

## Characterization and evaluation of catechol oxygenases by twelve bacteria, isolated from oil contaminated soils in Malaysia

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### Abstract

**Introduction:** Catechol is a common intermediate compound in aromatic degradation process. Some microorganisms have this potentiality to degrade aromatic hydrocarbons by catechol dioxygenases to less toxic compounds with ability of entering the tricarboxylic acid cycle. In the present study, the catechol oxygenase activity was measured for 12 crude oil degrader bacteria.

**Materials and methods:** Catechol oxygenase activity of two enzymes includes catechol 1, 2 dioxygenase and catechol 2, 3 dioxygenase were determined using spectrophotometer at 260 nm and 375 nm, respectively.

**Results:** The highest enzyme activity for catechol 1, 2 dioxygenase by *Bacillus cereus* UKMP-6G was (0.07 U/mL) and about catechol 2, 3 dioxygenase was 0.031 U/mL by *Rhodococcus ruber* UKMP-5M during the first minute of incubation. Catechol 1, 2 dioxygenase and catechol 2, 3 dioxygenase followed the *ortho* and *meta* pathway, respectively.

**Discussion and conclusion:** The enzyme assay results showed that among 12 examined bacteria, only *R. ruber* UKMP-5M has the ability to use *meta* pathway for degradation and produce 2-hydroxymuconic acid. The other isolates use *ortho* pathway and create *cis*, *cis*-muconic acid.

**Key words:** Catechol oxygenase, Degradation, Toluene, Enzyme assay

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## Introduction

Many bacteria have the ability to degrade aromatic hydrocarbons of contaminated sites. The soil microorganisms are potential catalytic sources for biodegradation of organic compounds. A number of bacteria are able to biodegrade toxic organic compounds that improved clean-up of different environments including water, soil and wastewater in aerobic and anaerobic conditions (1). Aromatic hydrocarbons could be degraded to less toxic compounds via conversion into dihydroxylated intermediates such as catechol or substituted catechols and then metabolized by intradiol or extradiol dioxygenases (2).

The aerobic degradation of hydrocarbon pathways consist three steps; in the first step, two hydroxyl groups are introduced into aromatic ring and catalyzed by mono- or dioxygenase to produce dehydroxy aromatic compounds which are usually catechols. Catechols are substrates for the next step of the catabolism by ring cleavage via catechol 1, 2 dioxygenase and catechol 2, 3 dioxygenase (Fig. 1) (3). Subsequently, catechol oxygenases have an important role in fundamental pathway in the carbon biochemical cycle and a high biotechnological potential in treatment of liquid wastes contaminated with aromatics compounds (4).

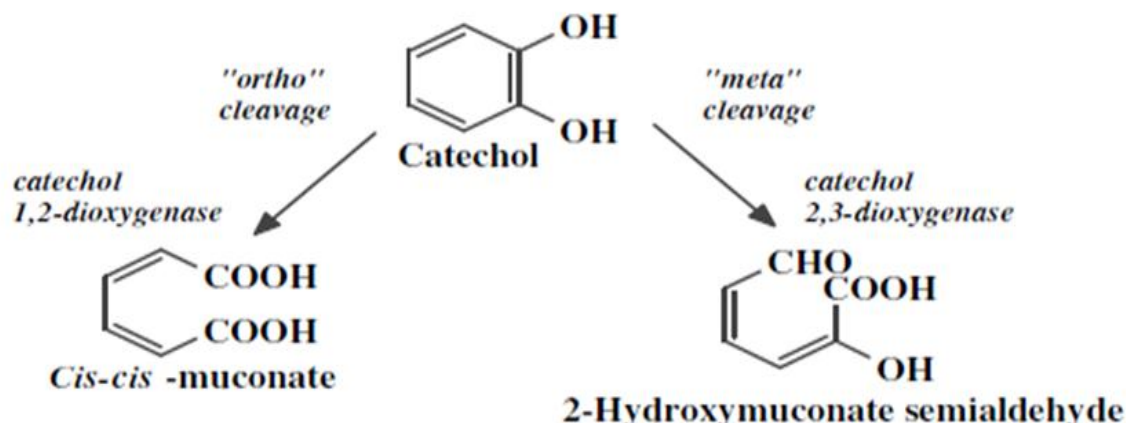


Fig. 1- Catechol degradation via *ortho* and *meta* pathway (7)

