Performance of Laccase Ps. NR22 in Crude Oil Degrading Activity: Kinetic Study

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***Abstract*— Due to the hazardous and toxic chemicals which contaminated soil, ground water, sediments, surface water and air give unfavorable effect to our ecosystem. Therefore, microbial degradation enzyme based is introduced in this study. Michealis Menten is a mathematical model used to prove the objectives. At the end of study, bacterial laccase can degrade almost 70 percent of hexane with 1.0986 m/s within 24 hours. As conclusion, performance of bacterial laccase based on kinetic study and ability are proven. In future, analysis different types of bacterial enzyme on asphaltenes component since asphaltenes were major fraction composition in crude oil.**

Keywords*— Ps. NR22, *Laccase, crude oil, hexane, performances, kinetic model and degradation.

I. INTRODUCTION

Laccase enzyme is a versatile oxidoreductase enzyme produced by *Pseudomonas. aeruginosa* NR22 when degrading crude oil as its alternative carbon source after completing nutrient broth. It catalyses oxidation of phenolic and non-phenolic aromatic compounds [1] which converting oxygen molecule to water followed by four-electron reduction [2]. In terms of performance of laccase, unit activity and laccase ability are parameters will be determined to achieve research of study.

Remediation strategies is a technique to treat water pollution in ocean which affect mainly by oil and gas industries. Remediation strategies are divided into two types, one is bioremediation and another one is phytoremediation. In this study, in-situ bioremediation using microbial enzymes is the main highlight in this study. Bioremediation is a strategy to treat water pollution caused by oil spill by using microorganisms. Microorganisms helps to clean up oil spill in a faster rate by breaking the toxic organic pollutant into non-toxic substances. Therefore, sustainability of the environment can be protected because microorganisms does not cause harm to our environment.

Microorganisms are easy to clone, take a short time to produce enzyme and have substrate specifically. Those advantages of bioremediation which it has become most favorable strategies compare to other strategies such as chemicals formulation and coagulant. Bioremediation also have two classes including in-situ and ex-situ. In situ is defined as the waste is treat on the spot at the origin place while ex-situ the waste is treat after removing contaminant waste to a treatment area. Oil and gas industry are always the main contributor to the oil spill which it will pollute most part of oceans. This is because, petroleum is extracted from the oil reservoir deep in the ocean and transported through oil pipeline to the processing plant. Throughout the transportation process, the oil spill will occur. Not than that, illegal oil spill also always occurs at the ocean results from the human action. It will severely damage most of the marine life because of toxic organic compounds in the crude oil. Different hydrocarbon compounds that have been found in the crude oil. For instance, paraffins, naphthenes, aromatics, sulphur, organic nitrogen, and phenols compounds which include in 30 percent polyaromatic hydrocarbons [3]. This type of crude oil has three different types of viscosity. The first one is low viscosity which it tends to float on the surface and medium viscosity either can float or sink in the water. For high viscosity of crude oil, it tends to sink in the water. The low and medium type of crude oil can be treated with physical treatment such as oil trap but for high viscosity, it must use membrane technology to treat the water. Unfortunately, fouling problems will occur if we use membrane technology. To overcome this problems, chemical treatment has been used. Chemical treatment can overcome these problems but at the end, it will cause more pollution to our environment especially to marine system. Therefore, these serious problems initiate the development and execution of bioremediation strategy to treat the oil pollutions naturally.

Scenario of Global Oil Spill

Figure 1 shows database extracts from International Tanker Owners Pollution Federation Limited (ITOPF) [4], there are 20 major spills occurred over the world in between year 1970 to year 2007. Atlantic Empress disaster in 1979 have the highest number oil spill, which is 287,000 tonnes near Off Tobago, West Indies. Hebei Spirit 2007 in South Korea was recorded as lowest number of oil spill which is only 11,000 tonnes.

