METABOLIC ENGINEERING OF *ZYMOMONAS MOBILIS* FOR IMPROVED PRODUCTION OF ETHANOL FROM LIGNOCELLULOSES

A Dissertation
Presented to
The Academic Faculty

by

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In Partial Fulfillment
of the Requirements for the Degree
PhD in Chemical & Biomolecular Engineering in the
School of Chemical & Biomolecular Engineering

Georgia Institute of Technology
May 2012

METABOLIC ENGINEERING OF *ZYMOMONAS MOBILIS* FOR IMPROVED PRODUCTION OF ETHANOL FROM LIGNOCELLULOSES

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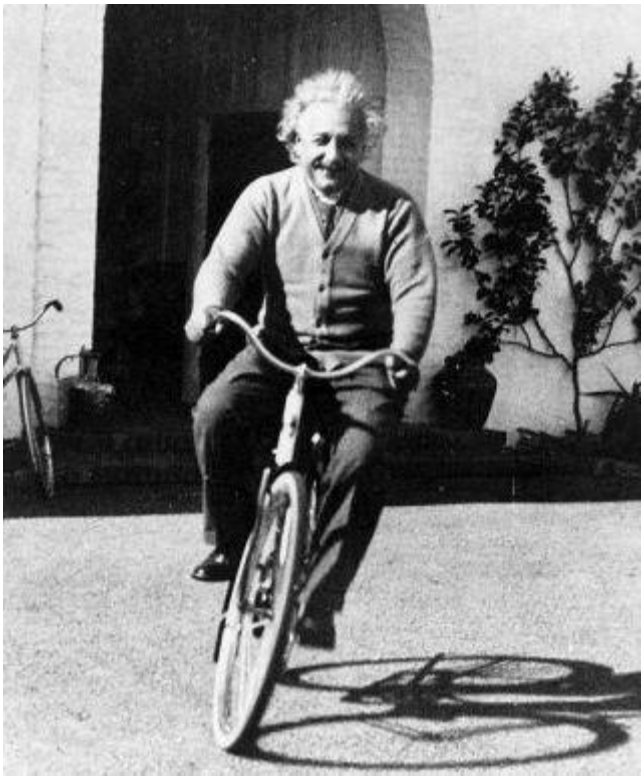

In Santa Barbara, 1933

Life is like riding a bicycle.
To keep your balance you must keep moving.

—Albert Einstein

To my loving family
None of this would be possible without you

ACKNOWLEDGEMENTS

From the bottom of my heart, I thank hundreds of people who have directly and indirectly helped me along the way to complete this dissertation work. I would like to mention each and every one of them, but due to space constraints, I am mentioning only a significant few.

First and foremost, I would like to thank my thesis advisor Dr. Rachel Chen. She has been an amazing teacher and has taught me a lot especially during my early days when I joined this department more as a chemical engineer with little knowledge of biology. Under her able guidance, I have matured from a student to a researcher. She has provided support, encouragements and ideas when my experiments would not work as expected and more importantly, taught me to always be on the lookout for an alternate line of action in case things do not work as planned. She has given lots of great ideas during my PhD project and it is her vision which has helped structure this project in such a productive and meaningful way. She has taught me the capability to multi-task which I now realize is such an indispensible tool for a successful researcher. Without doing things in parallel, it is nearly impossible to achieve goals in the correct time frame.

More importantly, I have learned the importance of patience, endurance and tolerance from Dr. Chen, values that are of paramount importance for a robust all-round personality. She has been a great advisor giving rough direction and leaving the rest of the project to me to explore which had helped me learn my biking better through my falls. She has even been a strict boss when necessary when she finds that I am slacking in my work. In a nut shell, she has been an amazing personality who will remain an

inspiration to me in my days ahead. I truly wish a wonderful life blessed with health and happiness for her and her family.

My doctoral committee members - Dr. Christopher Jones, Dr. Jay Lee, Dr. Hang Lu, Dr. Ronald Smith and Dr. Eberhard Voit - have guided me throughout this thesis. Despite their tight schedules, they always found time to answer my queries and give valuable advices. They are instrumental in shaping me as better scientist.

I owe a deep gratitude to Dr. Zichao Mao who was a post-doc in Dr. Chen's Biomolecular Engineering lab. My PhD project would not have been possible without him because he helped start the Chevron project and he kindly shared his ideas and work with me on which my PhD project is built. I want to thank Dr. Hyun-Dong Shin, another post-doc in my lab. I am sure that however much I acknowledge his contribution; it is not enough for the kind of support I have received from him. Without his support, extensive molecular biology experience and amazing scientific insight, it will be difficult to achieve all the things I accomplished during my PhD. He has helped on several occasions to make my experiments work. At times, he has given me great ideas in designing experiments. He always finds time for you when you ask him for a help.

My thanks are also to my previous undergraduate mentors Dr. Gummadi and Dr. Chandraraj at IIT Madras who showed me the interesting world of biology and created a thirst in me to do doctoral studies in biosciences.

My special thanks to all my lab mates. I would specially like to thank Dr. Anne Ruffing. She taught me the importance of being careful when doing experiments. Her remarkable success in the lab has been a source of great inspiration. Her disciplined style of work approaching a near perfection is a great thing to emulate. I would also like to

thank Ramanan Sekar with whom I interacted on regular basis to discuss about our experiments. I really appreciate the way he patiently hears my experimental design and then in an interactive process, helps on improving the design or plugging the loopholes. I would like to thank Charles and Lindsay for creating a lively atmosphere in the lab and office with their smiles and jokes and instilling a desire for superior scientific achievements by their questions and suggestions. I have also learnt a lot from Dr. Shara, Justin Harper and also from all other past and current lab members. Thank you so much. I cherish all the moments that I have shared with you. Without all of you people, this journey would not have remained happy.

Outside of the laboratory, I would like to thank my classmates in the department namely Ashish, Anna, Divya, Dhaval and Kayode. I have spent several hours with them going through the courses and it was the interaction with these people in the early days of PhD which convinced me that the journey to achieving a PhD would be a wonderful one. I would also like to thank my past and current roommates, Nagesh, Shreekrishna, Ajay, Sandeep who have made the stay at home convenient by cooking great food and sharing great moments after I returned from the lab. I would like to thank my dear friends, Gokul, Deepthi Adiga, Pramod (Bhai), Prashant, Prabuddh, and Anil who have made my stay at Georgia Tech a wonderful one.

Last but not the least; I would like to thank all my family members. I like to thank my dad who encouraged me to pursue PhD. Mom and dad- I know you always trust me and believe that one day I would make you proud – I promise that will happen and the completion of PhD program has propelled me further in fulfilling those dreams of ours.

I would like to thank my loving spouse, Dipti and in-laws who have been extremely understandable and supportive during my PhD program. Emotional support from Dipti, the person to whom I confide everything, is a wonderful gift from Almighty.

Finally I would also like to acknowledge the generous funding of a major portion of my research by Chevron Corporation, USA.

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LIST OF SYMBOLS AND ABBREVIATIONS

K_m	Michaelis-Menten constant
U	enzyme unit
V_{max}	maximum specific rate of an enzymatic reaction
α-KG	α-ketoglutarate
A(D/M/T)P	adenosine (di/mono/tri)phosphate
Amp	ampicillin
BPG	1,3-bisphosphoglycerate
CFE	cell-free extract
Cm	chloramphenicol
DCW	dry cell weight
DHA	dihydroxyacetone
DHAP	dihydroxyacetone phosphate
DTT	dithiothreitol
E4P	erythrose 4-phosphate
ED	Entner-Doudoroff
EDA	Entner-Doudoroff aldolase
EDD	Entner-Doudorof f dehydratase
EDTA	ethylenediaminetetraacetic acid
EMP	Embden-Meyerhof-Parnas
F6P	fructose 6-phosphate
FBP	fructose 1,6-bisphosphate
G6P	glucose 6-phosphate
GAP	glyceraldehyde 3-phosphate

GC-MS	gas chromatography – mass spectrometry
his	histidine
IPTG	isopropyl β-D-1-thiogalactopyranoside
kb	kilobase pairs
KDPG	2-keto-3-deoxy-phosphogluconate
MC	main culture
MIC	minimum inhibitory concentration
MM	mating medium
MTBE	methyl tertiary butyl ether
m/z	mass / charge
NAD	nicotinamide adenine dinucleotide
NAD(P)H	reduced nicotinamide adenine dinucleotide (phosphate)
OAA	oxaloacetic acid
P / Pi	phosphate (inorganic)
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PIPES	piperazine-N,N′-bis(2-ethanesulfonic acid)
PMSF	phenylmethylsulfonyl fluoride
PPi	pyrophosphate
PPP	pentose phosphate pathway
PSC	pre-seed culture
RM	rich medium
S7P	sedoheptulose 7-phosphate
SC	seed culture
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

Spec	spectinomycin
Str	streptomycin
Tc	tetracycline
TCA	tricarboxylic Acid
TES	tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
Tris	tris(hydroxymethyl)aminomethane
ZM27	Portion of a native plasmid pZMO3 containing replication origin
ZMGP	Portion of a native plasmid pZMO1 containing replication origin

LIST OF GENE AND ENZYME NOMENCLATURE

adh	alcohol dehydrogenase
Amp^R	ampicillin resistance cassette
ASeda	antisense of *eda*
Cm^R	chloramphenicol resistance cassette
eno	enolase
fba	fructose biphosphate aldolase
G3PDH	glycerol 3-phosphate dehydrogenase
GAPDH	glyceraldehyde 3-phosphate Dehydrogenase
GLK	Glucokinase
Gnd	6-phosphogluconate dehydrogenase
LDH	L-lactic dehydrogenase
ori	origin of replication
Padh	promoter of alcohol dehydrogenase
Peno	promoter for enolase
PFK	6-phosphofructokinase
Pgap	promoter of glyceraldehyde 3-phosphate
PGI	phosphoglucose isomerase
PGL	6-phosphogluconolactonase
PGM	phosphoglucomutase
Ppdc	promoter of pyruvate decarboxylase
$Spec^R$	spectinomycin resistance cassette
Str^R	streptomycin resistance cassette
Tc^R	tetracycline resistance cassette

talB	transaldolase
tklB/tktA	transketolase
TPI	triose phosphate isomerase
XR	xylose reductase
xylA	xylose isomerase
xylB	xylulokinase
ZWF	glucose 6-phosphate dehydrogenase

SUMMARY

Ethanol from lignocellulosic biomass is a promising alternative to rapidly depleting oil reserves. However, natural recalcitrance of lignocelluloses to biological and chemical treatments presents major engineering challenges in designing an ethanol conversion process. Current methods for pretreatment and hydrolysis of lignocelluloses generate a mixture of pentose (C_5) and hexose (C_6) sugars, and several microbial growth inhibitors such as acetic acid and phenolic compounds. Hence, an efficient ethanol production process requires a fermenting microorganism not only capable of converting mixed sugars to ethanol with high yield and productivity, but also having high tolerance to inhibitors. Although recombinant bacteria and yeast strains have been developed, ethanol yield and productivity from C_5 sugars in the presence of inhibitors remain low and need to be further improved for a commercial ethanol production.

The overarching objective of this work is to transform *Zymomonas mobilis* into an efficient whole-cell biocatalyst for ethanol production from lignocelluloses. *Z. mobilis*, a natural ethanologen, is ideal for this application but xylose (a C_5 sugar) is not its 'natural' substrate. Back in 1995, researches at National Renewable Energy Laboratory (NREL) had managed to overcome this obstacle by metabolically engineering *Z. mobilis* to utilize xylose. However, even after more than a decade of research, xylose fermentation by *Z. mobilis* is still inefficient compared to that of glucose. For example, volumetric productivity of ethanol from xylose fermentation is 3- to 4- fold lower than that from glucose fermentation. Further reduction or complete inhibition of xylose fermentation occurs under adverse conditions. Also, high concentrations of xylose do not get

metabolized completely. Thus, improvement in xylose fermentation by *Z. mobilis* is required.

In this work, xylose fermentation in a metabolically engineered *Z. mobilis* was markedly improved by applying the technique of adaptive mutation. The adapted strain was able to grow on 10% (w/v) xylose and rapidly ferment xylose to ethanol within 2 days and retained high ethanol yield. Similarly, in mixed glucose-xylose fermentation, the strain produced a total of 9% (w/v) ethanol from two doses of 5% glucose and 5% xylose (or a total of 10% glucose and 10% xylose). Investigation was done to identify the molecular basis for efficient biocatalysis. An altered xylitol metabolism with reduced xylitol formation, increased xylitol tolerance and higher xylose isomerase activity were found to contribute towards improvement in xylose fermentation. Lower xylitol production in adapted strain was due to a single mutation in ZMO0976 gene, which drastically lowered the reductase activity of ZMO0976 protein. ZMO0976 was characterized as a novel aldo-keto reductase capable of reducing xylose, xylulose, benzaldehyde, furfural, 5-hydroxymethyl furfural, and acetaldehyde, but not glucose or fructose. It exhibited nearly 150-times higher affinity with benzaldehyde than xylose. Knockout of ZMO0976 was found to facilitate the establishment of xylose fermentation in *Z. mobilis* ZM4.

Equipped with molecular level understanding of the biocatalytic process and insight into *Z. mobilis* central carbon metabolism, further genetic engineering of *Z. mobilis* was undertaken to improve the fermentation of sugars and lignocellulosic hydrolysates. These efforts culminated in construction of a strain capable of fermenting

glucose-xylose mixture in presence of high concentration of acetic acid and another strain with a partially operational EMP pathway.

CHAPTER 1

INTRODUCTION

In this chapter, the process for conversion of lignocelluloses to ethanol is described. *Zymomonas mobilis* ZM4 is a 'natural' ethanologen which is used to carry out the fermentation step of this conversion process. A discussion follows about *Z. mobilis*, its importance as an ethanologen and its history, physiology and metabolism relevant to ethanol production. Deficiencies in *Z. mobilis* that result in this bacterium being less attractive for use in a commercial cellulosic ethanol production plant are mentioned. The removal of some of these deficiencies to facilitate the industrial use of this bacteria helps to outline the research goals. Towards the end of this chapter, the approach taken for achieving these research goals is mentioned.

1.1 Conversion of Lignocelluloses to Ethanol

Lignocellulose is the most abundant source of renewable carbon in the biosphere (Claassen et al. 1999). Biofuels from lignocellulosic biomass can be an alternative to the limited fossil fuels reserves that remain in the world (Hahn-Hagerdal et al. 2006). Oil reserves we have will finish up in the next 35 years at current usage rate (Shafiee and Topal 2009). Lignocelluloses can be converted to ethanol, a versatile transportation fuel. The demand for ethanol, as a substitute for gasoline, has been rising because of concerns related to national security, economic stability and environmental impact. To meet this demand, 'The Renewable Fuels Standard' sets an annual goal of 36 billion gallons of renewable fuel by 2022 in the US energy independence and security act of 2007 (Sissine 2007). Unlike fossil fuels, ethanol is a renewable energy source produced through fermentation of sugars. Ethanol is widely used as a partial gasoline replacement in the US. These gasoline fuels contain up to 10% ethanol by

volume. As a result, the US transportation sector consumed about 4540 million liters of ethanol annually (Saricks et al. 1999). Automobile manufacturers have started rolling out significant numbers of flexible-fueled vehicles that can use an ethanol blend as high as E85 (85% ethanol and 15% gasoline by volume). 25 million ethanol flexible-fuel vehicles which include automobiles, motorcycles and light duty trucks have already been sold worldwide by June 2011. Using ethanol-blended fuel for automobiles can significantly reduce petroleum use (Saricks et al. 1999). Unlike fossil fuels, lignocellulose derived fuels, such as ethanol, are environmentally benign. Their usage does not add to the amount of existing carbon dioxide (CO_2) in atmosphere since CO_2 released during biomass conversion and ethanol combustion was originally from atmosphere for formation of lignocellulosic biomass. If not converted to ethanol, this biomass would ultimately undergo natural degradation to release the absorbed CO_2 back to the atmosphere. Ethanol is also a safer alternative to methyl tertiary butyl ether (MTBE), the most common additive to gasoline used to provide cleaner combustion. MTBE is a toxic chemical compound and has been found to contaminate groundwater (Sun and Cheng 2002).

Most of ethanol produced today is obtained from corn starch or from sucrose contained in sugarcane and sugar beet (Goldemberg 2007). However, converting food crops like corn and sugarcane to ethanol raises both ethical and economic concerns. A drastic increase in ethanol production using the current starch- or sucrose-based technology may not be practical because corn, sugarcane and sugar beet production for ethanol will compete for the limited agricultural land needed for food and feed production. In contrast, if lignocelluloses present in wood residues, agricultural residues, grasses and paper wastes are to be used, both concerns can be addressed. Lignocelluloses are abundant and widely available. Compared to crude oil and food

crops such as corn, lignocellulose supply is the most assured. Thus, use of lignocelluloses shields a commercial ethanol manufacturing plant from any fluctuations in raw material supply.

Lignocelluloses are composed of cellulose, hemicellulose and lignin. Cellulose $(C_6H_{10}O_5)_n$ is a polysaccharide consisting of a linear chain of several $\beta(1\rightarrow4)$ linked D-glucose units (Figure 1).Cellulose is the structural component of the primary cell wall (O'Sullivan 1997). Hemicellulose is a branched heteropolymer made from several C_6 and C_5 sugar monomers namely glucose, xylose, mannose, galactose, rhamnose, and arabinose (Figure 1). It is present along with cellulose in almost all plant cell walls. While cellulose is crystalline, strong, and resistant to hydrolysis, hemicellulose has a random, amorphous structure with little strength. It is easily hydrolyzed by dilute acid or base as well as myriad hemicellulase enzymes. Unlike cellulose, hemicellulose consists of shorter chains - 500-3,000 sugar units as opposed to 7,000 - 15,000 glucose molecules per polymer seen in cellulose (Saha 2003). Lignin is a cross-linked racemic macromolecule with molecular masses in excess of 10,000 Da. It is relatively hydrophobic and aromatic in nature. The molecule consists of various types of substructures that appear to repeat in a haphazard manner as shown in Figure 1. Lignin is a complex and unusual biopolymer. Unlike cellulose, it lacks a defined primary structure and possesses heterogeneity in its structure. Lignin provides the support structure needed for strengthening of wood (xylem cells) in trees (Lebo et al. 2000).

The compositions of lignocelluloses vary with the sources as shown in Table 1 . It is highly desirable to design a commercial cellulosic ethanol plant that can process most of these different lignocellulosic sources. This would reduce the dependence of the manufacturing plant on a particular lignocellulose.

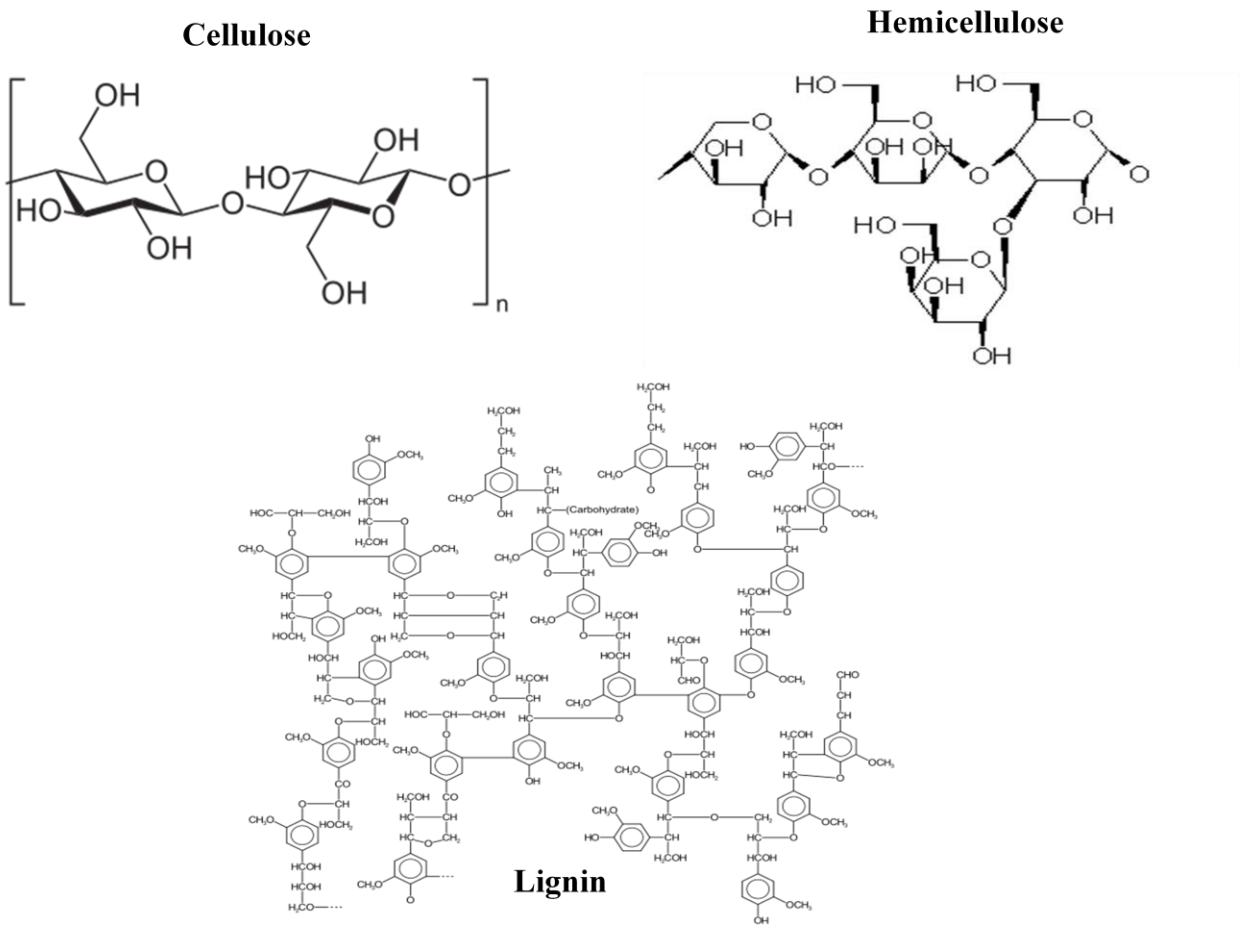

Figure 1: Structure of lignocellulose (Wikipedia 2012)

Table 1: Compositions of lignocelluloses from various sources (Sun and Cheng 2002)

The contents of cellulose, hemicellulose, and lignin in common agricultural residues and wastes[a]

Lignocellulosic materials	Cellullose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods stems	40–55	24–40	18–25
Softwood stems	45–50	25–35	25–35
Nut shells	25–30	25–30	30–40
Corn cobs	45	35	15
Grasses	25–40	35–50	10–30
Paper	85–99	0	0–15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15–20	80–85	0
Cotton seed hairs	80–95	5–20	0
Newspaper	40–55	25–40	18–30
Waste papers from chemical pulps	60–70	10–20	5–10
Primary wastewater solids	8–15	NA[b]	24–29
Swine waste	6.0	28	NA[b]
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0

[a] Source: Reshamwala et al. (1995), Cheung and Anderson (1997), Boopathy (1998) and Dewes and Hünsche (1998).
[b] NA – not available.

The general scheme for conversion of lignocelluloses to ethanol (Figure 2) is similar to that of conversion of starch. Chopped lignocellulose is pretreated thermochemically, which depolymerizes and solublizes hemicellulose, and thus exposes the cellulosic structure. Solublized hemicellulose is further hydrolyzed enzymatically into its monomers, xylose being the predominant product. Similarly, cellulose is converted to glucose using cellulases. Thus after hydrolysis of lignocelluloses, a mixture of C_5 and C_6 sugars is obtained which is put into a fermenter. Whole-cell biocatalysts such as recombinant yeast, *Escherichia coli* or *Zymomonas mobilis* then convert these mixed sugars to ethanol. They are genetically engineered to produce only ethanol and none of the fermentation by products like lactic acid, acetate and xylitol.

However, there still exists no commercial process for converting lignocelluloses to ethanol (Rogers et al. 2007). Lignocelluloses present several problems in designing an economically feasible conversion process to ethanol. They are naturally more recalcitrant and hence require severe chemical pretreatment. Besides C_6 sugars, they contain C_5 sugars and

hydrolysis of lignocellulose produces a number of microbial growth inhibitors such as acetic acid and various phenolic compounds (Badger 2002; Himmel et al. 2007; Stephanopoulos 2007). These challenges require use of novel pretreatment steps and a robust microbe that can survive in the hostile milieu of lignocellulosic hydrolysate and convert all the sugars completely and rapidly to ethanol.

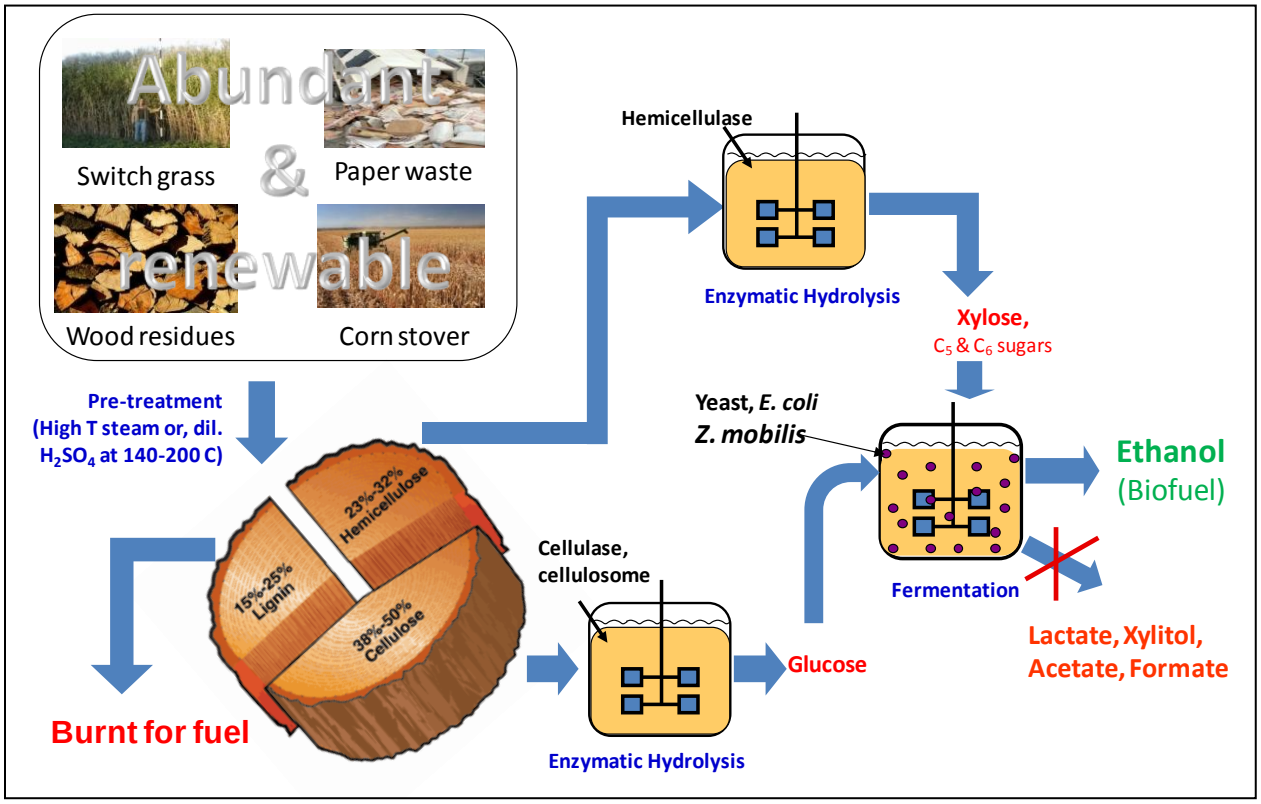

Figure 2: A general scheme for ethanol fermentation from cellulosic feedstock

1.2 *Z. mobilis* as an Ethanol Producer – Advantages and Disadvantages

Sugars released from lignocellulosic biomass after pretreatment and saccharification (Figure 2) can be fermented to ethanol by microorganisms. The yeast *Saccharomyces cerevisiae* is the commonly employed microbe (Inlow et al. 1988) for the fermentation step but recently,

there has been a steep rise in interest in using *Zymomonas mobilis*, which is a 'natural' ethanol producer (Panesar et al. 2006). In nature, *Z. mobilis* is found in high sugar content solutions (for example, cider, cell sap) fermenting sugars to ethanol in a high yield and high productivity process. It offers several advantages over yeast. Ethanol yield of *Z. mobilis* is as high as 97% of theoretical yield when glucose or fructose is the substrate. For sucrose, the yield is approximately 90% due to levan and sorbitol formation. One of the reasons for the high yield of ethanol is that *Z. mobilis* uses a small fraction of the substrate (2.0 – 2.6%) for biomass formation. It has a high specific ethanol productivity which is up to 2.5 times higher than yeast (Rogers P L 1982). It can tolerate up to 40% (v/v) glucose (Swings and De Ley 1977), up to 16% (v/v) ethanol (Swings and De Ley 1977) and up to 0.8% (w/v) acetic acid (at pH 6) (Kim et al. 2000b). Its ability to grow at lower pH (5.0-5.5) helps to reduce the aseptic requirements for fermentation, thus rendering the fermentation process more economical.

However, in spite of these attractive advantages, several factors prevent the commercial usage of *Z. mobilis* in cellulosic ethanol production. The foremost hurdle is that its substrate range is limited to glucose, fructose and sucrose. Wild-type *Z. mobilis* cannot ferment C_5 sugars like xylose and arabinose which are important components of lignocellulosic hydrolysates. Also, the low specific growth rate of *Z. mobilis* prevents it from reaching high cell density in fermenters in small duration. Like *E. coli* and yeast, *Z. mobilis* cannot tolerate toxic inhibitors present in lignocellulosic hydrolysates such as acetic acid and various phenolic compounds (Doran-Peterson et al. 2008). Concentration of acetic acid in lignocellulosic hydrolysates can be as high as 1.5% (w/v) (Takahashi et al. 1999), which is well above the tolerance threshold of *Z. mobilis* (Joachimsthal et al. 1998).

Several attempts have been made to engineer *Z. mobilis* to overcome its inherent deficiencies. National Renewable Energy Laboratory (NREL), USA has made significant contributions in expanding its substrate range to include C_5 sugars like xylose and arabinose (Deanda et al. 1996; Mohagheghi et al. 2002; Zhang et al. 1998; Zhang et al. 1995). Acetic acid resistant strains of *Z. mobilis* have been developed by rational metabolic engineering efforts (Baumler et al. 2006), mutagenesis techniques (Joachimsthal et al. 1998) or adaptive mutation (Chen et al. 2009). However, when these engineered strains metabolize mixed sugars in presence of inhibitors, the yield and productivity are much lower (Doran-Peterson et al. 2008), thus preventing their industrial application.

1.3 History, Physiology and Metabolic Pathway of *Z. mobilis*

Zymomonas mobilis is a gram negative, cylindrically-shaped, non-spore forming bacterium with polar flagella. In nature, bacteria belonging to the genus *Zymomonas* occur in tropical areas of America, Africa, and Asia, where they have been traditionally used to ferment plant saps to prepare local alcoholic beverages. In Europe, these bacteria are found growing in and spoiling beer, fermented apple juice (cider), and pear juice (perry) (Swings and De Ley 1977).

In the beginning of the 20^{th} century, there was demand for a clear, sweet cider, instead of a rough and dry farm-made cider. The manufacturers encountered great difficulties, as sweet ciders easily develop a secondary fermentation called the "cider sickness", perhaps due to an infection from fruit. Barker and Hillier (Barker and Hillier 1912) were the first to study cider sickness extensively, and they gave a description of the bacterium responsible for the typical aroma and flavor. The earliest symptoms are frothing and abundant gas formation. In a few days, the gas pressure causes the bottle to explode. The growing bacteria change the aroma and flavor

of the cider and reduce its sweetness. The cider attains a marked turbidity, which clears afterwards, with the formation of a heavy deposit. From the complex microflora of sick cider, Barker and Hillier (Barker and Hillier 1912) isolated and purified a bacterial strain (strain A (Barker 1948)) in 1911. The organisms caused the typical strong aroma and flavor upon re-infection in sterile cider. They were motile rods, single or joined in pairs, 2 by 1 µm, with rounded ends; in old cultures, the cells were frequently longer; involution forms were frequently up to 200 µm long, and their ends may be globular or dumb-bell shaped, with a diameter of 25 µm; spores were absent; they were facultative anaerobic, showing limited and slow growth on solid media; growth was creamy white and slimy; they vigorously fermented glucose and fructose with formation of ethanol and CO_2. This bacterial strain discovered by Barker and Hillier was the first *Zymomonas* known to mankind. Prior to being christened as *Zymomonas mobilis*, they were also known by the names *Thermobacterium mobilis and Pseudomonas linderi* (Doelle et al. 1993).

In the early 1950s, the genus *Zymomonas* had incited considerable excitement among biochemists by the discovery of Gibbs and DeMoss (Gibbs and Demoss 1951; Gibbs and Demoss 1954) that the anaerobic catabolism of glucose in *Zymomonas* follows the Entner-Doudoroff (ED) mechanism. This was indeed a revolutionary discovery since *Zymomonas* was the first example (and still is one of the very few) of an anaerobic organism using a pathway occurring mainly in strictly aerobic bacteria (Kersters and Deley 1968). In 2005, the genome sequence of *Zymomonas mobilis* ZM4 was reported. This strain is among the best known ethanologens in the genus *Zymomonas* (Seo et al. 2004). The complete genome of *Z. mobilis* ZM4 consists of a single circular chromosome of 2,056,416 bp with an average GC content of 46.33% (Seo et al. 2004).

The central carbohydrate metabolism pathway of *Z. mobilis* is shown in Figure 3. Wild-type *Z. mobilis* ferments its natural substrates (glucose, fructose and sucrose) exclusively through the ED pathway. EMP pathway, the more commonly employed carbohydrate degrading route in bacteria and eukaryotes, is not operational since the gene encoding for a key enzyme of the pathway, phosphofructokinase (PFK) (Figure 3) is absent. However, *Z. mobilis* has genes encoding for all other enzymes of EMP pathway (Seo et al. 2004). These enzymes are used for gluconeogenesis which is a complete reversal of EMP pathway. Gluconeogenesis is a metabolic pathway that results in biosynthesis of glucose when cell is feeding on non-carbohydrate carbon substrates such as pyruvate, lactate, glycerol and amino acids.

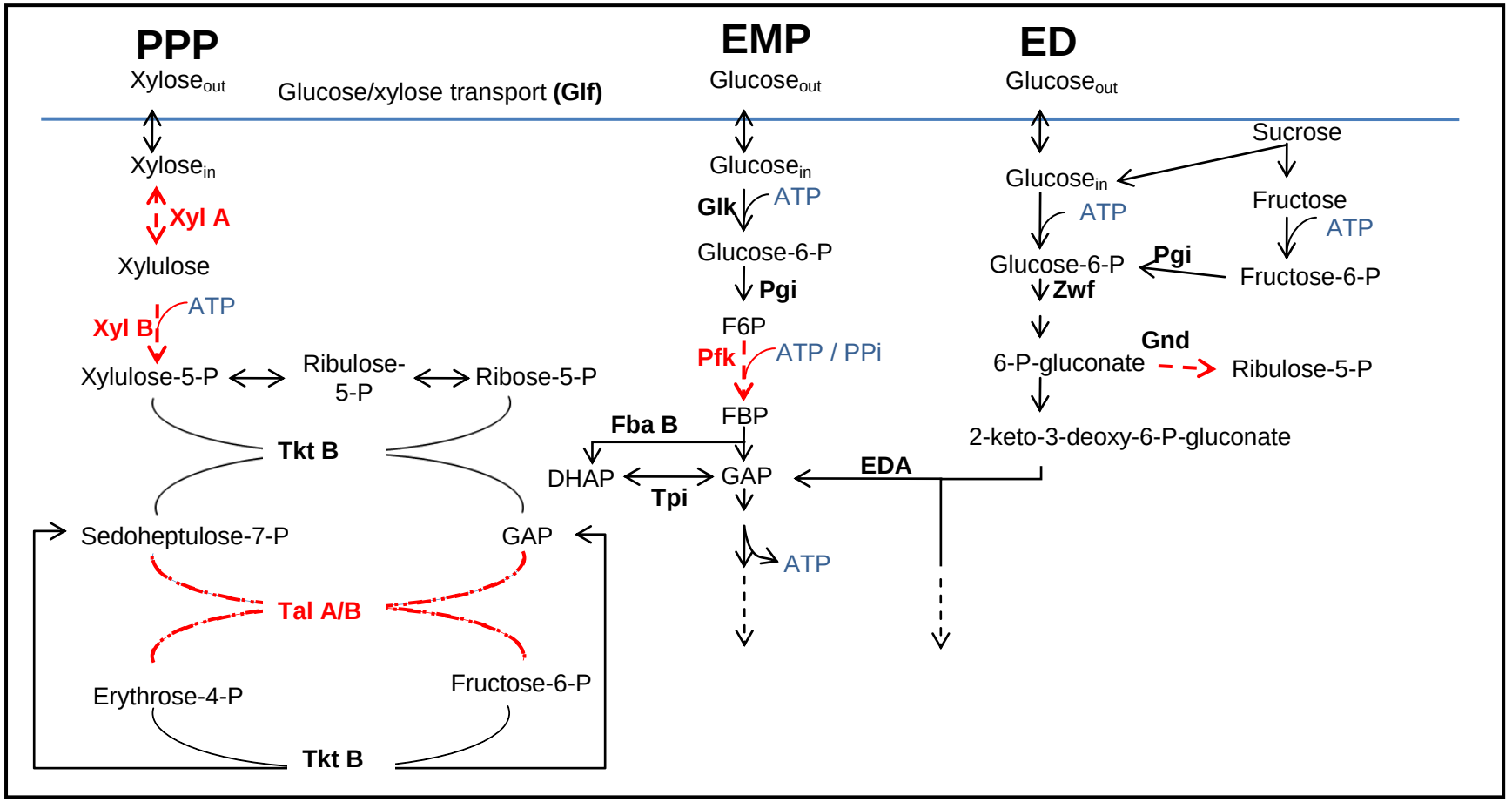

Figure 3: The central metabolic pathways of *Z. mobilis*. Biochemical reactions and genes which are absent in *Z. mobilis* are shown in red. Only relevant enzymes and metabolites have been shown

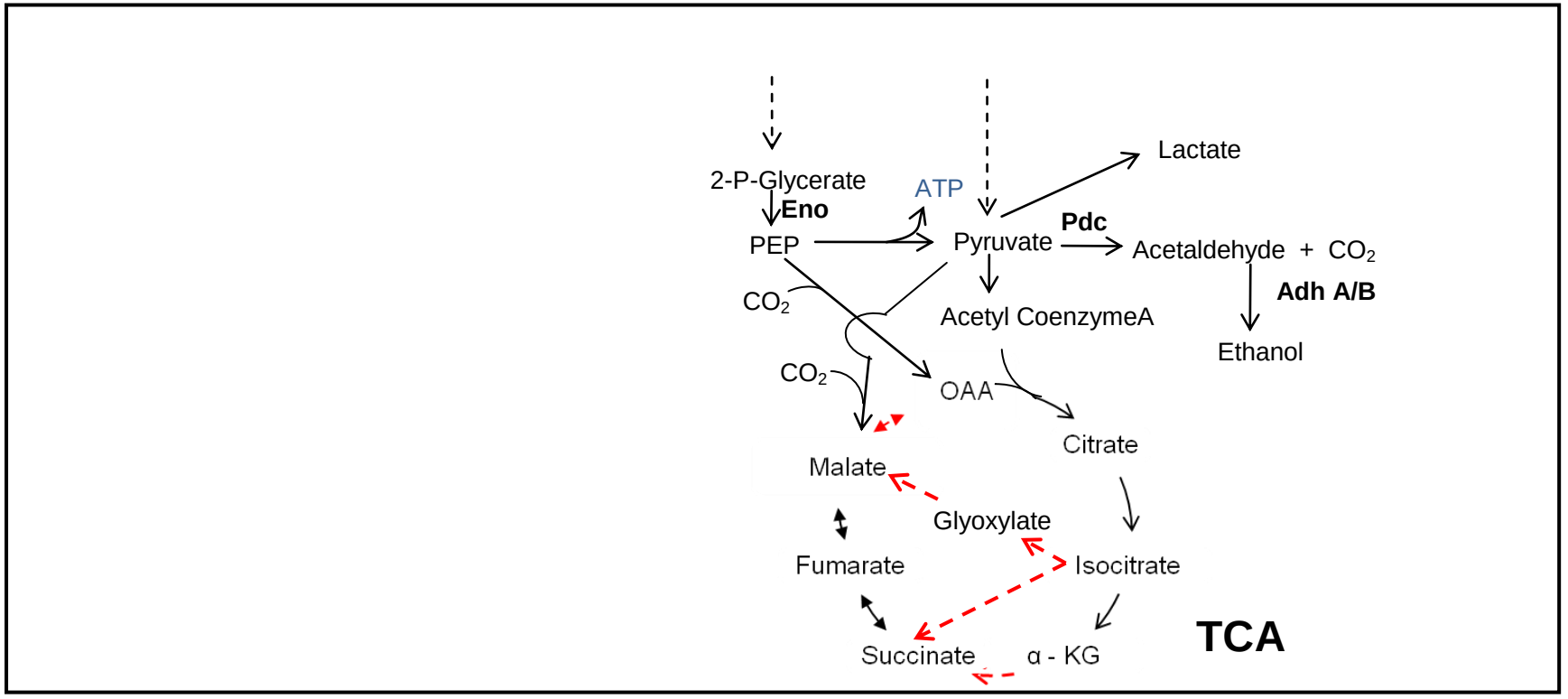

Figure 3 continued

From Figure 3, it can be seen that glucose metabolized through EMP pathway requires 1 or 2 mole of ATP (1 for pyrophosphate-dependent (PPi) PFK or 2 for ATP-dependent PFK) to generate 4 moles of ATP, thus resulting in an ATP yield of 2 or 3. However, in the ED pathway, since KDPG breaks down directly into pyruvate and GAP, a net generation of only one mole of ATP occurs. Thus, EMP pathway produces more energy (at least one extra mole of ATP per mole of glucose metabolized) than ED pathway.

