MICROBIAL ESTERASE AND THE DEGRADATION OF PLASTICIZERS

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Introduction

Plastic materials are playing an increasingly important role in our everyday lives. As a consequence, they are more frequently exposed to the environment; and the impact they or their additives might have on the latter becomes an important concern.

Plasticizers are the most common additives found in plastics. They are used to improve on their physical properties (e.g. flexibility, workability). They are frequently found in PVC, where they can make up to 67% of the total weight of plastics [1]. They are encountered in a wide variety of products including building materials, blood bags, toys, cosmetics, inks, insect repellent and electrical wiring insulation [2-5]. Di-ester plasticizers represent the most common class of plasticizers. Of those, di-ester phthalates, in particular di-2-ethylhexyl phthalate (DEHP), are the most widely produced and used [1]. Plasticizers are not covalently bonded to the plastic polymer matrix and, consequently, have the ability to leach out of the material. This, along with the omnipresence of plastic materials, explains why plasticizers have been found in landfill leachates [6, 7], open waters [4, 8-10], food [11], drinking water, precipitations and soils [9]. In fact, *Ribbons et al.* stated that DEHP was found in 42% of the environmental samples where it was sought [12]. Plasticizers are therefore considered ubiquitous contaminants of aqueous and soil environments. In fact, the extent of the environmental release has lead to the inclusion of six phthalates, including DEHP, in the United States Environmental Protection Agency list of priority pollutants [13].

Due to their stability, biodegradation is the most likely means of plasticizer degradation in the environment. Studies conducted with pure strains [12, 14-16] or consortia [17, 18] of microorganisms have shown that di-ester plasticizers could be readily degraded by many organisms under a wide array of conditions. However, some recent studies have shown that the degradation of compounds such as DEHP and di-2-ethylhexyl adipate (DEHA) was only partial and that the resulting metabolites were recalcitrant and increased the toxicity of the aqueous system [16, 19, 20]. These metabolites have been found in diverse environmental samples [9]. It then becomes important to understand the factors influencing the degradation mechanisms of plasticizers and their long term impact on the environment.

The first two steps in the biodegradation of di-ester plasticizers involve the hydrolysis of the ester bonds by esterase or lipase enzymes. Previous studies have isolated the esterases involved in the hydrolysis of di-ester phthalates and adipates from different microorganisms [21-23]. However, these studies were concentrating on the characterization of the enzymes and not on the factors affecting the kinetics of hydrolysis. The present research focused on the identification of an esterase from a bacterium, and the identification of the main factors influencing the associated rates of hydrolysis.

Materials and Methods

Bacterium and Medium

The bacterium used for this research, *Rhodococcus rhodochrous* ATCC 13808, was obtained from the American Type Culture Collection. It was selected for its ability to degrade hydrophobic compounds including di-ester plasticizers [16, 24, 25].

The bacteria were grown in 8 g/L nutrient broth or in growth media composed of mineral salt medium at pH 7 (4-g/L NH_4NO_3, 4-g/L KH_2PO_4, 6-g/L Na_2HPO_4, 0.2-g/L $MgSO_4 \cdot 7H_2O$, 0.01-g/L $CaCl_2 \cdot 2H_2O$, 0.01-g/L $FeSO_4 \cdot 7H_2O$, and 0.014-g/L Na_2EDTA), 0.1-g/L yeast extract and a carbon source. The carbon source was either hexadecane, adipic acid, DEHA, or a combination of these. It should be noted that NaOH was added to medium containing adipic acid to maintain a neutral pH.

Growth Conditions

The bacterium was grown at room temperature (~20°C) in either a 2-L New Brunswick glass

cylindrical batch reactor or a 4-L glass cyclone reactor with working volumes of 1.5L and 1L respectively. In both cases the medium was circulated through a centrifugal pump to optimize mixing. Sterile air was supplied at a rate of 1 L/min and 0.2 L/min for each reactor.

The microorganisms were grown in a sequential batch mode in the cyclone reactor. This mode of operation, in which half of the culture is discarded and replaced by fresh medium at the end of the exponential growth phase, allowed for rapid growth, abundant and reproducible levels of biomass.

The biomass concentration was measured by optical density at 500nm (OD_{500}) using a UV spectrometer (Varian, Cary 50 Bio) and by dry cell weight. The latter method involved centrifuging broth for 10 min at 10,000g (IEC centrifuge, B-22M), resuspending the pellet in distilled water and drying the resuspended biomass for 12-16h at 80°C.

Esterase Activity Assays

Two assays were used to measure esterase activity at 30°C. The o-nitrophenyl acetate hydrolysis assay was derived from *Krebsfanger et al.* [26]. 100 μL of methanol solution containing 0.052g/L of o-nitrophenyl acetate were added to 1.9mL of sample in a glass optical cell. The production of o-nitrophenol was monitored at a wavelength of 412nm using a UV-spectrophotometer (Varian, Cary 100 Bio). The initial rate of o-nitrophenol production was converted to give esterase activity units of mmol o-nitrophenyl acetate hydrolysed per litre per min.

