of the essential nutrients needed by any microorganism to support their cell growth [2]. Thus, higher amount of cell mass (Cell Dry Weight, CDW) has carried out higher biodegradation rate of crude oil since the cells have gained enough nutrients from the surrounding. By referring to the Table 1 below, this finding is relevant with the data obtained as 5% (v/v) crude oil produced highest cell growth and highest rate of crude oil degradation of 94.1%.

Table 1. Percentage of degraded oil at the end of the study

Concentration of crude oil (%, v/v)	Oil Degradation (%)
2	50.02
5	**94.1**
7	67.17

Degradation of crude oil

Percentage of degraded was determined by using a very simple formula from Latha and Kalaivani [10] which is by dividing the value of difference in weight of crude oil (initial and final) with its initial weight (Table 1) It was expected that, as the volume of crude oil added increase, the rate of crude oil degradation by bacterial species will decrease.

This was due to thick layer of undispersed crude oil was formed on the surface of the medium forming heterogeneous mixture of oil and water that inhibited the degradation process of crude oil and lowering cell growth by reducing mass transfer of nutrient or by causing limitation of oxygen. Oxygen was crucial since Ps.NR.22 was an aerobic species [12].

Research findings by Rahman [13], showed that after 20 days of fermentation, not only the rate of biodegradation has decreased from 66% to 27% but also has affected the bacterial cell count negatively from 2.1x102 CFU/g to 1.7x1011 CFU/g. This result was recorded with an increment of the crude oil concentration from 1% to 10% (v/v).

On the other hand, the data obtained in this study was totally contradicted from the hypothesis or the finding by Rahman [13]. By referring to Table 1, the biodegradation rate was directly proportional to the volume of crude oil used. The highest degradation was recorded in a glass containing 5% (v/v) of crude oil. There were some factors that needed to be investigated; to explain this outcome. First, the ecosystem where the bacteria species has been isolated and the location of crude oil sampling was done. Different ecosystems might result into a different metabolism, hence the mechanism of the bacterial cells which produced different kind of product in different time range. The crude oil contained more long chain hydrocarbon compared to the common carbon source that Ps.NR.22 has been living with.

Secondly, the bacterial strain activeness in producing enough bio-surfactant was correlated to the concentration of crude oil added. It can also assist the cells in overcoming all the limitations during high volume of crude oil involved. Figure 4 show the condition of the medium after 21 days fermentation (21DFM) with 2, 5 and 7% of crude oil added. By observing the physical conditions/appearance of the medium after the fermentation period, it could be highlighted that the highest amount of surfactant production was in beaker (b), followed by (c) and (a). This was due, no formation of undispersed of crude oil layer on the medium surface (in beaker b and c) and the hydrophobic oil mostly managed to mix well with the medium until a homogenous mixture of oil and medium formed (especially in beaker b) [14]. The physical appearance of the broth corresponding to the numerical results obtained which was the highest degradation rate will be beaker (b) followed by (c) and (a).

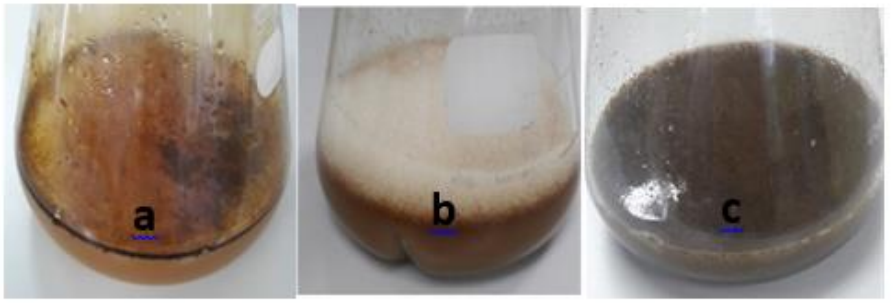

Fig. 4. Condition of the medium after 21 days fermentation (a) 2% of crude oil added (b) 5% of crude oil added (c) 7% of crude oil added

Production of laccase enzyme

Figure 5 shows the percentage of enzyme that has been produced within the 21 days of fermentation period.

Theoretically, high crude oil degradation was expected due to high/sufficient production of enzyme excreted by the Ps.NR.22 cells. From the figure, it clearly shown that the medium containing 7% (v/v) of crude oil concentration gave the highest production of enzyme followed by 2% (v/v) and 5% (v/v). Laccase enzyme was secreted during stationary phase.

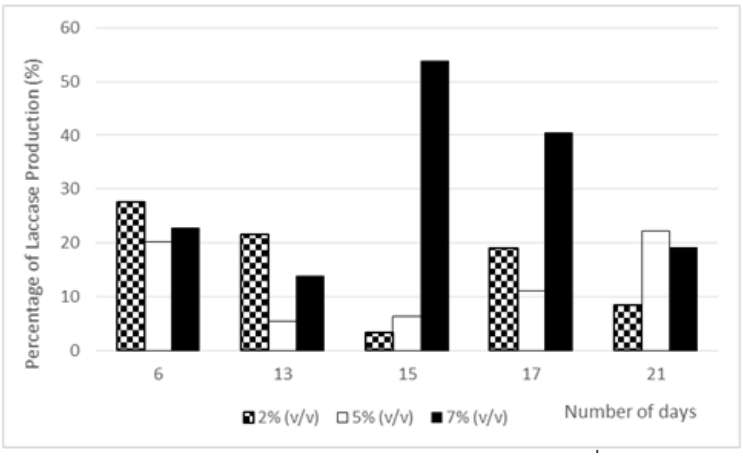

Fig. 5. Percentage production of laccase enzyme within fermentation period

Cells in beaker containing 7% (v/v) crude oil have the most stable and longest stationary phase. Thus, this could explain why the percentage of enzyme production in this beaker higher compares to others especially on day 15 (stationary phase). Beaker containing 5% (v/v) have the highest rate of crude oil degradation which supposedly the percentage of enzyme production in this beaker also will be the highest. But, from figure above, cells in this medium recorded the lowest enzyme production at early stage of the fermentation but later, the production of enzyme started to constantly increase. If the fermentation period is longer, the production of enzyme in this beaker will be high as well.

Conclusions

Pseudomonas aeruginosa NR. 22 (Ps.NR.22) isolated from one of the polluted lakes in Shah Alam, Malaysia has proven able to survive and degraded different concentrations of petroleum hydrocarbon. This ability was contributed by its bio-surfactant that was needed for the emulsification process. The enzyme involved in the mechanism was laccase that helped to initiate the breaking of hydrocarbon bonds in the crude oil. The best mechanism of crude oil biodegradation was recorded in medium with 5% (v/v) of crude oil concentration. It was with high crude oil degradation (94%) and high cell growth (up to 1.3598g/ml). Although the production of laccase enzyme in this medium was low, but great amount of bio-surfactant produced during the fermentation period has assisted the cell in degrading crude oil by reducing the interfacial tension (IFT) and increase the bioavailability of the oil to be attacked by cells of Ps.NR.22. This finding was suggesting that even it is needed, the laccase enzyme was not the main tool for the crude oil degradation but combination of both laccase and biosurfactant made it worked.

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