REVIEW ARTICLE

Degradability and Biochemical Pathways of the Endocrine - disrupting Plasticizers Phthalate Esters in Plastics by Microorganisms

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Abstract: Phthalate esters (PAEs) are a group of endocrine-disrupting organic chemicals commonly used as additives in the manufacturing of a wide range of plastics. Large quantities of different phthalate esters have been used in specific products for quality and performance by the manufacturing industries, and they pose a significant risk to human health and the ecological quality of the environments due to leaching out of phthalates from plastic products and their high mobility. Since phthalate esters are most removed efficiently through biodegradation by microorganisms in the environments, it is important to understand the efficiency, microorganisms involved, biochemical transformation processes and mechanisms of phthalate metabolism by the specific microorganisms. This article addresses the degradation of endocrine-disrupting phthalates and their fates by an integrative comparison and analysis on efficient PAEs-degrading microorganisms, the microbial metabolism, and the biochemical processes and limitation. The comparison reveals that no significant difference is evident on efficiencies between single strains of bacteria or the mixed bacterial consortia when degradation can be carried out. However, there are a few important characteristics among the efficiencies of the PAEs-degrading bacteria. The microorganisms shall utilize the specific phthalate ester as the sole source of carbon and energy. They shall mineralize the substrates, including the original compound and its degradation intermediates to achieve a complete removal. In addition, it is of practical importance for the bacteria to adapt and survive in a range of temperatures, salinity and pH as well as in the presence of indigenous microorganisms in bioremediation of contaminated sites or wastewater treatment. This review also reveals that caution should be given to both the presentation and interpretation of the degradation results for a comprehensive knowledge, particularly data on bacterial growth, extraction and analysis of residual PAEs, and the confounding use of surfactants or co-substrate in the research. The public awareness of plasticizers as an environmental pollutant is mostly due to its increasing quantities being used, constant contacts with human population on a daily basis and potential health hazards. Its toxicity shall be address more focused on reproductive biology meaningfully than the traditional mortality test in toxicology for the significant effects on animals including human.

Keywords: Biodegradation, endocrine-disrupting chemicals, microorganisms, phthalates, phthalate acid esters, environmental pollutants

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Received: April 7, 2021; **Accepted:** May 12, 2021; **Published Online:** May 17, 2021.
Citation: Tsz Ching Mak and Ji-Dong Gu, 2021. Degradability and Biochemical Pathways of the Endocrine-disrupting Plasticizers Phthalate Esters in Plastics by Microorganisms *Applied Environmental Biotechnology*, 6(1): 16-30. http://doi.org/10.26789/AEB.2021.01.003

1 Introduction

Phthalate acid esters (PAEs or phthalates) are a group of endocrine-disrupting organic compounds, made up of alkyl aryl esters or dialkyl esters of 1, 2-benzene dicarboxylic acid (Gu, 2018a, 2020a; Prasad and Suresh, 2012). A clear increase in the use and quantities consumed of PAEs by the manufacturing industries has been evident for the past half-century. PAEs are commonly used as plasticizers during the manufacturing of plastic products, e.g., polyvinyl chloride (PVC) pipes (Gao and Wen, 2016) and also personal care products and food additives. Despite being a common material used by manufacturing industries, phthalates are hazardous chemicals that warrant global public health and environmental concern due to the increasing consumption and also close contacts with human population due to the release from products and high mobility. PAEs are widely detected in different ecosystems from anthropogenic sources (Gu, 2017b; 2018a; 2020a). As phthalates are not bonded chemically to the plastic polymeric resin matrix, they leach out from products easily into the surrounding environment during the use or after disposal of the plastics (Gu, 2018a; Tang et al., 2016). Consequently, phthalates have been detected in a diverse range of environments, from river water to soil and marine sediments (Yang et al., 2018). Highest concentrations of them have been reported in landfill leachate (Kleerebezem et al., 1999). Numerous studies have found that phthalates and their metabolites interfere with the endocrine system and

have carcinogenic, teratogenic and hepatotoxic toxicity to animals (Zhao et al., 2016a).

Due to the health and ecological threats that phthalates pose, both the China National Environment Monitoring Center and the United States Environmental Protection Agency (US EPA) have listed dimethyl phthalate, diethyl phthalate, dibutyl phthalate, di-2-ethylhexyl phthalate and di-*n*-octyl phthalate as priority pollutants for control (Yang et al., 2018). Although phthalates undergo degradation under abiotic conditions such as hydrolysis and photochemical degradation, they are most degraded efficiently by the biodegradation of different microorganisms (Benjamin et al., 2015) through biochemical metabolism (Vert et al., 2012). Some issues are still obvious in the information available. One of them is that the intermediate metabolites produced during the biodegradation of phthalate esters, the mono-phthalate esters in particular, are much more toxic than the parent di-phthalate esters (Gu, 2018a). Another one is the mono-phthalate esters are weak acids and the accumulation of them result in low pH value to inhibit the subsequent transformation by the microorganisms. Therefore, there is an urgent need to identify the microorganisms that can eliminate PAEs from the environment effectively and efficiently by mineralization for a completely removal and clean up.

Microorganisms are consumers in ecosystems and they are the most important degraders of different phthalates. Available research information on phthalate-degrading microorganisms has been restricted to investigation on the efficiency by any single microorganism or consortium, and there are limited reviews on a comparison of the efficiencies or advantages of different microorganisms comprehensively. A previous review on remediation of phthalates compiles information on microorganisms and their enzymes that could efficiently transform phthalates (Benjamin et al., 2015), but it failed to address the extent of biodegradation, including mineralization and degradation pathway to offer any further insightful details. Similarly, Tang et al. (2016) compared the efficiency of *Rhizobium* sp. LMB-1 against microorganisms capable of degrading similar substrates. Although strain LMB-1 is able to degrade DMP and DEP, the comparison of efficiencies was limited to biodegradation of di-butyl phthalate and di-2-ethylhexyl phthalate only.

The objectives of this article are to contribute to the field of research on bacterial biodegradation of phthalates by reviewing and synthesis recent research, and by comparison of the degradation processes to offer a comprehensive understanding of the biodegradation mechanism and efficiencies by different microorganisms. Endocrine-disrupting phthalates and their fates will be addressed from the following perspectives: (1) the description of prevalent PAEs, (2) the endocrine-disrupting effects of PAEs, (3) the degradation of PAEs, (4) the comparison between the efficiency and mechanism of different bacteria for biodegradation of prevalent PAEs, and (5) a discussion on the insights, limitations and future research perspectives.

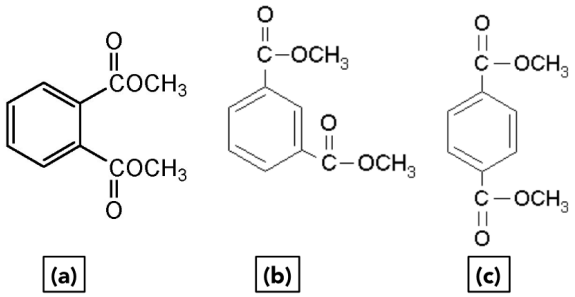

Figure 1. The three isomers of dimethlyphthalate esters with (a) *ortho*-, (b) *meta*-, and (c) *para*- positioned of the two methyl groups with ester bonds to the aromatic structure on each of the chemical structure.

2 Phthalates as Plasticizer

1, 2-Benzene dicarboxylic acid has 3 isomers, *ortho*, *para*, and *meta* (Benjamin et al., 2015; 2016) and phthalates are the corresponding esters with a variety of the ester bonded side chains (Figure 1). The chemical structures of phthalates vary according to the number of the side chains, which can be aryl, alkyl or di-alkyl groups linked to the aromatic ring, and the positions of them. Phthalic acid (PA) is *ortho*-1, 2-benzene dicarboxylic acid, and its esters constitute the majority of phthalates produced and used in different industries, particularly as plasticizers for the synthesis of PVC as one example (Benjamin et al., 2015).

