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Desulfurization of Dibenzothiophene by *Pseudomonas aeruginosa* Isolated from Iraqi Soils

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Abstract: A dibenzothiophene (DBT) utilizing bacteria, as the sole source of sulfur, were isolated from soil samples contaminated with hydrocarbons. In this study, an effective enrichment technique was applied to isolate bacterial isolates with capabilities to utilize dibenzothiophene (DBT) as a model compound of polyaromatic sulfur heterocyclic compounds (PASHs). From forty samples, sixty three bacterial isolates were obtained and three of them were characterized as biodesulfurising *Pseudomonas aeruginos*a. A convenient spectrophotometric assay (Gibbs' assay) was used to determine the quantity of desulfurized product (Hydroxybiphenyl HBP) by these isolates, also these isolates were capable to utilize ethanol as C- source.

Key words: Biodesulfurization, dibenzothiophene, *Pseudomonas aeruginosa*, Gibbs' assay.

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Introduction

Accidental and deliberate crude oil spills have been, and still continue to be, a significant source of environmental pollution, and poses a serious environmental problem, due to the possibility of air, water and soil contamination (1). Combustion of petroleum-derived fuels leads to the release of vast amount of sulfur dioxide (SO_2) into the atmosphere, which is a principle source of acid rain and air pollution. Thus, most countries have imposed strict regulations to control these releases mainly by enforcing stringent restrictions on the levels of sulfur in transportation fuels. However, a common problem, petroleum refineries are facing around the world is that crude oil reserves being used as feedstock for refining process are becoming heavier day after day with elevated sulfur contents (2, 3).

Currently, hydrodesulfurization (HDS) is employed to remove sulfur from fossil fuels. HDS operates at high temperature and pressure, and besides it cannot remove completely sulfur from the heterocyclic organosulfur compounds such as dibenzothiophene (DBT) (4). Biodesulfurization (BDS) through microbial activities can solve this problem (5). Compared with HDS process, the biodesulfurization (BDS) process using microorganisms and/or enzymes could be carried out more safely, under mild conditions (6). This process of microbial desulphurization or biodesulfurization is expected to overcome the technical and economic problems associated with HDS as it has the potential benefits of lower capital

and operating costs and will produce lesser greenhouse gases (7).

Research on biodesulfurization using dibenzothiophene has resulted in the elucidation of two different biochemical pathway, named Kodama and 4S. Kodama pathway is considered unsuitable because in this pathway water-soluble sulfur compounds are produced, which are then unavailable for burning and are therefore forfeited from the caloric value of the fuel. through 4S-pathway, dibenzothiophene is transformed to 2-hydroxybiphenyl and sulfite as end-products. Microbial systems have been reported to selectively take up the sulfur from the DBT molecule by consecutive enzymatic steps, leaving intact the carbon skeleton of DBT is released intact as 2-hydroxybiphenyl (2-HBP); therefore, fuel value is not lost (8, 9).

Therefore this study was aimed to isolating and identifying bacteria with ability to utilize DBT as a sole source of sulfur.

Materials and Methods

Bacterial Isolation

The microbial selection procedure was performed in basal salt medium (BSM) (sulfur-free) consisting of: K_2HPO_4 (4 g); Na_2HPO_4 (4 g); NH_4Cl (2 g); $MgCl_2.6H_2O$ (0.2 g); $CaCl_2.2H_2O$ (0.001 g) and $FeCl_3.6H_2O$ (0.001 g) per liter of distilled water (pH 7.5). This medium was used for isolation and growth of the microorganisms under sulfur deficient conditions (10). cells shapes and Gram reaction. Biochemical characterization of the isolates was based on the results of Catalase, Oxidase, Indole, Methyl red, Voges-Proskauer, Citrate utilization and Motility tests, in addition to growing the Glycerol (10 mM) was used as the carbon source. Soil samples and subsequently isolated bacteria were inoculated in BSM supplemented with 0.1 mM DBT. The sulfur source was added to the medium from sterile stock solutions before inoculation (100 mM DBT in ethanol). All cultures were incubated at 30°C with shaken at 150 rpm.

