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Biodegradation of plasticizers by *Rhodococcus rhodochrous*

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Abstract

Rhodococcus rhodochrous was grown in the presence of one of three plasticizers: bis 2-ethylhexyl adipate (BEHA), dioctyl phthalate (DOP) or dioctyl terephthalate (DOTP). None of the plasticizers were degraded unless another carbon source, such as hexadecane, was also present. When *R. rhodochrous* was grown with hexadecane as a co-substrate, BEHA was completely degraded and the DOP was degraded slightly. About half of the DOTP was degraded, if hexadecane were present. In all of these growth studies, the toxicity of the media, which was assessed using the Microtox assay, increased as the organism degraded the plasticizer. In each case, there was an accumulation of one or two intermediates in the growth medium as the toxicity increased. One of these was identified as 2-ethylhexanoic acid and it was observed for all three plasticizers. Its concentration increased until degradation of the plasticizers had stopped and it was always present at the end of the fermentation. The other intermediate was identified as 2-ethylhexanol and this was only observed for growth in the presence of BEHA. The alcohol was observed early in the growth studies with BEHA and had disappeared by the end of the experiment. Both the 2-ethylhexanol and 2-ethylhexanoic acid were shown to be toxic and their presence explained the increase of toxicity as the fermentations proceeded. The appearance of these intermediates was consistent with similar degradation mechanisms for all three plasticizers involving hydrolysis of the ester bonds followed by oxidation of the released alcohol.

Introduction

In addition to their polymeric components, most plastics contain a variety of low molecular weight additives called plasticizers, which are needed to produce workable final products. The most commonly used plasticizer is dioctyl phthalate, which was introduced in 1933 (Kyrides 1933) and is currently produced in quantities as high as 500 million kilograms per year in North America (Tepper 1973). Another commonly used plasticizer is bis 2-(ethylhexyl) adipate (BEHA), which is added to polymers when flexibility is a major factor in the end use of plastic film (Keith et al. 1992). It is commonly used in polyvinyl chloride (PVC) films when flexibility must be maintained over a wide range of temperatures, such as in the wrapping of refrigerated food products (Graham 1973).

Human populations are subject to significant exposure to these compounds as a direct result of the widespread use of various types of plasticizers in many products such as construction materials, automotive parts, medical supplies, household products, toys and packaging. For example, it has been shown that BEHA and other plasticizers readily leach out of plastic film when in contact with food products (Castle et al. 1988). When this and other routes of exposure are considered together, the average ingestion rate of plasticizers has been estimated to be on the order of 8 mg/person/day (Loftus et al. 1993). This ingestion rate is problematic given the results of recent studies in which it was concluded that the endocrine-disrupting action of phthalate esters could cause the early onset of

puberty in human females (Jobling et al. 1995; Colon et al. 2000). In addition, other interactions of plasticizers, including BEHA and phthalate esters, with non-human organisms have been documented (Albro 1975; Baker 1978; Takahashi et al. 1981; Lhuguenot et al. 1985; Elcombe et al. 1986; Schmezer et al. 1988; Dirven et al. 1993). These include reports of hydrolysis of the ester bonds of the plasticizers in the intestinal tracts of rats and other mammals to release 2-ethylhexanol (Albro et al. 1973; Albro 1975; Rowland et al. 1977; Takahashi et al. 1981; Barber et al. 1994), which has been reported to be further oxidized in the intestine resulting in an accumulation of 2-ethylhexanoic acid (Albro 1975). This compound has been linked to peroxisome proliferation and tumor growth (Lhuguenot et al. 1985). In another study, the accumulation of mono-ethylhexyl phthalate from the partial degradation of DOP also caused the proliferation of peroxisome (Lhuguenot et al. 1985; Albro et al. 1989).

Plasticizers will gradually migrate from plastics into the environment and come into contact with naturally occurring organisms (Choudhry et al. 1994; Faouzi et al. 1994; Staples et al. 1997). Since there are important health implications associated with the phthalate esters, the accumulation of these compounds in the environment could be problematic. The magnitude of this problem should be related to the degree to which microbial populations degrade the phthalate esters. However, surprisingly little work has been reported on the microbial degradation of these plasticizers.

