Biosynthesis of Long-Chain Dicarboxylic Acid Monomers From Renewable Resources

Final Technical Report

David P. Mobley

**GE Corporate Research and Development
One Research Circle
Niskayuna, NY 12309**

Date Published – April 1999

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1. Executive Summary

Long-chain aliphatic α,ω -dicarboxylic acids (diacids) are used in a wide variety of plastics and other chemical applications. Long-chain diacids are almost exclusively produced by chemical conversion processes that suffer a number of disadvantages, including limitations in the range of products, use of multi-step conversion processes, dependence on non-renewable feedstocks, and generation of unwanted and hazardous byproducts. Biotechnology offers an innovative way to overcome the limitations and disadvantages of the chemical processes to make diacids. Yeast biocatalysts are able to convert long-chain fatty acids, which are readily available from renewable agricultural products, directly to long-chain diacids. The biocatalyst can produce a variety of diacid products and produces no hazardous byproducts. However, development efforts are needed to realize the potential of this biosynthetic approach to the production of long-chain diacids.

The objective of this program was to develop and demonstrate key biocatalyst and bioprocess technology to produce cost-competitive long-chain diacids from agricultural products, and to demonstrate the suitability of the diacids as comonomers in a high melt flow polymer application.

Benefits

This DOE-sponsored program leads to the following energy, environmental, and economic benefits:

- Expanded markets for U.S. farm or forestry products to provide plant oils or animal fats and corn syrup as feedstocks.
- Avoidance of increased use of non-renewable petrochemical resources as chemical feedstocks, avoiding demand for petrochemical feedstocks with heating energy content of over 1×10^{12} Btu/yr.
- Avoidance of hazardous wastes and unwanted byproducts associated with the chemical production of diacids.
- Creation of new business for diacid production and the plastics industry, with cost savings to improve the productivity and global competitiveness of the U.S. chemicals and plastics industry.
- Technology for the development of new materials with applications ranging from plastics to fragrances.

Technical Accomplishments

The following summarizes key accomplishments in the three major task areas of the project—Biocatalyst Development, Bioprocess Development, and Application Development.

Biocatalyst Development—The focus of this task was to increase the rate of conversion of fatty acid feedstocks into the corresponding dicarboxylic acids through the ω -oxidation pathway in the yeast biocatalyst (*Candida tropicalis*). The approach was to identify genes in the ω -oxidation

pathway, select the genes that are important to conversion of the fatty acid substrates of choice, and construct an organism with amplified expression of the key genes.

Cytochrome P450 monooxygenase (*CYP*) and Cytochrome P450 NADPH reductase (*CPR*) genes code for the enzymes that constitute the putative rate-limiting step in the ω -oxidation pathway. Eight *CYP* genes and the two alleles of the *CPR* gene were cloned and sequenced.

A reverse transcriptase polymerase chain reaction (RT-PCR) assay was developed and used to measure the expression of selected genes during the course of the fermentation, allowing gene expression to be correlated with the production of diacids. This information was used to select those *CYP* genes whose expression is important for conversion of selected fatty acid feedstocks.

To complement the RT-PCR assay, an assay is needed to measure the amounts of CYP and CPR proteins in the cells. To meet this need we used a synthetic peptide approach to develop a series of antibodies designed to bind selectively to unique CYP proteins.

Two selected *CYP* genes were integrated and amplified into *C. tropicalis* ATCC 20962. Initial fermentations with the *CYP* amplified strains produced promising diacid productivities. More work is needed to determine whether amplification has affected *CYP* mRNA levels or CYP protein levels.

Bioprocess Development—The bioprocess development focused on reducing the cost of manufacture of diacids through improving the bioreactor productivity and through identification of suitable low-cost fatty acid feedstocks (substrates) for the bioreaction. In addition, we have carried out initial screening of process options for recovery and purification of the diacid product from the bioreactor broth, and conducted engineering economic analyses of the process and process improvements.

The bioreactor productivity was improved by optimizing the conditions for growth of the yeast biocatalyst, using statistical experimental design methods. Use of the optimized conditions increased the peak bioreactor productivity by 50% with respect to the productivity at the outset of the project. The bioreactor productivity was further improved by optimizing the operating conditions for the bioconversion phase of the batch process. Together with the optimized growth conditions, the peak productivity was increased by 80% and the overall bioreactor productivity was improved by 65% with respect to the values at the outset of the project.

A number of fatty acid materials were tested as substrates for the bioconversion for two reasons: (1) to identify low-cost starting material compatible with the bioprocess, and (2) to produce a variety of diacid materials for testing in the Application Development task. We tested fatty acid methyl esters fatty acid mixtures of different chain lengths and degrees of saturation, comparing relative conversion rates and operability in the bioconversion. Single and mixed diacids were isolated from the laboratory bioreactor broths and purified for the Application Development studies.

Purdue University conducted studies on separation processes for the recovery of diacids from bioreactor broth, with particular attention to screening of adsorption processes. The Purdue group developed high throughput screens and used them to identify suitable solvents and adsorbents from over 70 different adsorption mobile phase (solvent) candidate combinations and 30 stationary phase (adsorbent) candidates.

MBI International constructed an engineering economic model for the bioconversion. The model was used to estimate the economic effect of advances made during the project. The combined effect of these advances (increased bioreactor productivity, use of a lower cost substrate, and reductions in raw materials usage) was an estimated 40% reduction in the cost of manufacture.

Application Development—The test application of the diacid monomers was a copolymer in which the diacid improves the melt flow of the copolymer relative to the homopolymer while retaining the useful mechanical properties of the homopolymer. Increased melt flow yields a polymer that is more easily processed and can be used for thin-walled parts.

The effects of carbon chain length, degree of unsaturation, and source of the diacid on copolymer properties were determined by preparing copolymers from chemically synthesized diacids of different chain lengths (in both diacid and diacid chloride forms), individual biosynthetic diacids, and mixed biosynthetic diacids over a range of diacid monomer concentrations. All polymers from biosynthetic diacids showed acceptable properties (comparable to a chemically synthesized diacid controls) as judged by a high degree of incorporation, low byproduct linkage levels, appropriate glass transition temperature, molecular weight, and melt viscosity, and good melt stability. A variety of polymerization conditions were found to work well with biosynthetic diacids. Results obtained on a small laboratory scale were confirmed by scaling the polymerizations to 2000 g per batch.

Outlook

Sponsorship of this cost-shared project by the DOE Office of Industrial Technology brought together GE and Henkel Corporation as industrial partners to pursue the objectives and benefits outlined above. Other participants included Purdue University and MBI International. The technical successes of this project have led GE and Henkel to pursue a continued partnership for further development of bioprocesses for low-cost production of chemical intermediates.

2. Introduction

(David P. Mobley, GE CRD)

2.1 Background

Diacid industrial uses and production

Aliphatic α,ω -dicarboxylic acids (diacids) of the type addressed by this program (Figure 2-1) are used in a wide variety of plastics and other chemical applications. For example, azelaic (C9) and sebacic (C10) acids are used to make aliphatic polyesters which, in turn, are used in fibers, films, casting resins, plasticizers, synthetic lubricants, and adhesives [1,2]. Brassylic acid (C13) is used in the manufacture of synthetic musk as well as nylon 13,13. The diacid produced in the largest quantity (>40 MM lb/yr) as a pure chemical intermediate is dodecanedioic acid (C12); it is used in polyamides such as nylon 6,12, which is noted for high moisture resistance.

Diacids are almost exclusively produced by chemical conversion processes. Dodecanedioic acid is manufactured through the nickel-catalyzed cyclic trimerization of butadiene, followed by hydrogenation to cyclododecane, air oxidation to a mixture of cyclododecanone and cyclododecanol, and, finally, nitric acid oxidation to dodecanedioic acid (Figure 2-2) [3].

Chemical processes for the production of diacids have a number of limitations and disadvantages. All the chemical processes are restricted to the production of diacids of specific carbon chain lengths, limiting the range of available materials. The dodecanedioic acid process is a good example. Since the basic starting material is butadiene, the resulting product diacids are limited to multiples of four-carbon lengths. In practice, only dodecanedioic acid is made by this process. Dodecanedioic acid is the longest straight-chain diacid currently available from an industrial chemical process. Diacids with carbon numbers greater than 13 are difficult to chemically synthesize and are not available on a large scale, so the market for these materials is not established.

The dodecanedioic process is based on non-renewable petrochemical feedstocks. The multi-step conversion process produces unwanted byproducts such as cyclooctadiene and vinyl cyclohexene, which result in yield losses. The nitric acid oxidation step yields NO_x , which is either released to the atmosphere or must be destroyed in a reduction furnace. The manufacture of dodecanedioic acid produces 0.2 lb N_2O /lb diacid, as estimated from the analogous nitric acid oxidation step in the manufacture of adipic acid [4]. Finally, at approximately \$2/lb [1], the market price of dodecanedioic acid is relatively high for a bulk monomer.

The biotechnology alternative

Biotechnology offers an innovative way to overcome the limitations and disadvantages of the chemical processes to make diacids. Certain yeasts, including *Candida tropicalis*, are able to oxidize terminal aliphatic carbons to carboxylic acids. This makes it possible to convert long-chain fatty acids directly to long-chain dicarboxylic acids (Figure 2-3). Long-chain fatty acids are readily available from renewable agricultural and forest products such as soybean oil, tallow, corn oil, or tall oil.

The biological conversion accepts a wide variety of starting materials (i.e., fatty acid substrates), providing a range of diacid products that are otherwise unavailable. The biological conversion process doesn't yield polluting byproducts such as NO_x .

A biological conversion process for diacids is already used commercially in Japan [1]. However, due to limitations with current biocatalysts, this bioprocess is used only to make high-priced specialized diacid products, such as brassylic acid for fragrance applications. The biochemical basis of this process reveals the reasons that it is limited to high-priced products. The current process does not lend itself to further cost reduction, as explained below.

C. tropicalis and similar yeasts produce diacids from alkanes or fatty acids through the ω -oxidation pathway. The first step in this pathway is generally accepted to be rate-limiting [5]. This step is mediated by the ω -hydroxylase complex consisting of a cytochrome P450 monooxygenase and an associated NADPH cytochrome reductase.

Wild type *C. tropicalis* also efficiently metabolizes fatty acids and the dicarboxylic acid intermediates through a second pathway—the β -oxidation pathway—enabling the organism to grow on fatty acids or alkanes as the sole carbon source. β -oxidation proceeds through the successive chain-shortening of the diacid by two-carbon units at a time [6,7].

Classical strain improvement techniques have been used to develop strains that are partially deficient in their ability to grow on diacids, fatty acids, or alkanes. While these strains show enhanced production of diacids, they also produce diacids that are shorter by one or more pairs of carbon atoms than the alkane or fatty acid substrate and form unsaturated or 3-hydroxy-dicarboxylic acid byproducts due to residual activity of the β -oxidation pathway [8–10]. Partial metabolism of the substrate also results in costly yield losses.

Improved biocatalyst

Through genetic modification, Henkel Corporation developed a yeast biocatalyst that overcomes a number of deficiencies in the biocatalysts currently used for diacid production [11,12]. A unique approach was used to inactivate the genes in *C. tropicalis* which code for acyl-CoA oxidase, the catalyst for the first reaction in the β -oxidation pathway (Figure 2-4). Because of the diploid nature of the organism and the existence of two isozymes, four acyl-CoA oxidase genes were sequentially disrupted. Because acyl-CoA oxidase activity was completely eliminated, the transformed strain showed 100% efficiency of conversion of substrate to products and 100% retention of chain length [12]. This strain was also shown to accept a variety of fatty acid substrates, both saturated and unsaturated. While the β -oxidation blocked strain showed good productivity, the productivity was further enhanced by amplifying the P450 reductase gene [12].

In batch fermentations, the genetically modified *C. tropicalis* yielded high concentrations of diacid (80–100 g/L in the final broth) and peak bioreactor productivities of over 1 g/L/hr with some substrates for part of the conversion cycle. In spite of these technical breakthroughs, this biocatalyst was not developed to commercialization because the bioprocess was still not able to compete directly with existing sources of diacids and because of the lack of market demand for the new diacid materials it made available.

New application opportunity for diacids

Recently, it has been found that incorporation of a diacid as a comonomer into certain engineering thermoplastic resins produces copolymer products with desirable properties. The copolymer retains the useful mechanical properties of the homopolymer resins, such as high impact strength, while offering a lower melt viscosity than the conventional resin. Lower melt viscosity is very important to the plastics processor because it means that the molten plastic will flow into existing molds more quickly, reducing the processor's cycle time and increasing his productivity. It is also important to designers who use plastics, because it means that thinner-walled and lighter weight parts can be made for applications such as laptop computers. The diacid-containing copolymer resin will be referred to in this discussion as high-flow resin.

Because of its special combination of properties, the high-flow resin has the potential to command a significant share of the large global market for the resin. The rate of growth of the high-flow resin market is, however, limited by the availability and cost of suitable diacid comonomers. The cost of the cheapest suitable diacids available in bulk quantities is above the average selling price of the conventional resins. This means that the high-flow resin must be sold at a premium, which limits its market penetration. Long chain diacids produced biosynthetically, either as individual chain lengths or as mixtures of diacids, could potentially yield the same properties as chemically synthesized diacids. It has been estimated that, if biotechnology could provide a cheaper source of diacid monomer for the high-flow resin, sales of the high-flow resin could rise dramatically.

Bioprocess economics

The potential of biotechnology to provide diacids for applications such as the high-flow resin was sufficiently interesting to warrant a closer examination of the demonstrated and potential bioprocess economics. A preliminary economic analysis of the biological production of diacid was carried out prior to this project, assuming use of the genetically modified *C. tropicalis* and batch bioprocess technology demonstrated at that time. The total conversion cost was broken down into cost components.

Work undertaken prior to this project had demonstrated that raw materials costs, a large contributor to the overall cost, could be minimized by use of low-cost fermentation medium components for growth and maintenance of the yeast biocatalyst. Further opportunities for reduction of raw materials costs were identified in the use of low-cost fatty acid substrates and in the reduction of the rates of use of antifoam and of the base used for pH control.

The cost of fatty acid substrates is dependent on their source and composition. Pure fatty acid substrates are generally priced higher than \$0.70/lb. However, mixed fatty acids as derived directly from plant or animal sources are significantly cheaper. For example, tallow fatty acid sells for less than \$0.30/lb. Based on the results of prior studies, it appeared it may be possible to use the mixed diacid product that would result from a cheaper mixed fatty acid substrate.

Assuming that raw materials costs could be minimized, the preliminary economic analysis indicated that capital-related costs and utilities costs were the next most significant cost components. This analysis also suggested that the most effective way to reduce conversion costs was to increase the overall bioreactor productivity (amount of product formed per time per

bioreactor volume, also called diacid productivity). Increases in bioreactor productivity reduce capital costs (since smaller bioreactors will do the same job) and utility costs (since less bioreactor volume needs to be stirred and aerated for less time). As Figure 2-5 shows, the conversion cost was predicted to be strongly dependent on bioreactor productivity. Bioreactor productivity can be increased by improving the specific productivity of the biocatalyst and by optimizing the fermentation process conditions.

The importance of bioreactor productivity is reinforced by an examination of existing bioprocesses. Biological conversion is a proven cost-competitive method for producing a number of bulk chemicals that sell for less than \$1/lb (Table 2-1). As this table illustrates, economically successful biological processes for bulk chemicals share two characteristics: high product concentrations and high bioreactor productivities. The diacid bioprocess already demonstrates high product concentrations; further development must focus on increasing productivity.

2.2 Development Needs

The preliminary economic analysis and comparison with bioprocesses that already provide low-cost bulk chemicals indicated that, with further technical development, the bioprocess to produce diacids could be cost-competitive. In order to reach this objective, the technical development needs fall into three areas: 1) **biocatalyst development**, in order to increase the diacid productivity of the organism, and thus the bioreactor productivity, 2) **bioprocess development**, in order to make efficient use of the biocatalyst and reduce the fatty acid substrate and processing costs, and 3) **application development**, to define the diacid product requirements for high-flow resin and ensure that the biocatalyst and bioprocess development meet these requirements. The specific tasks undertaken in each of these development areas and the results of the investigations are detailed in the chapters below.

2.3 Benefits

This program directly addresses the goal of the DOE Alternative Feedstocks Program by demonstrating a new pathway to chemicals production using resources from agricultural processes as feedstocks. This program leads to the following energy, environmental, and economic benefits.

The successful commercialization of the technology developed in this program will aid the U.S. agricultural industry by expanding market demand for farm and forestry products. The projected demand for diacid monomers for the high-flow resin and other applications translates to over 50 million lb/yr of natural plant oils or animal fats (e.g., tallow, or seed oil), to be converted to fatty acids, and over 1 million bushels/yr of corn to provide corn syrup as a nutrient for the biocatalyst.

The use of agricultural products as feedstocks will avoid increased demand for non-renewable petrochemical resources as chemical feedstocks. Using renewable resources to meet projected demand for diacids for high-flow resin and other applications will avoid demand for over 60 million lb/yr of butadiene (derived from petroleum), which, with a heat of combustion of 19,200 Btu/lb, has a heating energy content of over 1×10^{12} Btu/yr.

The diacids bioprocess is an example of a clean pathway to fulfill America's chemical needs. Meeting the demand for diacids for high-flow resin with the bioprocess technology will avoid

production of over 10 million lb/yr NO_x associated with chemical production of dodecanedioic acid.

The commercialization of this technology will benefit U.S. industry. It will create new business in the oleochemicals industry in the form of diacid monomer production. The supply of diacids for high-flow resin will leverage sales in the plastics industry that are several times the value of the diacids alone. Diacids for this and other plastics applications will be delivered at a lower cost than current competitive materials. These cost savings will improve the productivity and global competitiveness of the U.S. plastics industry.

This technology provides increased productivity in the plastics processing industry. High-flow resins incorporating diacids from renewable resources offer lower melt viscosities than conventional resins, resulting in faster cycle times for the plastics molding industry.

The success of this program will also provide the technology for the follow-on development of a range of long-chain diacid and related materials not available from current process technologies. These new materials will be suitable for a wide variety of applications in a number of industries, ranging from plastics to fragrances.

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Figure 2-1. General formula for saturated aliphatic α,ω -dicarboxylic acids (diacids) – building blocks of the thermoplastics addressed in this effort and a wide range of other commercial products. Long-chain diacids have a total carbon number of 12 or greater.

Figure 2-2. The chemical route for manufacture of dodecanedioic acid suffers a number of limitations:

- No flexibility in diacid length
- Multi-step process
- Petrochemical feedstock
- Harmful byproduct (NO_x)

Figure 2-3. The route to diacids developed in this effort – direct conversion of a fatty acid catalyzed by a microorganism – has several advantages:

- Wide range of diacid products
- Single-step process
- Renewable feedstock
- No NO_x byproduct

Figure 2-4. Prior to this program, a novel approach was used to disrupt the POX genes in *C. tropicalis* that code for the first step in β -oxidation, resulting in a strain that yields high levels of diacids.

Figure 2-5. A preliminary economic analysis predicted that, after reducing raw materials costs, additional cost reductions could be realized by increasing bioreactor productivity.

Table 2-1. Bioprocesses for Chemicals. A number of low-cost chemicals are already produced at large scale by biological processes. Successfully bioprocesses have

- High product concentrations
- High bioreactor productivities

By comparison, the biological route to diacids has already yielded product concentrations equivalent to commercial processes. This program emphasizes bringing productivity up to the commercially successful range.

<i>Chemical</i>	<i>Price (\$/lb)</i>	<i>U.S. Consumption (MM lb/yr)</i>	<i>Biological Production^a (MM lb/yr)</i>	<i>Concentration from Bioreactor (g/L)</i>	<i>Bioreactor Productivity (g/L/hr)</i>
Acrylamide	0.76	120	12 ^b	120	
Citric acid	0.82	330	330	120–150	1.3–2
Ethanol	0.18	6900	6500	80	3.3
Lactic acid	0.80	40	40 (>250) ^c	90	2–3
Monosodium glutamate	0.87	90	90 ^d (>300) ^e	100	1.4–2.8
Long-chain diacids	–	–	–	80–100	0.5–0.7

^a U.S. production unless otherwise noted.

^b Production by bioprocess in Japan, competes successfully with chemical process.

^c U.S. fermentation capacity under construction.

^d U.S. consumption entirely supplied by imports, produced by fermentation.

^e Worldwide biological production.

3. Biocatalyst Development (Task 1)

(C. Ron Wilson, Henkel Corporation, Chemicals Group)

3.1 Introduction

In 1987 scientists at Henkel Research Corporation undertook a three year-project to develop alternative sources of long chain dicarboxylic acids (diacids) to be derived from non-petroleum based feedstocks. Certain yeasts, including *Candida tropicalis*, are known to excrete dicarboxylic acids (diacids) when cultured on alkanes or fatty acids. However, the productivity and selectivity of these yeasts are too low for development of a commercial process. Genetic engineering was employed to redirect the normal carbon flow within this organism from substrate degradation to substrate conversion. Yeasts of the *Candida* family metabolize alkanes via an omega (ω)-oxidation pathway in which the first step is catalyzed by a membrane bound ω -hydroxylase complex composed of both a cytochrome P450 monooxygenase (CYP) and a NADPH cytochrome reductase (CPR). The hydroxylase complex carries out the oxidation of the terminal methyl group present in alkanes and fatty acids and is thought to be the rate-limiting step in diacid formation. Two additional enzymes, an alcohol oxidase and an aldehyde dehydrogenase, complete the oxidation of the alkane or fatty acid to form the corresponding diacid.

The formation of diacids from n-alkanes or from fatty acids is not the pathway of choice for yeasts, which prefer to use these substances as energy sources for growth. In wild type strains of *Candida tropicalis* almost all of the alkane is degraded via the β -oxidation pathway. This problem was eliminated by Henkel scientists by blocking the first step in the β -oxidation pathway. Using a proprietary transformation system, the four acyl CoA oxidase genes in *C. tropicalis* ATCC 20336 were inactivated, successfully diverting the alkane substrate toward the formation of diacids. The resulting *C. tropicalis* strain, designated (ATCC 20962), would not grow on alkanes or fatty acids, but did convert these substrates into diacids at 100% efficiency. However, the productivity of this strain was still not at a high enough level to satisfy commercialization needs.

The biocatalyst portion of the DOE program has been directed toward improving diacid productivity of *C. tropicalis* ATCC 20962 by the coordinate amplification of native CYP and CPR genes which are induced by the fatty acid feedstream of choice. Achieving this goal requires that multiple research tasks be successfully completed. These include:

- Cloning and characterization of a family of cytochrome P450's present in *C. tropicalis* ATCC 20336.
- Developing a method to identify which P450's are important for diacid formation.
- Integration of selected CYP and CPR genes into the genome of *C. tropicalis*.

3.2 Materials and Methods

1. Strains and plasmids

All relevant microbial strains and plasmids are described in Table 3-1.

2. Purification of genomic DNA from *C. tropicalis* ATCC 20336

a. For the construction of genomic libraries. 50 ml of YEPD broth (see Table 3-2) was inoculated with a single colony of *C. tropicalis* 20336 from YEPD agar plate and grown overnight at 30°C. 5 ml of the overnight culture was inoculated into 100 ml of fresh YEPD broth and incubated at 30°C for 4 to 5 hr with shaking. Cells were harvested by centrifugation, washed twice with sterile distilled water, and resuspended in 4 ml of spheroplasting buffer (1 M Sorbitol, 50 mM EDTA, 14 mM mercaptoethanol) and incubated for 30 min at 37°C with gentle shaking. 0.5 ml of 2 mg/ml zymolyase (ICN Pharmaceuticals, Inc., Irvine, CA) was added and incubated at 37°C with gentle shaking for 30 to 60 min. Spheroplast formation was monitored by SDS lysis. Spheroplasts were harvested by brief centrifugation (4,000 rpm, 3 min) and were washed once with the spheroplast buffer without mercaptoethanol. Harvested spheroplasts were then suspended in 4 ml of lysis buffer (0.2 M Tris/pH 8.0, 50 mM EDTA, 1% SDS) containing 100 µg/ml RNase (Qiagen Inc., Chatsworth, CA) and incubated at 37°C for 30 to 60 min.

Proteins were denatured and extracted twice with an equal volume of chloroform/isoamyl alcohol (24:1) by gently mixing the two phases by hand inversions. The two phases were separated by centrifugation at 10,000 rpm for 10 min, and the aqueous phase containing the high-molecular weight DNA was recovered. To the aqueous layer, NaCl was added to a final concentration of 0.2 M and the DNA was precipitated by adding 2 vol of ethanol. Precipitated DNA was spooled with a clean glass rod and resuspended in TE buffer (10 mM Tris/pH 8.0, 1 mM EDTA) and allowed to dissolve overnight at 4°C. To the dissolved DNA, RNase free of any DNase activity (Qiagen Inc., Chatsworth, CA) was added to a final concentration of 50 µg/ml and incubated at 37°C for 30 min. Then protease (Qiagen Inc., Chatsworth, CA) was added to a final concentration of 100 µg/ml and incubated at 55 to 60°C for 30 min. The solution was extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once with equal volume of chloroform/isoamyl alcohol (24:1). To the aqueous phase 0.1 vol of 3 M sodium acetate and 2 volumes of ice cold ethanol (200 proof) were added and the high molecular weight DNA was spooled with a glass rod and dissolved in 1 to 2 ml of TE buffer.

b. To be used for PCR amplification of CYP and CPR genes. Five 5 ml of YPD medium (Ditco Laboratories, Detroit, MI) was inoculated with a single colony and grown at 30°C overnight. The culture was centrifuged for 5 min at 1200 x g. The supernatant was removed by aspiration, and 0.5 ml of a sorbitol solution (0.9 M sorbitol, 0.1 M Tris-Cl pH 8.0, 0.1 M EDTA) was added to the pellet. The pellet was resuspended by vortexing, and 1 µl of 2-mercaptoethanol and 50 µl of a 10 µg/ml zymolyase solution were added to the mixture. The tube was incubated at 37°C for 1 hr on a rotary shaker (200 rpm). The tube was then centrifuged for 5 min at 1200 x g and the supernatant was removed by aspiration. The protoplast pellet was resuspended in 0.5 ml 1x TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA) and

transferred to a 1.5 ml microcentrifuge tube. The protoplasts were lysed by the addition of 50 μ l 10% SDS followed by incubation at 65°C for 20 min. Next, 200 μ l of 5M potassium acetate was added, and, after mixing, the tube was incubated on ice for at least 30 min. Cellular debris was removed by centrifugation at 13,000 x g for 5 min. The supernatant was carefully removed and transferred to a new microfuge tube. The DNA was precipitated by the addition of 1 ml 100% (200 proof) ethanol followed by centrifugation for 5 min at 13,000 x g. The DNA pellet was washed with 1 ml 70 % ethanol followed by centrifugation for 5 min at 13,000 x g. After partially drying the DNA under a vacuum, it was resuspended in 200 μ l of 1x TE. The DNA concentration was determined by ratio of the absorbance at 260 nm / 280 nm ($A_{260/280}$).

3. Construction of *C. tropicalis* 20336 genomic libraries.

Three genomic libraries of *C. tropicalis* were constructed, two by contract at Clontech Laboratories, Inc., (Palo Alto, CA) and one at Henkel Corporation (Cincinnati, OH).

a. Clontech Libraries. The first Clontech library was made as follows: Genomic DNA was prepared from *C. tropicalis* 20336 as described above, partially digested with *Eco*RI and size fractionated by gel electrophoresis to eliminate fragments smaller than 0.6 kb. Following size fractionation, several ligations of the *Eco*RI genomic DNA fragments with the lambda (λ) TriplExTM Clontech vector (Figure 3-1) arms with *Eco*RI sticky ends were packaged into λ phage heads under conditions designed to obtain one million independent clones. The second genomic library was constructed as follows: Genomic DNA was digested partially with *Sau*3A1 and size fractionated by gel electrophoresis. The DNA fragments were blunt ended using standard protocols¹: The strategy was to fill in the *Sau*3A1 overhangs with Klenow polymerase (Life Technologies, Grand Island, NY) followed by digestion with S1 nuclease (Life Technologies, Grand Island, NY). After S1 nuclease digestion, the fragments were end filled one more time with Klenow polymerase to obtain the final blunt-ended DNA fragments. *Eco*RI linkers were ligated to these blunt-ended DNA fragments followed by ligation into the λ TriplEx vector. The resultant library contained approximately 2×10^6 independent clones with an average insert size of 4.5 kb.

b. Henkel Library. The third genomic library was constructed at Henkel Corporation using λ ZAP ExpressTM vector (Stratagene, La Jolla, CA) (Figure 3-2). Genomic DNA was partially digested with *Sau*3A1, and fragments in the range of 6 to 12 kb were purified from an agarose gel after electrophoresis of the digested DNA. These DNA fragments were then ligated to *Bam*HI digested λ ZAP ExpressTM vector arms according to manufacturers protocols. Three ligations were set up to obtain approximately 9.8×10^5 independent clones. All three libraries were pooled and amplified according to manufacturer's instructions to obtain high-titre ($>10^9$ plaque forming units/ml) stock for long-term storage. The titre of packaged phage library was ascertained after infection of *E. coli* XL1Blue-MRF' cells (Stratagene). *E. coli* XL1Blue-MRF' were grown overnight in either LB medium or NZCYM (Table 3-2) containing 10 mM MgSO₄ and 0.2% maltose at 37°C or 30°C, respectively, with shaking. Cells were then centrifuged and resuspended in 0.5 to 1 volume of 10 mM MgSO₄. 200 μ l of this *E. coli* culture was mixed with several dilutions of

packaged phage library and incubated at 37°C for 15 min. To this mixture 2.5 ml of LB top agarose or NZCYM top agarose (maintained at 60°C) (see Table 3-2) was added and plated on LB agar or NCZYM agar (see Table 3-2) present in 82 mm petri dishes. Phages were allowed to propagate overnight at 37°C to obtain discrete plaques, and the phage titre was determined.

4. Screening of genomic libraries.

Both λ TriplExTM and λ ZAP ExpressTM vectors are phagemid vectors that can be propagated either as phage or plasmid DNA (after conversion of phage to plasmid). Therefore, the genomic libraries constructed in these vectors can be screened either by plaque hybridization (screening of lambda form of library) or by colony hybridization (screening plasmid form of library after phage to plasmid conversion). Both vectors are capable of expressing the cloned genes, and the main difference is the mechanism of excision of plasmid from the phage DNA. The cloning site in λ TriplExTM is located within a plasmid which is present in the phage and is flanked by *loxP* sites (Figure 3-1). When λ TriplExTM is introduced into *E. coli* strain BM25.8 (supplied by Clontech), the *Cre* recombinase present in BM25.8 promotes the excision and circularization of plasmid pTriplEx from the phage λ TriplExTM at the *loxP* sites. The mechanism of excision of plasmid pBK-CMV from phage λ ZAP ExpressTM is different. It requires the assistance of a helper phage such as ExAssistTM (Stratagene) and an *E. coli* strain such as XLOR (Stratagene). Both pTriplEx and pBK-CMV can replicate autonomously in *E. coli*.

a. Screening genomic libraries (plasmid form)

(1) Colony lifts. A single colony of *E. coli* BM25.8 was inoculated into 5 ml of LB containing 50 µg/ml kanamycin, 10 mM MgSO₄, and 0.1% maltose and grown overnight at 31°C, 250 rpm. To 200 µl of this overnight culture (~ 4 X 10⁸ cells), 1 µl of phage library (2–5 × 10⁶ plaque forming units) and 150 µl LB broth were added and incubated at 31°C for 30 min after which 400 µl of LB broth was added and incubated at 31°C, 225 rpm for 1 h. This bacterial culture was diluted and plated on LB agar containing 50 µg/ml ampicillin (Sigma Chemical Company, St. Louis, MO) and kanamycin (Sigma Chemical Company) to obtain 500 to 600 colonies/plate. The plates were incubated at 37°C for 6 to 7 hrs until the colonies became visible. The plates were then stored at 4°C for 1.5 h before placing a Colony/Plaque ScreenTM Hybridization Transfer Membrane disc (DuPont NEN Research Products, Boston, MA) on the plate in contact with bacterial colonies. The transfer of colonies to the membrane was allowed to proceed for 3 to 5 min. The membrane was then lifted and placed on a fresh LB agar (see Table 3-2) plate containing 200 µg/ml of chloramphenicol with the side exposed to the bacterial colonies facing up. The plates containing the membranes were then incubated at 37°C overnight in order to allow full development of the bacterial colonies. The LB agar plates from which colonies were initially lifted were also incubated at 37°C overnight and stored at 4°C for future use. The following morning the membranes containing bacterial colonies were lifted and placed on two sheets of Whatman 3M (Whatman, Hillsboro, OR) paper saturated with 0.5 N NaOH and left at room temperature (RT) for 3 to 6 min to lyse the cells. Additional treatment of membranes was as

described in the protocol provided by NEN Research Products.

(2) DNA hybridizations. Membranes were dried overnight before hybridizing to oligonucleotide probes prepared using a non-radioactive ECLTM 3'-oligolabelling and detection system from Amersham Life Sciences (Arlington Heights, IL). DNA labeling, prehybridization, and hybridizations were performed according to manufacturer's protocols. After hybridization, membranes were washed twice at room temperature in 5× SSC, 0.1% SDS (in a volume equivalent to 2 ml/cm² of membrane) for 5 min each followed by two washes at 50°C in 1× SSC, 0.1% SDS (in a volume equivalent to 2 ml/cm² of membrane) for 15 min each. The hybridization signal was then generated and detected with Hyperfilm ECLTM (Amersham) according to manufacturer's protocols. Membranes were aligned to plates containing bacterial colonies from which colony lifts were performed and colonies corresponding to positive signals on X-ray were then isolated and propagated in LB broth. Plasmid DNA's were isolated from these cultures and analyzed by restriction enzyme digestions and by DNA sequencing.

b. Screening genomic libraries (plaque form)

(1) λ library plating. *E. coli* XL1Blue-MRF' cells were grown overnight in LB medium (25 ml) containing 10 mM MgSO₄ and 0.2% maltose at 37°C, 250 rpm. Cells were then centrifuged (2,200 × g for 10 min) and resuspended in 0.5 volumes of 10 mM MgSO₄. 500 μ l of this *E. coli* culture was mixed with a phage suspension containing 25,000 amplified lambda phage particles and incubated at 37°C for 15 min. To this mixture 6.5 ml of NZCYM top agarose (maintained at 60°C, see Table 3-2) was added and plated on 80–100 ml NCZYM agar (see Table 3-2) present in a 150 mm petri dish. Phage were allowed to propagate overnight at 37°C to obtain discrete plaques. After overnight growth plates were stored in a refrigerator for 1-2 hr before plaque lifts were performed.

