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FINAL PROJECT REPORT

PRODUCTION OF SOLADIESEL RD® FROM CELLULOSIC FEEDSTOCKS

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solazyme

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PREFACE

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Production of Soladiesel RD™ from Cellulosic Feedstocks is the final report for the PIER Transportation Grant Solicitation (PIR-08-048) conducted by Solazyme, Inc. The information from this project contributes to Energy Research and Development Division's Transportation Program.

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ABSTRACT

Solazyme, Inc., is a renewable energy company focused on producing renewable fuels, chemicals, and foods using proprietary algae grown in high-cell density fermentors on a variety of sugar feedstocks. The research described in this report sought to determine if Solazyme's technology is transferable to sugars derived from cellulosic biomass. Cellulosic feedstocks contain a mixture of sugars made up primarily of glucose (60-75 percent) and xylose (25-40 percent), a sugar first isolated from wood. Most microorganisms, including algae, can metabolize glucose but not xylose. In addition, cellulosic feedstocks are generally enriched for compounds that can hinder the growth of most microorganisms, including algae. To address these issues, Solazyme adopted a parallel processing approach for algal strains focused on three principal areas: discovering new strains capable of metabolizing xylose, genetically engineering existing strains to metabolize xylose, and evolving strains to become more tolerant of inhibitors present in cellulosic feedstocks. From the standpoint of cellulosic sugars themselves, efforts were focused on the removal of inhibitory compounds, or substances that can slow or stop the necessary chemical reactions. This report demonstrates that algal strains have been discovered that are capable of using xylose as a carbon source. Researchers showed strains can be successfully engineered to metabolize xylose and produce oil from this sugar alone. Researchers successfully evolved strains to tolerate some of the most abundant inhibitors present in cellulosic feedstocks and developed methodologies to remove those same compounds from cellulosic sugars. The initial results were applied to larger-scale fermentations using these new strains grown on a variety of cellulosic feedstocks, producing oil-rich biomass. Finally, researchers produced and purified algal oil derived from cellulosic feedstocks. This project benefits California through the development of advanced alternative fuels which reduce greenhouse gas emissions, reduce dependence upon imported energy and increase California's energy security, and potentially provide California with new jobs in the rapidly expanding field of alternative fuels production.

Keywords: California Energy Commission, Solazyme, renewable, diesel, cellulosic, algae, oil, energy, xylose, fermentation

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EXECUTIVE SUMMARY

Solazyme is an industrial biotechnology company based in South San Francisco that uses standard industrial fermentation equipment to harness the prolific oil producing ability of microalgae. Solazyme's technology is feedstock flexible, meaning that it can utilize a wide variety of plant-based sugars, such as sugarcane-based sucrose, corn-based dextrose, and sugar from other biomass sources including cellulosics. Growing its microalgae in the dark using fermentation tanks to convert photosynthetic plant sugars into oil, Solazyme calls its technology "indirect photosynthesis."

This project was undertaken to develop an innovative method of creating advanced, renewable transportation fuels. Many alternative fuels processes depend upon dedicated energy crops, which compete with food crops for existing farmland. Microalgae, though, can be used to manufacture carbon-neutral sustainable biofuels without requiring large tracts of land. Solazyme's algal process for producing oils does not rely on photosynthesis, outdoor ponds, or photo bioreactors, but on *heterotrophic* algae that can grow and generate biomass in the dark. Most microalgae produce their own nutrients by using sunlight in a phototsynthetic process. However, Solazyme's proprietary microalgae are heterotrophic, meaning that they grow in the dark (in fermenters) by consuming sugars derived from plants that have already harnessed the sun's energy. These heterotrophic algae convert sugars into oil in standard industrial fermentation vessels. While sugars such as glucose or fructose are suitable feedstocks for conversion into oils by these algae, they can also use sugars derived from cellulosic materials such as municipal green waste, switchgrass, corn stover (the leaves and stalks of maize), beet pulp, wood waste, and so forth. The Soladiesel RD™ process utilizes this heterotrophic algal fermentation to make sustainable, cellulosic feedstocks.

The research was defined by three main activities:

- Identification of algal strains capable of converting cellulosic feedstocks into oil
- Procurement of cellulosic feedstocks followed by evaluation and optimization of strain performance on the feedstocks
- Conversion of these cellulosic feedstocks into oil using the best procedures and strains

The converted cellulosic-derived oil was then transformed using standard technologies into renewable Soladiesel$_{RD}$® in compliance with the specifications developed by the American Society for Testing and Materials specifications (ASTM).

Included among the fuels produced are Soladiesel$_{BD}$® an ASTM D6751 biodiesel, Soladiesel$_{RD}$® an ASTM D975 renewable diesel, Solajet ™ , the world's first 100 percent algal-derived jet fuel meeting all nonpetroleum military specifications for JP-5 jet fuel and Soladiesel$_{HRF-76}$® a renewable diesel for ships currently undergoing extensive testing and certification by the U.S. Navy as renewable F76 (the fuel normally used in shipboard diesels).

While technologies that convert cellulosic biomass into sugars are maturing rapidly and hold promise for reducing the cost of sugars used in industrial applications, there are numerous

challenges associated with this process including the types of sugars made and the level of growth-inhibiting compounds introduced during their manufacture.

As the U.S. Department of Energy and myriad private entities are investing heavily in cellulosic technologies, Solazyme's intent is to not duplicate these efforts. Instead, Solazyme has focused on developing a technology process that utilizes microbes to generate oils from a variety of feedstocks and can be deployed wherever and whenever suitable feedstocks are economically available. In the work described in this report, Solazyme has relied on third-party vendors to supply cellulosic feedstocks, while exploring ways to tailor its processes to use them in the most efficient way.

The problem of cellulosic feedstock usage and enhancement was approached from three perspectives. First, efforts focused on engineering or discovering algal strains, or species capable of using both classes of sugars (five and six carbon molecules, or C5 and C6) present in cellulosic feedstocks. Second, efforts were directed toward evolving strains to grow and produce oil even in the presence of inhibitors (substances that slow or stop a chemical reaction) produced during the generation of cellulosic sugars. Third, processes were developed to remove these inhibitory compounds effectively from cellulosic feedstocks. For this effort to be considered successful, it was not necessary to succeed on all of these processes in a single algal strain, but rather demonstrate that each approach was feasible and capable of affecting the overall process. Every aspect of this program was successfully accomplished.

Solazyme identified strains capable of using C5 sugars and producing oil. While their productivities were low when compared to lead fuels strains, they represent some of the first examples of C5 sugar usage by algae.

Solazyme successfully engineered the capacity to metabolize xylose into a specific strain of algae for fuels production, and demonstrated that these engineered pathways could divert carbon from xylose and use it to produce lipid. These results offer great promise and demonstrate the feasibility of investing in additional engineering efforts around the proprietary algal strains to improve lipid (fats and other organic compounds that are not water soluble).

Strain evolution efforts were also successful. Several strains exhibited superior performance on some of the principal inhibitors found in cellulosic feedstocks. One of the evolved strains, K2-5, produced more lipid than the parent Strain A under *all* conditions tested, including production in the control medium. As part of an internal strain improvement program, new, classically improved strains were identified that displayed performances on cellulosic derived feedstocks that were superior to existing lead fuels strain grown under *control* conditions at the start of this program.

Researchers procured and successfully detoxified five types of cellulosic feedstocks from three providers. They validated strain performance on each feedstock in the lab and developed a robust bench-scale process. This resulted in the successful completion of 14 7-liter fermentation runs ultimately resulting in the generation of a little more than 3 kilograms (kg) of refined and bleached algal oil produced entirely from cellulosic-derived sugars.

During the course of this project, researchers demonstrated the viability of using genetic engineering as well as classical strain improvement methods to perfect algal strains for cellulosic feedstock conversion. Highlighted by the study were areas that require further research and development for commercialization to become a reality.

CHAPTER 1: Introduction

1.1 Solazyme's Approach to Biomanufacturing Clean, Renewable Fuel

Solazyme is the only advanced biosynthetic transportation fuel production company that has manufactured an oil-based transportation fuel at commercial manufacturing scale that meets existing ASTM fuel standards. Solazyme utilizes algae to make oil, in this case a triglyceride, that is further refined into fuels. Among the fuels researchers have produced are Soladiesel$_{BD}$® an ASTM D6751 biodiesel, a Fatty Acid Methyl Ester (FAME), Soladiesel$_{RD}$® an ASTM D975 renewable diesel, Solajet ™, the world's first 100 percent algal derived jet fuel meeting all non-petroleum military specifications for JP-5 jet fuel and Soladiesel$_{HRF-76}$® a renewable diesel for ships currently undergoing extensive testing and certification by the Navy as renewable F76.

