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SCREENING OF CATECHOL DIOXYGENASE GENE AMONG BACTERIAL COMMUNITIES ISOLATED FROM ANTHROPOGENIC CONTAMINATED AREA IN PAHANG, MALAYSIA

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Abstract

The development of industrial, recreational, and urban areas in Pahang has introduced hydrocarbon pollution into the environment. The hydrocarbons impart a significant effect on the environment and human health, in both short- and long-term effects. Long-term exposure to these environmental pollutants may enhance the degradative abilities of the indigenous bacterial communities through the exhibition of catechol dioxygenase; an enzyme that can degrade hydrocarbons. In this study, bacteria communities that are tolerant towards hydrocarbons were successfully isolated and identified from two contaminated rivers and one pristine area. The presence of the catechol dioxygenase gene from the isolated bacterial samples was then screened using Polymerase Chain Reaction (PCR) amplification. A total of 33 hydrocarbon-tolerant bacteria were isolated from both contaminated and pristine areas. Five genera isolated from the contaminated areas; the *Bacillus*, *Lysinibacillus*, *Aeromonas*, *Shewanella*, and *Pseudomonas* strains were detected to harbour the catechol dioxygenase gene. Meanwhile, no catechol dioxygenase gene was detected in bacterial samples isolated from pristine area. Results obtained from the screening of the catechol dioxygenase gene can provide preliminary insight regarding the presence of catabolic enzymes particularly in different anthropogenic areas, which could provide a better understanding regarding the potential of catechol dioxygenase in eliminating toxic pollutants from the environment.

INTRODUCTION

In this era of the industrial revolution, anthropogenic activities including mining, refinery and petrochemical, and ship industries have become crucial to the country's economic development. Nevertheless, the introduction of wastewater effluents discharged from these industries into water bodies has turned into a great concern of pollution [1]. Pollution affects millions of people around the world direct or indirectly. One of the main pollutants introduced by anthropogenic activities including hydrocarbons. Hydrocarbons can be defined as a group of persistent, toxic, mutagenic, and carcinogenic substances that pose significant risks to human health and the environment [2,3]. The release

of hydrocarbon into the environment, either by accidental spillage or heavy rain runs off, can cause contamination in soil and water. It can affect the growth and development of terrestrial plants and also can exert acute toxic effects on marine organisms [4,5]. Exposure of hydrocarbons to humans also can affect the health, short-term, including eye and skin irritation [6], and long-term which can disturb the function of the immune system and internal organ damage [7].

Fortunately, the emergence of hydrocarbon-degrading bacteria that are able to produce a specific type of enzyme, such as the catechol dioxygenase enzyme has offered a convincing solution in eliminating hydrocarbons. This bacteria was a result of an adaptation in a hydrocarbon-

contaminated environment, which allows the exhibition of a new enzyme that is able to facilitate the degradation of toxic substances [8]. The catechol dioxygenase enzyme is responsible for cleaving hydrocarbon aromatic rings by incorporating two oxygen atoms and yielding a by-product that can be utilized by the bacteria as carbon and energy sources, converting hydrocarbon into a non-toxic compound [9]. This enzyme can be found in various bacteria species, such as *Pseudomonas* [10,11], *Sphingomonas*, *Acinetobacter*, *Nocardia*, *Rhodococcus*, and *Bacillus* sp. [12,13], which are commonly found in an anthropogenic contaminated area. As this enzyme is responsible for the degradation of hydrocarbons from the environment, the detection of the gene is vital to provide early hindsight into the potential of this enzyme as a bioremediation agent to restore contaminated sites into a better environment.

