Microbial Degradation of Di-ester Plasticizers in the Presence of Surfactants

by

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ABSTRACT

The degradation of common di-ester plasticizers, including di-2-ethylhexyl phthalate, di-2-ethylhexyl adipate and di-(propylene glycol) dibenzoate, by several strains of soil bacteria has been previously shown to lead to an accumulation of metabolites that are more toxic than the parent compounds. This research has shown that the pattern of degradation of these plasticizers can be significantly different in the presence of biosurfactants or synthetic surfactants. In particular, the additions of surfactin, sophorolipid or Pluronic L122 to cultures of *Bacillus subtilis* each resulted in increases in the quantities of the first metabolites in the plasticizer degradation pathway. One of these was 2-ethylhexanol, which had been previously shown to be the most toxic intermediate released during plasticizer degradation. The other was mono-2-ethylhexyl adipate, the mono-ester released from the hydrolysis of di-2-ethylhexyl adipate. This compound was isolated in this work and found to have a toxicity comparable to that of 2-ethylhexanol.

The cause of the significant accumulation of the first and most toxic plasticizer metabolites in the presence of surfactants was investigated. Results were obtained that suggested that the action of the surfactants was to sequester these initial metabolites. The effect of this would be to reduce the observed rates of subsequent degradation of these two intermediates to less toxic compounds.

RÉSUMÉ

Il a été précédemment démontré que la dégradation de certains plastifiants, tel le di-2-éthylhexyl de phthalate, le di-2-éthylhexyl d'adipate et le dibenzoate de di-(glycol de propylène), par certaines bactéries provenant des sols, peut mener à une accumulation de métabolites plus toxiques que les plastifiants initiaux. L'ouvrage présent a démontré que la présence de bio-surfactants ou de surfactants synthétiques peut affecter cette dégradation de manière significative. En particulier, des ajouts de surfactin, de sophorolipide, ou de Pluronic L122 à des cultures de *Bacillus subtilis* ont mené à des quantités élevées de métabolites provenant de la dégradation primaire de plastifiants. Le 2-éthylhexanol, métabolite présentant le niveau de toxicité le plus élevé lors de la métabolisation des plastifiants étudiés, a été identifié comme étant l'un de ces composés. Le mono-2-éthylhexyl d'adipate, un mono-ester obtenu par l'hydrolyse du di-2-éthylhexyl d'adipate, présentait aussi une concentration élevée. Ce dernier métabolite a été isolé et il a été démontré que sa toxicité était comparable a celle du 2-éthylhexanol.

L'augmentation des quantités de métabolites primaires, qui sont aussi les plus toxiques, a été étudiée plus en détail. Les résultats obtenus ont suggéré que les surfactants ajoutés rendaient ces métabolites moins accessibles aux microorganismes. La conséquence de cet effet serait une réduction des taux de dégradation du 2-éthylhexanol et du mono-2-éthylhexyl d'adipate.

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1. INTRODUCTION

1.1 Di-ester Plasticizers

Polymeric materials typically contain various additives that are incorporated in order to obtain desired structural properties. Plasticizers are a class of compounds added to plastic formulations in order to increase their workability, flexibility, softness and distensibility [1, 2].

Many compounds can be used as plasticizers, but the most common are di-ester plasticizers, particularly the phthalic acid ester di-2-ethylhexyl phthalate (D(EH)P) [1, 3, 4]. The principle use of D(EH)P is to plasticize PVC [3, 5], which is one of the most widely used plastics, making this plasticizer of great industrial importance. Adipic acid esters such as di-2-ethylhexyl adipate (D(EH)A) are also employed, often in packaging used for food [6, 7], but also as co-plasticizers in various plastic formulations [8]. Dibenzoate esters such as di-(propylene glycol) dibenzoate (D(PG)DB) are being considered as potential replacement plasticizers in PVC floor tiles [9] and toys [10]. All of these compounds are lipophilic with high octanol-water partitioning coefficients (K_{ow}), and they have low volatilities [5]. The chemical structures of D(EH)P, D(EH)A, D(PG)DB are shown in Figures 1-1, 1-2 and 1-3, respectively.

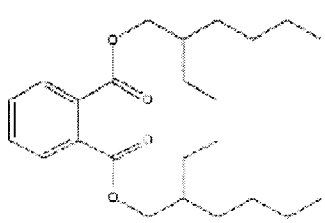

Figure 1-1 - The structure of di-2-ethylhexyl phthalate (D(EH)P).

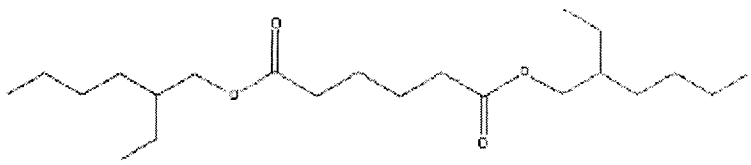

Figure 1-2 - The structure of di-2-ethylhexyl adipate (D(EH)A).

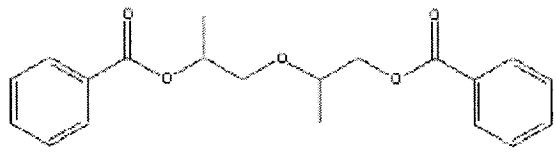

Figure 1-3 - The structure of di-(propylene glycol) dibenzoate (D(PG)DB).

1.2 Plasticizers in the Environment

Plasticizers are not covalently bound to the plastics to which they are added and are therefore susceptible to leaching [11-13]. Because plastic formulations, such as those used with PVC, can contain up to 40% plasticizer by weight [14], even a very gradual loss of plasticizer can become a very significant source of environmental contamination. A large portion of environmental release is thought to occur due to leaching and volatilization in and around landfills and dump sites as well as around plastics manufacturing facilities [3, 15]. Another major source is the continuous release of plasticizers from consumer products such as toys and food wrapping, and construction materials such as floor tiles and cable insulation, throughout their use prior to disposal [8, 16]. An annual rate of environmental release of phthalic acid di-ester plasticizers was estimated at 2.3×10^7 kg worldwide in 1975 [15]. In 1990, the estimate of annual environmental release of plasticizers, 93% of which were estimated to be phthalate di-esters, was 7.7×10^7 kg in Western Europe alone [3].

A study in Germany in 2002 found the average D(EH)P levels in some surface waters to be 2.27 μg/l and in some sediments to be 0.70 mg/kg [17]. Several phthalate di-esters and mono-esters were also detected in significant quantities in samples taken from landfill leachates throughout Western Europe [18]. In a study done in the United States, the median concentrations of D(EH)A and D(EH)P in rivers were estimated at 3 μg/l and 7 μg/l respectively [19]. These were the highest median concentrations of any pollutant found in that study.

Because of their hydrophobicity, it is not surprising that the highest concentrations of plasticizers have been detected in sediments. A contributing factor to their significant concentrations in aqueous environments may be the presence of surface-active agents. For example, rhamnolipids produced by the common bacterium *Pseudomonas aeruginosa* have been shown to solubilize and remove poorly soluble compounds such as phenanthrene and hexadecane from soil matrices in packed columns [20, 21].

Because such large amounts of plasticizers are released from a wide variety of sources and they are able to migrate once they are released, plasticizers have become ubiquitous contaminants in both soil and aqueous environments [5, 17, 19].

1.3 Health Risks of Di-ester Plasticizers

Because so many plasticized products are encountered in every day life, concern has been raised regarding the possible health effects of plasticizers. Examples of exposure to humans include ingestion of foods that had been PVC-wrapped [7], and blood transfusions due to significant leaching of D(EH)P from blood bags and tubing

[22]. The airborne presence 2-ethylhexanol, a fragment of D(EH)P, has been linked to an increased frequency of asthma symptoms in hospitals. It was suggested that this compound originated from plasticized PVC floor tiles [23].

It has also been shown that metabolites of di-ester plasticizers with a 2-ethylhexyl moiety, such as D(EH)P or D(EH)A, can act as peroxisome proliferators in rats and mice [24], which can lead to hepatic cancer in these animals. Other work has attributed endocrine disruption in rats to the D(EH)P metabolites mono-2-ethylhexyl phthalate and 2-ethylhexanoic acid [25]. A study performed with monkeys suggested that this was not the case for primates, however, as they appeared to show no ill effects when administered phthalate di-esters [26].

1.4 Biodegradation of Plasticizers

Because of the extent of environmental contamination by plasticizers, their fate upon release has been the subject of numerous studies. Non-biological mechanisms of degradation have been shown, such as photodecomposition [27] and alkaline hydrolysis [28], but the former required fairly intense radiation and the latter was a slow process with a predicted half-life of over 100 years. It appears that the likeliest mode of degradation to occur is also the most commonly studied – biodegradation by microorganisms.

The early work concerning plasticizer degradation focused on the removal of phthalate di-esters by activated sludge consortia [29-31]. Although these studies reported complete degradation of the plasticizers, it is unlikely that the efficiency of activated sludge systems would be reproduced in most environmental sites. It was also observed

that the more common phthalate esters with longer and/or branched alkyl side-chains, such as D(EH)P, were more difficult to degrade than those with shorter alkyl side-chains, like di-butyl phthalate [29, 30]. This was confirmed in more recent work under aerobic conditions using isolated soil consortia [32], and under anaerobic conditions using sewage sludge [4, 33].

Other researchers investigated the aerobic degradation of phthalate esters by individual strains of soil microbes and found that D(EH)P disappeared completely [34, 35]. These studies, however, were primarily concerned with the disappearance of the parent compound and did not consider the possibility of partial degradation and the possible accumulation of metabolites.

In a recent study, Nalli *et al* followed the degradation of several di-ester plasticizers by *Rhodococcus rhodochrous* [35]. It was observed that the bacterium could perform the initial hydrolysis of D(EH)A and D(EH)P, but could not degrade the metabolite, 2-ethylhexanoic acid, which accumulated in the medium. The authors proposed a pathway for the degradation of phthalate and adipate di-ester plasticizers, which is illustrated in Figure 1-4 for D(EH)A. The first step is the hydrolysis of one of the ester bonds, producing one molecule of 2-ethylhexanol and one of mono-2-ethylhexyl adipate. The second ester bond, in the monoester, is then hydrolyzed leaving adipic acid and 2-ethylhexanol. The 2-ethylhexanol can be further oxidized to 2-ethylhexanal and to the recalcitrant 2-ethylhexanoic acid, while the adipic acid can be consumed as a carbon source.

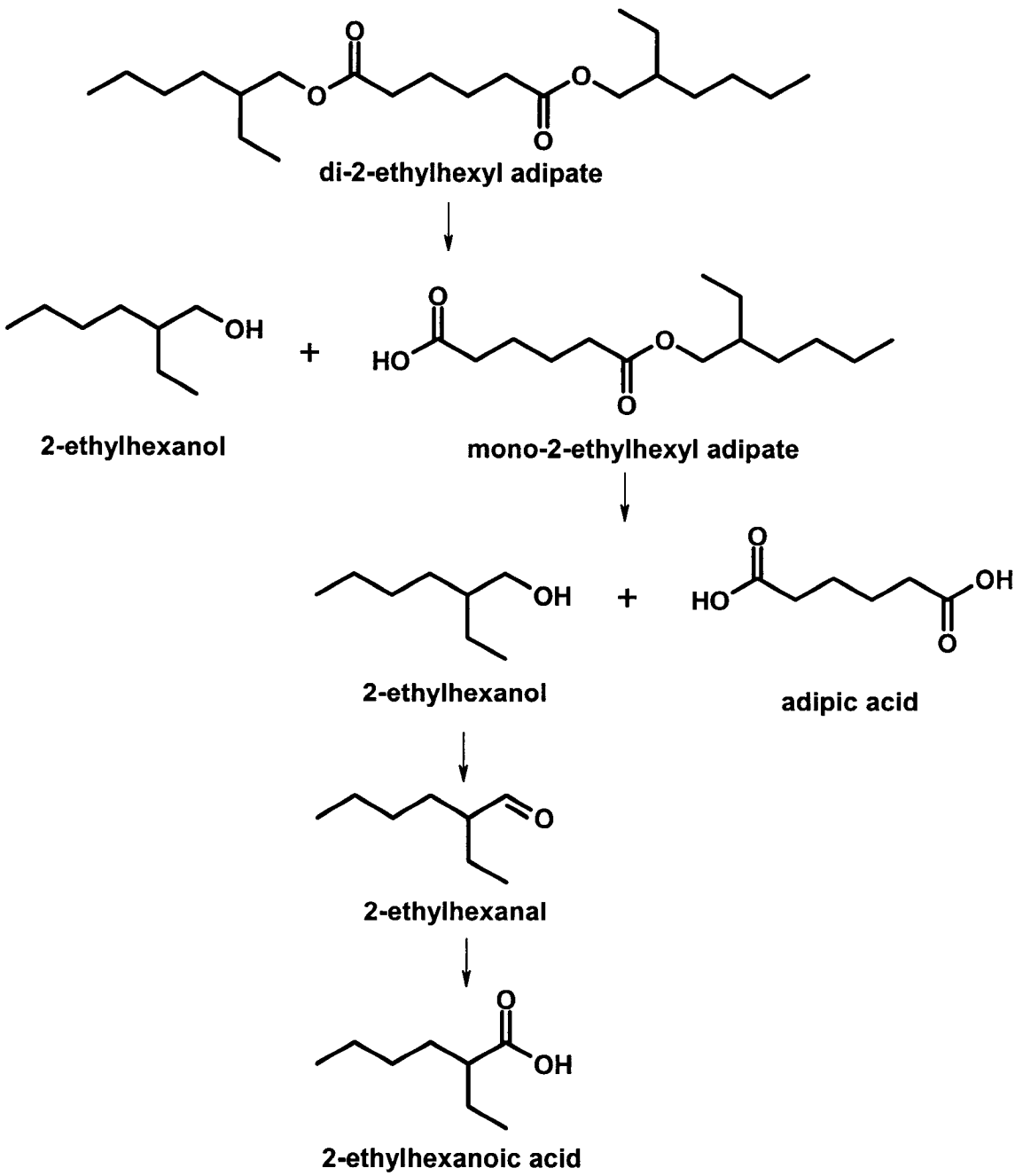

Figure 1-4 - D(EH)A degradation pathway proposed by Nalli *et al* [35].

A similar analysis was reported by Gartshore *et al* for the interaction of di-(propylene glycol) dibenzoate (D(PG)DB) with the yeast *Rhodotorula rubra* [36]. As this yeast grew on glucose in a mineral salt medium, D(PG)DB was partially hydrolyzed, resulting in the accumulation of di-(propylene glycol) monobenzoate in the broth (Figure 1-5).

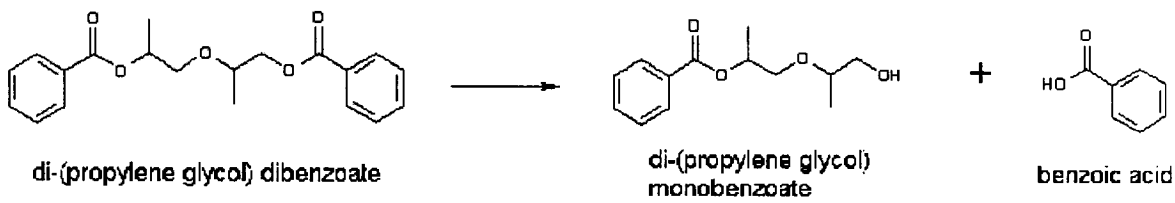

Figure 1-5 - Partial hydrolysis of D(PG)DB by R. rubra observed by Gartshore *et al* resulting in the accumulation of a mono-ester [36].

These studies were the first to report the accumulation of metabolites during di-ester plasticizer degradation. The significance of these findings lies in the fact that, in both cases, the metabolites that accumulated increased the toxicities of the growth media [35, 36]. The acute toxicities of the final metabolites, as well as those of most of the intermediates, were determined to be significantly greater than the toxicities of the original plasticizers [35, 36].

1.5 Biodegradation of Hydrocarbons in the Presence of Surfactants

The effects of surface-active agents on the degradation of hydrophobic substrates by microorganisms have been extensively studied but there has not been a clear consensus on the significance of the results. This is not surprising because, firstly, different species of microorganisms behave very differently in terms of their substrate

preference, enzymatic production and activity and physical characteristics of their membranes. Secondly, surfactants possess widely different structures and properties that can cause them to assemble into a variety of different complex aggregates in addition to micelles in the presence of certain compounds [37-39]. Finally, there are a wide range of different types of hydrophobic compounds. The three-way interaction between specific surfactants, substrates and microbes will determine the effects of the presence of those surfactants on the degradation of particular hydrophobic compounds.

Some work has been done previously describing the effects of surfactants on the degradation of di-ester plasticizers. Gibbons *et al* showed that the addition of a biosurfactant produced by a strain of *Mycobacterium* sp. enhanced the removal of D(EH)P from aqueous media by that bacterium as well as by a strain of *Nocardia* sp. [40].

Much more work on the effects of surfactants on the rate of biodegradation has been done with other types of hydrophobic compounds. In light of their environmental and health risks, poly-aromatic hydrocarbons have been the most commonly studied lipophilic compounds in that respect. The synthetic surfactant, Tween 80, was shown to improve the degradation of phenanthrene by *Sphingomonas paucimobilis* in aqueous media [41]. An increase in the minerlization rate of fluoranthene by the same organism was also reported in media supplemented with the non-ionic surfactant Triton X-100 [42]. In contrast, the addition of Triton X-100 to liquid cultures of *Pseudomonas saccharophila* resulted in no effect on the degradation of phenanthrene [43]. In another study, performed both in aqueous media and in soil suspensions, it was reported that Tween 80 improved the elimination of four- to six-ring poly-aromatic hydrocarbons by

Phanerochaete chrysosporium, but had no effect on the degradation of poly-aromatic hydrocarbons with fewer aromatic rings [44].

In previous work performed with the addition of the glycolipid sophorolipid, produced by *Candida bombicola*, greater removal of phenanthrene by *Sphingomonas yanoikuyae* was observed in both liquid cultures and soil suspensions with increasing biosurfactant concentration [45]. Sophorolipids from *Torulopsis bombicola* were also shown to stimulate the growth of that organism in the presence of alkanes [46]. The degradation rate of a hydrocarbon mixture that contained mostly alkanes was doubled by the addition of sophorolipids to a 10% soil suspension containing indigenous organisms [47]. The addition of rhamnolipids from *Pseudomonas aeruginosa* improved the degradation of hexadecane of *P. aeruginosa* in aqueous cultures and in soil columns [48]. In another study, however, the addition of either biosurfactants from *P. aeruginosa* or sodium dodecyl sulfate (SDS) resulted in decreased mineralization of poly-aromatic hydrocarbons by indigenous organisms in creosote-contaminated soil [49].

In all of the work cited above that reported improved hydrocarbon degradation in the presence of surfactants, the authors concluded that the effect was due to the solubilization of the hydrophobic substrate resulting in greater bioavailability to the organism. None of the surfactants shown to improve degradation were reported to be toxic to the microorganisms.

The solubilization of a hydrophobic compound by surfactants, however, does not necessarily increase its bioavailability. Guha *et al* studied the degradation of phenanthrene by a mixed culture in the presence of several surfactants including Triton X-100 [50]. The results showed that the degradation rate was unaffected when the

surfactant was added at a concentration near its critical micelle concentration, but decreased appreciably at higher surfactant concentrations. Based on these data, the authors developed a model that accounted for the decreased accessibility of a compound sequestered inside a surfactant micelle. Similar results were obtained in other work with different surfactants [51].

