OPTIMIZATION OF SUBSTRATE AND STARTER CELL CONCENTRATIONS FOR DIBENZOTHIOPENE BIODEGRADATION BY INDIGENEOUS MARINE BACTERIA *Mauricauda olearia* LBF-1-0009, *Alcanivorax xenomutants* LBF-1-0018, and *Stakelama pacifica* LBF-1-0031

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Abstract

Dibenzothiophene (DBT) and its derivatives are widely used-model of organic sulfur compounds for the biodegradation process of petroleum oil. The abilities of microorganisms to degrade pollutants are significantly influenced by various factors such as microbial species, nutrients and environmental parameters. In this research, we conducted a follow-up study to determine the optimum conditions of two parameters affecting DBT biodegradation, namely substrate and starter cell concentrations. Three indigenous marine bacteria isolated from Indonesia's sea environments potentially degrading DBT were examined. The isolates are belong to *Mauricauda olearia* strain CL-SS4 (99%), *Alcanivorax xenomutants* strain JC109 (99%), and *Stakelama pacifica* strain JLT832 (99%). The optimum DBT concentrations act as the growth substrate for all three isolates was 100 ppm, while the optimum cell concentrations for starter inocula was 20 of OD600 nm conversion units.

Keywords: optimization, substrate concentration, starter cell concentration, dibenzothiophene biodegradation, marine bacteria

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Introduction

Dibenzothiophene (DBT) is an organosulfur compound, consisting of two benzene rings fused to a central thiophene ring as illustrated in Figure 1. Therefore, this compound is categorized as one of policyclyc aromatic hydrocarbons (PAHs). DBT is a colourless solid that is chemically similar to anthracene. DBT and its derivatives have been widely used as model of organic sulfur compounds in petroleum (Tec, 2004).

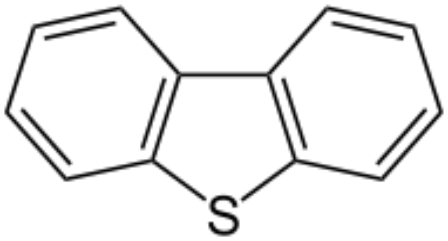


Figure 1. Molecular Structure of Dibenzothiophene

DBTs, along with its products, are normally located in groundwater, seawater, sediments, and soil sites contaminated with petroleum spill and wood preserving wastes (Xu *et al.*, 2006). Moreover, petroleum application in industrial processes, coal gasification, and creasote treatment of wood induce the outspread pollution of these compounds. As heterocyclic compounds, DBT and its derivatives are notably non biodegradable. Accordingly, they can persist in environment up to 3 (three) years after an oil contamination (Seo *et al.*, 2009). These sulfur-substituted compounds, in addition, are mutagenic and carcinogenic; thus pose serious health risks to the contaminated ecosystems (Jensen *et. al.*, 2003).

Dibenzothiophene and its higher molecular weight analogues are commonly found in higher molecular weight fractions of petroleum, but are not pyrogenic and biogenic PAH components. Whereas, the chemical profiles of sulfur heterocycles are well

documented in petroleum products, studies of their biodegradation in petroleum contaminated natural environments are particularly limited. Owing to the economic importance of desulfurization of unprocessed petroleum, detailed studies were performed with various bacterial species. It was discovered that catabolism of dibenzothiophene is catalyzed by distinct enzymes in two pathways (Seo *et al*., 2009).

During the past few years, most study regarding DBT degradation focused on desulphurization or oxidation of dibenzothiophene and its derivatives. Several bacteria from genus *Pseudomonas, Rhizobium, Shingomonas, Xanthobacter,* and *Sphingomonas* were reported as DBT-degrading bacteria. These bacteria were isolated from various crude oil reservoir tanks in Japan (Kitauchi *et al.*, 2004). Several marine bacteria have been reported to have the ability to degrade DBT. Rodrigues *et al.,* (2010) successfully isolated a marine bacterium belonging to the genus *Alkaligene*s that are capable of completing mineralization of dibenzothiophene.

