ENZYMATIC DEGRADATION OF PHTHALIC ACID ESTERS

**A Thesis Submitted to the
Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirement for the Degree of**

DOCTOR OF PHILOSOPHY

in Chemical Engineering

**by
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**August, 2013
İZMİR**

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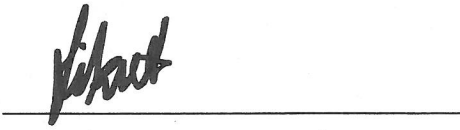**Examining Commitee Members:**

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ACKNOWLEDGEMENTS

ABSTRACT

ENZYMATIC DEGRADATION OF PHTHALIC ACID ESTERS

Endocrine disrupting compounds (EDCs) are environmental pollutants which interfere with the hormone system even at low concentrations resulting in adverse health effects on both human and wildlife. In this study, we aimed to investigate enzymatic degradation of diethylhexyl phthalate (DEHP) and diethyl phthalate (DEP) using both commercial porcine pancreas lipase and lipase from recombinant E.*Coli* strains that contain lipase genes from thermophilic *Bacillus sp*. isolated from Balçova Geotermal region in İzmir.

Incubation of 20 mg/L DEHP with 20,000 U/L PPL enzyme for 7 days resulted in formation of monoethyl phthalate (MEHP), phthalic acid (PA), and dimethyl phthalate (DMP) which are the possible metabolites of DEHP. The percent decrease in DEHP (20 mg/L) was found to be 92% compared to positive control samples. In the case of DEP, about 53% decrease was obtained after incubation with 20.000 U/L for 7 days. Hydrolysis constants for DEHP ranged between 0.13 and 0.22 d^{-1}, whereas those for DEP ranged 0.43 and 0.54 d^{-1}.

As a result of enzymatic hydrolysis of DEHP (1-20 mg/L) with 4000 U PPL enzyme, DEP was produced as hydrolysis product of DEHP after 44 h. In the case of DEP (1-20 mg/L) incubated with 4000 U crude lipase solution for 140 h, DMP was obtained as a possible product of transesterification reaction. The maximum rate (V_{max}) of enzymatic hydrolysis reaction for DEHP and DEP was calculated as 0.79 mg/L.h and 1.83 mg/L.h, respectively. The Michealis-Menten constants (K_m) for enzymatic hydrolysis of DEHP and DEP were calculated as 2.45 and 2.12 mg/L, respectively.

ÖZET

FİTALİK ASİT ESTERLERİNİN ENZİMATİK PARÇALANMASI

Endokrin sistemini bozucu kimyasallar düşük derişimlerde bile hormone sistemi ile girişim yaparak insanlarda ve hayvanlarda olumsuz sağlık etkilerine yol açmaktadırlar. Bu çalışmanın amacı, ticari domuz pankreası kaynaklı lipaz (PPL) ve Balçova jeotermal bölgesinden izole edilmiş termofilik *Bacillus* türüne air lipaz genini içeren recombinant *E. Coli* bakterisinden saflaştırılmış lipaz enzimlerini kullanarak dietilhekzil fitalat (DEHP) ve dietil fitalatın (DEP) enzimatik parçalanmasını araştırmaktır.

20 mg/L DEHP'ın 20,000 U/L PPL enzimi ile 7 gün süresince inkübe edilmesi sonucunda DEHP'ın parçalanma ürünleri olan monoetilfitalat, fitalik asit, ve dimetil fitalat oluşmuştur. Pozitif kontrol örnekleriyle karşılaştırıldığında DEHP'ın azalması yaklaşık %92 oranında olduğu belirlenmiştir. DEP için ise azalmanın 7 gün sonrasında yaklaşık %53 oranında olduğu görülmüştür. DEHP için hidroliz sabitinin 0.13 ve 0.22 gün^{-1}, DEP için ise 0.43 ve 0.54 gün^{-1} aralığında olduğu bulunmuştur. DEHP'ın (1-20 mg/L) 4000 U PPL enzimi ile 44 saatlik ile hidrolizi sonucunda DEP'ın hidroliz ürünü olarak oluştuğu görülmüştür. DEP (1-20 mg/L) ise 4000 U lipase ile inkübasyonu sonucunda transesterifikasyon reaksiyonu ürünü olarak DMP'ın oluştuğu görülmüştür. DEHP ve DEP için maksimum reaksiyon hızları sırasıyla, 0.79 ve 1.83 mg/L.saat olarak hesaplanmıştır. Michaelis menten denklemi sabiti (K_m) ise sırasıyla 2.45 ve 2.12 mg/L olarak bulunmuştur.

This dissertation is lovingly dedicated to my sister,
Bahar AKCAN. *Her support, encouragement, and constant love have sustained me throughout my life.*

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CHAPTER I

INTRODUCTION

The major function of the endocrine system is to maintain and control internal balance (homeostasis) of body systems in order to prevent functional disorders. This system controls the pivotal functions in human and animal body such as reproduction, blood pressure, and general metabolism, muscle or nervous system functions, and maintains normal levels of glucose or ions in the blood (Lintelmann et al. 2003).

In recent years, numerous studies have reported that some substances in the environment may interfere with the normal function of any hormone in the humans and wildlife (Depledge and Billinghurst, 1999; Fox 2005, Diamanti-Kandarakis *et al*., 2009). These substances are called as endocrine disrupting compounds (EDC) that are defined as "exogenous compounds that interfere with the synthesis, secretion, transport, binding action, or elimination of hormones in the body which are responsible for the maintenance or homeostasis, reproduction, development, and behavior" by the US Environmental Protection Agency (USEPA, 1998). Common EDC include some synthetic compounds such as pesticides, polychlorinated biphenyls (PCBs), phthalic acid esters (PAE), aromatic hydrocarbons, heavy metals, phenols and natural hormones. Removal of these compounds from the environment is important since they can indicate endocrine disrupting activity even at low concentrations

PAE are the dialkyl or alkyl aryl esters of 1,2-benzendicarboxylic acid (phthalic acid). The name phthalate derives from phthalic acid, which refers to three isomers, i.e., the ortho-isomer or phthalic acid (PA), para-isomer or terephthalic acid (TA), and metaisomer isophthalic acid (IA). Some common PAE are di (2-ethylhexyl) phthalate (DEHP), dietyl phtalate (DEP), di-*n*-butyl phthalate (DBP), and di-*n*-octylphthalate (DOP). These chemicals have been used commonly as plasticizers in plastic products like polyvinil chloride (PVC) resins, adhesives, and cellulose film coatings, flooring and wall covering, medical devices (e.g., tubes and blood bags), and furniture (Hashızume et al. 2002; Rank, 2005). It is known that when they are used as plasticizers, phthalates are not chemically bonded to the plastics polymer and therefore, eventually can migrate from the plastics into the environment (Liang et al. 2008).

PVC formulated with DEHP contain no covalent bond since DEHP exists as free molecules between the polymer fibers and therefore DEHP molecules can easily leave the plastic and migrate to surrounding environment (Rank, 2005). Because of their known adverse health effects such as developmental and reproductive disorders (Saillenfait et al., 2009) and changes in hormonal system (Svan and Davis, 2003), and their affinity to solid organic phase, biodegradation studies were concentrated on PAE removal from soil or waste sludge. Different bacterial strains were isolated and identified for biodegradation of DEHP which is the most persistent and the commonly used plasticizer. Some of these bacterial strains were *Bacillus Subtilis* (Quan et al. 2005), *Mycobacterium sp.* (Nakamiya et al. 2005), *Pseudomonas Fluoresence* (Zeng et al. 2002), *Sphigomonas sp.* (Chang et al. 2004), and *Acinetobacter lwoffii* (Hashizume et al. 2002). Biodegradation studies on PAE not only include bacterial degradation, but also there were enzymatic degradation studies in which either commercial or recombinant enzymes were used for PAE removal. Enzymes used in these studies include commercial porcine pancreas lipase (PPL) for DEHP degradation (Gavala et al. 2004), cutinase from *F. oxyprorum* for BBP degradation (Kim et al. 2002) and commercial *Candida cylidracea* lipase for DBP degradation (Tanaka et al. 2000).

Degradation of PAEs consists of different type of pathways, including de-esterification or dealkylation, β-oxidation and trans-esterification. De-esterification is the most common reaction in which phthalic diesters are serially converted to phtalic monoesters and phthalic acid (Shelton et al. 1984; Eaton and Ribbons 1982). β-oxidation reaction converts phthalates with longer side chains than DEP to those with shorter chains by removing ethyl group each time (Amir et al. 2005). Then, DEP is further converted to PA by two pathways, de-esterification and an alternative trans-esterification pathway.

DEP can be degraded by replacing an ethyl group with a methyl group in each step, producing ethyl-methyl phthalate and DMP, the process of which is termed as trans-esterification (demethylation) (Cartwright et al. 2000). In literature, degradation pathway for metabolism of phthalate diester (PDE) is proposed as hydrolysis of two ester bonds; first giving the monoester and free alcohol, followed by the second ester bond to give PA and alcohol (Feng et al. 2002; Saito et al. 2010).

Studies on enzymatic degradation of PAEs have reported that these compunds could be hydrolysed with lipase or esterase enzymes from various sources including bacteria (Albro and Latimer 1974, Kurane et al. 1980, Soontornchat et al. 1994), pancreatic lipase (Saito et al. 2010) mammalian enzymes, such as nonspecific lipase from rat pancreas (Duran and Esposito 2000, Sutherland et al. 2004, Chang et al. 2007), esterase from rat intestine (Soontornchat et al. 1994), carboxyl esterase from rat and human (Albro and Latimer 1974), and human salivary esterase. All these enzymes catalyze the hydrolysis of ester bonds in PAEs resulting in formation of corresponding monoesters and alcohols (Liang et al. 2008). Pancreatic lipase (EC 3.1.1.3) which is effective on ester hydrolysis reaction of ester bonds generally acts on water-insoluble triglycerides (Garner and Smith, 1972). Kurane et al. (1979) reported that DEHP could be hydrolyzed with various lipase and esterase by enzymatic hydrolysis reaction. After 18 h incubation of 2000 ppm DEHP with 1 mg/ml steapsin (pancreas) and esterase from pig liver resulted in 24% and 16.1% degradation of DEHP, respectively. They also reported that DEHP which is a slightly soluble PAE was degraded to free and water soluble phthalic acid by metabolism of *Nocardia Erythropolis.* Kurane et al. (1984) also analyzed the intermediates produced from DEHP hydrolysis by a purified phthalate ester hydrolase from *Nocardia erythropolis.* They found only PA and no MEHP and concluded that "the purified enzyme rapidly converts phthalate diesters into phthalic acid without phthalate monoesters accumulating. This implies that the hydrolysis is accomplished by the same enzyme, and that hydrolysis of the monoester is faster than of the diester. The biodegradability difference of phthalates is likely due to the steric effect of phthalates side ester chains which hinders the hydrolytic enzymes from binding to the phthalates and thereby inhibits their hydrolysis. This has been supported by Xia et al. (2004) in a study of quantitative structure-activity relationship analysis of phthalates and their aerobic biodegradability.

In this study, thermophilic lipase and PPL enzyme were used in order to investigate hydrolysis of DEHP and DEP. Thermophilic enzymes could be a potential use for removal of PAEs during anaerobic treatment for stabilization of waste sludge. This enzyme may also be applied during thermal pretreatment of waste sludge that is used for improvement of stabilization, enhancement of dewatering and methane potential of the sludge. Hence, environmental contamination of PAEs through wastewater and sludges could be reduced to protect environmental and human health.

This study also involves investigation of factors, i.e., pH, PPL, and PAE concentration on enzymatic hydrolysis of PAEs by PPL which was shown to have a potential to be an effective means but have not been optimized for improved removal. In addition, time course analysis of DEHP and DEP hydrolysis with PPL enzyme was studied in order to determine kinetics of two PAEs.

The main goal of this study was to investigate degradation of DEHP or DEP in the presence of enzyme purified from recombinant *E.coli* that contains thermopilic lipase gene from *Geobacillus* strains isolated from thermal waters, and to compare the effectiveness of this enzyme with commercial PPL. Specific objectives of this study were:

- Investigation of enzymatic hydrolysis of PAE by commercial PPL
- Investigation of biodegradability of PAEs by recombinant lipase from recombinant *E. coli*
- Determination the effect of various factors on enzymatic hydrolysis reaction: Enzyme concentration, substrate concentration, and pH.
- Comparison of enzymatic hydrolysis of DEHP and DEP to determine the effect of alkyl side chain length (molecular weight).
- Realization of kinetic parameters for both the recombinant and the commercial enzymes on PAE hydrolysis.

This thesis contains seven chapters. An overview and the main objectives of the study are presented in Chapter 1. Literature review included three main chapters; endocrine disrupting chemicals (Chapter 2), phthalic acid esters (Chapter 3), and enzyme (Chapter 4). Materials and methods section included enzymatic degradation experiments with commercial lipase while Chapter 6 is related to degradation experiments DEHP and DEP with the recombinant enzyme.

CHAPTER 2

ENDOCRINE DISRUPTING CHEMICALS

An endocrine-disrupting chemical (EDC) is defined by the U.S. Environmental Protection Agency (USEPA) as: "an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body which are responsible for the maintenance or homeostasis, reproduction, development, and/or behavior" (Nomura et al. 1992).

Endocrine disrupting chemicals include industrial synthetic chemicals such as polychlorinated biphenyls (PCBs), plasticizers (phthalates), alkylphenol ethoxylates, polycyclic aromatic hydrocarbons (PAHs), bisphenol A, polychlorinated dibenzodioxins dibenzofurans (PCDF), and organotin compounds. Potential endocrine disruptors in aquatic and terrestrial environments are gonodal steroids, phytoestrogens, synthetic steroids, pesticides and fungicides (vinclozolin) (Lintelman et al. 2003). A list of suspected and known EDCs and their major sources are given in Table 2.1

Table 2.1 Endocrine Disrupting Chemicals (Hester and Harrison,1999)

Compounds	Source
Natural Hormones	Naturals hormones augmented by hormonal drugs such those as oral contraceptives, are excreted by humans and animals and occur in sewage.
Phytoestrogens	Natural constituents of many foodstuffs including beans, sprouts, cabbage, spinach etc. The major classes are lignans and isoflavones.
Mycotoxins	Produced by fungi which can contaminate crops.
Polychlorinated biphenyls	Widespread persistent environmental contaminants
Alkylphenol polyethoxylates (APEs)	Non-ionic surfactants used in detergents, paints, herbicides, pesticides and plastics. Breakdown products, such as nonylphenol and octylphenol, are found in sewage and industrial effluents.
Dioxins	Products of combustion of many materials.
Phthalate esters	Widely used as plasticizers for PVC.
Bisphenol A	A component of polycarbonate plastics and epoxy resins used to line food cans

In 1998, USEPA initiated the Endocrine Disruption Screening Program (EDSP) for the determination of whether certain substances have an effect in humans that is similar to an effect produced by a naturally occurring hormone system. The aim of this program was to use validated methods for the screening and testing chemicals to identify potential endocrine disruptors, determine adverse effects and dose-response relationships, assess risks, and ultimately manage risks under current laws (USEPA, 2007a). In order to identify adverse health effects and measure concentrations of EDCs, USEPA's Office of Research and Development proposed Endocrine Disruptors Research Program which focused on: (1) reducing uncertainty regarding the effects, exposure, assessment, and management of EDCs, (2) determining the extent of the adverse impact of EDCs on humans, wildlife, and the environment; and (3) supporting the Agency's screening and testing program to identify endocrine active chemicals (USEPA, 2007). In conclusion, the EDCs are regarded with utmost importance.

2.1. Endocrine System and Mechanism of Disruption

An endocrine (hormone) system is found in nearly all animals, including mammals, non-mammalian vertebrates (e.g. fish, amphibians, reptiles and birds), and invertebrates (e.g. snails, lobsters, insects and other species). This system has a main function that provides balance of hormones (homeostasis) in the organism in order to prevent functional disorders. All biological processes in the body such as growth, development, and metabolism of the body; the electrolyte composition of bodily fluids; and reproduction are regulated by the hormones (Hiller-Sturmhöfel and Bartke 1998).

Hormones are produced by endocrine glands, including the hypothalamus, pituitary gland, adrenal glands, gonads, (i.e., testes and ovaries), thyroid gland, parathyroid glands, and pancreas (Hiller-Sturmhöfel and Bartke 1998). Hormones could be divided into three main categories according to their chemical structure (1) peptide hormones which are made of amino acid chains, (2) steroids which are made from cholesterol, and (3) amino acid derivatives (Duran and Esposito, 2000). Each class of the hormones has different general molecular structure and therefore their mechanisms of action are also different. For example, steroid and thyroid hormones act by entering target cells and stimulating specific genes (Hiller-Sturmhöfel and Bartke 1998). Hormones that are produced by the major endocrine glands in human body and their primary functions are given in Table 2.2.

Table 2.2. Hormones Produced by the Major Hormone-Producing Glands and Their Primary Functions (Adopted from Hiller-Sturmhöfel and Bartke 1998)

Endocrine Gland	Hormone	Primary Hormone Function
Hypothalamus	Corticotropin-releasing hormone (CRH)	Stimulates the pituitary to release adrenocorticotropic hormone (ACTH)
	Gonadotropin-releasing hormone (GnRH)	Stimulates the pituitary to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH)
	Thyrotropin-releasing hormone (GHRH	Stimulates the pituirary to release thyroid-stimulating hormone (TSH)
	Growth hormone-releasing hormone (GHRH)	Stimulates the release of growth hormone (GH) from the pituitary
	Dopamine	Inhibits the release of prolactin from the pituitary
Anterior pituitary gland	ACTH	Stimulates the release of hormones from the adrenal cortex
	LH	In women, stimulates the production of sex hormones (i.e., estrogens) in the ovaries as well as during ovulation; in men, stimulates testosterone production in the testes.
	FSH	In women, stimulates follicle development, in men stimulates sperm production.
	TSH	Stimulates the release of thyroid hormone
	GH	Promotes the body's growth and development
	Prolactin	Controls milk production
Posterior pituitary gland	Vasopressin	Helps control the body's water and electrolyte levels
	Oxytocin	Promotes uterine contraction during labor and activates milk ejection in nursing women
Adrenal cortex	Cortisol	Helps control carbohydrate, protein, and lipid metabolism; protects against stress
	Aldosterone	Helps control the body's water and electrolyte regulation
Testes	Testosterone	Stimulates development of the male reproductive organs, sperm production, and protein anabolism
Ovaries	Estrogen	(produced by the follicle) Stimulates development of the female reproductive organs
	Progesterone	(produced by the Prepares uterus for pregnancy and mammary glands for corpus luteum) lactation
Thyroid gland	Thyroid hormone	Controls metabolic processes in all cells and triiodothyronine [T3]
	Calcitonin	Helps control calcium metabolism (i.e., lowers calcium levels in the blood)
Parathyroid gland	Parathyroid hormone (PTH)	Helps control calcium metabolism (i.e., increases calcium
Pancreas	Insulin	Helps control carbohydrate metabolism (i.e., lowers blood sugar levels)
	Glucagon	Helps control carbohydrate metabolism (i.e., increases blood sugar levels)

2.1.1. Mechanism of Endocrine Disruption

EDCs affect hormone system by different mechanisms: They can either impair hormone production by inhibiting important enzyme-catalyzed reactions or lead to induction of hormone-metabolizing enzymes. For example, these chemicals can inhibit aromatase enzyme that catalyze the conversion of testosterone to estrogen leading to higher testosterone concentrations and to lower estrogen concentrations. Tributyltin compounds have been reported to result imposex (i.e., females with typical male sex characteristics) in marine neogastropods by inhibiting the enzyme aromatase and therefore increasing the level of testosterone in females (Sutherland et al. 2004). Production and activity of important group of hormone-metabolizing enzymes, cytochrome P450-group in the liver that plays important role in synthesis and degradation of steroid hormones, could be influenced by EDC such as PCB congeners and dioxins (Soontornchat et al. 1994, Sauvageau et al. 2009). Another disturbance of the EDCs on hormone system is the effect on the transport of the hormones via bloodstream to the target tissues and organs (Lintelman et al. 2003).

Reproductive hormone-receptor systems appear to be especially vulnerable to EDCs. Clinical and experimental observations on rats indicated effects of some specific EDCs on both male and female reproductive system. For instance, exposure to phthalates resulted in hypospadias and decreased testosterone synthesis in male rats and premature thelarche in female rats. PCBs have been reported to cause delayed puberty, decreased weight of sexual organs, and reproductive deficiencies on parentally and lactationally exposed rats. Carcinogenic effects like liver, stomach, and lung tumors have also been reported after PCB exposure (WHO, 2000).

Many studies were also reported health effects of endocrine disrupting pollutants have many reproductive effects such as reduced fertility, male and female reproductive tract abnormalities, and loss of fetus and menstural problems (Saillenfait et al. 2009). It is suggested that plasticizer di-*n*-hexyl phthalate (DnHP) is teratogenic and adversely affect the reproductive tract in male rat fetuses.

It is also reported that early exposure to DnHP caused permanent and dose-related alterations of the male rat reproductive development. Saillenfait et al. (2009) investigated the developmental toxic potential of di-*n*-hexyl phthalate (DnHP) and dicyclohexyl phthalate (DCHP) in rats. They found that DCHP leads to fetal growth retardation at 750 mg kg/L per day, as evidenced by significant reduction of fetal weight. They also reported that while DnHP showed clear embryo lethality and teratogenicity, while DCHP did not show such effects.

CHAPTER 3

PHTHALIC ACID ESTERS

Phthalic acid esters (PAEs) or phthalates are the dialkyl or alkyl aryl esters of 1,2-benzendicarboxylic acid, also known as o-phthalic acid (Figure 3.1). The name phthalate derives from phthalic acid, which refers to three isomers, i.e., the ortho-isomer or phthalic acid (PA), para-isomer or terephthalic acid (TA), and metaisomer or isophthalic acid (IA) (Liang et al. 2008). These compounds are used as plasticizers in plastic products like polyvinyl chloride (PVC) resins, adhesives, and cellulose film coatings. They are also used in cosmetics, insecticides, and propellants in small amounts (Hashizume et al. 2002).

3.1. Physical and Chemical Properties of PAEs

PAEs are characterized by low water solubility and high octanol/water partition coefficient, the physical end chemical properties defines the fate of chemicals in the environmental compartment. Physical and chemical properties of some common phthalates are given in Table 3.
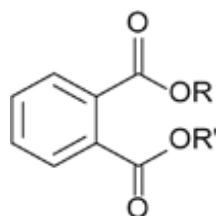
Figure 3.1. General structure of phthalates
(R and R" are the same or different alkyl or aryl groups)

PAEs can be categorized according to their molecular weight. PAEs with higher molecular-weight, such as DEHP, DiNP, and DiDP, are primarily used as plasticizers to soften polyvinyl chloride (PVC) products, whereas PAEs with lower-molecular-weight (ester side chain length, one to four carbons), such as DMP, diethyl phthalate (DEP), di-n-butyl phthalate (DBP), and butyl benzyl phthalate (BBzP), are widely used as solvents to hold color and scent in various consumer and personal care products (NRC, 2008).