Surfactants may have other effects that impact hydrocarbon degradation by microorganisms. Zhang *et al* found that the addition of rhamnolipids to octadecane-degrading *Pseudomonas aeruginosa* cultures had inhibitory effects for some strains, but increased the degradation rate for others [52]. It was demonstrated that the cells of the strains that showed improved degradation had become more hydrophobic as a result of the rhamnolipid addition. Another study later attributed this effect of the rhamnolipid to the removal of lipopolysaccharides from the cell membrane [53]. It was suggested that the effect of a surfactant on cell hydrophobicity was as significant as the dispersion of the hydrophobic substrate when considering the bioavailability of that substrate to the organism [52]. In a study not focusing on hydrocarbon degradation, surfactin, a lipopeptide biosurfactant produced by *Bacillus subtilis*, was also found to increase the hydrophobicity of certain strains of that species [54].

Biosurfactants and synthetic surfactants have been shown to alter the rates of degradation of many poorly soluble compounds. The majority of this work was performed with poly-aromatic hydrocarbons or alkanes, though none of these xenobiotic compounds are as prevalent in the environment as di-ester plasticizers. Plasticizers are oily compounds that are likely to interact with surface-active agents already present in the

environment, and this should be considered when assessing the potential for biodegradation.

1.6 *Bacillus subtilis* - a Potential Plasticizer-Degrading Bacterium

Based on the work cited above, many microorganisms may have the potential for degrading di-ester plasticizers. An organism that is of particular interest in this respect is the well-studied *Bacillus subtilis*. This bacterium is commonly found in nature and it is known to produce surfactin [55], a very powerful biosurfactant [56]. Its potential for di-ester plasticizer degradation is supported by results from a previous screening showing that two out of three strains of *B. subtilis* were capable of hydrolyzing 2-ethylhexyl butyrate [57]. Furthermore, esterases capable of hydrolyzing di-methyl phthalate were recently isolated from a strain of *Bacillus* sp. [58].

1.7 Research Objectives

Surfactants were shown to solubilize hydrophobic compounds that adhered to solid phases [20, 21, 48]. Similarly, surfactants in the environment may solubilize plasticizers that partition in organic-rich sediments, increasing their contact with microorganisms. The resultant interaction between surfactant-solublized plasticizers and microorganisms is of particular interest in light of recent work showing that the metabolites that accumulated as a result of partial microbial degradation are much more toxic than the plasticizers themselves [35, 36]. The aim of this research, therefore, was to observe the effects of surfactants on the degradation of di-ester plasticizers by common microorganisms. More specifically, the objectives were:

1) to investigate the effects of the presence of several biosurfactants and synthetic surfactants on the degradation of the most common di-ester plasticizers by a common microorganism, *Bacillus subtilis*;
2) to monitor the degradation of these plasticizers over time with and without the addition of surfactants to observe how the pattern of accumulation of toxic plasticizer metabolites would be affected; and
3) to assess how the problem of plasticizer contamination would be affected by the interaction of another type of compound found in the environment: surfactants.

2. MATERIALS & METHODS

2.1 Selected Organisms and Storage Conditions

The organisms used in this work were strains of *B. subtilis* obtained from American Type Culture Collections (ATCC). This species of organism was selected because it was known to produce the biosurfactant surfactin. The strains chosen were *B. subtilis* ATCC 21332 and *B. subtilis* ATCC 6633. As suggested by ATCC, the optimal growth temperature for both organisms was 30°C and the optimal growth medium was Difco Nutrient Broth [59]. The substrate selected for growth in the presence of plasticizers and surfactants was glucose (American Chemicals Ltd., Montreal, QC), at a concentration of 2.5 g/l.

Long term storage of the cultures was done in vials containing 20% glycerol (Sigma-Aldrich, Montreal, QC) and 20% Difco Nutrient Broth (Fisher Scientific, Montreal, QC) solution (18 g/l Difco Nutrient Broth in distilled water). The vials were kept at -70°C in a freezer (REVCO, Model ULT1386). Reconstitution, performed in a laminar fumehood (The Baker Company, Model VBM600), consisted of allowing a vial to thaw, then pouring its contents into a pre-autoclaved 500 ml Erlenmeyer flask containing 100 ml of optimal growth medium suggested by ATCC (8 g/l Difco Nutrient Broth in distilled water).

2.2 Selected Plasticizers

The diester plasticizers used were di-2-ethylhexyl adipate (D(EH)A) (99% purity), di-2-ethylhexyl phthalate (D(EH)P) (99% purity), and di-(propylene glycol)

dibenzoate (D(PG)DB) (98% purity). All plasticizers were obtained from Sigma-Aldrich, Montreal, QC.

2.3 Culture Growth in Shake Flasks

Shake flask cultures were grown in 500-ml Erlenmeyer flasks with sponge caps (Fisher Scientific, Montreal, QC) at 30°C in a rotary shaker at 150 rpm. Following reconstitution from the freezer and three days of growth, 1 ml of broth was transferred as an inoculum into each new flask as required by the specific experiment. The growth medium for the experimental flasks consisted of 100 ml of mineral salt medium (MSM) along with 2.5 g/l glucose (American Chemicals Ltd., Montreal, QC). The composition of MSM is listed in Table 2-1. Prior to inoculation, the flasks containing MSM and a separate solution of glucose in distilled water were autoclaved for 1 hour at 121°C and 14.7 psig (101 kPa). The glucose solution was then added to the flasks to obtain the required concentration. Plasticizer was also added to the flasks at a concentration of 2.5 g/l. This was done using a 0.5-ml glass syringe (Hamilton, Reno, NV) prior to autoclaving the flasks. Inoculation of shake flasks as well as any additions of compounds following autoclaving was done under sterile conditions in a laminar fumehood.

Table 2-1 - Composition of mineral salt medium (MSM) in distilled water [35].

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

2.4 Bioreactor Cultures

Cultures of *B. subtilis* ATCC 6633 were grown in a 1-litre cyclone reactor in the presence of D(EH)A with and without the addition of surfactants. A schematic representation of the setup is shown in Figure 2-1. The sterilization of the reactor and the reservoir containing the medium consisted of autoclaving each for 3 hours at 121°C and 14.7 psig (101 kPa).

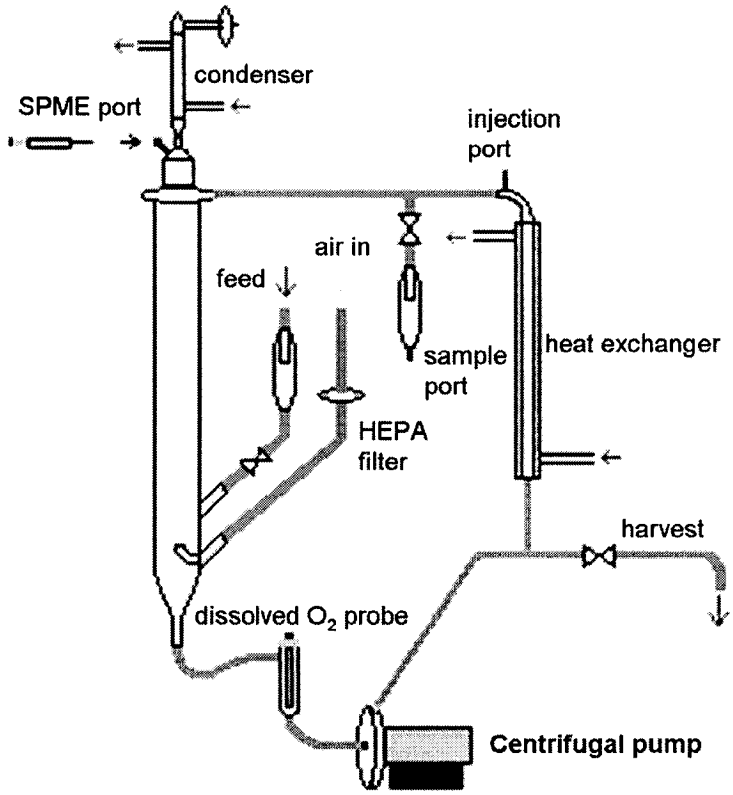

Figure 2-1 - The sequencing batch cyclone reactor used in plasticizer degradation experiments [60]. The flow of the broth in the system was counter-clockwise. The medium was fed into the reactor from a reservoir through the stream labelled "feed".

The contents of the reactor were continuously pumped through the glass cyclone by a 1/55 hp centrifugal pump, which provided excellent mixing. The temperature inside

the reactor was maintained at 30°C by circulating the broth through a glass counter-current flow heat exchanger. A condenser was fitted to the top of the cyclone in order to prevent volume loss arising from evaporation during longer runs. A dissolved oxygen probe (Cole-Parmer, Anjou, QC) was used to monitor growth and microbial activity and served as an indicator for when samples should be taken. The cyclone was aerated through an inline HEPA filter (Millipore Millex-FG50, 0.2 μl) and the flow of air was maintained constant at 213 ml/min with a rotameter (Cole-Parmer, Anjou, QC). Any liquid flow in or out of the reactor passed through isolators in order to prevent contamination. The separate components of the reactor were all connected by latex tubing (VWR Scientific, West Chester, PA).

The medium consisted of 2.5 g/l glucose in the MSM solution described in Table 2-1. It was fed to the reactor by gravity from a reservoir. Cycling was achieved by removing 90% of the reactor volume through the harvest port, then refilling with fresh medium up to 1 litre. The initial inoculum, the plasticizer, and any surfactant solution added (as required) were all injected with sterile syringes (Fisher Scientific, Montreal, QC) through the septum of the injection port. Samples of 10 ml were withdrawn from the sample port for analysis (see Section 2.6.1).

Typically, the culture was first grown on a medium containing only glucose as a carbon source. Glucose, dissolved oxygen, and total protein concentrations were used as indicators of the end of the exponential growth phase, at which point the reactor was cycled. The system was cycled repeatedly until a stable and reproducible growth pattern was achieved. The next cycle was carried out with the plasticizer and then with both the plasticizer and surfactant present. After the surfactant was added, the reactor was shut

down, cleaned, and re-sterilized in order that no residual surfactant remain in subsequent growth cycles.

Some fermentations were performed in parallel reactors in order to eliminate any possible acclimation effects on the organisms that may arise from previous exposure to plasticizer when surfactant was added. Plasticizer was added to one reactor and both plasticizer and surfactant were added to the other. The setup of both reactors was identical and both were fed from the same medium reservoir. After the parallel runs were completed, the experiment was repeated with the reactors switched.

2.5 Culture Analyses

2.5.1 Protein Analysis

The quantification of microbial growth was performed using the BIORAD DC Protein Assay (BIO-RAD Laboratories, Hercules, CA). For the analysis of shake flask cultures, 100 µl of broth was withdrawn for the assay directly from the flask under sterile conditions in a laminar fume hood. For reactor cultures, 100 µl was obtained from each 10-ml sample. The procedure was then followed as specified by BIO-RAD [60].

2.5.2 Glucose Analysis

The Thermo Trace Glucose Hexokinase Assay (Thermo Trace Ltd., Melbourne, Australia) was used to monitor the concentration of glucose during the fermentations. 100 µL was withdrawn from the broth and diluted 100 times in distilled water. 1 ml of diluted sample was then added to 2 ml of glucose hexokinase reagent, quickly vortexed, and incubated at 34°C for 3 minutes. The optical absorbance was then measured at a

wavelength of 340 nm. A calibration curve having an r^2 value of 1.000 was produced with known concentrations of glucose in MSM using the above method.

2.5.3 Surface Tension Measurements

For shake flask cultures, the surface tension of the entire content of the flask was measured using the Wilhelmy plate method with a Krűss K12 processor-tensiometer (Krűss USA, Charlotte, NC). In order to monitor the surface tension of the broth in the reactor, smaller samples of up to 5 ml were withdrawn and placed in Petri dishes (Fisher Scientific, Montreal, QC). The surface tension was measured using the DeNouy ring method with a Fisher Autotensiomat (Fisher Scientific, Montreal, QC).

2.6 Quantification of Plasticizer and Metabolites

2.6.1 Liquid-liquid Extraction

The aqueous concentrations of the plasticizer and its metabolites were analyzed by organic solvent extraction followed by gas chromatography.

For shake flask cultures, the contents of the flasks were poured into 250-ml separatory funnels. The pH was then adjusted to just below 2 by the addition of concentrated H_2SO_4. The solvent used for extraction was chloroform (Fisher Scientific, Montreal, QC) with n-pentadecane (American Chemicals Ltd., Montreal, QC) added as the internal standard. After vigorous shaking for 30 seconds and phase separation, a sample of the organic extract was analyzed using gas chromatography (Section 2.6.3).

The 10-ml reactor samples were withdrawn into test tubes directly from the sample port (Figure 2-1). After removing 5 ml of sample for protein, glucose, and surface tension measurements, 5 ml remained for the extraction of the plasticizer and its

metabolites. The pH of the samples was adjusted to below 2 with a drop of concentrated H_2SO_4. The extraction was performed directly in the test tubes with the addition of 5 ml of chloroform containing 1.5 g/l dissolved n-pentadecane as the internal standard. The test tubes were then vortexed for 30 seconds, the phases were allowed to separate, and samples of the organic phase were injected into the gas chromatograph (Section 2.6.3).

2.6.2 Metabolite Detection in the Gas Phase

Because 2-ethylhexanol and 2-ethylhexanal have relatively high volatilities compared to the plasticizer and the other metabolites, it was important to follow their concentrations in the gas phase as well as the aqueous phase. The technique used for this purpose was solid phase micro-extraction (SPME) followed by gas chromatography. SPME is based on the adsorption of compounds on to a solid fibre. The fibre can then be placed inside the injector of a gas chromatograph and the adsorbed compounds are volatilized onto the column.

The 70 μm Carbowax / DVB StableFlex SPME fibre assembly (Supelco, Bellefonte, PA) was used in conjunction with the Manual Holder (Supelco, Bellefonte, PA) and inserted at the top of the reactor as shown in Figure 2-1. The fibre was allowed to equilibrate for 30 minutes inside the reactor, and then was quickly placed into the injector of the gas chromatograph.

2.6.3 Gas Chromatography

The gas chromatograph (GC) used was a Varian CP-3800 (Varian, Walnut Creek, CA) equipped with a flame ionization dectector (FID). For the analysis of organic extracts from the shake flask and reactor samples, the column was a CP-Sil 5 (ID 0.53

mm) (Varian, Walnut Creek, CA). The operating conditions of the GC for the analysis of all samples other than SPME are presented in Table 2-2. Calibration curves for the plasticizers and their metabolites were produced with standards of known concentration.

Table 2-2 - GC operating conditions for plasticizer and metabolite quantification.

Operation Condition	Value
Injection temperature	250°C
Initial column temperature	60°C
Temperature ramp rate	**10°C/min** (60°C-150°C), **20°C/min** (150°C – 300°C)
Final column temperature	300°C
Detector (FID) temperature	300°C
Column flow rate	10 ml/min (constant)

The column used for SPME quantification was a Varian fused silica CP SIL 8CB (ID 0.32 mm). The operating conditions for the GC for SPME injections are described in Table 2-3. A calibration curve was developed for 2-ethylhexanol in the gas phase by measuring its aqueous phase concentration during an abiotic reactor run. The decrease in concentration due to evaporation over a specific time period was then correlated to the size of the peak obtained from the SPME chromatogram. The calibration curve and a more detailed explanation of the calibration procedure are presented in Appendix A.2.

Table 2-3 - Operating conditions of the gas chromatograph for SPME injections.

Operation Condition	Value
Injection temperature	250°C
Initial column temperature	60°C (1 minute hold time)
Temperature ramp rate	15°C/min
Final column temperature	280°C (4 minute hold time)
Detector (FID) temperature	300°C
Column pressure	1.2 psi (8.3 kPa)

2.6.4 Metabolite Identification by GC/MS

The identification of the metabolites and their peak positions on the chromatographs was initially performed by injection of commercial standards using the same GC method. As a verification step, the organic extracts from the samples were also analyzed by gas chromatography / mass spectrometry (GC/MS). This was particularly necessary for the mono-2-ethylhexyl adipate, as no commercial standard was available. The GC/MS (TRACE GC 2000/ Finnigan POLARIS, Thermo Quest, Montreal QC) was equipped with a Restek RTX-5 MS column (Fisher Scientific, Montreal, QC) with an internal diameter of 0.25 mm. The operating conditions of the GC/MS are tabulated in Table 2-4.

Table 2-4 - GC/MS operating 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

2.7 Surfactants Added to Microbial Cultures

2.7.1 Synthetic Surfactants

The synthetic surfactants added to the growth experiments with *B. subtilis* are listed in Table 2-5 along with their sources.

Table 2-5 - List of surfactants selected for addition to microbial cultures and their respective sources.

Surfactant	Source
Pluronic L122	BASF Corp., Wyandotte, MI
SDS	Mallinckrodt Inc., Paris, KY
Tween 80	Atlas Chemical Industries, Brantford, ON
Triton X-100	Sigma-Aldrich, Montreal, QC

2.7.2 Biosurfactants

The two biosurfactants added to plasticizer-degrading cultures were surfactin isolated from *B. subtilis* ATCC 21332 and sophorolipid isolated from *C. bombicola* ATCC 22214.

Surfactin was produced in shake flasks by growing *B. subtilis* ATCC 21332 on 5 g/l glucose in MSM, at 30°C, in a rotary shaker set to 150 rpm. After four to five days of culture, the contents of the flasks were transferred into 30 ml Teflon centrifuge tubes (Fisher Scientific, Montreal, QC) and centrifuged (IEC Model B-22M, Fisher Scientific, Montreal, QC) for 15 minutes at 10,000 rpm at room temperature. The supernatants were then poured into separatory funnels and the pH was adjusted to just below 2 with concentrated H_2SO_4. Chloroform was used as the organic solvent for extraction in a 1:2 (solvent:broth) volume ratio. The funnels were vigorously agitated for 1 minute and the organic phase was withdrawn into a pre-weighed vial. The extraction was performed a

second time and the organic phase was combined with that from the first extraction. The chloroform from the final extract was then evaporated under a gentle stream of nitrogen gas (MEGS, St. Laurent, QC.), leaving the dry crude surfactin crystals to be weighed. Sophorolipid lactone crystals were obtained as described in [61].

Both biosurfactants were stored at 4°C until ready for use. Prior to their addition to microbial cultures, they were re-suspended in MSM and then filter-sterilized. This was done by vacuum filtration using 0.22 μm Millipore mixed-cellulose membrane filters (Fisher Scientific, Montreal, QC). The vacuum filtration setup was autoclaved for 1 hour at 121°C and 14.7 psig (101 kPa) before filtering in order to ensure that the filtrate remains sterile following the procedure. Sterile transfer pipettes and sterile syringes (Fisher Scientific, Montreal, QC) were then used to add the biosurfactant to shake flask and reactor cultures, respectively.

