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Biodegradation of Plasticizers: Characterization and Toxicity of their Metabolites

By

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ABSTRACT

More than one billion pounds of plasticizers are produced each year to supply the plastics industry. Some of these plasticizers, particularly phthalates, are suspected endocrine disruptors. However, few studies have been conducted to determine if they are susceptible to biodegradation by naturally occurring bacteria once they are released into the environment.

Six organisms were tested for their ability to grow in the presence of six different industrial plasticizers. Two bacteria, *Rhodococcus rhodochrous* and *Arthrobacter paraffineus*, grew well in media containing n-hexadecane and one of the plasticizers.

Fermentations in a 2-liter reactor were performed with *Rhodococcus rhodochrous* and three plasticizers: bis 2-ethylhexyl adipate, dioctyl phthalate and dioctyl terephthalate. The organism degraded all of the adipate, half of the terephthalate was degraded and the phthalate was degraded slightly.

In these growth studies, the toxicity of the media increased as the organism grew. This trend was linked to the accumulation of metabolites from the partial degradation of the plasticizer. The two major metabolites were identified as 2-ethyl hexanol and 2-ethyl hexanoic acid. The alcohol was only observed part way through the growth in the presence of the adipate. Its concentration decreased as it was oxidized to the acid and it was not present at the end of the fermentation.

The acid was observed for all three types of plasticizers and it was present in high concentrations at the end of every experiment. The nature and pattern of production of the metabolites were consistent with a pathway for the degradation of all three plasticizers by hydrolysis of the ester bonds.

The accumulation of toxic metabolites indicates that biodegradation may not be a solution to reducing environmental impacts associated with plasticizers that have leached into the environment.

RÉSUMÉ

Six bactéries ont été testées pour leur capacité de croissance en présence de six plastifiants industriels. Deux bactéries, soit *Rhodococcus rhodochrous* et *Arthrobacter paraffineus* ont connu une bonne croissance dans un bouillonte culture contenant du n-hexadecane et un plastifiant. Des fermentations dans un réacteur de 2 litre ont été obtenues à l'aide de la bactérie *Rhodococcus rhodochrous* et de trois plastifiants soit 2-ethyl hexyl adipate, dioctyl phthalate et dioctyl terephthalate. L'adipate fut complètement degrade par la bactérie alors que le terephthalate le fut à moitié et le phthalate à pèine.

Lors des études de croissance, la toxicité des bouillons du culture a augmenté avec la croissance de la bactérie. Ce phenomène est relié à l'accumulation des métabolites provenant de la degradation partielle du plastifiant. Les deux principaux métabolites identifiés furent 2-éthyl hexanol et l'acide de 2-éthyl hexanoic. De l'alcool fut observe durant la croissance, en présence de l'adipate. L'alcool a diminué en concentration en s'oxidant à l'acide et n'était plus présent à la fin de la fermentation.

L'acide à été observée pour les trois types de plastifiant et était présent en grande concentration à la fin de chaque expérience. La nature et la modèle de production des métabolites furent conformes avec le mécanisme de dégradation de chacun des trois plastifiants par l'hydrolyse de la liaison de l'éther.

ACKNOWLEDGEMENTS

I must begin by thanking Dr. Cooper for all the help through the projects and Master's degree. His patience and wisdom guided me through hard times. I would also like to thank Dr. Nicell for helping me throughout the project.

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Well, back to the lab!

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1. INTRODUCTION

Plastics have gradually been replacing traditional materials such as wood and iron in construction and domestic materials. The ease of production and forming the plastic makes it an attractive material to work with. All plastic materials contain a number of small molecular weight additives as well as polymers. A major component of plastics is a group of compounds called plasticizers, which is needed to produce workable final products. They are incorporated in many different plastic products, including those of the following industries; construction, automotive, household products, toys, packaging and medical supplied[1].

All of these plastics contain plasticizers in their formulations. While the polymers, by definition, are very long hydrocarbon chains, the plasticizers are orders of magnitude smaller. They are usually small hydrocarbons and often contain a single aromatic group. The pure compounds are usually liquids at room temperature.

This research employed plasticizers such as bis-2-ethylhexyl phthalate esters. These compounds and other types of phthalate esters have recently been implicated as endocrine disrupters[2, 3, 4, 5]. Recent studies have shown that there is evidence that the endocrine-disrupting action of phthalate esters can cause early onset of puberty in human females[6]. Notably, the government of United States recently announced a recall of cosmetics containing di-butyl phthalate[7]. As a group, the phthalate esters have many important commercial applications and their use is widespread. This means that it is almost impossible for anyone to avoid coming in contact with these plasticizers in day-to-day life. For example, it has been shown that bis 2-ethylhexyl adipate readily leaches out

of plastic film when in contact with different foods[8]. Their migration into food has lead to an estimated ingestion rate of plasticizer of 8.2 mg/person/day[9].

Because the plasticizers can leach out of the plastics[10, 11, 12], it is important to study their fate upon release into the environment. Since there are important health implications associated with the phthalates and probably other plasticizers[13, 14], it is likely that they will be a problem if they tend to accumulate in the environment. Such accumulation would only be likely to occur if the plasticizers resist biodegradation. However, surprisingly little work has been done on the microbial degradation of plasticizers. Thus, it is important to determine if these compounds are degraded and, if they are degraded, the nature of the breakdown products.

1.1 Review

1.1.1 Plasticizers Used in Industry

There are many types of plasticizers that are used by industries to produce different types of plastics. The most commonly used plastic in the construction industries is polymerized vinyl chloride (PVC), which was first introduced in 1931[1]. This polymer needs a plasticizer as an additive to enhance certain types of characteristics such as flexibility, extensibility and workability. Several different types of plasticizers are used depending on the demands of the applications. The plasticizer most commonly used in PVC production is di-(2-ethylhexyl) phthalate; commonly called dioctyl phthalate (DOP) and shown in Figure 1-1. This plasticizer was introduced in 1933[15] and industry now uses approximately 500 million kilograms (1 billion pounds) per year[16] accounting for 50% of all phthalate plasticizers used in plastic production[17].

Another common plasticizer called dioctyl terephthalate (DOTP) is an isomer of DOP. Dioctyl terephthalate (Figure 1-2) was introduced to the industry as a substitute for DOP because of the possibility of producing the mono-ester (mono-ethylhexyl phthalate) after incomplete biodegradation of DOP[18, 19]. Compounds like this mono-ester have been linked to different types of cancer in higher organisms[20].

Another type of plasticizer used in industry is bis 2-(ethylhexyl) adipate (Figure 1-3). It is added primarily when efficiency (or flexibility) is a major factor in the function of the plastic film[21]. This application usually involves PVC films where

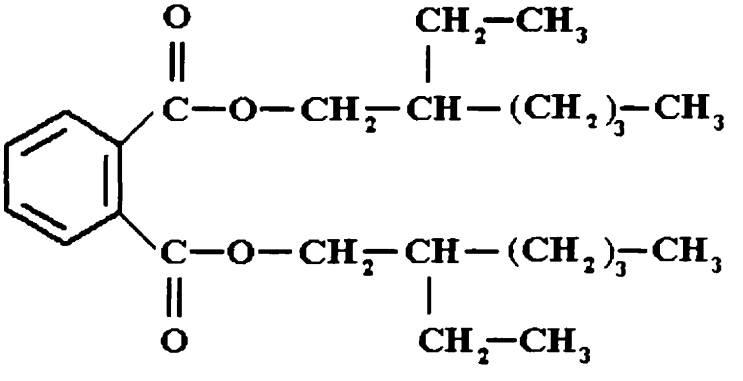

Figure 1-1. The structure of dioctyl phthalate (DOP).

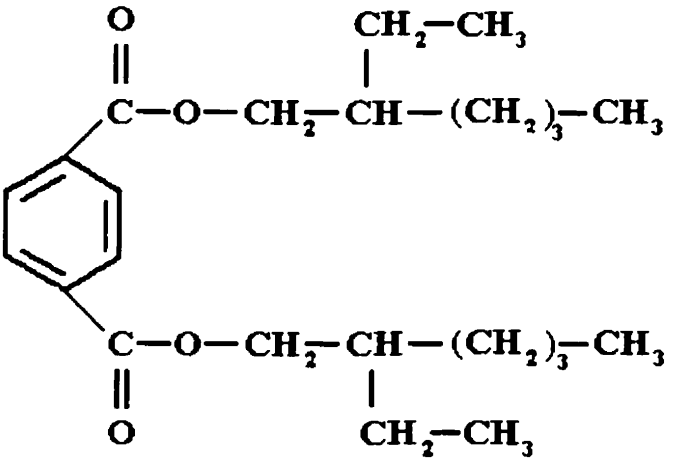

Figure 1-2. The structure of dioctyl terephthalate (DOTP).

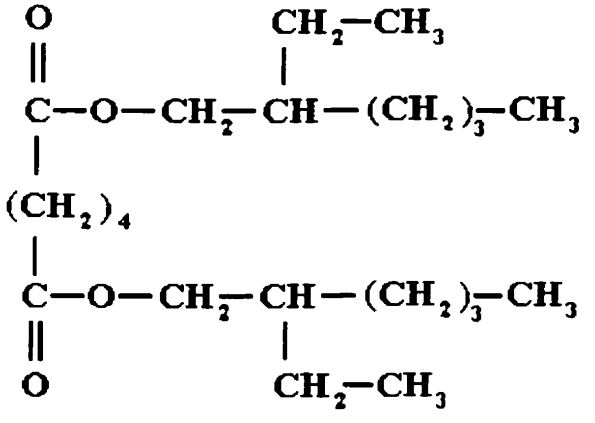

Figure 1-3. The structure of bis 2-ethylhexyl adipate (BEHA).

flexibility must be maintained over a wide range of temperatures, such as in wrapping of refrigerated food products[1].

Two other plasticizers, di-benzoate, and tri cresyl phosphate are sometimes added to plasticizers to obtain certain characteristics such as fire resistance and decreased volatilization of plasticizer.

1.1.2 Mobility of Plasticizers

A major concern associated with all of the plasticizers is the problem of the gradual loss of plasticizers from plastic formulations. The migration and leaching problems are significant because of the high concentrations of plasticizer in the solid matrix. For example, polyvinyl chloride plastics contain up to 40% by weight as plasticizer[1]. Because all of these plasticizers are small compounds relative to the polymers, once they leach out of the plastic, they can freely move into the environment. Plasticizers have very low solubility in the aqueous phase; the solubility decreases as the functional groups increase in length[22]. However, their entrance into the aqueous phase is enhanced by the addition of surfactants and emulsifiers[23]. It seems that plasticizers and their breakdown products are very soluble in fatty tissues and could accumulate in these sites over time[24].

The leaching of the different plasticizers is dependent on many factors. Certain plasticizers leach out of the plastic matrix at different rates than others. For example, the adipate plasticizer has a particularly high mobility. The rate of decrease in concentration of this plasticizer in a polymer by volatilization and oil and water extraction is greater than that observed for any other industrially significant plasticizer.

The material in contact with plastic could influence the rate of migration of plasticizer[1]. When dealing with a container of PVC, the type of material or liquid in the container could affect the rate of migration of the plasticizer to the other phase. Blood bags made of PVC have been a cause for concern because high levels of leached plasticizer were found to accumulate in the lung, liver and spleen of blood transfusion patients[12]. Other types of plasticizer migration are found in foods wrapped in PVC film. This is a particular concern for fatty foods such as dairy products[25]. Other authors of research papers on the subject of plasticizer mobility[26, 27, 28] have come to the common conclusion that a significant quantity of plasticizers can leach into the environment.

The phthalate plasticizers seem to be quite stable molecules. This means that, if there is a spill or leaching of the compound it will stay in the soil for an appreciable time[22]. Traces of plasticizers have been found in water tables, riverbeds and lakes throughout industrial areas[29].

1.1.3 Interaction of Microorganisms with Plasticizers

Microorganisms in the environment can come into contact with a particular type of plasticizer once it leaches out of the plastic. These organisms can interact with the plasticizer in many ways. The microorganism may use the plasticizer as a carbon source and completely convert it to biomass and carbon dioxide (mineralize the plasticizer)[30, 31]. Other studies have shown the incomplete degradation of the plasticizer, leaving a residue of metabolites such as the breakdown products of an ester hydrolysis[31]. It is also possible that a plasticizer could be biologically inert meaning that the plasticizer has no interaction with the organism[32].

Some work on the degradation of plasticizers involved phthalate esters. Activated sludge systems were able to remove these plasticizers from the aqueous phase. There seemed to be complete mineralization of the plasticizer to carbon dioxide[33, 34, 35]. Unfortunately, most of the plasticizers being leached into the environment will not be degraded in as efficient a system as an activated sludge process.

The bacterium *Micrococcus* sp. strain 12B was used to remove dibutyl phthalate from an aqueous mixture[31]. It was noted that this organism is able to use this compound as the sole carbon source by hydrolyzing the functional group into butanol and completely oxidizing the phthalate component. The alcohol (butanol), a product of the hydrolysis, did not degrade while the organism was growing on the plasticizer. This alcohol is an example of a product that may accumulate in the environment, depending on the organisms in the environment, as a result of the breakdown of a plasticizer through interaction with a microorganism.

Reports have shown that there is evidence of degradation of the phthalate plasticizers in soil[36]. Organisms that are typically found in common garden soil seemed to have the proper enzymes to completely mineralize the plasticizer (i.e., DOP) without leaving any by products. Neither the pathway of the degradation nor the organisms involved in this degradation were identified.

Another study found that the phthalate plasticizer (DOP) adsorbed irreversibly to soil and became recalcitrant[32]. The organism was not able to degrade the plasticizer in the soil due to the binding. The organisms were not identified, but tests were performed to see the relative change in population of the organisms in the soil and it was found that there was no effect on the population.