Mechanical properties of plastic products can be improved by the addition of PAEs, which fill up interstitial spaces between polymeric chains to yield flexibility for processing (Benjamin et al., 2015). Low PAE concentrations result in hard plastic products while high PAE concentrations result in softer and more flexible plastic products (He et al., 2015). In addition, phthalates are used as additives to many industrial as well as consumer and personal care products, including food containers, cosmetics, toys, paints, insecticides, adhesives, shampoo, skin lotion, and medical therapy devices (Gao and Wen, 2016; Tang et al., 2016).

3 PAEs as Endocrine-disrupting Pollutants

With a steady and increasing demand for plastic products by our society (Gu, 2017b; 2020a), the worldwide production of PAEs is also growing at a steady pace, and more than 8 million tons of PAEs were produced in 2011 (Gao and Wen, 2016). However, there are two main concerns regarding the use of PAEs. First, the chemical bonding between aromatic structure and the ester part of the side chains is not covalent in the plastic polymers, therefore PAEs leach out easily from plastic products to the surroundings and have been detected in a diverse range of environments including air, water, sediments, and soils (Zhao et al., 2016a, b). Di-2-ethylhexyl

phthalate DEHP) and di-n-butyl phthalate (DBP) are the most prevalent PAEs in the environment as they are the most abundantly produced and used in industrial production of daily use plastic products (Kumar et al., 2017).

PAEs and their degradation intermediates have potential detrimental effects on the health of human and animals. PAEs are endocrine-disrupting chemicals and could interfere with the hepatic, reproductive and renal systems of humans (Gu, 2018a; 2015), and may lead to developmental and reproductive disorders (Prasad and Suresh, 2012). As a result, many congeners of PAEs such as dimethyl phthalate (DMP), diethyl phthalate (DEP), DBP and DEHP have been classified as priority pollutants for control by US EPA and similar agencies of other countries (He et al., 2015). DMP interferes with the normal development, endocrine system and reproductive system of humans and animals (Prasad, 2017), and DEP induces abnormalities in sexual differentiation (Prasad and Suresh, 2012). As one of the most common phthalates produced and used in the anthropocene, DBP is one of the most significant pollutants detected in different ecosystems, especially sediments. DBP is a potential carcinogenic, teratogenic, and hepatotoxic pollutant (Zhao et al., 2016a), and has been found to decrease the production and motility of human sperm, reduce pregnancy rates and increase the chances of miscarriage (Prasad and Suresh, 2012). Furthermore, a common degradation intermediate of the majority of PAEs, the mono-phthalate ester, is also associated with cancer and kidney damage (Gu, 2018a; Zhao et al., 2016a). Therefore, information on the microbial transformation and degradation of PAEs is required for a better understanding of this subject and also for remediation strategies to eliminating them in the environment.

4 Biodegradation of Phthalates

Biodegradation is defined as the decomposition of organic chemicals, such as organic pollutants, to harmless products including H_2O, CO_2, and CH_4 via biotic processes of organisms or microorganisms, including archaea, algae, bacteria, fungi and yeasts (Gu, 2016; 2017b; 2018a; 2020b)(Figure 2). PAEs are refractory compounds that have half-lives from weeks to more than a year under different environmental conditions subject to abiotic processes such as photolysis and hydrolysis, and the critical process to eliminate PAEs from the ecosystems remains biodegradation by microorganisms (Gu, 2016; Prasad and Suresh, 2012). Microorganisms capable of utilizing and degrading PAEs are commonly isolated from activated sludge (Gu and Wang, 2013; Gu et al., 2005; Li et al., 2012; Wang et al., 2003a, b, 2004, 2017a), mangrove wetland (Gu and Wang, 2013; Li and Gu, 2006a, b, 2007; Li et al., 2005a, b; Wang et al., 2008, 2012, 2017a, 2018), and deep ocean sediments (Gu, 2018a; Gu et al., 2009; Wang and Gu, 2006a, b; Wang et al., 2008, 2017a). In the process of isolation of microorganisms capable of degrading these chemicals, an enrichment culturing and transferring

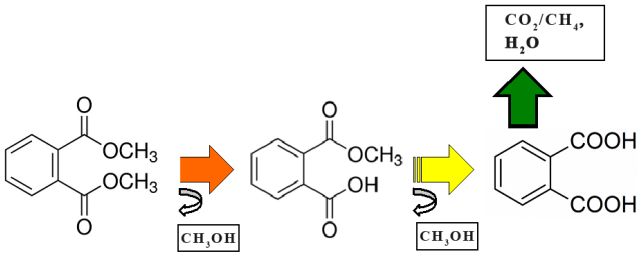

Figure 2. Microbial mineralization of *ortho*-dimethylphthalate through hydrolysis reaction to removal the two methyl groups by ester hydrolysis before formation of mineralization products

technique shall be implemented correctly to allow subsequent confirmation of the biotransformation and mechanisms analyzed (Gu, 2016; 2021). Currently, there are many who did not perform this technique correctly in the reported research, and the results and conclusion are questionable or incorrect.

PAEs are a good source of carbon and energy for a wide range of microorganisms because of the relatively simple chemical structure and also the ester bonded side chains. Biodegradation of PAEs generally involves the initial hydrolysis of the two ester bonds to form phthalic acid after cleavage of both of them (Gu, 2017b; 2018a; Gu and Wang, 2013; Zhang et al., 2018). The biodegradation of PAEs can occur via aerobic and anaerobic pathways to support growth, enzymatic activities and production of metabolic intermediates (Cheung et al., 2007; Gu, 2018a; Gu and Wang, 2013). Microorganisms such as yeasts and fungi can degrade short side-chained phthalate esters at low concentrations (20 to 50 mg/L) in 2 to less than 20 days (Luo et al., 2009, 2011; Wang et al., 2012, 2017a, 2018). In contrast, numerous bacterial species are much more efficient in metabolism of phthalates and can degrade more than 100 mg/L of phthalate within a week (Table 1). The hydrolytic cleavage of the ester bonds can be achieved by a single species of bacteria or by a cooperation between two different genera or species of bacteria (Gu, 2018a; Li and Gu, 2006b, 2007; Gu and Wang, 2013), indicating the substrate selectivity by the same class of enzymes for slightly different modified structures of the substrates (Li and Gu, 2006a; Li et al., 2005a, 2007). Degradation studies typically use selective culture medium technique to isolate enriched the bacteria for further investigations and some caution shall be observed (Gu, 2018a). Isolated microorganisms with the ability to degrade a selective substrate are then cultured in minimum salt medium (MSM) supplemented with the phthalate ester substrate as the sole source of carbon and energy, and monitored for biodegradation performance through detection of the substrate concentrations and also at the same time the microbial population over time of incubation (Gu, 2021). A sequence of biochemical reactions is involved in transforming and degrading the substrate with formation of intermediates prior to its mineralization. However, it is essential to note that some intermediate metabolites of phthalates are more toxic than the original parent compounds, e.g., mono-phthalate

Table 1. DMP and DEP biodegradation efficiencies by microorganisms

Phthalate	Bacterial Name	Conc. (mg/L)	Time (h)	Mineralization	Reference
DMP	*Variovorax* sp BS1	600	30	Yes	Prasad, 2017
	Bacillus thuringiensis	400	72	No	Surhio et al., 2014
	Variovorax sp BS1, *Achromobacter denitrificans*	300	24	Yes	Prasad, 2017
	Sphingomonas yanoikuyae DOS01	180	35	No	Gu et al., 2009
	Rhizobium sp. LMB-1	100	45	Yes	Tang et al., 2016
	Cyanothece sp. PCC7822	20	96	Yes	Zhang et al., 2016
	Trichosporon sp. DMI-5-1	21.5	576	No (68.8% degraded)	Luo et al., 2011
DEP	*Acinetobacter* sp. JDC-16	500	27	Yes	Liang et al., 2010
	Sphingomonas sp. C28242	450	120	No	Fang et al., 2017
	Variovorax sp.	300	18	Yes	Prasad and Suresh, 2012
	Sphingobium yanoikuyae SHJ	170	4	Yes	Wang et al.,2018
	Pseudomonas fluoresences FS1	100	36	Yes	Zeng et al., 2004

esters (Gu, 2017b; 2018a; 2021). Therefore, degradation research and bioremediation strategies should be focused on the complete degradation of phthalates without accumulation of such intermediates, which minimizes the adverse effects of toxic intermediates on the environment and also to human and animals of the ecosystems.

