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

Final Report

May 1996

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

For
U.S. Department of Energy
Office of Industrial Technologies
Washington, DC

By
AlliedSignal, Inc.
Des Plaines, Illinois

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Prepared for
U.S. Department of Energy
Under DOE Albuquerque Operations Office
Sponsored by the Office of the Assistant Secretary
for Energy Efficiency and Renewable Energy
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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 were successful in isolating, from natural soil and water samples, four 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 were developed. These blocked-mutants are used to convert cyclohexane into caprolactone but, because of the block, are unable to metabolize the caprolactone further and excrete it as a final end product.

Several factors changed the focus of this project during phase II research. A new nylon carpet recycling process and the long time frame required for instituting a totally new bioprocess led to the abandonment of the cyclohexane to caprolactone bioprocess program by AlliedSignal. A modified and limited study was conducted to evaluate whether a simplified bioprocess to convert cyclohexanol into cyclohexanone or caprolactone was feasible. These studies included the measurement of growth rates and key enzymes levels in a collection of microorganisms that metabolize cyclohexanol to determine if their bioactivity is high enough to support an economical cyclohexanol bioprocess.

Although these microorganisms had sufficient levels of bioactivity, they were only capable of tolerating low levels (<1%) of cyclohexanol and were thus not suitable for development of a cost effective bioprocess because of the high cost of dilute product recovery.

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1.0 INTRODUCTION

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 X 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 to the environment 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 was 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 was 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 was to select microorganism strains that have the highest rates of cyclohexane conversion and greatest cyclohexane solvent resistance. Mutant production and genetic isolation techniques were designed to isolate metabolically blocked mutant strains that can convert cyclohexane into caprolactone but cannot metabolize it further. The key for these enzymatic reactions can be found in Section 7 at the end of the report.

Several factors changed the focus of this project during phase II research. A new process to obtain over 100 million pounds of caprolactam per year by extracting recycled used carpeting was examined and found to be economically and socially attractive (Waste Carpet Recycling: Continuous Pressure Hydrolysis Process for Nylon-6 (DE-FC02-94CE41118)) Final Report by Inara Brubaker, AlliedSignal Research and Technology

and Techno-economic Analysis of the Pressure Hydrolysis Process and the NREL Fluid Bed Pyrolysis Process (Waste Carpet Recycling: Comparison of the Pressure Hydrolysis Depolymerization Process ((Task 7, DE-FC02-94CE41118) submitted by Inara Brubaker and John Hermes AlliedSignal Fibers Engineering). In addition, the slow progress and long time frame required for instituting a totally new bioprocess led to the abandonment of the cyclohexane to caprolactone bioprocess program by AlliedSignal. A modified and limited study was conducted to evaluate if a simplified bioprocess to convert cyclohexanol into cyclohexanone or caprolactone was feasible. These studies included the measurement of growth rates and key enzyme levels in a collection of microorganisms that metabolize cyclohexanol to determine if their bioactivity is high enough to support an economical cyclohexanol bioprocess.

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.

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 [1,2,3,4]. 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 Section 7 for a 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 [5,6]. 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 [7]. 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-Villager like enzymatic reaction that converts cyclohexanone into caprolactone and uses a NADPH

cofactor has also been described in microorganisms [8,9]. 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 [10]. 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 proposed 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 water. We proposed that the microbial cells would be immobilized in a fixed bed bioreactor and used as a biological catalyst for cyclohexane conversion. The caprolactone that is produced in the bioreactor would 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.

1.3 The Modified Cyclohexanol to Caprolactam Process

The proposed bioprocess for conversion of cyclohexane into caprolactone followed by chemical conversion using ammonia to form caprolactam is a major change in the process for the synthesis of nylon-6. The primary force driving this change was the inability to expand nylon production without the burden of producing more byproducts like low cost ammonium sulfate and acetone. Attempts by AlliedSignal to acquire more caprolactam capacity by acquisitions have not been successful, but a new process to recover over 100 million pounds from recycled carpeting has been proposed. This would make the need for a new bioprocess unnecessary. In addition, the lack of progress in identifying good caprolactone production stains has led to the decision to stop research on the

cyclohexane process. An alternate and limited program that could use the expertise and microbial isolates of the current project to examine a modified conversion of cyclohexanol into either cyclohexanone or caprolactone was begun. This process would use microbes to oxidize cylohexanol to cyclohexanone using the enzyme cyclohexanol dehydrogenase (Figure 3). The cyclohexanone could be used directly in our current chemical process thus eliminating a very high temperature chemical oxydation and expensive steam distillation to separate cyclohexanol from cyclohexanone. Or, further oxidation of cyclohexanone by the biocatalyst would yield caprolactone by using the enzyme cyclohexanone monooxygenase. The caprolactone could be used to form caprolactam by treatment with ammonia as described in the original bioprocess.

2.0 EXPERIMENTAL TECHNIQUES SECTION

2.1 Microbial Growth Procedures

2.1.1 Dual Substrate Growth Medium

Bacterial strains were treated with different concentrations of mutagen (NTG) and the cells that survive were screened for mutants. The indicator plates contain only 0.05% yeast extract or only 2mM Na Acetate and 0.1% caprolactone (Table 1). The normal cells utilize caprolactone as a rapid growth substrate and give large white colonies while the negative mutants only grow slowly on the limited amount of yeast extract and produce petite clear colonies. Different methods for growing and evaluating the mutated cells were also evaluated.

2.1.2 Colored Dye Growth Medium

The bacterial isolates were treated with a chemical mutagen and plated out on solid agar media that contains a small amount of nutrient, and caprolactone was provided in the vapor phase as the major growth substrate. In addition, a metabolic dye, tetrazolium, was added to the medium (Table 2). Under these conditions, the parent strain of bacteria yields a large red colony. A potential mutant that is missing a key enzyme would not metabolize caprolactone and can only yield a small white colony. Several thousand colonies were rapidly screened by this method and small white colonies were selected for further testing. These colonies were tested on all the intermediates of cyclohexane metabolism in order to determine the metabolic step that is blocked

2.2 Genetic Selection Techniques

2.2.1 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 (Table 3).

2.3 Enzyme Assay Procedures

2.3.1 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 hydroxylation of cyclohexane to form cyclohexanol uses molecular oxygen (O_2) and the enzymatic reaction can be followed by measuring oxygen uptake from solution. Clark type polarographic oxygen probes and a biological oxygen monitor were used to develop a whole cell hydroxylation assay. Our strain has a cyclohexane dependent oxygen uptake rate of 0.443% O_2 per minute per ml. The decrease in O_2 as a function of cyclohexane addition is documented in Table 4, and the rates of change are described in Figures 4,5 and 6.

Two assays to detect cyclohexane hydroxylase enzyme activity in cell-free enzyme extracts were performed. Two liter cultures of cyclohexane grown cells were harvested and broken open to prepare concentrated enzyme extracts. A NADPH coupled assay that measures the oxygen and cyclohexane dependent oxidation of NADPH and an electrode assay that measures the cyclohexane and NADPH dependent consumption of oxygen were unable to measure the low levels of cyclohexane hydroxylase in our cell extracts. The reaction mixtures are mixed in a 1 ml cuvette at 30°C and contain: KH_2PO_4-Na_2HPO_4 buffer at 50mM and pH 6.5, NADPH at 0.5 mM, 2 uL cyclohexane and 10 to

200 uL of cell extract. The percent O_2 is tabulated in Table 5 and the rates of reaction are described in Figure 7. No increase in reaction rate was seen upon addition of cyclohexane and an unexpected rise in oxygen levels was observed.

2.3.2 GC-MS Caprolactone Assay

The conversion of cyclohexane into caprolactone requires the use of whole bacterial cells. An efficient method to extract and measure these chemical intermediates is needed to monitor the progress of the biochemical reactions. We have found that incubation of whole cells in reaction broth with a one half volume of ethyl acetate during 20 minutes of controlled mixing at ambient temperature followed by centrifugation of the sample to precipitate the cells is capable of extracting the organic intermediates. The extracts were analyzed with a gas chromatograph using a methyl siloxane column to separate the individual components. The negative control samples have very little background interference and cyclohexane, cyclohexanol, cyclohexanone and caprolactone are easily separated and quantified. The hydrolyzed chemical intermediate, 6-hydroxyhexanoate, is not soluble in neutral ethyl acetate and does not interfere with the gas chromatograph assay. The individual peaks that were separated on the gas chromatograph were analyzed using a mass selective spectrometer detector. The mass fragmentation patterns of the individual components were compared to standards and the identities of each chemical intermediate was confirmed. Timed samples of the biotransformation reactions were taken to confirm the metabolism of cyclohexane.

3.0 RESULTS AND DISCUSSION

3.1 Task 1.0 Phase II Design Methods to Produce Genetic Mutants

The first task in the second phase of the program was to design efficient methods to produce metabolically blocked production mutants. Those strains that were identified in phase one as having cyclohexane and cyclohexanol utilization activity and some solvent resistance were used to develop these genetic selection techniques. Every different bacterial isolate has optimal conditions for generating genetic mutations. We used different chemical and physical mutagens and different amounts and times of exposure to treat our isolates. The optimal conditions for mutagenesis were determined by measuring kill rates and by using an internal control for mutation to antibiotic resistance.

3.1.1 Mutagenesis of Strain ESM 0059

Bacterial strain 0059 is capable of using various organic compounds as the source of metabolic carbon and energy for growth. Measurement of the rate of growth tells us how rapidly the organic compounds are being metabolized and converted into intermediates and how long it would take to prepare large quantities of bacterial cells as we scale up the size of our bioreactions. The growth rate is defined as the time required to achieve a doubling in cell mass. Because strain 0059 can be grown in liquid culture as a uniform cell suspension, the growth of cells can be easily measured as an increase in optical density of the suspension. We have determined that the growth rate of this strain utilizing cyclohexane (20 hours doubling time) is similar to the rate with more common organics such as acetate (17 hr.)and ethanol (15 hr.) under aerobic conditions at 30 C (Figure 8,9 and 10).

The selection of caprolactone blocked mutants of strain 0059 was attempted. We made several changes to our genetic selection procedures because of poor cell survival after mutagenic treatments and inefficient cell plating on solid mutant selective media. Three

new mutagenic experiments were performed. We lowered the dose of mutagen to 5, 10 or 15 micrograms per milliliter for one hour. The cells were rescued in a new medium using either ethanol and yeast extract or a combination of nutrient broth extract with yeast extract. In addition, the cells were diluted in a nutrient broth and yeast extract prior to plating the cells out on solid selective media. Finally, we delayed the addition and lowered the concentration of caprolactone in the selective plates to favor the survival of mutants.

