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**A BIOLOGICAL/CHEMICAL PROCESS FOR REDUCED
WASTE AND ENERGY CONSUMPTION
CAPROLACTAM PRODUCTION**

Phase I: Select Microorganisms and Demonstrate Feasibility

Final Report

**By
Edward J. St.Martin**

August 1995

Work Performed Under Contract No. DE-FC04-93AL94462

**For
U.S. Department of Energy
Office of Industrial Technologies
Washington, D.C.**

**By
AlliedSignal, Inc.
Des Plaines, Illinois**

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ABSTRACT

A novel biological/chemical process for converting cyclohexane into caprolactam was investigated. Microorganisms in a bioreactor would be used to convert cyclohexane into caprolactone followed by chemical synthesis of caprolactam using ammonia. The proposed bioprocess would be more energy efficient and reduce byproducts and wastes that are generated by the current chemical process. We have been successful in isolating from natural soil and water samples two microorganisms that can utilize cyclohexane as a sole source of carbon and energy for growth. These microorganisms were shown to have the correct metabolic intermediates and enzymes to convert cyclohexane into cyclohexanol, cyclohexanone and caprolactone. Genetic techniques to create and select for caprolactone hydrolase negative-mutants are being developed. These blocked-mutants will be used to convert cyclohexane into caprolactone but, because of the block, be unable to metabolize the caprolactone further and excrete it as a final end product.

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1.0 INTRODUCTION

This report describes the technical progress for Phase I, Select Microorganisms and Demonstrate Feasibility, covering the period from May 1993 through August 1994. It is the first of three phases in a cost-shared project between AlliedSignal and the U.S. Department of Energy, Assistant Secretary for Energy Efficiency and Renewable Energy, Office of Industrial Technologies, under DOE Albuquerque Field Office Cooperative Agreement DE-FC04-93AL94462.

The current nylon 6 manufacturing process uses caprolactam as feed stock. The chemical conversion of cumene to caprolactam is a multi-step complex process that requires high temperatures and pressures. It is energy intensive (consumes 4×10^{12} BTU per year) and generates considerable waste. The AlliedSignal Frankford plant alone lost 0.1 million pounds of waste cumene and 0.36 million pounds of phenol last year. The Hopewell plant produces 14 million pounds of ammonium sulfate and 0.7 million pounds of acetone low-valued byproduct/waste per year. Nylon 6 can also be produced from caprolactone and ammonia in a very efficient process using known chemistry. However, at present, caprolactone's market price renders this route uneconomical. The proposed biological process is aimed at providing a one step cost effective production process for caprolactone. In addition to being a nylon precursor, caprolactone can also be used as feed stock for biodegradable polyester.

The goal of this project is to establish the technical feasibility of a biomanufacturing process for the conversion of cyclohexane into caprolactone by optimizing and scaling up a laboratory-demonstrated bioprocess to pilot-plant scale.

The approach will be to select microorganism strains that have the highest rates of cyclohexane conversion and greatest cyclohexane solvent resistance. Mutant production and genetic isolation techniques will be used to isolate metabolically blocked

mutant strains that can convert cyclohexane into caprolactone but cannot metabolize it further. Concurrent development of a product separation and purification process will also be performed. A pilot plant will be constructed to measure the performance, long term stability, economics, and environmental benefits of the caprolactone biomanufacturing process. The key for enzymatic reactions can be found in Section 7 at the end of the report.

1.1 The Current Chemical Process for Caprolactam Production

Many alternate chemical routes to caprolactam synthesis have been described in the literature. The chemical process, shown in Figure 1, starts with the catalytic hydrogenation of benzene, uses phenol as an intermediate, and has the highest selectivity and yield. However, it consumes significant energy and also produces a significant amount of waste. AlliedSignal's Frankford, Pennsylvania plant purchases cumene to convert into phenol. In this process cumene is oxidized to cumene hydroperoxide with air at 105°C and 5 psig pressure. The oxidation product is separated, mixed with sulfuric acid and maintained at 77°C while the hydroperoxide splits into phenol and an equal mole of acetone.

The phenol from the Frankford plant is shipped to AlliedSignal's plant in Hopewell, Virginia where it is converted into caprolactam. The phenol is first converted into cyclohexanone using 75% hydrogen gas at 155°C and pressures of 80 to 220 psig using a palladium on carbon catalyst. The cyclohexanone is then treated with hydroxylamine sulfate in the presence of aqueous ammonia in an oximation reaction to form cyclohexanone oxime and ammonium sulfate. It is important to point out that the hydroxylamine sulfate is prepared by the Rashig method from ammonia, air, ammonium carbonates and sulfur dioxide, and that a mole of ammonium sulfate byproduct is produced for every mole of

hydroxylamine sulfate. The cyclohexanone oxime is then treated with fuming sulfuric acid and undergoes a Beckman rearrangement to yield caprolactam and sulfuric acid. By this chemical process, a total of 4.4 pounds of ammonium sulfate is produced for every pound of caprolactam product.

Figure 1 Chemical Caprolactam Process

Chemical Caprolactam Process

1.2 The Proposed Biological/Chemical Process

The use of biological catalysts, shown in Figure 2, is very different. The proposed biological process begins with cyclohexane that is commercially available and produced from benzene by catalytic hydrogenation. No high priority hazardous chemicals such as cumene and phenol would be produced or released by this biological route. Microorganisms that can metabolize cyclohexane have previously been described. One of these microorganisms was found to use an enzyme complex that directly oxidizes cyclohexane to cyclohexanol with no side reactions and 100% conversion efficiency (please refer to Figure 8 and Table 14 for definition of enzymatic reactions). The cyclohexane hydroxylase enzyme has been isolated from this microbial isolate and shown to be a cytochrome P450 type monooxygenase that uses oxygen and a NADPH cofactor. NAD (nicotinamide adenine dinucleotide) and its phosphorylated derivative NADP are electron accepting chemicals that can exist in the oxidized or reduced form as NADH and NADPH. They exist in every living organism and are used by enzymes as a cofactor in performing biological oxidation and reduction reactions. In addition, this enzyme catalyzed reaction occurs at room temperature and at atmospheric pressure. Subsequent enzymatic reactions that completely oxidize cyclohexanol into cyclohexanone using a NAD cofactor linked dehydrogenase enzyme has also been described in several microorganisms. By comparison, direct chemical oxidation of cyclohexane to cyclohexanol and cyclohexanone on a metal catalyst is possible but overall yields are only 75-80% at 10% cyclohexane conversion. A subsequent microbial Baeyer-Villiger like enzymatic reaction that converts cyclohexanone into caprolactone and uses a NADPH cofactor has also been described in microorganisms. It is important to point out that all three enzyme reactions occur sequentially in one microorganism and no intermediate separations and isolations are required. In natural cyclohexane-utilizing microbes, that are isolated from nature, the cyclohexane that is converted into