(2) Plaque lift and DNA hybridizations. Magna LiftTM nylon membranes (Micron Separations, Inc., Westborough, MA) were placed on the agar surface in complete contact with λ plaques, and transfer of plaques to nylon membranes was allowed to proceed for 5 min at RT. After plaque transfer the membrane was placed on two sheets of Whatman 3MTM (Whatman, Hillsboro, OR) filter paper saturated with a 0.5 N NaOH, 1.0 M NaCl solution and left for 10 min at RT to denature DNA. Excess denaturing solution was removed by blotting briefly on dry Whatman 3M paper. Membranes were then transferred to two sheets of Whatman 3MTM paper saturated with 0.5 M Tris-HCl (pH 8.0), 1.5 M NaCl and left for 5 min to neutralize. Membranes were then briefly washed in 200–500 ml of 2× SSC, dried by air, and baked for 30–40 min at 80°C. The membranes were then probed with labeled DNA.

Membranes were prewashed with a 200–500 ml solution of 5× SSC, 0.5% SDS, 1 mM EDTA (pH 8.0) for 1 - 2 hr at 42°C with shaking (60 rpm) to get rid of bacterial debris from the membranes. The membranes were prehybridized for 1 - 2 hr at 42°C with (in a volume equivalent to 0.125 - 0.25 ml/cm² of membrane) ECL GoldTM buffer (Amersham,) containing 0.5 M NaCl and 5% blocking reagent. DNA fragments that were used as probes were purified from agarose gel using a QIAEX IITM gel extraction kit (Qiagen Inc., Chatsworth, CA) according to manufacturers protocol and labeled using an Amersham ECLTM direct

nucleic acid labeling kit (Amersham). Labeled DNA (5 - 10 ng/ml hybridization solution) was added to the prehybridized membranes and the hybridization was allowed to proceed overnight. The following day membranes were washed with shaking (60 rpm) twice at 42°C for 20 min each time (in a volume equivalent to 2 ml/cm² of membrane) in a buffer containing either 0.1 (high stringency) or 0.5 (low stringency) × SSC, 0.4% SDS and 360 g/l urea. This was followed by two 5 min washes at room temperature in (in a volume equivalent to 2 ml/cm² of membrane) 2 × SSC. Hybridization signals were generated using the ECLTM nucleic acid detection reagent and detected using Hyperfilm ECLTM (Amersham).

Agar plugs which contained plaques corresponding to positive signals on the X-ray film were taken from the master plates using the broad-end of a Pasteur pipette. Plaques were selected by aligning the plates with the X-ray film. At this stage, multiple plaques were generally taken. Phage particles were eluted from the agar plugs by soaking in 1 ml SM buffer¹ overnight. The phage eluate was then diluted and plated with freshly grown *E. coli* XL1Blue-MRF' cells to obtain 100–500 plaques per 85 mm NCZYM agar plate. Plaques were transferred to Magna Lift nylon membranes as before and probed again using the same probe. Single well-isolated plaques corresponding to signals on X-ray film were picked by removing agar plugs and eluting the phage by soaking overnight in 0.5 ml SM buffer.

c. Conversion of λ clones to plasmid form. The lambda clones isolated were converted to plasmid form for further analysis. Conversion from the plaque to the plasmid form was accomplished by infecting the plaques into *E. coli* strain BM25.8. The *E. coli* strain was grown overnight at 31°C, 250 rpm in LB broth containing 10 mM MgSO₄ and 0.2% maltose until the OD₆₀₀ reached 1.1–1.4. Ten milliliters of the overnight culture was removed and mixed with 100 μ l of 1 M MgCl₂. A 200 μ l volume of cells was removed, mixed with 150 μ l of eluted phage suspension and incubated at 31°C for 30 min. LB broth (400 μ l) was added to the tube and incubation was continued at 31°C for 1 hr with shaking, 250 rpm. 1–10 μ l of the infected cell suspension was plated on LB agar containing 100 μ g/ml ampicillin (Sigma, St. Louis, MO). Well-isolated colonies were picked and grown overnight in 5 ml LB broth containing 100 μ g/ml ampicillin at 37°C, 250 rpm. Plasmid DNA was isolated from these cultures and analyzed. To convert the λ ZAP ExpressTM vector to plasmid form *E. coli* strains, XL1Blue-MRF' and XLOR were used. The conversion was performed according to the manufacturer's (Stratagene) protocols for single-plaque excision.

5. Protocol for transformation of *C. tropicalis ura*

5 ml of YEPD was inoculated with *C. tropicalis* ATCC 20962 *ura*- from a frozen stock and incubated overnight on a New Brunswick shaker (New Brunswick Scientific Co., Edison, NJ) at 30°C and 170 rpm. The next day, 10 ml of the overnight culture was inoculated into 100 ml YEPD and growth was continued at 30°C, 170 rpm. The following day the cells were harvested at an OD₆₀₀ of 1.0 and the cell pellet was washed one time with sterile ice-cold water. The cells were resuspended in ice-cold sterile 35 % Polyethylene glycol (4,000 MW) to a density of 5×10⁸ cells/ml. A 0.1 ml volume of cells was utilized for each electroporation. The following electroporation protocol was followed: 1.0 μ g of transforming DNA was added to 0.1 ml cells,

along with 5 µg denatured, sheared calf thymus DNA, and the mixture was allowed to incubate on ice for 15 min. The cell solution was then transferred to an ice-cold 0.2 cm electroporation cuvette, tapped to make sure the solution was on the bottom of the cuvette and electroporated. Following electroporation, 0.9 ml SOS media (1M Sorbitol, 30% YEPD, 10 mM CaCl₂) was added to the suspension. The resulting culture was grown for 1 hr at 30°C, 170 rpm. Following the incubation, the cells were pelleted by centrifugation at 1500 x g for 5 min. The electroporated cells were resuspended in 0.2 ml of 1M sorbitol and plated on synthetic complete media minus uracil (SC - uracil) (Table 3-2). In some cases the electroporated cells were plated directly onto SC-uracil. Growth of transformants was monitored for five days. After three days, several transformants were picked and transferred to SC-uracil plates for genomic DNA preparation and screening.

6. Plasmid DNA isolation.

Plasmid DNA were isolated from *E. coli* cultures using a Qiagen plasmid isolation kit (Qiagen Inc., Chatsworth, CA) according to manufacturer's instructions.

7. DNA sequencing and analysis.

DNA sequencing was performed at Sequetech Corporation (Mountain View, CA) using Applied Biosystems automated sequencer (Perkin Elmer, Foster City, CA). DNA sequences were analyzed with MacVector™ and GeneWorks™ software packages (Oxford Molecular Group, Campbell, CA).

8. PCR protocols.

PCR amplification was carried out in a Perkin Elmer Thermocycler using the AmpliTaqGold enzyme (Perkin Elmer Cetus, Foster City, CA) kit according to manufacturer's specifications. Following successful amplification, in some cases, the products were digested with the appropriate enzymes and gel purified using QiaexII™ (Qiagen, Chatsworth, CA) as per manufacturer's instructions. In specific cases the Ulta™ Taq polymerase (Perkin Elmer Cetus, Foster City, CA) or the Expand Hi-Fi™ Taq polymerase (Boehringer Mannheim, Indianapolis, IN) was used per manufacturer's recommendations or as defined in the text.

9. RNA preparation.

The first step of this protocol involves the isolation of total cellular RNA from cultures of *C. tropicalis*. The cellular RNA was isolated using the Qiagen RNeasy Mini Kit™ (Qiagen Inc. 9600 De Soto Avenue, Chatsworth CA 91311). Two milliliter samples of *C. tropicalis* were collected in standard 2 ml screw capped Eppendorf style tubes at various times before and after the addition of the selected substrate. Cell samples were immediately frozen in liquid nitrogen or a dry-ice/alcohol bath after their harvesting from the fermentor. To isolate total RNA from the samples, the tubes were allowed to thaw on ice and the cells pelleted by centrifugation in a microfuge for 5 min at 4°C and the supernatant discarded while keeping the pellet ice-cold. Cell

rupture was achieved by adding ice-cold Zirconia/Silica beads (0.5 mm diameter) and ice-cold RLT (buffer included with the Qiagen RNeasy Mini Kit) buffer followed by homogenization. The samples were cooled in an ice water bath between several cycles of homogenization. The homogenized cells samples were microfuged at full speed for 10 min and 700 µl of the RNA containing supernatant removed and transferred to a new eppendorf tube. 700 µl of 70% ethanol was added to each sample followed by mixing by inversion. This and all subsequent steps were performed at room temperature. Total RNA was isolated from the ethanol treated sample using a Qiagen RNeasy spin column as per manufacturer instructions. RNA eluted in the water flow through was collected for further purification.

10. Quantitative competitive reverse transcription polymerase chain reaction (QC-RT-PCR) protocol)

QC-RT-PCR is a technique used to quantitate the amount of a specific RNA in a sample. This technique employs the amplification of a specific DNA molecule that is complementary to an RNA molecule in the original sample. By the addition of various amounts of a competitor RNA molecule to the sample, one can determine the concentration of the RNA molecule of interest (in this case the mRNA transcripts of the *CYP* and *CPR* genes). The levels of specific mRNA transcripts were assayed over time in response to the addition of substrates to the growth medium for the identification and characterization of the genes involved in the oxidation of these substrates. This approach can be used to identify genes involved in the oxidation of any given substrate.

a. Primer design. The first requirement for QC-RT-PCR is the design of the primer pairs to be used in the reverse transcription and subsequent PCR reactions. These primers need to be unique and specific to the gene of interest. As there is a family of genetically similar *CYP* genes present in *C. tropicalis*, care had to be taken to design primer pairs that would be discriminating and only amplify the gene of interest, in this example the *CYPB* gene. Due to the high level of homology between the family of genes, the most variable regions of coding sequence were targeted for the design of the primer pairs. In Figure 3-3, a portion of coding region for the *CYPB* allele is shown. The boxed sequences in Figure 3-3 are the sequences of the forward and reverse primers used to quantitate expression of this gene.

b. Design and synthesis of the competitor DNA template. The competitor RNA is synthesized *in vitro* from a competitor DNA template that has the T7 polymerase promoter and carries a deletion relative to the native target RNA sequence. The DNA template for the *in-vitro* synthesis of the competitor RNA is synthesized using PCR primers that are approximately 40 to 60 nucleotides in length. In this example, the primer pair for the synthesis of the *CYPB* competitor DNA is derived from the two primers boxed in Figure 3-3. The forward primer also contains the T7 promoter consensus sequence. The Reverse Primer is followed by a deletion and then another short region of upstream sequence. The forward primer was used with the corresponding reverse primer to synthesize the competitor DNA template. The primer pairs were combined with genomic DNA in a standard *Taq* Gold™ polymerase PCR reaction according to the manufacturer's recommended conditions (Perkin-Elmer/Applied Biosystems, 850 Lincoln Center Drive, Foster City CA 94404). The reaction mixture was placed in a thermocycler for 25 to 35 cycles using the highest annealing

temperature possible in order to assure a homogeneous PCR product. The PCR products were either gel purified or filtered purified to remove unincorporated nucleotides and primers. The competitor template DNA was then quantified using the ($A_{260/280}$) method.

c. *Synthesis of the competitor RNA.* The competitor template DNA was transcribed *in vitro* to make the competitor RNA using the Megascript T7 kit from Ambion Biosciences (Ambion Inc., 2130 Woodward St. Suite 200, Austin, Texas, 78744-1832) according to the directions provided by the manufacturer. The resulting RNA preparations were then checked by gel electrophoresis for the conditions giving the highest yields and quality of competitor RNA. The DNA template was then removed using DNase I as described in the Ambion kit. Serial dilutions of the RNA were made for use in the QC-RT-PCR reactions and the original stocks stored at -70°C .

d. *QC-RT-PCR reactions.* QC-RT-PCR reactions were performed using rTth polymerase from Perkin-Elmer(Perkin-Elmer/Applied Biosystems, 850 Lincoln Center Drive, Foster City CA 94404) according to the manufacturer's recommended conditions. The reverse transcription reaction was performed in the presence of the reverse primer. A series of 8 to 12 of the QC-RT-PCR reaction mixes were aliquoted to different reaction tubes. Serial dilutions of competitor RNA were added to the series of QC-RT-PCR reaction mixes. The QC-RT-PCR reactions were mixed and incubated at 70°C for 15 min according to the manufacturer's recommended times for reverse transcription to occur. At the completion of the 15 minute incubation, the sample temperature was reduced to 4°C to stop the reaction and the PCR reaction mix was added to each of the RT reactions. The reaction mixtures were placed in a thermocycler (Perkin-Elmer GeneAmp PCR System 2400, Perkin-Elmer/Applied Biosystems, 850 Lincoln Center Drive, Foster City CA 94404) and the following PCR cycle performed: 94°C for 1 min followed by 94°C for 10 sec followed by 58°C for 40 sec for 17 to 22 cycles. The PCR reaction was completed with a final incubation at 58°C for 2 min followed by 4°C . The number of cycles performed was enough to yield roughly 0.5 ng of PCR products. In some reactions where no PCR product was produced, the samples were returned the thermocycler for additional cycles, this process was repeated until enough PCR products were produced to quantify using HPLC. The number of cycles necessary to produce enough PCR product is a function of the amount of the target mRNA present in the total cellular RNA. The lower the concentrations of the target mRNA present the more PCR cycles are required to produce a detectable amount of product.

e. *HPLC quantification.* Upon completion of the QC-RT-PCR reactions, the samples were analyzed and quantitated by HPLC using a reverse phase ion-pair column. A linear aqueous/acetonitrile gradient was used to resolve and quantitate the PCR products from the QC-RT-PCR reactions. The amounts of the QC-RT-PCR products were plotted and quantitated with an attached Waters Corporation 745 data module. The log ratios of the amount of target mRNA QC-RT-PCR product (U) to competitor QC-RT-PCR product (C), as measured by peak areas, was plotted, and the amount of competitor RNA required to equal the amount of target mRNA product determined.

11. Development of Antibodies

Antibodies specific for CYP and CPR proteins would be valuable tools for correlating expression of these genes at the cellular level. However, cytochrome P450 monooxygenase and NADPH oxidoreductase proteins are localized in the membranes and are difficult to purify. In the case of *Candida* species, purification of a single P450 enzyme away from the myriad of P450's present is almost impossible. Efforts to develop antibodies specific for the different P450 enzymes was therefore directed toward the use of synthetic peptides. A series of synthetic peptides suitable as antigens which could potentially result in the formation of antibodies capable of distinguishing between the closely related *Candida* CYP proteins were designed and antibodies prepared. IgG fractions of samples with activities toward the antigenic peptides were purified and provided for further analysis.

3.3 Results

1. Cloning and characterization of *C. tropicalis* cytochrome P450 monooxygenase (CYP) and cytochrome P450 NADPH oxidoreductase (CPR) genes

To clone CYP and CPR genes several different strategies were employed. Available CYP amino acid sequences were aligned and regions of similarity were observed (Figure 3-4). These regions corresponded to described conserved regions seen in other cytochrome P450 families.^{2,3} One region corresponded to the HR2 domain containing the invariant cysteine residue near the carboxyl terminus which is required for heme binding, while the other region corresponded to the central region of the I helix thought to be involved in substrate recognition (Figure 3-4). Degenerate oligonucleotide primers corresponding to these highly conserved regions of the CYP52 gene family were designed and used to amplify DNA fragments of CYP genes from *C. tropicalis* 20336 genomic DNA. These discrete PCR fragments were then used as probes to isolate full-length CYP genes. In a few instances oligonucleotide primers corresponding to highly conserved regions were directly used as probes to isolate full-length CYP genes from genomic libraries. In the case of CPR, a heterologous probe based upon the known DNA sequence for the CPR gene from *C. tropicalis* ATCC 750 was used to isolate the *C. tropicalis* 20336 CPR gene.

a. Cloning of the CPR gene from *C. tropicalis* 20336

(1) *Cloning of the CPRA allele.* Approximately 25,000 phage particles from the first genomic library of *C. tropicalis* 20336 were screened with a 1.9 kb *Bam*HI-*Nde*I fragment from plasmid pCU3RED⁴ containing most of the *C. tropicalis* ATCC 750 CPR gene. Five clones that hybridized to the probe were isolated and the plasmid DNA from these lambda clones was rescued and characterized by restriction enzyme analysis. The restriction enzyme analysis suggested that all five clones were identical and that all contained a truncated CPR gene.

Since the first Clontech library yielded only a truncated CPR gene, the second library prepared by Clontech was screened to isolate a full-length CPR gene. Three putative CPR

clones were obtained. All three clones, having inserts in the range of 5–7 kb, were characterized by PCR using degenerate primers, and all were found to encode truncated *CPR* genes. All three clones were partially sequenced, and this analysis confirmed that two of the clones carried the 3' region of the *CPR* gene that was missing from a clone isolated from the initial screen. An intact *CPRA* gene, encoding a putative 679 amino acid protein, was isolated using this sequence information. The *CPRA* protein, when analyzed by the protein alignment program of the GeneWorksTM software package (Oxford Molecular Group, Campbell, CA), showed extensive homology to CPR proteins from *C. tropicalis* 750 and *C. maltosa*.

(2) Cloning of the *CPRB* allele. To clone the second *CPRB* allele, the third genomic library, prepared by Henkel, was screened using DNA fragments from *CPRA* as probes. Five clones were obtained and sequenced. One clone contained the full length *CPRB* allele encoding a protein that differed from *CPRA* by nine amino acids.

b. Cloning of *C. tropicalis* 20336 (*CYP*) genes

(1) Clones carrying *CYPA*, *CYPB*, *CYPC*, *CYPE* and *CYPG* were isolated from the first and second Clontech genomic libraries using an oligonucleotide probe (HemeB1) whose sequence was based upon the amino acid sequence for the highly conserved CYP heme binding region (Table 3-3). The first and second libraries were converted to the plasmid form and screened by colony hybridizations using the HemeB1 probe.

(2) Cloning of Additional *CYP* Genes. Two additional *CYP* genes were isolated from the third genomic library using PCR fragments as probes. One PCR probe was generated after PCR amplification of 20336 genomic DNA using oligonucleotide primers based upon the amino acid sequence for all available CYP proteins (accessed from National Center for Biotechnology Information (www.nlm.ncbi.gov)). Degenerate primers were designed based upon a conserved amino acid sequence from the Helix 1 and Helix 2 regions. These primers were used in pairwise combinations in a PCR reaction with Stoffel *Taq* DNA polymerase (Perkin-Elmer Cetus, Foster City, CA) according to the manufacturer's recommended procedure. A PCR product of approximately 450 bp was obtained. This product was purified from agarose gel using Gene-cleanTM (Bio 101, LaJolla, CA) and ligated to the pTAGTM vector (Figure 3-5) (R&D systems, Minneapolis, MN) according to the recommendations of the manufacturer. No treatment was necessary to clone into pTAG because it employs the use of the TA cloning technique. Plasmids from several transformants were isolated and their inserts were characterized. One plasmid contained the PCR clone intact. The PCR fragment was used as a probe to screen the third genomic library, and one clone containing a full-length *CYP* gene was obtained. The clone encoded a CYP protein of 523 amino acids which was designated *CYPF*.

A second PCR probe was generated using primers for highly conserved sequences of *CYP52A* genes of *C. tropicalis* ATCC 750. The reverse primer (P-750R) was designed based on the highly conserved heme binding region (Table 3-3). The design of the forward primer (P-750F) was based upon a sequence conserved near the N-terminus of the *CYP52A* genes from *C. tropicalis* 750 (Table 3-3). Amplification of 20336 genomic DNA with these two primers gave a mixed PCR product. The DNA sequence of one of the PCR fragments

exhibited 85% identity to the DNA sequence for a *CYP52D* gene of *C. tropicalis* 750. When this PCR product was used to screen the third genomic library, one clone was identified that contained a full-length *CYP* gene. A putative CYP protein of 512 amino acids was encoded. This *CYP* gene was designated as *CYPD*.

The screening of the second genomic library with the HemeB1 primer (Table 3-3) yielded a clone that contained what appeared to be a truncated gene. A fragment from this clone was used as a probe to screen the third genomic library for a full length *CYP* gene. One clone, designated *CYPH*, was isolated and found to encode a full-length CYP protein having 499 amino acids.

2. Identification of CYP and CPR genes induced by selected fatty acid and alkane substrates

Genes whose transcription is turned on by the presence of selected fatty acid or alkane substrates have been identified using the QC-RT-PCR assay. This assay was used to measure (*CYP*) and (*CPR*) gene expression in fermentor grown cultures *C. tropicalis*. This method involves the isolation of total cellular RNA from cultures of *C. tropicalis* and the quantification of a specific mRNA within that sample through the design and use of sequence specific QC-RT-PCR primers and an RNA competitor. Quantification is achieved through the use of known concentrations of highly homologous competitor RNA in the QC-RT-PCR reactions. The resulting QC-RT-PCR amplified cDNA's are separated and quantitated through the use of ion pairing reverse phase HPLC. Genes which were induced were identified by the calculation of their mRNA concentration at various times before and after induction. Figure 3-6 provides an example of how the concentration of mRNA for *CYPB* can be calculated using the QC-RT-PCR assay. The log ratio of unknown (U) to competitor product (C) is plotted versus the concentration of competitor RNA present in the QC-RT-PCR reactions. The concentration of competitor which results in a log ratio of U/C of zero represents the point where the unknown messenger RNA concentration is equal to the concentration of the competitor. Figure 3-6 allows for the calculation of the amount of a specific *CYP* message present in total RNA isolated from cell samples taken at times T_0 , T_1 and T_2 after the addition of the substrate. From this analysis, it is possible to determine the concentration of the *CYP* mRNA present in a known amount of total cellular RNA. The data in Figure 3-6 show that the selected *CYP* gene is significantly induced after the addition of the substrate. Figure 3-7 shows the relative amounts of *CYP* expression with and without a substrate. Clearly, no induction of the selected *CYP* gene occurs in the absence of appropriate substrate. This type of analysis was used to establish the induction patterns of *CYP* and *CPR* genes for various types of fatty acid and alkane substrates. Figure 3-8 shows the relative induction of the *CYP* and *CPR* genes by a fatty acid substrate. *CYPB* and *CYPC* are significantly induced. The induction, if present, of other *CYP* genes (A, G & H) was below the detection limit of the assay.

3. Integration of selected CYP and CPR genes into the genome of C. tropicalis.

In order to integrate selected genes into the chromosome of *C. tropicalis* 20336 or its descendants, there has to be a target DNA sequence, which may or may not be an intact gene, into which the genes can be inserted. There must also be a method to select for the integration

event. In some cases the target DNA sequence and the selectable marker are the same, and, if so, then there must also be a method to regain use of the target gene as a selectable marker following the integration event. In *C. tropicalis* and its descendants, one gene which fits these criteria is *URA3A*, encoding orotidine-5'-phosphate decarboxylase. Using it as a target for integration, *ura⁻* variants of *C. tropicalis* can be transformed in such a way as to regenerate a *URA⁺* genotype via homologous recombination (Figure 3-9). Depending upon the design of the integration vector, one or more genes can be integrated into the genome at the same time. Moreover, because of the high sequence similarity between *URA3A* and *URA3B* genes, integration of the construct can occur at both the *URA3A* and *URA3B* loci. Subsequently, an oligonucleotide designed with a deletion in a portion of the *URA* gene based on the identical sequence across both the *URA3A* and *URA3B* genes, can be utilized to yield *C. tropicalis* transformants which are once again *ura⁻* but which still carry one or more newly integrated genes of choice (Figure 3-9). *ura⁻* variants of *C. tropicalis* can also be isolated via other methods such as classical mutagenesis or by spontaneous mutation. Using well established protocols, selection of *ura⁻* strains can be facilitated by the use of 5-fluoroorotic acid (5-FOA).⁵ The utility of this approach for the manipulation of *C. tropicalis* has been well documented.⁶⁻⁹

a. Construction of a *URA* integration vector. Integration vectors were constructed which carried *CYP* and *CPR* genes, either singly or in combination, flanked by *URA* sequences (Figure 3-9).

b. Cloning of *CYP* and *CPR* Genes into *pURAI*. Selected *CYP* and *CPR* genes were cloned into the *pURAI* integration vector using unique restriction endonuclease sites. (Figure 3-9). The ligation mixture was transformed into *E. coli* XL1 Blue MRF' (Stratagene), and resistant colonies were selected and screened for correct constructs, which should contain vector sequence, the inverted *URA3A* gene, and the selected *CYP* or *CPR* gene. Restriction digests were used to identify correct clones. Plasmid inserts were sequenced and compared to the original target genes to confirm that the clones were correct.

c. Confirmation of *CYP* integration into the Genome of *C. tropicalis*. Based on the known DNA sequence for both the *URA* and *CYP* and *CPR* genes, it was possible to detect integration events by restricting genomic DNA with the appropriate restriction enzymes and then performing Southern hybridizations using appropriately labeled probes. DNA to be used for Southern hybridization was labeled using a ECL direct labeling and detection system (Amersham) and the Southern was processed according to the ECL kit specifications. The blots were exposed for 16 hours (hr) as recommended.

Integration was confirmed by hybridization of the probes to specific restriction fragments of known size from the genomic DNA of the transformants but not from the *C. tropicalis* 20336 control.

4. Effects of CYP Amplification in the genome of *C. tropicalis*

The goal of the project is to improve diacid production by the integration and amplification of selected *CYP* genes into the genome of *C. tropicalis* strain ATCC 20962 (blocked for beta oxidation). Our belief is that the NADPH oxidoreductase (*CPR*) gene will also need to be amplified in conjunction with the selected *CYP* gene(s). To date we have succeeded in identifying multiple *CYP* genes which are induced by fatty acid and alkane substrates. Several of these *CYP* genes have been integrated into the genome of *C. tropicalis*. Integration in both single and multiple copies was accomplished. Initial fermentations of the *CYP* amplified strains produced promising diacid productivities. More fermentation conditions need to be examined and more samples from various times need to be assayed to determine whether or not amplification has affected *CYP* mRNA levels. The amount of *CYP* protein present during the production of diacids also needs to be determined. This will be accomplished by both biochemical assays and by the use of antibodies whose development was initiated as part of this project. The ultimate goal is to simultaneously integrate both a selected *CYP* gene and a *CPR* gene into the *C. tropicalis* genome in multiple copies and to accomplish a coordinated increase in the expression of these proteins. Efforts to accomplish this are continuing.

5. Formation of antibodies

At the conclusion of the DOE sponsored program this task was not complete. Initial results indicate that this approach has been successful in developing antibodies that can distinguish certain peptides from several of the *CYP* proteins. However, final evaluation will require the production of the individual *CYP* proteins using expression vectors in clean systems such as *E. coli* or *Saccharomyces*. This will be completed in research to be conducted outside of the DOE sponsored program.

3.4 References

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Figure 3-1. Diagrammatic representation of cloning vector pTriplEx from Clontech™ Laboratories, Inc. Selected restriction sites within the multiple cloning site are shown.

Figure 3-2. Diagrammatic representation of lambda ZAP Express™ vector available from Stratagene, LaJolla, CA.

