Biological Co-Fermentation of Carbon Dioxide and Methane to Malate

CCEMC Grand Challenge Project #K130103

PI: Derek Greenfield

1250 45th St Ste 150

Emeryville, CA 94608-2901 USA

dgreenfield@imicrobes.com (650) 731-5499

Project Advisor: Vicki Lightbown

Project Completion Date:	5/31/2016
Project Budget:	$500,000
Funds Received:	$350,000
Hold Back:	$150,000
Submission Date:	12/5/2016

Table of Contents

List of Figures

Executive Summary

The Grand Challenge launched our efforts to convert greenhouse gases into valuable products. Industrial Microbes proposed building a novel biological method to consume two greenhouse gases and chemically combine them to produce high-value chemicals. Our process occurs inside an engineered organism acting as a chemical factory.

The technology developed in this project can be deployed to reduce greenhouse gas emissions and produce many different high-value products. Industrial Microbes has identified dozens of chemical and fuel products that could be manufactured cost-competitively from greenhouse gases (GHGs), all with reduced life cycle emissions compared to current methods.

The key to this project is the co-utilization of carbon dioxide and methane as raw materials. Methane is the lowest-cost feedstock that can also provide chemical energy to fix carbon dioxide. Other co-feedstocks can be used to power carbon dioxide fixation to produce malate, including traditional sugars, starches, cellulosic sugars, sugar alcohols, and alcohols. However, the economics of the process vary considerably depending on the co-feedstock; methane from natural gas or biogas is the lowest-cost co-feedstock on a per-energy or per-carbon basis.

The goal of this project was to build a prototype process for making malate, a chemical building block used in a wide variety of applications. We have designed an enzyme-based chemical assembly line inside a living yeast cell to combine methane, oxygen, and CO_2 into malate. Methane provides the energy needed to activate CO_2. In the final process, the four-carbon product malate will consist of three carbon atoms from methane and one from CO_2. Malate can be used to make biodegradable plastics, synthetic fibers for clothing, synthetic rubber for running shoes, coatings for furniture, and ingredients in candy and pharmaceuticals (Figure 1).
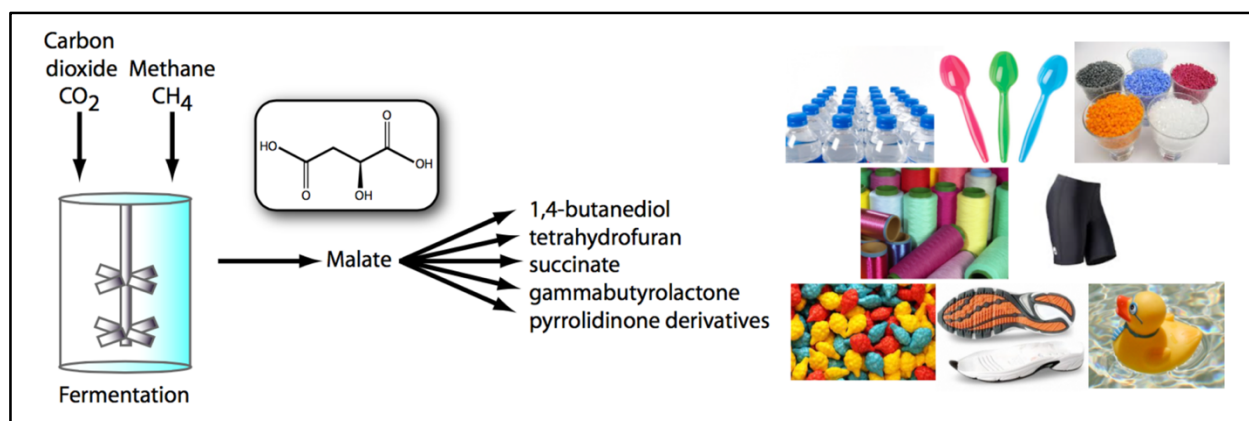

Figure 1. Technology Overview.
Industrial Microbes has developed fermentation technology toward transforming greenhouse gases CO_2 and methane into malate (malic acid), which is used in a wide range of products. Fermentation is performed by engineered yeast microorganisms. Products include plastics, resins, fibers, rubber, and food additives.

Industrial Microbes has made significant breakthroughs toward a green fermentation platform that can reduce carbon pollution at scale. Project tasks were to establish malate production from carbon dioxide, discover enzymes that could consume methane, and combine the two parts in one microbe into a prototype process. Industrial Microbes achieved most of the breakthroughs needed to build the prototype. Malate was produced and the efficiency of carbon dioxide fixation was estimated at half of maximum.

Despite lower crude oil prices, we model that we will be able to produce malate at less than its current cost today. Novel chemical manufacturing processes must be cost-competitive with current production methods. Carbon dioxide and methane are inexpensive, which can enable production of chemicals such as malate at lower cost than current petrochemical methods. This cost advantage provides us an opportunity to rapidly increase adoption of green chemical production, replacing petroleum-derived products and reducing the associated carbon pollution.

This process at scale could directly capture 1 tonne of CO_2 for every 3 tonnes of malate produced. Unlike existing petrochemical manufacturing processes, which emit greenhouse gases, our process can consume these gases.

Grand Challenge support was leveraged to raise private investment and obtain additional funding for the project. Industrial Microbes raised a seed funding round in 2015 with investors including Y Combinator, SV Tech, CLI Ventures, Point Reyes Capital Management, Fundamental Ventures, and individual angel investors. The company received four Phase I SBIR grants from United States Federal agencies: the National Science Foundation, the Environmental Protection Agency, the Department of Energy, and the Department of Agriculture. These grants build upon CCEMC support or are synergistic with this project. Our focus remains on reducing carbon emissions by developing low-cost green chemical production methods.

Two development partners will be needed to commercialize the malate technology in Alberta. Malate has major applications as a chemical and polymer feedstock, but the overlap between carbon emitters and chemical manufacturers was smaller than expected. Two distinct partners can fill these roles toward commercialization in Alberta.

1. Project Description

1.1 Introduction and Background

What if a fire *consumed* carbon dioxide instead of *releasing* it? Fire allowed early humans to survive. Today, fire – in the form of burning fossil fuels – continues to provide much of the energy necessary to our modern society. But fire is a rapid and uncontrolled reaction. Is it possible for a similar reaction to consume fuel in a different way – *to make new bonds rather than break them, and to capture CO_2 rather than release it?* Such a reaction would combine CO_2 with fuel to build new and useful molecules. In fact, reactions like these power the engines of many living cells. Enzymes in such cells can combine different kinds of fuels in a controlled manner; some enzymes can consume CO_2. By designing these cells to produce the right enzymes in the right combinations, it is possible to create organisms that perform specific chemical reactions. Using the tools of modern synthetic biology, we have designed an enzyme-controlled reaction that burns fuel and oxygen, and consumes CO_2.

Biological fermentation is an excellent method to inexpensively and efficiently remove massive amounts of carbon dioxide from the atmosphere. Plants, algae, and bacteria already fix over 57,000 megatonnes of carbon dioxide annually, 10-fold more carbon than emitted by all human activities [1]. Unlike the energy-intensive, harsh conditions in industrial chemical plants, these organisms have evolved to convert carbon dioxide into key biomolecules at atmospheric temperature and pressure, with minimal energy input. Using the tools of modern synthetic biology and metabolic engineering, nature's most efficient enzymes and pathways can be engineered into industrial microorganisms to convert CO_2 into commercially useful molecules.