In Pahang, two different anthropogenic sites have been identified, which are Sungai Tunggak and Sungai Lembing. Sungai Tunggak is located in Gebeng Industrial Park which is surrounded by a number of chemical and petrochemical industries that are producing lots of effluents, especially hydrocarbons and the effluents discharged into this adjacent river resulting in the contamination of the river [14] become a strategic site for the manifestation of hydrocarbon-degrading bacteria to harbour the catechol dioxygenase. Meanwhile, Sungai Lembing is located at an abandoned mining site which is commonly contaminated with heavy metal. However, few studies found the presence of the catechol dioxygenase gene in bacteria species isolated from heavy-metal contaminated sites [15] indicating the possibility of heavy metal-tolerant bacteria to also manifest the same degrading enzyme as hydrocarbon-degrading bacteria. Even though some studies have reported concerning the various contamination event in Sungai Tunggak and Sungai Lembing [16,17], however not much study has been conducted regarding catechol dioxygenase enzyme caused it to have an unclear gap on the presence of this enzyme in bacteria isolated from these anthropogenic areas, thus led to the objective of this study. This study was aimed to screen for the catechol dioxygenase gene, mainly catechol 2,3-dioxygenase in bacteria isolated from water and sediment samples collected from these two different anthropogenic contaminated areas in Pahang as a preliminary study of the variability of catechol dioxygenase gene in degrading various types of contaminants.

MATERIALS AND METHODS

Sample Collections

In this study, water and sediment samples were collected from two different sites that represent the anthropogenic area in Pahang. The sites including Sungai Tunggak, located in Gebeng Industrial Park (between 30° 56' 06" to 30° 59' 44" N and 103° 22' 42" to 103° 24' 47" E) [18], and Sungai Lembing, located at abandoned mining site (3° 54' 23" N and

103° 2' 30" E) [16]. A control sample was also collected from Lang Tengah Island, as a representative of the unpolluted pristine area. Water samples were collected from 3 checkpoints for each site using HDPE sterile sample bottle. Sediment samples from the same checkpoints were also collected at a depth of 1 -10 cm from the surface and stored in a sterilized zip lock bag. All collected samples were taken to the laboratory and stored at 4°C before processing. All samples were processed inside the laminar flow for sterile conditions, and all equipment used during sampling and prior sample processing was sterilized by autoclave or wiped using ethanol to minimize contamination.

Isolation of Bacterial Isolates

Sediment Sample

Five grams of sediments were suspended in a minimal salt medium (MSM) prepared according to the method described by Boboye, et. al. (2010) [19] with some modification. The medium contained (g \cdot L $^{-1}$): Na₂HPO₄·12H₂O 3.78, KH₂PO₄ 0.5, NH₄Cl 5.0, MgSO₄·7H₂O 0.2 and yeast extract 0.01. As much as 1% engine oil was supplemented into the medium as sole carbon source and incubated overnight at 37°C with agitation at 150 rpm [20]. Serial dilution up to 10 $^{-6}$ was performed for all overnight suspensions. As much as 50 μ l of sample from dilution factor 10 $^{-4}$, 10 $^{-5}$, and 10 $^{-6}$ was plated on MSM agar plate supplemented with 1% engine oil and were incubated for 24h, 48h, 72h, and 96h. The growth and the morphology of bacterial isolates on each plate were observed and recorded.

Water Sample

A vacuum filtration technique was implemented to filter 2L of water samples collected. The water sample was filtered using a 0.22 μ m membrane filter to isolate as many bacterial colonies as possible. The membrane filter containing filtered bacterial samples was placed onto a nutrient agar plate and was incubated at 37°C for 24h and 48h. Colonies grown from each plate were sub-cultured onto a fresh nutrient agar plate. The newly sub-cultured agar plates were incubated at 37°C overnight. The colony of bacterial samples from the nutrient agar was then sub-cultured on an MSM agar plate supplemented with 1% engine oil at 37°C to screen for bacteria colonies that were able to grow on hydrocarbon. The growth of the bacteria that were able to grow on hydrocarbon was recorded.

Bacterial Identification

Morphological Characterisation

Morphological characteristics of a single colony obtained were observed using a dissecting microscope (Stemi DV4,

ZEISS). The colour, margin, form, and elevation of the colony were observed and recorded.