5 Biodegradation of DMP by a Mixture of Bacteria

Prasad (2017) investigated the degradation efficiency of DMP by *Achromobacter denitrificans*, *Variovorax* sp. strain BS1 and a mixture of them. The bacteria were isolated from plastic-contaminated soils near the Powai Lake of Bombay in India. *Variovorax* species is a Gram-negative, aerobic or facultative aerobic bacterium (Willems et al., 1991), while *A. denitrificans* is a Gram-negative and aerobic bacterium (Sgrelli et al., 2012). The mixture of them could degrade 300 mg/L of DMP by more than 99% in less than 24 hours, accompanied by cell growth measured by spectrophotometer at 600 nm (OD_{600}). It was found that the rate of DMP degradation by *A. denitrificans* was highest, the binary mixture had the second highest degradation rate, followed by *Variovorax* species. *A. denitrificans* degraded 250 mg/L of DMP in 15 hours, which was the highest rate of DMP degradation among the three types of cultures used. It shall be mentioned here that the water solubility of DMP is near 100 mg/L as the upper limit and the concentrations used were higher than this critical value. Because of this physical characteristic of the chemical, the concentrations reported during the experiment are oversaturated and could not be determined reliably by sampling and chemical analysis (Gu, 2018a; 2021). However, MMP was found to accumulate to more than 200 mg/L during the time of incubation, with 50 mg/L of MMP remaining even after 24 hours. *A. denitrificans* was inefficient in degrading MMP. As MMP has a much higher endocrine-disrupting activity than DMP (Prasad, 2017), the accumulation of MMP during degradation is undesirable and be due to self-inhibition during the transformation. In contrast, *Variovorax* sp. and the mixture of the two could effectively degrade DMP under aerobic conditions without the accumulation of intermediate metabolites within 24 hours. These results suggested that *Variovorax* sp. played an more important role in the breakdown of MMP in particular.

Variovorax sp. strain BS1 could degrade up to 300 mg/L of DMP in less than 30 hours of incubation (Prasad and Suresh, 2012), with a half-life of 2.4 hours. Moreover, *Bacillus thuringiensis* is one of the strains with high DMP-transforming efficiency (Surhio et al., 2014), but it was not able to degrade the metabolites such as MMP and PA. In another study, *Sphingomonas yanoikuyae* DOS01 isolated from ocean sediment degrades 180 mg/L of DMP in 35 hours (Gu et al., 2009), but it could not degrade PA. In contrast, freshwater cyanobacteria were far less efficient in DMP degradation. The cyanobacterium *Cyanothece* sp. PCC7822 took 96 hours to degrade only 20 mg/L of DMP, and was not able to utilize the degradation metabolite, PA (Zhang et al., 2016). As such, the mixture of *Variovorax* sp. BS1 and *Achromobacter denitrificans* showed higher DMP biodegradation capability of DMP and its metabolites than individual strains of microorganisms. In addition, the current study shows that each bacterial strain may play a specific role in the degradation pathway by utilizing different substrates and/or transformation products. Therefore, the degradation dynamics should be further studied by investigating the metabolic processes of the pathways by each species and the corresponding genes involved in esterase production to make meaningful contribution to the research and development on this subject area (Gu, 2021).

6 Biodegradation of DEP

Acinetobacter sp. JDC-16 is a Gram-negative, aerobic bacterium isolated from river sediment in Hebei, China. It is capable of degrading 500 mg/L of DEP completely in 27 hours (Liang et al., 2010). In this study, the substrate concentration used is a major issue to interpreta the result because it far exceeds the solubility in water. Various bacterial strains such as *Rhodococcus* sp. L4 (Lu et al., 2009) and *Gordonia alkanivorans* YC-RL2 (Nahurira et al., 2017) degrade DEP, but they took 6-8 days to completely degrade 100 mg/L of DEP. A relatively efficient species is *Pseudomonas fluoresences* FS1, which degrads 100 mg/L of DEP in 36 hours (Zeng et al., 2004). In comparison, *Acinetobacter* sp. JDC-16 is much more efficient. In addition, strain JDC-16 has the advantage of degrading a range of PAES, including PCA, PA,

DBP, DEP, and DMP.

Sphingobium yanoikuyae strain SHJ is a Gram-negative, facultative aerobic bacteria isolated from coastal sediments and grows optimally at 30°C and pH 7.2 (Wang et al., 2018). It is highly efficient in degrading DEP and can utilize DMP, DEP, and DBP as sources of carbon and energy for growth. Under aerobic condition, *S. yanoikuyae* SHJ can completely degrade 170 mg/L of DEP in 4 hours, and the half-life of DEP was 0.85 hours (Wang et al., 2018). However, there is a co-substrate in the experiment because methanol of 0.5% (v/v) was present in the DEP standard solutions as a solvent to enhance the solubilization of DEP. Fang et al. (2017) reported that low concentrations of methanol led to high activity of a bacterial esterase, but the biochemistry base for this is unclear. Hence, the salience of degradation results obtained by Wang et al. (2018) should be verified in future studies. Further studies should investigate the ability of *S. yanoikuyae* strain SHJ to utilize methanol, and effect of methanol concentrations on the growth of bacteria. If methanol acted as a supplementary source of carbon for microbial growth or enhanced esterase activity, the actual degradation efficiency of DEP as a sole carbon source could be lower. Although strain SHJ appears to be highly efficient at DEP degradation, its efficiency in bioremediation of environments without methanol is yet to be confirmed.

7 Biodegradation of DBP by different bacteria

7.1 DBP degradation by *Acinetobacter* sp. strain LMB-5

Acinetobacter sp. strain LMB-5 is a Gram-negative bacterium isolated from soil in a greenhouse for vegetable production in Nanjing, China (Fang et al., 2017). Strain LMB-5 breaks down 300 mg/L of DBP in 60 hours. The recombinant esterase, Est3563, was expressed successfully in *Escherichia coli* strain BL21 and then isolated for further characterization. The metabolites detected during DBP degradation are butyl methyl ester (BME), 2-benzenedicarboxylic acid, DMP, and PA. The presence of such metabolites suggested that DMP was degraded through the removal of its side chains, and de-esterified to form PA. PA was then transferred to the tricarboxylic cycle to be broken down. However, the detection of shorter side chain DMP and butyl methyl ester has no good explanation on biochemical basis and mechanism known.

The bacterial esterase Est 3563, involved in the biodegradation process, transforms DBP with a high level of catalytic activity at 40°C and pH 7.0 to 9.0 for optimal performance. The successful expression of Est 3563 in *E. coli* implies that the esterase has potential in biotechnology development for recombinant protein production and bioremediation application. The enzyme is characterized by a catalytic triad of Ser-Asp-His residue, which is a common characteristic of esterase (Sun et al., 2014). More importantly, the study identified a consensus motif (Gly-X-Ser-X-Gly) which surrounds the Ser residue at the active site, and suggested its role for ester bond cleavage (Zhang et al., 2014). The optimum range of esterase activity was at pH 7.5-9.0 and 40C, but Est 3563 does not show tolerance for temperatures higher than 50°C, which could be explained from the lack of Trp and Phe to maintain the stability of the protein structure (Fang et al., 2017).