Once a plasticizer enters the natural environment, microorganisms could interact with it in several ways. Firstly, it is possible that a plasticizer is biologically inert and would tend to accumulate in the environment. For example, a study was conducted in which soil organisms were placed in an aqueous phase with different plasticizers and observations revealed that most of the plasticizers resisted degradation (Cartwright et al. 2000). Secondly, under the appropriate conditions microorganisms could use the plasticizer as a carbon source and completely convert it to biomass and carbon dioxide (Keyser et al. 1976; Eaton et al. 1982; Webb et al. 2000). Some organisms that are typically found in soil seemed to have the proper enzymes to completely mineralise dioctyl phthalate without leaving any byproducts (Kurane et al. 1980; Shanker et al. 1985). Activated sludge systems have also been reported to be able to accomplish the complete mineralization of phthalate esters (Saeger et al. 1976; O'Grady et al. 1985). However, plasticizer degradation that occurs in an activated sludge process is not likely to reflect the degradation under the non-optimal conditions in the natural environment. Finally, a third possibility is that there could be incomplete degradation of a plasticizer. For example, the bacterium Micrococcus sp. strain 12B was observed to use aqueous dibutyl phthalate as a sole carbon source by hydrolyzing the functional group and then completely oxidizing the phthalate component. However, the butanol byproduct was not degraded (Eaton et al. 1982).

Six bacteria were selected because they were all soil microorganisms known to grow on hydrocarbons. These were *Rhodococcus rhodochrous* ATCC 21766 (Syldatk et al. 1988), *Arthrobacter paraffineus* ATCC 19558 (Duvnjak et al. 1983), *Pseudomonas flourescens* TEXACO (Sepic et al. 1996), *Rhodococcus* sp. (Chaineau et al. 1999), *Corynebacterium* sp. ATCC 21511 (Dahlstrom et al. 1973) and *Mycobacterium convolutum* ATCC 29671 (Beam et al. 1974). These were grown under aerobic conditions in media containing one of the commonly used plasticizers; namely, DOP DOTP and BEHA. Based on the results of preliminary experiments, *Rhodococcus rhodochrous* was selected for further investigation.

Materials and methods

Growth of bacteria

All of the growth studies were done with mineral salt medium (MSM) containing 4 g/l NH_4NO_3, 4 g/L KH_2PO_4, 6 g/l Na_2HPO_4, 0.2 g/l $MgSO_4{\cdot}7H_2O$, 0.01 g/l $CaCl_2{\cdot}2H_2O$, 0.01 g/l $FeSO_4{\cdot}7H_2O$, 0.014 g/l and Na_2EDTA (A&C Produits Chimiques Americain, St-Laurent, QC). The first set of screening studies contained one of the plasticizers BEHA 99%, DOP 99% or DOTP 98% (Sigma-Aldrich, St. Louis MO) at a concentration of 1 g/l. Two types of experiments were done. Either no other carbon source was present or 0.25% of hexadecane was added. The experiments were conducted in 500 ml Erlenmeyer flasks using 100 ml of MSM. The organisms grew in a rotary incubator shaker (New Brunswick Scientific, Series 25) set at 250 RPM. *Rhodococcus rhodochrous*, *Arthrobacter paraffineus*, *Pseudomonas flourescens* TEXACO and *Rhodococcus* sp. were grown at 30 °C. *Corynebacterium* sp and *Mycobacterium convolutum* were grown at 37 °C.

For growth experiments in the batch reactor with the bacterium, *R. rhodochrous*, the media were supplemented with 0.25% v/v hexadecane (Sigma Aldrich, St. Louis MO) and 0.05 g/l yeast extract (DIFCO, Detroit, Michigan). The 2-l New Brunswick Scientific reactor was maintained at 30 °C using a recirculating water bath and a stainless steel tube in a tube heat exchanger. The air inlet was fitted to an inline air filter (Millipore Millex-FG50, 0.2 μl). The reactor containing the 1.5 l of media was steam sterilized for a period of 2 hours. The reactor was inoculated with 10 ml of broth containing cells in the exponential phase of growth. 30 ml samples were obtained via a sampling port on the reactor and introduced into 50 ml borosilicate vials with Teflon coated caps. Samples were stored in a refrigerator at 4 °C for periods not exceeding 24 hours. All samples were shaken before splitting into smaller samples for subsequent sample preparation and analyses, as described below.

Protein concentration

Protein tests were carried out by placing a 10 ml sample in a 30-ml Teflon centrifuge tube (Fisher Brand), which was centrifuged at 10,000 rpm at room temperature for 10 minutes. The supernatant was decanted and the pellet was washed twice with MSM. The final pellet was resuspended in 5 ml of MSM and the protein concentration of the biomass, related to the final biomass concentration, was measured using the BIORAD DC Protein Assay (BIO-RAD Laboratories Inc., Mississauga, ON) (Oh et al. 1994).