Z. mobilis does not use the oxidative pentose phosphate pathway (PPP) since it does not have the gene encoding for 6-phosphogluconate dehydrogenase (Gnd). Its genome also does not contain the transaldolase (Tal) gene, but still it is able to synthesize all the PPP metabolites (Figure 3), which are precursors for nucleotides and amino acids. *Z. mobilis* is a facultative fermenting anaerobe since it misses several enzymes of the tricarboxylic acid (TCA) cycle and hence cannot oxidize glucose completely to CO_2 and H_2O under aerobic conditions. Compared to anaerobic conditions, yields of biomass and ethanol are low under aerobic conditions (Sootsuwan et al. 2008).

1.4 Project Objectives

The overarching aim of the project is to transform the wild-type *Z. mobilis* into an efficient whole cell biocatalyst for commercial cellulosic ethanol production. Glucose and xylose are among the principal sugars produced by depolymerization of lignocelluloses (Howard et al. 2003). Thus, for an economically feasible ethanol production, both sugars should be rapidly and completely fermented. To achieve this, the following objectives were established for the project –

1) Improve xylose fermentation in engineered *Zymomonas* using adaptive mutation
2) Develop a bio-molecular level understanding for the efficient xylose fermentation
3) Apply the understanding gained to further improve fermentation of sugars and lignocellulosic hydrolysates

1.5 Strategy

Heterologous xylose metabolizing genes can be expressed in *Z. mobilis* to enable the bacteria to ferment xylose besides its natural ability to ferment glucose. This technique was pioneered by National Renewable Energy Laboratory (NREL) (Zhang et al. 1995). Though this technique succeeded in giving xylose-fermenting *Zymomonas*, the xylose fermentation was inefficient compared to the glucose fermentation. The volumetric ethanol productivity was 3- to 4-fold lower on xylose compared to glucose (Lawford and Rousseau 2000; Viitanen et al. 2011a) and high concentration of xylose did not undergo complete conversion. For example, greater than 1% (w/v) xylose remained on fermentation of 6% (w/v) xylose by engineered *Zymomonas* (Lawford et al. 1999). Several research efforts have been made to improve xylose fermentation in *Z. mobilis* by rational metabolic engineering. For example, overexpression of xylulokinase gene (Jeon et al. 2005) which encodes for the enzyme catalyzing the second step in xylose fermentation by *Zymomonas* (Figure 3). However, the control architecture of metabolic pathways that has evolved over thousands of years may resist the desired flux increase (Stephanopoulos and Vallino 1991). The production of desirable compounds from microbes can often require a complete reprogramming of their innate metabolism (Alper et al. 2006). Hence, one way of solving this problem would be to carry out genetic modifications that theoretically lead to improvement in xylose fermentation, study the response of the metabolic pathways and based on this response, introduce more genetic modifications. Several cycles of genetic modification experiments are usually required to obtain the ‘desired’ strain (Bailey 1991).

Instead of the above approach, we tackled the problem of inefficient xylose fermentation by adding the technique of extended evolutionary engineering or adaptive mutation to the original rational metabolic engineering technique. As detailed in chapter 2, adaptive mutation was used to adapt a non-xylose fermenting rationally engineered *Zymomonas mobilis* ZM4/pZMETX to obtain a xylose fermenting strain - A1, which was further adapted to obtain additional improvement.

Adaptation has been successfully used for obtaining industrially relevant strains (Fong et al. 2003; Kuyper et al. 2005; Lawford et al. 1998; Lawford et al. 1999; Meijnen et al. 2008; Rosenberg 2001; Viitanen et al. 2008; Zhang et al. 2002). Adaptation is a powerful technique requiring little knowledge of the underlying metabolic pathways and regulations that may be difficult to control by the rational metabolic engineering efforts. As shown in Figure 4, adaptive mutation is the technique of repeatedly growing cells under a selection pressure till the cells attain stable growth and fermentation rates. The selection pressure has to be carefully chosen. In our case, we had a rationally engineered *Z. mobilis* with all the machinery for xylose metabolism, still due to some unknown reasons it was unable to grow in xylose. Rationally engineered Z. *mobilis* has the inherent ability to ferment glucose. Hence, an appropriate selection pressure in this case will be to make cells grow in a glucose-xylose mixture. Under a selection pressure, cells may mutate but only those cells that develop beneficial mutations which enable xylose utilization will survive and populate the adaptation media. This concept is very similar to survival of the fittest concept.

As further discussed in chapters 2 and 3, a bio-molecular level investigation can be done to identify the mutations that are responsible for improvement in xylose fermentation in the adapted strains. This process is facilitated by comparing the rationally engineered strain ZM4/pZMETX, less adapted ‘A1’ and more adapted strain ‘A3’ at genetic and metabolic levels. Once these mutations were discovered, as discussed in chapter 4, wild-type ZM4 was genetically engineered to include these mutations in an effort to develop strains comparable to adapted strains. This exercise also confirmed the necessity of identified mutations for improvement in xylose fermentation. Further, these mutations, essential for establishing and improving xylose fermentation, were also included in the construction of a xylose-fermenting strain from an acetate resistant strain ZM6014.

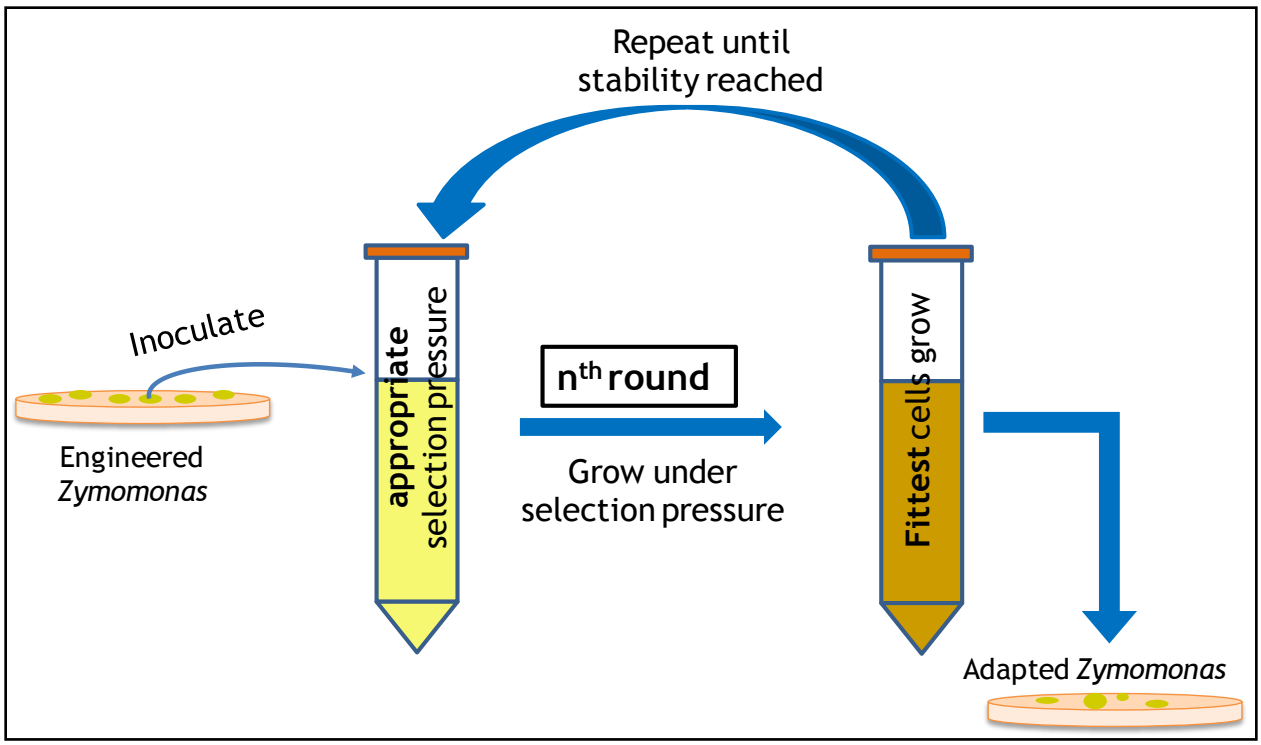

Figure 4: Technique of adaptive mutation

After successfully improving the ability of *Zymomonas* to metabolize xylose and creating the best reported xylose-fermenting strains so far (Agrawal et al. 2011), efforts were focused on improving glucose metabolism in *Zymomonas* by a rational metabolic engineering approach. The metabolic engineering strategy chosen was to introduce a new route (EMP pathway) for glucose metabolism in addition to the existing ED pathway (see Figure 3). The additional sugar metabolism capability through the EMP pathway may lead to an enhanced sugar uptake and consequently higher specific ethanol productivity. The new pathway will extract 2-3 times more energy from glucose than the existing pathway. The increased energy (ATP) availability would enable cells to spend energy, when needed, on increased cell maintenance required under 'stress' conditions (for example, high concentration of acetic acid and ethanol) as reported by several researchers (Joachimsthal et al. 1998; John Fieschko 1983; Lawford and Ruggiero 1990; Rogers P L 1982). Additionally, the improved energy status is likely to drive xylose (non-natural

substrate) metabolism as the extra energy can be used to ease the protein burden caused by the overexpression of xylose metabolizing genes.

Glycolytic and fermentative enzymes of *Z. mobilis* comprise approximately 50% of cytoplasmic proteins in exponential phase cells (An et al. 1991). Most of these enzymes are non-allosteric. Thus, high levels of these enzymes coupled with minimal allosteric control ensure considerably high glycolytic flux of approximately 1μmol glucose/min/mg cell protein in *Z. mobilis* (Arfman et al. 1992). Several researchers have tried overexpressing the enzymes of the ED pathway for increasing the glycolytic flux. However, the results are not very encouraging as the glycolytic flux could be increased only by a maximum of 17%. This increase in glycolytic flux is not commensurate with the expensive nutritional requirements of recombinant microbe compared to wild-type thus rendering no economic value to this achievement. Hence, it is widely believed that the naturally evolved ED pathway in *Z. mobilis* operates under optimal conditions (Snoep et al. 1996). Besides, most of the control of glycolytic flux is vested with the enzymes in the initial steps of glucose degradation like glucokinase (GLK) and glucose 6-phosphate dehydrogenase (ZWF) (Snoep et al. 1996). Thus if an EMP pathway is established in *Z. mobilis*, it will provide an additional route for glucose conversion to pyruvate, which is the rate limiting step in *Z. mobilis* for glucose conversion to ethanol.

As the only missing gene of the EMP pathway in *Z. mobilis* ZM4 is phosphofructokinase, expression of a heterologous pyrophosphate-dependent 6-phosphofructokinase (PPi-PFK) will complete the EMP pathway (Figure 3). However, this alone may not be sufficient to switch on the EMP pathway in *Z. mobilis* because there can exist factors such as (a) insufficient expression of EMP-associated native enzymes which limits the glycolytic flux through the EMP pathway and (b) unknown regulatory elements inhibiting the EMP pathway. Several strategies as discussed in chapter 5 were employed to functionalize the EMP pathway. Overexpression of the enzymes that limit the glycolytic flux through the EMP pathway, partial blockage of ED pathway and adaptation was carried out. All these efforts resulted in only partial operation of the EMP pathway.

CHAPTER 2

ADAPTATION YIELDS A HIGHLY EFFICIENT XYLOSE-FERMENTING *ZYMOMONAS MOBILIS* STRAIN

Portions of this chapter have been reproduced from (Agrawal et al. 2011).

2.1 Abstract

Zymomonas mobilis is a superb ethanol producer with productivity exceeding yeast strains by several fold. Although metabolic engineering was successfully applied to expand its substrate range to include xylose, xylose fermentation lagged far behind glucose. In addition, xylose fermentation was often incomplete when its initial concentration was higher than 5%. Improvement of xylose fermentation is therefore necessary. In this work, we applied adaptation to improve xylose fermentation in metabolically engineered strains. As a result of adaptation over 80 days and 30 serial transfers in a medium containing high concentration of xylose, a strain, referred as A3, with markedly improved xylose metabolism was obtained. The strain was able to grow on 10% (w/v) xylose and rapidly ferment xylose to ethanol within 2 days and retained high ethanol yield. Similarly, in mixed glucose-xylose fermentation, a total of 9% (w/v) ethanol was obtained from two doses of 5% glucose and 5% xylose (or a total of 10% glucose and 10% xylose). Further investigation reveals evidence for an altered xylitol metabolism in A3 with reduced xylitol formation. Additionally xylitol tolerance in A3 was increased. Furthermore, xylose isomerase activity was increased by several times in A3, allowing cells to channel more xylose to ethanol than to xylitol. Taken together, these results strongly suggest that altered xylitol metabolism is key to improved xylose metabolism in adapted A3 strain. This work further

demonstrates that adaptation and metabolic engineering can be used synergistically for strain improvement.

2.2 Introduction

Lignocellulose is the most abundant source of renewable carbon in the biosphere (Claassen et al. 1999). Utilizing this vast renewable resource for energy production requires innovative technologies. Although lignocellulosic ethanol has long been considered as a viable replacement for gasoline as transportation fuel, its large scale production is still hampered by the high production cost. *Zymomonas mobilis* is one of the best ethanol producers found in nature. Its high ethanol yield (97% of the theoretic value), higher productivity than the common yeast strain, make it particularly attractive as biocatalyst in ethanol fermentation (Panesar et al. 2006; Rogers P L 1982; Swings and De Ley 1977). However, these values only pertain to its ability to use glucose. *Z. mobilis* naturally does not ferment xylose. An important breakthrough came when Zhang et al successfully applied metabolic engineering to expand its substrate range to xylose (Zhang et al. 1995). In the ensuing decade, however, xylose metabolism continued to be a focal point of research as it was found that the ability of engineered strains to metabolize xylose was considerably lower than that of glucose and improvement was needed (De Graaf et al. 1999; Gao et al. 2002; Jeon et al. 2005; Kim et al. 2000a).

The most significant difficulties with xylose fermentation by engineered *Z. mobilis* were lower xylose consumption rate, which led to a long fermentation time and incomplete xylose utilization when xylose concentration was high. Much research over the past decade has been devoted to identify potential bottlenecks in xylose fermentation. Studies using NMR techniques suggested that xylose-metabolizing cells were in a less energized state compared to glucose-metabolizing cells (Kim et al. 2000a). A 1992 study by Feldmann et al. (1992) was the first to

identify that xylitol formed during xylose metabolism was a significant limiting factor. Besides detracting from ethanol yield, xylitol was further converted to xylitol phosphate, a potent growth inhibitor, by heterologous xylulokinase, a necessary enzyme in xylose metabolism (Akinterinwa and Cirino 2009; Feldmann et al. 1992). Additionally, xylitol was shown as a competitive inhibitor for xylose isomerase isolated from an *Arthrobacter* strain with K_i 0.3 mM (Smith et al. 1991). This was likely to be true for xylose isomerase from *E. coli* (Viitanen et al. 2008). Suppressing xylitol formation, therefore, could lead to improved xylose metabolism. Indeed, as described in a US patent (Viitanen et al. 2008), a strain ZW800 with reduced xylitol formation was generated by knocking out the gene for glucose-fructose oxidoreductase (GFOR), removing one of the mechanisms responsible for xylitol synthesis. The mutant showed significantly improved xylose metabolism, compared to its parental strain. An added advantage for the mutant strain was the ability to tolerate acetate. For example, in a mixed sugar fermentation with 92 g/L glucose and 97 g/L xylose, in the presence of 7.2 g/L acetate and 10mM sorbitol, a majority of xylose was consumed and xylose concentration was reduced to about 30 g/L over a fermentation period of 50 hours, producing about 72.3 g/L ethanol (Viitanen et al. 2008).

In this work, we used an alternative approach, adaptation or adaptive mutation, to improve xylose utilization in a rationally engineered strain. This evolutionary approach has been proven in recent literature as a powerful tool for strain improvement, especially combined with rational metabolic engineering (Fong et al. 2003; Kuyper et al. 2005; Lawford et al. 1998; Lawford et al. 1999; Meijnen et al. 2008; Rosenberg 2001; Viitanen et al. 2008; Zhang et al. 2002). For example, an adapted recombinant *Zymomonas* strain was obtained using continuous culture in a medium containing hardwood hydrolysate (Lawford et al. 1999). In a more recent study, xylose fermentation was significantly improved in an adaptation involving about 40

transfers for *Pseudomonas putida* (Meijnen et al. 2008). This approach is easy to implement and widely applicable as it requires no detailed knowledge of the bottlenecks of the target metabolism. The latter aspect is relevant to xylose fermentation in *Z. mobilis* as even in improved strains, xylose fermentation still lags behind glucose and the reasons for this are not completely clear. A good example for the incomplete picture of xylose fermentation in this organism is a mechanism for xylitol formation. Besides GFOR, a NADPH-dependent xylose reductase was the second enzyme responsible for xylitol formation (Feldmann et al. 1992; Viitanen et al. 2008), yet this enzyme remains elusive. While an adaptation is relatively easy to set up, the specific conditions need to be selected carefully in order to yield strains with desired traits. As previously metabolically engineered strains appeared to have difficulties in dealing with high concentration of xylose, we chose 5% xylose as the sole carbon source in the adaptation experiment. This condition was not previously used in adaptation for *Zymomonas.*

We present here a comparative study of two adapted strains, referred as A1 and A3, respectively. A1 was obtained by transforming ZM4 (ATCC 31821) with an expression vector carrying four *E. coli* xylose metabolic enzymes (Figure 7(B)), followed by a brief adaptation involving only 4 transfers. This brief adaptation was necessary in order to obtain a clone that grew on xylose as sole carbon source. The need for an initial adaptation was reported by others (Viitanen et al. 2008; Zhang et al. 2002). To further improve xylose fermentation, an extended adaptation involving 31 transfers was carried out for about 80 days, resulting in a markedly improved strain, designated as A3. We show that A3 outperforms A1 in xylose consumption rate by several fold, and compares favorably with other reported *Zymomonas* strains in the ability to ferment high concentration of xylose, demonstrating the power of using extended adaptation in

improving engineered strains. Further analysis of the two strains indicates altered xylitol metabolism is, at least partially, responsible for the improvement.

2.3 Materials and Methods

2.3.1 Materials

Molecular biology reagents, such as mini - and maxi - plasmid purification kit, gel DNA recovery kit, Taq DNA polymerase, iProof High-Fidelity DNA Polymerase, PCR primers, restriction enzymes, Quick T4 DNA ligase, SDS-PAGE ready gel, RNA extraction and RT-PCR kits were purchased from commercial sources, including Zymoresearch, Qiagen, Invitrogen, Promega, Stratagene and BioRad. Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich or Fisher Scientific. Unless otherwise indicated, all commercial enzymes required for enzymatic assays were purchased from Sigma-Aldrich.

2.3.2 Organisms and Media

Zymomonas mobilis ZM4 transformants were grown in RM medium (1% yeast extract, 0.2% KH_2PO_4) supplemented with 100 μg/ml chloramphenicol and different amounts of glucose or xylose (as indicated) as carbon source. *Escherichia coli* strains (W3110 and JM109) were grown in Luria-Bertani (LB) medium supplemented with 100 μg/ml ampicillin. Agar plates contained the same media except with additional 1.5% agar.

All concentrations in % are percentages in weight per volume unless otherwise indicated.

2.3.3 Culture Conditions

E. coli cells were grown at 37°C in culture tube or shake flask on a biological shaker shaken at 250 rpm. *Z. mobilis* was grown at 30°C. Pre-seed culture (PSC) and seed culture (SC) of *Z. mobilis* were cultivated in 15-ml centrifuge tube and 100-ml Pyrex screw-cap bottle,

respectively, filled to 60% volume and shaken at 250 rpm. PSC was prepared by inoculating a single colony from an agar plate into the liquid medium. For 5% glucose-5% xylose fermentation, PSC contained 2.5% glucose-2.5% xylose while for 10% xylose fermentation, PSC contained 5% xylose. PSC was grown to the stationary phase. SC was prepared by inoculating it to an optical density (OD) of 0.1 using the stationary phase PSC. SC contained the same amount of sugars as the fermentation media in bioreactors. Appropriate amount of cells were harvested from SC at exponential phase, resuspended in fresh medium and used to inoculate the fermenter to get a starting OD of 0.1. Before the inoculation, fermenter was purged with N_2 gas at 0.2 lpm and pH was adjusted to 5.75 by addition of 1.7M KOH and 1M H_3PO_4. During fermentation, acid was not used for pH control. However, it was observed that the pH in the vessel was in a narrow range of 5.75 to 6.0. To maintain anaerobic conditions, the exhaust tube was immersed in a water column to prevent atmospheric oxygen from diffusing into the fermenter vessels. Dissolved oxygen was monitored and was found to remain close to 0% throughout the fermentation. There was negligible loss of ethanol due to evaporation in this set up. Three replicates were done for each experiment starting from three colonies on an agar plate.

2.3.3.1 Fermenter Setup

The fermenter used (Infors HT Multifors, Switzerland) had 4 vessels each with a working volume of 500 ml (Figure 5). The fermenter was stirred at 300 rpm. Condensers for the fermenter were kept at 1°C using a chiller. Since fermentation by *Z. mobilis* should be done in absence of oxygen, anaerobic conditions in the fermenter are maintained by sparging with N_2 gas as shown in Figure 5. However, the exiting N_2 gas also carries ethanol vapors with it. Besides this, ethanol evaporation also occurs. Both these factors together result in a significant loss of produced ethanol. Since one does not know how much ethanol loss has occurred, this results in

reporting of inaccurate results such as ethanol yield and productivity data. Some researchers have used a GC-MS to measure the ethanol loss (Bandaru et al. 2006). However, a continuous online GC-MS measurement has to be done to find out the total amount of ethanol loss. Some researchers have modeled the rate of ethanol evaporation from the fermenter and applied this correction to their results (Petschacher and Nidetzky 2008). However, even after accounting for the ethanol loss, an important factor still gets overlooked. Due to the loss of ethanol, which is the product, there is mitigation of product inhibition and to get the right measure of the biocatalytic efficiency, this also needs to be accounted for. Hence, the original set up was modified as shown in Figure 6. In the modified set up, a tube was attached to the exhaust, the other end of which was immersed at the bottom of a water column. This set up prevented the diffusion of atmospheric oxygen as it now has to partition into the water phase, diffuse through the water column, again partition into the gas phase and then move through the tube to reach the fermenter vessel. This provides an excellent barrier to O_2 diffusion and thus anaerobic conditions are maintained even without sparging N_2 gas. As N_2 purging is not done, ethanol loss does not occur due to carry over by N_2 gas. Any ethanol evaporation can be recovered in the water column as well. However, during the experiments, it was seen that negligible amount of ethanol was recovered in the water column suggesting that this set up prevents any loss of ethanol.

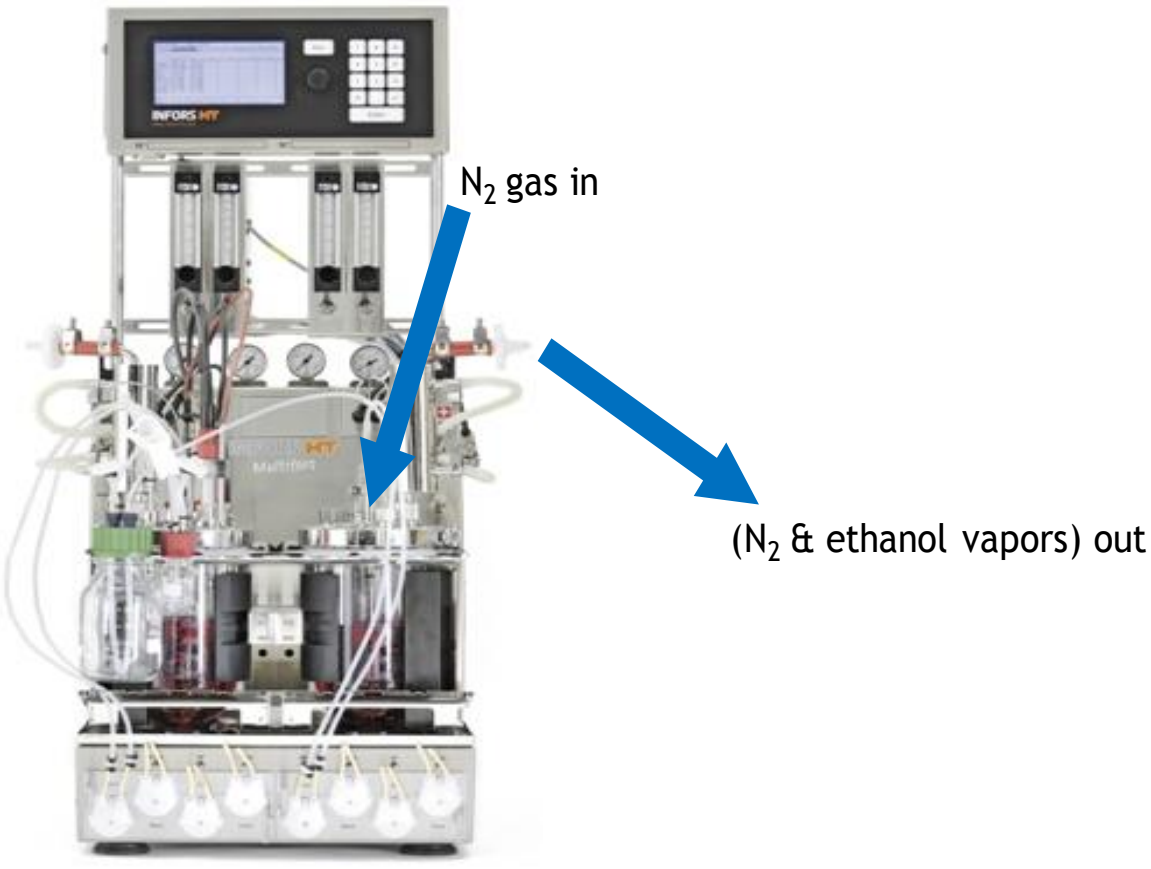

Figure 5: Original fermenter setup commonly used

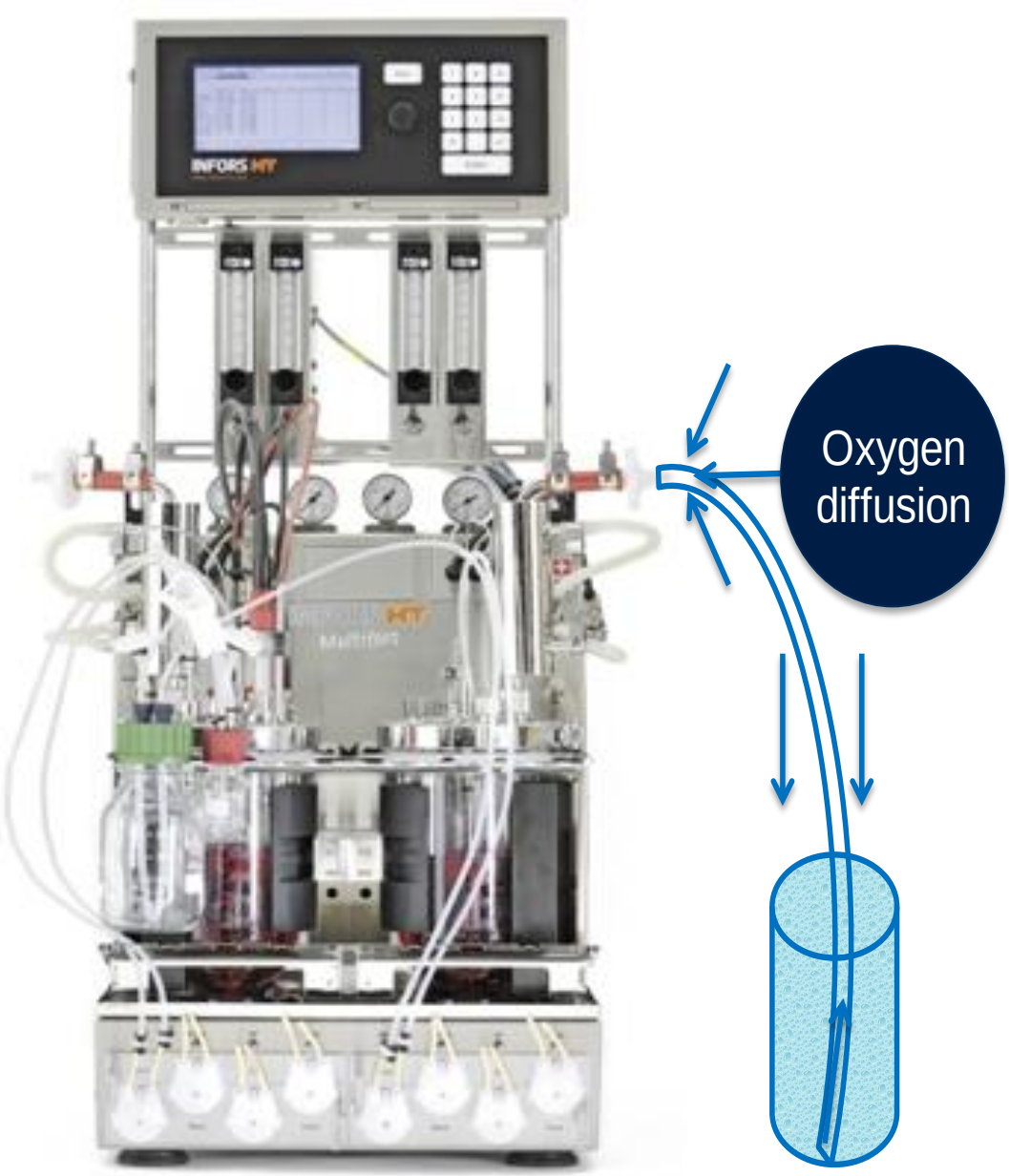

Figure 6: Modified fermenter set up. Water column acts as a barrier for oxygen diffusion

2.3.4 Analytical Methods

Cell growth was determined by measuring optical density (OD) at 600nm. One OD_{600} on our spectrophotometer (Beckman Coulter DU 530) corresponds to 0.4 mg dry cell weight (DCW) of *Z. mobilis* per ml culture broth. Samples taken during fermentation were spun down

by centrifugation and supernatants were analyzed for concentrations of glucose, xylose, lactic acid, glycerol and ethanol using high-performance liquid chromatography (Agilent Technologies) instrument equipped with Supelcogel column H. All concentrations in units of % should be read as percentages in weight per volume unless otherwise mentioned. Overlapping between xylose and xylitol peaks was sometimes observed. Xylitol concentration was determined enzymatically. The reaction was carried out in 42 mM Tris-HCl buffer at pH 8.5, 5 mM NAD, 10 mM $MgSO_4$, 4 U/ml sorbitol dehydrogenase (SDH) (Roche Diagnostics) and 10 µl of xylitol containing sample. Total amount of NADH produced by xylitol containing samples were compared to that produced by xylitol standards for xylitol estimation.

2.3.5 Enzymatic Assays

Sample preparations were executed according to a published procedure (Akinterinwa and Cirino 2009). Briefly cells were harvested by centrifugation at 3,000 g at 4°C for 15 min. Cells were washed once with extraction buffer [10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Washed cell pellets were stored at -20°C until use. Cell pellets were resuspended to an OD_{600} of 50 in the extraction buffer and sonicated (8 cycles of 10 s with 30s cooling period). Cell debris was discarded after centrifugation at 53,300 g for 20 min at 4°C. Supernatant was used as cell-free extract for enzymatic assays. Bradford Assay was used for estimation of protein concentration in the extract.

All enzymatic assay reactions were carried out in a final volume of 200µl on a microplate. NAD(P)H absorbance was measured at 340 nm using Spectramax M5 Plus spectrophotometer (Molecular devices).

Xylose isomerase (XI) activity was assayed in a reaction mixture containing 1 mM triethanolamine (TEA) (pH 7.5), 0.3 mM NADH, 100 mM xylose, 10 mM $MgSO_4$, 4 U/ml SDH (Roche Diagnostics) and 10 µl of crude cell extract (Gao et al. 2002; Schellenberg et al. 1984). Xylulokinase was assayed by a method described in (Akinterinwa and Cirino 2009). A solution containing 87 mM TEA (pH 8.5), 17 mM EDTA, 4.2 mM fructose-6-phosphate (F6P), 0.3 mM NADH, 2.5 U/ml glycerol-3-phosphate dehydrogenase, 10 U/ml triosephosphate isomerase (TPI) and 10 µl crude extract was used for transaldolase assay. Reaction was started by adding 25 µl of 12 mM erythrose-4-phosphate. 42 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$, 1 mM thiamine pyrophosphate (TPP), 0.5 mM ribose-5-phosphate, 0.2 mM NADH, 2.5 U/ml glycerol 3-phosphate dehydrogenase, 10 U/ml TPI and 10 µl crude extract were used for transketolase assay. Reaction was started by adding 20 µl of 5 mM xylulose 5-phophate. Phosphoglucose isomerase (PGI) was assayed in a 50 mM Tris-Cl buffer (at pH 8) containing 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, 3 mM NAD, 2 mM F6P, 1 U/ml glucose-6-phosphate dehydrogenase and 10 µl crude extract. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assayed in a 42 mM Tris-Cl buffer (at pH 8.5) containing 10 mM trisodium phosphate, 1 mM NAD, 3 mM β-mercaptoethanol, 1 mM DL-glyceraldehyde-3-phosphate (GAP) and 10 µl crude extract. This method is similar to that described in (Iddar et al. 2002). Putative xylose reductase was assayed in McIlvaine buffer at pH 7.2 (prepared by adding 16.5 ml of 0.2 M sodium phosphate dibasic to 3.5 ml of 0.1 M citric acid) containing 0.35 mM NADPH, 0.26 M xylose and 5 µl crude cell extract (Viikari and Korhola 1986).

Statistical significance of the enzymatic assay data was determined using Microsoft Excel's t-test: Two-sample assuming equal variance.

2.3.6 Cloning of *E. coli* Xylose Metabolizing Genes into *Z. mobilis*

A plasmid pZMETX was designed and constructed to express four *E. coli* xylose metabolic enzymes, *xylA* (xylose isomerase), *xylB* (xylulokinase), *talB* (transaldolase) and *tktA* (transketolase). The expression was driven by two *Z. mobilis* promoters (Figure 7 (B)).

Promoter-less *xylAB* operon was cloned from *Escherichia coli* K-12 substr. W3110 and fused to the 500bp pyruvate decarboxylate promoter (Ppdc) cloned from *Z. mobilis* ZM4. *talB* and *tktA* (along with its ribosome binding site), were cloned from W3110 in separate steps and fused together after the 500bp enolase promoter (Peno) cloned from ZM4. The two constructed operons – Ppdc-xylA-xylB and Peno-talB-tktA – were then ligated separately into two pGem®Teasy vectors (Promega) and then subcloned into vector pBluescript KS (Stratagene) to obtain pETX. 1.7kb ZM27 replication origin was PCR amplified from ORF1 of 2.7 kb native plasmid pZMO3 of *Z. mobilis* ATCC 10988 (Afendra et al. 1999). ZM27 replication origin enables the plasmid to be maintained and replicated within *Z. mobilis* ZM4 cells. ZM27 was then ligated into commercially available vector pSTV28 (Takara Bio Inc., Japan) which has a chloramphenicol resistance gene and p15A origin of replication to obtain pSTVZM27. pSTVZM27 was electroporated into ZM4 to obtain control strain ZM4/pSTVZM27 using *E. coli* JM109 as an intermediate host. ZM4/pSTVZM27 thus does not contain xylose metabolizing genes. pETX and pSTVZM27 were ligated together to obtain pZMETX (Figure 7 (B)). pZMETX was first transformed into *E. coli* JM109 to yield JM109/pZMETX. Plasmid extracted from JM109/pZMETX was then electroporated into *Z. mobilis* ZM4 to obtain ZM4/pZMETX.

2.3.7 RNA Extraction and RT-PCR

RNA extraction was done using RNeasy Protect Bacteria Mini Kit (Qiagen). Extracted RNA was treated with Ambion® TURBO DNA-*free*™ to get rid of genomic DNA contamination.

Complimentary DNA (cDNA) synthesis was done using SuperScript™ First-Strand Synthesis System (Invitrogen). Quantitative PCR (qPCR) was then carried out for *xylA* and *xylB* genes and housekeeping genes rpoD and 16S rRNA. Brilliant II SYBR Green (Stratagene) was used to track quantitative PCR carried out in Stratagene Mx3005P real time PCR system.

2.4 Results

2.4.1 Drastic Improvement in Xylose Fermentation by Adaptation

To develop a strain capable of fermenting xylose, an expression plasmid, pZMETX, was constructed to introduce four *E. coli* xylose-metabolism genes into *Z. mobilis* ZM4 (Figure 7). Despite successful detection of all four enzymatic activities, however, the transformant of ZM4 was unable to grow on xylose as sole carbon source. This was different from what was reported for similarly engineered strains (De Graaf et al. 1999; Zhang et al. 1995). ZM4/pZMETX was subsequently subjected to a brief adaptation. First, cells were grown in RM medium containing 4% glucose-1% xylose as carbon source and were subsequently subcultured in RM medium with mixed sugars with the ratio of glucose to xylose changed to 2.5%-2.5%, 1%-4%, and finally 5% xylose. The need for an initial adaptation was also reported by others (Viitanen et al. 2008; Zhang et al. 2002). The strain designated as A1 was the first strain obtained from the adaptation process, which was capable of growing on xylose as sole carbon source. However, xylose

metabolism of this strain was sluggish and much slower than what was reported by a similarly engineered strain (Zhang et al. 1995) and much slower than its glucose metabolism.

To improve xylose fermentation, A1 was subjected to an adaptation process by serial dilution and subculturing in RM medium containing 5% xylose as sole carbon source. This process was carried out for 80 days involving 30 serial transfers. Initially, it took the cells approximately 96 hours to reach late exponential phase (LEP) and an OD_{600} of approximately 0.6. As adaptation progressed, a gradual reduction in time taken to reach LEP and an increase in LEP OD_{600} were observed. By the 18^{th} serial transfer, LEP OD_{600} reached at about 1.2 (double the initial value), and no further increase in LEP OD_{600} in subsequent transfers was observed. The adaptive mutation process was terminated after 30^{th} transfer and the adapted strain was designated as A3. The time taken for A3 to reach LEP was about 60 hours, significantly lower than the initial 96 hours, suggesting a significant acceleration in xylose metabolism.

Several experiments were run in bioreactors (see Material and Methods) to characterize the adapted strain A3 and compared to its parental strain A1. In a mixed sugar fermentation containing 5% glucose and 5% xylose (5%G-5%X), a typical diauxie behavior was observed in both strains (Figure 8 (A)). Glucose was consumed first, at an almost identical rate for the two strains. In contrast, the xylose fermentation between the two strains was drastically different. For A3, xylose was nearly consumed at about 32 hours (residual xylose concentration at 0.11%), whereas for A1, the consumption of xylose was not complete even after more than 110 hours (residual xylose concentration at 0.31%). As shown in Table 2, maximum specific rate of xylose consumption by A3 was 1.8 g xylose/g DCW/ h, four times higher than that for A1 (0.45 g/g/h).

Both strains exhibited identical growth curve on glucose, indicating glucose metabolism was not altered by adaptation. A3 was able to maintain the same growth rate upon transition

from glucose to xylose, whereas A1 grew on xylose with much diminished rate. The highest dry cell mass reached was 2.8 mg/ml and 2.0 mg/ml for A3 and A1, respectively (Figure 7 (A)). The difference suggests significant metabolic changes during xylose fermentation. Ethanol yield based on total sugars consumed was slightly higher for A3 (96.6%) than for A1 (93.7%) (Table 2). Other fermentation byproducts were produced but at significantly lower level than ethanol. At the end of fermentation, the total amount of byproducts (lactic acid plus glycerol) produced were 0.2% and 0.15% for A3 and A1, respectively. The amount of xylitol produced by A3 was 0.06%, less than half of the amount produced by A1 (0.13%).