Other work has shown that the organism *Rhodococcus erythropolis* could degrade different plasticizers both in soil[37, 38] and in an aqueous phase[39]. The plasticizer used in the study was DOP and the organism mineralized the plasticizer completely, leaving no metabolites as residue.

Soil organisms were placed in the aqueous phase with different plasticizers and *Rhodococcus rhodochrous* was identified as one of the organisms that degraded diethyl terephthalate[40]. This organism was able to hydrolyze and degrade the terephthalate while being unable to use diethyl phthalate as a carbon source. This shows that the position of the functional groups of the phthalate ester is an important factor for the degradation of the plasticizer.

1.1.4 Other Biological Interactions With Plasticizers

The most common interactions studied in previous work deal with the interaction of the plasticizer, both the adipate and phthalate, with higher organisms[19, 24, 41, 42, 43, 44, 45]. This interaction occurs in the intestinal tract of rats and other mammals. In the intestines of these organisms the ester bonds in both the phthalate and adipate were hydrolyzed, producing 2-ethyl hexanol[18, 24, 43, 46, 47]. This product could then be further oxidized into the carboxylic acid of the same carbon backbone. The ethyl side chain inhibits the complete oxidation, thus the 2-ethyl hexanoic acid was found to be an end product of the degradation[43]. This accumulation of 2-ethyl hexanoic acid has been linked to peroxisome proliferation[21]. Further work showed that lipase is involved in the initial breaking of the ester bond, producing the phthalic acid and 2-ethyl hexanol from the original DOP[17].

It has also been shown that these metabolites of both the phthalic and adipic esters (2-ehtyl hexanol and 2-ethyl hexanoic acid) are involved in the proliferation of liver cells called hepatocellular carcinoma in both rats and mice[21]. The carcinomas lead to tumor production and eventual death of the animal. The exact mechanism of this proliferation is unknown and it seems that the higher organisms, including monkeys and humans, show no symptoms of this carcinoma[48]. Other studies have shown that the peroxisome proliferation (causing death to rats and mice) was directly caused by the metabolites of the hydrolysis of 2-ethylhexyl adipate[21]. The same type of proliferation was observed with the metabolites from the degradation of DOP. The mono-ethlyhexyl phthalate produced by the incomplete degradation of DOP acts as a peroxisome proliferator like the previously mentioned compounds[13, 19].

Aquatic life seems to be susceptible to the concentrations of plasticizers in water systems. DOP and DBP were found to accumulate in the tissues of fish over time, particularly in industrialized areas, with accumulations observed between 0.2 to 10 μg plasticizer/g fish mass[50]. At low concentrations of plasticizers, the growth and reproduction of fish were inhibited, thus the plasticizer appears to act as an endocrine disrupter[50]. Endocrine disruptors are molecules that interfere with hormonal functions thus disrupting some endocrine pathways[3]. Notably, it has been shown that dibutyl phthalate (DBP) affects the timing of maturity in humans. This plasticizer is commonly used in the cosmetic industry, thus it is suspected that it could cause girls in their early teen years to mature at an accelerated rate[6]. This endocrine disruption characteristic is cause for concern, thus the study of the environmental impact of common plasticizers may shed some light on this issue.

Due to the fact that toxic effects are not seen in higher organisms, the exact toxicity to humans and the environment is difficult to conclude. Due to some inconclusive results from higher organisms, the acute toxicity of the plasticizers has not been finalized.

1.2 Objectives

The release of plasticizers into the environment is of concern due to their potentially negative impacts. However, currently, little is known about the fate of these compounds following their release into the soil and water column through the disposal of industrial wastes and leaching from existing plastics. In addition, little is known about the ability of common microorganisms to degrade these compounds, nor about the identity and impact of the products of biodegradation, if and when it occurs. Therefore, the main thrust of this study is to begin the process of assessing the potential environmental impact of plasticizers that have been released into the environment. Specifically, the objectives of this work are:

(1) investigate the ability of a variety of different organisms to degrade commonly used plasticizers;

(2) conduct a detailed study of the process of microbial biodegradation of selected plasticizers in order (a) to evaluate the rate and degree of biodegradation in batch cultures over time; (b) to monitor the growth of the organism; (c) to characterize the

metabolites and their toxicity and (d) to postulate a mechanism of degradation of the plasticizer.

(3) provide an preliminary assessment of whether the release of plasticizers should be an issue of major concern.

2. Materials and Methods

2.1 Organisms

The organisms were obtained from the sources described in Table 2-1. These organisms were stored in vials containing 20% glycerol (Sigma-Aldrich Co.) and 20% Difco Nutrient Broth mixture (18 g/L Difco Nutrient Broth in distilled water). The organisms were conserved for a period of 1 year at –70 ° C in a freezer (REVCO, Model ULT1386). After this period new culture vials were made with fresh broth.

Table 2-1. Organism name, optimal growth temperature and source.

Organism	Optimal Growth (°C)	Source
***Arthrobacter Paraffineus* ATCC 19558**	30	ATCC
***Corynebacterium Sp* ATCC 21511**	37	ATCC
***Mycobacterium* OFS**	37	J.J. Perry, USA
Pseudomonas flourescens TEXACO	30	H. Leskovsek, Slovenia
***Rhodococcus* ISO1**	30	J. Oudot, France
***Rhodococcus rhodochrous* ATCC 21766**	30	ATCC

2.2 Culture Maintenance

The organisms were maintained on Difco Nutrient Broth agar plates at 4 ° C (Fisher Brand ISOTEMP fridge). Every 2 to 3 weeks new plates were made by streaking organisms that were growing in 100 mL of Nutrient Broth mixture in Erlenmeyer flasks (500 mL) as described below. Samples of the strains being used were kept at –70 ° C.

The same preparation for the inoculum was used for the experiments for all six bacteria. The first inoculum taken from the –70 ° C freezer grew in Difco Nutrient Broth mixture for a period of 3 days at the optimum temperature as tabulated in Table 2-1. The culture was then transferred to a medium of modified mineral salt solution (MMSM) and n-hexadecane (10 g/L) using sterile techniques in a laminar fume hood (The Baker Company, Model VBM600). The composition of MMSM is shown in Table 2-2. After 1 week of growth the inoculum was ready for both the screening experiments and the growth study. The inoculum was then transferred to the shake flasks for the screening test and through the inoculation port for the reactor using a 5-mL plastic sterile syringe (Fisher Brand).

Table 2-2. Composition of modified mineral salt solution

Compound	Concentration (g/L)
NH_4NO_3	4.0
KH_2PO_4	4.0
Na_2HPO_4	6.0
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2 \cdot 2H_2O$	0.01
$FeSO_4 \cdot 7H_2O$	0.01
Na_2EDTA	0.014

2.3 Reactor

The batch growth studies were carried out in a 2-L New Brunswick Scientific batch reactor (Figure 2-1). The reactor was maintained at constant temperature using a recirculating water bath (Haake, model FE2) and a stainless steel tube in a tube heat exchanger (New Brunswick Scientific). The air inlet was fitted to an inline air filter

(Millipore Millex-FG50, 0.2 μL). A modified cover for the batch reactor sealed the reactor. This cover was machined from a 1" thick Teflon slab with the ports drilled into it. This cover was necessary to ensure that no plasticizers would be leached into the reactor.

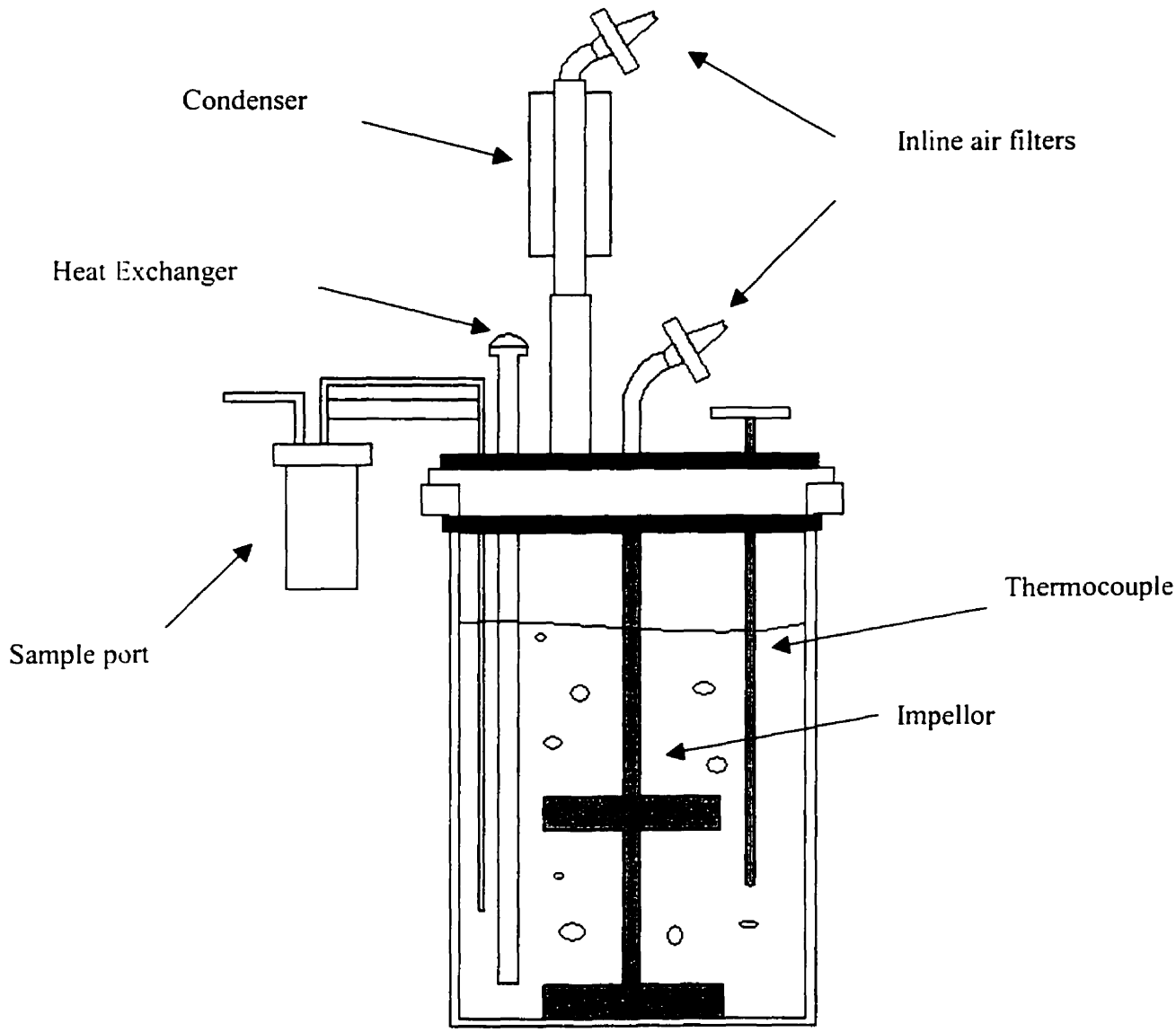

Figure 2-1. Schematic of the batch reactor.

2.4 Growth Conditions

The screening experiment involved the following three conditions: (1) plasticizer as a sole carbon source; (2) plasticizer with yeast extract; and (3) plasticizer with n-hexadecane. A positive result was obtained after the organism grew in the medium after three transfers. Each transfer was allowed to grow for 2 weeks.

The shake flask screening experiments were all conducted using MMSM with the addition of plasticizers alone or in combination with yeast extract or hexadecane as the substrates. The flasks were incubated at different temperatures specified in Table 2-1. The organisms grew in a rotary incubator shaker (New Brunswick Scientific, Series 25) set at 250 RPM. Two shakers were used, one set at 30 °C and the other at 37 °C to accommodate the different optimal growing conditions of the 6 organisms. In the batch reactor, the temperature of the reactor was controlled by a hot water recirculatory bath. This temperature was kept at 30 °C, which is the optimum temperature for *Rhodococcus rhodochrous*.

The steam sterilization conditions for the Erlenmeyer flasks were 121 °C and 20 psig for approximately 30 minutes. The batch reactor was sterilized over a 2-hour period under the same conditions.

The hexadecane and the different plasticizers were added using a 10-mL sterile glass pipette (Fisher Brand) to the sterilized Erlenmeyer flask in the laminar fume hood, or they were injected through a port of the batch reactor using a 5-mL sterile needle (Fisher Brand). N-hexadecane and all of the plasticizers were obtained from Sigma-Aldrich Co.

The samples were removed from the batch reactor through a sampling port while the reactor was kept at a constant temperature and mixing conditions. The sample was collected in a 30-mL glass screw top vial with a Teflon seal (Fisher Brand). The samples were stored for 1 day at 4 °C until three samples were collected for analysis.

2.5 Dry Weight Measurement

The samples in the 30-mL vials were shaken and 8 mL were removed to make biomass measurements. The standard dry weight analysis[50] was used to measure the biomass. The samples were placed in 30-mL Teflon centrifuge tubes (Fisher Brand) and centrifuged (IEC, Model B-22M) for 10 min at 10,000 RPM at room temperature. The supernatant was then decanted and the pellet was washed twice with MMSM. After the two centrifugation steps, the final pellet was resuspended in distilled water and placed in a tared aluminum dish that had previously been dried overnight. The dishes were then placed in an oven (Fisher Isotemp Oven 100 series, model 126G) at 105 °C for a period of 48 hours. The dishes were cooled and the mass was obtained using an analytical balance (Mettler, model AE 160). The final measure was recorded as grams of dry biomass per liter of fermentation broth.

2.6 Protein Concentration of Biomass

The 30 mL sample was shaken and a volume of 10 mL was removed and placed in a 30 mL Teflon centrifuge tube (Fisher Brand). The sample was centrifuged at 10,000 RPM at room temperature. The supernatant was decanted and the pellet was washed two

times with MMSM solution (10 mL). The final pellet was resuspended in 5 mL of MMSM and the protein concentration of the biomass mixture was obtained. The protein concentration was measured using the BIORAD DC Protein Assay. The procedure was followed as stated by BIO-RAD (BIO-RAD Laboratories Inc.).