Catechol 1, 2 dioxygenase (*ortho* pathway) that also known as  $\beta$ -keto adipate pathway, is  $\text{Fe}^{3+}$  enzymes and catechol 2, 3 dioxygenase (*meta* pathway) is  $\text{Fe}^{2+}$  enzymes. During the last step, the ring cleavage products convert to small aliphatic compounds which metabolized to carbon dioxide during tricarboxylic acid cycle (TCA cycle) (5). Catechol 1, 2 dioxygenase

is an intradiol dioxygenase that incorporate two oxygen atoms between the vicinal hydroxyl groups, while an extradiol dioxygenase, such as catechol 2, 3 dioxygenase, cleaves the aromatic ring of the substrate with two hydroxyl groups and produces muconic semialdehyde (6). Catechol 2, 3 dioxygenase has an important role on biodegradation of alkyl substitution

aromatic hydrocarbons such as xylene and is usually encoded by plasmids (7). Catechol oxygenases are the common biomarker for evaluation of hydrocarbon biodegradation by bacteria. The objective of this study was assessment of catechol dioxygenases in cell-free extracts by 12 bacterial isolates in order to find the appropriate candidate for bioremediation in contaminated sites. All the used bacteria in this study were isolated from groundwater and oil contaminated soils samples which were collected from the Petronas crude oil refinery in Terengganu, Malaysia.

### Materials and methods

**Chemicals:** Toluene and catechol were obtained from Merck Company.

**Isolates and culture conditions:** Twelve isolates had been identified from crude oil contaminated soils in Terengganu, Malaysia. The samples were obtained from culture collection of School of Bioscience and Biotechnology, University Kebangsaan Malaysia (UKM). All the isolates were identified using commercial kit Microbact 24E (Oxoid) according to the manufacture's instruction. The isolates were more confirmed by polymerase chain reaction (PCR) assay targeting 16S rDNA gene. Several isolates (UKMP-5T, UKMP-7T, and UKMP-8T) were also analyzed morphologically and their biochemical and physiological properties were determined (8).

**Preparation of standard inoculums:** The bacterium was grown in nutrient broth and the culture was incubated at 37°C in an orbital shaker (Infors Ht multitron

Germany) at 150 rpm for 18 hours. The culture was harvested by centrifugation (Eppendorf centrifuge 5810 R) at 4000 rpm for 15 minutes at 4°C. The pellet was washed and resuspended with 50 mM phosphate buffer to obtain an optical density (OD) ~ 0.5 at wavelength 550 nm using spectrophotometer (BiowaveII WPA England) (9). This served as the standard inoculums for the subsequent study.

**Sample preparation for catechol oxygenase assay:** The catechol oxygenase activity was performed according to Farrell and Quilty method (10). The activity was measured for two enzymes, catechol 1, 2 dioxygenase and catechol 2, 3 dioxygenase. Catechol 1, 2 dioxygenase activity was determined by increasing the absorbance at 260 nm by spectrophotometer due to the cleavage of catechol into *cis, cis*-muconate (11). Catechol 2, 3 dioxygenase was catalyzed the conversion of catechol to 2-hydroxymuconic semi-aldehyde which increased the absorbance at 375 nm. The culture preparation for catechol oxygenase assay was as follows: Ten percent culture from standard inoculums (v/v) and 0.25 mL of 100 mM toluene as an aromatic hydrocarbon source added into 50 mL Minimal salt medium (MSM) which containing (K<sub>2</sub>HPO<sub>4</sub> 1.8 g, KH<sub>2</sub>PO<sub>4</sub> 1.2 g, NH<sub>4</sub>Cl 4.0 g, NaCl 0.1 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2 g, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.01 g in 1 L distilled water (9) and pH adjusted to 6.5 in 250 mL conical flask. The culture was incubated at 30°C, with shaking at 150 rpm for 5 days. At the end of the incubation, the bacterial cells were centrifuged at 4000 rpm for 20 minutes at 4°C. The pellet was washed

twice and resuspended with 0.33 mM Tris-HCl buffer (pH 7.6). The OD<sub>550nm</sub> of the cells was adjusted to 0.6. The cells were put in ice and sonicated (Sonic material vibra cell) for 20 seconds for 3 times to break the cells and centrifuged at 4°C, 20000 rpm for 20 minutes. The supernatant (cell free extract) was collected and kept in ice to use for catechol dioxygenase assay (10).