RT-PCR primers for the coding sequence of *Candida tropicalis* CYPB

Forward Primer

```

AAGAAGAGGGCAGGGCTCAAGAGTACAAC-----
TTCTTTCTCCCGTCCCGAGTTCTCATGTTG-----

-----GTTAGGTGATGGGATCTTCACATTGGACGG
-----CAATCACTACCCTAGAAGTGTAACCTGCC

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Reverse primer

Figure 3-3. A portion of the double-stranded DNA sequence of the coding region of the *CYPB* gene. The boxed sequences represent the forward and reverse primers used in the QC-RT-PCR quantification of the expression of this gene.

Figure 3-4. Diagrammatic representation of highly conserved regions of CYP and CPR gene protein sequences. Helix 1 represents the putative substrate binding site and HR2 represents the heme binding region. The FMN, FAD and NADPH binding regions are indicated below the CPR gene.

Figure 3-5. pTAg PCR product cloning vector. Commercially available from R&D Systems, Minneapolis, MN.

Figure 3-6. Calculation of mRNA(U) concentration in a QC-RT-PCR reaction.

Figure 3-7. Relative induction of a *C. tropicalis* ATCC 20962 CYP B by the addition of a fatty acid substrate.

Figure 3-8. Induction of *C. tropicalis* ATCC 20962 *CYP* and *CPR* genes by fatty acid feed. *CYP* genes *CYP A*, *CYP G* and *CYP H* are expressed at levels below the detection level of the QC-RT-PCR assay.

Table 3-1. List of *Escherichia coli* and *Candida tropicalis* strain

<i>E. Coli</i> STRAIN	GENOTYPE	SOURCE
XL1Blue-MRF'	<i>endA1, gyrA96, hsdR17, lac⁻, recA1, relA1, supE44, thi-1, [F' lacP⁺ZΔM15, proAB, Tn10]</i>	Stratagene, La Jolla, CA
BM25.8	<i>SupE44, thi Δ(lac-proAB) [F' traD36, proAB⁺, lacP⁺Z ΔM15] λimm434 (kan^R)P1 (cam^R) hsdR (r_{kl2}-m_{kl2}-)</i>	Clontech, Palo Alto, CA
XLOR	<i>Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 thi-1 recA1 gyrA96 relA1 lac [F' proAB lacP⁺ZΔM15 Tn10 (Tet^r) Su⁻ (nonsuppressing λ'(lambda resistant))</i>	Stratagene, La Jolla, CA

<i>C. tropicalis</i> STRAIN	GENOTYPE	SOURCE
ATCC20336	Wild-type	American Type Culture Collection, Rockville, MD
ATCC750	Wild-type	American Type Culture Collection, Rockville, MD
ATCC 20962	<i>ura3A/ura3B, pox4A::ura3A/pox4B::ura3A, pox5::ura3A/pox5::URA3A</i>	Henkel
ATCC 20962 ura-	<i>ura3A/ura3B, pox4A::ura3A/pox4B::ura3A, pox5::ura3A/pox5::URA3A, ura3-</i>	Henkel

Table 3-2. Media Compositions

LB Broth	
Bacto Tryptone	10 g
Bacto Yeast Extract	5 g
Sodium Chloride	10 g
Distilled Water	1,000 ml
LB Agar	
Bacto Tryptone	10 g
Bacto Yeast Extract	5 g
Sodium Chloride	10 g
Agar	15 g
Distilled Water	1,000 ml
LB Top Agarose	
Bacto Tryptone	10 g
Bacto Yeast Extract	5 g
Sodium Chloride	10 g
Agarose	7 g
Distilled Water	1,000 ml
NZCYM Broth	
Bacto Casein Digest	10 g
Bacto Casamino Acids	1 g
Bacto Yeast Extract	5 g
Sodium Chloride	5 g
Magnesium Sulfate (anhydrous)	0.98 g
Distilled Water	1,000 ml
NZCYM Agar	
Bacto Casein Digest	10 g
Bacto Casamino Acids	1 g
Bacto Yeast Extract	5 g
Sodium Chloride	5 g
Magnesium Sulfate (anhydrous)	0.98 g
Agar	15 g
Distilled Water	1,000 ml
NZCYM Top Agarose	
Bacto Casein Digest	10 g
Bacto Casamino Acids	1 g
Bacto Yeast Extract	5 g
Sodium Chloride	5 g
Magnesium Sulfate (anhydrous)	0.98 g
Agarose	7 g
Distilled Water	1,000 ml

Table 3-2. Media Compositions (cont'd)

YEPD Broth	
Bacto Yeast Extract	10 g
Bacto Peptone	20 g
Glucose	20 g
Distilled Water	1,000 ml
YEPD Agar¹⁰	
Bacto Yeast Extract	10 g
Bacto Peptone	20 g
Glucose	20 g
Agar	20 g
Distilled Water	1,000 ml
SC - uracil¹⁰	
Bacto-yeast nitrogen base without amino acids	6.7 g
Glucose	20 g
Bacto-agar	20 g
Drop-out mix	2 g
Distilled Water	1,000 ml

Drop-out mix			
Adenine	0.5 g	Alanine	2 g
Arginine	2 g	Asparagine	2 g
Aspartic acid	2 g	Cysteine	2 g
Glutamine	2 g	Glutamic acid	2 g
Glycine	2 g	Histidine	2 g
Inositol	2 g	Isoleucine	2 g
Leucine	10 g	Lysine	2 g
Methionine	2 g	para-Aminobenzoic acid	0.2 g
Phenylalanine	2 g	Proline	2 g
Serine	2 g	Threonine	2 g
Tryptophan	2 g	Tyrosine	2 g
Valine	2 g		

Table 3-3. Primer table for PCR amplification to construct gene integration vectors, to generate probes for gene isolation and detection, and to obtain DNA sequence of constructs. (A- deoxyadenosine triphosphate [dATP], G- deoxyguanosine triphosphate [dGTP], C- deoxycytosine triphosphate [dCTP], T- deoxythymidine triphosphate [dTTP], Y- dCTP or dTTP, R- dATP or dGTP, W- dATP or dTTP, M- dATP or dCTP, N- dATP or dCTP or dGTP or dTTP)

Target gene(s)	Primer Name	Sequence (5' to 3')
<i>CYP</i>	HemeB1	ATTCAACGGTGGTCCAAGAATCTGTTTGG
<i>CYP</i>	2,3,5P	GAGCTAGTTGAGACCACAGTTTGC
<i>CYP</i>	2,3,5M	CTTCAGTTAAAGCAAATTGTTTGGCC
pTriplEx vector	Triplex5'	CTCGGGAAGCGCGCCATTGTGTTGG
pTriplEx vector	Triplex3'	TAATACGACTCACTATAGGGCGAATTGGC

4. Bioprocess Development

4.1 Selection of Low-Cost Fatty Acid Substrate (Task 2.1)

(David P. Mobley and Gary K. Shank, GE CRD)

4.1.1 Introduction

The fatty acid substrate cost will be a significant proportion of the total diacid production cost for the bioconversion process. Hence a key goal of this program is the identification of suitable low-cost fatty acid substrates.

The selection of the source of fatty acids for the bioprocess has an effect on all of the major development tasks in the program. The improved biocatalyst under development (see Chapter 3) must be designed to have optimum activity for the selected fatty acid substrate. The bioprocess must be designed to handle this substrate and the resulting diacids. Finally, the selected fatty acid substrate must produce diacid that is suitable for the polymer application (see Chapter 5). The goal is to use the fatty acid source that yields the lowest overall diacid production cost and that yields diacids acceptable for the high-flow resin application.

The mixtures of fatty acids that are derived directly from hydrolysis of natural oils and fats are significantly cheaper than single fatty acids or fatty acid esters. For example, lauric acid, a pure fatty acid, sells for more than \$0.75/lb, while tallow fatty acids, the mixture of fatty acids from tallow, sells for around \$0.30/lb. The cheapest fatty acid source may not be the best substrate, however. For example, a cheap fatty acid source that is slowly converted in the bioprocess may not be as cost-effective overall as a somewhat more expensive substrate that is more rapidly converted.

Some fatty acid substrates may require particular operating conditions. For example, some fatty acids may have toxic effects on the biocatalyst above a critical concentration. Substrates containing these acids can still be used, however, by metering the substrate addition rate such that the substrate concentration in the fermentor is kept low.

In this program, our approach was to select candidate substrates from a list of sources of fatty acids costing under about \$0.50/lb. Example substrates are the fatty acid mixtures from tallow, tall oil, soybean oil or cottonseed oil. Table 4.1-1 shows the composition of fatty acids derived from a number of these plant oil and animal fat sources. Tallow and tall oil are particularly interesting as potential substrate sources because they are cheap, high-volume byproducts of the meat processing and paper pulping industries, respectively. Annual U.S. production of beef tallow, for example, exceeds 3 billion pounds.

We measured the relative rates and extent of conversion of the candidate substrates in batch fermentations, using existing diacid-producing strains of *C. tropicalis* and current bioprocess technology. 100 to 500 g quantities of diacids from the selected fatty acid substrates were isolated for use in Applications Development. Product diacids were separated and purified. Selected individual diacids were also synthesized for use in Applications Development, when these diacids were not available commercially.

4.1.2 Materials and Methods

Organism—*Candida tropicalis* ATCC 20987, a recombinant yeast developed by Henkel Corporation, was used throughout this study. In this strain, the genes coding for enzymes in the first step of fatty acid β -oxidation have been disrupted so that the yeast can no longer use fatty acids as a carbon source, and multiple copies of cytochrome P450 and reductase genes of the ω -hydroxylase system have been introduced.^{1,2}

Materials—The following were used as received. Medium components: ammonium sulfate, unrefined 95% dextrose corn syrup, refined 95% dextrose corn syrup, α -D-glucose (dextrose), corn steep liquor, Hodag M-10™ antifoam, dibasic potassium phosphate, monobasic potassium phosphate, salts and trace elements as found in Difco® Yeast Nitrogen Base (Difco Laboratories, Detroit, MI) (see trace elements solution below), YM Broth (Difco), Yeast Nitrogen Base (Difco), and Yeast Extract (Difco). Substrates: methyl myristate, methyl palmitate, methyl stearate, tallow fatty acids, modified tallow fatty acids, commercial stearic acid, mixed fatty acids enriched for oleic acid, and commercial linoleic acid. Analytical standards:

12-hydroxydodecanoic acid, 97%; 16-hydroxyhexadecanoic acid, 98%; methyl laurate, 99.5%; methyl myristate, 99%; methyl palmitate, 99+%; lauric acid, 99.5%; myristic acid, 99.5+%; palmitic acid, 99%; pentadecanoic acid, 99+%; 1,12-dodecanedioic acid, 99%; 1,14-tetradecanedioic acid, 99%; and 1,16-hexadecanedioic acid, 98%.

Media—DCA4 and OPT1 growth media had the compositions listed in Tables 4.1-2 and 4.1-3, respectively. Trace elements solution contained, per liter aqueous solution: 500 mg H₃BO₃, 40 mg CuSO₄·5H₂O, 100 mg KI, 200 mg FeCl₃·6H₂O, 400 mg MnSO₄·H₂O, 200 mg Na₂MoO₄·2H₂O, and 400 mg ZnSO₄·7H₂O. Separate filter-sterilized concentrated solutions of magnesium sulfate, sodium chloride, and calcium chloride were prepared for use in the OPT1 medium.

Stock Cultures, Precultures, and Main Cultures—Stock culture was prepared by inoculating 25 ml Difco YM Broth in a 250 ml conical flask with 2.0 ml of seed culture of *C. tropicalis* ATCC 20987 obtained from Henkel Corporation and incubating at 30°C and 250 rpm for 24 hours. By serial culture in this manner, enough of a master feed stock culture was prepared to last for the entire course of this study. The master feed stock culture was preserved in 1.5 ml aliquots by mixing 0.75 ml of the culture with an equal volume of sterile glycerol solution (50% in water) and storing immediately in a freezer at -80°C.

Preculture was prepared by inoculating 20 ml Difco YM Broth in a 250 ml conical flask with 1.5 ml of thawed master feed stock culture and incubating at 30°C and 250 rpm for 24 hours. Main culture was prepared by inoculating 500 ml DCA4 medium in a 2 liter baffled conical flask with 5 ml of preculture (1% inoculum) and incubating at 30°C and 250 rpm for 24 hours.

Stirred Fermentor Equipment and Methods—Bioconversions were carried out in both Biostat E® (B. Braun Biotech, Inc., Allentown, PA) and BioFlo III® (New Brunswick Scientific Co., Edison, NJ) fermentors, each with 5 liters nominal working liquid capacity, equipped with mechanical stirring, temperature and pH control, antifoam addition, filter-sterilized air sparging, and dissolved oxygen probe. Each Biostat E® fermentor had a glass-sided, rounded-bottom fermentor vessel with a diameter of 15 cm and a liquid height at 5 liters of 30 cm. At the starting

volume of 2.5 to 3.0 liters, two of the three impellers were submerged with the top impeller at the level of 4.25 liters of culture. The vessel was unbaffled, but internal heat exchange coils provided some baffling. Each BioFlo III[®] fermentor had a glass-sided, rounded-bottom fermentor vessel with a diameter of 17 cm and a liquid height at 5 liters of 23.5 cm. The two impellers were both submerged at the 2.5 liter starting volume. The vessel was equipped with four radial baffles.

The fermentation process was composed of two phases, a growth phase and a conversion phase. Growth was initiated by inoculating OPT1 medium (Table 4.1-3) in a fermentor with main culture (10% inoculum) to obtain a volume after inoculation of 2.5 to 3.0 liters. Either all of the glucose was added initially or half of the total glucose was added to the medium initially and the remaining glucose was added in fed-batch manner during the course of the growth. The culture was grown for up to 18 hours at the selected temperature and pH (using 6N NaOH or 6N KOH and 4N H₂SO₄ for pH control), stirring at 900 rpm, and aerating at 1.2 vvm. At the end of the growth phase, cell count measurements typically showed about 10⁹ viable cells per ml.

The conversion phase was begun at the end of the growth phase by starting continuous feeds of fatty acid substrate (added at a rate sufficient to be in excess of the conversion) and glucose (cosubstrate, 50% (w/v) solution). Antifoam was added during the conversion as needed. The glucose feed rate was set to selected values above 1 g glucose/liter initial volume/h. The pH was increased to selected values above 7 and adjusted as needed through the conversion to maintain the solubility of the diacid products. The temperature was adjusted to the selected value. Stir speed and aeration remained at the same values as in the growth phase.

During the conversion phase, samples were taken to measure substrate and product concentration, viable cell count, optical density (0 h only), and glucose.

Product Isolation and Purification—(Method 1): The fermentor contents from a bioconversion of methyl myristate were adjusted to pH 10.3 by addition of 6N NaOH, diluted with 2 parts water per 3 parts fermentor contents, and centrifuged (80 min at 900Xg). The clear supernatant was removed and the centrifuge pellet was reslurried with water (1 part per 2 parts pellet) and centrifuged as before. The clear supernatant was combined with the first supernatant and acidified to pH 2.3 with 4N H₂SO₄. The resulting precipitate was isolated by centrifugation (30 min at 900Xg), washed with water and recentrifuged. The centrifuge pellet was vacuum filtered to remove liquid and the resulting crude diacid filter cake was dried in a vacuum oven. The pellet from centrifugation at high pH was found to contain unrecovered product, so it was reslurried with water, adjusted to pH 10 to 11, and centrifuged as before, repeating the procedure until little product remained in the centrifuge pellet. The combined supernatants from these high pH centrifugations were acidified to precipitate diacid product, which was isolated by centrifugation, filtration, and drying as above. The crude diacid products were purified by recrystallization from ethanol. The product was further purified by recrystallization from a 3:2 mixture of heptane and ethyl acetate and recrystallization from ethyl acetate, using activated carbon to decolorize the hot solution.

(Method 2): The fermentor contents from a bioconversion of methyl palmitate were diluted two-fold with water and adjusted to pH 9 by addition of 6N NaOH. The mixture was filtered at 50 to 70°C with a laboratory scale cross-flow microfilter unit (Membralox[®] 1T1-70, U.S. Filter Corp., Warrendale, PA) equipped with a 0.5 µm ceramic membrane until about 55% of the starting volume was collected as filtrate. The filter retentate was centrifuged (30 min at 7000Xg).

Analysis of the filtrate and centrifuge supernatant showed they contained the same concentration of diacid product, so they were combined and acidified with H_2SO_4 . The precipitated crude diacid was recovered by centrifugation (40 min at 7000Xg) and dried. The crude product was recrystallized from ethyl acetate, using activated carbon to decolorize the hot solution.

(Method 3): The fermentor contents from a bioconversion of methyl stearate were diluted two-fold with water, adjusted to pH 9 by addition of 6N NaOH, and centrifuged (30 min at 7000Xg). The pellet was reslurried with water, adjusted to pH 9 and centrifuged as before. The combined supernatants were acidified to pH 3 with H_2SO_4 and centrifuged (30 min at 7000Xg). The centrifuge pellet was vacuum dried. To recover more diacid product, the pellet from the high pH centrifugations was acidified to pH 3 with H_2SO_4 and centrifuged as before. The resulting pellet was vacuum dried. The dry solids were extracted with ethanol (1 liter ethanol per 100 g solids), separated by centrifugation, re-extracted with half the first volume of ethanol, and separated again by centrifugation. The combined ethanol extracts were concentrated to one third the starting volume. The concentrate was mixed with an equal volume of diethyl ether and allowed to cool, yielding a crude diacid product. The crude product was recrystallized from ethyl acetate, using activated carbon to decolorize the hot solution.

(Method 4): The fermentor contents from a bioconversion of commercial stearic acid were acidified to pH 3.5 with H_2SO_4 and centrifuged (30 min at 7000Xg). The resulting pellet was dried and extracted twice with hot ethanol, separating the extract from the solids by centrifugation. The combined ethanol extracts were concentrated in a rotary evaporator to a damp solid, which was mixed with heptane at reflux. The hot heptane solution was decanted and allowed to cool, yielding crystalline diacid product.

(Method 5): The fermentor contents from a bioconversion were adjusted to pH 8.5 to 9 by addition of 6N KOH and centrifuged (30 min at 900Xg). The biomass pellet was reslurried to the original volume and centrifuged as before. The combined supernatant solutions were acidified to pH 3 with HCl. The acidified mixture was autoclaved (121°C for 30 min). Upon cooling, a solid layer of crude diacid product formed at the top of the mixture. The solid layer was removed and the cloudy liquid lower phase was centrifuged (40 min at 7000Xg) to recover the remaining precipitated diacid product. The combined crude diacid product was recrystallized from heptane to obtain purified product.

Analysis for Substrate and Product Concentrations—Substrate and product concentrations were determined by gas chromatography (GC). Samples were prepared by acidifying a 1.0 g fermentor broth sample with 0.4 ml 6N HCl, diluting with acetone (J.T. Baker, ACS reagent grade, 20 ml) and shaking at 250 rpm for about 15 min. A drying agent was then added to the sample (anhydrous MgSO_4 , 2 g), and it was shaken again for about 15 min. The sample was allowed to settle for a few minutes after shaking, and equal volumes of the sample solution, internal standard solution (4.0 g/l pentadecanoic acid in methyl *t*-butyl ether), and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Supelco, Inc., Bellefonte, PA) were mixed to derivatize the carboxylic acids. The derivatized samples were analyzed along with the appropriate standards on a Hewlett-Packard gas chromatograph (Model 5890) equipped with an autosampler (Model 7673), flame ionization detector, and HP-5 capillary column (Hewlett Packard, 30 m length, 0.32 mm I.D., 0.25 m film thickness; He carrier at 2 ml/min; 130°C (hold for 2 min), ramp to 170°C at $20^\circ\text{C}/\text{min}$, ramp to 280°C at $10^\circ\text{C}/\text{min}$, hold at 280°C for 2 min).

With this procedure it is necessary to derivatize the sample soon after the prior work-up in order to avoid interference from side reactions.

Optical Density—Samples of fermentor culture were diluted 200-fold and absorbance was measured at 625 nm (Spectronic® 20 Spectrophotometer (Milton Roy Co., Rochester, NY), 1 cm path length, 1.5 ml disposable cuvette).

Viable Cell Count—Samples of fermentor culture were serially diluted in sterile water and 1×10^{-5} and 1×10^{-6} dilutions were used to inoculate plates (100 x 15 mm) prepared with Difco YM Agar (21 g/l YM Broth and 18 g/l Bacto® Agar). An inoculation volume of 50 ml per plate was used.

Glucose Measurements—Glucose measurements were made with an Accu-Chek® III Blood Glucose Monitor (Model 766, Boehringer Mannheim Corp., Indianapolis, IN). A drop of fermentor culture was placed onto a colorimetric test strip and after 60 sec the test strip was wiped and inserted into the calibrated electronic monitor for a reading.

4.1.3 Results

Laboratory scale bioconversions were carried out with the fatty acid substrates listed in Tables 4.1-4 and 4.1-5. As the tables indicate, these substrates encompass a range of carbon chain lengths and degree of unsaturation. The fatty acids are mixtures representative of commercially available low-cost starting materials. The methyl esters are essentially single-component materials. The methyl esters were used to test the effects of chain length on the conversion and to provide individual diacid products for use in Applications Development.

Some of the substrates, such as methyl myristate, are liquid at the bioconversion process temperature, while others, such as tallow fatty acids, are waxy solids at the process conditions. The solid substrates were heated and fed to the process in the molten state.

Figures 4.1-1 through 4.1-3 compare typical results of diacid product concentration as a function of conversion time for the test fatty acid substrates. Relative conversion results are presented for methyl ester substrates using sodium hydroxide for pH control, and for free fatty acid substrates using either sodium hydroxide or potassium hydroxide for pH control.

For the methyl ester substrates (Figure 4.1-1), the methyl myristate was converted at a higher rate and to a higher final product concentration than the methyl palmitate or the methyl stearate. This may be related to the physical state of the substrates. The melting points of the methyl palmitate and methyl stearate are above room temperature. These substrates were fed into the fermentor as a melt, but became a dispersed solid phase in the fermentor.

For the mixed fatty acid substrates using sodium hydroxide as the base (Figure 4.1-2), the rate of conversion was similar for all the substrates except the modified tallow fatty acids, which exhibited a lower conversion rate. The rates of conversion under these conditions were noticeably lower than that of the methyl myristate, and were more similar to that of the methyl palmitate and methyl stearate.

The use of potassium hydroxide for pH control with the mixed fatty acid substrates resulted in improved conversion rates (Figure 4.1-3). The rate of conversion obtained with mixed fatty acids enriched for oleic acid together with potassium hydroxide was similar to that obtained with

methyl myristate. The rate of conversion and final product concentration with linoleic acid was lower than that of the mixed acids enriched for oleic acid.

The mixed fatty acid substrates produced significantly more foaming during the bioconversion than the methyl ester substrates. This is not especially surprising since the bioconversions were carried out in the alkaline pH range. Under these conditions, the free fatty acids exist as the salt, or soap, form while the methyl esters are nonionic compounds. The mixed fatty acid substrates required the addition of significant amounts of antifoam to control foaming.

When the bioconversions were operated in such a way that there was little residual unreacted substrate left at the end of the conversion, it was found that the different components in the mixed substrates were converted in proportion to their relative concentrations in the substrate. For example, in the case of tallow fatty acid and stearic acid conversion experiments listed in Table 4.1-6, the compositions of the diacid products in the fermentor were, within experimental error, what would be predicted from complete conversion of the component mixtures in the substrates. The differences between the predicted and observed compositions of the diacid products were greatest for the minor components, where the relative analytical error is also the greatest.

In the conversion experiment for modified tallow fatty acids presented in Table 4.1-6, the residual monoacid concentration was high relative to the product diacid concentration. In this case, the product diacid was deficient in the saturated C18 diacid relative to the amount of this component expected from the composition of the substrate. This suggests that the C18 monoacid component was converted more slowly than other components. This could be due to physical effects; the C18 monoacid has a higher melting point and lower solubility than the other components, so it could have been less accessible to the biocatalyst. On the other hand, it is possible that the C18 monoacid reacted more slowly than other components due to substrate selectivity of the biocatalyst.

The conversion of commercial linoleic acid also resulted in a diacid component distribution that was significantly different from that expected from the substrate component distribution. In this case the C18:2 diacid should have been the principal component; instead it was only 31.5% of the total product diacid. It is possible that the C18:2 diacid was unstable under the bioconversion conditions, undergoing oxidation and polymerization reactions in the aerated conditions of the fermentor. If so, this is apparently an effect that is seen at high C18:2 concentrations in the substrate, but not when the C18:2 monoacid is a minor constituent of the substrate. For example, in the case of the mixed fatty acids enriched for oleic acid, in which the C18:2 acid is below 10% of the total substrate, the C18:2 component in the diacid product was at the level expected from the composition of the substrate (cf. Table 4.1-6).

The diacid products from several bioconversions were isolated and purified for synthesis of polymer products, using the methods described above (Table 4.1-7). The diacid products of the C14, C16, and C18 methyl esters provided a series of saturated diacids to study the effect of carbon number alone on the polymer properties. The diacid product of the commercial stearic acid provided a fully saturated mixed diacid to compare the polymer properties obtained from a mixed diacid with those of polymer prepared from single diacid components. The diacid product from the 90% C18:1 acid provided a material that was substantially a single unsaturated diacid component. This was useful for comparison of the polymer properties resulting from an

unsaturated diacid (the C18:1 diacid) with the corresponding saturated diacid (the C18 diacid). The diacid product from the other mixed acids enriched for oleic acid (cf. Table 4.1-5) is representative of the product obtained from an inexpensive oleic acid source. The diacid products from the two mixtures enriched for oleic acid are useful for determining the effects of the minor constituents of the mixed unsaturated diacid on the polymer properties.

The results of GC analysis of the isolated diacids are shown in Table 4.1-7. The materials were obtained as white to very slightly colored, finely divided crystalline materials. All of the isolated diacids contained greater than 96% total diacid content by GC analysis. The monoacid content of all these materials was 1.3% or less, which was deemed acceptable for the polymer application testing in this work.

4.1.4 References

1. Picataggio, S., K. Deanda and J. Mielenz. 1991. Determination of *Candida tropicalis* acyl coenzyme A oxidase isozyme function by sequential gene disruption. *Molecular and Cellular Biology* **11**(9): 4333-4339.
2. Picataggio, S., T. Rohrer, K. Deanda, D. Lanning, R. Reynolds, J. Mielenz, and L.D. Eirich. 1992. Metabolic engineering of *Candida tropicalis* for the production of long-chain dicarboxylic acids. *Bio/Technology* **10**: 894-898.

Figure 4.1-1. Profiles of conversion of methyl ester substrates to diacids. Diacid concentration in the fermentor broth as a function of conversion time is shown for methyl myristate (◇), methyl palmitate (□), and methyl stearate (△) substrates. Sodium hydroxide was used for pH control in all cases.

Figure 4.1-2. Profiles of conversion of mixed fatty acid substrates to diacids. Total diacid concentration in the fermentor broth as a function of conversion time is shown for tallow fatty acid (◇), modified tallow fatty acid (□), stearic acid (△), mixed fatty acids enriched for oleic acid (◆), and linoleic acid (■) substrates. Sodium hydroxide was used for pH control in all cases.

Figure 4.1-3. Profiles of conversion of commercial mixed fatty acid substrates to diacids, using potassium hydroxide for pH control. Total diacid concentration in the fermentor broth as a function of conversion time from the first introduction of substrate is shown for mixed fatty acids enriched for oleic acid (□), and linoleic acid (◇).

Table 4.1-1. Typical Fatty Acid Composition* of Common Fats and Oils (By GC Analysis)

	Carbon Number [†]	Lard	Beef Tallow (Bleachable Fancy)	Coconut	Corn	Cottonseed	Palm	Palm Kernel Oil	Soybean	Tall Oil
Caproic	C6							0.3		
Caprylic	C8			7.5				3.9		
Capric	C10			7.0				4.0		
Lauric	C12	0.5		48.0				49.6		
Myristic	C14	1.5	3.0	16.5		1.0	1.0	16.0		
Myristoleic	C14:1		0.5							
Palmitic	C16	26.0	26.0	8.0	11.5	25.0	47.0	8.0	10.5	
Palmitoleic	C16:1	4.0	2.5	1.0		1.0				
Margaric	C17	0.5	0.5							
Heptadecenoic	C17:1	0.5	0.5							
Stearic	C18	13.5	22.5	4.0	2.0	3.0	4.0	2.4	3.0	2.0
Oleic	C18:1	43.0	43.0	5.0	26.5	17.0	37.5	13.7	22.5	59.5
Linoleic	C18:2	9.0	1.5	2.5	59.0	53.0	10.0	2.0	54.5	37.0
Linolenic	C18:3	0.5			1.0				8.5	
Arachidic	C20							0.1		0.5
Arachidonic	C20:1	1.0					0.5		1.0	0.5

*Percentage by weight

[†]C14:1, for example, indicates a total of 14 carbon atoms and one unsaturated linkage.

Table 4.1-2. DCA4 Medium Composition

Medium Component	Concentration, g/l
Ammonium sulfate	3
Yeast Extract (Difco)	3
Yeast Nitrogen Base (Difco)	6.7
Glucose, reagent grade	60
Potassium phosphate (dibasic)	1
Potassium phosphate (monobasic)	1
Antifoam (Hodag M-10)	4

Table 4.1-3. OPT1 Medium Composition

Medium Component	Concentration, g/l
Ammonium sulfate	8
Potassium phosphate (dibasic)	1
Potassium phosphate (monobasic)	2
Corn steep liquor	9
Glucose, from corn syrup	40
MgSO ₄	0.5
NaCl	0.1
CaCl ₂	0.1
Trace elements solution	1 ml/l
Antifoam (Hodag M-10)	4

Table 4.1-4. Typical Composition of Methyl Ester Substrates Used in This Study

Methyl Ester	Methyl Myristate	Methyl Palmitate	Methyl Stearate
Melting Point, °C	17	27	36
<u>Saturated Esters</u>	Composition, wt%		
	3		
	95	2	
	2	95	4
		3	95
<u>Unsaturated Esters</u>			
C18:2			1

Table 4.1-5. Composition of Mixed Fatty Acid Substrates Used in This Study

Commercial Fatty Acid	Tallow Fatty Acid	Modified Tallow Fatty Acid	Stearic Acid	Mixed Acids Enriched for Oleic Acid	Linoleic Acid
Melting range, °C	36-44	44-53	54.5-55.5	< 5	< 5
<u>Saturated Acids</u>	Composition, wt%				
	1.7	3.1	1.9	2.6	0.1
	0.1	0.4	0.5	0.2	
	25.6	27.5	49.0	4.4	3.2
	0.5	1.1	2.1	1.6	
	14.9	27.1	46.5	0.9	1.2
<u>Unsaturated Acids</u>					
C14:1				0.7	
C16:1	2.9	2.4		5.0	0.2
C18:1	46.7	38.2		76.8	37.2
C18:2	7.5			7.8	58.0

Table 4.1-6. Composition of Diacid Products of Mixed Fatty Acids in Fermentor Samples

Fatty Acid Substrate	Tallow Fatty Acids		Modified Tallow Fatty Acids		Stearic Acid		Mixed Acids Enriched for Oleic Acid		Linoleic Acid	
	Diacid wt%		Diacid wt%		Diacid wt%		Diacid wt%		Diacid wt%	
Component	Predicted	Found	Predicted	Found	Predicted	Found	Predicted	Found	Predicted	Found
C14	1.7	3.0	3.2	6.2	2.0	4.3	2.7	4.2	0.1	0.0
C14:1							0.7	0.0		
C15	0.1	1.3	0.4	3.7			0.2	1.2		
C16	25.8	25.2	27.7	26.9	50.5	48.9	4.4	8.4	3.2	9.9
C16:1	2.9	3.1	2.4	2.5			5.0	5.3	0.2	1.0
C17	0.5	1.7	1.1	4.7			1.6	1.6		
C18	14.9	16.8	27.0	15.0	47.4	46.8	0.9	2.0	1.2	3.1
C18:1	46.6	42.6	38.1	39.2			76.7	70.0	37.2	54.6
C18:2	7.5	6.4	0.0	1.8			7.8	7.3	58.1	31.5

Table 4.1-7. Composition of Isolated Biosynthetic Diacids

Fatty acid substrate	Methyl Myristate	Methyl Palmitate	Methyl Stearate	Stearic Acid	Mixed Acids Enriched for Oleic Acid	Mixed Acids Enriched for Oleic Acid (90%)*
Components (wt%)						
Diacids						
C12	0.2					
C14	98.5	1.0	0.3	1.9	3.2	0.2
C15		1.3		0.9		
C16	1.1	86.5	1.0	45.7	4.9	2.3
C16:1					3.2	0.2
C17		2.8	0.5	2.5	0.9	0.3
C18		5.6	93.5	45.8	0.7	2.3
C18:1 (cis)		0.5	0.2	0.5	74.1	86.9
C18:1 (trans)		0.1			4.9	5.6
C18:2					5.1	1.1
C20		0.2	1.0	1.0	0.9	
Total diacid (%)	99.8	98.0	96.5	98.3	98.2	98.9
Total monoacid (%)		0.4	1.3		0.4	0.3
Other components (%)	0.2	0.4	1.4	0.2		
Total identified components	100.0	98.8	99.2	99.0	98.6	99.2

* Prepared from mixed fatty acids containing 90% C18:1 monoacid.

4.2 Bioprocess Optimization (Task 2.2)

(David P. Mobley and Gary K. Shank, GE CRD)

4.2.1 Introduction

As described in Chapter 2, the preliminary bioprocess economic analysis indicated that the conversion costs are strongly dependent on bioreactor productivity (the amount of product formed per time per bioreactor volume). Conversion costs decrease significantly as bioreactor productivity increases (see Figure 2.5). In this project, improvement of the bioreactor productivity was approached through a combination of biocatalyst development (Chapter 3) and bioprocess development.

In this section, we describe optimization of the batch fermentation conditions to yield higher bioreactor productivity with biocatalyst strains available at the outset of the study. An objective of this study was to increase the overall fermentor cycle productivity by at least 60% through optimization of the operating conditions, while maintaining high final product concentrations. Future work can combine optimized bioprocess conditions with improved strains of the biocatalyst to achieve still higher bioreactor productivity.

The batch bioprocess can be divided into two phases: growth and conversion. The growth phase is initiated by inoculating a nutrient medium with the yeast biocatalyst. The growth phase continues for a selected period of time, after which the fatty acid substrate is added to initiate the conversion phase. The fatty acid substrate, which induces the ω -oxidation activity of the biocatalyst, is added continuously during the conversion phase at a rate sufficient to be in excess of the conversion rate. Glucose is also added throughout the conversion phase to provide an energy source for the yeast.

