Sains Malaysiana 40(11)(2011): 1231–1235

Detection of Toluene Degradation in Bacteria Isolated from Oil Contaminated Soils

(Pengesanan Pencuraian Toluena oleh Bakteria yang dipencilkan dari Tanah yang dicemari Minyak)

AINON HAMZAH, AREZOO TAVAKOLI & AMIR RABU*

ABSTRACT

Toluene (C_7H_8) a hydrocarbon in crude oil, is a common contaminant in soil and groundwater. In this study, the ability to degrade toluene was investigated from twelve bacteria isolates which were isolated from soil contaminated with oil. Out of 12 bacterial isolates tested, most of Pseudomonas *sp. showed the capability to grow in 1 mM of toluene compared with other isolates on the third day of incubation. Based on enzyme assays towards toluene monooxygenase,* Pseudomonas *aeruginosa UKMP-14T and* Bacillus cereus *UKMP-6G were shown to have the highest ability to degrade toluene. The toluene monoxygenase activity was analysed by using two calorimetric methods, Horseradish peroxidase (HRP) and indole-indigo. Both of the methods measured the production of catechol by the enzymatic reaction of toluene monooxygenase. In the HRP assay, the highest enzyme activity was 0.274 U/mL, exhibited by* Pseudomonas aeruginosa *UKMP-14T. However, for indole-indigo assay,* Bacillus cereus *UKMP-6G produced the highest enzyme activity of 0.291 U/ml. Results from both experiments showed that* Pseudomonas aeruginosa *UKMP-14T and* Bacillus cereus *UKMP-6G were able to degrade toluene.*

Keywords: Degradation; horseradish peroxidase indigo (HRP); indole; toluene

ABSTRAK

Toluena (C_7H_8) hidrokarbon dalam minyak mentah adalah bahan pencemar yang sering terdapat dalam tanah dan air bawah tanah. Dalam kajian ini, kebolehan untuk mencuraikan toluena oleh 12 pencilan bakteria yang dipencilkan daripada tanah tercemar minyak dikaji. Daripada 12 pencilan bakteria yang diuji, kebanyakan Pseudomonas *sp. menunjukkan keupayaan untuk hidup di dalam 1 mM toluena berbanding dengan pencilan lain pada hari ketiga pengeraman. Berdasarkan pengasaian enzim toluena monooksigenase,* Pseudomonas aeruginosa *UKMP-14T dan* Bacillus cereus *UKMP-6G menunjukkan keupayaan tertinggi untuk mencuraikan toluena. Aktiviti toluena monooksigenase diasai menggunakan dua kaedah kalorimetri, iaitu peroksida horseradish (HRP) dan indole–indigo. Kedua-dua kaedah ini mengukur pembentukan katekol oleh tindak balas enzim toluena monooksigenase. Melalui asai HRP, aktiviti enzim yang tertinggi ialah 0.274 U/ml, yang dihasilkan oleh* Pseudomonas aeruginosa *UKMP-14T. Manakala untuk asai indole-indigo,* Bacillus cereus *UKMP-6G menghasilkan aktiviti enzim yang tertinggi iaitu 0.291 U/mL. Hasil kedua-dua eksperimen ini menunjukkan* Pseudomonas aeruginosa *UKMP-14T dan* Bacillus cereus *UKMP-6G berupaya untuk mencuraikan toluena.*

Kata kunci: Indigo; indol; pencuraian; peroksida Horseradish (HRP); toluena

INTRODUCTION

Toluene is a methyl substitution on the aromatic benzene ring, is distributed in water, soils and industrial effluents (Witzig et al. 2006). This compound and some of its derivatives are toxic but microorganisms like *Bacillus, Pseudomonas* and Actinomyces were able to survive in high toluene concentrations and used toluene as a carbon source. Toluene degrading bacteria use different pathways for toluene consumption (Harayama et al. 1999). The oxidative microbes degrade toluene via hydroxylation of the aromatic ring to a mixture of catechols and cresols (Figure 1) (Chaillan et al. 2004).