Gas chromatography

A 2-ml sample of broth was placed in a test tube with 3 ml of chloroform containing 0.01% (mass/volume) of pentadecane as an internal standard. This was mixed for two minutes and the organic phase was removed by a transfer pipette and placed in a 5-ml glass vial 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 (HP5890 Series II) had a SPB-5 column (Supelco). The settings of the GC were: injector temperature of 250 °C, initial column temperature of 60 °C, temperature ramp rate of 10 °C/min, final column temperature of 300 °C, detector temperature of 370 °C, ramp hold time of 2.5 min, and final hold time of 0.1 min. The gas chromatograph/mass spectrophotometer (Thermo Quest model TRACE GC 2000/ Finnigan POLARIS) had a RTX-5 MS column (Restek) with an internal diameter of 0.25 mm. The column conditions for the GC/MS were the same as those for the GC. The mass spectrophotometer settings were 50–600 molecular weights mass spec range, the transfer line was maintained at 275 °C and the ion source was maintained at 200 °C. Standard samples of 2-ethylhexanol 99%+, 2-ethylhexanoic acid 99% and 2-ethylhexanal 96% were purchased from Sigma (Sigma Aldrich, St. Louis MO).

Partitioning experiment

Six separatory funnels (Pyrex Brand 60 ml with Teflon plug) were filled with 50 ml MSM solution. Different amounts of 2-ethylhexanol, *n*-hexadecane and bis 2-ethylhexyl adipate were added. The mixtures were vigorously shaken and left to settle for a period of 24 hours. A 2 ml sample was removed from the bottom of the separatory funnel and extracted with 2 ml of the chloroform/internal standard solution previously described. The concentrations of the different organic compounds were obtained from GC analysis.

Toxicity assessment

The Microtox™ acute toxicity assay was selected as a means of measuring relative changes in toxicities of solutions arising from microbial interactions. This test was chosen because it is inexpensive, rapid and has been reported to be more sensitive than other microbiological assays, such as the nitrification assay (Reynolds et al. 1987) or the activated sludge respiration assay (Elnabarawy et al. 1988). A good correlation was also found between the Microtox™ EC_{50} and the corresponding rat and mouse LD_{50} values (Kaiser et al. 1994). Thus, it has been concluded that the Microtox™ test is sensitive and correlates with toxic effects on other species.

Acute Microtox™ toxicity was determined using the 5-minute assay with the Model 500 Toxicity Analyzer according to the procedures for the Basic Test recommended by the instrument manufacturer (Azur Environmental, Carlsbad, CA). The assay involves the recording of the light output of the luminescent marine bacterium *Photobacterium phosphoreum*, after 5 minutes incubation with various dilutions of the sample. The samples were obtained by centrifuging 10 ml of the 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) and stored at −15 °C until a batch of samples was ready for toxicity testing. Four serial 1:2 dilutions of

this sample with diluent (2% NaCl in reagent water) were prepared and allowed to stabilize to 15 ± 0.5 °C. 0.5 ml aliquots of these sample dilutions were added each to 0.51 ml of bacteria suspension (also prepared in diluent and pre-incubated at 15 ± 0.5 °C). The light output of the bacteria was recorded immediately before and 5 minutes after the addition of the sample to the bacteria suspension. A control was run concurrently with each test to account for the normal drop of the light output during the 5 minutes incubation time. The volume fraction of a sample that causes a 50% decrease in light output is reported as the effective concentration, or EC_{50} value. For pure samples of known toxicant concentration, the EC_{50} is converted to concentration units by multiplying the EC_{50} expressed as a volume fraction by the sample concentration. In this study toxicities were expressed in toxicity units (TU_{50}), which is the inverse of EC_{50} when expressed as a volume fraction. TU_{50} was used because it is proportional to toxicant concentration with a high TU_{50} reflecting a high toxicity.