Cell suspensions were inoculated (1: 200) in DBT liquid medium for sulfur bioavailability assay (10,11). Positive and negative controls consisted of BSM with or without MgSO4. After two days of incubation, subcultures were made in fresh media (1: 500) and shaken for four more days (12). Cultures with significant growth in DBT media as compared to the negative controls were centrifuged, the supernatants were used for Gibbs assay and the pellets were streaked on LB (Luria-Bertani) agar plates for colony isolation. Individual colonies were then inoculated in DBT medium and incubated at 37°C with shaking (150 rpm). Samples were taken during a period of 2 to 4 days and tested with Gibbs assay.

Identification of the Isolate(s)

The selected isolates were identified based on their morphological and biochemical characteristics. The morphological characterization involved culturing the isolates on LB (Luria-Bertani) agar plates for studying appearance of the colonies. Following that, smears from the colonies were Gram stained to study the isolate isolates on the King A, King B and cetrimide agar. These tests were performed according to the Bergey's Manual of Determinative Bacteriology (13).

Gibb's Assay

The cell- free culture supernatant was obtained at different time intervals from bioavailability assay (as mentioned above). 1ml of each cell-free supernatant was transferred to clean eppendorf and adjusted to pH 8 by adding 60 µl of 1 M $NaHCO_3$ (14) or by adding 3 µl of 4 M of Na OH. Detection of the end products of the desulfurization of thiophene compound, was carried out in microtiter plates by adding 3 µl of Gibb's reagent to 300 µl of adjusted cell- free supernatant (1:100) and mixture was kept at room temperature for 30 min. Bacterial culture capable of desulfurizing thiophene compound, accumulated phenolic compound in cultural supernatant and gave blue color in the presence of Gibb's reagent.

Utilization of Ethanol and MgSO4 as Alternative Carbon and Sulfur Sources by DBT Biodesulfurizing Bacteria

Since DBT was added to BSM from stock solution of DBT (100 mM) in ethanol, the utilization of ethanol as an alternative carbon source was tested in the presence of an inorganic sulfur source such as $MgSO_4$. The growth of DBT biodesulfurizing bacterial isolates at 37^o C in BSM containing 0.1 mM DBT plus 6 mM ethanol (control flask) was compared to the growth in medium containing 6 mM ethanol and 0.2 mM $MgSO_4$. Flasks (250 ml) containing 100 ml of the respective medium were inoculated and incubated under shaking (150 rpm) at 37 ^oC. At different time intervals, samples (2 ml) of bacterial cultures were withdrawn and the growth was measure at 580 nm.

Results and Discussion

Identification of Isolates

A DBT utilizing bacteria were isolated on the basis of its ability to utilize DBT as the sole source of sulfur. Forty soil samples were collected from different oil refineries in Iraq. Sixty three isolates were obtained but only three of these isolates were able to utilizing DBT as sole source of sulfur. These isolates were tentatively identified as *Pseudomonas* sp. on the basis of a number of morphological, cultural and physiological characteristics. The isolates were non-spore former, gram negative, aerobic, coccobacilli bacteria with unipolar motility (15). Colonies which were suspected to be belong to the genus *Pseudomonas* sp. were grown on LB agar plates (figure1), and characterized by produce pyocyanian (blue-green) and have a grape like odor. Also, the shape of the colony appears like a fried egg shape, smooth in shape with flat edges and elevated center, green in color, these results are reasonable with the result demonstrated by (16,17).