Toluene monooxygenase, benzyl alcohol dehydrogenase, benzaldehyde dehydrogenase and catechol-2,3-dioxygenase are enzymes involved in the degradation of toluene and are organized in two different pathways. The upper pathway codes enzymes for the conversion of aromatic alcohol to acid, while the lower pathway enzymes involved in the aromatic acid metabolism via an *ortho* and *meta* pathway (Powlowski & Shingler 1994). The second pathway involves ring hydroxylation, yielding methyl catechols as the metabolic intermediate. The key enzyme involved in this pathway is toluene dioxygenase (Harayama et al. 1999).

Catechol derivatives generated from both the side-chain and aromatic ring hydroxylation routes enter the TCA cycle by the meta-cleavage pathway (Basu et al. 2003). In anaerobic conditions, toluene is converted to phenol, cresols and aromatic alcohols with initial oxidation by ring hydroxylation or methyl oxidation (Grbic-Galic & Vogel 1987). The aim of this study was to determine the ability of local bacterial isolates in degrading toluene. Two simple methods were used in assaying toluene dioxygenase activity, which are horseradish peroxidase (HRP) and indole as the key compounds that monitor by color intensity or florescent products.

Materials and Methods

Bacterial Isolates

Twelve bacteria isolates that have been isolated from soil contaminated with oil were used in this study. These isolates included the genus of *Pseudomonas, Rhodococcus, Acinetobacter,* and *Bacillus* sp. (Table 1). The isolates were identified using biochemical tests and sequencing of the 16s rDNA.

Bacterial Growth and Culture Conditions

The bacterial isolate was inoculated in 100 mL nutrient broth (NB) in a 250 mL conical flask. After an overnight incubation at 37°C with shaking at 150 rpm, the culture was centrifuged at 5000 rpm for 15 min. The pellet was added with normal saline for preparation of standard inoculums (Ainon et al. 2010). Subsequently, 10% (v/v) of standard inoculums of each bacterium were transferred into 100 mL of minimal salt media (MSM) with addition of 1.0% (v/v) Tapis crude oil as a sole carbon and energy source.

Determination of Growth Curve

Each of the bacterial isolate was cultured in MSM containing 0.5 mM and 1 mM toluene and incubated for four days at 30°C in an orbital shaker at 150 rpm. The growth was monitored by measuring the absorbance at 550 nm (A_{550}) using a UV- spectrophotometer. The experiment was done in triplicate.

Toluene Oxygenase Assay by HRP Method

The toluene oxygenase activity was assay according to the method of Zhaohui et al. (2003). In this method, after incubation, the cells were centrifuged at 4000 rpm (Eppendorf centrifuge 5810R) for 15 minutes at 4°C and the pellet was washed twice with 50 mM sodium phosphate buffer (pH 7.0). Then, the cells were resuspended in 50 mM sodium phosphate buffer to adjust its optical density at 600 nm to a value of 1.0 using a UV-spectrophotometer. The enzyme assay contains 500 µL cells suspension, 450 µL sodium phosphate buffer (50 mM) and 50 µL toluene. The negative control contains 500 µL cells suspension, 450 µL sodium phosphate buffer and 50 µL distilled water. The mixture was incubated in a screw-capped bottle at 30°C with shaking for 3 h at 150 rpm.

After incubation, the sample was centrifuged at 14,000 rpm for 5 min to separate the cells from the supernatant. The supernatant was transferred into sterile eppendorf tubes, and after the addition of 5 µL of 0.1 mL H_2O_2 and 10 units of horseradish peroxidase (HRP), a reddish brown color was observed. The color production was allowed to proceed at room temperature for 30 minutes and intensity of the mixture was measured at 420 nm using a spectrophotometer (Zhaohui et al. 2003).

