BIODEGRADATION OF PHTHALATE ESTERS BY *BACILLUS* SP. AND *PSEUDOMONAS* SP. ISOLATED FROM SOIL

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การย่อยสลายทางชีวภาพของทาร์เลตเอสเทอร์โดย แบคทีเรียสายพันธุ์ *BACILLUS* และ *PSEUDOMONAS* ที่คัดแยกจากดิน

นางสาว อาริยา นาวาเจริญ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ทาร์เลตเอสเทอร์เป็นสารที่นิยมใช้อย่างกว้างขวางเพื่อทำให้พลาสติกมี ความนิ่มหรือยืดหยุ่นในอุตสาหกรรมพอลิเมอร์และยังใช้ในการผลิตสี กาว สารหล่อลื่น ยา เครื่องสำอาง และ ยาฆ่าแมลง อีกด้วย เนื่องจากความต้องการบริโภคในปริมาณมาก ทาร์เลตเอสเทอร์ถูกผลิตขึ้นทั่วโลกเป็นจำนวนหลายล้านตันต่อปี ผลกระทบที่ตามมาคือการรั่วไหลของทาร์เลตเอสเทอร์ลงสู่สิ่งแวดล้อม ทั้งจากกระบวนการผลิต การใช้และทิ้งผลิตภัณฑ์ โรงงานบำบัดน้ำเสียจากอุตสาหกรรมและชุมชน และการรั่วไหลออกมาจากหลุมฝังกลบ ซึ่งเหล่านี้ก่อให้เกิดอันตรายต่อสิ่งแวดล้อม เนื่องจากทาร์เลตเอสเทอร์ สามารถก่อมะเร็ง ทำให้เกิดความบกพร่องของการพัฒนาทางกายของทารกในครรภ์ และทำให้เกิดความผิดปกติของต่อมไร้ท่อได้ เพราะฉะนั้นการย่อยสลายทาร์เลตเอสเทอร์ จึงมีความจำเป็นอย่างยิ่ง มีการศึกษาพบว่าทาร์เลตเอสเทอร์สายสั้นสามารถถูกย่อยสลายได้โดยปฏิกิริยา Hydrolysis หรือ Photolysis หรือ การย่อยสลายโดยจุลินทรีย์ การศึกษาในครั้งนี้พบว่า *Bacillus* สายพันธุ์ 3c3 เป็นแบคทีเรียที่มีความสามารถในการทนสารละลายอินทรีย์ ที่คัดแยกจากดินบ่อน้ำพุร้อนในจังหวัดกระบี่ ประเทศไทย และ *Pseudomonas aeruginosa* สายพันธุ์ DB-9 ซึ่งคัดแยกจากดินที่มีประวัติปนเปื้อนขยะชุมชน แสดงความสามารถในการย่อยสลายทาร์เลตเอสเทอร์ เช่น ไดเมทิล ทาร์เลต, ไดเอทิล ทาร์เลต, ไดโพรพิล ทาร์เลต, ไดบิวทิล ทาร์เลต และ เบนซิล บิวทิล ทาร์เลต นอกจากนี้ผู้วิจัยได้ศึกษาอิทธิพลของการเติมแหล่งของคาร์บอนและพลังงาน, อุณหภูมิ, พีเอช, สารลดความตึงผิว, ความเข้มข้นของทาร์เลตเอสเทอร์ และปฏิกิริยาระหว่างทาร์เลตเอสเทอร์ต่อการย่อยสลายโดย *Bacillus* สายพันธุ์ 3c3 และ *P. aeruginosa* สายพันธุ์ DB-9 พบว่า *Bacillus* สายพันธุ์ 3c3 สามารถย่อยสลายไดเอทิล ทาร์เลตที่ 0.45 มิลลิโมลาร์ ได้ถึงร้อยละ 68 ภายใน 2 วัน การเติมสารสกัดยีสต์ช่วยส่งเสริมการเจริญของแบคทีเรียและเพิ่มความสามารถในการย่อยสลายไดเอทิล ทาร์เลตได้มากที่สุดถึงร้อยละ 40 นอกจากนั้นการเจริญของแบคทีเรียและประสิทธิภาพการย่อยสลายเบนซิล บิวทิล ทาร์เลต ของ *P. aeruginosa* สายพันธุ์ DB-9 ในสภาวะที่มีสารสกัดยีสต์ได้เพิ่มสูงขึ้นถึงร้อยละ 25 และร้อยละ 2 ตามลำดับ สภาวะที่เหมาะสมที่สุดสำหรับการย่อยสลายทาร์เลตเอสเทอร์ โดย *Bacillus* สายพันธุ์ 3c3 และ *P. aeruginosa* สายพันธุ์ DB-9 คือที่อุณหภูมิห้อง (ระหว่าง 35 ถึง 37 องศาเซลเซียส) และพีเอช 7.0 Tween 80 ที่ 2 กรัมต่อลิตร คือสารลดแรงตึงผิวชนิดปราศจากประจุที่ถูกเลือกมาเพื่อช่วยเพิ่มความสามารถในการละลายของทาร์เลตเอสเทอร์ และส่งเสริมการเจริญของแบคทีเรียสายพันธุ์ DB-9 ถึงแม้ว่าจะมีพิษต่อสายพันธุ์ 3c3 เพียงเล็กน้อย นอกจากนั้น *Bacillus* สายพันธุ์ 3c3 สามารถย่อยสลายทาร์เลตเอสเทอร์ที่ความเข้มข้นต่างๆได้อย่างสมบูรณ์ แต่ความสามารถในการย่อยสลายจะลดลงที่ความเข้มข้นที่สูงขึ้น *P. aeruginosa* สายพันธุ์ DB-9 สามารถย่อยสลายไดบิวทิล ทาร์เลต และ เบนซิล บิวทิล ทาร์เลตได้อย่างสมบูรณ์ ขณะที่ไดเมทิล ทาร์เลต, ไดเอทิล ทาร์เลต และไดโพร พิล ทาร์เลต สามารถถูกย่อยสลายได้บ้าง นอกจากนี้ *Bacillus* สายพันธุ์ 3c3 และ *P. aeruginosa* สายพันธุ์ DB-9 ยังสามารถย่อยสลายทาร์เลตเอสเทอร์ที่อยู่ในสภาวะผสมได้อีกด้วย

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต .....
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AREEYA NAVACHAROEN: BIODEGRADATION OF PHTHALATE ESTERS BY *BACILLUS* SP. AND *PSEUDOMONAS* SP. ISOLATED FROM SOIL. THESIS ADVISOR: ASSOCIATE PROFESSOR ALISA VANGNAI, Ph.D., 105 pp.

Phthalate esters have been widely used not only as a plasticizer for polymer industries, but also used in the production of paint, glue, lubricant, pharmaceutics, cosmetics and pesticides. Due to a high demand, millions of tons have been globally produced per year. Consequently, they may leak into the environment from manufacturing, product use/disposal, industrial and municipal wastewater treatment plants and also be leached out of landfills causing a threat to aquatic environment. Since phthalate esters may result in carcinogenic, teratogenic and endocrine effects, degradation is therefore necessary. Recent studies have reported that short chain phthalate esters can be degraded either by hydrolysis or photolysis or by microorganisms. In this study, *Bacillus* sp. strain 3C3 which was isolated from hot spring soil in Krabi province, Thailand, as an organic-solvent tolerant bacterium and *Pseudomonas aeruginosa* strain DB-9 which was isolated from soil with a history of municipal waste exposure exhibited the ability to biodegrade a wide range of PEs, i.e. DMP, DEP, DPrP, DBP and BBP. The effect of additional carbon and energy source, temperature, pH, surfactant, PEs concentration and PEs interaction towards PEs biodegradation by *Bacillus* sp. strain 3c3 and *P. aeruginosa* strain DB-9 were investigated. Initially, *Bacillus* sp. strain 3C3 was able to slowly degrade DEP (0.45 mM) to the maximum at 68% within 2 days. The addition of yeast extract stimulated bacterial growth and enhanced the highest DEP biodegradability by 40%. Furthermore, bacterial growth rate and BBP degradation efficiency of *P. aeruginosa* strain DB-9 in the presence of yeast extract was increased by 25% and 2%, respectively. The optimal conditions for PEs degradation of *Bacillus* sp. strain 3c3 and *P. aeruginosa* strain DB-9 were at room temperature (35-37 °C) and pH 7.0. Tween 80 at 2 g L^{-1} was nonionic surfactant selected for enhancing PEs solubility and supporting bacterial growth of the strain DB-9, though it was slightly toxic to the strain 3c3. Moreover, *Bacillus* sp. strain 3c3 exhibited ability to completely degrade PEs at various concentrations, but the degradation capacity was decreased at higher concentrations. The *P. aeruginosa* strain DB-9 could completely degrade DBP and BBP at various concentrations, while DMP, DEP and DPrP could be degraded to some extents. In addition, the ability of *Bacillus* sp. strain 3c3 and *P. aeruginosa* strain DB-9 to degrade PEs in mixed conditions was also feasible.

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LIST OF ABBREVIATIONS

BBP	Butylbenzyl phthalate
CMC	Critical micelle concentration
DBP	Dibutyl phthalate
DEHP	Di (2-ethylhexyl) phthalate
DEP	Diethyl phthalate
DMP	Dimethyl phthalate
DOP	Dioctyl phthalate
DPrP	Dipropyl phthalate
EPA	Environmental Protection Agency
HPLC	High Performance Liquid Chromatography
OD	Optical density
PEs	Phthalate esters
Ppm	part per million
PVC	Polyvinylchloride
PWB	Printed wiring board
rpm	Revolution per minute
WWTP	Wastewater treatment plant

CHAPTER I

INTRODUCTION

1.1 Statement of problem

Phthalate esters (PEs) is a group of synthetic chemicals that have been used as plasticizers that imparts flexibility to polyvinyl chloride resins and other polymers such as polyvinyl acetate, cellulose nitrate, polystyrene and polyurethane (Staples *et al*., 1997). They are also presented as additives in a wide variety of products such as insecticides, paints, insulators in electric appliance, adhesives, toys, cosmetic, covering, teeth rings, clothes and food packaging, etc (Fromme *et al*., 2002). As industrially important chemicals, PEs are produced in extremely large volumes, e.g. the worldwide production of 4.3 million tons was reported in 2006 and at a constant production level for more than 20 years (Peijnenburg& Struijs, 2006).

Since PEs are not chemically bound to the polymer matrices, they have been released into the environment during the production, the widespread use and the discharge from sewage sludge and landfill leachate after disposal and accumulated into soil, sediment, natural water and groundwater (Staples *et al*., 1997). Industrial and municipal wastewater treatment plants (WWTP) are considered to be the important sources releasing phthalate esters into the environment (Roslev *et al*., 2007). For example, the sludge from waste water treatment plant (WWTP) of electronic industries, e.g. Printed wiring board (PWB) manufacturers, semiconductor component assembly facilities was found to be one of the major released sources of PEs in Thailand, China, and Philippine (Brigden *et al*., 2007). Interestingly, PEs have been found in discharged effluent from the concentrated latex factories which is one of the leading economic

industries in Thailand (Kumlanghan *et al*., 2008). However, several reports have shown that the major diffused sources of PEs were not only from the industrial areas, but the highest phthalate concentrations were also observed in wastewater and sludge from residential areas, e.g. in France, United Kingdom, Canada, and the Netherlands, the significant amount of PEs were detected from municipal wastewater treatment plant where they were discharged from highly populated area as well as car wash, and several laundry factories (Dargnat *et al*., 2009).

Moreover, PEs have been detected in different environmental compartments at concentration ranging from 0.3 to 77 ng m^{-3} in the atmosphere, from 0.3 to 98 µg L^{-1} in surface water, from 0.2 to 8.4 mg kg^{-1} dry weight (dw) in sediment, and from 28 to 154 mg kg^{-1} dw in sewage sludge (Fromme *et al*., 2002; Staples *et al*., 1997). Due to low water solubility, PEs tend to precipitate and accumulate in sludge (Staples *et al*., 1997), so the use of sludge in agriculture for soil amendment might be an important input source of the pollutants to the environment. The PEs contamination in the environment leads to the accumulation of such compounds in living organisms and subsequently affect human through food chain. There have been reports of PEs accumulation in freshwater fish species: the bream (*Abramis brama*) 1900–3120 ng g^{-1} dw of DEHP and 720–800 ng g^{-1} dw of DEP, in marine species: the flounder (*Platichthys flesus*) 40–70 ng g^{-1} dw of DEHP and 100–200 ng g^{-1} dw of DEP (Dargnat *et al*., 2009), and in human as DEHP was detected in human blood at 15-83.2 µg L^{-1} (Inoue *et al*., 2005). The contamination and accumulation of PEs in the environment have raised the human health and environment safety issue because PEs have been found to be potentially harmful to human due to their hepatotoxic, teratogenic, carcinogenic characteristics (Matsumoto *et al*., 2008), and their endocrine disrupting effects to fish and mammals (Moore, 2000).

Since PEs has been stated that it tends to be concentrated in the sewage sludge from the precipitation of wastewater in either industrial or domestic WWTP and then the sewage sludge is subsequently used as a soil amendment in agriculture, so human and living organisms can be directly exposed to PEs. In order to ensure human health safety and protect soil and/or water resources and ecosystem quality, reduction of PEs content in the sewage sludge is a great importance for further sludge disposal and/or agricultural utilization. Furthermore, development of the efficient treatment technique of PEs contaminated in soil and sludge is necessary. Abiotic processes such as hydrolysis, oxidation and photolysis are unlikely to play significant roles in the environmental fate of PEs. On the other hand, a biological treatment through microorganism has been stated as the major mechanism for remedy the PEs contaminated in soil and sludge (Staples *et al*., 1997). The biological treatment for contaminated soil has interestingly increased in recent years because it is simple to maintain, cost-effective, and applicable over large area. However, the feasibility and effectiveness of bioremediation is influenced by many factors such as the presence of suitable microorganisms, nutrient availability, temperature, pH and the alkyl chain length of the PEs (Di Gennaro *et al*., 2005; O'Grady D *et al*., 1985). Until now, the biodegradation of PEs has been widely studied in both gram-positive and gram-negative bacteria which exhibited ability to degrade PEs in the concentration range from 5 to 610 ppm as presented in Table 2. In addition, the microbial community analysis according to the DEP enrichment in compost medium revealed that the community change was associated with the genera *Sphingomonas*, *Pseudomonas*, and *Actinomycetes* (Kapanen *et al*., 2007). Therefore, this study aims to further screen and isolate the different genera of bacteria capable of completely biodegrade PEs, optimize the biodegradation conditions in order to employ them in the treatment of soil and/or

sludge contaminated with PEs and determine if the isolated PEs-degrading bacteria were able to degrade PEs at higher concentrations than previous findings.

1.2 Objectives

To reduce the toxicity of hazardous chemicals contaminated in the environment, the biodegradation is one of the important alternatives to biotransform the hazardous chemical such as phthalate esters (PEs). Thus, in this research, the biodegradation of PEs focusing on phthalate with short esters side chain, i.e. DMP, DEP, DPrP, DBP, BBP, was investigated. This research was consisted of four objectives:

1.2.1 To screen, isolate and identify the bacteria that had ability for utilizing phthalate esters as a sole carbon and energy source.

1.2.2 To optimize the conditions, i.e. supplementary nutrient, temperature, pH, for phthalate esters biodegradation and to determine degradation efficiency and bacterial growth rate.

1.2.3 To investigate the effect of nonionic surfactant on solubility of phthalate esters and bacterial growth.

1.2.4 To investigate the interaction among phthalate esters during the biodegradation.

1.3 Hypothesis

1.3.1 The isolated phthalate-degrading bacteria might have ability to degrade phthalate esters.

1.3.2 The optimal conditions would be beneficial for further application to remedy phthalate esters that contaminated in the environment.

1.3.3 The interaction among the phthalate esters might enhance the bacterial ability to completely degrade phthalate esters.

1.4 Scope of study

1.4.1 Screening, isolation and identification of phthalate esters degrading bacteria

1.4.2 Characterization of the isolated phthalate esters-degrading bacteria

1.4.2.1 Effect of nutrient on phthalate esters biodegradability

1.4.2.2 Effect of temperature on phthalate esters biodegradability

1.4.2.3 Effect of pH on phthalate esters biodegradability

1.4.2.4 Effect of surfactant on solubility of phthalate esters and bacterial growth

1.4.2.5 The biodegradability of the isolated phthalate esters-degrading bacteria towards other phthalate esters (substrate range)

1.4.2.6 The biodegradability of the isolated phthalate esters-degrading bacteria in mixture of phthalate esters (substrate interaction)

CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 Phthalate esters properties

The seven commercially important phthalate esters (PEs) are listed in Table 1, where the abbreviations used for the various PEs are presented. Phtalate esters (PEs) are dialkyl or aryl esters of 1, 2-dibenzenedicarboxylic acids (phthalic acid) with the general structure shown in Figure 1 where R^1 and R^2 can be various combinations of straight and/or branched alkyl chain or aryl group (Ogunfowokan *et al*., 2006)

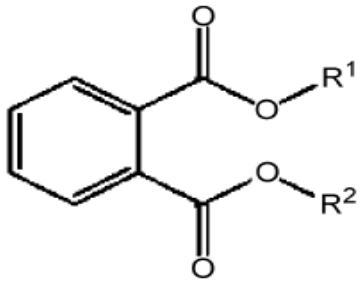


Figure 1 General structure of the phthalates

Physical properties of phthalate esters are shown in Table 1 The longer the alkyl side chain represent the lower the solubility and the higher the octanol/water partition coefficient (log K_{ow}) indicating greater hydrophobicity (Staples *et al*., 1997).

Table 1 Physical properties seven commercial phthalate esters (Staples *et al*., 1997)

Phthalate ester	Abbreviation	Formular	Alkyl chain length	Molecular weight	Specific gravity (20°C)	log Kow	Water solubility (mgL^{-1})
Dimethyl phthalate	DMP	$C_{10}H_{10}O_4$	1	194.2	1.192	1.46-1.90 (1.61)[b]	2179-4320 (4200)
Diethyl phthalate	DEP	$C_{12}H_{14}O_4$	2	222.2	1.118	2.21-3.0 (2.38)	260-7028 (1100)
Dipropyl phthalate	DPrP	$C_{14}H_{18}O_4$	3	250.3	-		
Dibutyl phthalate	DBP	$C_{16}H_{22}O_4$	4	278.4	1.042	3.74-5.15 (4.45)	1.5-13 (11.2)
Butylbenzyl phthalate	BBP	$C_{19}H_{20}O_4$	4,6[a]	312.4	1.111	3.57-4.91 (4.59)	5.1-20.3 (20.0)
Di(2-ethylhexyl) phthalate	DEHP	$C_{24}H_{38}O_4$	8	390.6	0.986	4.20-8.39 (7.50)	0.00024-0.09 (0.0003)
Di-n-octyl phthalate	DnOP	$C_{24}H_{38}O_4$	8	390.6	0.978	5.22-8.54 (8.06)	0.00046-3 (0.00005)

[a] Aromatic ring
[b] ( ): recommended value based on Staples *et al.*, 1997

2.2. PEs production and usage

PEs are extensively used in chemical industries as additives which provide flexibility in polyvinylchloride (PVC) resins in plastic products such as food packaging, building materials, home furnishings, clothing, automobile parts, tools, toys, and electric insulations. PEs are also used as constituents in the production of paint, glue, lubricant, cosmetics, insecticides, and aspirin. Additionally, the PEs are used as plasticizer in polyvinyl acetate, polyvinylidene chloride, polystyrene, ethyl cellulose, cellulose nitrate, acetate and acetate butyrate, chlorinated rubber, polyesters, and polyurethane resins (Staples *et al*., 1997).

As the major industrial chemicals, PEs are produced in extremely large volumes, e.g. the worldwide production of 4.3 million tons was reported in 2006, and at a constant level for more than 20 years (Peijnenburg & Struijs, 2006).

2.3 Sources of contamination and environmental fate

Since PEs are not a part of the chain of polymers making up the plastics, it can be released quite easily from these products (Staples *et al*., 1997). They may enter into the environment either intentionally or unintentionally from consumer products through manufacturing, product use & disposal, industrial and municipal wastewater treatment plants, or leaching out of landfill into soil, water and groundwater. Industrial and municipal wastewater treatment plants (WWTP) are considered to be the important sources releasing phthalate esters into the environment (Roslev *et al*., 2007). For example, the sludge from waste water treatment plant (WWTP) of electronic industries, e.g. Printed wiring board (PWB) manufacturers, semiconductor component assembly facilities was found to be one of the major released sources of PEs in Thailand, China, and Philippine (Brigden *et al*., 2007). Interestingly, the PEs investigation in wastewater

and sludge in France, United Kingdom, Canada, and Netherlands showed significant amount of PEs were discharged from highly populated area as well as several laundry factories (Dargnat *et al*., 2009).

PEs have been detected in different environmental compartments at concentration ranging from 0.3 to 77 ng m^{-3} in the atmosphere, from 0.3 to 98 µg L^{-1} in surface water, from 0.2 to 8.4 mg kg^{-1} dry weight (dw) in sediment, and from 28 to 154 mg kg^{-1} dw in sewage sludge (Fromme *et al*., 2002; Stales *et al*., 1997). Due to low water solubility, PEs tend to precipitate and accumulate in sludge (Staples *et al*., 1997), so the use of sludge in agriculture for soil amendment might be an important input source of the pollutants to the environment. The PEs contamination in the environment leads to the accumulation of such compounds in living organisms and subsequently affect human through food chain. There have been reports of PEs accumulation in freshwater fish species: the bream (*Abramis brama*) 1900–3120 ng g^{-1} dw of DEHP and 720–800 ng g^{-1} dw of DEP, in marine species: the flounder (*Platichthys flesus*) 40–70 ng g^{-1} dw of DEHP and 100–200 ng g^{-1} dw of DEP (Dargnat *et al*., 2009), and in human as DEHP was detected in human blood at 15-83.2 µg L^{-1} (Inoue *et al*., 2005).