Solazyme focuses on the production of biofuels through the heterotrophic fermentation of algae (in the dark) using standard industrial fermentation equipment. Algae possess internal biochemical pathways that synthesize oil more efficiently than any other known natural or engineered process. Under the right conditions, some algal species can produce oil to over 80 percent of the dry weight of the cells. In fact, the world's petroleum deposits consist largely of the fossilized remains of prehistoric algal blooms. Solazyme has previously developed technology to produce renewable oil using a very high-cell-density fermentation process that generates algae with very high lipid content. The resulting algal oil, when extracted, has a composition ideal as a feedstock for conversion into FAME-based biodiesels, hydrotreated renewable diesels as well as a variety of oleochemicals (chemicals derived from plants and animal fats) for use in the food, personal healthcare, cleaning and electrical distribution industries, all of which can be carried out using standard technologies. Many groups are trying to exploit algae as a potential source of renewable biofuel, mostly focusing on *phototrophically* grown algae, which rely directly on photosynthesis to grow and on algal ponds or photo bioreactors in which to accumulate algal biomass. Solazyme's innovative algal process does not rely on photosynthesis or outdoor ponds/photoreactors in which productivities are very low, but on *heterotrophic* algae that can grow and generate biomass in the dark. These heterotrophic algae ingest organic (carbon-containing) molecules present in feedstocks (mono-di-tri and even tetra-saccharides) and convert these carbons in to oil in standard industrial fermentation vessels. The feedstocks can be in the form of readily-available sugars obtained from cane or beets, as well as newly-emerging cellulosic sugars derived from sustainable, non-food, waste plant matter such as municipal green waste, switchgrass, corn stover, beet pulp, etc. All of these feedstocks in turn are derived from plants that grow and fix carbon using photosynthesis. Solazyme's process thus exploits "indirect photosynthesis" — a term that differentiates the company's process from photosynthetic algal cultivation yet emphasizes that it fully preserves the carbon neutrality benefits of photosynthesis.

Emerging technologies that convert cellulosic biomass—such as fast-growing grasses or agricultural residue—to sugars are maturing rapidly and hold out the promise of reducing the cost of industrial sugar. These cellulosic technologies have been exploited primarily to serve the production of ethanol, but Soladiesel RD® will also utilize this sugar source and reap the same cost and environmental benefits. The United States government and many private companies are investing heavily in cellulosic technologies. Solazyme does not intend to duplicate these efforts in converting cellulosic biomass to sugar. Instead, Solazyme intends to tailor its process, allowing its proprietary algae to *consume* sugars derived from myriad processes. The research for optimizing this process was carried out using cellulosic feedstocks obtained from third-party vendors that already have existing technologies for converting cellulosic biomass into sugars

1.1 Project Goals and Objectives

The objective of this project was to carry out research that would enable Solazyme's commercial oil biomanufacturing process to use sustainable, non-food derived, cellulosic feedstocks. The research covered by this grant can be broadly defined by three main activities:

1. Identification of an algal strain that will convert cellulosic feedstocks into oil.
2. Procurement of cellulosic feedstocks and evaluation/optimization of strain performance on the feedstocks.
3. Conversion of cellulosic feedstocks into oil using optimized procedures and an optimized strain.

Converted oil will be processed to form in spec ASTM D975 renewable Soladiesel RD™ using standard technologies to produce a fuel with broad infrastructure options. As a separate document, commercialization plan will also be presented for bringing the technologies developed under this project to market.

CHAPTER 2:
Identification of an Algal Strain that will Convert Cellulosic Feedstocks into Oil

Any microalga selected for bioconversion of cellulosic feedstocks to oil has to satisfy several unique criteria. Unlike pure sugar feedstocks, cellulosic feedstocks have unique properties that present various difficulties for their subsequent utilization by microorganisms. Most microorganisms can metabolize glucose. However, many (including yeasts used to make ethanol) cannot utilize C5 sugars such as xylose, which can comprise up to 40 percent of the sugars present. Obviously, a microorganism that can also convert the carbons present in xylose to fuel is highly desirable, not simply from an economic perspective but also from a technical standpoint, as high concentrations of non-metabolized (C5) sugars can build up during fermentations and become toxic. In addition, the harsh conditions (extremes of pH, temperature and pressure) used to convert lignocellulosic materials into fermentable sugars also lead to the generation of toxic by-products such as furfural, hydroxymethyl furfural and high salt concentrations. The tolerance of any fuels organism to these toxins is critical.

Therefore, desired properties for an optimal strain include an ability to metabolize xylose and some level of tolerance to known cellulosic toxins. These properties, while desirable, do not trump the most important property of all: an ability to make large amounts of oil by bioconversion of all carbons available in cellulosic feedstocks, principally glucose and xylose.

Researchers engaged multiple, parallel strategies to identify the best strain for bioconversion of cellulosic carbons, keeping in mind that the final strain chosen will produce the most oil from cellulosic feedstocks and may not necessarily possess each and every desired attribute. Toward this end researchers:

- used directed genetic engineering to engineer strains with synthetic pathways that can potentially convert xylose into oil.
- used directed strain evolution to select for strains with increased tolerance to cellulosic toxins.
- screened our existing strain collection as well as new, improved strains from our independent strain improvement program for the best overall cellulosic oil producers.

2.1 Genetic Engineering Xylose Metabolism

The lead fuels strain at the start of the project (Strain A) was not able to metabolize xylose. Researchers embarked on a genetic engineering approach to insert pathways that would allow this strain to metabolize xylose and convert it into oil.

Xylose utilization in algae requires three steps. First, xylose has to enter the cell's cytoplasm from the growth media. Second, intracellular xylose must then be biochemically converted into xylulose-5-phosphate. Third, xylulose-5-phosphate must be available to enter the pentose

phosphate pathway, present in all organisms, where it can finally participate in the general metabolic flux carried out within the cell.

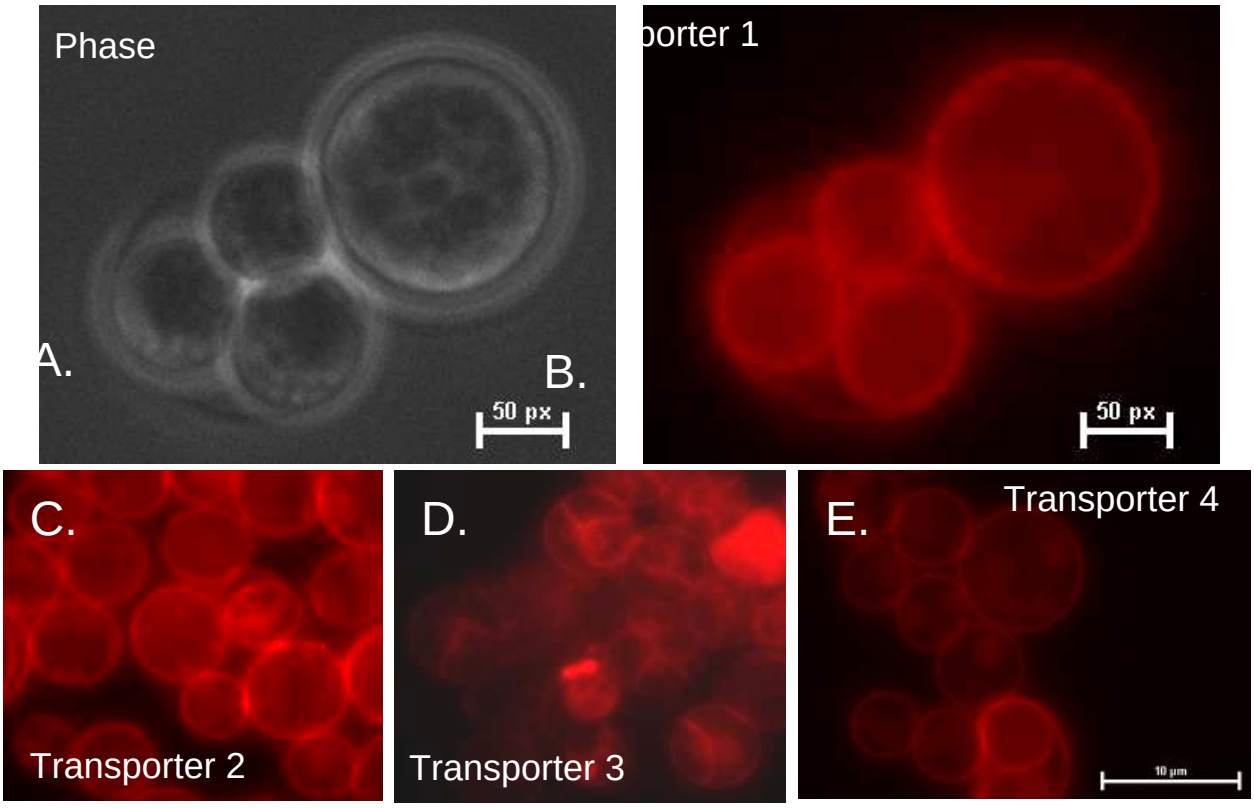
2.1.1 Entry of Xylose into the Cell

Microorganisms that possess the intrinsic ability to metabolize xylose express sugar transporters embedded in their cell membrane that act as conduits for xylose to travel from the extra-cellular environment into the cytoplasm. These transporters can be either promiscuous in that they can transport a variety of C6 or C5 sugars (including xylose) into the cell, or they can be quite specific for transporting xylose alone. In addition, transporters can either passively provide a gateway into the cell by passive diffusion of xylose down a concentration gradient, or be actively engaged in transporting xylose by expending energy to drive the movement of xylose into the cell against a concentration gradient.