caprolactone is metabolized further by a caprolactone hydrolase that hydrolyses the lactone to an acid-alcohol, 6-hydroxy-hexanoic acid. This product would be metabolized further to CO_2 and H_2O , while providing carbon and energy to support the growth of the microorganisms. We propose to isolate mutants of these microorganisms that are missing caprolactone hydrolase enzyme activity. Thus, the caprolactone that is formed from cyclohexane conversion by the mutant microbes will accumulate and be excreted as a final end product. The additional reducing power to regenerate the second NADPH cofactor will be supplied by co-metabolism of acetate to CO_2 and H_2O .

We propose that the microbial cells will be immobilized in a fixed bed bioreactor and used as a biological catalyst for cyclohexane conversion. The caprolactone that is produced in the bioreactor will be isolated and converted directly into caprolactam, the nylon-6 precursor, by reaction with ammonia at 300°C and 300 atm. The direct conversion reaction is an established AlliedSignal technology. In addition, the caprolactone itself can serve as a monomer for polymerization. The polyester that is produced is a biodegradable plastic.

Figure 2 Biological/Chemical Caprolactam Process

Biological / Chemical Caprolactam Process

1.3 Innovative Aspects and General Applicability of Biomanufacturing Concepts

The proposed redesign of the AlliedSignal caprolactam chemical production process, with a combined biological/chemical synthesis, is a totally new concept. The efficiency of the biological oxidation reactions allow direct conversion of cyclohexane into caprolactone without the co-synthesis of acetone and ammonium sulfate and wasteful synthesis of side-reaction products. The biological/chemical route to caprolactam also avoids the use of cumene and phenol as hazardous synthetic intermediates.

The use of immobilized cells as catalysts for chemical synthesis is an emerging technology in the chemical industry. Whole microbial cells can internally recycle biological oxidation-reduction cofactors and, therefore, can be used to perform chemical oxidations. The use of genetic techniques to construct blocked-mutant strains allows these microbes to be used as specific biocatalysts to perform selected chemical conversions and produce end products. Although the selectivity and efficiency of biosynthetic reactions make them less wasteful than classical chemical reactions, very few bioprocesses have been commercialized in the past. Bioprocesses have been considered to be unstable, to only work in dilute water solutions and to be hard to engineer. However, the recent progress in the commercialization of bioprocesses for the production of classical organic chemicals such as acrylamide by Nitto, muconic acid by Celgene and benzene cis-glycol by ICI indicates that many of the hurdles that prevented bioprocess development in the past can now be overcome. Basic research in microbial genetics, microbial solvent resistance, enzymology in concentrated organic solvents and bioseparations has provided many new technologies that can be used to engineer viable commercial bioprocesses. The new technologies that will be developed for bioproduction of caprolactam will also have an impact on similar industrial processes that used organic solvents at high temperatures and pressures and produce waste. Basic knowledge of

blocked-mutant strain isolation, selection for solvent resistance, bioreactor design and product isolation techniques can be used to design other bioprocesses to replace existing wasteful processes and to efficiently synthesize totally new products.

2.0 EXPERIMENTAL TECHNIQUES SECTION

2.1 Microbial Growth Procedures

2.1.1 Liquid and Solid General Growth Media

The following liquid and solid growth media were used.

Table 1. Hydrocarbon Minimal Media Recipe (HCMM2)

Applied & Environmental Microbiology	
Nov. 1990, p3565-3575	
Ingredients	Grams/liter of Distilled Water
KH_2PO_4	1.36
Na_2HPO_4	1.42
KNO_3	0.5
$(\text{NH}_4)_2\text{SO}_4$	2.38
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.05
$\text{CaCl}_2/\text{CaCl}_2 \cdot 5\text{H}_2\text{O}$	0.01
Trace solution	1.0ml
(Recipe follows)	
Agar	15

Table 2. Trace Element Solution(1000x)

Ingredients per 100 mls	
H_3BO_3	0.286 gram
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0.154 gram
$\text{Fe}(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$	0.353 gram
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	3.9 mg
ZnCl_2	2.1 mg
CoCl_2	4.1 mg
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	2.5 mg
Adjust the pH to 7.2	

Table 3. Trower's Liquid Mineral Media(MM)

APPLIED & ENVIRONMENTAL MICROBIOLOGY	
May, 1985 Vol. 49 p1282-1289	
INGREDIENTS	PER LITER OF DISTILLED WATER
NaH ₂ PO ₄	4.5 grams
KH ₂ PO ₄	1.0
(NH ₄) ₂ SO ₄	1.8
MgSO ₄ .7H ₂ O	0.2
CaCl ₂ .2H ₂ O	0.1
Trace elements	1.0 ml

Table 4. Soybean Flour Media

INGREDIENTS	PER LITER OF DISTILLED WATER
glucose	20.0 grams
NaCl	5.0
K ₂ HPO ₄	5.0
yeast extract	5.0
soybean flour	5.0
adjust pH to 7.0 with 6 N HCl	

2.1.2 Selective Enrichment Growth Media

The selection of microbes that are capable of utilizing cyclohexane as a source of carbon and energy for growth has only been reported in a limited number of research papers. ^(1,2,3,4) Cyclohexane is an organic solvent that kills most cells that come in contact with it. In addition, cyclohexane is not very soluble in water (0.006%). All culture media used for primary isolation of cyclohexane utilizing microbes contained trace metals and 0.01% yeast extract to serve as a general source of nutrients. In order to isolate cyclohexane utiliziers from natural soil and water samples, specialized media and glassware were utilized. All labware has to be protected from cyclohexane because it can dissolve and react with most plastic laboratory supplies. All test tubes and flasks require

teflon lined caps and seals. Glass petri dishes or aluminum-lined plastic petri dishes were used for solid media. All samples were incubated in sealed galvanized one gallon paint cans to control solvent vapors. When solvents were added to sealed tubes and flasks, the solvent was applied to filter paper disks that were attached to the teflon caps. In some enrichments, the solvents were put into polyethylene cryo-vials that allow the cyclohexane to slowly diffuse into the growth medium. In other experiments, the cyclohexane was put onto cyclohexane washed XAD hydrophobic resin beads that allowed a controlled release of cyclohexane.

