Toluene–degrading activity of local bacteria isolated from contaminated sea of North Sumatera

W Lestari1*, N Priyani2, E Munir2, A Hartanto2, K Warsito2

1Department of Agrotechnology, Sekolah Tinggi Ilmu Pertanian Labuhan Batu, Rantauprapat, Indonesia
2Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan, Indonesia

*Email: widyalestari1688@gmail.com

Abstract. Hydrocarbon contamination in soil and water is still a major concern and considered as a serious environmental issue to various natural habitats. Microbial degradation of hydrocarbon compounds is considered as one of eco-friendly method to overcome this issue of contamination. Toulene-degrading bacteria were successfully recovered from contaminated marine seawater of Belawan and Tanjung Balai, North Sumatera. Three bacterial isolates identified as *Pseudomonas aeruginosa* TJB01, *Pseudomonas* sp. TJB05, and *Acinetobacter* sp. SBG05 were subjected to degradation test by evaluating their hydrocarbon-degrading activities towards Toluene as one of selected hydrocarbon compounds of BTEX. Parameters observed in this study were bacterial cell density (Colony-forming unit/ mL) by using Total Plate Count method and hydrocarbon residues (ppm) quantified by using Gas Chromatography. Initial concentration of Toluene was detected 173.10 ppm. Isolate *P. aeruginosa* TJB01 produced the highest cell density (log 7.94 CFU.mL\(^{-1}\)) and produced the highest toluence degradation (%) at 59.46% than other isolates and bacterial consortium during 12 days of incubation period. Further investigations are needed to optimize consortium potential in exhibiting better degradation activities.

1. Introduction

Toluene is commonly known as chemical contaminants within a group consists of benzene, toluene, ethylbenzene and xylenes or BTEX compounds [1]. The contamination may occur due to leakage or flow access of toluene compounds in the form of petrochemicals and other derived products. In addition, marine environments are prone to various source of toluene contamination through harbor or ferry dock activities, accidental oil spills and any activities using petrochemicals [2].

Bioremediation is an eco-friendly technique to overcome this contamination issue by integrating biodegradative bacteria to produce biosurfactant and enzymes involved in biodegradation which result in a less toxic compound being exposed back to the environment [1, 3, 4]. Application of biodegradative bacteria have reported mostly successful laboratory results through tolerance and degradation tests. Several strains of *Bacillus* spp. were reported to utilize crude oil as C-source in fermentation medium with high biomass after incubation period [5]. *Pseudomonas putida* was reported to perform a normal growth under stress of high toluene in culture medium [6, 7].

Hence, experiment on evaluating other bacteria, especially indigenous strains is worth conducted to obtain new candidates as biodegradative agents. In the present study, we tested the ability of three biosurfactant-producing bacteria strains isolated from marine source while their degradation ability or
tolerance were determined such as their growth under certain toluene concentration and degradation performance in fermentation medium.

2. Materials and Methods

2.1. Bacteria culture and condition
Biosurfactant-producing isolates were obtained from culture collection in Laboratory of Microbiology, Department of Biology, Universitas Sumatera Utara identified as *Pseudomonas aeruginosa* TJB01, *Pseudomonas* sp. TJB05 and *Acinetobacter* sp. SBG05 from previous study [8]. Bacteria cultures were grown in Mineral Salts medium (MSM), 1.0 g NaCl, 1.0 g K$_2$HPO$_4$, 1.0 g NH$_4$H$_2$PO$_4$, 3.0 g KNO$_3$, 1.0 g (NH$_4$)$_2$HPO$_4$, 0.2 g MgSO$_4$ in 1,000 mL distilled water (w/v). Bacteria cultures were grown by streaking culture into new MSM to obtain freshly growing cultures. Toluene concentration used as initial concentration in experiment was obtained by measuring representative diesel oil contaminated sites or ferry dock water in North Sumatra using Hewlett-Packard 6890 series Gas Chromatography.

2.2. Measurement of bacterial cell density
Direct suspension method was used to obtain bacterial suspension similar to McFarland standard No. 0.5 $\approx 1.5$–$2 \times 10^8$ Colony forming unit (CFU).mL$^{-1}$. Bacterial consortium was prepared by mixing three isolates within a ratio of 1:1:1 to obtain a 1-mL suspension. One millilitre of bacterial suspension was inoculated into 50 mL MSM broth (2% v/v) supplemented with initial concentration of toluene (173.10 ppm, v/v) and grown under agitation of 160 rpm at ambient temperature for 12 days. Aliquot of diluted cultures were spread on Plate Count Agar (PCA) during interval of 4, 8 and 12 days of observations. Colonies of bacteria were manually counted by using colony counter and expressed in log CFU.mL$^{-1}$ [5].

2.3. Measurement of toluene degradation
Remaining toluene content in MSM medium was measured during interval of 4, 8 and 12 days of observations. Standard curve of toluene was made by plotting various concentration of toluene versus its surface areas. Toluene was extracted using hexane solvent (1:10 v/v) for 15 min until immiscible layers were formed. Top layer was used for analytical measurement by using gas chromatography [9].