Methane is an ideal energy source to power CO_2 fixation. CO_2 is the most oxidized form of carbon, so converting it into other compounds requires an energy source with significant reducing power. In our process, methane provides both the chemical energy and reducing electrons needed to convert CO_2 into new products. Methane is the best source of reducing power because it is cheap, abundant in Alberta, energy-dense, and available year-round. The ecological benefits of sequestering CO_2 also apply to methane, as it is also a greenhouse gas, and is in fact 20-fold more potent than CO_2. Methane makes up >90% of natural gas, and can be obtained renewably from biogas sources such as landfills and wastewater treatment plants. Methane is a superior energy source to hydrogen because it is far less expensive per BTU, 3-fold cheaper compared to natural gas-derived hydrogen [2] (a process that generates massive amounts of CO_2), and 8 to 15-fold cheaper than hydrogen produced from renewable electrolysis [3]. A further advantage of methane is that it can power carbon fixation faster than photosynthesis, which translates into more carbon fixed per year per chemical plant. Whereas sunlight-driven photosynthesis relies on the slow Calvin Cycle, methane can power the fastest carbon-fixing carboxylases, which have 10-fold higher specific activity than RuBisCO from plants, algae, and cyanobacteria [4].

We proposed to manufacture malate (malic acid), a building-block dicarboxylic acid that can feed into a huge plastic and polymer feedstock market. Malate is a valuable product that requires low energy inputs for production. It was identified by the U.S. Department of Energy as a top 12 feedstock chemical that can be converted into a diverse array of products such as plastics,

resins, fibers, and rubber [5]. Malate is a safe, natural product found in fruits such as apples and cherries, and is produced in people, animals and plants. Malic acid can already be produced at high yield in engineered bacteria using sugar and CO_2. Malate is a four-carbon dicarboxylic acid (diacid) in a family of related chemicals that also includes succinate, fumarate, and maleic anhydride, which are chemically similar and can be easily interconverted using well-known biological and chemical processes. This market size for this family of chemicals and derivatives is large enough to remove 1 megatonne/year of CO_2 from the atmosphere.

Malate is currently made from petroleum in a carbon-intensive chemical process. Production of malate starts with butane, a product of crude oil refining [6]. Butane is oxidized to maleic anhydride in an inefficient process that wastes significant carbon [7]. Maleic anhydride is further processed to malate [8].

Malate can be produced efficiently in engineered microorganisms, but it is not cost-competitive due to the expensive sugar feedstock. This process can consume carbon dioxide, however it is not operated commercially because it is more expensive than the current method of malate production [9][10]. For malate production using microorganisms to be commercially viable, a lower-cost raw material is needed as a co-substrate to CO_2.
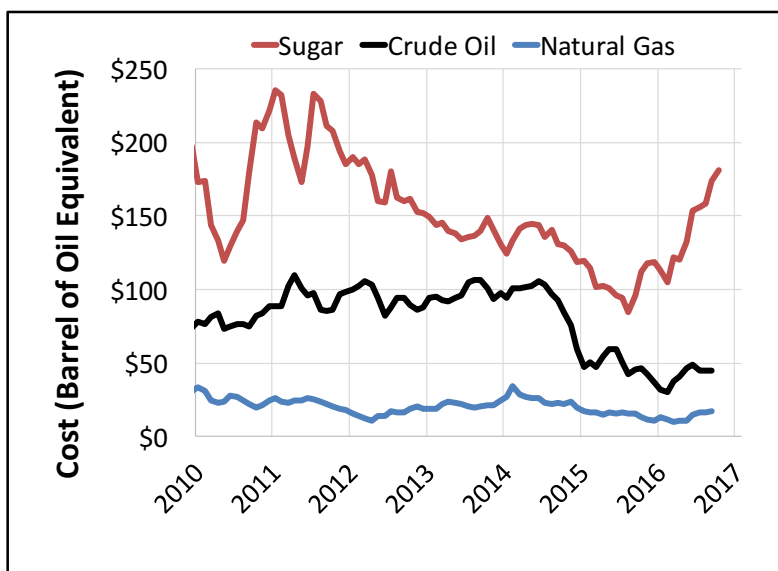
Figure 2. Malate feedstock cost comparison.
Traditional manufacture of malate begins with butane, from crude oil. Sugar and natural gas are alternative feedstocks, however natural gas is significantly lower-cost on an energy basis (shown), mass basis, and carbon basis.

We have designed a microbe that can consume methane and carbon dioxide to produce cost-competitive malate. There are no microbes that can consume methane and carbon dioxide to produce malate, or any related chemicals, at commercial yields. All the enzymes needed for this transformation exist in nature and have been well-characterized; the challenge is to combine these parts into one microbe and balance the activities of each enzyme. Because the production and consumption of NADH is exactly balanced when making malate from methane, almost every

carbon atom of methane and CO_2 ends up in the final product. Our solution removes atmospheric carbon, whereas traditional production of malic, succinic, and fumaric acids uses petroleum-derived maleic anhydride, and increases atmospheric CO_2 [11].

Malate from carbon dioxide and methane must be less expensive than the current method to make a large impact on carbon emissions. The market size of carbon-fixing malate is dependent on its cost of production. At low cost, it can serve as a feedstock for other chemicals including 1,4-butanediol, tetrahydrofuran, gamma-butyrolactone, pyrrolidinone derivatives, and linear aliphatic esters. The exact cost reduction target depends on factors including feedstock price and trends, market dynamics, scale-up risk, product purity, and compatibility with existing chemical processes. We estimate that a 10-30% decrease in production costs, relative to the current butane/maleic anhydride process, is needed for widespread adoption. We base this estimate on customer interviews for a variety of chemical products.

The manufacture of malate from captured CO_2 is ideally suited to Alberta. Alberta is a leader in the world carbon economy and has invested in research and infrastructure for carbon capture. The carbon tax incentivizes emitters to reduce emissions by investing in carbon capture infrastructure. Alberta also has abundant natural gas reserves to supply methane for co-fixation. The ecosystem of funding and support organizations, such as the ERA and Alberta Innovates, is invaluable for starting and growing clean technology companies.

1.2 Technology Description

Industrial Microbes designed a novel enzyme pathway to consume methane and CO_2 and produce malate. The team specializes in designing, building, and optimizing engineered microbes to consume gas feedstocks. Our strategy is to combine enzymes and enzyme pathways into a single microorganism that can be used at industrial scale. The designed malate production pathway is shown in Figure 3. This pathway is composed of three modules that can be optimized independently: (1) Methane assimilation to pyruvate via methane oxidation and glycolysis. (2) Carbon fixation by pyruvate carboxylase, yielding oxaloacetate; we have chosen pyruvate carboxylase as the key carbon-fixing enzyme because it is one of the fastest known carboxylating enzymes under typical substrate concentrations. (3) Malic acid biosynthesis; oxaloacetate enters the tricarboxylic acid (TCA) cycle and high levels of malic acid, a TCA cycle intermediate, can be generated via a series of well-understood enzyme modifications.

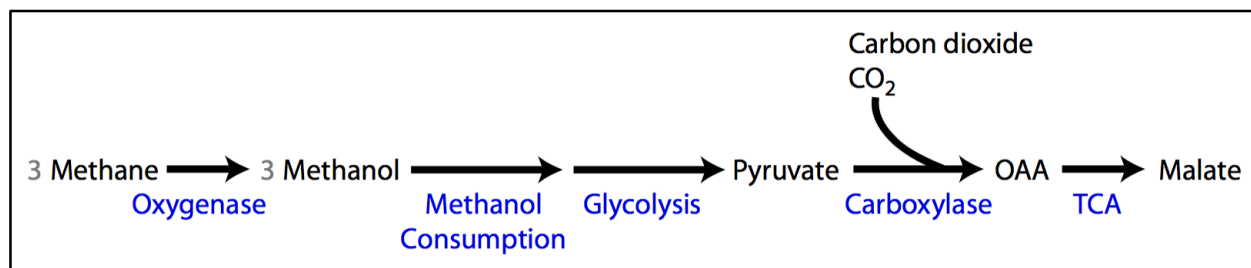

Figure 3. Enzyme Pathway Overview.
CO_2 can be efficiently assimilated into central metabolism using methane or methanol (rather than sugar) as a source of both energy and reducing power. OAA = oxaloacetate; TCA = tricarboxylic acid cycle.