2.7 Chemical Analyses

The following procedure was used to measure the concentration of n-hexadecane, plasticizers and metabolites in the various samples.

The samples were well mixed and a 2 mL volume was removed with a sterile pipette (Fisher Brand 10 mL Sterile disposable pipettes). This was placed in a test tube with 3 mL of a solution containing an internal standard. This solution consisted of chloroform with a 0.01% (mass/volume ratio) of pentadecane. The mixture of sample and chloroform was mixed for two minutes. The organic phase was removed by a transfer pipette into a 5-mL glass vial (Fisher Brand) and stored at −15 °C until the samples were injected into the gas chromatograph (GC) or the gas chromatograph/mass spectrophotometer (GC/MS).

The gas chromatograph (GC) (HP5890 Series II) contained a SPB-5 column (Supelco). The settings used for the GC are summarized in Table 2-3. The calibration curves for n-hexadecane, the plasticizers and the different metabolites were obtained. Examples are shown in Figure 2-2 and 2-3.

The gas chromatograph/mass spectrophotometer (GC/MS) (Thermo Quest model TRACE GC 2000/ Finnigan POLARIS) contained a RTX-5 MS column (Restek) with an internal diameter of 0.25 mm. The settings of the GC/MS are tabulated in Table 2-4.

Table 2-3. Gas chromatography operation conditions

Operation Conditions	Value
Injection temperature	250°C
Initial column temperature	60°C
Temperature ramp rate	10°C/min
Final column temperature	350°C
Detector temperature	370°C
Ramp hold time	2.5 min
Final hold time	0.1 min

Table 2-4. Gas chromatography/mass spectrophotometer operation conditions

Operation Conditions	Value
Injection temperature	250°C
Initial column temperature	65°C
Temperature ramp rate	10°C/min
Final column temperature	320°C
Final hold time	2.50 min
Ramp hold time	0.1 min
Start Mass Spec	2.2 min
Mass Spec Range	50-600
Transfer Line	275°C
Ion Source	200°C

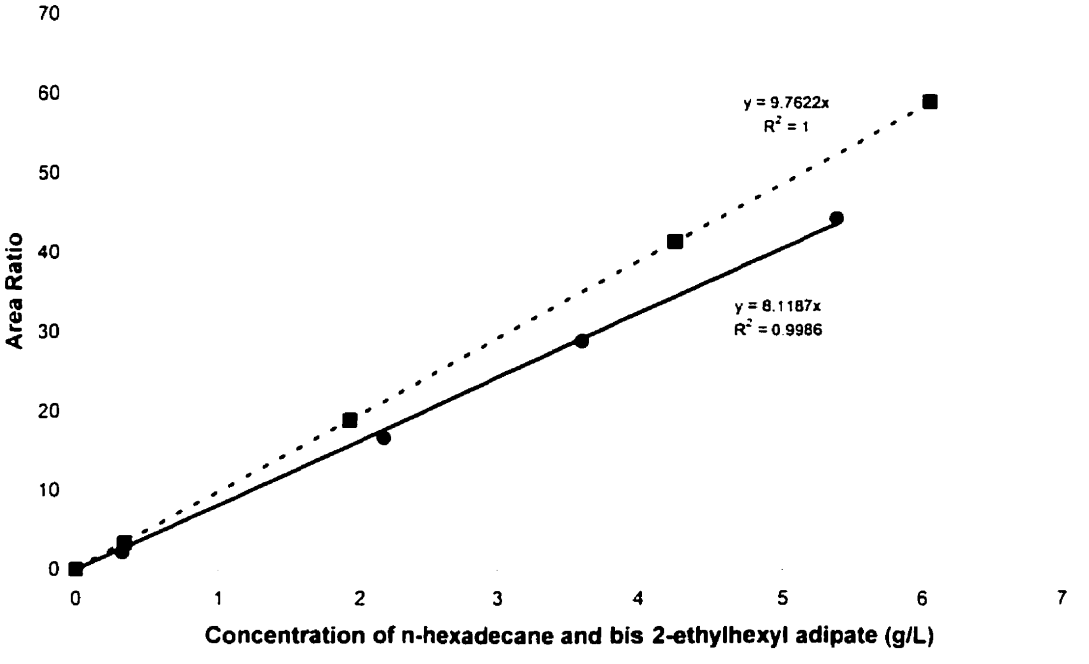

Figure 2-2. Calibration curves for n-hexadecane (■) and bis 2-ethylhexyl adipate (●).

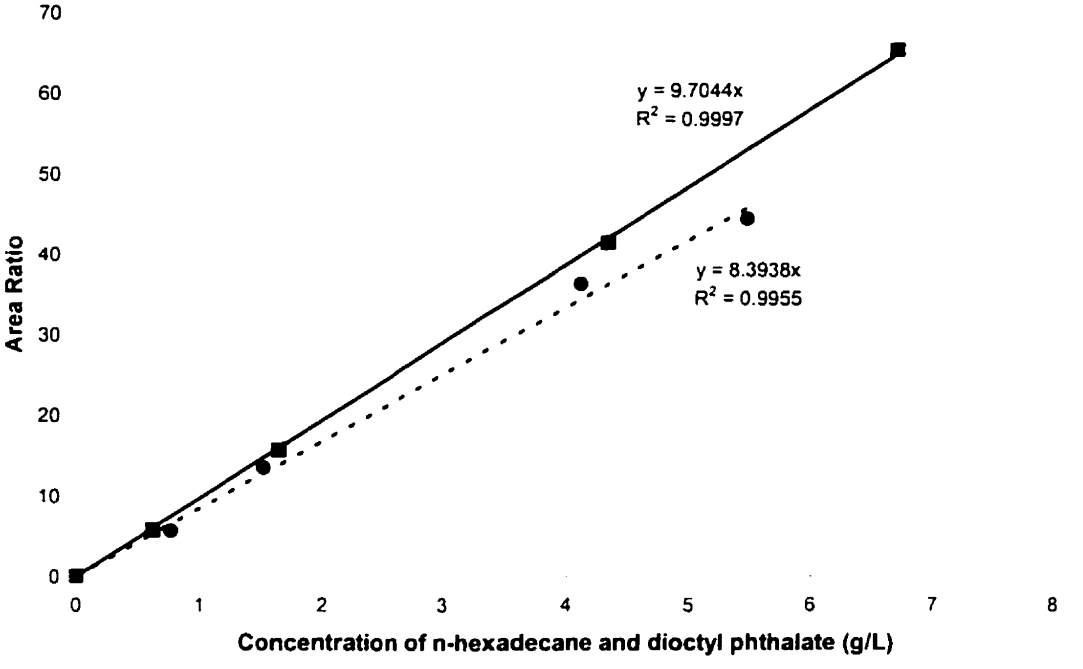

Figure 2-3. Calibration curves for n-hexadecane (■) and dioctyl phthalate (●).

2.8 Toxicity Measurements

There are many ways to find the toxicity of a solution; some of these include test organisms such as trout, protozoa and other marine organism. These test involve the exposure of the organism to rather large volumes of sample and are rather expensive and time-consuming to conduct. As an alternative to these test procedures, in the last few decades, researchers have been using microorganisms as the basis for the screening of toxicity[51, 52]. In particular, a toxicity test called the Microtox assay is based on the use of a marine organism *Vibrio fischeri* NRRL B-11177 in small test vials[51, 52, 53]. This procedure requires only 3 mL of sample volume and allows 3 samples to be tested for toxicity within 45 min. Due to these facts the test is an attractive alternative to the other methods previously mentioned.

The marine organism uses the enzyme luciferase to produce light in the following reaction[51, 52, 53].

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{Luciferase}} FMN + H_2O + RCOOH + \text{light}$$

The enzyme, luciferase, has been linked to the respiration pathway by nicotinamide adenine dinucleotide (NADH) and flavin nucleoide[53], thus the light emitted by the organism can be considered to be representative of the respiration of the organism. Thus a lower rate of respiration corresponds to a higher solution toxicity. The measures of toxicity obtained from this test are EC50 (effective concentration causing a 50% decrease of light output) and TU50 (100/EC50). The EC50 is obtained by plotting the concentration of the compound in aqueous phase versus the response on a logarithmic

scale. The response is the relative change in light emitted by the organism. This parameter is called gamma, which is a ratio of light lost to the light remaining after the set time. When gamma is one, there is a 50% decrease in light emitted. This response corresponds to the effective concentration (EC50). The lower is the EC50, the higher is the toxicity, i.e. for a low EC50, a low concentration of the toxicant is required to elicit the 50% response from the test organism. The TU50 provides a measure of toxicity that is directly proportional to the concentration of the toxicant. Thus, a high TU50 corresponds to a high toxicity; i.e., a solution of a toxicant must be diluted significantly to reduce its impact upon the organism to the 50% response level.

The organism that is used in the Microtox bioassay has been shown to have the same biochemical pathways of higher organisms. Tests have been conducted in which the response of the Microtox organism has been compared to the toxicity response of higher organisms such as rainbow trout. The correlations obtained between the tests have shown that there is a definite agreement between the toxicity values of the Microtox and other commonly used toxicity tests[54, 55, 56]. The organism that is used in the Microtox bioassay has been shown to have the same biochemical pathways of higher organisms. Due to the large number of toxicity measurements that were required in this investigation, and due to the low sample volume and low expense of the Microtox assay, this assay was used as a measure of toxicity in this work.

The toxicities of samples analyzed in this study were expressed as TU50's (in dilutions) due to the fact that the concentrations of the toxins in the mixtures were unknown. Thus, the TU50's quoted in this work represent the number of times that a sample had to be diluted to elicit a 50% reduction in the light output of the test organism.

The use of the Microtox assay can also be justified by the fact that it was the relative change in toxicity that was important in this investigation. Thus, the measurement of TU50 can be efficiently used to measure the relative change in toxicity of the broth over time.

The samples were obtained by centrifuging 10 mL of sample from the reactor at 10,000 RPM for 10 minutes. The supernatant was decanted into an 8 mL glass sample vial with a Teflon seal (Fisher Brand). The samples were then stored at -15 °C for eventual toxicity testing (12 samples per run of 1 reagent). The samples were then transferred into the 5-mL cuvettes designed for the Microtox Model 500 (Azur Environmental, formally Microbics Corporation). All the toxicity measures used the Basic Test setup in the Microtox software.

3. Results

3.1 Preliminary Growth Studies

Tables 3-1, 3-2 and 3-3 include the data for six different bacteria grown in media that all contain one of seven different plasticizers. These bacteria were chosen for their ability to readily grow on long chain hydrocarbons.

Table 3-1 contains the results of experiments in which the plasticizer was the only carbon source present. No growth was observed when the sole carbon source was a plasticizer that had a complex hydrocarbon structure.

Table 3-2 demonstrates that all of the bacteria could grow on yeast extract as the carbon source in the presence of one of mineral oil, dioctyl phthalate or dioctyl terephthalate. *Rhodococcus ISO1* and *Mycobacterium OFS* also grow in the presence of either the adipate or di-ethylene glycol dibenzoate. *Rhodococcus rhodochrous* and *Arthrobacter paraffinues* grew in the presence of all of the plasticizers. *R.rhodochrous* showed particularly significant growth in all of these experiments. *Pseudomonas flouresens* TEXACO and *Corynebacterium sp.* were the most sensitive microorganisms and their growth was inhibited by four of the seven plasticizers.

Table 3-3 contains data for a study of the two most vigorous bacteria, identified from the previously mentioned study in Table 3-2, for growth in a medium containing hexadecane and a plasticizer. The bacterial grew very well using the n-hexadecane as a substrate for both organisms in the study. *Rhodococcus rhodochrous* showed the heaviest growth. These results indicated that this was the most convenient choice of medium for studying the effect of bacterial growth on another substrate in the presence of the plasticizers.

From the above results, it was possible to select one system for the study of the biodegradation of plasticizers. Therefore, all of the rest of the results are for the growth of *R. rhodochrous* on media containing hexadecane and a plasticizer.

Table 3-1 Growth of bacteria in media containing a plasticizer as the only carbon source[1].

Organism	MO[2]	DOP[3]	DOTP[4]	BEHA[5]	DEDB[6]	DPDB[7]	TCP[8]
Rhodococcus rhodochrous	+	-	-	-	-	-	-
***Rhodococcus* ISO1**	+	-	-	-	-	-	-
Arthrobacter paraffineus	+	-	-	-	-	-	-
***Pseudomonas flourescens* TEXACO**	+	-	-	-	-	-	-
Corynebacterium species	+	-	-	-	-	-	-
Mycobacterium OFS	+	-	-	-	-	-	-

1. growth (+); no growth (-)
2. MO = Mineral Oil
3. DOP = Dioctyl phthalate
4. DOTP = Dioctyl terephthalate
5. BEHA = Bis 2-ethylhexyl adipate
6. DEDB = Di-ethyleneglycol di-benzoate
7. DPDB = Di-propalene glycol di-benzoate
8. TCP = Tri cresyl phosphate

Table 3-2 Growth of bacteria on media containing yeast extract and plasticizer[1].

Organism	MO[2]	DOP[3]	DOTP[4]	BEHA[5]	DEDB[6]	DPDB[7]	TCP[8]
Rhodococcus rhodochrous	++	++	++	++	++	++	++
***Rhodococcus* ISO1**	++	+	+	+	+	-	-
Arthrobacter paraffineus	++	+	+	+	+	+	+
***Pseudomonas flourescens* TEXACO**	+	+	+	-	-	-	-
Corynebacterium species	+	+	+	-	-	-	-
Mycobacterium OFS	+	+	+	+	+	-	-

1. Heavy Growth (++); Light Growth (+); No Growth (-)
2. MO = Mineral Oil
3. DOP = Dioctyl phthalate
4. DOTP = Dioctyl terephthalate
5. BEHA = Bis 2-ethylhexyl adipate
6. DEDB = Di-ethyleneglycol di-benzoate
7. DPDB = Di-propalene glycol di-benzoate
8. TCP = Tri cresyl phosphate

Table 3-3 Growth of *R.rhodochrous* and *A.paraffineus* on media containing plasticizer and hexadecane[1].

