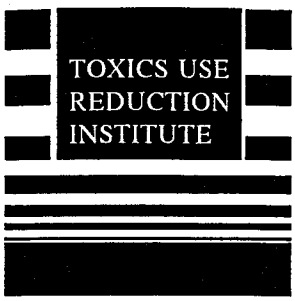

THE MASSACHUSETTS
TOXICS USE REDUCTION INSTITUTE

BIOLOGICAL SYNTHESIS OF CHEMICALS AND MATERIALS:

PRODUCTION OF SUBSTITUTED PARA-POLYPHENYLENE

Technical Report No. 2 **1992**

University of Massachusetts Lowell

BIOLOGICAL SYNTHESIS OF CHEMICALS AND MATERIALS:

PRODUCTION OF SUBSTITUTED PARA-POLYPHENYLENE

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The Toxics Use Reduction Institute Research Fellowship Program

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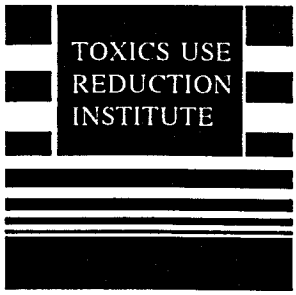


The Toxics Use Reduction Institute is a multi-disciplinary research, education, and policy center established by the Massachusetts Toxics Use Reduction Act of 1989. The Institute sponsors and conducts research, organizes education and training programs, and provides technical support to governments to promote the reduction in the use of toxic chemicals or the generation of toxic chemical byproducts in industry and commerce. Further information can be obtained by writing the Toxics Use Reduction Institute, University of

PREFACE

In 1991 the Toxics Use Reduction Institute established the Research Fellows Program at the University of Massachusetts Lowell (UML). The Research Fellows Program funds toxics use reduction research projects performed by a graduate student and their advisor. The goals of the Research Fellows Program are:

- to develop technologies, materials, processes, and methods for implementing toxics use reduction techniques,
- to develop an understanding of toxics use reduction among UML graduate students and faculty,
- to facilitate the integration of the concept of toxics use reduction into UML research projects,
- to provide UML faculty with "incubator" funding for toxics use reduction related research, and
- to act as a liaison between Massachusetts industries and UML faculty.

The types of projects funded through the Research Fellows Program are technology, methods, and policy research projects. Each final project report is published by the Institute. The opinions and conclusions expressed in this Research Fellow report are those of the authors and not necessarily those of the Toxics Use Reduction Institute.

Introduction

The long-term goal of this project is the investigation of biological processes for the production of chemicals and materials. This **specific** project is a two-year effort focused on the production of substituted para-polyphenylene. The *key intermediate* in this process (4,5-dihydroxy-4,5-dihydrophthalate) is produced by a mutant *Psedomonas* (CWL-phth-UV6) isolated in this work. Sodium phthalate is used as a starting material replacing benzene that has previously been used in the production of para-polyphenylene. Successful completion of the experimental studies will lead to an economical production system for a high strength and heat resistant polymer. Successful development of this process will reduce toxic chemical use in production as well as providing a product that can be used as an asbestos substitute.

The specific goals of this two-year project are:

1. Isolation of a microorganism that can grow on phthalic acid as a sole carbon source

2. Isolation of a mutant strain that produces the polymer intermediate 4,5-dihydroxy-4,5-dihydrophthalate.

3. Conduct a systematic evaluation of parameters (carbon source, energy source, pH, temperature, trace nutrient, etc.) that will optimize the production of 4,5-dihydroxy 4,5-dihydrophthalate.

4. Develop purification techniques to recover pure 4,5-dihydroxy-4,5-dihydrophthalate from fermentation media.

5. Develop immobilized microbial cell reactors to increase productivity.

6. Conduct a systematic experimental study of the radical polymerization of 4,5-dihydroxy-4,5-dihydrophthalate.

Research Methodology

Culture Isolation

Aerobic organisms that utilize phthalic acid as a sole carbon source were isolated by utilizing oil and solvent contaminated soil (UMASS Lowell Paint Shop) spread onto minimal media plus 0.1% phthalate agar plates and incubated at 25 C for four days. Minimal media, adjusted to pH 7.0 with NaOH, contained the following (g/L): NH_4SO_4, 1; $MgSO_4$, $7H_2O$, 0.5; NaCl, 0.25; $CaCl_2$, 0.25; $FeCl_3$, 0.02; $MnSO_4$, 0.002; $ZnSO_4$, 0.002; $CuSO_4$, 0.002.