Although it is possible that our strain already expresses promiscuous sugar transporters capable of moving xylose into the cytoplasm, it was reasoned that the additional expression of more specific xylose transporters could only improve the utilization of xylose in our lead strain. Various specific xylose transporters from yeast and plants have been described in the literature. Four such transporters were chosen, some of which transport xylose actively and some of which allow passive diffusion of xylose into the cell, to be expressed as transgenes in our lead fuels strain.

The gene for each xylose transporter was inserted into the genome of Strain A under the control of a proprietary promoter regulatory element. Subsequently, it was confirmed that each of the four genes was indeed expressed (transcribed) in our strain. Because transporter proteins must fold and insert into the cell membrane properly to be functional, researchers wanted to verify that the protein products of these inserted transgenes were indeed localizing to the cell membrane. To this end, DNA sequences encoding a fluorescent protein were fused in-frame to the carboxyl terminus (end) of each of the transporter transgenes and observed cells expressing the constructs utilizing fluorescence microscopy. Figure 1 contains representative micrographs of cells expressing transporters 1, 2, 3 or 4 with a fluorescent protein fused to their carboxyl terminus. Figure 1A shows the phase contrast image of a field of cells. Figure 1B shows the fluorescence due to the expression of Transporter 1 fused to the fluorescent reporter protein in the same field of cells. The main fluorescent signal is seen around the periphery of the cell membrane, strongly suggesting that Transporter 1 is expressed and localized properly and therefore likely to be functional. Similar results were obtained for Transporters 2, 3 and 4 (Figure 1C, D, E). Our cellular localization data suggest that all four transgenic transporters, when introduced into our lead Strain A, are expressed and localized properly. Although these studies are not capable of determining if they are fully active, they are certainly encouraging.

Figure 1: Expression and Localization of Transgenic Transporters 1, 2, 3 and 4 in Lead Strain A



Source: Solazyme, Inc.

The next two sub-sections describe the engineering of additional pathways into Strain A.

2.1.2 Biochemical Conversion of Xylose to Xylulose-5-phosphate

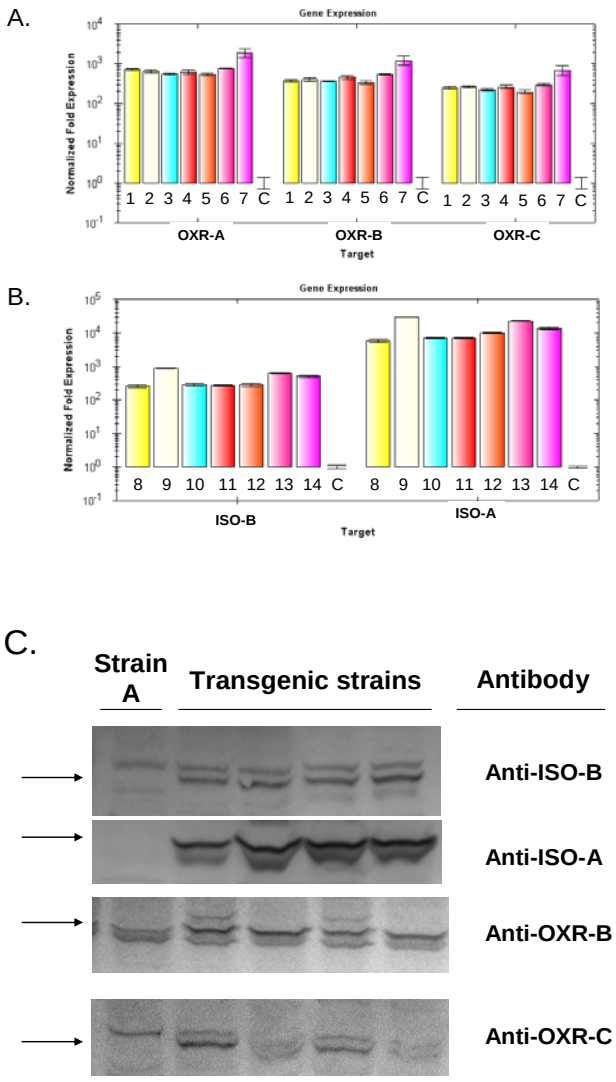
The biochemical conversion of xylose to xylulose-5-phosphate is known to proceed via two distinct pathways. The first, termed the oxido-reductase pathway, is predominantly found in fungi, and utilizes three enzymes (encoded by genes that are designated as OXR-A, OXR-B and OXR-C) to convert xylose via a series of intermediates to xylulose 5-phosphate, which is in turn metabolized via the pentose phosphate pathway present in all organisms. The second is termed the isomerase pathway, is found predominantly in bacteria, and utilizes two enzymes (encoded by genes that are designated as ISO-A and ISO-B) to convert xylose to xylulose 5-phosphate.

Each of these genes was codon-optimized and inserted into Strain A. Transgenic strains were engineered containing OXR-A, OXR-B and OXR-C or ISO-A and ISO-B, corresponding to each alternative pathway for the biochemical conversion of xylose to xylulose-5-phosphate. Researchers verified that, within the corresponding transgenic strains, each introduced gene was transcribed (expressed) at the RNA level. Figure 2A demonstrates that the transgenes OXR-A, OXR-B and OXR-C from the oxidoreductase pathway are indeed expressed in

transgenic strains 1-7 whereas no expression could be discerned from the original untransformed parent, C. Figure 2B demonstrates that transgenes ISO-A and ISO-B from the isomerase pathway are all expressed in transgenic strains 8-14 but not in the untransformed parent, C.

In addition, researchers generated antibodies against each gene product so they could further monitor expression at the protein level using Western blot analysis. Proteins corresponding to the expected size band (arrows) were observed in extracts made from transgenic strains containing the corresponding inserted transgenes, but not the untransformed parental Strain A (Figure 2C).

Figure 2: Expression of Transgenic RNA (A, B) and Protein (C)



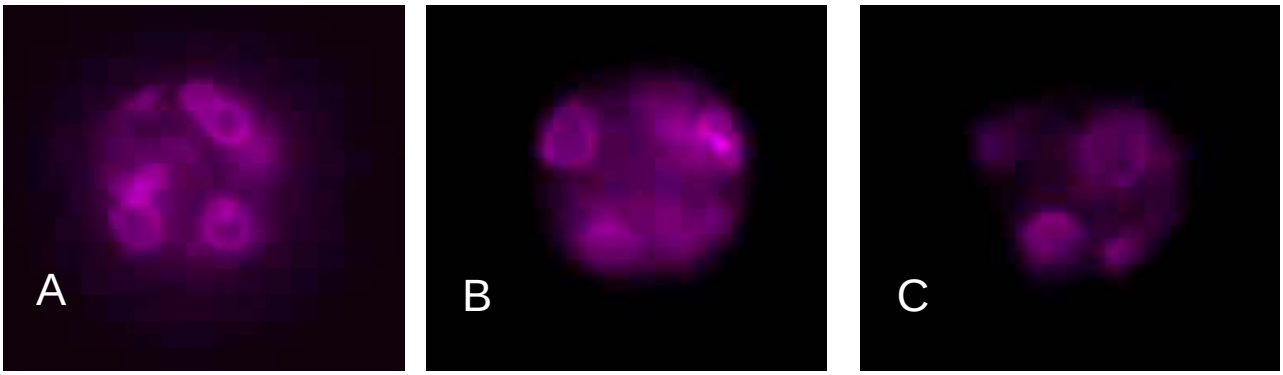
Source: Solazyme, Inc.