Organism	MO[2]	DOP[3]	DOTP[4]	BEHA[5]	DEDB[6]	DPDB[7]	TCP[8]
Rhodococcus rhodochrous	++	++	++	++	++	++	++
Arthrobacter paraffineus	++	++	++	++	++	++	+

1. Heavy Growth (++); Light Growth (+)
2. MO = Mineral Oil
3. DOP = Dioctyl phthalate
4. DOTP = Dioctyl terephthalate
5. BEHA = Bis 2-ethylhexyl adipate
6. DEDB = Di-ethyleneglycol di-benzoate
7. DPDB = Di-propalene glycol di-benzoate
8. TCP = Tri cresyl phosphate

3.2 Batch Fermentations

3.2.1 Heterogeneous Sampling Error

Problems were encountered during the sampling of the reactor broth because it had two liquid phases. The media contains plasticizer and n-hexadecane, both of which have a very low solubility in water. Figure 3-1 shows the GC data for a typical system containing both hexadecane and plasticizer. Initially the reactor contained appreciable amounts of the oil phase but this was not apparent in the early data because of poor mixing conditions resulting in a heterogeneous mixture; i.e., the samples were not representative of the makeup of the reactor. This produced scattered data in the first few samples of all of the growth studies as demonstrated in Figure 3-1. This situation dramatically improved once the biomass concentration began to increase in the reactor. The biomass of microorganisms that can grow on insoluble substrates often acts as an

emulsifier bringing small droplets of the oil phase into the aqueous phase[23]. This results in a more homogenous mixture.

For this work, consistent results were usually observed after twenty hours after the commencement of a typical growth study. In all of the growth studies presented in the next subsection, the GC data for the plasticizers and hexadecane are not reported for the first few samples. After about 20 hours, the trends became reproducible and it was deemed reasonable to include these data in subsequent analyses.

3.2.2 Absorbance of Protein for Biomass Representation

The degradation and toxicity studies were all carried out in batch fermentations. All of the following data were reproducible but only some examples of the duplicate fermentations are included. Each of the fermentation media contained 3% hexadecane and 3% of one plasticizer, expressed in volumetric ratios. The fermentation was checked for contamination every 2 days.

Figure 3-2 shows the growth of *R. rhodochrous* on hexadecane in the presence of dioctyl phthalate. It demonstrates that the measurement of the amount of protein in the biomass is at least as good an indication of growth as dry weight measurements. Dry weight measurements are notoriously difficult and inaccurate for systems containing an insoluble hydrocarbon[57, 58] due to the entrainment of oil in the pellet for the dry weight measurement. For these reasons, all of the other graphs show only the amount of protein in the biomass pellet as an indication of growth.

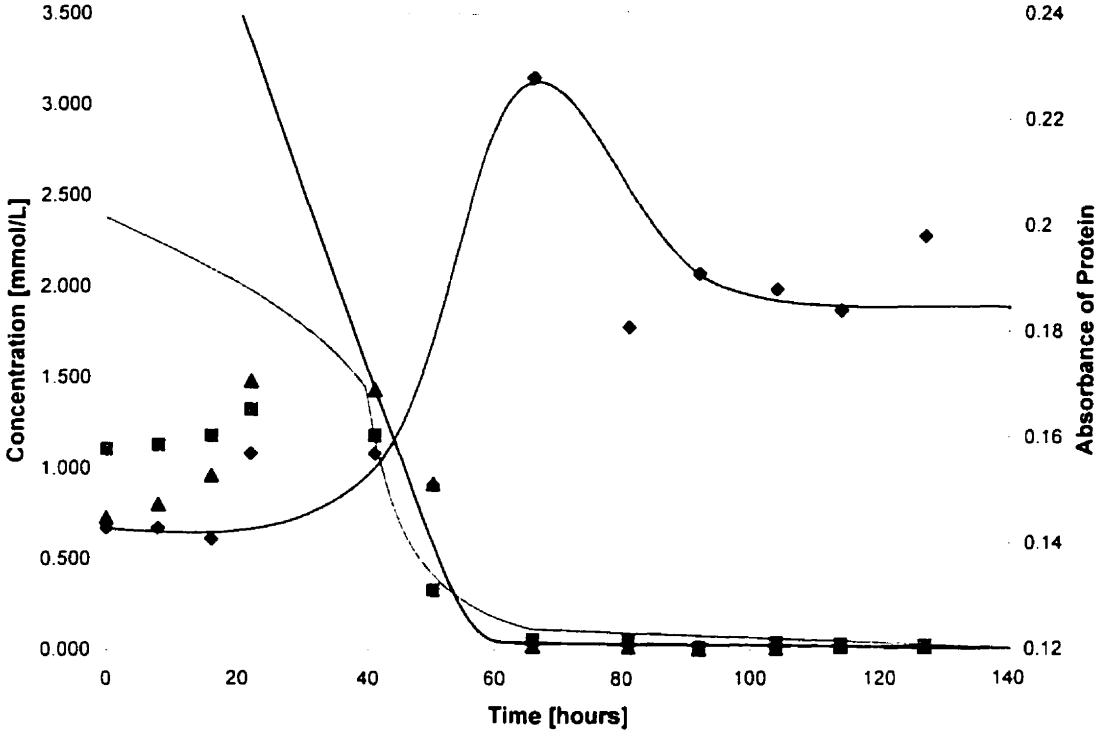

Figure 3-1. Growth of *R. rhodochrous*, expressed as protein absorbance (◆), on a medium containing bis 2-ethylhexyl adipate (▲) and hexadecane (■).

3.2.3 Decrease in Biomass after Degradation of Plasticizer

Figure 3-3 contains data for a fermentation in which the plasticizer present was bis 2-ethylhexyl adipate. As expected, the hexadecane was depleted as the amount of biomass increased. During the time of the degradation of the hexadecane there was complete removal of the plasticizer from solution. It can be seen that the amount of biomass, as determined by protein concentration, decreased sharply after growth was finished. This was the most dramatic example of this phenomenon but this general trend was found to be reproducible.

3.2.4 Metabolite Production During Fermentation

The concentrations of the hydrocarbons were all determined by gas chromatography. As the plasticizers were degraded, new peaks appeared in these chromatographs.

The results are shown in Figures 3-4 and 3-5 for two different growth studies in the presence of bis 2-ethylhexyl adipate. From the pattern with time, it appears reasonable to conclude that these must be metabolites from either the utilization of the hexadecane or the degradation of the plasticizer. Metabolite # 1 appeared in the samples after the concentrations of both hexadecane and the plasticizer had started to decrease. It then disappeared at about the same time that the concentrations of both of the original compounds had finished a precipitous decline. As the concentration of metabolite #1 started to decrease, that of metabolite # 2 began to increase sharply and remained high at the end of the experiment.

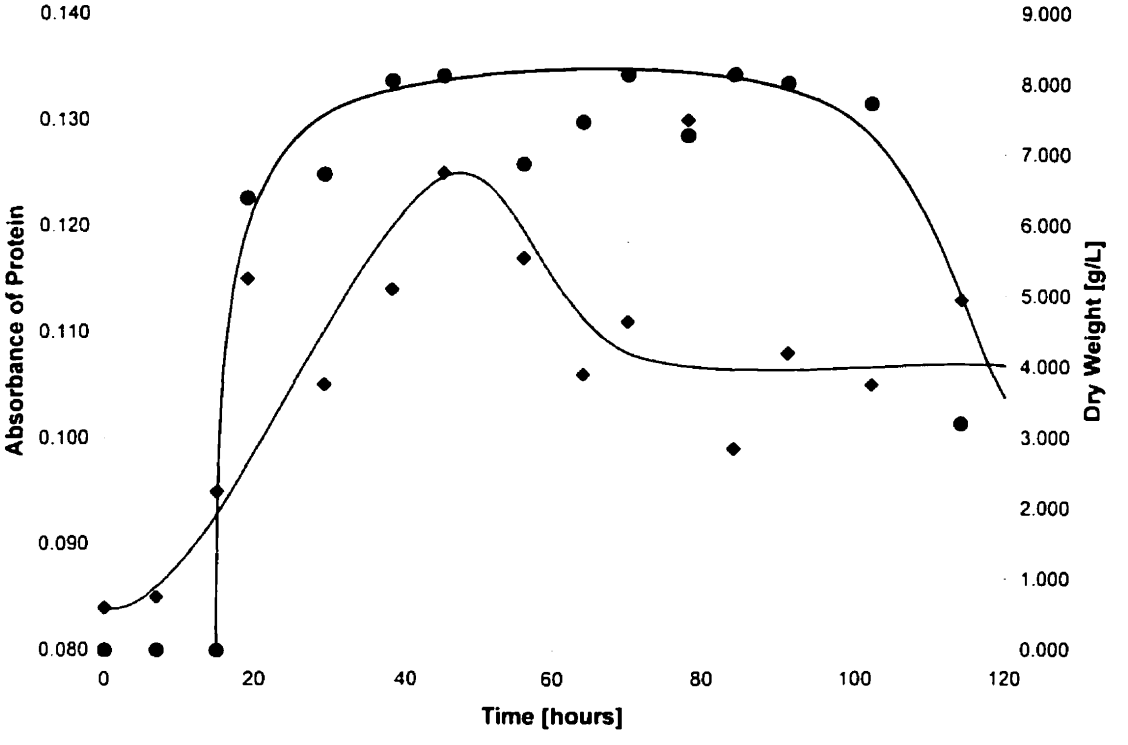

Figure 3-2. Dry weight (●) and protein absorbance (◆) for the growth of *R. rhodochrous* in a medium containing dioctyl phthalate and hexadecane in MMSM.

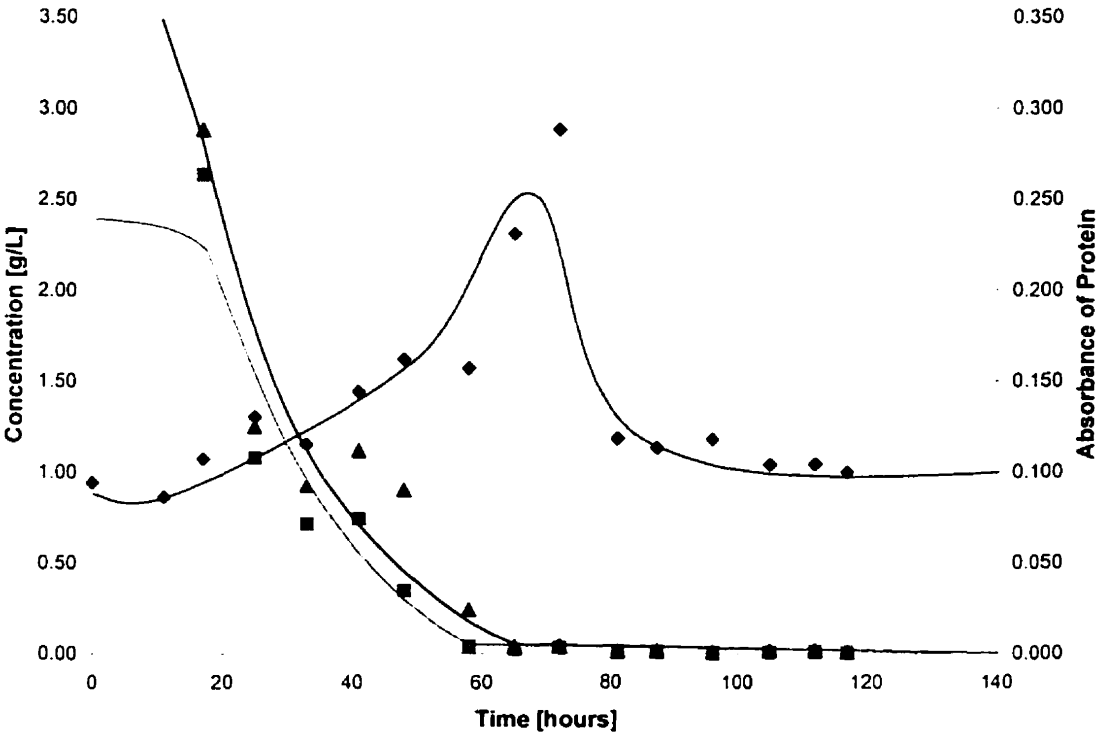

Figure 3-3. Growth of *R. rhodochrous*, expressed as protein absorbance (◆), on a medium containing bis 2-ethylhexyl adipate (▲), hexadecane (■).

Figure 3-6 contains toxicity data for samples of the broth after the cells had been removed. It also contains data for the two unidentified compounds. The toxicity started low and then began to increase at the time of appearance of metabolite #1. It quickly reached a maximum TU50 of approximately 2000 and then remained high.

The same trends were observed with the other plasticizers studied. In Figure 3-7 the degradation of dioctyl phthalate was minimal, but was still observable as a trend. At the same time, there was a definite increase in toxicity of the broth. As the plasticizer was degraded, evidence of a metabolite began to appear in the GC traces and its retention time was identical to that of metabolite #2. There was no peak for metabolite #1 in any of the samples taken for any of the experiments with DOP degradation (Figure 3-8).

Figure 3-9 shows the increase in toxicity of the broth when the organism was growing in the presence of dioctyl terephthalate. *R.rhodochrous* degraded more than 50% of the plasticizer. The toxicity of the samples from this growth study increased during the exponential phase of growth (i.e., the first 60 hours). The toxicity then decreased and finally increased again by the end of the fermentation.

As was observed for the systems containing DOP, metabolite # 2 was observed in the fermentation broth after the concentration of the DOTP started to decrease (Figure 3-10). The results of the two systems were also similar in that there was no observation of metabolite #1.

