ENGINEERED MICROBIAL CONSORTIUM FOR THE EFFICIENT CONVERSION OF BIOMASS TO BIOFUELS

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Current energy and environmental challenges are driving the use of cellulosic materials for biofuel production. A major obstacle in this pursuit is poor ethanol tolerance among cellulolytic *Clostridium* species. The first objective of this work was to establish a potential upper boundary of ethanol tolerance for the cellulosome itself. The hydrolytic function of crude cellulosome extracts from *C. cellulolyticum* on carboxymethyl cellulose (CMC) with 0, 5, 10, 15, 20 and 25% (v/v) ethanol was determined. Results indicated that the endoglucanase activity of the cellulosome incubated in 5% and 10% ethanol was significantly different from a control without ethanol addition. Furthermore a significant difference was observed in endoglucanase activity for cellulosome incubated in 5%, 10%, 15%, 20% and 25% ethanol in a standalone experiment. Endoglucanase activity continued to be observed for up to 25% ethanol, indicating that cellulosome function in ethanol will not be an impediment to future efforts towards engineering increasing production titers to levels at least as high as the current physiological limits of the most tolerant ethanologenic microbes.

The second objective of this work was to study bioethanol production by a microbial co-culture involving *Clostridium cellulolyticum* and a recombinant *Zymomonas mobilis* engineered for the utilization of oligodextrans. The recombinant *Z. mobilis* ZM4 pAA1 and wild type ZM4 were first tested on RM medium (ATCC 1341) containing 2% cellobiose as the carbon source. Ethanol production from the recombinant *Z. mobilis* was three times that observed from the wild type *Z. mobilis*. Concomitant with ethanol production was the reduction in OD from 2.00 to 1.580, indicating the consumption of cellobiose. No such change in OD was observed from the wild type.

The recombinant ZM4 was then co-cultured with *C. cellulolyticum* using cellobiose and microcrystalline cellulose respectively as carbon sources. Results indicate that the recombinant ZM4 acted synergistically with *C. cellulolyticum* to utilize 2.0 g L^{-1} cellobiose, producing as much as 0.40 mM concentration of ethanol whereas only 0.20 mM ethanol was detected for the wild type ZM4 co-cultured with *C. cellulolyticum* under the same conditions. A co-culture of the recombinant ZM4 and *C. cellulolyticum* using 7.5 g L^{-1} microcrystalline cellulose gave lower ethanol yield than when using cellobiose. In the latter case, the recombinant began producing ethanol in 5 days whereas the wild type required 10 days to produce detectable ethanol. Future efforts will concentrate on identifying the correct concentration of cellulosic substrate at which synergy will be observed using the recombinant ZM4 and other cellulose degrading microorganisms, as well as optimizing medium formulations to better support both organisms.

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INTRODUCTION

The continual depletion of fossil fuel reserves, increasing world's population currently estimated at 7 billion, environmental catastrophes associated with crude oil drilling as evidenced by recent oil spillages around the globe, price fluctuations and artificial scarcities created by political unrest in some of the world's largest oil producing countries, the increasing emission of greenhouse gases (GHG) and the need to preserve the environment through sustainable practices have called for an aggressive search for alternative fuels with bioethanol leading the "pack" amongst other choices. Ethanol is perhaps considered to be the most promising to use as it is an alternative liquid fuel, and it can be readily produced from a variety of agriculture-based renewable materials like sugarcane juice, molasses, potatoes, corn and barley [1]. The potential of ethanol as a transportation fuel was conceived as early as 1890 [2]. The yeast *Saccharomyces cerevisiae* is used all over the world as a major ethanol-producing microorganism but despite its extensive use, it has a number of disadvantages such as high aeration cost, high biomass production, low temperature and low ethanol tolerance [3].

Zymomonas mobilis is a facultative anaerobic Gram-negative bacterium belonging to the alpha subdivision of the phylum Proteobacteria, class Alpha-Proteobacteria, order Sphingomonadales and family Sphingomonadaceae. It is rod shaped with dimensions 1.0-2.0 x 4.0-5.0 μm, motile, does not sporulate, and does not produce capsules, intracellular lipids or glycogen. The optimal pH range for growth is 6-7.0 and the optimal temperature range is 25-31ºC. The G + C content of the cellular DNA is about 47.5-49.5% with an average *T*m of 89.3-89.5ºC [4]. It was first isolated in tropical countries from alcoholic beverages like the African palm wine and the Mexican "pulque" [5]. It was a contaminant of the cider ("cider sickness") or beer in

European countries [5]. This microorganism is an efficient glucose, fructose and sucrose degrader, employing the Entner-Doudoroff (ED) pathway with a net yield of one ATP per mole of glucose [6]. The 50's and 60's witnessed some upheaval in the study of *Z. mobilis* but it was not until 1979 at the height of the petroleum crisis that a group of Australian researchers led by P.L Rogers reported on the great potential of *Z. mobilis* for ethanol production [7]. *Z. mobilis*, though classified as an anaerobic microorganism, also grows well under aerobic conditions but with less production of ethanol [8].

Clostridium cellulolyticum ATCC 35319 formerly identified as strain H_{10} was isolated in the fall of 1982 from decayed grass compost packed for 3 to 4 months at the Université de Nancy, France. It is a Gram-positive, straight to slightly curved rod 3 to 6 µm long by 0.6 to 1.0 µm wide, with a mean G + C content of 41% and forms spores in cultures of cellulose media three or more days old [9]. It belongs to the phylum *Firmicutes,* class *Clostridia,* order *Clostridiales* and in the family *Clostridiaceae.* Colonies appear after 5 to 6 days on cellulose agar medium at an incubation temperature of 35ºC, with the maximum growth temperature as 45ºC and the minimum at 25ºC [9]. *C. cellulolyticum* does not grow on adonitol, amygdalin, dulcitol, erythritol, glycerol, glycogen, inositol, lactose, maltose, mannitol, melezitose, raffinose, rhamnose, salicin, sorbitol, sorbose, sucrose and trehalose [9]. Carbon dioxide, hydrogen, acetate, ethanol, lactate, and formate are produced from the breakdown of cellulose because *C. cellulolyticum* produces several cellulases, which are regrouped into an extracellular enzymatic complex called cellulosomes [10]. Cellulolytic activities allow the release of soluble cellodextrins from cellulose, which in return permits microbial growth [10]. The cellulosome is of particular interest since it permits a highly efficient degradation of crystalline cellulose and offers exceptional potential biotechnological applications [11]. The cellulosomes are present on the bacterial cell surface and are dedicated to

cellulose depolymerisation [10]. The advantages of the biosynthesis of the cellulosomes are (i) a direct and specific adhesion to the substrate of interest permitting efficient competition with other microorganisms present in the same biota and (ii) the proximity of the cell to the cellulose ensures an efficient cellular uptake of the soluble cellodextrins by avoiding their diffusion in the extracellular milieu [12].

1.1 Lead Hypotheses

1) *Z. mobilis* is an efficient ethanologen capable of producing up to 16% v v^{-1} ethanol; however, it is only able to utilize simple sugars such as glucose, fructose and occasionally sucrose with the extracellular production of levan. This drawback is a major reason why it has not been used extensively for bioethanol production at an industrial scale.

2) *C. cellulolyticum* is excellent at cellulose utilization due to the presence of cellulosomes, a complex mass of enzymes comprised of an array of cellulases and hemicellulases, but unlike *Z. mobilis* has a low yield of ethanol production. It has been suggested that there is also an overflow of pyruvate higher than the rate of procession of pyruvate ferridoxin oxidoreductase (PFO) and lactate dehydrogenase (LDH) [141].

1.2 Specific Aims of the Project

Specific Aim 1A: Determine the ethanol tolerance of cellulosomes from *C. cellulolyticum* to establish the maximum potential conversion efficiency for cellulose to ethanol conversion.

1.1a) Isolate active cellulosomes from stationary phase cultures of *C. cellulolyticum* grown on cellulose medium.

1.2a) Test activity of the cellulosomes in media containing increasing concentrations of ethanol.

Specific Aim 1B: *Z. mobilis* will be genetically modified to increase its sugar utilization range from simple sugars to larger oligodextrins.

1.1b) The endoglucanase genes *celY* and *celZ* of the plant pathogen *Erwinia chrysanthemi* will be cloned and expressed in *Z. mobilis.* These two genes work synergistically to degrade larger oligodextrins into cellobiose, cellotriose, cellotetrose etc.

1.2b) The *beta-glucosidase* gene of the cow rumen bacterium *Ruminococcus albus* will be similarly introduced to degrade the resulting cellobiose and other short oligodextrins to glucose. The *glucose-fructose oxidoreductase* gene in *Z. mobilis* has a leader peptide sequence for exporting the matured glucose-fructose oxidoreductase protein. This leader sequence contains the twin arginine translocase (Tat) transport system recognition sequence facilitating its export to the extracellular milieu. This *gfor* gene leader sequence will be translationally fused to the *beta-glucosidase* gene sequence and resulting in export of the mature protein.

Specific Aim 1C: *Z. mobilis* will be modified to express the CipC protein on its surface in order to facilitate direct incorporation of *C. cellulolyticum* produced cellulolytic enzymes.

1.1c) The scaffoldin gene *cipC* of *C. cellulolyticum* contains the cellulose binding domain (CBD) for attaching the bacterium to cellulose, as well as several cohesin domains upon which the dockerin-bearing cellulases, hemicellulases etc would be anchored.

1.2c) The ice nucleation gene *inaZ* of *Pseudomonas syringae* S203 is responsible for ice formation and is expressed on the cell surface. The internal repeating units of the gene, which are responsible for ice formation, would be truncated and the N terminal and C terminal regions transcriptionally fused to the *cipC* gene and introduced into *Z. mobilis.* With this done, the cipC protein would be anchored on the cell surface of *Z. mobilis.* The free cellulases released in the medium would be anchored via their dockerin domains on the cohesin cipC protein.

1.3 Anticipated Results and Expected Benefits of the Engineering Effort

Furthermore, as there is no documented evidence of microbial interrelationship studies involving these two microorganisms in co-culture, this work will serve as a starting point for further investigation on any previously unknown pathways, the genes that are likely to be turned on and the proteins made in the face of an unusual carbon source for *Z. mobilis* and an unexpected higher than normal ethanol concentration for *C. cellulolyticum.*

1.4 Physiological Characteristics of *Zymomonas Mobilis* under Aerobic Conditions

The reduced growth and ethanol production rate under aerobic conditions can be explained

by the presence of two key enzymes of the *Z. mobilis* metabolism: a type 2 NADH oxidoreductase and an NADH oxidase [13]. The type 2 NADH oxidoreductase is relevant because it does not pump protons during electron transport under aerobic respiration, unlike the more common type 1 NADH oxidoreductase [14]. Therefore, type 2 NADH oxidoreductase does not contribute to the proton gradient of the cellular membrane, which is the driving force in generating ATP. Other membrane proteins such as cytochrome bc1 complex, electron transfer flavoprotein and ubiquinone proteins are present to generate the proton gradient and thereby drive ATP generation under aerobic conditions. *Z. mobilis* can grow under aerobic conditions at a slower rate due to decreased supply of ATP. NADH oxidase catalyzes the oxidation of NADH (NADH + 0.5 O_2→ NAD) under aerobic conditions. Because of this enzyme, the pool of NADH, which is used for ethanol production, is decreased, resulting in a decrease in capacity to produce ethanol under aerobic conditions [14]. In the study of the transcriptomic and metabolic profiling of *Z. mobilis* during aerobic and anaerobic fermentations, Yang et al. [15] made the following observations:

1. In the absence of oxygen, ZM4 consumed glucose more rapidly, had a higher growth rate and ethanol was the major end-product.

2. Greater amounts of other end-products such as acetate, lactate and acetoin were detected under aerobic conditions and at 26 hours there was only 1.7% (v v^{-1}) of the amount of ethanol present anaerobically.

3. In the early exponential growth phase, significant differences in gene expression were not observed between aerobic and anaerobic conditions via microarray analysis.

4. HPLC and GC analyses revealed minor differences in extracellular metabolite profiles at the corresponding early exponential phase time point and

5. Transcripts for Entner-Doudoroff pathway genes (*glk, zwf, pgl, pgk and eno)* and gene *pdc,* encoding a key enzyme leading to ethanol production, were at least 30-fold more abundant under anaerobic conditions in the stationary phase based on quantitative-PCR results.

1.5 Sugar Metabolism in *Zymomonas mobilis*

The central carbon metabolism of *Z. mobilis* is different compared to other known Gram-negative microorganisms such as *Escherichia coli* [13]. *Z. mobilis* metabolizes only glucose, fructose and sucrose through the Entner-Doudoroff pathway, producing ethanol and CO_2 and is unable to utilize the glycolytic pathway due to the absence of 6-phosphofructokinase, which converts fructose-6-phosphate into fructose-1, 6-bisphosphate [16]. *Z. mobilis* ZM4 also lacks two enzymes in the tricarboxylic acid (TCA) cycle: 2-oxoglutarate dehydrogenase and malate dehydrogenase [13]. Despite the absence of these enzymes, *Zymomonas mobilis* is still able to produce important building blocks including oxaloacetic acid, malic acid and fumaric acid through alternative metabolic pathways: phosphoenolpyruvate carboxylase (phosphoenolpyruvate + CO_2 → oxaloacetate + orthophosphate) and citrate lyase (citrate → acetate + oxaloacetate) for oxaloacetate production, and malic enzyme (malate ↔ fumarate) for fumaric acid production. Transport of D-glucose and D-fructose is by facilitated diffusion but rapid growth can be attained only by metabolizing considerable amount of sugars [17]. The conversion of glucose-6-phosphate to 6-phosphogluconate and of 3-phosphoglycerate to 2-phosphoglycerate are the rate-limiting steps in the sugar metabolism of *Z. mobilis* [18]. The phosphofructokinase and the allosteric hexokinase, which are the key regulatory enzymes in glycolysis, are absent in the Entner-Doudoroff pathway of *Z. mobilis*. However, the latter step in the two pathways is similar (from glyceraldehyde-3-phosphate to pyruvate and ethanol) [1]. Pyruvate decarboxylase (*pdc)*, an enzyme generally found in yeast and molds, is present in *Z. mobilis* [19]. It catalyzes the non-oxidative decarboxylation of pyruvate to produce acetaldehyde and carbon dioxide. Two alcohol dehydrogenase isozymes are present in *Z. mobilis* and they catalyze the reduction of acetaldehyde to ethanol during fermentation, accompanied by the oxidation of $NADH^+$ to NAD^+ [20]. Fructose

when used can also be phosphorylated by a constitutive kinase that is highly specific for it and ATP [1]. The ethanol yield obtained from fructose in batch fermentation was generally lower (90% of theoretical maximum) than that from glucose (95%) [21], the formation of byproducts such as dihydroxyacetone, mannitol, glycerol, sorbitol, acetoin, acetaldehyde, lactic acid and acetic acid are in part responsible for the reduction in the ethanol yield [21]. In the presence of oxygen, production of acetaldehyde by *Z. mobilis* was due to increased NADH oxidase activity, resulting in the decreased availability of NADH for the reduction of acetaldehyde to ethanol by alcohol dehydrogenase [1]. The ability to hydrolyze sucrose is not a common feature of *Z. mobilis* but it is rather strain dependent [6], with the enzyme levansucrase firmly established to be involved in its hydrolysis [22]. During the fermentation of sucrose to ethanol, *Z. mobilis* produced as much as 11% sorbitol, 70% small molecular weight oligomers and 2% levan from the original carbon source [23], which is a (2→6) linked β-D-fructose polymer with a molecular weight of 10^7 KDa corresponding to 60,000 fructose units [1]. Desalted molasses which is the non-crystallizable residue remaining after sucrose purification can be utilized by *Z. mobilis* for ethanol production [1]. A study found out that fermentation of diluted molasses using mutant strains of *Z. mobilis* BL4 suggested that 25% molasses as optimal for maximum ethanol production [24]. Another study revealed that amongst different substrates (*e.g.* sucrose, cane juice and molasses substrates) evaluated for ethanol production, cane sugar was fermented more efficiently at 185 g sugar L^{-1}, yielding 0.50 g ethanol g^{-1} sugar, whereas ethanol yields were lower (0.39 g g^{-1} sugar) from molasses and medium at similar sugar concentrations [25]. Other substrates include mannitol whose metabolism is oxygen dependent and therefore reduces ethanol yield but increases lactic acid production [26]. Corn/maize, potatoes, maltrin, wheat, milo, cassava and sago have been tested for ethanol production using *Z. mobilis* [27]. Industrial trials have also been made using wet

and dry milled corn as well as corn, milo, and wheat mixtures as feedstock. Corn was found easier to ferment than milo at production rates in excess of 0.25% h^{-1} ethanol with fermentation completed within 36-40 hours in comparison to a typical yeast fermentation of 52-70 hours [28]. Murugan and Rajendran [29] compared ethanol production by *Saccharomyces cerevisiae* and *Z. mobilis* using *Agave* leaves. Results indicated that the *Agave* leaves containing *Z. mobilis* yielded maximum ethanol (5%) whereas only 4% ethanol was recorded with *Agave* leaves containing *S. cerevisiae.* An unpublished study by Anieto and Allen also compared ethanol yield of *Z. mobilis* and *S. cerevisiae* when using waste cola syrup as substrate and preliminary results suggest that *Z. mobilis* utilizes the cola syrup faster, yielding up to 7% ethanol from 25% waste cola syrup within 72 hours while *S. cerevisiae* grows slower [30]. Currenty there exist two industrial processes of ethanol production using this microorganism: the 'Glucotech Process' by the Australian research group at the University of Queensland and the 'Bio-Hol Process' by the Canadian research group at the University of Toronto [31]. Figure 1.1 below shows the central metabolic pathway of *Z. mobilis* as well as the engineered pathways. Blue represents the engineered pathway while red represents missing enzymes from the ZM4 sugar metabolic pathway. Figure 1.2 shows the Entner-Doudoroff pathway of sugar metabolism.

