Microbial Esterase and the Degradation of Plasticizers

by

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ABSTRACT

Previous studies have shown that the biodegradation of di-ester plasticizers can lead to the accumulation of toxic recalcitrant metabolites. *Rhodococcus rhodochrous* ATCC 13808 is a bacterium known to degrade plasticizers. The first steps of the biodegradation mechanism consist of esterase-mediated hydrolyses. The present study focused on characterizing the esterase produced by *R. rhodochrous* and defining its impact on the rate of hydrolysis of different di-ester plasticizers.

By means of esterase activity and growth studies, it was possible to determine that the esterase produced by *R. rhodochrous* was constitutive and bound to the cell membrane. Treatment with a non-ionic surfactant, Triton X-100, caused solubilization of the enzyme. The esterase exhibited high stability, retaining activity for more than 48 hours, even after separation from the cell. Esterase activity was highest at 30°C but observed at temperatures as low as 4°C.

The comparison of the rates of hydrolysis of different esters showed that the solubility of the substrate had an important impact, with the less soluble compounds generally having lower rates. However, steric hindrance also appeared to play an important role in the determination of the rate of hydrolysis. The most common plasticizer, di(2-ethylhexyl) phthalate, had the slowest rate of hydrolysis. Therefore, given the increasing and widespread use of DEHP and other di-ester plasticizers, such plasticizers will continue to accumulate in the environment. This growing pool of plasticizers will undergo slow biodegradation, resulting in the increasing production of toxic metabolites.

SOMMAIRE

Il a été démontré, lors d'études précédentes, que la biodégradation de plastifiants di-esters peut mener à l'accumulation de métabolites toxiques et récalcitrants. La bactérie *Rhodococcus rhodochrous* ATCC 13808 a la capacité de dégrader ces plastifiants. Les premières étapes liées au mécanisme de leur biodégradation consistent en hydrolyses catalysées par des estérases. La présente étude porte sur la caractérisation de l'estérase produite par *R. rhodochrous* et la détermination de son impact sur la biodégradation de plastifiants di-esters.

Par des études d'activité enzymatique et de croissance, il a été démontré que l'estérase produite par *R. rhodochrous* était constitutive et située sur la membrane cellulaire. Des traitements au Triton X-100, un détergent non-ionique, ont permis de solubiliser l'enzyme. L'estérase a démontré une grande stabilité, conservant de l'activité enzymatique après plus de 48 heures, et ce même lorsque extraite de la membrane cellulaire. L'activité enzymatique était plus élevée à 30°C, mais a été observée à des températures aussi basses que 4°C.

La comparaison des taux d'hydrolyse de différents esters a démontré que la solubilité des substrats avait un effet important. Les composés les moins solubles possédaient généralement des taux moins élevés. Par contre, il a été démontré que l'exclusion stérique jouait aussi un rôle important dans la détermination du taux d'hydrolyse. Le plastifiant le plus commun, le di(2-éthylhexyl) de phthalate, possédait le taux d'hydrolyse le plus bas. Conséquemment, en tenant compte de l'augmentation de leur production et de leur utilisation, de tels plastifiants continueront de s'accumuler dans

l'environnement. Ce pool croissant de plastifiants sera lentement biodégradé, ce qui résultera en une présence croissante de leurs métabolites toxiques.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1 Di-ester Plasticizers

Plastics play an increasingly important role in many aspects of everyday life. With their increased production and utilization comes the concern of the impact they and their additives might have on the environment. Plasticizers represent a class of compounds added to plastics in order to improve on their extensibility, flexibility, softness and workability [1-3]. Since these compounds are widely used and sometimes account for as much as 67% of the total weight of plastics [4], it is important to assess their importance relative to the environment.

Typically, plasticizers are liquids at room temperature, and usually possess low water solubility and volatility [2, 3]. They are not covalently bound to the polymer matrix of plastics. This is significant because it allows them to migrate out of the material [5-7].

Di-ester plasticizers represent the largest and most common type of plasticizers in use. Phthalic acid esters (phthalates) are particularly important with an annual production of 4.2 million tonnes in 1994 [4]. The most common phthalate, di(2-ehtylhexyl) phthalate (DEHP), whose structure is shown in **Figure 1-1a,** accounts for approximately 50% of the total annual plasticizer production [4]. These compounds are mostly used with polyvinyl chloride (PVC) plastics. Adipic acid esters (adipates) and benzoic acid esters (benzoates) are also widely used. The chemical structures of di(2-ethylhexyl) adipate (DEHA) and di(propylene glycol) dibenzoate (DPGDB) are shown in **Figures 1-1b** and **1-1c**, respectively. The adipates are often found in plastic films used for food packaging [8-10] or as co-plasticizers in other plastics [2]. The use of benzoates is a

recently introduced plasticizer that is being promoted as a possibly safer plasticizer for use with PVC [11].

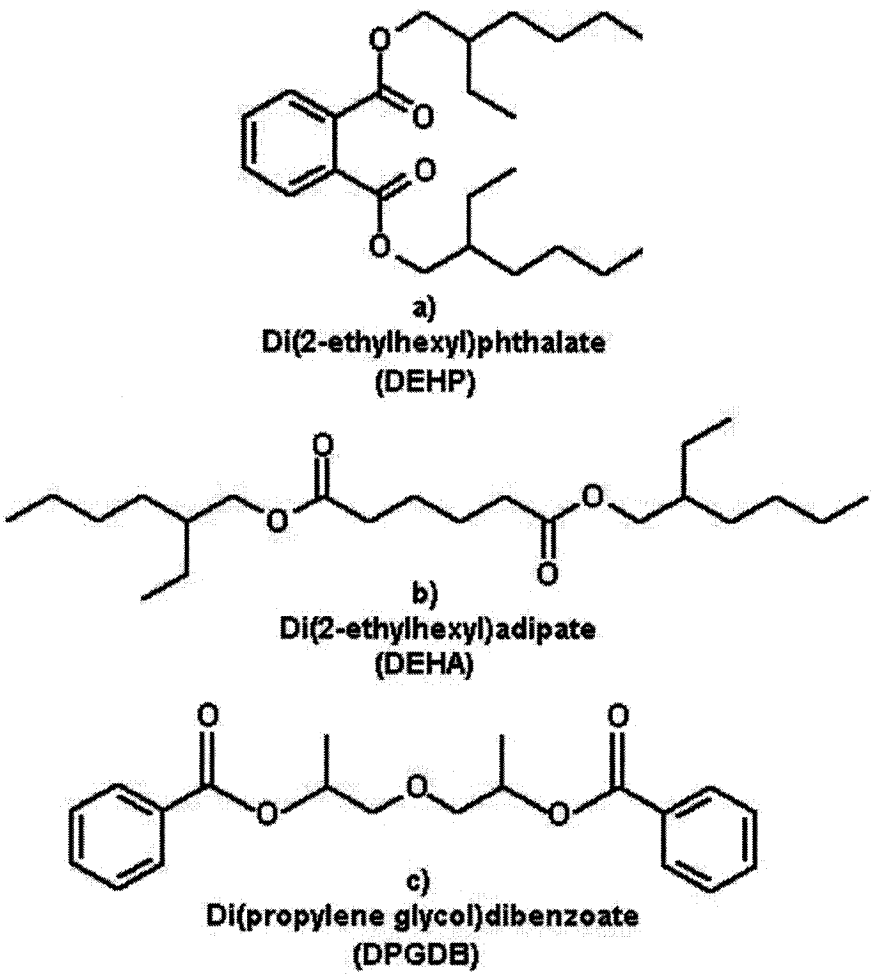

Figure 1-1 — Chemical structures of common di-ester plasticizers: a) di(2-ehtylhexyl) phthalate, b) di(2-ethylhexyl) adipate, and c) di(propylene glycol) dibenzoate.

Since plasticizers are used in so many different plastic formulations, they are found in many consumer items. Therefore, one or more of the di-ester plasticizers are encountered in a wide variety of products including building materials [1], blood bags

[12, 13], toys [1], cosmetics [14], inks[14], insect repellent [15] and electrical wiring insulation [1].

1.2 Di-ester Plasticizers in the Environment

Plasticizers are prone to leach out of plastics since they are not chemically bound to the polymer matrix. This, combined with the omnipresence of plastic materials, translates into a continuous release of plasticizers into the environment. The extent of the environmental release has lead to the inclusion of six phthalates, including DEHP, in the United States Environmental Protection Agency list of priority pollutants [16]. In fact, plasticizers are considered ubiquitous contaminants of aqueous and soil environments [5, 17]. In 1975, the estimated worldwide release into the environment of phthalates alone was estimated to be 23 000 tonnes [18]. In 1990, a study conducted in Western Europe estimated that 77 000 tonnes of plasticizers were released in that region alone [19]. Phthalic acid di-esters were estimated to account for 93% of this release.

The main sources of the plasticizers released are leachates from landfills and dump sites [18, 19]. Other common sources are directly from consumer products or infrastructures (e.g., PVC water drains), and manufacturing plants [18, 19].

Plasticizers have been found in soils [7, 20-22], open waters [14, 23-25], riverbeds [22, 24, 26], snow [24], drinking water [24] and food [10]. In fact, *Ribbons et al.* [27] stated that DEHP was encountered in 42% of environmental samples for which the appropriate analyses were done.

1.3 Biodegradation of Di-ester Plasticizers

While some studies have shown that degradation of di-ester plasticizer was possible by means of photodecomposition [28] or alkaline hydrolysis [29], these mechanisms are neither practical nor prevalent. In fact, photodecomposition requires high levels of radiation and most plasticizers have a half-life of a few hundred years under alkaline conditions [5]. Therefore, the most likely means of plasticizer degradation in the environment remains biodegradation.

Microorganisms able to degrade plasticizers have been found in many different environments including soils [30-32], rivers and marine regions [33, 34]. Similarly biodegradation occurs under many different conditions. These include aerobic [27, 30, 35, 36] or anaerobic conditions [4, 37], and in presence of pure organisms [27, 35, 36] or complex mixtures of organisms found in activated sludge systems [33, 37, 38].

Complete removal of di-ester plasticizers has been observed when they are encountered in activated-sludge systems [39, 40]. However, because plasticizers are generally released directly into the environment, without the possibility of treatment, and because environmental sites do not have the microbial richness found in activated-sludge systems, complete degradation does not readily occur in nature. It is important to note that the studies referenced here, as well as others conducted with microbial consortia or pure strains [41], were all concerned with the disappearance of the initial plasticizers and did not consider the possible accumulation of metabolites.

Two recent studies conducted with pure microbial strains focused on determining the extent of plasticizer biodegradation and identifying the metabolites produced. In the first study, *Nalli et al.* [42] screened several bacteria, including *Rhodococcus*

rhodochrous, for their ability to degrade commonly used plasticizers (adipates and phthalates). The microorganisms that could degrade DEHA and DEHP were only partially successful in that metabolites from the original plasticizers were found in the spent media. Furthermore, each of these metabolites exhibited varying degrees of aqueous toxicity. The proposed pathway for the degradation of DEHA is illustrated in **Figure 1-2** showing the metabolites being produced. Similar pathways were proposed for the degradation of DEHP and di(2-ethylhexyl) terephthalate.

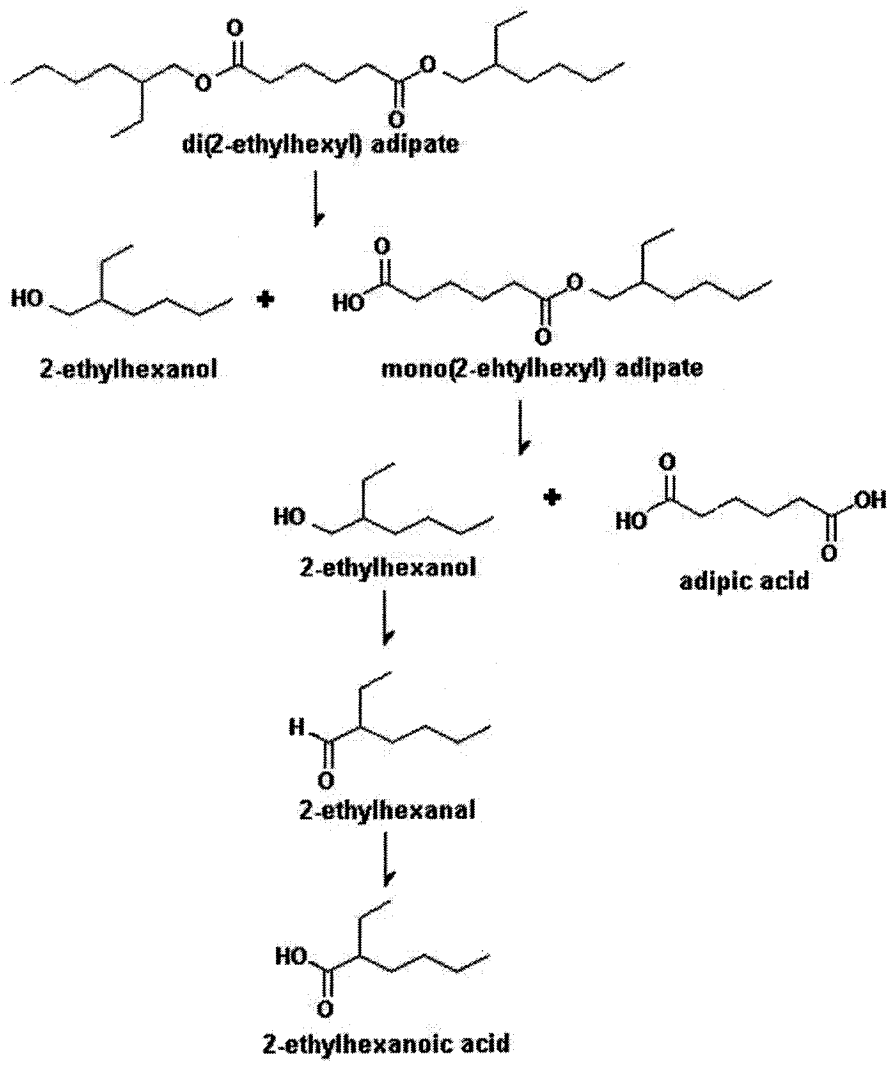

Figure 1-2 — Pathway for the partial biodegradation of di(2-ethylhexyl) adipate [42].

The initial step in the biodegradation pathway was the partial hydrolysis of DEHA to an alcohol (2-ethylhexanol) and a mono-ester (mono(2-ethylhexyl) adipate). The mono-ester can be further hydrolyzed to release more of the same alcohol and an acid (adipic acid). It is important to note that both of these hydrolysis steps would be catalyzed by esterases. The pattern is similar for DEHP and di(2-ethylhexyl) terephthalate leading to the same alcohol, 2-ethylhexanol, and either phthalic acid or terephthalic acid [42]. Each of the released acids is mineralized by many different microorganisms. However, the 2-ethylhexanol either accumulates [4, 42, 43] or is further oxidized: first to an aldehyde (2-ethylhexanal) and then to an acid (2-ethylhexanoic acid). 2-ethylhexanoic acid was also found to accumulate in many systems [42, 44]. In some cases, the mono-ester was found to accumulate [43].

In a second study, *Gartshore et al.* [45] investigated the biodegradation of DPGDB by the yeast *Rodotorula rubra*. Once again, the initial hydrolysis steps were catalyzed by esterases. Partial biodegradation to release metabolites was also observed for this system but the toxic compound which accumulated was the monoester di(propylene glycol) mono-benzoate. The proposed pathway is illustrated in **Figure 1-3**.

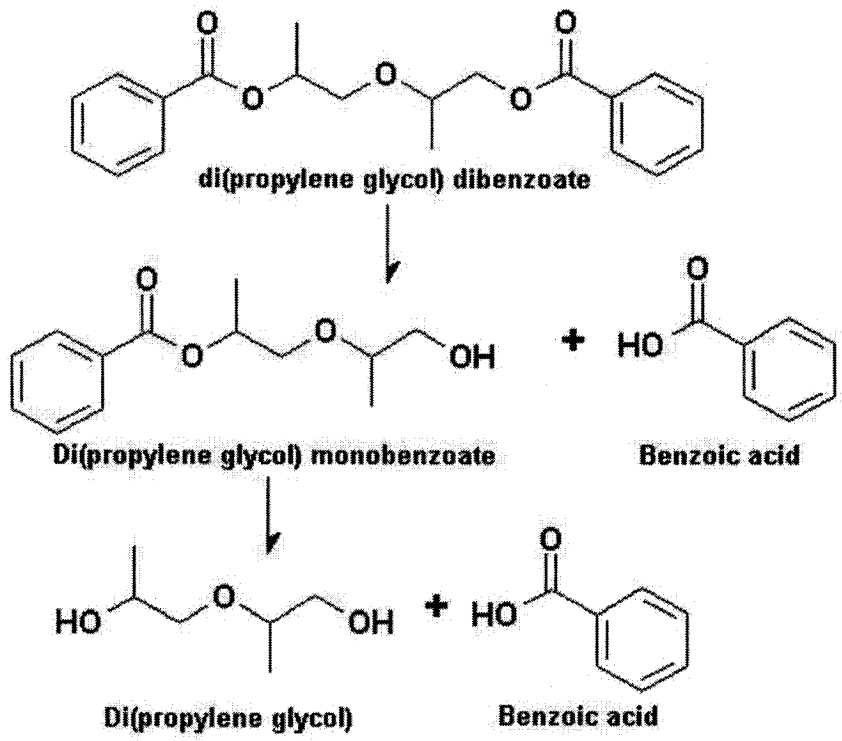

Figure 1-3 — Pathway for the partial biodegradation of di(propylene glycol) dibenzoate [45].