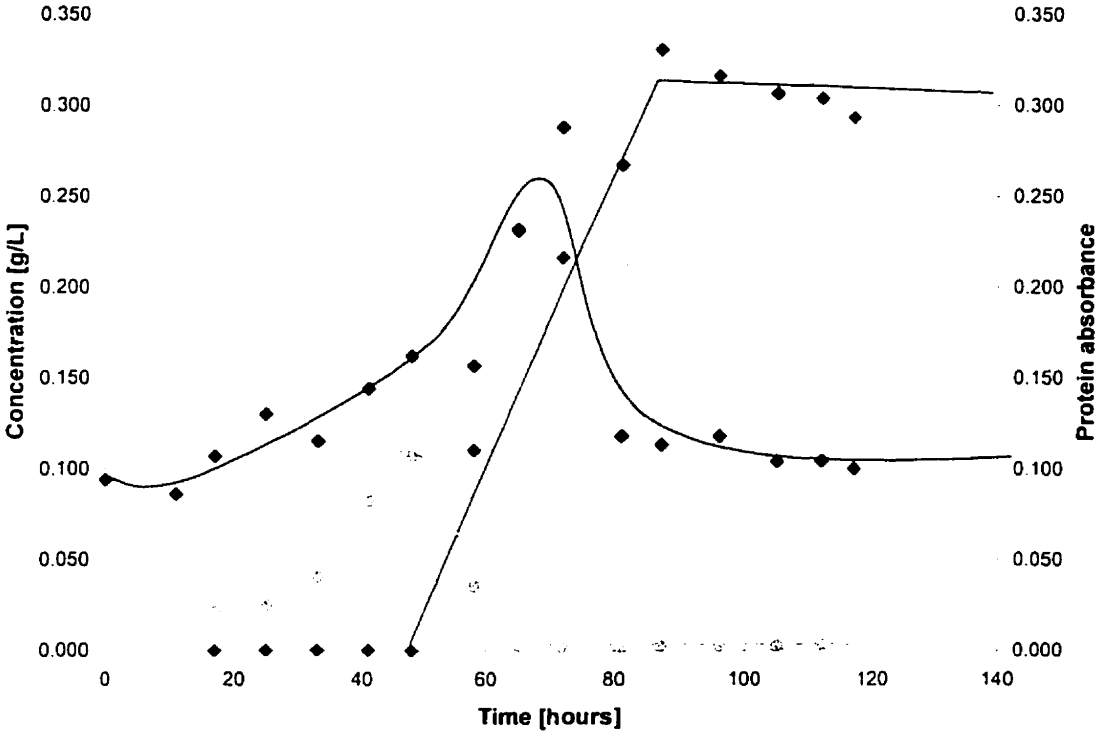

Figure 3-4: Protein absorbance (◆), metabolite #1 (❀) metabolite #2 (◆) for system shown in Figure 3-3.

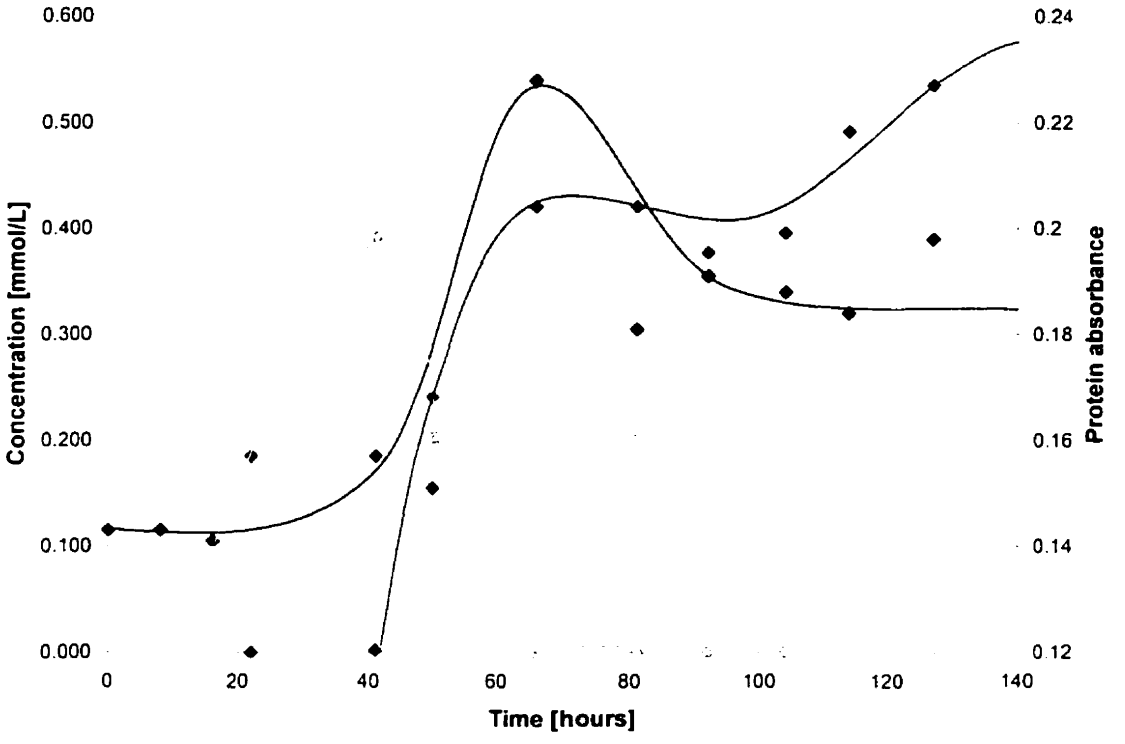

Figure 3-5. Protein absorbance (◆) metabolite #1 (◆) and metabolite #2 (◆) from the repeat of the experiment represented by Figures 3-4 and 3-3.

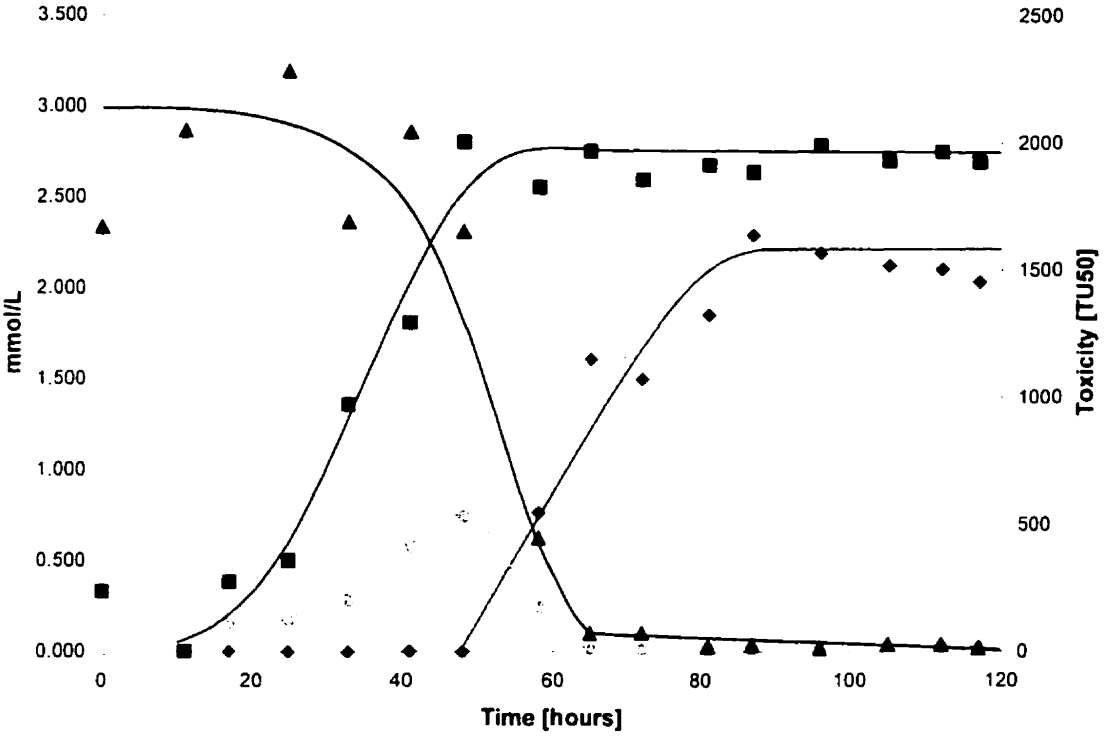

Figure 3-6. Toxicity (■), concentration of metabolite #1 ( ), metabolite #2 (◆) and bis 2-ethylhexyl adipate (▲) for system represented in Figures 3-3 and 3-4.

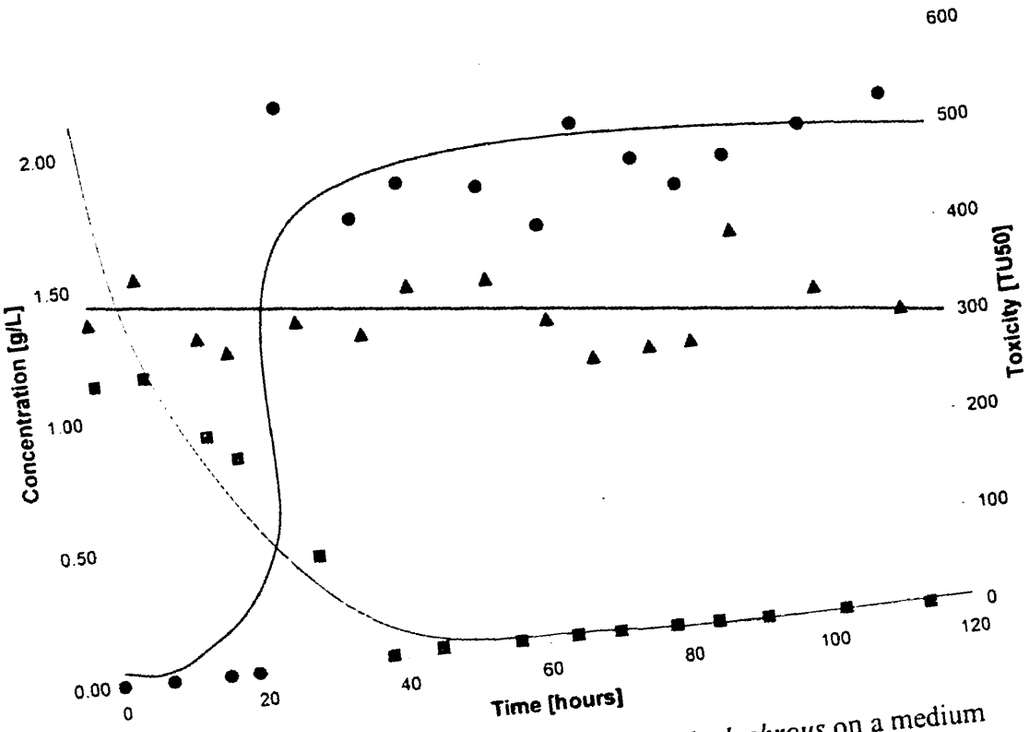

Figure 3-7. Toxicity data (■) for growth of *R. rhodochrous* on a medium containing dioctyl phthalate (▲) and hexadecane (■).

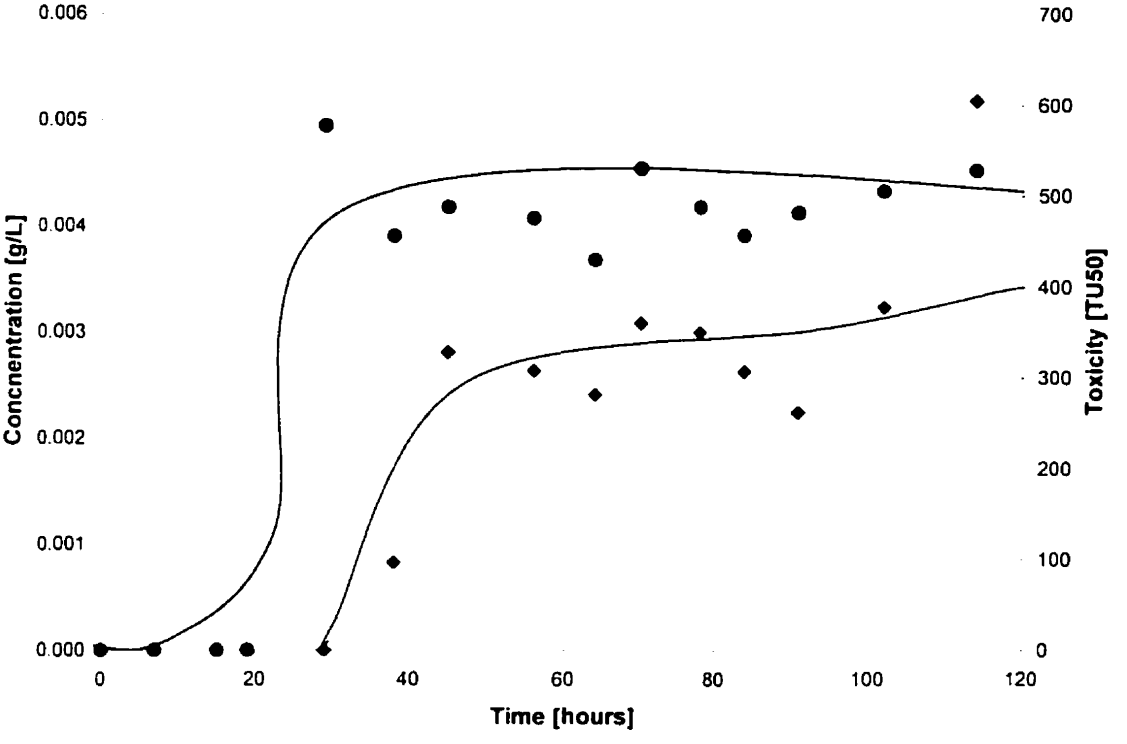

Figure 3-8. Toxicity (●) and concentration of metabolite #2 (◆) for the system represented in Figure 3-7.