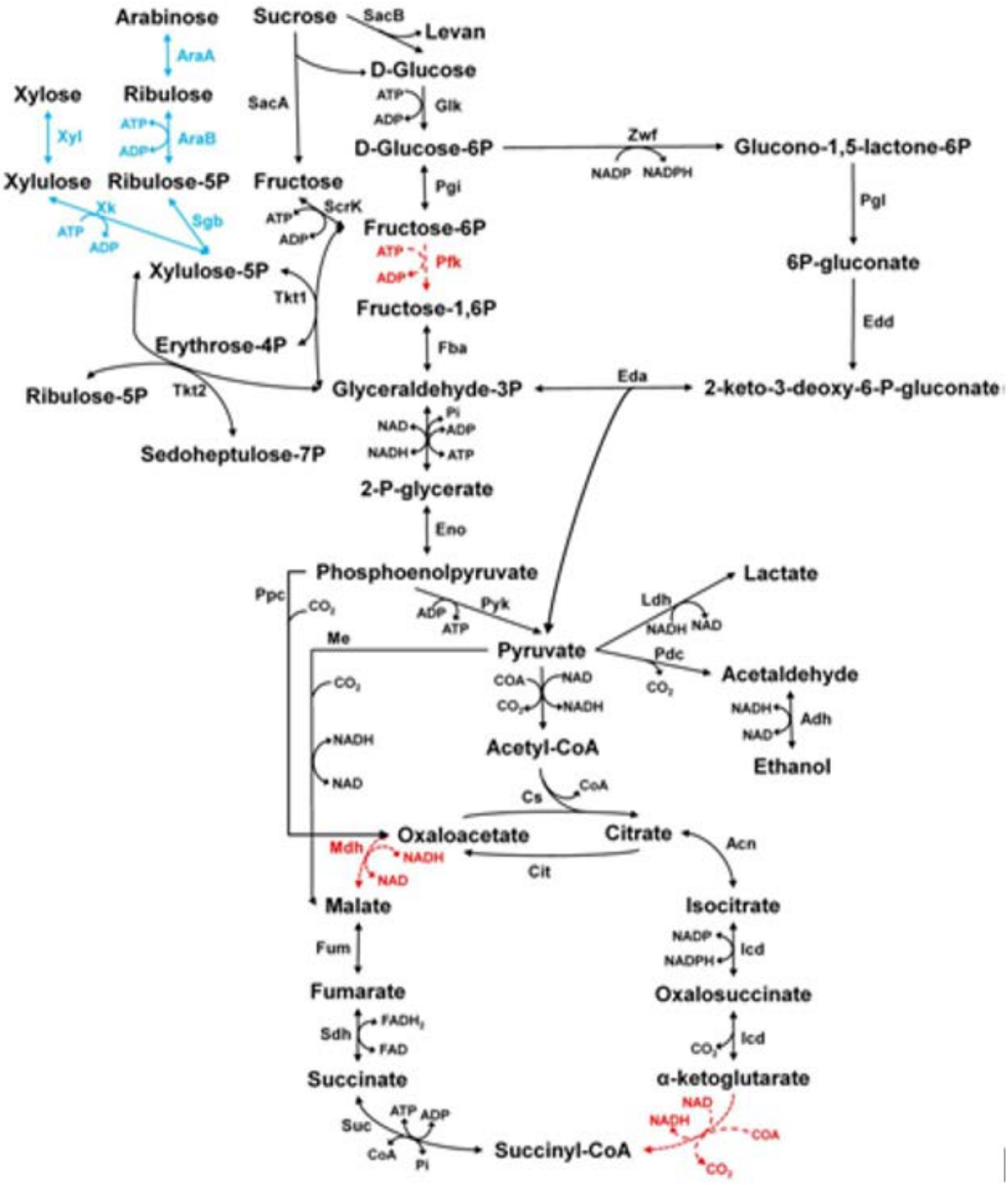


Figure 1.1: Central metabolism in *Zymomonas mobilis*. Figure from [13].

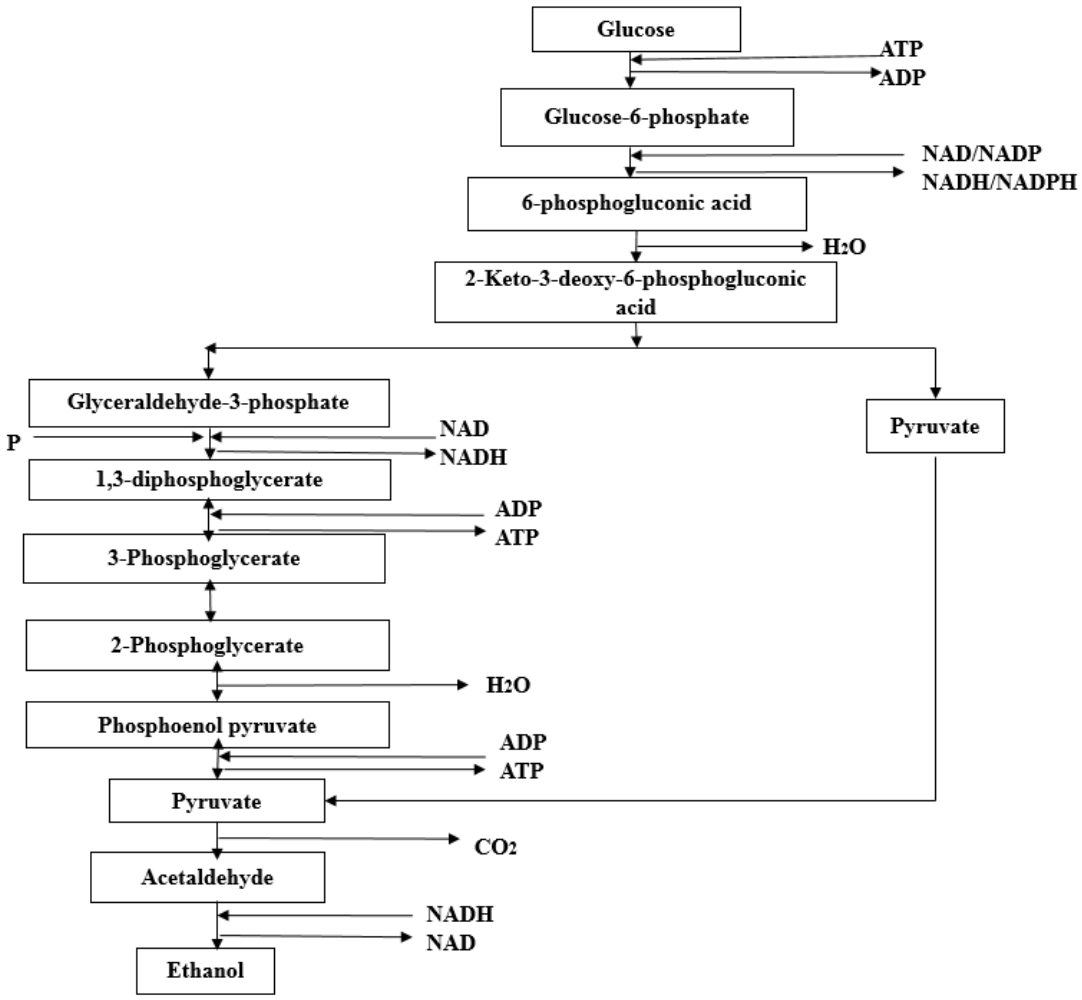


Figure 1.2: Entner-Doudoroff pathway in *Z. mobilis.* Adapted from [1]

1.6 Glucose-Fructose Oxidoreductase (GFOR)

Glucose-fructose oxidoreductase (GFOR) is an NADP-containing enzyme that is unique to *Z. mobilis* [32]. The enzyme whose matured version is located in the periplasm converts glucose to gluconolactone and fructose to sorbitol. Gluconolactone is then converted by gluconolactonase, another periplasmic enzyme, to gluconic acid [33, 34, and 35]. The physiological role of GFOR is the provision of sorbitol, a novel compatible solute for bacteria [33]. When cells are stressed with high sugar concentrations (either physiological sugars or maltose, which is not taken up or metabolized), they accumulate sorbitol intracellularly up to 1M [36] to counteract the detrimental

effects of dehydration exerted by high osmolarity. A mutant strain isolated as not producing sorbitol from sucrose was recently shown to be GFOR-deficient and unable to grow in the presence of 1M concentration of sucrose unless sorbitol was added [37].

1.7 Strain Improvement through Mutagenesis and Metabolic Engineering in *Zymomonas mobilis* through Recombinant DNA Technology

Strain improvement depends upon the nature, quality and quantity of the product desired and the amenability of the microorganism [38]. *Z. mobilis* has been found relatively resistant to mutagenesis, but ultraviolet (UV) light and 1-methyl-3-nitro-1-nitrosoguanidine (NTG) are effective mutagens for this microbe [39]. Using NTG, another strain of *Z. mobilis*, ZM 482, has been isolated which can tolerate higher concentrations of $Na^{+,}$ Mg^{+} and Cl^{-} ions. Application of the resultant strain helped in preventing the desalting of such medium ions from the fermentation media and made the fermentation process more economical [40]. A *Z. mobilis* mutant capable of simultaneous ethanol and ice nuclei production from agricultural byproducts such as sugar beet molasses, in steady-state continuous culture, has been constructed [41]. Tao and coworkers [42] reported ethanol fermentation by an acid-tolerant *Z. mobilis* under non-sterilized condition. The *Z. mobilis* was mutated with NTG, fermentation began at pH 4.5; the same ethanol yield of 73.1 g L^{-1} was achieved compared with the filter sterilization process of the medium. A new mutant has been developed which can metabolize xylose more rapidly than sucrose in mixed glucose/xylose mixtures [43]. For expanding the substrate range, especially for the utilization of plant biomass-derived polymers, several plant cell wall degrading enzyme (PCDE) genes have been cloned and expressed in *Z. mobilis*. However, similar to other Gram negative bacteria such as *E. coli*, the presence of an outer membrane results in inefficient protein secretion, which is a major technical challenge in engineering cellulolytic *Z. mobilis* [44].

Various attempts have been made to widen the substrate range of *Z. mobilis* to include industrially attractive feedstock such as whey, starch and cellulose [1]. Recombinant strains were usually selected using resistance and sensitivity to antimicrobial agents. Feldmann [45] and Zhang [46] were first to report on the engineering of pathways for xylose and for mannose metabolism. The cellulase gene from *Erwinia chrysanthemi* encoding endoglucanase, cloned into *Z. mobilis* with the help of plasmid RP4, expressed 40% cellulase activity in culture media [47]. The cloning of the cellulase gene of *Acetobacter xylinum* in *Z. mobilis* IFO 13756 resulted in a ten fold greater level of gene expression than that in *Escherichia coli* [48]. The substrate range of *Z. mobilis* ATCC 39676 was also expanded to include L-arabinose by the introduction of genes encoding L-arabinose-isomerase, L-ribulokinase, L-ribulose-phosphate-4-epimerase, transaldolase and transketolase of *Escherichia coli*. The engineered strain with plasmid pZB 206 grew on L-arabinose as the carbon source and produced ethanol at 98% of the theoretical yield [49].

Several genomic DNA-integrated strains of *Z. mobilis* have been developed through the insertion of all seven genes necessary for xylose and arabinose fermentation into the *Zymomonas* genome and were tested for stability by repeated transfer in a non-selective medium [50]. Liang and Lee [51] reported a successful transformation of *Z. mobilis* with plasmid pKT230 by electroporation. In this study, optimal field strength of 7.5 kV cm^{-1} produced the best transformation efficiency. Yanase et al. [52] also described transformation of *Z. mobilis* with pZA31, pZA32 and pZA33 shuttle vectors using the spheroplasts method. The frequency of transformation obtained was 10^4 to 10^5 transformants μg^{-1} of DNA for *Z. mobilis* IFO13756 (Z-6). Other reported methods of transformation include conjugation [53] and $CaCl_2$ procedure by Su and Goodman [54]. Yanase et al. [55] introduced genes encoding mannose and xylose catabolic enzymes from *Escherichia coli*; the introduction of *E. coli manA* into *Z. mobilis* chromosomal

DNA conferred the ability to co-ferment mannose and glucose producing 91% of the theoretical yield of ethanol within 36 h. They further introduced a recombinant plasmid harboring the genes encoding *E. coli xylA, xylB, tal* and *tktA* and broadened the range of fermentable sugar substrates for *Z. mobilis* to include mannose and xylose as well as glucose with 89.8% theoretical yield of ethanol within 72 hours. The *β*-glucosidase gene from *Xanthomonas albilneans* or *Ruminococcus albus* was expressed in *Z. mobilis* [56, 57]. Yanase et al. [56] expressed a *β*-glucosidase gene from *Ruminococcus albus* fused with the Tat (twin arginine translocation) signal peptide of a periplasmic enzyme, glucose-fructose oxidoreductase (Gfor), or Sec-dependent secretion signal peptide of gluconolactonase (Gln). They found that the enzyme thus produced was secreted into both the periplasmic and extracellular space. With the Tat and Sec signal peptides, 4.7% and 11.2% of the *β*-glucosidase activity, respectively, were detected in the extracellular space of the recombinant *Z. mobilis*. However, these genes could not support the growth of recombinant *Z. mobilis* on cellobiose as the sole carbon source. Linger et al. [58] heterologously expressed two cellulolytic enzymes E1 (endo-*β*-1, 4-glucanase) and GH12 (broad substrate range endo-*β*-1,4-glucanase activity) from an *Acidothermus* species by using two different secretion signals of *Z. mobilis* genes, *phoC* gene and ORFZM0331. The lack of a secretion signal in their gene resulted in the localization of 96% of GH12 activity within the cytoplasm, whereas the addition of the *phoC* secretion signal resulted in the localization of approximately 26% of the enzyme activity in the periplasmic space and 13% in the extracellular space. For E1 with the *phoC* secretion signal, approximately 20% of the E1 activity was found in the extracellular medium, 30% in the periplasmic space and 50% in cytoplasm. To develop *Z. mobilis* as a viable platform host organism for cellulosic biofuel production, more studies are needed to engineer strains that secrete multiple PCDEs into the extracellular space necessary for the degradation of plant biomass [44].

1.8 Ethanol Tolerance in *Zymomonas mobilis* and the Function of Hopanoids

The mechanism of resistance to high concentration of ethanol in *Z. mobilis* can be attributed in large part to its membrane structure. *Z. mobilis* contains fatty acid chains in its membrane lipids that have more than four carbon atoms longer than those of most bacteria, with the increase in chain length corresponding to increased ethanol tolerance [59]. Over 70% of the fatty acyl chains in the phospholipids of *Z. mobilis* are *cis*-18:1 (Δ11) fatty acid (vaccenic acid) [60]. Ethanol-dependent changes in the membranes of *Z. mobilis* include a decrease in the phospholipid content, an increase in the proportion of the cardiolipin and phosphatidylcholine and an increase in the proportion of hopanoids (evolutionary precursors of sterols) [61]. Biological membranes are of critical importance for cell function such as cell growth and integrity; they are composed predominantly of proteins and lipids that form heterogeneous, two-dimensional assemblies of molecules [62].

Many bacteria contain hopanoids, which are sterol analogues in bacteria. These pentacyclic triterpenoid lipids are synthesized from isopentenyl units, which are formed through a biosynthetic route leading to isopentenyl diphosphate. Six C_5 units are joined to form squalene, the immediate precursor in hopanoid synthesis. Hopanoids are membrane components involved in regulating membrane fluidity and stability [62]. A range of techniques (Langmuir film balance, calorimetric techniques, black lipid membranes, stop flow permeability techniques, solid-state magnetic resonance spectroscopy, etc.) have shown that hopanoids regulate membrane fluidity and induce order in the phospholipid matrix of membranes. Hopanoids increase order at physiological temperature (above the phase transition temperature at which increased molecular disorder threatens membrane stability and cell survival) [63]. Studies on liposome membrane permeability have shown that hopanoids can reduce molecular permeability and increase liposome membrane

stability [64]. *Z. mobilis* is rendered sensitive to ethanol in the presence of inhibitors preventing hopanoid biosynthesis [65]. *Frankia* bacteria, which form symbiotic nodules on a number of mainly shrubby plants, contribute another twist to hopanoid function [66]. These bacteria express the oxygen-sensitive nitrogenase in vesicles that are surrounded by extracellular layers of lipids. The lipids layers are largely composed of hopanoids and it has been suggested that these hopanoid layers are part of the oxygen-protection mechanism for the nitrogenase [66]. Figure 1.3 shows elongated hopanoids condensing action on phospholipids in bilayer membranes while figure 1.4 shows the different forms of the hopanoids.

While investigating the effect of ethanol and oxygen on the growth of *Z. mobilis* and the levels of hopanoids and other membrane lipids, Moreau et al. [67] reported that *Z. mobilis* was more tolerant to higher ethanol concentrations when grown anaerobically. Addition of ethanol to the medium caused complex changes in the levels of hopanoids and other lipids but with no significant increase in any of the hopanoid lipid classes as the level of ethanol increased. In ethanol-tolerant microorganisms, adaptation to ethanol stress involves an increase in the mean chain length of incorporated fatty acids, thereby thickening the hydrophobic membrane core. Often the proportion of monosaturated fatty acid (*cis*-vaccenic acid) also increases [60]. In another study, Hermans et al. [68] observed that variations of the growth rate of *Z. mobilis* between 0.08 and 0.40 h^{-1} did not show a marked effect on the hopanoid content neither did the variations of ethanol concentration on the composition of extracted lipids.

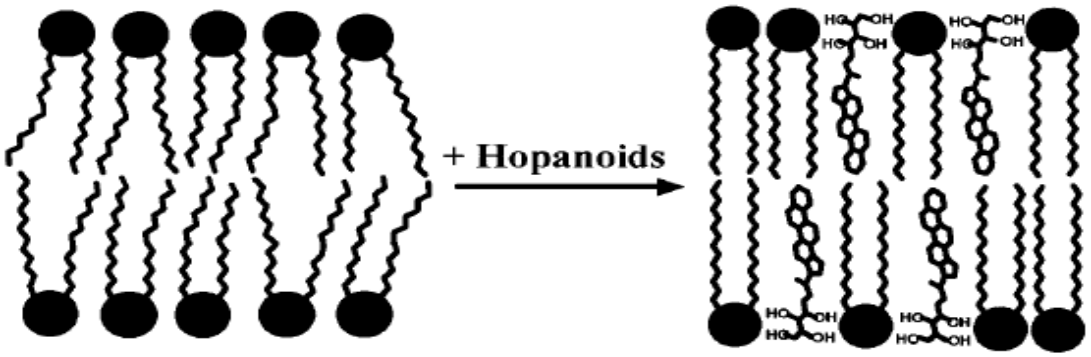


Figure 1.3:Schematic representation of the condensing action imposed by elongated hopanoids on phospholipids in bilayer membranes. Taken from [62].