In the above work, the presence and accumulation of mono-esters, 2-ethylhexanol and/or 2-ethylhexanoic acid had a critical impact on the toxicity of the systems studied. In fact, all these metabolites have a higher aqueous toxicity than their parent plasticizers [24, 42]. In the cases of 2-ethylhexanol and di(propylene glycol) mono-benzoate, the aqueous toxicities are, respectively, 7 and 10 times that of phenol on a molar basis.

Many recent studies have reported the presence of some of these metabolites in different environmental samplings [24, 46-48]. 2-ethylhexanol, which is a volatile organic carbon, is the most commonly encountered as it was found in open waters, snow precipitation, riverbeds, soil samples [24], as well as in the indoor air of different buildings (e.g., hospitals [49]). 2-ethylhexanoic acid was found in open waters, riverbeds, and drinking water [24]. Mono(2-ethylhexyl) phthalate has been observed in a

sample from a riverbed [24]. These compounds were also found in the urine of patients receiving blood as a result of the partial metabolism of DEHP contained in blood bags [50, 51].

1.4 Health Risks of Di-ester Plasticizers and their Metabolites

Although most di-ester plasticizers exhibit low acute toxicities [24, 42], there are other associated health risks. Plasticizers have been found in the tissues of many higher organisms [2, 5, 52-55]. The main examples are several species of fish and amphibians in which bioaccumulation and bioconcentration occurred [5, 53]. In some cases, plasticizers inhibited reproduction and were also proven to be carcinogenic [2, 5, 53].

Experiments conducted on rats showed that DEHP was partially metabolized to 2-ethylhexanol, mono(2-ehtylhexyl) phthalate and 2-ethylhexanoic acid [52, 55]. The plasticizer and its metabolites were found in the urine and the blood of the specimen as well as in the liver, the intestines and other tissues [52]. Other studies have linked the presence of these compounds to endocrine disruption [56]. Mono(2-ethylhexyl) phthalate and 2-ethylhexanoic acid have also been identified as peroxisome proliferators in rats and mice [55].

It is known that humans only partially degrade DEHP and DEHA [57-59] but studies conducted on monkeys showed no evidence that di-ester plasticizers or that their metabolites were carcinogenic to primates [60]. However, it has been shown that DEHP and di(n-butyl)phthalate may cause endocrine disruption and affect the timing of puberty in humans [61, 62].

2-ethylhexanol, 2-ethylhexanoic acid, mono(2-ethylhexyl) phthalate and mono(2-ethylhexyl) adipate display higher acute aquatic toxicity levels than DEHP or DEHA [24, 42]. Moreover, 2-ethylhexanol is considered to be a volatile organic carbon and has been linked to asthma symptoms in hospitals [49].

1.5 *Rhodococcus rhodochrous* ATCC 13808

Rhodococcus rhodochrous ATCC 13808 is a gram positive bacterium isolated from soil. The *Rhodococcus* genus consists of microorganisms exhibiting broad metabolic diversity. More specifically, many *Rhodococcus* species are known to metabolize alkanes and di-ester plasticizers [42, 63-66]. Screenings and studies have been conducted on *R. rhodochrous* with phthalates, adipates, benzoates and terephthalates [63], which showed that this organism could metabolize these compounds to different extents.

R. rhodochrous ATCC 13808, as with most species of this genus, produces a long-chain lipid called mycolic acid [67-70] (see **Figure 1-4**). This compound is produced in the highest amounts in the presence of hydrophobic substrates. The mycolic acid is attached to the outer-membrane of the microorganism but the two hydrophobic tails are free to increase the hydrophobicity of the surface of the microbe [71]. The increased hydrophobicity of the cells represents an advantage for the *Rhodococcus* genus when degrading hydrocarbons. Most hydrocabons are themselves hydrophobic and are characterized by low bioavailability in aqueous media. The mycolic acid increases the tendency of a microorganism to preferentially stay at the water-hydrocarbon interface. This in turn allows the bacterium to overcome, to a certain level, the problem of the low bioavailablity of hydrophobic substrates through conventional dissolution mechanisms.

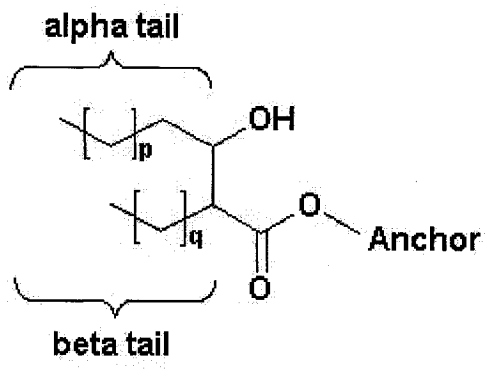

Figure 1-4 — General structure of mycolic acids. The alpha tail is longer than the beta tail and is characteristic to microbial species. The chains can be composed of as many as 90 carbons [71].

1.6 Esterases

Esterases are a class of enzymes that are capable of catalyzing the hydrolysis or the esterification of ester bonds. Esterases are often compared to or grouped with lipases. While lipases act on lipids that are strongly hydrophobic, esterases are more likely to be active with more soluble short-chain esters and lipids; i.e., approximately 6 carbons [72]. Generally, esterases do not require co-factors and are considered stable [72, 73]. They also exhibit broad substrate specificity [72-76] and are thought to be part of many primary metabolic pathways [72].

The active site of esterases is often a catalytic triad composed of the amino-acids serine, aspartic acid, and histidine [72, 77]. Other conformations are possible; however, in most cases serine is present and plays a major role in the reaction [72]. The mechanism for the hydrolysis of an ester catalyzed by an esterase is illustrated in **Figure 1-5**. First, the compound binds to the serine site, yielding a hemiacetal complex stabilized by the aspartic acid and the histidine sites (not shown). An alcohol is then

released and an acyl-enzyme complex is formed. Hydrolysis then occurs with the release of a carboxylic acid and the recovery of the initial enzyme.

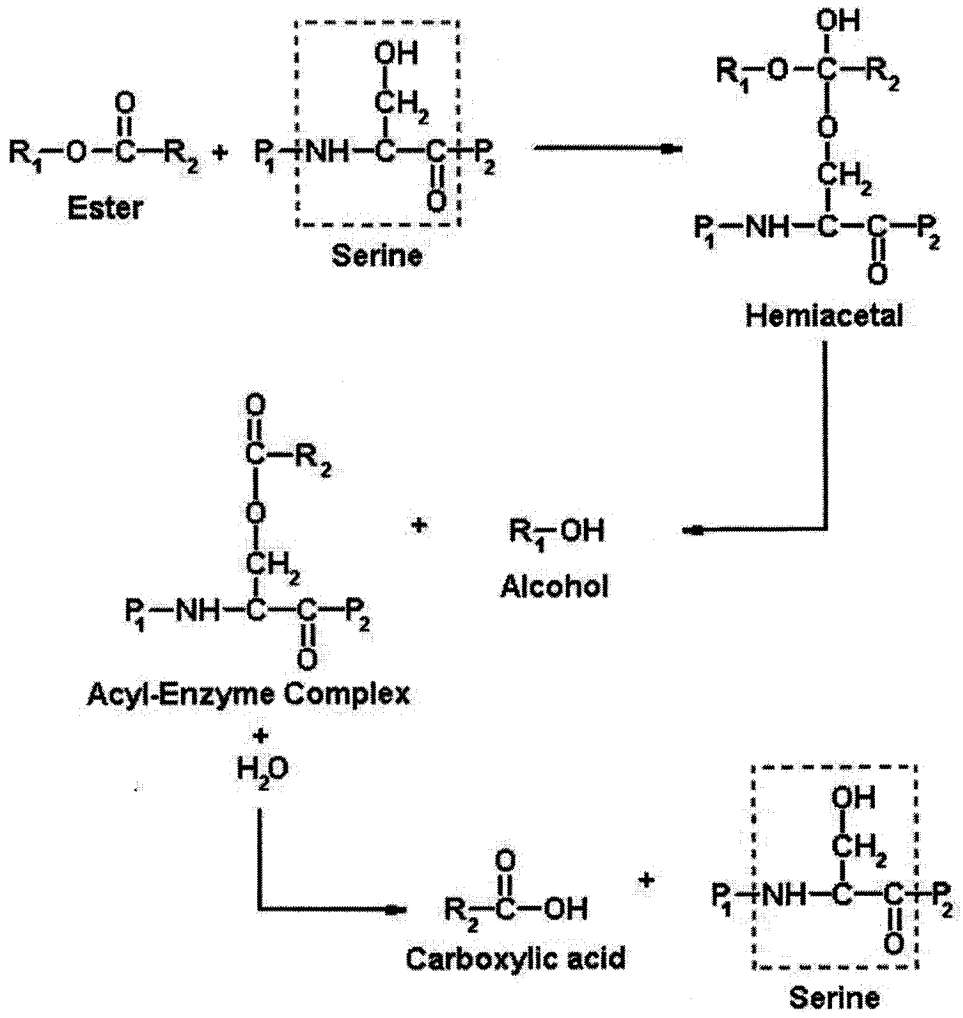

Figure 1-5 — Mechanism for the hydrolysis of ester bonds by an esterase. The active site is composed of a serine amino-acid. P_1 and P_2 represent chains of amino-acids.

Previous studies have investigated the role of esterases in the biodegradation of di-ester plasticizers. In fact, the role of esterases in the catabolism of phthalates and adipates was known by the end of the 1970's [35, 78] although the enzymes themselves

had not been isolated. In the early 1980's, Kurane et al. [76] isolated and characterized an esterase from a strain of *Rhodococcus erythropolis* that had been selected for its affinity for phthalic acid esters. The enzyme found displayed lipase characteristics and showed broad substrate specificity. More recent studies revealed similar results with different plasticizers and microorganisms [79, 80]. However these studies only focused on the isolation of esterases from microorganisms known to degrade di-ester plasticizers. Kinetic studies have been conducted to determine the rates of degradation of different di-esters [76, 81]. Although the length of side-chains and the solubility of di-esters have been linked to the rate of degradation [4, 81], researchers have failed to quantify their effect or to clearly demonstrate the impact of the enzyme on the rates.

1.7 Research Objectives

Many di-ester plasticizers are considered ubiquitous environmental contaminants and can yield recalcitrant toxic metabolites when biodegraded. The first steps in the degradation of these plasticizers involve an esterase-catalyzed hydrolysis. Thus, it is of particular interest to gain an understanding of the role of the esterase and the factors influencing the hydrolysis of these compounds. *Rhodococcus rhodochrous* ATCC 13808 is a bacterium able to degrade di-ester plasticizers. The esterase from this organism was selected for characterization and for the investigatation of the role that this enzyme plays in the degradation of different di-ester plasticizers. More specifically, the objectives of this study are as follows:

1) to characterize the enzyme with respect to its location in the cell, its substrate specificity, its stability and its reactivity at different temperatures;

2) to quantify the differences in the rates of hydrolysis of different di-ester plasticizers and other esters by this esterase; and
3) to determine some of the factors influencing the rate of hydrolysis of di-ester plasticizers.

This will provide a basis for increased insight into the potential environmental impacts of di-ester plasticizers, particularly those arising from their interactions with esterases.

2. MATERIALS AND METHODS

2.1 Selection of Microorganism, Storage, and Maintenance

The microorganism selected for this study was *Rhodococcus rhodochrous* ATCC 13808. It is considered the type strain of *R. rhodochrous*. This bacterium is a common soil organism and was selected because it is known to degrade hydrocarbons and, more specifically, di-ester plasticizers [42]. According to the American Type Culture Collection (ATCC), the ideal growth temperature is 26°C and the ideal medium for growth is Brain Heart Infusion (BHI).

The bacterium was stored in 1.5-mL plastic vials (Fisher Scientific, Montreal, QC) in a freezer (Revco, Model ULT1386, Fisher Scientific, Montreal, QC) at -70°C for a maximum period of one year before use. The vials contained a mixture of 20% glycerol (Sigma-Aldrich, Montreal, QC) and 20% BHI (Difco brand, Fisher Scientific, Montreal, QC) (37 g/L BHI in distilled water). To recover the organism, a vial was thawed in a laminar fume-hood (Baker Company, Model VBM600) and its contents was poured into a 500-mL Erlenmeyer flask fitted with a sponge cap (Fisher Scientific, Montreal, QC) containing 100 mL of 20% BHI. Before introduction of the organism, the Erlenmeyer flask and its contents were sterilized for 30 min at 121°C and 17 psig in an autoclave (AMSCO, Model SG-116 or AMSCO, Model 3021-S). After inoculation, the flask was incubated in a rotary incubator shaker (New Brunswick Scientific, Model G-25) at 30°C and 200 rpm. The microorganism was grown for 5-7 days, at which point 2-mL samples were transferred to another Erlenmeyer flask containing sterilized fresh medium.

The microorganism was maintained in such a manner, with transfers being performed once a week. All transfers were performed in a laminar fume-hood using sterile techniques.

2.2 Plasticizers and Other Chemicals

All chemicals used in this study were chemical reagent grade. A list of plasticizers and other compounds used in this study —as well as some of their physical properties, purity, and the supplier details — is shown in **Table 2-1**.

Table 2-1a — Plasticizers and other chemicals, physical properties and suppliers.

Compound	Molecular Weight	Density[a]	Solubility	Supplier
	g/mol	g/mL	mg/L	
Butyl butyrate (98%)	144.21	0.871	390[b]	Sigma-Aldrich Montreal, QC
o-Nitrophenyl acetate (99%)	181.15	-	1050[b]	Sigma-Aldrich Montreal, QC
o-Nitrophenol	139.11	-	-	A&C American Chemicals Montreal, QC
Di(2-ethylhexyl) adipate (99%) (DEHA)	370.58	0.925	0.78[b]	Sigma-Aldrich Montreal, QC
Di(methyl) phthalate (99%)	194.19	1.191	4290[c]	Sigma-Aldrich Montreal, QC
Di(ethyl) phthalate (99%)	222.24	1.118	928[c]	Sigma-Aldrich Montreal, QC
Di(n-butyl) phthalate (99%)	278.35	1.043	10[c]	Sigma-Aldrich Montreal, QC

a – All densities obtained from suppliers.
b – Values from the SRC PhysProp Database [82]
c – Values from *Leyder et al.* [83]

Table 2-1b — Plasticizers and other chemicals, physical properties and suppliers.

Compound	Molecular Weight g/mol	Density[a] g/mL	Solubility mg/L	Supplier
Di(n-hexyl) phthalate	334.45	0.998	0.05[b]	TCI Japan
Di(2-ethylhexyl) phthalate (99%) (DEHP)	390.56	0.973	0.04[c]	Sigma-Aldrich Montreal, QC
n-Hexadecane (99%)	226.40	0.78	-	Sigma-Aldrich Montreal, QC
n-Pentadecane (99%)	212.42	0.77	-	A&C American Chemicals Montreal, QC
Adipic acid	146.16	-	-	Ficher Scientific Montreal, QC
Sodium hydroxide (98.6%) (NaOH)	40.00	-	-	Fisher Scientific Montreal, QC
2,2,4,4,6,8,8-Heptamethylnonane	226.40	-	-	Sigma-Aldrich Montreal, QC
Chloroform (99.9%)	119.38	1.47	-	Fisher Scientific Montreal, QC
Triton X-100	-	-	-	Sigma-Aldrich Montreal, QC

a – All densities obtained from suppliers
b – Value from NTP report [84]
c – Value from *Leyder et al.* [83]

2.3 Bioreactors and Growth Conditions

2.3.1 Shake Flasks

500-mL Erlenmeyer flasks with sponge caps were inoculated with 2 mL of cell broth obtained from a BHI medium. Inoculation was performed using sterile techniques in a laminar fume-hood. The 100 mL of medium in the shake flask was previously autoclaved and contained either 8.0-g/L nutrient broth (Difco brand, Fisher Scientific, Montreal, QC) in distilled water; 2.0-g/L hexadecane in mineral salt medium (MSM); 1.0-g/L hexadecane and 2.5-g/L di(2-ethylhexyl) adipate (DEHA) in MSM; 2.0-g/L

adipic acid and 0.1-g/L yeast extract (Difco brand, Fisher Scientific, Montreal, QC) in MSM; or 1.0-g/L adipic acid, 2.5-g/L DEHA and 0.1-g/L yeast extract in MSM. The composition of MSM is summarized in **Table 2-2**. Sodium hydroxide (NaOH) (Fisher Scientific, Montreal, QC) was added to all media containing adipic acid. It was added in a 1:2 NaOH to adipic acid weight ratio. This was done to compensate for the addition of acid and to ensure that the pH would stay close to pH 7.

