ENGINEERING *E. COLI* TOWARD CONSOLIDATED BIOPROCESSING OF CELLULOSE

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ENGINEERING *E. COLI* TOWARD CONSOLIDATED BIOPROCESSING OF CELLULOSE

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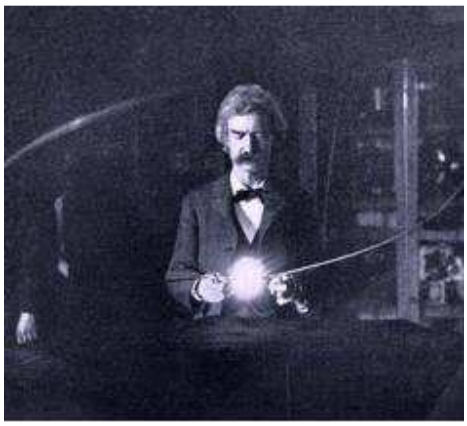

All you need is ignorance and confidence
and the success is sure.

-Mark Twain

To my wonderful wife, Steph
Whose love cannot be quantified

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LIST OF ABBREVIATIONS

ABC	ATP Binding Cassette
AFEX	Ammonia Fiber Explosion
ATP	Adenosine Triphosphate
BDO	2,3-*meso*-butanediol
BTU	British Thermal Units
CBP	Consolidated Bioprocess
DNS	Dinitrosalicylic Acid
DP	Degree of Polymerization
EDTA	Ethylenediaminetetraacetic Acid
GFP	Green Fluorescent Protein
HPLC	High Performance Liquid Chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K_M	Michaelis-Menten Constant
LB	Luria Broth
MES	2-(N-morpholino)ethanesulfonic Acid
MFS	Major Facilitator Superfamily
OD	Optical Density
PAF	Platelet Activating Factor
PASC	Phosphoric Acid Swollen Cellulose
PCBP	Pseudo-consolidated Cellulose Bioprocessing
PCR	Polymerase Chain Reaction

PHB	Poly-hydroxy Butyrate
PHBV	Poly-hydroxy Butyrate-Valerate
PHV	Poly-hydroxy Valerate
PLA	Poly-Lactic Acid
PMSF	Phenylmethylsulfonyl Fluoride
PNPG	*para*-nitrophenyl glucopyranoside
PTS	Phosphotransferase System
PSL	Polyserine Linker
SSF	Simultaneous Saccharification and Fermentation
UDP	Uridine diphosphate
V_{max}	Maximum Reaction Velocity

LIST OF GENE AND ENZYME NOMENCLATURE

BDO operon	*alsA* and *alsD* from *B. subtilis* and *budC* from *K. peumoniae*
Bgl3C/*bgl3C*	Family 3 glycosyl hydrolase from *S. degradans*
Ced3A/*ced3A*	Family 3 cellodextrinase from *S. degradans*
Cel5H/*cel5H*	Family 5 endoglucanase from *S. degradans*
Cel9R/*cel9R*	Family 9 endoglucanase from *C. thermocellum*
Cel48S/*cel48S*	Family 48 cellobiohydrolase from *C. thermocellum*
Cep94A/*cep94A*	Family 94 cellobiose phosphorylase from *S. degradans*
llp	Major Membrane Lipoprotein

SUMMARY

Cellulosic biomass represents a major untapped resource capable of replacing many products derived from fossil fuels. Cellulose, the major component of cellulosic biomass, is composed entirely of glucose and as such conversion of cellulose to glucose would permit formation of any bioproduct with glucose as the precursor. Enzymes exist that are capable of hydrolyzing cellulose and further generating glucose. One approach to reducing the cost associated cellulose bioprocessing is to develop a consolidated bioprocess in which enzymes are produced, cellulose is hydrolyzed, and products are formed in a single reaction must be developed. The three main objectives of this dissertation are to develop the components essential to a consolidated cellulose bioprocess: (1) a minimal set of enzymes capable of extensive cellulose hydrolysis under physiological conditions, (2) characterization of proteins capable of transporting hydrolysis intermediates into the cytoplasm, and (3) a system for rapid conversion of cellodextrins into glucose.

A minimal set of cellulase enzymes, Cel5H, Cel9R, and Cel48S, was selected for cellulose hydrolysis. Cel9R and Cel48S from *C. thermocellum* have been shown to synergistically hydrolyze cellulose. These enzymes, one endoglucanase and one cellobiohydrolase, take advantage of the typically observed endo-exo cellulase synergy. Addition of a second endoglucanase, Cel5H from *S. degradans* takes advantage of the endo-endo type synergy that can be observed between processive endoglucanases. The performance of the system was evaluated at conditions compatible with *E. coli*

fermentation. It was observed that these three enzymes are capable of extensive hydrolysis of cellulose across a broad range of compositions. Furthermore, substantial product formation was observed when this mixture was used during fermentation of cellulose. With all three enzymes acting in concert, product titers as high a 0.2% w/v were obtained and action of Cel5H alone was even capable of generating product as much as 0.1%. This system, upon initial investigation, is shown to achieve higher hydrolysis rates in-vivo than those developed by other researchers.

Three transporter enzymes were identified and characterized in their capacity for transport of cellobiose, a major product of cellulose hydrolysis, across the cell membrane. All three were shown to be suitable for fermentation of cellobiose by *E. coli*. Further conversion of cellodextrins produced by cellulases to glucose was achieved by two enzymes, Ced3A and Cep94A from *S. degradans*. Expression of Ced3A led to complete consumption of all glucose oligomers with a DP higher than 2 in a mixture of cellodextrins and expression of Cep94A generated rapid metabolism of cellobiose that was left behind by Ced3A. Together these enzymes proved capable of rapid conversion of all the products of cellulose hydrolysis to fermentable glucose. When combined, the three components developed and characterized in this dissertation represent all that is needed for a consolidated bioprocess in which cellulose is converted into bioproduct in a single step reaction.

CHAPTER 1

INTRODUCTION

1.1 Cellulosic Biomass

Biomass is biological material derived from living or recently living organisms. As it pertains to renewable energy it typically comes from plants. In 2012 the U.S produced 8.130 quadrillion BTUs of energy from renewable sources with 45% of that derived from biomass[1]. Cellulose is a major component of plant biomass and is the most abundant organic polymer on the planet. The abundance of cellulose is so high as to be considered inexhaustible with current technologies. Its availability and lack of importance as a major food source makes plant biomass an attractive, renewable material for carbon neutral industrial applications.

1.1.1 Sources of Cellulosic Biomass

Biomass has a variety of different applications. Currently, sugars from corn and cane sources are used to produce ethanol as a biofuel while seed oils, especially soybean oil, are used as precursors for biodiesel production. Major sources of cellulose include energy crops, forestry products and wastes [2], as well as wastes from agricultural, industrial and residential processes[3].

While the sources of cellulose are abundant very few of these sources represent pure cellulose. Plant matter is composed of a mixture of lignin, hemicellulose, and cellulose with the relative abundance of each species varying widely from species to species [4] and is even affected by factors such as geography, climate, storage conditions,

and processing techniques[5-7]. In general, cellulose comprises between 40 and 60 percent of plant biomass. In this biomass the cellulose fibers are wrapped in hemicellulose polymers which are all held together by lignin molecules[8].

Hemicellulose is a complex polymer of xylose and glucose that is very highly substituted with many functional groups including pentoses, hexoses, and carboxylic acids[9]. Lignin is another complex heterogeneous polymer composed of a variety of phenylpropanoid groups that are cross-linked by hydroxycinnamic moieties[10-12]. Hemicellulose helps to protect the cellulose fibers from enzymatic degradation and lignin is very hydrophobic further protecting the construct from degradation by inhibiting diffusion of water soluble chemicals and proteins into the cellulose fiber core[13, 14]. This superstructure causes this material to be very recalcitrant, greatly retarding the depolymerization of this carbon reserve.

Cellulose itself is attractive because it is a polymer of D-glucose molecules joined by β, 1-4, glycosidic bonds and complete depolymerization will generate glucose: the most widely known fermentable sugar. Cellulose molecules can vary in degree of polymerization anywhere from 300 in wood pulps to 10,000 in bacterial cellulose[15]. These chains will interact with each other via both Van der Waals and hydrogen bonding to form fibers[16-18]. These bundles can arrange themselves in a variety ways resulting in non-uniform crystallinity between cellulose from different sources[19, 20].

The composition of cellulose makes it incredibly attractive for a wide variety of applications. Because it is made entirely of glucose monomers it can be used for production of any chemical that has glucose as a starting substrate. Glucose is the most widely usable sugar in the microbial world[21] and is a precursor to the formation of

products by both aerobic and anaerobic processes in model organisms such as *E. coli*, *Z. mobilis* and *S. cerivisiae* as well as less studied species with more specific applications[22-25]. Products include biofuels as well as other high-value chemicals which can replace petroleum based alternatives. In employing biological processes we can couple the production of high-value chemicals with lower value fuels and commodities to make cellulose bioprocessing a profitable industrial endeavor.

1.1.2 Biofuels

A wide variety of biofuels have been explored for replacement of petroleum based fuels. These include but are not limited to ethanol, butanol, and biodiesel. Ethanol and butanol are obtained through anaerobic fermentations in many bacterial and yeast strains. Strains used for production of ethanol are either naturally well performing ethanologens like *Zymomonas mobilis* or *Saccharomyces cerevisiae* [26, 27], or metabolically engineered to remove the capacity for formation of any other fermentation products (*E. coli* and *S. cerivisiae*)[28]. In the case of butanol, heterologous enzymes from other bacterial species must be added to complete the butanol generating metabolic pathway in industrially relevant organisms[29, 30].

Additional efforts to improve the conversion of biomass to these products include improving pathways by supplementing heterologous enzymes or introducing upstream pathways to broaden substrate ranges to include arabinose, and xylose, the pentose monosaccharide components of hemicellulosic biomass[31-33]. Finally, improvement of the tolerance to stress generated by overproduction of organic solvents has resulted in the

emergence of organisms suitable to production of high amounts of butanol and ethanol[34, 35].

Biodiesel is generated through a simple transesterification reaction using glycerol and lipids obtained from plant sources[36]. Traditionally the major sources of these were plant oils derived mostly from seed crops such as soy and palm[37]. Recently, however, microalgae have been in major consideration as a source for the biodiesel precursors. The ability to use more traditional bioreactors to generate the biodiesel as well as the fact that cultivation does not require arable land offer several advantages over plant oils[38].

Currently ethanol can be added to gasoline up to 10% serving as an oxygenating species for combustion. Ethanol, however, cannot be transported with existing infrastructure[39, 40]. Butanol is less hygroscopic and has a higher energy density than ethanol making it less challenging to transport and more economically feasible than ethanol[41]. These liquid fuels represent an immediate replacement for gasoline and diesel fuel and offer a future improvement over current compounds used as alternative liquid fuels and most importantly offer alternatives for resources obtained from foreign sources.

In addition to liquid fuels, several fuel gases can be generated through biological processes. A variety of different organisms, both phototrophic and chemotrophic, are capable of generating hydrogen from biomass. Photosynthetic organisms can generate hydrogen from water alone as well as from simple sugars and organic acids[42-44]. Non-photosynthetic biological processes generate hydrogen from substrates ranging from simple sugars and complex carbohydrates to liquid and solid sewage waste[45, 46]. Methane can also be obtained from biological processes. This fuel gas is generated from

metabolism of a wide variety of residential and other waste residues including crop resideus, slaughterhouse waste, waste activated sludge, energy crops and fertilizer wastes by a wide variety of methanogenic bacteria[47]. Coal can also be converted to methane gas by using a microbial consortia of a multitude of archae species [48]. Gas fuels from biomass conversions can be directly utilized for generation of electricity by turbine as well as heat generation and use in combustion technologies.

1.1.3 Commodity Chemicals

1.1.3.1 Lactic Acid

Lactic acid is a carboxylic acid produced by the oxidation of pyruvate by the lactate dehydrogenase enzyme[49]. Also called milk acid it is abundant in dairy products. There are several species of bacteria that are naturally very efficient at producing lactic acid as a major product[50]. Other bacterial species, including *E. coli* have been metabolically engineered to product lactic acid as the sole anaerobic growth product[51]. It is of industrial relevance as a precursor to the biodegradable polymer polylactic acid, or PLA. Because lactic acid is present in two different enatiomeric forms the polymer is tunable in many relevant properties for a wide variety of applications[52]. All reports of significant lactic acid generation during fermentation use monosaccharides, usually either glucose or xylose, as the fermentation feedstock. Unlike many other fermentation products, lactic acid contains carbons and its production does not result in the loss of carbon via carbon dioxide.

1.1.3.2 Butanediol

2,3-butanediol is a product of the fermentation metabolism of a variety of organisms including *B. polymxa* and *K. pneumonia*[53]. Due to its chemical nature it has a very broad variety of applications ranging from energy to valuable precursors. The energy density is very similar to ethanol and methanol and as such can be used as a liquid fuel[54]. Perhaps more interestingly it can be converted to a number of different molecules through simple chemical reactions. 1,3 butadiene, the precursor to synthetic rubber, can be produced by a simple dehydration reaction[55]. Methyl ethyl ketone, a fuel additive, is also produced by dehydration[56]. Finally, esterification generates molecules that can be further converted to polyurethanes that are used in pharmaceuticals and other health care products[57]. Because of the massive potential of this bioproduct pathways for its production have been introduced into *E. coli* with great success[58]. Three proteins, acetolactate synthase and acetolactate decarboxylase from *B. susbtilis* and acetoin reductase from *K. pneumonia* were expressed in *E. coli* and strains were able to produce BDO up to 0.42 g/g glucose (theoretical yield is 0.5 g BDO/g glucose). Operation of this foreign in *E. coli* requires low oxygen or anaerobic conditions.

1.1.3.3 Poly-hydroxybutyrate

Many valuable bioproducts are simple molecules that are secreted and must be purified from the extracellular milieu. Microbes also produce valuable products that cannot be secreted, especially polymers accumulated by the cell for energy storage. One of the most interesting of these is polyhydroxyalkanoate, a biodegradable and biocompatible thermoplastic produced by bacterial species[59]. Microbes produce these when the carbon/nitrogen ratio is high[60]. The monomer units are butyric acid or valeric

acid with some organisms producing polymers of only butyrate (PHB), some producing polymers of only valerate (PHV), and others producing a copolymer (PHBV)[61, 62]. As a thermoplastic with a high melting point, this molecule which is not water soluble like many other biopolymers, represents a replacement for petroleum derived polymers with industrial applications and its biocompatibility makes it attractive for medical applications[63, 64].

Metabolism of the two major components of lignocellulosic biomass, glucose and xylose, is achieved by glycolysis, resulting in formation of pyruvate and acetyl-CoA. The products presented above, among others, are of interest for consolidated bioprocessing because they are all produced by pathways that use the products of glycolysis as a starting material. Furthermore, introduction of these pathways requires minimal genetic manipulation and recombinant protein expression making modifications suitable for enhance cellulose degradation easier to realize.

1.1.4 Biomass Processing, Pretreatment and Hydrolysis

As mentioned above, cellulosic biomass exists in nature as a complex structure of a variety of different compounds that is very resistant to degradation. As such, after harvesting the material it must be broken down so that it can be utilized. This process represents one of the most intensive parts of biomass utilization. It is estimated that nearly 20% of the cost of cellulosic ethanol can be attributed to pretreatments[65]. Typically a variety of pretreatments are employed in an effort to remove disrupt the mechanical superstructure, remove lignin, preserve the chemical integrity of hemicellulose and cellulose and reduce the crystallinity of the cellulose fraction. In order

for the resulting material to be usable for bioprocessing this must all be done without producing compounds inhibitor to cell growth and metabolism. The three major types of pretreatment technologies currently used are physical, chemical, or microbial processes[66].

1.1.4.1 Physical Pretreatments

A variety of physical pretreatment technologies exist and are mainly employed for the removal of lignin and the reduction of volume of the biomass to help increase the accessible surface area. The simplest form of physical pretreatment is milling. Milling can be done in wet or dry conditions with a variety of ways including ball milling, grinding, hammer milling, and roll milling. These forms of treatment tend to decrease crystallinity and increase surface area while making very few chemical modifications to the substrate.

Other forms of physical treatment employ water in its various phases to disrupt the biomass. Uncatalyzed steam explosion uses high pressure steam to rapidly heat the substrate. Once heated, the pressure is released and rapid decompression or expansion occurs causing disruption of the substrate's superstructure[67, 68]. Liquid hot water can also be used to pretreat biomass. This method involves boiling the biomass in water at high temperatures. These treatments using water have been reported to cause increased digestibility in more herbaceous feedstocks such as corn stover and sugarcane bagasse[69]. Additionally, these processes are able to at least partially hydrolyze hemicellulose and remove many of the side groups such as acetic and uronic acid that can lead to formation of inhibitory compounds under acidic pretreatment conditions. Just as importantly, these pretreatments are capable of solubilizing a majority of the biomass

including lignin and report recovery of monosaccharides between 55 and 90 percent depending on the identity of the biomass treated[66, 70, 71].

1.1.4.2 Chemical Pretreatments

Many different methods for chemical pretreatment of cellulosic biomass have been employed by the paper and pulp industry long before the interest in biofuels. The major technologies developed thus far include acid, alkaline, ammonia, and ionic liquid treatments. These chemicals are generally inexpensive, however their chemical nature may require specialized equipment which is expensive as well as extensive recycle in order to make the processes cost feasible. Additionally, hydrolysates generated by these processes require downstream treatments before they can be effectively used in bioprocesses.

1.1.4.2.1 Acid Pretreatment

Dilute acid pretreatments employing sulfuric acid, nitric acid, hydrochloric acid, phosphoric acid, and peracetic acid have been developed for a wide range of biomass substrates. These processes generally use dilute acid concentrations (up to 1% acid) and high temperatures (120-180 °C) in many reactor types including batch, plug flow, or countercurrent operations. In general, sulfuric acid is the most widely applicable to a range of different biomass sources, however other acids listed above have been shown to be more effective on some substrates[70, 72].

When biomass substrates are treated with these dilute acids the hemicellulose molecules are hydrolyzed and generally the lignin and cellulose portions remain intact with minor disruption of the lignin fraction. Removal of hemicellulose allows an

increased accessibility of the cellulose which leads to increased digestibility[66]. Depending on the reaction conditions and the nature of the substrate the process can also yield a high abundance of the monosaccharide components of the hemicellulose or conversely can lead to formation of oligomers which can are transformed into inhibitory compounds such as carboxylic acids, acetate, and furfural in the acidic environment[73].

Acid pretreatment is an attractive technology because it has been successfully applied to biomass ranging from hardwoods to energy crops to municipal solid waste. Additionally, near complete hydrolysis of hemicellulose is possible under these conditions[70]. Drawbacks of the technology, however, include the need for downstream treatment and the formation of inhibitory compounds that would need to be removed before bioprocessing.

1.1.4.2.2 Alkaline Pretreatments

Alkaline pretreatments are done using basic compounds such as sodium hydroxide, calcium hydroxide, aqueous ammonia, and ammonium hydroxide. Processes generally operate with chemical concentrations between 5% and 10% at room temperature. This method is generally more effective on substrates with lower lignin fractions such as agriculture residues or hardwoods[66, 70].

Alkaline pretreatments are effective in removing lignin as well as solubilizing hemicellulose. The hydroxide ions work in saponification of ester linkages between the xylan in hemicellulose as well as the ester bonds that are abundant in lignin[74]. While the effects on lignin and hemicellulose help to make the cellulose more accessible, this process also removes the carboxylic acid substituents which further increases accessibility to the substrate[75]. Ultimately, alkaline pretreatment results in increased

surface area and accessibility due to disruption of lignin as well as decreased DP and crystallinity[76].

Alkaline pretreatments are attractive because the processes require much less extreme conditions of temperature and pressure. The reactions are much slower, however, as a result and pretreatment using this technology takes much longer than other approaches. Additionally, the compounds used can be incorporated into the substrate as salts which requires removal downstream before the substrate can be fermented[77].

1.1.4.2.3 Ammonia Fiber Explosion (AFEX)

Ammonia Fiber Explosion pretreatments are done by exposing substrates to hot liquid ammonia at high pressure. Reactions are generally carried out at a 1:1 mass ratio of ammonia to substrate at nearly 100 °C. Use of pure ammonia results in reactions with pH higher than 12. Much like alkaline pretreatments, AFEX is generally more effective with substrates having lower amounts of lignin[70].

After incubation at conditions above the pressure is dropped and the substrate expands rapidly, altering its structure and increasing the digestibility. This process results in removal of lignin, solubilization of hemicellulose, and decrystalization of the cellulose fraction[66]. Unlike many other pretreatment forms, the chemical composition of the substrate is nearly unchanged by the processing despite significant superstructural changes[71]. AFEX is attractive due to the fact that very few, if any, side products are generated that would potential cause formation of inhibitory species. The cost of ammonia and toxicity of ammonia make it such that extensive recovery must be performed.

Table 1.1: Advantage and Disadvantages of Different Pretreatments[76]

Pretreatment method	Advantages	Disadvantages
Biological	– Degrades lignin and hemicellulose – Low energy consumption	– Low rate of hydrolysis
Milling	– Reduces cellulose crystallinity	– High power and energy consumption
Steam explosion	– Causes lignin transformation and hemicellulose solubilization – Cost-effective – Higher yield of glucose and hemicellulose in the two-step method	– Generation of toxic compounds – Partial hemicellulose degradation
AFEX	– Increases accessible surface area – Low formation of inhibitors	– Not efficient for raw materials with high lignin – High cost of large amount of ammonia
CO_2 explosion	– Increases accessible surface area – Cost-effective – Do not imply generation of toxic compounds	– Does not affect lignin and hemicelluloses – Very high pressure requirements
Wet oxidation	– Efficient removal of lignin – Low formation of inhibitors – Minimizes the energy demand (exothermic)	– High cost of oxygen and alkaline catalyst
Ozonolysis	– Reduces lignin content – Does not imply generation of toxic compounds	– High cost of large amount of ozone needed
Organosolv	– Causes lignin and hemicellulose hydrolysis	– High cost – Solvents need to be drained and recycled
Concentrated acid	– High glucose yield – Ambient temperatures	– High cost of acid and need to be recovered – Reactor corrosion problems – Formation of inhibitors
Diluted acid	– Less corrosion problems than concentrated acid – Less formation of inhibitors	– Generation of degradation products – Low sugar concentration in exit stream

Chemical pretreatments for cellulosic biomass can be both rapid and quite effective at increasing substrate digestibility. Unfortunately, all of these processes use chemicals that must later be removed before bioprocessing can continue. In some cases additional toxic compounds are produced. Because of this, enzymatic as well as whole cell microbial treatments are an attractive alternative.

1.1.4.3 Microbial Pretreatment

Microbial pretreatment of biomass employs naturally occurring fungal species to breakdown its individual components. Many fungal species are capable of degrading each of the components of biomass. Some species of white-rot fungi like *C. subvermispora* are capable of fully degrading lignin while leaving cellulose and hemicellulose components

relatively unaltered. They produce and secrete three major types of enzymes, lignin peroxidase, manganese peroxidase, and laccase which oxidize lignin. The products of this oxidation are then ultimately metabolized by the fungus. Brown-rot and soft-rot fungi, on the other hand, are capable of significantly reducing the cellulose fraction of biomass while having a minimal effect on lignin. Because of these characteristics, white-rot fungi have been the most well studied because they can remove and metabolize lignin to make the cellulose and hemicellulose more accessible while leaving the cellulose intact for metabolism by microbes capable of producing valuable biopoducts[78].

In these processes the substrate is inoculated and incubated between 25 and 30 °C for several weeks. Types of innocula include liquid cultures, cells grown on grains, or even preseeded lignocellulosic biomass[79]. Moisture content is an important parameter in this process, with most showing optimum degradation between 60 and 80% moisture[71, 80]. Microbial pretreatments are attractive because delignification can be very significant, energetic requirements are minimal, and the resulting product does not contain any toxic compounds that need to be removed prior to fermentation. Unfortunately, it also has the longest reaction time of all the methods discussed, several weeks in most cases, making it questionable for large scale industrial applications.

1.1.5 Enzymatic Hydrolysis of Treated Biomass

As mentioned above, the main goal of pretreatment of biomass is to enhance its propensity to be digested enzymatically. This enzymatic digestion of cellulose, often called saccharification, converts the cellulose molecules into oligosaccharides called cellooligomers. Current industrial processes utilize enzyme cocktails gathered from

cellulolytic fungal species to obtain maximal saccharification of cellulose[81]. Once generated, the cellooligomers can then be metabolized by organisms capable of producing valuable bioproducts. While some processes using strong acids are capable of generating monosaccharides from pretreated cellulose enzymatic approaches are widely preferred because the product streams can be sent straight to fermentation processes without an cleaning or treatment steps[82]. Microbial cellulolytic species are incredibly abundant and as such, a cornucopia of potential enzymes applicable to saccharification is available.

1.1.5.1 Enzymes used in cellulose Hydrolysis

Two major classes of enzymes are required to hydrolyze cellulose completely to cello-oligomers. Different species use different families of these same enzymes to achieve their goals. Fungal species, for example use a family 6, 7, and 48 enzymes while bacterial cellulotrophs use family 5, 9, and 48 enzymes[83]. Despite the structural differences between the families of enzymes both types of enzymes have preserved mechanisms of action on cellulose.