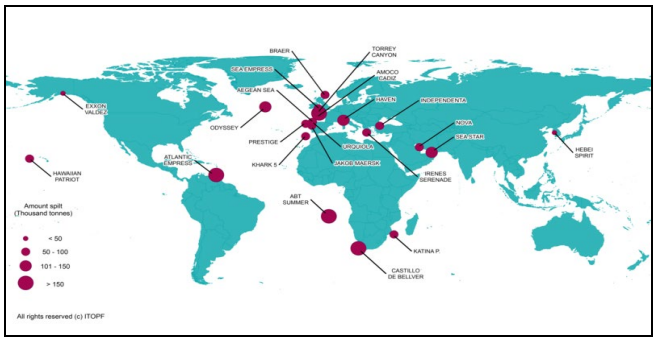

Figure 1: Location of major spills

Causes of Oil Spills

Oil spills are defined as leakage of crude oil into the environment specifically onto the large area covered by waters such as oceans, lakes and river. There are a few factors which contribute to the causes of the oil spills. Most commonly results from oil and gas industries which involving tankers, barges, pipelines, refineries, drilling rigs and storage facilities. The spills of the crude oil can cause in many ways. For instance, it results from human error that being careless while handling the crude oil, breakdown of equipment, natural disasters and illegal dumpers by irresponsible person.

Drilling rig is a place where the extraction of crude oil happened. To illustrate the general overview of the process of how oil spillage occurs, first, geologist must run 3D seismic survey to locate which wells contain a lot of oil and gas approximately. After the wells have been found, the wells were drilled down to a certain depth to get the oil and gas by using derrick. All wells that have been drilled were connected to a pipeline which it will work as delivering the oil and gas from wells to the processing facilities.

Once the oil and gas are completely refined, they are sent to the downstream section for distribution to sites such as petrol station. For example, massive industrial disaster about 4.9 million barrels of crude oil was happened in the Gulf of Mexico near Mississippi 2010 [5].

When it comes to natural disasters likes hurricanes and earthquake, there are no ways to control because it can happen at any time. This factor caused tanker ship accidents or leakage of underground pipelines. According accident near Mozambique 1992 [6], due to the unexpected natural disaster, the oil tanker ship broke and released about 67,000 metric tons of crude oil into the ocean.

Discarding waste in an improper manner where it damages the environment are called illegal dumping. Some people or industries does not follow rules and regulations to dump used oil. Based on regulation 3 of Environment Quality Act 1974, the effluent discharge volume of 60 m^3/day or more [7]. Industries used to dump oil waste onto the oceans because their discharge volume may not follow the regulations.

Crude Oil

Dark, oily liquid which usually found naturally in underground reservoirs, pools, and tar sands is definition of crude oil or also known as petroleum. Crude oil is refined with different boiling point to make diesel fuel, gasoline, fuel oil, and other sources of energy for consumer needs. Not only that, crude oils also can made waxes, ink, crayons and personal care products.

Crude oil can be divided into two types which is light oils and heavy oils. Example for light oils are gasoline and diesel fuel while heavy oils are bunker oils which are used for ships. Light oils are can stay for a temporary of time in aquatic because they are volatile while heavy oils remain for a long time.

Both have significant hazard. For light oils, they are flammable and considered toxic. Even though it is only light oil, but they can kill many living things. In contrast, heavy oils have less toxicity compare to light oils. They will affect organism for long term period such as chronic health.

Crude oil consists of 83 to 87 percent of carbon and 12 to 14 percent of hydrogen and having complex hydrocarbon mixture such as paraffins, aromatic hydrocarbons, naphthenes, gaseous hydrocarbons which is from CH_4 to C_4H_{10} [8]. Besides, crude oil also contains small number of non-hydrocarbons and minerals. Example of non-hydrocarbons are sulphur, nitrogen, and oxygen compounds.

Paraffin also known as alkanes which have straight carbon chain. Methane, ethane, propane, pentane and hexane are major hydrocarbons contain in paraffin. As the number of carbon increase, the boiling point also increase. Hence, paraffin becomes waxy as the number of carbon reach 25 to 40. While, iso paraffins or iso alkanes are made of branched carbon chain. It has similar hydrocarbons as paraffins, but the structures are different. For iso paraffins, the number of carbons increases makes the number of possible isomers also increases as in geometric progression.

Next, composition of crude oil based on hydrocarbons are olefins or alkenes which attached by double bond. It consists of ethylene and propylene. Olefins does not present in crude oil, but it will form during the process. Because of their low reactivity makes they are not desire in the finished product. Olefins with low molecular weight have good antiknock properties.

