Efficiency of Di-(2-Ethylhexyl) Phthalate Degrading Bacteria Isolated fom Mangrove Sediment and Landfill Soil

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Abstract

Phthalate esters (PAEs) are widely used as plasticizers to improve plasticity and flexibility of polymeric materials. Di-(2-ethylhexyl) phthalate (DEHP), one of the commonly used PAEs in plastic industries, has been ranked the top priority pollutants by the United States Environmental Protection Agency (USEPA). Biodegradation is considered a promising technology for DEHP removal. Consequently, the objectives of this study were to isolate efficient DEHP-degrading bacteria from contaminated mangrove sediment and landfill soil. The efficiencies of DEHP-degrading activities among the isolated bacteria were subsequently compared. The mangrove sediment and landfill soil samples were respectively collected from Samut Prakan and Rayong provinces, Thailand. Both locations have chronic exposure to plastic waste. In this study, five and seven DEHP-degrading bacterial isolates were obtained from mangrove sediment and landfill soil, respectively, by enrichment approach. For each isolate, the degradation efficiencies of 200 mg/l DEHP were monitored after 7 days. The DEHP degradation efficiencies of those retrieved from mangrove sediment (SPK) and landfill soil (RY) ranged from 71.34% to 96.70% and 9.19% to 30.65%, respectively. The results indicated that DEHP-degrading bacteria isolated from mangrove sediment were able to more efficiently degrade DEHP than those obtained from landfill soil. Mangrove sediment impacted by plastic pollution could provide a valuable source of efficient DEHP-degrading bacteria. Further study will focus on the development of DEHP-degrading bacterial consortium composing of a variety of bacterial characteristics, including the ability to degrade other PAEs. Bioremediation technology for plastic waste can be built upon initial findings gained from this study.

Keyword: Di-(2-ethylhexyl) phthalate (DEHP)/ Biodegradation/ DEHP-degrading bacteria/ Mangrove sediment/ Landfill soil/ Plastic waste

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1. Introduction

The worldwide production of plastic waste significantly increased over recent decades (Akhbarizadeh et al., 2020). Phthalate esters (PAEs) are known as plasticizers which are a composition of plastic. PAEs are widely used in plastic industries to improve plasticity, versatility, fluidity, malleability and flexibility of several materials, such as polyvinylchloride (PVC). Due to the lack of chemical bonding between the plasticizer and the polymer chain, PAEs can be easily discharged into the environment during manufacture, weathering, evaporating, and disposal processes. Consequently, PAEs can be found in soil, sediments, waters, and air (Fernández et al., 2012).

Di-(2-ethylhexyl) phthalate (DEHP) is one of the most commonly used plasticizers and it is mainly used in the production of PVC plastics. DEHP has been detected in drinking water, surface water, ground water, sludge, soil, and sediments. Consequently, humans potentially exposure to DEHP via ingestion, inhalation and dermal absorption (Malem et al., 2019; Zhu et al., 2020). Numerous toxicological studies have demonstrated that DEHP are endocrine disrupting chemicals (EDCs). Due to its toxicity such as carcinogenic, estrogenic, hepatotoxic agents, and contributors to chronic health effects, DEHP has been classified as a priority pollutant by the United States Environmental Protection Agency (USEPA), the European Union (EU) and the Chinese National Environmental Monitoring Center (CNEMC) (Hu et al., 2021; Lu et al.,

2020; Wang et al., 2020; Yuan et al., 2010). Previous studied reported that the concentration of DEHP at Pradu Bay, Rayong province, Thailand was 0.31-0.91 μg/l in seawater but it was below detection limit to 1.65 μg/g in sediment samples (Malem et al., 2019). Another study reported that the concentrations of DEHP in urban soil and agricultural soils collected from Guangzhou, China, were 264 μg/g dw and 29.4 μg/g dw, respectively (Zhu et al., 2020). The prevalence of DEHP in environments are now received high attention because it is relevant to health problems.