Cellobiohydrolases, sometimes referred to as exocellulases, hydrolyze 1,4-β-glycosidic bonds to form cellooligomers from the chain ends of the cellulose molecule. While each individual enzyme is end specific, this class of enzymes can hydrolyze either the reducing end or non-reducing end of the cellulose molecule[84]. These types of enzymes tend to prefer more crystalline types of cellulose substrates and often times show no hydrolysis toward soluble or amorphous cellulose. Cellobiohydrolases, falling into families 6 and 48 in bacterial species, generate cellobiose as their major hydrolysis product with small amounts of cellotriose also produced[85].

Endoglucanses hydrolyze 1,4-β-glycosidic bonds to release cellooligomers from the internal regions of a cellulose molecule. These enzymes are also reducing or non-reducing end specific with the end specificity varying from enzyme to enzyme. This class of enzymes prefers more amorphous types of cellulose and is often completely inactive on crystalline cellulose. Endoglucanases fall within the families 5 and 9 and generate cellooligomers ranging from cellobiose to cellotetraose[86-88].

Each of these enzyme classes can perform in a processive manner. Processive enzymes work by catalyzing more than one hydrolytic event per each association and dissociation event, proceeding along the molecule to catalyze product formation. During hydrolysis by processive enzymes it is possible for the initial hydrolytic event to release a product with a different degree of polymerization than the subsequent processive hydrolyses. Like activity, the processivity of an enzyme is dependent on the substrate on which it is acting[89-91].

1.1.5.2 Synergy with Cellulases

Because of the nature of cellulose as a substrate and the functionality of the enzymes capable of hydrolyzing it several enzymes must work in tandem to fully and completely hydrolyze cellulose. Naturally cellulolytic organisms produce dozens of different enzymes in varying quantities to metabolize cellulose[92, 93]. Synergy between cellulase enzymes is well studied and somewhat intuitive. The mechanism of endoglucanase action removes internal portions of the molecule, leaving behind two new chain ends on which cellobiohydrolase enzymes can act. Cellobiohydrolases remove portions at the end of the molecule exposing regions that an endoglucanase can attack.

Without both types of enzymes each will exhaust its available sites before complete hydrolysis is achieved.

Because of this requirement, cellulase synergy has been very widely studied. The synergism between pairs of enzymes expressed by *Clostridium* species has shown good synergy between family 9 endoglucanases and family 48 cellobiohydrolases with hydrolysis rates depending on the relative amounts of each enzymes[94, 95]. Furthermore, enzymes from different organisms demonstrate the same synergistic effects[96, 97]. It has also been shown that removal of a single cellulolytic enzyme from the genome can result in much less rapid rates of cellulose degradation[98]. In studies characterizing the performance of three different cellulase molecules it is seen that inclusion of very small amounts of one of the enzymes can double the cellulose hydrolysis rate compared to reactions with only two cellulases[99, 100].

1.1.5.3 Mechanisms of Cellulolytic Organisms

Among the known cellulolytic organisms a wide variety of cellulolytic systems exist. The total number of cellulase enzymes and the relative amounts of each class can vary widely as can the families into which these enzymes fall. In some organisms a single cellulase enzyme dominates (>50%) the cellulolytic system while other species express similar amounts of many different types of enzymes. *S. degradans* does not express any cellobiohydrolase enzymes and relies solely on endoglucanases, especially processive ones, to completely hydrolyze cellulose[101].

Aside from the identities of the components of an organism's cellulolytic complex two major strategies are employed by cellulolytic organisms to maximize substrate degradation. In the first approach enzymes are expressed in high amounts and secreted

into the extracellular space to freely associate with substrates. In the second approach the enzymes assemble themselves into nanostructures called cellulosomes that remain cell associated.

Cellulolytic organisms that grow aerobically have an abundance of energy that allows them to produce an abundance of proteins without significant metabolic burden. Organisms that secrete free enzymes are generally fungi. *T. reesei* has such an effective secreted cellulase system that it is used in industrial applications[102]. Anaerobes, on the other hand, employ a system to keep the cellulase they do produce associated with the outer membrane, resulting in more energetically economic protein production[103]. This technique allows enzymes that may act synergistically to be in close proximity of each other and any products formed will be generated closer to the cell for more rapid translocation. Many cellulolytic bacteria are ruminal or soil bacteria that are strict anaerobes and many of them utilize cellulosomal enzymes. It is interesting to note that some species that utilize cellulosomal cellulases also produce free cellulase enzymes[104]. Studies have been done to convert cellulosomal cellulase to free cellulases and vice versa. This was not shown to drastically alter the enzymes cellulolytic activities, indicating that the cellulosomal incorporation likely improves enzyme synergy and substrate uptake[94, 105].

1.1.6 Utilization of Cellooligomers

No matter the enzymes used or their cellular localization, the products formed during enzymatic hydrolysis of cellulose are cellooligomers ranging from cellobiose to as high as cellohexose. Naturally cellulolytic organisms use a number of different enzymes

to further reduce these oligomers down to glucose which can then be used in central carbon metabolism and energy production. Cellulolytic organisms often express as many of these enzymes as they do cellulases[106-108]. This indicates that this process is not only critical for cellulose metabolism, but also occurs in many different ways. It is known that cellooligomers act as inhibitors to cellulase enzymes so removal of these will hasten cellulase hydrolysis[109, 110]. Additionally, more rapid glucose generation will increase carbon flux and result in more available energy and carbon to be used for cell growth and enzyme production.

1.1.6.1 Mechanisms of Cellooligomer Utilization

In order to further depolymerize the products of enzymatic cellulose hydrolysis all the way to glucose, two mechanisms are employed. The first mechanism is a simple hydrolysis in which the enzyme uses water to hydrolytically cleave the 1,4-β-glycosidic bond to release a single glucose unit from the oligomer, reducing the DP by 1[111]. Enzymes that catalyze hydrolysis of cellooligomers are called β-glucosidases. In the case of hydrolysis glucose molecules will be phosphorylated by hexokinase, which uses ATP to achieve this end. The glucose-6-phosphate generated can then be used in glycolysis.

The second mechanism uses inorganic phosphate and water to phosphorolytically cleave the β-1,4-glycosidic bond to release a single glucose-1-phosphate molecule, leaving behind an oligomer with DP reduced by 1[112]. Enzymes that perform these reactions are called cellodextrin or cellobiose phosphorylases. The phosphorolytic mechanism generates phosphorylated glucose, eliminating the need for ATP in phosphorylation. By using this mechanism the only time ATP is used for phosphorylation is when glucose is generated from cellobiose. Glucose-1-phosphate must be converted to

glucose-6-phosphate by phosphoglucomutase before it can be used in glycolysis. Glucose-1-phosphate is also the substrate for UDP-glucose pyrophosphorylase which is the first enzyme in the glycogen synthesis pathway.

It is apparent that the phosphorolytic mechanism offers energetic savings compared to the hydrolytic mechanism in the form of unspent ATP. This is enhanced as the DP of the cellooligomers increases as each phosphorolytic step saves one ATP that would otherwise be necessary under the hydrolytic mechanism. While this savings of ATP could ultimately lead to more rapid substrate utilization, cell growth, and product formation it is important to note that the glucose-1-phosphate produced must be converted to glucose-6-phosphate to be used in glycolysis. Furthermore the products of phosphorolytic cleavage can be shunted to glycogen generation, removing it from the immediately available carbon pool. The hydrolytic mechanism, on the other hand, produces only glucose which can be used directly in glycolysis for energy production and product formation. The relative physiological benefits of each mechanism have yet to be thoroughly explored.

1.1.6.2 Phosphorolytic and Hydrolytic Enzymes

Cellooligomer phosphorylase and hydrolase enzymes are annotated as such because of their ability to break β-1,4-glycosidic bonds. These enzymes also have specificity for cellooligomers of specific DP. Many enzymes capable of utilizing cellobiose as a substrate have drastically reduced or even no activity on longer cellooligomers and enzymes that can degrade longer oligos may not be able to degrade cellobiose. An enzyme's specificity can be difficult to predict and does not depend on the enzyme family. Additionally these enzymes may have reduced activity on oligomers

containing other glycosidic bonds such as maltodextrin(α-1,4 glycosidic bonds) and β-glucan(β-1,3 glycosidic bonds) among others[113-115].

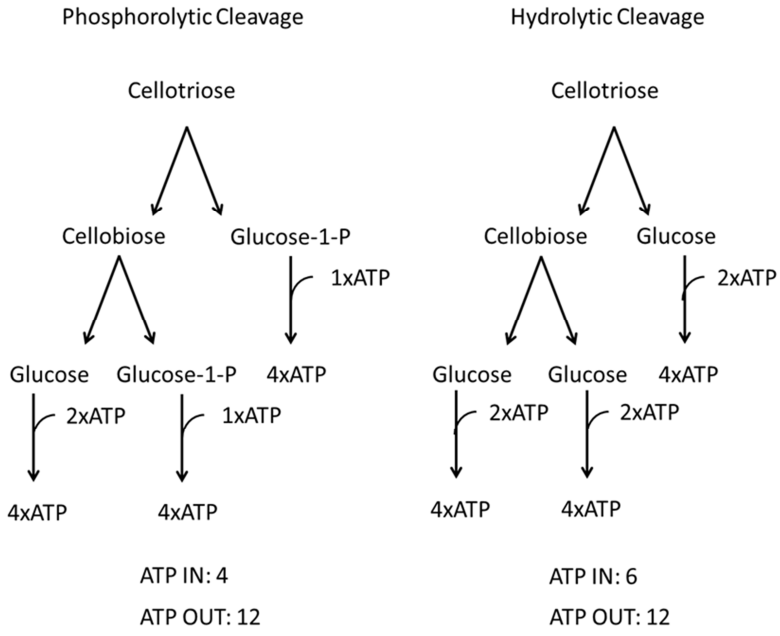

Figure 1.1: Hydrolytic and phosphorolytic mechanisms

1.1.6.3 Expression of Phosphorolytic and Hydrolytic Enzymes

As mentioned above cellulase enzymes must be secreted out of the cell in order to hydrolyze cellulose. Many glucosidases are also secreted to the extracellular space but some will remain within the cell, resulting in generation of glucose within the cytoplasm[111, 116, 117]. Interestingly, none of the phosphorylase enzymes are known to have secretion signal sequences meaning that they will remain within the cytoplasm[114, 118, 119]. This is likely because phosphorylase enzymes require inorganic phosphate of which a pool is maintained within the cells and an abundance of extracellular phosphate is unlikely to be present. Additionally, generation of extracellular

glucose-1-phosphate would need to then be transported into the cell. Furthermore, any glucose equivalents generated extracellularly would be available to other microorganisms whereas organisms capable of degrading cellodextrins intracellularly will generate glucose equivalents immediately available for metabolism by the host strain. Regardless of cellular localization, metabolism of cellooligomers removes cellulase inhibitors and generates molecules that can be readily metabolized by many microorganisms.

1.1.6.4 Transport of Cellodextrins

As mentioned above, many cellooligomer degrading enzymes are expressed and remain in the cytoplasm. In this case, cellooligomers must be translocated across the cell membrane before they can be converted to glucose or glucose-1-phosphate. Two major classes of different proteins are responsible for transporting cellooligomers into the cytoplasm. ABC transporters, or ATP binding cassette transporters, are proteins that use ATP to transport molecules into the cytoplasm. ABC transporters use two different subunits with one subunit responsible for substrate binding and another responsible for ATP binding and cleavage[120, 121]. Because substrate transport by these proteins is coupled with energy generation from ATP bond cleavage transport can also occur against a concentration gradient. Illustrations of these two mechanisms can be found in figure 1.2.

Permease enzymes are capable of passive transport of oligomers along a concentration gradient. Permease enzymes fall into the Major Facilitator Superfamily class of transport proteins[122]. Permease proteins do not require energy in the form of ATP but rather utilize naturally occurring gradients for transport[123]. Permeases often

utilize a symporter or antiporter mechanism to take advantage of energy associated with ion gradients to transport other chemicals[124].

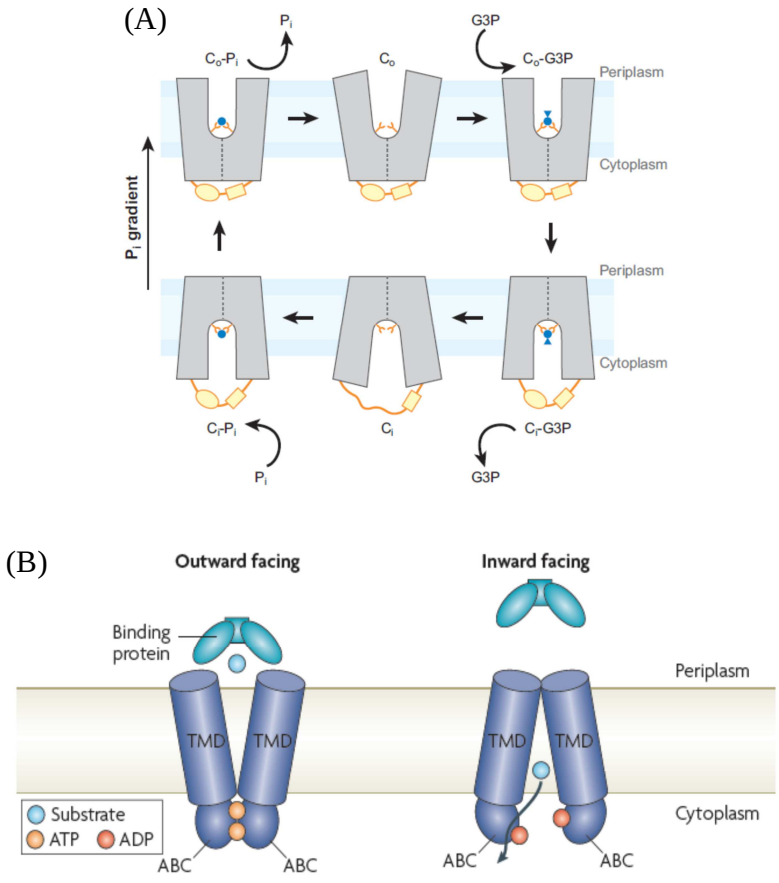

Figure 1.2: Mechanisms of (A) MFS [125]and (B) ABC permease transport[126]

Much like glucosidase and phosphorylase enzymes transport proteins can have a range of substrate specificity. Lac12 of *K. lactis* and LacY of *E.* coli are known lactose permease proteins which also facilitate transport of cellobiose[127, 128]. Cdt1 and Cdt2 from *N. crassa* transport cellobiose, cellotriose, and cellotetraose[129]. Alternatively, the

ABC transporter CbpA from *C. thermocellum* binds only cellotriose and nothing else[130]. While both ABC transporters and permease proteins are capable of translocating cellooligomers across the cell membrane, permease enzymes are capable of doing so without any energy spent in the form of ATP. This lack of ATP requirement makes permease proteins much more attractive for consolidated bioprocessing applications. Regardless of energetics of transport cellooligomers must be transported into the cytoplasm to capitalize on the energetic benefits of phosphorolytic cleavage.

The current paradigm of cellulose bioprocessing involves three separate steps (1) production of cellulase enzymes, (2) hydrolysis of cellulose, (3), metabolism of hydrolysis products. Cellulose is an extremely low cost, renewable feedstock, but in order to make products produced from cellulose more economically these three processes must be consolidated into a single process in which enzymes are produced, hydrolysis occurs and product is formed. Much work has been done already regarding cellulase enzymes and their activities and as such strategies for improved hydrolysis rates and cellulase degradation have been developed. Strategies for metabolism of the products of enzymatic cellulose hydrolysis must be developed in order to develop an efficient consolidated bioprocess.

1.2 Project Objectives

The work presented in this dissertation focuses on three objectives: (1) characterization of a new cellodextrinase enzyme capable of hydrolyzing a wide range of cellooligomers and its application to improved fermentation of sugars produced during enzymatic cellulose hydrolysis, (2) characterization of proteins suitable for the transport

of cellobiose into *E. coli* during consolidated bioprocessing and (3) development of a minimal set of cellulases capable of extensive cellulose hydrolysis.

1.2.1 Characterization of a Cellodextrinase and Its Application to Improved Fermentation of Sugars

During enzymatic degradation of cellulose multiple different cellooligomers are produced ranging from cellobiose up to cellohexose. Conversion of these oligomers into glucose equivalents is essential if *E. coli* is to be used as a whole cell catalyst for consolidated cellulose bioprocessing. In the interest of energetics and minimizing complexity of the system it would be ideal to employ as few enzymes as possible to achieve complete and rapid conversion of cellooligomers to something that *E. coli* can metabolize. *S. degradans*, a marine bacterium capable of degrading a wide array of complex polysaccharides including cellulose, expresses five annotated β-glucosidase enzymes. Three of these, Bgl1A, Bgl1B, and Bgl3C, are cellobiases while Ced3A and Ced3B are annotated as cellodextrinases. Ced3A is shown to be expressed when avicel, carboxymethylcellulose, and xylan are used as the carbon source while Ced3B is only seen during growth on xylan. Ced3A seems to be the enzyme responsible for the majority of cellooligomer hydrolysis in *S. degradans* and as such is an attractive candidate for expression in an *E. coli* strain to be used for consolidated bioprocessing. In characterizing the activity of this enzyme on a range of cellooligomers and its performance during fermentation we can evaluate its suitability for this application.

As mentioned above, xylose is a major component of the hemicellulose portion of cellulosic biomass. Because of this it is likely that during consolidated bioprocessing of realistic cellulosic substrates xylose generated from hemicellulose may be present along

with the cellooligomers generated from enzymatic hydrolysis of cellulose. In this case it would be essential to be able to rapidly ferment both sugars with the same whole cell catalyst. Verification of the capacity for cellodextrinase to allow for cofermentation of cellobiose and xylose would make this enzyme incredibly attractive for consolidated bioprocessing.

1.2.2 Selection of Cellobiose Transporters

Many of the enzymes capable of degrading cellobiose are expressed cytoplasmically. This obviously requires transport of the cellobiose across the cell membrane in order for *E. coli* to metabolize cellobiose. Three permease proteins, LacY from *E. coli* and CP1 and CP2 from *S. degradans* have been selected as potential candidates for cellobiose transport in *E. coli*. By characterizing the kinetics of cellobiose and their performance during cellobiose fermentation we will be able to select the optimal protein for cellobiose translocation for consolidated cellulose processing applications.

1.2.3 Development of a Minimal Set of Cellulases Capable of Extensive Cellulose Hydrolysis

A key component of a consolidated cellulose bioprocess is the cellulases used to hydrolyze cellulose. Three enzymes, Cel5H from *S. degradans* and Cel9R and Cel48S from *C. thermocellum* were selected for their demonstrated synergy, high individual activities, and apparent importance in their respective cellulolytic system. By using these enzymes we will be able to capitalize on endo-exo synergy as well as endo-endo synergy that have been observed. Additionally, use of only 3 enzymes will help to maintain low

complexity of the system allowing better control and understanding of its behavior. By characterizing the performance of a mixture of these enzyme at physiological conditions relevant to *E. coli* fermentation we can determine the suitability of this system for generation of hydrolysis intermediates in a consolidated bioprocess that uses *E. coli* as the microbial chassis.

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CHAPTER 2

PERIPLASMIC EXPRESSION OF A *SACCHAROPHAGUS* CELLODEXTRINASE ENABLES *E. COLI* TO FERMENT CELLODEXTRINS

2.1 Abstract

Metabolic engineering has been successful in generating highly efficient *E. coli* catalysts for production of biofuels and other useful products. However, most of these engineered biocatalysts are only effective when glucose is used as the starting substrate. Strategies to overcome this limitation in the past almost exclusively relied on extracellular secretion or surface display of a β-glucosidase. We show here, for the first time, a periplasmic expression of a *Sacchrophagus degradans* cellodextrinase (Ced3A, EC 3.2.1.21) as a successful strategy to enable *E. coli* to use cellodextrin. The engineered strain was able to grow with cellodextrin as sole carbon source. Additionally, we show that penetration of cellodextrin into periplasmic space was enhanced by using a mutant with leaky outer membrane. Furthermore, we demonstrate that the catalyst can efficiently ferment cellodextrin to lactic acid with about 80% yield. The ability of a biocatalyst to use cellodextrin should make it useful in consolidated bioprocessing of cellulose.

2.2 Introduction

Cellulosic materials are abundant renewable feedstock potentially useful for production of biofuels and other molecules. Their effective use could alleviate environmental concerns associated with petroleum feedstock and reduce the reliance of imported oil. The prevailing cellulosic technology requires cocktails of enzymes to

completely de-polymerize cellulose to glucose before microbial fermentation. This requirement stems from the inability of microbial catalysts to use cellulose polymer directly. In fact, most microbial catalysts are unable to use even the much smaller partial hydrolysis products collectively known as cellodextrin (or glucose polymer with DP of 2 or higher). The concerted action of cellulases (endoglucanases and exoglucanases) yields a mixture of cellodextrin, whose further breakdown requires a β-glucosidase which releases a glucose molecule from cellobiose. As commercial cellulases are typically not adequate in β-glucosidase activities to produce sufficient glucose, its supplement is often found to be necessary [1, 2]. Overall, the demand of large amounts of enzymes is one of the most important obstacles in commercializing cellulosic technology [3, 4].

Several approaches were used to develop microbial catalysts to assimilate (as opposed to hydrolysis to glucose first) cellodextrin directly. Researchers aimed to reduce the amount required for β-glucosidase, and generate a microbe capable of utilizing cellobiose, were reported for yeast [5] and other eukaryotes [6]. In most cases, a β–glucosidase was expressed extracellularly or displayed on cell surface to avoid the need to transport cellobiose into cells. Only limited success was achieved. While cells thus engineered were able to use cellobiose, the rate of product formation did not match what was from glucose [7, 8]. This may be due to the extra burden on cells for synthesis of glucosidase and limited extracellular expression or displayed enzyme. An alternative approach for direct assimilation of cellodextrin in yeast was reported recently, in which cellobiose intracellular assimilation was enabled by co-expression of a fungal MFS transporter and β-glucosidase [9]. When used in simultaneous saccharification and fermentation (SSF), it increased consumption rates of glucose and cellobiose

significantly, relative to a control without the transporter. Additionally, in a follow-up study, the ability to assimilate cellobiose intracellularly was shown advantageous in mixed sugar fermentation, allowing cells to simultaneously convert cellobiose and xylose to ethanol [10]. Among the efforts to engineer bacteria such as *E. coli* [11, 12], *Zymomonas mobilis*, *Klebsiella oxytoca* [13], the work on *E. coli* from Ingram's lab is most noteworthy. To eliminate the need for extracellular β-glucosidase, the cellobiose operon from *Klebsiella oxytoca* was cloned into *E. coli* and expressed intracellularly, which encodes proteins in the PTS cellobiose uptake system and a phospho-β-glucosidase (catalyzing the hydrolysis of cellobiose-P into glucose and glucose-6-P). The resulting strain was able to ferment cellobiose into ethanol with about 90% yield without exogenous β-glucosidase supplement [14]. However, cellodextrin with DP greater than two was not utilized due to the limitation of the PTS system.

In this work, we demonstrate a successful strategy significantly different from these previous attempts. Instead of a β-glucosidase, a *Saccharophagus* cellodextrinase, exhibiting broad substrate specificity with higher activity on larger cellodextrin molecules is used. Expressing the enzyme with its native signal peptide, the cellodextrinase is localized in the periplasm. We show that periplasmic expression of the enzyme is sufficient to enable cell growth on cellodextrin and additionally, to convert cellodextrin into lactic acid with high yield. With the availability of outer membrane permeable mutant, periplasmic expression offers an alternative to make active recombinant enzymes accessible to substrate molecules that are permeable to the outer membrane but not to the inner membrane, as is the case for cellodextrin.

2.3 Results

2.3.1 Expression of *Saccharophagus* Cellodextrinase Ced3A in *E. coli*

Saccharophagus degradans is a marine bacterium, adept at degrading of a variety of polymers existing in Nature, including cellulose [15, 16]. Cellodextrinase, Ced3A, is one of the enzymes expressed and secreted when *S. degradans* was grown on crystalline cellulose, suggesting its importance in cellulose degradation [16]. The gene of 3208 bp, *ced3A*, encodes a catalytic domain of family 3 hydrolase and a catalytic domain of an acetylesterase[17]. To evaluate the impact of the heterologous expression of the gene on *E. coli*, both the full length gene and truncated gene containing only the structural gene (designated as mature form) were cloned into *E. coli* via a low-copy number plasmid pSTmCED and pSTfCED, respectively. The mature form was additionally cloned into the pQE80L plasmid which has a His tag at the N-terminus to allow for its facile purification.

Initial analysis using synthetic substrates, para-nitrophenyl glucopyranoside (PNPG) showed that both the mature form and full-length form were functionally expressed. Purified mature protein was used to determine Michaelis-Menten kinetic parameters of the cellodextrinase on cello-oligomers from cellobiose (G2) to cellopentose (G5). As shown in Table 2.1, Ced3A was active on all substrates tested. Based on the value of V_{max} as well as catalytic efficiency, defined as the ratio of the turnover number to the K_m value of the enzyme, the highest activity was observed with cellotetraose. The measured V_{max} and K_m values on cellotetraose are 6.2 $\pm$ 1.2 Units min^{-1} mg^{-1} and 1310 $\pm$ 300 μM, respectively. Overall, the enzyme was more active on longer oligomers (G4 and G5) than on shorter ones (G2 and G3). This is consistent with the annotation.

