

1) Narrative

Introduction

A sustainable energy future is critical for environmental and strategic reasons. Fossil fuel use has increased greenhouse gas emissions, and continued consumption could adversely change global climate. In addition, the United States must rely on foreign petroleum suppliers, leading to unfavorable trade deficits, instability, and conflict.¹ One leading alternative to petroleum used for transportation is ethanol derived from cellulosic biomass.² A major barrier for biological-based biomass conversion is a cost effective method of releasing sugars from recalcitrant cellulosic biomass by enzymatic hydrolysis. Thermophilic, anaerobic bacteria offer a potential solution, as they produce efficient native hydrolytic enzymes.³ However, all thermophilic bacteria isolated to date convert sugars to organic acids in addition to ethanol, which makes them impractical for cellulose conversion.

The anaerobic, saccharolytic, thermophilic bacteria are a class of organisms with unique properties relevant for bioconversion of low cost cellulosic biomass feedstocks. The rate and efficiency of their cell associated enzymes to hydrolyze insoluble cellulose and xylan hold top values in the reported literature. In this regard, they hold a distinct advantage over the microorganisms that currently dominate biotechnological applications, which are unlikely to match the native hydrolytic ability of thermophilic bacteria due to the complexity of engineering highly efficient hydrolytic enzymes and the thermodynamic rate advantage of hydrolysis at higher temperatures.

The branched fermentation pathways of these organisms, which produce organic acids in addition to solvents, are the primary obstacles for their use in an industrial process. Other challenges, such as product tolerance and fermentation robustness also need to be addressed, but low product yields above all else preclude their consideration for a commercial process. The establishment of a preliminary genetic system in *Thermoanaerobacterium saccharolyticum* JW/SL-YS485, a xylanolytic thermophile, opens the possibility for the establishment of this strain as a tractable model organism for anaerobic thermophilic bacteria. *T. saccharolyticum* also holds applied value due to its ability to ferment insoluble xylan and biomass derived sugars. The overall objective of this thesis is to establish high yield ethanol production in this strain through metabolic pathway engineering.

The central objective of this project was to demonstrate that a thermophilic bacteria could be engineered to produce ethanol as sole end product. This was

¹ Greene N, Celik FE, Dale B, Jackson M, Jayawardhana K, Jin H, Larson ED, Laser M, Lynd L, MacKenzie D et al.: Growing energy: how biofuels can help end America's oil dependence. Natural Resources Defense Council, New York NY; 2004.

² Lynd LR, Gerngross TU, Wyman CE: Biocommodity engineering. *Biotechnol Prog* 1999, 15:777-793.

³ Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews* 2002. 66(3):506-577.

undertaken by gene disruption of metabolic pathways leading to acetic and lactic acid. In addition, the central fermentative pathways of *T. saccharolyticum* were investigated at the enzymatic and genomic level, and an alternative metabolic engineering strategy for high yield ethanol production was attempted by deletion of hydrogenase genes.

Results

Key enzymes involved in end-product formation were identified in *Thermoanaerobacterium saccharolyticum* JW/SL-YS485, a thermophilic anaerobic bacterium under consideration as a biological catalyst for the conversion of cellulosic biomass to ethanol. Based on enzymatic assays and genome sequence analyses, pathways were identified that would lead to the generation of all major products from xylose fermentation: lactate, acetate, ethanol, hydrogen, and carbon dioxide. Pyruvate ferredoxin oxidoreductase is the primary pyruvate decarboxylating enzyme, producing carbon dioxide, reduced ferredoxin, and acetyl-CoA, and ferredoxin is likely oxidized by a specific hydrogenase. It is concluded that enzymes are present in this organism that could theoretically produce ethanol from carbohydrates at high yield.