Biochemical Characterisation

Gram staining for each colony was conducted to differentiate the bacteria based on the Gram reaction. The staining was done according to the standard Gram staining procedure. The colony stained was observed under a light microscope using 1000X magnification.

Molecular Characterisation

Bacterial genomic DNA was extracted using a GF-1 bacterial DNA extraction kit (Vivantis, Selangor, Malaysia) according to manufactured procedures. Extracted DNA was used as a template for 16s rRNA identification using a set of universal primers that are highly conserved among prokaryotes. The universal primers used were as follows, forward primer: 5' – GAT AGG ATG ATG AAA GGC TG – 3', and reverse primer: 5' – CCT TCA TTA AGG CCG CAA ACT – 3'. Reaction mixtures were set up on ice and consist of 12.5 µL of 2X GoTaq Green Mastermix (Promega, Wisconsin, US), 1.0 µL of each primer, <250 ng of DNA template, and sterile distilled water to the final volume of 25 µL. The mixtures were set up in thermocycler at 95°C for 4 min. Then, followed by 25 cycles reaction at the following temperature: 95°C for 2 min, 55°C for 1 min, 72°C for 1 min, and were finalized with 72°C incubation for 10 min. The amplified 16s rRNA product was visualized on 1% agarose gel for approximately 60 min run at 100v. Amplicon with 1500bp expected size was observed.

DNA Sequencing

The amplified DNA fragment was excised and purified from agarose gel using Gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany) following the provided procedure. The purified product was sent to Apical Scientific Sdn Bhd for sequencing services.

Phylogenetic Analysis

The sequence obtained was analysed using the Basic Local Alignment Search Tool (BLAST) provided by National Center for Biotechnology Information (NCBI) sequence database to obtain the sequence similarity. The sequence of all samples was aligned using the ClustalW algorithm and a phylogenetic tree was constructed based on the retrieved sequence by maximum-likelihood methods. Both the alignment and phylogenetic tree method was done using Molecular Evolutionary Genetics Analysis (MEGA) version 10.1.

Screening for Catechol dioxygenase Gene

Catechol 2,3-dioxygenase (C23O) gene was amplified from the samples using a pair of primers, forward primer: 2,3D_zewF (5' – ATG AAA AAA GGC GTA ATG CGC – 3') and reverse primer: 2,3D_zewR (5' – AGC ACG GTC ATG AAA CGT TCG TTC – 3') which was designed by Wojcieszynska et al. (2011) [12] based on high conserve sequence of catechol 2,3-dioxygenase, allowing the amplification of the entire gene with an expected size of 916 bp. The reaction was prepared to contain 12.5 µL of 2X GoTaq Green Mastermix (Promega, Wisconsin, US), with 2.5 µL of each primer (10 µM), <250ng of DNA template, and sterile dH₂O up to 25 µL final volume. The condition for the reaction was set up at 94°C for 5 min of initial denaturation, proceeded with 25 cycles of 94°C denaturation for 1 min, 50°C to 55°C of annealing for 30 sec, and 72°C of extension for 1 min. The reaction was finalised with 5 min of additional elongation at 72°C. The amplicon was observed on 1% agarose gel and the presence of catechol 2,3-dioxygenase gene among the isolates was identified at 916 bp expected size.

RESULTS AND DISCUSSION

Adaptation of indigenous bacteria in highly polluted conditions has an important role in the biodegradation of contaminants from the environment [16,18], making the detection of the catechol dioxygenase essential to further understand the degradation potential in these bacteria. Catechol dioxygenase is an important key enzyme for the aerobic degradation of hydrocarbons in various bacterial pathways [21]. In this study, a total of 33 bacterial isolates were successfully isolated from both contaminated and pristine study sites and molecularly characterised using 16s rRNA identification methods. Fifteen isolates were identified from Sungai Tunggak, 11 isolates from Sungai Lembing, and 7 isolates from Lang Tengah. The characterisation of all samples was summarised in Table 1, Table 2 and Table 3, respectively.