To further illustrate the superior performance by A3, following a mixed sugar fermentation as described above, a second dose of 5%G-5%X was fed to the cells when sugars were nearly exhausted. As shown in Figure 8 (B), A3 readily converted the second dose of sugars to ethanol, producing 9% ethanol with a combined yield of 93.4% from a total of 20% sugars. To our knowledge, this is the highest reported amount of ethanol produced by *Z. mobilis* in a mixed sugar fermentation. During the fermentation of the second dose, however, no significant cell growth was observed yielding only an additional cell mass increase of 0.445 g DCW/liter. Additionally, xylose fermentation was significantly slower than that of the first period while glucose consumption rates remained high. Much more xylitol was produced during the second period, resulting in a final xylitol concentration of 0.26%. These data suggest that the ability of xylose fermentation was deteriorated in the second period, possibly due to accumulation of inhibitive metabolites (xylitol and ethanol) and depletion of nutrients as only sugars were fed in the second dose.

Table 2: Summary of fermentation parameters for A1 and A3

Strain	Initial sugar conc. [% (w/v)]	Max sp. rate of xylose consumption (g/g/h)	Final ethanol titer [% (w/v)]	Ethanol yield (%)[a]	Final xylitol concentration [% (w/v)]
A1	Mixed sugar (5%G-5%X)	0.45 ± 0.05	4.73 ± 0.06	93.7 ± 1.8	0.13 ± 0.01
A3	Mixed sugar (5%G-5%X)	1.80 ± 0.05	4.99 ± 0.06	96.6 ± 4.8	0.06 ± 0.00
A3	Two doses of mixed sugar (5%G-5%X)	ND	9.02 ± 0.15	93.4 ± 2.8	0.27 ± 0.01
A3	10% xylose	3.44 ± 0.25	4.31 ± 0.04	88.2 ± 0.1	0.11 ± 0.00

[a]Calculated based on consumed sugar (as a percentage of theoretical yield).
ND - Not Determined

The superiority of A3 in xylose fermentation was further manifested in its ability to grow in 10% xylose as sole carbon source. As shown in Table 2 and Figure 8 (C), after 46 hours of fermentation, nearly all of 10% xylose was consumed with only 0.33% xylose remaining, and 4.31% ethanol was produced with a yield of 88.2%. The maximum specific rate of xylose utilization was 3.49 g/g/h. In contrast, A1 grew very slowly on 10% xylose and after 46 hours of fermentation, only 1% xylose was consumed. Even after 10 days, residual 3% xylose was measured. The final OD reached by A1 was five times lower than that of A3. These data are indicative of significantly improved ability in fermenting xylose at high concentration in A3. It is important to note that compared to mixed sugar fermentation, using xylose as sole carbon source poses significantly higher stress to cells. The higher the xylose concentration is, the more difficult it is for the cells to complete fermentation. This is due to primarily the accumulation of xylitol (see below sections). Thus, xylose fermentation at high concentration is a better measure for a strain's ability to use xylose than mixed sugar fermentation. Previously, the highest xylose concentration that supports cell growth was ~6% (Kim et al. 2000a; Lawford and Rousseau 1999), and in some cases, the fermentation was incomplete with significant amount of residual xylose (>1% (Lawford and Rousseau 1999)).

Taken together, the fermentation data clearly demonstrate that A3 outperforms A1 in xylose fermentation and its performance compares favorably with strains reported in the literature, illustrating the power of extended adaptation in strain improvement.

Both A1 and A3 were genetically stable as they exhibited the same growth and fermentation characteristics when cultured and tested under the same conditions over a two-year period since their isolation.

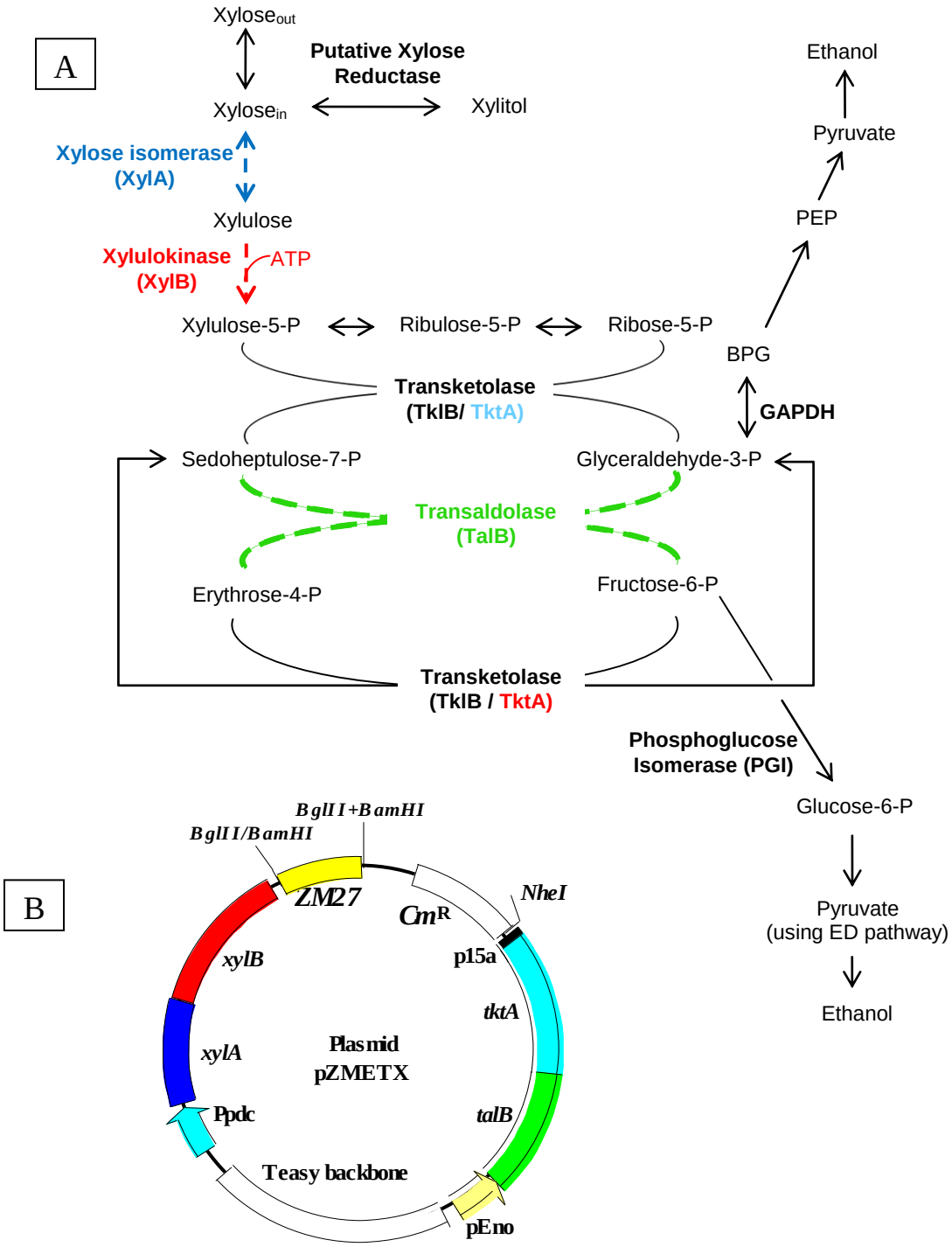

Figure 7: (A) Xylose metabolizing pathway in engineered *Z. mobilis*. Native enzymes (genes) are shown in black. Heterologous enzymes (genes) are shown in other colors. Engineered *Z. mobilis* has two transketolase genes – native *tklB* and heterologous *tktA*. (B) Plasmid map for pZMETX

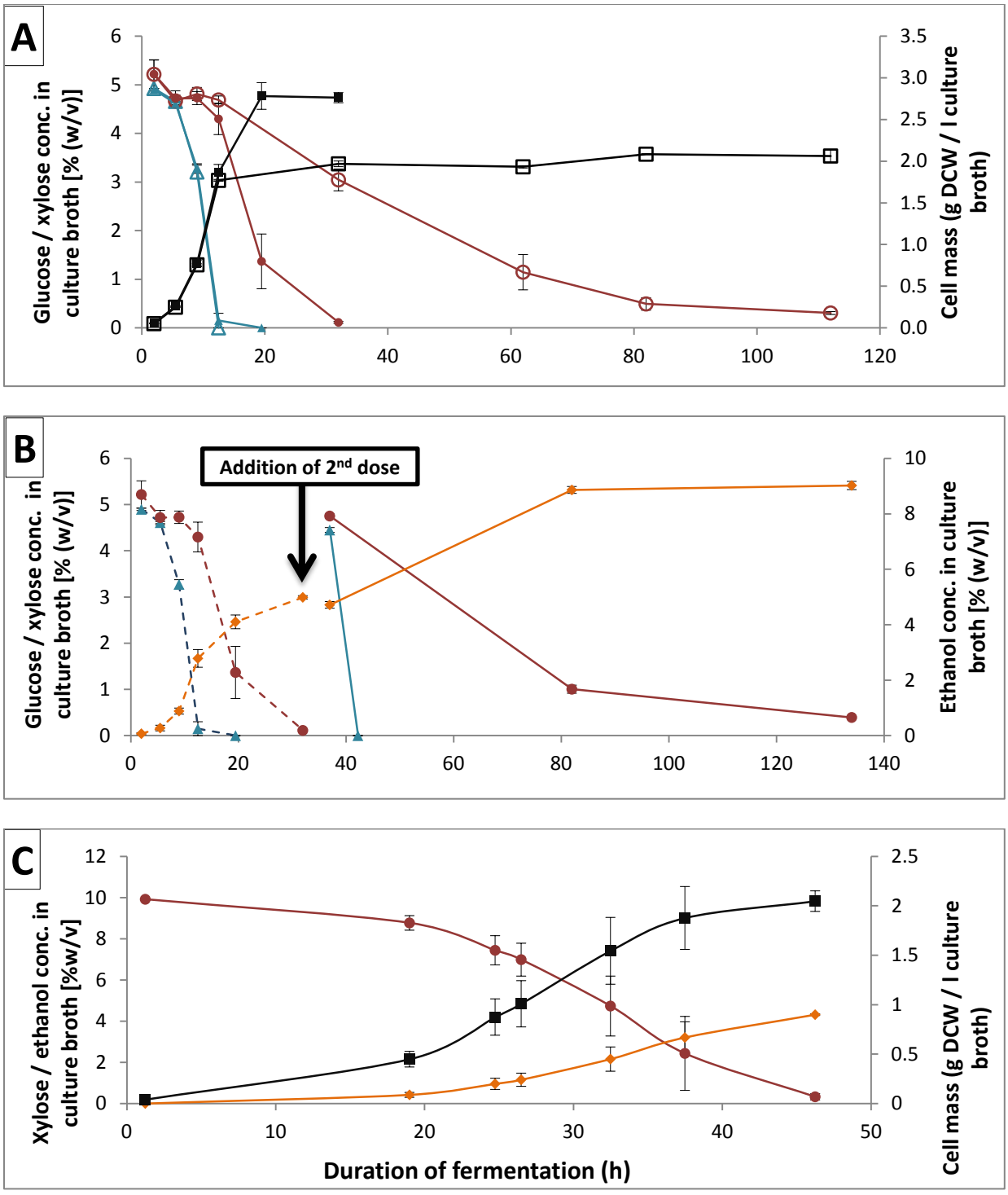

Figure 8: Fermentation profiles of A1 and A3. (A) Mixed sugar fermentation of 5% glucose-5% xylose mixture (B) Fermentation of two doses of mixed sugars (5%G-5%X) by A3. Fermentation of the 1st dose is shown as dashed lines; the arrow indicates the point of addition of the 2nd dose of 5%G-5%X (only sugars). (C) Fermentation of 10% xylose by A3. Black square (■) – cell biomass, blue triangle (▲) – glucose, red circle (●) – xylose, orange diamond (◆) – ethanol. Filled symbols represent A3 while open symbols represent A1

2.4.2 Reduced Xylitol Production and Increased Xylitol Tolerance in A3

The greatly improved xylose fermentation by A3 prompts us to investigate the mechanisms responsible for the metabolic changes. At a metabolite level, it was observed that A3 produced much less xylitol during xylose fermentation. As previously suggested by other researchers (Feldmann et al. 1992; Jeon et al. 2005; Kim et al. 2000a; Scangos and Reiner 1979), xylitol formation and its toxicity may be an important factor. This was investigated as a potential reason for the improvement in A3. Figure 9 shows xylitol formation as a function of residual xylose during a mixed sugar fermentation (5%G-5%X). At the beginning of vigorous xylose fermentation (coinciding with the exhaustion of glucose), xylitol formations were similar for both strains, at ~0.02%. However, during xylose fermentation the difference in xylitol formation between the two strains was significant (Figure 9). This is in spite of a much higher xylose consumption rate for A3 as shown in Figure 8 (A). There was an apparent acceleration of xylitol production in A1 toward the end of the xylose fermentation, which contributed to the widening of the difference in xylitol formation between the two strains. As a result, at the end of the fermentation, twice as much xylitol was produced by A1 as by A3 (0.13% vs. 0.06%). These data suggest that adaptation altered the metabolism to disfavor the formation of xylitol in A3.

Additionally, it was observed that adaptation enhanced xylitol tolerance. In 5% xylose fermentation with exogenous xylitol at a concentration of 0.1%, growth rate was less affected in A3, with a reduction of about 20% (Table 3), compared to a 50% decrease in specific growth rate for A1 under the same condition. The presence of xylitol at this concentration slows down xylose fermentation for both strains, but this is more so for A1 than A3, as indicated by the residual xylose at 42 hours of fermentation (Table 3). Sequence analysis of both xylose

isomerase and xylulokinase genes showed no mutation in their respective genes, ruling out functional mutation in these two enzymes in conferring tolerance.

Taken together, these data suggested that adaptation caused significant metabolic changes that lead to less xylitol formation and better xylitol tolerance in adapted strain A3, two factors crucial for efficient xylose utilization.

Table 3: Effects of exogenous xylitol (0.1%) on xylose fermentation

	Avg[a] specific growth rate (h^{-1})	**Reduction in sp. growth rate for cells cultured in 0.1% xylitol**	**Residual xylose[b] [% (w/v)]**
A1 (0% xylitol)	0.069 ± 0.002	47.8%	1.5 ± 0.2
A1 (0.1% xylitol)	0.036 ± 0.001		3.8 ± 0.1
A3 (0% xylitol)	0.094 ± 0.001	20.2%	0
A3 (0.1% xylitol)	0.075 ± 0.005		1.3 ± 0.1

Cells were grown on 5% (w/v) xylose in 20 ml capped vials. Three replicates were done for each condition.
[a] Averaged value over the first 42 hours of cell growth. Initial OD at ~0.05.
[b] Measured at 42 hours of fermentation.

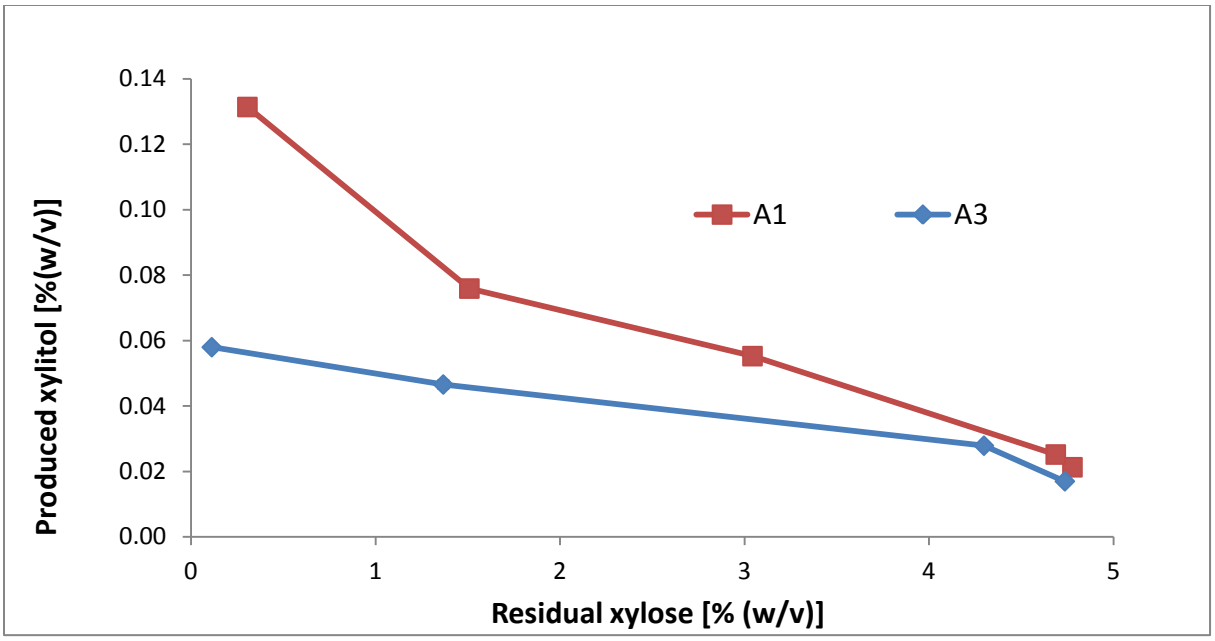

Figure 9: Xylitol produced by adapted strains as a function of residual xylose during mixed sugar (5%G-5%X) fermentation. Three replicates were done for each condition

2.4.3 Higher Xylose Isomerase Activity in A3

Activity assays were carried out for four xylose metabolizing enzymes (xylose isomerase (XI), xylulokinase (XK), transaldolase (Tal) and transketolase (Tkt)) and two enzymes linking the engineered pentose phosphate pathway to glycolysis, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and phosphoglucose isomerase (PGI) Figure 7. Table 4 shows the enzymatic activities measured in cell-free extracts of A1 and A3 under mixed sugar fermentation (5%G-5%X) at different stages of fermentation. Xylose isomerase activity in both strains lags far behind other five enzymes, from 2-3 folds up to 3 orders of magnitude. The significantly low XI activity relative to other heterologous xylose-metabolizing enzymes was previously observed by others (Zhang et al. 2002). It can be argued that based upon the results, XI may represent a metabolic bottleneck.

A comparison of XI activity between the two strains reveals that XI activity in A3 is significantly higher (4 – 8 times) than A1 when fermenting 5%G-5%X mixture (Table 4). Additionally, it appears the difference widens as fermentation progresses (Table 4). For other enzymes measured, the differences between the two strains were not as dramatic as XI (Table 4). The significance of smaller differences in these enzymes may not be as important as XI, as XI activity was the lowest. Apparently, adaptation resulted in an acceleration of the slowest step in xylose fermentation. It is important to note, however, even after the increased XI activity in A3, XI activity remains to be low, as compared to other measured enzyme activities.

Although xylose isomerase inhibition by xylitol was not experimentally shown for XI from *E. coli*, it is thought to be the case based on an *in vitro* study on *Arthrobacter* XI (Smith et al. 1991). As the significant difference in activities between the two strains was observed even when xylitol concentrations were similar (Ph1 and Ph2, Table 4), it is unlikely that the difference in XI activity between the two strains is due to inhibition of XI by xylitol in this case. To identify possible mutations responsible for the increased activity of XI, the recombinant plasmid pZMETX (Figure 7 (B)) in both strains was sequenced. Compared to the plasmid isolated from A1, the only mutation found from the plasmid isolated from A3 was within the Ppdc promoter region. The single base pair mutation, G to A, occurred at -23 bp (23 base pair upstream from the start codon of *xylA*). As the mutation occurred between the major transcription initiation site (-46 bp) and potential ribosome binding site (rbs) (-10 to -7) (Conway et al. 1987a), it is likely to affect the transcriptions of xylA-xylB. However, RT-PCR experiment failed to detect any significant changes for either *xylA* or *xylB* between A1 and A3 (data not shown). It is possible, however, that the mutation may affect some post-transcription events, leading to increased expression of XI. Unfortunately, changes in expression in XI could not be confirmed by SDS-

PAGE since low levels of XI in both A1 and A3 prevented detection by SDS-PAGE. Future studies are needed to clarify the role of the single mutation on its expression and activity.

Table 4: Enzymatic activities[a] (in mU/mg cell protein) in cell-free extracts of A1 and A3 harvested from mixed sugar fermentation (5%G-5%X)

Strain name (time of harvest)	**XI**	**XK**	**Tal**	**Tkt**	**GAPDH**	**PGI**
A1 (Ph 1)	**23 ± 0**	1700 ± 200	610 ± 30	350 ± 70	1200 ± 100	1900 ± 200
A3 (Ph 1)	**105 ± 7**	2300 ± 100	540 ± 40	430 ± 70	1500 ± 200	2400 ± 100
A1 (Ph 2)	**31 ± 1**	1800 ± 200	520 ± 70	280 ± 10	1500 ± 200	3300 ± 200
A3 (Ph 2)	**162 ± 28**	2200 ± 400	700 ± 40	380 ± 70	1800 ± 400	3500 ± 200
A1 (Ph 3)	**26 ± 4**	2300 ± 400	670 ± 70	450 ± 60	2000 ± 100	2900 ± 100
A3 (Ph 3)	**210 ± 26**	2300 ± 500	810 ± 20	440 ± 10	2500 ± 100	4400 ± 200

Cells were harvested during exponential growth on glucose (Ph 1), at the onset of xylose fermentation (Ph 2) and near the exhaustion of all xylose (Ph 3). XI-Xylose isomerase, XK-Xylulokinase, Tal-transaldolase, Tkt – transketolase, GAPDH - Glyceraldehyde-3-phosphate dehydrogenase, PGI - phosphoglucose isomerase.

[a]Three replicates were done for each condition. XI assay data for A1 and A3 significantly different ($p < 0.05$). For remaining enzymes, assay data not significantly different for A1 and A3 except for Ph 3 data for GAPDH & PGI.

2.5 Discussion

Although the feasibility of lignocellulosic ethanol has long been demonstrated, developing cost-effective technology to realize the potential on a large scale has been a challenge. In this work, we sought to improve ethanol production by enhancing the ability of biocatalyst to use xylose. Specifically, the ability to ferment xylose at high concentrations and reducing fermentation time are important engineering targets as they impact productivity and downstream processing. As demonstrated here, through an adaptation process, a strain (A3) with significantly improved traits was obtained. First the xylose consumption rate in A3 was significantly increased compared to its parental strain, A1, resulting in significantly reduced fermentation time in mixed sugar (5%G-5%X) fermentation, from over 110 hours to about 35 hours. Additionally, the improved xylose fermentation was manifested by its ability to ferment high concentration of xylose. In fact, A3 was able to grow with 10% xylose as sole carbon source, which was the highest concentration ever reported. The improved xylose fermentation and the ability to use high concentration of xylose can be used to achieve high product concentration. As demonstrated here, fermenting two doses of 5%G-5%X by A3 allowed ethanol to be accumulated to 9%, the highest ever shown for this organism in mixed sugar fermentation. The combined benefits of reduced fermentation time and increased ethanol concentration should generate a cost advantage for bioethanol processes employing A3.

Altered xylitol metabolism was responsible, at least partially, for the improvement. At least two pathways were implicated for xylitol formation in *Z mobilis*. The first is analogous to eukaryotic aldose reductase (Feldmann et al. 1992). The second is catalyzed by the glucose-fructose oxidoreductase (GFOR) (Viitanen et al. 2008; Zhang et al. 2009). Both enzymes are NADPH dependent but the cofactor in GFOR is tightly bound to the enzyme. An analysis of the

whole cell extract for the aldose reductase activity showed that in both A1 and A3, the aldose reductase activity was barely detectable, whereas the wild type strain ZM4 carrying an empty plasmid (without the xylose-metabolism heterologous genes) gave an activity of 22 mU/mg-proteins. The xylitol formation for the control was also 3 times higher at 0.06% than both A1 and A3. This suggests that adaptation cause a reduction of aldose reductase activity in both strains, which is at least partially responsible for the reduction of the xylitol formation in A1 and A3, although this does not explain the difference between A1 and A3. In light of a DuPont patent (Viitanen et al. 2008), the residual xylitol formation may be due to GFOR. However, we found no mutation in GFOR gene and its promoter region in either strain. Therefore, while evidence pointed to reduced xylitol formation in A3 as key for improvement, further studies are needed to clarify the role of various mechanisms in xylitol formation. It is possible that other chromosomal mutations contribute to the different rates of xylitol formation between the two strains. It is also possible that A3 could benefit from other chromosomal mutations unrelated to xylitol synthesis.

The work presented here illustrates the power of adaptation in strain improvement. While selection pressure applied in the adaptation is high concentration of xylose (5%), not xylitol, xylitol tolerance and reduced xylitol formation were selected. In addition, the increased XI activity favors the reduction of xylitol effects, by channeling more xylose to ethanol instead of xylitol. This further confirms what has been suggested by other researchers that xylitol metabolism is the key to efficient use of xylose in this organism. This study is thus a good example to demonstrate that adaptation not only is useful in improving strains but also could be equally well used to pinpoint bottlenecks in metabolically engineered strains. Identified targets from adaptation could be further improved through metabolic engineering. In this case, xylitol formation could be further reduced by knockout genes for GFOR and aldose reductase once

identified. Additionally, further increase of xylose isomerase expression will also be helpful in reducing xylitol formation. Furthermore, even in GFOR knockout strains, or reduced aldose reductase strains, xylitol formation was not eliminated. This may suggest a redox imbalance that could be addressed by metabolic engineering. Therefore, adaptation and metabolic engineering could be used synergistically in developing industrially useful strains.

CHAPTER 3

DISCOVERY AND CHARACTERIZATION OF AN ALDO-KETO REDUCTASE OF *Z. MOBILIS* ZM4

Portions of this chapter have been reproduced from (Agrawal and Chen 2011a).

3.1 Abstract

Although metabolic engineering has generated several *Zymomonas mobilis* strains capable of fermenting xylose, xylose utilization lags far behind glucose even in the best performing strains. Xylitol formed during xylose metabolism was suggested, a decade ago, as a major culprit, yet the postulated xylose reductase remains elusive. We report here the discovery of xylose reductase (XR) in *Zymomonas mobilis* ZM4 and its recombinant expression and characterization. Based on the phenotypic differences and homology search to known fungal xylose reductases, we identified an open reading frame, ZMO0976, as a likely XR. Subsequent analysis confirmed that ZMO0976 indeed encodes a xylose reductase. Interestingly, besides xylose and xylulose, the enzyme was found to be active towards benzaldehyde, furfural, 5-hydroxymethyl furfural (HMF), and acetaldehyde, but not glucose or fructose even at high concentration. Furthermore, benzaldehyde and furfural are much better substrates than xylose for the enzyme, with benzaldehyde exhibiting nearly 150 times higher affinity than xylose. The enzyme accepts both NADH and NADPH as cofactors but prefers the latter. The discovery of xylose reductase opens up possibilities to further improve xylose fermenting *Z. mobilis* strains. The activity of the enzyme to reduce furfural and HMF could be potentially used for

detoxification of lignocellulosic hydrolysate or improve microbial tolerance to these two major biomass derived inhibitors.

3.2 Introduction

Zymomonas mobilis, owing to its superior glucose fermenting capabilities, has received considerable scientific and commercial interests as a potential microbe for converting lignocellulose to ethanol (Panesar et al. 2006; Rogers P L 1982; Swings and De Ley 1977). However, wild-type *Z. mobilis* strains have a narrow range of substrates and can only utilize glucose, fructose, and sucrose. As xylose is the second major component in lignocellulose (Jeffries and Jin 2000), it is necessary to engineer microbial catalysts to use xylose. Over a decade ago, Zhang *et al* successfully constructed xylose-fermenting strains of *Z. mobilis* (Zhang et al. 1995). These strains were subsequently improved by integrating the xylose-metabolic genes into chromosome (Zhang and Chou 2008). However, despite intensive efforts, xylose fermentation in engineered strains still lags far behind glucose utilization. Formation of xylitol during xylose fermentation was widely recognized as one of the reasons (Feldmann et al. 1992; Jeon et al. 2005; Kim et al. 2000a; Scangos and Reiner 1979). At least two mechanisms are responsible for the formation of xylitol from xylose. First, glucose-fructose oxidoreductase (GFOR) was shown to accept xylose as a electron donor and reduce xylulose to xylitol (Viitanen et al. 2008; Zachariou and Scopes 1986). Additionally, a xylose reductase (XR) was postulated to be responsible for the conversion of xylose to xylitol, based on the cell extract activity using NADPH as electron donor (Feldmann et al. 1992). However, the enzyme was never isolated and the gene association was not identified. Thus the XR remains elusive (Viitanen et al. 2008; Zhang et al. 2009).

We report here the discovery and characterization of a xylose reductase from *Z. mobilis* ZM4. In the process of improving engineered *Zymomonas* strains for xylose fermentation, we isolated an adapted strain that produced much less xylitol than the wild type strain. The reduced xylitol production coincided with a much decreased XR activity in cell extract. Subsequent sequencing analysis showed a single base mutation within the gene ZMO0976, annotated as a putative aldose reductase. Further studies with the recombinant ZMO0976 confirmed its xylose reductase activity. Besides xylose, the enzyme ZMO0976 is active towards benzaldehyde, furfural, acetaldehyde, 5-hydroxymethylfurfural (HMF), and xylulose but not towards glucose and fructose.

3.3 Materials and Methods

3.3.1 Materials

Molecular biology reagents, such as mini - plasmid purification kit, gel DNA recovery kit, Taq DNA polymerase, iProof High-Fidelity DNA Polymerase, PCR primers, restriction enzymes, Quick T4 DNA ligase and SDS-PAGE ready gel were purchased from commercial sources, including Zymoresearch, Qiagen, Invitrogen, Promega, and BioRad. All chemicals used in this study were obtained from Fisher Scientific, Hampton, NH unless otherwise indicated.

3.3.2 Strains

Zymomonas mobilis ZM4 and A3 were used in this study. A3 was obtained by adaptation of a metabolically engineered *Z. mobilis* ZM4/pZMETX on 5% xylose. Details on adaptation were described in (Agrawal et al. 2011). *Escherichia coli* UT5600 (a K-12 strain) was used as host strain for expressing recombinant xylose reductase.

3.3.3 Cloning and Heterologous Expression of ZMO0976

ZMO0976 gene and its mutated form (designated mZMO0976) were cloned from *Z. mobilis* ZM4 and an adapted strain A3, respectively. The 5' – primer (5'-GCTATTGAC***GGTACC***ATGAACACTTCTACGCAAAAACC-3') contains a KpnI site (in bold italics) and a start codon (underlined). The 3' – primer (5'-TATTCGTACT***AAGCTT***TTATTTATCGCGTGGCGGGGGTG-3') contains a HindIII site (in bold italics) and a stop codon (underlined). The cloned gene was ligated into the commercially available high copy number plasmid pQE80L (QIAGEN) at restriction sites KpnI and HindIII. Resultant plasmids were designated as pQEZM976 (containing ZMO0976) and pQEmZM976* (containing mZMO0976) (Figure 10). Both plasmids contained in-frame N-terminal histidine $(His)_6$-tag before the start codon of the gene and IPTG (Isopropyl β-D-1-thiogalactopyranoside) - inducible T5 promoter for transcription of the gene. These plasmids were transformed into *E. coli* UT5600 to construct UT5600/pQEZM976 and UT5600/pQEmZM976*, respectively. A control strain UT5600/pQE80L was constructed by transforming UT5600 with the empty vector.

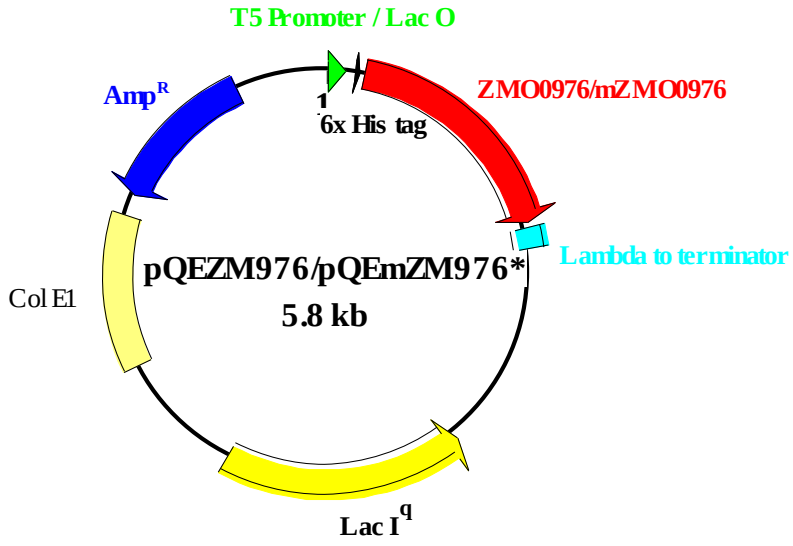

Figure 10: Plasmid map for pQEZM976 or pQEA976m

3.3.4 Culture Conditions

E. coli cells were grown Luria-Bertani (LB) medium supplemented with 100 µg/ml ampicillin at 37°C in culture tube or shake flask on a biological shaker shaken at 250 rpm. Cells were induced with 0.5 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) (Sigma-Aldrich, St. Louis, MO) at an OD of 0.4 – 0.6. Cells were incubated for four additional hours at reduced temperature of 30°C and then harvested for subsequent analysis.

All concentrations in % are percentages in weight per volume unless otherwise indicated.

Zymomonas mobilis cells were grown at 30°C in RM medium (1% yeast extract, 0.2% KH_2PO_4) containing different amounts of glucose or xylose (as indicated) as carbon source. Plasmid containing cells were grown in RM medium supplemented with 100 µg/ml chloramphenicol.

Cell culture conditions are described in (Agrawal et al. 2011). Briefly, pre-seed culture (PSC) and seed culture (SC) of *Z. mobilis* were cultivated in 15-ml centrifuge tube and 100-ml Pyrex screw-cap bottle, respectively, filled to 60% volume and shaken at 250 rpm. PSC was prepared by inoculating a single colony from agar plate into the liquid medium containing 2.5% glucose and 2.5% xylose. PSC was grown to the stationary phase. SC was prepared by inoculating it to an optical density (OD) of 0.1 using the stationary phase PSC. SC and fermentation media in bioreactors contained 5% glucose and 5% xylose (5%G-5%X). Appropriate amount of cells were harvested from SC at exponential phase, resuspended in fresh medium and were used to inoculate the bioreactor to get a starting OD of 0.1. The fermenter (Infors HT Multifors, Bottmingen, Switzerland) was stirred at 300 rpm, with minimum pH held at 5.75 by automatic addition of 1.7M KOH. N_2 purging was not done. To maintain anaerobic conditions, the exhaust tube was immersed in a water column to prevent atmospheric oxygen from diffusing into the fermenter vessels. Dissolved oxygen was monitored and was found to remain close to 0% throughout the fermentation.

Three replicates were done for each experiment starting from three colonies on an agar plate.

3.3.5 Preparation of Cell-Free Extract and Protein purification

E. coli cells were prepared according to a published procedure (Akinterinwa and Cirino 2009). Briefly cells were harvested by centrifugation at 5,000 g at 4°C for 30 min. Cells were washed twice with ice-cold extraction buffer [10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Washed cell pellets were stored at -20°C until use. Cell pellets were resuspended to an OD_{600} of 50 in the equilibration buffer (50 mM Na_2HPO_4, 300 mM NaCl and 10 mM imidazole at pH 8)

supplemented with 1 mM DTT and 1 mM PMSF and sonicated (8 cycles of 10 s with 30s cooling period). Cell debris was discarded after centrifugation at 16,000 g for 20 min at 4°C. Supernatant was used as cell-free extract (CFE) for enzymatic assays. Bradford Assay was used for estimation of protein concentration in the extract.

His-tagged protein in the cell-free extract was purified at 4°C using HIS-Select® HF Nickel Affinity Gel (Sigma-Aldrich, St. Louis, MO) which employs immobilized metal-ion affinity chromatography (IMAC). Manufacturer's protocol for large scale purification was followed. Briefly, 10 ml of cell-free extract was added to 1 ml of affinity gel in equilibration buffer. After overnight incubation, the gel was washed five times with equilibration buffer. The bound his-tagged protein was then eluted using 2 ml of elution buffer (50 mM Na_2HPO_4, 300 mM NaCl and 250 mM imidazole at pH 8). Imidazole was removed by overnight dialysis at 4°C using extraction buffer as the dialysis solution.

Cell-free extract for *Z. mobilis* was prepared as above except that extraction buffer was used for sonication and after which, cell debris were spun down at higher centrifugal force (53,300 g).

3.3.6 Xylose Reductase Assay

All enzymatic assay reactions were carried out in a final volume of 200 µl on a microplate. NAD(P)H (Sigma-Aldrich, St. Louis, MO) absorbance was measured at 340 nm using Spectramax M5 Plus spectrophotometer (Molecular devices, Sunnyvale, CA).

Xylose reductase was assayed in McIlvaine buffer at pH 7.2 (prepared by adding 16.5 ml of 0.2 M Na_2HPO_4 (sodium phosphate dibasic) to 3.5 ml of 0.1 M citric acid) containing 0.35 mM NADPH or NADH (as indicated) and an appropriate volume of sample (cell-free extract or purified protein) (Viikari and Korhola 1986). Xylose concentration in the assay mixture is

indicated in the text. For measuring the catalytic activity of xylose reductase towards other substrates, similar assay conditions were used. K_m and V_{max} were determined by varying the substrate concentrations (Benzaldehyde: 0.25 – 2.00 mM, furfural: 0.5 – 10.0 mM, xylose: 0.10 – 0.26 M) under constant concentrations of NADPH (0.35 mM) and purified protein (2 µg/200 µl reaction) in the assay mixture and fitting the data to Lineweaver-Burk equation: $1/v = (K_m/V_{max})[S] + 1/V_{max}$.

For product identification, enzymatic reactions were carried out in a final volume of 400 µl in an eppendorf tube incubated in a thermomixer. The reaction conditions were similar to those for microplate assay except that a high concentration of NADPH (15 mM) was used to produce sufficient amount of for identification using high-performance liquid chromatography (HPLC). Trichloroacetic acid (TCA) was added to a final concentration of 10% to stop the reaction.

3.3.7 Analytical Methods

Cell growth was determined by measuring optical density (OD) at 600nm using spectrophotometer (Beckman Coulter DU 530, Brea, CA). Xylitol concentration was determined enzymatically. The reaction was carried out with a total volume of 200µl in 42 mM Tris-HCl buffer at pH 8.5, 5 mM NAD (Sigma), 10 mM $MgSO_4$, 4 U/ml sorbitol dehydrogenase (Roche Diagnostics, Indianapolis, IN) and 10 µl of sample. Xylitol concentration was estimated based on the amount of NADH produced by xylitol containing samples compared to that produced by xylitol standards. The formation of xylitol by xylose reductase was confirmed by HPLC (Agilent Technologies) instrument equipped with an Aminex HPX-87H column (Bio-Rad). 5 mM H_2SO_4 at a flow rate of 0.4 ml/min was used as the mobile phase. SDS-PAGE and Coomassie blue staining were used to confirm the expression of ZMO0976 and mZMO0976 proteins. 15 µg of

cell-free extract protein and 1 µg of purified protein were loaded on a 12% Tris-HCl gel (Bio-Rad, Hercules, CA).

3.4 Results

3.4.1 Discovery of Xylose Reductase in *Zymomonas mobilis* ZM4

Adaptation by serial dilution was used to improve xylose fermentation in metabolically engineered strains of *Z. mobilis* (Agrawal et al. 2011). As a result, a strain, referred to as A3, with markedly improved xylose metabolism was obtained. A3 produced less xylitol than the control strain ZM4/pSTVZM27 (wild type ZM4 carrying an empty plasmid) when fermenting a 5% (w/v) glucose – 5% (w/v) xylose mixture (Agrawal et al. 2011). Enzymatic assay for xylose reductase (XR) in cell-free extracts gave an activity of 22 mU/mg protein for the control strain, comparable to a previous report by Feldmann *et al* for *Z. mobilis* strain CP4 (Feldmann et al. 1992), whereas no activity could be detected in the cell extract of A3. Thus, the absence of XR activity in the adapted strain A3 suggested that the xylose reductase(s) may have been mutated.

Using the NCBI's sequence analysis program tblastn, the amino acid sequences of characterized and putative XRs from several fungi (including *Aspergillus niger* (Prathumpai et al. 2005), *Candida guilliermondii* (Handumrongkul et al. 1998), *Kluyveromyces lactis* (Billard et al. 1995)) were aligned against the *Z. mobilis* ZM4 genome. One gene, ZMO0976, annotated as aldose reductase, showed significant sequence identity (22% - 31%) to these fungal XRs. An example of protein sequence alignment of ZMO0976 to a characterized fungal xylose reductase is shown in Figure 11. Subsequent cloning and sequencing of the ZMO0976 gene from both the wild-type strain ZM4 and adapted strain A3 were carried out. A comparison of sequence showed a single base pair mutation, C874T, for the gene cloned from A3, which resulted in a single mutation from arginine to cysteine.