2.7.3 Determination of Surfactant Critical Micelle Concentration

Solutions of the surfactants in MSM were prepared and then diluted with MSM to a wide range of surfactant concentrations. The surface tension was measured at each concentration using the Wilhelmy plate method with a Krűss K12 processor-tensiometer (Krűss USA, Charlotte, NC). A plot of surface tension versus the logarithm of surfactant concentration revealed the point at which the surface became saturated with surfactant. Surfactants were always added to microbial cultures at concentrations slightly higher than their experimentally determined CMCs.

2.8 Isolation of mono-2-ethylhexyl adipate

Mono-2-ethylhexyl adipate was isolated from the broth at the end of a 1-litre batch growth in the presence of D(EH)A. The contents of the reactor were harvested when no more residual plasticizer was detected. This was done in batches of 30 ml in Teflon centrifuge tubes (Fisher Scientific, Montreal, QC) that were then centrifuged (IEC Model B-22M, Fisher Scientific, Montreal, QC) for 15 minutes at 10,000 RPM at room temperature. The supernatants were combined in separatory funnels. The pH was then adjusted to 2 with concentrated H_2SO_4 and chloroform was added in a 1:2 (solvent:broth) volume ratio for the extraction. The collected organic phase was then placed under a stream of nitrogen gas (MEGS, St. Laurent, QC) in order to vaporize the chloroform as well as the residual 2-ethylhexanol.

The crude product was re-suspended in chloroform and washed with phosphate buffer at pH 7 to remove residual surfactin. The monoester isolate was spotted on a thin layer chromatography (TLC) plate (Silica Gel GF Redi/plate, Fisher Scientific, Montreal, QC) to check for purity. The solvent mixture used was a 65:15:2 volume ratio of chloroform, methanol, and water, respectively. The visualization was performed by spraying the plate with H_2SO_4 and then drying it in an oven at 100°C for approximately 10 minutes.

2.9 Toxicity Tests

2.9.1 Toxicity of mono-2-ethylhexyl adipate

The toxicity of the isolated mono-2-ethylhexyl adipate was measured using the Microtox assay. The assay is based on the toxic effect of the test compound on the marine organism *Vibrio fischeri* NRRL B-11177. The organism uses the enzyme, luciferase, to produce light in the following reaction [62].

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

Luciferase has been linked to the respiration pathway [63]; thus the light emitted by the organism can be considered to be representative of the respiration of the organism. A higher solution toxicity would result in a lower rate of respiration. The measure of toxicity obtained from this test was the EC_{50}, or the effective concentration that causes a 50% decrease in light output. A lower EC_{50} corresponds to a higher solution toxicity.

The isolated mono-2-ethylhexyl adipate was re-suspended in MSM at 0.1 g/l and transferred into 0.5-ml cuvettes designed for the Microtox Model 500 (Microbics Corp., Carlsbaad, CA). The procedure was then followed as stated by Microtox, using the Basic Test setup in the Microtox software.

2.9.2 The Toxic Effect of 2-ethylhexanol on the Growth of *B. subtilis*

The toxicity of 2-ethylhexanol to *B. subtilis* ATCC 6633 was assessed by the addition of the compound to cultures growing on glucose in MSM, and observing the decrease in growth at different concentrations of the alcohol. With a sterile transfer pipette in a laminar fume hood, 2-ethylhexanol (Sigma-Aldrich, Montreal, QC) was

added at different concentrations to autoclaved flasks containing 100 ml MSM. Glucose was added in the same fashion to a concentration of 2.5 g/l from a pre-autoclaved solution of glucose in distilled water. The flasks were then inoculated from a culture grown on Nutrient Broth. The protein assay (Section 2.1) was used to quantify the growth of the organism at each different concentration of 2-ethylhexanol after two days.

A second set of flasks was inoculated without the immediate addition of 2-ethylhexanol. The cultures were allowed to grow on 2.5 g/l glucose in MSM for two days to reach the stationary phase, at which point their growth was measured using the protein assay. The 2-ethylhexanol was then added at different concentrations, the cultures were allowed to grow for another day, and the protein assay was repeated.

The concentration of 2-ethylhexanol and any of its metabolites was followed by periodically withdrawing 2 ml of the broth, adjusting its pH to below 2 with a drop of concentrated H_2SO_4, and extracting it with 2 ml of n-pentadecane in chloroform solution. The organic phase was then analyzed by gas chromatography.

2.10 Biomass Adherence to Hydrocarbons (BATH) Assay

The cultures were grown in 500-ml Erlenmeyer flasks containing 100 ml of MSM solution. Di-2-ethylhexyl adipate was added to half of the flasks at a concentration of 2.5 g/l. The flasks were autoclaved for 1 hour at 121°C and 14.7 psig (101 kPa). A solution of glucose in distilled water was autoclaved separately and then it was added to the flasks so that the concentration of glucose was 2.5 g/l. A previously filter-sterilized surfactin solution in MSM was then added to half of the flasks at a concentration just above the

CMC of the biosurfactant, and the flasks were inoculated with *B. subtilis* ATCC 6633 previously grown on Nutrient Broth.

Samples of 1.5 ml were withdrawn at set times from the flasks for the BATH assay [52]. These samples were centrifuged in 1.5-ml Eppendorf microcentrifuge tubes (Fisher Scientific, Montreal, QC) at 10 000 rpm for 5 minutes in an IEC Micromax micro-centrifuge (Fisher Scientific, Montreal, QC). The supernatants were discarded and the pellets were re-suspended in 1.5 ml of distilled water. This was repeated twice in order to wash the cells and remove surfactin. After the second centrifugation and re-suspension of the pellets, the samples were transferred to test tubes to which another 1.5 ml of distilled water was added, bringing the total volume up to 3 ml. The optical density was measured at a wavelength of 450 nm. 3 ml of n-hexadecane (Sigma-Aldrich, Montreal, QC) was then added to the test tubes and they were vortexed for 1 minute. The phases were allowed to separate for 30 minutes and then the aqueous phase was withdrawn carefully with a Pasteur pipette. The optical density of the aqueous phase was again measured at 450 nm. The percent adherence to hydrocarbons was estimated based on the decrease in the optical density of the aqueous phase using the following formula:

$$\%\ adherence = \left(\frac{OD_{initial} - OD_{final}}{OD_{initial}} \right) \times 100$$

2.11 Evaporation Rate of 2-ethylhexanol

Shake flasks containing 100 ml of MSM were autoclaved and then 2-ethylhexanol (Sigma-Aldrich, Montreal, QC) was added at a concentration of 1 mM with a sterile transfer pipette. Filter-sterilized surfactin in MSM solution was then added to half of the

flasks so that the concentration of surfactin was just above its CMC. The flasks were placed in a rotary shaker at 150 rpm at room temperature. Samples of 2 ml were periodically withdrawn from the flasks and the concentration of 2-ethylhexanol was measured using the same method as described in Section 2.9.2.

2.12 Pluronic L122 – 2-ethylhexanol Interaction: Filtration Experiments

Solutions of 1 mM 2-ethylhexanol (Sigma-Aldrich, Montreal, QC) and various concentrations of Pluronic L122 (BASF Corp., Wyandotte, MI) were prepared in MSM. The surface tension of these solutions was measured A small sample (5 ml) of each solution was withdrawn and extracted with 5 ml of n-pentadecane in chloroform solution in a test tube. The initial 2-ethylhexanol concentration was determined with gas chromatography. 20 ml of each solution was then vacuum-filtered using 10 nm pore-size Millipore mixed-cellulose membrane filters (Fisher Scientific, Montreal, QC) for 20 minutes. The surface tension of the filtrate was measured and its 2-ethylhexanol concentration was analyzed using the same method as for the initial solution.

The membrane filters were also extracted to obtain an amount of 2-ethylhexanol retained on the filter. Following filtration, they were carefully placed in 40-ml capped glass vials and extracted with 10 ml of n-pentadecane in chloroform solution for 1 minute. The concentration of the 2-ethylhexanol in the extract was then analyzed by gas chromatography.

3. RESULTS

3.1 Critical Micelle Concentrations

Surfactin was isolated from *B. subtilis* ATCC 21332 cultures grown in shake flasks as described in Section 2.7.2. A typical yield was 0.06 g per 100 ml of culture medium which contained 5 g/l glucose initially. The critical micelle concentration (CMC) of the isolated biosurfactant was then determined. Figure 3-1 shows the surface tension drop with increasing surfactin concentration, and Figure 3-2 shows the corresponding logarithmic plot typically used to determine the CMC. The final CMC obtained for surfactin was an average of these estimated values from triplicate experiments.

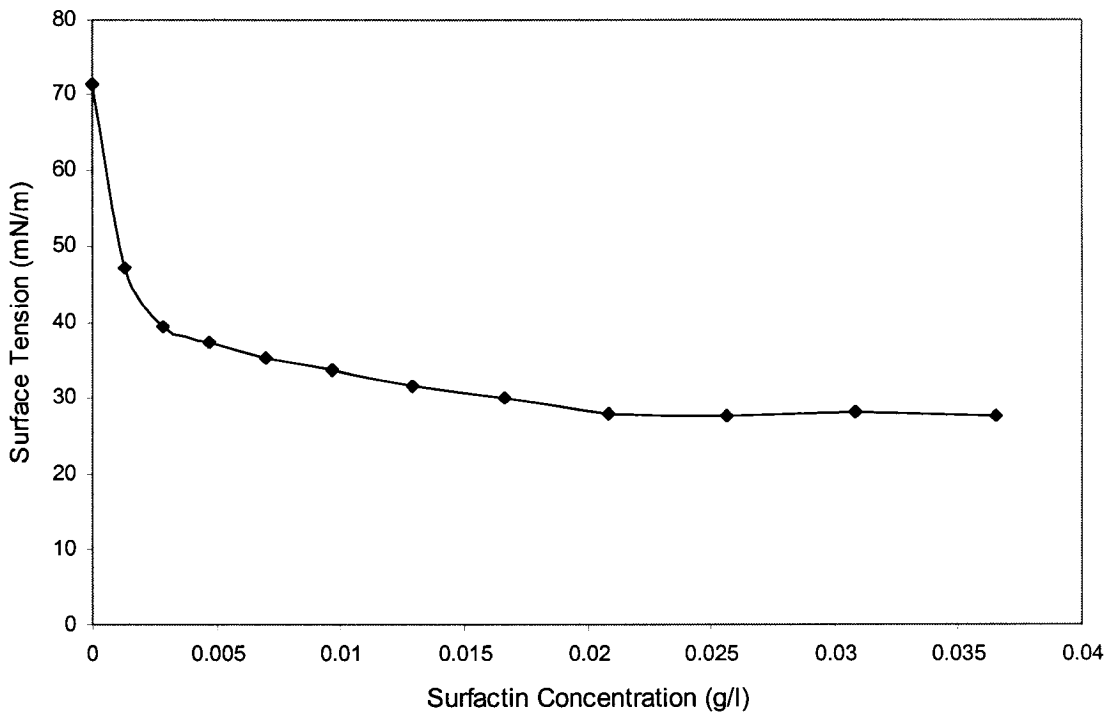

Figure 3-1 - The effect of surfactin concentration on the surface tension of MSM.

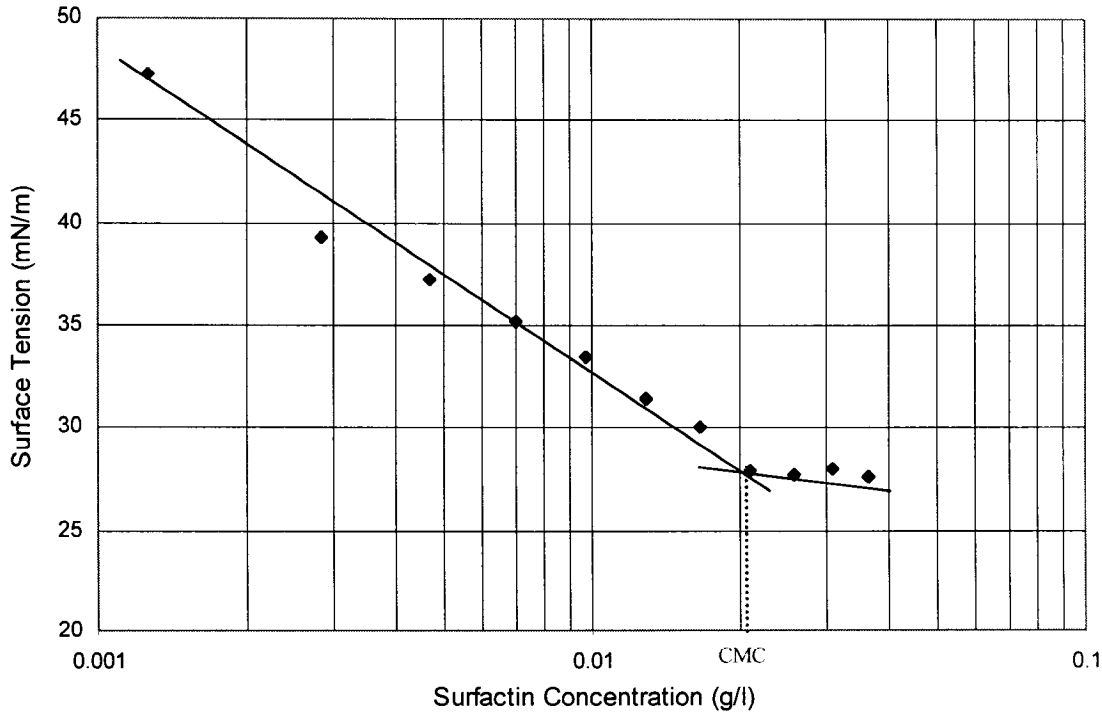

Figure 3-2 - The determination of the critical micelle concentration (CMC) of surfactin.

The CMC was determined for all the surfactants used. Table 3-1 summarizes these results and compares them to values obtained from literature. The experimental values were used to select the concentrations to add to the media.

Table 3-1 - CMC values of surfactants measured in the present study and also reported in the literature.

Surfactant	Measured CMC in MSM (g/l)	Reported CMC (g/l)
Surfactin	0.024	0.025[a]
Sophorolipid	0.041	0.004[b] – 0.082[c]
Triton X-100	0.070	0.043[d]
SDS	0.74	1.6[e]
Tween 80	0.15	0.036[f]
Pluronic L122	0.15	n/a[g]

[a]Cooper *et al* [64], [b]Schippers *et al* [45], [c]Christofi *et al* [65], [d]Guha *et al* [50], [e]Bramwell *et al* [51], [f] Zheng *et al* [66], [g]n/a – not available.

3.2 Effect of Surfactants on Plasticizer Degradation

3.2.1 Preliminary Experiments with a Surfactin-Producing Strain of *B. subtilis*

Table 3-2 contains the results of a preliminary analysis of the growth of surfactin-producing *B. subtilis* ATCC 21332 in the presence of three different plasticizers. These results showed a correlation between surface tension and the degree of plasticizer degradation, which implies that with increased amounts of surfactin, the amount of degradation is also increased.

Table 3-2 - Degradation of three plasticizers by *B. subtilis* after 5 days of culture.

Plasticizer	Transfer[a]	Plasticizer Remaining (mM)	Broth Surface Tension (mN/m)
D(EH)P	1	0.9	26.8
	2	1.8	36.9
	3	4.4	41.8
	4-NB[b]	5.3	39.9
	4	2.3	46.8
D(EH)A	1	0.0	28.1
	2	1.6	41.3
	3	3.3	43.1
	4-NB[b]	0.3	26.2
	4	0.2	26.3
D(PG)DB	1	-0.3	26.7
	2	0.9	41.3
	3	2.2	45.9
	4-NB[b]	3.4	55.2
	4	2.2	52.2

[a] Transfer 1 was inoculated from a culture grown on Nutrient Broth; all subsequent transfers were inoculated from the previous transfer.

[b] Transfer 4-NB was inoculated from transfer 3 and supplemented with 0.025 g/l Nutrient Broth.

This trend is illustrated more clearly in plots in Figure 3-3, which shows greater removal of all three plasticizers when the surface tension of the medium was lower. A surface tension of 26 to 28 mN/m indicated that surfactin was present in the medium at a concentration at or above its CMC.

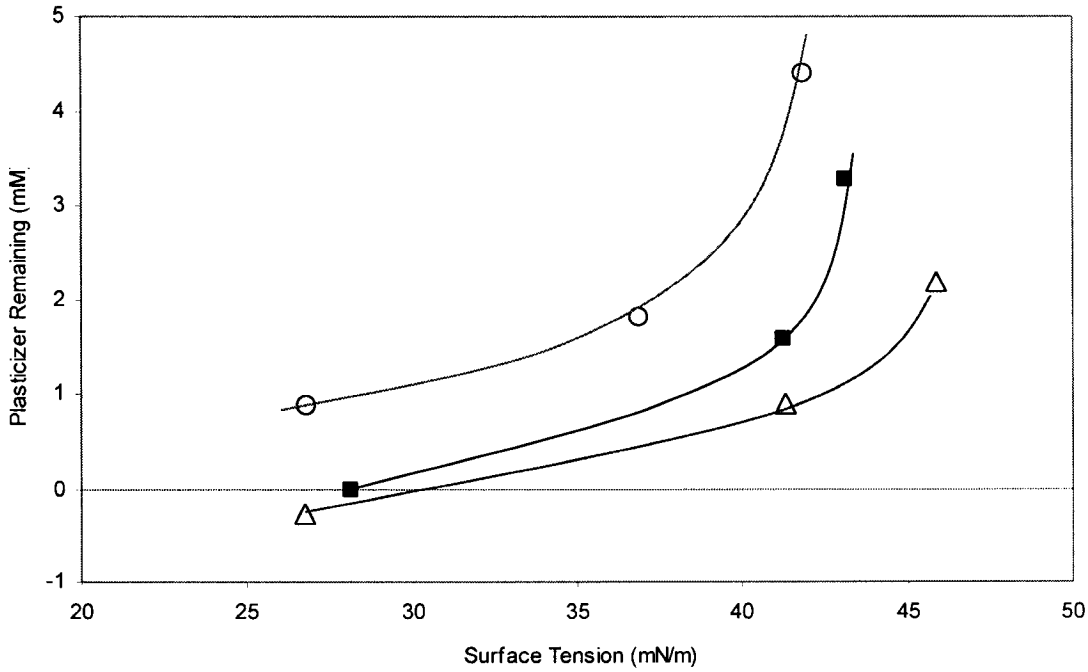

Figure 3-3 - The removal of D(EH)A (■), D(EH)P (○), and D(PG)DB (Δ) from aqueous culture by *B. subtilis* ATCC 21332 after 5 days versus the final surface tension of the medium. The initial concentrations were 6.8 mM D(EH)A, 6.4 mM D(EH)P or 7.3 mM D(PG)DB.

The time course of the degradation of one of the plasticizers, D(EH)A, is shown in Figure 3-4. The decrease in surface tension in the medium can also be seen occurring at the same time as the appearance of the metabolites, 2-ethylhexanol and mono-2-ethylhexyl adipate. The concentrations of D(EH)A and its metabolites were always

obtained by GC analysis. A typical gas chromatogram showing the peak positions of the plasticizer and its metabolites is presented in Appendix A.1.

