JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2013. Vol. **7**(2), p. 881-890

Isolation and Identification of Catechol Degrading Bacteria

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(Received: 11 January 2013; accepted: 02 February 2013)

This research involves the isolation, identification, determination of bacterial growth, degradation rate and enzyme activity of catechol dioxygenase in bacteria. Eight bacteria were isolated from 3 locations: the oxidation pool, refinery pool and soil at the PETRONAS Oil Refinery Plant in Kerteh, Terengganu, Malaysia. Out of the eight isolated bacteria, seven were Gram negative and one Gram positive, bacilli. Two bacteria were chosen based on correlation from the screening results of spread plate method and optical density method. Both bacteria were identified as *Pseudomonas pickettii* (88.3%) and *Agrobacterium radiobacter* (99.8%) via the API Kit 20NE. Based on the growth profile of bacteria, the growth rate of *A.radiobacter* was higher than *P. pickettii*, with 0.045 h^{-1} compared to 0.011 h^{-1}. *A.radiobacter* showed higher degradation rate than *P.pickettii*, with 1.037 mgL^{-1} h^{-1} for *A.radiobacter* and 0.910 $mgL^{-1}h^{-1}$ for *P.pickettii*. Enzyme assay of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase was conducted on both bacteria and the result was a higher enzyme activity in *A.radiobacter* than *P.pickettii*. Enzyme activity and specific activity of catechol 1,2-dioxygenase in *A.radiobacter* was 0.085 μmol min^{-1} mL^{-1} and 0.167 μmol min^{-1} mg^{-1} respectively while *P. pickettii* was 0.042 μmol min^{-1} mL^{-1} and 0.115 μmol min^{-1} mg^{-1}. Enzyme activity and specific activity of catechol 2,3-dioxygenase in *A.radiobacter* was 0.002 μmol min^{-1} mL^{-1} and 0.004 μmol min^{-1} mg^{-1} respectively while *P. pickettii* was 0.001 μmol min^{-1} mL^{-1} and 0.003 μmol min^{-1} mg^{-1}. The overall results of this study showed that *A.radiobacter* is a better candidate of the two bacteria in degrading catechol.

Key words: Catechol, *Agrobacterium, Pseudomonas*, Catechol dioxygenase.

Bioremediation is a biological process which involves usage of biological agents such as bacteria, fungi and yeast to remove polluted material such as oil waste from air, water, and land to break it down into inorganic compounds such as carbon dioxide, water and methane gas (Sarkar *et al*. 2004). It is the cheapest method to remove oil spills that does not require special equipment or technology. There are two kinds of bioremediation; the engineered and intrinsic form of bioremediation. Engineered bioremediation involves utilization of genetically modified microorganism to decompose pollutants. Characteristics of surroundings such as temperature, pH, nutrients resources, concentration and type of pollutant are factors which can influence degradation rate by microbes (Atlas 1995; Ripley *et al*. 2001). Intrinsic bioremediation on the other hand allows biodegradation to happen naturally in environment but over a long period of time. This method though cost effective and safe, is not the preferred method as the duration taken to clean up spills is longer than favored.

In this study we looked at isolating microorganisms that are able to breakdown catechol. Catechol is an organic material with the chemical formula, $C_6H_6O_2$. It is crystalline in room temperature, odorless, colorless, and dissolves in water. It is synthesized on a large scale by

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industries to generate products such as insecticides and perfumes (Helmut 2002). However, it is toxic, carcinogenic and is a waste product from industries such as pharmacy, cosmetic, textile and petroleum refinery (Kumar *et al*. 2005). Due to its solubility in water (IPCS & CEC 2005), the presence of catechol in water can result in death to aquatic life when the concentration exceeds 5 part per million (ppm) based on Environmental Quality Act (1974) (Kumar *et al*. 2005). Catechol was classed as a pollutant that is carcinogenic to mankind based on experiments conducted on laboratory mammals (IARC 1999). Reports indicated that intake of catechol in food will encourage formation of adenocarcinoma in rat's stomach (IARC 1999). In addition catechol is a genotoxic material in *in vivo* and *in vitro* assay, causing gene mutation, DNA strand decision, chromosome aberration, aneuploidy and cell transformation to occurs within non-human mammal cells (Brandt 1986; do Ceu Silva *et al*. 2003). There were also reports that certain animals were deformed from exposure to 2000 or 2800 mg / m^3 1,2-benzenediol (Flickinger 1976).