To confirm proper translocation of Ced3A in *E. coli*, subcellular fractionation was carried out and the extracellular, periplasmic, cytoplasmic, and insoluble fraction was analyzed for enzyme activity with the synthetic chromogenic substrate PNPG (Figure 2.1). The cells containing the empty plasmid showed no activity in any cellular fraction, as expected. The cells expressing the mature Ced3A has most of the enzyme activities in the cytoplasmic fraction whereas the cells expressing full length of the gene has most of the enzyme activities in the periplasm, suggesting that the signal peptide is needed for translocating the recombinant protein through the inner member. There was no activity in the insoluble fraction, indicating neither inclusion body nor significant membrane association of the enzyme under the conditions investigated. Since there was no extracellular activity, the full length recombinant cellodextrinase was therefore expressed as a soluble periplasmic protein.

Table 2.1: Activity of Mature Ced3A on Cellobiose, Cellotriose, Cellotetraose, and Cellopentose

Substrate	V_{max} (Units/min/mg)	K_m (μM)	k_{cat}(min^{-1})	Efficiency (min^{-1} μM^{-1})
Cellobiose	0.27 ± 0.03	192 ± 8.70	5040	26.1
Cellotriose	0.54 ± 0.20	406 ± 15.6	9970	24.5
Cellotetraose	6.2 ± 1.2	1310 ± 300	114000	86.7
Cellopentose	3.4 ± 0.7	1230 ± 225	62200	50.7

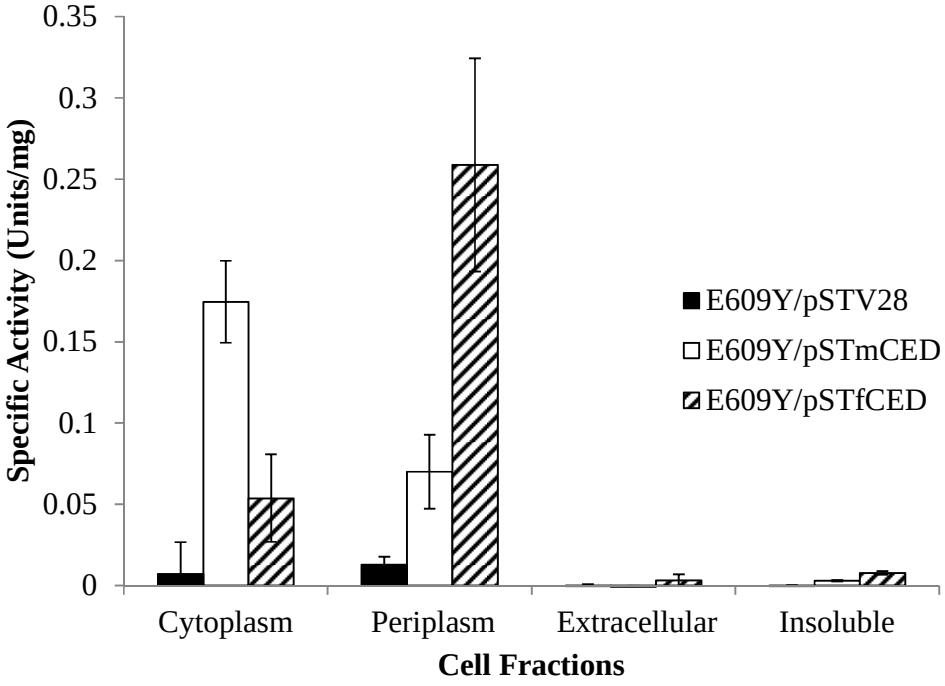

Figure 2.1: Activity of cellular fractions on PNPG measured spectrophotometrically

2.3.2 Expression of Full-Length Ced3A in *E. coli* Enabled Growth on Cellodextrin

Upon confirmation that Ced3A is active on cellodextrin of varying chain lengths and that it can be properly translocated, cell growth experiments were performed to determine the capacity of *E. coli* to grow on cellodextrin. In anticipating diffusion of large cellodextrin molecules through outer membrane may be limiting the cell growth. The two recombinant plasmids were transformed into a host strain, E609Y, which carries an lpp deletion. This deletion mutant was previously developed in the Chen lab and extensively characterized with significant increase in outer membrane permeability [18]. The two recombinant strains E609Y/pSTVmCED and E609Y/pSTVfCED along with a control strain E609Y/pSTV28 were cultivated in M9 media containing either cellobiose or a cellodextrin mixture (containing G2 to G5 as main components prepared in house as

described in Materials and Methods section) as sole carbon source at concentration of 0.5 w/v %, and IPTG at 1.0 mM to induce the synthesis of the recombinant enzyme. As shown in Figure 2.2, cells expressing the full-length cellodextrinase were able to grow on cellobiose and cellodextrins while cells expressing the mature-form cellodextrinase exhibited no growth in either case, confirming the importance of presence of the enzyme in periplasmic space for the growth phenotype. The growth rates of E609Y/pSTVfCED on glucose, cellobiose, and cellodextrin were 0.25 ± 0.02 hr^{-1}, 0.20 ± 0.02 hr^{-1}, and 0.30 ± 0.04 hr^{-1} respectively. The slightly lower growth rate of the recombinant strain on cellobiose, relative to cellodextrin, is consistent with the enzyme kinetics showing cellobiose is the least favorable substrate (Table 2.2).

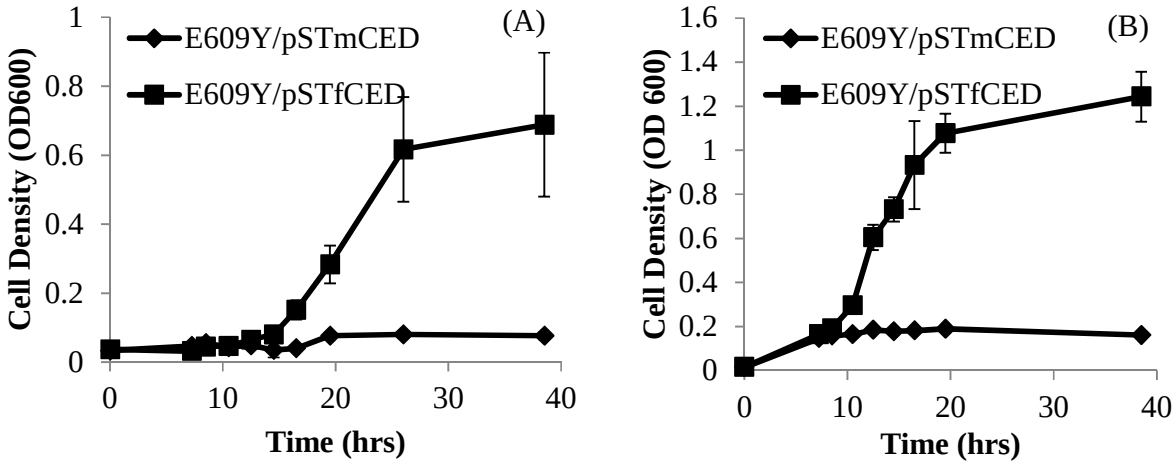

Figure 2.2: Growth of plasmid containing E609Y strains on (A) Cellobiose and (B) Cellodextrin

To evaluate the effect of outer membrane permeability on cell growth, the same plasmids were transformed into a host strain, E609, the parental strain of E609Y,

resulting in the two recombinant strains E609/pSTmCED and E609/pSTfCED. Growth experiment was carried out as above. As before, expression the mature protein did not result in cell growth whereas expressing the full length enzyme enabled a robust growth on both cellobiose and cellodextrin. While this result is qualitatively the same with those from studies from E609Y, a careful examination of growth rates on cellodextrin between the two host strains, E609 and E609Y, showed a significant difference, 0.12 vs. 0.30 h^{-1} (Table 2.2). Thus, apparently, the *lpp* deletion mediated outer membrane permeability increase help the cells gain access of cellodextrin in the periplasm.

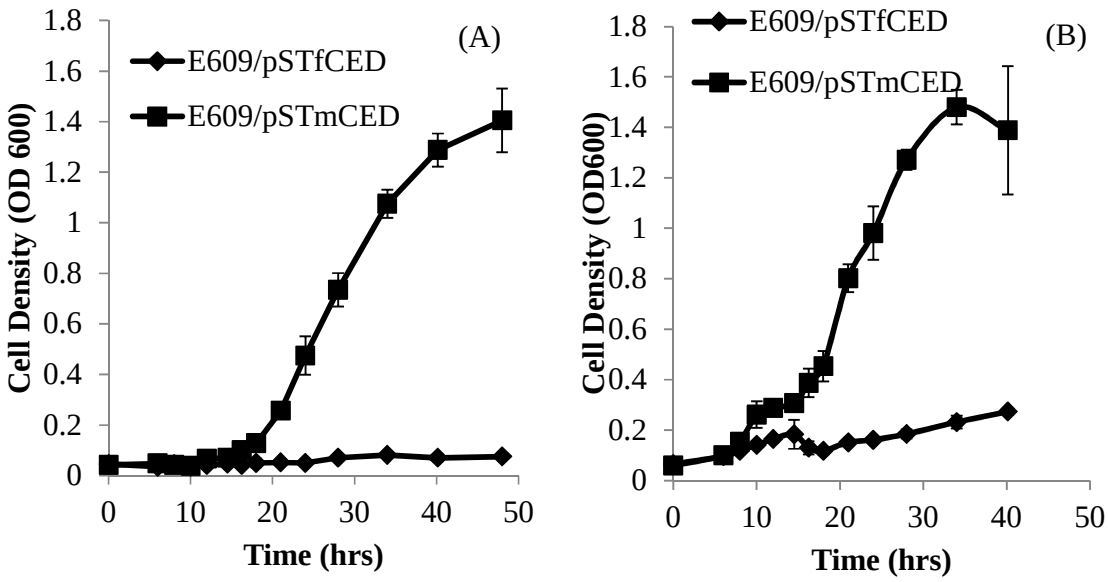

Figure 2.3: Growth of plasmid containing E609 strains on (A) cellobiose and (B) cellodextrin

Table 2.2: Average Growth Rates of E. Coli E609 and E609Y on Cello-oligomers (hr-1)

Strain	Substrate	Plasmid		
E609		pSTV28	pSTVmCED	pSTVfCED
	Glucose	0.41 ± 0.03	0.31 ± 0.04	0.40 ± 0.03
	Cellobiose	N/A	N/A	0.17 ± 0.02
	Cellodextrin	N/A	N/A	0.12 ± 0.03
E609Y				
	Glucose	0.30 ± 0.02	0.25 ± 0.02	0.25 ± 0.02
	Cellobiose	N/A	N/A	0.20 ± 0.02
	Cellodextrin	N/A	N/A	0.30 ± 0.04

2.3.3. Periplasmic Expression of Ced3A Allowed *E. coli* to Ferment Cellodextrins to Lactic Acid

To illustrate that *E. coli* cells engineered to express a periplasmic cellodextrinase are useful as catalyst in biorefinery application, additional experiments were carried out to evaluate the ability of *E. coli* cells to produce lactic acid from cellodextrin. SZ63 strain (obtained from Ingram Lab), which has been engineered to produce optically pure lactic acid as the sole product of fermentation [19], was modified by the *lpp* deletion[18] and the resulting SZ63Y was transformed with both pSTV28 and pSTVfCED vectors, and these strains were used for fermentation of cellodextrin mixtures. A two stage process was used. Cells were first grown aerobically in LB medium and induced for recombinant protein synthesis with IPTG at concentration of 1.0 mM. After 16 hour induction, cells were harvested and were suspended into M9 media containing 0.5% carbon source to an OD of 2.5 and cultivated anaerobically. For the strain expressing the full length Ced3A, significant cellodextrin hydrolysis was evident (Figure 2.4a). Reducing sugar concentration measured by a DNS method (details in Materials and Methods section) during the fermentation showed a brief transition period during the first

two hours with an increase of sugar concentration (Figure 2.4A). This was followed by a rapid decrease in sugar concentration until about 14 hours, when the hydrolysis was leveled off, resulting in an overall conversion about (58%). For the strain expressing the mature enzyme, only about (20%) conversion was observed, indicating the importance of periplasmic expression for the hydrolysis of cellodextrin. Figure 2.4B shows that strains expressing full length Ced3A were able to convert the cello-oligomers to lactic acid while those expressing the mature form of Ced3A produced no lactic acid. Lactic acid formation from cells expressing the full-length Ced3A peaked at 10 hours, with accumulation of lactic acid to about 2.3 g/L (0.23%) from initial 5 g/L(0.5%) cellodextrin that was reduced to 2.1 g/L (0.21%), achieving about 76% of the theoretical yield based on the consumed sugars. The reason for the initial increase in sugar concentration was further investigated by analyzing the sugar profiles during the fermentation. As shown in Figure 2.5, the cellodextrin mixture was quickly reduced to one dominated by glucose and cellobiose during the first two hours of fermentation. The increase in reducing sugar concentration could be explained by the faster hydrolysis to generate more monomer and dimeric sugar than the cells could use. After this initial period, the reducing sugar concentration decreased with time (Figure 2.4A). Examining the chromatograms taken between 2 and 14 hours (Figure 2.5), the decrease in glucose was more significant than cellobiose. At 14 hours, most of glucose was consumed whereas significant amount of cellobiose remain, which explains incomplete conversion of collodextrin (58%). Overall, the preferred use of longer cellodextrin over cellobiose is consistent with the enzyme kinetics shown in Table 2.1.

Thus, periplasmic expression of recombinant cellodextrin allows cells to use cellodextrin as feedstock for production of valuable products.

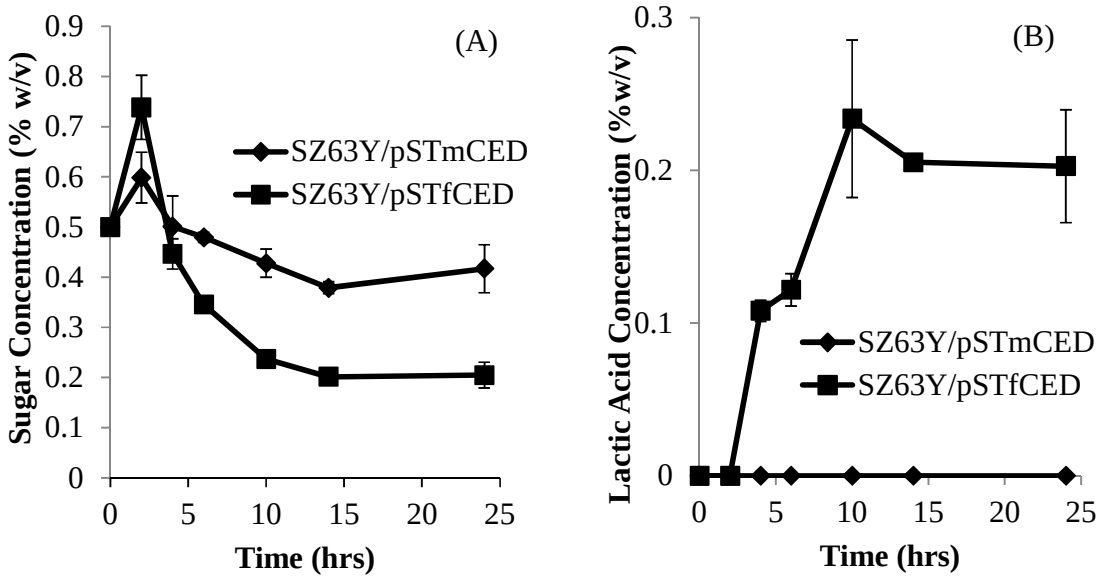

Figure 2.4: (A) Sugar and (B) Lactic Acid profiles of fermentation of cellodextrin by SZ63Y strains expressing Ced3A

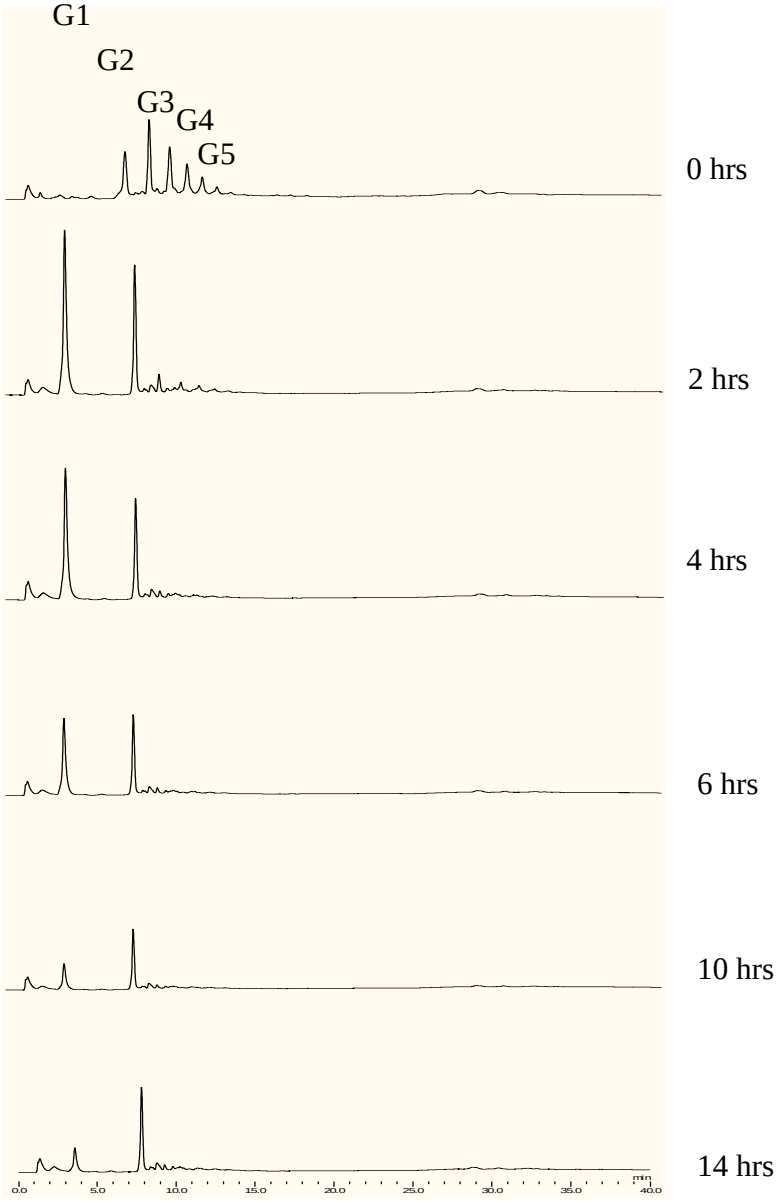

Figure 2.5: HPLC Sugar Profile During Fermentation of Cellodextrin by SZ63Y/pSTfCED

2.4 Discussion

In the present study, we cloned, expressed, and characterized a recombinant cellodextrinase from *Saccharophagus degradans.* We have shown that the heterologous protein can be properly translocated across the inner membrane when a native signal peptide sequence is included with the structural gene, and the presence of the recombinant enzyme in the periplasm is necessary to enable *E. coli* cells to grow on cellobiose and cellodextrins and ferment these substrates anaerobically to lactic acid.

In addition to the N-terminal sequence, this gene contains a family 3 glycoside hydrolase catalytic domain as well as a Platelet-Activating Factor (PAF) acetylesterase-like domain in the C-terminal region. A carbohydrate binding module is not present. This gene represents one of the two annotated cellodextrinase genes present in *S. degradans.* The gene product has been detected during growth on avicel, carboxymethylcellulose, and xylan, while Ced3B has only been detected during growth on xylan,[16] suggesting that Ced3A is a critical component of the cellulolytic and hemicellulolytic system of this bacterium. Cellodextrinases and β-glucosidases are enzymes that cleave cellodextrin with release of glucose. Cellodextrinases are enzymes exhibiting higher activity on longer cello-oligosaccharides than they do on cellobiose and shorter cello-oligosaccharides while β-glucosidase enzymes show the opposite preference [17, 20, 21]. Kinetic studies with Ced3A show a higher activity on cellotetraose and cellopentose than on cellobiose and cellotriose, which is consistent with the annotation of cellodextrinase. Additionally, when hydrolyzing cellodextrins and cellobiose, the final product is glucose, confirming the annotation. It is not clear, however, the function of C-terminal acetylesterase domain but its removal rendered the enzyme inactive (data not shown).

Subcellular fractionation and subsequent evaluation of enzyme activities associated with the periplasmic and cytoplasmic fractions provided solid evidence that the recombinant Ced3A in its full length had it signal sequence properly recognized which resulted in translocation of the enzyme to the periplasm. Enzyme activity, however, was not observed in the membrane fraction indicating the enzyme may not be acylated upon translocation. Therefore, the recombinant enzyme appeared to exist as a soluble enzyme in the periplasm, unlike in its native host, which exists as a lipoprotein. It is unknown what differences between *E. coli* and *Saccharophagus degradans* are responsible for the lack of acylation.

We demonstrated here that expression of the full form of Ced3A from a low-copy-number plasmid enables *E. coli* cells with and without a leaky outer membrane phenotype to grow on cellobiose as well as cellodextrin mixture, suggesting sufficient hydrolysis of oligomers. Hydrolysis was in fact so rapid in the E609Y strain that growth on cellodextrin was as fast as that on glucose. E609 strains did not show this trend, but rather demonstrated much slower growth on both cellobiose and cellodextrins compared to glucose. This suggests that the leaky outer membrane allows for more rapid diffusion of cello-oligomers into the periplasmic space where the hydrolytic enzyme resides. Without this increased permeability the transport of cello-oligomers is clearly hindered to the point of limiting growth rates.

The similar growth of E609Y on glucose and cellodextrins is interesting because it indicates that under these conditions the diffusion and hydrolysis of oligomers to glucose generates a carbon flux comparable to the simple diffusion of glucose (Table 2.1). Additionally, the growth of E609Y on cellobiose is only slightly slower than the

growth on glucose indicating that even though cellobiose is the least preferred substrate for this enzyme its hydrolysis rate is adequate, making it useful for SSF applications, in which cellobiose is the major intermediate from cellulose hydrolysis by cellulases.

The mature form of Ced3A containing no signal peptide remains in the cytoplasm and is unable to access the potential substrates outside the cytoplasm. Attempts at growing strains expressing this form of the enzyme on cellobiose and cellodextrins failed. This observation is consistent with numerous studies that indicate wild-type *E. coli* strain is incapable of transporting cellodextrin into cells under normal growth conditions [3, 22, 23]. Periplasmic expression of a cellodextrinase allows cells to expand its substrates to include multiple cellodextrin molecules, including cellobiose. This is a distinct advantage over the strategy when a β-glucosidase is used, which has more narrowly defined substrate specificity. Further, as cellodextrin assimilation is through glucose metabolism, there is no alteration in the intracellular endogenous carbon metabolism and regulations. This metabolic engineering strategy is also advantageous in its simplicity, as the only genetic modification can be achieved by using gene fusing of a suitable signal sequence to a structural gene of an enzyme of interest. Additionally, compared to outer surface display, cellodextrinase periplasmic expression allows cells to access glucose while keeping its extracellular concentration very low, reducing the chance for contamination, a non-trivial issue for industrial applications. On the other hand, periplasmic expression and outer surface display are not mutually exclusive. The periplasmic expression technique could complement the widely used outer surface display to increase the concentration of recombinant proteins per cell basis. Alternatively, outer surface display of one enzyme and periplasmic expression of another could be used synergistically to

engineer a more efficient whole-cell catalyst. This should open up new opportunities for metabolic engineering. This may be particularly important for cellulose degradation, as cellulases and other associated enzymes are notoriously inefficient and multiple enzymes are needed for complete hydrolysis. Maximizing the amount of enzymes displayed and exploiting their synergy could be important to increase cellulose degradation by engineered microbial catalysts. Therefore, periplasmic expression of enzymes may find broad applications as a metabolic engineering strategy.

2.5 Material and Methods

2.5.1 Strains and Plasmids

Strains and plasmids used in this study are listed in Table 2.3. *E. coli* JM109 was used for cloning and expression of both full-length and mature form of *ced3A* for *in-vitro* characterization. SZ63 is a gift from Dr. Ingram (University of Florida) and was further modified by a one-step PCR deletion method [24] to yield SZ63Y. *E. coli* strains E609 and E609Y were used for all growth studies. SZ63Y strain was used in lactic acid fermentation.

Table 2.3: Strains and Plasmids

Strains or Plasmids	Description	Source
Strains		
E609	*HfrCpps* isogenic parent of E609Y	Miller et al. (1998); Yem et al. (1978)
E609Y	*Lpp* deletion strain of *E. coli* E609	Ni et al. (2007)
JM109	Expression host for *ced3A* for in-vitro characterization	Yanisch et al. (1985)
SZ63Y	*Lpp* deletion strain of SZ63	This Study
Plasmids		
pQE80L	Amp^R, *T5* promoter, ColE1 ori	Qiagen
pSTV28	Cml^R, Lac promoter, p15 ori	Takara
pQECED	pQE80L vector with structural gene of *ced3A* from *S. degradans* inserted into BamHI and SalI sites	This Study
pSTVfCED	pSTV28 vector containing *ced3A* with lipoprotein signal sequence from *S. degradans* inserted into PstI and SacI Sites	This Study
pSTVmCED	pSTV28 vector containing *ced3A* without lipoprotein signal sequence from *S. degradans* inserted into BamHI and SalI sites	This Study
Genomic DNA		
Saccharophagus degradans 2-40T ATCC 43961	*S. degradans* genomic DNA	ATCC

2.5.2 Construction of Recombinant Plasmids

2.5.2.1 Plasmid pSTfCED for expression of full-length cellodextrinase

To construct the expression plasmid pSTfCED, the cellodextrinase (*ced3A*) gene was amplified from the genomic DNA of *Saccharophagus degradans* by PCR using two primers, FCED-F and FCED-R (Table 2.4). PCR reactions were performed using iProof™ High Fidelity DNA Polymerase (BIO-RAD). Melting temperature of 65°C and elongation times of 105 seconds were used. This amplified gene fragment was digested with PstI and SacI and subsequently ligated into pSTV28 vector to generate pSTfCED Direction of the cloned *ced3A* was verified by PCR using the same primers used for cloning.