2.4 Impacts of PEs contamination on environmental quality

Due to PEs are not chemically bound to the polymers matices, plasticizers in food packing readily leach out of the plastic film when contact with food products (Castle *et al*., 1988). Loftus *et al*. (1993) reported the average ingestion rate of plasticizers has been estimated to be on the order of 8 mg/person/day (Loftus *et al*., 1993) In addition, the contamination of DEP in drinking water of several communities in USA was also reported.

2.5 Toxicity of PEs

The environmental fate of phthalate esters gains more attention because of potential health effects in human and animal. Staples *et al*. (1997) reported that the lower molecular weight phthalate esters are both acutely and chronically toxic at concentrations below their solubility level and toxicity increases with increasing alkyl chain length up to four carbon atoms (Staples *et al*., 1997). Some PEs such as DEHP, DEP, DBP and BBP are suspected to be mutagens and carcinogens (Zeng *et al*., 2008). Moreover, there are several study reported that low molecular weight phthalates are liver carcinogenic (Kapanen *et al*., 2007).

The potential of PEs to have adverse health effects at the levels encountered during normal use, or at environmental exposure levels, their toxicity may be settled by disruption of normal endocrine control of reproductive tract tissue development (CSIRO, 2001). For example, Puerto Rican girls with premature breast development had higher levels of blood phthalates compared to other girls, suggesting an association between phthalate exposure and abnormal reproductive development (Colon *et al*., 2000). In summary, exposure to the phthalate esters may result in carcinogenic, teratogenic and endocrine effects.

2.6 Legislation

Six PEs, namely DMP, DEP, DBP, BBP, DEHP, and DOP, were classified as priority pollutants for risk assessment by the US Environmental Protection Agency (US EPA), mandating the reduction and control of phthalate pollution (USEPA, 1992 and update). US EPA set up the water criteria of 3 μg L^{-1} phthalates recommended for the protection of fish and other aquatic life in water and the Suggested No-Adverse Effect Levels (SNAEL) of 7.5–38.5 μg L^{-1} for drinking water (USEPA, 1994).

US manufactures and processors of certain phthalate esters are required to report estimates of the amounts released to surface waters to the toxics release inventory (TRI). The identity and characteristics of four PEs, namely DMP, DEP, DBP, and BBP, are currently part of the TRI chemical reporting list (SARA, 1985).

In the European community (DCE 2000/60/CE) (2000), DEHP has been listed among the 33 hazardous substances to be controlled in surface water. Besides, the European Directive of May 7, 2007, proposed a norm for environmental quality (NQE) of 1.3 μg L^{-1} DEHP in surface water. At the present, the European legislation recommends to reduce the use of phthalates in different products, namely cosmetics and food packaging (Dargnat *et al*., 2009).

2.7 Treatment

2.7.1 Physical and chemical treatments

Abiotic processes such as hydrolysis, oxidation, and photolysis of phthalate esters are unlikely to play important role in the environment fate of PEs (Staples *et al*., 1997). Lewis *et al*. (1984) found that DEP was virtually untransformed by photolysis (<1%), and only about 5% of its initial concentration of 191 μg L^{-1} was lost by hydrolysis in 12 h at pH 10

2.7.2 Biological treatment

Bioremediation as a treatment strategy for contaminated soil has interestingly increased in recent years because it is simple to maintain, cost-effective, and applicable over large area. However, the feasibility and effectiveness of bioremediation is influenced by many factors such as the presence of suitable microorganisms, nutrient availability, temperature, and pH (Di Gennaro *et al*., 2005). Many studies have been reported in recent

years on the biodegradability of PEs by bacteria, fungi, algae, and activated sludge culture. So far, various studies have showed that microorganisms play the major roles in PEs degradation in the environment under different conditions (e.g. sewage, soil, sediment, and surface water) (Staples *et al*., 1997). Microorganisms that have PEs biodegradability can be aerobic, anaerobic, or anoxic, but their activity is more effective under aerobic condition than others (Chao & Cheng, 2007). The biodegradable properties of phthalate esters vary depending on the structure of the compound. An increase in molecular weight and alkyl chain length of the molecule decreases the biodegradability. As several studies stated, PEs with shorter alkyl chains (i.e. DEP, DBP) are very easily biodegraded, while PEs with longer alkyl chains (i.e., DOP, DEHP) are poorly degraded under aerobic and anaerobic conditions (O'Grady D *et al*., 1985). Although some individual microbes are able to completely mineralize phthalate esters, a mixed microbial community, which is typically case in the environment, appears to have a more efficient PEs degradability (Staples *et al*., 1997).

2.8 Biodegradation of PEs in the environment

2.8.1 The chemical structure and biodegradability of PEs

The basic structure of phthalate is benzene dicarboxylic acid with two side chain, which can be a combination of straight and branched alkyl chain or aryl group (Staples *et al*., 1997). Studies have demonstrated that PEs with shorter ester chains like DMP, DEP, DPP, DBP, and BBP can be readily biodegraded and mineralized. In contrast, PEs with longer ester chains like DEHP and DOP are less susceptible to biodegrade (Chang *et al*., 2004). Moreover, different phthalate isomers also show the different biodegradation rate and phthalate-hydrolyzing enzymes are structurally specific. This stated is related to the

environmental abundance of organisms with the specific ability to degrade the phthalate isomers which are directly exposed to them (Liang *et al*., 2008).

2.8.2 Biodegradation of PEs by pure culture

Bacterial activity is more effective under aerobic condition than anaerobic and facultative condition. So, most PEs-degrading bacterial isolates are aerobes as presented in Table 2. The reported bacteria are mainly isolated from activated sludge and soil with a history of PEs contamination which have ability to degrade PEs in the concentration range from 5 to 610 ppm. The study of a microbial community analysis revealed that community change in an enriched compost medium with a high concentration of DEP (10–100 g kg^{-1}) was associated with the genera *Sphingomonas*, *Pseudomonas*, and *Actinomycetes* (Kapanen *et al*., 2007). So far, anaerobic PEs-degrading bacteria were little known, as the study of Chang *et al*. (2005) found three isolates; one *Clostridium* sp. and two *Bacillus* sp. strains were solely capable of degrading phthalates under anaerobic condition (Chang *et al*., 2005). In addition to bacteria, a few fungi species, including *Aspergillus niger* AG-1 (Ganji *et al*., 1995) and *Fusarium oxysporum* (Kim *et al*., 2005), as well as green microalga, such as *Closterium lunula* (Yan & Pan, 2004) and *Chlorella pyrenoidosa* (Yan *et al*., 2002), can also degrade PEs.

Table 2 Aerobic phthalate-degrading bacteria species

Bacterial species	**Isolated from**	**Phthalate esters**	**Concentration (ppm)**	**Degradation efficiency**	**References**
Sphingomonas sp. DEP-AD1	Activated sludge	DEP	610	100% in 150 hours	Fang *et al*., 2007
Pseudomonas fluorescens B-1	Mangrove sediment	BBP	2.4-20.8	100% in 45 hours	Xu *et al*., 2007
Corynebacterium nitrilophius G11	Soil	DBP	100	20% in 3 days	Chao *et al*., 2006
Rhodococcus rhodochrous G2,G7	Soil	DBP	100	100% in 2-3 days	Chao *et al*., 2006
Corynebacterium sp. O18	Activated sludge	DEP, DPrP, DBP and BBP	5	100% in 2, 2, 4 and 3 days	Chang *et al*., 2004
Sphingomonas sp. DK4	Activated sludge	DEP, DPrP, DBP and BBP	5	100% in 2, 2, 2 and 2 days	Chang *et al*., 2004
Pseudomonas fluorescens FS-1	Activated sludge	DMP, DEP and DBP	100	more than 99% in 3 days	Feng *et al*., 2004
Arthrobacter sp.	Soil	DMP	52.4	100% in 20 hours	Vega & Bastide, 2003
Acinetobacter lwoffii	River water	DBP	20	100% in 5 days	Hashizume *et al*., 2002

2.8.3 Biodegradation of PEs by mixed culture

In nature, complete degradation of complex organics, like phthalates, is always carried out by several members of microorganisms. The biodegradation of PEs primarily involves the sequential hydrolysis of ester linkage, which results in monoesters and subsequently PA, while forming alcohols simultaneously. For example, under methanogenic conditions, DEP is hydrolyzed to MEP and PA. So, the microbial assimilation of PEs requires diverse metabolic genes and enzymes, indicating a single organism is unlikely able to completely mineralize PEs (Staples *et al*., 1997).

So far, mixed culture consortia have been shown to completely mineralize DMP (Wang *et al*., 2004), BBP and their metabolites (Chatterjee & Dutta, 2008). In addition, co-bacterial culture can also enhance PEs degradation rate. Chang *et al*. (2005) reported an increased degradation rate of the eight PEs in the presence of both *Sphingomonas* sp. DK4 and *Corynebacterium* sp. O18 (Chang *et al*., 2005).

2.8.4 Biodegradation of PEs under various environmental conditions

Wastewater treatment processes

Discharge from sewage works is a major source of PEs into the environment. Comparison to other PEs, DEP and DEHP, in the ranges of 1–74 and 28– 122 μg L^{-1}, respectively, have been found in higher concentrations in raw sewage. Actually, PEs are not readily mineralized in the wastewater treatment processes because their high hydrophobicity and low solubility allow them to be adsorbed to suspended organic matter and subsequently transferred to settled sludge (Gavala *et al*., 2003). Furthermore, Oliver *et al*. (2007) studied PEs degradation in reactors. For instance, compared to the continuous flow mode, the recirculating flow mode of a trickling filter microcosm

improved the DEP and DEHP removal efficiency to 99% in 48 h and 88% in 96 h, respectively (Oliver *et al*., 2007).

Sludge

Sewage sludge contains relatively high concentrations of PEs as a result of accumulation from the urban runoff, drainage, and domestic and industrial discharges. Thus, DEHP is considered as a major pollutant at high concentration and its concentrations range from several to hundreds of mg kg^{-1} in dry weight (dw) (Cheng *et al*., 2000). Moreover, Gavala *et al*. (2003) reported that high concentrations of DEHP (more than 60 mg L^{-1}) in digested sludge have a negative effect on degradation of other phthalates, such as DBP, DEP, and DMP (Gavala *et al*., 2003).

Freshwater and sediment

PEs are ubiquitously detected in freshwater and tend to adsorb on sediment. Chang *et al*. (2005) reported that *Clostruium* sp. and *Bacillus* sp. which have been isolated from river sediment had ability to degrade DEP, DBP, and DEHP under anaerobic condition (Chang *et al*., 2005).

Soil and slurry

Mohan *et al*. (2006) studied DEP degradation by using a slurry-phase reactor bioaugmented with the outlet from an effluent treatment plant. The result showed that DEP were almost completely degraded within 48 h and the half-life in soil and aqueous phase were 1.19 and 2.52 days, respectively (Mohan *et al*., 2006).

Landfill sites

As previously reported, microorganisms in landfill environments have a lower phthalate degrading potential as those in the anaerobic reactors. Ejlertsson *et al*. (1996) found DEP and PA (50–250 mg L^{-1}) could be completely degraded into CH_4 and CO_2 in an anaerobic digester for municipal solid waste (Ejlertsson *et al*., 1996).

2.8.5 Biodegradation pathways of PEs

Generally, the phthalates biodegradation pathways consist of two processes: primary biodegradation from phthalate diesters (PDEs) to phthalate monoesters (PMEs) and then to PA and ultimate biodegradation from PA to CO_2 and/or CH_4 (Staples *et al*., 1997).

Primary degradation pathway

Primary degradation consists of different type of pathways, including de-esterification or dealkylation, β-oxidation and trans-esterification, as shown in Figure 2 (Liang *et al*., 2008).

- De-esterification: the most common one is the de-esterification of PDEs serially to form PMEs and PA.
- β-oxidation: Phthalates with longer side chains than DEP are occasionally converted to those with shorter chains by β-oxidation, which removes one ethyl group each time (Amir *et al*., 2005).
- Transesterification (or demethylation): DEP can be degraded by replacing a ethyl group with a methyl group in each step, producing ethyl-methyl phthalate and DMP (Cartwright *et al*., 2000).

Ejlertsson *et al*. (1996) and Jonsson *et al*. (2003) found that DEP under different landfill conditions was hydrolyzed to monoethyl phthalate (MEP) and PA, and then PA was further transformed to CO_2 and/or CH_4 (Ejlertsson *et al*., 1996; Jonsson *et al*., 2003). In addition, Jackson *et al*. (1996) reported that DEP may also be directly degraded into PA without intermediates MEP by *Aureobacterium saperdae* NRRL B-14840 (Jackson *et al*., 1996).

Ultimate degradation pathway

The pathways of ring cleavage of PA are different under aerobic and anaerobic conditions (Cartwright *et al*., 2000), as shown in Figure 3. Eaton and Ribbon (1982) proposed PA degradation pathway under aerobic condition that PA can be degraded by two dioxygenase-catalyzed pathways, forming the common intermediate protocatechuate (3, 4-dihydroxy benzoate). Protocatechuate is further metabolized through either ortho- or meta-cleavage pathway by ring cleavage enzymes (Eaton & Ribbon, 1982).

Kleerebezem *et al*. (1999) reported anaerobic degradation of PA through decarboxylation to benzoate. Then benzoate is then cleaved and degraded via β-oxidation, to hydrogen, carbon dioxide, and acetate (Kleerebezem *et al*., 1999).

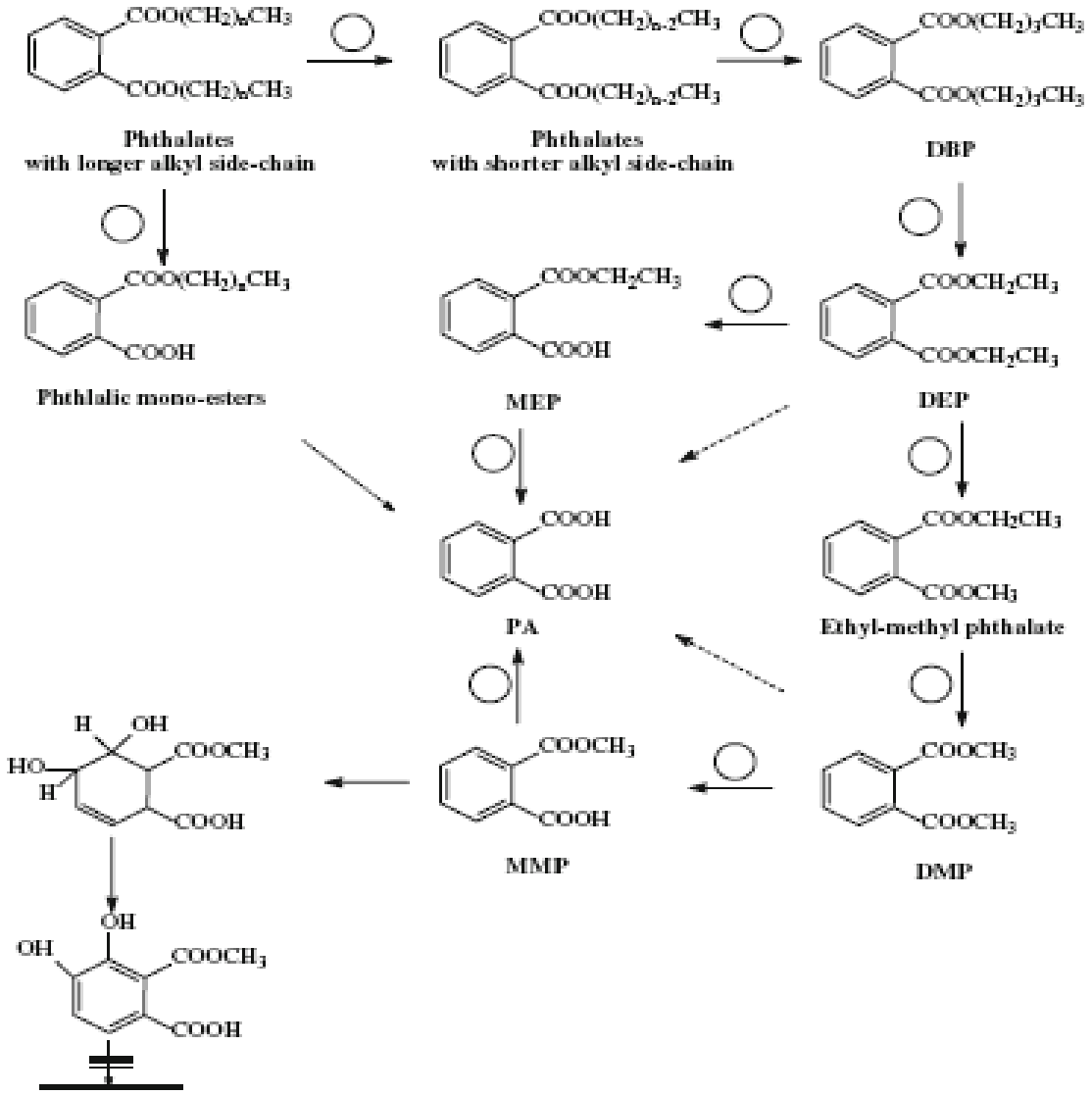


Figure 2 Primary degradation pathway of PEs (Liang *et al*., 2008)

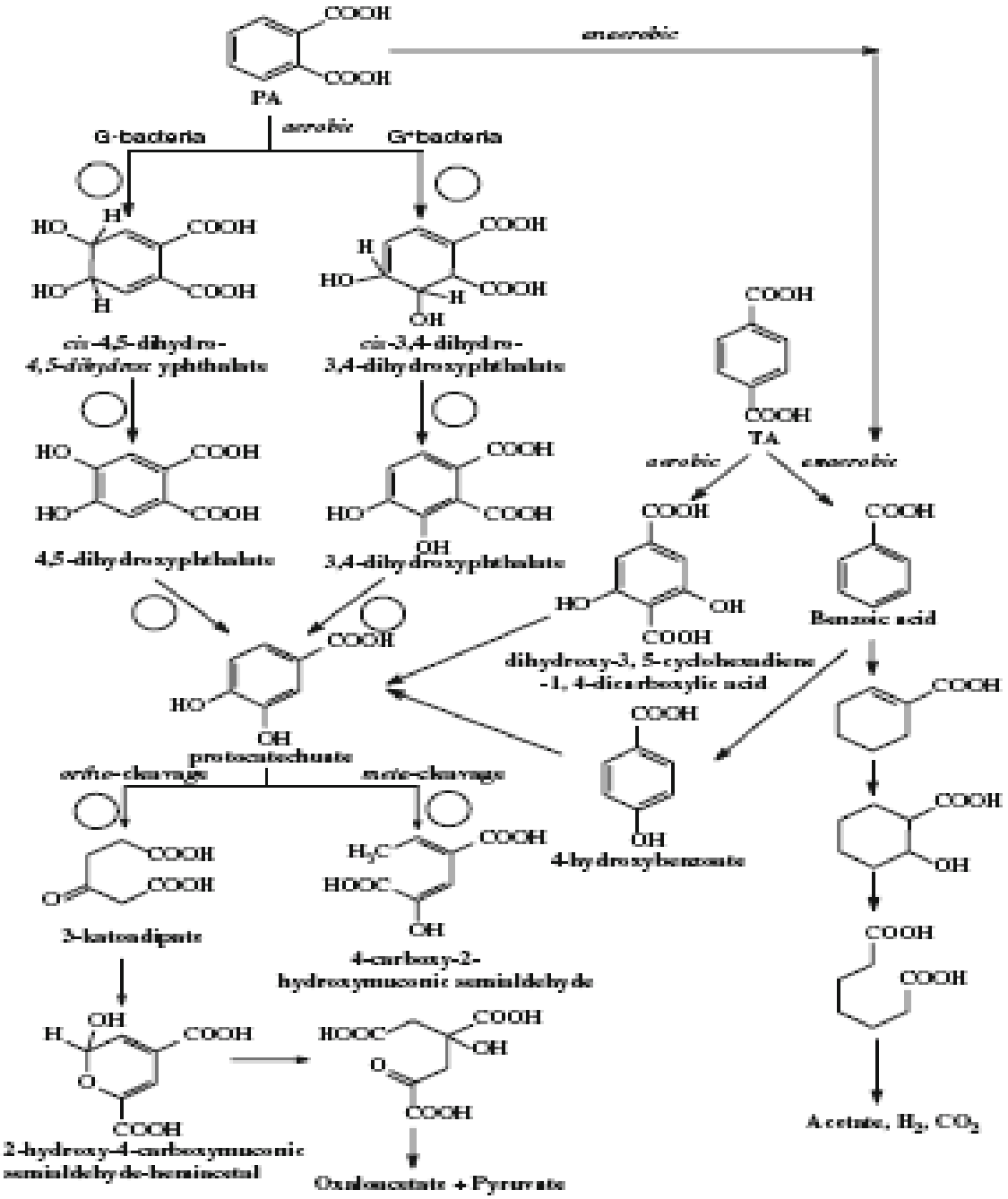


Figure 3 Ultimate degradation pathway of PEs (Liang *et al*., 2008)

2.8.6 The optimal condition of PEs biodegradation

Supplementary nutrient

There is several studies indicated that addition of supplementary nutrients could enhance the PEs degradation rate; for example, *Bacillus benzoevorans*, *Sphigomonas* sp. and *Corynebacterium* sp. which their PEs degradation rate were enhanced by addition of yeast extract (Chang *et al*., 2007).

Temperature and pH

Phthalate degradation efficiency might be decreased in cold (approximately 5 °C) environment (Staples *et al*., 1997). As the previous reports, the optimal conditions for PEs degradation of *Enterococcus* sp. strain OM1, *Bacillus banzoevorans* strain S4, *Sphigomonas* sp. strain O18 and *Corynebacterium* sp. strain DK4 were 30 °C and pH 7.0 (Chang *et al*., 2004). Park *et al*. (2008) also stated that the optimal conditions for phthalate esters degradation of *Sphingobium chungbukense* were 30 °C and pH 7.0 (Park *et al*., 2008).