Catechol can be generated from decomposition of phenol, toluene, naphthalene, benzoic acid, and benzene. Decomposition of catechol involves two types of pathway which is *ortho*-cleavage and *meta*-cleavage pathway. *Cis-cis* muconate which is formed through *ortho*-cleavage by catechol 1,2-dioxygenase can be used to synthesize adipic acid and the acid plays an important role in manufacturing nylon 6-6, insecticide, and antibacterial compounds (Yuan *et al*. 2004). *Cis-cis* muconate can also be used as raw material in producing resin which is strong and is durable against heat, thereby producing thermoplastic material of suitable quality for application in electrical appliances and automotive (Wu *et al*. 2006).

Catechol dioxygenase enzyme is a major enzyme for microorganisms that enables it to degrade aromatic compounds due to its ability to cleave aromatic compounds which are stable in the ring structure. It is a type of intracellular enzyme that consists of non-heme iron protein. Presence of iron (Fe) atom in this enzyme is important to detect diatom gas, shift electrons and bind diatomic gases, such as O, to itself. Therefore, enzyme catechol dioxygenase able to add two oxygen atoms into catechol and encourage *ortho*-cleavage (intradiol) and *meta*-cleavages (extradiol) to happen and thereby producing cis-cis muconate and 2-hydroxymuconic semialdehyde. Catechol 1,2-dioxygenase contains atom Fe(III) in the enzyme's active base while catechol 2,3-dioxygenase contains atom Fe(II) in the enzyme's active base. Different oxidation level of the Fe atom contributes to the difference in cleavage mechanism in catechol dioxygenase (Joan 1999).

MATERIALS AND METHODS

Bacterial Sample

As much as fifteen different bacterial colonies were obtained from soil (SL), seventeen from the refinery ponds (KR) and fourteen from oxidation ponds (KA) at the Petronas Oil Refinery Plant in Kerteh, Terengganu, Malaysia. These colonies were isolated by screening against catechol (50ppm) in mineral salt medium (MSM). The final screening of these colonies resulted in only eight (8) colonies being selected for further studies. They are 1-KR, 2-KA, 3-KA, 4-KR, 5-KR, 6-SL, 7-SL, and 8-SL.

Medium for Bacteria Isolation

Isolation of bacteria was conducted in mineral salt medium (Zajic & Supplison 1972) which was added with catechol (500 ppm). The medium was adjusted to pH 7.0.

Preparation of Stock Culture

Isolated bacteria were sub-cultured on nutrient agar and incubated for 24 hours at 37°C. Then, single colonies from the nutrient agar were streaked onto agar slants and incubated for 24 hours at 37°C. Following this, paraffin oil was added to overlay the bacteria and kept at 4°C. Every three months the stock culture was transferred to newly prepared agar slants.

Preparation of Standard Inoculum

Eight different bacteria were labeled and cultured on nutrient agar and incubated for 24 hours at 37°C. Then, a single colony of each bacteria was inoculated into nutrient broth and incubated at 37°C in an orbital shaker (HOTECH:722) for 24 hours at 150 rpm. Then, the broths were centrifuged at 4000 rpm (Eppendorf Centrifuge 5810R) at 4°C for 15 minutes. Supernatants were decanted and pellets containing bacterial cells were centrifuged with 0.85% NaCl twice to ensure removal of all broth components.

The supernatant was thrown away and the pellet was centrifuged again with 10 mL of 0.85% solution NaCl. The concentration of each standard inoculum was measured using a spectrophotometer (Jenway 6505 UV / VIS Spectrophotometer) to get 0.5 optical density (Azmy and Hamzah 2007) in 550 nm wavelength. The above standard inoculum will be used in the following step

Screening Bacteria

As much as 10% standard inoculums of eight bacteria were inoculated into 100 mL conical flask containing 20 mL mineral salt medium (Zajic and Supplison 1972) and 500 ppm catechol. Each sample was assayed in replicates. The samples were incubated at 150 rpm for 4 days, at 37°C. Both methods such as optical density (OD) in 550nm method and spread plate method was conducted on test samples for day 0 and day 4 of incubation. The plates from spread plate method were incubated at 37°C for 24 hours. Observation was made by calculating colony forming unit (CFU) formed on the plates above. Two bacteria which showed the best growth were chosen from the correlation of both methods above.