The PAEs can be degraded by both biotic and abiotic processes (Meng et al., 2015). Biodegradation is more likely to be the most effective process to remove PAEs from the environments due to its prominent advantages, including the complete degradation and the less production of environmental disturbance (Fernández et al., 2012; Hu et al., 2021). Numerous studies have revealed that microorganisms such as bacteria, yeast, and fungi were able to degrade DEHP under aerobic or anaerobic conditions. Bacterial strains isolated from various environments, including *Rhodococcus jostii* (Annamalai & Vasudevan, 2020), *Rhodococcus rhodochrous* (Chao & Cheng, 2007), *Bacillus subtilis* (Quan et al., 2005), *Pseudomonas fluorescens* (Xu et al., 2007), *Sphigomonas* sp. and *Corynebacterium* sp. (Chang et al., 2004) were capable of degrading PAEs. Previous studies reported that *Rhodococcus pyridinivorans* XB isolated from activated sludge degraded high-concentration of DEHP (200 mg/l) with a degradation efficiency of 98% within 48 hours (Zhao et al., 2018). Thus, the ability of microorganisms in degrading DEHP has high potential for the development of bioremediation technology. Although previous studies mostly focused on the degradation of DEHP using pure bacterial culture, bacterial consortium showed better adaption to harsh environments (Chang et al., 2004). Bacterial consortium also exhibited more efficient metabolisms. There have been several reports about the complete mineralization of specific PAEs by a mixed culture consortium (Wang et al., 2004; Wu et al., 2010). The halotolerant bacterial consortium LF1 enriched from activated sludge contained *Gordonia* sp., *Rhodococcus* sp.and *Achromobacter* sp. as predominant species could degrade 93.84% of 1000 mg/l DEHP after 48 hours incubation (Li et al., 2018). However, more information on the degradation of DEHP by bacterial consortium is still needed.

This study aims 1) to compare the efficiencies of DEHP-degrading activities among isolated bacterial strains and 2) to verify the DEHP-degrading activity of bacterial consortium. DEHP-degrading bacteria were enriched and isolated from mangrove sediment and landfill soil. Mangrove sediment was collected from Ban Seelong coastal area connected to the Gulf of Thailand in Bang Bo District, Samut Prakan, Thailand. Landfill soil was collected from Mueang Rayong District, Rayong, Thailand. Both sampling locations have been impacted by plastic wastes, including plastic bottle, plastic bag and food packaging. The concentration of DEHP used in this study was 200 mg/l, representing the impact of high levels of the pollutant. The knowledge obtained from this study will provide the information for the development of in situ remediating of PAEs from a contaminated environment.

2. Methodology

2.1 Enrichment media

A modified mineral salt medium (MSM) was applied for the bacterial enrichment contains the following ingredients (all concentrations in gram per litter): KH_2PO_4, 2.2; $Na_2HPO_4 \cdot 12H_2O$, 0.8; NH_4Cl, 3; $CaCl_2 \cdot 2H_2O$ 0.05, 0.1; $MgSO_4 \cdot 7H_2O$, 0.1; and $FeCl_3 \cdot 6H_2O$, 0.05 (Yuan et al., 2010). The pH of the medium was adjusted to 7 before autoclaving at 121 ^oC. DEHP with >98% purity was purchased from Applied Chemical and Instrument Co., Ltd. (Bangkok, Thailand). A stock solution of DEHP was prepared by dissolving the DEHP in dichloromethane to a concentration of 10,000 mg/l. Then, it was subsequently diluted with dichloromethane to a final concentration of 200 mg/l prior to use.

2.2 Sampling site description and sample collection

The mangrove forest area located in Ban Seelong coastal area near the Gulf of Thailand in

Bang Bo District, Samut Prakan, Thailand (13.48105ºN, 100.84848ºE). This sampling site is a small fishing community with the abundance of shrimp, crabs, fish and various aquatic animals. This area used to be a plentiful mangrove forest. Later the plastic pollution occurred by the accumulation of waste from the ocean. A lot of plastic wastes especially plastic bottles and food packaging have been found around the sampling site (Figure 1). So, the mangrove sediment likely has been exposed to chronic contamination of PAEs, including DEHP.

Landfill soil was collected from a landfill area from Mueang Rayong District, Rayong, Thailand (12.668819ºN, 101.250638ºE). The landfill has been operated by the local administrative organizations since 1995. The total amount of waste received is 3,600 tons/month (ONEP). A lot of wastes, including plastic material, have been found around the sampling site. So, the landfill soil may also expose to chronic contamination of PAEs, including DEHP.

The mangrove sediment and landfill soil samples used in the present study were randomly collected around the sampling site. The sediment samples and landfill soil samples were pooled on site and kept in a sterile Ziplock bag. The samples were kept on ice during transportation.