The growth and conversion conditions for the bioprocess were optimized separately, since the best conditions for these two phase were not necessarily the same. First the growth conditions (temperature, pH, nutrient addition rates, etc.) were optimized to obtain the highest initial bioreactor productivity in the conversion phase, using methyl myristate as the test substrate. Methyl myristate was chosen because it is a liquid at room temperature (which simplifies delivery to the bioreactor) and it yields a single diacid product (1,14-tetradecanedioic acid). Design of experiment techniques were used to minimize the number of experimental runs [1]. Next, the conversion conditions were optimized, holding the growth conditions within the optimum ranges identified in the first step. The optimization of conversion conditions was again carried out with methyl myristate as the test substrate. After verifying the rate of conversion under the optimized conditions to high final diacid concentrations with methyl myristate, the optimized conditions were then tested with a lower cost substrate identified in the parallel task summarized in Section 4.1. Finally, the operating conditions were refined as necessary to accommodate the lower cost substrate.

4.2.2 Materials and Methods

Organism—*Candida tropicalis* strains ATCC 20987 and ATCC 20962 developed by Henkel Corporation were used. In strain ATCC 20962, the genes coding for enzymes in the first step of fatty acid β -oxidation have been disrupted so that the yeast can no longer use fatty acids as a

carbon source. In strain ATCC 20987, multiple copies of cytochrome P450 and reductase genes of the ω -hydroxylase system have been introduced into the β -oxidation disrupted strain [2,3].

Materials and Media—Chemicals, medium components, and medium compositions were as described in Section 4.1.

Culture Methods—Methods for growth and maintenance of stock cultures, precultures, and main cultures were as described in Section 4.1. Equipment and methods for carrying out the bioconversion in stirred fermentors were also as described in Section 4.1; the operating conditions for the growth and conversion stages are discussed below.

Analyses—Analytical methods for product and substrate concentrations by gas chromatography, and characterization of cultures by optical density, viable cell count, and glucose concentration were as described in Section 4.1.

4.2.3 Results

Optimization of Growth Conditions—The operating conditions for the growth phase of the fermentation cycle that were examined were: temperature, pH, batch or fed-batch mode of glucose addition during growth, presence or absence of substrate during growth, length of the growth phase, and the rate of glucose feed in the case of fed-batch growth. In the following analysis of the experimental results, these variables are designated by the letters A through F. Values of the continuous variables have been normalized to fall within the range of 0 to 1, the limits corresponding to minimum and maximum values of their ranges. The discrete variables (batch or fed-batch; absence or presence of substrate) were assigned the binary values of 0 or 1.

Methyl myristate was used as the test substrate, using *C. tropicalis* ATCC 20987. After growth of the culture at the selected conditions, the conversion phase was carried out at a standard set of conditions for a time period in which the accumulation of product was linear with time. The diacid product appears quickly after substrate addition. Bioreactor productivity was calculated by determining the slope of a plot of diacid concentration vs. conversion time for each fermentor experiment (see Figure 4.2-1). Two productivity calculations were carried out—one for the diacid product alone and one for the sum of the diacid product and ω -hydroxy acid intermediate. The hydroxy acid intermediate results from the initial step in the ω -oxidation pathway (see Introduction chapter). While the ultimate objective was to increase the rate of production of diacid product, the productivity calculation that includes the hydroxy acid accounts for the total rate of conversion through the first step of the pathway.

In the first phase of optimization of growth conditions, the effects of variables A through D on bioreactor productivity were tested at constant values of E and F. Table 4.2-1 lists the results of this initial series of experiments. The first ten experiments were designed as a screening tool and were determined by randomized selection of values of the independent variables. The next eight experiments were designed to provide more detailed information about variables C and D; these experiments used a full factorial design in C and D at two selected combinations of variables A and B.

Response surface plots were prepared to visualize the effects, if any, of variables A through D on productivity. Figure 4.2-2 shows a plot for this round of experiments of the response surface

defined by A and B. The normalized productivity and values of the variables C and D are listed next to each data point. Similarly, Figure 4.2-3 shows the response surface with respect to variables C and D, listing values of productivity and variables A and B next to each point.

The response surface plots revealed no simple effects of the variables tested. For example, A appeared to be relatively unimportant, since reasonably high productivity values were obtained throughout the entire range of A. Statistical analysis supported this conclusion. Applying a linear least squares model with A as the independent variable and productivity as the dependent variable, the coefficient for A was not significantly different from zero (using the t-test, 95% confidence limit). Similarly, none of the other variables had a statistically significant effect on productivity when taken alone in a linear model.

The great majority of diacid productivity values fell within a fairly narrow range, and so they did not suggest strong effects of the variables tested. The few experiments with productivities outside of this range, however, indicated the importance of certain combinations of growth conditions.

The two experiments that yielded the worst productivities shared the particular combination of high B, C equal to 1, and D equal to zero. In subsequent experiments, the particular combination of conditions in these experiments was avoided. Removing any one of the conditions appeared to be sufficient to improve productivity. Examination of the data suggested that having C at 1 carried no benefit, so subsequent experiments were carried out with C at zero. Examination of fermentor operating data also suggested a link between B and E.

Based on these observations, a set of experiments was carried out at lower E than previously, in which B and D were varied, while A was constant and C was zero (see Table 4.2-2). The best productivity in this set was also the highest productivity of diacid plus hydroxy acid to date and occurred at high B and D equal to 1. This result was used as the basis for another round of experiments exploring the effects of B, E and F. A, C, and D were held constant ($C = 0$; $D = 1$). These experiments focused on the area defined by high B and low E because this was a preferable set of conditions to operate the fermentor.

Table 4.2-3 summarizes the results of this last round of experiments. Figures 4.2-4 and 4.2-5 show response surface plots of productivity of diacid alone and diacid plus hydroxy acid, respectively, as a function of B and E. Focusing on the window of conditions which gave the best productivity results, several observations can be made. First, peak diacid productivity values were improved more than 50% over the best values before this study. Second, productivity data taking the hydroxy acid into account remain relatively constant within this window. Third, diacid productivity alone (*i.e.*, without hydroxy acid) tended to increase with increasing F. This trend is more clearly represented in Figure 4.2-6, which shows productivity results in terms of B and F. A linear least squares model confirmed the statistical significance (95% confidence limit) of the relationship between F and productivity. Since F had no effect on productivity of diacid plus hydroxy acid, this means that the effect of this variable was on the selectivity toward diacid.

Optimization of Conversion Conditions—The operating conditions examined in the conversion phase of the process were pH, temperature, and glucose feed rate. For data analysis, these variables were assigned the variable names G through I. Values of these variables were normalized to fall within the range of 0 to 1, the limits corresponding to minimum and maximum values of their ranges. While examining the conversion phase conditions, the growth phase conditions were held constant at values within the optimum ranges identified above. Methyl

myristate was used as the fatty acid substrate and the biocatalyst strain was *C. tropicalis* ATCC 20987. Two types of high dextrose corn syrup were used as the source of glucose in this set of experiments. The glucose source was designated by the variable J, assigned the binary values of 0 or 1.

As in the experiments for optimization of growth conditions, the conversion phase was carried out for a time period in which the accumulation of product was linear with time. Productivity values for diacid alone and for diacid plus hydroxy acid were calculated from the slopes of the curves for product concentration as a function of time.

Table 4.2-4 summarizes the values of each of the independent variables and the resulting productivity values for each of the conversion phase optimization experiments. These data were analyzed by multiple linear regression to determine which of the independent variables had a significant effect on productivity and to determine the direction and magnitude of the effects that were significant. The data in Table 4.2-4 were fit to linear models of the form:

$$y = m_1x_1 + m_2x_2 + \cdots + b$$

using the least squares method.

Testing a model for diacid productivity including all the conversion phase variables (G through J), the variables that were significant by the t-test (single-tailed, 95% confidence limit) were H, I, and J. Variable G, within the range tested, was not significant with respect to the diacid productivity. The diacid productivity increased with decreasing H and with increasing I. When the dependent variable was taken to be the productivity of diacid plus hydroxy acid, the significant variables were G, I, and J. The productivity of diacid plus hydroxy acid increased with decreasing G and with increasing I. There was a significant difference between the two glucose sources (variable J) by both measures of productivity.

Removing the insignificant variables yielded models for productivity of diacid alone and of diacid plus hydroxy acid. The models, along with summary statistics, are presented in Tables 4.2-5 and 4.2-6, respectively. All the coefficients in these models are indicated to be significant by the fact that the value of the coefficient divided by the standard error (the t ratio) for these variables is greater than the t-critical value. The F statistic for these models is greater than the F-critical value (single-tailed, 95% confidence limit), indicating that we can reject the hypothesis that there is no relationship between diacid productivity and the independent variables. Regression models containing cross-product terms of the primary independent variables were also tested; none of the cross-product terms was statistically significant.

The productivity values predicted by the models at the conditions of each experiment were compared with the values that were measured in Figures 4.2-7 and 4.2-8. The coefficient of determination of these models is not high, indicating that there is variability in these experiments that is not being accounted for by the models or that the true behavior of the system is non-linear. This can be seen by the scatter in the figures. The figures also indicate, however, that these linear models can at least give reasonable predictions of expected productivity values. The magnitude of the coefficients in the model indicates the relative impact of changes in the dependent variables. For example, in the model for diacid productivity in Table 4.2-5, changing I from the minimum to the maximum value will have a larger effect than a similar change in H.

Three of the experiments in the data set were run with 200 – 300 ppm BHT (butylated hydroxy toluene or 2,6-di-*t*-butyl-4-methyl phenol) in the methyl myristate. This was done because BHT is added as a preservative to some commercial fatty acids. After accounting for the effect of the other variables, no effect of the BHT was observed above experimental error.

Conversion Under Optimized Conditions to High Product Concentration—To demonstrate the positive effect on the overall bioprocess of optimizing the growth and conversion conditions, some experiments with methyl myristate as substrate were continued for sufficiently long conversion times to reach high diacid product concentrations. The most promising conversion conditions were combined with the optimized growth conditions. The results with the bioprocess after optimization are compared with typical results before the optimization study in Figure 4.2-9.

To improve product solubility as the product concentration increased, the conversion pH was raised as the conversion phase progressed. The glucose feed rate was reduced during conversion to prevent glucose accumulation in the fermentation broth.

The example with optimized conditions yielded an overall diacid productivity 60% greater than the overall productivity before optimization. The peak diacid productivity after optimization was 80% greater than the peak diacid productivity observed before optimization and 80% greater than the overall productivity after optimization. The high peak productivity after optimization is encouraging because it indicates that the intrinsic conversion kinetics are not limited to the lower overall productivity level due to other effects such as transport phenomena. This suggests that it should be possible to increase the overall productivity to even higher values, through a combination of biocatalyst improvements and further process optimization.

Conversion Under Optimized Conditions with Lower Cost Substrate—The screening of a number of lower cost mixed fatty acid substrates is described in Section 4.1. Having optimized bioprocess conditions with methyl myristate as the model substrate, the optimized conditions were applied to a lower cost mixed fatty acid feed. As shown in Figure 4.2-10, the diacid productivity with this substrate was at least as good as that obtained with methyl myristate. The final diacid concentration was higher with the mixed fatty acid feed than with the methyl myristate.

An operating strategy that results in reductions of raw materials usages had been developed independent of this project. This strategy was tested in combination with the optimized bioprocess conditions for two biocatalyst strains, using a mixed fatty acid substrate. As shown in Figure 4.2-11, the overall diacid productivity compared favorably with the best results obtained previously.

Conversion with Strains Containing Multiple Gene Copies—Two strains containing multiple copies of a selected *CYP* gene integrated into the genome of *C. tropicalis* strain ATCC 20962 were tested in the bioreactor using the optimized bioprocess conditions and a mixed fatty acid substrate. As shown in Figure 4.2-12, the diacid productivity in these tests was promising. However, more work is needed to determine whether amplification affected *CYP* mRNA levels or *CYP* protein levels.

4.2.4 References

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2. Picataggio, S., K. Deanda and J. Mielenz. 1991. Determination of *Candida tropicalis* acyl coenzyme A oxidase isozyme function by sequential gene disruption. Molecular and Cellular Biology **11**(9): 4333-4339.
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Figure 4.2-1. Typical experiment showing accumulation of diacid and hydroxy acid products as a function of conversion time. Productivity of diacid equals the slope of the diacid concentration curve. Productivity of diacid plus hydroxy acid equals the sum of the slopes of the diacid and hydroxy acid curves.

Figure 4.2-2. Response surface plot showing productivity of diacid as a function of growth conditions A and B for the initial series of experiments (see Table 4.2-1). Values of productivity of diacid are listed next to each data point, followed by values of growth conditions C and D in parentheses.

Figure 4.2-3. Response surface plot showing productivity of diacid as a function of growth conditions C and D for the initial series of experiments (see Table 4.2-1). Values of productivity of diacid are listed next to each data point, followed by values of growth conditions A and B in parentheses.

Figure 4.2-4. Response surface plot showing productivity of diacid as a function of growth conditions B and E for the series of experiments summarized in Table 4.2-3. Values of productivity of diacid are listed next to each data point, followed by the value of growth condition F in parentheses.

Figure 4.2-5. Response surface plot showing productivity of diacid plus hydroxy acid as a function of growth conditions B and E for the series of experiments summarized in Table 4.2-3. Values of productivity of diacid plus hydroxy acid are listed next to each data point, followed by the value of growth condition F in parentheses.

Figure 4.2-6. Response surface plot showing productivity of diacid as a function of growth conditions B and F for the series of experiments summarized in Table 4.2-3. Values of productivity of diacid are listed next to each data point, followed by the value of growth condition E in parentheses.

Figure 4.2-7. Comparison of productivity of diacid predicted by the model in Table 4.2-5 with experimentally measured values.

Figure 4.2-8. Comparison of productivity of diacid plus hydroxy acid predicted by the model in Table 4.2-6 with experimentally measured values.

Figure 4.2-9. Comparison of the performance of the bioprocess before and after optimization of growth and conversion phase operating conditions using a test substrate.

Figure 4.2-10. Production of diacid from a mixed fatty acid substrate using optimized bioprocess conditions. Total diacid concentration in the fermentor broth as a function of time is shown from the first introduction of substrate for three experiments under similar conditions.

Figure 4.2-11. Production of diacid from a mixed fatty acid substrate using optimized conditions with *C. tropicalis* ATCC 20987 (□) and *C. tropicalis* ATCC 20962 (◇). Total diacid concentration in the fermentor broth as a function of time is shown from the first introduction of substrate.

Figure 4.2-12. Production of diacid from a mixed fatty acid substrate using optimized conditions and strains containing multiple copies of a selected *CYP* gene. (□, *C. tropicalis* Strain 1; ◇, *C. tropicalis* Strain 2). Total diacid concentration in the fermentor broth as a function of time is shown from the first introduction of substrate.

Table 4.2-1. Initial Set of Experiments for Optimization of Growth Phase Conditions

Experiment	Normalized Independent Variables ¹				Normalized Productivity of Diacid	Normalized Productivity of Diacid Plus Hydroxy Acid
	A	B	C	D		
1	0.58	0.42	0	1	0.47	0.47
2	0.96 ²	0.00	0	0	0.51	0.64
3	0.00	0.67	1	1	0.34	0.34
4	0.63	1.00	1	0	0.03	0.03
5	0.88	0.25	0	1	0.35	0.42
6	0.17	0.42	1	1	0.44	0.54
7	0.58	0.17	0	0	0.43	0.63
8	0.42	0.50	1	0	0.39	0.44
9	0.21	0.08	0	0	0.62	0.77
10	0.21	0.08	1	0	0.48	0.74
11	0.83	0.67	0	0	0.48	0.55
12	0.83	0.67	1	0	0.19	0.19
13	1.00	0.42	0	0	0.52	0.55
14	1.00	0.42	1	0	0.35	0.46
15	0.83	0.67	1	1	0.43	0.48
16	0.83	0.67	0	1	0.44	0.51
17	1.00	0.42	0	1	0.48	0.61
18	1.00	0.42	1	1	0.41	0.53

¹ Other growth conditions constant

² Setpoint; actual value drifted lower due to equipment malfunction

Table 4.2-2. Experiments Investigating the Interaction of Growth Conditions B, D and E

Experiment	Normalized Independent Variables*			Normalized Productivity of Diacid	Normalized Productivity of Diacid Plus Hydroxy Acid
	B	D	E		
19	0.25	0	0.43	0.34	0.71
20	0.25	1	0.43	0.35	0.54
21	0.67	0	0.43	0.51	0.59
22	0.67	1	0.43	0.59	0.80

* Other growth conditions constant

Table 4.2-3. Experiments for Optimization with Respect to Growth Conditions B, E, and F

Experiment	Normalized Independent Variables ¹			Normalized Productivity of Diacid	Normalized Productivity of Diacid Plus Hydroxy Acid
	B	E	F		
23	0.50	0.14	0.00	0.52	0.79
24	0.50 ²	0.14	0.43	0.84	0.86
25	0.83	0.14	0.29	0.55	0.70
26	0.83	0.14	1.00	0.80	0.88
27	0.67	0.14	0.04	0.55	0.88
28	0.67	0.14	0.43	0.59	0.89
29	1.00	0.00	0.00	0.47	0.72
30	1.00	0.00	0.61	0.65	0.93
31	0.50	0.19	0.36	0.48	0.89
32	0.83	0.20	0.36	0.80	0.80
33	1.00	0.14	0.43	0.59	0.87
34	0.50	0.43	0.25	0.25	0.43
35	0.83	0.27	0.32	0.53	0.78
36	1.00	0.29	0.29	0.62	0.75
37	0.83	0.43	0.25	0.38	0.56

¹ Other growth conditions constant

² Setpoint; actual value deviated upward due to equipment malfunction.

Table 4.2-4. Experiments for Optimization of Conversion Phase Conditions

Experiment	Normalized Independent Variables				Normalized Productivity of Diacid	Normalized Productivity of Diacid plus Hydroxy Acid
	G	H	I	J		
38	1.00	0.31	0.46	1	0.55	0.55
39	0.00	0.77	0.17	1	0.53	0.83
40	1.00	1.00	0.25	1	0.57	0.58
41	0.75	0.62	0.29	1	0.51	0.56
42	1.00	0.92	0.35	1	0.37	0.40
43	0.25	0.85	0.28	1	0.57	0.89
44	1.00	0.62	0.07	1	0.36	0.37
45	0.38	1.00	0.24	1	0.41	0.75
46	0.00	0.31	0.52	1	0.59	0.88
47	0.75	0.77	0.16	1	0.39	0.41
48	0.25	0.54	0.79	1	0.60	0.78
49	0.75	0.00	0.70	1	0.81	0.95
50	0.50	0.15	0.69	1	0.70	0.74
51	0.38	1.00	0.29	1	0.44	0.64
52	0.50	0.54	0.89	1	0.81	0.85
53	0.75	0.38	0.55	1	0.58	0.72
54	1.00	0.15	0.39	0	0.37	0.38
55	0.75	0.31	0.34	0	0.67	0.69
56	0.50	0.77	0.66	0	0.52	0.53
57	0.25	0.85	0.17	0	0.43	0.45
58	0.50	0.38	0.07	0	0.18	0.19
59	1.00	0.62	1.00	0	0.45	0.45
60	0.38	1.00	0.21	0	0.38	0.38
61	0.38	1.00	0.24	0	0.44	0.44
62	0.25	0.77	0.36	0	0.30	0.38
63	1.00	0.77	0.98	0	0.58	0.60
64	0.25	0.85	0.45	0	0.46	0.68
65	0.38	0.92	0.34	1	0.47	0.61
66	0.38	1.00	0.32	1	0.50	0.57
67	0.38	0.92	0.35	1	0.28	0.56
68	0.38	0.92	0.31	1	0.36	0.67
69	0.38	0.92	0.27	1	0.49	0.79
70	0.38	0.23	0.22	1	0.63	0.83
71	0.38	0.08	0.00	1	0.34	0.52
72	0.38	0.92	0.04	1	0.33	0.50
73	0.38	1.00	0.35	1	0.60	0.81
74	0.38	0.23	0.45	1	0.67	0.79
75	0.38	0.23	0.58	1	0.75	0.87

Table 4.2-5. Model for productivity of diacid as a function of significant conversion conditions

Model: Y = productivity of diacid = (m1)H + (m2)I + (m3)J + b				
	Coefficient	Std. error for Coeff.	t ratio	t critical
H	-0.1153	0.0552	-2.0895	1.69
I	0.3331	0.0712	4.6769	1.69
J	0.1112	0.0374	2.9703	1.69
Intercept	0.3629	0.0632	5.7409	1.69
Coefficient of determination: 0.5408				
Standard error for y estimate: 0.1032				
Degrees of freedom: 34				
F-observed statistic: 13.3493				
F-critical value: 2.89				
Regression sum of squares: 0.4262				
Residual sum of squares: 0.3618				

Table 4.2-6. Model for productivity of diacid plus hydroxy acid as a function of significant conversion conditions

Model: Y = productivity of diacid plus hydroxy acid = (m1)G + (m2)I + (m3)J + b				
	Coefficient	Std. error for Coeff.	t ratio	t critical
G	-0.2844	0.0682	-4.1709	1.69
I	0.3954	0.0793	4.9863	1.69
J	0.2202	0.0421	5.2286	1.69
Intercept	0.4546	0.0579	7.8585	1.69
Coefficient of determination: 0.6402				
Standard error for y estimate: 0.1162				
Degrees of freedom: 34				
F-observed statistic: 20.1622				
F-critical value: 2.89				
Regression sum of squares: 0.8162				
Residual sum of squares: 0.4588				

4.3 Product Recovery and Purification (Task 2.3)

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4.3.1 Summary

The bioconversion of alkanes and other long chain molecules to dicarboxylic acids can be achieved through fermentation processes. The literature gives examples of diacid production, and describes parameters which affect diacid generation, but report little on the recovery and purification of diacids from a fermentation broth which is chemically complex and contains proteins and other constituents as well as the diacid product. This report shows that diacids can be readily precipitated by a variety of agents, although the two acid groups on the molecule can result in both salting-in and salting-out behavior, which appears to be consistent with hydrophobic interaction theory. This work shows that diacids follow salting-in and salting-out behavior according to the concepts proposed by Cohn and embodied by the Cohn equation where $K_s = 5.2 \text{ L/mole}$ and $\beta = 0.14$ for HCl as the precipitating agent. A four step recovery procedure consisting of centrifugation to remove cells, ultrafiltration to remove protein, and precipitation to remove the DCA solid from the filtrate is described. A final adsorption step using a polymeric adsorbent is proposed, if the small amounts of diacids remaining are to be recovered by adsorption from the filtrate.

4.3.2 Background

Dicarboxylic acids are fatty acids used as chemical intermediates in the synthesis or formulation of polyurethanes, plasticizers, lubricants, polyamides, and perfumes, with long chain diacids being particularly useful for this type of application (1). The production of a C_{13} dicarboxylic acid (brassylic acid from an $n-C_{13}$ alkane using *Candida tropicalis* was recently demonstrated on a 20,000 L fermentation system. This system reportedly gave 150 tons of brassylic acid in a year, which is approximately the world's supply required to synthesize ethylene brassylate (musk oil). The microorganism used was derived from a wild type microorganism that was isolated from screening of 1,600 strains. Its ability to produce dodecane dioic acid (abbreviated DC-12) from the n -alkane was increased from 1.6 g/L to 95 g/L through a series of mutation and selection procedures. Screening of the mutated organism, known as strain M2030, showed that C_{11} through C_{18} dicarboxylic acids could be produced from their respective C_{13} to C_{18} n -alkanes, as well as n -alkenes.

The fermentation of $n-C_{13}$ to DC-13 occurred over a 110 hour aerated fermentation with a final concentration of 130 g/L being attained – starting from a 100 g/L concentration of the n -alkane. The authors [1] note that the fermentation almost completely consumes the n -alkane, and claim that no by-product remains at the end of it. This results in a separation and recovery section that is “extremely simplified,” although specifics of the recovery process are not given.

This section reports the recovery of a C_{18} diacid from an industrial fermentation broth. The processing of the broth required that the dicarboxylic acid be separated from cells, proteins, and other components present in the broth, and that the acid be recovered in a concentrated form at

high yield. The properties of the fermentation broth were characterized, and then a high-throughput screening technique was used to select the best composition of precipitate agents to maximize DCA recovery.

The precipitation characteristics of the DCA were correlated with Cohn's equation, which has the form [2]:

$$\ln \frac{W}{W_0} = \beta + K_s C_s \quad (1)$$

where

$\frac{W}{W_0}$	=	weight fraction of DCA in solution
W	=	weight of DCA in solution
W_0	=	initial weight of DCA in solution
K_s	=	salting-out constant
β	=	hypothetical solubility of DCA as ionic strength approaches zero
C_s	=	molar concentration of salt

This correlation was used to fit the data since the long chain dicarboxylic acids can be represented as two charged spheres of radius a , with a large distance, d , between the spheres such that $a/d \ll 1$. Then the electrostatic contribution to the activity coefficient is consistent with the Scatchard-Kirkwood equation, and the use of parameter K_s is justified since it can be viewed as accounting for both salting-in (electrostatic) and salting-out (hydrophobic) effects, the sum of which determines the solubility of the diacid at different salt concentrations [2, 3].

The solubility of DCA's decreases as the dielectric constant of the solvent increases [4]. Hence, isopropanol and other alcohols seemed to be logical as possible precipitating agents. The literature also indicated that the solubility of the DCA decreased in the presence of a cation such as Ca^{++} [4]. This observation helped to motivate studies in which the pH was adjusted with HCl, and $CaCl_2$ was added as a precipitating agent.

4.3.3 Materials and Methods

Chemicals

Calcium chloride dihydrate, ferric chloride, glycine, concentrated hydrochloric acid (HCl), hexane, 1-propanol, and tetrahydrofuran (THF) were obtained from Fisher Scientific Company (Fair Lawn, NJ). Potassium hydroxide, methanol, and HPLC grade isopropanol were obtained from Mallinckrodt Chemical, Inc. (Paris, KY). Pure ethanol (200 proof) was obtained from Midwest Grain Products of Illinois (Perkin, IL). Trizma base (tris(hydroxymethyl) aminomethane) and trizma hydrochloride were obtained from Sigma (St. Louis, MO). Diacid standards and fermentation broth were obtained from GE. Composition of the fermentation broth by GE analysis is shown in Table 4.3-1.

Liquid Phase Screening

Using 1.5 ml graduated micro-centrifuge tubes from DOT Scientific, Inc. (Burton, MI), a 200 microliter sample of the GE fermentation broth was dissolved in different solvents. The solvents used in this study were hexane, 1-propanol (normal propanol), isopropanol, ethanol, methanol, and tetrahydrofuran. The buffers used were 4.7 millimolar trizma base - 37 millimolar glycine (pH = 8.0), calcium chloride - hydrochloride (pH = 2.0), and 100 millimolar tris buffer (pH = 7.2).

After combining the solvent with the fermentation sample, each microfuge tube was mixed for 20 seconds on the Vortex-Genie™ mixer from TM Scientific Industries, Inc. (McGaw Park, IL). Following mixing, the samples were microfuged for 7 minutes at the maximum speed using a Marathon® Micro A microcentrifuge from Fisher Scientific Company (Pittsburgh, PA). The samples were then removed from the microfuge and the volume of precipitate in each tube was recorded.

Six standards were made by filling the microfuge tubes with a known volume of water. To determine the amount of precipitate which had formed, the level of precipitate in the microfuged samples was compared to the height of water in the standards. For precipitate levels less than 100 microliters, the uncertainty in the amount of precipitate is approximately ± 10 microliters. For precipitate levels greater than 100 microliters, the uncertainty in the amount of precipitate is on the order of 50 microliters.

Stationary Phase Screening

A stock solution of C18 diacid (cis 9 octadecene-1,18 dioic acid) was made by dissolving purified C18 diacid (cis 9, octadecene 1,18 dioic acid) standard in water. Using 1.0 M KOH and 6 M HCl solutions, the pH of the C18 diacid solution was adjusted to 2, 6, and 10 to make three diacid stock solutions. Below pH 8, the diacid was only partially soluble. The pH of the stock solutions was determined using pHydron pH paper from Micro-essential Laboratory (Brooklyn, NY). To keep the diacid in solution at low pH values, isopropanol was added to achieve a 50% solution of isopropanol. Upon addition of isopropanol, the solution became clear and colorless.

Thirty different adsorbents were tested for their ability to retain the C18 diacid at different pH values. Approximately 50 mg of each adsorbent was added to the top of a 0.45 μ m PVDF filtered microfuge tube from Whatman (Clifton, NJ). To each microfuge tube, 0.5 ml of diacid solution was added at pH 2, 6, or 10. After agitating the microfuge tube and letting it sit for 30 minutes, the samples were microfuged for 10 minutes using a Marathon Micro A microcentrifuge. The liquid was collected in the bottom of the tube and the solid stationary phase was retained in the top of the microfuge tube. Samples of the liquid were mixed with a 1% solution of FeCl₃ to precipitate any diacid remaining in the liquid phase. The samples were again microfuged and the amount of precipitate relative to a standard was determined. A schematic representation of this process is shown in Figure 4.3-1.

Adsorption Column Testing

Based on the results of the high throughput screen, several adsorbents were identified as viable candidates for adsorption of the diacid from solution. Amberlite XAD-2 (Rohm and Haas Co., Philadelphia, PA) was selected for a more involved adsorption study. A 0.771 cm ID x 45 cm long jacketed column was packed with 80-100 mesh XAD-2 adsorption media which had been swelled in isopropanol.

Both isocratic and gradient elution profiles were achieved using two Waters 510 HPLC pumps made by Millipore (Milford, MA). Samples were injected into the column with a Rheodyne Model 7125 injector (Cotati, CA). Two detectors were attached to the column, a Waters Model 996 photodiode array detector and a Waters Model 401 differential refractometer for detection of components exiting the column. Elution profiles were recorded on a computer using the Millennium 2010 Chromatography Manager, also made by Waters.

Cell and Protein Removal

