Final Technical Report: Improvement of *Zymomonas mobilis* for Commercial Use in Corn-based Biorefineries

Award Number: DE-FC36-07GO17056

Executive Summary

Between 2007 and 2010 DuPont conducted a program under DOE award DE-FC36-07GO17056 to develop and improve *Zymomonas mobilis* as an ethanologen for commercial use in biorefineries to produce cellulosic ethanol. This program followed upon an earlier DOE funded program in which DuPont, in collaboration with the National Renewable Energy Laboratory (NREL) had developed a *Zymomonas* strain in conjunction with the development of an integrated cellulosic ethanol process.

In the current project, we sought to maximize the utility of *Zymomonas* by adding the pathway to allow fermentation of the minor sugar arabinose, improve the utilization of xylose, improve tolerance to process hydrolysate and reduce the cost of producing the ethanologen. We undertook four major work streams to address these tasks, employing a range of approaches including genetic engineering, adaptation, metabolite and pathway analysis and fermentation process development.

Through this project, we have developed a series of strains with improved characteristics versus the starting strain, and demonstrated robust scalability to at least the 200L scale. By a combination of improved ethanol fermentation yield and titer as well as reduced seed train costs, we have been able to reduce the capital investment and minimum ethanol selling price (MESP) by approximately 8.5% and 11% respectively vs. our starting point. Furthermore, the new strains we have developed, coupled with the learnings of this program, provide a platform for further strain improvements and advancement of cellulosic ethanol technology.

Background and Objectives

Between 2003 and 2007, DuPont developed an improved recombinant *Zymomonas mobilis* strain as part of the development of an Integrated Corn Biorefinery (ICBR) under the DOE Cooperative Agreement 1435-04-030CA-70224. Within that program we developed an improved fermentation process and more efficient genetic tools for genetic engineering of *Zymomonas*, and constructed an improved recombinant strain that can meet commercial targets of yield, titer, and rate on clean mixed sugars of glucose and xylose and which when added to 80% corn stover hydrolysate derived from DuPont's dilute ammonia pretreatment process could convert all of the glucose and most of the xylose to ethanol at yields and titers higher than that reported in the literature for other hydrolysate fermenting strains. The *Zymomonas* strain available at the end of the ICBR program converted corn cob hydrolysates to ethanol at yields, titers, and rates suitable for pilot scale demonstrations; however, further improvement in rate, titer and yield were needed to achieve favorable overall process economics for commercial production of ethanol from cellulosic biomass.

This report summarizes the work and results of a project titled "Improving *Zymomonas mobilis* for Commercial Use in Corn-Based Biorefineries", performed by DuPont under DOE award GO 17056. This project was intended to build upon the success of the prior ICBR project and improve the *Zymomonas mobilis* strain to reach commercial goals. Key requirements in order to meet commercial economics included increased robustness in hydrolysate, and in particular improved xylose fermentation in the presence of inhibitors found in hydrolysates. Coupled with these strain improvements, this program also addressed improvements in the strain production and fermentation processes to optimize the cost and effectiveness of the organism and the process.

Summary of Technical Work

As envisioned in the Integrated Biorefinery concept and process developed in the ICBR program, the fermenting organism utilized in conversion of sugars released from cellulosic polymers by the pre-treatment and enzymatic hydrolysis steps must function under specific

conditions to maintain an economical process. The more important of these requirements in the process led to the set of goals and targets in this project and those goals in turn led to the set of tasks and sub tasks to be carried out to meet those goals.

The goals themselves and why they are important to the biorefinery to ethanol process are as follows:

1) The organism must ferment as many of the main sugars present in the hydrolysate as possible to keep the overall process yield as high as possible. The extent of utilization of each sugar must be high, the efficiency of conversion to ethanol must be high and the rate of conversion must be sufficient to keep the overall fermentation step inside the limits set by vessel size in the process.
2) This fermentation must take place in a hydrolysate broth that has had minimal or no cleanup steps performed on it after pre-treatment and hydrolysis. The cost of steps to remove inhibitors is usually excessive so tolerance of typical inhibitors by the organism is required by economics.
3) The ending ethanol titer must be high to keep the down stream process costs of concentrating the product ethanol from the primary fermentation beer reasonable. More significantly, titer must be high to minimize water use and costs of water re-cycle.
4) The scale of cellulosic ethanol plants will be large and the requirement of initial fermentation biocatalyst will be high so the initial cell mass must be produced economically and reliably in order to ferment variable quality and types of feedstock.