Figure 1. The mangrove forest area located in Bang Bo District, Samut Prakan, Thailand.

2.3 Enrichment of DEHP-degrading bacteria

DEHP-degrading bacteria from the mangrove sediment (SPK) and landfill soil (RY) were separately enriched. Five grams of sediment or soil sample were inoculated into the 250-ml Erlenmeyer flask containing 100 ml of the MSM supplement with DEHP at the concentration of 50 mg/l as the sole carbon and energy sources (Yuan et al., 2010). The suspension was incubated aerobically on an orbital shaker (200 rpm) in the dark at room temperature (30 ºC) for 7 days. One ml of the enrichment culture was serially transferred to 100 ml of fresh MSM with 100, 150, and 200 mg/l DEHP and incubated aerobically on an orbital shaker (200 rpm) in the dark at room temperature (30 ºC) for 7 days. Control cultures lacking a carbon source was performed under the same conditions.

2.4 Isolation of DEHP-degrading bacteria

After the enrichment was sub-cultured for four times, DEHP-degrading bacteria were isolated using a MSM agar plates containing 200 mg/l DEHP. The agar plates were incubated at room temperature (30 ºC) in the dark for 7 days. Colonies of candidate DEHP-degrading bacteria were picked up based on the differences in the colony morphology (i.e., sizes, shapes, margins and colors). Potential degraders were isolated and purified by repetitive streaking on MSM agar plates containing 200 mg/l DEHP. Bacterial strains SPK were isolated from mangrove sediment and bacterial strains RY were isolated from landfill soil. The characteristics of an individual colony of bacteria growing on MSM agar were observed based on their colony morphology (i.e., form, size, elevation, margin, surface, opacity, and color). The isolated strains

on MSM that were able to utilize DEHP for their growth were selected for further study.

2.5 Identification of DEHP-degrading bacteria

The identification of the isolates was based on 16S ribosomal RNA (rRNA) gene sequence analysis. The service of DNA extraction, purifying and sequencing was provided by Bionics in Korea. PCR of 16S rRNA gene amplicon was performed using 27F and 1492R primers, and sequencing was conducted using 518F and 800R primers. Finally, the Bioedit software was used to create a consensus DNA sequence from forward and reverse sequences. The similarity of the nucleotide sequence was determined by BLAST search against the National Center for Biotechnology Information databases (NCBI).

2.6 Verification of DEHP-degrading activity by isolated bacteria

All purified strains were tested for DEHP-degrading ability. The isolated strains were grown in the flask containing 50 ml of the 0.5X LB broth. Each flask was incubated aerobically on an orbital shaker (200 rpm) at room temperature (30 ºC) for 24 hr. Then, each culture was diluted to optical density approximately 1.0 with MSM. The microbial cell was centrifuged and washed three times with MSM before using as cell suspension for the biodegradation test. Microbial cell was resuspended with MSM. Then, 5 ml of cell suspension were transferred into a test tube and spiked with 200 mg/l DEHP. Sterile controls were prepared by autoclaving before the DEHP addition. The added DEHP was filtered through a PTFE filter sterile 0.22 μm membrane. All experiments were conducted in triplicate. Each flask was incubated aerobically on an orbital shaker (200 rpm) at room temperature (30 ºC) for 7 days. At day 0 and 7, the cultures were taken for the measurements of DEHP concentration.

2.7 Verification of DEHP-degrading activity by microbial consortia

The biodegradation of DEHP by mixed cultures was investigated. Four microbial consortia were formulated by mixing equal proportions of pure bacterial cultures that were isolated from mangrove sediment and landfill soil. The strains were selected based on their growth in MSM supplement with DEHP and their efficiency in degrading DEHP. Consortium 1 consisted of RY-01 and RY-04. Consortium 2 consisted of RY-01 and SPK-13. Consortium 3 consisted of RY-04 and SPK-12. Consortium 4 consisted of SPK-12 and SPK-13. The concentration of DEHP for the biodegradation test was 200 mg/l. All treatments were conducted in triplicate. Each flask was incubated aerobically on an orbital shaker (200 rpm) at room temperature (30 ºC) for 7 days. At days 0 and 7, the cultures were taken for measurement of DEHP concentration.