The screening of the catechol dioxygenase gene was also conducted using specific catechol 2,3-dioxygenase primers. From Sungai Tunggak samples, 8 bacterial isolates were detected to manifest catechol 2,3-dioxygenase gene (Figure 1) where 3 bacterial isolates were identified as *Bacillus* sp., 2 isolates were identified as *Aeromonas* sp., and 3 isolates were identified as *Pseudomonas* sp., *Rhodocyclaceae* bacterium, and *Shewanella* sp., respectively, as shown in the phylogenetic analysis (Figure 2). Out of all samples detected, *Bacillus* sp. and *Aeromonas* sp. were observed to be the most isolated species from Sungai Tunggak. Subsequently, our finding for Sungai Lembing samples shows positive results for catechol 2,3-dioxygenase screening in 3 bacterial isolates (Figure 3). As shown by the arrow in Figure 4, 2 isolates were identified as *Bacillus* sp., and 1 sample was identified as *Lysinibacillus* sp., which also originated from the same

family as *Bacillus*. For Lang Tengah control samples, 7 bacteria were isolated (Figure 5), but no catechol 2,3-dioxygenase was detected to be manifested in the isolated

bacteria (Figure 6) which as expected as Lang Tengah is not an anthropogenic area.

Table 1. Characterisation of Sungai Tunggak bacterial samples based on 16s rRNA identification and the presence of C23O gene

ID	16s rRNA identification	Query cover (%)	Percent similarity (%)	Presence of C23O
ST1	<i>Oceanimonas baumannii</i>	63	77.76	-
ST2	<i>Aeromonas veronii</i>	87	86.99	Yes
ST4	<i>Bacillus anthracis</i>	98	96.77	Yes
ST5	<i>Aeromonas tecta</i>	75	77.25	-
ST8	<i>Aeromonas</i> sp.	64	83.36	-
ST9	<i>Aeromonas veronii</i>	98	90.67	-
ST10	<i>Shewanella</i> sp.	95	93.76	Yes
ST11	<i>Aeromonas</i> sp.	73	91.03	Yes
ST12	<i>Bradyrhizobium</i> sp.	16	75.86	Yes
ST13	<i>Shewanella</i> sp.	87	78.77	-
ST14	<i>Bacillus subtilis</i>	47	90.09	Yes
ST16	<i>Rhodocyclaceae</i> bacterium	46	88.07	Yes
ST17	Uncultured aeromonadales	51	87.60	-
ST24	<i>Pseudomonas alcaliphila</i>	99	96.84	Yes
ST25	<i>Aeromonas veronii</i>	97	98.85	-

Table 2. Characterisation of Sungai Lembing bacterial samples based on 16s rRNA identification and the presence of C23O gene

ID	16s rRNA identification	Query cover (%)	Percent similarity (%)	Presence of C23O
SL2	<i>Bacillus</i> sp.	99	98.75	-
SL3	<i>Bacillus altitudinis</i>	95	99.08	-
SL4	<i>Bacillus subtilis</i>	99	97.53	-
SL7	<i>Lysinibacillus</i> sp.	98	97.44	Yes
SL8	<i>Bacillus</i> sp.	94	98.75	-
SL9	<i>Bacillus amyloliquefaciens</i>	87	84.93	-
SL10	<i>Bacillus thuringiensis</i>	99	99.77	Yes
SL11	<i>Bacillus altitudinis</i>	86	96.81	-
SL18	<i>Bacillus</i> sp.	85	80.64	Yes
SL20	Bacterium	94	97.46	-
SL21	<i>Ralstonia picketti</i>	74	92.05	-

Table 3. Characterisation of Lang Tengah bacterial samples based on 16s rRNA identification and the presence of C23O gene