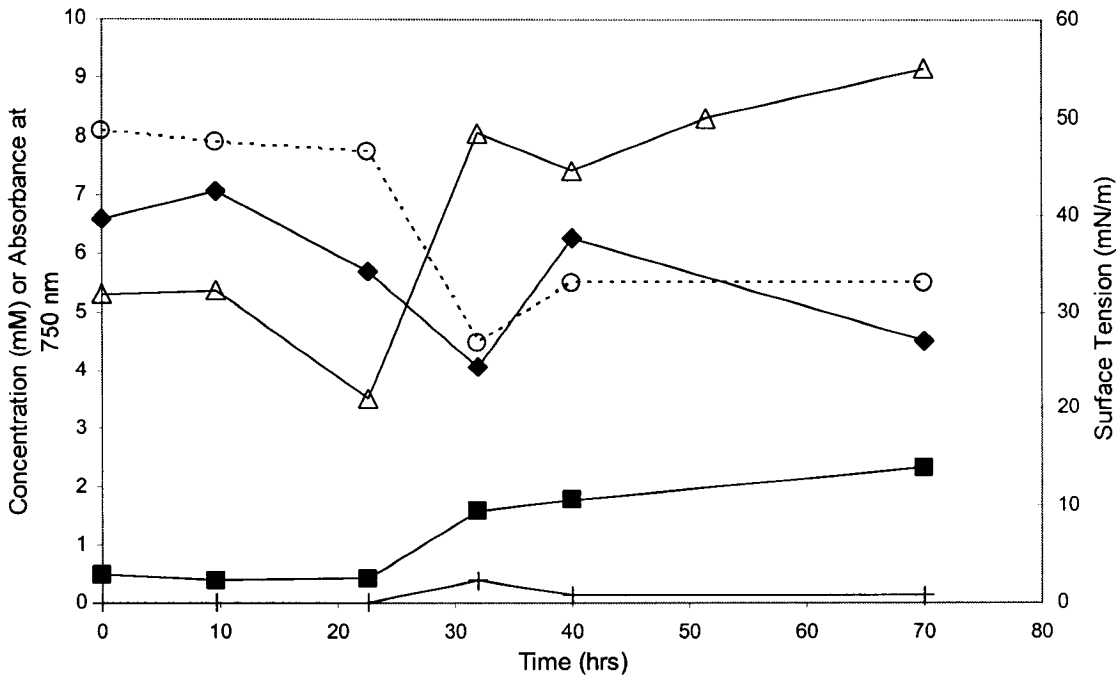

Figure 3-4 - The concentration of D(EH)A (♦) during growth of *B. subtilis* ATCC 21332 and the appearance of 2-ethylhexanol (■) and mono-2-ethylhexyl adipate (+). The protein concentration (Δ) (absorbance at 750 nm) and the surface tension of the medium (○) are also shown.

3.2.2 Effect of Surfactant Addition to Shake Flask Cultures

Surfactin was added to a strain of *B. subtilis* (ATCC 6633) that did not produce surfactin. Table 3-3 shows the amount of D(EH)A degraded after triplicate five-day cultures by *B. subtilis* ATCC 6633 with and without the presence of surfactin.

Table 3-3 - D(EH)A degraded and metabolites released by *B. subtilis* ATCC 6633 with and without surfactin added to the medium. The error is expressed as ± one standard deviation for triplicates.

Surfactin Added	D(EH)A Remaining (mM) [a]	Final Metabolite Concentration (mM)		Surface Tension (mN/m)	Total Protein [b]
		2-ethylhexanol	mono-2-ethylhexyl adipate		
None	2.9 ± 1.2	2.6 ± 0.6	1.3 ± 0.4	36.4 ± 0.5	0.09
0.19 g/l (added after 3 days)	2.9 ± 0.7	2.1 ± 0.1	1.1 ± 0.3	27.1 ± 0.3	0.11
0.19 g/l (to initial medium)	0.5 ± 0.1	2.7 ± 0.1	2.0 ± 0.0	27.1 ± 0.3	0.13

[a] The medium initially contained 6.8 mM D(EH)A

[b] Obtained with BioRAD Protein Assay and expressed as absorbance at a wavelength of 750nm; error between triplicates was insignificant

When surfactin was added to the medium prior to inoculation, much more plasticizer was degraded than in the control flasks. There was also significantly more mono-2-ethylhexyl adipate observed in the medium, but no significant difference in 2-ethylhexanol concentrations. The addition of surfactin following the completion of the growth phase (after 3 days) had no significant effect. Figure 3-5 shows the concurrent growth of *B. subtilis* ATCC 6633 with and without surfactin as well as the time course of the removal of the plasticizer in both cases. It is apparent from this figure that the degradation of D(EH)A occurs significantly faster when surfactin is present. Figure 3-6 reveals the tendency for greater accumulation of both 2-ethylhexanol and mono-2-ethylhexyl adipate when surfactin is added.

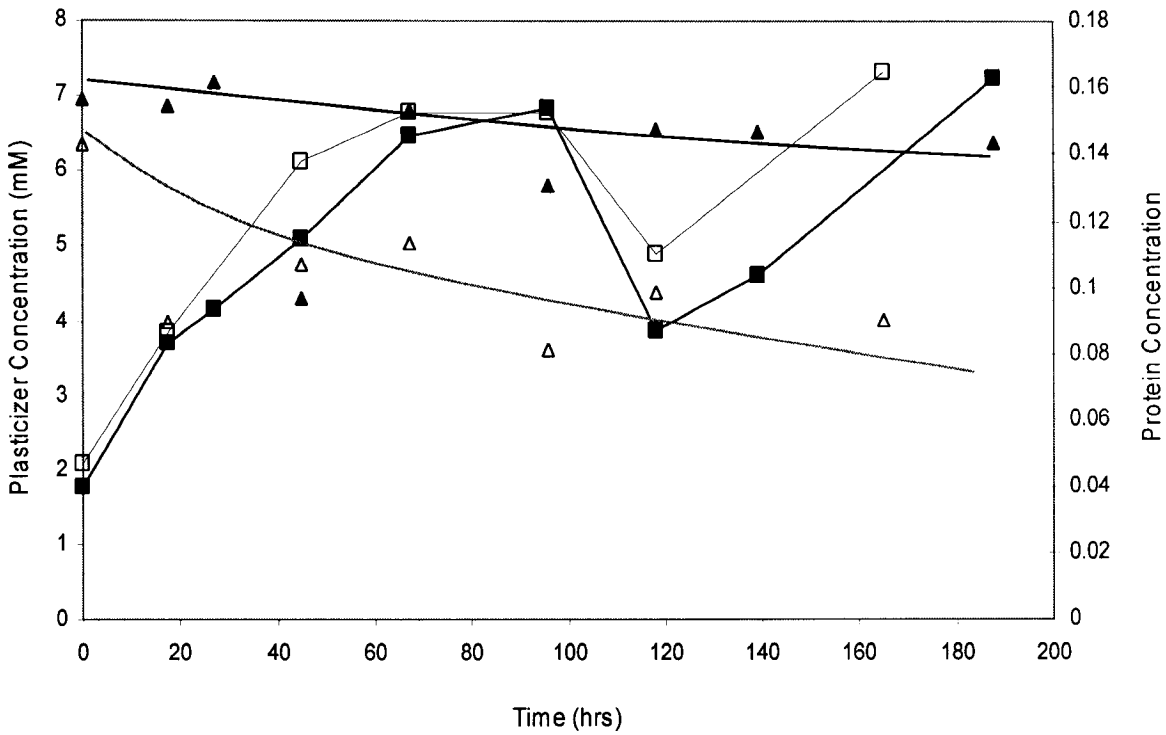

Figure 3-5 - D(EH)A concentration during the growth of *B. subtilis* ATCC 6633 with surfactin in the medium (Δ) and without (▲). The protein concentration, expressed as absorbance at 750 nm, is also shown when surfactin is present (□) and for the control flasks (■)

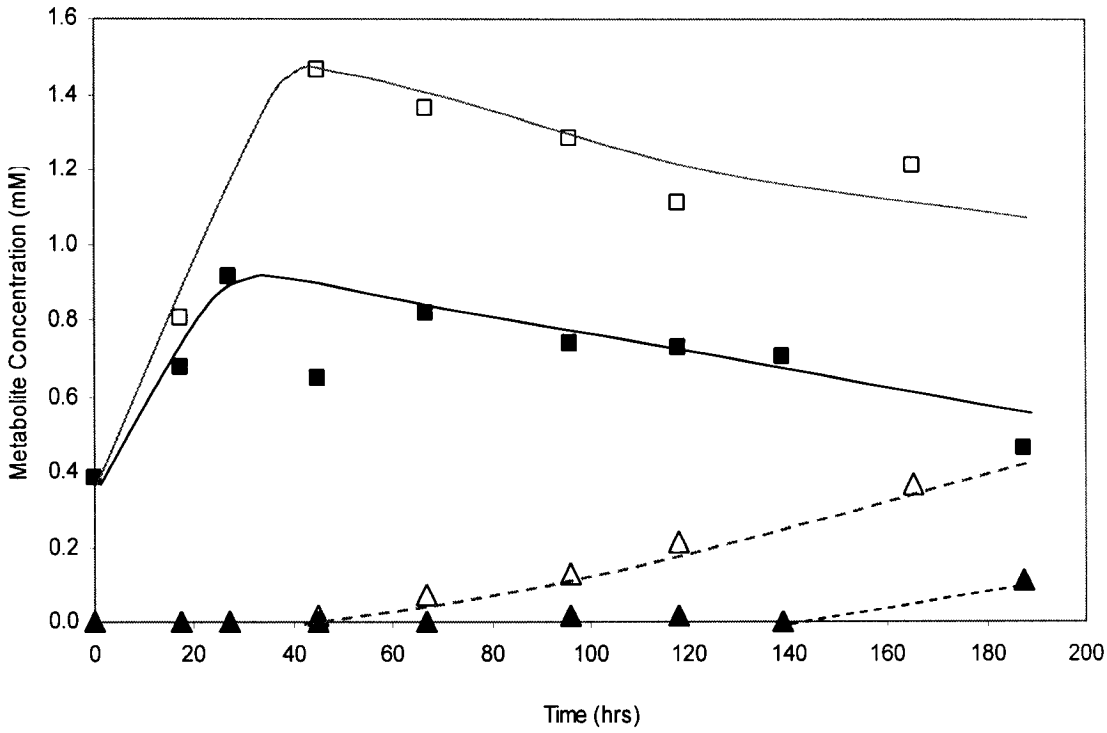

Figure 3-6 - The concentrations of 2-ethylhexanol (□) and mono-2-ethylhexyl adipate (Δ) arising when surfactin is added to *B. subtilis* ATCC 6633 cultures that are degrading D(EH)A. The concentrations of 2-ethylhexanol (■) and mono-2-ethylhexyl adipate (▲) in the absence of surfactin are also shown.

Triplicate cultures were also performed with *B. subtilis* ATCC 6633 in the presence of D(EH)A with the addition of other surfactants. Tables 3-4, 3-5 and 3-6 show the degradation of D(EH)A and the accumulation of metabolites when *B. subtilis* ATCC 6633 was grown on glucose in the presence of these surfactants for five days.

Table 3-4 - D(EH)A degraded by *B. subtilis* ATCC 6633 with synthetic surfactants present in the medium. The error is expressed as ± one standard deviation for triplicates.

Medium	D(EH)A Remaining (mM)	Final Metabolite Concentration (mM)		Surface Tension (mN/m)	Total Protein[b]
		2-ethylhexanol	mono-2-ethylhexyl adipate		
Control	4.5 ± 0.4	2.6 ± 0.3	0.31 ± 0.09	38.3 ± 0.6	0.134 ± 0.004
0.74 g/l SDS	6.7 ± 0.7	0.4 ± 0.0	0.04 ± 0.02	24.4 ± 0.3	0.065 ± 0.005
0.07 g/l Triton X-100	5.3 ± 1.5	1.1 ± 0.4	0.19 ± 0.06	38.9 ± 0.2	0.097 ± 0.005

[a] The medium initially contained 6.8 mM D(EH)A

[b] Obtained with BioRAD Protein Assay and expressed as absorbance at 750 nm

Table 3-5 - D(EH)A degraded by *B. subtilis* ATCC 6633 with surfactants present in the medium. The error is expressed as ± one standard deviation for triplicates.

Medium	D(EH)A Remaining (mM)[a]	Final Metabolite Concentration (mM)		Surface Tension (mN/m)[c]	Total Protein[d]
		2-ethylhexanol	mono-2-ethylhexyl adipate		
Control	5.5 ± 0.1	1.6 ± 0.4	0.03 ± 0.02	44 ± 1.0	0.126 ± 0.011
0.15 g/l Tween 80	5.8 ± 0.6	1.7 ± 0.4	0.08 ± 0.06	43 ± 2.3	0.135 ± 0.05
0.041 g/l sophorolipid[b]	4.8 ± 0.4	2.8 ± 0.2	0.10 ± 0.04	46 ± 5.5	0.129 ± 0.019

[a] The medium initially contained 6.8 mM D(EH)A

[b] Crystal Lactone Form.

[c] Obtained with DeNouy ring method.

[d] Obtained with BioRAD Protein Assay and expressed as absorbance at 750 nm

Table 3-6 - D(EH)A degraded by *B. subtilis* ATCC 6633 with and without the addition of Pluronic L122 to the medium. The error is expressed as ± one standard deviation for triplicates.

Medium	D(EH)A Remaining (mM)[a]	Final 2-ethylhexanol Concentration (mM)	Surface Tension (mN/m)[b]	Total Protein[c]
Control	4.6 ± 0.1	0.6 ± 0.1	48 ± 1.0	0.121 ± 0.010
0.16 g/l Pluronic L122	4.2 ± 0.7	1.2 ± 0.1	36 ± 2.6	0.113 ± 0.012

[a] The medium initially contained 6.8 mM D(EH)A

[b] Obtained with DeNouy ring method.

[c] Obtained with BioRAD Protein Assay and expressed as absorbance at 750 nm

Cultures in which Triton X-100 and SDS were introduced showed less D(EH)A degradation and lower metabolite concentrations than the cultures in the control flasks at the time of extraction. SDS was toxic to the microorganism at the concentration added, as indicated by the much lower total protein concentration for that surfactant listed in Table 3-4. The measurement of total protein in the broth was unaffected by the presence of Tween 80 or sophorolipid (Table 3-5). The Tween 80 had no effect on the degradation of D(EH)A or the final metabolite concentration. However, the addition of sophorolipid resulted in more plasticizer having been degraded and significantly higher 2-ethylhexanol and mono-2-ethylhexyl adipate concentrations than in the control flasks. The addition of Pluronic L122 did not significantly increase the amount of plasticizer hydrolyzed, but there was twice as much 2-ethylhexanol as in the control flasks (Table 3-6). This surfactant did not affect the growth of the organisms either.

3.2.3 Effect of Surfactants During Growth in a 1 litre Reactor

Because the plasticizers are hydrophobic compounds, they are not easily dispersed in aqueous media. It was therefore not possible to accurately measure the plasticizer concentration in an individual shake flask without sacrificing the entire flask. After the preliminary results, the work was continued with a sequencing batch reactor. The reactor was cycled several times with the bacteria growing on glucose. After the exponential growth phase became reproducible between cycles, the reactor was cycled and D(EH)A was added. When that run was completed, the reactor was cycled again and both D(EH)A and surfactin were added. The hydrolysis of D(EH)A with and without surfactin is shown in Figure 3-7, along with the glucose consumption for each of these cycles.

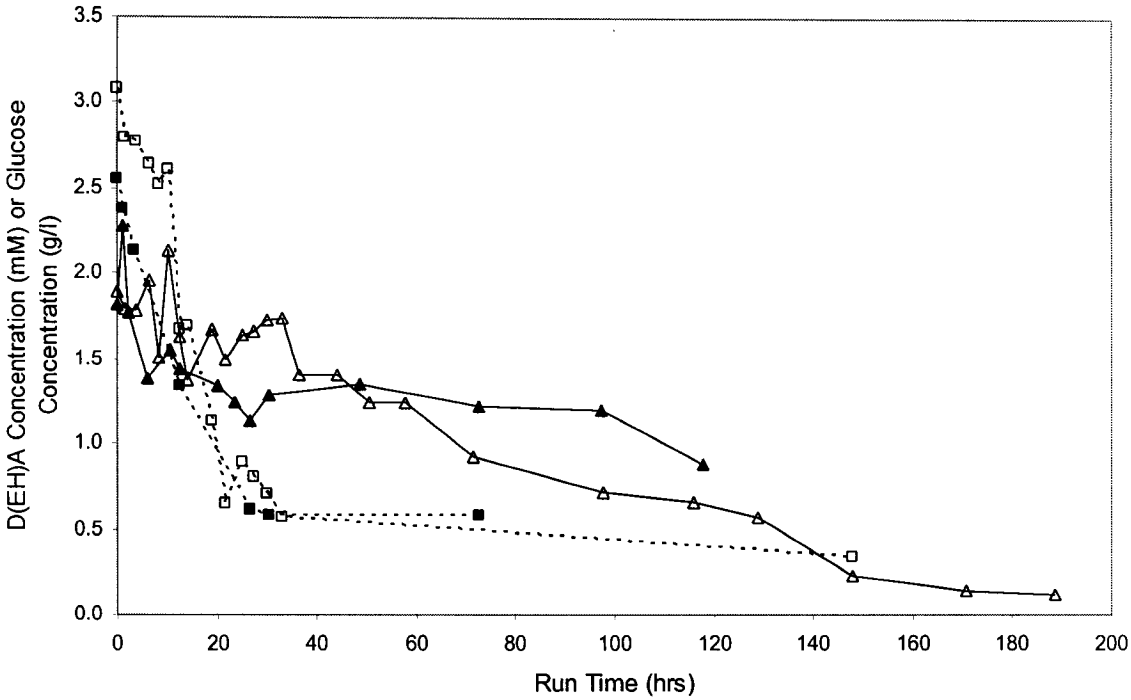

Figure 3-7 - The concentration of D(EH)A in fermentations performed with *B. subtilis* ATCC 6633 for single cycles with (Δ) and then without (▲) surfactin in the medium. The glucose concentration with (□) and without (■) surfactin is also shown.

In the reactor, the hydrolysis of D(EH)A occurred approximately at the same rate in the presence of surfactin as in the control run. Despite showing some variability early in the fermentation, the glucose concentration was the approximately the same in both cycles after the first twenty hours of culture. The growth of the organism, as determined by total protein concentration in the broth, was also approximately the same for both cycles, as shown in Figure 3-8.