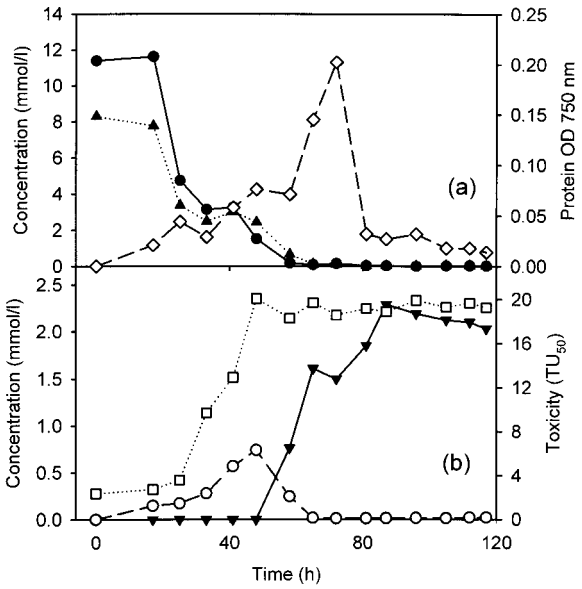

Figure 1. (a) Growth of *R. rhodochrous* represented by the protein concentration (◇) of the pellet after centrifugation and concentrations of bis 2-ethylhexyl adipate (▲) and hexadecane (●). (b) Concentrations of metabolite #1 (2-ethylhexanol) (○) and metabolite #2 (2-ethylhexanoic acid) (▼) and the Microtox toxicity (□) of the broth.

Results

Growth and toxicity data

Preliminary studies with the six bacteria showed that none of them were able to grow in a medium containing one of the three plasticizers as the sole carbon source. However, they were able to grow on other carbon sources, even if one of the plasticizers were present. In particular, *R. rhodochrous* grew very well on hexadecane when any of the plasticizers were added to the medium. Because of this, the rest of the experiments considered the growth of *R. rhodochrous* in the presence of hexadecane and one of the plasticizers.

As has been observed in previous work, the water insoluble substrates interfered with the use of the dry weight measurements (Marino et al. 1998). Therefore, growth was indirectly monitored through measurement of the amount of protein present in the pellet.

Figure 1 contains data for a typical batch growth study of *R. rhodochrous* growing in the presence of BEHA. The general trends shown in these figures were all reproducible. Even though this microbe would not grow when BEHA was the sole carbon source, by the time the hexadecane had been completely removed, there was no plasticizer left in the reactor. It can be seen that the amount of biomass, as determined by protein content of the pellet, reached a maximum after the hexadecane and BEHA had both disappeared and then dropped dramatically. Two new peaks appeared in the gas chromatographs of the samples from the broth, which were later shown to be metabolites. Metabolite #1 was observed early in the experiment but disappeared shortly before the hexadecane and plasticizer could no longer be detected. Metabolite #2 appeared later in the fermentation and continued to increase in concentration until growth had finished and then remained until the end of the experiment.

The toxicities of both hexadecane and BEHA were below detection (Table 1) and the fermentation broth initially exhibited no toxicity (Figure 1(b)). However, as growth commenced, there was an increase in toxicity that persisted to the end of the experiment, as did the concentration of metabolite #2.

R. rhodochrous was also grown on hexadecane in the presence of either DOP or DOTP. Figure 2 contains data for the degradation of DOTP and Figure 3 contains typical data for systems with DOP. All of the trends for each of these phthalate plasticizers were similar to those observed with BEHA. All three plasticizers can be degraded, if another carbon source is present. However, the microbe was not as efficient at removing the DOP and DOTP from the medium. That

Table 1. Toxicity and GC data for pure compounds and metabolites.[a]

Compounds	GC retention time (minutes)	Sample concentration (mmol/l)	Toxicity, as TU_{50} (dimensionless)	Toxicity, as EC_{50} (mmol/l)
Metabolite #1	1.9	–	–	–
Metabolite #2	2.2	–	–	–
2-ethylhexanal	1.5	1	17	0.06
2-ethylhexanol	1.9	1	17	0.06
2-ethylhexanoic acid	2.3	1	3.3	0.3
Phenol	–	1	5	0.2
Hexadecane	9.1	2.5	n/d	n/d
Dioctyl phthalate	16.6	2.5	n/d	n/d
Dioctyl terephthalate	17.6	2.5	n/d	n/d
Bis 2-ethylhexyl adipate	19.2	2.5	n/d	n/d

[a] n/d = toxicity not detected.

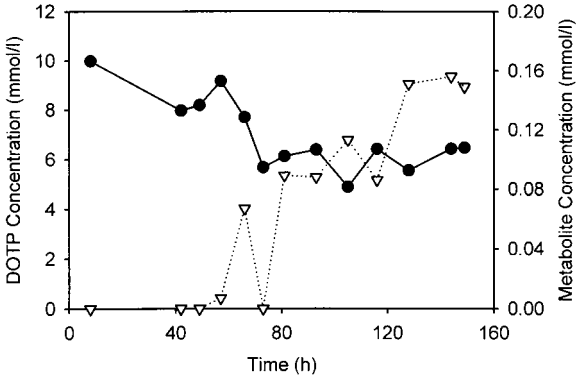

Figure 2. The concentration of metabolite #2 (2-ethylhexanoic acid) (▽) observed during growth of *R. rhodochrous* grown in a medium containing dioctyl terephthalate (●) and hexadecane.

