ENGINEERING A CELLULOLYTIC *ESCHERICHIA COLI* TOWARDS CONSOLIDATED BIOPROCESSING

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ENGINEERING A CELLULOLYTIC *ESCHERICHIA COLI* TOWARDS CONSOLIDATED BIOPROCESSING

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette transporter
Amp^R	Ampicillin resistant
ATP	Adenosine triphosphate
Cat^R	Chloramphenicol resistant
CBP	Consolidated bioprocessing
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic acid
DP	Degree of polymerization
DTT	Dithiothreitol
E.coli	*Escherichia coli*
ED	Entner–Doudoroff pathway
EDTA	Ethylenediaminetetraacetic acid
EMP	Embden-Meyerhof pathway
FRT	Flippase recognition site
G1	Glucose
G2	Cellobiose
G3	Cellotriose
G4	Cellotetraose
G5	Cellopentaose
GO	Glucose oxidase assay
HPAEC	High-performance anion-exchange chromatography

IPTG	Isopropyl β-D-1-thiogalactopyranoside
Km^R	Kanamycin resistant
MES	2-(*N*-morpholino)ethanesulfonic acid
MFS	Major facilitator superfamily
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced form of NADP
OD	Optical density
PCR	Polymerase chain reaction
Pi	Inorganic phosphate ion
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
PNPG	p-nitrophenyl- β-D-glucopyranoside
PTS	Phosphotransferase system
SDE	*Saccharophagus degradans*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSCF	Simultaneous saccharification and co-fermentation
SSF	Simultaneous saccharification and fermentation

LIST OF GENE AND ENYME NOMENCLATURE

Bgl	Operon for uptake of aryl-β-glucosides
CbP, Cep94A	*Cellobiose phosphorylase*
CdP. Cep94B	*Cellodextrin phosphorylase*
Cel	Operon for uptake of cellobiose, salicin & arbutin
LacY	*Lactose permease*

SUMMARY

The current energy crisis is exponentially growing and widening the chasm between demand and supply. Biofuels such as ethanol not only provide greener alternatives to fossil fuels but also have been shown to reduce emissions from vehicles, improving air and water quality. Biofuel production from sources such as cellulose is believed to be more sustainable due to its low cost, vast availability in nature and sources such as agricultural and industrial plant waste can be put to good use. However, the main obstacle is the absence of a low-cost technology for overcoming its recalcitrance. To overcome this, a concept called Consolidated Bioprocessing (CBP) has been put forward which proposes to integrate the production of saccharolytic enzymes, hydrolysis of the carbohydrate components to sugar molecules, and the fermentation of hexose and pentose sugars to biofuels into a single process. This process promises to lower the cost and improve the efficiency towards product formation. However, CBP demands adequate cellulase production in order to hydrolyze cellulose into utilizable sugars to maintain cell growth and the production of required enzymes and desired biofuels. In the recent past, biotechnological tools such as metabolic engineering have enabled the production of a large array of biocatalysts that are capable of converting substrates such as glucose into desirable compounds through recombinant cellulolytic strategies. This thesis work aims at engineering a bacterial strain for efficient conversion of cellodextrin to biofuels.

Upon hydrolysis of cellulose by enzymes such as cellulases, a mixture of cellodextrins is produced, which is required to be efficiently broken down to simple sugar molecules like glucose and finally to bioethanol. The three main objectives of this thesis are,

1) Engineer an *Escherichia coli* strain capable of uptaking cellodextrin molecules using energetically efficient transport systems.

2) Assimilate the transported cellodextrin molecules efficiently and convert them to produce bioethanol.

3) Investigate cellobiose utilization in *Escherichia coli*

In the first step, we successfully engineered an *E.coli* strain that is able to uptake cellodextrin molecules using energetically efficient transport (MFS transporter) systems. The intracellular transport of cellodextrin molecules enabled efficient hydrolysis to simpler sugars such as glucose through downstream recombinant enzymes like phosphorylases. The simple sugars were utilized by cells for growth and biofuel production. For this, two transporters belonging to the Major facilitator family (MFS) were identified from a cellulolytic microorganism. These transporters were cloned and expressed in recombinant *E.coli* and tested for cellodexrin uptake. When a transport system uptakes cellodextrin molecules, only one ATP is invested for the uptake of each oligomer containing multiple glucose molecules. This compares to a transport system that requires one ATP (or equivalent) for each glucose molecule transported. The energy saving in the transport is expected to be more significant when longer cellodextrin is transported. By spending only 1 ATP per cellodextrin molecule (equal to 2-7 glucose molecules) compared to 1 ATP per glucose molecule, this is considered to be an energetically more favorable method, which saves cellular energy that could be dispensed for other purposes such as cell growth and enzyme formation.

In the second step, the cellodextrin molecules that are transported into the *E.coli* cells are utilized efficiently and converted to glucose. To create a favorable condition for the cells, an efficient

cellodextrin assimilation pathway was engineered inside the cell once they have been transported. Cellodextrin molecules can be cleaved into two ways – Hydrolytic and phosphorolytic cleavage. Hydrolytic cleavage of cellodextrin requires an ATP molecule to phosphorylate before entering glycolysis. In comparison, a phosphorolytic cleavage of cellodextrins requires only an inorganic phosphate molecule to yield glucose-1-phosphate directly from higher cellodextrin oligomers. This method does not utilize ATP molecules unlike a hydrolysis reaction that also yields glucose. Therefore, it is bio-energetically more favorable to employ phosphorolytic cleavage of the transported cellodextrin oligomers. Accordingly, the cellodextrin molecules are therefore intracellularly broken down to glucose and glucose-1-phosphate. Glucose-1-phosphate is converted to glucose-6-phosphate by an endogenous enzyme phosphoglucomutase before entering glycolysis. Two phosphorylase enzymes, cellodextrin and cellobiose phosphorylase were successfully identified from *S.degradans,* cloned, expressed and characterized in *E.coli*.

The third section includes study of cellobiose utilization in *Escherichia coli*. In some cellulolytic organisms, cellobiose is the main product produced by cellobiohydrolase acting on longer cello-oligosaccharides. Enzymes such as β-glucosidases and cellobiose phosphorylase (intracellular) hydrolyse and phosphorylate cellobiose to glucose molecules. During extracellular degradation of cellulose in the bioethanol industry, the formation of glucose poses a risk of contamination in the medium. It has been shown that ethanol production yield was improved through intracellular uptake of cellobiose and hydrolysis leading to co-consumption of cellobiose and xylose. Therefore, understanding cellobiose utilization becomes utmost importance towards the efficient consumption of other sugars apart from glucose such as xylose and

ethanol production. The roles of cryptic gene operons *cel*, *bgl* and lactose permease gene in cellobiose uptake are investigated through respective gene mutations in the *E.coli* genome.

CHAPTER 1

INTRODUCTION

1.1 Background

1.1.1 Biofuel production from lignocellulosic biomass

High global energy demands, unstable and expensive petroleum sources and concern over global climate changes have led to the development of renewable clean energy that can displace fossil fuels[1]. Potential reductions in fossil fuel consumptions will result from in a multifaceted approach that includes nuclear, solar, hydrogen, wind and particularly biofuels, which many countries have already initiated extensive research and development programs in[2]. At present, the biofuel industry primarily produces bioethanol from cornstarch or cane sugar and biodiesel from vegetable oils and animal fats. However, these fuel resources are in competition with the food industry[3]. Resources such as starch from food crops are associated with certain political problems such as inadequate food supplies and other ethical issues[4]. Second generation of biofuels produced from lignocellulosic materials provides an alternative to the food-dependent biofuels[5].

Due to the continued growth in food prices, search for renewable resources to displace substantial amounts of nonrenewable fossil fuels rests on large amounts of low-cost biomass such as lignocelluloses[6, 7]. Lignocellulosic biomass, a promising alternative feedstock, is one of the most abundant renewable feedstock on the planet. More importantly, lignocellulose does not have food value and can grow on non-agricultural land. Although there are economic and environmental benefits of producing biofuels from lignocelluloses, currently, it is not as cost effective as compared to fossil fuels. The recalcitrance and complexity of biomass increase the

cost of production of hydrolyzed sugars. The molecular and polymeric structures make the resource highly resistant to chemical attack and solubilisation[1].

Consequently, a pretreatment process is used to open up cellulose structure and increase its surface area. Various methods such as acidic, alkaline and organic pulping have been considered[8]. This is followed by an enzymatic hydrolysis step in which a mixture of endo-cellulase and exo-cellulases are required to efficiently break down the cellulosic material to sugar monomers. Fermentation of these monosaccharides produces bioethanol. In the last decade or so, there has been a number of research works dealing with combining the various steps from lignocelluloses biomass to biofuel production to improve the overall yield and efficiency. Challenges such as overall cost control are still an issue. Therefore, several shortcomings of the use of lignocellulosic biomass have led to extensive development of bio-ethanol production.

1.1.2 Consolidated bioprocessing

There have been a number of options proposed to reduce the costs of the conversion of lignocellulose to ethanol including simultaneous saccharification and fermentation (SSF), which is combining the enzymatic hydrolysis of pretreated lignocellulose and fermentation in one step. In this process, the glucose produced by the hydrolyzing enzymes is consumed immediately by the fermenting microorganism present in culture. Another mode of operation is simultaneous saccharification and co-fermentation (SSCF), in which co-fermentation refers to the fermentation of both five-carbon and six-carbon sugars to ethanol. The hydrolyzed hemicellulose during pretreatment and the solid cellulose are not separated after pretreatment, allowing the hemicellulose sugars to be converted to ethanol together with SSF of the cellulose[9]. Consolidated Bioprocessing (CBP) has been proposed to integrate cellulase production, cellulose

hydrolysis, and product fermentation into one single step mediated by a single microorganism. Compared to other less integrated configurations, CBP is distinctive because it does not contain a separate and dedicated process step for cellulase production. This factor alone helps lower the cost of converting lignocellulose to ethanol[10].

Development of CBP microorganisms has been a key challenge ever since the concept of CBP was proposed in 1996. No such microorganism has been identified that singularly combines all these features (Cellulase production, cellulose hydrolysis and product fermentation). Such microorganisms could be developed by two strategies. The native cellulolytic strategy involves engineering naturally cellulolytic microorganisms to be ethanologenic. The recombinant cellulolytic strategy involves introducing heterologous cellulase genes into an organism whose product yield and tolerance credentials are well established[11]. Each strategy involves considerable uncertainty, and different strategies could prove advantageous for different products. The recombinant strategy is followed in this thesis work.

1.1.3 Recombinant strategy

Non-cellulolytic microorganisms with desired product formation properties are starting points for CBP organism development by the recombinant cellulolytic strategy. The primary objective of such developments is to engineer a heterologous cellulase system that enables growth and fermentation on pretreated lignocellulose. Table 1.1 lists characteristics of microorganism candidates popularly used for this strategy[12].

Table 1.1 Characteristics of microorganism candidates for recombinant CBP strategy

Characteristics	Yeast (*S. cerevisiae*)	Bacteria (*Z. mobilis*)
Cellulase production	Barely detectible activity for some enzymes from cloned genes	Unknown
Ethanol production	Up to 160 g/L of ethanol	Up to 130 g/L ethanol
Ethanol tolerance	Very high	High
Multi-sugar usage in native strains	No	No
Resistance to inhibitors in biomass hydrolysates	High	High
Amenability to genetic manipulation	Excellent	Good
Commercial acceptance	Very high	Acceptable

Although a large number of cellulose utilization microorganisms have been discovered from nature, attempts in creating recombinant cellulolytic microorganisms have not been successful because recombinant cellulolytic microorganisms must be capable of (i) overexpressing high level recombinant active cellulase, (ii) secreting or displaying most active cellulases outside the (outer) membrane so that the cellulase can hydrolyze insoluble cellulose, (iii) producing several cellulase components, (iv) regulating the expression of cellulase components for a maximal synergy for hydrolysis, (v) producing sufficient ATP for cellulase synthesis, and (vi) having the right sugar transport systems for soluble cellulolytic products. Recombinant cellulolytic microorganisms are not yet available because they cannot grow on cellulose by utilizing their own cellulase without the help of organic nutrients such as yeast extract, peptone, or amino acids[11].

The ability of *Escherichia coli* to utilize both hexoses and pentoses make this microbe an interesting host for biofuel production from lignocellulosic biomass[13]. *E.coli* has already been used for various other industrial processes for recombinant protein production. Through the expression of pyruvate decarboxylase and alcohol dehydrogenase from *zymomonas mobilis*, the engineering of *E.coli* was the first application of metabolic engineering for biofuel production[14]. *E.coli* has also been used for production of various other biofuels such as n-butanol and n-propanol[15].

1.2 Motivation and objectives

Consolidated bioprocessing is expected to enable significant reduction in production costs and improve process efficiency[11]. Enzyme-microbe synergy can be exploited to achieve maximum level of hydrolysis of substrate through eliminating product inhibition. CBP requires adequate production of cellulase enzymes in order to hydrolyze cellulose into sugars. Although there exists naturally occurring cellulolytic microbes such as *Clostridium thermocellum*, they are not capable of producing products of high yield. Through the technique of metabolic engineering, there have been vast developments in this field. Since recombinant systems are non-cellulolytic, the process demands efficient pretreatment methods and high enzyme concentrations that calls for an overall expensive process compared to the petroleum industry. Therefore, the aim of this project is to engineer a bacterial strain for efficient conversion of cellodextrin to biofuels. Upon hydrolysis of cellulose by enzymes such as cellulases, a mixture of cellodextrins are produced which is required to efficiently be broken down to simple sugar molecules like glucose and finally to bioethanol. This could be achieved through a two-step process.

In the first step, we will engineer an *E.coli* strain to uptake cellodextrin molecules using energetically efficient transport (MFS transporter) systems. The intracellular transport of cellodextrin molecules will enable efficient hydrolysis to simpler sugars such as glucose through downstream recombinant enzymes like phosphorylases. The simple sugars will be utilized by cells for growth and biofuel production. For this, two transporters belonging to the Major facilitator family (MFS) were identified from a cellulolytic microorganism. These transporters were cloned and expressed in recombinant *E.coli* and tested for cellodexrin uptake. When a transport system uptakes cellodextrin molecules, only one ATP is invested for the uptake of each oligomer containing multiple glucose molecules. This compares to a transport system that requires one ATP (or equivalent) for each glucose molecule transported. The energy saving in the transport is expected to be more significant when longer cellodextrin is transported. By spending only 1 ATP per cellodextrin molecule (equal to 2-7 glucose molecules) compared to 1 ATP per glucose molecule, this is considered to be an energetically more favorable method, which saves cellular energy that could be dispensed for other purposes such as cell growth and enzyme formation.

