Final Technical Report

Project Title: Development of Biocatalyst for the Fermentation of Agricultural Feedstocks to Chemicals

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Recipient: Cargill Dow LLC

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Cost-Sharing Partners: None

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Project Title: Development of Biocatalyst for the Fermentation of Agricultural Feedstocks to Chemicals

Project Objective: Project objective is to develop a xylose fermenting biocatalyst for the production of lactic acid and ethanol by genetically engineering Cargill Dow's proprietary lactic acid producing biocatalysts to ferment xylose.

Executive summary:

Xylose is a pentose sugar, which is a common component of biomass carbohydrates. For efficient utilization of biomass, it is essential to have technology to convert xylose to desirable products at economically attractive rates and yields. Many microorganisms can naturally utilize xylose as a food source, but none have been found that can economically convert xylose to useful products (via fermentation). In this project, yeast strains have been engineered to convert xylose to lactic acid and/or ethanol at high rates of conversion and at high yields; which is a major breakthrough towards the establishment of commercial biorefineries. This has the potential to benefit the US public in several ways: Increased jobs and revenue in rural areas of the United States through construction and operation of biorefineries, reduced use of imported petroleum, and increased revenue into the United States through international licensing of this technology.

This project was successful in accomplishing all the planned goals. Milestones were mostly completed on schedule, as can be seen in the chart below. The final milestone was completed ahead of schedule. A time line of task specific activity is presented below, following the task summaries. Performance targets at the beginning of the project were a rate of 1g/L*h at a yield of 80% for conversion of xylose to lactic acid. These targets were achieved. The best strain had an average rate of 1.14 g/L*h and an average yield of 77%, which exceeded the target on an economic basis.

Updated Gantt Chart/Roadmap:

ID Number	Task / Milestone Description	Planned Completion	Actual Completion	Comments
1	Performance targets for xylose to lactic conversion set through cost modeling	1/31/03	3/26/03	Cost modeling showed that originally set target performance numbers are feasible and will remain as target performance of strains constructed in this project.
2	Key xylose pathway genes from two proprietary hosts cloned and sequenced	1/31/03	1/31/03	
3	Most promising genetic engineering route(s) to construct strain(s) for efficient xylose to lactic acid conversion identified	7/31/03	7/31/03	

ID Number	Task / Milestone Description	Planned Completion	Actual Completion	Comments
4	Decision to continue or drop work on third host	10/31/03	7/31/03	Will continue work on 3 rd host
5	Biochemical characteristics of key xylose pathway enzymes of M1 and N1 determined	10/31/03	Not planned to be completed	No need for this data due to a different path taken successfully
6	Microorganism capable of meeting the performance targets ready	7/31/04	6/22/04	Biocatalyst met targets for lactate rate/yield from Xylose

Background: Cargill Dow has developed efficient biocatalysts for conversion of dextrose to lactic acid. The next step towards a more sustainable and lower cost lactic acid production process is to move from dextrose to biomass sugars as feedstock. In addition to dextrose, the main sugar from biomass would be xylose. The current biocatalysts, although naturally capable of xylose utilization, are not efficient in converting it to fermentation products. Preliminary tests before this project started have shown that biocatalysts *Kluyveromyces marxianus* (code name M1) and *Candida sonorensis* (code name N1) can convert xylose to lactic acid with high yield (70% in the best case), but very slowly. The objective of this project is to improve the efficiency, both yield and productivity, by genetic engineering.

The chart below shows the basic biochemical routes for the conversion of xylose to lactic acid and/or ethanol.

In this project, two different approaches were taken to improve the rate of conversion of xylose to products. One of the approaches makes use of the xylose utilization pathway: xylose reductase (XR) – xylitol dehydrogenase (XDH) – xylulokinase (XK) and the other makes use of xylose isomerase (XI) to overcome possible redox imbalance in the pathway.