Several mutagenic treatments of strain 0059 were performed in order to select for caprolactone negative mutants. The growth and recovery of the cells after treatment with chemical mutagens was very difficult and only a few bacteria survived and could be plated out as individual colonies. Selection for a more robust isolate from this strain has not yet been successful. The use of alternate growth media, special diluents and delayed addition of organic substrates did not totally removed the cell recovery problem. An alternate strain ESM 0057 was isolated and selected as a strain that has gained better growth in liquid culture on cyclohexane and excellent recovery of individual colonies on caprolactone to perform genetic selection for caprolactone blocked mutants.

3.1.2 Mutagenesis of Strain ESM 0057

Mutagenic treatment (NTG) of our new bacterial isolate that has an improved growth rate on cyclohexane was attempted. One method of determining how much mutagen to use is to measure the extent of cell killing. If the cells are sensitive to the mutagen, it should cause enough cell DNA damage to kill some of the cells. The culture was grown in the presence of different amounts of mutagen, 0,25,100 and 200 ug/ml and incubated for 1 hr followed by centrifugation and washes to remove the mutagen. These cells were then diluted out and plated on solid agar plates to determine how many cells survived the chemical treatment. At 25 ug/ml of mutagen 90% of the cells were killed, 99% at 50 ug/ml and 99.9% at 100 ug/ml. No cells survived at 200 ug/ml. The optimal level of

killing to ensure that the majority of cells have been mutated is between 99 and 99.9% kill.

3.2 Task 2.0 Phase II Develop Blocked Mutant Selection Methods

The second task was to develop efficient selection tools to identify the desired blocked-mutants. In order to select and identify those rare, one in a few thousand, blocked-mutants, specific enrichment and selection methods are required. Blocked- mutants can sometimes be identified by selecting for cells that are resistant to toxic chemical analogs of the intermediates of interest. Different fluorinated and chlorinated analogs of cyclohexane can be used to determine if analog resistance yields blocked-mutants. Negative selection methods that use antibiotics such as penicillin, D-cycloserine and energy dependent poisons like streptozotocin can also be used to selectively kill cells that can metabolize cyclohexane and enrich for the survival of blocked-mutants. Metabolic indicators such as colored tetrazolium dyes can also be used to screen thousands of bacterial colonies on solid agar media for the loss of cyclohexane metabolic activity.

3.2.1 Strategy for Detecting Mutant Strains

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. 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. 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. 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. 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.

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.

3.2.2 Mutant Screening of Strain ESM 0057

Five rounds of mutagenesis and screening for cyclohexane negative mutants were conducted with strain ESM 0057. The results are summarized in Table 6.. Approximately 50,000 colonies were examined and 29 presumptive negatives were examined for the presence of cyclohexane metabolizing enzymes and the ability to excrete caprolactone.

3.3 Task 3.0 Phase II Develop Enzymatic Assays to Identify Mutant Strains

The third task was to screen the presumptive blocked mutants for the presence and level of cyclohexane metabolic enzymes. These enzyme assays, that were developed in phase one and the beginning of phase two, were used to confirm the block "loss" of the enzyme caprolactone hydrolase. The enzyme assays were also used to determine the optimal growth conditions for induction of high levels of the cyclohexane hydroxylation and oxidation enzymes.

3.3.1 Identification of Mutant Strain 5711F4

Twenty nine presumptive cyclohexane-negative mutants were examined. These isolates were grown in 100 uL microtiter wells and transferred using mechanical replicators onto agar plates that contained: cyclohexane, cyclohexanol, cyclohexanone, caprolactone, 6-hydroxyhexanoate, adipic acid, succinic acid and a nutrient broth positive control. Twenty of the isolates were very slow to grow on cyclohexane and the metabolic intermediates. Eight isolates did not grow at all on cyclohexane but they were also negative or very slow to grow on all intermediates including 6-hydroxyhexanoate, adipic acid and succinic acid. One isolate did not grow at all. These results suggest that the majority of the presumptive-mutants are only defective in the ability to regulate the induction of cyclohexane metabolic enzymes. The eight stable cyclohexane-negatives appear to be defective in some general metabolic step and they are not specific to the enzymes for cyclohexane metabolism.

Several presumptive mutants were examined to determine if they had a mutation that blocked caprolactone hydrolase activity. The results presented in Table 7 show that most isolates had hydrolase activity except for isolate designated 5711F4. In addition, as seen in Table 8 this isolate still retained activity for the two earlier metabolic step enzymes cyclohexanol dehydrogenase and cyclohexanone monooxygenase.

3.3.2 Excretion of Caprolactone by Mutant Strain 5711F4

Because this mutant appeared to have the necessary enzymes to synthesize caprolactone from cyclohexane but did not have a caprolactone hydrolase, we examined if this strain could excrete caprolactone when incubated with cyclohexane for different time periods. The incubation times and growth curves of strain 5711F4 and control parent strain ESM 0057 are shown in Table 9 and Figure 11. Table 10 shows that caprolactone was excreted by the mutant strain at 15 min, 8 hours and 24 hours. Figure 12 shows that the unknown product had the same retention time as authentic caprolactone as determined by

gas chromatography. In addition, Figure 13 shows that the unknown peak has a mass fragmentation pattern identical to caprolactone as determined by mass spectrometry analysis.

These results were repeated and 27 other presumptive mutants were examined. GC/MS analysis of the reaction extracts revealed that ten of the isolates could metabolize cyclohexane but none of them excreted any detectable caprolactone. The positive results with isolate 5711F4 could not be repeated even though longer induction and more complete extraction procedures were used. We assume that the block mutation in strain 5711F4 was very unstable and this strain easily mutates back to a cyclohexane positive strain.

3.4 Task 4.0 Phase II (Modified) Conduct A Short Term Evaluation To Determine If A Limited Bioprocess To Convert Cyclohexanol Into Caprolactone Is Feasible.

The last task in phase two of the program was to have been the optimization of the caprolactone production process using shake flask experiments. We planned to examine the effect of many different production media, substrate and co-substrate levels, temperature and aeration rates on the level of caprolactone production. At this time, the use of immobilized cells and two phase reaction media were also to be investigated.

Because of a change in direction in company goals for caprolactam production and a lack of technical progress in isolating suitable caprolactone production strains, the third phase of the project, that was to involve scale-up and pilot plant evaluations, was terminated. A two month laboratory project to evaluate if a simplified bioprocess to convert cyclohexanol into caprolactone is feasible was conducted in its place. These studies include the measurement of growth rates and key enzyme levels in a collection of microorganisms that metabolize cyclohexanol to determine if their bioactivity is high enough to support an economical bioprocess.

3.4.1 Determination of Cyclohexanol Metabolic Rates

Seven different microbial isolates from our culture collection were grown using cyclohexanol as a growth substrate and examined in both liquid and solid media to confirm that the cultures only contained one pure strain. Two cultures that did not grow well on repeated transfer in a cyclohexanol medium were discarded. Three of the remaining five culture that had the fastest growth rates were chosen for more detailed growth analysis. The most active strain was 38 with a growth rate of 0.514 generations/hr at 17 mM and could tolerate 46 mM cyclohexanol (Figures 14,15). Strain 39 had a growth rate of 0.167 generations/hr at a concentration of 12.5 mM and could tolerate cyclohexanol levels of 45 mM (Figures 16,17). Strain 61 had a growth rate of 0.111 generations per hour. The fastest growth was measured at a 4.2 mM cyclohexanol concentration. The maximum level of cyclohexanol that strain 61 could tolerate was 25 mM (Figures 18,19).

Large cultures of three different bacterial strains were then cycled on increasing levels of cyclohexanol to select for isolates that have adapted to higher levels of tolerance to cyclohexanol. Strain 38 was adapted to 42 mM cyclohexanol (Table 11,12), strain 39 to 61 mM (Table 13) and strain 61 could grow at 15 mM (Table 14). The highest level at 61 mM represents a 0.64% solution of cyclohexanol.

Although the rate of growth and the maximum level of substrate that a bacterium can tolerate are good measures of its ability to metabolize cyclohexanol, a determination of the two key enzymes, cyclohexanol dehydrogenase and cyclohexanone monooxygenase are a more direct measurement of that microbes potential for converting cyclohexanol into caprolactone. Strain 39 had a specific enzyme activity (umole/min/mg cell protein) of 0.032 for dehydrogenase and 0.417 for monooxygenase. Strain 61 had 0.244 dehydrogenase and 0.607 monooxygenase enzyme activity. The best strain was 38 with 0.708 and 0.900 specific activity enzyme units (Table 15). These results can be used to

calculate the maximum conversion rates for synthesis of caprolactone or cyclohexanone from cyclohexanol.

3.4.2 Cost Estimates for Cyclohexanol Bioreactor

If a 1% solution of cyclohexanol can be fed to the bioreactor:

10 g/L X 3.8 L/gal = 38 g/gal

Then: 38 g/gal X 0.0022 lb/g= 0.0836 lb/gal

If the enzymatic activity in cell extracts is approximately 1 umole/ min/ mg protein: assume that cells grown to an optical density of 1.0 have 0.25 mg protein /ml and that these are concentrated 40 fold then mixed with 50% acrylamide to form catalyst beads that occupy 60% of the bioreactor volume. 1 umole/min/mg X .25 mg/ml X 40 X 50% X 60% = 3 umole / min/ml of reactor volume.

Then: 3 umole/ min/ml X 3785 ml/gal = 11355 umole or 0.0114 mole X 110 MW cyclohexanol = 1.25 g/min/gal

Then: 1.25 g/min/gal X 0.0022 lb/g X 60 min/hr = 0.164 lb/hr/gal

Then: A 10,000 gal reactor with a 30 min. residence time operating 24 hr /day for approximately one year could produce: 0.164 lb/hr/gal X 10,000 gal X24 hr/day X 350 days/yr = 13.78 million lb/yr

The cost for the biocatalyst , acrylamide support, bioreactor and operation can be approximated. The major cost of preparing the biocatalyst is the cost of the acrylamide support beads. If we assume that 66% of the reactor volume is biocatalyst beads and that the beads contain 15% acrylamide and use 0.1% ammonium persulfate for polymer initiation, the beads will cost $0.65 per gal of reactor. The cost of the cells is low because the growth substrates are inexpensive and the actual weight of the biocatalyst on a dry weight basis is small. If 40 X concentrated cells are used in a 50/50 mix with acrylamide solution and 20% of the growth substrate is converted into cells, the cost of

the cells is only $0.05 per gal of reactor material. The total cost of biocatalyst is therefore $0.70 per gallon for materials.

The operational cost for a 10,00 gallon reactor can also be calculated. We assume a stainless steel reactor using a pump and a heat exchanger. Cooling water from the existing plant would be used to drop the temperature form 50 to 35 C. The cost of a stainless 316 reactor $40,800, heat exchanger $12,000 and pump $3,600 gave a simplified total of $57,100 for capital equipment. The cost of power was estimated at $14,100 per year. If we assume 70% of equipment cost for installation and plumbing, the final cost for capital is $97, 000.

