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Production of Malonic Acid through the Fermentation of Glucose

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Abstract
The overall process to produce malonic acid has not drastically changed in the past 50 years. The current process is damaging to the environment and costly, requiring high market prices. Lygos, Inc., a lab in Berkeley, California, has published a patent describing a way to produce malonic acid through the biological fermentation of genetically modified yeast cells. This proposed technology is appealing as it is both better for the environment and economically friendly.

For the process discussed in this report, genetically modified *Pichia Kudriavzevii* yeast cells will be purchased from the Lygos lab along with the negotiation of exclusive licensing rights to the technology. The cells will be grown in fermentation vessels, while being constantly fed oxygen, glucose and fermentation media. The cells will excrete malonic acid in the 101 hour fermentation process. In order to meet a production capacity of 10M pounds of malonic acid a year, 236 total batches are needed. The fermentation broth will then be fed continuously to a downstream process which includes vacuum filtration, reverse osmosis, and crystallization to produce a solid malonic acid powder. After drying, malonic acid crystal powder of 99.9% purity will be sold to the cosmetic, pharmaceutical and petrochemical industries.

The design requires an initial investment of $23.1M. The investors' rate of return (IRR) is 52.6%, the return on investment (ROI) is 46.9% in year three, and the net present value (NPV) is $59.6M in 2018. Sensitivity analyses on the licensing fee and price of cells concluded that these prices are negotiable with Lygos, Inc. This design is recommended based on the process specifications and economic viability of the process, but the success of this project largely depends on the agreement that can be reached with the originators of the technology, Lygos.

Disciplines
Biochemical and Biomolecular Engineering | Chemical Engineering | Engineering

Dear Dr. John Vohs and Professor Bruce Vrana,

The enclosed reports contains a proposal for the design of a process to produce 10 Million pounds per year of Malonic Acid from the biological fermentation of glucose using genetically engineered *Pichia Kudriavzevii* yeast cells.

Our process will depend largely on a partnership with Lygos, Inc., a biotechnology company that has developed the technology to genetically modify yeast cells to produce large amounts of Malonic Acid. Vials of cells will be bought from this company and put into a seed fermentation line consisting of three seed fermenters for 16 hours each and finally into a production fermenter for 50 hours. To meet our production goal of 10 Million pounds per year, we will require three sets of seed lines and production fermenters. 236 batches will be produced in 47 operating weeks, or 329 days, equaling about 5 batches per week. Each batch will then be sent to a downstream process consisting of vacuum belt filtration, reverse osmosis, and crystallization to result in a final solid Malonic Acid product of 99.9% purity.

To determine the economic viability of this proposed process, our team conducted a thorough economic analysis. We decided pursuing exclusive licensing rights with Lygos, Inc. would be the best route to pursue due to Lygos' existing expertise in the technology and our desire to keep costs as low as possible. The economic feasibility of our process depended largely on the potential purchase price and licensing fee we would be able to negotiate with Lygos, and our base case is based on a purchase price of $5,000/vial of cells with an annual licensing fee of $1,000,000. With these values and a selling price of $5.00 per pound of Malonic Acid, as stated in our problem statement, our process will require a $23.1 Million total investment, providing an IRR of 52.6% and a third-year ROI of 46.9%. Sensitivity analyses on these costs and on selling price was performed, providing additional support to our conclusion of economic feasibility. The current price of Malonic Acid on the market ranges from $10-$100 per pound, giving us a significant competitive advantage if an agreement with Lygos can be reached. Thus, our team recommends investment in this process design.

Sincerely,

Emily Peters	Gabrielle Schlakman	Elise Yang

Production of Malonic Acid through the Fermentation of Glucose

Emily Peters | Gabrielle Schlakman | Elise Yang

Project Submitted to Mr. Bruce Vrana and Dr. John Vohs

Project Proposed by Mr. Bruce Vrana

Department of Chemical and Biomolecular Engineering

School of Engineering and Applied Science

University of Pennsylvania

April 17, 2018

Table of Contents

Section 1: Abstract

The overall process to produce malonic acid has not drastically changed in the past 50 years. The current process is damaging to the environment and costly, requiring high market prices. Lygos, Inc., a lab in Berkeley, California, has published a patent describing a way to produce malonic acid through the biological fermentation of genetically modified yeast cells. This proposed technology is appealing as it is both better for the environment and economically friendly.

For the process discussed in this report, genetically modified *Pichia Kudriavzevii* yeast cells will be purchased from the Lygos lab along with the negotiation of exclusive licensing rights to the technology. The cells will be grown in fermentation vessels, while being constantly fed oxygen, glucose and fermentation media. The cells will excrete malonic acid in the 101 hour fermentation process. In order to meet a production capacity of 10M pounds of malonic acid a year, 236 total batches are needed. The fermentation broth will then be fed continuously to a downstream process which includes vacuum filtration, reverse osmosis, and crystallization to produce a solid malonic acid powder. After drying, malonic acid crystal powder of 99.9% purity will be sold to the cosmetic, pharmaceutical and petrochemical industries.

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Section 2: Introduction

2.1 Background Information and Introduction

Malonic Acid is a standard component for many products and processes in the petrochemical, pharmaceutical, and cosmetics industry. Due to its unique structure, Malonic Acid, more formally known as Propanedioic Acid, is currently synthetically produced through nonrenewable petroleum feedstocks and further processed through esterification of the carboxylic acids with an alcohol. While this is the standard route for production of malonic acid at this point in the industry, it tends to be low-yielding and environmentally damaging due to the use of cyanide as an intermediate.

Current developments in the petrochemical industry have shown an increase in the use of biorefining processes to replace petroleum-derived chemicals. These processes not only propose lower costs of production, but also lower levels of carbon dioxide emissions.

A research-based start-up, Lygos, Inc., has developed technology to genetically engineer cells that can produce high yields of malonic acid. These cells are also able to withstand, low pH environments, and in turn, greater amounts of acid, which ensures their survival during long batch fermentation periods. The yield is proposed to be high enough to replace the current industry standard for malonate production at lower costs.

The patent, found in section 21.1 of the appendix, filed by Lygos describes an invention that provides recombinant host cells, materials, and methods for the biological production of malonate in addition to methods for determining the levels of malonate in the host cells. It additionally discusses downstream processing options, such as purification and conversion of malonate to other industrially used components. Lygos has developed a process to provide recombinant host cells with a heterologous nucleic acid encoding an acyl-CoA hydrolase that

catalyzes conversion of malonyl-CoA to malonic acid. The list of possible recombinant cells is extensive, yet their previous research has proven yeast to be the greatest producer of malonic acid through fermentation.

The process described in this report is an expansion upon patent example #48, "Bio-Reactor Based Production of Malonate." A strain of yeast, Y-134, *Pichia Kudriavzevii* (*P. Kudriavzevii*), was genetically modified to produce larger quantities of malonic acid. In this report, the laboratory-scale process described in the patent is modified to scale up the process for industrial-scale manufacturing.

This report describes the design of an industrial process capable of producing 10M lbs of malonic acid annually. The process is broken down into two main sections: fermentation and purification. Malonic acid is excreted by the cells during a 101 hour fermentation process, along with other undesired side products. The fermentation broth is then purified and separated in a downstream process consisting of vacuum belt filtration, reverse osmosis, and crystallization. This report describes the process synthesis, mass and energy balances, and equipment required to make the plant viable. The production of malonic acid through this biological route is much more environmentally friendly than the current method of malonic acid production through cyanide. An economic analysis is also presented, including price sensitivities, net present value, and returns on investments. Ultimately, it was determined that the best course of action to take is to acquire exclusive licensing rights with Lygos for the genetically modified cells in order to make this process viable at the lowest cost possible.

2.2 Objective Time Chart

Project Name	Production of Malonic Acid Through the Fermentation of Glucose
Project Champions	Mr. Bruce Vrana and Dr. John Vohs
Project Leaders	Emily Peters, Gabrielle Schlakman, and Elise Yang
Specific Goals	Design a process to produce malonic acid through the fermentation of glucose. Determine an agreement and payment scheme with Lygos, Inc.
Project Scope	In-scope: ● Produce 10M lb/year of malonic acid ● Production of malonic acid through the fermentation on glucose ● Purification of malonic acid to 99.9% purity ● Mass and energy balances of the overall process ● Economic analyses to determine project feasibility and the best route to obtain the patented genetically modified cells from Lygos ● Consideration of environmental and safety concerns Out-of-scope: ● Malonic acid production through other chemical means ● Detailed understanding of genetically modified organism ● Detailed procedure for cleaning and sterilizing the plant between batch processing ● Detailed description of plant startup
Deliverables	● Design report including Business Opportunity Assessment and Technical Feasibility Assessment ● Presentation of findings and conclusions

Timeline	Initiated January 10th, 2018 Completed April 17th, 2018 Presented April 24th, 2018

Section 3: Innovation Map

This section is not applicable for this report.

Section 4: Market and Competitive Analysis

The industrial method of malonic acid production has not been changed drastically in over 50 years. Classically, malonic acid is produced from chloroacetic acid. A sodium salt, generated from sodium carbonate, reacts with sodium cyanide to provide a cyano acetic salt. The nitrile group is then hydrolyzed by sodium hydroxide to sodium malonate. Acidification is then used to produce malonic acid (Weiner 1938). Figure 1 shows the reactions required to make malonic acid from chloroacetic acid. This method often does not produce a pure enough product or the pure product has an extremely low yield. Industrially, malonic acid is also produced by hydrolyzing dimethyl malonate or diethyl malonate. This manufacturing method is able to bring about a higher yield and purity, but the organic synthesis of malonic acid through these processes is extremely costly and environmentally hazardous. The chemicals currently used in these processes are identified as a hazard for the environment (EPA 2006).

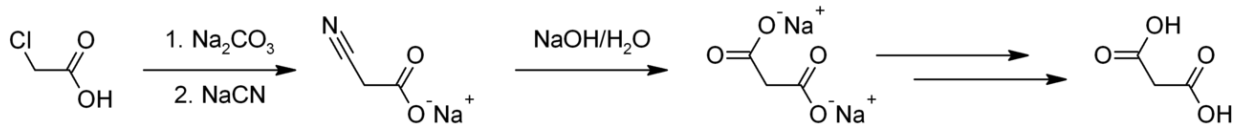

Figure 1. Preparation of malonic acid from chloroacetic acid. This equation describes the previous method of producing malonic acid.

Malonic acid is used in a diverse group of industries. It is used as a precursor in specialty polyesters and polymers, as it can be converted to 1,3 propanediol. By 2021 the market size for this is projected to reach $621 million. Malonic acid is also a component in alkyd resins. These are utilized in different coating applications to protect against UV light, oxidation, and corrosion damage. The global coatings market for automobiles was estimated to be around $18.5 billion in 2014 (James 2015).

Malonic acid also has applications as a more high value, specialty chemical in some industries. These include the electronic, flavors and fragrance, specialty solvents, polymer

crosslinking, and pharmaceutical industries. Specifically, in food and drugs, malonic acid is used to control acidity by either acting as an excipient in pharmaceutical drug formulations or as a natural preservative additive for foods. It is used to synthesize gamma-nonalactone and cinnamic acid in the flavor and fragrance industry and valproate in the pharmaceutical industry (Hilbrand 2001). The Eastman Kodak company uses malonic acid and its derivatives as surgical adhesives. Malonic acid has also been used to cross-link corn and potato starches to produce biodegradable thermoplastics. Starch based polymers comprise around 38% of the global biodegradable polymer market. According to the SIDS initial assessment report for malonic acid diesters in 2004, the global annual production of malonic acid and related diesters was over 20,000 tons. There is room for major growth in these markets as industrial biotechnology strives to displace petroleum-based chemicals for industrial use.

Malonic acid is being produced by numerous manufacturing plants globally. There are plants in North America (USA, Canada, and Mexico), Europe (Germany, France, UK, Russia and Italy), Asia-Pacific (China, Japan, Korea, India and Southeast Asia), South America (Brazil, Argentina and Columbia), and the Middle East and Africa (Saudi Arabia, UAE, Egypt, Nigeria and South Africa). Manufacturing companies include Lonza, Trace Zero, Tateyamakasei, Shanghai Nanxiang Reagent, and Medicalchem. Most of the malonic acid sold by these companies is sold as a powder with greater than 99% purity. The price of malonic acid ranges from around $10-$100 per kilogram of malonic acid (Malonic Acid Market Research 2017).

Figure 2 demonstrates the raw material cost to produce a kilogram of different products. Each compound can be made at a maximum theoretical yield from sugar, demonstrated on the x-axis. More sugar is required to produce a kilogram of a low-yield product, biodiesel, relative to a high-yield product, malonic acid. This also demonstrated that the higher yielding products are less sensitive to changes in raw material pricing. Therefore it is much more advantageous to use malonic acid in the chemical industry over other classically used chemicals (Dietrich).

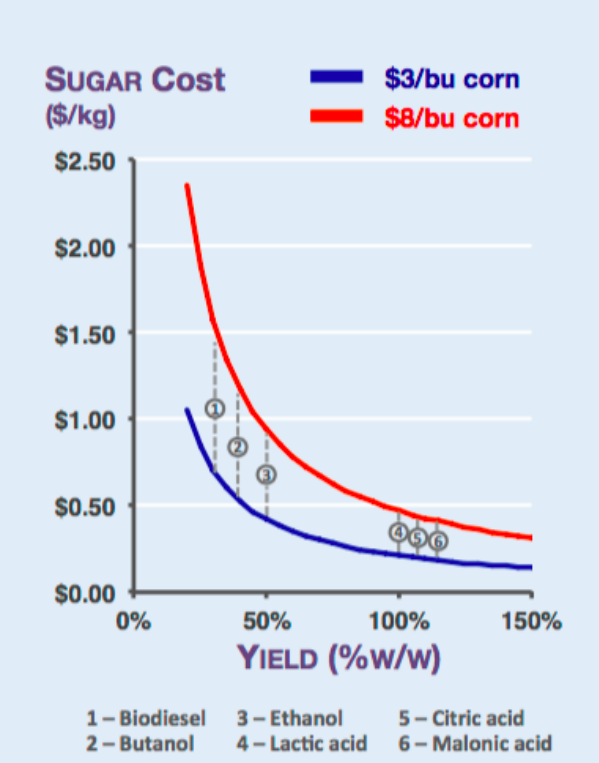

Figure 2. The Raw Material Cost Verses the Maximum Yield of Sugar.

Malonic acid has the capability to be used in a wider variety of applications and more commonly used in existing areas. However, demand has been held back by malonic acid's high cost or production and harm to the environment. The biologic production of malonic acid provides an attractive alternative to classical malonic acid production methods from an economic, environmental, and technical perspective (Dietrich). The fermentation of glucose to produce malonic acid is a higher yielding route that offers environmental benefits through the elimination of cyanide, chlororacedic acid, and carbon sequestration. The US Department of Energy listed malonic acid as one of the top 30 chemicals able to be produced from biomass (Werpy 2004).

Section 5: Customer Requirements

This section is not applicable for this report.

Section 6: Critical to Quality Variables

Malonic Acid is a component used as a stabilizer in many high-end cosmetic and pharmaceutical products. In order to ensure sufficient amounts of product, this process will focus on these larger, high-end industries. These targeted markets require that the product must be of at least 99.9% purity. It is expected that any further required purification will be done by the customers themselves. The malonic acid of 99.9% purity produced through this process will be sold as a powder in tubs and distributed to customers who will process and refine the solid further to fit their specific needs.

Section 7: Product Concept

Founded in 2010, Lygos, Inc. is providing biotechnology solutions for today's renewable chemical challenges. They have developed genetically engineered yeast cells for the biological production of malonic acid through the fermentation of glucose. They have determined that malonic acid is an ideal biological product as it lies on the carbon superhighway and is compatible with various low-cost feedstocks, such as glucose. The biological production method has the potential to provide higher yields, reduce costs compared to the current chemical processes used, and significantly reduce greenhouse emissions. Lygos has engineered yeast to increase expression of genes that lead to the production of Malonic Acid and to decrease production of side-products such as Succinic Acid. The company filed patent PCT/US2013/029441 in 2013 and successfully scaled the process to a laboratory pilot plant in 2017. Lygos' technology provides a compelling opportunity to produce Malonic Acid on a larger scale at much lower costs than traditional methods that exist in industry today.

Section 8: Superior Product Concept

This section is not applicable for this report.

Section 9: Competitive Analysis

This section is not applicable for this report. Everything regarding competitors is included in the Market and Competitive Analysis Section.

Section 10: Preliminary Process Synthesis

This section of the report describes the decisions made and steps taken throughout the design process. There were a number of different factors and alternatives that were considered before the final design of the process was determined. Ultimately, the process to produce malonic acid is broken down into two main sections: fermentation and purification. Genetically modified yeast cells are grown in a seed fermentation line and then a large production fermenter in order to yield malonic acid. The fermentation broth is purified into 99.9% pure malonic acid through vacuum belt filtration, reverse osmosis, and crystallization. The following sections discuss some of the alternatives and reasoning behind the selection of this process.

10.1 Yeast Cells

There were many options that were considered when designing this process, including the type of cells to use to model the process, the size of the vessels, the methods of purification, and if the process would be batch or continuous. Ultimately decisions were made to ensure the highest quality product and the minimal overall cost. Below, some of the alternative options are described along with the rationale for the final selection of the process.

The Lygos patent on the production of malonic acid through the fermentation of yeast, describes many different strains of cells that can be genetically modified to produce malonic acid. After assessing some of the different yeast and bacteria cells, yeast cell were determined to be the best option, specifically the *Pichia Kudriavzevii* strain. The *Pichia Kudriavzevii* strain was successfully used by Lygos in their pilot scale production plant. This strain can handle a high acidic environment and its conditions for fermentation was detailed in the patent.

In order to determine cell growth rates and malonic acid production rates, data on the concentrations of the broth components were taken from the patent. The patent provided information specifically about the optical density (OD) readings and malonic acid concentration over time. By graphing the $\ln(OD/OD_0)$ versus time and the molar concentration of malonic acid versus time, the growth rates and malonic acid yield were deduced from the patent. Figure 3 and 4 show the growth rates and malonic acid production rates from the patent.

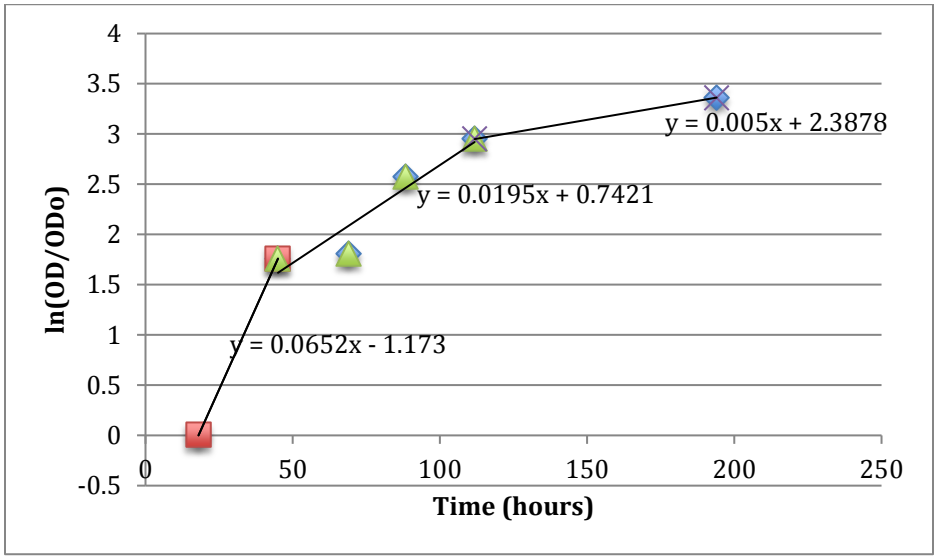

Figure 3. Growth curve of yeast cells. This graph demonstrates the growth of the cells over time based on the optical density readings found in the patent.

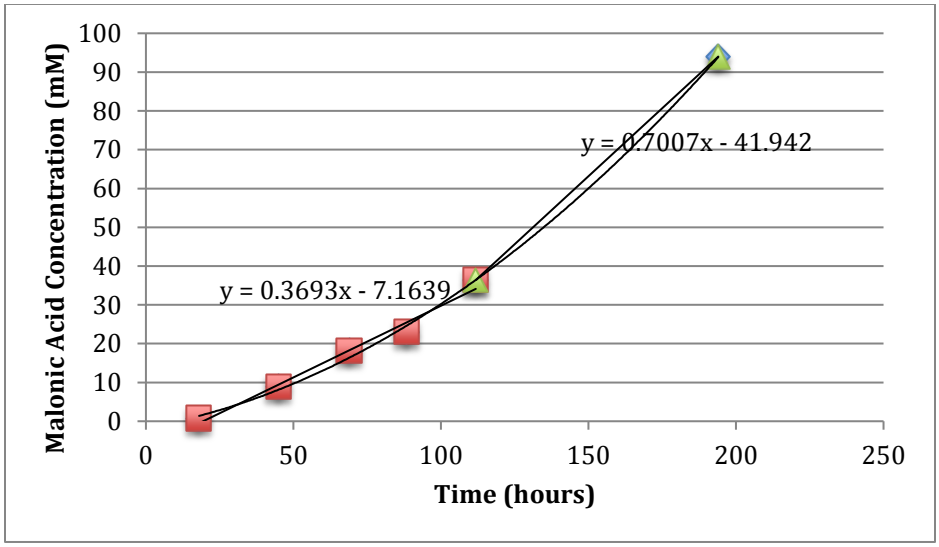

Figure 4. Malonic Acid Concentration. This graph demonstrates the concentration of malonic acid in the batch over time as a growth associated product.

Upon initial calculation, it was determined that these values would not be sufficient to be used to scale a manufacturing plant. The growth rate of the yeast cells was much too slow, with a doubling time of around 36 hours. Genetically modified cells are known to grow slower than the wild strain, but the growth rate found in the patent would require either a too large number of cells to begin fermentation or a very long fermentation period. Therefore, instead of using the numbers from the patent, information was collected about baker's yeast, due to the wide availability of data, and adjusted slightly to account for the genetic modification of the organism. The average growth rate from baker's yeast was found to be around 0.3 $\frac{1}{hr}$. With these corrected numbers, the production of significant amounts of malonic acid became more viable. Therefore, the lab must be able to further genetically modify the organism to be able to reach the required growth rates, yield, and titer used throughout this report in order to make this process viable. Labs often times are in a hurry to file to patent, and therefore do so before perfecting any one strain. Since the filing of their first patent, Lygos has produced a second patent that has improved the yield by 2X. Therefore it is reasonable to assume that given another few years, Lygos will be able further genetically modify the cells to achieve the yields assumed in this report.

The original genetically modified yeast cell by Lygos produces malonic acid intracellularly. The majority of the patent highlighted these type of cells. When preliminary calculations were performed assuming intracellular growth, the mass of cells required to produce 10M lb of malonic acid a year was too high. Additionally, having the product being trapped inside the cells meant that a step to lyse the cells before downstream processing would be necessary. However, the patent also mentions that Lygos had created a way to further genetically modify the cells to produce malonic acid extracellularly. The model discussed in this report

assumes that Lygos will further genetically modify the *Pichia Kudriavzevii* strain to secrete malonic acid as a growth associated product. With this assumption, the amount of cells required and sizes of reactors required to maintain cell growth are within reasonable ranges.

10.2 Production Fermenter Sizing

Another variable that was considered was the size of vessels for fermentation. A seed fermentation train is needed in order to grow up the cells to production volume. The 1 mL vial of cells that is purchased from the lab cannot be dumped directly into a large 200,000L vessel. Proper scale up through the train is required for effective malonic acid production in the production fermenter. According to industry standards, the seed fermentation line should increase by around a factor of 10 from vessel to vessel. Therefore once the size of large production fermenter was determined, the sizes of the fermenters in the seed train can be determined. Various sizes of fermenters, including 100,000 L, 200,000 L, 400,000 L, and 600,000 L vessels, were considered. Vessels of sizes greater than 200,000L were found to be extremely expensive, produced a lower final concentration of malonic acid, and generated a lot of heat. Vessels under 200,000 L would require too many batches to be produced a year to meet demand. A 200,000 L vessel was found to have the optimal balance of pricing, heat generation, malonic acid concentration, and number of batches per year.

10.3 Batch vs Continuous Downstream Processing

Once the upstream process was finalized, the downstream methods of separation and purification of the product needed to be determined. Upon assessing all of the different options to achieve a final pure product, the initial decision to be made was whether the downstream process

should be completed as a batch or a continuous process. While the upstream process is completed in batches, a continuous process was developed for the downstream section. The appeal of the continuous process is shorter run times, smaller vessels, and easier scheduling. Initial research concluded that all of the steps in required in the downstream process could be completed continuously. In order to convert the initial batch process to continuous processing, a harvest tank will be used after the final batch fermenters in order to collect the fermentation broth from each batch and hold it until it is pumped out of the tank into the continuous downstream process.

10.4 Removal of Biomass from Fermentation Broth

As mentioned above, the yeast cells will be genetically modified to secrete the malonic acid. This removes the need to lyse the yeast cells after fermentation to access the malonic acid. However, in order to further purify the malonic acid, the biomass needs to be removed from the broth. Initially, a centrifugation route was to be taken. However, due to the large size of the yeast cells, the final production line will use a vacuum belt filter to remove the solid biomass into a wet cake. This option lowers the cost of this step, compared to centrifugation, in addition to reducing the large amount of time and energy centrifugation required to completely remove the biomass. A vacuum belt filter will take the fermentation broth, press the biomass into a wet cake, and remove most of the valuable liquid that will need further purification. It is also assumed the growth media in the broth will be removed as part of the wet cake.

10.5 Filtration Rinse

Another decision that needed to be made was about the need for a displacement wash of the wet cake on the second half of the belt. This displacement wash will help to collect greater amounts of the product as opposed to losing it in the wet cake. This displacement wash water will be recycled from the fluids removed in the reverse osmosis step that is next in the downstream process. Washing the wet cake with a mixture of water plus some removed side products will not dilute the filtered fluid as much as it would with just a water rinse. The addition of a displacement wash lowered the wet cake liquid percentage from 40% to 20%. This rinse during the filtration step will prevent the loss of valuable malonic acid which is at this point still mixed in with the water and other side products in a liquid mixture.

10.6 Increasing the Malonic Acid Concentration before Crystallization

Malonic acid is naturally a crystalline solid at 30°C, so the purpose of the downstream process is to crystallize a 99.9% pure form of malonic acid. However, before the crystallization of the acid can occur, the concentration of malonic acid needs to be increased in order to allow for more of the product to be crystallized. This is achieved by a reverse osmosis step. The fluid that was filtered out from the vacuum belt filter will be pumped through a pressurized reverse osmosis filter. By using a membrane pore filter of 0.3 nm, 63% of the water will be removed in addition to around 5% of the other fluid components. This continuous filtration step will increase the mass percentage of malonic acid from 12% to 23% in the broth. This increase in malonic acid concentration will allow for a greater amount of malonic acid to be solidified during the crystallization step that is next in the downstream process. Additionally, as mentioned above, a

portion of the liquid removed from the reverse osmosis step is recycled into the vacuum belt filter in the previous step as the displacement wash.

10.7 Crystallization of Malonic Acid

In order to solidify the pure malonic acid, crystallization was chosen to achieve the final product. Specifically, crystallization by temperature will effectively produce enough malonic acid to meet production requirements. Other separation processes considered were chemical crystallization or esterification, but these methods required further processing to purify malonic acid from these chemical products and was deemed to consume too many additional resource to be effective. Running the remaining broth through a crystallizer that will be cooled to 15°C by a cooling jacket surrounding the vessel will lower the solubility of malonic acid to 1.12 g/L of solvent. This will allow for more than enough malonic acid to be solidified from the broth. A solubility curve of malonic acid in water was used to model the crystallization step as the broth only contains 0.4 mass percent of ethanol and 0.5 mass percent of succinic acid. Thus, in order to accurately model the solubility of malonic acid, the minute amounts of ethanol and succinic acid were neglected.

After crystallization, the vessel will produce a wet slurry of solidified malonic acid within the remaining fluid. This slurry will then be placed on another vacuum belt filter, this time to retain the solidified malonic acid. Just as in the first vacuum belt filter, there will be a displacement wash to clean off the slurry. This wash will be performed with pure DI water in order to not add more ethanol and succinic acid to the final product

10.8 Drying of Final Product

As with the first vacuum belt filter, the filter after the crystallizer will produce a 20% wet cake of solidified malonic acid after a displacement wash. This wet cake will need to be dried for future packaging and shipment. A dryer belt was considered, however due to the large quantity of product in each batch, the belt was deemed too inefficient and would require too large of a surface area. Instead, a fluidized bed dryer was chosen. The product will be run through a bed that will flow air through the solid in order to dry the surface area of the product which will be emptied into bins for packaging once it is completely dry.

10.9 Downstream Vessel Sizing

Another variable considered was the flow rate of the continuous process and the sizing of the crystallizer. The flow rate of the overall process was determined from the residence time of the crystallizer as this is the longest part of the downstream process. The rest of the downstream time was based off the flow rate required into the crystallizer. A flow rate of 12,000 L/hr for a six hour process was determined to be the optimal flow rate in order to be able to separate and purify a final batch by the time the next fermentation batch is ready for purification. Based on this flow rate and an assumed filling capacity of 80%, a 15,000 L crystallizer was chosen to be the optimal size of the crystallization vessel to be able to handle the necessary flow rate of liquid in the process.

For the belt filters and reverse osmosis components, simple industrial standards were chosen for vessel sizes and the timing was calculated based on the size as these are quick components in the process.

Lastly, the fluidized dryer was sized based on a 40% air capacity in order to dry the solid acid quickly and completely. Using a residence drying time of 30 mins, a 3,000 L dryer was determined to be the desired size of the vessel.

10.10 Downstream Pumps

The final consideration for the downstream process was the pumps between each vessel. The large quantities of broth need to be moved efficiently between vessels for continuous processing. Centrifugal pumps were added between all vessels in the first half of the downstream process. While pumps were considered to be added in the second half, the movement of the product will be gravitational as after the crystallizer there will be a solidified slurry, which can clog the pumps. The potential destruction or inefficiency of the pumps was enough to change the process to gravitational movement once solids are formed in order to prevent future repairs or inefficiencies in the final steps of the process.

Section 11: Assembly of a Database

11.1 Material and Energy Components

Table 1. Chemical Properties. This table includes the molecular weight, density, and specific heat capacity that were used throughout calculations in both mass and energy balances.

	Molecular Weight [g/mol]	Density [kg/m^3]	Specific Heat Capacity [kJ/kgK]
Water	18.02	1000.0	4.18
Malonic Acid	104.06	1620.0	2.87
Ethanol	46.07	785.1	2.46
Succinic Acid	118.09	1560.0	1.30
Air	28.97	1.225	1.00
Oxygen	15.99	1.429	0.92
Nitrogen	28.01	1.165	1.04
CO_2	44.01	1.980	0.85
NaOH	39.99	1250.0	0.71

Table 2. Heat of Formations. This table includes the heat of formation of components that were used to calculate the energy balances for the reactions in the fermenters.

	Heat of Formation [kJ/mol]
Water	-285.8
Malonic Acid	-891.0
Ethanol	-277.7
Succinic Acid	-939.0
Oxygen	0.0
Nitrogen	0.0
CO_2	393.5

These values were used throughout the project to for material and energy calculations.

The values were taken from NIST, the National Institute of Standards and Technology.

Table 3. Heat of Formation of *P. Kudriavzevii* in Glucose. This table finds the heat of formation of the yeast cells that were used based on their growth properties in glucose.

Calculation of Heat of Formation of *P. Kudriavzevii* Cells	
$y_{x/s}$ [g cells/mol glucose]	90.0
$y_{x/O2}$ [g cells/g oxygen]	0.97
ΔH_c [kJ/g cell]	21.2
ΔH_s [g glucose/kJ]	0.101
Y_h [g cell/kJ]	-0.047

The values used to calculate the heat of formation of the *P. Kudriavzevii* cells shown in Table 3 were taken from the textbook, *Bioprocess Engineering*, by Shuler and Kargi, 2002.

The heat of solidification of malonic acid (28.3 kJ/mol) was taken from *Xie, Sun, 2017* where the heat of solidification was determined for adipic acid. Adipic acid is considered a comparable substance for calculations throughout the project as there is limited literature on malonic acid itself.

11.2 Economic Components

The values used for the economic analysis were determined through the Equipment Costing and Profitability Analysis Spreadsheet. Additional equipment costs were found from vendors, such as Pollution Systems, Inc. and RPA Process Inc. Other pricing considerations were found from *Product and Process Design Principles* by Seider, Lewin, and Seader

Section 12: Process Description, Process Flow Diagram, and Material Balance

12.1 Overall Process

This section of the report presents the process flow diagram, material balance, and process description for the production of malonic acid. The block flow diagram in Figure 5 depicts the two main sections of the process: fermentation and purification.

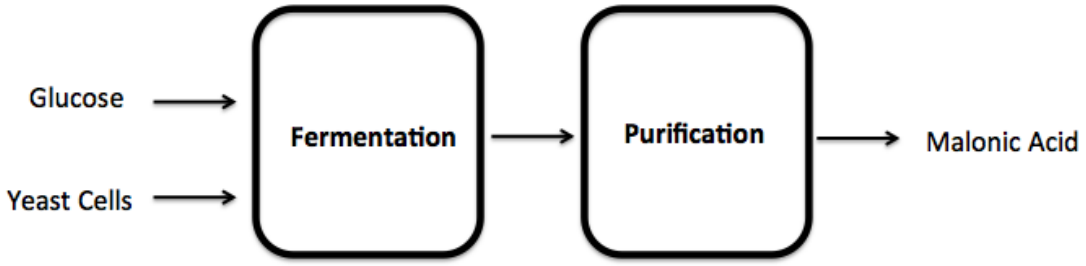

Figure 5. Block Flow Diagram

The detailed flow diagram is depicted in Figure 6 along with an accompanying description. The process is then divided into two main sections where each of these sections has their own detailed process flow diagrams, process descriptions, and materials balances. The majority of the calculations were performed using Microsoft Excel due to the limitations associated with Aspen and Superpro, specifically when dealing with solids and the unique equipment required in this process. Visio was used to create the flowsheet and diagram of the overall process due to the limitations in number of vessels available in Aspen and Superpro.

The overall process can be described briefly as follows. The plant receives a 1 mL vial of genetically modified yeast cells from the Lygos lab. The vial of cells is grown up in the lab through a chain of 4 small glass seed fermenters. The final lab scale fermenter is then used to begin the manufacturing scale fermentation. The cells are grown for 48 hours in a seed line

consisting of 3 seed fermenters. The fermentation broth is then sent to the final production fermenter for 50 hours. The vessels are also fed with glucose, various growth medium, and oxygen to produce malonic acid at the end of the production fermentation process. In order to produce 10M lbs of malonic acid a year, 236 batches are required. The fermentation broth is stored in a harvest tank once ready to be purified in the downstream processing. Unlike the upstream process, the downstream process is continuous. The fermentation broth is sent to a vacuum belt filter where the wet cake, consisting of the biomass, is removed. The remaining liquid undergoes reverse osmosis to concentrate the desired malonic acid, by removing large amounts of excess water. Finally 99.9% pure malonic acid is produced through crystallization followed by filtration and drying. The malonic acid is then l to be shipped off to the customer.

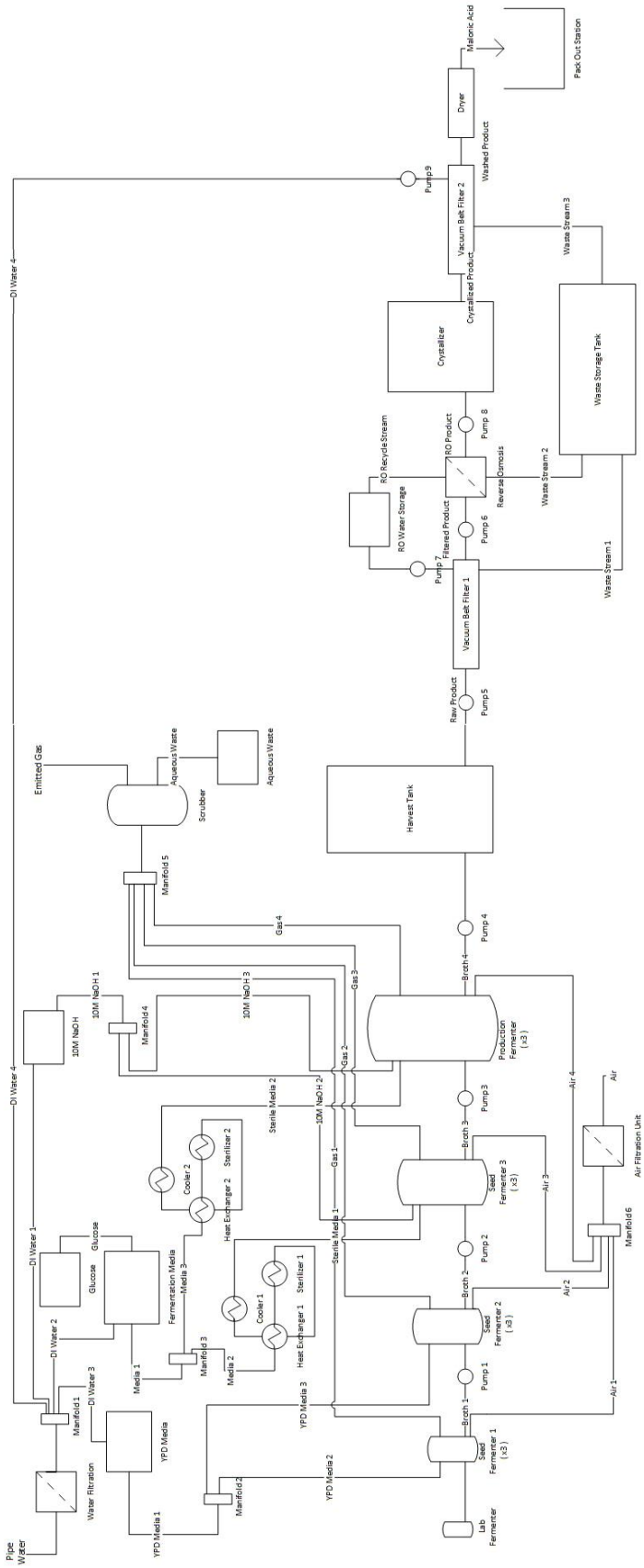

Figure 6. Detailed Process Flow Diagram. This process flow diagram depicts both the upstream and downstream process required to produce 99% pure malonic acid. The cells are first grown in a seed fermentation train and then malonic acid is produced in the large production fermenter. The broth is stored in a harvest tank and is fed continuously to the downstream processing. The broth then undergoes vacuum belt filtration, reverse osmosis, and crystallization to produce the final product.

12.2 Fermentation

12.2.1 Lab Fermentation

Before the cells can be placed in the first seed fermenter, they must be grown up to the desired concentration from the 1 mL vial received from the Lygos laboratory. Figure 7 demonstrates the laboratory scale up of the yeast cells. A factor of 10 is commonly used in industry for scaling up fermenters. The fermentation will be conducted at 30°C and grown overnight. The final concentration of cells after this process is 8.04 mg/L.

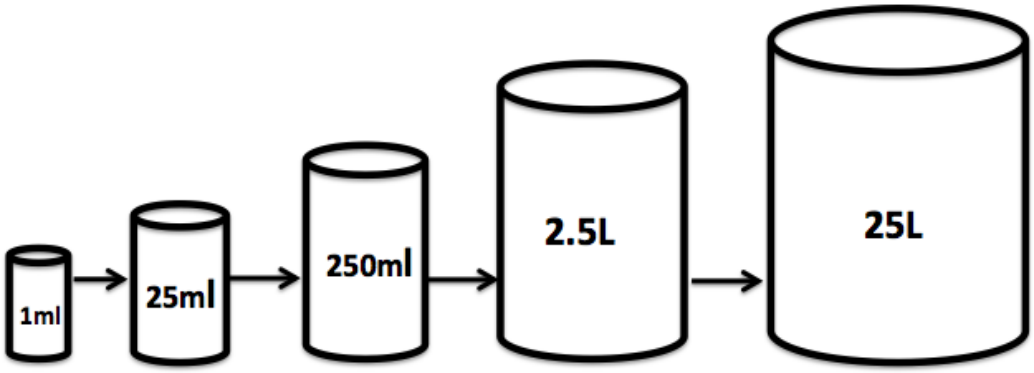

Figure 7. Lab Scale Fermentation. This image represents the lab scale up fermentation process. Lab scale up is required to turn the 1ml vial into a large volume, which can be fed into the larger scale manufacturing line.

12.2.2 Fermentation Reactions

There are four main reactions that occur during the fermentation process. The first reaction produces malonic acid, the second reaction produces succinic acid, the third reaction produces ethanol, and the final reaction produces more biomass. The first reaction produces the desired product, while the second and third reactions represent undesired side products. The last reaction is required for cell growth. As seen from these reactions, oxygen is required, and the process is aerobic. Figure 8 provides the four reactions, properly balanced according to stoichiometry.

$$Rxn\ 1: 2C_6H_{12}O_6 + 6O_2 \rightarrow 6H_2O + 3CO_2 + 3C_3H_4O_4$$

$$Rxn\ 2: 2C_6H_{12}O_6 + 7O_2 \rightarrow 3H_2O + 6CO_2 + 3C_2H_6O_4$$

$$Rxn\ 3: 5C_6H_{12}O_6 + 19O_2 \rightarrow 12H_2O + 6CO_2 + 36H_6O_4$$

$$Rxn\ 4: C_6H_{12}O_6 + 0.2775O_2 \rightarrow 1.925H_2O + CO_2 + 2.633C_{1.9}H_3O$$

Figure 8. Reactions Occurring During Fermentation. These 4 reactions describe the chemical reactions that take place during fermentation. The first reaction produces malonic acid, the second reaction produces succinic acid, the third reaction produces ethanol, and the final reaction produces more biomass.

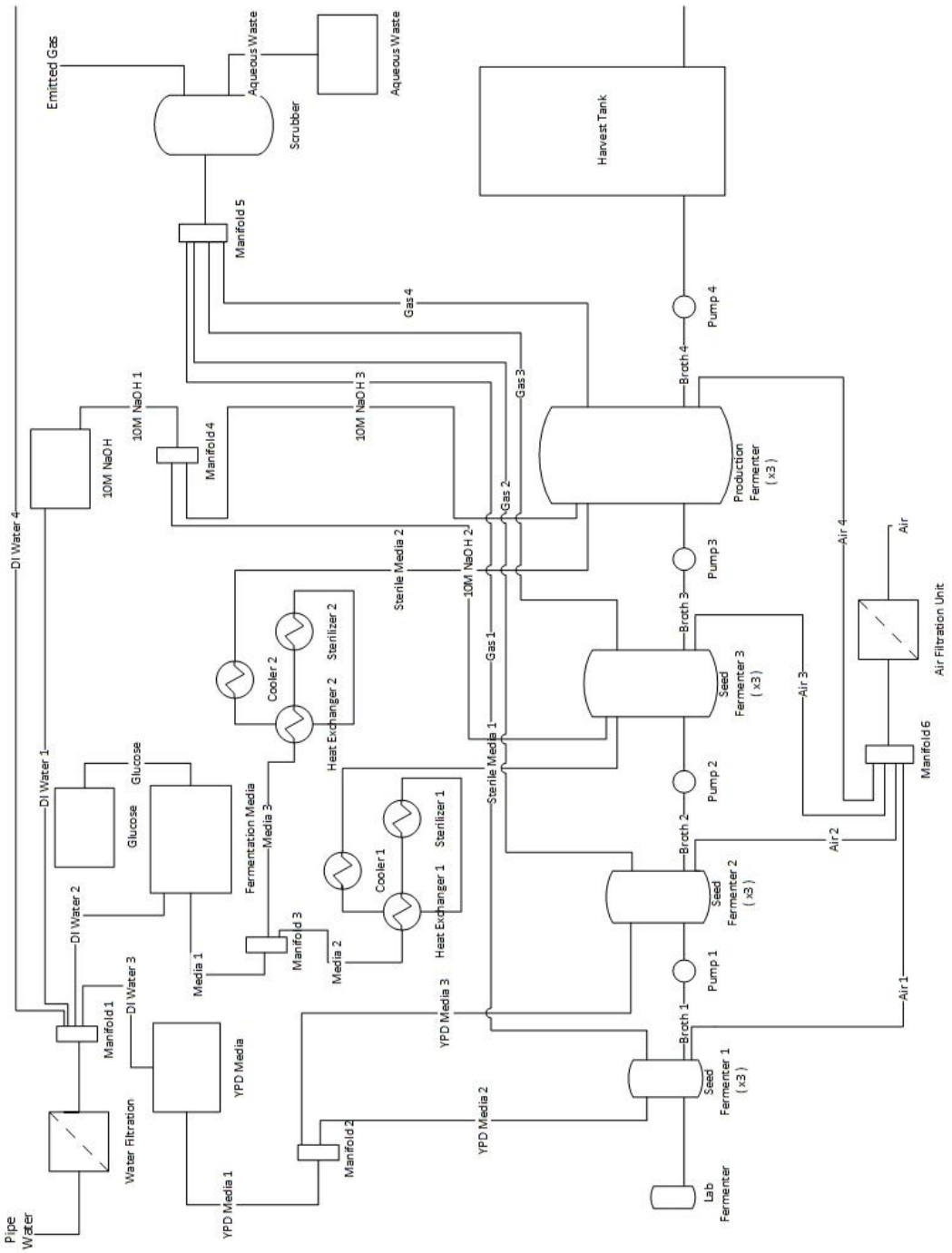

Figure 9. Process Flow Diagram for Fermentation. This figure depicts just the upstream process, consisting of a seed fermentation train and a large production fermenter. This process is all batch processing. The fermenters are fed with media and oxygen to allow for optimal growth and malonic acid production. One fermentation batch takes 101 hours.

12.2.3 Seed fermenter 1

Figure 9 demonstrates the complete process for fermentation. The final lab vessel will be dumped into seed fermenter 1. Additionally, utility water is purified in the water filter where the stream (DI Water 3) is added to the YPD mixture in the YPD Media tank. The tank mixes the media continuously at 300 rpm. The stream (YPD Media 1) is split, by Manifold 2, into two streams and YPD Media 2 is sent directly into seed fermenter 1. Once in the vessel, the media is sterilized by first heating the liquid to 121°C and then cooling it back down to 30°C. The media is sterilized in the vessel, by the jacket, instead of in an external heat exchanger because of its small volume. The YPD media is added to the cells in the fermenter to help facilitate growth. The important components of YPD media are water, glucose, and yeast extract. The exact composition of the media can be found in the section 21.2 in the appendix. Since the process is aerobic, oxygen is also continuously flowed into the vessel. Air is filtered in the air filtration unit and the stream of filtered air (Air 1) is sent to seed fermenter 1 at a rate of 1VVM. Much more oxygen is sent to the fermenters than required because the percent of mass transfer of oxygen to the cells is limited. The broth remains in the first seed fermenter for 16 hours and has a growth rate of 0.3 $\frac{1}{hr}$. The fermenter is kept at 30°C by a cooling jacket and the broth is constantly mixing and agitated. The gases produced during this reaction are constantly being removed through a stream (Gas 1) and sent to the scrubber. Under these conditions the majority of the glucose is used to grow the cells. The exact yield of each reaction are 20%, 3%, 2%, and 75% respectively. Using these values, a mass balance on the first seed fermenter was conducted, as seen in Table 4. Table 4 also shows the final volume, cell concentration, and malonic acid concentration.

Table 4. Mass Balance on Seed Fermenter 1. This table shows the mass balance for the first seed fermenter as well as the final working volume, cell concentration, and malonic acid concentration. This fermentation process takes place over 16 hours.

Mass Balance on Seed Fermenter 1

Stream ID	Lab Fermenter	YPD Media 1	Air 1	Gas 1	Broth 1
Total [lb/batch]	35	417	616	615	453
Component Flow [lb/batch]					
Glucose	0	0.087	0	0	0
Oxygen	0	0	143	142	0
Nitrogen	0	0	473	473	0
Cells	0.00035	0	0	0	0.043
Water	35.3	417	0	0	452
Malonic Acid	0	0	0	0	0.015
Succinic Acid	0	0	0	0	0.001
Ethanol	0	0	0	0	0.001
CO2	0	0	0	0.025	0
NaOH	0	0	0	0	0
YPD	0	0.022	0	0	0.022
YNB	0	0	0	0	0
Concentration of Cell [g/L]				0.09	
Concentration of Malonic Acid [g/L]				0.03	
Total Volume [L]				216	

12.2.4 Seed Fermenter 2

The content in seed fermenter 1 (Broth 1) is pumped into seed fermenter 2. Identical to the process for seed fermenter 1, the stream (YPD Media 3) is then sent directly into seed fermenter 2 where the media is sterilized by heat and cooling through the jacket. The YPD media is added to the fermentation broth 1 to help facilitate growth in seed fermenter 2. Air is filtered in the air filtration unit and the stream of filtered air (Air 2) is sent to seed fermenter 2 at a rate of 1VVM. The broth remains in seed fermenter 2 for 16 hours with a growth rate of 0.3 $\frac{1}{hr}$. The fermenter is held at 30°C using a cooling jacket. The fermentation broth in seed fermenter 2 is constantly being mixed and agitated. The gases produced during fermentation are constantly being removed through a stream (Gas 2) and sent to the scrubber. Under these conditions the majority of the glucose is used to grow the cells with the yield of each reaction being 20%, 3%, 2%, and 75% respectively. Table 5 demonstrates the complete mass balance on the second seed fermenter as well as the final working volume, cell concentration, and malonic acid concentration.

Table 5. Mass Balance on Seed Fermenter 2. This table shows the mass balance for the second seed fermenter as well as the final working volume, cell concentration, and malonic acid concentration. This fermentation process takes place over 16 hours.

Mass Balance on Seed Fermenter 2

Stream ID	Broth 1	YPD Media 2	Air 2	Gas 2	Broth 2
Total [lb/batch]	450	4,170	6,160	6,150	4,630
Component Flow [lb/batch]					
Glucose	0	10.6	0	0	0
Oxygen	0	0	1,430	1,420	0
Nitrogen	0	0	4,730	4,730	0
Cells	0.043	0	0	0	5.23
Water	452	4,160	0	0	4,620
Malonic Acid	0.015	0	0	0	1.85
Succinic Acid	0.001	0	0	0	0.168
Ethanol	0.001	0	0	0	0.123
CO2	0	0	0	3.01	0
NaOH	0	0	0	0	0
YPD	0.022	0.22	0	0	0.242
YNB	0	0	0	0	0
Concentration of Cell [g/L]				1.1	
Concentration of Malonic Acid [g/L]				0.38	
Total Volume [L]				2,220	

12.2.5 Seed Fermenter 3

The content in seed fermenter 2 (Broth 2) is pumped into seed fermenter 3. Additionally, utility water is purified in the water filter. The water stream (DI Water 2) and the glucose stream (Glucose) are added to a YNB mixture in the Fermentation Media tank. The tank mixes the fermentation media continuously at 300 rpm. The stream (Media 1) is split (Manifold 3) and the remaining stream (Media 2) is then sent through a heater exchanger network (Heat Exchanger 1, Sterilizer 1, and Cooler 1) to sterilize the media before the stream (Sterile Media 1) enters the third seed fermenter. The media is sterilized in an external heat exchanger network because the volume of the fluid is too great to sterilize in the vessel. The fluid is heated to 121°C and then cooling it back down to 30°C to properly sterilize it. The exact specification of the heat exchanger can be found in the Energy and Utilities section of this report. The YNB is added to the cells in the fermenter to help facilitate growth and malonic acid production. The important components of fermentation media are water, glucose, and ammonium sulfate. The composition of the YNB media can be found in section 21.2 of the appendix. Since the process is aerobic, oxygen is also continuously added to the system. A stream of filtered air (Air 3) is sent to seed fermenter 3 at a rate of 1VVM. The broth ferments in the third seed fermenter for 16 hours and has a growth rate of 0.2 $\frac{1}{hr}$. The fermenter is kept at 30°C using a cooling jacket and the broth is constantly mixed and agitated. The desired pH range of the fermentation process is between 2.5 and 3. Since more malonic acid is produced in seed fermenter 3 than in the previous two, base is added to control the pH. Purified water (DI water 1) is mixed with NaOH to produce a 10M NaOH solution in the 10M NaOH mixing vessel. The tank mixes the NaOH soluion continuously at 300 rpm. The stream (NaOH 1) is then added to the third seed fermenter. Based on the nature that microorganisms cannot grow in this concentration of sodium hydroxide, the stream does not

need to be sterilized before entering the vessel. The gases produced during fermentation are constantly removed through a stream (Gas 3) and sent to the scrubber. Under these conditions the majority of the glucose is used to produce malonic acid. The exact yield of each reaction are 90%, 3%, 2%, and 5% respectively. Using these values, a mass balance on the production fermenter was conducted, as seen in Table 6. Table 6 also shows the final working volume, cell concentration, and malonic acid concentration.

Table 6. Mass Balance on Seed Fermenter 3. This table shows the mass balance for the third seed fermenter as well as the final working volume, cell concentration, and malonic acid concentration. This fermentation process takes place over 16 hours.

Mass Balance on Seed Fermenter 3

Stream ID	**Broth 2**	**Sterile Media 1**	**Air 3**	**10M NaOH 2**	Gas 3	Broth 3
Total [lb/batch]	4,620	33,400	61,700	1	61,500	38,200
Component Flow [lb/batch]						
Glucose	0	392	0	0	0	0
Oxygen	0	0	14,400	0	14,050	0
Nitrogen	0	0	47,300	0	47,300	0
Cells	5.23	0	0	0	0	128
Water	4,620	32,900	0	0.684	0	37,700
Malonic Acid	1.85	0	0	0	0	307
Succinic Acid	0.168	0	0	0	0	6.33
Ethanol	0.123	0	0	0	0	4.63
CO2	0	0	0	0	145	0
NaOH	0	0	0	0.007	0	0.0068
YPD	0.242	0	0	0	0	0.24
YNB	0	126	0	0	0	126
Concentration of Cell [g/L]				3.3		
Concentration of Malonic Acid [g/L]				8.0		
Total Volume [L]				17,400		

12.2.6 Production Fermenter

The content in seed fermenter 3 (Broth 3) is pumped into the production fermenter. Identically to seed fermenter 3, the stream (Media 3) is sent through a heat exchanger network to sterilize the media before the stream (Sterile Media 2) enters the production fermenter. The YNB is added to the cells in the fermenter to help facilitate growth and malonic acid production. A stream of filtered air (Air 4) is sent to the production fermenter at a rate of 1VVM. The broth ferments in the production fermenter for 50 hours and has a growth rate of 0.1 $\frac{1}{hr}$. The fermenter is kept at 30°C using a heat exchanger and the broth is constantly mixed and agitated. Because the majority of the malonic acid is produced in the production fermenter, base is added to maintain the pH between 2.5-3. Purified water (DI water 1) is mixed with NaOH to produce a 10M NaOH solution in the 10M NaOH mixing vessel. The tank mixes the solution continuously at 300 rpm. The gases produced during fermentation are constantly removed through a stream (Gas 4) and sent to the scrubber. Under these conditions the majority of the glucose is used to produce malonic acid. The exact yield of each reaction are 90%, 3%, 2%, and 5% respectively. Table 7 demonstrates the mass balance on the production fermenter. Table 7 also shows the final volume, cell concentration, and malonic acid concentration. After 50 hours, a concentration of 125 g/L of malonic acid is achieved. From each batch, 47,300 lbs of malonic acid are produced. In order to manufacture a sufficient amount of malonic acid through fermentation to produce 10M lbs after downstream processing, 236 batches are needed a year. Therefore the total yearly production of malonic acid from the fermentation process is 11.2M lbs in order to account for the 7.3% of malonic acid lost in downstream processing and provide a final amount of 10M lbs. The broth (Broth 4), containing the desired malonic acid, is pumped into a harvest tank where it awaits downstream processing.

Table 7. Mass Balance on Production Fermenter. This table shows the mass balance for the production fermenter as well as the final working volume, cell concentration, and malonic acid concentration. This fermentation process takes place over 50 hours. This process is where the majority of malonic acid is produced.

Mass Balance on Production Fermenter

Stream ID	Broth 3	Sterile Media 2	Air 4	10M NaOH 3	Gas 4	Broth 4
Total [lb/batch]	38,200	347,000	1,540,000	902	1,530,000	395,000
Component Flow [lb/batch]						
Glucose	0	60,200	0	0	0	0
Oxygen	0	0	356,00	0	325,000	0
Nitrogen	0	0	1,183,000	0	1,183,000	0
Cells	128	0	0	0	0	19,000
Water	37,700	285,000	0	893	0	325,000
Malonic Acid	307	0	0	0	0	47,000
Succinic Acid	6.33	0	0	0	0	954
Ethanol	4.63	0	0	0	0	697
CO2	0	0	0	0	22,300	0
NaOH	0.0068	0	0	8.94	0	0
YPD	0.24	0	0	0	0	0.24
YNB	126	1,220	0	0	0	1,220
Concentration of Cell [g/L]					51	
Concentration of Malonic Acid [g/L]					126	
Total Volume [L]					170,000	

12.2.7 Scheduling

In order to ensure that the goal of the production capacity of 10M lbs of malonic acid per year would be met, the fermenters were scheduled, taking into account the required fermentation time in each vessel, time to clean and sterilize each vessel after each batch, and time to transfer all media and the fermentation broth in and out of each vessel.

One batch takes about 101 hours from the inoculation of the first seed fermenter to the end of fermentation in the large production fermenter. To meet the requirement of the production of 10M lbs of malonic acid in a year, 236 batches must be completed in a span of 47 operating weeks, which translates to about five batches per week. Based on the amount of malonic acid produced from each batch, only 231 batches are required to produce 10M lbs of malonic acid, but 236 batches is determined as the final number in order to account for inefficiencies, maintenance, and potential problems that may arise causing downtime and delayed production. This requires a total of three seed fermentation lines and three production fermenters. A new batch is started about every 18 hours in order to ensure constant production of malonic acid to be sent to the continuous downstream process.

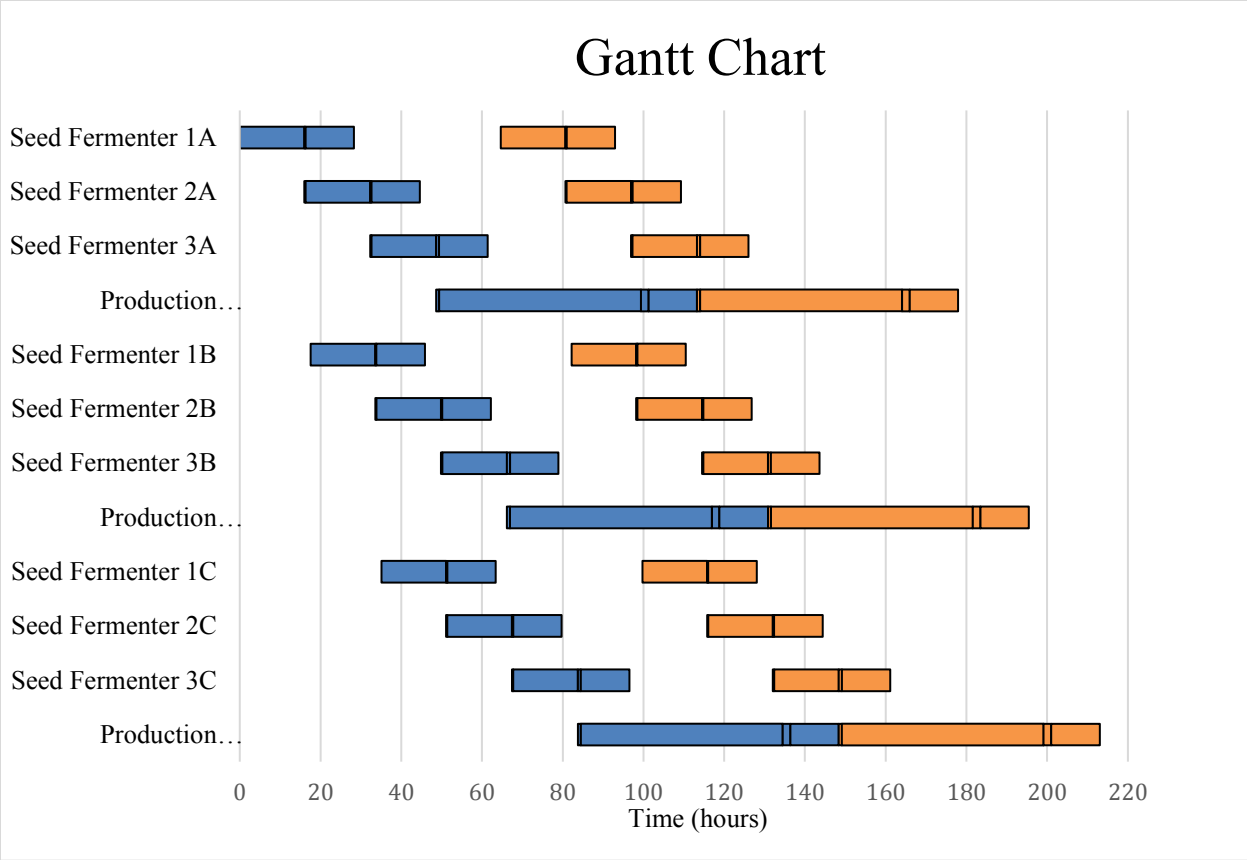

Figure 10. Gantt Chart of Upstream Processing. This image shows the scheduling for the upstream fermentation of the broth for two runs using the three production lines. The downstream process was not scheduled as it is a continuous process that is timed to run between each batch.

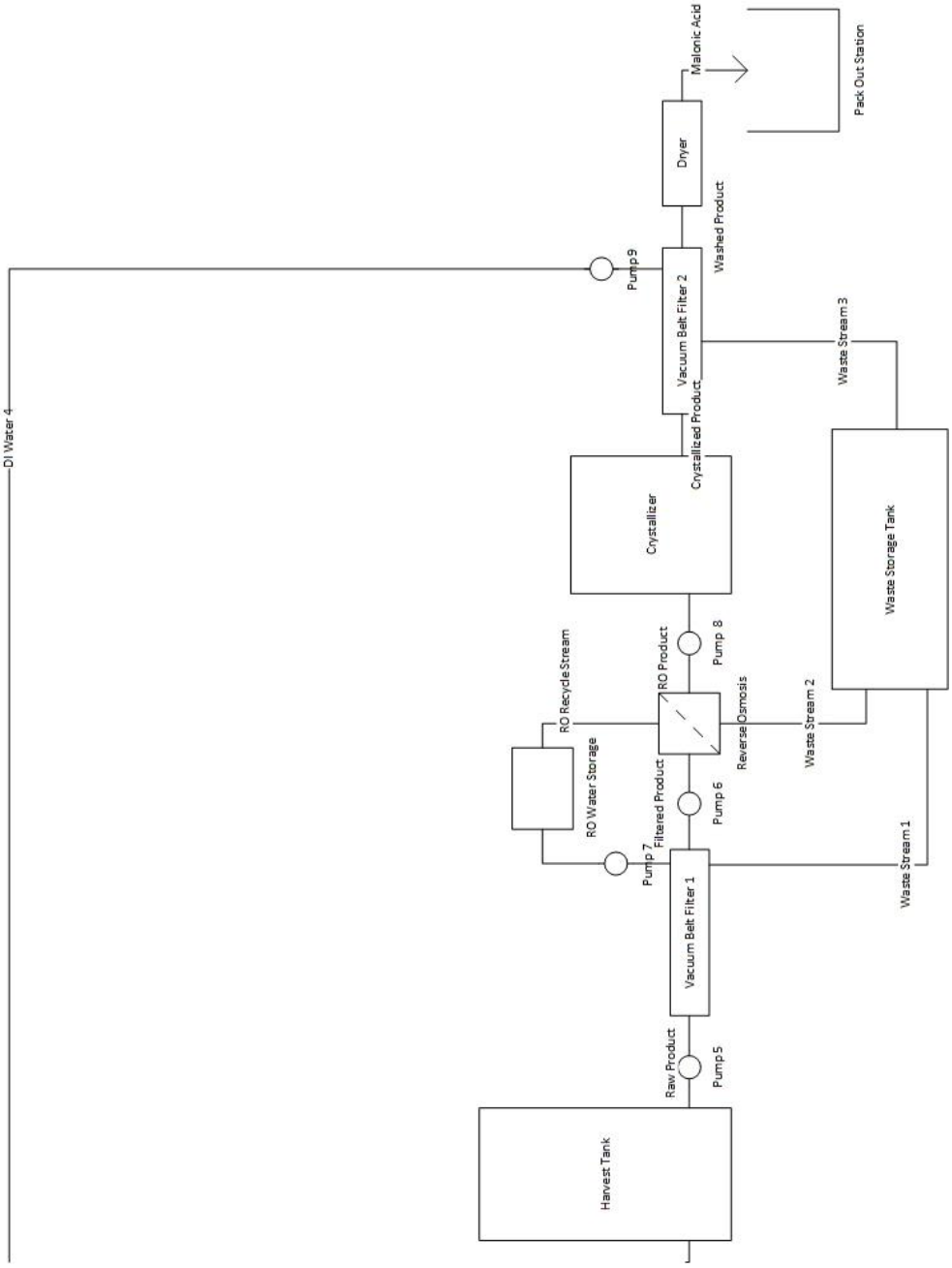

Figure 11: Downstream Processing. This figure shows the steps that are taken from the fermentation broth to separate the broth and product 99% pure solid malonic acid.

12.3 Separation and Purification

12.3.1 Vacuum Belt Filter

The broth stored in the harvest tank is pumped into the first vacuum belt filter to remove the biomass and growth media from the broth. The broth is sent through a centrifugal pump at 400 gpm onto the vacuum belt filter. The vacuum belt filter boasts a capacity to filter 480,000 lb a day. Using this rate and the 33,000 lb of fermentation broth that needs to be filtered, the vacuum belt filter will run for 1.7 hours to filter the full contents of a single batch. At the second half of the belt filter, a displacement wash is sent over the filter. This wash, recycled from part of the reverse osmosis removal stream, will be 25% of the fluid removed from reverse osmosis. This will come from a 35,000 L storage tank that will initially be filled with clean water but will fill up with removed reverse osmosis fluid as the plant begins to run. The displacement wash will consist of 30,000 L of water per batch in addition to 160 L of malonic acid, 5 L of ethanol and 3.5 L of succinic acid. This filtering process will remove all of the biomass and growth media in addition to 3.25% of the fluid as it will produce a 20% in mass wet cake. The overall mass balance of the vacuum belt filter is shown in Table 8. This table also shows the final volumes and concentrations of the fluid components after filtration. From each batch, 8.5 million g of biomass will be removed. This biomass will be sent into a waste storage tank that will eventually be sold to a waste management facility. The liquid remaining from this filtration is then pumped to a reverse osmosis filter to remove water in order to increase the concentration of malonic acid in the liquid for future crystallization.

Table 8. Vacuum Belt Filter 1 Mass Balance. This table shows the mass of the streams entering and leaving the first vacuum belt filter in the downstream process where the wet cake of biomass is removed.

	Raw Product	Displacement Wash	Waste Stream 1	Filtered Product
Total [lb/batch]	409,000	66,600	35,000	440,000
Biomass	19,000	0	19,000	0
YPD	0.24	0	0.24	0
YNB	1,200	0	1,200	0
Water	340,000	66,000	13,000	390,000
Malonic Acid	47,000	570	1,600	46,000
Ethanol	700	8	33	675
Succinic Acid	950	12	31	931

12.3.2 Reverse Osmosis

The filtered liquid is pumped at 400 gpm into a reverse osmosis filter in order to remove some water to attain a higher mass concentration of malonic acid. The reverse osmosis system will process the fluid at 400 gpm for 2 hours. By using a pore membrane size of 0.3nm, the water in the broth will be removed. A model was used to determine that 63% of the water will be removed to increase the malonic acid mass concentration from 12% to 23%. In the removal of the water, it was also assumed that 5% of the other valuable liquid will be lost as well. This waste stream will then be split, where 25% of the stream will be recycled back into a storage tank to be used as the displacement wash for the first vacuum belt filter while the other 75% will be sent into the waste storage tank for future processing. Table 9 shows the mass balance for the reverse osmosis system. For each batch 120,000 L of liquid will be removed from the overall broth during this step. The remaining 80,000 L desired stream that is rich in malonic acid is sent to the crystallizer.

Table 9. Reverse Osmosis Mass Balance. This table shows the mass balance of the reverse osmosis system which removes water to increase the malonic acid concentration in the target stream.

	Filtered Product	RO Recycle Stream	Waste Stream 2	RO Product
Total [lb/batch]	440,000	66,600	188,000	195,400
Water	390,000	66,000	186,000	147,000
Malonic Acid	46,000	570	1,700	44,000
Ethanol	675	8	25	640
Succinic Acid	930	12	34	890

12.3.3 Crystallizer

The liquid mixture that is rich in malonic acid from the reverse osmosis system is sent to the crystallizer to solidify the pure malonic acid that will eventually be the final product. The liquid is pumped into the continuous crystallizer at 200 gpm. Using a flow rate of 13,000 L/hr and a residence time of 1 hour, the continuous crystallization will take 6 hours to crystallize an entire batch. Based on an 80% filling capacity, the crystallizer will by 15,000 L. The mixture entering the crystallizer is 23% malonic acid by weight, 76% water, and the remaining 1% is the side products of ethanol and succinic acid. The side products present a negligible concentration in the liquid so the solubility of malonic acid in water was used to model the crystallizer. Section 21.5 in the appendix shows the solubility curve between malonic acid and water. From this curve, the targeted temperature of 15°C was chosen to crystallize the malonic acid. This temperature is low enough to solidify enough malonic acid without freezing the total mixture and it will be achieved with a cooling brine around the vessel. At this temperature, the solubility of malonic acid in water is 1.13 g/L. Under these conditions, 99% of the malonic acid will be solidified. The crystallizer will not dispose of any waste as the stream is now a slurry containing solids. This slurry will be sent to a second vacuum belt filter to remove the malonic acid crystals from the mixture. The mass balance for the continuous concentration is shown in Table 10.

Table 10. Crystallization Mass Balance. This table shows the mass balance of the crystallizer where 99.6% of the malonic acid is crystallized.

	RO Product	Crystallized Product
Total [lb/batch]	195,000	195,000
Water	150,000	150,000
Malonic Acid	44,000	170
Ethanol	650	650
Succinic Acid	890	890
Solidified Malonic Acid	0	43,800

12.3.4 Vacuum Belt Filter

The crystallization slurry is gravitationally moved onto a vacuum belt filter to dry and clean the malonic acid crystals in time with the crystallizer. This belt filter is timed to run 5 hours in order to begin drying the slurry as soon as it begins to leave the crystallizer at a lower capacity of 2,500 lb/hr. Just as with the first vacuum belt filter, there will be a displacement wash on the second half of the belt. However, this belt filter will be washed with pure DI water in order to prevent further contamination of the malonic acid crystals. This 1,200 L/hr wash will come directly from the plant's water filtration system. This wash will remove all excess water in order to end with a 20% wet cake of malonic acid crystals. The removed liquid will be sent to the waste storage tank for further processing. The overall mass balance of the second vacuum belt filter is shown in Table 11. After filtration, the solid malonic acid wet cake will be sent to a fluidized bed dryer to produce a dry, 99.9% pure product of malonic acid.

Table 11. Vacuum Belt Filter 2 Mass Balance. This table shows the mass balance for the second vacuum belt filter which removes the solidified malonic acid from the slurry that leaves the crystallizer.

	Crystallized Product	Displacement Wash	Washed Product	Waste Stream 3
Total [lb/batch]	195,000	13,000	57,000	151,000
Water	150,000	13,000	12,000	149,000
Malonic Acid	170	0	13	150
Ethanol	650	0	50	600
Succinic Acid	900	0	68	800
Solidified Malonic Acid	44,000	0	44,000	0

12.3.5 Fluidized Dryer

The 20% wet cake of malonic acid crystals is dried simultaneously as the rest of the batch is being filtered before storage. The cake will move gravitationally to the fluidized dryer at a flow rate of 3,500 L/hr from the vacuum belt filter. Using a residence drying time of 30 minutes and a 40% air capacity, the dryer will be sized at 3,000 L. In the fluidized dryer, air will be sent through the dryer at 3,000 L/min (1 VVM). In this dryer, the 20% of the wet cake that consists of remaining water, dissolved malonic acid, ethanol and succinic acid will be dried off and removed from the crystals. The mass balance for the fluidized drying of the malonic acid crystals can be viewed in Table 12. The air will leave the dryer and the final 99.9% pure malonic acid product will moved into bins for storage, future purity testing, and eventually shipment.

Table 12. Fluidized Bed Dryer Mass Balance. This table determines the final amount of malonic acid that is dried and how much air it takes to dry the crystals.

	Washed Product	Air In	Malonic Acid Out	Air Out
Total [lb/batch]	57,000	2,400	44,000	15,400
Water	12,000	0	0	12,000
Malonic Acid	13	0	0	13
Ethanol	50	0	0	50
Succinic Acid	68	0	0	68
Solidified Malonic Acid	44,000	0	44,000	0
Air	0	2,400	0	2,400

12.3.6 Scheduling

The downstream process is not scheduled as it is a continuous process, but it is timed to fit the 16 hours between each batch. The first vacuum belt filter will take 1.9 hours (112 minutes), reverse osmosis will take 2 hours, crystallization will take 6 hours, and the final vacuum belt filter and dryer will run for 5 hours simultaneously will the crystallizer once the stream begins to leave the vessel after its 1 hour residence time for crystallization. The pumps will also add some time to the downstream process but due to the continuous nature of the streams, they will run simultaneously with the vessels.

Section 13: Energy Balance and Utility Requirement

13.1 Energy Balance

This section of the report describes the energy requirements associated with the production of malonic acid. Energy is required to mix the media mixing tanks, agitate the fermenters, pump the fluids between vessels, and run the water filter, air filter, scrubber, vacuum belt filters, and dryer air. Energy is also produced by the four reactions that occur in the fermenters.

13.1.1 Mixing Tanks

In this process there are 3 main mixing tanks; YDP Media, Fermentation Media, and 10 M NaOH. These tanks are required as they mix the desired raw materials together. For examples, the Fermentation Media tank mixes YNB, water, and glucose. The tanks are mixed at 300 rpm using a pitch blade. Using equation 9 found in the 21. 3 of the appendix, the total power required to mix the tanks was determined. Table 13 demonstrates the total energy required to mix these tanks per batch and well as the total annual energy based on the number of batches required to produce 10M lbs of malonic acid a year.

Table 13. Mixing Tank Energy. This table represents the amount energy associated with mixing the tanks containing the YPD media, fermentation media, and base.

	YPD Media	Fermentation Media	10M NaOH
Mixing Constant	1.3	1.3	1.3
Fluid Dynamic Viscosity [Ns/m2]	798	798	798
Revolutions per Second	5	5	5
Impeller Diameter [m]	0.50	1.88	0.29
Number of Impellers	2	3	2
Mixing Constant	1.3	1.3	1.3
Power for Mixing [kW/batch]	6.6	518	1.30
Annual Energy for Mixing [kJ]			125,000,000

13.1.2 Heat of Reactions in Fermenters

During the fermentation process, there are multiple reactions occurring in each tank. The reactions produce a large amount of heat. Using the heat of reactions to form malonic acid, succinic acid, and ethanol and the heat of the oxygen uptake of yeast cells, the overall heat generated per vessel per batch was calculation. Equations 3,4, and 5 found in section 21. 3 of the appendix were used. These values can be seen in Table 14. Since the fermenters should be kept at a constant temperature of 30°C, the heat must be removed by either jackets or heat exchangers. These calculations and values can be found in the Utilities subsection of the Energy Balance and Utility Requirement section.

Table 14. Heat of Reaction for Fermentation. This table depicts the heat of the four reactions that occur in the 4 fermenters.

	Seed Fermenter 1	Seed Fermenter 2	Seed Fermenter 3	Production Fermenter
RX 1 [kJ]	-78	-9,480	-1,580,000	-243,000,000
RX 2 [kJ]	1	129	4,780	734,000
RX 3 [kJ]	-4	-534	-19,800	-3,040,000
Rx 4 [kJ]	-108	-12,800	-17700	-1,080,000
Energy [kJ/batch]	-189	-22,700	-1,610,000	-246,000,000

13.1.3 Fermenter Agitation

The fermenter must be continuously agitated in this aerobic process. Agitation ensures that a uniform suspension of microbial cells is achieved in a homogenous nutrient medium. Agitation increases the rate of oxygen transfer from the air bubble to the liquid medium. This in an important component of the fermenter but also requires energy and produces a large amount of heat. The heat generated from agitation must be accounted for in addition to the heat generated from the reaction when designing the jackets and heat exchanges to maintain fermentation at 30°C. Table 15 demonstrates the heat generated from the agitation and the power required to run the system. The energy to agitate the first two reactor was negligible compared to the energy generated by the heat of the reaction and therefore it was not included in the table. The energy required to run the agitation was based on the oxygen uptake rate of the yeast cells (OUR) and the sizing of the fermentation vessel. Section 21.7 of the appendix shows the information obtained about fermenters based on OUR.

Table 15. Energy of Agitators in the Fermenters. One of the major energy contributions of the fermenter is the agitator which is dictated by both oxygen uptake rate and the working volume of the fermenter.

	Seed Fermenter 3	Production Fermenter
OUR [mmol/hrL]	34	211
Working Volume [m3]	20	160
Power [kW/batch]	18.8	596
Energy [kJ/batch]	18,000	572,160
Energy Annually [kJ]		139,000,000

13.1.4 Pumps between fermenters

The fermentation broth will be pumped between all the fermenters. There is a pump between each of the fermenters as well as between the last fermenter and the harvest tank. Depending on the volume of the tank, different sized pumps are used. Additionally, the time required to pump the fluid to the next vessel was taken into account. According to industry standards, the time to transfer the broth should be between 15 minutes to 2 hours. The energy required to operate these pumps was determined for each batch as well as the total energy required to operate these pumps to produce 10M lbs of malonic acid a year. The exact power can be found in Table 16.

Table 16. Power for the Pump between Fermenters. This table demonstrates the 4 pumps required in the upstream processing along with their capacities, time for transfers, and energy. The energy is calculated per batch along with the annual energy.