Two strains were available at the beginning of the project. One strain contained the xylose pathway and had been selected for a high xylose use rate. This was the subject strain included in DuPont's proposal for the current work. In the interim between submission of our proposal and start of work, we developed an improved strain, derived by extensive adaptation of the strain described in our program proposal to growth in media containing ammonium acetate at the level expected to be encountered in process hydrolysate. This new strain had substantially

improved xylose utilization, which resulted in a >25% improvement in final ethanol titer. Both strains were carried forward into this program, although with agreement of DOE, the newer, adapted strain was designated as the starting point reference strain.

All the work done in this program on the ethanologen strain and fermentation process was done in the context of DuPont's dilute ammonia pretreatment process developed during our earlier ICBR program, with corn cobs as the feedstock substrate. The dilute ammonia process involves a mild pretreatment of the biomass with steam and ammonia, followed by enzymatic saccharification. DuPont had developed and scaled this process with the intent of minimizing the formation of inhibitory compounds such as furfurals which are known to form in acidic pretreatment processes, as well as minimizing the formation of acetic acid, another inhibitory compound. By using this milder pretreatment philosophy the pretreated biomass can be used directly in saccharification and fermentation without any neutralization, washing or "detoxification" steps, thereby reducing the cost and capital associated with the front end of the process. All adaptations and any performance assessments of the strains derived in this project were done using hydrolysate produced via the dilute ammonia process.

To achieve the program goals, we executed four major work streams: 1) adding the arabinose pathway, 2) genetic modification of the strain to improve uptake and allow simultaneous utilization of glucose, xylose and arabinose, 3) strain adaptation to improve hydrolysate resistance, and 4) development of reduced cost, scalable strain production and fermentation processes.

Task 1:

Task 1 was to integrate and optimize the arabinose metabolic pathway in *Zymomonas.* By the time work started on this project we had completed two types of adaptation of strains produced in the previous grant period and found adaptation to be very useful in producing a high performance strain. To follow this developmental path we explored alternative ways of introducing arabinose metabolism and optimizing for maximum use rate.

The three genes in the pathway could be added by either of two methods. Upon identifying the rate limiting activity of the three genes, the order of the genes in the operon was changed to maximize expression of the rate limiting gene. After adding the pathway genes and a short adaptation to growth on arabinose a few strains met the required rate and extent of arabinose use in arabinose only media. The strains fell short of the targets for arabinose use and rate in actual process hydrolysate but provided a good platform host in other carrier work.

Task2:

Task 2 was to improve sugar transport to allow co-fermentation of glucose, xylose and arabinose. We employed multiple strategies including modifying the native transporter in *Z. mobilis* as well as incorporating transporters requiring energy. These approaches produced strains that exhibited near simultaneous use of xylose and glucose and which had somewhat improved transport of arabinose.

Task 3:

Task 3 was to improve robustness of the production strain in hydrolysate. This task included all of the pathway engineering, adaptation and sequencing to understand adaptation and metabolite analysis work. It also included subtasks on screening *Z. mobilis* over and under expression libraries.

Beginning with the sequencing of earlier strains of *Zymomonas mobilis*, the information from sequencing of adapted strains was very useful in rational design of the introduced pathways. We were able to identify the most limiting activity in xylose metabolism. This observation led to a sequence diversity screen that found a more effective version of this enzyme which has been incorporated into strains in this project. Following this, we identified the next rate limiting step, and increased that activity as well. Upon increasing the activity of the second

rate limiting step, we found that further increases in the activity of the first rate limiting step provided an additional improvement in xylose utilization.

Strains in this series that incorporated an improved transporter from Task 2 showed us that the total sugar use rate was limited by a step or steps that are common to the glucose and xylose pathways. We redirected some effort in the final year of the project towards recovering the glucose utilization rate. Successful strains were obtained by starting from both an early recombinant strain and wild type.