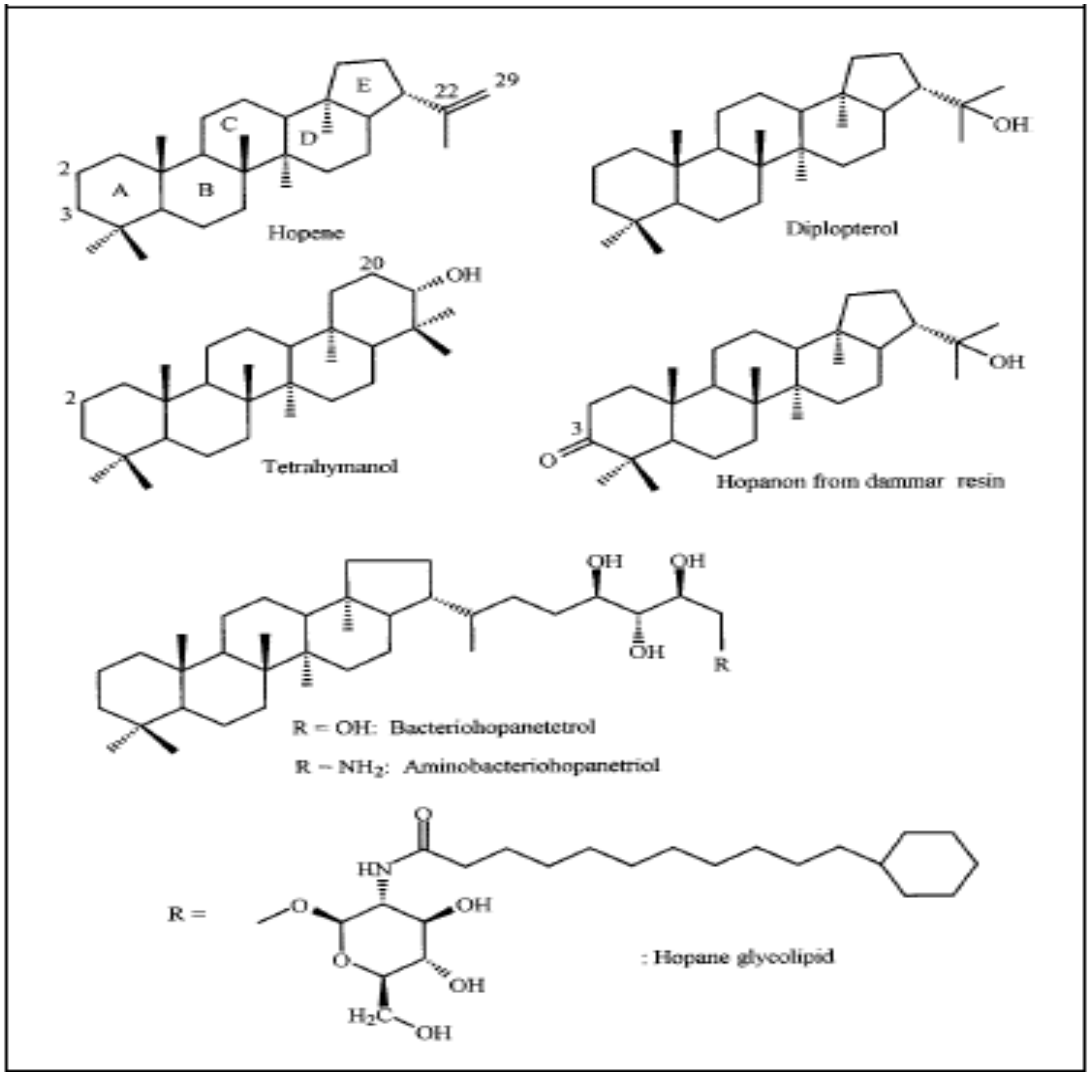


Figure 1.4:Examples of non-elongated and elongated hopanoids and tetrahymanol. Taken from [62].

1.9 Transcriptomic Profiling of *Zymomonas mobilis* Under Ethanol Stress [69]

Ethanol acts as an inhibitor of the cell growth and metabolism in *Z. mobilis*, thus resulting in the decrease of the rate of sugar conversion to ethanol [69]. Michel et al. [70] found that differential expression of related proteins are involved in ethanol-shock response, and the first genome sequence of *Z. mobilis* ZM4 by Jeffries [71] suggested that sigma factor (σE ZMO4104) may play an important role in resisting ethanol stress. In a recent study by He et al. [69], microarray technology was used to investigate the expression profiling of the ethanologenic *Z. mobilis* in response to ethanol stress. 1800 gene fragments were amplified by PCR and spotted onto a glass slide and using the microarray, the global transcriptional response of *Z. mobilis* ZM4 to ethanol stress was examined at 24 h post-inoculation under normal (media with no ethanol) and stress conditions (media with 5% ethanol). Of the 1800 genes examined by microarray analysis, 127 genes (7% of the total number of open reading frames represented on the array) were identified as being significantly up- or down-regulated (fold change $\geq$ 2.0, $P \leq 0.05$) during ethanol stress condition. Eighty-nine genes were up-regulated after 24 h post-inoculation under ethanol stress condition and 38 genes were down-regulated. Approximately 34% of the genes down-regulated in the presence of ethanol were related to metabolism. In the presence of ethanol, about 62% of the genes related to regulation, cell processes, transport and unknown function showed greater expression as compared to normal conditions. Nearly 24% of the genes, including plasmid encoding genes, showed greater expression under stress condition. Twenty-three Entner-Doudoroff pathway mRNAs such as *glk, zwf, pgl, pgk* and *eno,* as well as ethanol fermentation-related genes like *pdc* and *adhB* were shown to be less abundant under stress conditions but at levels not considered significant. The ORFs related to stress shock-responsive molecular chaperone complex proteins, such as DnaK, DnaJ, GrpE, HSP-33, etc. were also not affected

significantly under ethanol stress. There are five open reading frames in ZM4 (designated as *hpnA-E)* associated with hopanoid biosynthesis in a close arrangement with the squalene-hopene cyclase (*shc*) gene. The genes *hpnC* and *hpnD* involved in the hopanoid biosynthesis pathway were shown to be down-regulated (0.8-fold) in the presence of 5% ethanol after 24 h incubation. However, *hpnA, hpnB* and s*hc* were shown to be up-regulated (nearly 1.4-fold) under the same condition. Fifty-four transcriptional activators and repressors were identified in *Z. mobilis* genome and 33 were slightly down-regulated under ethanol stress while only 3 transcriptional regulators showed significant differential expression.

1.10 The Genome Sequences of the Ethanologenic Bacteria *Zymomonas mobilis* ZM4 and *Zymomonas mobilis* (ATCC 10988)

The complete genome of *Z. mobilis* ZM4 (ATCC 31821) consists of a singular circular chromosome of 2,056,416 bp with an average G+C content of 46.33%. The 1998 predicted coding ORFs cover 87% of the genome, and each ORF has an average length of 898 bp. Among these, 1,346 (67.4%) could be assigned putative functions, 258 (12.9%) were matched to conserved hypothetical coding sequences of unknown function and the remaining 394 (19.7%) showed no similarities to known genes [72]. *Z. mobilis* (ATCC 10988) has also been sequenced and was found to be smaller than ZM4 by 34,590 bp but with an average of 98% sequence identity to ZM4 [73]. The entire genome contains 1,875 protein-encoding genes, 48 tRNA, and 6 rRNA genes (two rRNA clusters) in the chromosome.

CHAPTER 2

THE CELLULOSOMES OF Clostridium cellulolyticum

The concept of the cellulosome was first introduced with the thermophilic cellulolytic anaerobic bacterium *Clostridium thermocellum* [74]. Cellulolytic activities allow the release of soluble cellodextrins from cellulose, which permits in return microbial growth [10]. The final products of cellulose digestion are water and CO_2 in aerobic conditions, and short chain fatty acids in anaerobiosis [75]. Since the first step in cellulose metabolism or degradation involves the action of cellulases, a lot of research has been focused on these enzymes in the past few years [76]. The cellulosomes received particular attention since it permits a highly efficient degradation of crystalline cellulose and offers exceptional biotechnological applications [77]. Cellulosomes are present on the bacterial cell surface and are dedicated to cellulose depolymerisation [10]. The biosynthesis of the cellulosomes has several advantages for the bacterial cell, which are

1. A direct and specific adhesion to the substrate of interest permitting efficient competition with other microorganisms present in the same environment.
2. The proximity of the cell to the cellulose ensures an efficient cellular uptake of the soluble cellodextrins by avoiding their diffusion in the extracellular medium [78].

The cellulosome from an enzymatic viewpoint (i) allows optimum concerted activity and synergism of the cellulases, (ii) avoids non-productive adsorption of the cellulases, (iii) limits competition between cellulases for the sites of adsorption, and (iv) allows optimal processivity of the cellulose along the cellulose fiber [79].

Most of the cellulosomal genes of *C. cellulolyticum* are clustered in an approximately 26 kb long DNA fragment in which 12 genes have been identified, i.e. *cipC-cel48F-celBC-cel9G-cel9E-orfX-cel9H-cel9J-man5K-cel9M-rgl11Y-cel5N* (figure 2.1 below)

Figure 2.1:Schema of the *cel* cluster in *C. cellulolyticum*. Adapted from [10].

This cluster represents the largest *cel* cluster described in cellulosome-producing *Clostridia* [80]. The *cipC* known as the scaffolding gene or scaffoldin is the first gene of the *cel* cluster. It encodes a specialized integrating protein without any catalytic activity [10]. Cellulosome integrating protein C (CipC) permits binding of the different catalytic cellulosomal components [10]. CipC has a modular organization consisting of eight cohesin domains of type 1 (Coh_I) numbered from 1 to 8 from the N- to the C-terminus, permitting the tight binding of complementary dockerin domain of type 1 (Doc_I) borne by the cellulosomal enzymes [10]. Figure 2.2 below shows the schematic organization of the cellulosome of *Clostridium cellulolyticum.*

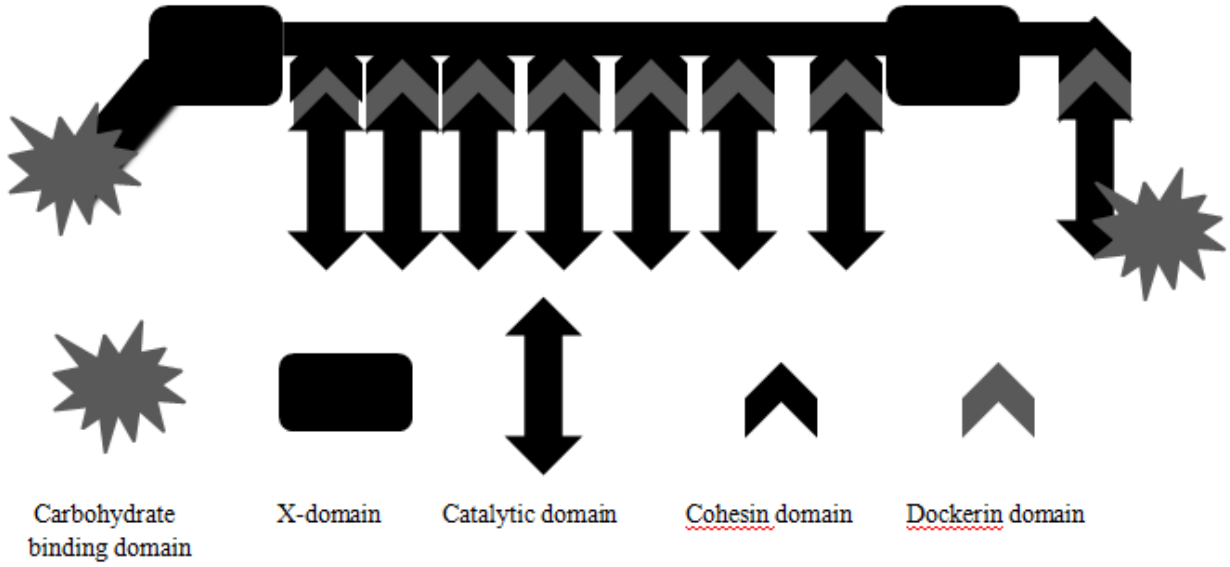


Figure 2.2:Schema showing the organization of the *Clostridium cellulolyticum* cellulosome around scaffoldin protein cipC. Adapted from Desvaux [10].

A carbohydrate-binding module (CBM) permitting adhesion to the cellulose and two X-modules are also present [81, 82]. The CBM of *cipC* belongs to the family IIIa and allows the tight binding of the entire cellulosome to the cellulosic substrate [10]. The exact function of X-module is still speculative and also the crystallization of the cipC has proved extremely difficult [83].

2.1 Cellulosomal Enzymes

As of the year 2005, 12 cellulosomal enzymes had been identified but only the following were biochemically characterized (1) Cc-Cel5A, (2) Cc-Cel5D, (3) Cc-Cel8C, (4) Cc-Cel48F, (5) Cc-Cel9G, (6) Cc-Cel9E, (7) Cc-Cel9M and (8) the rhamnogalacturonase Cc-Rgl11Y [10]. The common feature of all cellulosomal enzymes is the presence of dockerin domains of type I, which interacts with one of the cohesin domains of the scaffoldin [10]. A non-cellulosomal enzyme Cc-Cel5I is the only cellulase so far identified that does not belong to the cellulosome [84]. The three main classes of enzymes in the cellulosome are the *cellulases,* which can hydrolyze the β-1,4-glycosidic bonds of the cellulose by general acid hydrolysis [85], the *hemicellulases* with Cc-Man5K as the main enzyme under this class and the *pectinases* with Cc-Rg111Y as the only cellulosomal pectinase characterized in *C. cellulolyticum* [84]. Little is known about protein secretion in *C. cellulolyticum* even though bioinformatic analyses of the signal peptides of the cellulosomal components suggest that they are all secreted by the Sec (secretion) apparatus [86].

The way the *C. cellulolyticum* cellulosome is attached to the bacterial cell surface is currently unknown, but in *C. thermocellum,* the scaffoldin cellulosome integrating protein A (CipA) possesses a type II dockerin domain (Doc_{II}) which interacts with a type II cohesin domain (Coh_{II}) of the scaffoldin [86]. Blouzard et al. [87] reported the existence of approximately 30 dockerin-containing proteins by *C. cellulolyticum* grown on cellulose with the majority belonging

to glycosyl hydrolyses (GH). The breakdown of cellulose involves a synergistic mechanism of the cellulosomal complex; a mixture of different cellulosomal fractions tagged F1, F3 and F6 where the most divergent cellulosomal composition showed the most synergistic effects and the highest level of activity on straw, which was the most heterogeneous substrate tested [88]. In another study on the analysis of cohesin-dockerin interaction using mutant dockerin proteins, Sakka et al. [89] revealed that *Clostridium thermocellum* Xyn11A dockerin which has a typical dockerin sequence in which two amino acid residues are specifically conserved within the two segments of the dockerin modules, can recognize *Clostridium josui* cohesin modules in a non-species-specific manner. This is evidentiary to the fact that cellulosomal enzymes of *Clostridia* may not be cohesin specific. Tolonen et al. [90] discovered that the deletion of the single-family 9 cellulase gene in *Clostridium phytofermentans* prevents growth on cellulose although the mutant strain grows very well on glucose. Whilst this has not been investigated in *C. cellulolyticum* it can be said, albeit with caution, that such deletion could affect the entire activity of the cellulosomal enzymes since all cellulolytic *Clostridia* share close sequence similarities. A designer minicellulosome with specific functions has also been created; this has been accomplished by using a miniscaffoldin protein containing cohesins that would interact specifically with designated enzymes containing cognate dockerins [91]. To date, the three most important factors for efficient degradation of plant cell walls are (a) efficient binding of substrate, (b) multiplicity of enzymes types and (c) synergy between enzymes [92].

Other research interests on *Clostridium spp.* and their cellulosomes include a study by Tracy et al. [93] on the importance of their exceptional substrate and metabolic diversity for biofuel and biorefinery applications. With the current challenges in managing municipal solid waste (MSW), cellulases and cellulosomes could have great potential [94]. In a recent study by Li et al.

[95], a mobile group II intron originating from the *Lactoccous lactis*, the L1.LtrB intron, was used to disrupt both the paralogous L-lactate dehydrogenase and L-malate dehydrogenase genes, distinguishing the overlapping substrate specificities of these enzymes. Both mutations were then combined into a single strain, resulting in a substantial shift in fermentation toward ethanol production. This double mutant produced 8.5-times more ethanol than wild-type cells growing on crystalline cellulose.

2.2 The Endoglucanases (*celZ* and *celY*) from *Erwinia chrysanthemi*

Erwinia chrysanthemi is a pathogenic enterobacterium, which causes soft-rot disease in plants [96]. It secretes into the extracellular medium several enzymes, which allow it to digest the plant cell walls [97]. CelY and CelZ proteins are two different glucanases from this microorganism. With carboxymethyl cellulose (CMC) as a substrate, 95% of the total endoglucanase activity was attributed to CelZ while only 5% of the activity attributed to CelY, but synergy was observed when both enzymes were used on the substrate that resulted in an increase in activity of 1.8-fold [98]. In another related study, genes *celY* and *celZ* from *Erwinia chrysanthemi* were both functionally integrated into the chromosome of *Klebsiella oxytoca* P2 using surrogate promoters from *Zymomonas mobilis* for expression. This resulted in the secretion into the extracellular milieu of more than 20,000 endoglucanase units (carboxymethyl cellulose activity) per liter of fermentation broth [99]. Another strain of *Klebsiella oxytoca* M5A1 containing chromosomally integrated genes for ethanol production from *Zymomonas mobilis* and endoglucanase genes from *Erwinia chrysanthemi (celY, celZ)* produced 20,000 U of endoglucanase L^{-1} [100].

2.3 Ice Nucleation Proteins of *Pseudomonas syringae*

Ice nucleation in bacteria was first described in *P. syringae* in 1974 [101]. Other microorganisms with the ability to catalyze ice formation in supercooled water include *Erwinia herbicola, Pseudomonas fluorescens* and *Xanthomonas campestris* pathovar translucens [102, 103, and 104]. Bacterial ice nuclei can incite plant frost injury to many plant species [94]. Most plant species can supercool to about -5ºC and frost-sensitive plants species can avoid damaging ice formation above -5ºC if ice bacterial species are absent [105]. Bacterial ice nuclei active at warm subfreezing temperatures may potentially play important roles in artificial snow production, weather modification and in freezing of certain food products [105]. Ice nucleation activity has also proved to be useful as the basis for a reporter gene system whereby a promoterless ice nucleation gene is fused with a gene of interest to monitor transcriptional activity [105]. Ice nucleation activity can be quantified conveniently by a droplet freezing assay, in which environmental samples are suspended without prior preparation in an aqueous solution; small droplets from dilutions of the suspensions are cooled to an assay temperature such as -5ºC and the fraction of the droplets that freeze are recorded [105]. Ice bacterial species are common epiphytic inhabitants of healthy plants in nature [106]. The expression of ice nucleation activity in bacteria is not metabolically demanding, with only approximately 300 IceC protein molecules produced even in actively expressing cells of *P. syringae* [107].

The ice-nucleation protein (InaZ) of *P. syringae* is an outer membrane protein capable of imparting ice crystal formation on the supercooled water [108]. It is a monomeric protein composed of slightly more than 1200 amino acid residues with a deduced molecular weight of 118 KDa [109]. The specific amino acid residues of its N-terminal region (INPN) seem to interact with the phospholipid moiety of the outer membrane [110]. The major part of the central region INP

(81%) consists of a series of hierarchically organized, repeated amino acid sequences with lengths of 8, 16, and 48 amino residues, which act as templates for ice crystal formation [111]. The specific C-terminal region (INPC) is highly hydrophilic and exposed to the outermost cell surface [112]. INP (InaZ) as a surface anchoring motif has several characteristics, which are:

1. The INP resides in the outer membrane and is stably expressed at the stationary phase of the culture [112, 113],
2. The internal repeating domain is modulatable in length from the surface [114], and
3. The gene encoding INP can endow various Gram-negative bacteria such as *Escherichia coli* with ice nucleation activity [115].

As a result, of these innate qualities of the *ina*Z gene, it has been used by several researchers to anchor genes of interest on the cell surface and has also served as a reporter gene. Anchoring of cellulases on the cell surface would confer on an engineered microorganism the ability to degrade cellulose and larger oligodextrins in a manner analogous to that of cellulosome-producing Clostridia. Jung et al. [116] demonstrated the expression of *Bacillus subtilis* carboxymethylcellulase (CMCase) on the surface of *Escherichia coli* by using *Pseudomonas syringae* ice nucleation protein. The researchers also demonstrated that INP deleted from the central repeating domain could still direct CMCase to the cell surface. Figure 2.3a and 2.3b show the different modulations of the INP with 2.3b showing the attachment of the CMCcase expressed externally.