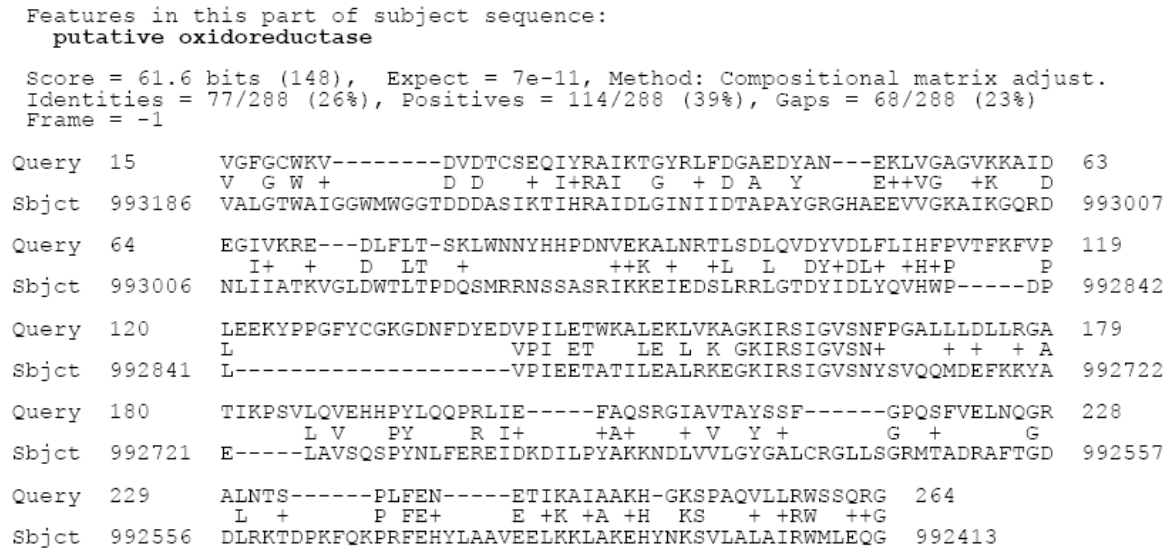

Figure 11: Protein sequence alignment of ZMO0976 to xylose reductase of *Pichia stipitis* (GenBank Accession # CAA42072.1)

To facilitate further studies of the function of ZMO0976 and to assess the effect of the single amino acid mutation, both wild type ZMO0976 gene and the mutated form, mZMO0976 (GenBank Accession # HQ890327), were expressed in *Escherichia coli* UT5600. Cell-free extracts of both strains – UT5600/pQEZM976 and UT5600/pQEmZM976* - along with the control strain UT5600/pQE80L, were tested for NADPH-dependent xylose reductase activity. As shown in Table 5, the XR activity in the CFE for cells expressing ZMO0976 was high, 460 mU/mg protein, whereas cells expressing mZMO0976 exhibited a much diminished activity of 8 mU/mg protein. As expected, no activity was detected from cell extract of the control, UT5600/pQE80L.

The native and mutated forms of the recombinant enzyme were purified based on the N-terminal His-tag. The purified proteins ZMO0976 and mZMO0976 had an expected molecular weight of ~38 kDa as observed on SDS-PAGE (figure not shown). XR activities of the purified

proteins are 3400 and 140 mU/mg for ZMO0976 and mZMO0976, respectively. Thus, the single mutation in mZMO0976 caused a significant reduction of XR activity.

Table 5: Xylose reductase activity of purified protein and cell-free extract (CFE)

Samples	**Activity (mU/mg protein)**
UT5600 expressing ZMO0976 (CFE)	460 ± 50
UT5600 expressing mZMO0976 (CFE)	8 ± 2
Purified recombinant ZMO0976	3400 ± 200
Purified recombinant mZMO0976	140 ± 50

Xylose concentration in the assay mixture was 260mM.

To confirm xylitol as the product from xylose in a XR-catalyzed reaction, reaction mixtures were analyzed by an HPLC method. As shown in Figure 12, a new peak emerged at 2 hr at a retention time coinciding with the xylitol standard, indicating the product from the enzymatic reaction is indeed xylitol. The xylitol peak is relatively small as compared to xylose. This is due to the low cofactor NADPH concentration used (15 mM). Xylitol concentration from purified XR was estimated to be 0.18% (or 11.8 mM) at the end of a two-hour reaction. Similarly, whole-cell catalyzed reactions using transformant (UT5600/pQEZM976) were also analyzed by HPLC. Xylitol was detected at the same retention time as the standard whereas cells containing only the empty plasmid did not generate a xylitol peak (data not shown).

Altogether, these data indicate that ZMO0976 encodes the elusive xylose reductase that contributes to xylitol formation in *Zymomonas mobilis*.

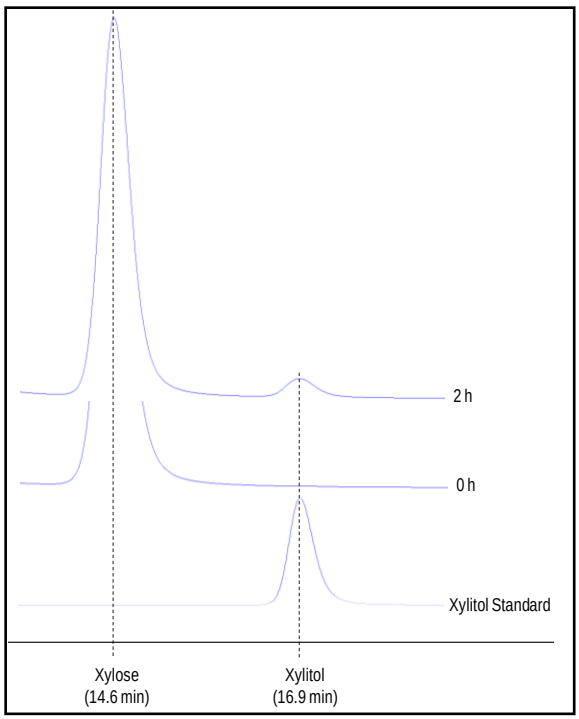

Figure 12: HPLC chromatograms of a reaction containing 260 mM xylose catalyzed by purified ZMO0976. From top to bottom - chromatogram for samples taken at 2 h, 0 h and xylitol standard

3.4.2 Cofactor Requirement and Substrate Specificity of ZMO0976

As an early study by Feldmann et al. (1992) suggested XR activity was NADPH dependent, our initial experiments used NADPH as the cofactor. Subsequently, NADH was tested with purified ZMO0976 and 260 mM xylose. The results showed the enzyme could accept NADH as cofactor but with reduced efficiency. The activity measured with NADH was at least one order of magnitude lower than that with NADPH (Table 6). For this reason, all subsequent studies and thus all data presented here used NADPH as cofactor.

Table 6: Fold reduction in ZMO0976 activity with NADH as cofactor compared to NADPH as cofactor

Furfural (10mM)	10
Acetaldehyde (260mM)	53
Xylose (260mM)	18

0.35 mM of either cofactors were used in the assay mixtures. Substrate concentration in assay mixture is shown within parentheses.

Early in our study, we observed that a high concentration of xylose (such as 260 mM) was necessary for the detection of XR activity. This prompted us to test other sugars and other molecules as possible substrates for the enzyme. Of all the substrates tested, the highest affinity was found with benzaldehyde. Activity was obvious with only 2 mM of benzaldehyde whereas with xylose, it was evident only at concentration as high as 260 mM, suggesting that benzaldehyde was a much better substrate than xylose. The enzyme was also active toward acetaldehyde but required a significantly higher concentration, 260 mM, compared to 2 mM with benzaldehyde. No activity toward glucose and fructose was observed even when similarly high concentration (260 mM) was used. Interestingly, furfural and 5-hydroxymethylfurfural (HMF) were both good substrates for the enzyme. A comparison of activities on various substrates, relative to benzaldehyde, is shown in Table 7.

Table 7: Aldo-keto reductase activities of recombinant ZMO0976 with different substrates

Substrate	**Concentration (mM)***	**Relative activity**
Benzaldehyde	2	1
Acetaldehyde	260	0.62
Furfural	5.2	0.81
HMF	10	0.11
Xylose	260	0.83
Xylulose	260	0.49
Glucose	260	0
Fructose	260	0

*Concentration of the substrate in assay mixture.
Enzymatic activity of 4030 ± 250 mU/mg protein was observed with 2 mM benzaldehyde

To evaluate the single amino acid mutation on the enzyme activity toward substrates other than xylose, the mutated form of ZMO0976 was also purified and tested against furfural, benzaldehyde and acetaldehyde. As shown in Table 8, activity of mZMO0976 is only 1% - 7% of that of the wild type, indicating, as with xylose, a drastic impact of the single mutation on the activity. This effect was apparently independent of the substrates.

Table 8: Specific aldo-keto reductase activities (mU/mg protein) of ZMO0976 and mZMO0976

	Furfural (10mM)	**Benzaldehyde (2mM)**	**Acetaldehyde (520 mM)**
ZMO0976	5470 ± 60	4030 ± 250	2500 ± 400
mZMO0976	180 ± 30	40 ± 10	170 ± 30

Substrate concentration in assay mixture is shown within parentheses.

For three representative substrates, xylose, benzaldehyde, and furfural, respective Michaelis-Menten kinetics parameters were determined. For xylose, K_m and V_{max} were 258 mM and 6.9 U/mg protein, respectively. The high K_m value is consistent with the early observation that detectable activity requires a high concentration of xylose. The K_m for benzaldehyde was 1.77 mM, about 150 fold lower than xylose. The K_m for furfural, 4.15 mM, was also much lower than that of xylose (Table 9). These data indicate that ZMO0976 is more active on benzaldehyde and furfural than xylose.

Table 9: Apparent K_m and V_{max} of ZMO0976 for benzaldehyde, furfural and xylose in presence of 0.35mM NADPH

	Benzaldehyde	**Furfural**	**Xylose**
K_m (mM)	1.77 ± 0.11	4.15 ± 0.18	258 ± 43
V_{max} (mU/mg protein)	7200 ± 200	5000 ± 1300	6900 ± 1200

3.5 Discussion

The existence of a xylose reductase in *Zymomonas mobilis* was suggested as early as 1992 by Feldmann et al. NADPH-dependent XR activities in the cell extract were shown as the

indication of the activity that correlated with xylitol formation during xylose fermentation (Feldmann et al. 1992). However, the gene encoding for the enzyme was never found. In this study, the phenotypic difference exhibited by an adapted strain A3 and its parental strain ZM4 in xylitol formation was used to identify the elusive xylose reductase. The sequenced *Z. mobilis* genome (Seo et al. 2004) was searched for candidates based on homology to known eukaryotic XRs. Sequencing analysis of the gene isolated from A3 indicated one single amino acid substitution in ZMO0976, which apparently was responsible for the reduction of cell extract XR activity and about three fold reduction in xylitol formation. Subsequent characterization after recombinant expression provided further evidence supporting the notion that the ZMO0976 indeed encodes a xylose reductase.

Besides xylose and xylulose, ZMO0976 readily reduces aromatic aldehydes. In fact, benzaldehyde and furfural are much better substrates than xylose. The affinity to these two aromatic aldehydes is one to two orders of magnitude higher than that of xylose. Intriguingly, neither glucose nor fructose, the two sugars that the microorganism ferments naturally, is a substrate for the enzyme. This raises an interesting question about its true physiological substrate as the microorganism is not known to use xylose or aromatic aldehydes. There has been only one report of a bacterial furfural reductase, but it does not have any activity toward xylose (Gutierrez et al. 2006). Therefore the enzyme discovered from this work appears to be novel.

As shown in this study, cells with reduced XR activities produced less xylitol, suggesting XR is one of the major routes of xylitol synthesis. It is conceivable that xylose metabolism may be benefited by eliminating the XR activity in its entirety. This work, by identifying the XR gene, paves the way for further improvement of xylose fermentation in *Zymomonas* through

metabolic engineering. For example, if the XR gene can be deleted, along with the GFOR gene (Viitanen et al. 2008), xylitol formation could be reduced further and xylose fermentation could be further improved without the impedance of the toxic byproduct. In addition, the increased availability of NADPH cofactor may result in higher biosynthetic activity promoting faster cell growth (Miller et al. 2010).

The finding that ZMO0976 reduced furfural and HMF is of significance from a very different perspective. The enzyme potentially provides a detoxification mechanism for cells fermenting lignocellulose for production of ethanol and other products. Furfural and HMF are produced during hydrolysis of lignocellulosic biomass and are potent inhibitors of microbial growth (Gutierrez et al. 2006; Palmqvist and Hahn-Hagerdal 2000). A pre-fermentation step employing recombinant ZMO0976 could be envisioned to reduce both furfural and HMF to concentrations tolerable to a fermenting microorganism in the subsequent process. Alternatively, a microbial strain (not necessarily *Z. mobilis*) could be engineered to overexpress the ZMO0976, thus endowing cells the ability to better tolerate these two biomass derived inhibitors. The activity with benzaldehyde may also be of interest as the enzyme was suggested to be involved in lignin degradation (Muheim et al 1991).

CHAPTER 4

ADAPTATION INSPIRED ENGINEERING OF *ZYMOMONAS MOBILIS* FOR XYLOSE FERMENTATION IN PRESENCE OF HIGH CONCENTRATION OF ACETIC ACID INHIBITOR

4.1 Abstract

Metabolic engineering efforts are being continuously made to improve the fermentation of lignocellulosic hydrolysates by *Zymomonas mobilis*. One focus of these efforts is to increase the efficiency of xylose fermentation and make it at par with glucose fermentation. We previously reported a highly efficient xylose-fermenting *Zymomonas mobilis* strain obtained by adaptation (Agrawal et al. 2011). Genetic analysis of the adapted strains identified one chromosomal mutation in ZMO0976 encoding a xylose reductase (XR), and one single base mutation on the plasmid in the promoter region for heterologous expression of xylose metabolism genes, resulting in lower xylitol formation and higher xylose isomerase activities than the control, respectively. Accordingly, in this study, we modified the genetic make-up of wild-type strain *Z. mobilis* ZM4 to rationally construct a strain similar to the adapted strain. This process re-confirmed the importance of the identified genetic mutations towards improvement in xylose fermentation. Among the genetic modifications done were – (a) the knockout of an aldo-keto reductase ZMO0976 and (b) electroporation of mutated plasmid (A3-pZMETX*) carrying the xylose metabolic genes. We demonstrated that knockout of ZMO0976 results in a lower xylitol production that enables the establishment of xylose fermentation. The single mutation in the promoter of xylose isomerase gene carried by A3-pZMETX* results in acceleration of xylose

fermentation accompanied by a lower xylitol production. Additionally, similar genetic modifications were carried out in an acetate resistant *Z. mobilis* strain. The strain was able to ferment all the glucose and nearly 70% of the xylose in presence of a high concentration of acetic acid (1.4%). The strain fermented a 5% glucose-5% xylose mixture in presence of 1% acetic acid at pH 5.8 to completion at an ethanol yield of 93.4% (of the maximum theoretical yield). The xylose fermentation capability in presence of this inhibitor is among the highest ever reported for *Zymomonas mobilis*, demonstrating the importance of applying molecular understanding in strain improvement.

4.2 Introduction

Adaptation is a powerful technique for strain development that yielded a highly efficient xylose-fermenting *Zymomonas mobilis* strain (Agrawal et al. 2011) as discussed in chapter 2. Although rational metabolic engineering has generated several *Z. mobilis* strains capable of fermenting xylose, xylose utilization lags far behind glucose even in the best performing strains (Caimi et al. 2012; Kahsay et al. 2012; Mohagheghi et al. 2002; Rogers et al. 2007; Viitanen et al. 2008; Viitanen et al. 2009; Viitanen et al. 2011a; Zhang 2003; Zhang et al. 1995). Xylose fermentation was established and improved in our rationally engineered strain by subjecting it to an adaptation process (Agrawal et al. 2011). Adaptation of ZM4/pZMETX, a *Z. mobilis* strain that has been rationally engineered for xylose fermentation, yielded two strains –

a) A1 – 'less' adapted strain obtained after 4 serial transfers
b) A3 – 'more' adapted strain obtained after 30 serial transfers

A3 ferments xylose at a faster rate than its parent strain A1 (Figure 8 (A)). A comparison of the non-xylose fermenting ZM4/pZMETX, sluggish xylose fermenting 'A1' and highly efficient xylose fermenting 'A3' resulted in two major findings –

a) An Aldo-keto reductase, ZMO0976, functioning as a xylose reductase in *Z. mobilis* – Xylose reductase activity of protein ZMO0976 contributes to xylitol production in *Z. mobilis* cultured on xylose. Both the adapted strains - A1 and A3 - were found to have a single mutation in ZMO0976. This mutation drastically lowered the reductase activity of ZMO0976 (Agrawal and Chen 2011a). Mutated ZMO0976 led to lower xylitol production in adapted strains A1 and A3 compared to the control strain. Since xylitol gets converted to toxic compounds within the cells (Feldmann et al. 1992; Jeon et al. 2005; Kim et al. 2000a; Scangos and Reiner 1979), the lowering of xylitol formation is essential for improving xylose fermentation in *Z. mobilis*.

b) Xylose isomerization is the rate-limiting step of xylose fermentation in *Z. mobilis* - Due to the adaptation process, a selective increase of xylose isomerase (XI) activity was seen in A3 compared to A1. Since XI activity is the lowest among the heterologous enzymes required for xylose fermentation, xylose isomerization acts as a rate-limiting step during xylose fermentation. The higher level of XI activity in A3 results in improved xylose fermentation. Increased XI activity in A3 was linked to a mutation in the promoter of XI gene carried by the plasmid pZMETX (Agrawal et al. 2011). Other researchers have also reported improvement in xylose fermentation in *Z. mobilis* by increasing XI activity (Kahsay et al. 2012; Kahsay et al. 2011; Viitanen et al. 2009).

The above findings, obtained from the comparison of adapted strains and rationally engineered parent strain, can be verified using rational metabolic engineering to establish conclusively their contribution towards improving xylose fermentation. Further improvement in these two attributes may lead to more efficient xylose fermentation. For example, mutated ZMO0976 still has some residual xylose reductase activity which may be completely eliminated by knocking

out ZMO0976. This may lead to still lower xylitol production and consequently better xylose fermentation.

In this study, it was verified that indeed these two attributes lead to better xylose fermentation in *Z. mobilis.* To begin with, knock out of ZMO0976 which functions as a xylose reductase (XR) was done in wild-type *Z. mobilis* ZM4. Knockout of ZMO0976 resulted in lower production of xylitol compared to the wild-type and adapted strains. The knockout facilitated the establishment of xylose fermentation in *Z. mobilis*. The knockout strain (ZM4 ΔXR) bearing the heterologous xylose metabolic genes managed to grow directly in xylose medium without any adaptation. Mutation on the plasmid was synergistic to that occurred on the chromosome and together resulted in a strain exhibiting 80% of xylose fermentation capability of the adapted strain, suggesting that these two mutations were major but other unidentified mutations could also contribute to the improvement.

In addition, these two attributes were also introduced in an acetate resistant *Z. mobilis* ZM6014 (Chen et al. 2009; Wang 2008) in an attempt to construct an efficient xylose-fermenting acetate resistant *Z. mobilis* strain. ZM6014 is an acetate resistant strain developed in our lab by a graduate student, Yun Wang, as part of her MS thesis work (Wang 2008). ZM6014 is capable of fermenting RM medium in presence of 1.4% acetic acid at pH 6. Acetic acid tolerance of ZM6014 is comparable to reported acetate resistant *Z. mobilis* strains (Jeon et al. 2002; Joachimsthal et al. 1998; Joachimsthal and Rogers 2000; Lawford et al. 1999; Viitanen et al. 2011a). Yun Wang had developed this strain by use of adaptive mutation alone and her MS thesis work concluded that "adaptive mutation alone is sufficient to develop acetic acid tolerance in *Z. mobilis*". In fact, efficient xylose-fermenting strain of *Z. mobilis*, A3, was also developed using adaptive mutation alone and was found to ferment xylose more efficiently than reported

strains in the literature (Agrawal et al. 2011). Thus, the above statement made in Yun Wang's MS thesis may be extended to include xylose fermentation as follows – "adaptive mutation alone is sufficient to develop acetic acid tolerance and efficient xylose fermentation in *Z. mobilis*".

One of the objectives of the project is to apply the molecular-level understanding gained from the adaptation process to further improve fermentation of lignocellulosic hydrolysates. Lignocellulosic hydrolysates usually contain high concentrations of xylose and acetic acid. Acetate is present as acetylated xylose residues in the hemicellulose fraction of lignocellulosic biomass. Even very mild pretreatment of lignocellulosic biomass releases acetate. Concentration of acetic acid in hemicellulose hydrolysates from a variety of biomass/waste materials was found to be in the range 0.2 % -1.0 % (Lawford and Rousseau 1993). However, since acetate is not metabolized by any ethanol fermenting microbe, it may soon reach levels well above 1 % when hydrolysates are concentrated to provide high final ethanol concentrations or where process water is recycled (Joachimsthal et al. 1998). Acetate inhibits growth and fermentation by microbes (Diez-Gonzalez and Russell 1997; Joachimsthal and Rogers 2000; Warnecke and Gill 2005). Although removing the acetate from lignocellulosic hydrolysate is one way to resolve the inhibition problem, incorporating this step would substantially increase the cost of manufacturing cellulosic ethanol as this step is chemically or energy intensive (Viitanen et al. 2011a; Yang et al. 2010a). Consequently, being able to engineer *Z. mobilis* strains to provide higher tolerance for acetate and ferment glucose and xylose at high ethanol yield will be a desirable improvement. Even without acetate, xylose metabolism is 3- to 4-fold slower than glucose metabolism (Lawford and Rousseau 2000; Viitanen et al. 2011a). The difference gets further amplified under stress conditions such as acetic acid. Slower carbon flux during xylose metabolism also results in a lower steady-state level of ATP (Kim et al. 2000a), and as a result *Z. mobilis* is far more

susceptible to stress and inhibitors when it is grown on xylose (Joachimsthal and Rogers 2000; Kim et al. 2000b).

A strain capable of fermenting xylose at a faster rate in the presence of acetic acid may be obtained by combining the efficient xylose-fermenting capability of A3 strain with the acetate resistance of ZM6014. In this study, such a strain was created by rational metabolic engineering of ZM6014 for xylose fermentation similar to the one done for wild-type *Z. mobilis* ZM4. ZMO0976 was knocked out from ZM6014 and it was then transformed with A3-pZMETX*. As a result of knockout, ZM6014 ΔXR was found to produce similar low levels of xylitol as ZM4 ΔXR. ZM6014 ΔXR/A3-pZMETX* was also capable of separately fermenting RM medium containing 5% xylose and RM containing 2% glucose - 1.4% acetic acid at pH 5.8. Additionally, it could ferment a mixture of glucose and xylose in the presence of 1% or 1.4 % acetic acid at pH 5.8.

4.3 Materials and Methods

4.3.1 Materials

Molecular biology reagents, such as mini and maxi – prep plasmid extraction kit, gel DNA recovery kit, Taq DNA polymerase, iProof High-Fidelity DNA Polymerase, PCR primers, restriction enzymes, Quick T4 DNA ligase and SDS-PAGE ready gel were purchased from commercial sources, including Zymoresearch, Qiagen, Invitrogen, Promega, and BioRad. All chemicals were obtained from Fisher Scientific, Hampton, NH unless otherwise indicated.

4.3.2 Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 10.

Table 10: Bacterial strains and plasmids used in this study

Strains/Plasmids	**Relevant Characteristics**	**Source**
ZM4	Wild-type *Zymomonas mobilis* ZM4	ATCC 31821
JM109	Wild-type *Escherichia coli* JM109	Promega
JM110	Wild-type *Escherichia coli* JM110 used as cloning host for preparation of non-methylated plasmids. Possesses streptomycin resistance (Str^R)	Stratagene
ZM6014	ZM4 adapted for acetic acid tolerance	(Chen et al. 2009; Wang 2008)
ZM4 ΔXR	ZM4 with a ZMO0976 knock out	This study
ZM6014 ΔXR	ZM6014 with a ZMO0976 knock out	This study
pTeasy	Commercial vector containing ampicillin resistance (Amp^R) cassette and ColE1 *ori*	Promega
pTspecXR	pTeasy vector containing spectinomycin resistance ($Spec^R$) cassette and homologous regions flanking ZMO0976	This study
pSTV28	Commercial plasmid containing chloramphenicol resistance (Cm^R) cassette and p15A ori	TaKaRa Bio Inc. (Japan)
pSTlsXR	pSTV28 containing $Spec^R$ and homologous regions flanking ZMO0976	This study
pZMETX (A1)	Plasmid obtained from A1 containing xylose metabolic genes (Figure 7 (A))	(Agrawal et al. 2011)
A3-pZMETX*	Mutated pZMETX obtained from A3 containing xylose metabolic genes (Figure 7 (A))	(Agrawal et al. 2011)

4.3.3 Culture Conditions

E. coli cells were grown in Luria-Bertani (LB) medium at 37°C in culture tube or shake flask on a biological shaker shaken at 250 rpm. Ampicillin (100 µg/ml) and chloramphenicol (50

μg/ml) were added to the LB medium for plasmid maintenance. Streptomycin (50 μg/ml) was used for confirming the presence of JM110.

All concentrations in % are percentages in weight per volume unless otherwise indicated.

Zymomonas mobilis cells were grown at 30°C in RM medium (1% yeast extract, 0.2% KH_2PO_4) containing different amounts of glucose or xylose (as indicated) as carbon source. pH of RM medium containing acetic acid was adjusted to 5.8 using 50% NaOH. ZM6014 and its derived strains were grown in different amounts of acetic acid as indicated. Chloramphenicol (50 or 100 μg/ml) and spectinomycin (200 μg/ml) were added to the RM medium as needed for plasmid maintenance and knock out respectively. A nutrient-rich medium called mating medium was used for recovery and selection of cells after electroporation. It comprised of 5% glucose, 1% yeast extract, 0.5% tryptone, 0.25% ammonium sulfate, 0.02% potassium phosphate dibasic and 1mM magnesium sulfate. The pH of mating medium was adjusted to 6.0 using phosphoric acid and it was filter sterilized.

1.5% agar was used for preparing media plates.

Pre-seed culture (PSC) and seed culture (SC) of *Z. mobilis* adapted strains - A1 and A3 – and knockout strains – ZM4 ΔXR/pZMETX (A1) and ZM4 ΔXR/A3-pZMETX* - were cultivated in 15-ml and 50-ml centrifuge tubes, respectively, filled to 60% volume and shaken at 250 rpm. PSC was prepared by inoculating a single colony from agar plate into the liquid medium. PSC was grown to the stationary phase. SC was prepared by inoculating it to an optical density (OD) of 0.1 using the stationary phase PSC. Appropriate amount of cells were harvested from SC at exponential phase, resuspended in fresh medium and were used to inoculate the main culture (MC) to a starting OD of 0.1. MC was grown in 100-ml Pyrex screw-cap bottle filled to

60% volume and shaken at 250 rpm. RM containing 5% xylose with appropriate antibiotic(s) was used for culturing PSC, SC and MC.

Pre-seed culture (PSC) and seed culture (SC) of acetate resistant ZM6014 ΔXR/A3-pZMETX* were cultured in RM containing 5% glucose - 5% xylose (5%G - 5%X) and 1% or 1.4% acetic acid. Same media was used for the main fermentation in the fermenter. Pre-seed culture (PSC) and seed culture (SC) of *Z. mobilis* were cultivated in static 15-ml centrifuge tube and 100-ml Pyrex screw-cap bottle, respectively, filled to 60% volume. PSC was prepared by inoculating a single colony from agar plate into the liquid medium. SC was prepared by inoculating it to an optical density (OD) of 0.1 using the stationary phase PSC. Appropriate amount of cells were harvested from exponential phase SC, resuspended in fresh medium and were used to inoculate the fermenter to get a starting OD of 0.1. The fermenter used (Infors HT Multifors, Switzerland) had vessels with a working volume of 500 ml. The fermenter was stirred at 300 rpm. Condensers for the fermenter were kept at 5°C using a chiller. Before the inoculation, fermenter was purged with N_2 gas at 0.2 lpm and pH was adjusted to 5.8 by addition of 1.7M KOH. During fermentation, acid was not used for pH control. However, it was observed that the pH in the vessel was in a narrow range of 5.8 to 6.0. To maintain anaerobic conditions, the exhaust tube was immersed in a water column to prevent atmospheric oxygen from diffusing into the fermenter vessels. Dissolved oxygen was monitored and was found to remain close to 0% throughout the fermentation. There was negligible loss of ethanol due to evaporation in this set up.

Three replicates were done for each experiment starting from three colonies on an agar plate.

4.3.4 Knockout of ZMO0976 in *Z. mobilis*

Knockout of ZMO0976 was done in *Z. mobilis* according to a published procedure (Viitanen et al. 2011a). pTspecXR was constructed to carry out the knockout of ZMO0976 (Figure 13). pTeasy backbone of the plasmid pTspecXR was obtained by EcoRI and SphI double digestion of a plasmid pTadh-fba. pTadh-fba has an ~2 kb operon Padh-fba ligated to the commercial pTeasy vector (Promega) using A-T cloning (see chapter 5). Alternatively, pTeasy backbone of pTspecXR can be obtained by direct double digestion of pTeasy vector with EcoRI and SphI. Sequence of spectinomycin resistance ($Spec^R$) cassette which includes the promoter was obtained from GenBank (accession # X03043). Synthetic DNA was used for $Spec^R$ cassette (Genscript). $Spec^R$ cassette was ligated to pSTV28 vector at BamHI site to obtain plasmid pSTVls. The 5' homologous region (HR) for XR was cloned from ZM4 genome using primers – (5'-TAATGCAA**GCATGC**CCTTATATGGTCTGACGTTG-3') and (5'-GCGATTC**GTCGAC**TGATATTGATACCAAGATCAATC-3'). Restriction sites are depicted in bold underlined fonts. The 5' homologous region extends from 1 kb upstream of ZMO0976 to 0.2 kb downstream of the 5'-end of ZMO0976. 5' HR was ligated to pSTVls vector at SalI and SphI restriction sites to get pSTls5'XR. Similarly, the 3' HR was cloned using primers – (5'-ATATTA**CCCGGG**CTATCCGCTGGATGTTGGAG-3') and (5'-ATTATT**GAATTC**GGACGGACGATCAGAGAAGCG-3'). The 3' HR extends from 0.2 kb upstream of the 3'-end of ZMO0976 to 1 kb downstream of ZMO0976. 3' HR was ligated to pSTls5'XR vector at XmaI and EcoRI restriction sites to get the suicide vector pSTlsXR. EcoRI and SphI were used to double digest pSTlsXR to obtain two DNA fragments of size 3.0 and 3.4 kb. The 3.4 kb DNA fragment consists of the $Spec^R$ cassette flanked by homologous regions of ZMO0976. This fragment was ligated to the EcoRI and SphI digested pTeasy backbone

mentioned above to obtain the suicide vector pTspecXR (Figure 13). JM110 was used as cloning host so that methylation-free plasmids can be obtained for efficient electroporation in *Z. mobilis* ZM4.

Several primers were used to confirm the knockout of ZMO0976 from ZM4 genome. Primers (5'- GCTATTGACGGTACCATGAACACTTCTACGCAAAAACC-3') and (5'-TATTCGTACTAAGCTTTTATTTATCGCGTGGCGGGGGTG-3') bind to the ends of ZMO0976 and amplify the entire ZMO0976 gene. Primers - (5'-GCCATCAAAGGTCAACGCGATAATTTGATTATTGCG-3') and (5'-CCAGATAATGTTCAAAGCGCGGTTTCTGGAATTTC-3') amplify only the middle portion of the ZMO0976 gene. The middle portion of ZMO0976 will be absent from the genome of a ZMO0976 knockout. Primers (5'-AAAGAAACCAATCCCGTTGTTGTCAAAGCCAAAGTC-3') and (5'- CCATTGAAGAATGGCATGCTTTGACCGATGAAGC-3') amplified the genomic region flanking ZMO0976.

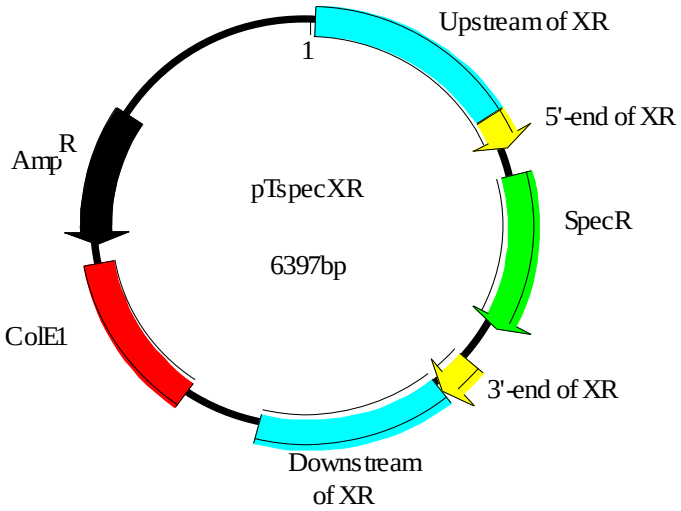

Figure 13: Plasmid map of pTspecXR, a pTeasy derived suicide vector for knockout of ZMO0976

4.3.5 Electroporation in *Z. mobilis*

Preparation of electrocompetent cells and electroporation in *Z. mobilis* ZM4 were done in accordance with published procedures (Picataggio et al. 1996; Zhang 2003). Electrocompetent wild-type *Z. mobilis* ZM4 and acetate resistant ZM6014 were prepared by inoculating a single colony from RM plate in 9 ml of RM contained in 15-ml centrifuge tube to obtain the seed culture (SC). SC was grown without shaking at 30°C for 24 hrs. SC was used at an inoculum size of 1% (v/v) for inoculating 108 ml of main culture contained in 180 ml screw-cap bottles. The main culture was grown at 30°C without shaking to get an OD of 0.5. The culture was placed on ice for 30 min and subsequently centrifuged at 3,000 g for 10 min at 4°C. Cells were washed twice with one volume of pre-chilled filter sterilized 10% (w/v) glycerol. Cells were washed one more time with 0.5 volume of 10% glycerol. Finally, cells were suspended in an appropriate volume of 10% glycerol such that the final OD is 20. Electrocompetent cells were stored at -80°C and were found to be usable for more than 1 year. Electrocompetent cells for engineered *Z. mobilis* were prepared using appropriate antibiotic during the cell culture.

Electroporation was carried out in an electroporation cuvette having a 0.1 cm gap. 40 µl of the electrocompetent cells were mixed with up to 10 µl of concentrated plasmid to achieve a final plasmid concentration between 0.01 µg/µl and 0.1 µg/µl. MicroPulser (Bio-Rad) with a capacitance of 10 µF and a resistance of 600 Ω was used to deliver an electroporation voltage of 16 kV/cm for a time constant of 4.0 – 5.5 ms. After electroporation, 1 ml of mating medium was added to the electroporation mixture and the cells were allowed to recover at 30° C for 1- 3 hours. The recovered cells were plated on solid mating media containing the appropriate antibiotic(s). Successful transformants usually appeared within 2 – 3 days of incubation at 30°C.

4.3.6 Analytical Methods

Cell growth was determined by measuring optical density (OD) at 600nm using spectrophotometer (Beckman Coulter DU 530, Brea, CA). Samples taken during fermentation were spun down by centrifugation and supernatants were analyzed for concentrations of glucose, xylose, xylitol, acetic acid and ethanol using high-performance liquid chromatography (Agilent Technologies) instrument equipped with an Aminex HPX-87H column (Bio-Rad). 5 mM H_2SO_4 at a flow rate of 0.4 ml/min was used as the mobile phase.

4.4 Results

4.4.1 Knockout of ZMO0976 in *Z. mobilis*

Non-methylated pTspecXR was electroporated in wild-type *Z. mobilis* ZM4 and acetate resistant ZM6014 at a concentration of 0.05 µg/µl. Initial screening of the transformants was done using colony PCR employing forward primer (5'-GCTATTGACGGTACCATGAACACTTCTACGCAAAAACC-3') and reverse primer (5'-TATTCGTACTAAGCTTTTATTTATCGCGTGGCGGGGGTG-3'). These primers bind respectively to the 5'-end and 3'-end of the ZMO0976 gene. If ZM4 genome is used as a template, this primer set yields a 1 kb band corresponding to the ZMO0976 gene. Instead, if ZM4 genome with a ZMO0976 knockout is used as a template, a 1.5 kb band corresponding to $Spec^R$ cassette flanked by 0.2 kb long 5'- and 3'-ends of ZMO0976 is obtained (Figure 13). Majority of the transformants yielded a strong 1.5 kb band and only a very faint 1 kb band indicating an incomplete knockout. Besides, there were some transformants that yielded only the 1.5 kb strong band indicating a complete knockout. Further screening of these transformants using other primer sets as mentioned in section 4.3 was done to confirm the presence of the knockout. Two of the transformants that had incomplete knockout were serial cultured for ~15

generations. The final culture was then plated and single colonies from the plate yielded only the 1.5 kb strong band and not the faint 1 kb band indicating a complete knockout. Presence of the knockout was confirmed by other primer sets as well. Thus serial culturing helped to convert incomplete knockout into complete knockout. This observation is consistent with that mentioned in (Viitanen et al. 2011a) where they serial cultured their transformant to convert the single cross-over to double cross-over.

pSTV28 derived suicide vector pSTlsXR having the same 3.4 kb DNA fragment for ZMO0976 knockout as pTspecXR was unable to knockout ZMO0976 from the ZM4 genome (Figure 13). The most successful transformant using the pSTlsXR had a very strong 1 kb band corresponding to the wild-type ZMO0976 and a faint 1.5 kb band corresponding to $spec^R$ flanked by ZMO0976 ends, indicating a negligible knockout. Even after serial culturing for 100 generations, the wild-type ZMO0976 band was still present. This is opposite to the results obtained by knockout using pTspecXR. Several reported knockouts in *Z. mobilis* ZM4 have been done using pTeasy or pTeasy-similar vectors (Kerr et al. 2011; Viitanen et al. 2008; Viitanen et al. 2011a; Viitanen et al. 2011b) and there is no report of knockout using pSTV28 derived vectors. Thus, pTeasy vector and not pSTV28 vector is suitable for knockout in *Z. mobilis*.

4.4.2 ZMO0976 Knockout Leads to Reduced Xylitol Production

Knockout of ZMO0976 led to a decrease in xylitol production. As shown in Figure 14, both ZM4 and ZM6014 with a ZMO0976 knockout (designated as ZM4 ΔXR and ZM6014 ΔXR respectively) produced 0.015% xylitol by fermentation of RM medium containing 5% glucose and 5% xylose as the carbon source. This is four-fold lower than that produced by the wild-type strain ZM4 or acetate resistant strain ZM6014. This is also 25% lesser than the amount of xylitol produced by adapted strains A1 and A3 at the beginning of vigorous xylose fermentation

(coinciding with the exhaustion of glucose). Both the adapted strains A1 and A3 have a single mutation in the aldo-keto reductase ZMO0976 which drastically reduces its reductase activity (Agrawal and Chen 2011a). Since xylitol production is not eliminated completely in ZM4 ΔXR and ZM6014 ΔXR, this indicates that other enzymes besides ZMO0976 contribute to xylitol formation in *Z. mobilis* ZM4. For example, glucose-fructose oxidoreductase (GFOR) has been reported to reduce xylose to xylitol in *Z. mobilis* (Viitanen et al. 2008).

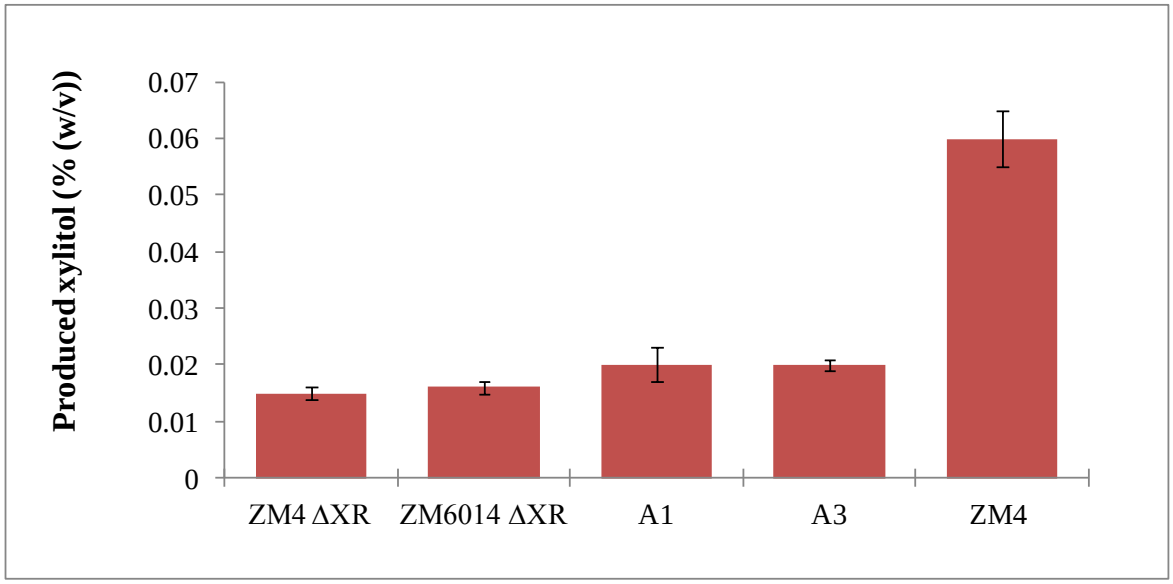

Figure 14: Xylitol produced by different strains when grown in RM containing 5% glucose - 5% xylose as carbon source. For xylose metabolizing A1 and A3 strains, the produced xylitol is at the beginning of vigorous xylose fermentation (coinciding with the exhaustion of glucose)

4.4.3 Construction of Xylose-Fermenting Strains from ZM4 ΔXR

Knockout of ZMO0976 results in lower xylitol formation. Several researchers have reported inhibition of xylose fermentation in *Z. mobilis* by xylitol (Agrawal et al. 2011; Feldmann et al. 1992; Viitanen et al. 2008). Hence, knockout of a xylose reductase will lower the xylitol inhibition and facilitate the establishment of xylose fermentation in *Z. mobilis*. For example, Viitanen et al. generated a strain ZW800 with reduced xylitol formation by knocking

out the gene for glucose-fructose oxidoreductase (GFOR) (Viitanen et al. 2008). GFOR knockout resulted in significantly improved xylose fermentation, compared to strain without GFOR knockout. As ZMO0976 possessed XR activity (Agrawal and Chen 2011a), knockout of ZMO0976 can also result in improvement in xylose fermentation. This was confirmed by expressing xylose metabolic genes in ZM4 ΔXR. pZMETX was constructed in our lab that carries the four genes required for xylose metabolism (Figure 7 (A)). As mentioned in Chapter 2, pZMETX was transformed in ZM4 to construct a xylose metabolizing strain, ZM4/pZMETX. Since ZM4/pZMETX failed to grow on xylose as sole carbon source, it was adapted to yield xylose-fermenting strains A1 and A3. Due to the adaptation process, Ppdc-xylAB operon in pZMETX underwent a single mutation in A3 strain. Unmutated pZMETX was extracted from A1 (designated pZMETX (A1)) and mutated pZMETX was extracted from A3 strain (designated A3-pZMETX*). It was previously reported by us that single mutation in pZMETX results in a higher xylose isomerase activity based on the enzymatic assays of cell extracts of A1 and A3 (Agrawal et al. 2011). In this study, this result was cross-verified using rational metabolic engineering.