The second assay was based on the hydrolysis of butyl butyrate. The latter was added to a 5-mL sample in a test tube to a concentration of 3mmol/L. The test tube was incubated in a shaker (New Brunswick Scientific, Model G-25) at 30°C and 250rpm for 10 min. After this period, the remaining butyl butyrate was extracted using 5mL of chloroform containing pentadecane as an internal standard. Analysis was performed by gas chromatography (Varian, CP-3800, Supelco SPB-5 column). The units were converted to mmol of butyl butyrate hydrolysed per litre per minute.

Preparation of Cell Fractions

In order to identify the location of the esterase activity in the microorganism, cells were separated in three fractions.

Broth was collected and centrifuged (10,000g, 10min). The supernatant phase was recovered and kept as the extracellular fraction. The pellet was resuspended in mineral salt medium (pH 7) and cells were lysed using a Bioneb® Cell Disruption system. The sample obtained was centrifuged as stated above. The supernatant was recovered and kept as the intracellular fraction. The pellet was resuspended in mineral salt medium (pH 7) and kept as the membrane-bound fraction.

Solubilization of Esterases

It was possible to extract the enzymes involved in the hydrolysis of di-esters from the cell membrane by treatment with the non-ionic detergent Triton X-100. Samples containing living biomass were centrifuged as stated above. The supernatant was discarded and the pellet was resuspended in phosphate buffer (0.1M, pH 7) containing 0.1% w/v Triton X-100. The samples containing detergent were mixed for 30min at room temperature using a magnetic stir plate. They were then centrifuged and the cell-free supernatants were tested for esterase activity.

Comparing Rates of Hydrolysis

A method similar to the butyl butyrate hydrolysis assay was developed for different di-esters: DEHA, di-methyl phthalate, di-ethyl phthalate, di-n-butyl phthalate, di-n-hexyl phthalate, and DEHP. Enough substrate was added to 5-mL samples of membrane-bound cell fraction to obtain a concentration of 3mmol/L. The samples were then incubated for a predetermined hydrolysis time (8h for DEHA, 45min for di-methyl phthalate and di-ethyl phthalate, 4h for di-n-butyl phthalate, 20h for di-n-hexyl phthalate, and 100h for DEHP — see Table 1) before being extracted with chloroform containing pentadecane. The extracted samples were analysed by gas chromatography.

Samples containing different quantities of esterases were obtained through dilutions or by sampling at different stages of growth. For each 10-mL sample obtained, 5mL were tested with one of the substrates mentioned above and the remaining 5mL were tested with butyl butyrate. It was then possible, by using butyl butyrate as a standard, to obtain a relative rate of hydrolysis for each of the substrates tested. This relative rate of hydrolysis was considered an appropriate tool for comparison between substrates; it had units of mmol substrate hydrolysed per mmol butyl butyrate hydrolysed.

Results and Discussion

Location of the Esterase Activity

An esterase activity sample was performed on a broth sample containing 1g/L of biomass. Its activity was assessed a value of 100%. Cells from the same sample were separated into extracellular, intracellular and membrane-bound fractions.

Esterase assays were performed on each fraction and their activities were quoted relative to that of the

broth sample. By comparing these results (Figure 1), it was determined that the enzymes responsible for the esterase activity were located in the membrane of the cell. The low levels of esterase activity seen in the extracellular and intracellular fractions were likely due to carry over of cell debris. It is important to note that similar trends were seen independently of the substrate used for growth. Moreover, the same trends were observed at all stages of growth in a reactor set-up (data not shown). These two results seemed to indicate that the enzymes of interest were constitutive. They were produced at all times by the bacteria and did not require a specific substrate for their production or activation.

R. rhodochrous is an organism that is known to interact with hydrophobic compounds. In fact, the organism itself can display hydrophobic characteristics [27, 28]. For example, in the presence of an oily compound, it will have a tendency to stay at the water/oil interface. This could partially explain why *R. rhodochrous* can readily degrade most di-ester plasticizers — many of which have very low solubilities in water. This, along with the fact that the enzymes hydrolysing the ester bonds of plasticizers are located on the cell membrane, could represent an advantage when facing the problem of bioavailability of hydrophobic compounds.

While the fact that the enzymes of interest could hydrolyse hydrophobic compounds(DEHP, di-n-hexyl phthalate, DEHA) would normally be associated with lipases, the same enzymes were hydrolysing small water soluble esters (butyl butyrate, o-nitrophenyl acetate, di-methyl phthalate) faster (Table 1). This lead to the conclusion that the enzymes observed were in fact esterases.