Project Summary:

The project was organized as follows:

Task 1: Improve xylose to lactate fermentation of strain *K. marxianus* (M1), by utilizing the native pathway genes XR-XDH-XK through identification and removal of metabolic “bottlenecks”.

Task 2: The same as task 1 except with *Candida sonorensis* (N1).

Task 3: Create a xylose to lactic fermenting strain of a benchmark organism *Pichia stipitis* (P1).

Task 4: Identify and isolate eukaryotic xylose Isomerases (XI) for use in Task 5

Task 5: Construct strains incorporating XI and test for functionality

Task 6: Biochemical characterization of xylose pathway enzymes (e.g. determine rate constants, inhibitors, expression levels, etc.)

Task 7: Develop tools for genetic transformations of target strains

Task 8: Genetic engineering of new host C1 for xylose to lactic acid fermentation

Task 9: Improvement cycles on most promising strains from tasks 1,2,5, or 8.

The approximate project timeline was as follows:

Timelines

Task	1 st quarter	2 nd quarter	3 rd quarter	4 th quarter	5 th quarter	6 th quarter	7 th quarter	8 th quarter	9 th quarter
1. Improve xyl-lactate of M1	—					→			
2. Improve xyl-lac of N1	—					→			
3. Xyl-lactic P1	—				→				
4. Isolation of novel enzymes	—	—	—	→					
5. Constr. of yeasts	—				→				
6. Biochem char. of xyl enzymes	→	→	→	→					
7. Tools for most promising strains				→					
8. Genetic engineering of new hosts		—	—	—	—	—	—	→	
9. Improvement of most promising strains				—	—	—	—	—	→

Project details by task are as follows:

Tasks 1 and 2 Improve xylose to lactate fermentation of strain *Kluyveromyces marxianus* (M1) and *Candida sonorensis* (N1).

Initial efforts focused on the identification, sequencing, and cloning of the native enzymes xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK) in the target strains. All three xylose pathway genes, XR, XDH and XK from a benchmark microorganism were also cloned into vectors for expression in target strains.

The DNA sequences for the isolated *C. sonorensis* genes were finalized in the second quarter. The entire predicted protein coding regions and 700-1000 bp of the promoters and 500 bp of the terminators were sequenced. The predicted XR, XDH and XK proteins are 319, 354, and 585 aa long, respectively. The highest overall identities of the predicted amino acid sequences with known XR, XDH and XK sequences are 74%, 56%, and 50%, respectively. *K. marxianus* genes for XR, XDH and XK were also isolated and sequenced.

Once the genes were identified and cloned, a variety of strains were developed with overexpression of various combinations of the 3 enzymes (native or from the benchmark organism), with the goal of determining if one particular step was acting as a metabolic bottle neck, if overexpression of the entire set of enzymes would improve xylose conversion to fermentation products, or if some other metabolic barrier was present (e.g. redox imbalance).

For each construct, transformation was confirmed by PCR analysis. Enzyme assays were used to confirm the expression and activity of each enzyme. Shake flask studies were conducted to determine the effects of expression on xylose utilization and conversion to product.

Generally, lactate production from xylose by these strains was not greatly improved when compared to the untransformed hosts, although some changes in the product profile were observed. This indicated that enzyme concentration of XR, XDH, and XK may not be limiting the rate of xylose fermentation; some other step or metabolic constraint appears more limiting.

This task was completed in quarter 6, with the most promising *K. marxianus* and *C. sonorensis* strains retained for possible further analysis and modification under Task 9.

Task 3 Construction of xylose to lactic fermenting *Pichia stipitis* (P1) strain as benchmark strain:

Ldh-gene containing derivatives of *Pichia stipitis* were constructed and shown to produce lactic acid from glucose and xylose in an initial +/- test. These strains were then characterized in shake flasks. Xylose utilization rate and lactic production rate were found to be significantly higher than in similar *K. marxianus* or *C. sonorensis* strains produced in tasks 1 & 2, but still significantly below project targets. Furthermore, changes in co-product profile in comparison to similar *K. marxianus* or *C. sonorensis* were observed. This task was completed at the end of the fifth quarter.