Both types of pZMETX were electroporated in ZM4 ΔXR to construct ZM4 ΔXR/pZMETX (A1) and ZM4 ΔXR/A3-pZMETX*. Both ZM4 ΔXR/pZMETX (A1) and ZM4 ΔXR/A3-pZMETX* were able to grow on xylose as sole carbon source without any adaptation. This is contrary to our previous experience where ZM4/pZMETX failed to grow on xylose as sole carbon source. ZM4/pZMETX has wild-type ZMO0976 present in its genome. ZM4/pZMETX had to be adapted on a glucose-xylose mixture initially to establish xylose fermentation and later on xylose to improve xylose fermentation (Agrawal et al. 2011). This observation was put to test again by electroporating pZMETX (A1) and A3-pZMETX* in wild-

type *Z. mobilis* ZM4. ZM4/pZMETX (A1) and ZM4/A3-pZMETX* were grown in RM medium containing 2% and 5% xylose as sole carbon source. No growth was observed even after one week of cultivation. After one week, ZM4/pZMETX (A1) cultured in 5% xylose showed growth. The OD was measured to be 0.14 which is more than ten-fold lower than the final OD for ZM4ΔXR/pZMETX (A1) usually observed after 3 days of cultivation. Only 0.6% xylose was consumed by ZM4/pZMETX (A1) to produce 0.2% ethanol and 0.05% xylitol. No further growth and fermentation were seen for ZM4/pZMETX (A1). This delayed growth may be a result of spontaneous mutation, but even the spontaneous mutation did not result in a strain as good as ZM4ΔXR/pZMETX (A1). As we had done in the past for ZM4/pZMETX, such strain needs to be adapted on xylose to facilitate better xylose fermentation. Thus, knockout of ZMO0976, which results in lower production of toxic xylitol, facilitates the establishment of xylose fermentation.

A comparison of rates of growth and xylose fermentation was done between the adapted strains and the knockout strain (Figure 15). A3 had the highest rates of growth and xylose fermentation among the four strains (Figure 15). It was followed by ZM4 ΔXR/A3-pZMETX* which exhibited better growth and fermentation than ZM4 ΔXR/pZMETX (A1). Also as shown in Figure 16, ZM4 ΔXR/A3-pZMETX* produced two-fold lower xylitol than ZM4 ΔXR/pZMETX (A1). The only genetic difference between the two strains is a single mutation in the Ppdc-xylAB operon of pZMETX. Thus, this mutation results in better xylose fermentation accompanied with a lower xylitol formation. We had earlier reported that this mutation in Ppdc-xylAB manifests in a higher xylose isomerase (XI) activity in A3 (Agrawal et al. 2011). Since xylose isomerization is a rate-limiting step in xylose fermentation, higher XI activity leads to a higher xylose flux resulting in higher rate of xylose fermentation. As shown in Figure 15, ZM4

ΔXR/pZMETX (A1) showed slightly higher rate of xylose fermentation than A1. ZM4 ΔXR/pZMETX (A1) also produced lower xylitol at 0.11% while A1 produced 0.15% (Figure 16). Thus the knockout of ZMO0976 resulted in a lower xylitol production, which resulted in lesser inhibition of the xylose fermentation. It is interesting to note that A1 and ZM4 ΔXR/pZMETX (A1) are comparable strains even though they were obtained by different methods – adaptation and rational metabolic engineering respectively.

Since the growth and fermentation by ZM4 ΔXR/A3-pZMETX* lags behind A3, this indicates that there are other attributes besides lower xylitol production and higher xylose isomerization that result in better xylose fermentation. Overall, the data helps to conclude that knocking out of ZMO0976 and mutation in pZMETX leads to better xylose fermentation.

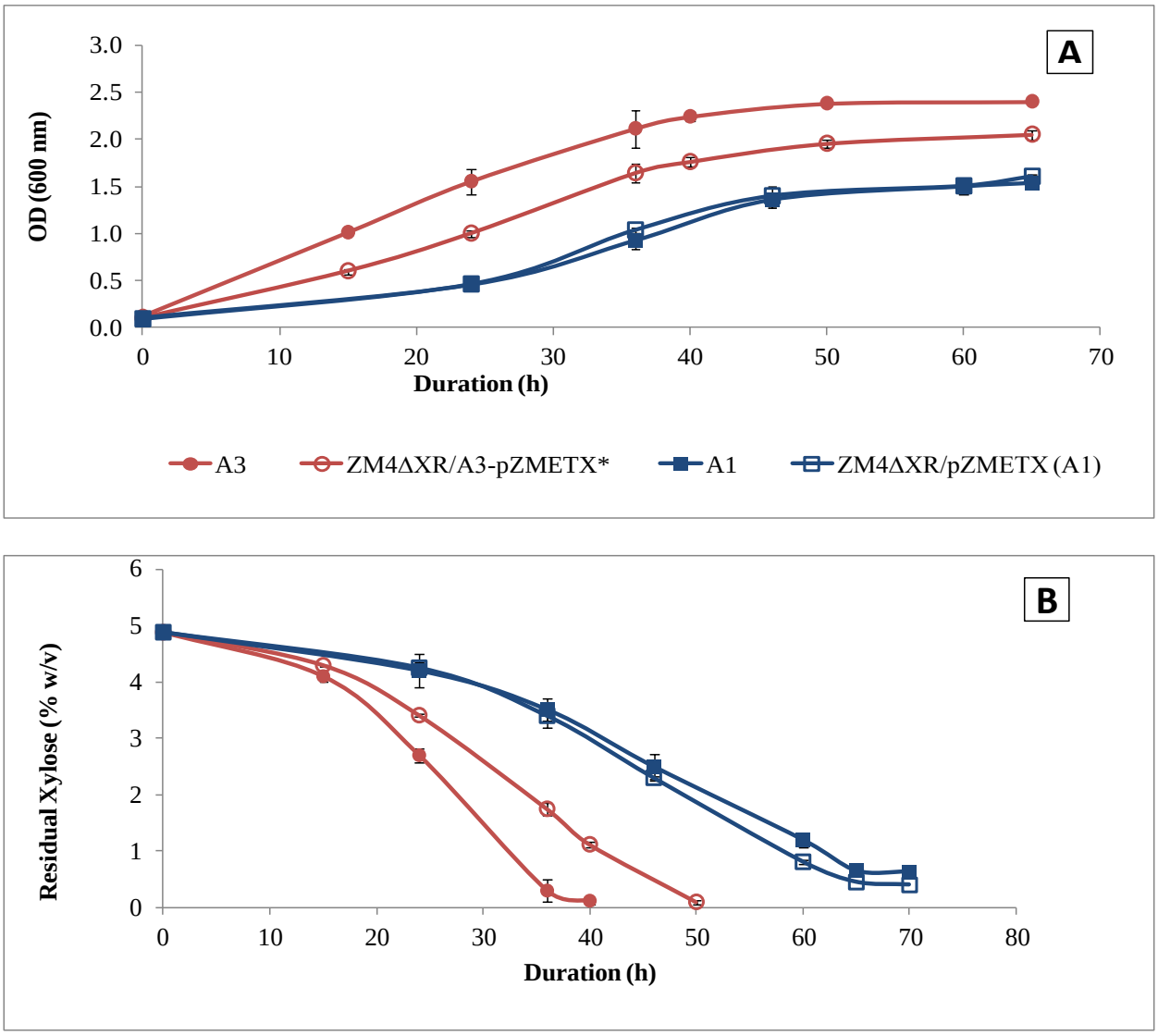

Figure 15: Comparison of growth and xylose fermentation of knockout and adapted strain. Cells were grown in 5% xylose

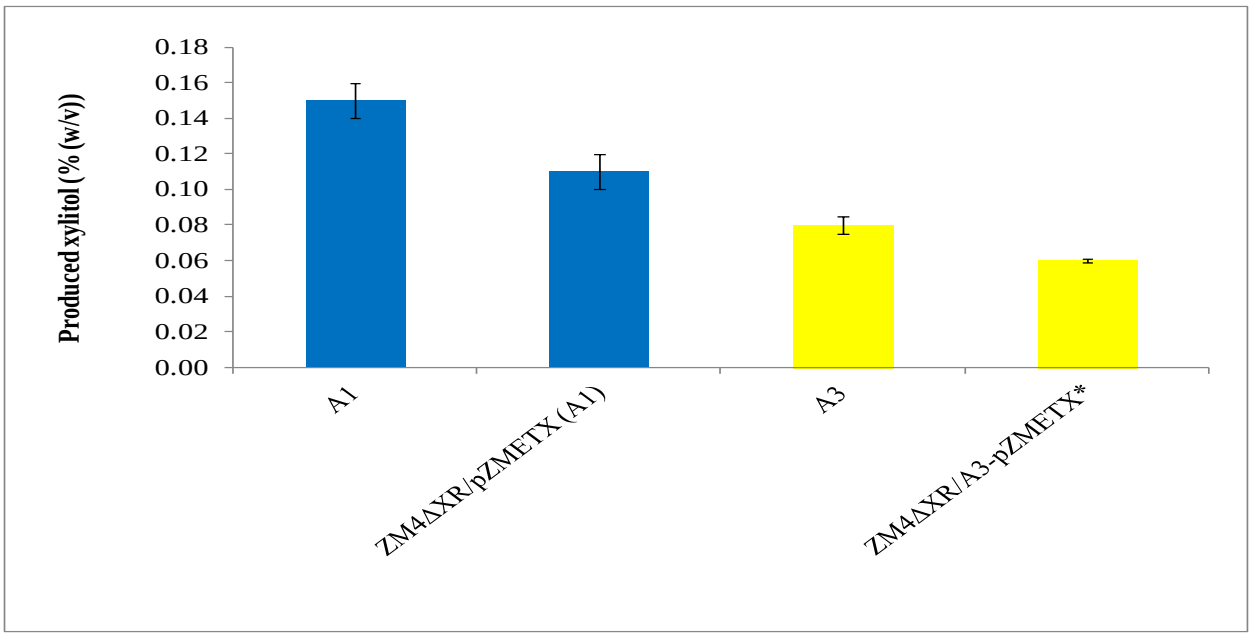

Figure 16: Comparison of xylitol production by the adapted and knockout strains fermenting 5% xylose

4.4.4 Xylose-Fermenting, Acetate Resistant Strain - ZM6014 ΔXR/A3-pZMETX*

An Acetate resistant strain of *Z. mobilis* obtained using chemical mutagenesis has been reported in literature which is able to ferment glucose in the presence of sodium acetate up to 20 g/ l (equivalent to 1.4% acetic acid) (Joachimsthal et al. 1998). In our lab, such a strain was developed by adaptation alone (Chen et al. 2009; Wang 2008). Also, an efficient xylose-fermenting *Z. mobilis* strain was developed in our lab by adaptation on high concentration of xylose (Agrawal et al. 2011). In this study, we combined the two traits – acetate resistance and efficient xylose fermentation - that had been developed separately using adaptation. It may not be possible to design an adaptation process to combine the two traits. Since acetate leads to increase in maintenance energy for the cells (Jeon et al. 2002; Joachimsthal et al. 1998; Kim et al. 2000b) and xylose fermentation is not as energy efficient as glucose fermentation (Kim et al.

2000a; Kim et al. 2000b), it will be difficult for the cells to cope up with acetate using xylose as the sole energy source.

Fortunately, our previous work with A1 and A3 strains (Agrawal et al. 2011) and present work with ZM4ΔXR/pZMETX (A1) and ZM4ΔXR/A3-pZMETX* strains have conclusively shown that reduced xylitol formation and higher xylose isomerization lead to improvement in xylose fermentation. Both these attributes can also be incorporated into any *Z. mobilis* strain such as the acetate resistant ZM6014 using rational metabolic engineering to establish and improve xylose fermentation.

A3-pZMETX* was electroporated in ZM6014 ΔXR to construct a xylose fermenting strain. ZM6014 ΔXR/A3-pZMETX* was able to ferment RM medium containing 1.4% acetic acid at pH 6. This suggests that ZM6014 is a stable acetate resistant strain as acetate was not added to the culture media used for preparation of electrocompetent cells, cell recovery and selection after electroporation. Acetate resistant ZM6014 ΔXR/A3-pZMETX* was found to produce similar low levels of xylitol as ZM4 ΔXR/A3-pZMETX* (Figure 14). Just like ZM4 ΔXR/A3-pZMETX*, ZM6014 ΔXR/A3-pZMETX* was able to ferment RM containing 5% xylose without any adaptation. However, ZM6014 ΔXR/A3-pZMETX* failed to ferment RM containing 5% xylose and 1.4% acetic acid at pH 5.8. Presence of acetic acid results in a considerable stress on cells (Joachimsthal et al. 1998; Kim et al. 2000b) which perhaps inhibits their growth on xylose which is a non-natural substrate and generates lower energy than glucose (Kim et al. 2000a; Kim et al. 2000b). Cell growth in such a situation was achieved by adding glucose to the fermentation media as ZM6014 is adapted to grow on glucose in presence of up to 1.4 % acetic acid. In the presence of glucose, ZM6014 ΔXR/A3-pZMETX* was able to ferment xylose in the presence of acetic acid.

ZM6014 ΔXR/A3-pZMETX* was able to ferment a mixture of 5% glucose and 5% xylose (5%G-5%X) in presence of 1.4% acetic acid at pH 5.8. As shown in Figure 17, at the end of four days of fermentation, only 1.7% xylose remained. 3.9% ethanol was produced at a yield of 91.5% of the maximum theoretical yield based on total sugars consumed. 0.3% xylitol was produced which may have inhibited any further fermentation. As mentioned before, it is desirable to engineer fermentation microbes with acetic acid tolerances well above 1% so that the buildup of acetic acid resulting for example, from hydrolysate concentration does not become inhibitory to cell growth (Joachimsthal et al. 1998).

On decreasing the acetic acid concentration to 1% at pH 5.8, ZM6014 ΔXR/A3-pZMETX* was able to ferment 5%G-5%X to completion in 58 h (Figure 18). Several acetate resistant xylose-fermenting *Z. mobilis* strains reported in literature fail to ferment xylose completely if acetic acid concentration is ~1%. For example, (a) 0.5% xylose remained for prehydrolyzate adapted ATCC 39676/pZB4L fermenting 0.8% glucose-4% xylose in presence of 1% acetic acid at pH 6 (Lawford et al. 1999). (b) 0.5% residual xylose for ZW801-4::ΔhimA fermenting a 10% glucose-10% xylose mixture at pH 5.8 in presence of 0.95% acetate (Viitanen et al. 2011a). (c) 0.7% residual xylose for ZM4/AcR (pZB5) fermenting 4% glucose-4% xylose in presence of 1.2% sodium acetate (equivalent to 0.88% acetic acid) at pH 5 (Jeon et al. 2002). ZM6014ΔXR/A3-pZMETX* is capable of fermenting glucose in a glucose-xylose-acetate mixture at a faster rate than those reported in literature. Maximum volumetric rate of glucose consumption for ZM6014ΔXR/A3-pZMETX* is 6.4 g/l/h which is higher than for example, (1) 1.0 g/l/h for prehydrolyzate adapted ATCC 39676/pZB4L (Lawford et al. 1999), (2) 5.50 g/l/h for ZW801-4::ΔhimA (Viitanen et al. 2011a) and (3) 3.7 g/l/h for ZM4/AcR (pZB5) (Jeon et al. 2002).The efficient glucose-fermenting trait in presence of acetic acid was acquired by wild-type

ZM4 due to its extensive adaptation in RM medium containing 1.4% acetic acid (Chen et al. 2009; Wang 2008). ZM6014 so obtained is a stable acetate resistant strain since it did not lose acetate resistance during the rational metabolic engineering process for constructing ZM6014 ΔXR/A3-pZMETX* when cells were cultured without acetate.

All our control strains which included ZM4, ZM4 ΔXR/pZMETX (A1), ZM4 ΔXR/A3-pZMETX*, A1 and A3 failed to grow on RM medium containing 5%G-5%X and 1% acetic acid at pH 5.8. Only ZM6014 grew on such a media but could not ferment xylose. This indicates that acetate resistance trait possessed by ZM6014 is essential for fermenting 5%G-5%X in presence of ≥ 1% acetic acid at pH 5.8.

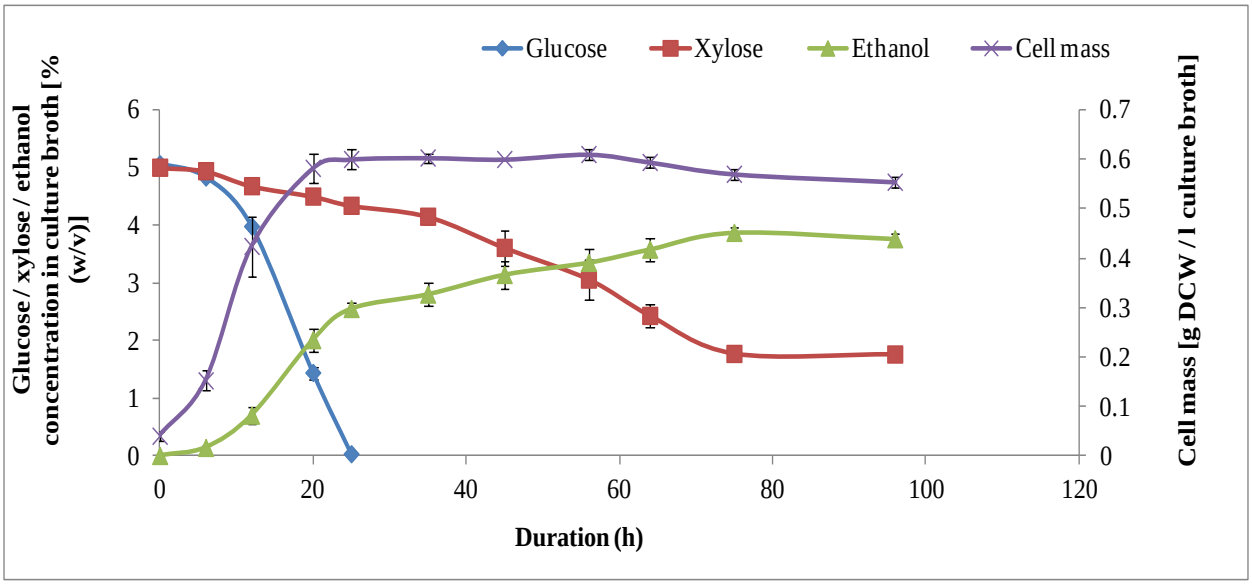

Figure 17: Fermentation of RM medium containing 5% glucose-5% xylose by ZM6014 ΔXR/A3-pZMETX* in presence of 1.4% acetic acid at pH 5.8

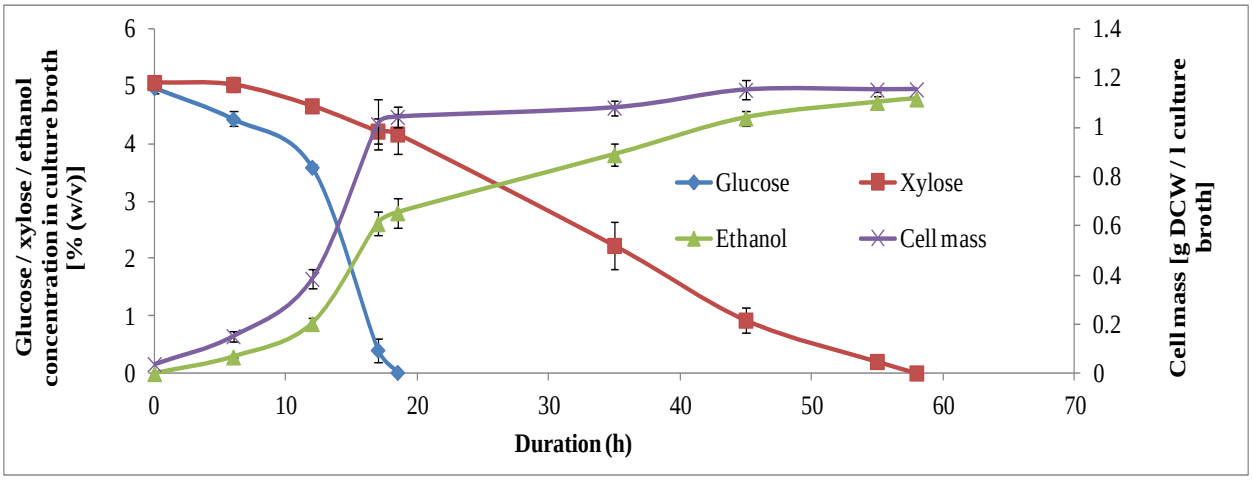

Figure 18: Fermentation of RM medium containing 5% glucose-5% xylose by ZM6014 ΔXR/A3-pZMETX* in presence of 1% acetic acid at pH 5.8

4.5 Discussion

We had earlier reported development of a highly efficient xylose-fermenting *Zymomonas mobilis* strain using adaptation (Agrawal et al. 2011). Taking a cue from the adapted and the parent strains, we attempted to develop strains similar to adapted strains using rational metabolic engineering. We demonstrated that lowering xylitol formation leads to establishment of xylose fermentation in *Z. mobilis* ZM4 and enhancement of xylose isomerase activity results in acceleration of xylose fermentation and reduction of xylitol formation.

The knockout of ZMO0976 in *Z. mobilis* resulted in a lower xylitol production. The knockout strains produced four fold lower xylitol than the wild-type strain ZM4. The knockout partially shields the strain from the toxic effects resulting from uncontrolled production of xylitol during xylose fermentation. This shield enabled the strains to ferment xylose directly without any adaptation when heterologous xylose metabolic genes were expressed in the knockout strains. ZM6014 ΔXR/A3-pZMETX* was able to consume as much as 3.3% xylose when fermenting 5%G-5%X in presence of 1.4% acetic acid, perhaps because its genome harbored a knockout of

ZMO0976. Still, accumulation of xylitol to growth inhibitory levels of 0.3% (Agrawal et al. 2011) was observed which may have been partially or completely responsible for halting of any further xylose fermentation by ZM6014 ΔXR/A3-pZMETX*. Without the ZMO0976 knockout, cells would have produced still more xylitol and xylose fermentation may have terminated much earlier. ZM4 ΔXR/pZMETX (A1) produced lower xylitol (Figure 16) and showed slightly higher rate of xylose fermentation (Figure 15) than the corresponding adapted strain A1. This reconfirms that the knockout of ZMO0976 results in a lower xylitol production, which leads to an improvement in xylose fermentation.

Even after the knockout of ZMO0976, xylitol formation could be seen in the knockout strains. This suggests that there are other enzymes which lead to the formation of xylitol in *Z. mobilis* ZM4. One of those enzymes is glucose-fructose oxidoreductase (GFOR). It was shown to accept xylose as an electron donor and reduce xylulose to xylitol (Viitanen et al. 2008; Zachariou and Scopes 1986). Though xylitol converts to toxic xylitol phosphate within the cells (Akinterinwa and Cirino 2009; Feldmann et al. 1992), formation of xylitol may be a necessary evil as it can be a way for the cells to maintain the redox balance.

While carrying out the knockout, it was observed that pTeasy is a suitable vector for the purpose. Several researchers have successfully used pTeasy or pTeasy similar vectors for knockout in *Z. mobilis* ZM4 (Kerr et al. 2011; Viitanen et al. 2008; Viitanen et al. 2011a; Viitanen et al. 2011b). It appears that pSTV28 is unsuitable for knockout in *Z. mobilis* ZM4. It is possible that pSTV28 has significant homology to the ZM4 genome or native plasmids. Presence of homologous regions will result in integration of the suicide plasmid in the cellular DNA prior to or after the double cross-over. In either case, the wild-type gene would continue to exist in the cell. pSTV28 vector had a maximum homology of 24/26 with an expect value of 0.17 while

pTeasy had a slightly lower homology of 17/17 with an 'expect value' of 2.0. The other possibility is that the pSTV28 derived suicide plasmid replicates stably in *Z. mobilis* ZM4. However, the suicide plasmid was not detected in the plasmid extracts of the transformants.

Adapted strain A3 showed better growth and fermentation than the rationally constructed ZM4 ΔXR/A3-pZMETX*. This is because A3 underwent extensive adaptation on 5% xylose which may have resulted in evolution of several efficient xylose-fermenting traits such as better tolerance to xylitol and better handling of osmotic pressure due to high sugar concentration. ZM4 ΔXR/A3-pZMETX* exhibited much better growth and fermentation on xylose than ZM4 ΔXR/pZMETX (A1) (Figure 15). Thus, single mutation in Ppdc-xylAB operon of pZMETX contributes to an improvement in xylose fermentation. This improvement is achieved by increasing xylose isomerase activity that pushes more xylose flux through the bottleneck in the xylose fermentation pathway (Agrawal et al. 2011). ZM4 ΔXR/pZMETX (A1) produced two fold higher xylitol than ZM4 ΔXR/A3-pZMETX*. It is possible that higher xylose isomerase activity due to A3-pZMETX* results in channeling more xylose towards ethanol instead of xylitol.

Establishment and improvement in xylose fermentation in wild-type *Z. mobilis* ZM4 was extended to an acetate-resistant strain ZM6014 derived from ZM4. ZM6014 ΔXR/A3-pZMETX* was able to ferment 5%G-5%X in presence of 1% acetic acid at pH 5.8 to completion in 58 hours. Compared to strains reported in literature, ZM6014 ΔXR/A3-pZMETX* exhibits better rate of glucose fermentation, ability to ferment xylose to completion and tolerate high concentration of acetic acid up to 1.4%. Thus, ZM6014 ΔXR/A3-pZMETX* has the potential of being employed for fermentation of lignocellulosic hydrolysates.

Adaptation is a powerful technique for strain development. We have shown in this study that rational metabolic engineering can be inspired from the information gained from study of adapted strains and used to mimic the improvements in the adapted strains. It is possible to replicate this information in a in a still better way thus creating rationally engineered strain better than the adapted strains.

CHAPTER 5

EMP PATHWAY IN *ZYMOMONAS MOBILIS*

5.1 Abstract

The ED pathway has evolved over centuries within *Zymomonas mobilis* to process optimum amounts of glucose, fructose and sucrose, which are the three natural substrates of *Z. mobilis*. *Z. mobilis* has been engineered for xylose fermentation by expressing heterologous enzymes that are able to route xylose into the ED pathway, thus making the fermentation of xylose to ethanol possible. Conventional methods such as removal of metabolic bottlenecks and reducing side-product formation result in improvement of xylose fermentation as discussed in chapter 2. A radical improvement in sugar fermentation was attempted in this study by establishing an additional glycolysis pathway generating more energy than the existing ED pathway. However, the new (EMP) pathway could be functionalized only to the level of the metabolites – dihydroxyacetone phosphate and glyceraldehyde 3-phosphate by overexpression of three enzymes namely PPi-PFK, FBP aldolase and TPI. Overexpression of putative FBP aldolase (ZMO0179) and *tpi* (ZMO0465) genes of *Z. mobilis* ZM4, which were found to encode for active enzymes, resulted in an alteration of metabolism with the production of a new byproduct dihydroxyacetone.

5.2 Introduction

Rational metabolic engineering of *Z. mobilis* for xylose fermentation, subsequent adaptation for establishment and improvement of xylose fermentation, and comparison of adapted and parent strains at genomic and metabolic levels provided a profound understanding of

Z. mobilis's xylose metabolism, in particular, and central carbon metabolism, in general. As several researchers have concluded in the past, we also soon came to the realization that the maximum carbon flux through the ED pathway had been reached (Arfman et al. 1992; Snoep et al. 1996). Increase of xylose flux through the rate limiting step, impediment of xylose flux to xylitol and reduction of inhibitory effects of xylitol, provide ideal conditions for pushing maximum xylose flux through the heterologous xylose fermentation and ED pathways.

The ED pathway has evolved over centuries within *Z. mobilis* for processing maximum glucose flux. High levels of glycolytic enzymes coupled with minimal allosteric control ensure considerably high glycolytic flux of approximately 1 μmol glucose/min/mg cell protein in *Z. mobilis* (Arfman et al. 1992). Several researchers have tried overexpressing the enzymes of ED pathway for increasing the glycolytic flux. However, the results are not very encouraging as the glycolytic flux could be increased only by a maximum of 17%. This increase in glycolytic flux is not commensurate with the expensive nutritional requirements of recombinant microbe compared to wild-type thus rendering no economic value to this achievement (Snoep et al. 1996). Hence, it can be concluded that a ceiling for increasing carbon flux through the ED pathway has been reached.

In this study, we tried to modify the metabolic network of *Z. mobilis* by establishing an additional glycolysis pathway. The metabolic engineering strategy chosen was to introduce an additional route (EMP pathway) for glucose metabolism in addition to the existing ED pathway (Figure 3). The increased sugar metabolism capacity is supposed to result in an enhanced sugar uptake and consequently higher specific ethanol productivity. The EMP pathway is more energy efficient than the existing ED pathway. The increased energy (ATP) availability would enable cells to spend energy, when needed, on increased cell maintenance required under 'stress'

conditions (for example, high concentration of acetic acid and ethanol) as reported by several researchers (Joachimsthal et al. 1998; John Fieschko 1983; Lawford and Ruggiero 1990; Rogers P L 1982). Additionally, the improved energy status is likely to drive xylose (non-natural substrate) metabolism once xylose metabolizing genes are expressed.

The attempt to establish an operational EMP pathway was begun by cloning and expression of pyrophosphate-dependent phosphofructokinase (PPi-PFK) in *Z. mobilis* ZM4. As the growth and fermentation profiles of engineered strain were similar to the control strain indicating an insignificant metabolic change, an investigation was undertaken that identified that the two enzymes downstream from PPi-PFK were possible limiting steps. As such, all the three genes namely PPi-PFK, fructose 1,6-bisphosphate (FBP) aldolase and triose phosphate isomerase (*tpi*) were overexpressed in *Z. mobilis* ZM4 to construct ZM4/pNGFPK/pMHGPTAF and ZM4/pEMP. However, the overexpression of three genes resulted in a reduction in rates of growth and fermentation, suggesting that the EMP pathway was still not operational. The EMP pathway got functionalized in these strains only up to the level of the metabolites – dihydroxyacetone phosphate and glyceraldehyde 3-phosphate - in the glycolysis pathway. Further attempts to completely functionalize the EMP pathway by partial silencing of a key enzyme of the ED pathway, ED aldolase (*eda*), and adaptation did not succeed.

5.3 Materials and Methods

5.3.1 Materials

Molecular biology reagents, such as mini - plasmid purification kit, gel DNA recovery kit, Taq DNA polymerase, iProof High-Fidelity DNA Polymerase, PCR primers, restriction enzymes, Quick T4 DNA ligase and SDS-PAGE ready gel were purchased from commercial sources, including Zymoresearch, Qiagen, Invitrogen, Promega, and BioRad. All commercial

enzymes were obtained from Sigma Aldrich, St. Louis, MO. All chemicals were obtained from Fisher Scientific, Hampton, NH unless otherwise indicated.

5.3.2 Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 11.

Table 11: Bacterial strains and plasmids used in this study

Strains/Plasmids	**Relevant Characteristics**	**Source**
ZM4	Wild-type *Zymomonas mobilis* ZM4	ATCC 31821
JM109	Wild-type *Escherichia coli* JM109	Promega
JM110	Wild-type *Escherichia coli* JM110 used as cloning host for preparation of non-methylated plasmids. Possesses streptomycin resistance	Stratagene
pSTV28	Commercial plasmid containing chloramphenicol resistance cassette (Cm^R) and p15A ori	TaKaRa Bio Inc. (Japan)
pSTVZM27	pSTV28 containing ZM27	(Agrawal et al. 2011)
pNGFPK	pSTVZM27 containing PPi-PFK gene	This study
pEMP	pNGFPK containing FBP aldolase and *tpi* genes	This study
pEMP (50, 300, 627)	pEMP containing 50, 300 or 627 bp long antisense of *eda* gene	This study
pMHE5Tc	pBBR1 replicon, broad-host-range expression vector backbone containing tetracycline resistance cassette (Tc^R)	(Fodor et al. 2004)
pMHEZMGP	pMHE5Tc containing ZMGP	This study
pQE-30 UA	Commercial plasmid containing ampicillin resistance cassette and T5 promoter	Qiagen
pQE30’	pQE-30 UA containing ~50bp of *Z. mobilis fba* gene	This study
pQEFBA	pQE-30 UA containing *Z. mobilis fba* (ZMO0179) gene	This study
pQETPI	pQE-30 UA containing *Z. mobilis tpi* (ZMO0465) gene	This study
pTeasy	Commercial vector containing ampicillin resistance (Amp^R) cassette and ColE1 *ori*	Promega
pTadh-fba	pTeasy containing operon Padh-fba	This study
pTpdc-tpi	pTeasy containing operon Ppdc-tpi	This study

5.3.3 Culture Conditions

E. coli cells were grown Luria-Bertani (LB) medium at 37°C in culture tube or shake flask on a biological shaker shaken at 250 rpm. *E. coli* cells were usually grown under aerobic conditions, wherein the shake flask was filled to 20% volume with media and mouth covered with a techni-cloth. Occasionally, cells were grown under semi-aerobic conditions where instead the flask was filled to 50% volume and mouth covered with an aluminum foil. Lesser background activity due to NADH oxidases was observed for cells grown under semi-aerobic conditions. Chloramphenicol (50 µg/ml) was added to the LB medium for plasmid maintenance. Streptomycin (Str) (50 µg/ml) was used for confirming the presence of JM110. Cells were induced with 0.5 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) (Sigma-Aldrich, St. Louis, MO) at an OD of 0.4 – 0.6. Cells were incubated for four additional hours at reduced temperature of 30°C and then harvested for subsequent analysis.

All concentrations in % are percentages in weight per volume unless otherwise indicated.

Zymomonas mobilis cells were grown at 30°C in RM medium (1% yeast extract, 0.2% KH_2PO_4) containing different amounts of glucose or gluconic acid (as indicated) as carbon source. If not indicated, RM medium contains 2% glucose. pH for RM medium containing gluconic acid was adjusted to 6.0 using NaOH. Chloramphenicol (Cm) (37.5, 50.0 or 100.0 µg/ml) and tetracycline (Tc) (7.5, 15 or 30 µg/ml) were added to the RM medium as needed for maintenance of plasmids in *Z. mobilis* cells. Cm 100 µg/ml was used for selection and subsequent culture of ZM4/pNGFPK. Tc 15 µg/ml was used for selection and Tc 30 µg/ml was used for subsequent culture of ZM4/pMHGPTAF. ZM4/pNGFPK/pMHEZMGP was selected and subsequently cultured in Cm 50 µg/ml and Tc 15 µg/ml. ZM4/pNGFPK/pMHGPTAF was selected on Cm 37.5 µg/ml / Tc 7.5 µg/ml and was subsequently cultured on Cm 50 µg/ml / Tc

15 µg/ml. Cm 50 µg/ml was used for selection and Cm 50 µg/ml or 100 µg/ml culturing of ZM4/pEMP, ZM4/pEMP50, ZM4/pEMP300 and ZM4/pEMP627. A nutrient-rich medium called mating medium was used for regeneration and selection of cells after electroporation. It comprised of 5% glucose, 1% yeast extract (Difco), 0.5% tryptone, 0.25% ammonium sulfate, 0,02% potassium phosphate dibasic and 1mM magnesium sulfate. The pH of mating medium was adjusted to 6.0 using phosphoric acid and it was filter sterilized.

1.5% agar was used for preparing media plates.

5.3.4 Cloning of PPi-PFK, FBP Aldolase and *tpi* Genes in *Z. mobilis*

Plasmid pNGFPK was constructed for overexpression of PPi-PFK in *Z. mobilis* (Figure 19). Detailed construction of pNGFPK and its corresponding empty plasmid, pSTVZM27, is mentioned in Appendix A. Briefly, 1.7 kb ZM27 replication origin was PCR cloned from pZMO3, a 2.7 kb native plasmid of *Z. mobilis* ATCC 10988 (Scordaki and Drainas 1987; Scordaki and Drainas 1990). ZM27 was then ligated into a BamHI digested pSTV28 to obtain plasmid pSTVZM27 of size 4.7 kb (Figure 19). Operon Pgap-PPi-PFK was ligated in pSTVZM27 to obtain pNGFPK.

1.7 kb ZMGP replication origin consists of the entire pZMO1 sequence which is a native plasmid of *Z. mobilis* ATCC 10988 (Arvanitis et al. 2000). Cloning of the linear DNA fragment, ZMGP from the circular pZMO1 was done in such a manner that the only open reading frame present in pZMO1 formed the 3'-end of ZMGP. ZMGP containing plasmid is maintained and replicated within *Z. mobilis* ZM4 cells. ZMGP was ligated to a broad host range expression vector pMHE5Tc at its BglII restriction site to obtain pMHEZMGP (Figure 20). pMHGPTAF was obtained by ligation of operon Ppdc-tpi to pMHEZMGP at restriction site AgeI followed by ligation of operon Padh-fba at XbaI and XhoI restriction sites (Figure 20). Operons Ppdc-tpi and

Padh-fba were obtained using overlap-extension PCR (OE-PCR). FP-Ppdc (5'-GATTAA**CCCGGG**AAACGTTTCCGCTTTG-3') and RP-Ppdc (5'-ATGAGCTTGCGTATTGTCATTGCTTACTCCA-3') were used to clone 550 bp of DNA sequence upstream of pyruvate decarboxylase (*pdc*) gene (ZMO1360) from *Z. mobilis* ZM4 genome. This DNA fragment contains the promoter of *pdc* (Ppdc). FP-tpi (5'-TGGAGTAAGCAATGACAATACGCAAGCTCAT-3') and RP-tpi (5'-GATAATATA**CCCGGG**CAAGCGCCGCTG-3') were used to clone the 750 bp long putative triose phosphate isomerase (*tpi*) gene (ZMO0465) along the 300 bp termination region downstream of *tpi* from *Z. mobilis* ZM4 genome. Thus, This DNA fragment contains the *tpi* gene along with its termination region (tpiT). AgeI compatible XmaI restriction site (shown in bold underlined fonts) was put on end primers - FP-Ppdc and RP-tpi. The two DNA fragments – Ppdc and tpiT – were joined using OE-PCR to construct operon Ppdc-tpi. RP-Ppdc and FP-tpi are complementary to each other to facilitate overlapping during OE-PCR. Operon Padh-fba was also obtained in a similar manner. FP-Padh (5'-GCGCGC**TCTAGA**TTGGAATGAATACCTAACAACTATG-3') and RP-Padh (5'-CTTCGGGAGTAATAGACATAGCTATAACCTCACCCTAC-3') were used to clone 595 bp of DNA sequence upstream of iron-containing alcohol dehydrogenase (adh) gene (ZMO1596) from *Z. mobilis* ZM4 genome. FP-fba (5'-GTAGGGTGAGGTTATAGCTATGTCTATTACTCCCGAAG-3') and RP-fba (5'-AATATA**CTCGAG**GCTCGATCACATCGTTTCCTTGGTC-3') were used to clone the 915 bp long putative fructose bisphosphate aldolase (*fba*) gene (ZMO0179) along with the 250 bp termination region downstream of *fba* from *Z. mobilis* ZM4 genome. XbaI and XhoI restriction sites (shown in bold underlined fonts) are present on FP-Padh and RP-fba respectively. Both the

operons Ppdc-tpi and Padh-fba were ligated to pTeasy vector using A-T cloning to construct pTpdc-tpi and pTadh-fba respectively. These plasmids were transformed in JM109. These plasmids were extracted from the transformed strains and then digested with restriction enzymes mentioned above for ligation in pMHEZMGP vector.