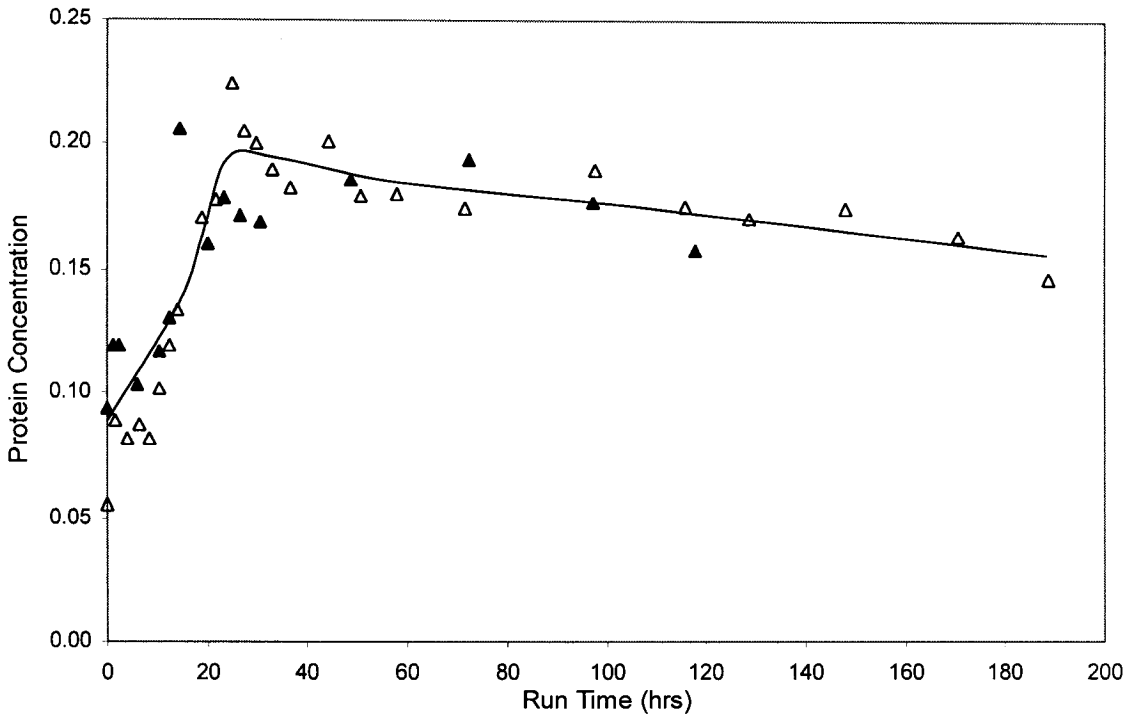

Figure 3-8 - The total protein concentration (expressed as absorbance at 750 nm) in the broth during the growth of *B. subtilis* in the presence of D(EH)A with (Δ) and without (▲) surfactin present in the medium.

The concentration versus time profiles of the metabolites are shown in Figure 3-9. 2-ethylhexanol reached dramatically higher concentrations when surfactin was present. Mono-2-ethylhexyl adipate, which was detected in only one sample during the control experiment, reached a concentration of over 0.7 mM after surfactin was added, and remained in the medium at significant concentrations until the end of the fermentation. The gas chromatogram shown in Appendix A.1 was obtained from a reactor sample during the late stages of the fermentation performed with surfactin.

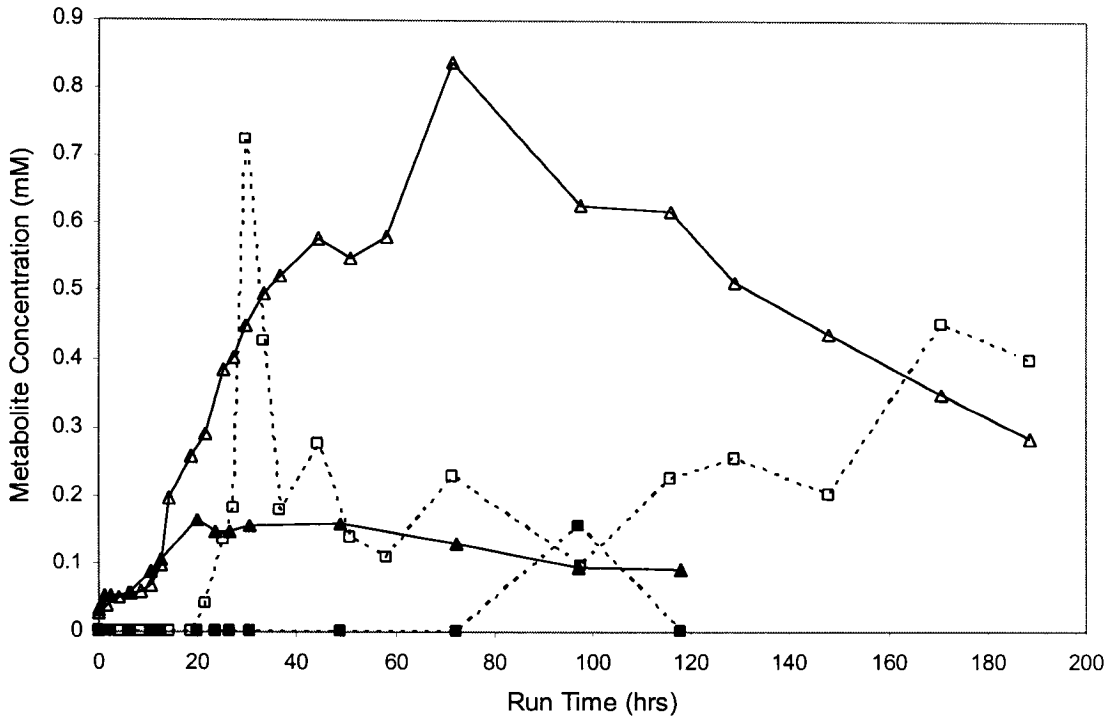

Figure 3-9 - The concentrations of metabolites during the degradation of D(EH)A by *B. subtilis*. Hollow symbols represent the concentrations of 2-ethylhexanol (Δ) and mono-2-ethylhexyl adipate (□) in the presence of surfactin, while filled symbols represent the concentrations of 2-ethylhexanol (▲) and mono-2-ethylhexyl adipate (■) in the control fermentation.

The dissolved oxygen concentration during these two experiments is shown in Figure 3-10. The first minimum in the profile, which occurred at the same time and had the same magnitude in both cycles, coincided with the peaks in protein concentration (shown in Figure 3-8). The second minimum occurred only in the presence of surfactin, and coincided with the maximum concentration of mono-2-ethylhexyl adipate in that cycle. The final dissolved oxygen concentration was lower for the cycle performed with surfactin added.

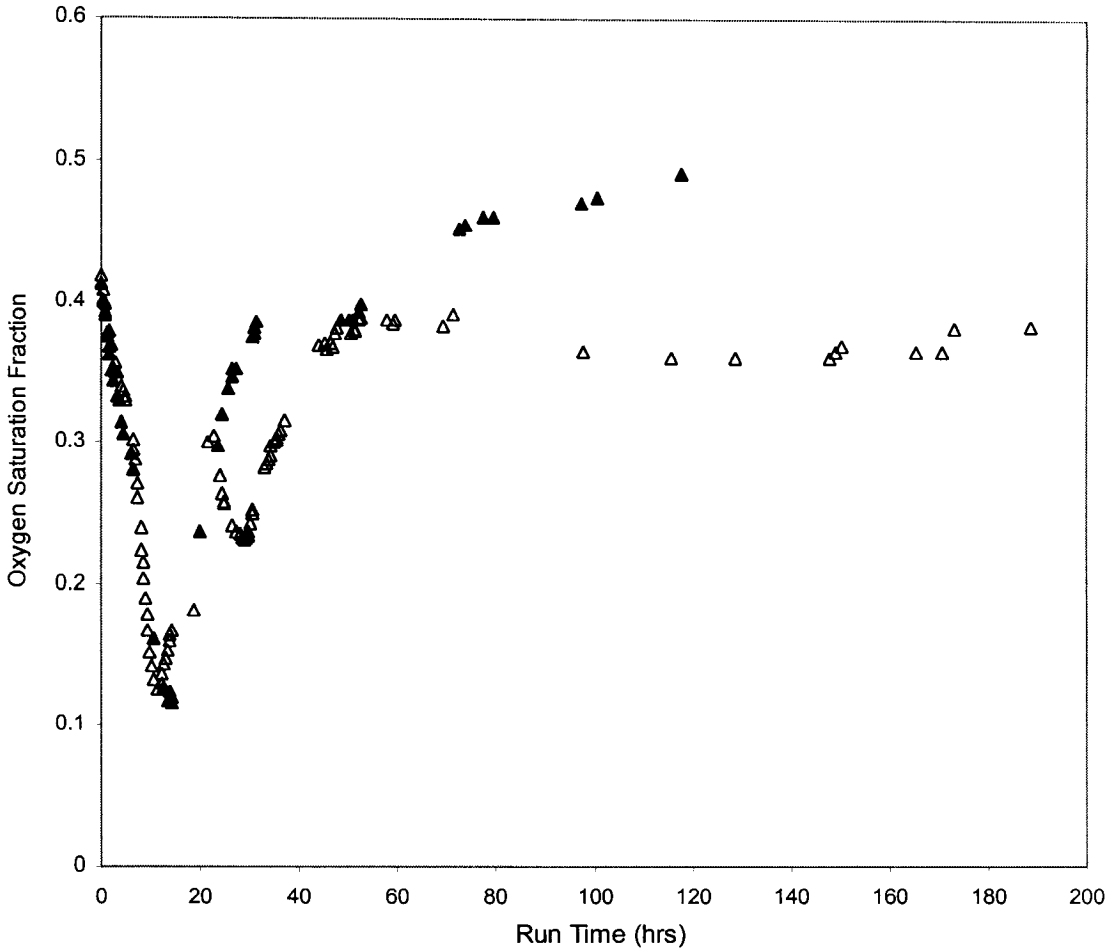

Figure 3-10 - The dissolved oxygen concentration in fermentations performed with by *B. subtilis* in the presence of D(EH)A with (Δ) and without (▲) surfactin added to the medium.

In order to eliminate any possible effect on metabolite accumulation due to the acclimation of the cells, *B. subtilis* ATCC 6633 was grown in parallel experiments in two cyclone reactors that were inoculated from the same medium. Surfactin was added to the medium in one reactor but not the other. The experiment was then repeated but the reactors used were switched. Figure 3-11 shows the glucose concentrations in both parallel fermentations. The consumption of glucose was not significantly affected by the presence of surfactin.

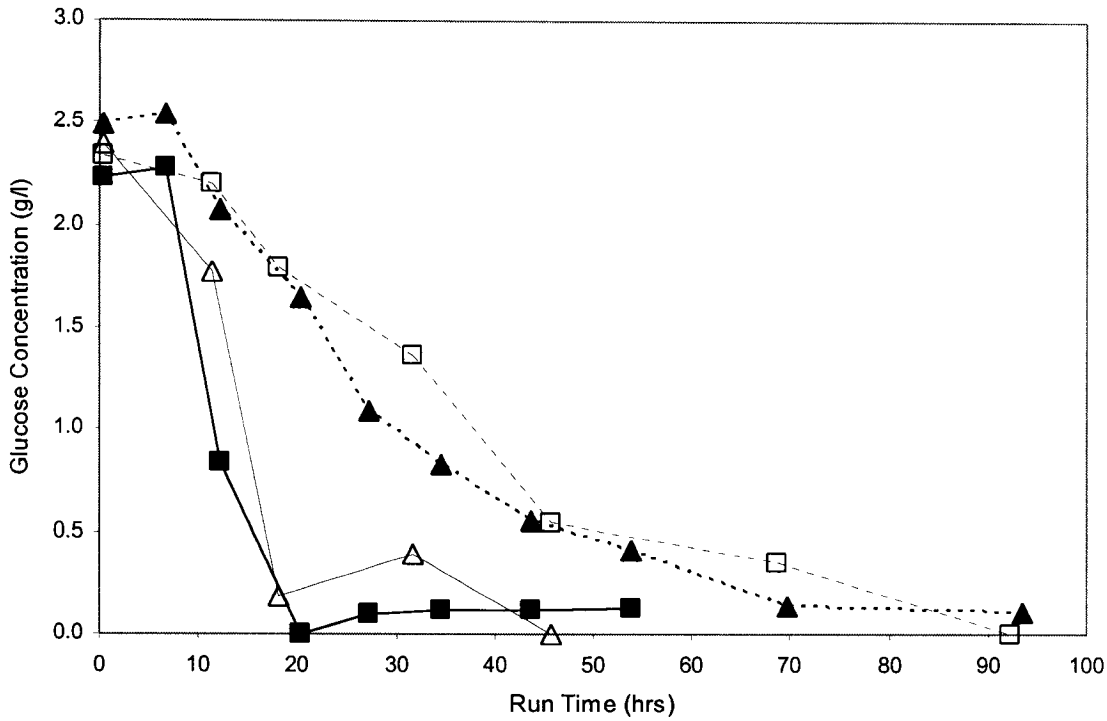

Figure 3-11 - Glucose concentration during the degradation of D(EH)A by *B. subtilis* in parallel reactors with and without surfactin. Runs 1A (Δ) and 2A (▲) were performed in the presence of surfactin. Runs 1B (□) and 2B (■) were the control fermentations performed in parallel with runs 1A and 1B respectively. The two line types (solid and dotted) represent each of the two reactors used.

The concentration of D(EH)A in these two parallel fermentations is shown in Figure 3-12. The plasticizer concentration in the reactors to which surfactin was added showed much higher initial values and apparently slower rates of hydrolysis. These results are misleading, however, because 5 mM D(EH)A was always added to the reactors, and the lower plasticizer concentrations in the control reactors were likely due to unrepresentative sampling due to heterogeneities in the mixture. Unlike in the sequencing batch cultures, the inoculation was performed by injection from a shake flask, resulting in considerably lower initial biomass concentrations. Previously, the biomass

concentration was high enough to solubilize the plasticizer by itself, whereas in this case it was not. This resulted in a medium that was not completely homogeneous.

Because of this uncertainty, it was important to verify that the plasticizer was actually degraded in the control reactor. Surfactin was therefore added at the end of the fermentation in order to solubilize any remaining D(EH)A that was previously not observable by sampling. As shown in Figure 3-13, no increase in plasticizer concentration was seen following the addition, which indicated that the measured D(EH)A concentration did indeed represent the amount of plasticizer left in the reactor. In fact, the concentration of the remaining plasticizer in the control reactor began to drop and was accompanied by an increase in 2-ethylhexanol concentration.

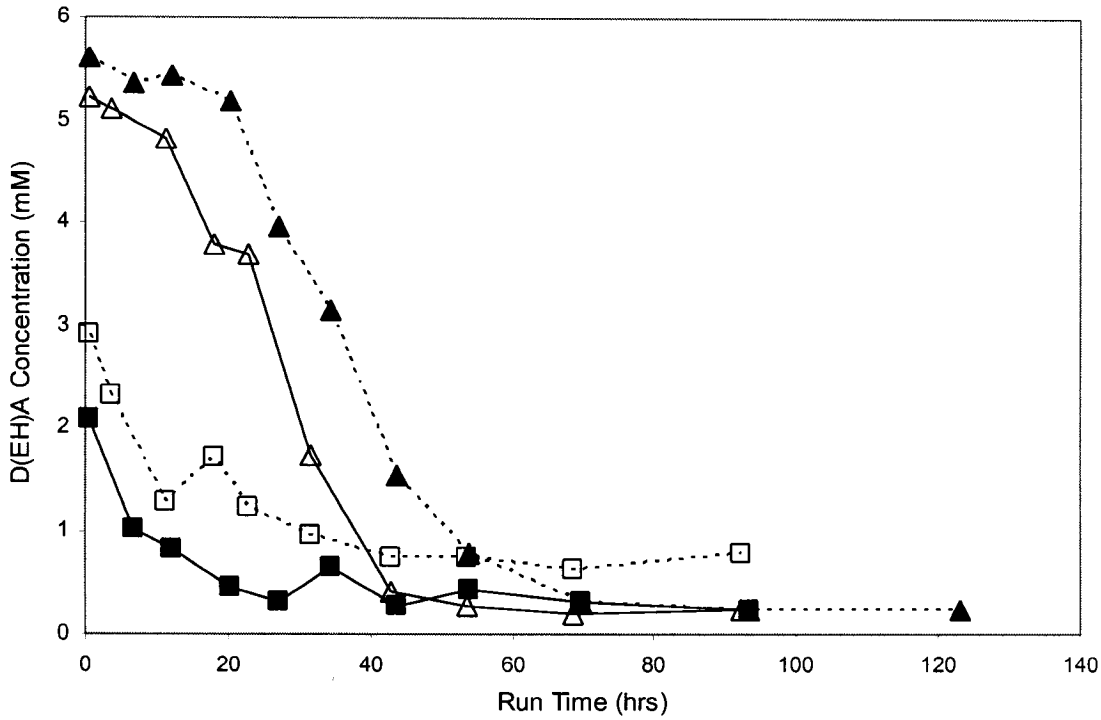

Figure 3-12 - Plasticizer concentration during the fermentation of *B. subtilis* in parallel reactors with and without surfactin. Runs 1A (Δ) and 2A (▲) were performed in the presence of surfactin. Runs 1B (□) and 2B (■) were the control fermentations without surfactin that were performed in parallel with runs 1A and 1B respectively. The two line types (solid and dotted) represent each of the two reactors used.

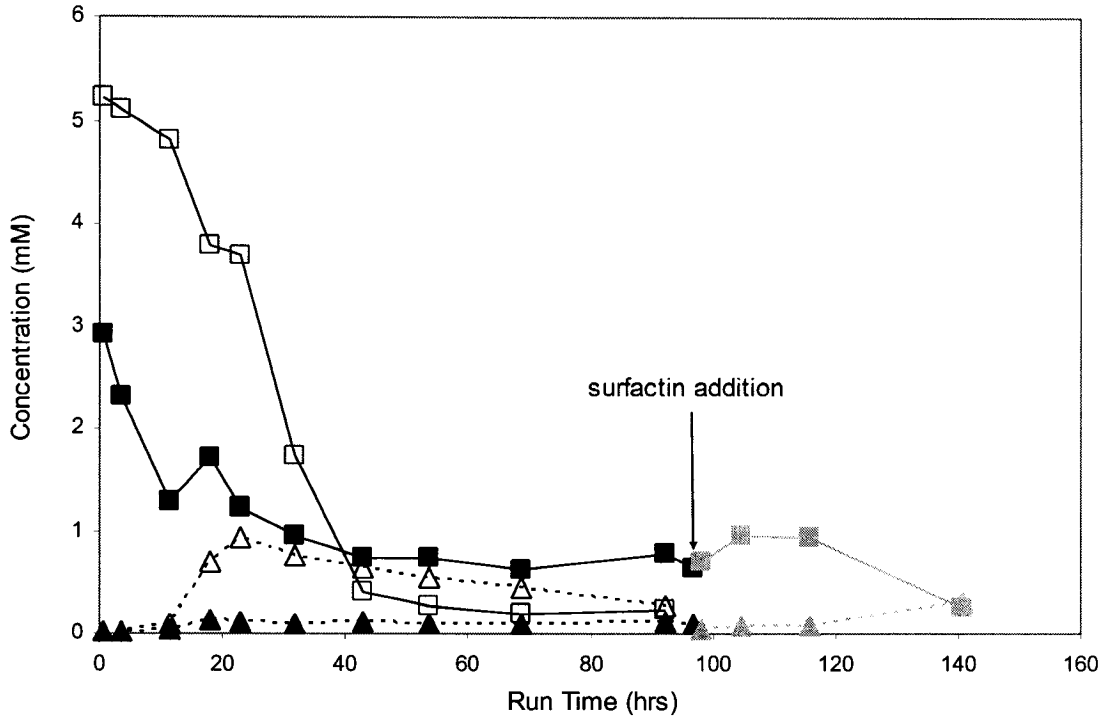

Figure 3-13 - The addition of surfactin to the control reactor to ensure that all of the plasticizer was degraded. The concentrations of D(EH)A (■,▩) and 2-ethylhexanol (▲,▲) are shown before the addition of surfactin (in black) and after (in red). Hollow symbols represent the concentrations of D(EH)A (□) and 2-ethylhexanol (Δ) in the fermentation to which surfactin wad added initially.