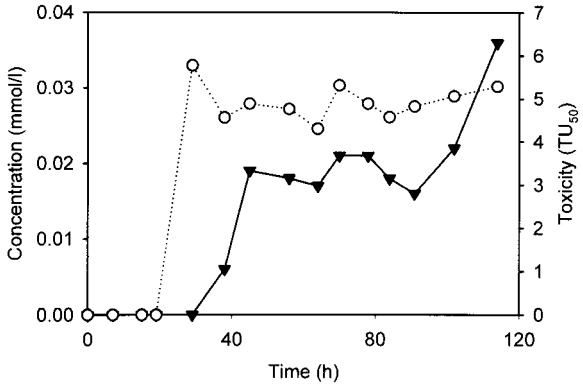

Figure 3. The Microtox toxicity (○) of the broth as the metabolite #2 (2-ethylhexanoic acid) (▼) increases in concentration when *R. rhodochrous* was grown in the presence of dioctyl phthalate and hexadecane.

is, BEHA was no longer detectable at the end of the fermentation (see Figure 1) but both DOP and DOTP were still present in significant quantities at the end of these fermentations. Metabolite #1 was not observed for any of the growth studies done in the presence of either DOP or DOTP. Both systems did generate detectable amounts of metabolite #2 but not as much as observed when BEHA was present.

Table 1 contains toxicity data for a number of pure compounds. Phenol, a common standard of toxicity is included for comparison and was used as a quality control check on toxicity measurements. The EC_{50} value determined in this study for phenol is consistent with toxicities previously reported (Wagner et al. 2002). None of the plasticizers or the hexadecane elicited toxic responses in the Microtox assay. Consistent with this, the media showed no appreciable initial toxicity.

Table 2 contains data arising from fermentation experiments with the three plasticizers. These data were averages of duplicates, excluding DOP, for all of the experiments of each type and include data from the fermentations presented in Figures 1 to 3. In every case there was a noticeable drop in biomass concentration after it reached a maximum late in the growth study. By this time, the medium had reached a stable toxicity level, which remained constant for as long as measurements were continued.

Characterization of metabolites

Assuming the unknown peaks are metabolites, possible candidates include 2-ethylhexanol and one of

Table 2. Summary of results from the degradation of plasticizers by *R. rhodochrous*.[a]

Plasticizer	Degradation (%)	Maximum concentration of metabolites (mmol/l)		Time of initial drop in biomass concentration (hours)	Final toxicity of the culture, as TU_{50} (dimensionless)
		2-ethylhexanol	2-ethylhexanoic acid		
Bis 2-ethylhexyl adipate	100 ± 0.1	1.9	2.9 ± 0.15	80	19 ± 0.37
Dioctyl terephthalate	73 ± 7	0	0.28 ± 0.033	90	5.4 ± 0.87
Dioctyl phthalate	48 ± 4	0	0.036 ± 0.009	70	5.3 ± 0.28

[a] Standard deviations (±) were calculated from series of data points with constant concentrations. This was not possible with 2-ethylhexanol where the maximum concentration was observed as a peak.

its oxidation products. A comparison of the GC retention times (Table 1) and fragmentation patterns in GC-MS between each of the metabolites and the same data for known compounds confirmed that metabolite #1 was 2-ethylhexanol and that metabolite #2 was 2-ethylhexanoic acid. The toxicities of these two compounds are included in Table 1. Also included in Table 1 are data for the unobserved, but possible intermediate, 2-ethylhexanal. Figure 4 includes two mass spectra, which are virtually identical. One is for 2-ethylhexanoic acid and the other is for metabolite #2. There is a very small parent molecular ion at atomic mass unit (amu) 145. The loss of either of the two alkyl branches results in amu 88 (loss of butyl group) or amu 116 (loss of ethyl group). The amu of 101 would represent the loss of CO_2.

The concentration of the hexadecane was about 1 g/l when the 2-ethylhexanol was observed in the batch experiments. The partitioning experiments showed significant extraction of the 2-ethylhexanol into hexadecane. The ratio of the molar concentration (hexadecane : water) was greater than 700. Based on the relative quantities of hexadecane and water in the batch experiment, the concentration of the alcohol dissolved in the water is predicted to be about half of the total amount measured in the chloroform extract.