2.5.2.2 Plasmids pQECED and pSTmCED for expression of mature form cellodextrinase

To construct the expression plasmid pQECED, the cellodextrinase (*ced3A*) gene was amplified from the genomic DNA of *Saccharophagus degradans* by PCR using the primers MCED-F and MCED-R. PCR reactions were performed using iProof™ High Fidelity DNA Polymerase (BIO-RAD). Melting temperature of 65°C and elongation times of 105 seconds were used. These primers were designed to amplify the region of the gene without the N-terminal signal sequence. This fragment was digested with BamHI and SalI restriction enzymes and ligated into the pQE80L vector that had undergone the same digestion. pQECED plasmids were then harvested and digested with BamHI and SalI and the *ced3A* portion was purified by gel extraction. This purified fragment as then ligated into the pSTV28 vector digested by BamHI and SalI to create the pSTmCED plasmid.

All transformations were performed by heat shock at 42 °C for 30 seconds, followed by incubation in SOC media for 1 hour and then plated on LB containing an appropriate antibiotic (ampicillin 100 μg/mL or chloramphenicol 25 μg/mL).

Table 2.4: Cloning Primers

Primer Name	DNA Sequence
FCED3-F	5'-CGGCGGGAGCTCATGAAAAATACTTTATCCTTTAAAACA
FCED3-R	5'-CGGCTGCTGCAGAAGTACTATGTACTATTCGCC
MCED3-F	5'- ATTGGGGGATCCTGTCAGGGTGTTAAACAGCAA
MCED3-R	5'- ATTCGGGTCGACCTATTCGCCCAGCATTTTTTT

2.5.3 Cultivation and Expression Conditions

Single colonies of plasmid bearing strains were inoculated into LB supplemented with an appropriate antibiotic and cultivated overnight. This overnight seed culture was used to inoculate up to 100 mL LB in Erlenmeyer flasks to OD_{600} of 0.1. When cell density reached between 0.3 and 0.4, isopropyl-β-D-thio-galactoside (IPTG) was added to a final concentration of 1.0 mM and flasks were transferred to a room temperature incubator for 16 hours to induce the expression of recombinant proteins.

2.5.4 Enzyme Purification

Ced3A to be used in kinetic characterizations were purified by column affinity using a Nickel-NTA resin. Elution was performed using 1 M imidazole. Eluent was dialyzed against HPLC grade water to remove salts.

2.5.5 Enzyme Assays

For determination of activity of crude lysates, cells were harvested after 16 hours of induction (induction condition as section 2.3) and lysed by ultrasonication in 50mM MES buffer (pH 6.0). All assays were performed in triplicate. Hydrolysis of pNP-β-glucoside and was determined by monitoring p-nitrophenol formation spectrophotometrically. Reaction mixtures (100 µL) contained 50 µL of crude lysate and 3 µg/mL of substrate with the balance 50mM MES buffer (pH 6.0). Assays were incubated at 25°C and the absorbance at 400nm was measured periodically to determine product formation.

By using anion-exchange chromatography, the hydrolysis of cellooligomers, cellobiose, cellotriose, cellotetraose, and cellopentose, was monitored. The reaction mixture (100 µL) contained 50mM MES (pH 6.0) and cellooligomer concentrations ranging from 5 µg/mL to 75 µg/mL. Reactions were initiated by addition of 0.5 µg of purified Ced3A. Reaction mixtures were incubated for 30 minutes at 25°C and terminated by the addition of 30 µL of 100 mM NaOH. Samples were diluted 10X and then analyzed by anion-exchange chromatography.

2.5.6 Cell Fractionation

Cells were harvested from culture that had been induced by 1mM IPTG for 16 hours. 25 mL of cell culture was centrifuged at 5,000g for 25 minutes and resuspended in 3 mL of shock buffer (100 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 M Sucrose and phenylmethylsulfonyl fluoride (PMSF) (20 µg/mL). 1 mL of this suspension was incubated on ice for 5 minutes and then centrifuged at 16,000g for 5 minutes. Pellets were warmed to room temperature and resuspended in 1.5 mL of ice-cold water. After 1

minute on ice, 85 μL of 20 mM $MgCl_2$ was added. Osmotically shocked cells were centrifuged at 16,000g for 5 minutes and the supernatant was saved as the periplasmic fraction. Remaining cells were lysed by ultrasonication and centrifuged at 16,000g for 5 minutes. Supernatant was saved as the cytoplasmic fraction and cell pellets were resuspended in 50mM MES and saved as the membrane fraction.

2.5.7 Metabolism of Cellodextrin

Innocula for cell growth experiments were prepared by harvesting cells that had been induced by 1mM IPTG for 16 hours in LB (as above) and washed with Phosphate Buffered Saline (pH 7.0) before inoculation. Inoculation was performed by resuspending the washed cell pellets in 10 mL of M9 media supplemented with glucose, cellobiose, or a cellodextrin mixture with varying concentrations as indicated. The cellodextrin mixture was prepared in house by a chemical method from cellulose following the method by Zhang et. al.[25]. The mixture contains G1 (2.8%), G2 (10.7%), G3 (26.1%), G4 (30.4%), G5 (21.8%), G6 (8.2). Antibiotic concentrations used were as follows: Ampicillin 100μg/mL(for strains containing pQE80L and pQECED vectors) and Chloramphenicol 25μg/mL(for strains containing pSTV28 and pSTfCED and pSTmCED). Upon inoculation into M9 media to initial OD of 0.1, IPTG was added to a final concentration of 1mM. All cultures were performed at 37°C and 250 rpm. Samples were taken at 3 hour intervals and the cell density (OD_{600}) was measured.

2.5.8 Fermentation of Cellobiose and Cellodextrins

Cells harvested from induced cultures (as above) were washed with Phosphate Buffered Saline (pH 7.0) and resuspended into M9 media to final cell density of OD 2.5. Anaerobic cultivation was carried out at 37 °C and 250 rpm in capped 20 mL scintillation vials with at least 10 mL of liquid volume. Samples were collected periodically and cell mass was measured spectrophotometrically as above, reducing sugar concentrations measured by DNS method (below) and lactic acid concentrations determined using a HPLC method.

2.5.9 DNS Method

In order to determine soluble reducing sugar concentrations, 100 μL of sample was added to 900 μL of DNS solution. DNS solution was prepared as follows: 0.75% 3,5-dinitrosalycylic acid, 1.4% sodium hydroxide, 21.6% potassium sodium tartrate, 0.55% phenol, 0.55% sodium metabisulfate, dissolved in water. These mixtures were then boiled for 5 minutes, centrifuged at 15,000g for 5 minutes and their optical density was measured.

2.5.10 Analytical method

Cell density (OD_{600}) and para-nitrophenol concentration was measured at 600 nm and 550 nm, respectively, on a UV/VIS spectrophotometer (DU530; Beckman Coulter, USA). Analysis of cellooligosaccharides was performed using High Performance Anion-Exchange Chromatography on a DIONEX system with an ED50 electro-chemical detector. Separation was achieved using a CarboPac PA-20 column. Detection was achieved by pulsed amperometry (waveform : t = 0.41 sec, p = -2.00 V; t = 0.42 sec, p = -

2.00 V; t = 0.43 sec, p = 0.60 V; t = 0.44 sec, p = -0.10 V; t = 0.50 sec, p = -0.10 V). The mobile phase consisted of a degassed solution A containing 100 mM sodium hydroxide and degassed solution B containing 500 mM sodium acetate and 100 mM sodium hydroxide. The mobile phase was continuously pressurized with helium gas to prevent dissolution of airborne oxygen and carbon dioxide. A flow rate of 0.5 mL/min was used. A linear gradient of acetate in the mobile phase was achieved as follows: t = 0 min, 100:0 (A:B); t = 30 min, 30:70; t = 35 min, 30:70; t = 45 min, 100:0; t = 55 min, 100:0.

The concentration of Lactic Acid was measured 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.

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CHAPTER 3

IMPROVED CELLOBIOSE UTILIZATION IN E COLI BY INDLUCING BOTH HYDROLYSIS AND PHOSPHOROLYSIS MECHANISMS

3.1 Abstract

Cellobiose is a major intermediate from cellulase hydrolysis of pretreated plant biomass. Engineering biocatalysts for direct use of cellobiose could eliminate the need for exogenous β-glucosidase. Additionally, rapid removal of cellobiose in a simultaneous saccharification and fermentation facilitates enzymatic hydrolysis as cellobiose is a potent inhibitor for cellulases. We report here improved cellobiose utilization by engineering *E. coli* cells to assimilate the disaccharide both hydrolytically and phosphorolytically(complete consumption occurring 4 h sooner). Additionally, we demonstrate that engineering intracellular cellobiose utilization could circumvent catabolite repression, allowing simultaneous fermentation of xylose and cellobiose, resulting complete sugar utilization. Using *meso*-2,3-Butanediol as model product, we further demonstrate that the accelerated carbon metabolism in turn led to an improved product formation (0.3% w/v vs. 0.26% w/v and 0.76% w/v vs. 0.61% w/v when fermenting 1% w/v and 2% w/v sugar respectively), illustrating the utility of the engineered biocatalysts in biorefinery applications.

3.2 Introduction

To utilize lignocellulosic requires a combination of pretreatment and enzymatic hydrolysis to overcome recalcitrance of the material [1-3]. This generates a mixture of

sugars containing cellooligosaccharides and monosaccharides, dominated by glucose, cellobiose, and xylose [4-8]. While wild type *E. coli* readily metabolizes many types of monosaccharides, including xylose [9, 10], *E. coli* strains are not able to use cellobiose and other cellooligosaccharides. Engineering *E. coli* cells for direct use of cellobiose is of interest as the disaccharide is a major intermediate from enzymatic hydrolysis. Direct use of cellobiose by a biocatalyst in a fermentation process could eliminate the need for exogenous β-glucosidase. Additionally, rapid removal of cellobiose in a simultaneous saccharification and fermentation (SSF) facilitates enzymatic hydrolysis as cellobiose is a potent inhibitor for cellulases [11-13].

Engineering *E. coli* for direct use of cellobiose has been attempted in the past. By surface display of a β-glucosidase, cellobiose was hydrolyzed into glucose, which was then taken up by cells and metabolized intracellularly [14]. Recently, we showed that a periplasmic expression of a *Saccharaphagus* cellodextrinase was also successful in generating a strain capable of utilizing cellodextrin including cellobiose [15]. Alternative to surface display or periplasmic expression of a hydrolase where the disaccharide is hydrolyzed outside of cytoplasm, cellobiose could be transported into cells by utilizing a transporter, such as LacY [16]. Once inside the cytoplasm, cellobiose could be hydrolyzed into glucose molecules by a recombinant hydrolase such as β-glucosidase [17, 18]. We have recently demonstrated that cellobiose could be alternatively metabolized via a phophorolysis mechanism [16] and this approach, instead of a hydrolase, a cellobiose phosphorylase is used, which splits a cellobiose molecule into one glucose molecule and one glucose-1-phosphate molecule using inorganic phosphate as donor.

The present study investigates whether a combination of hydrolysis and phosphorolysis could improve cellobiose utilization. We show that engineered *E. coli* cells with both hydrolysis and phosphorolysis mechanisms could readily convert cellobiose into *meso*-2,3-butanediol with high yield and conversion rate, demonstrating the utility of the improved biocatalyst in biorefinery.

3.3 Results

3.3.1 Cellobiose Metabolism in Engineered Strains

Previously, we constructed an *E. coli* strain capable of growth on cellobiose for lactic acid production by expressing, in its periplasm, a cellodextrinase, Ced3A, from *Saccharophagus degradans* [15]. In this strain, cellobiose was split into two glucose molecules in the periplasm, where they were uptaken for intracellular metabolism. We also constructed a strain that metabolizes cellobiose via phosphorolysis mechanism by expressing a cellobiose phosphorylase, Cep94A, from *Saccharophagus degradans* [16]. This strain was shown to be able to grow on cellobiose and additionally convert cellobiose to ethanol with high yield [16]. The goal of the present study was to investigate whether cellobiose metabolism could be accelerated by engineering a strain with both hydrolysis and phosphorolysis mechanisms. Additionally, we hope to demonstrate that the potential acceleration of cellobiose metabolism could lead to an enhanced production of a biorefinery product.

We chose to use meso-2,3-butanediol (BDO) as a model product. To this end, a MG1655 derivate, designated as MGLAP (Table 3.1), was used in this study. This strain was previously engineered to eliminate production of lactic acid and acetate production by knockout of genes associated with these two metabolites. As a result, the host strain

transformed with a plasmid containing enzymes for BDO production, pBBDO, produced (from glucose) 2,3-butanediol as the major fermentation product [19]. The strain was further modified by transforming it with pSTCED and pQECEP, expressing both the cellodextrinase (CED) and the cellobiose phosphorylase (CBP), respectively (Table 3.1). The resulting strain, capable of metabolizing cellobiose using both hydrolysis and phosphorolysis mechanisms is designated CED+CBP. Similarly, the control strains expressing either cellodextrinase or cellobiose phosphorylase, are designated as CED and CBP strains, respectively. Finally, an empty vector strain, designated as empty vector control, was also included in this fermentation study (Table 3.1). The four strains were compared with respect to their ability to utilize cellobiose (Figure 3.1A) and their ability to produce BDO (Figure 3.1B). As shown, during the 72-hour anaerobic fermentation of 1% cellobiose (detailed conditions in Materials and Methods), minimal consumption of cellobiose was observed for the empty vector control, consistent with the expectation. In contrast, significant consumption of cellobiose was observed for other three strains, with fast consumption evident for strains expressing CBP alone or both CBP and CED. For example, at 36 hours, the cellobiose concentrations for the two strains expressing single enzyme were 0.69% and 0.2% for the strain expressing CED and for the strain expressing CBP, respectively, indicating that the phosphorylase-expressing cells consumed cellobiose faster than the cellodextrinase-expressing cells. The lowest residual cellobiose concentration at 36 hours was found with the strain expressing both cellodextrinase and phosphorylase, with about 0.05% cellobiose remaining. These results show that hydrolysis and phosphorolysis are synergistic and cells with both mechanisms metabolize cellobiose much more rapidly. It can be seen in Figure 3.1A that CED+CBP and CEP

were able to completely utilize cellobiose in about 50 hour. In comparison, the CED strain expressing only cellodextrinase was able to utilize only 40% of the cellobiose by the end of the fermentation (72 hours).

Time profiles of meso-2,3-BDO production from cellobiose were shown in Figure 3.1B. Significant product formation was only observed with the strain expressing cellobiose phosphorylase and the strain expressing both cellobiose phosphorylase and cellodextrinase (Figure 3.1B), with the latter outpaced the former before the product concentration peaked at 48 hrs. Both strains reached the same maximum, 0.40 %, at 48 hours, representing an 80% of theoretical yield. The CED expressing strains produced slightly more BDO than the empty vector control strain to a maximum of 0.07%, suggesting that expressing Ced3A alone is not sufficient for significant cellobiose metabolism and BDO production.

These results suggest that cellobiose phosphorolysis is a more effective mechanism than cellodextrinase-mediated hydrolysis mechanism. These two mechanisms appear to be synergistic in terms of cellobiose consumption. While early faster production of BDO was observed, cells with both mechanisms did not result in higher product concentration over the fermentation cycle. Apparently, product yield is determined by factors more than the rate of cellobiose consumption. However, product yield of 80% from cellobiose[20], is close to what was achieved with glucose, (87%, [19]), indicating that cellobiose could be used as effectively as glucose for BDO production.

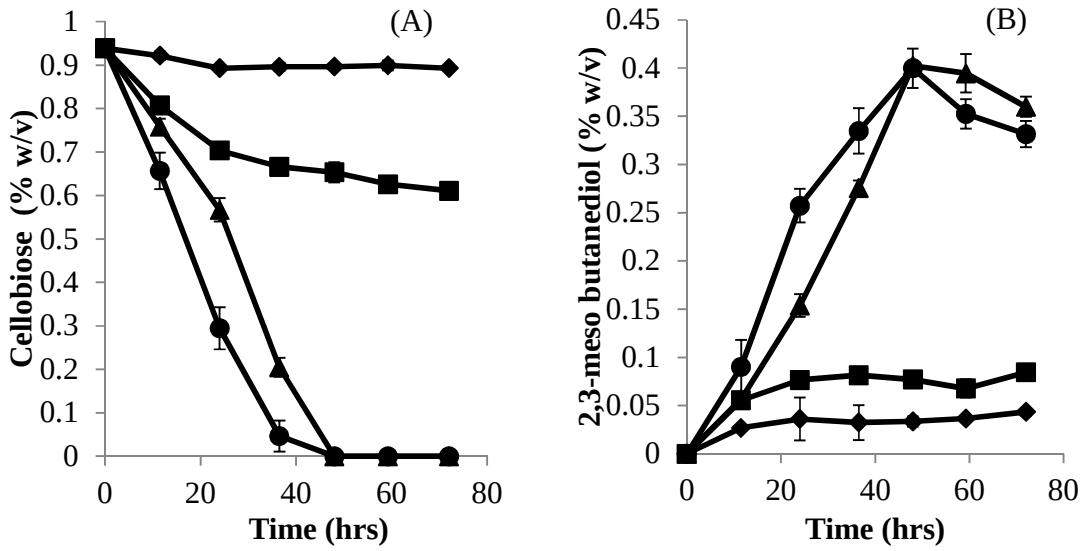

Figure 3.1: Cellobiose (A) and 2,3-butanediol (B) concentrations during the fermentation of LB with 1% cellobiose by MGLAP/pSTV28+pQE80L (♦), MGLAP/pSTCED+pQE80L (■), MGLAP/pSTV28+pQECEP (▲), and MGLAP/pSTCED+pQECEP (●)

3.3.2 Co-Fermentation of Cellobiose/Xylose for BDO Production

As shown above, cellobiose utilization could be improved by inclusion of both hydrolysis and phosphorylase mechanisms. To further investigate its utility in biorefinery, additional experiments were carried out under the condition of mixed sugar fermentation with cellobiose and xylose. We expect that fast intracellular metabolism of cellobiose by cellobiose phosphorylase may generate a condition that extracellular glucose concentration is sufficiently low to remove catabolite repression. If this is the case, simultaneous consumption of cellobiose and xylose will result and this should improve the overall carbon metabolism. To investigate this possibility, anaerobic mixed sugar fermentation (0.5% cellobiose and 0.5% xylose) were run for *E. coli* cells expressing both CED and CBP. This is compared to mixed monosaccharide fermentation

of the same concentration (0.5% glucose and 0.5 % xylose). Figure 3.2A shows that the strain exhibited similar total sugar (cellobiose plus xylose or glucose plus xylose) consumption rates. However, consumption of each individual sugar was considerably different. When cells were supplied with 0.5% glucose and 0.5% xylose, the utilization of glucose and xylose is biphasic (Figure 3.2B). The consumption of xylose began only when glucose was exhausted. Glucose was exhausted within the first 6 hours, and xylose metabolism started at 6 hours and exhausted at 15 hours. In contrast, in the case of cellobiose and xylose, a clear co-metabolism was evident from Figure 3.2C, with cellobiose concentration and xylose concentration decreased with time, starting from the very beginning. In fact, xylose was apparently metabolized faster than cellobiose. Xylose was completely consumed by 9 hours while cellobiose was exhausted in 15 hours. Despite the differences in the dynamics of sugar utilization, little differences were observed in final BDO titers.

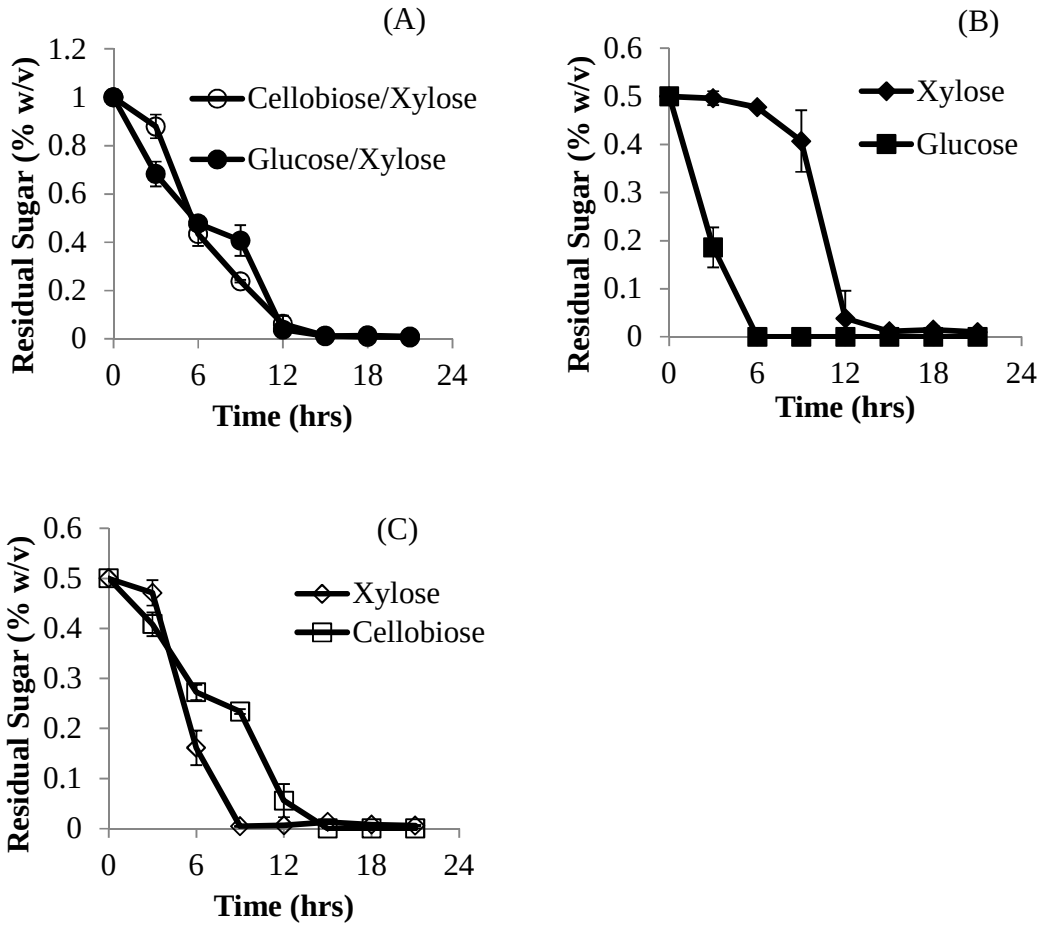

Figure 3.2: (A) total residual sugar, (B) residual glucose and xylose, and (C) residual cellobiose and xylose during fermentation of 1% sugars by MGLAP/pSTCED+pQECEP

In both cases, the final 2,3-butanediol concentration was 0.31 % w/v after 12 hours of fermentation (Figure 3.3A), representing a 60% yield. This is lower than 80% yield on cellobiose (Figure 3.1B). HPLC analysis showed that a precursor molecule, acetoine, was accumulated in both cases. The emergence of byproduct, acetoine, was previously observed [19], and was presumably due to the reversible nature of the last reaction step in the BDO synthesis. Typically, acetoine production is more pronounced

when sugar concentration is low. The combined concentration of BDO and Acetoine reached 0.4% and 0.38% for glucose/xylose mixture and cellobiose/xylose mixture, respectively (Figure 3.3B), suggesting the lost yield in BDO is accounted for by the byproduct acetoine.