Surfactant

Surfactants have been used effectively to increase biodegradability and bioavailability of PEs (Chao *et al*., 2006). Several studies investigated the ability of surfactant to increase the degradation efficiency; for example, PEs degradation was enhanced by the addition of Brij 30 or Brij 35 (Chang *et al*., 2007). Moreover, the effect of surfactant on bacterial growth was examined, the previous study reported that the addition of Brij 30 at 2 mg L^{-1} significantly improved DEHP degradation by *R. rhodochrous* G2, but slightly inhibited the degradation of DEHP by the isolate G1 and *R. rhodochrous* G7 (Chao & Cheng, 2007).

CHAPTER III

MATERIALS AND METHODS

3.1 Laboratory equipments and chemicals

3.1.1 Laboratory equipments

Laboratory equipment	Company	Country
Autoclave NLS-3020	Sanyo Electric Co.,Ltd	Japan
Autoclave HV-110	Hirayama	Japan
Biological safety cabinet, Forma Class II A2	Thermo Electron Corporation	USA
C18 Column (Hyperclone 5u BDS C18 130A, a 250 x 4.6 mm)	Phenomenex	USA
High Performance Liquid Chromatography (HPLC), LC-20	Shimazu	Japan
Incubator shaker, innova 4000	New Brunswick scientific	USA
Micropipette 20,100, 200, and 1000 µl	Gilson	France
Microscopy, SK-500	Seekscope	China
Protector Laboratory Hood	Science Technology	USA
Refrigerated Centrifuge Avanti ^{Tm} J-301	Beckman Coulter	USA
Refrigerated Centrifuge, 5804R	Eppendorf	USA
Spectrophotometer DU 650	Beckman	USA

3.1.2 Laboratory Chemicals

(1) Chemicals

Chemicals	**Company**	**Country**
Agar	Scharlau Chemic Microbiology	Spain
Bovine serum albumin (BSA)	Sigma	USA
Brij 35	Merck	Germany
$CaCl_2.2H_2O$	Merck	Germany
Citrate	Merck	Germany
Crystal violet	BDH	England
$CuSO_4.5H_2O$	Scharlau Microbiology	Spain
$FeCl_3.6H_2O$	BDH	England
Folin-Ciocalteu's reagent	Carlo Erba Reagenti	Italy
Glucose	Merck	Germany
Iodine crystal	BDH	England
KH_2PO_4	Carlo Erba Reagenti	Italy
K_2HPO_4	Riedel	Germany
$MgSO_4.7H_2O$	Carlo Erba Reagenti	Italy
NH_4Cl	May&Baker	England
NaCl	BDH	England
Na_2CO_3	BDH	England
NaOH	Merck	Germany
$Na_2HPO_4.12H_2O$	Fluka	Germany
Peptone	Merck	Germany
Safanin O	Fluka	Germany
Succinic acid	Merck	Germany

Tergitol type NP-10	Sigma-Aldrich	Germany
Triton x-100	Sigma-Aldrich	Germany
Tryptone	Himedia	India
Tween 80	Sigma-Aldrich	Germany
Yeast extracts	Scharlau Chemic Microbiology	Spain

(2) Analytical grade organic solvents

Organic solvents	**Company**	**Country**
Absolute ethanol (99.9% purity)	Merck	Germany
Butylbenzyl Phthalate (98% purity)	Sigma-Aldrich	Germany
Dibutyl Phthalate (99% purity)	Sigma-Aldrich	Germany
Diethyl Phthalate (98% purity)	Fluka	Germany
Dimethyl Phthalate (99% purity)	Sigma-Aldrich	Germany
Dipropyl Phthalate (98% purity)	Sigma-Aldrich	Germany
Phthalic acid (98% purity)	Sigma-Aldrich	Germany

(3) HPLC grade organic solvents

Organic solvents	**Company**	**Country**
Acetonitrile (99.9% purity)	Lab-scan	Thailand
Methanol (99.9 % purity)	Lab-scan	Thailand

3.2 Culture medium

3.2.1 The modified minimal salt medium (MSM)

The modified minimal salt medium will be used for screening, isolation, cultivation and substrate degradation experiments. The components of the medium are shown as follow (Chang *et al*., 2004).

$K_2HPO_4.3H_2O$	4.5648	g
KH_2PO_4	3.4023	g
$Na_2HPO_4.12H_2O$	5.3721	g
NH_4Cl	0.8024	g
$CaCl_2.2H_2O$	0.0368	g
$MgSO_4.7H_2O$	0.1232	g
$FeCl_3.6H_2O$	0.0014	g

The components will be dissolved in 1 liter of distilled water, adjusted pH to 7.0 and autoclaved at 121°C for 15 minutes.

3.2.2 Luria bertani (LB) medium

Tryptone	10	g
Yeast extracts	5	g
NaCl	10	g

The LB medium components will be dissolved in 1 liter of distilled water, adjusted pH to 7.0 and autoclaved at 121°C for 15 minutes. For solid media, 1.5 % (w/v) agar will be added to the LB media.

3.3 Preparation of PEs solution

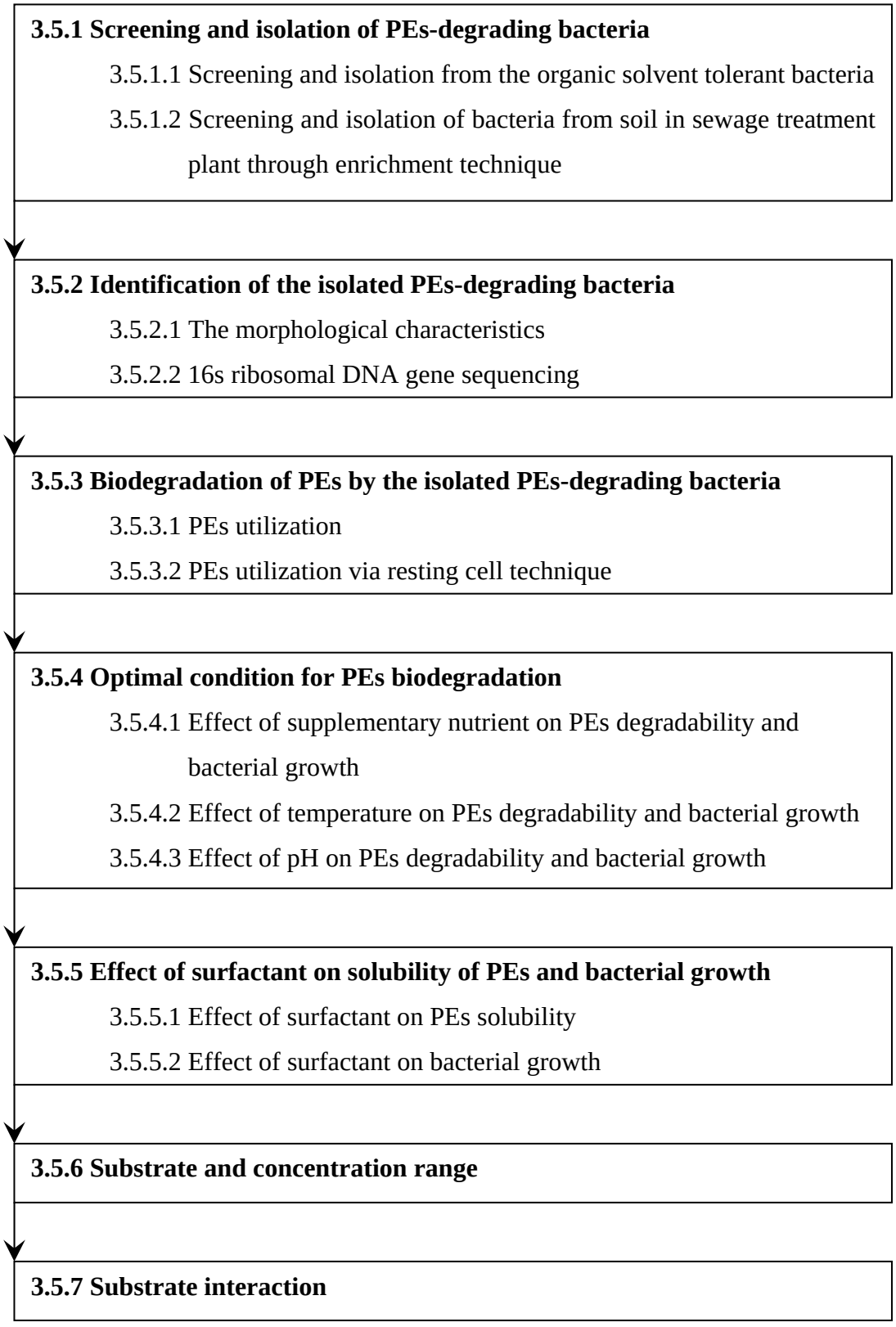
PEs, named DMP, DEP, DPrP, DBP and BBP, stock solution were prepared in absolute ethanol at a concentration of 100,000 ppm. PEs at each concentration was obtained by doing a serial dilution from PEs stock solution.

3.4 Preparation of surfactants

The nonionic surfactants, named Tergitol type NP-10, Triton x-100, Tween 80 and Brij 35, stock solution were prepared in DI water at a concentration of 10 g L^{-1}. Surfactant at each concentration was obtained by doing a serial dilution from a surfactant stock solution

3.5 Experimental procedures

The experimental procedures will be described in the order of experiment performed. All of the procedures used in this research can be simplified and shown in a flow chart below.



3.5.1 Screening and isolation of PEs-degrading bacteria

3.5.1.1 Screening and isolation from the organic solvent tolerant bacteria

According to Kongpol *et al*. (2008), the organic solvent tolerant bacteria were screened from soil samples taken from contaminated area, hot spring area, agricultural area and natural area in several provinces, Thailand by enriched with toluene and cyclohexane vapor. As shown in Table 3, many of them could tolerate and utilize diethyl phthalate (DEP) as a sole carbon source. Thus, those bacteria were initially tested the DEP biodegradability under the condition as described in topic 3.5.3.1.

Table 3 The organic solvent tolerant bacteria capable of utilizing DEP as a sole carbon source (Kongpol *et al*., 2008)

Bacterial specie	Bacterial isolate
Bacillus sp.	3c3
Bacillus sp.	4/1
Bacillus sp.	24
Bacillus sp.	25/1
Bacillus sp.	27/1
Bacillus sp.	36
Bacillus sp.	44
Bacillus sp.	45
Bacillus sp.	46

3.5.1.2 Screening and isolation of bacteria from soil in sewage treatment plant through enrichment technique

Soil samples were collected from the sewage treatment plant with a history of municipal waste exposure. Soil enrichment was performed by placing the soil sample (5g) in a 250-mL Erlenmeyer flask containing 100-mL of the modified minimal salt medium (MSM) and 50 ppm of DBP. After 7 days of incubation on a rotary shaker at 150 rpm at 30 °C , the soil suspension (5mL) was then transferred into 100-mL of minimal salt medium containing 50 ppm DBP. Then, a serial dilution of the enrichment culture was carried out and spread on the minimal salt medium agar plate supplemented with 50 ppm DBP. After 5-7 days of incubation, bacterial colonies formed on the plate were collected as DBP-degrading bacteria.

3.5.2 Identification of the isolated PEs-degrading bacteria

3.5.2.1 The morphological characteristics

Morphology of bacteria

Bacterial staining was used to study shape and size of bacteria and classify the bacteria into gram positive or gram negative. The bacteria were grown in LB broth (liquid medium) for 8-12 hours, stained, and visualized through the microscopy (staining solutions and protocol as shown in APPENDIX A).

Characteristics of bacterial colonies

Bacteria were grown on LB agar for 12-24 hours, and then the physiological feature on an agar plate was observed; for example, color, form, diameter, surface and edge.

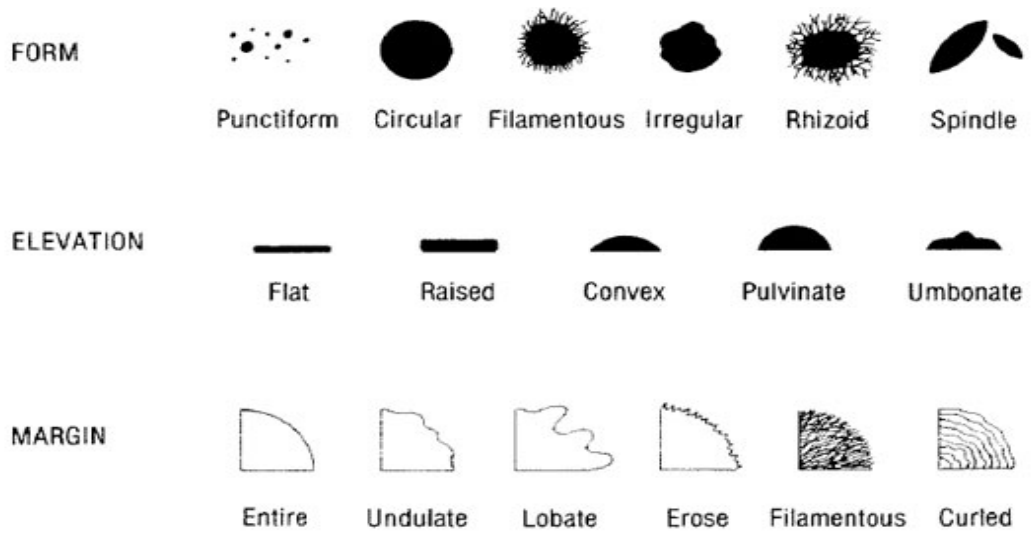


Figure 4 Characteristics of bacterial colony

3.5.2.2 16s ribosomal DNA gene sequencing

Genomic DNA from individual bacterial strains will be extracted by a standard method (Sambrook & Russell, 1989). The 16s gene fragment will be amplified from the genomic DNA of each bacteria by polymerase chain reaction (PCR) using the bacterium-specific primers: forward primer (63f) (5' CAGGCCTAACACATGCAAGTC 3') and a reverse primer 1387r (5'GGGCGGWGTGTACAAGGC 3') (Marchesi *et al*., 1998). A reaction mixture (total volume of 25 µL) contained 1X Taq buffer with $(NH_4)_2SO_4$, 0.2 mM dNTP, 2 mM $MgCl_2$, 0.4 µM of each primer, DNA sample 1µl (500 ng DNA) and Taq polymerase 5 units μL^{-1} (Fermentas, USA). The reaction mixture was subjected to pre-denaturation at 94°C for 3 minutes and 30 PCR cycles were conducted. Each cycles consisted of denaturation at 95°C for 1 minute, Annealing at 55°C for 1 minute, and chain extension at 72°C for 1.3 minutes with 5 minutes of an additional extension time at 72°C. Then, the fragment (1,300 bps) was cloned into pGEM-T Easy Vector (Promega, USA) and transformed into the competent cell of *Escherichia coli*

DH5α. Plasmid DNA (DNA 650 ng μL^{-1}) from the transformant was isolated using Gel/PCR DNA Fragment Extraction Kit (Cat. No. DF 100, Lot. No.Tl04901, Geneaid). After that, the insert was sequenced by the Macrogen Inc. (Seoul, Korea) and compared to the most similar sequences in NCBI database (http://www. ncbi.nlm.nih.gov).

3.5.3 Biodegradation of PEs by the isolated PEs-degrading bacteria

3.5.3.1 PEs utilization

The cell inoculums were prepared by transfer one loop of bacterial cell from LB agar plate into 20-mL bottle containing 5 mL LB medium, pH 7.0 and cultured on the rotary shaker at 150 rpm under room temperature (35-37 °C) for 12-24 hours. Then, the cell inoculums (5% v/v) were used to start a 100-mL bacterial culture grown in a minimal salt medium, pH 7.0 with 100 ppm of PEs on the rotary shaker at 150 rpm under room temperature. The 1 mL of sample was collected at different incubation times in order to determine cell growth by measuring cell optical density at 560 nm, the PEs residual by HPLC and cell protein by a modified Lowry method.

3.5.3.2 PEs utilization via resting cell technique

The cell inoculums were prepared by transfer one loop of bacterial cell from LB agar plate into 20-mL bottle containing 5 mL LB medium, pH 7.0 and cultured on the rotary shaker at 150 rpm under room temperature (35-37 °C) for 8-12 hours. Then, the cell inoculums (5% v/v) were used to start a 100-mL bacterial culture grown in a minimal salt medium, pH 7.0. The PEs at concentration 10 ppm was alternatively added as an inducer. After 12-24 hours of incubation on the rotary shaker at 150 rpm under room temperature, the cells were harvested by centrifugation for 15 min at 5,000 rpm 4 °C, washed twice with 0.85% (w/v) NaCl and suspended in minimal salt medium, pH 7.0

to an optical density of 1.0. The PEs at concentration 100 ppm was then added in order to test biodegradability of the isolates. The sample (200 µL) was collected at different incubation times to determine the PEs residual by HPLC and cell protein by a modified Lowry method. The degradation rate of each isolates was also calculated.

3.5.4 Optimal conditions for PEs biodegradation

The effect of supplementary nutrients, temperature, and pH towards DEP biodegradation and bacterial growth was investigated.

3.5.4.1 Effect of supplementary nutrient on PEs degradability and bacterial growth

According to the previous DEP biodegradation method (Topic 3.5.3.1), each additional nutrient source as followed was supplemented.

1. 4 mM citrate
2. 4 mM glucose
3. 4 mM succinate
4. 0.1% (w/v) peptone
5. 0.1% (w/v) tryptone
6. 0.1% (w/v) yeast extract

After the addition of nutrient source, the bacterial culture was incubated on a rotary shaker at 200 rpm, under room temperature for 2 days. Then, 1 mL culture medium was collected at different incubation time and determined for the remaining of DEP residual, cell growth, and cell protein concentration.

3.5.4.2 Effect of temperature on PEs degradability and bacterial growth

In this part, the experiment was carried out at three different temperatures, i.e. 30 °C, 35-37 °C (room temperature), 45°C, using the condition as previous described in topic 3.5.3.1 Bacterial culture was grown on a rotary shaker at 200 rpm for 2 days. The 1 mL culture medium was collected at different incubation time and then determined for the remaining of DEP residual, cell growth, and cell protein concentration.

3.5.4.3 Effect of pH on PEs degradability and bacterial growth

In this experiment, the pH values were varied to pH 7.0, 7.5 and 8.0 in culture medium. The pH values were adjusted with 1 M NaOH. The bacteria were cultured on a rotary shaker at 200 rpm under room temperature for 2 days using the cultured condition as described in topic 3.5.3.1. Then, the 1 mL culture medium was collected at different incubation time and determined for the remaining of DEP residual, cell growth, and cell protein concentration.

From these experiments, the suitable condition, considering from high percentage of total biodegradation and high bacterial growth rate, for the isolated PEs-degrading bacteria was selected for further study.

3.5.5 Effect of surfactant on solubility of PEs and bacterial growth

3.5.5.1 Effect of surfactant on PEs solubility

The experiment was performed in 250 mL flask containing 100 mL of minimal salt medium supplemented with 100 ppm PEs, i.e. DPrP, DBP and BBP. The four nonionic surfactants, named Tergitol type NP-10, Triton x-100, Tween 80 and Brij 35, were tested by using several concentrations (2, 1, 0.5, 0.25, 0.1, and 0.05 g L^{-1}). Then,

the flasks were equilibrated on the rotary shaker at 200 rpm under room temperature for 24 hours. The PEs concentration was analyzed through HPLC.

3.5.5.2 Effect of surfactant on bacterial growth

As the solubility limit of the phthalate esters, i.e. DPrP, DBP, BBP, surfactant was involved to improve degradability and bioavailability of them. The four nonionic surfactants, named Tergitol type NP-10, Triton x-100, Tween 80 and Brij 35, at concentration 2 g L^{-1} was studied under the cultured condition as described topic 3.5.3.1. Then, the 1 mL culture medium was collected at different incubation time to determine cell growth at OD 560 nm.

3.5.6 Substrate and concentration range

PEs, i.e. DMP, DEP, DPrP, DBP, BBP, are mostly found in the environment. As the literature review, PEs degradation ability of PEs-degrading bacteria was studied in the concentration range from 5 to 610 ppm. This experiment aimed to determine if the bacterial isolate was able to tolerate and degrade phthalate esters at higher concentrations. The degradation condition was performed through resting cell technique as described in topic 3.5.3.2. The five phthalate esters, named DMP, DEP, DPrP, DBP and BBP, were tested using concentrations of 50 ppm, 100 ppm, 200 ppm, 500 ppm and 1000 ppm. Then, 200 µL samples at different incubation times were collected and determine the PEs residual by HPLC and cell protein by a modified Lowry method. The degradation efficiency of each isolates was also calculated.

3.5.7 Substrate interaction

The effect each phthalate ester in mixed conditions was examined through resting cell technique as described in topic 3.5.3.2. The PEs, named DMP, DEP, DPrP, DBP and BBP, were combined in three categories (binary, ternary and quaternary) with concentration of each substrate at 100 ppm and 200 ppm. Then, 200 µL samples at different incubation times were collected and determine the PEs residual by HPLC and cell protein by a modified Lowry method. The degradation efficiency of each isolates was also calculated.

3.6 Analytical procedures

3.6.1 Cell growth determination

The cell growth was determined by a spectrophotometer at wavelength 560 nm using the culture medium as blank.

3.6.2 Cell protein determination

3.6.2.1 Protein extraction from cell

The sample (500 µL) was centrifuged at 10,000 rpm, 3-5 minutes. Then, cells were collected, washed twice and suspended in 0.85% (w/v) NaCl. Protein was extracted from cells by heating at 100 °C for 10 minutes and centrifugation at 10,000 rpm for 3-5 minutes. The cell-free supernatant (200-400 µL) was used to examine protein concentration.

3.6.2.2 Protein determination

Protein concentration was determined by a modified Lowry method (Lowry *et al.*, 1951). Bovine serum albumin (BSA) was used as a standard protein.