ID	16s rRNA identification	Query cover (%)	Percent similarity (%)	Presence of C23O
LT1	<i>Microbacterium oxydan</i>	99	97.29	-
LT2	<i>Cellulosimicrobium funkei</i>	98	97.01	-
LT3	<i>Bacillus pumilus</i>	63	92.72	-
LT4	<i>Bacillus altitudinis</i>	97	99.30	-
LT5	<i>Bacillus sporothermodurans</i>	99	98.82	-
LT6	<i>Brevibacillus antibioticus</i>	89	96.28	-
LT7	<i>Cellulosimicrobium sp.</i>	98	99.49	-

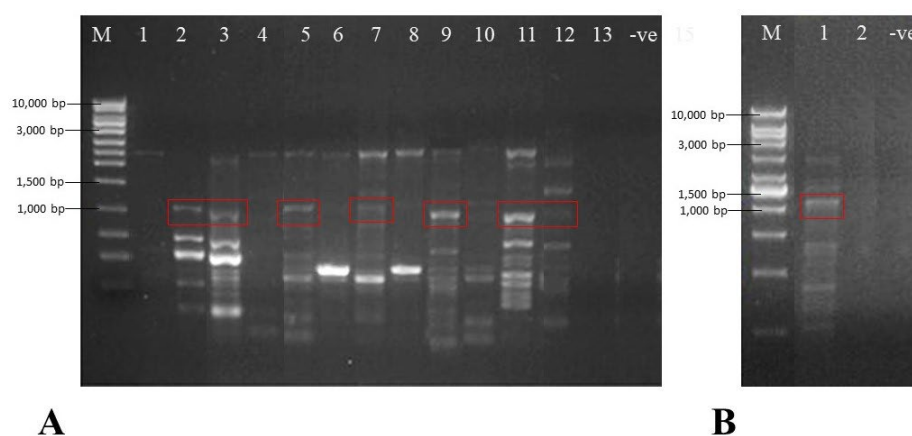


Figure 1. PCR products of the catechol 2,3-dioxygenase gene amplification of bacteria from Sungai Tunggak on 1% agarose gel stained with ethidium bromide at 1000 bp. In (A), lane M: 1kb marker, lane 1: sample ST1, lane 2: sample ST2, lane 3: sample ST4, lane 4: sample ST5, lane 5: sample ST8, lane 6: sample ST9, lane 7: sample ST10, lane 8: sample ST11, lane 9: sample ST12, lane 10: sample ST13, lane 11: sample ST14, lane 12: sample ST16, lane 13: sample ST13, lane -ve: Negative control. In (B), lane M: 1kb marker, lane 1: sample ST24, lane 2: sample ST25, lane -ve: Negative control. Isolates with catechol 2,3-dioxygenase gene shown in red box