Table 3.1. Physical and chemical properties of some PAEs (Adopted from Cao, 2010)

Phthalates	Molecular Weight	Density (g/mL)	Vapor Pressure 25 °C (Pa)	Water Solubiliy (mg/L) at 25 °C	LogKow at 25 °C
Dimethyl phthalate (DMP)	194.2	1.191	0.263	5220	1.61
Diethyl phthalate (DEP)	222.2	1.232	6.48×10^{-2}	591	2.54
Dipropylphthalate (DPP)	250.3	1.078	1.75×10^{-2}	77	3.40
Di-iso-buthyl phthalate (DiBP)	278.3	1.039	4.73×10^{-3}	9.9	4.27
Di-n-buthyl phthalate (DiBP)	278.3	1.043	4.73×10^{-3}	9.9	4.27
Buthylbenzyl phthalate (BBzP)	312.4	1.119	2.49×10^{-3}	3.8	4.70
Di-n-hexyl phthalate (DHP)	334.5	1.011	3.45×10^{-4}	0.159	6.00
Di-2-ethyl hexyl phthalate	390.6	0.985	2.52×10^{-5}	0.285^{*}	7.73
Di-n-octyl phthalate (DOP)	390.6	0.985	2.52×10^{-5}	2.49×10^{-3}	7.73
Di-iso-nonyl phthalate (DiNP)	419	0.972	6.81×10^{-6}	3.08×10^{-4}	8.60
Di-iso-decyl phthalate(DiDP)	446.66	0.966	1.84×10^{-6}	3.81×10^{-5}	9.46

3.2. Exposure Sources and Health Effects of PAEs

The major human exposure sources for phthalates include sources such as material related (building material, furniture, electronic devices), product related (cosmetics, packaging, paints, textiles, and medical devices), industrial (production, manufacturing, exhaust air), agricultural (insecticides, pesticides, and drugs), and wastes (landfills and waste sludge) (Wilkinson and Lamb 1999, Nakamiya et al. 2005, Wormuth et al. 2007).

DEHP, which is the most recalcitrant PAE compound, is found in a variety of products including from dialysis tubing, paints, adhesives, as well as in food products because of leaching during production or storage. Castle et al. (1990) measured DEHP levels in milk samples collected from a dairy in Norway in which plasticized tubing was used as milking equipment. The reported concentration of DEHP in milk samples collected from the milking chamber ranged from 30 µg/kg to 50 µg/kg, on the other hand it was 5 µg/kg in control samples obtained by hand milking. Studies suggested that daily exposures of infants and children to DEHP from ingestion of breast milk, cow's milk, and infant formula are in the range of 1–10 µg/kg/d (Mortensen et al. 2005, Zhu et al. 2006). Exposure to DEHP in patients with chronic renal failure undergoing maintenance hemodialysis was investigated by Dine et al. (2000) and the leached amount of DEHP from the plastic dialysis tubing was measured during a 4-h dialysis session. They reported that concentration of DEHP in blood plasma measured at the outlet of the dialysis machine (before the patient) found to be increased from 0.4 to 2.6 µg/ml during a 4 h dialysis session.

DEHP is also found in a variety of food products because of leaching during production and storage. DEHP, DBP, BBP and DEP were detected in both the packaging and the contacted foods Levels of DEHP was low (0.065 µg/g on average in beverages and 0–29 µg/g on average in foods) associated with the use of DEHP-plasticized cap or lid seals. DEHP DBP, BBP and DEHP were found in butter and margarine that were covered with aluminum foil and paper laminates. Migrated DEP from pie cartons was measured as 1.8 µg/g (average) (Page and Lacroix,1995).

PAEs belong to the class of endocrine disrupting pollutants and they show reproductive and developmental health effects on biological organisms. They can reduce concentrations of testosterone, an important androgen (or male sex hormone) that contributes to the development of male sex organs (Struve et al. 2009). Laboratory

studies on determination of developmental effect of phthalates on rats suggested that di-*n*-hexyl phthalate and dicyclohexyl phthalate are responsible for a reduction in fetal weight, a decrease in anogenital distance in female fetus, and undescended testis in male fetus (Saillenfait et al. 2009). Reddy et al. (2006) suggested that there is an association between occurrence of endometriosis disease and concentrations of PAEs in the plasma of women suffering from endometriosis. Researchers reported that correlation between the concentrations of PEs and different severity of endometriosis was strong and statistically significant ($p<0.05$) for di-n-butyl phthalate (DnBP), butyl benzyl phthalate (BBP), di-n-octyl phthalate (DnOP), and diethyl hexyl phthalate (DEHP).

Swan and Davis (2003) also reported that DEHP leads to changed serum cholesterol levels, decreased serum estradiol levels, prolonged estrous cycles in rats, and no ovulations in adult cycling rats. In addition to health effects of PAEs on endocrine system, some PAEs also have carcinogenic health effects on animals. For example, DEHP was shown to produce cancer in rodents after high-level lifetime exposures (Kluwe et al. 1982, Tickner et al. 2001). However, studies that investigated the mechanism of peroxisome proliferation suggested that animal studies are not relevant for humans, since humans are much less sensitive than rodents to PPAR-alpha mediated effects (Klaunig et al. 2003).

Table 3.2. Carcinogenity classification of PAE

Compound	Carcinogenity Class
Butyl benzyl phthalate	Group C
Di (2-ethylhexyl) phthalate	Group B2
Dimethyl phthalate	Group D
Dibutyl phthalate	Group D
Diethyl phthalate	Group D

B2: probable human carcinogen, C: possible human carcinogen
D: not classifiable as a human carcinogen

According to USEPA's carcinogenicity weight-of-evidence classification, DEHP was classified as B2, probable human carcinogen based on increased liver tumors in adult male and female rats. In addition, the diethyl phthalate was classified as class D which means not classifiable as to carcinogenicity (IRIS, 2013).

3.3. Environmental Concentrations of PAEs

Phthalates have been detected in various environments including air (Wensing et al. 2005), soil (Xu et al. 2008), sewage (Gavala et al. 2003), wastewater (Roslev et al. 2007), and natural waters as a result of the production, usage, and disposal of plastic products. Phthalates could easily migrate from plastics into the environment since they are not chemically bonded to the polymers. These chemicals are generally found in primary and secondary sludge of municipal wastewater treatment plants (WWTP) because of their high hydrophobicity that allows them to be adsorbed on suspended organic matter. The amount of PAEs in sewage sludge was found to be in the range of 12 to 1250 mg/kg-total solid (Gavala et al. 2003). PAE concentration from inlet of the municipal WWTP of Aalborg, Denmark was measured as 1.9, 20.5, 37.9 and 71.9 µg/L for DMP, DBP, BBP, and DEHP, respectively (Roslev et al. 2007). In other study the measured DBP and DEHP concentrations in soil samples were determined as 2.75 to 29.37 mg/kg soil (Xu et al. 2008). Yuan et al. (2002) measured concentrations of PAEs in 14 surface water and six sediment samples taken from rivers in Taiwan. The concentration of DEHP in the water and sediment samples was ranging from below detection limit (BDL) to 18.5 µg/l, and 0.5 to 23.9 µg/g, while DBP concentration was measured in the range of 1.0–13.5 µg/l and 0.3–30.3 µg/g in water and sediment samples, respectively. Sediment concentrations of PAEs were found to be higher than the levels found in water possibly due to hydrophobic property of these chemicals.

3.4. Biodegradation and Biotransformation of PAEs in the Environment

In the environment, removal of hazardous organic pollutants could occur by either biodegradation or biotransformation reactions. Biodegradation is defined as mineralization of the organic compound that results in formation of ultimate degradation products, CO_2, and water under aerobic conditions. In addition, ammonium (or nitrite), sulfate, phosphate, or chloride are also released if the compound contains nitrogen, sulfur, phosphorus, or chlorine. On the other hand, biotransformation refers to a single step mineralization process and the basic framework of the molecule remains essentially intact and this mechanism has an important role for removal of hazardous organic compounds from the environment (Neilson and Allard, 2008). Biodegradation/biotransformation of an organic compound is mainly affected by the bioavailability of the compound which is related to its solubility, dissolution rate, and sorption of dissolved part on particulate organic matter. Biochemical transformation reactions of organic compounds occur very slowly because of kinetic limitations. Microorganisms could increase the rate of these reactions via enzymes by lowering the activation energy of the reactions, resulting in about 10^9 or more times higher reaction rates. In addition, organisms could use these compounds as carbon and energy source, and convert them to more reactive species using oxygen as a biochemical reducing agent (Vogel et al. 1987).

The difference in biodegradability of phthalates is likely due to the steric effect of phthalates side ester chains, which hinders the hydrolytic enzymes from binding to the phthalates and thereby inhibits their hydrolysis (Liang et al. 2008). Studies have demonstrated that phthalates with shorter ester chains like DMP, DEP, DBP, DPP, DPrP, and BBP can be readily biodegraded and mineralized. On the other hand, phthalates with longer ester chains, such as dicyclohexyl phthalate and DEHP are less susceptible to biodegradation (Wang et al. 1996, Chang et al. 2007). Microbial or enzymatic degradation pathway of phthalates under both aerobic and anaerobic conditions generally consists of two processes: primary biodegradation from phthalate diesters (PDEs) to phthalate monoesters (PMEs), and then to PA. Finally, ultimate biodegradation from PA to CO_2 and/or CH_4 occurs (Peterson and Staples 2003, Juvancz et al. 2008).

Primary degradation process is very important for evaluating rate of decrease in the PAE concentration in the environment and also to evaluate environmental fate of the compound. Under aerobic conditions, further enzymatic degradation of the monoester proceeds via phthalic acid by either a 3,5- or 4,5-dihydroxyphthalate pathway to procatechuate. Aromatic ring cleavage of procatechnate can then occur via either an ortho pathway that results in the formation of pyruvate and oxaloacetate or a meta pathway yielding a 13-ketoadipate that is further degraded to acetyl CoA and succinate (Nomura et al. 1992, Eaton and Ribbons 1982). Although less is known about the pathways of anaerobic catabolism, it appears that the monoester is degraded to phthalic acid and then further degraded by the same pathway used for benzoate (Ejlertsson and Svensson, 1995). Benzoate has been shown to be readily degraded anaerobically (Shelton et al. 1984, Battersby and Wilson 1989).

3.4.1. Microbial metabolism of PAEs

Biological reactions carried out by microorganisms are of major significance since these reactions determine the fate and persistence of organic compounds in aquatic and terrestrial ecosystems. During degradation of xenobiotics by microorganisms, the ultimate metabolites, CO_2 and water, cannot be formed since a portion of the organic compound is used in synthesis (anabolic) reactions to produce energy required for growth and cell division (Neilson and Allard, 2008).

There are many studies on isolation of microorganisms metabolizing PAEs from contaminated soil (Nakamiya et al. 2005, Chao and Cheng 2007, Chang et al. 2007, Quan et al. 2005) and activated sludge (Chang et al. 2007, Zeng et al. 2004, Chen et al. 2007). Two major catabolic pathways have been identified for the bacterial degradation of PAEs. Some organisms could selectively hydrolyze only one ester bond, to give mono-alkylphthalate and alcohol, where the latter compound was then used for growth, while other organisms were capable of complete mineralization of either the mono-alkyl- or di-alkylphthalates (Quan et al. 2005). A summary of studies on bacterial degradation of PAE are presented in Table 3.3.

In a study by Chang et al. (2004), two aerobic PAE degrading bacteria strains, DK4 and O18, were isolated from a river sediment and petrochemical sludge, respectively.

Results of this study suggested that PAE with shorter alkyl-chains such DEP, DPrP, DBP, BBP, and DPP are very easily biodegraded, while longer alkyl-chains such as DCP, DHP, and DEHP are poorly degraded by the isolated strains. The isolated bacteria strains, DK4 and O18, were identified as *Sphigomonas* sp. and *Corynebacterium* sp., respectively.

Hashizume et al. (2002) isolated eight bacterial strains for biodegradation of five PAEs including DMP, DEP, DBP, BIBP, and DEHP. Among these compounds, DMP was not degraded by any of the isolated strains. However, DBP was almost completely degraded by one of the isolated strain (R3) which was identified as *Acinetobacter lwoffii.* The isolated strain R6 (*Acinetobacter lwoffii*) showed the highest biodegrading activity for DEHP with about 46% degradation. In addition, the crude enzyme solutions prepared from bacterial cells were prepared and tested for DEP, DBP, and DEHP degrading activity. Crude enzyme solution showed the highest ability for DEHP and enzymatic degradability of DEHP was in the range of 0.08-0.55 nmol/mg protein/min for 30 min incubation time at 37 °C.

A new bacterial strain that uses PAEs as sole carbon and energy source was isolated from activated sludge at a petrochemical plant. The bacterial strain was defined as *Pseudomonas fluoresences* FS1 and biodegradation kinetics of DMP, DEP, DnBP, DiBO, DnOP, and DEHP was studied with the new strain. Biodegradation of all PAEs followed a first order kinetic and biodegradation rates were greatly decreased with increasing alkyl chain length and alkyl branch chains (Zeng et al. 2004).

In another study, soil polluted with di-2-ethylhexyl phthalate was decontaminated by a bacterial strain, *Bacillus subtilis*, which was isolated from soil. After incubating the bacterial strain with soil containing DEHP for 5 days at 30 °C, strain degraded about 80% of 5 mM DEHP by adding 8% culture medium to soil. The strain was also able to utilize DnBP, DEP, DPP, and PA as sole carbon sources; and their biodegradation ratio was higher than 99% for an incubation period of 5 days (Quan et al. 2005).

Nakamiya et al. (2005) investigated biodegradability of DEHP with bacterial strains isolated from a garden soil. The researchers isolated four bacterial strains, one of which was coded as strain NK0301 identified as *Mycobacterium sp* from its 16S rDNA sequencing homology. At optimal cultivation conditions (30 °C; pH 6.8; DEHP, 0.1% (v/v)), this strain was able to degrade more than 98% DEHP in 21 h. The major degradation products of DEHP were determined by gas chromatography-mass

spectrometry as 2-ethylhexanol and 1,2-benzenedicarboxylic acid. Detection of these compounds indicated that biodegradation of DEHP was conducted by lipase-like enzymes. Strain NK0301 was also cultivated on polyvinyl chloride sheets containing DEHP as the plasticizer and after 3 d nearly 90% of DEHP was removed. After this treatment, the polyvinyl chloride sheets did not exude DEHP to artificial saliva. Zeng et al. (2004a) investigated biodegradability of DEHP with an isolated bacterial strain of *Pseudomonas Fluorescence FS1*. They reported that *Pseudomonas Fluorescence FS1* can utilize DEHP as the sole carbon and energy source. The degradation of DEHP was found to fit the first order Monod kinetic. For an incubation period of 60 days, 87% of DEHP removal was obtained with an initial concentration of 200 mg/l while 100% removal was achieved with 50 mg/L initial DEHP concentration. The optimum pH for degradation was found to be in the range of 6.5 to 8.0 and optimum temperature was 35 °C.

Table 3.3. Summary of studies on bacterial degradation of PAEs

Species	Isolated from	Phthalates	Concentration (mg/L)	Performance	References
Sphigomonas sp. DK4	Activated sludge (Petrochemical wastewater) or river sediment	DEP, DPrP, DBP, DHP, BBP, DEP, DPrP, DBP, DHP, BBP,	5 for each	Batch mode, 30°C, pH 7 DBP, DHP, BBP, DCP and DPP were completely degradation in 2, 2, 2, 4, 2, 4, and 2 days; DEHP was 90% degraded in 5 days	Chang et al. 2004
Pseudomonas fluorescens FS-1	Activated sludge	DMP, DEP, DnBP, DiBP, DnOP, and DEHP	50-400 (for each)	In 100 mg/L, more than 99% of DMP, DEP, DnBP, and DiBP, less than 30% of DnOP, and 20% of DEHP were removed in 3-days	Zeng et al. 2004a
Acinetobacter lwoffii	River water	DBP and DEHP	20	Complete degradation of DBP and 20% degradation of DEHP in 5 days	Hashizume et al. 2002
Mycobacterium sp. NK301	Garden soil	DEHP	1,000	30°C, pH 6.8, 98% was degraded in 21 h	Nakamiya et al. 2005
Microbacterium sp. CQ0110	Activated sludge (DEHP exposed)	DEHP	1,350	Complete degradation in 10 days, $t_{1/2}$=1.59 d	Chen et al. 2007
R. rhodochrous G2, G7	Soil	DEHP	100	98.4% degradation in 3 days by G2; 91.7% degradation in 5 days by G7	Chao and Cheng, 2007
Bacillus sp. S4	Sludge	DEHP	1,000	Degradation constant k=0.081, $t_{1/2}$=8.6; DEP, DBP, BBP can also be degraded.	Chang et al. 2007
Bacillus subtilis No.66	Soil	DEHP	3,900	81.6% degradation in 5 days	Quan et al. 2005

Another bacterial strain *Microbacterium sp.* CQ0110 was isolated from activated sludge and it was used for degradation of DEHP. Degradation of DEHP lower than 1,350 mg/L concentration with exponential model that fits the kinetics equation (ln $C=-0.4087t+A$). The half-life of the DEHP in wastewater was determined as 1.59 days. Optimum pH and temperature for degradation of DEHP in wastewater was reported as 6.5–7.5, 25–35 °C, respectively (Chen et al. 2007).

Chao and Cheng (2007) studied biodegradation of DEHP in aqueous medium using four previously isolated DBP degraders identified as isolate G1 and *Rhodococcus rhodochrous* G2. Both isolated strains could degrade 100 mg/L DEHP in three days more than 100% efficiency. The researchers also reported that addition of 2 mg/L DEHP significantly increased the degradation of DEHP by *Rhodococcus rhodochrous* G2 and more than 90% of the DEHP was degraded within 24 h.

Biodegradation of eight PAEs was tested by two bacterial strains isolated from river sediment and petrochemical sludge, respectively. The isolated bacterial strains were identified as *Sphigomonas sp.* and *Corynebacterium sp.*, respectively. Except DEHP, almost complete degradation was observed with *Sphigomonas sp.*, for an incubation period of 7-days. The optimum conditions for biodegradation were determined as 30 °C and pH 7.0. The removal ratio of DEHP, the most recalcitrant PAE compound, was about 89% at the optimum conditions (Chang et al. 2004).

3.4.2. Enzymatic Biodegradation/Biotransformation of PAEs

Enzymatic treatment of organic contaminants has many advantages compared to conventional biological waste treatment methods. For example, enzymes are more resistant to toxic and shock loading effects, and there is no need for acclimation. In addition, enzymes could be applied to a wide range of organic compound concentrations, pH, temperature, and salinity (Duran and Esposito, 2000). Bioremediation of polluted sites using enzymes may be very advantageous compared to classical methods in which degrading microorganisms are inoculated or stimulated in the polluted sites with the supplies of their nutrients since inoculation and control of microorganism to the environment is difficult and addition of nutrients to the polluted site may cause the increase of the chemical oxygen demand of the water environment.

However, application of enzymes to a polluted site, a process known as enzymatic bioremediation, will diminish those defects of the bioremediation by microorganisms. Enzymes could be isolated and applied to the polluted site and provide a rapid remediation (Sutherland et al. 2004). A summary of studies on enzymatic degradation of PAEs are presented in Table 3.4.

Major enzymes that are involved in metabolism of PAEs are phthalate oxygenase, phthalate dioxygenase, phthalate dehydrogenase, and phthalate decarboxylase (Nomura et al. 1992, Kurane et al. 1980). In addition, lipase and esterase enzymes from bacteria (Kurane et al. 1980, Albro and Latimer, 1974) or animals (Saito et al. 2010) were also used in first step hydrolysis of PAEs that includes formation of corresponding monoesters and alcohols.

Kurane et al. (1980) conducted a study that involves enzyme induction from *Nocardia erythropolis* and enzymatic hydrolysis of DEHP by commercial lipases from microbial sources. They reported that induction of phthalate ester-hydrolyzing enzymes from the *N. erythropolis* in the presence of 4000 mg/L DEHP. They also suggested that phthalate esterase activity was observed in cells and cell free extracts even microorganism was grown in medium containing olive oil as the sole source of carbon and energy. This finding suggested that phthalate esterase is a kind of lipase or esterase with broad substrate specificity. Removal ratio for DEHP by 1 mg/ml enzyme (30 $^{\circ}$C and 18 h) microbial lipases from *Rhizopus delemar,* Steapsin (Pacreas)*, Rhizopus arrhizus*, *Pseudomonas* and esterase from pig liver was found as 80.4, 24.8, 36.6, 19.1, 16.1%, respectively.

Enzymatic degradation of DBP by lipase enzyme purified from *Candida Cylindracia* was investigated in the study of Tanaka et al. (2000). In order to obtain model contaminated sediment, DBP was adsorbed on sea sand (1.5 µmol/0.5 g-sea sand). After 3-day incubation at pH 5, about 90% removal was obtained for DBP. The degradation of DBP was mainly due to hydrolysis of ester bonds by lipase enzyme.

Fungal cutinase purified from *F. oxyprorum* and commercial *Candida cylidracea* esterase enzymes were used for testing biodegradation of BBP. 500 mg/L BBP was mixed with enzyme solutions of cutinase and esterase, and incubated 3 days in a shaking incubator (30 $^{\circ}$C, 200 rpm). The cutinase enzyme (10 mg/L) degraded about 60 % of the BBP within 7.5 h. However, only 10% of the initial BBP was removed after 3 days incubation with esterase enzyme (Kim et al. 2002).

In the study of Gavala et al. (2004), anaerobic degradation of primary sludge containing DEP, DBP, and DEHP as well as enzymatic degradation of these PAEs with commercial lipase was investigated. The sludge was pretreated at 70 $^{\circ}$C and subsequent anaerobic biodegradation was applied at 37 °C. The pretreatment of sludge at high temperature negatively influenced biodegradability of PAEs. In addition, enzymatic treatment of the sludge with commercial lipase resulted in one to two orders of magnitude higher DEHP removal than under normal mesophilic conditions. The percent removal of DEHP (7 mg/L) after 100 h incubation at 28 °C with commercial lipase was about 85%.