In the second step, the cellodextrin molecules that are transported into the *E.coli* cells are utilized efficiently and converted to glucose. To create a favorable condition for the cells, an efficient cellodextrin assimilation pathway will be engineered inside the cell once they have been transported. Cellodextrin molecules can be cleaved into two ways – Hydrolytic and phosphorolytic cleavage. Hydrolytic cleavage of cellodextrin requires an ATP molecule to phosphorylate before entering glycolysis. In comparison, a phosphorolytic cleavage of cellodextrins requires only an inorganic phosphate molecule to yield glucose-1-phosphate directly from higher cellodextrin oligomers. This method does not utilize ATP molecules unlike

a hydrolysis reaction that also yields glucose. Therefore, it is bio-energetically more favorable to employ phosphorolytic cleavage of the transported cellodextrin oligomers in order to save on ATP molecules that can be utilized by cells for growth and enzyme synthesis. The cellodextrin molecules are therefore intracellularly broken down to glucose and glucose-1-phosphate. Glucose-1-phosphate is converted to glucose-6-phosphate by an endogenous enzyme phosphoglucomutase and therefore enters glycolysis directly.

1.3 Scope of thesis

The overall objective is to develop cellulolytic *Escherichia coli* capable of directly fermenting cellulose into biofuels, providing microbial biocatalysts for consolidated bioprocessing. The engineering strategy emphasizes on energetic favorability of designed strains for biofuel production. The work described in this thesis fulfills the following objective: Developing *Escherichia coli* to successfully metabolize cellodextrin oligomers through intracellular transport by employing recombinant MFS and ABC transport systems, reducing cellodextrins to simple sugars through energetically favorable phosphorylation reactions and finally produce bioethanol. In combination, these energy saving strategies ensure that cells grow on the cellodextins have enough cellular energy to produce recombinant enzymes and bioethanol. Specifically, chapter two includes cloning and screening of two MFS cellodextrin transporters from a cellulolytic bacterium. Chapter three includes the expression and characterization of two enzymes, cellodextrin and cellobiose phosphorylase from *S.degradans*. Chapter four includes the study of the role played by two cellobiose transport operons (Cel, Bgl) and lactose permease (LacY) in cellobiose utilization in *Escherichia coli.* The results in this thesis work is part of a larger goal that would enable the consolidated bioprocessing of biomass that would lead to less dependence

on expensive cellulases and complete hydrolysis of cellulose and cellodextrins. This work addresses the cause of cost effectiveness in biofuel production and delivers a large benefit of cost advantage.

CHAPTER 2

IDENTIFICATION OF CELLODEXTRIN TRANSPORTERS FROM A *CELLULOLYTIC BACTERIUM*

2.1 Introduction

This chapter investigates engineering an *E.coli* strain that will be able to uptake and assimilate cellodextrin molecules using energetically efficient transport (MFS transporter) system. The intracellular transport of cellodextrin molecules would enable efficient hydrolysis to simpler sugars such as glucose through downstream recombinant enzymes like phosphorylases. The simple sugars are utilized by cells for growth and biofuel production. For this, transporters such as Major facilitator family (MFS) that can efficiently transport cellodextrin mixtures into the cells have to be identified from cellulolytic micro-organisms, cloned and expressed in recombinant *E.coli* and establish an efficient method of cellodextrin utilization[16].

The bioethanol industry uses bacteria such as *Escherichia coli* to ferment sugars derived from cellulose. Plant cell walls and other industrial wastes provide with abundant sugar source but are largely unused[17]. At present, the plant biomass largely utilizes cellulase cocktails to produce glucose[1]. *Escherichia coli* cannot breakdown certain cellodextrin products from cellulases and require the presence of higher concentrations of enzymes such as β-glucosidases to improve glucose production[18]. In comparison, certain cellulolytic bacteria show higher transcription levels of certain transporters such as major facilitator superfamily (MFS) sugar transporters. In one study, a deletion of one of the MFS transporters resulted in slow growth on cellulose[19]. Such studies suggest that cellodextrin transport systems are an essential tool required for utilization of cellulose-derived sugars.

Sugar transporters in microbes are usually mediated by permeases, phosphoenolpyruvate-dependent phosphotranferase systems (PTS), ATP-binding cassette (ABC) transport systems, or major facilitator superfamilies[20]. Mono and disaccharides are commonly transported by the PTS systems, whereas ABC and MFS systems are involved in transport of longer cellodextrins. There have been well-characterized cellodextrin transport systems in cellulolytic microbes such as *Clostridium thermocellum* and *Streptomyces reticuli* as ABC transport systems [21, 22]. ABC transporters are known to be highly efficient and broad substrate specificity[23]. An ABC transporter consists of four components, two of which are trans-membrane proteins forming a transport channel. Two other components are inside the cytoplasm and carry the ATP binding site. A binding protein outside cytoplasm provides the substrate specificity of the transport system[22]. Four of the five ABC transporter operons found in *Clostridium thermocellum* are involved in cellodextrin transport. In most cellulolytic organisms, the transported cellodextrins undergo preferably phosphorolytic cleavage. Since there are only 2 ATP molecules consumed per sugar molecules, the microbes save on more ATP molecules as the cellodextrin chain length increases[20]. This energy saving mechanism is employed in this study by expressing such cellodextrin transport systems from a cellulolytic bacterium. The transporter candidates were identified and screened for cellodextrin uptake based upon their locations and proximity towards cellulose binding and glucosidase proteins in the genome. As wild type *E. coli* is unable to use cellodextrin (DP >2), co-expression of a cellodextrin transporter and cellodextrinase allowed cells to grow on cellodextrin. This was used as the basis for screening.

Six MFS and one ABC protein were selected from this bacterial strain. The KO11 Δ*cel* Δ*bgl* strain was used as a host for screening cellodextrin candidates (Chapter 4). In order to breakdown cellodextrins to glucose that would enable cell growth, a cellodextrinase (Ced3A),

was expressed in a high copy number plasmid under a T5 promoter. This form of Ced3A lacks a signal peptide that causes the enzyme to be functional intracellularly.

2.2 Materials and methods

2.2.1 Bacterial strains, plasmids and chemicals:

E.coli KO11 (ATCC#55124) was used as a vehicle for cloning vectors and *E.coli* KO11 Δ*cel*Δ*bgl* (described in chapter 4) was used as a host for cellodextrin utilization experiments (Table 2.1). Plasmids pQE80L (Invitrogen, Carlsbad, CA) and pBBR122 was used for cloning. Luria Broth rich medium and M9 minimal media mix (1X M9 salts, 1%(w/v) cellodextrin mixture, 1ml/l $MgSO_4$, 0.05% yeast extract, 100µl/l $CaCl_2$) (Fisher Scientific, Pittsburg, PA) were used for aerobic cultivations. Antibiotics namely amphicillin (70µg/ml) and kanamycin (50µg/ml) (Fisher Scientific, Pittsburg, PA) were added to cultures of plasmid-bearing *E.coli* strains. Isopropyl β-D-1-thiogalactopyranoside (IPTG)(Zymo Research, Irvine, CA) was used to induce gene expression.

Table 2.1 Strains and plasmids used in this study

Strains	Description	Source
E.coli KO11	W Δfrd *pflB*::pdc_{Zm} $adhB_{Zm}$ $ldhA^{+}$ *cat*	ATCC
E.coli KO11Δ*cel*Δ*bgl*	*E.coli* KO11 strain, *cel* & *bgl* operons deleted	This study

Plasmids	Description	Source
pBBQH	pBBR122 vector with T5 promoter	This study
pBBR122	Broad-host-range vector	MoBiTec
pQE80L-Ced3A	Plasmid under T5 promoter and cellodextrinase gene inserted	This study

2.2.2 Cloning of Cellodextrin transport candidates from cellulolytic bacterium:

The candidate genes were designated as candidate genes 1-7. Primers were designed to amplify the candidate gene/operons for cellodextrin utilization from the cellulolytic bacterial genome via PCR. The amplified gene fragments were ligated into a linearized pBBQH vector, which was used to transform into *E. coli* KO11. pBBQH is a modified form of pBBR122 vector with the T5 promoter. The transporter candidates were expressed under a T5 promoter induced by isopropyl β-D-1-thiogalactopyranoside (IPTG).

2.2.3 Screening of transporter candidates for cellodextrin uptake:

To screen the transporter candidates for cellodexrin uptake, the recombinant cellodextrin transporter plasmids and pQE80L-*ced3A*, plasmid expressing a cellodextrinase, were transformed into KO11 Δ*cel*Δ*bgl* mutant strain. The recombinant strains were grown in M9 minimal media mix with 1% cellodextrin mixture as carbon source and cultivated aerobically

with an initial OD_{600} = 0.01 at 37°C and 250 rpm for 5 days. The strains with empty plasmids, pQE80L and pBBQH and one just expressing cellodextrinase Ced3A were used as controls. The cell growth was measured using a UV-Spectrophotometer and the total residual sugar was measured using a high-performance anion-exchange chromatography (HPAEC) using a Dionex system. The total sugars were calculated by the sum of G1 (Glucose) through G7 cellodextrin oligomers from the dionex analysis.

2.2.4 Growth profile and sugar characteristics of selected cellodextrin transporter candidates:

To study the growth and sugar characteristics of the selected cellodextrin transport candidates, the cell growth and sugar concentrations of strains expressing the transporters were monitored in the presence of cellodextrin mixture. The recombinant strains were grown in M9 minimal media mix with 1% cellodextrin mixture as carbon source and cultivated aerobically with an initial OD_{600} = 0.01 at 37°C and 250 rpm for 7 days. The strains with empty plasmids, pQE80L and pBBQH and one just expressing cellodextrinase Ced3A were used as controls. The cell growth was measured using a UV-Spectrophotometer and individual cellodextrin oligomer concentration was measured using a high-performance anion-exchange chromatography (HPAEC) using a Dionex system. The total sugar concentration was calculated by the sum of G1 through G7 cellodextrin oligomers.

2.3 Results

2.3.1 Cloning and screening of transporter candidates for cellodextrin uptake:

The genes encoding for the cellodextrin transport candidates were identified through RT-PCR studies (Data not shown). These genes were PCR amplified and inserted into the respective restriction sites of the pBBQH vector under a T5 promoter. To screen for cellodextrin uptake, the cloned recombinant plasmids were expressed along with a cellodextrinase Ced3A and cultivated in M9 minimal media mix in the presence of 1% cellodextrin mixture as the carbon source for 5 days. For this evaluation, a cellodextrin sample was prepared from cellulose following a protocol by Zhang[24]. In order to evaluate the residual cellodextrin concentration, samples were taken at the end of four and five days and analyzed by high performance anion-exchange chromatography (HPAEC). As shown in Table 2.2, the aerobic cultivation resulted in negligible cell growth and utilization of cellodextrins (1-3%) in the control strains. Similarly, candidates gene 1, 2, 5 and 6 also showed negligible cell growth and cellodextrin uptake. However, the expression of MFS candidates gene 3 resulted in cell growth of OD_{600} = 0.986 and 47.8% cellodextrin utilization after 5 days, and gene 4 resulted in cell growth of OD_{600} = 1.532 and 62.7% cellodextrin utilization. These results provide experimental evidence that the candidates' gene 3 and 4 effectively transport cellodextrin oligomers into the cell. To further investigate the cellodextrin transport capabilities of these candidates, growth and sugar profiles were studied in the presence of 1% cellodextrin mixture.

Table 2.2 Cell growth and cellodextrin utilization by *Escherichia coli* KO11 Δ*cel* Δ*bgl*

Strains	4 days		5 days	
	A_{600}	ΔCellodextrin (%)	A_{600}	ΔCellodextrin(%)
pQE80l+pBBR122	0.31	1.2	0.167	3.4
Ced3A	0.27	3.1	0.121	3.2
Ced3A + gene 5	0.28	3.8	0.136	4.5
Ced3A + gene 1	0.11	2.1	0.106	3.4
Ced3A + gene 2	0.11	2.6	0.117	4.4
Ced3A + gene 3	1.16	31.5	0.986	47.8
Ced3A + gene 4	1.99	43.5	1.532	62.7
Ced3A + gene 6	0.6	21	0.8	21

2.3.2 Growth and sugar characteristics of cellodextrin transporter candidates:

Following initial screening of transport candidates for cellodextrin uptake, genes 3 and 4 were selected for further study based upon cell growth and cellodextrin utilization. The growth and sugar characteristics were analyzed in the presence of cellodextrin oligomers. The recombinant strains (transporter candidates + cellodextrinase, Ced3A) were cultivated in the presence of 1% cellodextrin mixture for a period of 7 days and sugar concentrations were analyzed every 24 hours by high performance anion-exchange chromatography (HPAEC). Total sugar concentration was calculated as the sum of G1-G7 compounds through HPAEC. As shown in fig 2.1, the expression of genes 3 and 4 resulted in significant increase in cell growth (1.5 and 1.3 respectively) and sugar consumption (93% and 86% respectively) after 7 days. The MFS candidates also showed high initial growth rate and sugar consumption up to 3-4 days whereas the controls with empty vectors and the one only expressing cellodextrinase (Ced3A) showed no

indication of cell growth and the sugar profile remained constant. The transporters were cloned under a T5 promoter induced by IPTG.

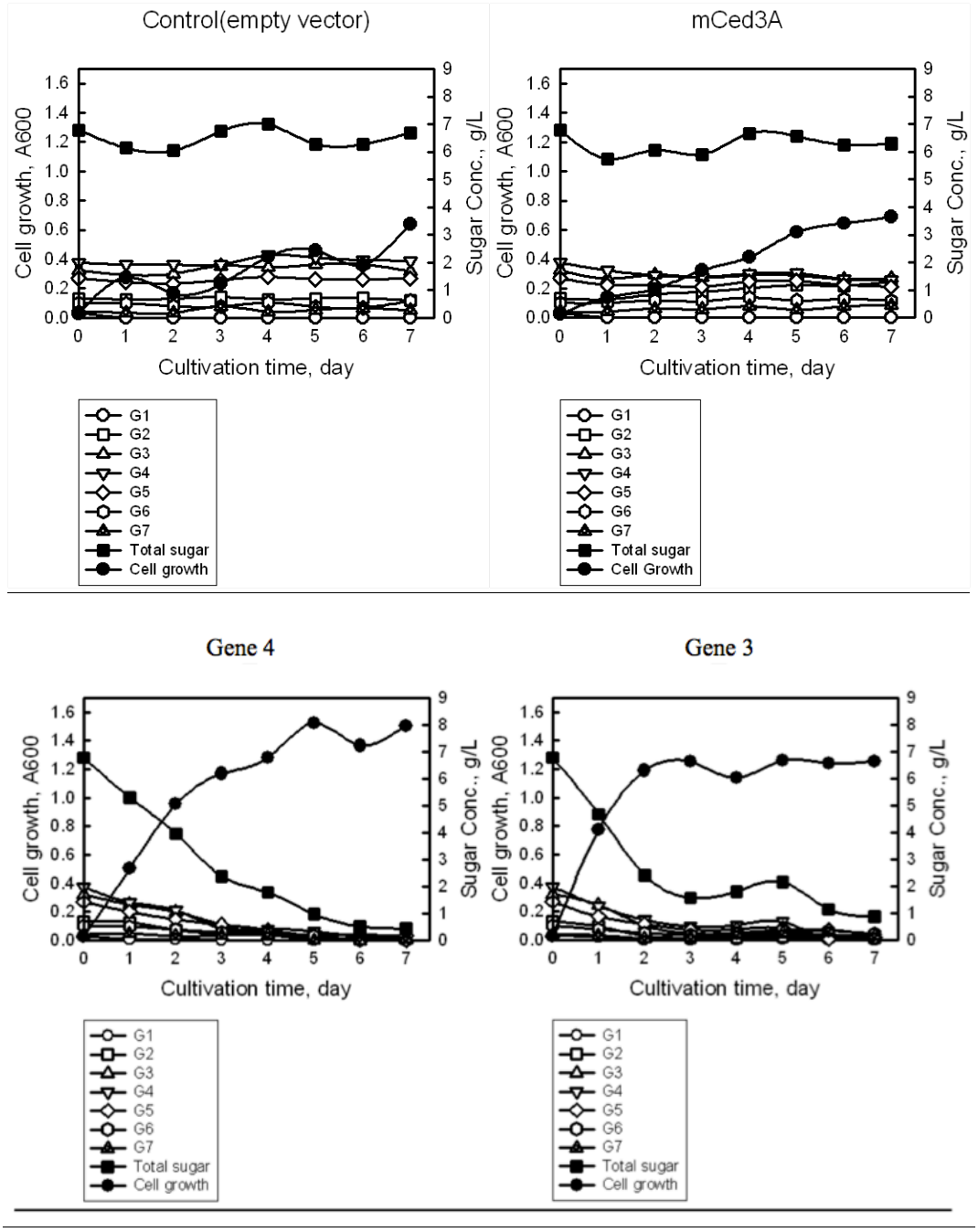

Figure 2.1 Growth and sugar characteristics of selected cellodextrin transporter candidates