Discussion

Growth of bacteria in the presence of plasticizers

Initial experiments using one of the plasticizers as the sole carbon source were unsuccessful but *R. rhodochrous* grew very well when hexadecane was included in the medium. In each of these experiments, the hexadecane was completely degraded within the first sixty hours, which corresponded to the exponential growth phase of the organism. Each of the three plasticizers was at least partially degraded during this period of exponential growth. The most easily degraded plasticizer was BEHA, which completely disappeared over the course of the first sixty hours (Figure 1(a)). However, as the concentration of BEHA decreased two new compounds were observed on the GC traces of the samples (Figure 1(b)).

Although neither DOTP nor DOP were as easily degraded as BEHA, a significant amount of DOTP disappeared in the first 60 hours of the growth studies. By the end of each experiment about a quarter of the original DOTP remained (Table 2). DOP was the most intractable of the three plasticizers with about half of the initial amount still present at the end of the experiments. The degradation of both of these plasticizers produced only one metabolite, 2-ethylhexanoic acid. There was no evidence of the presence of 2-ethylhexanol in any of the samples taken during experiments with either DOP or DOTP.

Metabolites from plasticizer degradation

Metabolites #1 and #2 were identified as 2-ethylhexanol and 2-ethylhexanoic acid, respectively. Note that 2-ethylhexanol is often referred to as octyl alcohol, which is the basis for the naming of the plasticizers DOP and DOTP. Based on the identities of the two metabolites, it is possible to propose logical mechanisms for the appearance of the metabolites during the growth studies on all three plasticizers which all contain two esters of 2-ethylhexanol and a dicarboxylic acid.

Figure 5 illustrates a proposed mechanism for the degradation of BEHA by *R. rhodochrous*. The first step is the hydrolysis of the ester bonds in BEHA to release 2-ethylhexanol. This alcohol appears early in the fermentation and then disappears as it is oxidized to 2-ethylhexanoic acid. The bacterium is not able to degrade this compound and the concentration