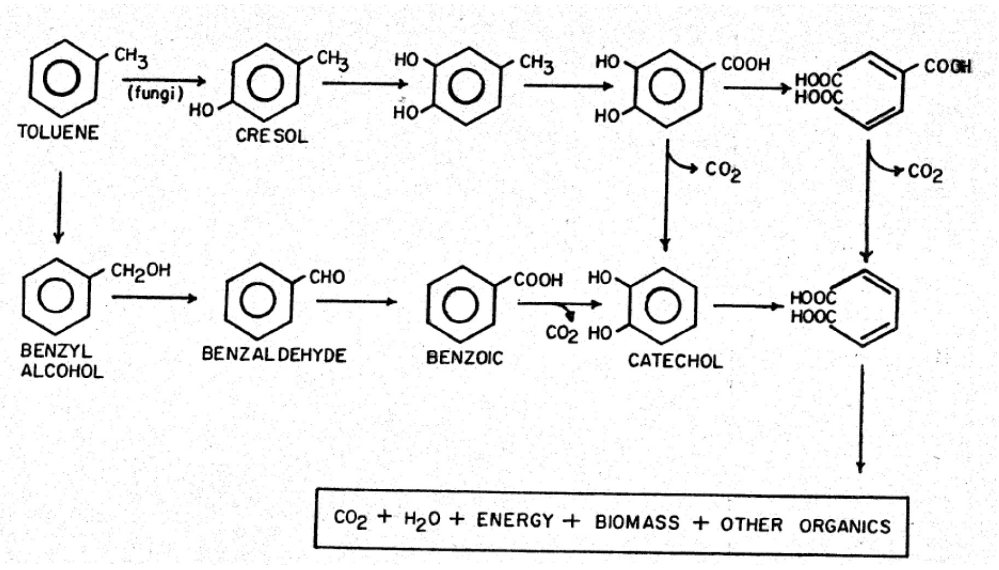

FIGURE 1. Mechanisms of toluene metabolisms

TOLUENE OXYGENASE ASSAY BY INDOLE-INDIGO METHOD

In this technique, at the end of incubation time, the optical density for 1 mL of the culture was determined. The samples were transferred to a micro centrifuge tube immediately. The cells were harvested and resuspended with 50 mM phosphate buffer solution (pH 7.0). Enzyme reaction was started by the addition of 5 µL of 100 mM indole in N,N'dimethyl formamide into the cells and the formation of indigo was monitored using a spectrophotometer at 600 nm (O'Connor & Hartmans 1998; Woo et al. 2000; Zaki 2006).

Results and Discussion

In the present study, the ability of 12 bacterial isolates was determined for their ability to grow in the presence of toluene. Their abilities for toluene degradation were monitored using two methods, horseradish peroxidase (HRP) and indole-indigo method. In the first method, the horseradish peroxidase (HRP) was coupled with hydroxylation of aromatic substrates which give colored and/or fluorescent compounds. The products were easily detected with spectrophotometer. In this experiment, toluene was converted into catechol when it was added to the cells suspension. Catechol reacted with H_2O_2 and also HRP, which produced products that can be monitored by spectrophotometer at wavelength 420 nm. The intensities of color generation indicated the total activity of the oxygenase (Joo et al. 1999; Zhaohui et al. 2003). In indole-indigo method, toluene oxygenase oxidized indole to indoxyl which was spontaneously converted to indigo, a dark blue compound which is easily detected on media. The indoxyl can be monitored at OD 600 nm by using spectrophotometer (O'connor et al. 1997). In dioxygenase-expressing bacteria, the spontaneous elimination of water to yield indoxyl is the general mechanisms for indigo production (Allen et al. 1997).

Twelve bacterial isolates isolated from oil contaminated soil were identified with biochemical and molecular techniques. Five out of 12 isolates were from the *Pseudomona*s species (Table 1). They are known for their ability in aromatic compound degradation. In addition, their ability for toluene degradation was reported by several researchers (Bhushan et al. 2000; O'connor et al. 1997; Woo et al. 2000). The *Pseudomonas* sp. has various metabolisms, which associated to the presence of degradative plasmids such as ALK (alkanes), OCT (octane), XYL (xylene), CAM (alcanphour), NAH (naphthalene), TOL (toluene) and SAL (salicylic acid) (Pérez Silva et al. 2006). It was believed that the presence of TOL plasmid also contributes to the ability to degrade toluene in *Pseudomonas* sp. In addition, toluene is degraded by bacteria via five different pathways (Harayama et al. 1999).