Characterization

Isolated cultures were characterized by Gram stain and by Oxi/Ferm Tube (Roch Diagnostic Systems). One isolate (CWL-phth) was further studied.

Mutant Isolation

The isolate (CWL-phth) was grown to log phase at 30 C on minimal media plus 0.1% yeast extract and 0.1% phthalic acid. The culture was exposed to UV irradiation for various times (1, 5, & 10 minutes) in petri dishes. A one ml sample, of the irradiated culture, was then used to innoculate a flask of minmal media plus 1.0% succinate and inncubated overnight.

The culture was harvested by centrifugation and washed twice with minimal media. One ml of the culture was resuspended in 100 mls of minimal media plus 0.1 % phthalic acid and incubated at 30 C until the culture reached an OD of 1.0 (absorbtion @ 540 nm). Penicillin G was then added (10,000 units/ml) and the culture was incubated overnight at 30 C. The culture was harvested by centrifugation, washed twice with minimal media and resuspended in minimal media. The culture was then plated out onto minimal media plus 1.0% succinate plates. The colonies that grew were replica plated to plates containing minimal media plus phthalate or succinate. Those colonies failing to grow on the phthalate plates were investigated further.

Analytical Procedures

Metabolites were assayed with a high-performance liquid chromatography (HPLC) gradient elution system consisting of a Bio-Gel TSK-DEAE-5-PW column (BIORad). The separation conditions were: Buffer A 0.1 M Tris pH 7.0, Buffer B 0.1 M Tris pH 7.0 plus 1.0 M NaCl, with a gradient of 0 to 100% B in fifteen minutes.

Immobilization Procedure

A 100 ml overnight culture of CWL-phth-UV6 grown on minimal media plus 0.1 % yeast extract and 1.0 % glucose was harvested by centrifugation and resuspended in one ml of minimal media. An equivalent volume of 3% agar solution (42 C) was added and mixed to obtain a homogeneous solution. Dry porous silica particles (Hi-Sil 210, PPG Industries, 20/30 mesh size) were added and mixed rapidly until all particles were evenly coated.

All materials used were sterilized in an autoclave, except the support material, which was placed in a 200 C oven overnight.

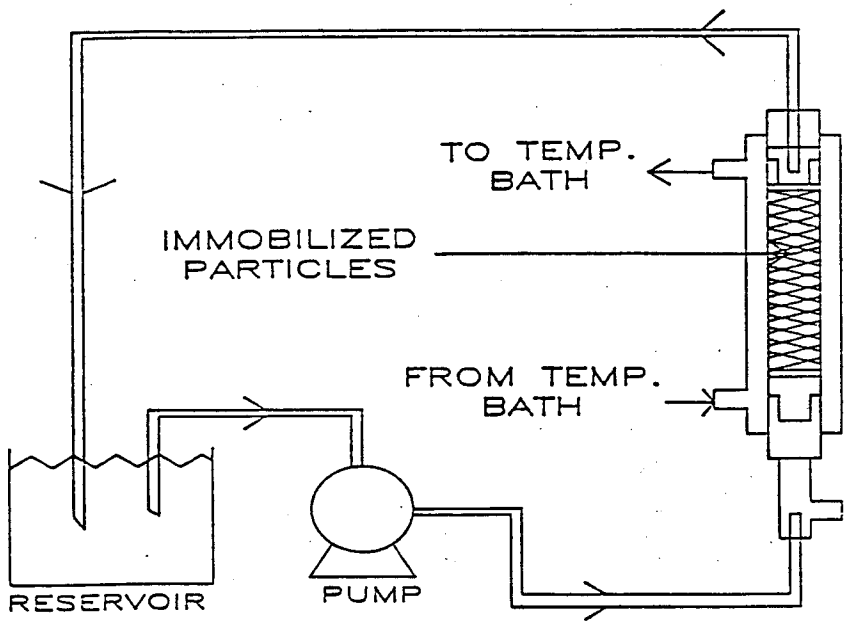
Shake Flask Fermentation

Corner-baffled shake flasks containing minimal media plus carbon sources (occupying 5% of flask volume) were incubated at 30 C in a gyrorotatory bath. Samples were removed periodically and either assayed immediately or stored at -20 C.