1.3 Project Goals

We proposed to develop a strain that could co-ferment methane and CO_2 into malate. We divided this project into three technical tasks; the first two tasks were advanced in parallel, then combined in the third task. Our approach was to introduce diverse enzymes into the ideal production host, then optimize the entire enzyme pathway.

Task 1: Construct a CO_2-fixation Pathway in Yeast to Produce Malate
Researchers have constructed a yeast strain that produces malate at high titer and moderate yield by introducing three enzymes, however their strain requires expensive sugars to power CO_2 fixation and is not suited for methane consumption. We will introduce those three metabolic enzymes into a commercial yeast strain suitable for methane consumption and demonstrate that this strain can produce malate.

Task 2: Identify a High Activity Methane-Fixation Enzyme
The malate producing strain from Task 1 is missing the enzymes needed to assimilate methane into central metabolism. We will test multiple enzyme pathways and identify a set of enzymes that will enable our yeast strain to consume methane.

Task 3: Combine CO_2 and Methane Fixation to Produce Malate, and Demonstrate Ability to Increase CO_2 Fixation
To construct the full enzymatic pathway, we will combine the malate production strain from Task 1 with the functional methane oxidation catalyst from Task 2. The initial conversion efficiency may be low due to insufficient enzyme activity or the presence of side reactions. We will use the tools of synthetic biology and metabolic engineering to optimize the full pathway using multiple approaches.

2. Outcomes and Learnings

2.1 Experimental Approach for CO_2-Based Malate Production

Yeast is the ideal organism to produce malate because yeast strains have been successfully used at large scale to produce chemicals, and they are compatible with low-pH fermentation conditions. The production of an acidic product, such as malate, results in a pH drop during the fermentation, which yeast is uniquely suited to tolerate. Bacteria can also be used to produce malate, though the pH must be controlled by adding stoichiometric quantities of base. This adds a large amount of byproduct waste that must be separated after the fermentation [12]. Yeast fermentation avoids these byproducts, and the acidic pH conditions lower the chance of bacterial contamination. Finally, yeast naturally produces malate, and genetic engineering strategies have been very successful in increasing its production [9].

We selected the industrial yeast *Pichia pastoris* because it is well-studied and genetically tractable. Its genome is sequenced and tools for genetic manipulations are easy to use and commercially available [13]. In addition, *P. pastoris* is a sexual yeast that can live in either a haploid or diploid form, with established protocols for mating and sporulation. *Pichia pastoris* is related to the model yeast *Saccharomyces cerevisiae*, which has been successfully engineered to produce a wide range of products. *P. pastoris* is generally regarded as safe (GRAS), and has been successfully used in industrial fermentations for years due to its exceptional ability to express heterologous proteins at high levels [14]. *P. pastoris* can also grow to exceptionally high density, which can result in high product titer and productivity.

Pichia pastoris can grow on methanol (but not methane) as a sole carbon source via a well-understood, high flux pathway [13], [15]. The enzyme alcohol oxidase (AOX) converts methanol into formaldehyde, which is subsequently either dissimilated into formate and CO_2 for energy or assimilated into building block molecules.

Malate has been successfully produced in a strain of *S. cerevisiae*, in a glucose-fed fermentation [9], [16]. The previous metabolic engineering strategy focused on three genetic manipulations, which we constructed in *Pichia pastoris* (shown in Figure 4). This high yield pathway uses a pyruvate carboxylase enzyme (Pyc2p) to fix a CO_2 molecule in converting pyruvate into oxaloacetate. Reducing the oxaloacetate to malate requires the overexpression of malate dehydrogenase (Mdh3p) in the cytoplasm. Finally, the malate transporter from *S. pombe* (SpMae1p) is constitutively expressed in order to facilitate malate export from the cell.

We initially focused on producing malate in *P. pastoris* using glucose as the carbon source, then planned on transitioning to methanol. The rationale was that glucose is rapidly metabolized and would produce more malate compared to methanol. Based on the successful strategies used in *S. cerevisiae*, we overexpressed PYC2, MDH3 and SpMAE1 using constitutive promoters, from both plasmids and integrated genomic loci. We tested these strains for malate production in both glucose- and methanol-fed shake flask scale fermentations.
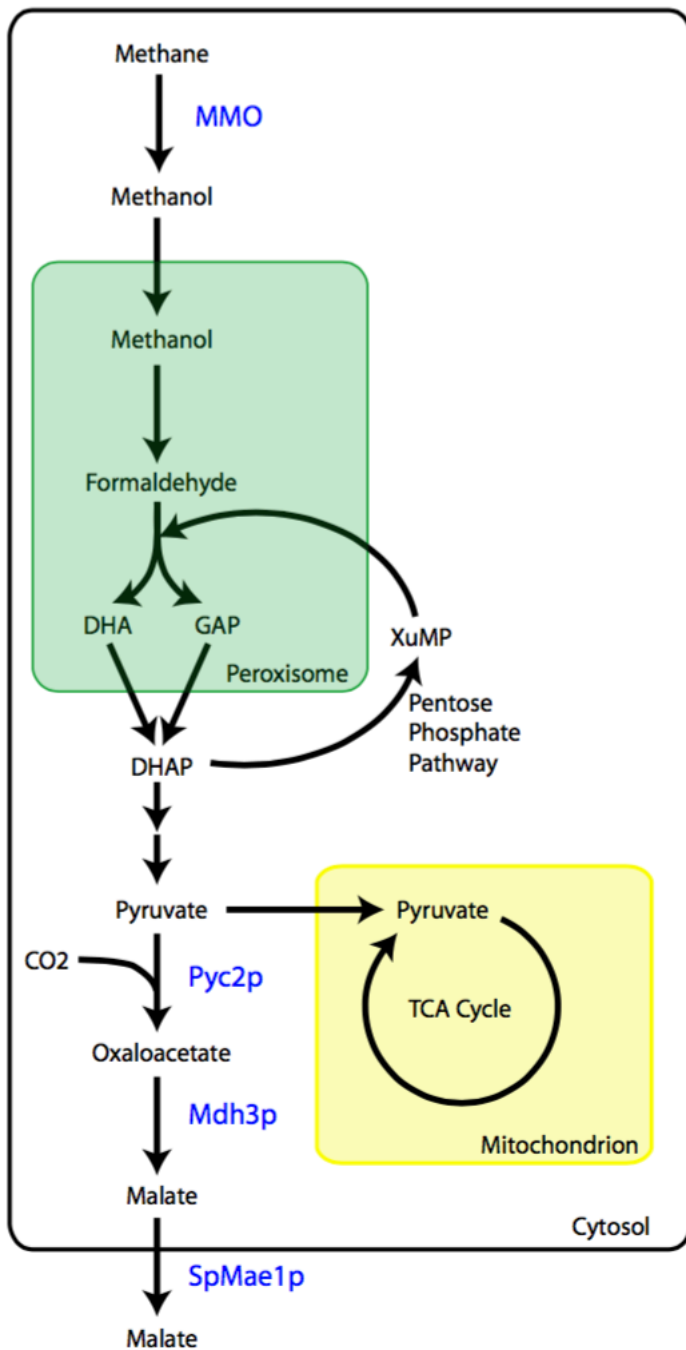
Figure 4. Genes Targeted in Yeast.
Industrial Microbes metabolic pathway for malate production in yeast *Pichia pastoris*. Blue enzymes indicate engineering targets. Pyc2p (pyruvate carboxylase), Mdh3p (malate dehydrogenase), and SpMae1p (malate transporter) are targeted for expression in Task 1.

2.2 Experimental Results for CO_2-Based Malate Production

Strain Construction for Malate

There are three steps to convert the central metabolite pyruvate into malate, each catalyzed by a separate enzyme. The conversion of pyruvate to oxaloacetate fixes carbon dioxide. The genes encoding these three enzymes were cloned into a single plasmid, each with its own strong promoter and terminator. All plasmids were sequence-verified by Sanger sequencing. This plasmid, which we designed for this specific purpose, can integrate the genes directly into the host genome with a single copy or multiple copies in the same genomic location. This feature allows us to vary the expression level of our enzymes. By testing many unique colonies, we identified a range of expression levels that lead to different amounts of malate production.