Having said that microbial degradation is a complex process. The abilities of microorganisms to degrade pollutants are significantly affected by various factors such as microbial species, nutrients and environmental parameters (El-Tarabily, 2002; Tabari *et al*., 2010). Only a handful studies have been carried out related to optimization of DBT biodegradation. Lin *et al.* (2014) investigated microbial degradation of DBT by *Pseudomonas* sp. LKY-5 using Response Surface Methodology (RSM) observing some variables such as substrate concentrations, temperatures, pHs, and agitation rates. It is demonstrated that, in PAH biodegradation process by marine bacteria, substrate and cell concentrations are important factors for the rate of biodegradation. These two variables primarily influence the rate of contaminant desorption from environment and the rate of degradation by the microorganisms (Haritash and Kaushik, 2009). Furthermore, these factors can be optimized gradually.

In previous study, we have investigated biodiversity of marine bacteria capable of degrading PAHs including DBT (Yetti *et al*., 2016). In the present study, we conducted a follow up study to determine optimal conditions for DBT biodegradation by indigenous marine bacteria from Indonesia through modulation of substrate and starter cell concentrations.

Materials and Methods

Microorganisms and Culture Condition

The bacterial strains used in this study were LBF-1-0009, LBF-1-0018, and LBF-1-0031; from culture collections of Laboratory of Biocatalyts and Fermentation (LBF), Research Center for Biotechnology, Indonesian Institutes of Sciences (LIPI). The microorganisms previously were isolated from Pari Island, Indonesia. The isolates were revived on Marine Agar (MA) and incubated for 24 hours, at 30^{o}C. For the experiments, the isolates were cultured in broth Artificial Sea Water (ASW) medium.

Qualitative Assay for DBT Degradation

Degradation capability of isolates on dibenzothiophene was conducted by sublimation methods as described by Alley and Brown (2000). In sublimation method, bacterial isolates were innoculated on ASW agar and sublimed by DBT powder at 95°C for 3 (three) minutes. Inoculated isolates on media without DBT and non-inoculated media with DBT were used as controls. The samples were then incubated for 7 (seven) days at 30^{o}C. The isolates capacity for degrading DBT was determined by surrounding clear zone and/or color change of medium to orange. To confirm the degrading capacity, we performed growth assays by growing the isolates in liquid ASW media containing 50 ppm of DBT.

Optimization of Substrates Concentration for Biodegradation of DBT

The isolates were inoculated to broth ASW medium containing various concentrations of dibenzothiophene i.e. 0, 50, 100, 500, and 1000 ppm. Dibenzopthiophene stock solution was prepared by solving DBT into dimethil sulphoxide (DMSO). The bacterial growth was monitored using UV-Vis spectrophotometer on wavelength 600 nm (Koch, 1970).

Optimization of Starter Cell Concentrations for Biodegradation of Dibenzothiophene

To determine the optimum cell concentrations for DBT biodegradation, the isolates LBF-1-009, LBF-1-0018, and LBF-1-

0031 were innoculated in various cell concentrations into ASW media containing 100 ppm of DBT (Tanjung *et al*., 2016). Variation in cell concentrations was converted based on OD 600 nm measurement, namely 0, 20, 30, and 50 (Tanjung *et al.*, 2016). One ml of each growing cultures was taken at a regular interval within 7 days of cultivation. The changes of absorbance of each sample were monitored using spectrophotometer on wavelength 600 nm (Koch, 1994; 1970).