**Catechol 1, 2 dioxygenase assay:** The activity of catechol 1, 2 dioxygenase was prepared by mixing 2 mL of 50 mM Tris-HCl buffer (pH 8.0), 0.7 mL distilled water, 0.1 mL of 100 mM 2-mercaptoethanol, 0.1 mL cell free extract and 0.1 mL catechol from 1 mM (Sigma) in sterile tube. The sample was read in a quartz cuvette. A control was prepared by replacing the catechol with distilled water. The formation of *cis, cis* muconic acid from *ortho*-cleavage pathway was monitored using spectrophotometer at wavelength 260 nm for 10 minutes at 1 minute interval. The extinction coefficients for catechol at OD<sub>260nm</sub>=16.800 l/mol/cm (10 and 12).

**Catechol 2, 3 dioxygenase assay:** For the assay, the mixture was prepared in a cuvette as follows: 2 mL (50 mM) Tris-HCl buffer (pH 8.0), 0.6 mL distilled water, 0.2 mL cell-free extract and 0.2 mL of 100 mM catechol. The formation of 2-hydroxymuconic semi-aldehyde from *meta* cleavage pathway was monitored for 10 minutes at 1 minute interval using spectrophotometer at wavelength 375 nm. The extinction coefficients for catechol at

OD<sub>375nm</sub> = 36.000 l/mol/cm (10, 11).

The catechol oxygenase activity was calculated based on formula (13) as follows:

$$\text{Enzyme unit/mL sample} = \frac{\text{Measured Value} \times \text{Test Volume} \times \text{Dilution Factor}}{(\text{Time} \times \text{Concentration Constant} \times \text{Enzyme Volume})}$$

One unit is defined as 1 μmol product (*cis, cis*-muconate or 2-hydroxymuconic semi-aldehyde by catechol 1, 2 dioxygenase or catechol 2, 3 dioxygenase) per minute in one milliliter assay (2 and 14).

## Results

In this study, twelve isolates were used to determine the catechol oxygenase activities (Table 1).

Table 1- Bacteria used in this study

No	Specific name	Genus
1	UKMP-5T	<i>Pseudomonas haemolytica</i>
2	UKMP-8T	<i>Pseudomonas aeruginosa</i>
3	UKMP-1M	<i>Pseudomonas multivorans</i>
4	UKMP-14T	<i>Pseudomonas aeruginosa</i>
5	UKMP-5G	<i>Pseudomonas aeruginosa</i>
6	UKMP-7T	<i>Bacillus sp</i>
7	UKMP-10T	<i>Bacillus sp</i>
8	UKMP-6G	<i>Bacillus cereus</i>
9	UKMP-2M	<i>Acinetobacter baumannii</i>
10	UKMP-12T	<i>Acinetobacter baumannii</i>
11	UKMP-5M	<i>Rhodococcus ruber</i>
12	UKMP-1G	<i>Exiguobacterium lactigenes</i>

**Catechol 1, 2 dioxygenase assay:** The catechol 1, 2 dioxygenase activity for all 12 isolates was measured at 260 nm over a period of 10 minutes at one minute interval (Fig. 2).