Two methods were used to detect utilization of cyclohexane by either pure or mixed bacterial cultures. When cyclohexane was the major source of organic carbon, the 10 ml test tubes or special 250 ml flasks with side arms were examined directly for an increase in bacterial growth. The optical density was recorded using a Sequoia-Turner model 340 visible spectrophotometer and 600 nm wavelength light.

When complex growth media that only had cyclohexane as a minor component was used, cyclohexane utilization was measured by analyzing the cyclohexane vapors in the headspace of sealed 20 ml serum tubes and 160 ml serum vials. A 50 uL gas sample was injected into a EGG Chandler gas chromatograph with 10% SP-2100 on a 80/100 Supelcoport (1-2429) 6', 1/8" stainless steel column. Helium was used as the carried gas at a flow of 25 ml/min at 70°C. The cyclohexane standards had a retention time of 1.2 minutes.

2.1.3 Bacterial Cell Harvesting and Disruption

The cell cultures were incubated in HCMM2 medium with 0.01 % yeast extract and 0.04-0.2 % caproic aldehyde or alternate growth substrates for

approximately one or two days on a shaker incubator at 30°C until the cell density is around 0.6 to 0.9 optical density units at 600 nm.

The resulting cultures were centrifuged at 8000 rpm in a Sorval RC-5B centrifuge for 30 minutes at 20°C. The cell pellet was suspended in 10 mM (K) phosphate buffer at pH 7.0. Usually, the cells are concentrated 20X. The cells were sonified with a Heat Systems sonicator at one second intervals for 3 to 6 minutes until the majority of the cells are broken as determined by observation in a light microscope at 300-1000X magnification. The cells were kept cold using an ice and water bath. The sonified cell preparation was centrifuged in a Sorval RC-5B centrifuge for 20 minutes at 15,000 rpm at 20°C to separate the cell debris from the liquid extract. The supernatant liquid was collected and checked for the enzyme activities as listed below.

Table 5. Bio-Rad Protein Assay

Reagents:

10 mM (K) Phosphate buffer pH

Bio-Rad reagent

Protein Sample

Protein Standard sample (Bovine Serum Albumin)

Reaction Mixture Contains:	
100-800	uliters of unknown sample
3100-2400	uliters of diluent = 10 mM (K) phosphate buffer
3200	uliters Total Volume
800	uliters of Bio-Rad reagent
Incubation time	20 minutes at room temperature
Range for this assay	between 0-20 micrograms protein per ml.
Read absorbance	595nm against calibration curve

2.2 Enzyme Assay Procedures

2.2.1 Cyclohexane Hydroxylase

Cyclohexane hydroxylase can be measured by following the NADPH-dependent oxidation of cyclohexane with a Clark-type oxygen electrode at 30°C ^(5,6). The decrease in oxygen is detected using a two-channel YSI biological oxygen monitor. The cyclohexane hydroxylase enzyme activity is very complex and unstable and composed of three separate components. The enzyme system is comprised of a ferredoxin reductase, a ferredoxin and a cytochrome P-450 as a terminal electron acceptor. The reaction mixtures are mixed in a 1 ml cuvette at 30°C and contain: $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ buffer at 50mM and pH 6.5, NADPH at 0.5 mM, 2 uL cyclohexane and 10 to 200 uL of cell extract.

Figure 3. Cyclohexane Hydroxylase

2.2.2 Cyclohexanol Dehydrogenase

Cyclohexanol dehydrogenase activity was measured in cell extracts spectrophotometrically (Beckman DU-70) by following the cyclohexanol, NAD and extract dependent production of reduced nicotinamide adenine nucleotide (NADH₂) at 340 nm wavelength light ⁽⁷⁾. The enzyme catalyzes the removal of two electrons and the transfer of two protons from cyclohexanol to the NAD cofactor. The increase in absorbance per minute was converted to umole of product by using the extinction coefficient of 1 molar NADH₂ at 340 nm of 6,230 absorbance units. Control reactions that exclude either substrate or enzyme extract were used to correct for any nonspecific changes in absorbance.

The reaction mixtures were in 1 ml cuvettes and run at a constant temperature of 30°C. The reaction contained: glycine-NaOH buffer at pH 10.6 (20mM), NAD (0.2mM), cyclohexanol (0.5mM) and 10 to 200 μ L of cell extract.

Figure 4. Cyclohexanol Dehydrogenase

2.2.3 Cyclohexanone Monooxygenase

Cyclohexanone monooxygenase was measured spectrophotometrically as the cyclohexanone, oxygen and enzyme extract dependent oxidation of NADPH to NADP at 30°C ^(8,9).

The enzyme uses reduced NADPH to activate oxygen and transfer the oxygen to cyclohexanone to form an internal lactone, caprolactone. The decrease in absorbance per minute was converted to umole of product using the extinction coefficient of 1 molar NADPH at 340 nm of 6,230 absorbance units. Control samples that omitted either substrate or enzyme extract were run in order to correct for endogenous reactions such as NADPH oxidase activity.

The one ml reaction mixtures contained: KH_2PO_4 -KOH buffer at pH 7.0 (22mM), NADPH (0.16 mM), cyclohexanone (0.66mM) and 10 to 200 μ L of cell extract.