In another study, Drainas et al. [115] transferred the ice nucleation gene *inaZ* in *Z. mobilis* and quantified its expression under the control of three different promoters. The result showed that the *inaZ* gene can serve as an easily assayable reporter gene in this bacterium and that a significant proportion of ice nuclei active at temperatures of above -7ºC may be released into the culture medium. Jung et al. [117] demonstrated the utility of the Inp surface display by fusing levansucrase (LevU) to the C-terminus of Inp for the bioconversion of sucrose to levan.

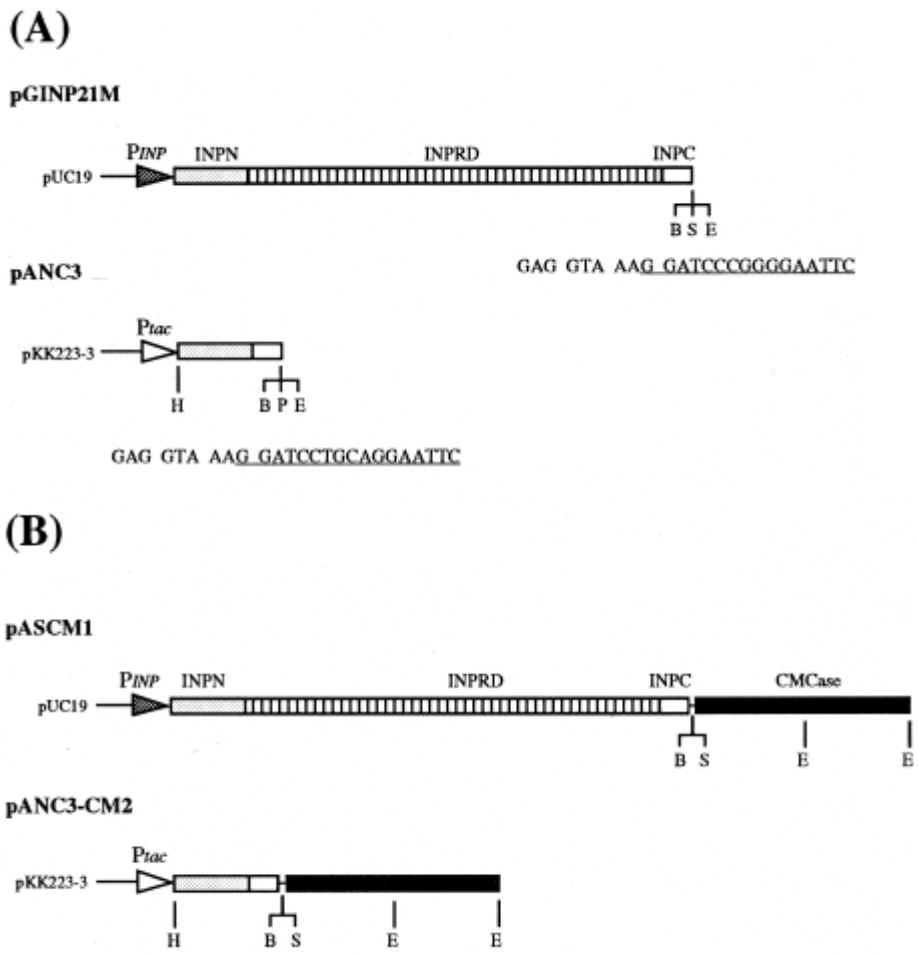


Figure 2.3: (A): The structure of the constructed surface anchoring proteins in plasmids pGINP21M and pANC3 [116]. (B): Abbreviations of B, S, E, P, and H denote recognition sites for *Bam*HI, *Sma*I, *Eco*RI, *Pst*I and *Hind*III, respectively [116].

The cells expressing Inp-LevU were found to retain both the ice-nucleation and whole-cell levansucrase enzyme, indicating the functional expression of Inp-LevU hybrid protein on the cell surface. Savvides [118] reported the simultaneous ethanol and bacterial ice nuclei production from sugar beet molasses by a *Z. mobilis* CP4 strain expressing the *inaZ* gene of *P. syringae* in continuous culture. Results showed greater tolerance to high sugar concentration due to their phospholipid and fatty acid contents. Therefore, anchoring genes of interest on the cell surface with an *inaZ* fusion gene could contribute to the stability of the cell membrane in *Z. mobilis* and in turn could result in greater ethanol tolerance. Overall, microbial cell-surface display has many

potential applications which include live vaccine development, peptide library screening, bioconversion using whole cell biocatalysts and bioadsorption [119].

2.4 Synthetic Biology, Microbial Consortia and Coculturing

The term synthetic biology is almost as old as the term genetic engineering [120]. However, synthetic biology has recently become a field of its own mostly driven by the advances in DNA synthesis, sequencing and systems biology [120]. Synthetic biology has broad applications in agricultural, medical, chemical and food industries. Examples of landmark accomplishments include microbial production of artemisinic acid, a key precursor of the commonly used antimalarial drug artemisinin [121], commercial manufacture of bio-derived 1.3-propanediol (an industrial chemical with a variety of applications in solvents, adhesives, resins, detergents and cosmetics [122], and the reconstruction of a complete microbial genome by the J. Craig Venter Institute [123]. Synthetic biology can be used to engineer recombinant microorganisms capable of efficiently converting plant biomass to biofuels such as long-chain alcohols [124], alkenes [125], biodiesel and jet fuels [126]. Atsumi et al. [124] engineered an *E. coli* strain with the *n*-butanol biosynthetic pathway from Clostridia. This engineered strain produced 552 mg L^{-1} of *n*-butanol whereas an engineered strain of *S. cerevisiae* harboring a similar *n*-butanol biosynthetic pathway can only produce 2.5 mg L^{-1} of n-butanol [126]. Most recently, Steen et al. [127], through extensive metabolic engineering, increased *n*-butanol production in *E. coli* to 30 g L^{-1} and yield to 0.287 g g^{-1} glucose, which exceeds *n*-butanol production in the native *Clostridia* host. Keasling and coworkers engineered recombinant *E. coli* strains to overproduce free fatty acids through cytosolic expression of a native *E. coli* thioesterase and deletion of fatty acid degradation genes [128]. After the introduction of ethanol production genes from *Zymomonas mobilis* and overexpression of

endogenous wax-ester synthase, direct production of fatty acid ethyl esters (FAEEs) was achieved at 674 mg L^{-1}, which was 9.4% of the theoretical yield. In addition to biofuels, a wide variety of value-added compounds can also be produced by recombinant microorganisms engineered by synthetic biology and metabolic engineering strategies [129]. For example the Staphanopoulos group engineered a recombinant *E. coli* strain capable of producing taxadiene, the precursor of the widely used anticancer drug taxol [130]. Lee et al. [131] engineered an *E. coli* strain to produce succinic acid, and Raab et al. [132] also engineered *S. cerevisiae* for the production of succinic acid. The engineered *E. coli* strains can produce succinic acid from glucose with up to 99.2 g L^{-1} whereas the engineered *S. cerevisiae* strain can only produce 3.6 g L^{-1} succinic acid. In another study, a recombinant *E. coli* strain was engineered to produce another platform chemical, xylitol, from a mixture of hemicellulose sugars [133].

Zuroff and Curtis [134] defined consortia as interactive groupings of microorganisms ranging from defined dual species communities to undefined, multi-species aggregations. Natural consortia has been extensively studied by a number of scientists including Brune [135] who pointed out that consortia in the gut of various termite species are capable of efficient lignocelluloses utilization. Chaffron and von Mering [136] added that elucidation of the mechanisms of termite lignocelluloses utilization can be applied to the development of biofuel production systems. On this note, Warnecke et al. [137] using metagenomic analysis revealed that spirochetes and *Fibrobacter* species are responsible for cellulose and hemicelluloses utilization in the "higher" *Nasutitermes* species. Brune [135], Rouland-Lefèvre and Bignell [138] reported that the ability of other termites (specifically, Macroterminitae) to achieve high conversion of lignocelluloses is due to an exosymbiosis with fungus from the genus *Termitomyces.* Schink [139] noted that organic material breakdown in these natural systems is dependent on a symbiotic

interaction between anaerobic bacteria and methanogenic or sulfate-reducing bacteria which consume hydrogen as it is produced to drive the process forward. An example of this symbiosis is the breakdown of cadavarine; an aliphatic amine intermediate produced proteinaceous organic matter degradation [134].

There has been much progress in the development of genetically engineered consortia to perform unique tasks, and these approaches may be applicable to biofuel-producing consortia [134]. Shin et al. [140] genetically engineered two *E. coli* strains for xylan utilization. One strain was engineered to co-express two hemicellulases to hydrolyze xylan into xylooligosaccharides and the second imports the xylooligosaccharides to produce ethanol. The ethanol yield for this co-culture on purified xylan was approximately 55% of the theoretical yield and upon addition of three purified hemicellulases; a yield of about 71% was achieved suggesting that additional improvements should be possible by introducing another engineered *E. coli* strain. Shou et al. [141] demonstrated a slightly more ideal cooperation using two engineered *Saccharomyces cerevisiae.* One strain requires adenine and overproduces lysine while the other requires lysine and overproduces adenine. In this mutualistic relationship, adenine is released as senescence is approached which supports the growth of the partner that, in turn, provides the lysine requirement of the first strain to sustain the dual culture system. In another study by Bayer et al. [142] a combination of genetic engineering and natural capabilities was used to establish a cooperating dual culture able to convert cellulose to methyl halides. The cellulolytic bacterium, *Actinotalea fermentans,* is inhibited by alcohols and organic acids produced during hydrolysis and fermentation of cellulose. *S. cerevisiae* was engineered to utilize these compounds to produce methyl halides and, when grown in co-culture, alleviated the feedback inhibition on *A. fermentans* hydrolysis. The study confirmed that the yeast would not grow on carboxymethyl cellulose without

A. fermentans and the addition of yeast boosted the growth of *A. fermentans*. As noted by Zuroff and Curtis [134], these examples demonstrate the possibility of using genetic engineering to create a symbiotic cooperation between microorganisms for chemical production. In a similar manner, combining the capabilities of natural consortia with genetic engineering could be used to develop efficient biofuel producing consortia.

The ideal biofuel producing microorganism (IBPM) must possess a number of independent characteristics:

(i) It must be able to hydrolyze cellulosic materials effectively, with minimal requirement for pre-processing

(ii) It must be able to convert the sugars released into molecules useful as liquid fuels and/or chemical industry feedstocks.

(iii) It must be able to produce these molecules at a high concentration without poisoning itself, in order to minimize downstream processing costs; and

(iv) It must be capable of rapid growth in a bioreactor and suitable in other aspects for use in an industrial context. [143]. Synthetic biology aims to construct novel biological systems from smaller components [143].

This program outlined above is shown in the concept of BioBricks [144]: modular, interchangeable DNA components, which due to the use of a combination of restriction sites generating compatible and incompatible sticky ends, can be assembled in any order and in any desired number to generate complex multi-gene systems. A great deal has been learned about the nature of microbial biomass degradation systems. Efforts to transfer such systems to heterologous hosts have not been completely successful, though some progress has been made [145].

Commercial biomass conversions are principally of two types, which are (i) The Simultaneous Saccharification and Fermentation (SSF) system in which cellulases are added to a bioreactor with cellulose degradation and is concurrent with glucose assimilation and product formation and (ii) Consolidated Bioprocessing (CBP) in which a single organism produces

cellulases, assimilates the sugars released and produces the desired product [143]. Unfortunately, no known naturally occurring microorganism possesses the necessary combination of characteristics with sufficient product yield for commercial viability [143]. To this end several; synthetic biology approaches have been made towards increasing efficiency and capacity of biomass conversion. The field of biofuel production is ripe with opportunities to use co-cultures, where a bioprocessing approach that converts biomass to biofuel in a single reactor has significant potential for producing low-cost biofuel [145]. Shin et al. [146] engineered two strains of *E. coli* to transform xylan. One strain secretes two hemicellulases while the other uses the released sugars to produce ethanol. The control single culture containing the expression of both parts proved to have a lower yield of ethanol compared to the binary culture. Chen and coworkers [147] used synthetic yeast consortia to produce ethanol from cellulose and demonstrated the potential of a division of labor approach. In this experiment, three different yeast strains were developed to secrete three different proteins with docking tags enabling their assembly onto an extracellular scaffold. The three specific heterologous enzymes were an endoglucanase (AT), an exoglucanase (CB) and a β-glucosidase (BF) that together are capable of cellulose degradation. The consortia population was modulated by adjusting the inoculation ratio of each of the four strains including the Scaf-ctf producing strains (SC). The final reported ratio was 7:2:4:2 of SC:AT:CB:BF. This optimized ratio produced 87% of the theoretical ethanol production value from phosphoric acid swollen cellulose (PASC) and was 3-fold higher than a similar consortium producing the secreted enzymes only with a control strain (CE) in place of SC.

Cho et al. [148] reported the integration of multiple copies of the bifunctional endo/exoglucanase of *Bacillus* sp. DO4 and β-glucosidase of *Bacillus circulans* into the chromosomal DNA of *S. cereviasiae.* Enzyme activity was detected and there appeared to be

somewhat enhanced growth in the presence of cellooligosaccharides, but it was concluded that higher expression levels would be required to allow effective growth and ethanol production. Fujita et al. [149] reported generation of a recombinant strain of *S. cerevisiae* expressing *T. reesei* endoglucanase II and cellobiohydrolase II together with β-glucosidase I of *Aspergillus aculeatus,* the three enzymes were expressed as fusion proteins attached to the cell surface. The recombinant strain was able to produce about 3 g L^{-1} ethanol from amorphous cellulose. Joachimsthal and Rogers [150] transformed pZB5 into *Z. mobilis* ZM4 and demonstrated higher ethanol tolerance than CP4 derivatives on high concentrations of glucose/xylose (65 g L^{-1}) mixtures. However when sugar concentration was further increased to 75 g L^{-1} of each, ZM4 (pZB5) fermentation stalled after the ethanol concentration rose to 67 g L^{-1}. Naturally, high concentrations of pure cellulosic substrates are unfavorable to *C. cellulolyticum* and under this condition, nutrients or products have accumulated to toxic levels [151]. To improve growth and ethanol production in *C. cellulolyticum* an expression system (pMG8) which constitutes the *Z. mobilis pdc* and *adh* genes under the control of a strong ferridoxin gene promoter of *Clostridium pasteurianum* was introduced [152]. The resultant recombinant strain Cc-pMG8 was able to grow on cellulose medium with higher specific growth (0.049 g L^{-1} h^{-1}) than that of the parent strain (0.044 g L^{-1} h^{-1}) and produces two fold more ethanol (20 mM). This higher ethanol production was attributed to intracellular utilization of excess pyruvate. Den Haan et al. [153], described the generation of a strain of *S. cerevisiae* co-expressing endoglucanase EG1 of the filamentous fungus *T. reesei* and β-glucosidase BGL1 of the yeast *Saccharomyces fibuligera*. They demonstrated that the recombinant strain could grow with amorphous cellulose (phosphoric acid swollen cellulose, PASC) as the sole carbohydrate source and produce ethanol, though for some reasons entirely unclear, only 1 g L^{-1} ethanol was produced. Various organisms in nature can secrete all the necessary cellulase components, but these

organisms are often difficult to manipulate genetically [154]. Attempts to engineer more genetically tractable microorganisms to secrete all of the cellulase components heterologously have not yet been successful and this could be because of the heavy metabolic burden associated with expression of the cellulase-associated proteins which inhibits cell growth, or because intracellular assembly of the cellulosomal complexes interferes with their excretion [155]. Arai et al. [156] constructed two engineered strains of *Bacillus subtilis*—one secreting the scaffold and the other secreting an endoglucanase or a xylanase that binds to the scaffold to become active—which exhibited the predicted enzymatic activity in co-culture.

Ethanol production by fermentation of starches and cellulosic materials is gaining increasing interest because of the increasing cost and price fluctuations of petroleum [157]. The utilization of cellulosic materials for the production of ethanol is currently hampered by lack of adequate progress in producing monosaccharides from cellulose and in efficient utilization of all the sugars formed [157]. Because products of hydrolysis often cause feedback inhibition of hydrolytic enzymes, simultaneous hydrolysis and fermentation has been proposed [157]. Abate et al. [158] described ethanol production by a co-culture of *Z. mobilis* and *Saccharomyces sp.* with higher yields and production rates than with either microorganism in pure culture. Szambelan et al. [159] described the utilization of inulin from artichoke as a substrate for ethanol production in which they achieved a theoretical maximum yield of 94%. An advantage of simultaneous hydrolysis and fermentation of cellulose lies in avoiding the accumulation of glucose and disaccharides hence, no product inhibition of the cellulolytic enzymes occurs [160].

No single microorganism (wild type or recombinant) with a high cellulolytic activity and a simultaneous high yield and production rate of ethanol is known [161]. There have been improvements with co-cultures for ethanol production from cellulose in recent times. Qian et al.

[162] reported about a conversion rate of 96.1% within 48 h using softwood hydrolysate as substrate in co-culture fermentation of *Saccharomyces cerevisiae, Pachysolen tannophilus* and a recombinant *Escherichia coli* strain. Kato et al. [163] reported a co-culture system of the anaerobic cellulolytic bacterium *Clostridium straminisolvens* and an aerobic bacterium resulted in a strong increase in cellulase activity. Holtzapple et al. [164] developed a stable mixed fermentation system 'MixAlco' for the production of ethanol using unsterile biomass and waste materials as substrates.