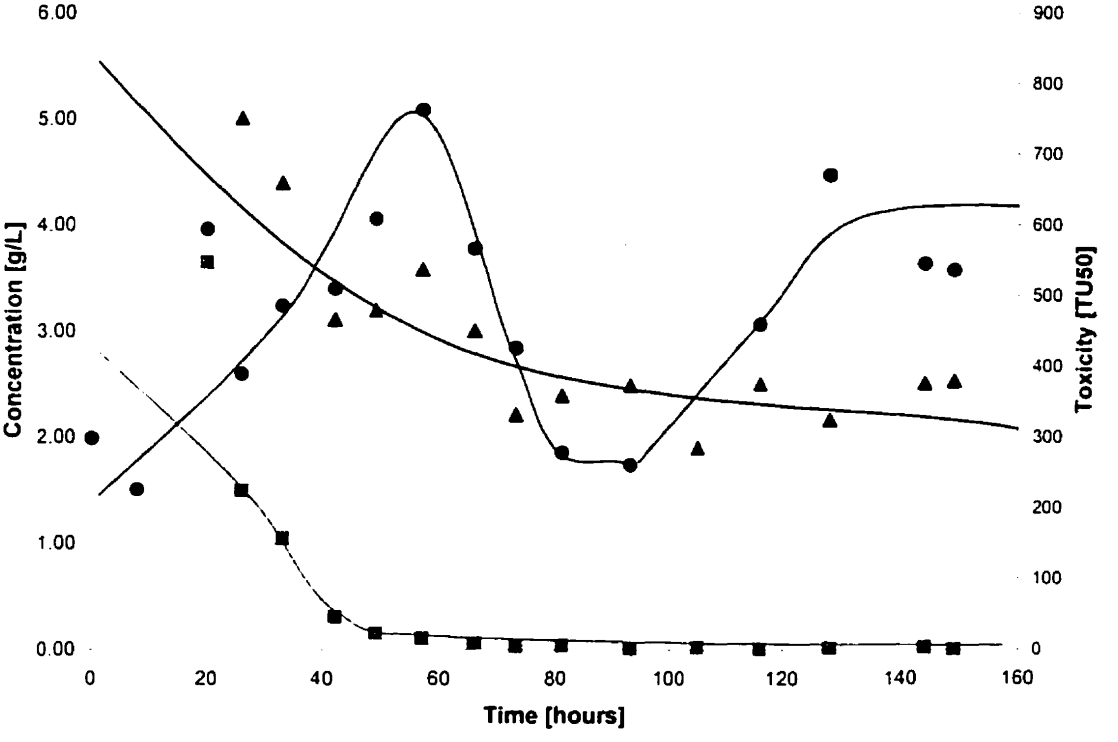

Figure 3-9. Toxicity data (■) for growth of *R. rhodochrous* on a medium containing dioctyl terephthalate (▲) and hexadecane (■).

3.2.5 Characterization of Metabolites

The positions of the two new peaks observed in the GC traces after growth was underway are reported in Table 3-4. It can be seen that these peaks are different from the hexadecane and the original plasticizers. Metabolite #2 was observed for the growth studies of all three types of plasticizer.

Metabolite #2 was subjected to GC-MS analysis. The database of the instrument listed 2-ethyl hexanoic acid as the most likely identification for this unknown (probability of 72%). Figures 3-11 (a) and 3-11 (b) show the MS fragmentation patterns for metabolite #2 and a sample of 2-ethyl hexanoic acid, respectively. Both show the same major fragments. Table 3-4 shows that both had similar retention times on the columns as well. The small difference in the retention times is probably due to the fact that the GC behaviour of organic acids is complicated by the carboxylic acid function. Small differences in the pH of the samples being extracted can result in small changes in retention times.

The GC-MS trace for metabolite #1 did not lead to any one obvious compound when it was compared to the database. However, one of the logical possibilities was 2-ethyl hexanol and this identification was confirmed by comparing GC-MS traces (Figure 3-12 (a) and Figure 3-12 (b)) and retention times (Table 3-4).

The retention times for 2-ethyl hexanal are listed in Table 3-4. This compound was not observed in the chromatographs of any of the samples taken.

Table 3-4. Gas chromatography and gas chromatograph/mass spectrophotometer data for plasticizers and other data.

Compounds	GC Retention Time (min)	GC/MS Retention Time (min)
Metabolite #1	**1.9**	**6.6**
Metabolite #2	**2.2**	**8.5**
2-ethyl hexanal	**1.5**	**5.2**
2-ethyl hexanol	**1.9**	**6.7**
2-ethyl hexanoic acid	**2.3**	**9.1**
pentadecane	**7.8**	**13.7**
hexadecane	**9.1**	**14.7**
bis 2-ethyhexyl adipate	**16.6**	**22.4**
dioctyl phthalate	**17.6**	**23.7**
dioctyl terephthalate	**19.2**	**25.1**

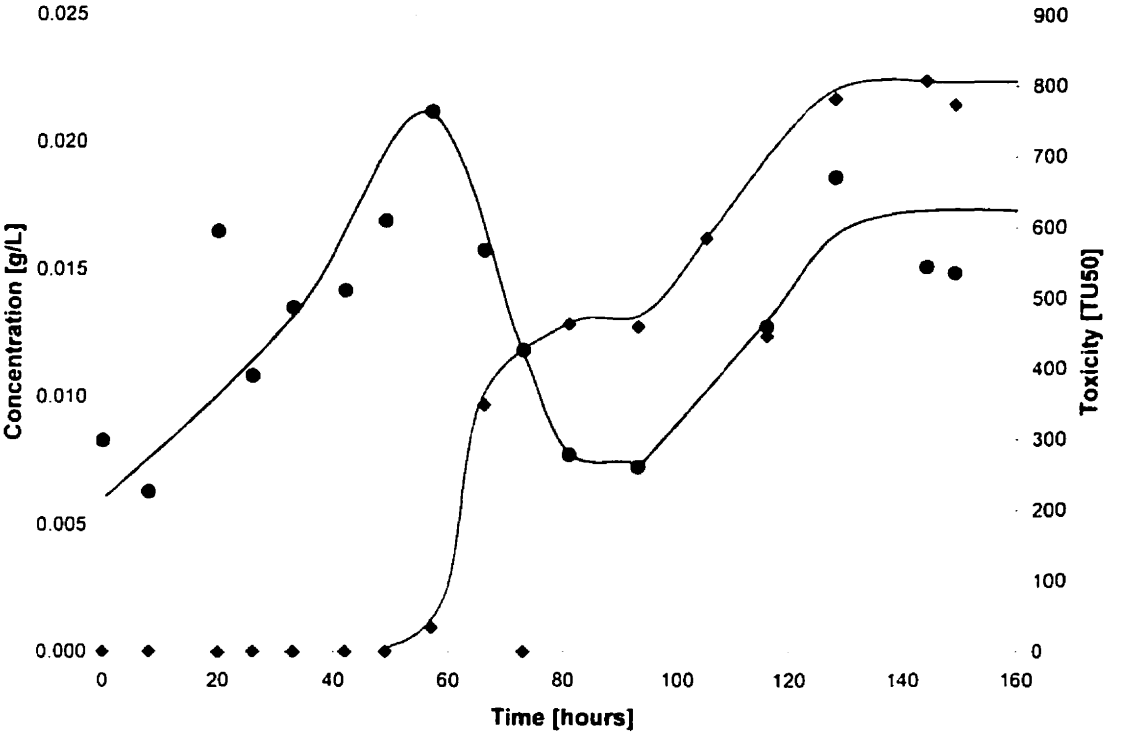

Figure 3-10. Toxicity data (●) and concentration of metabolite #2 (◆) for the system represented in Figure 3-9.

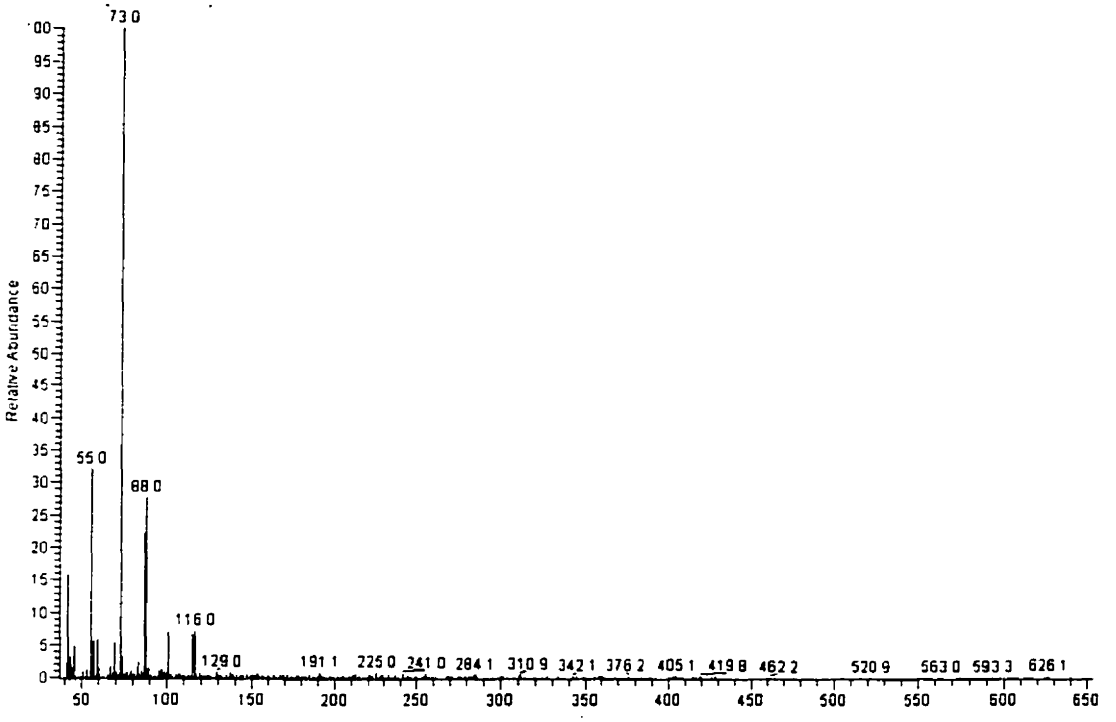

Figure 3-11 (a). Mass spectrum of metabolite #2 from end samples of dioctyl terephthalate degradation.

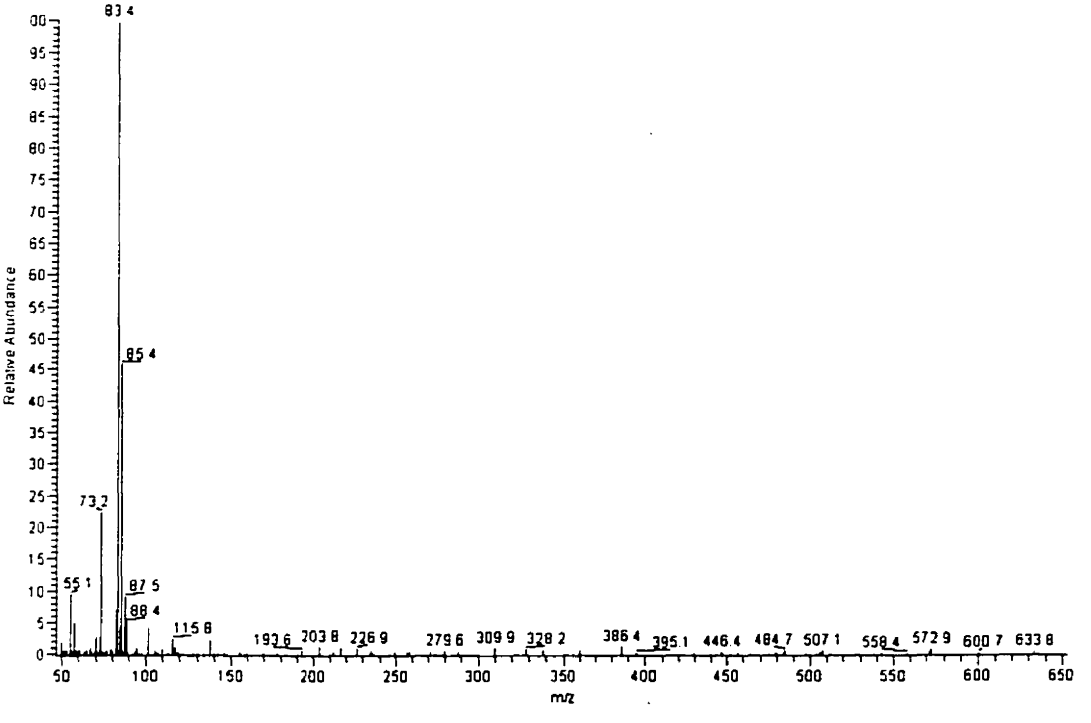

Figure 3-11 (b). Mass spectrum of 2-ethyl hexanoic acid.