Molecular Identification of Dibenzothiophene Degrading Bacteria and Construction of Phylogenic Tree

The isolates LBF-1-0009, LBF-1-0018, and LBF-1-0031 were identified based on analysis of partial 16S rRNA gene. Genomic DNA (gDNA) was extracted using Wizard Genomic DNA Purification Kit (Promega). Amplification of 16s rRNA gene was carried out using PCR Mix Go Taq MM Green (Promega). The primers used in this experiment were universal primers, namely 9F (5'- GAGTTTGATCCTGGCTCAG-3') dan 1541R (5'- GCTACCTTGTTACGACTT-3'). PCR reaction was performed following these conditions: 95^0C, 2 minutes (1 cycle); 95^0C 30 seconds; 65^0C 1 minute; 72^0C, 2 minutes (10 cycles); 95^0C 30 seconds, 55^0C 1 minute, 72^0C 2 minutes (30 cycles) as described by Yetti *et al.*, (2016). Hereafter, the amplification products were purified and sequenced. Analysis of base sequence was carried out using BLAST program based on data banks in NCBI.

The phylogenetic trees were constructed using the neighbour-joining method of Mega 3.1 ABI *sequencer software* (Kumar *et al*., 2004). Nearly full-length of 16S rDNA sequences of the most phylogenetically related strains were selected from the Gene Bank database as reference strains.

Results

Qualitative Assay for Dibenzothiophene Degradation

Qualitative assay for dibenzothiophene degradation was carried out by sublimation method. Figure 2 shows the appearance of clear zone surrounding the isolates LBF-1-1-0009, LBF-1-0018, and LBF-1-0031. In addition, they also changed the colour of agar media to orange indicating dibenzothiophene degradation.

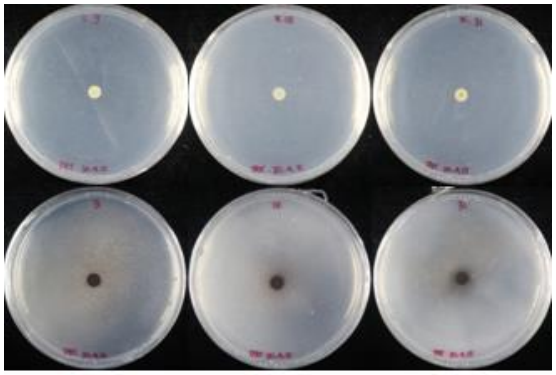


Figure 2. Sublimation assay on dibenzothiophene results of marine bacteria in question after 7 days of incubation. Isolates (A) LBF-1-0009, (B) LBF-1-0018, and (C) LBF-1-0031 and their respective controls.

Optimization of Substrates Concentrations for Biodegradation of Dibenzothiophene

Three isolates exihibited distinct growth pattern type of their growth in medium containing different concentrations of DBT. Visual assessment of LBF-1-0018 isolate, which is identified as *Bacillus subtilis*, exhibited faster growth than the other two. It was indicated by pronounced colour change of the media, even on day 0. The LBF-1-0009 isolate, belong to *Bacillus subtilis*, produced less pronounced changes in colour, compared to LBF-1-0031 isolate, which is identified as *Novosphingobium sp.* (Figure 3). Isolate LBF-1-0018 changed colour of the media from off-white colour to orangish. These changes had been noticeable, even since the day 0 and had become more prominent after 7 days of incubation. This alteration in colour was observable in culture media containing DBT from 50 until 1000 ppm. Correspondingly, LBF-1-0031 isolate exhibited similar behavior changing colour of the media on the day 0. Nevertheless, the colour intensity generated by LBF-1-0031 isolate was less prominent than LBF-1-0018 isolate. By contrast, LBF-1-0009 isolate exhibited distinct behavior and no colour change could be observed on the day 0.