Batch Reactor Studies

The immobilized cells were plased in a temperature controlled bioreactor, diagramed in Figure 1. Production media (minimal media +/- NH_4SO_4, phthalic acid, various carbon sources) was added to the bioreactor and operated in batch mode for 24 hours. Samples were removed periodically and either assayed immediatedly or stored at -20 C.

Figure 1



Bioreactor

Ion Exchange Chromatography

Large samples of 4,5-dihydroxy-4,5-dihydrophthalate were recovered from the fermentation media by ion-exchange chromatography. The cells were removed by centrifugation and discarded. The supernatant was applied to a 30 x 50 cm chromatography column containing 45 grams of Amberlite IRA-938 resin (Rohm and Haas). The column was washed with 10 volumes of distilled water. Two liters of supernatant were applied to column and washed with two volumes of distilled water to remove impurities. The product, 4,5-dihydroxy-4,5-dihydrophthalate, was eluted with one volume of 1.0 M NaCl.

Results

Culture Isolation and Characterization

A *Pseudomonas sp.* (hereafter refered to as CWL-phth) was isolated from oil and solvent contaminated soil. The soil was in a spill area next to the UMASS Lowell Paint Shop. Enrichment cultures were used to isolate organisms that could grow on phthalic acid as a sole carbon and energy source. Twenty-nine strains were isolated and one (CWL-phth) was further characterized. The Gram stain was negative. The results of the Oxi/Ferm Tube test are listed in Table 1. From the Gram stain reaction and the biochemical tests the culture has tentatively been designated a *Pseudomonad.*

Table 1

Test	Reaction	Test	Reaction
1. Ana-Dex	neg	6. xylose	neg
2. Arginine	pos	7. Aer-Dex	pos
3. N_2 Gas	neg	8. Urea	neg
4. H_2S	pos	9. Citrate	neg
5. Indole	neg		