Zeng et al. (2004) reported that hydrolysis of DEHP to PA by esterase from *Norcardia erythropolis*. The researchers suggested that initial degradation of DMP by esterase from *Bacillus* species. There are also some studies on PAE hydrolysis by mammalian enzymes, such as nonspecific lipase from rat pancreas (Duran and Esposito 2000, Sutherland et al. 2004, Chang et al. 2007), esterase from rat intestine (Soontornchat et al. 1994), and carboxyl esterase from rat and human (Albro and Latimer 1974), and salivary esterase from human.

Liang et al. (2008) reported that primary degradation of PAEs consists of different type of pathways, including de-esterification or dealkylation, â-oxidation and trans-esterification. De-esterification is the most common way of PAE degradation in which phthalate diesters are serially converted to phthalate monoester and phthalic acid. This degradation pathway is the same under both aerobic and anaerobic conditions (Shelton et al. 1984). â-oxidation involves biodegradation of phthalate diesters by removing one ethyl group at each time (Amir et al. 2005). After that, DEP is further converted to PA by two pathways, de-esterification and an alternative trans-esterification pathway (demethylation) which is the replacement of ethyl group of DEP with methyl group (Figure 3.2). Transesterification reaction normally requires a high temperature and pressure however in the presence of a biological catalyst it can proceed under ambient conditions. Demethylation reaction which is the replacement of each ethyl group with a methyl group followed by transesterification reaction that results in cleavage of relatively strong C-C bond within the ethyl group is cleaved resulting in EMP and then DMP (Cartwright et al. 2006).
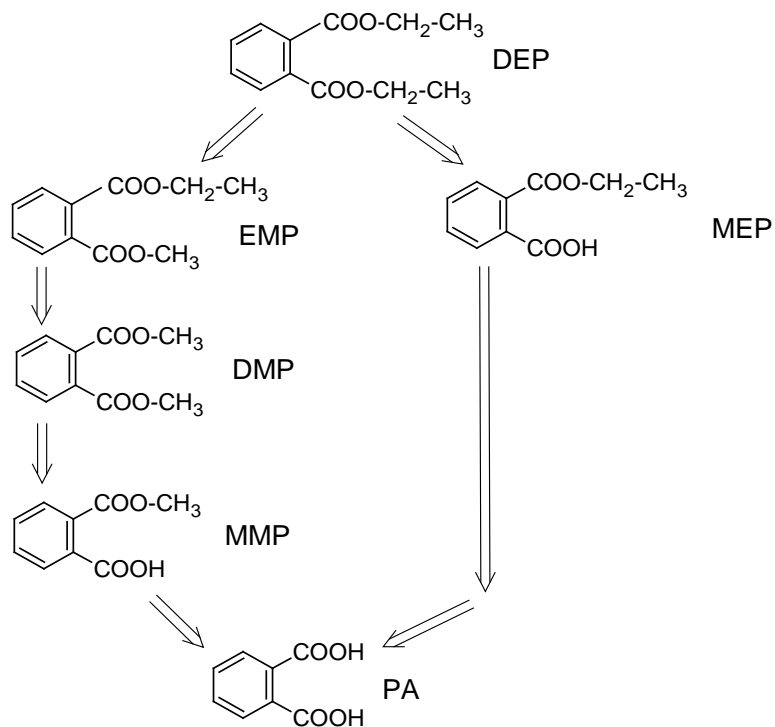
Figure 3.2. Biodegradation of DEP by the indigenous soil microbial community in soil co-contaminated with methanol (Adopted from Cartwright et al. 2000)

Saito et al. (2010) studied hydrolysis of DEHP using commercial crude lipase from porcine pancreas (Type II, 147 U). The researchers reported that, incubation of 100 mg/L DEHP with 1470 U lipase solution at 37 $^{\circ}$C for 24 h resulted in 93% decrease in initial concentration. However, optimization of conditions for a better removal performance has not been studied for PPL

Enzymatic degradation studies on PAEs generally studied at high concentrations ranging from 5 to 2000 mg/L. Lipase and esterase enzymes were commonly used for the removal of PAEs since these enzymes are known to be effective on ester bonds. Degradation efficiency for PAEs ranged from 10 to 90% depending on the PAE concentration and the type of the enzyme used. Results of the studies indicate that removal of PAEs by using enzymes is an effective way and there is no study on application of thermophilic lipase enzyme for degradation of PAEs.

Table 3.4. Summary of Studies on Enzymatic Degradation of PAEs

Compound	Concentration	Enzyme	Performance	Reference
DEHP	6-10 mg/L	100 and 1000 U/L commercial esterase from porcine liver (15 U/mg)	Esterase, $t_{1/2}$ was reduced to 198 days to 1 day	Gavala et al. 2004
BBP	500 mg/L	100 mg/L , Fungal cutinase from *F. oxysporum f. sp. pisi*	60% decrease in initial concentrationin 7.5 h	Kim et al. 2002
		100 mg/L, yeast esterase *Candida Cylindracia*	10% decrease in initial concentrationin 3 days	
DBP	1.5 µmol/ 0.5 g-sea sand	360U/ml, Lipase from *Candida cylindracia*	3d incubation at pH 5, about 90% removal of DBP	Tanaka et al. 2000
DEHP	100 mg/L	1470 U Crude lipase from porcine pancreas	93% was degraded by crude lipase within 24 h	Saito et al. 2010
DEHP	2000 mg/L	Lipase from *Rhizopus delemar* Lipase from Steapsin(Pancreas) Lipase from *Rhizopus arrhizus* Lipase from *Pseudomonas* Esterase from pig liver	(Removal ratio for 1 mg/ml enzyme, 30 $^{\circ}$C and 18 h) 80.4% 24.8% 36.6% 19.1% 16.1%	Kurane et al. 1980

3.4.3. First Order Degradation Kinetic of PAE Degradation in the Environment

Primary biodegradation of PAEs is generally followed first-order kinetics (Gavala et al. 2003, Gavala et al. 2004, Zeng et al. 2004, Cheng et al. 2008). Biodegradation of DEHP in sewage sludge during thermopilic composting phase was described by first-order kinetics. The kinetic constant for DEHP was ranged between 0.27 and 0.40 d^{-1} for three different composting reactors that have initial DEHP concentration of 213 to 296 mg/kg TS (Cheng et al. 2008). Zeng et al. (2008) studied biodegradation kinetics of dimethylphthalate (DMP), diethylphthalate (DEP), di-*n*-butylphthalate (DnBP), diisobutylphthalate (DIBP), di-*n*-octylphthalate (DnOP), and di(2-ethylhexyl)phthalate (DEHP) by a novel bacterium, *Pseudomonas fluoresences* FS1 isolated from activated sludge at a petrochemical factory which has the ability to utilize PAEs as the sole source of carbon and energy under aerobic conditions. They reported that biodegradation of DMP, DEP, DnBP, DIBP, DnOP, and DEHP by *P. fluoresences* FS1 followed first order degradation kinetics and biodegradation rate is strongly affected by the alkyl chain length and alkyl branch chains. Half lives of DEP for a initial concentration range of 25 to 400 mg/L ranged from 7.75 to 12.49 h. In the case of DEHP, half lives for an initial concentration range of 12.5 to 200 mg/L was reported between 9.59 to 16.59 d. Gavala et al. (2003) reported that biodegradation of 5 mg/L DEP and 7 mg/L DEHP in primary sludge in anaerobic digestion process resulted in hydrolysis constant (K_h) of 0.08 and 0.0036 d^{-1} for DEP and DEHP, respectively.

First-order kinetic constant for aerobic biodegradation of PAEs in surface water and sediment was reported in the range of 0.2 to 2 for both low and high molecular weight PAEs. First-order kinetic constant for low molecular weight PAEs that present in water phase waste water was reported as 24 d^{-1} while that for high molecular weight PAEs that present slurry phase was 0.75 d^{-1} (Peterson and Staples 2003).

CHAPTER 4

ENZYMES

Enzymes play an important role in biochemical reactions since they acts as catalyst in almost all of the chemical reactions in living organisms. Enzymes are made of amino acids which are the basis of protein. Unlike proteins they have an active site that substrate molecule binds for product formation. Most of the enzymes contain more than one sununits such as coenzymes and cofactors which affects the enzyme activity (Shuler and Kargı, 2002). Cofactors are metal ions such as Mg, Zn, Mn, and Fe, whereas coenzymes are complex organic molecules like NAD, FAD, CoA and some vitamins. Enzymes are specific to the substrates and are grouped along with the reaction that they catalyze. The major class of the enzymes and the type of the reaction they catalzye are presented in Table 4.1.

Table 4.1. Classification of the enzymes based on the reaction they catalyze
(Adopted from, Shuler and Kargı, 2002)

Class	EC	Chemical Reaction Catalyzed	Examples
Oxidoreductase	1	Oxidation-reduction where oxygen and hydrogen are gained or lost	Cytochrome, oxidase, lactate, dehydrogenase
Transferase	2	Transfer of functional groups, such as an amino group, acetyl group or phosphate group	Acetate, kinase, alanine, deaminase
Hydrolase	3	Hydrolysis (addition of water)	Lipase, sucrase
Lyase	4	Removal of groups of atoms without hydrolysis	Oxalate decarboxylase. isocit rate lyase
Isomerase	5	Rearrangement of atoms within a molecule	Glucose-phosphate, isomerase, alanine racemase
Ligase	6	Joining of two molecules (using energy usually derived from the breakdown of ATP)	Acetyl-CoAsynthetase, DNA ligase

EC: Enzyme commission number

Except some enzymes such as pepsin, rennin, and trypsin which are originally studied, most enzymes named by adding the suffix –ase to the end of the substrate they catalyze (Shuler and Kargı, 2002). The enzyme amount that catalyze the transformation of 1µmol of substrate per minute under specified conditions (temperature and pH) is defined as one unit of enzyme (Eisenthal and Danson, 1993).

4.1. Enzyme Kinetics

Enzymes reduce the activation energy (ΔE) of the reaction catalyzed by binding the substrate and forming and enzyme-substrate complex. Enzymes increase the rate of the biochemical reactions by lowering the activation energy (ΔE). Although ΔE is decreased, there is no change in the free-energy and the equilibrium constant. For example the activation energy of the uncatalyzed reaction for decomposition of hydrogen peroxide at 20 °C is 18 kcal/moles, whereas that for the chemically and enzymatically (catalase) catalyzed reaction are 13 and 7 kcal/mol, respectively (Shuler and Kargı, 2002). The enzyme catalyse increases the reaction rate by a factor of about 10^8. Substrate binding and formation of ES complex is described with the lock-and-key model, in which enzyme represents the lock and the substrate represents the key.

Enzyme kinetics defines the mechanism of enzyme catalyzed reactions. Kinetics of the enzyme catalyzes reactions are generally defined by Michealis – Menten kinetic or saturation kinetics. For the saturation of the enzyme and substrate reaction there are two chain reactions; a reversible reaction enzyme-substrate complex formation and dissociation of ES complex. When the all the active sites are occupied with substrate at high enzyme concentration the enzyme becomes saturated. When the enzyme is saturated, ES complex is assumed to be formed rapidly, thus the rate of the reverse reaction of the second step is ignored. It is assumed that the second irreversible reaction only occurs when product accumulation is negligible at the beginning of the reaction (Shuler and Kargı, 2002).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} E + P \qquad (4.1)$$

The rate constant k_2 is often denoted as k_{cat} in biological literature. The rate of variation of ES complex is

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES] \qquad (4.2)$$

The initial rate of product formation;

$$v_o = k_2[ES] \qquad (4.3)$$

Rapid equilibrium assumption was used for derivation of Henri and Michaelis-Menten equations. Assuming, a rapid equilibrium between the enzyme and substrate to form an $[ES]$ complex, the equilibrium coefficient can be used to express $[ES]$ in terms of $[S]$. Based on the assumption of $k_{-1} \gg k_2$, the first step (formation of [ES]) can be treated as rapid equilibrium process. The equilibrium constant K_m is given by

$$Km = \frac{k_{-1} + k_2}{k_1} = \frac{[E][S]}{[ES]} \qquad (4.4)$$

Since the enzyme is not consumed, the conservation equation on the enzyme yields

$$[E] = [E_0] - [ES] \qquad (4.5)$$

So that;

$$K_m = \frac{([E_0] - [ES])[S]}{[ES]} \qquad (4.6)$$

Solving for [ES] we obtain

$$[ES] = \frac{[E_0][E]}{K_m + [S]} \qquad (4.7)$$

Substituting eq. 4.7 into eq. 4.3 yields

$$v = \frac{d[P]}{dt} = k_2 \frac{[E_0][E]}{K_m + [S]} = \frac{V_m[S]}{K_m + [S]} \tag{4.8}$$

where $V_m = k_2[E_0]$ is the maximum rate of the reaction.

The reaction rate increases with increasing the substrate concentration [S] asymptotically, approaching its maximum rate V_m when all enzyme is bound to substrate.

4.2. Lipases

Lipases are triacylglycerol acylhydrolases (EC 3.1.1.3) that catalyze both hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids. Unlike esterase, lipases are activated only when adsorbed to an oil-water interface (Martinelle et al. 1995, Svendsen, 1997) and they do not hydrolyze dissolved substrates in the bulk fluid. In eukaryotes, lipases are involved in various stages of lipid metabolism including fat digestion, absorption, reconstitution, and lipoprotein metabolism. In plants, lipases are found in energy reserve tissues (Balashev et al. 2001). From the X-ray structure of cocrystals between lipases and substrate analogues, there is strong indirect evidence that, when contact occurs with a lipid/water interface, this lid undergoes a conformational rearrangement which renders the active site.

Major application areas of lipases are organic synthesis, hydrolysis of fats and oils, modification of fats, and flavor enhancement in food processing (Sharma et al. 2001). Novel enzyme technology studies make it possible that producing more stable lipase enzyme which could be active at extreme conditions such as temperatures exceeding 45 °C (Sharma, et al. 2001). Thermostable lipases have an importance for catalysis of some fats that undergo desirable physical changes at high temperatures.

Isolation of thermostable enzymes as well as improving their performance in certain industrial applications is possible with recombinant DNA technology (Boston et al. 1997, Svendsen et al. 1997, Schmid and Verger 1998). Janssen et al. (1994) reported production of thermostable lipase from thermophilic *Bacillus sp*. strain Wai 28A 45 in the presence of tripalmitin at 70 °C.

In another study, a thermophilic bacterium, *B. thermoleovorans* ID-1, isolated from hot springs in Indonesia, showed extracellular lipase activity and high growth rates on lipid substrates at elevated temperatures (Lee et al. 1999). Tekedar and Şanlı (2011) studied cloning of thermoalkalophilic esterase genes from three different *Geobacillus* strains isolated from thermal environmental samples in Balçova (Agamemnon) geothermal site. All three enzymes were found to be stable up to 70 °C and was also stable at high pH values.

4.2.1. Application of Lipases in Wastewater Treatment

In wastewater treatment lipase enzymes are generally used for removal of fats and oil that increases the COD load of biological treatment. There are several studies that have described the use of microorganisms and/or enzymes pools developed in the laboratory for the biological treatment of effluents with high fat and oil concentrations. Masse et al. (2001) reported that pretreatment with pancreatic lipase PL-250 reduced the average particle size to a maximum of 60 % of the initial particle size. The bacterial lipase LG- 1000 was also found to be efficient in reducing the average fat particle size in slaughterhouse wastewater, but high doses (>1000 mg/L) were required to obtain a substantial reduction after 4 h of pretreatment.

CHAPTER 5

MATERIALS & METHODS

5.1. Enzymatic Degradation Experiments

Porcine pancreas lipase (20 U/mg, Applichem) was used for enzymatic degradation of bis (2-ethylhexyl) phthalate (Sigma, $\geq$ 99%) and diethyl phthalate (Sigma, $\geq$ 99%). PPL enzyme was dissolved in 100 mM sodium phosphate at pH 7.4. DEHP and DEP stock solutions of 1000 mg/L concentration were prepared in methanol (Suprasolv, Merck). Appropriate volume of stock solution was spiked into enzyme solution to obtain the final working concentration. Heat denaturated enzyme solutions were used as positive control in order to check any abiotic degradation. In order to stop the enzymatic reaction, 1N HCl was added to reaction mixture to final concentration of 10% (v/v) as proposed by Nakamiya et al. (2005) and Saito et al. (2010). Immediately after collecting samples from the reaction mixture, samples were extracted with equal volume of ethyl acetate manually shaking for 5 min. After waiting for 20 minute, about 1 ml of solvent phase was removed for GC-MS analysis. All samples and controls were prepared in triplicate and incubated in a shaking incubator (Certomat BS-1) operated at 37 °C and 120 rpm. In the case of degradation experiments with recombinant lipase enzyme from recombinant *E.coli*, incubation temperature was 55 °C which is the optimum temperature for this enzyme.

5.1.2. Kinetic Studies

Time course analysis for enzymatic hydrolysis of 5 mg/L DEHP and DEP was investigated using PPL at 10, 100, 1000, and 2000 U/L. All samples and positive controls were incubated for 12 and 25 days for DEP and DEHP, respectively. PAE peak areas were measured in solvent extracts and analyzed for PAEs and hydrolysis products. Remaining concentration of PAEs was calculated based on the percent of PAE peak area at each sampling time compared to the initial peak area.

In order to determine the Michaelis-Menten kinetic parameters K_m and V_{max} values of recombinant lipase enzyme, Lineweaver- Burk plots were used assuming that the reactions followed a simple Michaelis-Menten kinetics. Lineweaver-Burk curves were obtained for both DEP and DEHP hydrolysed with 4000 U recombinant lipase enzyme dissolved in 100 mM sodium phosphate buffer at pH 7.4. Concentrations of DEP and DEHP were selected as 1, 5, 10, 15, and 20 mg/L. After 0, 1, 2, 3, and 4 h incubation time, 800 µl sample was collected from each enzyme solution and 1N HCl was added to terminate the enzymatic reaction. After that, all samples were extracted with equal volume of ethyl acetate and analyzed with GC-MS instrument. Remaining concentrations of PAEs were calculated based on the percent of PAE peak area at each sampling time compared to the initial peak area. Remaining enzyme activites were measured according to spectrophotometric enzyme assay using 50 mM paranitrophenyl palmitate as substrate.

5.2. GC-MS Analysis

GC analysis of the solvent phase was carried out with gas chromatograph (Thermoscientific, Trace GC ULTRA) with a mass selective detector (DSQ II). In order to increase sensitivity of the instrument, all samples were analyzed at selective ion monitoring (SIM) mode that enables to gather data for masses of interest rather than looking for all masses over a wide range. SIM program was set based on characteristic ions of possible DEHP metabolites provided from the literature (Feng et al. 2002, Quan et al. 2005). The signal to noise (S/N) ratio for all compounds were selected as 10 for increasing the sensitivity of the peak detection. Retention time of possible degradation products were determined by full scan mode analysis of GC-MS instrument.

Retention time and m/z values for metabolites of DEHP and DEP are given in Table 5.1 and 5.2, respectively. Operating conditions for GC for detection of metabolites of DEHP and DEP are presented in Table 5.3 and 5.4, respectively.

Table 5.1. Expected retention time and m/z values of the characteristic fragments in mass spectra of common metabolites of DEHP

Compound	m/z	Expected retention time (min)
MEHP	163, 149, 133	8.39
DMP	163, 135, 149	4.45
2-Ethylhexanol	148, 104, 76, 50	5.45
2-ethyl-hexadecanoic acid	104, 76, 56, 50	7.43
DEHP	149,167, 150	10.81
PA	104, 76, 18	3.75

Table 5.2. Expected retention time and m/z values of the characteristic fragments in mass spectra of common metabolites of DEP

Compound	m/z	Expected retention time(min)
DEP	149, 177	5.92
DMP	163, 147, 77	5.05
MEP	148, 104, 76	*
PAH	104, 76, 56	*
PA	104, 76, 18	4.20

*Peak identification was based on ion chromatogram; PAH: Phthalic anhydrate

Table 5.3. Operating conditions for GC for detection of DEHP degradation products

Instrument / Condition	Description
Gas Chromatography	Thermo Scientific (Trace GC ULTRA)
Column	HP-5 MS 5% phenyl Methyl Siloxane, film thickness: 0.25 µm, diameter: 250 µm.
Carrier gas and flow rate	Helium at 1.5 ml/min
Injection Mode	Splitless
Injection Volume	1 µl
Inlet Temperature	300 °C
Detector Temperature	150 °C
Temperature Program	0.5 min at 80 °C 80 °C to 160 °C at 30 °C per min. 160 °C to 280 °C at 15 °C per min.

Table 5.4. Operating conditions for GC for detection of DEP degradation products

Instrument / Condition	Description
Gas Chromatography	Thermo Scientific (Trace GC ULTRA)
Column	HP-5 MS 5% phenyl Methyl Siloxane, film thickness: 0.25 µm, diameter: 250 µm.
Carrier gas and flow rate	Helium at 1.5 ml/min
Injection Mode	Splitless
Injection Volume	1 µl
Inlet Temperature	300 °C
Detector Temperature	150 °C
Temperature Program	0.5 min at 80 °C 80 °C to 210 °C at 20 °C per min.

5.3. Optimization of Extraction Procedure

In order to determine the best solvent for extraction of DEHP, three different solvents were tested for extraction of DEHP from the enzyme solution. DEHP was spiked into PPL enzyme solutions at 10, 100, 1000, and 2000 U/L which are previously denatured by boiling for 10 min. Extraction efficiency was calculated as percent extraction recovery based on the peak areas measured in solvent samples with the same DEHP concentration. In addition, effect of PPL and DEHP concentration on extraction recovery was investigated for PPL and DEHP concentration at two levels which are 5 and 20 mg/L for DEHP and 10 U/L and 2000 U/L for PPL enzyme. Statistical evaluation of these factors on extraction recovery was performed with two-way ANOVA test.

The effect of HCl addition on extraction recovery was also evaluated since enzymatic reaction between PPL and PAE was terminated by adding 1 N HCl to each sample to a final concentration of 20% (v/v) before applying extraction procedure. 4 ml of heat denatured PPL solution (2000 U/L) was spiked with DEHP to a final concentration of 20 mg/L. Subsequently, 1 ml of 1 N HCl was added to stop the enzymatic reaction. After that, samples were extracted with 5 ml ethyl acetate and solvent phase was analyzed with GC-MS instrument. Effect of HCl addition on extraction efficiency was investigated by comparing the extraction efficiencies of HCl added samples and control samples

5.3.1. Repeatability of Solvent Extraction Procedure

Repeatability of solvent extraction procedure was investigated for PPL concentrations of 10, 100 , 1000 U/L, and 2000 U/L. Five samples were prepared for each DEHP concentration ranging from 5 to 20 mg/L. After applying solvent extraction procedure, DEHP peak areas were quantified by GC-MS analysis and percent RSD values for each DEHP concentration were calculated (Table 5.5). According to results obtained repeatability of the five samples with the 5, 10, 15, and 20 mg/L DEHP was good since all RSD% values were found to be lower than 15%. In addition, linearity for the GC-MS analysis of DEHP in solvent extracts was evaluated for a concentration range of 5 to 20 mg/L.