The concentrations of 2-ethylhexanol and mono-2-ethylhexyl adipate in these parallel fermentations are shown in Figures 3-14 and 3-15. The maximum amount of 2-ethylhexanol and the amount remaining at the end of the growth studies were both significantly higher in the presence of surfactin, regardless of the reactor used. Similarly, the concentration of mono-2-ethylhexyl adipate was always much higher in the presence of surfactin and was not dependent on the reactor (Figure 3-15).

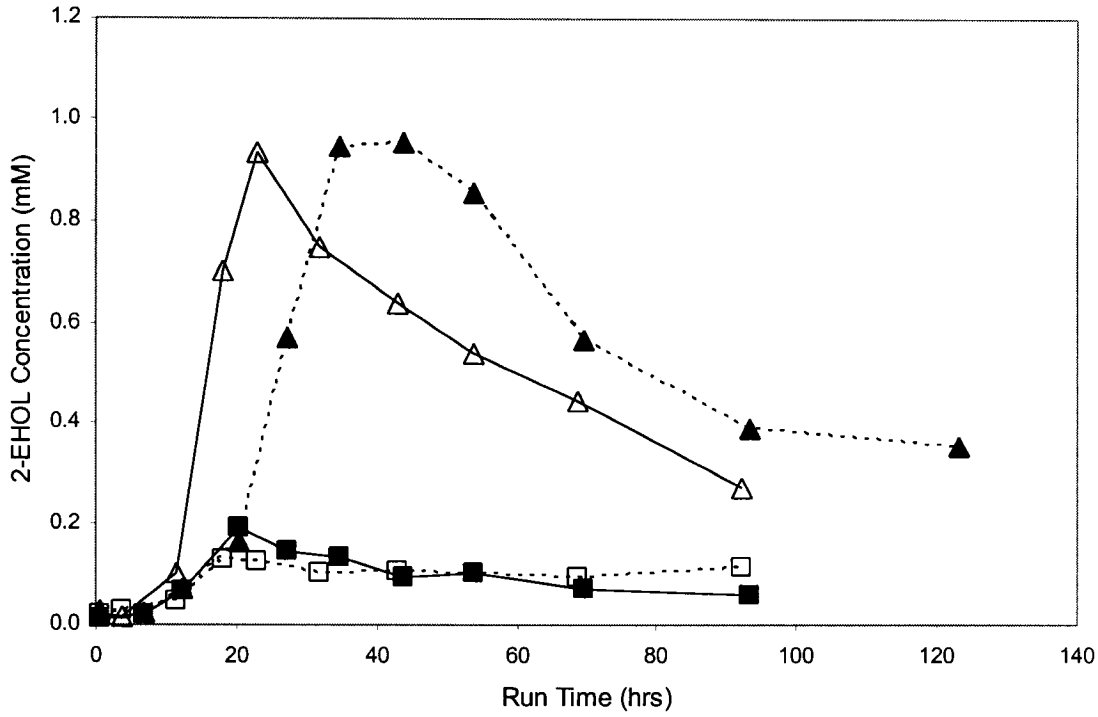

Figure 3-14 - 2-ethylhexanol concentration during the fermentation of *B. subtilis* in parallel reactors with and without surfactin. Runs 1A (Δ) and 2A (▲) were performed in the presence of surfactin. Runs 1B (□) and 2B (■) were the control fermentations without surfactin that were performed in parallel with runs 1A and 1B respectively. The two line types (solid and dotted) represent each of the two reactors used.

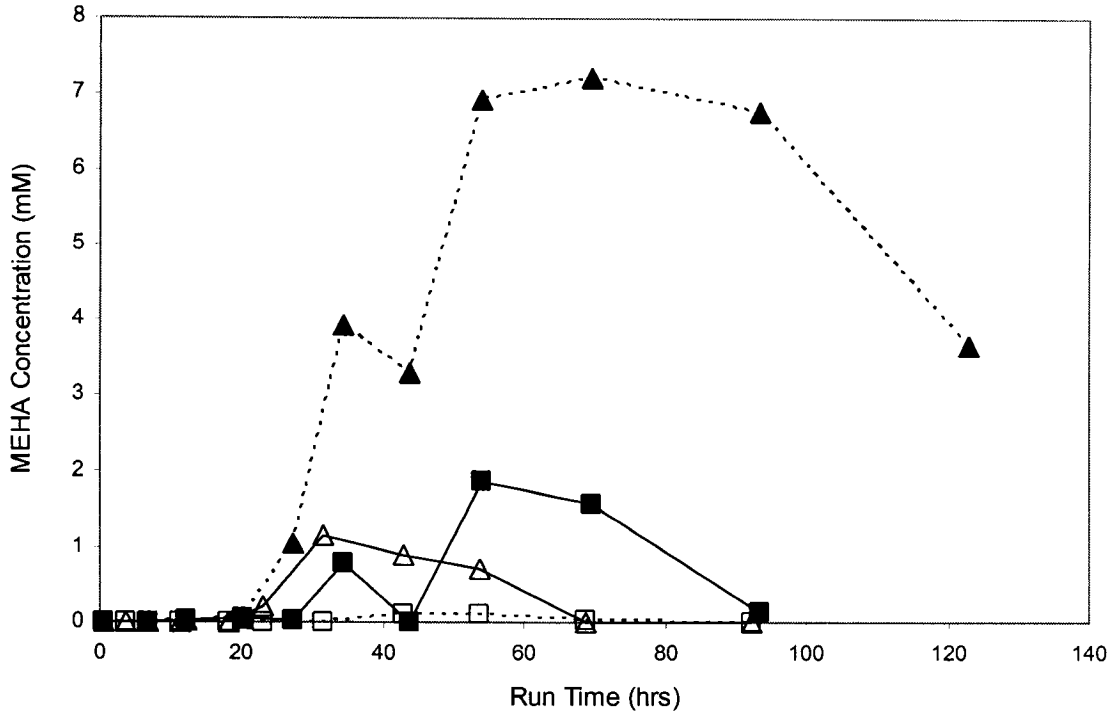

Figure 3-15 - Mono-2-ethylhexyl adipate concentration during the growth of *B. subtilis* in parallel reactors with and without surfactin. Runs 1A (Δ) and 2A (▲) were performed in the presence of surfactin. Runs 1B (□) and 2B (■) were the control fermentations without surfactin that were performed in parallel with runs 1A and 1B respectively. The two line types (solid and dotted) represent each of the two reactors used.

The parallel fermentations were repeated with the synthetic surfactant Pluronic L122. The D(EH)A and glucose concentrations are shown in Figure 3-16. As before, the reactor containing the surfactant showed significantly higher 2-ethylhexanol concentrations, as shown in Figure 3-17. Figure 3-17 also includes the concentrations of 2-ethylhexanol in the gas phase. These values are much lower than those in the aqueous phase but it can be seen that the amount of the metabolite in the gas phase was significantly higher when the surfactant was present in the medium.

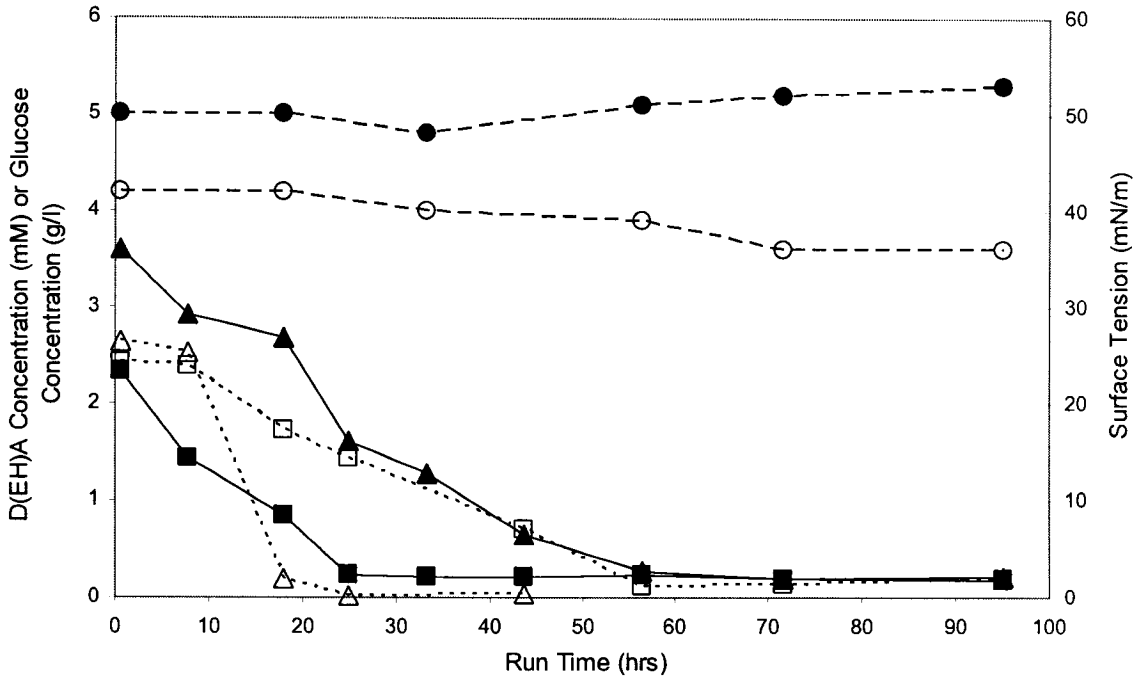

Figure 3-16 - D(EH)A concentration during the growth of *B. subtilis* in parallel reactors with (▲) and without (■) Pluronic L122 in the medium and the corresponding glucose concentration with (Δ) and without (□) the surfactant. The surface tension data are for media with surfactant added (○) and for the control experiment (●).

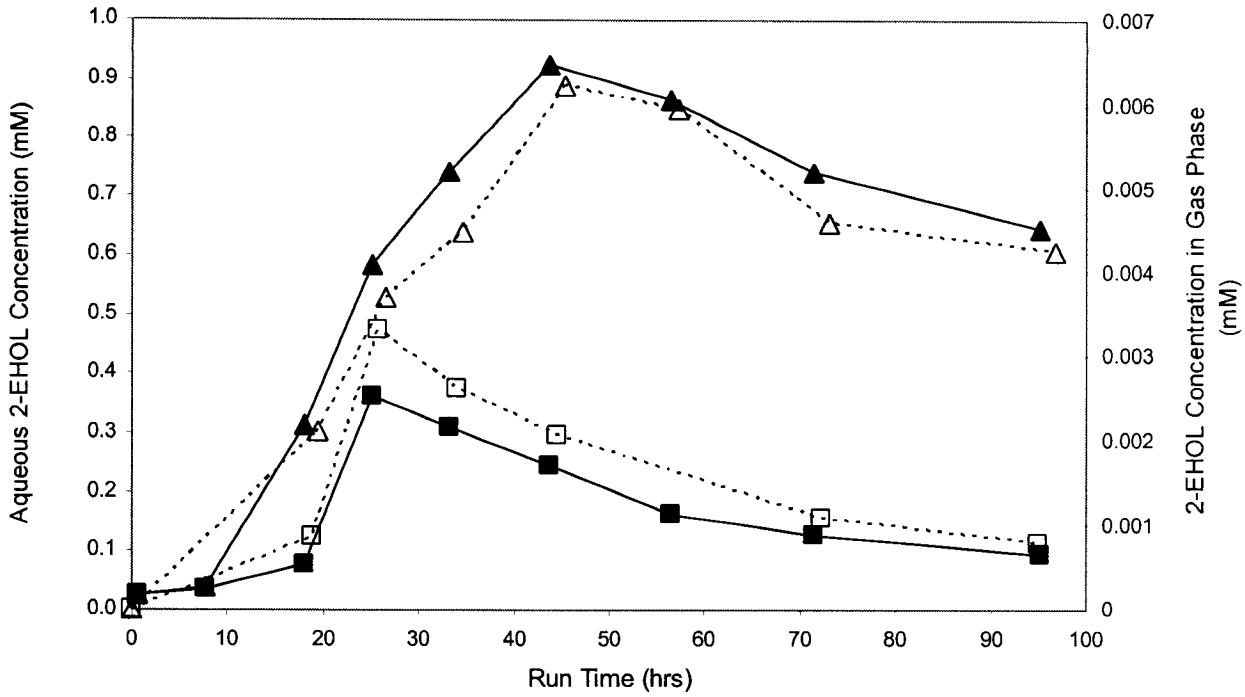

Figure 3-17 - 2-ethylhexanol concentration during the growth of *B. subtilis* in the presence of D(EH)A in parallel reactors with and without Pluronic L122. Shown are the 2-ethylhexanol concentrations in the aqueous phase with (▲) and without (■) Pluronic L122 in the medium. The concentration in the gas phase is also shown with (Δ) and without (□) Pluronic L122 in the medium.

3.3 Isolation of mono-2-ethylhexyl adipate

The isolation of mono-2-ethylhexyl adipate was performed after collecting the broth at the end of the fermentation shown in Figure 3-15, as described in Section 2.8. Analysis by GC revealed that the supernatant of the final harvest contained both 2-ethylhexanol and mono-2-ethylhexyl adipate. A surface tension of 20 mN/m at the end of the indicated that surfactin was also present. The small amount of 2-ethylhexanol present was removed under reduced pressure with the extraction solvent and this was verified by GC analysis. The mono-ester was extracted with chloroform from the aqueous phase at pH 7. Thin layer chromatography (TLC) showed that this did not

extract any of the small amount of surfactin present. The R_F of mono-2-ethylhexyl adipate for the solvent system used was determined to be 0.62.

In order to confirm its identity, the isolated mono-2-ethylhexyl adipate was analyzed by GC/MS. The mass spectrum of the compound, shown in Figure 3-18, was identified by the GC/MS spectrum library software (Qual Browser, Thermo Electron Corp., Mississauga, ON) as that of mono-2-ethylhexyl adipate.

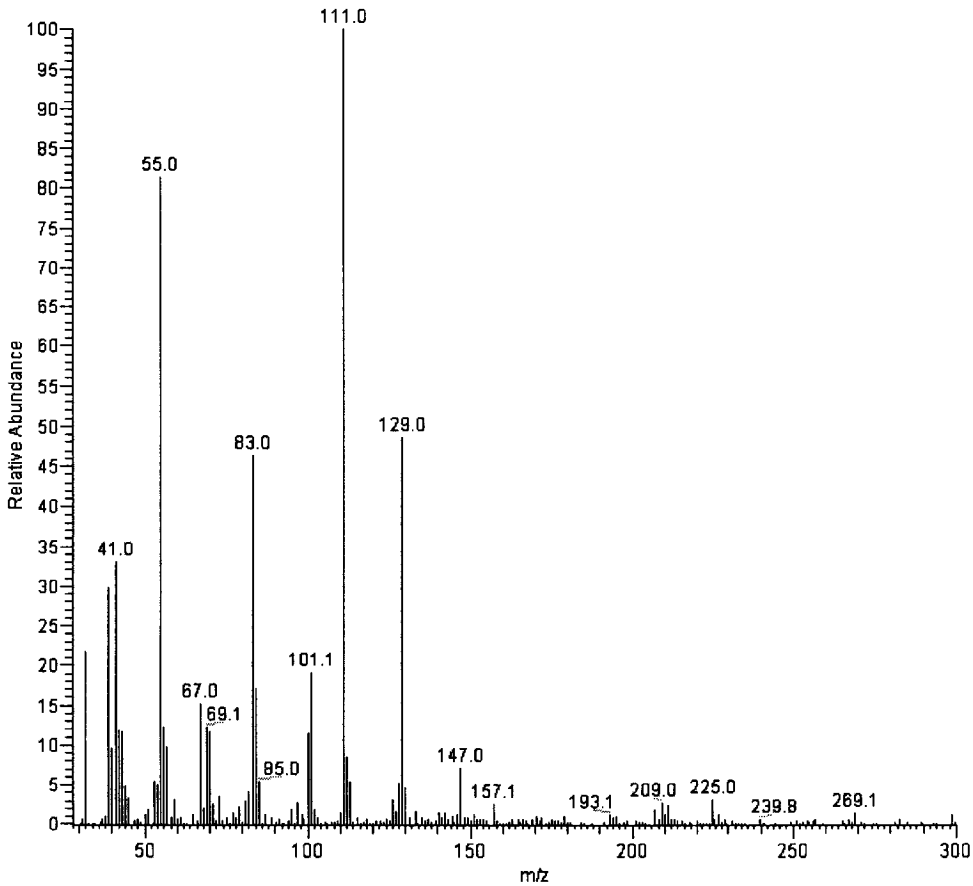

Figure 3-18 - Mass Spectrum of mono-2-ethylhexyl adipate.

3.4 Toxicity Results

The toxicity of the isolated mono-2-ethylhexyl adipate was obtained with the Microtox assay. The result is tabulated in Table 3-7 along with the toxicities of the other D(EH)A metabolites obtained previously by Nalli *et al* using the same method [35].

Table 3-7 - The toxicities of D(EH)A metabolites as obtained with the Microtox assay.

Compound	Concentration of Test Solution in MSM (g/l)[a]	EC_{50} (mM)
2-ethylhexanal	0.1	0.703[a]
2-ethylhexanol	0.1	0.054[a]
2-ethylhexanoic acid	0.1	0.729[a]
mono-2-ethylhexyl adipate	**0.1**	**0.079**

[a] Nalli *et al* [35].

The toxicity of 2-ethylhexanol to the growth of *B. subtilis* was also assessed. This was done by culturing the bacteria in media containing different concentrations of the compound and then observing the effect on growth as measured by protein concentration. Figure 3-19 shows the changes in protein concentration of *B. subtilis* cultures two days after the addition of 2-ethylhexanol to the initial media as well as one day after the addition of the alcohol to cells at the end of exponential phase that were previously grown in the absence of the compound. There was a small toxic effect on growing cells upon the addition of 2-ethylhexanol, and the effect increased gradually as the 2-ethylhexanol concentration was increased up to 4 mM, at which point there was almost no growth at all. This inhibition was less dramatic for cultures that had already completed their growth phase, with only the highest concentration of 2-ethylhexanol causing a decrease in protein concentration.