The total cost for just the bioreactor can then be calculated. If capital costs are depreciated over five years, the capital expenditure is $20,000 per year. Operation of utilities costs $14,000 and the biocatalyst beads cost $7,000. The total is $41,000 per year. The most sensitive factor is the stability of the catalyst. If the $7,000 biocatalyst must be replaced every month the cost escalates to $118,000 per year.

The cost for separation and purification of product has been deliberately omitted because the dilution of the cyclohexanol to only a 1% solution for biocatalysis is impractical. The cost of recovering the product cyclohexanone or caprolactone from 99% water is not economically feasible. The biocatalytic synthesis could only be practiced if the cells could tolerate greater than 90% cyclohexane in the reactor.

4.0 CONCLUSIONS AND RECOMMENDATIONS

Microorganisms that can metabolize cyclohexane, cyclohexanol, cyclohexanone, caprolactone and 6-hydroxyhexanoate can be isolated form natural sources. The most difficult isolations were for microbes that can metabolize the chemically unreactive cyclohexane molecule. We were able to document the metabolic path for the metabolism of cyclohexane by three techniques. Feeding of chemical intermediates confirmed that microorganisms that metabolize cyclohexane can always also metabolize the intermediates cyclohexanol, cyclohexanone, caprocltone and 6-hydroxyhexanoate. The use of gas chromatography and mass spectroscopy also detected the presence of the cyclohexanol and caprolactone intermediates at low levels in some cells that were actively metabolizing cyclohexane. Finally, we were able to develop specific enzyme assays and measure all the enzyme activities involved in the metabolism of cyclohexane to 6-hydroxyhexanoate: cyclohexane hydroxylase, cyclohexanol dehydrogenase , cyclohexanone monooxygenase and caprolactone hydrolase. These results confirm that the major pathway, that we proposed early in the research program, was present in all the microorganisms that we isolated and examined.

The ability to generate, isolate and identify cyclohexane-negative mutants of these strains was also demonstrated. Not all of the strains could be successfully mutated and selected because of their difficult growth patters. Some strains did not give reproducible growth on cyclohexane and the other chemical intermediates while some strains were very sensitive to the action of the chemical mutagens that we used and did not recover form the selective treatments.

The most disappointing factors were the inability to isolate a stable caprolactone hydrolase negative-mutant that could consistently excrete caprolactone and the very low level of chemical resistance of our microorganisms to cyclohexane and cyclohexanol. We

had been successful earlier in easily isolating microbes that were resistant to concentrated solutions of both cyclohexane and cylohexanol but these microbes were not good cyclohexane or cylohexanol metabolizing strains. The ability to both metabolize and have good resistance to cyclohexane is crucial to the development of efficient and economical bioreactors. Both attributes must be present in the same cells. Dual selections for new strains from nature that have both abilities would be required; or the genetic construction of engineered strains that have been given the genes for the solvent resistance and cylohexane and cylohexanol metabolism might be necessary.

The economics of petrochemical synthesis of both commodity and specialty chemicals requires the use of concentrated solutions and some harsh conditions such as solvent, salt and temperature resistance. The next breakthrough in organic synthesis using microbes will require the selection of new microbes that come from extreme environments such as thermal vents and deep oil wells. The use of these naturally selected "extremophiles" will ensure the survival of the biocatalyst and a robust bioreactor design.

5.0 ACKNOWLEDGMENTS

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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

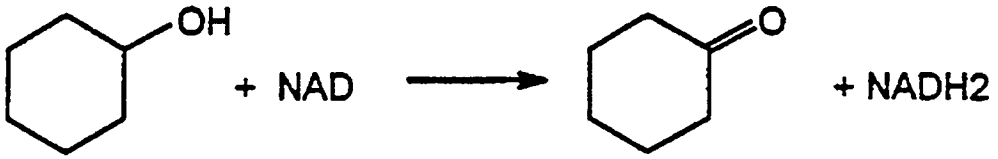
- **CAPROLACTONE HYDROLASE**: (capro) a six carbon chain acid (lactone) C-O-C=O in a carbon to oxygen to carbon ring (hydrol) H-O-H water adding (ase) enzyme

- **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)

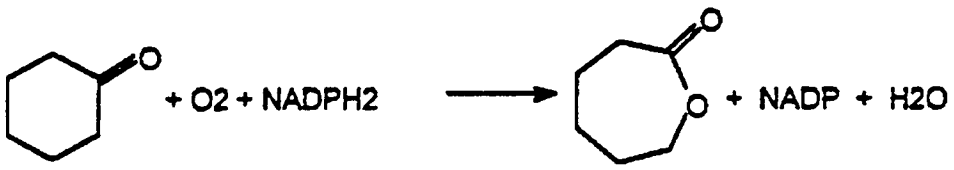
Enzymatic Reactions

FEED STOCK - CYCLOHEXANE

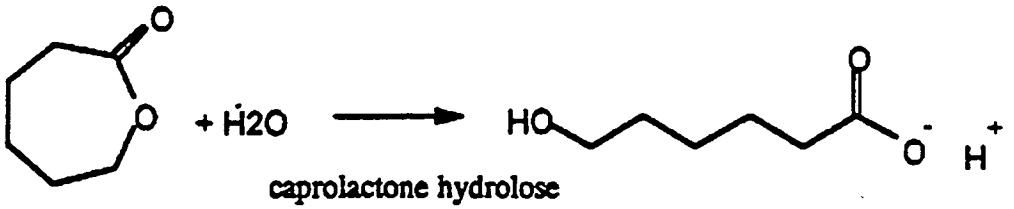
cyclohexane hydroxylase



cyclohexanol dehydrogenase



cyclohexanone monooxygenase



caprolactone hydrolose

DESIRED PRODUCT - CAPROLACTONE

6-Hydroxy-Hexanoate dehydrogenase

Table 1a **Duel Substrate Growth Medium**

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
$(NH_4)_2SO_4$	2.38
$MgSO_4$ $7H_2O$	0.05
$CaCl_2/CaCl_2$ $5H_2$	0.01
Yeast Extract	0.5
Na acetate	2 mM
Trace solution	1.0ml
(Recipe follows)	
Agar	15

Table 1b. **Trace Element Solution(1000x)**

Ingredients per 100 mls	
H_3BO_4	0.286 gram
$MnSO_4.H_2O$	0.154 gram
$Fe(NH_4)_2SO_4.6H_2O$	0.353 gram
$CuSO_4.5H_2O$	3.9 mg
$ZnCl_2$	2.1 mg
$CoCl_2$	4.1 mg
$Na_2MoO_4.2H_2O$	2.5 mg
Adjust the pH to 7.2	

Table 2 **Colored Dye Growth Medium**

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
$(NH_4)_2SO_4$	2.38
$MgSO_4$ $7H_2O$	0.05
$CaCl_2/CaCl_2$ $5H_2$	0.01
Yeast Extract	0.5
Tetra Nitro blue tetrazolium chloride	0.1
Trace solution	1.0ml
(Recipe follows)	
Agar	15

Table 3 **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

Table 4 Cyclohexane Hydrolase Activity in Whole Cells of ESM 0057-1

Minutes	% Oxygen	Comments
0	98	Water
1	91.8	
2	91.7	
3	91.5	
4	91.1	
6	89.8	
9	88.2	
10	87.8	
15	85.6	
18	84.3	
20	83.9	
22	82.9	5 ul of(5 ml buffer + 5 ul cyclohexane)
25	81.4	
28	80.3	
30	79.7	
35	77.6	
36	77.4	5 ml buffer + 5+5 ul cyclohexane)
40	74.5	
45	71.9	
50	70.9	
55	68.7	
60	67.4	cyclohexane i ul straight
61	67.2	
62	66	
63	65	
64	64.1	
65	63.3	
66	62.5	
67	61.6	
68	61	
69	60.3	
70	59.7	cyclohexaane 1 ul straight
72	58.7	
76	55.9	
80	53.9	

Table 4 Cyclohexane Hydrolase Activity in Whole Cells of ESM 0057-1 (cont'd)

85	50.7	
86	50	
88	48.6	
90	47.4	
94	44.5	
100	39.5	
103	36.9	
105	35.4	
107	33.9	
108	32.9	
110	31.4	

Table 5 Cyclohexane Hydroxylase activity in Cell Extracts of ESM 0057-1

Time Minutes	% Oxygen	Comments
0	100.5	
1	100.6	
3	100.1	
4	99.9	
6	99.4	
10	98.6	
13	98.1	
15	98.2	
18	98.2	
19	98	
20	98	
23	97.7	
25	97.4	
26	97.3	Added 60 ul of NADPH
27	97.1	
29	96.4	
32	95.7	
33	95.5	
36	94.8	
39	94	
40	93.9	Added 0.5 microliter cyclohexane
41	93.3	
49	93.1	
50	93.1	
53	93.5	
56	93.5	
57	93.6	
60	94.2	
70	96.4	
80	99.1	
86	100.8	

Table 6. Summary of Strain ESM 0051 Mutant Selection

ESM 0057 Strains NTGed	0057-1	572K	5722	5731	5723	Total
Total colonies screened	28,800	8708	1464	3040	5040	47,052
Petites colonies picked	400	264	120	250	350	1384
Cyclohexane negative mictotiter	400	250	100	200	300	1250
Condirmed cyclohexane negatives	40	12	7	52	18	129
Caprolactone negatives						29
Confirmed caprolactone negatives						8
Reconfirmed caprolactone negative						0

Table 7. Caprolactone Hydrolase Activity of ESM 0057 Mutant Strains

Mutant Strains of 57-1	Specific Activity umoles/min./ml.	Difference in O.D./ Min. between 0-2 Minutes
57-1 Control	0.39	-0.056
5711D4	0.89	-0.066
5711E3	0.21	-0.028
5711F4	0.0	0.0
5712D4	1.45	-0.103
5712F3	1.08	-0.123
5713D3	0.149	-0.039
5713D5	0.160	-0.021
5713E4	0.235	-0.032

Table 8. Enzyme Analysis of Strain 5711F4

Enzyme	Specific Activity (umole/min/mg protein)
Cyclohexanol dehydrogenase	0.015
Cyclohexanone monooxygenase	0.065

Table 9. Optical density of Test and Control Cultures of 5711F4

TIME (HRS)	C0'	C0	C4	C8	C24	C48
0	1.2	1.1	1.22	1.14	1.115	1.218
2			1.167	1.143	1.149	1.16
4			1.156	1.128	1.123	1.117
6				1.125	1.122	1.128
8				1.153	1.147	1.167
24					1.028	1.067
48						0.982