The microorganism was grown for 4-7 days in a rotary incubator shaker at 30°C and 200 rpm. It was transferred twice before actual testing to allow for acclimatization to the new medium. Analyses could be performed at all stages of growth.

Table 2-2 — Salt concentrations of mineral salt medium (MSM) in distilled water.

Compound	Concentration g/L	Supplier
Ammonium Nitrate NH_4NO_3	4.0	Fisher Scientific Montreal, QC
Potassium Phosphate KH_2PO_4	4.0	Fisher Scientific Montreal, QC
Sodium Phosphate Dibasic Na_2HPO_4	6.0	Fisher Scientific Montreal, QC
Magnesium Sulfate Heptahydrate $MgSO_4 \cdot 7H_2O$	0.2	A&C American Chemicals Montreal, QC
Calcium Chloride Dihydrate $CaCl_2 \cdot 2H_2O$	0.01	Fisher Scientific Montreal, QC
Iron Sulfate Heptahydrate $FeSO_4 \cdot 7H_2O$	0.01	Fisher Scientific Montreal, QC
Disodium Edetate Na_2EDTA	0.014	Fisher Scientific Montreal, QC

2.3.2 Circulating Batch Reactor

Because mixing is an issue when working with hydrophobic substrates (*e.g.* hexadecane and DEHA), a circulating batch reactor system (**Figure 2-1**) was used for some experiments. This type of system also allowed for better aeration.

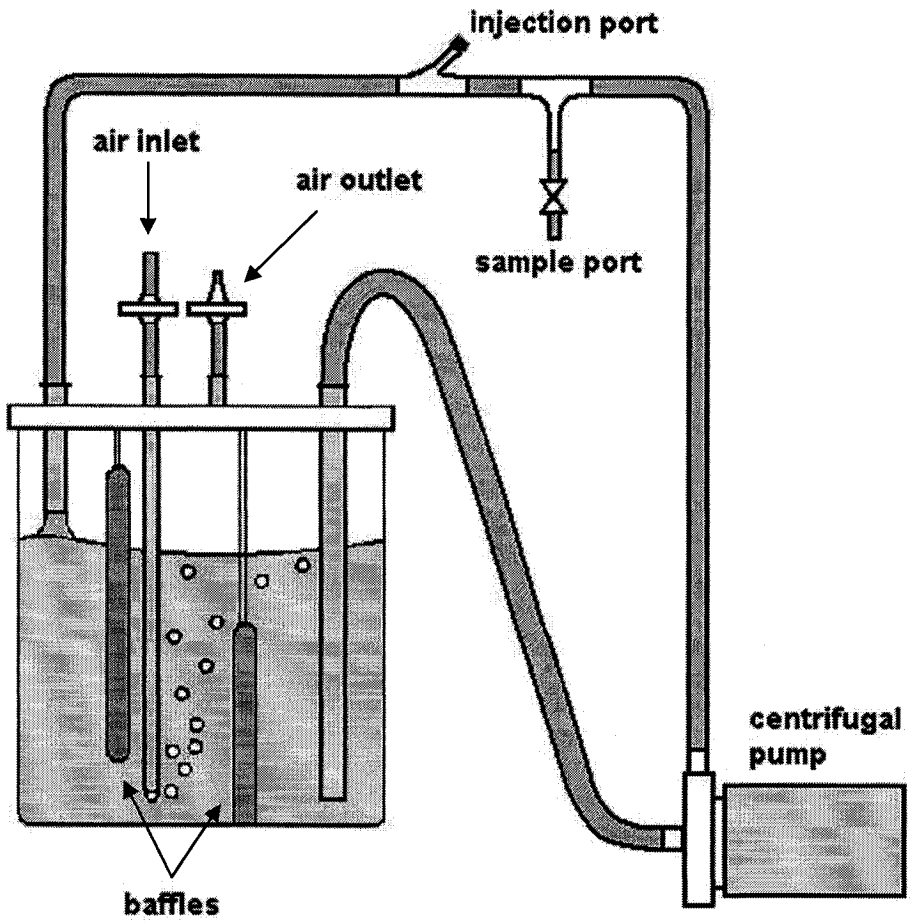

Figure 2-1 — Circulating Batch Reactor.

A 2-L New Brunswick glass batch reactor was used for the circulating batch reactor setup. The cover was made of Teflon (2.54 cm thick) to prevent the possibility of any plasticizer leaching from a stopper. The broth was continuously pumped through the system by a 1/55 hp centrifugal pump (Magnetek, Model MDX). This, along with baffles

incorporated into the reactor, ensured better mixing and prevented the formation of dead zones in the reactor. Saturated moist air was supplied to the bottom of the reactor. The aeration rate was 0.67 vvm. This allowed for both better aeration and improved mixing. Both the air inlet and the air outlet were passed through Hepa-Vent inline filters (Fisher Scientific, Montreal, QC) to prevent contamination.

The reactor was autoclaved for a period of 1 hour at 121°C and 17 psig. 1.5 L of MSM containing the soluble substrates (if being used) were autoclaved simultaneously in a glass bottle and poured in the reactor as soon as the sterilization was finished. The hydrophobic substrates were autoclaved separately under similar conditions. After cooling of the reactor and MSM, hydrophobic substrates were added through the injection port using a 5-mL disposable sterile syringe (Fisher Scientific, Montreal, QC). The system was then inoculated with 20 mL of a broth of *R. rhodochrous* ATCC 13808 grown in shake flasks. The inoculation was performed through the injection port using a 30-mL disposable sterile syringe (Fisher Scientific, Montreal, QC). The reactor was operated at room temperature (~ 20°C). Samples were obtained from the sample port and analyzed immediately.

The substrate concentrations used were either 1.0-g/L hexadecane and 2.5-g/L DEHA; or 1.0-g/L adipic acid, 2.5-g/L DEHA and 0.1-g/L yeast extract. As in the case of the shake flasks, NaOH was added to medium containing adipic acid to control its pH.

2.3.3 Cyclone Batch Reactor

The use of a cyclone batch reactor system (**Figure 2-2**) allowed for a quick and efficient provision of biomass. The exceptionally efficient mixing of the liquid phase

made the growth rapid and reliable. The system was composed of a 4-L glass cyclone reactor, an autoclavable dissolved oxygen probe (Ingold, Model IL531), a 1/55 hp centrifugal pump, and an in-line heat exchanger. The dissolved oxygen probe was connected to a strip-chart recorder to monitor growth. The in-line heat exchanger was used only as a support, because the kinetics of growth were not of interest for the experiments. Air was supplied at an aeration rate of 0.2 vvm. Both the air inlet and the air outlet were passed through Hepa-Vent inline air filters to avoid contamination. A 10-L carboy reservoir (Fisher Scientific, Montreal, QC) was situated on top of the reactor and supplied medium by gravity flow through the feed inlet. The feed was passed through an isolator to avoid contamination, and its flow was controlled by a manual valve. The reactor and the reservoir were sterilized in an autoclave at 121°C and 17 psig for 1 h and 3 h, respectively. Samples could be taken at all times from the isolator/sample port.

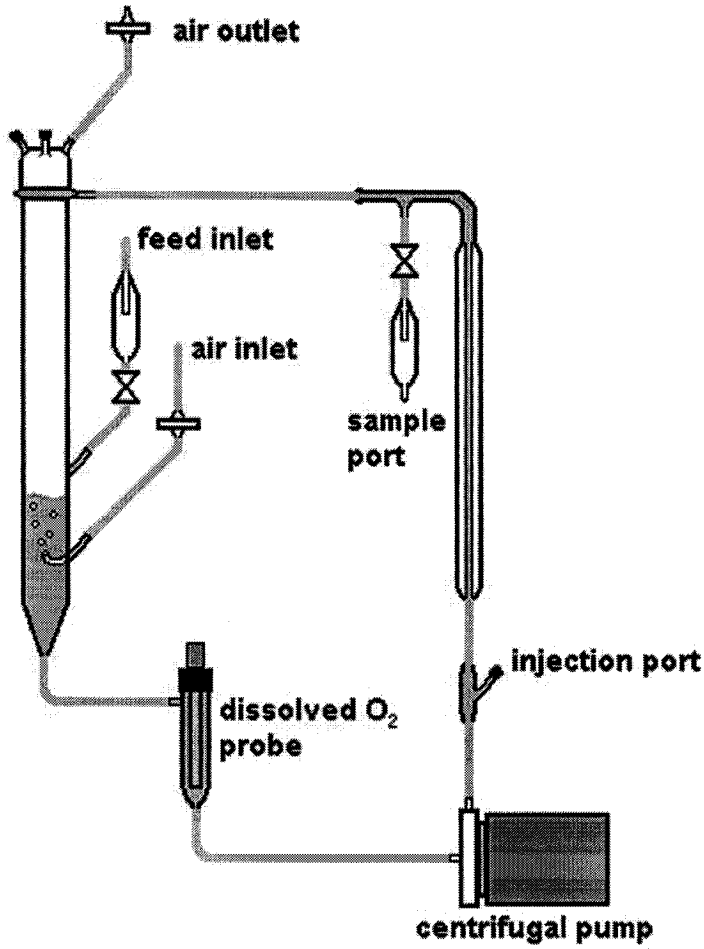

Figure 2-2 — Cyclone Circulating Batch Reactor.

The reservoir contained 2.0-g/L adipic acid, 0.1-g/L yeast extract, and 0.5-g/L NaOH in 10.0 L MSM. Initially, 1.0 L of medium was poured in the reactor. The reactor was then inoculated with 20 mL of *R. rhodochrous* ATCC 13808 broth grown in shake flasks. This was performed using a 30-mL disposable sterile syringe. When the stationary phase was reached, the concentration of biomass was approximately 1 g/L. To start a new cycle, half of the broth (0.5 L) was drained through the sample port. This was performed with the pump functioning. The medium was then replenished with 0.5 L of fresh medium fed from the reservoir. This corresponded to a single cycle and means the

starting biomass concentration was approximately 0.5 g/L. A cycle would then last one doubling time, which corresponded to approximately 5 hours. If a double cycle was performed, the initial biomass concentration was 0.25 g/L and the cycle time would last approximately 10 hours, corresponding to two doubling times. Similarly, a triple cycle would last 15 hours. It is important to note that the final biomass concentration was always approximately 1 g/L no matter the type of cycle performed. In order to minimize foaming, 0.1 mL of heptamethylnonane was added to the medium when necessary using a 1-mL disposable sterile syringe (Fisher Scientific, Montreal, QC). Heptamethylnonane was degraded very slowly by the microorganism and acted only as an anti-foam agent.

The "cycling" mode of operation lead to two major advantages: the predictability of the period of growth and a constant biomass concentration at the stationary phase. The predictability allowed for planning of sample times for growth studies and for consistency in sample preparation. The constant biomass concentration was a great asset when comparing the enzyme activity levels at different conditions or for different substrates.

2.4 Biomass Concentration Measurements

2.4.1 Dry Weight Measurement

The standard dry weight analysis was used to determine the biomass concentration [85]. A 20-mL or 30-mL sample was collected from a bioreactor for analysis. The sample was placed in 30-mL Teflon centrifuge tubes (Fisher Scientific, Montreal, QC) and centrifuged for 10 min at 10 000 x *g* at room temperature (IEC centrifuge, Model B-22M). The supernatant was discarded and the pellet was washed

with MSM. After a second centrifugation, the pellet was re-suspended in distilled water and poured into a tared aluminum weighing dish (Fisher Scientific, Montreal, QC). The sample was dried in an oven (Fisher Isotemp Oven 100 series, model 126G) for 48 h at 80°C. After cooling, the dish containing the sample was weighed using an analytical balance (Denver Instruments, Pinnacle Series, Model P214). The biomass concentration was quoted in g of dried biomass per litre of broth (g/L).

2.4.2 Optical Density Measurement

In order to have a rapid method for the determination of biomass concentration when working with the cyclone batch reactor, the optical density of the sample was used as an indirect measure. A 1-mL broth sample was put in a glass optical cell (Fisher Scientific, Montreal, QC) using a 1000-μL automatic pipetter (Gilson Pipetman, Model P1000). A 2.0 mL volume of distilled water was added to the sample. The optical cell was then introduced in a UV-spectrophotometer (Varian, Cary 50 Bio) set to measure absorbance at a wavelength of 500 nm. Distilled water was used as a reference zero. Five absorbance measurements were taken per sample and their average was reported as the optical density (OD_{500nm}).

To obtain the biomass concentration, a correlation was established between optical density at 500 nm and dried biomass per litre of broth, as given by the dry weight method. **Figure 2-3** gives an example of such a calibration curve. A calibration curve was performed for every new start-up of the cyclone reactor since the optical properties of the broth were observed to vary slightly between runs.

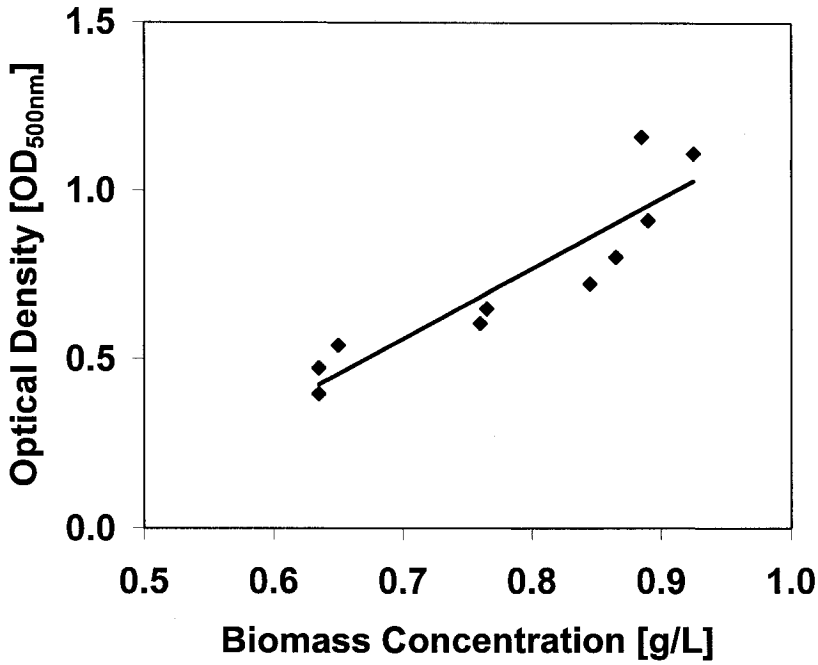

Figure 2-3 — Calibration curve relating optical density at 500 nm to biomass concentration as determined by dry weight measurement. The samples were diluted 1:2 in distilled water before measurements of optical density were taken.

2.5 Quantification of Compounds Using Gas Chromatography

The quantification of butyl butyrate, DEHA, and all phthalates was performed using gas chromatography and the following extraction method.

A solution of chloroform containing 500 mg/L of pentadecane as an internal standard was added to the sample to be analyzed in a 1:1 volume ratio. This was mixed for 1 min and allowed to settle. The organic phase was removed using a gas-tight 1-mL glass syringe (Hamilton brand, Fisher Scientific, Montreal, QC). The extract was stored in a 1.5-dram glass vial (Fisher Scientific, Montreal, QC) in a 4°C refrigerator (Fisher Isotemp) for a maximum period of 4 days before analysis. The samples were not affected by this storage period.

The extract was brought to room temperature before being injected in the gas chromatograph (GC) (Varian, Model CP-3800) using a 100-μL injection syringe (Hamilton brand, Fisher Scientific, Montreal, QC). The column used was a Supelco SPB-5 (30 m X 0.32 mm, 0.25 μm film) and the detector was a flame ionization detector (FID). The operating conditions for the GC are summarized in **Table 2-3**.

Table 2-3 — Operating conditions for gas chromatography.

Operating Condition	Value
Injection Temperature	250°C
Initial Column Temperature	40°C
Temperature Ramp #1	12°C/min
Temperature for Change of Ramp	120°C
Temperature Ramp #2	30°C/min
Final Column Temperature	300°C
Detector Temperature	320°C

The concentration of each compound could be determined from the ratio of the area under the peak of the compound of interest to the area under the peak of pentadecane. A calibration curve relating the concentration to the area ratio was established for each compound. Examples of such curves can be found for butyl butyrate and DEHP in **Figure 2-4**.