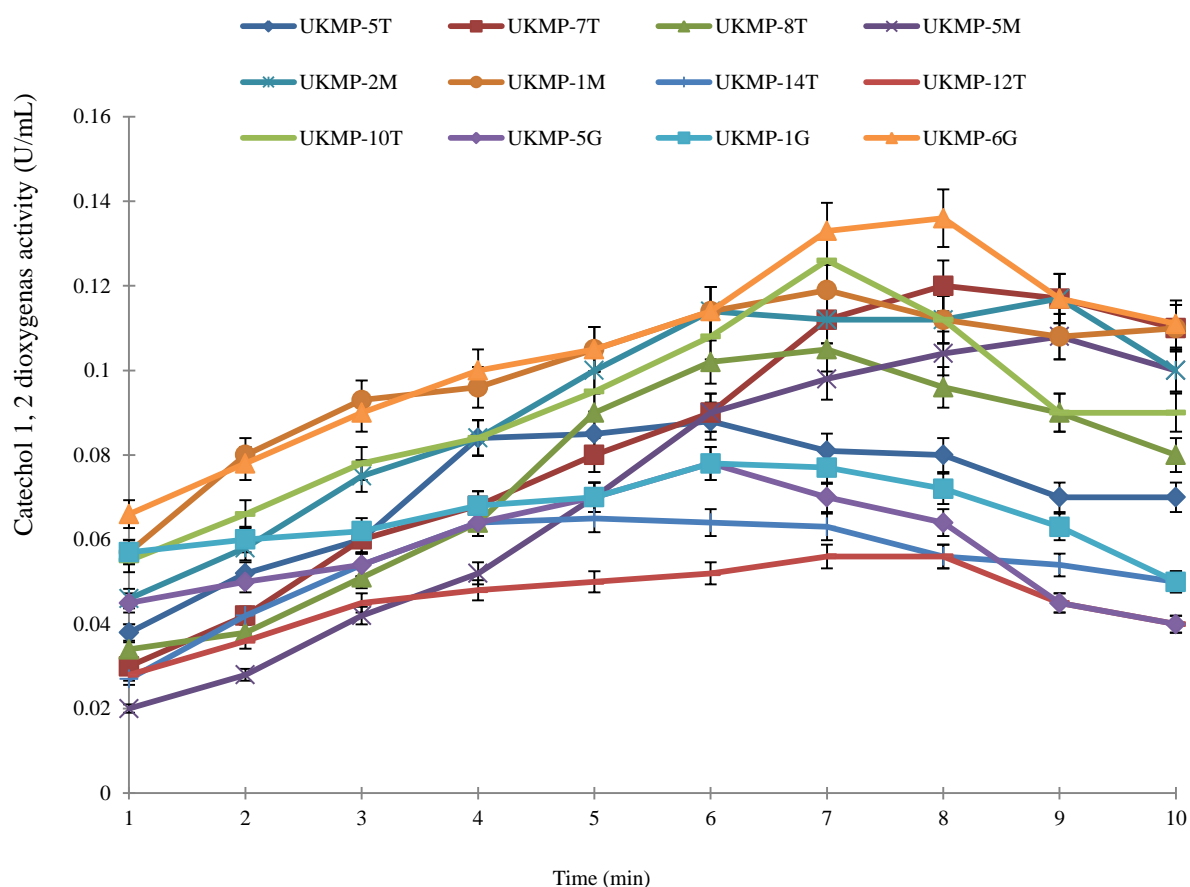


Fig. 2- The catechol 1, 2 dioxygenase activity for all isolates using catechol as substrate

The highest enzyme activity for catechol 1, 2 dioxygenase was from *B. cereus* UKMP-6G (0.07 U/mL) at the first minute while the lowest enzyme activity was elicited in *A. baumannii* UKMP-12T (0.04 U/mL) after 10 minutes reaction. The optimum activity for each one of the isolates was different at range 5 to 9 minutes. For example, the highest catechol 1, 2 dioxygenase activity for *P. aeruginosa* UKMP-14T was 0.07 U/mL after 5 minutes incubation and for *P. haemolytica* UKMP-5T was 0.09 U/mL after 6 minutes. The

highest catechol 1, 2 dioxygenase activity by *Bacillus sp* UKMP-10T was 0.13 U/mL produced for 7 minutes and for *B. cereus* UKMP-6G was 0.14 U/mL after 8 minutes. The catechol 1, 2 dioxygenase activity by *A. baumannii* UKMP-2M was 0.12 U/mL after 9 minutes.

**Catechol 2, 3 dioxygenase assay:** The catechol 2, 3 dioxygenase activities for all 12 isolates was measured at 375 nm over a period of 10 minutes at one minute interval (Fig. 3).