TIME (HRS)	T0'	T0	T4	T8	T24	T48
0	1.171	1.202	1.197	1.178	1.185	1.195
2			1.119	1.126	1.122	1.117
4			1.116	1.097	1.073	1.086
6				1.085	1.09	1.067
8				1.098	1.073	1.089
24					0.948	0.981
48						0.846

C= Control cultures 57-1
T= Test cultures 5711F4 caprolactone negative mutant of 57-1
C0= 6 ul cyclohexane, incubated for 15 minutes in the shaker incubator before extraction for cyclohexane to reach equilibration in liquid phase.
C0'= No cyclohexane

Table 10. Summary of Caprolactone Production by 5711F4

Cultures 57-1	Cyclohexane ug/L	Cyclohexanol ug/L	Cyclohexanone ug/L	Caproic aldehyde ug/L	Caprolactone ug/L
C0'	ND	ND	ND	ND	ND
C0	2571	ND	ND	ND	ND
C4	2106	ND	1.79	1.46	NT
C8	1890	ND	ND	ND	ND
C24	1967	ND	ND	ND	ND
C48	1928	ND	ND	ND	ND

Cultures 5711F4 Mutant	Cyclohexane ug/L	Cyclohexanol ug/L	Cyclohexanone ug/L	Caproic aldehyde ug/L	Caprolactone ug/L
T0'	ND	ND	ND	ND	ND
T0	2166	ND	ND	ND	2.47
T4	1819	ND	ND	ND	ND
T8	1695	ND	ND	ND	1.76
T24	1712	ND	ND	ND	1.16*
T48	2827	ND	ND	ND	ND

*=Below quntitation level but above detection level.
ND= Not detected

Table 11. Growth and Tolerance of ESM 0038 on Cyclohexanol(A)

Date	Time	Hours	Cyclohexanol concentration ul				
			0	3.6	10.8	14.4	21.6
11/20/95	9:30 AM	0	0.016	0.017	0.023	0.016	0.017
	2:00 PM	4.5	0.240	0.246	0.230	0.187	0.149
	4:30 PM	7	0.406	0.560	0.482	0.500	0.340
11/21/95	12:30 PM	27	0.324	0.677	0.984	0.874	0.774
	5:00 PM	31.5	0.318	0.655	0.978	0.852	0.823
11/22/95	10:00 PM	48.5	0.295	0.635	1.005	0.830	1.017

Date	Time	Hours	Cyclohexanol concentration ul			
			0	180	220	300
11/22/95	4:30 PM	0	0.124	0.141	0.126	0.134
11/26/95	12:30 PM	92	0.316	1.370	0.742	0.081

Inoculum from 180 ul cyclohexane flask of 11/22/95 2 ml.

Date	Time	Hours	Cyclohexanol concentration ul		
			0	220	350
11/29/95	5:30 PM	0	0.046	0.046	0.051
12/01/95	9:30 AM	64	0.245	0.088	0.021 clumpy
12/04/95	12:30 PM	99	0.171	0.056	0.022

Table 12. Growth and Tolerance of ESM 0038 on Cyclohexanol (B)

Inoculum from 180 ul cyclohexanol flask final O.D. 1.370

Date	Time	Time Hours	Cyclohenol 180 ul
12/07/95	9:00AM	0	0.201
	10:00 AM	1	0.247
	12:30 PM	3.5	0.258
	3:00 PM	6	0.248
12/08/95	8:30 PM	23.5	0.890
	1:30 PM	28.5	1.021
	5:30 PM	32.5	1.081
12/11/95	1:00 PM	110.5	1.372
12/12/95	9:00 AM	131.5	1.743

Inoculum from 180 ul cyclohexanol flask of 12/07/95 final O.D.1.743

Date	Time	Hours				
			0	220	270	320
12/13/95	2:00 PM	0	0.215	0.280	0.272	0.249
12/14/95	4:30 PM	26.5	0.460	0.408	0.246	0.216
12/15/95	9:00 AM	42	0.440	0.629	0.240	0.215
Additions						
*Inoculum			Nothing	Nothing	10 ml of 180 ul flask 12/07/95	10 ml of 180 ul flask 12/07/95
*cyclohexanol			Nothing	Nothing	270 ul	320 ul
12/15/95	1:30 PM	47.5	0.432	0.653	0.602	0.568
	4:00 PM	50	0.430	0.648	0.602	0.572
12/18/95	1:00 PM	119	0.395	0.803	0.582	0.560
12/19/95	10:00 AM	140	0.382	0.784	0.619	0.560
12/20/95	9:00 AM	163	0.370	0.768	0.601	0.560
12/21/95	9:00AM	187	0.362	0.824	0.600	0.568

Table 13 Growth and Tolerance of ESM 0039 on Cyclohexanol

Date	Time	Hours	Cyclohexanol concentration ul				
			0	20	60	80	120
11/20/95	9:30 AM	0	0.124	0.124	0.124	0.129	0.117
	2:00 PM	4.5	0.187	0.188	0.195	0.218	0.171
	4:30 PM	7	0.248	0.252	0.26	0.251	0.209
11/21/95	12:30 PM	27	0.345	0.976	1.015	0.822	0.486
	5:00 PM	31.5	0.332	0.96	1.015	0.822	0.486
11/22/95	10:00 PM	48.5	0.317	0.944	1.15	1.382	0.6

Date	Time	Hours	Cyclohexanol concentration ul			
			0	180	220	300
11/22/95	4:30 PM	0	0.109	0.111	0.101	0.214
11/26/95	12:30 PM	92	0.6	1.189	1.305	0.101

Date	Time	Hours	Cyclohexanol concentration ul			
			0	300	400	500
11/29/95	5:30 PM	0	0.259	0.315	0.216	0.214
12/01/95	9:30 AM	64	0.615	0.262	0.151	0.131
12/04/95	12:30 PM	99	0.628	0.314	0.12	0.118

Table 14. Growth and Tolerance of ESM 0061 on Cyclohexanol

Date	Time	Hours	Cyclohexanol Concentration ul				
			0	20	60	80	120
			Optical Density at 600 nm				
11/20/95	10:00 AM	0	0.06	0.059	0.071	0.064	0.062
	2:00 PM	4	0.085	0.092	0.088	0.091	0.098
	4:30 PM	6.5	0.095	0.102	0.101	0.102	0.112
11/21/95	12:30 PM	26.5	0.17	0.265	0.191	0.164	0.161
	5:00 PM	31.5	0.176	0.328	0.211	0.172	0.172
11/22/95	10:00 AM	48	0.175	0.679	0.269	0.172	0.172
11/26/95	12:30 PM	192	0.156	0.631	0.666	1.132	0.212

Date	Time	Hours	Cyclohexanol mM	
			120	220
			Optical Density at 600 nm	
11/29/95	5:30 PM	0	0.06	0.06
12/01/95	9:30 PM	60	0.135	0.09
12/04/95	12:30 PM	142	0.146	0.07

Table 15 Enzyme Analysis of Strains ESM 0038, 39, 61

Specific Activity uM/minute/mg protein		
Strains	Cyclohexanol dehydrogenase	Cyclohexanone monooxygenase
ESM 0038	0.708	0.900
ESM 0039	0.032	0.417
ESM 0061	0.244	0.607