The use of cellulolytic materials would ensure that there is no competition between food and fuel production, one of the major drawback of first generation biofuels. For lignocelluloses degradation co-culture fermentation processes offer the possibility to implement all necessary enzymatic conversions in one bioreactor and in some cases, a stepwise fermentation process is suggested [165]. The simultaneous conversion of glucose and xylose to ethanol by the co-culture of *Z. mobilis* and *Pichia stipitis* was reported by Fu et al. [166]. In another study involving the fermentation of cassava starch, a mixed culture of *Endomycopsis fibuligera* NRRL 76 and *Zymomonas mobilis* ZM4 produced 10.5% v v^{-1} ethanol from 22.5% w v^{-1} cassava starch [167]. Maki et al. [168] reported about several applications of *Clostridium thermocellum* together with other strains of *Clostridium* sp. or *Thermoanaerobacterium saccharolyticum.* Li et al. [169] reported the co-culture of *Saccharomyces cerevisiae* and *Pichia stipitis* in bioethanol production from rice straw. Results showed that heat inactivation of *S. cerevisiae* cells before addition of *P. stipitis* resulted in high xylose fermentation efficiency. Ho et al. [170] reported the simultaneous saccharification and fermentation of cellulosic bioresources to biofuels, using a stable rumen-mimicking bacterial consortium referred to as the "Functional Rumen Bacterial Consortium" (FRBC). Results showed that rumen consortia have the ability to produce cellulosic biofuels. He et al. [171] reported the co-culturing of *C. thermocellum* with non-cellulolytic

Thermoanaerobacter strains (X514 and 39E) significantly improved ethanol production by 194-440% with strain X514 enhancing ethanol fermentation much more than strain 39E. Bioethanol has also been produced from raw starchy material of sweet potato using mixed-cultures of *Paenibacillus* sp. and *Z. mobilis,* a yield of 6.89 g L^{-1} ethanol was achieved in culture of small volume [172]. Jiao et al. [173] reported the syntrophic metabolism of a co-culture containing *Clostridium cellulolyticum* and *Rhodopseudomonas palustris* for hydrogen production. When cultured alone, *C. cellulolyticum* degraded only 73% of the supplied cellulose. However, in co-culture *C. cellulolyticum* degraded 100% of the total cellulose added (5.5 g L^{-1}) and at twice the rate of *C. cellulolyticum* monocultures. The H_2 production in the co-culture was 1.6-times higher than that of *C. cellulolyticum.* The co-culturing also resulted in a 2-fold increase in the growth rate of *C. cellulolyticum* and a 2.6-fold increase in the final cell density. The major metabolites present in the co-culture medium include lactate, acetate and ethanol, with acetate serving as the primary metabolite transferring carbon from C. *cellulolyticum* to *R. palustris.* They concluded that the stimulation of bacterial growth and cellulose consumption under the co-culture conditions is likely caused by *R. palustris's* removal of inhibitory metabolic by-products (e.g., pyruvate) generated during cellulose metabolism by *C. cellulolyticum.*

Ecosystem dynamics and stability are often modulated through interactions between organisms [174]. These interactions can be direct communication using signaling molecules or cell-cell contact or they can be indirect, such as through the sharing of nutrients. One of the grand challenges in synthetic biology has been the ability to send signals between cells as to coordinate population-level behaviors [175]. An important practical constraint of employing microbial communities for bioprocessing is the inability to reliably generate stable or dynamic community behavior and ecosystem composition. [175]. One approach to enabling coexistence is to engineer

beneficial interactions between each individual population. Several efforts have shown that mutualism can be achieved using combinations of auxotrophs. Shou et al. [176] engineered two yeast strains that each coexist by supplying an essential metabolite to the other. A mathematical model was built to analyze the requirements and constraints of the system. The initial growth rates and survival rate of both strains and their metabolites production rate were found to be critical for cooperative interactions to occur. Wintermute and Silver [177] reported the use of a series of 1035 *E. coli* auxotroph pairs to elucidate how different pairings can prove beneficial while others are not. They showed that cross feeding of the metabolites yielded a significant metabolic synergy in 17% of the pairings and constructed a quantitative model to describe and predict these synthetic interactions. Hu and coworkers [178] recently combined the tuning of genetics, cell-cell communication and the environment to produce a range of population dynamics in a synthetic ecosystem where two strains of *E. coli* directly modulate each other's growth via two AHL-based QS signal transduction. Here they employed a combination of computation and experiments to successfully identify combinations of AHL and antibiotics that produced specific dynamic ecosystem behaviors, including extinction, obligatory mutualism, facultative mutualism and commensalism.

Chen [179] outlined the conditions for a stable co-culture, which are:

1) The two strains must be compatible and able to grow together. This concept had been studied in-depth by Laplace et al. [180]

2) Fermentations conditions such as pH, temperature, and oxygen supply, for the two strains should be compatible. For example, *Z. mobilis* ferments glucose at pH 7 and temperature of 37ºC, but these conditions are not compatible with those of xylose-fermenting yeasts (*P. stipitis* and *C. shehatae*) which need pH 5 and temperature 30ºC.

The interactions between microorganisms in mixed culture environments may not always lead to desirable consequences; therefore, understanding the interactions between associated strains in a co-culture system is very important [179]. He further pointed out that very little research has

been done so far, primarily due to the complex nature of systems containing multiple microorganisms. French [143] also noted that the economic conversion of cheap, abundant, renewable biomass to valuable products would require the combination of a range of different characteristics that do not naturally occur in any one organism. Furthermore, it is obvious that metabolic pathways for product formation can be transferred from one host to another, and synthetic biology offers improved methods for investigating synergy between the many gene products involved in biomass degradation and solvent tolerance [143]. Bader et al. [157] concluded by outlining the different applications of co-culture fermentation which are foods, food additives, pharmaceuticals, enzymes, bulk and fine chemicals, bioremediation and degradation of lignocelluloses. Co-culturing offers the opportunity to use cheap substrates, increase yields and product quality. Additionally all aspects, including process parameters that produced and secreted substances and possibly the occurring biotransformations, may provide an opportunity to control growth and product formation during co-culture fermentation process [180].

2.5 Construction of Recombinant DNA

2.5.1 Contruction of the Recombinant DNA pAA1

The first construct involves the endoglucanse genes *celY* and *celZ* of *E. chrysanthemi*, the leader sequence of the glucose-fructose oxidoreductase gene of *Z. Mobilis* ZM4 and the β-glucosidase gene of *Ruminococcus albus*. This is a four fragment cloning, with each fragment having a specific role. *celY* and *celZ* genes work synergistically in degrading oligosaccharides. The leader sequence of the glucose-fructose oxidoreductase is an export protein which would be attached to the β-glucosidase gene and the function is export the latter to the outside of the cell. The latter when exported, would also degrade oligosachharides, principally cellobiose.

An alternative approach to the bioengineering bottleneck would be to clone the *pdc* and *adh* genes of *Z. mobilis* into *C. cellulolyticum*. This approach has been successfully implemented in *E. coli* and *C. cellulolyticum* and in each case; greater ethanol yield were reported. *Z. mobilis* has been chosen because it is the best known ethanologen and with an exceptionally high ethanol tolerance. Whilst there are several literatures detailing the anchoring of surface expression genes and general re-engineering of *Z. mobilis;* in the event of a failure to transform *Z. mobilis* by electroporation or the spheroplast method and also a failure to achieve the cloning and expression of the *pdc* and *adh* of *Z. mobilis* in *C. cellulolyticum*, the mutant *E. coli* (Δ*chbG*) JW-1722 obtained from the Yale University *E. coli* Genomic Stock Center lacking the chb operon for cellobiose degradation would be employed to serve as the surrogate for the expression of the *celZ-celY-gfor-betaglucosidase* gene sequences.

2.5.2 Construction of the Recombinant DNA pAA2

The second construct involves the ice nucleation gene *inaZ* of *P. syringae,* the cellulosome integrating protein gene *cipC* of *C. cellulolyticum* and the *phoZ* gene of *Streptococcus pyogenes.* The *inaZ* gene serves to anchor the *cipC* gene on the on the surface of the cell. This would enable the latter to bind to released cellulosomes in the medium. The *phoZ* has no catalytic function besides serving as a detection mechanism for the successful expression of the *cipC* gene on the outside of the cell.

CHAPTER 3

MATERIALS AND METHODS

3.1 Determination of the Ethanol Tolerance of Purified Cellulosomes

3.1.1 Bacterial Strain and Media

C. cellulolyticum (ATCC 35319) was purchased from the American Type Culture Collection. The culture was first grown in *C. cellulolyticum medium* (ATCC1368) using glucose as the carbon source. Medium preparation involved the addition of resazurin to indicate for the presence of oxygen. The medium was boiled under pressure, with the removal of oxygen using a vacuum pump installed. At the point at which the resazurin changed color from blue to pink, the boiling was stopped and the medium allowed to cool down to room temperature. The medium was tranfered into the Coy Anaerobic chamber where 10 ml each were dispensed into sterile serum bottles. The bottles were sealed to prevent any introduction of oxygen and the medium sterilized using standard autoclave conditions. Thereafter, the *C. cellulolyticum* culture was aseptically transferred into the 10 ml medium, gassed for 1 minute using 90% Hydrogen and 10% carbon gas mix. The culture was incubated for 24 hours in 37°C shaking incubator at 225 revolutions per minute. After 24 hours, they were routinely transferred into *Clostridium cellulolyticum* medium (ATCC medium 1368) containing microcrystalline cellulose as carbon source and incubated for 72 hours under the same conditions.

3.1.2 Cellulosome Purification

Cultures were purified according to the methods of Gal et al. [181] as described. Cells were cultivated anaerobically in two 500-ml flasks at 37ºC and 160 r.p.m. in *C. cellulolyticum* medium. After 5 days of growth, cells and residual cellulose were harvested by centrifugation at 13000 g

for 20 minutes. The pellets were washed five times in 50 ml of 25 mM Tris-HCl (pH 8.0), resuspended in the same medium and filtered through a 3-µm poresize glass filter (glass microfiber filter GF/D; Whatman) to remove cellulose fibers, washed on the filter, first with 30 pellet volumes of 25 mM Tris-HCl (pH 8.0) and second with 30 pellet volumes of 12.5 mM Tris-HCl (pH 8.0) and the cellulosome eluted with 150 ml of water on the filter. The eluted fraction was then filtered on a 0.2-µm pore size nylon membrane filter and concentrated using Amicon Ultra centrifugal filters (30 KDa MWCO). The final 1-ml sample of filtrate (eluate) extract containing the crude cellulosome preparation was subjected to vacuum drying for 20 minutes to concentrate the dilute cellulosomes extracts to approximately 0.5 ml.

3.1.3 Substrates

Medium-viscosity carboxymethyl cellulose (CMC) originally purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio) was used.

3.1.4 Hydrolysis with Crude Cellulosome Preparation

Reactions were carried out according to the method of King et al. [182] for the quantification of free reducing ends of released mono and oligosaccharides as described below. Each tube contained 90 µL of 0.25% CMC in 0.1 M sodium acetate buffer pH 5.5. Five tubes were set up as follows: (a) 90 µL crude cellulosome with 90 µL of water (negative control) (b) 90 µl CMC substrate with 90 µL of water (negative control) (c) 90 µL CMC substrate in 0% ethanol with 90 µL of crude cellulosome (d) 90 µL CMC substrate in 10% ethanol with 90 µL of crude cellulosome (5% final ethanol concentration), and (e) 90 µL CMC substrate in 20% ethanol with 90 µL of crude cellulosome (10% final ethanol concentration), these were incubated at 37ºC first

for 24 hours and then for another 24 hours. These time points were chosen to allow for adequate hydrolysis of the substrate and release of the reducing sugars. This exact procedure was repeated two additional times under the same conditions. A stand alone sample was analyzed and it included 15%, 20% and 25% final ethanol concentrations in the set to gauge the upper ethanol concentration limit of activity.

3.1.5 Protein Quantification of Cellulosome Preparations

Total protein concentrations of crude cellulosome extracts were determined by the Bradford assay using Bovine serum albumin (BSA) as a standard. Bovine serum albumin standards at concentrations of 0, 20, 40, 80 and 100 μg ml^{-1} were prepared in 0.1 M sodium acetate buffer pH 5.5, the standard and cellulosome extracts incubated at 37ºC for 30 minutes, and Absorbance readings were taken at 595 nm on a Synergy 2 Multi-Detection Microplate Reader (BioTek Instruments Inc., Winooski VT).

3.1.6 Reducing Sugar Quantification

The DNS reagent was prepared according to standard procedure [183]. The DNS reagent is non-specific and reacts with both five and six carbon reducing sugars [182]. Glucose standards of 0, 0.25, 0.50, 0.75, and 1.0 mg ml^{-1} were prepared in 0.1 M sodium acetate buffer pH 5.5 with additional sets of the same standards containing 10% and 20% ethanol, respectively. The glucose standards prepared under different conditions were used to determine if the presence of ethanol would interfere with quantification of reducing sugars. 60 μL of each standard or reaction hydrolysis product was added to 120 μL DNS reagent in a 2.0 ml PCR tube for a total volume of 180 μL. The DNS reactions were carried out in thermo cyclers (Bio-Rad) by heating at 95ºC for 5

minutes followed by cooling to 4ºC for 1 minute and holding at 20ºC. A 36 µL aliquot of each completed DNS reaction was added to 160 µL of deionized water in a flat bottom, 96-well microplate and mixed thoroughly using the micropipette. Absorbance was immediately determined at 540 nm.

3.1.7 Statistical Analyses

The data obtained from the glucose standard curve assay and the endoglucanase activities were analyzed using ANOVA and the t-test of means from the statistical software package R programming language (www.r-project.org) and Sigma plot (www.sigmaplot.com).

3.1.8 SDS-PAGE

SDS-PAGE was performed by the procedure of Laemmli [184] by preparing 10% polyacrylamide gels. The samples used for the SDS-PAGE analyses were boiled in sample buffer before use. The SDS-PAGE were run under a constant flow of cold water using the Hoefer slab apparatus for approximately 3 hours.

3.1.9 Cloning, Construction and Expression

3.1.9.1 Amplification of pBBR1 MCS-3

The vector backbone was linearized with the restriction enzyme KpnI, the linearized sequence verified for correctness on DNA gel to give a band size of 5.2 kb and without any smear on the gel. The fragment was PCR amplified using Phusion DNA polymerase (NEB) and the condition was 98°C for 1 minute, 98°C for 30 seconds, 56°C for 30 seconds for annealing

extension temperature of 72°C for 90 seconds, the cycle was repeated 35 times through the second step to the fourth step, a final extension for 5 minutes at 72°C and a hold at 4°C.

3.1.9.2 Amplification of *celZ* and *celY* Genes from *Erwininia chrysenthemi*

As stated earlier, the endoglucanase genes *celY* and *celZ* have been cloned from *Erwinia chrysanthemi* and expressed in *Klebsiella oxytoca* SZ21 [100]. The *celY* gene and *celZ* genes with the ZM4 promoter from pLOI 2352 (kindly provided by Professor L. Ingram, University of Florida) were individually amplified using Phusion DNA polymerase (NEB). For the *celY* gene the PCR condition was 98°C for 1 minute, 98°C for 30 seconds, 72°C for 45 secs (to include annealing and extension), cycle repeated 35 times through the second step to the merged, a final extension for 5 minutes at 72°C and a hold at 4°C. Similar conditions were used for the *celZ* gene but with the annealing temperature set at 54°C for 30 seconds and extension time for 45 seconds. The amplicons were verified by DNA electrophoresis and sequenced for correctness. The sequences were matched with the sequences in the NCBI database.

3.1.9.3 Amplification of Glucose-Fructose Oxidoreductase Leader Sequence of *Z. mobilis* and *β-glucosidase* Gene of *Ruminococcus albus* in *Z. mobilis*

The *β-glucosidase* gene from *Ruminococcus albus* has been cloned and translationally fused to the glucose-fructose oxidoreductase leader sequence of *Z. mobilis* for export*;* this resulted in 61% secretion and 0.49 g ethanol yield per g cellobiose [56]. To amplify the leader sequence of the glucose-fructose oxidoreductase leader gene, the reverse primer was designed to include 10 base of the forward primer of the *B-glucosidase* gene and used to amplify the sequence of 159 base pairs. Similarly the forward primer of the *B-glucosidase* gene was designed to include 10 base pairs of the complimentary sequence of the gfor leader reverse primer sequence without the

inclusion. The *β-glucosidase* gene from the genomic DNA of *Ruminococcus albus* (kindly provided by Professor P. Weimer, University of Wisconsin) and the Leader sequence of the *gfoR* gene of *Z. mobilis* for protein folding, which was fused to the *β-glucosidase* gene, were amplified using synthetic oligonucleotide primers. The PCR condition used was the same as previously described, however the annealing temperature was 65°C. The amplicons were verified by DNA electrophoresis and sequenced for correctness. The sequences were matched with the sequences in the NCBI database.

3.1.9.4 Cloning and Assembly of Plasmid pAA1

The cloning and expression of these three genes was to expand the substrate utilization range of *Z. mobilis* to include larger oligodextrins and amorphous cellulose by cellulosomes. These three fragments, ZM4 promoter with *celZ, celY* and *gfoR-betaglucosidase* fusion were cloned into the KpnI site of the broad host range vector pBBR1MCS3 (tc^r) constructed by Kovach et al. [186]. The cloning was performed using the Life Technologies Gene Art Seamless Cloning and Assembly kit [187]. This kit is optimized to clone up to 4 DNA fragments with a total vector and insert size of 13 kb.

Table 3.1: List of Primers

Genes amplified	Size in base pairs	Primers used
pBBR1mcs-3 forward for pAA1 clone	5228	AGGGATAAGGTACCGGGCCCCCC
pBBR1mcs-3 for pAA1 clone reverse	5228	GGTTGATCCAGCTTTTGTTCCCTTT
celZ forward with ZM4 promoter	2735	AAAAGCTGGATCAACCGGCAATTT
celZ reverse with ZM4 promoter	2735	CTCCTTCTTCAATTAGTTACAGCTACCAA

celY forward	1012	CTAATTGAAGAAGGAGAATGAATGGGAAA GCC
celY reverse	1012	CTCCTTCTTTATTTACCGCGCGCCAACATCAC
gfoR-betaGLC fusion forward	2434	GTAAATAAAGAAGGAGTAAGAATGACGAACAA
gfoR-betaGLC fusion reverse	2434	CCGGTACCTTATCCCTCTAACATGGAATTCAG
pBBR1 mcs 3 forward for pAA2 clone	5228	TTGGTAAAGGTACCGGGCCCCC CCTCGAGGTC
pBBR1 mcs 3 reverse for pAA2 clone	5228	ATAAGCTTCAGCTTTTGTTCCCTT TAGTGAGG
inaZ(NC) fusion forward	774	AAAAGCTGAAGCTTATAAATGTGAGCG GATAA
inaZ(NC) fusion reverse	774	TTTTTACGCTTTACCTCTATCCAGTCATCGTC
cipC forward	4635	CGTAAAAAGTCTTTAGCATTT TTG
cipC reverse	4635	TTTTTTGATTAAGTTTTGCACTTCCG TTTGT
phoZ forward	1338	AACTTAATCAAAAAAGCGGCGAAA AACAAACA
phoZ reverse	1338	CCGGTACCTTTACCAATACCTTTATC TTTAAT

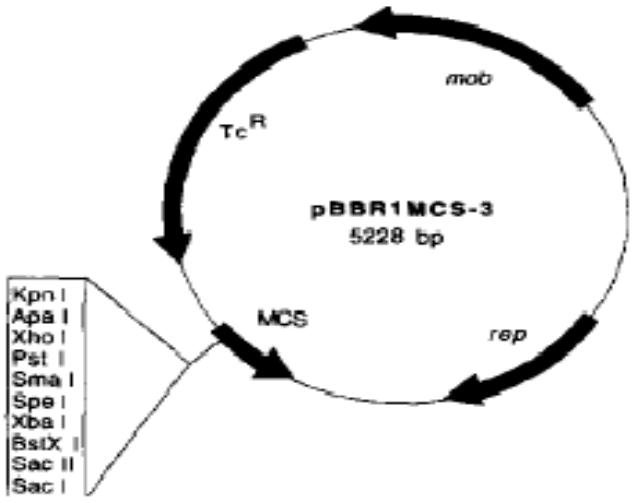


Figure 3.1: Diagram of the pBBR1 MCS-3 showing the Kpn I cut site [186]

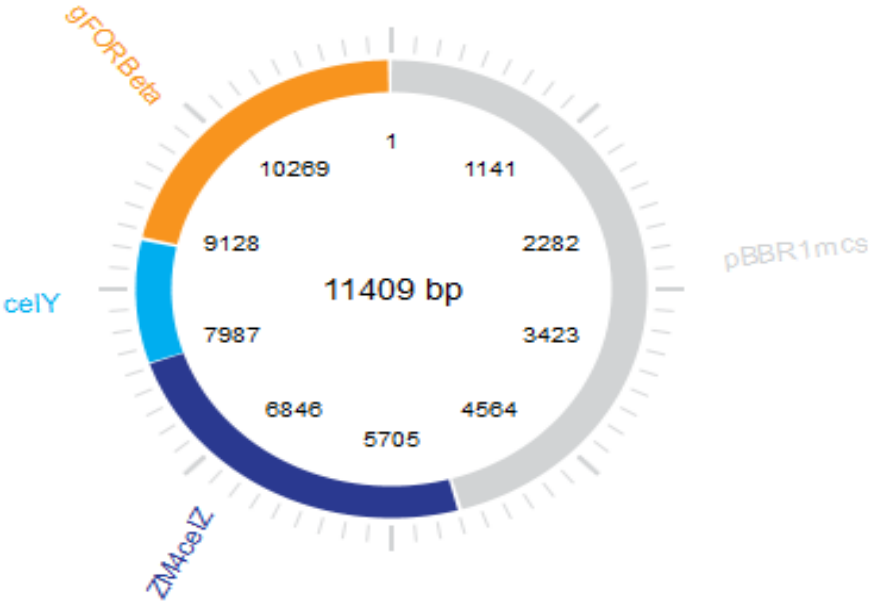


Figure 3.2: The arrangement of the DNA fragments (*celZ* with ZM4 promoter, *celY, gfoR* leader sequence fused to the beta-glucosidase (gFORBeta) in constructed plasmid pAA1.