Bacteria Identification

Identification was done on two chosen bacteria based on morphological features. Gram staining was carried out and macroscopic features of bacteria were observed. Biochemical tests such as indole test, catalase test, oxidase test, Methyl Red Test (MR), Voges Proskauer Test (VP), oxidative-fermentative (OF) test, and growth test on selective agar such as MacConkey (MCA) was also conducted to assist with the identification of isolates. In addition the API Kit 20NE was also used for bacteria identification.

Growth Profile of Bacteria

As much as 10% of standard inoculums of each bacterium was added into 50 mL of MSM and mixed with catechol (50 ppm) in a 250 mL conical flask. Each sample was replicated. Culture samples were incubated at 37°C for 4 days at 150 rpm. Color changes of medium were observed on the 0, 1st, 2nd, 3rd and 4th day of incubation. Bacterial growth has occurred when the medium turns cloudy. Bacterial growth was measured to obtain OD readings at 550 nm wavelength and the spread plate method was carried out at 24 hour intervals. Then, the plates were incubated at 37°C for 24 hours. Observations were made by calculating the number of colonies (CFU) formed on the spread plate.

Preparation of Catechol Standard Curve

According to Paulo *et al*. (2005), catechol solution of 500 ppm concentration was (0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL, 0.5 mL) mixed with 1 mL of 4-aminoantipyrine and 1 mL of NaOH solution. Fifteen minutes later, the solutions turned reddish-brown in color and the mixtures were measured at 555 nm using UV-VIS spectrophotometer. Concentration of catechol was calculated using formula $M_1 V_1 = M_2 V_2$. A graph of OD readings versus catechol concentration was plotted to be used as reference.

Catechol Degradation

As much as 10% standard inoculum of bacteria was added into 50 mL of MSM which was then mixed with catechol (50 ppm) in every conical flask. Each sample was replicated. Culture samples were incubated at 37°C for 4 days, at 150 rpm. On the 0, 1st, 2nd, 3rd and 4th day of incubation, 1 mL of culture was centrifuged at 14000 rpm for 15 minutes. Then supernatants were added with 1 mL of 4-aminoantipyrine and 1 mL of NaOH solution (Paulo *et al*. 2005) and measured at 555 nm using UV-VIS spectrophotometer. A graph of catechol concentration versus incubation period was plotted. Degradation rate of catechol, Qs ($mgL^{-1} h^{-1}$) was calculated from the steepness of graph at exponential phase (Pirt 1975). The steeper the graph, the higher the value of Qs.

Enzyme Activity Assay

Preparation of Supernatant

As much as 10 mL of the culture was taken from the catechol degradation test above when 50% degradation was achieved of catechol. Then, cultures were centrifuged at 4000 rpm using Eppendorf Centrifuge 5810R at 4°C for 15 minutes. Supernatant was decanted and the pellet was mixed with 4 mL of phosphate buffer 33 mM (pH 7.0). Mixtures were vortexed and sonicated at 130 Volts for 1 minute in ice to prevent denaturation of enzyme and maintain its activity. Then, the mixture was centrifuged at 4000rpm at 4°C for 15 minutes.

Catechol 1,2-dioxygenase Enzyme Assay

According to Briganti *et al*. (1997), as much as 970 µL of Tris-HCl 50 mM (pH 7.5), 10 µL of supernatant, and 20 µL of catechol 10 mM were mixed in a test tube and placed in water bath at 25°C. OD reading at 260 nm was recorded at an

interval of 5 minutes to detect the presence of *cis-cis* muconate. A graph of OD readings against time was plotted. One unit of enzyme activity equals to the amount of enzymes which turns 1 µmol of catechol into *cis-cis* muconate per minute. Coefficient extinction of catechol, ε in 260 nm is 16000 $M^{-1}cm^{-1}$.