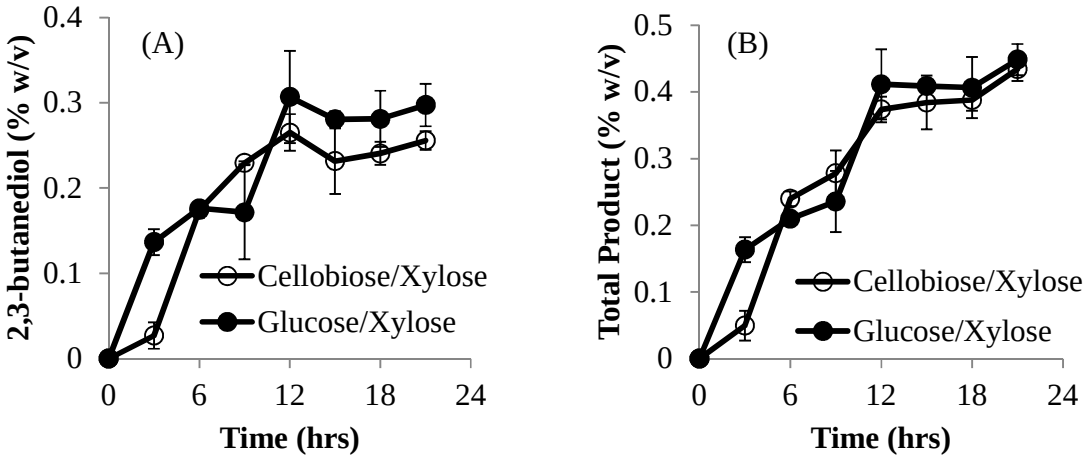

Figure 3.3: (A) total 2,3-butanediol concentration and (B) total product concentration during fermentation of 1% sugars in a 1:1 ratio by MGLAP/pSTCED+pQECEP

The above mixed sugar experiment was repeated with higher concentration of carbon source, 1% cellobiose and 1% xylose, compared to 1% glucose and 1% xylose. As shown in Figure 3.4A, initial rates of total sugar consumption were identical for both cases but diverged after 12 hours, with the monosaccharide fermentation lagged behind and about 0.5% sugar remained at the end of the 48 hr fermentation. This compares to a complete fermentation of cellobiose/xylose by 32 hours, indicating the ability of use cellobiose directly in this strain improved the mixed sugar fermentation. More careful

examination of sugar profiles showed that fermentation of 1% glucose and 1% xylose was biphasic as was the case with lower concentrations with minimal xylose utilization until glucose was exhausted at 12 hours. In this case, however, the xylose was not exhausted by the end of the fermentation, with 47% of the xylose remaining after 48 hours (Figure 3.4B). Fermentation of 1% cellobiose and 1% xylose resulted in simultaneous utilization of both sugars in this case with the exhaustion of xylose occurring after 22 hrs and the exhaustion of cellobiose occurring after 32 hours (Figure 3.4C). Consistent with the sugar concentration profiles, product formation during fermentation of the cellobiose/xylose mixture was more rapid than the glucose/xylose mixture with a maximum level of BDO reaching 0.72 %w/v at 26 hours (Figure 3.5A), whereas the glucose/xylose fermentation achieved a BDO concentration of 0.50 % w/v at the same time, and the maximum of BDO concentration was not reached until after 36 hours, which is 0.61%, lower than the case with cellobiose/xylose mixture (Figure 3.5A). The combined BDO and acetoine concentration reached 0.74% w/v and 0.91% w/v for 2% glucose/xylose mixture and 2% cellobiose/xylose mixture, respectively (Figure 3.5B).

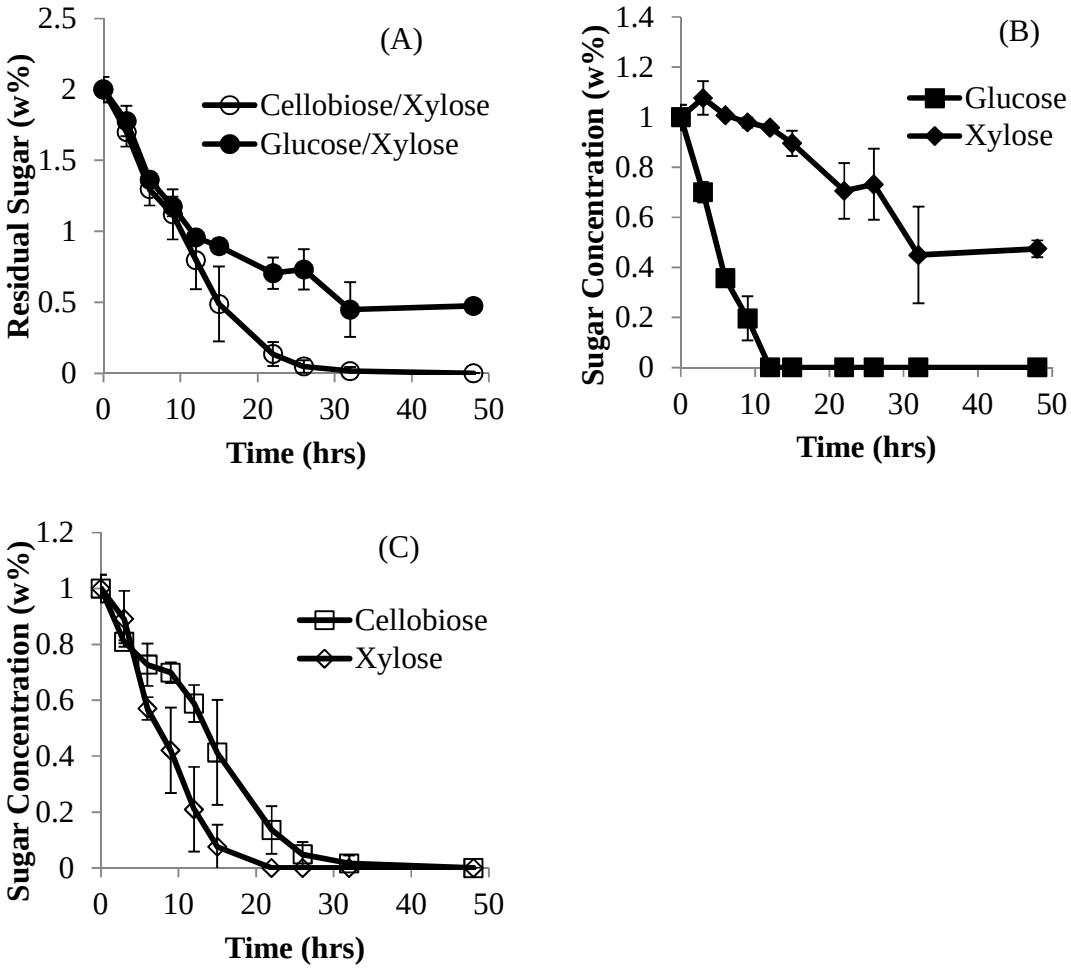

Figure 3.4: (A) total residual sugar, (B) residual glucose and xylose, and (C) residual cellobiose and xylose during fermentation of 2% sugars by MGLAP/pSTCED+pQECEP

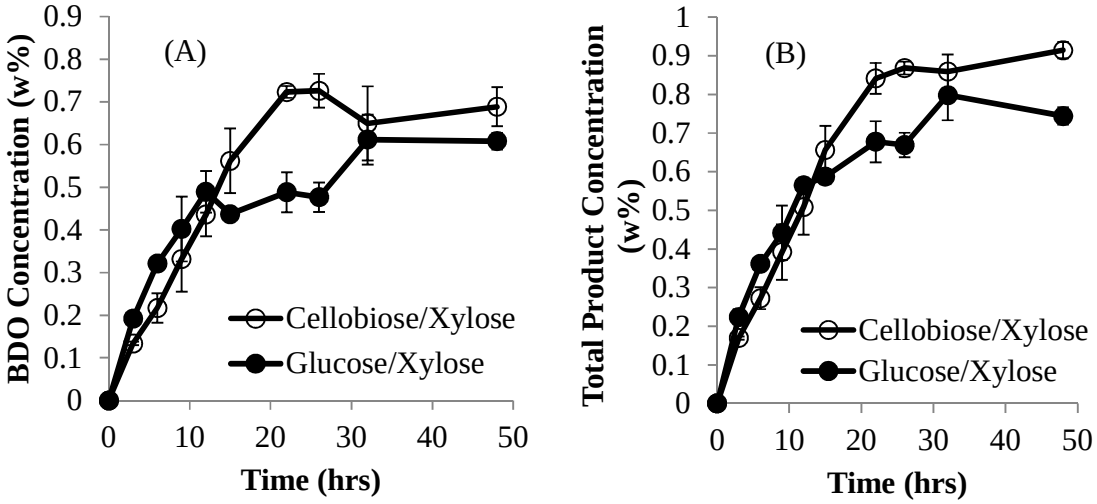

Figure 3.5: (A) total 2,3-butanediol concentration and (B) total product concentration during fermentation of 2% sugars in a 1:1 ratio by MGLAP/pSTCED+pQECEP

These results clearly demonstrate that engineering intracellular cellobiose utilization could circumvent catabolite repression, allowing simultaneous use of xylose and cellobiose. As a result, overall carbon metabolism was improved and product concentration and yield were also improved.

3.4 Discussion

In this study, we constructed a strain with the ability to metabolize cellobiose through both hydrolysis and phosphorolysis mechanisms. We showed that while phosphorolysis was more effective than cellodextrinase-mediated hydrolysis, an improvement in cellobiose utilization was observed by combining the two mechanisms. Additionally, intracellular metabolism of cellobiose circumvented catabolite repression. Consequently, engineered biocatalysts are capable of fermenting xylose and cellobiose concurrently, resulting in improved carbon consumption. The accelerated carbon

metabolism in turn led to an improved product formation. Demonstrated with BDO as model product, increases in product concentration, yield, and productivity with mixed cellobiose/xylose fermentation relative to mixed glucose/xylose fermentation suggest that the *E. coli* strains capable of cellobiose utilization can be advantageously used in biorefinery applications.

3.5 Material and methods

3.5.1 Strains and Plasmids

Strains and plasmids used in this study are listed in Table 3.1. *Escherichia coli* MGLAP is a derivative of MG1655, previously constructed to overproduce pyruvate [19]. Three expression plasmids used in this study, pBBDO, pSTCED, and pQECEP harbors genes for production of meso-2,3-butanediol (BDO), the cellodextrinase(CED), and cellobiose phosphorylase(CEP), respectively (Table 3.1).

All transformations were performed by heat shock at 42 °C for 30 s, followed by incubation in SOC media for 1 hour and then plated on LB containing an appropriate antibiotic (ampicillin 100 μg/ml or chloramphenicol 25 μg/ml or kanamyacin 50 μg/ml).

Table 3.1: *E. coli* Strains and Plasmids

Strains or Plasmids	Description	Source
Escherichia coli Host Strains		
MGLAP	MG1655, F^{-} λ^{-} *ilvG*$^{-}$*rfb*$^{-}$50 *rph*$^{-1}$;Δ *poxB,* Δ *ldhA,* Δ *ackA,* and Δ *pta*	Shin, et. al. [19]
Plasmids		
pQE80L	Amp^{R}, *T5* promoter, ColE1 ori	Qiagen
pSTV28	Cml^{R}, Lac promoter, p15 ori	Takara
pQECEP	pQE80L vector with structural gene of *cep94A* from *S. degradans*	Sekar, et. al. [15, 16]
pSTCED	pSTV28 vector containing *ced3A* with lipoprotein signal sequence from *S. degradans*	Rutter, et. al[15]
pBBDO	pBBR122 derivative replaced Cm^{R} gene with T5 expression cassette of pQE80L. Containing *als*S and *als*D of *Bacillus subtilis* 168 and *bud*C gene of *Klebshiella peumoniae*	Shin, et. al. [19]
E. coli transformants		
MGLAP/pBBDO/pQE80L/pSTV28	*Empty vector control*	*This study*
MGLAP/pBBDO/pQE80L/pSTCED	CED: expressing cellodextrinase	This study
MGLAP/pBBDO/pQECEP/pSTV28	CBP: expressing cellobiose phosphorylase	This study
MGLAP/pBBDO/pQECEP/pSTVCED	CED+CBP: expressing both Cellodextrinase and cellobiose phosphorylase	This study

3.5.2 Cultivation and Expression Conditions

Single colonies of plasmid bearing strains were inoculated into Luria Broth (LB) supplemented with an appropriate antibiotic and cultivated overnight. This overnight seed culture was used to inoculate up to 100 ml LB in Erlenmeyer flasks to OD_{600} of 0.1. When cell density reached between 0.3 and 0.4, IPTG was added to 0.2 mM and flasks were incubated for 16 h to induce the expression of recombinant proteins. MGLAP strains were induced at 18 °C.

3.5.3 Enzyme Assays

For verification of activity of crude lysates of strains expressing Ced3A and Cep94A, cells were harvested after 16 h of induction (induction condition as above) and lysed by ultrasonication in 50 mM MES buffer (pH 6.0). Hydrolysis of cellobiose was determined by monitoring glucose formation using the Sigma Glucose (GO) Assay Kit. Reaction mixtures (100 µl) contained 10 µl of crude lysate and 90 µl of phœsphate-buffered saline (PBS) containing 1% cellobiose. Assays were incubated at 25 °C for 30 minutes and the GO reagent was added 1:1 to the reaction mixture. The mixture was then incubated at 37 °C for 30 min and absorbance at 540 nm was measured.

3.5.4 Fermentation Conditions

Cells harvested from induced cultures (as above) were washed with PBS (pH 7.0) and resuspended into LB medium containing 1% w/v substrate along with the appropriate antibiotics to initial cell density of OD_{600} 0.05. Anaerobic cultivation was carried out at 37 °C and 250 rpm with 0.2 mM IPTG in capped 20 ml scintillation vials with at least 10 ml of liquid volume. Samples were collected periodically and residual sugar, 2,3-

butanediol and acetoine concentrations determined using a HPLC method. Cell mass was measured spectrophotometrically.

3.5.5 Analytical Method

The concentration of Cellobiose, Glucose, Xylose, Acetoine, and 2,3-butanediol was measured by HPLC 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.

3.6 References

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CHAPTER 4

CHARACTERIZATION OF THREE CELLOBIOSE PERMEASE ENZYMES AND THEIR USE IN CELLOBIOSE FERMENTATION

4.1 Abstract

During enzymatic hydrolysis of cellulose, cellobiose is generated as a major product. In order to further convert cellobiose to valuable bioproducts it must be further converted to glucose equivalents that can be used in glycolysis and subsequent product formation pathways. Many enzymes capable of this conversion are expressed in the cytoplasm and require transport of the cellobiose into the cell by proteins to act. We selected three permease enzymes to evaluate for their ability to transport cellobiose intracellularly. LacY from *E. coli* and CP1 and CP2 from *S. degradans* were characterized kinetically as well as by their performance during fermentation using cellobiose as the sole carbon source. All three proteins were found to have affinity for cellobiose and their expression allowed adequate cellobiose uptake to allow cell growth and product formation during cellobiose fermentation.

4.2 Introduction

Hydrolysis of cellulose by cellulase enzymes yields a mixture of cellooligomers ranging between cellobiose and cellopentose[1, 2]. Almost all cellulases generate cellobiose from cellulose with a majority of them producing cellobiose as the major product of hydrolysis[3-5]. As such, conversion of cellobiose to glucose equivalents is a critical step in bioconversion of cellulose to valuable products. This conversion can be

achieved by a number of different enzymes using either a hydrolytic or a phosphorolytic mechanism[6, 7]. Expression of these enzymes in both bacterial and yeast systems has led to the ability of those organisms to metabolize cellobiose, resulting in cell growth and formation of a wide variety of fermentation products[8, 9].

Many studies have been done using extracellular β-glucosidase enzymes to convert cellooligomers to glucose extracellularly[10]. Studies have shown, however, that conversion to glucose within the cytoplasm allows *E. coli* to ignore any catabolite repression by glucose resulting in more rapid carbon flux[11]. In order to take advantage of this metabolic phenomenon, however, we require transport of cellobiose across the cell membrane into the cytoplasm. Two major types of proteins are responsible for transport of cellobiose. ABC transporters, or ATP binding cassette transporters, are proteins that use ATP to transport molecules into the cytoplasm[12]. Permease enzymes fall into the Major Facilitator Superfamily class of transport proteins and often couple sugar transport with transport of ions down a gradient to replace the requirement of energy in the form of ATP[13, 14].

Three permease proteins were identified as putative cellobiose transporters. LacY from *E. coli* is a lactose permease that has been shown to have lactose transport inhibited by the presence of cellobiose. Additionally, deletion of *lacY* from the genome was shown to abolish growth on cellobiose by *E. coli* expressing a cytoplasmic cellobiose phosphorylase[9]. Several permease genes were identified in the *S. degradans* genome by their homology to the Major Facilitator Superfamily of proteins. Putative cellobiose transporters were selected based on the proximity to other genes responsible for cellulose and cellobiose metabolism. CP1 is adjacent to a gene coding for a β-glucosidase enzyme

and CP2 is located near the gene coding for the cellulase Cel5H which is shown to be a major component of the cellulolytic system[15].

In this study we evaluate the performance of three permease proteins, LacY from *E. coli* and CP1 and CP2 from *S. degradans*, for their ability to transport cellobiose across the cell membrane. Michaelis-menten parameters were determined for each protein expressed in a whole cell microbial catalyst. Additionally, cells expressing each transporter along with a cytoplasmic enzyme capable of conversion of cellobiose to glucose equivalents had their performance during fermentation of cellobiose evaluated. In so doing we can identify proteins suitable for transport of cellobiose into the cell for conversion to bioproducts.

4.3 Results

4.3.1 Kinetic Characterization of Cellobiose Transporters

Previous work has suggested that the lactose transporter protein, LacY, in *E. coli* has some activity towards cellobiose. In order to investigate this further, *ΔlacY* strains were constructed and *lacY* was complimented on the plasmid pBBR122. Additionally, two other major facilitator superfamily cellobiose transporter candidates, CP1 and CP2, were identified in *S. degradans* based on their proximity to cellobiose utilizing genes in the genome. These genes were all cloned with N-terminal GFP fusions in order to quantify individual expression levels. Polyserine linkers were used between the GFP and structural regions to insure proper incorporation of the protein into the membrane.

Kinetic characterization was carried out using the oil-stop method. Substrate concentrations ranging between 0.2 and 10 mM cellobiose with 10μM tritiated cellobiose as the radioactive label were used to characterize LacY and CP1. Substrate

concentrations ranging between 0.1 and 200 μM tritiated cellobiose were used to characterize CP2. The control strain in both cases was the KY strain expressing GFP alone from pBBR122.

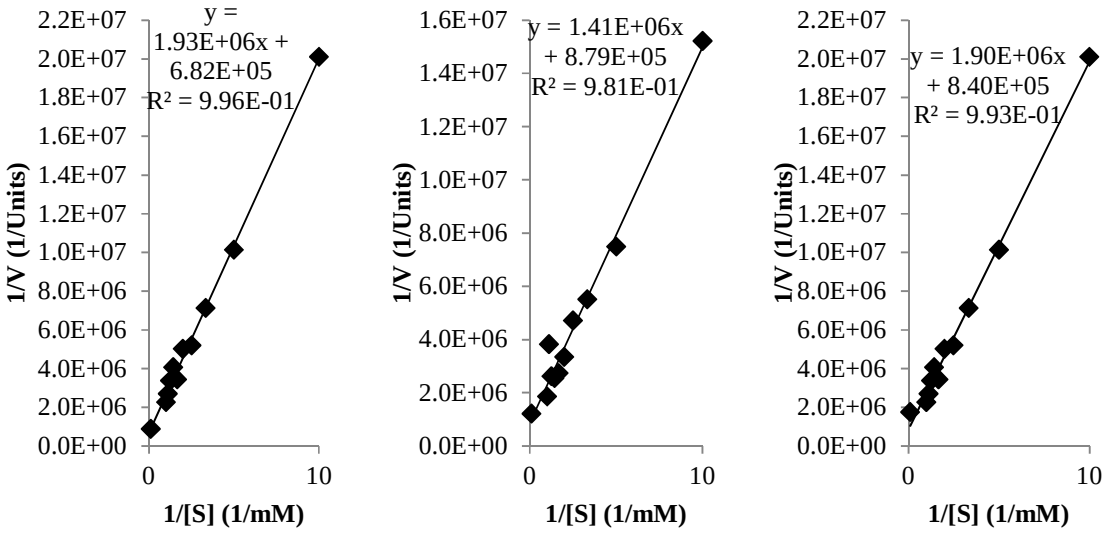

Figure 4.1: Lineweaver-Burk Analysis for LacY

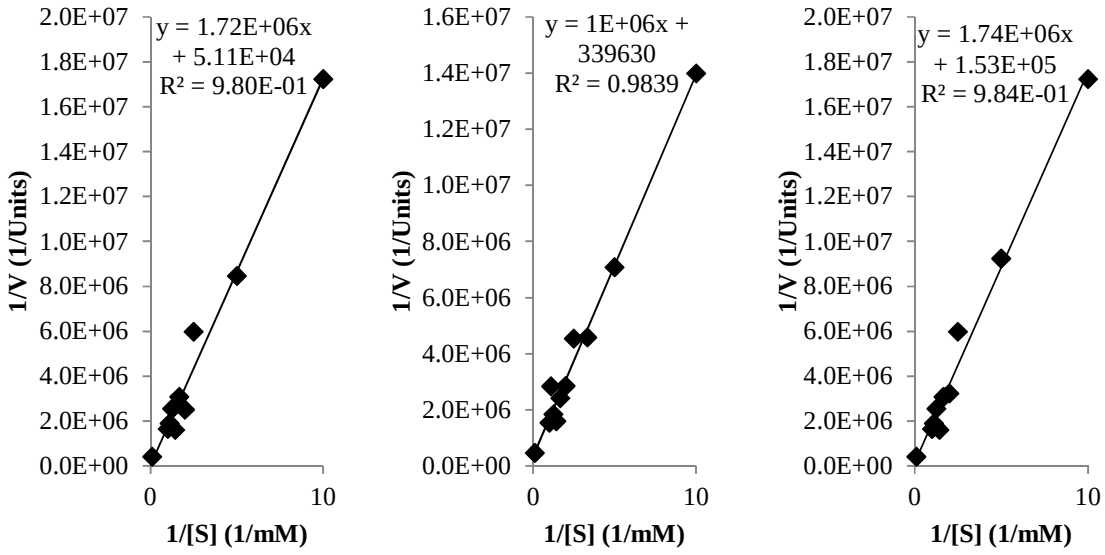

Figure 4.2: Lineweaver-Burk Analysis for CP1

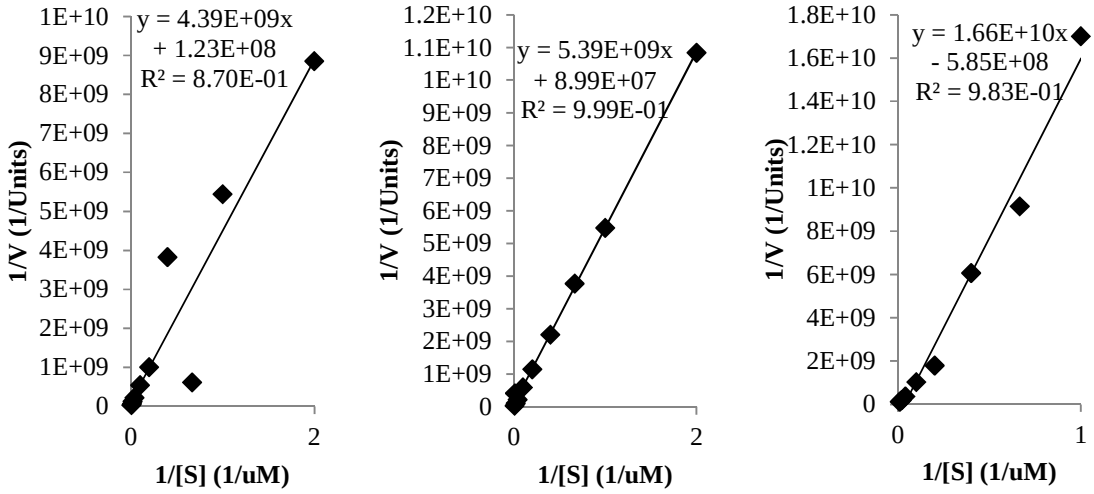

Figure 4.3: Lineweaver-Burk Analysis for CP2

As can be seen in Table 4.1 all proteins are capable of transporting cellobiose across the *E. coli* membrane. CP2 has the highest affinity with a K_m of 0.038 mM which is 100 fold lower than the 2.32 mM and 4.56 mM K_m values measured for LacY and CP1 respectively. LacY and CP1 showed similar V_{max} values at 0.032 U/min/mg and 0.035 U/min/mg respectively. The control strain demonstrated Vmax values of 0.004 U/min/mg and 0.0002 U/min/mg under high concentration and low concentration reaction conditions respectively, each roughly 10% of measured values for transporter proteins.

Table 4.1: Kinetic Parameters of LacY, CP1, and CP2

	Vmax (U/min/mg)	Km (mM)
LacY	0.032 ± 0.006	2.32 ± 0.88
CP1	0.035 ± 0.014	4.56 ± 1.61
CP2	0.0017 ± 0.0003	0.0384 ± 0.014

4.3.2 Aerobic Fermentation of Cellobiose by *E. coli* Strains Expressing Transporters

As shown above, all three transport proteins are capable of transporting cellobiose into the cytoplasm of *E. coli*. In order to investigate the industrial utility of these enzymes, these strains were used for fermentation of cellobiose. One of two cytoplasmic cellobiose utilizing enzymes, Bgl3C or Cep94A from *S. degradans*, were ligated into pHCE plasmid and transformed into transporter-gfp fusion expressing strains. Strains expressing Bgl3C and LacY, CP1 and CP2 will be referred to as KY/pBBRGLACY/pHCEBGL, KY/pBBRGCP1/pHCEBGL, and KY/pBBRGCP2/pHCEBGL respectively. Strains expressing Cep94A and LacY, CP1 and CP2 will be referred to as KY/pBBRGLACY/pHCECEP, KY/pBBRGCP1/pHCECEP, and KY/pBBRGCP2/pHCECEP respectively. Strains were then grown aerobically in M9

minimal medium containing 0.5% cellobiose. Cell growth and substrate consumption were measured.