Figure 2 indicates the results of the bacterial growth in 0.5 mM and 1mM of toluene. *Pseudomonas* sp. had a better ability to grow in higher concentrations of toluene than other isolates. *Pseudomonas hemolytica* UKM-5T showed highest growth in the presence of 0.5 mM toluene while *Pseudomonas aeruginosa* UKM-5G in 1 mM of toluene. The highest growth rate for the most of the bacteria was on the third day of the incubation.

TABLE 1. Identification of bacterial isolates by biochemical tests and 16S rDNA sequencing

No	Sample Code	Bacterial Name
1	UKMP-5T	*Pseudomonas hemolytica*
2	UKMP-7T	*Bacillus* sp.
3	UKMP-8T	*Pseudomonas aeruginosa*
4	UKMP-5M	*Rhodococcus ruber*
5	UKMP-2M	*Acinetobacter baumanii*
6	UKMP-1M	*Pseudomonas multivorens*
7	UKMP-14T	*Pseudomonas aeruginosa*
8	UKMP-12T	*Acinetobacter baumanii*
9	UKMP-10T	*Bacillus* sp.
10	UKMP-5G	*Pseudomonas aeruginosa*
11	UKMP-1G	*Exiguobacterium lactigenes*
12	UKMP-6G	*Bacillus cereus*

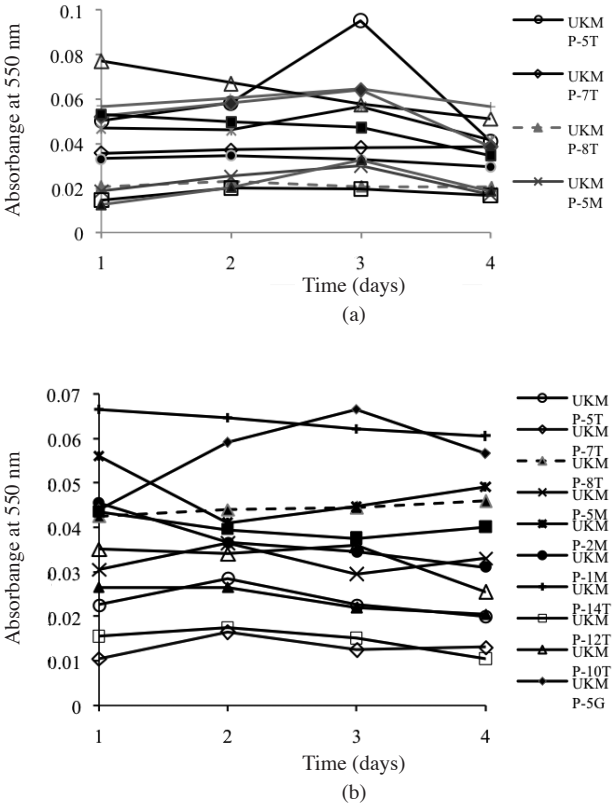

FIGURE 2. The growth at A_{550} of 12 bacterial isolates in the presence of 0.5 mM (a) and 1 mM (b) of toluene concentrations

Table 2 illustrates the results for HRP and indole–indigo assay. The highest enzyme activity was shown by *P. aeruginosa* UKMP-14T using HRP method followed by two *Bacillus* isolates. Meanwhile for the indole-indigo method, the highest toluene oxygenase activity was shown by *Bacillus cereus* UKMP-6G.