DEHP peak area versus concentration graphs for 10 and 2000 U/L are presented in Figure 5.1a and 5.1b, respectively. Coefficients of determination values for 10 U/L and 2000 U/L PPL were found as 0.996 and 0.978, respectively.

Table 5.5. Repeatability of solvent extraction procedure for DEHP

DEHP (mg/L)	PPL (U/L)			
	10	100	1000	2000
5	12.3	14.4	14.3	9.21
10	8.76	10.7	9.52	6.83
15	6.26	9.85	8.54	7.82
20	11.8	13.6	6.21	5.93

Values in the table are percent relative standard deviation (%RSD)
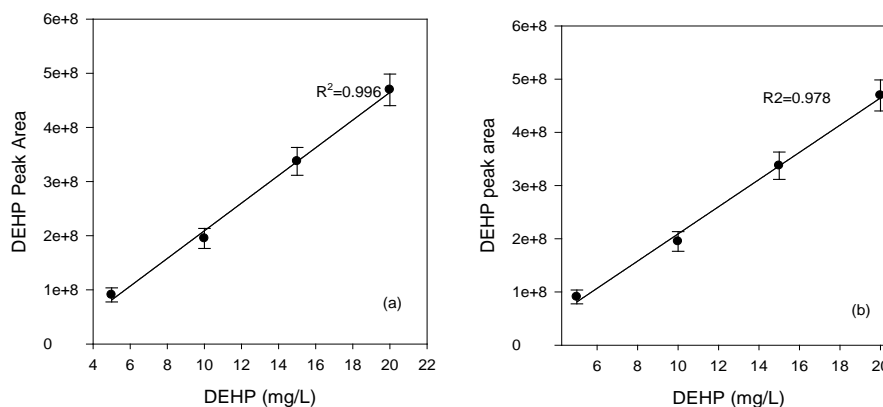
Figure 5.1. Graphical presentations of linearity plot of DEHP (5-20 mg/L)
a) 10 U/L b) 2000 U/L PPL enzyme (n=5)

5.4. HPLC Analysis

Water phase samples were analyzed with high performance liquid chromatography (Agilent, 1200) for detection of water soluble degradation products of DEHP. HPLC method was a modification of the method proposed by Shintani (2001) and gradient elution program was used for detection PA, MEHP, and DEHP. The properties of the HPLC system and operating conditions for analysis are presented in Table 5.6.

Table 5.6. Properties and operating conditions for HPLC

Units and parameters	Properties and conditions	
Column	Inertsil ODS-3V	
Column length	4.6 x 250 mm	
Particle	5 µm	
Mobile Phase(s)	A= Acetonitrile	
	B= NaAc Buffer (pH:3)	
Elution program	Time (min)	%B
	0	40
	25	10
	25.01	5
	0	60
	50	60
	0	60
Flow rate	1 ml/min	
Temperature	30 °C	
Pressure	Max pressure set 200 bar	
Detector type	Diodarray (DAD)	
Wavelength	254 nm	
Injection volume	40 µl	

5.4.1. Linearity of PAEs for HPLC Analysis

In order to evaluate the linearity of the HPLC analysis of DEHP, PA, MEHP, a standard solution of three compounds were prepared in a concentration of 0.5 to 5 mg/L.Three-compound samples were prepared and analzyed with HPLC. Concentration versus peak area graph for each PAE is presented in Figure 5.2. All three compounds were found to be linear in a concentration range of 0.5 to 5 mg/L, since R^2 levels for DEHP, MEHP, and PA were 0.998, 0.999, and 0.983, respectively.
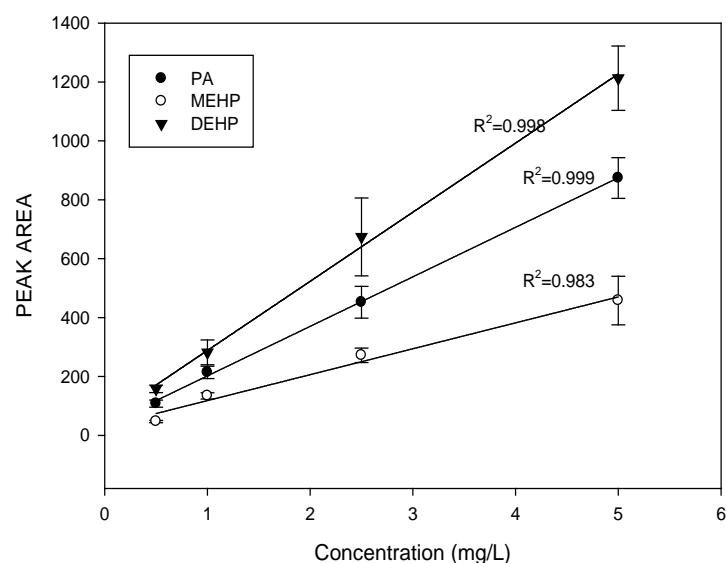
Figure 5.2. Linearity of DEHP, MEHP, and PA for concentration of 0.5 to 5 mg/L

5.5. Partial Purification of Lipase Enzyme

Purification of lipase enzyme from recombinant *E.coli* that includes lipase gene from different thermoalkalophilic bacteria (*Geobacillus* strains) isolated from thermal environmental samples in Balçova (Agamemnon) geothermal site (Yavuz et al. 2004). Experimental methods reported by Tekedar and Şanli (2011) were followed for expression and purification of lipase enzyme. Purification was carried out using recombinant *E. coli* strain BL21 (DE3) which has lipase genes in the pET-28a (+) expression vector.

The expression of lipase gene was induced by addition of the synthetic lactose isopropyl-β-Dthiogalactopyranoside (IPTG) which depresses the lac operator and allows the expression of T7 RNA polymerase and transcribes the target gene. All media included 30 µg/ml kanamycin since pET expression vector contained kanamycin resistant marker. E. *coli* strains numbered as 21 and 33 which refer isolates from uncontrolled thermal leak and isolate from reinjection water, respectively, were used for lipase enzyme expression. The strains that stored at -80 °C were spread on two LB plates containing kanamycin and incubated overnight at 37 °C. After that, a single colony was chosen from the plate and inoculated in 10 ml LB broth containing 30 µg/ml kanamycin and incubated at 37 °C, 100 rpm for 22 h.

Following day cultures were diluted 1:10 into 100 ml media and grown at 37 °C to an optical density of about 1 at 600 nm which corresponds to mid-logarithmic phase. At that point, IPTG (Fermentas) was added into each culture to 100 µM concentration for expression of lipase gene and incubated for 4 h. After that, cells were harvested by centrifugation at 4000 rpm and 4 °C for 20 min. The harvested cell pastes were stored frozen at –20 °C until purification process. Purification of lipase was performed using His-taq nickel affinity gel column (Sigma) and low pressure liquid chromatography system (Visco). The cell pellets stored at -20 °C were dissolved in 10 ml of 50 mM sodium phosphate buffer at pH 7. Subsequently, cells were lysed by a sonicator (Bendelin UW 2070) for 5 min. Insoluble material was removed after centrifugation at 4000 rpm and 4 °C for 20 min and the supernatant was loaded onto a 2.5 cm x 10 cm column previously equilibrated with phosphate buffer (50 mM sodium phosphate buffer at pH 7). Then column was washed with 100 ml phosphate buffer including 0.3 M NaCl in order to remove unbound protein and elution of the lipase was performed with 250 mM imidazole in phosphate buffer including 0.1 M NaCl. The fractions collected from the column were analyzed with nanodrop (Thermo Scientific, 8000) in order to determine approximate proteins concentration by measuring the absorbance values at 280 nm. Then, collected fractions were pooled and dialysed against 50 mM sodium phosphate buffer at pH 7 overnight at 4 °C to remove imidazole from the solution

5.6. Enzyme Assay

Activity of recombinant lipase was measured sprectrophotometrically using *p*-nitrophenyl palmitate (Sigma) as a substrate. The assay was based on hydrolytic cleavage of *p*-nitrophenyl ester by enzyme to release *p*-nitrophenol and palmitic acid.

The assay mixture (1 ml) contained 10 µl of 50 mM PnPP dissolved in acetonitrile (Reidel, HPLC grade), 10 µl enzyme solution, and 980 µl reagent-A (100 mM sodium phosphate buffer with 150 mM sodium chloride, and 0.5% (v/v) Triton X-100, pH 7.4). Absorbance measurements were carried out at 400 nm and 55 °C. Blank samples which contained ultrapure water instead of substrate and the absorbance change of the samples, and then activity was calculated according to following formula,

$$U/ml \quad enzyme = \frac{\left[\left(\Delta A400\right)_{test} - \left(\Delta A400\right)_{blank}\right]\left(1ml\right)\left(df\right)}{\left(0.018\right)\left(0.01\right)} \tag{5.1}$$

where 1 ml is the volume of assay; df is dilution factor; 0.0148 is micromolar extiction coefficient of paranitrophenol at 400 nm; 0.01 is the volume (in ml) of enzyme used.

The assay was performed using a thermostated spectrophotometer (Shimadzu UV-2450) at 55 °C which is optimum temperature for the enzyme. One unit of lipase activity was defined as the amount of enzyme releasing 1.0 micromole of p-nitrophenol per minute at pH 7.4 at 55 °C using p-nitrophenyl palmitate as a substrate.

5.7. Determination of Molecular Mass of Recombinant Lipase

Homogeneity and molecular weight of recombinant lipase was determined by sodium dodecyl sulfate-polyacyrlamide gel electrophoresis (SDS-PAGE). An SDS-12% polyacyrlamide gel was prepared by the method of Laemmli (Laemmli, 1970). SDS gels contained 10% (w/v) SDS and 30% acrylamide mixture, 1.5 M Tris-HCl buffer, pH 8.8, 10% (w/v) ammonium per sulfate and tetramethylethylenediamine (TEMED). 30 µl of protein samples were added into 30 µl of sample buffer containing 3 ml of distilled water, 1 ml of 0.5 M Tris-HCl at pH 6.8, 1.6 ml glycerol, 1.6 ml 10% SDS, 0.4 ml mercaptoethanol and 0.4 ml 0.5%(w/v) bromophenol blue. After that all samples were heated to 95 °C for 10 min.

A protein molecular marker (Fermentas, #SM0431) contained β-Galactosidase (116.0), bovine serum albumin (66.2), Ovalbumin (45), Lactase dehydrogenase (35), Rease Bsp 981 (25), and β-lactoglobulin (18.40 kDa) were used as reference and loaded on the gel. Electrophoresis was carried out with a vertical mini-gel system (Bio-Rad, Miniprotean Tetra) by applying 100 volts for 2 h. After that gel was removed from the tank when the blue dye reached to bottom of the gel. Then gel was placed in staining solution containing 0.05% coomasie blue and waited for 24 h at 40 rpm. Destaining was performed with a solution containing 13% trichloroacetic acid (Reidel), 5% methanol (Reidel), and 82% distilled water. At the end of these processes, the photo of the gel was taken using a gel imaging system (Bio-Rad, Versadoc 4000 MP).

5.8. Determination of Protein Concentration

Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method (Bradford, 1976). Bovine serum albumin was used as a standard protein. Protein samples and protein standards are processed in the same manner by mixing them with assay reagent and using a spectrophotometer to measure the absorbance. A standard curve was prepared for protein concentration ranging from 0.03 to 0.5 mg/ml. Protein concentration of the enzyme samples were determined using the equation obtained from the standard curve Absorbance values measured at 595 nm and predicted protein concentration for six crude lipase solution aliquated in 2-ml eppendorf vials.

CHAPTER 6

RESULTS AND DISCUSSION

6.1. Optimization of Analytical Methods

6.1.1. Optimization of Solvent Extraction Procedure

Results of the experiments that were performed to determine the best solvent for extraction procedure suggested that ethylacetate has the highest percent recovery for extraction of DEHP (Figure 6.1). Relative percent standard deviation values for ethyl acetate, dichloromethane, and hexane were 14.81, 17.20, and 24.94%, respectively. Supporting this result, Pietrogrande et al. (2003) reported that ethyl acetate is the best solvent for extraction of PAEs since this solvent resulted in a high recovery (> 95%) with a good reproducibility (%RSD < 5, n=3).
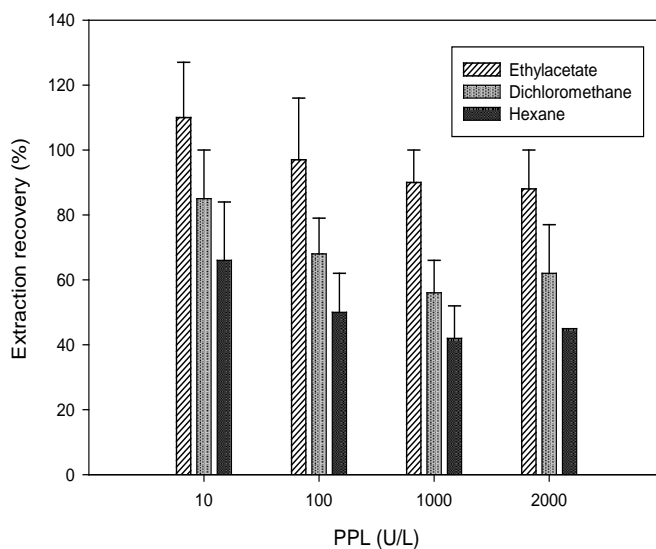
Figure 6.1. Effect of solvent type on extraction recovery of DEHP

6.1.2. Effect of PPL and DEHP Concentration on Extraction Efficiency

Two-way ANOVA test was performed to determine the effect of PPL and DEHP concentration on solvent extraction recovery. Results of the two way-ANOVA test suggested that the differences in mean recovery values among different DEHP and PPL concentrations were not significant since all p-values were found to be higher than 0.05 (Table 6.1). Therefore, it can be concluded that, percent recoveries for solvent extraction procedure are acceptable and does not affected by both enzyme and DEHP concentration.
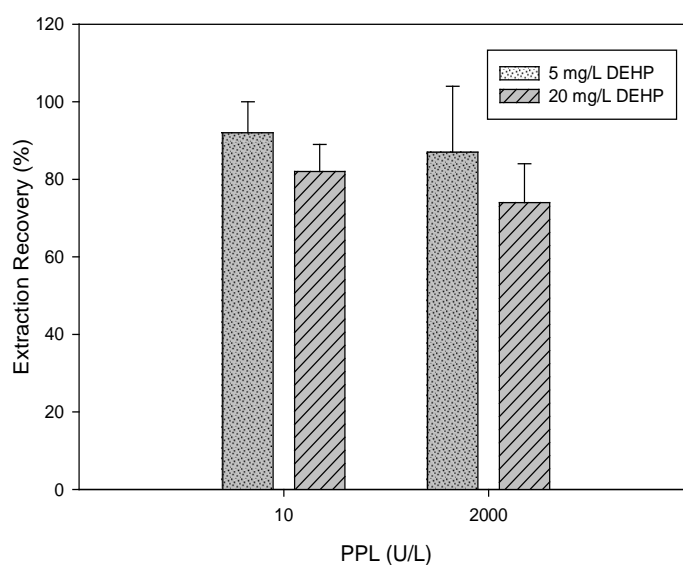
Figure 6.2. Effect of PPL and DEHP concentration on extraction recovery
(Error bars show one standard deviation, n=5)

Table 6.1. Results of two-way ANOVA: R% versus DEHP, PPL

Source	DF	SS	MS	F	P
DEHP	1	132.25	132.25	58.78	**0.083**
PPL	1	42.25	42.25	18.78	**0.144**
Error	1	2.25			
Total	3	176.75			
S = 1.5		R-Sq = 98.73%		R-Sq(adj) = 96.18%	

6.1.3. Effect of HCl Addition on Extraction Recovery

Effect of HCl addition on extraction efficiency was investigated by comparing the extraction efficiencies of HCl added samples and control samples. Integration results for DEHP peak area, % RSD and percent recovery (%R) values are presented in Table 6.2. Control samples and HCl added samples were compared in terms of extraction recovery applying two sample t-test and the results suggested that addition of HCL did not show a significant effect on percent recovery ($p=0.095$).

Table 6.2. Effect of HCl addition on solvent extraction efficiency

Sample	Average DEHP peak area[‡]	SD[*]	%RSD	%R
Control samples[a]	895190553	31492709	3.52	90
HCl added samples[b]	969693701	50071093	5.16	97
5 ml ethyl acetate[c]	994967844	52666411	5.29	-

[a] Samples that do not contain HCL, [b] 1N HCl added samples (final concentration 20%)
[c] 5 ml ethyl acetate spiked to 20 mg/L DEHP (n=5) [*]Standard Deviation

6.1.4. Effect of Pretreatment Method on HPLC Analysis of DEHP

In order to remove particulate material from the enzyme solution before HPLC analysis, filtration is generally applied as pretreatment method. In order to investigate the effect of filtration on HPLC analysis of PA, MEHP, and DEHP, 5 ml of sodium phosphate buffer (100mM, pH: 7.4) was spiked with PA, MEHP, and DEHP to a final concentration of 5 mg/L. Then all samples were filtered through a 0.45 µm PTFE syringe filter (Rotilabo) prior to HPLC analysis. In addition, buffer solutions without enzyme, were also spiked with PA, MEHP, and DEHP for comparison with the filtered samples. HPLC analysis results for the filtered and buffer spiked with PA, MEHP, and DEHP are presented in Figure 6.3.
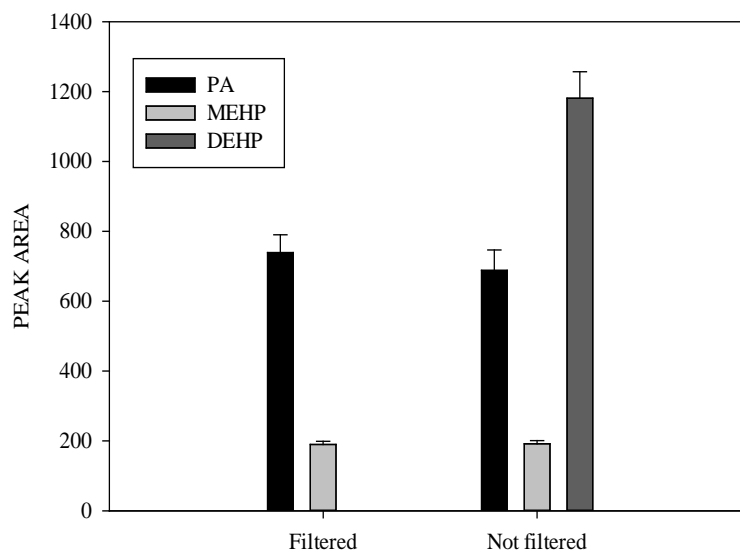
Figure 6.3. Effect of filtration on HPLC analysis of PA, MEHP, and DEHP

The results suggested that, after filtration through 0.45 µm PTFE filter DEHP peak was disappeared while PA was detected with the same amount as the samples that are not filtered. This could be attributed to higher hydrophobicity of DEHP compared to MEHP and PA. Hydrophobic property of these compounds could result in adsorption on the filter media. For this reason, all water phase samples were pretreated by centrifugation at 10,000 rpm for 10 min. before HPLC analysis.

6.2. Degradation of PAEs by PPL

Studies on enzymatic degradation of PAEs have reported that these compunds could be hydrolysed with lipase or esterase enzymes from various sources including bacteria (Albro and Latimer 1974, Kurane et al. 1980, Soontornchat et al. 1994), pancreatic lipase (Saito et al. 2010) mammalian enzymes, such as nonspecific lipase from rat pancreas (Duran and Esposito 2000, Sutherland et al. 2004, Chang et al. 2007), esterase from rat intestine (Soontornchat et al. 1994), carboxyl esterase from rats and humans (Albro and Latimer 1974), and human salivary esterase. All these enzymes catalyze the hydrolysis of ester bonds in PAEs resulting in formation of corresponding monoesters and alcohols (Liang et al. 2008).

In order to investigate enzymatic hydrolysis of DEHP and DEP, commercial porcine pancreas enzyme (E.C. 3.1.1.1) was used in this study. Effect of experimental parameters such as pH and enzyme concentration on enzymatic hydrolysis of PAEs was studied. Time course of DEHP and DEP degradation was evaluated with different enzyme concentrations to determine the kinetics of degradation mechanisms.

6.2.1. Identification of Degradation Products of DEHP

In order to determine enzymatic degradation products, 20 mg/L DEHP was incubated with 20,000 U/L PPL enzyme for 7 days. PPL and DEHP concentration was selected at high concentration based on Gavala et al. (2004) who studied 1000 and 10,000 U/L enzymatic units of esterase per liter for removal of PAEs from contaminated waste sludge. GC-MS analysis of the solvent extracts indicated that enzymatic hydrolysis products PA, DMP, and MEHP were produced as a result of enzymatic hydrolysis reaction (Figure 6.4).

Supporting this finding, formation of PA and MEHP via enzymatic hydrolysis or bacterial metabolism of DEHP was reported in the literature (Kurane et al. 1980, Ejlertsson and Svensson 1995, Zeng et al. 2004, Nakamiya et al. 2005, Saito et al. 2010).GC-MS chromatogram for 20 mg/L DEHP incubated with 20,000 U/L PPL is presented in Figure 6.4. Among all degradation products, the peak area of the MEHP which is the first hydrolysis product of DEHP was higher than the other compounds. Further hydrolysis of MEHP resulted in PA formation; however, all MEHP could not be converted to PA possibly due to steric hindrance of monoethylhexyl phthalate that have carboxyl groups inhibiting enzyme to bind the substrate (Liang et al. 2008). None of the degradation products were detected in positive control samples suggesting that there was no physical or chemical degradation of DEHP during the incubation time. Compared to positive control samples, about 92% decrease was observed in DEHP peak areas. In addition to MEHP and PA, DMP was detected after 7-d of incubation time. DMP was possibly formed via transesterification reaction which is defined as the nucleophilic substitution of one alcohol by another alcohol from an ester (Morrison, 1992). Supporting this result, Okamato et al. (2011) reported that phthalic acid esters undergo transesterification reaction in the presence of methanol.