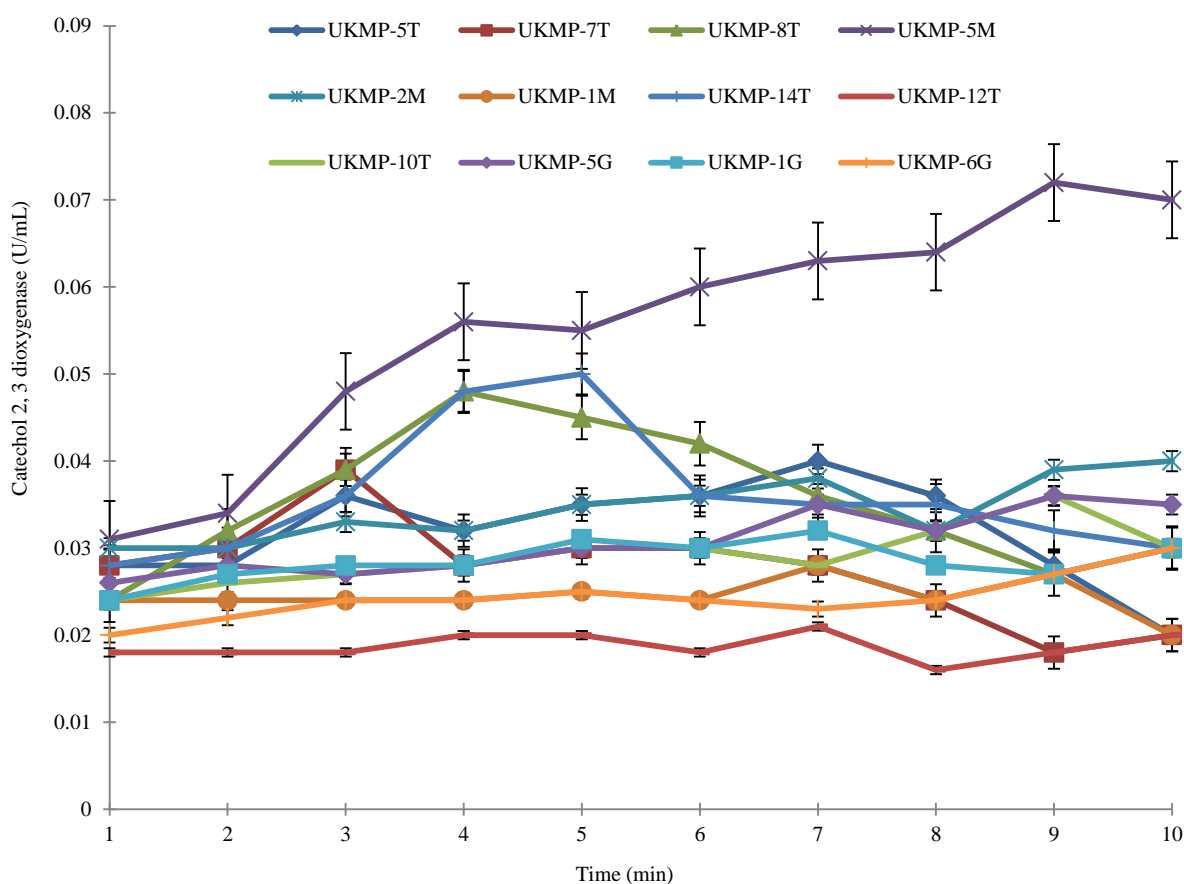


Fig. 3- The catechol 2, 3 dioxygenase activity for all isolates using catechol as substrate.

The highest catechol 2, 3 dioxygenase activity was by *R. ruber* UKMP-5M (0.031 U/mL) at the first minute of reaction, while the lowest was by *A. baumannii* UKMP-12T (0.018 U/mL) after 10 minutes reaction. The optimum activity for each of the isolates was different at ranges 4 to 10 minutes. For example, the highest catechol 2, 3 dioxygenase activity for *P. aeruginosa* UKMP-8T was 0.05 U/mL after 4 minutes incubation and *P. aeruginosa* UKMP-14T was 0.05 U/mL after 5 minutes incubation and *R. ruber* UKMP-5M at 0.08 after 9 minutes. The highest catechol 2, 3 dioxygenase activity by *B. cereus* UKMP-6G was 0.03 U/mL produced for 10 minutes.

The catechol 1, 2 dioxygenase and catechol 2, 3 dioxygenase followed the *ortho* and *meta* pathway, respectively.