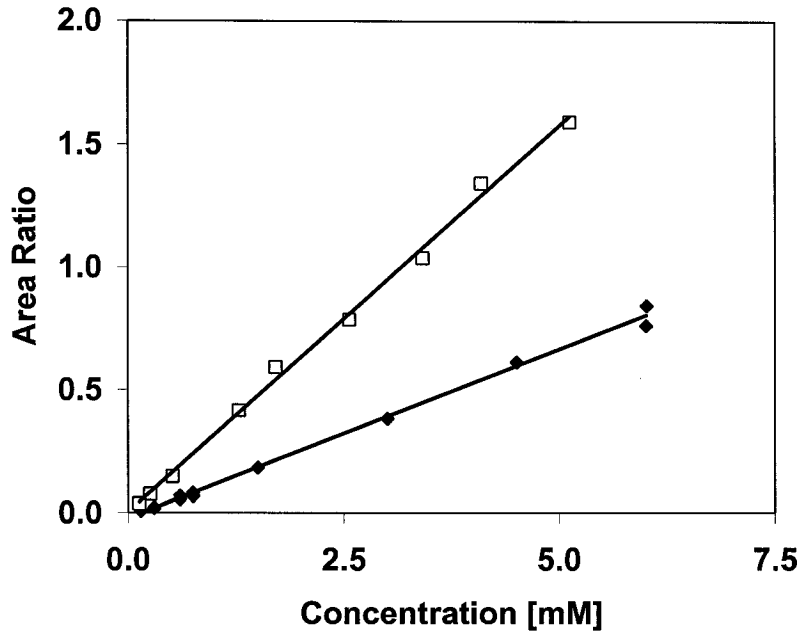

Figure 2-4 — Calibration curves relating area ratio to concentration in mM (mmol/L) for butyl butyrate (♦) and DEHP (□).

2.6 Esterase Activity Assays

2.6.1 o-Nitrophenyl Acetate/Esterase Assay

The esterase activity assay based on the hydrolysis of o-nitrophenyl acetate was derived from the method used by *Krebsfanger et al.* [86]. When catalyzed by an esterase, o-nitrophenyl acetate hydrolyses to acetic acid and o-nitrophenol. The latter absorbs light at 412 nm. It is then possible to follow its production using a UV-spectrophotometer.

The UV-spectrophotometer (Varian, Cary 100 Bio) was set at a wavelength of 412 nm and zeroed with distilled water. The sample to be tested was separated into two 1.5-mL plastic micro-centrifuge tubes (Fisher Scientific, Montreal, QC). A 0.5 mL volume of a solution of methanol containing 0.052 g/L of o-nitrophenyl acetate was also placed in a micro-centrifuge tube. All three tubes were incubated in a water bath (Lauda, Model E-100) at 30°C for a period of 5 min. A 1000-µL automatic pipetter was used to

transfer 950 μL of sample into an optical glass cell. A 100 μL volume of o-nitrophenyl acetate reagent solution was added using a 200-μL automatic pipetter (Gilson Pipetman, Model P200). Subsequently, another 950 μL of sample were rapidly added and the optical cell was put in the UV-spectrophotometer for absorbance measurement. Measurements were taken every 0.1 s for a period of 5-10 min. An example of the output from the UV-spectrophotometer is shown in **Figure 2-5**.

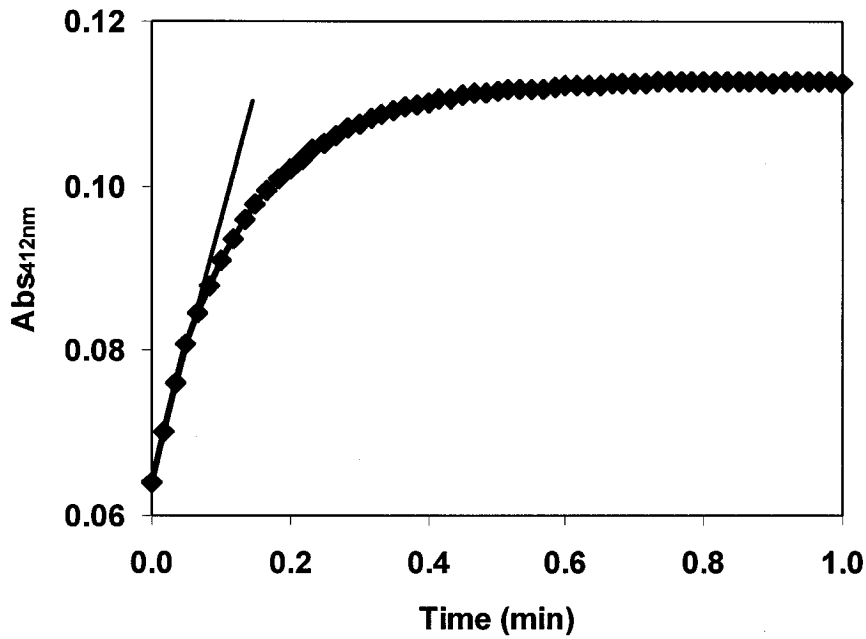

Figure 2-5 — Absorbance of light at a wavelength of 412 nm during the hydrolysis of o-nitrophenyl acetate to o-nitrophenol and acetic acid. The relevant measurement was the initial slope.

The esterase activity was then determined according to the initial slope of the trend. The units of the slope could be converted from $\Delta Abs_{412nm} \cdot min^{-1}$ to mM o-nitrophenol produced$\cdot min^{-1}$ based on a calibration curve relating absorbance to

concentration. Finally, based on a stoichiometric ratio of 1:1, the activity could be given as mM o-nitrophenyl acetate reacted·min^{-1}.

Controls were performed on distilled water, MSM, and autoclaved broth with and without o-nitrophenyl acetate reagent solution.

2.6.2 Butyl Butyrate/Esterase Assay

Another method used to determine the esterase activity was developed for this project. It was based on the hydrolysis of butyl butyrate.

A 5.0 mL volume of sample was placed in a disposable glass test tube (Fisher Scientific, Montreal, QC) using a 10-mL disposable glass pipette (Fisher Scientific, Montreal, QC). The test tube was then placed in a water bath at 30°C for 5 min. Butyl butyrate was added to the sample using a 20-µL automatic pipetter (Gilson Pipetman, Model P20) to reach a concentration of 3.0 mM (2.5 µL added for 5.0 mL of sample). The contents of the test tube were mixed for 10 s using a vortexer (Fisher Vortex, Model Genie 2). The test tube was covered using parafilm (Fisher Scientific, Montreal, QC) and it was incubated for 10 min in a rotary incubator shaker operating at 30°C and 200 rpm. The reaction was stopped by adding 5 mL of chloroform containing 500 mg/L of pentadecane. The mixture was vortexed for 1 min. The organic phase was recovered using a gas-tight glass syringe and stored in a 1.5-dram glass vial. It was then analyzed for concentration of butyl butyrate by gas chromatography, according to the method explained in **Section 2.5**.

The esterase activity was determined according to the amount of butyl butyrate reacted over the 10-min period of the assay. Units were converted to mM of butyl butyrate reacted·min^{-1}.

Controls were performed to determine the accuracy of the addition of butyl butyrate and the error of the assay. Further controls were performed to test for volatilization, and adsorption to the test tube walls or cells walls. These parameters were proven to be negligible.

No changes in the concentration of butyl butyrate were observed after the addition of the chloroform/pentadecane solution. This control confirmed that the reaction was stopped by the presence of chloroform and pentadecane,

2.7 Cell Lysis

2.7.1 Viable Cell Count

Six disposable glass test tubes and 100 mL of MSM were sterilized in an autoclave at 121°C and 17 psig for 30 min. In a laminar fume-hood, 9.0 mL of MSM were placed in each test tube using a 10-mL disposable glass pipette. A 1.0-mL broth sample was then placed in the first test tube using a sterile 1000-µL automatic pipetter. The mixture was mixed using a vortexer, and 1 mL was transferred to the following test tube. Mixing and transfers were repeated until the initial sample was diluted 10^6 times.

100 µL were sampled from the test tubes containing the 10^4, 10^5 and 10^6 times dilutions. Each sample was spread on individual agar/nutrient broth plates (18 g/L agar technical (Difco brand, Fisher Scientific, Montreal, QC) and 8 g/L nutrient broth). The plates were then taken out of the fume-hood and incubated for 4 days in an incubator

oven (Fisher Isotemp 500 series, Model 516D) at 30°C. The amount of colonies on each plate was then counted, with each colony assumed to arise from a single cell. Measurements of the number of cells per volume of broth (cells/mL) could be derived from the dilution factor.

2.7.2 Determination of Cell Lysis Conditions

Cell lysis was performed by shear using a Bioneb® Cell Disruption system (Glas-Col, Terre Haute, IN). Nitrogen was supplied to the apparatus from a gas cylinder. The pressure was adjusted to 90 psig using a regulator. The liquid flow rate was adjusted to 18 $mL \cdot min^{-1}$.

A 22-mL sample was taken from a *R. rhodochrous* ATCC 13808 broth at stationary phase. An initial sample of 2 mL was kept in a 1.5-dram sterilized glass vial in a 4°C refrigerator. The remaining 20 mL were continuously passed through the Bioneb®. Periodically, 2-mL samples were taken using a 1000-µL automatic pipetter. They were immediately placed in sterile glass vials in the refrigerator. The Bioneb® was stopped after 15 min. All collected samples were then taken to the laminar fume-hood to proceed to the viable cell count manipulations.

Cell lysis was determined according to the decrease in viable cells per volume of broth over time. To account for the sample volume, the time was normalized to the average residence time of the sample (volume of sample divided by its flowrate), yielding the number of passes through the apparatus. The volume losses encountered while using the Bioneb® were taken into account.

2.7.3 Cell Lysis Conditions

For all further experiments, samples were passed a minimum of 10 times through the Bioneb® to ensure proper cell lysis. The gas pressure from the tank was set to 90 psig and the liquid flow rate was adjusted to 18 $mL \cdot min^{-1}$.

2.8 Separation of Cell Fractions and Location of Esterase in the Cell

In order to locate the esterase, the cell was separated into three major cell fractions: extracellular, intracellular, and membrane.

Samples of *R. rhodochrous* ATCC 13808 were obtained at different growth conditions. 50 mL of sample were used. Of those, 20 mL were placed in a 30-dram glass vial (Fisher Scientific, Montreal, QC) stored in a refrigerator at 4°C, and labelled as "Broth". Using a disposable 10-mL glass pipette, the remaining 30 mL of sample were separated equally into two 30-mL Teflon centrifuge tubes. The sample was then centrifuged at 10 000 x *g* for 10 min at room temperature. The supernatant was recovered in a 30 dram glass vial using a 10-mL glass pipette, labelled as "Extracellular fraction" and stored at 4°C until further testing. The pellet was re-suspended in an equal volume of MSM, and cells were lysed according to the method specified in **Section 2.7.3**. The lysed broth was centrifuged under the same conditions as stated above. The new supernatant was recovered, stored at 4°C, and labelled as "Intracellular fraction". The pellet was re-suspended in an equal volume of MSM, labelled as the "Membrane-bound fraction" and stored at 4°C.

Each sample was then tested for esterase activity based on either the o-nitrophenyl acetate/esterase assay (**Section 2.6.1**) or the butyl butyrate/esterase assay (**Section 2.6.2**). It was then possible to determine in which cell fraction the esterase was located.

2.9 Protein Solubilization

In an attempt to isolate the esterase, the enzyme was extracted from the cell membrane by treatment with the synthetic non-ionic surfactant Triton X-100.

A sample was obtained from a *R. rhodochrous* ATCC 13808 broth at stationary phase. A 5 mL volume of sample was labelled "Cells" and tested for esterase activity (using the butyl butyrate/esterase assay, see **Section 2.6.2**). The remainder of the sample was centrifuged at 10 000 x *g* for 10 min at room temperature. The supernatant was discarded and the pellet was re-suspended in an equal volume of phosphate buffer (0.1 M, pH 7) containing 0.1% (w/v) Triton X-100. A 5-mL sample was labelled "Cells + Triton X-100" and immediately tested for esterase activity. The remainder of the mixture was placed in a glass beaker (Fisher Scientific, Montreal, QC) and stirred at room temperature for 30 min at approximately 100 rpm. The sample was then centrifuged under the same conditions as stated above. The supernatant was recovered, labelled as "Extracted protein", and tested for esterase activity. The pellet was re-suspended in phosphate buffer without Triton X-100, and was then labelled as "Treated cells" and tested for activity.

Controls demonstrated that the esterase activity was preserved and that Triton X-100 alone did not account for higher activity levels. The measurements for esterase activity were then compared and the solubilization efficiency could be established.

Visual analysis was also performed using an optical microscope (Leitz, Diaplan). 100 μL were taken from the "Cells" and from the "Extracted protein" samples and spread on microscope slides using a sterile 200-μL automatic pipetter.

2.10 Esterase Stability

The stability of the esterase was tested for samples in which the enzyme was attached to the membrane of the cell or solubilized.

At least 30 mL of membrane-bound sample (obtained from the method detailed in **Section 2.8)** or of extracted protein sample (obtained from solubilization by Triton X-100, see **Section 2.9**) was divided into smaller samples of 5 mL. These were placed in disposable glass test tubes. The test tubes had been previously autoclaved at 121°C and 17 psig for 30 min. Parafilm was used to close the test tubes. One test tube was immediately tested for esterase activity (using the butyl butyrate/esterase assay, see **Section 2.6.2**) to determine the initial activity. The other test tubes were placed in a temperature controlled rotary incubator shaker at 200 rpm. Tests were done at three temperatures: 30°C, 20°C, and 4°C. For the 4°C tests, a test tube rack was fixed to a vortexer which was placed in a 4°C refrigerator. Periodically, test tubes were taken out of the shakers and tested for esterase activity up to 48 h. The deactivation and the stability of the esterase could then be determined over time.

It is important to note that the butyl butyrate/esterase assay includes a 30°C pre-heating stage in a water bath for all samples. This normalized all results independently of the temperature of storage.

2.11 Effect of Temperature on the Esterase Activity

A membrane-bound sample of 45 mL (obtained according to the method in **Section 2.8**) was separated equally in 9 disposable glass test tubes. The test tubes were covered with parafilm and placed at different temperatures in groups of three. One group was placed in a rotary incubator shaker at 30°C, another group at 20°C, and finally a third group at 4°C in a vortexer/refrigerator setup (as described in **Section 2.10**). After a period of 15 min, the test tubes were taken out, and 2.5 μL of butyl butyrate were added to each sample using a 20-μL automatic pipetter. The test tubes were placed back in their respective incubators. After 10 min, the reaction was stopped by adding 5 mL of chloroform containing 500 mg/L of pentadecane using a 10-mL disposable pipette. This was done for all test tubes. The mixture was vortexed for 1 min, and the organic phase was recovered using a 1-mL gas-tight syringe. Extracts were kept in 1.5-dram vials at 4°C until they were analyzed by gas chromatography (**Section 2.5**). Activity measurements were expressed as mM of butyl butyrate reacted·min^{-1}.

2.12 Determination of Effective Hydrolysis Time

The following method was used to determine the effective hydrolysis time for butyl butyrate, DEHA, dimethyl phthalate, diethyl phthalate, di(n-butyl) phthalate, di(n-hexyl) phthalate and DEHP.

At least 30 mL of a membrane-bound sample (**Section 2.8**) was divided into 5-mL samples and placed in disposable test tubes. 3 mM of the substrate of interest (i.e., butyl butyrate, or DEHA, or dimethyl phthalate, etc.) were added to the contents of each test tube using a 20-μL automatic pipetter. Parafilm was used to close the test tubes, and

the mixture was vortexed for 10 s. The samples were placed in a rotary incubator shaker at 30°C and 200 rpm. The first sample was tested immediately and the rest were tested over the time of the experiment. The reaction was stopped by adding chloroform containing 500 mg/L of pentadecane, and mixing it for 1 min in the vortexer. The organic phase was extracted with a 1-mL gas-tight glass syringe, and stored in a 1.5-dram glass vial. The vial was kept in a refrigerator at 4°C for further analysis. This was done at various times. The samples were analyzed for substrate concentration using gas chromatography (**Section 2.5**).

2.13 Comparison of Rates of Hydrolysis

The rates of hydrolysis of all compounds tested (i.e., o-nitrophenyl acetate, DEHA, dimethyl phthalate, diethyl phthalate, di(n-butyl) phthalate, di(n-hexyl) phthalate, and DEHP) were compared to that of butyl butyrate. Membrane-bound samples were obtained according to the method found in **Section 2.8**. Two types of samples were used for these comparisons: namely, membrane-bound samples obtained at different stages of growth, and dilutions from a membrane-bound sample obtained at stationary phase. The dilutions were performed using MSM, and their ratio of sample to MSM ranged from 1:0 to 1:19.