Naphthenes have 5 to 6 carbon atoms in ring structure which consists cyclopentane, methyl cyclopentane, dimethyl cyclopentane, cyclohexane and 1,2 dimethyl cyclohexane. It covers about 50 percent of the crude oil [9]. Last composition of hydrocarbons in crude oil is aromatics. Aromatics build up from 6 carbon atom in ring with three around linkage. It includes benzene, toluene, xylene, ethyl benzene, cumene and naphthaline. Aromatics are not desirable in kerosene and lubrication oil.

For non-hydrocarbons composition in crude oil are sulphur, nitrogen and oxygen. Sulphur are made of hydrogen sulphide and mercaptans. Due to foul odor, it become undesirable. Next, quinotine, pyradine, pyrole, indole and carbazole are compounds which contains in nitrogen. Nitrogen can degrade the color of product on exposure to sunlight if it is present in the gasoline and kerosene. While oxygen contains naphthenic acids and phenols compound. At various stages of processing, these acids can cause corrosion and gives pollution problems.

Products that refined from crude oil have its properties which is harmful for environment. For example, gasoline have high volatile products that easily evaporate. It only takes 1 to 2 days to evaporate to the air. Due to its narrow-cut fraction with no residue and low viscosity, gasoline spreads quickly and form thin sheen on water or on the land. For living things in marine system, they are very toxic which it can penetrate the substrate since it is non-adhesive.

For diesel, it has low-to-moderate volatile products that can evaporate to the air moderately. It also contains no residue and it spreads rapidly to form a thin film. Sometimes diesel also can act moderate-to-high. It is very toxic to biota and able to penetrate substrate to marine ecosystem if there is oil spill.

Intermediate product such as lube oil and fuel oil are less volatile. The toxicity varies depends on its boiling point components. They also can penetrate into the ecosystem due to its moderate-to-high viscosity properties.

Fuel oil have medium viscosity makes it is unstable. The fuel oil tends to separate when spill onto water. It either can buoyant or sink in the water depends on water density. The sunken oil has highest possibility to accumulate and suspended on the bottom under calm condition.

Residual products are able to evaporate. In contrast, when it spills, the area contamination is intertidal, and the surface become persistent. It is long-term contamination. Commonly, residual products always have high viscosity which is semisolid texture. It becomes less viscous when the temperature is high.

All of those products have same affect towards environment. If the crude oil or the products spill in the ocean, it can last longer in the ocean and threaten the species [10]. Therefore, a new natural technique that will sustain environment without any pollution.

Bioremediation

To degrade and detoxify environment contaminants, bioremediation strategies is relevant compare to current technologies. It is biological treatment which are cost-effective compare to chemical and physical treatment. The uses of microorganisms or microbial processes are definition of bioremediation. Even though bioremediation is new technology but naturally at the site, there are right microorganisms known as indigenous microorganisms which have physiological and metabolic capabilities to degrade the contaminants.

Bioremediation can be done on site makes minimum exposed to disruption. Transportation cost, liabilities, waster permanently and long-term liability can be eliminated. This is because bioremediation often in situ application. Contaminants such as crude oil will decompose permanently into carbon dioxide and water where it does not harm environments.

Since sites are frequently contaminated with complex mixtures of organic compounds such as crude oil. To have successful bioremediation, interdisplicinary approach such as engineering, microbiology, ecology, geology, and chemistry must united to overcome complexities that present at the sites.

Bioremediation can be divided into two types which are ex situ technologies and in situ technologies. Ex situ technologies is defined as technology to treat water pollution by physical removal of the contaminated material. For instance, bioreactors, landfarming, and composting. Bioreactors is method to degrade contaminant or treat liquid in container such as slurry while landfarming use solid-phase treatment systems for contaminated soils. Composting involved aerobic microorganism which uses oxygen to react. It was thermophilic treatment process which means the contaminant materials mixed with a bulking agent. This technology commonly used in continuously fed reactors.

In situ technologies are treatment that degrade the contaminant at that current place such as bioventing and biostimulation. Method to treat contaminated soils by supply oxygen into the soil for stimulation of microbial growth and activity are called bioventing. While, biostimulation used indigenous microorganisms to degrade contaminant. It can be done either ex or in situ.

All those technologies can be done if the contaminants are biodegradable which can be degraded by capable microorganisms, but if the contaminated area cannot be degraded by microorganism, biology infused with engineering techniques can be used. For instances, addition of biosurfactant to absorbed contaminants at the subsurface of water.