	Pump 1	**Pump 2**	**Pump 3**	**Pump 4**
Transfer Broth [L]	216	2,214	17,388	169,834
Pump Capacity [GPM]	3	30	100	400
Time to transfer fluids [min]	19	20	46	112
Power [kw/batch]	-	0.5	3.8	15.3
Energy [kJ/batch]	-	579	10,500	103,000
Total Energy [kJ]				26,900,000

13.1.5 Water Filter

A water filter is required to turn the utility water into DI water. There is a cost associated with deionizing the water, but this cost is much less than the cost associated with buying the proper amount of deionized water since the volume of water requires is so large. Around 2 HP or 1.5 kW are required to run this device. Since most of the plant requires water, this system will be running during most of the time the plant is operating. Therefore the total amount of energy required to run the water filtration system annually is 42,638,400 kJ.

13.1.6 *Air Filter*

Two types of filters are required to properly filter the air for fermentation. The first filter removes large particles and the second removes smaller contaminants. Table 17 demonstrates the total amount of energy required to run the air filtration system annually.

Table 17. Power and Energy of Air Filter. The annual energy required to operate the two air filters is calculated in this table.

	Particle Air Filter	Submicron Air Filter	Total Air Filtration
Power [kW]	3.7	4.1	7.8
Energy [kJ]	105,174,720	116,544,960	221,719,680

13.1.7 *Scrubber*

A scrubber is required to remove the undesired particulates from the gas removed from fermentation. The total amount of energy required to run the 45 kW scrubber, including the fan, is 1,270,000,000 kJ annually.

13.1.8 Downstream Pumps

The fermentation broth will be pumped through the first half of the downstream process. Initially, it will be pumped from the harvest tank into the first vacuum belt filter. After the filter, it will be pumped into the reverse osmosis system and from that system to the crystallizer. There will also be pumps for the displacement washes on each vacuum belt filter at very low capacities. There is only a small volume of wash for each filter, so those specific pumps will be at 30 gpm, while the other pumps for the larger vessels are between 400-200 gpm. The timing of the pumps were taken into account when considering the timing of the downstream continuous process. Once the liquid mixture has been crystallized, the slurry will move through a gravitational feed into the second vacuum belt filter and into the dryer. The lack of pumps for the final vessels is due to the fact that the slurry contains solids which could clog up the pumps and create inefficiencies and time delays that will cost energy and money for the plant. For each batch, the energy required to operate the pumps was calculated as well as the amount of energy needed for the pumps annually. The pump energies can be found in Table 18.

Table 18. Energy Consumption for Pumps in the Downstream Process. This table shows the capacity and energy consumption for each pump in the downstream process needed to move the liquid between vessels. It also shows the complete annual consumption of energy by the pumps in the downstream process.

	Pump 5	**Pump 6**	**Pump 7**	**Pump 8**	**Pump 9**
Transfer Broth [L]	169,800	193,000	30,000	80,000	6,000
Pump Capacity [GPM]	400.0	400.0	100.0	200.0	30.0
Time to transfer fluids [min]	112	128	80	105	53
Power [kW/batch]	2.5	2.5	0.6	1.2	0.19
Energy [kJ/batch]	16,700	19,000	2,900	7,800	590
Total Energy [kJ]					11,089,640

13.1.9 Vacuum Belt Filters

The vacuum belt filters used to remove the biomass and then to isolate the solidified malonic acid need a lot of energy to function through the vacuum, which has been modeled like a reverse pump, and the motor to move the belt. The first vacuum belt filter is expected to use more energy as it will be handling a greater quantity of broth in a shorter amount of time. To find the energies for the belt filters, the energy needed to move the fluid away from the belt was calculated similarly to the calculations for a pump. Additionally, the motor to move the belt was calculated based on the velocity and size of the belt. The energy consumptions for the vacuum belt filters are shown in Table 19.

Table 19. Energy Consumption by Vacuum Belt Filters. This table shows the energy consumption of the two vacuum belt filters from the vacuum and belt in addition to the annual energy consumption these components.

	Vacuum Belt Filter 1	Vacuum Belt Filter 2
Transfer Broth [L]	193,000	67,900
Pump Capacity [GPM]	400.0	100.0
Time to transfer fluids [min]	128	180
Vacuum Power [kW/batch]	5.6	1.1
Area [ft^2]	1059	1059
Motor Run Time [min]	105	300
RPM	0.48	0.02
Velocity [lb/min]	28.7	0.98
Belt Power [kW]	0.92	0.14
Total Energy [kJ/batch]	48,700	14,300
Total Annual Energy [kJ]		14,868,000

13.1.10 Dryer Air

The fluidized bed dryer will consume energy in the pressure of air that is needed to dry the malonic acid crystals. Using the force of air needed to achieve 3,000 L per air per minute, in addition to an area of 38 ft^2 where the air will be blown, the dryer is expected to consume 650,000 kJ annually for 236 batches. Calculations for the dyer energy can be found in the section 21.4 in the appendix.

13.2 Utility Requirements

This section of the report describes the utility requirements associated with the production of malonic acid. In this process, water is required as a raw material and as a coolant in heat exchangers on the fermenters, fermenter jackets, and crystallization. A large amount of water is required to run the plant. Additionally, steam is required to properly sterilize the fluid in the fermenter. Design calculations were conducted to minimize the amount of utility water and steam required. The exact specifications can be found in the proceeding sections.

13.2.1 Sterilization of YPD Media through the Jacket

The media that goes into seed fermenter 1 and seed fermenter 2 must be properly sterilized. This means heating the fluid first to 121°C and then cooling it back down to 30°C, the fermentation temperature. For the first two seed fermenters the media is sterilized through steam and cooling water in a jacket. The amount of steam and water required as utility for this was determined. Table 20 portrays the amount of steam and water required per batch and per year.

Table 20. Steam and Water Requirements to Sterilize YPD Media in Seed Fermenter 1 and 2. This table demonstrated the temperatures, amounts, and area of heat transfer to properly heat and cool the YPD media within the fermentation vessels through vessel jackets.

Heating Jacket				
	Seed Fermenter 1		**Seed Fermenter 2**	
Fluid	YPD Media	Steam	YPD Media	Steam
Original Temperature [C]	25	170	25	170
Final Temperature [C]	121	130	121	130
Amount of Fluid [lb]	417	2,096	4,175	20,980
Area [ft^2]		4.38		43.8
Cooling Jacket				
	Seed Fermenter 1		**Seed Fermenter 2**	
Fluid	YPD Media	Water	YPD Media	Water
Original Temperature [C]	121	25	121	25
Final Temperature [C]	30	29	30	29
Amount of Fluid [lb]	417	9,490	4,175	94,980
Area [ft^2]		7.95		79.6

13.2.2 Sterilization of YNB Media through a Heat Exchanger Network

The volume of the media sent into seed fermenter 3 and the production fermenter is too great to heat with the fermenter jackets. Therefore, an external heat exchanger network is required to sterilize the media before fermentation. At first, the network was designed with 2 heat exchangers: a heater with steam and a cooler with water. The required amount of steam and water as a utility cost was too high with this initial design. Therefore a heat exchanger network consisting of three heat exchangers as seen in the overall process flow diagram was designed. The media fluid itself is used to preheat and precool the fluid to lower the utility requirement of steam and water. Designing the network in this fashion decreased the amount of steam required by 2.7 fold and water by 3.0 fold. Table 21 demonstrates the overall steam and water requirement to sterilize the media properly.

Table 21. Water and Steam Requirements to Sterilized Fermentation Media in External Heat Exchanger Network. This table demonstrated the temperatures, amounts, and area of heat transfer to properly heat and cool the fermentation media before it enters seed fermenter 2 and the production fermenter.

Heat Exchanger				
	Seed Fermenter 3		**Production Fermenter**	
Fluid	Fermentation Media	Fermentation Media	Fermentation Media	Fermentation Media
Original Temperature [C]	25	121	25	121
Final Temperature [C]	86	60	86	60
Amount of Fluid [lb]	33,440	33,440	346,800	346,800
Area [ft^2]		1145		11873
Sterilizer				
	Seed Fermenter 3		**Production Fermenter**	
Fluid	YPD Media	Steam	YPD Media	Steam
Original Temperature [C]	86	170	86	170
Final Temperature [C]	121	130	121	130
Amount of Fluid [lb]	33,440	61,280	346,800	635,470
Area [ft^2]		182		1890
Cooler				
	Seed Fermenter 3		**Production Fermenter**	
Fluid	YPD Media	Water	YPD Media	Water
Original Temperature [C]	60	25	60	25
Final Temperature [C]	30	29	30	29
Amount of Fluid [lb]	33,440	250,800	346,800	2,601,000
Area [ft^2]		313		3240

13.2.3 Cooling the Fermentation Vessels

The heat generated by the reactions taking place in the fermentation vessels and the heat added by agitation needs to be offset by cooling water in order to maintain fermentation at 30°C. A jacket around the fermenter is enough to keep seed fermenter 1 seed fermenter 2, and seed fermenter 3 at a constant temperature. However, for the production fermenter heat exchangers are also required for additional cooling water to properly offset the heat generated in the system due to its large volume. The jacket will remove 47% of the heat. Table 22 demonstrates the amount of cooling water required for each fermentation vessel. For these calculations, it was assumed that the heat generated by the reactions were constant throughout the process. However, during the peak heat production time, the amount of water flow should be around doubled.

Table 22. Cooling Water Required for Fermenters. This table demonstrates the amount of cooling water required to keep the fermentation vessels at a constant 30C. Heat is produced from the reactions and from the agitator that must be offset by cooling water. Seed fermenter 3 and the production fermenter are too large for cooling just by the jacket and therefore heat exchangers are also required to cool 25% of the fluids.

	Seed Fermenter 1	**Seed Fermenter 2**	**Seed Fermenter 3**	**Production Fermenter**
Total Heat Removed [kJ/batch]	189	22,700	1,630,000	246,700,000
Jacket Required	X	X	X	X
Heat Exchanger			X	X
Cooling Water [lb/batch]	25	2,990	21,5000	32,500,000
Area [ft^2]	-	-	6.13	928

13.2.4 Water as a Raw Material

Water is required in many of the process units. It is needed as a raw material in the fermentation media and base solution as well as to operate reverse osmosis and wash the biomass cake. The plant has its own water purification system that deionizes the water and removes any contaminants. Therefore, purified water does not need to be purchased but instead can be

considered as a utility. Table 23 demonstrated the amount of water required for each process in the plant.

Table 23. Raw Material Water. This table demonstrates the amount of water required as raw material for both the upstream and the downstream process. The total amount of water is calculated per bath and annually.

	Water [lb/batch]	Water [lb/year]
Seed Fermenter 1	420	99,120
Seed Fermenter 2	4,160	981,760
Seed Fermenter 3	32,900	7,764,400
Production Fermenter	286,000	67,496,000
Vacuum Belt Filter 2	13,170	3,108,120
Total	336,650	79,449,400

13.2.5 Cooling of the Crystallizer

The crystallizer needs to be cooled to 15°C in order to decrease the solubility of the malonic acid in the water. The heat generated by solidification needs to be counteracted by cooling water. Chilled water at 5°C will be run through a coiled jacket around the vessel for the duration of the crystallization process at 14,000 L/hour. Annually, this requires 22 million liters of chilled water. The calculations to determine the required chilled water for the crystallization vessel are shown in section 21.4 in the appendix.

Section 14: Equipment Lists, Unit Description and Specification

This section of the report describes the equipment used in the process to produce malonic acid described in this report. Equipment lists and descriptions can be found in this section. The specification sheets for each piece of equipment can be found in section 21.6 of the appendix.

14.1 Fermenters

The manufacturing plant to produce malonic acid will have numerous seed fermentation and production fermenter of different sizes. Table 24 below shows the dimensions, volume and number of a given type of fermenter.

Table 24. Dimensions of Fermentation Vessels. This table shows the volumes, dimensions and quantity of the 4 fermentation vessels. An H/D of 3 was used to determine the dimensions in order to optimize heat transfer.

	Seed Fermenter 1	Seed Fermenter 2	Seed Fermenter 3	Production Fermenter
Volume [L]	250	2,500	25,000	200,000
Diameter [ft]	1.6	3.3	7.2	12.6
Height [ft]	4.7	10.0	21.6	56.7
Quantity	3	3	3	3

Each of the fermenters will be made of 316SS, a high grade stainless steel. This material, which includes the alloy molybdenum, enhances corrosion resistance, specifically against industrial solvents. Additionally, the material is nonreactive. The fermenter will also have a mill finish. The finish ensures the vessel is smooth and no contaminants can get caught in the vessels in between washes. The fermenters will each have a jacket, used for heating and cooling, of the vessel. Seed fermenter 3 and the production fermenter will also have external heat exchangers to provide additional cooling. Each fermenter will have an agitator to properly mix the fluid and provide sufficient oxygen to the cells. The agitators are an essential component of the fermenter.

These specifications for each fermenter were considered when determining the pricing of each vessel in later sections of this report.

14.2 Heat Exchangers

All the heat exchangers are shell in tube countercurrent heat exchangers. They are made of stainless steel. Table 25 shows the specifications of each heat exchanger.

Table 25. Heat Exchanger Sizing. This table represents the area of heat transfer for all the heat exchangers in the process.

Heat Exchanger	**Area of Heat Transfer [ft^2]**
Heat Exchanger 1	1150
Cooler 1	313
Sterilizer 1	182
Heat Exchanger 2	11873
Cooler 2	3243
Sterilizer 2	1890
Batch Fermenter 3 Heat Exchanger	6.0
Production Fermenter Heat Exchanger	928

14.3 Pumps

All the pumps in the system are standard centrifugal pumps. The pumps vary in the capacity. Table 26 presents all capacities of the pumps.

Table 26. Pump Capacity and Transfer Times. This table shows the specifications of each pump in the plant. Pump capacities were determined based on the amount of broth transferred and the amount of time it would take to transfer the liquid.

Pump Number	Capacity [gpm]	Transfer Time [min]
1	3	19
2	30	20
3	100	46
4	400	112
5	400	112
6	400	128
7	100	80
8	200	105
9	30	44

14.4 Mixing Vessels

The mixing vessels for fermentation medium and base for the fermenters will be made of stainless steel. This material will be able to handle proper steam in place and clean in place systems. They each have pitch blade down pumping impellers that rotate at 300 rpm. Table 27 demonstrates all the specifications of these three mixing vessels.

Table 27. Dimensions of Mixing Vessels. This table shows the volumes and dimensions of the 3 mixing vessels. The table also shows the mixing impeller diameter, which is half the diameter of the actual vessel.

	YPD Media	Fermentation Media	10M NaOH
Tank Volume [L]	2400	167000	470
Diameter [ft]	3.3	12.3	1.9
Length [ft]	9.9	37.0	5.8
Impeller Diameter [ft]	1.7	6.2	1.0
Number of Impellers	2	3	2

14.5 Harvest Tank

The Harvest Tank will be a large steel vessel that is 1.5 times the size of the production fermenter. The vessel will have a volume of 300,000 L, a diameter of 12.5 ft, and a height of 37 ft. This vessel will hold the fermentation broth from the 3 production fermenters and feed the broth continuously into downstream process. There will be one of these vessels on site.

14.6 Air Filter

There will be two type of air filters onsite. The first will be a MAXFLO D-7- series industrial air cleaner. This air filter improves the air quality by around 70% by removing large particulates and contaminants. The air filter has an airflow of 7000 CFM. The exact specifications can be found in the section 21.9 of the appendix. Given the processes' sensitivity to contamination, the air must also go through submicron filtration to further remove smaller materials before each fermenter. The TRIDENT Cleansweep Submicron Filters can remove particulates up to 0.01 um. The exact specifications can be found in section 21.9 of the appendix.

14.7 Water Filter

The water must be deionized before it can be used in the process to produce malonic acid. The Pure Aqua Dual Bed Deionizers model DM48-Q-FRP will be able to deionize water at a peak of 135 GPM or continuous 15 GPM. The exact specifications can be found section 21.9 of the appendix.

14.8 Scrubber

A scrubber is required to clean the gas streams coming off the fermenters. A Packed Bed Chemical Scrubber, model CS-17, produced by Pollution Systems will be used to efficiently remove the gas contaminants from a continuous process stream through a chemicals reaction. The exact specification sheet can be found in section 21.9 of the appendix.

14.9 Vacuum Belt Filters

Both vacuum belt filters will be constructed identically. They each will have a filtration area of 1059 ft^2 with a cake thickness of 6 inches. The frame will be constructed of carbon steel with a belt made of monofilaments, multifilaments, and felt. The belts will be 60 feet long by 17 feet wide. They are able to withstand a temperature up to 110 °C and a vacuum up to -650 mm HG. Each belt is able to process 480,000 lbs a day. The specific sizing can be found in Table 28.

Table 28. Sizing of the Vacuum Belt Filters. This table shows the sizing of the vacuum belt filters needed to process the necessary streams.

	Vacuum Belt Filter 1	**Vacuum Belt Filter 2**
Volume [L]	750,000	73,000
Belt Area [ft^2]	1059	1059
Cake Thickness [ft]	0.5	0.5
Length [ft]	60	60
Width [ft]	17.5	17.5

14.10 Reverse Osmosis System

A reverse osmosis system will be used to remove water before the crystallization process. The system will use a membrane pore size of 0.3 nm to remove 62.8% of the water. The system will be 19 inches wide, 23 inches long, and 46 inches high in stainless steel and have a capacity to process 400 gpm of fluid.

14.11 Reverse Osmosis Storage Tank

A 35,000 L stainless steel storage tank will be used to hold fluid for the displacement wash in the first vacuum belt filter. This tank will be 24 feet high by 8 feet wide. Before the initial run of the plant, the tank will be filled with DI water for the first wash, but once the system begins to run, it will fill up with around 30,000 L of recycled fluid from the reverse osmosis system from each batch. Here the fluid will be held until the next time a displacement wash is needed on the first vacuum belt filter.

14.12 Crystallizer

A 15,000 L continuous crystallizer will be used to solidify the 99.9% pure malonic acid product. The crystallizer will be able to handle 13,000 L/hr with a residence time of 1 hour and an 80% filling capacity. It will be 18 feet tall with a diameter of 6 feet in order to handle the capacity. It will also have a cooling jacket surrounding the surface area of the crystallizer will chilled water to cool the liquid mixture to 15°C. The calculations for the size of the crystallizer can be found in section 21.4 of the appendix.

14.13 Fluidized Bed Dryer

A fluidized bed dryer will be used to dry the solidified malonic acid continuously. The dryer will be 3,000 L and made out of stainless steel. Air will be pumped into the dryer at 1 VVM with a 40% air capacity. It will be 10 feet long by 3 feet wide. The necessary capacity was determined from the solid malonic acid flow rate with a 30 minute residence time. Section 21.4 of the appendix shows the calculations for the sizing of the dryer.

14.14 Waste Storage Tank

Similar to the harvest tank, a waste storage tank will me a large vessel to contain the removed biomass, acid, and water before further processing. The vessel will be 300,000 L with a height of 37 ft and a diameter of 12.5 ft.

Section 15: Equipment Cost Summary

The purchase and bare-module costs for all equipment units in this process was calculated using the appropriate design specifications. The purchase costs of the fermenters were estimated based on industry standards due to the need for fabrication of highly specific units. All other units were modelled with the Equipment Costing Spreadsheet or correlations from the Product and Process Design Principles text. A CE Index of 595.7 was used in order to project to the date of actual purchase in 2019. All equipment was modelled as being made of stainless steel to prevent corrosion due to the highly acidic nature of this process. The purchase costs and bare-module costs are summarized in Table 29. The total bare-module cost for all equipment amounts to $11,313,600. Fabricated equipment makes up the bulk of the total cost of equipment (67%), totaling $7,518,000. About 58% of this cost of fabricated equipment comes from the three seed fermentation lines and three production fermenters, which are crucial to the upstream process, with the rest of the cost going to the specialized equipment units in the downstream process.

Table 29. Equipment Cost Summary. This table presents the purchase costs and bare-module costs of all equipment necessary for the process. These prices are based on a CE Index of 595.7.

Equipment	# Units	Purchase Cost	Bare-Module Cost
Seed Fermenter 1	3x	$1,700	$5,300
Seed Fermenter 2	3x	$16,500	$53,000
Seed Fermenter 3	3x	$165,000	$529,700
Production Fermenter	3x	$1,320,000	$4,237,200
Water Filtration Unit	1x	$5,000	$11,600
Air Filtration Unit	1x	$5,000	$11,600
Pump 1	3x	$3,400	$11,100
Pump 2	3x	$33,700	$111,300
Pump 3	3x	$27,100	$89,300
Pump 4	3x	$32,100	$106,000
Pump 5	1x	$9,200	$30,400
Pump 6	1x	$9,200	$30,400
Pump 7	1x	$9,600	$31,800
Pump 8	1x	$9,000	$29,900
Pump 9	1x	$13,100	$43,300
Sterilizer 1	1x	$43,800	$138,900
Sterilizer 2	1x	$100,500	$318,400
Cooler 1	1x	$48,200	$152,800
Cooler 2	1x	$142,100	$450,400
Heat Exchanger 1	1x	$43,800	$138,900
Heat Exchanger 2	1x	$413,300	$1,310,200
Manifold	6x	$12,000	$12,000
Gas Scrubber	1x	$66,400	$213,200
Harvest Tank	1x	$67,800	$135,700
YPD Storage Tank	1x	$6,800	$13,600
Glucose Storage Tank	1x	$8,600	$17,300
Fermentation Media Storage Tank	1x	$53,700	$107,400
10M NaOH Storage Tank	1x	$4,300	$8,500
Waste Storage Tank	1x	$67,800	$135,700
RO Storage Tank	1x	$67,800	$135,700
Vacuum Belt Filter	2x	$1,035,200	$2,401,700
RO Unit	1x	$21,900	$21,900
Crystallizer	1x	$115,000	$237,000
Dryer	1x	$15,700	$32,400
Total			**$11,313,600**

Section 16: Economic Analysis

The economic viability of this design is largely dependent on the agreement that can be reached with Lygos, Inc. due to dependence on the proprietary technology developed and owned by this company. Multiple methods of partnership were put into consideration during the development of this process, including acquisition of the company, acquisition of the technology, joint venture, and licensing of the technology. After careful consideration, exclusive licensing of the technology was concluded to be the most viable option. Acquisition and joint venture were ruled out due to our team's lack of expertise in genetic engineering and bioprocessing on the laboratory scale. Lygos, Inc. has a team of skilled scientists and engineers and have already been working on this technology for multiple years.

The following economic analysis in sections 16.1-3 is based on the assumption that Lygos Inc. will agree to allow us exclusive licensing rights at an annual fee of $1 Million and to the purchasing of vials of cells at a price of $5,000 per each 1 mL vial. In section 16.4, sensitivity analyses show how our economic analysis is affected at different licensing fees and cell prices.

16.1 Variable Costs

Total variable costs at 100% capacity consists of general expenses, raw materials, and utilities. A summary of all variable costs is shown in Table 30.

Table 30. Summary of Variable Costs. Annual variable costs were projected by factoring in all raw material costs, utility costs, and estimation of general expenses based on industry standards.

Variable Cost Summary

Variable Costs at 100% Capacity:			
General Expenses			
Selling / Transfer Expenses:		$	155,000
Direct Research:		$	2,481,500
Allocated Research:		$	258,500
Administrative Expense:		$	1,034,000
Management Incentive Compensation:		$	646,200
Total General Expenses		$	5,971,100
Raw Materials	$0.55 per lb of Malonic Acid		$5,637,100
Byproducts	$0.00 per lb of Malonic Acid		$0
Utilities	$0.20 per lb of Malonic Acid		$2,021,400
Total Variable Costs		**$**	**13,629,700**

16.1.1 General Expenses

The cost of general expenses totals $5,971,100 annually, making up 44% of the annual variable cost. This category includes selling/transfer expenses (3.0% of sales), direct research (4.8% of sales), allocated research (0.50% of sales), administrative expense (2.0% of sales), and management incentive compensation (1.3% of sales).

16.1.2 Raw Materials

The cost of raw materials totals $5,637,100 annually making up 41% of the annual variable cost. The raw materials necessary for this process include YPD, YNB, Glucose, concentrated NaOH, and cells. A breakdown of the cost of the individual materials is shown in Table 31 and Figure 12. Glucose contributes the largest amount to the annual raw material cost (58%), at a price of $0.20/lb due to this processes heavy dependence on the fermentation of

glucose to produce the malonic acid product. Purchase of YNB is the next largest contributor to the annual cost as large amounts of this media is necessary to grow cells from small vials into a volume great enough produce enough malonic acid to meet the production goal. If Lygos, Inc. agrees to sell us cells at a price of $5,000/vial, the cost of cells will make up 21% of the raw material cost. The sensitivity of profitability to this cost is examined in section 16.4. At the prices listed in Table 31, raw materials will cost a total of $0.55 per pound of Malonic Acid produced.

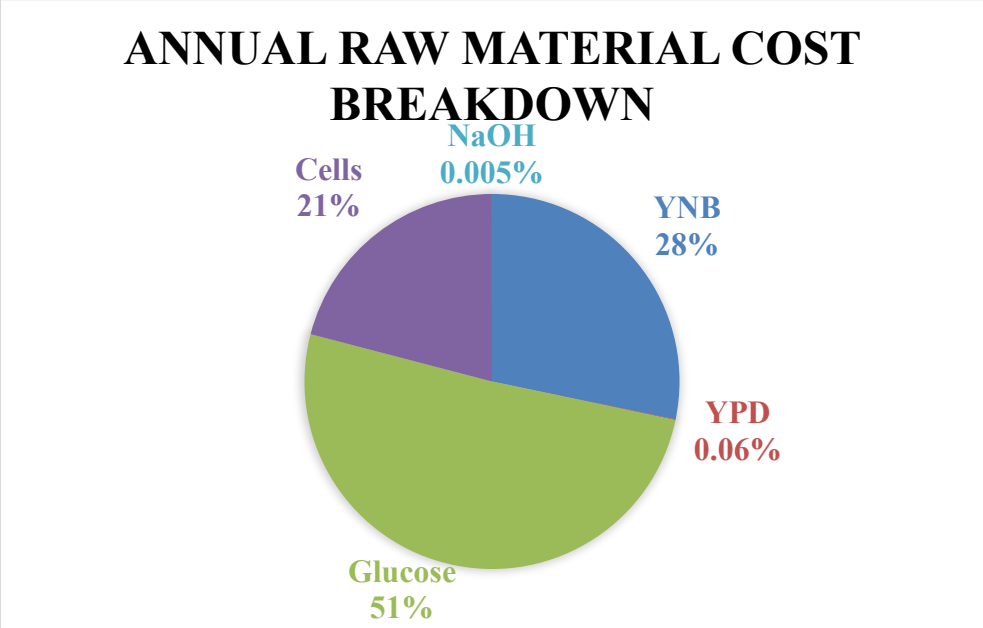

Figure 12. Breakdown of Annual Raw Material Costs**.** This figure shows the percentage of contribution of each raw material to the total annual raw material cost.

Table 31. Annual Raw Material Costs. The annual cost of each raw material necessary for this process are shown. Prices for each component were determined based on industrial and market standards.

Raw Material	Price	Annual Cost
YPD	$60.00/lb	$3,400
YNB	$5.00/lb	$1,592,100
Glucose	$0.20/lb	$2,861,300
NaOH	$0.13/lb	$300
Cells	$5,000/vial	$1,180,000
Total		**$5,637,100**
Cost per lb of Malonic Acid		**$0.55**

16.1.3 Utilities

The cost of utilities totals $1,813,900 annually making up 15% of the annual variable cost. As shown in Table 32 and Figure 13, the largest contributor to the cost of utilities is steam. This is within reason due to the great amount of steam needed to sterilize all raw materials going into any of the fermenters to prevent contamination and any unwanted growth. Through heat integration in the sterilizers, the total cost of steam was reduced 2-fold. The cost of steam makes up 56% of the annual utility cost.

Wastewater treatment and landfill of biomass also contribute significantly to utility costs due to the large amounts of organic waste being produced throughout the process. This cost was included in utilities as we plan to send all waste to outside companies that are willing to handle this type of organic waste. All of the water removed during reverse osmosis and used to wash the product must be sent to wastewater treatment due to the presence of organics. Annually, this will cost $232,100. Landfill of the biomass waste that results from filtration of cells after fermentation and before downstream processing will cost $359,600 annually.

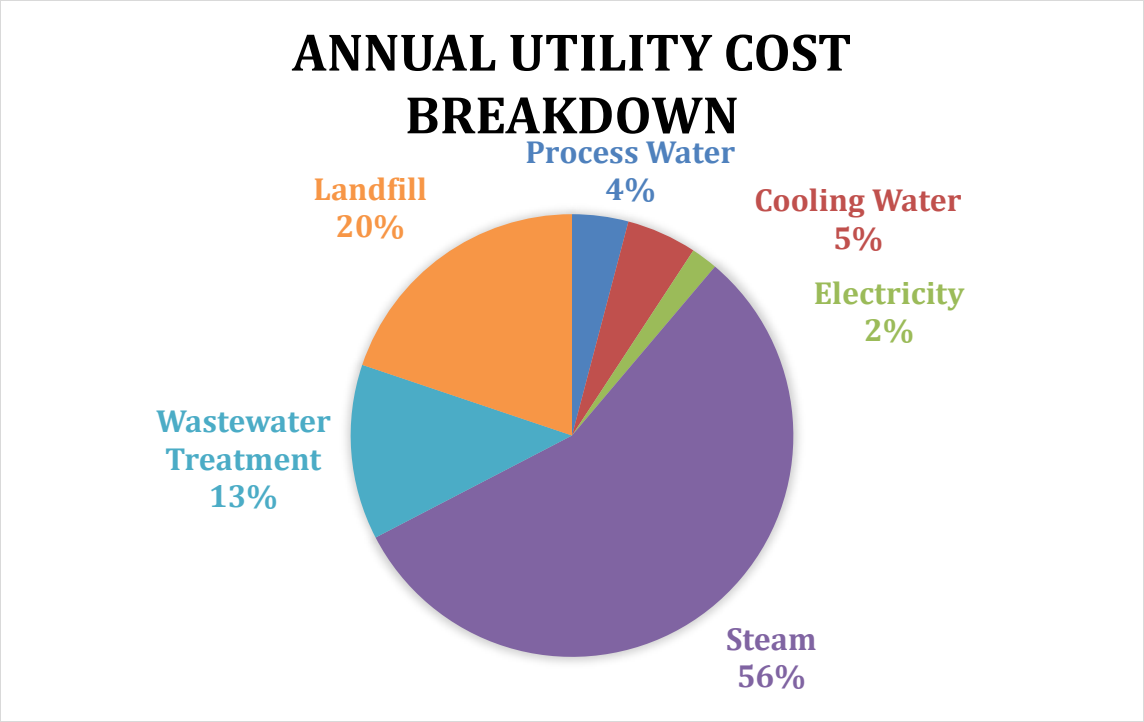

Figure 13. Breakdown of Annual Utility Costs. This figure shows the percentage of contribution of each utility to the total utility cost.

Table 32. Annual Utility Costs. The annual utility cost for the process is shown. Prices for all utilities were found from the Product and Process Design Principles (4th edition) text.

Utility	Price	Annual Cost
Process Water	$0.80/1,000 gal	$74,500
Cooling Water	$0.10/1,000 gal	$93,100
Electricity	$0.07/kW-hr	$35,200
Steam	$6.00/1,000 lbs	$1,019,300
Wastewater Treatment	$0.15/organic removed	$232,100
Landfill	$0.08/dry lb	$359,600
Total		**$1,813,800**
Cost per lb of Malonic Acid		**$0.20**

16.2 Fixed Costs

Total fixed costs will total $6,319,000 per year and take into account operations, maintenance, overhead, taxes, and licensing fees. A summary of all fixed costs can be seen in Table 33.

Table 33. Summary of Fixed Costs. Total annual fixed cost was determined using methods from Product and Process Design Principles (4th edition) and also includes the licensing fee expected to be paid to Lygos, Inc. for use of their technology.

Fixed Cost Summary

Operations			
	Direct Wages and Benefits	$	1,123,200
	Direct Salaries and Benefits	$	168,500
	Operating Supplies and Services	$	67,400
	Technical Assistance to Manufacturing	$	810,000
	Control Laboratory	$	877,500
	Total Operations	**$**	**3,046,600**
Maintenance			
	Wages and Benefits	$	652,900
	Salaries and Benefits	$	163,200
	Materials and Services	$	652,900
	Maintenance Overhead	$	32,600
	Total Maintenance	**$**	**1,501,700**
Operating Overhead			
	General Plant Overhead:	$	149,700
	Mechanical Department Services:	$	50,600
	Employee Relations Department:	$	124,400
	Business Services:	$	156,000
	Total Operating Overhead	**$**	**480,600**
Property Taxes and Insurance			
	Property Taxes and Insurance:	$	290,200
Other Annual Expenses			
	Rental Fees (Office and Laboratory Space):	$	-
	Licensing Fees:	$	1,000,000
	Miscellaneous:	$	-
	Total Other Annual Expenses	**$**	**1,000,000**
Total Fixed Costs		**$**	**6,319,000**

16.2.1 Operations and Maintenance

Operating costs total $3,046,600 annually, which includes direct wages and benefits at $40/operator-hour, direct salaries and benefits (15% of direct wages and benefits), operating supplies and services (6% of direct wages and benefits), technical assistance to manufacturing ($60,000 per year for each operator per shift), and control laboratory costs ($65,000 per year for each operator per shift). This process will require a total of four operators per shift, two for upstream and two for downstream, assuming three shifts per day. We will also require one laboratory technician to manage the successful transfer of cells from vials to the first seed fermenter, which is crucial to the process.

Maintenance costs will total $1,501,700 annually and include wages and benefits, salaries and benefits, materials and services, and maintenance overhead.

16.2.2 Operating Overhead & Taxes

Operating overhead totals $480,500 annually and property taxes and insurance total $290,200 annually.

16.2.3 Licensing Fees

A crucial cost that is factored into the fixed costs is the licensing fee paid to Lygos, Inc. in order to ensure exclusive licensing of their technology. This cost was estimated to be $1 Million based on industry standards for the purpose of economic analysis. This value is further analyzed in section 16.4.3.

16.3 Profitability Analysis

16.3.1 Capital Investment

We estimated that construction would take one year to complete due to the small size of the plant. The total bare-module cost totals $11,313,600. The total direct permanent investment will be $12,295,800, factoring in cost of site preparations (5% of Total Bare Module Costs), cost of service facilities (5% of total bare module costs), contingencies and contractor fees (18% of direct permanent investment), cost of plant startup (10% of total depreciable capital), and the cost of land (2% of total depreciable capital).

Table 34. Summary of Total Investment. The total investment was calculated using the bare module equipment costs, direct permanent investment, and total depreciable capital.

Investment Summary			
Total Bare Module Costs:			
Fabricated Equipment	$	7,518,100	
Process Machinery	$	3,241,700	
Spares	$	-	
Storage	$	418,300	
Other Equipment	$	-	
Catalysts	$	-	
Computers, Software, Etc.	$	-	
Total Bare Module Costs:			$ 11,178,000
Direct Permanent Investment			
Cost of Site Preparations:	$	558,900	
Cost of Service Facilities:	$	558,900	
Allocated Costs for utility plants and related facilities:	$	-	
Direct Permanent Investment			$ 12,295,800
Total Depreciable Capital			
Cost of Contingencies & Contractor Fees	$	2,213,242	
Total Depreciable Capital			$ 14,509,000
Total Permanent Investment			
Cost of Land:	$	290,200	
Cost of Royalties:	$	-	
Cost of Plant Start-Up:	$	1,450,900	
Total Permanent Investment - Unadjusted			$ 16,250,100
Site Factor			1.25
Total Permanent Investment			$ 20,312,600

The total capital investment is about to $23.1 Million with $3.4 Million being attributed to the present value of the current working capital requirements.

Table 35. Working Capital Summary. The working capital for the first three years and the total capital investment are shown.

Working Capital

	2019	2020	2021
Accounts Receivable	$ 2,124,600	$ 424,900	$ 424,900
Cash Reserves	$ 301,700	$ 60,300	$ 60,300
Accounts Payable	$ (314,700)	$ (63,000)	$ (62,900)
Malonic Acid Inventory	$ 283,300	$ 56,700	$ 56,700
Raw Materials	$ 15,400	$ 3,100	$ 3,100
Total	**$ 2,410,200**	**$ 482,000**	**$ 482,000**
Present Value at 15%	*$ 2,095,900*	*$ 364,500*	*$ 317,000*

Total Capital Investment **$ 23,090,000**

16.3.2 Cash Flow

A projection of cash flows beginning in 2019 and ending in 2034 is shown in Figure 14. Cash flow is only negative in 2019 when the plant is constructed and the capital investment is made. After the first year of production in 2020, with the plant operating at 50%, cash flow becomes positive and continues to increase in the following years as plant operating capacity is increased to 100% within five years. This presents this process as economically viable as we project to achieve a positive cash flow by the end of 2020.

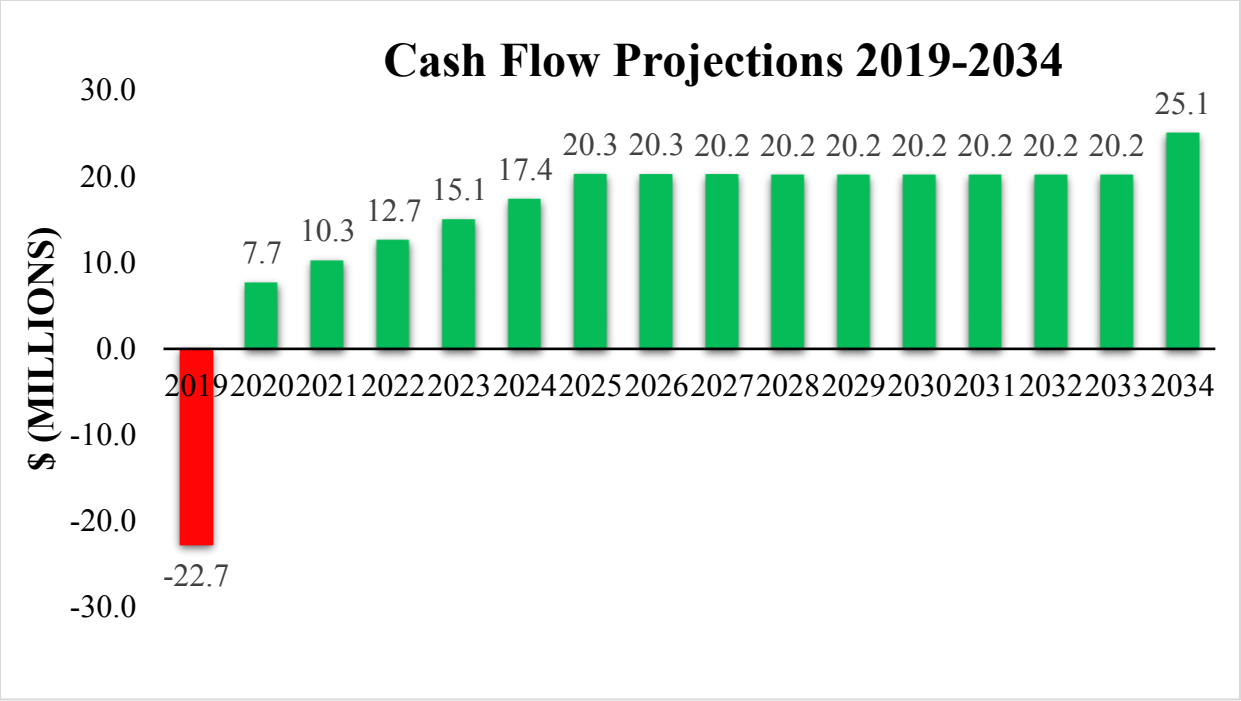

Figure 14. Cash Flow Projections from 2019 to 2034. Cash flow is negative only in the first year when the plant is being constructed. As the plant reaches 100% capacity over the next five years, positive cash flow increases.

16.3.3 Profitability Metrics

Table 36 provides a summary of the profitability metrics for the process in the third year of production. Over 15 years of production, we estimate we will achieve an IRR of 52.6% and an ROI of 46.9% with a Net Present Value of $59.6 Million in 2018. These metrics indicate the economic viability of this process and a positive outlook on investment into this process.

Table 36. Summary of Profitability Measures. The ROI is presented for the third year of production. Both the IRR and ROI present this process as economically viable.

Profitability Measures	
The Internal Rate of Return (IRR) for this project is	52.3%
The Net Present Value (NPV) of this project in 2018 is	$ 59,599,000

ROI Analysis (Third Production Year)

Annual Sales	**36,188,700**
Annual Costs	**(15,859,800)**
Depreciation	**(1,625,000)**
Income Tax	**(6,920,400)**
Net Earnings	**11,783,500**
Total Capital Investment	**25,133,100**
ROI	**46.9%**

16.4 Sensitivity Analysis

Sensitivity analyses of the Return on Investment (ROI) in the third-year (performing at 100% plant capacity) were performed with three different factors: the selling price of Malonic Acid, the price of cells purchased from Lygos, Inc., and the annual licensing fee paid to Lygos, Inc. Based on these, it was concluded that the ROI is most sensitive to changes in selling price. Variations in prices for cells and the annual licensing fees did not have great effects on the ROI values, which proves advantageous for us as we can be flexible in determining the agreement terms with Lygos, Inc.

16.4.1 Selling Price

Malonic acid is currently sold in industry for anywhere between $10-$100 per pound. This process provides an economic advantage by reducing the cost of production, allowing a selling price much lower than current market prices. In sections 16.1-3, a base case selling price of $5.00/lb Malonic Acid was used with the base case variable and fixed cost amounts, resulting

in an ROI of 46.9%. Keeping variable and fixed costs constant, the selling price was varied from $2 to $20 per pound of Malonic Acid. The results are shown in Figure 15. Increasing the selling price by 100% from $5.00/lb to $10.00/lb can increase the ROI by almost 60%, while still keeping the price at the lower end of the spectrum of malonic acid products on the market.

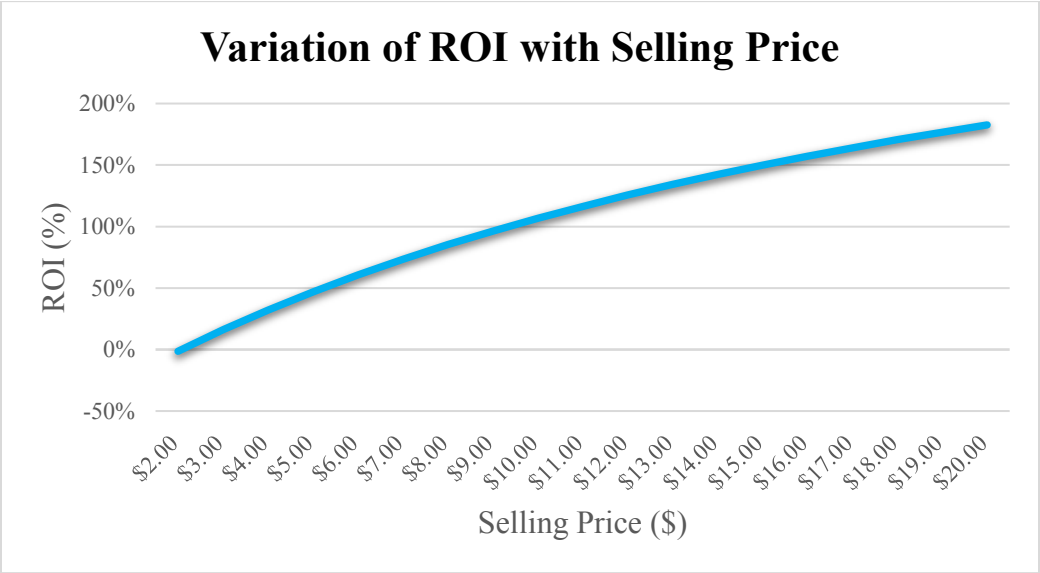

Figure 15. Sensitivity Analysis of Selling Price. The effect of selling price per pound of Malonic Acid on the third year ROI shows a significant increase in ROI as the price is increased from $2/lb to $20/lb. The current market price varies from $10-$100/lb.

16.4.2 Variable Cost (Cells)

In sections 16.1-3, the price for cells that would be purchased from Lygos, Inc. was estimated to be $5,000/vial which gave us an ROI of 46.9% at the base case selling price of $5.00/lb of Malonic Acid. This value was based on industry standards, but an accurate price could not be obtained from the company at this time. Thus, an analysis of how the price of cells can affect the economics was performed by varying the price of cells from $1,000 to $15,000 per vial and is shown in Figure 16. A 15-fold increase in cell price from $1000/vial to $15,000/vial only reduces the ROI by about 5.4%. The cost of cells has little effect on overall ROI indicating great flexibility in negotiating the price of purchase with Lygos, Inc.

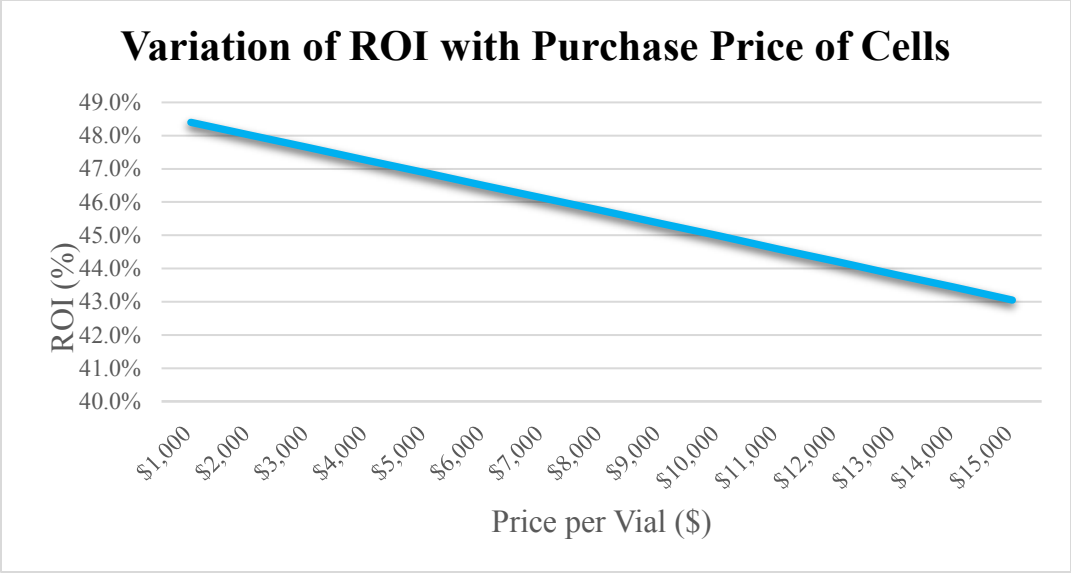

Figure 16. Sensitivity Analysis of Purchase Price of Cells. Cells need to be purchased from Lygos, Inc. and can alter the total annual variable cost. The ROI is not affected significantly by changes in this price.

16.4.3 Fixed Cost (Licensing Fee)

In addition to the purchase cost of cells, we anticipated that Lygos, Inc. would charge a fixed annual licensing fee for exclusive rights to their technology. In sections 16.1-3, the base case value of the licensing fee was estimated to be $1,000,000, giving us an ROI of 46.9% at a selling price of $5/lb of Malonic Acid. To see how this annual fee would affect the ROI, the price of th fee was varied from $500,000 to $10 Million. Similar to the purchase price of the cells, the effect on the ROI was small compared to the change in the cost, as seen in Figure 17. This gives us additional flexibility in negotiations with Lygos, Inc. and makes the exclusive licensing of the technology very plausible, validating the decision to pursue licensing over acquisition or joint venture.

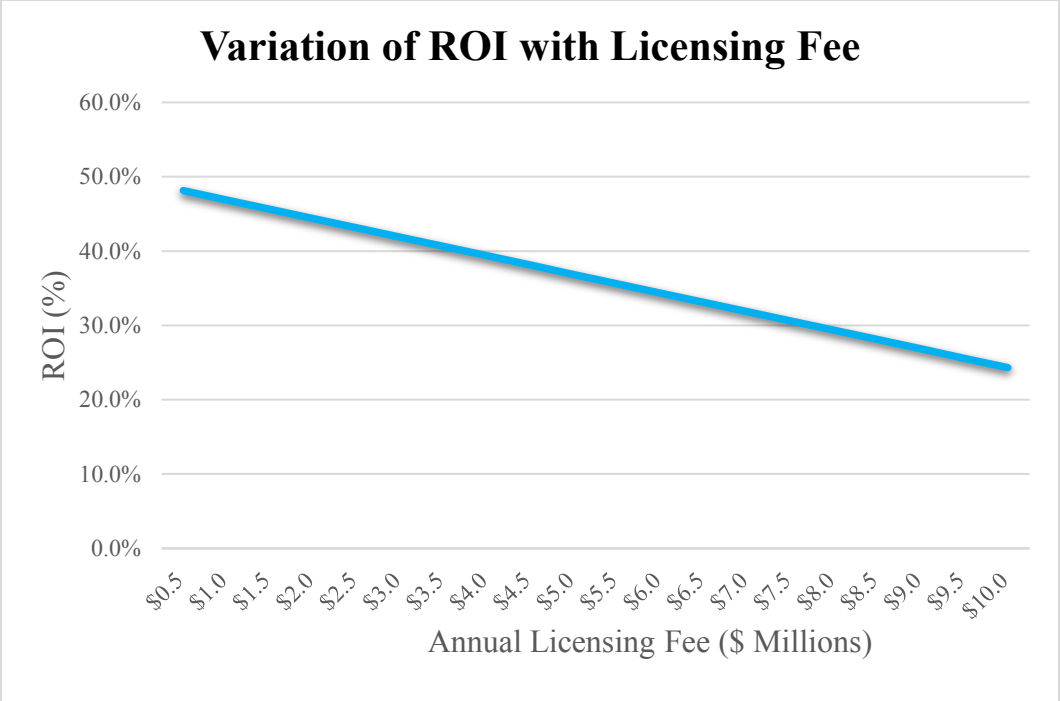

Figure 17. Sensitivity Analysis of Licensing Fee. A licensing fee will be paid to Lygos, Inc. for exclusive rights to their technology and can alter the annual fixed costs. The ROI changes very slightly due to changes in this fee.

Section 17: Other Important Considerations

17.1 Plant Location, Layout, and Startup

The production of malonic acid through the process described in this report relies on the production of genetically modified yeast cells to produce high concentrations of malonic acid. This yeast cells have been genetically modified by Lygos, a lab in Berkeley, California. Because the process is dependent on these cells, it is imperative that the plant has a continuous and reliable supply. To avoid issues inherent in long distance transportation, the plant will be built close to the existing Lygos Laboratory. The manufacturing facility cannot be built right near the laboratory, as this real estate would be extremely expensive in this highly populated area. Taking land costs into consideration, the manufacturing facility would be built in more rural California but still in close driving distance to the Berkeley area.

Since malonic acid has applications in such a diverse group on industries, prioritizing location based on the customer does not seem logical. Regardless of the choice of location, malonic acid will need to be transported.

Plant layout is important for this process. Contamination is a major concern in any biologic process and therefore the facility must be built to avoid such contamination. The areas in which fermentation occurs should be kept at a higher pressure to ensure that contaminants do not enter into the space (Bohn 2015). Additionally, there will a gowning room for the workers, where they must gown up before entering into the fermentation space. There will also need to be a smaller lab area on the plant to complete the lab scale fermentation process.

Detailed startup for the plant was not developed fully as it was largely outside of the scope of this project. Factors like instillation time and initial purchasing of equipment were all considered in the financial section of this report.

17.2 Contamination

Contamination is of major concern of in this process. Contamination can have a major financial impact on manufacturing as raw materials must be changed, entire batches may need to be thrown out, time must be spent to determine the root cause of the issue, and delays to the production schedule add to diminished productivity and potentially the inability of the plant to meet production demands. Therefore contamination by bacteria and phage can greatly affect the productivity and financial viability of a plant (Hutchinson). Proper measures must be taken to limit the probability of contamination.

The plant will be designed in such a way that reduces the risk of contamination. The rooms where fermentation occurs will be kept at a higher pressure than the other rooms to not allow foreign contaminant to enter. All the staff working in the plant will also be required to wearing proper gowning. Humans are the largest contributors to contamination. Gowning, which involves the covering of exposed hair and skin, contains human contaminants and prevents cross contamination from room to room (Bohn 2015).

Within the process all the streams will be sterilized before transfer into fermentation vessels. High heat, followed by cooling, helps to kill off the foreign matter that could be living and growing within the raw materials. It is imperative that these streams are sterilized so that no contaminant enters into the fermentation vessels.

Routine cleaning between batches is also a key factor in limiting contamination. Each of the fermentation vessels will be cleaned and steamed in place between batches to ensure that there is nothing in the vessels before the next batch is introduced. Two methods will be used to clean the vessels; Clean in Place (CIP) and Steam in Place (SIP). CIP is an automated system for cleaning process equipment without disassembly. Processes that use bioreactors and fermenters commonly employ this cleaning method. With CIP cleaning is faster, less labor intensive, more repeatable, and poses less of a risk of chemical exposure to people (McNulty 2016). CIP removes in process residues, controls bioburden, and reduces endotoxin levels within processing equipment. The CIP system is comprised of a pre-rinse or grey water tank, caustic solution tank, a final rinse tank, a transfer pump, and a steam-heated heat exchanger.

SIP is also required for this process. It is a commonly adopted method for in-line sterilization of processing equipment (Capia 2004). This process is widely used in the food, beverage, and biotechnology industries to kill organisms. It uses thermal energy of condensing steam to sterilize the system. SIP should always be done after CIP is completed (Process Industry Forum 2014). The process to properly clean the vessels in between batches, completing both CIP and SIP, will take approximately 12 hours. The exact calculations for the amount of SIP and CIP needed along with the vessels are out of the scope of this project.

7.3 Product Purity

As mentioned above, the target purity for the malonic acid solids is 99.9%. This is due to the needs of the consumers in cosmetics and pharmaceuticals who need highly pure components before they continue to process them. Based on the calculations, the final solidified malonic acid

should be 99.9% pure but further purity testing will be required in the lab, perhaps using mass spectrometry or another method.

17.4 Waste Management

When designing this plant it is important to take into account proper waste management. The wet cake, made primarily of biomass, removed through filtration is a source of waste. There were two options considered for dealing with the biomass waste. The first involved selling the biomass to a farm for animal feed and the second was disposing of it in a landfill. The first option could either bring in revenue for the plant or at least be an option of getting rid of the waste for free. Ultimately this option was discarded because of the high cost associated with the testing required to prove the safety of the genetically modified cells. Disposing of the waste through means of a landfill would on average cost $0.08/dry lb. Since the plant is relatively small, and not that much biomass is produced annually, it is logical to dispose of the waste through safe waste disposal. This would be much less expensive than conducting laboratory studies.

Waste management must also be considered for the liquid waste stream. This stream is a combination of the waste streams collected from reverse osmosis, crystallization, and scrubbing. This liquid contains chemicals that are highly diluted in water. The streams will be collected in a tank that will be picked up by a waste management company who will properly dispose of the waste. The cost associated with this waste management is accounted for in the financial section of this paper.

17. 5 Further Considerations with Genetically Modified Cells

There was limited information accessible about the yield, titer, and growth rates of the genetically modified organism that was chosen. The industrial consultants provided reasonable numbers for these values. For the purposes of this project it was assumed that the genetically modified yeast cells would be able to be further modified by Lygos to meet these specifications. Additionally, from the four reactions presented in the report that occur in the reactor, no information was available about the yield of each reaction from this genetically modified organism. Reasonable assumptions were made for these numbers in both the seed fermenters and the batch fermenter and validated by the industrial consultants. Further research and studies would need to be conducted to confirm these assumptions.

Calculations were also conducted assuming that all the biomass produced would replicate and grow to produce more genetically modified yeast cells. Often times, however, modified cells over time revert back to their wild type. Further testing should be conducted to determine if this occurs with this strain of yeast and, if so, at what rate.

17.6 Safety and Health

This plant uses and produces chemicals that can be harmful if inhaled, injected, or come in contact with the skin. Malonic acid is harmful if swallowed and can cause skin irritation, eye damage, and respiratory irritation. Succinic acid exposure can cause serious eye damage and irritation. Ethanol is harmful if swallowed and can also cause eye irritation and damage to organs. Ethanol is also highly flammable both as a liquid and a vapor and can easily be ignited by heat, sparks or flames. The lower flammable limit is 3.3% by volume, which is reached in some of the vessels in this process. Sodium hydroxide can cause severe skin burns, eye damage,

and skin irritation. NaOH can also be highly corrosive to metals. YNB can cause irritation to the skin and eyes and affect the respiratory system. YPD might cause slight skin and eye irritation but is not classified as a hazardous chemical. Water and glucose are not chemicals with high safety concerns for this process. Little is known about the exact information about the genetically modified yeast cells. Information about the safety and toxicity was taken for normal yeast cells. Yeast cells can cause slight eye irritation and skin irritation. They are also combustible at high temperatures. More research should be done on the genetically modified organism to fully understand its safety risks.

To protect against eye irritation, skin irritation, and other issues caused by inhalation, all plant workers should wear proper protective wear. This gear consists of gloves, goggles, and clothing that covers all parts of the body. The materials that are flammable need to be stored in cool, well ventilated, and segregated areas away from heat and flames to reduce the risk of explosions

17.7 Environmental Considerations

Careful consideration was placed on the environmental implication of the plant. It was found that a few of the raw materials and chemical products used in the production of malonic acid pose some environmental risk. The environmental hazard of sodium hydroxide is caused by the hydroxide ion, pH effect. A high concentration in water will result in toxic effects for aquatic organism like fish. Additionally acids, like malonic acid and succinic acid, should be kept out of sewers, storm drains, surface water and soil. Therefore the wastewater and waste stream collected from the process will be sent to a waste management treatment center. The expense of this was included in financial calculations.

A scrubber system will be used to remove some particulates and gases from the industrial exhaust streams coming off of the fermenters. The gas being removed from the fermenters will contain mostly oxygen, nitrogen, and carbon dioxide. The scrubber will remove the hazardous components, and therefore reduce the environmental hazard associated with letting off the gases directly.

Overall this process is much better for the environment than the alternative method of making malonic acid. The chemicals used and produced in this process are overall much better for the environment than the cyanide and other harmful chemicals produced by the alternate method. Therefore this process is a major improvement, in terms of environmental considerations.

17.8 Process Controls and Instrumentation

Temperature, pH, and contamination control in the fermenters is critical to the fermentation process. The temperature of the fermenter must be kept around 30°C throughout fermentation in order for the cells to grow and produce malonic acid according the model described above. Yeast cells are greatly affected by temperature. If the cells are too cold they will go dormant and if they are too hot they undergo fermentation uncontrollably. Additionally, high temperatures promote the production of fusel alcohols, which are much heavier alcohols which can affect the fermentation broth (Palmer 2015). Therefore it is imperative that the temperature remain around 30°C throughout the process. Heat exchangers are used to cool the fluid in the fermenters. Thermocouples should be used to monitor the temperature of the fermentation broth to make sure it does not go above the desired temperature. If it does, more fluid should be sent through the heat exchangers to cool the broth further.

Throughout the downstream process, the fluid will be kept at 30°C until it reaches the crystallizer. Here, the crystallization of the malonic acid will produce heat due to the heat of solidification, so a cooling jacket around the vessel must be used to control the temperature of the crystallizer. In addition, the crystallizer must be cooled to 15°C to increase the percent of solidification of malonic acid in the vessel. Thus, there needs to be a significant amount of cooling around the crystallizer for the 6 hours it is run for each batch.

pH is also an important variable to control. The yeast strain used in the design of this process was chosen partially because of its ability to handle a large amount of acid. Although yeast cells, like baker's yeast, require a pH around 4, the *Pichia Kudriavzevii* strain can grow in a lower pH range. The ideal range of pH for fermentation was found to be between 2.5-3. Even though the strain can handle a lower pH, controlling the pH is still vital. A pH out of the ideal range, can negatively affect the process by causing a prolonged yeast lag phase, affecting accumulated mass loss, changing the consumption rate of the total sugar, increasing the amount of side product, and decreasing the amount of desired product (Liu et al. 2015). To control the pH, a base, 10M NaOH, is added to the fermenters to control the acidity of the fermentation broth. Even with the addition of a base, monitoring of the acidity throughout the process, especially in the large batch fermenter, is important. Controlling the pH by adding an acid is not necessary in this process because based on the amount of acid produced in this fermentation process, the broth tends to be lower than the desired pH and never above.

Section 18: Conclusion and Recommendation

This initial report suggests that this project is worth pursuing. Based on initial analysis, this process is able to meet the production goal of 10M lbs of malonic acid annually. However in order to be considered for implementation, the process needs to be further tested and refined.

Many of the assumptions made in this report are based on research papers and common trends, so further investigation and actual lab testing is required to support the findings presented. Additionally, assumptions about Lygos' capabilities for genetic modification were made that need to be supported by the lab itself.

If the process were to be pursued, it could perhaps be optimized by increasing the growth rate of the cells used, which could cut down on total batch time, decrease the amount of batches needed, or increase the amount of malonic acid produced annually. Next, the need for 3 fermentation lines should be considered. This number of lines were used to ensure that the batch production fermenters are always being used. It should be further analyzed if it is more cost efficient to decrease the number of fermenters and increase the number of batches produced. Another consideration is the annual cost of steam. While the steam needs for the plant are high and costly, this is not a deterrent from pursuing the process due to the relatively low cost in other raw materials and high selling cost of malonic acid.

The economic feasibility of the proposed process depends largely on the agreement that can be reached with Lygos, Inc. for the exclusive licensing rights to the technology and the purchase price of the genetically modified yeast cells per vial. With the assumption of an annual licensing fee of $1 Million and a purchase price of $5,000 per vial, this process is an economically attractive opportunity at a selling price of $5.00 per pound of malonic acid, requiring a total capital investment of $23.1 million, providing an IRR of 52.6% and a third-year

ROI of 46.9%. Cash flow becomes positive within the first year of production due to the low cost of production. These conclusions are also based on the assumption that market prices for the raw materials and utilities used throughout the process stay relatively stagnant. These costs should be monitored for drastic changes that may alter the profitability of this process. Additionally, the process is still feasible at variations of the cell costs and licensing fees, providing a good position to negotiate a suitable agreement with Lygos. The selling price of $5.00 per pound of malonic acid puts this process at a competitive advantage as compared to current market prices ranging from $10-$100 per pound, making price increase a possible topic of further investigation. If a favorable agreement with Lygos can be reached, we recommend the pursuance of investment in this design.

Section 19: Acknowledgments

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Section 21: Appendix

21.1 Lygos Patent

Recombinant host cells for the production of malonate

Abstract

Systems and methods for the production of malonate in recombinant host cells.

Classifications

C12P7/46 Dicarboxylic acids having four or less carbon atoms, e.g. fumaric acid, maleic acid
View 5 more classifications
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Description

RECOMBINANT HOST CELLS FOR THE PRODUCTION OF MALONATE

Claims

1. A recombinant host cell that comprises a heterologous malonyl-CoA hydrolase.
2. The recombinant host cell of claim 1 that is a yeast.

3. The recombinant host cell of claim 2 that is selected from the group consisting of Candida, Cryptococcus, Komagataella, Lipomyces, Pichia, Rhodospiridium, Rhodotorula, Saccharomyces, Trichosporon, and Yarrowia host cells.
4. The recombinant host cell of claim 3 that is selected from the group consisting of Saccharomyces cerevisiae and Pichia kudriavzevii.
5. The recombinant host cell of claim 1 that is selected from the group consisting of Bacillus, Clostridium, Corynebacterium, Escherichia, Pseudomonas, and Streptomyces host cells.
6. The recombinant host cell of claim 1 , wherein said heterologous malonyl-CoA hydrolase is an enzyme with an amino sequence that has at least 63% identity to consensus sequence SEQ ID NO:7 and is identical to SEQ ID NO:7 at positions corresponding to X_1 I88, G161, G162, G213, E236, and D244.
7. The recombinant host cell of claim 6, wherein said malonyl-CoA hydrolase is S. cerevisiae EHD3 containing mutation E124Xi.
8. The recombinant host cell of claim 1 , wherein said heterologous malonyl-CoA hydrolase is an enzyme with an amino sequence that has at least 86% identity to consensus sequence SEQ ID NO:8 and is identical to SEQ ID NO:8 at positions corresponding to Xi at position 91, G65, G66, G116, E139, and D147.
9. The recombinant host cell of claim 8, wherein said malonyl-CoA hydrolase is Bacillus cereus (strain Ql) 3-hydroxyisobutyryl-CoA hydrolase UniProt ID:B9IZZ9 containing mutation E91Xi.
10. The recombinant host cell of claim 9, wherein said E91Xi mutation is selected from the group consisting of E91S, E91N, E91Y, E91A, E91K, and E91T mutations.
11. The recombinant host cell of claim 1 , wherein said heterologous malonyl-CoA hydrolase is an enzyme with an amino sequence that has at least 75% identity to consensus sequence SEQ ID NO:9 and is identical to SEQ ID NO:9 at positions corresponding to Xi at position 95, G67, G68, G120, E143, and D151.
12. The recombinant host cell of claim 11, wherein said malonyl-CoA hydrolase is mutated Pseudomonas fulva (strain 12X) 3-hydroxyisobutyryl-CoA hydrolase UniProt ID: F6AA82 containing mutation E95Xi.
13. The recombinant host cell of claim 12, wherein said E95Xi mutation is selected from the group consisting of E95S, E95N, E95Y, E95A, E95K, and E95T mutations.
14. The recombinant host cell of claim 1 , wherein said heterologous malonyl-CoA hydrolase is an enzyme with an amino sequence that has at least 75% identity to consensus sequence SEQ ID NO: 10 and is identical to SEQ ID NO: 10 at positions corresponding to XI at position 128, G100, G101, G153, E176, and D184.
15. The recombinant host cell of claim 14, wherein said mutation is selected from the group consisting of E128S, E128N, E128Y, E128A, E128K, and E128T mutations.
16. The recombinant host cell of claim 1 , wherein said heterologous malonyl-CoA hydrolase is a mutated malonyl-CoA:ACP transacylase.
17. The recombinant host cell of claim 16, wherein said malonyl-CoA hydrolase is mutated Escherichia coli malonyl-CoA:ACP transacylase FabD.
18. The recombinant host cell of claim 17, wherein said malonyl-CoA hydrolase comprises one or more of the the following amino acid modifications: S92C, H201N, R117D, R117E, R117N, R117Y, R117G, R117H, Q11D, QUE, Q11N, Q11Y, Q11G, Q11H, L93A, L93V, L93I, L93F, L93S, and L93G.

19. The recombinant host cell of claim 18, wherein said malonyl-CoA hydrolase contains a combination of mutations selected from the group consisting of S92C/L91V/R117H, L91I/R117Y/A246E, Q80L/L91S/R117G, and L91I/R117Y.
20. The recombinant host cell of claim 1 that further comprises a heterologous acetyl- CoA synthetase selected from the group consisting of S. cerevisiae ACS1 , S. cerevisiae ACS2, Salmonella enterica Acs, Escherichia coli AcsA, or Bacillus subtilis AcsA.
21. The recombinant host cell of claim 1 that further comprises a heterologous S. cerevisiae pyruvate dehydrogenase complex enzyme selected from the group consisting of PDA1, PDB1, LAT1, LPD1, and PDX1.
22. The recombinant host cell of claim 1 that further comprises a heterologous ethanol catabolic pathway enzyme.
23. The recombinant host cell of claim 1 that further comprises a heterologous ATP citrate lyase.
24. The recombinant host cell of claim 1 that further comprises a heterologous acetyl- CoA carboxylase.
25. The recombinant host cell of claim 24, wherein the acetyl-CoA carboxylase is Yarrowia lipolytica acetyl-CoA carboxylase ACC.
26. The recombinant host cell of claim 1 that further comprises a genetic modification that decreases malonate catabolism.
27. The recombinant host cell of claim 1 that further comprises a heterologous transport protein selected from the group consisting of PDR5, PDR10, PDRl l, PDR12, PDR15 and PDR18.
28. The recombinant host cell of claim 1 that further comprises a genetic modification that increases host cell tolerance to malonate.
29. The recombinant host cell of claim 1 that further comprises a genetic modification that improves host cell catabolism of carbon sources selected from the group consisting of ethanol, acetate, and sucrose.
30. A recombinant host cell comprising a malonate transcription factor biosensor comprising:
a heterologous nucleic acid encoding a malonate transcription factor that can bind to and activate a promoter; and
a promoter that is activated by the malonate transcription factor operably linked to a nucleic acid that encodes a heterologous reporter protein.
31. The recombinant host cell of claim 33, wherein the malonate transcription factor is an MdcY transcription factor, the promoter is a PM<1$_C$L promoter, and the host cell is Escherichia coli.
32. A method of screening for hosts cell with increased malonate production comprising the steps of:
culturing a recombinant host cell that comprises a heterologous nucleic acid encoding a malonate transcription factor that can bind to and activate a promoter and a promoter that is activated by the malonate transcription factor operably linked to a nucleic acid that encodes a heterologous reporter protein;
adding host cell fermentation broth containing malonate to the recombinant host cell; and
identifying cells with increased malonate production based on increased levels of heterologous reporter protein.
33. The method of claim 35, wherein the malonate transcription factor is an MdcY transcription factor and the promoter is a PM<I$_C$L promoter.
34. A method of producing malonate comprising the step of culturing a host cell of claim 1 under conditions that result in production of malonate.

35. A method of isolating malonate from host cell fermentation broth, wherein said method comprises the steps of:
culturing a recombinant host cell capable of producing malonate in fermentation broth and under conditions that result in malonate production; adding a calcium salt to the fermentation broth to precipitate calcium malonate; and separating the precipitated malonate from the fermentation broth to provide isolated malonate.
36. The method of claim 35, wherein the calcium salt is calcium carbonate, calcium hydroxide, or a mixture thereof.
37. A method of purifying malonic acid from host cell fermentation broth, wherein the method comprises the steps of:
culturing recombinant host cells capable of producing malonate in fermentation broth under conditions that result in the production of malonic acid;
separating the host cells from the fermentation broth
adjusting the pH of the fermentation broth to 1.5 or lower;
adding an aliphatic amine to the fermentation broth;
transferring malonic acid from the fermentation broth by phase transfer into an organic solvent or combination of organic solvents; and
removing aliphatic amine and organic solvent to provide purified malonic acid.
38. The method of claim 37, wherein said aliphatic amine is a tertiary amine selected from the group consisting of triethylamine, tripropylamine, tributylamine, tripentylamine, trihexylamine, triheptylamine, trioctylamine, trinonylamine, and tridecylamine, and the organic solvent is n-octanol.
39. A method of isolating dialkyl malonate from host cell fermentation broth, comprising the steps of:
culturing a recombinant host cell capable of producing malonate under conditions that result in malonate production;
separating the host cells from the fermentation broth;
adding an aliphatic alcohol and acid catalyst to the fermentation broth to form a dialkyl malonate; and
separating the resulting dialkyl malonate from the fermentation broth to provide isolated malonate.
40. The method of claim 39, wherein said aliphatic alcohol is ethanol and said dialkyl malonate is diethyl malonate.
41. A method of producing acrylate, comprising reacting malonate and paraformaldehyde in an organic solvent containing base at a temperature between 50°C and 90°C.
42. The method of claim 41, wherein the solvent and base are pyridine.
43. A method of producing hexanedioic acid, comprising reacting a monoalkyl malonate and 1 ,2-dichloroethane in a solution containing magnesium silicate.
44. A method of producing an alkyl 3-hydroxypropionate from a monoalkyl malonate by catalytic reduction with a borane catalyst.
45. The method of claim 44, further comprising the step of hydrolyzing the ester bond of said alkyl 3-hydroxypropionate to form 3-hydroxypropionate.
46. A method for producing heptanedioc acid, comprising the steps of:
reacting dialkylmalonate and glutarate semialedhyde to form 7-alkoxy-6- (alkoxycarbonyl)-7-oxohept-5-enoic acid;
acidifying 7-alkoxy-6-(alkoxycarbonyl)-7-oxohept-5-enoic acid to yield pent-l-ene- 1,1,5-tricarboxylic acid;

heating pent-l-ene-l,l,5-tricarboxylic acid to yield hept-2-enedioic acid; and catalytically hydrogenating hept-2-enedioic acid to yield heptanedioic acid (pimelic acid).
47. A method for producing 1,3 -propanediol acid, comprising the hydrogenation of dialkylmalonate.

GOVERNMENT INTEREST

[0001] This invention was made with government support under grant no. DE-SC0006469 awarded by the Department of Energy. The government has certain rights in this invention.

BACKGROUND OF THE INVENTION

[0002] The long-term economic and environmental concerns associated with the petrochemical industry has provided the impetus for increased research, development, and commercialization of processes for conversion of carbon feedstocks into chemicals that can replace those derived from petroleum feedstocks. One approach is the development of biorefining processes to convert renewable feedstocks into products that can replace petroleum-derived chemicals. Two common goals in improving a biorefining process include achieving a lower cost of production and reducing carbon dioxide emissions.

[0003] Propanedioic acid ("malonate", CAS No. 141-82-2) is currently produced from nonrenewable, petroleum feedstocks. Mono- or di-esterification of one or both carboxylic acid moieties of malonate with an alcohol (e.g. methanol or ethanol) yields the monoalkyl and dialkyl malonates, respectively. 2,2-dimethyl-l,3-dioxane-4,6-dione ("Meldrum's acid" CAS No. 2033-24-1) is produced from malonate using either acetone in acetic anhydride or isopropenyl acetate in acid.

[0004] Chemical synthesis is currently the preferred route for synthesis of malonate and malonate derived compounds. For example, dialkyl malonates are produced through either a hydrogen cyanide or carbon monoxide process. In the hydrogen cyanide process, sodium cyanide is reacted with sodium chloroacetate at elevated temperatures to produce sodium cyanoacetate, which is subsequently reacted with an alcohol/mineral acid mixture to produce the dialkyl malonate. Hildbrand et al. report yields of 75-85% (see "Malonic acid and Derivatives" In: Ullmann's Encyclopedia of Industrial Chemistry, Wiley- VCH, Weinheim, New York (2002)). In the carbon monoxide process, dialkyl malonates (also referred to herein as diester malonates) are produced through cobalt-catalyzed alkoxycarbonylation of chloroacetates with carbon monoxide in the presence of an alcohol at elevated temperatures and pressures.

[0005] The existing, petrochemical-based production routes to the malonate and malonate- derived compounds are low yielding, environmentally damaging, dependent upon nonrenewable feedstocks, and require expensive treatment of wastewater and exhaust gas. Thus, there remains a need for methods and materials for biocatalytic conversion of renewable feedstocks into malonate, purification of biosynthetic malonate, and subsequent preparation of downstream chemicals and products.

SUMMARY OF THE INVENTION

[0006] The present invention provides recombinant host cells, materials, and methods for the biological production of malonate, methods for detecting the presence of malonate and determining the levels of malonate (referred to herein as "sensing malonate") in malonate producing host cells, and methods for screening host cells for increased malonate production. In addition, the present invention provides methods for the purification of biologically produced malonate, and the methods for converting malonate to other industrially important chemicals.

[0007] In a first aspect, the invention provides recombinant host cells comprising a heterologous nucleic acid encoding an acyl-CoA hydrolase that catalyzes conversion of malonyl-CoA to malonic acid, as illustrated here: Malonyl-CoA Hydrolase

Malonyl-CoA Malonic Acid

These recombinant host cells produce more malonate than counterpart cells that do not comprise such a heterologous hydrolase. In various embodiments, the host cells can produce at least 10 g/L malonate under appropriate fermentation condtions, and in various embodiments, productions levels can be as high as 50 g/L to 100 g/L or higher. In some embodiments, the heterologous nucleic acid encodes a mutated form of an endogenously expressed enzyme; thus, the present invention provides a variety of mutated acyl-CoA hydrolases, nucleic acids encoding them, and recombinant expression vectors comprising those nucleic acids. In other embodiments, the heterologous nucleic acid codes for the overexpression of an endogenous enzyme. Further, in some embodiments the heterologous nucleic acid encodes a wild-type or mutant enzyme of an acyl-CoA hydrolase heterologous to (not natively expressed in) the host cell. In some embodiments the host cell is a yeast cell. In other embodiments, the host cell is a bacterial cell.

[0008] Thus, in various embodiments, the heterologous nucleic acids provided by the invention encode a wild-type or mutated form of an acyl-CoA hydrolase. Non-limiting examples of acyl-CoA hydrolases encoded by the nucleic acids provided by the invention and suitable for malonyl-CoA hydrolysis include wild-type and modified enzymes selected from the group consisting of 3-hydroxyisobutyryl-CoA hydrolases (EC 3.1.2.4), 3- hydroxypropionyl-CoA hydrolases (EC 3.1.2.4), acetoacetyl-CoA hydrolases (EC 3.1.2.11), methylmalonyl-CoA hydrolases (EC 3.1.2.17), propionyl-CoA hydrolases (EC 3.1.2.18), succinyl-CoA hydrolases (EC 3.1.2.3), and malonyl-CoA:ACP transacylases (EC 2.3.1.39) mutated as provided herein to have malonyl-CoA hydrolase activity.

[0009] In various embodiments, the invention provides a malonyl-CoA hydrolase that is a mutant of a 3-hydroxyisobutyryl-CoA hydrolase (EC 3.1.2.4). Suitable 3-hydroxyisobutyryl- CoA hydrolases can be obtained from both eukaryotic and prokaryotic, including both Gram positive and Gram negative, organisms. In various embodiments, the 3-hydroxyisobutyryl- CoA hydrolase is obtained from a yeast strain, a Bacillus species, and a Pseudomonas species.

[0010] In additional embodiments, the invention provides a malonyl-CoA hydrolase that is a malonyl-CoA:ACP transacylase (EC 2.3.1.39) mutated as provided herein to have malonyl- CoA hydrolase activity, encoded by a prokaryote. In various embodiments, the prokaryote is a Gram-negative bacterium. In various embodiments of the invention, the Gram-negative bacterium is an Escherichia.

[0011] In a second aspect, the invention provides recombinant expression vectors encoding a wild-type or mutated acyl-CoA hydrolase that catalyzes conversion of malonyl-CoA to malonate. In some embodiments, the expression vector is a yeast expression vector; in other embodiments, the expression vector is a bacterial expression vector. In various embodiments, the bacterial expression vector is an Escherichia coli expression vector.