Agrobacterium sp. JDC-49 is another bacterial strain found to degrade 200 mg/L of DBP in 48 hours, and the half-life of DBP at 100 and 200 mg/L was 10.4 and 17.2 hours, respectively (Wu et al., 2010a, b). However, the degradation of DBP by *Acinetobacter* strain LMB-5 is more efficient, with a half-life of DBP at 9.47 and 13.28 hours respectively. Additionally, *Arthrobacter* sp. strain C21 degrades up to 300 mg/L of DBP in 70 hours (Wen et al., 2014), while *Acinetobacter* sp. strain LMB-5, in contrast, only requires 60 hours. The above comparisons indicate that strain LMB-5 is the most efficient one in degrading DBP.

7.2 DBP degradation by *Providencia* sp. 2D

Providencia sp. 2D is a Gram-negative, facultatively anaerobic and rod-shaped bacterial strain isolated from farm compost in Guangzhou of China (Zhao et al., 2016a). It could completely degrade DBP at an initial concentration of up to 200 mg/L in 3 days. The half-life of DBP was 10.7 hours at the initial concentrations of 200 mg/L. The metabolites detected during the degradation of DBP are MBP, PA and benzoic acid (BA). All metabolites decrease their concentrations after 48 hours and completely disappear by 144 hours. However, substrate utilization tests indicate that strain 2D is not efficient in degrading PA at higher than 100 mg/L. At 500 mg/L, less than 44% of PA could be degraded after 144 hours. These findings are consistent with previous research, where PA produced from de-esterification of DBP acidified degradation conditions, which led to the decrease of metabolic activities and inhibited further biochemical reactions under the acidic conditions (Jin et al., 2012).

The general pathway suggested is that a hydrolysis reaction by esterase attack of DBP to form MBP and then PA. DBP may also be directly de-esterified to PA. Then, PA undergoes cleavage of the aromatic ring prior to mineralization. In the degradation of PA, the cleavage of the aromatic ring varies depending on either aerobic and anaerobic conditions (Sarkar et al., 2013). Firstly, under aerobic conditions, PA is metabolized through the 4,5-dihydroxyphthalate pathway to form PCA, which is then mineralized as CO_2 and H_2O. This pathway is similar to previous research on the DBP degradation pathways by Gram-negative bacteria (Iwaki et al., 2012). Although PCA could not be detected during PA degradation, strain 2D contains a gene which encodes for protocatechuate dioxygenase, implying that PCA might have been quickly degraded by *Providencia* sp. 2D after it is formed. Secondly, the presence of BA is an important indicator of a separate

anaerobic pathway occurring in DBP degradation by strain 2D (Liang et al., 2008). BA is produced from removing a carboxyl group from PA, then the aromatic ring of BA is cleavaged and mineralized to CO_2 and H_2O. These observations on the two pathways are further supported by the degradation kinetics. Strain 2D degrades PCA under aerobic condition, whereas it could degrade PA and BA under both aerobic and anaerobic conditions. Although other species of bacteria can metabolize BA to catechol or PCA under aerobic conditions (Fuchs et al., 2011), the BA degradation pathway by strain 2D was not investigated in the reported study.

An important point to note is that *Providencia* sp. 2D showed efficient DBP-degradation capability when applied to contaminated soil. Regardless of soil with or without compost amendment as supplementary nutrient supply, the addition of strain 2D was able to reduce the half-life of DBP from more than 10.3 days to less than 3.3 days. The reduction of time shows that strain 2D has potential in potentially DBP degradation for bioremediation of soils. It is also clear from this and similar reports for an on-site testing with inoculation of a microorganism into the soil that tracing and tagging of the microorganism is required to support the metabolic function of it in the environment, but many reports do not have such information to support their results or claim on a solid ground of science (Gu, 2016; 2018b; 2021).

7.3 DBP degradation by *Rhizobium* sp. strain LMB-1

Rhizobium sp. strain LMB-1 is a Gram-negative, obligate aerobic and rod-shaped bacterium. It could mineralize 50 mg/L of DBP in 48 hours, and the corresponding half-life is 16.1 hours (Tang et al., 2016). The degradation metabolites detected by gas chromatography-mass spectrometer (GC-MS) are DEP, DMP, mono-ethyl phthalate (MEP), mono-methyl phthalate (MMP), PA and tartaric acid. Although metabolites are detected, the concentrations of them were very low, which implies that the intermediates are not accumulated and are efficiently degraded by strain LMB-1 as one possibility while the alternative is some of these low concentrations of metabolites are actually contaminants of the instruments and also processing. The proposed degradation pathway is as follows: DBP is first hydrolyzed through β-oxidation to produce the intermediate metabolite DEP. Two possible routes of DEP biodegradation were proposed and they may co-occur. Firstly, DEP undergoes de-esterification reactions to form MEP and then PA. Alternatively, DEP can be de-methylated to form DMP, which is then de-esterified to MMP and then PA. The demethylation is a bold claim because biochemically this reaction requires a much high level of energy input comparing to ester hydrolysis (Gu and Wang, 2013). PA was not accumulated, and the presence of tartaric acid indicated that PA undergoes further degradation processes such as aromatic ring cleavage.

In comparison, *Enterobacter* sp. T5 shows similar efficiency for the degradation of 100 mg/L of DBP within 45 hours (Tang et al., 2016). However, strain LMB-1 has an advantage since the half-life for DBP is 17.2 hours, shorter than the 20.9 hours by strain T5. In addition, strain LMB-1 could also degrade DEHP (100 mg/L) in 120 hours. In comparison, *Sphingomonas* sp. DK 4 and *Corynebacterium* sp. O18 both degrade 100 mg/L of DEHP ind 168 hours (Chang et al., 2004; Fang et al., 2010). As no degradation pathway for DEHP is available in this study, degradation efficiency of strain LMB-1 for DEHP and its tolerance of different DEHP concentrations can be focused for more information. Results from the study indicates that strain LMB-1 may possess metabolic capabilities to degrade a wide range of substrates including tartaric acid, PA, MMP, MEP, DMP, DEP and DBP. Such a wide degradation spectrum makes LMB-1 a potential candidate for the bioremediation of environments polluted by PAEs, but the substantiation of such claim must be established prior to any initiatives to implement in bioremedation.

7.4 DBP degradation by *Sphingobium* sp.

Two strains of the *Sphingobium* genus degrade DBP with high efficiency. *Sphingobium yanoikuyae* strain P4, a Gram-negative bacterium, was isolated from a waste dumping site in the West Himalayas of India (Mahajan et al., 2019). It is able to degrade a high concentration of DBP of 1,000 mg/L in 24 hours at 28°C. Such high concentration is far above the solubility as pointed out above ((Gu, 2021). In addition to degrading DBP, strain P4 also utilizes an extensive range of substrates including di-isobutyl phthalate (DIBP), DEP, DMP, BBP, BA and PA. Although PA is produced during DBP degradation, no PA can be detected after 30 hours. The metabolites detected during degradation include mono-n-butyl phthalate (MnBP) and PA. According to the detected metabolites, DBP is first hydrolyzed to MnBP, which is further hydrolyzed to PA similar to others reported (Gu, 2018a; Gu and Wang, 2013; Li and Gu, 2007). PA is then degraded to PCA by dioxygenase before being mineralized in the TCA cycle. While the optimal temperature for most bacteria to degrade pollutants ranged between 30-38°C (Fang et al., 2010), strain P4 is psychrotolerant and could degrade DBP at temperatures of 15°C to 28°C. Notably, strain P4 could completely degrade 1,000 mg/L of DBP within 60 hours at temperatures as low as 15°C as claimed. Sequence analysis identified various catalytic centers associated with ester bond metabolism. Firstly, an intracellular carboxylesterase with conserved motifs matches previous reports on serine hydrolases (Zarafeta et al., 2016; Fang et al., 2017). The carboxylase has a pentapeptide motif of G182-X-S184-X-G186, in which X indicates non-conserved residues in the sequence, and aspartic acid, glutamic acid or glutamine as the first non-conserved residue, while a non-polar residue such as alanine is the second X. Previous reports on many phthalate hydrolases or esterases show that the serine residue within the pentapeptide motif plays a role in the catalytic processes (Zarafeta et al., 2016).