Reagents

Solution A: 2% sodium carbonate in 0.1 M sodium hydroxide containing 0.5 % sodium dodecyl sulfate (SDS)

Solution B: 0.5% copper sulfate in 1% potassium sodium tartrate

Solution C: Phenol reagent (Folin- Ciocalteu's reagent)

After preparation of sample (0.4 mL), 2 mL of mixed solution A and B (A: B, 50: 1, v/v) were added and rapidly mixed. The mixture was incubated at 30°C for 10 minutes. Subsequently, 0.2 mL of solution C were added, rapidly mixed, and incubated at 30°C for 30 minutes. Finally, the protein quantity was measured by spectrophotometer at wavelength 750 nm using H_2O as blank.

3.6.3 PEs residual determination

The phthalate esters residual were determined using High Performance Liquid Chromatography (HPLC)

3.6.3.1 Sample preparation for HPLC analysis

Cells in 500 µL sample were removed by centrifugation at 10,000 rpm for 3-5 minutes. Then, the cell-free supernatant was collected, mixed with acetonitrile at the ratio 1: 4 (v/v) and filtered through 0.45 µM nylon syringe filter.

3.6.3.2 HPLC analytical condition

PEs residual was analyzed using reverse phase HPLC equipped with a UV detector (at 226 nm). The separation was performed at 28.5 °C on a C18 column using an acetonitrile: water mixture (70:30, v/v) as a mobile phase at a flow rate of 1 mL min^{-1}. The injection volume was 20 µL. The retention time of DMP, DEP, DPrP, DBP and BBP

under the condition tested were 3.5, 4.3, 6.3, 10.4 and 9.2 minute, respectively as shown in APPENDIX E.

The mobile phase for separating PA was methanol: 1% acetic acid water (40:60, v/v), the flow rate was 0.5 mL min^{-1} and UV wavelength was 228 nm. The retention time under the tested condition was 8.4 min (Wu *et al*., 2010).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Screening, isolation and identification of phthalate esters-degrading bacteria

4.1.1 Screening and isolation from the organic solvent tolerant bacteria

The 9 *Bacillus* sp. isolates were initially isolated as organic solvent tolerant bacteria able to grow at comparatively high temperature, i.e. 45°C (Kongpol *et al.*, 2008). Their ability to biotransform aromatic hydrocarbons was subsequently examined. Interestingly, the preliminary results showed that they were able to biotransform DEP and utilize it for growth. Therefore, further investigation was carried out. Upon their growth on the minimal salt medium supplemented with 100 ppm (0.45 mM) DEP, *Bacillus* sp. isolate 3c3, 24, and 44 were able to slowly utilized DEP to the maximum degradation of 52.69%, 59.12% and 60.89%, respectively, as presented in Table 4.

Table 4 DEP biodegradability and maximum growth of the 9 *Bacillus* sp. isolates

Bacterial isolate	At 72 hours of incubation	
	% Total degradation	Maximum growth
3c3	52.69	0.048
24	59.12	0.044
44	60.89	0.028
45	18.56	0.108
46	23.83	0.155
4/1	9.65	0.080
25/1	26.46	0.010
27/1	17.63	0.172
36	24.04	0.091

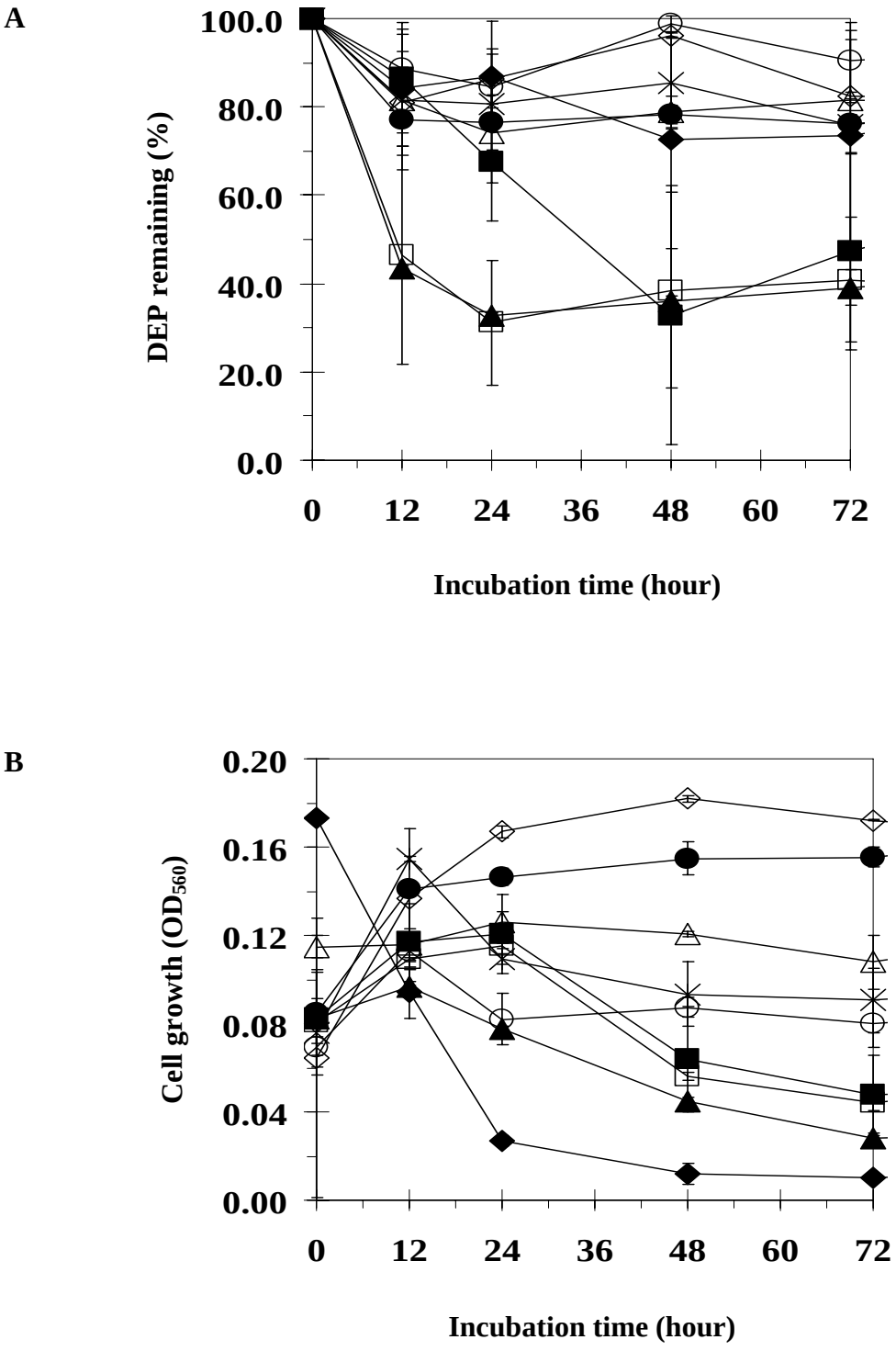


Figure 5 DEP degradation (**A**) and bacterial growth (**B**) of *Bacillus* sp. isolate 3c3 (■), 24(□), 44(▲), 45(△), 46(●), 4/1(○), 25/1(◆), 27/1(◇) and 36(∗). Cells were grown at 45 °C in the minimal salt medium in the presence of 100 ppm (0.45 mM) DEP.

In order to improve bacterial growth and degradation efficiency, the *Bacillus* sp. isolates 3c3, 24, and 44 were then cultured in minimal salt medium containing 0.1% (w/v) yeast extract and 100 ppm (0.45 mM) DEP at 45 °C. The result found that the isolate 3c3, 24 and 44 had similar degradation efficiency and maximum growth as shown in Figure 6. Only isolate 3c3 had the highest specific degradation rate at 0.0034 mmole (h.mg protein)$^{-1}$, while specific degradation rate of isolate 24 and 44 was comparatively similar, as shown in Table 5. Therefore, the *Bacillus* sp. isolate 3c3 was selected for further study.

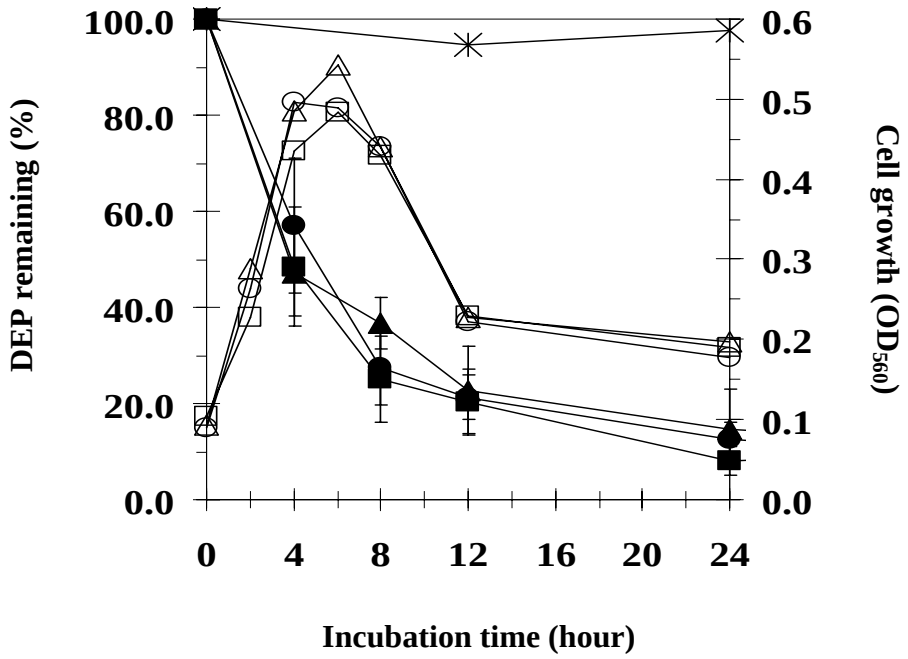


Figure 6 DEP degradation efficiency (closed symbol) and bacterial growth (opened symbol) of *Bacillus* sp. isolate 3c3 (■,□), 24 (●,○) and 44 (▲,△). Cells were grown at 45 °C in minimal salt medium in the presence of 100 ppm (0.45 mM) DEP with 0.1% (w/v) yeast extracts supplementation. Abiotic control was also examined (∗).

Table 5 Specific degradation rate of *Bacillus* sp. isolate 3c3, 24 and 44

***Bacillus* sp. isolate**	**Specific degradation rate [mmole (h .mg protein)$^{-1}$]**
3c3	0.0034
24	0.0024
44	0.0021

4.1.2 Screening and isolation of bacteria from soil in sewage treatment plant through enrichment technique

DBP is one of the phthalate esters that has been widely contaminated and accumulated in the environment especially in municipal landfill and leachate (Stales *et al*., 1997). DBP-degrading bacteria could be isolated from soil with a history of municipal waste exposure. Therefore, soil samples were collected from several waste contaminated areas in Thailand. Enrichment technique was applied to increase the quantity of bacteria capable of degrading DBP. By screening on the minimal salt medium agar plate supplemented with 100 ppm (0.36 mM) DBP, only 2 different types of bacterial colonies were isolated. The 2 bacterial isolates, designated as DB-6 and DB-9, were then separately grown in liquid minimal salt medium in the presence of 100 ppm (0.36 mM) DBP at room temperature (35-37 °C). The result of total degradation and maximum growth was showed in Table 6. The isolate DB-9 exactly showed higher degradation efficiency and maximum growth than isolate DB-6, as shown in Figure 7. Therefore, the bacterial isolate DB-9 was chosen for further study.

Table 6 BBP biodegradability and maximum growth of isolate DB-6 and DB-9

Bacterial isolate	At 48 hours of incubation	
	% Total degradation	Maximum growth
BB-6	40±1	0.408
BB-9	86±1	1.387

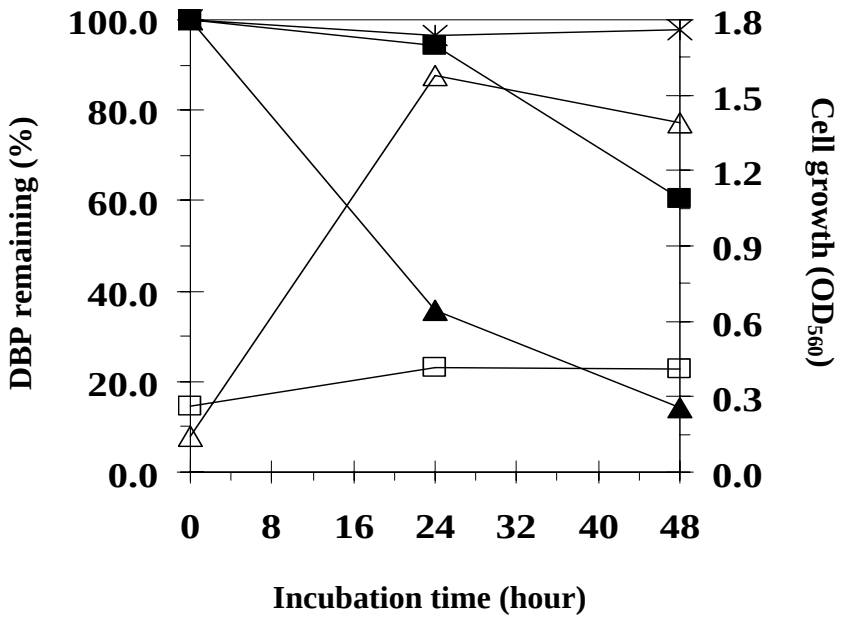


Figure 7 DBP degradation efficiency (closed symbol) and bacterial growth (opened symbol) of bacterial isolate DB-6 (■,□) and DB-9 (▲,△). Cells were grown at room temperature (35-37 °C) in minimal salt medium in the presence of 100 ppm (0.36 mM) DBP. Abiotic control was BBP-containing medium without bacterial cells (∗).

4.1.3 Identification of the PEs-degrading bacteria

4.1.3.1 The morphological characteristics

The two selected bacterial isolates, named 3c3 and DB-9, were identified for their morphological characteristics by bacterial gram staining (APPENDIX A), result as shown in Figure 8, 9. In addition, the characteristics of bacterial colony on LB agar (as described in Method Topic 3.5.2.1) had been shown in Table 7.

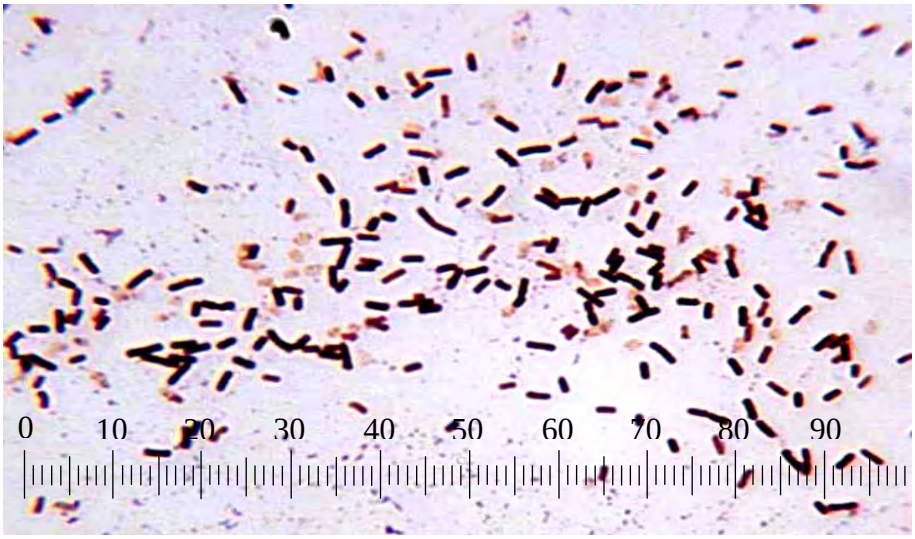


Figure 8 Gram staining and morphology of *Bacillus* sp. isolated 3c3 (100X)

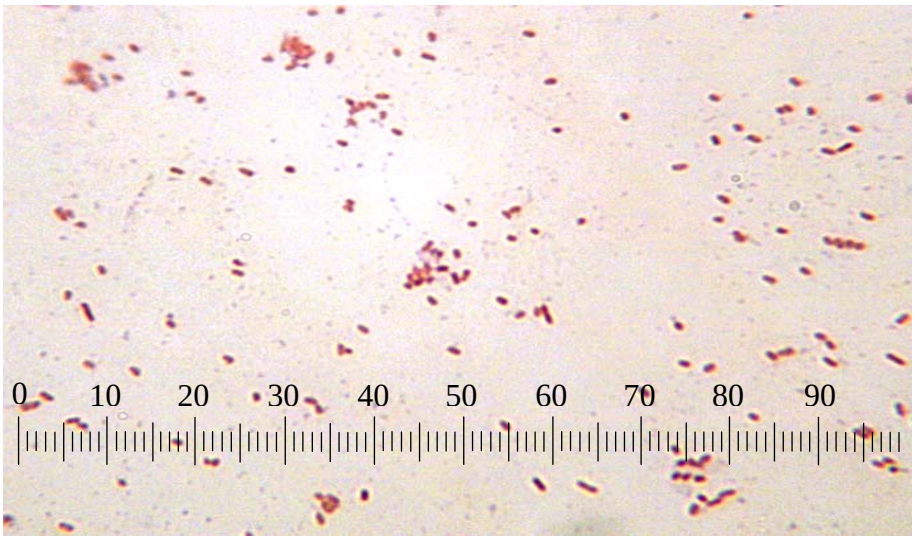


Figure 9 Gram staining and morphology of isolate DB-9 (100X)

4.1.3.2 16s ribosomal DNA gene sequencing

The 16s ribosomal DNA gene sequence was used to identify the phthalate ester-degrading bacteria. The specific primers (63f and 1387r) (Marchesi *et al*., 1998) were used to amplify the target sequence as described in Method Topic 3.5.2.2. These primers were found to be more useful for 16s rDNA gene of bacterial species and environment samples than PCR primers that were generally used. After PCR amplification, PCR products were analyzed by using 0.8% (w/v) agarose gel electrophoresis. The length of the amplified 16s rDNA fragment product was approximately 1.3 kbps. Subsequently, the blastN program was used to compare and analyze the 16s rDNA sequence against NCBI database (www. ncbi.nlm.nih.gov). The alignment result of 16s rDNA sequences were showed in APPENDIX B and summarized in Table 8.

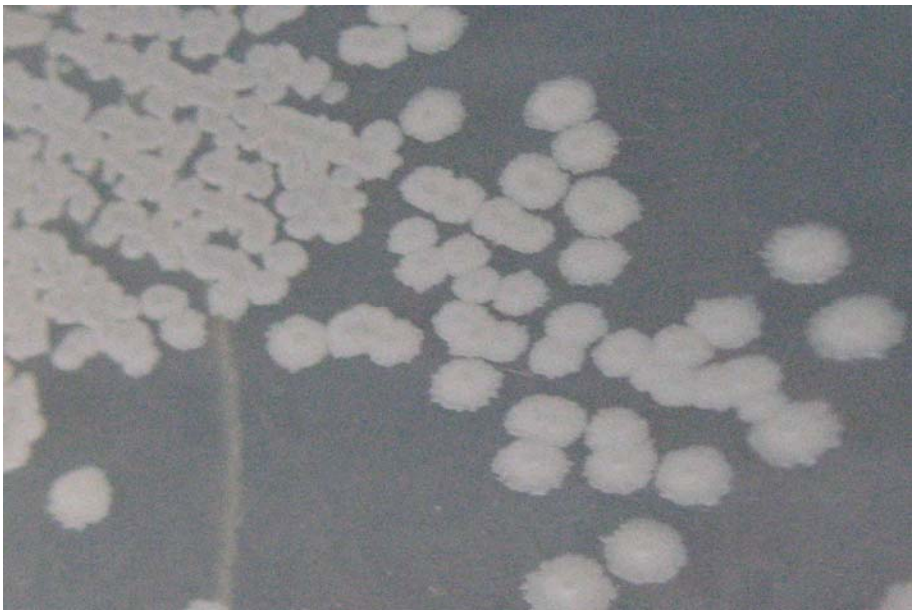


Figure 10 Characteristics of bacterial colony of *Bacillus* sp. strain 3c3 on LB agar

Figure 11 Characteristics of bacterial colony of *Pseudomonas aeruginosa* strain DB-9 on LB agar

Table 7 Morphological characteristics of bacteria

Bacterial isolate	Morphology		Colony characteristic				
	Gram stain	**Cell shape**	**Color**	**Form**	**Elevation**	**Margin**	**Surface**
3c3	Positive	Bacilli	Cream	Irregular	Umbonate	Lobate	Smooth
DB-9	Negative	Rod	Blue-Green	Circular	Convex	Entire	Smooth

Table 8 Identification of phthalate ester-degrading bacteria by 16s rDNA gene sequencing

Bacterial isolate	Blast result			Bacteria
	Sequence identity (%)	**Sequence accession no.**	**Organism**	
3c3	99	GU166746.1	*Bacillus subtilis* strain BS11	*Bacillus* sp.
	99	FJ889050.1	*Bacillus amyloliquefaciens* strain Bac3M5	
DB-9	98	GU339238.1	*Pseudomonas aeruginosa* strain EH8	*Pseudomonas aeruginosa*

Biodegradation was expected to be the dominant loss mechanism of PEs in surface waters, soils and sediments. Numerous studies indicated that phthalate esters were degraded by a wide range of bacteria under both aerobic and anaerobic conditions (Stales *et al*., 1997). In this study, gram positive and gram negative bacteria were isolated and identified using 16s rDNA sequencing as *Bacillus* sp. strain 3c3 and *Pseudomonas aeruginosa* strain DB-9, respectively. The isolated phthalate-degrading bacteria could completely degrade PEs by individual, although mixed microbial populations had better metabolism (Engelhardt *et al*., 1975).