[0012] In a third aspect, the invention provides recombinant host cells suitable for the biosynthetic production of malonate at levels enabling its isolation and use as a starting material for chemical synthesis of other useful products. In some embodiments, the host cell is a eukaryote. In some embodiments, the host cell is a yeast cell. In various embodiments, the yeast is a Candida, Cryptococcus, Hansenula, Issatchenkia, Kluyveromyces, omagataelia, Lipomyces, Pichia, Rhodosporidium, Rhodotorula, Saccharomyces or Yarrowia species. In some embodiments, the eukaryotic host cell is a fungus. In some embodiments, the cell host is an algae.

[0013] In other embodiments, the host cell is a bacterial cell. In various embodiments, the host cell is a bacterial cell selected from the group consisting of Bacillus, Clostridium, Corynebacterium, Escherichia, Pseudomonas, and Streptomyces. In some embodiments, the host cell is an E. coli cell.

[0014] Generally, the recombinant host cells of the invention have been genetically modified for improved malonate yield, titer, and/or productivity. In various embodiments, the host cells have been modified for increased malonate biosynthesis through one or more host cell modifications selected from the group consisting of modifications that result in increased acetyl-CoA biosynthesis, increased malonyl-CoA biosynthesis, decreased malonyl-CoA utilization, decreased malonate catabolism, increased secretion of malonate into the fermentation broth, increased host cell tolerance to malonate in the fermentation broth, and/or increased host cell catabolism of carbon sources (e.g. acetate, alginate, ethanol, fatty acids, lignocellulosic biomass, methanol, pentose sugars, and syn gas).

[0015] In a fourth aspect, the invention provides methods for producing malonate in a recombinant host cell, which methods generally comprise culturing the recombinant host cell in fermentation broth under conditions that enable it to produce malonate. In some embodiments, the host cell has been engineered to express more or less of an endogenous enzyme that results in the production of more malonate than a corresponding cell that has not been so engineered. In some embodiments, the method comprises culturing a recombinant host cell expressing a heterologous (foreign or non-native) enzyme that results in the increased production of malonate. In some embodiments, the host cell used in the method comprises one or expression vectors comprising heterologous malonyl-CoA hydrolase enzymes. In some embodiments of these methods, the fermentation broth is supplemented with carbon sources promoting malonate production and selected from the group consisting of cellodextrins, 5 carbon sugars, 6 carbon sugars, carbon dioxide, ethanol, methanol, glycerol, acetate, and/or fatty acids.

[0016] In a fifth aspect, the invention provides biosensors comprising a malonate transcription factor and a promoter responsive to said transcription factor operably linked to a marker gene. The invention also provides methods for "sensing" malonate, malonate production, and malonate producing host cells and methods for screening for host cells with increased malonate production. In various embodiments, said

methods comprise culturing a host cell expressing a malonate transcription factor and containing a promoter responsive to said transcription factor and operably linked to a marker gene, and selecting host cells with improved malonate production by screening for expression of the marker gene product and selecting those host cells that express higher levels of the marker gene product. In some embodiments, malonate is produced in one host cell, the fermentation broth from the first cell is contacted with (added to media containing) a second cell comprising a malonate transcription factor and a promoter responsive to said transcription factor operably linked to a marker gene, and host cells with improved malonate production are identified by identifying cells with the highest levels of expression of the marker gene product. In other embodiments, malonate is produced in a host cell comprising a malonate transcription factor and promoter responsive to malonate operably linked to a marker gene, and host cells with increased malonate production are screened for increased malonate production by screening for and identifying cells that express the highest levels of the marker gene product. In some embodiments, the transcription factor can bind malonate, which results in binding of the transcription factor to a cognate promoter and activation of the marker gene that is operably linked to the promoter. In some embodiments, the transcription factor is an MdcY transcription factor. In some embodiments, the method is practiced to screen or select for genetically modified host cells with improved malonate production relative to control cells.

[0017] In a sixth aspect, the invention provides purified malonate isolated from the fermentation broth of a host cell producing malonate, optionally a host cell of the invention. The invention also provides methods for purifying malonate from the fermentation broth of a host cell producing malonate, the methods generally comprising culturing a host cell in fermentation broth under conditions that enable the host cell to produce malonate, and purifying the malonate from the fermentation broth. In some embodiments of the invention, the concentration of malonate in the broth is increased by dewatering the fermentation broth during the purification process. In various embodiments of the inventon, the dewatering is achieved by reverse osmosis processing, evaporation, or a combination of the two. In various embodiments, the purification is achieved by adding one or more of the following: a divalent cation, a monovalent cation, ammonium, a monosubstituted amine, a disubstituted amine, a trisubstituted amine, a cationic purification resin, or an acid. In various embodiments of the invention, these agents are added in conjunction with one or more organic solvents. In some embodiments of the invention, a hydrophobic solvent is used in a liquid-liquid extraction of the fermentation broth. In other embodiments, malonate is purified from the fermentation broth by reactive extraction or distillation with an acid catalyst and an alcohol.

[0018] In a seventh aspect, the invention provides methods of making compounds derived from malonate and compounds produced by such methods. The methods generally comprise reacting malonate with one or more substrates to produce a compound. In some embodiments of these methods, chemicals with established synthetic routes from malonate are produced using biologically derived malonate. In other embodiments of these methods, new synthetic routes for the production of useful chemicals are provided that are suitable for use with either a synthetically or biologically derived malonate. In some embodiments, monoalkyl malonate esters are synthesized from biologically derived malonate. In other embodiments, dialkyl malonate esters are synthesized from biologically derived malonate. In some embodiments, an acrylate is synthesized from malonate or malonic acid. In other embodiments, acrylate is

synthesized from malonate monoesters or diesters. In other embodiments, dicarboxylic acids are produced from malonate. Illustrative dicarboxylic acids that can be produced in accordance with the methods of the invention include those selected from the group consisting of pentanedioic acid, hexanedioic acid, heptanedioic acid, octanedioic acid, nonanedioic acid, decanedioic acid, undecanedioic acid, dodecanedioic acid, and the corresponding monoalkyl and dialkyl esters of each. In other embodiments of the invention, dicarboxylic acids are produced from a malonate-derived compound. In other embodiments of the invention, ε-caprolactam is produced from malonate. In other embodiments of the invention, δ-valerolactam is produced from malonate.

[0019] These and other aspects and embodiments of the invention are illustrated in the accompanying drawings and described in more detail below.

BRIEF DESCRIPTON OF THE FIGURES

[0020] Figure 1 shows a dose-response curve for an E. coli MdcY malonate biosensor of the invention that utilizes the promoter PM<1$_C$L- The X-axis is the concentration of exogenous malonate added to the fermentation broth; the Y-axis is the cell culture density (OD_{600}) after 12 hours growth in medium with 25 μg/ml tetracycline. E. coli transformed with plasmid SI 4, comprising an MdcY transcription factor and a tetA gene under control of a PM<I$_C$L promoter, produced the tetracycline resistance protein TetA upon exogenous addition of malonate. The biosensor displayed malonate-dependent increases in tetracycline resistance as measured by the increase in OD_{600} with the increase in concentration exogenously added malonate as described in additional detail in Example 21.

[0021] Figure 2 is an HPLC chromatogram trace showing separation and detection of acrylic acid production from malonate according to the methods of the invention as described in Example 28. The X-axis shows elution of acrylic acid at approximately 17.5 minutes, and the Y-axis shows arbitrary units derived from detection of acrylic acid via a UV detector monitoring 210 nm. Acrylic acid produced from malonate according to the methods of the invention (dashed line) exhibited the same retention time as an authentic acrylic acid standard (solid line).

DETAILED DESCRIPTION OF THE INVENTION

[0022] The present invention provides recombinant host cells, materials, and methods for the biological production of malonate, screening malonate producing host cells for improved malonate production, purification of biologically produced malonate, and the synthetic conversion of malonate to industrially important chemicals.

[0023] While the present invention is described herein with reference to aspects and specific embodiments thereof, those skilled in the art will recognize that various changes may be made and equivalents may be substituted without departing from the invention. The present invention is not limited to particular nucleic acids, expression vectors, enzymes, host microorganisms, or processes, as such may vary. The terminology used herein is for purposes of describing particular aspects and embodiments only, and is not to be construed as limiting. In addition, many modifications may be made to adapt a particular

situation, material, composition of matter, process, process step or steps, in accordance with the invention. All such modifications are within the scope of the claims appended hereto.

[0024] All patents, patent applications, and publications cited herein are incorporated herein by reference in their entireties.

Section 1: Definitions

[0025] In this specification and in the claims that follow, reference will be made to a number of terms that shall be defined to have the following meanings.

[0026] As used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to an "expression vector" includes a single expression vector as well as a plurality of expression vectors, either the same (e.g., the same operon) or different; reference to "cell" includes a single cell as well as a plurality of cells; and the like.

[0027] Amino acids in a protein coding sequence are identified herein by the following abbreviations and symbols. Specific amino acids are identified by a single-letter abbreviation, as follows: A is alanine, R is arginine, N is asparagine, D is aspartic acid, C is cysteine, Q is glutamine, E is glutamic acid, G is glycine, H is histidine, L is leucine, I is isoleucine, K is lysine, M is methionine, F is phenylalanine, P is proline, S is serine, T is threonine, W is tryptophan, Y is tyrosine, and V is valine. A dash (-) in a consensus sequence indicates that there is no amino acid at the specified position. A plus (+) in a consensus sequence indicates any amino acid may be present at the specified position. Thus, a plus in a consensus sequence herein indicates a position at which the amino acid is generally non-conserved; a homologous enzyme sequence, when aligned with the consensus sequence, can have any amino acid at the indicated "+" position. At positions in a consensus sequence where one of a subset of amino acids can be present, the following abbreviations are used: B represents that one of the amino acids R, K, or H is present at the indicated position; J represents that one of the amino acids D or E is present at the indicated position; O represents that one of the amino acids I, L, or V is present at the indicated position; U represents that one of the amino acids S or T is present at the indicated position; and Xi represents that one of the amino acids A, D, R, H, K, S, T, N, Q, or Y (or a subset of those amino acids) is present at the indicated position. Illustrative subsets of Xi include Xi is A, D, K, S, T, N, or Y and Xi is S or N. Specific amino acids in a protein coding sequence are identified by their respective single-letter abbreviation followed by the amino acid position in the protein coding sequence where 1 corresponds to the amino acid (typically methionine) at the N-terminus of the protein. For example, El 24 in S. cerevisiae wild type EHD3 refers to the glutamic acid at position 124 from the EHD3 N- terminal methionine (i.e., Ml). Amino acid substitutions (i.e., point mutations) are indicated by identifying the mutated (i.e., progeny) amino acid after the single-letter code and number in the parental protein coding sequence; for example, E124A in S. cerevisiae EHD3 refers to substitution of alanine for glutamic acid at position 124 in the EHD3 protein coding sequence. The mutation may also be identified in parentheticals, for example EHD3 (E124A). Multiple point mutations in the protein coding sequence are separated by a backslash (/); for example, EHD3 E124A/Y125A indicates that mutations E124A and Y125A are both present in the EHD3

protein coding sequence. The number of mutations introduced into some examples has been annotated by a dash followed by the number of mutations, preceeding the parenthetical identification of the mutation (e.g. A5W8H3-1 (E95Q)). The Uniprot IDs with and without the dash and number are used interchangeably herein (i.e. A5W8H3-1 (E95Q) = A5W8H3 (E95Q)).

[0028] As used herein, the term "express", when used in connection with a nucleic acid encoding an enzyme or an enzyme itself in a cell, means that the enzyme, which may be an endogenous or exogenous (heterologous) enzyme, is produced in the cell. The term "overexpress", in these contexts, means that the enzyme is produced at a higher level, i.e. , enzyme levels are increased, as compared to the wild-type, in the case of an endogenous enzyme. Those skilled in the art appreciate that overexpression of an enzyme can be achieved by increasing the strength or changing the type of the promoter used to drive expression of a coding sequence, increasing the strength of the ribosome binding site or Kozak sequence, increasing the stability of the mRNA transcript, altering the codon usage, increasing the stability of the enzyme, and the like.

[0029] The terms "expression vector" or "vector" refer to a nucleic acid and/or a composition comprising a nucleic acid that can be introduced into a host cell, e.g., by transduction, transformation, or infection, such that the cell then produces ("expresses") nucleic acids and/or proteins other than those native to the cell, or in a manner not native to the cell, that are contained in or encoded by the nucleic acid so introduced. Thus, an "expression vector" contains nucleic acids (ordinarily DNA) to be expressed by the host cell. Optionally, the expression vector can be contained in materials to aid in achieving entry of the nucleic acid into the host cell, such as the materials associated with a virus, liposome, protein coating, or the like. Expression vectors suitable for use in various aspects and embodiments of the present invention include those into which a nucleic acid sequence can be, or has been, inserted, along with any preferred or required operational elements. Thus, an expression vector can be transferred into a host cell and, typically, replicated therein (although, one can also employ, in some embodiments, non-replicable vectors that provide for "transient" expression). In some embodiments, an expression vector that integrates into chromosomal, mitochondrial, or plastid DNA is employed. In other embodiments, an expression vector that replicates extrachromasomally is employed. Typical expression vectors include plasmids, and expression vectors typically contain the operational elements required for transcription of a nucleic acid in the vector. Such plasmids, as well as other expression vectors, are described herein or are well known to those of ordinary skill in the art.

[0030] The terms "ferment", "fermentative", and "fermentation" are used herein to describe culturing microbes under conditions to produce useful chemicals, including but not limited to conditions under which microbial growth, be it aerobic or anaerobic, occurs.

[0031] The term "heterologous" as used herein refers to a material that is non-native to a cell. For example, a nucleic acid is heterologous to a cell, and so is a "heterologous nucleic acid" with respect to that cell, if at least one of the following is true: (a) the nucleic acid is not naturally found in that cell (that is, it is an "exogenous" nucleic acid); (b) the nucleic acid is naturally found in a given host cell (that is, "endogenous to"), but the nucleic acid or the RNA or protein resulting from transcription and translation of this nucleic acid is produced or present in the host cell in an unnatural (e.g., greater or lesser than

naturally present) amount; (c) the nucleic acid comprises a nucleotide sequence that encodes a protein endogenous to a host cell but differs in sequence from the endogenous nucleotide sequence that encodes that same protein (having the same or substantially the same amino acid sequence), typically resulting in the protein being produced in a greater amount in the cell, or in the case of an enzyme, producing a mutant version possessing altered (e.g. higher or lower or different) activity; and/or (d) the nucleic acid comprises two or more nucleotide sequences that are not found in the same relationship to each other in the cell. As another example, a protein is heterologous to a host cell if it is produced by translation of RNA or the corresponding RNA is produced by transcription of a heterologous nucleic acid; a protein is also heterologous to a host cell if it is a mutated version of an endogenous protein, and the mutation was introduced by genetic engineering.

[0032] The terms "host cell" and "host microorganism" are used interchangeably herein to refer to a living cell that can be (or has been) transformed via insertion of an expression vector. A host microorganism or cell as described herein may be a prokaryotic cell (e.g. , a microorganism of the kingdom Eubacteria) or a eukaryotic cell. As will be appreciated by one of skill in the art, a prokaryotic cell lacks a membrane-bound nucleus, while a eukaryotic cell has a membrane-bound nucleus. [0033] The terms "isolated" or "pure" refer to material that is substantially, e.g. greater than 50% or greater than 75%, or essentially, e.g. greater than 90%, 95%, 98% or 99%, free of components that normally accompany it in its native state, e.g. the state in which it is naturally found or the state in which it exists when it is first produced.

[0034] A carboxylic acid as described herein can be a salt, acid, base, or derivative depending on the structure, pH, and ions present. The terms "malonate" and "malonic acid" are used interchangeably herein. Malonic acid is also called propanedioic acid ($C_3H_4O_4$; CAS# 141-82-2).

[0035] The term "malonate-derived compounds" as used herein refers to mono-alkyl malonate esters, including, for example and without limitation, mono-methyl malonate (also referred to as monomethyl malonate, CAS# 16695-14-0), mono-ethyl malonate (also referred to as monoethyl malonate, CAS# 1071-46-1), mono-propyl malonate, mono-butyl malonate, mono-teri-butyl malonate (CAS# 40052-13-9), and the like; di-alkyl malonate esters, for example and without limitation, dimethyl malonate (CAS# 108-59-8), diethyl malonate (CAS# 105-53-3), dipropyl malonate (CAS# 1117-19-7), dibutyl malonate (CAS# 1190-39- 2), and the like, and Meldrum's acid (CAS# 2033-24-1). The malonate-derived compounds can be produced synthetically from malonate and are themselves valuable compounds but are also useful substrates in the chemical synthesis of a number of other valuable compounds.

[0036] As used herein, the term "nucleic acid" and variations thereof shall be generic to polydeoxyribonucleotides (containing 2-deoxy-D-ribose) and to polyribonucleotides (containing D-ribose). "Nucleic acid" can also refer to any other type of polynucleotide that is an N-glycoside of a purine or pyrimidine base, and to other polymers containing nonnucleotidic backbones, provided that the polymers contain nucleobases in a configuration that allows for base pairing and base stacking, as found in DNA and RNA. As used herein, the symbols for nucleotides and polynucleotides are those recommended by the IUPAC-IUB Commission of Biochemical Nomenclature (Biochem. 9:4022, 1970). A "nucleic acid" may also be referred to herein with respect to its sequence, the order in which different nucleotides occur in the nucleic acid, as the sequence of nucleotides in a nucleic acid typically defines its

biological activity, e.g., as in the sequence of a coding region, the nucleic acid in a gene composed of a promoter and coding region, which encodes the product of a gene, which may be an RNA, e.g. a rRNA, tRNA, or mRNA, or a protein (where a gene encodes a protein, both the mRNA and the protein are "gene products" of that gene).

[0037] The term "operably linked" refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, ribosome-binding site, and transcription terminator) and a second nucleic acid sequence, the coding sequence or coding region, wherein the expression control sequence directs or otherwise regulates transcription and/or translation of the coding sequence.

[0038] The terms "optional" or "optionally" as used herein mean that the subsequently described feature or structure may or may not be present, or that the subsequently described event or circumstance may or may not occur, and that the description includes instances where a particular feature or structure is present and instances where the feature or structure is absent, or instances where the event or circumstance occurs and instances where it does not.

[0039] As used herein, "recombinant" refers to the alteration of genetic material by human intervention. Typically, recombinant refers to the manipulation of DNA or RNA in a cell or virus or expression vector by molecular biology (recombinant DNA technology) methods, including cloning and recombination. Recombinant can also refer to manipulation of DNA or RNA in a cell or virus by random or directed mutagenesis. A "recombinant" cell or nucleic acid can typically be described with reference to how it differs from a naturally occurring counterpart (the "wild-type"). In addition, any reference to a cell or nucleic acid that has been "engineered" or "modified" and variations of those terms, is intended to refer to a recombinant cell or nucleic acid.

[0040] As used herein, the term "transcription factor biosensor" refers to a system to detect a substance, e.g. , malonate, by activating expression of a "marker" or "reporter" gene where reporter gene expression is mediated by a transcription factor that is capable of binding to a promoter and activating transcription upon binding of that substance, e.g. , malonate. For example, malonate may bind to a transcription factor {e.g. , MdcY) and activate transcription from a promoter {e.g. , PM<I_CL)- A "malonate transcription factor" is a transcription factor that, when bound to malonate, can activate a promoter. Thus, MdcY is a malonate transcription factor.

[0041] The terms "transduce", "transform", "transfect", and variations thereof as used herein refers to the introduction of one or more nucleic acids into a cell. For practical purposes, the nucleic acid must be stably maintained or replicated by the cell for a sufficient period of time to enable the function(s) or product(s) it encodes to be expressed for the cell to be referred to as "transduced", "transformed", or "transfected". As will be appreciated by those of skill in the art, stable maintenance or replication of a nucleic acid may take place either by incorporation of the sequence of nucleic acids into the cellular chromosomal DNA, e.g., the genome, as occurs by chromosomal integration, or by replication extrachromosomally, as occurs with a freely-replicating plasmid. A virus can be stably maintained or replicated when it is "infective": when it transduces a host microorganism, replicates, and (without the benefit of any complementary virus or vector) spreads progeny expression vectors, e.g., viruses, of the

same type as the original transducing expression vector to other microorganisms, wherein the progeny expression vectors possess the same ability to reproduce.

Section 2: Malonyl-CoA hydrolase enzymes

[0042] In accordance with one aspect of the invention, malonate is produced through the action of a malonyl-CoA hydrolase catalyzing the conversion of malonyl-CoA to malonate. To date, no wild-type malonyl-CoA hydrolase gene has been identified, although the presence of a small amount of malonate in the fermentation media of non-engineered strains indicates that a wild-type enzyme with this activity may exist. The present invention provides a number of genes that are counterparts to wild type genes that have been mutated to confer malonyl-CoA hydrolase activity. The host cell making the malonyl-CoA hydrolase is a recombinant host cell; in many embodiments, the host cell has been genetically modified to comprise heterologous nucleic acid(s) encoding malonyl-CoA hydrolase enzyme(s) catalyzing hydrolysis of malonyl-CoA to malonate. In some embodiments, the recombinant host cell is a eukaryote. In various embodiments, the eukaryote is a yeast strain selected from the non-limiting example genera: Candida, Cryptococcus, Hansenula, Issatchenkia, luyveromyces, omagataelia, Lipomyces, Pichia, Rhodosporidium, Rhodotorula, Saccharomyces, or Yarrowia. Those skilled in the art will recognize that these genera broadly encompass yeast, including those distinguished as oleaginous yeast. In some embodiments, the host cell is Saccharomyces cerevisiae. In other embodiments, the host cell is Pichia kudriavzevii. In other embodiments of the invention, the eukaryotic host cell is a fungus or algae. In yet other embodiments, the recombinant host cell is a prokaryote selected from the non-limited example genera: Bacillus, Clostridium, Corynebacterium, Escherichia, Pseudomonas, Rhodobacter, and Streptomyces. In some embodiments, the host cell is E. coli.

[0043] The present invention results in part from the discovery that various acyl-CoA hydrolases and transacylases can be engineered to have malonyl-CoA hydrolase activity and so be useful for biological production of malonate. Non-limiting examples of acyl-CoA hydrolases suitable for modification for malonyl-CoA hydrolysis include any of those from the group consisting of 3-hydroxyisobutyryl-CoA hydrolases (EC 3.1.2.4), 3- hydroxypropionyl-CoA hydrolases (EC 3.1.2.4), acetoacetyl-CoA hydrolases (EC 3.1.2.11), methylmalonyl-CoA hydrolases (EC 3.1.2.17), propionyl-CoA hydrolases (EC 3.1.2.18), succinyl-CoA hydrolases (EC 3.1.2.3), and malonyl-CoA:ACP transacylases (EC 2.3.1.39) mutated as provided herein to have malonyl-CoA hydrolase activity. [0044] In some embodiments, the malonyl-CoA hydrolase used to produce malonate in accordance with the invention is a mutated S. cerevisiae EHD3 acyl-CoA hydrolase (see SEQ ID NO: l for the wild-type EHD3 amino acid sequence). One such mutant with altered substrate specificity is the E124V mutant (see Rouhier, "Characterization of YDR036C from Saccharomyces cerevisiae." Dissertation, Miami University, Miami University and OhioLINK (2011)), which, while previously reported, was not reported to have malonyl-CoA hydrolase activity. In some embodiments of the invention, an E. coli host cell expressing the El 24V mutant is used to produce malonate, which is then purified from the cell or fermentation broth. In other embodiments of the invention, a yeast cell expressing the E124V mutant is used to produce malonate in accordance with the invention. In yet another embodiment, an oleaginous yeast cell expressing the E124V mutant is used to produce malonate in accordance with the invention.

[0045] Prior attempts to produce the E124A mutant of EHD3 resulted in cell death upon induction of protein expression from a pET28a expression vector; the protein was unable to be purified (see Rouhier, supra). The present invention provides expression vectors for the E124A mutant that can be used in E. coli host cells, rendering them capable of producing malonate. These E. coli expression vectors are characterized in that, relative to the pET28a vector of Rouhier, the E124A mutant is produced at a lower, non-toxic, level. This is achieved, for example by employing expression vectors with a lower copy number or weaker promoter than used by Rouhier. One skilled in the art will also appreciate that translation can be modulated by the affinity of the ribosome binding site (RBS), or Kozak sequence, for the ribosome. Thus a weaker RBS or Kozak sequence can also be employed to reduce gene expression. Examples of lower copy number expression vectors include, but are not limited to pSClOl origin expression vectors, pl 5a origin expression vectors, and expression vectors that integrate into the chromosomal DNA. Examples of weaker promoters than the T7 promoter used by Rouhier include, but are not limited to the P_{Lac}oi, PTRC, and PBAD promoters. In some embodiments, the vector has a pSClOl origin of replication. In other embodiments, the promoter used for expression of the EHD3 E124A mutant coding sequence is the Pi_{ac}oi promoter. Additionally, the present invention provides vectors for yeast host cells that code for the expression of the E124A mutant. The genetically modified S. cerevisiae EHD3 E124A expression vectors of the invention can be used in vivo for the production of malonate in E. coli and S. cerevisiae, and the methods of the invention provide means for the subsequent purification of malonate from fermentation broth of these strains, and the synthetic conversion of malonate into derivative small-molecule compounds. [0046] The present invention also provides the E124S mutant of EHD3 for use as a malonyl-CoA hydrolase, vectors for expressing this mutant, and host cells that express this mutant and produce malonate (see Example 31). Wild-type S. cerevisiae EHD3 catalyzes the hydrolysis of 3-hydroxypropionyl-CoA (3HPA-CoA) and 3-hydroxyisobutyryl-CoA (3HIBA-CoA), and while this invention is not to be limited by theory, E124 is predicted to interact with the terminal hydroxyl moiety on 3HPA-CoA, stabilizing the substrate in the EHD3 active site (see Rouhier, supra). Certain aspects of this invention arise from the discovery that specific El 24 point mutations increase enzyme hydrolysis of malonyl-CoA, producing malonate. Mutation of E124 to a nucleophilic amino acid {e.g., S or T), basic amino acid {e.g., H, K, or R), or amide amino acid {e.g., N or Q) improves the binding of malonyl-CoA in the EHD3 active site over 3-hydroxypropionyl-CoA and increases malonate production (relative to the unmutated counterpart enzyme). The E124S, E124T, E124N, E124Q, E124H, E124K, and E124R mutations also decrease production of byproducts {e.g. , acetate, propionate, isobutyrate, and succinate) due to decreased hydrolysis of endogenous host cell acyl-CoA molecules. The E124S point mutation places a hydroxyl moiety in a position that promotes hydrogen bonding between the serine residue and the terminal carboxylate group of malonyl-CoA. The E124Q point mutation places the glutamine amide group in a position near the terminal carboxylate group of malonyl-CoA. The E124K point mutation places the lysine amine group in a position that promotes hydrogen bonding between the lysine residue and the terminal carboxylate group of malonyl-CoA. In contrast to the nucleophilic, amide, and basic E124 point mutations described above, mutations E124A and El 24V remove the presence of a charged amino acid at position 124; these mutations both eliminate hydrogen bonding between the terminal carboxylate on malonate and the EHD3 124 amino acid sidechain and open the EHD3 active site to promiscuous activity, increasing undesirable byproduct formation and decreasing malonate production.

[0047] In some embodiments of the invention, an E. coli host cell expressing the E124S mutant is used to produce malonate. In other embodiments of the invention, a yeast host cell expressing the E124S mutant is used to produce malonate. In other embodiments, an oleaginous yeast host cell expressing the E124S mutant is used to produce malonate. In some embodiments of the invention, an E. coli host cell expressing the E124Q mutant is used to produce malonate. In other embodiments of the invention, a yeast host cell expressing the E124Q mutant is used to produce malonate. In other embodiments, an oleaginous yeast host cell expressing the E124Q mutant is used to produce malonate. In some embodiments of the invention, an E. coli host cell expressing the E124K mutant is used to produce malonate. In other embodiments of the invention, a yeast host cell expressing the E124K mutant is used to produce malonate. In other embodiments, an oleaginous yeast host cell expressing the E124K mutant is used to produce malonate. In some embodiments of the invention, an E. coli host cell expressing the E124H mutant is used to produce malonate. In other embodiments of the invention, a yeast host cell expressing the E124H mutant is used to produce malonate. In other embodiments of the invention, an oleaginous yeast host cell expressing the E124H mutant is used to produce malonate. In some embodiments of the invention, an E. coli host cell expressing the E124R mutant is used to produce malonate. In other embodiments of the invention, a yeast host cell expressing the E124R mutant is used to produce malonate. In other embodiments of the invention, an oleaginous yeast host cell expressing the E124R mutant is used to produce malonate. In other embodiments, a recombinant host cell expressing an EHD3 El 24 nucleophilic amino acid point mutation (i.e., E124S or E124T) is used to produce malonate. In other embodiments, a recombinant host cell expressing an EHD3 E124 basic amino acid point mutation (i.e., E124H, E124K, or E124R) is used to produce malonate. In other embodiments, a recombinant host cell expressing an EHD3 E124 amide amino acid point mutation (i.e., E124N or E124Q) is used to produce malonate.

[0048] The present invention also provides a mutated EHD3 comprising a mutated active site, vectors for expressing the mutant, and host cells that express the mutant and produce malonate. Certain aspects of the present invention arose, in part, from the discovery that specific amino acids (i.e., F121, and F177) are important for acyl-CoA substrate binding, and introduction of specific point mutations increase malonyl-CoA hydrolysis and production of malonate. Introduction of mutation F121I or F121L increases malonyl-CoA access to the active site. Similarly, introduction of mutation F177I or F177L increases malonyl-CoA access to the active site. One or more point mutations at amino acid positions F121 or F177 can be introduced alone, or along with an E124 point mutation. In various embodiments, a F121 and/or F177 point mutation is introduced along with an E124 point mutation. In some embodiments, a recombinant host cell expressing an EHD3 F121I or F121L mutant is used to produce malonate. In other embodiments, a recombinant host cell expressing an EHD3 F177I or F178L mutant is used to produce malonate. In these embodiments, the recombinant host cell can be, without limitation, an E. coli or yeast, including but not limited to S. cerevisiae or other yeast, host cell.

[0049] The present invention also provides mutated EHD3 comprising a mutated mitochondrial targeting sequence, vectors for expressing the mutant, and host cells that express the mutant and produce malonate. In an S. cerevisiae host, wild-type EHD3 is localized in the mitochondria. Malonyl-CoA is found in both the mitochondria and the cytosol; EHD3 catalyzed hydrolysis of cytosolic malonyl-CoA requires localization of an EHD3 to the cytosol. Certain aspects of the present invention arose from the discovery

that mutations of the EHD3 mitochondrial targeting sequence can increase production of malonate. The EHD3 amino acids important for mitochondrial targeting include R3, K7, K14, K18, and R22, and mutation of one or more of these basic amino acids to a hydrophobic amino acid (i.e. , A or V) abrogates mitochondrial targeting. In some embodiments, a recombinant host comprising an EHD3 consisting of one or more mutations to A or V at amino acids selected from the group consisting of R3, K7, K14, K18, and R22 is used to produce malonate. In some embodiments, the recombinant host is a yeast strain. In other embodiments, the host is S. cerevisiae. In still further embodiments, the recombinant host cell contains one or more copies of an EHD3 with the mitochondrial targeting sequence unaltered (i.e. , wild-type) and one or more copies of an EHD3 with the mitochondrial targeting sequence mutated. Additional examples of mitochondrial targeting sequences useful in this aspect of the invention are: WT COX4, SynAl, SynA2, Syn Bl, and Syn B2, as outline for other applications by Allison & Schatz (Allison & Schatz (1986) PNAS 83:9011-9015). In further embodiments of the invention, peroxisomal targeting signal (e.g. PTS1 , PTS2), most often containing a Ser-Lys-Leu motif, is fused to the C-terminus of the malonyl-CoA hydrolase to result in localization of this protein to the peroxisome.

[0050] Thus, in one aspect of the invention, the recombinant host cell comprises a heterologous nucleic acid encoding a mutant S. cerevisiae EHD3 that results in increased production of malonate relative to host cells not comprising the mutant EHD3. In some embodiments, the mutant EHD3 is heterologously expressed in E. coli. In other embodiments, the mutant EHD3 is heterologously expressed in S. cerevisiae. In other embodiments, the mutant EHD3 is heterologously expressed in an oleaginous yeast cell. In some embodiments, the mutant EHD3 contains a point mutation at position E124. In some embodiments, the point mutation at residue E124 is either E124A or E124V. In some embodiments, the point mutation at E124 is E124S or E124T. In some embodiments, the point mutation at E124 is E124S. In some embodiments, the point mutation at E124 is a basic amino acid selected from the group consisting of E124H, E124K, and E124R. In some embodiments, the point mutation at E124 is E124H. In some embodiments, the point mutation at E124 is E124K. In some embodiments, the point mutation at E124 is E124R. In some embodiments, the point mutation at residue E124 is E124N or E124Q. In some embodiments, the point mutation at residue E124 is E124Q. In some embodiments, one or more EHD3 amino acids selected from the group consisting of F121 and F177 are mutated to I or L. In some embodiments, one or more EHD3 amino acids selected from the group consisting of R3, K7, K14, K18, and R22 are mutated to either A or V. [0051] In another aspect of the invention, an enzyme other than, or in addition to, EHD3 is utilized as a malonyl-CoA hydrolase to produce malonate in accordance with the invention. In some embodiments, Haemophilus influenzae YciA is heterologously expressed in a heterologous host to produce malonate in accordance with the invention (see Zhuang et al. Biochemistry 47: 2789-2796 (2008)). In other embodiments, the malonyl-CoA hydrolase is an acyl-CoA hydrolase endogenous to Rattus norvegicus (see Kovachy et al., J. Biol. Chem. 258: 11415-11421 (1983)). In other embodiments, the malonyl-CoA hydrolase is the acyl- CoA hydrolase from brown adipose tissue mitochondrial protein fraction from Mesocricetus auratus (see Alexson et al., J. Biol. Chem. 263: 13564-13571 (1988)).

[0052] Thus, in accordance with the invention acyl-CoA hydrolases other than, or in addition to, EHD3 (from S. cerevisiae or homologous enzymes from other organisms) can be used for biological synthesis of malonate in a recombinant host. In some embodiments, the recombinant host is S. cerevisiae. In other

embodiments, the recombinant host is E. coli. In other embodiments, the recombinant host is a yeast other than S. cerevisiae as described in additional detail below. In various embodiments, the host is modified to express a mutated enzyme selected from the group consisting of S. albicans EHD3, H. sapiens HIBCH (UniProt:Q6NVYl), A. thaliana CHY1 (UniProt:Q9LKJl), R. norvegicus HIBCH (UniProt:Q5XIE6), M. musculus HIBCH (UniProt:Q8QZSl), G. gallus HIBCH (UniProt:Q5ZJ60), B. taurus HIBCH (UniProt:Q2HJ73), D. rerio HIBCH (UniProt:Q58EB4), B. cereus Bch, P. aeruginosa Hich, E. coli YciA, H. influenzae YciA, M. musculus ACOT4, M. musculus ACOT8, S. enterica SARI_01218, A. pernix Kl, C. hutchinsonii Chut02003666, S. solfataricus P2 SS02287, S. acidocaldarius DSM 639 Saci_0145, P. aerophilum str. IM2 PAE3404, D. melanogaster CG1635, P. carbinolicus DSM 2380 Pcar_1366, A. dehalogenans 2CP-C 110, G. gallus ACOT9, and X. laevis MGC 114623.

[0053] Those of skill in the art will appreciate that one or multiple suitably mutated acyl- CoA hydrolases can be used in accordance with the invention to convert malonyl-CoA to malonate in a host cell. Moreover, acyl-CoA hydrolases other than those specifically disclosed herein can be utilized in mutated or heterologously expressed form, and it will be well understood to those skilled in the art in view of this disclosure how other appropriate enzymes can be identified, modified, and expressed to achieve the desired malonyl-CoA hydrolase activity as disclosed herein.

Consensus Sequences

[0054] Malonyl-CoA hydrolases of the invention include those that are homologous to consensus sequences provided by the invention. As noted above, any enzyme substantially homologous to an enzyme specifically described herein can be used in a host cell of the invention. One enzyme is homologous to another (the "reference enzyme") when it exhibits the same activity of interest and can be used for substantially similar purposes. Generally, homologous enzymes share substantial sequence identity. Sets of homologous enzymes generally possess one or more specific amino acids that are conserved across all members of the consensus sequence protein class.

[0055] The percent sequence identity of an enzyme relative to a consensus sequence is determined by aligning the enzyme sequence against the consensus sequence. Those skilled in the art will recognize that various sequence alignment algorithms are suitable for aligning an enzyme with a consensus sequence. See, for example, Needleman, SB, et al "A general method applicable to the search for similarities in the amino acid sequence of two proteins." Journal of Molecular Biology 48 (3): 443-53 (1970). Following alignment of the enzyme sequence relative to the consensus sequence, the percentage of positions where the enzyme possesses an amino acid (or dash) described by the same position in the consensus sequence determines the percent sequence identity. When a degenerate amino acid (i.e. B, J, O, U, "+") is present in a consensus sequence any of the amino acids described by the degenerate amino acid may be present in the enzyme at the aligned position for the enzyme to be identical to the consensus sequence at the aligned position. When a dash is present in a consensus sequence the enzyme must not have an amino acid present in the aligned position for the enzyme to be identical to the consensus sequence at the aligned position.

[0056] The present invention provides consensus sequences useful in identifying and constructing malonyl-CoA hydrolases of the invention. In various embodiments, these malonyl-CoA hydrolase consensus sequences contain active site amino acid residues believed to be necessary (although the invention is not to be limited by any theory of mechanism of action) for formation of an oxyanion hole responsible for stabilizing the enolate anion intermediate derived from a malonyl-CoA substrate as well as the amino acid residues important for malonyl-CoA binding, as described below. A malonyl-CoA hydrolase enzyme encompassed by a consensus sequence provided herein has an enzymatic activity that is identical, or essentially identical, or at least substantially similar with respect to ability to hydrolyze malonyl-CoA to that of one of the enzymes exemplified herein. A malonyl-CoA hydrolase enzyme may be found in nature or, more typically, is an engineered mutant of a wild-type enzyme modified in accordance with the invention to have malonyl-CoA hydrolase activity. A malonyl-CoA hydrolase enzyme may be identified or constructed from another enzyme by mutating the sequence of the other enzyme to create a sequence encompassed by a consensus sequence herein; if an enzyme shares substantial homology to a consensus sequence herein but has suboptimal, including no, malonyl-CoA hydrolase activity, then, in accordance with the invention, it is mutated to conform to a consensus sequence provided herein to provide a malonyl-CoA hydrolase of the invention.

[0057] The invention provides four malonyl-CoA hydrolase consensus sequences: (i) malonyl-CoA hydrolase based on EHD3 EC 3.1.2.4 (SEQ ID NO:7), (ii) malonyl-CoA hydrolase based on Bacillus EC 3.1.2.4 (SEQ ID NO:8), (iii) malonyl-CoA hydrolase based on Pseudomonas EC 3.1.2.4 (SEQ ID NO:9), and (iv) malonyl-CoA hydrolase based on both Bacillus and Pseudomonas EC 3.1.2.4 ((SEQ ID NO: 10). The consensus sequences provide a sequence of amino acids in which each position identifies the amino acid (if a specific amino acid is identified) or a subset of amino acids (if a position is identified as variable) most likely to be found at a specified position in a malonyl-CoA hydrolase of that class. Those of skill in the art will recognize that fixed amino acids and conserved amino acids in these consensus sequences are identical to (in the case of fixed amino acids) or consistent with (in the case of conserved amino acids) with the wild-type sequence(s) on which the consensus sequence is based. A dash in a consensus sequences indicates that suitable enzymes for mutation in accordance with the invention can be found in nature that may have an additional amino acid at the location of the dash in the sequence, but typically no amino acid is present at the location of a dash.

Malonyl-CoA hydrolase consensus sequence based on EHD3 EC 3.1.2.4 enzymes

[0058] The invention provides a malonyl-CoA hydrolase consensus sequence based on EHD3 EC 3.1.2.4 enzymes (SEQ ID NO:7), and in various embodiments, suitable malonyl- CoA hydrolases for use in the methods of the invention have at least 63% identity to this malonyl-CoA hydrolase consensus sequence. In various embodiments, enzymes suitable for mutation of the key glutamic acid residue to Xi in accordance with the methods of the invention to confer malonyl-CoA hydrolase activity have 65%, 70%, 80%, 90%, or 95% or more identity to SEQ ID NO:7. Proteins having significant homology to this consensus sequence include UniProt ID: C5DE94 (63% identity), UniProt ID: Q6CJH2 (64% identity), UniProt ID: G2WAE2 (66% identity), UniProt ID: J8Q6P9 (66% identity), UniProt ID: G8C0H0 (68% identity), UniProt ID: C5DX08 (68% identity), UniProt ID: P28817 (69% identity), UniProt ID: A7TTD5 (69% identity), UniProt ID: J7S9J9 (70% identity), UniProt ID: Q6FM09 (71% identity), UniProt ID:

I2H4L2 (71% identity), UniProt ID: H2AME2 (73% identity), UniProt ID: G8ZTJ4 (77% identity), UniProt ID: G0W4I8 (77% identity), UniProt ID: GOV818 (78% identity), and UniProt ID: J5S5X3 (79% identity). In some embodiments, a malonyl-CoA hydrolase with equal to or greater than 63% identity to the consenseus sequence SEQ ID NO:7 is expressed in a recombinant host cell and used to produce malonate in accordance with the invention.

[0059] In mutant and wild-type enzymes homologous to this consensus sequence (SEQ ID NO: 7), amino acids that are highly conserved are V101, R110, LI 14, R116, K119, L120, N121, A122, L123, L135, E137, Y138, K140, S141, S151, R156, C159, G161, G162, D163, V164, A168, F185, E188, Y189, S190, N192, A196, T197, K200, M206, G208, 1209, T210, M211, G212, G213, G214, V215, G216, H220, P222, F223, R224, T227, E228, T230, M234, P235, E236, D238, 1239, G240, F242, P243, D244, V245, F249, P252, Q263, Y267, L268, T271, G272, G277, G284, S287, H288, Y289, L298, R301, L302, E304, E333, F334, L352, V354, 1355, F359, L374, F391, L399, K402, S403, S406, N417, D429, L430, T432, A433, E449, F450, K457, L458, K461, W468, L494, T502, Y506, P507, L514, P515, and K561. In various embodiments, malonyl-CoA hydrolase enzymes homologous to this consensus sequence (SEQ ID NO:7) contain at least 25% of these conserved amino acids, often a majority (greater than 50%) of these conserved amino acids, and sometimes all of these conserved amino acids.

[0060] Some amino acids in this consensus sequence (SEQ ID NO:7) are essential for activity and conserved across all members of the class. Malonyl-CoA hydrolase enzymes encompassed by the EHD3 EC 3.1.2.4 based consensus sequence contain six active site residues important for hydrolase activity: (i) three active site amino acid residues (G161, G162, G213) in the consensus sequence believed to be necessary (although the invention is not to be limited by any theory of mechanism of action) for formation of an oxyanion hole responsible for stabilizing the enolate anion intermediate derived from the malonyl-CoA substrate; (ii) two amino acid residues (E236, D244) of the consensus sequence necessary for acyl-CoA hydrolysis; and (iii) an amino acid residue at position 188 (of SEQ ID NO:7) believed to be necessary for malonyl-CoA substrate binding. Of these six residues, then, five are present in the consensus sequence (SEQ ID NO:7) and in all malonyl-CoA hydrolases encompassed by that sequence, and the sixth, at position 188 (amino acid Xi in the consensus) is selected from the group consisting of polar or positively charged amino acids (R, H, K, S, T, N, Q, Y), as well as A and D, to provide a malonyl-CoA hydrolase of the invention capable of producing malonate in a recombinant host cell. The six essential residues from the consensus sequence sequence (G161, G162, G213, E236, D244, X_1 I88) correspond to G99, G100, G149, E172, D180, and E124 (typically mutated to Xi), respectively, in S. cerevisiae EHD3 used to illustrate the invention in example 31. Malonyl-CoA hydrolase consensus sequence based on Bacillus EC 3.1.2.4 enzymes

[0061] The invention provides a malonyl-CoA hydrolase consensus sequence (SEQ ID NO:8) based on Bacillus EC 3.1.2.4 enzymes, and in various embodiments, suitable malonyl- CoA hydrolases for use in the methods of the invention have at least 86% identity to this malonyl-CoA hydrolase consensus sequence. In various embodiments, enzymes suitable for mutation of the key glutamic acid residue to Xi in accordance with the methods of the invention to confer malonyl-CoA hydrolase activity have 90%, or 95% or more identity to SEQ ID NO:8. Proteins having significant homology to this consensus sequence include UniProt ID: C2TX63 (92% identity), UniProt ID: C2UV40 (91% identity), UniProt ID: C2QBT2

(93% identity), UniProt ID: C2XTU0 (93% identity), UniProt ID: C2PVQ0 (93% identity), UniProt ID: C3A5N3 (93% identity), UniProt ID: C2SJV4 (93% identity), UniProt ID: C2Z7U1 (92% identity), UniProt ID: C2VTI4 (97% identity), UniProt ID: B3Z9Y3 (97% identity), UniProt ID: B7JNH7 (97% identity), UniProt ID: Q63BK8 (97% identity), UniProt ID: B0Q3Q4 (97% identity), UniProt ID: B0AQX0 (97% identity), UniProt ID: B3YSW2 (97% identity), UniProt ID: C2NHG5 (97% identity), UniProt ID: B3ZIZ8 (97% identity), UniProt ID: C2QSV2 (97% identity), UniProt ID: C3C255 (97% identity), UniProt ID: B5UZZ1 (96% identity), UniProt ID: C2MKL7 (95% identity), UniProt ID: B9IZZ9 (95% identity), UniProt ID: F0PNG8 (95% identity), UniProt ID: Q738L0 (97% identity), UniProt ID: C2PEV7 (95% identity), UniProt ID: C2YRH7 (96% identity), UniProt ID: Q4MU30 (95% identity), UniProt ID: Q81DR3 (96% identity), UniProt ID: C2W7W8 (89% identity), and UniProt ID: A7GPH6 (86% identity). In various embodiments, a malonyl-CoA hydrolase with equal to or greater than 86% identity to the consenseus sequence SEQ ID NO:8 is expressed in a recombinant host cell and used to produce malonate in accordance with the invention. Sequences for B9IZZ9 (SEQ ID NO:46), C3ALI3 (SEQ ID NO:47), F0PNG8 (SEQ ID NO:49), Q63BK8 (SEQ ID NO:51), and Q81DR3 (SEQ ID NO:52) containing $X_!$ at the position of the key glutamic acid residue that is mutated in accordance with the invention to confer malonyl-CoA hydrolase activity are provided in the sequence listing.

[0062] In mutant and wildtype enzymes homologous to this consensus sequence (SEQ ID NO:8) amino acids that are highly conserved are Ml, T2, E3, V5, L6, F7, S8, G13, V14, A15, 117, T18, L19, N20, R21, P22, K23, A24, L25, N26, S27, L28, S29, Y30, M32, L33, 136, G37, K39, L40, K41, E42, W43, E44, 149, 152, V53, L54, K55, G56, A57, G58, K60, G61, F62, C63, A64, G65, G66, D67, 168, K69, T70, L71, Y72, E73, A74, R75, S76, N77, E78, A80, L81, Q82, A84, E85, F87, F88, E90, E91, Y92, 194, D95, T96, Y99, Y101, K103, P104, 1105, 1106, A107, C108, L109, DUO, Gi l l, 1112, V113, M114, G115, G116, G117, V118, G119, L120, T121, N122, G123, A124, R127, 1128, V129, T130, T133, K134, W135, A136, M137, P138, E139, M140, N141, 1142, G143, F144, F145, P146, D147, V148, G149, A150, A151, Y152, F153, L154, N155, A157, P158, G159, G162, V165, A166, L167, A169, L172, K173, A174, D176, V177, L178, 1180, A182, A183, D184, L192, F195, L196, W204, V210, L214, K215, L231, E236, H241, F242, E248, 1250, 1251, S253, L254, E255, F261, L269, L270, S271, K272, S273, P274, S276, L277, K278, V279, T280, L281, K282, Q283, G287, K290, S291, E293, C295, F296, A297, T298, D299, L300, L302, A303, K304, N305, F306, M307, R308, H309, D311, F312, F313, E314, G315, V316, R317, S318, V320, D322, K323, D324, Q325, N326, P327, Y329, K330, Y331, D336, V337, V342, N343, F345, F346, L348, and L349. In various embodiments, malonyl-CoA hydrolase enzymes homologous to this consensus sequence (SEQ ID NO:8) contain at least 25% of these conserved amino acids, often a majority (greater than 50%) of these conserved amino acids, and sometimes all of these conserved amino acids.

[0063] Some amino acids in this consensus sequence (SEQ ID NO:8) are essential for activity and conserved across all members of the class. Malonyl-CoA hydrolase enzymes encompassed by the Bacillus EC 3.1.2.4 based consensus sequence contain six active site residues important for hydrolase activity: (i) three active site amino acid residues (G65, G66, Gl 16) of the consensus sequence believed to be necessary (although the invention is not to be limited by any theory of mechanism of action) for formation of an oxyanion hole responsible for stabilizing the enolate anion intermediate derived from the malonyl-CoA substrate; (ii) two amino acid residues (E139, D147) of the consensus sequence necessary

for acyl-CoA hydrolysis; and (iii) a mutated amino acid (Xi91) (of SEQ ID NO:8) believed to be necessary for malonyl-CoA substrate binding. Of these six residues, then, five are present in the consensus sequence (SEQ ID NO: 8) and in all malonyl-CoA hydrolases encompassed by that sequence, and the sixth, Xi91 is necessary to provide a malonyl-CoA hydrolase of the invention capable of producing malonate in a recombinant host cell. The six essential residues from the consensus sequence sequence (G65, G66, G116, E139, D147, Xi91) correspond to G65, G66, G116, E139, D147, and E91 (typically mutated to Xi), respectively, in Bacillus thuringiensis subsp. finitimus (strain YBT-020) F0PNG8 used to illustrate the invention in example 31 (See SEQ ID NO: 49 containing mutation E91S).

[0064] Non-limiting examples of enzymes suitable for malonyl-CoA hydrolysis homologous to the consensus sequence (SEQ ID NO: 8) and encoded by cloned or synthesized nucleic acids provided by the invention include mutant enzymes containing at least one mutation illustrated by the group of mutant enzymes consisting of Bacillus cereus (strain Ql) B9IZZ9 (E91S), B9IZZ9 (E91A), B9IZZ9 (E91H), B9IZZ9 (E91K), B9IZZ9 (E91R), B9IZZ9 (E91Q), B9IZZ9 (E91T), B9IZZ9 (E91N), B9IZZ9 (E91Y), B9IZZ9 (E91D); Bacillus thuringiensis subsp. finitimus (strain YBT-020) F0PNG8 (E91S), F0PNG8 (E91A), F0PNG8 (E91H), F0PNG8 (E91K), F0PNG8 (E91R), F0PNG8 (E91Q), F0PNG8 (E91T), F0PNG8 (E91N), F0PNG8 (E91Y), F0PNG8 (E91D); Bacillus cereus (strain ATCC 14579 / DSM 31) Q81DR3, Q81DR3 (E91S), Q81DR3 (E91A), Q81DR3 (E91H), Q81DR3 (E91K), Q81DR3 (E91R), Q81DR3 (E91Q), Q81DR3 (E91T), Q81DR3 (E91N), Q81DR3 (E91Y), Q81DR3 (E91D); Bacillus cereus (strain ZK / E33L) Q63BK8, Q63BK8 (E91S), Q63BK8 (E91A), Q63BK8 (E91H), Q63BK8 (E91K), Q63BK8 (E91R), Q63BK8 (E91Q), Q63BK8 (E91T), Q63BK8 (E91N), Q63BK8 (E91Y), Q63BK8 (E91D).

Malonyl-CoA hydrolase consensus sequence based on Pseudomonas EC 3.1.2.4 enzymes

[0065] The invention provides a malonyl-CoA hydrolase consensus sequence based on Pseudomonas EC 3.1.2.4 enzymes (SEQ ID NO:9), and in various embodiments, suitable malonyl-CoA hydrolases for use in the methods of the invention have at least 75% identity to this malonyl-CoA hydrolase consensus sequence. In various embodiments, enzymes suitable for mutation of the key glutamic acid residue to Xi in accordance with the invention to confer malonyl-CoA hydrolase activity have 80%, 90%, or 95% or more identity to SEQ ID NO:9. Proteins having significant homology to this consensus sequence include: UniProt ID: F5KBQ4 (80% identity), UniProt ID: A6VAN3 (81% identity), UniProt ID: A4XS22 (81% identity), UniProt ID: F6AA82 (75% identity), UniProt ID: E2XN63 (84% identity), UniProt ID: F2KE35 (85% identity), UniProt ID: C3KDS5 (83% identity), UniProt ID: F8G3B7 (86% identity), UniProt ID: G8PYD2 (85% identity), UniProt ID: Q4KGS1 (82% identity), UniProt ID: Q3KGL5 (85% identity), UniProt ID: B0KV51 (86% identity), UniProt ID: B1J4J2 (86% identity), UniProt ID: A5W8H3 (86% identity), UniProt ID: Q88N06 (86% identity), UniProt ID: Q1I5T5 (84% identity), UniProt ID: F8H1A4 (77% identity), UniProt ID: A4VIV7 (77% identity), and UniProt ID: Q9I5I5 (81% identity). In some embodiments, a malonyl-CoA hydrolase with equal to or greater than 75% identity to the consenseus sequence SEQ ID NO:9 is expressed in a recombinant host cell and used to produce malonate in accordance with the invention. Sequences for A4XS22 (SEQ ID NO:45), F6AA82 (SEQ ID NO:50), and E2XN63 (SEQ ID NO:48), each containing Xi at the position of the key glutamic acid residue that is mutated in accordance with the invention, are included in the sequence listing.

[0066] Highly conserved amino acids in this consensus sequence (SEQ ID NO:9) are Ml, E6, G13, R15, 116, A19, L21, D22, A23, L27, N28, A29, L30, L32, P33, M34, 135, L38, W45, A46, C53, V54, L56, R57, G58, N59, G60, K62, A63, F64, C65, A66, G67, G68, V70, L73, C77, P81, G82, P85, L87, A88, F91, F92, Y96, R97, L98, H103, P106, K107, P108, Cl l l, W112, H114, G115, V117, G119, G120, G121, M122, G123, L124, Q126, R131, 1132, V133, T134, P135, R138, L139, M141, P142, E143, 1146, G147, L148, D151, V152, G153, S155, F157, L158, R160, P162, G163, L165, G166, L167, F168, L171, N177, D180, A181, D183, L184, L186, A187, D188, R189, Q195, Q196, L199, L203, Q205, N207, W208, E210, Q215, L216, S218, L219, A222, P232, L237, R239, R240, D244, L247, D248, A258, D267, L269, G280, P282, V288, W289, Q291, R294, R296, L298, S299, L300, E307, Y308, S311, L312, N313, C314, R316, H317, P318, F320, E322, G323, V324, R325, A326, R327, L328, D330, D332, P335, W337, W339, P346, A352, H353, and F354. In various embodiments, malonyl-CoA hydrolase enzymes homologous to this consensus sequence (SEQ ID NO:9) contain at least 25% of these conserved amino acids, often a majority (greater than 50%) of these conserved amino acids, and sometimes all of these conserved amino acids.

[0067] Some amino acids in this consensus sequence (SEQ ID NO:9) are essential for activity and conserved across all members of the class. Malonyl-CoA hydrolase enzymes encompassed by the Pseudomonas EC 3.1.2.4 based consensus sequence contain six conserved active site residues necessary for hydrolase activity (i) three active site amino acid residues (G67, G68, G120) of the consensus sequence believed to be necessary (although the invention is not to be limited by any theory of mechanism of action) for formation of an oxyanion hole responsible for stabilizing the enolate anion intermediate derived from an acyl- CoA substrate; (ii) two amino acid residues (E143, D151) of the consensus sequence believed to be necessary for acyl-CoA hydrolysis; and (iii) amino acid Xi95 (of SEQ ID NO:9) is believed to be necessary for malonyl-CoA substrate binding. Of these six residues, then, five are present in the consensus sequence (SEQ ID NO:9) and in all malonyl-CoA hydrolases encompassed by that sequence, and the sixth, Xi95 is necessary to provide a malonyl-CoA hydrolase of the invention capable of producing malonate in a recombinant host cell. In various embodiments of the invention, the key wild-type glutamic acid residue (E95) is (has been) mutated to a polar or positively charged amino acid (i.e. R, H, K, S, T, N, Q, Y), or A or D, to produce Xi95 and provide a malonyl-CoA hydrolase of the invention capable of producing malonate in a recombinant host cell. In some embodiments of the invention, amino acid E95 is (has been mutated to) an amino acid selected from the group consisting of K, S, T, N, Y, A, and D. In some embodiments of the invention, amino acid E95 is S or N. The six essential residues from the consensus sequence sequence (G67, G68, G120, E143, D151, Xi95) correspond to G67, G68, G120, E143, D151, and E95 (typically mutated to Xi), respectively, in Pseudomonas fulva (12-X) F6AA82-2 used to illustrate the invention in example 31 (see SEQ ID NO:50 containing mutations E95S/Q348A).

[0068] Non-limiting examples of enzymes suitable for malonyl-CoA hydrolysis homologous to the consensus sequence (SEQ ID NO:9) and encoded by cloned or synthesized nucleic acids provided by the invention include mutant enzymes containing at least one mutation illustrated by the group of mutant enzymes consisting of Pseudomonas fulva (strain 12-X) F6AA82 (E95S), F6AA82 (E95N), F6AA82 (E95A), F6AA82 (E95H), F6AA82 (E95K), F6AA82 (E95R), F6AA82 (E95Q), F6AA82 (E95D), F6AA82 (E95T), F6AA82 (E95Y) as demonstrated in example 43; Pseudomonas fluorescens WH6

E2XN63 (E95S), E2XN63 (E95N), E2XN63 (E95A), E2XN63 (E95H), E2XN63 (E95K), E2XN63 (E95R), E2XN63 (E95Q), E2XN63 (E95D), E2XN63 (E95T), E2XN63 (E95Y); Pseudomonas mendocina (strain ymp) A4XS22 (E95S), A4XS22 (E95N), A4XS22 (E95A), A4XS22 (E95H), A4XS22 (E95K), A4XS22 (E95R), A4XS22 (E95Q), A4XS22 (E95D), A4XS22 (E95T), E2XN63 (E95Y).

[0069] In various embodiments of the invention the malonyl-CoA hydrolase is F6AA82 (E95S) from Pseudomonas fulva (strain 12-X), E2XN63 (E95S) from Pseudomonas fluorescens WH6, A4XS22 (E95S) from Pseudomonas mendocina (strain ymp), as illustrated in Example 31.

[0070] As illustrated in Example 43, F6AA82 E95Xi mutations resulted in malonyl-CoA hydrolase activity. F6AA82 proteins containing mutations E95S, E95Y, E95T, E95N, E96K, E95A, and E95D produced significantly (t-test, $p<0.05$) more malonate than the wild type F6AA82 protein. F6AA82 proteins containing these mutations are suitable for use as malonyl-CoA hydrolases and production of malonate. Of the F6AA82 enzymes containing an Xi mutation conferring malonyl-CoA hydrolase activity, mutations E95S or E95N are preferred. Of the F6AA82 enzymes containing an Xi mutation conferring malonyl-CoA hydrolase activity, mutations E95A, E95T, E95K, E95Y and E95D are suitable. In various embodiments of the invention the malonyl-CoA hydrolase is F6AA82 (E95S). In other embodiments of the invention the malonyl-CoA hydrolase is F6AA82 (E95N). F6AA82 proteins containing mutations E95H, E95Q, or E95R did not result in increased malonate production under test conditions employed as described in the examples.

[0071] Where an enzyme with substantial homology to a consensus sequence herein has suboptimal or no malonyl-CoA hydrolase activity, then, in accordance with the invention, it can be mutated to conform to a consensus sequence provided herein to provide a malonyl- CoA hydrolase of the invention. For example, protein A5W8H3 shows 86% identity to malonyl-CoA hydrolase consensus SEQ ID NO:9 but does not exhibit malonyl-CoA hydrolase activity (example 31). One or more amino acids that differ between A5W8H3 and consensus SEQ ID NO:9 may be mutated to introduce malonyl-CoA hydrolase activity. Specifically A5W8H3 mutations T2N, C5F, V7J, L8B, G10U, D12B, P24J, A26U, N31U, Q41B, A72B, A740, Q75J, S83J, S90B, A94J, F100B, A101B, N129U, A159U, F1690, P175B, G185J, G192B, A198J, A213J, N217B, Q224J, C228B, A229J, W2360, H241Q, E242B, Q245J, A2520, R261A, Q264J, D272B, G274A, Q275B, Y297B, Q302J, Q305B, M310O, N333B, A347J, A355J, A3570, and/or G368U may be used to impart malonyl-CoA hydrolase activity.

Malonyl-CoA hydrolase consensus sequence based on bacterial EC 3.1.2.4 enzymes

[0072] Despite Bacillus and Pseudomonas being evolutionarily distant (i.e. Bacillus is Gram-positive and Pseudomonas is Gram-negative), there is significant sequence conservation between the Bacillus EC 3.1.2.4 and Pseudomonas EC 3.1.2.4 enzymes. The present invention provides a malonyl-CoA hydrolase consensus sequence based on these bacterial EC 3.1.2.4 acyl-CoA hydrolases (SEQ ID NO: 10). Malonyl-CoA hydrolase enzymes encompassed by this consensus sequence typically possess at least 25% (or a majority or all) of the highly conserved amino acids from this sequence, which conserved amino acids are selected from the group consisting of L53, L59, N60, L62, M66, L88, F97, C98, A99, G100, G101, F124, F125, Y129, K140, P141, G148, G152, G153, G154, G156, L157, T167, M174, P175, E176, 1179, G180,

D184, V185, G186, L191, L210, D219, A226, P333, N364, F375, E377, D385, and P390. A suitable malonyl-CoA hydrolase of the invention homologous to this consensus sequence includes the active site amino acids necessary for malonyl-CoA hydrolysis (G100, G101, G153, E176, and D184) of the consensus sequence, as well as a Xil28, where the key wild-type glutamic acid residue (E128) is (has been) mutated to a polar or charged amino acid (i.e. R, H, K, S, T, N, Q, Y), or D or A, and is capable of producing malonate in a recombinant host cell.

Malonyl-CoA hydrolases based on malonyl-CoA:ACP transacylases

[0073] In yet other embodiments of the invention, the malonyl-CoA hydrolase is a mutated malonyl-CoA:ACP transacylase (EC 2.3.1.39). The invention provides an E. coli FabD sequence (SEQ ID NO:53), and in various embodiments, suitable malonyl-CoA hydrolases for use in the methods of the invention have at least 50%, 60%, 70%, 80%, 90%, 95%, or more sequence identity when aligned relative to SEQ ID NO: 53 and contain one or more of the following amino acid mutations at the aligned positions: S92C, H201N, R117D, R117E, R117N, R117Y, R117G, R117H, Q11D, QUE, Q11N, Q11Y, Q11G, Q11H, L93A, L93V, L93I, L93F, L93S, L93G.

[0074] In some embodiments of this invention the malonyl-CoA hydrolase is a mutated E. coli FabD malonyl-CoA:ACP transacylase (see SEQ ID NO:53 for wild type sequence) with one or more mutation selected from the group consisting of S92C, H201N, R117D, R117E, R117N, R117Y, R117G, R117H, Q11D, QUE, Q11N, Q11Y, Q11G, Q11H, L93A, L93V, L93I, L93F, L93S, L93G. Example 35 illustrates recombinant yeast cells expressing a mutated E. coli FabD enzyme containing one of the following combinations of mutations S92C/L91V/R117H, L91I/R117Y/A246E, Q80L/L91S/R117G, and L91I/R117Y, and producing malonate at levels higher than wild type yeast without a mutated FabD enzyme. Section 3: Expression vectors

[0075] In various aspects of the present invention, the recombinant host cell has been modified by "genetic engineering" to produce a recombinant malonyi-CoA hydrolase enzyme and malonate. The host cell is typically engineered via recombinant DNA technology to express heterologous nucleic acids that encode a malonyl-CoA hydrolase, which is either a mutated version of a naturally occurring acyl-CoA hydrolase or transacylase or a non- naturally occurring malonyl-CoA hydrolase prepared in accordance with one of the consensus sequences provided herein or is a naturally occurring acyl-CoA hydrolase with malonyl-CoA hydrolase activity that is either overexpressed in the ceil in which it naturally occurs or is heterologously expressed in a cell in which it does not naturally occur.

[0076] Nucleic acid constructs of the present invention include expression vectors that comprise nucleic acids encoding one or more malonyl-CoA hydrolase enzymes. The nucleic acids encoding the enzymes are operably linked to promoters and optionally other control sequences such that the subject enzymes are expressed in a host cell containing the expression vector when cultured under suitable conditions. The promoters and control sequences employed depend on the host cell selected for the production of malonate. Thus, the invention provides not only expression vectors but also nucleic acid constructs useful in the construction of expression vectors. Methods for designing and making nucleic acid constructs and

expression vectors generally are well known to those skilled in the art and so are only briefly reviewed herein.

[0077] Nucleic acids encoding the malonyl-CoA hydrolase enzymes can be prepared by any suitable method known to those of ordinary skill in the art, including, for example, direct chemical synthesis and cloning. Further, nucleic acid sequences for use in the invention can be obtained from commercial vendors that provide de novo synthesis of the nucleic acids.

[0078] A nucleic acid encoding the desired enzyme can be incorporated into an expression vector by known methods that include, for example, the use of restriction enzymes to cleave specific sites in an expression vector, e.g., plasmid, thereby producing an expression vector of the invention. Some restriction enzymes produce single stranded ends that may be annealed to a nucleic acid sequence having, or synthesized to have, a terminus with a sequence complementary to the ends of the cleaved expression vector. The ends are then covalently linked using an appropriate enzyme, e.g., DNA ligase. DNA linkers may be used to facilitate linking of nucleic acids sequences into an expression vector. [0079] A set of individual nucleic acid sequences can also be combined by utilizing polymerase chain reaction (PCR)-based methods known to those of skill in the art. For example, each of the desired nucleic acid sequences can be initially generated in a separate PCR. Thereafter, specific primers are designed such that the ends of the PCR products contain complementary sequences. When the PCR products are mixed, denatured, and reannealed, the strands having the matching sequences at their 3' ends overlap and can act as primers for each other. Extension of this overlap by DNA polymerase produces a molecule in which the original sequences are "spliced" together. In this way, a series of individual nucleic acid sequences may be joined and subsequently transduced into a host cell simultaneously. Thus, expression of each of the plurality of nucleic acid sequences is effected.

[0080] A typical expression vector contains the desired nucleic acid sequence preceded and optionally followed by one or more control sequences or regulatory regions, including a promoter and, when the gene product is a protein, ribosome binding site, e.g., a nucleotide sequence that is generally 3-9 nucleotides in length and generally located 3-11 nucleotides upstream of the initiation codon that precede the coding sequence, which is followed by a transcription terminator in the case of E. coli or other prokaryotic hosts. See Shine et ah, Nature. 254:34 (1975) and Steitz, in Biological Regulation and Development: Gene Expression (ed. R. F. Goldberger), vol. 1, p. 349 (1979) Plenum Publishing, N.Y. In the case of eukaryotic hosts like yeast a typical expression vector contains the desired nucleic acid coding sequence preceded by one or more regulatory regions, along with a Kozak sequence to initiate translation and followed by a terminator. See Kozak, Nature 308:241-246 (1984).

[0081] Regulatory regions or control sequences include, for example, those regions that contain a promoter and an operator. A promoter is operably linked to the desired nucleic acid coding sequence, thereby initiating transcription of the nucleic acid sequence via an RNA polymerase. An operator is a sequence of nucleic acids adjacent to the promoter, which contains a protein-binding domain where a transcription factor can bind. Transcription factors activate or repress transcription initation from a promoter. In this way, control of transcription is accomplished, based upon the particular regulatory regions used and the presence or absence of the corresponding transcription factor. Non-limiting

examples for prokaryotic expression include lactose promoters (Lacl repressor protein changes conformation when contacted with lactose, thereby preventing the Lacl repressor protein from binding to the operator) and tryptophan promoters (when complexed with tryptophan, TrpR repressor protein has a conformation that binds the operator; in the absence of tryptophan, the TrpR repressor protein has a conformation that does not bind to the operator). Non-limiting examples of promoters to use for eukaryotic expression include pTDH3, pTEFl (as illustrated in Example 31), pTEF2, pRNR2, pRPL18B, pREVl, pGALl , pGALlO, pGAPDH, pCUPl , pMET3, pPGKl, pPYKl, pHXT7, pPDCl, pFBAl, pTDH2, pPGIl, pPDCl, pTPIl, pEN02, pADHl, and pADH2. As described in Example 44, the promoters for the genes HSP150, PGK1, PH05, SCT1, PRB 1, TPI1, ACH1, HXK2, ACOl, JEN1, MDH2, POX1, CITl, ALD4, ADH1, TDH3, ADH2, and SDH1 from S. cerevisiae strain BY4741 were demonstrated to be useful for the production malonic acid in accordance with the invention. As will be appreciated by those of ordinary skill in the art, a variety of expression vectors and components thereof may be used in the present invention.

[0082] Although any suitable expression vector may be used to incorporate the desired sequences, readily available expression vectors include, without limitation: plasmids, such as pESC, pTEF, p414CYCl, p414GALS, pSClOl, pBR322, pBBRlMCS-3, pUR, pEX, pMRlOO, pCR4, pBAD24, pUC19, pRS series; and bacteriophages, such as M13 phage and λ phage. Of course, such expression vectors may only be suitable for particular host cells or for expression of particular malonyl-CoA hydrolases. One of ordinary skill in the art, however, can readily determine through routine experimentation whether any particular expression vector is suited for any given host cell or protein. For example, the expression vector can be introduced into the host cell, which is then monitored for viability and expression of the sequences contained in the vector. In addition, reference may be made to the relevant texts and literature, which describe expression vectors and their suitability to any particular host cell. In addition to the use of expression vectors, strains are built where expression cassettes are directly integrated into the host genome.

[0083] The expression vectors are introduced or transferred, e.g. by transduction, transfection, or transformation, into the host cell. Such methods for introducing expression vectors into host cells are well known to those of ordinary skill in the art. For example, one method for transforming E. coli with an expression vector involves a calcium chloride treatment wherein the expression vector is introduced via a calcium precipitate.

[0084] For identifying whether a nucleic acid has been successfully introduced or into a host cell, a variety of methods are available. For example, a culture of potentially transformed host cells may be separated, using a suitable dilution, into individual cells and thereafter individually grown and tested for expression of a desired gene product of a gene contained in the introduced nucleic acid. For example, an often-used practice involves the selection of cells based upon antibiotic resistance that has been conferred by antibiotic resistance- conferring genes in the expression vector, such as the beta lactamase {amp), aminoglycoside phosphotransferase (neo), and hygromycin phosphotransferase (hyg, hph, hpt) genes.
[0085] Typically, a host cell of the invention will have been transformed with at least one expression vector. When only a single expression vector is used, the vector will typically contain a malonyl-CoA hydrolase gene. Once the host cell has been transformed with the expression vector, the host cell is

cultured in a suitable medium containing a carbon source, such as a sugar (e.g., glucose). As the host cell is cultured, expression of the enzyme(s) for producing malonate occurs. Once expressed, the enzyme(s) catalyzes the hydrolysis of the thioester bond of malonyl-CoA, thus releasing malonate and CoA.

[0086] If a host cell of the invention is to include more than one heterologous gene, the multiple genes can be expressed from one or more vectors. For example, a single expression vector can comprise one, two, or more genes encoding one, two, or more malonyl-CoA hydrolase enzyme(s) and/or other proteins providing some useful function, e.g. improved malonate yield, titer, and/or productivity. The heterologous genes can be contained in a vector replicated episomally or in a vector integrated into the host cell genome, and where more than one vector is employed, then all vectors may replicate episomally (extrachromasomally), or all vectors may integrate, or some may integrate and some may replicate episomally. Chromosomal integration is typically used for cells that will undergo sustained propagation, e.g., cells used for production of malonate for industrial applications. Example 45 illustrates the benefits of genomic integration of a malonyl-CoA hydrolase gene provided by the invention; in that example, a single integrated copy of the gene is shown to result in higher malonic acid titers than achieved when the same gene was expressed from a plasmid. This example also serves to demonstrate modulation of malonic acid production as provided by the invention, as an increase in the number of copies of the malonyl-CoA hydrolase encoding gene per cell results in an increase of malonic acid in the fermentation media. While a "gene" is generally composed of a single promoter and a single coding sequence, in certain host cells, two or more coding sequences may be controlled by one promoter in an operon. In some embodiments, a two or three operon system is used.

[0087] In some embodiments, the coding sequences employed have been modified, relative to some reference sequence, to reflect the codon preference of a selected host cell. Codon usage tables for numerous organisms are readily available and can be used to guide sequence design. The use of prevalent codons of a given host organism generally improves translation of the target sequence in the host cell. As one non-limiting example, in some embodiments the subject nucleic acid sequences will be modified for yeast codon preference (see, for example, Bennetzen et al., J. Biol. Chem. 257: 3026-3031 (1982)). In some embodiments, the nucleotide sequences will be modified for E. coli codon preference (see, for example, Nakamura et al., Nucleic Acids Res. 28:292 (2000)). In other embodiments, the nucleotide sequences are modified to include codons optimized for S. cerevisiae codon preference (see Example 42).

[0088] Nucleic acids can be prepared by a variety of routine recombinant techniques. Briefly, the subject nucleic acids can be prepared from genomic DNA fragments, cDNAs, and RNAs, all of which can be extracted directly from a cell or recombinantly produced by various amplification processes including but not limited to PCR and rt-PCR. Subject nucleic acids can also be prepared by a direct chemical synthesis.

[0089] The nucleic acid transcription levels in a host microorganism can be increased (or decreased) using numerous techniques. For example, the copy number of the nucleic acid can be increased through use of higher copy number expression vectors comprising the nucleic acid sequence, or through integration of multiple copies of the desired nucleic acid into the host microorganism's genome, as demonstrated in Example 45. Non- limiting examples of integrating a desired nucleic acid sequence onto the host chromosome include recA-mediated recombination, lambda phage recombinase-mediated

recombination and transposon insertion. Nucleic acid transcript levels can be increased by changing the order of the coding regions on a polycistronic mRNA or breaking up a polycistronic operon into multiple poly- or mono- cistronic operons each with its own promoter. RNA levels can be increased (or decreased) by increasing (or decreasing) the strength of the promoter to which the protein-coding region is operably linked. Illustrative techniques for plasmid design and assembly to afford malonate production are provided in Examples 1, 3, 31, and 35.

[0090] The translation level of a desired polypeptide sequence in a host microorganism can also be increased in a number of ways. Non-limiting examples include increasing the mRNA stability, modifying the ribosome binding site (or Kozak) sequence, modifying the distance or sequence between the ribosome binding site (or Kozak sequence) and the start codon of the nucleic acid sequence coding for the desired polypeptide, modifying the intercistronic region located 5' to the start codon of the nucleic acid sequence coding for the desired polypeptide, stabilizing the 3'-end of the mRNA transcript, modifying the codon usage of the polypeptide, altering expression of low-use/rare codon tRNAs used in the biosynthesis of the polypeptide. Determination of preferred codons and low-use/rare codon tRNAs can be based on a sequence analysis of genes derived from the host microorganism.

[0091] The polypeptide half-life, or stability, can be increased through mutation of the nucleic acid sequence coding for the desired polypeptide, resulting in modification of the desired polypeptide sequence relative to the control polypeptide sequence. When the modified polypeptide is an enzyme, the activity of the enzyme in a host may be altered due to increased solubility in the host cell, improved function at the desired pH, removal of a domain inhibiting enzyme activity, improved kinetic parameters (lower Km or higher Kcat values) for the desired substrate, removal of allosteric regulation by an intracellular metabolite, and the like. Altered/modified enzymes can also be isolated through random mutagenesis of an enzyme, such that the altered/modified enzyme can be expressed from an episomal vector or from a recombinant gene integrated into the genome of a host microorganism.

Section 4: Recombinant host cells

[0092] In one aspect, the invention provides recombinant host cells suitable for biological production of malonate. Any suitable host cell may be used in practice of the methods of the present invention. In some embodiments, the host cell is a recombinant host microorganism in which nucleic acid molecules have been inserted, deleted or modified (i.e., mutated; e.g., by insertion, deletion, substitution, and/or inversion of nucleotides), either to produce malonate, or to increase yield, titer, and/or productivity of malonate relative to a "control cell" or "reference cell". A "control cell" can be used for comparative purposes, and is typically a wild-type or recombinant parental cell that does not contain one or more of the modification(s) made to the host cell of interest.