Figure 5. Cyclohexanone Monooxygenase

2.2.4 Caprolactone Hydrolase

Caprolactone hydrolase activity was measured as the caprolactone and enzyme-extract dependent production of acid ⁽¹⁰⁾. The enzyme adds water to open the lactone ring and the product is an acid alcohol, 6-hydroxy-hexanoic acid. The change in pH was measured in a spectrophotometer at 620 nm using a bromothymol-blue pH indicator dye. The decrease in absorbance was converted to umole of acid using a change of 666 absorbance units at 620 nm per mole of added acid. Control samples that did not contain either substrate or enzyme were used to correct for any nonspecific acid production.

The one ml reaction contained: KH_2PO_4 - Na_2HPO_4 buffer at pH 7.5 (0.33mM), caprolactone (0.66 mM), bromothymol blue (16.7 ug/ml) and 10 to 200 uL of cell extract.

Figure 6. Caprolactone Hydrolase

2.2.5 6-Hydroxy-Hexanoate Dehydrogenase

The 6-hydroxy-hexanoic acid dehydrogenase enzyme activity was measured with a spectrophotometer at 30°C. The enzyme removes two electrons from the alcohol end of the molecule to form caproic aldehyde and transfers two protons to the NAD-cofactor to yield NADH. The increase in absorbance was converted into umole of product using the absorbance of 1M NADH of 6,230 absorbance units. Control samples that did not contain either substrate or enzyme extract were used to correct for any nonspecific change in absorption.

The 1 ml reaction mixture contained: glycine-NaOH buffer at pH 10.4 (20mM), NAD (0.3mM), 6-hydroxy-hexanoic acid (2mM) and 10 to 200 uL of cell extract.

Figure 7. 6-Hydroxy-Hexanoate Dehydrogenase

2.3 Genetic Selection Techniques

2.3.1 Optimization of Chemical Mutagenesis

The natural rate of mutation in bacteria is approximately one mutation in the gene of interest per one hundred million cell divisions. The frequency at which it can be detected is, therefore, one mutant cell in a background of 100 million normal cells. In order to identify and isolate a mutant, such as a cyclohexane blocked strain that is missing caprolactone hydrolase because of a genetic defect in the hydrolase gene, an efficient method for generating more mutants is required. Chemical mutagens can increase the rate of mutation so that only one in one thousand or ten thousand surviving cells might contain a genetic defect in the gene of interest. Three mutagens have been used by our lab for creating

blocked mutants: N-methyl-N-nitro-N-nitrosoguanidine (NTG), ethane methyl sulfonate (EMS) and ultraviolet light at 254 nm (UV).

The amount and time of mutagenic treatment must be determined for each microbial cell type. A direct method of measuring an increase in mutations in a population of cells is to measure the increase in mutation to antibiotic resistance. For example, plate out bacterial cells on solid agar media that contain an antibiotic like streptomycin before and after mutagen treatment and select the dose of mutagen that gives the greatest number of streptomycin resistant mutants. Although the mechanism of streptomycin sensitivity is well known for many microbes, many of the natural soil isolates are either not sensitive or not mutable to resistance and this method cannot be used to optimize mutagen treatment.

The optimization of mutagenic treatments can also be accomplished by measuring the amount of killing that a mutagen causes. The chemical and physical mutagens cause damage to DNA that is translated improperly during the next round of cell replication. If a large number of genes have been damaged, the cells cannot recover. Because bacterial cells contain a few thousand genes, if 99.9% of the cells are killed, most of the cells in that treated population must have sustained multiple mutations and some must have been in the gene of interest.

2.3.2 Selective Enrichment for Mutants

The selection of negative mutants requires that we isolate microbes that have lost the ability to grow on a substrate such as cyclohexane. Because it is a loss, there is no positive way to select for the mutants. The strategy behind negative enrichment is to use antimicrobial chemicals that selectively kill rapidly-growing microbial cells, but allow negative-blocked mutants to survive. For example,

we can use penicillin, cycloserine or streptozotocin to kill cells that are growing on caprolactone and thereby enrich the treated cell culture for rare mutants that are negative and blocked in caprolactone metabolism. If 99% of the cells are killed, the surviving cells would now be enriched 100 fold in mutant cells. After several rounds of this selective killing, the proportion of mutant versus positive cells would increase such that the mutants would now become the predominant cell type.

The strains that we have isolated must be tested for their ability to grow and be killed by the above antibiotics. Many soil and water cell isolates are naturally resistant to some antibiotics, and the above selective enrichment procedures cannot be used for them.

2.3.3 Design of Liquid and Solid Mutant Identification Media

The use of high efficiency mutagenesis and negative cultural enrichment can result in a culture that now contains mutants in a high enough proportion to be easily identified. For example, if the mutants represent one in one thousand cells, we could screen a few thousand cells and have a good probability of finding the desired mutant.

In order to identify these mutants, we can design culture media to differentiate a rare negative-mutant cell from the majority of positive cells. The cells can be plated out on solid media that contains high levels of the substrate caprolactone but only low amounts of a general substrate, such as acetate, that all the cells can grow on. Then, the caprolactone-positive cells will grow into large colonies but the negative-mutants can be identified because they remain small. In addition, we can add metabolic dyes such as tetrazolium red to the growth medium in order to further separate the large red positive-cell colonies from the small white colonies that are formed from blocked-mutant cells. The correct

concentration of positive and negative growth substrates and indicator dyes must be determined for each new microbial cell isolate.

3.0 RESULTS AND DISCUSSION

3.1 Task 1.0 - Confirm Cyclohexane Utilization By Microbes

The first sub-task in Phase I was a proof-of-principal for the isolation of microbes that can metabolize, "eat", organic solvents such as cyclohexane and cyclohexanol. We developed several technologies for the isolation of microbes that can both survive and metabolize in the presence of a strong organic solvent, such as cyclohexane. We have used both vapor and liquid phase cyclohexane with liquid and solid bacterial isolation media to select for these microbes. These microbes came from natural sources such as soil and water, and were selected for by their ability to survive and utilize cyclohexane or cyclohexanol as a source of carbon and energy for growth. Helper strains, that are resistant to cyclohexane and very active in cyclohexanol metabolism, were used to rescue and select for isolates that are slow cyclohexane oxidizers. Co-substrates such as acetate, cyclohexanol, toluene and gasoline were used to encourage the growth of organic solvent utilizing microbes. A search of culture collection strains from the Center for Biocatalysis and Bioprocessing at the University of Iowa was also attempted.