The cloned vector pAA1 (Figure 3.2) was transformed using standard transformation protocol into *E. coli* NEB-10 Beta competent cells as described by the manufacturer. The transformants were plated out on lysogeny medium (LB) containing 40 µL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) for blue/white screening and with 15 µg ml^{-1} of tetracycline. In order to screen for the right clone, the plasmid pAA1 and the vector backbone pBBR1MCS-3 were first digested using KpnI and NotI HF restriction enzymes (NEB) which cut at the positions within the vector backbone. Furthermore, pAA1 and pBBR1MCS-3 were digested with the restriction enzyme NdeI (NEB). NdeI cut site CAˆTATG exists within the insert that produced pAA1 but not on the vector backbone pBBRIMCS-3.

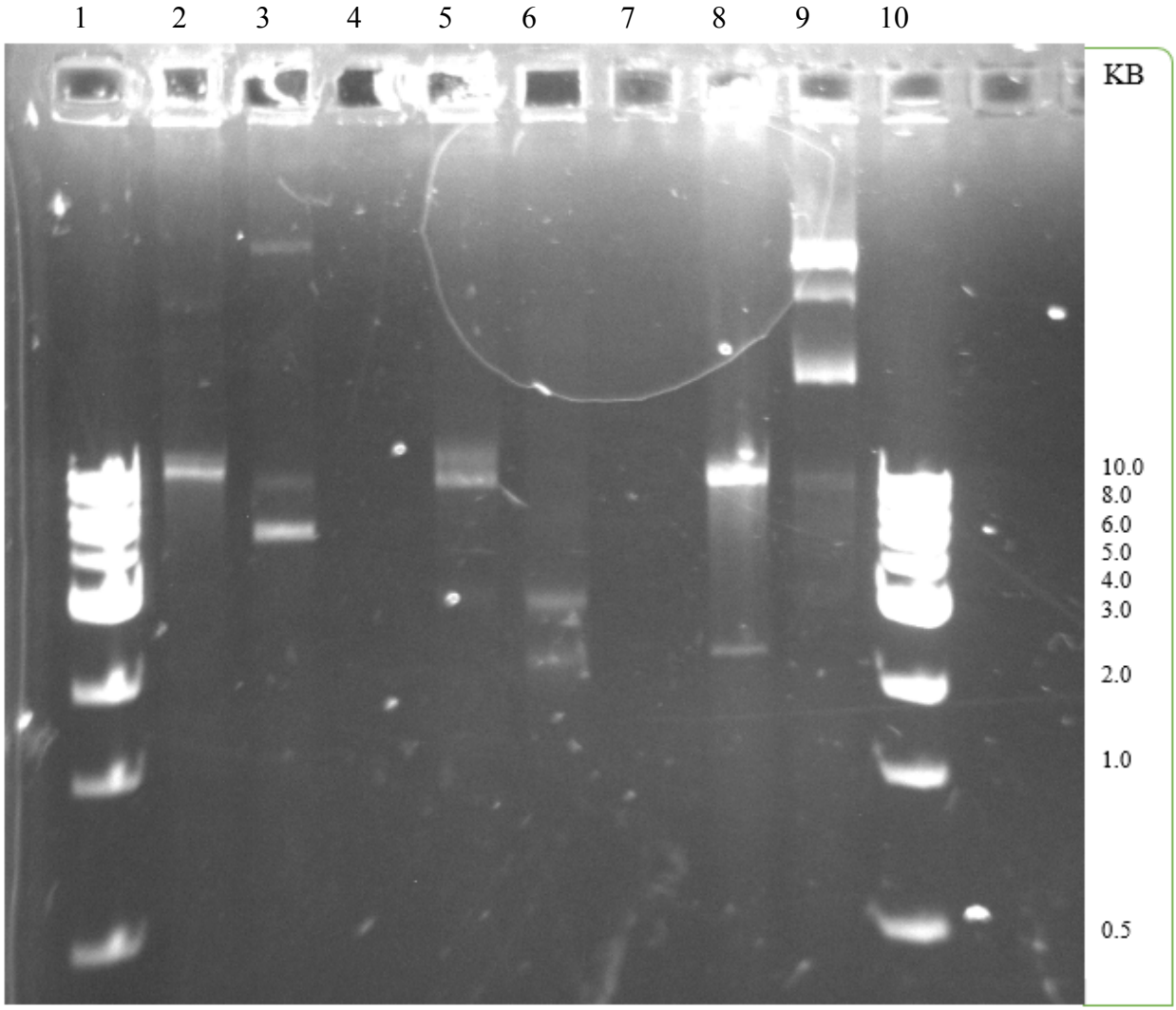


Figure 3.3: pAA1 and pBBRIMCS-3 digested with KpnI, NotI HF and NdeI restriction enzymes. Lanes 1 and 10 are the 1 KB ladder. From left lanes 2 and 3 are pAA1 and pBBRIMCS-3 respectively digested with KpnI, with pAA1 showing a band size at 11 KB while pBBRIMCS-3 appeared at 5 KB band size when digested with KpnI. From left lanes 5 and 6 are pAA1 and pBBRIMCS-3 respectively digested with NotI HF, with pAA1 showing band sizes at approximately 8.5 KB and 3.0 KB while pBBR1MCS-3 appeared at band sizes of 2.9 KB and 2.3 KB. With the inserts, the NdeI site was created in the clone pAA1 and this site does not exist in the pBBR1MCS-3 backbone. A restriction digest as seen in lanes 8 and 9 of pAA1 and pBBR1MCS-3 respectively shows a cut of the pAAI but no cut of the pBBR1MCS3, which does not have the cut site, indicating that the inserts are at the right position with the vector backbone.

The clone pAA1 was completely sequenced by Eurofins MWG operon using the Sanger sequencing techinique and verified for correctness and thereafter used for transformation of *Z. mobilis* ZM4 (WT) to create ZM4 pAA1 by electroporation as described by Liang and Lee [51].

The culture was grown in a stationary flask at 30°C to an absorbance (600 nm) of 0.3 to 0.4. The cells were harvested by centrifugation at 13000 g for 10 mins at 4°C. The cells from an original 100-ml culture were suspended in 10 ml of sterile 10% glycerol (supplemented with 0.85%) NaCl, centrifuged, and finally resuspended in 2-3 ml of 10% glycerol. The plasmind pAA1 was extracted from *E. coli* NEB-10 Beta, suspended in water and concentrated to 3000 ng DNA before electroporation. The apparatus used for generating exponential decay pulses was a Gene Pulser (Bio-Rad). A peak voltage of 1.5 kV and 25 μF capacitance and a parallel resistor of a specified ohm. A 200 μL aliquot of *Z. mobilis* was mixed with 10 μL of pAA1 in a chilled electroporation chamber with an electrode gap of 0.2 cm and held on ice for 5 minutes. Thereafter the mixture of cells and DNA was pulsed and immediately after pulsing, the cells were mixed with 1 ml of RM medium for outgrowing at 30°C for 4 hours. At the end of this outgrowth period, the cells were diluted with RM medium and plated on tetracycline (15 μg ml^{-1}) containing selective RM-agar The vector backbone pBBR1MCS-3 was also transformed into *Z. mobilis* ZM4 pBBR1MCS-3 simply to verify the expression of the antibiotic marker in a new host but without the inserts. *Z. mobilis* ZM4 pAA1 and ZM4 pBBR1MCS-3 were inoculated into RM broth containing 15 μg ml^{-1} of tetracycline and 30 μg ml^{-1} of gentamicin and incubated at 30ºC for 48 hours. The gentamicin was used because *Z. mobilis* is naturally resistant to gentamicin antibiotics up to 50 μg ml^{-1}, therefore most contaminants would not survive in the presence of gentamicin. Thereafter, OD was determined at 600 nm using the spectrophotometer (Spectronic 20D+).

Figure 3.4: The RM medium tubes inoculated with ZM4 pAA1, ZM4 (wild type) and ZM4 pBBR1MCS-3 respectively.

After forty-eight hours, there was no change in the ZM4 (wild type, middle) clearly showing the suppression of growth by tetracycline even in the presence of gentamicin, to which ZM4 is naturally resistant

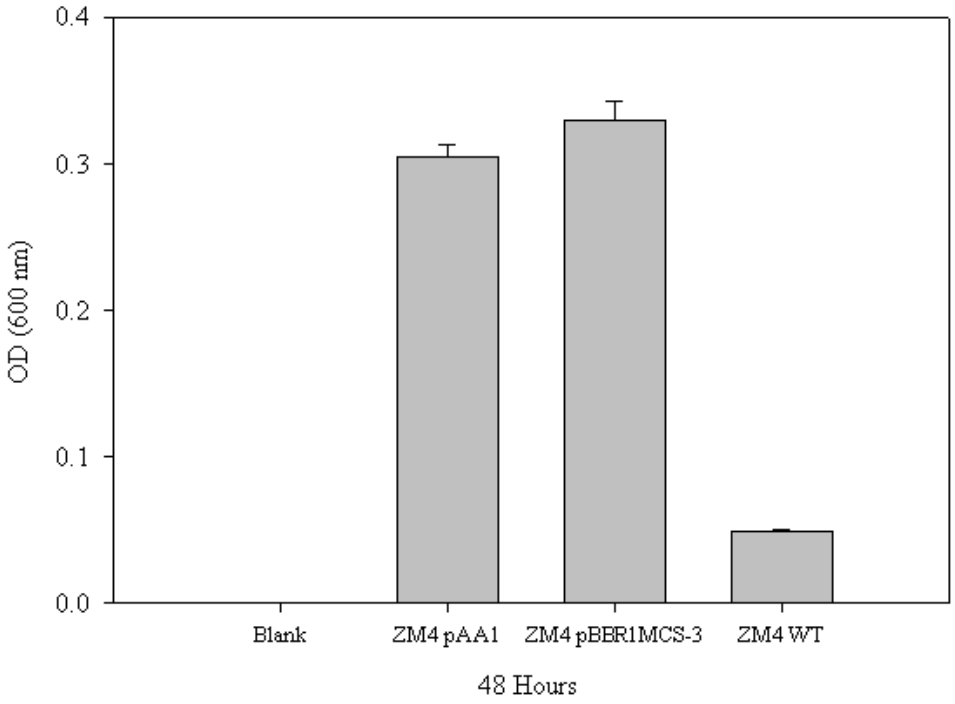


Figure 3.5: The growth of recombinant and wild type *Z. mobilis* in RM medium containing 15 µg ml^{-1} of tetracycline and 30 µg ml^{-1} of gentamicin with glucose as the carbon source.

In order to verify that the recombinant ZM4 (bearing pAA1) is indeed *Zymomonas mobilis*, the cells were once again grown in RM medium with 2 percent glucose as the carbon source and supplemented with 15 µg ml^{-1} of tetracycline. The wild type ZM4 was also grown in RM medium with 2 percent glucose as the carbon source and supplemented with 30 µg ml^{-1} of gentamicin. Cells were harvested after 48 hours and genomic DNA extraction performed using the FastDNA spin kit (MP Biomedicals). The leader sequence of the glucose-fructose oxidoreductase gene (gFOR) is unique to ZM4 and so the gene fragment was amplified from the genomic DNA extracted from ZM4 pAA1 and ZM4 wild type. The same *gfor* leader sequence which was originally fused to the beta-glucosidase gene from *Ruminococcus albus* in the pAA1 vector was also amplified using the same primers. Figure 3.6 below shows the amplification of the gFOR leader sequence with 100 base-pair ladder.

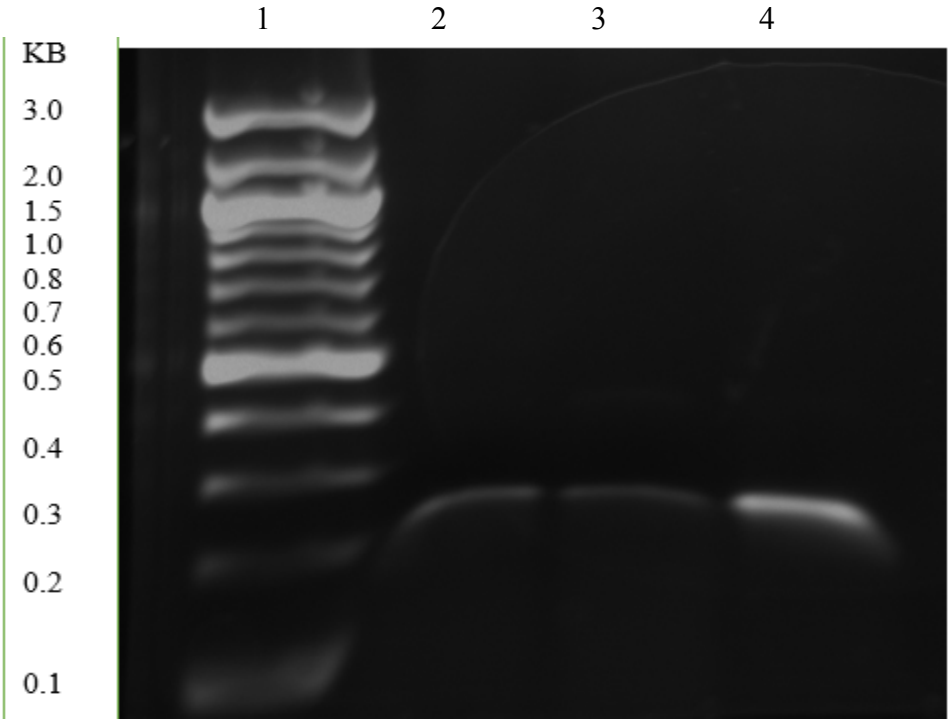


Figure 3.6: Amplification of the glucose-fructose oxidoreductase leader sequence from genomic DNA extracted from recombinant *Z. mobilis* ZM4 and wildtype ZM4 and from the plasmid vector pAA1. From left, lane 1 contains 100 bp ladder. Lane 2 contains gFOR leader sequence from ZM4 pAA1. Lane 3 contains same leader sequence from wild type ZM4 while Lane 4 is from the plasmid vector pAA1.

The recombinant *Z. mobilis* ZM4 transformed with pAA1 (tetracycline resistant) was grown in RM medium containing 2% glucose supplemented with 15 µg ml^{-1} of tetracycline. The pAA1 was then extracted from ZM4 pAA1 using the 5 Prime fast plasmid extraction kit (5 Prime) and the inserts of ZM4celZ, celY and gFOR-betaGLC were amplified from the plasmid extraction for comparison. PCR products were amplified using Q5 high fidelity DNA polymerase (New England Biolabs).

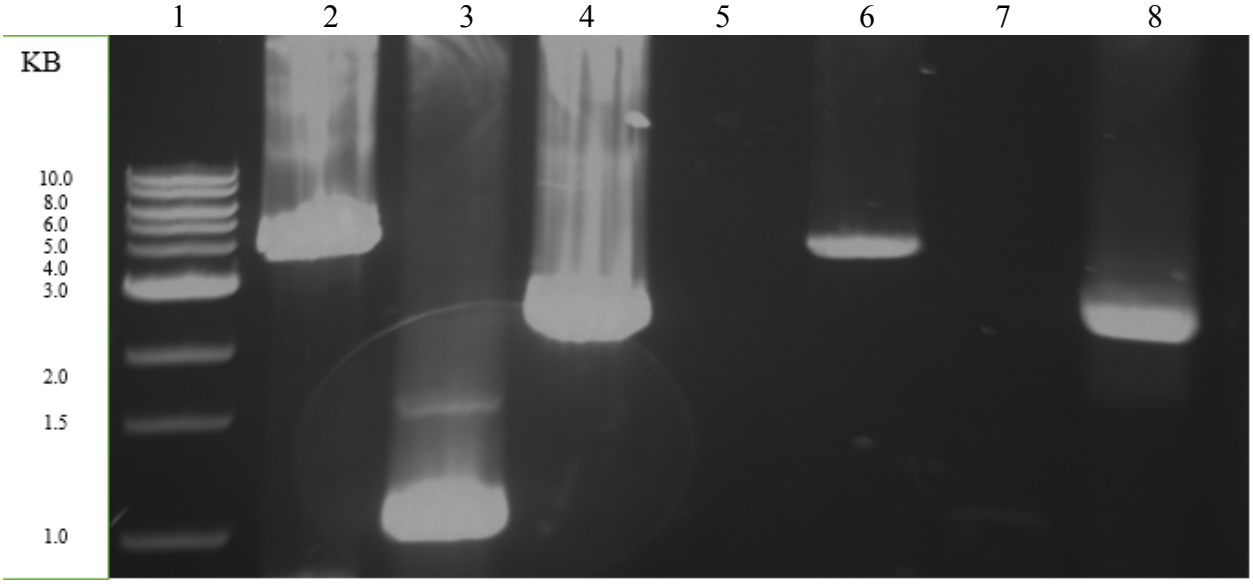


Figure 3.7: Amplified sequences of pAA1 taken from *E. coli* NEB 10 Beta and *Z. mobilis* ZM4, respectively.

From left, lane 1 is the 1 KB ladder, lanes 3 and 7 represents the band size of celY amplified from pAA1 taken from *E. coli* NEB 10 Beta and ZM4 respectively. Lanes 4 and 8 represent gFOR-betaGLC amplified from pAA1 taken from *E. coli* NEB 10 Beta and ZM4 respectively. Lanes 2 and 6 were amplified from pAA1 taken from *E. coli* NEB 10 Beta and ZM4 respectively using the primers for ZM4celZ, which has a band size of 2.7 KB, however the amplicon on the gel shows a band size of 4 KB, an apparent indication that the ZM4celZ forward or reverse primers are likely binding to a site of the vector backbone of the pBBR1MCS-3 or somewhere in the insert, respectively.

3.2 Cellulosome Capture: Expression of the *cipC* Gene of *C. cellulolyticum*

3.2.1 Amplicfication of pBBR1 MCS3

The vector backbone was linearized with the restriction enzyme KpnI, the linearized sequence verified for correctness on DNA gel to give a band size of 5.2 KB and without any smear on the gel. The fragment was PCR amplified using Phusion DNA polymerase (NEB) and the condition was 98°C for 1 minute, 98°C for 30 seconds, 56°C for 30 seconds for annealing extension temperature of 72°C for 90 seconds, the cycle was repeated 35 times through the second step to the fourth step, a final extension for 5 minutes at 72°C and a hold at 4°C.