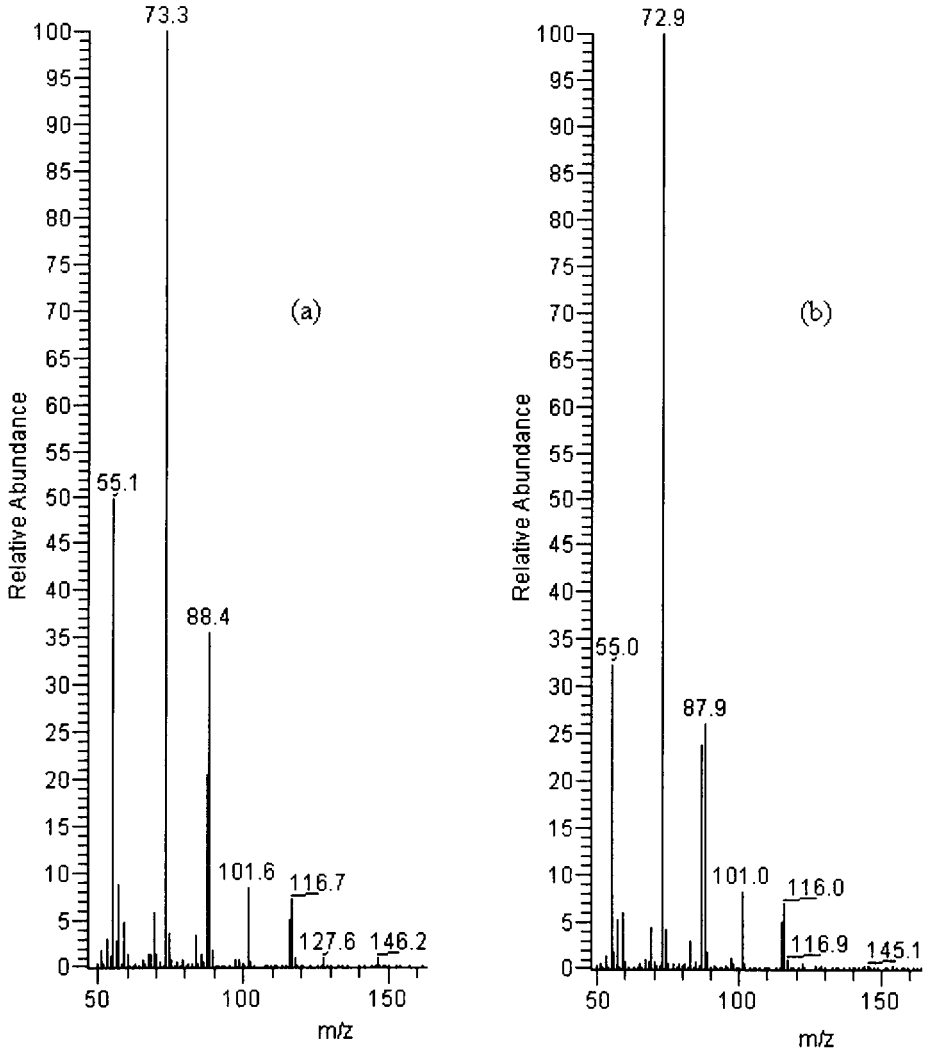

Figure 4. The mass spectra of (a) 2-ethylhexanoic acid standard and (b) a sample obtained from the degradation of BEHA by *R. rhodochrous*.

of acid remains stable until the end of the fermentation. It is reasonable to assume that 2-ethylhexanal is an intermediate in this process but it was not observed.

Similar mechanisms involving a sequence of hydrolysis and oxidation steps would explain the behavior of the DOTP and the DOP systems. However, the concentration of the alcohol never reached a sufficient level to be observed in the GC samples when these plasticizers were being degraded. This could be explained by considering relative reaction rates. Other studies have found that the degradation of the ester bond is the rate-limiting step for the degradation of both DOTP and DOP (Jackson et al. 1996). Thus, as the alcohol appears, it is rapidly oxidized to the acid. In contrast, BEHA is hydrolyzed more easily and there is an initial accumulation of the first intermediate before it can be converted to the acid. Observations of appreciable amounts of a metabolic intermediate, such as the alcohol during the degradation of BEHA are unusual (Shanker et al. 1985; Samsonowa et al. 1996; Cartwright et al. 2000).

R. rhodochrous is able to hydrolyze the ester bonds and oxidize the resulting alcohol but it is not able to metabolize the resulting 2-ethylhexanoic acid. The most likely explanation is that the ethyl branch in the 2 position blocks the alkane metabolism of carboxylic acids in *R. rhodochrous*. Other microorganisms have been shown to have difficulty degrading branched hydrocarbons (Fall et al. 1979; Schaeffer et al. 1979).

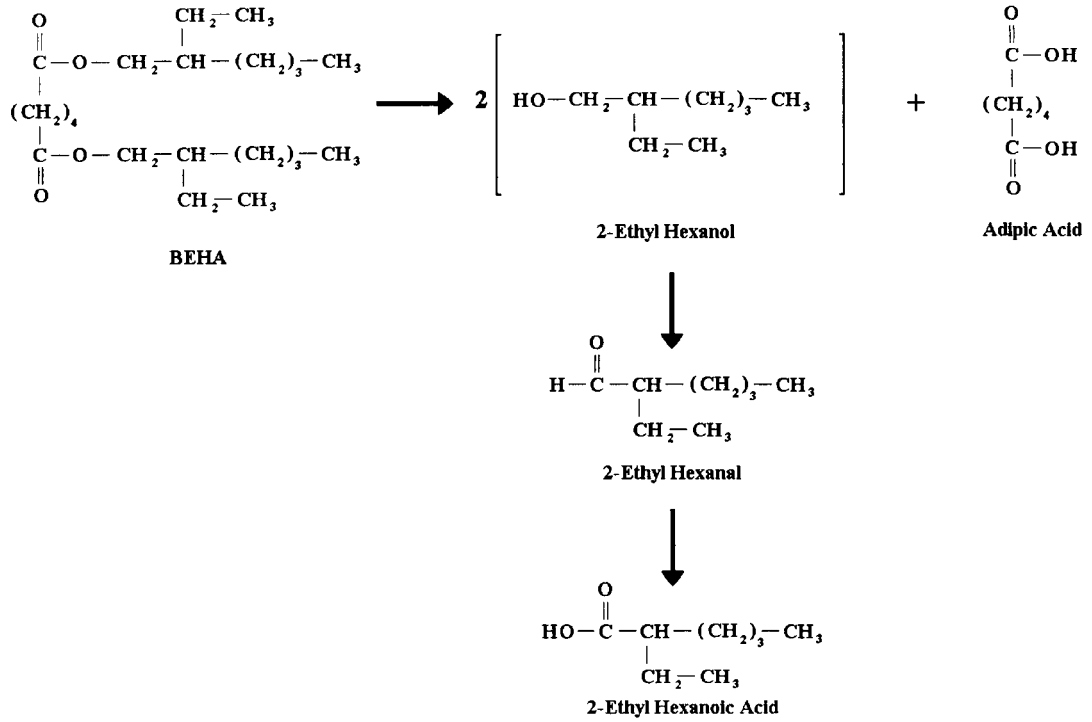

Figure 5. Proposed mechanism for the production of the 2-ethylhexanol and 2-ethylhexanoic acid from BEHA.