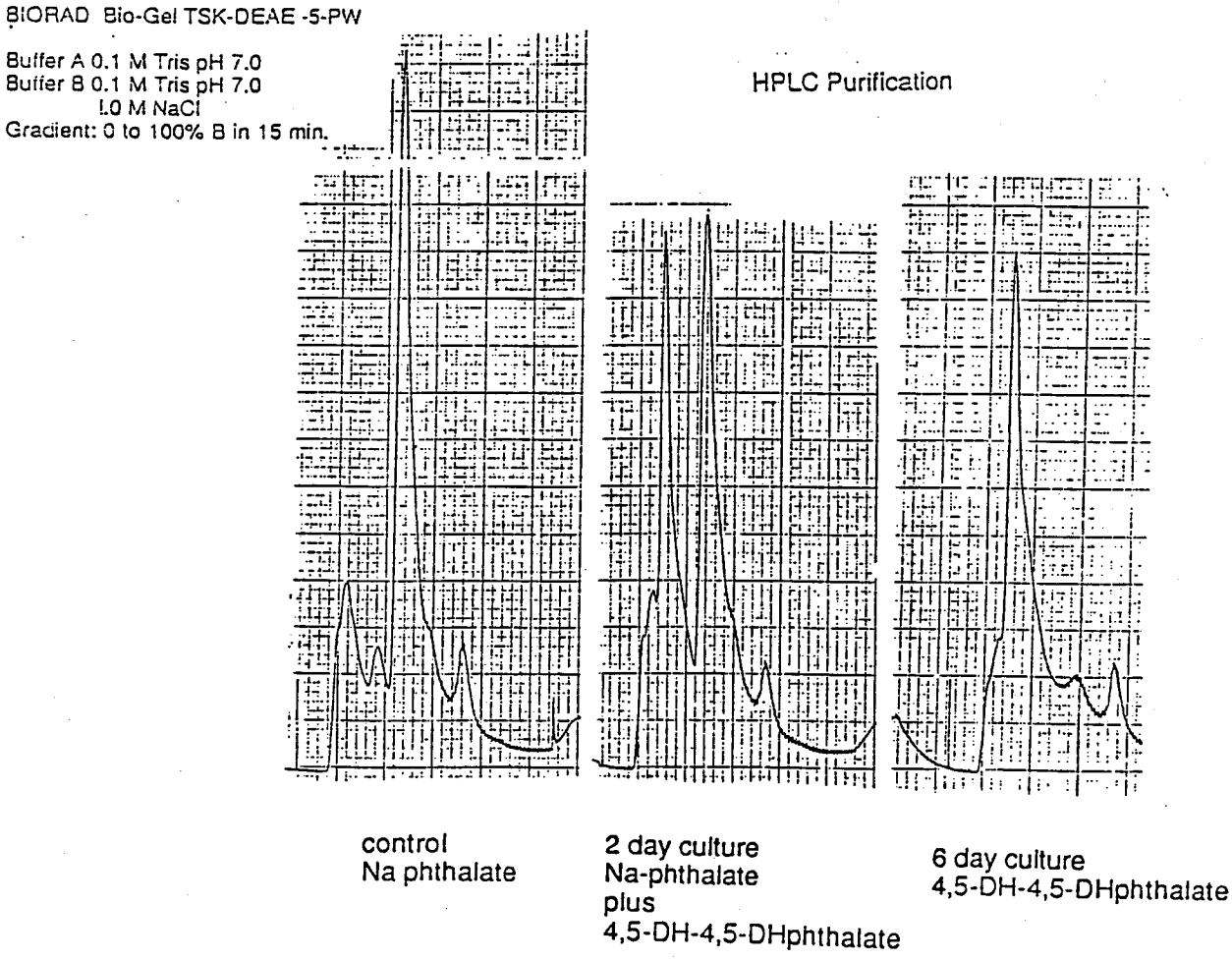
Mutant Isolation

CWL-phth was subjected to UV irradiation and penicllin counter-section to obtain a mutant that had lost the ability to grow on phthalic acid a sole carbon source. Nine mutants with this phenotype were isolated (9 out of 500) and further tested. Each mutant was innoculated into minimal media plus 0.1% phthalic acid and 1.0% glucose. Three colonies produced a red-brown product that was most likely a breakdown product of 4,5-dihydroxy phthalic acid. The remaining six colonies were assayed by HPLC chromatography to determine if 4,5-dihydroxy-4,5-dihydrophthalate was being produced. One colony (CWL-phth-UV6) produced material consistant with properties analogous to 4,5-dihydroxy-4,5-dihydrophthalate.

HPLC Chromatography

Early work (data not shown) utilizing C-18 reverse phase HPLC was inadiquate for charaterization and quantitation of 4,5-dihydroxy-4,5-dihydrophthalate due to acid conditions that led to metabolite breakdown and column deterioration. Therefore, a new method based on DEAE ion-exchange chromatography, was developed. We developed a separation theory based on the fact that starting material phthalic acid and product 4,5-dihyroxy-4,5-dihydrophthalate could be separated based on differences in acidity. The acid weaking effect of reduced unsaturation (lower resonance energy) combined with electron releasing hydroxyl groups should result in 4,5-dihydroxy-4,5-dihydrophthalte being a weaker acid. Figure 2 is a chromatogram utilizing DEAE chromatography and clearly shows that good separation results at pH 7.0. At pH values below 7.0 4,5-dihydroxy-4,5-dihydrophthalate will breakdown to 4-hydroxy phthalate.