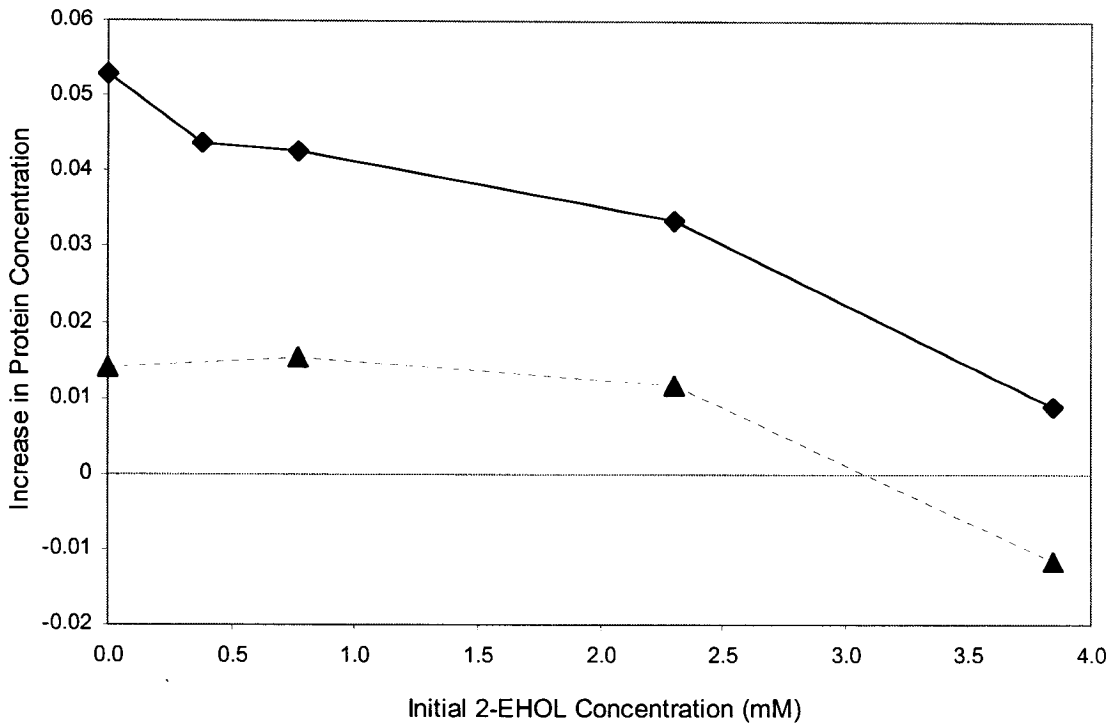

Figure 3-19 - The effect of the addition of 2-ethylhexanol on the growth of *B. subtilis*. Growth is reflected through an increase in protein concentration measured as a change in absorbance at 750 nm. 2-ethylhexanol was added to the initial medium (♦) and at the end of exponential phase (▲).

The concentration of 2-ethylhexanol was also measured over the course of the same experiment. Figure 3-20 shows that when less 2-ethylhexanol was added to the medium, a greater fraction of it was oxidized. For cells inoculated into a medium that already contained 2-ethylhexanol, the alcohol appeared to be more readily oxidized at concentrations below 0.8 mM. Though the concentration at which the alcohol became more difficult to oxidize was higher for cultures with more biomass (those having already completed their exponential growth phase), the trend was the same. Not surprisingly, Figure 3-20 also shows that more 2-ethylhexanol can be oxidized when more biomass is present.

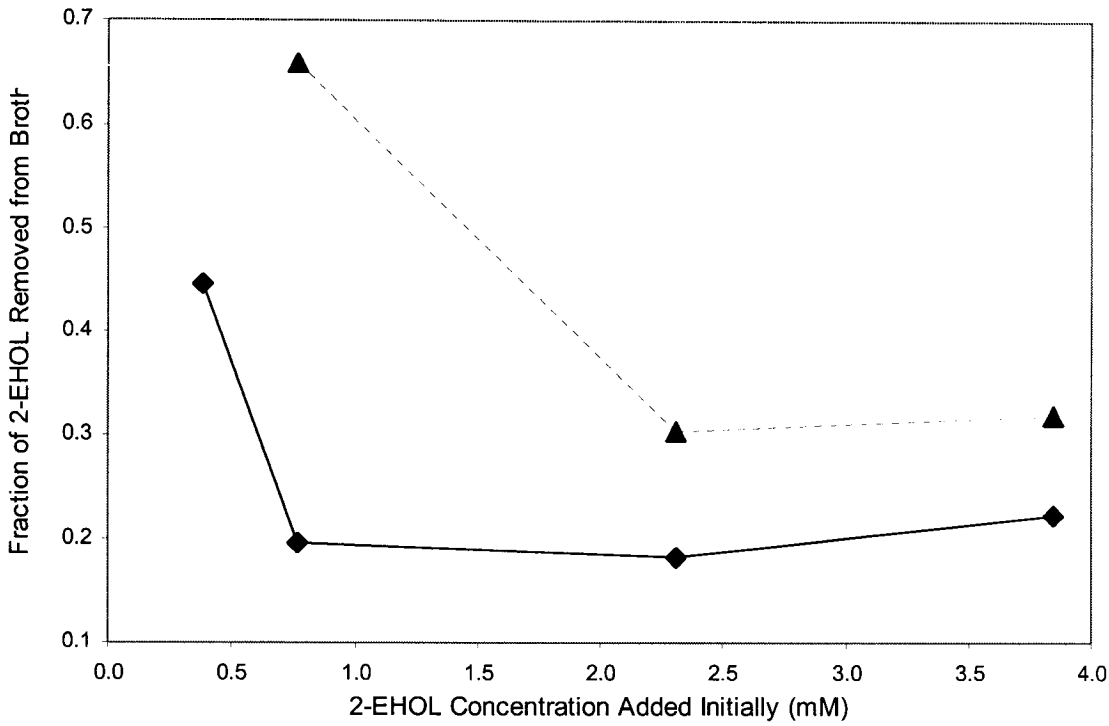

Figure 3-20 - The fraction of 2-ethylhexanol that was removed from *B. subtilis* cultures at different initial concentrations of the alcohol. 2-ethylhexanol was added to the initial media in some flasks (♦) and its concentration was measured after two days. It was added at the end of exponential phase (▲) to others, and its concentration was measured a day later.

3.5 Removal of 2-ethylhexanol in the Stream of Air

Figure 3-21 shows the results of the abiotic experiment in which 2-ethylhexanol had been added to sterilized shake flasks and allowed to evaporate. The evaporation rate of the 2-ethylhexanol, as can be seen in the figure, is approximately the same for the control flasks as for the flasks containing Pluronic L122.

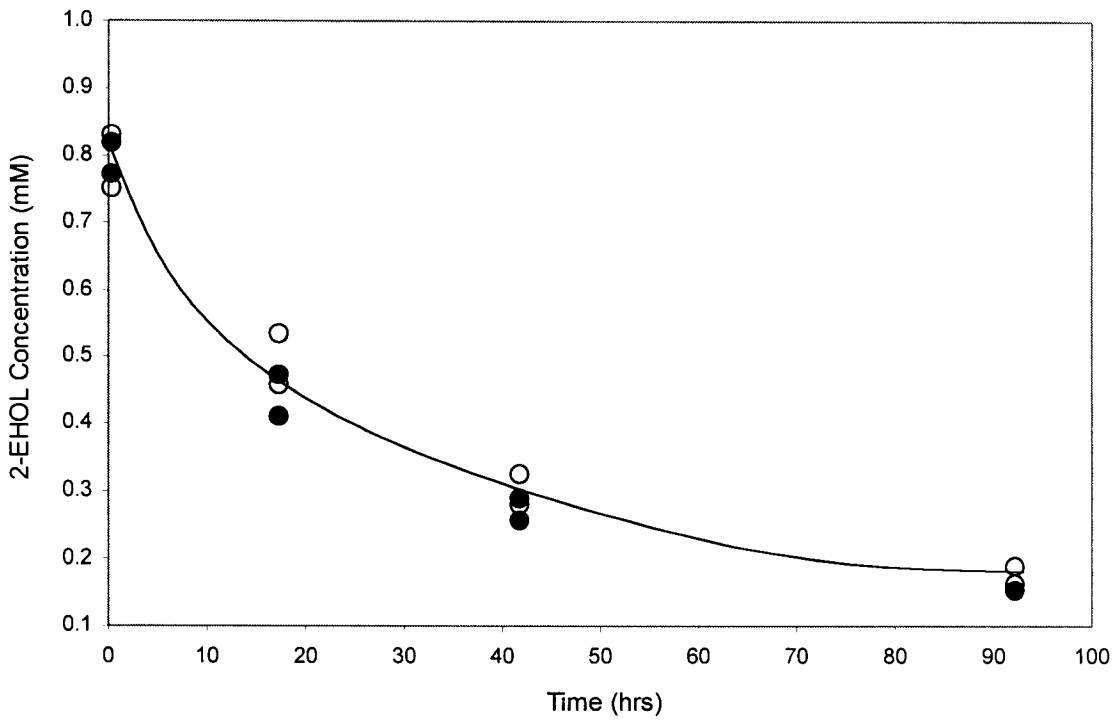

Figure 3-21 - Reduction in 2-ethylhexanol concentration due to vapourization from shake flasks at 25°C, 150 rpm with (○) and without (●) Pluronic L122 added.

3.6 Biomass Adherence to Hydrocarbons

The "biomass adherence to hydrocarbons" (BATH) assay was performed at two times in the growth of *B. subtilis* ATCC 6633 in shake flasks. The first time was at the point where the difference in observed metabolite concentrations was the largest and the second was at the end of the experiment. The results tabulated in Table 3-8 show that there was significantly more adherence to hexadecane in the presence of surfactin at the end of the fermentation. Earlier in the fermentation, however, the presence of surfactin had no effect on adherence to hexadecane when the organism was grown in a medium containing plasticizer.

Table 3-8 - BATH assay results for *B. subtilis* growing in the presence of D(EH)A and/or surfactin.

Surfactin Concentration	D(EH)A Concentration	% Adherence of Cells to C_{16} t = 46.5 hrs	t = 168 hrs
0 g/l	2.5 g/l	12.1 ± 4.6	1.8 ± 1.8
	0 g/l	3.6 ± 1.2	1.7 ± 1.7
0.04 g/l	2.5 g/l	13.0 ± 3.0	19.6 ± 2.8
	0 g/l	8.9 ± 2.0	30.7 ± 10.5

3.7 Filtration Experiments

Figure 3-22 shows the increasing amount of 2-ethylhexanol extracted from the filters following the filtration with increasing Pluronic L122 concentration in the solution. It should be noted that some 2-ethylhexanol was always retained in the filter even without any surfactant added, but the amount increased significantly when surfactant was present. The amount of the alcohol retained in the filters continued to increase gradually with more surfactant added, but the increase was less dramatic than the one observed for the

first addition of the surfactant. There was, nevertheless, a significant difference between 0.16 g/L surfactant (i.e., the concentration added to cultures) and the lowest concentration tested at 0.04 g/L. The differences in the concentrations of 2-ethylhexanol between the initial solution and the filtrate followed the same trend as seen in Figure 3-22 with increasing Pluronic L122 concentration (results not shown).

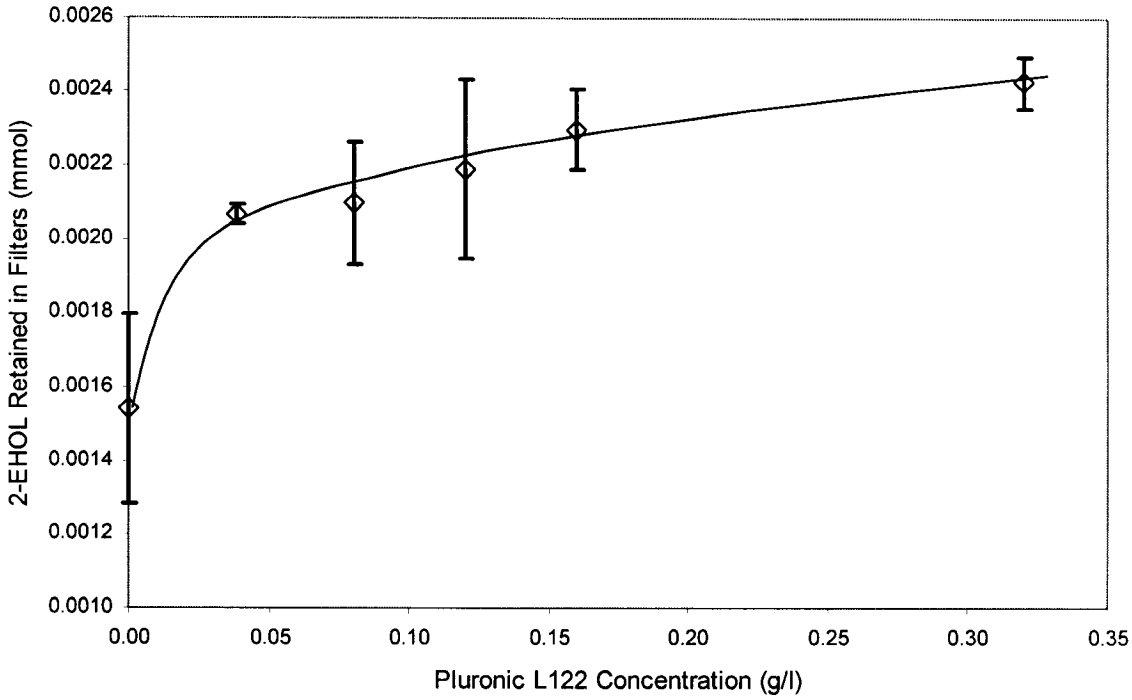

Figure 3-22 - The amount of 2-ethylhexanol trapped in the filters at different concentrations of Pluronic L122.

4. DISCUSSION

4.1 The Toxicity of Plasticizer Metabolites

Toxicities of metabolites from the degradation of di-ester plasticizers were previously determined by Nalli *et al* [35] and Gartshore *et al* [36] using the Microtox assay. Nalli *et al* measured the toxicities of all the metabolites from the degradation of D(EH)A (Figure 1-4) except for mono-2-ethylhexyl adipate. Those measured were all shown to be significantly more toxic than the parent plasticizer, which itself had a toxicity below the detectable limit [35]. In this work, high enough concentrations of mono-2-ethylhexyl adipate were attained to allow recovery of this compound. This was used to measure the toxicity.

Table 3-7 shows that both 2-ethylhexanol and mono-2-ethylhexyl adipate, the two compounds produced in the first step of the D(EH)A degradation pathway, have toxicities that are an order of magnitude greater than those of the other metabolites. The high toxicity of mono-2-ethylhexyl adipate is consistent with the result obtained by Gartshore et *al*, who found that the mono-ester metabolite of D(PG)DB was the most toxic intermediate in the degradation pathway of that plasticizer [36].

The toxicity of 2-ethylhexanol to the growth of the bacterium responsible for its release was also examined in this work. The addition of the alcohol at concentrations comparable to those observed during plasticizer degradation by *B. subtilis* did show inhibition of growth, although this was only observed for stationary phase cells at relatively high concentrations of the alcohol (Figure 3-19). However, 2-ethylhexanol at concentrations below these levels did cause inhibition of the oxidation of the alcohol to 2-ethylhexanal (Figure 3-20).

4.2 The Effects of Surfactants on Plasticizer Degradation

In previous work on the release and accumulation of plasticizer metabolites arising from microbial degradation [35], it was found that the first compounds produced from the hydrolysis of the ester bonds, 2-ethylhexanol and mono-2-ethylhexyl adipate, were either not observed or appeared briefly and then were further degraded (Figure 1-4). Only the 2-ethylhexanoic acid, the least toxic metabolite, accumulated at the end of the culture [35].

A very different pattern was observed for the work being presented here on the degradation of D(EH)A by *B. subtilis* in the presence of surfactants. There was a significant accumulation of the intermediates 2-ethylhexanol and mono-2-ethylhexyl adipate and these were often still present at the end of the experiments. These results are particularly significant because 2-ethylhexanol and mono-2-ethylhexyl adipate have been found to be the most toxic plasticizer metabolites tested to date (Table 3-7).

The use of surfactants is often considered as a method for improving the bioavailability of poorly soluble substrates [21, 40, 41, 45, 47, 48]. In the case of pollutants such as hydrocarbons, this is useful because their biodegradation removes potentially harmful compounds from the environment without adverse effects. The significant accumulation of 2-ethylhexanol and mono-2-ethylhexyl adipate, which was only observed in the presence of surfactants is, therefore, somewhat surprising. An increase in metabolite accumulation due to the addition of a surfactant was reported previously by Willumsen *et al* [42]. The authors found that an unidentified metabolite accumulated in the culture broth of *Sphingomonas paucimobilis* using fluoranthene as sole carbon source, but only in the presence of Triton X-100.

A possible factor that could have contributed to the larger concentrations of 2-ethylhexanol and mono-2-ethylhexyl adipate is that a larger amount of the plasticizer may have been hydrolyzed when a surfactant capable of solubilizing it was present. Surfactants have been shown to disperse similar insoluble compounds into water [20, 21, 42, 48]. Shake flask experiments are subjected to only a gentle agitation and in all of these experiments the plasticizer floated in large droplets at the air-water interface unless a surfactant was added to disperse it. When sufficient surfactant was added, the medium became a homogeneous emulsion. The solubilization of the plasticizer could increase its bioavailability to the microorganisms, which would subsequently increase its hydrolysis rate, thereby resulting in an increase in the release of 2-ethylhexanol and mono-2-ethylhexyl adipate.

Since the metabolites from the hydrolysis of the plasticizer (Figure 1-4) are considerably more water-soluble than the parent plasticizer, it would not be expected that the rates of their degradation would be significantly increased by the presence of a surfactant. An enhanced rate of production of the first intermediates coupled with an unaltered rate of further degradation would lead to an accumulation of these compounds. The results from the experiments performed in shake flasks with a strain of surfactin-producing *B. subtilis* support this argument. The amount of surfactin produced in each shake flask was variable but the relative amount present was directly related to the degree of lowering of surface tension in the media. Increased amounts of surfactin in the media resulted in a larger degree of removal of each of the three plasticizers (Figure 3-3). Furthermore, when surfactin was isolated and then added to cultures of the strain of *B. subtilis* that could not produce this biosurfactant, the organism appeared to hydrolyze the

plasticizer at a faster rate (Figure 3-5). This was accompanied by a greater accumulation of both 2-ethylhexanol and mono-2-ethylhexyl adipate (Figure 3-6), the first two metabolites of the degradation pathway (Figure 1-4).

This enhanced degradation rate of a potential carbon source might be expected to lead to more biomass. The concurrent protein curves in Figure 3-5 showed that the microbial growth was not affected by the presence of surfactant or the accompanying increase in plasticizer hydrolysis. It is likely that the culture was growing on glucose as the primary growth substrate. As such, a higher rate of D(EH)A hydrolysis would not lead to significantly more growth or enzyme production. In other words, more enzymes capable of further degrading plasticizer metabolites would not be produced simply because more plasticizer was hydrolyzed. The microbes appeared to be degrading the plasticizer fortuitously using constitutive enzymes. Thus, the hydrolysis of more plasticizer would not subsequently cause an increase in the rate of oxidation of 2-ethylhexanol once it was released. If surfactin increased the bioavailability of the plasticizer, an increase in the rate of D(EH)A hydrolysis would be expected. But without an accompanying increase in 2-ethylhexanol oxidation rate, this would lead to higher concentrations of the side chain intermediate when surfactin is present in the medium.

In light of the shake flask experiments it was surprising that the results of the larger scale growth studies did not indicate any effect of the presence of the biosurfactant on the rate of plasticizer hydrolysis (Figure 3-7). However, the concentrations of 2-ethylhexanol and mono-2-ethylhexyl adipate still reached much higher values when surfactin was present (Figure 3-9). In fact, the difference was even more dramatic than in shake flasks. The observation that the addition of surfactin had no effect on the

plasticizer hydrolysis rate for the larger scale experiments can be attributed to the much better degree of mixing in the cyclone reactor than in the shake flasks. The homogeneity of the medium was also increased by running the reactor in sequencing batch mode, which allowed higher initial biomass concentrations than by inoculating from flasks. Since the cells themselves act as an emulsifier, a homogeneous medium could be obtained even with poorly soluble substrates and no surfactant added, as long as the biomass concentration was sufficiently high. In shake flasks experiments, however, hydrophobic phases are not well distributed and their dispersal by a surfactant can significantly increase growth rates [40, 46, 47].