[0093] In an important embodiment, the present invention provides recombinant yeast cells suitable for the production of malonate at levels sufficient for subsequent purification and use as described herein. Yeast host cells are excellent host cells for construction of recombinant metabolic pathways comprising heterologous enzymes catalyzing production of small molecule products. There are established molecular biology techniques and nucleic acids encoding genetic elements necessary for construction of yeast

expression vectors, including, but not limited to, promoters, origins of replication, antibiotic resistance markers, auxotrophic markers, terminators, and the like. Second, techniques for integration of nucleic acids into the yeast chromosome are well established. Yeast also offers a number of advantages as an industrial fermentation host. Yeast can tolerate high concentrations of organic acids and maintain cell viability at low pH and can grow under both aerobic and anaerobic culture conditions, and there are established fermentation broths and fermentation protocols. The ability of a strain to propagate and/or produce desired product under low pH provides a number of advantages with regard to the present invention. First, this characteristic provides tolerance to the environment created by the production of malonic acid. Second, from a process standpoint, the ability to maintain a low pH environment limits the number of organisms that are able to contaminate and spoil a batch. Third, this characteristic also eliminates or at least reduces the need to add additional acid to facilitate purification of malonic acid by some methods provided by the invention (see Example 37). [0094] In some embodiments of the invention, the recombinant host cell comprising a heterologous nucleic acid encoding a malonyl-CoA hydrolase is a eukaryote. In various embodiments, the eukaryote is a yeast selected from the non-limiting list of genera; Candida, Cryptococcus, Ilansenuia, Issatchenki, Kluyveromyces, Komagataella, Lipomyces, Pichia, Rhodosporidium, Rhodotorula, Saccharomyces or Yarrowia species. In various embodiments, the yeast is of a species selected from the group consisting of Candida albicans, Candida etbanolica, Candida krusei, Candida methanosorbosa, Candida sonorensis, Candida tropicalis, Cryptococcus curvatus, Ilansenuia polymorpha, Issatchenki a orientalis, Kluyveromyces lactis, Kluyveromyces marxianus, Kluyveromyces thermotoleraas, Komagataella pastoris, Lipomyces starkeyi, Pichia angusta, Pichia deserticola, Pichia galeiformis, Pichia kodamae, Pichia kudriavzevii, Pichia membranaefaciens, Pichia methanolica, Pichia pastoris, Pichia salictaria, Pichia stipitis, Pichia thermotolerans, Pichia trehalophila, Rhodosporidium toruloides, Rhodotorula glutinis, Rhodotorula graminis, Saccharomyces bayanus, Saccharomyces bouiardi, Saccharomyces cerevisiae, Saccharomyces kiuyveri, and Yarrowia lipolytica. One skilled in the art will recognize that this list encompasses yeast in the broadest sense, including both oleaginous and non-oleaginous strains. Examples 46 and 47 illustrate the use of Pichia kudriavzevii strain Y-134 in accordance with the invention.

[0095] Alternative recombinant host cells are provided by the invention for biological production of malonate. illustrative examples include eukaryotic, prokaryotic, and archaea cells. Illustrative examples of eukaryotic cells include, but are not limited to: Aspergillus niger, Aspergillus oryzae, Crypthecodinium cohnii, Cunninghamella japonica, Entomophthora coronata, Mortierella alpina, Mucor circinelloides, Neurospora crassa, Pythium ultimum, Schizochytrium limacinum, Thrausiochytrium aureum, Trichoderma reesei and Xaniliophyllomyces dendrorhous. In general, if a eukaryotic ceil is used, a nonpathogenic strain is employed. Illustrative examples of non-pathogenic strains include, but are not limited to: Piciiia pastoris and Saccharomyces cerevisiae. In addition, certain strains, including Saccharomyces cerevisiae, have been designated by the Food and Drug Administration as Generally Regarded As Safe (or GRAS) and so can be conveniently employed in various embodiments of the methods of the invention.

[0096] Illustrative examples of recombinant prokaryotic host cells provided by the invention include, but are not limited to, Bacillus subtilis, Brevibacterium ammoniagenes, Clostridium beigerinckii, Enterobacter sakazakii, Lactobacillus acidophilus, Lactococcus lactis, Mesorhizobium loti, Pseudomonas

aeruginosa, Pseudoinonas putida, Rhodobacler capsulatus, Rhodobacter sphaeroides, Salmonella enterica. Salmonella typhi, Salmonella typhimurium, Shigella flexneri, Staphylococcus aureus, Sireptomyces ambofaciens, Streplomyces aureofaciens, Streptomyces aureus, Streptomyc.es fungkidicus, Streptomyces griseochromogenes, Streptomyces griseus, Streptomyces lividans, Streptomyces oiivogriseus, Streptomyces rameus, Streptomyces tanashiensis, and Streptomyces vinaceus. Certain of these cells, including Bacillus subtilis, Lactobacillus acidophilus, have been designated by the Food and Drug Administration as Generally Regarded As Safe (or G AS) and so are employed in. various embodiments of the methods of the invention. While desirable from public safety and regulatory standpoints, GRAS status does not impact the ability of a host strain to be used in the practice of this invention; hence, non-GRAS and even pathogenic organisms are included in the list of illustrative host strains suitable for use in the practice of this invention.

[0097] Escherichia coli is also an excellent prokaryotic host cell for metabolic pathway construction, and E. coli is also well utilized in industrial fermentation of small-molecule products. Unlike most wild type yeast strains, wild type E. coli can catabolize both pentose and hexose sugars as carbon sources. E. coli also has a shorter doubling time relative to yeast, enabling experiments to be conducted more rapidly. The present invention provides a wide variety of E. coli host cells suitable for the production of malonate as described herein. In various embodiments of the methods of the invention, the recombinant host cell comprising a heterologous nucleic acid encoding a malonyl-CoA hydrolase is an E. coli cell.

Section 5: Additional modifications and fermentation conditions for improved malonate production

[0098] In other aspects of the invention, increased malonate yield, titer, and/or productivity is achieved by employing host cells provided by the invention that have been genetically modified in ways other than, or in addition to, introduction of a heterologous malonyl-CoA hydrolase and/or by employing fermentation conditions provided by certain methods of the invention. In brief, the recombinant host cell of the invention comprise genetic modifications that increase acetyl-CoA biosynthesis, increase malonyl-CoA biosynthesis, decrease malonate catabolism, increase secretion of malonate from the host cell, increase host cell tolerance to malonate, and increase catabolism of various carbon sources.

Genetic modifications and fermentation conditions that increase acetyl-CoA biosynthesis

[0099] In accordance with the invention, increased malonate titer, yield, and/or productivity can be achieved by genetic modifications that increase acetyl-CoA biosynthesis, and the invention provides enzymes that increase acetyl-CoA biosynthesis, vectors for expressing enzymes that increase acetyl-CoA biosynthesis, host cells expressing enzymes that increase acetyl-CoA biosynthesis and increase malonate titer, yield, and/or productivity, and methods relating thereto. As described above, malonate is produced by hydrolysis of malonyl-CoA, which, can be produced from acetyl-CoA; thus, increases in acetyl-CoA biosynthesis can improve malonate production.

[0100] One route by which acetyl-CoA is produced is by an acetyl-CoA synthetase (EC 6.2.1.1), which catalyzes the formation of acetyl-CoA from acetate and coenzyme A (CoA). The invention provides recombinant host cells suitable for producing malonate in accordance with the methods of the invention

comprising one or more heterologous acetyl-CoA synthetase (ACS) enzymes that increase malonate titer, yield, and/or productivity relative to a host cell not comprising a heterologous acetyl-CoA synthetase. Non-limiting examples of suitable ACS enzymes are S. cerevisiae ACS1 (GenBank: AAC04979.1) and ACS2 (GenBank: CAA97725.1). In some embodiments, a recombinant host cell comprising S. cerevisiae acetyl-CoA synthetase ACS1 and/or ACS2 is used to increase malonate titer, yield, and/or productivity. In other embodiments, a recombinant host cell comprising an acetyl-CoA synthetase selected from the group consisting of Salmonella enterica Acs, Escherichia coli AcsA, and Bacillus subtilis AcsA is used to increase malonate yield, titer, and/or productivity. Those skilled in the art appreciate that other acetyl-CoA synthetases can be expressed in a recombinant host cell producing malonate in accordance with the invention to increase malonate yield, titer, and/or productivity. This modification is illustrated in Examples 9 and 46.

[0101] A second route through which acetyl-CoA is produced is by a pyruvate dehydrogenase complex, which catalyzes the formation of acetyl-CoA from pyruvate. The invention provides recombinant host cells suitable for producing malonate in accordance with the methods of the invention that comprise one or more heterologous pyruvate dehydrogenase complex enzymes that increase malonate titer, yield, and/or productivity relative to a host cell not comprising a heterologous pyruvate dehydrogenase complex enzyme. Non-limiting examples of suitable pyruvate dehydrogenase complex enzymes include S. cerevisiae PDAl, PDB l, LATl, LPDl, and PDXl. In some embodiments of the invention, malonate yield, titer, and/or productivity are increased in a recombinant host cell used to produce malonate by expressing one or more pyruvate dehydrogenase enzymes selected from the group consisting of S. cerevisiae PDAl, PDB l, LATl, LPDl, and PDXl. Those skilled in the art appreciate that other pyruvate dehydrogenase enzymes can be expressed in a recombinant host cell producing malonate in accordance with the invention to increase malonate yield, titer, and/or productivity. This modification is illustrated in Example 10.

[0102] A third route through which acetyl-CoA is produced is by a heterologous ethanol catabolic pathway comprising enzymes catalyzing the conversion of ethanol to acetyl-CoA. Compared to malonate, ethanol is a less expensive chemical, and host cells producing malonate and expressing an ethanol catabolic pathway can convert ethanol to malonate. An alcohol dehydrogenase (EC 1.1.1.1) catalyzes conversion of ethanol to acetaldehyde. Non- limiting examples of suitable alcohol dehydrogenase enzymes include those selected from the group consisting of S. cerevisiae ADH2, E. coli AdhP, H. sapiens ADH1A, H. sapiens ADH1B, and H. sapiens ADH1C. In addition to the alcohol dehydrogenase, an ethanol catabolic pathway also comprises either an acetaldehyde dehydrogenase (acylating; EC 1.2.1.10), or an aldehyde dehydrogenase (EC 1.2.1.3) and an acetyl-CoA synthetase (EC 6.2.1.1). An acetaldehyde dehydrogenase (acylating) catalyzes the conversion of acetaldehyde to acetyl-CoA, an aldehyde dehydrogenase catalyzes the conversion of acetaldehyde to acetate, and an acetyl-CoA synthase, as described above, catalyzes the formation of acetyl-CoA from acetate and CoA. Non-limiting examples of suitable acetaldehyde dehydrogenases (acylating) include those selected from the group consisting of E. coli MhpF, E. coli AdhE, Pseudomonas sp CF600 DmpF, and Pseudomonas putida TodL. Non-limiting examples of aldehyde dehydrogenases include S. cerevisiae ALD2, ALD3, ALD4, ALD5, and ALD6; and H. sapiens ALD1, ALD2, ALD4, and ALD10. Non-limiting examples of acetyl-CoA synthetase enzymes include S. cerevisiae ACS1, S. cerevisiae ACS2, and E. coli Acs.

[0103] The present invention provides recombinant host cells suitable for producing malonate in accordance with the methods of the invention comprising one or more heterologous ethanol catabolic pathway enzymes that increase malonate yield, titer, and/or productivity relative to host cells not comprising the heterologous ethanol catabolic pathway enzyme(s). In some embodiments, the heterologous ethanol catabolic pathway enzymes are an ethanol dehydrogenase and an acetaldehyde dehydrogenase (acylating). In some embodiments, the heterologous ethanol catabolic pathway enzymes are S. cerevisiae ADH2 ethanol dehydrogenase and E. coli MhpF acetaldehyde dehydrogenase (acylating). In some embodiments, a heterologous S. cerevisiae ADH2 and E. coli MhpF are expressed in recombinant E. coli expressing a heterologous S. cerevisiae EHD3 malonyl-CoA hydrolase. In other embodiments, a heterologous S. cerevisiae ADH2 and E. coli MhpF are expressed in recombinant S. cerevisiae expressing a heterologous S. cerevisiae EHD3 malonyl-CoA hydrolase. In other embodiments, a heterologous S. cerevisiae ADH2 and E. coli MhpF are expressed in a recombinant oleaginous yeast expressing a heterologous S. cerevisiae EHD3 malonyl-CoA hydrolase. In other embodiments, the heterologous ethanol catabolic pathway enzymes are S. cerevisiae ADH2 ethanol dehydrogenase and Pseudomonas sp. CF600 DmpF acetaldehyde dehydrogenase (acylating). In some embodiments, a heterologous S. cerevisiae ADH2 and Pseudomonas sp. CF600 DmpF are expressed in recombinant E. coli expressing a heterologous S. cerevisiae EHD3 malonyl-CoA hydrolase. In some embodiments, a heterologous S. cerevisiae ADH2 and Pseudomonas sp. CF600 DmpF are expressed in recombinant S. cerevisiae expressing a S. cerevisiae EHD3 malonyl-CoA hydrolase. In some embodiments, a heterologous S. cerevisiae ADH2 and Pseudomonas sp. CF600 DmpF are expressed in a recombinant oleaginous yeast expressing a heterologous S. cerevisiae EHD3 malonyl-CoA hydrolase. In other embodiments, the heterologous ethanol catabolic pathway enzymes are S. cerevisiae ADH2 ethanol dehydrogenase and Pseudomonas putida TodL acetaldehyde dehydrogenase (acylating). In some embodiments, a heterologous S. cerevisiae ADH2 and Pseudomonas putida TodL are expressed in recombinant E. coli expressing a heterologous S. cerevisiae EHD3 malonyl-CoA hydrolase. In some embodiments, a heterologous S. cerevisiae ADH2 and Pseudomonas putida TodL are expressed in recombinant S. cerevisiae expressing a heterologous S. cerevisiae EHD3 malonyl-CoA hydrolase. In some embodiments, a heterologous S. cerevisiae ADH2 and Pseudomonas putida TodL are expressed in a recombinant oleaginous yeast expressing a heterologous S. cerevisiae EHD3 malonyl-CoA hydrolase. In other embodiments, the heterologous ethanol catabolic pathway enzymes are one or more alcohol dehydrogenase selected from the group containing S. cerevisiae ADH2, E. coli AdhP, H. sapiens ADH1A, H. sapiens ADH1B, and/or H. sapiens ADH1C and one or more acetaldehyde dehydrogenase (acylating) selected from the group containing E. coli MhpF, E. coli AdhE, Pseudomonas sp CF600 DmpF, and Pseudomonas putida TodL. Those skilled in the art appreciate that other alcohol dehydrogenase enzymes and acetaldehyde dehydrogenase (acylating) enzymes can be expressed in a recombinant host cell suitable for producing malonate in accordance with the methods of the invention to increase malonate yield, titer, and/or productivity.

[0104] In other embodiments, the heterologous ethanol catabolic pathway enzymes are an ethanol dehydrogenase, an aldehyde dehydrogenase, and an acetyl-CoA synthetase. In some embodiments, the heterologous ethanol catabolic pathway enzymes are a S. cerevisiae ALD2 alcohol dehydrogenase, a S. cerevisiae ALD2 aldehyde dehydrogenase, and a S. cerevisiae ACS1 acetyl-CoA synthetase. In other embodiments, the heterologous ethanol catabolic pathway enzymes are a S. cerevisiae ALD2 alcohol

dehydrogenase, a S. cerevisiae ALD2 aldehyde dehydrogenase, and a S. cerevisiae ACS2 acetyl-CoA synthetase. In other embodiments, the heterologous ethanol catabolic pathway enzymes are a S. cerevisiae ALD2 alcohol dehydrogenase, a S. cerevisiae ALD6 aldehyde dehydrogenase, and a S. cerevisiae ACS1 acetyl-CoA synthetase. In other embodiments, the heterologous ethanol catabolic pathway enzymes are a S. cerevisiae ALD2 alcohol dehydrogenase, a S. cerevisiae ALD6 aldehyde dehydrogenase, and a S. cerevisiae ACS2 acetyl-CoA synthetase. In other embodiments, the heterologous ethanol catabolic pathway enzymes are one or more alcohol dehydrogenases selected from the group containing S. cerevisiae ADH2, E. coli AdhP, H. sapiens ADH1A, H. sapiens ADH1B, and/or H. sapiens ADH1C, one or more aldehyde dehydrogenases selected from the group containing S. cerevisiae ALD2, S. cerevisiae ALD3, S. cerevisiae ALD4, S. cerevisiae ALD5, S. cerevisiae ALD6, H. sapiens H. sapiens ALD1, H. sapiens ALD2, H. sapiens ALD4, and/or H. sapiens ALD10, and one or more acetyl-CoA synthetases selected from the group containing S. cerevisiae ACS1, S. cerevisiae ACS2, and/or E. coli Acs.

[0105] In some embodiments, recombinant host cells suitable for producing malonate according to the methods of the invention comprise a heterologous ethanol catabolic pathway enzyme and convert endogenously produced ethanol into acetyl-CoA and increase malonate yield, titer, and/or productivity. In other embodiments, ethanol is exogenously added to the fermentation broth and recombinant host cells suitable for producing malonate according to the methods of the invention comprise a heterologous ethanol catabolic pathway enzyme and convert exogenously added ethanol into acetyl-CoA and increase malonate yield, titer, and/or productivity. When exogenously added to the fermentation broth, ethanol is added to obtain a minimal concentration of 1% ethanol volume/volume, and is typically added to the fermentation broth to obtain a concentration between between 1-15% volume/ volume. Ethanol catabolism for improving malonate production in accordance with the invention is illustrated in Example 11.

[0106] Increased cytosolic pools of acetyl-CoA is a fourth route to increase malonate biosynthesis; in numerous plant and animal cells, but not S. cerevisiae, ATP citrate lyase (EC 2.3.3.8) is the primary enzyme responsible for cytosolic acetyl-CoA biosynthesis. In more detail, acetyl-CoA in the mitochondrion is condensed with oxaloacetate to form citrate through the activity of citrate synthase. Subsequently, citrate is transported from the mitochondrion into the cytosol where ATP citrate lyase catalyzes the formation of acetyl- CoA, oxaloacetate, and ADP. While S. cerevisiae does not contain a native ATP citrate lyase, suitable heterologous ATP citrate lyase enzymes have been described in oleaginous yeast strains (see, for example, Boulton et al., J. Gen. Microbiol. 127: 169-176 (1981)). The present invention provides recombinant host cells comprising one or more heterologous nucleic oleaginous yeast ATP citrate lyase enzymes. Non-limiting examples of oleaginous yeast ATP citrate lyase enzymes include those selected from the group of oleaginous yeasts consisting of Candida curvata, Cryptococcus albidus, Lipomyces lipofer, Rhodospiridium toruloides, Rhodotorula glutanis, Trichosporon cutaneum, Yarrowia lipolytica, and the like. In various embodiments, the recombinant host cell comprises a heterologous nucleic acid encoding an ATP citrate lyase. In various embodiments, the ATP citrate lyase is from an organism selected from the group consisting of Candida curvata, Cryptococcus albidus, Lipomyces lipofer, Rhodospiridium toruloides, Rhodotorula glutanis, Trichosporon cutaneum, Yarrowia lipolytica. This modification is illustrated in Example 12.

[0107] Acetyl-CoA biosynthesis can also be increased in accordance with the invention by altering expression of one or more nucleic acids encoding proteins affecting fatty acid storage or catabolism. The present invention provides host cells comprising genetic modifications of one or more nucleic acids encoding proteins affecting fatty acid storage and catabolism. In Saccharomyces cerevisiae, these proteins include SNF2, IRA2, PRE9, PHO90, SPT21, POX1, ANT1, FOX3, PAS1, PAS3, ARE1, ARE2, DGA1, LROl, ACL1, MAE1 , GLC3, GLG1, GLG2, PAT1, and PEX11. This modification is illustrated in Example 13.

[0108] In some embodiments of the invention, the host cell comprises genetic modifications affecting expression and/or activity of proteins involved in fatty acid catabolism. For example, most host cells will naturally degrade fatty acids, hydroxy fatty acids and many diacids through beta-oxidation pathways. Beta-oxidation occurs, in most cases, by activating free fatty acid groups to CoA thioesters with acyl-CoA ligases. The acyl- CoA intermediate is further oxidized and degraded - proceeding through a 2,3 enoyl-CoA, 3- hydroxyacyl-CoA, and 3-ketoacyl-CoA - and subsequent cleavage results in production of acetyl-CoA and an acyl-CoA shortened by two carbons relative to the initial substrate. The enzymatic activities required for beta-oxidation are known. The present invention provides host cells that possess increased catabolic pathway activity for medium (C4-C8)- and long (>C8)-chain fatty acids, hydroxyl fatty acids, and diacids compared to control host cells. For example, in yeast (e.g. , Saccharomyces cerevisiae), beta-oxidation occurs in the peroxisome; examplary, non-limiting, nucleic acid products affecting peroxisomal beta-oxidation are Saccharomyces cerevisiae PAT1 and PEX11. In some embodiments of the invention, a host cell modified for increased expression of PAT1 and/or PEX11 is provided for use in the methods herein for the production of malonate. This modification is illustrated in Example 14.

Genetic modifications and fermentation conditions that increase malonyl-CoA biosynthesis

[0109] In accordance with the invention, increased malonate titer, yield, and/or productivity can be achieved through increased malonyl-CoA biosynthesis, and the invention provides host cells, vectors, enzymes, and methods relating thereto. Malonyl-CoA is produced in host cells through the activity of an acetyl-CoA carboxylase (EC 6.4.1.2) catalyzing the formation of malonyl-CoA from acetyl-CoA and carbon dioxide. The invention provides recombinant host cells for producing malonate that express a heterologous acetyl-CoA carboxylase (ACC). In some embodiments, the host cell is a S. cerevisiae cell comprising a heterologous S. cerevisiae acetyl-CoA carboxylase ACCl or an enzyme homologous thereto. In some embodiments, the host cell modified for heterologous expression of an ACC such as S. cerevisiae ACCl is further modified to eliminate ACCl post-translational regulation by genetic modification of S. cerevisiae SNF1 protein kinase or an enzyme homologous thereto. The invention also provides a recombinant host cell suitable for producing malonate in accordance with the invention that is an E. coli cell that comprises a heterologous nucleic acid coding for expression of E. coli acetyl-CoA carboxylase complex proteins AccA, AccB, AccC and AccD or one or more enzymes homologous thereto. In accordance with the invention, additional acetyl-CoA carboxylases can be heterologously expressed to increase malonyl-CoA and malonate biosynthesis. This modification is illustrated in Example 15.

[0110] In various embodiments of the invention, expression of BirA, biotin-[acetylCoA carboxylase] holoenzyme synthetase, is coexpressed with E. coli acetyl-CoA carboxylase complex proteins AccA, AccB, AccC and AccD to enhance the acitivity of the ACC complex and result in an increase in malonyl-CoA and malonate production. In various embodiments of the invention, S. cerevisiae ACCl is further modified to eliminate ACCl post-translational regulation by introducing serine to alanine mutations at any, all, or any combination of the following residues; S10, S233, S430, S1114, S1145, S1148, S1157, S1159, S1162, S1163, SI 169. In some embodiments of the invention, the acetyl-CoA carboxylase used is from Yarrowia lipolytica CLIB122, herein referred to as Y1ACC. Example 42 illustrates how the inclusion of this enzyme in a malonate-producing host strain provided by the invention results in a doubling of the titer of malonate from that host after 120 hours of fermentation, relative to the same host fermented without this enzyme. In additional embodiments of the invention, this enzyme is coexpressed with a biotin-[acetyl-CoA carboxylase] holoenzyme synthetase, also derived from this organism (BPL1). In additional embodiments of the invention, the acetyl-CoA carboxylases and biotin-[acetylCoA carboxylase] holoenzyme synthetase encoding genes are dtsRl accBC and derived from Corynebacterium glutamicum. In additional embodiments of the invention, these genes are derived from a yeast strain including, but not limited to those of the genera, Candida, Pichia, or any of the other yeast herein. In various embodiments of the invention, the host cell producing malonate expresses any combination of these acetyl-CoA carboxylases and biotin-[acetyl-CoA carboxylase] holoenzyme synthetase enzymes.

[0111] In some embodiments of the invention, a host cell suitable for producing malonate according to the methods of the invention comprises genetic modifications affecting expression and/or activity of proteins involved in fatty acid biosynthesis. Malonyl-CoA is naturally a substrate in the biosynthesis of fatty acids, and diversion of malonyl-CoA to fatty acid production decreases the ability for the host cell to produce malonate. The invention provides recombinant host cells for producing malonate that express a heterologous fatty acid synthase (FAS) multienzyme complex. Temperature sensitive mutations of S. cerevisiae fatty acid synthase complex are known (see, Knobling et al., Eur. J. Biochem., 59:415-421 (1975)). Expression of a heterologous, temperature sensitive fatty acid synthase complex allows diversion of malonyl-CoA to fatty acid biosynthesis to be controlled by the temperature at which the host cell is cultured. In some embodiments, the host cell is a S. cerevisiae cell comprising S. cerevisiae fatty acid synthases FAS1 and FAS2 or enzymes homologous thereto. In some embodiments of the invention, FAS 1 and FAS2 enzymes are temperature-sensitive FAS1 or FAS2 enzymes.

[0112] In addition to genetic modification of the host cell, fatty acid biosynthesis can be decreased through addition of a FAS inhibitor to the cell culture media. For example, the FAS inhibitor cerulenin forms a covalent bond with the active site cysteine CI 305 in the S. cerevisiae ketoacyl synthase domain of the FAS complex, inhibiting enzyme activity (Johansson et al., PNAS, 105:12803-12808 (2008)). Cerulenin is not only effective in inhibiting S. cerevisiae FAS activity, but is generally an inhibitor of FAS complexes containing a Cys-His-His or Cys-His-Asn catalytic triad in the ketoacyl synthase domain. In some embodiments, cerulenin is added to the fermentation broth to a final concentration between 5 mg/1 and 100 mg/1 to inhibit fatty acid biosynthesis and increase malonate production in recombinant host cells producing malonate in accordance with the methods of the invention. In various embodiments of a method of the invention, a FAS inhibitor is added to fermentation broth containing recombinant host cells producing malonate. In some embodiments of a method of the invention, the FAS inhibitor is

cerulenin, as illustrated in Example 16. In some embodiments of the method of the invention, cerulenin is supplemented in the fermentation broth at a concentration between 5 mg/1 and 100 mg/1. In other embodiments of a method of the invention, the fatty acid synthase complex inhibitor is selected from a group consisting of platensimycin, thiolactomycin, and triclosan.

[0113] One of the substrates for acetyl-CoA carboxylase is carbon dioxide, and increasing the carbon dioxide partial pressure in the fermentation broth promotes formation of malonyl- CoA. The fermentation broth should contain a minimum dissolved carbon dioxide pressure of 0.01 atmospheres, and an increase in dissolved carbon dioxide partial pressure above this threshold is desirable. The fermentation broth should typically contain between 0.1 and 1 atmospheres dissolved carbon dioxide partial pressure. The dissolved carbon dioxide partial pressure in the fermentation broth may be increased to above saturating conditions, or above 1 atmosphere dissolved carbon dioxide. In some embodiments of a method of the invention, the dissolved carbon dioxide partial pressure in the fermentation broth is increased to between 0.1 and 1 atmospheres. In some embodiments of the method of the invention, carbon dioxide partial pressure is increased through addition of carbonates or bicarbonates to fermentation broth. For example, and without limitation, calcium carbonate can be added to the fermentation broth to increase dissolved carbon dioxide partial pressure. In other embodiments of the method of the invention, the fermentation is run in a pressurized vessel that contains carbon dioxide at above atmospheric pressure. In other embodiments of the method of the invention, carbon dioxide gas is sparged into the fermentation broth. The gas mixture being sparged may contain other gases if the added components do not interfere with host cell growth or malonate production. It may be advantageous to co-localize the source of the carbon dioxide gas with the malonate fermentation. For example, and without limitation, gaseous carbon dioxide resulting from various fermentation processes (e.g. , ethanol, isobutanol, 3-hydroxypropionate, etc.), chemical processes (e.g. , downstream malonate synthetic chemistry), or energy generation (e.g., coal or natural gas powerplants) may be pumped into fermentation broth from malonate producing host cells to increase the carbon dioxide partial pressure, as illustrated in Example 17.

Genetic modifications that decrease malonate catabolism

[0114] In accordance with the invention, increased malonate titer, yield, and/or productivity can be achieved by decreasing malonate catabolism, and the invention provides host cells, vectors, enzymes, and methods relating thereto. One metabolic pathway by which malonate is catabolized in a host cell is through the activity of an acyl-CoA synthetase catalyzing the conversion malonate and Coenzyme A to malonyl-CoA. In some embodiments of the invention, a recombinant host cell suitable for producing malonate in accordance with the methods of the invention comprises a genetic modification resulting in the deletion, attenuation, or modification of one or more nucleic acids encoding for an acyl-CoA synthetase. In some embodiments of the invention, the recombinant host cell is yeast and the one or more acyl-CoA synthetases are selected from the group consisting of FAA1, FAA2, FAA3, FAA4, LSC1, and LSC2. In other embodiments of the invention, the recombinant host cell is E. coli and the one or more acyl-CoA synthetases are selected from the group consisting of FadD, FadK, Fadl, SucC, SucD, and YahF. This aspect of the invention is illustrated in Example 18.

Genetic modifications that increase malonate secretion from the host cell

[0115] In accordance with the invention, increased malonate titer, yield, and/or productivity can be achieved by increasing malonate transport into the fermentation broth, and the invention provides host cells, materials, and methods relating thereto. In some embodiments of the invention, the recombinant host cell suitable for use in the methods of the invention is a S. cerevisiae cell that comprises a heterologous nucleic acid coding for expression of an S. cerevisiae transport protein selected from the group consisting of PDR5, PDR10, PDR11, PDR12, PDR15 and PDR18. In some embodiments of the invention, the recombinant host cell suitable for producing malonate in accordance with the methods of the invention is an E. coli cell that comprises a heterologous nucleic acid coding for expression of E. coli DcuC. This aspect of the invention is illustrated in Example 19.

Genetic modifications that increase host cell tolerance to malonate

[0116] In accordance with the invention, increased malonate titer, yield, and/or productivity can be achieved by increasing host cell tolerance to malonate, and the invention provides host cells, materials, and methods relating thereto. High concentrations of malonate can competitively inhibit succinate dehydrogenase (EC 1.3.5.1) activity (see Slater, Methods Enzymol. 10:48-57 (1967)). The present invention is based, in part, on the discovery that mutant succinate dehydrogenase enzymes exhibit a lower competitive inhibition by malonate. For example S. cerevisiae succinate dehydrogenase SDH1 residues E300, R331, and R442 are important for substrate (e.g., succinate) recognition. Increasing the size of the SDH1 active site decreases competitive inhibition by malonate while still allowing the enzyme to maintain activity toward the native substrate, succinate. In specific, introduction of one or more mutations selected from the group consisting of E300D, R331K or R331H, and R442K and R442H decreases competitive inhibition of SDH1 by malonate. In some embodiments, a recombinant host cell expressing an SDH1 with point mutation R300D is used to produce malonate in accordance with the invention. In other embodiments, a recombinant host cell expressing an SDH1 with point mutation R331K or R331H is used to produce malonate in accordance with the invention. In other embodiments, a recombinant host cell expressing an SDH1 with point mutation R442K or R442H is used to produce malonate in accordance with the invention. This aspect of the invention is illustrated in Example 20.

Genetic modifications that increase catabolism of various carbon sources

[0117] In the methods of the invention, carbon feedstocks are utilized for production of malonate. Suitable carbon sources include, without limitation, those selected from the group consisting of purified sugars (e.g., dextrose, sucrose, xylose, arabinose, lactose, etc.); plant- derived, mixed sugars (e.g., sugarcane, sweet sorghum, molasses, cornstarch, potato starch, beet sugar, wheat, etc.), plant oils, fatty acids, glycerol, cellulosic biomass, alginate, ethanol, carbon dioxide, methanol, and synthetic gas ("syn gas"). A given host cell may catabolize a particular feedstock efficiently or inefficiently. If a host cell inefficiently catabolizes a feedstock, then one can modify the host cell to enhance or create a catabolic pathway for that feedstock. For example, Example 47 shows production of malonate, in accordance with the invention, from a number of different carbon sources using Pichia kudriavzevii as the host strain. While this organism does not catabolize sucrose under the conditions tested, the invention provides the introduction of a sucrose invertase (beta-fructofuranosidase, EC 3.2.1.26) to facilitate the use of this carbon source. Example 30 also demonstrates the production of malonic acid in accordance with the

methods of the invention using a variety of carbon sources. Additional embodiments of the invention include the use of methanol catabolizing host strains. In some embodiments, the host is a yeast strain. In some embodiments, the host is selected from the Komagataella pastoris, Pichia methanolica, or Pichia pastoris.

[0118] The invention provides host cells comprising genetic modifications that increase malonate titer, yield, and/or productivity through the increased ability to catabolize non- native carbon sources. Wild type S. cerevisiae cells are unable to catabolize pentose sugars, lignocellulosic biomass, or alginate feedstocks. In some embodiments, the invention provides a an S. cerevisiae cell comprising a heterologous nucleic acid encoding enzymes enabling catabolism of pentose sugars useful in production of malonate as described herein. In other embodiments, the heterologous nucleic acid encodes enzymes enabling catabolism of lignocellulosic feedstocks. In yet other embodiments of the invention, the heterologous nucleic acid encodes enzymes increasing catabolism of alginate feedstocks.

[0119] Those skilled in the art will recognize that the individual manipulations to increase malonate production from a given host cell can be used in virtually unlimited combination and often confer simulatious benefits resulting in a much higher malonate output than a single manipulations and in cases, the sum of the individual manipulations. Thus, the invention encompasses not only the single manipulations described herein, it also is embodied by any combination of these perterbuations resulting in the increase of malonic acid produced the host strain.

Section 6: Detecting malonate producing host cells and screening host cells for improved malonate production

[0120] The present invention also provides a transcription factor biosensor system that can be used for the accurate sensing of malonate in liquid media. In many applications, this system is used to detect malonate produced within a host cell by sensing the presence of malonate in the fermentation media containing the malonate producing host cell. "Accurate sensing" refers to detecting the presence of and/or directly or indirectly determining the concentration of malonate; therefore, by accurately sensing malonate, this aspect of the invention has application in strain improvement, i.e., for increasing malonate production in host cells. In this system, malonate binds to a protein moiety present on a transcription factor. Binding of malonate to the malonate binding moiety results in either binding of the transcription factor to the promoter that is activated by the transcription factor, or in some embodiments, results in derepression of the promoter by the malonate-bound transcription factor.

[0121] Any number of transcription factors that bind to malonate, or are activated to bind to a promoter in response to a signal generated by binding of malonate to a binding moiety, are suitable for use in this system.

[0122] In some embodiments, the transcription factor can bind malonate, which results in binding of the transcription factor to a cognate promoter and activation of a gene that is operably linked to the promoter. Non-limiting examples of transcription factors that bind malonate include the transcription factors

Acinetobacter calcoaceticus MdcY (SEQ ID NO:3), Rhizobium leguminosarum MatR (SEQ ID NO:4), Klebsiella pneumoniae MauR (SEQ ID NO:5), and homologs thereof.

[0123] In some embodiments, a transcription factor used in the invention is an MdcY transcription factor. An MdcY transcription factor can directly bind malonate and regulate transcription mediated by promoters such as $PM_{<}1_{C}L$- MdcY and MdcY-responsive promoters are known in the art (see, e.g., Koo et al, J Bacteriol. 182:6382-6390 (2000)).

[0124] An example of an MdcY polypeptide sequence from Acinetobacter calcoaceticus is provided in SEQ ID NO: 3. MdcY polypeptides that can be employed in accordance with the invention include variants and homologs of the MdcY polypeptide sequence set forth in SEQ ID NO: 3. Thus, a MdcY transcription factor polypeptide may have an amino acid sequence that has at least 60% identity, typically at least 75%, 90%, 95%, 99% or greater amino acid sequence identity, preferably over a region of at least 100 or more amino acids, or at least 200 or more amino acids, or over the length of the entire polypeptide, to an amino acid sequence of SEQ ID NO:3. One of skill in the art understands in view of this disclosure that variants can also be employed, e.g., using the known sequences as guidance for selecting amino acid substitutions that will not result in loss of function.

[0125] In some embodiments, the MdcY transcription factor for use in the invention is naturally present in a host cell. In other embodiments, a host cell is genetically modified to express a foreign transcription factor by introducing a heterologous nucleic acid encoding the malonate transcription factor into the host cell. In some embodiments, the genetically modified host cell comprising a heterologous nucleic acid encoding the malonate transcription factor is an E. coli host cell. [0126] The MdcY transcription factor can bind to a number of promoters and activate expression of a gene operably linked to the promoter. An example of a MdcY-responsive promoter suitable for use in accordance with the invention is provided in SEQ ID NO: 6. In some embodiments, an MdcY-responsive promoter for use in the invention, typically comprise an operator sequence ATTGTATACAAT. In some embodiments, the promoter is at least 75% identical to the promoter sequence shown in SEQ ID NO:6. In some embodiments, the promoter comprises a subsequence of SEQ ID NO:6 comprising 10, 20, 25, 30, 35, or more, contiguous nucleotides of SEQ ID NO:6.

[0127] Thus, in one aspect, the present invention provides a method for accurately sensing malonate. In some embodiments, the sensing step comprises detecting, e.g., measuring the amount of a gene product of a reporter or marker gene {e.g. a fluorescent reporter gene). In some embodiments, the gene product of the reporter gene influences the growth rate of a host cell comprising the components of a malonate transcription factor biosensor of the invention. In some embodiments, the gene product of the reporter gene causes the modified host cell to become resistant or sensitive to a compound. For example, in some embodiments, the reporter gene is an antibiotic resistance gene {e.g. a tetA gene) where the presence of malonate in the culture medium induces antibiotic resistance such that the host cell exhibits improved growth in the presence of malonate when the antibiotic is present, as illustrated in Example 21. In some embodiments, a host cell that comprises the components of a transcription factor biosensor of the invention is a host cell that is capable of producing malonate. Example 36 illustrates practice of this aspect of the invention using both antibiotic resistance (tetA) and a colorimetric signal (lacZ) as outputs

that are correlated to malonic acid concentration, produced in accordance with other aspects of the invention, in fermentation media.

[0128] Generally, then, the present invention provides for a method for screening or selecting a host cell that produces malonate comprising: (a) providing a modified host cell of the present invention, (b) culturing the host cell, and (c) screening or selecting the host cell based on the expression of the reporter gene by the host cell.

[0129] In some embodiments of the present invention, the method for screening or selecting a host cell that produces malonate comprises: (a) providing a plurality of modified host cells of the present invention wherein the modified host cells of different modification are in separate cultures, (b) culturing each separate culture of host cell, (c) screening or selecting the host cell based the expression of the reporter gene by the host cell, and (d) comparing the expression of the reporter genes of the separate cultures. In some embodiments of the present invention, step (d) comprises identifying one or more cultures, and/or the corresponding host cell, that have an increased expression of the gene product of the reporter gene. It will be recognized by those skilled in the art that these cultures arise from a single transformant and represent a clonal population.

[0130] In some embodiments, the method of the invention is a method for selecting a host cell that produces malonate, wherein the selection is a positive selection. In a positive selection, the selecting step selects for host cells that have a higher expression of a reporter gene that increases the probability of remaining viable and doubling, and thus have a higher probability of remaining viable and doubling. For example, host cells producing malonate will remain viable and propagate at a rate faster than host cells not that do not produce malonate. Similarly, host cells with increased malonate production as compared to a control strain will propogate at a rate faster than the control strain.

[0131] In some embodiments of the present invention, the method for selecting an E. coli host cell that produces malonate comprises: (a) providing a plurality of modified E. coli host cells of the present invention wherein the modified host cells of different modification are in separate cultures, (b) culturing each separate culture of host cell, (c) positively selecting the host cell based the expression of the reporter gene by the host cell, and (d) comparing the expression of the reporter genes of the separate cultures.

[0132] In other embodiments of the present invention, the method for selecting an E. coli host cell that produces malonate comprises: (a) providing a plurality of modified E. coli host cells of the present invention wherein the modified host cells of different modification are in the same culture, (b) culturing the heterogenous mixture of modified host cells in growth medium containing a positive selecting agent such that the host cells exhibiting a higher production of malonate than the plurality of host cells will propogate at a rate faster than host cells exhiting lower production of malonate and (d) isolating the host cells exhibiting the highest production of malonate.

Section 7: Malonate purification

[0133] In a sixth aspect, the invention provides methods for purifying malonate from fermentation broth, the methods comprising: (a) culturing a host cell under conditions suitable for production of malonate, (b) and recovering (i.e. , purifying) the malonate from fermentation broth. The invention also provides purified malonate produced in accordance with the methods of the invention. Biosynthesized malonate can be produced intracellularly and/or secreted into the culture medium. Intracellulary produced malonate is typically secreted into the culture medium using a membrane transporter, as described above. Malonate not secreted can be removed from the host cell by chemical, enzymatic, or mechnanical cell lysis. Malonate can be recovered from the cells, from the fermentation broth, or both. If the cell is engineered to secrete malonate, one can opt to recover the malonate only from the fermentation broth or one can opt to recover it both from the media and from the cell (i.e., by lysing the cell). If the cell is not engineered to secrete malonate one can lyse the host cell to isolate the malonate therein.

[0134] The present invention provides methods to isolate malonate produced biologically. As used herein, "isolate", "purify", and "recover" are used to refer to separation of the malonate from other substances present. "Isolation", "purification", or "recovery" as used in this context is intended to convey a preparation of malonate that is enriched in malonate relative to the cell or fermentation broth that produced it but that may or may not be substantially (i.e., more than 50%) pure on a weight/weight (w/w) basis. Isolating malonate in accordance with these methods involves separating the malonate produced from at least part or all of the fermentation medium, host cells, and parts thereof, from which malonate is produced. Malonate may be purified, i.e., to more than 50% purity on a w/w basis, in accordance with the invention from the fermentation broth and/or from the producing cell in which any naturally occurring or recombinant host cell (e.g. , E. coli, S. cerevisiae, oleaginous yeast, and the like) producing malonate is grown, i.e., the host cell is not limited to a recombinant host cell of the invention. The isolated malonate may be free or essentially free of impurities from the host cells. The malonate is isolated or purified to a degree such that any impurities present do not interfere in the subsequent use of the malonate. For example, if the subsequent use is as an industrial chemical, such as a chemical to be used in a polymerization reaction, then the malonate is essentially free of impurities when any remaining impurities would not interfere with the use of the malonate in a polymerization reaction. Typically malonate used for polymerization reactions has a purity of at least 95% w/w or higher. If the malonate is to be used as a fuel, such as a fuel to be used in a combustion reaction, then the compound is essentially free of impurities when any impurities remaining would not interfere with the use of the malonate as a fuel. If the malonate is used as an animal feed, then the malonate is essentially free of impurities when any impurities remaining would not interfere with the use of the material as animal feed. When malonate is used as an animal feed, one may opt to recover the biomass containing malonate from the fermentation broth and use the biomass as animal feed.

[0135] In some embodiments of the purification methods of invention, the fermentation broth is concentrated to increase the working concentration of malonate and decrease the volume of liquid that requires processing. In various embodiments of the purification methods of the invention, this concentration is achieved by evaporation, including vacuum and heat, reverse osmosis, "high pass" membrane dewatering, and/or thin film evaporation. [0136] In some embodiments, the purification methods of the invention comprise the step of recovering the malonate produced, wherein the recovering step is concurrent or subsequent to the culturing step. In some embodiments, the malonate is purified

from the fermentation broth and the host cells. In other embodiments, the host cells are separated from the fermentation broth, lysed, and then malonate is recovered from the host cells. In other embodiments, the host cells are lysed in the fermentation broth and malonate is recovered from the lysed cells and fermentation broth. One method for recovering malonate from the fermentation broth provided by the invention is precipitation of malonate with a cation. In some embodiments this is a monovalent cation, in other embodiments it is a divalent cation. Typically the cation is added to the fermentation broth (or lysate) as a salt. For example, precipitation of calcium malonate from an aqueous solution, which may be fermentation broth or a cell lysate or a mixture of both, containing malonate in accordance with the invention is accomplished by the addition of a calcium salt (Weiner, Org. Synth. 18:50 (1938); Weiner, Org. Synth. Coll. 2:376 (1943)). Various calcium salts (e.g. , calcium hydroxide, calcium carbonate, calcium chloride) can be used in accordance with the invention to precipitate malonate from fermentation broth.

[0137] A calcium malonate salt forms when the when two malonate carboxylic acids are unprotonated and calcium is present in the fermentation broth. Calcium malonate is insoluble in aqueous solutions, e.g., fermentation broth, will precipitate, and can then be recovered from the fermentation broth. The pK_a values of the two malonate carboxylic acid moieties are 2.83 and 5.69; thus, when the fermentation broth pH is below 5.7, calcium malonate does not form and malonate remains dissolved in the fermentation broth. When the fermentation broth pH is above 5.7, calcium malonate can form and will precipitate from the fermentation broth. Carboxylic acids are weak acids, and the accumulation of malonate in the fermentation broth will decrease the pH, and if the pH falls below 5.7 the addition of calcium cations does not result in the formation of calcium malonate. One method of the invention to purify malonate from fermentation broth is to add a calcium salt where the calcium salt anion is a base (i.e. , calcium carbonate and calcium hydroxide) and addition of the calcium salt raises and/or maintains the fermentation broth pH above 5.7. In some embodiments of a method of the invention, calcium hydroxide is added to the fermentation broth to reach/maintain a pH between 5.69 and 7.5 and precipitate calcium malonate. In a second embodiment of a method of the invention, calcium carbonate is added to the fermentation medium to reach/maintain a pH between 5.69 and 7.5 and precipitate calcium malonate. In a third embodiment of the invention, calcium chloride is added to the fermentation broth when the fermentation broth pH is between 5.69 and 7.5 and precipitate calcium malonate. In accordance with the methods of the invention, other calcium salts can be added to the fermentation broth to precipitate malonate. Addition of calcium carbonate has the additional advantage of increasing the carbon dioxide partial pressure in the fermentation broth, promoting formation of malonyl-CoA through the activity of acetyl-CoA carboxylase, described above. Example 23 illustrates this aspect of the invention by describing the purification of malonate using a calcium salt.

[0138] One may add the calcium salt at the beginning of the fermentation (i.e. , before substantial malonate has accumulated), during the fermentation, or at the end of the fermentation. In some embodiments, calcium carbonate or calcium hydroxide is added to the fermentation broth before the host cells have begun producing malonate. In other embodiments, calcium carbonate or calcium hydroxide is added to the fermentation broth before malonate concentrations exceed 5, 10, 15, 20, 25, 30, 40, or 50 g/1. In other embodiments, calcium carbonate or calcium hydroxide is added the fermentation broth at the end

of the fermentation. One may choose to recover the calcium malonate precipitate from the fermentation broth concurrent or subsequent to the fermentation.

[0139] Because calcium malonate monohydrate solubility decreases with increasing temperature, recovery of malonate from the fermentation broth may be increased by raising the temperature to between 50°C and 100°C during the recovery step. In some embodiments of the method of the invention, a calcium salt (i.e. , calcium carbonate, calcium hydroxide, and/or calcium chloride) is added to fermentation broth, the temperature is increased to between 50°C and 100°C, and malonate is recoved from the fermentation broth. In additional embodiments of the invention, the temperature is increased above 100°C. However, thermal decomposition of malonic acid can occur at temperatures of about 100°C, accordingly, increasing the temperature to or above 100°C may negatively impact yield.

[0140] In some embodiments of the invention, malonate is purified from the production media by precipitation with a monovalent cation. Typically the monovalent cation is added to the fermentation broth (or lysate) as a salt. For example, precipitation of soduim malonate from an aqueous solution, which may be fermentation broth or a cell lysate or a mixture of both, containing malonate in accordance with the invention is accomplished by the addition of a sodium salt. Various sodium salts (e.g. , sodium hydroxide, sodium carbonate, sodium chloride) can be used in accordance with the invention to precipitate malonate from fermentation broth. A sodium malonate salt forms when the when two malonate carboxylic acids are unprotonated and sodium is present in the fermentation broth. Sodium malonate is soluble in aqueous solutions, e.g., fermentation broth, but can be forced out of solution by increasing the pH of the media above the higher pKa of malonic acid (5.69); see Example 32. Additional precipitation of the sodium malonate is achieved by increasing the concentration of the ions by evaporation of the solvent (water). As with the aspects of the invention associated with calcium malonate precipitation, the addition of sodium salts containing a base (e.g. sodium hydroxide or sodium carbonate) can be used to maintain the pH of the fermentation broth above 5.7, thus facilitating precipitation of the sodium salt. Separation of the precipitate from the fermentation broth provides purified malonate in accordance with the invention.

[0141] Another method of the invention for isolating malonate from the fermentation broth is through reactive extraction with an aliphatic primary, secondary, or tertiary amine or a primary, secondary, or tertiary alcohol. This method arises in part from the discovery that both primary alcohols and tertiary amines are highly effective agents for the selective removal of malonate from fermentation broth as is illustrated in Examples 24-26, 36-41. In some embodiments of the invention, malonate is purified from the fermentation broth by reactive extraction with trioctylamine in organic solvent. In other embodiments of in the invention, malonate is purified from the fermentation broth using a tertiary amine selected from the group consisting of triethylamine, tripropylamine, tributylamine, tripentylamine, trihexylamine, triheptylamine, trinonylamine, and tridecylamine. In additional embodiments of the invention, the amine compound is ammonia. In yet other embodiments, it is a dialkylamine including, but not limited to diethylamine, dipropylamine, diisopropylamine, dibutylamine, dipentylamine, dihexylamine, diheptylamine, dioctylamine, dinonylamine, didecylamine. Example 33 demonstrates the isolation of malonate from fermentation broth using diethylamine. In various embodiments of the invention the amino compound is added in conjuction with an organic solvent or mixture of solvents including, but not limited

to: pentanol, hexanol, heptanol, octanol, nonanol, decanol, hexane, decane, kerosene, ethers, ethylacetate or any combination thereof. In various embodiments, the solvent is a branched or unbranched, alkane, alcohol, diol, ester, ether, diether, or lactone containing four to twenty carbons In various embodiments, addition of solvents facilitates the transfer of amino compounds to the aqueous phase and the amine-coordinated malonates to the organic phase, thus improving purification.

[0142] A number of parameters can be adjusted in accordance with the invention to improve malonate extraction. Length of alkyl groups, ionic strength of the fermentation broth, pH, molar equivalents of trialkylamines, temperature, and cosolvent each have an impact on the extraction efficiency of trialky amines. The impact of alkyl group length is illustrated in example 36. Tripropyl-, trihexyl-, and trioctyl-amine are each capable of isolating malonic acid from the aqueous phase and solubilizing it into the 1 -octanol organic phase in accordance with the invention. The extraction efficiency of trioctylamine > trihexylamine > tripropylamine, indicating the longer of these chain lengths is the preferable embodiment (for high-yield) of the invention at low pH, with a 1-octanol cosolvent.

[0143] As with salt precipitation, pH has an impact on malonic acid extraction from an aqueous phase when using trialkylamines. Trialkylamine extraction has the highest efficiency when both acid moieties are carboxylated. Example 37 shows the extraction efficiencies of trioctylamine over a range of pH values. The highest level of extraction occurs at pH 1.5 and diminishes as pH is increased. Above the second pKa of malonic acid (5.69) there is virtually no extraction. While the invention provides malonate extraction from an aqueous phase using trialkylamines without limitation on pH, embodiments at pH of 2.0 or lower are preferred for maximum efficiency. In some embodiments of the invention, host cell growth and production of malonate at a low pH, as illustrate by Example 48, facilitates protonation of malonate to enhance extraction with trialkylamines. In other embodiments of the invention, exogenous acid is added to facilitate this protonation.

[0144] In various embodiments of the invention, different concentrations of trialkylamine are used. As reported in Example 38, linear relationship between extraction efficiency and trioctylamine :malonic acid mol fraction was observed; specifically the linear relationship existed between trioctylamine: malonic acid mol fractions of 0 to 1. Above 1 molar ratio, 100% of the malonic acid was extracted into the organic phase. Thus, to maximize extraction efficiency the amount of trialkylamine in the organic phase must be equimolar to the amount of malonic acid in the aqueous phase (e.g., fermentation broth). Ideally a greater than equimolar amount of trialkylamine will be added to the organic phase to compensate for decreased extraction efficiencies due to other organic acids and anions in the fermentation broth.

[0145] As illustrated in Example 39, trialkylamine extraction efficiency of malonic acid is negatively impacted by increasing the ionic strength (salt concentration) of the aqueous phase. Desalting of various ions can be achieved through a number of methods, including: addition of other agents to generate an insoluble salt, pH adjustment, and/or passage through various membranes.

[0146] In various embodiments of the invention, the reactive extraction is Fischer esterification of malonate at one or both carboxylic acids with an alcohol in the presence of an acid catalyst to result in the formation of monoalkyl and dialkyl malonate esters, respectively. For example, diethyl malonate can be

produced by Fischer esterification of malonate using ethanol. The monoalkyl and dialkyl malonate esters have low solubility in aqueous solutions and low volatility, enabling the malonate esters to be separated from water and other volatile components in the fermentation broth by distillation. In various embodiments of the invention, separation of the esters is achieved by phase partitioning into an organic solvent. In general, a primary or secondary aliphatic alcohol is suitable for Fischer esterification with malonate; for example, and without limitation, the alcohol may be selected from the group consisting of methanol (CAS# 67-56-1), ethanol (CAS# 64-17-5), 1-propanol (CAS# 71-23-8), 1-butanol (CAS# 71-36-3), isopropanol (CAS# 67-64-0) and isobutanol (CAS# 78-83-1), among others. A number of acid catalysts are suitable for Fischer esterification of an alcohol with malonate; for example, and without limitation, the acid catalyst may be selected from the group consisting of sulfuric acid, tofic acid, scandium(III) triflate, N,N'-dicyclohexylcarbodiimide and tetrabutyl ammonium tribromide. In various embodiments of the invention, malonate is purified from fermentation broth by reactive distillation using ethanol to convert malonate to diethyl malonate. In other embodiments of the invention, malonate is purified from the fermentation broth by reactive distillation using methanol to convert malonate to dimethyl malonate. In various embodiments of the invention, the acid catalyst is sulfuric acid.

[0147] As is illustrated in Examples 41 Fischer esterification to ethanol using sulfuric acid as a catalyst can result in >99% purification of malonic acid from an aqueous phase. In various embodiments of the invention, the excess ethanol used in this process is recovered under reduced pressure and recycled for use in subsequent fermentation batches. In various other embodiments of the invention, the catalyst is matrix based (e.g. amberlite resins) and is recovered, recharged, and reused in subsequent batches.

[0148] The economics of malonate purification by Fischer esterification can be improved in accordance with the invention by co-localization of the malonate biorefinery with an alcohol biorefinery or chemical refinery (used herein to refer to production of alcohol from renewable or petroleum feedstocks using synthetic chemistry and not a fermentation process). In some embodiments of the invention, a malonate biorefinery is co-localized with an alcohol biorefinery or chemical refinery to reduce the cost of monoalkyl or dialkyl malonate ester synthesis. In some embodiments of the invention, the alcohol resulting from the biorefinery or chemical refinery is of lower purity than typically sold and distributed for fuel and/or chemical applications.

[0149] In other embodiments, the method of the invention for isolating malonate from the fermentation broth involves liquid-liquid extraction using an organic solvent. The pK_a values of the two malonate carboxylic acid moieties are 2.83 and 5.69; thus, when the pH of the media is reduced below 2.8, greater than 50% of the molecules are not ionize. The affinity of these species is higher for organic solvent than for the aqueous fermentation broth. The separation of these molecules into the organic phase serves to drive the equilibrium toward the protonated species and thus progressively into the organic solvent. In some embodiments of the invention, the acid pH of the broth is the result of fermentation conditions. In other embodiments of the invention, the pH is lowered to the appropriate acidity by the addition of an acid. In various embodiments of the invention, the solvent is selected from, but not limited to, the group consisting of ethylacetate, dichlorome thane, dichloroethane, decane, dodecane, hexanes, octanol, pentanol, or mixtures of thereof. In some embodiments of the invention, host cell growth and production of malonate at a low pH facilitates protonation of malonate to enhance solubility of the malonate in the

organic solvent. In other embodiments of the invention, exogenous acid is added to facilitate this protonation. In some embodiments of the invention, the solvent is removed (wholly or partially) by vacuum distillation. In various embodiments of the invention, the concentrated malonic acid is esterified to an alcohol, as described elsewhere, and the ester purified by distillation.

[0150] In additional embodiments of the invention, malonate is removed from the fermentation broth by binding to an ion exchange resin. In various embodiments of the invention, the resin is selected from, but not limited to, the following: Lewatit® VP OC 1065, Lewatit® MP-64 chloride form, Toyopearl®, Dowex® 66 free base, Amberlite® IRA-67 free base, Amberlite® IRA-96 free base, Amberjet® 4200 chloride form, Lewatit® MonoPlus M 500 chloride form, Dowex® 1X8 chloride form, Amberlyst A26 hydroxide form, Amberlite® IRA958 chloride form. In various embodiments of the invention, malonic acid is eluted from the resin using a pH or salt gradient. In additional embodiments of the invention, one or more of these resins is used to remove impurities from the concentrated malonic acid

[0151] While the purification methods of the present invention can be used as single-step purification methods for purification of malonate from the fermentation broth, two or more purification methods can be used in series in accordance with the invention. For example, and without limitation, precipitation of malonate with calcium hydroxide may be followed by Fischer esterification of malonate with ethanol and further purification of the resulting diethyl malonate using distillation.

Section 8: Chemistry for using malonate to synthesize other chemicals

[0152] Malonate is a chemical precursor in a large number of important industrial reactions. The present invention enables, for the first time, the use of biologically derived malonate in these reactions. In any known reaction that utilizes malonate as a reactant, biologically derived malonate can be used. In any reaction using a monoalkyl malonate, dialkyl malonate, or Meldrum's acid, biologically derived malonate can be used following conversion to the appropriate substrate for the reaction. Furthermore, the invention provides new chemical synthesis methods in which malonate is used as a starting material. For these reactions, one can use malonate derived from any means, including biological production of malonate through enzyme catalyzed oxidation of malonate semialdehyde, synthetic oxidation of biologically- or synthetically-derived 3-hydroxypropionate, or synthetic oxidation of biologically- or synthetically-derived 1 ,3-propanediol.

[0153] Thus, in addition to providing biologically derived malonate for use in known chemical reactions, the invention provides new methods for using malonate and malonate- derived compounds to produce other useful compounds.

[0154] The invention arises in part from the fact that malonate and malonate-derived compounds can be used as substrates in the synthesis of acrylates (i.e., acrylate, methyl acrylate, ethyl acrylate, propyl acrylate, and butyl acrylate) by reaction with formaldehyde. The acrylates represent billions of dollars per year in sales and are used in products as wide ranging as diapers to shampoo to films and coatings. The invention provides two basic syntheses for production of an acrylate by reacting malonate or malonate-diesters with formaldehyde. In one method, malonate-diester is reacted with formaldehyde in the presence

of an appropriate base (e.g. diethylamine) to produce the diester of 2-methylenemalonate. Subsequent saponification of the esters and heating results in the production of one part acrylate, one part CO_2, and two parts alcohol. The composition of the ester portion of the malonate diester, the base, and the solvent used can vary widely. For example, and without limitation, dimethyl, diethyl, dipropyl, dibutyl, diisopropyl, or dihexyl esters can be utilized. Similarly, the base used can be one of many. In various embodiments, the base is a tri- substituted amine. Non-limiting examples of tri-substituted amines suitable for use according the methods of the invention include those selected from the group containing piperidine, trimethylamine, triethylamine, tripropylamine, triisopropylamine, tributylamine, or longer chain trialkylamine. In some embodiments of this aspect of the invention, diethyl malonate is reacted with formaldehyde in pyrrolidine to form acrylate in a Knoevenagel condensation and subsequently saponified and heated to yield one part acrylate, one part CO_2, and two parts ethanol. In other embodiments of the method of the invention, dimethyl malonate is reacted with formaldehyde in diethylamine to form dimethyl 2-methylenemalonate which is subsequently converted to acrylate, CO_2, and methanol.

[0155] In other embodiments, a magnesium salt catalyzes the condensation of a malonate- diester with formaldehyde to form a diester 2-methylenemalonate which is then worked up in the described manner. For example, and without limitation, magnesium-silicate is a suitable magnesium salt for this reaction. In some embodiments, diethyl-malonate is reacted with formaldehyde in the presence of a magnesium-silicate catalyst to form diethyl 2- methylenemalonate. In other embodiments, diisopropyl malonate is reacted with formaldehyde in the presence of a magnesium-silicate catalyst to form diisopropyl 2-methylenemalonate. A general scheme using a malonate mono-ester is illustrated here:

In this case, Ri is selected from the non-limiting examples: H, CH , CH_3CH_2, CH $(CH_2)_2$, $CH_3(CH_2)_3$, $(CH_3)_2CH_2$, or $(CH_3)_2CH_2CH_2$.

[0156] In another embodiment, a Doebner modification of the Knoevenagel condensation is employed and malonate is reacted with formaldehyde in pyridine to produce acrylate. Example 28 illustrates the production of acrylate from malonate in accordance with this embodiment of the invention. In other embodiments, monomethyl malonate is reacted with formaldehyde in pyridine to form methyl acrylate. In the scheme illustrated here, a malonate mono-ester serves as the starting material in a Doebner modification of the Knoevenagel condensation

selected from the non-limiting examples: H, CH_3, CH_3CH_2, $CH_3(CH_2)_2$, $CH_3(CH_2)_3$, (CH $)_2CH_2$, or (CH $)_2CH_2CH_2$. In other embodiments, monoethyl malonate is reacted with formaldehyde in pyridine to form ethyl acrylate.

[0157] In additional embodiments of the invention, malonate and malonate-derived compounds can be used as substrates in the synthesis of unsaturated dicarboxylic acids through reaction with various substrates, including, but not limited to, aldehydes, alkyl halides, dialdehydes, alkyl dihalides, terminal olefins, and combinations of the above (i.e. , difunctional substrates). In the case of reaction of malonate or malonate-derived compound with an aldehyde, an alkene is formed that can be subsequently hydrogenated to yield the saturated product. Generally, malonate and malonate-derived compounds can be used to synthesize C5-C12 straight chain, saturated or unsaturated diacids (i.e. , pentanedioic acid

(CAS 110-94-1), hexanedioic acid (CAS 124-04-9), heptanedioic acid (CAS 111-16-0), octanedioic acid (CAS 505-48-6), nonanedioic acid (CAS 123-99-9), decanedioic acid (CAS 111-20-6), undecanedioic acid (CAS 1852-04-6), dodecanedioic acid (CAS 693-23-2)) and their corresponding dialkyl esters. In various embodiments of the invention, malonate is used as a substrate in the chemical synthesis of a C5-C12 straight chain, saturated diacid. In various embodiments of the invention, malonate or a malonate-derived compound is used as a substrate in the chemical synthesis of pentanedioic acid, hexanedioic acid,heptanedioic acid, octanedioic acid, nonanedioic acid, decanedioic acid, undecanedioic acid, or dodecanedioic acid.

[0158] In other embodiments of the invention, or a compound derived from malonate provided by, or not provided by, the invention is used as a substrate in the chemical synthesis of pentanedioic acid. Pentanedioic acid can be formed in accordance with the invention through reaction of synthetically derived diethyl malonate and formaldehyde (see Ahluwalia et al, "Organic reaction mechanisms," 2nd ed. Alpha Science International: Harrow (2005) pgs 340-341); synthetically derived diethyl malonate and dichloromethane (see Perkin et al., J Am Chem Soc 59:990-995 (1891)); and synthetically derived Meldrum's acid and formaldehyde (see Hedge et al., J. Org. Chem. 26:3166-3170 (1961)).

[0159] In other embodiments of the invention, malonate or a malonate-derived compound is used as a substrate in the chemical synthesis of hexanedioic acid. Hexane dioic acid is one of two compounds used to produce Nylon 6,6 and represents billions of dollars in annual raw material sales. Nylon 6,6 is use in a wide range of durable consumer goods including, carpet, airbags for automobiles, and rope. Hexanedioic acid can be formed in accordance with the invention through reaction of magnesium-chelated malonate monoesters and 1,2- dichloroethane. Alkylation of dichloroethane and dimethyl malonate has been described (US Patent No. 6,262,298); however the undesired product dimethyl cyclopropane- 1,1-dicarboxylate is the only product reported at 99.3% purity and 83% theoretical yield. Formation of cyclopropane- 1,1-dicarboxylate results from intramolecular alkylation following the initial alkylation of 1 ,2-dichloroe thane. In contrast, the present invention provides materials and methods for using a magnesium-chelated malonate monoester that does not allow the undesired intramolecular alkylation to the cylopropane. A magnesium- chelated malonate monoester reacts with 1 ,2-dichloroethane through decarboxylation- facilitated enolate generation; the second intramolecular reaction (i.e. , to the cyclopropane dicarboxylate) is unable to proceed following decarboxylation. An examplary catalyst for use in accordance with this embodiment of the invention is magnesium-silicate. Those skilled in the art appreciate that various dihaloalkanes (e.g. 1,3-dihalopropane, 1 ,2-dihalopropane) can also be used with a magnesium-chelated malonate monoester to form a dicarboxylic acid product and that those halide groups can be I, CI, Br, or F. For example, and without limitation, a 1,3-dihalopropane can be reacted with a magnesium-chelated malonate monoester to produce heptanedioic acid.

[0160] The invention also provides methods for production of hexanedioic acid by reaction of malonate and malonate-derived compounds with ethanedial. The formation of alkenoic acids by Knoevenagel reaction of malonate-diesters and aliphatic mono-aldehydes is known (see, for example, Rao et al., J Am Oil Chem Soc, 70:297-299 (1993); and, Zhang et al., Synth Comm, 40:3093-3100 (2010)). The present invention arises in part from the discovery that a dialdhyde can be reacted with a malonate-derived compound to produce the corresponding tetraester l,3-diene-l,l,4,4-tetracarboxylate. For example, reaction

of diethyl malonate with ethanedial yields tetraethyl buta-l,3-diene-l,l,4,4-tetracarboxylate; similarly, the reaction of malonate with ethanedial (glyoxal) yields hexa-2,4-dienedioic acid. Hydrolysis of the ester groups, when desired, proceeds through addition of acid to the reaction mixture. Decarboxylation to form the unsaturated dicarboxylic acid (i.e., hexa-2,4-dienedioic acid) is thermally induced, and subsequent hydrogenation of the unsaturated dicarboxylic acid yields hexanedioic acid. In some embodiments of the invention, diethyl malonate and ethanedial are condensed to yield the dialkylated bis-malonate ethyl ester; subsequent saponification to hydrolyze the ester groups followed by thermally-induced decarboxylation yields the unsaturated dicarboxylic acid. Hydrogenation of the unsaturated dicarboxylic acid yields hexanedioic acid. This process is summarized in the following reaction scheme, in which the alkyl groups (Ri and R2) are chosen from the non-limiting examples: H, CH_3, CH_3CH_2, $H_3(CH_2)_2$, $CH_3(CH_2)_3$, $(CH_3)_2CH_2$, or $(CH_3)_2CH_2CH_2$.

Those skilled in the art appreciate that Knoevenagel condensation of various dialdehydes and malonate-derived compound can be used according to the methods of the invention to synthesize saturated and unsaturated (with the alkenes at the 2 and n-2 positions) dicarboxylic acids. For example, and without limitation, propanedial can be used to synthesize heptanedicarboxylic acid, butane- 1-4-dial is used to synthesize octanedicarboxylic acid, and pentane-l,5-dial can be used to synthesize nonanedicarboxylic acid.

[0161] In other embodiments of the invention, a malonate or a malonate-derived compound is used as a substrate in the chemical synthesis of heptanedioic acid. In addition to the dihaloalkane and dialdehyde routes to heptanedioic acid described above, the invention also provides methods for synthesis of heptanedioic acid through reaction of prop-2-enal (acrolein), which may or may not be derived from malonate as provided by the invention, and malonate. Michael addition of malonate to prop-2-enal yields a 2-(3-oxopropyl)malonic acid, and subsequent condensation to the aldehyde moiety with another molecule of malonate produces hex-l-ene-l,l,6,6-tetracarboxylic acid. Thermally induced decarboxylation followed by hydrogenation yields heptanedioic acid.

[0162] In another method of the invention, malonate is used as a substrate in the chemical synthesis of a lactam or lactone. In one embodiment, synthetic conversion of diethyl malonate to Delta- valerolactam though the following steps; Diethyl malonate is produced by Fischer esterification of malonate with ethanol. Michael addition of diethyl malonate to acrylonitrile affords diethyl 2-(2-cyanoethyl) malonate. Hydrogenation of the nitrile moiety of diethyl 2-(2-cyanoethyl)malonate yields an amino acid that is lactamized under mild conditions to yield to ethyl 2-oxopiperidine-3-carboxylate. Saponification of the ester moiety of ethyl 2-oxopiperidine-3-carboxylate followed by heating results in decarboxylation to afford delta- valerolactam. This non-limiting example is illustrated here:

[0163] In another method of the invention, mono-ethyl malonate is used as a substrate in the chemical synthesis of ethyl acetate. Heating of the mono-ethyl malonate results in thermally induced decarboxylation to form ethyl acetate. In some embodiments, mono-ethyl malonate is used to synthesize ethyl acetate. One skilled in the art will recognize that there are a number of established methods for making mono-alkyl malonates from the malonic acid provided by the invention.

[0164] In another method of the invention, a mono-alkyl malonate (e.g. mono-methyl malonate, mono-ethyl malonate, mono-propyl malonate, mono-butyl malonate, mono- isobutyl malonate) is used as a substrate in the chemical synthesis of 3-hydroxypropionate or its related alkyl esters. Borane will chemoselectively reduce the free carboxylic acid moiety of the mono-alkyl malonate and produce the corresponding alkyl 3-hydroxypropionate. Alkyl 3-hydroxypropionates are themselves valuable products. The ester can also be hydrolyzed to yield 3-hydroxypropionate. 3-hydroxypropionate can in turn be dehydrated to acrylic acid, a valuable commodity chemical describe in more detail herein. In some embodiments, a mono- alkly malonate is used to synthesize 3-hydroxypropionate. In some embodiments, the mono- alkyl malonate is mono-methyl malonate. In some embodiments, the mono-alkyl malonate is mono-ethyl malonate. [0165] In another method of the invention, malonate is used to synthesize a dialkylated 1 ,3- dicarbonyl compound. In various embodiments, malonate is used as a substrate in the chemical synthesis of a compound selected from the group consisting of diethyl 2,2- dimethylmalonate, diethyl 2,2-diethylmalonate, diethyl 2,2-dipropylmalonate, diethyl 2,2- dibutylmalonate, dimethyl 2,2-dimethylmalonate, dimethyl 2,2-diethylmalonate, dimethyl 2,2-dipropylmalonate, and dimethyl 2,2-dibutylmalonate. Synthesis of dialkylated 1 ,3- dicarbonyl compounds can be formed in accordance with the invention from synthetically derived dialkyl malonates using alkyl halides (see Gatterman et al., "The practical methods of organic chemistry" 3rd ed, The Macmillan Company: New York, pgs. 189-191 (1916)).

[0166] In other embodiments of the invention, the malonic acid or malonate-derived compound provided by the invention is reacted with urea to form barbituric acid, the core structure of approximate fifty related pharmaceuticals currently on the market, including phenobarbital.

[0167] In some embodiments of the synthetic methods of the invention, a highly purified malonate preparation is used for the synthetic reaction and/or associated downstream applications; in other embodiments, a less purified malonate is used. In still other cases, a crude lysate or other relatively unpurified malonate can be used for the synthetic reaction and/or downstream applications. For most synthetic chemistry reactions, malonate purity greater than 90% w/w is typically desired. In cases where malonate is used for polymerization reactions even greater purity (i.e. , 95% w/w or higher) may be desired. In other applications, however, one may employ a much less pure preparation of malonate. For example, malonate is useful as an animal foodstock, and for this purpose, one may employ the producing cells themselves or a crude lysate of them.

[0168] In another embodiment of the invention, malonate (produced by any means) is reacted with glutarate-semialdehyde (which can also be produced from malonate) to yield hept-2-enedioic acid. This is an additional example of a Knoevenagel condensation and can be conducted with the broad array of solvent and catalytic reagents associated with this reaction. Hept-2-enedioic acid is then reduced using catalytic hydrogenation to yield heptanedioic acid (pimelic acid). Pimelic acid has a wide variety of uses, including as a monomer in Nylon 5,7; as a fermentation media supplement used to enhance biotin biosynthesis; and as a plasticizer. Prior art methods for pimelic acid synthesis are costly and low yielding. The invention provides a new chemical synthesis for this important compound that may be derived from malonic acid as provided by the invention.

[0169] In other embodiments of the invention, vapor or liquid phase hydrogenation is used to convert dialkylmalonates into 1 ,3-propanediol. Due to the reactive nature of the number two carbon of malonate and malonate esters, polysubstituted derivatives of various compounds can be produced using the malonate provided by the invention. In some embodiments, neopentylglycol (2,2-dimethylpropane-l,3-diol), an important component of some polyesters, is produced by first generating 2,2-dimethyl-dialkyl malonate. This can compound can be produced using a variety of bases as catalysts and a methyl halide as the source of the methyl group. The subsequent hydrogenation of the ester groups results in the production neopentylglycol and the cognate alcohols from the ester groups. This represents a novel route to this important industrial compound. Furthermore, due to the highly reactive nature of the number two carbon of malonate and malonate derived compounds, other alkylhalides can be employed to result in new compounds with the potential for novel properties when incorporated into polymers.

[0170] The invention, having been described in detail, is illustrated by the following examples, which should not be construed as limiting the invention, given its diverse aspects, embodiments, and applications.

EXAMPLES

Example 1: Construction of recombinant nucleic acids encoding wild-type EHD3 and EHD3 mutants E124S, E124A, E124A/E308V, and E124V malonyl-CoA hydrolases and expression vectors for production of malonate in E. coli

[0171] The present invention provides methods for producing malonate in an E. coli host, as well as E. coli host cells that produce malonate and express an EHD3 mutant enzyme, including but not limited to E124S, E124A, and E124V. This example describes the construction of protein coding sequences for EHD3 and the mutant EHD3 proteins useful in the invention, expression vectors containing those coding sequences, and host cells comprising those expression vectors. The nucleic acid encoding wild type S. cerevisiae EHD3 was amplified by PCR from Baker's yeast using primer pair A93/A94. Point mutation E124A was introduced using primer pairs A93/A96 and A95/A94, mutation El 24V was introduced using primer pairs A93/A98 and A97/A94, and mutation E124S was introduced using primer pairs A93/A100 and A99/A94. The resulting nucleic acids were cloned into an E. coli expression vector containing the pSClOl origin of replication, a chloramphenicol resistance cassette, and a $PL_{BCO}I$ promoter using standard techniques. The resulting vectors were transformed into an E. coli DHlOb host and plated on Luria-Bertani (LB) agar plates containing 50 μg/ml chloramphenicol (Cm^{50}) and 2% w/v glucose. Individual colonies were then inoculated into 3 ml LB media in 48-well plates; following 6 hours growth, plasmids were isolated and the EHD3 protein-coding region sequenced. When sequencing plasmids derived from clones of EHD3 (E124A), it was discovered that a second, point mutation, E308V, stabilized the EHD3 (124 A) clone; the presence of an uncharged valine residue likely stabilizes protein folding. The EHD3 (E124A, E308V) strain was also used for malonate production (see Example 2). Primers A93-A100 are SEQ ID NO: 13-20, respectively.

Example 2: In vivo production of malonate in E. coli using EHD3 mutants E124S and E124A/E308V

[0172] This example describes the host cells and culture conditions resulting in the in vivo production of malonate using a heterologous EHD3 malonyl-CoA hydrolase in an E. coli host cell. E. coli strain K12 was transformed with vectors containing wild-type EHD3, EHD3 (E124A), EHD2 (E124V), EHD3 (E124S), EHD3 (E124A, E308V) or an empty vector negative control. Transformants were streaked on LB agar plates (Cm^{50}, 2% glucose). Following overnight growth at 37°C, individual colonies were inoculated into 3 ml LB (Cm^{50}, 2% glucose) in a 48 -well plate. Cultures were incubated on a plate shaker at 37°C for 6 hours, at which point each culture was inoculated 1% v/v into M9 minimal medium supplemented with Cm^{50} and a mixed carbon source (0.5% glycerol, 0.05% glucose, 0.2% lactose) in a 48-well plate. Cultures were incubated on a plate shaker at 30°C, and a 500 sample of the fermentation broth was removed for analysis after 48 hours incubation.

[0173] Samples were centrifuged (x6000g, 1 min) and the supernatant analyzed for malonate quantification. Chemical standards were prepared in 20 μM of water. The separation of malonate was conducted on a Shimadzu Prominence XR UPLC connected to a refractive index detector and UV detector monitoring 210nm. Product separation was performed on a Bio-Rad Aminex HPX-87h Fermentation Monitoring column. The UPLC was programmed to run isocratically using 5 mM H_2SO_4 as the eluant with a flow rate of 600 per minute. 10 were injected per sample, and the sample plate temperature was held at 4°C. Malonate standards began eluting at -19.8 minutes. Addition of the standard to samples containing malonate demonstrated a proportional increase in malonate peak area, confirming malonate production. Malonate concentrations (mg/1) were calculated by comparision to a standard curve prepared from authentic malonate.

[0174] For cultures harboring the empty vector control, no peak was observed at the same retention time as the malonate standard, and the integrated peak area was below the detection limit of the instrument (i.e., no malonate production was observed). Malonate production was observed in samples harboring wild-type EHD3, EHD3 (E124A), EHD3 (E124V), EHD3 (E124S), and EHD3 (E124A, E308V); malonate concentrations were (mean ± std dev; n=3): Wild-type EHD3, 6.0 ± 0.2 mg/1, EHD3 (E124A), 7.6 ± 0.7 mg/1, EHD3 (E124V), 0.28 ± 0.03 mg/1, EHD3 (E124S), 82.3 ± 7.8 mg/1, and EHD3 (E124A/E308V), 8.35 ± 2.5 mg/1. EHD3 (El 24V) resulted in decreased production of malonate relative to the wild- type protein, likely due to both poor malonyl-CoA substrate binding and high promiscuous activity toward other, endogenous acyl-CoA molecules. Similarly, EHD3 (E124A) yielded only a minor increase in malonate production relative to wild-type EHD3. EHD3 mutant E124S resulted in a significant ($p<0.05$, t-test) increase in malonate production over wild- type EHD3, EHD3 (E124A), and EHD (E124V), demonstrating the importance of E124S in increasing malonyl-CoA substrate binding and malonate production.