Majority of bioremediation system currently use are bacterial, to make a new future natural degradation, enzyme of bacterial are used to degrade organic compounds in water system was studied in this research.

Bacterial Enzyme

Bacterial enzyme is a type of biocatalyst which initiates or modifies the rate of chemical reaction. Bacterial enzyme in this study is laccase produced *Pseudomonas. aeruginosa* NR22.

There are a few general characteristics to illustrate biocatalyst. The most commonly is the range of reaction can be catalyzed is broad and its catalytic power is high. Biocatalyst catalytic power is up to 10^9 until 10^{12}. Compare to chemistry catalyst, biocatalyst is pollutionless and easily decomposable at any stage. This special characteristic makes they are more favorable to sustain environment.

Bacterial enzyme also has specific active site where a region for substrate to combine. The binding site of substrate will cause the products are formed. Although, it is used by the previous substrate, therefore, it will bind with another substrate for another reaction.

Based on [2], laccases are defined as versatile oxidoreductase enzyme which able to oxidize a wide area of phenolic and non-phenolic compounds by converting molecule to water or concomitant four-electron reduction. It can be found in glycoprotein from various fungi until higher plants. Due to its redox potential and stability at high temperature and pH, it is exploited for applications in industrial processes such as biobleaching, bioremediation, textile dye decolorization, pollutant degradation and biosensors.

Mechanism of Bacterial Laccase in Degrading Crude Oil

Catalytic properties of laccase play an important role to degrade compounds in crude oil to form water molecules on the concomitant electron loss of a single oxygen molecule. Since laccase is a multi-copper enzyme which form in central part when redox mechanism has been take place. It can be classified into three types which is type 1, 2 and 3 copper [1].

There are two different models explaining on mechanism of the enzyme to the substrate which is Lock and Key analogy and Induced Fit analogy. Both needs an enzyme and substrate, and both have substrate that will bind to its active site. The differences between both of them are the shape of enzyme while substrate bind to active site. For Lock and Key only used for correctly sized of substrate which fits into the active site of the enzyme while for induced fit, the substrate is the main role in changing its shape of the enzyme which is partially flexible.

According to analogy first postulated in 1894 by Emil Fischer [11], the key will represent substrate while lock represent enzyme. The basic mechanism is the enzyme will catalyze the substrate and convert into product. The binding site of the substrate will bind to the active site of the enzyme. A large number of interactions form with the amino acid residues in active site when substrate binds to active site of enzyme. In contrast, particular active site of enzyme is for specific substrate.

The shape of active site of enzyme will undergo conformational change when meet with substrate which makes the reactivity increased. Moreover, the enzyme has varied range of specificity. This is why Induced Fit proposed by Daniel Koshland [12] is different with Lock and Key theory. Similar to the Lock and Key theory, the basic mechanism is substrate bind to the active site of enzyme and catalytic reaction will take place.

Kinetic Study – Michealis Menten

Michealis Menten is a mathematical model regularly used to test the enzyme kinetics that have a special arrangement two-parameter rectangular hyperbola. Based on the graph Michealis Menten, it is enzymatic activity (rate) versus concentration of substrate. The equation of mathematical model is

$V = C (V_{max}) / C + K_m$ ---------- **Equation 1**

Where *V* is the dependent variable known as velocity rate of an enzyme reaction, *C* is the independent variable known as substrate concentration, and *Vmax* and *Km* are parameter to be estimated. *Vmax* is the maximum velocity and serves as a horizontal asymptote, *Km* the Michealis constant while *C* the results a velocity of *Vmax/2* [13].

Based on definition, the higher value of enzymatic activity is where determination of what enzyme have the best performance in degrading the substrate. From the graph also, the value of Km and Vmax can be obtained. Hence, this value will be used in Michealis Menten equation to study the kinetic of bacterial enzyme towards substrate.