2.4 Discussion:

In this study, we screened seven candidates and identified two Major facilitator family cellodextrin transporter genes 3 and 4 from a cellulolytic bacterium. We subsequently studied the growth and sugar consumption profiles of the selected candidates in the presence of a cellodextrin mixture. In order to screen the candidates, KO11 Δ*cel* Δ*bgl* strain was used as a host harboring an intracellularly functional cellodextrinase (Ced3A), which hydrolyzed the transported cellodextrins to glucose in turn utilized for cell growth. The data presented here showed that the two selected candidates, gene 3 and 4 effectively transport cellodextrin oligomers through the cell membrane of *E.coli*. Since the permease proteins are not required in high concentration in *E.coli*, an optimum of 0.2mM IPTG was used to induce expression of the permease proteins. Specifically, candidate gene 4 caused slow initial cell growth and sugar utilization whereas candidate gene 3 enabled high initial cell growth and cellodextrin transport. This result suggests that candidate gene 3 may have higher substrate specificity towards long chain oligomers in comparison to candidate gene 4. However, candidate gene 4 caused an overall higher cell growth and cellodextrin utilization after 7 day cultivation indicating that this permease shows better activity towards smaller cellodextrin molecules that form over time during cultivation due to natural degradation. Further characterization of these MFS permease proteins for their substrate specificity towards pure cellodextrins such as cellobiose, cellotriose and cellotetraose would provide deeper insight about their unique functions. However, this finding is significant since this is the first case that a cellodextrin transporter has been identified from this cellulolytic bacterium.

Biofuel production by consolidated bioprocessing (CBP) from cellulose demands highly efficient systems to ferment cellodextrin oligomers and reduce the extracellular accumulation of glucose and cellobiose. Since efficient hosts for biofuel production such as *E.coli* lack such machineries, identification of heterologous cellodextrin transporters become particularly useful during recombinant CBP strategies. The transport of longer chain oligomers would be bio-energetically highly advantageous during such a process that might lead to efficient biofuel production. The functional confirmation of two MFS permeases provides the basis for future studies aimed at characterization of these systems. Such cellodextrin transport systems in bioethanol producing strains would overcome a major bottleneck during cellulose degradation and fermentation and would make cellulosic ethanol economically viable[19].

CHAPTER 3

CHARACTERIZATION OF PHOSPHORYLASE ENZYMES FROM *SACCHAROPHAGUS DEGRADANS*

3.1 Introduction

In order to assimilate cellodextrin molecules by *Escherichia coli*, an energetically favorable method is needed to hydrolyze cellodextrins to glucose and subsequently to bioethanol. Once the cellodextrin molecules are transported inside the *E.coli* cells, a cleavage method is followed in which the glucose oligomers are phosphorolytically split rather than hydrolyzed, producing glucose-1-phosphate utilizing inorganic phosphate rather than ATP molecules. In this view, two enzymes cellodextrin and cellobiose phosphorylase are required. They have been identified from *Saccharophagus degradans*, a marine bacterium.

Saccharophagus degradans has been identified as a polysaccharide degrading bacterium. It is a pleomorphic, gram-negative, aerobic bacterium first isolated from salt marshes[25]. This organism has been show to degrade up to 10 different complex polysaccharides[26]. From the genome analysis of *S. degradans,* there are 12 β-1, 4-endoglucanases and one cellobiohydrolase. The seven cellulose accessory enzymes act on cellulose oligosaccharides and include two cellodextrinases, three cellobiases, a cellodextrin and cellobiose phosphorylase[27]. Cellobiose phosphorylase (CbP) and cellodextrin phosphorylase (CdP) are enzymes involved in cellulose degradation. Microbes in which at least one such phosphorylase is found include *Prevotella ruminicola*[28]*, Ruminococcus albus[29], Thermotoga maritima*[30], *Clostridium thermocellum*[31], *Cellovibrio gilvus*[32] and *Clostridium stercorarium*[33]. CdP catalyzes

phosphorolytic cleavage of a β-glucosidic bond within cellodextrin, yielding glucose-1-phosphate and reducing cellodextrin chain by one glucose unit (Eqn. 1), where P_i denotes inorganic phosphate.

$$(\text{Cellodextrin})_n + P_i \longrightarrow (\text{Cellodextrin})_{n-1} + \text{glucose-1-P} \qquad \text{Eqn.1}$$

The same CdP enzyme catalyzes the further reduction of cellodextrin chain until DP of cellodextrin is reduced to 2. Interestingly, cellobiose is not a substrate for any CdP characterized so far [31, 33] and its degradation requires cellobiose phosphorylase, an enzyme specific to cellobiose (Eqn.2) .

$$\text{Cellobiose} + P_i \longrightarrow \text{glucose} + \text{glucose-1-P} \qquad \text{Eqn.2}$$

Glucose-1-P from a phosphorolytic reaction is converted by an endogenous phosphoglucomutase to Glucose-6-P, which enters the glycolysis (such as the EMP pathway). Compared to hydrolysis, where cellodextrin is hydrolyzed into glucose, requiring ATP to phosphorylate before entering glycolysis, phosphorolytic cleavage yields glucose-1-P directly without an input of ATP, requiring only phosphate anion to phosphorylate all glucose units in cellodextrin except the last one (Eqn.2). Thus phosphorolytic cleavage of cellodextrin is energy-saving relative to the hydrolytic route. The number of ATP saved increases with the increase of DP of cellodextrins. For example, metabolizing cellotetraose through phosphorolytic mechanism would save three ATPs, compared to hydrolysis. Due to their distinct substrate specificity, cellulose metabolism through phosphorolytic mechanism requires two phosphorylases. Indeed, genomes of cellulolytic bacteria, such as *Clostridium thermocellum*, encode two phosphorylases, one for cellodextrin and one for cellobiose [34, 35].

While using phosphorolytic mechanism gives a clear advantage to anaerobic microbes in cellulose assimilation, the need to employ such a strategy in an aerobic microbe is less

compelling. Yet the genome of an aerobic cellulolytic marine bacterium, *Saccharophagus degradans,* encode two putative phosphorylases, annotated as cellobiose phosphorylase (CEP94A) and cellodextrin phosphorylase (CEP94B), respectively [33], suggesting that phosphorolytic cleavage of cellodextrin may have a role in cellulose metabolism in this microorganism even under aerobic conditions. Cloning and expression of the two phosphorylase genes using an *E. coli* expression system were successfully carried out. Furthermore, subsequent characterizations reveal novel substrate specificity for the cellodextrin phosphorylase (CEP94B).

3.2 Materials and methods:

3.2.1 Strains and materials:

The genomic DNA of *Saccharophagus degradans* ATCC 43961 was isolated using a bacterial genomic DNA extraction kit from ZymoResearch (Irvine, CA, USA) after growth of the strain in Marine broth 2216 (Becton Dickinson & Co, NJ, USA). *Escherichia coli* JM109 ATCC 53323 was used as host strain in cloning and expression experiments. Glucose (Fisher Scientific, MA, USA), Glucose-1-phosphate, Cellobiose, Cellotriose, Cellotetraose and Cellopentaose (Sigma-Aldrich, MO, USA) were substrates used for enzymatic assays. Cellodextrin mixture was prepared according to Zhang [24] and used for substrate specificity experiments[36].

3.2.2 Cloning and expression:

Primers Cep94A-F (5’ – CTCGCA*GGATCC*ATGAAATTTGGGCACTTTGACGACAA – 3’, *BamH*I site), Cep94A-R (5’ – CGATGC*CTGCAG*TTAGCCCAATGTAACTTCTACGTTAC – 3’, *Pst*I site), Cep94B-F (5’ – CTCGCA*GGATCC*ATGTTAAAAGCCATTAACAACGGCGA – 3’, *BamH*I site) and Cep94B-R (5’ – CGATGC*GTCGAC*TTAGTGTGTGGCAGGTAATAGAACG – 3’, SalI site) were used to

amplify the full genes CEP94A and CEP94B from the genome of *S.degradans* via PCR. The amplified gene fragments were ligated into *BamH*I-*Pst*I-linearized pQE80L and *BamH*I-*Sal*I-linearized pQE80L to obtain pQE80L-CEP94A and pQE80L-CEP94B respectively, which were used to transform into *E. coli* JM109. The resultant recombinant strains were cultured in LB media with 100µg/mL ampicillin at 37˚C and induced with 1mM IPTG at 25˚C for 24 hours for the expression of CEP94A and CEP94B. After resuspending the cell pellet in 50 mM sodium phosphate, 0.3 M NaCl buffer, pH 8.0, the cells were sonicated and the soluble fraction was collected using centrifugation. The target protein was purified using immobilized metal (nickel) affinity chromatography (HIS-Select™ Resin, Sigma-Aldrich) by eluting the protein from the resin with 50 mM sodium phosphate, 0.3 M NaCl, 250 mM imidazole buffer, pH 8.0, and dialyzed against 10 mM Tris-HCl, pH 7.5 for removal of imidazole for subsequent assays.

3.2.3 Enzymatic assays:

The phosphorylase activity of recombinant Cep94B was determined by measuring the amount of glucose-1-phosphate generated from cellodextrin and phosphate. The reaction mixture in a total volume of 200µL consisted of 50mM PIPES, 8mM phosphate pH 7.0, 5mM $MgSO_4$, 0.5mM NADP, 4U of phosphoglucomutase, 2U of glucose-6-phosphate dehydrogenase, 250µM pure cellodextrin and 50µL of enzyme solution. The mixture was incubated at 42°C for 10 minutes and heated to 95°C for 5 minutes to stop the reaction. The concentration of NADPH formed was determined by measuring the change in absorbance at 340nm. Glucose-1-phosphate formation was estimated assuming molar equivalence to the NADPH formed[37]. After establishing that cellobiose is a substrate of both enzymes, phosphorylase activity from the purified proteins were

also measured using cellobiose as substrate and liberated glucose was measured enzymatically or by HPAEC.

The phosphorylase activities of recombinant CEP94A and CEP94B were determined by measuring the amount of glucose released from cellobiose and phosphate as substrates. The reaction mixture in a total volume of 200µL consisted of 40mM MES buffer, pH 6.6 with 100mM cellobiose, 50mM phosphate and 50µL of enzyme solution and was incubated at 30°C for 30 minutes. The reaction was stopped by boiling the reaction mixture for 5 minutes and collecting the supernatant via centrifugation. The amount of D-glucose produced was measured by the glucose oxidase-peroxidase method using the Glucose (GO) Assay Kit (Sigma Aldrich, MO, USA). One unit of activity is defined as the amount of enzyme that releases 1µmol glucose per minute of reaction. Potential interference from β-glucosidase activities was ruled out as controls using cell lysate from transformant carrying empty plasmid showed no activity under the conditions used.

For substrate specificity assays of CEP94A and CEP94B enzymes, similar reactions were carried out by incubating the enzymes with cellodextrin mixture prepared according to Zhang[24] at 30°C for up to 18 hours. The residual oligosaccharides were determined through high-performance anion-exchange chromatography (HPAEC) using a Dionex system equipped with an ED50 electrochemical detector (Sunnyvale, CA, USA). Monosaccharides and oligosaccharides were separated on a CarboPac PA-20 column (Dionex, Sunnyvale, CA, USA). Also for CEP94B, substrate specificity with pure cellodextrin G2-G5 was carried out under the same conditions as phosphorylase activity assay except that the reaction mixture was incubated at 30°C for 4 hours. Samples were taken at t=0 and t=4 hours for product identification using high-performance anion-exchange chromatography (HPAEC). Detection was through pulsed

amperometry (waveform; for time [1-15, 17-35, 37-58] = 0.41 s, potential [1-3, 5-15, 17-35, 37-58] = -2.00 V; t = 0.42 s, p = -2.00 V; t = 0.43 s, p = 0.60 V; t = 0.44 s, p = -0.10 V; t = 0.50 s, p = -0.10 V). The mobile phase consisted of degassed solution A containing 100 mM sodium hydroxide and solution B containing 500 mM sodium acetate and 100 mM sodium hydroxide. The mobile phase was pressurized with inert gas (He) to prevent interference of airborne carbon dioxide. A flow rate of 0.5 ml/min was used. The following linear gradient was used: at t = 0 min, 100:0 (solution A/solution B); at t = 30 min, 30:70; at t =35 min, 30:70; at t = 45 min, 100:0; and at t = 55 min, 100:0. The retention times of standard compounds in this study is given in Table 3.1.

Table 3.1 Retention times of standard compounds in this study (HPAEC)

Compound	Retention time (minutes)
Glucose	3.6
Cellobiose	6.2
Cellotriose	9.2
Cellotetraose	11.45
Cellopentaose	12.79

To study the kinetics of forward reactions of proteins CEP94A and CEP94B, similar reactions were carried out with varying concentrations of substrate (0.33-2mM) and the residual oligosaccharide concentrations were determined through HPAEC analysis. Kinetics of reverse reactions of CEP94A and CEP94B were determined by measuring the amount of phosphate released from glucose-1-phosphate. The reaction mixture in a total volume of 200µL consisted of 50µL of enzyme solution, 40mM α-glucose-1-phosphate, 10mM dithiothreitol (DTT) and 50mM Tris-HCL buffer, pH 7.0 with varying concentrations of substrate (0.33-2mM) and incubating the mixture at 37°C for 15 minutes. The reaction was stopped by the addition of 1M acetate buffer.

The amount of phosphate was measured by a phosphate colorimetric assay kit (Biovision, Mountain View, CA, USA). One unit of activity is defined as the amount of enzyme that releases 1μmol phosphate per minute of reaction[35].