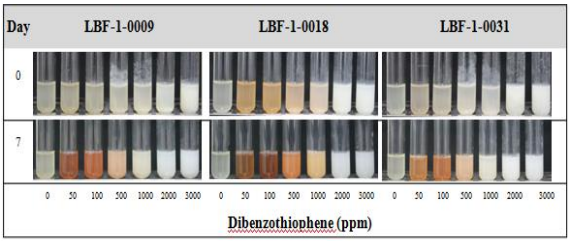
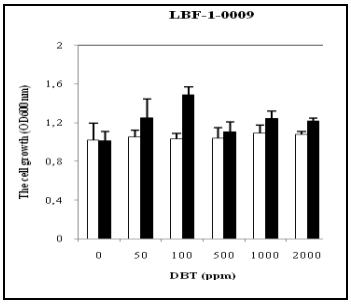
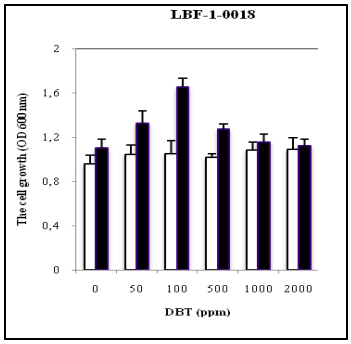


Figure 3. Performance of three distinct LBF isolates in growth assay for establishing the optimum substrate concentrations. The comparison between media on the day 0 and 7 was presented.

Further, Figure 4 shows the growth pattern of those three isolates in various concentrations of 0, 50, 100, 500, 1000 and 2000 ppm of DBT. From the figure, it can be concluded that LBF-1-0018 exhibited better growth on various DBT concentrations compare with the other two. While the LBF-1-0009 and LBF-1-0018 isolates were able to grow in DBT concentrations as high as 2000 ppm; the LBF-1-0031 isolate showed negative growth when growing in DBT concentrations of 2000 ppm. It can also be seen from Figure 4 that all isolates had the optimum growth in the concentration 100 ppm of DBT.





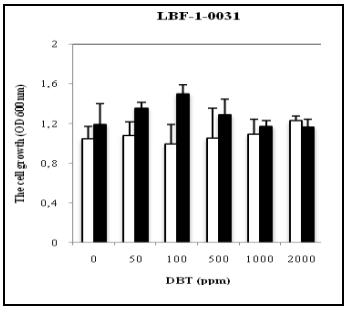


Figure 4. The growth pattern of three LBF isolates in several dibenzothiophene (DBT) concentrations incorporated in ASW media throughout 7 days of cultivation. ☐ day 0 and ■ day 7

Optimization of Starter Cell Concentrations for Biodegradation of Dibenzothiophene

To determine the optimum concentration of starter cells, three isolates namely LBF-1-0009, LBF-1-0018, and LBF-1-0031, each was innoculated to ASW media containing 100 ppm of DBT with various cell concentrations of 0, 20, 30, and 50. The cell concentrations were determined by value conversion from the measurement of optical density (OD) at 600 nm.

By visual observation, we obtained some information dealing with growth profile of each isolate cultured with various cell concentrations. On the day 0, cultures of LBF-1-0018 isolate with different concentrations from 20 to 50 showed earlier colour change of the medium than the others (Figure 6). While, all isolates were able to transform colour of the media containing 100 ppm of DBT from off-white colour to orangish; they showed distinct intensities of colour change. From this qualitative indicator, on the day 7, the highest conscentration of the LBF-1-0018 isolate to convert the media colour was as high as 20 of OD600 nm conversion units of cell concentrations. In constrast, as high as 30 of cell concentration of LBF-1-0009 isolate was able to change the media colour. Surprisingly, the LBF-1-0031 isolate showed colour-changing capability up to 50 of cell concentrations.

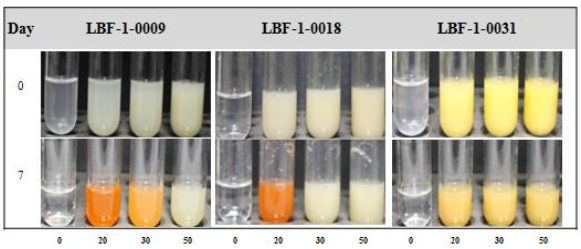
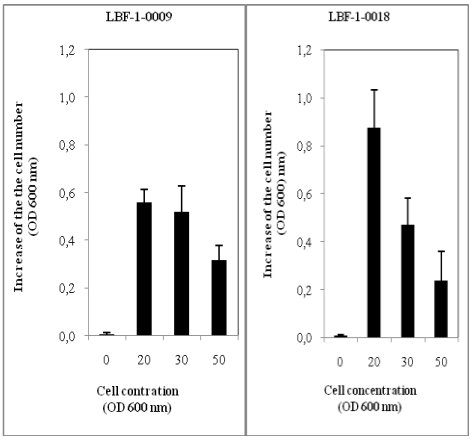


Figure 5. Performance of three LBF isolates in growth assay for establishing the optimum starter cell concentrations (based on OD600 nm). The comparison between media on the day 0 and 7 was presented.