The expression of enzymes responsible for conversion of xylose to xylulose-5-phosphate conferred the ability to metabolize xylose in Strain A. Isolate A had acquired the ability to grow on xylose. The original parent was unable to grow on xylose (data not shown). The ability of Isolate A to grow on xylose is further evidence that the transgenes are being expressed, and, retain their enzymatic activity in a heterologous organism and, most importantly, can confer on that organism the ability to utilize xylose.

2.1.3 Delivery of Xylulose-5-phosphate for Metabolism via the Pentose Phosphate Pathway

In most eukaryotes, the enzymes of the pentose phosphate pathway are present in the cytoplasm, ready to shunt xylulose-5-phosphate into endogenous metabolic pathways in the cell as it is made. However, plants and algae contain additional organelles (plastids) where the pentose phosphate pathway enzymes are often compartmentalized away from metabolic processes occurring in the cytoplasm. Various plastidic transporters provide a gateway for the exchange of metabolites between the cytoplasm and plastidic compartment. These plastidic transporters reside in the inner plastidic membrane and allow select metabolites to pass through the membrane. Phosphorylated sugars are actively transported by a gene family collectively known as phosphate translocators. These translocators, similar to sugar transporters, can have discrete substrate specificities, such that specific translocators may transport only one type of sugar and not another (for example, glucose-6-phosphate and not xylulose-5-phosphate). Researchers reasoned that since xylulose-5-phosphate is not normally a metabolite present in the cytoplasm of our lead Strain A, our strain probably did not possess an endogenous translocator that would specifically and efficiently transport the xylulose-5-phosphate that was being made into the plastids where the bulk of the pentose phosphate enzymes might reside. A previously-identified plant translocator that specifically transports xylulose-5-phosphate was chosen to be expressed in Strain A. Gene products designated for the plastid usually have specific sequences within their coding region (called transit peptides) directing proper export to their final cellular destination/compartment. Because transit peptide sequences are not well-conserved between species, it was decided to replace the transit peptide encoded by the plant translocator gene with one that researchers had identified from an endogenous translocator found in strain A, from the same family of genes. To determine if the chimeric gene was indeed localizing properly to the inner membrane of plastids, researchers again fused a fluorescent protein to the carboxyl terminus of our translocator chimera and examined the cells expressing the fusion protein utilizing fluorescence microscopy. Figure 3A, B and C are sections taken through the same cell. The fluorescent signal appears to be concentrated around the organellar-like structures within the cell, consistent with localization in the inner membrane of plastids. Compare the localization pattern of this fluorescent protein, with that of the sugar transporter-fluorescent protein fusions previously shown in Figure 1.

Figure 3: Localization of Translocator Chimaera to Organellar-like Structures within Strain A



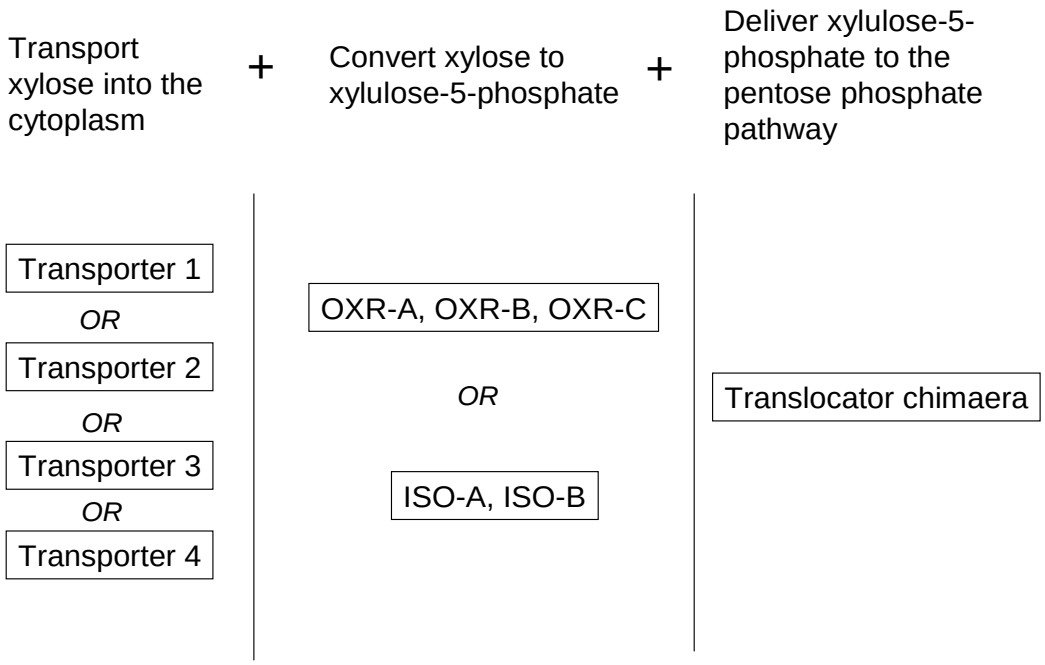
Source: Solazyme, Inc.

Our results suggest that the chimeric translocator gene that was constructed and expressed in the lead Strain A is localized properly to the plastid membrane. As such it is likely to be functional and capable of delivering xylulose-5-phosphate that is being made in the cytoplasm to the pentose phosphate pathway in plastids enabling it to enter general cell metabolism.

2.1.4 A Combinatorial Approach to Transgene Expression: Engineering Superior Xylose Metabolizers

Researchers successfully demonstrated that individual transgenes inserted into Strain A are expressed and either localized to the expected cellular compartment or actually demonstrated activity on a phenotypic level. While it was possible to derive the ability to grow very slowly on xylose by expressing ISO-A and ISO-B alone, our expectation is that combining all of the three crucial components for efficient xylose utilization in a single strain will result in a greatly improved strain. To this end, strains containing various combinations of transgenes were constructed and screened these strains to identify the best xylose metabolizers. Figure 4 shows the different combinations of genes that are possible to introduce into Strain A. Each transgene member of a crucial step is combined with two other genes or groups of genes belonging to the other two steps required for efficient xylose metabolism. Unique strains containing genotypic iterations were constructed.

Figure 4: All Possible Combinations of Different Classes of Transgenes Introduced into Strain A



Source: Solazyme, Inc.

Ten independent transformants of each unique genotype were clonally purified and assessed for growth on solid media containing 2.5 percent xylose as the sole carbon source. Potential positive clones were further tested in liquid culture containing xylose as the sole carbon source. Growth of the cells was monitored by measuring the optical density of the culture. At the end of the experiment, the culture supernatant was analyzed for sugar content. Table 1 shows the final growth and sugar consumption of the 10 best strains (Strains D to M) compared with negative controls which cannot metabolize xylose (Strains A, B and C). Strains D to M are clearly capable of consuming xylose, as all of the xylose in the culture media had disappeared by the end of the experiment. In contrast, the same amount of xylose that was present at the beginning of the experiment was still detected at the end of the experiment in culture medium hosting the negative control strains A, B and C. The disappearance of xylose was accompanied by increases in cell density, indicating that carbon from xylose was being used to support cell growth. In contrast, the negative controls did not increase in cell density throughout the course of the experiment, and at the end of the experiment, the culture media contained about the same amount of xylose that had been in the media at the beginning of the experiment, indicating that xylose was not consumed or very little xylose was consumed.

Table 1: Growth Inhibition of Our Strains in Crude versus Deionized Cellulosic Feedstocks

	Percent of highest OD reached	Xylose remaining in the media, g/L
A	5.0	27.1
B	5.7	24.4
C	5.7	26.4
D	80.1	0.0
E	68.6	0.0
F	100.0	0.0
G	81.9	0.0
H	62.8	0.0
I	67.9	0.0
J	81.8	0.0
K	74.4	0.0
L	82.9	0.0
M	92.0	0.0

Source: Solazyme, Inc.

Researchers were able to engineer xylose-metabolizing strains by inserting transgenes conferring each of the three steps required for efficient xylose utilization in our alga. Multiple strains with unique genotypes showed clear increases in biomass using xylose as the sole carbon source. The discovery of multiple transgenes or groups of transgenes that can transport xylose into the cell and biochemically convert xylose to xylulose-5-phosphate, as well as our successful expression of a chimeric translocator to deliver xylulose-5-phosphate to endogenous, central metabolic pathway has indeed proved successful. In so doing, these efforts reveal the fundamental biological requirements of our lead Strain A for utilizing sugars derived from cellulosic feedstocks.