3.2.4 Enzyme characterization:

To determine an optimal pH for CEP94A and CEP94B, enzyme activity was measured using cellobiose as a substrate as described above except the reaction was carried out in a pH range of 2.5 – 10.0 using the following buffers: 50mM sodium citrate buffer (pH 2.5-4), 50mM sodium acetate buffer (pH 4.0–5.5), MES buffer 50mM (pH 5-7), 50mM Tris–HCl buffer (pH 7–9.0), and 50mM glycine–NaOH buffer (pH 9.0–10.0). pH stability was determined by measuring the residual cellobiose activity (pH 6.6, 30°C) after incubating the enzyme in an appropriate buffer for 12 hours at 4°C. Optimal temperature was determined by performing the cellobiose activity assay at various temperatures, ranging from 30-65°C. The thermal stability was determined by incubating the enzyme at the designated temperature (30-65°C) for 30 minutes and proceeding to perform the cellobiose activity assay in 40mM MES buffer, pH 6.6, at 30°C.

3.2.5 Analytical methods:

Protein concentration was measured by the method described by Bradford using a protein reagent dye (Bio-Rad, Hercules, CA, USA), with bovine serine albumin as the standard. SDS-PAGE was performed using a Mini-protean TGX electrophoresis kit (Bio-Rad, Hercules, CA, USA) and stained with Bio-safe Comassie Blue (Bio-Rad, Hercules, CA, USA). A 10-KDa precision plus protein standard ladder was used as a standard mass protein marker. Cell density (OD_{600}) was measured on an UV/visible light spectrophotometer (Beckman Coulter, CA, USA)

3.3 Results:

3.3.1 Recombinant expression and purification of CbP and CdP:

The gene encoding CbP was PCR amplified from the *S. degradans* genomic DNA and inserted into the BamHI and PstI sites of a commercial *E. coli* expression vector pQE80L (Qiagen) as described in Materials and Methods. Similarly, the CdP gene was also cloned into the vector except different restriction sites (BamHI and SalI) were used. As a result, both enzymes are expressed as proteins with a His-tag at their respective N-terminus to facilitate purification. As shown in Fig 3.1, both recombinant phosphorylases were highly expressed and appeared on a SDS-PAGE gel as dominant bands corresponding to their expected molecular weight (~90kDa).

One step affinity purification based on the interaction of the N-terminal His-tag and Nickel affinity gel resulted in purified proteins without apparent impurity on a SDS-PAGE (Fig 3.1). The recovery yield was high, 87% for CbP and 66% for CdP (Table 3.2). Under the condition used, from 120 mL culture, about 10.5 mg CbP and 8 mg CdP were obtained in purified form for subsequent studies.

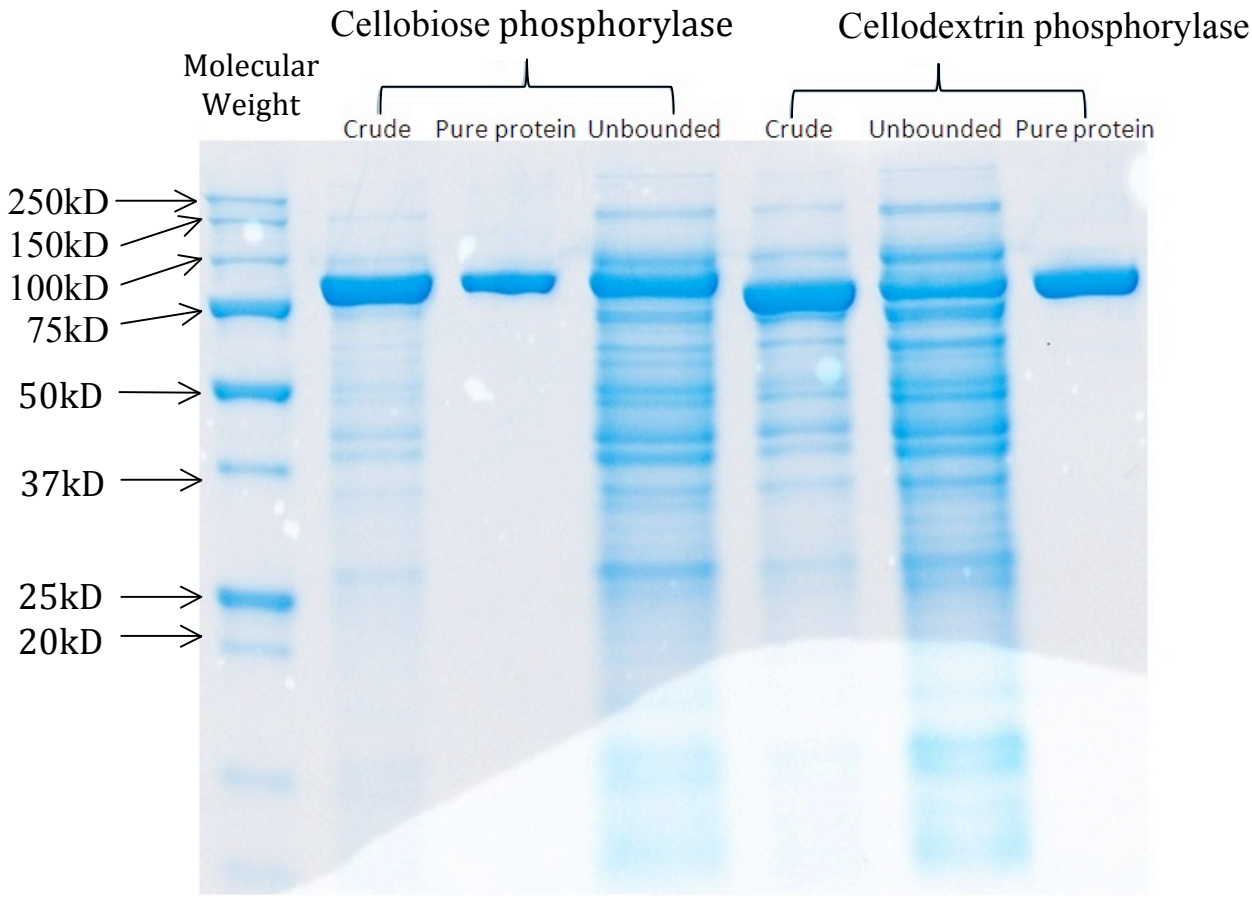

Figure 3.1 SDS-PAGE analysis of cell crude extract, unbound fraction and elute from immobilized metal (nickel) affinity chromatography of CbP and CdP

Table 3.2 Summary for CbP & CdP purification

Protein type	Total Activity (Units)	Total Protein (mg)	Specific activity (U/mg)	Purification fold	%Recovery
Crude extract (CbP)	4.25	33.2	0.13	1	100
Pure CbP	3.7	10.5	0.35	2.75	87
Crude extract (CdP)	0.3	28.45	0.01	1	100
Pure CdP	0.2	8	0.02	2.33	66

3.3.2 Substrate specificity and kinetics of CbP and CdP:

To investigate substrate specificity of CbP and CdP, a cellodextrin sample was prepared from cellulose following a protocol by Zhang[24]. Based on the HPAEC analysis, the mixture contains approximately 3.8% glucose (G1), 8.9% cellobiose (G2), 20% cellotriose (G3), 26% cellotetraose (G4), and 24.5% cellopentaose (G5), with the balance as unidentified due to lack of standards but presumably higher glucose oligomers. In an initial evaluation of substrate specificity, purified CbP and CdP was incubated with cellodextrin (prepared as above) and samples were taken periodically and analyzed by high performance anion-exchange chromatography (HPAEC) for G1 to G5 concentrations. The various oligomers were identified by their respective retention times (Table 3.1) and quantified from appropriate calibration curves. As shown in Fig 3.2a, cellodextrin incubation with purified CbP resulted in a significant decrease (60%) in cellobiose concentration, which accompanied a significant increase (80%) in glucose concentration from its initial value. Meanwhile, no significant change in concentrations of G3, G4, and G5 was observed during the 16 hour reaction. These results provide experimental evidence to support the annotation of the gene encoding a cellobiose phosphorylase and the activity profile conforms to the expectation of a typical cellobiose phosphorylation from previous studies of the enzymes from other microorganisms.

In contrast, incubation of cellodextrin with purified CdP resulted in significant decreases (40-60%) in concentrations of G2-G5 (Fig 3.2b), which was accompanied by an increase of 91% in glucose concentration. While the change of concentrations of G3-G5 is indication of activity of a cellodextrin phosphorylase, the change in G2 or cellobiose concentration was highly unusual as previous studies indicate that these two types of phosphorylase do not cross–react with their

respective substrates (G2 and Gn, where n>2)[33]. Therefore, the results seem to suggest the *S. degradans* CdP is novel in its activity with cellobiose.

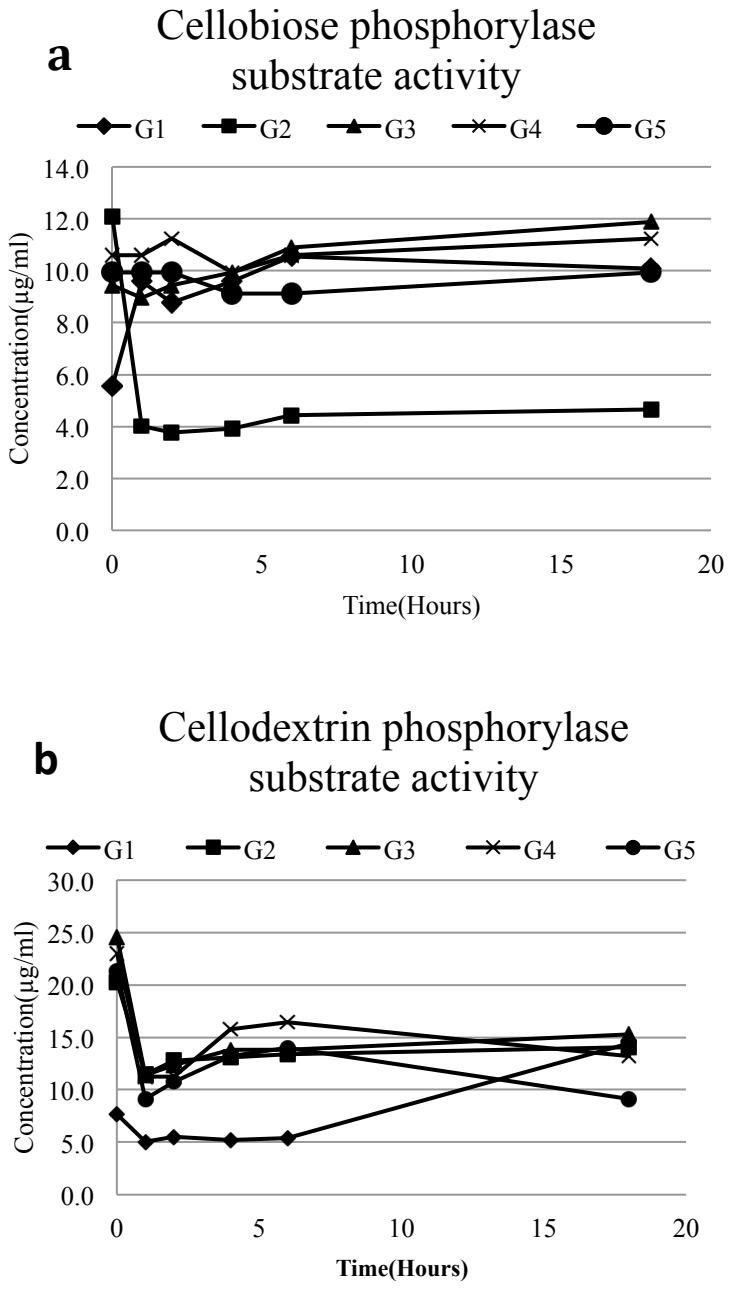

Figure 3.2 Sugar profiles of CbP and CdP enzymes on cellodextrin mixture.

G1-Glucose, G2-Cellobiose, G3-Cellotriose, G4-Cellotetraose, G5-Cellopentaose

To further investigate substrate specificity of CdP, each cellooligosaccharide (G2-G5) was incubated with purified Cep94B in a buffer containing 50 mM inorganic phosphate (P_i). Samples were taken for analysis using high performance anion-exchange chromatography (HPAEC) to identify products by comparing retention times with respective standards. As shown in Fig.3.3a and b, with G2 as substrate, a new glucose peak emerged as reaction preceded, indicating that the enzyme accepts cellobiose as a substrate and liberating glucose as one of the products. Similarly, spectra from reaction with cellotetraose as substrate showed a new peak corresponding to cellotriose at 4 hours (Fig.3.3c and d), indicative of its activity toward cellotetrose. Cellotriose and cellopentaose were tested in the similar manner, new peaks at retention times corresponding to cellobiose and cellotetraose were detected, respectively (data not shown). These results indicate that the enzyme is active for all cellodextrins tested and releases a lower cellodextrin (G_{n-1}) as one of the products. While this result confirms the annotation of Cep94B as a cellodextrin phosphorylase, the detection of glucose from cellobiose as a substrate was highly unusual as previous studies indicate that Cep94B and cellobiose phosphorylase do not cross–react with their respective substrates (G2 and Gn, where n>2) [33, 50, 56]. Alternative analysis was also carried out to corroborate these results. Measurement of G-1-P through an enzymatic assay [37] was carried out for reactions with G2-G5 as substrate. In each case, phosphorylase activity was confirmed whereas controls with lysate from transformant carrying empty plasmid, or with substrate only, showed no indication of phosphorylase activity. Therefore, we can conclude that the recombinant CdP is not only active with expected substrates G3-G5 but it is novel in its activity with cellobiose.

To confirm the preliminary finding and determine the kinetic parameters for the two enzymes, further studies were carried out with pure commercial cello-oligosaccharides at different

concentrations. Respective parameters of Michaelis–Menten kinetics were determined based on lineweaver-burk plots and are shown in Table 3.3&3.4. For the reaction with cellobiose, the Km values for CbP in the phosphorolytic reaction are 6.05 and 0.18 mM for cellobiose and phosphate, respectively. These values are comparable with those reported earlier for CdP[30, 33].

While the cellobiose phosphorylase activity is confirmed for CdP, the kinetic parameters are quite different from those for CbP. As shown in Table 3.3, for reaction with cellobiose, the Km value for phosphate for CdP is 0.57 mM, about 3 fold higher than that for CbP. There are two orders of magnitude differences in Vmax, with CdP much lower. On the other hand, the Km value for cellobiose for CbP is much higher than that for CdP, 6.05 mM vs. 0.32 mM (Table 3.3). Overall, the data presented in Table 3.3&3.4 shows that while CdP exhibits novel activity toward cellobiose, it is a very weak enzyme, compared to CbP, as far as cellobiose phosphorylase activity is concerned.

As cellobiose phosphorylase is also used for synthesis of oligosaccharides, the enzyme was also characterized in the reverse direction using glucose-1-phosphate and glucose as substrates. For CbP, the Km values are 3.73 mM and 1.77 mM for glucose-1-P and glucose, respectively. These values are higher than those for CdP, as observed for the forward reaction. For Vmax, although CbP exhibits higher Vmax than that for CdP as with the forward reaction, but the difference is not as dramatic as in the forward reaction. As a result, the catalytic efficiency is quite similar for both enzymes in the synthesis direction. Cellotriose synthesis activity was observed with CdP only (cellobiose and G-1-P as substrates), but was weak compared to cellobiose synthesis activity (Table 3.4).