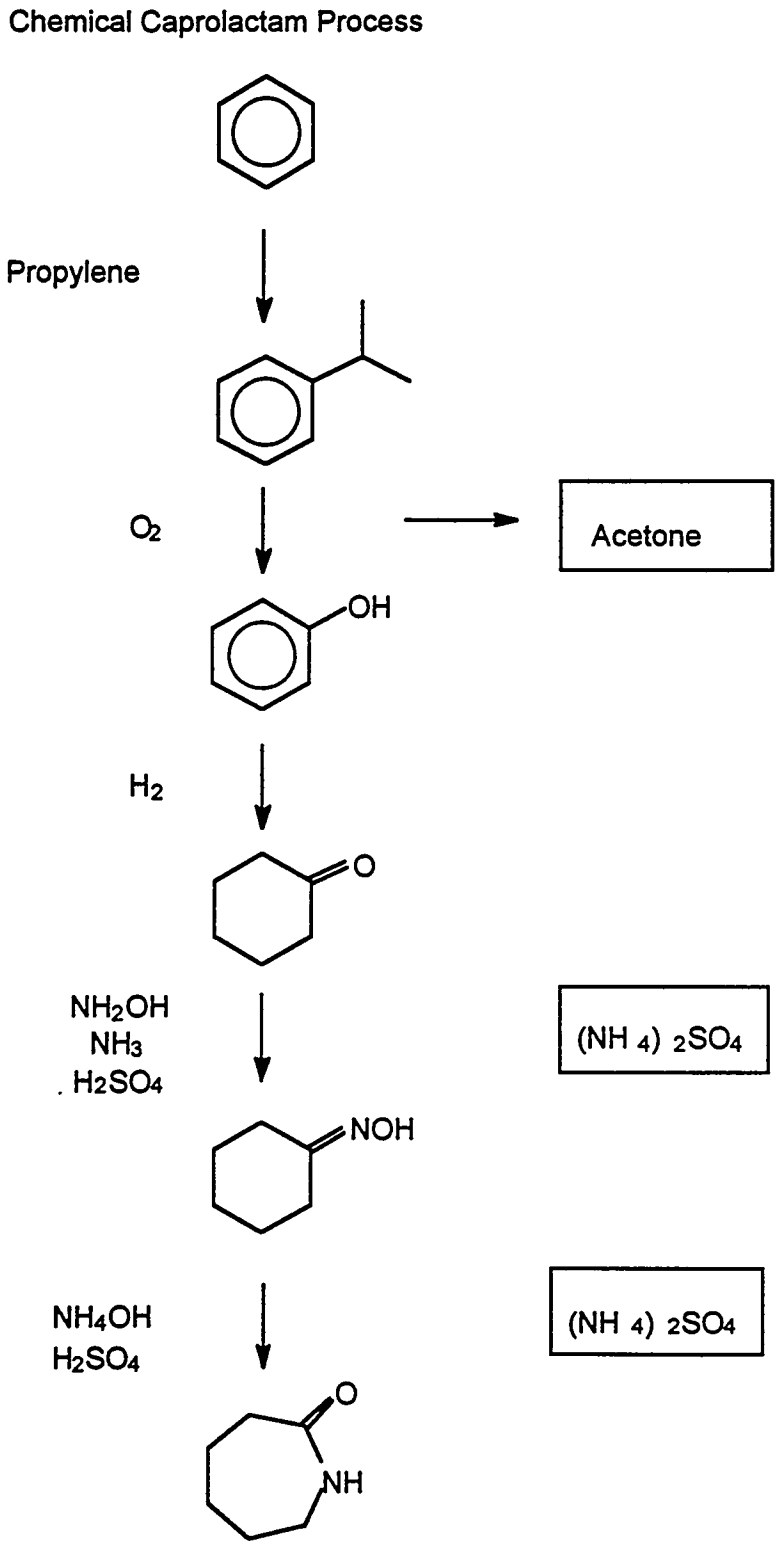


Figure 1 The Current Chemical Process for Caprolactam Production

Biological / Chemical Caprolactam Process

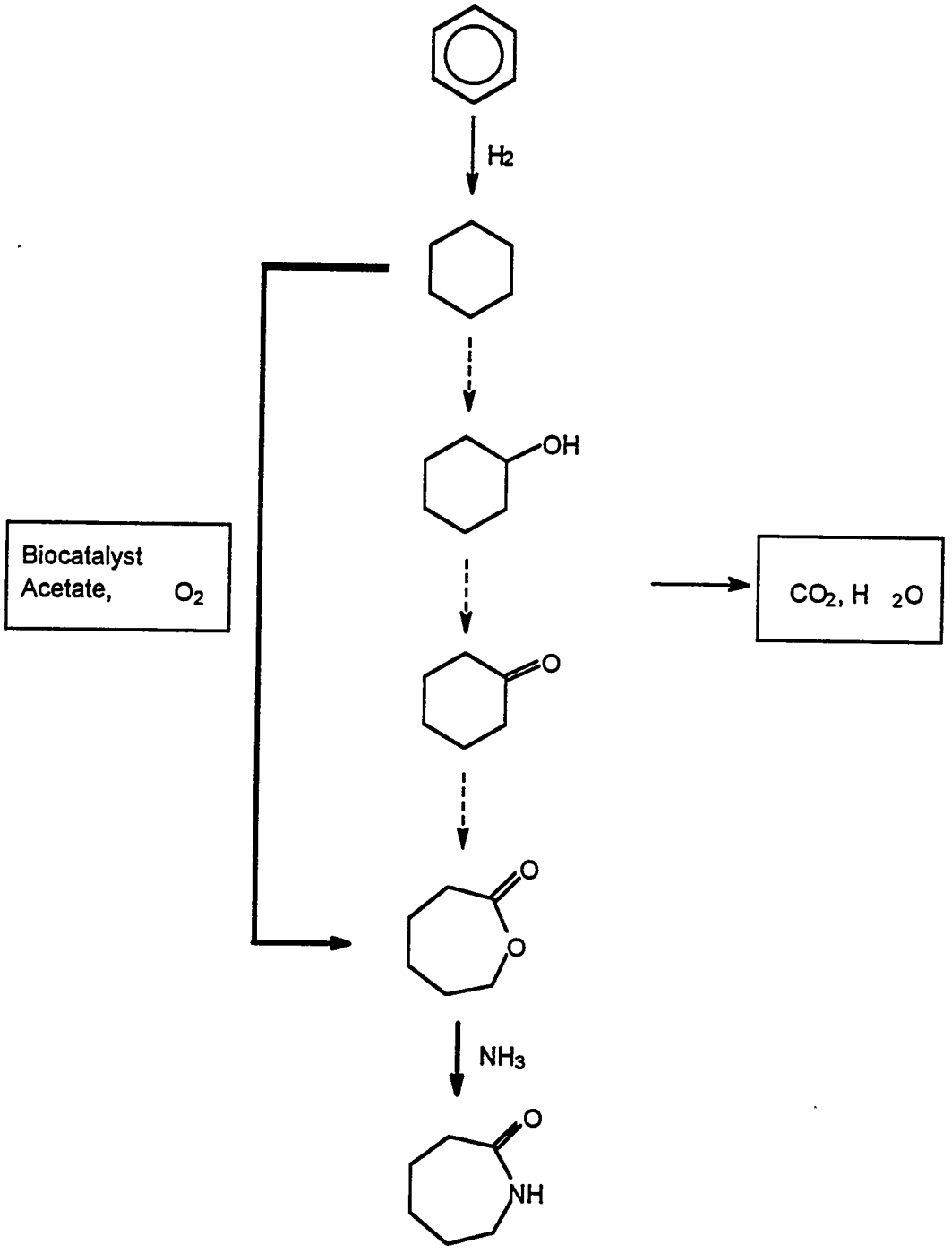


Figure 2 The Proposed Biological/Chemical Process

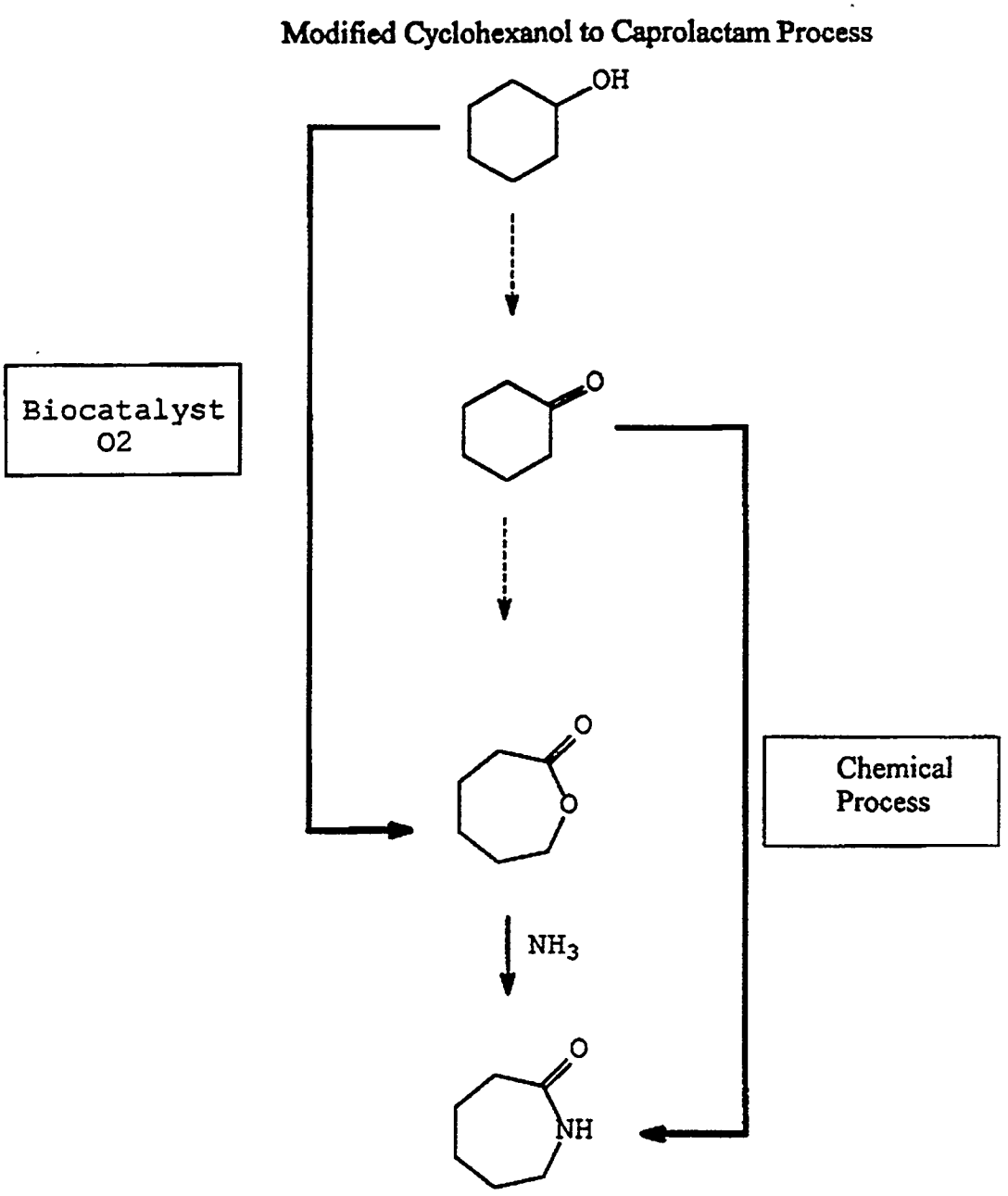


Figure 3 The Modified Cyclohexanol to Caprolactam Process

Figure 4 Oxygen uptake by whole cells of ESM 0057-1 (A)

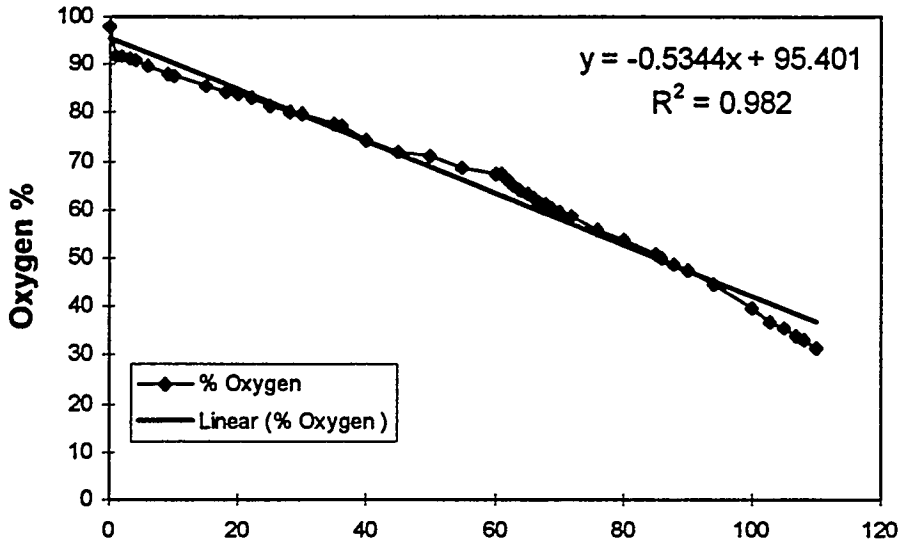


Figure 5 Oxygen uptake by whole cells of ESM 0057-1 (B)