### Discussion and conclusion

Many aromatic hydrocarbons such as BTEX (Benzene, Toluene, Ethylbenzene and Xylenes) are main compounds of petroleum crude oil (15). Aromatic hydrocarbons could be biodegraded to less toxic compounds by several steps which were described in bacteria with different pathways. Biodegradation using microbes is an applicable method to reduce hydrocarbons in polluted environments. Most of the microorganism were isolated from petroleum contaminated sites (water

or soil) have shown abilities for oil and hydrocarbon degradation (8). Using microbial enzyme is a simple and benefit method for bioremediation. Toluene as a common hydrocarbon was converted to catechols via toluene oxygenase in upper pathway. Catechol is an important intermediate compound which is degraded into Krebs cycle products during the lower pathway (12). The catechol oxygenase was determined in terms of catechol 1, 2 dioxygenase and catechol 2, 3 dioxygenase. The activity of catechol 1, 2 dioxygenase was determined by monitoring the formation of *cis*, *cis*-muconic acid from catechol through *ortho* cleavage. The Catechol 2, 3 dioxygenase activity was determined by monitoring the formation of 2-hydroxymuconic acid semialdehyde from catechol by *meta* cleavage and further conversion to form, acetaldehyde and pyruvate. The final products of both pathways are molecules which can enter the tricarboxylic acid (TCA) cycle. It is believed that *ortho* pathway catalyze completed degradation of hydrocarbons. The role of *ortho* pathway is more efficient than *meta* pathway for carbon conversion to cell mass (growth yield) (12). In contrast, *meta* pathway is known for incomplete metabolism due to production of dead-end or suicide-metabolites. Nevertheless, *meta* pathway is more capable than *ortho* pathway to degrade alkyl-compounds (10). In general, chromosomal genes encode the *ortho* pathway while plasmids such as TOL plasmids encode the *meta* pathway (16).

The highest catechol 1, 2 dioxygenase activity produced by *B. cereus* UKMP-6G

was 0.07 U/mL which this value is nearby catechol 1, 2 dioxygenase activity by *P. putida* PsU-0 0.083 U/mL and PsU-08 0.077 U/mL (17), but the enzyme activity was lower compared with catechol 1, 2 dioxygenase activity by *Stenotrophomonas maltophilia* strain KB2 that was 1.03 U/mL (18). The catechol 1, 2 dioxygenase activity for *Rhodococcus* sp. NCIM 2891 was 0.6 U/mL (19), *Rhodococcus erythropolis* AN-13 (0.54 U/mL) (20), *P. putida* GJ31 (4 U/mg) (21) and 22 U/mL was determined by *Pseudomonas* sp. JL1 (22). The highest catechol 2, 3 dioxygenase activity determined by *R. ruber* UKMP-5M was 0.031 U/mL which was nearby catechol 2, 3 dioxygenase activity by *P. putida* PsU-0 (0.024 U/mL) and PsU-E1 (0.028 U/mL) (17). However, the catechol 2, 3 dioxygenase activity from *R. ruber* UKMP-5M was much lower than catechol 2, 3 dioxygenase activity by *S. maltophilia* strain KB2 which was 0.37 U/mL (18), *Alcaligenes eutrophus* (0.64 U/mg) and 4/8 U/mL reported by *P. putida* GJ31 (21).

Several factors were involved in catechol oxygenase activities. The product of enzyme can be changed based on used substrate, for example, in *S. aromaticivorans* strain F199, catechol 1, 2 dioxygenase with *ortho* pathway was favored while the cells were utilized toluene and benzoate as substrate (23). *Rhodococcus* strain YU6 (24) and strain 19070 (25) were used benzene to induce the catechol 1, 2 dioxygenase (*ortho* pathway) with higher activity than catechol 2, 3 dioxygenase. In contrast, the bacteria follow the *meta* pathway using xylene (24)

or toluate (25). The growth of *P. putida* in methylcatechols leads to *meta* pathway induction but, the presence of benzoate or catechol induced the *ortho* pathway (17).

Generally chlorocatechols are mineralized via *ortho* pathways while methyl aromatics are commonly mineralized via *meta* pathways (21). Catechol oxygenase activities are decreased to a low level in anaerobic condition (26).