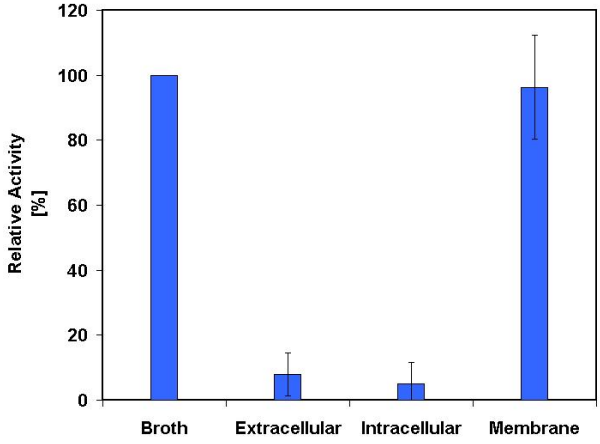

Figure 1: Relative esterase activity of different cell fractions for *R. rhodochrous* grown on hexadecane.

Solubilization of Esterases

In an attempt to further characterize the enzymes responsible for the hydrolysis of di-ester plasticizers, the former were extracted from the cell membrane by treatment with Triton X-100. The extracted protein samples displayed esterase activity (Figure 2), indicating solubilization by the non-ionic detergent. However, the solubilization was not complete as some activity remained in the treated cell samples. Moreover the extent of the solubilization was quite different from one extraction to the next. On the other hand, the esterase activity was not deterred by the solubilization process. The addition of the esterase activity of the treated cells to that of the extracted proteins was equal, within error, to the esterase activity of the non-treated cells in the presence of Triton X-100.

This solubilization technique allowed for a proper initial step for the purification and identification of the enzyme(s) responsible for the initial hydrolysis of di-ester plasticizers.

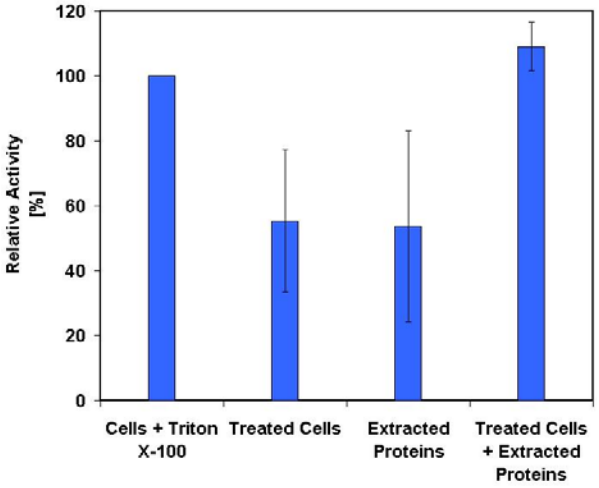

Figure 2: Relative esterase activity of *R. rhodochrous* cells before and after protein solubilization by treatment with Triton X-100 in phosphate buffer.

Comparing the Rates of Hydrolysis

The rate of hydrolysis assays were performed on membrane-bound cell fraction samples. This was done to discard any masking effect due to growth of the cells. Because the cells were lysed, preferential hydrolysis of one compound over another could not be attributed to it being a more appropriate substrate for the bacterium.

It is important to note that when comparing the rate of hydrolysis of a substrate to that of butyl butyrate, the correlations were always linear (data not shown). This led to believe that the same enzymes were involved in the hydrolysis of all the compounds tested. This was also in accordance with the fact

that many esterases exhibit broad substrate specificity.
The range of relative rates of hydrolysis observed covered as much as 4 orders of magnitude (Table 1), with butyl butyrate having the fastest rate and DEHP having the slowest. This wide range indicated that some factors differentiating each compound tested were playing an important role in determining these rates of hydrolysis.

Table 1: Characteristic hydrolysis times and relative hydrolysis rates for plasticizers and other esters tested.

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

Previous studies have stated that the solubility of di-ester plasticizers might be an important factor regulating their degradation [1, 29]. The results obtained in the present study were consistent with such observations. However, some discrepancies were seen from this trend; it was suspected that steric hindrance played an important role for compounds such as DEHP, mostly due to the presence of side-chains. This was also observed by *Eljertsson et al.* [1] when phthalate di-esters were biodegraded under methanogenic conditions.
These results imply that, considering their slow rates of hydrolysis, many di-ester plasticizers will continue to accumulate in the environment. Of most concern is the fate of DEHP and DEHA; both of these compounds are often only partially metabolized, yielding more toxic metabolites. Therefore, the results of the present study indicate that, assuming the current trends in production and use of these plasticizers will remain, the concentrations of both plasticizers and their metabolites will continue to increase in the environment.

Conclusion

The esterase activity associated with the hydrolysis of ester bonds of di-ester plasticizers by *R. rhodochrous* was found to be situated in the membrane of the bacterial cell. It was possible to partially solubilize the enzyme(s) responsible for the hydrolysis by treatment with the non-ionic detergent Triton X-100.
The rates of hydrolysis of different esters (including many di-ester plasticizers) were compared. It was found that the rates were greatly influenced by both solubility and steric hindrance. These results raise concern on the fate of plasticizers (particularly DEHP) and their metabolites in the environment. The findings implied that these compounds will continue to accumulate in the environment and that they will continue their release of recalcitrant metabolites.

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