Example 3: Construction of additional EHD3 E124 mutants and expression vectors for in vivo production of malonate in E. coli

[0175] Example 1 describes the construction of E. coli expression vectors for wild-type EHD3 and a subset of E124 mutations, specifically E124A, E124V, E124S, and E124A/E308V, and Example 2 describes their use to produce malonate in E. coli cells. This example describes construction of E. coli expression vectors for all EHD3 El 24 point mutations, i.e., E124G, E124T, E124C, E124L, E124I,

E124M, E124P, E124Y, E124W, E124D, E124N, E124Q, E124H, E124K, E124R, and E124F. These EHD3 mutants were constructed using an E. coli vector with pSClOl origin and PLacoi origin of replication, as described in Example 1. The forward PCR primer comprises nucleic acid sequence (5'-aatttttactgatNNNtattctttgaattttcaaatagc-3'), where sequence "NNN" is the three nucleotides encoding the desired E124 amino acid point mutation; likewise, the reverse PCR primer comprises nucleic acid sequence (5'-ttcaaagaataaNNNatcagtaaaaaatttgatggacttg-3'), where sequence "NNN" is complementary to the three nucleotides encoding the desired E124 amino acid point mutation. The forward PCR primer was used in conjuction with PCR primer A94 (SEQ ID NO: 14), and the reverse PCR primer was used in conjuction with PCR primer A93 (SEQ ID NO: 13) to produce two overlapping EHD3 gene fragments containing the desired point mutation. Amplification of the two EHD3 gene fragments with primers A93 and A94 yielded the full length EHD3 gene containing the desired point mutation. The expression vectors were constructed using standard cloning protocols and transformed into E. coli DHlOb and were subsequently isolated as described in Example 1 and verified by sequencing.

Example 4: In vivo production of malonate in recombinant E. coli using EHD3 E124 mutants

[0176] This example describes fermentation of a set of E. coli host cells, each member of the set containing one of the 19 possible EHD3 El 24 amino acid substitutions and determination of malonate levels produced. Specific El 24 point mutations should theoretically improve malonyl-CoA binding and malonate production relative to wild-type; in particular, point mutations E124T, E124N, E124Q, E124H, E124K, and E124R should improve malonate production, theoretically due to introducing amino acids containing functional groups that improve interaction with the terminal carboxylic acid moiety of malonate. Point mutations E124S, E124Q, and E124K have side-chains that are located in the EHD3 binding pocket in positions that should theoretically best coordinate malonate binding. Malonate production is performed and quantified substantially as described in Example 2. Example 5: Construction of YciA malonyl-CoA hydrolase expression vectors for in vivo production of malonate in E. coli

[0177] This example describes the use of E. coli YciA for the production of malonate in E. coli in accordance with the invention. Wild type E. coli acyl-CoA YciA is PCR amplified from the host genome using primers A120 (SEQ ID NO:21) and A121 (SEQ ID NO:22). The resulting nucleic acid is cloned behind the PLacoi promoter on an E. coli expression plasmid containing a pSClOl origin of replication and an ampicillin resistant gene. The control vector comprises an empty vector without the yciA gene insertion behind the PLacoi promoter. As described in Example 1, individual colonies are cultured, their plasmid isolated, and the coding region of the YciA gene insert sequenced.

Example 6: In vivo production of malonate in recombinant E. coli using heterologous YciA

[0178] This example describes the host cells and culture conditions resulting in in vivo production of malonate using an expression vector encoding a heterologous YciA malonyl- CoA hydrolase as described in Example 5 in an E. coli host cell. Wild type E. coli strain K12 is transformed with vectors containing E. coli YciA; wild-type E. coli harboring an empty vector serves as a negative control. Malonate production is performed and quantified as described in Example 2.

Example 7: Construction of engineered EHD3 malonyl-CoA hydrolase expression vectors for in vivo production of malonate in yeast

[0179] The present invention also provides expression vectors, host cells, and methods for in vivo production of malonate in a yeast cell. Yeast cells can, in general, tolerate higher concentrations of organic acids in the fermentation broth and possess better-established industrial fermentation protocols than E. coli.

[0180] The yeast expression vectors described in this example were generated in part from use of the E. coli expression vectors described in Examples 1 and 3 as PCR templates for the EHD3 genes. The yeast expression vectors contain a 2-micron origin of replication, ura3 auxotrophic marker, and TEF promoter; the vectors also contain a puc origin of replication and an ampicillin or chloramphenicol resistance cassette for vector propogation in E. coli. The plasmids were transformed into either a S. cerevisiae BY4741 or BY4742 background, both derivatives of the S288C parental strain.

[0181] Additional mutations can be introduced into the EHD3 coding sequence to abrogate mitochondrial targeting. The basic amino acids R3, K7, K14, K18, and R22 in the EHD3 coding sequence are important for mitochondrial targeting, and mutation of any one or more of them to A or V decreases mitochondrial EHD3 expression and increases cytosolic EHD3 expression. Mutations are introduced by PCR amplification of an EHD3 template with forward and reverse primers containing the desired point mutation. Mutations are introduced using primers matching the EHD3 gene with the exception that the nucleotide sequence at the position of the desired amino acid point mutations is altered to "gyt" (where y is either a cysteine or thymine nucleotide in a mixed population of oligonucleotide PCR primers). The gene fragements are first cloned into an E. coli expression vector haroboring a pSClOl origin of replication, pLacoi promoter, and chloramphenicol resistance marker, and the vector sequences confirmed as described in Example 1 ; following isolation of the desired mutant, the EHD3 gene is amplified and cloned into a yeast expression vector.

Example 8: In vivo production of malonate in recombinant yeast using engineered EHD3 malonyl-CoA hydrolase

[0182] S. cerevisiae BY4742 host cells are transformed with a yeast expression vector prepared substantially as described in Example 7 harboring heterologous EHD3 or an empty vector negative control plasmid using standard protocols. Transformants are streaked on synthetic complete dropout medium (SD) agar plates lacking uracil and cultured at 30°C; individual colonies are grown overnight in 3 mL SD media overnight at 30°C and subsequently diluted 1% v/v into 3 ml of SD lacking uracil. Strains are cultured at 30°C for 72 hours; 500 μΐ aliquots are sampled at 24, 48, and 72 hour timepoints for quantification of malonate production and OΌβoo- The TEF promoter is a constitutive promoter.

Example 9: Increasing malonate biosynthesis in engineered yeast through expression of heterologous acetyl-CoA synthetase

[0183] In addition to the methods, vectors, and host cells for expression of a heterologous malonyl-CoA hydrolase and in vivo production of malonate, as illustrated in Examples 1-8, the invention also provides methods and host cells for improved titer, yield, and/or productivity of malonate. In one aspect, malonate production is improved by increasing the biosynthesis of acetyl-CoA. This example describes heterologous expression of acetyl-CoA synthetase enzymes in a recombinant S. cerevisiae host comprising a malonyl-CoA hydrolase pathway and resulting improvement in malonate production. The five acetyl-CoA synthetase proteins illustrated are S. cerevisiae ACS1 and ACS2, E. coli AcsA, Salmonella enterica Acs, and Bacillus subtilis AcsA. All genes are PCR amplified from their respective hosts and cloned into a yeast expression vector harboring a 2-micron origin of replication, ura3 auxotrophic marker, and TEF promoter; the vectors also contain a puc origin of replication and ampicillin resistance cassette for vector propogation in E. coli.

Example 10: Increasing malonate biosynthesis in engineered yeast through expression of heterologous pyruvate dehydrogenase

[0184] While Example 9 describes increased acetyl-CoA biosynthesis through expression of heterologous acetyl-CoA synthetases, this example describes increased acetyl-CoA biosynthesis through expression of heterologous pyruvate dehydrogenase enzymes. In specific, S. cerevisiae pyruvate dehydrogenase enzymes PDA1, PDB 1, LAT1, LPD1, and PDX1 are heterologously expressed in recombinant S. cerevisiae comprising a malonyl-CoA hydrolase pathway. The genes are all PCR amplified from the S. cerevisiae chromosome and cloned into a yeast expression vector harboring a 2-micron origin of replication, ura3 auxotrophic marker, and TEF promoter; the vectors also contain a puc origin of replication and ampicillin resistance cassette for vector propogation in E. coli.

Example 11: Increasing malonate biosynthesis in E. coli and 5. cerevisiae by heterologous expression of an ethanol catabolic pathway

[0185] This example describes a third route to increase acetyl-CoA biosynthesis: heterologous expression of an ethanol catabolic pathway. An ethanol catabolic pathway comprises two or three enzymes. An alcohol dehydrogenase and an acetaldehyde dehydrogenase (acylating), or an alcohol dehydrogenase, acetaldehyde dehydrogenase (non- acylating), and an acetyl-CoA synthetase. The alcohol dehydrogenase enzymes S. cerevisiae ADH2, E. coli AdhP, and H. sapiens ADH1A, H. sapiens ADH1B, and H. sapiens ADH1C are combinatorially cloned with with an acetaldehyde dehydrogenase (acylating) or aldehyde dehydrogenase and acetyl-CoA synthetase. The acetaldehyde dehydrogenase (acylating) enzymes E. coli MhpF, E. coli AdhE, Pseudomonas sp CF600 DmpF, and Pseudomonas putida TodL are also all cloned combinatorially. In ethanol catabolic pathways utilizing an acetaldehyde dehydrogenase (non-acylating) S. cerevisiae ALD2, ALD3, ALD4, ALD5, and ALD6 are used. Acetyl-CoA synthetase enzymes used are S. cerevisiae ACS1 and ACS2, and E. coli Acs. All genes are PCR amplified from genomic DNA.

[0186] For E. coli host cells, the ethanol catabolic pathway is expressed from a vector backbone harboring pl5a origin of replication, ampicillin resistance marker, and $Pi_{ac}oi$ promoter. All combinations of all two and three gene pathways are constructed as single operons. E. coli K12 is co-transformed with an EHD3 expression plasmid and an ethanol catabolic pathway plasmid and streaked on LB agar plates (Cm^{50}, Cb^{50},

2% w/v glucose). Control strains harbor empty vector. Production cultures and analysis thereof are conducted as described in Example 2, with the notable exception of the addition of the second antibiotic require to maintain the second plasmid.

[0187] S. cerevisiae fermentations can be conducted using identical ethanol concentrations as the E. coli experiments. The yeast expression vectors harbor a 2-micron origin, ura3 auxotrophic marker, and CUP promoter, and all combinations of the ethanol catabolic pathways are constructed on this vector backbone; a plasmid absent an ethanol catabolic pathway serves as a negative control. An ethanol catabolic pathway plasmid is transformed into recombinant S. cerevisiae BY4742 comprising an engineered EHD3 malonyl-CoA hydrolase pathway on a yeast chromosome. All fermentations are conducted at 30°C in SD media without uracil. Ethanol catabolic pathway expression is induced with 100 μM copper sulfate after 12 or 24 hours growth. 500 μΐ aliquots are sampled at 24, 48, and 72 hours for quantification of ethanol, acetaldehyde, acetate, and malonate concentrations; OD_{600}measurments of cell density are also recorded at each timepoint. In addition to malonate titer, ethanol consumption is calculated.

Example 12: Increasing malonate biosynthesis in 5. cerevisiae by heterologous expression of an ATP citrate lyase

[0188] This example describes a fourth approach to increase acetyl-CoA biosynthesis and improve malonate production in recombinant S. cerevisiae. ATP citrate lyase (EC 2.3.3.8) catalyzes the formation of acetyl-CoA, oxaloacetate, and ADP in the cytosol from citrate. ATP citrate lyase enzymes from the oleaginous yeasts Candida curvata, Cryptococcus albidus, Lipomyces lipofer, Rhodospiridium toruloides, Rhodotorula glutanis, Trichosporon cutaneum, Yarrowia lipolytica, are PCR amplified from genomic DNA and cloned into a yeast expression vector behind a CUP promoter; the expression vector contains a 2-micron origin and leu2d auxotrophic marker. A plasmid absent an ATP citrate lyase enzyme serves as a negative control.

[0189] An ATP citrate lyase pathway plasmid is transformed into recombinant S. cerevisiae BY4742 comprising an engineered EHD3 malonyl-CoA hydrolase pathway on a yeast chromosome. All experiments are conducted at 30°C in SD media without uracil. ATP citrate lyase pathway expression is induced with 100 uM copper sulfate after 12 or 24 hours growth. Some cultures are also supplemented with 0.5, 1 , 2.5, or 5 g/1 citrate to provide an additional demonstraton of pathway activity. 500 μΐ aliquots are sampled at 24, 48, and 72 hours for quantification of citrate (where applicable) and malonate concentrations; Oϋβoo measurments of cell density are also recorded at each timepoint. In addition to malonate titer, citrate consumption can be calculated. Example 13: Increasing malonate biosynthesis in recombinant yeast through modification of host cell fatty acid storage

[0190] This example describes a fifth approach to increase acetyl-CoA biosynthesis and improve malonate production in recombinant S. cerevisiae. Fatty acid biosynthesis pathways compete with malonate production for acetyl-CoA and malonyl-CoA, and altering host cell fatty acid anabolism can increase malonate production. The present invention provides host cells comprising genetic modifications of one or more nucleic acids encoding proteins affecting fatty acid storage and catabolism. In Saccharomyces cerevisiae, the proteins SNF2, IRA2, PRE9, PHO90, SPT21, POX1, ANT1, FOX3,

EHD3, PAS1, PAS3, ARE1 , ARE2, DGAl, LROl, ACLl, MAEl, GLC3, GLGl, GLG2, PATl, and PEXl l are knocked out individually and combinatorially and the resulting strains cultured for malonate production. All S. cerevisiae strains constructed comprise an engineered EHD3 malonyl-CoA hydrolase pathway for production of malonate. Fermentations are performed as described in Example 8, and malonate is quantified as described in Example 2.

Example 14: Increasing malonate biosynthesis in recombinant yeast through increased beta-oxidase activity

[0191] This example describes a sixth approach to increase acetyl-CoA biosynthesis and improve malonate production in recombinant S. cerevisiae. In addition to decreasing host cell fatty acid anabolism, increasing host cell fatty acid catabolism can increase malonate production. The present invention provides host cells modified for increased expression of PATl and/or PEXl l. PATl and PEXl l are PCR amplified from genomic S. cerevisiae DNA and cloned into a yeast expression vector behind a CUP promoter; the expression vector contains a 2-micron origin and leu2d auxotrophic marker. A plasmid without a beta- peroxidase enzyme serves as a negative control.

[0192] A beta-oxidase pathway plasmid is transformed into recombinant S. cerevisiae BY4742 comprising an engineered EHD3 malonyl-CoA hydrolase pathway on a yeast chromosome. The engineered malonyl-CoA hydrolase is integrated onto the chromosome using standard recombination methods. The resulting strain serves as a base to test subsequent modifications and their impact on malonate production. All experiments are conducted at 30°C in SD media without uracil. Pathway expression is induced with 100 μM copper sulfate after 12 or 24 hours growth. Some cultures are also supplemented with 0.5, 1, 2.5, or 5 g/1 palmitic acid to provide an additional demonstration of pathway activity. 500 μΐ aliquots are sampled at 24, 48, and 72 hours for quantification of palmitic acid (where applicable) and malonate concentrations; OD_{600} measurments of cell density are also recorded at each timepoint. In addition to malonate titer, palmitic acid consumption can be calculated.

Example 15: Improving malonate biosynthesis in engineered yeast through increased acetyl-CoA carboxylase activity

[0193] In addition to the methods, vectors, and host cells for expression of a heterologous malonyl-CoA hydrolase and in vivo production of malonate, the invention also provides methods and host cells for improved titer, yield, and/or productivity of malonate. In one aspect, malonate production is improved by increasing the biosynthesis of malonyl-CoA. Malonyl-CoA is the penultimate intermediate in the biosynthesis of malonate from acetyl- CoA, and in S. cerevisiae, this reaction is catalyzed by acetyl-CoA carboxylase (ACCl).

[0194] Malonyl-CoA biosynthesis is increased by overexpression of S. cerevisiae ACCl. Toward this end, the ACCl gene is cloned using standard methods behind the CUP promoter on an S. cerevisiae expression plasmid containing a 2-micron origin of replication and ura3 auxotrophic marker. The control vector comprises an empty vector. S. cerevisiae host strains are engineered with chromosomal deletions of ACCl and SNF1 protein kinase responsible for ACCl phosphoregulation; chromosomal deletions are

constructed both independently and in combination. Host cells harboring expression plasmids or control plasmids are grown as described in Example 8 and malonate production quantified as described in Example 2.

Example 16: Improving malonate biosynthesis in host cells through supplementation of the fermentation broth with cerulenin

[0195] In this example, malonate production in a recombinant host cell expressing an EHD3-derived malonyl-CoA hydrolase is improved by supplementation of the fermentation broth with cerulenin. The malonate production plasmid A4, comprising S. cerevisiae EHD3 (E124S) under control of a PLacoi promoter, is transformed into an E. coli K12 host.

[0196] Individual colonies are inoculated into 3 ml LB medium (Cm^{50}, 2% w/v glucose) in 48-well plates and cultured for 6 hours at 37° C on a plate shaker. Strains are then subcultured 1% v/v into 3 mL of M9 minimal medium (Cm^{50}, 0.5% w/v glycerol, 0.05% w/v glucose, 0.2% w/v lactose) and cultured at 30°C on a plate shaker. Following 6 hours growth, one half of the cultures are supplemented with 10 mg/1 cerulenin. After 48 hours growth, malonate concentration in the supernatant is measured as described in Example 2.

Example 17: Improving malonate biosynthesis in engineered yeast through supplementation of fermentation broth with carbon dioxide

[0197] In this example, fermentation conditions are modified to increase the biosynthesis of malonyl-CoA. Enzymatic conversion of acetyl-CoA to malonyl-CoA by the enzyme acetyl- CoA carboxylase requires a stoichiometric amount of carbon dioxide, and supplementation of the growth media with carbon dioxide increases malonate production. Carbon dioxide is added to the growth media as either solid calcium carbonate or gaseous carbon dioxide.

[0198] Recombinant yeast cells harboring a malonyl-CoA biosynthetic pathway are grown in a defined minimal medium supplemented with between 0.1 - 10 g/1 calcium carbonate. Control cultures are not supplemented with calcium carbon. Malonate production is quantified as described in Example 2 over the course of 48 hours growth.

Example 18: Improving malonate biosynthesis in engineered yeast through decreased malonate catabolism

[0199] In this example, malonate production is increased by eliminating endogenous malonate catabolism in the host cell. S. cerevisiae contains multiple acyl-CoA synthetases, including FAAl, FAA2, FAA3, FAA4, LSCl, and LSC2; by deletion or modification of the nucleic acids on the host genome encoding these proteins, catabolism of malonate in the growth media can be decreased.

[0200] Malonyl-CoA knockout strains are constructed of each of the yeast acyl-CoA synthetases. The resulting strains are then cultured in a defined medium supplemented with 1- 5 g/1 sodium malonate. The

malonate concentration in the fermentation broth is monitored over the course of 48 hours and quantified as described in Example 2. Strains with multiple knockouts can be constructed following similar procedures.

Example 19: Improving malonate biosynthesis in engineered yeast through improved malonate secretion from the host cell

[0201] In this example, malonate production is improved by increasing secretion of malonate from the host cell. This is accomplished by overexpression of one or more of each of the S. cerevisiae pleiotropic resistant pumps, namely PDR5, PDR10, PDR11, PDR12, PDR15 and PDR18.

Example 20: Improving malonate biosynthesis in engineered yeast through decreased succinate dehydrogenase competitive inhibition by malonate

[0202] In this example, competitive inhibition of S. cerevisiae succinate dehydrogenase SDHl is decreased, enabling higher titers of malonate to be achieved. First, the S. cerevisiae is genetically modified for deletion of the native, chromosomal copy of succinate dehydrogenase (SDHl) using standard methods. The resulting strain is grown anaerobically to facilitate growth in absence of SDHl protein. The SDHl deletion strain is subsequently transformed with a vector harboring a genetically modified SDH expression cassette containing an E300D, R331K, R331H, R442K, R442H mutation, or a combination of these mutations. These mutant SDH genes are cloned behind a constitutive TEF promoter on a yeast backbone harboring 2-micron origin and ura3 auxotrophic marker. Vectors are subsequently transformed into S. cerevisiae strain encoding malonyl-CoA hydrolase on the chromosome and the resulting strains grown in SD media lacking uracil. The transformed strains are cultured for malonate production as described in Example 8 and malonate is quantified as described in Example 2 to quantify the impact of these SDH mutations on malonate production.

Example 21: MdcY malonate transcription factor biosensor using exogenously added malonate.

[0203] Plasmid S14 was used to demonstrate biosensor response to exogenously added malonate in an E. coli host cell. S14 employs the malonate responsive transcription factor, MdcY (SEQ ID NO:3), and the MdcY-responsive promoter, P_{Md}cL (SEQ ID NO:6) derived from Acinetobacter calcoaceticus. This biosensor of the invention was constructed using an E. coli vector backbone with ampicillin resistance marker and ColEl origin of replication; the tetA tetracycline resistance gene was placed under control of the $PM_{<}I_{C}L$ promoter. Transformation of plasmid S14 into an E. coli host resulted in a strain expressing the tetA gene product following supplementation of the fermentation broth with malonate, and the strain exhibits a malonate-dependent increase in tetracycline resistance.

[0204] Nucleic acids encoding for MdcY and TetA gene products, $PM_{<}I_{C}L$ promoter, and the E. coli vector backbone were synthetically produced; the biosensor vectors were than constructed by PCR amplification of the nucleic acids and subsequent cloning into the E. coli vector backbone. The plasmids were transformed into chemically competent E. coli DHlOb and the resulting clones plated on LB agar

plates containing 50 μg/ml carbenicillin (Cb^{50}). Individual colonies were grown overnight in 3 ml LB medium supplemented with antibiotic and the sequences of the purified plasmid were verified.

[0205] E. coli strain K12 was co-transformed with plasmids S14 and individual colonies isolated from LB agar plates (Cb). Colonies were grown in 25 ml LB broth (Cb) until reaching an optical density at 600 nm (OΌβοο) of approximately 0.50, at which point in time cell stocks were prepared and stored at -80°C; cell stocks were 0.5 ml cell culture and 0.5 ml of a 50% v/v glycerol solution.

[0206] All biosensor demonstrations were performed with malonate. An aliquot of biosensor cell stock was thawed and used to inoculate 50 ml of LB medium (Cb^{50}) in a 250 ml, baffled Erlenmeyer flask. Cultures were incubated for 2 hours at 37°C; subsequently, 0.6 ml biosensor culture was added to 48-well plates prepared with 2.3 ml LB medium (Cb^{50}) supplemented with tetracycline and malonate acid at the desired concentration (n=4). Plates were than grown at 30°C on an orbital titer plate shaker. Following 12 hours incubation, 200 μΐ samples were taken for ΌΟ oo measurement. [0207] Biosensor cultures harboring S14 displayed a dose-dependent response for malonate (Figure 1). The dynamic range (the maximum difference in OD_{600} values between the fully induced samples and those samples absent malonate supplementation) was 1.2 OD_{600} units, indicating the MdcY-$P_M$dcL based biosensor was highly responsive to exogenously added malonate. An increase in OD_{600} was observed between between 0.5-1 mM exogenously added malonate, providing a suitable range over which malonate can be quantified using this method.

Example 22. MdcY malonate transcription factor biosensor to detect biologically produced malonate in fermentation broth

[0208] In this example, a malonate transcription factor biosensor was used to detect the production of malonate from a yeast strain engineered as described in other aspects of the invention.

[0209] Malonic acid was produced using a genetically engineered yeast strain as follows. S. cerevisiae BY4741 yeast cells harboring a vector for expression of malonyl-CoA hydrolase comprising a CYC1 terminator, an ampicillin resistance cassette, a PMB 1 origin of replication, a CEN/ARS origin of replication, and a URA3 selection marker was used for fermentation. The F0PNG8-1 malonyl-CoA hydrolase (from Bacillus thuringiensis subsp. finitimus strain YBT-020; UniProt ID F0PNG8, with E91S mutation) and the F6AA82-2 malonyl-CoA hydrolase (from Pseudomonas fulva strain 12-X; UniProt ID F6AA82, with E95S and Q348A mutations) were each expressed from this plasmid under control of the TEF1 promoter. The culture medium described in Example 30 was used with 20 g/L glucose as a carbon source. Production was performed as follows. Two ml of culture medium in a 48- well plate was inoculated with 20 μΐ of a starter culture of the producer strain, in quadruplicate. The plate was covered with a breathable membrane, incubated on a plate shaker at 30°C, and sampled for HPLC and biosensor analysis of product accumulated after 142 h of growth. Cells and cell debris were removed from the culture media by centrifugation and filtered through 0.45 micron membrane prior to analysis by HPLC or biosensor.

[0210] E. coli cells harboring either plasmid pS14, encoding a tetracycline resistance gene (tetA) under expression control of the malonic acid-responsive P_md_CL promoter, or plasmid pS27, encoding a lacZ gene under expression control of the malonic acid-responsive P_md_CL promoter, were used as biosensor indicator strains. The vector pS27 was constructed in the same manner as described for pS14 in Example 21, with the lacZ gene, encoding a beta- galactosidase, inserted in place of the tetA gene. Biosensor strains were prepared as described in Example 21. [0211] Yeast spent media obtained from 96-well production plates were added to 96-well plates containing 120 ul of biosensor cell culture. For the TetA (pS14) biosensor, 10 ul of tetracycline stock solution (to provide a range of 20-35 ug/ml) were added to each well. For both the TetA (pS14) and lacZ (pS27) biosensor, the remaining volume of each well was filled with LB medium (Cb^{50}) to a final volume of 600 ul and grown as previously described. Samples (200 ul) were collected to 96 well plates after 2 h for S27 cultures and after 5-8 h for S14 cultures, and OD600 was measured. An ortho-nitrophenyl-P-galactoside (ONPG) assay was performed on samples from S27 (beta-galactosidase reporter) biosensor plates as follows. Cells were diluted 1 :4 in 25 ul lysis buffer and subsequently 90 ul of ONPG stock solution (10 mg/ml in deionized water) were added to each well. Contents of each well were completely mixed and left at 30°C for 4-16 hours. Optical densities were measured at 420 nm.

[0212] A dose-dependent response to malonate was observed. Specific malonic acid concentrations were also measured by HPLC, as described in Example 2, and quatified by comparison to a standard curve. Linear regression analyses between the quantifiable output of the biosensor, OD420 (for pS27) or OD600 (for pS14) and specific malonic acid concentrations measured by HPLC were calculated to have coefficients of determination (R) of 0.88492 plotting 37 OD420 samples and 0.89755 plotting 18 OD600 samples.

[0213] One skilled in the art will recognize that these very high coefficients of determination are indicative of the correlation between biosensor output and malonate concentration in the culture media. This aspect of the invention provides a tremendous advantage in both cost and time with regard to screening differential outputs in biological malonate production. Dilution of culture media used to challenge the biosensor can facilitate the extension of the dynamic response range of the sensor from zero to full solution saturation of malonate. The use of a plate based screen enables the screening of 96 samples in a few minutes in comparison to a time requirement of 2-20 minutes or more per sample for HPLC analysis. The savings in capital investment and solvent usage and disposal engendered by limiting or replacing HPLC altogether are also substantial.

Example 23. Precipitation of malonate from fermentation broth by addition of calcium hydroxide, calcium carbonate, and calcium chloride

[0214] In this example, malonic acid was purified from the fermentation broth by precipitation with a divalent cation, specifically calcium. The purification methods were demonstrated using synthetic metabolites exogenously added to the fermentation broth. A yeast culture of S. cerevisiae BY4741 was grown in 0.5 L of synthetic complete media for 72 h at 30°C, 200 rpm. After 72 h, 25 ml aliquots of whole-cell fermentation broth were used to dissolve 0, 0.5, 1, 5, 10, 25, 50, 75, 100 g/1 equivalents of malonic acid. Each 25 ml sample was divided into five- 5 ml aliquots, and the pH of one aliquot of each

concentration was adjusted to 5.5, 6.0, 6.5, 7.0, or 7.5. All samples were then centrifuged (x6000 rcf, 5 min, 25°C), and the supernatants were transferred to separate tube. HPLC analysis showed malonic acid only in the supernatant fraction. Next, calcium chloride, a representative divalent cation, was employed for precipitating malonic acid from the clarified supernatant fraction by addition in equimolar equivalents to 0.5, 1, 5, 10, 25, 50, 75, 100 g/1 of malonic acid of each sample at 25°C. The malonate concentration remaining in the supernatant and precipitate was then measured by HPLC as described in Example 2. At concentrations below 5g/L, the extraction effiency was 10% or less. It was also negligible once the pH was lowered to 5.5. However, at higher concentrations and pH values from 6-7.5 this method was quite effective at purifying malonate. The respective extraction efficiencies (percent isolated from fermentation broth) for this method at lOOg/L, 75g/L, 50g/l, 25g/L, and lOg/L are as follows: pH 7.5 = 89.8%, 88.5%, 83.3%, 70.1%, and 66.4%; pH 7.0 = 88.0%, 86.9%, 81.8%, 71.5%, and 63.9%; pH 6.5 = 80.5%, 79.0%, 75.8%, 65.0%, and 59.0%; pH 6.0 = 54.4%, 55.0%, 52.8%, 43.0%, 34.9%.

[0215] These results demonstrate that this method of the invention purified the malonic acid from the fermentation broth, separating it from both yeast cells and other dissolved chemicals.

Example 24: Purification of biologically derived malonate from fermentation broth by reactive extraction with ethanol and methanol

[0216] In this example, endogenously produced malonate from cultures of S. cerevisiae harboring a malonate biosynthesis pathway is purified from fermentation broth by reactive extractionb. The host cells are first removed from 50 mL of fermentation broth by centrifugation (x6000 rcf, 5 min). Formation of the diethyl and dimethyl ester in the fermentation broth is performed using methods adapted from: Gatterman L. and Babsinian VS. "The practical methods of organic chemistry" 3rd ed, The Macmillan Company: New York, pg. 161-162 (1916). In brief, sodium chloride is added to the fermentation broth; subsequently, ethanol and sulfuric acid are added leading to formation of the diethyl malonate ester.

Example 25: Reactive extraction of malonate from fermentation broth using tertiary amines

[0217] Yeast fermentation broth is prepared as described in Example 23. Malonic acid is exogenously added to the fermentation broth to a final concentration of 50 g/1. The solution pH is adjusted to a value <4.0 by addition of an acid. [0218] Three solutions of tertiary amines (TA) are prepared using 1-octanol as a diluent at 0.25, 0.5, and 0.75 mol-TA/kg 1-octanol. Tertiary amines used are triethylamine, tripropylamine, tributylamine, tripentylamine, trihexylamine, triheptylamine, trinonylamine, and tridecylamine.

[0219] An equal volume of organic solvent containing the amine and diluent are mixed with a fermentation broth containing malonate. The reaction is stirred at 1000 rpm, 25°C, for 2 hours; subsequently, the two phases are separated by centrifugation (x6000 rcf, 10 min). Malonate concentration in each phase can be measured by HPLC as described in Example 2. Example 26: Reactive distillation of malonate from fermentation broth using methanol and ethanol

[0220] This example describes reactive distillation of malonate from fermentation broth using methanol and ethanol; a cationic exchange resin, Amberlyst-15, is used as the solid catalyst. The resin is dried in a vacuum oven for 6 hours at 70 degrees C before use. The assembly consists of a glass column packed with ceramic attached to a collection container. Esterification takes place in a reactor connected to the bottom of the column. A condenser is placed at the top of the column for the condensation of low volatile vapors. The esterification reactor is fed with the fermentation broth and the ion-exchange resin, Amberlyst-15 (2% w/w), is added as a catalyst. Sufficient heat is applied to the reactor to vaporize the reaction mixture, and either methanol or ethanol is added to the reactor once the desired temperature is achieved. Samples are withdrawn from the drain valves of the esterification reactor and collection container. After completion of the reaction, volumes and masses of final reaction mixtures from the esterification reactor and collection containers are measured. Malonate consumption and product formation are measured as described in Example 2.

Example 27: Synthetic conversion of malonate to dimethyl malonate and diethyl malonate

[0221] Biologically derived malonate is produced from recombinant S. cerevisiae in 1 L culture flasks as follows: strains are streaked on synthetic complete dropout medium (SD) agar plates lacking uracil and cultured at 30°C; individual colonies are grown overnight in 3 mL SD media overnight at 30°C and subsequently diluted 1% v/v into 500ml of SD lacking uracil. Strains are cultured at 30°C for 72 hours. The resulting fermentation broth is centrifuged (x6000g, 10 min) and the supernatant separated from the cell pellet. Malonate is precipitated from the fermentation broth using calcium chloride (Refer to Example 23). The resulting calcium malonate is then converted to diethyl malonate by adding excess ethanol and equimolar sulfuric acid to catalyse a Fischer esterification. Example 28: Production of acrylate from malonate and formaldehyde by Doebner modification of the Knoevenagel condensation

[0222] Acrylate was produced in accordance with the invention through the condensation of malonic acid with paraformaldehyde in pyridine. The reaction was conducted in a 3- necked round bottom flask with a magnetic stirrer. Fifteen ml pyridine and 15 ml toluene were added to the flask, and 10 g powdered malonic acid were added in 5 equal parts; subsequently, 1.1 equivalents (3.2 g) of paraformaldehyde was added to the reaction vessel over a thirty minute period. The mixture was stirred vigorously to promote solubilization of the components. The temperature of the reaction was started at 0°C and then increased over the course of several hours to 50°C until the formation of carbon dioxide, evidenced by the formation of bubbles, was observed. After 2 hours of heating at 50°C, the flask was allowed to return to room temperature and an aliquot of the reaction was diluted 100-fold in water and analyzed by HPLC using the method described in Example 2. The sample of malonate- derived acylic acid co-eluted with an authentic standard at an elution time of approximately 17.5 minutes (see Figure 2).

[0223] While the Doebner modification of the Knoevenagel reaction has been used to produce many compounds, its use in acrylate production is novel and important. Over a billion kilograms of acrylic acid are used annually to make wide a range of products from diapers to films and coatings. It is currently sourced largely from petroleum and production using the method described in this invention provides a partially or optionally fully renewable route to this commodity chemical, depending on the source of paraformaldehyde. In addition, malonic acid produced through the methods of this invention will be

substantially less expensive than its petroleum derived counterpart. This facilitates production of sustainable or partially sustainable acrylic acid at a cost competitive with the incumbent petrochemical route.

Example 29: Production of pentanedioic acid from malonate and formaldehyde

[0224] Biologically derived malonate is produced from recombinant S. cerevisiae in one L culture flasks as described in Example 8. The resulting fermentation broth is centrifuged (x6000g, 10 min) and the supernatant separated from the cell pellet. Malonate is precipitated from the fermentation broth using calcium chloride and converted to diethyl malonate. The biologically derived diethyl malonate is then reacted with formaldehyde in pyridine and worked up to the desired acid according to literature methods (Hedge et al. (1961) JOC 26:3166-3170). Example 30: Bio-catalytic production of malonate from various carbon sources.

[0225] In accordance with the invention, S. cerevisiae BY4741 yeast cells harboring a malonyl-CoA hydrolase expresson vector comprising a CYC1 terminator, an ampicillin resistance gene, a PMB1 origin of replication, a CEN/ARS origin of replication, and a URA3 selection marker were grown in yeast fermentation media comprising 5 g/L ammonium sulfate, lg/L monopotassium phosphate, 0.5 g/L magnesium sulfate, 0.1 g/L sodium chloride, 0.1 g/L calcium chloride, 2 mg/L inositol, 0.5 mg/L boric acid, 0.4 mg/L calcium pentothenate, 0.4 mg/L niacin, 0.4 mg/L pyridoxine hydrochloride, 0.4 mg/L thiamine HC1, 0.4 mg/L zinc sulfate, 0.4 mg/L manganese sulfate, 0.2 mg/L p-aminobenzoic acid, 0.2 mg/L riboflavin, 0.2 mg/L sodium molybdate, 0.2 mg/L ferric chloride, 0.1 mg/L potassium iodide, 40 µg/L copper sulfate, 2 µg/L folic acid, 2 µg/L biotin, 10 mg/L adenine, 50 mg/L L- arginine HC1, 80 mg/L L-aspartic acid, 20 mg/L L-histidine HC1, 50 mg/L L-isoleucine, 100 mg/L L-leucine, 50 mg/L L-lysine HC1, 20 mg/L methionine, 50 mg/L L-phenylalanine, 100 mg/L L-threonine, 50 mg/L L-tryptophan, 50 mg/L L-tyrosine, and 140 mg/L L-valine (the base media). In different fermentations, each of the following was used as a sole carbon source: 20 g/L glucose, 2% v/v ethanol, or 2% v/v glycerol. [0226] In this example, the F0PNG8-1 malonyl-CoA hydrolase (from Bacillus thuringiensis subsp. finitimus strain YBT- 020; UniProt ID F0PNG8, with E91S mutation) was used under control of the TEF promoter. One and one-half ml of base media supplemented with 2% of the carbon source in a 48-well plate was inoculated with 50 µĭ of a saturated culture of the producer strain for culture in triplicate. The culture plate was covered with a breathable membrane, incubated on a plate shaker at 30°C, and sampled for HPLC analysis of product accumulation after 138 h of growth.

[0227] HPLC analysis of malonate accumulation was conducted as described in Example 2. The results were as follows: glucose as carbon source: 4.8 mM +/- 0.2 mM (standard deviation) malonate; ethanol as carbon source: 7.5 mM +/- 0.8 mM malonate; and glycerol as carbon source: 1.7 mM +/- 0.1 mM malonate. These results show that the carbon sources tested were all suitable for use in production of malonate in accordance with the invention. Example 31: Construction and expression of recombinant plasmid vectors encoding various malonyl-CoA hydrolases, and their use in the production of malonate in yeast

[0228] Nucleic acids encoding various malonyl-CoA hydrolases provided by the invention were amplified by PCR from plasmids using the primers listed as follows: EHD3 (E124S) primers Y1-11_A13-R (SEQ ID NO:23)/ Y1-11_A13-F (SEQ ID NO:24); B9IZZ9-1 primers YO012 (SEQ ID NO:25)/YO013 (SEQ ID NO:26); F0PNG8-1 primers YO014 (SEQ ID NO:27)/YO015 (SEQ ID NO:28); C3ALI3-1 primers YO018 (SEQ ID NO:29)/YO019 (SEQ ID NO:30); Q81DR3-1 primers YO020 (SEQ ID NO:31)/YO021 (SEQ ID NO:32); A4XS22-1 primers YO024 (SEQ ID NO:33)/YO025 (SEQ ID NO:34); E2XN63-1 primers YO026 (SEQ ID NO:35)/YO027 (SEQ ID NO:36); A5W8H3-1 primers YO028 (SEQ ID NO:37)/YO029 (SEQ ID NO:38); and F6AA82-1 primers YO030 (SEQ ID NO:39)/YO031 (SEQ ID NO:40). The purified PCR products were cloned downstream of the TEF1 promoter and upstream of the CYC1 terminator in a shuttle vector containing an ampicillin resistance cassette, a PMB 1 origin of replication, a CEN/ARS origin of replication and a URA3 selection marker. The resulting plasmids were transformed into E. coli competent host cells and selected on LB agar plates containing Cb^{50}. Following overnight incubation at 37°C, individual colonies were inoculated in 2 ml of LB-Cb^{50} in a 48-well plate and grown for 5 h at 37°C on a shaker before the plasmids were isolated and confirmed by sequencing. Upon sequencing the construct containing protein F6AA82 (E95S), an unintended point mutation, Q348A, was found; this mutation was attributed to an error during PCR amplification. The resulting protein, F6AA82 (E95S/Q348A), is also referred to as F6AA82-2 herein. The Q348A point mutation was not shown to be necessary to obtain protein activity.

[0229] S. cerevisiae BY4741 cells were used as host for the vectors for expression of the various malonyl-CoA hydrolases. The plasmid vectors were individually introduced into the yeast host cells using standard procedures. Transformants were selected on agar plates of the media described in Example 30, containing 2% glucose as the carbon source.

[0230] The eight hydrolase-expressing S. cerevisiae strains were cultured as described in Example 22 and analyzed by HPLC as described in Example 2. The relative concentrations of malonate in the fermentation media were as follows (expressed as integrated area under the malonate peak; mean ± S.D.; n=4): S. cerevisiae BY4741 (negative control) 48,865+9,345; EHD3 (E124S) 94,721 ± 8,115; B9IZZ9 (E91S) 261,717 ± 38,012; F0PNG8 (E91S) 216,654 ± 31,145; F6AA82 (E95S/Q348A) 212,096 ± 29,338; E2XN63 (E95S) 198,046 ± 35,084; Q81DR3 (E91S) 193,665 ± 37,898; Q63BK8 (E91S) 167,477 ± 8,110; and A5W8H3 (E95S) 52,047 ± 9,042. The identifiers are the Uniprot ID (http://www.uniprot.org/) followed by the mutation provided by the invention to result in malonate production. No malonate was detected in samples consisting of medium not inoculated with yeast cells.

[0231] In additional examples, C3ALI3 from Bacillus mycoides and A4XS22 from Pseudomonas medocina (strain ymp) containing E101S and E95S mutations, respectively, were utilized as malonyl-CoA hydrolases. Because the media conditions were varied slightly by buffering to pH 4.0, F0PNG8-1 and F6AA82-1 were included for comparison; all other fermentation, sampling, and analytical conditions were as described above. The results were as follows: C3ALI3 (E101S) 10+1 mM, A4XS22 (E95S) 7+1 mM, F0PNG8 (E91S) 11+2 mM, and F6AA82 (E95S/Q348A) 23+2 mM malonate. In the absence of a malonyl-CoA hydrolase protein, S. cerevisiae cells did not produce detectable concentrations of malonate.

[0232] This example demonstrates, in accordance with the invention, malonate can be produced in a yeast host cell expressing an enzyme containing an Xi mutation conferring malonyl-CoA hydrolase activity. The E to S active site mutations common to all the mutant hydrolases used in this example can be utilized in other members of these enzyme classes to provide similar results.

Example 32: Precipitation of malonic acid from cells and fermentation broth using a monovalent cation

[0233] In this example, malonic acid was purified from the fermentation broth by precipitation with a monovalent cation. The monovalent cation was sodium. The purification methods were demonstrated using synthetic malonic acid exogenously added to fermentation broth. A culture of S. cerevisiae BY4741 was grown up, prepared and seeded with malonic acids as described in example 23. Next, sodium chloride, a representative monovalent cation, was employed for precipitating malonic acid from the clarified supernatant fraction. Either 2 or 4 molar equivalents (as compared to malonate concentration) of sodium chloride was added to the supernatant fraction of each sample at 25°C. The malonate concentration remaining in the supernatant and precipitate was then measured by HPLC as described in Example 2. The concentration of malonate remaining in the media seeded with 100 g/L malonate and treated two molar equivalents of sodium chloride varied by pH as follows: pH 5.5 = 103%, pH 6.0 = 96%, pH 6.5 = 71%, pH 7.0 = 74%, pH 7.5 = 86%. The concentration of malonate remaining in the media seeded with 100 g/L malonate and treated four molar equivalents of sodium chloride varied by pH as follows: pH 5.5 = 92%, pH 6.0 = 86%, pH 6.5 = 66%, pH 7.0 = 81 %, pH 7.5 = 86%.

Example 33: Purification of malonate from cells and fermentation broth using diethyl amine

[0234] In this example, malonic acid was purified from the fermentation broth by addition of diethyl amine. The purification method was demonstrated using commercially obtained malonic acid exogenously added to the fermentation broth. Yeast fermentation media was prepared and known quantities of malonic acid added as described in a previous example. Diethyl amine, a representative di-substituted amine, was employed for purifying malonic acid from the clarified supernatant fraction. Between 0.5 and 100 g/1 of diethyl amine was added to the supernatant fraction of each sample at 25°C. The metabolite concentration remaining in the supernatant and precipitate was then measured as previously described via HPLC, as described in Example 2. The concentration of malonate remaining in the media seeded with 100 g/L malonate treated with 4 equivalents of diethylamine per equivalent of malonate varied by pH as follows: pH 5.5 = 100%, pH 6.0 = 86%, pH 6.5 = 65%, pH 7.0 67%, pH 7.5 = 57%.

Example 34: Synthesis of pimelic acid

[0235] This example describes the synthesis of pimelic acid via the condensation of glutarate semialdehyde with malonate and catalytic hydrogenation. In a 250 ml round bottom flask, 10 grams of glutarate semialdehyde are dissolved in 40 ml of pyridine. Nine grams of malonic acid are added, and the mixture is heated, with stirring at 80°C for 5 hours. The pyridine is removed using rotoevaporation, and the resulting material, containing the intermediate hept-2-en-l-7-dioic acid, is redissolved in hexanol. A Pd/C catalyst is added and the resulting mixture is stirred under a hydrogen atmophere for 24 h. The

catalyst is removed by filtration through Celite; the solvent is removed under reduced pressure; and the resulting pimelic acid is purified using by silica gel flash chromatography.

[0236] Pimelic acid is a key component of Nylon 5,7, which is used in fermentation to supplement biotin auxotrophy as well as in the production of several plastics. Prior art methods for pimelic acid synthesis are costly and low yielding. The invention provides a new chemical synthesis for this important compound that may be derived from malonic acid as provided by the invention.

Example 35: Construction and expression of recombinant vectors encoding additional malonyl-CoA hydrolases, and production of malonate in yeast

[0237] In this example, E. coli malonyl-CoA:ACP transacylate FabD (SEQ ID NO:53) was mutated to contain one or more of the following amino acid changes at the indicated positions S92C, H201N, R117D, R117E, R117N, R117Y, R117G, R117H, Q11D, QUE, Q11N, Q11Y, Q11G, Q11H, L93A, L93V, L93I, L93F, L93S, L93G and was employed as a malonyl-CoA hydrolase in S. cerevisiae. The nucleic acid encoding E. coli FabD was PCR amplified from E. coli strain K12 using primer Fl (5'-ATGACGCAATTTGCATTTGTGTTCCC -3') and F2 (5'- TTAAAGCTCGAGCGCCGCT- 3')· The amplified gene was then mutated using standard methods and inserted into a shuttle expression plasmid under the control of the TEF1 promoter and upstream of the CYC1 terminator. This vector contains an ampicillin resistance cassette, a PMB 1 origin of replication, a CEN/ARS origin of replication, and a URA3 selection marker. The individual mutational combinations assayed are listed with the results below.

[0238] Individual colonies were inoculated into 1 ml of the media described in Example 30 containing 2% glucose as a carbon source. The cultures were incubated on a shaker at 30°C for 24 h, and 20 μΐ of these cultures were used to inoculate production cultures of 2 ml of the same media. The production cultures were covered with a breathable membrane, incubated with shaking at 30°C and sampled for HPLC analysis of product accumulation after 96 h and 168 h of growth.

[0239] No malonate was detected in samples consisting of medium not inoculated with yeast cells. Wild type yeast produced less than 0.1 mM malonate following 168 h fermentation. Expressing any of the four FabD variants produced malonate at levels higher then cells not expressing these proteins. Malonate accumulation after 96 and 168 h of fermentation using various engineered FabD malonyl-CoA-ACP transacylase enzymes expressed in S. cerevisiae were as follows: FabD S92C/L93V/R117H 96h = l.OlmM, 168h = 2.49mM; FabD L93I/R117Y 96h = 1.47mM, 168h = 2.48mM; FabD L93S/R117G 96h = l. l lmM, 168h = 2.89mM; FabD L93I/ R117Y 96h = 1.64mM, 168h = 3.47mM.

Example 36: Reactive extraction of malonic acid from water with trialkylamines in 1- octanol.

[0240] In this example malonic acid was purified from water by reactive extraction with three trialkylamines; tripropylamine, trihexylamine, and trioctylamine. The purification method was demonstrated using authentic malonic acid added to distilled water. Malonic acid was added to water to a final concentration of 100 g/1; the pH of the solution was approximately 1.5.

[0241] 250 ul of the aqueous malonic acid solution was mixed with 250 ul of an organic phase consisting of 25% v/v trialkylamine and 75% v/v 1-octanol. One sample was prepared without addition of the organic phase; this sample provided a measurement of the initial concentration malonic acid in each sample. Samples were mixed by inversion for 18 hours, centrifuged (xl 8,000 g) for 1 minute, and the aqueous phase sampled for analysis of malonic acid concentration by HPLC.

[0242] For HPLC analysis of malonic acid in the aqueous phase we employed a Shimadzu XR HPLC system equipped with a UV detector. 5 μΐ of each sample was injected into the system and separated with an Aminex HPX-87h fermentation-monitoring column (Bio-Rad, Hercules, CA). The mobile phase was de-ionized water (pH 1.95 with sulfuric acid), flow rate was 0.6 ml/min, oven temperature was 50C and, the UV detector monitored 210 nm. Samples containing were monitored for malonic acid elution at -10 minutes post injection.

[0243] 33+2.6% of the malonic acid was extracted with tripropylamine, 73+4.4% of the malonic acid was extracted with trihexylamine, and 89+11.9% of the malonic acid was extracted with trioctylamine (n=3). Therefore, long chain length trialkylamines are preferred over short-chain length trialkylamines to increase extraction efficiency. Of the trialkylamines used in this example, trioctylamine is preferred to the shorter-chain length trihexylamine and tripropylamine.

Example 37: Decreasing aqueous phase pH to increase reactive extraction of malonic acid using trialkylamines.

[0244] In this example malonic acid was purified from water at different pH values by reactive extraction with trioctylamine in 1-octanol. A stock solution of 100 g/1 malonic acid was first prepared in water. The stock solution was then separated into working samples that were adjusted to the desired pH. Because addition of base diluted the malonic acid concentration at each pH value both malonic acid concentration before and after reactive extraction were taken. Calculation of the difference in malonate concentration between the pre- and post-extracted samples provided the percent yield at each pH value tested.

[0245] The reactive extraction was performed as follows. 250 ul of the aqueous malonic acid solution was mixed with 250 ul of an organic phase consisting of 25% v/v trioctylamine and 75% v/v 1-octanol. All samples were mixed by inversion for 18 hours, centrifuged (xl 8,000 g) for 1 minute, and the aqueous phase sampled for analysis of malonic acid concentration by HPLC as described in Example #36.

[0246] The extraction efficiencies at each pH were as follows; pH 1.5, 70%; pH 2.26, 57%; pH 2.93, 45%; pH 4.05, 30%; pH 4.62, 23%; pH 5.0, 15%; pH 5.5, 5%; pH 6.0, 0%; and pH 7.0, 3%.

[0247] Extraction efficiency decreased with increasing pH, and the highest extraction efficiency was achieved at pH 1.5. Above pH 6.0 extraction efficiencies were negligible. Thus, it is preferred that the pH of the fermentation broth be below 2.0 when extracting malonic acid using a trialkylamine.

Example 38: Increasing trialkylamine concentration in an organic phase to increase reactive extraction of malonic acid from an aqueous solution.

[0248] In this example malonic acid was purified from water using a trialkylamine/1- octanol organic phase overlay containing different concentrations trioctylamine. The purification method was demonstrated using synthetic malonic acid added to distilled water to a final concentration of 100 g/1; the pH of the solution was approximately 1.5.

[0249] 250 ul of the aqueous malonic acid solution was mixed with 250 ul of an organic phase consisting of the indicated amount of trialkylamine (expressed as the mol fraction relative to malonic acid in the aqueous phase) in 1-octanol. One sample was prepared without addition of the organic phase and was used to provide the measurement of the initial malonic acid concentration in the aqueous phase. [0250] Samples were mixed by inversion for 18 hours, centrifuged (xl8,000 g) for 1 minute, and the aqueous phase sampled for analysis of malonic acid concentration by HPLC as described in Example #36.

[0251] A linear relationship between extraction efficiency and trioctylamine:malonate mol fraction was observed; specifically the linear relationship existed between trioctylamine:malonic acid mol fractions of 0 to 1. Above 1 molar ratio, 100% of the malonic acid was extracted into the organic phase. Thus, the amount of trialkylamine in the organic phase must be equimolar to the amount of malonic acid in the aqueous phase (e.g., fermentation broth) to maximize extraction yield. Ideally a greater than equimolar amount of trialkylamine will be added to the organic phase to compensate for decreased extraction efficiencies due to other organic acids and anions in the fermentation broth.

Example 39: Increasing malonic acid reactive extraction yield from aqueous solutions at different ionic strengths by using equimolar amounts of trioctylamine in 1-octanol.

[0252] In this example malonic acid was purified from water at different ionic strengths by reactive extraction with trioctylamine; by increasing the molar ration of trioctylamine in the organic phase to malonic acid in the aqueous phase to 1: 1 we were able to improve the malonic acid extraction efficiency.

[0253] Malonic acid was added to water to a final concentration of 100 g/1. The ionic strength was adjusted to the indicated concentration by addition of sodium chloride. The final pH of all samples following addition of malonic acid and sodium chloride was approximately pH 1.5.

[0254] 250 ul of the aqueous malonic acid solution was mixed with 250 ul of an organic phase consisting of trioctylamine in 1-octanol. The trioctylamine volumes were calculated such that the triocytylamine:malonic acid molar ratio was as indicated; the remainder of the volume was 1-octanol. One sample was prepared without addition of the organic phase; this sample provided a measurement of the initial malonic acid concentration. Samples were mixed by inversion for 18 hours at 18°C, centrifuged (xl8,000 g) for 1 minute, and the aqueous phase sampled for analysis of malonic acid concentration by HPLC as described in Example 36.

[0255] Ionic strength (mM concentration) impacted the extraction of malonic acid using 0.59, 1.07, 1.61, and 2.14 molar equivalents of trioctylamine as follows; at an ionic strength of 0, 63.5%, 98.6%, 99.5%, and 99.4% of malonic acid was recovered respectively; at an ionic strength of 75, 60.3%, 91.3%, 93.7%, and 93.6% of malonic acid was recovered respectively; at an ionic strength of 150, 55.9%, 85.8%, 89.5%,

and 89.1 % of malonic acid was recovered respectively; at an ionic strength of 225, 52.4%, 83.5%, 86.0%, and 85.9% of malonic acid was recovered respectively; at an ionic strength of 300, 46.5%, 79.4%, 83.1 %, and 82.6% of malonic acid was recovered respectively; at an ionic strength of 375, 44.3%, 76.7%, 79.9%, and 80.1 % of malonic acid was recovered respectively; at an ionic strength of 450, 43.3%, 72.8%, 77.0%, and 78.1 % of malonic acid was recovered respectively; at an ionic strength of 500, 43.1 %, 72.2%, 75.1 %, and 76.6% of malonic acid was recovered respectively.

[0256] Increasing the trioctylamine: malonic acid molar ratio to 1 : 1 increased the malonic acid extraction efficiency from the aqueous solution at all ionic concentrations tested. No meaningful improvements in malonic acid extraction efficiency were obtained by increasing the trioctylamine: malonic acid molar ratio above 1 : 1. When extracting malonic acid from aqueous solutions it is preferable to use a trioctylamine :malonic acid molar ratio of at least 1 : 1 , and if there are no other organic acids a molar ratio of exactly 1 : 1 is preferred. If contaminating organic acids are present, a molar ratio higher than 1 : 1 may be preferred in order to improve the malonic acid extraction efficiencies.

Example 40: Increasing malonic acid back extraction yields from trialkylamine/1- octanol organic phase by increasing ionic strength of aqueous phase.

[0257] In this example we demonstrate methods to back extract malonic acid from an organic phase consisting of a trioctylamine in 1-octanol and into an aqueous phase.

[0258] Malonic acid was added to water to a final concentration of 100 g/1 and extracted into an organic phase consisting of trioctylamine (used at a 1 : 1 molar ratio to malonic acid) dissolved in 1-octanol. 10 ml of aqueous solution was extracted into 10 ml of organic phase at 18°C for 18 hours. The organic phase was then separated by centrifugation (x4000g, 5 min). The aqueous phase was also sampled to quantify the non-extracted malonic acid concentration.

[0259] 250 ul organic phase were then mixed with 250 ul aqueous solutions at the given ionic strength sodium chloride or sodium hydroxide. Samples were mixed by inversion at 18°C for 18 hours. The samples were than centrifuged (xl8,000 g, 1 min] and the aqueous phase sampled for analysis of malonic acid concentration by HPLC as described for Example 36.

[0260] The data presented below was normalized to the malonic acid concentration that was back-extracted into distilled water. Addition of either 0.5 molar sodium chloride or sodium hydroxide increased the yields of the back extraction reaction over 25-fold. Addition of sodium chloride above 1.5 M decreased back extraction yields; however, with sodium hydroxide back extraction yields continued to improve with further addition of sodium hydroxide. Back extraction yields would be further increased by running back extraction reactions at an elevated temperature (i.e. above 18°C]. Milimolar concentration of NaCl and fold improvement: OmM, 1; 0.5mM, 27.3; ImM, 43.1; 1.5mM, 74.5; 2mM, 63.4; 2.5mM, 66.5; 3mM, 69.4; 3.5mM, 63.7; 4mM, 57.8; 4.5mM, 53.3; and 5mM, 45.2. Milimolar concentrations of NaOH and fold improvement: OmM, 1; 0.5mM, 33.6; ImM, 46.5; 1.5mM, 57.5; 2mM, 52.7; 2.5mM, 56.4; 3mM, 62.6; 3.5mM, 58.2; 4mM, 68.5; 4.5mM, 67; 5mM, 74.2.

Example 41: Purification of malonic acid from aqueous solution by esterification and subsequent phase separation

[0261] In this example we demonstrated esterification of malonic acid and ethanol to form diethyl malonate and subsequent phase separation of the diethyl malonate into a hexane organic phase.

[0262] 500 μΐ aqueous solutions containing malonic acid at 100 g/1 concentration, ethanol at the indicated concentration, and sulfuric acid at the indicated concentration were prepared. Malonic acid and ethanol were the substrates for forming diethyl malonate; sulfuric acid was added as the catalyst. An organic overlay of 250 μΐ hexane was added to each of the samples, they were mixed by inversion for 18 hours at 25 °C and atmospheric pressure, centrifuged (xl 8,000 g) for 1 minute, and the aqueous phase sampled for analysis of malonic acid concentration by HPLC as described in Example 36.

[0263] Consumption of malonic acid was measured by the decrease in malonic acid concentration in the aqueous phase. The baseline malonic acid concentration (i.e. 100% unreacted malonic acid) in the aqueous phase was established from a sample containing 0% v/v ethanol and 0% v/v sulfuric acid.

[0264] The addition of both ethanol and sulfuric acid to the reaction mixture was necessary to catalyze malonic acid esterification. The most preferable reaction conditions, as measured by percent consumption of malonic acid, were achieved at high ethanol and sulfuric acid concentrations. Namely, greater than 40% v/v ethanol (7.14 molar ratio to malonic acid) and greater than 10% v/v sulfuric acid (1.94 molar ratio to malonic acid) were necessary to catalyze near complete consumption of malonic acid. The results of this demonstration are as follows: esterification reactions were conducted with 5, 10, 20, 30, 40, and 50% (V/V) ethanol. The malonic acid remaining in the aqueous phase was as follows: with 5% (V/V) sulfuric acid 78.9%, 61.6%, 43.2%, 43.2%, 28.2%, and 16.6%; 10%(V/V) sulfuric acid 83.5%, 70.3%, 49.5%, 28.6%, 16.6%, and 8.5%; 5%(V/V) sulfuric acid 71.8%, 53.2%, 28.1%, 11.8%, 4.4%, and 0.6%, respectively. The no acid control showed 95.1%, 93.2%, 85.8%, 69.1%, 63.0%, and 87.3% malonate remaining in the respective solutions with ethanol. The addition of acid with no ethanol present resulting in the following concentrations of malonic acid remaining in the aqueous phase: 96.1%, 92.7%, and 84.4% for 5, 10, and 20% (V/V) sulfuric acid, respectively.

Example 42: Improving malonate biosynthesis in engineered yeast through increased acetyl-CoA carboxylase activity

[0265] In addition to the methods, vectors, and host cells for expression of a heterologous malonyl-CoA hydrolase and in vivo production of malonate, the invention also provides methods and host cells for improved titer, yield, and/or productivity of malonate. In one aspect, malonate production is improved by increasing the biosynthesis of malonyl-CoA. Malonyl-CoA is the penultimate intermediate in the biosynthesis of malonate from acetyl- CoA, and in S. cerevisiae, this reaction is catalyzed by acetyl-CoA carboxylase (ACC1).

[0266] In this example, the ACC from the yeast Yarrowia lipolytica CLIB 122 (NCBI Reference Sequence: XP_501721) was back- translated to a DNA sequence using standard codon optimization

tables for expression in S. cerevisiae. The resulting in the YIACC DNA sequence is included below as SEQ ID NO:ll.

[0267] DNA encoding 50 nucleotide of upstream S. cerevisiae FAAl homolog followed by 300 base pairs of the S. cerevisae TEF1 promoter sequence followed by the YIACC sequence (SEQ ID NO: 11) and finally 50 nucleotides of downstream S. cerevisiae FAAl were synthesized as a single piece of DNA from oligonucleotides using standard protocols and amplified by PCR. The resulting, flanked, YIACC PCR product and a linear DNA construct containing the selectable URA3 gene and flanking FAAl homologous sequences, to direct recombination into the FAAl site on the chromosome, were co-transformed into BY4741 containing 2 copies of malonyl-CoA hydrolase F6AA82-2 provided by the invention (strain LYM004), using a standard lithium acetate transformation procedure, and selected for by plating on Synthetic Defined medium containing 2% glucose (SD) and lacking uracil (-Ura) plates. Clones and corresponding genomic DNA were isolated and verified by PCR to contain the YIACC integration into FAAl.

[0268] For in vivo malonate production, one individual colony for each transformant picked was inoculated in a 50 μΐ aliquot of SD-Ura medium in a 96-well plate. LYM004 was grown for comparison of yeast lacking expression of a heterologous YIACC. The plate was incubated on a shaker at 30°C for approximately 4 h and 25 μΐ of these pre-cultures were used to inoculate fermentation plates. In this example, 2 ml of SD medium in 48-well plates was used for the fermentation. The fermentation plates were covered with a breathable membrane, incubated on a plate shaker at 30°C and sampled for HPLC analysis of product accumulation after 72 and 120h of fermentation. HPLC analysis of malonate accumulation in the fermentation broth was conducted as described in example 36. [0269] No malonate was detected in samples consisting of medium only. LYM004 expressing Y1ACC produced 1.94 and 2.43-fold more malonate at 72 and 120h, respectively, than LYM004 alone. The highest concentration of malonate measured in the fermentation media were observed in samples collected after 120 h of fermentation.

Example 43: Production of malonate in S. cerevisiae using engineered F6AA82 malonyl- CoA hydrolase

[0270] In this example all twenty proteogenic amino acid point mutations were introduced at position E95 in protein F6AA82 (Q348A). Amino acid position 95 was believed to interact with the terminus of malonyl-CoA, and mutation of E95 would introduce malonyl-CoA hydrolase activity into the protein.

[0271] F6AA82 (Q348A) mutants at position 95 (i.e., all amino acids) were constructed using standard methods and cloned into a yeast plasmid containing a uracil auxotrophic marker, CEN/ARS origin of replication, TEF promoter and CYC terminator. The F6AA82 mutants were cloned behind the TEF promoter and directly upstream of the CYC terminator. All plasmids contained an ampicillin resistance marker and ColEl origin for propagation of the plasmid in E. coli.

[0272] Plasmids were transformed into S. cerevisiae using a lithium acetate procedure and transformants were selected on SD -Uracil agar plates at 30°C. Twelve colonies of each mutant were inoculated into pre-cultures of 500 μΐ SD -Ura medium in a 96-well plate and were incubated ~16h with shaking at 30°C.

A 5 μΐ aliquot of these pre-cultures was used to inoculate 96-well production plates containing 500 μΐ RD4 -Ura (IX YNB, 3X SC supplement, 2% glucose, 75 mM succinic acid buffer pH 4.0, uracil dropout) media. The production plates were incubated at 30°C with shaking for 3 days before sampling the fermentation broth. The samples were clarified by centrifugation and filtered on a 0.45 μιη membrane prior to HPLC analysis. HPLC analysis of malonate accumulation in the fermentation broth was conducted as described in example 36.

[0273] The production from each F6AA82 mutant (containing mutation Q348A in addition to the described E95 mutations), under these conditions, was as follows: F6AA82 (E95N) 8.03+0.14 mM, F6AA82 (E95S) 4.18+0.61 mM, F6AA82 (E95Y) 3.87+0.30 mM, F6AA82 (E95A) 2.33+0.50 mM, F6AA82 (E95K) 1.65+0.23 mM, F6AA82 (E95T) 1.16+0.62 mM, F6AA82 (E95D) 0.75+0.27 mM, F6AA82 (E95F) 0.17+0.13 mM, F6AA82 (E95V) 0.11+0.32 mM, F6AA82 (E95L) 0.11+0.12 mM, F6AA82 (E95G) 0.00+0.01 mM, F6AA82 (E95P) -0.31+0.78 mM, F6AA82 (E95R) -0.32+0.69 mM, F6AA82 (E95) -0.32+0.40 mM, F6AA82 (E95W) -0.34+0.73 mM, F6AA82 (E95Q) -0.37+0.74 mM, F6AA82 (E95H) - 0.53+0.70 mM, F6AA82 (E95C) -0.57+0.69 mM, F6AA82 (E95I) -0.64+0.81 mM, and F6AA82 (E95M) -0.77+0.79 mM. Negative concentrations of malonate were due to the production titers falling below the detectable level of the HPLC and indicate the absence of malonate production.

[0274] F6AA82 (Q348A) proteins containing mutations E95S, E95Y, E95T, E95N, E96K, E95V, and E95D produced significantly (t-test, $p<0.05$) more malonate than the F6AA82 (Q348A) protein. F6AA82 proteins containing these mutations are suitable for the hydrolysis of malonyl-CoA and production of malonate. F6AA82 mutations E95S and E95N are preferred for the hydrolysis of malonyl-CoA and production malonate.

[0275] E95Y, E95T, E95K, E95V, and E95D produced significantly (t-test, $p<0.05$) more malonate than the wild type F6AA82 protein. F6AA82 proteins containing these mutations are suitable for the hydrolysis of malonyl-CoA and production of malonate.

Example 44: Impact of promoter selection on the production of malonate in 5. cerevisiae

[0276] In this example, an expression plasmid backbone comprising a CEN/ARS origin of replication, a PMB 1 origin of replication, an ampicillin resistance marker, an URA3 marker and an hph marker was amplified from plasmid Y20. The malonyl-CoA hydrolase F6AA82-2 flanked by the CYC1 terminator was amplified by PCR from plasmid Yl- F6AA82-2 and assembled with the backbone using standard techniques. The resulting plasmid, pPLIB0-Q9I, was used as a backbone for cloning of the different promoters selected.

[0277] Ninety-six reference genes were selected from S. cerevisiae strain BY4741. Promoter sequences to these genes were generated by PCR amplifying, using S. cerevisiae strain BY4741 genomic DNA as template, nucleic acid fragments corresponding to approximately 750 base pairs immediately upstream of their open reading frame, including the start codon of the gene of interest. Using standard cloning techniques, these promoter sequences were cloned immediately upstream of F6AA82-2 (Start codon at

3739 in SEQ ID NO: 12) in pPLIB0-Q9I. The resulting plasmids were propagated in E. coli and the presence of the desired promoter was verified by sequencing.

[0278] The plasmids described above were used to transform S. cerevisiae BY4741 using standard lithium acetate procedures. Transformants were selected on agar plates of CSM medium without uracil at 30°C. One transformant harboring each plasmid was inoculated in a pre-culture plate consisting of 300 ul of RD -Ura medium (2X YNB, 3X SC-U, 2% glucose) in a 96-well plate. The pre-culture plate was incubated at 30°C approximately 20 h with shaking, and 30 μΐ of these pre-cultures was used to inoculate production plates consisting of 1.8 ml of RD4 -Ura medium (2X YNB, 3X SC-U, 2% glucose, 75 mM succinate buffer pH 4.0) in 48-well culture plates (1 production well for each pre-culture). The plates were incubated at 30°C with shaking for 144h before sampling the fermentation broth. The samples were clarified by centrifugation and filtered on a 0.45 μιη membrane prior to HPLC analysis.

[0279] HPLC analysis of malonate accumulation in the fermentation broth was conducted as described in example 36. Millimolar malonate concentration in the fermentation broth after 144h of fermentation with different promoters driving expression of F6AA82-2 are as follows: HSP150 8.8; PGK1 8.6; PH05 8.5; SCT1 7.8; PRB 1 7.6; TPI1 7.1 ; ACH1 7; HXK2 6.9; ACOl 6.9; JEN1 6.9; MDH2 6.8; POX1 6.6; CITl 6.6; ALD4 6.6; ADH1 6.5; TDH3 6.4; ADH2 6.4; SDH1 6.4; TDH1 6.1 ; MLS1 6.1 ; RPN6 6; GLK1 5.9; POT1 5.8; HSP26 5.8; FBA1 5.7; LPD1 5.7; CYC1 5.5; COX5a 5.5; TEF1 5.5; SHH4 5.5; GND2 5.5; TPS1 5.5; MDH1 5.4; PDC1 5.4; HXK1 5.3; TDH2 5.3; IDH2 5.3; DDR2 5.2; SLT2 5.2; EN02 5.1 ; COX6 5; CHOI 4.9; PH03 4.9; PFK1 4.9; ACS1 4.9; GUT2 4.8; PHM7 4.8; CIT2 4.7; ACS2 4.7; ALD2 4.5; IDH1 4.5; IDP2 4.5; FBP1 4.3; PH012 4.3; PDC5 4.2; PFY1 4.1 ; GDH1 4.1; PEX13 4.1 ; ICT1 4.1 ; YSA1 4; KGD2 3.9; GIM5 3.9; GAP1 3.9; DBP5 3.8; STE5 3.7; BI02 3.7; PDC6 3.6; HXT5 3.6; REG1 3.6; TPS 3 3.5; BI05 3.3; PH08 3.3; IRC7 3.2; GPM1 3.2; ALA1 3.1 ; KGD1 3.1 ; MIG1 3.1 ; YKT6 2.9; SN04 2.8; ARA1 2.8; PDR10 2.7; YBR139W 2.3; ERR2 2.2; CRC1 1.9; TSL1 1.3; ENOl 1.2; PFK2 1.1 ; RPL6A 1.1 As many of these promoters are unnamed, the letter number code in front of each number indicates the gene immediately downstream of the cloned promoter, in S. cerevisiae strain BY4741.

[0280] This example demonstrates that a wide variety of promoters can be used to control expression of a malonyl-CoA hydrolase to produce malonate in vivo in yeast. Furthermore, 40 different promoters resulted in titers of at least 5 mM malonate, and 7 promoters resulted in production of at least 7 mM in this experiment.

Example 45: Integration of a malonyl-CoA hydrolase encoding gene to the 5. cerevisiae genome for the production of malonate.

[0281] In this example, S. cerevisiae strain BY4741 is used as a host strain, for the genomic integration of the malonyl-CoA hydrolase afforded by the invention. Three sites on the S. cerevisiae genome were chosen for integration of the synthetic nucleic acid constructs in this strain and the three linear nucleic acids cassettes to be integrated each carried unique homology sites to target integration and a unique selectable marker encoded upstream of the malonyl-CoA hydrolase. In all three cases nucleic acid encoding the malonyl-CoA hydrolase F6AA82 (E95S/Q348A) flanked by the TEF1 promoter and the CYC1 terminator was utilized as the expression portion of the cassette.

[0282] In two cases the solo long terminal repeats YPRCdeltal5 and YORWdelta22, non- coding DNA of the chromosome, were targeted for integration. In the third case, a malonyl- CoA hydrolase cassette provided by the invention (F6AA82 (E95S/Q348A)) was integrated in place of the BUD21 gene. The sequences of these linear insertion cassettes are included below as SEQ ID NO:41, SEQ ID NO:42, and SEQ ID NO:43. The unique integration sites and selectable markers are YPRCdeltal5 (HIS3), YORWdelta22 (LEU2), and BUD21 (URA3), respectively.

[0283] These nucleic acid cassettes were constructed and transformed into S. cerevisiae BY4741 using standard protocols. Transformants were selected on agar plates made of SC medium with appropriate amino acid dropouts at 30°C. Several transformants for each construction were re-streaked on selective agar plates. Transformants were then screened and verified by production of malonate and by PCR. The following strains resulted from these integrations: LYM002 contains 1 copy of F6AA82 (E95S/Q348A) integrated at YPRCdeltal5 (HIS3); LYM004 carries 2 copies of F6AA82 (E95S/Q348A), one at YPRCdeltal5 (HIS3) the second at YORWdelta22 (LEU2); LYM007 is derived from LYM004 and has a third copy of F6AA82 (E95S/Q348A) integrated at the BUD21 locus.

[0284] For fermentation and malonate production, S. cerevisiae BY4741 carrying plasmid Y1-F6AA82 (E95S/Q348A) served as a positive control. Pre-cultures of 500 μΐ of RD4 medium in a 96-well plate were incubated 16-20h with shaking at 30°C. A 5 μΐ aliquot of these pre-cultures was used to inoculate 96-well production plates containing 500 μΐ of RD4 medium (IX YNB, 3X SC supplement, 2% glucose, 75 mM succinic acid buffer pH 4.0). The production plates were incubated at 30°C with shaking for 120h before sampling of the fermentation broth. The samples were prepared and analyzed by HPLC as described in Example 36.

[0285] A standard curve established with an authentic standard was used to determine malonate concentration in the fermentation broth. The malonic acid production levels from these fermentations were as follows S. cerevisiae BY4741 + Y1-F6AA82 (E95S/Q348A), 2.6+0.4 mM; LYM002, 3.2+0.2 mM; LYM004, 4.6+0.2 mM; LYM007, 7.7+ 0.3 mM.

[0286] This example illustrates the benefits of genomic integration of a malonyl-CoA hydrolase encoding gene provided by the invention in that a single integrated copy of the gene results in higher malonic acid titers than the same gene expressed from a plasmid. This example also serves to demonstrate modulation of malonic acid by the invention via the number of malonyl-CoA hydrolase encoding genes are present in the cell.

Example 46: Expression of acetyl-CoA synthetases for increased production of malonate from an engineered host cell.

[0287] Acetyl-CoA synthetases (ACSs) are common to many organisms. These enzymes produce acetyl-CoA using acetate and adenosine triphosphate (ATP) as substrates. In accordance with this invention, these enzymes can be used to convert acetate from any source, including endogenous metabolism, feedstock hydrolysis, or by feeding, into acetyl- CoA, the penultimate precursor in malonyl-CoA

biosynthesis. By increasing acetyl-CoA levels, one can increase malonyl-CoA, and hence, malonate, in host cells provided by the invention.

[0288] An illustrative ACS suitable for this purpose can be obtained from the bacteria Salmonella enterica. To express the S. enterica ACS (SeACS) in S. cerevisiae, the amino acid sequence was back-translated to a DNA sequence using codon optimization tables, and one residue determined to be involved in decreasing enzymatic activity was mutated (L641P). The resulting codon optimized DNA sequence provided by the invention is SEQ ID NO:44.

[0289] Nucleic acids encoding the SeACS 1 (L641P) were synthesized de novo from oligonucleotides and inserted into a yeast plasmid, Yl, under the control of the TEF1 promoter from S. cererivisiae using standard protocols. This plasmid also contains a CEN/ARS origin and a gene for conferring uracil prototrophy. Clones of LYM004 containing this plasmid were selected by plating on SD-uracil plates and verified to contain the SeACS 1(L64 IP) gene by sequencing.

[0290] LYM004 was used as host for the expression of the SeACS 1(L64 IP) acetyl-CoA synthetase. For in vivo malonate production, one individual colony for each transformant picked was inoculated in a 50 μΐ aliquot of SD-Ura medium in a 96-well plate. LYM004 was grown for comparison of yeast lacking expression of a heterologous SeACSl(L641P), but containing a Yl empty vector. The plate was incubated on a shaker at 30°C for approximately 48h, and 50 μΐ of these pre-cultures were used to inoculate fermentation plates. In this example, 500 uL of RD4U medium in 96-well plates were used for the fermentation. The fermentation plates were covered with a breathable membrane, incubated on a plate shaker at 30°C, and sampled for HPLC analysis of product accumulation after 120h of fermentation. HPLC analysis was conducted as described in Example 36.

[0291] No malonate was detected in samples consisting of medium not inoculated with yeast cells. Under the conditions tested, LYM004 expressing SeACS 1 produced 1.86-fold more (5.5+0.62 mM) malonate at 120h than LYM004 with an empty Yl control alone, thus illustrating the improvements in malonate production provided by this embodiment of the invention.

Example 47: Utilization of different carbon sources for production of malonate using a modified strain of Pichia kudriavzevii.

[0292] Strain LPK3003 was derived from Pichia kudriavzevii strain Y-134 (obtained from the USDA Agricultural Research Services, Peoria, IL) by genomic integration of a nucleic acid cassette encoding the hph hygromycin phosphotransferase (conferring resistance to hygromycin B) driven by the PkTEFl promoter, and the F6AA82 (E95S/Q348A) malonyl- CoA hydrolase driven by the PkTDHl promoter.

[0293] Seed cultures of LPK3003 were grown for 16-20h in YPD medium with shaking at 30°C. This seed culture was used to inoculate 1.1X YNB, which was then aliquoted at 450 μΐ per well in a 96-well plate. 50 μΐ of solutions of various carbon sources (glucose, sucrose, ethanol, glycerol or acetate from sodium acetate) were added to each well (in triplicate) to a final concentration of 2% (w/v). The plate was

incubated at 30°C with shaking for 115 h and the fermentation broth was sampled. Samples were prepared and analyzed by HPLC as described in Example 36.

[0294] In this example, there was little or no growth observed for sucrose or acetate carbon sources, and malonate accumulation was negligible in these samples. As described above, host cells can be modified to confer or enhance sucrose and/or acetate catabolism by introducing a sucrose invertase and/or acetyl-CoA synthase (ACS), respectively. The other carbon sources tested in this example resulted in malonate accumulation (average replicates ± S.D.): 4.6 ± 0.5 mM from glucose, 5.38 ± 0.05 mM from glycerol and 3.7 ± 0.3 mM from ethanol.

[0295] These results demonstrate that a variety of carbon sources can be used to produce malonate from an engineered Pichia kudriavzevii strain expressing a malonate-CoA hydrolase. Notably, glycerol provided the highest titers in this example.

Example 48: Bio-reactor based production of malonate.

[0296] In this example, yeast strain LYM004 (see Example 45 for construction details) was grown in fed-batch control in a 0.5 L bioreactor. A single colony of LYM004 was isolated from a SC plate and cultured in 5 mL of RD4 media (see Example 43 for recipe). The culture was maintained at 30°C overnight, shaking at 200 rpm. The 4 mL of culture was used to inoculate 50 mL of fresh RD4 media in a 250 mL non-baffled flask and grown overnight at 30°C, 200 rpm. The time zero OD 600 nm absorbance was 0.304. After overnight growth (16h), this culture was used to inoculate 1 L of RD4 media. This culture was split into 2 separate 500 mL aliquots and added to two separate bioreactors. Both fermentations were maintained at 30°C, with a single impeller run at 400 rpm, and sparge rate of 1 vessel volumes per minute (VVM) using compressed air. The cultures were grown overnight (21h) to allow for glucose consumption prior to starting the fed-batch phase. The feed (recipe below) was delivered for 2s, every 980s. 0.5 mL samples were taken daily and analyzed for production of malonic acid. After 4 days, the cultures had accumulated 34 mM of malonic acid and reached an OD 600nm of 16.2. After 9 days, the cultures had accumulated 116 mM of malonic acid and reached an OD 600nm of 52.1.