TABLE 2. Toluene oxygenase activities produced by bacterial isolates using HRP and indole-indigo method (U/mL)

Bacteria isolate	1	2	3	4	5	6	7	8	9	10	11	12
HRP method	0.101	0.166	0.137	0.173	0.173	0.093	0.274	0.173	0.253	0.253	0.166	0.260
Indole-indigo method	0.273	0.280	0.204	0.054	0.258	0.064	0.204	0.140	0.258	0.215	0.248	0.291

Figure 3 shows the absorbance at 420 nm of the HRP method corresponds to the enzyme activity of the 12 bacterial isolates. Based on the graph, most of the bacterial isolates (except UKMP-2M, UKMP-14T and UKMP-10T) showed increasing in absorbance in the first 90 minutes of incubation and gradually decreased at the end of 150 minutes. The absorbance of *Pseudomonas aeruginosa* UKMP-14T was the highest among other bacteria at 120 min. The range of absorbance of all the bacterial isolates was between 0.01-0.07 which was different from work carried out by Zhaohui et al. (2003). In their work, the range of absorbance was 0.1-0.2. This may be due to the sensitivity of their assays and equipments used.

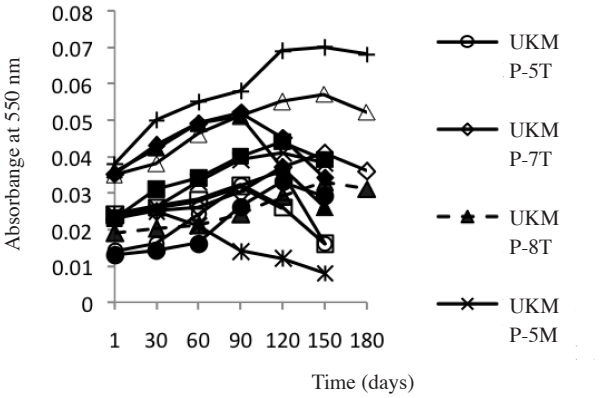

FIGURE 3. The absorbance of HRP assay from bacterial isolates measured at 420 nm

Figure 4 illustrates the absorbance at 600 nm of indole-indigo assay by 12 bacterial isolates. Most of the bacteria (except UKMP-14T) showed moderately increased in the absorption in the first 30 minutes after adding indole. The absorbance of isolate UKMP-14T was the highest (0.052) after 35 minutes of reaction. Study by Woo et al. (2000) showed the maximum absorbance for toluene dioxygenase from *Pseudomonas putida* F1 using indole-indigo method was 0.05 in 10 minutes. Based on these results, enzyme toluene dioxygenase from *Pseudomonas aeruginosa* UKMP-14T had the same activity but the reaction was much slower.

Based on these assays, *Pseudomonas aeruginosa* UKMP-14T and *Bacillus cereus* UKMP-6G were good candidates for toluene degradation. They are indigenous bacteria in soils and have different pathways for toluene degradation. Hence, further studies need to be carried out in understanding the toluene degradation pathways.

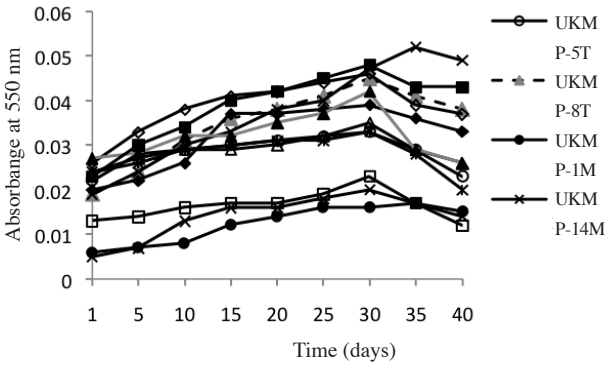

FIGURE 4. The absorbance of indole-indigo assay from 12 bacterial isolates measured at 600 nm

CONCLUSION

Two simple methods, HRP and indole-indigo assay were suitable to be used for determining toluene degradation. Both methods produced color that can be monitored by UV-spectrophotometer. These methods are broadly useful for screening oxygenases enzymes that act on aromatic substrates.

ACKNOWLEDGEMENTS

This research was funded by grant no STGL-003-2007 under Genome Molecular Biology Initiative from MGI, MOSTI and Science Fund 02-01-02-SF0408.