Figure 2

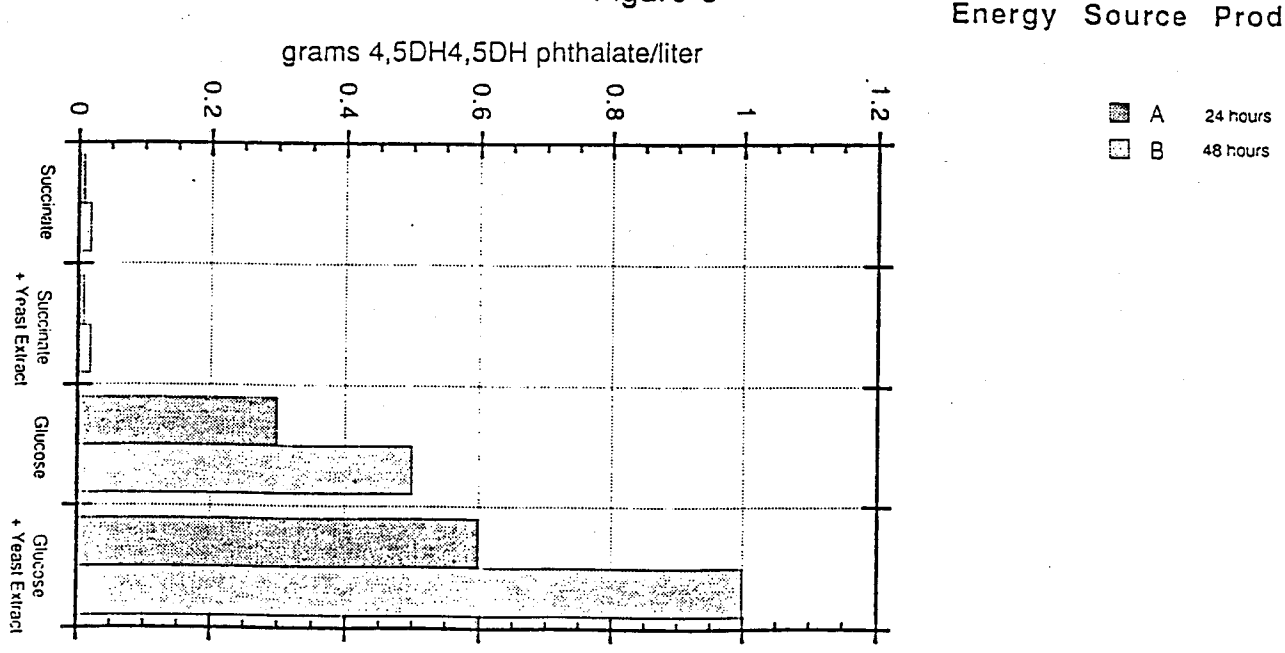


Shake Flask Fermentation

Carbon source optimization was performed utilizing shake flask fermentations. The cellular reaction that converts phthalic acid into 4,5-dihydroxy-4,5-dihydrophthalate is a combined oxidation and reduction. Since the organism can not fully breakdown phthalic acid a supplemental carbon source must be supplied that does not interfer with product formation. The following carbon sources were utilized and tested for product formation: ethanol, succinate, glucose, succinate plus yeast extract, and glucose plus yeast extract. Initial screening determine that product formation occured with all except ethanol.

The effect of carbon source on product formation (4,5-dihydroxy-4,5-dihydrophthalate) was further tested in the following experiment. An overnight culture of CWL-phth-UV6 was used to innoculate flasks containg minimal media plus one of the following four energy sources: 5% succinate, 5% glucose, 5% succinate plus 0.1% yeast extract, or 5% glucose plus 0.1% yeast extract. The results can be seen in Figure 3. Glucose was an effective energy source and succinate was not. The inability of succinate to serve as a carbon source may be due to catabolite repression. TCA cycle intermediates are known to repress carbon source utilization in *Pseudomonas*. The increased product formation with yeast extract was due to increased cell mass.