Task 4 Screen for and isolate target xylose isomerases (XI) enzymes:

At the beginning of the project 4 different XIs (A-XI, B-XI, L-XI and S-XI) had been isolated and cloned into expression vectors. During the project a 5th, *Piromyces* XI (P-XI), was synthesized from a published sequence. These five XIs represent different origins, plant, fungal and bacterial.

These XI's were cloned into expression vectors for testing in the target strains and benchmark organisms. Further, the native xylose utilization pathway in the test organisms was disrupted. This allowed testing XI functionality by testing growth on xylose. This task was completed at the end of the fourth quarter. Organisms were further modified and tested under tasks 5 and 9.

Task 5 Construction of xylose to lactate fermenting biocatalysts:

XI containing derivatives of *K. marxianus* were constructed and lactic production from xylose was shown in shake flask cultures with a strain carrying *Piromyces* XI. This task was completed at the end of the fifth quarter. Further organism development was conducted under task 9

Task 6 Rough biochemical characterization of chosen pathway enzymes

Cofactor dependency of XR from both *K. marxianus* and *C. sonorensis* was determined using cell free extracts. Like most XRs, both only showed activity with NADPH. As the XI containing strains showed much more promise for further development, no further work was done on characterization of the native pathway enzymes. This task was effectively completed at the end of the fourth quarter.

Task 7 New genetic engineering tools for most promising hosts:

This work was actually completed before the project started. By the end of the 4th quarter, it was clear that no additional tools would be needed so this task was deemed closed.

Task 8 Genetic engineering of new host species for xylose to lactate/ethanol fermentation:

Cargill Dow had identified one new promising host species, C1, before the project started. C1 derivatives were constructed expressing XI. To accomplish this, vectors containing homologous expression signals were constructed for C1 and a transformation protocol was developed. A transformation marker and a lactate dehydrogenase gene were placed under control of a glycolytic C1 promoter. LDH was then transformed into C1 and lactic acid production from glucose by the resulting strain was shown.

Work was then started towards elimination of co-product formation. A full-length copy of a target gene (to be deleted in order to achieve this) was isolated, vectors constructed and one copy of the gene deleted. It was further discovered that two copies of this gene are present in the genome and that both need to be deleted before co-product formation is eliminated. The second

copy of the target gene responsible for co-product formation was then deleted. Lactic acid production at low pH by the resulting C1 strain was evaluated in shake flask assay and compared to *K. marxianus* strains. The new C1 strain was capable of producing a very high titer of lactic acid with very high yield. The yield was similar to *K. marxianus* strains, but the maximum titer at low pH was even higher than what the current best *K. marxianus* strains reach in the same test (see Figure 1).

Piromyces XI was transformed into C1 in order to improve xylose fermentation. This strain was shown to have low levels of XI activity and showed slight improvements in xylose consumption rate and reduction in xylitol formation when compared to parent strains without XI.

Vectors with a second transformation marker gene were constructed and the second marker was successfully expressed in C1. The second marker enables further modifications of C1.

As modifications in target gene2 have been beneficial in *K. marxianus*, we also wanted to modify this gene in C1. Work to identify this gene from C1 revealed that C1 has two closely related genes. Both of them have now been isolated and fully sequenced from C1. Vector construction to delete one of these was completed.

Gene3 has been successfully used as a selectable marker in related organisms. Degenerate oligonucleotides were designed based on known sequences of this gene in related organisms and used to amplify part of the gene from C1. Sequence comparison of the amplified fragment showed high homology to known gene3 sequences. Full-length gene3 was isolated from C1. The coding region and about 1.5-kb of upstream and downstream was sequenced. This gene will be used to develop a re-usable marker system for C1.

A vector containing a phleomycin resistance gene was constructed for C1 transformations.

Task 8 was completed at the end of the ninth quarter.