[0297] The batch feed media consisted of 17g/L Difco YNB; 50 g/L ammonium sulfate; 49.8 g/L Synthetic Complete (SC) supplement lacking histidine, methionine, and leucine; 2.57 g/L methionine; 8.85g/L succinic acid, and 20 g/L glucose; the pH o was adjusted to 4.0.

[0298] This protocol was repeated a strain, LYM007, that expressed 3 copies of a malonyl- CoA hydrolase (see Example 45). In this example, a single impeller was run at 700 rpm and the composition of the feed was as follows: 68 g/L YNB (Sigma), 16.6 g/L SC-his-met-leu (Sunrise Science), 1.284 g/L methionine, 75 mM succinate buffer (pH 4.0), 400 g/L glucose. The feed rate used was cycle 5s on every 980s. 0.2 mL samples were taken daily and analyzed for production of malonic acid, acetic acid, succinic acid, pyruvic acid via HPLC. Growth was monitored by measuring optical density at 600nm (OD600). The OD600 and malonate concentration from each time point was as follows: 18h, OD = 6.9, 3.2 mM; 45 h, OD = 13.8, 14.0 mM; 69h, OD = 16.6, 24.2 mM; 88.5h OD = 24.4, 31.1 mM; 111.8h, OD = 36.9, 47.8 mM.

[0299] In a comparative example, LPK3003 (see Example 47) was grown in fed-batch control in a 0.5 L fermenter. A single colony of LPK3003 was isolated from a YPD plate and cultured in 5 mL of YPD media. The culture was grown at 30°C overnight, shaking at 200 rpm. The 5 mL of culture was used to inoculate 50 mL of fresh minimal media, containing 6.8 g/L YNB (Difco) and 2 g/L glucose and grown overnight at 30°C, 200 rpm. This culture was then used to inoculate 500 mL of 6.8 g/L YNB (Difco) media, 2 g/L glucose and grown in a 1 liter fermenter. Temperature was maintained at 30°C, a single impeller was run at 700 rpm, and sparge rate was set to 1 vessel volumes per minute (VVM) using compressed air. The cultures were grown overnight for 21h to allow for glucose consumption prior to starting the fed-batch phase. A feed, containing 68 g/L YNB and 400 g/L of glucose was initiated by setting the fermenter feed to cycle on every 980s for 5s. 200 uL samples were taken daily and analyzed for growth (OD600) and production of malonic acid (as described in Example 36). Foaming was controlled by addition of 200 uL of antifoam at every sampling interval. pH was measured and adjusted to 4.5 with 10N NaOH at every sampling interval. Production of malonic acid continued beyond 111 h, accumulating 36.4 mM of malonic acid. Importantly, this is the first demonstration of production without addition of amino acids, uracil, or adenine. The OD600, malonate concentration, and pH from each time point was as follows: 18h, OD = 2.1, 0.7 mM, pH = 2.3; 45h, OD = 12.2, 8.8 mM, pH = 2.0; 69h, OD =12.8, 18.1 mM, pH = 2.0; 88.5h, OD = 27.6, 22.9 mM, pH = 2.7; 111.8h, OD = 40.1, 36.4 mM, pH = 2.4; 194h, OD = 60.6 , 94 mM, pH = 3.3. [0300] While the titer of malonate was higher from LYM007, the conditions under which LPK3003 can be grown (minimal media, low pH) and the fact that it contains only a single copy of F6AA82-2 malonyl-CoA hydrolase make this a superior strain for additional engineering efforts.

Patent Citations (4)

Publication number	Priority date	Publication date	Assignee	Title
Family To Family Citations				
DE69707055D1	*1996-05-22	2001-11-08	Nippon Paint Co Ltd	A process for the preparation of acrylate monomers containing malonate
DE19752041A1	1997-11-24	1999-05-27	Huels Chemische Werke Ag	A process for the C-alkylation of malonic esters
JP2012525156A	*2009-04-30	2012-10-22	ゲノマチカ, インク.	Isopropanol, microorganisms for the production of n- butanol, and isobutanol
KR20120068021A	*2009-09-09	2012-06-26	게노마티카 인코포레이티드	Microorganisms and methods for the co-production of isopropanol with primary alcohols, diols and acids

* Cited by examiner, † Cited by third party

Cited By (4)

Publication number	Priority date	Publication date	Assignee	Title
Family To Family Citations				
EP3161142A1	*2014-06-26	2017-05-03	Lygos, Inc.	Recombinant host cells for the production of malonate
US20160230198A1	*2015-02-10	2016-08-11	GeneSys Consulting, LLC	Methylmalonic acid compositions, biological methods for making same, and microorganisms for making same
WO2016130597A1	*2015-02-10	2016-08-18	GeneSys Consulting, LLC	Methylmalonic acid compositions, biological methods for making same, and microorganisms for making same

KR101828551B1 *2016-01-112018-02-13한국과학기술원Recombinant Variant Microorganism Having a Producing Ability of Malonic Acid and Method for Preparing Malonic Acid Using the Same
* Cited by examiner, † Cited by third party, ‡ Family to family citation

Similar Documents

Publication	Publication Date	Title
US20100105955A1	2010-04-29	Methods and compositions for producing fatty aldehydes
US8530207B2	2013-09-10	Photosynthetic microorganisms comprising exogenous prokaryotic acyl-ACP thioesterases and methods for producing fatty acids
US20110125118A1	2011-05-26	Production of an Organic Acid and/or Related Chemicals
Litsanov et al.	2012	Efficient aerobic succinate production from glucose in minimal medium with Corynebacterium glutamicum
US20110171702A1	2011-07-14	Recombinant cell producing 2-hydroxyisobutyric acid
US8765433B2	2014-07-01	Alcohol dehydrogenases (ADH) useful for fermentive production of lower alkyl alcohols
US20110165644A1	2011-07-07	Production of alkenes by enzymatic decarboxylation of 3-hydroxyalkanoic acids
WO2009023493A1	2009-02-19	Methods and organisms for the growth-coupled production of 1,4-butanediol
WO2009113853A2	2009-09-17	Adipate (ester or thioester) synthesis
US20100298612A1	2010-11-25	Engineered biosynthesis of fatty alcohols
WO2012094425A2	2012-07-12	Biological methods for preparing adipic acid
WO2013003744A2	2013-01-03	Bioconversion process for producing nylon-7, nylon-7,7 and polyesters
WO2009135074A2	2009-11-05	Microorganisms for the production of methacrylic acid
WO2012052427A1	2012-04-26	Production of alkenes by combined enzymatic conversion of 3-hydroxyalkanoic acids
US20120077252A1	2012-03-29	Biological methods for preparing adipic acid
Krivoruchko et al.	2015	Microbial acetyl-CoA metabolism and metabolic engineering
WO2010111344A2	2010-09-30	Methods and microorganisms for production of c4-dicarboxylic acids
WO2010076324A1	2010-07-08	Method for the preparation of diols
WO2013096898A2	2013-06-27	Bioconversion process for producing nylon-7, nylon-7,7 and polyesters
WO2009076480A2	2009-06-18	Methylbutanol as an advanced biofuel
WO2011029166A1	2011-03-17	Microorganisms and process for producing n-propanol
US8765425B2	2014-07-01	In situ expression of lipase for enzymatic production of alcohol esters during fermentation
WO2010023206A1	2010-03-04	Enzymatic production of 2-hydroxy-isobutyrate (2-hiba)
WO2007047680A2	2007-04-26	Increasing the activity of radical s-adenosyl methionine (sam) enzymes
Jensen et al.	2013	Ornithine cyclodeaminase-based proline production by Corynebacterium glutamicum

Priority And Related Applications

Priority Applications (3)

Application	Priority date	Filing date	Title
US201216074792	2012-03-06	2012-03-06	*US Provisional Application*
US201216191122	2012-04-02	2012-04-02	*US Provisional Application*
PCT/US2013/029441	2012-03-06	2013-03-06	Recombinant host cells for the production of malonate

Legal Events

DateCodeTitleDescription
2015-01-14AKDesignated contracting states:
Kind code of ref document: A1

Designated state(s): AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR

2015-01-14AXRequest for extension of the european patent to
Extension state: BA ME

2015-01-1417PRequest for examination filed
Effective date: 20141002

2015-06-10DAXRequest for extension of the european patent (to any country) deleted
2015-10-28RIC1Classification (correction)
Ipc: C12N 1/19 20060101AFI20150924BHEP
Ipc: C07C 67/08 20060101ALI20150924BHEP
Ipc: C12N 1/21 20060101ALI20150924BHEP
Ipc: C07C 67/31 20060101ALI20150924BHEP
Ipc: C12N 9/16 20060101ALI20150924BHEP
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Ipc: C07C 51/38 20060101ALI20150924BHEP
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Ipc: C12N 1/19 20060101AFI20160125BHEP
Ipc: C07C 51/38 20060101ALI20160125BHEP
Ipc: C12N 1/21 20060101ALI20160125BHEP
Ipc: C12N 9/16 20060101ALI20160125BHEP
Ipc: C07C 67/31 20060101ALI20160125BHEP
Ipc: C07C 67/08 20060101ALI20160125BHEP
Ipc: C12P 7/46 20060101ALI20160125BHEP
2016-03-02RA4Despatch of supplementary search report
Effective date: 20160129

2017-01-2517QFirst examination report
Effective date: 20170103

Data provided by IFI CLAIMS Patent Services

21.2 Composition of YNB and YPD

Composition of Yeast Nitrogen Base (YNB)

Ingredient	**µg/L**
Biotin	2
Calcium Pantothenate	400
Folic Acid	2
Inositol	2,000
Niacin	400
p-Aminobenzoic acid	200
Pyridoxine hydrochloride	400
Riboflavin	200
Thiamine hydrochloride	400
Boric Acid	500
Copper Sulfate	40
Potassium Iodide	100
Ferric Chloride	200
Manganese Sulfate	400
Sodium Molybdate	200
Zinc Sulfate	400
Potassium Phosphate monobasic	1,000,000
Magnesium Sulfate	500,000
Sodium Chloride	100,000
Calcium Chloride	100,000

Composition of Yeast Extract Peptone Dextrose (YPD)

Ingredient	**g/L**
Peptic Digest of Animal Tissue	20
Yeast Extract	10
Dextrose	20

21. 3 Equation Sheet

Cell Growth and Heat

$$ln\frac{OD}{OD_o} = \mu_{net}t \quad (1)$$

$$\tau_D = \frac{\ln 2}{\mu_{net}} = \frac{0.693}{\mu_{net}} \quad (2)$$

$$\frac{\Delta H_s}{Y_{x/s}} = \Delta H_{cell} + \frac{1}{Y_H} \quad (3)$$

$$Q_{GR} = V_T \mu_{net} X \frac{1}{Y_H} \quad (4)$$

$$OUR = q_{O_2} X = \frac{\mu_g X}{Y_{X/O_2}} = \frac{\frac{dX}{dt}}{Y_{X/O_2}} \quad (5)$$

Heat Exchanger

$$Q = mC_p \Delta T \quad (6)$$

$$Q = UA \Delta T_{LM} \quad (7)$$

$$\Delta T_{LM} = \frac{\Delta T_1 - \Delta T_2}{ln\frac{\Delta T_1}{\Delta T_2}} \quad (8)$$

Power and Energy

$$P_{mixer} = k\mu n^2 D^3 \quad (9)$$

k=mixing constant

$\mu = fluid\ dynamic\ viscosity$

n= revolutions per second

D= impeller diameter

$$P_{pump(kW)} = \frac{q\rho hg}{3.6 * 10^6} \quad (10)$$

Q= flow capacity

$\rho = density\ of\ fluid$

G= gravity

H=differential head

21.4 Calculations for Mass, Energy, Utilities, and Sizing

21.4.1 Number of Cells in Fermentation Vessels

Fermentor	**Size [L]**	**Fermentation Time [hr]**	**Growth Rate [1/hr]**	**Final Number of Cells [g]**	**Initial Number of Cells [g]**
Fed Batch Fermentation	200,000	50	0.1	8640233.618	58217.43618
Seed 3	25000	16	0.2	58217.43618	2373.071009
Seed 2	2500	16	0.3	2373.071009	19.52977413
Seed 1	250	16	0.3	19.52977413	0.160725101

21.4.2 Energy for Agitation of Fermentation Vessels

	SF1	**SF2**	**SF3**	**PF**
Oxygen required for cells [mmol]	45,692	5,552,086	13,700,836	2,105,508,623
Time [hr]	16	16	16	50
Volume of Vessel [L]	250	2500	25000	200000
OUR	11	139	34	211
Working Volume [m^3]	0.2	2	20	160
Energy [kw]	-	-	18.75	596
Energy [kJ]	-	-	18000	572160
Energy [hp]			25	799

21.4.3 Energy from Heat of Reactions and Heat Exchangers for Fermenters

*All these numbers were taken from NIST	**Heat of Formation (kJ/mol)**		**saccharomyces**		
Gluocse	174		yx/s [g/mol]	90	
Oxygen	0		yx/o2 [g/g]	0.97	
Co2	393.5		delta Hc [kj/g cell]	21.2	
H20	-285.83		delta Hs glucose [g/kj]	0.1004784689	
Malonic Acid	-891		Yh	-0.04717229549	
Succinic Acid	-939				
Ethanol	-277.7				

	Rxn 1 (kj)	Rxn 2 (kj)	Rxn 3 (kj)	Overall Rxn for B	Overall Rxn for A
per rxn	-3555.48	322.41	-5000.49	-3290.2695	-1678.420789
per mol glucose	-1777.74	161.205	-1000.098		
	SF1	**SF2**	**SF3**	**BF**	
total amount glucose [g]	39.51763164	4801.80392	177740.5693	27314706.46	
total amount glucose [mol]	0.219542398	26.67668845	987.4476072	151748.3692	
RX 1 [kJ]	-78	-9485	-1579883	-242792231	
RX 2 [kJ]	1	129	4775	733878	
RX 3 [kJ]	-4	-534	-19751	-3035265	
Rx 4 [kJ]	-108	-12778	-17743	-1078484	
total energy [kJ] pos	189	22667	1630601	246744262	
total energy [kJ]	-189	-22667	-1612601	-246172102	
25% oftotal energy	-	-	407650.3311	61686065.44	
delta T	4				
cp of water (kJ/kgK)	4.18				
	SF1	**SF2**	**SF3**	**BF**	
amount of cooling water required (kg)	11.31420037	1355.684611	97524.00267	14757431.92	
Amount of cooling water required in L	11.3	1355.7	97524.0	14757431.9	
Time (min)	960	960	960	3000	
flow rate (L/m)	0.0118	1.412	102	4919	
Volume of inside tank	250	2500	25000	200000	
Volume of outside tank	261.3	3855.7	122524.0	14957431.9	
Amount of cooling water required [L/hr]	0.707137523	84.73028817	6095.250167	295148.6385	

U value [btu/Fft2hr]	100				
Tin	25				
Tout	29				
T liquid	30				
delta Tlm	4.106674673	39.39201441			
	SF1	**SF2**	**SF3**	**BF**	
Area (ft2)	-	-	6.13	927.64	

21.4.4 Energy for Sterilization Heat Exchangers and Coolers

Heating Fluid		**Cooling Fluid**		
Fluid original temp [C]	86	Fluid original temp [C]	60	
Fluid Temp Needed to Heat [C]	121	Fluid Temp Needed to Heat [C]	30	
Steam		Cooling Water		
Steam orignial temp [C]	170	Water original temp [C]	25	
Steam final temp [C]	130	water final temp [C]	29	
Cp [j/gc]	1.996	Cp [j/g C]	4.18	
	YNB Media			
Cp[j/gC]	4.18			
m SF1 [g]	0			
m SF2 [g]	0			
m SF3 [g]	15169848.94	33443.75237		
m BF [g]	157303688.7	346794.8582		
delta T heating	35			
delta T cooling	-30			
Amount of Steam Required				
	YNB Media			
SF3	27797456.16	61282.82781		
BF	288245612	635472.041		

Amount of Cooling Water Required				
	YNB Media			
SF3	113773867.1	250828.1428	old amount of steam needed	866860986.9
BF	1179777665	2600961.437	reduction	2.742857143
			old amount of water needed	3923772982
			reduction	3.033333333
	steam	**cooling water**		
Tlm [C]	46.45516255	14.25009459		
Tlm [F]	115.6192926	57.65017027		
U value [btu/Fft2hr]	100			
Area of Heat Transfer for Heater				
	YNB Media			
SF3	181.9357797			
BF	1886.58235			

21.4.5 Sterilizer Energy for First Two Fermenters

Heating Fluid		**Cooling Fluid**	
Fluid original temp [C]	25	Fluid original temp [C]	121
Fluid Temp Needed to Heat [C]	121	Fluid Temp Needed to Heat [C]	30
Steam		Cooling Water	
Steam original temp [C]	170	Water original temp [C]	25
Steam final temp [C]	130	water final temp [C]	29
Cp [j/gc]	1.996	Cp [j/g C]	4.18

	YPD Media
Cp[j/gC]	4.18
m SF1 [g]	189198.5167
m SF2 [g]	1893695.104
m SF3 [g]	0
m BF [g]	0
delta T heating	96
delta T cooling	-91
Amount of Steam Required	
	YPD Media
SF1 [g]	950921.6028
SF2	9517810.262
SF3	0
BF	0
Amount of Cooling Water Required	
	YPD Media
SF1 [g]	4304266.254
SF2	43081563.61
SF3	0
BF	0
	Steam
Tlm [C]	73.47730886
Tlm [F]	164.259156
U value [btu/Fft^{2}hr]	100
Area of Heat Transfer for Heater	
	YPD Media
SF1 (ft^{2}hr)	4.380851147
SF2	43.84810471
SF3	0
BF	0

Area of Heat Transfer for Cooler	
	YPD Media
SF1 [ft^{2}hr]	7.952758285
SF2	79.59945824
SF3	0
BF	0

21.4.6 Acid Base Chemistry for controlling the pH of the Fermenters

	Malonic Acid	**Succinic Acid**	
1st proton	2.83	4.2	pKa
2nd proton	5.69	5.6	pKa
Desired pH	3	2.6	
	Seed 3	**Batch Fermenter**	
Malonic Acid [g]	5.45066437	17874999.98	
Malonic Acid [mol]	0.05241023433	171874.9998	
Succinic Acid [g]	0.1101483709	360555.5554	
Succinic Acid [mol]	0.0009334607702	3055.555554	
Total volume [L]	6,575.01	177,202.32	
Malonic Acid [M]	0.000007971129918	0.9699365014	
Succinic Acid [M]	0.0000001419710705	0.01724331559	
Base Concentration for Malonic Acid [M]	0.00001179016512	0.5711409549	
Base Concentration for Succinic Acid [M]	0.000000008957768963	0.0004331325046	
Total Base Conc [M]	0.00001179912289	0.5715740874	
MW of Base	40	40	
Based Needed [g]	**3.10**	**4.05E+03**	
Conc of NaOH in water	10	10	
Water required [g]	310.3172584	**4.05E+05**	

21.4.7 Energy for the Upstream Pumps

	Pump 1	**Pump 2**	**Pump 3**	**Pump 4**	
Volume Exiting (L)	216.1	2,214.6	17,388.9	169,834.1	
Volume Exiting (gal)	57.1	585.0	4,593.7	44,865.4	
Pump capacity (gpm)	3.0	30.0	100.0	400.0	
Time to transfer fluids (min)	19.0	19.5	45.9	112.2	
Power [kw]	2.79E-02	0.5	3.8	15.3	5,180.44
Energy [kJ]	3.19E+01	579	10529	102833	26,897,421.74
Flow capacity [m^3/hr]	0.6818181818	6.818181818	22.72727273	90.90909091	
Density [kg/m^3]	1000	1000	1000	1000	
Gravity [m/s^2]	9.81	9.81	9.81	9.81	
differential head [ft]	15.0388212	26.62798465	61.68135464	61.68135464	

21.4.8 Sizing of Storage Tanks

	YPD Media	**YNB Media**	**NaOH**	**Surge Tank**	
Water [g]	2077932.457	144369076	4.05E+05	-	
Other Materials	0	612,014.63	4,054.47	-	
Volume in Tank [L]	2088.374329	145440.3197	409.78	-	
Tank Size [L]	2401.630478	167256.3677	471.2417388	300000	
Power for mixing the tank [kw]	6.608786479	517.7864934	1.296759039	-	
Annual Energy for Mixing [kj]	1570247.667	123026070.8	308109.9477		124904428.4
Volume of tank [ft3]	84.81285986	5906.608448	16.64176063	10594.41	
L/D	3	4	3	7	
D [ft]	3.301795646	12.34230013	1.918651268	12.4440845	
L [ft]	9.905386939	37.0269004	5.755953804	37.33225351	
mixing constant	1.3	1.3	1.3	-	
fluid dynamic viscosity [Ns/m^2]	798	798	798	-	
Revolutions per second	5	5	5	-	
Impeller Diamerer [m]	0.5031936565	1.88096654	0.2924024532	-	
Number of Impellers	2	3	2		

	1.7	6.2	1.0		

21.4.9 Calculations for Mass Balance of Vacuum Belt Filter 1

Vacuum Belt Filter 1				Percent Liquid Lost	0.0325	
In	**Weight (g)**	**Volume (L)**	**Weight (lb)**	**Volume Lost (L)**	**Weight Lost (g)**	**Weight Lost (lb)**
Cells/Biomass	8,640,233.62		19,048.43			
Malonic Acid	21,444,947.84	13,237.62	47,277.96	430.22	696,960.80	1,536.53
Succinic Acid	432,624.00	277.32	953.77	9.01	14,060.28	31.00
Ethanol	316,218.82	404.91	697.14	13.16	10,277.11	22.66
Water	154,812,608.18	155,489.17	341,302.97	5,053.40	5,031,409.77	11,092.35
YPD (minus glucose)	109.84		0.24			
YNB	554,638.26		1,222.77			
RO Recycle Water	29,869,464.00	30,000.00	65,850.82	975.00	970,757.58	2,140.15
RO Recycle Malonic	259,200.00	160.00	571.44	5.20	8,424.00	18.57
RO Recycle Ethanol	3,904.85	5.00	8.61	0.16	126.91	0.28
RO Recycle Succinic	5,460.00	3.50	12.04	0.11	177.45	0.39
Total	216,339,409.42	199,577.52	476,946.19			
Total without Wash	186,201,380.57	169,409.02	410,503.29			
Total Fluid Recycled	30,138,028.85	30,168.50	66,442.90			

Assuming 20% Wet Cake	
Liquid	0.20
Cake Percent	0.80
Total Cake Weight (g)	10,800,292.02
Fluid in Cake (g)	2,160,058.40

OUT			
Stream 1	**Volume (L)**	**Weight (g)**	**Weight (lb)**
Biomass		8,640,233.62	19,048.43
Lost Liquid	6,486.27	6,732,193.90	14,841.93
YPD (minus glucose)		109.84	0.24
YNB		554,638.26	1,222.77
Total		15,927,175.62	35,113.37
Stream 2	**Volume (L)**	**Weight (g)**	**Weight (lb)**
Malonic Acid	12,962.20	20,998,763.04	46,294.29
Succinic Acid	271.70	423,846.27	934.42
Ethanol	396.58	309,719.65	682.81
Water	179,460.77	178,679,904.84	393,921.29
Total	193,091.25	200,412,233.80	441,832.82

Timing	
15,927,175.62	g per batch
35,113.37	lb per batch
17.56	tons per batch
0.07	% of a day to process
1.76	hours to run full belt
105.34	minutes

21.4.10 Calculations for Mass Balance of Reverse Osmosis System

Reverse Osmosis	Percentage water removed	0.63	
In	**Weight (g)**	**Volume (L)**	**Weight (lb)**
Malonic Acid	20,998,763.04	12,962.20	46,294.29
Succinic Acid	423,846.27	271.70	934.42
ethanol	309,719.65	396.58	682.81
Water	178,679,904.84	179,460.77	393,921.29
Total	200,412,233.80	193,091.25	441,832.82
	Amount lost in water	0.05	
	Volume Lost (L)	**Weight Lost (g)**	**Weight Lost (lb)**
Malonic Acid	648.11	1,049,938.15	2,314.71
Succinic Acid	13.58	21,192.31	46.72
Ethanol	19.83	15,485.98	34.14
Out			
Stream 1	**Volume (L)**	**Weight (g)**	**Weight (lb)**
Water	82,701.37	82,341,516.24	181,531.75
Malonic Acid	488.11	790,738.15	1,743.28
Ethanol	14.83	11,581.13	25.53
Succinic Acid	10.08	15,732.31	34.68
Total	83,214.39	83,159,567.84	183,335.25
Stream 2	**Volume (L)**	**Weight (g)**	**Weight (lb)**
Malonic Acid	12,314.09	19,948,824.89	43,979.58
Succinic Acid	258.11	402,653.96	887.70
Ethanol	376.75	294,233.66	648.67
Water	96,759.41	96,338,388.60	212,389.54
Total	109,708.36	116,984,101.11	257,905.49
Stream 3 (25% Recycle)	**Volume (L)**	**Weight (g)**	**Weight (lb)**
Water	30,000.00	29,869,464.00	65,850.82
Malonic Acid	160.00	259,200.00	571.44

Ethanol	5.00	3,904.85	8.61
Succinic Acid	3.50	5,460.00	12.04
Total	30,168.50	30,138,028.85	66,442.90

Timing	
400.00	gpm
1,514.16	L/min
90,849.84	L/hr
2.13	hr

21.4.11 Calculations for Mass Balance of Crystallizer

Crystallization			
In	**Weight (g)**	**Volume (L)**	**Weight (lb)**
Malonic Acid	19,948,824.89	12,314.09	43,979.58
Succinic Acid	402,653.96	258.11	887.70
Ethanol	294,233.66	376.75	648.67
Water	96,338,388.60	96,759.41	212,389.54
Total	116,984,101.11	109,708.36	257,905.49
Out	**Weight (g)**	**Volume (L)**	**Weight (lb)**
Solidified Malonic Acid	19,838,958.30	12,246.27	43,737.36
Malonic Acid	109,866.58	67.82	242.21
Succinic Acid	402,653.96	258.11	887.70
Ethanol	294,233.66	376.75	648.67
Water	96,338,388.60	96,759.41	212,389.54
Total	97,145,142.81	97,462.09	214,168.12

Solubility at 15C	1.13	g/L	
		13,452.78	L/hr
Liters Fluid	97,394.27	15,000.00	L Crystallizer
g malonic acid dissolved	109,866.58		
g malonic left	19,838,958.30		

% Solidified	0.99		

Timing	
6.00	hours
1	Residence Time (hr)

21.4.12 Calculations for Mass Balance of Vacuum Belt Filter 2

Vacuum Belt Filter 2			
IN (g)	**Weight (g)**	**Volume (L)**	**Weight (lb)**
Malonic Acid Solids	19,838,958.30	12,246.27	43,737.36
Malonic Acid	109,866.58	67.82	242.21
Succinic Acid	402,653.96	258.11	887.70
Ethanol	294,233.66	376.75	648.67
Water	96,338,388.60	96,759.41	212,389.54
Water Wash	5,973,892.80	6,000.00	13,170.16
Total with Wash	122,957,993.91	115,708.36	271,075.65
Total Without Wash	116,984,101.11	109,708.36	257,905.49
Total Fluid Recycle	5,973,892.80	6,000.00	13,170.16
Amount Liquid Lost	0.076		
	Volume Lost (L)	**Weight Lost (g)**	**Weight Lost (lb)**
Malonic Acid	5.15	8,349.86	18.41
Succinic Acid	19.62	30,601.70	67.47
Ethanol	28.63	22,361.76	49.30
Water	7,353.72	7,321,717.53	16,141.60
Water Wash	456.00	454,015.85	1,000.93
OUT			
Stream 1	**Volume (L)**	**Weight (g)**	**Weight (lb)**
Malonic Acid Solids	12,246.27	19,838,958.30	43,737.36
Malonic Acid	5.15	8,349.86	18.41

Succinic Acid	19.62	30,601.70	67.47
Ethanol	28.63	22,361.76	49.30
Water	7,809.72	7,775,733.39	17,142.54
Total	20,109.39	27,676,005.01	61,015.07
Stream 2	**Volume (L)**	**Weight (g)**	**Weight (lb)**
Malonic Acid	62.66	101,516.72	223.81
Succinic Acid	238.50	372,052.26	820.23
Ethanol	348.12	271,871.91	599.37
Water	94,949.69	94,536,548.01	208,417.16
Total	95,598.97	95,281,988.90	210,060.58

Assuming 20% Wet Cake	
Wet Percentage	0.20
Cake Percent	0.80
Solid Cake	4,959,739.58
Amount of fluid	19,838,958.30

Timing	
27,676,005.01	g per batch
61,015.07	lb per batch
30.51	Tons per batch
0.13	% of a day to process
3.05	hours to run full belt

21.4.13 Calculations for Mass Balance of Fluidized Bed Dryer

Fluidized Bed Dryer			
In			
Stream 1	**Volume (L)**	**Weight (g)**	**Weight (lb)**
Malonic Acid Solid	12,246.27	19,838,958.30	43,737.36
Malonic Acid	5.15	8,349.86	18.41
Succinic Acid	19.62	30,601.70	67.47
Ethanol	28.63	22,361.76	49.30
Water	7,809.72	7,775,733.39	17,142.54
Total	20,109.39	27,676,005.01	61,015.07
Air	900,000.00	1,102,500.00	2,430.59
Assume purity	0.990		
Stream 2	**Volume (L)**	**Weight (g)**	**Weight (lb)**
Malonic Acid Solid	12,246.27	19,838,958.30	43,737.36
Stream 2	**Volume (L)**	**Weight (g)**	**Weight (lb)**
Malonic Acid	5.15	8,349.86	18.41
Succinic Acid	19.62	30,601.70	67.47
Ethanol	28.63	22,361.76	49.30
Water	7,809.72	7,775,733.39	17,142.54
Air	900,000.00	1,102,500.00	2,430.59
Total	907,863.12	8,939,546.71	19,708.30

21.4.14 Energy for Downstream Pumps

	Pump 5	**Pump 6**	**Pump 7**	**Pump 8**	**Pump 9**
Volume Exiting (L)	169,834.12	193,091.25	30,168.50	109,708.36	6,000.00
Volume Exiting (gal)	44,865.42	51,009.30	7,969.67	28,981.88	1,585.03
Pump capacity (gpm)	400.00	400.00	100.00	200.00	30.00
Time to transfer fluids (min)	112.16	127.52	79.70	144.91	52.83
Time to transfer fluids (sec)	6,729.81	7,651.40	4,781.80	8,694.56	3,170.06
Energy [kw]	2.48	2.48	0.62	1.24	0.19
Energy (kJ)	16,671.58	18,954.59	2,961.46	10,769.40	588.98
Flow capacity [m^3/hr]	90.91	90.91	22.73	45.45	6.82
Density [kg/m^3]	1,000.00	1,000.00	1,000.00	1,000.00	1,000.00
Gravity [m/s^2]	9.81	9.81	9.81	9.81	9.81
differential head [ft]	10.00	10.00	10.00	10.00	10.00

21.4.15 Calculations for Cooling Water in Crystallizer

Heat of Crystallization of Adipic Acid	
272.16	J/g
0.27216	kJ/g
28.32137784	kJ/mol of malonic

Malonic Acid Cooling	
Heat of Solidification (kJ/mol)	28.321
mass of batch (kg)	116,984.101
	Crystallizer
mol of malonic acid solidified	190,646.476
total energy [kJ] pos	5,399,370.891
total energy kJ	5,399,370.891
delta T	-15.000
Cp of water (kJ/kgK)	4.180
increase in batch temp from crystallization	11.042
Complete delta T	-26.042
Energy to cool water needed	-12,734,274.031
amount of cooling water required (kg)	116,984.101
Amount of cooling water required in L	116,984.101
Time (min)	360.000
flow rate (L/m)	324.956
Volume of inside tank	15,000.000
Volume of outside tank	131,984.101
Amount of cooling water required [L/hr]	19,497.350
U value [btu/Fft^{2}hr]	100.000

21.4.16 Energy Balance of Fluidized Bed Dryer

Dryer	
Volume Exiting (L)	900,000.000
Volume Exiting (gal)	237,754.800
Time to transfer (min)	300.000
gpm	792.516
force (lbs)	2,430.594
Area	5,462.534
Pressure (PSIG)	0.445
HP	0.206
Energy (kW)	0.153
Energy (kJ)	2,761.544

21.4.17 Energy Balance of Vacuum Belt Filters and RO System

	Vacuum 1	**RO**	**Vacuum 2**
Volume Exiting [L]	193,091.25	193,091.25	95,598.97
Volume Exiting [gal]	51,009.30	51,009.30	25,254.57
Pump capacity [gpm]	400.00	400.00	100.00
Time to transfer fluids [min]	127.52	127.52	252.55
Energy [kw]	5.61	1.71	1.09
Energy ([kJ]	42,932.15	13,110.26	16,563.41
Flow capacity [m^3/hr]	90.91	90.91	22.73
Density [kg/m^3]	1,000.00	1,000.00	1,000.00
Gravity [m/s2]	9.81	9.81	9.81
differential head [ft]	22.65	6.92	17.65
	Motor 1		Motor 2
Area (ft^2)	1,059.00		1,059.00
Distance (ft)	60.00		60.00
Cake thickness (in)	6.00		6.00
Cake thickness (ft)	0.50		0.50
amount of cake per 60 feet (ft^3)	529.50		529.50
L processed	199,577.52		115,708.36
ft3 processed	7,048.02		4,086.21
number of rotations	13.31		7.72
Time (hr)	1.76		5.00
Time (min)	105.34		300.00
rpm	0.13		0.03
% L process per rotation	0.08		0.13
grams processed each rotation	16,253,034.40		15,933,179.98
lb processed	35,831.76		35,126.61
Velocity	7.58		1.54
Horsepower	1.23		0.25
Energy (kw)	0.92		0.18
Energy (kJ)	5,819.87		3,307.76
Total System Energy (kW)	6.53	1.71	1.28
Total System Energy (kJ)	48,752.02	13,110.26	19,871.18

21.4.18 Sizing of Downstream Vessels

	Crystallizer	**Dryer**	**RO Storage**
Volume (L)	15,000.00	3,000.00	35,000.00
Volume (ft3)	529.72	105.94	1,236.01
L/D ratio	3.00	3.00	3.00
D (ft)	6.08	3.56	8.06
D actual (ft)	6.08	3.56	8.06
L actual (ft)	18.24	10.67	24.19

	Vacuum Belt Filters
Area (ft^2)	1,059.00
Length (ft)	60.00
Width (ft)	17.65

21.4.19 Total Energy, Water, and Steam Requirements

Upstream:

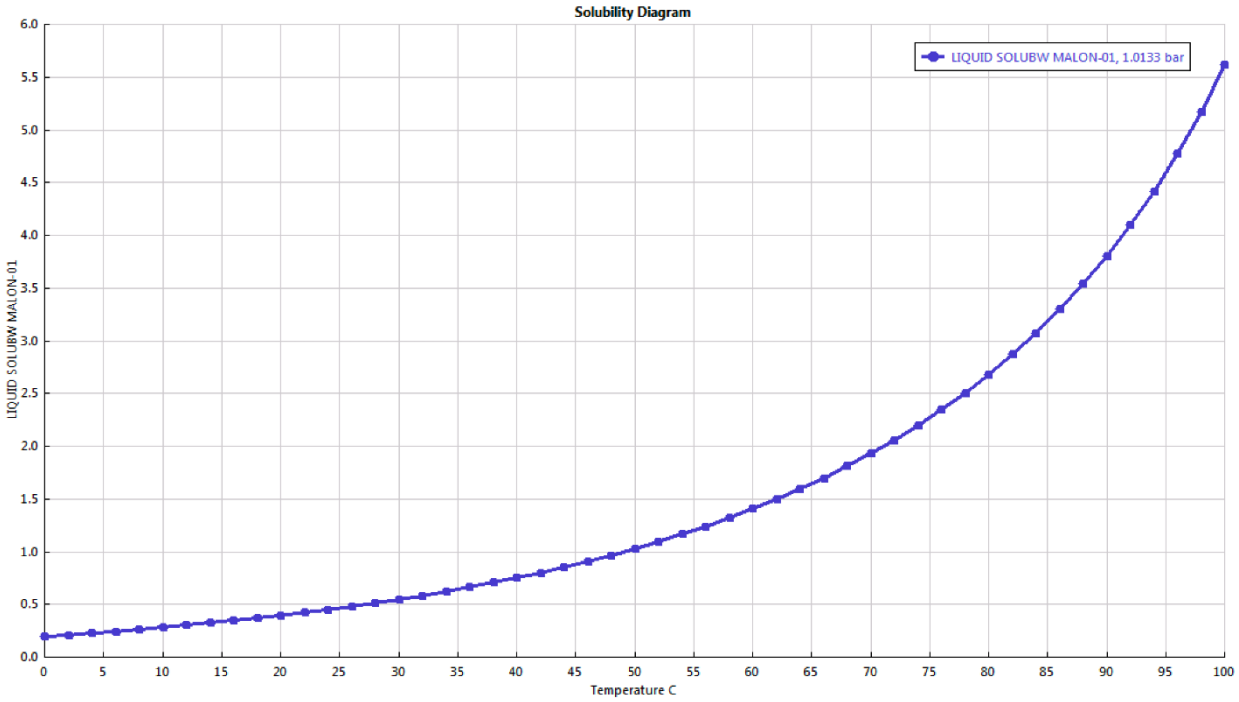
Energy	**kJ/yr**
scrubber	1,270,000,000
air filtration	221,719,680
Pumps for Ferm	26,897,422
agitation of ferm	139,000,000
mixing tanks	124,904,428
	1,782,521,530
Water	**L/yr**
sterilization	316,461,218
cool fermenter to keep at constant temp	
raw material	34,657,147
Steam	**lb/yr**
sterilization	169880928.9

Downstream:

Energy	**kJ**
VBF 1	11,505,478
RO	3,094,021
VBF 2	4,689,597
Dryer	651,724
Downstream Pumps	11,787,261
Water	L
Displacement Wash	1,416,000
cool Crystallizer	27,608,248

21.5 Solubility Curve

Solubility Curve of malonic acid in water. This curve was used to determine the amount of malonic acid solidified during the crystallization step.



21.6 Equipment Specification Sheets

21.6.1 Fermenters

Seed Fermenter One					
Identification:	Item		Seed Fermenter 1		
	No. Required		3		
Function:	Grow yeast cells				
Operation:	Batch				
Stream ID:	Inlet: *Lab Fermenter*	Inlet: *YPD Media 1*	Inlet: *Air 1*	Outlet: *Gas 1*	Outlet: *Broth 1*
Temperature(ºC)	30	30	30	30	30
Flow Rate (lb/batch)	35	417	616	615	453
Glucose	0	0.087	0	0	0
Oxygen	0	0	143	142	0
Nitrogen	0	0	473	473	0
Cells	0.00035	0	0	0	0.043
Water	35.3	417	0	0	452
Malonic Acid	0	0	0	0	0.015
Succinic Acid	0	0	0	0	0.001
Ethanol	0	0	0	0	0.001
CO2	0	0	0	0.025	0
NaOH	0	0	0	0	0
YPD	0	0.022	0	0	0.022
Design Data:	Fermentation Time (hours)				16
	Volume (L)				250
	Diameter (ft)				1.6
	Height (ft)				4.7
	Growth rate (hr^{-1})				0.3
	Working Volume (L)				216
	Oxygen Uptake Rate (mmol/hrL)				11
	Material				316SS Mill Finish
	Jacket and Agitator Included				

Heat generated (kj/batch):	189
Total Purchase Cost:	$1,650
Bare Module Cost:	$5,297

Seed Fermenter Two

Identification:	Item		Seed Fermenter 2		
	No. Required		3		
Function:	Grow yeast cells				
Operation:	Batch				
Stream ID:	Inlet: *Broth 1*	Inlet: *YPD Media 2*	Inlet: *Air 2*	Outlet: *Gas21*	Outlet: *Broth 2*
Temperature (ºC)	30	30	30	30	30
Flow Rate (lb/batch)	450	4,170	6,160	6,150	4,630
Glucose	0	10.6	0	0	0
Oxygen	0	0	1,430	1,420	0
Nitrogen	0	0	4,730	4,730	0
Cells	0.043	0	0	0	5.23
Water	452	4,160	0	0	4,620
Malonic Acid	0.015	0	0	0	1.85
Succinic Acid	0.001	0	0	0	0.168
Ethanol	0.001	0	0	0	0.123
CO2	0	0	0	3.01	0
NaOH	0	0	0	0	0
YPD	0.022	0.22	0	0	0.242

Design Data:	Fermentation Time (hours)	16
	Volume (L)	2,500
	Diameter (ft)	3.3
	Height (ft)	10.0
	Growth rate (hr^{-1})	0.3
	Working Volume (L)	2,220
	Oxygen Uptake Rate (mmol/hrL)	139
	Material	316SS
		Mill Finish
	Jacket and Agitator Included	

Heat generated (kj/batch): 22,700

Total Purchase Cost:	$16,500
Bare Module Cost:	$52,965

Seed Fermenter Three

Identification:	Item No.	Seed Fermenter 3 3				
Function:	Grow yeast cells and produce malonic acid					
Operation:	Batch					
Stream ID:	Inlet: *Lab Fermenter*	Inlet: *Sterile Media 1*	Inlet: *Air 3*	Inlet: *10M NaOH 2*	Outlet: *Gas 3*	Outlet: *Broth 3*
Temperature (ºC)	30	30	30	30	30	30
Flow Rate (lb/batch)	35	417		616	615	453
Glucose	0	392	0	0	0	0
Oxygen	0	0	14,400	0	14,050	0
Nitrogen	0	0	47,300	0	47,300	0
Cells	5.23	0	0	0	0	128
Water	4,620	32,900	0	0.684	0	37,700
Malonic Acid	1.85	0	0	0	0	307
Succinic Acid	0.168	0	0	0	0	6.33
Ethanol	0.123	0	0	0	0	4.63
CO2	0	0	0	0	145	0
NaOH	0	0	0	0.007	0	0.0068
YPD	0.242	0	0	0	0	0.24
YNB	0	126	0	0	0	126

Design Data:	Fermentation Time (hours)	16
	Volume (L)	25,000
	Diameter (ft)	7.2
	Height (ft)	21.6
	Growth rate (hr^{-1})	0.2
	Working Volume (L)	17,400
	Oxygen Uptake Rate (mmol/hrL)	34
	Material	316SS Mill Finish
	Jacket, Heat Exchanger, and Agitator Included	

Heat generated (kj/batch): Utilities Required (kJ/batch):	1,630,000 18,000
Total Purchase Cost: Bare Module Cost:	$165,000 $529,650

Production Fermenter

Identification:	Item No.	Required	Production Fermenter 3			
Function:	Grow yeast cells and produce malonic acid					
Operation:	Batch					
Stream ID:	Inlet: *Lab Fermenter*	Inlet: *Sterile Media 1*	Inlet: Air 3	Inlet: *10M NaOH 2*	Outlet: *Gas 3*	Outlet: *Broth 3*
Temperature (ºC)	30	30	30	30	30	30
Flow Rate (lb/batch)	4,620	33,400	61,700	1	61,500	38,200
Glucose	0	392	0	0	0	0
Oxygen	0	0	14,400	0	14,050	0
Nitrogen	0	0	47,300	0	47,300	0
Cells	5.23	0	0	0	0	128
Water	4,620	32,900	0	0.684	0	37,700
Malonic Acid	1.85	0	0	0	0	307
Succinic Acid	0.168	0	0	0	0	6.33
Ethanol	0.123	0	0	0	0	4.63
CO2	0	0	0	0	145	0
NaOH	0	0	0	0.007	0	0.0068
YPD	0.242	0	0	0	0	0.24
YNB	0	126	0	0	0	126

Design Data:		
	Fermentation Time (hours)	16
	Volume (L)	200,000
	Diameter (ft)	12.6
	Height (ft)	56.7
	Growth rate (hr^{-1})	0.1
	Working Volume (L)	170,000
	Oxygen Uptake Rate (mmol/hrL)	211
	Material	316SS
		Mill Finish
	Jacket, Heat Exchanger, and Agitator Included	

Heat generated (kj/batch): Utilities Required (kJ/batch):	246,700,000 572,160
Total Purchase Cost: Bare Module Cost:	$1,320,000 $4,237,200

21.6.2 Pumps

Pump 1		
Identification:	Item	Centrifugal Pump
	No. Required	3
Function:	Pump Broth 1 from Seed Fermenter 1 to Seed Fermenter 2	
Operation:	Continuous	
Stream ID:	*Broth 1*	
Temperature (ºC)	30	
Flow Rate (gpm)	3	
Design Data:	Run Time (hours)	0.32
	Head (ft)	15
	Material	Stainless Steel
Utilities Required (kJ/hr)		101
Total Purchase Cost:		$3,372
Bare Module Cost:		$11,128

Pump 2		
Identification:	Item	Centrifugal Pump
	No. Required	3
Function:	Pump Broth 2 from Seed Fermenter 2 to Seed Fermenter 3	
Operation:	Continuous	
Stream ID:	*Broth 2*	
Temperature (ºC)	30	
Flow Rate (gpm)	30	
Design Data:	Run Time (hours)	0.33
	Head (ft)	27
	Material	Stainless Steel
Utilities Required (kJ/hr)		1,780
Total Purchase Cost:		$33,729
Bare Module Cost:		$111,306

Pump 3		
Identification:	Item	Centrifugal Pump
	No. Required	3
Function:	Pump Broth 3 from Seed Fermenter 3 to Production Fermenter	
Operation:	Continuous	
Stream ID:	*Broth 3*	
Temperature (ºC)	30	
Flow Rate (gpm)	100	
Design Data:	Run Time (hours)	0.77
	Head (ft)	62
	Material	Stainless Steel
Utilities Required (kJ/hr)		13,800
Total Purchase Cost:		$27,060
Bare Module Cost:		$89,298

Pump 4		
Identification:	Item	Centrifugal Pump
	No. Required	3
Function:	Pump Broth 4 from Production Fermenter to Harvest Tank	
Operation:	Continuous	
Stream ID:	*Broth 4*	
Temperature (ºC)	30	
Flow Rate (gpm)	400	
Design Data:	Run Time (hours)	1.9
	Head (ft)	62
	Material	Stainless Steel
Utilities Required (kJ/hr)		55,000
Total Purchase Cost:		$32,136
Bare Module Cost:		$106,049

Pump 5		
Identification:	Item	Centrifugal Pump
	No. Required	1
Function:	Pump fluid onto first vacuum belt filter	
Operation:	Continuous	
Stream ID:	*Raw Product*	
Temperature (ºC)	30	
Flow Rate (gpm)	400	
Design Data:	Run Time (hours)	1.87
	Head (ft)	10
	Material	Stainless Steel
Utilities Required (kJ/hr)		8,915
Total Purchase Cost:		$9,218
Bare Module Cost:		$30,419

Pump 6		
Identification:	Item	Centrifugal Pump
	No. Required	1
Function:	Pump fluid into reverse osmosis system	
Operation:	Continuous	
Stream ID:	*Filtered Product*	
Temperature (ºC)	30	
Flow Rate (gpm)	400	
Design Data:	Run Time (hours)	2.13
	Head (ft)	10
	Material	Stainless Steel
Utilities Required (kJ/hr)		8,899
Total Purchase Cost:		$9,219
Bare Module Cost:		$30,419

Pump 7		
Identification:	Item	Centrifugal Pump
	No. Required	1
Function:	Pump displacement wash onto vacuum belt filter 1	
Operation:	Continuous	
Stream ID:	*RO Recycle Wash*	
Temperature (ºC)	30	
Flow Rate (gpm)	100	
Design Data:	Run Time (hours)	1.33
	Head (ft)	10
	Material	Stainless Steel
Utilities Required (kJ/hr)		2,227
Total Purchase Cost:		$9,648
Bare Module Cost:		$31,838

Pump 8		
Identification:	Item	Centrifugal Pump
	No. Required	1
Function:	Pump fluid into crystallizer	
Operation:	Continuous	
Stream ID:	*RO Product*	
Temperature (ºC)	30	
Flow Rate (gpm)	200	
Design Data:	Run Time (hours)	2.42
	Head (ft)	10
	Material	Stainless Steel
Utilities Required (kJ/hr)		4,450
Total Purchase Cost:		$9,048
Bare Module Cost:		$29,858

Pump 9		
Identification:	Item	Centrifugal Pump
	No. Required	1
Function:	Pump water wash onto vacuum belt filter 2	
Operation:	Continuous	
Stream ID:	*Filtered Product*	
Temperature (ºC)	30	
Flow Rate (gpm)	30	
Design Data:	Run Time (hours)	0.88
	Head (ft)	10
	Material	Stainless Steel
Utilities Required (kJ/hr)		669
Total Purchase Cost:		$13,134
Bare Module Cost:		$43,342

21.6.3 Mixing and Storage Tanks

YPD Media Mixing Tank		
Identification:	Item	YPD Media
	No. Required	1
Function:	Mixes the YPD Media with water for Seed Fermenter 1 and 2	
Stream ID:		*YPD Media 1*
Temperature (ºC)		25
Flow Rate (lb/batch)	4591	
Water		4581
YPD		11
Design Data:	Volume (L)	2,400
	Diameter (ft)	3.3
	Height (ft)	9.9
	Impeller Diameter (ft)	1.7
	Working Volume (L)	2,100
	Mixing Speed (rpm)	300
	Material	Stainless Steel
Utilities Required (kj/batch):		5,950
Total Purchase Cost:		$6,815
Bare Module Cost:		$13,630

Fermentation Media Mixing Tank		
Identification:	Item	Fermentation Media
	No. Required	1
Function:	Mixes the YNB Media with water and glucose for Seed Fermenter 2 and Production Fermenter	
Stream ID:		*Media 1*
Temperature (ºC)		25
Flow Rate (lb/batch)	380,000	
Water		318,00
YNB		1,350
Glucose		60,600
Design Data:	Volume (L)	167,000
	Diameter (ft)	12
	Height (ft)	37
	Propeller Diameter (ft)	6.2
	Working Volume (L)	145,000
	Mixing Speed (rpm)	600
	Material	Stainless Steel
Utilities Required (kj/batch):		466,000
Total Purchase Cost:		$53,724
Bare Module Cost:		$107,448

10M NaOH Mixing Tank		
Identification:	Item	10M NaOH
	No. Required	1
Function:	Mixes NaOH with water to create 10M NaOH for Seed Fermenter 2 and Production Fermenter	
Stream ID:		*10M NaOH*
Temperature (ºC)		25
Flow Rate (lb/batch)	903	
Water		893
NaOH		9.0
Design Data:	Volume (L)	470
	Diameter (ft)	1.9
	Height (ft)	5.8
	Propeller Diameter (ft)	1.0
	Working Volume (L)	410
	Mixing Speed (rpm)	300
	Material	Stainless Steel
Utilities Required (kj/batch):		1,200
Total Purchase Cost:		$4,260
Bare Module Cost:		$8,520

Harvest Tank		
Identification:	Item	Harvest Tank
	No. Required	1
Function:	Stores Broth 4 from upstream processing for downstream processing	
Stream ID:		*Broth 4*
Temperature (ºC)		30
Flow Rate (lb/batch)		395,000
Design Data:	Volume (L)	300,000
	Diameter (ft)	12.4
	Height (ft)	37
	Material	Stainless Steel
Total Purchase Cost:		$67,846
Bare Module Cost:		$135,692

Reverse Osmosis Recycle Storage		
Identification:	Item	RO Water Storage
	No. Required	1
Function:	Contain the recycle wash from reverse osmosis for the belt filter 1	
Stream ID:		*Washed Product*
Temperature (ºC)		30
Flow Rate (lb/hr)		31,200
Water		30,900
Malonic Acid		270
Succinic Acid		6
Ethanol		14
Design Data:	Volume (L)	35,000
	Height (ft)	24
	Diameter (ft)	8
	Material	Stainless Steel
Total Purchase Cost:		$67,846
Bare Module Cost:		$135,692

Waste Storage Tank			
Identification:	Item	Waste Storage Tank	
	No. Required	1	
Function:	Contain waste until further processing		
Stream ID:	Inlet: *Waste Stream 1*	Top Outlet: *Waste Stream 2*	Bottom Outlet: *Waste Stream 3*
Temperature (ºC)	30	30	25
Flow Rate (lb/hr)	20,000	55,150	68,900
Biomass	10.805	-	-
Growth Media	700	-	-
Malonic Acid Solids	-	-	-
Water	7,540	54,340	68,300
Malonic Acid	886	780	74
Succinic Acid	18	17	268
Ethanol	6	11	197
Design Data:	Height (ft) Diameter (ft) Material	37 12.5 Stainless Steel	
Total Purchase Cost:		$67,846	
Bare Module Cost:		$135,692	

21.6.4 Heat Exchangers

Heat Exchanger 1				
Identification:	Item	Heat Exchanger 1		
	No. Required	1		
Function:	Use the YPD Media to heat and cool itself			
Operation:	Continuous			
Materials Handles:	Cold In	Cold Out	Hot In	Hot Out
Temperature (ºC)	25	86	121	60
Flow Rate (lb/hr)	33,440	33,440	33,440	33,440
YPD Media	33,440	33,440	33,440	33,440
Water	0	0	0	0
Design Data:	Effective Area (ft^2)	1150		
	Hot Fluid on Tube side			
	Material	Stainless Steel		
Total Purchase Cost:		$43,807		
Bare Module Cost:		$138,868		

Heat Exchanger 2				
Identification:	Item	Heat Exchanger 2		
	No. Required	1		
Function:	Use the YPD Media to heat and cool itself			
Operation:	Continuous			
Materials Handles:	Cold In	Cold Out	Hot In	Hot Out
Temperature (ºC)	25	86	121	60
Flow Rate (lb/hr)	346,800	346,800	346,800	346,800
YPD Media	346,800	346,800	346,800	346,800
Water	0	0	0	0
Design Data:	Effective Area (ft^2)	11873		
	Hot Fluid on Tube side			
	Material	Stainless Steel		
Total Purchase Cost:		$413,300		
Bare Module Cost:		$1,310,161		

Sterilizer 1				
Identification:	Item	Sterilizer 1		
	No. Required	1		
Function:	Use steam to sterilize the Fermentation Media			
Operation:	Continuous			
Materials Handles:	Cold In	Cold Out	Hot In	Hot Out
Temperature (ºC)	86	121	170	130
Flow Rate (lb/hr)	33,440	33,440	61,280	61,280
YPD Media	33,440	33,440	0	0
Steam	0	0	61,280	61,280
Design Data:	Effective Area (ft^2)	182		
	Hot Fluid on Tube side			
	Material	Stainless Steel		
Total Purchase Cost:		$43,810		
Bare Module Cost:		$138,878		

Sterilizer 2				
Identification:	Item	Sterilizer 2		
	No. Required	1		
Function:	Use steam to sterilize the Fermentation Media			
Operation:	Continuous			
Materials Handles:	Cold In	Cold Out	Hot In	Hot Out
Temperature (ºC)	86	121	170	130
Flow Rate (lb/hr)	346,800	346,800	635,470	635,470
YPD Media	346,800	346,800	0	0
Steam	0	0	635,470	635,470
Design Data:	Effective Area (ft^2)	1890		
	Hot Fluid on Tube side			
	Material	Stainless Steel		
Total Purchase Cost:		$100,457		
Bare Module Cost:		$318,449		

Cooler 1				
Identification:	Item	Cooler 1		
	No. Required	1		
Function:	Use water to cool the Fermentation Media after sterilization			
Operation:	Continuous			
Materials Handles:	Cold In	Cold Out	Hot In	Hot Out
Temperature (ºC)	25	29	60	30
Flow Rate (lb/hr)	250,800	250,800	33,440	33,440
YPD Media	0	0	33,440	33,440
Water	250,800	250,800	0	0
Design Data:	Effective Area (ft^2)	313		
	Hot Fluid on Tube side			
	Material	Stainless Steel		
Total Purchase Cost:		$48,216		
Bare Module Cost:		$152,845		

Cooler 2				
Identification:	Item	Cooler 2		
	No. Required	1		
Function:	Use water to cool the Fermentation Media after sterilization			
Operation:	Continuous			
Materials Handles:	Cold In	Cold Out	Hot In	Hot Out
Temperature (ºC)	25	29	60	30
Flow Rate (lb/hr)	2,601,000	2,601,000	346,800	346,800
YPD Media	10,850	0	346,800	346,800
Water	2,601,000	2,601,000	0	0
Design Data:	Effective Area (ft^2)	3,240		
	Hot Fluid on Tube side			
	Material	Stainless Steel		
Total Purchase Cost:		$142,094		
Bare Module Cost:		$450,438		

21.6.5 Vacuum Belt Filters

Vacuum Belt Filter 1			
Identification:	Item	Vacuum Belt Filter	
	No. Required	1	
Function:	Remove biomass from fermentation broth		
Operation:	Continuous		
Stream ID:	Inlet: *Raw Product*	Top Outlet: *Filtered Product*	Bottom Outlet: *Waste Stream 1*
Temperature (ºC)	30	30	30
Flow Rate (lb/hr)	271,700	251,700	20,000
Biomass	10,850	-	10,805
Water	232,000	224,460	7,540
Malonic Acid	27,300	26,414	886
Succinic Acid	550	532	18
Ethanol	300	294	6
Growth Media	700	-	700
Design Data:	Run Time (hours)	1.76	
	Length (ft)	60	
	Width (ft)	17.5	
	Material	*Structure:* Carbon Steel *Belt:* Monofilaments/Felt	
Utilities Required (kJ/hr):		27,700	
Total Purchase Cost:		$517,610	
Bare Module Cost:		$1,200,855	

Vacuum Belt Filter 2			
Identification:	Item	Vacuum Belt Filter	
	No. Required	1	
Function:	Filter out solidified malonic acid		
Operation:	Continuous		
Stream ID:	Inlet: *Crystallized Product*	Top Outlet: *Washed Product*	Bottom Outlet: *Waste Stream 3*
Temperature (ºC)	15	25	25
Flow Rate (lb/hr)	88,900	20,000	68,900
Malonic Acid Solids	14,300	14,300	-
Water	73,900	5,600	68,300
Malonic Acid	80	6	74
Succinic Acid	290	22	268
Ethanol	213	16	197
Design Data:	Run Time (hours)	3.05	
	Length (ft)	60	
	Width (ft)	17.5	
	Material	*Structure:* Carbon Steel *Belt:* Monofilaments/Felt	
Utilities Required (kJ/hr):		6,515	
Total Purchase Cost:		$517,610	
Bare Module Cost:		$1,200,855	

21.6.6 Reverse Osmosis System

Reverse Osmosis System				
Identification:	Item	Reverse Osmosis System		
	No. Required	1		
Function:	Increase malonic acid concentration in liquid			
Operation:	Continuous			
Stream ID:	Inlet: *Filtered Product*	Side Outlet: *RO Product*	Top Outlet: *RO Recycle*	Bottom Outlet: *Waste Stream 2*
Temperature (ºC)	30	30	30	30
Flow Rate (lb/hr)	207,430	121,080	31,200	55,150
Water	184,940	99,700	30,900	54,340
Malonic Acid	21,700	20,650	270	780
Succinic Acid	440	417	6	17
Ethanol	320	305	4	11
Design Data:	Run Time (hours)	2.13		
	Length (ft)	1.9		
	Width (ft)	1.6		
	Height (ft)	3.7		
	Pore Size (nm)	0.3		
	Material	Stainless Steel		
Capacity (gpm):		400		
Utilities Required (kJ/hr):		6,159		
Total Purchase Cost:		$21,917		
Bare Module Cost:		$21,917		

21.6.7 Crystallizer

Crystallizer		
Identification:	Item	Crystallizer
	No. Required	1
Function:	Solidify Malonic Acid	
Operation:	Continuous	
Stream ID:	Inlet: *RO Product*	Outlet: *Crystallized Product*
Temperature (ºC)	30	15
Flow Rate (lb/hr)	43,000	43,000
Malonic Acid Solids	-	7,260
Water	35,400	5,600
Malonic Acid	7,300	40
Succinic Acid	148	148
Ethanol	108	108
Design Data:	Run Time (hours)	6
	Height (ft)	18
	Diameter (ft)	6
	Material	Stainless Steel
Cooling Water (L/hr)		19,500
Total Purchase Cost:		$115,031
Bare Module Cost:		$32,356

21.6.8 Fluidized Bed Dryer

Fluidized Bed Dryer				
Identification:	Item	Fluidized Bed Dryer		
	No. Required	1		
Function:	Dry the wet malonic acid solids			
Operation:	Continuous			
Stream ID:	Inlet: *Washed Product*	Air Inlet: *Air*	Outlet: *Malonic Acid*	Air Outlet: *Drying Air*
Temperature (ºC)	25	25	25	25
Flow Rate (lb/hr)	12,200	486	8,750	3,900
Malonic Acid Solids	8,750	-	8,750	-
Water	73,900	-	-	73,900
Malonic Acid	3,400	-	-	3,400
Succinic Acid	13	-	-	13
Ethanol	10	-	-	10
Air	-	486		486
Design Data:	Run Time (hours)	5		
	Length (ft)	11		
	Width (ft)	4		
	Height (ft)	3		
	Material	Stainless Steel		
Utilities Required (kJ/hr):		552		
Total Purchase Cost:		$517,610		
Bare Module Cost:		$1,200,855		

21.7 Fermentation Sizing and Energy based on Oxygen Uptake Rate

Large Scale Microbial Production of Advanced Biofuels: How big can we go?

By Gregory T. Benz, President, Benz Technology International, Inc.

Conventional biofuels, whether from starch, sugar or lignocellulosic materials, tend to be low energy density products such as ethanol or butanol. These are made using catabolic (anaerobic), thermodynamically "downhill" processes. Such processes have been proven feasible in rather large fermenter volumes, in excess of 1 million gallons (~ 4 million liters).

In order to make use of current infrastructure and engine design, DOE has shifted focus to higher energy density fuels that act more like oils or gasoline, such as hydrocarbons, long chain fatty acids, fatty alcohols, or terpene type molecules. These so-called "hydrocarbon biofuels" can be produced by a number of conversion pathways, including biochemical routes based on aerobic (anabolic; thermodynamically "uphill") submerged cultivation.

The principal technical issue for large scale fermentation is the need to provide sufficient oxygen to allow the organisms to thrive, and the size and power required of the agitators typically used to disperse air.

It is anticipated that the ability to economically maintain effective gas-liquid mass transfer will ultimately limit the size at which aerobic submerged cultivation processes for hydrocarbon production from sugars can be operated. Maximum bioreactor sizes will likely be smaller than what is possible for strictly anaerobic fermentation processes where aeration isn't necessary. A key enabler of cost competitive biological hydrocarbon production is the ability to economically aerate at large scales.

This author did a study for NREL detailing the size of equipment required for various scenarios, the feasibility of manufacturing such equipment, and approximate equipment costs. Both mechanically agitated and bubble column designs were looked at. The bubble column details will not be repeated here, as in most cases, the total power needed was much higher than for the mechanically agitated cases. However, a comparative summary is made at the end of this article.

Methodology:

For this study, we used 9 increments of volume, 4 increments of OTR (Oxygen Transfer Rate) and one empirical mass transfer correlation.

The volumes studied were 80, 280, 500,750, 1000, 1400, 2200, 3000 and 4000 kiloliters. The OTRs studied were 50, 100, 150 and 200 mmol/l-h. The mass transfer correlation used is the V'ant Riet correlation for ionic liquids (non-coalescing fluids.) This may be found in "Basic Bioreactor Design" (book), Chapter 2.3, page 251, K. van't Riet and J. Tramper, published 1991.

Some base conditions were assumed for all runs. These are as follows:

0.344 bar back pressure, unaerated aspect ratio (liquid height/tank diameter) of 2.5, plant site elevation of 2000' (0.91 bar barometric pressure), operating temperature of 30C, carbon evolution ratio of 0.95 moles CO2 produced per mole of O2 consumed, wetted material 316SS,

double mechanical shaft seal, sparge ring design based on 0.344 bar maximum pressure drop, generic impeller types as described below, electricity costs set at $0.07/kwh, 70% compressor efficiency, 95% agitator gear drive efficiency, total gas line pressure drop of 1.38 bar, safety factor of 1.3 applied to OTR to allow for correlation error band, agitator motor sized for at least 1.18 times shaft power at peak OTR, 80% operating cycle for figuring utility cost, minimum DO of 1 mg/l at top of vessel and viscosity less than 50 cP.

The principle mass transfer calculation is defined below:

1) OTR = kla* (driving force) where the driving force, in log mean terms, is ((Csat-C)in – (Csat-C)out)/ln((Csat-C)in/(Csat-C)out))

Agitator power is derived from the relationship:
2) kla = A(P/V)B(Us)C

The constants A, B and C are ideally empirically derived from broth-specific pilot studies. We will be using an ionic, non-coalescing liquid correlation found in reference 2. The values of the constants, based on units of 1/s, W/l and M/s, are 0.25 for A, 0.7 for B and 0.2 for C. There are many other correlations in the literature, and results in actual broth can vary considerably. Nonetheless, this correlation should give a reasonable idea of what is possible.

The mass transfer coefficient correlation for bubble columns was derived from equations in the same reference: kla = 0.32*(Us)^0.7*μ(cP)^-.84*1.025^(temp-20, degC)

Compressor brake power, in units of KW, with atm. for pressure and air flow in M3/min., and using a specific heat ratio for air of 1.394, equals 5.97(inlet pressure, atm.)(inlet flow, M3/min.)((pressure ratio)0.283-1)/efficiency fraction.

Capital cost estimates are based on a combination of vendor quotes, the author's data bases, and judgment, especially for the largest sizes, where there are little data to base cost estimates on. The author expresses gratitude to Rexnord, Lightnin Mixer, Chemineer, Inc. and Tom Reynolds of Rogers Equipment sales for budget estimates that were helpful in putting the capital cost estimates together.

The results are shown in the next sections.

Agitated results:

Working volume, M3	Tank diam., M	Liquid level, M	Tank SS, M	OTR, mmol/l-h	Airflow, nM3/h	Agitator motor size, kW	Compressor motor size, kW	Agitator speed, rpm	~ impeller size, 4 impellers, M	~Seal shaft diam., mm	~ Extension shaft diam, pipe size	Total brake power, kW	Annual power cost, $K	Agitator capital cost, $K
80	3.47	8.68	11.8	50	1100	75	75	125	1.07	96	6" sch80	122	75	160
80	3.47	8.68	11.8	100	2210	149	149	125	1.25	119	8" sch 80	255.9	157	230
80	3.47	8.68	11.8	150	3400	224	261	155	1.20	127	8" sch 80	394.9	242	330
80	3.47	8.68	11.8	200	4590	298	335	155	1.28	139	8" sch 120	536.7	329	420
280	5.27	13.17	18.0	50	3400	224	261	100	1.52	147	8" sch 120	399.2	245	380
280	5.27	13.17	18.0	100	6970	410	559	125	1.55	167	10" sch 120	833.1	511	590
280	5.27	13.17	18.0	150	10600	671	894	125	1.70	196	12" sch 120	1282	786	780
280	5.27	13.17	18.0	200	14300	894	1118	125	1.81	216	12" sch 120	1741	1068	1100
500	6.39	15.98	21.5	50	5870	335	559	100	1.69	168	10" sch 120	705.3	432	530
500	6.39	15.98	21.5	100	12100	745	1118	125	1.72	203	12" sch 120	1469	901	1200
500	6.39	15.98	21.5	150	18200	1118	1931	125	1.88	233	14" sch 160	2259	1385	1500
500	6.39	15.98	21.5	200	24500	1490	2235	125	2.01	256	16" sch120	3065	1879	1800
750	7.32	18.29	24.9	50	8500	559	745	84	2.01	211	12" sch 120	1038	637	840
750	7.32	18.29	24.9	100	17300	1118	1490	125	1.84	233	14" sch 80	2160	1325	1600
750	7.32	18.29	24.9	150	26200	1676	2235	125	2.02	266	16" sch 120	3317	2034	2000
750	7.32	18.29	24.9	200	35400	2235	2980	125	2.15	293	18" sch 120	4498	2758	2700
1000	8.05	20.13	27.2	50	11000	596	1118	84	2.11	216	14" sch 80	1368	839	860
1000	8.05	20.13	27.2	100	22600	1490	2235	100	2.21	276	16" sch 120	2840	1741	2000
1000	8.05	20.13	27.2	150	34300	2235	2980	125	2.11	293	18" sch 120	4360	2674	2700
1000	8.05	20.13	27.2	200	45900	2980	4470	125	2.26	323	18" sch 120	5908	3623	3600
1400	9.01	22.52	30.2	50	15100	894	1490	68	2.53	265	16" sch 120	1891	1160	1800
1400	9.01	22.52	30.2	100	30600	1676	2980	100	2.34	287	18" sch 120	3922	2405	2200
1400	9.01	22.52	30.2	150	46600	2608	4470	125	2.24	309	18" sch 120	6012	3687	3100
1400	9.01	22.52	30.2	200	62700	3725	5588	125	2.38	348	20" sch 80	8143	4993	3900
2200	10.47	26.19	35.2	50	23500	1490	2235	68	2.76	314	18" sch 120	2926	1794	3000
2200	10.47	26.19	35.2	100	47900	2608	4470	84	2.83	353	20" sch 120	6065	3719	4100
2200	10.47	26.19	35.2	150	72400	4470	6705	100	2.79	398	22" sch 120	9294	5699	5000
2200	10.47	26.19	35.2	200	97600	5588	8940	100	2.97	429	24" sch 120	12586	7718	5800
3000	11.61	29.04	38.8	50	30600	1490	3725	56	3.25	335	20" sch 120	4016	2464	3400
3000	11.61	29.04	38.8	100	62000	3725	6705	84	2.96	397	22" sch 120	8303	5091	4700
3000	11.61	29.04	38.8	150	94500	5588	11200	100	2.91	429	24" sch 120	12703	7789	6200
3000	11.61	29.04	38.8	200	1E+05	7450	14900	100	3.11	472	26" x 2"	17210	7823	7800
4000	12.78	31.96	42.4	50	40100	2235	4470	56	3.39	383	22" sch 120	5323	10554	4300
4000	12.78	31.96	42.4	100	81100	4470	8940	68	3.52	453	26" x 2"	10984	6735	6100
4000	12.78	31.96	42.4	150	1E+05	6705	14900	84	3.39	483	26" x 2"	16789	10295	7700
4000	12.78	31.96	42.4	200	2E+05	8940	18625	100	3.26	501	28" x 2"	22693	13915	9500

Commentary on Results:

As one can see, the equipment size and power requirements can become quite large. But can such equipment be built by today's vendors? The limitation is on agitator size, rather than vessel or compressor capacity.

Judgment on the maximum feasible agitator size is based on the following observations of existing equipment:

To the author's knowledge, the largest motor nameplate power ever built for a single fermenter agitator was 3500 Hp, but it was never started up. The highest power ever operated in a fermenter was about 1250 Hp. The highest power operating in any gas-liquid system is about 3500 Hp, in a TPA (Terephthalic Acid) process. The maximum torque ever applied to an agitator was in a unit used for a coal-water mixture, and it was 375 Hp at 7 rpm in a 30,000m^3 tank.

So, the author is confident that a 3000 Hp fermenter agitator could be built with today's technology and manufacturing capacity, with operating speeds of about 50-100 rpm. Confidence

diminishes as either power or torque goes up from there. Above 3000 Hp, every component must be custom designed. In some cases, new tooling or manufacturing methods would have to be used to build the parts, and much of the work would have to be subcontracted to manufacturers who are not in the agitator business.

It is not certain that the largest sizes estimated herein can actually be built with the tooling extant in the industry today. However, market forces drive the capabilities manufacturers have. If a market need developed for multiple units of greater than 3000 Hp (2200 kW) motor size, the manufacturers would find a way to meet the need. But unforeseen problems would undoubtedly occur in the first few installations of large sizes never built before.

Agitator Mounting Notes:

Large agitators create considerable loads on the mounting structure. In addition to weight, there is impeller thrust load, torque and bending moment. In an effort to reduce vessel cost, some fermenter designers have tried to isolate these loads to a structure independent of the vessel. Such efforts generally lead to added operating expenses, as additional bearings, gear couplings or universal joint jackshafts are required to allow for differential movement between the mounting structure and the vessel. These extra parts are all additional maintenance items.

Moreover, though such designs remove the gear drive weight from the vessel, the independent bearing support required still sees the wetted parts weight, the impeller thrust and the bending moment, and these are transmitted to the vessel nozzle. In addition, though torque is not normally transmitted (save for bearing and seal friction), the design must allow for what happens if a bearing seizes up, so full torque must be designed for.

The end result is that the capital cost savings are minimal, and the operating costs are higher. Therefore, this author recommends that the agitator drive be directly mounted to the vessel nozzle, with the vessel head appropriately designed to handle the resultant loads, as per the picture below. (The below picture is of a 186 KW unit, with this author. The units described in this report will be much larger, though the author hopefully won't be.)

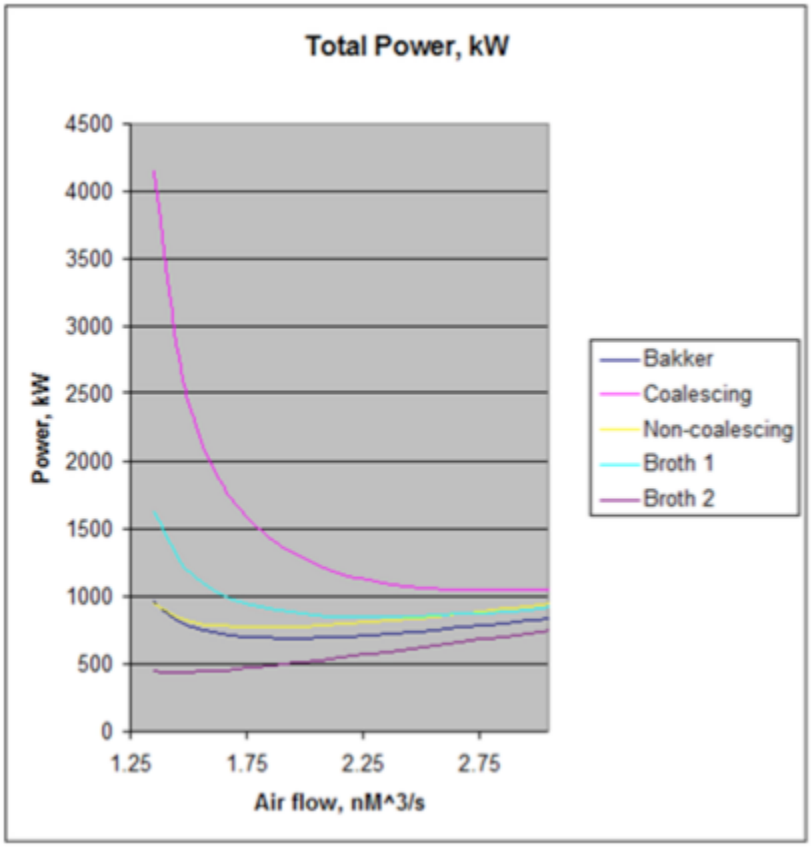
Vessel Costs:

Though vessel cost estimates were not formally a part of the scope of work for NREL, the author has seen several vessel quotes recently. A reasonable estimate of vessel cost for units built to be agitated fermenters, in 316SS, mill finish, is $2200 per cubic meter of working volume. So, we can roughly estimate the vessel costs as follows:

Vessel working volume, M^3	Vessel cost, $K
80	176
280	616
500	1100
750	1650
1000	2200
1400	3080
2200	4840
3000	6600
4000	8800

Comments on mass transfer correlations:

All of the results in this report were based on a single correlation for kla. Though it is from a very respected source, actual results in a broth can vary considerably. Below is a graph of required power as a function of airflow for a given size fermenter, using several published correlations (including the one we used here, in yellow) and a couple of correlations from actual broths.



Ideally, one should develop a broth-specific kla correlation. The author can assist in doing this.

Comparison to Bubble Column Results:

Below is a chart comparing selected results from the bubble column report done for NREL to the optimized agitated results presented here. It is evident that bubble columns are favored at low viscosity, low OTR and larger volumes, whereas the agitated case is better for smaller volumes, higher OTRs and higher viscosities, sometimes by more than an order of magnitude. The first column, "agitated", refers to the power developed in this report. The others refer to several bubble column correlations in the literature, including several different viscosities. We will not discuss details of bubble columns here, but note that they are very sensitive to viscosity.

NREL bubble column vs agitator comparison

Benz Technology International, Inc.

Agitated power is optimized.

Volume: 80 M^3

	Total brake power, kW					
OTR	Agitated	V'ant Riet 1 cP	V'ant Riet 5 cP	V'ant Riet 10 cP	V'ant Riet 20 cP	Shah, 1 cP
50	122	211	1221	2035	6817	244
100	256	549	3490	7936	18100	580
150	395	967	6185	14200	32400	957

Volume: 750 M^3

	Total brake power, kW					
OTR	Agitated	V'ant Riet 1 cP	V'ant Riet 5 cP	V'ant Riet 10 cP	V'ant Riet 20 cP	Shah, 1 cP
50	1038	1165	6231	13900	33200	1343
100	2160	2913	16500	37400	89300	3065
150	3317	5041	29400	66500	158000	5053

Volume: 4000 M^3

	Total brake power, kW					
OTR	Agitated	V'ant Riet 1 cP	V'ant Riet 5 cP	V'ant Riet 10 cP	V'ant Riet 20 cP	Shah, 1 cP
50	5323	4418	19600	43000	96600	5036
100	11000	10500	51800	115000	259000	11400
150	16800	17600	91400	203000	461000	18400

Conclusions:

Depending on OTR requirements, it is likely that mechanically agitated fermenters of 500-2000 M^3 can be built with today's technology and manufacturing capacity. Larger volumes combined with larger OTR requirements will require technology and manufacturing capability not currently available. Based on the correlations used, it appears that in most cases there is no

power cost advantage to using bubble column designs, though there is no doubt a capital cost advantage, and the possibility of building larger fermenters without new manufacturing technology. The full NREL reports are available by contacting this author.