From the above growth assay, we found that the optimum cell concentrations of all isolates for dibenzothiphene biodegradation were 20. The LBF-1-0018 isolate exhibited the most significant growth, which was indicated by the most pronounced colour change; when a cell concentration of 20 was introduced. This was followed by LBF-1-0031 and LBF-1-0009 isolates, respectively. Through OD measurement, in general, all the three isolates exhibited good capability to grow in enriched medium from 20 to 50 of OD600 nm cells (Figure 6). These results confirmed the qualitative (visual) assay conducted previously. From the OD quantification, the highest OD indicating the highest growth was exhibited by the cell concetrations of 20 of all three isolates. Moreover, the LBF-1-0018 isolate gave the best growth compared to the other two. From the Figure 6, it is also can be seen that higher cell concentrations do not always give better effect on microbial ability in biodegradation process.



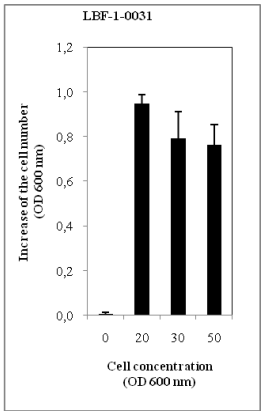


Fig 6. The cell growth of isolates with different starter concentrations in ASW medium after 7 days of incubation

Molecular Identification of Dibenzothiophene-Degrading Bacteria and Construction of Phylogenic Tree

Molecular identification of three isolates was carried out by analysis of partial 16S rRNA gene using BLAST programme (www.ncbi.nlm.nih.gov) compared with data in the Genbank. Results of BLAST analysis are presented in Table 1.

Table 1 provides information that LBF-1-0009, LBF-1-0018, and LBF-1-0031 isolate have the highest similarity with *Mauricauda olearia* strain CL-SS4, *Alcanivorax xenomutants* strain JC109, and *Stakelama pacifica* strain JLT832, respectively.

Table 1. Bacteria Identities of Potential Dibenzothiophene degrading Bacteria

Code of Isolates	Most closely related organism	BLAST Search Value (%)
LBF-1-0009	*Mauricauda olearia* strain CL-SS4 (KF724486.1)	99
LBF-1-0018	*Alcanivorax xenomutants* strain JC109 (NR_133958.1)	99
LBF-1-0031	*Stakelama pacifica* strain JLT832 (NR_116368.1)	99

The relationship of three potential isolates of dibenzothiophene-degrading bacteria was described by phylogenic tree as shown in Figure 7.

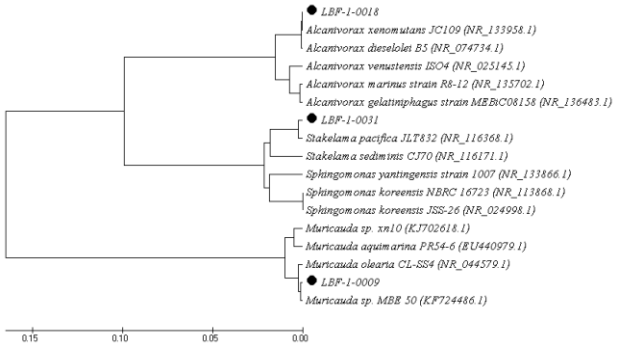


Figure 7. Phylogenetic tree derived from 16S rRNA gene sequence of 58 isolates capable of degrading PAHs. The NJ-tree was constructed using neighbour joining algorithm with Kimura 2 parameter distances in MEGA 3.1 software. Bar, 1% estimated sequence divergence