Adaptation to growth in hydrolysate was obtained by using a very small scale, automated continuous culture device. Adapted strains perform slightly better than their naïve precursors in hydrolysates which are relatively easy to ferment, but perform considerably better than their parent strains in hydrolysates with high inhibitor content. All adapted strains have been sequenced to determine the specific genetic changes which correlate to improved fermentation performance.

Task 4:

Task 4 was to develop a robust and economical fermentation process. A major part of achieving the goals of this task was to achieve a high xylose use rate to allow sufficient energy production during the xylose utilization phase of production fermentation to keep up with ATP demand for maintenance and ideally have energy left for growth. The additional work was to understand nutrient requirement for growth well enough to produce a cheap and simple media for seed growth. Initial development of *Zymomonas mobilis* had resulted in strains which achieved high yields and titers when seed was grown on a rich glucose media with supplemental nutrients. In order to achieve commercially viable economics, we needed to develop strains which ferment effectively when the seed is grown on a much leaner media, such as whole corn mash with minimal supplemental nutrients.

In the course of this task we identified the primary nutrient needed by *Z. mobilis* which is absent in corn mash, and were able to insert and express the genes required to produce this

nutrient. Upon insertion of this genetic modification as well as by adaptation, we were able to reduce the added nutrients required for a strong seed by 90%.

Summary of Accomplishments

As a result of the efforts described above, we developed a family of 12 key new recombinant *Zymomonas mobilis* strains, which provide performance improvements over the starting strain. Of these new strains, 8 include all of the currently know requisite elements for optimal xylose use rate, two of which have completed initial adaptation in process hydrolysate, and two of which also incorporate the arabinose pathway. The remaining four strains do not contain all of the known elements for high xylose use rate, but were developed from adapted strains and show significant promise as platforms for further development.

Compared to the strains available at the start of this project, these new strains are differentiated in various ways, depending on the specific strain. Some of the specific improvements incorporated in these strains include:

- Increased rate of glucose utilization
- Increased rate of utilization of xylose
- The ability to metabolize arabinose to ethanol
- Improved tolerance to corn cob dilute ammonia process hydrolysate
- Reduced nutrient requirement for seed production

At the end of the program, one of the arabinose utilizing strains was selected for a performance validation run at the 1L and 200L scales. In this campaign, we used a lean, low cost media with approximately 90% less of the added nutrients than used at the outset of the program. We used a common batch of process hydrolysate produced via the dilute ammonia process as the fermentation substrate. In this final comparison, the new strain outperformed the starting strain by over 6% in terms of net ethanol produced and final ethanol titer, and over 5% in fermentation process yield when tested side by side at the 1L scale. To test reliability of performance upon scale-up, we repeated the fermentation of the new strain in a 200L fermenter

using the same hydrolysate and same seed media. In this larger fermentation, we exceeded the 1L performance in terms of net ethanol and final titer by about 4%, indicating robust scalability of the strain and process beyond the laboratory.

It should be noted that the strain tested in the final evaluation had not been adapted for tolerance to process hydrolysate. We normally see some improvement in yield and titer at the end of adaptation, so as we complete adaptation of this strain we may see further gains beyond those demonstrated to date. Additionally, several of the resultant strains which were not tested in the evaluation show significant promise for further improvement, either through adaptation, or by integrating improvements across strains.

The improvement in yield and final titer and the ability to use a much leaner seed media combined to reduce both the cost and capital for cellulosic ethanol produced with the new strain. The capital required was reduced by approximately 8.5% and the minimum ethanol selling price was reduced by approximately 11%.

In addition to the improved technical and economic performance of the developed strains, this program has advanced our understanding of *Zymomonas mobilis* as an ethanologen platform. We have identified key rate limiting steps in the C5 pathways, developed “rules” for maximizing xylose utilization, developed efficient protocols for strain development, identified key nutrient needs, and enabled strains to produce major nutrients themselves. These learnings provide the basis for further development and exploitation of *Zymomonas mobilis* as a commercially viable C5/C6 fermentation host.