3.1.1 Selection of Natural Soil and Water Samples

Table 6. Pure cultures of hydroxylating microbes

6946-ATCC	<i>Arthrobacter simplex</i>
UI-315	<i>Aspergillus alliaceus</i>
X-172	<i>Aspergillus niger</i>
UI-1477	<i>Bacillus cereus</i>
14581-ATCC	<i>Bacillus megaterium</i>
7159-ATCC	<i>Beauveria sulfurescens</i>
16373-ATCC	<i>Caldariomyces fumago</i> (16273-AG)
5159-UI	<i>Candida intermedia</i>
2312-UI	<i>Candida tropicalis</i>
MR-141	<i>Chaetonium funiculum</i>
14004-ATCC	<i>Corynebacterium mediolarum</i>
3655B-NRRL	<i>Cunninghamella echinulata</i>
1393-NRRL	<i>Cunninghamella elegans</i> (-)
2178-NRRL	<i>Curvularia lunata</i>
11011-ATCC	<i>Cylindrocarpon radiculicola</i>
1086-NRRL	<i>Gliocladium deliquescens</i>
11395-ATCC	<i>Microsporum gypseum</i>
MR-56	<i>Mucor recurvatus</i>
Am-53378-UI	<i>Mycobacterium fortuitum</i>
B-3683-NRRL	<i>Mycobactrium species</i>
5646-NRRL	<i>Nocardia species</i>
21197-ATCC	<i>Nocardia butanica</i> (<i>Rhodococcus rhodochrous</i>)
MR-336	<i>Penicillium stipitatum</i>
60690-UI	<i>Pseudomonas aeruginosa</i> -var. Saleh
AM-670	<i>Pseudomonas fluorescens</i>
MR-RO	<i>Rhizopus oryzae</i>
MR-224	<i>Rhizopus species</i>
889-IFO	<i>Rhodotorula rubra</i>
MR-Sacch	<i>Saccharomyces cerevisiae</i>
13378-ATCC	<i>Sepedonium chrysospermum</i>
11796-ATCC	<i>Streptomyces griseolus</i>
13273-ATCC	<i>Streptomyces griseus</i>
SC-2831-UI	<i>Stysanus stemonites</i>
39115-ATCC	<i>Streptomyces viridosorum</i>
ESM 0037	
ESM 0038	
ESM 0022	

Table 7. Source of Samples for Isolation of Cyclohexane Degrading Microbes

01	Soil sample from wild mint grove
02	Soil sample from replanted grove
03	Soil sample from abandoned grove
04	Soil sample from active mint grove
05	Soil sample from citrus plant grove
06	Soil sample from AlliedSignal Des Plaines site duck pond
07	Soil sample from AlliedSignal Des Plaines site building # 6 and 7
08	Soil sample from AlliedSignal Des plaines site court yard (around juniper bushes and pine trees)
09	Iron-ton sludge
10	Soil sample site from Frankford plant
11	Sewage plant sludge
12	Soil sample from Beecher, IL. From gasoline storage tank area.
13	Iron-ton bioreactor solids
14	Soil sample from Wisconsin gasoline soaked soil
15	(chemical company) sample from various stages of a bioreactor from influent through effluent (Got # 8 and its mutants BE2 and GB5)
16	(oil refinery) site ground water (Got CX-2)
17	(oil refinery) site trench water
18	Soil sample 2 myristic acid isolate
19	Soil sample tetradecane isolate
20	Soil sample octadecane isolate
21	Soil sample pentadecane isolate
22	Soil sample Tet#3 strain
23	Activated sludge Lock Haven treatment plant

3.1.2 Liquid and Solid Media Cultural Enrichments

Several techniques were used to enrich and select for microbes that can metabolize cyclohexane.

Table 8. Microbial Enrichment and Selection Techniques

1.	Soil and water samples were incubated directly with cyclohexane in the liquid culture.
2.	Soil and water samples were incubated with cyclohexane vapors.
3.	Soil and water samples were pre-incubated with gasoline, toluene, acetate or cyclohexanol before incubation with cyclohexane vapors.
4.	Soil and water samples were incubated with plastic vials that allowed for slow cyclohexane permeation and release.
5.	(OMIT)
6.	(OMIT)
7.	Liquid cultures were monitored by measuring either cyclohexane utilization or an increase in optical density with time.
8.	Liquid cultures were diluted and re-incubated with fresh media for several cycles in order to detect any reproducible growth.
9.	Liquid cultures were plated out on solid agar plates and incubated with and without cyclohexane vapors in order to identify individual colonies that metabolize cyclohexane.
10.	Individual colonies were reisolated on solid agar plates several times in order to ensure that the isolates were pure clones and gave reproducible growth.

3.1.3 Isolation of Cyclohexane and Cyclohexanol Utilizers

Some thirty-three fungal and bacterial strains, that are known to have hydroxylating enzymes, were screened for their ability to hydroxylate cyclohexane and form cyclohexanol but no reproducible hydroxylating isolate was identified. Two strains, 5159-UI *Candida intermedia* and MR-36 *Mucor recurvatus*, showed some activity when they were incubated with cyclohexane and the soybean flour media, and the cyclohexane vapor levels were monitored for one month. Although the levels of cyclohexane dropped from 386 ppm to only 59 ppm for this one experiment, the experiment could not be repeated and we suspect leakage was responsible for the false result.

A mixed culture from a chemical company bioreactor that was treating a waste stream also demonstrated cyclohexane utilization when acetate was used as a primary growth substrate. After 7 days of incubation, the cyclohexane vapors dropped from 578 ppm to 388 ppm. We were successful in isolating a

cyclohexane utilizing bacterial isolate from this waste treatment bioreactor. The waste stream comes from a chemical plant that discharges a mixture of chemicals including cyclohexane. This isolate can utilize cyclohexane vapors in both liquid and solid media. Growth on cyclohexane in liquid culture is difficult to measure because the cells are very hydrophobic and remain on the liquid surface and cling to the glass tubes.