2.8 Analysis of DEHP concentration

Residual DEHP contents in culture media were determined according to previously published protocol (Chao, Lin, Shiung, & Kuo, 2006; Zhu et al., 2018). An equal volume of dichloromethane (5 ml) was added directly to each flask, followed by vortexing for 1 min. Dichloromethane layer was transferred into a new tube and the aqueous phase was further extracted with dichloromethane (5 ml, 2X). After the solvent being dried by left in the fume hood, the DEHP residues were eluted with 1 ml of dichloromethane. Then, the solution was passed through a 0.2-μm membrane filter into the GC vial and analyzed using a gas chromatograph equipped with flame ionization detector (GC-FID, Agilent USA).

2.9 Statistical analysis

A t-test was conducted to compare the significant differences ($p<0.05$) of the reduction of DEHP concentrations between the experimental treatments and controls (Excel software, Microsoft).

3. Results and Discussion

3.1 Biodegradation of DEHP by bacteria isolated from mangrove sediment

The results show that five DEHP-degrading bacterial isolates, SPK-03G, SPK-05, SPK-08, SPK-12, and SPK-13, were obtained from the mangrove sediment (Figure 2). The DEHP-degrading activities of these five isolates were monitored at days 0 and 7. The DEHP

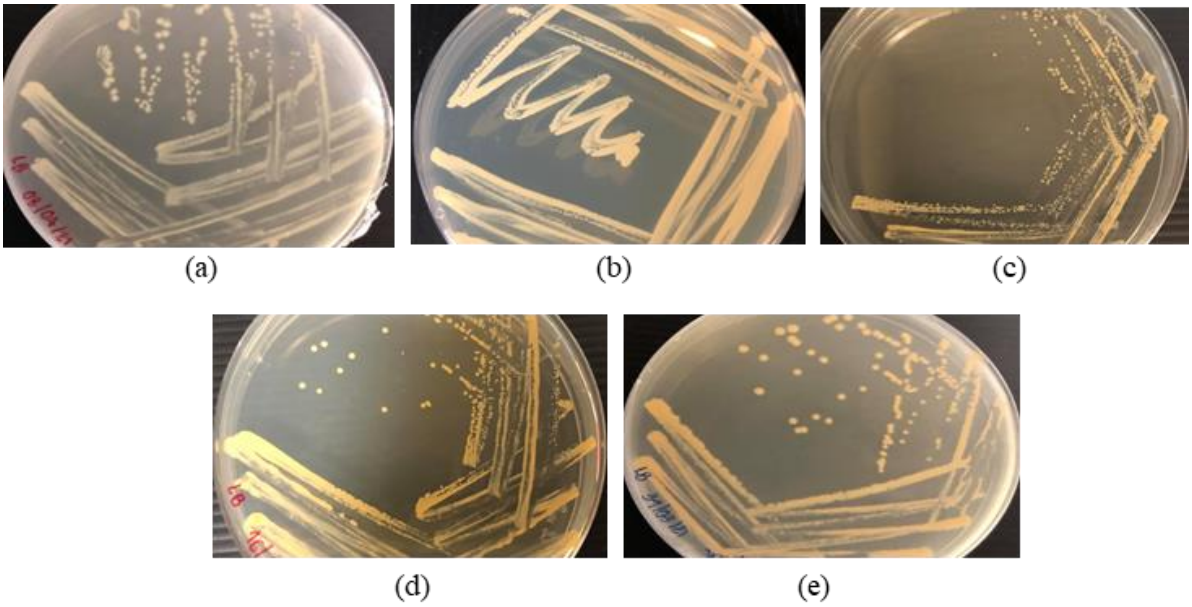

Figure 2. Five DEHP-degrading bacteria isolated from mangrove sediment on LB agar plate after incubated at room temperature in the dark for 2 days. SPK-03G (a), SPK-05 (b), SPK-08 (c), SPK-12 (d), and SPK-13 (e).

degradation efficiencies of all isolates obtained from the mangrove sediment were 71.34% to 96.70% (Table 1). Among the obtained isolates, only one isolate can be identified and it was closely related to *Bacillus megaterium* strain AK4. A previous study showed that *Bacillus* sp. was the dominant bacteria found in the mangrove sediment responsible for PAE aerobic degradation. A previous study also demonstrated that DEHP-degrading bacteria isolated from mangrove sediment were associated with *Bacillus pumilus*, *Bacillus catenulatus*, and *Bacillus amyloliquefaciens* (Yuan et al., 2010).