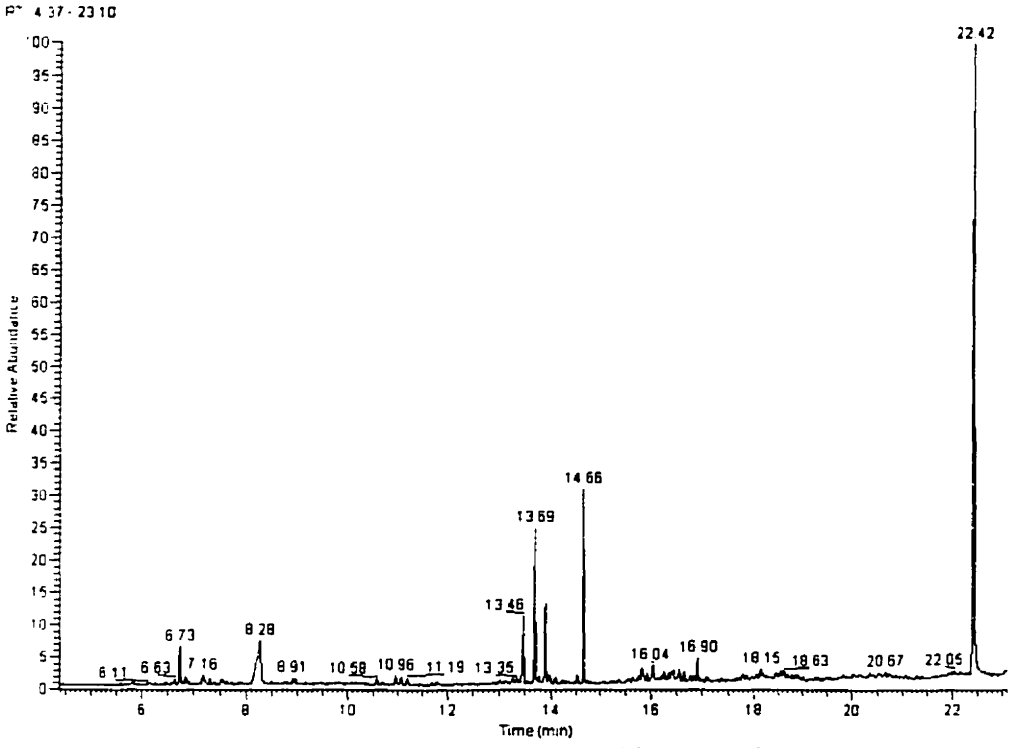

Figure 3-12 (a). Gas chromatograph of bis 2-ethylhexyl adipate reactor sample.

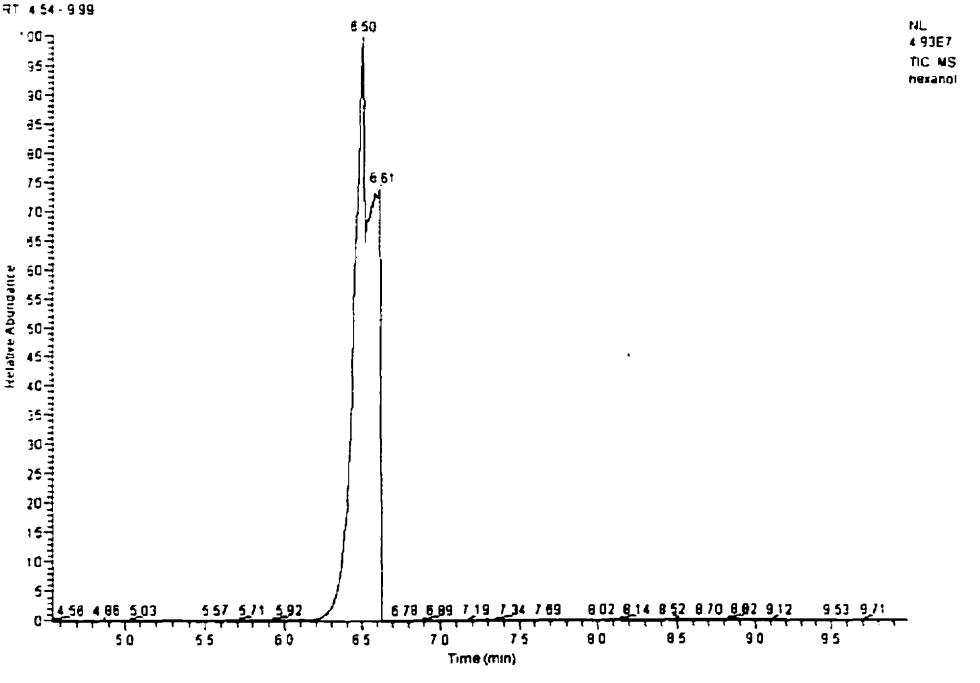

Figure 3-12 (b). Chromatograph of pure 2-ethyl hexanol.

3.3 Toxicity of Pure Compounds

Table 3-5 lists the toxicities of all of the individual compounds involved in this work as measured by Microtox toxicity assay. Each compound was added to an aqueous solution of MMSM described in the material and methods. The concentrations of the different pure compounds were selected to be close to those concentrations of compounds observed throughout the course of the growth studies. It was found that the Microtox organism was very sensitive to 2-ethyl hexanol, which had a toxicity of 14,400 TU50 at a concentration of 0.1 g/L. The aldehyde and carboxylic acid had the same range of toxicity as the phenol solution, which was used both as a standard and control for the Microtox assay. All the plasticizers involved in this research had toxicities below the level of detection of the Microtox assay.

Table 3-5. Toxicity of compounds in MMSM[1].

Compounds	Concentration of Compound	Toxicity in TU50
2-ethyl hexanal	0.1 g/L	1,100
2-ethyl hexanol	0.1 g/L	14,400
2-ethyl hexanoic acid	0.1 g/L	950
Phenol	0.1 g/L	2,000
hexadecane	1 g/L	Below detection
dioctyl phthalate	1 g/L	Below detection
dioctyl terephthalate	1 g/L	Below detection
bis 2-ethyhexyl adipate	1 g/L	Below detection

1. MMSM= minimum mineral salt medium as defined in materials and methods.

4. DISCUSSION

4.1 Growth of Bacteria in the Presence of Plasticizers

In the preliminary studies, attempts were made to grow several different bacteria on a variety of different plasticizers as the sole carbon source. In general, very little degradation was observed for any of the plasticizers. The only exception was the simplest of the plasticizers, mineral oil. However, this material is rarely used as a plasticizer.

In a real situation, a plasticizer leaching from flooring tiles or any other type of plastic material will be dispersed over a large area where a mixture of organisms may have the ability to degrade the plasticizer over time. Another scenario that could lead to degradation would be co-metabolism. Co-metabolism is the process of breakdown of a plasticizer without any energy or biomass production arising from the plasticizer. After leaching out of the plastic and into the aqueous environment, it is conceivable that the plasticizer could be mixed with different substrates such as hydrocarbons and sugars. These could act as primary substrates and at the same time the substrates would activate the pathways needed for the breakdown of the plasticizer.

Before investigating the possibility of co-metabolism, it was necessary to determine whether the plasticizers would inhibit the growth of the bacteria. The preliminary screening studies were repeated with easily utilized substrates, either yeast extract or hexadecane. Growth was observed for most of these systems and at least some of the bacteria tested could grow in the presence of all of the plasticizers. Since the main objective of this work was to study the biodegradation of plasticizers, the remainder of this work focused on the fate of plasticizers in the presence of bacteria growing on

another substrate. A small concentration of yeast extract was needed to enhance the growth so that considerable degradation could be seen within one week of growth. Two of the bacteria, *Rhodococcus rhodochrous* and *Arthrobacter paraffineus*, were able to grow particularly well in the presence of all of the plasticizers tested. Furthermore, this was observed for both types of carbon substrate, i.e., yeast extract and hexadecane.

4.2 Biodegradation of Plasticizers

The most definitive way to determine if biodegradation of the plasticizers was occurring was to follow the degradation of the plasticizers over a period of time. This could also lead to some understanding of the degradation mechanisms involved. The best growth observed in the preliminary studies was for *R. rhodochrous* and this was true for all of the plasticizers. *R.rhodochrous* is a commonly occurring soil organism[40]. Thus this is a good candidate for the interaction with the plasticizer in a real situation. This bacterium was selected for the detailed growth studies. To further narrow the field of the study for the remainder of the experiments, hexadecane was selected as the "easily-degraded" carbon source. Some initial work was done using each of the plasticizers listed earlier, but the preliminary results demonstrated that the systems were very complicated and were thus beyond the scope of the present study. Thus, it was decided that this study would concentrate on the degradation of three of the plasticizers dioctyl phthalate, terephthalate and bis 2-ethylhexyl adipate.

R. rhodochrous easily grew in the presence of all of the plasticizers. In every growth study, the hexadecane (the primary carbon source) was degraded completely within the first sixty hours. This sixty-hour period also corresponded to the exponential

growth phase of the organism, thereby accounting for most of the biomass increase. The organism exhibited different behavior depending on the plasticizer, as will be discussed below.

During the degradation of the bis 2-ethylhexyl adipate (BEHA), the organism completely degraded the plasticizer at the same time as the hexadecane (Figure 3-3). Complete metabolism of the plasticizer occurred within the exponential phase of growth. However, even though the original plasticizer was no longer present, this does not prove that the plasticizer was completely mineralized. Metabolites were observed in the gas chromatographs after growth had commenced and these can be shown to originate from the breakdown of the plasticizer. Metabolite #1 accumulated in the exponential phase and metabolite # 2 began to appear as the first metabolite disappeared from the reactor. Metabolite #2 appeared to be very stable and remained in the reactor until the end of the experiment without degrading.

Dioctyl terephthalate (DOTP) was not as easily degraded as the adipate. About 50% of the original plasticizer remaining at the end of the growth studies. Dioctyl phthalate (DOP) was even less degradable than DOTP and most of the original plasticizer remained at the end of the experiments. The slower rate of degradation of the DOP relative to the DOTP is attributed to the ortho- arrangement of the ester functional groups. It has been proposed that the closeness of the functional groups could inhibit the enzyme activity involved in hydrolysis of phthalate ester[59]. It is apparent that the adipate is more easily degraded than either of the phthalates. This could be related to the ease with which the ester bonds of the adipate are hydrolyzed relative to those of the

phthalates. This will be relevant when considering the pattern of the appearance and disappearance of the metabolites.

None of the experiments involving the growth of *R.rhodochrous* in the presence of either DOP or DOTP showed any evidence of the production of metabolite #1. However, both of types of experiments generated metabolite #2 - although the total amounts were less than the amounts observed for the experiments with the adipate. To understand these patterns observed for the phthalates, it is first necessary to consider the nature of these metabolites.

4.3 Characterization of the Metabolites from Plasticizer Degradation

Metabolite #2 was observed in the GC traces of the end-of-growth samples from the broth of all three plasticizer-degradation studies. It seemed reasonable to conclude that this was a metabolite of the degradation of either the plasticizers or hexadecane. Analysis of this unknown compound by GC-MS indicated that the most likely identification was 2-ethyl hexanoic acid. This was confirmed by comparison of the GC-MS trace of the unknown compound with that of a pure sample of this acid.

All three of the plasticizers used in this part of the study contain the alcohol, 2-ethyl hexanol, attached by an ester bond (Figures 1-1, 1-2 and 1-3). This particular alcohol is commonly referred to as octyl alcohol. It has the same carbon skeleton as 2-ethyl hexanoic acid.

The degradation of one of the plasticizers (bis 2-ethylhexyl adipate) produced a second metabolite, which only appeared for a short time and then disappeared as growth

continued. This unknown compound and a sample of pure 2-ethyl hexanol had identical GC-MS trace patterns.

Based on these observations, it becomes possible to postulate the degradation mechanism that leads to the appearance of the metabolites. This part of the degradation is identical for all three plasticizers (Figures 4-1 to 4-3). In each case, the microorganism causes the hydrolysis of the ester bonds. This releases 2-ethyl hexanol, which then undergoes further oxidation. In the case of the adipate, there is an early increase in the concentration of the alcohol followed by a decrease as it is oxidized. The other two plasticizers must undergo the same mechanism but there was no build-up in the concentration of the alcohol. It was not possible to detect even the smallest peak for the alcohol in the GC or GC-MS traces for any of these samples. In fact, it is not common to see significant amounts of intermediate products and the adipate is an unusual example[24]. It seems that in the cases of DOTP and DOP, the hydrolysis is the rate-limiting step, thus there is no accumulation of the alcohol in the reactor. This was also seen in other work where the rate-limiting step was the initial breaking of the ester bond of phthalate and terephthalate plasticizers[40]. This relatively facile hydrolysis of the adipate ester bonds would explain why this plasticizer is completely degraded during growth of *R.rhodochrous* on hexadecane while appreciable amounts of the phthalate plasticizers remain at the end of the growth studies.

It is also apparent that the other expected intermediate in the proposed mechanism (Figures 4-1 to 4-3), 2-ethyl hexanal, has a very short lifetime. The position for this aldehyde in the GC trace was determined but this peak was not observed for any of the

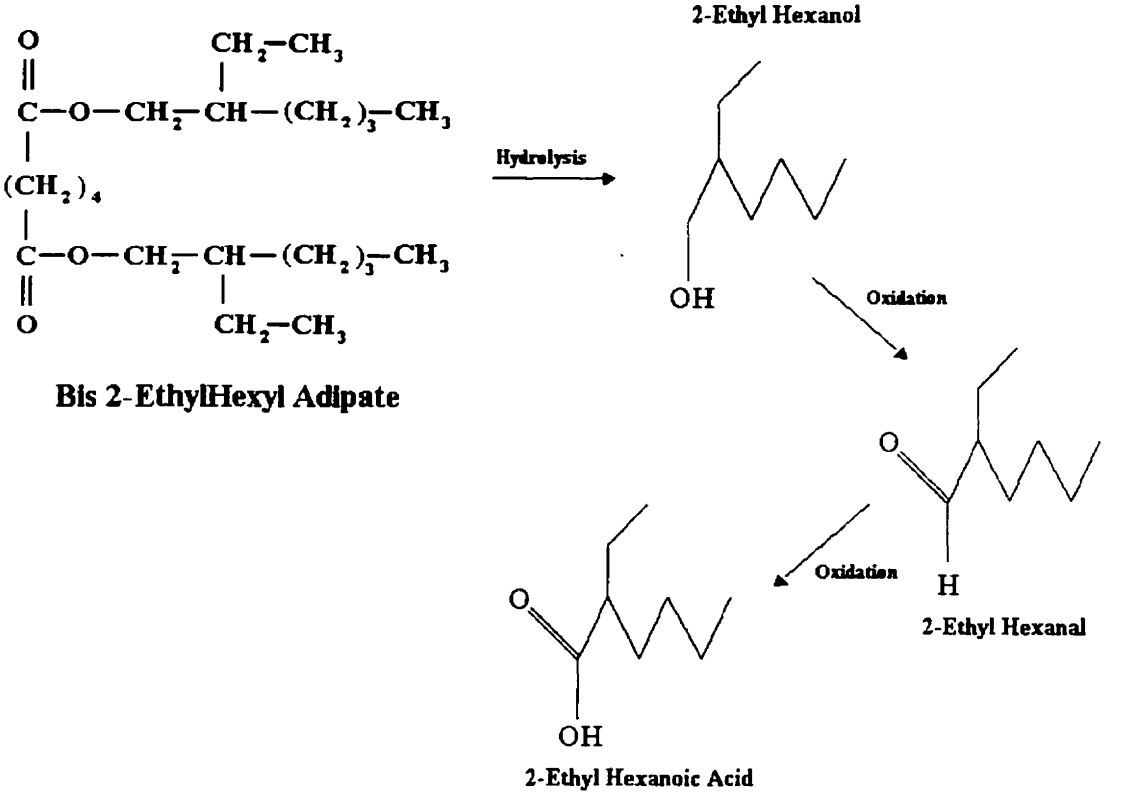

Figure 4-1. Proposed mechanism for the production of the 2-ethyl hexanol and 2-ethyl hexanoic acid from the degradation of bis 2-ethylhexyl adipate.