As shown in Figure 4.1, all strains expressing any of the transporters are capable of growth on cellobiose. Relative growth rates differ, however, between strains expressing Bgl3C and strains expressing Cep94A. In the case of Bgl3C, KY/pBBRGCP2/pHCEBGL grows the highest final OD of 2.6. KY/pBBRGCP1/pHCEBGL and KY/pBBRGLACY/pHCEBGL both reach a similar final OD ~ 1.7. Fermentations by Cep94A strains not only showed different growth trends between transporters but overall growth rates were slower than the Bgl3C strains. KY/pBBRGCP1/pHCECEP and KY/pBBRGLACY/pHCECEP strains reached the same final OD of 1.8 with KY/pBBRGCP1/pHCECEP strain reaching this more than 12 hours before KY/pBBRGLACY/pHCECEP. KY/pBBRGCP2/pHCECEP showed a similar growth rate to KY/pBBRGCP1/pHCECEP but the lag phase lasted nearly 6 hours longer and it reached a final OD of 2.5. Interestingly, while growth rates differ depending on the identity of the cellobiose utilizing enzyme each transporter allowed growth to the same OD regardless of cellobiase.

Table 4.2: Growth Rates of Transporter Strains During Aerobic Growth on Cellobiose

	Growth Rate (1/hr)	
	Bgl3C	Cep94A
Control	0.072 ± 0.024	0.058 ± 0.04
LacY	0.140 ± 0.016	0.093 ± 0.015
CP1	0.253 ± 0.015	0.226 ± 0.009
CP2	0.180 ± 0.020	0.087 ± 0.015

In addition to growth rates, substrate consumption was measured for each strain(Figure 4.2). Of the Bgl3C expressing strains, KY/pBBRGCP2/pHCEBGL and KY/pBBRGCP1/pHCEBGL consumed all the cellobiose after 18 hours and 33 hours respectively. KY/pBBRGLACY/pHCEBGL consumed nearly 80% of the cellobiose by the end of the fermentation. Of the Cep94A expressing strains, KY/pBBRGCP2/pHCECEP and KY/pBBRGLACY/pHCECEP completely consumed the cellobiose after 12 hours. KY/pBBRGCP1/pHCECEP consumed the cellobiose completely in 19 hours. In the case of Bgl3C strains cellobiose consumption rates match growth rates. Consumption of cellobiose by Cep94A strains, however, should show more rapid consumption by KY/pBBRGCP1/pHCECEP than others which is the opposite of what is seen.

4.3.3 Anaerobic Fermentation of Cellobiose by *E. coli* Strains Expressing Transporters

As shown above expression LacY, CP1, and CP2 in *E. coli* expressing cellobiose utilizing enzymes allows for consumption of cellobiose and subsequent cell growth. In order to further analyze whether these proteins are suitable for industrial applications, similar fermentations were carried out under anaerobic conditions. The same strains as above were grown anaerobically in M9 minimal medium with 0.5% cellobiose added. Cell growth, substrate consumption, and product formation was measured.

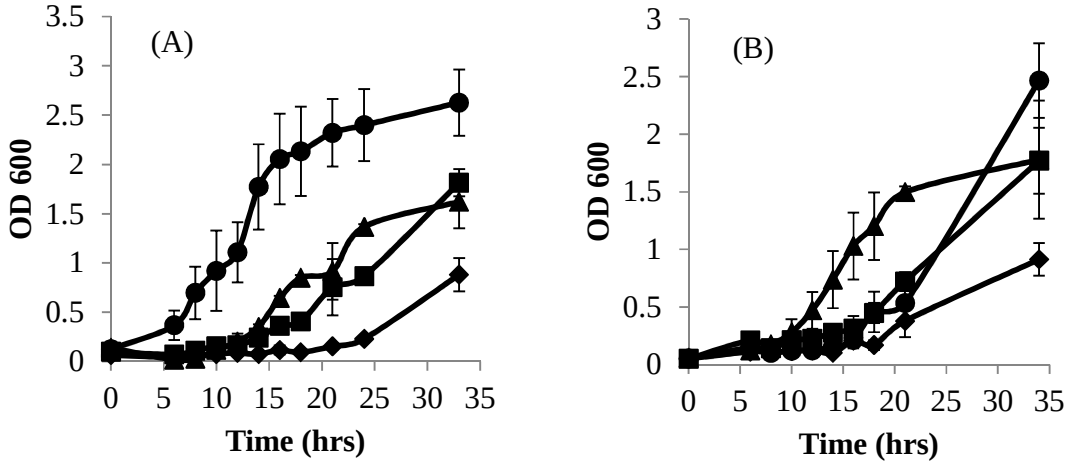

Figure 4.4: Cell growth profiles of (A) Bgl3C strains and (B) Cep94A strains expressing no transporter (♦), LacY (■), CP1 (▲), and CP2 (●) during aerobic growth

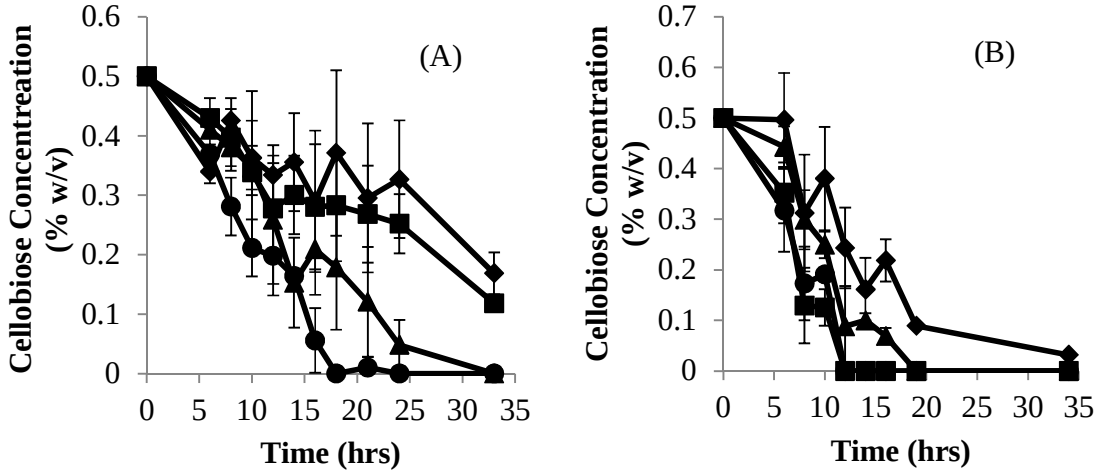

Figure 4.5: Cellobiose consumption by (A) Bgl3C strains and (B) Cep94A strains expressing no transporter (♦), LacY (■), CP1 (▲), and CP2 (●) during aerobic growth

As above, all strains expressing transporter proteins are able to grow anaerobically on cellobiose(Figure 4.3). All Bgl strains expressing transporters grew to a similar final OD of 1.6 with KY/pBBRGCP2/pHCEBGL reaching this OD faster than BglLacY or KY/pBBRGCP1/pHCEBGL. Cep94A strains expressing transporters all also

reach similar final OD of 1.6. However, in this case CepLacY reaches final OD more quickly than KY/pBBRGCP1/pHCECEP or KY/pBBRGCP2/pHCECEP. No growth was seen in KY strains expressing Bgl3C or Cep94A.

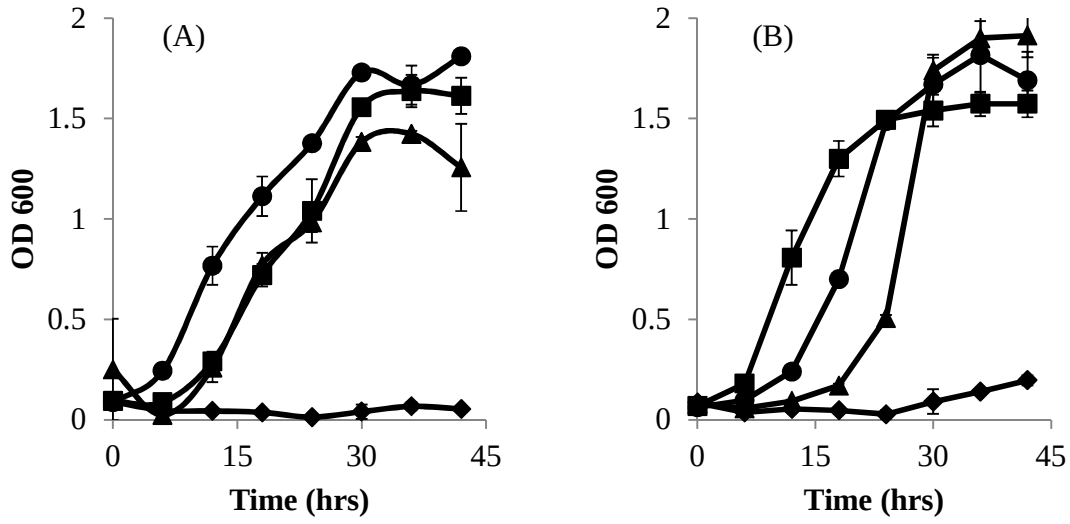

Figure 4.6: Cell growth by (A) Bgl3C strains and (B) Cep94A strains expressing no transporter (♦), LacY (■), CP1 (▲), and CP2 (●) under anaerobic conditions

In addition to growth rates, substrate consumption was measured for each strain (Figure 4.4). Of the Bgl3C expressing strains, BglLacY, KY/pBBRGCP1/pHCEBGL, and KY/pBBRGCP2/pHCEBGL all consumed a similar amount of cellobiose, but KY/pBBRGCP2/pHCEBGL showing a higher initial substrate consumption rate. Of the Cep94A producing strains, CepLacY shows a more rapid substrate consumption than KY/pBBRGCP1/pHCECEP or KY/pBBRGCP2/pHCECEP. In strains expressing either Bgl3C or Cep94A no substrate consumption was seen in KY strains expressing no transporter.

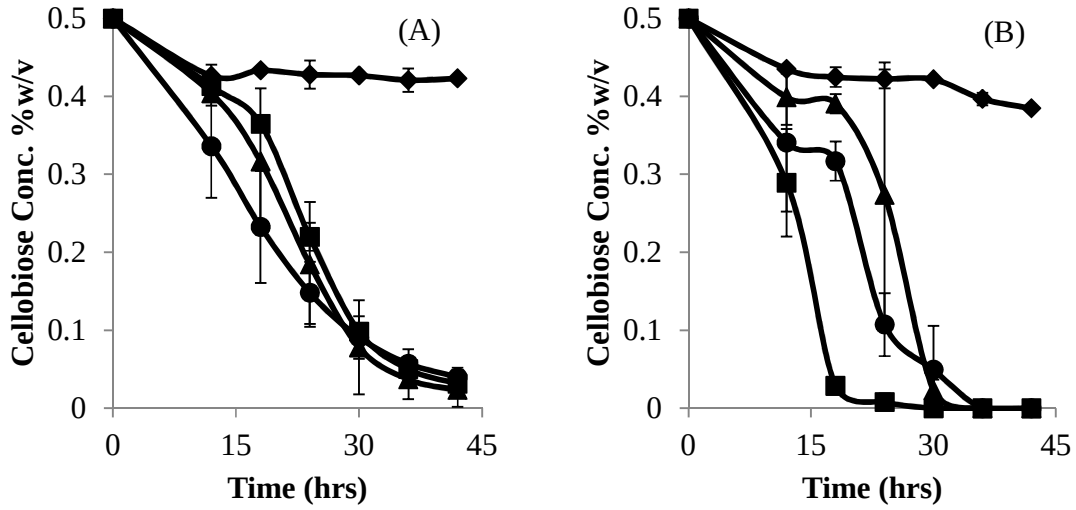

Figure 4.7: Cellobiose consumption by (A) Bgl3C strains and (B) Cep94A strains expressing no transporter (♦), LacY (■), CP1 (▲), and CP2 (●) under anaerobic conditions

Ethanol formation was measured for all strains tested. Bgl3C expressing strains all showed similar final ethanol titers of 0.13 with %w/v with the control strain (expressing no transporter) producing no ethanol. All Bgl3C strains reached final ethanol titers at the same time. Cep94A strains also showed similar final ethanol titers of 0.15 % w/v with the control strain (expressing no transporter) producing no ethanol. CepLacY however reached this final ethanol concentration after 18 hours with KY/pBBRGCP1/pHCECEP and KY/pBBRGCP2/pHCECEP produced maximal product concentrations after 30 hours.

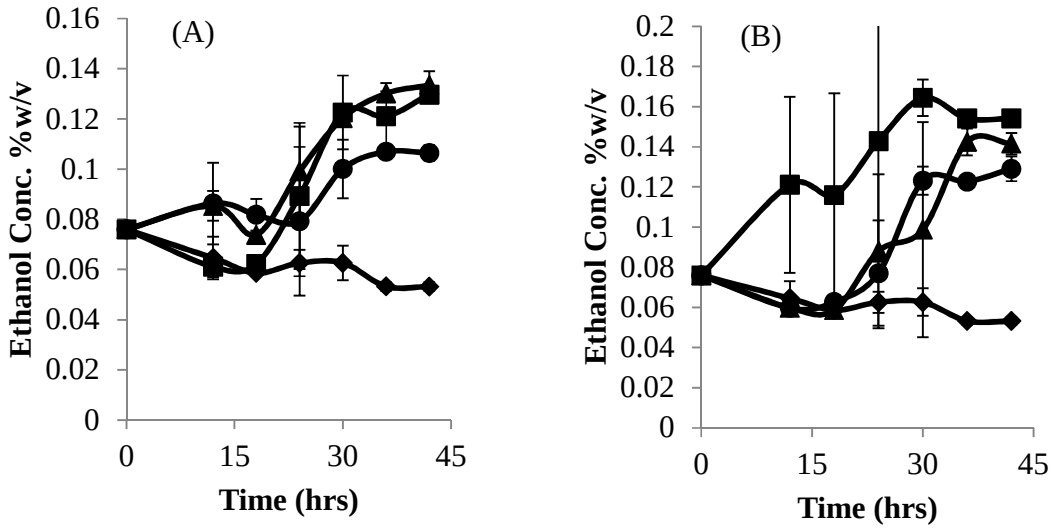

Figure 4.8: Ethanol production by (A) Bgl3C strains and (B) Cep94A strains expressing no transporter (♦), LacY (■), CP1 (▲), and CP2 (●) under anaerobic conditions

4.4 Discussion

In this study we cloned, expressed, and characterized three different putative cellobiose permease proteins. We have shown that LacY from *E. coli* and CP1 and CP2 from *S. degradans* are capable of transporting cellobiose across the cell membrane of *E. coli*. Results indicate that CP2 has the highest binding affinity for cellobiose.CP1 and LacY show similar cellobiose affinity to each other roughly 100x lower than that of CP2. CP1 and LacY also show similar maximum reaction velocities to each other. CP2 shows a maximum reaction velocity ten times lower than the other two proteins tested. These numbers indicate that CP2 has a catalytic efficiency (kcat/Km) ten-fold higher than either CP1 or LacY. This indicates that CP2 is likely the most suitable of the three for use in consolidated bioprocess where transient cellobiose concentrations will remain very low.

In addition to the ability of these proteins to transport cellobiose, this transport has been shown to be rapid enough to generate growth and metabolism during fermentation using cellobiose as the sole carbon source. When coupled with expression of either a β-glucosidase or a cellobiose phosphorylase expression of each of the transporters caused *E. coli* to grow, consume cellobiose nearly completely, and generates ethanol as a product. Interestingly, the dynamics of cell growth and substrate consumption varied based on the identity of the cytoplasmic cellobiase. In the case of strains expressing Bgl3C all strains grew and consumed cellobiose nearly identically with the CP2 showing slightly more rapid growth and consumption profiles. In strains expressing Cep94A, however, the LacY strain was able to grow and consume cellobiose more rapidly than the other strains. It is possible that because cellobiose is a likely native substrate of both CP1 and CP2 it is inhibited by the presence of cytoplasmic glucose-1-phosphate which is generated by Cep94A but not Bgl3C. Product formation was consistent between the two types of cellobiase enzyme, however, little difference was seen between strains expressing the different transporters. The low concentration of substrate combined with the 52% theoretical yield of ethanol combined with the low apparent conversion makes it likely that any differences that exist would be so low as to be difficult to detect analytically. Low product yields are likely a result of growth in minimal medium as it has been shown that fermentation in LB greatly improves ethanol yields compared to M9 medium.

Together this data demonstrates that the three proteins LacY, CP1, and CP2 are capable of using cellobiose as a substrate for translocation across the cell membrane of *E. coli*. This marks the first time these three proteins have been identified as cellobiose

transporters and had their transport kinetics characterized. Furthermore, expression of each of these proteins in *E. coli* allows growth and product formation using cellobiose as the substrate. As such these proteins are highly suited for consolidated cellulose bioprocessing, which generates cellobiose as a major intermediate.

4.5 Materials and Methods

4.5.1 Strains and Plasmids

All strains and plasmids used are listed in Table 4.3. KO11 *Δlacy*, annotated KY, was used for all kinetic characterizations and fermentations. All transformations were performed by heat shock at 42 °C for 30 s, followed by incubation in SOC media for 1 h and then plated on LB containing an appropriate antibiotic (ampicillin 100 μg/ml or chloramphenicol 25 μg/ml or kanamyacin 50 μg/ml).

4.5.2 pHCECEP Plasmid for Expression of Cep94A

To construct the expression plasmid pHCECEP, the cellobiose phosphorylase (*cep94A)* gene was amplified from the genomic DNA of *Saccharophagus degradans* by PCR using two primers, CEP-F and CEP-R (Table 4.3). PCR reactions were performed using iProof™ High Fidelity DNA Polymerase (BIO-RAD). Melting temperature of 60°C and elongation times of 105 seconds were used. This amplified gene fragment was digested with NdeI and SphI and subsequently ligated into pHCE vector to generate pHCECEP.

4.5.3 pHCEBGL Plasmid for Expression of Bgl3C

To construct the expression plasmid pHCEBGL, the β-glucosidase (*bgl3C)* gene was amplified from the genomic DNA of *Saccharophagus degradans* by PCR using two primers, BGL-F and BGL-R (Table 4.3). PCR reactions were performed using iProof™ High Fidelity DNA Polymerase (BIO-RAD). Melting temperature of 60°C and elongation times of 105 seconds were used. This amplified gene fragment was digested with NdeI and BamHI and subsequently ligated into pHCE vector to generate pHCEBGL.

4.3.4 Construction of GFP-Transporter Fusions

Green fluorescent protein was fused to the c-terminal of all three transporter, LacY, CP1, and CP2. These modules were joined by a 56 amino acid polyserine linker identified in the Cel5H protein from *S. degradans*. Each operon is under the control of the LacI promoter from *S. degradans.* Plasmids were constructed using Gibson assembly reaction (New England Biolabs) to insert the cassette into the pBBR122 plasmid. The ZraI restriction site was used to digest the plasmid.

4.5.5 pBBRGFP for Expression GFP

LacI was amplified using LacIP-F, and LacIP-R primers. LacIP-F has a region that overlaps the pBBR-F primer. GFP was amplified using GFP-F and GFP-R primers. GFP-R has a region that overlaps the pBBR-R primer and GFP-F has a region that overlaps the LacIP-R primer. Primers to amplify the pBBR plasmid nucleotides 5106-5125 and 3880-3900 are named pBBR-F and pBBR-R respectively. These individual

fragments were then mixed together and in a Gibson reaction (New England Biolabs) to create the LacI-GFP gene construct inserted at the ZraI site of pBBR122.

4.5.6 pBBRGLACY for Expression of LacY-GFP Fusions

LacI was amplified using LacIP-F, and LacIP-R primers. LacIP-F has a region that overlaps the pBBR-F primer. The LacY gene was amplified using the LacY-F and LacY-R primers. LacY-F has a region that overlaps the LacIP-R primer. GFP was amplified using pBsGFP-F and GFP-R primers. GFP-R has a region that overlaps the pBBR-R primer. Primers to amplify the pBBR plasmid nucleotides 5106-5125 and 3880-3900 are named pBBR-F and pBBR-R respectively. The PSL portion was amplified using sGFPPSL-R and LacYPSL-F primers. sGFPPSL-R has a region that overlaps with pBsGFP-F and LacYPSL-F has a region that overlaps with LacY-R. All fragments were amplified individually by PCR. These individual fragments were then mixed together and in a Gibson reaction (New England Biolabs) to create the LacI-LacY-PSL-GFP gene construct inserted at the ZraI site of pBBR122.

4.5.7 pBBRGCP1 for Expression of CP1-GFP Fusions

LacI was amplified using LacIP-F, and LacIP-R primers. LacIP-F has a region that overlaps the pBBR-F primer. The CP1 gene was amplified using the CP1-F and LCP1-R primers. CP1-F has a region that overlaps the LacIP-R primer. GFP was amplified using pBsGFP-F and GFP-R primers. GFP-R has a region that overlaps the pBBR-R primer. Primers to amplify the pBBR plasmid nucleotides 5106-5125 and 3880-3900 are named pBBR-F and pBBR-R respectively. The PSL portion was amplified using sGFPPSL-R and CP1PSL-F primers. sGFPPSL-R has a region that overlaps with

pBsGFP-F and CP1PSL-F has a region that overlaps with CP1-R. All fragments were amplified individually by PCR. These individual fragments were then mixed together and in a Gibson reaction (New England Biolabs) to create the LacI-CP1-PSL-GFP gene construct inserted at the ZraI site of pBBR122.

4.5.8 pBBRGCP2 for Expression of CP2-GFP Fusions

LacI was amplified using LacIP-F, and LacIP-R primers. LacIP-F has a region that overlaps the pBBR-F primer. The CP2 gene was amplified using the CP2-F and LCP2-R primers. CP2-F has a region that overlaps the LacIP-R primer. GFP was amplified using pBsGFP-F and GFP-R primers. GFP-R has a region that overlaps the pBBR-R primer. Primers to amplify the pBBR plasmid nucleotides 5106-5125 and 3880-3900 are named pBBR-F and pBBR-R respectively. The PSL portion was amplified using sGFPHL-R and CP2HL-F primers. sGFPHL-R has a region that overlaps with pBsGFP-F and CP2HL-F has a region that overlaps with CP2-R. All fragments were amplified individually by PCR. These individual fragments were then mixed together and in a Gibson reaction (New England Biolabs) to create the LacI-CP2-PSL-GFP gene construct inserted at the ZraI site of pBBR122.

4.5.9 Kinetic Characterization

Transporter kinetics were evaluated using the oil-stop method using tritiated cellobiose[15]. Labeled cellobiose at concentrations ranging from 0.5 to 200 μM was used for characterization of CP2. For characterization of SdeCP1 and LacY unlabeled cellobiose ranging from 1 to 10 mM was used with 10μM labeled cellobiose added to each concentration. Strains expressing the GFP-transporter fusions were induced with 0.2

mM IPTG and appropriate antibiotics for 20 hours at 18 °C. Cells were harvested by centrifugation at 16,000xg and resuspended to OD 20 in PBS. 50 μL of substrate solution was added to 50 μL of cells and incubated at room temperature for 90 seconds. This mixture was added on top of 50 μL of silicon oil and centrifuged at 16,000xg for 5 minutes and then put in an ethanol dry ice mixture to arrest metabolism. The pellets were then snipped off into scintillation vials containing Ecoscint Original (National Diagnostics) for counting.

4.5.10 Fermentation of Cellobiose

KY strains expressing the gap-transporter fusions were induced in LB medium with 0.2 mM IPTG and appropriate antibiotics at 18 °C for 20 hours. These were then inoculated to OD 0.05 into fresh M9 medium containing 0.5 % cellobiose and appropriate antibiotics and incubated at 37 °C and 250 rpm. Samples were collected periodically for analysis on HPLC.

4.5.11 Analytical Methods

Radioactive decay was measured by a Packard Tri-Carb 2900TR Liquid Scintillation Counter using energy channel divisors of 0-18.6, 18.6-256, and 256-2000 kEV. Cell density (OD_{600}) and was measured at 600 nm on a UV/VIS spectrophotometer (DU530; Beckman Coulter, USA). Fluorescence of GFP fusions was measured at 485nm and 510nm for excitation and fluorescence respectively on a microplate reader (M5; Spectramax, USA) The concentrations of ethanol and cellobiose were measured 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.