A series of different 5-mL samples (different stage of growth or dilution) were placed in disposable glass test tubes. However, for each particular sample condition, a similar sample was prepared to form a pair of identical samples (i.e., same origin, same dilution, etc.). For each pair, the butyl butyrate/esterase assay was performed on one sample, while the other substrate was tested on the second. To test the rate of hydrolysis

of other substrates, the method for the butyl butyrate/esterase assay was replicated with two major modifications. First, the volume of substrate added to the test tube had to be adjusted according to the molecular weight and density of the compound (e.g. 4 μL were added for DEHA). Finally, the time of the modified assay had to be based on the effective hydrolysis time (e.g. 8 h for DEHA). All other manipulations were identical.

The only exception to this was for the assays with o-nitrophenyl acetate. Since there was an existing efficient assay for this compound, and since this assay was very different from the butyl butyrate assay, the comparison of the rate of hydrolysis was based on their respective assay.

3. RESULTS

3.1 Butyl Butyrate/Esterase Assay

The hydrolyses of butyl butyrate and o-nitrophenyl acetate were useful for quick determinations of esterase activity. The protocol used for the o-nitrophenyl acetate/esterase assay was derived from the assay by *Krebsfanger et al.* [86].

The butyl butyrate/esterase assay was developed for this project. A short hydrolysis study was necessary to determine an appropriate effective hydrolysis time that could be applied throughout the project. **Figure 3-1** shows the hydrolysis of butyl butyrate by a sample containing 1 g/L *R. rhodochrous* ATCC 13808. The assay time chosen for the esterase assay was 10 minutes. The selection of this assay time allowed for a representative measure of the initial rate of hydrolysis while reducing the error due to manipulations.

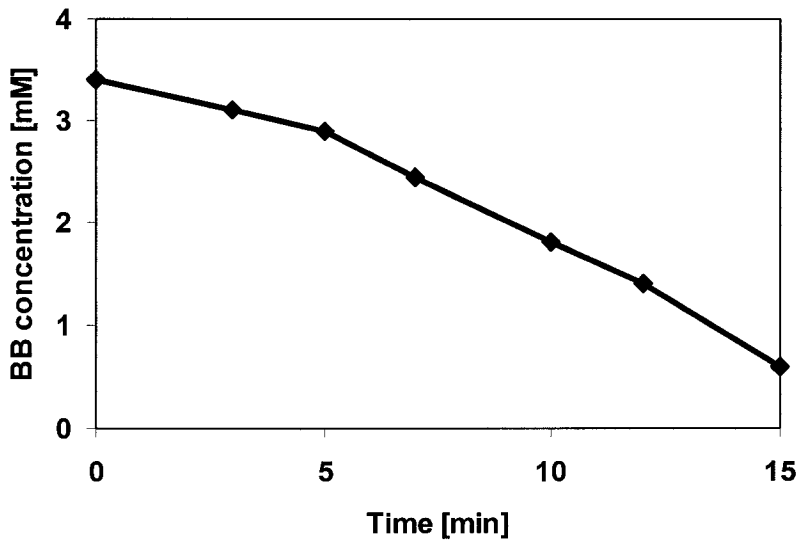

Figure 3-1 – Butyl butyrate hydrolysis by 1 g/L *R. rhodochrous* ATCC 13808.

Figure 3-2 shows the results from controls for the butyl butyrate/esterase assay. The broth sample contained 1.5 g/L *R. rhodochrous* ATCC 13808 grown in a circulating batch reactor on adipic acid (3 g/L) and yeast extract (0.1 g/L) in MSM. A portion of the broth samples were autoclaved to perform controls on dead biomass with inactive enzymes. The broth samples displayed an activity of 0.189 mM BB/min ± 0.009. The error is representative of the standard deviation. The MSM controls showed no activity for all of the samples, and the dead biomass controls had activity of 0.005 mM BB/min ± 0.007. The results from the controls led to the conclusion that in all activity measurements, the observed activity was the sole result of enzyme activity.

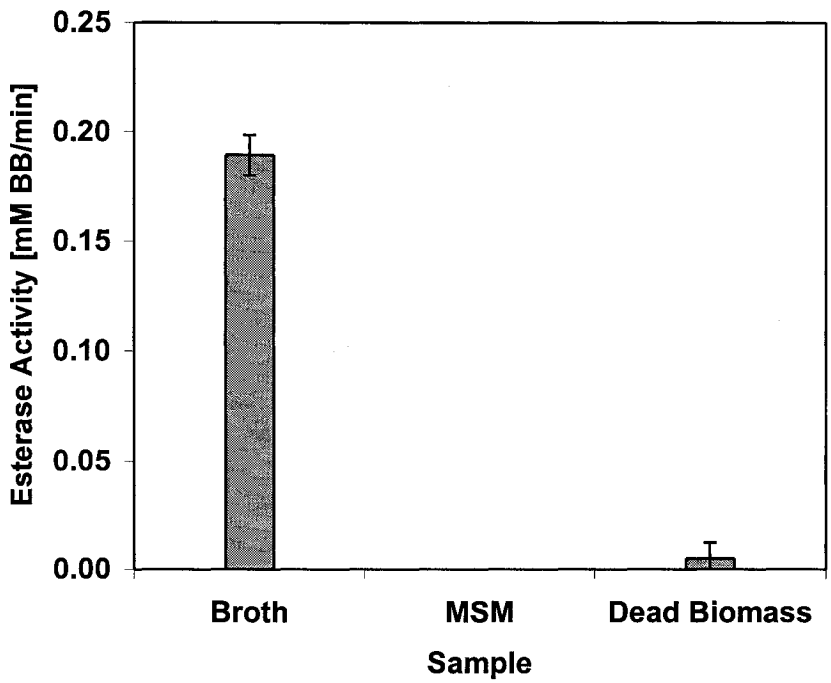

Figure 3-2 – Results from control samples assayed for the butyl butyrate/esterase assay. Broth and dead biomass samples contained 1.5 g/L *R. rhodochrous* ATCC 13808 in MSM.

3.2 Cell Lysis

In order to evaluate the lysing efficiency of the Bioneb® Cell Disruption System, a broth containing *Rhodococcus rhodochrous* ATCC 13808 was passed continuously through the apparatus. The concentration of living cells was monitored throughout the experiment by plating and counting the viable cells. **Figure 3-3** shows that the concentration of living cells decreased rapidly and then stabilized at roughly 25% of its initial value as the sample was continuously passed through the Bioneb® System.

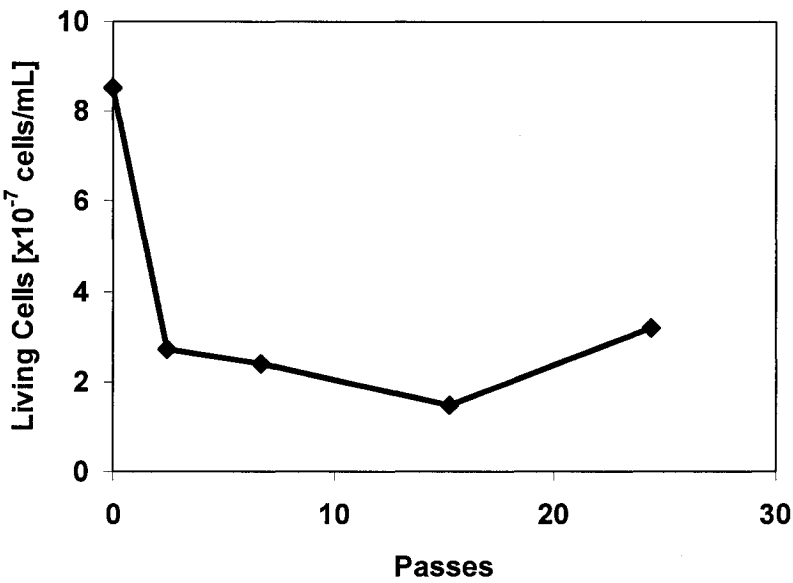

Figure 3-3 – *R. rhodochrous* ATCC 13808 cell lysis as a function of the number of passes through the Bioneb® System.

3.3 Characterization of the Esterase

3.3.1 Location of the Esterase in the Cell

Figure 3-4 shows the relative esterase activity for each of the separated cell fractions (extracellular, intracellular, and membrane) from *R. rhodochrous* ATCC 13808. The bacterium was grown on 2.5 g/L hexadecane in MSM. The cell fractions were obtained from a broth harvested at the stationary phase and separated from one another. The activity measurements obtained from the broth were taken to be 100%. The activity measurements from other fractions were reported relative to this. Both the o-nitrophenyl acetate/esterase and butyl butyrate/esterase assays resulted in similar values. The results show that practically all of the activity of the broth was contained in the membrane-bound cell fraction. The activity balance between the broth and the membrane samples closed to within 10%.

Similar results were obtained when the organism was grown on 1.0 g/L hexadecane and 2.5 g/L di(2-ethylhexyl)adipate; 1.0 g/L adipic acid, 2.5 g/L di(2-ethylhexyl) adipate and 0.1 g/L yeast extract; 2.0 g/L adipic acid and 0.1 g/L yeast extract; or 8.0 g/L nutrient broth. The organism could be grown in shake flasks or in a circulating batch reactor without influencing the observed activity partitioning.

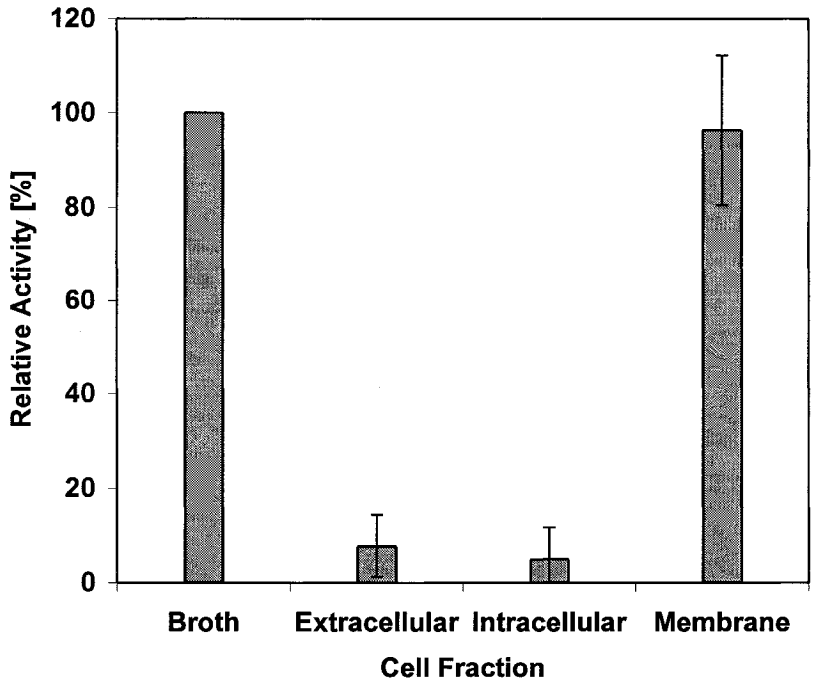

Figure 3-4 – Relative esterase activity of different cell fractions for *R. rhodochrous* ATCC 13808 grown on hexadecane.

3.3.2 Esterase Activity and Growth

In order to determine if the pattern of activity in the different cell fractions was dependent on the stage of growth, *R. rhodochrous* ATCC 13808 was grown in a circulating batch reactor and analyzed at different time intervals. **Figure 3-5** shows the results obtained for biomass concentration and esterase activity (based on the butyl butyrate/esterase assay) for each fraction of the bacterium grown with 2 g/L adipic acid and 0.1 g/L yeast extract in a cyclone circulating reactor. **Figure 3-6** shows similar results for a circulating reactor with medium containing 1 g/L adipic acid, 2.5 g/L di(2-ethylhexyl) adipate and 0.1 g/L yeast extract. The activity measurements were based on the o-nitrophenyl acetate/esterase assay.

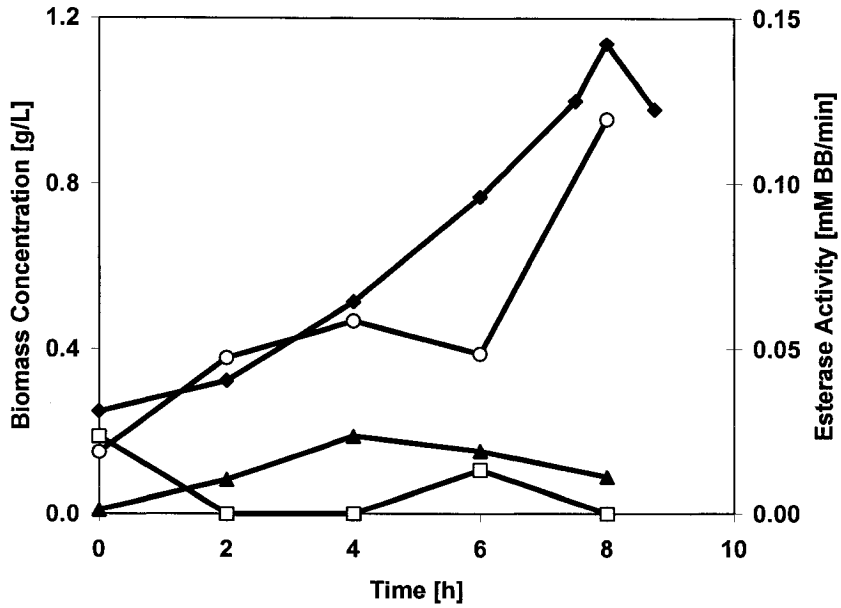

Figure 3-5 – Extracellular (□), intracellular (▲), and membrane-bound (○) esterase activity during growth of *R. rhodochrous* ATCC 13808 in a cyclone circulating batch reactor when adipic acid and yeast extract are used as substrates. Biomass concentration (♦) is also shown.

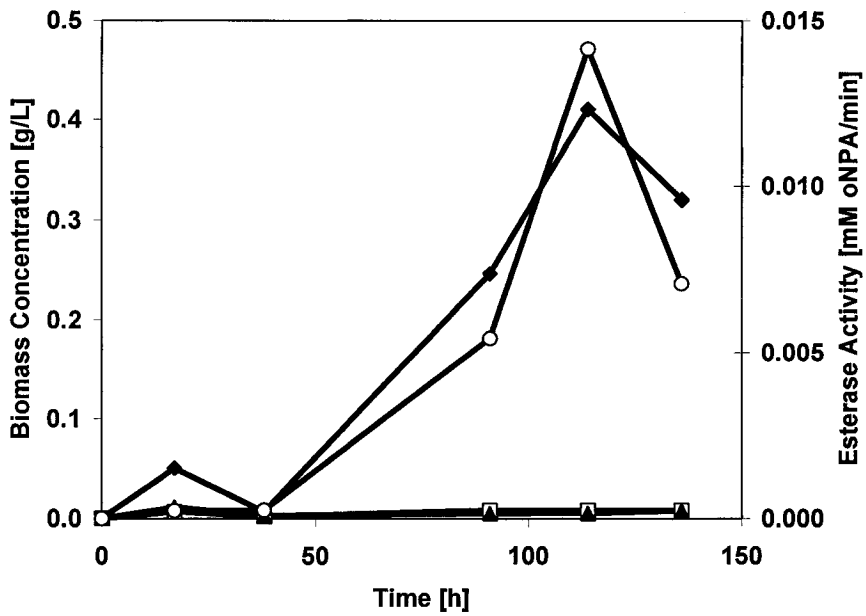

Figure 3-6 – Extracellular (□), intracellular (▲), and membrane-bound (○) esterase activity during growth of *R. rhodochrous* ATCC 13808 in a circulating batch reactor when di(2-ethylhexyl) adipate, adipic acid and yeast extract are used as substrates. Biomass concentration (♦) is also shown.

In both cases, the activity pattern was independent of the stage of growth. Moreover, the membrane-bound activity follows the trend of the biomass concentration.

Figure 3-7 shows the membrane-bound esterase activity for the bacterium grown with different substrates and sampled at different stages of its growth. All esterase activity assays were performed with butyl butyrate as the substrate. Results show a clear correlation between esterase activity and biomass concentration regardless of the substrate used for growth.

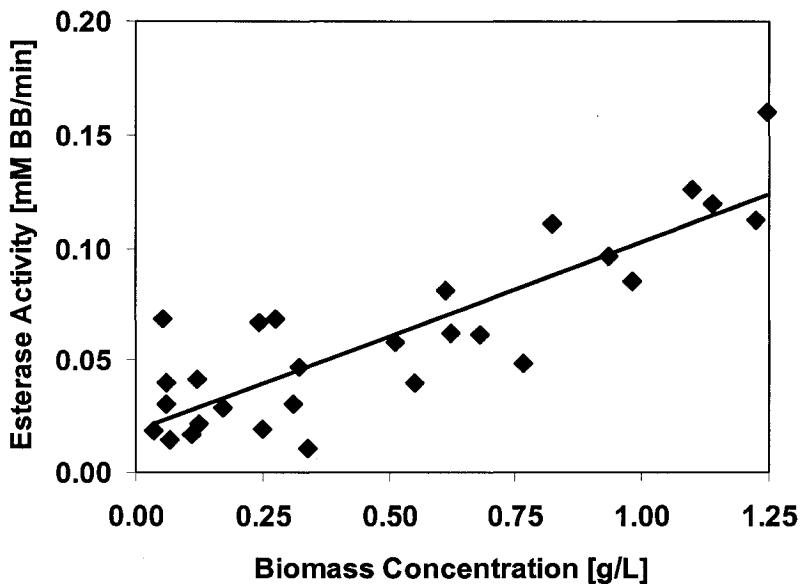

Figure 3-7 – Relating the membrane-bound activity from *R. rhodochrous* ATCC 13808 to the biomass concentration. Samples were taken at different stages of growth. The substrates used were hexadecane, hexadecane/di(2-ethylhexyl) adipate, adipic acid/ di(2-ethylhexyl) adipate/yeast extract, adipic acid/yeast extract, or nutrient broth. The correlation has an R^2-value of 0.73.