Attempts to remove solid material from the fermentation broth by filtration proved fruitless. Regardless of the filtration media selected, the filter would plug after a few minutes of operation. Since the fermentation solids settled to the bottom of the sample tube, centrifugation of the broth appeared to be the only viable option for removal of the solid material.

Ultracentrifugation

Fermentation broth was ultracentrifuged at 6,000 rpm using a Beckman J21C Centrifuge with a J14 rotor for 20 minutes. The cells and cell debris formed a solid cake at the bottom of the ultracentrifuge tube with a relatively clear liquid above the solids.

Ultrafiltration

After ultracentrifuging, the liquid was decanted from the ultracentrifuge tube and poured into a model 8400 Amicon pressurized stirred cell ultrafiltration unit from Millipore. Using a 76 mm Amicon Diaflo YM10 ultrafiltration membrane, protein with molecular weights above 10,000 were removed from the liquid. Pressure in the ultrafiltration unit was maintained at 55 psi using compressed nitrogen gas from BOC Group, Inc. (Murray Hill, NJ).

Precipitation

To 5 mL of filtrate, varying amounts of concentrated HCl (from 10 to 500 microliters) were added to several 10 mL disposable borosilicate glass culture tubes from VWR Scientific Products (McGaw Park, IL). Each HCl concentration was replicated once. The test tube was mixed for 10-15 seconds on a Vortex Genie mixer.

After mixing, the test tube was left at room temperature for at least 2 hours. Following this waiting period, precipitate was separated from the liquid using an Adams Analytical Centrifuge made by Clay Adams Company (Parsippany, NJ). After pooling the liquid from the two

replicates, the pH of the solution was measured using a Cole-Parmer refillable calomel reference electrode (Cole-Parmer Instrument Company, Vernon Hills, IL) attached to a Corning pH/Ion Meter 150 from Corning Science Products Division (Corning, NY).

Lyophilization of samples

Prior to drying, the remaining wet solids were weighed using a Mettler AE160 balance from Mettler-Toledo, Inc. (Hightstown, NJ). The solids were dried using a Labconco Freeze Drier 3 from Labconco Corp. (Kansas City, MO). Since the samples needed to be frozen prior to putting them in the freeze drier, the 10 mL test tubes were immersed in either a dry ice/acetone or dry ice/isopropanol bath. Both mixtures provide a slushy solution with a temperature around -78 °C. After freezing the sample, the test tubes were placed inside the Labconco freeze drier sampling tubes, which ranged in size from 80 to 1200 mL. Prior to adding the samples to the freeze drier, the temperature of freeze drier was around -80 °C. After freeze drying for at least 20 hours, the test tubes were removed from the freeze drier and reweighed on the Mettler balance.

Gas Chromatography

A CP-Sil 5CB fused silica WCOT capillary gas chromatography column with a 25m x 0.25 mm ID x 0.12 micron film thickness from Chrompack (Raritan, New Jersey) was used in a Varian 3400 gas chromatography system (Sugar Land, TX). Components were detected using a flame ionization detector (FID) with a sensitivity of 10^{-11} . See Table 2 for the temperature gradient and other operating conditions.

Prior to injecting samples into the capillary column, they were derivatized with either Sil-Prep, BSA (N,O -bis(trimethylsilyl) acetamide), or BSTFA (N,O -bis(trimethylsilyl) trifluoro acetamide) silylation reagents from Alltech Associates, Inc. (Deerfield, IL). Split ratios and flow rates were measured using a Digital Flow Check from Alltech. Samples were injected using a 75N Hamilton (Reno, Nevada) syringe, which has a total capacity of 5 microliters. Chromatograms were integrated using an HP3390A reporting integrator from Hewlett- Packer (Avondale, PA).

Melting Points

The melting points of precipitates were determined using a Thomas Hoover Capillary Melting Point Apparatus (Arthur H. Thomas Company, Philadelphia, PA.). A few milligrams of dry precipitate were placed in a capillary tube and inserted into the heated oil of the melting point apparatus. The solids were observed through a magnified site glass in the front of the heating unit. Once the solids started to melt, the temperature was read from a mercury thermometer which was immersed beside the capillary tubes in the heating oil.

4.3.4 Results and Discussion

Liquid Phase Screening

Over 70 different combinations of mobile phases were tested to determine solubility characteristics of the diacids. Most of these combinations were mixtures of alcohols with a buffer, which was either calcium chloride-hydrochloric acid buffer (pH 2), trizma base-glycine buffer (pH 7.2), or trizma buffer (pH 8). The amount of precipitate formed was determined as described earlier in the Materials and Methods section. It is important to note that the amount of precipitate formed is inversely related to the solubility of the broth components.

Figure 4.3-2 shows the effect of the pH on the amount of precipitate formed at a constant tetrahydrofuran concentration. At the lower pH, relatively large amounts of precipitate are formed compared to the higher pH. As the alcohol concentration increases, the solubility of the fermentation broth also increases at the lower pH. However, there does appear to be a slight decrease in solubility with increasing alcohol concentrations at the higher pH.

This phenomenon is most likely due to the multicomponent nature of the broth. Since the fermentation broth was used straight from the fermentor, it contained cells, proteins, unreacted substrate, and other components as well as the diacid products. The slight decrease in solubilities at the higher pH is believed to be caused by precipitation of proteins or other components instead of the diacids.

The fact that the two curves converge at higher alcohol concentrations is consistent with expectations. As the alcohol concentration increases, the molar concentration of the buffer in solution decreases. Thus the buffer should have less effect at the higher alcohol concentrations and the curves at the different pH values should converge.

The same pH trend observed in Figure 4.3-2 can also be seen by comparing Figures 4.3-3 and 4.3-4. The solubility of the fermentation broth components increases with increasing alcohol concentration at the lower pH and decreases with increasing alcohol concentration at the higher pH. Thus for the given alcohols, the same trend holds true.

Stationary Phase Screening

Results of the stationary phase screening are shown in Table 4.3-3. The adsorbents are organized by the type of stationary phase (i.e., polymeric, cation exchange, anion exchange, mixed bed, and "other"). The polymeric adsorbents and most of the "other" supports were tested with all three pH diacid solutions. Since the cation exchange resins were not stable at pH 10, they were only tested at pH 2 and pH 6. Likewise, the anion exchange resins were only tested at pH 6 and pH 10, due to their instability at pH 2. The mixed-bed resins were tested at all pH values, but the results at pH 10 were suspect due to precipitation on the resin. Mixed bed is a mixture of both cation and anion exchange resins supplied from the manufacturer. Since the beads were not washed prior to use, they may contain acids or other precipitation agents which might affect the results. Consequently, questionable results are excluded from the data shown in Table 4.3-3.

Of the 30 adsorbents tested, three of the polymeric adsorbents, as well as powdered charcoal (Darco G-60), were found to adsorb significant amounts of diacids. These adsorbents were XAD-2, XAD-4, and CG-162. It appears that these stationary phases will adsorb the diacid at

low pH and release the diacid at high pH. As a result of the stationary phase screening, a column was packed with XAD-2 for further testing.

Adsorption Column Testing

As expected from the stationary phase screen, the XAD-2 column adsorbed the diacids at low pH and released them at high pH. However, fatty monoacids were also adsorbed by the column at the low pH. The column does not appear to have a selectivity for the diacids over the monoacids. As a result, both the monoacids and diacids are retained at low pH and elute at the same point when the pH is raised. In an effort to selectively retain the diacids, several different mobile phase gradients were attempted. In each case, the monoacids and diacids eluted at the same time.

However, when a sample of fermentation broth was injected on the XAD-2 column, there was an extra unretained peak which eluted at a different time than the fatty acids. Using the multiple wavelength capabilities of the photodiode array detector, it was determined that the unretained peak adsorbed strongly at 254 nm and 280 nm. Since proteins adsorb strongly at these wavelengths, it appears that the fermentation broth may also contain some proteins.

To further ascertain the characteristics of the fermentation broth, titration studies were conducted on the broth.

Precipitation

Prior to adjusting the pH of the fermentation broth, the cells were removed by a high-speed ultracentrifuge. After ultracentrifugation, the titration data for the broth was obtained by adding HCl to several aliquots and determining the mass of dry precipitate which had formed. The Cohen plot for broth which has not been ultrafiltered is shown in Figure 4.3-5. Because of the amount of scatter in the data and results from the adsorption study, it was determined that the protein in the broth needed to be removed prior to precipitation.

From a series of other experiments, a 10,000 molecular weight ultrafiltration step was selected to remove any high molecular weight protein in the fermentation broth. The titration curve of the protein free broth is shown in Figure 4.3-6. The corresponding Cohen plot for ultrafiltered broth is shown in Figure 4.3-7. From linear regression on the low acid concentration data, the values of the constants in the Cohen equation can be determined. For the ultrafiltered broth, the value of K_s is 5.22 L/mole, and the value of β is 0.14.

An explanation for the increasing solubility at an HCl concentration of 0.45 molar still needs to be developed. It may be possible to anticipate such an effect from the analysis of salt effects on hydrophobic interactions in precipitation (of proteins) by Melander and Horvath [2], who showed that:

$$K_s = \Omega\sigma - \Lambda \quad (2)$$

where $\Omega\sigma$ denotes an intrinsic salting-out coefficient and $-\Lambda$ is a salting-in coefficient that accounts for hydrophobic and electrostatic interactions. This explanation would suggest that protonation of the carboxylic acid groups initially causes hydrophobic interactions between the dicarboxylic acid molecules, thus resulting in their precipitation until a maximum is reached at

pH 5, followed by a small extent of redissolution due to the chaotropic character of the Cl^- ion at a somewhat higher concentration. This explanation is somewhat speculative at this time, and needs further development.

The overall process concept of separation of DCA's from fermentation broth is shown in Figure 4.3-8. The process is relatively simple: Centrifugation removes cells; ultrafiltration removes protein; addition of a small amount of HCl causes the product to precipitate, with a subsequent filtration step resulting in solids recovery. Since a small amount of the diacids may remain in the filtrate, a final adsorption step may be necessary.

Gas Chromatography

Preliminary results from the gas chromatography studies indicate that the precipitate formed from addition of HCl is primarily C18 diacid. The precipitate contains only small amounts of oleic acid and non-detectable amounts of most of the other diacids. However, there are several peaks in the chromatograms which have not yet been identified. The exact composition of the precipitate is still to be determined.

4.3.5 Conclusions

The fermentation broth containing approximately 112 g/L diacids, cells, and residual broth constituent is readily processed in a four-step procedure to give purified dicarboxylic acids. The precipitation protocol was selected after screening 70 different combinations of precipitating conditions, and then studying the salting-out/salting-in characteristics of organic alcohols, THF, and HCl. HCl was chosen as the preferred precipitating agent since the handling of HCl after the precipitation step did not require recovery by distillation as could be the case for THF or alcohols. Adsorption may be necessary as a final step to recover small amounts of diacids from the filtrate resulting from the filtration step, depending on the value (and cost) of the diacid product.

4.3.6 References

1. Uemura, N., Taoka, A., Takagi, M., "Production of Dicarboxylic Acids by Fermentation," 1988, World Conference on Biotechnology for the Fats and Oils Industry, 148-152.
2. Melander, W., Horvath, C., "Salt Effects on Hydrophobic Interactions in Precipitation and Chromatography of Proteins: An Interpretation of the Lyotropic Series, 1977, *Arch. Biochem. Biophys.*, 183, 200-215.
3. Mironov, I. V., Sadofeev, I. G., "Constants of Equilibria Involving Dicarboxylic Acids: The Effect of the Ionic Composition of the Medium," 1995, *Russian Journal of Physical Chemistry*, 69(7), 1102-1108.
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Figure 4.3-1. Schematic representation of high throughput screen using adsorbent loaded on 0.45 micron PVDF membrane holder and microfuge tube. See text for explanation.

Figure 4.3-2. Effect of pH at constant tetrahydrofuran concentration.

Figure 4.3-3. Effect of organic solvent concentration at pH 2.

Figure 4.3-4. Effect of organic solvent concentration at pH 7.2.

Figure 4.3-5. Cohn plot showing decreasing solubility of fermentation broth components with increasing salt concentration. Precipitate includes both DCA and protein.

Figure 4.3-6. Change in pH and precipitation formed as a function of HCl concentration broth ultrafiltered through a 10,000 molecular weight cutoff membrane.

Figure 4.3-7. Cohn plot showing precipitation and then resolubilization of DCA's as a function of increasing HCl concentration.

Figure 4.3-8. Schematic diagram of recovery/purification sequence for DCA from fermentation broth.

Table 4.3-1. Composition of DCA's in broth.

Diacids	
C12	0.210 g / L
C14	3.371 g / L
C14:1	0.817 g / L
C15	0.345 g / L
C16	5.876 g / L
C16:1	6.007 g / L
C17	0.307 g / L
C17:1	1.460 g / L
C18	1.064 g / L
C18:1 (cis)	83.166 g / L
C18:1 (trans)	0.296 g / L
C18:2	9.290 g / L
C19	0.215 g / L
Total Diacids	112.424 g / L

Monoacids	
C16	0.132 g / L
C18	0.169 g / L
C18:1 cis	3.002 g / L
C18:1 trans	0.691 g / L
Total Monoacids	3.994 g / L

Derivatives	
C16-Methyl Ester	0.268 g / L
C16 - Hydroxy Acid	0.246 g / L
Total Derivatives	0.514 g / L

Table 4.3-2. Temperature profile and operating conditions for gas chromatography.

Column:	25 m x 0.25 mm ID Fused Silica WCOT CP-Sil 5CB (df=0.12 μ m)
Temperature Profile:	Start at 100°C 100°C $\rightarrow$ 155°C at 8°C/min Hold at 155°C for 1 min 155°C $\rightarrow$ 225°C at 5°C/ min 225°C $\rightarrow$ 300°C at 15°C/min Hold at 300°C for 5 min
Carrier Gas:	Helium (1.0 ml/minute), Inlet Pressure = 25 PSI
Split Ratio:	90:1 (Vent to column ratio)
Injector:	310°C
Detector:	FID, Attenuation = 8, Sensitivity = 10^{-11} , 310°C

Table 4.3-3. Results from adsorbent screen.

Chromatographic Supports	Fraction of Diacid Adsorbed		
	pH 2	pH 6	pH 10
Polymeric Adsorbents			
XAD 2	0.75	0.34	-
Amberchrom CG162	0.90	0.90	-
CG71	1.00	0.67	0.75
Amberlite XAD71	0.90	0.50	0.67
XAD 4	0.90	0.34	0.25
Ambersorb XEN 563	0.75	0.34	0.67
Ambersorb XEN 572	0.67	0.75	0.90
Cation Exchange			
AG50WX4	0.67	0.67	
Dowex HCR-S	0.67	0.50	
Express-ion C	0.67	0.50	
Amberlite IR-120 +	0.67	-	
Amberlite IR-124	0.67	-	
Dowex50WX8	0.67	-	
Express-ion S	0.67	-	
Aminex A-7	0.50	-	
Aminex Q-15S	-	0.50	
Anion Exchange			
Express-ion Q		-	0.50
Express-ion D		-	0.50
DEAE Cellulose		0.34	0.50
Mixed Bed			
Dowex SBR	0.75	-	0.50
Amberlite MB-1	-	-	
Dowex MR3	-	0.34	
Other Supports			
Darco G-60	0.90	0.90	1.00
Norton Support #08203	0.75	0.75	0.50
Hyflo super cel	0.67	-	0.25
Bio-sil A	0.75	-	-
Vydac C18	0.67	0.50	-
Solka Floc BW100	0.50	-	-
Adsorptive magnesia	0.67	0.34	
Cellulose Microspheres	0.50	0.34	

4.4 Process Economic Analysis

4.4.1 Summary

At the outset of the project, a process economic analysis was conducted on the conversion of methyl myristate to 1,14-tetradecanedioic acid (C14 diacid) using *Candida tropicalis* ATCC 20987 as the biocatalyst. The process was designed for the production of 44 MM pounds of diacid product on an annual basis at greater than 99.0% purity level. Key process steps included sterilization, fermentation, extraction, evaporative crystallization, and final packaging of the product. Auxiliary operations included a wastewater treatment area as well as a solvent recovery system.

The total capital investment was determined to be \$158 MM, while the annual total manufacturing cost was \$117 MM, corresponding to a manufacturing cost of \$2.65/lb. Raw material and other chemicals (64%) were the largest part of the total production cost, followed by fixed costs (24%). The cost of methyl myristate accounted for 37% of the total production cost.

For a 20% return on investment (R.O.I.) over 10 years, the projected selling price of the diacid product with the baseline process was \$5.89/lb. A sensitivity analysis was conducted on the effects of major system parameters on diacid selling price. Variations in equipment cost, followed by raw materials and chemicals had the largest effects on the diacid selling price for a 20% R.O.I. A 20% decrease in equipment cost brought about a 10% decrease in the selling price. These results indicated that the process is highly dependent upon changes in the capital investment cost as well as the direct production cost.

The initial economic analysis was updated to reflect performance improvements through the course of the project. The performance improvements were the optimization of the bioconversion conditions to improve bioreactor productivity, the substitution of lower-cost substrates for methyl myristate, and the reduction in usage rates for other raw materials. The combined effect of these process improvements reduced the estimated total capital investment to \$121 MM. The annual total manufacturing cost was reduced by 40% to \$71 MM, corresponding to a manufacturing cost of \$1.60/lb.

4.4.2 Process Overview

A simplified representation of the process is shown in Figure 4.4-1. The unabridged form of the flowsheet for the process is given in Appendix 1.

Overall—In the baseline process C14 diacid is produced from methyl myristate, via fermentation, using *Candida tropicalis*. The chemical composition required for yeast growth and bioconversion is the medium OPT1 composition (see Section 4.1). The diacid is recovered from the fermentor broth as a soluble salt, acidified, and extracted. This extract is purified by crystallization, and a dry, crystalline product is the end result. The solvent has been assumed to be hexane.

Raw Materials & Chemicals Handling (Sheets 1-3)—Raw materials are shipped in via truck or railcar. The salts and proteins must be put into solution before sterilization and use. The dextrose syrup and sodium hydroxide must be diluted before sterilization. Air and antifoam are continuously sterilized by filters prior to use. Unrefined dextrose syrup is handled in 304L

(chloride resistant) stainless steel (30L SS) equipment for sanitation purposes. Corn steep liquor is handled in 316L SS due to the sulfite and lactic acid concentrations. Sodium hydroxide (NaOH) is handled in carbon steel equipment. As long as it is >99%, sulfuric acid (H_2SO_4) can be handled with carbon steel equipment. Methyl myristate is also handled in carbon steel. Other salts and trace elements are received bagged and used, as needed, to make up "salt and protein blend" for the fermentors. Process air is piped through steel lines until the sterile filters. Then it is switched to 304L SS for sanitary purposes. Antifoam is stored and pumped in 304L SS for sanitary purposes.

Media Sterilization (Sheet 3 & Sheet 4)—The dextrose and proteins must be sterilized separately to avoid complexing. In each case, sterilization at $130^{\circ}C$ for 6 ± 1 minutes is used as the basis. Because corn steep liquor (CSL) is in the medium, the temperature or hold time may need to be raised slightly. CSL contains viable, sulfite-resistant *Lactobacillus* strains. Process water, the majority of which is added directly to the fermentors, is sterilized similarly.

Fermentation (Sheets 5 & 6)—Fermentation begins by growing up a batch inoculum from a flask in the Inoculum Fermentor (V-501) for eight hours. At that time, it is transferred to the Small Seed Fermentor (V-502) where it grows to about 10 times its previous volume over eight hours. This seed is transferred to the Large Seed Fermentor (V-503) and grown up for 8 hours to about ten times its original volume. After eight hours, the seed is ready to be charged into a Production Fermentor (V-601 $\rightarrow$ V-604). This train must be constantly going to supply the Production Fermentors with seed.

The seed is allowed to grow in the Production Fermentor without methyl myristate for 18 hours. After that, methyl myristate is metered into the fermentor and converted to C14 diacid by the organism. During the fermentation, the diacid is neutralized with NaOH to control the pH, so the product is actually a soluble salt at this stage. The fermentation is finished after an additional 114 hours. The conversion of methyl myristate to diacid is assumed to be 99%. The batch is dumped to a drop tank and the fermentor turned around for the next batch.

The fermentors are assumed to be 304L SS in construction. Carbon steel might be used if the fermentation products are benign and if the organism tolerates the iron levels that would be present. Chilled water is provided for temperature control, but it was assumed that steam would only be necessary for start-up purposes since the entering streams were all sterile. The four Production Fermentors and the Drop Tank are identical vessels. The Drop Tank simply serves as surge capacity feeding the Base Addition Tank and centrifuge so that the Production Fermentors can be turned around. The drop pumps (P-600A & B) are designed to empty the fermentors quickly (1-2 hours).

Recovery (Sheet 7)—The broth (703) is centrifuged (C-701) to remove cells and insoluble matter. The clarified broth is acidified with H_2SO_4 to convert the C14 salt to acid. The extraction process performs better with the product in the acid form. The cells removed are assumed to go to waste, but they may be recycled or used as an animal feed adjunct. The spent cells stream, at about 10% of the reactor volume, is assumed to contain 10% of the diacid product. Also, the unreacted methyl myristate was assumed to fully partition with the spent cells. For sanitary purposes, any recovery equipment in contact with viable cells is 30L SS. The acidification tank (V-703) and pump are of fiber-reinforced plastic (polyester) to prevent corrosion.

Purification & Product Preparation (Sheets 8 & 9)—While fermentation modeling data were fairly complete, analysis of the product purification was somewhat more approximating. The diacid product was taken to fully partition in the organic phase (taken as hexane). Solvent and acidified broth enter a continuous, counter current extractor (Podbielniak-type). One extraction stage is assumed in this case, but more may be needed. The organic phase is assumed to contain 95% of the diacid. The aqueous phase is sent to wastewater treatment. In the continuous crystallizer (E-801), it is assumed that the diacid product crystallizes out when half of the solvent is evaporated. The crystals are removed by a continuous belt-press filter and the mother liquor sent to waste treatment. The percent diacid product in the belt-press filter liquid outlet is assumed to be zero. The filtered crystals are dried in a tunnel dryer (E-802), ground, and sized to 3 ± 1 mm, and bagged. The product is assumed to contain 0.5% solvent. The purge of mother liquor and vent gas from the tunnel drier go to solvent recovery. The fines and overs from screening are recycled to the filter inlet. All of this equipment is assumed to be 304L SS. Depending on the real needs, carbon steel may suffice.

Solvent Recovery (Sheet 3)—The solvent system is assumed to consist of aqueous/organic phase separation. Evaporative solvent recovery is fed from the belt-filter filtrate and the condensed dryer air. Make-up solvent is assumed to be 1% of the solvent recovery system throughput.

Waste Disposal, Utilities & Other Concerns—An activated sludge waste treatment plant has been added to the site. Steam generation has not been added as the plant is assumed to be located near a cogeneration facility. The same goes for electrical generation. Process water comes from the local utility.

Mass and energy balance assumptions are given in Appendix 2. Appendix 4 contains process model spreadsheet description and output for the baseline process with 44 MM lb diacid production per year.

4.4.3 Economic Analysis

Basis and Assumptions

- 44 MM lbs per year diacid produced
- >99% product purity
- 8500 hours/year uptime
- 86% overall process yield
- 99% solvent recovery (on-site)
- Site is the U.S. Midwest located next to a cogeneration facility and near a corn wet-mill
- Air is compressed on site. Chilled water is prepared on site. Electricity and steam are purchased from the cogeneration facility. Waste treatment is on site.
- Raw materials are shipped in by truck or rail.

A detailed analysis of the economic estimates for the baseline process is given in Appendix 4. The total capital investment for the 44 MM lb/year production of diacid with the baseline process

was \$157.7 MM. The breakdown of this amount is given in Table 4.4-1. Complete details are given in Appendix 4.

A summary of the total manufacturing cost with the baseline process is given in Table 4.4-2. As can be seen from the table, variable costs (raw materials and chemicals) (64%) account for the majority of this cost, followed by fixed costs (24%).

For a 20% return on investment, the projected selling price for the diacid product from the baseline process is \$5.89/lb. Complete details for this calculation are given in Appendix 4.

A sensitivity analysis was conducted on the effects of major system variables on diacid selling price. The results are given in Table 4.4-3. Variations in equipment cost had the largest effect on the diacid selling price followed by variations in the raw materials and chemicals. A 20% decrease in equipment cost brought about an 10% decrease in the selling price necessary for a 20% R.O.I. Equipment cost is directly related to reactor productivity. Since a large part of the process involves fermentation, an increase in reactor productivity would decrease the fermentor size and subsequently decrease equipment cost. Also, because raw materials and other chemicals accounted for 57% of the total production cost, variation of this parameter significantly affected the diacid selling price.

The economic analysis of the baseline process was modified to reflect performance improvements through the course of the project. The basic process design was left unchanged for these estimates. The performance improvements were the optimization of the bioconversion conditions to improve bioreactor productivity, the substitution of lower-cost substrates for methyl myristate, and the reduction in usage rates for other raw materials (see Sections 4.1 and 4.2). The combined effect of these process improvements reduced the estimated total capital investment to \$121.6 MM (a 23% reduction). The annual total manufacturing cost was reduced by 40% to \$70.5 MM, corresponding to a manufacturing cost of \$1.60/lb. Table 4.4-4 breaks out the effects of the individual process improvements on the estimated manufacturing costs.

4.4.4 References

1. Bailey, J. E., and D. F. Ollis (1986). Biochemical Engineering Fundamentals, 2nd Edition, Pg. 293. McGraw-Hill, Inc.
2. Peters, M. S., and K. D. Timmerhaus (1991). Plant Design and Economics for Chemical Engineers, 4th Edition, McGraw-Hill, Inc.

Table 4.4-1. Estimation of Capital Investment Cost.

<i>COST</i>	<i>\$ MM</i>
Direct costs (equipment, piping, etc)	93.2
Indirect costs (engineering, construction)	43.9
Fixed Capital (Direct + Indirect)	137.1
Working capital	20.6
Total capital investment (Fixed + Working)	157.7

Table 4.4-2. Estimation of Annual Total Manufacturing Cost.

<i>COST</i>	<i>\$ MM</i>	<i>PRODUCTION COST (\$/LB)</i>	<i>% OF TOTAL PRODUCTION COST</i>
Variable Costs	74.7	1.70	64
Fixed Costs	28.2	0.64	24
Depreciation	13.7	0.31	12
Total Product Cost	116.6	2.65	100

Table 4.4-3. Sensitivity of Diacid Selling Price to Changes in Various Cost Factors.

Cost Factor	Selling price for 20% R.O.I. (\$/lb diacid product)		
	<u>Base Case</u>	<u>20% increase in cost factor</u>	<u>20% decrease in cost factor</u>
Equipment	5.89	6.49	5.29
Raw Materials & Chemicals	5.89	6.36	5.41
Utilities	5.89	5.95	5.82

Table 4.4-4. Effect of Process Performance Improvements on the Projected Manufacturing Cost (\$/lb)

	Baseline Process	Optimized Bioconversion (1)	1 Plus Lower- cost Substrate (2)	2 Plus Reduced Raw Material Usage
Variable Costs	1.70	1.60	1.34	0.91
Fixed Costs	0.64	0.57	0.53	0.45
Depreciation	0.31	0.27	0.26	0.24
Total Manufacturing Cost	2.65	2.44	2.13	1.60

5. Application Development

(Herman O. Krabbenhoft, GE CRD)

5.1 Screening and Selection of Diacids (Task 3.1)

5.1.1 Introduction

As described in Section 4.1, the selection of a suitable low-cost source of fatty acid substrate is important to the technical and economic success of the bioconversion process. The fatty acid substrate is to be selected to yield the lowest possible diacid production cost while delivering a diacid that is compatible with the high-flow resin application.

Fontana, et al. reported a synthesis process for polyestercarbonate copolymers (based on aliphatic dicarboxylic acids, such as dodecanedioic acid, and a bisphenol) which exhibited reduced glass transition temperatures relative to polycarbonates ("homopolymer") [1,2]. The lower T_g materials provide for improved processibility (i.e. lower molding temperatures and/or shorter molding cycles). Both aliphatic diacid chlorides and diacids were studied as a means of incorporating aliphatic segments in the polycarbonate backbone.

Dodecanedioic acid (DDDA) was a suitable dicarboxylic acid for the copolyestercarbonate. The detailed advantages or disadvantages of polycarbonates incorporating higher chain length diacids (e.g., C16 or C18) were not explored, because these materials were not available in bulk quantities.

The bioconversion of fatty acids makes the longer chain diacids available. The typical fatty acid compositions of some common vegetable fats and oils are given in Table 5.1-1. As can be seen, the 18-carbon chain generally predominates. Furthermore, the unsaturated materials — oleic acid, linoleic acid, and linolenic acid — comprise the bulk of the C18 fatty acids. The fatty acids derived from animal fats (e.g., beef tallow or lard) should also be suitable as renewable feedstocks for biosynthetic dicarboxylic acids. Table 5.1-1 includes these animal fats.

The first objective of this Task was the determination of the effect of diacid chain length and degree of unsaturation on the glass transition temperature of the derived copolyestercarbonates. Because C18 carbon chains are prevalent in many vegetable and animal fats and oils, it was deemed important to find out how C18 dicarboxylic acids (both saturated and unsaturated) compare to C12 diacid (DDDA) used in the copolyestercarbonate. In addition, it was thought to be worthwhile at the outset to include the C6 diacid system (i.e., adipic acid) for further comparison. However, adipic acid cannot be incorporated under the same conditions as C12 diacid [2]. Therefore, to render the C6, C12, and C18 comparison meaningful, it was decided to carry out the polymerization reactions with the corresponding diacid chlorides which allowed the same polymerization procedure to be utilized for each of the soft segment systems.

After determining the effects of chain length and degree of unsaturation on glass transition temperature with the diacid chlorides, the next objective was to explore the same effects, starting with the diacids themselves. These studies were carried out first with individual diacids (C12, C14, C16, and C18) prepared via classical organic synthesis. This was followed by studies with individual diacids (C14, C16, C18, and C18:1) prepared biosynthetically. Finally, in anticipation of the use of mixed fatty acid substrates in the bioconversion, the effects of mixed biosynthetic diacids on polymer properties were studied. In all of these experiments with different diacid

sources the key polymer characteristics that were measured were glass transition temperature and molecular weights.

5.1.2 Results and Discussion

Part 1—Preparation of Acid Chlorides

The acid chlorides were prepared from the corresponding diacids. The C6 and C12 diacids are commercially available. The saturated C18 diacid (C18) was prepared utilizing standard laboratory synthetic procedures. The unsaturated C18 diacid (C18:1) was available from biosynthesis (see Table 4.1-7, diacid from mixed fatty acids enriched for oleic acid (90%)).