Catechol 2, 3-dioxygenase Enzyme Assay

Based on Kataeva & Golovleva (1990), as much as 0.8 mL of Tris-HCl 50 mM (pH 7.5), 0.1 mL of supernatant, and 0.1 mL of catechol 10 mM was mixed in a test tube and put in a water bath at 25°C. OD reading at 375 nm was recorded at 5 minute intervals to detect the presence of 2-hydroxymuconic semi-aldehyde. A graph of OD readings against time was plotted. One unit of activity enzyme equals to the amount of enzyme which turns 1 µmol of catechol into products per minute. Coefficient of catechol degradation obtained at 375 nm is 33000 $M^{-1}cm^{-1}$.

Statistical analysis

Statistical analysis was carried out using "Student's T-test" with confidence level of 95% with P value that is less than $\alpha = 0.05$, showing significant differences between the 2 samples.

RESULTS AND DISCUSSION

Bacteria Identification

Macroscopic and Microscopic Observation

The eight bacterial cultures were grown on nutrient agar. The resulting pure cultures were observed for colony colour and shape. The macroscopic details were combined with the microscopic details of these organism. The results show that most of the isolated bacterial samples from the oxidation pond, refinery pond and soil in oil refinery plant are Gram negative and rod shaped.

Biochemical test

Biochemical test were conducted on the eight chosen bacteria which was isolated from the oxidation pond (KA), refinery pond (KR) and soil (SL) at PETRONAS Oil Refinery Plant in Kerteh, Terengganu, Malaysia. These isolates were labeled as 1-KR, 2-KA, 3-KA, 4-KR, 5-KR, 6-SL, 7-SL, and 8-SL. The biochemical test was carried out to identify the biochemical nature of bacteria and to ensure there was no duplicate bacteria among them (Table 1).

Catalase test carried out on all bacterial samples showed positive results, indicating that they are able to turn hydrogen peroxide into water and oxygen. Three bacterial samples (1-KR, 3-KA, 8-SL) gave negative results, whilst the remaining five bacterial samples (2-KA, 4-KR, 5-KR, 6-SL, 7-SL) were positive. It shows that five bacterial samples stated above are capable of synthesizing the enzyme cytochrome oxidase and oxidizing tetramethyl-p-phenylenediamine dihydrochloride substrate and forming a purple by-product, indophenol.

Based on the results of the indole test, none of the bacterial samples possessed the enzyme tryptophanase which is essential to breaks tryptophan into pyruvic acid, ammonia, and indole. In the Voges-Proskauer (VP) and Methyl Red (MR) test, three isolates (1-KR, 7-SL, 8-SL) gave positive results, whilst the VP test was positive for the other isolates. It shows that most of *Enterobacteriaceae* can only display one of the fermentation pathways and seldom display both pathways. The VP test shows that three of the bacterial samples stated above are able to carry out butanediol fermentation and thereby produce acetone that results in the red colour of α-naphthol in a alkaline environment. The five bacterial samples that gave positive results in MR test are 2-KA, 3-KA, 4-KR, 5-KR, and 6-SL. The MR test shows that they can undergo mixed acid fermentation pathways and produce acidic products that can be detected by methyl red indicator. The indicator turns red in color when pH is < 4.5.

Based on the results of the citrate test, all of the eight bacteria are able to degrade citrate thereby producing sodium ions which cause the pH of the medium to increase, turning the colour of the medium from green to blue. From the results of the oxidative-fermentative test, only two bacterial samples (1-KR and 4-KR) are able to aerobically and anaerobically degrade glucose. As much as six of the isolates are (2-KA, 3-KA, 5-KR, 6-SL, 7-SL, and 8-SL) oxidative.

The MacConkey test was conducted to support the results of Gram staining as Gram negative bacteria can be grow on MacConkey. This is due to the presence of crystal violet in the medium that inhibits the growth of Gram positive bacteria. Seven bacterial samples (1-KR, 3-KA, 4-KR, 5-KR, 6-SL, 7-SL, and 8-SL) showed growth on this selective medium and it shows that the

bacterial samples stated above are Gram negative bacteria and are able to undergo lactose fermentation.

Screening of Bacteria

The two screening methods used in this study are the spread plate method and the optical absorption method. The screening was carried out

Table 1. Results of Biochemical Test on Isolated Bacteria.