2.1.5 Determining the Lipid Production Capability of Xylose-consuming Strains

Researchers demonstrated the successful genetic engineering of Strain A, which could not originally grow using xylose, to metabolize xylose and use the resulting carbons to support cell growth. A very important goal of these genetic engineering efforts, however, is to produce a strain capable of converting xylose to *lipid* biomass. Therefore, it was important to determine if our xylose-metabolizing strains were capable of producing oil when grown using xylose as the sole carbon source.

The ten best xylose metabolizers were subjected to a lipid productivity assay, together with negative control strains that could not metabolize xylose. Cells were first grown up in media containing glucose, and then subjected to a lipid productivity phase using either glucose or xylose as the sole source of carbon. As sugar became depleted from the cultures, more of the corresponding sugar was fed to the cells until consumption ceased. At this point, the experiment was terminated and cells were analyzed for lipid content. Table 2 shows the results of the lipid analysis. All strains were normalized to Strain A's performance on glucose expressed as lipid as percent of dry cell weight. All engineered strains performed similarly to the parental, non-engineered control strains when glucose was fed to the cultures. The negative control strains (A, B, C), which could not metabolize xylose, produced negligible amounts of lipid when cultured on this carbon source (6-8 percent of the amount normally made by Strain A in glucose). In contrast, all ten xylose metabolizers were capable of producing significant amounts of lipid when cultured in xylose. The best strain, L, accumulated up to 60 percent of the amount of lipid normally made by Strain A when grown on glucose.

Table 2: Lipid Productivity of Xylose-metabolizing Strains Grown in Glucose or Xylose

Strain	Percent of control lipid production in glucose	Percent of control lipid production in xylose
A	100	8.9
B	93. 8	6.6
C	93.5	6.2
D	93.5	32.4
E	103.8	34
F	101	30.5
G	100.8	32.2
H	100	32.4
I	104.7	45.7
J	99.9	30.6
K	102.5	35
L	100.8	60.2
M	102	27.8

Source: Solazyme, Inc.

Researchers not only engineered the capacity to metabolize xylose in our lead Strain A, but, even more importantly, shown that the inserted pathways can metabolize carbon from xylose and utilize it to produce lipid. The results show great promise and demonstrate the feasibility of investing in additional engineering efforts around our proprietary algal strains to further improve lipid productivity when grown on sugars derived from cellulosic feedstocks.

2.2 Directed Evolution for the Selection of Strains Resistant to Cellulosic Toxins

Cellulosic feedstocks contain toxins such as furfurals and high salt concentrations that are inhibitory to most microorganisms. In the first attempt at directed evolution of the lead Strain A, researchers decided to select for increased salt tolerance in our quest to evolve strains exhibiting improved performance on cellulosic feedstocks.

The maximum growth rate of our parental strain was first determined, and then its growth rate in different concentrations of a salt challenge was measured. A salt concentration that reduced growth to about 33 percent of the maximal growth rate was chosen to be the selective pressure. Multiple independent cultures of Strain A were maintained and sub-cultured at this salt concentration until increases in the growth rate were observed. Cultures were obtained (K1 to K5) that could grow, in the presence of salt, at the same rate as the original parental strain grown without the salt challenge (Table 3).

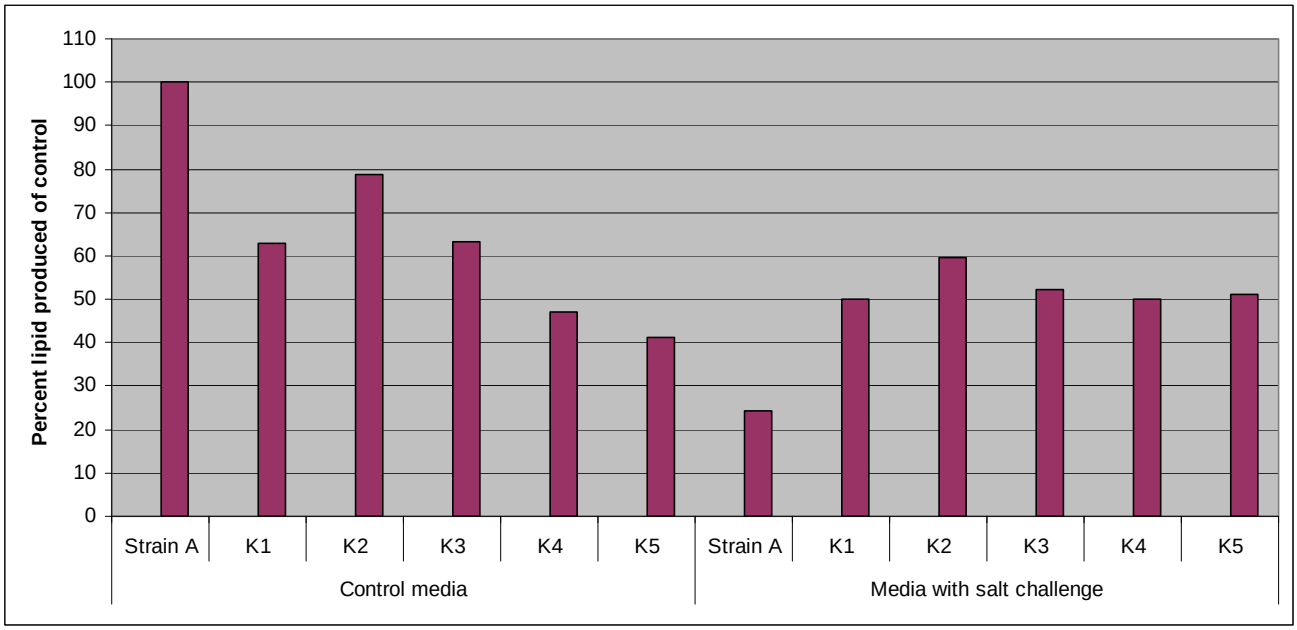
Table 3: Growth Rate of Evolved Cultures Compared to Control Strain A

Flask	Percent increase in growth rate
K1	106
K2	103
K3	110
K4	116
K5	125

Source: Solazyme, Inc.

While researchers succeeded in evolving cultures that could grow in the presence of a salt challenge, they also needed to demonstrate that these strains were indeed capable of producing oil under such conditions. Therefore, evolved cultures were evaluated for lipid production both in the absence and presence of high salt conditions. The results in Figure 5 demonstrate that all five cultures (K1 to K5) performed better than the original parental Strain A when grown under high salt conditions. Compared to the non-evolved Strain A, the evolved cultures produced more lipid under high salt conditions.

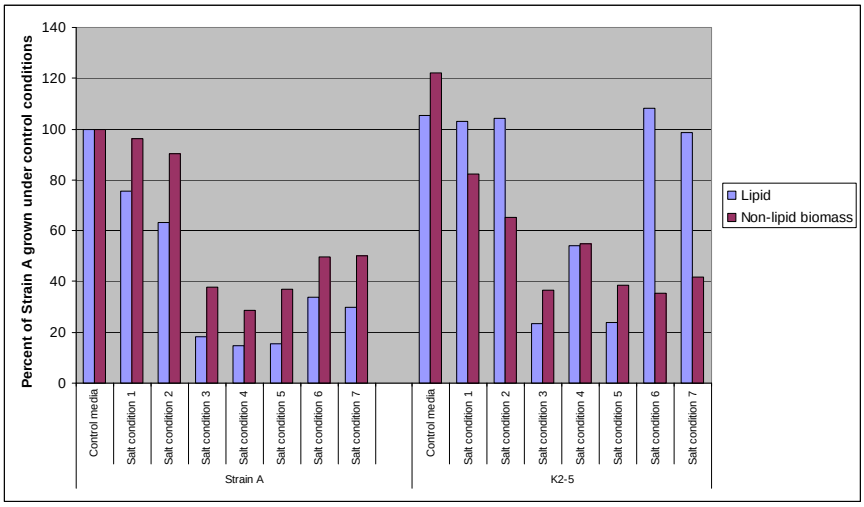
Figure 5: Lipid Productivity of Evolved Cultures Compared to Strain A Grown Under Control Conditions



Source: Solazyme, Inc.

Because of the strain evolution process, the evolved cultures contained heterogeneous cell mixtures and therefore the lipid and growth data for each culture is an average of all the cells in the population. Some cells in the population might perform worse than average, while other cells in the population might perform better than the average. Therefore, each evolved culture was plated out to single cells on solid media and individual colonies arising from each single cell was then assessed for growth and lipid productivity. A single isolate, which was designated as K2-5, demonstrated superior performance compared to all of the evolved cultures. This strain is resistant to many different salts, not just the original salt used for selection during strain evolution. Figure 6 shows that this strain produces more lipid than the original parental Strain A, not only when challenged with seven different types of high salt conditions, but even under the control regime where no additional salt challenge is applied.