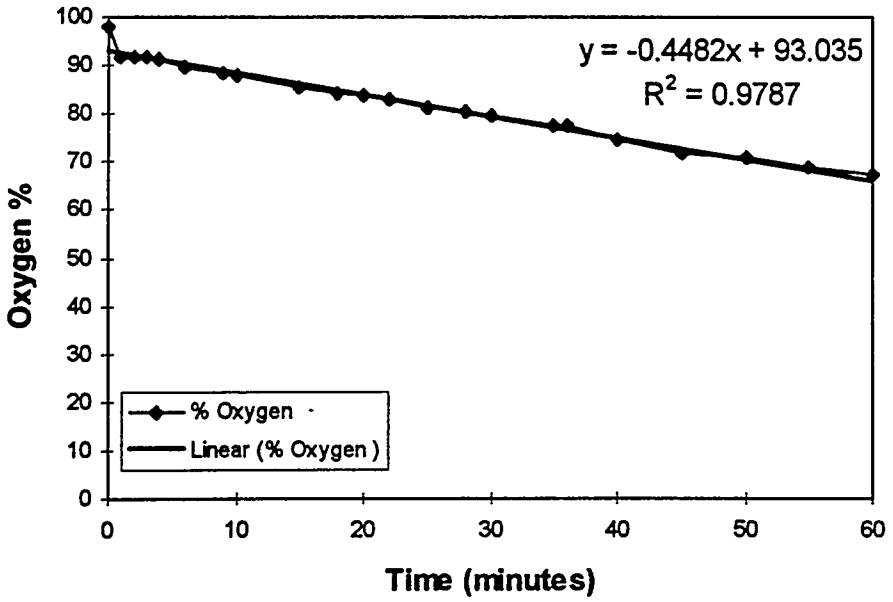


Figure 6 Oxygen uptake by whole cells of ESM 0057-1 (C)

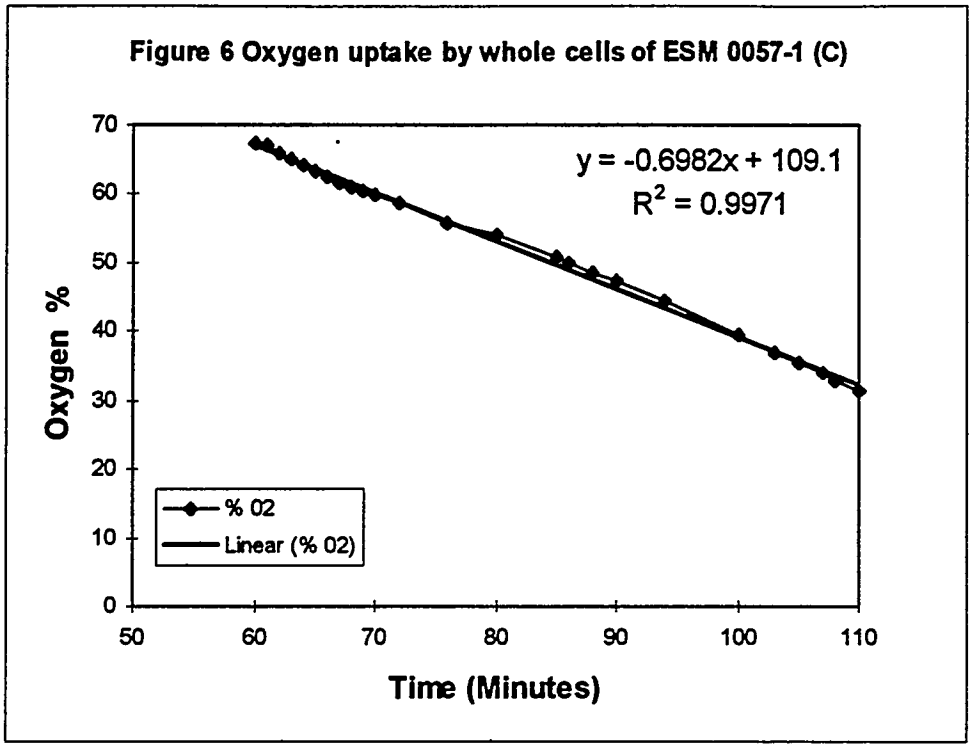


Figure 7 Oxygen uptake by cell extract of ESM 0057-1(D)

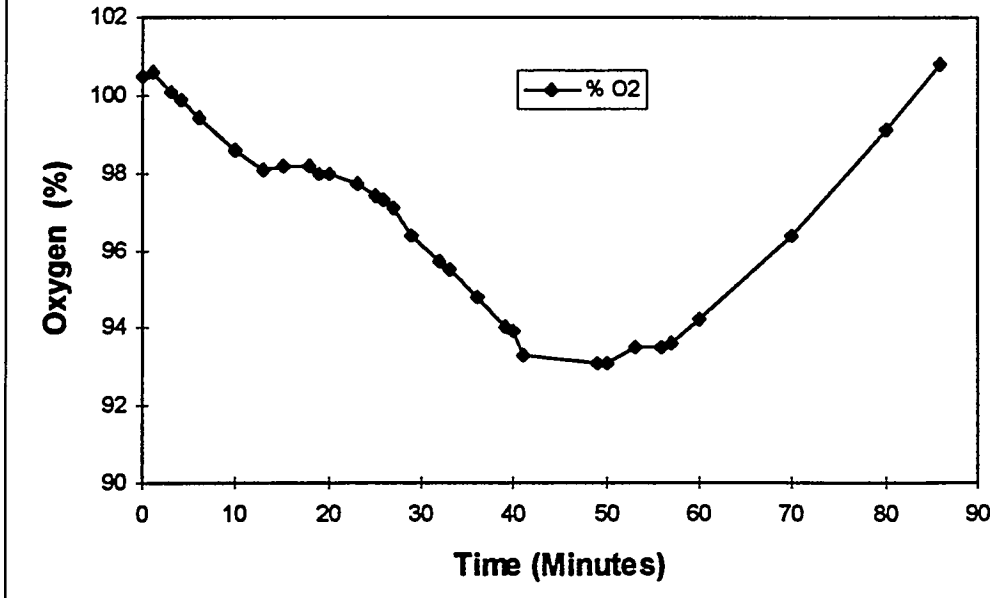


Figure 8 Growth of ESM 0059 on Cyclohexane

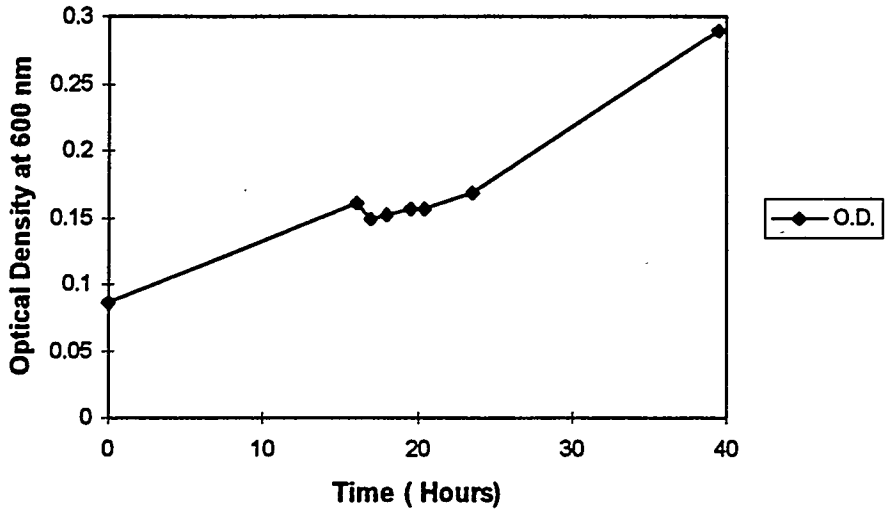
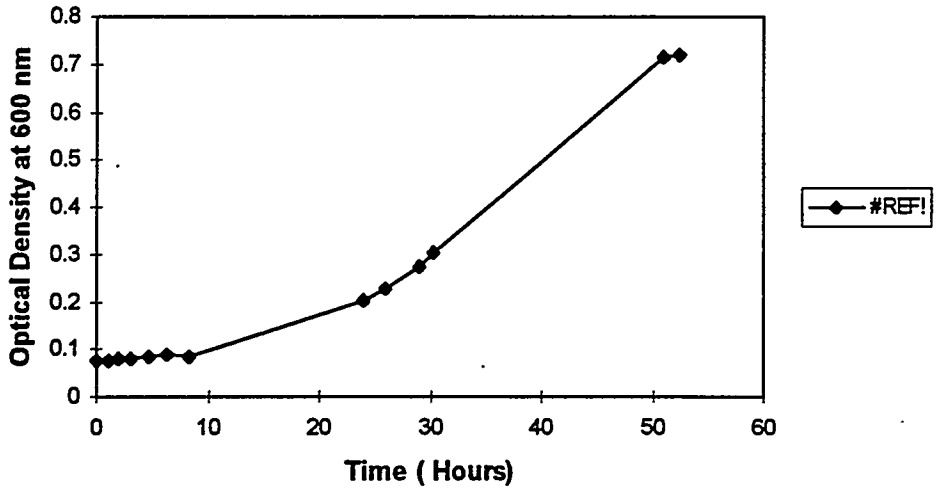


Figure 9 Growth of ESM 0059 on Na Acetate



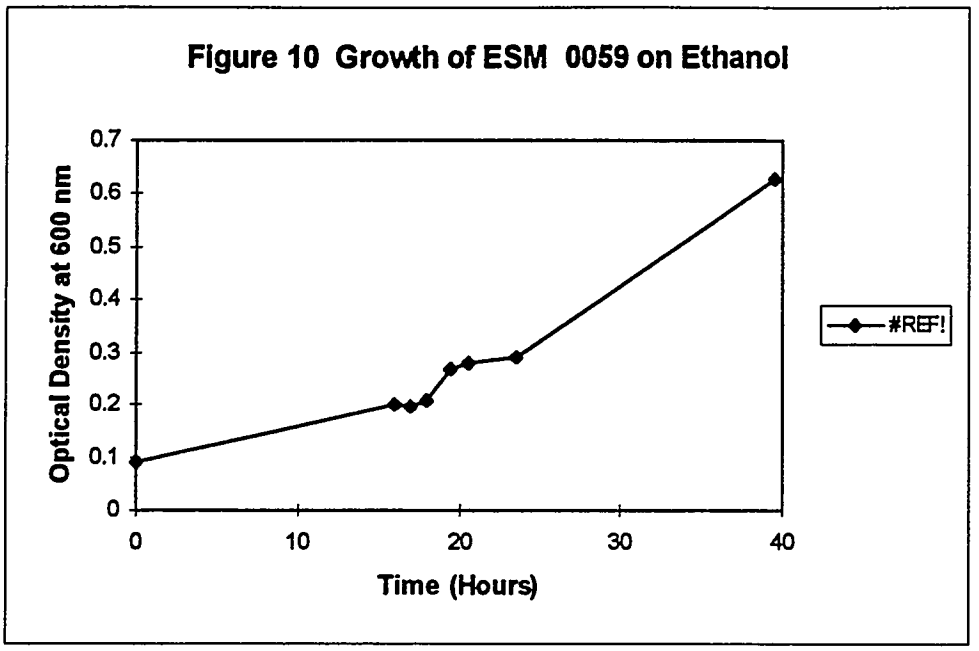


Figure 10 Growth of ESM 0059 on Ethanol
Optical Density at 600 nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
Time (Hours)
#REF!