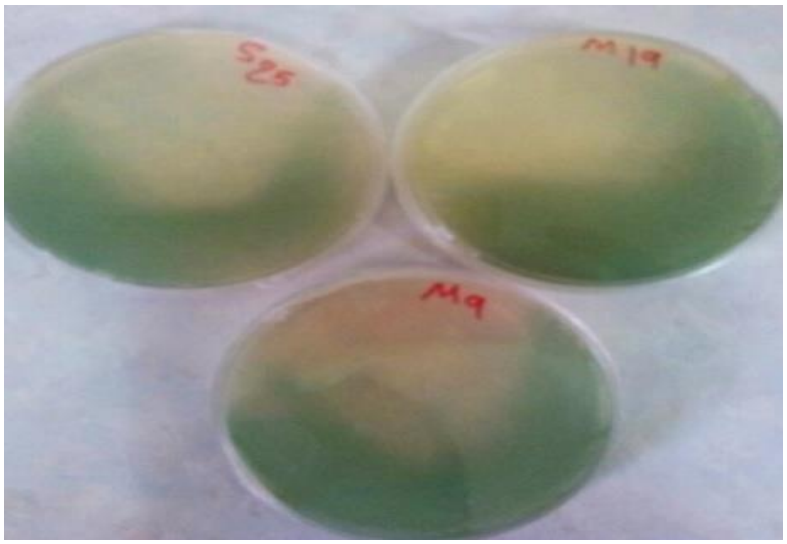

Figure (1): Growth of *Pseudomonas* sp on LB agar plates

Results of cultural, morphological and biochemical characteristics (table 1) of these isolates were in agreement with Bergey's Manual of Systematic Bacteriology (13). Depending on these results the isolates were classified as *Pseudomonas aeruginos*a.

Table (1): Biochemical characteristics of bacterial isolates

Test		S-25	M-9	M19
Catalase		+	+	+
Oxidase		+	+	+
Motility		+	+	+
Growth on cetrimide		+	+	+
urease		-	-	-
Gelatinase		+	+	-
Citrate utilization		+	+	+
Growth on King-A		+	+	+
Growth on King-B		+	+	+
MR		w	+	+
VP		w	-	w
Indol		-	-	-
TSI	**H_2S**	-	+	+
	CO_2	-	+	-
	Acid	Alk/ Alk	Alk /Acid	Alk/ Acid

+: Positive result (-): Negative result N: Not tested W: Weak test
TSI: Triple sugar iron agar MR: Methyl red VP :Vogas-Proskauer

Gibb's Assay- Guided Desulfurization Activity

The capability of DBT- desulfurizing bacteria to specifically break C-S-C bonds in DBTs liberating the phenolic end products 2-HBPs in the case of DBTs was testing using Gibb's reagent (2,6-dichloroquinone-4chromide). This reagent reacts with phenolic compound forming blue-color compound (2,6-dichloro benzenoneindophenol).
Accordingly, bacterial isolate capable of only desulfurizing DBTs (cleavage of C-S bond only) and accumulating phenolic end product could be distinguished from strain capable of degrading (C-C bond cleavage) and/or desulfurizing and degrading (C-S and C-C bond cleavage) thiophene compound .

The obtained result revealed the desulfurization (blue color) with all (three) *Pseudomonas aeruginos*a isolates grown on DBTs (fig 2). Only these isolates gave positive result from all DBT- grown cultures. This preliminary result suggests the involvement of the 4S pathway in the utilization of DBT via the specific cleavage of only the C-S bond by the bacterial cultures exhibiting positive results with Gibb's reagent (18).

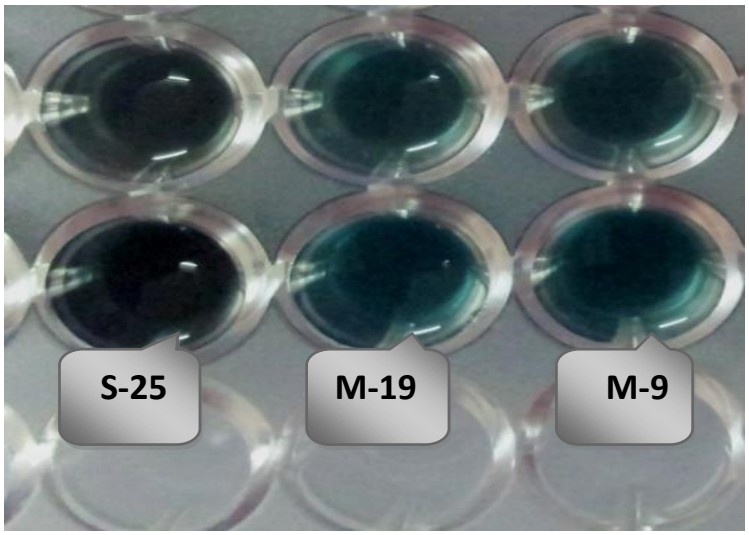

Figure (3): Gibb's assay guided desulfurization. The formation of blue color is an indication of desulfurization and formation of phenolic end products