Figure 6: Performance of Purified Strain K2-5 Grown in the Presence of Different Salt Challenges Compared to Un-evolved Strain A



Source: Solazyme, Inc.

The strain evolution effort described here, the first of its kind to be attempted on our lead Strain A, successfully produced a superior strain, K2-5, that produced more lipid than the original parent Strain A under *all* conditions tested, including production in the control media which has no salt. Researchers have successfully evolved salt tolerance in our lead fuels Strain A. The evolved strain K2-5 will be a strong contender for bioconversion of cellulosic feedstock into oil.

2.3 Screening Our Existing Strain Collection as well as Classically Improved Strains for the Best Overall Cellulosic Oil Producers

Solazyme has an extensive algal strain collection which has already been interrogated for high oil producers, which could potentially contain strains that are uniquely suited for bioconversion of cellulosic feedstocks into oil. In addition, an on-going classical strain improvement program in which lead Strain A is routinely mutagenized and screened for improvements in lipid production and growth. As discussed earlier, the strain utilized to convert cellulosic feedstocks into oil as part of this research will not necessarily possess all of the attributes desired in a cellulosic fuels strain. Rather it must be the strain capable of producing the most oil on our cellulosic feedstocks. Therefore, both the existing strain collection and classically improved strains derived from Strain A were subjected to further screening efforts. The existing collection was screened more extensively for strains that might utilize xylose more efficiently than our genetically engineered versions of Strain A. At the same time, researchers interrogated classically mutagenized derivatives of Strain A that exhibit increased oil production under standard conditions for their performance on cellulosic feedstocks.

2.3.1 Screening Solazyme's Strain Collection

Strains from Solazyme's collection were screened for growth (biomass production) and lipid productivity on solid media containing either glucose or xylose as the sole carbon source. 23 strains were identified as potentially able to grow on solid media containing xylose as the sole added sugar. Some organisms are capable of metabolizing even the meager amounts of sugar present in agarose, and can exhibit some growth even when, in reality, they are not capable of consuming the *added* carbon source (such as, xylose). As a result, false positives for carbon consumption can be frequent when using solid media to test for carbon utilization. Therefore, it was important to confirm the growth of candidate strains in completely defined liquid medium. Of the 23 initial positive strains, five were not recoverable from frozen stocks, and four could not grow, or grew too slowly in liquid culture to be of further use. The remaining 14 strains were subjected to growth and lipid analysis in liquid media containing either glucose or xylose. Table 4 shows the results.

Table 4: Growth and Lipid Productivity of Potential Naturally Xylose-metabolizing Strains Identified from Our Strain Collection

	Glucose		Xylose	
Strain	**Relative dry cell weight (percent)**	**Relative lipid production (percent)**	**Relative dry cell weight (percent)**	**Relative lipid production (percent)**
A	100	100	6.2	Below detection
N	95	95	6	Below detection
O	19	12	5.6	Below detection
P	65	67	4.2	Below detection
Q	17	7.4	11	Below detection
R	12	3.7	9	Below detection
S	17	8.5	12	Below detection
T	27	11.5	15	Below detection
U	8.2	3.3	3.6	Below detection
V	26	20.1	6.9	Below detection
W	70	78	3.3	Below detection
X	44	24.8	2.6	Below detection
Y	18	13.6	15	10
Z	6.6	1.8	2.6	Below detection

Source: Solazyme, Inc.

The 14 test strains are denoted as Strains N through Z, and are normalized to the lead Strain A. In glucose-containing media, Strain A was superior for both growth and lipid production

compared to all 14 strains. In xylose-containing media, the residual biomass generated by Strain A was used as a baseline against which to compare growth in xylose in the other cultures. The ability to generate biomass through the metabolism of xylose should result in sufficient biomass generation to exceed the 6.2 percent seen in the control strain-in essence the negative control. Table 4 shows that, except for Strain Y, none of the remaining 13 strains or Strain A could generate significant biomass or produce lipid in media containing xylose as the sole carbon source. While Strain Y could generate increased biomass and detectable lipid in xylose, the biomass and lipid generated were both about a tenth of that acquired by Strain A in glucose containing medium. Strain Y performed poorly compared to Strain A even in glucose-containing medium. Biomass production was only 18 percent of Strain A, and lipid production was a mere 13.6 percent of Strain A. Cellulosic feedstocks typically contain a mixture of about 60 percent glucose to 40 percent xylose. A xylose-consuming strain would still have to be able to convert glucose to oil efficiently. Although Strain Y could metabolize some xylose and even convert it to oil, its overall lipid productivity in both glucose and xylose was only about one-tenth of that exhibited by our lead fuels Strain A in glucose. Genetic engineering of xylose metabolism into our lead Strain A is the best option for full utilization of carbohydrates derived from cellulosic feed stocks for commercial fuel production. Researchers presented data in Section 2.1.5 and Table 2 in which the best engineered strain derived from Strain A retained the superior lipid productivity of Strain A in glucose and, in addition, could convert xylose to lipid at 60 percent of its productivity in glucose. This engineered strain is far superior to any of the non-genetically modified algal strains in the current strain collection.

2.3.2 Performance Evaluation of New, Improved Strains Derived from Our Lead Fuels Strain A on Cellulosic-derived Sugars

Solazyme has an on-going classical strain improvement program aimed at improving lipid titer and growth in our lead Strain A by classical mutagenesis. Several descendents of Strain A were isolated that exhibited improved characteristics when grown on glucose. Researchers reasoned that these strains might also perform well on cellulosic-derived feedstocks, possibly even outperforming Strain A in terms of lipid productivity and growth on these feedstocks. A total of four improved strains, designated A-1 through A-4, were tested for lipid production on two separate cellulosic feedstocks. K2-5, previously described in Section 2.2 as an evolved version of Strain A selected to be resistant to high salt concentrations, was also included in the analysis. Strain A was included as the benchmark for comparison. The results of the performance of all six strains on two different cellulosic feedstocks are presented in Table 5. Control, optimized media contains glucose and has been formulated to maximize lipid productivity in all of our strains. Lipid productivity is expressed as a percentage of the gram/liter lipid produced in Strain A under control (optimized) conditions. Cellulosic Feedstock 1 was derived from corn stover and Cellulosic Feedstock 2 was derived from green municipal waste; these two feedstocks were partially-deionized and concentrated. All of the Strain A derivatives including K2-5, evolved for tolerance to high salt concentrations, strains A-1 through A-4, mutagenized and screened for improved growth and lipid productivity, performed better than Strain A in control media. Performance of all strains were compromised in both cellulosic feedstocks compared to control optimized media, but the classically improved strains all produced

significantly more lipid than Strain A in both cellulosic media, even outperforming Strain A grown in optimized media in the case of Cellulosic Feedstock 2.

Table 5: Performance of Improved and Evolved Strains on Two Different Cellulosic Feedstocks

Strains	Lipid produced as a percentage of Strain A grown in control media		
	Control, optimized media	Cellulosic Feedstock 1	Cellulosic Feedstock 2
A	100	61	77
K2-5	130	82	114
A-1	136	83	105
A-2	141	78	117
A-3	160	91	134
A-4	173	88	143

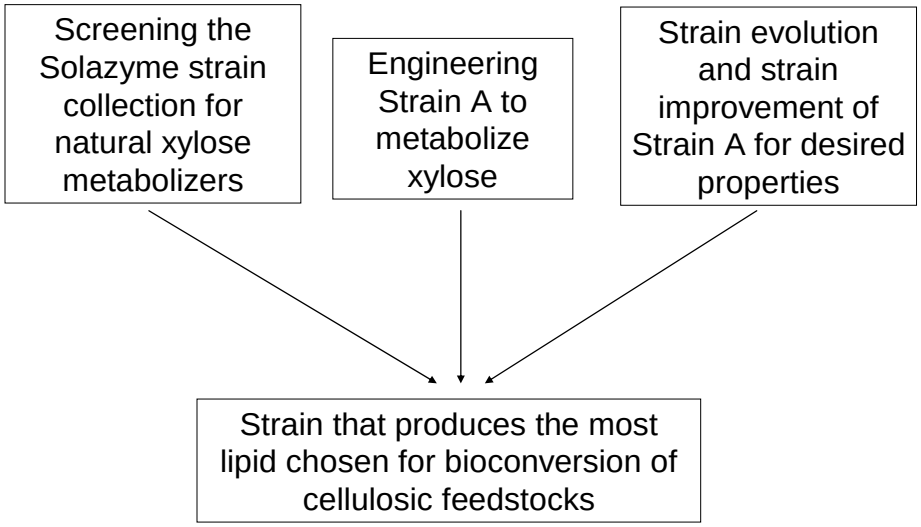
Source: Solazyme, Inc.