Table 4.3: Primers

Primer	Sequence
pBBR-F	5’-GACGTCAGGTGGCACTTTTCG
pBBR-R	5’-TCCCAGAGCCTGATAAAAACG
LacIP-F	5’-GAAAAGTGCCACCTGACGTCCATTTACGTTGACACCA TCGAATGG
LacIP-R	5’-AGCTGTTTCCTGTGTGAAATTGTTAT
sGFP-F	5’-ATAACAATTTCACACAGGAAACAGCTATGCGTAAAG G TGAAGAACTGTTCAC
sGFP-R	5’-GTTTTTATCAGGCTCTGGGATTATTATTTGTACAGTTC GTCCATACC
pBsGFP-F	5’-ATGCGTAAAGGTGAAGAACTGTTCAC
LacIP-R	5’-AGCTGTTTCCTGTGTGAAATTGTTAT
sGFPPSL-R	5’-TGAACAGTTCTTCACCTTTACGCATGTTTTCTGCTTCA ATGCGCGCGGG
LacY-F	5’-TAACAATTTCACACAGGAAACAGCTATGTACTATTTA AAAAACACAAACTTT
LacY-R	5’-AGCGACTTCATTCACCTGACGACG
LacYPSL-F	5’-GTCGTCAGGTGAATGAAGTCGCTGTTAAAAACTTAAT TAAAACATGGAACG
CP1-F	5’-TAACAATTTCACACAGGAAACAGCTATGTTGTCAGTA AAAGAAAAAGTAG
CP1-R	5’-GTTTACAGTTTTTAAATTTAGCGCTTG
CP1PSL-F	5’-AAGCGCTAAATTTAAAAACTGTAAACGTTAAAAACTT AATTAAAACATGGAACG
CP2-F	5’-TAACAATTTCACACAGGAAACAGCTATGGTCTCTCCA AACAGTCAAGTTAG
CP2-R	5’-TTTTCTGCGCTCGGCTAATTCTGCG
CP2HL-F	5’-CGCAGAATTAGCCGAGCGCAGAAAAATGGAGCTCCGT GGATCATCG
sGFPHL-R	5’-GTGAACAGTTCTTCACCTTTACGCATGATATCTCTAGA GTCGACACTAGTG
CEP-F	5’-ATGCATAGCATATGAAATTTGGGCACTTTGACGACAA
CEP-R	5’-CATCGATAGCATGCTTAGCCCAATGTAACT TCT
BGL-F	5’-GTACTAGACATATGATGCTGCTAAGCTTAAAAAACAC TCA
BGL-R	5’-GCATGCAGGATCCTTACTGCTGGTATTGGAAGCTAGT TT

Table 4.4: Strains and Plasmids

Strains or Plasmids	Description	Source
Escherichia coli Host Strains		
KY	KO11 Δ *lacy*	Sekar, et al. [9]
Plasmids		
pHCE	Amp^R, *HCE* promoter, ColE1 ori	Takara
pBBR122	Amp^R ,Cml^R, T7, Rep ori	Takara
pHCECEP	pHCE vector containing *cep94A* from *S. degradans*	This Study
pHCEBGL	pHCE vector containing *bgl3C* from *S. degradans*	This Study
pBBRG	pBBR122 vector containing *gfp*	This Study
pBBRGLACY	pBBR122 vector containing the *lacy-gfp* fusion	This Study
pBBRGCP1	pBBR122 vector containing the *CP1-gfp* fusion	This Study
pBBRGCP2	pBBR122 vector containing the *CP2-gfp* fusion	This Study
E. coli transformants		
KY/pBBRG	*Expressing GFP*	*This Study*
KY/pBBRGLACY	*Expressing GFP-LacY fusion*	*This Study*
KY/pBBRGCP1	*Expressing GFP-CP1 fusion*	*This Study*
KY/pBBRGCP2	*Expressing GFP-CP2 fusion*	*This Study*
KY/pBBRG/pHCECEP	*Expressing GFP and Cep94A*	*This Study*
KY/pBBRGLACY/pHCECEP	*Expressing GFP-LacY fusion and Cep94A*	*This Study*
KY/pBBRGCP1/pHCECEP	*Expressing GFP-CP1 fusion and Cep94A*	*This Study*
KY/pBBRGCP2/pHCECEP	*Expressing GFP-CP2 fusion and Cep94A*	*This Study*
KY/pBBRG/pHCEBGL	*Expressing GFP and Bgl3C*	*This Study*
KY/pBBRGLACY/pHCEBGL	*Expressing GFP-LacY fusion and Bgl3C*	*This Study*
KY/pBBRGCP1/pHCEBGL	*Expressing GFP-CP1 fusion and Bgl3C*	*This Study*
KY/pBBRGCP2/pHCEBGL	*Expressing GFP-CP2 fusion and Bgl3C*	*This Study*

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CHAPTER 5

EVALUATION OF A MINIMAL SET OF CELLULASES FOR CONSOLIDATED BIOPROCESSING OF CELLULOSE

5.1 Abstract

One approach to reduce the cost associated with multi-step cellulose bioprocessing is to develop a consolidated system in which enzymes are produced and cellulose is hydrolyzed under conditions conducive to product formation. A key component of this type of system is a system of cellulase enzyme capable of extensive and rapid hydrolysis of cellulose. Selection of a minimal set of enzymes to achieve this goal will result in a system low in complexity and therefore much easier to understand and manipulate. Three cellulases were selected: Cel5H from *S. degradans* and Cel9R and Cel48S from *C. thermocellum* and studied for their performance under conditions physiologically relevant to *E. coli*. This system is shown to achieve upwards of 15% PASC hydrolysis under at least half of the compositions tested. Furthermore, optimized mixtures used in a simultaneous saccharification process using a whole cell biocatalyst engineered for conversion of cellobiose to 2,3-*meso*-butanediol were capable of converting 20% of the substrate to product in 72 hours.

5.2 Introduction

In order to utilize cellulosic biomass in a cost-effective way a consolidated bioprocess in which cellulases are produced cellulose is hydrolyzed and products are

formed must be developed. The major bottleneck is this sort of process is often the slow rate of cellulose hydrolysis by the enzymes produced. Research has shown that complete degradation of cellulose requires several types of enzymes acting simultaneously. Two major categories of enzymes are responsible for hydrolyzing the cellulose molecule. Endo (1,4) –β-D-glucanases (EC 3.2.1.4) hydrolyze cellulose at internal regions of the molecule and cellobiohydrolases (EC 3.2.1.19) hydrolyze cellulose by releasing cellobiose from the chain end[1-3].

Engineering a microbial platform for consolidated cellulose bioprocessing requires the development of a system of cellulases capable of extensive and rapid cellulose hydrolysis under conditions also suitable for fermentation[4]. It has been shown that binary cellulase systems are capable of synergistic degradation of cellulose. These studies have shown that the types of enzymes as well as the enzymatic family to which they belong are critical factors in achieving high degrees of synergy. High synergy can be observed between endoglucanase and cellobiohydrolase enzymes[5]. Some studies report synergy between two endoglucanases while almost no instances of synergy between two cellobiohydrolases have been seen[6]. Furthermore, high synergy is often seen between family 48 and family 9 enzymes as well as between family 5 and family 6 enzymes. It has also been shown that the relative abundance of each enzyme in the mixture can alter the hydrolysis rate and synergy drastically[7-10].

While hydrolysis using a cellobiohydrolase and an endoglucanase enzyme has been shown to be effective, addition of a second endoglucanase enzyme has the potential to capitalize on both exo-endo synergy as well as endo-endo synergy to improve cellulose hydrolysis rate and extent. In this study we present data on the performance of a ternary

mixture of Cel5H from *S. degradans* and Cel9R and Cel48S from *C. thermocellum* during hydrolysis of cellulose under *E. coli* physiological conditions. This mixture is capable of extensive hydrolysis in-vitro across a wide range of enzyme compositions. This performance translates well into in-vivo performance during a pseudo-consolidated bioprocess to produce butanediol in abundance and hydrolyzing 20% of cellulose, an extent never before reported.

5.3 Results

5.3.1 Hydrolysis of Cellulose by Three Cellulases

Sugars released during hydrolysis of 2% PASC by mixtures of Cel5H, Cel9R, and Cel48S at a range of enzyme ratios was measured by DNS after 24 hours of incubation at 37 °C and pH 6.0. Extent of hydrolysis and enzymatic activity was calculated from sugars released for each enzyme composition. A 1:2:1 mass ratio of Cel5H to Cel9R to Cel48S showed the most extensive hydrolysis of 1% amorphous cellulose at 22% total hydrolysis. This 22% hydrolysis translates to an activity of 1.3 mU. All compositions tested demonstrated > 5% hydrolysis of amorphous cellulose. Additionally maximal hydrolysis of binary interactions was observed with a 3:1 composition of Cel5H and Cel9R showed 16% hydrolysis at a rate of 0.95 mU and a 1:6 ratio of Cel9R to Cel48S showed 16% hydrolysis at a rate 0.93 mU.

Table 5.1: Activity, Extent of Hydrolysis, and Synergy of a Ternary Enzyme Mixture Acting on PASC

Fracton of Enzyme			Activity	Extent of Hydrolysis	Synergy
Cel5H	Cel9R	Cel48S	mU	%	
0.5	0.5	0	0.824 ± 0.035	13.9 ± 0.6	1.59
0.5	0	0.5	0.540 ± 0.049	9.16 ± 0.8	2.13
0	0.5	0.5	0.886 ± 0.057	15.0 ± 0.9	1.84
0.75	0.25	0	0.995 ± 0.015	16.8 ± 0.3	2.46
0.25	0.75	0	0.842 ± 0.045	14.2 ± 0.1	1.33
0.166667	0.833333	0	0.911 ± 0.042	15.4 ± 0.7	1.36
0.83	0.16	0	0.934 ± 0.015	15.8 ± 0.3	2.59
0.25	0	0.75	0.465 ± 0.022	7.89 ± 0.4	1.98
0.166667	0	0.833333	0.398 ± 0.058	6.75 ± 0.9	1.74
0.75	0	0.25	0.501 ± 0.065	8.48 ± 1.0	1.84
0.833333	0	0.166667	0.542 ± 0.086	9.19 ± 1.4	1.95
0	0.25	0.75	0.859 ± 0.109	14.5 ± 1.8	2.47
0	0.166667	0.833333	0.928 ± 0.065	15.7 ± 1.0	3.05
0	0.75	0.25	0.595 ± 0.016	10.0 ± 0.3	0.97
0	0.833333	0.166667	0.688 ± 0.075	11.6 ± 1.3	1.04
0.33	0.33	0.33	0.597 ± 0.035	10.1 ± 0.6	1.44
0.5	0.25	0.25	0.975 ± 0.040	16.5 ± 0.6	2.53
0.25	0.5	0.25	1.30 ± 0.033	22.0 ± 0.5	2.60
0.25	0.25	0.5	0.704 ± 0.012	11.9 ± 0.2	1.92
0.4	0.4	0.2	0.915 ± 0.002	15.5 ± 0.1	2.00
0.2	0.4	0.4	0.663 ± 0.024	11.2 ± 0.4	1.50
0.4	0.2	0.4	0.687 ± 0.025	11.6 ± 0.4	1.95
0.714286	0.142857	0.142857	0.728 ± 0.097	12.3 ± 0.2	2.11
0.142857	0.714286	0.142857	1.09 ± 0.056	18.5 ± 1.0	1.81
0.142857	0.142857	0.714286	0.554 ± 0.059	9.38 ± 0.6	1.83
0.090909	0.454545	0.454545	0.891 ± 0.011	15.1 ± 0.2	1.92
0.454545	0.090909	0.454545	0.789 ± 0.032	13.3 ± 0.6	2.65
0.454545	0.454545	0.090909	0.966 ± 0.032	16.3 ± 1.4	1.97
0.166667	0.333333	0.5	0.779 ± 0.006	13.2 ± 0.1	1.92
0.333333	0.166667	0.5	0.702 ± 0.030	11.9 ± 0.5	2.13
0.5	0.333333	0.166667	0.93 ± 0.029	15.7 ± 0.5	2.17
0.166667	0.5	0.333333	0.839 ± 0.005	14.2 ± 0.1	1.70
0.333333	0.5	0.166667	0.887 ± 0.010	15.0 ± 0.2	1.76
0.5	0.166667	0.333333	0.902 ± 0.047	15.3 ± 0.7	2.64

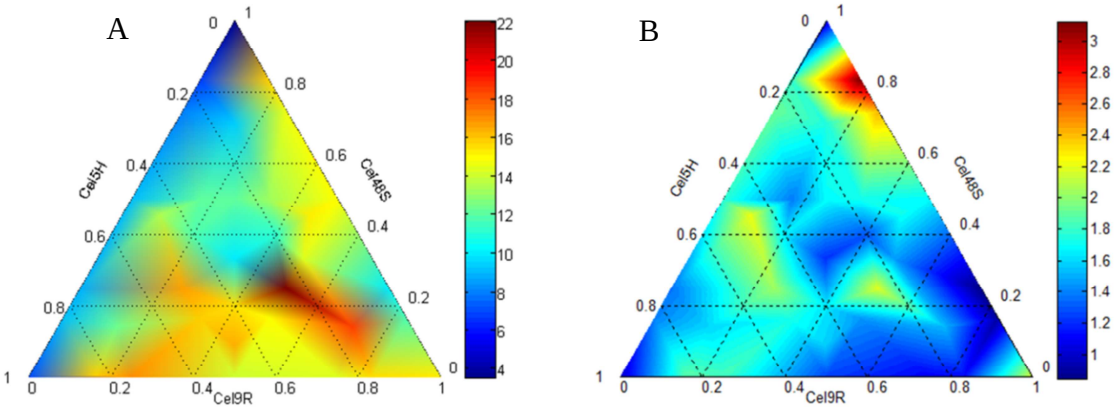

Figure 5.1: (A) Extent of hydrolysis (%) and (B) degree of synergy of Cel5H/Cel9R/Cel48S mixtures during hydrolysis of PASC

While maximum cellulase activity was observed at a 1:2:1 ratio of Cel5H, Cel9R, and Cel48S, maximal enzymatic synergy value of 3.0 was observed with a 1:6 ratio of Cel9R to Cel48S. The Cel5H/Cel9R binary system showed maximum synergy of 2.6 at a 6:1 enzyme ratio and the Cel5H/Cel48S binary system showed a maximum synergy of 1.9 at a 1:4 enzyme ratio. Maximal synergy for the ternary system of 2.65 was observed for a 4:1:4 mixture of Cel5H: Cel9R: Cel48S. All compositions demonstrated a synergy value greater than 1 with the exception of a 3:1 mixture of Cel9R to Cel48S showing a synergy of 0.97..

5.3.2 Removal of Cellobiose by a Whole Cell Biocatalyst

It is known that cellobiose inhibits cellulase activity[11]. Because of this any system developed for consolidated cellobiose processing must be capable of rapid conversion of cellobiose into product to enhance the cellulose hydrolysis rate. In this case we grew strains shown previously to rapidly convert cellobiose into butanediol by using a periplasmic cellodextrinase and a cytoplasmic cellobiose phosphorylase (MGLAP/pBBDO/pQECEP/pSTVCED) alongside a strain engineered for improved expression and secretion of Cel5H (BL21/sCel5H) using the native signal sequence of the protein and a strong promoter to achieve high extracellular titers. As can be seen in Figure 5.2 addition of the cellobiose consuming strain resulted in increased hydrolysis of cellulose by the Cel5H enzyme. The system with BL21/sCel5H alone was capable of generating cellobiose to a final concentration of 0.20% w/v while the system with both BL21/sCel5H and MGLAP/pBBDO/pQECEP/pSTVCED generated product such that at least 0.27% w/v cellobiose must have been generated. Both systems reached maximal hydrolysis after 96 hours.

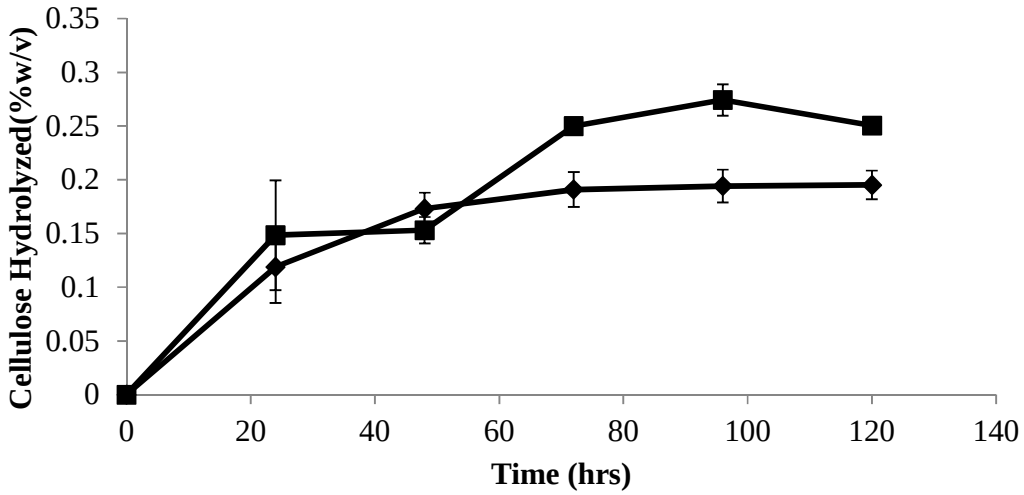

Figure 5.2: Cellulose hydrolyzed during fermentation of PASC by (♦) BL21/sCel5H and (■) BL21/sCel5H + MGLAP/BDO

5.3.3 Fermentation of Cellulose

To test the performance of this ternary cellulase system in-vivo fermentations were carried out using a BDO producing strain. A strain engineered for secretion of Cel5H was grown in the same culture with a strain previously engineering for cellodextrin fermentation to 2,3-*meso*-butanediol. PASC was added to a final concentration of 2% w/v along with exogenously produced Cel9R and Cel48S to generate the following enzyme compositions based on enzymatic activities: (1) 1:2:1 Cel5H:Cel9R:Cel48S, (2) 3:1 Cel5H:Cel9R, (3) 1:5 Cel5H:Cel48S and (4) Cel5H alone. Anaerobic fermentations were carried out and the formation of BDO was monitored in each case.

As can be seen in Figure 5.3 the ternary mixture of enzymes and the Cel5H/Cel9R binary mixture produced 2,3-*meso*-butanediol and acetoin combined to a concentration of 0.2% w/v while the Cel5H/Cel48S binary mixture and the Cel5H alone produced just

under 0.14% w/v butanediol and acetoin. If a 100% theoretical yield is assumed this indicates the mixtures are capable of attaining 20% and 14% hydrolysis of cellulose, respectively, after 72 hours.

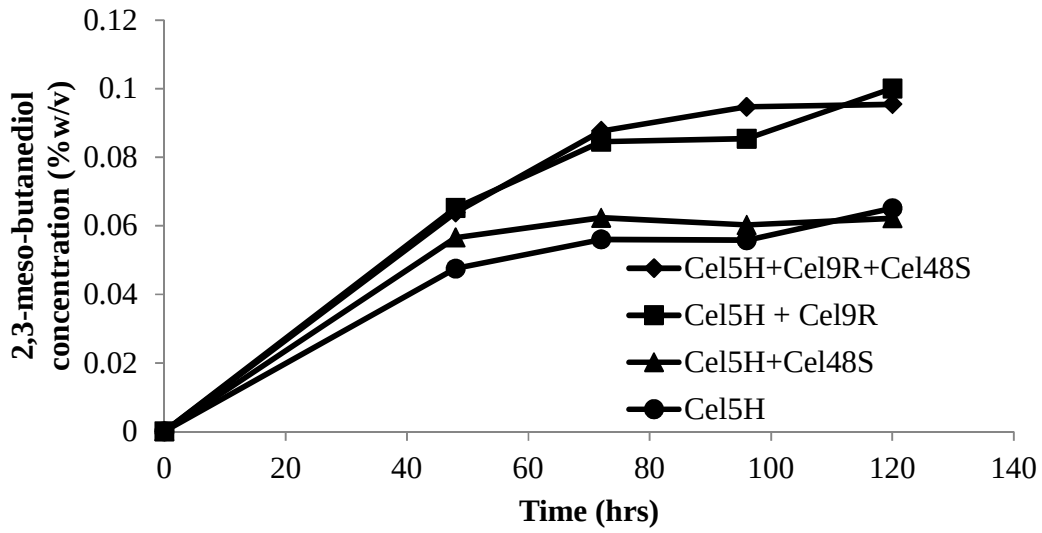

Figure 5.3: BDO product generation during pseudo-consolidated bioprocessing of PASC using (♦) Cel5H/Cel9R/Cel48S mixture, (■) Cel5H/Cel9R, (▲) Cel5H/Cel48S, and (●) Cel5H

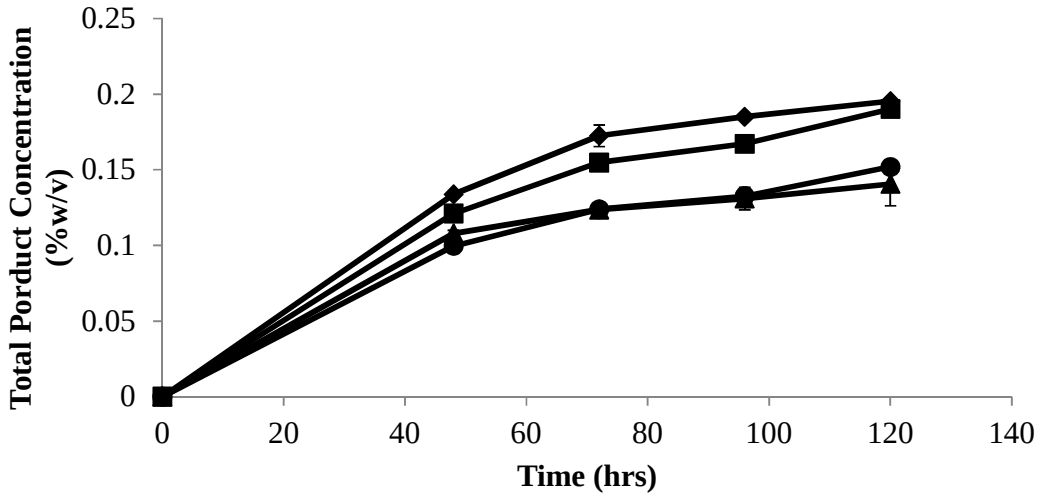

Figure 5.4: Total product generation during pseudo-consolidated bioprocessing of PASC using (♦) Cel5H/Cel9R/Cel48S mixture, (■) Cel5H/Cel9R, (▲) Cel5H/Cel48S, and (●) Cel5H

5.4 Discussion

In this study the performance of a mixture of three different cellulases, Cel5H from *S. degradans*, and Cel9R and Cel48S from *C. thermocellum* was evaluated in-vitro at physiological conditions as well as in vivo during anaerobic fermentation. This has given insight into the behavior of not only the ternary system, but also into the three different binary systems. Results show that a mixture of the three enzymes at a 1:2:1 ratio of Cel5H to Cel9R to Cel48S can achieve maximal hydrolysis of acid swollen cellulose at pH 6.0 and 37 °C. More interestingly over 50% of the compositions tested were capable of achieving greater than 60% of the maximum activity. This indicates extremely tight control of relative enzyme amounts is likely unnecessary to ensure good performance. Furthermore, performance of this system is the worst when Cel9R concentrations are low

with extent of hydrolysis dropping below 10% when Cel9R comprises less than 20% of the mixture. This suggests that Cel9R must represent more than 20% of the protein in order to achieve desired performance of this system.

The binary systems of Cel5H and Cel9R or Cel9R and Cel48S are also capable of achieving hydrolysis close to the maximum observed however, the Cel5H/Cel48S system achieves comparatively poor hydrolysis (<10% hydrolysis when Cel9R is omitted). The binary systems that include Cel9R are all capable of achieving a maximum hydrolysis rate roughly 75% of the maximum observed with the ternary mixture. These are achieved at a 3:1 ratio of Cel5H to Cel9R and a 1:5 ratio of Cel9R to Cel48S (Table 5.1). It is important to note that the composition of these binary mixtures has a much larger effect on the hydrolysis rate than is observed with the ternary mixture. It is seen in figure 5.1 that the Cel5H/Cel48S system especially shows a rapid decrease in hydrolysis rate as the composition diverges from the maximum, likely due to the lack of presence of Cel9R in the mixture. The other binary mixtures appear to be a bit more robust with major decreases in performance seen only far away from the maximum composition.

All synergy values measured were greater than one with a maximum synergy observed with the binary mixture of Cel9R to Cel48S at a 1:3 ratio. Interestingly, this enzyme ratio has been observed during the growth of *C. thermocellum* on crystalline cellulose, suggesting that this composition is in fact ideal for maximal efficient cellulose hydrolysis. Synergy is affected much more greatly by the composition of the enzyme mixture, with most compositions showing synergy values much lower than the maximum. Maximum synergy by a system including all three enzymes, however, is observed at a composition coinciding with the maximal observed hydrolysis (Table 5.1).

Before using these systems in a consolidated fermentation we were able to verify that a whole cell *E. coli* biocatalyst engineered for cellobiose consumption by expression of a cellodextrinase (Ced3A) and cellobiose phosphorylase (Cep94A) (MGLAP/pBBDO/pQECEP/pSTCED) was able to improve the hydrolysis rate by Cel5H being secreted by a different strain of *E. coli* (BL21/sCel5H). The addition of the cellobiose consuming strain improved the extent of PASC hydrolysis by Cel5H by 35% compared to the performance achieved with Cel5H alone. This indicates that the MGLAP/pBBDO/pQECEP/pSTCED strain is well suited for consumption of cellobiose generated during a consolidated cellulose bioprocess to remove a significant inhibitor of cellulase activity.

A pseudo-consolidated bioprocess (PCBP) was developed using *E. coli* MGLAP/pBBDO/pQECEP/pSTCED for conversion of cellodextrins to BDO and *E. coli* BL21/sCel5H for expression and secretion of Cel5H. The other two enzymes, Cel9R and Cel48S, were purified and added to the fermentation exogenously. Both strains of *E. coli* were grown simultaneously in the presence of PASC and BDO formation was measured. Using this ternary system of cellulases to produce sugars during a PCBP resulted in significant product formation and hydrolysis of at least 20% of the cellulose substrate. This is achieved by both the ternary system as well as the Cel5H/Cel9R binary mixture. Hydrolysis by Cel5H alone as well as the Cel5H/Cel48S binary mixture resulted in 14% hydrolysis. This was achieved after 72 hours of hydrolysis after which point, hydrolysis seemed to stop. Hydrolysis may have ceased due to the degradation of the cellulase enzymes or possibly due to the exhaustion of substrate that the enzymes are still capable of hydrolyzing.