[A] 1,18-octadecanedioyl dichloride (**6**)—The reaction sequence shown in Scheme 1 was employed for the preparation of the C18 diacid chloride. Sebacic acid (**1**) was converted to dimethyl sebacate (**2**) in 99% yield via the Fischer esterification procedure [3]. The dimethyl ester **2** was transformed into the corresponding monomethyl ester **3** in 75% distilled yield by treatment with anhydrous barium hydroxide in methanol following the procedure of Durham, McLeod, and Corn [4]. Next, the methyl hydrogen sebacate **3** was subjected to Kolbe electrolysis to provide a 63% recrystallized yield of dimethyl 1,18-octadecanedioate (**4**) [5]. The dimethyl ester **4** was then saponified to the corresponding diacid **5**, which was isolated in 87% yield [6]. Finally, **5** was converted into its analogous diacid chloride **6** (90% isolated yield) by treatment with thionyl chloride [7]. The overall yield for the preparation of 1,18-octadecanedioyl dichloride (**6**) from sebacic acid (**1**) was 37% (5 steps).

[B] 1,18-*cis*-octadec-9-enedioyl dichloride (**9**)—The sequence of reactions given in Scheme 2 was utilized to effect the preparation of the unsaturated diacid chloride **9**. So that the derived acid chloride **9** would have a similar immediate chemical history to that of the saturated diacid chloride **6**, the C18:1 unsaturated diacid **7** was first converted (in 83-85% distilled yield) into its dimethyl ester **8**, which was then transformed back to **7** (in 98% yield) via saponification with potassium hydroxide in refluxing aqueous ethanol. The diacid **7** was finally treated with thionyl chloride to give the diacid chloride **9** in 92% isolated yield.

[C] 1,6-hexanedioyl dichloride (adipoyl chloride) (**11**)—Treatment of adipic acid (**10**) with thionyl chloride provided adipoyl chloride (**11**) in 95% yield; see Equation 1.

[D] 1,12-dodecanedioyl dichloride (**13**)—Treatment of dodecanedioic acid (DDDA, **12**) with thionyl chloride afforded the corresponding diacid chloride **13** in 95% yield; see Equation 2.

An attempt to vacuum-distill diacid chloride **6** resulted in its complete thermal decomposition. Accordingly, each of the diacid chlorides **6**, **9**, **11**, and **13** was isolated by removing the excess thionyl chloride under vacuum and then subjecting the acid chloride to prolonged (~16 hours) high vacuum. Diacid chlorides **6**, **11** and **13** were light yellow liquids; **6** solidified to a “white” waxy material upon standing; **9** was a light brown liquid.

Part 2—Preparation and Properties of the Polyestercarbonates from Diacid Chlorides

Several polyestercarbonates were prepared from each diacid chloride by catalyzed reaction with a bisphenol, phosgene, and a chain-stopper. Tables 5.1-2 through 5.1-5 present the pertinent information — i.e., the relative levels of diacid, the molecular weights of the copolyestercarbonates (weight average, number average, and peak values), and the glass transition temperatures of the polymers.

It was determined by NMR spectroscopy that about 94% of the dicarboxylic groups were incorporated into the polymer. While this value was in good agreement with NMR analysis of a control copolyestercarbonate, we had expected nearly quantitative incorporation with the diacid chlorides.

High molecular weight materials were obtained in each experiment. In general, as expected, the weight average molecular weights within a specific diacid chloride series increased as the amount of the soft segment increased. Furthermore, considering copolyestercarbonates based on the same mole percent of the various diacid chlorides, the longer the diacid chloride (i.e., the higher its molecular weight), the higher the weight average molecular weight of the corresponding copolyestercarbonate.

However, the weight average molecular weights of the copolyestercarbonates derived from the unsaturated C18:1 diacid chloride were unusual — they were much higher than those of the corresponding saturated C18 diacid chloride. One would have anticipated that the M_w values for the analogous C18 and C18:1 copolyestercarbonates would have been similar, since the molecular weights of the two diacids (as well as the two diacid chlorides) differ by only two atomic mass units. With that in mind, it is pointed out that the corresponding peak molecular weight values (M_p) for the C18 and C18:1 copolymers are in good agreement. It is also noted that

number average molecular weight values (M_n) for the corresponding pairs of C18 and C18:1 derived copolymers are quite similar.

The substantial differences between M_w and M_p for the polyestercarbonates derived from the C18:1 diacid chloride (compared to the relatively small differences between M_w and M_p for the polymers based on the C6, C12, and C18 diacid chlorides) suggest that these products could contain a small amount of some very high molecular weight species (which would have a significant effect on M_w). Such a very high molecular weight component is consistent with a branched copolymer which would be produced by a tetra-acid chloride such as **14**. The tetra-acid chloride **14** could have been formed as a side product in the conversion of **7** to **9** (Scheme 2) since hydrogen chloride (liberated as a by-product) is known to catalyze the dimerization of olefins.

Semi-quantitative evidence consistent with the presence of branched polymer for the C18:1 derived materials was obtained from GPC viscometry (which also provides molecular weight information). Thus, it was found that the branching index values (g') for representative pairs of substrates invariably had lower values (indicating more branching) for the C18:1 based polymer than the C18 based polymer or the C12 based polymer in the pairing of experiments 67 and 68; see Table 5.1-6 [8]. Other support for the presence of a slight amount of branched polymer was gained from a plot of the storage modulus versus shear rate at 200°C. The glass transition temperatures of C18:1 based polyestercarbonates are not affected by the presence of the very slight amount of branched material.

The effect of diacid chloride chain length on the glass transition temperature (T_g) of the derived polyestercarbonate is shown in Tables 5.1-2 through 5.1-5. In order to employ meaningful comparisons regarding the length of the dicarboxylic acids and the glass transition temperatures of the derived polyester carbonates, plots of T_g versus the level of diacid chloride were generated first; these are shown in Figures 5.1-1 through 5.1-4. Because the correlation coefficients (R^2) were high for the C6, C12, C18, and C18:1 systems, comparisons of the T_g values should be reliable. The zero-intercepts were in relatively good agreement with the T_g of polycarbonate ("homopolymer").

Utilizing the equations given in Figures 5.1-1 through 5.1-4, one can calculate the glass transition temperature for polyestercarbonates based on the various diacid systems. At a constant level of incorporation, the longer the diacid chloride, the lower the T_g of the polyestercarbonate. Taking an alternative approach, it is seen that the longer the diacid chloride, the less is needed to achieve a particular T_g .

Another observation that emerges from inspection of Tables 5.1-4 and 5.1-5 and Figures 5.1-3 and 5.1-4 is that the C18:1 diacid chloride is somewhat more effective than the C18 diacid chloride in lowering the T_g of the polyester carbonate. This situation was consistent with the effects of chain length and unsaturation on the melting points of dicarboxylic acids [9]. Thus, as shown in Figure 5.1-5, the saturated even-numbered diacids tend toward a melting point of ~125°C as the chain length is increased up to about 16 to 20 carbon atoms. On the other hand, the C14 to C18 cis-symmetrical-monounsaturated dicarboxylic acids have melting points in the range of 69 to 74°C — some 50°C lower than their saturated counterparts. It was anticipated that this melting point lowering effect seen with cis-symmetrical-monounsaturated dicarboxylic acids would also be manifested in copolyester carbonates based on them. As for the specific reason that the cis-symmetrical-monounsaturated diacids (and the corresponding derived copolyester carbonates) have lower melting points (and lower glass transition temperatures) than their saturated analogs, it is hypothesized that the cis-double bond simply imparts a “kink” in the chain which renders ordering of the chains in the solid state more difficult.

Lastly, because the distinguishing feature of the copolyester carbonate is its relatively low melt viscosity, which provides for better processability (moldability), we examined the melt viscosity of one copolyester carbonate made from each of the four dicarboxylic constituents. The melt viscosity was examined from two perspectives: (1) as a function of oscillatory shear rate (from 0.1 to 400 radians per second) at 200°C; and (2) at 1.000 radian per second as a function of time (from 0 to 30 minutes) at 200°C.

Table 5.1-7 presents the data for the viscosity versus shear rate for the polyester carbonates prepared with the C6, C12, C18, and C18:1 diacid chlorides. Figures 5.1-6 through 5.1-9 present the corresponding viscosity versus shear rate plots. As can be seen, the shapes of the viscosity versus shear rate plots for the C18 derived polyester carbonates are very similar to that of the C12 based polyester carbonate, suggesting comparable shear sensitivity and processability for these systems. (The fact that the C18:1 based polyester carbonate displays slightly more shear sensitivity than the C18 derived copolymer is attributable to the slight amount of branched material present.)

With regard to the viscosity versus time measurements, inspection of Table 5.1-8 reveals that each of the saturated polyester carbonates (i.e. those derived from the C6, C12, and C18 diacid chlorides) displayed excellent melt stability, as judged by the slight changes in melt viscosity after 30 minutes. In contrast, the unsaturated polyester carbonate (experiment 64) showed an 18.74 % increase in melt viscosity. However, additional studies with analogous unsaturated copolyester carbonates (subsequently prepared with the C18:1 diacid; see below) have shown that these unsaturated materials possess excellent melt viscosity stability.

Part 3—Use of Individual Diacids made via Classical Organic Synthesis

A series of four polyester carbonates was prepared using saturated aliphatic dicarboxylic acids of increasing chain length — dodecanedioic acid (C12), tetradecanedioic acid (C14), hexadecanedioic acid (C16), and octadecanedioic acid (C18). Each of the diacids was prepared via classical organic synthesis [10]. With regard to the specific formulations, the amount of diacid was adjusted to yield a constant T_g, based on the earlier results. Incorporation of each diacid was indicated to be complete since acidification of the brine solution resulted in no

insoluble diacid precipitated. Furthermore, the proton NMR spectrum of each polyester carbonate showed no signals attributable to the presence of carbonate-anhydride linkages in the polymer backbone.

With regard to the molecular weights and glass transition temperatures of these polyester carbonates, Table 5.1-9 presents the relevant information. As can be seen, a high molecular weight polyester carbonate was obtained from each saturated aliphatic long-chain diacid utilized. The glass transition temperatures are approximately the same — 127.9 to 128.8°C. The conclusion arrived at in the experiments with diacid chlorides was substantiated — the longer the diacid, the less diacid is required (to achieve a particular Tg).

Therefore, the individual C12, C14, C16, and C18 diacids — each made via classical organic synthesis — are suitable for incorporation into the polycarbonate backbone to give high molecular weight copolyester carbonates with reduced glass transition temperatures.

Part 4—Use of Individual Diacids made via Biosynthesis

A series of four polyester carbonates were prepared using long-chain dicarboxylic acids (C14, C16, C18, and C18:1) prepared from the corresponding fatty acid via a biosynthetic process (see Section 4.1). In addition, a polyester carbonate based on the C12 diacid (prepared via classical organic synthesis) was made as a control. Each biosynthesized diacid was completely incorporated into the polycarbonate backbone based on the observations that no diacids precipitated upon acidification of the brine solution. Furthermore, proton NMR spectra indicated that in each case there was no carbonate-anhydride carry-over into the final copolyester carbonate final product. And, as indicated in Table 5.1-10, the molecular weight data show that high molecular weight polyester carbonates were prepared from diacids made via biosynthesis. The glass transition temperatures of the polyester carbonates prepared from the biodiacids are consistent with the Tg values measured for the polyester carbonates prepared from diacids made via classical organic synthesis.

Thus, the individual C14, C16, C18, and C18:1 diacids — each made via biosynthesis — are suitable for incorporation into the polycarbonate backbone to give high molecular weight copolyester carbonates with reduced glass transition temperatures.

Part 5—Use of Mixed Diacids made via Biosynthesis

A mixture of long-chain dicarboxylic acids was also available via biosynthesis from commercial stearic acid (see Section 4.1). The composition (based on GC analysis) of this diacid mixture was C14 (1.9%), C15 (0.9%), C16 (45.7%), C17 (2.5%), C18 (45.8%), C18:1 (0.5%), C20 (1.0%). Five polyester carbonate preparations were carried out with this diacid mixture. Table 5.1-11 collects the pertinent information for the polyester carbonates.

In each preparation of the polyester carbonate from the mixed diacids satisfactory incorporation of the diacids was suggested by acidification of the brine solution — a clear solution resulted, indicating no precipitation of diacids. The polyester carbonates from experiments 193 and 194 were subjected to detailed NMR analysis which revealed incorporation values of 94.3% and 99.5%, consistent with prior results. The NMR spectra indicated the absence of carbonate-

anhydride linkages. A high molecular weight copolyestercarbonate was obtained from each experiment. Similarly, the polyestercarbonates displayed appropriate Tg values (~128°C).

5.1.3 Conclusions

In this investigation we determined that the glass transition temperatures of copolyestercarbonates based on aliphatic dicarboxylic acid chlorides or dicarboxylic acids are inversely dependent upon the chain length at equivalent levels of incorporation. Furthermore, a *cis*-symmetrical monounsaturated dicarboxylic acid (*cis*-octadec-9-enedioic acid, C18:1) or its diacid chloride effects a lower Tg than does its saturated counterpart (C18) upon incorporation into the polycarbonate backbone. Both of the polyestercarbonates derived from C18 diacid chlorides displayed viscosity versus shear rate curves in line with that of polyestercarbonate prepared with the C12 diacid. Furthermore, the polyestercarbonate based on the C18 and C18:1 dicarboxylic acids exhibit melt viscosity stability in line with the analogous C12 system.

High molecular weight copolyestercarbonates can be prepared with longer-chain dicarboxylic acids (carbon number 12 to 18). The longer-chain dicarboxylic acids can be prepared via classical organic synthesis or via biosynthesis from renewable feedstocks such as fatty acids derived from natural fats and oils. Moreover, mixtures of long-chain diacids (biosynthesized from natural fatty acid mixtures) also appear to be suitable as co-monomers.

The results of the screening of different diacids in the polymer indicate that a variety of fatty acid substrates will produce polymer with the desired molecular weight and Tg properties. This means that the fatty acid substrate selection can be based on other factors, such as compatibility with the bioconversion process, availability, and cost.

5.1.4 Experimental

Materials—Sebacic acid (1), adipic acid (10), and dodecanedicarboxylic acid (12) were obtained from commercial suppliers and used as received. The unsaturated C18:1 diacid (7) was available via biosynthesis (see Table 4.1-7, diacid from mixed fatty acids enriched for oleic acid (90%)). Thionyl chloride, obtained from Aldrich Chemical Co., was distilled immediately prior to use. Three of the diacids made via classical organic synthesis — dodecanedioic acid, tetradecanedioic acid, and hexadecanedioic acid — were obtained from Aldrich Chemical Co. and used as received [10]. The other diacid prepared via classical organic synthetic methods — octadecanedioic acid — was made as described below. The diacids made via biosynthesis — C14, C16, C18, C18:1, and the mixture of diacids comprised primarily of C16 and C18 — were prepared as described in Section 4.1.

Dimethyl Sebacate (2)—[See Scheme 1] In a one-necked 2 l round-bottomed flask was placed 215 g (1.0 mole) of 94% sebacic acid and 800 mL of anhydrous methanol. Then, 8 mL of concentrated sulfuric acid was added dropwise with swirling. Next, the mixture was heated at reflux for 4 hours; the solution (which formed within ~30 minutes of heating) was stirred by means of a Teflon® coated magnetic stirbar. After being allowed to cool to room temperature, the solution was poured into a 4 L beaker containing 2.5 L of water. The contents were transferred to a 4 L separatory funnel and the layers separated. The upper aqueous layer was extracted with methylene chloride (2 X 300 mL), the extracts being combined with the lower organic layer. The combined organic layers were washed with water (1 X 300 mL), 5% aqueous

sodium carbonate (1 X 300 mL), and water (1 X 300 mL). The resulting organic solution was dried over anhydrous MgSO_4 , filtered, and concentrated under vacuum using a rotary evaporator to give 229.81 g (99.8% crude yield) of clear, light yellow liquid. The ^1H NMR spectrum (CDCl_3) had signals (ppm) at 3.65 (singlet, OCH_3), 2.28 (triplet, $\text{CH}_2\text{CH}_2\text{CO}_2$), 1.57 (quintet, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}_2$), and 1.27 (envelope, $(\text{CH}_2)_n$).

Methyl Hydrogen Sebacate (3)—[See Scheme 1] *This reaction was carried out in a drybox.* In a 2 L Erlenmeyer flask was placed 930 mL of 0.492 M methanolic barium hydroxide solution. Then, 210.92 g (0.916 mole) of the dimethyl sebacate prepared above was added with swirling. Within a couple of minutes the solution had become a little cloudy; within 5 minutes a heavy precipitate had formed and settled at the bottom of the flask. After allowing the reaction to stand overnight (~20 hours), the barium salt was isolated by filtration and washed with anhydrous methanol; the total volume of the filtrate was ~1400 mL. The wet barium salt (380.39 g) was then placed in a 4 L beaker containing 750 mL of diethyl ether. With agitation provided by a mechanical stirrer, 1 L of aqueous hydrochloric acid solution (prepared from 600 mL of water and 400 mL of concentrated hydrochloric acid) was added dribblewise. Following completion of the addition of the hydrochloric acid solution, the mixture was stirred for another 10 minutes. Next, the resulting liquid mixture was transferred to a 2 L separatory funnel; the aqueous barium chloride slurry remaining in the beaker was extracted with ether (3 X 250 mL), the extracts being added to the ethereal solution in the separatory funnel. The layers were separated and the upper organic phase was washed with water (3 X 200 mL), dried over anhydrous magnesium sulfate, filtered, and concentrated under vacuum with a rotary evaporator to give 149.30 g of white solid.

The ^1H NMR spectrum (CDCl_3) indicated that the material was about 98.5% **3** and about 1.5% sebacic acid. The product was distilled to give 113.92 g (57.5% yield) of white solid (bp 125–128°C @ ~50 m Hg; mp 41.5–43°C) [11]. The ^1H NMR spectrum displayed the following signals (ppm): 10.8 (broad singlet, CO_2H), 3.63 (singlet, OCH_3), 2.30 (triplet, $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), 2.26 (triplet, $\text{CH}_2\text{CH}_2\text{CO}_2\text{CH}_3$), 1.57 (multiplet, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$ and $\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{CH}_3$), and 1.26 (envelope, $(\text{CH}_2)_n$). The ^{13}C -NMR spectrum (CDCl_3) consisted of signals at 180.57, 174.83, 51.89, 34.47, 29.42, 29.36, 25.29, and 25.02 ppm. A repeat of this preparation using 415.0 g (1.80 mole) of dimethyl sebacate afforded 291.22 g (75.0% yield) of **3** (bp 140–142°C @ 100 m Hg; mp 38–40°C) whose molecular weight was determined by titration with 0.1 M sodium hydroxide to be 217.10 (theoretical molecular weight of **3** is 216.28); the proton NMR spectrum was identical to that of the first batch of **3**.

Dimethyl Octadecanedioate (4)—[See Scheme 1] Using an electrolysis cell assembled exactly as described in Reference 7, 108.27 g (0.500 mole) of **3** dissolved in 250 mL of absolute methanol to which 0.55g (0.025 g-atom) of sodium had been added was subjected to a potential of 50–60 volts (which resulted in a current flow of 1.25–1.60 amperes) for 12.5 hours. The reaction mixture was acidified with 10 mL of glacial acetic acid. The methanol was removed under vacuum with a rotary evaporator using a hot water bath. The residue (which solidified upon cooling) was dissolved in 700 mL of anhydrous diethyl ether and filtered to remove insoluble polymeric side products. The filtrate was concentrated on a rotary evaporator and then taken up in 500 mL of methylene chloride and washed with 5% aqueous sodium bicarbonate (3 X 150 mL) and water (1 X 100 mL), dried over anhydrous magnesium sulfate, filtered, and concentrated under vacuum with a rotary evaporator to give 83.82 g of a yellow solid. Two other electrolysis reactions were conducted using 108.1 g (0.500 mole) and 74.23 g (0.343 mole) of **3**

to afford 81.85 g and 58.74 g of crude product. The three crude products were combined and recrystallized from methanol to provide 103.0 g (44.8% yield) of **4** as a white solid (mp 58-59°C) [12]. The ¹H NMR spectrum (CDCl₃) consisted of signals (ppm) at 3.63 (singlet, OCH₃), 2.27 (triplet, CH₂CH₂CO₂), 1.63 (quintet, CH₂CH₂CH₂CO₂), and 1.20 (envelope, (CH₂)_n). The carbon-13 NMR spectrum (CDCl₃) displayed signals at 174.62, 51.75, 34.45, 30.03, 30.03, 29.98, 29.84, 29.65, 29.54, and 25.29 ppm. The combined sodium bicarbonate washes from above were acidified with dilute hydrochloric acid and extracted with ether (3X 100 mL). The combined ether extracts were dried over anhydrous magnesium sulfate, filtered, and concentrated under vacuum with a rotary evaporator to provide 18 g of recovered **3**. The methanolic mother liquor from the recrystallization of **4** was concentrated under vacuum with a rotary evaporator to provide 90.44 g of an amber liquid which upon fractional distillation afforded 19.15 g of a mixture (approximately 1:1 according to proton and carbon-13 NMR analysis) of methyl non-8-enoate (**15**) and methyl nonanoate (**16**) (cuts # 1 and 2, bp 35-40°C @ ~200 m Hg), 16.67 g of primarily dimethyl sebacate (cut # 3, boiling point 108-115°C @ ~350 m Hg), and 49.36 g of **3** (cut # 4, bp 138-140°C @ 200 m Hg) [13]. Taking into account the combined amounts of recovered **3** results in an overall 63.0% isolated yield of **4**.

Octadecanedioic Acid (5)—[See Scheme 1] In a 2 L three-necked round-bottomed flask, fitted with a mechanical stirrer, a reflux condenser, and an addition funnel, was placed 100.0 g (0.292 mole) of **4** and 300 mL of water. With stirring the mixture was heated until the diester melted. Next, 100 mL of ethanol was added followed by the dropwise addition of 250 mL of 45% aqueous potassium hydroxide from the addition funnel. The resulting mixture was heated at reflux over night (~15 hours). Workup of a 20 mL aliquot of the reaction mixture indicated that the saponification was complete. The remainder of the reaction mixture was worked up as follows. After allowing the reaction mixture to cool to room temperature, the reaction mixture was further cooled by means of an ice/water bath. Next, with stirring, concentrated hydrochloric acid was added dropwise until the mixture was acidic. The mixture was then filtered under vacuum. The filter cake was then slurried with 1000 mL of distilled water and filtered again. This process was repeated until the filtrate did not give a precipitate with aqueous silver nitrate solution, indicating that there was no chloride ion (either as the potassium salt or the acid) present in the product. The final filter cake was then dried to constant weight in a vacuum oven to provide 80.16 g (87% yield) of **5** (mp 124-126°C) [14]. The proton NMR spectrum (d₆-DMSO) consisted of signals (ppm) at 11.72 (broad singlet, CO₂H), 1.93 (triplet, CH₂CH₂CO₂H), 1.23 (quintet, CH₂CH₂CH₂CO₂H), 1.0 (envelope, (CH₂)_n). The carbon-13 NMR spectrum (d₆-DMSO) displayed signals at 175.28, 34.53, 30.02, 30.02, 29.99, 29.89, 29.72, 29.51, and 25.39 ppm. In another preparation of **5** from 46.8 g (0.137 mole) of **4** using this saponification procedure, 43.0 g (100% yield) of **5** (mp 124-126°C) was obtained.

Octadecanedioyl Dichloride (6)—[See Scheme 1] In a 250 mL three-necked round-bottomed flask, equipped with an addition funnel (fitted at the top with a nitrogen purge inlet) and a reflux condenser (the efflux end of which was connected to a mineral oil bubbler), was placed 35.0 g (0.111 mole) of diacid **5**. The flask was immersed in an oil bath maintained at 70°C. The addition funnel was charged with 53 mL (85.86 g, 0.722 mole) of freshly distilled thionyl chloride. With stirring (by means of a Teflon® coated magnetic stirbar) the thionyl chloride was added dropwise. Subsequently an additional 15-20 mL of thionyl chloride was added via a pipette to rinse down the solid that accumulated on the sides of the flask. After a total of 3 hours,

everything was in solution (a pale yellow color) and there was no gas evolution. The addition funnel was replaced with a distillation set-up and the excess thionyl chloride was distilled. After no more thionyl chloride distilled, the flask was hooked up to a high vacuum system (~10 m Hg) for 24 hours at room temperature. During this period, the pale yellow liquid solidified to a "white" waxy material which weighed 35.29 g (90% isolated yield) of **6** whose ¹H NMR spectrum (CDCl₃) displayed signals (ppm) at 2.90 (triplet, CH₂CH₂COCl), 1.72 (quintet, CH₂CH₂CH₂COCl), and 1.3 (envelope, (CH₂)_n). The C-13 NMR spectrum (CDCl₃) consisted of signals at 174.30, 47.56, 30.06, 30.04, 29.96, 29.76, 29.51, 28.87, and 25.50 ppm. In an earlier preparation of **6**, the diacid chloride underwent complete decomposition upon attempted high vacuum distillation of the product.

Dimethyl *cis*-Octadec-9-enedioate (8)—[See Scheme 2] Utilizing the Fischer esterification procedure employed for the conversion of **1** into **2**, 50.5 g (0.162 mole) of **7** was transformed into 46.7 g (85% yield) of distilled **8** (bp 170-177°C @ 100 m Hg). The proton NMR spectrum (CDCl₃) of **8** displayed signals (ppm) at 5.12-5.02 (multiplet, CH₂CH=CHCH₂), 3.39 (singlet, OCH₃), 2.04 (triplet, CH₂CH₂CO₂CH₃), 1.80-1.65 (multiplet, CH₂CH₂CH=CHCH₂CH₂), 1.36 (quintet, CH₂CH₂CH₂COCH₃), and 1.02 (envelope, (CH₂)_n). The carbon-13 NMR spectrum (CDCl₃) displayed signals at 174.58, 130.24, 51.78, 34.44, 30.06, 29.55, 29.51, 29.47, 27.45, and 25.33 ppm. Distinguishing absorption signals in the infrared spectrum (neat) were at 3003, 2928, 2855, 1742, 1437, 1362, 1198, and 1173 cm⁻¹. Another preparation of **8** was carried out in 83% distilled yield (bp 171-176°C @ 100 m Hg).

***cis*-Octadec-9-enedioic Acid (7)**—[See Scheme 2] Following the same procedure as that utilized for the saponification of **4** to give **5**, 136.9 g (0.402 mole) of **8** was converted into 122.6 g (97.6% isolated yield) of **7** (mp 69-71°C) whose ¹H NMR spectrum (d₆-DMSO) consisted of signals (ppm) at 11.69 (broad singlet, CO₂H), 5.11-5.01 (multiplet, CH₂CH=CHCH₂), 1.92 (triplet, CH₂CH₂CO₂H), 1.74-1.72 (multiplet, CH₂CH₂CH=CHCH₂CH₂), 1.24 ("quintet," CH₂CH₂CH₂COCH₃), and 1.01 (envelope, (CH₂)_n). The carbon-13 NMR spectrum (d₆-DMSO) consisted of signals at 175.26, 130.37, 34.52, 30.03, 29.59, 29.50, 29.47, 27.50, and 25.39 ppm.

***cis*-Octadec-9-enedioyl Dichloride (9)**—[See Scheme 2] Employing the same procedure for the preparation of **6** from **5**—except that the reaction was carried out at room temperature instead of refluxing thionyl chloride—resulted in the transformation of 35.0 g (0.112 mole) of **7** into 36.14 g (92% isolated yield) of **9** (a light brown liquid) whose ¹H NMR spectrum (CDCl₃) displayed signals (ppm) at 5.19-5.08 (multiplet, CH₂CH=CHCH₂), 2.68 (triplet, CH₂CH₂COCl), 1.88-1.72 (multiplet, CH₂CH₂CH=CHCH₂CH₂), 1.51 ("quintet," CH₂CH₂CH₂COCH₃), and 1.10 (envelope, (CH₂)_n). The carbon-13 NMR spectrum (CDCl₃) consisted of signals at 174.25, 130.27, 47.55, 30.03, 29.41, 28.84, 27.58, and 25.48 ppm. Distinguishing absorption signals in the infrared spectrum (neat) were at 3005, 2928, 2857, 1800, 1464, 1404, 955, and 723 cm⁻¹. In another preparation of **9** from 26.0 g (0.083 mole) of **7**, a quantitative yield (30.94 g) of **9** was obtained.

Adipoyl Chloride (11)—[See Equation 1] Using the same procedure as that employed for the preparation of **6**, 73.0 g (0.500 mole) of adipic acid (**10**) was converted into 86.9 g (95% isolated yield) of adipoyl chloride (**11**).

Dodecanedioyl Dichloride (13)—[See Equation 2] Using the same procedure as that employed for the preparation of **6**, 116.25 g (0.500 mole) of dodecanedioic acid (**12**) was converted into 126.9 g (95% isolated yield) of dodecanedioyl dichloride (**13**).

Copolyestercarbonates from Diacid Chlorides—A 500 mL 5-necked round-bottomed flask, charged with bisphenol, chainstopper, solvent, water, and catalyst, was fitted with a gas inlet tube, a mechanical stirrer, a caustic addition tube, a 50 mL addition funnel, and a pH electrode connected to a pH controller interfaced with a pump for delivering the caustic solution (aqueous 25% sodium hydroxide). The top of the addition funnel was connected to two caustic traps in series. The container of aqueous sodium hydroxide solution was placed on a balance and tared. The pH controller was set to the initial value. The addition funnel is charged with the specified amount of the diacid chloride dissolved in solvent. With a slow purge of nitrogen through the reaction system (indicated to be leak-free by the appearance of bubbles in the two potassium hydroxide traps), the addition funnel was discharged into the stirred reaction mixture. After the addition of the diacid chloride was completed, the addition funnel was first rinsed with solvent and then exchanged for a condenser utilizing aqueous ethylene glycol at 40°F as the heat exchange fluid. The pH controller was reset to the polymerization value. The nitrogen purge was turned off and the phosgene tank enabled. The phosgene delivery system, previously programmed to deliver phosgene at the specified rate, was then turned on. When the specified amount of phosgene had been delivered, the phosgene delivery automatically stopped. The nitrogen purge was turned back on and the reaction mixture stirred for an additional 5 minutes. When the nitrogen purge period was completed, the reaction was processed by washing with aqueous hydrochloric acid and distilled water and precipitating with antisolvent. The polymer was isolated by filtration and dried in a vacuum oven. Isolated yields of the copolyestercarbonates generally ranged from 94 to 98%.

Copolyestercarbonates from Dicarboxylic Acids — A 500 mL 5-necked round-bottomed flask, charged with bisphenol, chainstopper, the specified amount of the particular dicarboxylic acid, solvent, water, and catalyst, was fitted with a gas inlet tube, a mechanical stirrer, a caustic addition tube, a condenser utilizing aqueous ethylene glycol at 40°F as the heat exchange fluid, and a pH electrode connected to a pH controller interfaced with a pump for delivering the caustic solution (aqueous 33% sodium hydroxide). The top of the condenser was connected to two caustic traps in series. The container of aqueous sodium hydroxide solution was placed on a balance and tared. The pH controller was set to the initial value. Prior to starting the phosgenation, a slow purge of nitrogen was effected through the reaction system (indicated to be leak-free by the appearance of bubbles in the two potassium hydroxide traps). The nitrogen purge was then turned off and the phosgene tank enabled. An equivalent of caustic was added to the reaction and flask and the mixture stirred. Then, the phosgene delivery system, previously programmed to deliver phosgene at the specified rate, was turned on. The pH controller was adjusted to specified values through the polymerization. When the specified amount of phosgene had been delivered, the phosgene delivery automatically stopped. The nitrogen purge was turned back on and the reaction mixture stirred for an additional 5 minutes. When the nitrogen purge period was completed, the reaction was processed by washing with aqueous hydrochloric acid and distilled water, using centrifugation for phase separation, and precipitating with antisolvent. The polymer was isolated by filtration and dried in a vacuum oven.

GPC analyses—were carried out according to the standard procedure for polycarbonates.

DSC measurements—were made with a Perkin Elmer DSC 7 Differential Scanning Calorimeter. The measurements were performed in a nitrogen purged oven using approximately 10 mg of material; the heating rate was 20°C per minute. The data were analyzed with a Perkin Elmer 7 Series Thermal Analysis System.