3.2.2 Amplification of the *cipC* Gene of *C. cellulolyticum*

The *cipC* gene of *C. cellulolyticum* was amplified from its genomic DNA using similar conditions as earlier described but at an annealing temperature of 63°C and an extension time of 75 seconds. The amplicon was verified by DNA gel electrophoresis for the correct size and thereafter sequenced for correctness.

3.2.3 Amplification of N Terminal and C Terminal Sequences of the *inaZ* Gene of *Pseudomonas syringae*

The *inaZ* gene in pPROBE TI (kindly provided by S. Lindow) had been expressed in *Escherichia coli* Top 10 by cloning it directly in front of the strong promoter P_{Llac01} in our laboratory. The N terminal sequence with the promoter P_{Llac01} and C terminal sequences without the stop codon of *inaZ* gene from *Pseudomonas syringae* S203 were individually amplified from plasmid pProbe TI and fused together as described by Jung et al. [116]. The entire internal repeating sequences for ice making containing approximately 900 amino acids in length were truncated as they are not needed for surface anchoring of proteins and could ultimately prove bulky

for expression efficiency. The annealing temperature used was 65°C and extension time was set at 20 seconds. The amplicon was verified on DNA gel electrophoresis for the correct size and thereafter sequenced for correctness.

3.2.4 Amplification of the Alkaline Phosphatase Gene (*phoZ)* of *Streptococcus pyogenes*

The alkaline phosphatase gene *phoZ* from the plasmid pMNN (kindly provided by Professor Melody Neely) is used in determining the successful expression of genes on the cell surface. *phoZ* gene is only active outside the cell and can be measured colorimetrically in the presence of bromo-4-chloro-3-indolyl phosphate [188]. The gene was amplified using synthetic primers at an annealing temperature of 55°C and an extension time of 25 seconds. The amplicon was verified on DNA gel electrophoresis for the correct size and thereafter sequenced verified for correctness by sequencing.

3.2.5 Cloning and Expression of the Amplicons

Attempts were made to successfully fuse the individual inserts but with no success and so this aspect was shelved for future investigation. The efficacy of this construct for the consortium would have been assessed by the cultivation of *Z. mobilis* in co-culture with *C. cellulolyticum* on microcrystalline cellulose relative to a wild-type control [189].

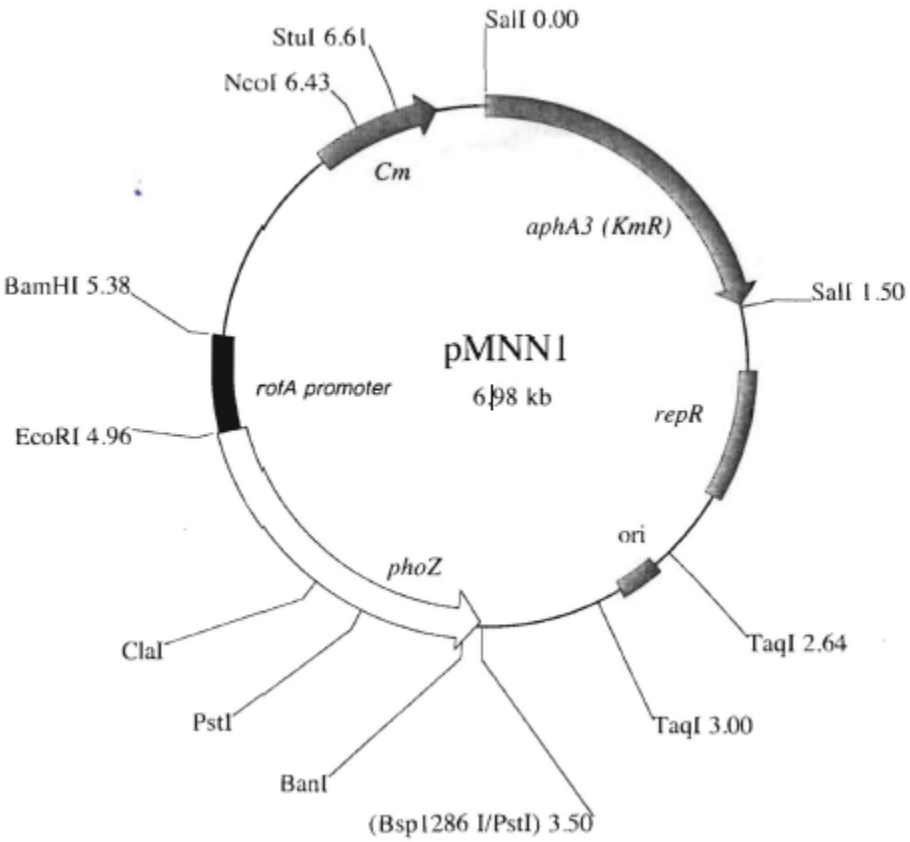


Figure 3.8: The vector pMNN 1 showing the *phoZ* gene [188].

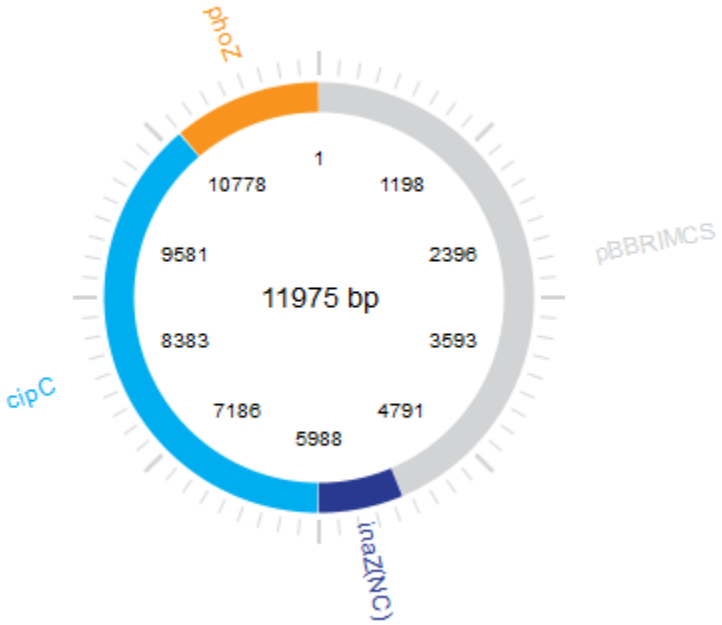


Figure 3.9: The *in silico* arrangement of the DNA fragments (*inaZ (NC), cipC* and *phoZ)* within the vector pBBRI mcs3.

3.3 Expected Results, Potential Obstacles and Alternative Plans

The *inaZ* gene has been previously cloned and expressed in *Escherichia coli* Top 10 as described by Miller et al. [190] to serve as a positive control in ice nucleation experiments. However, the objective here is to anchor the cellulosomes on the cell surface of *Z. mobilis*. Truncation of the *inaZ* gene is not expected to hamper its ability to anchor our cellulosomes on the surface as this procedure has been routinely carried out by previous researchers. The optimal temperature range for *Z. mobilis* is 30ºC while *C. cellulolyticum* grow best at 35ºC. An extensive literature search revealed that several researchers have successfully grown *Z. mobilis* up to 32ºC and *C. cellulolyticum* as low as 25ºC. With this overlapping growth conditions, it is expected that these two microorganisms would grow well in the medium provided the temperature range is maintained.

3.4 List of Combinations Tested for Ethanol Production

1. *C. cellulolyticum* alone
2. Wild type *Z. mobilis* alone
3. Recombinant *Z. mobilis* alone
4. *C. cellulolyticum* with recombinant *Z. mobilis*
5. *C. cellulolyticum* with wild type *Z. mobilis*

3.5 The Parameters Measured

1. Ethanol tolerance of the purified cellulosome and endoglucanase activity
2. Ethanol concentration
3. Reducing sugars released

CHAPTER 4

RESULTS AND DISCUSSION

4.1 SDS-PAGE

The cellulosome extracts were run on SDS-PAGE containing 0.25% CMC to determine the likely fractions of the cellulosome retained in the extract. The largest fraction at approximately 150 KDa suggests the presence of the scaffoldin protein CipC. The individual enzymes of the cellulosome range between 40 and 90 KDa in size. Figure 4.1a shows the SDS-PAGE with the inclusion of 0.25% CMC.

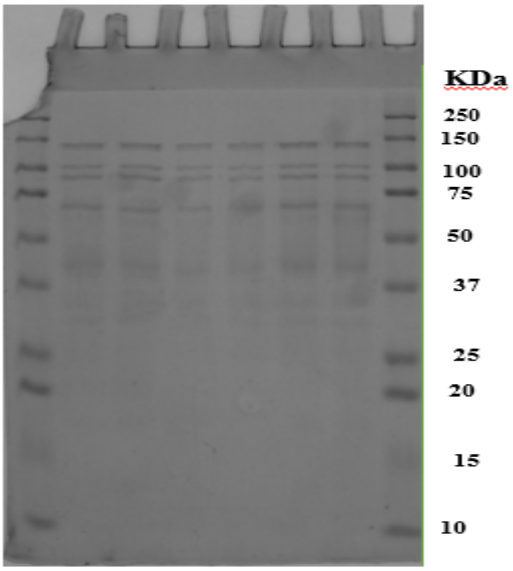


Figure 4.1: SDS-PAGE with 0.25% CMC without ethanol. Wells 1 and 8 contain 250 KDa protein ladder. Wells 2-7 are replicates of the cellusomal proteins.

Table 4.1: Expected cellulosome enzyme subunit sizes expected on the SDS-PAGE gel shown in figure 4.1.

	Name of the cellulosome subunit	Expected size (KDa)	Observed size (KDa)
1	Cellulosome Integrating Protein (CipC)	160.0	150.0 (approx.)
2	Cellobiohydrolase (Cel9E)	93.8	100.0 (approx.)
3	Putative cellulase (Cel9J)	81.3	85.0 (approx.)
4	Putative cellulose (Cel9H)	80.0	85.0 (approx.)
5	Pectinase (Rgl11Y)	70.8	70.0 (approx.)
6	Endoglucanase (Cel5D)	63.4	65.0 (approx.)
7	Endoglucanase (Cel8C)	47.2	47.0 (approx.)

4.2 Glucose Standard Curve

Figure 4.2 below shows a plot of the glucose standard curve without ethanol, with 10% ethanol, and with 20% ethanol. The regression values under the three conditions were determined using the R programme to be 0.992, 0.993 and 0.992 respectively. This experiment was repeated two more times and designated as batches 1, 2 and 3 with each batch consisting of glucose standard 0%, 10% and 20% ethanol. To further determine whether the addition of ethanol had any significant effect on the measurement of Absorbance in the reducing sugar reaction for the standard curves, ANOVA was applied to each batch ($\alpha = 0.05$) and the values obtained were 0.9994, 0.9962 and 0.9916, respectively. From the values obtained, it was concluded that ethanol up to 20% did not interfere with the reducing sugar quantification reaction and would therefore not affect the determination of the endoglucanase activity of the crude cellulosomes.

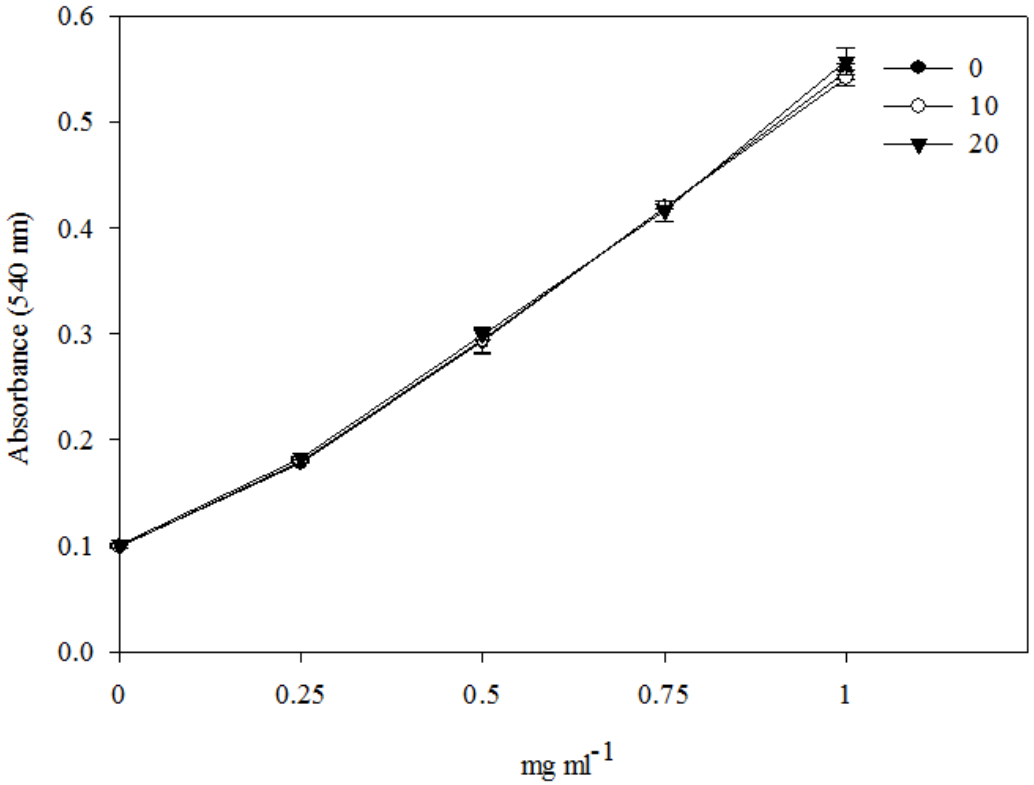


Figure 4.2: Glucose standard curve in 0%, 10%, and 20% ethanol concentrations.

Ethanol does not interfere with quantification of free-reducing ends of carbohydrates. Error bars represent standard deviations among three replicates.

4.3 Measurement of Endoglucanase Activity

To determine endoglucanase activity of the cellulosome from the Absorbance values obtained at 540 nm, the standard endoglucanase determination formula for carboxymethyl cellulose was calculated using the method of Xaio et al. [191]:

$$\text{IU ml}^{-1} = (A_{540}\,\text{sample}\ A_{540}^{-1}\ \mu\text{g}^{-1}\ \text{standard})\ (1/180\ \mu\text{g}\ \mu\text{mol}^{-1}\ \text{glucose})\ (1/30\ \text{min})\ (1/x\ \text{ml})$$

Where one international unit (IU) is defined as an average of one μmol of glucose equivalents released per min in the assay reaction. A_{540} sample is the absorbance obtained from the reducing sugar assay for CMC at $\lambda = 540$ nm; $A_{540}\ \mu\text{g}^{-1}$ standard is the absorbance for one μg of glucose as derived from the glucose standard curve. 180 μg μmol^{-1} glucose is the amount of glucose in one μmol; 30 min is the assay incubation time; and *x* ml is the volume of the enzyme used in the assay [191], (in our case 0.09 ml). Tables 4.2 below shows the calculated values for the endoglucanase activity under the different ethanol concentrations after 24 and 48 hours, the standard deviations of triplicate samples are shown in parenthesis.

Table 4.2: Endoglucanase activities measured from CMC per batch determined at 24 and 48 hours of incubation. The numbers in parentheses represent the standard deviations of triplicate values.

Samples (Batch 1)	Endoglucanase activity (10^{-6}, IU ml^{-1}) after 24 hours	Endoglucanase activity (10^{-6}, IU ml^{-1}) after 48 hours
Cellulosome without substrate	3.000	3.000
CMC without cellulosome	3.000	3.000
CMC in 0% ethanol with cellulosome	11.22 (1.025)	10.80 (0.177)

(table continues)

CMC in 5% ethanol with cellulosome	8.72 (0.537)	10.76 (0.042)
CMC in 10% ethanol with cellulosome	8.28 (0.170)	8.90 (0.113)
Samples (Batch 2)	Endoglucanase activity (10^{-6}, IU ml^{-1}) after 24 hours	Endoglucanase activity (10^{-6}, IU ml^{-1}) after 48 hours
CMC in 0% ethanol with cellulosome	8.19 (0.244)	9.24 (0.100)
CMC in 5% ethanol with cellulosome	8.66 (0.330)	9.83 (0.127)
CMC in 10% ethanol with cellulosome	8.17 (0.140)	9.25 (0.159)
Samples (Batch 3)	Endoglucanase activity (10^{-6}, IU ml^{-1}) after 24 hours	Endoglucanse activity (10^{-6}, IU ml^{-1}) after 48 hours
CMC in 0% ethanol with cellulosome	7.55 (0.138)	8.85 (0.052)
CMC in 5% ethanol with cellulosome	7.10 (0.216)	8.46 (0.132)
CMC in 10% ethanol with cellulosome.	6.97 (0.261)	8.15 (0.360)

Table 4.3: Endoglucanase activities measured from CMC per batch determined at 24 and 48 hours of incubation. The numbers in parentheses represent the standard deviations of triplicate values.

Samples (Batch 4)	Endoglucanase activity (10^{-6}, IU ml^{-1}) after 24 hours	Endoglucanase activity (10^{-6}, IU ml^{-1}) after 48 hours
CMC in 5% ethanol with cellulosome	4.0775 (0.03)	4.210 (0.04)
CMC in 10% ethanol with cellulosome	3.960 (0.08)	4.09 (0.111)
CMC in 15% ethanol with cellulosome	3.820 (0.09)	3.905 (0.03)
CMC in 20% ethanol with cellulosome	3.7175 (0.02)	3.845 (0.02)
CMC in 25% ethanol with cellulosome	3.68 (0.04)	3.63 (0.05)

Figure 4.3 shows the bar plot of endoglucanase activity for batch 1, 2, and 3 determined after 24 hours and 48 hours. The bar plots clearly indicate an improvement in reducing sugars released after 48 hours (5% and 10%, respectively) and the extracts incubated in substrates

containing 10% ethanol showed slightly lower values of reducing sugars released overall when compared with those without ethanol and those with 5% ethanol concentration.