Kim et al. (2006) also reported that ethyl methyl phthalate (EMP) and dimethyl phthalate (DMP) are produced as methylated intermediates of DEP in the presence of 0.1% (v/v) methanol. Similarly, Quan et al. (2005) reported that DMP is formed as a result of methylation of insoluble degradation products of DEHP after solvent extraction from the reaction mixture.
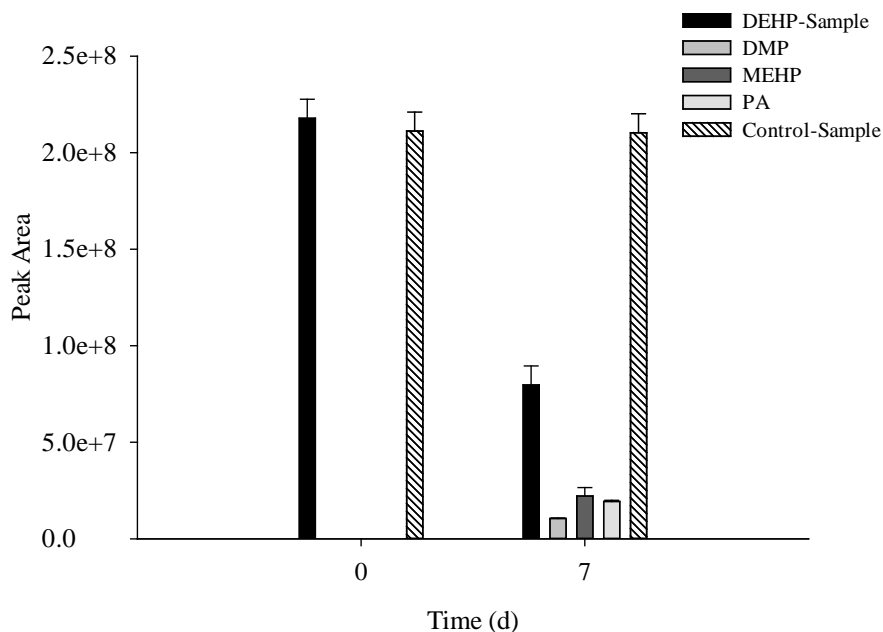
Figure 6.4. GC-MS analysis results for degradation of 20 mg/L DEHP with 20,000U/L PPL

HPLC analysis was performed for detection of water soluble degradation products of DEHP. After 7 d of incubation time, DEHP degradation products were not detected in water phase due to possibility of having not enough amount accumulated in water phase for the detection of HPLC. Therefore all samples were incubated for an additional 7 days and HPLC analysis results for 14-d water phase samples are presented in Figure 6.6. In this case MEHP and DEHP were detected in water phase samples, unfortunately PA peak was not observed. The results of HPLC analysis of water phase indicated that percent decrease in DEHP peak area was about 30% after 14 days of incubation time. However, GC-MS analysis results showed that about 92% decrease after 7 days. This difference could be due to low water solubility of DEHP that leads to difference in GC-MS and HPLC analysis.

Another possible reason is that before HPLC analysis samples were centrifuged and this may lead to adsorption of some PAE on the enzyme particles and removed from the reaction mixture. In addition, 20 mg/L DEHP was not completely degraded after incubation with 20,000 U/L PPL for 14-d possibly due to the presence of 2-ethyl side-branches in DEHP that limits the access of the enzyme to the ester bonds and therefore limits the overall rate of hydrolysis (Sauvageau et al. 2009). Supporting this result Schaeffer et al. (1979) also reported that action of enzymes causing β-oxidation are inhibited by 2-methyl or 2-ethyl side-branch of the DEHP. As a result of this experiment, it was decided that a lower environmentally relevant DEHP concentration should be selected for further experiment.
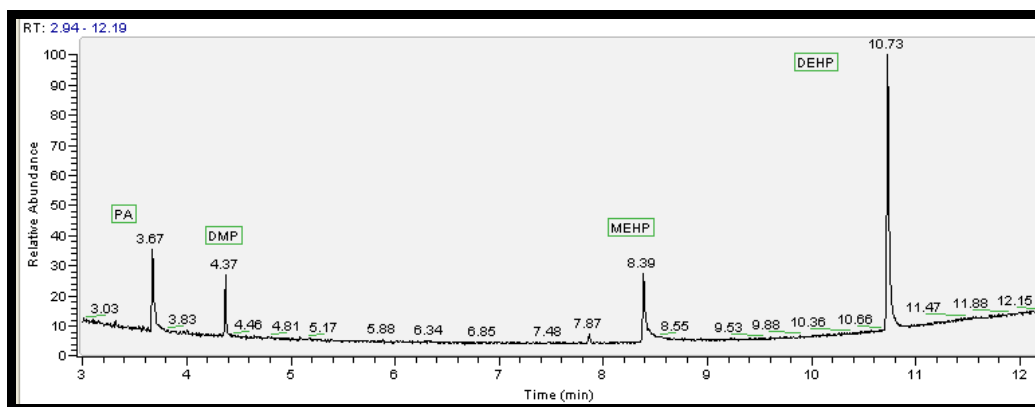
Figure 6.5. GC-MS chromatogram for 20 mg/L DEHP incubated with 20,000 U/L PPL
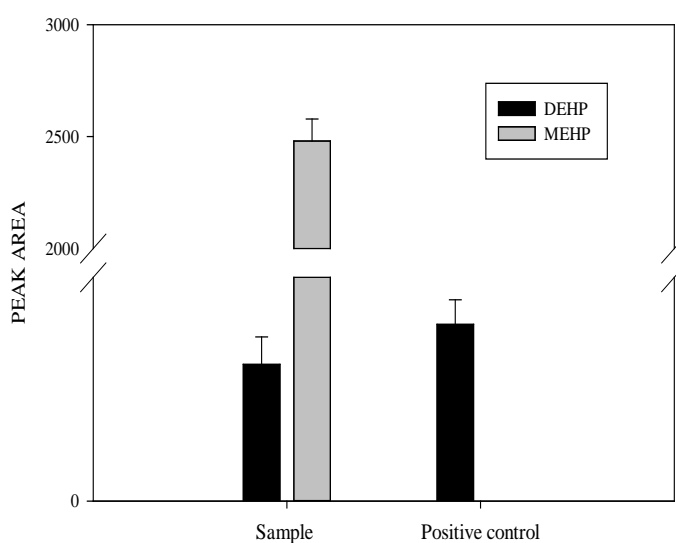
Figure 6.6. HPLC analysis results for 20 mg/L DEHP incubated with 20,000 U/L PPL

6.2.2. Identification of Degradation Products of DEP

In order to determine enzymatic degradation products of DEP, 20,000 U/L PPL was incubated with 20 mg/L DEP at 37 °C and 120 rpm for 7 days. GC-MS analysis of the samples was performed after 0 and 7 d for detection of DEP and hydrolysis products. The peak areas quantified in samples and controls are presented in Figure 6.7. DMP and DEP peaks were detected on the GC-MS chromatograms (Figure 6.8). The mass spectrum of detected DMP peak is presented in Figure 6.9. After 7 days of incubation time, about 53% decrease in DEP peak areas was observed compared to positive control samples. DMP was produced as a result of a possible transesterification reaction of DEP in the presence of methanol. However, enzymatic hydrolysis products of DEP, EMP, and PA was not detected after 7 days possibly due to insufficient incubation time or inhibition effect of PPL enzyme at high concentration. The reason for not detecting hydrolysis products of DEP could also be related to its molecular structure that readily undergo transesterification reaction in the presence of methanol and producing DMP.
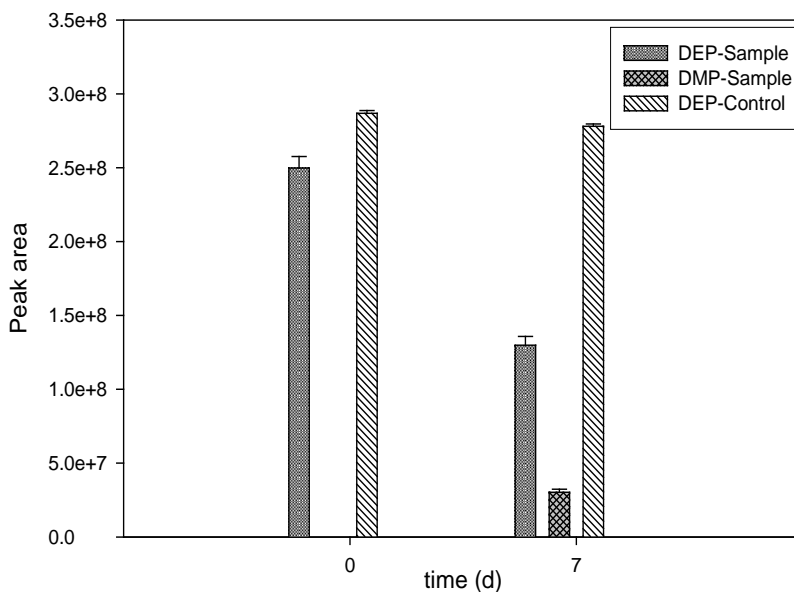
Figure 6.7. GC-MS analysis results for degradation of 20 mg/L DEP
with 20,000 U/L PPL
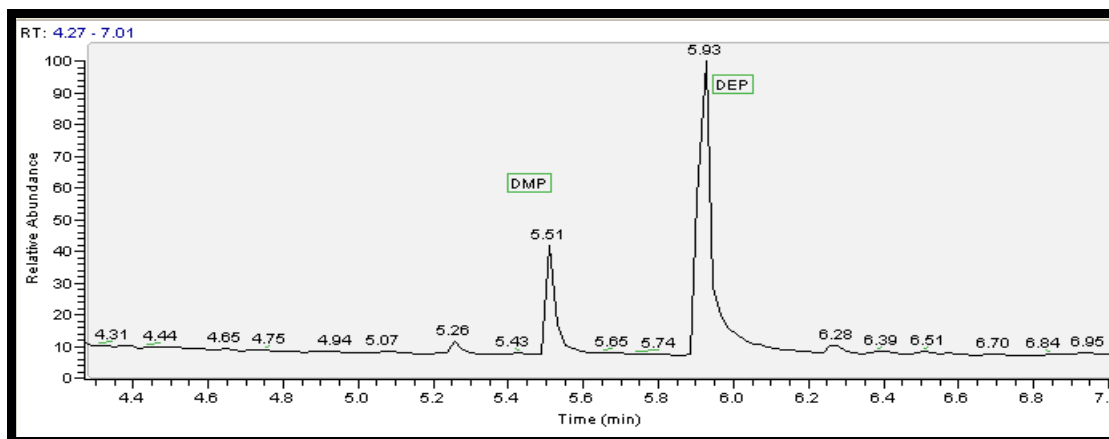
Figure 6.8. GC-MS chromatogram of 20 mg/L DEP with incubated with
20,000 PPL (DMP: 5.51 min, DEP: 5.93 min)

6.3. Two-Way ANOVA Design: Effect of PPL and DEHP Concentration

In this experiment effect of PPL enzyme and DEHP concentration on degradation of DEHP was investigated using each factor at two levels with three replicates. Experimental variables and factors levels are presented in Table 6.3. Percent degradation was calculated based on DEHP peak areas obtained from positive control samples. Samples with 1000 U/L resulted in percent degradation of 93 and 96% for 5 mg/L and 0.1 mg/L lipase concentrations, respectively.

In the case of 20,000 U/L PPL, 74 and 55% degradation was obtained for 5 mg/L and 0.1 mg/L DEHP, respectively (Figure 6.10). Results of the ANOVA analysis suggested that PPL concentration significantly affects the percent degradation of DEHP ($p<0.0001$). However, DEHP concentration resulted in a p-value of 0.14 suggesting that there is no significant effect on degradation efficiency of DEHP (Table 6.3). Results of the ANOVA analysis also suggested that PPL concentration significantly affects the percent degradation of DEHP ($p<0.0001$). Increasing enzyme concentration from 1000 U/L to 20,000 U/L decreases degradation efficiency possibly due to inhibition effect of the enzyme at these concentrations. For this reason, further enzymatic degradation experiments were performed at PPL concentration range of 10-2000 U/L. Contour plot for DEHP and PPL concentration showed that, PPL concentration below 2500 U/L results in higher than 90% degradation of DEHP (Figure 6.9).

However, about 92% degradation was obtained after incubation of 20 mg/L DEHP with 20,000 U/L PPL for 7 days. These results suggest that when the enzyme concentration increased substrate concentration should also be increased to prevent inhibition effect of enzyme.

Table 6.3. Factors and levels used in ANOVA design[*]

| Factor setting | Experimental Variables | |
	Enzyme (U/L)	DEHP (mg/L)
High (+)	20,000	5
Low (-)	*1000*	*0.1*

[*]t= 7 days; 37 °C and 120 rpm, n=5

Table 6.4. Results of two-way ANOVA design for effect of
PPL and DEHP concentration on degradation

Source	DF	SS	MS	F	P
DEHP	1	102.73	102.73	2.73	**0.137**
PPL	1	3585.60	3585.60	95.38	**0.000**
Interaction	1	23.49	23.49	0.62	**0.452**
Error	8	300.74	37.59		
Total	11	4012.56			
$S = 6.131$ $R^2 = 92.50\%$ R^2 (adj) = 89.69%					

DF: Degrees of freedom, SS: Sum of squares, MS: Mean square, F:F-values, P: P-value
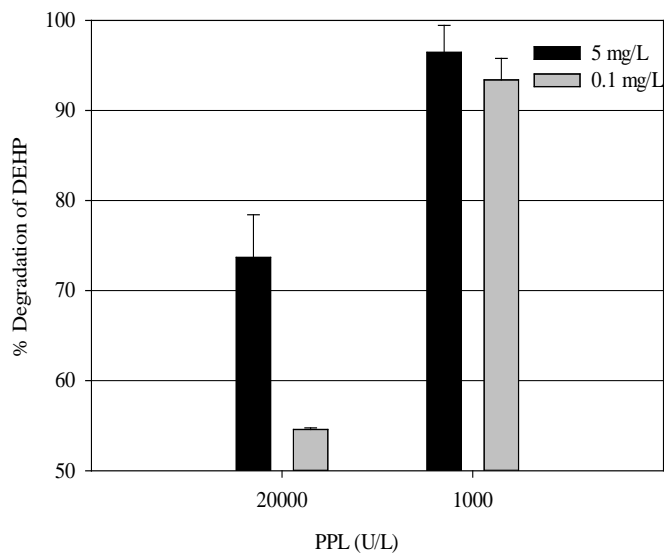
Figure 6.9. Effect of concentration of PPL and DEHP on percent degradation
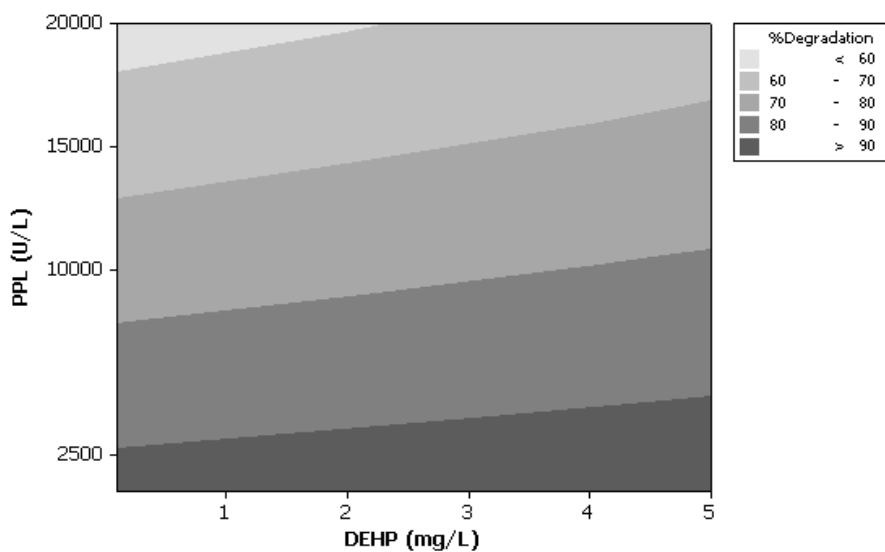
Figure 6.10. Contour plot for percent degradation (%D) of DEHP as a function
PPL (U/L) and DEHP (mg/L) concentration

6.4. Effect of PPL Concentration on Enzymatic Degradation of PAEs

After identification of possible degradation products of DEHP and DEP as a result of enzymatic hydrolysis with PPL enzyme, the effect of enzyme concentration on PAE degradation was investigated with 10, 100, 1000, and 2000 U/L PPL enzyme.

Concentration of DEHP was chosen as 5 mg/L. In this experiment, both DEHP and DEP were evaluated in terms of enzymatic hydrolysis with PPL in order to compare the degradability of these two PAEs with different molecular structure.

6.4.1. Effect of PPL Concentration onEnzymatic Degradation of DEHP

After incubation of 5 mg/L DEHP with 10, 100, 1000, and 2000 U/L PPL enzyme, DEHP peak areas in the control samples were found to be nearly constant for all PPL concentrations (Figure 6.12). In the case of samples, DEHP peak areas decreased by increasing the PPL concentration. Percent decrease in the peak areas compared to positive control samples were determined as 57, 55, 58, and 67% for 10, 100, 1000, and 2000 U/L PPL, respectively.

This experiment suggested that the highest degradation of DEHP was obtained with 2000 U/L PPL enzyme and the decrease in DEHP peak area was about 67% for 7 days of incubation time. Saito et al. (2010) reported that 93% of the 100 mg/L DEHP was hydrolysed by 1470 U/ml crude lipase enzyme within 24 h incubation time. Hydrolysis time for DEHP was much lower than the levels used in this study since the enzyme concentration was about 1500 times greater than the concentration used in this study. In addition, the difference in percent degradation may be related to DEHP concentration difference. In another study, half-life of DEHP incubated with 1000 U/L commercial esterase from porcine liver was reported as 6 days (Gavala et al. 2004). Similary, 55% degradation was obtained with 1000 U/L PPL enzyme after 7 days of incubation time in this study. There was a small difference between these two studies in terms of degradation efficiency possibly associated with the distinction between the interfacial activation of esterase and lipase enzymes. Unlike lipase enzyme esterase enzyme hydrolyzes water soluble short acyl chain esters, this enzyme may be inactive against water‐insoluble long chain triacylglycerol (Chahinian and Sarda, 2009, Mita et al. 2010).

MEHP, which is the first metabolite of DEHP hydrolysis, was detected in samples that contain 5 mg/L DEHP incubated with 10 U/L PPL enzyme for 7 days. When PPL concentration was increased to 100 U/L and 1000 U/L, MEHP peak was not detected, however PA peak was observed possibly due to further hydrolysis of MEHP (Figure 6.13 and 6.14).

Enzyme concentration of 10 U/L was probably not sufficient for complete hydrolysis to PA in 7 days, whereas it was sufficient at 100 and 1000 U/L PPL levels. In consequence, at least 100 U/L PPL is needed for substantial degradation of DEHP. It was reported that sequential hydrolysis of PAEs, in which phthalate diesters are serially converted to phthalate monoester and PA, occurs via de-esterification reaction (Shelton et al. 1984). Formation of PA also reported in many studies as hydrolysis product of DEHP via bacterial metabolism (Feng et al. 2002, Quan et al. 2005, Chen et al. 2007, Liang et al. 2008) or enzymatic degradation (Saito et al. 2010).

Figure 6.12. Effect of PPL concentration on DEHP degradation with PPL enzyme
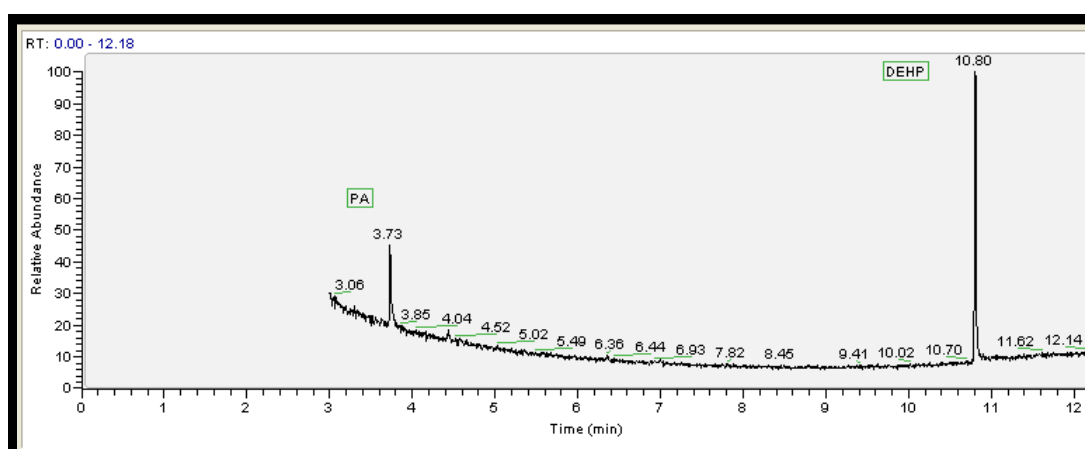
Figure 6.13. GC-MS chromatogram for 5 mg/L DEHP incubated with 1000 U/L PPL enzyme (RT of PA: 3.73 min, DEHP: 10.80 min)

6.4.2. Effect of PPL Concentration on Enzymatic Degradation of DEP

Effect of PPL enzyme on enzymatic degradation of DEP was also investigated in order to evaluate the effect of molecular structure on enzymatic hydrolysis of PAEs. For this purpose DEP, which has a short alkyl side chain compared to DEHP, was used in enzymatic degradation experiments.