Similar to Fang et al. (2017), a conserved catalytic triad associated with esterases (Sun et al., 2014) has been identified in addition to the pentapeptide motif, consisting of Asp-Ser184-His101. Furthermore, a conserved His-Gly-Gly-Gly tetrapeptide sequence is identified in strain P4, which is a motif included in the α/β hydrolase family. The sequence has been previously reported in mono-alkyl and di-alkyl phthalate ester hydrolysis (Mahajan et al., 2019). The positively charged residue in the motif may play an essential role in neutralizing the carboxylate anions to prevent repulsion between the catalytic center and the anion near the ester bond of the phthalate (Mahajan et al., 2019).

Such sequencing results support the observation that strain P4 has an outstanding ability in degrading DBP as compared to other efficient DBP-degrading bacteria such as *Sphingobium* sp. strain TJ (Jin et al., 2013). As strain P4 has an outstanding potential to be applied in bioremediation, it would be meaningful for future studies to verify such findings for the efficiency of this strain. Furthermore, it is also meaningful to investigate the mechanisms and properties of enzymes involved in DBP degradation in more detail as the enzymes are active at lower temperatures, which is markedly different from the usual range of optimal degradation temperatures of 30°C to 38°C (Jin et al., 2013). *Sphingobium* sp. strain TJ is a Gram-negative bacterium isolated from water samples of the Haihe estuary in China (Jin et al., 2013). The strain could degrade 500 mg/L of DBP in 32 hours and could also utilize DMP, DEP, MBP, and benzoic acid (BA) for growth. However, strain TJ could not use PAEs with longer sidechains, e.g., DOP and di-isooctyl phthalate. Such results suggested that the ability of strain TJ in degrading phthalates is affected by the length of phthalate side chains due to structural compatibility between the enzyme and the substrate. These results are similar to that of *Ochrobactrum* JDC-41 (Wu et al., 2010a, b). In addition, JDC-41 could also degrade 500 mg/L of DBP, but requires much longer time than TJ, which shows that strain TJ is more efficient in degrading DBP.

Strain TJ is tolerant to high salinity levels of up to near 40‰ of NaCl. Previous research shows that *Burkholderia cepacia* strain DA2 is capable of DMP degradation within 10‰ of salinity (Wang et al., 2008), indicating that strain TJ is a more promising candidate for bioremediation of salt-containing wastewater. One drawback of strain TJ is that it is not capable of degrading PA (Jin et al., 2013). Therefore, future studies may consider using strain TJ in mixed cultures with other bacteria capable of efficiently degrading PA to achieve quick degradation of DBP and mineralization of its metabolites.

7.5 Biodegradation of DBP by bacterial consortium

Consortium LV-1 was isolated from the enrichment of river sludge in Henan, China ((Wang et al., 2017a). It is able to degrade 500 mg/L of DBP to non-detectable levels within 72 hours and could tolerate high DBP concentrations of up to 1,000 mg/L. DBP, DMP, DEP, and PA are utilized by the consortium. There is a total of 48 genera found in the consortium, with the relative abundance of *Brucella* spp. and *Sinobacter* spp. being the dominant ones at 62.78% and 14.83%, respectively in the population. The detected intermediates are mono-butyl phthalate (MBP), mono-ethyl phthalate (MEP) and PA. The presence of mono-ethyl phthalate indicates that DBP was proposed as possibly degradation through reactions alternating between β-oxidation and ester hydrolysis. The study suggests two possible degradation pathways. The sources of the detected degradation intermediates remain to be confirmed to allow the claim to be established.

In the first pathway, BEP is first formed by removing an ethyl group from DBP through β-oxidation, then MBP is formed by ester hydrolysis of BEP. However, BEP could not be detected in the study. The β-oxidation of the terminal side chain is unlikely because the ester bond between the aromatic moiety and the side chain is far weaker than the C-C bond within the side chain. The second degradation pathway follows that no BEP is produced during DBP degradation. Instead, MBP is the first metabolite formed through ester hydrolysis of DBP, then β-oxidation and ester hydrolysis result in the formation of PA. In contrast to the multiple metabolites generated during degradation by consortium LV-1, preceding research confirms that PA is the only metabolite detected during the DBP degradation (He et al., 2015; Yang et al., 2018). Wang et al. (2017a) suggested that the bacterial composition of LV-1 may explain the results. The relative abundance of *Sinobacter* spp. and *Brucella* spp. with above 78% in total for their role on oil and phenol degradation, but not on phthalate degradation. As the metabolic pathway of *Brucella* spp. and *Sinobacter* spp. in DBP degradation remains unclear, the substrate utilization ability of each of the genera should be further studied to identify the biochemical roles with specific genes expressed to advance the current understanding on the mechanisms or synergistic interactions in degradation.

In comparison, single strain bacteria such as *Ochrobactrum* sp. JDC-41 is able to completely degrade the same initial concentrations DBP within the same time frame of 3 days (Wu et al., 2010a, b). However, JDC-41 could not tolerate higher concentrations (>500 mg/L) of DBP, while LV-1 exhibited a high degradation ratio of 97.6% at 1000 mg/L of DBP, indicating its tolerance of high DBP concentrations. The real concentration in solubility term shall be revisited to judge the validity of the claim. Furthermore, the diversity of consortium LV-1 may be advantageous for the survival of bacteria in application to environmental remediation. Compared to consortium HD-1 which has 3 genera (He et al., 2013), consortium LV-1 contains 48 genera (Wang et al., 2017a; 2018), where 8 genera among the 48 have a relative abundance higher than 1%. A higher diversity of bacteria could enhance the ratio of survival of microorganisms across a range of environments (He et al., 2013), which gives an ad-

vantage of using LV-1 in bioremediation. In addition, as high initial concentrations of 1,000 mg/L did not appear to show toxic inhibition on the degradation activity of LV-1. Therefore, LV-1 shows potential to be applied in DBP-polluted environments. However, one drawback of consortium LV-1 is that it contains the genus *Brucella*, which is a pathogenic species that could infect humans and animals (Wang et al., 2017a). In order to lower the risk that pathogens pose to humans, there is a need for disinfection of the bioremediated medium, which may limit the practicality of applying LV-1 in bioremediation.

Consortium HD-1 was isolated from enriched activated sludge of a wastewater treatment plant (He et al., 2013). The consortium could degrade 1,200 mg/L of DBP by >90% within 48 hours. *Gordonia* sp., *Burkholderia* sp. and *Achromobacter* sp. are identified using denaturing gradient gel electrophoresis. HD-1 hashigher growth rates and degradation efficiencies in alkaline than acidic conditions. The optimal pH and temperature for HD-1 to degrade DBP is at pH 8 to 9 and 25-35°C. Similar to consortium B1 (Yang et al., 2018), PA is the only major metabolite detected during DBP degradation by HD-1. It grows well using phenol or PA as the sole source of carbon and energy. This shows that in addition to the ability to utilize PA, HD-1 could also degrade simple aromatic compounds. However, He et al. (2013) noted that the lower biomass and degradation efficiency under acidic condition might be due to the accumulation of PA during DBP degradation. Since HD-1 is able to utilize PA at a limited efficiency and encounters hindrance in growth and degradation rates due to acidic conditions, future studies may also consider investigating the tolerance of HD-1 for different PA concentrations. Moreover, application of a microorganism with efficient PA-degrading abilities along with consortium HD-1 may help to enhance bioremediation.