Rheology measurements—were made with a Rheometrics Dynamic Spectrometer Model 7700. The measurements were performed in a nitrogen purged oven using a 25 mm parallel plate test geometry. Data were obtained over a frequency range of 0.1 to 400 radians per second and a temperature of 200°C. The viscosity versus time measurements (which were carried out after two viscosity versus shear rate sweeps on the sample) were made at a frequency of 1 rad/sec and a temperature of 200°C.

NMR measurements—were made on a General Electric Omega 300WB NMR spectrometer. The proton spectra were obtained in a 5 mm carbon-proton dual probe. About 20 mg of polymer were dissolved in 0.5 ml of CDCl₃. Thirty-two scans were accumulated with 90° pulses, waiting 30 seconds between pulses. The carbon spectra were obtained on a 10 mm broadband probe. About 250 mg of sample along with about 50 mg of Cr(acac)₃ were dissolved in 3.5 ml CDCl₃. About 2000 scans were accumulated with 90° pulses. Gated, broadband ¹H decoupling was applied during the acquisition of the free induction decay. Six scans were acquired per minute.

5.1.5 References and Notes

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8. "GPC/V Software User's Guide," Section 5.3, Waters (1994); "Waters GPC/Viscometry System Supplement," pp 18-19 (1989).
9. The melting points for the dicarboxylic acids included in Figure 5.1-5 were taken from (a) the "Aldrich Catalog Handbook of Fine Chemicals," (1996-1997) [C6.0, C8.0, C10.0, C12.0, C14.0, and C16.0]; (b) P. Chuit, *Helv. Chim. Acta*, **9** 268 (1926) [C18]; (c) the "CRC Handbook of Chemistry and Physics," 50th Edition (1969-1970) [C20.0]; (d) F. Bennington

and R.D. Morin, *J. Org. Chem.*, **26** 5210 (1961) [C6:1]; (e) A. Barco, S. Benetti, G.P. Polini, and R. Taddia, *Org. Prep. Proc. Int.*, **6** 217 (1974) [C8:1]; (f) D.J. Cram and N.L. Allinger, *J. Amer. Chem. Soc.*, **78** 7518 (1956) [C10:1]; (g) B.W. Baker, R.W. Kierstead, R.P. Linstead, and B.C.L. Weedon, *J. Chem. Soc.*, 1804 (1954) [C12:1]; (h) N. Jayasuriya, S. Bosak, and S.L. Regen, *J. Amer. Chem. Soc.*, **112** 5844 — supplementary material — (1990) [C14:1, C16:1, C18:1, C20:1]; (i) H. Hunsdiecker, *Chem. Ber.*, **77B** 185 (1944).

10. (a) The C12 diacid was prepared from the trimerization of butadiene to cyclododecatriene which was hydrogenated to cyclododecane which was in turn oxidized to a mixture of cyclododecanone and cyclododecanol which was further oxidized to dodecanedioic acid. (b) The C14 diacid was prepared from 1,12-dibromododecane (synthesized from dodecanedioic acid via reduction to 1,12-dodecanediol followed by bromination) by conversion to the corresponding dinitrile which was then saponified to tetradecanedioic acid. (c) The C16 diacid was prepared from 1,12-dibromododecane via the classical malonic ester synthesis. (d) The C18 diacid was prepared as described in Experimental Section. The author thanks Kari Hermansen of Aldrich Chemical Co. for providing the details on the preparations of the C12, C14, and C16 diacids.
11. The reported ["Aldrich Catalog Handbook of Fine Chemicals," p 1310 (1996-1997)] boiling and melting points are 168-170°C @ 3 mm Hg and 42-43°C, respectively.
12. The reported [Reference 5] melting point is 57-58°C.
13. The presence of the side product methyl esters **15** and **16** is accounted for as follows: during electrolysis, the methyl hydrogen sebacate **3** undergoes decarboxylation to generate the free radical **17** which couples with itself to produce the desired **4**. Alternatively, **17** can abstract a hydrogen atom from the methylene group beta to the radical center to produce both **15** and **16**. The dimethyl sebacate (**2**) was probably present as an impurity in the starting **3**.

14. The reported [P. Chuit, *Helv. Chim. Acta*, **9** 268 (1926)] melting point is 124.6-124.8°C.

Scheme 1

Scheme 2

Figure 5.1-1. Plot of Tg vs hexanedioic acid level (C-6)

Figure 5.1-2. Plot of Tg vs dodecanedioic acid level (C-12)

Figure 5.1-3. Plot of Tg vs octadecanedioic acid level (C-18.0)

Figure 5.1-4. Plot of Tg vs cis-octadec-9-enedioic acid level (C-18.1)

Figure 5.1-5. Melting points of corresponding saturated and cis-symmetrical-monosaturated linear dicarboxylic acids

Figure 5.1-6. Plot of melt viscosity at 200 °C of polyestercarbonate from C-6 diacid (exp. 66).

Figure 5.1-7. Plot of melt viscosity at 200 °C of polyestercarbonate from C-12 diacid (exp. 70).

Figure 5.1-8. Plot of melt viscosity at 200 °C of polyester carbonate from C-18.0 diacid (exp. 65).

Figure 5.1-9. Plot of melt viscosity at 200 °C of polyester carbonate from C-18.1 diacid (exp. 64).

Table 5.1-1. Typical fatty acid composition of common vegetable and animal fats/oils

fatty acid	Corn Oil	Soy-bean Oil	Cotton-seed Oil	Palm Oil	Palm Kernel Oil	Tall Oil	Coco-nut Oil	Beef Tallow	Lard
C-6					0.3				
C-8					3.9		7.5		
C-10					4.0		7.0		
C-12					49.6		48.0		0.5
C-14			1.0	1.0	16.0		16.5	3.0	1.5
C-14.1								0.5	
C-16	11.5	10.5	25.0	47.0	8.0		8.0	26.0	26.0
C-16.1			1.0				1.0	2.5	4.0
C-17								0.5	0.5
C-17.1								0.5	0.5
C-18	2.0	3.0	3.0	4.0	2.4	2.0	4.0	22.5	13.5
C-18.1	26.5	22.5	17.0	37.5	13.7	59.5	5.0	43.0	43.0
C-18.2	59.0	54.5	53.0	10.0	2.0	37.0	2.5	1.5	9.0
C-18.3	1.0	8.5							0.5
C-20					0.1	0.5			
C-20.1		1.0		0.5		0.5			1.0
Σ(C-18)	88.5	88.5	73.0	51.5	18.1	98.5	11.5	67.0	66.0

Table 5.1-2. Copolyestercarbonates based on the C-6 [hexanedioic] system

EXP	Diacid level charged	Diacid level – % found ^a	M _w (weight)	M _n (number)	M _p (peak)	T _g (°C)
58	0.258	91.7	54,800	19,000	59,600	144.7
62	0.381	93.3	56,100	19,400	60,400	142.3
46	0.489	96.9	56,300	22,100	60,400	141.2
69	0.492	92.7	56,500	19,900	61,200	140.0
54	0.733	96.0	55,800	19,100	60,400	134.8
66	0.899	93.8	57,700	19,900	61,300	129.6

^a Diacid found by NMR as a percent of diacid level charged**Table 5.1-3.** Copolyestercarbonates based on the C-12 [dodecanedioic] system

EXP	Diacid level charged	Diacid level – % found	M _w (weight)	M _n (number)	M _p (peak)	T _g (°C)
59	0.258	91.7	56,300	20,800	60,400	139.0
63	0.376	93.9	57,500	20,600	61,300	132.4
48	0.488	96.4	58,400	25,400	61,300	126.5
55	0.488	97.0	58,800	20,700	62,200	127.8
70	0.489	95.7	58,700	21,300	63,000	127.6
67	0.899	99.0	60,800	21,000	64,400	105.2

Table 5.1-4. Copolyestercarbonates based on the C-18.0 [octadecanedioic] system

EXP	Diacid level charged	Diacid level – % found	M _w (weight)	M _n (number)	M _p (peak)	T _g (°C)
61	0.257	93.8	57,000	19,900	61,600	131.8
57	0.370	94.3	58,600	20,400	62,500	124.3
65	0.377	94.5	59,200	20,800	62,500	124.6
52	0.489	92.6	59,400	21,500	62,400	119.2
71	0.489	93.1	60,400	21,500	63,400	116.4

Table 5.1-5. Copolyestercarbonates based on the C-18.1 [cis-octadec-9-enedioic] system

EXP	Diacid level charged	Diacid level – % found	M _w (weight)	M _n (number)	M _p (peak)	T _g (°C)
60	0.259	92.2	64,200	20,300	61,300	131.3
72	0.359	91.7	67,500	21,000	61,300	124.1
56	0.366	91.6	71,300	20,900	64,400	126.8
64	0.379	91.6	70,900	20,700	64,400	120.7
50	0.489	93.9	77,000	19,200	64,400	116.8
68	0.908	95.6	118,200	22,500	70,700	86.2

Table 5.1-6. Results from GPC viscometry analysis of selected polymers

Pair	Diacid level	M _w	M _n	M _p	IV	g'
(61) 18.0	0.257	27,800	10,300	28,500	0.557	1.001
(60) 18.1	0.259	33,800	10,300	29,200	0.570	0.969
(57) 18.0	0.370	29,200	10,500	29,400	0.555	0.998
(56) 18.1	0.366	39,400	10,700	31,400	0.585	0.962
(52) 18.0	0.489	28,800	10,600	29,000	0.580	1.001
(50) 18.1	0.489	45,000	11,000	32,600	0.609	0.954
(67) 12.0	0.899	29,700	10,600	29,600	0.587	1.005
(68) 18.1	0.908	79,700	12,500	34,900	0.744	0.910

IV is intrinsic viscosity; g' is the branching index

Table 5.1-7. Viscosity vs. shear rate for selected polycarbonatecarbonates

EXP (diacid)	viscosity (poise) at indicated shear rate (rad/sec) ^a				
	0.1000	1.0000	10.001	100.01	400.00
66 (C-6)	3.422	3.288	2.297	0.839	0.343
70 (C-12)	3.694	3.379	2.230	0.839	0.343
65 (C-18.0)	2.851	2.801	2.036	0.780	0.323
64 (C-18.1)	3.327	2.545	1.579	0.629	0.280

[a] Measurements carried out at 200°C.

Table 5.1-8. Viscosity vs. time for selected polycarbonatecarbonates at 200°C

EXP	viscosity at 0 min	viscosity at 30 min	% change in viscosity
66 (C-6)	327,890	329,180	0.39
70 (C-12)	337,930	353,250	4.53
65 (C-18.0)	281,030	279,480	-0.55
64 (C-18.1)	254,990	302,790	18.74

Table 5.1-9. Polycarbonatecarbonates made from *individual* dicarboxylic acids prepared via chemical synthesis.

EXP	DIACID	Level	M _w	M _n	M _w /M _n	T _g (°C)
183	C-12	0.478	55,200	19,400	2.85	127.9
184	C-14	0.404	53,300	19,000	2.81	128.2
185	C-16	0.350	56,600	18,800	3.01	128.8
186	C-18.0	0.310	61,300	21,200	2.89	128.4

Table 5.1-10. Polyester carbonates made from *individual* dicarboxylic acids prepared via biosynthesis.

EXP	DIACID	Level	M _w	M _n	M _w /M _n	T _g (°C)
187	C-14	0.429	49,200	17,900	2.75	126.1
188	C-16	0.391	53,600	19,400	2.76	126.6
190	C-18.0	0.358	52,900	19,500	2.71	128.3
191	C-18.1	0.360	60,300	21,400	2.82	126.4
192	C-12 ^a	0.478	63,200	22,700	2.78	130.2

^a This diacid was prepared by chemical synthesis

Table 5.1-11. Polyester carbonates made from *mixed* dicarboxylic acids prepared via biosynthesis from a mixture of fatty acids.

EXP	DIACID ^a	Level	M _w	M _n	M _w /M _n	T _g (°C)
189	C-16/C-18.0	0.373	54,000	18,800	2.87	127.1
193	C-16/C-18.0	0.373	64,800	21,600	3.00	128.4
194	C-16/C-18.0	0.373	65,900	23,900	2.75	128.4
195	C-16/C-18.0	0.373	61,700	24,700	2.50	126.0
196	C-16/C-18.0	0.373	71,100	27,900	2.54	129.7

^a The principal components were C-16 and C18 diacid; see text for other constituents

5.2 Preparation and Testing of Polymer from Biosynthetic Diacids (Task 3.2)

5.2.1 Introduction

The studies detailed in Section 5.1 demonstrated that longer-chain dicarboxylic acids can be used to prepare copolyestercarbonate with the desired molecular weight and Tg properties. Polymer with the desired properties can be prepared from individual longer-chain diacids, mixtures of diacids, chemically synthesized or biosynthetic diacids, and either saturated or unsaturated diacids.

The objectives of this Task were to optimize the polymerization process using selected biosynthetic diacids, and to further characterize the properties of polymers resulting from the biosynthetic diacids.

Mixed diacids were used in these studies. The principal diacids were the mixed diacid resulting from the bioconversion of mixed fatty acids enriched for oleic acid (hereafter referred to as C18:1 diacid) and the mixed diacid resulting from the hydrogenation of the C18:1 diacid (hereafter referred to as C18 diacid). The earlier studies had indicated acceptable polymer properties from diacids derived from a variety of fatty acid sources, including mixed fatty acids enriched for oleic acid. The mixed fatty acids substrate offers a balance of cost, availability, and compatibility with the bioconversion process. The corresponding hydrogenated product was used in order to further explore the effects of the degree of unsaturation of the diacid on the polymer properties.

5.2.2 Results and Discussion

The polymerization procedure discussed in Section 5.1 appears to have worked well in that (1) incorporation of the diacids was complete; (2) high molecular weight copolyestercarbonates were obtained; and (3) there was no carbonate-anhydride carry-over into the final copolyestercarbonate product. Moreover, the Tg values of the polymers were generally in accord with expectations.

Beyond this polymerization procedure, we also examined some modifications. For example, we began the phosgene addition immediately after adding the initial caustic solution. Also, we investigated the possibility of providing additional time to ensure that the conversion was complete. We achieved this (in experiments 193-195, Table 5.2-1) by adjusting the pH profile and by turning off the phosgene a period of time during the polymerization. However, as shown by subsequent proton NMR data, these additional periods of time were not required to produce good quality copolyestercarbonate.

Next, focusing on the C18 and C18:1 dicarboxylic acids, we employed the following modifications to the parameters: (a) higher initial percent solids, (b) lower phase ratio of solvent to water, (c) a staged phosgene delivery profile, and (d) a modified pH profile.

These modifications were effected in experiments 196, 200, and 202. Two parallel experiments (experiments 201 and 203), in which the above-described "phosgene off" approach was utilized, were also conducted using the same initial percent solids and initial phase ratio. The pertinent information for the copolyestercarbonates prepared in these experiments is summarized in Tables 5.2-1 (experiment 196) and 5.2-2 (experiments 200-203).

As can be seen, high molecular weight polyestercarbonate was produced via both phosgenation procedures (i.e. "on" and "off"). The glass transition temperatures for the C18:1 based copolyestercarbonates were lower than those of the C18 materials.

Next, we focused on a polymerization procedure for making copolyestercarbonate where the key modification to the last procedure was a yet higher initial percent solids. These conditions were used in experiments 205-207. Again, the focus was on the C18 and C18:1 diacids prepared via biosynthesis. Also included (as a control) in this set of experiments was C12 diacid made via chemical synthesis. Table 5.2-3 presents the pertinent information for the copolyestercarbonates obtained in these experiments.

As can be seen, high molecular weight copolyestercarbonates were again produced. The Tg values were in line with previous findings — the C18:1 diacid provides a lower Tg copolyestercarbonate than the C18 diacid than the C12 diacid.

The final set of small-scale experiments encompassed the following features with respect to the previous experiments: (a) an initial percent solids intermediate between the previous two sets, (b) the same phase ratio as the last two sets, and (c) a reduced phosgene delivery rate. We also carried out a parallel set of small-scale experiments using the "phosgene off" procedure.

Tables 5.2-4 through 5.2-6 present the pertinent results for these two sets of experiments; Figures 5.2-1 through 5.2-4 are plots of the data given in Table 5.2-6. Also included for comparative purposes are the corresponding data obtained from a representative sample of copolyestercarbonate made with C12 diacid.

The findings from these small-scale polymerizations clearly demonstrated several important points regarding the process for making the polyestercarbonates and the properties of the polyestercarbonates:

- (1) The extents of diacid incorporation were high regardless of the polymerization procedure used. Moreover, the incorporation of the C18 and C18:1 diacids was in line with that measured for the representative copolyestercarbonate made with C12 diacid. Furthermore, NMR analysis indicated the absence of signals attributable to carbonate-anhydride moieties. Thus, these polymerization procedures should be entirely for preparing high molecular weight copolyestercarbonates based on long-chain biodiacids.
- (2) The nature of the dicarboxylic acid (i.e. the chain-length and unsaturation) exerts a significant influence on the glass transition temperatures of the copolyestercarbonates. Thus, at an equivalent level of incorporation, the C18:1 diacid is more effective than the C18 diacid, which is more effective than the C12 diacid in lowering the glass transition temperature.
- (3) The thermal stability of the copolyestercarbonates derived from long-chain biosynthetic diacids — either saturated (C18) or unsaturated (C18:1) — is good as judged by (a) the small changes in melt viscosity or molecular weight after 30 minutes at 270°C and (b) the small differences between the first and second sweep plots of the melt viscosity (at 250°C) versus the shear rate (0.1 - 400 rad/sec).

The viscosity versus shear rate curves for the copolyestercarbonates prepared by the two different polymerization procedures (e.g., Figures 5.2-1a and 5.2-1b) are virtually superimposable on one

another. Also, the small-scale polyestercarbonates display viscosity versus shear rate curves (Figures 5.2-1 through 5.2-3) essentially the same as that of a representative control copolyestercarbonate (Figure 5.2-4).

Based on the small-scale preparations, we synthesized the copolyestercarbonates on an approximately 2000 g scale. Table 5.2-7 summarizes the relevant characterization results.

According to carbon-13 NMR analysis of the copolyestercarbonates, incorporation of the dicarboxylic acid into the polymer backbone was essentially complete (96-102% of the charged amount). These spectral findings are consistent with the observations that no precipitation of diacid resulted from acidification of the caustic aqueous brine solution separated from the polymerization reaction.

However, it is seen that the isolated yields of the copolyestercarbonates varied considerably. While the yield of the C12-based copolyestercarbonate was in line with that generally obtained in previous small-scale preparations (90-91%), the yields of the copolyestercarbonates derived from the C18:1 and C18 diacids were lower. The lower yields realized for the copolyestercarbonates based on the long-chain biosynthetic diacids may be attributable to loss of product due to incomplete separation of the aqueous and organic phases during isolation of the polymer.

In spite of the relatively low isolated yields of the biodiacid-derived copolyestercarbonates, high molecular weight products were obtained. It is noted, however, that the molecular weight (M_w) of the second batch of the copolyestercarbonate based on the C18:1 diacid was lower than expected.

The glass transition temperatures of the copolyestercarbonates prepared on a 2000 g scale were in line with expectations based on our extensive studies with small-scale preparations. That is, the glass transition temperatures of the C18:1-based copolyestercarbonates were lower than that of the C18-based copolyestercarbonate, which was lower than that of the C12-based copolyestercarbonate.

Finally, according to proton NMR analysis, there was greater anhydride carry-over in the case of the copolyestercarbonate based on the C18 diacid (0.66 mole %) than with the other copolyestercarbonates (0.14-0.23 mole %). Proton NMR analysis of a control C12 copolyestercarbonate sample indicated 0.46 mole % anhydride carry-over. Because of thermal instability, anhydride carry-over should be minimized.

In addition to the characterization results presented in Table 5.2-7, we also examined the thermal stability of the 2000 g scale copolyestercarbonates; the pertinent results are collected in Tables 5.2-8a and 5.2-8b. As can be seen (Table 5.2-8a), the copolyestercarbonates based on the C18:1 bioacid showed good thermal stability based on the small changes in melt viscosity or weight average molecular weight after exposure to 1 rad/sec oscillatory shear at 270°C for 30 minutes. In contrast, however, the copolyestercarbonate derived from the biosynthetic C18 dicarboxylic acid experienced a substantial decrease in melt viscosity.

As shown in Table 5.2-8b, based on thermogravimetric analysis, the C18:1 unsaturated biodiacid-based copolyestercarbonate was less thermally stable than the C18 saturated biodiacid based material. The C18 biodiacid-based copolyestercarbonate exhibited percent weight loss temperatures very similar to those of the C12 based materials. Conducting the TGA in air resulted in the same trends, although the specific temperatures were lower.

5.2.3 Conclusions

The copolyestercarbonates based on long-chain biosynthetic dicarboxylic acids can be prepared utilizing a basic polymerization procedure and modified procedures. The polyestercarbonates based on the C18 and C18:1 dicarboxylic acids exhibit properties (molecular weight, T_g, melt viscosity and melt viscosity stability) in line with the analogous C12 system.

Thus, the longer-chain diacids (e.g. C18 and C18:1) that can be produced via biosynthesis from low-cost renewable fatty acid substrates appear to be possible alternatives to the diacid made through chemical synthesis.

5.2.4 Experimental

Materials — The diacids made via biosynthesis — C18, C18:1, and the mixture of diacids comprised primarily of C16 and C18 — were prepared as described in Section 4.1. Synthetic C12 diacid, bisphenol, chainstopper, and catalyst were used as received. 33% caustic solution was prepared by appropriate dilution of commercially available 50% NaOH solution.

Copolyestercarbonates from mixed dicarboxylic acids prepared via biosynthesis — A 500 mL 5-necked round-bottomed flask, charged with bisphenol, chainstopper, the specified amount of the particular dicarboxylic acid, solvent, water, and catalyst, was fitted with a gas inlet tube, a mechanical stirrer, a caustic addition tube, a condenser utilizing aqueous ethylene glycol at 40°F as the heat exchange fluid, and a pH electrode connected to a pH controller interfaced with a pump for delivering the caustic solution (aqueous 33% sodium hydroxide). The top of the condenser was connected to two caustic traps in series. The container of aqueous sodium hydroxide solution was placed on a balance and tared. The pH controller was set to the initial value. Prior to starting the phosgenation, a slow purge of nitrogen was effected through the reaction system (indicated to be leak-free by the appearance of bubbles in the two potassium hydroxide traps). The nitrogen purge was then turned off and the phosgene tank enabled. An equivalent of caustic was added to the reaction and flask and the mixture stirred. Then, the phosgene delivery system, previously programmed to deliver phosgene at the specified rate, was turned on. The pH controller was adjusted to specified values through the polymerization. When the specified amount of phosgene had been delivered, the phosgene delivery automatically stopped. The nitrogen purge was turned back on and the reaction mixture stirred for an additional 5 minutes. When the nitrogen purge period was completed, the reaction was processed by washing with aqueous hydrochloric acid and distilled water, using centrifugation for phase separation when necessary, and precipitating with antisolvent. The polymer was isolated by filtration and dried in a vacuum oven.

As discussed in the text, the polymerization procedure incorporated one or more of the following modifications in some experiments: starting phosgenation immediately after the initial caustic addition; adjusting the pH profile; turning phosgene off for a period of time; adjusting the initial percent solids; changing the solvent to water phase ratio; and adjusting the phosgene delivery rate.

When performed, proton NMR analysis indicated no signals due to the carbonate-anhydride moiety. A portion of the aqueous layer at the end of reaction was acidified with hydrochloric acid and then examined visually for the precipitation of any unreacted dicarboxylic acid; in each experiment, no precipitates were detected.

Copolyestercarbonates synthesized at 2000 g scale—The polymerization conditions used for Experiments 210-212 (see discussion above) were carried out on a larger scale. The copolyestercarbonates were isolated in a Henschel mixer charged with antisolvent, operated at high speed while the polymer solution was added. The precipitated copolyestercarbonate was isolated by filtration and then returned to the Henschel mixer along with antisolvent and rechopped. The copolyestercarbonate was then isolated by filtration and dried in a vacuum oven.

GPC analyses — were carried out according to the standard procedure for polycarbonates.

DSC measurements — were made with a TA Instruments 2920 calorimeter. The sample area was purged with dry house nitrogen at a rate of 20 cc/min. Cooling was effected using a refrigerated cooling system. The instrument was calibrated using the melt onset of high purity indium and zinc and the heat of fusion of indium under the same instrument conditions. Samples were heated and cooled twice at a rate of 20°C/min between 0°C and 225°C.

Rheology measurements — were made with a Rheometrics Dynamic Spectrometer Model 7700. The measurements were performed in a nitrogen purged oven using a 25 mm parallel plate test geometry. Data were obtained over a frequency range of 0.1 to 400 radians per second and a temperature of 200°C. The viscosity versus time measurements (which were carried out after two viscosity versus shear rate sweeps on the sample) were made at a frequency of 1 rad/sec and a temperature of 200°C.

NMR measurements—were made on a General Electric Omega 300WB NMR spectrometer. The proton spectra were obtained in a 5 mm carbon-proton dual probe. About 20 mg of polymer were dissolved in 0.5 ml of CDCl₃. Thirty-two scans were accumulated with 90° pulses, waiting 30 seconds between pulses. The carbon spectra were obtained on a 10 mm broadband probe. About 250 mg of sample along with about 50 mg of Cr(acac)₃ were dissolved in 3.5 ml CDCl₃. About 2000 scans were accumulated with 90° pulses. Gated, broadband ¹H decoupling was applied during the acquisition of the free induction decay. Six scans were acquired per minute.

Figure 5.2-1a. Plot of melt viscosity at 250°C of polyester carbonate from C-12 diacid (exp 210).

Figure 5.2-1b. Plot of melt viscosity at 250°C of polyester carbonate from C-12 diacid (exp 215).

Figure 5.2-2a. Plot of melt viscosity at 250°C of polyester carbonate from C-18.0 diacid (exp 211).

Figure 5.2-2b. Plot of melt viscosity at 250°C of polyester carbonate from C-18.0 diacid (exp 216).

Figure 5.2-3a. Plot of melt viscosity at 250°C of polyestercarbonate from C-18.1 diacid (exp 212).

Figure 5.2-3b. Plot of melt viscosity at 250°C of polyestercarbonate from C-18.1 diacid (exp 217).

Figure 5.2-4. Plot of melt viscosity at 250°C of control polycarbonate from C-12 diacid (exp 220).

Table 5.2-1. Polyestercarbonates made from *mixed* dicarboxylic acids prepared via biosynthesis

EXP	DIACID	Level	M _w	M _n	M _w /M _n	T _g (°C)
189	C-16/C-18.0	0.373	54,000	18,800	2.87	127.1
193	C-16/C-18.0	0.373	64,800	21,600	3.00	128.4
194	C-16/C-18.0	0.373	65,900	23,900	2.75	128.4
195	C-16/C-18.0	0.373	61,700	24,700	2.50	126.0
196	C-16/C-18.0	0.373	71,100	27,900	2.54	129.7

Table 5.2-2. Polyestercarbonates made from C-18.0 and C-18.1 dicarboxylic acids prepared via biosynthesis

EXP	COCl ₂	DIACID	Level	M _w	M _n	M _w /M _n	T _g (°C)
200	on	C-18.0	0.358	64,700	26,700	2.42	128.4
201	off	C-18.0	0.358	64,000	25,600	2.50	128.4
202	on	C-18.1	0.360	71,100	28,000	2.54	126.1
203	off	C-18.1	0.360	72,200	29,900	2.41	126.3

Table 5.2-3. Polyestercarbonates made from C-18.0 and C-18.1 dicarboxylic acids prepared via biosynthesis

EXP	DIACID	Level	M _w	M _n	M _w /M _n	T _g (°C)
205	C-12 ^a	0.481	69,000	29,000	2.38	131.0
206	C-18.0	0.321	76,000	30,900	2.46	129.5
207	C-18.1	0.323	69,800	30,100	2.32	127.6

^a This diacid was prepared by chemical synthesis**Table 5.2-4.** Molecular weights and glass transition temperatures of polyestercarbonates made from C-18.0 and C-18.1 dicarboxylic acids prepared via biosynthesis

EXP	COCl ₂	DIACID	Level	M _w	M _n	M _w /M _n	T _g (°C)
210	on	C-12	0.478	75,100	26,000	2.89	131.3
211	on	C-18.1	0.361	73,600	25,000	2.94	126.6
212	on	C-18.0	0.358	68,800	25,600	2.69	127.3
215	off	C-12	0.478	76,800	25,900	2.97	131.1
216	off	C-18.1	0.361	75,400	28,900	2.61	126.8
217	on	C-18.0	0.358	70,100	26,000	2.70	128.8
220	on	C-12	0.478	66,500	20,900	3.18	130.3

Table 5.2-5. Diacid incorporation, melt viscosity stability, and molecular weight stability of polyestercarbonates made from C-18.0 and C-18.1 diacids prepared via biosynthesis

EXP	Diacid level charged	Diacid level found	visc _i (poise)	visc _f (poise)	Δ visc (%)	M _{wi} (dalton)	M _{wf} (dalton)	Δ Mw (%)
210	0.478	0.474	28,000	28,800	2.9	75,100	73,600	2.0
211	0.358	0.366	25,000	26,200	4.8	73,600	74,100	0.7
212	0.361	0.323	17,700	17,600	0.6	68,800	64,300	6.5
215	0.478	0.474	28,500	28,400	0.4	76,800	69,500	9.5
216	0.358	0.353	27,700	27,900	0.7	75,400	72,800	3.4
217	0.361	0.339	21,300	21,800	2.3	70,100	70,400	0.4
220	0.478	0.453	18,400	20,100	9.2	66,500	63,200	5.0

The visc_i and visc_f columns report initial and final viscosities after 30 minutes; the Δ visc column gives the percent change in melt viscosity. The M_{wi} and M_{wf} columns report the molecular weight before and after the melt viscosity measurements; the Δ Mw column gives the percent change in molecular weight.

Table 5.2-6. Melt viscosity versus shear rate for polyestercarbonates made from C-18.0 and C-18.1 diacids prepared via biosynthesis

EXP (diacid)	viscosity (poise x 10 ⁻⁴) at indicated shear rate (rad/sec)				
	0.100	1.0000	10.001	100.01	400.0
210 (C-12)	5.458	5.324	4.937	3.057	1.658
	5.327	5.379	4.976	3.063	1.663
211 (C-18.1)	5.076	4.977	4.418	2.735	1.532
	4.965	5.000	4.374	2.690	1.512
212 (C-18.2)	3.649	3.547	3.407	2.369	1.397
	3.559	3.458	3.318	2.310	1.366
215 (C-12)	5.711	5.715	5.283	3.201	1.713
	5.678	5.611	5.180	3.149	1.691
216 (C-18.1)	5.580	5.451	4.824	2.915	1.597
	5.404	5.428	4.781	2.880	1.579
217 (C-18.0)	4.180	4.108	3.925	2.648	1.518
	4.105	4.082	3.875	2.618	1.507
220 (C-12)	3.598	3.504	3.325	2.245	1.324
	3.560	3.566	3.319	2.213	1.302

Two values are given for each shear rate: the upper value is from the first sweep; the lower value is from the second sweep.

Table 5.2-7. Characterization of Copolyestercarbonates made at 2000 g Scale

Exp	Diacid	Diacid level charged	Diacid level found	Yield (g)	Yield (%)	M _w (dalton)	M _n (dalton)	M _w /M _n	T _g (°C)	anhyd (%)
260	C12	0.478	0.487	2141	89.7	65,400	25,500	2.57	131.5	0.23
261	C18:1	0.361	0.347	1480	61.3	62,400	24,700	2.53	126.3	0.14
262	C18:1	0.361	0.365	1967	81.5	53,200	21,300	2.49	125.4	0.22
263	C18	0.358	0.344	1896	78.6	61,800	24,800	2.49	129.0	0.66

Table 5.2-8a. Thermal Stability of Copolyestercarbonates made at 2000 g Scale

Exp	Diacid	initial viscosity (poise)	final Viscosity (poise)	viscosity change (%)	initial Mw (dalton)	final Mw (dalton)	Mw change (%)
260	C12	26,500	27,300	+3.0	65,400	68,800	+5.2
261	C18.1	25,600	25,300	-1.1	62,400	64,200	+2.9
262	C18.1	10,700	11,000	+2.8	53,200	54,800	+3.0
263	C18.0	18,000	15,700	-12.8	61,800	60,300	-2.4

(a) The initial and final values for viscosity and weight average molecular weight refer to before and after 30 minutes.

Table 5.2-8b. Thermal Stability of Copolyestercarbonates made at 2000 g Scale

Exp	Diacid	atmosphere	T @ 1% wt loss (°C)	T @ 2% wt loss (°C)	T @ 5% wt loss (°C)	T @ 10% wt loss (°C)	T @ 15% wt loss (°C)
260	C12	N ₂	431.5	451.9	475.0	492.2	503.5
261	C18:1	N ₂	366.8	390.1	416.3	437.8	451.2
262	C18:1	N ₂	397.3	409.9	427.3	447.0	461.0
263	C18:0	N ₂	438.8	456.3	478.7	498.3	511.3
Standard	C12	N ₂	421.4	446.2	471.0	490.4	502.6
260	C12	air	380.8	407.8	443.8	465.0	479.5
261	C18:1	air	334.7	362.0	393.2	416.2	429.8
262	C18:1	air	361.8	387.2	419.7	441.8	456.0
263	C18:0	air	367.7	401.9	437.8	468.2	488.2
Standard	C12	air	374.3	398.1	434.6	457.1	468.4

Appendix 1: Process Flow Diagrams

GE CORPORATE R & D - C14 DIACID MANUFACTURING - PROCESS FLOWSHEET

Sheet 1: Raw Material Handling A

MBI International - 2 Jul, 1998 - JRV R - Rev. 1 - Proj. 1103-001

Drawing2 - Page-1 - JRV R - 07/02/98 - Sheet 1

GE CORPORATE R & D - C14 DIACID MANUFACTURING - PROCESS FLOWSHEET

Sheet 2: Raw Material Handling B

MBI International - 2 Jul, 1998 - JRVR - Rev. 1 - Proj. 1103-001

appx1.doc - Page-1 - JRVR - 07/02/98 - Sheet 1

GE CORPORATE R & D - C14 DIACID MANUFACTURING - PROCESS FLOWSHEET

Sheet 3: Chemicals Preparation

MBI International - 2 Jul, 1998 - JRV R - Rev. 1 - Proj. 1103-001

apx1.doc - Page-1 - JRV R - 07/02/98 - Sheet 1

GE CORPORATE R & D - C14 DIACID MANUFACTURING - PROCESS FLOWSHEET

Sheet 4: Sterilization

MBI International - 2 Jul, 1998 - JRVF - Rev. 1 - Proj. 1103-001

appx1.doc - Page-1 - JRVF - 07/02/98 - Sheet 1

GE CORPORATE R & D - C14 DIACID MANUFACTURING - PROCESS FLOWSHEET
Sheet 5: Seed and Inoculum Train
 MBI International - 2 Jul, 1998 - JRVR - Rev. 1 - Proj. 1103-001

F-501, 502, 503 STERILE OFF-GAS FILTERS

H-501, 502, 503 OFF-GAS CONDENSORS

H-511, 512, 513 OFF-GAS HEATERS

GE CORPORATE R & D - C14 DIACID MANUFACTURING - PROCESS FLOWSHEET

Sheet 6: Fermentation Train

MBI International - 2 Jul, 1998 - JRVB - Rev. 1 - Proj. 1103-001

F-601, 602, 603, 604 STERILE OFF-GAS FILTERS

H-601, 602, 603, 604 OFF-GAS CONDENSORS

H-611, 612, 613, 614 OFF-GAS HEATERS

P-600 A&B
Fermentor Drop Pumps

GE CORPORATE R & D - C14 DIACID MANUFACTURING - PROCESS FLOWSHEET

Sheet 7: Recovery

MBI International - 2 Jul, 1998 - JRVB - Rev. 1 - Proj. 1103-001

appx1.doc - Page-1 - JRVB - 07/02/98 - Sheet 1

GE CORPORATE R & D - C14 DIACID MANUFACTURING - PROCESS FLOWSHEET

Sheet 8: Purification

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appx1.doc - Sheet 8 - JRVR - 07/02/98 - Sheet 8

GE CORPORATE R & D - C14 DIACID MANUFACTURING - PROCESS FLOWSHEET

Sheet 9: Product Preparation

MBI International - 2 Jul, 1998 - JRVR - Rev. 0 - Proj. 1103-001

appx1.doc - Page-1 - JRVR - 07/02/98 - Sheet 1

Appendix 2: Mass Balance Estimates

- The model assumes a seed time of 8 h for seed fermentors (flask on up) and 18 h for each production fermentor. Conversion time is taken as 114 h and turnaround time as 6 h.
- 80 g/l product in the fermentor is the basis.
- A fermentor liquid capacity of 80% of its entire volume was used.
- Inoculation volumes of 3% of each subsequent seed fermentor stage were taken.
- 3% of the total cell growth was assumed in the seed fermentors.
- Substrate conversion occurred only in the production fermentors.
- Fermentation broth densities were taken as 1.02 times that of water.
- The water content of corn syrup and corn steep liquor were both taken as 40% by weight.