Analytical Chemistry to Detect and Measure Malate

We grew the engineered *P. pastoris* in glucose minimal media, and measured malate production by HPLC (Varian 9012 HPLC, 9050 UV Detector, and 4.6 mm x 15 cm Phenomenex Synergy Hydro RP 5μ column). A reference standard solution of malate was prepared at 1 mg/mL. Malate was identified in the samples by retention time. The concentration of malate was calculated by dividing the peak area of malate in the sample by the peak area of the malate in the reference standard and multiplying by the concentration of the standard.

Figure 5 shows data from our strains that have been engineered to produce malate. These are overlaid traces from our best *P. pastoris* malate production strain, NH38, under various fermentation conditions. By optimizing the fermentation conditions, including aeration, media buffering capacity, and glucose feeding intervals, we increased production to 6.05 g/L of malate from glucose. This is the first reported production of malate from *P. pastoris*.
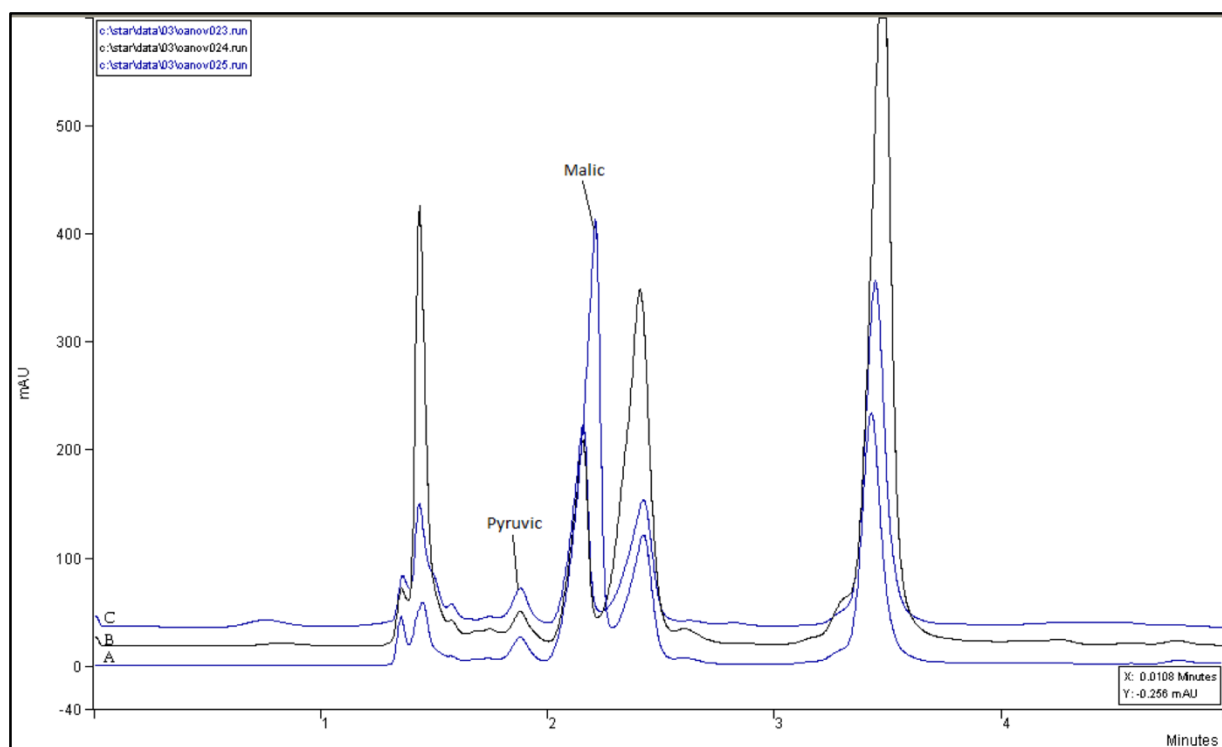
Figure 5. Malic acid produced from glucose.
Malic acid was produced by engineered yeast from glucose as a starting material. Traces A, B, and C show data from the same strain, NH38, under different fermentation conditions. The malate (malic acid) peak was identified by comparison with the retention time of a reference standard, and by the presence of a single peak when samples were spiked with additional malate.

Attempted Production of Malate from Methanol and Carbon Dioxide

Industrial Microbes engineered yeast successfully produced malate using glucose as a carbon source. While malate produced from either glucose or methane would fix CO_2, production from methane would have dramatically lower feedstock costs. Therefore, one of our project goals was to produce malate from methanol, the metabolic intermediate that links methane metabolism with malate production.

In addition to the synthetic biology approaches we outlined above, we varied chemical and physical fermentation conditions to produce malate from methanol. We tested a matrix of 24 conditions including methanol concentration, oxygen concentration, temperature, nitrogen source, CO_2 concentration, media buffer, and biotin concentration. We also attempted to co-feed our methanol fermentations with either glucose or glycerol.

Efficiency of Carbon Dioxide Fixation

Industrial Microbes technology can produce commercially-relevant concentrations of malate because it utilizes the central metabolite pyruvate. Both glucose and methanol are converted inside

the cell to pyruvate. The amount of carbon dioxide fixed by malate production in this way is not a fixed quantity, however, because there are two metabolic pathways to produce malate from pyruvate. The first is through the enzyme pyruvate carboxylase, which fixes carbon dioxide, and the second is through the enzyme pyruvate decarboxylase, which does not fix carbon dioxide. The total malate produced is therefore not a perfect measure of the quantity of carbon dioxide fixed.

The simplest method for estimating the carbon dioxide fixed suggests that fixation occurs at 45% of maximum efficiency. To estimate this efficiency, we measured the flux through the two different enzyme pathways by constructing yeast strains with combinations of the three pathway enzymes SpMAE1, MDH3, and PYC2. PYC2 is the pyruvate carboxylase mentioned above. Interestingly, strains incorporating a subset of these three enzymes alone could produce malate, although the strain combining all three enzymes produced the highest titer (Figure 6). The addition of the PYC2 gene almost doubled the malate titer, suggesting that the current Industrial Microbes process fixes carbon dioxide at approximately half of its theoretical potential. This estimate assumes that the addition of the PYC2 enzyme adds an additional pathway for malate production that is independent of the MDH+MAE pathway. This assumption is reasonable but untested. If PYC2 expression diverts flux away from the non-carboxylation pathway, the actual carbon fixation efficiency may be higher. Further work will be required to identify any indirect metabolic effects of expression of the PYC2 enzyme that might reduce this carbon fixation efficiency.
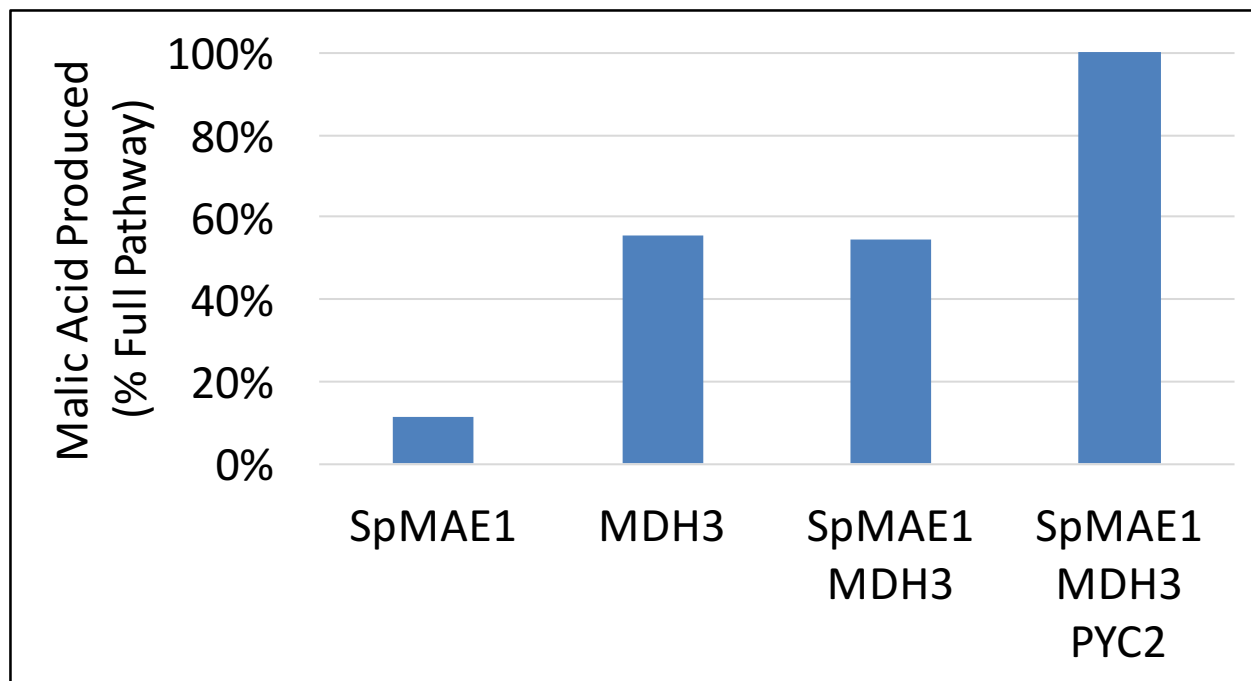
Figure 6. Estimate of carbon fixation efficiency.
Carbon dioxide fixation efficiency is estimated at 45% of theoretical maximum, based on the increased malate production from PYC2 expression. SpMAE1 = malate transporter; MDH3 = malate dehydrogenase; PYC2 = pyruvate carboxylase.