Our results also revealed that the SPK-12 and SPK-13, mixed culture, showed the very high efficiencies of the DEHP-degradation (Table 1). Both enrichment cultures may contain cooperative DEHP-degrading bacteria. However, both SPK-12 and SPK-13 will be further isolated and identified. The relationship among the cooperative DEHP-degrading bacteria will also be further investigated.

3.2 Biodegradation of DEHP by bacteria isolated from landfill soil

In this study, seven DEHP-degrading bacterial isolates, RY-01, RY-02, RY-03, RY-04, RY-06, RY-08, and RY-09, were obtained from the landfill soil (Figure 3). The isolated bacteria were monitored their DEHP-degrading activities at days 0 and 7 (Table 1). The results showed that DEHP-degrading bacteria obtained from the landfill soil were able to degrade DEHP ranged from 9.19% to 30.65%. The isolated RY-01, closely related to *Bartonella* sp. B43870, showed the highest DEHP-degradation efficiency (Table 1). The majority of the isolates (RY-03, RY-06, RY-08, and RY-09) was closely related to *Luteimicrobium* sp. G6 and another was affiliated with *Rhodococcus hoagii* RYA5. Overall, the obtained isolates showed relatively low DEHP degradation efficiency. Previously identified DEHP-degrading bacteria isolated from landfill or activated sludge were *Rhodococcus pyridinivorans* XB (an initial concentration = 200 mg/l DEHP, degradation efficiency = 98% within 2 days; Zhao et al., 2018), *Microbacterium* sp. J-1 (an initial concentration = 200 mg/l DEHP, degradation efficiency =96% within 5 days; Zhao et al., 2017), and *Agromyces* sp. MT-O (an initial concentration = 200 mg/l DEHP, degradation efficiency =90% within 4 days; Zhao et al., 2016). Overall, the DEHP degradation efficiencies shown by previous studies are higher than those obtained by this study. Enrichment conditions will be further optimized to enhance the growth of DEHP-degrading bacteria in the analyzed landfill soil.