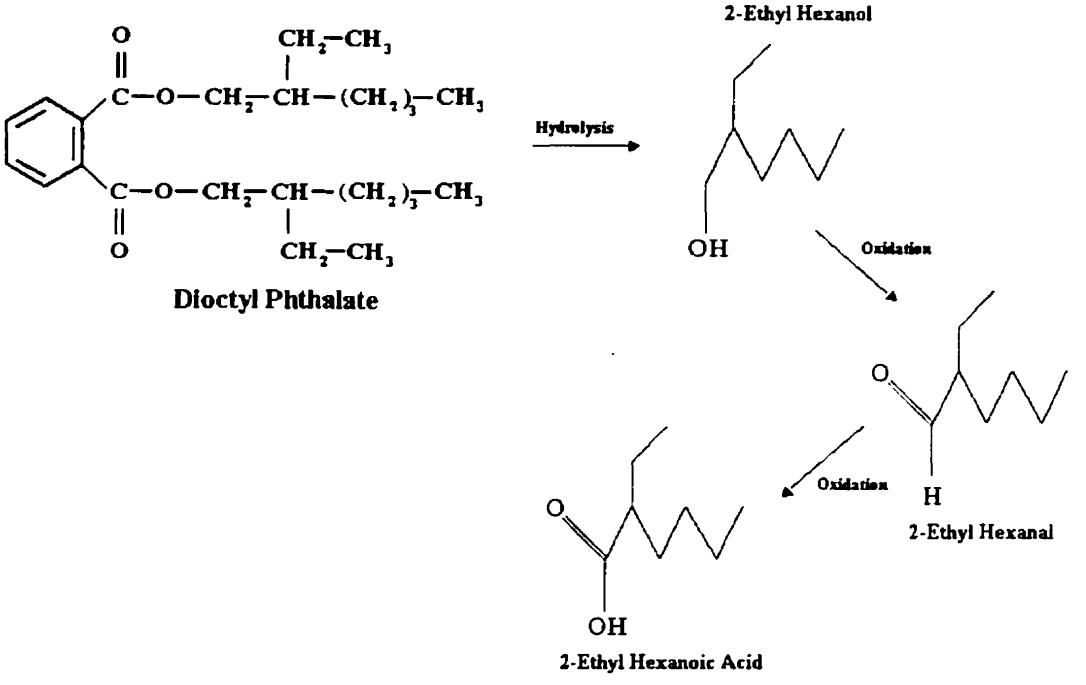

Figure 4-2. Proposed mechanism for the production of 2-ethyl hexanoic acid from the degradation of dioctyl phthalate.

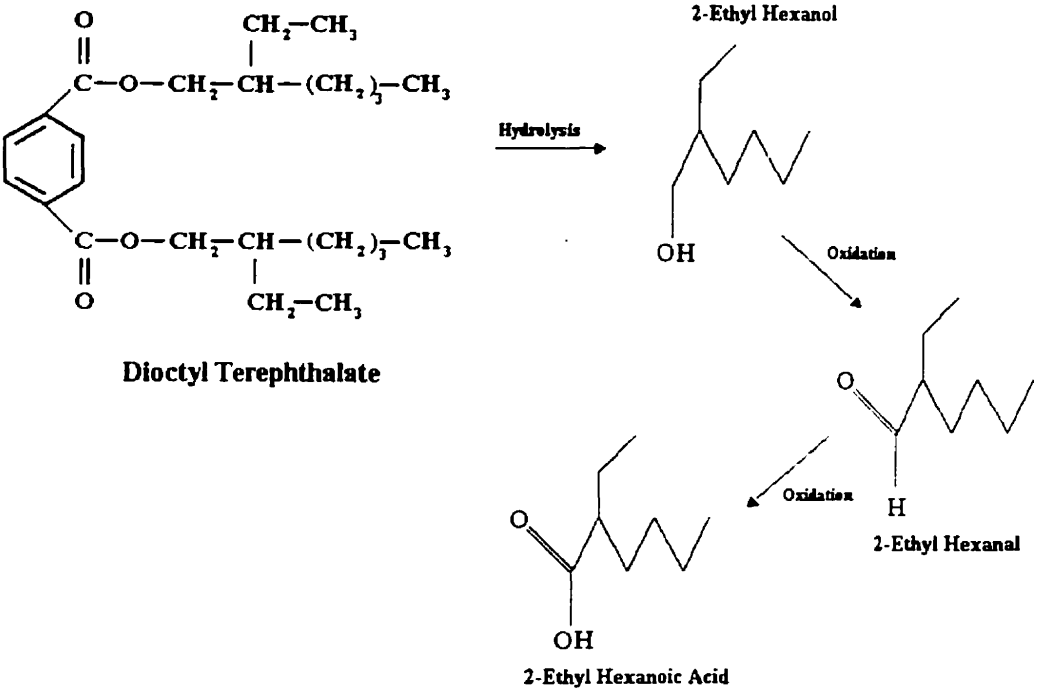

Figure 4-3. Proposed mechanism for the production of 2-ethyl hexanoic acid from the degradation of dioctyl terephthalate.

plasticizer studies. As soon as the aldehyde is formed by oxidation of the alcohol, it is further oxidized to the acid.

In all of the studies, there are significant amounts of 2-ethyl hexanoic acid present after growth has finished. This is consistent with the bacterium, *R. rhodochrous*, being unable to utilize this acid. This is probably due to the position of the ethyl branch on the carbon skeleton. Some organisms have difficulty degrading branched hydrocarbons stopping the degradation at the branch and leaving an end product[60, 61] such as the one observed in this work.

4.4 Toxicity Studies

The biochemical pathways of the Microtox organism affected by the toxins are shared with higher organisms[52, 53]. This test has also been correlated to other tests commonly used[54, 55, 56]. With these facts the toxicity data obtained by the Microtox are considered to be a fair representation of a toxic response that could be seen in the environment.

Normally, biodegradation is considered to be a means for degrading pollutants in the environment while accomplishing a reduction in toxicity. In order to determine if this was actually happening in the case of the plasticizer, the toxicities of the batch fermentations were monitored. Unfortunately, it was not possible to isolate all of the components in the fermentation broth and test each of these toxicity individually. Instead, measurements were made of the toxicity of the cell-free broth samples to see how these would change if and when the plasticizers were degraded. Thus, the toxicity

values reflect the toxicity of the mixture of compounds in the broth, rather than the toxicity of individual components.

The initial toxicities of the non-degraded plasticizer/hexadecane mixtures were not high for any of the growth studies (below a TU50 of 300). This is consistent with the relatively low toxicities of all of the plasticizers as well as hexadecane (below detection). From Figures 3-6 to 3-9 it can be seen that the toxicity of the broth samples increased over the course of the growth experiments. This is presumably due to the accumulation of metabolites in the reactor. These Microtox data are supported by the measurements of biomass. In most of the experiments, there is a noticeable decrease in biomass as the concentration of the acid increases.

The most interesting case is that of the adipate plasticizer, since two metabolites are observed and both were shown to be very toxic. The solution of 1.5% adipate begins as a non-toxic mixture. However, after the degradation of the plasticizer the solution becomes very toxic compared to the phenol standard. The toxicity of the alcohol is much higher than the toxicity of the acid. This is why the toxicity of the solution is high even when a relatively small amount of alcohol has been produced and before any of the acid has appeared. When the concentration of the alcohol decreases and that of the acid increases, the toxicity of the solution stabilizes at a TU50 of 2000.

However, despite the very high levels of toxicity, the values are lower than expected. For example, at the maximum observed concentration of the alcohol (approximately 0.1 g/L), according to the data in Table 3-5 the toxicity of the solution should correspond to a TU50 of 14,400. However, the observed TU50 is approximately 2100. One reason that can be suggested to account for this discrepancy is that there may

be interactions between solution components that are reducing the observed toxicity. In particular, the alcohol is soluble in hydrocarbons and at this point in the fermentation, there is an appreciable amount of hexadecane still present. This could reduce the effect of the alcohol on the test system because a significant amount of the toxic agent could conceivably be "tied up" in the non-aqueous phase. In any case, there is still an appreciable increase in the toxicity of the broth samples and the values are large enough to be of concern.

By the time that the concentration of the acid has reached a maximum, there is no hexadecane or alcohol present. If the extraction argument for the alcohol is correct, the toxicity of these broth samples should correspond to the value calculated from the data in Table 3-5 because all of the acid will be in the aqueous solution. This is what is observed. The maximum concentration of the acid is 0.293g/L, thus the expected TU50 is 2784. This is in reasonable agreement with the observed TU50 value in Figure 5 of 2000.

The alcohol is very toxic to the microbe used in the Microtox test but it does not seem to significantly inhibit the growth of *R. rhodochrous*. This is not too surprising since *R.rhodochrous* was selected for this study because it was a bacterium that was known to be able to grow in the presence of hydrocarbons and their metabolites. In addition, if appreciable amounts of the alcohol were being extracted into the hydrocarbon phase, the effective concentration of this toxic compound might be relatively low. However, the acid is toxic to *R. rhodochrous* as can be seen in the significant decrease in the amount of biomass, in most of the growth studies, as the concentration of 2-ethyl hexanoic acid increases.

The phthalate plasticizers have low toxicities and all of experiments show a significant increase in toxicity while the original compounds are partially degraded. The phthalate systems might be more complex than the degradation of the adipate because appreciable amounts of the original plasticizer are still present as the concentration of 2-ethyl hexanoic acid builds up in the broth samples. It is known that synergic effects can occur in the presence of more than one toxic compound with Microtox[62]. However, even with this qualification, it seems reasonable to conclude that growth of this bacterium in the presence of either of these plasticizers will result in the appearance of appreciable amounts of a toxic metabolite that is not degraded further.

4.5 Potential Impact

Plasticizers can be introduced into the environment through the leaching of plastics and subsequent migration through water transport. It is likely that these liberated plasticizers will come into contact with different organisms that can partially degrade the plasticizers and produce metabolites. Initially, it might appear that the introduction of plasticizers into the environment would not be of serious concern due to their low toxicity and gradual biodegradation. However, in recent work[3,6], it has been shown that the most serious problem associated with these mobile plasticizers may be their long-term effects as endocrine disrupters; i.e., hormone mimickers that can result in the disruption of the normal embryonic development and the reproductive success of organisms. It might be hoped that this serious problem could be mitigated by biodegradation of the plasticizers as they encounter native microorganisms in the soil-water environment. However, the work presented here shows that this hoped-for solution cannot necessarily be relied upon

to resolve the environmental impacts associated with plasticizers. It is quite possible that the original problem associated with endocrine disruption may be resolved by biodegradation only to be replaced by a new problem associated with the acute toxicity of the metabolites.

5. CONCLUSION

5.1 Conclusions

It was found that *Rhodococcus rhodochrous* and *Arthrobacter paraffineus* grew on n-hexadecane as a substrate in the presence of plasticizers.

Rhodococcus rhodochrous was used to observe the growth of a soil bacterium in the presence of different plasticizers (bis 2-ethylhexyl adipate, dioctyl phthalate and terephthalate). The toxicity of the broth was found to increase over the course of the fermentation while the concentrations of the different plasticizers were decreasing. This increase in toxicity was linked to an increase in the concentration of metabolites from the degradation of the original plasticizers.

The metabolites involved in the breakdown of bis 2-ethylhexyl adipate were identified as 2-ethyl hexanol and 2-ethyl hexanoic acid. It was demonstrated that both of these metabolites are the source of acute toxicity. Only the acid was observed for the breakdown of the phthalate plasticizers. All of the observations were consistent with similar mechanisms for the degradation of the plasticizers. The first step was the hydrolysis of the ester bonds followed by oxidation of the released 2-ethyl hexanol to 2-ethyl hexanoic acid.

5.2 Recommendations

R.rhodochrous degraded some plasticizers commonly used in industry and produced metabolites which are toxic. Further studies should be conducted on the degradation of plasticizers in the presence of other microorganisms. Other soil bacteria

should be tested for their ability to degrade the plasticizers and to observe any metabolites that are produced during the degradation. This is necessary in order to establish whether or not the results with *R.rhodochrous* represent a general phenomenon. If other microbes cause the partial breakdown of plasticizer to toxic metabolites, then it will demonstrate that there is significant cause for concern about the fate of these plasticizers in the environment.

It would also be useful to look at the fate of other, less commonly used plasticizers. If the three plasticizers being considered in the current study prove to lead to the production of toxic metabolites, it will be necessary to consider alternatives. As a consequence, these other plasticizers may become industrially more important.

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