- Growth and bioconversion medium composition was taken as:

Growth	g/(l final fermentor volume) required
Ammonium sulfate	4.5
Corn steep liquor	5.1
Corn syrup	22.6
Salts and trace elements	0.4
Potassium phosphate (di)	0.6
Potassium phosphate (mono)	1.1
Hodag antifoam	2.3
Bioconversion	
Corn syrup	83.9
Sodium hydroxide	43.5
Hodag antifoam	5.6

- Aeration rate of 1 slpm/l bioreactor (1 VVM).
- Gas flowrates are relative to STP
- Assume off-gas moisture content is only water (80% r.h.) at 86 degrees F and off-gas is dried in initial moisture content (30% r.h.) at 77 degrees F
- 0.38 lb sulfuric acid/lb product
- The sodium hydroxide and corn syrup streams were diluted 2-fold with process water.

- 100% of the product precipitates at 50 wt % (product and solvent)
- Assume product precipitate is 60% solid and 40% liquid
- Unreacted methyl myristate partitions 100% to the cells
- Fines and overs were taken as 5% of product
- The solvent rate was 1 lb per 1 lb acid broth.
- Conversion of corn syrup and corn steep liquor to cellular mass was taken as 100%
- Sodium hydroxide, and sulfuric acid were all assumed to end up in the salts and trace elements category.
- Antifoam was unconverted.
- "Other gases" refers to the mixture of gases liberated within the fermentors, the flowrate of which equals that of the incoming air
- Lose 1% solvent in solvent recovery.
- Since sodium hydroxide is utilized in the bioconversion stage, it is added to the production fermentors rather than after the fermentors.
- Corn syrup is the sum of dextrose and water components of the UDS streams.
- The liquid reference point for enthalpies was 77°F (25°C). Steam tables, however, were used for steam condensate.
- Enthalpies of gases were taken from psychrometric charts and steam tables (steam and condensate).
- The total sterile air flow rate into the reactors was 30m³/min, with 30% r.h. Conditions for the air coming out of the fermentors were 86°F, and 80% r.h.
- Chilled water flowrate for the fermentors was approximated from Bailey and Ollis [1] by estimating the fermentor heat load as 6 kcal/(liter fermentor*hour).
- Air flow in the dryer was set at twice that of the solvent outlet flow.
- Heats of mixing were not accounted for.

Appendix 3: Equipment Notes

- Equipment sizing was based on 3 days storage tank capacity and 8 h intermediate tank capacity.
- Empty head space was taken as 20% for tanks and 30% for fermentors.
- The nominal discharge head for pumps was taken as 15 psig.
- Pump efficiency was 20%, and the motor efficiency was 80%, resulting in an overall pump efficiency of 16%.
- Heat exchangers (pre-heaters, sterilizers) utilized an overall heat transfer coefficient of 75 $\text{btu}/(\text{h}\cdot\text{ft}^2\cdot^\circ\text{F})$.
- Hold coils were insulated.
- For the condensers, Nusselt #'s were calculated for vertical tubes.
- A 1 h residence time was taken for the crystallizer.

Appendix 4: Spreadsheet Description and Output

Spreadsheet Description:

Process variables for the C14 diacids fermentations are:

1. Product basis (lb product/year).
2. Fermentation yield (%).
3. Centrifugation yield (%).
4. Extraction yield (%).
5. Solvent (w/w) in dried acid.
6. Product in the filtrate liquid phase (%).

The process modelling begins with the mass balance. Looking at the production fermentors as a whole, given the annual production rate, the product concentration, growth, bioconversion, and turnaround times, overall efficiency ($100/(\text{fermentor yield (\%)})*100/(\text{centrifuge yield (\%)}*(100/(\text{extraction yield (\%)}*100/(\text{product in the filtrate liquid phase (\%)}))$), substrate uptakes, and cell growth conditions, the size of the fermentors and substrate flowrates can be back calculated. Next, water addition was balanced in the fermentors, knowing how much water was in the substrates, how much water was added to dilute the substrates, and the overall liquid volume needed to produce the given annual production rate. At this point, all of the mass balance variables (excluding those for energy requirements) are known up through the production fermentors. Next, the downstream mass balances can be calculated.

Following solution of the mass balance, energy usage, namely steam and cooling water requirements can be easily calculated.

Spreadsheet Output:

Includes:

1. Process flow diagram calculations (material and energy balances).
2. Equipment specifications and list.
3. Manufacturing costs summary.
4. Capital cost estimates.
5. Return on investment calculations.

[illegible]

MBI International Material and Energy Balance C14 DIACIDS													
Prepared by:				JRVR/MJB									
Date:				OCT. 1996									
Basis Year:				1996									
0.05 %													
99 %													
0 %													
Material Balance:													
142	201	211	Stream No.	221	231	251	252	253	254	301	302	303	304
MM	Amm Sulf	NaPO4, di	Component (lb/hr)	NaPO4,m	S&TE	Salts	Proteins	S&PB	S&PB	Proc Air	Proc Air	Proc Air	Ster Air F
5404			Methyl Myristate										
			Dextrose										
			CSL (minus water)				232	232	232				
	341		Ammonium Sulfate			341		341	341				
			Salts & Trace Elements		30	30		30	30				
		45	Sod. Phos, dibasic			45		45	45				
			Sod. Phos, monobasic	83		83		83	83				
			Sodium Hydroxide										
			Antifoam										
			Other gases										
			Air							240262	240262	1202	239060
			Sulfuric Acid										
			Solvent										
			Diacid										
			Cells										
			Water				654	654	654	396	396	5	391
5404	341	45	Total Stream	83	30	499	885	1385	1385	240658	240658	1207	239451
0.8671	1.5000	1.5000	Specific Gravity				1.2550	1.0000	1.0000	0.0013	0.0013	0.0013	0.0013
739			Flow rate, gph				84	164	164				
			Flow rate, cu ft /hr							2978442	2978442	14941	2963501
Energy Balance													
142	201	211	Stream No.	221	231	251	252	253	254	301	302	303	304
MM	Amm Sulf	NaPO4, di	Composition	NaPO4,m	S&TE	Salts	Proteins	S&PB	S&PB	Proc Air	Proc Air	Proc Air	Ster Air F
77	77	77	Temperature(F)	77	77	77	77	77	77	77	77	77	77
60	15.0	15.0	Pressure (psig)	15.0	15.0	15.0	15	60	60	60	60	60	60
0.60	1.50	1.50	Cp (BTU/lb-deg F)		1.00		1.00	1.00	1.00				
0	0	0	Enthalpy (BTU/lb)	0	0	0	0	0	0	25	25	25	25
5404	341	45	Flow Rate (lb/hr)	83	30	499	885	1385	1385	240658	240658	1207	239451
0	0	0	Total Enthalpy (BTU/hr)	0	0	0	0	0	0	5980353	5980353	29999	5950354

MBI International Material and Energy Balance C14 DIACIDS													
Prepared by: JRVR/MJB													
Date: OCT. 1996													
Basis Year: 1996													
Material Balance:													
305	311	312	313	320	320a	321	322	325	Stream No.	401	402	403	404
Ster Air	Antifoam	Antifoam	Ster AF	Fr Solvent	Fr Solvent	Solvent	Sol Recyc	Sol Waste	Component (lb/hr)	Dex a	Dex b	Dex c	Dex d
									Methyl Myristate				
									Dextrose	4836	4836	4836	4836
									CSL (minus water)				
									Ammonium Sulfate				
									Salts & Trace Elements				
									Sod. Phos, dibasic				
									Sod. Phos, monobasic				
									Sodium Hydroxide				
	598	598	598						Antifoam				
									Other gases				
239060									Air				
									Sulfuric Acid				
				37	37	3451	3414	34	Solvent				
									Diacid				
									Cells				
391									Water	4836	4836	4836	4836
239451	598	598	598	37	37	3451	3414	34	Total Stream	9672	9672	9672	9672
0.0013	1.0000	1.0000	1.0000	0.8000	0.8000	0.8000	0.8000	0.8000	Specific Gravity	1.1700	1.1700	1.1700	1.1700
	71	71	71	5	5	511	506	5	Flow rate, gph	980	980	980	980
2963501									Flow rate, cu ft /hr				
Energy Balance													
305	311	312	313	320	320a	321	322	325	Stream No.	401	402	403	404
Ster Air	Antifoam	Antifoam	Ster AF	Fr Solvent	Fr Solvent	Solvent	Sol Recyc	Sol Waste	Composition	Dex a	Dex b	Dex c	Dex d
77	77	77	77	77	77	77	77	77	Temperature(F)	167	266	266	176
60	15.0	60	60	15.0	15.0	60	60	60	Pressure (psig)	60	60	60	60
	1.00	1.00	1.00	0.60	0.60	0.60	0.60	0.60	Cp (BTU/lb-deg F)	1.00	1.00	1.00	1.00
25	0	0	0	0	0	0	0	0	Enthalpy (BTU/lb)	90	189	189	99
239451	598	598	598	37	37	3451	3414	34	Flow Rate (lb/hr)	9672	9672	9672	9672
5950354	0	0	0	0	0	0	0	0	Total Enthalpy (BTU/hr)	870464	1827975	1827975	957511

[illegible]

nce													MBI International Material and Energy Bal C14 DIACIDS
JRVV/MJB													Prepared by:
OCT. 1996													Date:
1996													Basis Year:
													Material Balance:
502	503	520	521	522	523	416	531	532	533	540	541	542	Stream No.
AF SS	AF LS	Dex ST	Dex I	Dex SS	Dex LS	S&PB	S&PB I	S&PB SS	S&PB LS	Flask	Inoculum	Sm Seed	Component (lb/hr)
		145	0	4	141								Methyl Myristate
						7	0	0	7				Dextrose
						10	0	0	10				CSL (minus water)
						1	0	0	1				Ammonium Sulfate
						1	0	0	1				Salts & Trace Elements
						2	0	0	2				Sod. Phos, dibasic
													Sod. Phos, monobasic
1	17										0.02	1	Sodium Hydroxide
													Antifoam
													Other gases
													Air
													Sulfuric Acid
													Solvent
													Diacid
		145	0	4	141	20	0	1	19	0	0	1	Cells
												2	67 Water
1	17	290	0	9	281	42	0	1	40	0	2	68	Total Stream
1.0000	1.0000	1.1700	1.1700	1.1700	1.1700	1.0000	1.0000	1.0000	1.0000	1.0200	1.0200	1.0200	Specific Gravity
0	2	29	0	1	28	5	0	0	5	0	0	8	Flow rate, gph
													Flow rate, cu ft/hr
													Energy Balance
502	503	520	521	522	523	416	531	532	533	540	541	542	Stream No.
AF SS	AF LS	Dex ST	Dex I	Dex SS	Dex LS	S&PB	S&PB I	S&PB SS	S&PB LS	Flask	Inoculum	Sm Seed	Composition
77	77	86	86	86	86	86	86	86	86	86	86	86	Temperature(F)
60	60	60	60	60	60	60	60	60	60	15	15	15	Pressure (psig)
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	Cp (BTU/lb-deg F)
0	0	9	9	9	9	9	9	9	9	9	9	9	Enthalpy (BTU/lb)
1	17	290	0	9	281	42	0	1	40	0	2	68	Flow Rate (lb/hr)
0	0	2611	2	78	2531	374	0	11	362	1	18	614	Total Enthalpy (BTU/hr)

[illegible]

MBI International													
Material and Energy Balance													
C14 DIACIDS													
Prepared by:	JRVR/MJB												
Date:	OCT. 1996												
Basis Year:	1996												
Material Balance:													
Stream No.	584	600	601	602	603	604	610	611	612	613	614	615	616
Component (lb/hr)	OG LS d	AF FT	AF 1	AF 2	AF 3	AF 4	NaOH FT	NaOH 1	NaOH 2	NaOH 3	NaOH 4	S&PB	S&BP1
Methyl Myristate													
Dextrose													
CSL (minus water)												225	56
Ammonium Sulfate												330	83
Salts & Trace Elements												29	7
Sod. Phos, dibasic												44	11
Sod. Phos, monobasic												81	20
Sodium Hydroxide							3292	823	823	823	823		
Antifoam		580	145	145	145	145							
Other gases	6950												
Air													
Sulfuric Acid													
Solvent													
Diacid													
Cells													
Water	5.56E+01						9876	2469	2469	2469	2469	634	159
Total Stream	7.01E+03	580	145	145	145	145	13168	3292	3292	3292	3292	1343	336
Specific Gravity	0.0013	1.0000	1.0000	1.0000	1.0000	1.0000	1.2550	1.2550	1.2550	1.2550	1.2550	1.0000	1.0000
Flow rate, gph		69	17	17	17	17	1244	311	311	311	311	159	40
Flow rate, cu ft /hr	86705												
Energy Balance													
Stream No.	584	600	601	602	603	604	610	611	612	613	614	615	616
Composition	OG LS d	AF FT	AF 1	AF 2	AF 3	AF 4	NaOH FT	NaOH 1	NaOH 2	NaOH 3	NaOH 4	S&PB	S&BP1
Temperature(F)	77	77	77	77	77	77	86	86	86	86	86	86	86
Pressure (psig)	18	60	60	60	60	60	60	60	60	60	60	60	60
Cp (BTU/lb-deg F)		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Enthalpy (BTU/lb)	25	0	0	0	0	0	9	9	9	9	9	9	9
Flow Rate (lb/hr)	7006	580	145	145	145	145	13168	3292	3292	3292	3292	1343	336
Total Enthalpy (BTU/hr)	174094	0	0	0	0	0	118514	29628	29628	29628	29628	12090	3023

[illegible]

[illegible]

MBI International												
Material and Energy Balance												
C14 DIACIDS												
Prepared by:												
Date:												
Basis Year:												
Material Balance:												
662	Stream No.	664	671	672	674	681	682	684	690	691	692	694
OG 1b	Component (lb/hr)	OG 1d	OG 2a	OG 2b	OG 2d	OG 3a	OG 3b	OG 3d	Off-Gas FT	OG 4a	OG 4b	OG 4d
Methyl Myristate												
Dextrose												
CSL (minus water)												
Ammonium Sulfate												
Salts & Trace Elements												
Sod. Phos, dibasic												
Sod. Phos, monobasic												
Sodium Hydroxide												
Antifoam												
57972	Other gases	57972	57972	57972	57972	57972	57972	57972	231888	57972	57972	57972
Air												
Sulfuric Acid												
Solvent												
Diacid												
Cells												
347.83	Water	347.83	1217.41	347.83	347.83	1217.41	347.83	347.83	1391.33	1217.41	347.83	347.83
58320	Total Stream	58320	59189	58320	58320	59189	58320	58320	233280	59189	58320	58320
0.0013	Specific Gravity	0.0013	0.0013	0.0013	0.0013	0.0013	0.0013	0.0013	0.0013	0.0013	0.0013	0.0013
Flow rate, gph												
721781	Flow rate, cu ft /hr	721781	732543	721781	721781	732543	721781	721781	2887123	732543	721781	721781
Energy Balance												
662	Stream No.	664	671	672	674	681	682	684	690	691	692	694
OG 1b	Composition	OG 1d	OG 2a	OG 2b	OG 2d	OG 3a	OG 3b	OG 3d	Off-Gas FT	OG 4a	OG 4b	OG 4d
86	Temperature(F)	77	86	86	77	86	86	77	77	86	86	77
18	Pressure (psig)	18	18	18	18	18	18	18	18	18	18	18
Cp (BTU/lb-deg F)												
25	Enthalpy (BTU/lb)	25	44	25	25	44	25	25	25	44	25	25
58320	Flow Rate (lb/hr)	58320	59189	58320	58320	59189	58320	58320	233280	59189	58320	58320
1457997	Total Enthalpy (BTU/hr)	1449249	2604336	1457997	1449249	2604336	1457997	1449249	5796996	2604336	1457997	1449249

MBI International													
Material and Energy Balance													
C14 DIACIDS													
Prepared by:	JRVR/MJB												
Date:	OCT. 1996												
Basis Year:	1996												
Material Balance:													
Stream No.	702	704	705	706	799	801	802	803	804	805	806	807	811
Component (lb/hr)	BA Broth	Clar Broth	Acid Broth	Cells	CO2	Ex Sol Feed	Extract	Cryst Feed	Cryst Slur	Filter Feed	Filter Cake	Dry Diacid	Ex Aq Ph
Methyl Myristate	54			54									
Dextrose													
CSL (minus water)													
Ammonium Sulfate													
Salts & Trace Elements	3292	2963	5033	329									5033
Sod. Phos, dibasic													
Sod. Phos, monobasic													
Sodium Hydroxide													
Antifoam	598	538	538	60									538
Other gases													
Air													
Sulfuric Acid													
Solvent						69452	69452	69452	3451	3451	604	3	
Diacid	6054	5449	5449	605			5176	5176	5176	5435	5435	5435	272
Cells	757			757									
Water	64924	58432	58432	6492									58432
Total Stream	75679	67382	69452	8298		69452	74629	74629	8627	8886	6039	5438	64276
Specific Gravity	1.0200	1.0200	1.0200	1.0600		0.8000	0.8069	0.8069	0.8600	0.8612	0.8900	0.9000	1.0200
Flow rate, gph	8795	7831	8071	928		10291	10963	10963	1189	1223	804	716	7470
Flow rate, cu ft/hr													
Energy Balance													
Stream No.	702	704	705	706	799	801	802	803	804	805	806	807	811
Composition	BA Broth	Clar Broth	Acid Broth	Cells	CO2	Ex Sol Feed	Extract	Cryst Feed	Cryst Slur	Filter Feed	Filter Cake	Dry Diacid	Ex Aq Ph
Temperature(F)	86	86	86	77	86	77	77	77	100	100	77	167	77
Pressure (psig)	20	20	20	15	20	20	20	20	20	20	20	15	20
Cp (BTU/lb-deg F)	1.00	1.00	1.00	1.00		0.60	0.60	1.00	1.00	1.00	1.50	1.50	1.00
Enthalpy (BTU/lb)	9	9	9	0	9	0	0	0	23	23	0	135	0
Flow Rate (lb/hr)	75679	67382	69452	8298		69452	74629	74629	8627	8886	6039	5438	64276
Total Enthalpy (BTU/hr)	681115	606435	625071	0		0	0	0	198431	204387	0	734132	0

		MBI International											
		Material and Energy Balance											
		C14 DIACIDS											
		Prepared by:	JRVR/MJB										
		Date:	OCT. 1996										
		Basis Year:	1996										
		Material Balance:											
812	815	Stream No.	814	821	822	823	910	911	912	913	998	999	1110
Cryst Solv	Cryst Solv	Component (lb/hr)	Solv Rec	Hot Air	Solv Air	Solv Air	Gr Diacid	DA Fines	Sc Diacid	DIACID	Total OG	Total Wste	Proc Water
		Methyl Myristate											
		Dextrose											
		CSL (minus water)											
		Ammonium Sulfate											
		Salts & Trace Elements										5033	
		Sod. Phos, dibasic											
		Sod. Phos, monobasic											
		Sodium Hydroxide											
		Antifoam										538	
		Other gases									239060		
		Air		1202	1202	1202							
		Sulfuric Acid											
66001	66001	Solvent	2847		601	601	3	0	3	3			
		Diacid	0				5435	259	5176	5176		272	
		Cells										757	
		Water									956	58432	8196
66001	66001	Total Stream	2847	1202	1804	1804	5438	259	5179	5179	240016	65033	8196
0.8000	0.8000	Specific Gravity	0.8000	0.0013	0.0013	0.0013	0.9000	0.9000	0.9000	0.9000	0.0013	1.0000	1.0000
9780	9780	Flow rate, gph	422				716	34	682	682		7709	972
		Flow rate, cu ft /hr		14881	22322	22322					2970498		
		Energy Balance											
812	815	Stream No.	814	821	822	823	910	911	912	913	998	999	1110
Cryst Solv	Cryst Solv	Composition	Solv Rec	Hot Air	Solv Air	Solv Air	Gr Diacid	DA Fines	Sc Diacid	DIACID	Total OG	Total Wste	Proc Water
156	77	Temperature(F)	77	167	160	77	120	100	80	77	77	77	77
20	20	Pressure (psig)	20	20	15	15	15	15	15	15	18	15	20
0.60	0.60	Cp (BTU/lb-deg F)	1.00				1.50	1.50	1.50	1.50		1.00	1.00
47	0	Enthalpy (BTU/lb)	0		66	0	65	35	5	0	25	0	0
66001	66001	Flow Rate (lb/hr)	2847	1202	1804	1804	5438	259	5179	5179	240016	65033	8196
3128462	0	Total Enthalpy (BTU)	0	0	118973	536	350752	8934	23306	0	6000406	0	0

								MBI International					
								Material and Energy Balance					
								C14 DIACIDS					
								Prepared by:	JRVR/MJB				
								Date:	Oct-96				
								Basis Year:	1996				
								Material Balance:					
1111	1112	1210	1300	1301	1302	1303	1999	Stream No.	2301	2302	2411	2412	2421
PW NaOH	PW UDS	PW S&PB	Proc water	Proc water	Proc water	Proc water	Total PW	Component (lb/hr)	ChWS SR	ChWR SR	ChWS DS	ChWR DS	ChWS PS
								Methyl Myristate					
								Dextrose					
								CSL (minus water)					
								Ammonium Sulfate					
								Salts & Trace Elements					
								Sod. Phos, dibasic					
								Sod. Phos, monobasic					
								Sodium Hydroxide					
								Antifoam					
								Other gases					
								Air					
								Sulfuric Acid					
								Solvent					
								Diacid					
								Cells					
6584	1612	499	49558	49558	49558	49558	66450	Water			17409	17409	2492.879
6584	1612	499	49558	49558	49558	49558	66450	Total Stream	0	0	17409	17409	2493
1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	Specific Gravity	1.0000	1.0000	1.0000	1.0000	1.0000
780	191	59	5875	5875	5875	5875	7877	Flow rate, gph	0	0	2064	2064	296
								Flow rate, cu ft /hr					
								Energy Balance					
1111	1112	1210	1300	1301	1302	1303	1999	Stream No.	2301	2302	2411	2412	2421
PW NaOH	PW UDS	PW S&PB	Proc water	Proc water	Proc water	Proc water	Total PW		ChWS SR	ChWR SR	ChWS DS	ChWR DS	ChWS PS
77	77	77	77	167	266	266	77	Temperature(F)	50	75	50	100	50
20	20	20					20	Pressure (psig)					
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	Cp (BTU/lb-deg F)	1.00	1.00	1.00	1.00	1.00
0	0	0	0	90	189	189	0	Enthalpy (BTU/lb)	-27	-2	-27	23	-27
6584	1612	499	49558	49558	49558	49558	66450	Flow Rate (lb/hr)	0	0	17409	17409	2493
0	0	0	0	4460245	9366515	9366515	0	Total Enthalpy (BTU)	-27	-2	-27	23	-2

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

MANUFACTURING COST SUMMARY							
Product:	C14 DIACIDS				CAPITAL INVESTMENT (\$MM)	Major Equipment	28.16
Process:	FERMENTATIONS					Direct Cost	93.22
Capacity, MM Lbs/Yr:	44					Project Cost	137.11
Basis Year:	1996					Working Capital	20.57
Stream Hrs/Yr:	8500					TOTAL INVESTMENT	157.68
Est. No.:	1						
Date:	OCT. 1996						
Prepared by:	JRVR/MJB	Revised by:	DPM				
ITEM	UNITS	STREAM REF.	QUANT. (UNITS PER)		UNIT PRICE	COST PER	
			Hour	Lb. Produced	Cents	Year \$MM	Lb. Produced Cents
RAW MATERIALS							
Unrefined 95 DE Dextrose, d.b.	Lbs.	101	8060	1.5570	8.00	5.48	12.46
Corn Steep Liquor, 50% d.s.	Lbs.	111	386	0.0746	19.00	0.62	1.42
Methyl Myristate, 99+%	Lbs.	141	5738	1.1085	76.00	37.07	84.24
Subtotal Raw Materials						43.17	98.12
CATALYSTS, SUPPLIES, & CHEMICALS							
Ammonium Sulfate, anhyd.	Lbs.	201	341	0.0658	24.00	0.69	1.58
Sodium Hydroxide, d.b.	Lbs.	121	3294	0.6363	32.00	8.96	20.36
Sulfuric Acid, 100%	Lbs.	131	2071	0.4000	4.00	0.70	1.60
Solvent, 100% (hexane assumed)	Lbs.	321	695	0.1342	85.00	5.02	11.40
Nutrients & Minerals, d.b.	Lbs.	231	30	0.0058	23.00	0.06	0.13
Potassium Phosphate, dibasic, anhydrous	Lbs.	211	42	0.0081	77.00	0.27	0.62
Potassium Phosphate, monobasic, anhydrous	Lbs.	221	83	0.0161	106.00	0.75	1.70
Antifoam	Lbs.	311	598	0.1155	130.00	6.61	15.01
Subtotal CS&C						23.07	52.42
VARIABLE UTILITIES							
Steam, 50 psig	M lbs	3998	26	0.0050	250.00	0.55	1.25
Electricity	KWHR		17590	3.3981	5.00	7.48	16.99
Process Water	M Gals	1999	8	0.0015	300.00	0.20	0.46
Cooling Water	M Gals	2999	168	0.0325	20.00	0.29	0.65
Subtotal Variable Utilities						8.51	19.35
TOTAL VARIABLE COSTS						74.75	169.89
Operating Labor & Benefits							
	FTEs	14.0			1820.00	2.17	4.92
Supervision	% of Oper. Labor	20.0				0.43	0.98
Laboratory	% of Oper. Labor	20.0				0.43	0.98
Maintenance	% of Fixed Cap. Inv	3.0				4.73	10.75
Operating Supplies	% of Fixed Cap. Inv	0.8				1.18	2.69
Subtotal Direct Costs						8.95	20.33
Plant Overhead							
	% of Labor & Sup.	75.0				1.95	4.43
Insurance & Taxes	% of Fixed Cap. Inv	3.3				5.20	11.83
Administration	% of Labor & Sup.	15.0				0.39	0.89
Sales, R&D	% of Man. Cost	10.0				11.66	26.50
Subtotal Ovhd., Ins., Taxes, Sales, R&D						19.20	43.64
TOTAL FIXED COSTS						28.15	63.97
TOTAL CASH COSTS						102.90	233.86
Depreciation	Straight-Line	10.0 % fixed cap.				13.71	31.16
TOTAL MANUFACTURING COSTS						116.61	265.00

R.O.I.

Return on Investment			
Selling Price (\$/lb)			5.89
Total pounds sold/year			44000000
Total sales			2.59E+08
Discounts, distribution, freight (30%)			77748000
Net sales			1.81E+08
Cost of goods sold			1.17E+08
Gross profit			64800872
Profit (yrs 2 - 10)			64800872
Start up costs (10% capital)			15767774
Profit (first year)			49033098
Net earnings after taxes (50%), year 1			24516549
Net earnings after taxes, years 2 - 10			32400436
Working capital			20566662
Original fixed capital investment			1.37E+08
Return on investment (%)			20.04852

Equipment

MBI International Equipment List					
FLOW-					
SHEET	TAG	EQUIPMENT DESCRIPTION	MATLS. OF	EQUIPMENT SIZE & TYPE	UNIT COST
NUMBER	NUMBER		CONS.		Mar-96
1	P-101A	UDS PUMP	304 L SS	18 gpm, 0.8 hp	\$6,200
1	P-101B	UDS PUMP	304 L SS	18 gpm, 0.8 hp	\$6,200
1	P-102	CSL PUMP	316 L SS	44 gph, 0.1 hp	\$8,400
1	P-103	NaOH PUMP	Carbon Steel	11 gpm, 0.6 hp	\$6,000
1	P-104	H ₂ SO ₄ PUMP	Carbon Steel	2.5 gpm, 0.1 hp	\$1,600
1	P-105	MM PUMP	Carbon Steel	13.0 gpm, 1.0 hp	\$1,600
1	P-111A	DILUTE UDS PUMP	304 L SS	35 gpm, 10 hp	\$8,000
1	P-111B	DILUTE UDS PUMP	304 L SS	35 gpm, 10 hp	\$8,000
1	P-113	NaOH DILUTION PUMP	Carbon Steel	22.5 gpm, 1.5 hp	\$1,700
1	T-101A	UNREFINED DEXTROSE (UDS) STORAGE TANK	304 L SS	40,000 gal, 19'd x 19'h	\$79,900
1	T-101B	UNREFINED DEXTROSE (UDS) STORAGE TANK	304 L SS	40,000 gal, 19'd x 19'h	\$79,900
1	T-102	CORN STEEP LIQUOR (CSL) STORAGE TANK	316 L SS	conical bottom, 3150 gal, 6.1'd x 18.4'h	\$15,000
1	T-103	50% SODIUM HYDROXIDE (NaOH) STORAGE TANK	Carbon Steel	47000 gal, 20'd x 20'h	\$58,800
1	T-104	100% SULFURIC ACID (H ₂ SO ₄) STORAGE TANK	Carbon Steel	14,500 gal, 13.5'd x 13.5'h	\$8,400
1	T-105	METHYL MYRISTATE (MM) STORAGE TANK	Carbon Steel	71,500 gal, 23'd x 23'h	\$71,500
1	T-111A	UDS DILUTION TANK	304 L SS	19827 gal, 15'd x 15'h, 20.3 hp agitator	\$110,000
1	T-111B	UDS DILUTION TANK	304 L SS	19827 gal, 15'd x 15'h, 20.3 hp agitator	\$110,000
1	T-113	NaOH DILUTION TANK	Carbon Steel	10,200 gal, 12'd x 12'h, 102 hp agitator	\$22,100
2	P-221A	S&PB PUMP	FRP	3.0 gpm, 1 hp	\$4,700
2	P-221B	S&PB PUMP	FRP	3.0 gpm, 1 hp	\$4,700
2	T-221A	SALT & PROTEIN BLEND (S&PB) TANK	FRP	1560 gal, 5.1'd x 10.2' h, 1.6 hp agitator	\$24,100
2	T-221B	SALT & PROTEIN BLEND (S&PB) TANK	FRP	1560 gal, 5.1'd x 10.2' h, 1.6 hp agitator	\$24,100
3	F-301A	STERILE AIR FILTER	304 L SS	0.2-micron, 55,000 lb/hr	\$8,500
3	F-301B	STERILE AIR FILTER	304 L SS	0.2-micron, 55,000 lb/hr	\$8,500
3	F-311A	STERILE AF FILTER	304 L SS	0.2-micron, 139 lb/hr	\$4,100
3	F-311B	STERILE AF FILTER	304 L SS	0.2-micron, 139 lb/hr	\$4,100
3	H-321	SOLVENT CONDENSOR	304 L SS	1000 sq ft, shell & tube	\$14,700
3	P-300	AIR COMPRESSOR	Carbon Steel	55,000 lb/hr, 50 psig, 3020 hp	\$579,000
3	P-311	AF PUMP	304 L SS	47 gpm, 0.1 hp	\$10,000
3	P-321	SOLVENT PUMP	304 L SS	1.0 gpm, 0.1 hp	\$4,300
3	T-311	ANTIFOAM (AF) STORAGE TANK	304 L SS	6050 gal, 10.1'd x 10.1'h	\$25,000
3	T-321	FRESH SOLVENT STORAGE TANK	304 L SS	5880 gal, 10'd x 10'h	\$26,300
3	V-301	AIR ACCUMULATOR	Carbon Steel	36,800 cu ft, 25'd x 75'h	\$24,700
4	H-401	DEXTROSE PREHEATER	304 L SS	100 sq ft, tube-in tube, 6'd	\$3,000
4	H-402	DEXTROSE STERILIZER	304 L SS	100 sq ft, tube-in tube, 6'd	\$3,000
4	H-403	DEXTROSE COOLER	304 L SS	100 sq ft, tube-in tube, 6'd	\$3,000
4	H-411	S&PB PREHEATER	316 L SS	20 sq ft, tube-in tube, 2'd	\$2,500
4	H-412	S&PB STERILIZER	316 L SS	20 sq ft, tube-in tube, 2'd	\$2,500
4	H-413	S&PB COOLER	316 L SS	20 sq ft, tube-in tube, 2'd	\$2,500
3	H-421	PW PREHEATER	316 L SS	1000 sq ft, shell in tube	\$10,000
3	H-422	PW STERILIZER	316 L SS	1000 sq ft, shell in tube	\$10,000
3	X-421	PROCESS WATER HOLD COIL	316 L SS	2' d x 600'l, tubular	\$6,500
4	X-401	DEXTROSE HOLD-COIL	304 L SS	12 cu ft, 2'd x 6.1'l, tubular	\$29,900
4	X-411	S&PB HOLD COIL	316 L SS	2.52 cu ft, 2'd x 115'l, tubular	\$3,700
5	P-541	PUMP, INOCULUM FERMENTOR	304 L SS	1.0 gpm, 0.1 hp	\$4,300
5	P-542	PUMP, SMALL SEED FERMENTOR	304 L SS	1.0 gpm, 0.1 hp	\$4,300
5	P-543	PUMP, LARGE SEED FERMENTOR	304 L SS	5.0 gpm, 1.0 hp	\$5,000
5	A-501	AGITATOR, INOCULUM FERMENTOR	304 L SS	1.0 hp	\$8,400
5	A-502	AGITATOR, SMALL SEED FERMENTOR	304 L SS	4.0 hp	\$10,800
5	A-503	AGITATOR, LARGE SEED FERMENTOR (price included with vessel)	304 L SS	35 hp	\$0
5	F-501	STERILE OFF-GAS FILTER, INOCULUM FERMENTOR	304 L SS	0.03 lb/hr, 0.2-micron	\$1,300
5	F-502	STERILE OFF-GAS FILTER, SMALL SEED FERMENTOR	304 L SS	1 lb/hr, 0.2-micron	\$1,300
5	F-503	STERILE OFF-GAS FILTER, LARGE SEED FERMENTOR	304 L SS	36 lb/hr, 0.2-micron	\$2,600
5	H-501	OFF-GAS CONDENSOR, INOCULUM FERMENTOR	304 L SS	1 sq ft, tube-in-tube	\$1,800
5	H-502	OFF-GAS CONDENSOR, SMALL SEED FERMENTOR	304 L SS	8 sq ft, tube-in-tube	\$1,800
5	H-503	OFF-GAS CONDENSOR, LARGE SEED FERMENTOR	304 L SS	72 sq ft, tube-in-tube	\$2,300
5	V-501	INOCULUM FERMENTOR	304 L SS	476 gal, 3'd x 9'h, 85 sq ft jacket	\$15,200
5	V-502	SMALL SEED FERMENTOR	304 L SS	3820 gal, 6'd x 18'h, 255 sq ft jacket	\$23,300
5	V-503	LARGE SEED FERMENTOR	304 L SS	34,300 gal, 12.5'd x 37.5'h, 13,800 sq ft jacket	\$242,000
6	A-601	AGITATOR, PRODUCTION FERMENTOR	304 L SS	2400 hp	\$817,000
6	A-602	AGITATOR, PRODUCTION FERMENTOR	304 L SS	2400 hp	\$817,000
6	A-603	AGITATOR, PRODUCTION FERMENTOR	304 L SS	2400 hp	\$817,000
6	A-604	AGITATOR, PRODUCTION FERMENTOR	304 L SS	2400 hp	\$817,000
6	F-601	STERILE OFF-GAS FILTER, PRODUCTION FERMENTOR	304 L SS	0.2-micron, 1200 lb/hr	\$8,500
6	F-602	STERILE OFF-GAS FILTER, PRODUCTION FERMENTOR	304 L SS	0.2-micron, 1200 lb/hr	\$8,500
6	F-603	STERILE OFF-GAS FILTER, PRODUCTION FERMENTOR	304 L SS	0.2-micron, 1200 lb/hr	\$8,500
6	F-604	STERILE OFF-GAS FILTER, PRODUCTION FERMENTOR	304 L SS	0.2-micron, 1200 lb/hr	\$8,500
6	H-601	OFF-GAS CONDENSOR, PRODUCTION FERMENTOR	304 L SS	1000 sq ft, shell & tube	\$14,700
6	H-602	OFF-GAS CONDENSOR, PRODUCTION FERMENTOR	304 L SS	1000 sq ft, shell & tube	\$14,700
6	H-603	OFF-GAS CONDENSOR, PRODUCTION FERMENTOR	304 L SS	1000 sq ft, shell & tube	\$14,700
6	H-604	OFF-GAS CONDENSOR, PRODUCTION FERMENTOR	304 L SS	1000 sq ft, shell & tube	\$14,700
6	P-600A	PRODUCTION FERMENTOR DROP PUMP	304 L SS	1000 gpm, 25 hp	\$14,000
6	P-600B	PRODUCTION FERMENTOR DROP PUMP	304 L SS	1000 gpm, 25 hp	\$14,000
6	V-601	PRODUCTION FERMENTOR	304 L SS	477,000 gal, 30'd x 90'h, 6360 sq ft jacket, 4440 sq ft coil	\$1,670,000
6	V-602	PRODUCTION FERMENTOR	304 L SS	477,000 gal, 30'd x 90'h, 6360 sq ft jacket, 4440 sq ft coil	\$1,670,000
6	V-603	PRODUCTION FERMENTOR	304 L SS	477,000 gal, 30'd x 90'h, 6360 sq ft jacket, 4440 sq ft coil	\$1,670,000
6	V-604	PRODUCTION FERMENTOR	304 L SS	477,000 gal, 30'd x 90'h, 6360 sq ft jacket, 4440 sq ft coil	\$1,670,000
7	A-701	AGITATOR, DROP TANK	304 L SS	50 hp	\$52,500
7	A-703	ACIDIFICATION TANK AGITATOR	FRP	111.5 hp	\$8,400
7	C-701A	CENTRIFUGE	316 L SS	3230 L/hr sludge, 34620 L/hr feed, 125 hp (Westalia SA-160)	\$631,000
7	C-701B	CENTRIFUGE	316 L SS	3230 L/hr sludge, 34620 L/hr feed, 125 hp (Westalia SA-160)	\$631,000
7	P-702	CENTRIFUGE FEED PUMP	304 L SS	150 gpm, 20 hp	\$6,400
7	P-703	EXTRACTOR FEED PUMP	FRP	150 gpm, 20 hp	\$6,400
7	V-701	DROP TANK	304 L SS	477,000 gal, 30'd x 90'h, 6360 sq ft jacket, 4440 sq ft coil	\$1,670,000
7	V-703	ACIDIFICATION TANK	FRP	1120 gal, X'd x X'h	\$12,500
8	E-801	EVAP. CRYSTALLIZER (including pumps & vacuum jets)	304 L SS	7620 gal, 190 sq ft evaporator, 100 mbar, 40 C, 422 lpd	\$841,000
8	E-802	TUNNER DRYER	304 L SS	4.4 m w x 2.5 m l	\$63,100
8	F-801A	BELT-PRESS FILTER	304 L SS	4 m w x 36 m l	\$5,320,000
8	F-801B	BELT-PRESS FILTER	304 L SS	4 m w x 36 m l	\$5,320,000
8	H-801	AIR HEATER	304 L SS	480 sq ft, shell-&-tube	\$8,400
8	H-805	CONDENSOR	304 L SS	Condensor (66,000 lb/hr), 2000 sq ft, shell and tube	\$25,000
8	H-806	CONDENSOR	304 L SS	1000 sq ft, shell & tube	\$14,700
8	S-801	CONTINUOUS SOLVENT EXTRACTOR/SEPARATOR	304 L SS	48 lph feed (Westalia TA-140)	\$325,000
9	G-901	HAMMER MILL	304 L SS	50 hp, 5700 lb/hr	\$43,800
9	S-901	SCREENER	304 L SS	7 sq ft, continuous	\$25,000
9	X-901	PRODUCT BAGGER	304 L SS	5400 lb/hr, 100-lb bags	\$28,000
9	X-900	WASTEWATER TREATMENT		0.174 million gal/day (activated sludge)	\$800,000
3	F-320	SOLVENT RECOVERY	304 L SS	Condensers for streams 811, 812, & 822, distillation, tanks, etc., (7,000 lb/hr)	\$500,000
TOTAL CAPITAL EQUIPMENT COST					\$28,162,900

Capital Cost

Estimation of Capital Investment Cost				\$MM
1. Direct costs				
A. Equipment Items				
1. Purchased Equipment				28.16
2. Installation (35% of purchased equipment)				9.86
3. Instrumentation and Controls installed (15% of purchased equipment cost)				4.22
4. Piping, installed (50% of purchased equipment)				14.08
5. Electrical, installed (25% of purchased equipment)				7.04
B. Buildings, process and auxiliary (40% of purchased equipment)				11.27
C. Service facilities and yard improvements (60% of purchased equipment)				16.90
D. Land (6% of purchased equipment)				1.69
			Total	93.22
2. Indirect costs				
A. Engineering and supervision (20% of direct costs)				18.64
B. Construction expenses (15% of direct costs)				13.98
C. Contingency (40% of purchased equipment)				11.27
			Total	43.89
3. Fixed-capital investment				
			Total	137.11
4. Working capital (15% of fixed-capital investment)				20.57
5. Total capital investment				157.68

Case Report - C1999041600329

Site/Caller Summary:

Site ID: GERD140601
Site Name/Address: GERD-Niskayuna - NY
One Research Circle
Niskayuna, NY 12309

Time Zone: EST
Caller Name: Madlyn N Salamone
Caller Phone: 518-387-6297
Alternate Phone:

Alt. Site ID:
Alt. Site Name/Address:
Alt. Contact Name:

Case Summary:

Case Title: User is unable to connect to FoxPro database from 4th floor.
ID: C1999041600329
Call Type: Connectivity Issue
Severity: SL3
Priority: n/a
Condition/Status: Open-Reject/Researching
Part Description: MS Windows NT
Part Number: MS Windows NT
Product Serial Number:
Contract:

Case History:

*** PHONE LOG 04/16/99 09:47:21 AM mlkeown
User is calling regarding ticket number c1999041300155. This ticket is closed however the one on the fourth floor is still not working properly. The followin information was copied and pasted from the above ticket number:
*** PHONE LOG 04/13/99 08:58:33 AM sgkilgore
mn: k13a60A, running windows nt, user can't access the PDMS database. used with AIM.(FoxPro program). user says there are two computers setup to access the database. one is on the the 3rd floor the other is on the 4th. when trying to access the database getting error message: network failure or server not available
*** NOTES 04/13/99 12:12:23 PM tmartinez Action Type:Manager review
No Network Issues found. Dispatching to WINTEL
*** NOTES 04/14/99 03:48:02 PM rkretzschar Action Type:Manager review
Remapped drives to point to the proper programs
Please close case

Dispatching to wintel ATTN: R. Kretzschar.

*** RETURN 04/16/99 10:20:55 AM spearson
It looks like this case should be closed by looking at the notes. If this is not the case, please state why this case is being dispatched,

Scott

*** PHONE LOG 04/16/99 12:23:20 PM mlkeown Action Type:Incoming call
The computer located on the fourth floor is still not connecting to the FoxPro database. This was stated in the first part of the ticket. Redispatching to wintel.

*** RETURN 04/16/99 01:50:31 PM spearson
Drive mapping is a desktop issue, not server. Please dispatch to desktop,

Scott

Activity Summary:

Activity	Date/Time	Originator	Additional Information
Return	04/16/99 01:50:31 PM	spearson	from Queue GERD Winte...

Activity Summary (Continued):

Activity	Date/Time	Originator	Additional Information
Rule Action	04/16/99 01:09:09 PM	sa	Action Call escalator...
Rule Action	04/16/99 12:54:05 PM	sa	Action Queue Supervis...
Rule Action	04/16/99 12:24:32 PM	sa	Action Queue Members ...
Dispatch	04/16/99 12:23:46 PM	mlkeown	from WIP default to Q...
Phone Log	04/16/99 12:23:20 PM	mlkeown	Start = 04/16/99 12:2...
Return	04/16/99 10:20:55 AM	spearson	from Queue GERD Winte...
Rule Action	04/16/99 10:18:10 AM	sa	Action Queue Supervis...
Rule Action	04/16/99 09:47:39 AM	sa	Action Queue Members ...
Dispatch	04/16/99 09:47:25 AM	mlkeown	from WIP default to Q...
Phone Log	04/16/99 09:47:21 AM	mlkeown	Start = 04/16/99 09:4...
Set Support Pro	04/16/99 09:47:21 AM	mlkeown	Contract ; Line No 1.
Set Product	04/16/99 09:47:21 AM	mlkeown	Contract ; Line No 1.
Modify	04/16/99 09:47:21 AM	mlkeown	into WIP default and ...
Set Support Pro	04/16/99 09:47:05 AM	mlkeown	Contract ; Line No 1.
Set Product	04/16/99 09:47:05 AM	mlkeown	Contract ; Line No 1.
Create	04/16/99 09:40:50 AM	mlkeown	Contact = Madlyn N Sa...