Symbols

C Dissolved oxygen concentration, mass or moles/volume (e.g., mg/l or mmol/l)
Csat Dissolved oxygen concentration at saturation (mg/l or mmol/l)
D Impeller diameter, M
DO Dissolved oxygen concentration, general term (mg/l or mmol/l)
kla Overall mass transfer coefficient, 1/time (1/s)
OTR Oxygen Transfer Rate, mass or moles per volume-time), e.g. mg/l-h or mmol/l-h
P Power (e.g., W)
P/V Specific Power: agitator invested power/(mass or volume) of liquid (e.g., W/M3)
Us Superficial Gas Velocity, distance/time (M/s)
T Tank diameter, M
VVM Volume of gas/volume of liquid/minute at standard conditions (min-1)
Z Liquid level, M

21.8 Material Safety Data Sheets

Material Safety Data Sheet

Malonic acid

ACC# 13575

Section 1 - Chemical Product and Company Identification

MSDS Name: Malonic acid
Catalog Numbers: S80069, S80069A, A170-100
Synonyms: Carboxyacetic Acid; Dicarboxymethane; Methanedicarboxylic Acid; Propanedioic Acid.
Company Identification:
Fisher Scientific
1 Reagent Lane
Fair Lawn, NJ 07410
For information, call: 201-796-7100
Emergency Number: 201-796-7100
For CHEMTREC assistance, call: 800-424-9300
For International CHEMTREC assistance, call: 703-527-3887

Section 2 - Composition, Information on Ingredients

CAS#	Chemical Name	Percent	EINECS/ELINCS
141-82-2	Malonic acid	>99	205-503-0

Section 3 - Hazards Identification

EMERGENCY OVERVIEW

Appearance: white solid.
Warning! Causes eye, skin, and respiratory tract irritation. May be harmful if swallowed.
Target Organs: Respiratory system, eyes, skin.

Potential Health Effects
Eye: Causes eye irritation.
Skin: Causes skin irritation.
Ingestion: May cause gastrointestinal irritation with nausea, vomiting and diarrhea. May be harmful if swallowed.
Inhalation: Causes respiratory tract irritation.
Chronic: Effects may be delayed.

Section 4 - First Aid Measures

Eyes: Immediately flush eyes with plenty of water for at least 15 minutes, occasionally lifting the upper and lower eyelids. Get medical aid imme diately.
Skin: Get medical aid immediately. Immediately flush skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes.
Ingestion: Never give anything by mouth to an unconscious person. Get medical aid immediately. Do NOT induce vomiting. If conscious and alert, rinse mouth and drink 2-4 cupfuls of milk or water.
Inhalation: Get medical aid immediately. Remove from exposure and move to fresh air immediately. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Do NOT use mouth-to-mouth resuscitation.
Notes to Physician: Treat symptomatically and supportively.

Section 5 - Fire Fighting Measures

General Information: As in any fire, wear a self-contained breathing apparatus in pressure-demand, MSHA/NIOSH (approved or equivalent), and full protective gear. Dusts at sufficient concentrations can form explosive mixtures with air. During a fire, irritating and highly toxic gases may be generated by thermal decomposition or combustion.
Extinguishing Media: For small fires, use water spray, dry chemical, carbon dioxide or chemical foam. Use water spray, dry chemical, carbon dioxide, or appropriate foam.
Flash Point: Not applicable.
Autoignition Temperature: Not applicable.
Explosion Limits, Lower: Not available.
Upper: Not available.
NFPA Rating: (estimated) Health: 2; Flammability: 1; Instability: 0

Section 6 - Accidental Release Measures

General Information: Use proper personal protective equipment as indicated in Section 8.
Spills/Leaks: Clean up spills immediately, observing precautions in the Protective Equipment section. Wear a self contained breathing apparatus and appropriate personal protection. (See Exposure Controls, Personal Protection section). Sweep up, then place into a suitable container for disposal. Avoid generating dusty conditions.

Section 7 - Handling and Storage

Handling: Wash thoroughly after handling. Remove contaminated clothing and wash before reuse. Use with adequate ventilation. Minimize dust generation and accumulation. Do not get in eyes, on skin, or on clothing. Keep

container tightly closed. Do not ingest or inhale.
Storage: Store in a tightly closed container. Store in a cool, dry, well-ventilated area away from incompatible substances.

Section 8 - Exposure Controls, Personal Protection

Engineering Controls: Facilities storing or utilizing this material should be equipped with an eyewash facility and a safety shower. Use adequate ventilation to keep airborne concentrations low.
Exposure Limits

Chemical Name	ACGIH	NIOSH	OSHA - Final PELs
Malonic acid	none listed	none listed	none listed

OSHA Vacated PELs: Malonic acid: No OSHA Vacated PELs are listed for this chemical.
Personal Protective Equipment
Eyes: Wear appropriate protective eyeglasses or chemical safety goggles as described by OSHA's eye and face protection regulations in 29 CFR 1910.133 or European Standard EN166.
Skin: Wear appropriate gloves to prevent skin exposure.
Clothing: Wear appropriate protective clothing to prevent skin exposure.
Respirators: Follow the OSHA respirator regulations found in 29 CFR 1910.134 or European Standard EN 149. Use a NIOSH/MSHA or European Standard EN 149 approved respirator if exposure limits are exceeded or if irritation or other symptoms are experienced.

Section 9 - Physical and Chemical Properties

Physical State: Solid
Appearance: white
Odor: none reported
pH: Acidic in solution.
Vapor Pressure: Negligible.
Vapor Density: Not available.
Evaporation Rate: Negligible.
Viscosity: Not available.
Boiling Point: 284 deg F
Freezing/Melting Point:276 deg F
Decomposition Temperature:284 deg F
Solubility: Completely soluble in water.
Specific Gravity/Density:1.62 (water=1)
Molecular Formula:C3H4O4
Molecular Weight:104.0256

Section 10 - Stability and Reactivity

Chemical Stability: Stable under normal temperatures and pressures.
Conditions to Avoid: Incompatible materials, dust generation, excess heat.
Incompatibilities with Other Materials: Oxidizing agents, reducing agents, bases.
Hazardous Decomposition Products: Carbon monoxide, carbon dioxide.
Hazardous Polymerization: Has not been reported.

Section 11 - Toxicological Information

RTECS#:
CAS# 141-82-2: OO0175000
LD50/LC50:
CAS# 141-82-2:
Draize test, rabbit, eye: 100 mg Severe;
Draize test, rabbit, skin: 500 mg/24H Mild;
Inhalation, rat: LC50 = >8900 mg/m3/1H;
Oral, mouse: LD50 = 4 gm/kg;
Oral, rat: LD50 = 1310 mg/kg;
.

Carcinogenicity:
CAS# 141-82-2: Not listed by ACGIH, IARC, NTP, or CA Prop 65.

Epidemiology: No information found
Teratogenicity: No information found
Reproductive Effects: No information found
Mutagenicity: No information found
Neurotoxicity: No information found
Other Studies:

Section 12 - Ecological Information

No information available.

Section 13 - Disposal Considerations

Chemical waste generators must determine whether a discarded chemical is classified as a hazardous waste. US EPA guidelines for the classification determination are listed in 40 CFR Parts 261.3. Additionally, waste generators must consult state and local hazardous waste regulations to ensure complete and accurate classification.
RCRA P-Series: None listed.
RCRA U-Series: None listed.

Section 14 - Transport Information

	US DOT	Canada TDG
Shipping Name:	Not regulated as a hazardous material	No information available.
Hazard Class:		
UN Number:		
Packing Group:		

Section 15 - Regulatory Information

US FEDERAL

TSCA
CAS# 141-82-2 is listed on the TSCA inventory.
Health & Safety Reporting List
None of the chemicals are on the Health & Safety Reporting List.
Chemical Test Rules
None of the chemicals in this product are under a Chemical Test Rule.
Section 12b
None of the chemicals are listed under TSCA Section 12b.
TSCA Significant New Use Rule
None of the chemicals in this material have a SNUR under TSCA.
CERCLA Hazardous Substances and corresponding RQs
None of the chemicals in this material have an RQ.
SARA Section 302 Extremely Hazardous Substances
None of the chemicals in this product have a TPQ.
SARA Codes
CAS # 141-82-2: immediate.
Section 313 No chemicals are reportable under Section 313.
Clean Air Act:
This material does not contain any hazardous air pollutants.
This material does not contain any Class 1 Ozone depletors.
This material does not contain any Class 2 Ozone depletors.
Clean Water Act:
None of the chemicals in this product are listed as Hazardous Substances under the CWA.
None of the chemicals in this product are listed as Priority Pollutants under the CWA.
None of the chemicals in this product are listed as Toxic Pollutants under the CWA.
OSHA:
None of the chemicals in this product are considered highly hazardous by OSHA.
STATE
CAS# 141-82-2 is not present on state lists from CA, PA, MN, MA, FL, or NJ.

California Prop 65

California No Significant Risk Level: None of the chemicals in this product are listed.

European/International Regulations

European Labeling in Accordance with EC Directives

Hazard Symbols:

XN

Risk Phrases:

R 22 Harmful if swallowed.
R 36/37/38 Irritating to eyes, respiratory system and skin.

Safety Phrases:

S 26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
S 28 After contact with skin, wash immediately with...

WGK (Water Danger/Protection)

CAS# 141-82-2: 1

Canada - DSL/NDSL

CAS# 141-82-2 is listed on Canada's DSL List.

Canada - WHMIS

This product has a WHMIS classification of D2B.

This product has been classified in accordance with the hazard criteria of the Controlled Products Regulations and the MSDS contains all of the information required by those regulations.

Canadian Ingredient Disclosure List

Section 16 - Additional Information

MSDS Creation Date: 12/12/1997
Revision #5 Date: 12/03/2003

The information above is believed to be accurate and represents the best information currently available to us. However, we make no warranty of merchantability or any other warranty, express or implied, with respect to such information, and we assume no liability resulting from its use. Users should make their own investigations to determine the suitability of the information for their particular purposes. In no event shall Fisher be liable for any claims, losses, or damages of any third party or for lost profits or any special, indirect, incidental, consequential or exemplary damages, howsoever arising, even if Fisher has been advised of the possibility of such damages.

Material Safety Data Sheet
Glucose Standard Solution 1% (Aq.)

ACC# 89827

Section 1 - Chemical Product and Company Identification

MSDS Name: Glucose Standard Solution 1% (Aq.)
Catalog Numbers: S76789
Synonyms:
Company Identification:
Fisher Scientific
1 Reagent Lane
Fair Lawn, NJ 07410
For information, call: 201-796-7100
Emergency Number: 201-796-7100
For CHEMTREC assistance, call: 800-424-9300
For International CHEMTREC assistance, call: 703-527-3887

Section 2 - Composition, Information on Ingredients

CAS#	Chemical Name	Percent	EINECS/ELINCS
50-99-7	Glucose	1.0	200-075-1
65-85-0	Benzoic acid	<1.0	200-618-2
7732-18-5	Water	Balance	231-791-2

Section 3 - Hazards Identification

EMERGENCY OVERVIEW

Appearance: clear, colorless liquid.
Caution! May cause allergic respiratory reaction. May cause allergic skin reaction. May cause eye and skin irritation. May cause respiratory and digestive tract irritation.
Target Organs: None known.

Potential Health Effects
Eye: May cause eye irritation.
Skin: May cause skin irritation. May cause skin sensitization, an allergic reaction, which becomes evident upon re-exposure to this material.

Ingestion: May cause gastrointestinal irritation with nausea, vomiting and diarrhea. May cause allergic reaction.
Inhalation: May cause respiratory tract irritation. May cause asthmatic attacks due to allergic sensitization of the respiratory tract.
Chronic: No information found.

Section 4 - First Aid Measures

Eyes: Immediately flush eyes with plenty of water for at least 15 minutes, occasionally lifting the upper and lower eyelids. Get medical aid imme diately.
Skin: Flush skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes. Get medical aid if irritation develops or persists.
Ingestion: If victim is conscious and alert, give 2-4 cupfuls of milk or water. Never give anything by mouth to an unconscious person. Get medical aid.
Inhalation: Remove from exposure and move to fresh air immediately. Get medical aid if cough or other symptoms appear.
Notes to Physician: Treat symptomatically and supportively.

Section 5 - Fire Fighting Measures

General Information: As in any fire, wear a self-contained breathing apparatus in pressure-demand, MSHA/NIOSH (approved or equivalent), and full protective gear.
Extinguishing Media: Substance is noncombustible; use agent most appropriate to extinguish surrounding fire.
Flash Point: Not available.
Autoignition Temperature: Not available.
Explosion Limits, Lower: Not available.
Upper: Not available.
NFPA Rating: Not published.

Section 6 - Accidental Release Measures

General Information: Use proper personal protective equipment as indicated in Section 8.
Spills/Leaks: Absorb spill with inert material (e.g. vermiculite, sand or earth), then place in suitable container.

Section 7 - Handling and Storage

Handling: Wash thoroughly after handling. Use with adequate ventilation. Avoid contact with eyes, skin, and clothing. Keep container tightly closed. Avoid ingestion and inhalation.

Storage: Store in a tightly closed container. Store in a cool, dry, well-ventilated area away from incompatible substances.

Section 8 - Exposure Controls, Personal Protection

Engineering Controls: Use adequate ventilation to keep airborne concentrations low.
Exposure Limits

Chemical Name	ACGIH	NIOSH	OSHA - Final PELs
Glucose	none listed	none listed	none listed
Benzoic acid	none listed	none listed	none listed
Water	none listed	none listed	none listed

OSHA Vacated PELs: Glucose: No OSHA Vacated PELs are listed for this chemical. Benzoic acid: No OSHA Vacated PELs are listed for this chemical. Water: No OSHA Vacated PELs are listed for this chemical.
Personal Protective Equipment
Eyes: Wear appropriate protective eyeglasses or chemical safety goggles as described by OSHA's eye and face protection regulations in 29 CFR 1910.133 or European Standard EN166.
Skin: Wear appropriate protective gloves to prevent skin exposure.
Clothing: Wear appropriate protective clothing to prevent skin exposure.
Respirators: Follow the OSHA respirator regulations found in 29 CFR 1910.134 or European Standard EN 149. Use a NIOSH/MSHA or European Standard EN 149 approved respirator if exposure limits are exceeded or if irritation or other symptoms are experienced.

Section 9 - Physical and Chemical Properties

Physical State: Liquid
Appearance: clear, colorless
Odor: odorless
pH: Not available.
Vapor Pressure: Not available.
Vapor Density: Not available.
Evaporation Rate: Not available.
Viscosity: Not available.
Boiling Point: 100 deg C
Freezing/Melting Point:0 deg C
Decomposition Temperature: Not available.
Solubility: Not available.
Specific Gravity/Density:~1.0
Molecular Formula: Not available.
Molecular Weight: Not available.

Section 10 - Stability and Reactivity

Chemical Stability: Stable.
Conditions to Avoid: Incompatible materials, strong oxidants.
Incompatibilities with Other Materials: Oxidizing agents.
Hazardous Decomposition Products: Carbon monoxide, carbon dioxide.
Hazardous Polymerization: Will not occur.

Section 11 - Toxicological Information

RTECS#:
CAS# 50-99-7: LZ6600000
CAS# 65-85-0: DG0875000
CAS# 7732-18-5: ZC0110000
LD50/LC50:
CAS# 50-99-7:
Oral, rat: LD50 = 25800 mg/kg;
.

CAS# 65-85-0:
Draize test, rabbit, eye: 100 mg Severe;
Draize test, rabbit, skin: 500 mg/24H Mild;
Inhalation, rat: LC50 = >26 mg/m3/1H;
Oral, mouse: LD50 = 1940 mg/kg;
Oral, rat: LD50 = 1700 mg/kg;
Skin, rabbit: LD50 = >10 gm/kg;
.

CAS# 7732-18-5:
Oral, rat: LD50 = >90 mL/kg;
.

Carcinogenicity:
CAS# 50-99-7: Not listed by ACGIH, IARC, NTP, or CA Prop 65.
CAS# 65-85-0: Not listed by ACGIH, IARC, NTP, or CA Prop 65.
CAS# 7732-18-5: Not listed by ACGIH, IARC, NTP, or CA Prop 65.

Epidemiology: No data available.
Teratogenicity: No data available.
Reproductive Effects: No data available.
Mutagenicity: No data available.
Neurotoxicity: No data available.
Other Studies:

Section 12 - Ecological Information

No information available.

Section 13 - Disposal Considerations

Chemical waste generators must determine whether a discarded chemical is classified as a hazardous waste. US EPA guidelines for the classification determination are listed in 40 CFR Parts 261.3. Additionally, waste generators must consult state and local hazardous waste regulations to ensure complete and accurate classification.
RCRA P-Series: None listed.
RCRA U-Series: None listed.

Section 14 - Transport Information

	US DOT	Canada TDG
Shipping Name:	Not regulated as a hazardous material	No information available.
Hazard Class:		
UN Number:		
Packing Group:		

Section 15 - Regulatory Information

US FEDERAL

TSCA
CAS# 50-99-7 is listed on the TSCA inventory.
CAS# 65-85-0 is listed on the TSCA inventory.
CAS# 7732-18-5 is listed on the TSCA inventory.
Health & Safety Reporting List
None of the chemicals are on the Health & Safety Reporting List.
Chemical Test Rules
None of the chemicals in this product are under a Chemical Test Rule.
Section 12b
None of the chemicals are listed under TSCA Section 12b.
TSCA Significant New Use Rule
None of the chemicals in this material have a SNUR under TSCA.
CERCLA Hazardous Substances and corresponding RQs
CAS# 65-85-0: 5000 lb final RQ; 2270 kg final RQ
SARA Section 302 Extremely Hazardous Substances
None of the chemicals in this product have a TPQ.
SARA Codes
CAS # 50-99-7: Not controlled.
CAS # 65-85-0: immediate.
Section 313 No chemicals are reportable under Section 313.
Clean Air Act:

This material does not contain any hazardous air pollutants.
This material does not contain any Class 1 Ozone depletors.
This material does not contain any Class 2 Ozone depletors.

Clean Water Act:

CAS# 65-85-0 is listed as a Hazardous Substance under the CWA.
None of the chemicals in this product are listed as Priority Pollutants under the CWA.
None of the chemicals in this product are listed as Toxic Pollutants under the CWA.

OSHA:

None of the chemicals in this product are considered highly hazardous by OSHA.

STATE

CAS# 50-99-7 is not present on state lists from CA, PA, MN, MA, FL, or NJ.
CAS# 65-85-0 can be found on the following state right to know lists: California, New Jersey, Pennsylvania, Massachusetts.
CAS# 7732-18-5 is not present on state lists from CA, PA, MN, MA, FL, or NJ.

California Prop 65

California No Significant Risk Level: None of the chemicals in this product are listed.

European/International Regulations

European Labeling in Accordance with EC Directives

Hazard Symbols:

Not available.

Risk Phrases:

Safety Phrases:

WGK (Water Danger/Protection)

CAS# 50-99-7: 0
CAS# 65-85-0: 1
CAS# 7732-18-5: No information available.

Canada - DSL/NDSL

CAS# 50-99-7 is listed on Canada's DSL List.
CAS# 65-85-0 is listed on Canada's DSL List.
CAS# 7732-18-5 is listed on Canada's DSL List.

Canada - WHMIS

not available.

This product has been classified in accordance with the hazard criteria of the Controlled Products Regulations and the MSDS contains all of the information required by those regulations.

Canadian Ingredient Disclosure List

CAS# 65-85-0 is listed on the Canadian Ingredient Disclosure List.

Section 16 - Additional Information

MSDS Creation Date: 2/09/1998
Revision #4 Date: 11/20/2008

The information above is believed to be accurate and represents the best information currently available to us. However, we make no warranty of merchantability or any other warranty, express or implied, with respect to such information, and we assume no liability resulting from its use. Users should make their own investigations to determine the suitability of the information for their particular purposes. In no event shall Fisher be liable for any claims, losses, or damages of any third party or for lost profits or any special, indirect, incidental, consequential or exemplary damages, howsoever arising, even if Fisher has been advised of the possibility of such damages.

Material Safety Data Sheet
Sodium hydroxide, solid

ACC# 21300

Section 1 - Chemical Product and Company Identification

MSDS Name: Sodium hydroxide, solid
Catalog Numbers: BP359-212, BP359-500, S318-1, S318-10, S318-100, S318-10LC, S318-3, S318-3LC, S318-5, S318-50, S318-500, S318-50LC, S320-1, S320-10, S320-3, S320-50, S320-500, S392-12, S392-12LC, S392-212, S392-50, S392SAM1, S392SAM2, S392SAM3, S399-1, S399-212, S399-50, S399-500, S612-3, S612-3500LB, S612-50, S612-500LB, S613-10, S613-3, S613-50, S613-500LB
Synonyms: Caustic soda; Soda lye; Sodium hydrate; Lye.
Company Identification:

Fisher Scientific
1 Reagent Lane
Fair Lawn, NJ 07410

For information, call: 201-796-7100
Emergency Number: 201-796-7100
For CHEMTREC assistance, call: 800-424-9300
For International CHEMTREC assistance, call: 703-527-3887

Section 2 - Composition, Information on Ingredients

CAS#	Chemical Name	Percent	EINECS/ELINCS
1310-73-2	Sodium hydroxide	95-100	215-185-5
497-19-8	Sodium carbonate	<3.0	207-838-8

Section 3 - Hazards Identification

EMERGENCY OVERVIEW

Appearance: white solid.
Danger! Causes eye and skin burns. Causes digestive and respiratory tract burns. Hygroscopic (absorbs moisture from the air).
Target Organs: Eyes, skin, mucous membranes.

Potential Health Effects
Eye: Causes eye burns. May cause blindness. May cause chemical conjunctivitis and corneal damage.

Skin: Causes skin burns. May cause deep, penetrating ulcers of the skin.
Ingestion: May cause severe and permanent damage to the digestive tract. Causes gastrointestinal tract burns. May cause perforation of the digestive tract. Causes severe pain, nausea, vomiting, diarrhea, and shock.
Inhalation: Irritation may lead to chemical pneumonitis and pulmonary edema. Causes severe irritation of upper respiratory tract with coughing, burns, breathing difficulty, and possible coma. Causes chemical burns to the respiratory tract.
Chronic: Prolonged or repeated skin contact may cause dermatitis. Effects may be delayed.

Section 4 - First Aid Measures

Eyes: In case of contact, immediately flush eyes with plenty of water for a t least 15 minutes. Get medical aid immediately.
Skin: In case of contact, immediately flush skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes. Get medical aid immediately. Wash clothing before reuse.
Ingestion: If swallowed, do NOT induce vomiting. Get medical aid immediately. If victim is fully conscious, give a cupful of water. Never give anything by mouth to an unconscious person.
Inhalation: If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical aid.
Notes to Physician: Treat symptomatically and supportively.

Section 5 - Fire Fighting Measures

General Information: As in any fire, wear a self-contained breathing apparatus in pressure-demand, MSHA/NIOSH (approved or equivalent), and full protective gear. Use water spray to keep fire-exposed containers cool. Use water with caution and in flooding amounts. Contact with moisture or water may generate sufficient heat to ignite nearby combustible materials. Contact with metals may evolve flammable hydrogen gas.
Extinguishing Media: Substance is noncombustible; use agent most appropriate to extinguish surrounding fire. Do NOT get water inside containers.
Flash Point: Not applicable.
Autoignition Temperature: Not applicable.
Explosion Limits, Lower: Not available.
Upper: Not available.
NFPA Rating: (estimated) Health: 3; Flammability: 0; Instability: 1

Section 6 - Accidental Release Measures

General Information: Use proper personal protective equipment as indicated in Section 8.
Spills/Leaks: Vacuum or sweep up material and place into a suitable disposal container. Avoid runoff into storm sewers and ditches which lead to waterways. Clean up spills immediately, observing precautions in the Protective Equipment section. Avoid generating dusty conditions. Provide ventilation. Do not get water on spilled substances or inside containers.

Section 7 - Handling and Storage

Handling: Wash thoroughly after handling. Do not allow water to get into the container because of violent reaction. Minimize dust generation and accumulation. Do not get in eyes, on skin, or on clothing. Keep container tightly closed. Avoid ingestion and inhalation. Discard contaminated shoes. Use only with adequate ventilation.
Storage: Store in a tightly closed container. Store in a cool, dry, well-ventilated area away from incompatible substances. Keep away from metals. Corrosives area. Keep away from acids. Store protected from moisture. Containers must be tightly closed to prevent the conversion of NaOH to sodium carbonate by the CO2 in air.

Section 8 - Exposure Controls, Personal Protection

Engineering Controls: Facilities storing or utilizing this material should be equipped with an eyewash facility and a safety shower. Use adequate general or local exhaust ventilation to keep airborne concentrations below the permissible exposure limits.
Exposure Limits

Chemical Name	ACGIH	NIOSH	OSHA - Final PELs
Sodium hydroxide	2 mg/m3 Ceiling	10 mg/m3 IDLH	2 mg/m3 TWA
Sodium carbonate	none listed	none listed	none listed

OSHA Vacated PELs: Sodium hydroxide: No OSHA Vacated PELs are listed for this chemical. Sodium carbonate: No OSHA Vacated PELs are listed for this chemical.
Personal Protective Equipment
Eyes: Wear chemical splash goggles and face shield.
Skin: Wear butyl rubber gloves, apron, and/or clothing.
Clothing: Wear appropriate protective clothing to prevent skin exposure.
Respirators: Follow the OSHA respirator regulations found in 29 CFR 1910.134 or European Standard EN 149. Use a NIOSH/MSHA or European Standard EN 149 approved respirator if exposure limits are exceeded or if irritation or other symptoms are experienced.

Section 9 - Physical and Chemical Properties

Physical State: Solid
Appearance: white
Odor: Odorless
pH: 14 (5% aq soln)
Vapor Pressure: 1 mm Hg @739 deg C
Vapor Density: Not available.
Evaporation Rate: Not available.
Viscosity: Not available.

Boiling Point: 1390 deg C @ 760 mmHg
Freezing/Melting Point:318 deg C
Decomposition Temperature: Not available.
Solubility: Soluble.
Specific Gravity/Density:2.13 g/cm3
Molecular Formula: NaOH
Molecular Weight:40

Section 10 - Stability and Reactivity

Chemical Stability: Stable at room temperature in closed containers under normal storage and handling conditions.
Conditions to Avoid: Moisture, contact with water, exposure to moist air or water, prolonged exposure to air.
Incompatibilities with Other Materials: Water, metals, acids, aluminum, zinc, tin, nitromethane, leather, flammable liquids, organic halogens, wool.
Hazardous Decomposition Products: Toxic fumes of sodium oxide.
Hazardous Polymerization: Will not occur.

Section 11 - Toxicological Information

RTECS#:
CAS# 1310-73-2: WB4900000
CAS# 497-19-8: VZ4050000
LD50/LC50:
CAS# 1310-73-2:
Draize test, rabbit, eye: 400 ug Mild;
Draize test, rabbit, eye: 1% Severe;
Draize test, rabbit, eye: 50 ug/24H Severe;
Draize test, rabbit, eye: 1 mg/24H Severe;
Draize test, rabbit, skin: 500 mg/24H Severe;
.

CAS# 497-19-8:
Draize test, rabbit, eye: 100 mg/24H Moderate;
Draize test, rabbit, eye: 50 mg Severe;
Draize test, rabbit, skin: 500 mg/24H Mild;
Inhalation, mouse: LC50 = 1200 mg/m3/2H;
Inhalation, rat: LC50 = 2300 mg/m3/2H;
Oral, mouse: LD50 = 6600 mg/kg;
Oral, mouse: LD50 = 6600 mg/kg;
Oral, rat: LD50 = 4090 mg/kg;
.

Carcinogenicity:
CAS# 1310-73-2: Not listed by ACGIH, IARC, NTP, or CA Prop 65.
CAS# 497-19-8: Not listed by ACGIH, IARC, NTP, or CA Prop 65.

Epidemiology: No information found

Teratogenicity: No information found
Reproductive Effects: No information found
Mutagenicity: See actual entry in RTECS for complete information.
Neurotoxicity: No information found
Other Studies:

Section 12 - Ecological Information

No information available.

Section 13 - Disposal Considerations

Chemical waste generators must determine whether a discarded chemical is classified as a hazardous waste. US EPA guidelines for the classification determination are listed in 40 CFR Parts 261.3. Additionally, waste generators must consult state and local hazardous waste regulations to ensure complete and accurate classification.
RCRA P-Series: None listed.
RCRA U-Series: None listed.

Section 14 - Transport Information

	US DOT	Canada TDG
Shipping Name:	SODIUM HYDROXIDE, SOLID	SODIUM HYDROXIDE SOLID
Hazard Class:	8	8
UN Number:	UN1823	UN1823
Packing Group:	II	II

Section 15 - Regulatory Information

US FEDERAL

TSCA
CAS# 1310-73-2 is listed on the TSCA inventory.
CAS# 497-19-8 is listed on the TSCA inventory.
Health & Safety Reporting List
None of the chemicals are on the Health & Safety Reporting List.
Chemical Test Rules
None of the chemicals in this product are under a Chemical Test Rule.

Section 12b
None of the chemicals are listed under TSCA Section 12b.
TSCA Significant New Use Rule
None of the chemicals in this material have a SNUR under TSCA.
CERCLA Hazardous Substances and corresponding RQs
CAS# 1310-73-2: 1000 lb final RQ; 454 kg final RQ
SARA Section 302 Extremely Hazardous Substances
None of the chemicals in this product have a TPQ.
SARA Codes
CAS # 1310-73-2: immediate, reactive.
CAS # 497-19-8: immediate.
Section 313 No chemicals are reportable under Section 313.
Clean Air Act:
This material does not contain any hazardous air pollutants.
This material does not contain any Class 1 Ozone depletors.
This material does not contain any Class 2 Ozone depletors.
Clean Water Act:
CAS# 1310-73-2 is listed as a Hazardous Substance under the CWA.
None of the chemicals in this product are listed as Priority Pollutants under the CWA.
None of the chemicals in this product are listed as Toxic Pollutants under the CWA.
OSHA:
None of the chemicals in this product are considered highly hazardous by OSHA.
STATE
CAS# 1310-73-2 can be found on the following state right to know lists: California, New Jersey, Pennsylvania, Minnesota, Massachusetts.
CAS# 497-19-8 is not present on state lists from CA, PA, MN, MA, FL, or NJ.

California Prop 65

California No Significant Risk Level: None of the chemicals in this product are listed.

European/International Regulations

European Labeling in Accordance with EC Directives
Hazard Symbols:
C
Risk Phrases:
R 35 Causes severe burns.

Safety Phrases:
S 26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
S 37/39 Wear suitable gloves and eye/face protection.
S 45 In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

WGK (Water Danger/Protection)
CAS# 1310-73-2: 1
CAS# 497-19-8: 1
Canada - DSL/NDSL
CAS# 1310-73-2 is listed on Canada's DSL List.
CAS# 497-19-8 is listed on Canada's DSL List.
Canada - WHMIS
This product has a WHMIS classification of E.
This product has been classified in accordance with the hazard criteria of the Controlled Products Regulations and the MSDS contains all of the information required by those regulations.

Canadian Ingredient Disclosure List

CAS# 1310-73-2 is listed on the Canadian Ingredient Disclosure List.
CAS# 497-19-8 is listed on the Canadian Ingredient Disclosure List.

Section 16 - Additional Information

MSDS Creation Date: 12/12/1997
Revision #10 Date: 2/15/2008

The information above is believed to be accurate and represents the best information currently available to us. However, we make no warranty of merchantability or any other warranty, express or implied, with respect to such information, and we assume no liability resulting from its use. Users should make their own investigations to determine the suitability of the information for their particular purposes. In no event shall Fisher be liable for any claims, losses, or damages of any third party or for lost profits or any special, indirect, incidental, consequential or exemplary damages, howsoever arising, even if Fisher has been advised of the possibility of such damages.

Material Safety Data Sheet
Succinic Acid, 99%

ACC# 95199

Section 1 - Chemical Product and Company Identification

MSDS Name: Succinic Acid, 99%
Catalog Numbers: AC158740000, AC158740025, AC158740030, AC158745000, NC9119816, NC9135536, NC9135540, XXAC15874-1GR, XXAC15874-1KG, XXAC15874-9KG
Synonyms: Amber Acid; Butanedioic Acid; Ethylenesuccinic Acid; 1,2-Ethanedicarboxylic Acid.
Company Identification:

Fisher Scientific
1 Reagent Lane
Fair Lawn, NJ 07410

For information, call: 201-796-7100
Emergency Number: 201-796-7100
For CHEMTREC assistance, call: 800-424-9300
For International CHEMTREC assistance, call: 703-527-3887

Section 2 - Composition, Information on Ingredients

CAS#	Chemical Name	Percent	EINECS/ELINCS
110-15-6	Succinic Acid	99%	203-740-4

Section 3 - Hazards Identification

EMERGENCY OVERVIEW

Appearance: white solid.
Warning! Causes respiratory tract irritation. Causes eye and skin irritation. May cause digestive tract irritation.
Target Organs: No data found.

Potential Health Effects
Eye: Causes eye irritation. May cause chemical conjunctivitis.
Skin: Causes skin irritation. Contact with heated material can cause skin burns and irritation.
Ingestion: May cause gastrointestinal irritation with nausea, vomiting and diarrhea.
Inhalation: Causes respiratory tract irritation. Can produce delayed pulmonary edema.
Chronic: Effects may be delayed.

Section 4 - First Aid Measures

Eyes: Immediately flush eyes with plenty of water for at least 15 minutes, occasionally lifting the upper and lower eyelids. Get medical aid imme diately.
Skin: Get medical aid. Flush skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes. Wash clothing before reuse.
Ingestion: Never give anything by mouth to an unconscious person. Get medical aid immediately. Do NOT induce vomiting. If conscious and alert, rinse mouth and drink 2-4 cupfuls of milk or water.
Inhalation: Remove from exposure and move to fresh air immediately. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical aid.
Notes to Physician: Treat symptomatically and supportively.

Section 5 - Fire Fighting Measures

General Information: As in any fire, wear a self-contained breathing apparatus in pressure-demand, MSHA/NIOSH (approved or equivalent), and full protective gear. Dusts at sufficient concentrations can form explosive mixtures with air. During a fire, irritating and highly toxic gases may be generated by thermal decomposition or combustion.
Extinguishing Media: For small fires, use water spray, dry chemical, carbon dioxide or chemical foam. Use water spray, dry chemical, carbon dioxide, or appropriate foam.
Flash Point: 206 deg C (402.80 deg F)
Autoignition Temperature: 630 deg C (1,166.00 deg F)
Explosion Limits, Lower: Not available.
Upper: Not available.
NFPA Rating: (estimated) Health: 2; Flammability: 0; Instability: 0

Section 6 - Accidental Release Measures

General Information: Use proper personal protective equipment as indicated in Section 8.
Spills/Leaks: Clean up spills immediately, observing precautions in the Protective Equipment section. Sweep up, then place into a suitable container for disposal. Avoid generating dusty conditions. Provide ventilation.

Section 7 - Handling and Storage

Handling: Wash thoroughly after handling. Remove contaminated clothing and wash before reuse. Use with adequate ventilation. Minimize dust generation and accumulation. May form flammable dust-air mixtures. Avoid contact with eyes, skin, and clothing. Keep container tightly closed. Avoid ingestion and inhalation. Wash clothing before reuse.

Storage: Store in a tightly closed container. Keep from contact with oxidizing materials. Store in a cool, dry, well-ventilated area away from incompatible substances.

Section 8 - Exposure Controls, Personal Protection

Engineering Controls: Facilities storing or utilizing this material should be equipped with an eyewash facility and a safety shower. Use adequate ventilation to keep airborne concentrations low.
Exposure Limits

Chemical Name	ACGIH	NIOSH	OSHA - Final PELs
Succinic Acid	none listed	none listed	none listed

OSHA Vacated PELs: Succinic Acid: No OSHA Vacated PELs are listed for this chemical.
Personal Protective Equipment
Eyes: Wear appropriate protective eyeglasses or chemical safety goggles as described by OSHA's eye and face protection regulations in 29 CFR 1910.133 or European Standard EN166.
Skin: Wear appropriate protective gloves to prevent skin exposure.
Clothing: Wear appropriate protective clothing to prevent skin exposure.
Respirators: A respiratory protection program that meets OSHA's 29 CFR 1910.134 and ANSI Z88.2 requirements or European Standard EN 149 must be followed whenever workplace conditions warrant a respirator's use. Follow the OSHA respirator regulations found in 29 CFR 1910.134 or European Standard EN 149. Always use a NIOSH or European Standard EN 149 approved respirator when necessary.

Section 9 - Physical and Chemical Properties

Physical State: Solid
Appearance: white
Odor: none reported
pH: 2.7 (0.1 M sol.)
Vapor Pressure: Negligible.
Vapor Density: Not applicable.
Evaporation Rate: Negligible.
Viscosity: Not available.
Boiling Point: 455 deg F
Freezing/Melting Point:363 deg F
Decomposition Temperature:455 deg F
Solubility: Moderately soluble in water.
Specific Gravity/Density:1.6 (water=1)
Molecular Formula:C4H6O4
Molecular Weight:118.0396

Section 10 - Stability and Reactivity

Chemical Stability: Stable at room temperature in closed containers under normal storage and handling conditions.
Conditions to Avoid: High temperatures, incompatible materials, excess heat.
Incompatibilities with Other Materials: None reported.
Hazardous Decomposition Products: Carbon monoxide, carbon dioxide.
Hazardous Polymerization: Has not been reported.

Section 11 - Toxicological Information

RTECS#:
CAS# 110-15-6: WM4900000
LD50/LC50:
CAS# 110-15-6:
Draize test, rabbit, eye: 750 ug Severe;
Oral, rat: LD50 = 2260 mg/kg;<br.

Carcinogenicity:
CAS# 110-15-6: Not listed by ACGIH, IARC, NTP, or CA Prop 65.

Epidemiology: No information available.
Teratogenicity: No information available.
Reproductive Effects: No information available.
Mutagenicity: No information available.
Neurotoxicity: No information available.
Other Studies:</br.

Section 12 - Ecological Information

Ecotoxicity: No data available. No information available.
Environmental: Succinic acid is not expected to volatilize from water surfaces.
Physical: Vapor-phase succinic acid is degraded in the atmosphere by reaction with photochemically-produced hydroxyl radicals; the half-life for this reaction in air is estimated to be about 5.8 days. Particulate-phase succinic acid may be physically removed from the air by wet and dry deposition.
Other: An estimated BCF value of 0.21 was calculated for succinic acid, using a measured log Kow of -0.59 and a recommended regression-derived equation. According to a classification scheme, this BCF value suggests that bioconcentration in aquatic organisms is low.

Section 13 - Disposal Considerations

Chemical waste generators must determine whether a discarded chemical is classified as a hazardous waste. US EPA guidelines for the classification determination are listed in 40 CFR Parts 261.3. Additionally, waste generators must consult state and local hazardous waste regulations to ensure complete and accurate classification.

RCRA P-Series: None listed.
RCRA U-Series: None listed.

Section 14 - Transport Information

	US DOT	Canada TDG
Shipping Name:	Please contact Fisher Scientific for shipping information	No information available.
Hazard Class:		
UN Number:		
Packing Group:		

Section 15 - Regulatory Information

US FEDERAL

TSCA
CAS# 110-15-6 is listed on the TSCA inventory.
Health & Safety Reporting List
None of the chemicals are on the Health & Safety Reporting List.
Chemical Test Rules
None of the chemicals in this product are under a Chemical Test Rule.
Section 12b
None of the chemicals are listed under TSCA Section 12b.
TSCA Significant New Use Rule
None of the chemicals in this material have a SNUR under TSCA.
CERCLA Hazardous Substances and corresponding RQs
None of the chemicals in this material have an RQ.
SARA Section 302 Extremely Hazardous Substances
None of the chemicals in this product have a TPQ.
SARA Codes
CAS # 110-15-6: acute.
Section 313 No chemicals are reportable under Section 313.
Clean Air Act:
This material does not contain any hazardous air pollutants.
This material does not contain any Class 1 Ozone depletors.
This material does not contain any Class 2 Ozone depletors.
Clean Water Act:
None of the chemicals in this product are listed as Hazardous Substances under the CWA.
None of the chemicals in this product are listed as Priority Pollutants under the CWA.
None of the chemicals in this product are listed as Toxic Pollutants under the CWA.
OSHA:
None of the chemicals in this product are considered highly hazardous by OSHA.
STATE

CAS# 110-15-6 is not present on state lists from CA, PA, MN, MA, FL, or NJ.

California Prop 65

California No Significant Risk Level: None of the chemicals in this product are listed.

European/International Regulations

European Labeling in Accordance with EC Directives

Hazard Symbols:

XI

Risk Phrases:

R 36/37/38 Irritating to eyes, respiratory system and skin.

Safety Phrases:

WGK (Water Danger/Protection)

CAS# 110-15-6: 0

Canada - DSL/NDSL

CAS# 110-15-6 is listed on Canada's DSL List.

Canada - WHMIS

This product has a WHMIS classification of D2B.

Canadian Ingredient Disclosure List

CAS# 110-15-6 is listed on the Canadian Ingredient Disclosure List.

Section 16 - Additional Information

MSDS Creation Date: 9/02/1997
Revision #4 Date: 3/18/2003

The information above is believed to be accurate and represents the best information currently available to us. However, we make no warranty of merchantability or any other warranty, express or implied, with respect to such information, and we assume no liability resulting from its use. Users should make their own investigations to determine the suitability of the information for their particular purposes. In no event shall Fisher be liable for any claims, losses, or damages of any third party or for lost profits or any special, indirect, incidental, consequential or exemplary damages, howsoever arising, even if Fisher has been advised of the possibility of such damages.

Material Safety Data Sheet
Ethanol, Absolute

ACC# 89308

Section 1 - Chemical Product and Company Identification

MSDS Name: Ethanol, Absolute
Catalog Numbers: NC9602322
Synonyms: Ethyl Alcohol; Ethyl Alcohol Anhydrous; Ethyl Hydrate; Ethyl Hydroxide; Fermentation Alcohol; Grain Alcohol; Methylcarbinol; Molasses Alcohol; Spirits of Wine.
Company Identification:

Fisher Scientific
1 Reagent Lane
Fair Lawn, NJ 07410

For information, call: 201-796-7100
Emergency Number: 201-796-7100
For CHEMTREC assistance, call: 800-424-9300
For International CHEMTREC assistance, call: 703-527-3887

Section 2 - Composition, Information on Ingredients

CAS#	Chemical Name	Percent	EINECS/ELINCS
64-17-5	Ethanol	ca.100	200-578-6

Section 3 - Hazards Identification

EMERGENCY OVERVIEW

Appearance: colorless clear liquid. Flash Point: 16.6 deg C.
Warning! Causes severe eye irritation. **Flammable liquid and vapor.** Causes respiratory tract irritation. This substance has caused adverse reproductive and fetal effects in humans. May cause central nervous system depression. May cause liver, kidney and heart damage. Causes moderate skin irritation.
Target Organs: Kidneys, heart, central nervous system, liver.

Potential Health Effects
Eye: Causes severe eye irritation. May cause painful sensitization to light. May cause chemical conjunctivitis and corneal damage.
Skin: Causes moderate skin irritation. May cause cyanosis of the extremities.
Ingestion: May cause gastrointestinal irritation with nausea, vomiting and diarrhea. May cause systemic toxicity

with acidosis. May cause central nervous system depression, characterized by excitement, followed by headache, dizziness, drowsiness, and nausea. Advanced stages may cause collapse, unconsciousness, coma and possible death due to respiratory failure.
Inhalation: Inhalation of high concentrations may cause central nervous system effects characterized by nausea, headache, dizziness, unconsciousness and coma. Causes respiratory tract irritation. May cause narcotic effects in high concentration. Vapors may cause dizziness or suffocation.
Chronic: May cause reproductive and fetal effects. Laboratory experiments have resulted in mutagenic effects. Animal studies have reported the development of tumors. Prolonged exposure may cause liver, kidney, and heart damage.

Section 4 - First Aid Measures

Eyes: Get medical aid. Gently lift eyelids and flush continuously with wate r.
Skin: Get medical aid. Wash clothing before reuse. Flush skin with plenty of soap and water.
Ingestion: Do not induce vomiting. If victim is conscious and alert, give 2-4 cupfuls of milk or water. Never give anything by mouth to an unconscious person. Get medical aid.
Inhalation: Remove from exposure and move to fresh air immediately. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical aid. Do NOT use mouth-to-mouth resuscitation.
Notes to Physician: Treat symptomatically and supportively. Persons with skin or eye disorders or liver, kidney, chronic respiratory diseases, or central and peripheral nervous sytem diseases may be at increased risk from exposure to this substance.
Antidote: None reported.

Section 5 - Fire Fighting Measures

General Information: Containers can build up pressure if exposed to heat and/or fire. As in any fire, wear a self-contained breathing apparatus in pressure-demand, MSHA/NIOSH (approved or equivalent), and full protective gear. Vapors may form an explosive mixture with air. Vapors can travel to a source of ignition and flash back. Will burn if involved in a fire. Flammable Liquid. Can release vapors that form explosive mixtures at temperatures above the flashpoint. Use water spray to keep fire-exposed containers cool. Containers may explode in the heat of a fire.
Extinguishing Media: For small fires, use dry chemical, carbon dioxide, water spray or alcohol-resistant foam. For large fires, use water spray, fog, or alcohol-resistant foam. Use water spray to cool fire-exposed containers. Water may be ineffective. Do NOT use straight streams of water.
Flash Point: 16.6 deg C (61.88 deg F)
Autoignition Temperature: 363 deg C (685.40 deg F)
Explosion Limits, Lower:3.3 vol %
Upper: 19.0 vol %
NFPA Rating: (estimated) Health: 2; Flammability: 3; Instability: 0

Section 6 - Accidental Release Measures

General Information: Use proper personal protective equipment as indicated in Section 8.
Spills/Leaks: Absorb spill with inert material (e.g. vermiculite, sand or earth), then place in suitable container.

Remove all sources of ignition. Use a spark-proof tool. Provide ventilation. A vapor suppressing foam may be used to reduce vapors.

Section 7 - Handling and Storage

Handling: Wash thoroughly after handling. Use only in a well-ventilated area. Ground and bond containers when transferring material. Use spark-proof tools and explosion proof equipment. Avoid contact with eyes, skin, and clothing. Empty containers retain product residue, (liquid and/or vapor), and can be dangerous. Keep container tightly closed. Keep away from heat, sparks and flame. Avoid ingestion and inhalation. Do not pressurize, cut, weld, braze, solder, drill, grind, or expose empty containers to heat, sparks or open flames.
Storage: Keep away from heat, sparks, and flame. Keep away from sources of ignition. Store in a tightly closed container. Keep from contact with oxidizing materials. Store in a cool, dry, well-ventilated area away from incompatible substances. Flammables-area. Do not store near perchlorates, peroxides, chromic acid or nitric acid.

Section 8 - Exposure Controls, Personal Protection

Engineering Controls: Use explosion-proof ventilation equipment. Facilities storing or utilizing this material should be equipped with an eyewash facility and a safety shower. Use adequate general or local exhaust ventilation to keep airborne concentrations below the permissible exposure limits.
Exposure Limits

Chemical Name	ACGIH	NIOSH	OSHA - Final PELs
Ethanol	1000 ppm TWA	1000 ppm TWA; 1900 mg/m3 TWA 3300 ppm IDLH	1000 ppm TWA; 1900 mg/m3 TWA

OSHA Vacated PELs: Ethanol: 1000 ppm TWA; 1900 mg/m3 TWA
Personal Protective Equipment
Eyes: Wear appropriate protective eyeglasses or chemical safety goggles as described by OSHA's eye and face protection regulations in 29 CFR 1910.133 or European Standard EN166.
Skin: Wear appropriate protective gloves to prevent skin exposure.
Clothing: Wear appropriate protective clothing to prevent skin exposure.
Respirators: A respiratory protection program that meets OSHA's 29 CFR 1910.134 and ANSI Z88.2 requirements or European Standard EN 149 must be followed whenever workplace conditions warrant a respirator's use.

Section 9 - Physical and Chemical Properties

Physical State: Clear liquid
Appearance: colorless
Odor: Mild, rather pleasant, like wine or whis
pH: Not available.
Vapor Pressure: 59.3 mm Hg @ 20 deg C
Vapor Density: 1.59

Evaporation Rate: Not available.
Viscosity: 1.200 cP @ 20 deg C
Boiling Point: 78 deg C
Freezing/Melting Point:-114.1 deg C
Decomposition Temperature: Not available.
Solubility: Miscible.
Specific Gravity/Density:0.790 @ 20°C
Molecular Formula:C2H5OH
Molecular Weight:46.0414

Section 10 - Stability and Reactivity

Chemical Stability: Stable under normal temperatures and pressures.
Conditions to Avoid: Incompatible materials, ignition sources, excess heat, oxidizers.
Incompatibilities with Other Materials: Strong oxidizing agents, acids, alkali metals, ammonia, hydrazine, peroxides, sodium, acid anhydrides, calcium hypochlorite, chromyl chloride, nitrosyl perchlorate, bromine pentafluoride, perchloric acid, silver nitrate, mercuric nitrate, potassium-tert-butoxide, magnesium perchlorate, acid chlorides, platinum, uranium hexafluoride, silver oxide, iodine heptafluoride, acetyl bromide, disulfuryl difluoride, tetrachlorosilane + water, acetyl chloride, permanganic acid, ruthenium (VIII) oxide, uranyl perchlorate, potassium dioxide.
Hazardous Decomposition Products: Carbon monoxide, irritating and toxic fumes and gases, carbon dioxide.
Hazardous Polymerization: Will not occur.

Section 11 - Toxicological Information

RTECS#:
CAS# 64-17-5: KQ6300000
LD50/LC50:
CAS# 64-17-5:
Draize test, rabbit, eye: 500 mg Severe;
Draize test, rabbit, eye: 500 mg/24H Mild;
Draize test, rabbit, skin: 20 mg/24H Moderate;
Inhalation, mouse: LC50 = 39 gm/m3/4H;
Inhalation, rat: LC50 = 20000 ppm/10H;
Oral, mouse: LD50 = 3450 mg/kg;
Oral, rabbit: LD50 = 6300 mg/kg;
Oral, rat: LD50 = 7060 mg/kg;
Oral, rat: LD50 = 9000 mg/kg;<br.

Carcinogenicity:
CAS# 64-17-5: Not listed by ACGIH, IARC, NTP, or CA Prop 65.

Epidemiology: Ethanol has been shown to produce fetotoxicity in the embryo or fetus of laboratory animals. Prenatal exposure to ethanol is associated with a distinct pattern of congenital malformations that have collecetively been termed the "fetal alcohol syndrome".
Teratogenicity: Oral, Human - woman: TDLo = 41 gm/kg (female 41 week(s) after conception) Effects on Newborn - Apgar score (human only) and Effects on Newborn - other neonatal measures or effects and Effects on Newborn - drug dependence.

Reproductive Effects: Intrauterine, Human - woman: TDLo = 200 mg/kg (female 5 day(s) pre-mating) Fertility - female fertility index (e.g. # females pregnant per # sperm positive females; # females pregnant per # females mated).
Neurotoxicity: No information available.
Mutagenicity: DNA Inhibition: Human, Lymphocyte = 220 mmol/L.; Cytogenetic Analysis: Human, Lymphocyte = 1160 gm/L.; Cytogenetic Analysis: Human, Fibroblast = 12000 ppm.; Cytogenetic Analysis: Human, Leukocyte = 1 pph/72H (Continuous).; Sister Chromatid Exchange: Human, Lymphocyte = 500 ppm/72H (Continuous).
Other Studies: Standard Draize Test(Skin, rabbit) = 20 mg/24H (Moderate) Standard Draize Test: Administration into the eye (rabbit) = 500 mg (Severe).</br.

Section 12 - Ecological Information

Ecotoxicity: Fish: Rainbow trout: LC50 = 12900-15300 mg/L; 96 Hr; Flow-through @ 24-24.3°CFish: Rainbow trout: LC50 = 11200 mg/L; 24 Hr; Fingerling (Unspecified)Bacteria: Phytobacterium phosphoreum: EC50 = 34900 mg/L; 5-30 min; Microtox test When spilled on land it is apt to volatilize, biodegrade, and leach into the ground water, but no data on the rates of these processes could be found. Its fate in ground water is unknown. When released into water it will volatilize and probably biodegrade. It would not be expected to adsorb to sediment or bioconcentrate in fish.
Environmental: When released to the atmosphere it will photodegrade in hours (polluted urban atmosphere) to an estimated range of 4 to 6 days in less polluted areas. Rainout should be significant.
Physical: No information available.
Other: No information available.

Section 13 - Disposal Considerations

Chemical waste generators must determine whether a discarded chemical is classified as a hazardous waste. US EPA guidelines for the classification determination are listed in 40 CFR Parts 261.3. Additionally, waste generators must consult state and local hazardous waste regulations to ensure complete and accurate classification.
RCRA P-Series: None listed.
RCRA U-Series: None listed.

Section 14 - Transport Information

	US DOT	Canada TDG
Shipping Name:	Not reviewed.	No information available.
Hazard Class:		
UN Number:		
Packing Group:		

Section 15 - Regulatory Information

US FEDERAL

TSCA
CAS# 64-17-5 is listed on the TSCA inventory.
Health & Safety Reporting List
None of the chemicals are on the Health & Safety Reporting List.
Chemical Test Rules
None of the chemicals in this product are under a Chemical Test Rule.
Section 12b
None of the chemicals are listed under TSCA Section 12b.
TSCA Significant New Use Rule
None of the chemicals in this material have a SNUR under TSCA.
CERCLA Hazardous Substances and corresponding RQs
None of the chemicals in this material have an RQ.
SARA Section 302 Extremely Hazardous Substances
None of the chemicals in this product have a TPQ.
SARA Codes
CAS # 64-17-5: acute, chronic, flammable.
Section 313 No chemicals are reportable under Section 313.
Clean Air Act:
This material does not contain any hazardous air pollutants.
This material does not contain any Class 1 Ozone depletors.
This material does not contain any Class 2 Ozone depletors.
Clean Water Act:
None of the chemicals in this product are listed as Hazardous Substances under the CWA.
None of the chemicals in this product are listed as Priority Pollutants under the CWA.
None of the chemicals in this product are listed as Toxic Pollutants under the CWA.
OSHA:
None of the chemicals in this product are considered highly hazardous by OSHA.
STATE
CAS# 64-17-5 can be found on the following state right to know lists: California, New Jersey, Pennsylvania, Minnesota, Massachusetts.

California Prop 65
WARNING: This product contains Ethanol, a chemical known to the state of California to cause developmental reproductive toxicity.
California No Significant Risk Level: None of the chemicals in this product are listed.

European/International Regulations

European Labeling in Accordance with EC Directives
Hazard Symbols:
F
Risk Phrases:
R 11 Highly flammable.

Safety Phrases:
S 16 Keep away from sources of ignition - No smoking.
S 33 Take precautionary measures against static discharges.
S 7 Keep container tightly closed.
S 9 Keep container in a well-ventilated place.

WGK (Water Danger/Protection)
CAS# 64-17-5: 0
Canada - DSL/NDSL
CAS# 64-17-5 is listed on Canada's DSL List.
Canada - WHMIS
This product has a WHMIS classification of B2, D2A.
Canadian Ingredient Disclosure List
CAS# 64-17-5 is listed on the Canadian Ingredient Disclosure List.

Section 16 - Additional Information

MSDS Creation Date: 7/27/1999
Revision #4 Date: 3/18/2003

The information above is believed to be accurate and represents the best information currently available to us. However, we make no warranty of merchantability or any other warranty, express or implied, with respect to such information, and we assume no liability resulting from its use. Users should make their own investigations to determine the suitability of the information for their particular purposes. In no event shall Fisher be liable for any claims, losses, or damages of any third party or for lost profits or any special, indirect, incidental, consequential or exemplary damages, howsoever arising, even if Fisher has been advised of the possibility of such damages.

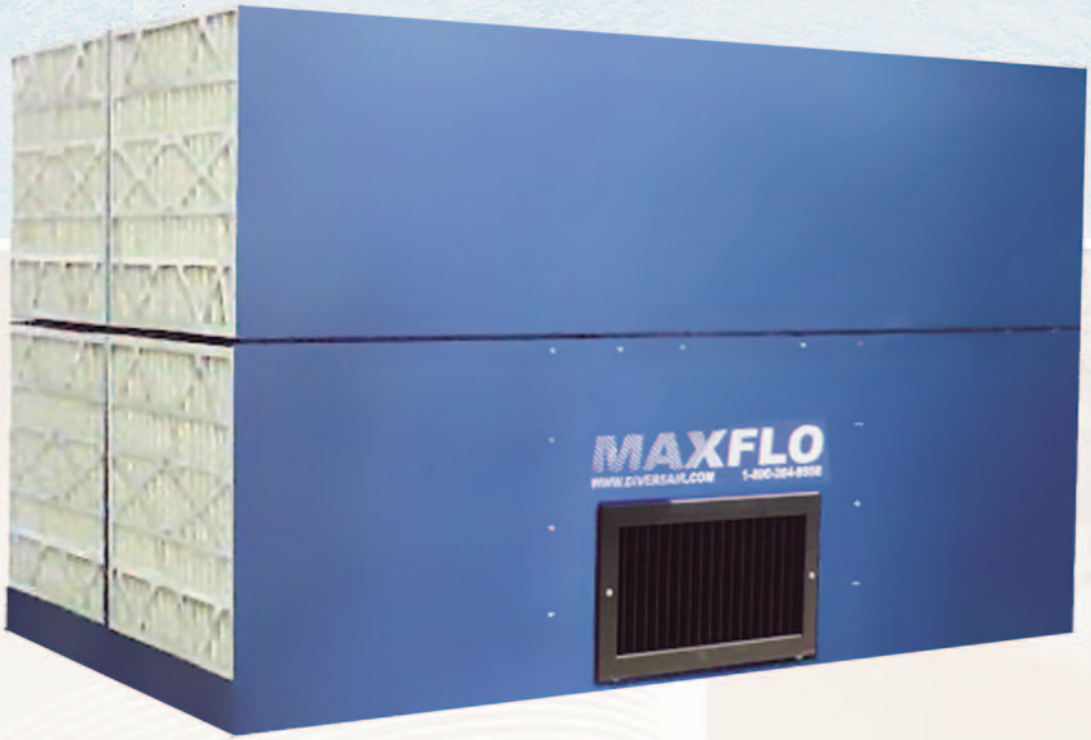
21.9 Equipment Fact Sheets

DIVERSIFIED AIR SYSTEMS, INC.

MAXFLO D-70™ series

Industrial Air Cleaners

- *Economical way to improve air quality 65% to 85%*
- *Helps meet or exceed OSHA standards*
- *Recirculates clean air, eliminating the need for expensive make-up air*



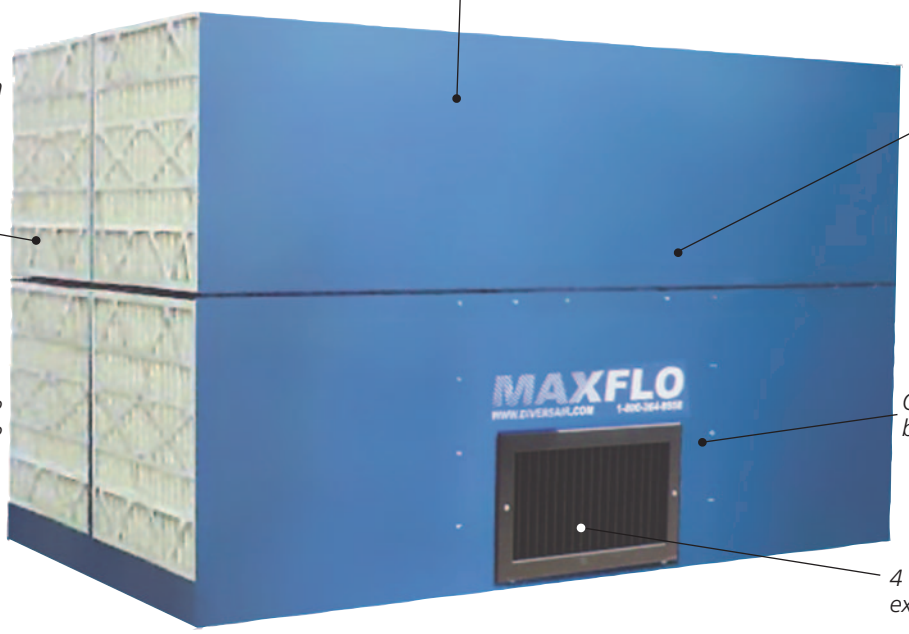
- *Reduces housekeeping costs and improves productivity*
- *Minimal installation required, ceiling or side wall mounted*
- *Ships fully assembled, 7000 to 7600 CFM range*

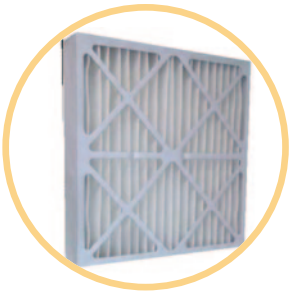
Superior technology generating substantial operating savings

Typical applications for the MAXFLO ambient air cleaners

• Welding Smoke • Grinding Dust • Sanding Dust • Oil Smoke • Coolant Mist • Powders • Odors, etc...

Outstanding MAXFLO D series features

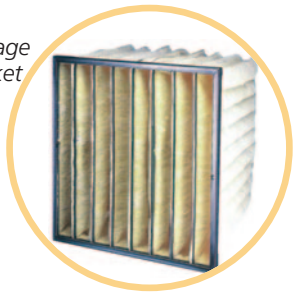




First stage Pleated Prefilters

Made in USA
3 YEAR WARRANTY

Second stage Multi Pocket bag filter

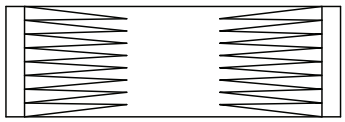


Ideal for automotive industry, welding shops, vocational schools, training facilities, laboratories, wood working shops, metal transforming industries, batching and powder processes.

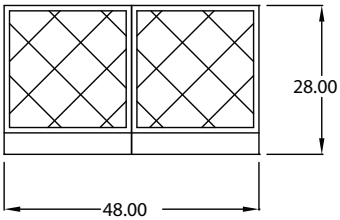
Call today: 800-264-8958

MAXFLO D-70 SERIES SPECIFICATIONS

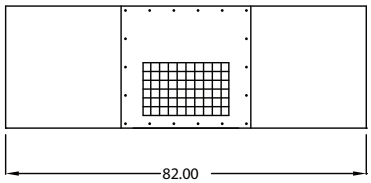
16 GAUGE STEEL CABINET FINISHED WITH A DARK BLUE CHEMICAL RESISTANT POLYURETHANE PAINT, FOUR WAY INDIVIDUALLY ADJUSTABLE BLADES, AND A SINGLE SPEED, BELT DRIVEN MOTOR WIRED TO JUNCTION BOX ON UNIT.



TOP VIEW

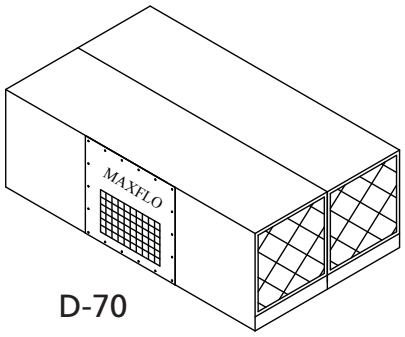


SIDE VIEW



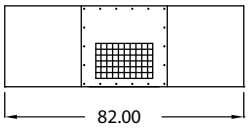
FRONT VIEW

SPECIFICATIONS				
AIRFLOW:	D-70	7000 CFM	PREFILTERS: (Depends on config.)	(4) 4'' Pleated Std. (4) Aluminum Mesh, Opt.
	D-75	7500 CFM		
	D-76	7600 CFM		
ELECTRICAL:	5 HP 208-230/460/3, 14.0-13.0/6.5 Amps		MAIN FILTERS: (Depends on config.)	(4) 22'' 8 pocket 65%, Std. (4) 22'' 8 pocket 95%, Opt.
WARRANTY:	3 Years on all parts except filters		NOISE:	82 dBA
OPTIONS:	Filter replacement gauge, Mounting bracket, Silencer			

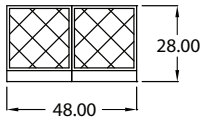


D-70

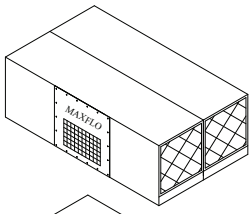
MAXFLO D-70 SERIES OPTIONAL MULTIPLE CABINET CONFIGURATION



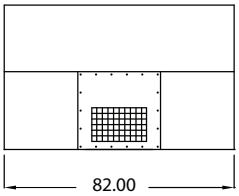
FRONT VIEW



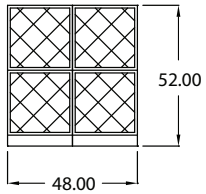
SIDE VIEW



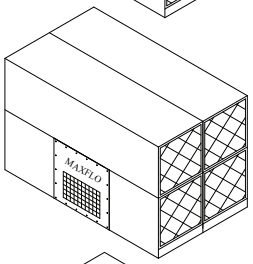
D-70



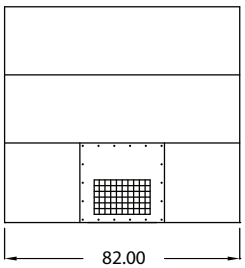
FRONT VIEW



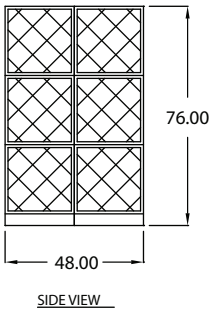
SIDE VIEW



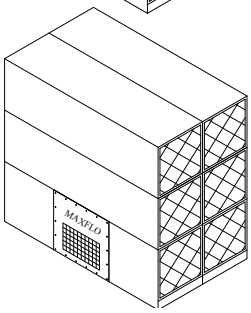
D-75



FRONT VIEW



SIDE VIEW



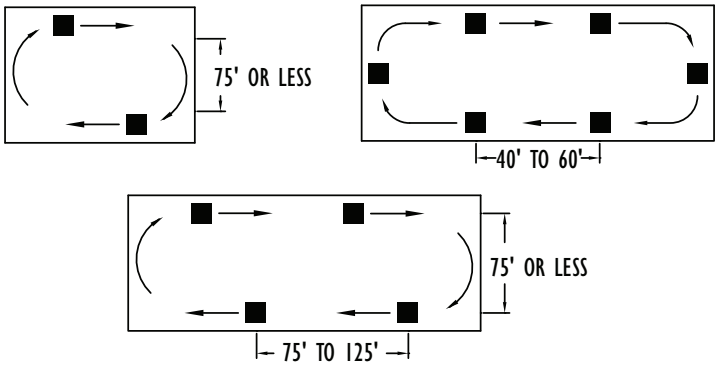
D-76

Call today: 800-264-8958

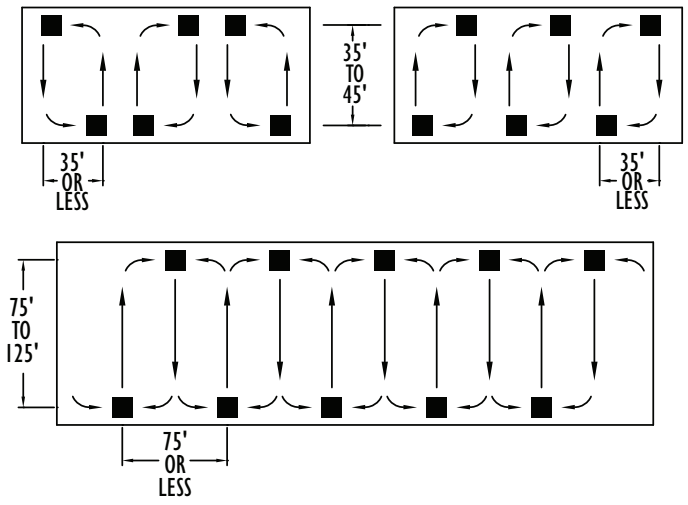
Free Hanging Unducted Air Cleaning Systems

Unducted air cleaning systems consist of one or more interior air cleaners positioned in the overhead plant space to create a planned air circulation airflow pattern. This method effectively cleans the overall ambient air. Unducted systems rely on constant cleaning and mixing of the entire area volume to remove contaminants. Unducted systems never remove 100% of the contaminants. The objective of this method is to achieve a substantial contaminant reduction at steady-state levels. Unducted systems will typically provide an estimated 65 to 85% contaminate reduction. The percentage reduction is base on the number of air changes provided by the unducted air cleaning system.

Recommended Air Flow Patterns



CIRCULAR AIR PATTERNS



CROSS-BAY PATTERNS

TRIDENT

engineering quality air &

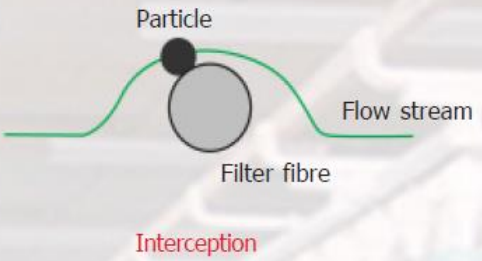
- Very low installation clearance - install anywhere
- Oil removal (coalescing)
- Flow from 60 scfm to 1065 scfm
- Particle removal 0.01 µm
- Maximum oil carryover 0.003 mg/m³

CE

Submicron Filters

Cleansweep

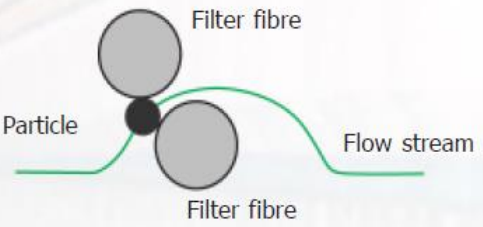
Cleansweep Microfilters are compressed air filtration devices manufactured for industrial use and supported by Trident Pneumatics. Contaminants regularly found in air lines include dust, oil, rust and liquid water. Cleansweep Microfilters remove all these contaminants, thereby ensuring that the products made by you and the services offered by you are not adversely affected by them.



Interception

How Cleansweep Microfilters Work

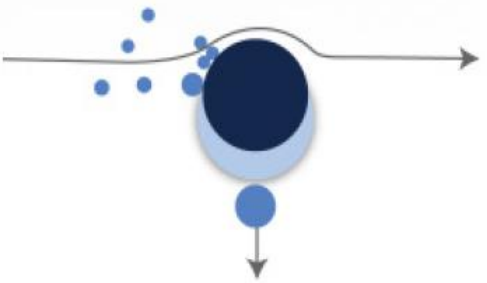
Within the housing of each Cleansweep Microfilter is a filter element. This element has a specially designed multi-layered structure. Compressed air from your supply passes through the various layers sequentially, moving outwards from the centre of the element. Typically, air from an industrial compressor contains oil, condensed water and solid particles such as rust. As the air passes through a Cleansweep filter element, the contaminants are removed through three mechanisms:



Diffusion

Interception

Larger contaminant particles, of size around 10 microns or more, are blocked by a fine-pore medium.


Coalescence

Diffusion

Finer (sub-micron) particles that are carried further into the filter element are enmeshed in fibres. The fibres are made of borosilicate. The trapping action is a result of the labyrinthine pathways through the fibrous medium.

Coalescence

Water and oil particles adhere initially to fibres on impinging on them. They merge to form droplets and are held in the fibrous medium by surface tension.

The contaminant particles are trapped both at the inner surface of the filter element and within the medium. The liquid drops that coalesce in the element flow down, accumulating at the bottom of the housing. A drain valve is provided to discharge the liquids. Clean, contaminant-free air flows out of the Cleansweep Microfilter to your application. Cleansweep Microfilters are ideal for providing compressed air for a very wide range of applications. The quality of the air delivered by a Cleansweep Microfilter is of the level required for sensitive CNC machinery, painting and powder coating systems, packaging machines, textile and garment machinery, instrumentation, pharmaceutical industries, dental clinics, the telecom industry (repelling moisture from underground cables to avoid short circuits), pneumatic control systems, zeolite-type oxygen and nitrogen generators, garages and machine tools.

THE CLEANSWEEP ADVANTAGE

Convenience

Trident Pneumatics has designed Cleansweep Microfilters with a unique design featuring a knock-off type element. This makes Cleansweep filters stand apart from filters produced by other manufacturers, which need to be installed with a significant bottom clearance. This translates to convenience and economy of space for you. You can provide Cleansweep Microfilters with even a very low clearance at the bottom.

Very low installation clearance

Trident filters

Other filters

Economy

The flow path of air in the filter head of a Cleansweep Microfilter is smooth and curved, optimised to offer low resistance. As a result, the pressure drop across the filter is just 0.03 to 0.1 bar. This makes for greater economy, with lower bills for compressed air. Further, the filter element has a large surface area, as a result of which it has a long life, and so the frequency of replacement is low. Cleansweep Microfilters are provided with easy-to-read meters that indicate when filter elements should be replaced (when the pressure drop is greater than 0.4 kg/cm2).

Replacement elements

Safety

All Cleansweep Microfilters have the CE, CRN approval marking. They are all rated for 16 bar operation for extra safety for you and your equipment.

Ergonomics

The shape and size of Cleansweep Microfilters have been designed for convenient installation and maintenance.

Selecting your Cleansweep Microfilter

Cleansweep Microfilters are available in six models, each with four options for the filter element and two drain options so that you can choose the right filtering solution for your industry. Specify the model, element grade and drain option when ordering your Cleansweep Microfilter.

Step 1

The first step in determining the right Cleansweep Microfilter for your requirement is to select the model. Identify the pressure correction factor corresponding to the air pressure of your application from the pressure correction factor table. Divide the flow rate of your application by the pressure correction factor. For example, if the pressure is 73 psi, the pressure correction factor is 0.82. For a flow rate of 60 scfm, the pressure-corrected flow rate is 60/0.82=73 scfm. Choose the model corresponding to the closest flow rate (higher) from the air flow table. For this example, the choice would be the T 250.

Pressure correction factor table

Pressure (Psi)	44	73	102	131	160	189	218	232
Correction factor	0.63	0.82	1	1.18	1.36	1.55	1.73	1.82

Air flow table

Model	T100	T250	T600	T851	T1210	T1810
Flow rate (scfm)	60	150	350	500	710	1065
Weight (Pounds)	2.77	6.6	8	17.1	19	36.5

Step 2

The next step is to determine the grade of the filter element required by you. Use this filter element table to select the grade of element that matches your air quality requirements:

Filter Element Table

Element grade	P	X	Y	A
Filter element	Borosilicate	Borosilicate	Borosilicate	Activated carbon
Particle removal (µm)	5	1	0.01	0.01
Maximum oil carry-over (mg/m^2)	5	0.05	0.01	0.003
Initial pressure loss	0.03	0.06	0.1	0.06
Colour of end cap	Green	Red	Yellow	Black

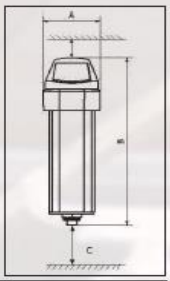
Step 3

The last step is to choose the type of drain valve. Cleansweep Microfilters are fitted with either electro-adjustable (EA) or internal automatic (IA) drain valves.

Drain valve	IA	EA (Optional)**
System	Mechanical (float)	Electrical (energised coil)
Operation	A float opens the drain valve whenever the oil-and-water level crosses a limit.	Periodically discharges accumulated liquids. The interval between discharges may be adjusted.

** (fitting for electric drain valve connection only)

Using the specification matrix provided here, determine the ordering code of the Cleansweep Microfilter corresponding to your requirements and choice. If, for example, you are looking for a filter for an air flow of 60 scfm, you would select the T 250. If the element grade corresponding to your air quality requirements is X and you opt for an IA drain valve, the code you would need to specify is PF128A.



Specification matrix

Model	Element Grade	Item Code		Pipe Size NPT	Housing Dimensions (inch)			Filter Spares**	
		(EA) Drain Type	(IA) Drain Type		A	B	C	Item Code	Weight Pounds
T 100	P	PF151	PF152					AS703	
	X	PF151A	PF152A	1/2"	3.4	11.5	1.96	AS702	0.44
	Y	PF151B	PF152B					AS701	
	A	PF151C	PF152C					AS704	
T 250	P	PF153	PF154					AS695	
	X	PF153A	PF154A	1"	4.4	15.7	1.96	AS694	0.66
	Y	PF153B	PF154B					AS693	
	A	PF153C	PF154C					AS696	
T 600	P	PF155	PF156					AS699	
	X	PF155A	PF156A	1½"	4.4	18.6	1.96	AS698	0.88
	Y	PF155B	PF156B					AS697	
	A	PF155C	PF156C					AS700	
T 851	P	PF168	PF166					AS715	
	X	PF168A	PF166A	2"	5.8	26.2	1.96	AS716	2.20
	Y	PF168B	PF166B					AS717	
	A	PF168C	PF166C					AS718	
T 1210	P	PF179	PF178					AS719	
	X	PF179A	PF178A	2"	5.8	28.9	1.96	AS720	2.64
	Y	PF179B	PF178B					AS721	
	A	PF179C	PF178C					AS722	
T 1810	P	PF171	PF169					AS723	
	X	PF171A	PF169A	3"	8.3	30	1.96	AS724	3.96
	Y	PF171B	PF169B					AS725	
	A	PF171C	PF169C					AS726	

* Available in BSP

Differential pressure gauge as a standard scope.

Element kit includes Element & O-Ring
Float drain assembly AS470
Differential Pressure Gauge AF047

P = 5 micron; X = 1 micron; Y = .01 micron Coalescing;
A = .01 micron Activated Carbon

** Filter spares include element and O-Ring.

Dual-Bed Deionizers

Large Sized Deionization Applications

DM-6100
SERIES

Engineered for large-sized deionization (DI) applications, these dependable units feature the same precision electronics and performance characteristics as their large-scale counterparts, all in a smaller, top mounted package. Applications cover the full spectrum of industrial, commercial, medical and agricultural uses.

Pure Aqua supplies a full line of standard and fully customizable dual bed deionizer systems, all of which are engineered using advanced 3D computer modeling and process design software for accurate and customized solutions.