Discussion

In the present work, the two factors affecting biodegradation levels, namely substrate and cell concentrations, were optimized for efficient dibenzothiophene biodegradation. There are six factors affecting biodegradation by microbial cells, encompassing: (1) amount of substrate; (2) amount of bacterial inoculum (cell concentration); (3) filtration of inoculum, (4) type of basal salts medium, (5) initial pH of basal salts medium, and (6) flask closure (Lin *et al.*, 2014). It also applies to the dibenzothiophene biodegradation by marine bacteria. The degrading strains may not be efficient to metabolize DBT despite abundant available carbon and energy sources. The abilities of microorganisms to degrade pollutants are significantly influenced by various factors such as microbial species, nutrients and environmental parameters (Huang *et al.,* 2008*;* Rigas *et al.*, 2007). Consequently, the two factors, specifically substrate and cell concentrations were examined in this study.

The results of the study to optimize substrate concentrations showed that three isolates have distinct capabilities to grow in various dibenzothiophene concentrations. The LBF-1-0009 and LBF-1-0018 isolates showed ability to grow on all assayed DBT concentrations, while LBF-1-0031 presented ability to grow on DBT-contaning media up to 1000 ppm. It can be originated from limitation ability of each bacterial isolate to consume the subtrate for their metabolism. This study is in accordance with the former experiment conducted by Lin *et al.*, (2014), demonstrating that an increase in substrate concentration significantly decrease the DBT removal during the microbial degradation process.

The optimal growths of all isolates in the DBT-containing media were achieved when the cell concentration of 20 used as starter inocula. The growths of isolates were accompanied with an increase in turbidity (OD600). Previously, the effect of cell concentrations or microbial amount on biofegradation process was stated by some researcher. In most cases, higher concentrations of substrate degrader do not always correlate with higher biodegradation process. On the contrary, low number of microbial cell concentrations had good effect on PAH biodegradation (Shihag *et al.*, 2014).

The LBF-1-0009 and LBF-1-0018 isolates are identified to have high similarity to *Mauricauda olearia* strain CL-SS4 and *Alcanivorax xenomutants* strain JC109, respectively, with 99% homology result. There are still very limited reports related with biodegradation of dibenzothiophene by these genera. In several reports, most of the potential genuses known for their DBT-biodegradaging ability are *Pseudomonas*, *Brevibacterium* or *Bacillus* (Kitauchi *et al.*, 2004; Kilbane and Bielaga, 1990; Buzanello *et al.*, 2014; and Verma *et al.*, 2016). In addition, LBF-1-0031 isolates has 99% similarity to *Stakelama pacifica* strain JLT832. In the preceeding study, we presented that these three bacterial genera were belong to PAH degrading bacteria from Indonesian marine environment (Yetti *et al.*, 2016).

Acknowledgements

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References

Alley, J.F. and L.R. Brown (2000). Use of sublimation to prepare solid microbial media with water-insoluble substrates. *Appl Environ Microbiol.* l66 1: 439.

Buzanello, B. E., R.P. Rezende, F.M.O Sousal, E.L. S. Marques and L.L. Loguercio. (2014). A novel Bacillus pumilus-related strain from tropical landfarm soil is capable of rapid dibenzothiophene degradation and biodesulfurization. *BMC Microbiology*. 14: 257

El-Tarabily, K.A. (2002). Total microbial activity and microbial composition of a mangrove sediment are reduced by oil pollution at a site in the arabian gulf. *Canadian journal of microbiology*. 48(2): 176- 182.

Haritash and Kaushik. (2009). Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *Journal of Hazardous Materials*. 169:1–15.