Additionally, further investigations are needed on the abundance of microorganisms in the consortium, especially on the activity-based analysis than genomic DNA. For example, RNA extraction, reverse transcription to cDNA and then amplification of 16S rRNA gene before Illumina or Next-generation sequencing (Wang et al., 2017a) can be used to identify the bacterial members in the community. Consortium HD-1 has high capability and efficiency for degrading DBP compared to consortium LV-1. HD-1 could degrade 1,200 mg/L of DBP by >90% within 48 hours, while consortium LV-1 could only degrade 500 mg/L of DBP in 3 days, which was comparable to the degradation performance by single strains of *Ochrobactrum* sp. JDC-41 and *Sphingobium* sp. TJ.

Consortium B1 was isolated from activated sludge collected from a sewage treatment plant (Yang et al., 2018). In the study, the most abundant genera in consortium B1 are *Pandoraea* followed by *Microbacterium*, *Flavisolibacter*, *Gordonia*, and *Comamonas*. The culture of consortium B1 removes 100 mg/L of DBP almost completely in 40 hours. The consortium could degrade DBP most efficiently at 30°C with optimum pH range at 5.5-8.5. There are marked changes in the relative abundance of *Microbacterium* and *Pandoraea*. The relative abundance of *Microbacterium* decreases in day 1 of the incubation, but increases in day 2; *Pandoraea* shows a reverse trend. *Flavisolibacter*, *Gordonia*, and *Comamonas* show a slight decrease in relative abundance throughout the experiment. The only DBP degradation metabolite detected is PA, and concentrations of PA decreased from day 1 to day 2. In addition, B1 shows high growth in acidic condition compared to JDC-49, HD-1, and *Sphingobium* sp. (He et al.,2013, 2015; Jin et al., 2013; Wu et al., 2011). The results of this study corroborate with previous findings showing that *Gordonia* sp. and *Comamonas* sp. in consortium B1 could utilize PA (Jin et al., 2012; Wang et al., 2003a). Similarly, a previous report found that it was easier to detect metabolites in pure culture than in mixed cultures (Hudcova et al., 2011). It is suggested that different strains of bacteria in the mixed culture could utilize metabolic intermediates to lead to difficulties in detecting intermediates other than PA (Li and Gu, 2006a, 2007; Li et al., 2005a; Yang et al., 2018). It is clear that consortium B1 has the merits of utilizing and degrading a range of metabolic intermediates from DBP degradation, and also phthalates under acidic conditions, which makes it a strong candidate for bioremediation.

Yang et al. (2018) further tested the degradation ability of consortium B1 by inoculating the consortium into soil spiked with DBP (100 mg/kg). Kinetic parameters showed that the degradation of DBP in soil by consortium B1 followed the First-order model. The addition of consortium B1 to soil could increase DBP degradation of indigenous microorganisms in the non-sterilized soil, with a half-life of DBP in soil decreasing from 12.21 days to 2.14 days. Such results indicate that consortium B1 shows potential particularly in application to DBP-contaminated soils, as the bacteria in the consortium act synergistically with indigenous soil bacteria for DBP degradation. This type of study has several pitfalls which are discussed previously (Gu, 2016; 2020b; 2021).

8 Biodegradation of PAEs with long side-chain

PAEs are diverse structurally in the positions of the ester bonds and also the nature of the side chains, either the same or different. PAEs with short-chains undergo biodegradation more readily than those with long-chains (Liang et al., 2010). With the changes in positions and the length of the side chain, the physical and chemical properties of the respective PAEs vary greatly. The difference in biodegradability of PAEs may be due to the steric hindrance by the side chains of PAEs, which interfere with the effective binding of enzymes to PAEs, thus affecting the degradation process (Liang et al., 2008). Because of their more recalcitrant nature and low biodegradation rates compared with those of short-alkyl-chain phthalates, long-alkyl-chain phthalates, such as DEHP, D-*n*-OP, and DPP, are considered posing higher risk to the environment and human health (Cousins et al., 2003).

8.1 Degradation of DEHP by single strain of bacteria

Mycobacterium sp. strain YC-RL4 was isolated from petroleum - contaminated soil (Ren et al., 2016). It utilizes 50 mg/L of DEHP in 72 hours. The optimal temperature and pH are 30°C and pH 8, respectively. The metabolites detected during the biodegradation of DEHP include mono-ethylhexyl phthalate (MEHP), PA, and BA, and strain YC-RL4 utilizes the degradation metabolites for growth. The proposed degradation pathway involved the initial hydrolysis of the DEHP ester bonds to form MEHP and then PA. BA is then formed by decarboxylation of PA. An important finding of the study is on the cell surface properties of bacteria. Notably, the cell surface hydrophobicity (CSH) changed across both incubation time and substrate. The CSH is correlated with the assimilation and biodegradation of DEHP and CSH reachs a maximum when bacterial growth and phthalate degradation are in exponential phase. An increase in CSH allows bacterial cells to adapt and adhere to hydrophobic pollutants for a better access to sources of carbon and nutrients, thus increasing biodegradation (Ren et al., 2016).

In contrast, consortium LF could degrade >92% of 1,000 mg/L of DEHP in 72 hours (Li et al., 2018). Consortium LF consists of seven genera in total, and three major genera are identified as *Gordonia* sp., *Rhodococcus* sp. And *Achromobacter* sp. With a relative abundance of 54.93%, 9.92% and 8.47%. The degradation metabolites identified are 2-ethylhexyl pentyl phthalate, butyl 2-ethylhexyl phthalate, MEHP and MBP. These intermediates indicate that DEHP and its intermediates are metabolized by β-oxidation and hydrolysis. Although PA is not observed, substrate utilization tests of PA suggest that consortium LF could efficiently degrade PA. Results suggested that consortium LF can successfully metabolize DEHP and its intermediates. An important feature of consortium LF is that it tolerates to high salt conditions of 4% of NaCl, similar to the halotolerance DBP-degrading *Sphingobium* sp. TJ (Jin et al., 2013). Seawater has approximately 3% (3‰) salt concentration, which indicates that the consortium LF is suitable for application in the bioremediation of saline environments.

8.2 Degradation of long-chain PAE by bacterial consortium

Gordonia sp. strain JDC-2 and *Arthrobacter* sp. strain JDC-32 were Gram-positive bacteria isolated from activated sludge and utilize PAEs as their sole source of carbon and energy (Wu et al., 2010a, b). *Gordonia* sp. strain JDC-2 could efficiently degrade 500 mg/L of DOP within 60 hours. Additionally, substrate utilization tests show that strain JDC-2 could utilize DOP, DBP, DEP, and DMP. However, the PA concentration accumulates up to 93.26 mg/L and could not be degraded after 30 days. Such results imply that while *Gordonia* sp. strain JDC-2 is capable of degrading DOP to PA, it fails to further utilize or break down the aromatic ring of PA. On the other hand, *Arthrobacter* sp. strain JDC-32 could completely degrade 500 mg/L of PA after incubation for 20 hours, and the biomass increases from 0.012 to 0.441 (OD_{600}). However, JDC-32 could only degrade DOP to a small extent, and there was no detectable increase in biomass during incubation in the presence of DOP. Strain JDC-32 could efficiently utilize and degrade phthalates with shorter alkyl chains such as DBP, DEP, DMP, PA, and PCA.