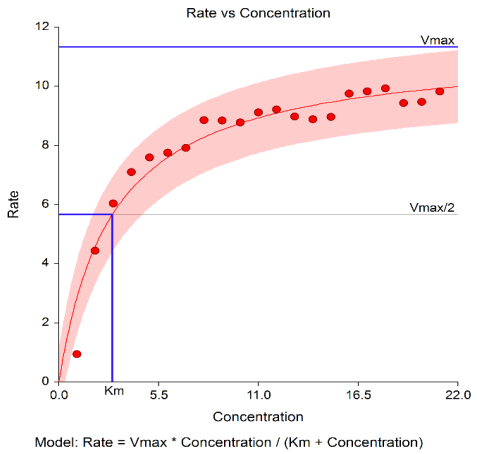

Figure 2: Graph of Michealis Menten [13].

II. METHODOLOGY

A. Materials

As listed were all materials and chemicals used which collected from various sources. Pseudomonas agar (Microbiology), nutrient broth (Merck), modified ammonium phosphate monobasic (79546, ≥98% $(NH_4)_2HPO_4$, Sigma-Aldrich) and potassium dihydrogen phosphate (V90004, Vetec™ reagent grade, 99% KH_2PO_4).

B. Media Preparation

34.5 g of Pseudomonas agar powder was weighed and transferred into a beaker. 1000 ml of distilled water were added successively. To completely dissolved, the solution was stirred on the magnetic stirrer for 10 minutes. After the solution was completely dissolved, the solution was put in the autoclave for 20 minutes at 121°C [14]. After 20 minutes, the solution is taken out from the autoclave machine, and were kept for 15 minutes under laminar fume hood.

C. Subculture from pure culture Ps. NR22

To subculture bacteria, pure culture of Ps. NR 22 was used. Swab inoculating loop into the pure culture with aseptic technique and the cover of the petri dish was removed followed by inoculation using a streaking technique on the agar. The cover of petri dish was covered immediately. The petri dishes were sealed and kept in the incubator at 37°C [15]. The same steps were repeated once a week to make sure the microorganism gets enough nutrient to continue growth.

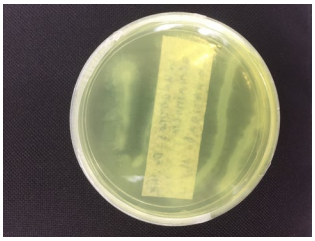

Figure 3: Subculture of *Pseudomonas. aeruginosa*

D. Bacterial Laccase production

Crude oil sample from Dulang field were prepared for 2% (v/v), 5% (v/v) and 7% (v/v) in conical flasks. Different volume percent of crude oil, 25 ml of inoculum and 250 ml sterilized nutrient broth was poured into the conical flasks. The steps were repeated except for a control flask. Aseptic technique was applied during transferring procedure. All the sample were kept in the shaking incubator at 37°C and 150 rpm [16].

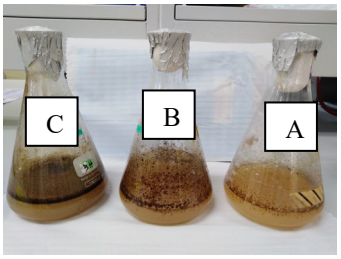

Figure 4: Bacterial Laccase production; different in percentage of crude oil; 2% (A), 5% (B) & 7%(C)

E. Extraction of Bacterial Laccase

After 21 days, the three conical flasks were transferred into Falcon tubes and were centrifuged twice at 4°C, 5000 rpm for 30 minutes. After 30 minutes, the supernatant was bacterial laccase was extracted and transferred into a close container.

Figure 5: Extracted bacterial Laccase

F. Ability of Bacterial Laccase to Degrade Hexane

Five different concentrations of hexane were prepared in glass container. Initial absorbance value was recorded at 210 nm. Then, 1 ml of enzyme and buffer were added into each container and left for 2 hours in an incubator with 110 rpm at 25°C. Then after 2 hours, final absorbance value was recorded. By using the mathematical calculation, final absorbance minus with initial absorbance have been performed. Therefore, the finalized absorbance value versus different types of concentration of hexane solution were plotted.

G. Perform a Calibration Curve

Chromatography–mass spectrometry (GCMS) data that was sent at the first stage of our research was analyzed. From the data, hexane component was 923,7000 m^2 while the total area was 1,286,672,484 m^2. After mathematical calculation was performed, the percentage of hexane in the crude oil was about 0.79 percent. Five different concentrations of hexane in between range 0 to 0.79 percent were prepared. The absorbance value was recorded. Absorbance value versus different concentration of hexane were plotted and 0.999 of R^2 was obtained.