Huang, L, T. Ma and D. Li. (2008). Optimization of nutrient component for diesel oil degradation by *Rhodococcus erythropolis*. *Marine Pollution Bulletin*. 56: 1714-1718

Jensen, A.M., K.W. Finster and U. Karlson. (2003). Degradation of carbazole, dibenzothiophene, and dibenzofuran at low temperature by *Pseudomonas* sp. strain C3211. *Environ. Toxicol. Chem*. 22:730–735

Khashayar, Tabari and Mahasa. (2010). Biodegradation potential of petroleum hydrocarbons by microbial diversity in soil. *World applied sciences journal*. 8(6): 750-755.

Kilbane, J. J., and B. A. Bielaga. (1990). Toward sulfur-free fuels. *Chemtech*. 20: 747–75

Kitauchi, F., S. Hirano, M. Haruki, T. Imanaka, M. Morikawa and S. Kanaya. (2005). Comparison of Dibenzothiophene-Degrading Bacteria under Low Oxygen Conditions. *Journal of Environmental Biotechnology.* 4 (2): 117–120

Koch, A.L. (1994). Growth Measurement," Methods for General and Molecular Bacteriology, Gerhardt, P *et al*., (ed) American Society for Microbiology, Washington, DC. *p. 248-277*

Koch, A.L. (1970). Turbidity Measurement of Bacterial Culture in Some Available Commercial Instrument. *Analytical Biochemistry.* 38: 252-59

Lin, L., Z. Chaocheng, L. Qiyou, Z. Yunbo, L. Chunshuang and X. Jianliang. (2014). Optimization for Microbial Degradation of Dibenzothiophene by *Pseudomonas* sp. LKY-5 Using Response Surface Methodology. *China Petroleum Processing and Petrochemical Technology*. 16 (1): 19-26

Rigas, F., K. Papadopoulou and V. Dritsa (2007). Bioremediation of a soil contaminated by lindane utilizing the fungus *Ganoderma australe* via response surface methodology. *Journal of Hazardous Materials*. 140: 325-332

Rodrigues, J., A. Madhukar, N. Kumar and U.M.X. Sangodkar. (2011). Isolation and Characterization of a marine bacterium belonging to the genus *Alkaligenes* capable of the complete mineralization of dibenzothiophene. *Indian J. Mar. Sci volume.* 40 (3): 391-397

Sihag, S., H. Pathak and D.P. Jaroli. (2014). Factors Affecting the Rate of Biodegradation of Polyaromatic Hydrocarbons. *Int. J. Pure App. Biosci.* 2 (3): 185-202

Su Seo, J., Y.S. Keum and Q.X. Li. (2009). Bacterial Degradation of Aromatic Compounds. *Int. J. Environ. Res. Public Health*. 6: 278-309

Tanjung, P. N, E. Yetti, A. Thontowi, A. Supriadi , S. Purwantisari , and Yopi. (2016). Karakterisasi Biodegradasi Senyawa Poliaromatik Dibenzothiophene Oleh Bakteri Laut Novosphingobium mathurense LBF-1-0061. *Jurnal Biologi Indonesia* 12 (2): 257-264

Teh, C.H. (2004). Deep HDS of diesel fuel: chemistry and catalysis. *Catalysis Today Volume.* 98: 3-18.

Thontowi, A. and Yopi. (2013). Diversity of alkane and hydrocarbon degrading bacteria in Pari Island, Jakarta. *JBI.* 9 (1): 137-146.

Verma, N., R. Sharma and R. Kaur. (2016). Microbial Desulphurization Study of Dibenzothiophene and Crude Oil by a Soil Isolate. *Ijsrm.Human.* 4 (4): 133-145.

Xu, P., B. Yu, F. L. Li, X. F. Cai, and C. Q. Ma. **(**2006). Microbial degradation of sulfur, nitrogen, and oxygen heterocycles. *Trends Microbiol.* 14**:** 398–405

Yetti, E., A. Thontowi and Yopi. (2016). Polycyclic aromatic hydrocarbon degrading bacteria from the Indonesian Marine Environment. *Biodiv.* 17 (2): 857-864