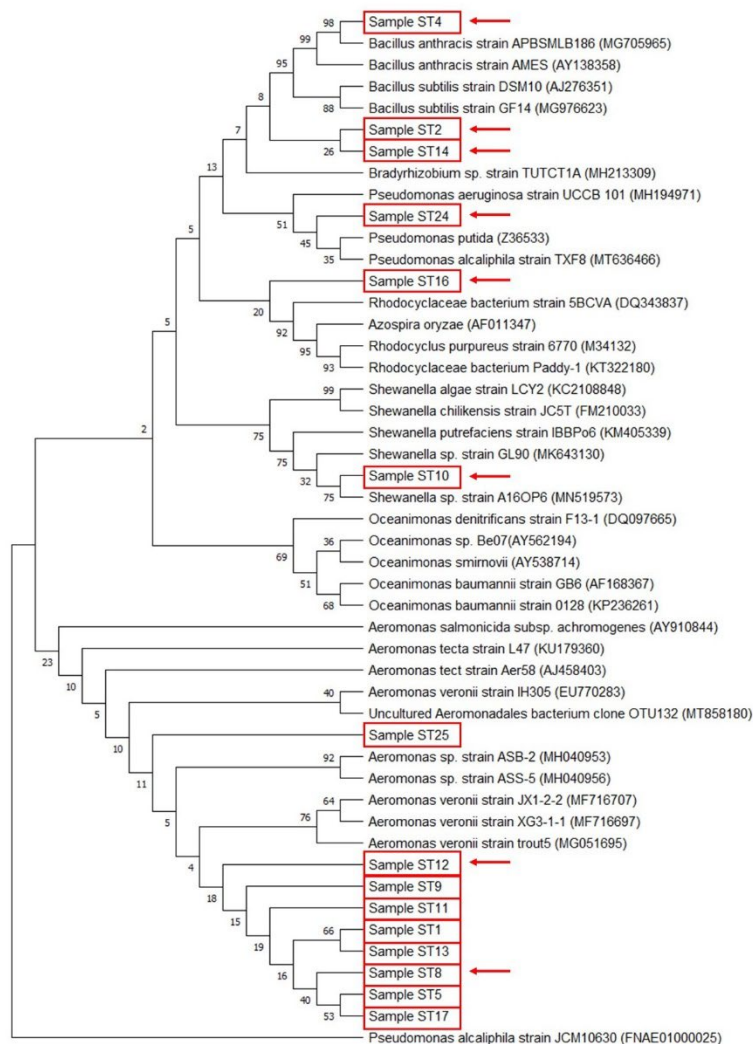


Figure 2. Phylogenetic analysis of 16S rRNA gene sequence for bacteria isolated from Sungai Tunggak samples (showed in boxes) using Maximum-likelihood (ML) method. Arrow indicates isolates with catechol 2,3-dioxygenase gene

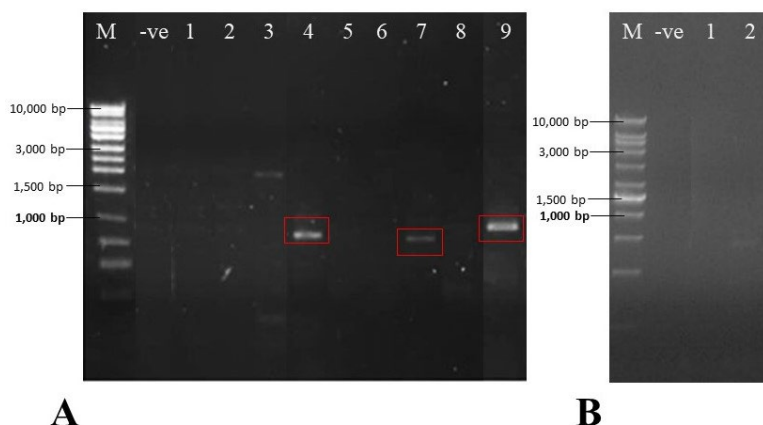


Figure 3. PCR products of the catechol 2,3-dioxygenase gene amplification of bacteria from Sungai Lembing on 1% agarose gel stained with ethidium bromide at 1000 bp. In (A), lane M: 1kb marker, lane -ve: Negative control, lane 1: sample SL2, lane 2: sample SL3, lane 3: sample SL4, lane 4: sample SL7, lane 5: sample SL8, lane 6: sample SL9, lane 7: sample SL10, lane 8: sample SL11, lane 9: sample SL18. In (B), lane M: 1kb marker, lane -ve: Negative control, lane 1: sample SL20, lane 2: sample SL21. Isolates with catechol 2,3-dioxygenase gene shown in red box