The DNA fragment containing both Ppdc-tpi operon and Padh-fba operon was subcloned from plasmid pMHGPTAF using primers 5'-GCTACG**GCATGC**TTGGAATGAATACCTAACAAC-3' and 5'-ATCTAGA**GTCGAC**AAACGTTTCCGCTTTGATAGC-3'. The DNA fragment was ligated to pNGFPK at SphI and SalI (shown in bold underlined fonts) restriction sites to construct plasmid pEMP as shown in Figure 21.

Figure 19: Plasmid map of pNGPFK (overexpresses PPi-PFK in *Z. mobilis*) and corresponding empty vector (pSTVZM27)

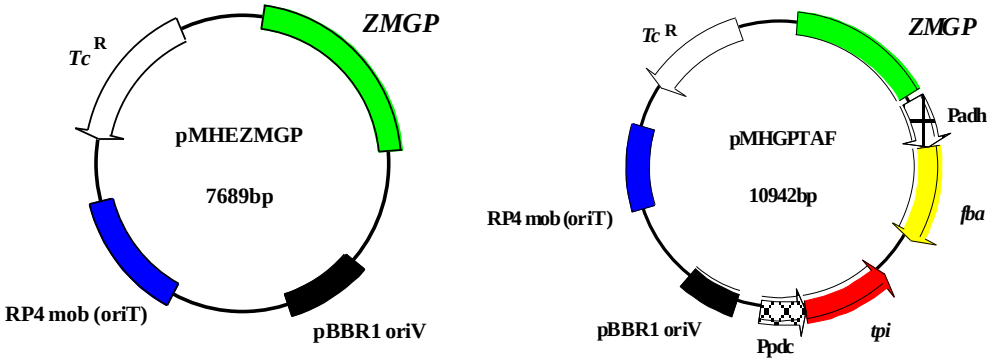

Figure 20: Plasmid map for pMHGPTAF (overexpresses FBP aldolase & tpi in *Z. mobilis*) and corresponding empty vector (pMHEZMGP)

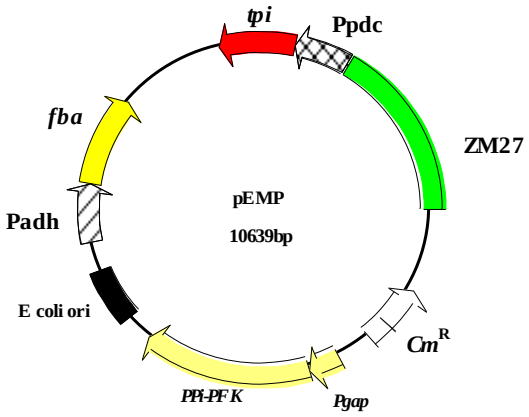

Figure 21: Plasmid map of pEMP (overexpresses three genes - PPi-PFK, FBP aldolase and *tpi* in *Z. mobilis*)

5.3.5 Cloning of FBP Aldolase and *tpi* Genes in *E. coli*

Putative genes annotated for fructose 1,6-bisphosphate aldolase (*fba*) and triose phosphate isomerase (*tpi*) were cloned from the genome of *Z. mobilis* ZM4. Primers used for cloning *fba* were (5'-CGCATGTCTATTACTCCCGAAGTAAAAG-3') and (5'-GCCGCGTCAATCTTTCTTCTTATAAATCTCG-3'). Primers used for cloning *tpi* were (5'-

ATGACAATACGCAAGCTCATCG-3') and (5'-TTACTAGGCTAAACGTCCCCC-3'). Adenine 'A' was added to the 3'-ends of the PCR products and they were ligated to commercial pQE-30 UA vector to generate pQEFBA (carrying *fba* gene) and pQETPI (carrying *tpi* gene) using A-T cloning. Control vector – pQE30' - had a small fragment (~50bp) of *Z. mobilis fba* gene ligated to it (Figure 22). All the three vectors were transformed into JM109. IPTG inducible T5 promoter and histidine tag in pQE-30 UA enabled gene expression and protein purification respectively.

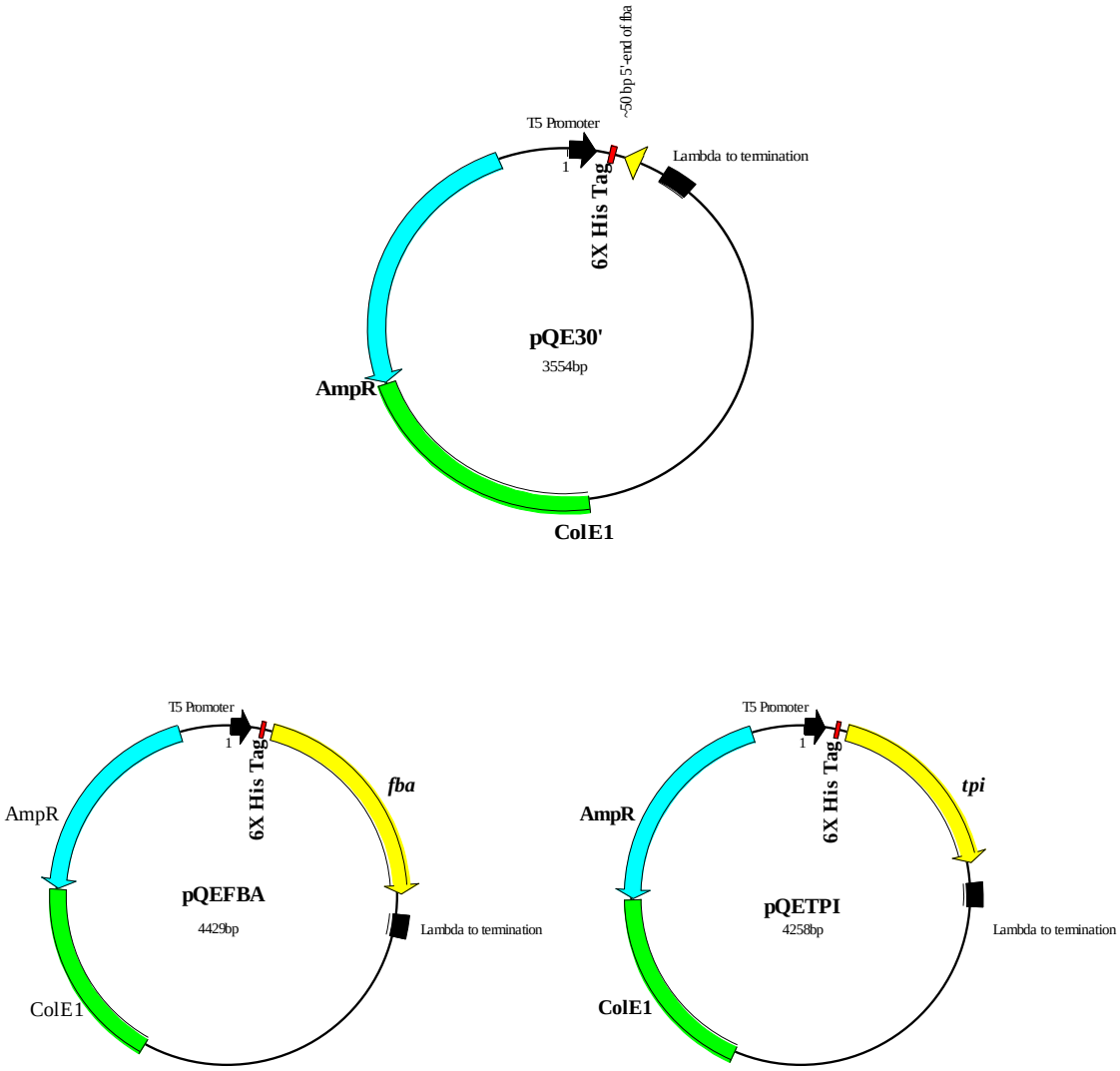

Figure 22: Plasmid map for pQEFBA (contains FBP aldolase gene), pQETPI (contains *tpi* gene) and control plasmid pQE30'

5.3.6 Knockout of ED Aldolase (*eda*)

A suicide vector, pTspecEDA (Figure 23), for the knockout of *eda* was constructed in a similar manner as the suicide vector, pTspecXR, for knockout of ZMO0976 in *Z. mobilis* ZM4 (see chapter 4). Only the homologous regions differ between the two suicide vectors. The 3' HR was cloned using primers – (5'- ATATTA**ACCGGT**AAACCCGTCCATTCTTTGCGTCG-3') and (5'- GCCTGAA**CAATTG**AAAGTGCTTACCCACGTATTGC-3'). Restriction sites are depicted in bold underlined fonts. The 3' HR extends from 0.1 kb upstream to 1 kb downstream of the 3'-end of *eda*. AgeI and MfeI digested 3' HR was ligated to pSTVls vector at XmaI and EcoRI restriction sites to get the suicide vector pSls3'EDA. The 5' homologous region (HR) for *eda* was cloned from ZM4 genome using primers – (5'-GCGACTA**GACGTC**ATTGTTGAGAATAAAGACAATCCG-3') and (5'-ATGTAT**CTCGAG**GTGCGAAGCGTTACTTCAAGAACG-3'). The 5' homologous region extends from 1 kb upstream to 0.1 kb downstream of the 5'-end of *eda*. XhoI and BsaHI digested 5' HR was ligated to pSls3'EDA vector at ClaI and SalI restriction sites to get pSlsEDA.

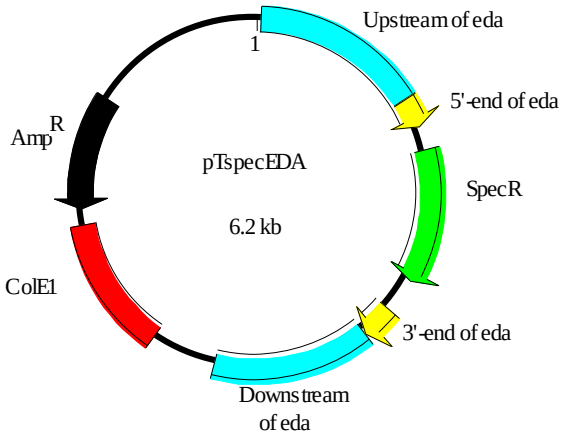

Figure 23: Plasmid map of pTspecEDA, suicide vector for knockout of *eda*

5.3.7 Cloning and Expression of Antisense of *eda*

Three antisense of *eda* (ASeda) of lengths - ~50 bp, ~300 bp and 627 bp - were respectively ligated to pEMP vector at restriction sites SgrAI and SacII to construct three vectors – pEMP50, pEMP300 and pEMP627. ASeda of length approximately 50 bp was obtained by hybridizing primers (5'-ATTTAT**CGCCGGTG**TTAGGCAACAGCAGCGCGCTTGAAAGCAGAAGCTTCTTTAGCGAGTGCCG**CCGCGG**TTAATA-3') and (5'-TATTAA**CCGCGG**CGGCACTCGCTAAAGAAGCTTCTGCTTTCAAGCGCGCTGCTGTTGCCTAA**CGCCGGTG**ATAAAT-3'). Restriction sites are depicted in bold underlined fonts. ASeda of lengths ~300 bp and 627 bp were cloned from genome of *Z. mobilis* ZM4 using forward primer (5'-ATTTAT**CGCCGGTG**TTAGGCAACAGCAGC-3') and respectively reverse primers (5'-TATTAT**CCGCGG**CCAGGTGTTGCTAATGC-3') and (5'-TATGTA**CCGCGG**ATGCGTGATATCGATTCC-3'). The 5'-end of all the three ASeda start from the stop codon of *eda* gene.

5.3.8 Electroporation in *Z. mobilis*

Preparation of electrocompetent cells and electroporation in *Z. mobilis* ZM4 was done as mentioned in chapter 4.

5.3.9 Measuring Intracellular PPi Concentration

Perchloric acid extract of cells was prepared for solubilization of intracellular pyrophosphate (PPi) as described by Mijakovic et al (Mijakovic et al. 2002). PPi concentration in the cell extract was estimated using a colorimetric assay method (Heinonen et al. 1981). Culture broth was rapidly chilled to 4°C using an ethanol / dry ice bath. Chilled cells were harvested by centrifugation at 3,000 g for 10 min at 4°C. Cells were rinsed with perchloric acid ($HClO_4$) by

pouring 0.6 M cold $HClO_4$ over the cells and aspirating it out. Cells were resuspended in five-fold volume of 0.6 M cold perchloric acid. The resuspended solution was kept on ice for 20 min. Precipitated protein and cell debris were removed by centrifugation at 35,000 g for 15 min at 4°C. pH of the supernatant was adjusted to 7.4 with a solution of 0.6 M KOH in 100 mM Tris-HCl (pH 7.4). Precipitated $KClO_4$ was removed by centrifugation at 16,000 g for 10 min at 4°C. Supernatant was removed and an appropriate amount of H_2SO_4 was added such that the final concentration of H_2SO_4 in the supernatant was 0.25 M. 1 ml of MT solution (contains 4 ml of 40 mM ammonium heptamolybdate, 1 ml of 2.5 M H_2SO_4 and 50 µl of triethylamine) was added. After mixing and allowing the solution to stand for at least 15 min, centrifugation was done at 2,000 g for 5 min to get rid of the voluminous yellowish precipitate of phosphomolybdate-triethylamine. Supernatant was removed and 0.5 ml of 2.5 M H_2SO_4 was added to it. After mixing, centrifugation was done at 2,000g for 5 min to get rid of the precipitate that may have come from the first centrifugation. Supernatant was removed and 0.15 - 0.25 ml of 1 M 2-mercaptoethanol solution was added. After mixing and letting the color develop for 15 - 90 min depending on amount of 2-mercaptoethanol added (0.25ml - 0.15ml), absorbance was measured at 700 nm. The absorbance value for the sample was compared to those obtained for pyrophosphate standards to estimate the concentration of pyrophosphate in the sample.

5.3.10 Enzymatic Assays

Cell-free extracts of *Z. mobilis* and *E. coli* were prepared according to a published procedure (Deng et al. 1999). Briefly, cells were harvested by centrifugation at 3,000 g at 4°C for 15 min. Cells were washed twice with ice-cold extraction buffer [5 mM PIPES (piperazine-N,N′-bis(2-ethanesulfonic acid)), 0.1 mM EDTA at pH 7.0, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. EDTA, which is a chelating agent, was not added to the

extraction buffer that was used for preparing cell extracts for FBP aldolase assay. Washed cell pellets were stored at -20°C until use. Cell pellets were resuspended to an OD_{600} of 50 in the extraction buffer and sonicated (8 cycles of 10 s with 30 s cooling period). Cell debris was discarded after centrifugation at 53,300 g for 20 min at 4°C. Supernatant was used as cell-free extract for histidine (his)-tagged protein purification or / and enzymatic assays. Bradford Assay was used for estimation of protein concentration in the extract.

His-tagged protein in the *E. coli* cell-free extract was purified at 4°C using HIS-Select® HF Nickel Affinity Gel (Sigma-Aldrich, St. Louis, MO) which employs immobilized metal-ion affinity chromatography (IMAC). Manufacturer's protocol for large scale purification was followed. Briefly, 10 ml of cell-free extract was added to 1 ml of affinity gel in equilibration buffer. After overnight incubation, the gel was washed five times with equilibration buffer. Gel-bound his-tagged protein was then eluted using 2 ml of elution buffer (50 mM Na_2HPO_4, 300 mM NaCl and 250 mM imidazole at pH 8). Imidazole was removed by overnight dialysis at 4°C using extraction buffer as the dialysis solution.

All enzymatic assay reactions were carried out in a final volume of 200 µl containing 10 µl of cell extract on a microplate at 30°C. NADH absorbance was measured at 340 nm using Spectramax M5 Plus spectrophotometer (Molecular devices).

Pyrophosphate-dependent phosphofructokinase (PPi-PFK) activity was assayed in a reaction mixture containing 20 mM Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (pH 7.2), 1 mM EDTA, 0.3 mM NADH, 1 mM fructose 6-phosphate (F6P) and 6 U/ml each of commercial FBP aldolase, TPI and glycerol 3-phosphate dehydrogenase (G3PDH). Reaction was started by adding sodium pyrophosphate to a final concentration of 1 mM (Deng et al. 1998; Deng et al. 1999). One unit of PPi-PFK activity was defined as the amount of enzyme

producing 1 µmol FBP per minute under the assay conditions. TPI was assayed in a similar reaction mixture consisting of 20 mM TES (pH 7.2), 1 mM EDTA, 0.3 mM NADH and 6 U/ml of G3PDH. Reaction was started by adding DL-glyceraldehyde 3-phosphate solution to a final concentration of 5.25 mM. One unit of TPI activity was defined as the amount of enzyme producing 1 µmol dihydroxyacetone phosphate per minute under the assay conditions. FBP aldolase was assayed in a medium free of EDTA. The assay medium consisted of 80 mM Tris-HCl (pH 7), 0.3 mM NADH and 6 U/ml each of commercial TPI and G3PDH. Metallic ions as indicated were added to the assay medium at a final concentration of 3 mM. Reaction was started by adding FBP to a final concentration of 2.25 mM (Baldwin and Perham 1978). One unit of FBP aldolase activity was defined as the amount of enzyme cleaving 1 µmol FBP per minute under the assay conditions. Phosphoglucose isomerase (PGI) was assayed in a 50 mM Tris-Cl buffer (at pH 8) containing 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, 3 mM NAD, 2 mM F6P and 1 U/ml glucose 6-phosphate dehydrogenase.

The apparent K_m values for PPi-PFK were obtained by fixing the concentration of one of the substrates – PPi or F6P – at saturating concentration of 1.6 mM and fitting the data to Lineweaver-Burk equation: $1/v = (K_m/V_{max})\,[S] + 1/V_{max}$.

20 mM potassium phosphate buffer (pH 6.5) containing 1 mM PMSF was used as extraction buffer for 2-keto-3-deoxy-phosphogluconate (KDPG) aldolase assay. KDPG aldolase activity was assayed in a reaction mixture containing 20 mM potassium phosphate buffer (pH 7.5), 0.3 mM NADH and 105 U/ml L-lactic dehydrogenase (LDH) (Griffiths et al. 2002). Reaction was started by adding KDPG to a final concentration of 2 mM. One unit of KDPG aldolase activity was defined as the amount of enzyme cleaving 1 µmol of KDPG per minute under the assay conditions.

5.3.11 Analytical Methods

Cell growth was determined by measuring optical density (OD) at 600 nm using spectrophotometer (Beckman Coulter DU 530, Brea, CA). Samples taken during fermentation were spun down by centrifugation and supernatants were analyzed for concentrations of glucose, gluconic acid, acetic acid, ethanol and dihydroxyacetone (DHA) using high-performance liquid chromatography (Agilent Technologies) instrument equipped with an Aminex HPX-87H column (Bio-Rad). 5 mM H_2SO_4 at a flow rate of 0.4 ml/min was used as the mobile phase. The ratio of labeled and unlabeled ethanol was determined using gas chromatography-mass spectrometry (GC-MS) by measuring the ratio of peak areas corresponding to mass / charge (m/z) of 47 and 46 and applying the correction for natural abundance. This m/z ratio corresponds to the positively charged molecular ion of ethanol obtained from electron impact (EI) of ethanol molecule. Sample preparation was similar to that for HPLC. Supernatant was heated in a vial and the headspace was fed into the GC-MS (Agilent Technologies) for analysis. The instrument was equipped with an Agilent J&W DB-624 fused silica capillary column. Helium gas at a flow rate of 1.1 ml/min was used as the mobile phase.

SDS-PAGE and Coomassie blue staining were used to confirm the expression of FBP aldolase and triosephosphate isomerase (TPI) proteins. 15 µg of cell-free extract protein and 1 µg of purified protein were loaded on a 12% Tris-HCl gel (Bio-Rad, Hercules, CA).

5.4 Results

5.4.1 PPi-PFK Overexpression in *Z. mobilis*

Recent genome sequencing of *Z. mobilis* ZM4 reveals that the only missing gene for the EMP pathway is phosphofructokinase (*pfk*) (Seo et al. 2004). Hence, EMP pathway can be completed in ZM4 by expression of a phosphofructokinase (PFK). As shown in Figure 24, PFK

catalyzes the conversion of fructose-6-phosphate (F6P) to fructose 1,6-bisphosphate (FBP), which is more amenable to enzymatic reactions due to its symmetricity, by using the phosphoanhydride bond energy stored in adenosine triphosphate (ATP) or PPi (Mertens 1991). Thus, PFK can be of two types –

a) ATP – dependent (EC 2.7.1.11): predominantly irreversible ($\Delta G° = -4.41$ kcal/mole)

b) PP_i – dependent (EC 2.7.1.90): reversible ($\Delta G° = -2.08$ kcal/mole)

Even after the expression of a heterologous *pfk* gene in *Z. mobilis*, the EMP pathway may not get switched on. There can be several factors such as inadequate expression of EMP-associated genes or unknown repressors of the EMP pathway which can hinder flux through it. Thus PFK is a necessary condition for switching on EMP but may not be a sufficient condition. PPi-PFK was chosen over ATP-PFK since PPi-PFK will result in more ATP production. Also, if ATP-PFK is used, the extra ATP it generates may be lost in futile recycling involving ATP-PFK and fructose-1,6-biphosphatase, which catalyzes the conversion of FBP to F6P (Steinbuchel 1986). PPi-PFK from *Borrelia Burgdorferi* B31 was chosen since it is a non-allosteric form of PPi-PFK. As most of the glycolytic enzymes in *Z. mobilis* are non-allosteric, it is apt and rather convenient to use a non-allosteric enzyme. The heterologous enzyme PPi-PFK was successfully expressed in *Z. mobilis* ZM4 to construct ZM4/pNGFPK.

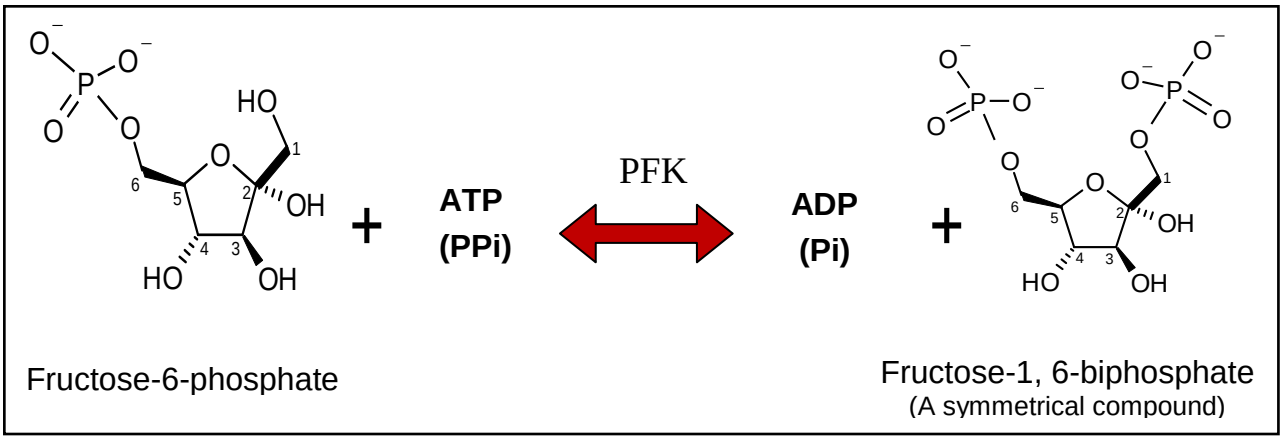

Figure 24: Reaction catalyzed by phosphofructokinase. ATP-PFK uses ATP while PPi-PFK uses PPi for converting F6P to FBP

5.4.1.1 PPi-PFK Activity

In vitro activity assay for PPi-PFK was performed to confirm the *in vivo* activity of PPi-PFK, expressed in the cells carrying plasmid pNGFPK. As depicted in Figure 25, engineered strain showed significant amount of PPi-PFK activity reaching a maximum of 3 U/mg cell proteins. No activity was observed for the control strain. Activity of PPi-PFK in the engineered strain is comparable to the activity of native PPi-PFK in *E. histolytica* (Deng et al. 1998). However, it is an order of magnitude smaller than the activity in recombinant *E. coli* expressing IPTG-inducible PPi-PFK from B burgdorferi (Deng et al. 1999). This occurs since induction by IPTG leads to a significantly higher expression of the PPi-PFK gene.

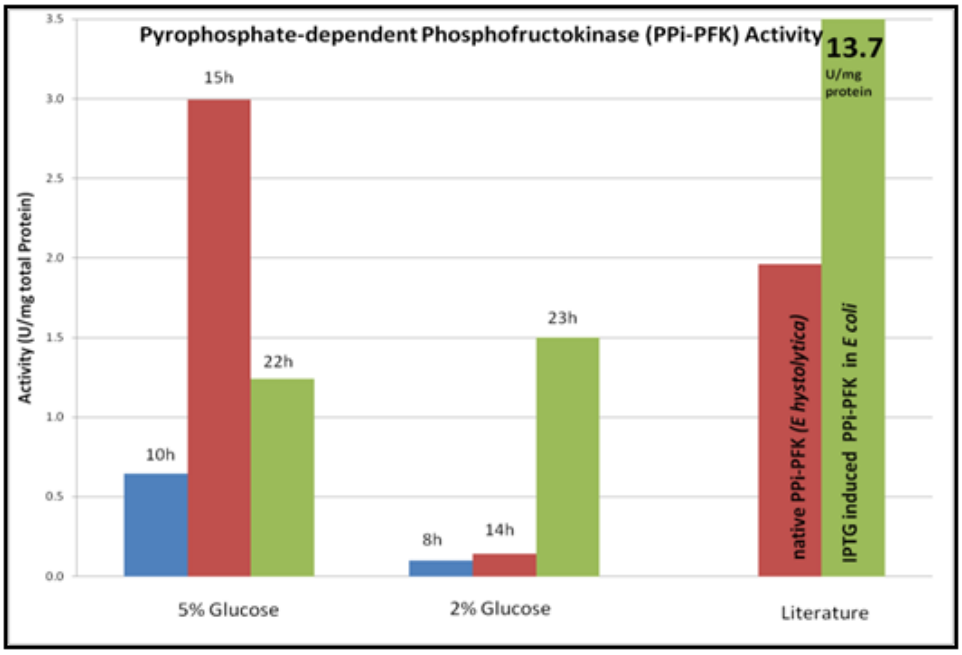

Figure 25: PPi-PFK activity assay in the engineered strain. Engineered cells were grown in RM medium containing either 5% glucose or 2% glucose. The time at which the cells were harvested is indicated at the top

5.4.1.2 Kinetic Parameter (K_m) for Enzyme PPi-PFK

PPi and F6P are the two substrates of PPi-PFK (Figure 24). Both should be present *in vivo* in adequate quantities (greater than their apparent Michaelis constant 'K_m') to ensure that PPi-PFK is operating close to its maximum velocity (V_{max}). This would ensure sufficient flux through the reaction step and consequently through the EMP pathway. Kemp and Tripathi had concluded that low levels of PPi may be responsible for the inability of an *E. coli* mutant deficient in ATP-PFK but transformed with heterologous PPi-PFK to grow in minimal media supplemented with mannitol (Kemp and Tripathi 1993). Hence, the apparent K_m values need to be determined and compared with the *in vivo* concentrations. As shown in Table 12, the experimentally determined apparent K_m^{F6P} is approximately two times higher than the value reported in the literature. Data for K_m^{PPi} is comparable to reported data in literature. This can be

due to the fact that crude extract of *Z. mobilis* was used while crude extract of *E. coli* was used in the literature. There can be metabolites present within the crude extract affecting the activity of the enzyme PPi-PFK and hence the K_m values.

Table 12: Apparent K_m (F6P and PPi) values for enzyme PPi-PFK. All values are in μM

	$\mathbf{K_m^{PPi}}$	$\mathbf{K_m^{F6P}}$
Recombinant *Z. mobilis* (crude extract, experiment)	22	223
Recombinant *E. coli* (crude extract, PPi-PFK from *B. Burgdorferi*) (Deng et al. 1999)	15	109
E. histolytica (crude extract) (Deng et al. 1998)	39	100
E. histolytica (purified enzyme) (Deng et al. 1998)	40	85

5.4.1.3 Intracellular PPi Concentration in *Z. mobilis*

Intracellular concentrations of both PPi and F6P in *Z mobilis* have not been reported in literature. Glucose 6-phosphate (G6P) is a metabolite of the ED pathway (Figure 3); hence it must be present in adequate concentration. Conversion of G6P to F6P is catalyzed by PGI which is usually expressed in wild-type *Z mobilis* at sufficient levels as determined by Hesman et al and also by us in this study (Hesman et al. 1991). Thus, intracellular concentration of F6P should be sufficient. However, if intracellular concentration of PPi is less than the apparent K_m^{PPi}, then it will be difficult to increase the concentration of PPi. PPi gets degraded very quickly in the cells especially by pyrophosphatase to ensure the irreversibility of biosynthetic pathways. Since PPi is part of the energy pool (which also includes ATP, ADP, AMP and Pi) of the cell, its metabolism is tightly regulated and hence cannot be manipulated easily. Fortunately, intracellular PPi

concentrations in control and engineered strains were measured to be at least 10 times more than the K_m^{PPi} value (Table 13) and of the same order as that reported for *E. coli* in literature (Kukko and Heinonen 1982). From these experimental data, it can be concluded that PPi is present *in vivo* in sufficient quantity to drive PPi-PFK at V_{max}.

Table 13: Intracellular pyrophosphate concentration for control and engineered strains and their comparison to *E. coli*

	Intracellular PPi concentration	
	µM	**nmol/mg total protein**
ZM4/pSTVZM27 (Control)	367 ± 88	2.6 ± 0.1
ZM4/pNGFPK (Engineered)	301 ± 9	4.8 ± 0.5
***Escherichia coli* (Kukko and Heinonen 1982)**	500	2.5

5.4.2 Effects of Overexpression of PPi-PFK in *Z. mobilis*

5.4.2.1 Effect on Growth and Fermentation Characteristics

ZM4/pNGFPK and the corresponding control (ZM4/pSTVZM27) exhibited similar rates of growth, glucose consumption and ethanol production (data not shown). Compared to the control, the engineered strain is at a disadvantage due to constitutive expression of PPi-PFK at high levels (because of the gap promoter). Expression of the extra protein may result in a decrease in glycolytic flux and growth rate depending on the amount of overexpressed protein. This is due to competition of the overexpressed gene with expression of all other genes or simple dilution of all other proteins due to the overexpressed protein (Snoep et al. 1995). If there is no flux through EMP pathway, expression of PPi-PFK simply adds to the 'protein burden'. PPi-PFK

behaves as a gratuitous (non-functional, non-toxic) protein (Kurland and Dong 1996) in this case and its accumulation can result in reduced growth of engineered strain as compared to control. The lack of significant differences between the two strains suggests that there is unlikely a large flux through EMP under the condition tested.

5.4.2.2 Performance of Engineered Strain under Stress Conditions

If the EMP pathway is operational in the engineered strain – ZM4/pNGFPK, then more ATP production will possibly equip it to better tolerate 'stress' conditions. The maintenance energy coefficient for *Z. mobilis* cells has been found to increase under stress conditions, such as high concentrations of acetic acid (Joachimsthal et al. 1998) and ethanol (John Fieschko 1983; Rogers P L 1982), high temperature (Rogers P L 1982) and low pH (Lawford and Ruggiero 1990) . As an example, Beyeler et al. reported a maintenance energy coefficient of 1.6 g glucose/g DCW/h for ZM4 cultured in a 100g glucose/l medium without acetate in a continuous fermenter with complete cell recycling (Beyeler et al. 1984). This coefficient is approximately 2.5 times smaller than that reported by Joachimsthal et al which was 3.9 g/g/h for ZM4 cultured in 111.4 g/l glucose medium under 'stress' of 20 g sodium acetate/l in a similar fermenter (Joachimsthal et al. 1998). The following stress conditions were tested to see if cells expressing PPi-PFK exhibit better tolerance. However, insignificant differences in terms of growth and fermentation capability were observed between the control and engineered strains.

a) High acetic acid concentration (1%) – Additional ATP availability in the cells may confer increased resistance to high concentrations of acetic acid. Cells usually deal with high concentrations of acetic acid by pumping H^+ out of the cell, by expressing stress genes or by changing membrane permeability (Warnecke and Gill 2005). All of these processes require energy.

b) High glucose concentrations (5%, 10% and 17.5%) – Increased osmotic pressure would require more ATP as maintenance energy to maintain the rigidity of cell membranes.

c) Cu^{2+} ions (0.5mM to 3mM) – Cupric ions inhibit 2-keto-3-deoxy-phosphogluconate (KDPG) aldolase enzyme of ED pathway *in vitro* at a concentration of 1 mM (Knappmann et al. 1995). Thus, by subjecting cells to high concentration of Cu^{2+} (between 0.5 and 3 mM), it was expected that the flux through ED pathway will get blocked and only EMP would be operational. Thus, only the engineered strain will be able to grow while the control would not. However, increasing concentrations of Cu^{2+} progressively inhibited growth to similar levels in both control and engineered strains (data not shown).

5.4.2.3 Investigation on Reasons for Non-Observance of Differences between Control and Engineered Strains

As the control and engineered strains exhibited similar growth and fermentation profiles, this means that the extracellular fluxes and rate of biosynthesis remained the same even though an additional element (heterologous enzyme PPi-PFK) was introduced in the central carbon metabolism. Even under stress conditions, no difference was observed between the control and engineered strains in terms of growth and fermentation rates. During stress conditions, since maintenance energy requirements increase, less ATP is available for growth. Thus, the experimental observation suggests that ATP production in engineered strain is not significantly different from that in the control. Hence, EMP pathway may not be operational or the EMP flux is very small. The reasons for insignificant flux through EMP can be –

a) Insufficient expression of EMP-associated native enzymes which limits the glycolytic flux through the EMP pathway.

b) Unknown regulatory elements inhibiting the EMP pathway.

5.4.2.4 Identification of Rate-Limiting Steps for EMP Operation

The activity of other enzymes in EMP pathway was measured to evaluate if they were getting expressed at a level comparable to that of PPi-PFK. The enzymes which are common to ED and EMP pathways were not assayed since as ED pathway is functional in cells, these common enzymes must be expressed at sufficient levels. As shown in Figure 26, the three enzymes - phosphoglucose isomerase (PGI), FBP aldolase and triose phosphate isomerase (TPI) - are unique to the EMP pathway. *Z. mobilis* has the genes encoding these enzymes but when degrading glucose through the ED pathway, they are not necessarily expressed as their activities are not needed.

a) PGI – PGI catalyzes the inter-conversion between G6P and F6P. The activity for PGI (Figure 27) compared well with the literature values (Hesman et al. 1991). The values varied between 1 and 2 U/mg total protein in crude extract. Thus it is of the same order as PPi-PFK activity.

b) FBP Aldolase - The activity varied between 1-5 mU/mg total cell protein which is 2 orders of magnitude smaller than that of PPi-PFK. In some samples, it was difficult to distinguish the background activity from the FBP aldolase activity i.e. the activity was too low to measure. Background activity is usually high in crude extract due to plenty of contaminants (Kruger 1995). Literature is ambiguous about the activity of this enzyme in *Z. mobilis* (Dawes et al. 1966; Horbach et al. 1994). Even when the values are reported, they are still very low compared to PGI and PPi-PFK activity.

c) TPI - The activity varied between 10-100 mU/mg total cell protein which is an order of magnitude smaller than that of PPi-PFK. Activities of TPI reported in literature (Algar and Scopes 1985) are within the same range.

It is possible that low activities of FBP aldolase and TPI may not be able to drive glycolytic flux through EMP pathway. Some studies have shown that glucose metabolism in *Z. mobilis* occurs at the rate of 1μmol glucose/min/mg cell protein (Arfman et al. 1992) which is equivalent to at least 1 U of *in vivo* activity for any EMP enzyme if glucose metabolism occurred solely through the EMP pathway. In summary, low activities of FBP aldolase and TPI are likely limiting the carbon flux through the EMP pathway.

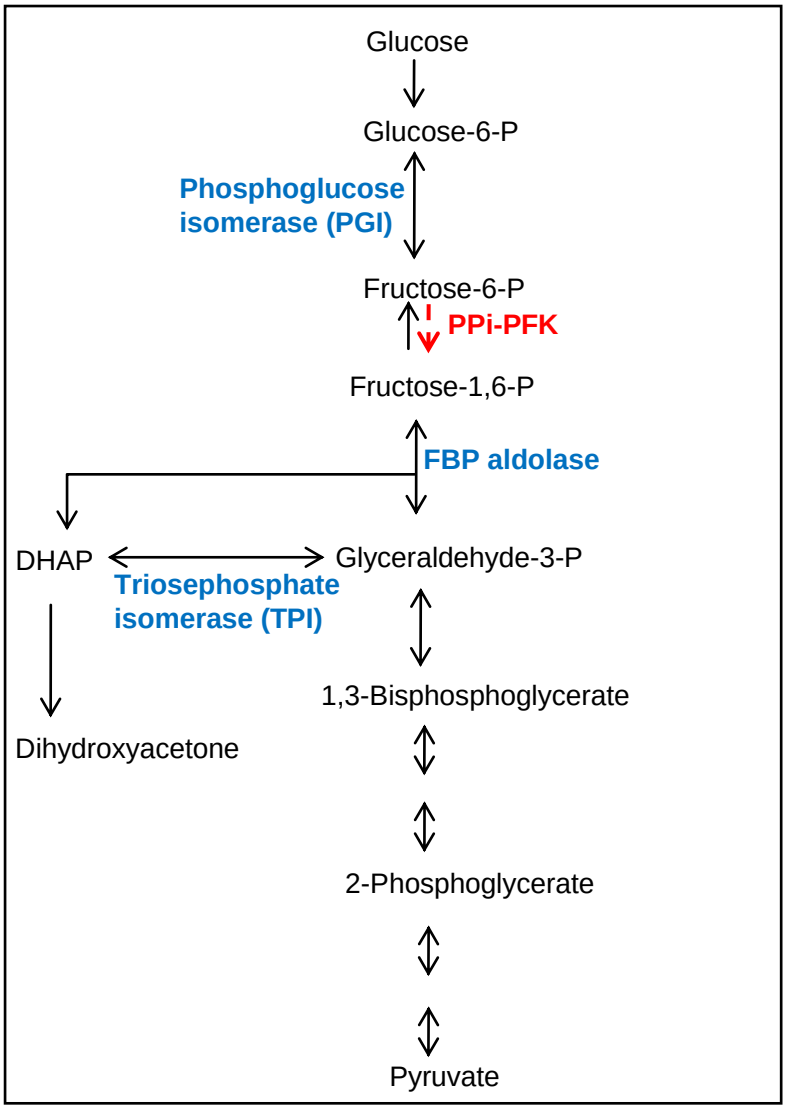

Figure 26: EMP pathway in *Z. mobilis*

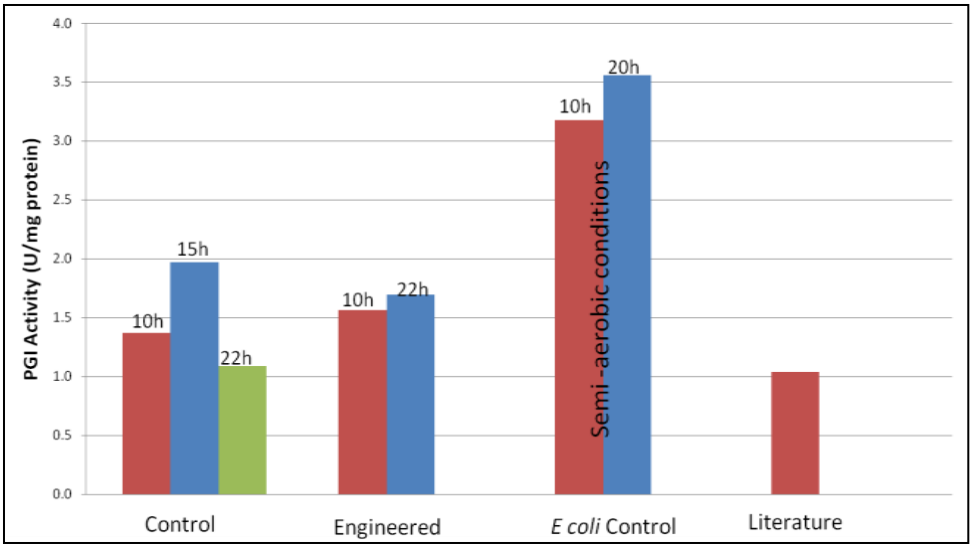

Figure 27: Comparison of PGI activities. *Z. mobilis* cells were grown in RM (5% glucose). *E. coli* cells were grown in LB media under semi-aerobic conditions. Time at which the cells were harvested is indicated at the top of the bars

5.4.3 FBP Aldolase and *tpi* of *Z. mobilis* Encode for Active Enzymes

Putative genes of *Z. mobilis* ZM4 encoding for FBP aldolase (FBA) and TPI were chosen for overexpression in ZM4 to facilitate the establishment of EMP pathway. Our experiments showed that cell-free extract of ZM4 possessed both these activities indicating that the putative *fba* and *tpi* of *Z. mobilis* may encode for active enzymes. Use of native genes is desirable since it will lead to easier expression in *Z. mobilis* ZM4 known to possess a strong restriction-modification system, which makes electroporation of heterologous DNA difficult (Kerr et al. 2011; Phillips 2006).