Utilization of Ethanol and $MgSO_4$ as Alternative Carbon and Sulfur Sources

To confirm that the isolates able to utilize ethanol as C-source, all bacterial isolates were grown in BSM containing DBT plus ethanol and in BSM containing ethanol plus $MgSO_4$. Results indicated that the growth density ($O.D_{580}$) of bacterial isolates was a little more in medium containing ethanol plus $MgSO_4$ compared to the second media. his mean that these isolates were

capable to utilize ethanol as C- source. The utilization of ethanol as a carbon source was reported for *Rhodococcus erythropolis* KA2-5-2 (19) and *Gordonia* sp. CYKSI (20), which is DBT desulfurizing bacteria. The cultures containing 0.1 or 1.0 % ethanol exhibited a shorter lag time and more rapid exponential growth than cultures grown with glucose alone. However, the presence of ethanol in media at concentrations higher than 1.0 % produced progressively decreased exponential growth rates and slightly reduced extents of growth (21). The little difference in the capability of these isolates in utilizing both DBT and $MgSO_4$ as S-source, could be related to the fact that $MgSO_4$ is a simple source of sulfur compared to DBT. However, the presence of ethanol increase the solubility (bioavailability) of DBT. It was suggested that NADH, which is produce by the biochemical reaction of NAD with ethanol catalyzed alcohol dehydrogenase, might contributed to the conversion of FMN to $FMNH_2$, which is coenzyme for the activities of desulfurization enzymes (22). It was reported that the addition of DBT dissolved in ethanol provided more rapid growth and desulfurization than DBT powder only (23).

Conclusions

1-The DBT- desulfurizing bacteria isolated in this study showed broad spectrum for desulfurization of organosulfur model (DBT).

2-DBT is attacked via the specific cleavage of only the C-S bond resulting in formation of 2- HBP which is the end product of the common 4S biodesulfurization pathway. Thus, not carbon loss is observed.

3-Our DBT- biodesulfurizing bacterial isolates possess some promising features that make them potential candidates for developing a biocatalytic desulfurization process for fuels.

References

1. Trindade, P.V.O.; Sobral, L.G.; Rizzo, A.C.L.; Leite, S.G.F. and Soriano, A.U. (2005). Bioremediation of a weathered and a recently oil-contaminated soils from Brazil: a comparison study. *Chemosphere*, 58: 515-522.
2. Bhatia, S. and Sharma, D.K. (2012). Thermophilic desulfurization of dibenzothiophene and different petroleum oils by *Klebsiella*sp. 13T; *Environ. Sci. Pollut*, 19(8): 3491- 3497.
3. Li, W. and Jiang, X. (2013). Enhancement of bunker oil biodesulfurization by adding surfactant; World J. *Microbiol. Biotechnol*, 29(1):103-108.
4. Sumedha, B. and Sharma, D.K. (2010). Biodesulfurization of dibenzothiophene, its alkylated derivatives and crude oil by a newly isolated strain Pantoeaagglomerans D23W3. *Biochem. Eng*, 50:104–109.
5. Oldfield, C.; Pogrebinsky, O.; Simmonds, J.; Olson, E. S. and Kulpa, C. F. (1997). Elucidation of the metabolic pathway for dibenzothiophene desulfurization by *Rhodococcus* sp. strain IGTS8 (ATCC 53968). *Microbiology*, 143:2961–2973.
6. Chen, H.; Cai, YB.; Zhang, WJ. and Li, W. (2009). Methoxylation pathway in biodesulfurization of model organosulfur compounds with *Mycobacterium* sp., *Bioresour. Technol*, 100: 2085–2087.
7. Li, W.; Zhang, Y.; Dong, W., M. and Shi, Y. (2005). Biodesulfurization of dibenzothiophene and other organic sulfur compounds by a newly isolated *Microbacterium* strain ZD-M2. FEMS *Microbbiology Lett*, 247:45-50.
8. Pokethitiyook, P.; Tangaromsuka, J.; Kruatrachue, M.; Kalambahetib, C. and Borole, A. (2008). Biological removal of organic sulfur by bacteria strains isolated in Thailand. *Science Asia*, 34: 361-366.
9. Mohebali, G.; Ball, A.; Rasekh, B. and Kaytash, A. (2007). Biodesulfurization potential of a newly isolated bacterium, *Gordonia alkanivorans* RIPI190A. *Enzyme and Microbial. technol.*, 40: 578-584.