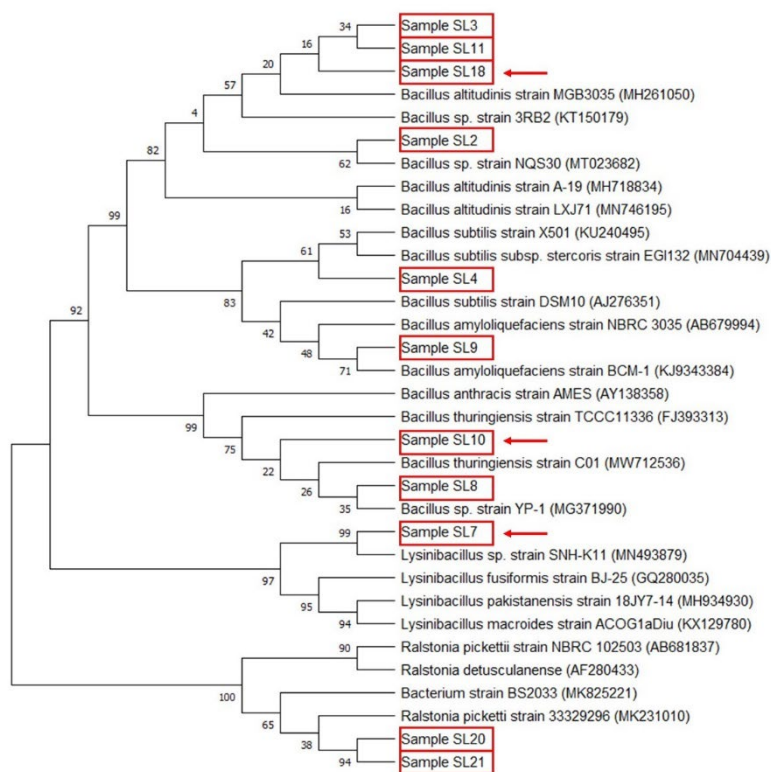


Figure 4. Phylogenetic analysis of 16s rRNA gene sequence for bacteria isolated from Sungai Lembing samples (showed in boxes) using Maximum-likelihood (ML) method. Arrow indicates isolates with catechol 2,3-dioxygenase gene

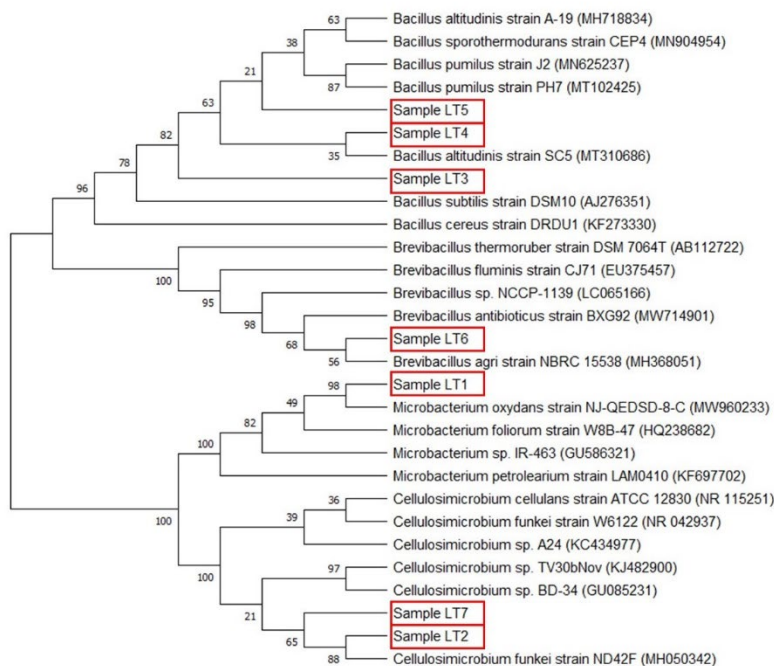


Figure 5. Phylogenetic analysis of 16s rRNA gene sequence for bacteria isolated from Lang Tengah samples (showed in boxes) using Maximum-likelihood (ML) method