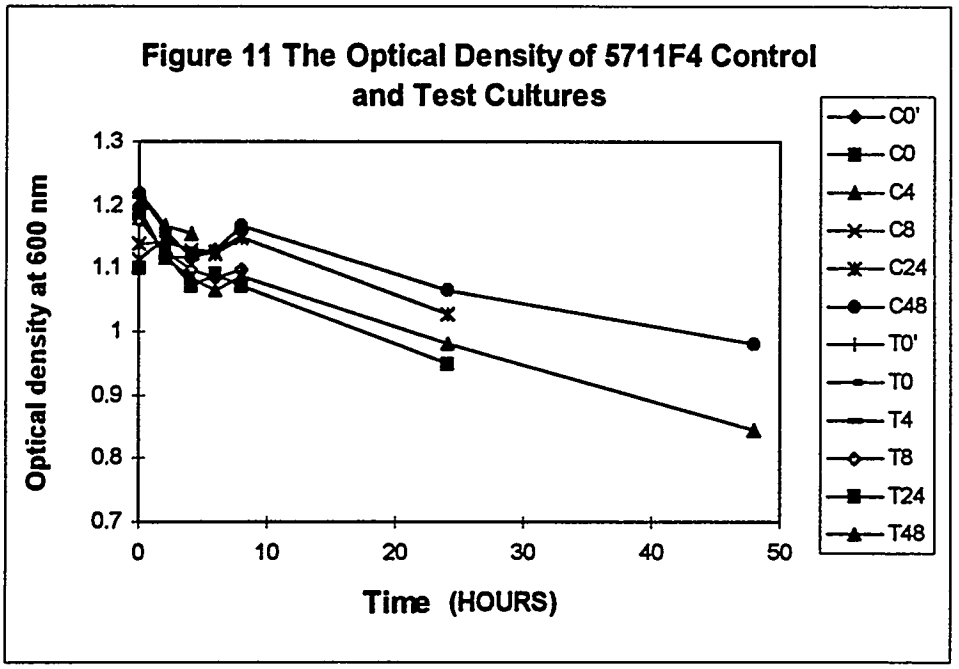


Figure 11 The Optical Density of 5711F4 Control and Test Cultures
Optical density at 600 nm
1.3
1.2
1.1
1
0.9
0.8
0.7
0
10
20
30
40
50
Time (HOURS)
C0'
C0
C4
C8
C24
C48
T0'
T0
T4
T8
T24
T48

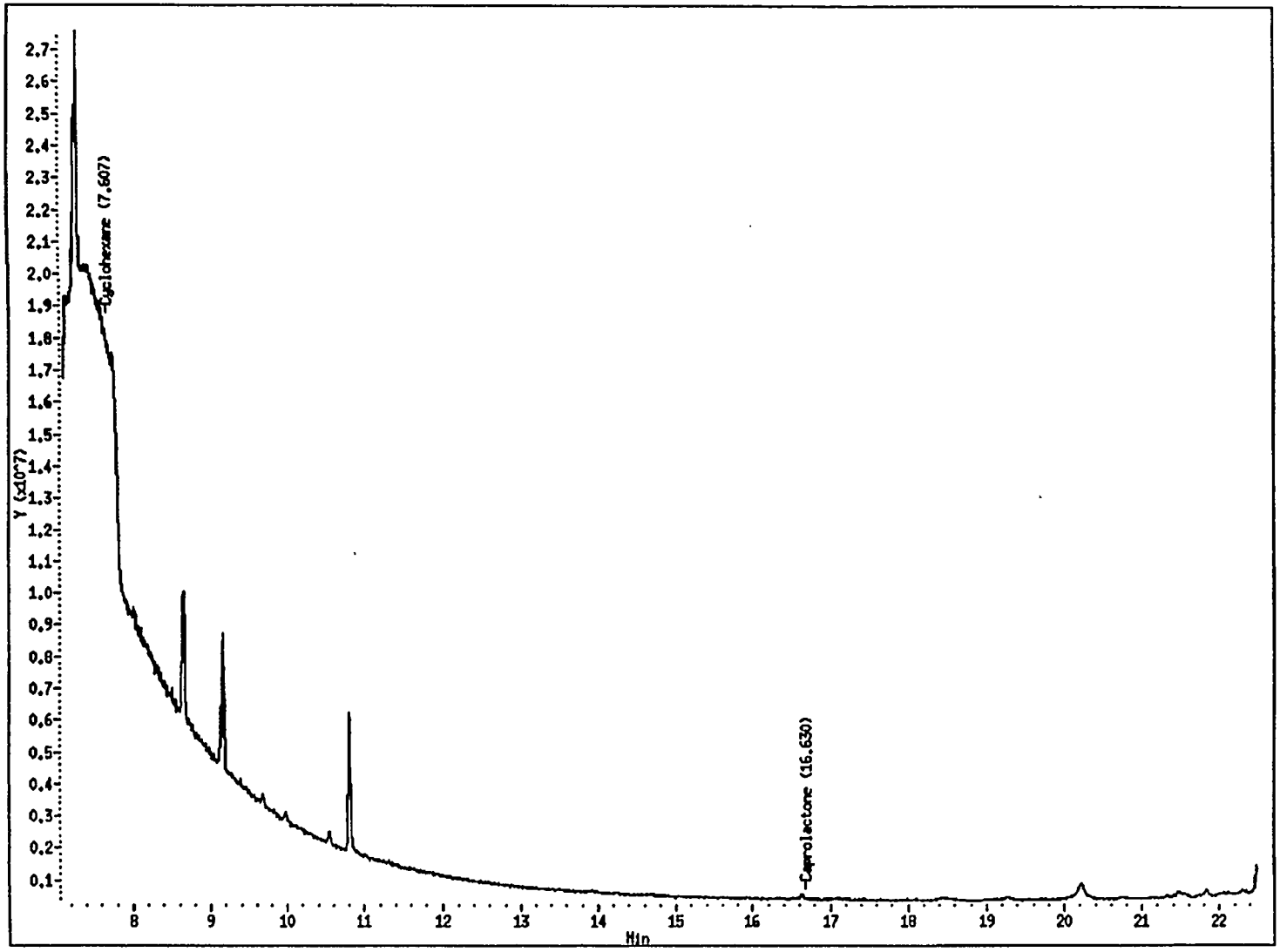


Figure 12 Gas Chromatography Analysis of 5711F4

CONCENTRATIONS

RT	EXP RT	DLT RT	MASS	RESPONSE	ON-COL (ug/L)	FINAL (ug/L)	TARGET RANGE	RATIO
1 Cyclohexane						CAS #: 110-82-7		
7.607	7.534	0.073	840	30587840	2166	2166		100.00(M)
7.544	7.534	0.010	69	13444926			24.20 -64.20	43.96
7.586	7.534	0.052	56	45376573			135.08- 175.08	148.35
5 Caprolactone						CAS #: S02-44-3		
16.630	16.643	-0.013	114	8958	2.47	2.47		100.00
16.630	16.643	-0-013	84	8277			197.43- 237.43	92.40
16.635	16.643	-0.008	55	104466			1122.29-1162.29	1166.08

Figure 13 Mass Spectroscopy Analysis of 5711F4

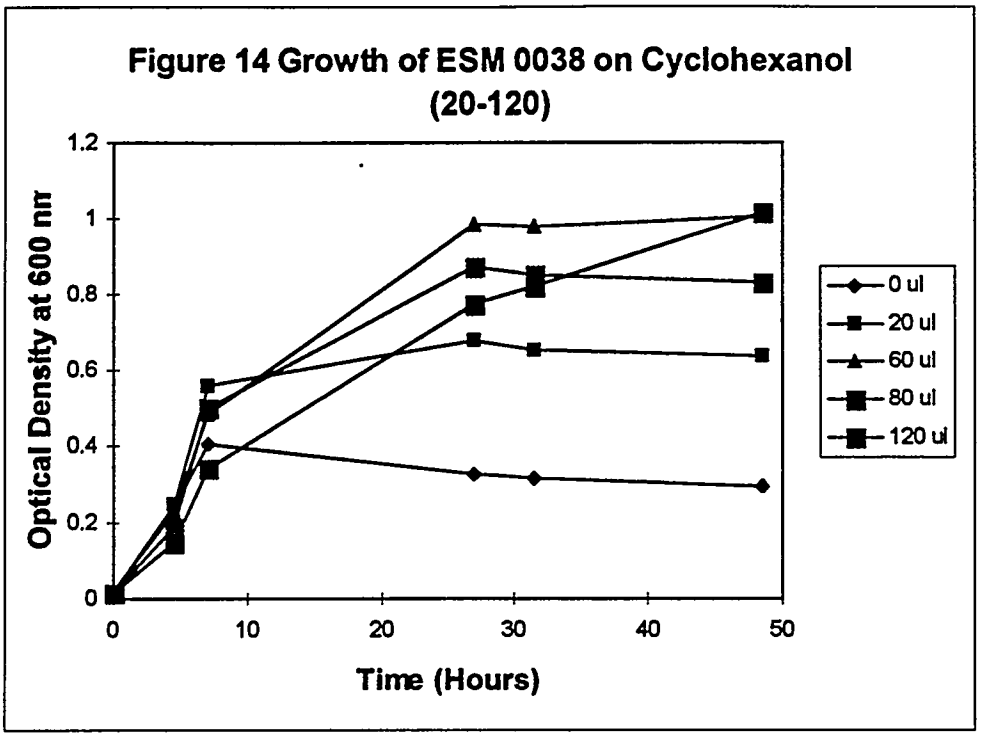


Figure 14 Growth of ESM 0038 on Cyclohexanol (20-120)
Optical Density at 600 nm
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
Time (Hours)
0 ul
20 ul
60 ul
80 ul
120 ul