We were also successful in isolating a cyclohexane utilizing bacteria from the contaminated ground water from an old oil refinery site. Unlike the isolate above, this strain is capable of better growth in liquid culture. Both isolates were difficult to isolate because they are part of a consortium of bacteria that combine to utilize cyclohexane. Repeated subculturing on liquid and solid media was used to encourage the selective growth of the cyclohexane utilizer. Neither culture can grow well on cyclohexane alone and require some trace amounts of nutrients for growth. One strain can grow on cyclohexane if the vitamin biotin is provided. We routinely use 0.01% yeast extract to provide trace nutrients to our cultures. Although the first task was accomplished, the search continues for new and better cyclohexane utilizers with improved cyclohexane utilization activity. This includes the selection of new strains from soil and water samples as well as the construction and isolation of mutants from strains tested earlier.

3.2 Task 2.0 - Demonstrate Cyclohexane Metabolism To Caprolactone

The second task was the demonstration that cyclohexane utilization by these microbes proceeds through conversion to cyclohexanol, then cyclohexanone, and finally to caprolactone. We have demonstrated this metabolic route by two methods. Our cyclohexane and cyclohexanol utilizing microbes were tested for their ability to metabolize and grow using the metabolic intermediates: cyclohexanol, cyclohexanone, caprolactone, 6-hydroxy-hexanoate and caproic-

aldehyde. Because our bacterial isolates can utilize all of these intermediates for growth, they must possess the complete metabolic pathway for cyclohexane utilization. A second method was the actual detection and measurement of the enzymes responsible for the conversion of cyclohexane into caprolactone. We prepared enzyme extracts of our bacterial strains and measured the oxidation of cyclohexanol into cyclohexanone, oxidation of cyclohexanone into caprolactone and the hydrolysis of caprolactone into 6-hydroxy-hexanoate. We have two genera of bacteria that were assayed. A gram-negative small motile rod, "pseudomonas-like", had specific activity levels for the three enzymes of : 0.664, 0.377 and 0.034 micromoles of product formed per minute per milligram of cell extract protein measured at 30°C. Two of our isolates are gram-positive pleiotrophic rods "arthrobacter-like" and had enzyme levels that were approximately one tenth as active. Our best cyclohexane utilizing isolate is a small gram positive rod, and it has higher enzyme levels as reported below.

3.2.1 Confirm Utilization of all Metabolic Intermediates

Strain 0057 was examined in order to confirm that it could utilize chemical intermediates of cyclohexane metabolism. The final optical absorbance of the liquid cultures was used to determine if the intermediates had been utilized for growth as compared to a control culture that did not receive added chemicals. Because the culture could metabolize and grow on cyclohexane, cyclohexanol, and caprolactone it must possess all the necessary enzymes to convert cyclohexane into caprolactone.

Table 9. Utilization of Carbon Sources for Growth by Strain 0057.

SUBSTRATE	Final Absorbance at 600nm
n-acetyl glucosamine	0.028
Glucosamine	0.014
n-caproic acid	0.629
Adipic acid	0.915
Caprolactam	0.028
Glucose	0.028
Glycerol	0.048
Na citrate	0.024
Na succinate	0.691
6 amino caproate	0.029
cyclohexane	0.064
cyclohexanol	0.386
caproic aldehyde	0.437
caprolactone	0.601
media control	0.036

3.2.2 Detection of Required Enzyme Reactions

The best cyclohexane utilizing isolate CX-2 is a small gram positive rod that can grow well on both liquid and solid media. This culture was grown with cyclohexane vapors as the sole source of carbon and energy for growth. The cells were broken open and a cell-free extract was prepared for enzyme analysis. Four of the key enzyme activities were detected at good levels i. e. over 0.100 specific activity units. The cyclohexane hydroxylase enzyme is composed of several sub-units and is unstable. Therefore, the complex assay system for this enzyme has not been developed yet.

Table 10. Enzyme Analysis of Strain CX-2

ENZYME	SPECIFIC ACTIVITY (umole/min/mg protein)
Cyclohexanol dehydrogenase	(OMIT)
Cyclohexanone monooxygenase	(OMIT)
Caprolactone hydrolase	(OMIT)
6-Hydroxyhexanoate dehydrogenase	(OMIT)
Bio-Rad protein assay	0.810 mg protein/ml

3.3 TASK 3.0 - DEVELOP ANALYTICAL TECHNIQUES

The third task is the development of analytical techniques. New assays are developed as they are needed for the project.

3.3.1 Design of Enzyme Assays

After cyclohexane and cyclohexanol utilizing bacteria were isolated, we developed individual enzyme assays for the metabolism and conversion of cyclohexane into the product caprolactone. We developed an NAD-coupled continuous spectrophotometric assay for cyclohexanol dehydrogenase, a NADPH- coupled assay for cyclohexanone mono-oxygenase, and a pH-dye based assay for the caprolactone hydrolase. A 6-hydroxyhexanoate dehydrogenase assay was also designed. We are currently developing the assay to measure hydroxylation of cyclohexane by using a set of sensitive oxygen electrodes.

3.3.2 Intermediate Product Extraction and Analysis

A rapid gas chromatography assay that can measure cyclohexane levels in the gas phase was used as a first-screen to detect cyclohexane utilization by bacteria during our primary cultural enrichments. This assay is set to detect 500 ppm of cyclohexane in the vapor phase. A gas chromatography assay for caprolactone detection in liquid cultures will also be developed. Techniques for cell and culture extraction of similar chemical-intermediate products using solvents such as ethyl acetate have previously been developed by our laboratory.