One can more accurately monitor carbon fixation by measuring carbon dioxide concentration in a sealed fermentation vessel during malate production. To perform this measurement, we used a commercially available carbon dioxide sensor in a custom 3D-printed enclosure that could attach

to 125 mL fermentation bottles. Several factors made it challenging to obtain a reproducible measurement: the vessel must remain sealed during an extended fermentation; carbon dioxide is produced during normal respiration; carbon dioxide dissolves in the cellular broth; and some carbon dioxide sensors are sensitive to humidity. Future work could incorporate an off-gas analyzer to continuously measure carbon dioxide concentration in a fed-batch process.

The most sensitive method to determine carbon fixation is to use isotopically labeled carbon dioxide. In this way, the fate of ^{13}C-labeled carbon dioxide can be tracked at the molecular level. This requires more sophisticated analytical methods, and the experiment must be designed to account for unlabeled carbon dioxide produced from cellular respiration and subsequently fixed into malate. Future work to measure carbon fixation will focus on isotopic labeling.

2.3 Experimental Approach for Methane Utilization

Many industrial fermentation processes utilize yeast, however yeast are limited to methanol and sugars as raw materials. Yeasts such as *Pichia pastoris* can grow on methanol as a sole energy source [15], but no naturally occurring yeast has been found to produce an enzyme that catalyzes the single-step oxidation of methane to methanol [17], [18]. *Pichia pastoris* can grow on methanol as a sole carbon source via a well-understood, high flux pathway, which is transcriptionally up-regulated by the presence of methanol. The enzyme alcohol oxidase (AOX) converts methanol into formaldehyde, which is subsequently either dissimilated into formate and carbon dioxide for energy, or assimilated into building block molecules. The assimilation pathway condenses formaldehyde with xylulose-5-phosphate in a cyclic pathway that progresses through the intermediates dihydroxyacetone and glyceraldehyde-3-phosphate. One net molecule of glyceraldehyde-3-phosphate is produced for every three turns of the cycle.

The chemistry required to oxidize methane into methanol is performed in methanotrophic bacteria using two different types of monooxygenases. Monooxygenases are a class of enzyme that add a single hydroxyl group to a substrate, using molecular oxygen as a co-substrate. To screen methane oxidation catalysts, we integrated the candidate pathway genes into the *Pichia pastoris* genome and tested for growth on methane

Changes to Experimental Approach

Our original approach focused entirely on a yeast host for screening enzymes that could consume methane, using a growth-based screen. We chose to expand this approach in two ways. First, we screened enzymes in both yeast and bacteria. Bacteria served us well as a rapid-prototyping system for testing candidate enzymes. This is because the sources of methane-consumption enzymes are frequently from bacteria, and would need to be refactored for expression in yeast but not for expression in bacteria. Our hypothesis was that we could quickly find enzymes that have high activity in bacteria and then transfer those enzymes into yeast. Second, we developed methane-oxidation assays that were more sensitive than a growth-based selection.

2.4 Experimental Results for Methane Utilization

Construction of Initial Test Strains

In our initial experiments, we expressed the methane-oxidation enzymes in our yeast strain. First, the rationally-designed enzyme was cloned into an expression plasmid and integrated into a yeast chromosome. This enzyme was tested with a range of tags to improve solubility and/or activity.

We designed codon-optimized DNA sequences and synthesized the DNA for all the genes. Next, we cloned all these genes into plasmids with a unique promoter or terminator for each gene and integrated them into the host genome. This procedure was performed for three different enzymes -- chosen specifically to represent different branches of the evolutionary tree, in the hope that one of the enzymes might be more amenable to heterologous expression in our yeast strain. Methane oxidation enzymes have never been actively expressed in yeast.

Industrial Microbes was awarded 500,000 bases of DNA synthesis as part of the Gen9 G-Prize [19]. We used a portion of this prize to design and synthesize DNA constructs for the expression of additional methane oxidation enzymes in yeast. We also built strains that target enzymes to different intracellular compartments, where the biochemical environment might be more amenable to their functional expression.

Testing Growth on Methane

To test our strains and enzymes for growth on methane as a sole carbon and energy source, we developed the following protocol. Strains are grown up to stationary phase in standard rich media (YPD) and then transferred to a minimal media containing methanol as the sole carbon source. The culture is then grown for 1-2 days in this minimal media to induce the expression of the pathway to convert methanol into central metabolites. The cells are washed in minimal media containing no carbon source (i.e. no sugar or methanol) and then diluted into the same minimal media without a carbon source. The culture is transferred into a sterile 125 mL serum bottle, and sealed with a butyl stopper. At this point, the culture is in an air-tight environment so the gaseous methane can be injected into the headspace above the culture. In a control serum bottle, only air is injected instead of methane. After an appropriate amount of time, the two cultures are compared to detect any growth of the yeast strain by utilizing the methane gas as a carbon source. Any difference in cell density indicates cell growth -- this can be measured either by measurements of optical density or by spreading a defined amount of the culture on solid media (e.g. rich media plus agar in Petri dishes) and counting the number of colony forming units.

A similar assay involves essentially the same steps, with the exception that we add a growth-limiting amount of methanol (0.05%) to the minimal media in the serum bottle. This accounts for the possibility that the cells are not able to detect small amounts of methanol being created (slowly) by a functional (but weak) methane monooxygenase enzyme.

2.5 Discussion and Lessons Learned

Many breakthroughs were achieved during this project. Malate was produced at a relatively high titer.

During this project, we learned two important lessons. The first lesson is that availability of a high-quality assay is crucial both for enzyme discovery and enzyme improvement. The quality of an assay can determine whether milestones are achievable. The second lesson is that contingency plans are essential for key tasks, and backup experiments need to be planned in order to begin them with enough time to achieve the milestone.

3. Greenhouse Gas and Other Impacts

3.1 Greenhouse Gas (GHG) Benefits

GHG emission reduction benefits at the 1 megatonne scale will occur when the process is implemented at commercial scale (see discussion in Section 3.2).

Industrial Microbes malate process will reduce greenhouse gas (GHG) emissions in two ways:
1. It fixes CO_2 from an emissions source directly into malate.
2. Industrial Microbes malate replaces GHG-intensive petroleum-based malate, at a net emissions reduction as indicated by life-cycle analysis.