H. Performance Test on Bacterial Laccase – Activity

A unit which indicate the rate of reaction catalyzed by that enzyme expressed as micromoles of substrate transformed (or product formed) per minute was known as enzymatic activity. An enzyme unit is the amount of enzyme that will catalyses the transformation of 1 mmol of substrate or min under specified conditions of pH and temperature. The specific activity of an enzyme is expressed as the number of units per milligram of protein [17]. It can be performed by using Michealis Menten equation.

$v = C (V_{max}) / C + K_m$ ---------- **Equation 1**

0.79 percent of 10 mL diluted hexane was prepared in a glass container. Then, 0.3 mL of Laccase was added into the sample. Buffer solution was added with the same amount of hexane solution. The absorbance value was recorded every 1 hour, and graph was directly plotted in the Microsoft Excel to observe the trend. The absorbance value was stop recorded when the graph had reached the decreasing value.

I. Performance Test on Bacterial Laccase – Ability

Ability is defined as the capability of the extracellular enzyme to degrade hydrocarbon [18]. Based on data obtained from 3.8.3, initial and final absorbance value were recorded. Percentage of degradation of hydrocarbon component was performed by using mathematical calculation

% of degradation = [(Final - Initial)/Final] x 100 --- **Equation 2**

Where initial value was at 0^{th} reading and final value was at 7^{th} reading using graph from Figure 3.

III. RESULTS AND DISCUSSION

A. Characterization of Crude Oil

1) Gas Chromatography-Mass Spectrometry (GCMS)

From the figure shown above, crude oil was run for GCMS to obtain the percentage of each component. From the results, there are 83 components in the crude oil. For instances, component that have in the crude oil was p-xylene, tridecane, 1-hexadecyne, hexane and many more.

From the figure 6, it shows that retention time versus area. Each of the components in the crude oil have their own retention time and area. From our observation, the highest peak obtained in the graph was at minute 22 which is contain sulfurous acid, 2-ethylhexyle isohexyl ester, pentane, 3,3-dimethyl, tetrazolo (1,5-b) 1,2,3-triazine, 5,6,7,8-tetrahydro-6,7-dimethyl and octane, 2,3,7-trimethyl. While the lowest peak in the graph, component that can be found was at minute 29, 36 and 41. Some of the components are 2-butyl-1,2-azaborolidine, 3-undecene, 6-methyl and pentane, 3,3-dimethyl.

Among all the components, the only available chemical that can replace crude oil were hexane. Therefore, by using figure 7, mathematical calculation was performed to determine the composition of hexane in crude oil.

Total Area = 128,667,248,4 m^2
Area of Hexane = 352,8000 m^2
Area of Cyclohexane = 570,9000 m^2

Composition of Hexane
= (Area of hexane + area of cyclohexane) / Total area
= (352,800 + 570,9000) / 128,667,248,8
= 0.79%

From the graph shown below, retention time for hexane component was at minute 3.884 while retention time for cyclohexane was at minute 7.2986.

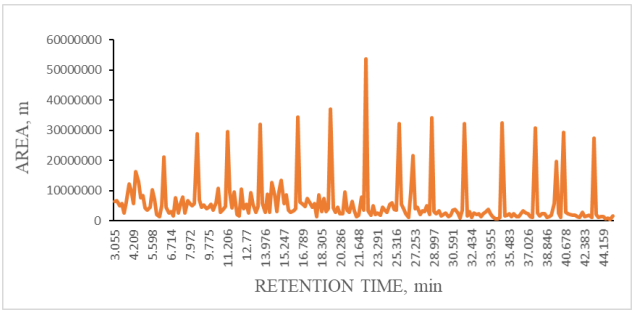

Fig. 6: GC-MC data crude oil from Dulang field; overall overview

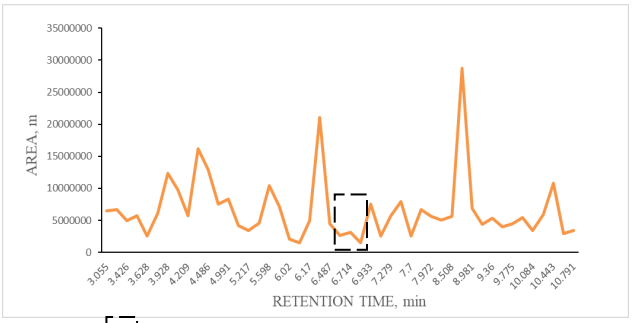

Figure 7: Zoom in the GC-MS data crude oil from Dulang field; component hexane is highlighted