This data, taken together, indicates that mixtures of the three enzymes studied, Cel5H from *S. degradans*, and Cel9R and Cel48S from *C. thermocellum* are capable of extensively hydrolyzing a model insoluble, amorphous cellulose substrate at conditions suitable for *E. coli* fermentation. This can be accomplished over a wide range of compositions removing the need for strict control of the system. The hydrolysis rate is high enough to facilitate product formation during fermentation and generating the highest product titers after only 72 hours. Maximum in-vivo hydrolysis reaches 10% with both the ternary system and the binary system including Cel5H and Cel9R. To our knowledge hydrolysis this extensive has not yet been achieved in a consolidated process in which cellulase and fermentation product is generated simultaneously.

5.5 Materials and Methods

5.5.1 Strains and Plasmids

All strains and plasmids used are listed in Table 5.2. *E. coli* JM109 was used for expression of Cel9R and Cel48S. Butanediol production was achieved by the MGLAP strain previously characterized [13]. All transformations were performed by heat shock at 42 °C for 30 s, followed by incubation in SOC media for 1 h and then plated on LB containing an appropriate antibiotic (ampicillin 100 μg/ml or kanamyacin 50 μg/ml).

5.5.2 Construction of pQTCEL9 for Cel9R expression

To construct the expression plasmid pQTCEL9, the endoglucanase gene (*cel9R)* was amplified from the genomic DNA of *Clostridium thermocellum* by PCR using two primers, CEL9-F and CEL9-R (Table 5.3). PCR reactions were performed using iProof™

High Fidelity DNA Polymerase (BIO-RAD). Melting temperature of 60°C and elongation times of 105 seconds were used. This amplified gene fragment was digested with BamHI and SacI and subsequently ligated into pQTH vector to generate pQTCEL9.

5.5.3 Construction of pQTCEL48 for Cel48S expression

To construct the expression plasmid pQTCEL48, the endoglucanase gene (*cel48S)* was amplified from the genomic DNA of *Clostridium thermocellum* by PCR using two primers, CEL48-F and CEL48-R (Table 5.1). PCR reactions were performed using iProof™ High Fidelity DNA Polymerase (BIO-RAD). Melting temperature of 60°C and elongation times of 105 seconds were used. This amplified gene fragment was digested with BamHI and HindIII and subsequently ligated into pQTH vector to generate pQTCEL48.

5.5.4 Protein Expression and Purification

Cel5H strain was induced in LB medium with 0.2 mM IPTG at 18 °C for 48 hours. JM109/pQTCEL9C and JM109/pQTCEL48 strains were induced in LB medium with 1.0 mM IPTG at 37 °C for 24 hours. Cells were harvested by centrifugation at 5,000xg for 30 minutes resuspended to 10x concentration in PBS and lysed by ultrasonication. Cel5H crude lysate was purified by cobalt affinity using Cobalt Resin (Clontech). Cel9R was purified by nickel affinity using His-Buster Nickel Resin (Clontech). Cel48S was purified by incubation at 80 °C for 30 minutes followed by centrifugation at 16,000 x g for 5 minutes. Cel48S remained in the supernatant.

5.5.5 Hydrolysis of PASC in vitro

PASC was prepared from Avicel by [13]. PASC was added to phosphate buffer pH 6.0 to a final concentration of 1%. Enzymes were then added in varying ratios to a final loading of 0.6mg enzyme/mg cellulose. Reactions were incubated at 37 °C with shaking at 250 rpm for 24 hours. Sugars produced were measured by DNS method.

5.5.6 DNS Method

In order to determine soluble reducing sugar concentrations, 100 μL of sample was added to 900 μL of DNS solution. DNS solution was prepared as follows: 0.75% 3,5-dinitrosalycylic acid, 1.4% sodium hydroxide, 21.6% potassium sodium tartrate, 0.55% phenol, 0.55% sodium metabisulfate, dissolved in water. These mixtures were then boiled for 5 minutes, centrifuged at 15,000g for 5 minutes and optical density of the supernatant at 550 nm was measured. Reducing sugar concentrations were calculated using glucose as standards.

5.5.7 Fermentation of PASC

The soluble Cel5H strain obtained from collaborators was inoculated into LB containing 50μg/mL and induced with 0.1 mM IPTG at 30 °C for 24 hours. Extracellular activities were measured before transferring 10 mL of culture to a 20 mL scintillation vial containing 0.2 grams of PASC. Purified Cel9R and Cel48S were added to the same vials to appropriate enzyme ratios to achieve a final enzyme loading of 0.6 mg enzyme/mg PASC. These were then incubated at 37 °C anaerobically for 24 hours. After 24 hours a butanediol producing strain engineered for cellobiose consumption that had been induced by 0.2 mM IPTG for 20 hours at 18 °C was added to the vials to a final OD of 2.0. Vials

were capped and incubated for 1 week at 37 °C with sampling occurring every 24 hours. Butanediol formation was measured by HPLC.

5.5.8 Analytical Methods

Cell density (OD_{600}) and was measured at 600 nm on a UV/VIS spectrophotometer (DU530; Beckman Coulter, USA). The concentrations of butanediol were measured 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.

Table 5.2: Strains and Plasmids

Strains or Plasmids	Description	Source
Escherichia coli Host Strains		
JM109	Expression host for recombinant protein production	Sekar, et. al.[15]
MGLAP/BDO	Butanediol producing strain engineered for cellobiose metabolism	Rutter et. al.[12]
Plasmids		
pQTH	Amp^R, *T5*, ColE1 ori, N-terminal TAT sequence	Takara
pBBR122	Amp^R ,Cml^R, T7, Rep ori	Takara
pQTCEL9	pQTH vector containing *cel9R* from *S. degradans*	This Study
pQTCEL48	pHCE vector containing *cel48S* from *S. degradans*	This Study
E. coli transformants		
JM109/pQTCEL9	*Expressing Cel9R*	*This Study*
JM109/pQTCEL48	*Expressing Cel48*	*This Study*
BL21/sCel5H	*Expressing Cel5H*	*This Study/Gift from Dr. Kim at Korean Institute of Advanced Technology*

Table 5.3: Primers for expression of cellodextrinase genes in E. coli

Primer	DNA Sequence
CEL9-F	5’- GCGATTGGATCCGCAGACTATAACTATGGAGA
CEL9-R	5’- GGCGCCGAGCTCGTATGAATAGTCTGTAGA
CEL48-F	5’- GCATACTAGATCTATGAACAATAACGATCTCTTTCAG
CEL48-R	5’-ATAGTACAAGCTTTCAGTGGTGATGGTGATGGTGGTGGTTC TTGTACGGCAATGTATCTA

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CHAPTER 6

CONCLUSIONS SIGNIFICANT CONTRIBUTIONS AND FUTURE DIRECTIONS

6.1 Conclusions

The work presented in this dissertation has accomplished the three major objectives stated in the introduction: (1) characterization of a new cellodextrinase enzyme capable of hydrolyzing a wide range of cellooligomers and its application to improved fermentation of sugars produced during enzymatic cellulose hydrolysis, (2) selection of proteins suitable for the transport of cellobiose into *E. coli* during consolidated bioprocessing and (3) development of a minimal set of cellulases capable of extensive cellulose hydrolysis.

6.1.1 Characterization of a Cellodextrinase and its Application of Improved Fermentation of Relevant Sugars

Complete characterization of Ced3A, a cellodextrinase from *S. degradans* [1], as it pertains to cellulose metabolism was completed. Kinetic analysis showed this enzyme was capable of generating glucose from cellooligomers ranging from DP 2 up to DP 5. The protein both with and without its native N-terminal signal sequence was studied to determine the role of the lipobox containing leader peptide[2]. It was determined that the native signal sequence was recognized by *E. coli*, resulting in translocation of the enzyme across the inner membrane into the periplasm. Removal of the signal sequence from the gene caused the enzyme to remain within the cytoplasm. Furthermore, only when the

enzyme was expressed in the periplasm were *E. coli* strains capable of growing on cellobiose or a mixture of cellodextrins as the sole carbon source. This suggests that not only are cellodextrins able to diffuse through the outer membrane of *E. coli* but also that this diffusion rate is rapid enough to allow generation of glucose by the cellodextrinase at a rate adequate to support cell growth. Fermentation of cellobiose and cellodextrin by similar product forming strains led to lactic acid and 2,3-*meso*-butanediol yields upward of 80%.

While expression of cellodextrinase alone broadened the substrate range of *E. coli* to include cellobiose and longer cellodextrins, metabolism of cellobiose was much slower than longer oligomers, resulting in incomplete consumption of cellobiose, leaving behind up to 60% of initial cellobiose provided during fermentation. Because cellobiose represents the major product of cellulose hydrolysis it is critical that it be metabolized rapidly. To remedy this, the expression of cellodextrinase was coupled with expression of a cytoplasmic cellobiose phosphorylase (Cep94A) that has been shown to have high activity on cellobiose. *E. coli* expressing both Ced3A and Cep94A was shown to completely metabolize cellobiose 10 hours sooner than when Cep94A alone was expressed. This improved cellobiose metabolism ultimately led to more rapid product formation with 60% more BDO present after 24 hours of fermentation.

Additionally, we were able to show that conversion of cellobiose into glucose within the cytoplasm allows cells to ignore catabolite repression that would otherwise occur when glucose is generated extracellularly. Fermentations of cellobiose/xylose mixtures showed that both cellobiose and xylose were consumed simultaneously while fermentations of glucose/xylose mixtures showed classic diauxic behavior. Furthermore,

when fermenting 2% total sugar the cellobiose/xylose mixture was completely consumed while roughly half of the initial xylose remained during fermentation of a glucose/xylose mixture. Cellobiose and xylose, taken together, represent upwards of 70% of sugars that would be generated from cellulosic biomass and their complete utilization is critical. Furthermore, myriad cellular processes are affected by catabolite repression [3] and the ability to ignore this phenomenon has the potential to drastically improve product formation during consolidated cellulose bioprocessing.

6.1.2 Characterization of Three Cellobiose Permeases

Three transport proteins were identified to be cellobiose permeases suitable for cellobiose transport during fermentation. LacY, lactose permease, from *E. coli* [4]and two proteins never before studied from *S. degradans*, CP1 and CP2[5], were shown to transport cellobiose across the cell membrane of *E. coli*. Evaluating the kinetics of each protein showed that LacY and CP1 had Vmax and Km values of 0.03 Units/min/mg and 3 mM respectively while CP2 had a Vmax of 0.002 Units/min/mg and a Km of 0.04 mM. Furthermore, when coupled with a cytoplasmic cellobiase individual expression of each of these proteins in *E. coli* allowed consumption of cellobiose leading to cell growth and product formation. Interestingly, the identity of the cytoplasmic cellobiase determined which permease protein allowed for the best substrate consumption and cell growth. When coupled with a cellobiose phosphorylase all permease proteins grew and consumed cellobiose at the same rate. When coupled with β-glucosidase, however, LacY outperformed the other two proteins. The catalytic efficiency of CP2 is ten times higher than the other two proteins which indicates that at low cellobiose concentrations that will

be generated during consolidated cellobiose processing CP2 is likely more suitable for this type of bioprocess than the other two permeases characterized in this work.

6.1.3 Development of a Minimal Set of Cellulases for Consolidated Bioprocessing

Successful hydrolysis of acid-swollen cellulose at conditions amenable for *E. coli* growth and fermentation was achieved both in-vitro and in-vivo by the three cellulases Cel5H from *S. degradans* [6] and Cel9R [7] and Cel48S [8]from *C. thermocellum.* Together in a 1:2:1 ratio of Cel5H:Cel9R:Cel48S these enzymes hydrolyzed 22% of acid-swollen cellulose in 24 hours in-vitro. More importantly, over half of the enzyme compositions tested were capable of achieving 75% of the maximum observed hydrolysis. This broad range of compositions that show performance close to the maximum indicates that tight control of enzyme composition, which can be difficult to achieve in-vivo, is not critical for performance of the cellulolytic system.

High hydrolytic activity in-vitro was easily translated into in-vivo performance. First, we demonstrated that the previously engineered strain capable of rapid conversion of cellobiose to butanediol, when grown in binary culture with strains secreting Cel5H, was able to increase cellulose hydrolysis by up to 35% compared to hydrolysis by the Cel5H strain alone. Next, this binary system of whole cell biocatalysts was used to evaluate the in-vivo performance of the optimum conditions observed in-vitro. The optimized ternary mixture outperformed the Cel5H alone as well as the binary mixture of Cel5H and Cel48S. The performance of the Cel5H/Cel9R binary system however, was on par with the ternary mixture. Top performers were capable of conversion of 20% of cellulose to product. Optimized ternary mixtures of Cel5H, Cel9R, and Cel48S as well as

binary mixtures of Cel5H and Cel9R are capable of extensive cellulose hydrolysis in a consolidated bioprocess to generate valuable bioproducts.

6.2 Significant Contributions

This dissertation provides significant contributions to the development of cost-effective conversion of cellulose to valuable bioproducts. First, an *E. coli* whole cell biocatalyst for conversion of all cello-oligomers produced during enzymatic cellulose hydrolysis, from cellobiose all the way to cellopentose, to the bioproducts ethanol, lactic acid, and butanediol was developed. This was achieved by expression of only two enzymes, a cellodextrinase and a cellobiose phosphorylase, neither of which were secreted from the cell. This leads to generation of glucose within the cell which allows cells to ignore catabolite repression that would be seen if glucose was produced. This enables coutilization of xylose and glucose equivalents in the form of cellobiose. This catalyst was shown to work in tandem to improve cellulose hydrolysis in-vivo by removing cellobiose, a major cellulase inhibitor, from the culture medium.

This dissertation also expanded on the knowledge of transport of cellobiose across the cell membrane. Three permeases LacY from *E. coli* and CP1 and CP2 from *S. degradans* were shown to have affinity toward cellobiose. Of these three CP2 had the highest catalytic efficiency, likely making it ideal for use in consolidated bioprocesses in which transient cellobiose concentrations will be very low. Furthermore, expression of each of these proteins along with a cellobiase enzyme in *E. coli* caused rapid growth and fermentation of cellobiose. Each protein allowed complete consumption of cellobiose after only 36 hours. When compared with an average rate of cellobiose generation by cellulase enzymes (0.22 %w/v produced in 24 hours) it is apparent that cellobiose uptake

and conversion, as high as 0.7 %w/v consumed in 24 hours, is adequately rapid so as to not be the rate-limiting step of the consolidated bioprocess.

Finally, this dissertation contributes to the knowledge of systems of cellulases as applied to rapid cellulose hydrolysis. We have demonstrated a minimal system of three cellulase enzymes capable of rapid and extensive cellulose hydrolysis at conditions that match the optimal fermentation conditions for *E. coli*. Cel5H, Cel9R, and Cel48S, when acting in unison are capable of extensive hydrolysis of cellulose across a wide range of compositions. Furthermore, when this system is used in a consolidated bioprocess in which enzyme production, cellulose hydrolysis, and product formation are achieved simultaneously, cellulose is hydrolyzed rapidly enough to allow significant growth and product formation by *E. coli*. This represents the first time that a ternary system of cellulases has been used in a consolidated bioprocess with *E. coli*.

Together, this dissertation presents improvements to all three major components required for consolidated cellulose bioprocessing, cellulose product and hydrolysis, transport of the hydrolysis intermediates, and conversion of those intermediates into valuable products. Our advancements in all three of these processes operate effectively individually but more importantly they have been shown to operate in tandem under the same process conditions such as temperature, pH, and dissolved oxygen. As such we have developed novel *E. coli* biocatalysts that constitute a consolidated bioprocess in which multiple cellulase enzymes substantially hydrolyze cellulose which leads to formation of a variety of valuable bioproducts.

6.3 Recommendations for Future Directions

Three objectives were accomplished in this dissertation: (1) characterization of a system for metabolism of intermediates of enzymatic cellulose hydrolysis by a combination of two enzymes, (2) identification of three proteins suitable of cellobiose transport, and (3) characterization of a minimal set of three enzymes capable of extensive cellulose hydrolysis both in-vitro and in-vivo. Data indicates that these three components, when acting in concert, can be used to produce bioproducts during fermentation in a consolidated bioprocess using cellulose as the only substrate. However, in this instance performance on only a single substrate, phosphoric acid swollen cellulose, a model amorphous cellulose substrate was characterized. Additionally, only one cellulase, Cel5H, was produced endogenously while the other two proteins, Cel9R and Cel48S, were supplemented into the consolidated system. Future directions into further developing this system toward a completely consolidated bioprocess involve exploration of performance on other substrates, development of strains for high level secretion of Cel9R and Cel48S, and evaluation of the three transporters during consolidated bioprocessing. Furthermore, characterization of this system has offered insights into critical elements in the development of other consolidated bioprocesses.

6.3.1 Performance on a Range of Substrates

As mentioned previously, cellulose requires pretreatment before it is optimally suitable for hydrolysis by enzymes. While extensive pretreatment can almost completely convert biomass into monosaccharides, the cleanup necessary after these processes makes them unattractive. PASC represents a model amorphous cellulose substrate but it is unfortunately generated through acid treatment which and as such requires subsequent

cleaning steps before it is suitable for fermentation. While valuable insights can be obtained from studies done with PASC application of a system to an industrial process requires adequate performance on more realistic substrates such as Avicel or α-cellulose. Although both of these substrates have much higher crystallinity than PASC they require no form of chemical pretreatment to generate and are more ideal for consolidated bioprocessing on the industrial scale. Because Cel48S has an exo mode of action it has low activity on amorphous cellulose and as a result work with PASC has shown, both in-vitro and in-vivo, that Cel48S has a minimal contribution to hydrolysis. By increasing the crystallinity of the substrate by using α-cellulose (40% crystalline) or Avicel (100% crystalline) it is likely that Cel48S will play an increasingly important role in achieving extensive hydrolysis. As one of the three enzymes becomes more important, the landscape of the activity-composition map will change and more importantly, the range over which high levels of hydrolysis can be achieved may be reduced. Additionally, due to the increased recalcitrance of crystalline cellulose, hydrolysis rates are likely to decrease. Exploration of the behavior on less pretreated substrates is critical for providing insight into the capacity for this system, and others like it, to perform in a completely consolidated cellulose bioprocess.

6.3.2 Engineering *E. coli* for Secretion of Cellulases

A critical step in successful consolidated bioprocessing of cellulose is secretion of high amounts of protein by the whole-cell biocatalyst. While it is difficult to achieve high secretion in *E. coli* data here demonstrates a strain engineered for secretion of high levels of Cel5H secretion can be used in consolidated bioprocessing to convert cellulose into valuable bioproduct. This strain, obtained from collaborators, was engineered for

improved secretion without any major changes to the genome or metabolism and is able to achieve hydrolysis levels significantly higher than those previously reported during consolidated bioprocessing. Success in secretion is achieved, in this case, by use of a foreign signal sequence that is recognized by the native *E. coli* machinery. These results indicate that successful development of strains capable of secreting Cel9R and Cel48S in large quantities is possible.

There are two potential approaches for improvement of cellulase expression and secretion. The first approach is manipulation of genetic elements to improve expression. Options include exploring the use of consituitive, or inducible promoters as well as altering the ribosomal binding site to increase expression. The second approach involves altreration of signal sequences to accelerate secretion. Signal sequences from many different species and proteins can be matched with proteins to identify sequences that result in high secretion from *E. coli*. Armed with strains capable of secretion of the Cel5H, Cel9R, and Cel48S cellulases a truly consolidated process in which all cellulase enzymes are produced by the same strains responsible for cellodextrin fermentation to product can be realized.

6.3.3 Evaluation of Cellobiose Permeases

A major challenge associated with developing components of a system for consolidated bioprocessing is the difficulty in mimicking the conditions present in a consolidated bioprocess without all the necessary components working in tandem. This makes evaluation of the individual components very difficult. More specifically, because hydrolysis intermediates will be consumed as they are produced, concentrations of cellodextrins in a consolidated system will remain very low throughout the course of

fermentation. Characterization at higher substrate concentrations is certainly useful for identifying which permease proteins are responsible for transport of individual sugars, however, little can be gleaned regarding their behavior at lower concentrations. All data presented in this dissertation regarding cellobiose permease proteins was collected using relatively high levels of sugar substrates and as such, the results may not be directly translatable into performance while transient sugar concentrations are as low as can be expected in during CBP. By combining the existing system for cellodextrin metabolism with the cellulase secreting strains to be developed above, the performance of each permease protein under true CBP conditions can be evaluated.

6.3.4 Future Directions for Consolidated Bioprocessing

6.3.4.1 Realistic Evaluation of System Performance

The work presented in this dissertation shows that a ternary mixture of cellulase enzymes can rapidly hydrolyze a cellulose substrate. This is achieved by high levels of expression and secretion of recombinant proteins. Data also indicates that after some time, generally after 36 to 48 hours, the rate of cellulose hydrolysis slows down substantially. The cause of this phenomenon is yet to be completely understood however, several factors such as reduction in recombinant protein expression efficiency, exhaustion of usable substrate, and degradation of enzymes can help explain the loss of activity. Keeping this in mind, it is suggested that instead of evaluating the degree of hydrolysis or product formation after long time (greater than 96 hours), a better metric of the performance of a system can be obtained from the initial rate data (first 24 hours). This time frame represents a much more reasonable time scale for industrial applications and it would be relatively simple to invoke many of the commonly used engineering practices

such as recycle and in-situ product capture to capitalize on the relatively fast initial hydrolysis rates compared to those observed in later stages of fermentation. Over these shorter time frames it is much easier to predict and control the composition of the celluloytic system and rates of formation of growth-associated products will be higher, both of which are critical for optimal fermentation performance.

6.3.4.2 Development of Systems for CBP of Cellulose/Hemicellulose Mixtures

Work in presented in this dissertation is done with pure cellulose and the products of cellulose hydrolysis alone. A realistic consolidated system would more likely use a much more complicated substrate containing more than one of the major components of lignocellulosic biomass. In this process a wide array of different sugars will be produced including mono and oligosaccharadies of glucose, xylose, mannose, arabinose, and galactose as well as many organic acids. In order to maximize carbon flux through the microbial catalyst used in this system, coutilization of sugars is likely necessary. One way to facilitate this is to metabolize oligomers, especially those of glucose, within the cytoplasm to reduce the potential for catabolite repression of sugar utilization pathways by other sugars. By generating glucose and/or xylose within the cytoplasm, the microbe will metabolize those sugars without repressing the uptake and metabolic pathways for other sugars or carboxylic acids present.

6.3.4.3 Control of Cellulolytic Systems In-Vivo

It is well known that the composition and relative abundance of cellulases in a mixture can drastically alter the cellulose hydrolysis rate. The system presented in this dissertation was capable of hydrolysis close to the maximum over a broad range of

compositions, however, a 25% increased performance was observed at the optimum compared to nearby compositions. The behavior on less ideal substrates or by different cellulolytic systems may show an even more drastic difference between the optimum compositions and those nearby. Maintaining operation of our system as close to the maximum as possible will generate more rapid carbon flux and product formation by the whole-cell biocatalyst. This maintenance requires tight control over relative amounts of protein present in the extracellular space.

A lot of work has been done identifying the activities of cellulase enzymes and how they work on various substrates both individually and in concert. Optimal enzyme compositions have been identified in-vitro, however, in-vivo studies make no attempts to control relative expression levels. Many tools exist that have the potential to help control relative expression levels, and many tools can be developed to do so as well.

Targets for regulation of protein expression exist at the genetic, transcript, protein, and metabolite level. Different types of promoters including constituitive, inducible, and repressible types are known to have different strengths. One potential approach to controlling relative levels of proteins is to use promoters with relative strengths corresponding to the desired relative abundance of each protein. Promoters must be selected carefully because some are known to be “leaky” resulting in poor control of transcript levels[10]. Another tool for controlling protein expression is the ribosomal binding sequence present in the mRNA transcript. Much like promoters, RBS can have different relative strengths rising from factors such as binding strength RNA secondary structure[11]. Another technology that can be used to control relative expression levels is the construction of gene fusions. By fusing copies of genes downstream of a single

promoter in the same ratios we hope to attain among the proteins we can guarantee that the ratio is maintained at least at the transcript level. This is likely to be most valuable in systems where desired enzyme-to-enzyme ratios are low as achieving higher ratios would require a prohibitively long RNA transcript.

One final opportunity for engineering a control system for protein expression level lies in responding to the products of each enzyme's hydrolysis. This system would be much more complicated than the others mentioned here as it would require discovery or development of signal proteins responsive to specific cellooligomers and a cellulolytic system in which each cellulase has a product profile unique enough to distinguish it from the others. Additionally, this approach would require a cellodextrin metabolism such as the one developed in this dissertation in which glucose is generated intracellularly as extracellular depolymerization of cellodextrin will remove the capacity for sensing. If all above conditions are met, expression of each protein could be under control of a promoter that responds positively to the products of a different protein in the system. A transient increase in one enzyme's products caused by an increase in abundance of that protein will induce a signal to increase the expression of other enzymes to maintain the desired balance. Furthermore, all of the approaches and techniques mentioned above can potentially be combined to create a more sensitive or dynamically responsive system to improve control of relative expression levels.

The suggestions mentioned here have the potential to improve, in the short term, the system for consolidated cellulose bioprocessing developed and presented in this dissertation. Additionally we have offered results as well as suggestions that will allow for improvement of any CBP systems that may be of interest in the future.

6.4 References

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