REFERENCES

Ainon Hamzah, Amir Rabu, Raja Farzarul Hanim Raja Azmy & Noor Aini Yussoff. 2010. Isolation and characterization of bacteria degrading Sumandak and South Angsi Oils. *Sains Malaysiana* 39(2): 161-168.

Allen, C.C., Boyd, D.R., Larkin, M.J., Reid, K.A., Sharman, I. & Wilson, K. 1997. Metabolism of naphthalene, 1-naphthol, indene, and indole by *Rhodococcus* sp. strain NCIMB 12038. *Applied and Environmental Microbiology* 63(1): 151-155.

Basu, A., Dixit, S.S. & Phale, P.S. 2003. Metabolism of benzyl alcohol via catechol ortho-pathway in methylnaphthalene-degrading *Pseudomonas putida* CSV86. *Applied Microbiology and Biotechnology* 62: 579-585.

Bhushan, B., Samanta, S.K. & Jain, R.K. 2000. Indigo production by naphthalene-degrading bacteria. *Letters in Applied Microbiology* 31: 5-9.

Chaillan, F., Flèche, A.L., Bury, E., Phantavong, Y.H., Grimont, P. & Saliot, A.O. 2004. Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms. *Research in Microbiology* 155: 587-595.

Grbic-Galic, D. & Vogel, T.M. 1987. Transformation of toluene and benzene by mixed methanogenic cultures. *Applied and Environmental Microbiology* 53(2): 254-260.

Harayama, S., Kishira, H., Kasai, Y. & Shutsubo, K. 1999. Petroleum biodegradation in marine environments. *Journal of Microbial Biotechnology* 1(1): 63-70.

Joo, H., Arisawa, A., Lin, Z. & Arnold, F.H. 1999. A high-throughput digital imaging screen for the discovery and directed evolution of oxygenases. *Chemistry and Biology* 6: 699-706.

O'connor, K.E., Dobson, A.D.W. & Hartmans, S. 1997. Indigo formation by microorganisms expressing styrene monooxygenase activity. *Applied and Environmental Microbiology* 63(11): 4287-4291.

O'Connor, E.K. & Hartmans, S. 1998. Indigo formation by aromatic hydrocarbon-degrading bacteria. *Biochemistry Letters* 20(3): 219-223.

Pérez Silva, R.M., Rodríguez, A.A. & Gómez Montes de Oca, J.M. 2006. Biodegradation of crude oil by *Pseudomonas aeruginosa* AT18 strain. *Tecnologia Quimica XXVI* (1): 70-77.

Powlowski, J. & Shingler, V. 1994. Genetics and biochemistry of phenol degradation by *Pseudomonas* sp. CF 600. *Biodegradation* 5: 219–236.

Witzig, R., Junca, H. Hecht, H.H. & Pieper, D.H. 2006. Assessment of toluene/biphenyl dioxygenase gene diversity in benzene-polluted soils: links between benzene biodegradation and genes similar to those encoding isopropylbenzene dioxygenases. *Applied and Environmental Microbiology* 72(5): 3504-3514.

Woo, H.J., Sanseverino, J., Cox, C.D., Robinson, K.G. & Sayler, G.S. 2000. The measurement of toluene dioxygenase activity in biofilm culture of *Pseudomonas putida* F1. *Journal of Microbiological Methods* 40: 181-191.

Zaki, S. 2006. Detection of *meta*- and *ortho*-cleavage dioxygenases in bacterial phenol-degraders. *Applied Science and Environmental MTG* 10(3): 75-81.

Zhaohui, X., Mulchandani, A. & Chen, W. 2003. Detection of Benzene, Toluene, Ethyl Benzene, and Xylenes (BTEX) using toluene dioxygenase-peroxidase coupling reactions. *Biotechnology Progress* 19: 1812-1815.

Molecular Biology Laboratory
School of Biosciences and Biotechnology
Faculty Science and Technology
Universiti Kebangsaan Malaysia
43600 UKM Bangi, Selangor D.E.
Malaysia

*Corresponding author; email: amirrabu@gmail.com

Received: 18 June 2010
Accepted: 15 August 2010