Bacteria	Cat	Oxi	Ind	VP	MR	Cit	OF	MCA
1-KR	+	-	-	+	-	+	O&F	+
2-KA	+	+	-	-	+	+	O	-
3-KA	+	-	-	-	+	+	O	+
4-KR	+	+	-	-	+	+	O&F	+
5-KR	+	+	-	-	+	+	O	+
6-SL	+	+	-	-	+	+	O	+
7-SL	+	+	-	+	-	+	O	+
8-SL	+	-	-	+	-	+	O	+

+ = positive, - = negative, Cat = catalase test, Oxi = oxidation test, Ind = indole test, VP = Voges-Proskauer test, MR = methyl red test, Cit = citrate test, OF = oxidation-fermentation test, MCA = Mac Conkey agar

Table 2. Identification of Isolate 3-KA and 7-SL by API Kit 20NE

Bacteria	Bacteria Code	Species	Accuracy Percentage
3-KA	1647741	*Pseudomonas pickettii*	88.3%
7-SL	1041473	*Agrobacterium radiobacter*	99.8%

Table 3. Specific growth rate of *Pseudomonas pickettii* and *Agrobacterium radiobacter*

Bacteria	Specific Growth Rate, μ (h^{-1})
Pseudomonas pickettii	0.011
Agrobacterium radiobacter	0.045

Table 4. Catechol degradation rate of *Pseudomonas pickettii* and *Agrobacterium radiobacter*

Bacteria	Catechol Degradation Rate, Qs($mgL^{-1}h^{-1}$)
Pseudomonas pickettii	0.910
Agrobacterium radiobacter	1.037

Table 5. Specific enzyme activity of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase in *Pseudomonas pickettii* and *Agrobacterium radiobacter*

Bacteria	Catechol 1,2-dioxygenase			Catechol 2,3-dioxygenase		
	Activity (μmol min^{-1} mL^{-1})	Protein Concentration (mg/mL)	Specific Activity (μmol min^{-1} mg^{-1})	Activity (μmol min^{-1} mL^{-1})	Protein Concentration (mg/mL)	Specific Activity (μmol min^{-1} mg^{-1})
Pseudomonas pickettii	0.042	0.364	0.115	0.001	0.364	0.003
Agrobacterium radiobacter	0.085	0.508	0.167	0.002	0.508	0.004

for four days to determine the capability of the bacteria to degrade catechol. The spread plate method is more accurate compared to the optical absorption method, as it accounts only for the living organisms as opposed to the optical absorption method which is based on turbidity and can be generated by living or non-living entities.

Both of these methods used catechol as the source of carbon for the growth of bacteria. However, catechol is not a good source of carbon as it cannot efficiently be degraded by all microorganisms. According to Cheng *et al*. (2002), the growth rate of bacteria with catechol as the carbon source is much slower as only 30% of catechol was degraded into carbon dioxide during a period of six months compared to the growth of microbes on media containing glucose as the carbon source. This is due to the fact that the degradation of aliphatic hydrocarbons such as glucose is much faster than the degradation of aromatic hydrocarbons such as catechol due to its stable ring structure. Efficiency of bacteria in catechol degradation will determine its usage as a degrading agent in bioremediation during oil spills.

Spread Plate Method

Among the eight isolated bacteria, six bacteria showed significant increase ($p<0.05$) in growth after 4 days of incubation. The growth of bacteria 1-KR, 3-KA, and 7-SL increased the most within four days as 1.645 log CFU / mL, 1.485 log CFU / mL, and 1.275 log CFU / mL (Fig. 1). The remaining bacteria 4-KR, 6-SL, and 8-SL had grown as much as 0.665 log CFU / mL, 1.285 log CFU / mL, and 0.395 log CFU / mL respectively. Bacteria 2-KA and 5-KR had shown decrease as much as 2.170 log CFU/ mL and 0.020 log CFU / mL in growth.