Compared to cellobiose, longer cellodextrins are much better substrates for CdP (Table 3.4). The Km values for cellotriose, cellotetraose, and cellopentaose are several fold lower than that for cellobiose, while Vmax are not significantly different. Thus, lower Km values with cellodextrins are largely responsible for the increased catalytic efficiency toward longer cellodextrins. The lowest Km is found with cellotetraose, exhibiting the highest kcat/Km, or specificity constant, although the differences between G4 and either G3 or G5 are rather small (Table 3.4).

Table 3.3 The kinetic parameters for enzymes CbP and CdP with substrates of DP until 2

	Cellobiose phosphorylase				Cellodextrin phosphorylase			
Forward reactions	K_m(mM)	V_{max}(U/mg)	k_{cat}(s^{-1})	k_{cat}/K_m(s^{-1} M^{-1})	K_m(mM)	V_{max}(U/mg)	k_{cat}(s^{-1})	k_{cat}/K_m(s^{-1} M^{-1})
Phosphate	0.176 ± 0.01	8.94 ± 0.22	13.30 ± 0.462	75580 ± 3712	0.565 ±0.08	0.025 ± 0.003	0.037 ± 0.006	65 ± 11
Cellobiose	6.050 ± 0.13	13.55 ± 0.17	20.16 ± 0.357	3330 ± 83	0.324 ± 0.04	0.010 ± 0.002	0.015 ± 0.011	46 ± 19
Reverse reactions	K_m(mM)	V_{max}(U/mg)	k_{cat}(s^{-1})	k_{cat}/K_m(s^{-1} M^{-1})	K_m(mM)	V_{max}(U/mg)	k_{cat}(s^{-1})	k_{cat}/K_m(s^{-1} M^{-1})
Glucose-1-Phosphate	3.73 ± 0.01	4.62 ± 0.03	6.87 ± 0.06	1841 ± 22	0.535 ± 0.02	0.322 ± 0.022	0.481 ± 0.046	899 ± 87
Glucose	1.77 ±0.03	1.11 ± 0.01	1.65 ± 0.022	930 ± 17	0.975 ± 0.04	0.495 ± 0.017	0.740 ± 0.092	758.8 ± 111

Table 3.4 The kinetic parameters for enzyme CdP with cellodextrins of DP > 2

	Cellodextrin phosphorylase			
Forward reactions	K_m(mM)	V_{max}(U/mg)	$k_{cat}(s^{-1})$	$k_{cat}/K_m(s^{-1} M^{-1})$
Cellotriose	0.065 ± 0.001	0.013 ± 0.001	0.019 ± 0.002	292 ± 31
Cellotetraose	0.031 ± 0.001	0.007 ± 0.001	0.011 ± 0.002	355 ± 23
Cellopentaose	0.090 ± 0.003	0.016 ± 0.002	0.023 ± 0.004	256 ± 11
Reverse reactions	K_m(mM)	V_{max}(U/mg)	$k_{cat}(s^{-1})$	$k_{cat}/K_m(s^{-1} M^{-1})$
Cellobiose	0.665 ± 0.01	0.006 ± 0.001	0.009 ± 0.002	13.48 ± 2.25

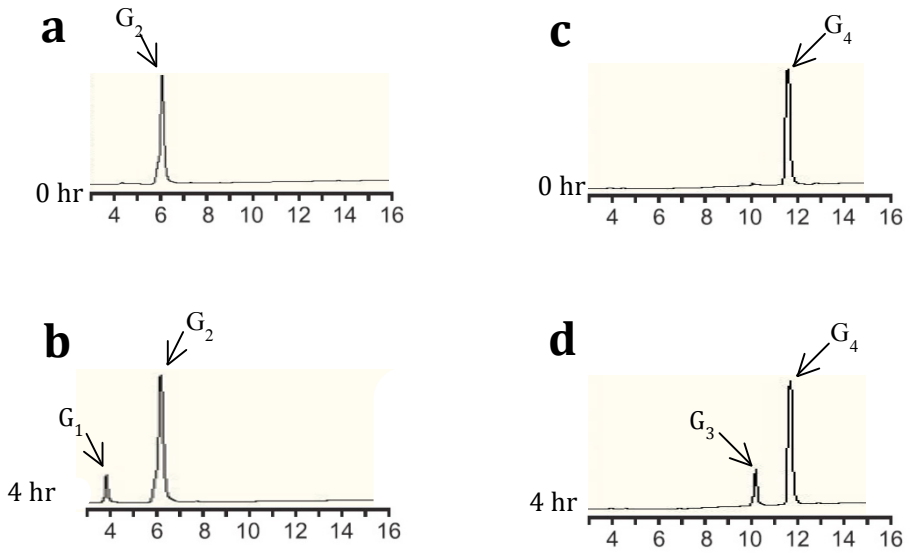

Figure 3.3 High-Performance Anion-Exchange Chromatography (HPAEC) spectra of reaction contents with either cellobiose or cellotetraose as substrates, at two different times with CdP

A- Cellobiose at t = 0 B- Cellobiose at t = 4 hours
C- Cellotetraose at t = 0 D-Cellotetraose at t = 4 hours

Taken together, these data provide first experimental confirmation for the annotation of the two phosphorylase genes in this microorganism. While CbP exhibits a usual activity profile, CdP appears to be the first of the kind in its activity with cellobiose. As with *Clostridium stercorarium* CdP [33], the best substrate for *Saccharophagus* CdP is G4.

3.3.3 The pH and temperature characteristics of CbP and CdP

The effect of pH and the stability of both enzymes were studied by measuring the activities of each enzyme at different pH values and determining their relative activities. For better comparison, cellobiose phosphorylase activity was used in the characterization for both enzymes.

CbP exhibited a broad optimum pH range. The enzyme is active over a pH range of 5 to 8.5 (Fig 3.4a). The enzyme was stable over a pH range of 5 to 7, retaining about 90% of its activity (Fig 3.4b). It was also found stable after storage in the same range of pH for 12 hours at 4˚C.

In contrast, the pH profile of CdP is atypical. The enzyme activity gradually increases with the increase of pH. The enzyme activity peaks at pH about 7.5 followed by a sharp decrease for pH above 7.5 (Fig3.4c). The enzyme was stable at pH 7-8, retaining about 90% of its activity (Fig 3.4d). The temperature characteristics of both enzymes were also investigated. As shown in Fig. 3.5a and 3.5c, both CbP and CdP exhibited their respective maximum activity at a temperature of 40-45˚C. Both enzymes are stable up to 45˚C. Interestingly, after an initial drop of activity when temperature increases from 45˚C to 50˚C, no further decrease of enzyme activities as temperature increases to 65˚C. At this temperature, CbP retained about 65% of its activity whereas CdP retained only 35% of its activity (Fig. 3.5b and 3.5d).

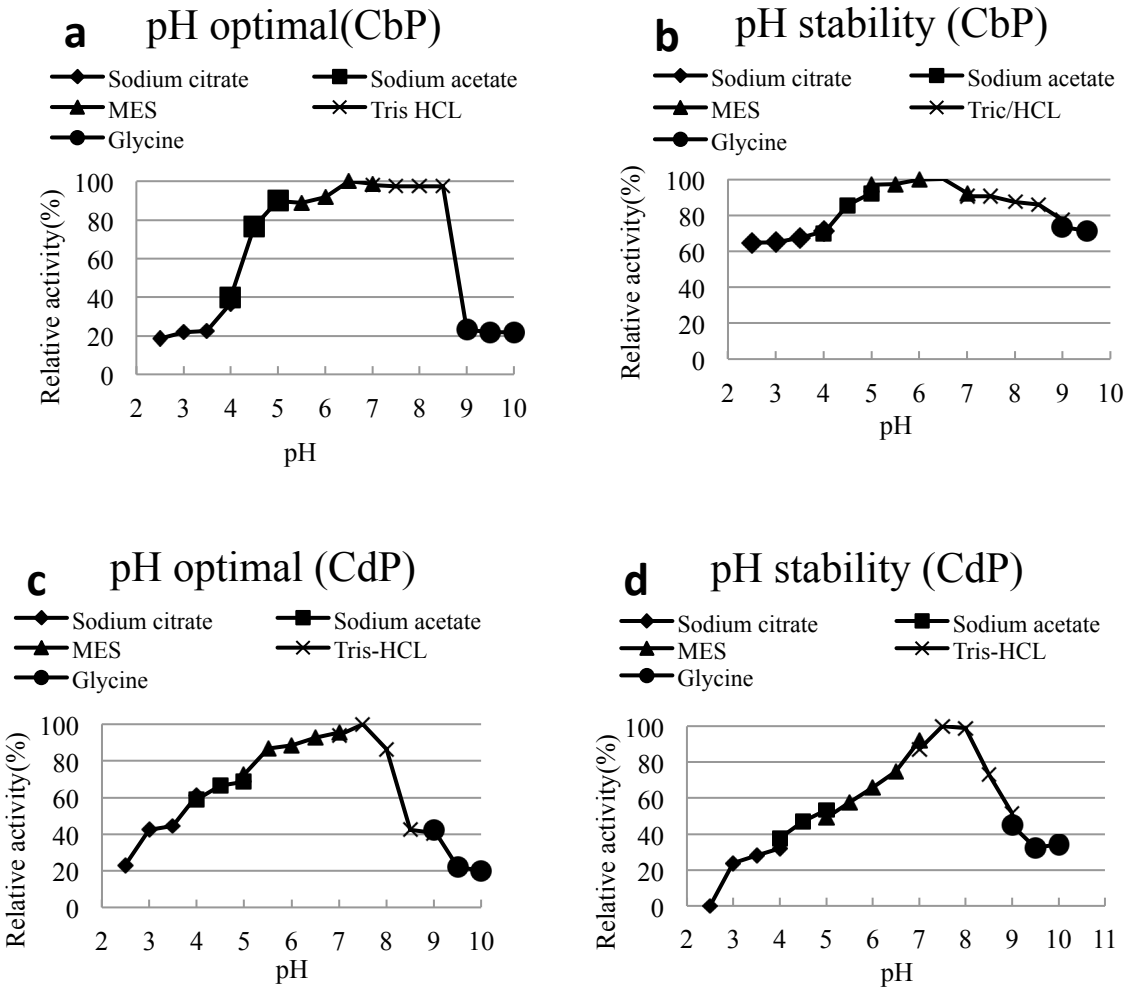

Figure 3.4 The pH optimal (a, c) and stability profile (b, d) of purified recombinant CbP & CdP

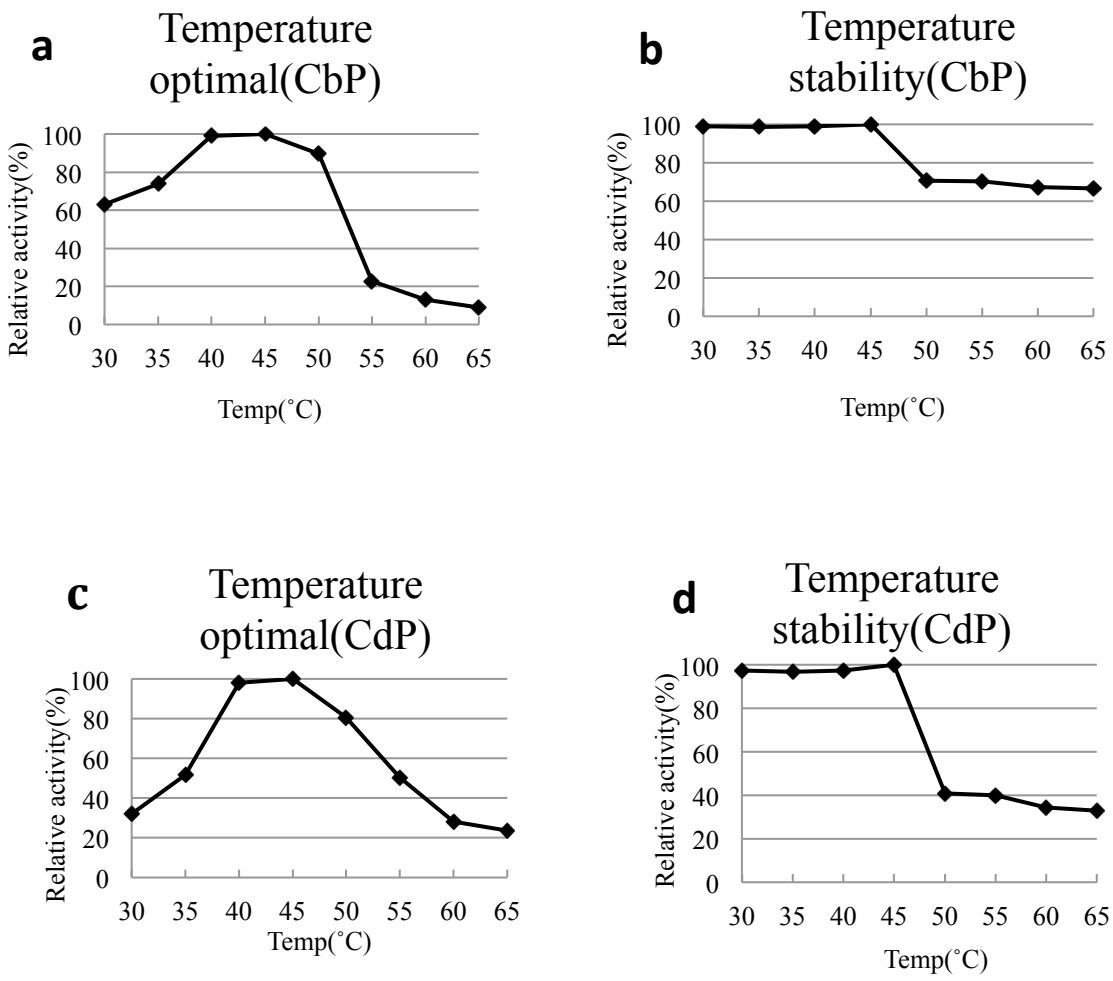

Figure 3.5 The temperature (a, c) and stability (b, d) of purified recombinant CbP and CdP

3.4 Discussion:

In this study, we carried out cloning of the genes (CEP94A and CEP94B) for putative cellobiose phosphorylase and cellodextrin phosphorylase from a cellulolytic marine bacterium *Saccharophagus degradans*, subsequently overexpressed the two recombinant enzymes in an *E. coli* system, and characterized the purified recombinant enzymes with respect to the substrate specificity, pH and temperature characteristics. The data presented here provided experimental

confirmation for the respective function of the enzymes. As such, CEP94A is a typical cellobiose phosphorylase (CbP), active only with cellobiose in the phosphorolytic direction. Additionally, the annotation of CEP94B as a cellodextrin phosphorylase was confirmed. However, CdP appears to be novel in its cross activity with cellobiose. This finding is significant as this is the first case that a CdP was shown to exhibit activity toward cellobiose, a substrate for CbP. In most microorganisms from which phosphorylase is found, the two phosphorylases co-exist but with diverge substrate specificity. Examples include well-characterized phosphorylases from *Clostridium thermocellum*, whose genome encodes one cellobiose phosphorylase and cellodextrin phosphorylase and they do not overlap with their substrates [34, 35, 38]. Despite the novelty, the activity of *Saccharophagus* cellodextrin phosphorylase with cellobiose is two orders of magnitude lower than that of cellobiose phosphorylase under the same conditions. Based on the results, it seems more reasonable to envision that each enzyme acts on its own substrate (i.e. Cep94A with cellobiose and Cep94B with cellodextrin) and cross-activity is not important *in vivo.* The pH and temperature profiles for the enzymes do not differ substantially to suggest an in vivo scenario that CdP's cellobiose phosphorylase activity is important relative to the true cellobiose phosphorylase. Compared to Cep94A, the enzyme is less stable. In fact, the unstable nature of Cep94B led to an inactive protein, as reported by others[37]. We observed that fresh preparation of the enzyme was necessary for reproducible data as reported in this study. Storage at low temperature at -20^{o}C was necessary to keep the enzyme active for up to 48 hours.