The mixture of *Gordonia* sp. JDC-2 and *Arthrobacter* sp. strain JDC-32 show a synergistic effect in degrading DOP into PA. Analysis of degradation metabolites indicate that strain JDC-2 might degrade DOP via β-oxidation to remove ethyl groups from the side chains to form BOP and subsequently DBP. Although metabolites such as butyl octyl phthalate (BOP), DBP and DEP are detected during the degradation of DOP by the bacterial consortium, the concentrations are much lower than the detected levels from single bacterial strain JDC-2, and MMP and PA were not detected. Cooperation of two different bacteria to achieve complete degradation of phthalate ester has been elucidated (Gu, 2018a; Gu and Wang, 2013; Li and Gu, 2007). Thus, it is suggested that the role of *Gordonia* sp. was responsible for the initial degradation of DOP, and the generated metabolites, such as PA, are utilized and degraded by *Arthrobacter* sp. JDC-32. Such results indicated that the consortium could degrade DOP more efficiently than using either species alone.

9 Biodegradation pathways and degradation efficiency

Both single bacterial strains and consortia may efficiently degrade PAEs based on efficiencies in degradation studies above. The common and established reactions so far is hydrolysis of the ester linkages to release the side chains. Though β-oxidation and demethylation are also proposed, the bases for such reaction to take place by microorganisms are still not strong enough on a thermodynamically and biochemistry bases. However, there are certain common characteristics that bacteria for biodegradation of PAEs should ideally possess. Firstly, the bacterial inoculum should utilize the targeted phthalate and its degradation intermediates as their carbon and energy source to achieve mineralization of the parent compound (Gu, 2016; 2021). In this aspect, mixed cultures have the following advantages. The number of catabolic pathways available for the degradation of pollutants is increased in mixed cultures, in which different bacterial strains contribute differently to biodegradation pathways to increase the overall degradation efficiency (Prasad, 2017). At the same time, metabolic intermediates from one bacterial strain can be utilized by another strain more efficiently for mineralization (He et al., 2013; Wu et al., 2010a).

When considering the efficient removal of phthalates in situ in bioremediation, bacteria should adapt and survive in a

wide range of temperatures and pH conditions. PAEs often exist with other pollutants simultaneously in natural environments (Ren et al., 2016). Because of the ageing and sequestration by physical matrices, bioavailability of the target contaminants need to be delineated clearly for any bioremediation to be carried out (Gu, 2018a; 2021). Although some single species of bacteria could utilize and degrade phthalates with short alkyl chains (Liang et al., 2010; Wen et al., 2014; Wu et al., 2010a), they failed to utilize those with long side chains. The merit of diverse species in a microbial consortium is the higher chances to tolerate toxic substances and degrade a range of pollutants (Yang et al., 2018; Wu et al., 2010a), which increases success rates under different environmental conditions (He et al., 2013). Moreover, consortia LV and B1 show efficient degradation over a wide range of pH conditions (Wang et al., 2017a; 2018), the effectiveness of bioremediation is dependent on the selection and application of suitable bacteria to selected environmental conditions (Santisi et al., 2015) and knowledge on the site characteristics and the microbial biochemical capabilities (Gu, 2016; 2021). In other words, bacteria growth requirements and the environmental conditions shall be matched for best performance and results. The selection of halotolerant *Sphingomonas* sp. strain TJ or consortium LF (Jin et al., 2013; Li et al., 2018) may result in greater efficiency and effectiveness of phthalate removal for the bioremediation in saline environments.

Bacteria with the ability to enhance biodegradation of indigenous bacteria show practical value. *Providencia* sp. strain 2D and consortium B1 show an increase in the phthalate degradation capacity of the indigenous microorganisms in soil (Zhao et al., 2016b; Yang et al., 2018). This may simply due to the 'priming effects'. *Providencia* sp. strain 2D has the advantage of degrading DBP completely, including its metabolite, PA, under both aerobic and anaerobic conditions, but the broad claim has serious challenge because the mechanisms for its survival under both aerobic and anaerobic are not provided. These strains of bacteria may have potentials for bioaugmentation applications as amendment by inoculation of PAE-degrading bacteria to contaminated sites.

Interestingly, the induction of single strains of bacteria by substrates can increase the efficiency of DEP and DBP degradation (Wu et al., 2010a; Liang et al., 2010). Induction of *Acinetobacter* JDC-16 with DEP results in a shorter lag phase initially and improve DEP esterase activity (Liang et al., 2010). Jin et al. (2012) also found that expression levels for an esterase increased after induction.

Enzymes have high specificities for substrates including phthalate esters, and different enzymes may be involved in each hydrolying step. For example, esterases catalyzing dialkyl phthalates may not transform mono-alkyl phthalates (Hara et al., 2010; Li and Gu, 2006a; Luo et al., 2012; Whangsuk et al., 2015), which indicates that dialkyl phthalates are hydrolyzed by two different esterases (Xu et al., 2017). Similar results were reported earlier (Li and Gu, 2007; Gu, 2021). Moreover, genes encoding enzymes of ester bond hydrolysis are usually different from genes for PA utilization and catabolism (Whangsuk et al., 2015). It is, therefore, of practical significance to compare the genetic sequences of novel phthalate-degrading bacterial isolates to available libraries of genes and gene clusters for characterization and analysis of the catabolic process. Identification and comparison of genes related to phthalate degradation will enrich current understanding of the synergistic mechanisms between mixed bacterial population in cooperative degradation of phthalates (Li and Gu, 2007; Gu, 2021). Moreover, esterases have been successfully produced from the expression of recombinant DNA, such as Est 3563 of *Acinetobacter* sp. strain LMB-5 (Fang et al., 2017; Li et al., 2016; Wang et al., 2012). The production of recombinant esterases could serve as a crucial starting point for genetic engineering and enhancing biodegradation of microorganisms for selective substrates in biotechnological application.

10 Cautions and Improvements

Firstly, to provide convincing evidence that microorganisms are responsible for the degradation of phthalates, the changes of phthalate concentration and growth curves of bacteria should be simultaneously presented as a basic requirement (Gu, 2016). However, some studies do not present data on microbial growth simultaneously with the concentration changes of phthalates (Wu et al., 2011; Surhio et al., 2014) or lack the presentation of data on growth entirely. For example, *Gordonia alkivorans* strain YC-RL2 is a DEHP-degrading bacterium that showed high degradation efficiency over a range of temperatures, salinity and substrate concentration (Nahurira et al., 2017), but information on growth, such as biomass, was absent in the publication.

Other studies present many growth curves of OD_{600} measurements respectively to different initial phthalate concentrations (Wen et al., 2014), which does not allow a quantitative analysis of the results. There are two suggestions to mediate this problem. First, both phthalate degradation and microbial growth under optimal conditions, such as the growth curve of optimum temperature, pH value and concentration of phthalate against the corresponding OD_{600} values for the most essential can be shown. Additionally, bacterial growth data can be analyzed and presented in values indicative of bacterial growth characteristics, namely maximum yield of biomass, specific growth rate, and the lag phase to allow a meaningful comparison (Gu, 2017a; 2021).

Substrate extraction and quantification require additional attentions. Most current experimental methods for extracting residual phthalates from environmental matrices ignore the adsorption and sequestration of PAEs as a confounding variable affecting biodegradation results in experiments (Gu, 2021). There is also a possibility of adsorption of PAEs onto biomass, especially in laboratory condition (Gu, 2016),

which result in over-estimation of the biodegradation ability by bacteria. Most current studies obtain recovery of PAEs by using solvents to dissolve phthalates into an aqueous phase for extraction and then quantitative analysis. The presence of cell biomass is often neglected. The method used by Babu and Wu (2010) involves the application of diethyl ether to extract possible residual PAEs from centrifuged cell pellets and this step can be considered in future studies.