Spectrophotometric Method (Optical Density)

Based on the results (Fig. 2), only four of the eight bacterial samples grew significantly ($p<0.05$) after 4 days of incubation. Bacteria 3-KA, 7-SL, and 8-SL had grown the most; as much as 4.480 log CFU / mL, 6.560 log CFU / mL, and 8.085 log CFU / mL respectively but bacteria 1-KR, 4-KR, 5-KR, and 6-SL had just grown as much as 1.345 log CFU / mL, 1.100 log CFU / mL, 2.410 log CFU / mL, and 4.310 log CFU / mL respectively. However, the growth of bacteria 2-KA decreased to 0.659 log CFU / mL.

According to the correlation of screening results from the spread plate method and the optical absorption method, bacteria 3-KA and 7-SL are better at adapting themselves compared to the rest of the bacteria and they utilize catechol to generate acetyl-CoA as it is important for the growth of bacteria in the Tricarboxylic acid cycle (TCA) to generate ATP and CO, as final products (Barrrios-Martinez *et al*. 2006). However, catechol has become a growth inhibitor to bacteria 2-KA due to the toxicity of catechol to bacterial cells (Park *et al*. 2001).

Bacteria Identification by API Kit 20NE

Two bacterial isolates were chosen from the screening results from the spread plate and optical absorption methods. These isolates were successfully identified using the API 20NE Kit (Table 2). The results obtained from the kit identified these isolates as belonging to the *Pseudomonas* and *Agrobacterium* sp (Helmut 2002; Koutny *et al*. 2003).

Growth Profile of Bacteria

Growth of bacteria *Pseudomonas pickettii* and *Agrobacterium radiobacter* was studied further. From the results of the experiment (Fig. 3), there is no lag phase that can be seen from the growth of both bacteria but only an exponent phase, stationary phase and death phase can be observed. *A.radiobacter* shows a far more apparent exponential phase compared to *P.pickettii*. The increase of growth is higher compared to *P.pickettii* in the first day of incubation. It shows that *A.radiobacter* can produce primary metabolites which help in fast growth by degrading catechol (Barrios-Martinez *et al*. 2006). The stationary phase of *P.picketti* starts on the second day but the stationary phase of *A.radiobacter* starts on the first day of incubation. This means that *P.pickettii* needs a longer period of time to adapt itself for survival. The death phase of *A.radiobacter* starts on the second day but the death phase of *P.pickettii* starts on the third day. Due to the insufficient catechol detected it was believed that most of the catechol had been photooxidized and degraded by the bacteria.

The specific growth rate of bacteria was determined from the steepness of the exponential phase from the graph above (Fig.3). Specific growth rate of *P.pickettii* and *A.radiobacter* are as much as 0.011 h^{-1} and 0.045 h^{-1} respectively (Table 3). It shows that the rate of cell division in *A.radiobacter*

is 4 times faster than in *P.pickettii* and therefore the biomass of the cellin *A.radiobacter* is 4 times higher than in *P.pickettii*. It proves that *A.radiobacter* can efficiently turn catechol into cell biomass compared to *P.pickettii*.

Degradation of Catechol

The graph below (Fig. 4) shows the capability of *P.picketti* and *A.radiobacter* in catechol degradation over a duration of 4 days. Catechol concentration in *P.picketti* and *A.radiobacter* cultures drop significantly on the 2nd day of incubation and it drops as much as 21.83 mg / L for *P.pickettii* and 24.889 mg / L for *A.radiobacter*. It proves that *A.radiobacter* can degrade catechol faster compared to *P.pickettii*. It is related to the growth rate of *A.radiobacter* which is 4 times faster than *P.pickettii*. Therefore, *A.radiobacter* is more efficient in catechol utilization compared to *P.pickettii* and this is in agreement with the study done by Struthers *et al.* (1998) that reported *A.radiobacter* was efficient in degrading aromatic compounds.

According to the graph above (Fig. 4), catechol degradation rate, Qs (mgL-¹h-¹) of *P.pickettii* and *A.radiobacter* are 0.910 mgL-¹h-¹ and 1.037 mgL-¹h-¹ respectively (Table 4). It shows the degradation rate of *A.radiobacter* is higher than *P.pickettii* and faster in breaking catechol into simple substances such as ATP and CO.