It is known according literature that major degradation products of DEP are monoethyl phthalate (MEP), phthalic acid (PA), and further hydrolysis oxidation product; 3,4-dihdroxy benzoic acid (Amir et al. 2005, Cartwright et al. 2006). After 7 days of incubation of 5 mg/L DEP with PPL enzyme, peak areas of DEP and hydrolysis products were determined by GC-MS analysis and results are presented in Figure 6.15. Quantification of the peak areas suggested that the highest PA formation was obtained with 2000 U/L PPL enzyme concentration. DMP peak was detected with 1000 and 2000 U/L PPL enzyme the peak areas was substantially low compared to DEP and PA. When enzyme concentrations were compared in terms of PA formation it was found that there is a statistical difference between the peak areas (*p=0.003*). GC-MS chromatogram for the 5 mg/L DEP incubated with 1000 U/L PPL for 7 days is presented in Figure 6.14. The highest PA was produced with 2000 U/L PPL.
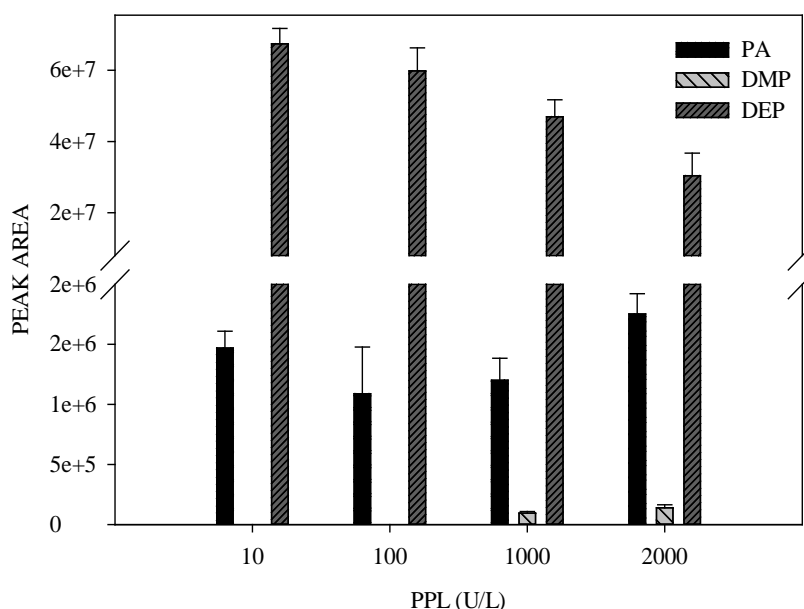
Figure 6.14. GC-MS analysis results for the effect of PPL
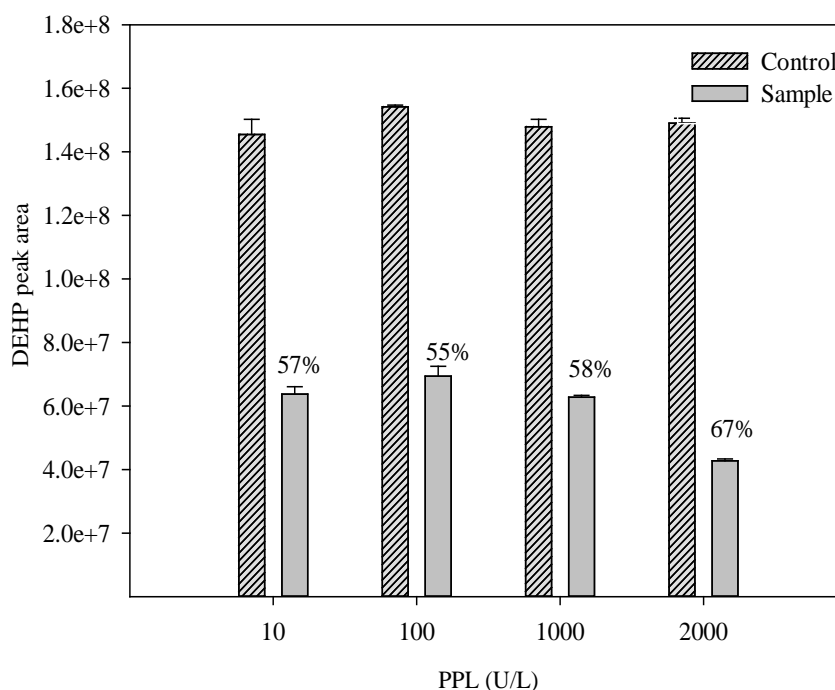concentration on degradation of 5 mg/L DEP
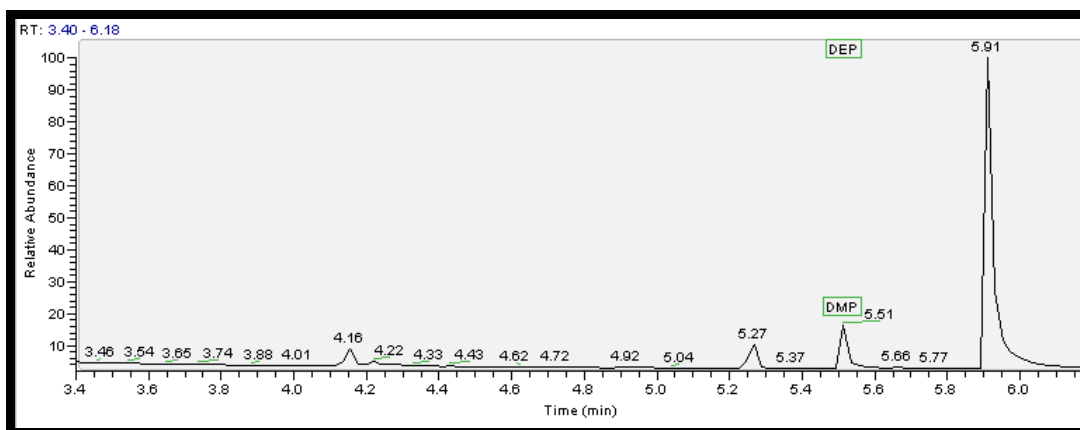
Figure 6.15. GC-MS chromatogram for 5 mg/L DEP incubated with
1000 U/L PPL for 7 days

Peak areas of DEP were also measured in positive control samples that were run parallel with other samples. After quantification of the peak areas in both samples and positive controls, percent decrease in DEP peak area was found as 80, 82, 87, and 92% for 10, 100, 1000, and 2000 U/L PPL concentrations, respectively (Figure 6.16). According to results obtained with this experiment, incubation of 5 mg/L DEP with 2000 U/L PPL results in 92% degradation for 7 days of incubation time, a better degradation performance compared to DEHP, the longer alkyl side chain PAE.
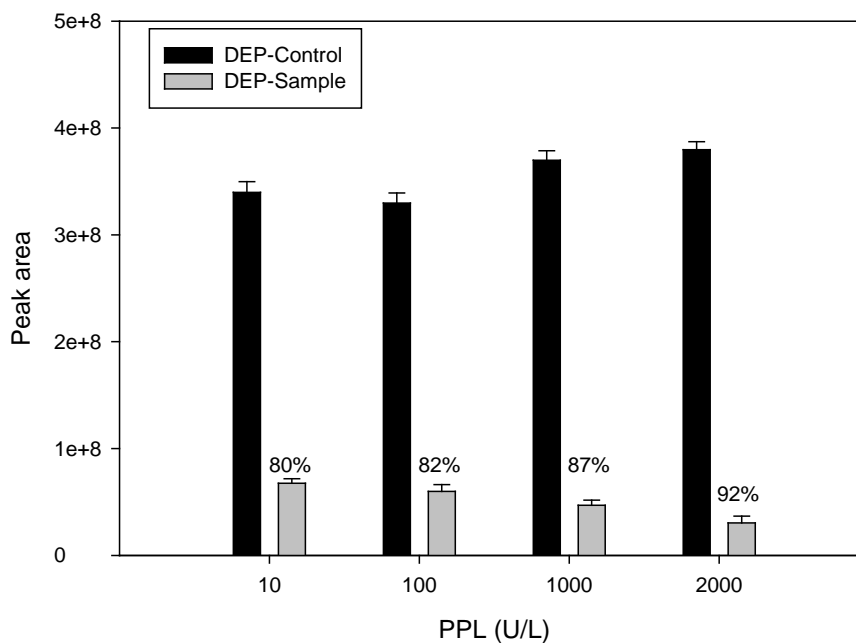
Figure 6.16. GC-MS analysis results for 5 mg/L DEP incubated with
PPL enzyme

6.5. Effect of pH on Enzymatic Degradation of DEHP

The pH of the surrounding environment is important for the enzymes since the protein structure of the enzyme molecule is stable at a specific pH value that provides a constant total net charge. Therefore, the changes in charges with pH affect the activity, structural stability, and solubility of the enzyme. According to studies on enzymatic degradation of PAEs, optimum pH level for hydrolysis of PAEs with microbial lipase or esterase enzyme ranges between 7 and 7.5 (Kurane et al. 1980, Kim et al. 2002, Kim et al. 2007, Gavala et al. 2004, Saito et al. 2010). In addition, degradation studies on PAEs based on isolation of micoorganims from contaminated areas generally performed at pH level ranging from 6.5 to 8 (Chang et al. 2004, Zeng et al. 2004, Nakamiya et al. 2005, Chen et al. 2007). It is also known that the optimum pH level for PPL enzyme for hydrolysis of triaclyglycerols is 8 and one unit of lipase activity is defined as the enzyme amount that releases 1 µeq H^+ per min at 37 °C at pH 8 (Garner and Smith,1972). To determine the optimum pH level for hydrolysis of DEHP with PPL enzyme, percent decrease in DEHP peak area was evaluated at pH levels of 6, 6.5, 7.0, 7.5, and 8. Percent decrease in peak area of DEHP was determined by comparing the peak areas in samples and positive controls (Figure 6.17). Percent degradation of DEHP was found to be higher than 95% at pH levels ranging between 6.5 and 8. Statistical comparison of the pH levels of 6.5, 7, 7.5, and 8 suggested that there is no statistical difference between these pH levels in terms of percent degradation in DEHP peak area (p= *0.082*).

Effect of pH on enzymatic degradation of DEHP was evaluated at pH levels of 7.4 and 8. The pH level of 8 was the optimum pH level for hydrolysis of tributryin with PPL enzyme. Therefore, effect of pH on both percent decrease in DEHP peak areas and product formation was evaluated at pH levels of 7.4 and 8. DEHP and PPL enzyme levels were 5 mg/L and 1000 U/L, respectively. After 7 days of incubation time integrated peak areas for DEHP and PA are presented in Figure 6.18. Although percent decrease in peak area was nearly same for pH 7.4 and 8, PA formation was higher at pH 7.4 compared to pH 8. When the peak areas at pH 7.4 and 8 were compared it was observed that PA formation at pH 7.4 was about 2 times higher than pH 8. Therefore, the optimum pH level for hydrolysis of DEHP was determined as 7.4.
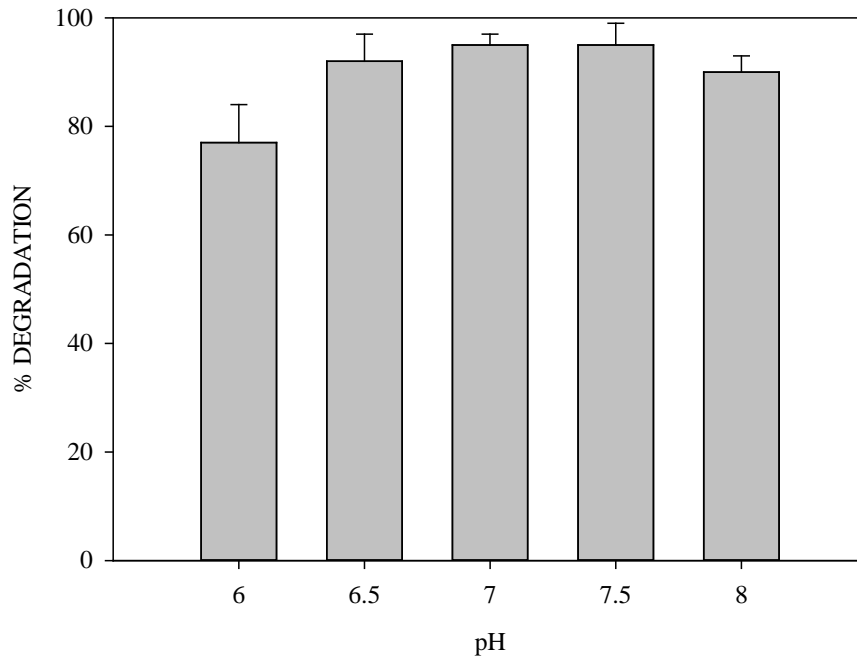
Figure 6.17. Effect of pH on enzymatic degradation of DEHP
(5 mg/L DEHP and 1000U/L PPL, 7-d at 37 °C)
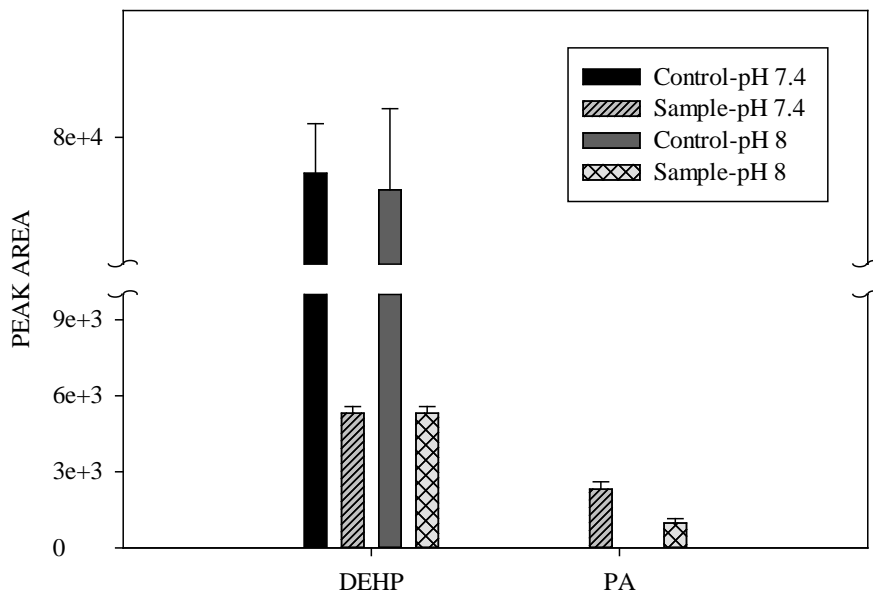
Figure 6.18. Effect of pH on enzymatic degradation of DEHP by PPL enzyme
(5 mg/L DEHP and 1000 U/L PPL, 7 days at 37 °C)

HPLC analysis of water phase samples at pH 7.4 and 8 was performed after 7 and 15d incubation time. Analysis results showed that MEHP which is the first hydrolysis product of DEHP was detected in samples at pH 7.4. The quantification of the MEHP peak areas indiciated that peak areas in samples was nearly same after 7 and 15 days of incubation time (Table 6.5). In addition, MEHP formation was not observed in water phase samples at pH 8 after both 7 and 15 days incubation time.

Table 6.5. HPLC analysis results for the effect of pH on enzymatic degradation of DEHP[*]

Incubation time (d)	pH	Sample code	MEHP peak area	DEHP Peak area
7	7.4	S	138.83	419.13
		C	0	458.00
15		S	135.97	436.00
		C	0	438.00
7	8	S	0	420.73
		C	0	426.93
15		S	0	432.00
		C	0	418.00

[*]5 mg/L DEHP and 2000 U/L PPL, 7 days at 37 °C, 120 rpm; S: Sample, C: Control

6.6. Effect of pH on Enzymatic Degradation of DEP

According to the results obtained in the previous experiment, the highest degradation for DEHP was obtained at pH 7.4. Therefore, pH effect on enzymatic hydrolysis of DEP (5 mg/L) was evaluated using 2000 U/L PPL at pH levels of 7.4 and 8. Results of this experiment suggested that both percent DEP degradation and DMP formation was higher at pH level of 7.4 compared to pH 8 (Figure 6.19). In addition, this peak was not detected in none of the control samples suggesting that there is no physical or chemical degradation of DEP during the incubation time. According to literature it is known that enzymatic hydrolysis reaction of DEP proceeds by sequential hydrolysis of C-O bonds linking the carboxyl group of the phthalate to the ethyl chain, forming the MEP and then PA (Cartwright et al. 2006).

Then, formation of ethyl methyl phthalate (EMP), dimethyl phthalate (DMP), and monomethyl phthalate (MMP) could occur via demetylation or transesterification reactions. Okamato et al. (2011) reported that PAEs undergo both hydrolysis and transesterification reactions in the presence of an alcohol. Dipropyl phthalate was transformed to monopropyl phthalate, dimethyl phthalate, and monomethyl phthalate via transesterification with methanol. Kim et al. (2002) also reported formation of buthyl methyl phthalate and as a result of biodegradation of benzyl buthyl phthalate by fungal cutinase and yeast esterase in the presence of methanol (0.1% v/v).
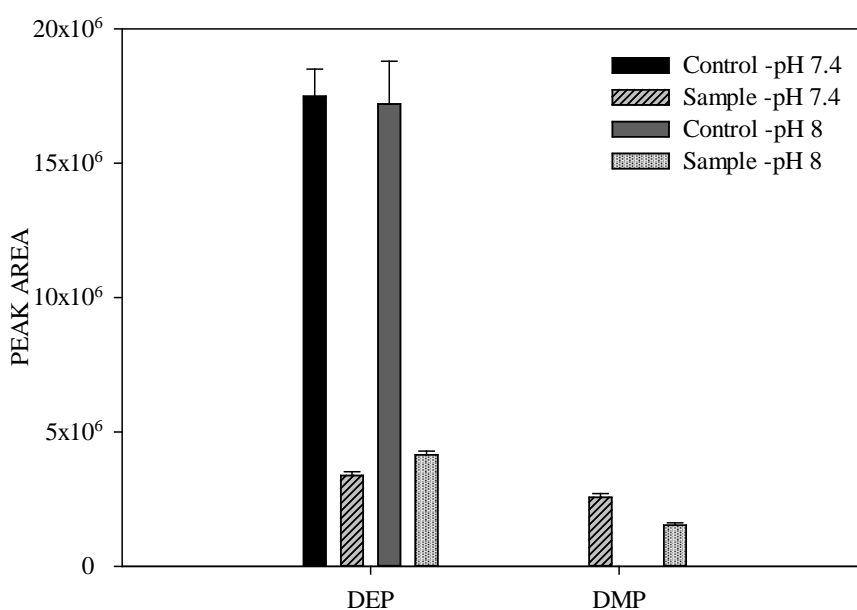
Figure 6.19. Effect of pH on enzymatic hydrolysis of DEP by PPL enzyme
(5 mg/L DEP and 1000U/L, 7 days at 37 °C)

In this study methanol content in the reaction medium was about 0.5% for 5 mg/L PAE dissolved in 100 ml enzyme solution and therefore transesterification reaction that results in formation of methylated hydrolysis products could be possible at this level. As it can be observed from the overlaid GC-MS chromatogram of 5 mg/L DEP and 2000 U/L PPL enzyme (Figure 6.20), DMP was detected in samples at 5.67 min; however this peak does not exist in the positive controls. The peak detected at the retention time of 6.07 min was identified as DEP comparing the retention time of the standard solution and it was detected in both samples and positive controls. Therefore it can be concluded that DEP was not degraded completely in 7 days of incubation time. Percent degradation of DEP peak area was determined as 81% compared to peak area of DEP in positive control.
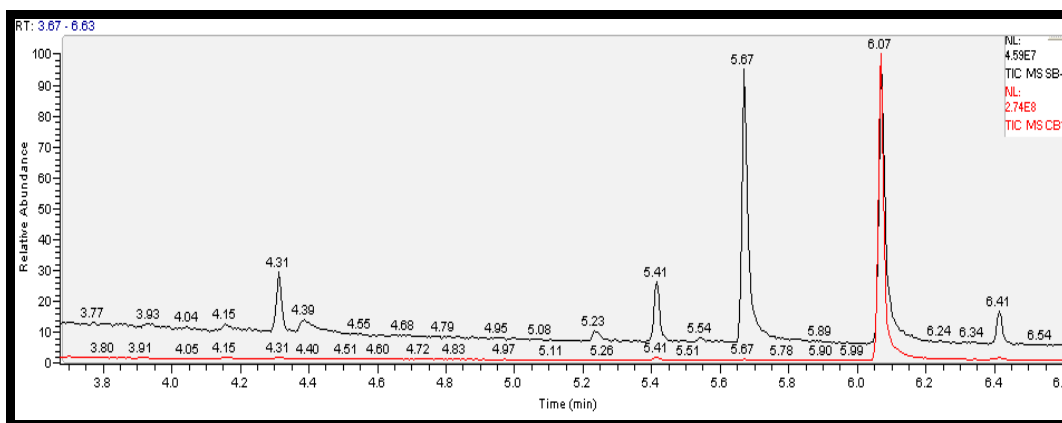
Figure 6.20. Overlaid GC-MS chromatogram for 5 mg/L DEP degradation
(Red line: control; black line: sample)

In the case of DEP, percent decrease in peak areas were estimated as 81% and 76% for pH 7.4 and 8, respectively. Results of the pH optimization experiment suggested that the highest degradation was obtained at pH 7.4 for both DEHP and DEP. Supporting this result, most of the studies on enzymatic or bacterial degradation of PAEs was performed at nearly neutral pH level between 7 to 7.5 (Gavala et al. 2004, Saito et al. 2010). Statistical comparison of the pH 7.4 and 8 in terms of degradation efficiency indicated that there was a statistically significant difference between these pH levels (p=*0.033*). Formation of degradation products was also found to be higher at pH 7.4 compared to pH 8.

6.7. Time Course Analysis of Enzymatic Hydrolysis of PAEs

Time course analysis for enzymatic hydrolysis of 5 mg/L DEHP and DEP was investigated using PPL at 10, 100, 1000, and 2000 U/L. Time course graphs for both DEP and DEHP are plotted up to the time where the PAE concentrations are nearly zero compared to initial concentration.