Applications

- Paints
- Chemicals
- Cosmetics
- Electronics
- Textiles
- Plating
- Ice Plants
- Printing
- Boiler feed
- Film processing
- Electrodeposition
- Metalworking lubricants
- Washes - cars to aircrafts
- Humidification control
- Testing and materials
- Research and development
- Glassware rinse
- Hospitals/Medical
- Horticulture/Greenhouse
- Food/Beverage processing

Standard Features

- Constant monitoring of water quality
- No untreated by-pass water
- Compact, non-corrosive components
- Fiberglass tanks for corrosion resistance
- Auto regeneration on preset water quality limit
- Solid-state reliability for trouble-free service
- Automatic shut down during power failure
- Programmable purge prior to regeneration
- 12 volt operation (120V/1pH/60Hz transformer incl.)
- Convenient, modular construction
- Easy, economical installation
- Optional recirculation pumps
- Parallel or Series regeneration

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Dual-Bed Deionizers

Large Sized Deionization Applications

DM-6100 SERIES

System Features

- Custom programmable for automatic precision and simplicity.
- Delivers duplex DI water more economically than any other deionizer.
- The most advanced DI control package available.
- No other DI controls offer so much performance at such a low price.
- The digital-type meter displays both water quality and regeneration time remaining.
- Tank polisher can be accommodated under the single control to pick up sodium or silica leakage, allowing you to easily attain higher purity water at less expense.
- Single set of printed circuit boards control both simultaneous and sequential regeneration.
- All programming features are easily accessible on the front panel.
- Indicator lights show at a glance which phase of the regeneration cycle the system is in.
- Direct eduction of chemicals through multiport valves eliminates additional valves.
- Automatic pre-rinse prior to regeneration prevents false regeneration and preserves chemicals.
- Monitored final rinse rids the system of residual chemicals before the deionizer returns to service.
- Test ports allow sampling of decationized and final DI water to check system performance.
- Modular construction reduces downtime and simplifies troubleshooting and service.
- Rugged NEMA 12 electrical enclosures, standard on all controls, meet or exceed NEMA showering arc (ICS 2-230), surge withstand (IEEE 587) and electrostatic discharge (MIL-STD-88380).
- Automatic shutoff during power failure stops resins from being exhausted past quality endpoint.
- NOVRAM backup saves all data during a power failure, then returns the display to the last data point when power is restored. No batteries are required.
- A low level sensor can be easily wired into the control to warn of low chemical volumes.
- Provisions for a remote control panel and auxiliary "START REGENERATION" source allow regeneration to be initiated and controlled from a remote location.
- Level controlled shutoff can be utilized with the addition of a float switch in the storage tank.
- Relays and fuses on the power circuit allow for the operation of recirculation and supply pumps.

Model #	Nominal Capacity (Grains)	Service Flow (GPM)		Pipe Size	Mineral Tank Size	Resin Quantity (ft^3)		Shipping Weight (lbs)
		Cont.	Peak			Cation	Anion	
DM24-Q-FRP	200,000	5	35	2"	24"x72"	10	10	2100
DM30-Q-FRP	300,000	7	50	2"	30"x72"	15	15	2900
DM36-Q-FRP	400,000	9	70	2"	36"x72"	20	20	4800
DM42-Q-FRP	600,000	12	100	3"	42"x72"	30	30	6600
DM48-Q-FRP	800,000	15	135	3"	48"x72"	40	40	8500

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Air Control Systems

Packed Bed Chemical Scrubber, Model CS-17

The Model CS-17 is a Packed Bed Chemical Scrubber designed to efficiently remove the gas contaminant from a continuous process stream through a chemical reaction. This system includes fully automated controls to minimize operator interaction. Exhaust gas enters the scrubber and passes through a bed of packed media where it contacts a scrubbing solution to capture the pollutant. The scrubbing solution is introduced in counter-current flow by a liquid distribution spray nozzle.

This model, specifically designed for acid removal, uses dilute caustic as a neutralizing reagent to react with the acid and produce non-volatile, soluble salts and water. A chemical reagent pump adds caustic to fresh water to create a scrubbing solution. This water is then conveyed by the recirculation pump to the spray header to flood the packing where it will interface with the process stream.

The buildup of salts in the scrubbing solution is limited using fresh makeup water and blowdown. The cleaned exhaust stream then passes through a mist eliminator where water droplets are removed. Finally, the cleaned air stream is discharged to the atmosphere.

Base System Components

Stainless Steel Construction
Engineered Internal Packing
Recirculation Pump
Carbon Steel Interconnecting Ductwork
Carbon Steel Process Blower
Carbon Steel Exhaust Stack
NEMA 4 Control Panel
Touchscreen Operator Interface
Liquid Level Controls
Pressure Gauges and Transmitters
Chemical Metering Pump
pH Probe and Analyzer
Immersion Heater (as needed)

Specifications

Removal Efficiency:	95%
Air Flow Capacity:	17,500 ACFM
Pollutant Loading:	32 lbs/hr
Inlet Connection:	42” x 36”
Stack Height:	36’
Stack Diameter:	28”
Scrubber Process Fan:	30 HP, TEFC Motor
Recycle Pump:	150 GPM
Power Requirements:	480 V/ 3 ph / 60 Hz, 53 FLA

Pollution Systems
2170 Buckthorne Place
Suite 160
The Woodlands, Texas 77380

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Fax (713) 456-2666
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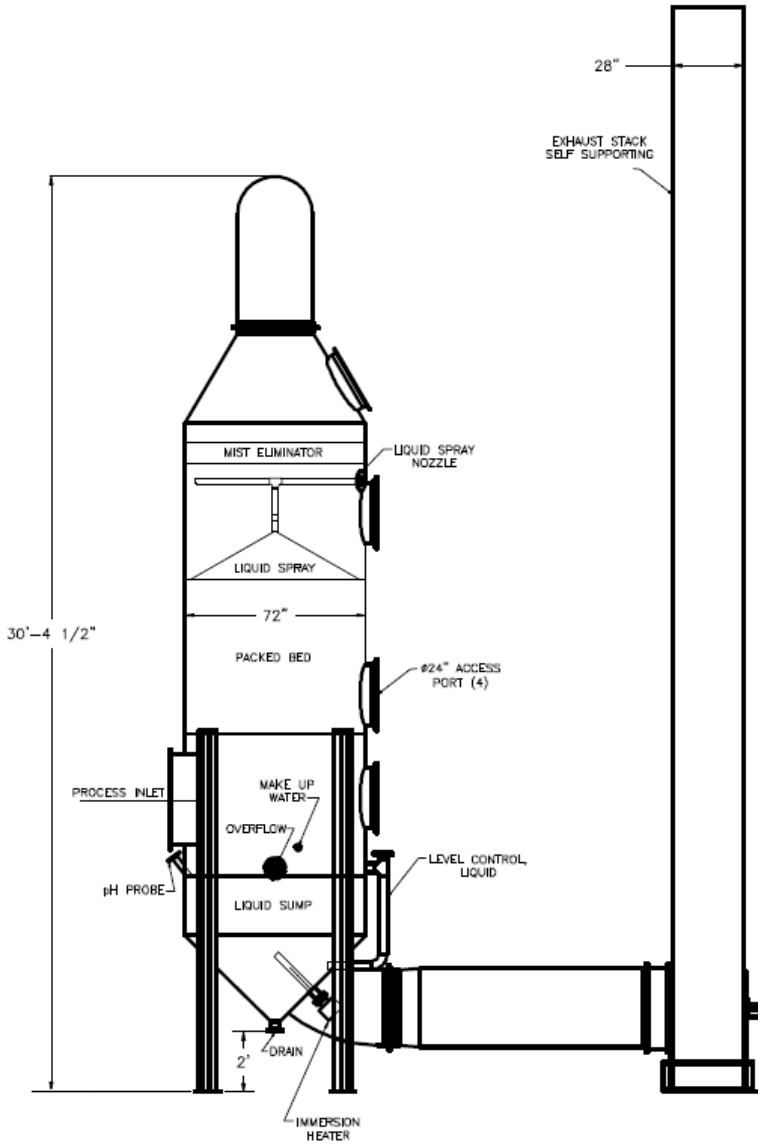

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FILTRES PHILIPPE®

Function :

At HASLER Group, our comprehensive line of Filtres Philippe® horizontal belt filters provide an ideal solution for your mineral or bulk chemical processing applications. New technologies and years of expertise guarantee 98% uptime to ensure you meet your processing goals.
In a filter cloth and rubber belt system, the belt is not only a key operating component, but is also a significant portion of the cost of the system. With this in mind, HASLER Group manufactures and tests the belts in dedicated facilities. Each belt is run on a proprietary robotic machine to remove residual stresses, true edges, cut grooves and bore drain holes. Our belts feature patented chemically bonded belt curbs, and are factory spliced and hot vulcanized to ensure a long operating life.

Key features :

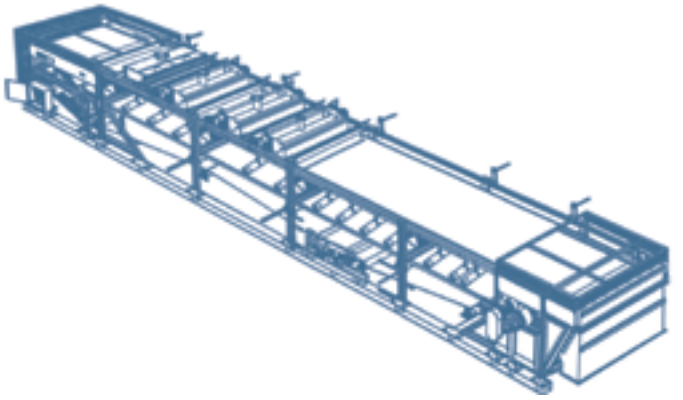
- Available in a wide range of sizes from lab-scale units of 0.25 m² (2.69 ft²) to large production systems of 200 m² (2,152 ft²)
- Multiple washing and drying zones
- Rubber-lined tail and head pulleys with a single steel shaft to ensure corrosion resistance and stiffness that eliminates torsion effects
- Innovative belt support system with water-lubricated endless wear belts and replaceable slides to reduce friction and ensure that the belt remains flat and in contact with the vacuum box
- Cloth tensioning and guiding system features easy adjustment for optimum running efficiency and long cloth life.
- Optimised cloth and belt cleaning to reduce water consumption and extend operating life

Why Filtres Philippe® beltfilters?

Experience and personalised options make Filtres Philippe® the leading choice on the market for your filtration and separation needs. Designing and engineering filters for over a century, we have successfully installed filtration systems in hundreds of leading mineral processing and bulk chemical production facilities worldwide.

At HASLER Group, we focus on increasing productivity and profitability by reducing product loss, operating and maintenance costs and optimising utility usage. We provide a total package with engineering and installation support for all civil and mechanical works.

Series	Filtration area	
Series 1	Minimum m^2 (ft^2)	Maximum m^2 (ft^2)
Series 2	0.25(2.69)	1.40(15.26)
Series 3	(10.76)	15(161.45)
Series 4	18(193.75)	142(1528.48)
Series 5	75(807.3)	200(2152.8)



Vacuum box

rubber belt

Cake discharge

Product specifications

Frame materials

- Standard: Painted carbon steel
- Optional: 316, 316L, 304L stainless steel. Vacuum belt construction
- Standard : SBR (HT ot LT), hot vulcanized with cold bonded curbs (Natural rubber)
- Option : belt : EPDM or Butyl rubber, curbs : EPDM

Filter cloth media

- Materials: monofilament, multifilament, and felt
- Weave style: plain, twilled, or satin
- Wide range of mesh sizes, porosities and breaking strengths available

Operating parameters

- Temperature: up to 230° F (110° C)
- Vacuum: -100mm Hg up to -650mm Hg
- Belt speed: 3.28 ft./minute (1m/minute) to 164 ft./minute (50m/minute)
- Cake thickness: .12 in. (3 mm) to 4.72 in. (120 mm)
- Final moisture content: as low as 4%
- Minimum Temp : - 5°C
- Minimum environment Temp : +5°C

Standard features

- Factory-vulcanized endless belt with patented belt curbs that flex and follow pulleys closely
- Structural steel frame with no welded parts
- Easily accessible pneumatic tilting vacuum box
- Belt support system featuring water-lubricated endless wear belts and replaceable HDPE slide rails
- Rubberized pulleys with one-piece steel shaft
- Rigid filtrate/gas pipes
- Fully automatic cloth tracking system
- Pneumatically driven cloth tension system with no counterweights
- Drip-proof discs to protect pulley and roller bearings

Optional features

- Choice of single or multiple discrete wash-dry zones
- Wide range of feeder styles available to suit your slurry and process
- Washing boxes and mobile dams to meet your application needs
- Available high-pressure and acid washing of filter cloth
- Dry or wet cake discharge

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CRYSTAL QUEST®
Thunder Reverse Osmosis

Installation and Maintenance Instructions

Models

4000, 5000, & 7000

Introduction

Your Thunder commercial reverse osmosis system is a durable piece of equipment which, with proper care, will last for many years. This User's Manual outlines installation, operating, maintenance, and troubleshooting details vital to the sustained performance of your system.

The test results which are included with this User's Manual indicates your system's permeate (product) and rejection test results.

If your system is altered at the site of operation or if the feed water conditions change, please contact your local dealer or distributor to determine the proper recovery for your application. **NOTE:** In order to maintain warranty, an operating log must be maintained. Copies must be sent to your local dealer or distributor for review.

NOTE: Prior to operating or servicing the Thunder commercial reverse osmosis system, this User's Manual must be read and fully understood. Keep it and other associated information for future reference and for new operators or qualified personnel near the system.

Safety

The Safety section of this User's Manual outlines the various safety headings used throughout this manual's text and are enhanced and defined below:

NOTE: Indicates statements that provide further information and clarification.

CAUTION: Indicates statements that are used to identify conditions or practices that could result in equipment or other property damage.

WARNING: Indicates statements that are used to identify conditions or practices that could result in injury or loss of life. **FAILURE TO FOLLOW WARNINGS COULD RESULT IN SERIOUS INJURY OR EVEN DEATH.**

Labeling

Do not under any circumstance; remove any Caution, Warning, or other descriptive labels from the system.

System Specifications

Model	**Thunder 4000**	**Thunder 5000**	**Thunder 7000**
Gallons Per Day	4000	5000	7000
Dimensions (approx.)	30" x 38" x 47"	30" x 38" x 47"	30" x 38" x 47"
Weight (approx.)	235 lbs.	250 lbs.	265 lbs.
Element Size (in.)	HF1-4040	HF1-4040	HF1-4040
Elements (qty.)	**2**	**3**	**4**
Pressure	150 psi	150 psi	150 psi
Motor HP	1	1.5	1.5
Voltage	120/220 Volt 1 Phase	120/220 Volt 1 Phase	120/220 Volt 1 Phase
Hertz	60	60	60
Feed Connection	1"	1"	1"
Product Connection	1"	1"	1"

Waste Connection	1"	1"	1"
Minimum Feed (gpm)	6	7	10
Recovery*	33%-50%	33%-50%	33%-50%

* Recovery ratio may vary between 33% - 50% and up to 75% if system projections have been provided.

Feed Water & Operation Specifications

Nothing has a greater effect on a reverse osmosis system than the feed water quality. NOTE: It is very important to meet the minimum feed water requirements. Failure to do so will cause the membranes to foul and void the warranty.

Feed Water Specifications

Free Chlorine	<0 ppm	Manganese	<0.05 ppm
Total Dissolved Solids	<2000 ppm	Organics	<1 ppm
Turbidity (SDI)	<5	Silica	<1 ppm
pH	3-11	Iron	<2 ppm in Concentrate
Temperature	40°F-105°F	Hardness	<I5gpg

The manufacturer has provided you with the suggested operation specifications. These specifications should be met in order to have the reverse osmosis system perform optimally. All operation specifications are based on the test conditions listed below.

Operating Specifications

Minimum Feed Pressure	35 psi	Minimum NaCI Rejection	96%
Nominal % Rejection	98.5%	Maximum Hardness	I5gpg
Maximum TDS	2000 ppm	Operating Pressure	150 psi
pH Range	3-11	Maximum Temperature	105°F

Test Conditions: Permeate flow and salt rejection based on 550 ppm , 150 psi, 77°F (25°C), pH 7, and 50% recovery as indicated. **NOTE:** Higher TDS and/or lower temperatures will reduce the system's production.

Rejection, Recovery, & Flow Rates

Thunder reverse osmosis systems are designed to produce purified water at the capacities indicated by the suffix in the system's name under the conditions listed above. For example, the Thunder 7000 produces 7000 gallons per day of purified water @ 77°F.

The amount of total dissolved solids (TDS) rejected by the membrane is expressed as a percentage. For example, a 99% rejection rate means that 99% of total dissolved solids do not pass through the membrane. To calculate the % rejection, use the following formula:

% Rejection = [(Feed TDS - Product TDS) / Feed TDS] x 100

NOTE: All TDS figures must be expressed in the same units, usually parts per million (ppm) or milligrams per liter (mg/l).

Thunder commercial reverse osmosis systems are designed to reject up to 98.5% NaCI, unless computer projections have been provided and state a different rejection percentage.

The amount of purified water recovered for use is expressed as a percentage. To calculate % recovery, use the following formula:

% Recovery = (Product Water Flow Rate / Feed Water Flow Rate) x 100

NOTE: All flow rates must be expressed in the same units, usually gallons per minute (gpm).

System Requirements & Operation Guidelines

Plumbing

The membranes and high pressure pumps used on Thunder systems require a continuous flow of water with a minimum feed pressure of 35 psi, which does not exceed 105℉.

The piping for the feed line is a 1" Solenoid Valve This is connected to the filter housing.

The piping used for the concentrate line is 1" FNPT and should be run to an open drain in a free and unrestricted manner (no backpressure).

The piping used for the permeate line is 1" FNPT and can be run to the holding tank or directly to the point-of-use application with PVC pipe, or another FDA approved material This is so the material being used does not dissolve into the permeate water. Be certain that all of the components of the feed water are soluble at the concentrations attained in the system. A system operating at 50% recovery, concentrates all impurities by two times the amount.

CAUTION: Any restrictions or blockage in the drain line can cause backpressure, which will increase the system's operating pressure. This can result in damage to the system's membranes and components.

Electrical

The motors used on Thunder 4000, 5000, & 7000 systems are pump and motor combinations. The standard Thunder 4000, 5000 and 7000 are available in 1 phase120/220 volt 50/60 hertz. The systems may be upgraded to 220/460 volt 3 phase 50/60 hertz.

Please ensure that the electrical circuit supplying the system is compatible with the requirements of the specific Thunder model you are installing.

NOTE: We recommend that a licensed electrician wire your system in accordance with local and national electrical codes (NEC).

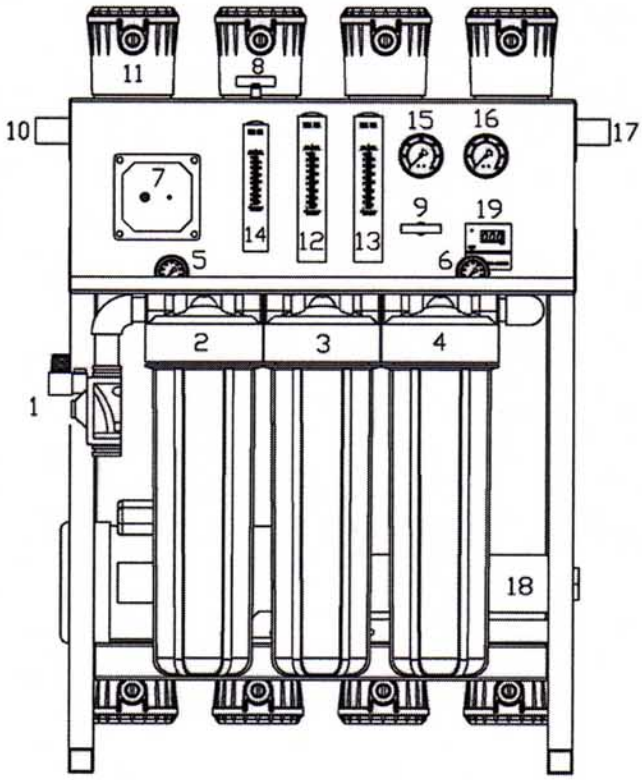
WARNING: To reduce the risk of electrical shock, the incoming power supply must include a protective earth ground.

Each Thunder system is equipped with a 5 foot electrical cord.

Pre-Filtration

Thunder systems are supplied with a 5 micron sediment filter a 10 micron carbon block and a 1 micron sediment filter. Change the cartridge at least every month or when a 10-15 psi differential exists.

NOTE: The system must be operated on filtered water only. Do not attempt to clean used filter cartridges.



Number Identification

1. Solenoid Valve – Turns On/Off Feed Water
2. 5 micron Sediment – Removes particulates
3. 10 Micron Carbon Block - Removes Chlorine
4. 1 micron Sediment- Removes particulates
5. Pressure Gauge- Measures feed pressure
6. Pressure Gauge- Measures pressure after filters
7. RO Computer - Controls RO system
8. Recycle Valve – Recycles concentrate back to feed (If Applicable)
9. Concentrate Valve – Controls flow of waste (concentrate) water to the drain
10. Permeate Out- Connect this to your storage tank (POU)
11. Pressure Vessels – Holds Membranes
12. Flow Meter – Measures flow of permeate water
13. Flow Meter – Measures flow of concentrate water
14. Flow Meter - Measures flow of concentrate recycle (If Applicable)
15. Pressure Gauge – Measures Pump feed pressure
16. Pressure Gauge – Measures Concentrate pressure
17. Concentrate Out - Connect this to an open drain
18. RO Pump- Pressurizes RO System
19. Conductivity Meter – Measures Quality of Permeate

NOTE: Some permeate will be produced during the auto flush; therefore, an overflow for the permeate storage tank is recommended.

Pump Bypass Valve

The pump bypass valve is installed as a standard feature on the Thunder 4000, 5000, & 7000 reverse osmosis systems. It provides an adjustment for pump pressure, which will vary as the required system pressure changes. Note that with a multi-stage pump, the pump flow decreases as the operating pressure increases.

As the feed water temperature decreases, and/or the feed water TDS increases, the system will require a higher operating pressure to produce the specified permeate flow. A Thunder system installed in Florida may provide the specified permeate flow of 3.47 gpm at 150 psi; however the same system installed in Maine - much colder feed water - may require 190 psi to produce the same amount of permeate. The system in Florida would have a higher concentrate flow to the drain because of the lower operating pressure, which would result in poor system recovery.

% Rejection = (Feed TDS - Product TDS)/(Feed TDS) x 100

Membrane Removal & Replacement

Changing membranes in pressure vessels is an easy process if you have the proper information and tools at hand. Please refer to the following instructions when removing and replacing membrane elements:

1. Remove the end caps from the top of the membrane housings. This is done by removing the white Nylon snap ring of the Champ housing or unscrewing the bolts of the housing, which holds on the clamp.

2. Remove the membrane bag containing the membrane element from the shipping box.

3. Cut the bag open as close as possible to the seal at the end of the bag, so the bag may be re used if necessary.

4. Remove the membrane element from the bag and remove the black core tube protectors from each end of the membrane.

5. Remove parts from the parts container (if included) and inspect. Make sure that all parts are clean and free from dirt. Examine the brine seal, and permeate tube for nicks or cuts. Replace the 0-rings or brine seal if damaged.

6. Flow directions should be observed for installation of each element in each housing.

As time progresses, the efficiency of the membrane will be reduced. In general, the salt rejection does not change significantly until two or three years after installation when operated on properly pretreated feed water. The permeate flow rate will begin to decline slightly after one year of operation, but can be extended with diligent flushing and cleaning of the system. A high pH and/or precipitation of hardness can cause premature loss in rejection of membrane elements in the system.

To replace the membrane elements:

1. Remove all of the membrane element(s) from the membrane element housings from the top of the housing. Heavy-duty pliers and channel lock pliers may be necessary to pull the old membrane element out of the membrane element housing.

2. Install the brine seal side of the membrane elements first. When the housings have a direction of flow from bottom to top, the brine seal should be located on the end of the membrane element at the bottom of the housing.

3. Lubricate the brine seal with a food grade lubricant.

4. At a slight angle insert membrane while slightly rotating element being careful not to tear or flip the brine seal. Re-lube the brine seal if necessary.

5. With a smooth and constant motion, push the membrane element into the housing so that the brine seal enters the housing without coming out of the brine seal groove. A slow twisting motion should be used to insert the membrane element, to ensure that the brine seal stays in place.

6. Re-install the end caps by gently twisting the end cap while pushing it onto the housing. Ensure that you do not pinch or fatigue any 0-rings while pushing the end plug on. Push the end plug on until the outer diameter of the plug is flush with the outer diameter of the membrane housing.

7. Insert nylon snap ring until fully seated. Snap ring must be able to be spun in place if fully seated. If you are using stainless steel housing, Install the clamps halves, and tighten bolts until the clamp halves meet.

8. Reconnect any fittings that may have been disconnected when the membrane element housings were disassembled.

9. To Start-Up the system, refer to Start-Up

CAUTION: New or factory cleaned membranes are shipped in a preservative solution. New or cleaned membranes must be flushed for at least 1 hour to remove the preservative from the membrane. Discard all of the permeate and concentrate, which is produced during the flush.

Membrane Cleaning

Periodic cleaning of the membrane(s) can improve system performance. In normal operation, mineral scale, biological matter, colloidal particles, and organic substances can foul the membranes.

WARNING: Cleaning chemicals are dangerous and can cause injury and damage to the environment. Read and comply with all safety and disposal precautions listed on the Material Safety Data Sheets (MSDS's). It is the user's responsibility to comply with all applicable federal, state, and local regulations.

Organic Foulant Cleaning

The following cleaning procedures are designed specifically for membranes that have been fouled with organic matter. Review the general cleaning instructions for information that is common to all types of cleaning such as suggested equipment, pH and temperature limits, and recommended flow rates.

Safety Precautions

1. When using any chemical indicated here in subsequent sections, follow accepted safety practices. Consult the chemical manufacturer for detailed information about safety, handling and disposal.

2. When preparing cleaning solutions, ensure that all chemicals are dissolved and well mixed before circulating the solutions through the membrane elements.

3. It is recommended the membrane elements be flushed with good-quality chlorine-free water after cleaning. Permeate water is recommended; but a de-chlorinated potable supply or pre-filtered feed water may be used, provided that there are no corrosion problems in the piping system. Operate initially at reduced flow and pressure, to flush the bulk of the cleaning solution from the elements before resuming normal operating pressures and flows. Despite this precaution, cleaning chemicals will be present on the permeate side following cleaning. Therefore, permeate must be diverted to drain for at least 10 minutes or until the water is clear when starting up after cleaning.

4. During recirculation of cleaning solutions, the temperatures must not exceed 50°C at pH 2-10, 35°C at pH 1 -11, and 30°C at pH 1 -12.

5. For membrane elements greater than six inches in diameter, the flow direction during cleaning must be the same as during normal operation to prevent element telescoping, because the housing thrust ring is installed only on the reject end of the housing. This is also recommended for smaller elements.

Cleaning Procedures

There are seven steps in cleaning membrane elements with organics.

1. Make up the cleaning solution listed from Table 1.

Table 1: Organic Cleaning Solution

- Preferred 0.1% (wt) Soda Ash
- PH 12, 30°C maximum
- Preferred 0.1% (wt) NaOH 0.025% (wt)
- « PH 12, 30°C maximum

Notes:

(wt) Denotes weight percent of active ingredient.
Cleaning chemical symbols in order used: NaOH is sodium hydroxide.

Cleaning the Organics from Membrane Elements

2. Low-flow pumping. Pump mixed, preheated cleaning solution to the vessel at conditions of low flow rate (about half of that shown in Table 2) and low pressure to displace the process water. Use only enough pressure to compensate for the pressure drop from feed to concentrate. The pressure should be low enough that essentially no permeate is produced. A low pressure minimizes re-deposition of dirt on the membrane. Dump the concentrate, as necessary, to prevent dilution of the cleaning solution.

Table 2: Recommended Feed Flow Rate Per Housing During High Flow Rate Re-Circulation

- Dependent on number of elements in pressure vessel.
- « 4-Inch full-fit elements should be cleaned at 12-14 gpm (2.7-3.2 m3/hr).

3. Re-circulate. After the process water is displaced, cleaning solution will be present in the concentrate stream that can be recycled to the cleaning solution tank. Recycle the cleaning solution for 15 minutes or until there is no visible color change. If a color change occurs, dispose of the cleaning solution and prepare a new solution as described in step 2.

4. Soak. Turn the pump off and allow the elements to soak. Soak the elements for 1 -15 hours (soaking overnight will give best results). To maintain temperature during an extended soak period, use a slow recirculation rate (about 10 percent of that shown in Table 2). Soak time will

vary depending on the severity of the fouling. For lightly fouled systems, a soak time of 1-2 hours is sufficient.

5. High-flow pumping. Feed the cleaning solution at the rates shown in Table 2 for 45 minutes. The high flow rate flushes out the foulants removed from the membrane surface by the cleaning. If the elements are heavily fouled, using a flow rate that is 50 percent higher than shown in Table 2 may aid cleaning. At higher flow rates, excessive pressure drop may be a problem. The maximum recommended pressure drops are 15 psi per element or 50 psi per multi-element vessel, whichever value is more limiting.

6. Flush out. Prefiltered raw water can be used for flushing out the cleaning solution, unless there will be corrosion problems (e.g., stagnant seawater will corrode stainless steel piping). To prevent precipitation, the minimum flush out temperature is 20°C. The system should be flushed for 1 hour.

7. The system should be restarted. Elements and the system need to stabilize before taking any data. The stabilization period will vary depending on the severity of the fouling. To regain optimum performance, it may take several cleaning and soak cycles.

NOTE: Recommendations made here are specifically designed for the membrane elements inserted in the Titan™ reverse osmosis and nanofiltration elements. These recommendations, such as cleaning procedures and chemicals employed, may not be compatible with other brands of membrane elements. It is your responsibility to ensure the suitability of these recommendations and procedures if they are applied to membrane elements other than those which come with your system.

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Additional Information

By experience, the cleaning solution of Na4EDTA with caustic has been found to be slightly less effective than a standard caustic solution or a solution of caustic and Na-DSS. For any solution, contact time is critical. Several overnight soaks may be necessary to restore the system performance. After the elements are clean it is very beneficial to clean one additional time in order to clean off the last remaining biofilm layer on the surface of the membrane. Any remaining biofilm will tend to attract and trap dirt, so an extra cleaning will increase the time between cleanings. For industrial systems where the permeate or product water is not used for drinking, a non-oxidizing biocide can be used prior to step 1 of the cleaning procedure to kill any bacteria or biofilm in the system. Please refer to separate instructions on methods for sanitizing membrane systems (i.e., "Sanitization with DBNPA - Tech Facts"). If the only choice for a sanitizing agent is an oxidant, such as hydrogen peroxide, the system must be cleaned before sanitization.

Inorganic Foulant Cleaning

The following cleaning procedures are designed specifically for membranes that have been fouled with organic matter. Review the general cleaning instructions for information that is common to all types of cleaning such as suggested equipment, pH and temperature limits, and recommended flow rates.

Safety Precautions

1. When using any chemical indicated here in subsequent sections, follow accepted safety practices. Consult the chemical manufacturer for detailed information about safety, handling and disposal.

2. When preparing cleaning solutions, ensure that all chemicals are dissolved and well mixed before circulating the solutions through the membrane elements.

3. It is recommended the membrane elements be flushed with good-quality chlorine-free water after cleaning. Permeate water is recommended; but a de-chlorinated potable supply or pre-filtered feed water may be used, provided that there are no corrosion problems in the piping system. Operate initially at reduced flow and pressure, to flush the bulk of the cleaning solution from the elements before resuming normal operating pressures and flows. Despite this precaution, cleaning chemicals will be present on the permeate side following cleaning. Therefore, permeate must be diverted to drain for at least 10 minutes or until the water is clear when starting up after cleaning.

4. During recirculation of cleaning solutions, the temperatures must not exceed 50°C at pH 2-10, 35°C at pH 1-1 1 , and 30°C at pH 1-12.

5. For membrane elements greater than six inches in diameter, the flow direction during cleaning must be the same as during normal operation to prevent element telescoping, because the housing thrust ring is installed only on the reject end of the housing. This is also recommended for smaller elements.

Cleaning Procedures

There are seven steps in cleaning membrane elements with Inorganics. 1 .

Make up the cleaning solution listed from Table 1 . Table 1: Inorganic

Cleaning Solution

- Preferred 2.0% (wt) Citric Acid PH 2, 45°C maximum
- Alternate Muriatic Acid

Notes:

(wt) denotes weight percent of active ingredient.
Cleaning chemical symbols in order used: HCI is hydrochloric acid (Muriatic Acid).

Cleaning the Inorganics from Membrane Elements

- Alternative 1.0%
- Alternative 0.5% HsPCU

Notes:

1 (wt) denotes weight percent of active ingredient.
2 Cleaning chemical symbols in order used: HCI is hydrochloric acid (Muriatic Acid).

2. Low-flow pumping. Pump mixed, preheated cleaning solution to the vessel at conditions of low flow rate (about half of that shown in Table 2) and low pressure to displace the process water. Use only enough pressure to compensate for the pressure drop from feed to concentrate. The pressure should be low enough that essentially no permeate is produced (approx. 60 psi). A low pressure minimizes redeposition of dirt on the membrane. Dump the concentrate, as necessary, to prevent dilution of the cleaning solution.

Table 2: Recommended Feed Flow Rate Per Housing During High Flow Rate Re-Circulation

Element Diameter	PSI	GPM
2.5 Inches	20 - 60	3 - 5
4 Inches	20-60	8-10

- Dependent on number of elements in pressure vessel.
- 4-Inch full-fit elements should be cleaned at 12-14 gpm (2.7-3.2 m3/hr).

3. Re-circulate. After the process water is displaced, cleaning solution will be present in the concentrate stream that can be recycled to the cleaning solution tank. Recycle the cleaning solution for 10 minutes or until there is no visible color change. If at anytime during the circulation process there is a change in pH or a color change, dispose of the solution and prepare a new solution as described in step 2. A pH of 2 must be maintained for the cleaning to be effective.

4. Soak. Turn the pump off and allow the elements to soak. Soak the elements for 1-15 hours (soaking overnight will give best results). To maintain temperature during an extended soak period, use a slow recirculation rate (about 10 percent of that shown in Table 2). Soak time will vary depending on the severity of the scaling. For lightly scaled systems, a soak time of 1-2 hours is sufficient.

5. High-flow pumping. Feed the cleaning solution at the rates shown in Table 2 for 10 minutes. The high flow rate flushes out the foulants removed from the membrane surface by the cleaning. If the elements are heavily fouled, using a flow rate that is 50 percent higher than shown in Table 2 may aid cleaning. At higher flow rates, excessive pressure drop may be a problem. The maximum recommended pressure drops are 15 psi per element or 50 psi per multi-element vessel, whichever value is more limiting.

6. Flush out. Prefiltered raw water can be used for flushing out the cleaning solution, unless there will be corrosion problems (e.g., stagnant seawater will corrode stainless steel piping). To prevent precipitation, the minimum flush out temperature is 20°C. The system should be flushed for one hour.

7. The system should be restarted. Elements and the system need to stabilize before taking any data. The stabilization period will vary depending on the severity of the fouling. To regain optimum performance, it may take several cleaning and soak cycles.

NOTE: Recommendations made here are specifically designed for the membrane elements inserted in the Thunder reverse osmosis and nanofiltration elements. These recommendations, such as cleaning procedures and chemicals employed, may not be compatible with other brands of membrane elements. It is your responsibility to ensure the suitability of these recommendations and procedures if they are applied to membrane elements other than those which come with your system.

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Additional Information

Never recirculate the cleaning solution for longer than 20 minutes. With longer recirculation, the carbonate scale can reprecipitate and end up back on the membrane surface, making it more difficult to

clean. Carbonate scale reacts with HCl releasing carbon dioxide gas. Depending on the severity of the fouling, it may take repeated cleanings to remove all the scale. Cleaning severe scale may not be economical and element replacement may be the best choice.
Citric acid was originally used as a cleaner for cellulose acetate membranes and is not as effective with thin film composite chemistry. Further, it has a disadvantage of being a nutrient source for systems, which have biological fouling. It is, however, easier to handle than HCl and is included as a primary cleaner for that reason.

Flushing the System

The system should be flushed weekly to remove sediment from the surface of the membranes. To manually flush the system following the preceding steps:

1. The system must be running during the flushing procedure.

2. Fully open the concentrate valve
3. Allow the system to run for 10 to 20 minutes.

4. After 10 to 20 minutes, close the concentrate valve to its previous position, raising the operating pressure to 150 psi. Ensure the proper concentrate flow rate is going to the drain.

5. The system is now ready to operate.

Draining the System for Shipment

Prior to shipping or storing your system, the system should be cleaned with an appropriate cleaner, flushed with water, and protected from biological attack with an appropriate solution for membrane elements. The membrane housing(s) and plumbing lines of the system must be completely drained. Any water remaining in the plumbing of a system may freeze, causing serious damage. The party shipping or storing the system is responsible for any damage resulting from freezing.

To drain the system:

1. Disconnect the inlet, concentrate, pre-filter, and permeate plumbing.

2. Drain all water from the pre-filter cartridge housings by unscrewing the housings, removing the pre-filter cartridges, and drain the water from the housings.

3. Disconnect the tubing from the connector on the permeate and concentrate inlets and outlets.

4. Fully open the concentrate valve.

5. Drain the flow meters by disconnecting the tubing from the bottom fitting of each meter.

6. Allow the system to drain for a minimum of eight hours or until the opened ports quit dripping.

7. After draining is complete, reconnect all of the plumbing.

Troubleshooting

If the system production declines or the system stops working, check the mechanical components for any visual problems. Listed below are the items to check for any visual problems. Listed below are the items to check for two of the most commonly encountered problem conditions: Low system pressure and abnormal permeate flow. Also refer to the reverse osmosis troubleshooting matrix on the next page.

Low System Pressure

Low system pressure occurs when sufficient feed water pressure and flow are not obtained. This causes the high-pressure reverse osmosis pump to cavitate. Failure to provide the proper feed will result in lower system pressure that may result in low production and poor rejection. Check the following components:

1. **Pump:**

   Isolate the pump and determine how much pressure can be achieved. This can be determined by checking the pump discharge pressure gauge at this point. If the system is not equipped with this gauge, disconnect the hose that runs from the pump to the pressure vessel. Install a pressure gauge. The pressure of the pump must reach at least 190 psi when the flow is restricted.

2. **Pre-Filter:**

   Check the differential in the pre-filter gauges to determine if the filter needs to be replaced. If the system is not equipped with these gauges, examine the pre-filter cartridge to make sure that it is not clogged and does not restrict feed flow to the pump. Replace, if necessary.

3. **Low Feed Water Flow Rate:**

   Determine that the system is getting a sufficient volume of feed water. Disconnect the feed water hose from the system and place it in a one gallon bucket. Measure the time it takes to fill the bucket to determine the feed flow. (Feed flow is measured in gallons per minute, so divide 1 gallon by the time in minutes to obtain the flow rate). Refer to the System Specifications for the required feed flow.

4. **Inlet Solenoid Valve:**

   Feed water enters the system through an automatic solenoid shut-off valve, which is normally closed. Ensure that the solenoid opens when the reverse osmosis pump starts. The system can be operated without the solenoid for troubleshooting. Remove the solenoid to see if it is contributing to the problem. Normally, cleaning the solenoid diaphragm will correct any malfunction of the solenoid.

5. **Electric:**

   Check to ensure that there are no electrical fuses blown and that all electrical connections are secure. Use a voltmeter to verify that the motor is getting sufficient power.

6. **Pressure Gauge:**

   Check for foreign matter on the gauge fitting. Remove any visible matter and replace the fitting. Verify that the tube is not pushed too far inside the fitting. This could restrict flow and cause an inaccurate display. If the fitting and tube are fine and the pressure gauge is still malfunctioning, the gauge should be replaced.

7. **Concentrate Control Valve**:

   The concentrate control valve may have a tear in the diaphragm. Remove the valve, inspect the diaphragm, and replace if necessary.

Pump

The pumps used on Thunder 4000, 5000, & 7000 systems are pump and motor combinations. They are multi-stage centrifugal pumps. Follow these guidelines to ensure proper operation of the pump:

- The pump must NEVER be run dry. Operating the pump without sufficient feed water will damage the pump.
- ALWAYS feed the pump with filtered water. The pump is susceptible to damage from sediment and debris.
- If any damage occurs to your system's pump a re-build kit may be available. Contact your local dealer or distributor and inform them of your system's model and pump size.

Mounting

The free standing system should be bolted down in compliance with local regulation standards or securely fastened.

Membrane Elements

Thunder reverse osmosis systems come pre-loaded with Thin Film Composite (TFC) High Flow Low Energy membranes, unless otherwise specified. General membrane element performance characteristics are listed below:

	Membrane Element Characteristics		
Operating Pressure	150psi	Maximum Pressure	400 psi
Nominal % Rejection	98.5%	Chlorine Tolerance	<1 ppm
Maximum Temperature:	110°F	Turbidity	1 NTU
Silt Density Index	<5 SDI	pH Range	3-11

Test Conditions: Permeate flow and salt rejection based on 550 ppm, 150 psi, 77°F (25°C), pH 7, and 15% recovery. NOTE: Higher TDS and/or lower temperatures will reduce the membrane's production.

Start-Up

Unless otherwise indicated, these instructions cover the Thunder 4000, 5000, & 7000 reverse osmosis systems. Please refer to the flow diagrams and exploded view diagrams found in this User's Manual for additional information and reference.

Installation

Thunder reverse osmosis systems must be securely mounted and placed on an even floor, so that the system will not vibrate or move. If this occurs, place the system on a rubber mat to reduce the vibrations and movement.

Carefully inspect your system before start-up. Check that all plumbing and electrical connections are not loose or have not come undone during shipment. A User's Manual, Test Results, and Filter Housing Wrench will accompany your Thunder reverse osmosis system

System Purging:

NOTE: Leave the power to the system off for this procedure.

1. Redirect permeate water to the drain for this procedure.
2. Fully open the concentrate valve #9 (Counter Clockwise).
3. Fully close the recycle valve # 8 (Clockwise)(If Applicable).
4. Fully close the bypass valve mounted on the RO pump.
5. Offset the position of the bypass white lever on the solenoid valve #1.
6. Turn the feed water on and let the system purge until no visible bubbles appear from concentrate flow meter #13.
7. See page 6 for Number Identification. Use this procedure when starting up a new system or when membranes are being replaced.

Initial Start-Up (After Purging):

1. Fully open concentrate valve #9 (Counter Clockwise)
2. Fully close recycle valve # 8 (Clockwise)(If Applicable)
3. Fully open bypass valve mounted on RO pump.
4. Return position of bypass white lever on the solenoid valve #1.
5. Turn RO system on #7 and adjust bypass valve, concentrate (waste) valve #8 and recycle valve # 9(If Applicable) to designed flow and pressure.
6. Inspect for leaks and after one hour shut down system. Re-direct permeate back to tank or POU and then turn sytem back on.
7. Record readings daily for a week and after a week record readings once a week.
8. See Page 6 for Number Identification. See below for detailed startup procedures.

Start-Up

1. Locate the 1" FNPT Solenoid Valve feed water inlet next to the filter housing.

2. Attach the inlet piping to the 1" FNPT Solenoid Valve feed water inlet. Always maintain a smooth and sufficient flow of feed water during operation.

3. Locate the 1" FNPT permeate outlet.

4. Attach the permeate piping to the permeate outlet. Make sure that the permeate water can flow freely and that there is no backpressure. Backpressure can cause damage to the membrane elements.

   CAUTION: The plumbing in the permeate line can contaminate the high quality water produced by the system; ensure that the components are compatible with the application. The pH of the reverse osmosis permeate water will normally be 1-2 points lower than the feed water pH. A low pH can be very aggressive to some plumbing materials such as copper piping.

5. Locate the concentrate outlet on the drain side of the concentrate control valve.

6. Attach the drain line to the concentrate outlet. Water must be allowed to run freely, without any restrictions or blockage in the drain line. Make sure that no backpressure exists on the concentrate line.

7. Ensure that the electrical power requirements of the Thunder system match your electrical power supply.

8. Thunder systems are typically controlled with a liquid level switch in a storage tank. The liquid level switch turns the system on when the water level in the tank drops, and off when the tank is full. If your reverse osmosis system is equipped with an electrical control box, the level control is connected to the level control connections in the control box. The level control connections are dry contacts and never apply power to these connections. Liquid level switches can be obtained by your local dealer or distributor. If a liquid level switch is to be used, install it at this time.

9. Disconnect the permeate line to the storage tank or point-of-use application and direct it to the drain. Flush the system for approximately 15 minutes with the feed water by fully opening the concentrate valve counter-clockwise. This will assist in removing any impurities or preservative within the system. At this time check for any wetted parts which may have been damaged during shipment. After 15 minutes shut down the feed water.

10. Turn on the power to the system.

11. Follow any specified system projections supplied.

12. Make sure that no backpressure exists on the permeate line.

13. Locate the concentrate control valve and the concentrate pressure gauge.

14. Turn the concentrate control valve until the designated recovery is acquired. The concentrate pressure will increase as the concentrate control valve is closed. The exact operating pressure may vary depending on the temperature and TDS of your feed water. It may be necessary to re adjust the system if there is a major change in feed water temperature and/or TDS.

15. Turn the pump bypass valve until the correct pump pressure is achieved. For example a Thunder 5000 should be adjusted until it produces about 5000 GPD or 3.47 GPM of permeate or product water at 77°F.

    GPM = GPD/1440

    WARNING: Never exceed the maximum pressure rating of your pressure vessels.

Operation & Maintenance

The reverse osmosis process causes the concentration of impurities. The impurities may precipitate (come out of solution) when their concentration reaches saturation levels. **NOTE:** Precipitation can scale or foul membranes and must be prevented.

Check your feed water chemistry and pre-treat the water and/or reduce the system's recovery as required. If necessary, consult with your local dealer or distributor.

Pre-Filter Pressure Gauges

These gauges measure the feed water pressure when it enters and exits the pre-filter housings. A pressure differential of 10 -15 psi or more on the two pressure gauges indicates that the pre-filters require servicing. For example, if the inlet pressure is 40 psi, the filter should be changed when the outlet pressure is 30 psi or below.

Product (Permeate) Flow Meter & Waste (Concentrate) Flow Meter

These flow meters indicate the flow rates of the permeate and concentrate water. The measurements, when added together, also indicate the feed water flow rate or (total flow rate); if the system is not equipped with a concentrate recycle valve.

Waste (Concentrate) Recycle Valve

This valve allows you to recycle some of the concentrate water back into the feed of the pump. This will increase the recovery of the Thunder system. An optional waste recycle flow meter allows you to measure how much concentrate is recycled. The amount of waste water recycled is limited by the TDS of the feed water. The drawback of using a concentrate recycle is that there will be an increase of total dissolved solids in the permeate water.

CAUTION: Excessive recycling may cause premature fouling or scaling of the membrane elements.

RO Controller

The RO Controller is located on the front panel of your Thunder system. There is a low pressure shut-off switch which monitors the system's feed water pressure and that will shut off the system and sound alarm if pressure loss is detected. It will also shut down the system when the tank is full using the liquid level switch.

Auto Flush

The auto flush option bypasses the concentrate control valve, reducing the concentrate pressure and increasing the flow of feed water across the membrane. The auto flush removes foulants that may have attached to the surface of the membrane. By removing these foulants before they crystallize on the surface of the membrane, the system can operate longer without cleaning and/or replacing the membrane elements. The following are instances in which the auto-flush should be utilized:

1. When injecting antiscalant chemicals. These chemicals keep scaling ions in solution up to a higher concentration so the ions don't precipitate, and scale the membrane elements. If the solubility concentration is exceeded, the ions may precipitate and scale the membrane.

2. For feed water with a high scaling potential (hard water) in addition to the auto flush, pre-treat the water with an anti scalant or a water softener.

3. Where minimal maintenance is important, auto flush can increase the time between membrane cleanings.

4. For high TDS (total dissolved solids) applications where the TDS exceeds 550 ppm.

5. For higher recovery applications (that use a recycle valve).

6. For systems that may remain inoperative for long periods of time, auto-flush should be installed. The auto-flush will not operate if the electrical power is removed from the system.

The auto flush feature includes an adjustable setting that regulates its operation. In the auto flush mode the water will flush across the membrane in the same direction as the water flows in normal operation; this is not a backwash flow.

8. Motor:

   The motor may not be drawing the correct current. Use a clamp-on amp meter to check the current draw.

9. Leaks:

   Check the system for leaks, as this can result in low pressure.

Abnormal Permeate Flow

Permeate flow should be within 15% of the rated production, after correcting the feed water temperatures above or below 77°F. Check your permeate flow meter to determine the permeate flow rate. If the system does not have a permeate flow meter, measure the time it takes to fill a 1 gallon container then calculate the permeate flow rate at gallons per minute or gallons per day.

NOTE: To determine the temperature correction factor, locate the Temperature Correction Table in this User's Manual and follow the directions.

1. Causes of Low Permeate Flow:

   - Cold Feed Water
   - Low Operating Pressure
   - Defective Membrane Brine Seal
   - Fouled or Scaled Membrane

2. Causes of High Permeate Flow:

   - Defective Product Tube O-Rings
   - Defective or Oxidized Membrane

3. Causes of Poor Permeate Quality:

   - Low Operating Pressure
   - Defective Product Tube O-Rings
   - Defective or Oxidized Membrane

Reverse Osmosis System Troubleshooting

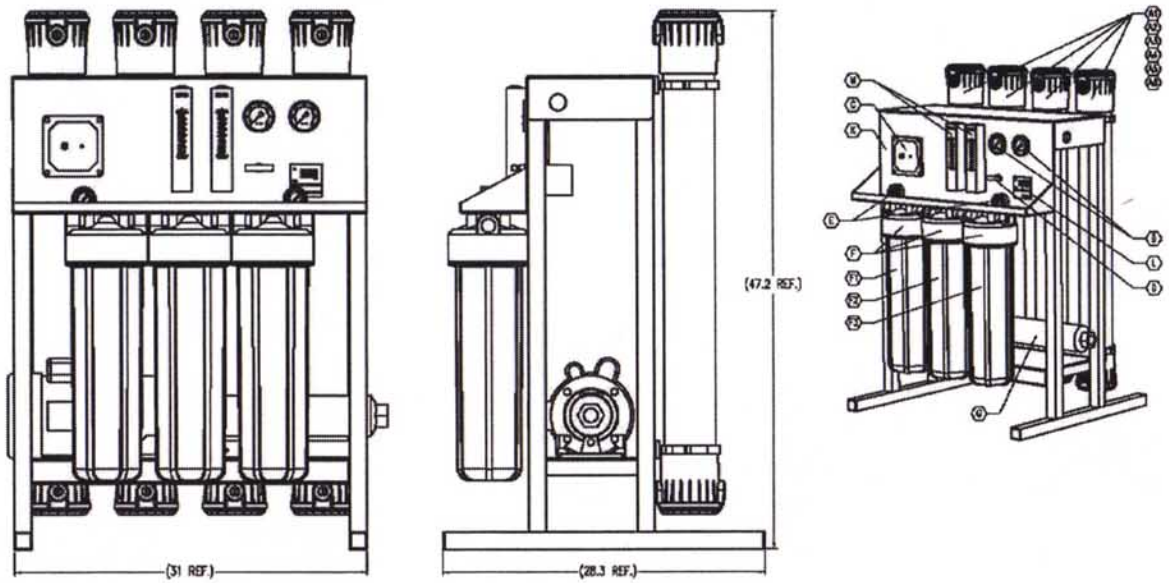
Symptoms			Location	Possible Causes	Verification	Corrective Action
Salt Passage	Permeate Flow	Pressure Drop				
Normal to Increased	Decreased	Normal to Increased	Predominately First Stage	Metal Oxide Fouling	Analysis of Metal Ions in Cleaning Solution.	Improve pretreatment to remove metals. Clean with Acid Cleaners.
Normal to Increased	Decreased	Increased	Predominately First Stage	Colloidal Fouling	SDI Measurement of Feed Water.	Optimize pretreatment for colloid removal. Clean with high pH anionic cleaners.
Increased	Decreased	Increased	Predominately First Stage	Scaling (CaS04, CaSOS, BaSO4, SiO2)	Analysis of metal ions in cleaning solution by checking LSI of reject. Calculate max. solubility of CaSO4, BaS04, SiO2 in reject.	Increase acid addition and antiscalant dosage for CaVOS and CaCO4. Reduce recovery. Clean with Acid Cleaners.
Normal to Moderate Increase	Decreased	Normal to Moderate Increase	Any Stage	Biological Fouling	Bacteria count in permeate and reject. Slime in pipes and pressure vessels.	Shock dosage of Sodium Bi-Sulfate. Continuous feed of Sodium Bi-Sulfate at reduced pH. Formaldehyde disinfection. Chlorination and de-chlorination. Replace cartridge filters.
Decreased or Slightly Increased	Decreased	Normal	Any Stage	Organic Fouling	Destructive Element Testing.	Activated Carbon or other pretreatment. Clean with high pH cleaner.
Increased	Increased	Decreased	Most Severe in First Stage	Chlorine Oxidation	Chlorine Analysis of feed water. Destructive element test.	Check Chlorine feed equipment and de-chlorination system.
Increased	Increased	Decreased	Most Severe in First Stage	Abrasion of membrane by Crystalline Material	Microscopic solids analysis of feed. Destructive element test.	Improve pretreatment. Check all filters for media leakage.
Increased	Normal to Increased	Decreased	At Random	O-Ring Leaks, End or Side Seal Leaks	Probe test. Vacuum test. Colloidal material test.	Replace 0-Rings. Repair or replace elements.
Increased	Normal to Low	Decreased	At Random	Recovery Too High	Check Flows and Pressure Against Design Guidelines.	Reduce the recovery rate. Calibrate and/or add sensors.

Operation Log

Company:_____ Date of Start-Up: _____
Location: _____ Date of Last Cleaning:_____
Week Of:_____ Cleaning Formulation:_____
System Serial #:_____

Date		
Time		
Hours of Operation		
Cartridge Filter Inlet Pressure (psi)		
Differential Pressure (psi)		
Permeate Pressure (psi)		
Feed Pressure (psi)		
Concentrate Pressure (psi)		
Differential Pressure (psi)		
Pump Discharge Pressure (psi)		
Permeate Flow (GPM)		
Concentrate Flow (GPM)		
Feed Flow (GPM)		
Recovery %		
Feed Temperature		
Feed Conductivity (mg/L)		
Permeate Conductivity (mg/L)		
Rejection %		
FeedpH		
Permeate pH		
Scale Inhibitor Feed (ppm)		
Acid Feed (ppm)		
Sodium Bisulfite Feed (ppm)		
Feed Water:		
Iron (mg/L)		
Free Chlorine (mg/L)		
Hardness (ppm CaCOS)		
Turbidity (NTU)		

Notes:



Dimenensions are in inched:

Tolerances:

Fractional ±

Angular Mach ±

Bend ±

Two Place Decimal ±

Three Place Decimal ±

CRYSTAL QUEST
Water Filters

Thunder
4000, 5000 & 7000

Do not scale drawing

Thunder 4000

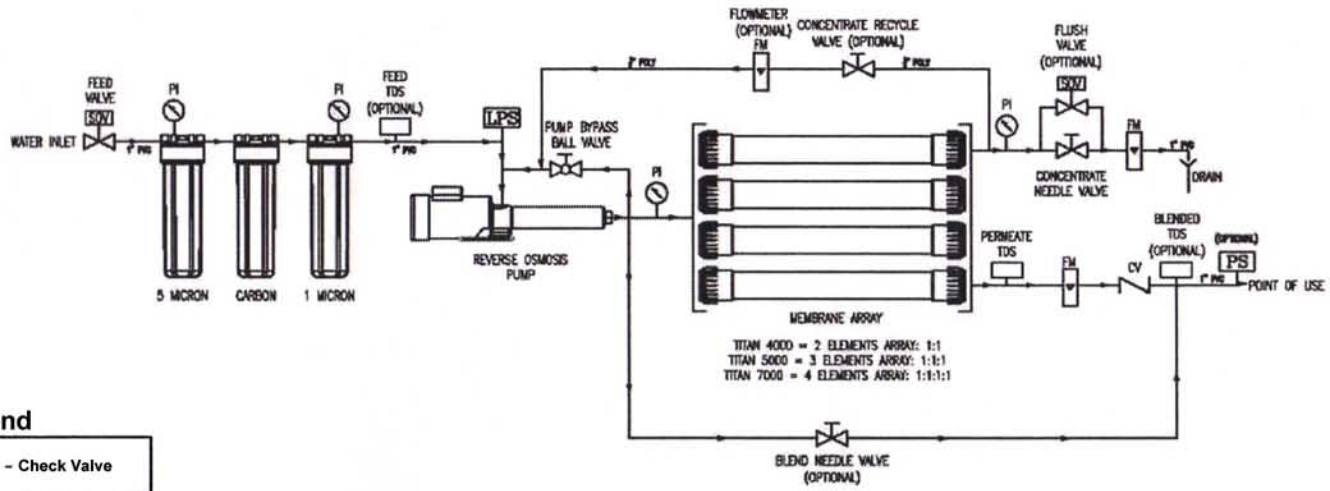
	2	4040 CHAMP 1/2" NPT PART# 200527	A1
	2	THIN FILM COMPOSITE MEMBRANE 4040 TFC HF1 PART# 200379	A2
	4	CHAMP 4" END PLUG PART# 200596	A3
	4	CHAMP 4" SNAP RING PART# 200598	A4
	4	CHAMP 4" O-RING SET PART# 200599	A5
	1	2.5" 0-300 PSI PANEL MOUNT GYLCERINE FILLED GAUGE PART# 200904	B
	1	COMPUTER CONTROL MINITROL PART# 200857 120VAC/ 200858 220VAC	C
	1	1/2" STAINLESS STEEL NEEDLE VALVE PART# 201006	D
	2	0-160 PSI BOTTOM MOUNT GAUGE PART# 200901	E
	2	20" BIG GREY DOUBLE O-RING FILTER HOUSING 1" NPT PART# 200731	F
	1	4.5" X 20" BIG GREY 5 MICRON POLY-PRO (6 PIECES PER CASE) PART# 200640	F1
	1	4.5" X 20" BIG GREY 10 MICRON CARBON BLOCK (6 PIECES PER CASE) PART# 200663	F2
	1	4.5" X 20" BIG GREY 1 MICRON POLY-PRO (6 PIECES PER CASE) PART# 200639	F3
	1	GOULDS 1 HP PUMP & MOTOR 1 PHASE PART# 200788	G
	1	SOLENOID VALVE 1" NPT PART# 200914 120VAC/ 200915 220 VAC	I
	1	LOW PRESSURE SWITCH PART# 200905	J
	1	POWDER COATED EPOXY FRAME PART# N/A	K
	1	HM DIGITAL COMMERCIAL PANEL MOUNT TDS METER PART# 200877	L
	2	0-10 GPM FLOW METER PART# 200899	M
OPTIONAL	1	1" ASD ANTI-SCALING BAR PART# 200107	N
OPTIONAL	1	AUTO-FLUSH SYSTEM OPTION PART# 201343	O
OPTIONAL	1	CONCENTRATE RECIRCULATE SYSTEM OPTION PART# 200046	P

Thunder 5000

	3	4040 CHAMP 1/2" NPT PART# 200527	A1
	3	THIN FILM COMPOSITE MEMBRANE 4040 TFC HF1 PART# 200379	A2
	6	CHAMP 4" END PLUG PART# 200596	A3
	6	CHAMP 4" SNAP RING PART# 200598	A4
	6	CHAMP 4" O-RING SET PART# 200599	A5
	1	2.5" 0-300 PSI PANEL MOUNT GYLCERINE FILLED GAUGE PART# 200904	B
	1	COMPUTER CONTROL MINITROL PART# 200857 120VAC/ 200858 220VAC	C
	1	1/2" STAINLESS STEEL NEEDLE VALVE PART# 201006	D
	2	0-160 PSI BOTTOM MOUNT GAUGE PART# 200901	E
	2	20" BIG GREY DOUBLE O-RING FILTER HOUSING 1" NPT PART# 200731	F
	1	4.5" X 20" BIG GREY 5 MICRON POLY-PRO (6 PIECES PER CASE) PART# 200640	F1
	1	4.5" X 20" BIG GREY 10 MICRON CARBON BLOCK (6 PIECES PER CASE) PART# 200663	F2
	1	4.5" X 20" BIG GREY 1 MICRON POLY-PRO (6 PIECES PER CASE) PART# 200639	F3
	1	GOULDS 1.5 HP PUMP & MOTOR 1 PHASE PART# 200789	G
	1	SOLENOID VALVE 1" NPT PART# 200914 120VAC/ 200915 220 VAC	I
	1	LOW PRESSURE SWITCH PART# 200905	J
	1	POWDER COATED EPOXY FRAME PART# N/A	K
	1	HM DIGITAL COMMERCIAL PANEL MOUNT TDS METER PART# 200877	L
	2	0-10 GPM FLOW METER PART# 200899	M
OPTIONAL	1	1" ASD ANTI-SCALING BAR PART# 200107	N
OPTIONAL	1	AUTO-FLUSH SYSTEM OPTION PART# 201343	O
OPTIONAL	1	CONCENTRATE RECIRCULATE SYSTEM OPTION PART# 200046	P

Thunder 9000

	4	4040 CHAMP 1/2" NPT PART# 200527	A1
	4	THIN FILM COMPOSITE MEMBRANE 4040 TFC HF1 PART# 200379	A2
	8	CHAMP 4" END PLUG PART# 200596	A3
	8	CHAMP 4" SNAP RING PART# 200598	A4
	8	CHAMP 4" O-RING SET PART# 200599	A5
	1	2.5" 0-300 PSI PANEL MOUNT GYLCERINE FILLED GAUGE PART# 200904	B
	1	COMPUTER CONTROL MINITROL PART# 200857 120VAC/ 200858 220VAC	C
	1	1/2" STAINLESS STEEL NEEDLE VALVE PART# 201006	D
	2	0-160 PSI BOTTOM MOUNT GAUGE PART# 200901	E
	2	20" BIG GREY DOUBLE O-RING FILTER HOUSING 1" NPT PART# 200731	F
	1	4.5" X 20" BIG GREY 5 MICRON POLY-PRO (6 PIECES PER CASE) PART# 200640	F1
	1	4.5" X 20" BIG GREY 10 MICRON CARBON BLOCK (6 PIECES PER CASE) PART# 200663	F2
	1	4.5" X 20" BIG GREY 1 MICRON POLY-PRO (6 PIECES PER CASE) PART# 200639	F3
	1	GOULDS 1.5 HP PUMP & MOTOR 1 PHASE PART# 200789	G
	1	SOLENOID VALVE 1" NPT PART# 200914 120VAC/ 200915 220 VAC	I
	1	LOW PRESSURE SWITCH PART# 200905	J
	1	POWDER COATED EPOXY FRAME PART# N/A	K
	1	HM DIGITAL COMMERCIAL PANEL MOUNT TDS METER PART# 200877	L
	2	0-10 GPM FLOW METER PART# 200899	M
OPTIONAL	1	1" ASD ANTI-SCALING BAR PART# 200107	N
OPTIONAL	1	AUTO-FLUSH SYSTEM OPTION PART# 201343	O
OPTIONAL	1	CONCENTRATE RECIRCULATE SYSTEM OPTION PART# 200046	P



Symbol Legend

PI - Pressure Indictor	CV - Check Valve
LPS - Low Pressure Switch	- Ball Valve
SOV - Solenoid	PS - Tank Switch
FM - Flow Meter	

Membrane Array

Thunder 4000 = 2 Elements: 1:1
Thunder 5000 = 3 Elements: 1:!:!
Thunder 9000 = 4 Elements: 1:1:1

Dimenensions are in inched:

Tolerances:
Fractional ±
Angular Mach ±
Bend ±
Two Place Decimal ±
Three Place Decimal ±

CRYSTAL QUEST Water Filters

Flow Diagram

Do not scale drawing

Models
4000, 5000 and 7000

Temperature Correction Factors For Membrane Flux

TEMPERATURE •C	TEMPERATURE •F	CA (Cellulose Acetate)	TF (Thin Film Composite}
10°	50°	1.468	1.71!
11°	52°	1.429	1.648
12°	54°	1.391	1.588
13°	55°	1.355	1.530
14°	57°	1.320	1.475
15°	59°	1.286	1.422
16°	61°	1.253	1.371
17°	63°	1,221	1.323
18°	64°	1.190	1.276
19°	66°	1.160	1.232
20°	68°	1.132	1.189
21°	70°	L1O4	1.148
22°	72°	1.O76	1.109
23°	73°	1.050	1.071
24°	75°	1.025	1.O35
25°	**77°**	**1.000**	**1.000**
26°	79°	0.988	0.971
27°	81°	0.976	0.943
28°	82°	O.964	0.915
29°	84°	O.953	0.889
30°	86°	0.942	O.863

To use: Simply multiply the actual flux to obtain temperature corrected flax.

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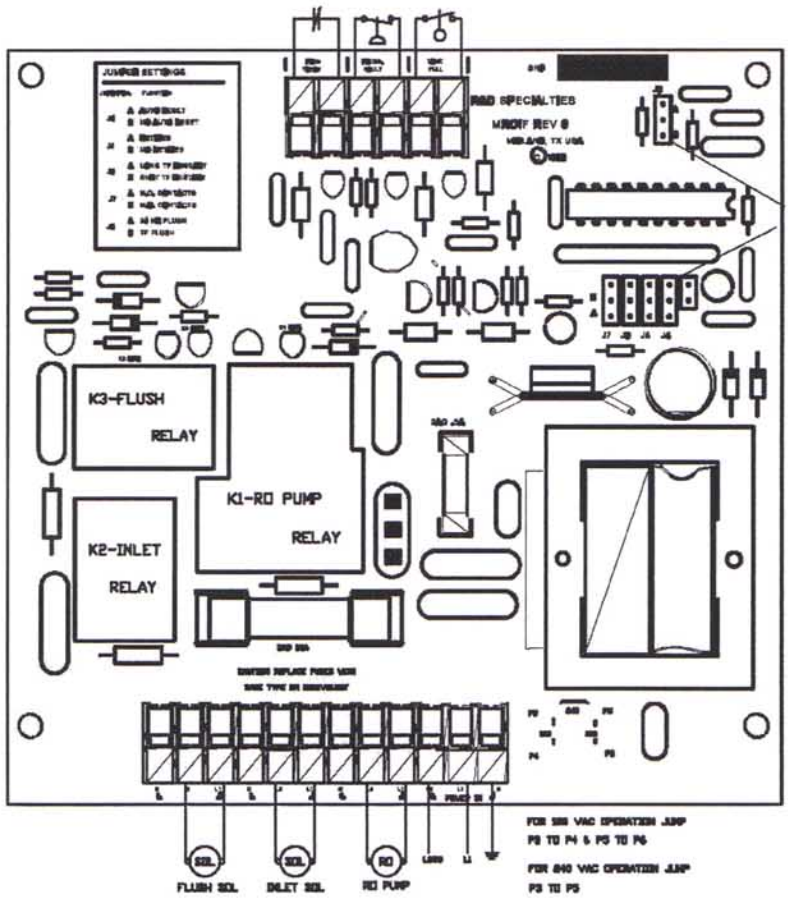

FIGURE 1

JUMPER	POSITION B	POSITION A
J4	AUTO RESET DISABLED	AUTO RESET ENABLED
J5	RETRIES DISABLED	RETRIES ENABLED
J6	2 SEC RESTART	15 MIN RESTART
J7	N.O. SWITCHS	N.C. SWITCHS
J8	TANK FULL FLUSH	24 HOUR FLUSH

TABLE 1

NOTES

ALL SWITCHS MUST BE DRY CONTACT ONLY!! IF VOLTAGE IS
APPLIED TO THESES INPUTS, DAMAGE TO CONTROLLER WILL RESULT
FOR POWER WITH NEUTRAL AND HOT LEADS, L1 IS HOT AND L2 IS NEUTRAL

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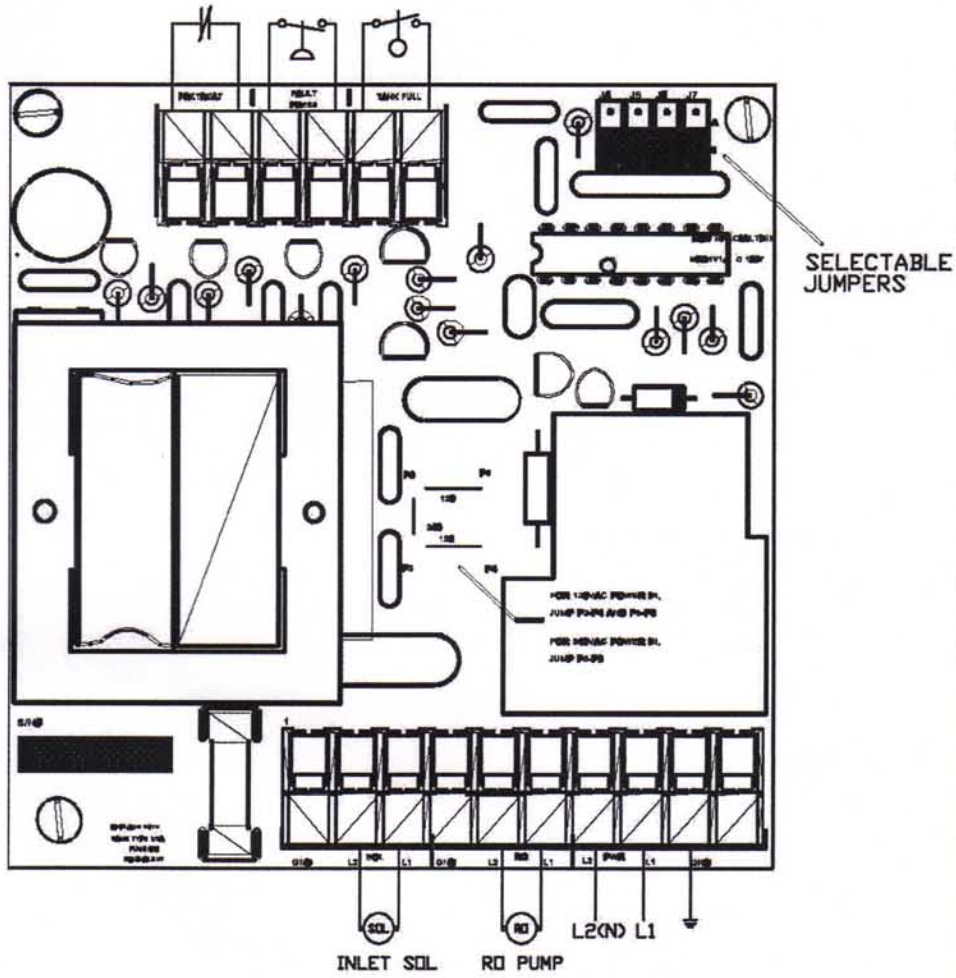

FIGURE 1

JUMPER	POSITION B	POSITION A
J4	AUTO RESET DISABLED	AUTO RESET ENABLED
J5	RETRIES DISABLED	RETRIES ENABLED
J6	2 SEC RESTART	15 MIN RESTART
J7	N.O. SWITCHS	N.C. SWITCHS

NOTES

ALL SWITCHS MUST BE DRY CONTACT ONLY!! IF VOLTAGE IS APPLIED TO THESE INPUTS, DAMAGE TO CONTROLLER WILL RESULT
FOR POWER WITH NEUTRAL AND HOT LEADS, L1 IS HOT AND L2 IS NEUTRAL

Service Assistance

If service assistance is required, take the following steps:

1. Call your distributor.
    a. Prior to making the call, have the following information available:
        i. Machine installation date
        ii. Serial number (found on left-hand side of front panel)
        iii. Daily Log Sheets
        iv. Current operating parameters (i.e., flow, operating pressures, pH, etc.)
        v. Detailed description of problem

Warranty & Guarantee

Reverse Osmosis System One-Year Limited Warranty

Warranty Terms

Subject to the terms and conditions set forth hereinafter, Seller warrants to the original purchaser (hereafter the "Buyer") that the products manufactured by Seller are free from defects in material and in workmanship for twelve (12) months from the Warranty Commencement Date (as defined below) only when used strictly in accordance with the applicable operating instructions and within the range of the operating conditions specified by Seller for each such product.

This Warranty does not extend to equipment or components manufactured by others into which a Seller product has been incorporated or to equipment or components which have been incorporated into a Seller product but, if allowable, Seller hereby assigns, without warranty, to the Buyer its interest, if any, under any warranty made by the manufacturer of such equipment or component. This Warranty does not cover disposable items such as fuses, lamps, filters, cartridges, or other such disposable items, which must be replaced periodically under the normal and foreseeable operating conditions of the goods warranted hereby.

Warranty Commencement Date

The Warranty Commencement Date for each Seller product shall be the later of the date of: (1) receipt by the Buyer, or (2) the date of installation at the Buyer's premises provided that such installation must occur within three (3) months of shipment from the Seller's manufacturing facility in California. In no event shall the Warranty Commencement Date exceed three (3) months from the shipment from the Seller's manufacturing facility. The Buyer shall provide proof of purchase in order to exercise rights granted under this Warranty. If requested by manufacturer, the Buyer must also provide proof of the installation date. Proof of installation shall be returned by Buyer to Seller within thirty (30) days after installation by virtue of supplying a Warranty Validation Card supplied with each Seller product fully completed and signed in ink by Buyer and the authorized installer of the product.

Warranty Service

SELLER'S OBLIGATION UNDER THIS WARRANTY IS LIMITED TO THE REPAIR OR REPLACEMENT (AT SELLER'S SOLE DISCRETION) OF ANY PRODUCT, OR COMPONENT THEREOF, PROVED TO BE DEFECTIVE IN MATERIAL OR WORKMANSHIP WITHIN THE COVERED WARRANTY PERIOD.

The buyer, at the Buyer's risk and expense, shall be responsible for returning such product or component, only after obtaining a Return Goods Authorization (RGA) number from the Seller, arranging for freight

prepaid, and in conformance with any special packaging and shipping instructions set forth on the operation documentation or RGA instructions, or as otherwise reasonably required, to the Seller's address set forth below, together with (1) RGA number issued by Seller at Buyer's request; (2) proof of purchase and, if necessary, proof of installation date; (3) a Return Good Authorization Form; (4) a description of the suspected defects; (5) the serial number of the Seller product alleged to be defective; and (6) a description of the type of water and pretreatment equipment which has been utilized in connection with the product, if any. Seller shall, in Seller's reasonable discretion, be the sole judge of whether a returned product or component is defective in material or workmanship. Required or replaced products or components shall be returned surface freight. In genuine emergency situations, the Seller will (at Seller's sole discretion) forward replacement parts to Buyer without waiting for authorized return of the questionable part(s). In such cases, Buyer will issue a purchase order or other payment guarantee prior to shipment. If the returned part is found to have been misused or abused, the defective part is not received by Seller within thirty (30) days; the Buyer will be invoiced for the replacement part(s) provided. This Warranty does not cover or include labor and/or travel to the Buyer's premise or location or any other location. Charges of $1000 per day plus associated travel expenses will be incurred by the Buyer in providing the Warranty Service at any location other than Seller's main headquarters; that is if the Seller deems that the product is not covered by said Warranty. The Seller reserves the right to precondition such travel to Buyer's premises upon prepayment of Seller's anticipated costs of attending such premises.

Voidability of Warranty

This Warranty shall be void and unenforceable as to any Seller product which has been damaged by accident, mishandling, abuse or has been repaired, modified, altered, disassembled or otherwise tampered with by anyone other than Seller or an authorized Seller service representative; or, if any replacement parts are not authorized by Seller have been used, or, the product has not been installed, operated and maintained in strict accordance and adherence with the operating documentation and manuals for such product. Any expressed warranty, or similar representation of performance set forth in the operation documentation for a reverse osmosis, nanofiltration, or ultrafiltration membrane incorporated into a Seller product shall be void and unenforceable unless the feed water requirements set forth in the operating documentation for such product are unequivocally and strictly adhered to.

Limitations and Exclusions

THIS WARRANTY AND REMEDIES DESCRIBED HEREIN AND HEREINABOVE ARE EXCLUSIVE AND IN LIEU OF ANY AND ALL OTHER WARRANTY OR REMEDIES, EXPRESSED OR IMPLIED, INCLUDING WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. IN NO EVENT SHALL THE SELLER BE LIABLE FOR ANY CONSEQUENTIAL, INCIDENTAL OR OTHER SIMILAR TYPES OF DAMAGES, FOR DAMAGES FOR THE LOSS OF PRODUCTION OR PROFITS, OR INJURY TO PERSON OR PROPERTY. NO PERSON HAS ANY AUTHORITY TO BIND SELLER TO OTHER THAN WHAT IS SET FORTH ABOVE.

THIS WARRANTY GIVES THE BUYER SPECIFIC LEGAL RIGHTS AND THE BUYER MAY ALSO HAVE OTHER RIGHTS WHICH VARY FROM JURISDICTION TO JURISDICTION. THE PARTIES RECOGNIZE AND AGREE, THAT IN ALL RESPECTS THE LAWS OF THE STATE OF CALIFORNIA SHALL APPLY TO AND SHALL GOVERN ANY INTERPRETATION OR LEGAL SIGNIFICANCE OF THIS DOCUMENT.

NO WARRANTY OR OTHER LIABILITY OF SELLER TO BUYER UNDER THIS AGREEMENT OR OTHERWISE WILL IN ANY EVENT EXCEED THE COST OF REPLACEMENT OF THE APPLICABLE SELLER PRODUCT, PART, OR ACCESSORY THAT IS SUBJECT TO ANY BREACH OF SELLER'S WARRANTY. SELLER WILL NOT BE LIABLE FOR ANY DAMAGE TO ANY PROPERTY OF BUYER OR TO BUYER'S CUSTOMERS FOR ANY CONSEQUENTIAL, INCIDENTAL, OR ECONOMIC LOSS OR COMMERCIAL DAMAGE WHATSOEVER. REMEDIES HEREIN PROVIDED ARE EXPRESSLY MADE THE SOLE AND EXCLUSIVE REMEDIES FOR BREACH OF ANYWARRANTY OR OTHER OBLIGATION HEREUNDER EXPRESS OR IMPLIED OR FROM THE OPERATION OF LAW.