According to the study of Struthers *et al.* (1998), *A.radiobacter* can degrade atrazine which is a more complex aromatic compound compared to catechol in the presence of sucrose and citrate as the main carbon source. The ability of

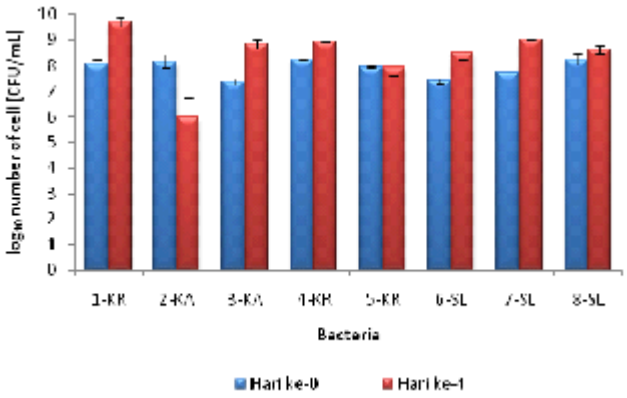

Fig. 1. Growth profiles of eight isolated bacteria. Observation was carried out on the 0th and 4th day of incubation and standard deviation is in the range of ±0.014 to ±0.34

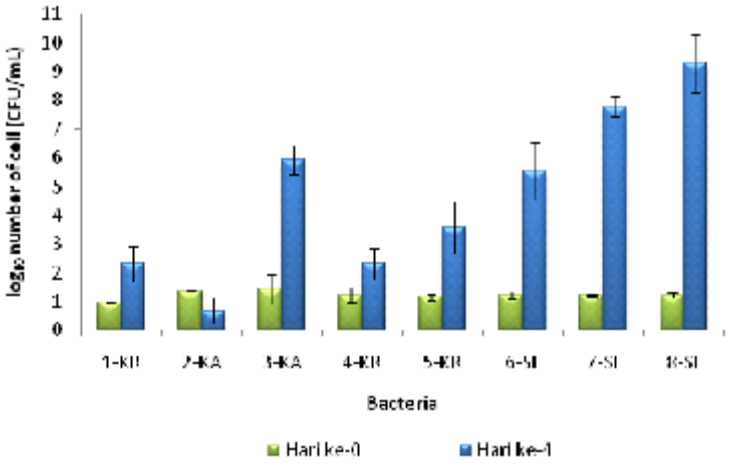

Fig. 2. Growth profile of bacteria on optic adsorption method.Observation on the 0th and 4th day of incubation was done and standard deviation is in the range of ±0.021 to ±1.0

A.radiobacter in degrading aromatic hydrocarbon compounds proves its potential role as a bioremediation agent in catechol decomposition. Aside from that, the study of Koutny *et al*. (2003) has also proven the ability of some microbes such as *Pseudomonas, Agrobacterium, Bukholderia, Acinetobacter, Ralstonia, Klebsiella, Bacillus, Rhodococcus* and *Rhizobium* in degrading phenolic compounds such as catechol thus supporting the outcome of this study where *Agrobacterium* sp. and *Pseudomonas* sp .are able to degrade catechol efficiently.

Enzyme Assay of Catechol Dioxygenase

Based on the results of enzyme assay (Table 5), both bacteria *P.pickettii* and *A.radiobacter* can utilize catechol as the main carbon source due to its ability to produce intracellular enzymes such as catechol 1,2-dioxygenase (C12D) and catechol 2,3-dioxygenase (C23D). C12D enzymes can degrade catechol through *ortho*-cleavage pathway and produce *cis-cis* muconate and other products such as succinate and acetyl-CoA which is necessary in the TCA cycle. On the other hand, C23D enzymes are able to produce 2-hydroxymuconic semialdehyde from catechol through *meta*-cleavage pathway and produce pyruvate and acetaldehyde.

Enzyme activity of C12D detected in *P.pickettii*is 0.042 µmol min^{-1} mL^{-1} and its specific enzyme activity is 0.115 µmol min^{-1} mg^{-1}. Enzyme activity of C23D in *P.pickettii* is 0.001 µmol min^{-1} mL^{-1} and its specific activity is 0.003 µmol min^{-1} mg^{-1}.