Hydrolysis rate of PAEs was described by first order kinetics according to equation 6.1.

$$\frac{dS}{dt} = K_h \cdot S \qquad (6.1)$$

where K_h is the hydrolysis constant (d^{-1}); S is PAE concentration (mg/L)

Half-life of the DEHP and DEP was also calculated according to equation 6.2.

$$t_{1/2} = \frac{\ln 2}{K_h} \qquad (6.2)$$

Experimental results showed that DEHP was decreased to 99% of its initial concentration with 10, 100, 1000, and 2000 U/L PPL enzyme for an incubation time of 22 days (Figure 6.21). After 7 days of incubation time, percent decrease in DEHP concentration was 66, 87, 87, 81% for 10, 100, 1000, and 2000 U/L PPL enzyme. In a previous experiment, compared to 81% decrease observed in this experiment, about 67% degradation was obtained in 7 days of incubation time when 2000 U/L PPL was used for degradation of 5 mg/L DEHP (see Section 6.3.1). This difference could be related to experimental conditions such as difference in extraction recovery of samples and controls, or enzyme activity change during the incubation time.
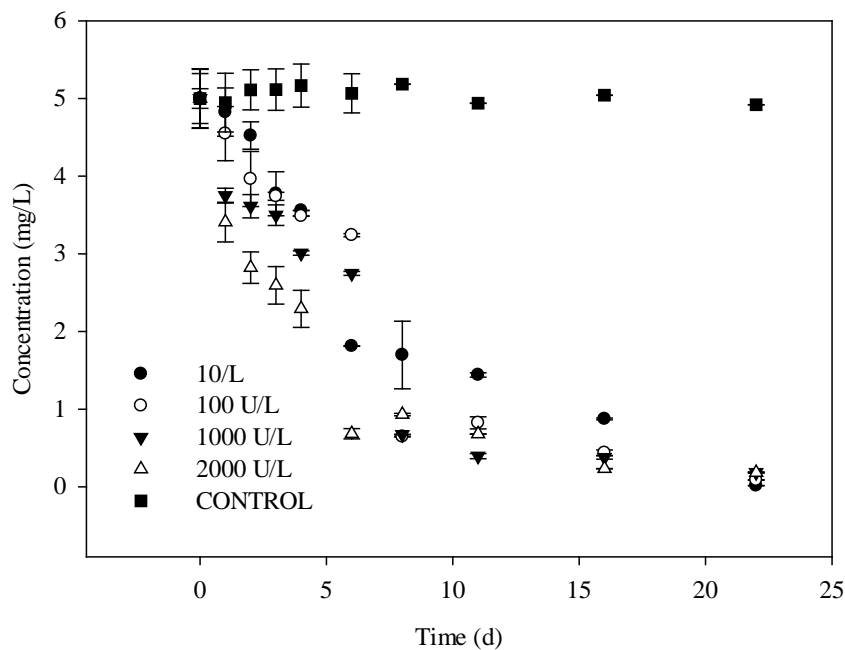
Figure 6.21. Time course of 5 mg/L DEHP degradation with PPL enzyme

Experimental and modeled concentration profiles for DEHP are presented in Figure 6.22. The performance of the models was evaluated according to their coefficient of determination values (R^2). R^2 values of the fitted models were all > 0.90 indicating good fits (Table 6.6). The average hydrolysis constant and half-life for DEHP was calculated as 0.16 d^{-1} and 4.5 d^{-1}, respectively.
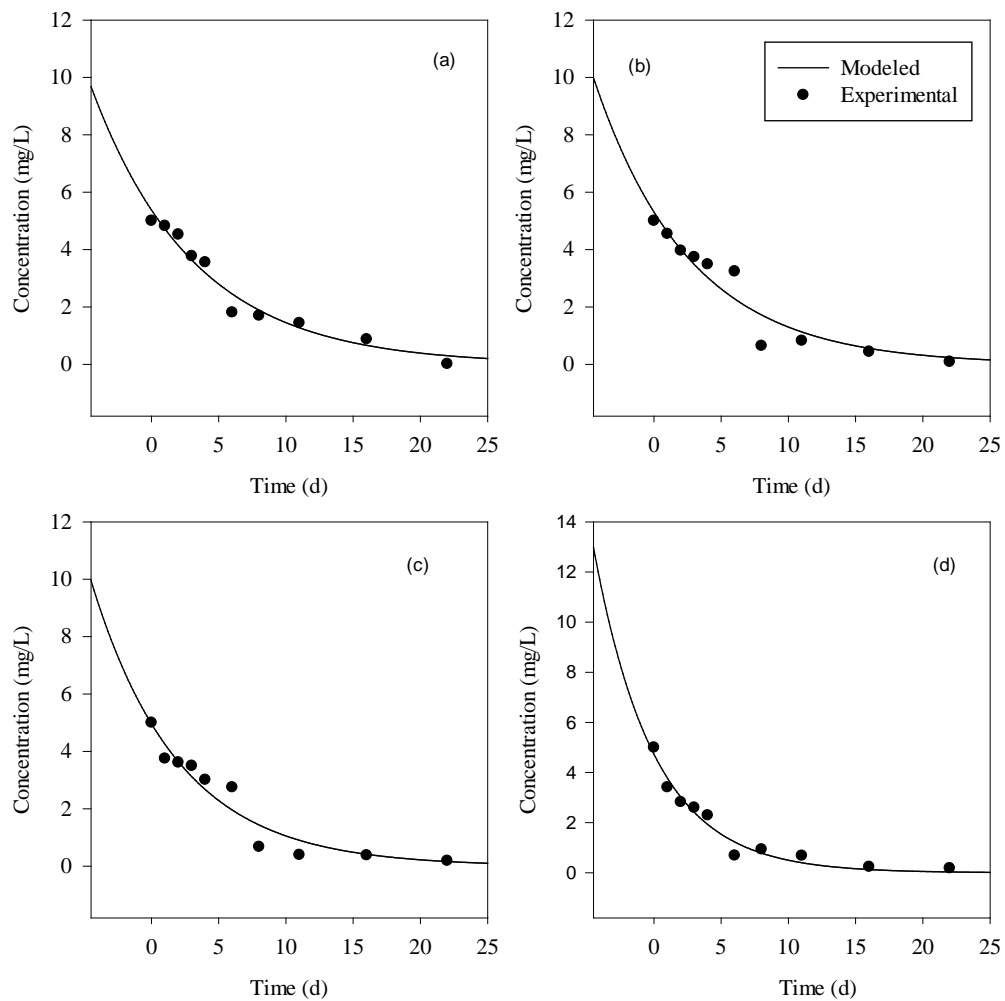
Figure 6.22. Experimental and modeled concentration profile for DEHP
incubated with a) 10 U/L b) 100 U/L c) 1000U/L d) 2000 U/L

Table 6.6. Summary of the first order decay models obtained for DEHP

PPL (U/L)	K_h (d^{-1})	$t_{1/2}$ (d)	R^2	SE*	p-value
10	0.13	5.31	0.96	0.36	0.0001
100	0.14	4.93	0.92	0.57	0.0004
1000	0.15	4.62	0.92	0.50	0.0002
2000	0.22	3.15	0.96	0.32	<0.0001

*Standard error of estimate

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Experimental results for time course analysis of DEP incubated with 10, 100, 1000, and 2000 U/L PPL enzyme for an incubation time of 12 days are presented in Figure 6.23. Experimental and modeled concentration profiles for DEP are presented in Figure 6.24. The performance of the models was evaluated according to their coefficient of determination values. R^2 values of the fitted models were all ≥ 0.95 indicating very good fits to the experimental data (Table 6.7). The average hydrolysis constant and half-life for DEP was calculated as 0.47 d^{-1} and 1.51 d^{-1}, respectively (Table 6.7).
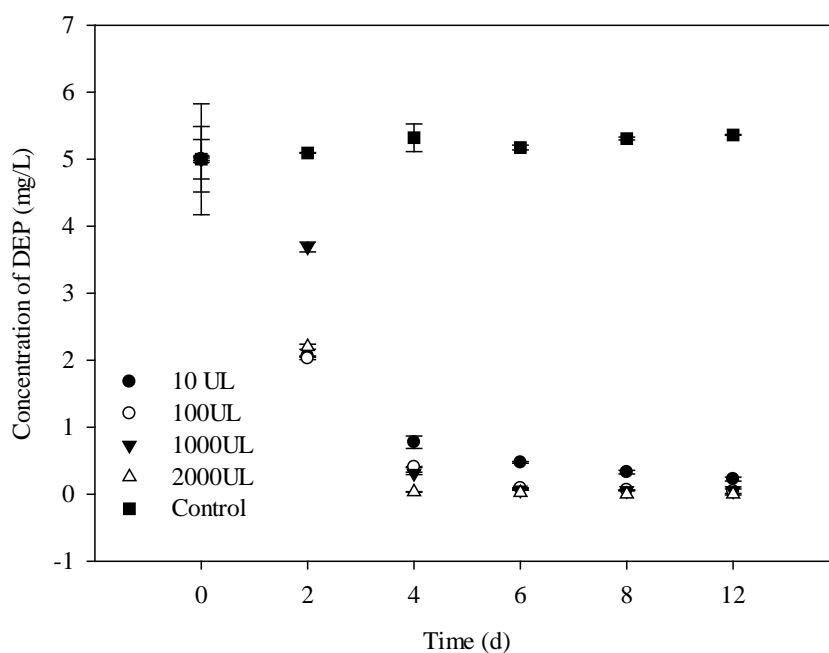
Figure 6.23. Time course of 5 mg/L DEP degradation with PPL enzyme

Table 6.7. Summary of the first order decay models obtained for DEP

PPL (U/L)	K_h (d^{-1})	$t_{1/2}$ (d)	R^2	SE*	p-value
10	0.43	1.60	0.99	0.15	<0.0001
100	0.52	1.35	0.99	0.18	0.0002
1000	0.38	1.82	0.90	0.80	0.0278
2000	0.54	1.28	0.97	0.38	0.0041

*Standard error of estimate
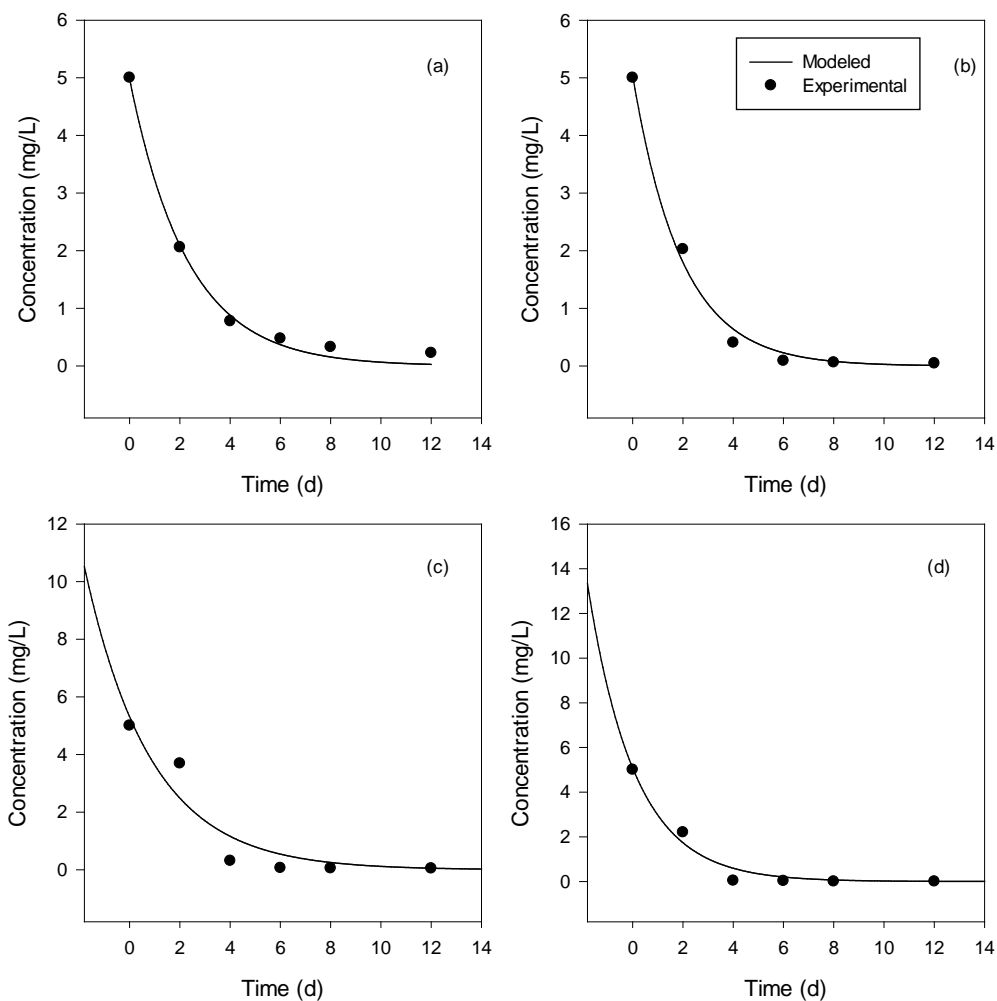
Figure 6.24. Experimental and modeled concentration profile for DEP incubated with
a) 10 U/L b) 100 U/L c) 1000U/L d) 2000 U/L

Primary biodegradation of PAEs is generally followed first-order kinetics
(Gavala et al. 2003, Gavala et al. 2004, Zeng et al. 2004, Cheng et al. 2008). Gavala et
al. (2004) reported that hydrolysis of DEHP in a pretreated primary sludge incubated
with esterase from porcine liver followed first order kinetics. The hydrolysis constants
(K_h values) for DEHP and DEP incubated with 1000 U/L was reported as 0.12 and 8.2
d^{-1} respectively. The K_h value calculated for DEHP in this study was about three times
higher than that was reported by Gavala et al. (2004). However, K_h value obtained for
DEP was much lower than the level reported by Gavala et al. (2004). This difference
could be related to use of esterase instead lipase enzyme that could show different
activity on different substrates; esterase is active on water soluble triglycerides whereas
lipase shows more activity on water insoluble triglycerides.

In another study of Gavala et al. (2003) biodegradation of 5 mg/L DEP and 7 mg/L DEHP in primary sludge during mesophilic anaerobic digestion process resulted in hydrolysis constant (K_h) of 0.08 and 0.0036 d^{-1} for DEP and DEHP, respectively. These levels were much lower than the levels found in this study. First-order degradation constants of DEHP obtained in this study was comparable with the levels reported by Cheng et al. (2008). The researchers reported that kinetic constant for DEHP ranged between 0.27 and 0.40 d^{-1} for three different composting reactors that have initial DEHP concentration between 213 and 296 mg/kg TS (Cheng et al. 2008).

6.8. Experimental Results for Purification of Lipase Enzyme from Recombinant *E.coli*

6.8.1. Results for Purification of the Lipase in *E.coli*

Recombinant *E.coli* strains that contain lipase genes from thermophilic bacteria isolated from Balçova Geothermal Region in Izmir was used for expression of lipase protein (Yavuz et al. 2004). Experimental procedure reported by Tekedar and Şanlı (2011) was followed for expression and purification of the lipase enzyme from recombinant *E.coli*. Clarified cell pastes obtained from 80 ml and 160 ml bacterial culture, which were pre-incubated with 1 mM IPTG for expression of lipase gene, were dissolved in 5 ml sodium-phosphate buffer. After sonication and centrifugation, supernatant phase was loaded on to His-Select Nickel affinity gel column (Sigma). Collected fractions from elution peaks were analyzed with nanodrop (ThermoScientific, 8000) and absorbance values measured at 280 nm for each fraction given in Figure 6.27.
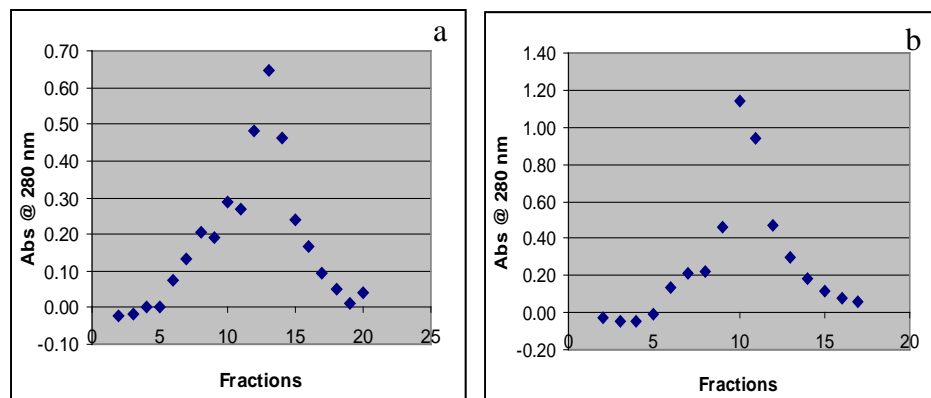
Figure 6.27. A_{280} values for the eluted fractions with Ni-affinity column
a) 80 ml culture b) 160 ml culture

The maximum absorbance value obtained for 160 ml bacteria culture was about two times higher than that was obtained for 80 ml culture. Since one absorbance at 280 nm corresponds to 1 mg/ml protein concentration, total protein amount for each purification step was 3.39 mg and 4.33 mg, approximately. Compared to results reported by Tekedar and Şanlı (2011), purified protein amounts were reasonable since the amount total esterase protein (20-30 mg) that can be obtained from 1.0 L of bacteria culture that contain esterase gene from *Geobacillus* strains.

6.8.2. SDS-PAGE Analysis

Fractions with the similar protein content were pooled and SDS-PAGE analysis was performed in order to determine homogeneity and molecular weight of the lipase protein. After pooling the similar fractions, 17 fractions were obtained and they were run on 12% SDS-PAGE (Figure 6.28). SDS gel analysis of the lipase enzyme indicated a protein band which corresponds to a molecular weight of 40 kDa.
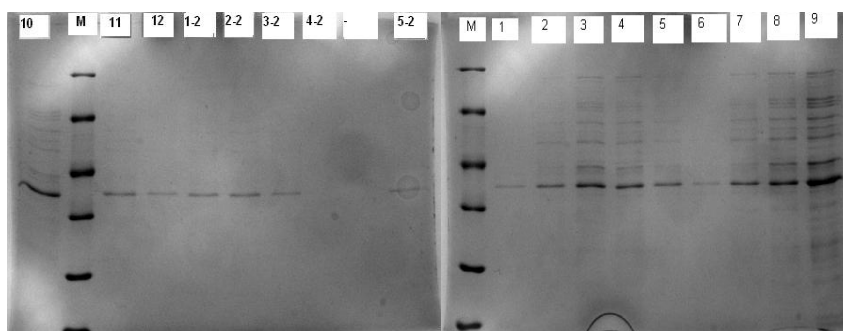
Figure 6.28. SDS-PAGE analysis of selected fractions. M: molecular mass marker from top to bottom, 116.0, 86, 45, 35, 25, and 18.40 kDa.

6.8.3. Determination of Protein Content

Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method (Bradford, 1976). Bovine serum albumin was used as a standard protein. Protein samples and protein standards are processed in the same manner by mixing them with assay reagent and using a spectrophotometer to measure the absorbance. A standard curve was prepared for protein concentration ranging from 0.03 to 0.5 mg/ml. Protein concentration of the enzyme samples were determined using the equation obtained from the standard curve (Figure 6.29). Absorbance values

measured at 595 nm and predicted protein concentration for six crude lipase solution aliquated in 2-ml eppendorf vials are given in Table 6.8. According to these results, average protein concentration of the crude lipase solution was 0.75 mg/mL. All percent RSD values were lower than 10 indicating that repeatability of the measurements were satisfactory.
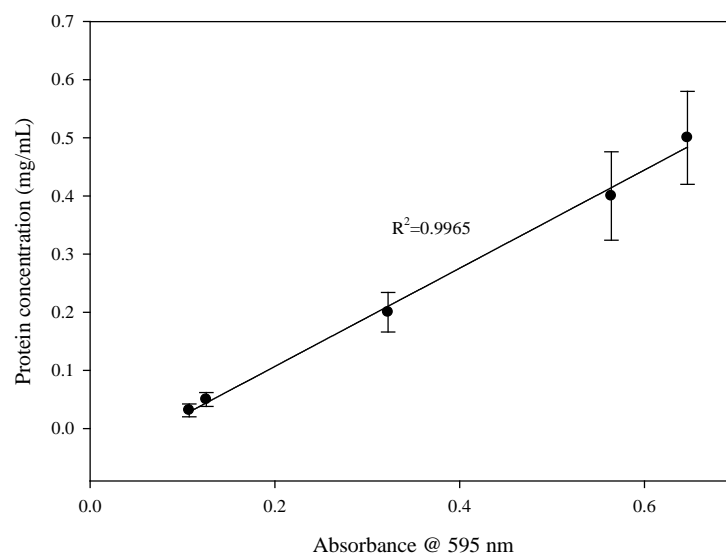
Figure 6.29. Standard calibration curve for BSA (mg/L)
(Error bars show one standard deviation, n=3)

Table 6.8. Results of the protein concentration measurement for
recombinant lipase solution

	Enzyme Samples					
Absorbance at 595 nm	**1**	**2**	**3**	**4**	**5**	**6**
	0.741	1.067	1.217	0.861	0.870	0.843
	0.820	1.189	1.400	0.852	0.816	0.852
	0.823	1.098	1.380	0.842	0.853	0.794
Average (n=3)	0.795	1.118	1.332	0.852	0.846	0.830
RSD (%)	5.91	0.01	0.01	1.06	3.31	3.73
Predicted conc. (mg/mL)	0.609	0.881	1.063	0.657	0.652	0.638

6.8.4. Results for Measurement of Enzyme Activity

Spectrophotometric assay for measurement of activity of recombinant lipase enzyme was also performed for six crude lipase solutions aliquated in 2-ml eppendorf vials (Table 6.9) Activity was measured for the lipase enzyme obtained from two purification process performed at different times.

For each enzyme sample three measurements were carried out and enzyme activity was calculated as unit per ml enzyme.According to these results, average enzyme activity of the recombinant lipase enzyme was 7085 U/ml. All RSD values were lower than 10% suggesting that repeatability of the activity measurements is satisfactory.

Table 6.9. Activity measurement results for crude lipase enzyme

	Enzyme Samples					
	1	**2**	**3**	**4**	**5**	**6**
Activity (U/mL)	7269	7580	6555	5037	6550	8877
	7860	7325	7502	4860	6919	8886
	6898	7552	7095	4985	6987	8787
Average (n=3)	7342	7486	7051	4961	6819	8850
RSD (%)	7	2	7	2	3	1

6.8.5. Effect of Methanol on Enzyme Activity

Stability of crude lipase against methanol was investigated since phthalate standards were prepared in methanol. Enzyme activities were measured using PnPP as substrate upon 15 min incubation of the enzyme in each solvent and the assay conditions were pH 7.4 and 55 °C. Enzyme activity was measured in the presence of methanol content ranging from 0.5 to 20% (v/v). Measured activities were expressed as percent relative activity and results are presented in Figure 6.30. Results showed that lipase enzyme lost its 75% and 80% of activity in the presence of 10 and 20% methanol, respectively.

Enzyme activity was decreased by increasing the methanol content possibly forming of active center blockage because of the polarity strength of methanol, which tends to strip the water from the enzyme's active site leading to enzyme deactivation (Fu and Vasudevan, 2009).
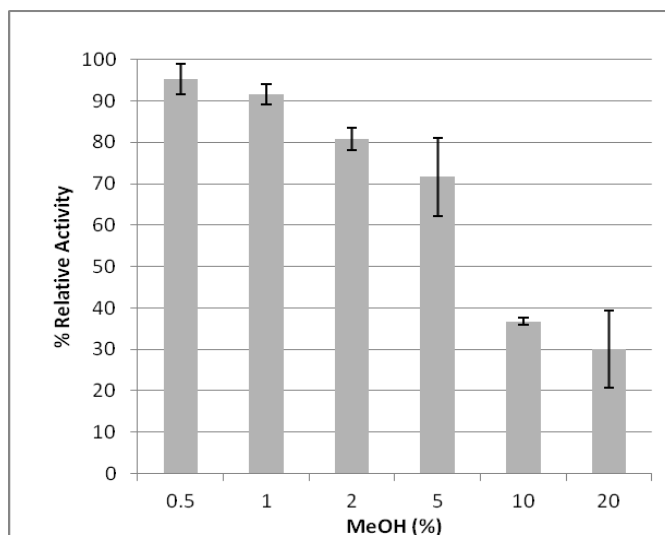
Figure 6.30. Effect of methanol on activity of recombinant lipase enzyme

However, influence of methanol on lipase activity was not higher than 30% in the presence of 5% and lower methanol addition. In this study, methanol content in enzyme solution containing 5 and 20 mg/L PAE corresponds to 0.5% and 1% methanol, respectively. At these levels, loss of enzyme activity was determined as 5 and 9% of initial activity. Therefore, it can be concluded that inhibition effect of methanol on enzyme activity at the studied concentrations of PAEs are not significant.