B. Production of Bacterial Laccase

1) Growth Curve of Pseudomonas. aeruginosa NR22

Based on figure 7 below, it shows the growth curve of *Pseudomonas. aeruginosa* with different concentration of crude oil. The growth curve is obtained by plotting absorbance value versus time in day. Absorbance value is obtained from the UV visible equipment. In order to achieve research objectives, laccase enzyme have to be extracted from the Ps. cells.

The degradation of petroleum hydrocarbons can be mediated by specific enzyme system. The initial on xenobiotics by oxygenase. Attachment of microbial cells to the substrates will produce biosurfactant. This is because in order to degrade crude oil, the microcells have to emulsify for homogenize the layer of crude oil [19]. Therefore, laccase was produced.

There are four phases of bacterial growth curve in general. Lag phase is where the initial phase take place. It characterized by cellular activity but not growth. A small group of cells are placed in a nutrient rich medium that allows them to synthesize proteins and other molecules necessary for replication. The cells increase in size but no cell division [20].

Next, exponential phase is where bacteria cells are dividing by binary fission and doubling in numbers after each generation time. The DNA, RNA and cell wall components and other substances have to grow make its metabolic activity also high [21].

At stationary phase, the population growth experienced in the exponential phase begins to decline as the available nutrients become depleted and waste products start to accumulate [22]. At this stage where production of extracellular enzyme was produced. The bacteria tend to find another hydrocarbon as alternative nutrient source in order to survive in the process.

As nutrients become less available and waste products increase, the number of dying cells continues to rise. This phase called as death phase. The number of living cells decreases exponentially and population growth experiences a sharp decline [20].

To compare between three different percentage of crude oil, it clearly saw that all have four phases mentioned earlier. The important phase is stationary phase where laccase enzymes were produced. Between 2% and 7% they have same period of time where it takes 4 days for production of laccase. But 7% have a sharp decline for death phase compared to the 2%. Therefore, 7% was chosen for the main experiment.

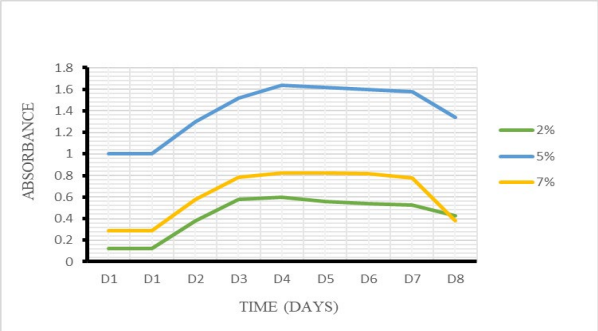

Figure 8: Kinetic growth of *Pseudomonas. aeruginosa* at 2%, 5% and 7%

C. *Percentage of Degradation of Hexane*

Based on the graph shown in Figure 8 below proved that Laccase are available in the system and valid to use in the next experiment because 80% of component hexane has been degraded in 2 hours. The working condition was at 110 rpm and 27°C.

From the previous research, *Pseudomonas aeruginosa* was known as hydrocarbon-degrading bacteria [23]. The results of their study indicate that the *Pseudomonas. aeruginosa* has the ability to degrade various hydrocarbons such as hexadecane, crude oil and fractions A5 and P3 of crude oil and is efficient in rhamnolipid production and hydrocarbon emulsification [24].

Because the potential of bacteria for bioremediation application is highly dependent on biotic and abiotic soil parameters *Pseudomonas*. sp strain make it promising agent for cleaning up environments contaminated with petroleum compounds [24].

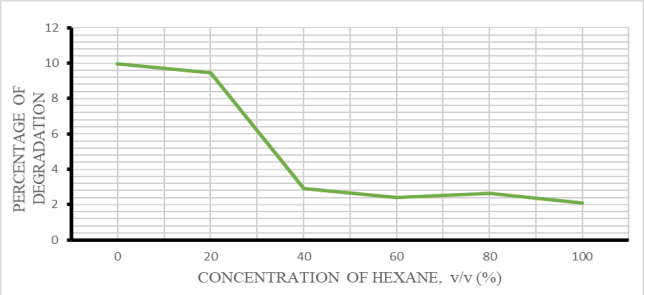

Figure 9: Graph of percentage of hexane degraded by Laccase

D. *Calibration Curve*

Calibration curve is defined as a general method for determining the concentration of a substance in an unknown sample by comparing the unknown to a set of standard samples of known concentration [25].