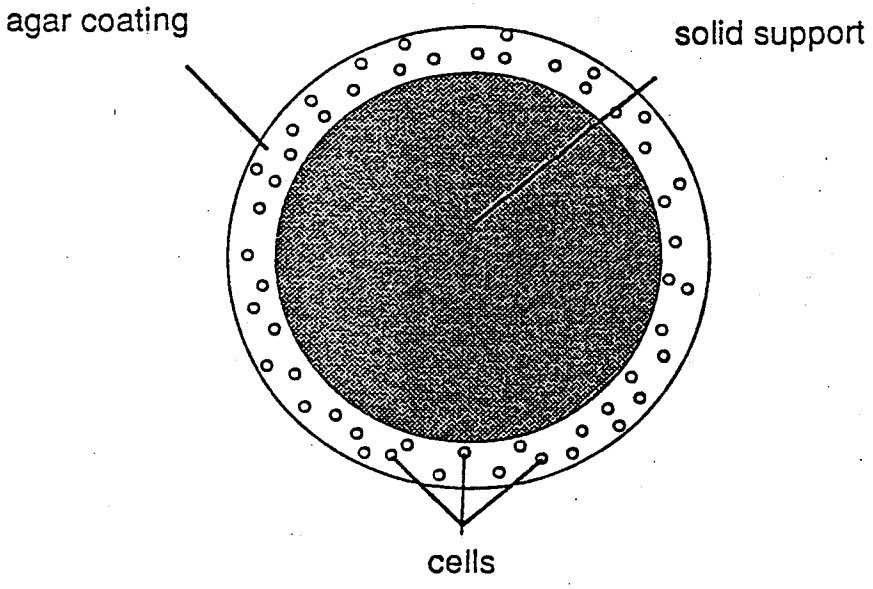
Figure 3



Batch Reactor Studies with Immobilized Cells

Cells of CWL-phth-UV6 have successfully been immobilized utilizing the technique of Lawton[1] . The cells are entraped in a thin agar coating on a solid particle. A schematic of this system can be found in Figure 4. Because of the superior mechanical properties, this immobilized biocatalyst can be scaled-up to large scale for industrial development[2].

Figure 4



Immobilized Biocatalyst

Immobilized particles were first placed in flasks containing minimal media (+ or - NH_4SO_4) plus 0.1% phthalate and 5.0% glucose, inncubated at 30 C for 24 hours, to determine their ability to produce 4,5-dihydroxy 4,5-dihydrophthalic acid. In each case 100% of phthalic acid was converted. Minimal media minus NH_4SO_4 was used to ensure that conversion was due only to immobilized cells and not free cells.

The immobilized cells were next placed in a temperature controlled bioreactor as diagramed in Figure 1. Two different production media were tested for

product formation (minimal media + or - NH_4SO_4, 0.1 phthalic acid and 5.0% glucose). The reactors were run for 24 hours and in each case there was a conversion of 33% of phthalic acid into product.

Conclusions

Experimental

Four of the stated six two-year goals have successfully been completed. They are:

1. *Pseudomonas* CWL-phth has been isolated from oil and solvent contaiminated soil that can grow on phthalic acid as a sole carbon

2. A mutant strain of *Pseudomonas* CWL-phth-UV6 has been isolated that produces the polymer intermediate 4,5-dihydroxy-4,5-dihydrophthalic acid

4. A DEAE ion-exchange chromatographic technique has been developed for the purification of 4,5-dihydroxy-4,5-dihydrophthalic acid. This eliminates the need for toxic solvents used in similar processes.

5. An immobilized cell technology has been developed that can be used for the industrial production of this substituted para polyphenylene intermediate, 4,5-dihydroxy-4,5-dihydrophthalic acid.

A systematic evaluation of energy sources has begun to determine which material is the most effective in converting phthalic acid into 4,5-dihydroxy-4,5-dihydrophthalate. Of the materials tested, glucose appears to be the most effective. In the second year of this project, the remaining parameters (carbon source, pH, temperature, trace nutrient, etc.) will be evaluated.

A systematic experimental study of the radical polymerization of 4,5-dihydroxy-4,5-dihydrophthalate has begun. Parameters to be evaluated are : protecting groups, temperature of reaction, initiators, solvent systems, etc.

Toxic Use Reduction

This projects impacts toxic use reduction in three direct ways in the production of a high strength and heat resistant polymer. Phthalic acid is used as a starting material for substituted para-polyphenylene instead of benzene that has been

used by ICI for para-polyphenylene[3]. Ion-exchange chromatography is used to purify the polymer intermediate instead of methylene chloride[3]. Successful development of this process will also reduce toxic chemical use by providing a product that can be used as an asbestos substitute.

Goals 3 and 6 will be completed in the second year of this two-year project.

References

1. Lawton, C.W., et al., *Biotechnology and Biochemical Engineering Sym.* 17:507 (1987)

2. Bunning, T. J., et al., *Bioprocess Engineering*, 7:71 (1991)

3. Ballard, D.G.H., et al, *Macromolecules*, 21:294, (1988)