ZMO0179 gene of *Z. mobilis* ZM4 has been annotated as a Class I (metal-independent) fructose 1,6-bisphosphate aldolase (*fba*) of archaeal type based on homology with known *fba*. Similarly, ZMO0465 gene has been annotated as triose phosphate isomerase (*tpi*). *fba* and *tpi* of *Z. mobilis* are 915 bp and 750 bp respectively.

Both these genes were cloned from ZM4 and expressed in *E. coli* JM109. As shown in Figure 28, cell-free extract (CFE) of JM109/pQEFBA had an activity of 40 mU/mg total cell protein while the control strain JM109/pQE30' had four fold lower activity at 10 mU/mg total cell protein. Both these activities were obtained in the presence of Mg^{2+} in the assay medium. Without the addition of Mg^{2+}, FBP aldolase activity in CFE of JM109/pQEFBA and JM109/pQE30' reduced to 5 mU/mg total protein. Using Mn^{2+} and Ca^{2+} instead of Mg^{2+}, resulted in 50% higher and 25% lower activities respectively, indicating that Mn^{2+} is the best metallic cofactor of FBP aldolase of ZM4 among the three metallic ions. With Zn^{2+}, Na^{+} and Fe^{3+}, an activity of only 5 mU/mg protein was observed for both JM109/pQEFBA and the corresponding control, JM109/pQE30'. All these observations suggest that the extra metal-dependent FBP aldolase activity observed in CFE of *E. coli* JM109/pQEFBA must be due to the overexpressed FBP aldolase of ZM4. His-tagged FBP aldolase protein was purified from the CFE of JM109/pQEFBA. Purified FBP aldolase had an activity of 60 mU/mg protein with Mg^{2+} ions. Purified FBP aldolase activity showed a similar response to Mn^{2+} and Ca^{2+} as the CFE. Purified FBP aldolase showed no activity if Zn^{2+}, Na^{+} and Fe^{3+} were used as a metal co-factor. Activity could not be recovered in the assay reactions even after the addition of the right metal co-factors such as Mg^{2+}, Mn^{2+} and Ca^{2+}. Thus, FBP aldolase is a metal-dependent enzyme, belonging to Class II rather than the annotated class I.

As shown in Figure 29, cell-free extract (CFE) of JM109/pQETPI had an activity of 10 U/mg total cell protein while that of the control strain, JM109/pQE30', had only half of the activity. Purified TPI had an activity of 60 U/mg protein. Thus, the expression of FBP aldolase and *tpi* cloned from ZM4 in *E. coli* confirmed that both these genes encode for active enzymes. The molecular weight of FBP aldolase and TPI obtained from its amino acid sequence is 33 and

26 kDa respectively. This is consistent with the observation on SDS PAGE (figure not shown), where the bands corresponding to the purified proteins were present between 25 and 37 kDa.

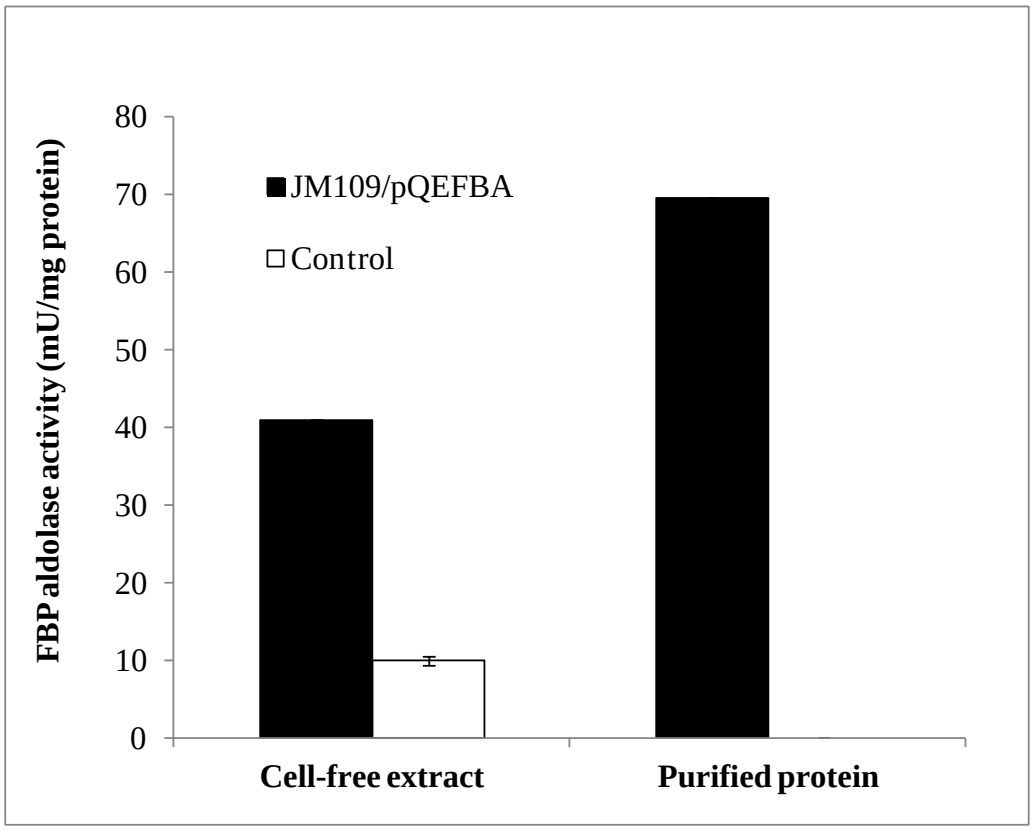

Figure 28: FBP aldolase activity in cell-free extract and his-tagged purified protein

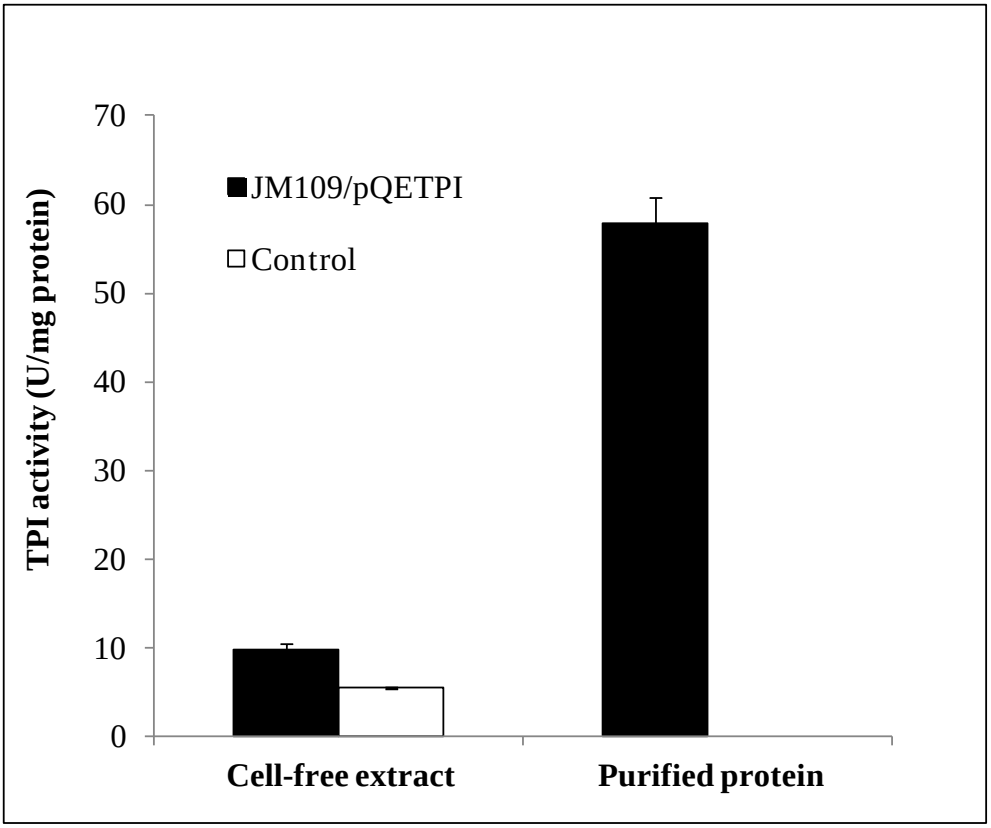

Figure 29: TPI activity in cell-free extract and his-tagged purified protein

5.4.4 FBP Aldolase and *tpi* Overexpression in *Z. mobilis*

FBP aldolase and *tpi* were overexpressed in *Z. mobilis* ZM4 using native promoters – Padh and Ppdc respectively - to construct ZM4/pMHGPTAF. Both of these promoters have been used in *Z. mobilis* to drive expression of homologous or heterologous genes (Conway et al. 1987b; Douka et al. 2001; Mackenzie et al. 1989). Cell-free extract of ZM4/pMHGPTAF had an FBP aldolase activity of 20 mU/mg cell protein and TPI activity of 300 mU/mg cell protein. The corresponding control ZM4/pMHEZMGP had an FBP aldolase activity of 1-5 mU/mg cell protein and TPI activity of 16 mU/mg cell protein. Thus, the overexpression of FBP aldolase and *tpi* yields up to twenty fold higher activity compared to the native levels. As shown in Figure 30,

fermentation of 2% glucose present in RM medium by ZM4/pMHGPTAF resulted in a production of 0.08% dihydroxyacetone (DHA). No dihydroxyacetone was produced by the control ZM4/pMHEZMGP or the wild-type strain ZM4. Thus, the overexpression of FBP aldolase and *tpi* results in alteration of metabolism of *Z. mobilis* ZM4.

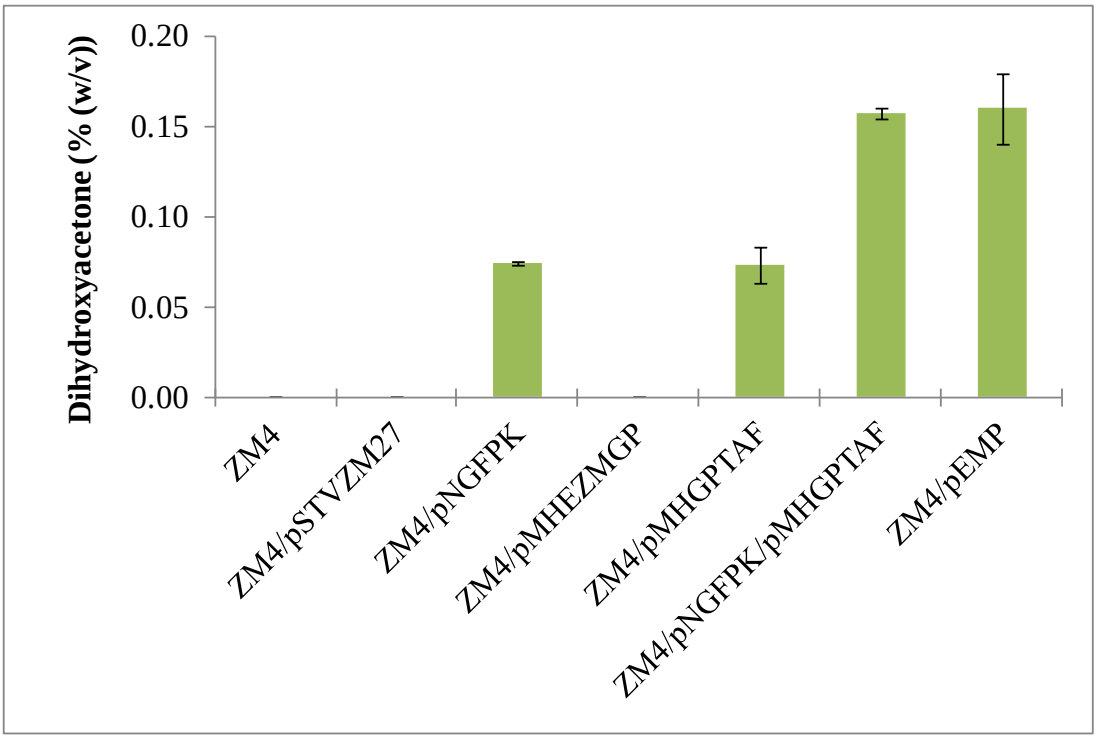

Figure 30: Dihydroxyacetone production from 2% glucose in *Z. mobilis*

5.4.5 Simultaneous Overexpression of PPi-PFK, FBP Aldolase & *tpi* in *Z. mobilis*

PFK catalyzes the missing step, and FBP aldolase and TPI catalyze the bottleneck steps of the EMP pathway in *Z. mobilis* ZM4. Thus, all the three genes – PPi-PFK, FBP Aldolase & *tpi* – must be simultaneously overexpressed in *Z. mobilis* to allow a significant carbon flux through the EMP pathway. The PPi-PFK gene is present in the plasmid pNGFPK. FBP aldolase & *tpi*

genes are present in plasmid pMHGPTAF. Simultaneous overexpression of the three genes was done in two ways –

a) ZM4/pNGFPK/pMHGPTAF – pMHGPTAF was electroporated in electrocompetent ZM4/pNGFPK
b) ZM4/pEMP – A new plasmid pEMP was constructed that contains all the three genes.

5.4.5.1 Lowering Antibiotic Concentration Resulted in Successful Transformation

Even after several attempts, positive clones could not be obtained by electroporation of pMHEZMGP or pMHGPTAF in electrocompetent ZM4/pNGFPK. Concentrations of antibiotics used for selection of electroporated cells were 100 µg/ml for chloramphenicol and 15 µg/ml for tetracycline. 100 µg/ml for chloramphenicol (Cm) and 10 - 20 µg/ml for tetracycline (Tc) have been commonly used concentrations as selection pressure for electroporated *Z. mobilis* (Arfman et al. 1992; Kerr et al. 2011; Zou et al. 2011b). However, only spontaneous mutants were obtained when Cm 100 µg/ml and Tc 15 µg/ml were used for electroporation of pMHEZMGP or pMHGPTAF in ZM4/pNGFPK after incubation for more than a week. However, when the concentrations of antibiotics were lowered to Cm 37.5 µg/ml and Tc 7.5 µg/ml, positive clones appeared. The concentration of antibiotics used is only 50% higher than the minimum inhibitory concentrations (MIC) of Cm and Tc, which are 25 µg/ml and 5 µg/ml respectively (Skotnicki et al. 1984). Though using antibiotic concentration close to MIC can result in several negative clones, but at the same time, lower concentrations also impose less stress on the cells, thereby helping the electroporated cells to recover. Similarly, ZM4/pEMP was obtained by selection on Cm 37.5 µg/ml. Indeed, throughout this project, when it was difficult to obtain a positive clone for *Z. mobilis*, antibiotic concentrations at levels 50 or 100% higher than MIC were successfully employed to counter the low transformation efficiency. Several researchers have observed

extremely low transformation efficiencies in *Z. mobilis* (Dong et al. 2011; Kerr et al. 2011; Lam et al. 1993; Zou et al. 2011a). Once the positive clones were obtained, interestingly, they were able to grow in solid and liquid media at the commonly used higher antibiotic concentrations.

5.4.5.2 Dual Plasmid System in *Z. mobilis* ZM4

To our knowledge, there is no report of *Z. mobilis* ZM4 harboring more than one engineered plasmids. There is only one report of the presence of two engineered plasmids in *Z. mobilis* CP4 (De Graaf et al. 1999). We have shown in this study that two engineered plasmids can be stably maintained in *Z. mobilis* ZM4. Our study shows that ZM27 replication origin (carried by pNGFPK) and ZMGP replication origin (carried by pMHGPTAF) are compatible in *Z. mobilis* ZM4.

5.4.5.3 Growth and Fermentation by *Z. mobilis* Overexpressing PPi-PFK, FBP Aldolase & *tpi*

A growth and fermentation study for the two strains - ZM4/pNGFPK/pMHGPTAF and ZM4/pEMP – was done in RM medium to evaluate the effects of the simultaneous overexpression of the three genes – PPi-PFK, FBP aldolase and *tpi*. However, no growth and fermentation advantage could be seen for ZM4/pNGFPK/pMHGPTAF or ZM4/pEMP compared to the wild-type strain ZM4. This can be an indication that the overexpression did not switch on the EMP pathway. It is unlikely that a large flux through EMP exists since the energy-efficient EMP pathway is expected to result in higher rates of growth and fermentation. Also, *Zymomonas mobilis* has the lowest molar growth yield reported for a micro-organism since it only uses the ED pathway (Swings and De Ley 1977). Hence, operation of EMP pathway in *Z. mobilis* is expected to result in an increase in growth yield making it comparable to other micro-organisms who are capable of using EMP pathway such as *E. coli*.

Overexpression of only PPi-PFK or only FBP aldolase and *tpi* in ZM4 resulted in a final titer of 0.08% dihydroxyacetone from RM medium which contains 2% glucose (Figure 30). Thus, when all the three genes are overexpressed, a higher titer of dihydroxyacetone can be expected. Indeed, as shown in Figure 30, ZM4/pNGFPK/pMHGPTAF or ZM4/pEMP produced 0.16% dihydroxyacetone which is interestingly equal to the sum of that produced individually by ZM4/pNGFPK and ZM4/pMHGPTAF. Even though only a small amount (0.16%) of dihydroxyacetone is produced, it indicates a significant alteration of metabolism, since *Z. mobilis* ZM4 converts all glucose to ethanol and produces insignificant amount of other byproducts such as lactic acid, glycerol and acetic acid (Swings and De Ley 1977). It can be seen in Figure 26 that the overexpression of the three genes may result in an increased amount of dihydroxyacetone phosphate. This dihydroxyacetone phosphate gets secreted as dihydroxyacetone in the culture media in ZM4/pNGFPK/pMHGPTAF or ZM4/pEMP. Control strains or wild-type ZM4 did not produce any dihydroxyacetone.

5.4.6 Expression of Antisense of *eda* in *Z. mobilis*

As the overexpression of the genes corresponding to missing and rate-limiting steps of the EMP pathway did not appear to switch on the pathway, another strategy was employed. If the carbon flux through the native ED pathway can be partially or completely blocked in ZM4/pEMP, cells may try to push the restricted ED carbon flux instead through the engineered EMP pathway.

The ED pathway may be blocked by knocking out any of the several enzymes of the ED pathway (Figure 3). ED aldolase (*eda*) was chosen for knockout. ED aldolase or KDPG aldolase is a key enzyme of the ED pathway that cleaves 2-keto-3-deoxy-phosphogluconate (KDPG) into pyruvate and glyceraldehyde 3-phosphate (GAP) (Figure 3). The metabolite, KDPG, has been

reported to be toxic to cells (Conway et al. 1991; Lorra K Fuhrman 1998). Hence, the knockout of ED aldolase would lead to the accumulation of toxic KDPG. This would result in death or growth inhibition of those cells that channel carbon flux through the ED pathway but those cells that can channel the flux through the EMP pathway will survive as the EMP pathway does not produce any KDPG (Figure 3).

However, this essential gene could not be knocked out in ZM4/pEMP even though the rescue plasmid pEMP was present, which would have rescued the cells by re-routing the carbon flux through the EMP pathway once the ED pathway was shut down. The experimental design for *eda* knock out was similar to the one used for successful knockout of xylose reductase (ZMO0976) from the ZM4 genome. Failure of the knockout experiment suggests that the knockout of *eda* may be lethal to the cells.

An alternate strategy for silencing the *eda* gene was employed which involved the expression of the antisense of *eda* (ASeda). This strategy can do a partial knock down of *eda* expression, thus safeguarding cells against the lethal effects of a complete *eda* knockout. The antisense of *eda* was placed after the PPi-PFK gene in the plasmid pEMP (Figure 21). This facilitated expression of ASeda using Pgap which is a native constitutive promoter of ZM4. Transcription from Pgap promoter will generate an mRNA which is a fusion of PPi-PFK gene and ASeda. Three different lengths - ~50 bp, ~300 bp and 627 bp of antisense of *eda* gene (which is 627 bp long) were used to construct three vectors respectively – pEMP50, pEMP300 and pEMP627. The three different vectors will silence the *eda* gene in the cells to different extents. For example, 627 bp long ASeda would silence the gene to a greater extent than the 50 bp ASeda. It has been reported that the silencing of the gene by its antisense is proportional to the length of the antisense (Oddone et al. 2009; Sturino and Klaenhammer 2004).

A growth experiment was conducted for all the three constructs using ZM4/pEMP as a control in RM medium supplemented with Cm 50 µg/ml. As shown in Figure 31, growth profile of ZM4/pEMP50 is nearly similar to that of the control strain ZM4/pEMP. This indicates that the expression of ASeda of length 50 bp has negligible effect on cells. ASeda of length 50 bp is less than 10% of the length of *eda* gene (627 bp). The growth rates for both ZM4/pEMP300 and ZM4/pEMP627 were similar, but much slower than that for the control strain. The maximum specific growth rates for ZM4/pEMP300 and ZM4/pEMP627 were approximately 40% less than that of the control strain. Thus, expression of antisense of *eda* of lengths 300 and 627 bp resulted in repression of cell growth, perhaps due to a decrease in the activity of ED aldolase. This was confirmed by measuring the ED aldolase activity in the cell-free extracts of the four strains. For the control strain and ZM4/pEMP50, ED aldolase activities were nearly similar at 1.6 U/mg cell protein. For ZM4/pEMP300, ED aldolase activity is 1.1 U/mg cell protein which is 34% lower than that for the control strain. For ZM4/pEMP627, activity was still lower at 1.0 U/mg cell protein. This is 39% lower than that for the control strain. The lower activities observed for ZM4/pEMP300 and ZM4/pEMP627 confirm the effectiveness of ASeda of lengths 300 and 627 bp in silencing the *eda* gene partially. Also, ASeda of length 50 bp was not able to silence the *eda* gene, perhaps because of the smaller length of the antisense. Little difference in ED aldolase activity and cell growth (Figure 31) are observed for ZM4/pEMP300 and ZM4/pEMP627. This indicates that ASeda greater than 300 bp results in similar level of *eda* silencing as ASeda of length 300 bp.

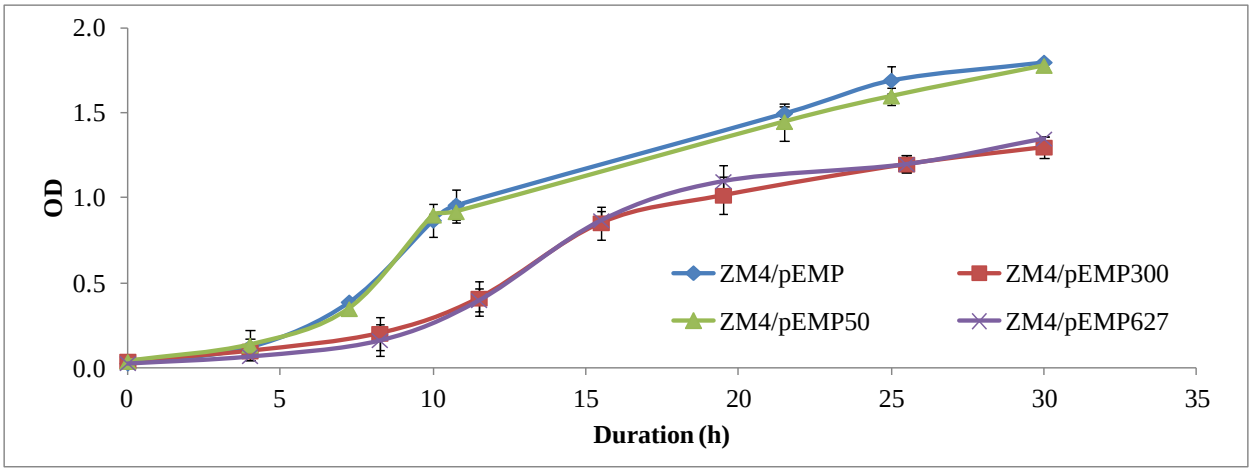

Figure 31: Comparison of growth of *Z. mobilis* expressing different lengths of antisense of *eda*

5.4.7 *Z. mobilis* ZM4 Can Grow on Gluconic Acid

Z. mobilis ZM4 is capable of growth on gluconic acid (GA) as the sole carbon and energy source. Ethanol followed by acetic acid was the major product of gluconic acid fermentation. Cells grew up only to an optical density of 0.17 in 1 week, which is more than ten-fold lower than the optical density from growth on 2% glucose. Unlike fermentation of glucose, fructose and sucrose, GA fermentation is very slow and does not complete.1.5% GA out of the initial 2% GA was fermented in 1 week to produce 0.6% ethanol and 0.2% acetic acid (Figure 32). To our knowledge, this is the first report of growth of *Z. mobilis* ZM4 on gluconic acid. There has been only one report about the ability of *Z. mobilis* ATCC 29191 (Z6) to degrade gluconate and its inability to use gluconate as the sole energy and carbon source.

Conversion of gluconate to acetic acid results in the generation of three moles of NADH while conversion of gluconate to ethanol requires one mole of NADH (Figure 33). Thus, to maintain the redox balance, 3 moles of ethanol should be produced per mole of acetic acid. However, 3.8 moles of ethanol were produced per mole of acetic acid from the fermentation of 2% GA by ZM4. Higher ethanol production can be due to the ability of cells to carry out the

redox balance by employing pathways other than the acetic acid formation pathway and due to the natural preference of *Z. mobilis* for producing ethanol rather than acetic acid.

From the mole balance observed during the length of the experiment and the theoretical redox balance, it can be concluded that the fermentation of gluconic acid by *Z. mobilis* ZM4 occurs primarily according to the equation below –

$$2C_6H_{12}O_7 \rightarrow CH_3COOH + 3C_2H_5OH + 4CO_2 + H_2O$$

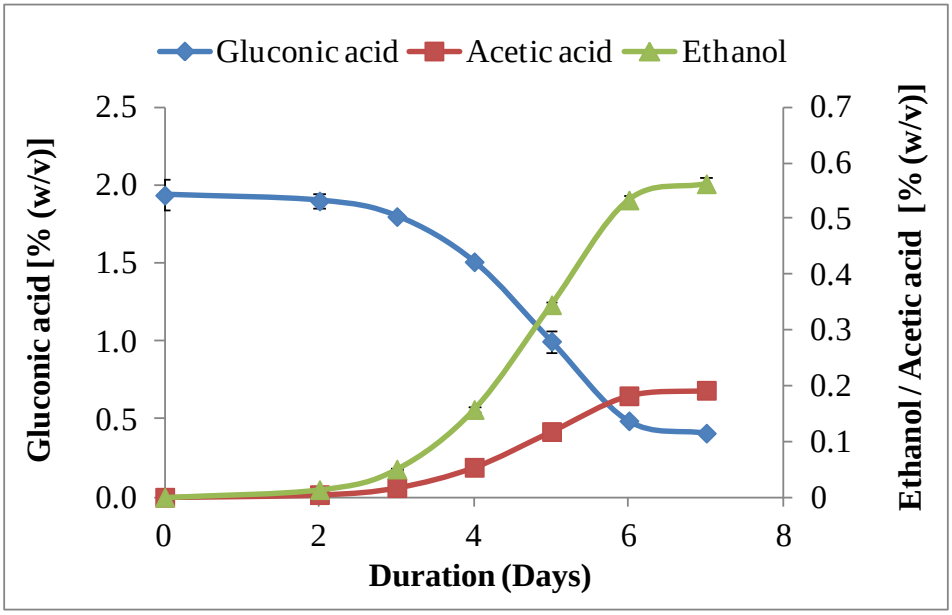

Figure 32: Fermentation of gluconic acid by *Zymomonas mobilis* ZM4

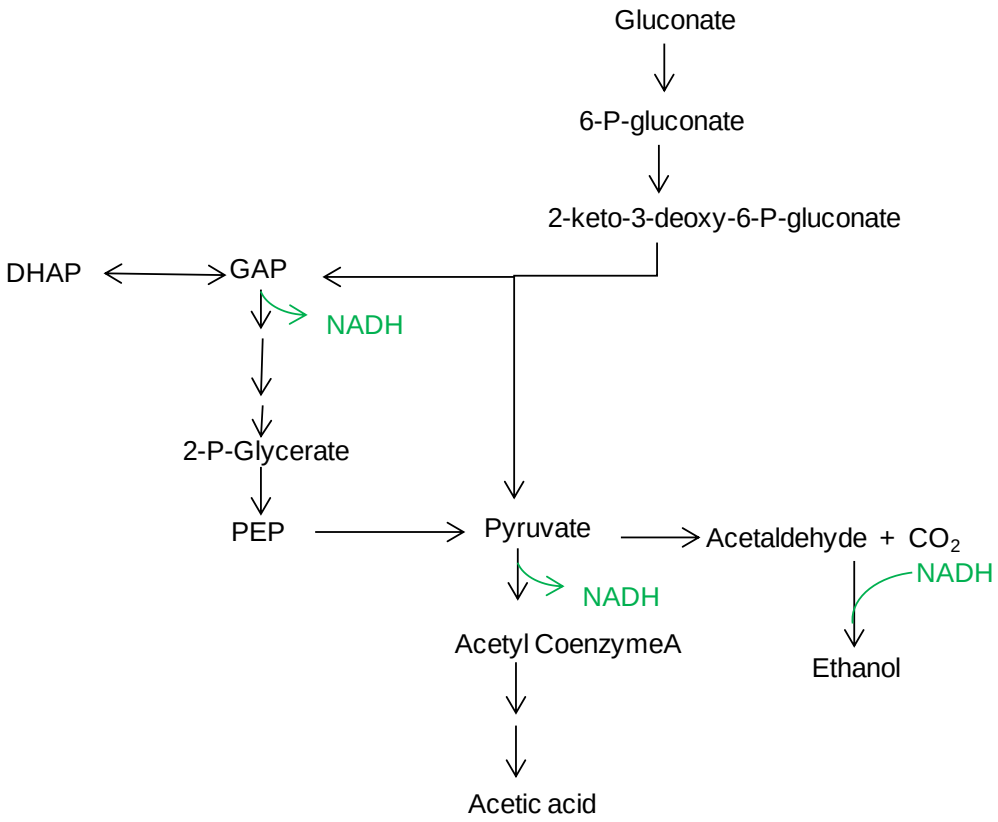

Figure 33: NADH production and consumption during conversion of gluconate to acetic acid and ethanol

5.4.8 Adaptation of *Z. mobilis* Expressing Antisense of *eda* in Gluconic Acid

Since the expression of the antisense of *eda* in *Z. mobilis* did not result in any growth and fermentation advantage compared to the control strain (Figure 31), it indicates that EMP pathway is still not operating. Earlier, we had successfully employed adaptive mutation technique in our lab for developing an acetate-resistant strain and a highly efficient xylose-fermenting strain of *Z. mobilis* (Agrawal et al. 2011; Chen et al. 2009; Wang 2008). Hence, we attempted to establish the EMP pathway by subjecting the three strains – ZM4/pEMP50, ZM4/pEMP300 and ZM4/pEMP627 – along with the control strain ZM4/EMP to an adaptation process. Both ZM4/pEMP300 and ZM4/pEMP627 have significantly reduced flux through the ED pathway

because of reduced ED aldolase activity. The reduced ED aldolase activity results in reduction of cell growth rate. To restore the carbon flux to previous levels, these strains may use the alternative EMP pathway since the missing enzyme and the enzymes catalyzing the rate-limiting steps of the EMP pathway are overexpressed in these strains.

However, a good adaptation strategy is needed to put pressure on these engineered strains to channel glucose flux through the 'new' EMP pathway instead of the 'old' ED pathway, which has been partially blocked by the ASeda expression. One way of doing this can be growing cells on a mixture of glucose and gluconic acid. Cells can grow to a high cell density on glucose and gluconic acid can act as a selection pressure for the EMP pathway. Gluconic acid or gluconate is oxidized form of glucose. In *Z. mobilis*, glucose is phosphorylated and oxidized to 6-phosphogluconate, which is then dehydrated to 2-keto-3-deoxy-6-phosphogluconate (KDPG) (Figure 34). Similarly, gluconate (non-phosphorylated form of 6-phosphogluconate) may also be converted to KDPG by *Z. mobilis*. Indeed, as mentioned in this study, wild-type *Z. mobilis* ZM4 can grow on gluconate as the sole carbon and energy source. Since only ED pathway is functional in ZM4, it suggests that gluconate is also metabolized through the ED pathway, thus yielding KDPG as a glycolytic metabolite.

Feeding gluconate to the *Z. mobilis* cells such as ZM4/pEMP300 and ZM4/pEMP627 may lead to accumulation of KDPG. If these engineered cells choose to metabolize glucose through the ED pathway, this will result in further accumulation of KDPG, but if the cells choose EMP pathway, there is no further increase in the KDPG pool. Also, it is very difficult for the cells to get rid of accumulated KDPG since ZM4/pEMP300 and ZM4/pEMP627 have significantly reduced ED aldolase activity due to expression of ASeda. The toxicity of

accumulated KDPG will thus help to get rid of cells channeling glucose through the ED pathway and will favor those engineered cells or mutants that channel glucose through the EMP pathway.

However, the adaptation of ZM4/pEMP300 and ZM4/pEMP627 on 2% glucose and 2% gluconate did not show any improvement in growth rate even after 50 generations.

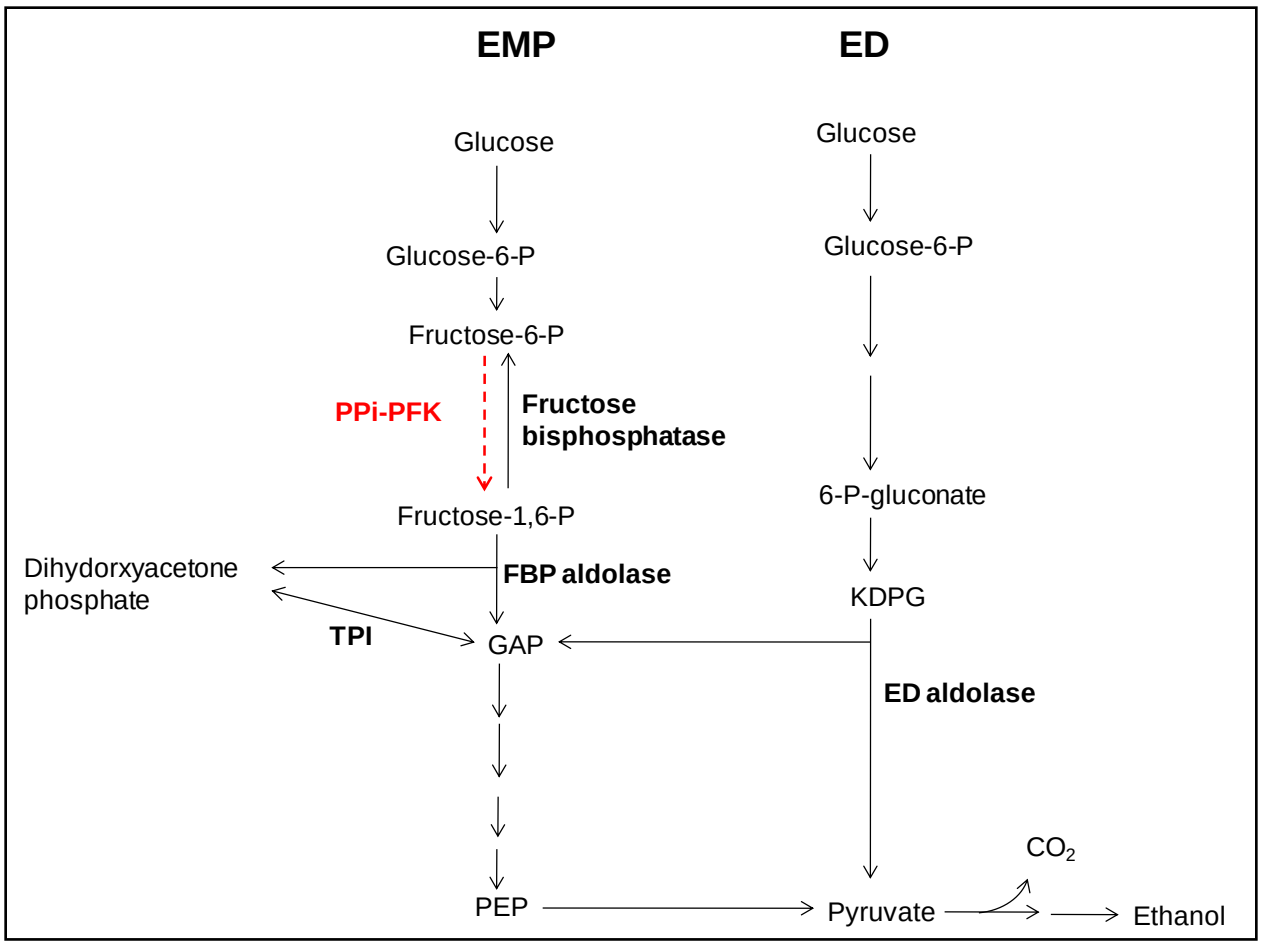

Figure 34: ED and EMP pathways in *Zymomonas mobilis*

5.4.9 GC-MS Analysis for Detection of EMP Operation

5.4.9.1 A Simple Technique for Determining Ratio of Flux through ED and EMP Pathways

A simple technique was developed to determine the ratio of glucose flux through the ED and EMP pathways in *Z. mobilis*, which enabled quick confirmation of the operation of the EMP pathway. Most of the techniques reported in literature to determine the ratio of flux through different metabolic pathways are quite involved. The sample preparation usually involves a time consuming derivatization process. Thereafter, large volumes of data generated by mass-

spectrometry (MS) have to be analysed, which again requires a mathematically involved treatment (Christensen et al. 2001; Fischer and Sauer 2003; Fuhrer et al. 2005).

We determined the flux ratio by using ^{13}C (carbon isotope) labeling experiments and subsequent analysis of produced ethanol using gas chromatography – mass spectrometry (GC-MS). Cells were fed with [1-^{13}C] glucose in a batch culture. If glucose is metabolized using the ED pathway, the labeled carbon of [1- ^{13}C] glucose is used for CO_2 production and hence, ethanol so produced is unlabeled. However, if cells are using the EMP pathway, labeled ethanol will be produced (Figure 35). Using a GC-MS, labeled and unlabeled ethanol in the culture broth were detected. The ratio of amounts of labeled to unlabeled ethanol, after correction for natural abundance, will give the ratio of metabolic flux between the EMP and ED pathways. Additionally, the rate of ATP production can be obtained from the rate of glycolytic flux through the EMP and ED pathways.

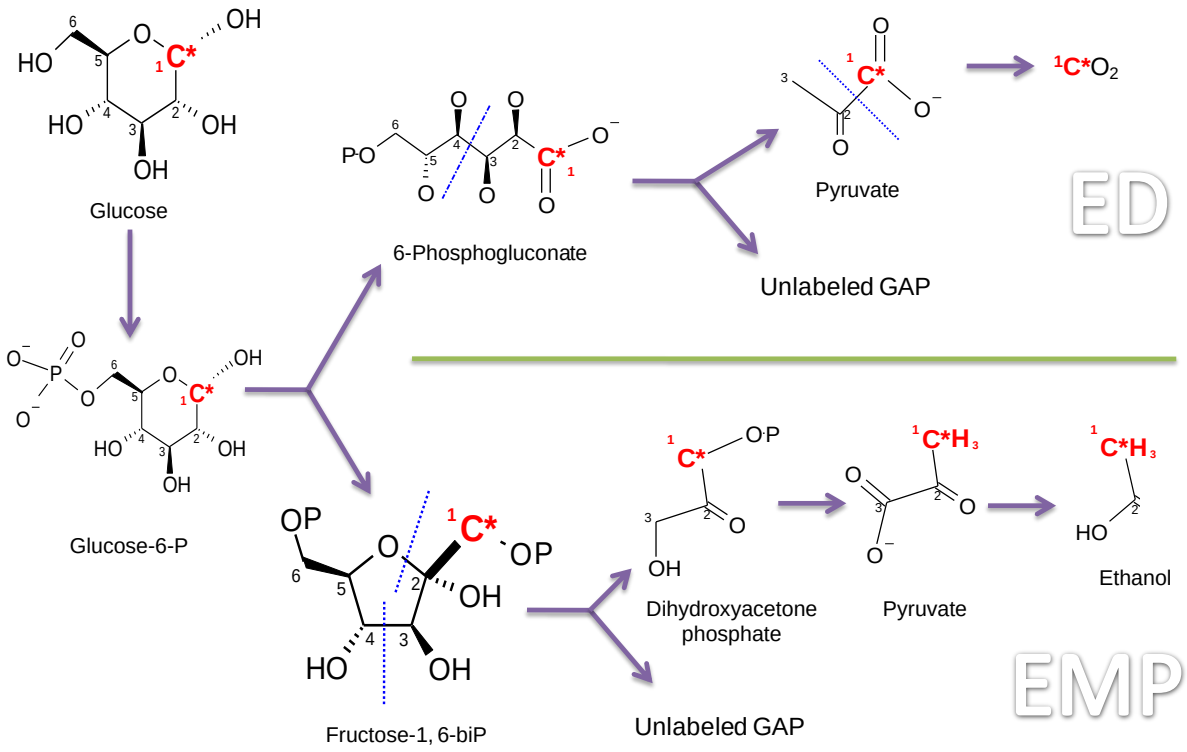

Figure 35: Metabolism of [1-^{13}C] glucose using ED or EMP pathway

5.4.9.2 Determination of Flux Ratio Using GC-MS

The ratio of labeled and unlabeled ethanol was measured using GC-MS in three strains – *E. coli* KO11, ZM4/pEMP and ZM4/pEMP627. KO11 is a genetically engineered *E. coli* capable of producing ethanol at high yields (Yomano et al. 1998). KO11 and engineered *Z. mobilis* were grown in LB and RM medium respectively containing 2% [1- ^{13}C] glucose. Unlabeled ethanol was obtained from the fermentation of RM containing 2% glucose by wild-type *Z. mobilis* ZM4. The ratio of labeled and unlabeled ethanol in supernatant of the positive control, *E. coli* KO11, was estimated to be 41% indicating that both the ED and EMP pathways operate at significant levels in KO11. The ratio of labeled and unlabeled ethanol for ZM4, ZM4/pEMP and ZM4/pEMP627 were 3.26%, 3.45% and 3.37% respectively. This indicates that only the ED

pathway operates in ZM4/pEMP and ZM4/pEMP627 just like the wild-type *Z. mobilis* ZM4. Thus, the overexpression of the three genes – PPi-PFK, FBP aldolase and *tpi* coupled with nearly 40% reduction in expression of the native ED aldolase failed to switch on the EMP pathway in *Z. mobilis*.