The results also show that the enzyme activity of C12D detected in *A.radiobacter* is 0.085 µmol min^{-1} mL^{-1} and the specific enzyme activity is

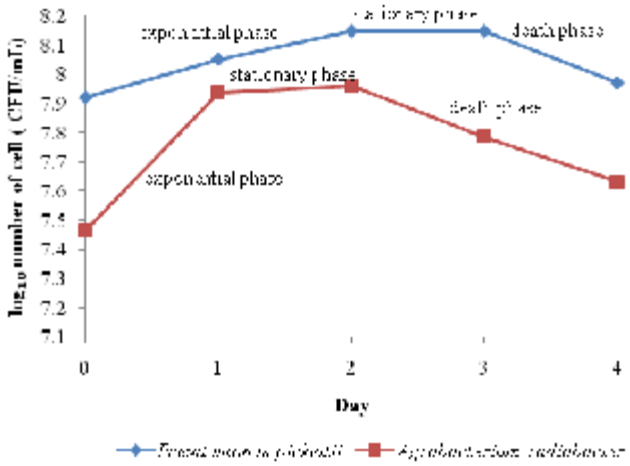

Fig. 3. Growth profiles of *Pseudomonas pickettii* and *Agrobacterium radiobacter* observed over 4 days

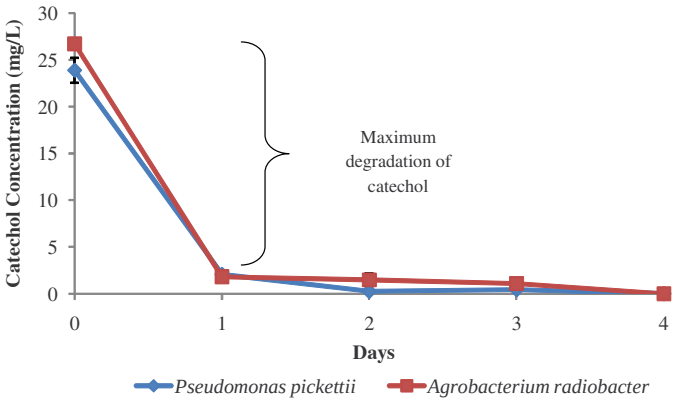

Fig. 4. Catechol degradation of *Pseudomonas pickettii* and *Agrobacterium radiobacter* in 4 days.

0.167 µmol min^{-1} mg^{-1}. Enzyme activity of C23D in *A.radiobacter* is 0.002 µmol min^{-1} mL^{-1} and the specific activity is 0.004 µmol min^{-1} mg^{-1}.

Enzyme activity and the specific activity of both C12D and C23D in *A.radiobacter* is higher than *P.pickettii*. It shows that *A.radiobacter* is much more effective in degrading catechol due to the higher amount of enzymes that convert 1 µmol of catechol into *cis-cis* muconate or 2-hydroxymuconic semialdehyde per minute. Besides that, C12D enzyme can mostly be found in microorganisms (Broderick & O'Halloran 1991) and it supports the results of this study which states that the degradation of catechol in both bacteria is more to *ortho*-cleavage pathway than *meta*-cleavage pathway due to the presence of C12D enzymes in higher amounts.

Presence of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase in both bacteria proves their capability in degrading catechol aerobically through *ortho* and *meta*-cleavage pathway (Barrios-Martinez *et al*. 2006).

CONCLUSION

Eight bacteria were isolated from three different locations at the PETRONAS Oil Refinery Plant in Kerteh, Terengganu. Two bacteria were chosen from the correlation results from spread plate and optical density method as possible candidates for cathecol degradation. Both bacteria were identified as *Pseudomonas pickettii* (88.3%) and *Agrobacterium radiobacter* (99.8%) via API Kit 20NE. Based on the growth profile and enzyme activity and specificity of the two isolates, *A.radiobacter* was found to be a better candidate in degrading catechol.

ACKNOWLEDGMENTS

The researchers would like to thank Prof Dr Ainon Hamzah for allowing use of her laboratory. We would also like to thank University Kebangsaan Malaysia for the provision of an Industry Grant (INDUSTRI-2011-025- Isolation and Characterization of Phenol Degrading Bacteria from Oil Contaminated Soil) to Prof Dr Ainon Hamzah and Assoc Prof Dr Kalaivani Nadarajah for the purchase of research material to conduct the research.

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