Researchers identified new, improved strains with greater lipid productivity to use in place of the original lead fuels Strain A for fermentation using a variety of cellulosic feedstocks. Because the lipid productivity of Strain A-4 was consistently higher than those of the other strains, this strain was chosen for further work on optimizing the process for final bioconversion of cellulosic sugars to oil. In some cases, this strain demonstrates productivity in cellulosic feedstock that exceeds even that of the original Strain A in optimized, non-cellulosic medium.

2.4 Strain Identified for Bioconversion of Cellulosic Feedstock into Oil

A three-pronged, parallel-processing approach was used to identify the optimal strain for conversion of cellulosic-derived sugars into oil, as illustrated in Figure 7 below. The optimal strain is expected to produce large quantities of oil in cellulosic feedstocks, which contain a mixture of glucose and xylose as well as toxins inhibitory to growth.

Figure 7: Our Approach to Identifying a Strain for Bioconversion of Cellulosic Feedstocks



Source: Solazyme, Inc.

Researchers screened the extensive strain collection for strains that could produce oil in glucose or xylose, but did not identify any candidates that could produce as much oil as Strain A in glucose. The one strain identified that could produce very modest amounts of oil in xylose was a poor oil-producer in glucose, when compared to Strain A, hence it would be of little immediate use on cellulosic feedstocks which are comprised of anywhere from 60 to 75 percent glucose.

The genetic engineering was a resounding success in that researchers were able to engineer transgenic strains capable of utilizing xylose for growth and lipid production, but clearly more genetic engineering is required to make these strains fermentation-ready for cellulosic feedstocks, which contain a mixture of glucose and xylose. The current transgenic strains are capable of metabolizing xylose. Further engineering efforts might involve engineering Strain A-5 to better metabolize xylose, along with improving the flux of the carbons derived from xylose through the general metabolic pathways of the cell.

Strain evolution efforts were very successful in producing an improved version of Strain A (Strain K2-5) that could produce more oil under conditions of high salt. In a parallel effort at strain improvement, Strain A was mutagenized by classical means and its descendents were screened for improved lipid productivity and growth profiles. This parallel processing

approach produced four other strains to be considered together with K2-5 for converting cellulosic feedstocks into oil.

The final optimal strain chosen for bioconversion, Strain A-4, is an improved version of Strain A. It was derived from Strain A through a regimen of classical strain improvement and subsequent screening for the desired properties of a lead fuels strain. This strain consistently performed the best of all the improved strains. Strain A-4 produced more oil in cellulosic feedstock medium than Strain A in optimized media. As a consequence, Strain A-4 was chosen for the conversion of cellulosic feedstocks into oil. In summary, researchers successfully identified a strain that will be used to produce oil from cellulosic feedstocks. This strain performs significantly better than our previous lead fuels Strain A and will produce the most oil from cellulosic feedstocks out of all the strains currently available.

CHAPTER 3:
Procurement of Cellulosic Feedstocks and Evaluation/Optimization of Strain Performance

3.1 Conversion of Cellulosic Biomass into Cellulosic Feedstock

Cellulosic biomass can potentially provide a sustainable raw material for bioconversion to fuels, as it is derived from plant matter considered waste, such as agricultural residues (corn stover, bagasse, etc.), paper (recycled newspaper, etc.), wood (wood chips, sawdust, etc), and green municipal wastes (leaves, grass clippings, etc). The plant matter that makes up cellulosic biomass contains sugars that are locked up in crystalline and semi-crystalline lattices. As a result, the sugars contained in cellulosic biomass are not readily bio-available without considerable processing. The fibrous plant matter that makes up cellulosic biomass is composed primarily of cellulose, hemicellulose and lignin. Cellulose and hemicellulose contain polymers of sugars that must be hydrolyzed into useable monomers – glucose in the case of cellulose and C5 sugars, primarily xylose, in the case of hemicellulose. Lignin and hemicellulose form a protective sheath around the cellulose and both must be detached from the cellulose before sugars can be released. The first challenge in biomass processing requires disentangling of these materials from their natural rigid structure in plant woody matter. Various technologies have been developed to liberate cellulose and hemicellulose from its rigid seal with lignin. This process, termed pre-treatment involves using both physical and chemical means to disrupt the plant material. Physically, the biomass is first reduced to a fine size to make it more amenable to chemical treatment. Chemical treatment is then employed to disrupt the rigid lignocellulosic structure thereby releasing cellulose and hemicellulose fractions for further processing. Chemical treatment options include acid or base hydrolysis in the presence of heat and pressure to force the fibers apart. The pretreatment step can result in release of toxins that can be problematic for the subsequent growth of micro-organisms.

Following pretreatment, the released cellulose and hemicellulose are broken down to their component sugars, glucose and xylose. This can be achieved by acid hydrolysis, caustics, or a less harsh method employing enzymes that can break down cellulose and hemicellulose. The use of acids or caustics either in the pretreatment stage or during the hydrolysis stage, ultimately results in the generation of significant levels of salts in the feedstock. In addition to the salts naturally present in plant material, the treatment can also elevate the salt content of the final saccharified material. Thus, the high salt concentrations and toxins can subsequently inhibit the growth of micro-organisms used to bioconvert the cellulosic-derived sugars to biofuels.

3.2 Procurement of Cellulosic Feedstocks

The U.S. government and many private companies are investing heavily in cellulosic technologies. Solazyme intends to tailor its process to allow its proprietary algae to consume sugars from such cellulosic technologies. To fulfill the objectives of this grant, Solazyme has

procured cellulosic feedstocks from third parties to use for research activities related to bioconversion.

Procurement of adequate cellulosic feedstocks were the main bottleneck for the progression of this project. The processing of cellulosic biomass to release sugars that can be used for fermentation remains, for the most part, an area of research that is still under active development as part of various government and privately-sponsored enterprises. As a result, there are no commercial entities from which cellulosic feedstocks can be easily purchased. Solazyme has had to first identify third party vendors capable of supplying cellulosic feedstocks, visit the vendors' facilities, explain our requirements and then develop special agreements and contracts with these vendors to obtain the feedstocks. After agreements have been signed, it can take long periods of time before adequate feedstocks are delivered. Furthermore, some cellulosic feedstocks obtained contained low or no detectable monosaccharide. While Solazyme has conducted research demonstrating the feasibility of using algae for bioconversion of cellulosic feedstocks, any commercialization of this work is contingent upon the eventual development of a steady supply of cellulosic feedstock in a form utilizable by our technology. Nonetheless, for the purposes of this project, feedstocks were procured for strain testing and optimization of oil production. Commercial production of oil from cellulosic feedstocks will require progress in technologies that can reliably supply enough cellulosic feedstocks for large-scale fermentations.

To date, 8 different types of cellulosic feedstocks have been procured. Table 6 shows the origin of each feedstock. Feedstock 1, 3 and 5 are derived from corn stover from two different sources; feedstock 2 is derived from green municipal waste; feedstock 4 and 6 are derived from forage sorghum from two different sources; feedstock 7 contains pooled cellulosic sugars from disparate sources; and feedstock 8 is derived from maple wood pulp.

Table 6: Cellulosic Feedstocks Procured

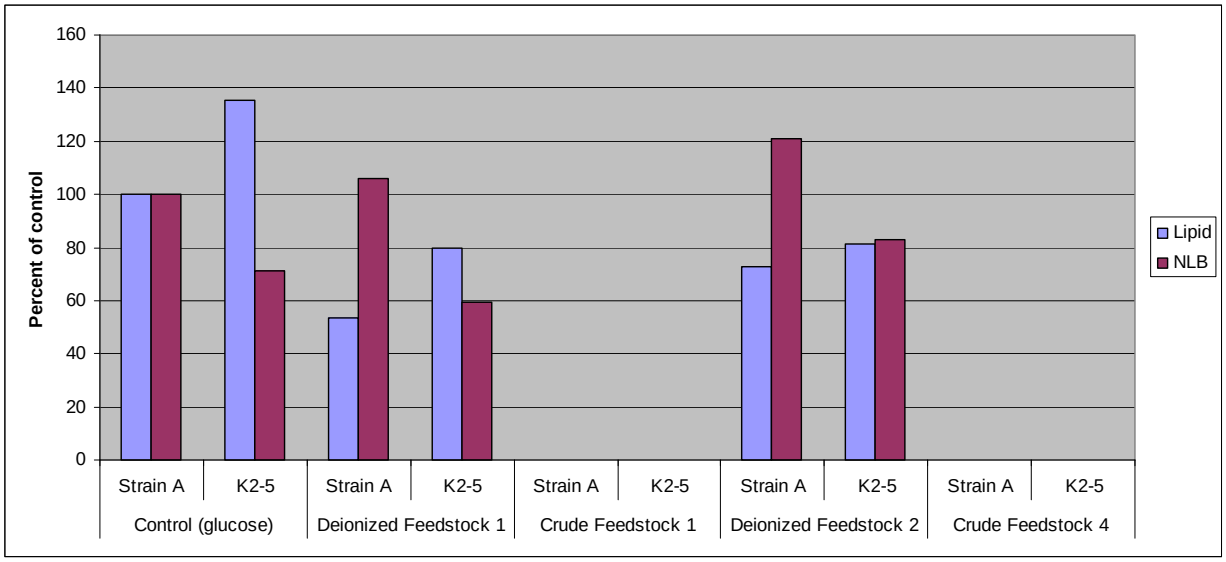
Cellulosic Feedstock	Description
1	Corn stover #1
2	Green municipal waste
3	Corn stover #2
4	Forage sorghum #1
5	Corn stover #3
6	Forage sorghum #2
7	Pooled cellulosics
8	Maple wood pulp

Source: Solazyme, Inc.