Cellulose, being a recalcitrant substrate, requires significant input of cellular energy in its full degradation. A metabolic strategy that minimizes the energy expenditure is commonly employed by those truly cellulolytic microorganisms such as strict anaerobe *Clostridium thermocellum*. The confirmation that the genome of an aerobe *Saccharophagus degradans*

encodes two phosphorylases suggests energy-saving may still be important for microbes under aerobic conditions. Additionally, the results presented here show that the best cellodextrin substrate for CdP is G4, reminisce to *C. thermocellum*[33]. However, many questions remain as to the exact mode of cellulose metabolism in this organism. First, as phosphorylase is cytoplasmic, cellodextrin and cellobiose assimilation requires a transport system. In *Clostridium thermocellum*, an ABC transporter is believed to uptake cellodextrin(i.e. N> 2)[20, 55, 58]. Yet a transport system for cellodextrin uptake in *S. degradans* has not been reported so far. Second, while the presence of phosphorylase suggests the use of phosphorolytic mechanism, it is more likely this is only one of the ways how cellodextrin is assimilated as the genome also encodes competing pathways. There are at least two cellodextrinases and several β-glucosidases in this organism. Additionally, a previous study seems to suggest that the organism assimilates cellulose through cellobiose [27] casting doubts about the *in vivo* significance of the cellodextrin phosphorylase. The functional confirmation of phosphorylases and characterization of the two phosphorylases properties provide a basis for future studies aimed at a more quantitative understanding of the contribution of each metabolic cellulose assimilation mechanism.

CHAPTER 4

CELLOBIOSE UTILIZATION IN *ESCHERICHIA COLI* EXPRESSING CELLOBIOSE PHOSPHORYLASE FROM *SACCHAROPHAGUS DEGRADANS*

4.1 Introduction

In some cellulolytic organisms, cellobiose is the main product from cellobiohydrolase activity when supplied with cello-oligosaccharides [11, 48]. Enzymes such as β-glucosidases and cellobiose phosphorylase (intracellular) hydrolyse and phosphorylate cellobiose to glucose molecules respectively. During extracellular degradation of cellulose in the bioethanol industry, the formation of glucose poses a risk of contamination in the medium. It has been shown that ethanol production yield was improved through intracellular uptake of cellobiose and hydrolysis leading to co-consumption of cellobiose and xylose[53]. Therefore, understanding cellobiose utilization is of importance for the efficient degradation of lignocellulose.

In *Escherichia coli*, two operons, *cel* and *bgl* encode genes associated with cellobiose uptake and metabolism. However, these two operons are cryptic under normal cultivation conditions[52]. Cryptic genes are phenotypically silent DNA sequences that are not normally expressed in the organism but are expressed through a mutation. The *bgl* operon consists of three genes *bglG*, *bglF* and *bglB*. The gene *bglB* encodes a phospho-β-glucosidase that acts upon aryl-β-glucosides. The expression of the operon *celABCDF* enables the utilization of compounds such as cellobiose, arbutin and salicin[59-63]. The genes *celB* and *celC* are involved in the phosphoenolpyruvate-dependent transport and phosphorylation of cellobiose, arbutin and salicin[64-67]. Although, *E.coli* is unable to grow on cellobiose as a sole carbon source, studies have shown that on

prolonged incubation in cellobiose-based medium, *cel* operon mutants were obtained which could grow on cellobiose as the carbon source. A recent study showed that a yeast lactose permease could mediate transport of cellobiose[53]. Lactose permease is a lactose/proton symporter that transports lactose across the cytoplasmic membrane[68]. It is not clear, however, that LacY from *E.coli* could transport cellobiose. This chapter describes the study of role of *cel, bgl* operons and the lactose permease (*lacY*) gene in cellobiose uptake in *Escherichia coli*. The study of cellobiose metabolism by *Escherichia coli* is undertaken through the expression of cellobiose phosphorylase (*cepA*) discussed in chapter 3. Cell growth is believed to be aided by *cepA* through breakdown of cellobiose to glucose from cellobiose in the media and thereby utilized as a carbon source by the cells.

4.2 Materials and methods

4.2.1 Bacterial strains, plasmids and chemicals:

E.coli KO11 (ATCC#55124) and *E.coli* KO11 Δ*cel*Δ*bgl* was used as a vehicle for cloning vectors and for cellobiose utilization experiments. Genes alcohol dehydrogenase (*adh*) and pyruvate decarboxylase (*pdc*) involved in ethanol production are genomically integrated in *E.coli* KO11[54]. Plasmids pQE80L (Invitrogen, Carlsbad, CA) was used for cloning and pKD4, pCP20 and pKD46 were used for gene knockout (Table 4.1). Luria Broth rich medium and M9 minimal media mix (1X M9 salts, 1% cellobiose, 1ml/l $MgSO_4$, 0.05% yeast extract, 100µl/l $CaCl_2$) (Fisher Scientific, Pittsburg, PA) were used for cell cultivations. Antibiotics amphicillin (100µg/ml) and kanamycin (50µg/ml) (Fisher Scientific, Pittsburg, PA) were added to cultures of plasmid-bearing *E.coli* strains. Isopropyl β-D-1-thiogalactopyranoside (IPTG), used to induce gene expression was purchased from Zymo Research (Irvine, CA). Protein concentration was measured by the method by Bradford (Bio-Rad, Hercules, CA, USA), with bovine serine

albumin as the standard. Cell density (OD_{600}) was measured on an UV/visible light spectrophotometer (Beckman Coulter, CA, USA)

Table 4.1 Strains and plasmids used in this study

Strains	Description	Source
E.coli KO11	W Δfrd *pflB*::pdc_{Zm} $adhB_{Zm}$ $ldhA^{+}$ *cat*	Laboratory
E.coli KO11 Δ*cel* Δ*bgl*	*E.coli* KO11 strain, *cel* & *bgl* operons deleted	stock
E.coli KO11 Δ*lacY*	*E.coli* KO11 strain, *LacY* gene deleted	This study

Plasmids	Description	Source
pKD46	Plasmid harboring the λ Red Recombinase system, Amp^{R}	Laboratory stock
pCP20	Plasmid harboring the flipase antibiotic marker disruption system, Amp^{R}	Laboratory stock
pKD3	Plasmid harboring Kan^{R} resistance marker flanked by Flipase recognition target sites (FRT)	Laboratory stock
pKD4	Plasmid harboring Str^{R} resistance marker flanked by Flipase recognition target sites (FRT)	Laboratory stock
pQ*cepA*	Plasmid pQE80L under T5 promoter and cellobiose phosphorylase gene inserted into BamHI and PstI sites	This study

4.2.2 Construction of *cel*/*bgl*/*lacY* operon knockouts strains in *E.coli* strains:

The knockout strains were constructed using the gene inactivation method developed by Wanner and coworkers [43]. The FRT-flanked kanamycin resistance gene was PCR amplified using primers given in Table 4.2 with 40-nucleotide extensions homologous to the adjacent regions of the respective genes/operons. Homologous recombination was mediated by the plasmid-borne (pKD46) phage λ Red recombinase. The kanamycin gene was then excised by helper plasmid

pCP20 containing the FLP recombinase. Finally, both plasmids were cured by growth at above 37°C as they contain temperature-sensitive replicons. The overall strategy for knockout is illustrated in Fig 4.1[69].

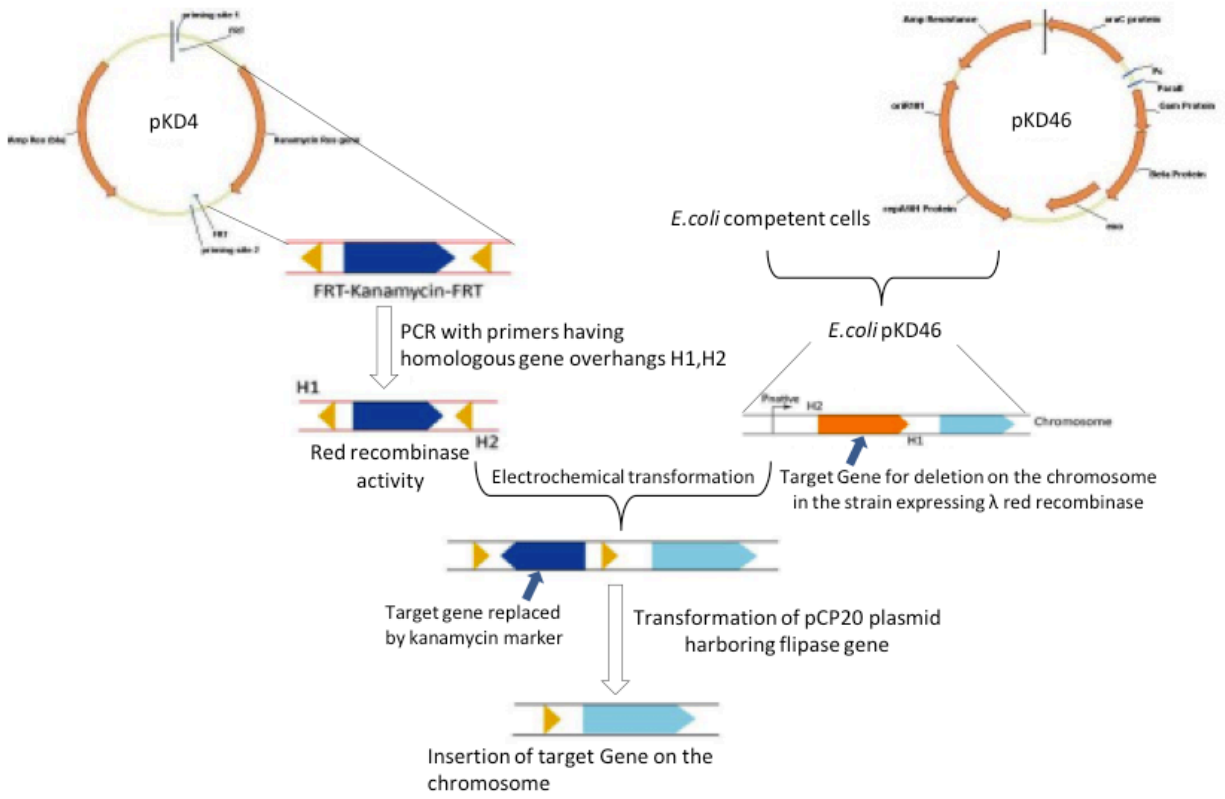

Figure 4.1 Experimental protocol for gene deletion

4.2.3 Cloning and expression of Lactose permease (LacY):

Primers were designed to amplify the full gene lactose permease (LacY) from the genome of *Escherichia coli* KO11 via PCR (Table 4.2). The amplified gene fragment was ligated into *Pst*I-*Nco*I linearized pQE80L-*cepA* to form the cloned plasmid, pQE80L-*cepA*-*lacY*, which was later transformed into *E. coli* KO11 Δ*lacY* strain. To analyze the expression of *lacY* gene in engineered *E.coli*, the uptake of lactose by KO11 Δ*lacY* pQ*cepA*-*lacY* was compared to KO11

Δ*lacY* pQ*cepA* as a control through analysis of residual lactose concentration in the extracellular medium. This extracellular lactose measurement assumes that decrease in lactose concentration was only due to cellular uptake and not due to lactose degradation in the extracellular medium[70].

Table 4.2 Primers used in this study

Operon/Gene	Primer name	Sequence (5'-3')
celABCDF	celkckoutfwd	ATGGAAAAGAAACACATTTATCTGTTTTGTTCTGCGGGCA
	celkckoutrev	GTGTAGGCTGGAGCTGCTTC
	celchkfwd	TTAATGTGCTTTTTTAAGCTCTGCGATGCAGTCGGCAAAG
	celchkrev	ATGGGAATTAGCCATGGTCC
		TCTGTTTTGTTCTGCGGGCATGTCTACCTCTTTACTGGTA
		GAATGCACCAACGGACTAAGGTTTAGCGCCAGTAACACAT
bglGFB	bglkckoutfwd	ATGAACATGCAAATCACCAAAATTCTCAACAATAATGTTG
	bglkckoutrev	GTGTAGGCTGGAGCTGCTTC
	bglchkfwd	TTATTTTTTTAATGACAGCCCCCGCGTTTTGATCACTTCT+
	bglchkrev	ATGGGAATTAGCCATGGTCC
		CAGCGGGAAAAAGTCGTCATGGGGCGCGGAATTGGCTTTC
		TGTATCGAACCATCCGCTTCAACGCTGTCTTTTGCGCCTA
lacY	lacY_Knkfwd	ATGTACTATTTAAAAAACACAAACTTTTGGATGTTCGGTT
	lacY_Knkrev	GTGTAGGCTGGAGCTGCTTC
	lacY_chkfwd	TTAAGCGACTTCATTCACCTGACGACGCAGCAGGGAAAGC
	lacY_chkrev	ATGGGAATTAGCCATGGTCC
		CCAGTTGGTCTGGTGTCAAAAATAA
		TCGGTCATTGGCATGTTCAA
lacY (Clone)	lacYfwd_PstI	CTCGCACTGCAGAAAGAGGAGAAATTAACTATGTACTATT
	lacYrev_NcoI	TAAAAAACACAAAC
		CGATGCCCATGGTTAAGCGACTTCATTCACCTG

4.2.4 Localization of cellobiose phosphorylase/β-glucosidase activity in KO11 Δ*cel*Δ*bgl* strain:

KO11 Δ*cel*Δ*bgl* mutant strain bearing pQ*cepA* plasmid was cultured anaerobically in M9 minimal media mix in the presence of 1% cellobiose as the carbon source at 250 rpm at 37°C for 72 hours. Antibiotics were added as appropriate (ampicillin 100µg/ml). The cells were induced with IPTG to a final concentration of 0.2mM to induce expression of *cepA* gene. Extracellular, periplasmic and intracellular fractions were prepared according to the method described in the pET system manual (EMD Chemicals, San Diego, CA). 3 ml culture was harvested and re-suspended in 1.5 ml 30 mM Tris– HCl, pH 8.0, 20% sucrose and EDTA was added to a final concentration of 1 mM. The cell suspension was incubated at room temperature for 10 min and pelleted by centrifugation at 10,000g at 48°C for 10 min. The cells were re-suspended in 0.2 ml ice-cold 5mM $MgSO_4$ and incubated on ice for 10 min. The cells were pelleted by centrifugation as above, and the supernatant was collected as a periplasmic fraction. The pellet was re-suspended in 0.2 ml PBS solution, sonicated, and the supernatant was saved as a cytoplasmic fraction. Each fractional sample was analyzed at the end of 24, 48 and 72 hours for cellobiose phosphorylase and β-Glucosidase activity.