The *Bacillus* sp. strain 3c3 was gram positive bacteria isolated from hot spring soil in Krabi province, Thailand by organic solvents enrichment (Kongpol *et al*., 2008) and *Pseudomonas aeruginosa* strain DB-9 was gram negative bacteria isolated from soil with a history of municipal waste exposure using enrichment technique. Other gram positive bacteria have been reported as phthalate-metabolizing strains, although they could degrade PEs at low concentration; for example, *Gordonia* sp. MTCC 4818 isolated from creosote contaminated soil could degrade DBP at concentration 24 ppm (Chatterjee & Dutta, 2003), *Arthrobacter* sp. isolated from soil could degrade DMP at concentration 52.4 ppm (Vega & Bastide, 2003), *Rhodococcus ruber* CQ0301 isolated from landfill soil could degrade DBP (Liang *et al*., 2008), *Corynebacterium* sp. O18 isolated from activated sludge of petrochemical wastewater had ability to degrade a wide range of phthalate esters at concentration 5 ppm (Chang *et al*., 2004), and *Corynebacterium nitrilophius* G11 and *Rhodococcus rhodocchrous* G2 were isolated from soil as DBP-degrading bacteria (Chao *et al*., 2006).

On the other hand, gram negative phthalate-degrading bacteria were also stated. For instance, Chang *et al*. (2004) was successful on isolating *Sphigomonas* sp. DK.4 from activated sludge of petrochemical wastewater exhibiting biodegradability of various

phthalate esters at concentration 5 ppm, *Pseudomonas aeruginosa* P1 was isolated from bioaugmented slurry reactor as DBP-degrading bacteria (Liang *et al*., 2008). *Acinetobacter lwoffii* isolated from river water could degrade DBP at concentration 20 ppm (Hashizume *et al*., 2002), and *Pseudomonas fluorescens* B-1 isolated from mangrove sediment had ability to degrade DBP and BBP at concentration 2.5-20.8 ppm (Xu *et al*., 2007).

Staples *et al*. (1997) stated that anaerobic degradation of phthalates is generally much slower than aerobic degradation. So far, only three strains, one *Clostridium* sp. and two *Bacillus* sp. strains were capable of degrading phthalates anaerobically (Staples *et al*., 1997). Nevertheless, *Bacillus* sp. strain 3c3 and *Pseudomonas aeruginosa* strain DB-9 could degrade PEs under aerobic condition.

4.2 Optimal conditions for PEs degradation

4.2.1 Effect of supplementary nutrient on PEs biodegradability

4.2.1.1 *Bacillus* sp. strain 3c3

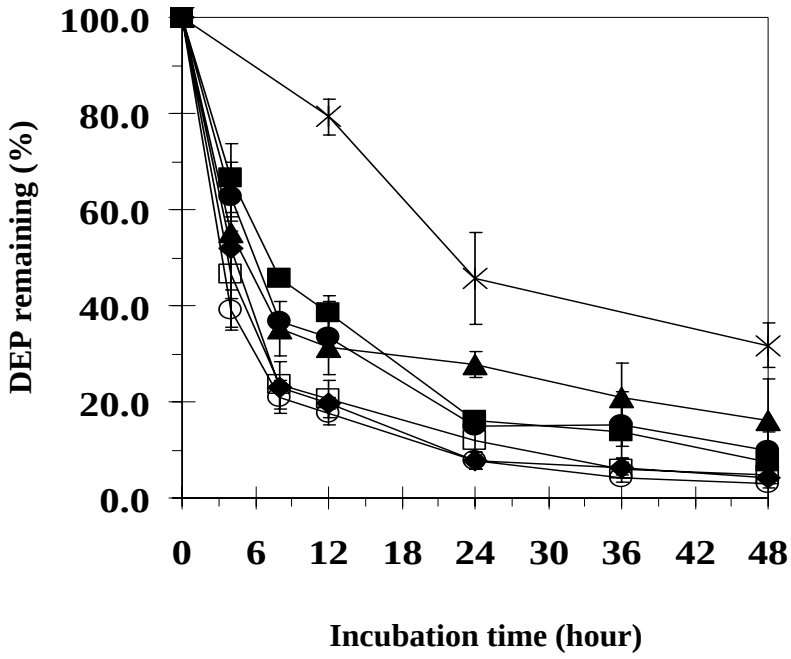
PEs can serve as a sole carbon source for *Bacillus* sp. strain 3c3 exhibiting the degradation of 68±5% with the maximum growth at 0.123 in 48 hours of incubation under room temperature (35-37 °C). The optimal conditions for growth were attempted and investigated in order to increase degradation efficiency. Type of additional carbon and energy source was varied to promote cell growth and cell degradability. The result found that the total DEP degradations of *Bacillus* sp. strain 3c3 in minimal salt medium in the presence of 100 ppm (0.45 mM) DEP containing 4mM citrate, 4mM glucose, 4mM succinate, 0.1% (w/v) peptone, 0.1% (w/v) tryptone and 0.1% (w/v) yeast extract were higher than the total DEP degradation in minimal salt medium without nutrient, as presented in Figure 12. When supplemented with various nutrients, the growth rate of *Bacillus* sp. strain 3c3 was significantly increased (Figure 12). Total degradation and growth rate were summarized in Table 9.

Table 9 Effect of various nutrients on DEP degradation efficiency and bacterial growth of *Bacillus* sp. strain 3c3

Degradation condition	Growth rate (h^{-1})	% DEP degradation (48 hours)
Culture medium [a]	0.001	68±5
Culture medium [a] + 4 mM citrate	0.031	90±4
Culture medium [a] + 4 mM glucose	0.054	93±3
Culture medium [a] + 4 mM succinate	0.054	79±5
Culture medium [a] + 0.1% (w/v) peptone	0.032	92±6
Culture medium [a] + 0.1% (w/v) tryptone	0.070	94±2
Culture medium [a] + 0.1% (w/v) yeast extract	0.088	95±4

[a] Culture medium was minimal salt medium containing 100 ppm (0.45 mM) DEP

A



B

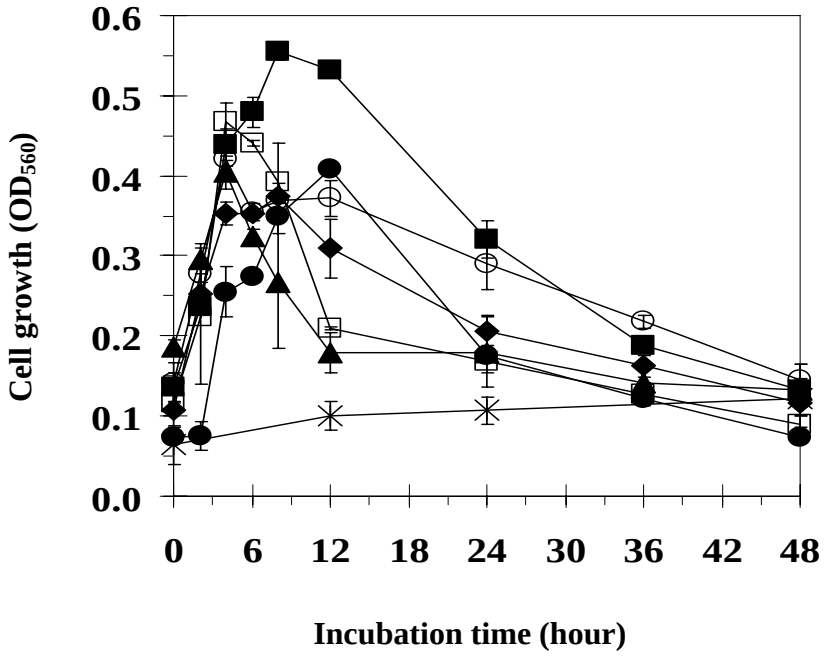


Figure 12 DEP degradation (**A**) and bacterial growth (**B**) of *Bacillus* sp. strain 3c3 in minimal salt medium in the presence of 100 ppm (0.45 mM) DEP supplemented with 4 mM citrate (●), 4 mM glucose (■), 4 mM succinate (▲), 0.1% (w/v) peptone (◆), 0.1% (w/v) tryptone (○) and 0.1% (w/v) yeast extract (□) at room temperature (35-37 °C). DEP degradation in the absence of additional carbon source was also examined (∗).

4.2.1.2 *Pseudomonas aeruginosa* strain DB-9

PEs can serve as a sole carbon source for *Pseudomonas aeruginosa* strain DB-9 exhibiting the degradation efficiency of 90±2% and growth rate of 0.062 h^{-1} in 12 hours of incubation under room temperature (35-37 °C). The optimal conditions for growth were investigated in order to increase degradation efficiency. Type of additional carbon and energy source was varied to promote cell growth and cell degradability. The result found that degradation efficiency of *Pseudomonas aeruginosa* strain DB-9 in the minimal salt medium in the presence of 100 ppm (0.32 mM) BBP and 2 g L^{-1} Tween 80 containing 0.1% (w/v) yeast extract were comparatively similar with minimal salt medium without yeast extract (Table 10). Nonetheless, growth rate of *Pseudomonas aeruginosa* strain DB-9 in minimal salt medium containing 2g L^{-1} Tween 80 was higher than the minimal salt medium alone and also higher than minimal salt medium in the presence of 100 ppm (0.32 mM) BBP and 2 g L^{-1} Tween 80 (Table 10). This result showed that Tween 80 was considered as a co-substrate which could increase the bacterial growth rate, while BBP was another substrate that toxic to the bacterial cells. Thus, the addition of yeast extracts in the presence of 100 ppm (0.32 mM) BBP and 2 g L^{-1} Tween 80 in minimal salt medium was then carried out in order to increase growth rate and degradation efficiency. The result showed that supplementation of yeast extract at 0.1% (w/v) increased the bacterial growth rate by 25% and increased degradation efficiency by 2%.

Table 10 Growth rate and degradation efficiency of *Pseudomonas aeruginosa* strain DB-9

Condition	Growth rate (h^{-1})	% Total degradation (12 hours)
MSM	0.002	NA
MSM + 2gL^{-1} Tween 80	0.178	NA
MSM + 2gL^{-1} Tween 80 + 100 ppm BBP	0.062	90±2
MSM + 0.1% (w/v) yeast extract	0.102	NA
MSM + 0.1% (w/v) yeast extract +2gL^{-1} Tween80	0.166	NA
MSM + 0.1% (w/v) yeast extract +2gL^{-1} Tween80 + 100 ppm BBP	0.077	91±2

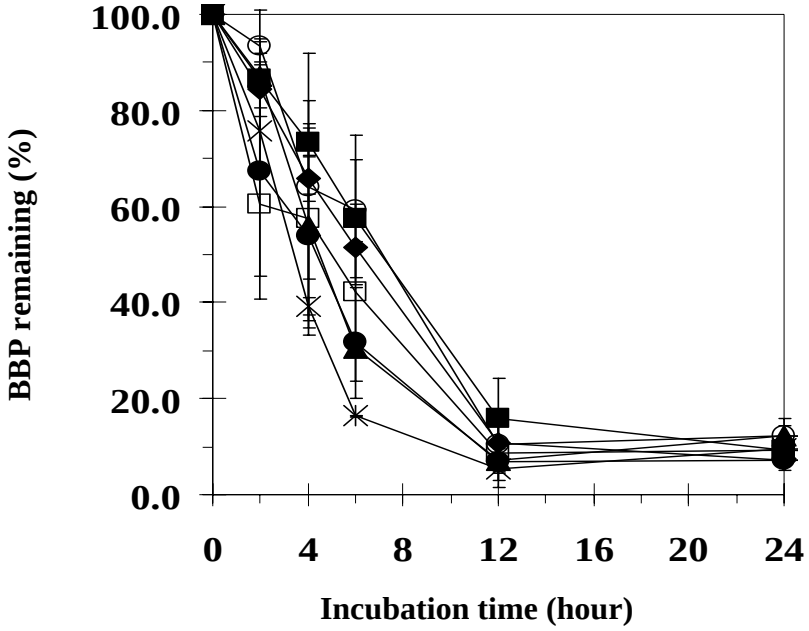
NA = Not Analyze

Table 11 Effect of various nutrients on bacterial growth and BBP degradation efficiency of *P. aeruginosa* strain DB-9

Degradation condition	**Growth rate (h^{-1})**	**% Total degradation (24 hours)**
Culture medium [a]	0.062	90±2
Culture medium [a] + 4 mM citrate	0.074	93±2
Culture medium [a] + 4 mM glucose	0.073	91±2
Culture medium [a] + 4 mM succinate	0.066	88±4
Culture medium [a] + 0.1% (w/v) peptone	0.077	93±1
Culture medium [a] + 0.1% (w/v) tryptone	0.083	88±2
Culture medium [a] + 0.1% (w/v) yeast extract	0.077	91±2

[a] Culture medium was mineral medium containing 100 ppm (0.32 mM) BBP with 2 g L^{-1} Tween 80

A



B

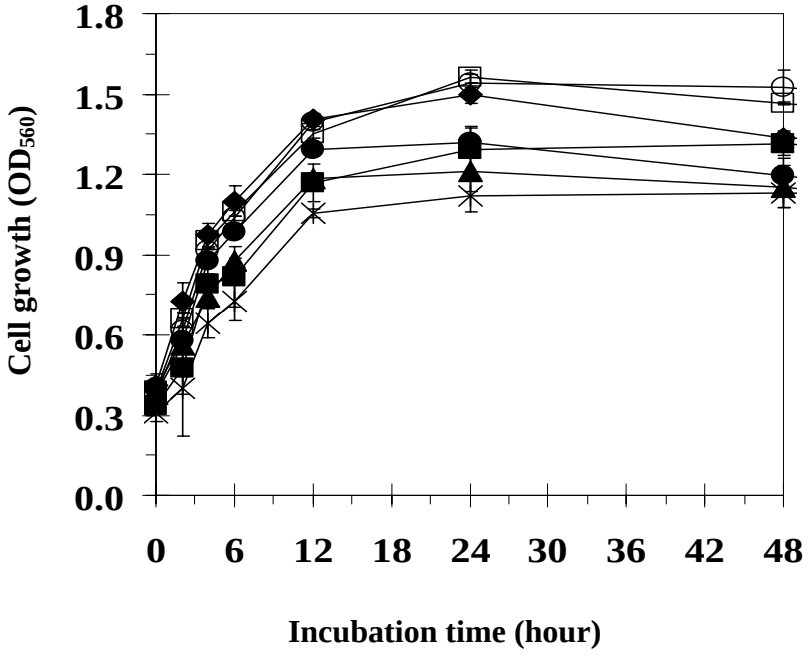


Figure 13 BBP degradation (**A**) and bacterial growth (**B**) of *Pseudomonas aeruginosa* strain DB-9 in minimal medium in the presence of 100 ppm (0.32 mM) BBP supplemented with 4 mM citrate (●), 4 mM glucose (■), 4 mM succinate (▲), 0.1% (w/v) peptone (◆), 0.1% (w/v) tryptone (○) and 0.1% (w/v) yeast extract (□) at 30 °C. BBP-containing medium without nutrient was also examined (∗).

PEs degradation efficiency might be decreased in poor nutrient environment (Staples *et al*., 1997). Upon bacterial growth in the minimal salt medium supplemented with 100 ppm (0.45 mM) DEP, *Bacillus* sp. strain 3c3 was able to slowly utilize DEP to maximum degradation at 68±5% after 48 hours of incubation under room temperature 35-37 °C. Additional carbon and energy sources in the presence of DEP were optimized in order to increase degradation efficiency and/or improve cell growth as well as cell degradability. In this study, the addition of 0.1% (w/v) yeast extract not only exhibited the highest growth rate, but also significantly enhanced degradation efficiency. Thus, yeast extract was a suitable additional carbon source for *Bacillus* sp. strain 3c3. This result corresponded to the report of Chang *et al*. (2007) who isolated *Bacillus benzoevorans*, *Sphigomonas* sp. and *Corynebacterium* sp. which their phthalate esters degradation rate were enhanced by addition of yeast extract. DEP degradation efficiency of *Bacillus* sp. strain 3c3 after supplemented with 0.1% (w/v) yeast extract was 95±4%, while the degradation efficiency of *Bacillus* sp. S4 isolated from sludge after supplemented with 0.1% (w/v) yeast extract was lower than 50% (Chang *et al*., 2007) in 48 hours of incubation.

In the presence of 100 ppm (0.32 mM) BBP with 2 g L^{-1} Tween 80 but without addition of any carbon and energy sources, *Pseudomonas aeruginosa* strain DB-9 could degrade BBP at 90±2% with growth rate of 0.062 h^{-1} in 12 hours of incubation under room temperature. When supplemented with 0.1% (w/v) yeast extract, its degradation efficiency and growth rate were comparatively similar to that without yeast extract. It might be possible that Tween 80 was also considered as co-substrate that could increase the bacterial growth rate and degradation efficiency. Tween 80 was not only served as surfactant but also considered as an additional carbon source. According to the result in Table 10, growth rate of minimal salt medium with 2 g L^{-1} Tween 80 was significantly

higher than minimal medium alone and also higher than minimal medium containing 2 g L^{-1} Tween 80 and 100 ppm (0.32 mM) BBP. When 0.1% (w/v) yeast extract was supplemented in minimal medium, the bacteria had better growth rate than the medium alone and also had better growth rate than minimal medium containing 2 g L^{-1} Tween 80 and 100 ppm (0.32 mM) BBP. It could be concluded that BBP at 100 ppm (0.32 mM) had toxicity to bacterial cells, though the bacteria could use it as carbon source. Moreover, the bacteria could use Tween 80 as their co-substrate. So *Pseudomonas aeruginosa* strain DB-9 needed the additional carbon and energy sources to enhance the bacterial growth rate and degradation efficiency which was similar to the result of Vamsee-Krishna *et al*. (2006) that *Pseudomonas aeruginosa* PP4 and *Pseudomonas* sp. PPD isolated from soil enriched with terephthalate (phthalate isomer) was capable of utilizing phthalate isomers at concentration 1000 ppm as the carbon and energy source when cultured in minimal medium supplemented with 0.25% (w/v) glucose (Vamsee-Krishna *et al*., 2006). Moreover, *Pseudomonas fluoresences* FS1 isolated from sludge of petrochemical factory had ability to degrade a wide range of PEs at concentration 50-400 ppm when the bacterial cells were grown in enrichment medium containing inorganic salts, 0.5% (w/v) malt extract and 1.0% peptone (Zeng *et al*., 2004).

4.2.2 Effect of temperature on PEs biodegradability

The effect of temperature on bacterial growth and degradation efficiency at DEP concentration of 100 ppm (0.45 mM) in the minimal salt medium supplemented with 0.1% (w/v) yeast extract, pH 7.0 was carried out by various temperatures at 30 °C, 35-37 °C (room temperature) and 45 °C. The result showed that growth rate of *Bacillus* sp. strain 3c3 in the presence of 100 ppm (0.45 mM) DEP was maximum when temperature was at 35-37 °C (Table 12).While degradation efficiency was slightly decreased when temperature was increased (Figure 14). In conclusion, DEP degradation at 35-37 °C (room temperature) showed the highest growth rate, while total degradation efficiency at temperature 30 °C, 35-37 °C and 45 °C was comparatively similar at approximately 90%.

Table 12 Effect of temperature on bacterial growth and DEP degradation efficiency of *Bacillus* sp. strain 3c3

Temperature (°C)	**Growth rate (h^{-1})**	**% Total degradation (24 hours)**	**Specific degradation rate [mmole(min .mg protein)$^{-1}$]**
30	0.030	98±1	2.31E-04±1.03E-04
35-37*	0.086	98±2	3.91E-04±7.21E-05
45	0.078	93±2	4.42E-05±7.25E-06

* Room temperature was approximately 35-37 °C.

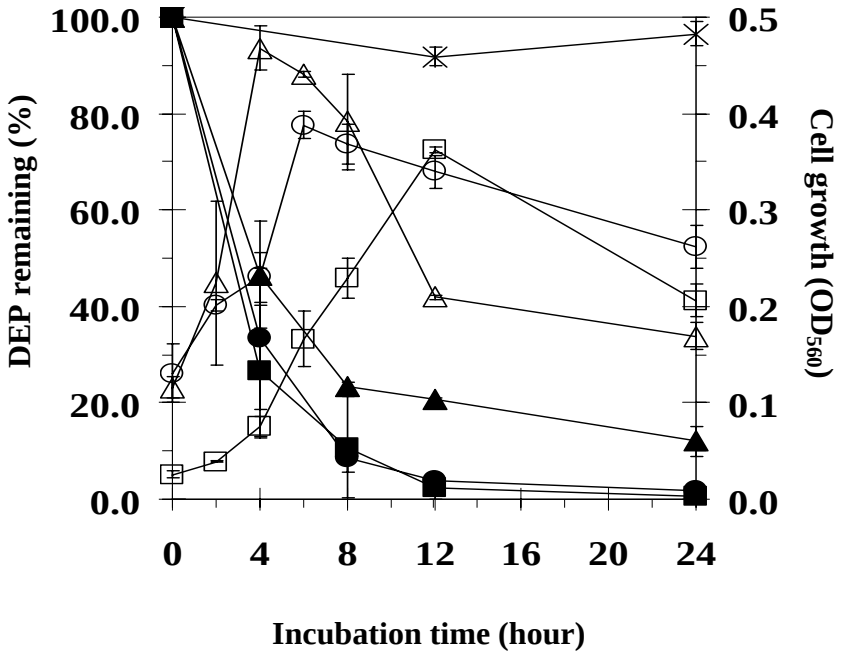


Figure 14 DEP degradation efficiency (closed symbol) and bacterial growth (opened symbol) of *Bacillus* sp. strain 3c3 at temperature 30 °C (■,□), room temperature (35-37 °C) (●,○) and 45 °C (▲,△) was cultured in minimal salt medium in the presence of 100 ppm (0.45 mM) DEP containing 0.1% (w/v) yeast extract. Abiotic control was DEP-containing medium without bacterial cells (∗).