5.5 Discussion

Establishment of EMP pathway in *Z. mobilis* ZM4 may add to the sugar processing ability of this natural ethanologen and enhance its ability to tolerate stress by producing more ATP per mole of sugar metabolized. Thus, engineering the EMP pathway in *Z. mobilis* may lead to improved production of ethanol from lignocellulosic hydrolysates. Phosphofructokinase is the only missing enzyme in the EMP pathway of *Z. mobilis* ZM4 (Seo et al. 2004). However, expression of pyrophosphate-dependent phosphofructokinase (PPi-PFK) alone in *Z. mobilis* did not appear to switch on the EMP pathway. Further investigation revealed that the native expression levels of two enzymes downstream of PPi-PFK namely FBP aldolase and TPI, are respectively two order and one order of magnitude lower than PPi-PFK and other glycolytic enzymes of the ED pathway such as phosphoglucose isomerase (this study) and glyceraldehyde 3-phosphate dehydrogenase (Agrawal and Chen 2011a). Thus, low expression levels of FBP aldolase and TPI are possibly limiting the flux through the EMP pathway. Putative FBP aldolase and *tpi* genes of *Z. mobilis* were chosen for overexpression as they were found to encode for active enzymes. Also, use of native genes for overexpression may result in easier transformation of expression vector as *Z. mobilis* possesses a strong restriction-modification system (Kerr et al. 2011; Phillips 2006), and possibly better expression.

The overexpression of the three genes – PPi-PFK, FBP aldolase and *tpi* did not result in any growth and fermentation advantage indicating that EMP pathway may not be operational.

Further confirmation of non-operation of EMP pathway was done using GC-MS. However, overexpression did result in an alteration of metabolism. 0.16% (w/v) dihydroxyacetone (DHA) was produced from 2% (w/v) glucose. Thus, 8% of the total glucose fed to the cells was converted to dihydroxyacetone (DHA). Overexpression of only PPi-PFK or only FBP aldolase and *tpi* resulted in conversion of 4% of the glucose to DHA. Wild-type *Z. mobilis* converts 97% of the glucose to ethanol and CO_2 using the ED pathway. The remaining 3% of the glucose is also channeled through the ED pathway to produce byproducts such as lactic acid, glycerol and acetic acid in addition to biosynthetic precursors required for cell growth (Rogers P L 1982; Swings and De Ley 1977). Thus, appearance of a new byproduct, DHA, suggests that the overexpression of the three genes or only PPi-PFK has forced at least 4% of the glucose through the EMP pathway up to the level of metabolites - dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) (Figure 26). GC-MS analysis showed that glucose does not get metabolized all the way up to ethanol through the EMP pathway. Hence, we managed to switch on the EMP pathway in *Z. mobilis* at least up to the level of metabolites - DHAP and GAP (Figure 34). It is possible that futile cycles involving fructose bisphosphatase prevent GAP from being further metabolized to ethanol (Steinbuchel 1986). The enzymes downstream of GAP may not be able to immediately process the extra GAP flux coming from EMP pathway. This would encourage overexpressed FBP aldolase to re-convert GAP and DHAP back to FBP, which would then be converted by fructose bisphosphatase back to F6P.

Construction of ZM4/pNGFPK/pMHGPTAF strain, which stably maintains two engineered plasmids, was more difficult than construction of *Z. mobilis* strains carrying only one engineered plasmid. Electroporation of pMHGPTAF in electrocompetent ZM4/pNGFPK did not yield any positive clones initially. Usage of a high antibiotic concentration resulted in appearance

of only spontaneous mutants, carrying only the antibiotic resistance cassette and not the electroporated plasmid, pMHGPTAF. On decreasing the antibiotic concentration close to the MIC, positive clones (one that stably maintains the electroporating plasmid) could be obtained. After electroporation, selection by employing a high antibiotic concentration may be promoting integration of the antibiotic resistance cassette of the electroporating plasmid into the genome or the native plasmids, thus resulting in non-maintenance of the electroporating plasmid. The integration of antibiotic cassette may be resulting in less plasmid burden compared to maintenance of two engineered plasmids. However, once a positive clone was obtained, it was able to grow in high antibiotic concentration, without losing the plasmids. Also, the two replication origins – ZM27 and ZMGP – are compatible with each other.

To completely functionalize the EMP pathway in *Z. mobilis* strain overexpressing PPi-PFK, FBP aldolase and *tpi*, knockout of *eda* was attempted in ZM4/pEMP. However, *eda* could not be knocked out even though a similar experimental design was successfully used for the knockout of xylose reductase (ZMO0976) (chapter 4). Thus, *eda* gene, of which only one copy is present in the genome, is an essential gene for *Z. mobilis* ZM4. Also, no other enzyme seems capable of playing the role of ED aldolase *in vivo*. Since *eda* could not be knocked out, an alternate strategy of partially silencing the *eda* gene using its anti-sense was employed. Expression of antisense of *eda* (ASeda) of lengths ~300 bp and 627 bp was able to reduce ED aldolase activity up to 40% which resulted in almost similar level of growth reduction. Little difference in ED aldolase activity and cell growth (Figure 31) was observed for ZM4/pEMP300 and ZM4/pEMP627. Also, ED aldolase activity and cell growth were similar for ZM4/pEMP50 and the control strain ZM4/pEMP. This indicates that ASeda of length 50 bp is ineffective in silencing the *eda* gene. Also, ASeda of length 300 bp is nearly as effective in silencing the *eda*

gene as AS627. Silencing of a gene by its antisense is achieved by *in vivo* binding of the antisense to the mRNA of the gene thus preventing the translation. Thus, ASeda of length 300 bp binds the mRNA of *eda* with nearly the same effectiveness as ASeda of length 627 bp. It has been reported that the silencing of the gene by the antisense is proportional to the length of the corresponding antisense (Oddone et al. 2009; Sturino and Klaenhammer 2004). Though this statement appears true from a statistical point of view, but our *in vivo* results obtained for three different lengths of antisense do not support the reported proportionality.

ZM4/pEMP627 also did not have a functional EMP pathway as confirmed by GC-MS analysis. Further adaptation on gluconic acid also did not functionalize the EMP pathway.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

6.1 Conclusions

The experimental results and analysis presented in chapters 2 - 5 accomplished the three objectives outlined in the introduction chapter –

1) An efficient xylose-fermenting strain of *Zymomonas mobilis* was developed by the use of an adaptive mutation technique.
2) Factors at bio-molecular level responsible for improved xylose fermentation were identified.
3) The knowledge and understanding of the metabolism of *Z. mobilis* acquired during strain development was put to use in an attempt to further improve fermentation of sugars and lignocellulosic hydrolysates.

6.1.1 Development of a Highly Efficient Xylose-Fermenting *Z. mobilis* Strain

An adaptation process spanning over 80 days and involving 30 serial transfers was employed for the development of a highly efficient xylose-fermenting *Z. mobilis* strain, A3. Extensive adaptation on xylose improved the fermentation of xylose considerably. When fermenting a 5% glucose - 5% xylose mixture, our 'less' adapted strain A1, obtained early on during the adaptation process, consumed xylose four times slower than A3. A3 fermented nearly all of the 10% (w/v) xylose in two days while A1 could consume only 7% (w/v) xylose even after 10 days. Extensive adaptation on xylose did not alter glucose metabolism of A3 since an

almost identical rate of glucose fermentation was observed for both A1 and A3. In a mixed glucose-xylose fermentation, A3 produced a total of 9% (w/v) ethanol from two doses of 5% glucose and 5% xylose (or a total of 10% glucose and 10% xylose).

A3 was able to couple the cell growth to xylose fermentation much better than A1, which results in a larger cell mass and hence faster volumetric rate of xylose fermentation. Factors that led to greatly improved xylose fermentation by A3 included lower xylitol formation, better xylitol tolerance and higher xylose isomerase activity.

6.1.2 Molecular Level Understanding of Improved Xylose Fermentation

Adaptation is not only useful in improving strains, but is equally useful for pinpointing key bottlenecks in metabolically engineered strains. A molecular level investigation of factors that lead to improvement in xylose fermentation was facilitated by a comparison of the control strain ZM4/pSTVZM27, parent strain ZM4/pZMETX, A1 (less adapted) and A3 (more adapted) strains. Product concentrations in the fermentation broth, enzymatic activities in the cell extract and selected DNA sequences were compared. The investigation revealed evidence for altered xylitol metabolism in A3 with reduced xylitol formation. A3 produced half of the xylitol produced by A1 at the end of 5% glucose-5% xylose fermentation. Both the adapted strains produced three fold lower xylitol than the control strain at the beginning of vigorous xylose fermentation (coinciding with the exhaustion of glucose). The reduction in xylitol formation results in improvement of xylose fermentation since cell growth and fermentation are subjected to a less amount of inhibition (Akinterinwa and Cirino 2009; Feldmann et al. 1992). Reduction in xylitol formation was due to the reduction in xylose reductase activity. An analysis of the whole cell extract for the xylose reductase activity showed that in both the adapted strains, the xylose reductase activity was barely detectable, whereas the control strain gave an activity of 22 mU/mg

cell proteins. These phenotypic differences and homology search to known fungal xylose reductases led to the discovery of a putative aldo-keto reductase, ZMO0976 functioning as a xylose reductase in *Z. mobilis*. Both the adapted strains carried a single base pair mutation, C874T in the gene ZMO0976, which resulted in a single mutation from arginine to cysteine. This mutation drastically lowers the reductase activity of ZMO0976. Xylose reductase activities of 1 mg of purified proteins are 3400 and 140 mU for ZMO0976 and mZMO0976, respectively. Besides xylose and xylulose, the enzyme was found to be active towards benzaldehyde, furfural, 5-hydroxymethylfurfural (HMF), and acetaldehyde, but not glucose or fructose even at high concentrations. Furthermore, benzaldehyde and furfural are much better substrates than xylose for the enzyme, with benzaldehyde exhibiting nearly 150 times higher affinity than xylose. Involvement of protein ZMO0976 in *in vivo* xylose reductase activity in *Z. mobilis* ZM4 was confirmed by knocking out ZMO0976 from the genome. The knockout strain, ZM4 ΔXR, produced four-fold lower xylitol than the wild-type strain ZM4 indicating a significant decrease of *in vivo* xylose reductase activity. The knockout of ZMO0976 facilitated the establishment of xylose fermentation in *Z. mobilis*. The knockout strain (ZM4 ΔXR) bearing the heterologous xylose metabolic genes managed to grow directly without any adaptation in RM medium containing xylose as the sole carbon source. On the other hand, wild-type strain ZM4 bearing the heterologous xylose metabolic genes failed to grow in such a medium. Spontaneous mutant arose in one of such cultures after extended incubation but spontaneous mutant could utilize only a fraction of xylose and failed to grow any further. The optical density reached by spontaneous mutant was less than 10% of that reached by ZM4 ΔXR bearing the xylose metabolic genes. Such spontaneous mutants need to be adapted on xylose to facilitate better xylose fermentation.

In addition to the alteration in xylitol metabolism with reduced xylitol formation, adaptation led to an increase in xylitol tolerance. In 5% xylose fermentation with exogenous xylitol at a concentration of 0.1%, growth rate was less affected in A3, with a reduction of about 20% compared to a 50% decrease in specific growth rate for A1 under the same condition. The presence of xylitol at this concentration also slowed down xylose fermentation for both strains, but this is more so for A1 than A3. After 42 hours of fermentation, 3.8% xylose remained to be fermented for A1 while for A3, only 1.3% xylose was left. Sequence analysis of both xylose isomerase and xylulokinase genes showed no mutation in their respective genes, ruling out functional mutation in these two enzymes in conferring tolerance. Hence, the genetic basis for xylitol tolerance is unknown.

Xylose isomerization was found to be the rate-limiting step during xylose fermentation in *Z. mobilis*. Adaptation resulted in enhancement of xylose isomerase (XI) activity 4-8 times in the A3 strain compared to A1 strain. This enabled the A3 strain to push more xylose flux through the bottleneck thus resulting in faster xylose fermentation. Enhanced XI activity also results in channeling xylose to ethanol instead of xylitol leading to lower xylitol production. At the end of 5% glucose – 5% xylose fermentation, A3 produced two-fold lower xylitol than A1. Enhanced XI activity in A3 was linked to a single mutation in the promoter of XI gene carried by plasmid pZMETX. This mutation was not present in pZMETX carried by A1 strain. The effect of this mutation on improvement of xylose fermentation was confirmed by electroporating unmutated pZMETX extracted from A1 (designated pZMETX (A1)) and mutated pZMETX extracted from A3 strain (designated A3-pZMETX*) in ZM4 ΔXR. ZM4 ΔXR/pZMETX (A1) grew at a similar rate as A1, but fermented xylose slightly better due to the knockout of ZMO0976. ZM4 ΔXR/A3-pZMETX* had higher rates of growth and fermentation than ZM4 ΔXR/pZMETX

(A1), thus establishing that the mutation in pZMETX leads to an improvement in xylose fermentation. Also, the mutation leads to a lower xylitol production. Growth and fermentation by ZM4 ΔXR/A3-pZMETX* lagged behind A3. This indicates that there are other attributes besides lower xylitol production and higher xylose isomerization that result in better xylose fermentation. Overall, knocking out of ZMO0976 and a mutation in pZMETX contribute towards better xylose fermentation in *Z. mobilis*.

Both these attributes were also incorporated in an acetate resistant ZM6014 using rational metabolic engineering to establish and improve xylose fermentation. The strain was able to ferment all the glucose and nearly 70% of the xylose in presence of a high concentration of acetic acid (1.4%). The strain fermented 5% glucose - 5% xylose mixture in presence of 1% acetic acid at pH 5.8 to completion at an ethanol yield of 93.4%.

6.1.3 Further Improvement in Fermentation of Sugars and Lignocellulosic Hydrolysates

ED pathway is optimized by years of evolution for fermentation of glucose in *Z. mobilis*. *Z. mobilis* has been engineered for xylose fermentation by expressing heterologous xylose metabolic enzymes, followed by adaptation to establish and improve xylose fermentation. The heterologous xylose fermenting enzymes channel xylose to ED pathway which converts it to ethanol. Acceleration of xylose isomerization, a rate-limiting step, reduced byproduct (xylitol) formation and better tolerance to xylitol result in improvement of xylose fermentation.

We tried to further improve the rates of sugar fermentation by establishing the EMP pathway in *Z. mobilis*. We only succeeded partially in establishing this pathway. The only missing gene of the EMP pathway – pyrophosphate-dependent 6-phosphofructokinase (PPi-PFK) was expressed in wild-type *Z. mobilis* ZM4. A maximum *in vitro* PPi-PFK activity of approximately 3 U/mg cell proteins was measured. The enzyme had an apparent Michaelis-

Menten constant (K_m^{PPi}) of 22 µM for pyrophosphate. Intracellular pyrophosphate (PPi) concentration was 301 µM. Thus, PPi is present *in vivo* in sufficient quantity to drive PPi-PFK at V_{max}. Two enzymes – FBP aldolase and triose phosphate isomerase (TPI) were found to be expressed at very low levels, thus creating bottleneck in EMP operation. Putative genes of *Z. mobilis* ZM4 corresponding to these enzymes were found to encode for active enzymes. Purified FBP aldolase had an activity of 60 mU/mg protein. Metallic cofactors such as Mn^{2+}, Mg^{2+} and Ca^{2+} were required for FBP aldolase activity. Purified TPI had an activity of 60 U/mg protein.

Both these enzymes along with PPi-PFK were overexpressed in wild-type *Z. mobilis* ZM4. The resulting strain showed an altered metabolism with the appearance of a new byproduct dihydroxyacetone (DHA), which was not produced by the wild-type *Z. mobilis* ZM4. The engineered strain produced 0.16% DHA from 2% glucose. This indicates that the EMP pathway in *Z. mobilis* has been switched on at least up to the level of metabolites - glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate - in the glycolytic pathway, albeit with a very low glucose flux. GC-MS analysis showed that the EMP pathway was not completely operational all the way up to ethanol. The ED aldolase (*eda*) gene could not be knocked out; and partial silencing of *eda* did not lead to establishment of the EMP pathway. Adaptation was also done on media containing gluconic acid to select for mutants with operational EMP. However, the EMP pathway could not be switched on in *Z. mobilis*. While testing the suitability of gluconic acid for adaptation experiments, it was discovered that *Z. mobilis* ZM4 can grow on gluconic acid as the sole carbon source. Cells consumed 1.5% gluconic acid to produce 0.6% ethanol and 0.2% acetic acid. Gluconic acid is only the fourth substrate besides glucose, fructose and sucrose on which *Z. mobilis* ZM4 can grow.

6.2 Significant Contributions

Lignocellulosic biomass can be an alternative to the limited fossil fuels reserves that remain in the world (Hahn-Hagerdal et al. 2006). Lignocelluloses can be converted to ethanol, a versatile transportation fuel. However, there still exists no commercial process for converting lignocelluloses to ethanol (Rogers et al. 2007; Sainz 2011). Along with technological improvements in the pretreatment and hydrolysis, the improved fermentation will make cellulosic ethanol process much more competitive. This work thus contributes to the world-wide efforts in converting vast, abundant renewable biomass into biofuel. We have developed an improved microbial strain for ethanol production from lignocelluloses. Our efforts were focused on metabolic engineering of *Zymomonas mobilis*. Numerous research groups around the world such as NREL-DuPont consortium and Dr. Lonnie O. Ingram's research group in US, Dr. Hugh G. Lawford's group in Canada, Dr. P. L. Rogers's group in Australia and Dr. Georg Sprenger's group in Germany, have carried out research on *Z. mobilis* for several years. In spite of the extensive work done by these groups in the past several years and the research that has been done on *Z. mobilis* for slightly more than a century, we were still able to contribute towards improving biocatalytic properties of *Z. mobilis* and better understanding of its metabolism.

We showed that adaptation can be used to transform *Z. mobilis* into an efficient biocatalyst that can be employed for cellulosic ethanol production. The strain was able to grow on 10% (w/v) xylose and rapidly ferment all the xylose to ethanol within 2 days and retained high ethanol yield. In a mixed glucose-xylose fermentation, the strain produced a total of 9% (w/v) ethanol from two doses of 5% glucose and 5% xylose (or a total of 10% glucose and 10% xylose).

Our investigation of efficient xylose-fermenting A3 strain showed that xylose isomerization and xylitol are key factors for improving xylose fermentation in *Z. mobilis*. Reduction in xylitol formation and increase in xylitol tolerance result in lesser inhibition of cell growth and fermentation. We discovered a novel aldo-keto reductase (ZMO0976) in *Z. mobilis* ZM4. NADPH-dependent ZMO0976 functions as a xylose reductase, knockout of which lowers xylitol production and thus improves xylose fermentation. Besides xylose, the enzyme was found to be active towards xylulose, benzaldehyde, furfural, 5-hydroxymethyl furfural (HMF), and acetaldehyde, but not towards glucose or fructose even at high concentration. Furthermore, benzaldehyde and furfural are much better substrates than xylose for the enzyme, with benzaldehyde exhibiting nearly 150 times higher affinity than xylose. The activity of the enzyme to reduce furfural and HMF could be potentially used for detoxification of lignocellulosic hydrolysate or improve microbial tolerance to these two major biomass derived inhibitors.

We observed that increase in xylose isomerase expression improves xylose fermentation by pushing more xylose flux through the fermentation pathway instead of xylitol. We discovered a mutation in the promoter of xylose isomerase that results in a higher xylose isomerase activity by some post-transcription event.

In spite of a mammoth amount of metabolic engineering that has been carried out on *Z. mobilis* for improving ethanol production (Caimi et al. 2012; Kahsay et al. 2012; Mohagheghi et al. 2002; Rogers et al. 2007; Viitanen et al. 2008; Viitanen et al. 2009; Viitanen et al. 2011a; Zhang 2003; Zhang et al. 1995), we were still able to extend the horizon by applying the adaptive mutation technique to improve fermentation of xylose to ethanol. We have conclusively shown that adaptation is a powerful technique for strain improvement and results in improvement based on selection pressure. While an adaptation is relatively easy to set up, the

specific conditions need to be selected carefully in order to yield strains with desired traits. For example, the selection pressure applied in the adaptation was high concentration of xylose (5%), not xylitol, but xylitol tolerance and reduced xylitol formation were selected.

We have demonstrated that adaptation can also help to identify genetic targets for further strain improvement. Genetic mutations discovered in the adapted strains were mimicked to construct rational strains comparable to adapted strains. These rationally constructed strains, having a known genetic makeup, are better suited for further metabolic engineering rather than the adapted strains. Also, the two desirable traits – acetate resistance and improved xylose fermentation - both developed by adaptation were combined together using rational metabolic engineering to construct a useful strain, ZM6014 ΔXR/A3-pZMETX*. This strain was able to ferment all the glucose and nearly 70% of the xylose in presence of a high concentration of acetic acid (1.4%). On decreasing the acetic acid concentration to 1%, ZM6014 ΔXR/A3-pZMETX* fermented 5% glucose -5% xylose to completion in 58 h. Maximum volumetric rate of glucose consumption for ZM6014ΔXR/A3-pZMETX* was 6.4 g/l/h, which is the highest reported so far. Thus, industrially useful strains can be developed by using adaptation and metabolic engineering synergistically.

Further, we also tried to alter the central carbon metabolism of *Z. mobilis* ZM4 by establishing a new glycolytic pathway, the EMP pathway, capable of generating three-fold higher energy than the ED pathway. Our experiments show that it may not be possible to establish EMP pathway in *Z. mobilis* ZM4 perhaps because of strict preference for ED pathway by the micro-organism. ED pathway may be preferred by *Z. mobilis* since the second step of the pathway results in production of NADPH, a biosynthetic cofactor. This cofactor is not produced in the EMP pathway. However, we were partially successful in switching on the EMP pathway.

We were able to operate the EMP pathway at least up to the level of metabolites - GAP and DHAP - but were not able to functionalize it all the way up to ethanol.

While attempting to establish the EMP pathway, we made several discoveries. We discovered that the putative genes for FBP aldolase and *tpi* encode for active enzymes. Contrary to the homology based annotation, our experiments showed that FBP aldolase is a metal-dependent enzyme. Wild-type *Z. mobilis* ZM4 was found to grow on gluconic acid as the sole carbon source. Wild-type ZM4 can only grow on glucose, fructose and sucrose. Thus, gluconic acid is only the fourth known substrate on which ZM4 manages to grow. However, unlike glucose, fructose and sucrose, growth on gluconic acid is very slow. The final cell density is more than ten-fold lower.

6.3 Recommendations for Future Work

6.3.1 Adaptation on Other Sugars

Commercial application of *Z. mobilis* for cellulosic ethanol production requires that it ferments all the sugars present in lignocellulosic hydrolysate rapidly and completely. Besides glucose and xylose, other sugars present in lignocellulosic hydrolysates are mannose, galactose, rhamnose and arabinose. As an example, amounts of various sugars present in the hemicellulosic fraction of agricultural residues are shown in Table 14. Wild-type *Z. mobilis* ZM4 can only ferment glucose and so far, it has been engineered to ferment xylose, arabinose and mannose (Deanda et al. 1996; Lawford and Rousseau 2002; Mohagheghi et al. 2002; Weisser et al. 1996; Zhang et al. 1998; Zhang and Chou 2008). However, fermentation of non-glucose sugars by *Z. mobilis* is usually slow and commercially repealing. Just as an adaptation on xylose was used to improve xylose fermentation in this work, adaptation on other sugars such as arabinose and mannose can be carried out for improving their fermentation as well. Adaptation may not only

yield efficient sugar fermenting strains but may also provide genetic targets and metabolism insights for further metabolic engineering of *Z. mobilis*.

Table 14: Sugar composition of hemicellulosic hydrolysates from agricultural residues

Agricultural residue	Sugar composition (% of total)				Total sugars (g/l) in hydrolysate
	Glucose	Xylose	Galactose	Arabinose	
Corn hulls + fibers	27	39	11	23	100 – 140
Corn stover	19	61	7	12	80 – 130
Bagasse	6	89	0	14	70 – 110

6.3.2 Genetic Basis of Xylitol Tolerance

Adaptation on high concentration of xylose not only led to a decrease in xylitol formation, but also increased xylitol tolerance, so that that the toxic effects of xylitol could be mitigated. Though experiments clearly showed higher xylitol tolerance in A3 compared to A1, this experimental observation could not be connected to the corresponding genetic factor. Feldmann et al. had reported that one of their xylitol tolerant mutants of *Z. mobilis* had a partial deletion of the heterologous xylulokinase gene that results in lower accumulation of toxic intracellular xylitol phosphate. Hence, the mutated xylulokinase imparts higher xylitol tolerance (Feldmann et al. 1992). However, sequence analysis of both xylose isomerase and xylulokinase genes of A3 showed no mutation in their respective genes, ruling out functional mutation in these two enzymes in conferring xylitol tolerance. Besides, xylulokinase activity was similar for the cell extracts of both A1 and A3. Hence, there are gene(s) other than xylulokinase responsible for higher xylitol tolerance in A3. Discovery of this gene may help in further increasing xylitol

tolerance, which will result in lesser inhibition of xylose fermentation. Increased xylitol tolerance will be especially helpful when cells are fermenting xylose in the presence of inhibitors such as acetic acid that result in higher xylitol production. Additionally, the gene associated with xylitol tolerance may also be related to tolerance of other toxic compounds present in lignocellulosic hydrolysates. For example, *Z. mobilis* regulator *hfq* was found to contribute towards tolerance against multiple lignocellulosic pretreatment inhibitors such as acetate, vanillin, furfural and hydroxymethylfurfural (Yang et al. 2010b).

One way of identifying the gene(s) associated with xylitol tolerance is a comparison of the entire genomes of A1 and A3. As A3 has higher xylitol tolerance than A1, genome of A3 will be harboring mutation(s) in the gene(s) or promoter(s) that result in higher xylitol tolerance. The genomic level comparison of A1 and A3 may also identify other genetic mutations that lead to improved xylose fermentation.

6.3.3 Industrial Applications of Aldo-keto Reductase, ZMO0976

Furfural and HMF reductase activity of ZMO0976 can be used to detoxify lignocellulosic hydrolysate. However, the ability of ZMO0976 to reduce xylose to xylitol may inhibit the xylose fermentation in xylitol-sensitive microbes such as *Z. mobilis* and *E. coli* (Akinterinwa and Cirino 2009). This can be tackled in several ways. For example, ZMO0976 can be expressed in a fermentation microbe using an inducible promoter. During the initial stages of fermentation, the promoter can be induced to overexpress ZMO0976. After detoxification of the hydrolysate is complete, a repressor can be added to shut down the expression of ZMO0976.

Z. mobilis may be engineered for xylitol production in a similar fashion as done by Cirino et al. for *E. coli* (Cirino et al. 2006). Xylitol is an industrially important chemical currently used as a natural, nutritive sweetener and as a food additive (Cirino et al. 2006). It has also been

identified as one of the top twelve value-added materials to be produced from biomass (Werpy et al. 2004). To facilitate xylitol production by *Z. mobilis*, ZMO0976 needs to be overexpressed in wild-type *Z. mobilis* ZM4. Simultaneous utilization of glucose and xylose also needs to be engineered. Alternatively, ZMO0976 can be overexpressed in microbes such as *E. coli* that have already been engineered for xylitol production (Cirino et al. 2006).

More industrial applications of ZMO0976, which is a novel aldo-keto reductase, have been outlined in our patent (Agrawal and Chen 2011b)

6.3.4 Presence of yet Another Xylose Reductase in *Z. mobilis* ZM4

We suspect that there is at least one more xylose reductase present in *Z. mobilis* ZM4 besides ZMO0976 and glucose-fructose oxidoreductase (GFOR). The knockout of ZMO0976 in ZM4 did not result in a complete elimination of xylitol formation. 0.015% xylitol was produced by ZM4 ΔXR from RM medium containing 5% glucose and 5% xylose. Since ZM4 ΔXR does not contain xylose isomerase, this xylitol formation may not be due to GFOR. GFOR has been shown to accept xylose as an electron donor and reduce xylulose to xylitol (Viitanen et al. 2008; Zachariou and Scopes 1986). Thus, in the absence of xylulose, GFOR may not be contributing to xylitol production. Identification of other xylose reductases and their knockout will further lower the xylitol production. Reduction in xylitol formation will lower the inhibition of xylose fermentation and cell growth. It will be especially desirable in situations for example, the fermentation of 5% glucose - 5% xylose in presence of 1.4% acetic acid by ZM6014 ΔXR/A3-pZMETX*, where the fermentation terminated because of buildup of xylitol to high levels.

6.3.5 Establishment of a Completely Functional EMP Pathway in *Z. mobilis*

We tried to establish a completely new glycolysis pathway in *Z. mobilis*, but we had limited success only. Our experiments showed that it may not be possible to establish EMP

pathway in *Z. mobilis* ZM4 perhaps because of strict control over the glycolytic flux by the micro-organism. We tried several strategies which included overexpression of enzymes catalyzing missing and rate-limiting steps, knockout of a key enzyme of ED pathway, partial silencing of the same enzyme by expressing its anti-sense followed by adaptation on gluconic acid containing media. Though we failed to functionalize the EMP pathway all the way up to ethanol, we did functionalize the EMP pathway till the half way mark, up to the level of GAP and DHAP in the glycolysis pathway.

There are lots of possibilities that remain to be explored for establishing EMP pathway in *Z. mobilis*. Among them are – (a) Shutting down ED pathway partially or completely by choosing a gene other than ED aldolase and (b) Adaptation conditions that favor operation of EMP. Success in establishing EMP will not only improve ethanol production but also open up further opportunities for metabolic engineering. For example, the presence of an energy efficient EMP pathway in *Z. mobilis* would allow metabolic engineers to make use of surplus ATP to introduce ATP-consuming pathways.

6.3.5.1 Conversion of Acetic Acid to Ethanol

Acetic acid can be converted to ethanol to potentially increase yield and meanwhile eliminate the inhibition. This conversion, however, requires energy as shown in Figure 36. For *Z. mobilis* cells with the ED pathway only, the limited energy resource (ATP availability) makes it impossible for this conversion to succeed. The energy surplus created by the new EMP may make it possible for this ATP-consuming conversion to proceed. Thus, further metabolic engineering could be applied to engineer the cells to use acetate for ethanol production.

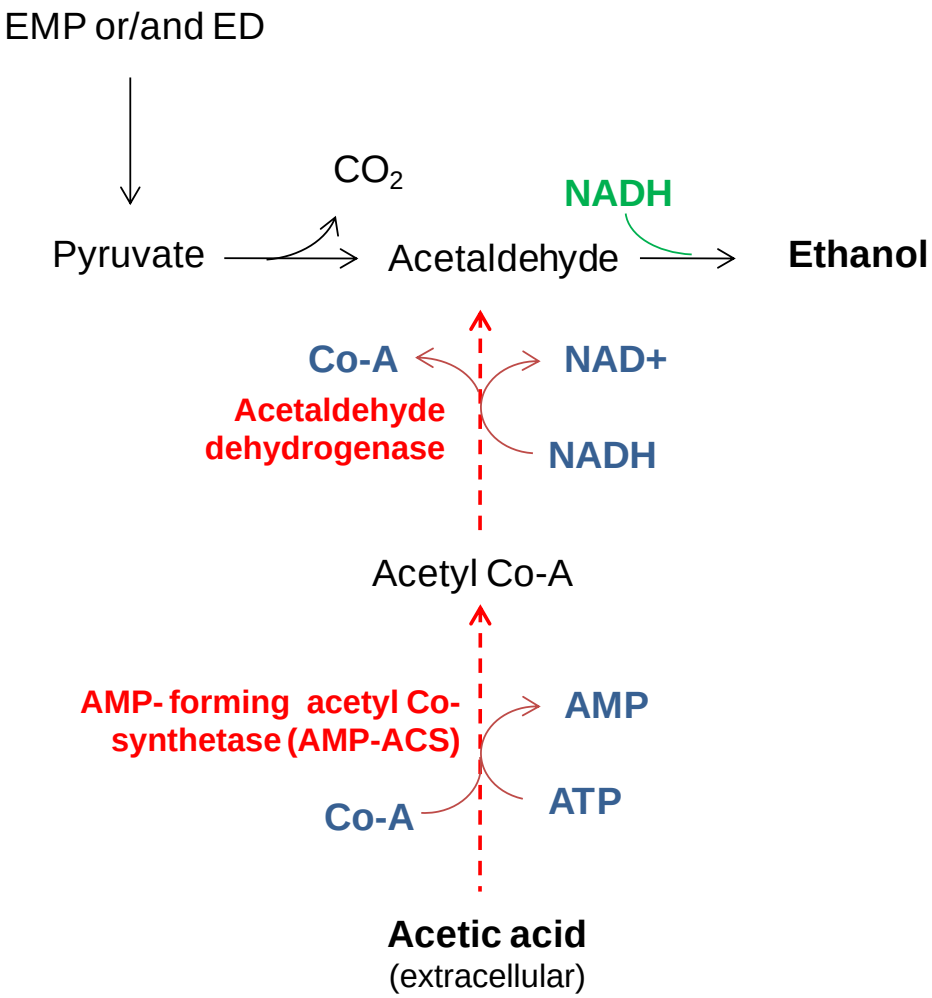

Figure 36: A possible route for conversion of acetic acid to ethanol in *Z. mobilis* with a functional EMP

APPENDIX A

AN EXAMPLE OF CONSTRUCTION OF ENGINEERED PLASMIDS FOR *Z. MOBILIS*

Several plasmids were constructed for gene expression in *Z. mobilis* ZM4. The construction of an engineered plasmid for *Z. mobilis* differs from that for an *E. coli*. In *Z. mobilis*, unlike *E. coli*, origin of replications such as ColE1 and p15A do not work. Hence, plasmid origin of replications for *Z. mobilis* ZM4 such as ZM27 and ZMGP were chosen to be fragments of the native plasmids of *Z. mobilis* ATCC 10988. Procedure for construction of a plasmid pNGFPK and corresponding empty plasmid pSTVZM27 are mentioned below to better illustrate the difference in the plasmid construction.

Plasmids pSTVZM27 and pNGFPK were constructed as shown in Figure 37. 1.7 kb ZM27 replication origin was PCR cloned from pZMO3, a 2.7 kb native plasmid of *Z. mobilis* ATCC 10988 (Scordaki and Drainas 1987; Scordaki and Drainas 1990). ZM27 is a DNA fragment which contains the origin of replication and necessary information for the maintenance of plasmid inside a *Z. mobilis* cell. While cloning, two restriction sites - both BglII - were introduced on either side of the ZM27 replication origin. ZM27 was then ligated into a BamHI digested medium copy number plasmid pSTV28 to obtain plasmid pSTVZM27 of size 4.7kb. The commercially available plasmid pSTV28 contains p15A, an origin of replication in an *E. coli* cell (*E. coli ori* as shown in Figure 37), and chloramphenicol resistance gene.

Promoter of glyceraldehyde-3-phosphate (Pgap) was PCR cloned from genomic DNA of *Z. mobilis* ZM4. Restriction site SpeI was attached to the 5'-end of this DNA fragment by using appropriate primers for PCR. PPi-PFK gene was PCR cloned from genomic DNA of *B.*

Burgdorferi B31 and restriction site NcoI provided at its 3'-end. Using overlap PCR, these two DNA fragments were combined to yield PPi-PFK gene on Pgap. This gene was then inserted in correct orientation into pTeasy vector to obtain T(+)GFPK. T(+)GFPK was digested with SpeI while pSTVZM27 with NheI. The digested plasmids were then ligated to get pNGFPK of 6.8 kb size.

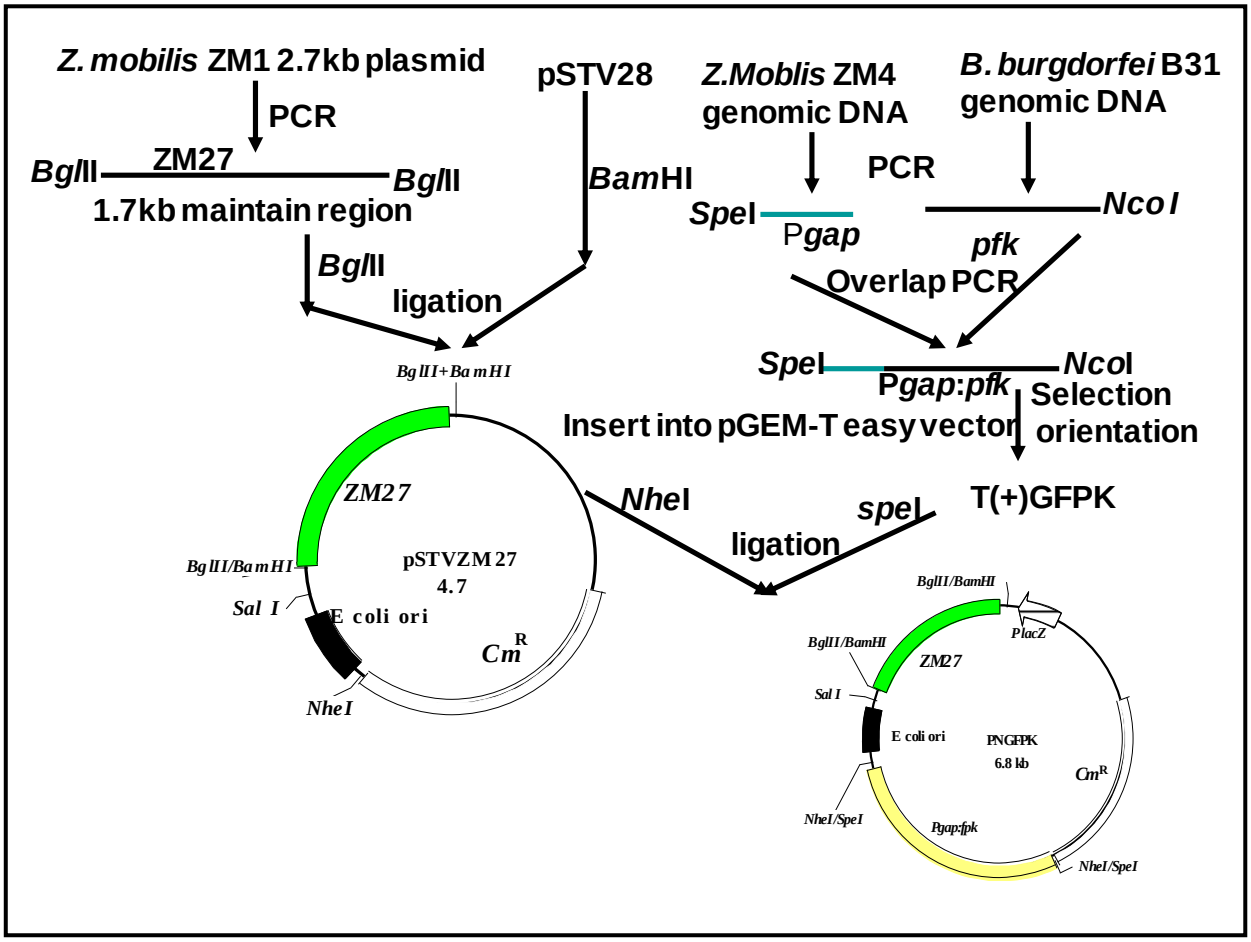

Figure 37: PPi-PFK overexpression plasmid pNGFPK and corresponding empty plasmid pSTVZM27

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