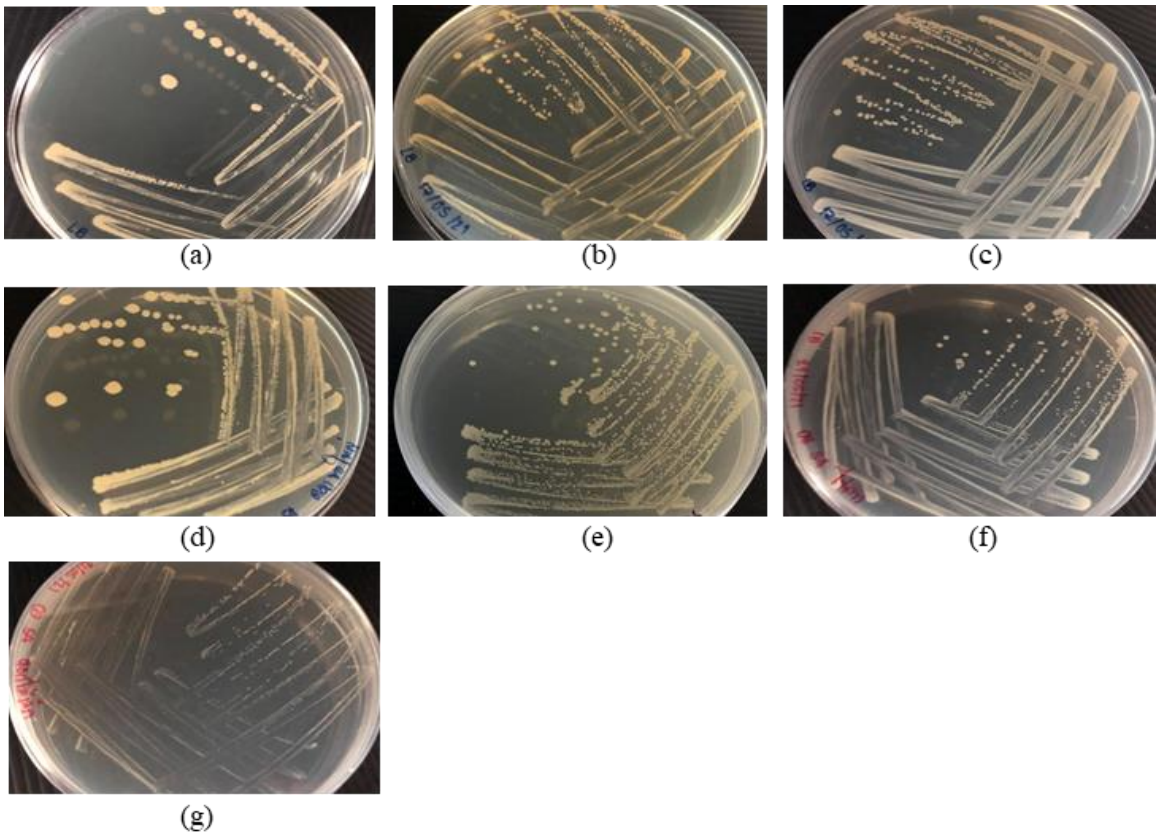

Figure 3. DEHP-degrading bacteria isolated from landfill soil on LB agar plate after incubated at room temperature in the dark for 2 days. RY-01 (a), RY-02 (b), RY-03 (c), RY-04 (d), RY-06 (e), RY-08 (f), and RY-09 (g).

Table 1: DEHP degrading activity of bacteria isolated from mangrove sediment and landfill soil.

Isolate	Degradation efficiency (%)	Closely related bacteria	Similarity (%)	Length (bp)	Accession
Mangrove sediment					
SPK-03G	75.93 ± 1.63	Unidentified	N/A	N/A	N/A
SPK-05	71.34 ± 2.09	Unidentified	N/A	N/A	N/A
SPK-08	77.43 ± 2.24	*Bacillus megaterium* strain AK4	99.93%	1383	MK966390
SPK-12	91.00 ± 2.34	Mixed	N/A	N/A	N/A
SPK-13	96.70 ± 0.47	Mixed	N/A	N/A	N/A
Landfill soil					
RY-01	30.65 ± 2.83	*Bartonella* sp. strain B43870	95.54%	830	MN504692
RY-02	28.02 ± 2.26	*Rhodococcus hoagii* strain RYA5	100.00%	1352	MT549098
RY-03	16.23 ± 4.28	*Luteimicrobium* sp. strain G6	100.00%	1363	MT993404
RY-04	28.55 ± 3.56	Mixed	N/A	N/A	N/A
RY-06	16.78 ± 5.94	*Luteimicrobium* sp. strain G6	100.00%	1348	MT993404
RY-08	9.19 ± 2.39	*Luteimicrobium* sp. strain G6	100.00%	1362	MT993404
RY-09	22.73 ± 4.90	*Luteimicrobium* sp. strain G6	100.00%	1366	MT993404

N/A: Not available

3.3 Comparison of DEHP degradation efficiency

The DEHP degradation efficiencies among the isolated bacteria obtained from mangrove sediment and landfill soil were compared (Table 1 and Figure 4). The DEHP degradation efficiencies of bacteria retrieved from the mangrove sediment were higher than those obtained from the landfill soil. Each ecosystem may contain unique microbial communities, including DEHP-degrading microorganisms. The mangrove sediment analyzed in this study has been influenced by the long-term contamination of plastic pollution. Therefore, plastic pollution could enhance the activity of DEHP-degrading bacteria. Indigenous bacteria in the impacted mangrove sediment may well adapt to use DEHP for their growth. Furthermore, the incubation condition conducted in this study (i.e., pH, DEHP concentration, and temperature) might be more suitable for promoting DEHP-degrading bacteria in the mangrove sediment than those in the landfill soil.