4.2.3 Effect of pH on PEs biodegradability

The effect of pH on DEP degradation efficiency at concentration 100 ppm (0.45 mM) in the minimal salt medium supplemented with 0.1% (w/v) yeast extract was investigated by various pH values of 7.0, 7.5 and 8.0, at room temperature. The result found that degradation efficiency at pH 7.0, 7.5 and 8.0 after 24 hours of incubation was comparatively similar as shown in Figure 15. Growth rate was decreased when pH was increased. Maximum growth rate was 0.086 at pH 7.0. On the contrary, degradation efficiency was relatively similar, as presented in Table 13.

Table 13 Effect of pH on bacterial growth and DEP degradation efficiency of *Bacillus* sp. strain 3c3

pH	Growth rate (h^{-1})	% Total Degradation (24 hours)	Specific degradation rate [mmole(min .mg protein)$^{-1}$]
7.0	0.086	98±1	3.91E-04±7.21E-05
7.5	0.072	98±2	1.48E-04±3.17E-05
8.0	0.050	98±2	1.31E-04±2.89E-05

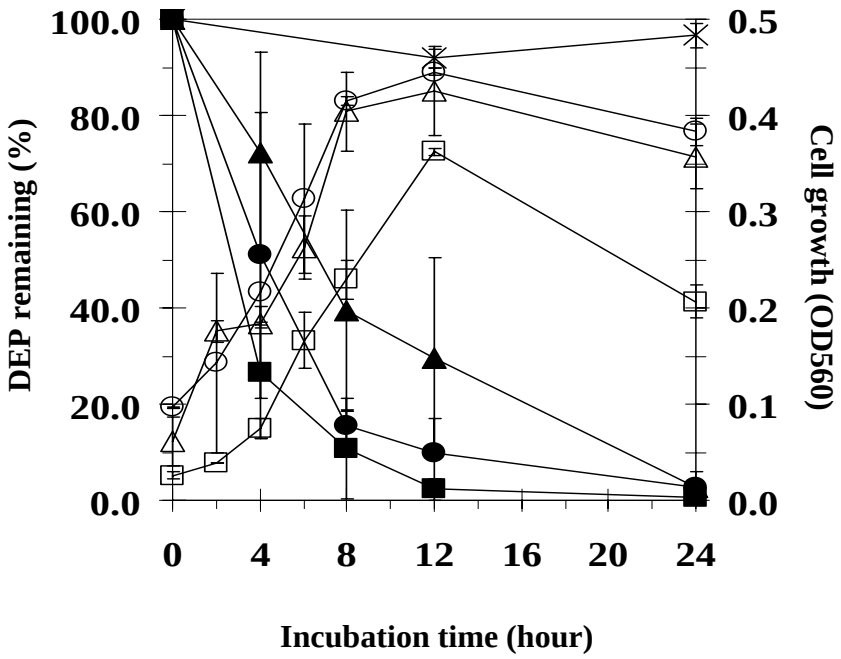


Figure 15 DEP degradation efficiency (closed symbol) and bacterial growth (opened symbol) of *Bacillus* sp. strain 3c3 at pH 7.0 (■,□), 7.5 (▲,△) and 8.0 (●,○) cultured in minimal medium in the presence of 100 ppm (0.45 mM) DEP containing 0.1% (w/v) yeast extract at 30 °C. Abiotic control was DEP-containing medium without bacterial cells (∗).

Phthalate degradation efficiency might be decreased in cold (approximately 5 °C) environment (Staples *et al*., 1997). In this study, DEP degradation of *Bacillus* sp. strain 3c3 could be carried out at 30 °C, 35-37 °C (room temperature) and 45 °C. The degradation efficiency at the defined temperatures was decreased when temperature was increased up to 45 °C. In contrast, growth rate was significantly increased when temperature was increased. DEP degradation of the strain 3c3 at room temperature (35-37 °C) showed the highest growth rate and specific degradation rate at pH 7.0. In addition to temperature, pH also affected degradation efficiency and bacterial growth. The DEP degradation efficiency at pH 7.0, 7.5 and 8.0 was relatively similar at approximately 98% in 24 hours of incubation at room temperature (35-37 °C), while bacterial growth at pH 7.0 had the highest growth rate than the bacterial growth at pH 7.5 and 8.0. In addition, bacterial growth at pH 7.0 showed the highest specific degradation rate. Thus the optimal conditions for PEs degradation of strain 3c3 were at room temperature (35-37 °C) and pH 7.0. Moreover, the strain 3c3 was capable of utilizing phthalate at the wide range of temperature and pH. The previous reports showed that the optimal conditions for phthalate esters degradation of *Enterococcus* sp. strain OM1, *Bacillus banzoevorans* strain S4, *Sphigomonas* sp. strain O18 and *Corynebacterium* sp. strain DK4 were 30 °C and pH 7.0 (Chang *et al*., 2004). Park *et al*, (2008) also stated that the optimal conditions for phthalate esters degradation of *Sphingobium chungbukense* were 30 °C and pH 7.0 (Park *et al*., 2008).

For *Pseudomonas aeruginosa* strain DB-9, the cultured condition was performed at room temperature (35-37 °C) and pH 7.0. The strain DB-9 could not grow at 45 °C. As the previous reports, the optimal temperature and pH for biodegradation of *Pseudomonas fluoresences* FS1 isolated from sludge of petrochemical factory were 20-35 °C and pH 6.5-8.0 (Zeng *et al*., 2004). Beside, *Pseudomonas aeruginosa* PP4 and *Pseudomonas* sp.

PPD isolated from soil enriched with terephthalate (phthalate isomer) was also cultured at 30 °C and pH 7.0.

4.3 Effect of surfactant on PEs solubility and bacterial growth

4.3.1 Effect of surfactant on PEs solubility

Due to low water solubility of PEs, i.e. DPrP, DBP BBP, they can easily adsorb to soil and sediment (Staples *et al*., 1997). Surfactants have been used effectively to increase the bioavailability of these hydrophobic pollutants (Chao *et al*., 2006). So, effect of four surfactants, named Tergitol type NP-10, Triton x-100, Tween 80 and Brij 35, on solubility of PEs was investigated. The concentration of surfactant was varied to 0.05 g L^{-1}, 0.10 g L^{-1}, 0.25 g L^{-1}, 0.50 g L^{-1}, 1.0 g L^{-1} and 2.0 g L^{-1}. The concentration at CMC of each surfactant was showed in Table 14. The result found that DPrP, DBP and BBP were more soluble when concentration of surfactant was increased to 2 g L^{-1}, as presented in Figure 16.

Table 14 Critical Micelle Concentration (g L^{-1}) of four nonionic surfactants (Kim *et al*.)

Surfactant	**Molecular weight (g $mole^{-1}$)**	**CMC g L^{-1}**
Tergitol type NP-10	683	0.14
Triton x-100	625	0.06
Tween 80	1310	0.02
Brij 35	1200	0.11

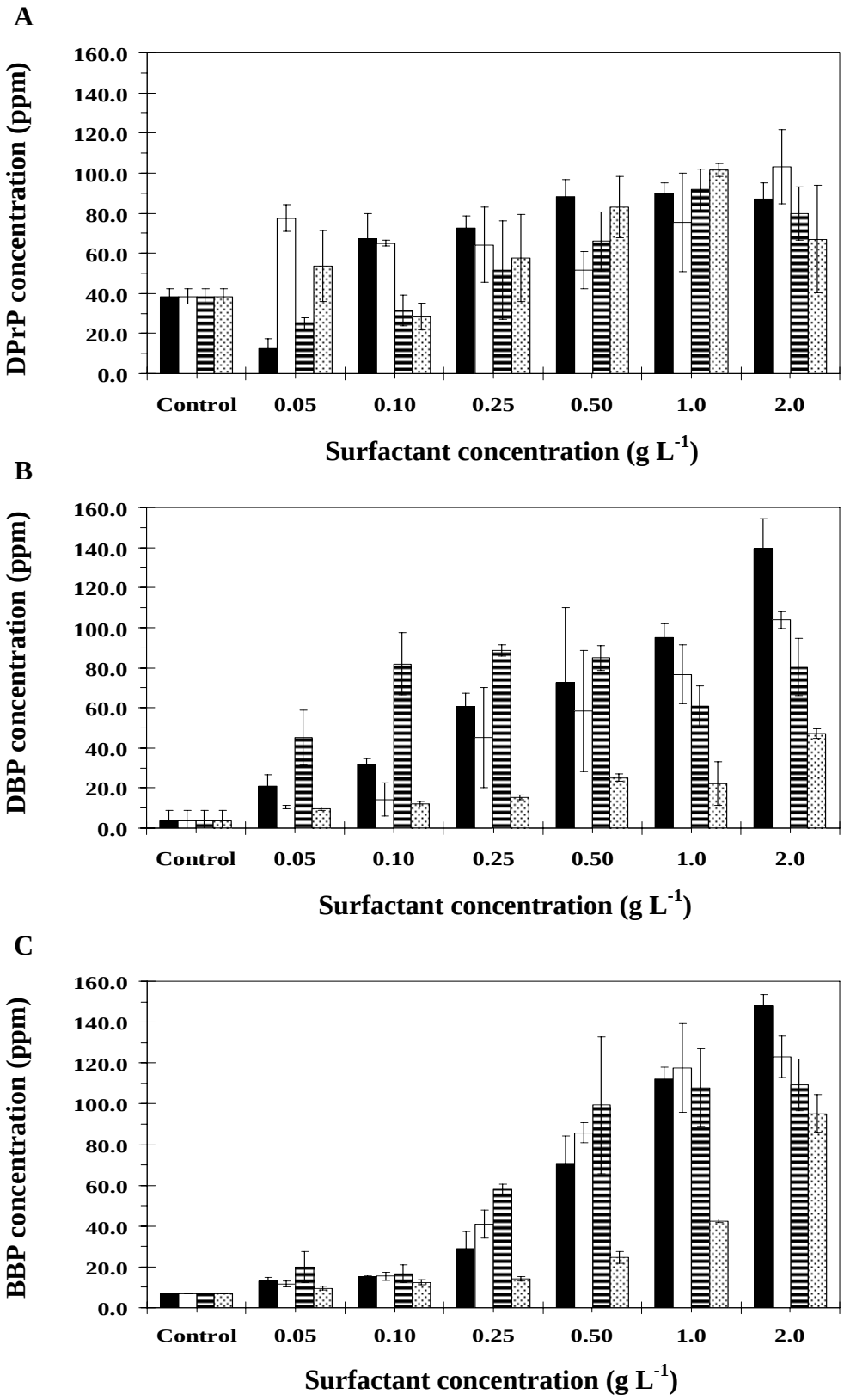


Figure 16 Effect of nonionic surfactants at various concentrations on solubility of DPrP (**A**), DBP (**B**), and BBP (**C**). Control was PEs-containing medium without surfactant.

4.3.2 Effect of surfactant on bacterial growth

Due to the solubility limit of the PEs, i.e. DPrP, DBP, BBP, surfactant was concerned to enhance biodegradability and bioavailability of PEs.

4.3.2.1 *Bacillus* sp. strain 3c3

The four nonionic surfactants, named Tergitol type NP-10, Triton x-100, Tween 80 and Brij 35, at concentration 2 g L^{-1} in minimal salt medium supplemented with 0.1% (w/v) yeast extract, at room temperature (35-37 °C) strongly inhibited the bacterial growth when compared to the condition that without surfactant with cell growth at 0.829. However, Tween 80 had the least toxicity to the bacterial cells with cell growth at 0.592, as shown in Figure 17.

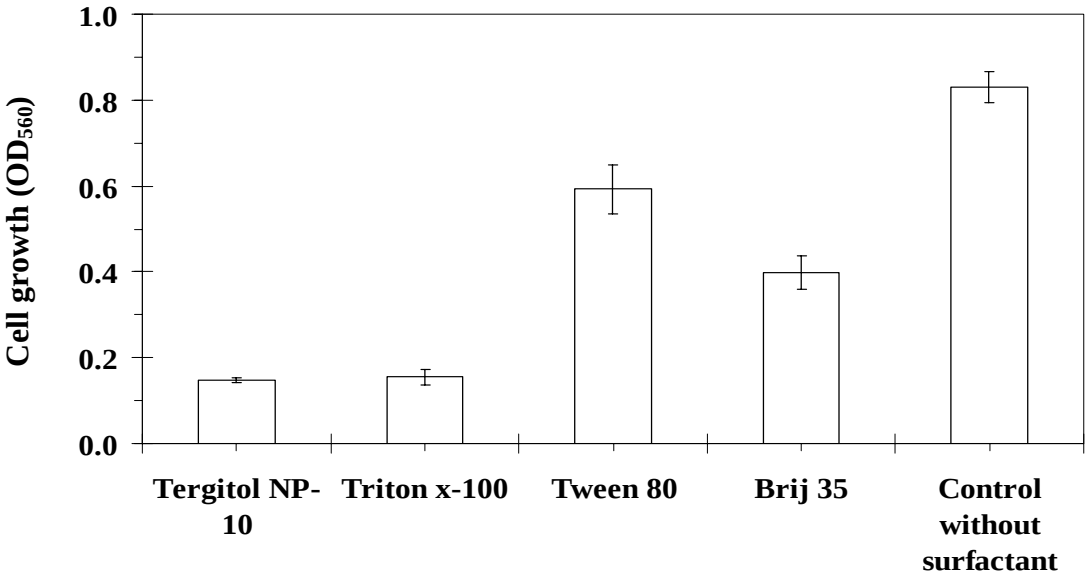


Figure 17 Effect of nonionic surfactants on cell growth of *Bacillus* sp. strain 3c3. The bacterial growth in the absence of surfactant was also examined.

4.3.2.2 *Pseudomonas aeruginosa* strain DB-9

The four nonionic surfactants, named Tergitol type NP-10, Triton x-100 and Brij 35, at concentration 2 g L^{-1} in minimal salt medium supplemented with 0.1% (w/v) yeast extract, at room temperature (35-37 °C) slightly inhibited the bacterial growth when compared to the condition that without surfactant with cell growth at 0.657. Interestingly, Tween 80 at 2 g L^{-1} not only increased bioavailability but also increased bacterial cells. Cell growth in the presence of Tween 80 is higher than the absence of surfactant, as presented in Figure 18.

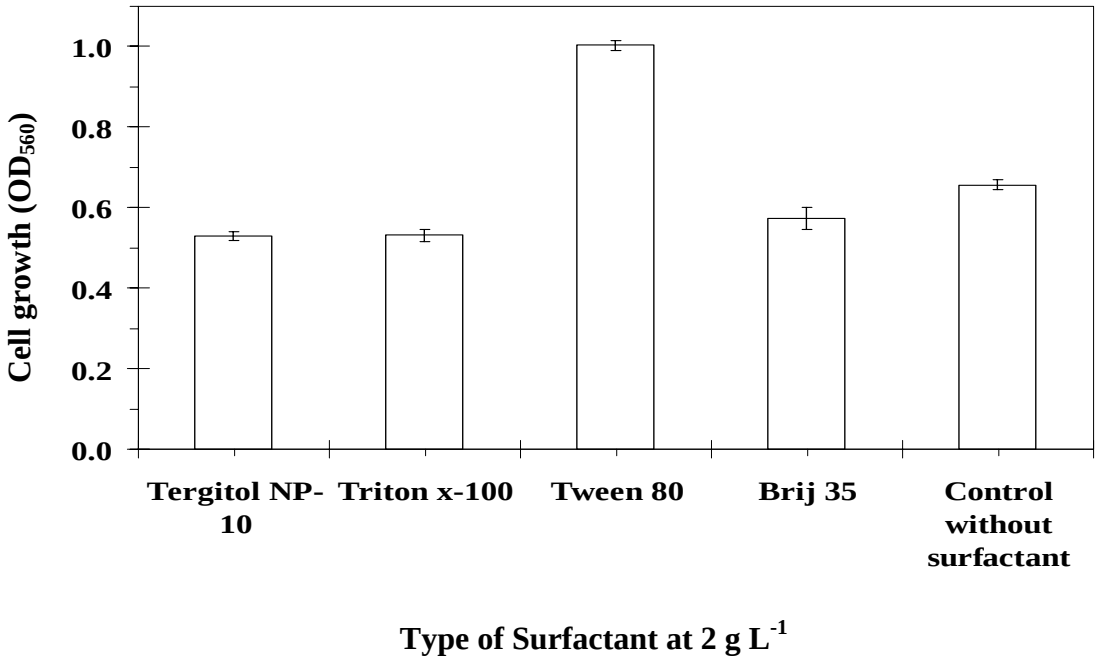


Figure 18 Effect of nonionic surfactants on cell growth of *Pseudomonas aeruginosa* strain DB-9. The bacterial growth in the absence of surfactant was also examined.

Due to low water solubility of PEs, i.e. DPrP, DBP BBP, they can easily adsorb to soil and sediment (Staples *et al*., 1997). Surfactants have been used effectively to increase biodegradability and bioavailability of these hydrophobic pollutants (Chao *et al*., 2006). Thus, the effect of nonionic surfactants on solubility of PEs and bacterial growth was investigated. The nonionic surfactants, namely Tergitol type NP-10, Triton x-100, Tween 80 and Brij 35, were studied with concentration at 0.05 g L^{-1}, 0.10 g L^{-1}, 0.25 g L^{-1}, 0.50 g L^{-1}, 1.0 g L^{-1} and 2.0 g L^{-1}. As the result of this study, solubility of DPrP, DBP and BBP at concentration 100 ppm were increased when concentration of surfactant was increased to 2.0 g L^{-1}. So, the use of surfactant had potential to increase the degradation efficiency of phthalate esters that prevalent contaminated in environment by increasing the total aqueous solubility of these compounds (Edwards *et al*., 1991). In addition, the nonionic surfactants at concentration 2.0 g L^{-1} affected on cells growth of *Bacillus* sp. strain 3c3 and *Pseudomonas aeruginosa* strain DB-9 was examined. According to the result in this study, surfactants increased total aqueous solubility of PEs; however, cell growth of the strain 3c3 was strongly inhibited when surfactant was added. As the previous report, Chao *et al*, (2006) who studied effect of six surfactants (Brij 35, Triton 101, Tergitol type NP-10, Triton x-100, SDS and Brij 30) at 1 CMC on bacterial growth found that the surfactants strongly inhibited growth of three gram positive bacteria (G1, G2 and G11). On the contrary, surfactants had positive effect on cell growth of the strain DB-9. Cell growth of the strain DB-9 in the presence of Tween 80 is higher than the absence of surfactant. This result corresponded to result of Topic 4.2.1.2 that Tween 80 considered as an additional carbon source for increasing bacterial growth and degradation efficiency. Thus, the effect of surfactants was inconsistent with both inhibition and improvement of degradation efficiency and bacterial growth depending on bacterial species (Chen *et al*., 2000; Mulligan *et al*., 2001; Volkering *et al*., 1997).

In conclusion, Tergitol type NP-10 and Triton x-100 were excellent surfactants for increasing solubility of DPrP, DBP and BBP, but they strongly inhibited bacterial growth. Though, Tween 80 increased solubility of these PEs less than Tergitol type NP-10 and Triton x-100, it had the least toxicity to bacterial cell of strain 3c3 and also increased cell growth of strain DB-9. Tween 80 was selected for further study as similar to the previous report that 1.0 mL of Tween 80 was added in the cultured medium as surfactant to maintain a homogeneous system for accurate quantification of the DBP residual (Zhou *et al*., 2009).

4.4 Substrate and concentration range

PEs, i.e. DMP, DEP, DPrP, DBP, BBP, are abundant in the environment (Staples *et al*., 1997). It was interested to investigate ability of the isolated strains to utilize other PEs as their substrate. So far, PEs degradation ability of PEs-degrading bacteria was studied in the concentration range from 5 to 610 ppm (Liang *et al*., 2008). Further investigation was to determine if the bacterial isolate was able to tolerate and degrade PEs at higher concentrations. The substrates including DMP, DEP, DPrP, DBP and BBP were tested at concentration of 50 ppm, 100 ppm, 200 ppm, 500 ppm and 1,000 ppm. For example, DEP degradation at 50 ppm, cells were precultured in mineral medium containing 0.1% (w/v) yeast extract supplemented with 10 ppm DEP. Then, the substrate degradation was studied through resting cell technique as described in Topic 3.5.3.2. Alternatively, Tween 80 at concentration 2 g L^{-1} was added in the cultured medium when the degradation condition had DPrP, DBP and/or BBP as a substrate. Others degradation condition could follow the steps as previous described.

4.4.1 *Bacillus* sp. strain 3c3

The results of degradation efficiency were showed in Figure 19 and summarized in Table 15 and 16.

4.4.2 *Pseudomonas aeruginosa* strain DB-9

The results of degradation efficiency were showed in Figure 20 and summarized in Table 17.