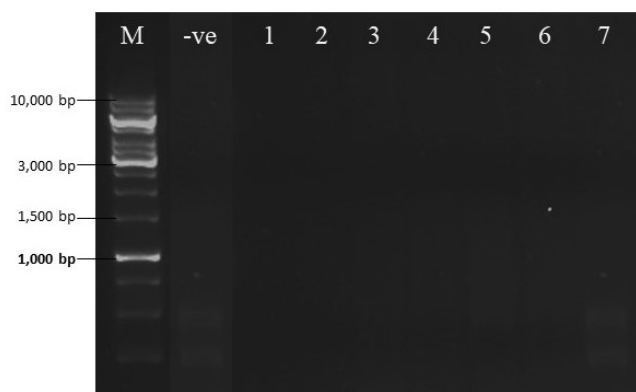


Figure 6. PCR products of the catechol 2,3-dioxygenase gene amplification of bacteria from Lang Tengah pristine area on 1% agarose gel stained with ethidium bromide at 1000 bp. Lane M: 1kb marker, lane -ve: Negative control, lane 1: sample LT1, lane 2: sample LT2, lane 3: sample LT3, lane 4: sample LT4, lane 5: sample LT5, lane 6: sample LT6, and lane 7: sample LT7

Based on the results obtained from both anthropogenic contaminated sites, Sungai Tunggak has been shown to exhibit the highest number of isolates detected with the catechol 2,3-dioxygenase gene compared to Sungai Lembing samples. This is because catechol dioxygenase has been reported to mainly degrade hydrocarbons [8] which as expected as Sungai Tunggak is mainly surrounded by petrochemical industries causing it to expose to a huge number of toxic effluents, especially hydrocarbons [14]. Nevertheless, Sungai Lembing is located at an abandoned mining site and has been reported to be contaminated with heavy metal instead of hydrocarbons [20,21] also shows the manifestation of the catechol 2,3-dioxygenase gene in the isolated bacteria samples. This finding might indicate the ability of catechol dioxygenase to also tolerate other types of contaminants besides hydrocarbon which can be supported by a previous study that shows the presence of catechol dioxygenase gene in a bacteria isolated from a heavy metal-polluted site [15]. Another study also stated that heavy metal contamination [24], which could lead to the assumption that Sungai Lembing might have a trace of hydrocarbon deposited in the river. However, future study needs to be done to prove the presence of hydrocarbon trace in this abandoned mining area.

Bacillus sp. was observed to be the most dominant species with the catechol 2,3-dioxygenase gene detected from both Sungai Tunggak and Sungai Lembing contaminated sites. This finding can be supported by a study conducted by Yan, et. al. (2013) where this bacterial species is able to demonstrate the high activity of catechol 2,3-dioxygenase enzyme reaction when induced with hydrocarbons [25]. A study by Li, et. al. (2020) also reported the high regulation of catechol 2,3-dioxygenase was expressed in the same type of bacteria species after being

induced with petroleum hydrocarbon. Several studies also reported that *Bacillus* sp. has been frequently isolated from different types of anthropogenic contaminated areas, commonly in crude oil drilling and petroleum refinery sites [26] as well as for ore mining sites [27] indicates the abundancy of *Bacillus* sp. as pollutant-degrading bacteria.

In conclusion, a total of 11 indigenous bacteria isolated from 2 different anthropogenic areas in Pahang were found to possess the catechol 2,3-dioxygenase gene that is responsible for the degradation of hydrocarbons pollutant which indicates the development of the particular catabolic enzyme responsible for the degradation activity among bacterial communities in these anthropogenic areas. Catechol dioxygenase also was detected to be manifested in bacteria samples from mining sites which might indicate the ability of the enzyme to degrade various types of pollutants. Based on the identified samples, *Bacillus* sp. was found to be the most abundant in both contaminated areas, which is as expected according to many biodegradation studies conducted. Thus, the findings obtained in this study can be used as a preliminary benchmark for future experimentation where advanced studies need to be pursued to further understand the potential of catechol dioxygenase to eliminate pollutants with the hope to restore Pahang's anthropogenic contaminated area.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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