3. Results and Discussion

3.1. Bacterial consortium and axenic culture growth in fermentation medium containing Toluene as C-source
The viable cell number expressed in log CFU.mL$^{-1}$ from each tested bacteria is presented in Figure 1. All isolates produced cell densities reaching log 7–8 CFU.mL$^{-1}$ in the end of incubation periods (12 days). Among growth of axenic cultures, isolate *Acinetobacter* sp. SBG05 produced the less number of cell density followed by isolate *Pseudomonas* sp. TJB05. Isolate *Pseudomonas aeruginosa* TJB01 and bacterial consortium produced similar cell densities reaching log 8 CFU.mL$^{-1}$. All isolates showed a decline in growth except in bacterial consortium and isolate TJB01 which might be prolonged due to efficient carbon assimilation.
Figure 1. Bacterial consortium and axenic cultures growth performance in culture medium containing toluene as C-source within 12 days of incubation period

3.2. Toluene degradation by bacterial consortium and axenic culture

Residue of Toluene (ppm) measured by gas chromatography is presented in Table 1. The percentage of toluene degradation is presented in Figure 2. There was a slight decrease of toluene concentration in control medium during incubation periods. Among tested isolates, isolate *P. aeruginosa* TJB01 produced the highest toluene degradation with 59.46% followed by isolate TJB (47.90%), SBG05 (46.69%) and bacterial consortium (39.84%). Remaining toluene residue from fermentation medium of TJB01 was 70.17 ppm in 12 days of incubation period.

Table 1. Toluene residue as result of biodegradation by bacterial consortium and axenic culture

| Toluene concentration (ppm) within incubation period (Day) | 0      | 4      | 8      | 12     |
|-----------------------------------------------------------|--------|--------|--------|--------|
| Control                                                   | 173.10 | 172.90 ± 0.61 | 172.33 ± 0.03 | 171.75 ± 0.99 |
| Consortium                                                | 173.10 | 135.08 ± 0.70  | 128.31 ± 1.60  | 104.13 ± 1.80  |
| TJB01                                                    | 173.10 | 128.87 ± 2.10  | 86.29 ± 1.65   | 70.17 ± 1.85   |
| TJB05                                                    | 173.10 | 144.91 ± 1.60  | 127.32 ± 4.80  | 92.27 ± 2.12   |
| SBG05                                                    | 173.10 | 151.47 ± 0.63  | 133.15 ± 2.78  | 90.18 ± 4.90   |
Figure 2. Percentage of toluene biodegradation in culture medium by bacterial consortium and axenic cultures within 12 days of incubation period

Toluene biodegradation has been reported and reviewed by numerous studies with different strain and culture condition in laboratory experimentation. Toluene may be digested in aerobic condition by bacteria through enzymatic reaction from oxygenase family, dioxygenases and monooxygenases [1]. During aerobic degradation, toluene was first degraded into oxygen followed by further degradation into nitrate under reducing condition. The conversion of toluene into nitrate under anaerobic condition was facilitated by benzylsuccinate synthase (bss) as reported from denitrifying bacterium, *Thauera* sp. [10]. A study on their enzymes may be explored further to obtain information on their biodegradation mechanisms *in vitro*. In nature, *Pseudomonas* was reported as a genus of toluene-tolerant bacteria within 3,000 ppm or 0.3% (v/v) of tolerance range [3]. In this study, our tested *Pseudomonas* spp. was able to withstand toluene in culture medium by producing considerable increases of cell density (CFU.mL\(^{-1}\)) under 0.1% (v/v) of toluene.

The isolates used in this study were recovered from near-surface marine water in ferry dock water contaminated with fossil fuel. The identified isolates were considered among potential strains, i.e. *Acinetobacter*, *Pseudomonas*, and *Rhodococcus* which were reported as frequent genera or bacterial inhabitants in toluene polluted habitats [4,11,12]. The toluene-degrading bacteria were considered as important member in degradation of BTEX compounds in nature with different degradation abilities reported by previous studies. However, our toluene-tolerant strains were not as tolerant as other strains ever reported. Isolate *Pseudomonas putida* DOT1 was able to tolerate 10,000 ppm of toluene (v/v) while other strains of *P. putida* namely S12, Idaho, and PpG1 were greatly tolerate 50,000 ppm of toluene (v/v) in laboratory test surpassing 100× to our tested concentration (173.10 ppm) [6,7]. Further optimization in culture condition of potential *P. aeruginosa* TJB01 is highly possible to improve its biodegradation capacity.

The growth of *P. aeruginosa* TJB01 matched the growth of bacterial consortium. However, toluene degradation by bacterial consortium were observed being the lowest among axenic cultures tested. Bacterial consortium may exhibit lower degradation ability due to spatial pattern of degradation or metabolism zone exhibit by different species within micrometer scale [13]. In aqueous environment, a low toluene concentration in culture medium may also hinder the degradation process by actively growing bacteria. This may due to the inability of large bacterial inoculum to maintain optimum metabolic activity from the very little toluene concentration, leading to a slower rate of toluene
degradation in nature [14]. Hence, our findings on degradation ability by these three marine isolates may be subjected into further investigation for a better understanding of their degradation abilities.

4. Conclusion
Three bacteria, *Pseudomonas aeruginosa* TJB01, *Pseudomonas* sp. TJB05 and *Acinetobacter* sp. SBG05 isolated from ferry dock water contaminated with toluene, are reported as toluene-tolerant and -degrading bacteria with potential for further optimization. Axenic cultures of *P. aeruginosa* TJB 01 produced a higher toluene degradation with 59% than other axenic cultures and bacterial consortium from an initial concentration of 173.10 ppm of toluene.

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