3.3 Performance Evaluation of Crude Cellulosic Feedstocks and Further Processing of Crude Feedstocks

The crude feedstocks that were procured directly from third parties contained very high concentrations of salts. During evaluation of our strains on these feedstocks, it became apparent that further processing was necessary to reduce the growth-inhibitory salts before bioconversion was possible. Figure 8 shows the results of an experiment to determine the growth and lipid productivity of Strain A and the evolved salt tolerant strain K2-5 on control, optimized media containing lab-grade glucose, and media containing crude or deionized cellulosic feedstocks. The growth (non-lipid biomass, NLB, accumulated) and lipid produced for each strain is shown as a percentage of that observed in the lead Strain A in control, optimized media. While the evolved, salt-tolerant K2-5 strain produced more oil than Strain A in control media and deionized feedstock, neither it nor Strain A was able to grow in this particular crude, non-deionized feedstock.

Figure 8: Growth Inhibition of Our Strains in Crude versus Deionized Cellulosic Feedstocks



Source: Solazyme, Inc.

Analysis of the feedstocks for salt content confirmed that crude feedstocks contained very high salt concentrations. Deionization of the crude feedstocks effectively reduced the high salt content by several orders of magnitude.

This initial performance evaluation of the strains on crude and small-batch deionized feedstocks demonstrates that further processing of crude feedstocks is necessary before bioconversion to oil by our strains is possible. Researchers then deionized all of the remaining crude feedstocks

procured. Following deionization, the diluted cellulosic feedstocks required further concentration to allow for higher cell-density, higher sugar-feed fermentations. These final processed feedstocks were used for further strain performance evaluation and fermentations at 7 L scale.

3.4 Performance Evaluation of Strain A-4 in Flasks on Final Processed Cellulosic Feedstocks

The performance of Strain A-4, previously chosen to be used for bioconversion of cellulosic-derived sugars as outlined in 2.4, was assessed in flask cultures using production medium with one of the various deionized, concentrated cellulosic feedstocks (Cellulosic Feedstocks 1 through 8) as the carbon source. Cellulosic Feedstocks 1, 3 and 5 were derived from corn stover procured from different sources, and Cellulosic Feedstocks 4 and 6 were derived from forage sorghum procured from different sources. Cellulosic Feedstock 2 was derived from green municipal waste. Cellulosic Feedstock 7 was a pool of sugars derived from all of the previously listed feedstocks, as well as pine chips. Cellulosic Feedstock 8 was derived from maple wood pulp. The lipid productivities of Strain A-4 in these different cellulosic feedstocks were compared to that of original lead fuels Strain A in control, optimized media. As presented in Table 7, Strain A-4 was able to grow and produce oil on all of the feedstocks that were tested. Lipid productivity was highest with Cellulosic Feedstock 3, which reached 160 percent of that observed in original lead fuels Strain A grown in optimized, control media. In fact, Strain A-4 was able to produce even more oil than Strain A under optimized conditions in five out of eight cellulosic feedstocks tested.

Table 7: Performance of Strain A-4 on Eight Different Cellulosic Feedstocks Compared to that of Strain A on Optimized Control Media

Cellulosic Feedstock	Percent Lipid Productivity (relative to control)
1	88
2	144
3	160
4	126
5	90
6	81
7	112
8	135

Source: Solazyme, Inc.

3.5 Performance Evaluation of Strain A-4 in Lab Scale Fermentors on Final Processed Cellulosic Feedstocks

Previous flask experiments have validated the ability of deionized, concentrated cellulosic feedstocks to support growth and lipid production in Strain A-4. At this point, researchers wanted to optimize fermentation conditions to obtain the maximal amount of oil from bioconversion of these cellulosic feedstocks.

Optimization was conducted using deionized, concentrated Cellulosic Feedstock 1 using three different fermentation conditions (conditions A, B and C). After a fermentation run, the lipid content of the cells as well as the total amount of lipid produced in the particular run was quantified. Table 8 shows the results of fermentation optimization.

Table 8: Optimization of Fermentation Conditions

Fermentation ID	Fermentation condition	Final Lipid Content (percent)	Total Oil Produced (percent of best performer)
214	A	66	37
215	A	67	39
232	B	47	63
250	C	55	100

Source: Solazyme, Inc.

Although condition A produced cells containing a higher lipid content, condition C produced the greatest total amount of oil from an individual fermentation run. Therefore, it was decided to run fermentations using the rest of the cellulosic feedstocks with both conditions A and C for bioconversion of cellulosic sugars into oil.

CHAPTER 4:
Conversion of Cellulosic Feedstocks Into Oil Using Optimized Procedures And An Optimized Strain

As described in section 3.4, researchers chose two different fermentation conditions to convert batches of cellulosic feedstocks into oil, using Strain A-4 identified in section 2.4. Conditions A and C, determined to produce the greatest percentage of oil and the greatest total amount of oil respectively, were used to ferment final processed cellulosic feedstocks into oil. Table 9 shows the final lipid content (lipid as percent dry cell weight) achieved by Strain A-4 in each of the feedstocks under various conditions, as well as the oil produced for each run (percentage of best run). Fermentation ID 232, described in section 3.4 as one of the conditions used during optimization, is included in the table as well.

Table 9: Conversion of Cellulosic Feedstock-Derived Sugars into Oil

Fermentation ID	Cellulosic Feedstock	Condition	Final Lipid Content (percent)	Total Oil Produced (percentage of best run)
214	#1	A	66	26
215	#1	A	67	27
232	#1	B	47	44
250	#1	C	55	70
247	#2	C	70	56
248	#2	C	64	54
230	#4	C	59	42
231	#4	C	65	42
249	#4	C	58	100
307	#5	A	62	51
308	#5	A	57	55
309	#6	C	49	65
304	#7	A	63	31
305	#7	A	63	31

Source: Solazyme, Inc.

After harvesting biomass, washing and drying, oil was recovered from dried biomass, and subsequently refined and bleached. Cellulosic Feedstock #8, maple wood pulp, was not used in the fermentations because quantities are currently limited. As noted before, procurement of cellulosic feedstocks was a major hurdle in the execution of this project and certainly impacted the ability to make oil from cellulosic sugars in a routine manner.

Solazyme experienced challenges in the final conversion of cellulosic derived RB oil into ASTM certified D975 fuel within the project term. As Solazyme routinely produces thousands of gallons of renewable fuels, meeting a variety of military and ASTM standards, researchers did not proceed with the conversion to fuel within the project term. The real technical accomplishments and value generated through the work described in this report represent tremendous progress in strain engineering and xylose metabolism, evolution and selection of strains capable of superior performance on cellulosic feedstocks, optimization of cellulosic feedstocks to support growth of lead fuels strains and development of fermentation process on these feedstocks leading to the production of algal oil.

In summary, a foundation has been established for further work exploring the production of renewable diesel fuel from cellulosic feedstocks using an optimized algal strain. During the course of the project researchers demonstrated the viability of using both genetic engineering as well as classical strain improvement methods to optimize proprietary algal strain for cellulosic feedstock bioconversion. The research has paved the way for future work and optimization around multiple aspects of this process. The research has also highlighted areas that require further development for commercialization of such a process to become reality. Currently, there are no reliable, commercial suppliers of large quantities of cellulosic feedstocks. In addition, further improvements in both the process of generating cellulosic feedstocks and further development of strains for tolerating them will be required.