3.3.3 Solubilization of the Esterase

Because the esterase is located in the membrane, many analyses cannot be performed without a pretreatment step. Unlysed cells were suspended in a phosphate buffer (0.1 M, pH 7) solution containing Triton X-100, a non-ionic detergent. The detergent allows proteins to detach themselves from the membrane and to be solubilized in the solution. Results from the solubilization of the esterase are shown in **Figure 3-8** for two different sets of extractions conducted under the same conditions. The activity measurements obtained from the cells in phosphate buffer were given a value of 100% and all other measurements were reported relative to this.

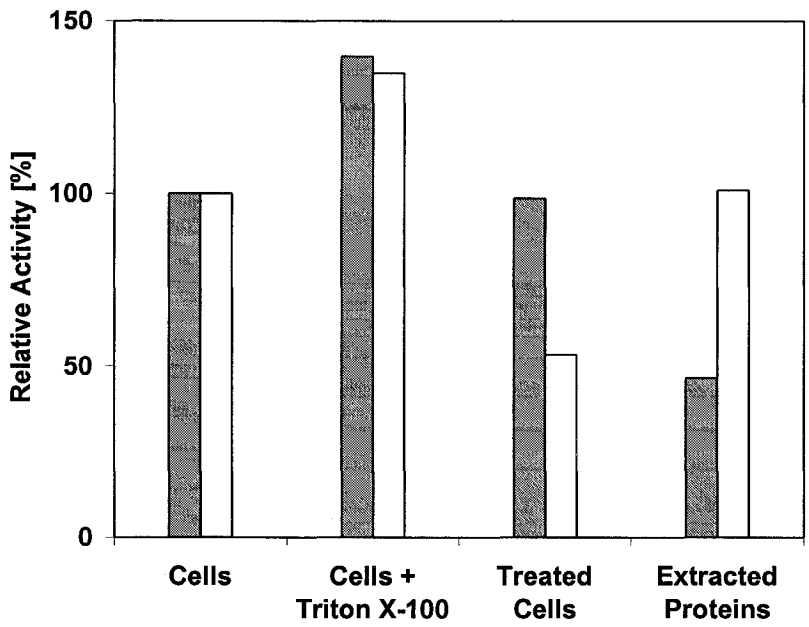

Figure 3-8 – Solubilization of a membrane-bound esterase from *R. rhodochrous* ATCC 13808 using the non-ionic detergent Triton X-100 in phosphate buffer. Data are given for cells in phosphate buffer alone, for cells in phosphate buffer containing Triton X-100, for treated cells after separation, and for the extracted solubilized proteins. Data for two different extractions under the same conditions are shown.

The results show an increase in activity when the cells are in contact with Triton X-100. It can also be seen that the activity levels for treated cells and for extracted protein samples vary greatly from one extraction to the next. However, in both sets of data, the sums of the activities of the components correspond to within 3% and 14% respectively to the activities of the original cells suspended in phosphate buffer containing Triton X-100.

Figure 3-9 shows photographs and activities of samples before and after solubilization. The solubilized proteins sample retained 66% of the esterase activity without any cells present.

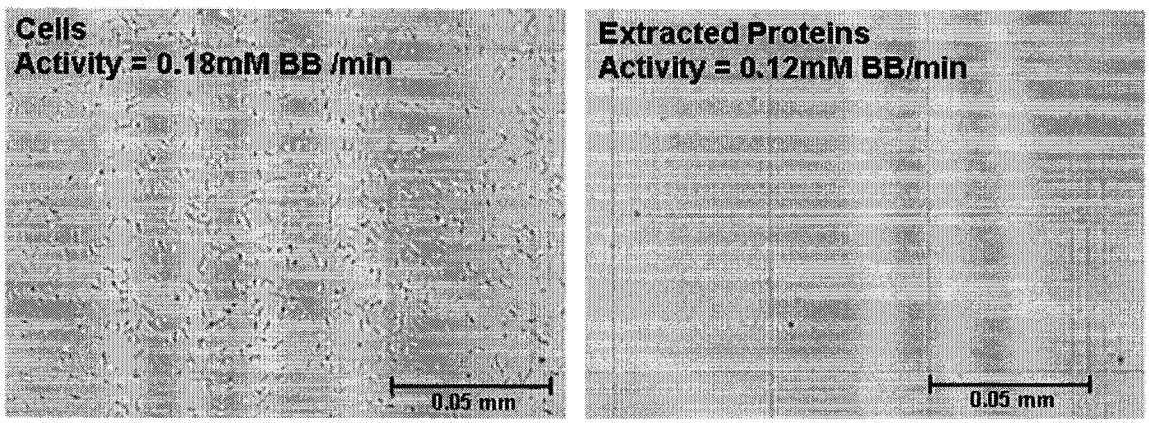

Figure 3-9 – Microscope photographs and activity measurements of a *R. rhodochrous* ATCC 13808 cell broth and its extracted protein sample. The protein extraction was conducted with Triton X-100 in phosphate buffer.

3.3.4 Esterase Deactivation

The esterase is expected to be deactivated over time. Because the temperature and the environment in which an enzyme is located can have an important influence on its stability, deactivation studies were conducted at various temperatures (i.e., 30°C, 20°C, and 4°C) for both the membrane-bound and the solubilized enzyme. **Figure 3-10** and **Figure 3-11** show the results for each respective situation. The results were reported as relative activity measurements, with the initial sample given a value of 100%. All activity measurements were based on the butyl butyrate/esterase assay.

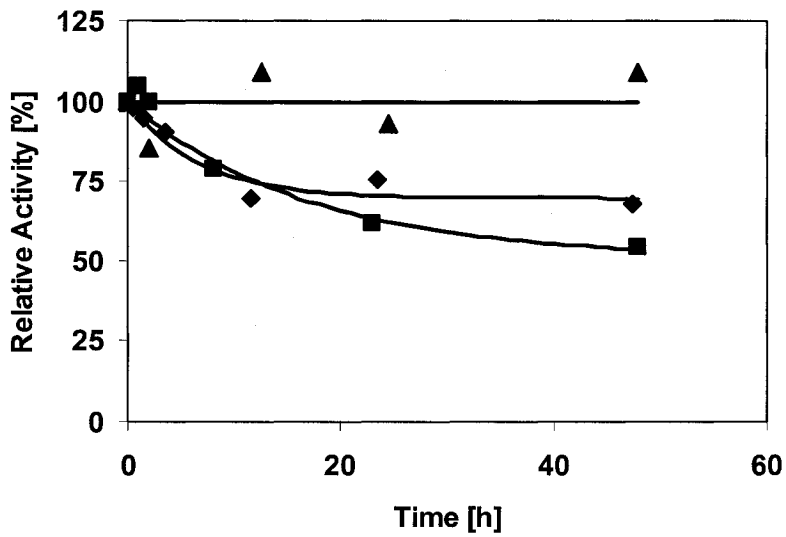

Figure 3-10 – Deactivation at 30°C (♦), 20°C (■), and 4°C (▲) for the membrane-bound esterase from *R. rhodochrous* ATCC 13808.

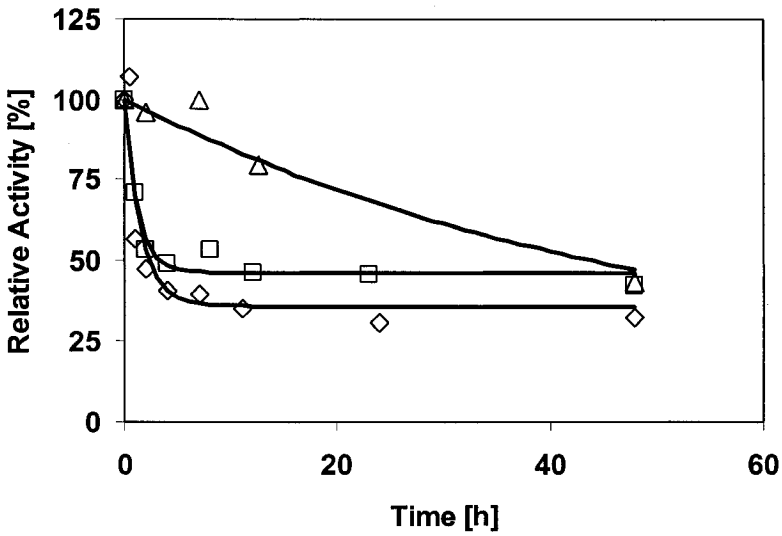

Figure 3-11 – Deactivation at 30°C (◊), 20°C (□), and 4°C (Δ) for the extracted membrane-bound esterase from *R. rhodochrous* ATCC 13808. Extraction performed with Triton X-100 in phosphate buffer.

As expected, a decrease in activity was seen over time, and this decrease was slower with lower temperatures. A model (**Equation 3.1**) was fit for each set of data based on a least-square analysis.

Equation 3.1: $$A = A_{st} + (100\% - A_{st})e^{-t/\tau}$$

where A = Relative Activity [%]
A_{st} = Steady-state Relative Activity [%]
t = time [h]
τ = time constant [h]

The regressions are shown in **Figure 3-10** and **Figure 3-11** as solid lines and the corresponding constants can be found in **Table 3-1**.

Table 3-1 – Constants and standard error from the least-square model for the deactivation of the esterase from *R. rhodochrous* ATCC 13808 at 30°C, 20°C, and 4°C. The model is shown in **Equation 3.1**.

Enzyme	Temperature	Steady-state Activity A_{st}	Time Constant τ	Standard Error
	[°C]	[%]	[h]	[%]
Membrane-bound	30	69.9	6.5	1.3
	20	50.7	17.0	1.8
	4	99.5	45.0	4.7
Extracted	30	35.9	1.5	3.4
	20	46.2	1.2	1.0
	4	14.1	50.0	2.7

For all cases, the steady-state activity was greater than zero. This indicates that the esterase was not completely deactivated under the conditions studied. The variations between the steady-state activities at 30°C and at 20°C were relatively small and could be mostly attributed to error. However, at 4°C the value was much higher for the membrane-bound esterase (99.5%), indicating that the enzyme retained its initial level of activity over long periods of time. The value of 14.1% for the solubilized enzyme could be an artifact of the model; the last data point strongly influences the fit of the model parameters since there are no data points between 12 hours and 48 hours. An error in measurement at 48 hours would then have an important impact on the values of the constants of the model.

The time constant indicates the rate of deactivation of the esterase. As expected the values increased with lower temperatures. The time constants of 1.5 h and 1.2 h obtained for the solubilized enzyme at higher temperatures should be considered equal

within experimental error. In this case, the error could be introduced by the deactivation that occurred during the pre-heating step preceding the butyl butyrate/esterase assay.

3.3.5 Activity and Temperature

The butyl butyrate/esterase assays were conducted at different temperatures to determine the effect of this parameter on the reactivity of the enzyme. The tests were conducted on membrane-bound samples at temperatures of 30°C, 20°C, and 4°C. **Figure 3-12** shows that the activity was the highest at 30°C and decreased with temperature. However, it also shows that the enzyme retained close to 40% of its activity at a temperature of 4°C.

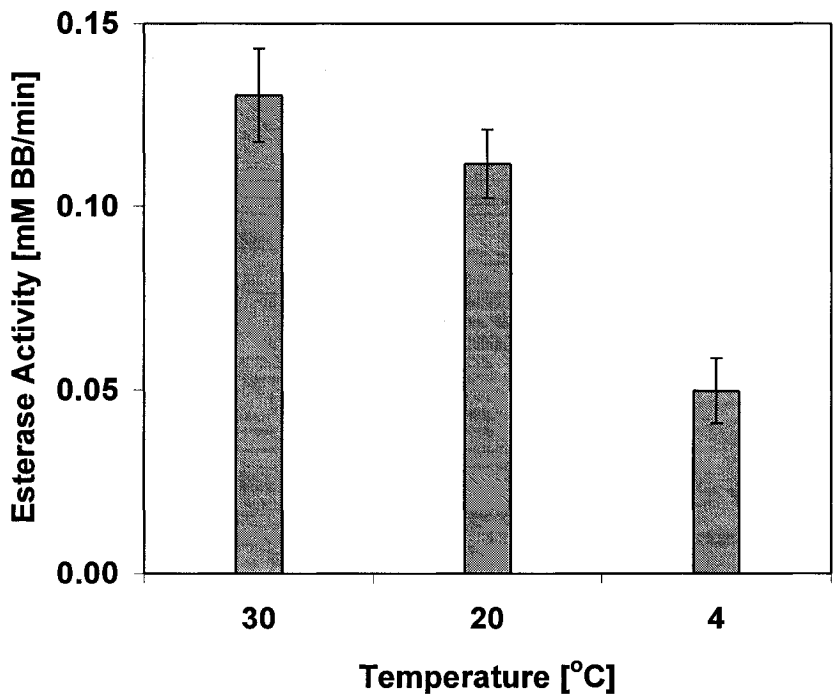

Figure 3-12 – Esterase activity at different temperatures (30°C, 20°C, and 4°C) for the membrane-bound esterase from *R. rhodochrous* ATCC 13808.

3.4 Hydrolysis of Plasticizers and Other Esters

3.4.1 Effective Hydrolysis Time

The methodology used to determine the interaction of the membrane-bound esterase with different compounds allowed for direct comparisons of their rate of hydrolysis. The only parameter changing from substrate to substrate was the effective hydrolysis time. The latter was determined, for each compound, according to their rate of degradation by a membrane-bound sample obtained from a 1 g/L *R. rhodochrous* ATCC 13808 broth. Controls were performed with and without dead biomass to evaluate the effect of volatilization and adsorption of the compounds to the biomass. In all cases the influence of these parameters was negligible for the periods allowed for hydrolysis. The hydrolysis results for all compounds tested are presented in **Figure 3-13**. The selected effective hydrolysis time for each substrate is presented in **Table 3-2**. They ranged from 0.1 minute for o-nitrophenyl acetate to 100 hours for DEHP.

Two main factors influenced the selection of the effective hydrolysis time: it had to be representative of an initial rate —the reaction had to be in its early, almost linear stage; and a 15% change in concentration had to be detected to overcome error at low activity levels. Two exceptions were allowed. The hydrolysis time selected for butyl butyrate (10 min) was representative of a 50% change in concentration. Although this value is much higher than that of other substrates, it was selected mostly for practicality: too short an assay would increase errors due to manipulations. Moreover, the reaction remained linear up until that point and represented an initial rate equally as well as earlier measurements.

On the other end of the spectrum, the hydrolysis time for o-nitrophenyl acetate (0.1 min) was representative of a 1% change in concentration; this was due to the higher sensitivity of the UV-spectrophotometer assay compared to the GC-based assays. Measurements of changes in concentration were much more precise for the former based on the minimum changes detectable. The hydrolysis time had to be selected accordingly.

All further experiments concerning the rate of hydrolysis of the compounds tested were then conducted according to their respective effective hydrolysis time.

3.4.2 Relative Rates of Hydrolysis

In order to properly incorporate o-nitrophenyl acetate in the rate of hydrolysis analysis and give the latter a semi-quantitative value, hydrolysis assays were prepared for each compound. The units for the rates of hydrolysis were converted to millmoles of substrate reacted (i.e., $mmol \cdot L^{-1} \cdot min^{-1}$ or $mM \cdot min^{-1}$) for all compounds tested.

To improve on the accuracy of this comparison, and to ensure that the latter was independent of the selection of the effective hydrolysis time, all hydrolysis measurements were compared to a reference compound, namely butyl butyrate. Membrane-bound samples were then tested for both butyl butyrate hydrolysis and any of the other substrates. The samples tested were taken at different stages of growth or would be dilutions of a sample harvested at stationary phase.