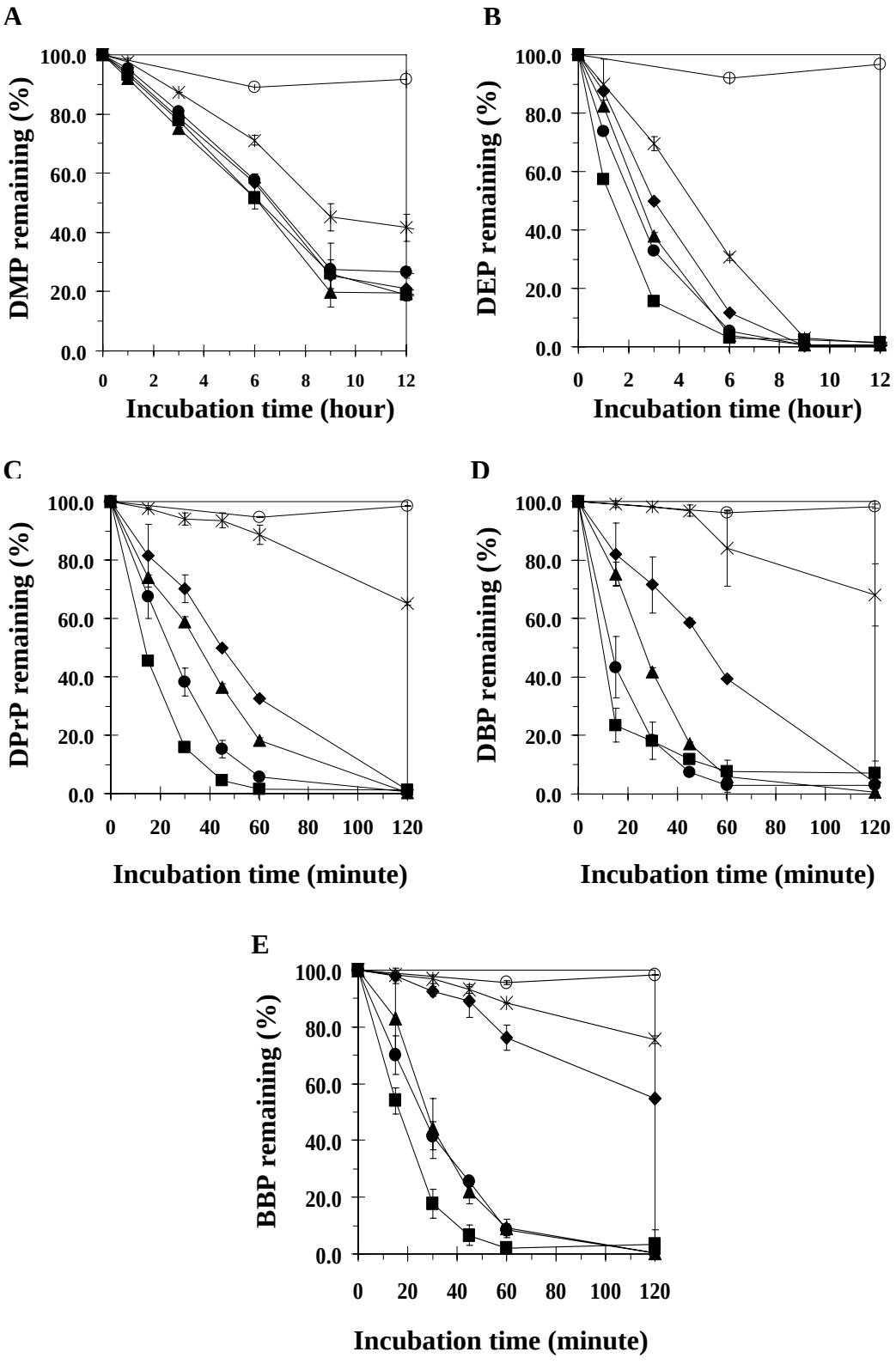


Figure 19 Biodegradation efficiency of the strain 3c3 towards DMP (**A**), DEP (**B**), DPrP (**C**), DBP (**D**) and BBP (**E**) at concentration 50 ppm (■), 100 ppm (●), 200 ppm (▲), 500 ppm (◆) and 1,000 ppm (∗). Abiotic control (○) was also determined.

Table 15 Degradation efficiency of *Bacillus* sp. strain 3c3 towards DMP and DEP at various concentrations

Substrate	**Concentration (ppm)**	**% Degradation (12 hours)**
DMP	50	89±4
	100	87±2
	200	93±1
	500	92±1
	1000	67±5
DEP	50	98±1
	100	99 ±1
	200	100±1
	500	100±1
	1000	100±1

Table 16 Degradation efficiency of *Bacillus* sp. strain 3c3 towards DPrP, DBP and BBP at various concentrations

Substrate	**Concentration (ppm)**	**% Degradation (2 hours)**
DPrP	50	99±1
	100	99±1
	200	100±1
	500	99±1
	1000	35±1
DBP	50	93±4
	100	97±2
	200	99±1
	500	96±1
	1000	32±11
BBP	50	97±5
	100	100±1
	200	100±1
	500	45±1
	1000	25±1

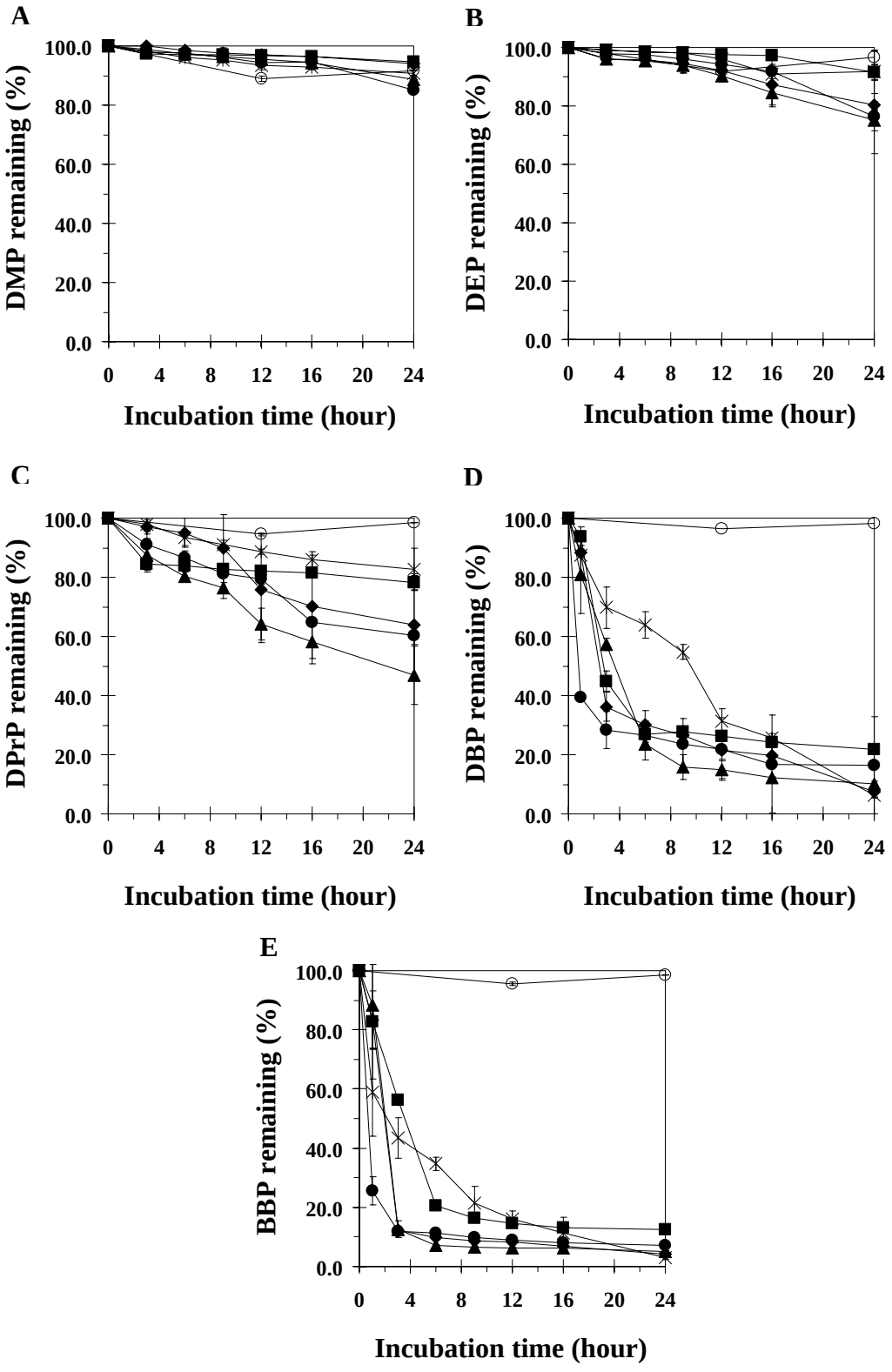


Figure 20 Biodegradation efficiency of the strain DB-9 towards DMP (**A**), DEP (**B**), DPrP (**C**), DBP (**D**) and BBP (**E**) at concentration 50 ppm (■), 100 ppm (●), 200 ppm (▲), 500 ppm (◆) and 1,000 ppm (∗). Abiotic control (○) was also determined.

Table 17 Degradation efficiency of *P. aeruginosa* strain DB-9 towards DMP, DEP, DPrP, DBP and BBP at various concentrations

Substrate range	**Concentration (ppm)**	**% Degradation (24 hours)**
DMP	50	5±1
	100	5±1
	200	11±3
	500	6 ±1
	1000	9 ±1
DEP	50	9±7
	100	24±13
	200	25±4
	500	20±1
	1000	8±3
DPrP	50	22±2
	100	40±3
	200	53±10
	500	36±17
	1000	17±7
DBP	50	78±1
	100	84±16
	200	90±1
	500	93±2
	1000	94±1
BBP	50	87±1
	100	93±1
	200	95±1
	500	96±1
	1000	97±1

PEs, i.e. DMP, DEP, DPrP, DBP, BBP, are abundant in the environment due to extensively use in production of paint, glue, lubricant, cosmetic, insecticide and the production of plastic products, i.e. plastic bottle, plastic bag, food packaging (Staples *et al*., 1997). The previous result showed that the strain 3c3 was capable of degrading DEP and the strain DB-9 was capable of degrading DBP and BBP to some extents. It was interested to investigate their ability to utilize other PEs as their substrate. Until now, PEs degradation ability of PEs-degrading bacteria was studied in the concentration range from 5 to 610 ppm (Staples *et al*., 1997). Therefore, this experiment was to determine if the isolated strains were able to degrade substrate range of PEs at various concentrations. The result found that the strain 3c3 had ability to degrade a wide range of substrates and concentrations as presented in Table 15 and 16. This bacterial strain could degrade DEP concentration at 50 ppm, 100 ppm, 200 ppm, 500 ppm and 1,000 ppm approximately more than 99% within 12 hours of incubation, while DMP was degraded approximately 90% at concentration of 50 ppm, 100 ppm, 200 ppm and 500 ppm and the degradation efficiency was decreased at concentration 1,000 ppm within 12 hours of incubation. Surprisingly, the strain 3c3 was able to almost completely degrade DPrP and DBP at concentration of 50 ppm, 100 ppm, 200 ppm and 500 ppm, while the degradation efficiency at 1,000 ppm was significantly decreased within 2 hours of incubation. BBP concentration at 50 ppm, 100 ppm and 200 ppm was nearly 99% degraded, and then degradation efficiency was subsequently decreased in 2 hours of incubation when concentration was increased to 500 ppm and 1,000 ppm. Liang *et al*. (2008) had demonstrated that PEs with shorter ester chains like DMP, DEP, DPrP, DBP and BBP could be readily biodegraded and mineralized (Liang *et al*., 2008). However, DMP, DPrP, DBP and BBP at high concentration, i.e. 1,000 ppm, adversely affected the degradation efficiency. Similar result was reported by Chang *et al*, (2004) that at higher PEs

concentration, degradation efficiency was decreased and level of toxicity to bacterial cells was increased. Nevertheless, the strain 3c3 exhibited greater degradation ability than *Corynebacterium* sp.O18 which is gram positive bacteria that could completely degrade DEP, DPrP, DBP and BBP at concentration 5 ppm within 2, 2, 4 and 3 days, respectively (Chang *et al*., 2004) and *Arthrobacter* sp. was another gram positive bacterium that could completely degrade DMP at concentration 52.4 ppm in 20 hours of incubation (Vega & Bastide, 2003).

In this study, *Pseudomonas aeruginosa* strain DB-9 exhibited excellent DBP and BBP degradation efficiency as presented in Table 17. When DBP concentration was increased form 50 ppm to 1,000 ppm, degradation efficiency was increased approximately from 78% to 94%. Similar to its BBP degradation that when BBP concentration was increased from 50 ppm to 1000 ppm, degradation efficiency was increased approximately from 87% to 97%. This bacterial strain could effectively degrade DBP and BBP at high concentration, i.e. 1000 ppm. However, this bacterial strain could slightly degrade DMP, DEP and DPrP with 11±3%, 25±4% and 53±10% maximum degradation efficiency at concentration 200 ppm in 2 hours of incubation, respectively and then degradation efficiency was decreased at higher concentration, i.e. 500 ppm, 1,000 ppm. This result was different from Zeng *et al*, (2004) that biodegradation efficiency of *Pseudomonas fluoresences* FS1 was decreased when the alkyl chain length of PEs increased (Zeng *et al*., 2004). Liang *et al*, (2008) stated that different phthalate isomers showed different biodegradation efficiency and phthalate-hydrolyzing enzymes were structurally specific (Liang *et al*., 2008).

4.5 Substrate interaction

The effect each PEs in mixed conditions was examined through resting cell technique as described in topic 3.5.3.2.

4.5.1 *Bacillus* sp. strain 3c3

The PEs, named DMP, DEP, DPrP, DBP and BBP, were combined in three categories (binary, ternary and quaternary) with concentration of each substrate at 100 ppm and in quaternary with concentration of each substrate at 200 ppm. The result of degradation efficiency was concluded in Table 18 and 19.

4.5.2 *Pseudomonas aeruginosa* strain DB-9

The five phthalate esters, named DMP, DEP, DPrP, DBP and BBP, were combined in two categories (binary and ternary) with concentration of each substrate at 100 ppm. The result of degradation efficiency was summarized in Table 20.

Table 18 Degradation efficiency of the strain 3c3 towards DEP, DPrP, DBP and BBP at concentration 100 ppm with individual and mixed substrate conditions

Combination	**% Degradation (2 hours)**			
	DEP	**DPrP**	**DBP**	**BBP**
Single				
DEP	15±4			
DPrP		100±1		
DBP			97±2	
BBP				100±1
Binary				
DEP+DPrP	16±1	99±1		
DEP+DBP	18±3		100±1	
DEP+BBP	10±2			99±1
DPrP+DBP		99±1	99±1	
DPrP+BBP		98±1		100±1
DBP+BBP			100±1	100±1
Ternary				
DEP+DPrP+DBP	13±2	97±1	100±1	
DEP+DPrP+BBP	15±1	96±1		100±1
DEP+DBP+BBP	7±3		99±1	98±1
DPrP+DBP+BBP		97±1	100±1	100±1
Quaternary				
DEP+DPrP+DBP+BBP	14±3	97±1	98±1	100±1

Table 19 Degradation efficiency of the strain 3c3 towards DEP, DPrP, DBP and BBP at concentration 200 ppm with individual and mixed substrate conditions

Combination	**% Degradation (2 hours)**			
	DEP	**DPrP**	**DBP**	**BBP**
Single				
DEP	17±1			
DPrP		100±1		
DBP			99±1	
BBP				100±1
Quaternary				
DEP+DPrP+DBP+BBP	6±9	57±3	85±1	62±2

Table 20 Degradation efficiency of the strain DB-9 towards DMP, DEP, DPrP, DBP and BBP at concentration 100 ppm with individual and mixed substrate conditions

Combination	% Degradation (24 hours)				
	DMP	**DEP**	**DPrP**	**DBP**	**BBP**
Single					
DMP	5±1				
DEP		24±13			
DPrP			40±3		
DBP				84±16	
BBP					93±1
Binary					
DBP+DMP	5±1			89±1	
DBP+DEP		13±1		88±4	
DBP+DPrP			72±3	95±1	
BBP+DMP	5±2				96±1
BBP+DEP		14±7			95±1
BBP+DPrP			60±1		96±1
DBP+BBP				96±3	94±3
Ternary					
DBP+BBP+DMP	5±3			93±1	93±1
DBP+BBP+DEP		16±2		92±4	94±2
DBP+BBP+DPrP			61±1	90±1	93±1

PEs are extensively used in the production of paint, glue, lubricant, cosmetic, insecticide and the production of plastic products, i.e. plastic bottle, plastic bag, food packaging, through, they may leak from product use and/or disposal or leaching out of landfill into soil ,water and/or groundwater and became as prevalent environmental contaminations, usually in mixtures (Staples *et al*., 1997). Therefore, it was interested to examine whether *Bacillus* sp. strain 3c3 and *P. aeruginosa* strain DB-9 could utilize PEs, i.e. DMP, DEP, DPrP, DBP, BBP, in mixture as well as individually. This experiment was observed interaction during phthalate esters by conducting into four categories (single, binary, ternary, and quaternary). The result of *Bacillus* sp. strain 3c3 at initial concentration 100 ppm of each phthalate ester found that the degradation efficiency of the phthalate esters in mixture was similar to the phthalate esters individually as presented in Table 18. However, total degradation efficiency was decreased in mixture of quaternary when initial concentration of each phthalate was increased to 200 ppm as shown in Table 19. This result was different from the report of Chang *et al*. (2004) that the presence of all phthalate esters could enhance degradation efficiency by providing more carbon and energy sources for the bacteria.

The substrate interaction of *P. aeruginosa* strain DB-9 was investigated in three categories (single, binary and ternary) with initial concentration of each phthalate at 100 ppm as presented in Table 20. The result found that degradation efficiency of DMP, DBP and BBP in mixture was similar to individual, while degradation efficiency of DEP in binary and ternary mixture of DBP and BBP was less than individual. So, DBP and BBP had inhibitory effect on DEP degradation. On the contrary, total degradation efficiency of DPrP was enhanced in the binary and ternary mixture of DBP and BBP. The similar result was obtained by Chang *et al*. (2004).

CHAPTER V

CONCLUSIONS

The two newly discovered strains of PEs-degrading bacteria were isolated. First, *Bacillus* sp. strain 3c3 was Gram positive bacteria isolated from hot spring soil in Krabi province, Thailand by organic solvent enrichment. It could degrade DEP with total degradation of 68±5% when cultured in minimal salt medium containing 100 ppm (0.45 mM) DEP. Moreover, the addition of carbon and energy source which increased DEP degradation by 32%, 37%, 16%, 35%, 38% and 40% were 4mM citrate, 4mM glucose, 4mM succinate, 0.1% (w/v) peptone, 0.1% (w/v) tryptone and 0.1% (w/v) yeast extract, respectively. Addition of yeast extract showed the maximum increasing of total DEP degradation. The optimal conditions for PEs degradation of strain 3c3 were at room temperature (35-37 °C) and pH 7.0. Interestingly, the isolated strain could effectively degrade DMP, DEP, DPrP, DBP and BBP at high concentration, i.e. up to 1,000 ppm. Thus, this bacterium is newly discovered strain exhibited ability to completely degrade PEs with higher concentrations than other Gram positive PEs-degrading bacteria as shown in Table 2. Moreover, this strain also showed a great ability to degrade PEs in mixed substrate conditions which are prevalently found in activated sludge, wastewater and leachate.

Second, *Pseudomonas aeruginosa* strain DB-9 was Gram negative bacteria isolated from soil with a history municipal waste exposure. It could degrade BBP with the percentage of total degradation of 86±1% when cultured in minimal salt medium with 2 g L^{-1} Tween 80 containing 100 ppm BBP. In the presence of 100 ppm BBP and 2 g L^{-1} Tween 80 in minimal salt medium, Tween 80 was not only a solubilizing agent but also

considered as a co-substrate which increased the bacterial growth rate, while BBP was utilized as a substrate which caused partial toxicity to the bacterial cells. The additions of yeast extract in the presence of 100 ppm BBP and 2 g L^{-1} Tween 80 increased growth rate by 25% and increased degradation efficiency by 2%. The optimal conditions for PEs degradation of strain DB-9 were at room temperature (35-37 °C) and pH 7.0. Interestingly, the isolated strain could completely degrade DBP and BBP, while it slightly degraded DMP, DEP and DPrP to different extents. Though, its PEs biodegradability was greater than other Gram negative bacteria as presented in Table 2. In addition, this strain was able to degrade PEs under various mixed substrate conditions.

The investigation of nutrient supplementation, pH, temperature and surfactants provided the preliminary information for each bacterial isolate, which may be useful for further bioremediation application of PEs-contaminated sited.

CHAPTER VI

SUGGESTIONS AND FUTURE WORKS

Since it was stated that PEs tend to be concentrated in sewage sludge from the precipitation of wastewater in WWTP and the sludge is subsequently used as a soil amendment in agriculture , therefore it could be directly harmful to human and living organisms. Bioaugmentation of a comparatively high density of bacterial cells capable of PEs biodegradation as a bacterial seeding into soil and sludge is one of the efficient alternatives to clean up the PEs toxicity. In this research, the two newly bacterial strains were isolated and characterized the ability to biodegrade PEs as well as investigated the factors affecting the PEs biodegradation in order to further apply the bacteria for cleaning up PEs contaminated and accumulated in soil and sewage sludge.

The addition of nutrients and surfactants used in this study affected the degradation of PEs depending on type of microorganism. Other conditions should be further study to improve degradation efficiency.

As a consequence, the two isolated bacteria capable of PEs biodegradation obtained from this study will provide an alternative as a bacterial seeding to develop a bioaugmentation system for the complete PEs biodegradation in wastewater, sludge and soil. The achievement of this study will be beneficial to a bioremediation of PEs-contaminated sites, human health and environmental quality.

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APPENDICES

APPENDIX A

Gram staining

A.1 Dye and chemical solution for bacterial staining

Gram stain is staining technique that widely used in bacteriology developed in 1884 by Hans Christain Gram. At differential stain procedures, bacteria will give different results depending on their cell wall chemistry. The gram stain divides bacteria into two groups based on their reaction to the stain. After the last step, gram positive bacteria will be purple and gram negative will be red. The size, shape, and arrangement of the organisms can also be determined from a stained specimen using microscope.

1. **Gram's Crystal violet**

Solution A:	Crystal violet	2	g
	Ethyl alcohol	20	g
	Distilled water	100	ml
Solution B:	Ammonium oxalate	0.8	ml
	Distilled water	100	ml

Solution A and solution B will be mixed and filtrated in order to remove dregs before using.

2. **Gram's Iodine**

Iodine	1	g
Potassium iodide (KI)	2	g
Distilled water	300	ml

KI will be dissolved in distilled water and added iodine before using.

3. **Gram's Alcohol**

Ethyl alcohol	98	ml
Acetone	2	ml

All solutions will be mixed before using.

4. **Gram's Safranin**

Safranin O (2.5% solution in 95% ethyl alcohol)	10	ml
Distilled water	100	ml

All solutions will be mixed before using.