Many bacteria have been used *meta* pathway for hydrocarbons biodegradation such as *Gordonia polyisoprenivorans* (1), *Rhodococcus* sp. strain DK17 (27), *Rhodococcus* sp. strain YU6 (24), *Bacillus* S-5 (28), *P. putida* F1 (29) and *A. calcoaceticus* (30) that were degrade catechol and derivatives by catechol 2, 3 dioxygenase. In contrast, in *Rhodococcus* sp NCIM 2891 (19), *Acientobacter* strain DF4 and W-17 (28), and *P. putida* mt-2 strain paW8 (31) *ortho* pathway was favored.

In most cases, the induction of one catechol oxygenase has an influence to another catechol oxygenase. The catechol metabolism by *meta* pathway are to be active if the *ortho* pathway enzymes are unable to prevent its intracellular accumulation (17). The reverse effect for catechol oxygenase activates showed by *S. maltophilia* (18). The *ortho* pathway enzymes are produced in most strains when the *meta* pathway enzymes have not been induced and then catechol has been accumulated (32). In this study, both catechol 1, 2 dioxygenase and catechol 2, 3 dioxygenase were produced simultaneously but the catechol 1, 2 dioxygenase (*ortho*

pathway) showed higher activity for 11 isolates. In contrast, Bayly and Mckenzie study showed the catechol induction of catechol 2, 3 dioxygenase by *P. aeruginosa* lead to elicit of 1, 2 dioxygenase activity (17).

The maximum catechol oxygenase activity for most of 12 isolates was at 5 to 7 minutes of incubation. In a similar study, the activity of catechol 1, 2 dioxygenase and catechol 2, 3 dioxygenase by *Gordonia polyisoprenivorans* (1) and *Mycobacterium fortuitum* (4) were reduced after 10 minutes of incubation. Study done by Zaki showed catechol 1, 2 dioxygenase activity was the highest after 10 minutes of incubation (28). But the higher time for catechol 2, 3 dioxygenase activity, was reported by *P. fluorescens* was 12 hours (20). In summary, the present study clearly indicates that the described catechol dioxygenases make it useful for bioremediation applications.

#### Acknowledgment

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## ویژگی‌ها و ارزیابی کاتکول اکسیژناز در دوازده باکتری جدا شده از خاک‌های آلوده به نفت در مالزی

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### چکیده

**مقدمه:** کاتکول یک ترکیب حد واسط در مسیر تجزیه ترکیبات آروماتیک است. در برخی از میکروارگانیسم‌ها، کاتکول اکسیژناز هیدروکربن‌های حلقوی را به ترکیبات کم‌خطرتر تجزیه کرده و محصولات ایجاد شده در مسیر چرخه کربس قرار می‌گیرند. در این بررسی، میزان فعالیت کاتکول اکسیژناز برای ۱۲ باکتری تجزیه‌کننده نفت خام ارزیابی شد.

**مواد و روش‌ها:** فعالیت کاتکول اکسیژناز با ارزیابی دو آنزیم کاتکول ۱،۲ دی اکسیژناز و کاتکول ۲،۳ دی اکسیژناز با استفاده از اسپکتروفتومتر در طول موج ۲۶۰ و ۳۷۵ نانومتر تعیین شد.

**نتایج:** بیشترین فعالیت کاتکول ۲،۱ دی اکسیژناز در باسیلوس سرئوس UKMP-6G (۰/۰۷ U/mL) و کاتکول ۳،۲ دی اکسیژناز در رودوکوکوس روبر UKMP-5M (۰/۰۳۱ U/mL) در نخستین دقیقه بررسی شد. این دو آنزیم به ترتیب بیانگر مسیرهای ارتو و متا هستند.

**بحث و نتیجه‌گیری:** نتایج سنجش آنزیمی نشان داد که در میان ۱۲ باکتری ارزیابی شده فقط باکتری رودوکوکوس روبر UKMP-5M از مسیر متا تجزیه را انجام می‌دهد که ۲- هیدروکسی میکونیک اسید تولید می‌شود. سایر باکتری‌های جدا شده با استفاده از مسیر ارتو ماده cis,cis- میکونیک اسید ایجاد می‌کنند.

**واژه‌های کلیدی:** کاتکول اکسیژناز، تجزیه، تولوئن، سنجش آنزیمی

\* نویسنده مسؤل مکاتبات

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