These benefits are almost entirely due to our manufacturing technology which uses low-energy fermentation and purification. Published life cycle analyses of succinate, a chemical similar to malate and produced in a similar manner, shows that fermentation-based production has enormous energy and GHG emissions reductions compared to the petrochemical-based production process [20], [21].

The life cycle of our green malate production process includes natural gas recovery/transport, natural gas processing, fermentation, and purification into commercial-grade malate (Figure 7 right). Our fermentation process directly fixes CO_2 and methane into malate. This green chemistry fermentation process avoids high-temperature, high-pressure conditions and instead relies on enzymes in yeast cells that operate near room temperature and at atmospheric pressure. For this reason, the largest source of GHG emissions during green malate production is energy required for purifying malate from the fermentation broth. We have developed an acidic fermentation process
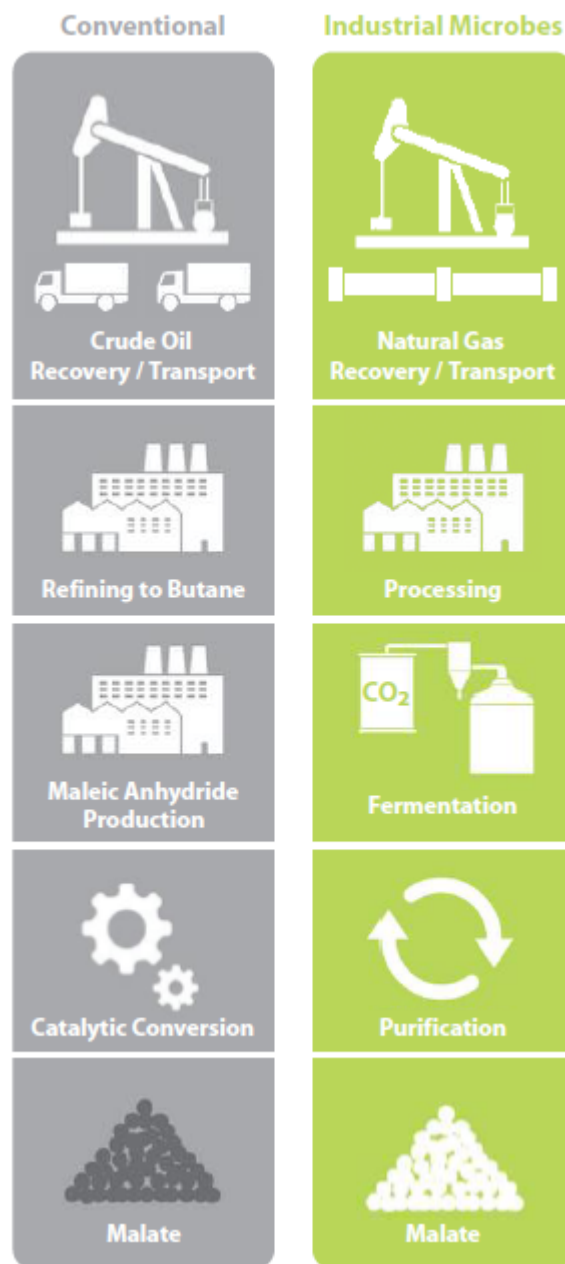

Figure 7. Boundary conditions for life cycle analysis. Malate production steps are shown from conventional feedstock petroleum (left) and Industrial Microbes carbon dioxide fixation (right). A life cycle analysis indicates that our process can significantly reduce GHG. Figure adapted from [21].

specifically to lower the energy needed for malate purification, by selecting and engineering a low-pH tolerant yeast strain.

The conventional production of malate from petroleum causes significant CO_2 emissions. The full life cycle of conventional malate production includes extracting and refining crude feedstock into butane, chemical processing into maleic anhydride, and catalytic conversion to malate (Figure 7, left). The largest source of GHG emissions is the energy-intensive production of the intermediate chemical maleic anhydride. In this reaction, butane feedstock is partially oxidized using a vanadium catalyst. This process is inefficient, converting only 60% of butane into maleic anhydride; 40% of the feedstock is burned to carbon monoxide and CO_2, generating 1.2 kg CO_2e/kg malate [7]. The process operates at 400 C, which requires significant energy and generates additional CO_2 [7], [12]. This energy requirement can be reduced somewhat if the manufacturer uses a cogeneration plant, however many older plants do not utilize this technology. In addition, some older petrochemical plants use the original benzene oxidation method, which emits even more CO_2 than the modern butane method.

Malate can sequester carbon dioxide for long periods, depending on the specific use. Plastics and fibers made from malate will sequester carbon for 50-100 years or more.

3.2 Quantification of GHG Benefits

In the Industrial Microbes green malate production process, one molecule of CO_2 is combined with three molecules of methane to produce one molecule of malate. At maximum carbon fixation efficiency, the mass of malate is composed of 32.8% carbon dioxide, by mass.

Theoretical maximum carbon fixation in malate, by mass:
MW CO_2 (44.01 g/mol) / MW Malate (134.09 g/mol) = 32.8%

This is equivalent to 3.05 tonnes of malate for each tonne of carbon dioxide fixed. These calculations are based on a stoichiometric analysis using the molecular weights of malate (134.09 g/mol) and CO_2 (44.01 g/mol). The balanced chemical equation for the production process is:
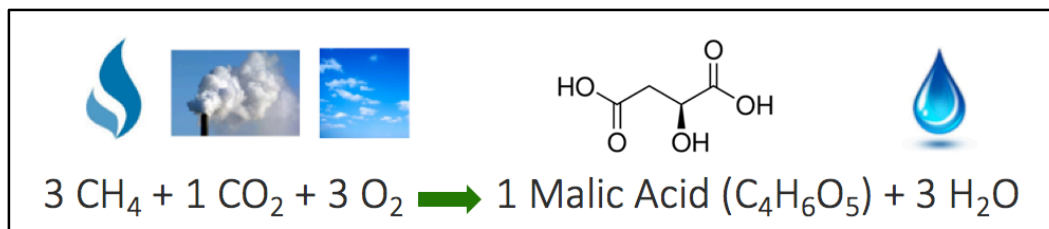
Figure 8. Balanced chemical equation for malate (malic acid) production.
Feedstocks are methane from natural gas, carbon dioxide from treated flue gas, and oxygen from air. Malic acid and water are the products.

The maximum titer of malate observed with the first generation of strains is 6.05 g, which has a theoretical maximum carbon fixation of 6.05 g x 32.8% = 1.98 g carbon dioxide. We estimate that our current strains and process at laboratory scale can fix 45% of the maximum theoretical amount

of carbon dioxide per molecule of malate (see section 2.3). Thus, the total estimated carbon dioxide fixed by this experiment is 1.98 g x 45% = 0.89 g carbon dioxide, or 14.7% of the mass of malate. At this early stage of technology development, the fixed carbon dioxide was derived both from the atmosphere in the headspace above the fermentation broth and from cellular metabolism.

There remain challenges for Industrial Microbes' process at the end of this feasibility project. The bench-scale process needs significant improvement in titer, yield, and productivity to compete with petroleum-derived malate on price.

3.3 GHG Benefits at Commercial Scale

A small 35,000 tonne/year commercial malate plant would fix up to 35,000 x 32.8% = 11,480 tonnes/year of carbon dioxide, depending on the efficiency of the plant and the microbe. This estimation does not include emissions benefits from reduced lifecycle emissions in replacing petroleum-derived malate, nor does it account for additional emissions due to plant operations. Several dozen 100,000 tonnes/year plants, producing a total of 3.05 megatonnes of malate per year, could be constructed to meet the Grand Challenge goal of fixing one megatonne of carbon dioxide annually.