The more important observation during the reactor runs was the very large accumulation of the first metabolites of the plasticizer degradation pathway. Because the rate of hydrolysis of the first ester bond was the same with and without surfactin, the greater accumulation of 2-ethylhexanol and mono-2-ethylhexyl adipate in the reactor could only be explained by a lower rate of removal of these compounds once they were released.

A possible explanation for the negative effect on the rate of degradation of the first metabolites could be some specific interaction of the small lipopeptide, surfactin, with the enzymes involved in the subsequent steps of the plasticizer degradation pathway. An alternative explanation would be that the surfactant properties of surfactin were the cause of the phenomenon. If the first explanation were true, it would not be expected that the effect of other surfactants would be similar to that of surfactin. As Tables 3-5 and 3-6 show, however, several other surfactants also caused the build-up of the first

intermediates. This suggested that the increased concentrations of the early metabolites were likely due to a different type of interaction.

Alcohols, particularly long chain alcohols, have been shown to interact closely with surfactants in an aqueous medium [67-74]. As they have a somewhat hydrophilic OH group and a hydrophobic aliphatic chain, it is not surprising that they can act as co-surfactants. They have been shown to form mixed micelles with surfactant molecules and subsequently change the surface chemistry of the mixture [67-72]. Such an alcohol-surfactant interaction could have lowered the availability of 2-ethylhexanol to the enzymes responsible for its oxidation. A similar explanation was suggested by Willumsen *et al* for the increased accumulation of fluoranthene metabolites in the presence of Triton X-100. It was suggested that the metabolites released during the degradation of fluoranthene may have associated with surfactant micelles and therefore become less bioavailable [42].

Figure 3-22 shows that the interactions between 2-ethylhexanol and Pluronic L122 form stable aggregates such that 2-ethylhexanol is filtered from the solution despite the fact that its concentration is below the solubility limit. This verified that an interaction between the surfactant and the 2-ethylhexanol occurred. The addition of alcohols was previously shown to change the CMC, CMT, and cloud points of several surfactants [68, 70, 72], as well as changing micellar size and stability [67, 69, 73]. 2-ethylhexanol, more specifically, possesses antifoaming properties when added to some surfactant solutions [75]. It has also been suggested that it interacts with bis-2-ethylhexyl sulfosuccinate micelles and affects their degree of packing [76].

It is therefore reasonable to propose that the 2-ethylhexanol interacted with the surfactants in such a way that they were sequestered from the enzymes that would have oxidized them to the next metabolite in the proposed pathway. The enzymes responsible for this step most likely belong to either the alcohol dehydrogenase or cytochrome p450 families [77-79], and these tend to be membrane-bound or intracellular enzymes. A 2-ethylhexanol molecule bound inside a large Pluronic L122 aggregate would be difficult to access for membrane-bound enzymes and probably completely inaccessible to intracellular enzymes.

A similar effect could easily occur with the mono-2-ethylhexyl adipate, which consists of a hydrophobic chain with an acid group at one end. A surface tension drop was almost always observed in the fermentations when mono-2-ethylhexyl adipate was detected (e.g., as seen in Figure 3-4). This revealed that mono-2-ethylhexyl adipate itself possessed surfactant properties and therefore could also interact with the added surfactants, thereby decreasing its bioavailability.

If an interaction of the metabolites with the surfactants decreased the rates of their further degradation, it raised the question of why this effect was not observed for the parent plasticizer. The reason for this is likely that the interaction between the intermediates and the surfactants is different from the solubilization of an oil by a surfactant. The presence of these surfactants has always either increased or not affected the primary degradation rate of the plasticizer. Therefore, the esterases responsible for the initial hydrolysis step always had at least as much access to the plasticizer when the surfactants were present as when they were not.

Though all three surfactants that led to higher intermediate concentrations possess different chemical structures, they do have a common property that may elucidate why there is no negative effect on plasticizer hydrolysis: All three have been shown to deviate from the behaviour of classic micelle-forming surfactants [38, 80, 81]. For example, surfactin has been observed self-assembling in β-pleated sheet structures [38]. It has been suggested that the lactone form of the sophorolipid used in this work assembles into insoluble monolayers at interfaces and does not form micelles [80]. Pluronic L122, a PEO-PPO-PEO block co-polymer, is known to form aggregates in solution before the air-liquid interface is saturated with the surfactant [81]. It has been postulated that these block co-polymer surfactants form micelle-like structures through folding, with the hydrophobic PPO segments tending towards the core of the structure and the more hydrophilic PEO segments tending towards the outside [82]. Bi-layer vesicles with hydrophilic cores have also been proposed [83]. In all three cases, it can be expected that the solubilized compound, such as an oily plasticizer, would be contained within the hydrophobic "pockets" of the aggregates. Due to the complexity in structure and packing, the aggregates formed by these surfactants may be larger and more loosely packed than classical micelles and therefore would not create a significant barrier for hydrolyzing enzymes to penetrate and attack the solubilized plasticizer.

A surfactant-alcohol interaction seemed the most likely explanation for the increased accumulation of 2-ethylhexanol, but several other possibilities were also considered. One of these is a possible increase in cell hydrophobicity induced by the added surfactant. During all the fermentations performed in reactors, 2-ethylhexanol was always released in concentrations below its solubility level. This means that a water-

soluble compound was released upon transformation of a hydrophobic one. A more hydrophobic cell would have a greater tendency to adhere to the oily plasticizer rather than be dispersed in the aqueous phase along with the water-soluble metabolites. This could decrease the accessibility of those metabolites for further degradation, causing them to reach higher concentrations.

The results of the BATH assay experiment shown in Table 3-8 showed that the surfactin did increase the hydrophobicity of the biomass significantly, but only late in the fermentation (i.e., only after the growth phase had been completed). The large difference in 2-ethylhexanol concentration between fermentations with and without surfactin occurred considerably earlier in the culture. At this point, the surfactin did not affect cell adherence to hydrocarbon when D(EH)A was present, and only slightly when there was no plasticizer in the medium. It follows that an increase in cell hydrophobicity was not responsible for the decrease in 2-ethylhexanol oxidation in the reactor runs when surfactant was added.

Another possible explanation for a surfactant causing an alteration in the relative concentrations of the various intermediates is its potential effect on the abiotic removal of these compounds by volatilization into the gas phase. 2-ethylhexanol is volatile and disappeared fairly quickly from the aqueous phase both in the reactor and in flasks, even without any microorganisms present. During the actual growth studies, it was the only intermediate detected in significant concentrations in the gas phase of the reactor. Figure 3-21 shows the removal of 2-ethylhexanol from abiotic flasks occurring at approximately the same rate, both with and without Pluronic L122 added. This result revealed that there was no significant decrease in the evaporation rate of 2-ethylhexanol

in the presence of the surfactant that would account for the greater accumulation of the alcohol in the aqueous phase.

The surfactants could have an indirect action on the degradation of plasticizers if they inhibited growth of the cells. For example, sophorolipids were previously shown to be toxic to the growth of a strain of *B. subtilis* [84]. But in all of the experiments performed, the surfactants that led to higher intermediate concentrations did not adversely affect the growth of *B. subtilis* ATCC 6633. The resultant increased concentrations of these intermediates also did not affect the growth of the cells, as can be seen from the nearly identical protein concentration profiles in the broth observed during the fermentations. As shown in Figure 3-19, the concentration at which 2-ethylhexanol became toxic to stationary phase cells (approximately 3 mM) is considerably higher than the concentrations typically reached during plasticizer degradation (approximately 1 mM).

The binding of the early intermediates to surfactant aggregates as they were released from the hydrolysis of the plasticizer does offer an explanation for their greater accumulation in the presence of surfactants. This significant increase in 2-ethylhexanol and mono-2-ethylhexyl adipate accumulation has important environmental implications because surface active agents are present throughout the environment [85-87]. These could be synthetic surfactants or biosurfactants produced by various microorganisms – especially in sites containing hydrophobic waste materials. Thus, there will be many situations in which both plasticizers and surface active compounds are both present. As microorganisms degrade the plasticizers, the properties of some of these surface-active agents could lead to an accumulation of the most toxic metabolites resulting from the

hydrolysis of the ester bonds. There would be a build up of 2-ethylhexanol and mono-2-ethylhexyl adipate instead of the less acutely toxic 2-ethylhexanoic acid.

5. CONCLUSION

While surface-active agents can be useful in the biodegradation of hydrophobic pollutants, the presence of diester-plasticizers in these wastes could dramatically reduce this benefit. It has already been established that some of the metabolites from the biodegradation of these plasticizers are significantly more acutely toxic than the original compounds. The work presented here shows that if certain surfactants are added to the system, the concentrations of the most toxic intermediates of the plasticizer degradation pathway can be increased significantly by inhibiting the rate of conversion of these initial intermediates to the next intermediates in the breakdown pathway.

Thus, surfactants can be beneficial by increasing plasticizer bioavailability and thus raise the likelihood of partial degradation. Their presence, however, can also be detrimental by increasing the relative concentrations of 2-ethylhexanol and mono-2-ethylhexyl adipate.

6. REFERENCES

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A. APPENDIX

A.1 Typical Gas Chromatogram

An example of a gas chromatogram is shown in Figure A-1. This spectrum was from the extract of a sample tken in the final stages of a fermentation performed in a 1-litre cyclone reactor with *B. subtilis* ATCC 6633 in the presence of surfactin. The operating conditions of the GC used are described in Section 2.6.3. The chromatogram shows the relative peak positions of the plasticizer, D(EH)A, as well as the two metabolites of primary interest, 2-ethylhexanol and mono-2-ethylhexyl adipate. The first and largest peak was the extraction solvent, chloroform, and the peak occurring at 5.5 minutes was the internal standard, n-pentadecane. The mono-2-ethylhexyl adipate assignment was made on the basis of GC-MS analysis.

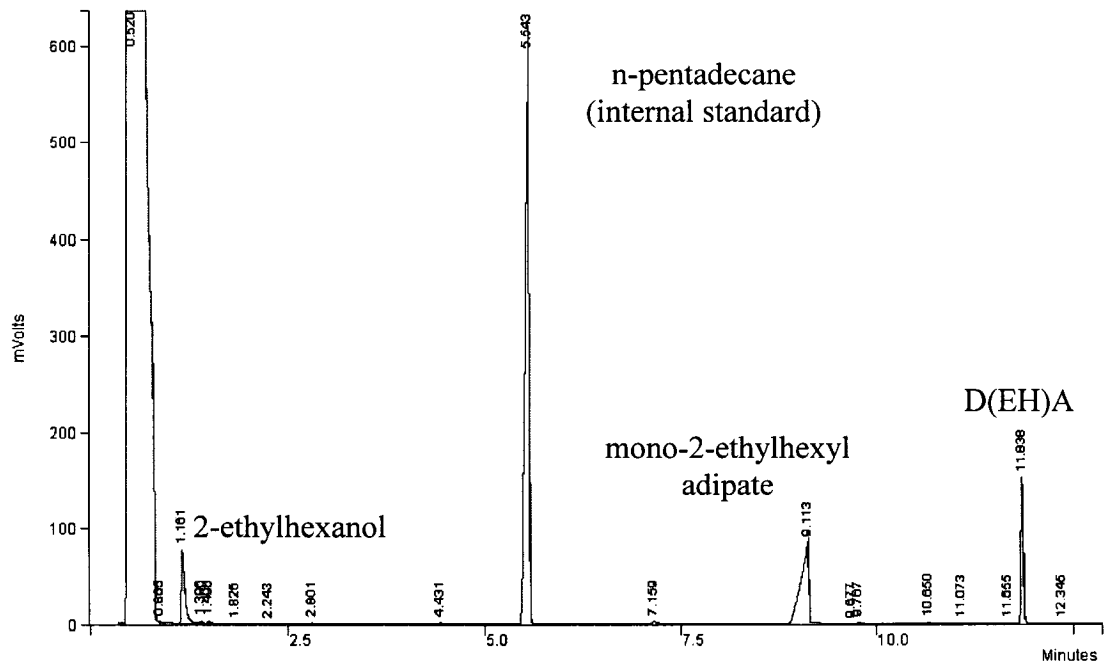

Figure A-1 - Gas chromatogram of the chloroform extraction of a sample taken from the reactor

A.2 SPME Calibration

The SPME calibration (Figure A-2) for the concentration of 2-ethylhexanol in the gas phase was developed using a cyclone reactor. 2-ethylhexanol was added to the sterile MSM medium in the reactor and its aqueous concentration was measured over time as described in Section 2.6.1 for reactor samples. Samples were taken in pairs, one shortly after the other, and an SPME measurement of the gas phase was taken in between the two (as described in Section 2.6.2).

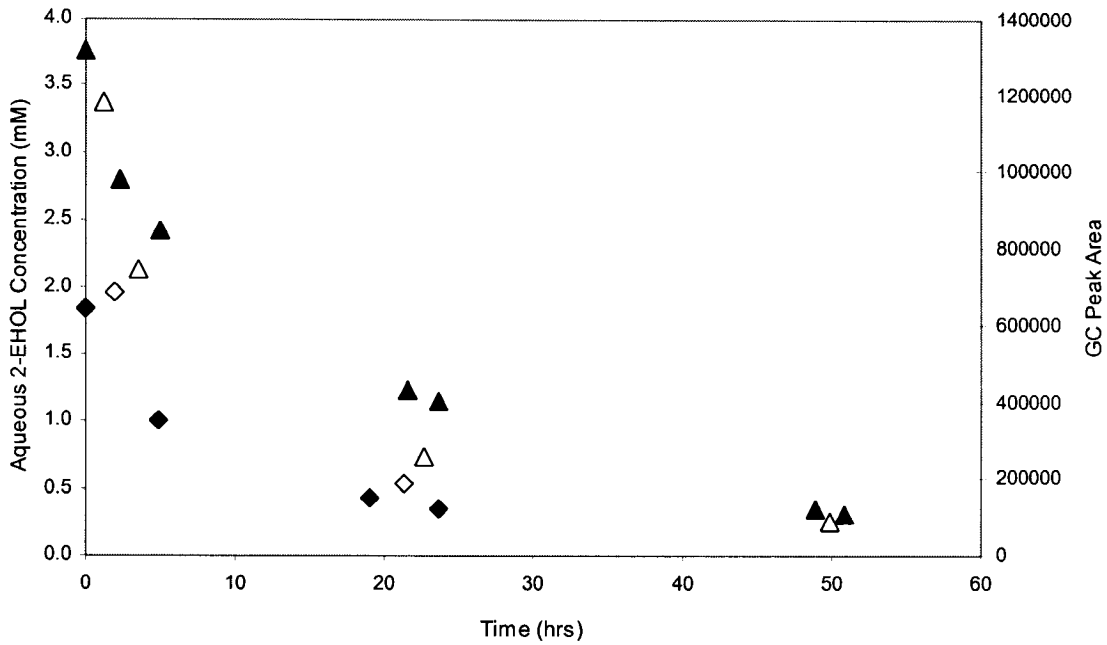

Figure A-2 - The aqueous phase concentration of 2-ethylhexanol in a cyclone reactor and the corresponding GC peak areas obtained by SPME from the gas phase. The aqueous concentration of 2-ethylhexanol is shown for two experiments (▲, ♦) with the respective SPME data (Δ, ◊).

The concentration of 2-ethylhexanol in the aqueous phase could be assumed to decrease linearly between samples taken in close succession. The GC peak area obtained from SPME analysis between the samples, therefore, was assumed to represent a constant gas phase concentration of the alcohol that would result from this linear decrease in aqueous concentration.

The flow of air into the reactor was maintained constant at 213 ml/min. Because the reactor was sealed tightly except for the outlet at the top of the condenser (Figure 2-1) and because the aqueous medium was saturated with air, it was assumed that the airflow through the reactor and at the outlet was also 213 ml/min. Given the volume of the reactor (V), the change in the concentration of 2-ethylhexanol in the aqueous phase (ΔC_{aq}) during the time between aqueous samples (Δt), and the airflow through the reactor (Q_{air}), the gas phase concentration of the alcohol (C_{gas}) was calculated using the following equation:

$$C_{gas}[mmol/l] = \frac{\Delta C_{aq}[mmol/l]}{V[ml] \times \Delta t[\text{min}]} \times Q_{air}[ml/\text{min}]$$

The calculated gas phase concentrations were then plotted versus the peak areas from the corresponding SPME chromatograms to produce the calibration curve, shown in Figure A-3.

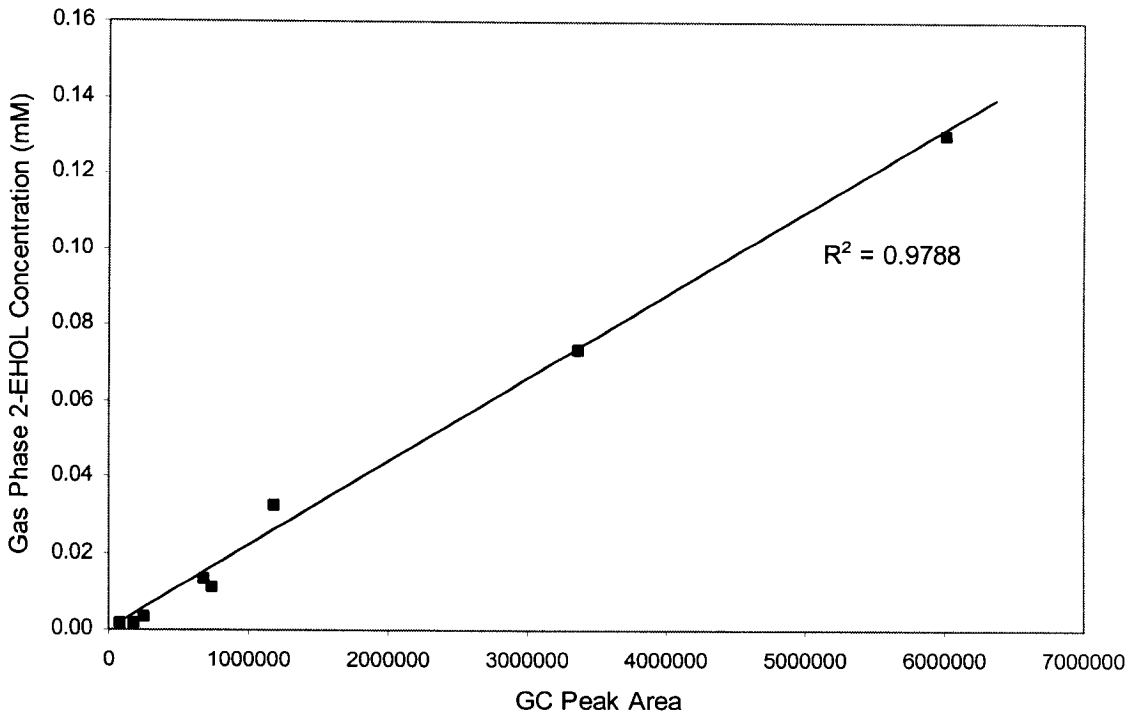

Figure A-3 - SPME calibration curve for the gas phase concentration.