10. Kilbane, J. J. (1990). Sulfur-specific microbial metabolism of organic compounds. *Resour. Conserv. Recyc*, 3: 69-79.
11. Krawiec, S. (1999). Bacterial desulfurization of thiophenes :screening techniques and some speculations regardingthe biochemical and genetic bases. *In Dev. Indust. Microb.* Vol. 31, suppl. 5: 103-14.
12. Wang, P. and Krawiec, S. (1994). Desulfurization of dibenzothiophene to 2-Hydroxybiphenyl by some newly isolated bacterial strains. *Arch. Microbiol*, 161: 266-71.
13. Bergey, D.H. and Holt, J.G. (2000). "Bergey's manual of determinative bacteriology"; 9th edn., Lippincott Williams and Wilkins, Philadelphia.
14. Konishi, J.; Ishii, Y.; Onaka, T.; Okumura, K. and Suzuki, M. (1997). Thermophili carbon-sulfur-bond-targeted biode-sulfurization. *Appl. Environ. Microbiol*, 63 (8): 3164-9.
15. Ryan, K.J. (2004). Ray CG (editors). Sherris Medical Microbiology (4th ed.). McGraw Hill. ISBN 0-8385-8529-9.
16. Collee, J. G.; Fraster, A. G.; Marmion, B. P. and Simon A. S. (1996). Laboratory Strategy in the diagnosis of infective syndromes. In practical Med. Microbial. (eds. Mackie and Mccartiny (14th ed.) Churchill living stone. D.
17. Holt, J. G.; Kreig, N. R. and Williams, S. T. (1994). Bergyy's Manual of Determination bacteriology. (9thed) Williams and Wilkins, USA. P:141-163.
18. Gupta, N.; Roychoudhury, P. K. and Deb, J. K. (2005). Biotechnology of desulfurization of diesel: Prospects and challenges. *Appl. Microbiol. Biotechnol*, 66: 356–366.
19. Tanaka, Y., Yoshikawa, O.; Maruhashi, K. and Kurane, R. (2002). The cbs mutant strain of *Rhodococcus erythropolis* KA2-5-1 expresses high levels of Dsz enzymes in the presence of sulfate. *Arch. Microbiol*, 178:351-357.
20. Choi, O. K.; Cho, K. S.; Ryu, H. W. and Chang, Y. K. (2003). Enhancement of phase separation by the addition of de-emulsifiers to three-phase (diesel oil/biocatalyst/aqueous phase) emulsion in diesel biodesulfurization. *Biotechnol. Lett*, 25:73-77.
21. Yoshikawa, O.; Ishii, Y.; Koizumi, K.I.; Ohshiro, T.; Izumi, Y. and Maruhashi, K. (2002). Enhancement and stabilization of desulfurization activity of Rhodococcus erythropolis KA2-5-1 by feeding ethanol and sulfur components. Biosci. Bioeng, 94, 447-452.
22. Yan, H.; Kishimoto, M.; Omasa, T.; Katakura, Y.; Suga, K.I.; Okumura, K. and Yoshikawa, O. (2000). Increase in desulfurization activity of Rhodococcus erythropolis KA2-5-1 using ethanol feeding. Biosci Bioeng 89: 361–366.
23. Setti, L.; Farinelli, P.; Di Martino, S.; Frassinetti, S.; Lanzarini, G. and Pifferi, P. G. (1999). Developments in Destructive and Non-destructive pathways for Selective Desulfurizations in Oil-biorefining processes. App. Microbiol. Biotechnol, 52, 111.