The phosphorylase activity of recombinant *cepA* was determined by measuring the amount of glucose-1-phosphate generated from cellobiose and phosphate. The reaction mixture in a total volume of 200µL consisted of 50mM PIPES buffer, 8mM phosphate pH 7.0, 5mM $MgSO_4$, 0.5mM NADP, 4U of phosphoglucomutase, 2U of glucose-6-phosphate dehydrogenase, 250µM pure cellobiose and 50µL of enzyme solution. The mixture was incubated at 42°C for 10 minutes and heated to 95°C for 5 minutes to stop the reaction. The concentration of NADPH formed was

determined by measuring the change in absorbance at 340nm. Glucose-1-phosphate formation was estimated assuming molar equivalence to the NADPH produced[37].

The β-Glucosidase activity was determined through the hydrolysis of *p*NP-β-glucoside. This was determined by monitoring the formation of p-nitrophenol spectrophotometrically. The reaction mixture (500µl) contained 20mM PIPES, pH 6.5, and 0.15mM *p*NP-β-glucoside initiated by the addition of enzyme fraction. After incubation at 37°C for the indicated time, the reaction was quenched by the addition of 500µl of 1M Tris base. The absorbance at 400 nm was used to calculate product formation. The protein concentration is determined through the Bradford assay. One unit of activity is defined as the amount of enzyme that releases 1µmol product per minute of reaction.

4.2.5 Optimization of cellobiose concentration for growth of *E.coli* KO11/pQ*cepA* and KO11 Δ*cel*Δ*bgl*/pQ*cepA*:

To determine the optimum cellobiose concentration of *E.coli* mutant strains, the cell growth of strains expressing cellobiose phosphorylase was monitored in the presence of varying concentration of cellobiose as the carbon source. The recombinant strains were grown in M9 minimal media mix anaerobically with 1-5% cellobiose as carbon source and cultivated with an initial OD_{600} = 0.01 at 37°C and 250 rpm for 3 days. Antibiotics were added as appropriate (ampicillin 100µg/ml). The cells were induced with IPTG to a final concentration of 0.2mM to induce expression of *cepA* gene. The cell growth was measured using a UV-Spectrophotometer and residual cellobiose concentration was measured using the dinitrosalicylic acid method (DNS)[42]. Samples taken during fermentation were spun down by centrifugation and supernatants were analyzed for concentrations of ethanol using high-performance liquid chromatography (Agilent Technologies, Santa Clara, CA) instrument equipped with Supelcogel

column H. The following formula was used to calculate the ethanol yields[71]: ethanol yield (%) = ([ethanol]/ ([maximum glucose content of substrate]×0.51)×100.

4.2.6 Growth characteristics of *E.coli* KO11 Δ*cel*Δ*bgl* and KO11 Δ*lacY* expressing cellobiose phosphorylase (*cepA*):

To study the growth characteristics of *E.coli* mutants, the cell growth of strains expressing cellobiose phosphorylase (*cepA*) was monitored in the presence of cellobiose as the carbon source. The recombinant strains were grown in M9 minimal media mix anaerobically with 1% cellobiose as carbon source and cultivated with an initial OD_{600} = 0.01 at 37°C and 250 rpm for 48 hours. Antibiotics were added as appropriate (ampicillin 100µg/ml, kanamycin 50µg/ml). The cells were induced with IPTG to a final concentration of 0.2mM to induce expression of *cepA* gene. The strains with empty plasmid pQE80L were used as control. Samples were analyzed after 24 and 48 hours. The cell growth was measured using a UV-Spectrophotometer and residual cellobiose concentration was measured using the dinitrosalicylic acid (DNS) method[42].

4.2.7 Growth characteristics, sugar and ethanol profiles of KO11 wild type and KO11 Δ*lacY* expressing cellobiose phosphorylase (*cepA*):

To study the growth characteristics, residual sugar and ethanol profiles of all recombinant strains, the strains expressing cellobiose phosphorylase (*cepA*) were subjected to anaerobic fermentation in M9 minimal media mix with 1% cellobiose (optimized G2 concentration) as the carbon source with an initial OD_{600} = 0.01 at 37°C and 250rpm for 72 hours. Antibiotics were added as appropriate (ampicillin 100µg/ml, kanamycin 50µg/ml). The cells were induced with

IPTG to a final concentration of 0.2mM to induce expression of *cepA* gene. Samples were analyzed every 8 hours during day 1, 12 hours during day 2 and once at the end of 72 hours. KO11 wild type with the empty plasmid pQE80L was used as the control strain. The cell growth was measured using a UV-Spectrophotometer and residual cellobiose concentration and ethanol concentration were measured using the high-performance liquid chromatography (Agilent Technologies, Santa Clara, CA) instrument equipped with Supelcogel column H.

4.2.8 Improving ethanol production yield of KO11 pQ*cepA* and KO11 Δ*lacY* pQ*CepA-lacY*:

To improve the ethanol yield of the engineered cellobiose utilizing strains KO11 pQ*cepA* and KO11 Δ*lacY* pQ*cepA-lacY*, the strains were cultivated in Luria bertani media anaerobically with 2% cellobiose (optimized G2 concentration) as the carbon source with an initial OD_{600} = 0.01 at 37°C and 250rpm for 72 hours. Antibiotics were added as appropriate (ampicillin 100µg/ml, kanamycin 50µg/ml). The cells were induced with IPTG to a final concentration of 0.2mM to induce expression of *cepA* gene. Samples were analyzed at the end of 72 hours. The cell growth was measured using a UV-Spectrophotometer and residual cellobiose concentration and ethanol concentration were measured using the high-performance liquid chromatography (Agilent Technologies, Santa Clara, CA) instrument equipped with Supelcogel column H.

4.3 Results

4.3.1 Localization of cellobiose phosphorylase/β-glucosidase activity in KO11 Δ*cel*Δ*bgl*/*cepA* strain:

In order to investigate the location of activity of cellobiose phosphorylase and β-glucosidase in the recombinant strains, the KO11 Δ*cel*Δ*bgl*/*cepA* strain was cultured anaerobically in the presence of 1% cellobiose as the carbon source. The various cell fractions (extracellular, periplasmic, cytoplasmic and cell pellet) were analyzed for cellobiose phosphorylase/β-glucosidase activity. Phosphorylase activity was determined by measuring glucose-1-phosphate produced and β-glucosidase activity was determined through the hydrolysis of pNP-β-glucoside. No β-glucosidase activity was observed in any of the cell fractions. Cellobiose phosphorylase activity was observed only in the cytoplasmic fraction up to 2143.5 U/mg after 48 hours. However, there was no detectable *cepA* activity observed in the other cell fractions. These observations provide experimental evidence that *cepA* was functional only intracellularly and there was no β-glucosidase background activity (to cleave cellobiose to glucose) in *cepA* activity measurements during cultivations.

4.3.2 Growth characteristics of *E.coli* KO11 Δ*cel*Δ*bgl* and KO11 Δ*lacY* expressing cellobiose phosphorylase (*cepA*):

The growth characteristics of *E.coli* mutant strains expressing *cepA* were studied in the presence of cellobiose as the carbon source. The recombinant strains were cultivated anaerobically in the presence of 1% cellobiose in M9 minimal media and the cell density and residual cellobiose concentration were determined. As shown in Table 4.3, the expression of *cepA* in wild type KO11 strain enabled cellobiose assimilation of up to ~94% at the end of 48 hours. On deleting

the cellobiose PTS transport operons *cel* and *bgl* from the genome, the strain was still able to utilize up to ~98% cellobiose for cell. However, the deletion of lactose permease gene (*lacY*) led to the inability of the strain KO11 to efficiently assimilate cellobiose (~13%). On complementation of lactose permease gene onto KO11 Δ*lacY* strain expressed under T5 promoter, the strain regained the ability to assimilate cellobiose(~73%). To analyze the expression of *LacY* gene in engineered *E.coli*, the uptake of lactose by KO11 Δ*lacY* pQ*cepA-lacY* was compared to KO11 Δ*lacY* pQ*cepA* as a control through analysis of residual lactose concentration in the extracellular medium. The rate of lactose assimilation in KO11 Δ*lacY* pQ*cepA-lacY* was ~47% greater than that of KO11 Δ*lacY* pQ*cepA* indicating successful expression and activity of LacY (data not shown). These observations show the significance of lactose permease towards cellobiose transport in *E.coli* in comparison to *cel* and *bgl* operons. All recombinant strains expressing the empty plasmid pQE80l showed negligible growth and cellobiose utilization.

Table 4.3 Growth characteristics in *E.coli* KO11 Δ*cel*/Δ*bgl* and KO11 Δ*lacY* with expression of *cepA*

Strains	24 h		48 h	
	A_{600}	ΔCellobiose (%)	A_{600}	ΔCellobiose (%)
KO11 pQE80L	0.139	4.3	0.154	5.3
KO11/*cepA*	1.196	72.5	3.06	93.7
KO11 Δ*cel*Δ*bgl*/pQE80L	0.108	4.3	0.114	4.8
KO11 Δ*cel*Δ*bgl*/*cepA*	0.909	49	3.09	97.7
KO11 Δ*lacY/*pQE80L	0.081	9.3	0.122	10.4
KO11 Δ*lacY/cepA*	0.05	11.7	0.14	13
KO11 Δ*lacY/cepA-lacY*	1.19	31.43	3.04	72.38

4.3.3 Optimization of cellobiose concentration for growth of *E.coli* KO11/pQ*cepA* and KO11 Δ*cel*Δ*bgl*/pQ*cepA*:

The optimum cellobiose concentration for growth of recombinant *E.coli* KO11 was determined by studying the growth profile and ethanol production. The recombinant strains were cultivated anaerobically in the presence of 1-5% cellobiose concentration in minimal media. The cell growth, residual cellobiose and ethanol concentrations were determined after 72 hours. As shown in Table 4.4 and 4.5, there was a decrease in cellobiose utilization with increase in initial cellobiose concentration. Cellobiose was nearly completely consumed at 1% (w/v) in both recombinant strains. Similarly, ethanol yield was efficient at lower cellobiose concentrations. At 2% cellobiose, the ethanol yield was similar when comparing KO11 wild type and KO11

Δ*cel*Δ*bgl* (~33%). However, the yield was not consistent with 1% cellobiose concentration. Therefore these results show that the recombinant strains cultured at 2% initial cellobiose concentration as carbon source resulted in stable ethanol production.

Table 4.4 Effect of cellobiose concentration for growth of *E.coli* KO11 *cepA*

Cellobiose (%, w/v)	Cell growth (A_{600})	Residual Cellobiose (g/L)	Ethanol (g/L)	Ethanol yield (%)
1.0	2.85±0.13	0.93±0.01	2.34±0.01	45.95
2.0	2.89±0.10	4.61±0.17	3.40±0.13	33.37
3.0	2.84±0.10	14.12±0.75	2.89±0.09	18.90
4.0	3.08±0.07	24.99±0.61	2.96±0.06	14.51
5.0	2.96±0.11	32.36±2.30	2.65±0.12	10.40

Table 4.5 Effect of cellobiose concentration for growth of *E.coli* KO11 Δ*cel*/Δ*bgl cepA*

Cellobiose (%, w/v)	Cell growth (A_{600})	Residual Cellobiose (g/L)	Ethanol (g/L)	Ethanol yield (%)
1.0	2.87±0.13	0.96±0.02	1.66±0.01	32.64
2.0	3.28±0.10	2.59±0.30	3.26±0.02	31.93
3.0	3.34±0.05	12.31±0.92	3.32±0.25	21.69
4.0	3.25±0.04	20.66±0.53	3.26±0.36	15.98
5.0	3.29±0.08	31.17±0.20	2.86±0.05	11.22

4.3.4 Growth characteristics, cellobiose and ethanol profiles of KO11 wild type and KO11 Δ*lacY* expressing cellobiose phosphorylase (*cepA*):

The growth characteristics, cellobiose and ethanol profiles of *E.coli* recombinant strains expressing *cepA* were studied in the presence of cellobiose as the carbon source. The recombinant strains were cultivated anaerobically in the presence of 1% cellobiose in M9 minimal media and the cell density, residual cellobiose concentration and ethanol production were determined. The expression of *cepA* in wild type KO11 enabled cellobiose utilization of 100% and ethanol yield of 66.56% after 72 hours (Fig 4.2B&C). However, deletion of lactose permease (*lacY*) reduced the cellobiose uptake drastically to 4.92% and negligible ethanol yield (0.98%) (Fig 4.2B&C). Complementation of *lacY* gene expression enabled *E.coli* KO11 to again utilize cellobiose (100%) and ethanol yield of ~50%. These observations again indicate that lactose permease is significant for cellobiose transport in *E.coli*. KO11 wild type strains expressing the empty plasmid pQE80l showed negligible growth and cellobiose utilization.