Figure 1. Lactic acid production at low pH by C1 and *K. marxianus* (M1) strains

Task 9 Improvement of most promising strains:

K. marxianus derivatives expressing XI in several host backgrounds with different combinations of deleted and overexpressed genes for XR, XDH, XK were constructed. Their ability to produce ethanol from xylose in shake flask assays was assessed. One specific combination of these genes, deletion of genes for XR and XDH and overexpression of XK and *Piromyces* XI was found to be common for superior xylose to ethanol conversion (see Figure 2). The strain was capable of converting xylose to ethanol under a variety of fermentation conditions. A similar set of strains, expressing XI, was constructed in the *C. sonorensis* host as well.

Figure 2: Effects of different sets of genetic modifications on *K.marxianus* (M1) xylose fermentation.

Xylose to ethanol conversion by *C. sonorensis* derivatives expressing XI in several host backgrounds with different combinations of deleted and over expressed genes for pathway enzymes XR, XDH, XK were evaluated in shake flasks. The same combination of these four genes that was previously found to be superior for xylose to ethanol conversion by *K. marxianus*, was also the best combination in *C. sonorensis*. These *C. sonorensis* strains were shown to convert xylose to ethanol and lactate in a variety of fermentation conditions.

Novel XI genes from 3 different genres, *Cyellamyces aberensis*, *Bacteroides thetaiotaomicron*, and N1 were expressed in *K. marxianus*. The first two of these were shown to lead to xylose to ethanol fermentation in *K. marxianus*.

Milestone 6, “Microorganism capable of meeting the performance targets for xylose to lactic conversion”, was met using a *K. marxianus* derivative carrying 5 different modifications (XR-, XDH-, XK++, XI+, LDH+) related to xylose to lactic acid conversion. Figure 3 shows the results of 5 shake flask experiments with this strain for production of lactic acid from xylose. This strain achieved 114 % +/- 6% of the targeted rate (1 g/L*h) and 96 % +/-4% of the targeted yield (80%), when taking the average of the 5 experiments. This performance level on an economic basis exceeds achieving 100% performance for both objectives. The results of one experiment (#5 in Figure 3) exceeded 100% on both targets.

Two genes were identified in the *K. marxianus* genome that could be responsible for by-product formation in xylose fermentations. Strains over expressing one or the other of these genes as well as strains where one or the other was deleted were constructed. One of the genes was shown to code for the reaction producing the by-product. Deletion of this gene in xylose to ethanol or lactic fermenting strains may thus improve yield of the main fermentation product and reduce the by-product level. The enzymatic activity coded by the other gene studied remains unclear. This task was completed at the end of the ninth quarter.

Figure 3. Xylose to lactate conversion rates and yields

Patents: *A cumulative list of patents applied for or resulting from the award.*

Title: Genetically Modified Yeast Species, Constructs For Introducing Exogenous Genes into Yeast Species, and Fermentation Processes Using Genetically Modified Yeast

Cargill Dow Case #: 1182

Provisional US patent application Filed: 5/2/03; converted to case 1182A below.

Inventors: Vineet Rajgarhia, Christopher Miller, and Aristos Aristidou

Title: Genetically Modified Yeast Species, and Fermentation Processes Using Genetically Modified Yeast

Cargill Dow Case # 1182A

PCT Application number 60/467,727 Filed 5/3/04 (claiming priority to above US provisional application). PCT publication number WO04099381A2.

Inventors: Rajgarhia, Vineet; Koivuranta, Kari; Penttila, Merja; Ilmen, Marja; Suominen, Pirkko; Aristidou, Aristos; Miller, Christopher; Olson, Stacey; Ruohonen, Laura

Other Inventions reported to DOE:

A patent application is being drafted for biocatalyst C1 (Task 8). This will constitute an invention disclosure, which will be reported to the DOE Chicago Field office as required.

Publications / Presentations: *[Identify and attach all publications and presentations made for industry or government groups resulting from the award during.]*

None