We report engineering *Thermoanaerobacterium saccharolyticum*, a thermophilic anaerobic bacterium that ferments xylan and biomass-derived sugars, to produce ethanol at high yield. Knockout of genes involved in organic acid formation (acetate kinase, phosphate acetyltransferase, and L-lactate dehydrogenase) resulted in a strain able to produce ethanol as the only detectable organic product, and also resulted in substantial changes in electron flow relative to the wild-type. Ethanol formation in the engineered strain (ALK2) involves pyruvate:ferredoxin oxidoreductase with electrons transferred from ferredoxin to NAD(P), a pathway different from that in previously-described microbes with a homoethanol fermentation. The homoethanologenic phenotype was stable for over 150 generations in continuous culture. The growth rate of strain ALK2 was similar to the wild-type, with a reduction in cell yield proportional to the decreased ATP availability resulting from acetate kinase inactivation. Glucose and xylose are co-utilized, and utilization of mannose and arabinose commences before glucose and xylose are exhausted. In work led by fellow graduate student Kara Podkaminer, strain ALK2 was shown in at 50°C to have a 2.5-fold reduction in cellulase loading compared to *Saccharomyces cerevisiae* at 37°C in simultaneous hydrolysis and fermentation experiments. The maximum ethanol titer produced by strain ALK2, 37 g/L, is the highest reported thus far for a thermophilic anaerobe, although further improvements are desired and likely possible.

Three putative hydrogenase enzyme systems in *T. saccharolyticum* were investigated at the genetic, mRNA, enzymatic, and phenotypic levels. A new Fe-only hydrogenase gene cluster, provisionally termed *hfs* (hydrogenase-Fe-S), was found to be the main enzymatic catalyst of hydrogen production. It is composed of four genes containing Fe-S clusters, and has several conserved homologues among clostridial saccharolytic, cellulolytic, and pathogenic bacteria. A second hydrogenase gene cluster, *hyd*, exhibited methyl viologen-linked hydrogenase enzymatic activity and is likely specific to the transfer of electrons from NAD(P)H to hydrogen. A third hydrogenase gene cluster, *ech*, did not exhibit hydrogenase activity under any of the conditions tested. Deletion of the *hfs* and *hydA* genes result in a loss of detectable methyl viologen-linked

hydrogenase activity. Strains carrying a deletion of the *hfs* genes show a 97-95% reduction in hydrogen and acetic acid production. A *hfs⁻*, *L-ldh⁻* strain exhibited an increased ethanol yield from consumed carbohydrates, and represents an alternate strategy to engineer high ethanol yields in *T. saccharolyticum*.

Conclusion

The results of this thesis advance the fundamental understanding of the branched metabolic pathways in anaerobic, thermophilic organisms, and advance the applied goal of using thermophilic bacteria to produce ethanol from cellulosic biomass. Although difficult to quantify exactly, the ability to utilize cellulolytic and xylanolytic thermophilic bacteria for cellulosic biomass conversion is one of the largest foreseeable cost reductions in the biomass to ethanol process, and could make the process economically competitive with production of petroleum based gasoline. A cost neutral renewable replacement for gasoline would be a significant benefit to society, especially at the scale that cellulosic biomass offers. Cellulosic biomass utilization could offset a significant amount of greenhouse emissions, as well as provide a sustainable and more equitable energy supply for society.

2) Published and submitted journal articles that received support from the Link fellowship.

Shaw A. J., Jenney F. E., Adams M. W. W. & Lynd L. R. (2008) End-product pathways in the xylose fermenting bacterium, *Thermoanaerobacterium saccharolyticum*. *Enzyme Microb Technol* 42:453-458.

Shaw, A. J., K. K. Podkaminer, S. G. Desai, J. S. Bardsley, S. R. Rogers, P. G. Thorne, D. A. Hogsett, and Lynd, L. R. (2008) Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. *PNAS* 105:13769-13774.

Shaw, A. J., Hogsett, D. A., and Lynd, L. R. (2009) Identification of the [FeFe] hydrogenase responsible for hydrogen generation in *Thermoanaerobacterium saccharolyticum*. (2009) *submitted*

3) Discretionary funds were used to purchase consumable laboratory materials used to perform biochemistry and molecular biology during the course of this work. In addition, the Link fellowship helped defray the travel expenses the Metabolic Engineering VI conference in Noordwijkerhout, The Netherlands.

4) Receiving the Link energy fellowship was a not only a financial benefit to me; it also increased my confidence that the project I was pursuing would be well received by the academic and engineering communities. In the second month of my graduate study I attended the Metabolic Engineering V conference; I recall being very impressed with almost every presentation I heard and poster I saw at this high quality conference. Two years later, it was a privilege to be able to not only attend, but also present my work at

Metabolic Engineering VI. The Link energy fellowship helped make this possible, and I greatly appreciate the opportunity it afforded me.