6.9. Degradation of PAEs with Recombinant Lipase Enzyme

6.9.1. Inhibition Effect of PAEs on Recombinant Lipase

To investigate inhibition effect of DEHP on recombinant lipase enzyme, 1 ml of 7085 U recombinant lipase enzyme was incubated with 1, 2.5, 5, 10, and 20 mg/L DEHP for 24 h. Then the remaining activities were measured and results were expressed as relative percent activity (Figure 6.31).

Percent remaining activities in control samples that include the same methanol content with the samples was found to be lower than 60% except for the control sample of 2.5 mg/L DEHP. In addition, relative enzyme activities in samples were found to be lower compared to controls possibly due to inhibition effect of DEHP on recombinant enzyme. Decrease in enzyme activity due to inhibition effect of DEHP was estimated as the difference between relative percent activities of sample and controls. For 24 h incubation time, decrease in the enzyme activities was calculated as 15, 29, 33, 42, and 45% for 1, 2.5, 5, 10, and 20 mg/L DEHP, respectively.
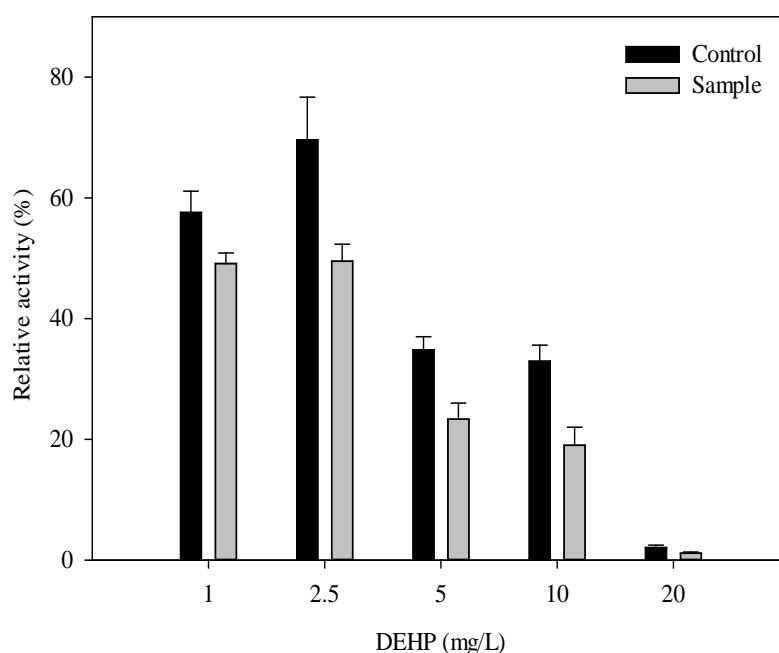
Figure 6.31. Inhibition effect of DEHP on recombinant lipase
(Initial enzyme activity: 7085 U/ml, 24h)

6.9.2. Degradation of DEHP with Recombinant Lipase

In order to evaluate enzymatic hydrolysis of DEHP (5 mg/L) with recombinant lipase enzyme,two different concentrations from crude lipase solution (1290 U and 80 U) were prepared in 2 ml 100 mM sodium phosphate buffer at pH 7.4. Remaining activity of DEHP incubated with crude lipase enzyme was measured after 1, 42, 86 and 128 h, and results were expressed as relative activity (Figure 6.32). Activity of the enzyme was reduced to below 50% of its initial value after 42-h of incubation time.

The activity loss after 128 h of incubation time was found as 80% and enzymatic reaction was terminated and samples were analyzed with GC-MS after application of solvent extraction procedure.
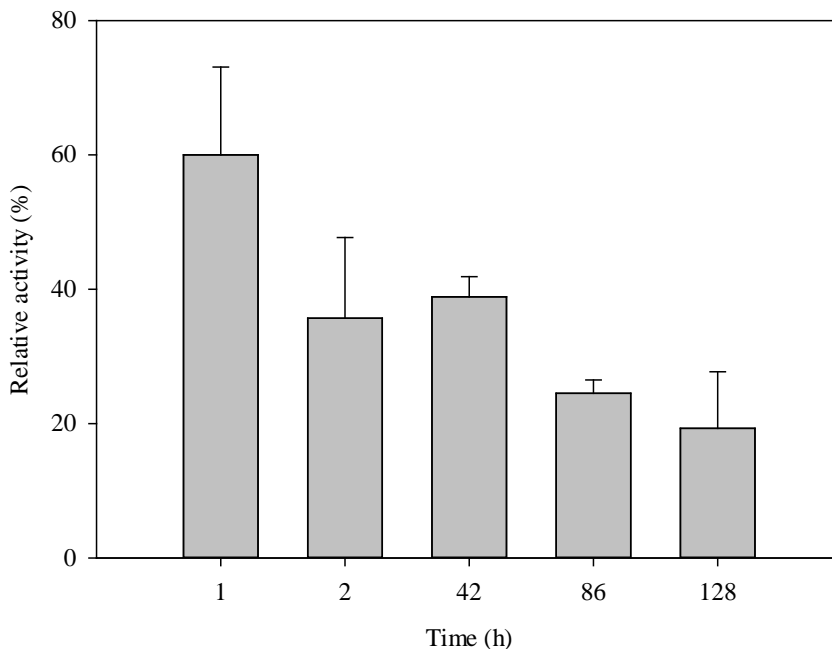
Figure 6.32. Percent relative activity of recombinant lipase (1290 U)
incubated with 5 mg/L DEHP

Analysis results showed that peak area of DEHP was decreased 67 and 86% compared to positive control samples after incubation with 1290 U and 80 U recombinant enzyme for 128 h. Although, MEHP and PA were produced as a result of enzymatic hydrolysis with PPL enzyme, these products were not detected after incubation with recombinant lipase.

Supporting this result, Saito et al. (2010) reported that bacterial cholesterol esterase (CEase) from *Pseudomonas aeruginas* did not show hydrolytic activity on DEHP while both bovine and porcine pancreatic cholesterol esterase hydrolyzed to their corresponding monoesters. Hexadecanoic acid peaks were detected in both samples and positive controls after 128 h incubation at 55 °C (Table 6.10). Formation of these products could be related to transesterification reaction of DEHP metabolites in the presence of methanol. Ming et al. (2010) also reported that fatty acid methyl esters, i.e., hexadecanoic acid methyl ester and octodecanoic acid methyl esters, are produced as a result of reaction between fatty acids and methanol.

Crude lipase enzyme has a optimum activity at 55 °C which could promote transesterification reaction of DEHP. This reaction normally requires high temperature and/or pressure, but can proceed under ambient conditions in the presence of a biological catalyst (Morrison, 1998).

Table 6.10. GC-MS Analysis results for crude lipase incubated with DEHP

Sample/Control		DEHP AREA	HA AREA
1290 U	S1	55426	1344515
	S2	53705	1282426
	C1	162965	243337
	C2	164277	243337
80 U	S1	58819	206024
	S2	58124	189305
	C1	409119	1508016
	C2	409119	1554587

Incubation for 128 h at 55 °C and 120 rpm; HA: Hexadecanoic acid S: Sample, C: Control

Recombinant lipase enzyme with higher activity (4000 U) was also used for investigating degradation of 1, 5, 10, and 20 mg/L DEHP, since 5 mg/L DEHP was not hydrolyzed with 1290 and 80 U crude lipase enzyme. During the incubation time, samples were taken from reaction solution after 1, 2, 4, 6, 22, and 44 h. Enzyme activities were measured and results were expressed as percent relative activity (Figure 6.33). After 44 h incubation time, percent decrease in enzyme activity for 1, 5, 10, and 20 mg/L DEHP was calculated as 3, 4, 9, and 11%, respectively. In order to evaluate methanol effect on enzyme activity, 1 ml of lipase enzyme solution containing 2% of methanol was also incubated under same conditions. After 44 h of incubation, activity of the lipase enzyme solution containing 2% of methanol was decreased to 601 U which corresponds to 20% of the initial activity.
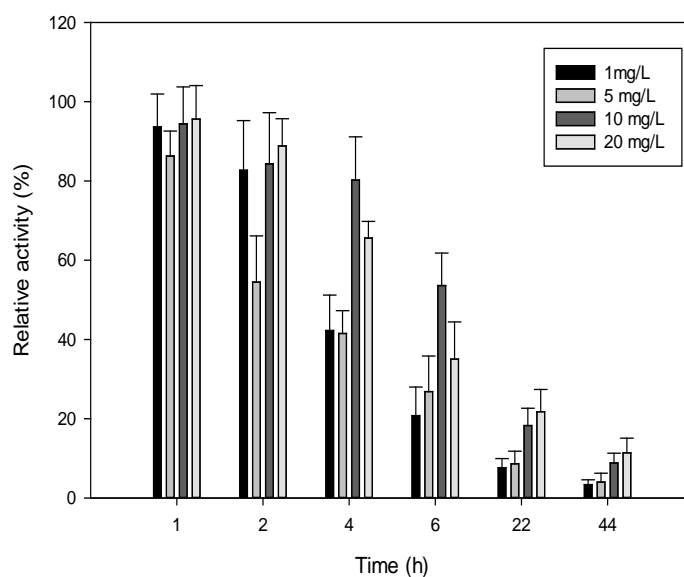
Figure 6.33. Relative activity (%) of recombinant lipase (4000 U)
incubated with DEHP

GC-MS analysis of the samples suggested that DEP was produced in samples containing 5, 10, and 20 mg/L DEHP after 44 h incubation time (Table 6.11). Among these samples, the highest DEP formation was obtained with 5 mg/L DEHP. It is known according to the literature that PAEs with longer side chain converted to DEP by β-oxidation reaction which removes one ethyl group each time (Cartwright et al. 2000, Amir et al. 2005). After 44 h incubation of DEHP (1 to 20 mg/L) with 4000 U crude lipase enzyme, MEHP and PA which are hydrolysis products of DEHP were not detected in any of the samples. This could be related to molecular structure of the DEHP that has longer alkyl side chain than other PAEs that hinders hydrolytic enzymes from binding to the compound (Xia et al. 2004, Liang et al. 2008).

Table 6.11. GC-MS analysis results for recombinant lipase incubated with DEHP

Concentration (mg/L)	DEHP[*]	SD	DEP[*]	SD
20	133206026	28880969	42263	2063
10	55843018	561034	26761	1951
5	56751483	2379656	25040	1085
1	29922327	3585346	0	0

[*]Average peak area, n=3, 4 ml, 4000U enzyme solution incubated at 55 °C
and 120 rpm for 44h

6.9.3. Degradation of DEP with Recombinant Lipase

Enzymatic degradation experiments with recombinant lipase enzyme were also performed with DEP that has shorter alkyl side chain compared to DEHP. In order to compare biodegradability of DEP and DEHP, 4000 U recombinant lipase enzyme was incubated with 1, 5, 10, and 20 mg/L DEP. Enzyme activities were measured after 22, 94, and 116 h of incubation time, and results are expressed as relative percent activity for each enzyme concentration (Figure 6.34).

After 140 h incubation of 1, 5, 10, and 20 mg/L DEP with crude lipase enzyme, 28, 14, 11, and 14% of relative activities were observed, respectively. When the relative activities of crude lipase enzyme incubated with DEP for 22 h were compared to those for DEHP, the activities were found to be higher than 60% for DEP while they were below 20% for all DEHP concentrations.

In addition, GC-MS analysis of the solvent extracts suggested that DMP was produced in samples with 5, 10, and 20 mg/L DEP. Supporting this result, formation of methylated hydrolysis products of PAEs such as DMP in the presence of 0.1% methanol was reported in the literature (Kim et al. 2002, Cartwright et al. 2006). Compared to peak area of positive control samples 25, 67, 81, and 97% decrease in DEP peak areas were obtained for 20, 10, 5, and 1 mg/L DEP, respectively (Table 6.10).
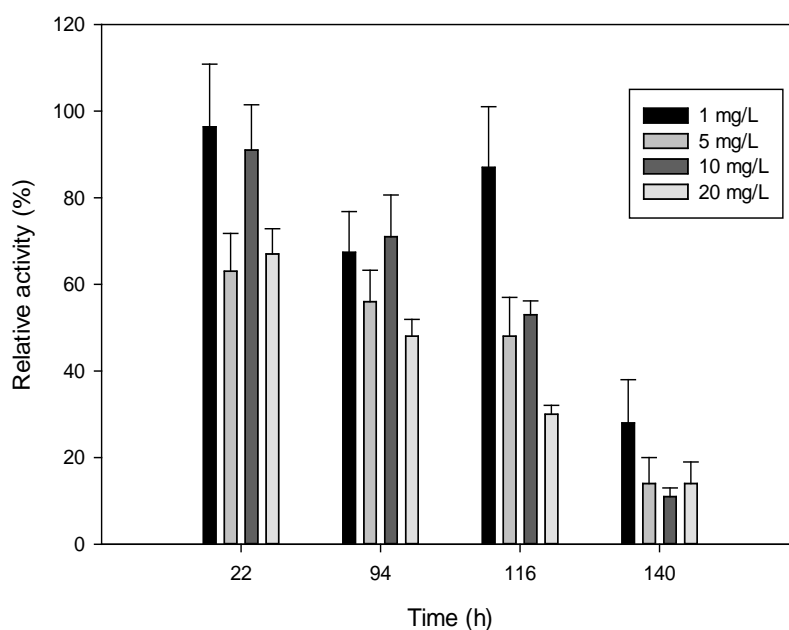
Figure 6.34. Relative activity results for 4000 U crude lipase incubated
with DEP for 22, 94, 116 and 140 h.

Table 6.12. GC-MS analysis results for recombinant lipase (4000 U) incubated with DEHP

DEP (mg/L)	DEP[*]	SD	DMP[*]	SD
20	419418877	19059815	3943273	23210
10	181782682	7071068	766509	63640
5	103577413	998158	273641	19114
1	15854667	89429	148965	12596

[*]Average peak area, n=3, Incubation conditions: 1 ml of 4000 U crude lipase for 140 h at 55 °C and 120 rpm

Experimental results obtained with recombinant lipase enzyme suggested that 5 mg/L DEHP incubated with 1290 U lipase enzyme for 128 h resulted in 67% decrease in DEHP peak area compared to positive control samples. However, MEHP and PA peaks were not detected. HA was detected in samples and controls as a result of a possible transesterification reaction in the presence of methanol at high incubation temperature (55 °C). When 4000 U recombinant lipase was used for degradation of DEHP, 44 h incubation time resulted in DEP formation. The highest DEP was obtained with 5 mg/L DEHP. In the case of DEP incubated with 4000 U recombinant lipase enzyme, DMP was observed after 140 h incubation time. The highest degradation was obtained for 1 mg/L DEP that resulted in 97% decrease in peak areas.

6.9.4. Enzyme Kinetics for Hydrolysis of DEP and DEHP with Recombinant Lipase

After 4 h incubation of 1, 5, 10, 15 and 20 mg/L DEHP and DEP with 4000 U recombinant lipase enzyme, remaining enzyme activities were measured spectrophotometrically and results are presented in Table 6.13. Remaining activities for DEP ranged from 65 to 77% for DEP, whereas it was ranged from 61 to 82% for DEHP.

Table 6.13. Remaining enzyme activity of 4000 U recombinant lipase incubated
with DEHP and DEP

Concentration (mg/L)	DEP[*] % Remaining Activity	DEHP[*] % Remaining Activity
1	67	68
5	73	61
10	77	76
15	65	82
20	74	79

*Average percent remaining activity after 4 h incubation at 55 °C and 120 rpm (n=3)

Remaining concentrations of PAEs were calculated based on the percent of PAE peak area at each sampling time compared to the initial peak area. Kinetic parameters for enzymatic hydrolysis of DEHP and DEP were calculated using Lineweaver-Burk plot for both DEHP and DEP (Figure 6.35 and 6.36). The maximum rate (V_{max}) of enzymatic hydrolysis reaction for DEHP and DEP was calculated as 0.79 mg/L.h and 1.83 mg/L.h, respectively. The Michealis-Menten constants (K_m) for enzymatic hydrolysis of DEHP and DEP were calculated as 2.45 and 2.12 mg/L, respectively. The K_m value for DEHP was higher than that was calculated for DEHP suggesting that; recombinant lipase enzyme shows higher affinity for DEP compared to DEHP.
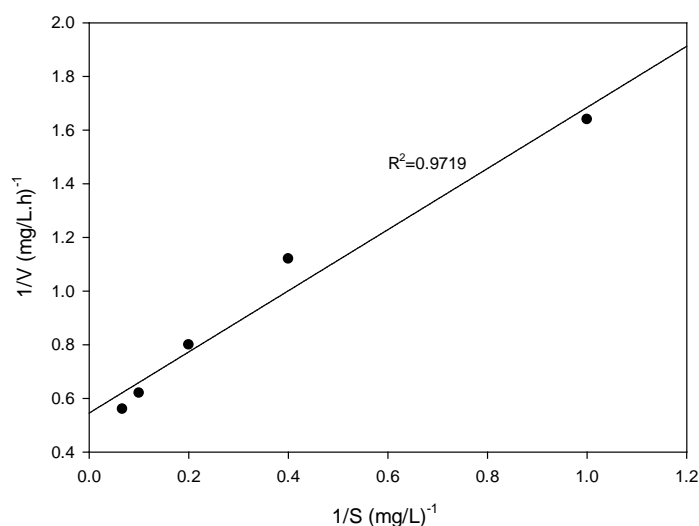

Figure 6.35. Lineweaver-Burk Plot analysis for hydrolysis of DEHP
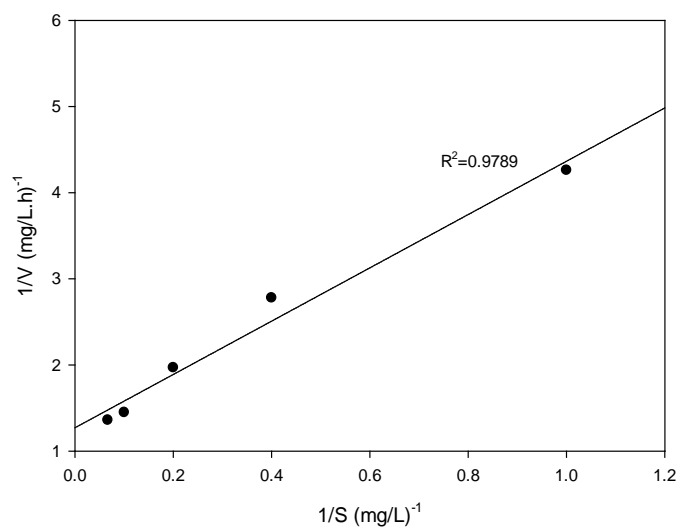with recombinant lipase enzyme

Figure 6.36. Lineweaver-Burk Plot analysis for hydrolysis of DEP
with recombinant lipase enzyme

CHAPTER 7

CONCLUSIONS

Enzymatic hydrolysis products of PAEs with PPL was identified for 20 mg/L DEHP incubated with 20 mg/L DEP and DEHP for 7 days of incubation time. GC-MS analysis results showed that MEHP, PA, and DMP were formed as enzymatic hydrolysis products of DEHP. The percent decrease in DEHP (20 mg/L) was about 92% compared to positive control samples. In the case of DEP, about 53% decrease was obtained after incubation with 20,000 U/L for 7 days. Two-way ANOVA design for PPL and DEHP effect on enzymatic degradation of DEHP suggested that >90% degradation of DEHP (0.1-5 mg/L) could be obtained with 5000 U/L and lower PPL enzyme. Percent decrease in the DEHP peak areas was 57, 55, 58, and 67% for 10, 100, 1000, and 2000 U/L PPL, respectively. In the case of DEP percent decrease values were about 80, 82, 87, and 92% for 10, 100, 1000, and 2000 U/L PPL concentrations, respectively.

Characterization of crude lipase recombinant from *E. coli* indicated that average protein concentration of the crude lipase solution was 0.75 mg/mL and enzyme activity was 7085 U/ml. The enzyme lost its 75% and 80% of activity for 15 min incubation in the presence of 10 and 20% methanol, respectively. Inhibition effect PAEs on enzyme activity was also studied for 24 h incubation time and decrease in the enzyme activity was 15, 29, 33, 42, and 45% for 1, 2.5, 5, 10, and 20 mg/L DEHP, respectively. 4000 U recombinant lipase incubated with DEHP (1-20 mg/L) resulted in formation of DEP that is known to have no neither mutagenic nor carcinogenic health effects based on animal studies. In the case of DEP (1-20 mg/L) incubated with 4000 U crude lipase solution for 140 h, DMP was obtained as a possible product of transesterification reaction. Percent decrease in DEP peak area was found to be 25, 67, 81, and 97% for 20, 10, 5, and 1 mg/L DEP, respectively.

Experimental data obtained for enzymatic degradation of both DEHP and DEP were fitted to first order reaction kinetics, and hydrolysis constants were estimated for a PPL concentration range of 10 to 2000 U/L. K_h values for enzymatic degradation of DEHP ranged between 0.13 and 0.22 d^{-1}, while those for DEP ranged from 0.43 to 0.54 d^{-1}.

In conclusion, recombinant lipase enzyme partially hydrolyzed DEHP to DEP which has shorter alkyl side chain and less toxic PAE compared to DEHP. Time course analysis of PAE degradation suggested that 5 mg/L DEP was almost completely degraded after 4 d of incubation with 2000 U/L PPL. However, DEHP was decreased about 54% of its initial concentration after 22 days of incubation with 2000 U/L PPL enzyme. The differences in degradation of the two PAEs could be related to water solubility and alkyl side chain length that effect binding of the compound to the enzyme. PPL concentrations of <5000 U/L resulted in >90% degradation of DEHP (0.1-5 mg/L). The optimum pH level for enzymatic hydrolysis of PAEs was determined as 7.4 for both DEHP and DEP hydrolysis with PPL enzyme. However, there were some inconsistencies in experimental results. For example, 53% decrease in peak area was obtained after incubation of 5 mg/L DEP with 2000 U/L PPL enzyme, whereas, degradation efficiency was almost 99% in 7 days of incubation during time course analysis. This could be related to activity change of enzyme or difference between extraction recoveries of the two experiment sets. Nevertheless, the inconsistencies point to the need for improvement in the methods to reduce variability in the results.

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