Calibration curve is one approach to the problem of instrument calibration. The concentration of the standards must lie within the working range of the technique used [26]. The calibration curve is ready to use when the R^2 is 0.99.

These are few procedures to produce a calibration curve:

1. Making the standards: Serial dilutions
2. Run the samples for the calibration curve and the unknown
3. Making the calibration curve
4. Results

For this research, calibration curve was performed by preparing five different concentrations of hexane. Next, the value of absorbance was recorded at 201nm. Repeat the same step with different concentration until the R^2 obtain 0.99.

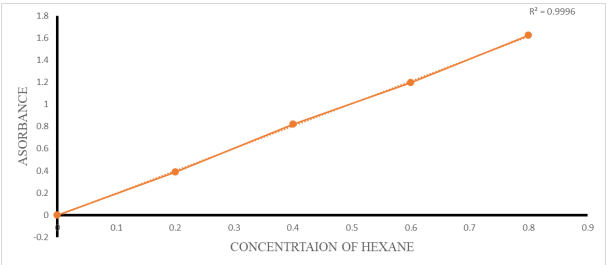

Figure 10: Calibration Curve for Hexane

E. *Performance test on Bacterial Laccase – Activity*

From figure 10 below, the data for V_{max}, $V_{max}/2$ (C) and K_m were extracted. By using equation 1, the mathematical calculation was performed

$v = C (V_{max}) / C + K_m$
$= 0.7 (1.4) / 0.7 + 0.192$
$= 1.0986$ mole/h

Therefore, the reaction velocity for the enzyme is 1.0986. When substrate is low, the rate equation is first order in substrate. When substrate is high, the equation for rate is zero order in substrate. The Michaelis-Menten equation describes a rectangular hyperbolic dependence of v on substrate [27].

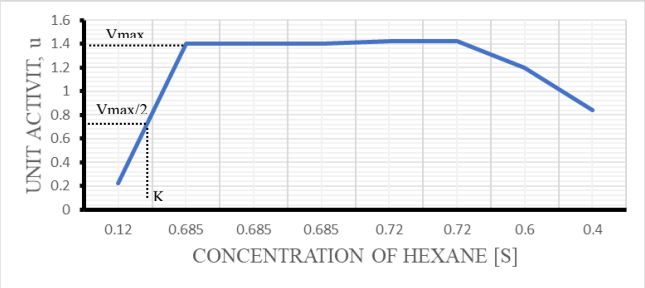

Figure 11: Activity of Bacterial Laccase on Hexane

F. Performance test on Bacterial Laccase – Ability

Table 1: Data from the activity of Laccase in degrading crude oil with different concentration of hexane

Time (hour)	Concentration of hexane, [S]	Unit Activity, u
0	0.12	0.22
1	0.685	1.4
2	0.685	1.4
3	0.685	1.4
4	0.72	1.42
5	0.72	1.42
6	0.6	1.2
7	0.4	0.84

In order to obtain the percentage of degradation, mathematical calculation must be performed. By using equation 2:

Percentage of degradation = [(0.4-0.12)/0.4] x 100
= 70%

IV. Conclusion

In conclusion, the kinetic study of performance of Laccase Ps NR22 in crude oil degrading activity and ability was well recorded. From the results obtained, we can conclude that the reaction velocity for the laccase to degrade crude oil is 1.0986 mole/h and 70% of hexane component were degraded in 7 hours. Therefore, we have confirmed the extracellular enzyme, Laccase secreted from Ps. NR22 is suitable to degrade hazardous and complex materials less in than 24 hours.

Acknowledgment

The author gratefully acknowledges the Faculty of Chemical Engineering Universiti Teknologi MARA and Dr Nik Raikhan Nik Him for the research to take place and the great opportunity for this paper to be published. Humongous thanks go to Syuhada Badrul Hizam as well as the lab assistant of Faculty of Chemical Engineering who has been supporting the work and this research.

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