3.4 Task 4.0 Select Caprolactone Production Strain

The final task for Phase I is the selection of a strain that can be used to produce caprolactone from cyclohexane or cyclohexanol during metabolism. The strain will be screened for the ability to produce the caprolactone intermediate by analyzing the culture medium of our strains during the metabolism of cyclohexane or cyclohexanol. Intermediate levels of caprolactone are expected to be low in these strains. Because of our early success in isolating strains, and in analytical tool development, we have been working on genetic manipulation of our current microbes during Phase I. Genetic manipulation of the microbes is required in order to block the further metabolism beyond the desired product caprolactone. We have identified the enzyme, caprolactone hydrolase, that is responsible for degrading caprolactone and are developing methods to select mutants that are missing this enzyme. Our strains have been treated with a chemical mutagen and have been plated out on solid indicator media to detect individual colonies that appear to be blocked in caprolactone utilization. Several thousand bacterial colonies have been screened. Presumptive blocked mutants are currently being analyzed to determine which enzymes are present or missing. We will use these blocked mutants to actually demonstrate and measure the amount of caprolactone production. The end of Phase I has provided two bacterial isolates that metabolize cyclohexane and all intermediates, and the technology to measure key enzymes has been developed. The development of mutagenesis and genetic selection for blocked strains was begun during Phase I.

3.4.1 Results of Chemical Mutagenesis

The concentration of mutagen (NTG) and duration of treatment was determined for all our isolates by measuring the amount of cell killing. The results for a

one-hour treatment of strain CX-2 are presented below. The 100 ug/ml treatment was closest to our target of 99% kill.

Table 11. Determination of Optimal Mutagen Concentration

NTG ug/ml	Colony Forming Units /ml.
0	1.5×10^9
25	1.6×10^8
50	6.0×10^7
100	1.0×10^7
200	Non detected at 10^{-4} dilution

3.4.2 Identification of Presumptive Blocked-Mutants

The strategy for detecting mutants after the cells are treated with mutagens requires several liquid culture growth transfers and individual colony plating on solid agar media.

1. After the cells are treated with the mutagen, they are transferred into a rich growth medium in order to rescue the cells that survive the treatment.
2. After growth, the cells are diluted into fresh minimal media, with succinate as growth substrate, in order to enrich for healthy cells and leave behind sick slow-growing mutants. This step is repeated until good growth is observed.
3. The cells are diluted into fresh media with the permissive substrates 6-hydroxyhexanoate or caproic aldehyde in order to discourage the growth of any mutants that are blocked after the caprolactone hydrolase reaction.

4. The cells are then diluted out to between five and seven fold in order to obtain a culture that contains only 100 to 300 individual cells per 0.10 ml sample. this sample is spread out on solid agar plates that contain the high levels of caprolactone with a trace of nutrients (0.01 % yeast extract). The normal cells produce large colonies because they can metabolize the caprolactone. Any presumptive caprolactone negative mutants will only produce a small colony by using the small amount of yeast extract for growth.
5. Several thousand colonies are screened and the small colonies are picked, using sterile toothpicks, into fresh agar plates. The colonies are also picked into caprolactone plates to confirm that they are not able to grow, and into 6-hydroxyhexanoate or caproic aldehyde to show that they can grow. Thus, if the cells cannot grow on caprolactone but can grow on 6-hydroxyhexanoate they must be missing the caprolactone hydrolase enzyme activity.

Table 12. Results of Mutagenesis and Screening of Strain 0058

Total colonies screened	22,240
petit colonies picked	1,056
presumptive negative	2
reconfirm negative	1

3.4.3 Enzymatic Analysis of Mutants

After picking and testing several hundred petite presumptive hydrolase negative mutants, only a few of the presumptive negative-mutants were left. These cells were grown in large batch cultures in order to assay for key enzyme activities. In order to properly prepare cells, we must use caproic aldehyde as the major growth substrate and then use cyclohexanol to induce the synthesis of the cyclohexane degradation enzymes. The cells were broken open and the cell

extract assayed for the loss of caprolactone hydrolase and the induction and presence of cyclohexanol dehydrogenase and cyclohexanone monooxygenase. All four of our cyclohexane and cyclohexanol utilizing strains have been treated with mutagens and presumptive negative mutants have been screened. Because the strains have different growth characteristics the mutagenic and selection process must be customized for each isolate.

Three presumptive negatives of strain 0038 were analyzed and found to be missing all the cyclohexane catabolic enzymes. The high frequency of detection of these mutants after mutagenic treatment strongly suggests that the genes are unstable and easily lost, as would occur if they were on a separate plasmid element. We have not used this strain for further experiments due to this genetic instability. One isolate of strain 0039 was analyzed and found to retain the caprolactone hydrolase as well as the other enzymes.

The cyclohexane positive strain 0058 was treated with mutagens four times and nine separate presumptive mutants were isolated and analysed. Most of the isolates retained their enzymes as seen with mutant BE2 that is described below. One isolate GB5 is no longer able to induce cyclohexanol dehydrogenase (0.007) but has elevated levels of the caprolactone hydrolase (0.490) as compared to the parent strain. We suspect that the mutation that gives loss of the dehydrogenase may have also effected the regulation of the other cyclohexane enzymes. Because the hydrolase is overproduced, these cells rapidly hydrolyse caprolactone and may be inhibited by the build-up of 6-hydroxy-hexanoate and toxicity of its degradation products.

Table 13. Enzymatic Analysis of 0058 Mutants BE2 and GB5

Strain	cyclohexanol uninduced	caprolactone induced	dehydrogenase uninduced	hydrolase induced
0058	(OMIT)			
BE2	(OMIT)			
GB5	(OMIT)			

3.5 Performance Projections

3.5.1 Energy Savings

The current chemical process for converting cyclohexane into caprolactone requires ammonia at 300°C and 300 atm, and consumes \$0.075 in utilities per pound of product. The major costs are for electrical power, and high and low pressure steam. In the biological process, the reactions occur at room temperature and atmospheric pressure, and occur simultaneously in one reaction vessel.

Exact efficiencies will be determined, but it is expected that 50% of the current energy usage, or \$0.038 per pound of caprolactam, will be saved. Assuming \$0.04/kWh, and current caprolactam production grows at 2%/yr from 640 million lb/yr in 1993 to about 896 million lb/yr in year 2010, then, the total potential energy savings will be about 9×10^{12} Btu/yr in year 2010. For 60% of this potential being produced by the bioprocess, the energy saved would be about 5×10^{12} Btu/yr in year 2010.