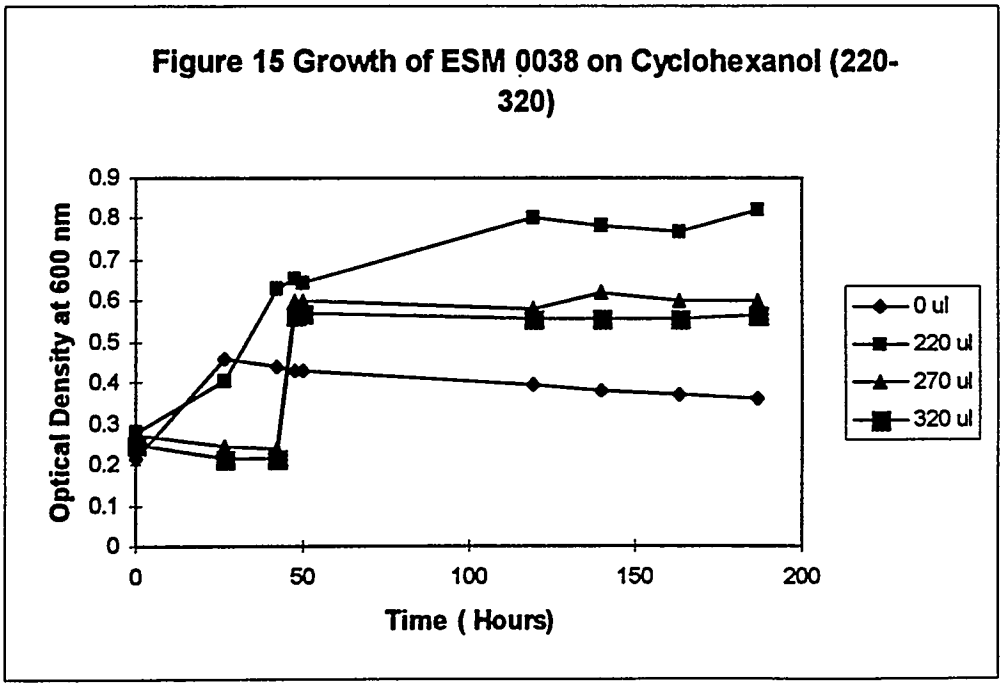


Figure 15 Growth of ESM 0038 on Cyclohexanol (220-320)
Optical Density at 600 nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
50
100
150
200
Time (Hours)
0 ul
220 ul
270 ul
320 ul

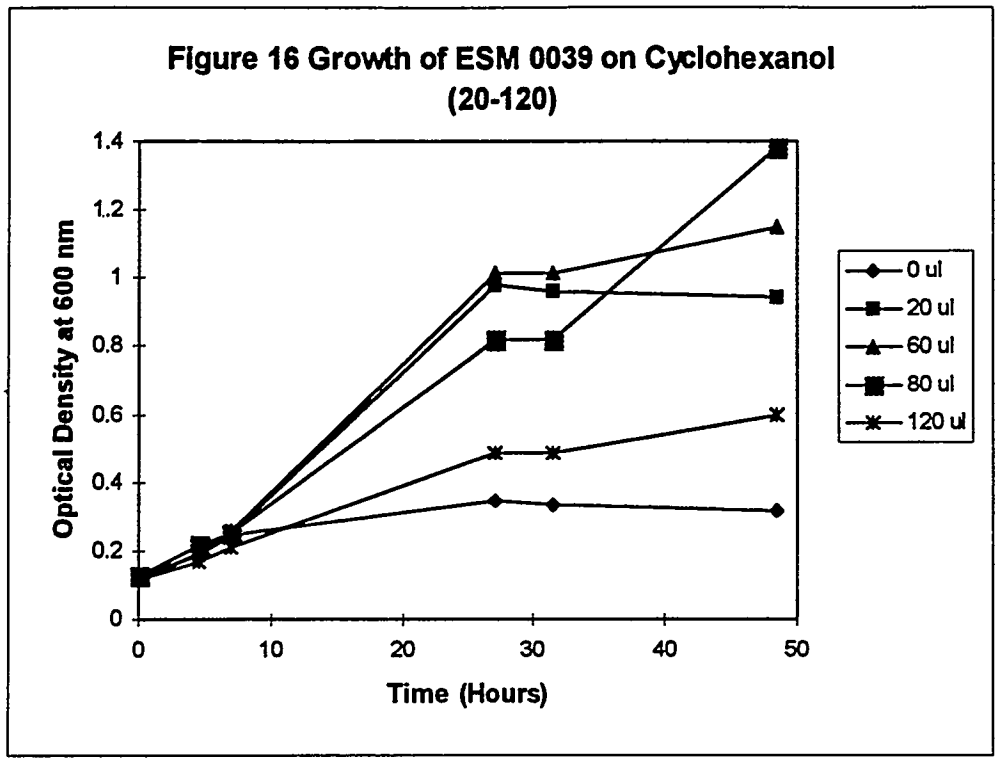


Figure 16 Growth of ESM 0039 on Cyclohexanol (20-120)
Optical Density at 600 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
Time (Hours)
0 ul
20 ul
60 ul
80 ul
120 ul

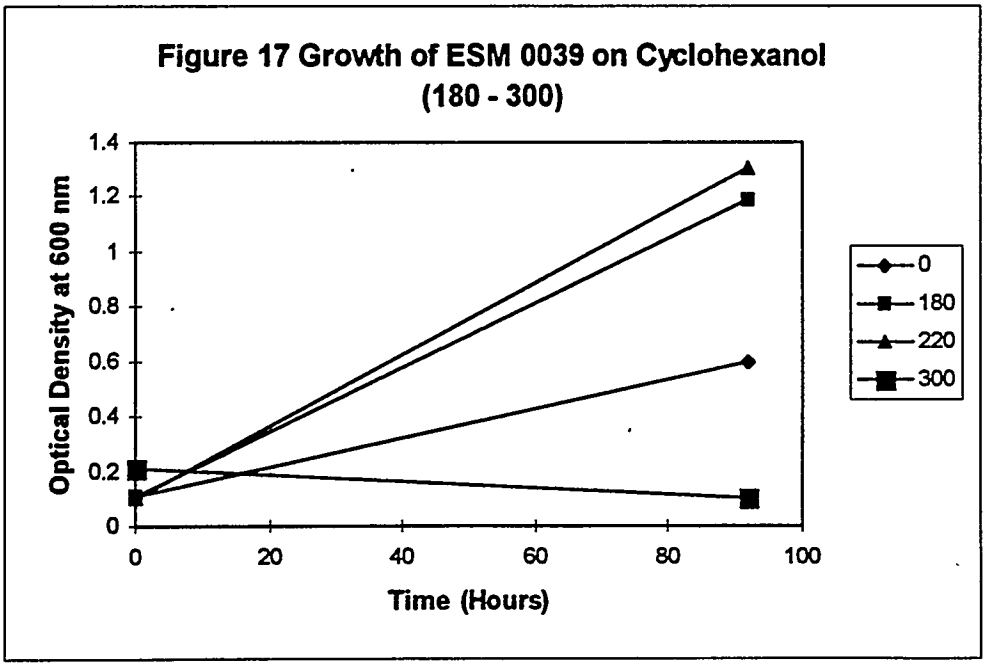


Figure 17 Growth of ESM 0039 on Cyclohexanol (180 - 300)
Optical Density at 600 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Time (Hours)
0
180
220
300

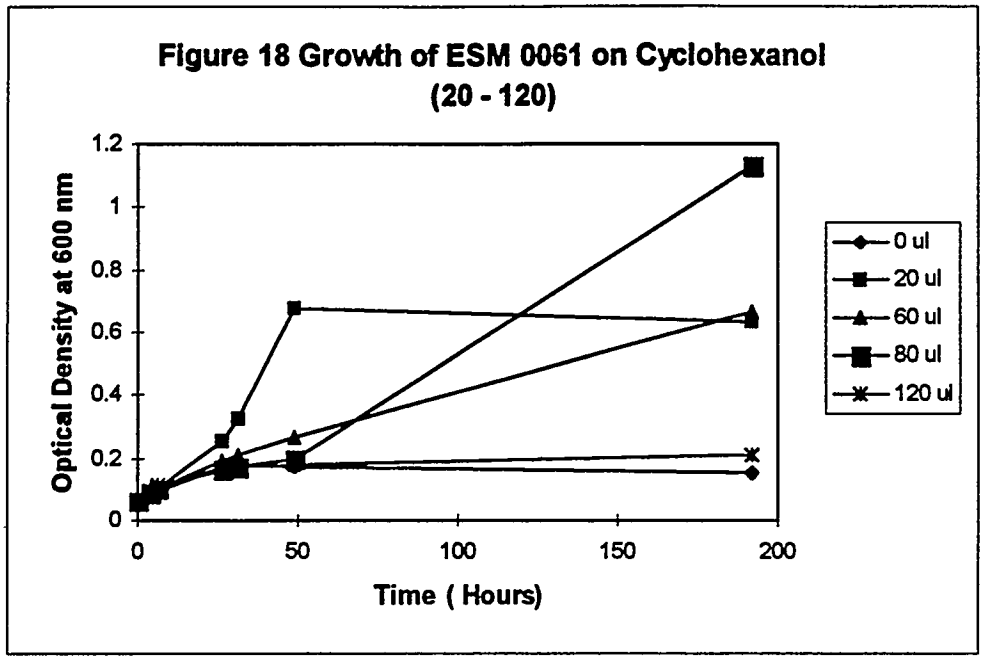


Figure 18 Growth of ESM 0061 on Cyclohexanol
(20 - 120)
Optical Density at 600 nm
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
Time (Hours)
0 ul
20 ul
60 ul
80 ul
120 ul

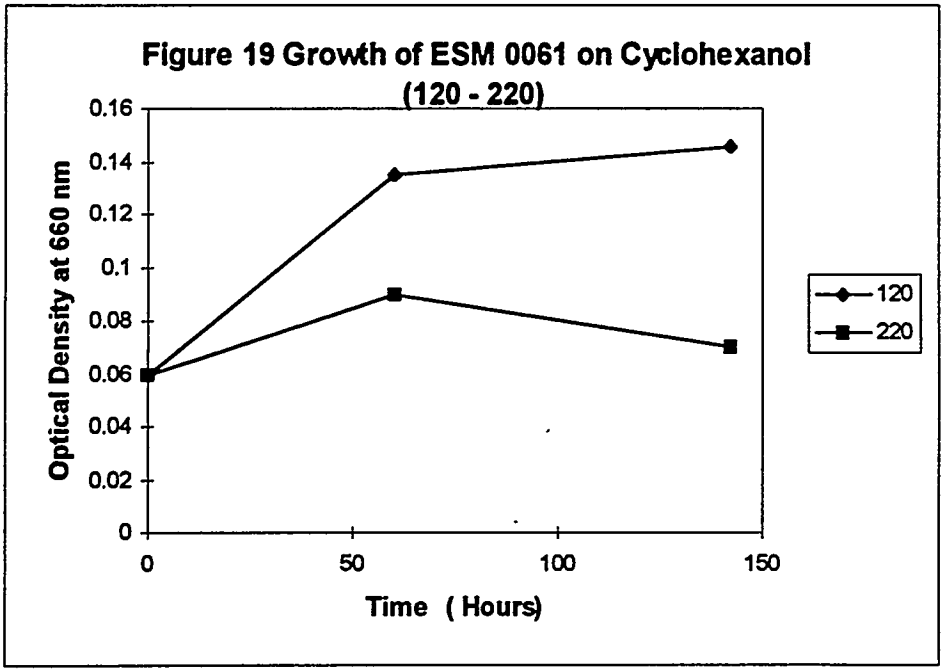


Figure 19 Growth of ESM 0061 on Cyclohexanol
(120 - 220)
Optical Density at 660 nm
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
50
100
150
Time (Hours)
120
220