The relative ease of hydrolysis of the BEHA explains the accumulation of the first intermediate, 2-ethylhexanol, but it also explains why BEHA is the only one of the three plasticizers to be completely hydrolyzed. Another comparison can be made between the amount of degradation of DOP and DOTP. DOP has an ortho-arrangement of the ester bonds and in DOTP these bonds are in the para-position. It has been proposed that the closeness of the functional groups in DOP could inhibit the enzyme activity involved in hydrolysis of phthalate esters due to steric hindrance (Albro et al. 1974).

Toxicity studies

The initial toxicities of the plasticizer/hexadecane media were below detection for all of the growth studies. This is consistent with the relatively low toxicities of all of the individual plasticizers as well as hexadecane (Table 1). However, all of the experiments demonstrated that the toxicity of the broth samples increased over the course of the growth experiments. This increase is attributed to the production of toxic metabolites in the reactor. A good example of this correlation is seen in Figure 3 in which the pattern of the increase in the toxicity of the broth resembles that of the accumulation of the metabolite 2-ethylhexanoic acid.

The most interesting systems were those that initially contained BEHA, because two metabolites were observed and as their concentration increased the solutions became increasingly toxic. In fact, the toxicity of the medium is high even when only a relatively small amount of alcohol has been produced and before any of the acid has appeared. This is consistent with the higher toxicity of the alcohol relative to the acid (Table 1). Later in the fermentation, the concentration of the alcohol decreases and that of the acid becomes significantly greater. The overall result is that by the end of the fermentation the toxicity of the medium had stabilized at a TU_{50} of 20.

The observed toxicity of the solution is not likely to be simply equivalent to the summation of the toxicities of the individual components shown in Table 1. Complications can arise because of the presence of multiple compounds. For example, there could be partitioning of toxic components among organic and aqueous phases and the biomass. This would explain the seeming inconsistency between the toxicity results of the alcohol in the fermentation broth. That is, at the maximum observed concentration of the alcohol

(approximately 1.9 mmol/l), the solution should have a TU_{50} of 32, according to the data in Table 1. However, the observed TU_{50} is approximately 21. A reasonable explanation for this discrepancy is that at this point in the fermentation about 1 g/l of hexadecane is still present into which some of the 2-ethylhexanol would partition, as was confirmed in the partitioning experiment. To prepare samples for the Microtox test, it was necessary to remove the biomass and this unavoidably removed significant amounts of hexadecane. This would significantly decrease the effective amount of the alcohol to which the Microtox organism would be exposed. On the other hand, when measuring the concentration of the alcohol in the fermentation, samples of the whole broth were extracted with chloroform and this would have included all the alcohol that was present. The overall effect is that the alcohol in the medium samples appears to elicit a toxic response that is lower than that which would be predicted according to the total alcohol concentration.

The results of the Microtox test are supported by observations of the biomass concentrations. The presence of the plasticizers does not prevent growth of the microbe. However, for all of the growth studies, there was a marked decrease in the amount of biomass shortly after both growth and degradation of the plasticizer had been completed. This decrease in biomass could be attributed to the accumulation of the toxic metabolites. A similar pattern of decrease in biomass has been reported for experiments involving organisms grown in soil in the presence of DOP (Cassidy et al. 1999). These authors did not look for metabolites, but it is reasonable to conclude that their system experienced a similar phenomenon to that reported here.

It should be noted that large concentrations of carboxylic acids would not be observed for an organism such as *R. rhodochrous* growing on a normal alkanes or alcohols. It is the ethyl branch at the 2-position of a relatively short acid that leads to a problem for the microorganism.

Conclusions

Plasticizers can be introduced into the environment through the leaching of plastics and subsequent migration by means of water transport. 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, recent work has 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. Generally, it is hoped that these and other potentially serious problems associated with contaminants could be mitigated by biodegradation when the compounds are exposed to the action of native microorganisms in the soil-water environment. Our preliminary work, as well as observations of earlier researchers, showed that the plasticizers could be difficult for microorganisms to degrade. More seriously, we have shown that the growth of bacteria on a more easily metabolized substrate can result in the partial degradation of the plasticizers. In the example presented here, one of the metabolites, 2-ethylhexanoic acid, was intractable to further degradation and very toxic. The net effect may be to trade a long-term problem such as endocrine disruption for acute toxicity.

Acknowledgements

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