3.5.2 Emission Reduction

The AlliedSignal Frankford plant, alone, lost 0.1 million pounds of cumene and 0.36 million pounds of phenol into the environment in 1991. The Hopewell plant, released 14 million pounds of ammonium sulfate and 0.7 million pounds

of acetone by-product waste that year. The proposed biological/chemical route would not release any of these priority pollutants and would yield a combined reduction of 9,680 ton/yr in year 2010.

3.5.3 Economic Penetration

Substantial cost savings are expected in converting from the chemical process to the biological/chemical process of producing caprolactam. The three biological oxidation reactions occur sequentially in one reaction vessel at low temperature and pressure and intermediate separations are not required. The reactions go to completion and costly byproduct separations will not be required. The last intermediate in the biological process is caprolactone and it can be converted to caprolactam in a single step. It is estimated that the largest cost savings will be a 50% reduction in capitol costs from \$0.30/lb to \$0.15/lb. An estimated 50% decrease in utility energy costs would reduce those costs from \$0.075/lb to \$0.038/lb of caprolactam product. Based on a current selling price of about \$0.92/lb this would be a 4% decrease in energy costs, with more substantial processing cost reductions to be determined from project results.

3.5.4 Scale Up - Commercialization

AlliedSignal has elected to provide 100% of the non-DOE cost share, and act as their own participant in the production of caprolactam which will be used internally as a feedstock for producing nylon. AlliedSignal is one of America's 30 largest industrial firms, and is the largest producer of type 6 nylon fibers in the world. Therefore, AlliedSignal is its own customer for the bioproducted caprolactone which is converted to caprolactam and then used as a feedstock in nylon production. AlliedSignal plans to substitute the caprolactam produced by the biological/chemical process for the caprolactam it is now producing by purely chemical methods. It will be a transparent substitution of the feedstock

for nylon production by AlliedSignal. Because it will be used by AlliedSignal internally, no detailed marketing plan is required; however, AlliedSignal would license the process to other manufactures for a reasonable fee as a return on leadership and risk investment. AlliedSignal management initiated the development of the new process, and will have followed and guided it to assure that the result is compatible with nylon feedstock requirements.

4.0 CONCLUSIONS AND RECOMMENDATIONS

The first task of this research project was to demonstrate that microorganisms, that can utilize caprolactone as a sole source of carbon and energy for growth, can be isolated from nature. These microorganisms can be isolated. But, as we expected, this was a very long and difficult process. Although natural isolates that metabolize cyclohexanol, cyclohexanone and caprolactone can be easily isolated from many environmental samples, we have, so far, only been successful in isolating cyclohexane utilizers from two sites. Both sites, a specialty chemical-plant treatment works and an oil refinery, are known to have significant amounts of cyclohexane released into the environment. In addition, when natural enrichments were obtained, the majority of microorganisms in the bacterial consortium were not capable of metabolizing cyclohexane alone. The use of specialized industrial site samples, different vapor-phase enrichments and many rounds of liquid- and solid-media transfers and selections are required to isolate cyclohexane utilizing microbes.

When cyclohexane or cyclohexanol utilizing microbes are isolated, we have found that they are all capable of utilizing all the intermediates of the proposed cyclohexane catabolic pathway. In addition, the key enzymes for converting cyclohexane and cyclohexanol into caprolactone are present and inducible in these strains.

The technology for assaying the individual enzymes and accurately measuring their levels was developed for all but the unstable hydroxylase. New equipment and the improved growth of our latest microbial isolate will allow us to complete the assay development. A rapid gas chromatography method for detecting vapor-phase cyclohexane utilization was developed.

Our success in isolating two cyclohexane and cyclohexanol utilizers allowed us to begin mutagenic treatment and mutant selection in Phase I of our research program. The first isolate strain 0058 was put through 4 rounds of selection. One mutant that is missing cyclohexanol dehydrogenase was confirmed. Although this first isolate was not blocked in just the hydrolase enzyme, the process for creating mutations and selecting for cyclohexane pathway blocked mutants has been demonstrated with these strains. These results suggest that construction of more mutants will lead to the caprolactone hydrolase blocked mutants that we are looking for.

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7.0 KEY FOR ENZYMATIC REACTIONS

- Enzymes are natural chemical catalysts. All living organisms synthesize enzymes to perform chemical reactions for the metabolism of food and its conversion into energy and building blocks for growth. Enzymes are made of long chains of amino acids that fold upon themselves to create a complex protein structure. Each kind of enzyme is very specific for the kind of chemicals it can bind to and what type of chemical reaction it can help catalyze.
- NAD: nicotinamide adenine dinucleotide and its phosphorylated derivative NADP are natural electron accepting chemicals that can exist in the oxidized or reduced form as NADH and NADPH. Enzymes utilize them as co-factors to help catalyze oxidation and reduction reactions
- CYCLOHEXANE HYDROXYLASE: (cyclo) a carbon ring (hex) six carbons (ane) single carbon to carbon bonds (hydroxyl) -O-H adding an oxygen and hydrogen side chain (ase) enzyme
- CYCLOHEXANOL DEHYDROGENASE : cyclohex(anol) -O-H hydroxyl or alcohol (de) removing (hydrogen) -H (ase) enzyme
- CYCLOHEXANONE MONOOXYGENASE: cyclohex(anone) C=O double bond to oxygen or ketone (mono) single (oxygen) -O- adding (ase) enzyme
- CAPROLACTONE HYDROLASE: (capro) a six carbon chain acid (lactone) C-O-C=O in a carbon to oxygen to carbon ring (hydrol) H-O-H water adding (ase) enzyme
- 6-HYDROXY-HEXANOATE DEHYDROGENASE: (6-hydroxy-) a -O-H hydroxyl on the sixth carbon (hexanoate) a six carbon acid -COO⁻ H⁺ (de) removing (hydrogen) -H (ase)

Enzymatic Reactions

FEED STOCK - CYCLOHEXANE

DESIRED PRODUCT - CAPROLACTONE