Surfactants or solubilizing agents are frequently used in degradation studies and this practice should be cautiously implemented as the results become complex for interpretation. Most environmental pollutants are low in water solubility by nature due to their hydrophobic properties. As the chemicals do not dissolve well in culture medium, microorganisms may have limited access to chemicals for assimilation and then metabolic processes, including transformation and degradation. Degradation studies may use Tween-80, a solubilizing agent, to enhance the solubility of phthalates. For example, *Acinetobacter* sp. strain SN-13 (Xu et al., 2005; 2006; 2017) degrades more than 90% of 100 mg/L of DEHP within 5 days and *Bacillus subtilis* strain 3C3 was also found to degrade phthalates efficiently, but Tween-80 is supplemented in both degradation experiments (Navacharoen and Vangnai, 2011).

Although solubilizing agents increase the accessibility of hydrophobic substrates to be utilized, and do not serve as a source of carbon and energy for bacteria (Xu et al., 2017), the results do not reflect the natural or indigenous phthalate utilization capability by bacterial strains. Moreover, surfactants such as Tween-80, Triton X-100, Brij-35 and Tergitol-NP10 have toxic and adverse effects on bacteria (Navacharoen and Vangnai, 2011). Toxic effects are not desirable because it has a direct detrimental effect on the phthalate degrading activity of bacteria, and may also negatively affect any indigenous bacteria in the environments. The practicality of applying solubilizing agents is restricted because application of such chemicals on a large-scale, including contaminated sites or wastewater treatment plants, is not advised. Therefore, there is a limited significance to using surfactants in bioremediation or treatment applications.

However, studies have shown that bacterial cells can attach onto droplets of DEHP and break it into micro-droplets upon utilization of the phthalate (Benjamin et al., 2016). SEM analysis in Benjamin et al. (2016) study showed the auto-aggregation of cells around DEHP droplets, which may be an important phenomenon for the biodegradation of hydrophobic phthalates.

Bacteria are able to produce biosurfactants, which could affect the cell surface hydrophobicity and allow them to adapt to environments with hydrophobic substances and utilize hydrophobic substrates more efficiently (Ren et al., 2016). While studies have suggested that bacteria may secrete biosurfactants to enhance the assimilation and degradation of phthalates, most PAE degradation studies have not investigated such properties of the isolated bacteria. Hence, such properties are worth addressing in future studies, particularly with bacteria which were previously found to degrade

Table 2. DBP biodegradation efficiencies of bacteria

Phthalate	Bacterial	Conc. (mg/L)	Time (h)	Complete degradation	Reference
DBP	Sphingobium yanoikuyae P4	1000	60	Yes	Mahajan et al., 2019
	Ochrobactrum sp. JDC-41	500	70	Yes	Wu et al., 2010a
	Sphingobium sp. TJ	500	32	Yes	Jin et al., 2013
	Acinetobacter sp. LMB-5	300	60	Yes	Fang et al., 2017
	Arthrobacter sp. C21	300	70	Yes	Wen et al., 2014
	Agrobacterium sp. JDC-49	200	48	Yes	Wu et al., 2011
	Enterobacter sp. T5	200	60	Yes	Fang et al., 2010
	Providencia sp. 2D	200	72	Yes	Zhao et al., 2016a
	Rhizobium LMB-1	50	48	Yes	Tang et al., 2012
	Consortium HD-1 (major species) Gordonia sp., Burkholderia sp. and Achromobacter sp.	1200	48	Yes	He et al., 2013
	”Consortium LV-1 (main genera:) Brucella spp. (62.78%) and Sinobacter spp. (14.83%).”	500	72	Yes	Wang et al, 2017b
	Consortium B1 Pandoraea sp. and Microbacterium sp.	100	> 40	Yes	Yang et al., 2018
	Anabaena flos-aquae (Cyanobacteria)	50	96	Yes	Babu and Wu, 2010
	Microcystis aeruginosa SM (Cyanobacteria)	50	120	Yes	Babu and Wu, 2010
	M. aeruginosa 2396	30	120	Yes	Babu and Wu, 2010

Table 3. DOP and DEHP biodegradation efficiencies of bacteria

Phthalate	Bacterial Name	Conc. (mg/L)	Time (Day)	Mineralization	Reference
DOP	Gordonia sp. JDC-2	500	2.5	No	Wu et al., 2010a
	Gordonia sp. JDC-2, Arthrobacter sp. JDC-32	500	2	Yes	Wu et al., 2010a
DEHP	Mycobacterium sp. YC-RL4	50	3	Yes	Xu et al., 2017
	Gordonia sp. HS-NH1	500	2.5	Yes	Li et al., 2016
	Gordonia alkanivorans YC-RL2	100	7	Yes	Nahurira et al., 2017
	A consortium LF 54.93% of Gordonia sp.; 9.92% of Rhodococcus sp.; 8.47% of Achromobacter	1000	3	Yes	Li et al., 2018

phthalates with lower water solubility.

11 Conclusion and Future Perspectives

Plastics and plasticizers will remain with us (Gu, 2017b; 2018a; 2020a, b) and they are a new addition to the physical ecosystems (Gu, 2020a). Because of the increasing quantities of both plastics and plasticizers used by our society, their impact on the ecosystems cannot be eliminated easily. Biodegradation is a natural means to facilitate the natural cycling of elements, and the efficiencies of degradation shall be focused either using single bacterial strains or bacterial consortia (Table 2 and Table 3). A few important characteristics shall be known before a decision can be made. First, the bacterium as inoculum should utilize the targeted phthalate as the sole source of carbon and energy (Gu, 2021). Second, the bacteria should have the complete repertoire of genes and enzymes to utilize the substrate, and also its degradation intermediates to achieve mineralization of the original compound. Third, for the efficient removal of phthalates in situ, bacteria should be robust enough to adapt and survive in a range of temperatures and pH conditions to maintain or enhance biodegradation in the presence of indigenous bacteria under the natural or polluted conditions. The challenges are many in implementation of successful bioremediation of this class of chemicals and the better change is with engineering systems for better control and management than natural ecosystems including contaminated soils.

Future research and studies shall consider the following issues and perspectives for investigations. First, it is important to analyze the complete genetic information of phthalate-degrading bacteria to gain insights and elucidate the biodegradation mechanisms at molecular level (Jin et al., 2016). The identification of microbial gene operons of efficient phthalate degraders is a useful basis for biotechnological and bioremediation applications such as the bioengineering of the whole set of genes involved for biocatalysis and improvement of such system to be applied in bioremediation (Cartwright et al., 2000; Gu, 2018b, 2020b). Second, microbial cell properties such as the cell surface hydrophobicity and production of biosurfactants should be investigated so that cell possess characteristics can be best utilized for application. Such properties are important in the biodegradation of phthalates with relatively high hydrophobicity, such as DBP and DEHP. Third, it would be meaningful to scale up laboratory degradation studies to encompass variables related to bioremediation applications. For example, concerning wastewater treatments, studies can compare the biodegradation ability of free cells commonly used in experiments and immobilized cells. For the bioremediation of contaminated sites, phthalate-degrading microorganisms can be inoculated into soil or sediment samples.

Fundamental research questions are still awaiting to be solved by further research, which shall be conducted with a solid foundation of both chemistry and microbiology to make advances in this area of research (Gu, 2021). A weak or lack of good research hypothesis will prevent high quality research as output and also the advancement in this area of breakthrough.

Acknowledgements

Research in this laboratory is supported by Natural Science Foundation of China (No. 92051103) and Guangdong Provincial Department of Education.

Conflict of Interest

Author declares that there is no conflict of interest in the information presented here.

Ethical approval

This article does not contain any studies with human participants or animals performed by the author involved.

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