Our proposed process uses methane to power carbon fixation, instead of burning methane for energy, which creates carbon emissions. Our process is thus synergistic with renewable energy production. Each tonne of malate production can displace almost a tonne of energy-related carbon emissions. The calculation is as follows: a tonne of malate produced utilizes 359 kg of methane (1000 kg x 48.12 g/mol methane consumed / 134.09 malate produced). Each 359 kg of methane consumed prevents 985 kg of carbon dioxide production via combustion (359 x 44.01 g/mol carbon dioxide produced / 16.04 g/mol methane consumed). In a life cycle analysis, the electricity or heat that would have been produced by the combustion is replaced by electricity from the grid with a corresponding carbon intensity. If the carbon intensity is low, for example by the widespread use of renewable energy, the GHG benefits of our process are amplified.

3.4 GHG Benefits and Market Adoption

Malate and other dicarboxylic acids are building-block molecules for dozens of chemical and consumer products with sufficient market size to consume a megaton/year of CO_2. The four-carbon dicarboxylic acids are precursors to 1,4-butanediol, tetrahydrofuran, γ-butyrolactone, adipic acid, pyrrolidinone derivatives, and linear aliphatic esters [22]. These chemicals are key components of a wide range of products, such as green solvents, water soluble polymers, fibers (lycra), biobased polymers (nylons, polyesters, PBS), surfactants, ion chelators, food acidulants, beverage additives, plasticizers, resins, and pharmaceutical additives [5], [23], [24]. The total market for these products is 3-4 megatonnes and growing. The costs of chemically converting dicarboxylic acids to other products such as 1,4 butanediol are low, because hydrogenation chemical operations are widely practiced and are similar to the conversion of petroleum-derived maleic anhydride to the same compounds [5].

The current malate price is \$1800-1900/tonne [25], [26], which limits the market to specialty food applications. The current market is estimated to be a modest 60,000 tonnes, representing a market size of CAD\$125M [27]. However, research firms and consultants predict that a lower diacid price will translate into a dramatically larger market, since diacids are building block chemicals [28]–[30]. We estimate the market size for products that could be derived from \$1000/tonne malate is 3-4 megatonnes and growing. The annual market for 1,4-butanediol alone is around one megatonne and expanding at an 18% annual rate [31]. Malate derivatives are used in dozens of chemical and consumer products with sufficient market size to consume a megatonne of CO_2.

The Industrial Microbes process has the potential to make malate at lower cost than current methods, which is crucial for commercialization. This is due to the low cost of our raw material inputs: carbon dioxide, methane, and oxygen/air. Since only 36% of the mass of malic acid is derived from methane, the process requires 360 kg of methane to make one tonne of malic acid; this cost is less than \$60/tonne malate at the current natural gas price of \$3/MMBtu. The remaining production costs are from capital depreciation, electricity, and product purification.

Commercialization of malate relies on one or more development partners to invest in the technology in exchange for a license. Industrial Microbes malate will compete with petroleum-derived malate so obtaining these development partners, and our level of profitability in Alberta, will depend on the relative prices of oil and natural gas, as well as carbon taxes and policies. Although our technology can produce malate cost-competitively with 2015-2016 oil prices, the roughly decade-low current prices do reduce incentive to invest in technology development. This is because lower-cost oil translates into lower-cost petrochemical feedstocks, which decreases the cost-savings that our technology provides.

3.5 Other Benefits

Our proposed process to fix CO_2 into malate has minimal environmental impact, no hazardous waste streams, minimal energy consumption, and no harmful emissions. Biological fermentation is one of the greenest technologies for production of industrial chemicals [32] because enzymes are highly selective, efficient catalysts that do not require toxic metals [33]. The inputs to our fermentation process are water, salts, methane, air, and a waste stream of dilute CO_2. Water is a chemical product of the process and can be used locally or for plant operations. No harmful emissions are expected.

Vertical integration of oil and gas operations into plastics or development of a plastics industry can help insulate the Alberta economy from commodity price swings. The development of a dicarboxylic acid-to-plastics industry in Alberta will also create high-paying jobs in biotech research, industrial fermentation, chemical recovery, plant construction, and plant operations. Canada is already a net exporter of malate [34], and Alberta's export of chemicals and plastics is growing at 6-7% annually to meet global demand. Malate production has the potential to contribute up to 1% of Alberta's total export market. Although exports are currently dominated by the energy sector [35], there is a growing opportunity to export CO_2-based materials such as malate.

4. Overall Conclusions

The project was successful in achieving several key results:

- Malate was produced in engineered yeast cells at reasonable titer.
- Malate production fixed an estimated half of the maximum amount of carbon dioxide theoretically possible.
- Patents were filed to protect key results and enable commercialization.

The following challenges have been identified:

- The price of malate dropped significantly during the project period due to the fall in crude oil prices, reducing the financial incentive to produce malate.
- No commercial partners were identified in Alberta that had significant carbon emissions and a current business related to malate manufacturing.
- Commercialization will likely require two partners in Alberta: a carbon dioxide emitter and a malate manufacturer.

5. Scientific Achievements

Patent Applications

PCT Patent Application
"SYNTHETIC METHANOTROPHIC AND METHYLOTROPHIC MICROORGANISMS"
WO 2015/160848 A1
Publication Date: 10/22/2015

Presentations

Turning greenhouse gases into building-block chemicals
Derek Greenfield, Zero 2014
April 16, 2014

Natural Gas is the New Sugar
Derek Greenfield, ABLC Next 2014
November 10, 2014

Upgrading Methane to Valuable Chemicals Using Biotech
Elizabeth Clarke, Waste Carbon Manufacturing, SynBioBeta
November 14, 2014

Using Synthetic Biology to Convert Low-cost Feedstocks into Valuable Chemical Products
Noah Helman, informEX
February 3, 2016

Methane to Chemicals using Biotech
Derek Greenfield, Cluster IAR
April 15, 2016

6. Next Steps Forward

Further technology development is needed to build the full prototype. The next steps in the technology development are:
1. Optimize the fermentation conditions for higher malate titer.
2. In resting cells, measure the carbon dioxide fixation of the full pathway.

We will commercialize our malate production technology via licensing to partners, including large emitters. We will work with partners to develop the malate process. Partners will receive licenses to our technology for specific applications or regions, and we will receive upfront payments and royalties. We are early in the development cycle; our strategy, validated using customer interviews, is to receive commercialization support from initial partners via joint development. The in-licensing of new technologies is common at oil and gas producers.

To sign joint development license agreements, we need to achieve titer and productivity milestones at bench scale. These milestones were developed from customer interviews. To commercialize the malate process, we must demonstrate the feasibility and value of our technology at increasingly larger scales.

The technology developed in this project has enormous potential. Dozens of other chemical products can be manufactured with the same technology and microorganisms. Many of those products would have improved life cycle carbon emissions compared to their current baseline commercial production processes. Succinate and fumarate can be made using the same platform and would also directly fix carbon dioxide into the chemical product.

7. Communications Plan

Industrial Microbes is committed to sharing results via patent applications, presentations at conferences, and in publications in scientific journals as appropriate.

Press

CCEMC Grand Challenge: Industrial Microbes
Cheryl Croucher, Innovation Anthology #615,
May 29, 2014
http://www.innovationanthology.com/programs.php?id=639

The Bio Incredibles
Jim Lane, Biofuels Digest
May 29, 2014
http://www.biofuelsdigest.com/bdigest/2014/05/29/the-bio-incredibles/

CO₂ to Malic Acid by Industrial Microbes
Doris de Guzman, Green Chemicals Blog
August 19, 2014
http://greenchemicalsblog.com/2014/08/19/co2-to-malic-acid-by-industrial-microbes/

Open Innovation
Omar Mouallem, Energy Exchange
January 5, 2015
http://www.energy-exchange.net/open-innovation/

Scientists Seek to Engineer Microbes to Make Simple Chemicals
Yarrow Madrona, Synapse
January 9, 2015
http://synapse.ucsf.edu/articles/2015/01/09/scientists-seek-engineer-microbes-make-simple-chemicals

YC-Backed Industrial Microbes Is Engineering Bacteria To Produce Chemicals From Natural Gases
Kim-Mai Cutler, TechCrunch
March 16, 2015
https://techcrunch.com/2015/03/16/industrial-microbes/

LS9 Alumni Found Industrial Microbes and Win G-Prize Contest
Conor McClune, SynBioBeta
September 17, 2015
http://synbiobeta.com/news/ls9-alumni-found-industrial-microbes-and-win-g-prize-contest/

Synthetic biology lures Silicon Valley investors
Erika Check Hayden, Nature News
November 4, 2015
http://www.nature.com/news/synthetic-biology-lures-silicon-valley-investors-1.18715

8. References

[1]   A. King and L. Dilling, "The first state of the carbon cycle report (SOCCR): The North American carbon budget and implications for the global carbon cycle," 2007.