Figure 3-14 shows the results obtained for four substrates. The relative rate of hydrolysis of each compound was determined from such graphs. The butyl butyrate activity was set as having a reference relative activity of 1. The relative rate of hydrolysis of other compounds would then be determined by the slope of the linear

correlation relating their rate of hydrolysis to that of butyl butyrate. The corresponding measurement has units of mmol substrate per mmol butyl butyrate. Values higher than 1 would correspond to a rate of hydrolysis faster than that of butyl butyrate, and slower for values lower than one. The relative rate of hydrolysis of each compound can be found in **Table 3-2**. All relative rates found were smaller than or equal to one, ranging from 0.063 for dimethyl phthalate to 0.0003 for di(2-ethylhexyl) phthalate. Dimethyl phthalate, diethyl phthalate, o-nitrophenyl acetate, and di(n-butyl) phthalate each had a relative rate of hydrolysis two orders of magnitude slower than that of butyl butyrate. DEHA and di(n-hexyl) phthalate were three orders of magnitude slower, while DEHP had a four-orders of magnitude slower hydrolysis rate.

3.4.3 Rate of Hydrolysis and Solubility

Because most di-ester plasticizers display low solubility, it was hypothesized that this factor would have a major influence on their hydrolysis rates. **Figure 3-15** shows the log-log plot of the relative rate of hydrolysis of the substrates tested as a function of solubility. Although the relative hydrolysis rate increased linearly on a log-log scale with increasing solubility, at least two compounds, namely butyl butyrate and DEHP, were outliers. This indicates that a factor other than solubility could, at least in some cases, play a major role in influencing the rate of hydrolysis.

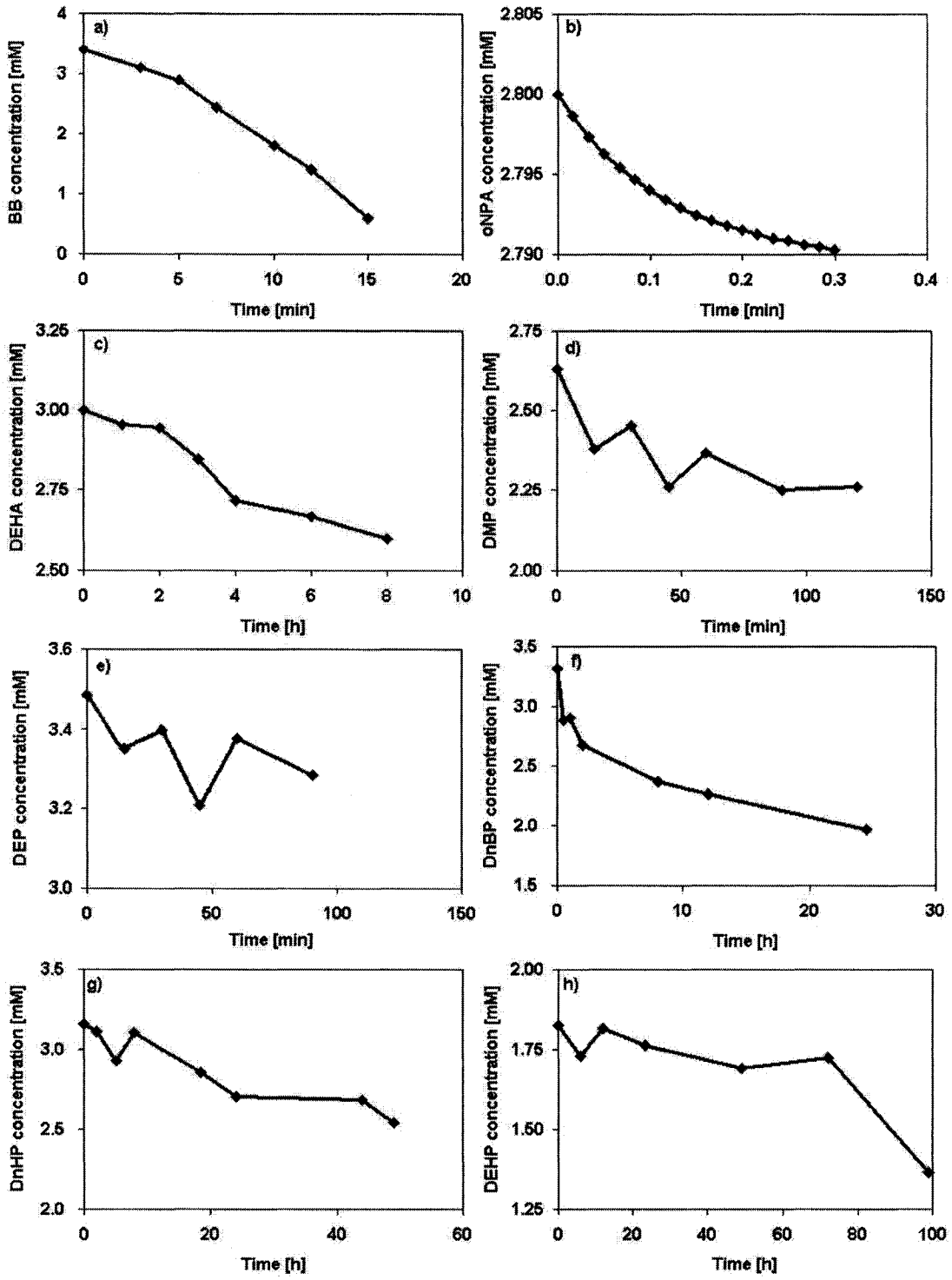

Figure 3-13 – Hydrolysis of esters by the membrane-bound esterase from 1g/L *R. rhodochrous* ATCC 13808 broth samples. Components shown are a) butyl butyrate, b) o-nitrophenyl acetate, c) di(2-ethylhexyl) adipate, d) di(methyl) phthalate, e) di(ethyl) phthalate, f) di(n-butyl) phthalate, g) di(n-hexyl) phthalate, and h) di(2-ethylhexyl) phthalate.

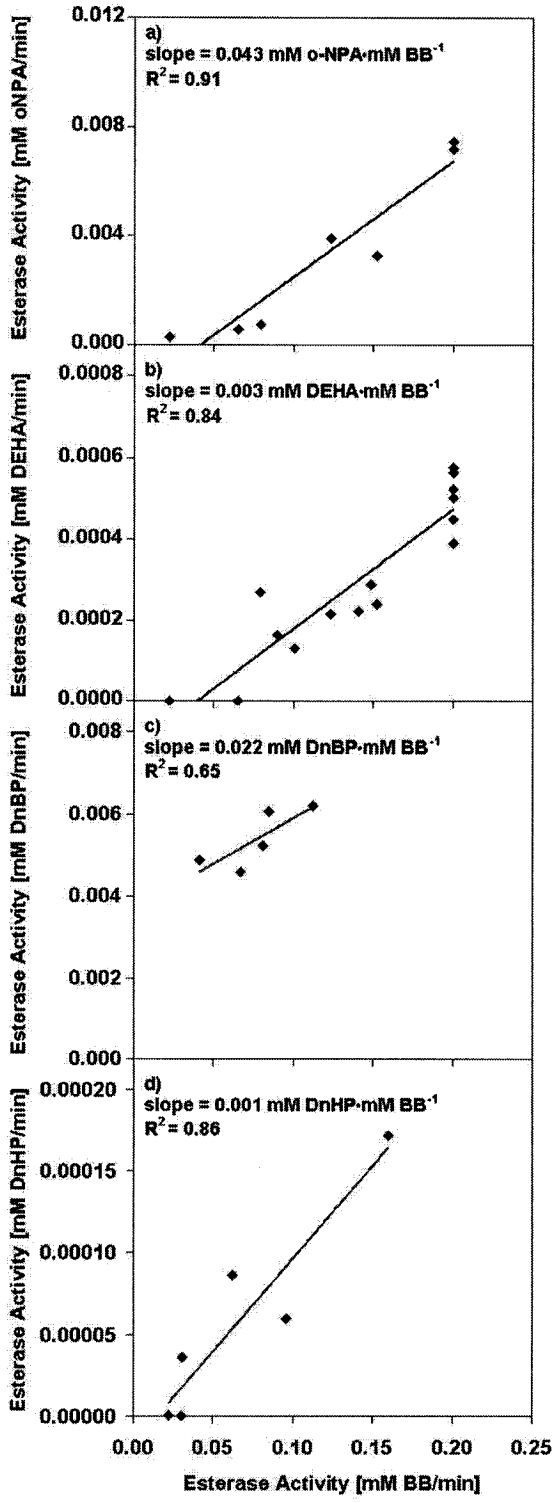

Figure 3-14 – Determining the relative rate of hydrolysis for the membrane-bound esterase from *R. rhodochrous* ATCC 13808 reacting with a) o-nitrophenol acetate, b) di(2-ethylhexyl) phthalate, c) di(n-butyl) phthalate, and d) di(n-hexyl) phthalate. Butyl butyrate is used as a reference. The R^2-values are displayed for each correlation.

Table 3-2 – Effective hydrolysis times and relative hydrolysis rates for plasticizers and other esters tested.

Compound	Effective Hydrolysis Time [h]	Relative Rate of Hydrolysis [mmol substrate/mmol BB]
BB Butyl butyrate	0.167 (10 min)	1
oNPA o-Nitrophenyl acetate	0.00167 (0.1min)	0.043
DEHA Di(2-ethylhexyl) adipate	8	0.003
DMP Di(methyl) phthalate	0.75	0.063
DEP Di(ethyl) phthalate	0.75	0.049
DnBP Di(n-butyl) phthalate	4	0.022
DnHP Di(n-hexyl) phthalate	20	0.001
DEHP Di(2-ethylhexyl) phthalate	100	0.0003

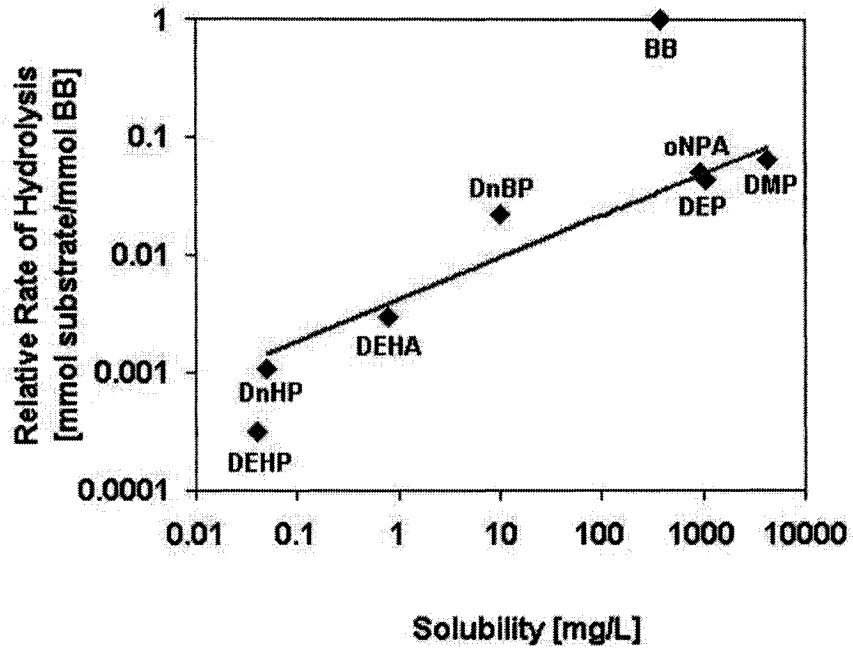

Figure 3-15 – The effect of solubility on the hydrolysis of plasticizers and other esters by the membrane-bound esterase from *R. rhodochrous* ATCC 13808. Butyl butyrate is used as a reference substrate.

4. DISCUSSION

4.1 Butyl Butyrate/Esterase Assay

It was necessary to develop a reliable assay to determine the esterase activity of the enzyme involved in this project. As seen from **Figure 3-2**, the error on the assay was of the order of 0.01 mM butyl butyrate·min^{-1}. Most activity measurements obtained during this project were on the order of 0.1 mM butyl butyrate·min^{-1}. The results for the MSM controls showed no significant adhesion or volatilization of the substrate during the assay time of 10 minutes. The activity measurements obtained from the dead biomass samples were very low. Since the enzymes were not active after being exposed to the high temperature/high pressure environment of the autoclave, this small activity level was attributed to adhesion of butyl butyrate to the cell surface. It is important to note that this low level of activity was not significant and was of the same order as the assay error. Since it was possible to obtain direct, rapid, and accurate measurements, the butyl butyrate/esterase assay was an efficient method for the determination of esterase activity.

4.2 Esterase Characterization

The enzyme from *R. rhodochrous* ATCC 13808 characterized in this study would be best categorized as a carboxylesterase (E.C. 3.1.1.1). The enzyme of interest could catalyze the hydrolysis of short-chains esters and lipids (i.e., approximately 6 carbons) such as DEHP but it showed higher activity with water-soluble compounds such as butyl butyrate, o-nitrophenyl acetate, etc. **Section 4.3** discusses in more detail the broad substrate specificity of the esterase. The high stability of the enzyme, shown in **Figures 3-10** and **3-11**, is also a common characteristic of carboxyl-esterases [72, 73].

The enzyme could also fit the description of a more specific category of esterase called di(2-ethylhexyl) phthalate esterase (E.C. 3.1.1.60). However, one should be careful in using this category. Although the description of E.C. 3.1.1.60 enzymes stipulates that DEHP is their natural substrate, the fact that this compound is a xenobiotic makes this category suspect. In fact, *Krell et al.* [87] first referred to the E.C. 3.1.1.60 esterase in "Plant biochemistry of xenobiotics. Purification and properties of a wheat esterase hydrolyzing the plasticizer chemical, bis(2-ethylhexyl)phthalate."; a publication from which was proposed the creation of the E.C. 3.1.1.60 category. Moreover, in this publication they reported that the esterase in question catalyzed the hydrolysis of other compounds such as 4-nitrophenyl acetate. There is no evidence that DEHP is the "natural" substrate for the enzyme or that the latter differs from the E.C. 3.1.1.1 category.

4.2.1 Esterase Location in the Cell

An important factor when trying to determine the location of the esterase in the cell was the use of an appropriate cell lysis technique. The use of a BioNeb® Cell Disruption system allowed for the enzyme to retain its activity even after lysis. This can be seen in **Figure 3-4**. The sum of the activities for the extracellular, the intracellular and the membrane-bound cell fractions corresponds, within 10%, to the activity level of the broth sample.

Assuming that the number of living cells is representative of the unlysed cells, **Figure 3-3** shows that 75% of the cells were lysed after 4 passes through the apparatus. It could then be argued that this lysis technique was appropriate for the separation of cell fractions and lead to samples which were representative of each cell fraction.

Since 96% of the relative activity was found in the membrane-bound fraction, which contained, at most, 25% of the living biomass of the broth sample, then most of the esterase must be membrane-bound. The low activity values for the extracellular and intracellular fractions could probably be attributed to carry-over from the membrane fraction. The data found in **Figures 3-5** and **3-6** also support the conclusion that the enzyme was membrane-bound during all phases of growth.

Other clues about the location of the esterase came from the extraction of the enzyme by Triton X-100. Enzyme extraction with non-ionic detergents is considered a mild non-destructive technique which mostly targets peripheral enzymes. It has been shown by *Kreit et al.* [88] that the treatment of *Rhodococcus sp.* cells with Triton X-100 leads to no difference in cell appearance, indicating that, in that case, cell lysis probably does not occur. Since, in the case at hand, the esterase was extracted from whole cells by a non-ionic detergent (**Figures 3-8** and **3-9**), the enzyme could be a peripheral enzyme.

The fact that the esterase was membrane-bound raises an interesting point concerning the hydrolysis of di-ester plasticizers. As stated before, *R. rhodochrous* ATCC 13808 is thought to be aided in hydrocarbon degradation by the hydrophobic surface created on its membrane by mycolic acids [70]. Many di-ester plasticizers such as DEHA and DEHP are also hydrophobic and possess very low aqueous solubility [83, 84] (**Table 2-1**). In a system containing plasticizers, the bacterium would preferentially stay at the water-plasticizer or water-oil phase interface. The esterase situated on the membrane of the bacterium would then be in direct contact with the di-ester plasticizer. This would eliminate the step to transport the hydrophobic plasticizers through the

membrane before they could be degraded. This could also explain the efficiency of *R. rhodochrous* at degrading plasticizers compared to other bacteria [63].

4.2.2 Constitutive Esterase in *R. rhodochrous* ATCC 13808

The activity pattern found for each cell fraction (**Figure 3-4**) was similar when the bacterium was grown on either hydrophobic substrates (i.e., hexadecane, di(2-ethylhexyl) adipate) or hydrophilic substrates (i.e., adipic acid, yeast extract, nutrient broth). This was true whether the growth was on these substrates alone or in combination. Because the presence of plasticizer was not necessary to obtain the esterase, its production did not seem to be induced by a particular substrate.

Moreover, the enzyme seemed to be present at a constant level in the microorganism no matter the stage of growth (**Figures 3-5** and **3-6**). This leads to the conclusion that the esterase from *R. rhodochrous* ATCC 13808 is a constitutive enzyme. The linear correlation between esterase activity and biomass concentration found in **Figure 3-7** is strong support for this argument. The amount of esterase per biomass is constant, and the higher the concentration of biomass present in a system the higher the esterase activity will be.