A.2 Gram staining procedures

The four steps of the gram stain can be summarized as followed:

1. **Primary stain**: Cover the smear with crystal violet for thirty seconds. All bacteria will take up this dye and appear purple. Rinse off the excess dye with distilled water.
2. **Mordant**: Gram iodine will be added on the smear to interact with crystal violet for thirty seconds. This complex will be difficult to remove from certain bacteria during the next step. The excess iodine will be rinsed off by distilled water.
3. **Decolorization**: 95% ethanol will be shortly applied (10-20 seconds) to the smear. Then, ethanol will be rinsed off by distilled water.
4. **Counterstain**: Safranin will be added for 20 seconds in order to dye any decolorized cells. It will not change the color of the cells that retain the crystal violet.

APPENDIX B

16s rDNA sequence

The following information is the sequence fragment of 16s rDNA sequencing of *Bacillus* sp. strain 3c3 (Figure B1) and *Pseudomona*s aeruginosa strain DB-9 (Figure B2) with forward primer (63f) and reverse primer (1387r).

A

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> 3C3_F 16s RNA sequence
ATGGGAGCTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCT
GTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCAT
GGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGC
TAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATC
GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTT
CCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCG
TAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTA
CCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTANGTGGCAA
GCGTTGTCCGGAATNATNGGGCGTAAAGGGCTCGCAGGCGGTNTCTTAAGTCTGATGTGA
AAGCCCCCGGCTCAACCGGGGAGGGTCATNGGAAACTGGGGANCTTGAGTGCAGAAGANG
AGAGTGGANTCCACGTGTAGCNGTGAAATGCGTAGAGATGTGAGGANANCAGTGCGAGGC
GACTCC
//


> 3C3_R 16s RNA sequence
CCCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGA
CTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCC
TTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGAC
GTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATG
CTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACAC
GAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCT
AGGATTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATNAAACCA
CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGT
ACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAANCCNCCTAA
CACTTAGCACTCATCGTNTACNGCGTGGACTACCAGGGTATCTATTNCTGTNCGCTNNCA
CGCTNTCGCTCNCAGCGTCAGTTACAGANCAGAGAGTCGCCTCGNCACNNGGTGNTTCCT
CNCATNTCTAC
//
```

Figure B1 Partial 16s rDNA sequence of *Bacillus* sp. strain 3c3

B

>#9_R 16S (625 bps)
TTCTGATTCACGATTACTAGCTATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCC
GGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTACCG
ACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCC
ACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCCGAGGTGCTGGTAAC
TAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCTCACGACACGAGCTGAC
GACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTT
CTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCC
ACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCA
GGCGGTCGACTTATCGCGTTAGCTGCGCCACTAAGATCTCAAGGATCCCAACGGCTAGTC
GACATCGTNTACGGCGTGGACTANCAGGGTATCTANTNCTGTTNGCTCCCACGCNTTCGC
ACNCAGTGTCAGTATCAGTCAGGTG
//

>#9_F 16S (576 bps)
GCTGCTCCTAGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTG
GGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGG
ATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAA
GGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTG
AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAG
CCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTT
GGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACC
GGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTAC
TGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACC
TGGGAACTGCATCCAAAACTACTGAGCTAGAGTACG
//

Figure B2 Partial 16s rDNA sequence of *Pseudomona*s *aeruginosa* strain DB-9

APPENDIX C

PEs standard curve

Standard curve was used to analyze chromatogram of HPLC result in order to calculate the concentration of substance. The PEs standard was freshly prepared in acetonitrile as a stock solution at 1000 ppm. Then, PEs at concentration of 15.625 ppm, 31.25 ppm, 62.50 ppm, 125 ppm, 250 ppm and 500 ppm was obtained by doing a serial dilution from PEs stock solution. The prepared standard solutions were then filtered through 0.45 µm nylon filter. The separation was performed by using the same method as described in topic 3.6.3.2. The standard curve of DMP, DEP, DPrP, DBP and BBP was showed in Figure C1, C2, C3, C4 and C5, respectively.

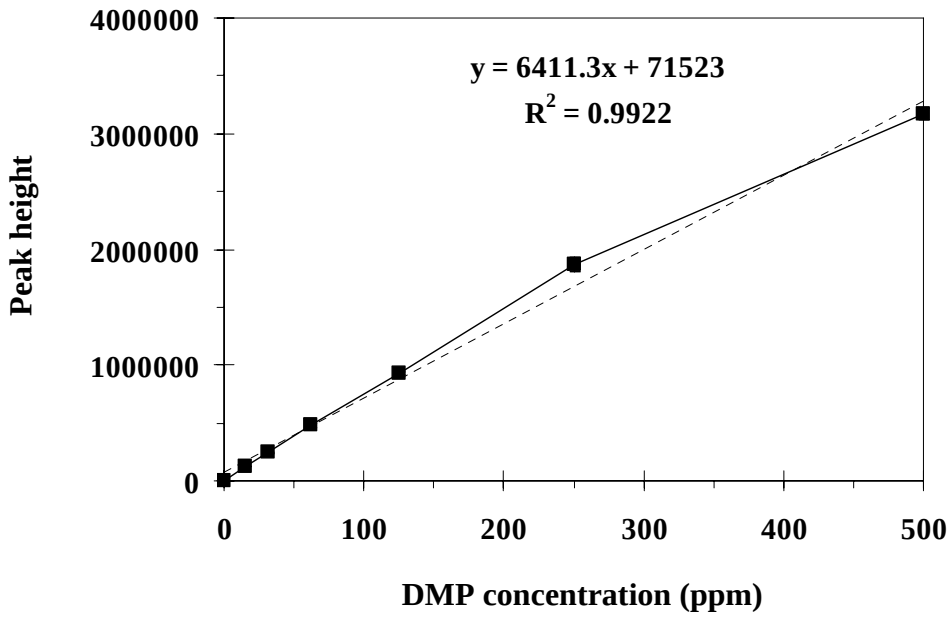

Figure C1 DMP standard curve for calculation of DMP concentration

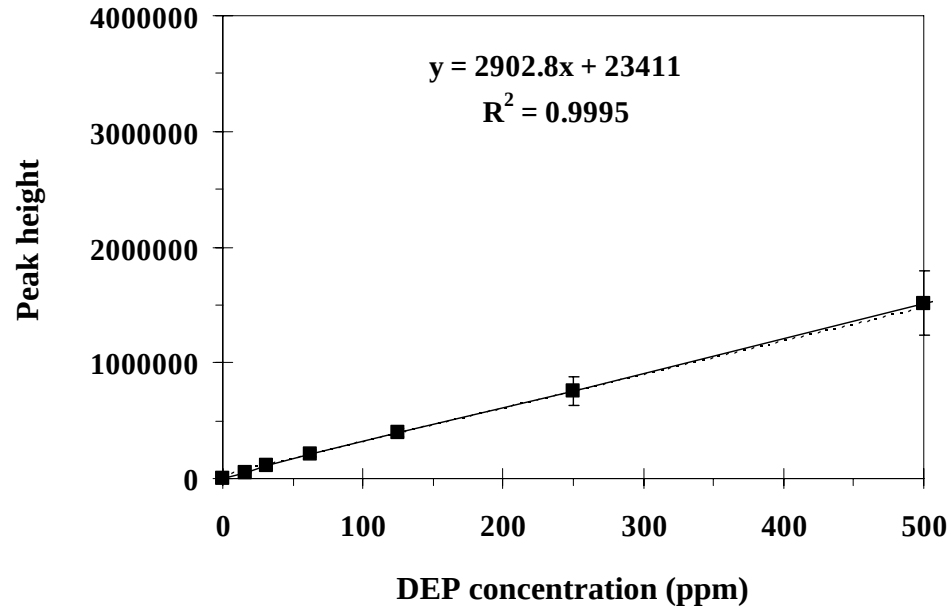

Figure C2 DEP standard curve for calculation of DEP concentration

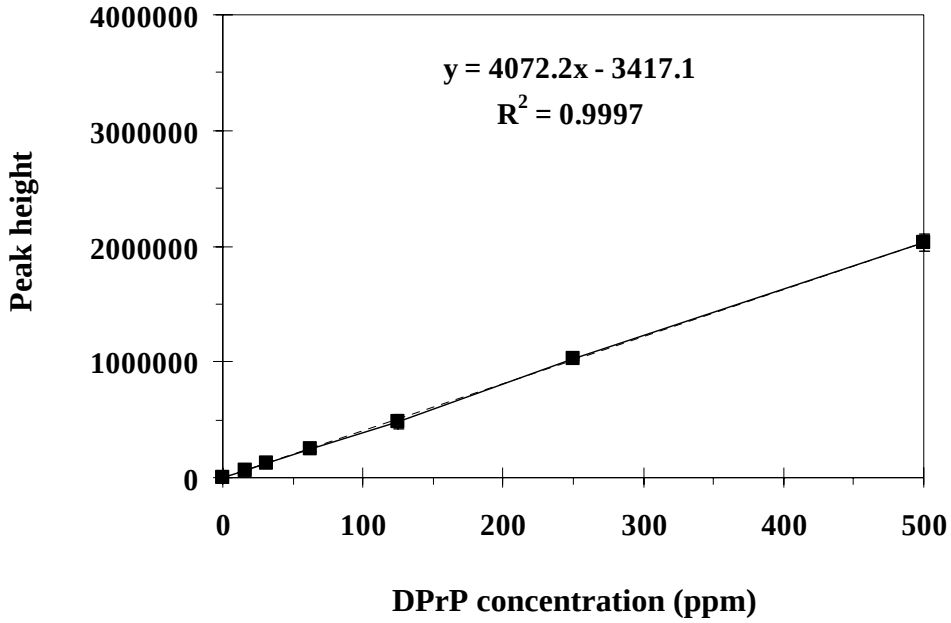

Figure C3 DPrP standard curve for calculation of DPrP concentration

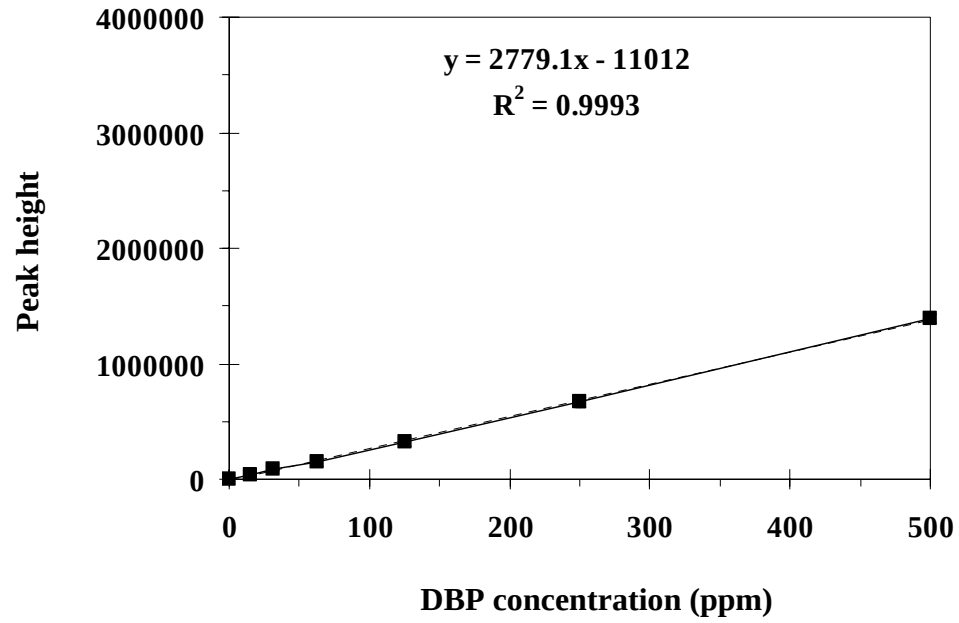

Figure C4 DBP standard curve for calculation of DBP concentration

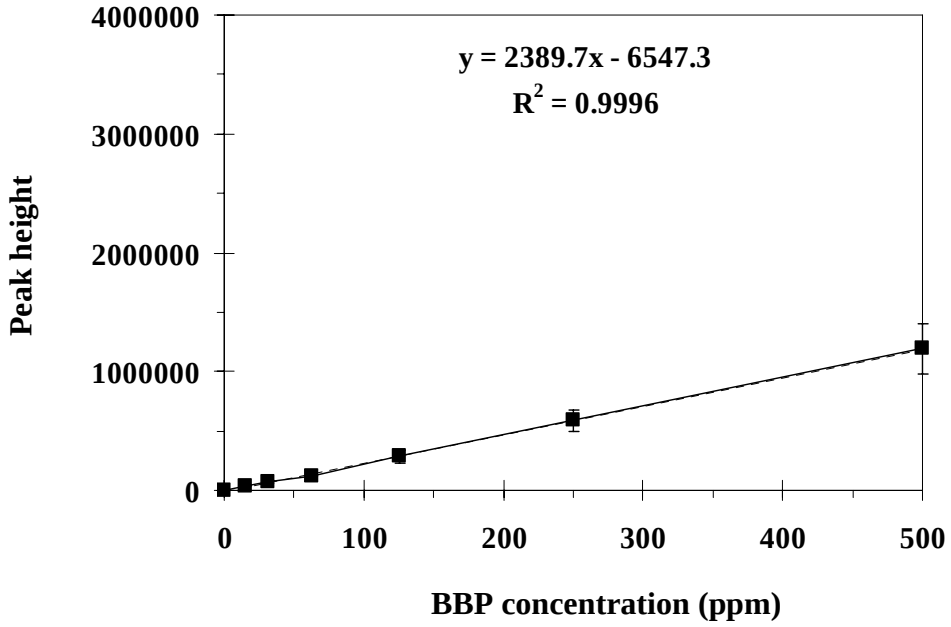

Figure C5 BBP standard curve for calculation of BBP concentration

APPENDIX D

Calculation of PEs residual

Determination PEs remaining and PEs degradation

Percentage of remaining PEs was calculated from HPLC peak height when compared to that of standard curve (APPENDIX C). The following equations were used to calculate the percentage of PEs degradation. For example:

A. Percentage of PEs remaining after 24 hours of incubation was

$$100 \text{ x } \frac{\text{Peak height reported at 24 hours}}{\text{Peak height reported at 0 hours}}$$

B. Percentage of PEs degradation after 24 hours of incubation was

$$100\text{-}100 \text{ x } \frac{\text{Peak height reported at 24 hours}}{\text{Peak height reported at 0 hours}}$$

APPENDIX E

Retention time of PEs

PEs was analyzed using reverse phase HPLC equipped with a UV detector (at 226 nm). The separation was performed at 28.5 °C on a C18 column using an acetonitrile: water mixture (70:30, v/v) as a mobile phase at a flow rate of 1 ml/min. The injection volume was 20 µl. The retention time of DMP, DEP, DPrP, DBP and BBP under the condition tested were 3.51, 4.38, 6.35, 10.42 and 9.23 minute, respectively as shown in Figure E1-E5.

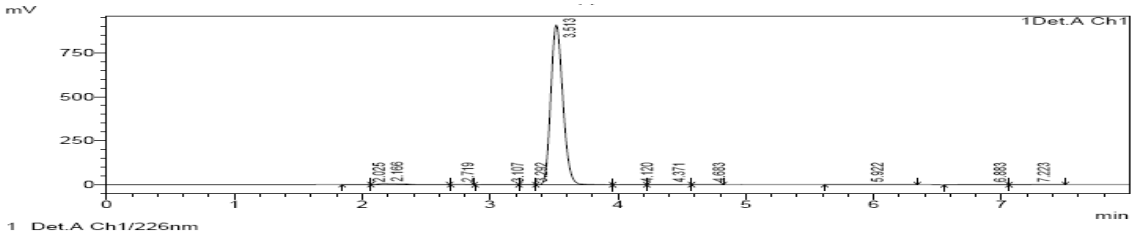

Figure E1 HPLC chromatogram of DMP at concentration 250 ppm. Theretaention time of DMP was 3.51.

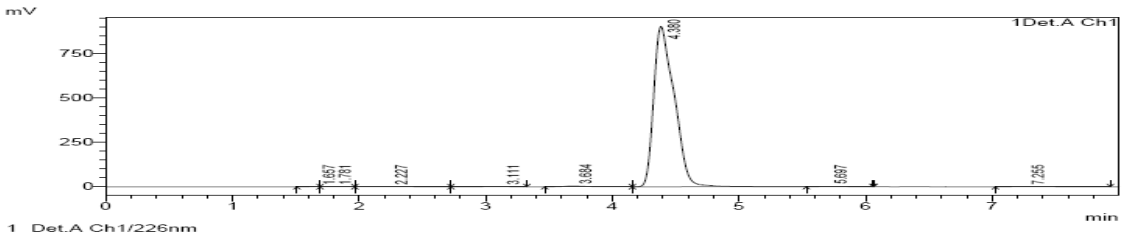

Figure E2 HPLC chromatogram of DEP at concentration 250 ppm. The retention time of DEP was 4.38.

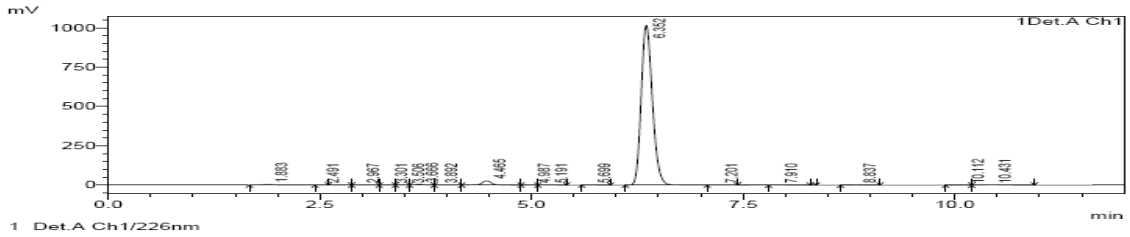

Figure E3 HPLC chromatogram of DPrP at concentration 250 ppm. The retention time of DPrP was 6.35.

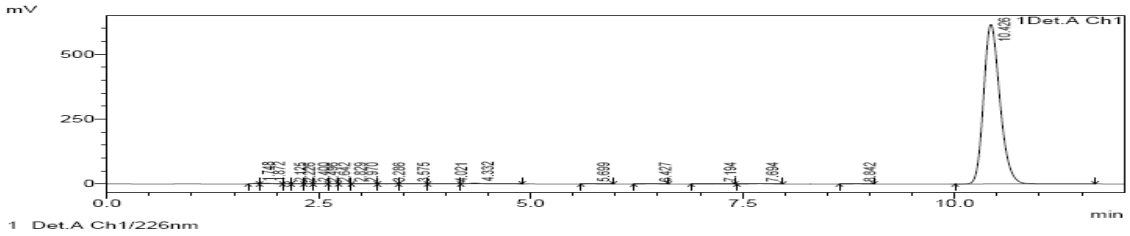

Figure E4 HPLC chromatogram of DBP at concentration 250 ppm. The retention time of DBP was 10.42.

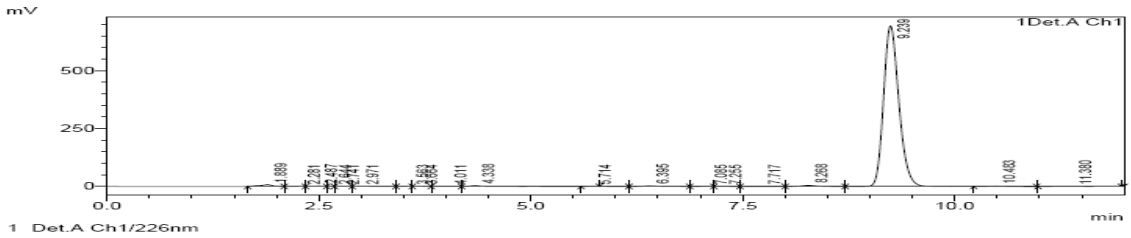

Figure E5 HPLC chromatogram of BBP at concentration 250 ppm. The retention time of BBP was 9.23.

APPENDIX F

Protein calibration curve

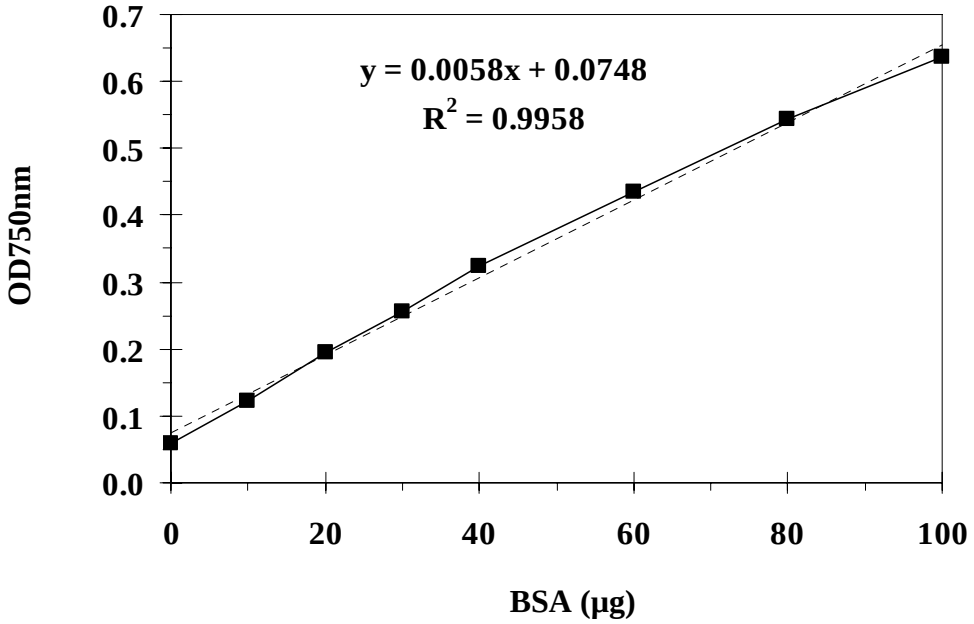

Figure F1 Standard curve of modified Lowry method used to determine cell protein.

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