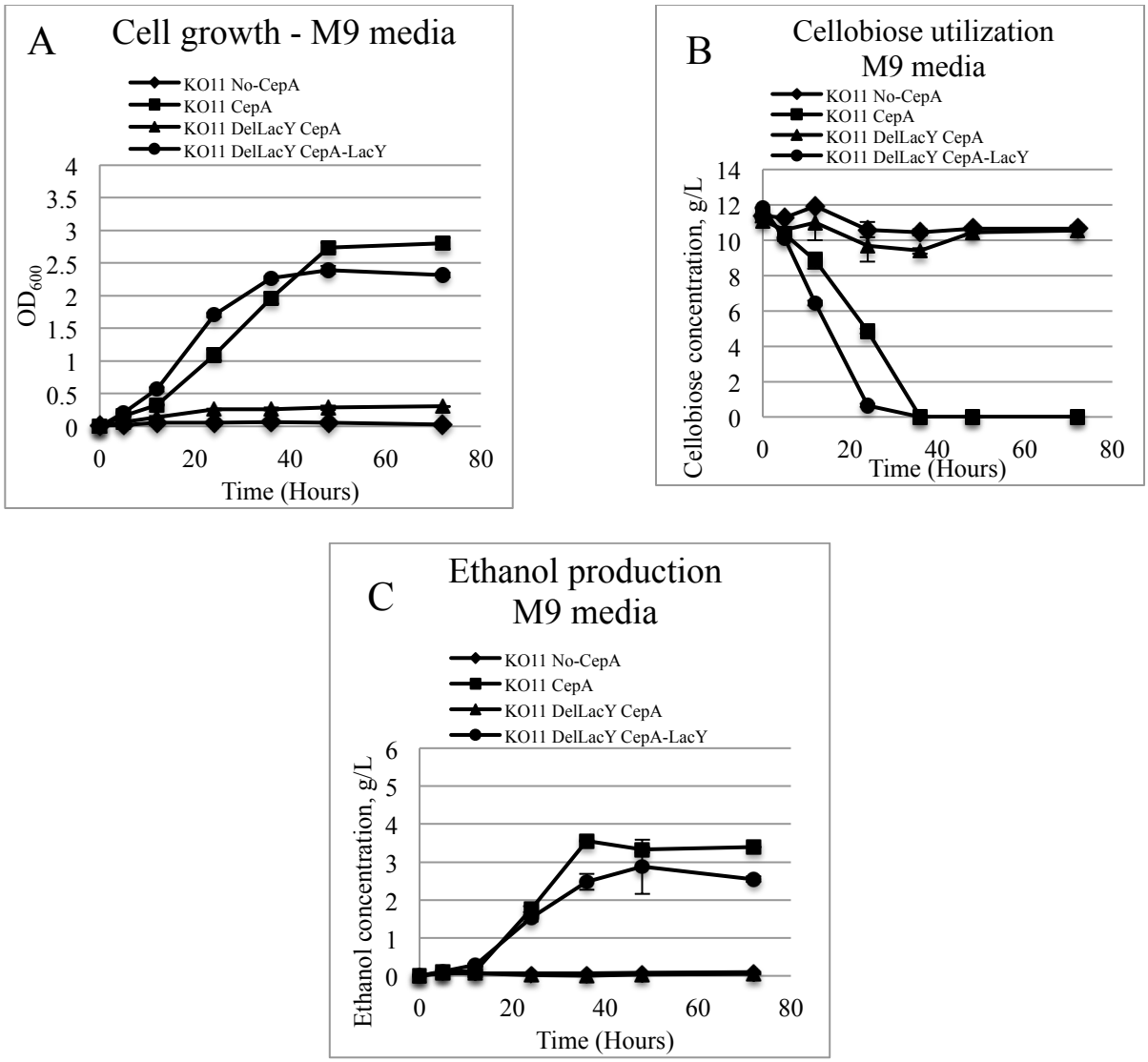

Figure 4.2 Growth characteristics, cellobiose and ethanol profiles of KO11 wild type and KO11 Δ*lacY* expressing cellobiose phosphorylase (*cepA*)

4.3.5 Improving ethanol production yield of KO11 pQ*cepA* and KO11 Δ*lacY* pQ*cepA-lacY*:

To improve the ethanol yield of the engineered cellobiose utilizing strains KO11 pQ*cepA* and KO11 Δ*lacY* pQ*cepA-lacY*, the strains were cultivated in Luria bertani media anaerobically with 2% cellobiose (optimized G2 concentration) as the carbon source for 72 hours. As shown in

Table 4.6, cultivation in LB media resulted in an increase in ethanol production yield by 2.57 and 2.78 fold in KO11 pQ*cepA* and KO11 Δ*lacY* pQ*cepA-lacY* respectively in comparison to cultivation M9 minimal media. This resulted also correlated with high G2 utilization (98.61% & 71.85% in KO11 pQ*cepA* and KO11 Δ*lacY* pQ*cepA-lacY* respectively). The low ethanol yield in M9 media compared to LB media indicates the lack of nutrients such as peptones and vitamins, which are required for cell maintenance and enzyme production. However, this result also indicates that the deletion and complementation of lactose permease (LacY) in the KO11 genome did not influence the ability of ethanol production in the engineered strain.

Table 4.6 Comparison of ethanol production yield in LB & M9 minimal media

In LB media:

Strain	Cell growth (A_{600})	Residual Cellobiose (g/L)	Ethanol (g/L)	Ethanol yield (%)
KO11 pQ*cepA*	2.9±0.02	0.28±0.07	9.72±0.56	95.29
KO11 Δ*lacY* pQ*cepA-lacY*	2.75±0.07	5.63±0.68	7.15±0.54	70.04

In M9 media:

Strain	Cell growth (A_{600})	Residual Cellobiose (g/L)	Ethanol (g/L)	Ethanol yield (%)
KO11 pQ*cepA*	2.88±0.04	8.16±0.44	3.776±0.14	37.02
KO11 Δ*lacY* pQ*cepA-lacY*	2.95±0.16	9.32±0.13	2.56±0.13	25.16

4.4 Discussion:

In this study, we demonstrated the importance of cellobiose transport operons *cel* & *bgl* and lactose permease gene (*lacY*) in the cellobiose utilization in *Escherichia coli*. We carried out gene knockouts of the proposed genes in *E.coli* KO11, an efficient ethanol [72]producer and co-expressed cellobiose phosphorylase (CepA) from *Saccharophagus degradans* and lactose permease (*lacY*) in order to enable growth on cellobiose as the sole carbon source. We subsequently studied the growth, sugar consumption and ethanol production of the recombinant strains in the presence of cellobiose as the carbon source. The data presented here indicates that lactose permease gene plays a key role in cellobiose transport in *E.coli.*

E.coli KO11 expressing an empty plasmid pQE80l was unable to grow on cellobiose media. By introducing cellobiose phosphorylase, the cells were able to utilize up to 94% of cellobiose after 48 hours of cultivation. It is understood from several reports that the cryptic operons like *cel* and *bgl* were involved in cellobiose transport in *E.coli*[52, 65, 73]. Through the study of localization of cellobiose phosphorylase gene in the cell, CepA was functional intracellularly, therefore, requiring a transporter across the cell membrane to enable cellobiose uptake and assimilation. Successful gene knockouts *cel* and *bgl* operons were carried out on the genome of *E.coli* KO11. Following the deletion of the two cryptic operons, *E.coli* KO11 co-expressing CepA was still able to utilize up to 98% cellobiose after 48 hours. A recent study demonstrated that fungal lactose permease gene a functional cellobiose transporter in *Saccharomyces cervisiae*. Suspecting the involvement of LacY, the gene was deleted from KO11 genome. This led to failure in growth on cellobiose media. When LacY and CepA was co-expressed on KO11 Δ*lacY*, the cells regained the ability to uptake up to 72% of cellobiose. This finding is significant since

this is the first demonstration that the lactose permease is the most essential gene responsible for cellobiose transport across the cell membrane in *E.coli*.

During cellulose hydrolysis for biofuel production, cellobiose is one of the main products formed during extracellular degradation by cellulose cocktails[11, 48]. The application of enzymes such as β-glucosidases increase the concentration of extracellular glucose that could intern be an energetic burden for cells to uptake the produced glucose during fermentation and also a risk for contamination of media during biofuel production. Utilization of cellobiose is essential for efficient cellulosic degradation and product formation. Wild type *Escherichia coli* is naturally unable to utilize the cellulose-derived disaccharide cellobiose as a carbon source. There have been various reports on the study of mutant strains of *cel* and *bgl* operons that enabled cellobiose utilization through the activation of the cryptic genes[74]. However, there are no convincing reports in the past regarding the essential genes that are involved in cellobiose uptake in *E.coli*. Here, we show that the gene lactose permease (*lacY*) is the most important gene responsible for cellobiose transport across the cell membrane. The efficient growth on cellobiose after the deletion of *cel* and *bgl* operons demonstrated that these genes do not affect the ability of *E.coli* to up take cellobiose. However, a *lacY* gene deletion caused *E.coli* incapable of growth. The expression of lactose permease was able to regain cellobiose utilization in a *lacY* mutant *E.coli* thus confirming the above conclusion made. A recent report showed the capability of a fungal LacY to transport one or more disaccharides in yeast[53]. It is also interesting to note that there is no significant homology between *lacY* gene and known cellobiose uptake operons. This demonstration of the involvement of lactose permease in disaccharide transport provides a basis for further studies aimed at a more detailed understanding through protein engineering of the transport mechanism of cellobiose.

CHAPTER 5

CONCLUSIONS

5.1 Conclusions

The experimental results and analysis presented in this thesis accomplish the three main objectives outlined in the introduction (Chapter 1):

1) *Escherichia coli* strain was engineered to uptake cellodextrin molecules using energetically efficient transport systems.

2) The transported cellodextrin molecules were efficiently assimilated and converted to produce bioethanol.

3) Investigate cellobiose utilization in *Escherichia coli*

5.1.1 Identification of cellodextrin transporters from a cellulytic bacterium

Two cellodextrin transporters from a cellulolytic bacterium were successfully identified through the metabolic engineering of *Escherichia coli* KO11, an efficient ethanol producer. The cloned transporters, candidate gene 3 resulted in ~48% cellodextrin uptake respectively. The second cloned transporter, candidate gene 4 resulted in ~63% cellodextrin uptake. Additional studies of growth and sugar consumption profiles of the recombinant strains were conducted. The strain KO11 Δ*cel* Δ*bgl* was used as the host for screening the transporter candidates. Operon knockouts of *cel* and *bgl* from the genome of KO11 were constructed using a gene inactivation method developed by Wanner and coworkers[43]. The recombinant strain also expressed an intracellularly functional cellodextrinase (Ced3A), which hydrolyzed cellodextrins to glucose.

The results show that candidate gene 4 is specific towards smaller cellodextrin molecules compared to candidate gene 3.

The identification of cellodextrin transporters from cellulolytic microorganisms using engineered *Escherichia coli* demonstrates the potential application for the consolidated bioprocessing of biofuel production. Additionally, these transporters offer bioenergetic advantages during the production process, which saves cellular energy in the form of ATP that could be utilized for cell growth, enzyme synthesis and product production. Unlike, a regular sugar transport system like PTS in *E.coli,* where one ATP molecule is required to transport each glucose molecule, the energy saved for transport of a cellodextrin molecule (~2-7 glucose molecules) is more favorable. Furthermore, the ability of these transporters to transport cellodextrins reduces the cost of cellulose cocktails required to hydrolyze cellulose molecules to simple sugars such as glucose extracellularly. In this work, we show that the employment of such cellodextrin transporters can be an essential tool for the assimilation of cellulose-derived sugars. While the identification of such transporters is significant in itself, this thesis also demonstrates the utilization of the transported cellodextrin molecules intracellularly through efficient assimilation pathways such as phosphorolytic cleavage in the presence of inorganic phosphate molecules.

5.1.2 Characterization of phosphorylase enzymes from *Saccharophagus degradans*

Two phosphorylase enzymes were successfully cloned, expressed and characterized from cellulolytic *Saccharophagus degradans*. The heterologous enzymes were expressed in *Escherichia coli* and the annotations of both enzymes were confirmed. With respect to consolidated bioprocessing, these phosphorylase enzymes create a favourable condition for cells to hydrolyze the transported cellodextrins through the activity of the identified cellodextrin transporters (Chapter 2). Phosphorolytic cleavage of cello-oligomers utilize inorganic phosphate

molecule instead of ATP for enzyme activity. Therefore, ethanol-producing cells save more ATP as the DP of cellodextrins transported increases. This energy could be utilized for cell growth, enzyme synthesis etc. The byproduct Glucose-1-phosphate is converted to glucose-6-phosphate by an endogenous enzyme phosphoglucomutase thus entering glycolysis directly. Therefore, this energy-saving strategy is essential to be incorporated in a CBP design.

Although CbP was only active towards cellobiose, CdP appeared to have cross activity with cellobiose. This finding is significant since this is the first case that a CdP has shown to be active toward cellobiose. Similar phosphorylases have been characterized from various organisms but with different substrate specificity[30, 34]. Cellulose, known for its properties required significant energy for degradation by cellulolytic organisms. The identification of CbP and CdP from *S.degradans* suggests the same although it is an aerobic microorganism. The results in this study provide a basis for further studies regarding the enzyme mechanism of cellodextrin phosphorylase from *S.degradans* with cellobiose as the substrate.

5.1.3 Cellobiose utilization in *Escherichia coli*

The importance of the lactose permease gene (*lacY*) towards uptake of the disaccharide, cellobiose in *Escherichia coli* was successfully demonstrated in this study. The potential candidates for cellobiose transport genes were deleted from *E.coli* KO11 genome using the λ red recombinase system. The deletion of *cel* and *bgl* operons did not influence the ability of cellobiose uptake. However, the deletion of lactose permease gene (*lacY*) led to failure of growth in cellobiose media. The complementation of *lacY* regained the ability of *E.coli* to uptake G2, suggesting the significant of this gene for G2 transportation. This is the first demonstration that *lacY* is the most essential gene involved in G2 uptake in *E.coli*. Although there have been past

reports about the inability of bacteria to recognize cellobiose as a substrate on mutating lactose permease[72], this report gives a direct experimental evidence of this finding.

Cellobiose is one of the important products of cellulose degradation in CBP process for biofuel production. Therefore, the utilization of G2 for conversion to glucose in ethanol producing organisms is essential during the process. It is also seen that there is no homology between LacY and other known cellobiose transporters. Thus, this finding provides important contributions to the understanding of G2 assimilation in *Escherichia coli* and will likely guide future research endeavors in the field.

5.2 Recommendations for future work

The three main objectives of this dissertation were achieved: *Escherichia coli* was successfully engineered to take up cellodextrin molecules using energetically efficient transport systems, transported cellodextrin molecules were efficiently assimilated and converted to produce ethanol, and cellobiose utilization in *E.coli* was investigated. With the engineering of a bacterial strain for efficient conversion of cellodextrin to biofuels, future research efforts will focus on two areas: 1) characterization of cellodextrin permeases for substrate specificity towards pure cello-oligomers and 2) engineer efficient cellulose enzymes to degrade cellulose to cellodextrins.

5.2.1 Characterization of cellodextrin permeases

Two cellodextrin transporters from a cellulolytic bacterium were successfully identified through the metabolic engineering of *Escherichia coli* KO11. The identification of cellodextrin transporters from cellulolytic microorganisms using engineered *Escherichia coli* demonstrates the potential application for the consolidated bioprocessing of biofuel production. Further,

detailed characterization of the identified cellodextrin permeases towards specific sugar-binding characteristics would provide insight about preferential utilization of certain sugars by these permease proteins. In order to achieve this, I suggest a detailed transcriptional analysis of the identified MFS permease proteins in order to identify a potential promoter sequences such as -10 and -35 regions and terminator sequences and thermodynamic properties of these cellodextrin-binding proteins. Studies using isothermal titration calorimeter could be performed to determine the abilities to bind different sugar molecules such as cellobiose, cellotriose, cellotetraose and cellopentaose. This analysis would provide key information such as binding constant, free energy and the binding stoichiometry of each protein. The results of such thermodynamic and kinetic data are useful in designing bioreactors for utilizing these proteins for bioethanol production from cellodextrin mixtures.

5.2.2 Engineer efficient cellulase enzymes to produce cellodextrins from cellulose

This dissertation demonstrates bio-ethanol production from cellodextrin oligomers as substrates. The results can be extended towards production of cellulase enzymes by *E.coli* so that crystalline cellulose can be hydrolyzed into oligomers of 2-7 glucose units and eventually to bio-ethanol. Suitable candidates of cellulose genes identified from cellulolytic bacteria should be cloned into recombinant plasmids and proteins should be tested for activity on a cellulose substrate. In particular, the cellulolytic enzymes from *Saccharophagus degradans* are of particular interest. Its genome encodes for several hydrolases, which enable this organism to grow on cellulose in nature. The organism also encodes for one cellobiohydrolase and four endoglucanase. A study also shows that it performs cellodextrin metabolism indicating that the secreted extra-cellular enzymes hydrolyze cellulose into cellodextrins[27]. A combination of multiple enzymes will be required for partial cellulose hydrolysis utilizing synergetic effects of individual enzymes.

Enzymes would be required to be secreted to the extracellular space, which required the addition of a signal sequence, which directs the expressed proteins to the periplasmic space.

CHAPTER 6

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