Finally, carboxylesterases are often thought to be an important enzyme in many metabolic pathways [72], mostly because of their broad substrate specificity and because esters are fairly common compounds. This would also be consistent with the enzyme being constitutive. If the enzyme is used in many common situations, it makes sense from an evolutionary perspective for the organism to have it available at all times.

4.2.3 Esterase Solubilization

The extraction of the esterase from the membrane without denaturing it was possible by treatment with the non-ionic detergent Triton X-100. It can be seen in **Figure 3-9** that the activity found in the extracted protein samples was not due to carry-over of biomass from the initial broth. No cells were detected in the extracted samples under microscope investigation. As well, no activity was detected for butyl butyrate/esterase assays performed on controls containing Triton X-100 in phosphate buffer with or without dead biomass. The activity of the extracted protein samples is, therefore, due to the solubilized esterase.

The extraction resulted in free enzymes with activity but the amount of activity was variable (**Figure 3-8**). The variation in activity of extracted protein samples can be explained by the fact that the solubilization of proteins by a detergent is a competitive reaction. The detergent forms complexes not only with the different proteins contained in the membrane, but also with some of the lipids found at the surface of the cells. In other words there are a number of possible mixed micelles such as protein-detergent, lipid-detergent, and protein-lipid-detergent [89, 90]. Considering that membrane proteins form roughly 30% of the total cellular proteins and that there is a low copy number of each individual membrane protein [91], practically speaking, the solubilization of one specific enzyme becomes uncontrollable.

It has been shown that increasing the concentration of detergent does not always improve the extraction, and that it can in some cases decrease the enzymatic activity of a sample as a result of the segregation of the enzyme in the micelle [92]. This was observed in the present study and addition of Triton X-100, above 0.1% v/v, was not

beneficial. At concentrations lower than the critical micelle concentration (CMC) of the detergent (0.016% w/v for Triton X-100 [88]), individual or groups of detergent molecules bind to the protein and can allow it to detach from the membrane. At concentrations slightly above the CMC, protein-detergent mixed micelles are formed and the enzyme is solubilized. In both cases the bioavailability of the enzyme is improved and an increase in activity can be observed [88, 90]. On the other hand, when the concentration of detergent is too high, there can be segregation of the enzyme in the micelle. Therefore, if the addition of Triton X-100 can increase the enzymatic activity, there is a maximum concentration above which the activity decreases. There is a trade-off between protein extraction and active enzyme extraction.

As stated above, there were significant fluctuations in the proportion of esterase extracted between experiments. However, it is important to note that the sum of the activities of the treated cells and of the extracted proteins corresponded within 14% to the activity level of the cells exposed to Triton X-100. This activity balance indicates that no important deactivation occurred during the protocol, and that all the initial esterase activity could be recovered.

4.2.4 Deactivation and Stability

The deactivation of an enzyme is an important characteristic. This is especially true when assessing the environmental impact of the esterase being studied. It is well known that enzymes have a tendency to deactivate outside the context of a living cell. This is usually due to the loss of the active, or most active, protein conformation.

After lysing the biomass, the esterase remained bound to the membrane. It can be seen (**Figure 3-10)** that the enzyme retained significant activity at temperatures at least up to 30°C. The fact that more than 50% of the initial activity remained after incubation at 20°C or 30°C for 48-hour periods indicates that the esterase is robust. It is unclear if the decrease in activity was due to the complete deactivation of a portion of the esterase present, or if it was due to the refolding of the enzyme to a less active conformation. It is also possible that more than one esterase was present and that the deactivation patterns would be different for each of them. The steady-state activities extrapolated from the least-square models at 20°C and 30°C seem roughly equal within error.

The slower deactivation rate at 20°C, as reflected by the higher time constant determined from the least-square model (**Table 3-1**), is in agreement with common deactivation theory. In fact, the activity retained after an incubation period of 48 hours at 4°C was close to 100%. This temperature is often used for the short-term storage of enzymes and seems appropriate as such for the membrane-bound esterase.

After solubilization by Triton X-100, the esterase exhibited deactivation patterns similar to those observed for the membrane-bound form (see **Figure 3-11**). However in this case, the rates of deactivation were faster (i.e., with lower time constants). This should be expected from a solubilized membrane-bound enzyme. In its natural state, the enzyme interacts with the membrane, sometimes being an integral part of it, and often uses this medium as a stabilizer or anchor for the active conformation, preventing premature deactivation [88]. It is then expected that, once outside the context of the cell membrane, many membrane-bound proteins will deactivate faster.

Although the free enzyme was initially less stable, there was still a non-zero steady-state activity for all temperatures tested. This indicates that the enzyme can maintain its active conformation over long periods of time. This might be true for the pure solubilized enzyme, however the presence of Triton X-100 is probably masking this effect. In fact, the creation of protein-detergent and protein-lipid-detergent complexes or micelles can help the enzyme retain its active conformation. *Sojo et al.* [90] speculated that, for solubilized membrane-bound proteins, the presence of detergent "[...] may provide an environment resembling that of cell membranes or cell walls". If this is the case, it can be argued that the steady-state activity would be, at least in part, a result of the Triton X-100 present in the samples. It is possible that such a plateau would exist for the pure solubilized esterase, yet no concrete evidence could be put forth from the data collected.

The results obtained for both the membrane-bound and the solubilized esterase show that the esterase from *R. rhodochrous* ATCC 13808 can act upon compounds such as plasticizers even after the organism dies, and this for long periods of time. It is also important to note that this phenomenon can occur even if the enzyme is detached from the membrane, or at least when it is in contact with certain detergents. Considering the fact that biological and chemical surfactants are commonly found in the environment, it is plausible that some of them might allow enzymes, such as the one studied here, to retain their active conformation over a longer period of time.

4.2.5 Activity and Temperature

The ability of the enzyme to retain a fairly high level of activity at lower temperatures (**Figure 3-12**) is an important factor when considering the environmental impact of the esterase from *R. rhodochrous* ATCC 13808. This means the enzyme can hydrolyze significant amounts of compounds such as plasticizers for long periods under different temperature conditions. Considering many of these compounds are considered ubiquitous and that many of their metabolites — including the products of their initial hydrolysis (e.g. 2-ethylhexanol, mono-2-ethylhexyl adipate, mono-2-ehtylhexyl phthalate) — are toxic and environmentally persistent, the robustness and versatility of the esterase from *R. rhodochrous* ATCC 13808 becomes more a concern than a solution to the plasticizer environmental problem. The fact that this versatility and robustness is likely found in other common microorganisms, would indicate that the problem could be of a greater scale than expected.

4.3 Hydrolysis of Plasticizers and Other Esters

All of the compounds tested for hydrolysis were degraded by the esterase from *R. rhodochrous* ATCC 13808. Considering the variety of these compounds (structure, solubility, etc.), the organism seems to display a broad substrate specificity.

It was not possible to clearly determine if this low selectivity could be attributed to one esterase alone. It is possible that there were more than one ester-hydrolyzing enzymes attached to the membrane. SDS-PAGE and Native-PAGE gels were performed to try to determine the amount of proteins and ester-degrading enzymes present in the extracted protein samples. These two gel electrophoresis techniques allow for

identification of proteins based on their molecular weight and enzyme activity, respectively. Unfortunately, bacteria normally contain very low amounts of each type of membrane-bound enzymes and it was not possible to resolve this question from these experiments. However, the linear correlations in esterase activity between pairs of esters tested seem to be in agreement with the presence of only one esterase to account for most or all of the activity. In other words, the enzyme acts upon each compound in a similar manner.

In the context of this research, an important point is that the esterase activity was membrane-bound. The hydrolysis study was concerned with the environmental context of microorganisms, such as *R. rhodochrous* ATCC 13808, interacting with compounds such as the ones tested and their overall effect.

The effective hydrolysis times (**Table 3-2**) show there was a significant variation among the substrates. In fact, for all the substrates except o-nitrophenyl acetate, the effective hydrolysis time gave an indication of the relative rate of hydrolysis. In other words, a greater value for effective time translated into a slower rate of hydrolysis. This comparison was possible because the methodology was identical for all the substrates but o-nitrophenyl acetate.

When looking at the differences in hydrolysis rates among the compounds studied, solubility seemed to be somewhat correlated to the relative hydrolysis rates (**Figure 3-15**). This was especially noticeable for the phthalates. Orders of magnitude differentiated the rates of hydrolysis of the different substrates. The least soluble ester, DEHP, had by far the slowest rate of hydrolysis with a value 4 orders of magnitude lower than that of butyl butyrate. The effect of solubility has been suggested as a possible

factor in plasticizer degradation [4, 81], however its influence has never been quantitatively evaluated. It was shown in **Figure 3-15** that, in fact, solubility correlated to some extent to the relative rate of hydrolysis. Therefore in most cases solubility can be considered as a major factor affecting the rate of hydrolysis.

However, while there was a linear trend, at least two compounds stood out as not strictly following this trend. These were butyl butyrate and DEHP. This implies that at least one additional factor was influencing the rates of hydrolysis. When looking at the structures of the compounds studied (**Figure 4-1**), it can be seen that butyl butyrate is a simpler molecule than the others and would be expected to have the lowest steric hindrance. This would make the ester bond more accessible to the enzyme. For example, diethyl phthalate, which has a solubility comparable to that of butyl butyrate, exhibited a much slower rate of hydrolysis. However, its two ester bonds are on an aromatic ring and in the ortho position to each other. This would be expected to cause significant steric hindrance and a slower rate of hydrolysis.

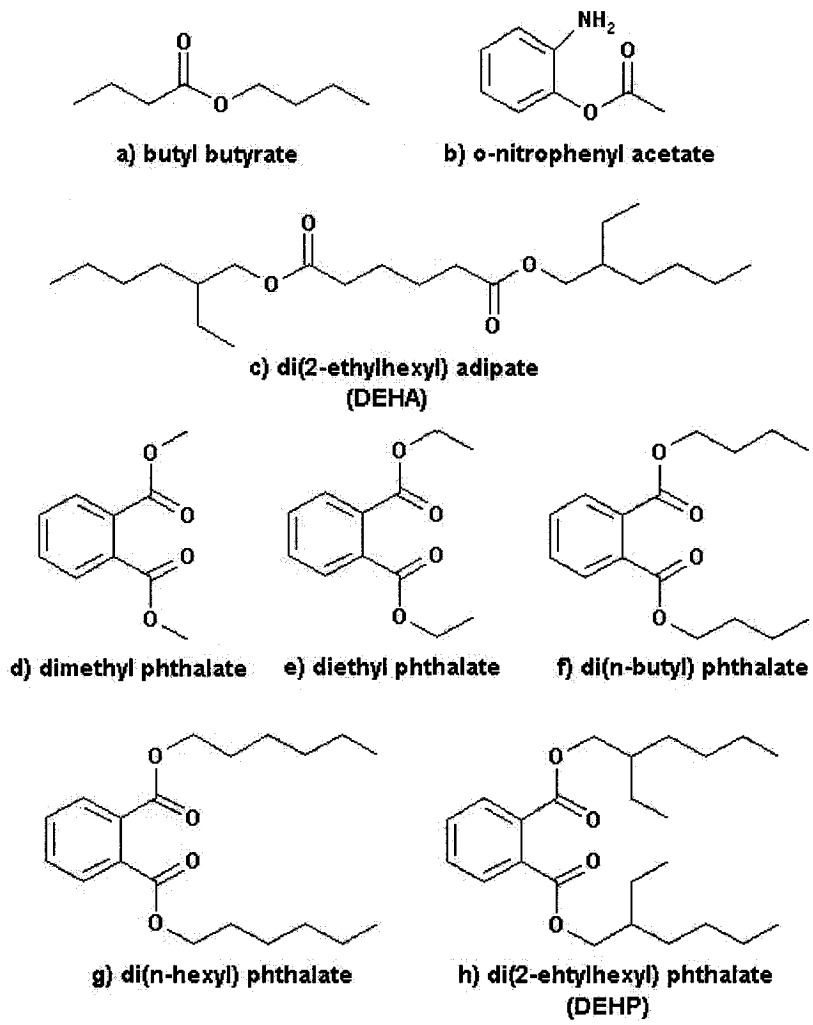

Figure 4-1 — Chemical structure of di-ester plasticizers and other esters: a) butyl butyrate, b) o-nitrophenyl acetate, c) di(2-ethylhexyl) adipate, d) dimethyl phthalate, e) diethyl phthalate, f) di(n-butyl) phthalate, g) di(n-hexyl)phthalate, and h) di(2-ethylhexyl) phthalate.

The steric hindrance argument can also explain the divergence of DEHP from the trend line. DEHP and di(n-hexyl) phthalate have very similar solubilities. However, there was an order of magnitude difference between their relative rates of hydrolysis. Once again, a consideration of the structure of the molecules leads to an explanation. The esters are very similar except that DEHP has 2-ethyl branches on the alcohols of the

ester bond (in the α-position). This would make it harder for the esterase to interact with the substrate.

DEHP is a xenobiotic industrial compound. In other words, it is not biologically produced. It has been demonstrated that side-chains such as the 2-ethyl group are poorly metabolized by microorganisms and this can lead to recalcitrant compounds [93]. In fact, this was part of the reason why the 2-ethyl side-chains were selected in the original choice of a plasticizer as it was desirable to prevent premature biodegradation [1].

The presence of the side-chain could then partially explain why the rate of hydrolysis would be slower. Many enzymes are very specific displaying very high selectivity. While it was demonstrated above that this is not the case for the enzyme from *R. rhodochrous* ATCC 13808, the presence of a 2-ethyl side-branch close to an ester bond could not only block its access but also perturb the anchoring of the enzyme. The same argument is seen with DEHA, which also has 2-ethyl side-chains in the α-position next to the ester bonds. While the molecule is linear, its rate of hydrolysis was comparable to that of a phthalate with equal solubility. For a similar compound without 2-ethyl branches (di(n-hexyl)adipate), a higher relative rate of hydrolysis would be expected.

The rate of hydrolysis of di-ester plasticizers is an important environmental concern. Many of these compounds are considered ubiquitous in the environment and it is not surprising that six different phthalates, including DEHP, are on the USEPA list of priority pollutants. The large and increasing production and use of plasticizers means that problems arising from their presence in the environment will continue to grow. Now that it has been shown how slowly they will be degraded by microorganisms, it is certain

that the environmental accumulation of plasticizers will continue. Although the production of some phthalates exhibiting acute toxicity, such as di(n-butyl) phthalate, has been halted in some countries, even these will remain environmental concerns for many years because of the slow rate of hydrolysis.

Other compounds such as DEHP and DEHA are said to have low acute toxicities. However, their long term effects are significant and include endocrine disruption [56, 61, 62] and carcinogen properties for some species [2, 5, 53, 55]. If the presence of these compounds is of concern, recent work has shown that the products of their biodegradation must also be considered [24, 42, 49, 55]. Metabolites such as 2-ethylhexanol, 2-ethylhexanoic acid, mono-2-ehtylhexyl adipate, and mono-2-ethylhexyl phthalate have high aqueous toxicity and have been found to accumulate in the environment [24]. For example, DEHP —the most commonly used plasticizer and the slowest to be hydrolyzed— represents a long-term environmental threat on its own. However, even after its disappearance, the partial products of degradation could actually increase the toxicity of a system [42, 43, 45]. The same phenomenon would be observed with DEHA, which is degraded at a slightly faster rate. It is then important that the polluting and toxic potentials of metabolites be taken into account when determining such parameters for plasticizers. The environmental impact of such compounds extends beyond their existence in the original form.

5. CONCLUSION

An esterase produced by *R. rhodochrous* ATCC 13808 and capable of hydrolyzing di-ester plasticizers was characterized. The enzyme was found to be bound to the cell membrane and constitutive. It could be extracted from the cell membrane by treatment with Triton X-100, a non-ionic detergent.

The esterase retained activity for more than 48 hours. Deactivation was faster at higher temperatures. The deactivation and stability patterns were similar when the enzyme was bound to the cell membrane or outside the cell. The enzyme displayed reasonable activity levels, even at temperatures as low as 4°C. The esterase should be able to hydrolyze di-ester plasticizers over long periods of time, under various environmental conditions and even after the death of the cells.

A quantitative analysis of the rates of hydrolysis of di-ester plasticizers was achieved with enzymatic assays specific to each compound. The solubility of compounds was found to be an important factor regulating their rate of hydrolysis. However, it was also demonstrated that steric hindrance is an important factor. Di(2-ethylhexyl) phthalate (DEHP), one of the most common environmental contaminants, was found to have the lowest rate of hydrolysis of all plasticizers studied.

Since the production and release of DEHP and other di-ester plasticizers are increasing, these problematic compounds will continue to accumulate in the environment. As a consequence of the slow biodegradation of this growing pool of di-ester plasticizers, the presence of acutely toxic metabolites, such as 2-ethylhexanol and 2-ethylhexanoic acid, will become of increasing environmental concern.

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