[2]   A. T-Raissi and D. Block, "Hydrogen: automotive fuel of the future," *IEEE Power Energy Mag.*, 2004.

[3]   R. Lemus and J. M. Duart, "Updated hydrogen production costs and parities for conventional and renewable technologies," *Int. J. Hydrogen Energy*, vol. 35, pp. 3929–3936, 2010.

[4]   A. Bar-Even and E. Noor, "Design and analysis of synthetic carbon fixation pathways," *Proc. Natl. Acad. Sci.*, vol. 107, no. 19, 2010.

[5]   T. Werpy and G. Petersen, "Top Value Added Chemicals from Biomass. Volume I - Results of Screening for Potential Candidates from Sugars and Synthesis Gas," 2004.

[6]   S. H. Brown, L. Bashkirova, R. Berka, T. Chandler, T. Doty, K. McCall, M. McCulloch, S. McFarland, S. Thompson, D. Yaver, and A. Berry, "Metabolic engineering of Aspergillus oryzae NRRL 3488 for increased production of L-malic acid.," *Appl. Microbiol. Biotechnol.*, Aug. 2013.

[7]   H. Chen and D. Shonnard, "Systematic framework for environmentally conscious chemical process design: early and detailed design stages," *Ind. Eng. Chem. Res.*, 2004.

[8]   Polynt Specialty Solutions, "Malic acid."

[9]   R. Zelle and E. De Hulster, "Malic acid production by Saccharomyces cerevisiae: engineering of pyruvate carboxylation, oxaloacetate reduction, and malate export," *Appl. Environ. Microbiol.*, 2008.

[10]   X. Zhang and X. Wang, "L-Malate production by metabolically engineered Escherichia coli," *Appl. Environ. Microbiol.*, vol. 77, no. 2, pp. 427–434, 2011.

[11]   S. Moon, S. Hong, T. Kim, and S. Lee, "Metabolic engineering of Escherichia coli for the production of malic acid," *Biochem. Eng. J.*, vol. 40, pp. 312–320, 2008.

[12]   B. Cok and I. Tsiropoulos, "Succinic acid production derived from carbohydrates: An energy and greenhouse gas assessment of a platform chemical toward a bio-based economy," *Biofuels, Bioprod. Biorefining*, 2013.

[13]   B. K.-S. Chung, M. Lakshmanan, M. Klement, C. B. Ching, and D.-Y. Lee, "Metabolic reconstruction and flux analysis of industrial Pichia yeasts.," *Appl. Microbiol. Biotechnol.*, vol. 97, no. 5, pp. 1865–73, Mar. 2013.

[14]   J. L. Cereghino and J. M. Cregg, "Heterologous protein expression in the methylotrophic yeast Pichia pastoris.," *FEMS Microbiol. Rev.*, vol. 24, no. 1, pp. 45–66, Jan. 2000.

[15]   G. P. Lin-Cereghino, L. Godfrey, B. J. de la Cruz, S. Johnson, S. Khuongsathiene, T. I., M. Yan, J. Lin-Cereghino, M. Veenhuis, S. Subramani, and J. M. Cregg, "Mxr1p, a key regulator of the methanol utilization pathway and peroxisomal genes in Pichia pastoris," *Mol. Cell. Biol.*, vol. 26, no. 3, pp. 883–897, 2006.

[16]   R. Zelle and E. De Hulster, "Key process conditions for production of C4 dicarboxylic acids in bioreactor batch cultures of an engineered Saccharomyces cerevisiae strain," *Appl. Environ. Microbiol.*, 2010.

[17]   I. J. Higgins, D. J. Best, R. C. Hammond, and D. Scott, "Methane-Oxidizing Microorganisms," vol. 45, no. 4, pp. 556–590, 1981.

[18]   T. Nagahama, E. Takahashi, Y. Nagano, M. A. Abdel-wahab, and M. Miyazaki, "Molecular evidence that deep-branching fungi are major fungal components in deep-sea methane cold-seep sediments," vol. 13, pp. 2359–2370, 2011.

[19]   Gen9, "G-Prize Check-in: How Industrial Microbes Used 500kb of Synthetic DNA," 2016. [Online]. Available: https://blog.gen9bio.com/synthetic-dna-award-for-synthetic-biology-industrial-microbes.

[20] Reverdia and W. van den Tweel, "Sustainable Succinic Acid," 2010.

[21] "BioAmber LCA Summary," 2013. [Online]. Available: http://www.bio-amber.com/bioamber/en/sustainability/life_cycle_analysis_lca. [Accessed: 01-Oct-2013].

[22] J. Zeikus, M. Jain, and P. Elankovan, "Biotechnology of succinic acid production and markets for derived industrial products," *Appl. Microbiol. Biotechnol.*, vol. 51, pp. 545–552, 1999.

[23] Y. Cao and X. Lin, "Metabolically engineered Escherichia coli for biotechnological production of four-carbon 1, 4-dicarboxylic acids," *J. Ind. Microbiol. Biotechnol.*, vol. 38, pp. 649–656, 2011.

[24] C. Engel and A. Straathof, "Fumaric acid production by fermentation," *Appl. Microbiol. Biotechnol.*, vol. 78, pp. 379–389, 2008.

[25] Grand View Research, "Malic Acid Market Analysis By End-Use (Beverage, Confectionery, Personal Care) And Segment Forecasts To 2024," 2016. [Online]. Available: http://www.grandviewresearch.com/industry-analysis/malic-acid-market.

[26] Zauba, "Detailed Import Data of DL Malic Acid," 2016. [Online]. Available: https://www.zauba.com/import-dl-malic-acid-hs-code.html.

[27] "Novozymes develops new process for renewable malic acid." [Online]. Available: http://www.biofuelsdigest.com/bdigest/2012/08/16/novozymes-develops-new-process-for-renewable-malic-acid/.

[28] Nexant, "Maleic Anhydride," 2005.

[29] M. Sauer, D. Porro, D. Mattanovich, and P. Branduardi, "Microbial production of organic acids: expanding the markets.," *Trends Biotechnol.*, vol. 26, no. 2, pp. 100–8, Feb. 2008.

[30] "Bioamber, Inc. S1/A SEC Filing," 2012.

[31] "Bio-Based Chemicals Value Added Products from Biorefineries, IEA Bioenergy." 2010.

[32] N. Ran, L. Zhao, Z. Chen, and J. Tao, "Recent applications of biocatalysis in developing green chemistry for chemical synthesis at the industrial scale," *Green Chem.*, vol. 10, pp. 361–372, 2008.

[33] M. Alcalde, M. Ferrer, F. Plou, and A. Ballesteros, "Environmental biocatalysis: from remediation with enzymes to novel green processes," *TRENDS Biotechnol.*, vol. 24, no. 6, 2006.

[34] "Industry Canada." [Online]. Available: http://www.ic.gc.ca/app/ccc/srch/nvgt.do?lang=eng&prtl=1&sbPrtl=&estblmntNo=253007320000&profile=cmpltPrfl&profileId=201&app=sold.

[35] "Export Development Canada." [Online]. Available: http://www.edc.ca/EN/About-Us/News-Room/News-Releases/Pages/alberta-international-exports.aspx.