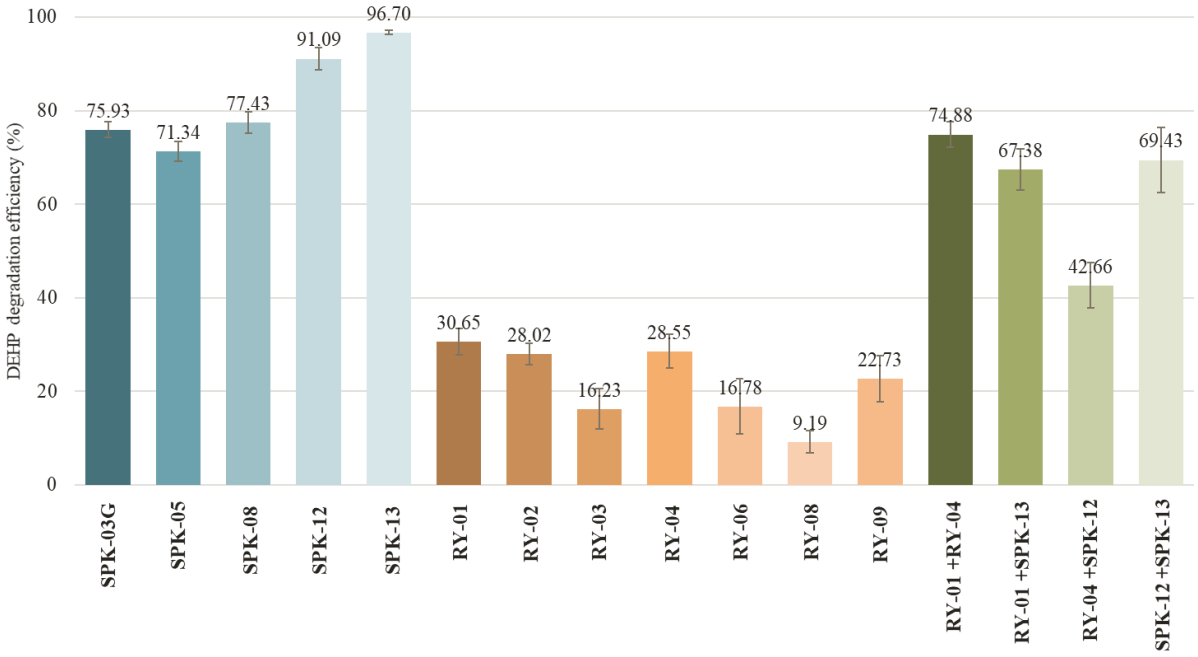

Figure 4. Comparison of DEHP degradation efficiencies among the isolated and mixed bacterial cultures.

3.4 Biodegradation of DEHP by bacterial consortium

Based on their efficiency in degrading DEHP, four bacterial isolates were selected to construct 4 bacterial consortia. Consortium 1 that was composed of RY-01 and RY-04 showed the highest DEHP-degrading efficiency (Figure 4). Although RY-01 and RY-04 solely exhibited low DEHP-degradation efficiency (Table 1), the combination of RY-01 and RY-04 increased the DEHP-degradation efficiency. This is possibly due to synergetic interaction and enzymatic system of bacteria in the Consortium 1. Individual microorganism can degrade only a limited range of hydrocarbon substrates, so mixed microorganisms with multiple metabolic capacities may increase relevant catabolic pathways associated with biodegradation (Naloka et al., 2021; Wanapaisan et al., 2018).

However, the Consortium 2 (RY-01 and SPK-13), Consortium 3 (RY-04 and SPK-12), and Consortium 4 (SPK-12 and SPK-13) could not enhance the DEHP-degradation efficiency compare to the individuals (Table 1 and Figure 4). It is possible that toxic intermediates produced by one DEHP-degrading bacteria are harmful to others (Wu et al. 2010). Shariati et al. (2021) reported that the consortium An6 (*Pseudomonas putida* ShA and *Gordonia alkanivorans* Sh6) could degrade 97.65%% of 500 mg/l DEHP within 3 days. The halotolerant bacterial consortium LF1 (*Gordonia* sp., *Rhodococcus* sp.

and *Achromobacter* sp.) enriched from activated sludge could degrade 93.84% of DEHP (1000 mg/l) after 48-hour incubation (Li et al., 2018). Further investigation on bacterial characteristics and physiological functions and an optimal ratio of inocula is needed for the construction of highly efficient DEHP-degrading bacterial consortium.

4. Conclusions

Five and seven DEHP-degrading bacterial isolates were retrieved from the mangrove sediment and the landfill soil, respectively. Although the landfill soil was a potential source of DEHP-degrading bacteria, the mangrove sediment impacted by plastic pollution was considered the valuable source of highly efficient DEHP-degrading bacteria. Among the obtained cultures, the SPK-12 and SPK-13 showed the particularly high degradation efficiencies of DEHP at 200 mg/l within 7 days. The results suggested that the SPK-12 and SPK-13 can be used as potential candidates for further development of bioremediation technology for DEHP removal. Although each isolate obtained from the landfill soil showed low DEHP degradation efficiencies, the combination of RY-01 and RY-04 enhanced the DEHP degradation efficiency. Further study will focus on the development of DEHP-degrading bacterial consortium composing of a variety of bacterial characteristics, including the ability to degrade other PAEs.

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