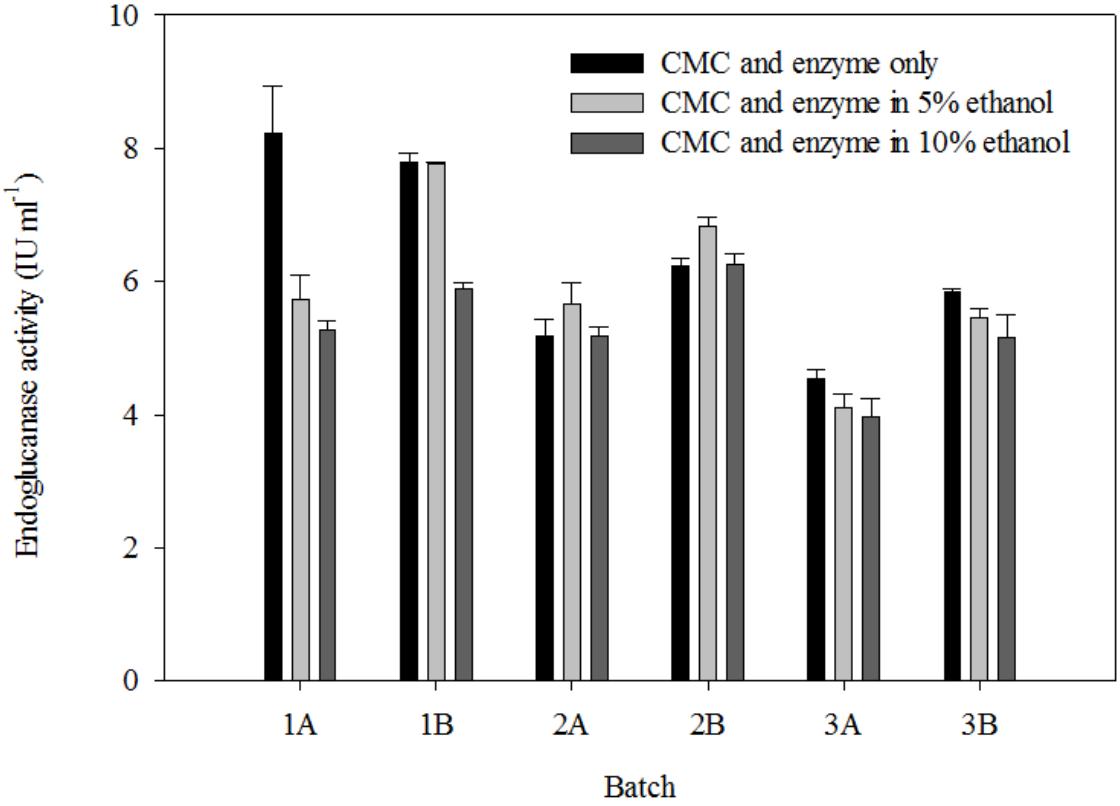


Figure 4.3: Endoglucanase activities for 0%, 5% and 10% ethanol concentrations. A, 24-hour incubation; and B, 48-hour incubation. Error bars represent standard deviations among three replicates.

The values obtained from the three independent experiments for 0%, 5% and 10% conditions were normalized before plotting and subjected to statistical analyses using ONE-WAY ANOVA. There is a statistical significant difference in the amount of reducing sugar released under the three conditions, $p = 0.033$ ($\alpha = 0.05$). Additionally, the t-test of means (using the Mann-Whitney Ranked Sum Test) was applied to the values to determine whether any difference existed between the 24 hours samples and the 48 hours sample. The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference ($P = <0.001$) (figure 4.4). The data indicate that the reaction was highly

affected by the addition of ethanol up to 10% and that endoglucanase activity progressed beyond could have continued beyond 48 hours.

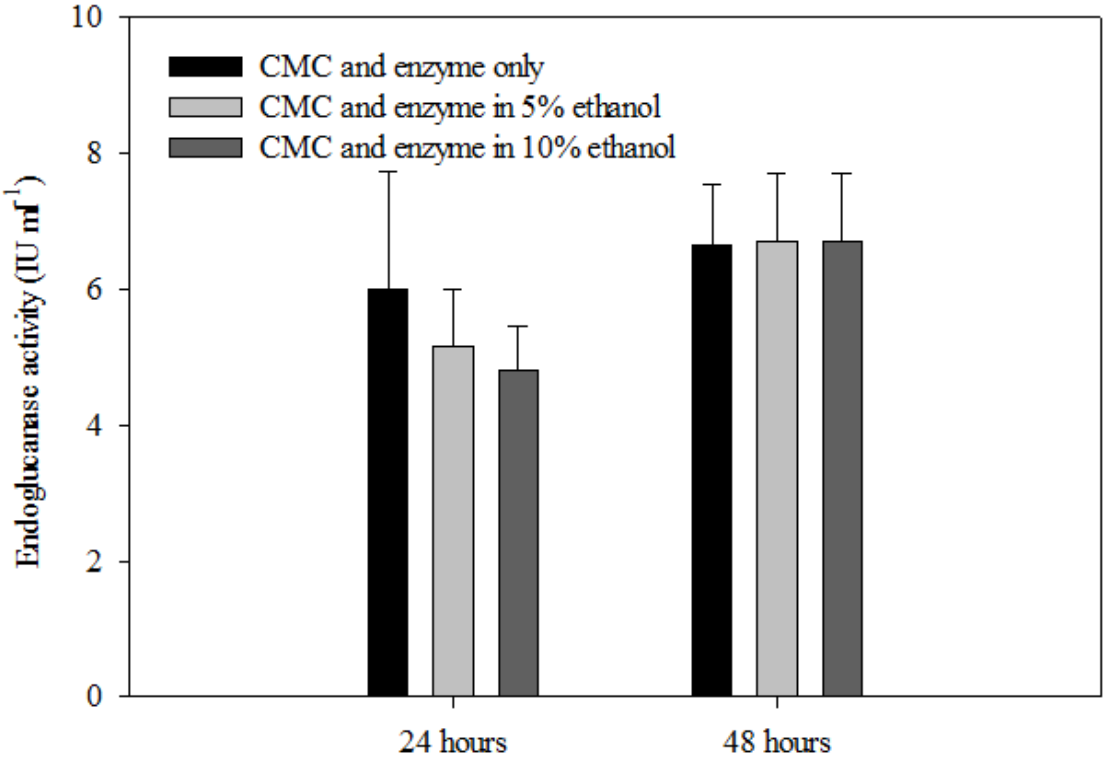


Figure 4.4: Endoglucanase activities with 0%, 5% and 10% ethanol concentrations. Error bars represent standard deviations among three replicates.

Figure 4.5 below shows the bar plot of a standalone batch 4 with 15%, 20% and 25% ethanol concentrations included for 24 hours and 48 hours incubation time. Replicates of the sample were statistically analyzed separately from other batches using the Kruskal-Wallis One Way Analysis of Variance on Ranks. The differences in the median values among the ethanol concentrations are greater than would be expected by chance; there is a statistically significant difference ($P = <0.001$).

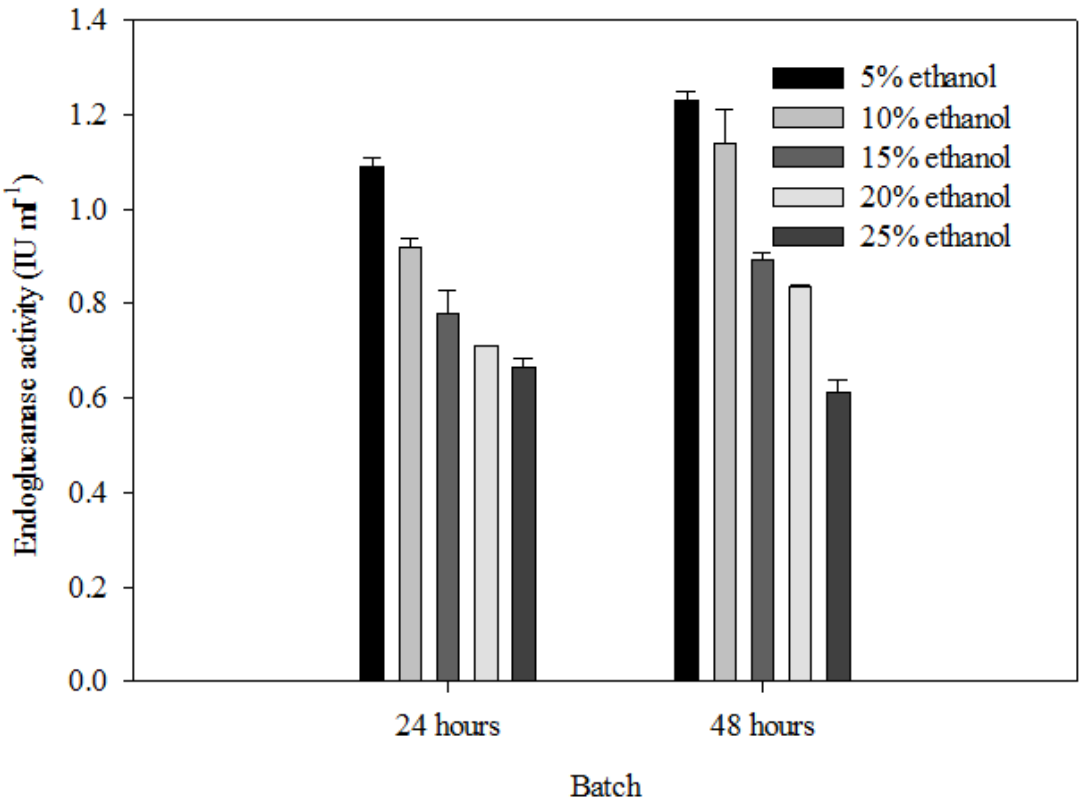


Figure 4.5: Endoglucanase activities of the independent batch 4 samples with 0%, 5% 10%, 15%, 20% and 25% ethanol concentration. Error bars represent standard deviations among three replicates.

The result of the statistical analysis obtained for the batch 4 supports the result earlier seen in batch 3 even though the standalone batch 4 did not produce the same level of endoglucanase activity as previously observed with the first 3 batches that were analyzed. Cellulosomal activity could be highly dependent upon other extraneous factors, such as pH, temperature, presence of chemical elements in the aqeous medium etc. For batch 4, samples from 5% to 25% followed a similar pattern with the previously analyzed batche 1, 2 and 3 and a decline was observed in endoglucanase activity with increasing ethanol concentration. An attempt was made to analyze higher ethanol concentration above 30% and the result (not shown) was highly inconsistent and therefore was dropped. The Mann-Whitney Ranked Sum Test was performed for the 24 hours and 48 hours reading of batch 4. The difference in the median values between the two groups is not

great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.228). This is not consistent with the previously observed result for the first three batches and could be as a result the low endoglucanase activities obtained.

The actual values for the endoglucanase activities determined could however be a function of the concentration of the cellulosome, the physiological state of the cells in the culture medium, the culturing conditions used, the purification, handling and processing conditions etc.

4.4 Section Conclusions

The cellulosomes of *C. cellulolyticum* retained endoglucanase activity in the presence of ethanol in some cases up to 25%. Enzyme activity seemed improved after 48 hours of incubation with CMC for 5% 10%, 15%, 20% and 25% samples, even though activity decreased as the ethanol concentration increased. However, at no point was activity totally eliminated. In a similar study by Skovgaard and Jorgensen [192], a mixture of mesophilic and thermostable lignocelluloytic enzymes were exposed to a temperature of 55^{o}C to 65^{o}C and up to 5% ethanol (w v^{-1}), the thermostable and mesophilic mixture remained active at up to 65^{o}C. When the enzyme mixtures reached their maximum temperature limit, ethanol had a remarkable influence on enzyme activity, e.g., the more ethanol, the faster the inactivation. During hydrolysis, it has been found that ethanol is a non-competitive inhibitor binding to the allosteric site of the enzyme, which results in reversible denaturation because of the solvent properties of ethanol [193]. Furthermore, ethanol destroys the tertiary hydrophobic interactions in the enzyme, breaking or loosening the compact structure of the enzyme complex [194, 195]. From these preliminary results, the various bioengineering attempts to increase ethanol production in microbes will not likely be limited by

cellulase activity but further investigation will need to be carried out to determine whether a "ceiling" exists for ethanol stress that could be tolerated by the cellulosomes. Additionally, the actual activity of cellulosomes *in vivo* could be different from that observed under the *in vitro* conditions tested here. This could occur if, for example, other yet-to-be annotated genes and their protein products may contribute to the resilience of the cellulosomes *in vivo*. Cellulosomes, in spite of their size and complexity, are remarkably robust complexes. It is likely that these structures can withstand various degrees of other environmental stresses in addition to those discussed here. Future efforts should explore tolerances to alternative biofuel products, such as biodiesel, biobutanol, etc.

CHAPTER 5

DETERMINATION OF ETHANOL PRODUCTION USING RECOMBINANT AND WILD TYPE *Zymomonas mobilis* ZM4 AND *Clostridium cellulolyticum*

This describes the various ethanol determination assays involving both single and co-cultures of both the wild-type and recombinant forms of *Z. mobilis* ZM4 and *C. cellulolyticum*

An ethanol standard curve was determined and used to calculate the ethanol produced in culture samples. Ethanol concentration was measured by gas chromatography on a Shimadzu model GC-14A outfitted with an SP1200 packed column (6 feet) column according to the methods of Weimer et al. [196]. The GC was set at an initial temperature of 50ºC, initial time of 2 minutes, program rate of 5ºC per minute, final temperature of 80ºC and final time of 1 minute. Range was set at 3, polarity at 1, injector temperature at 180ºC and detector temperature at 200ºC.

Cultures containing wild type ZM4, *C. cellulolyticum* and wild type ZM4 + *C. cellulolyticum* were grown in anaerobic tubes with microcrystalline cellulose as the carbon source. Cultures were incubated for 5 days and 10 days. Samples were taken from the cultures for the first 5 days and again after the 10^{th} day. The time points were chosen to allow sufficient time for *C. cellulolyticum* to grow. The samples collected were analyzed using the gas chromatograph and the ethanol concentration calculated from the pre-determined ethanol standard. This experiment was repeated two more times under similar conditions. Plots of the ethanol standard curve and the ethanol concentration of the samples are shown in figures 5.1-5.4.

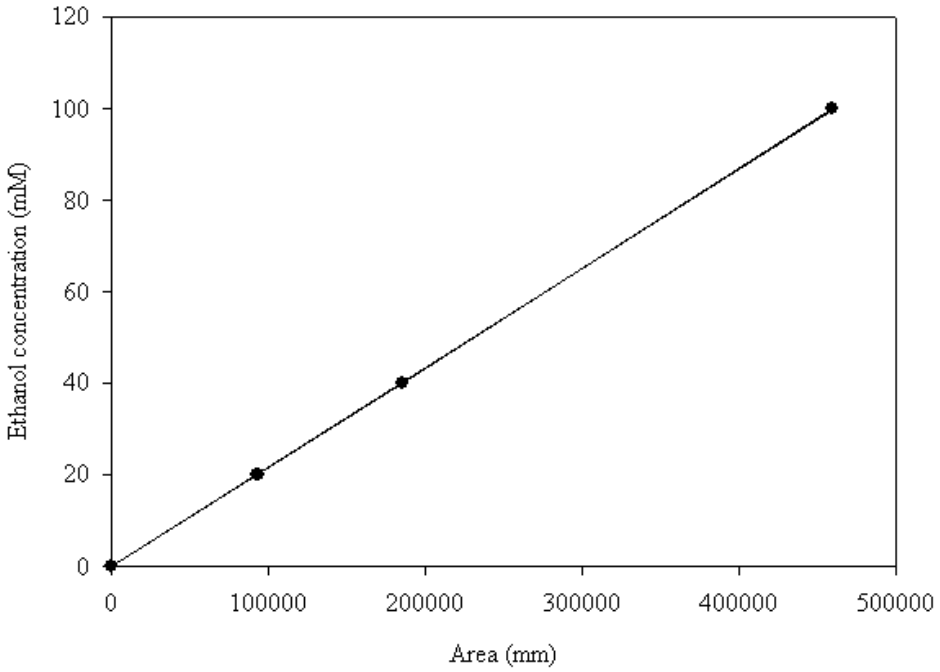


Figure 5.1: Ethanol standard curve (mM) as determined by gas chromatography.

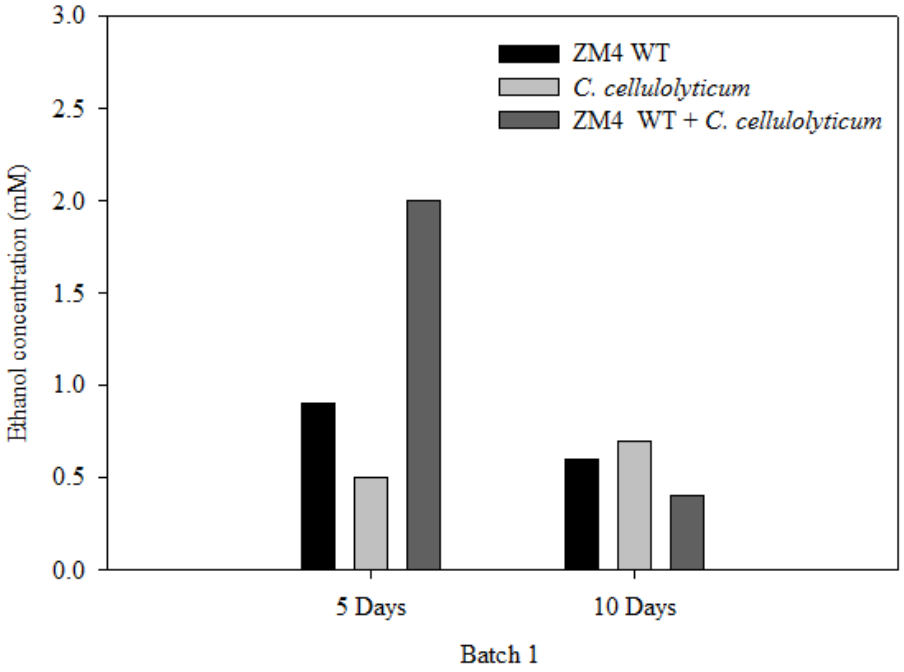


Figure 5.2: Ethanol production from microcrystalline cellulose after 5 and 10 days incubation.

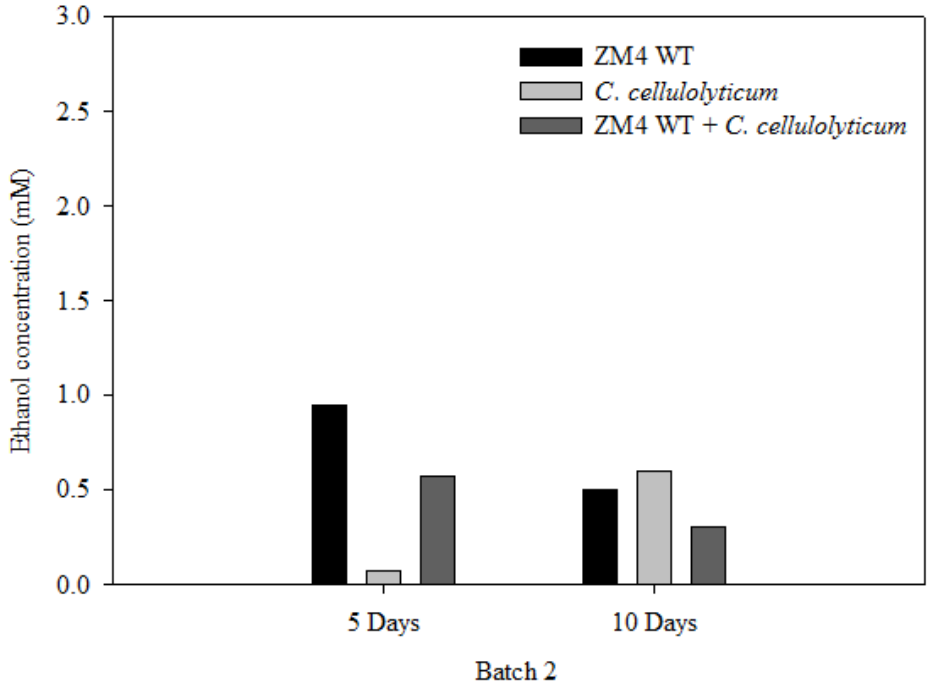


Figure 5.3: Ethanol production from microcrystalline cellulose.

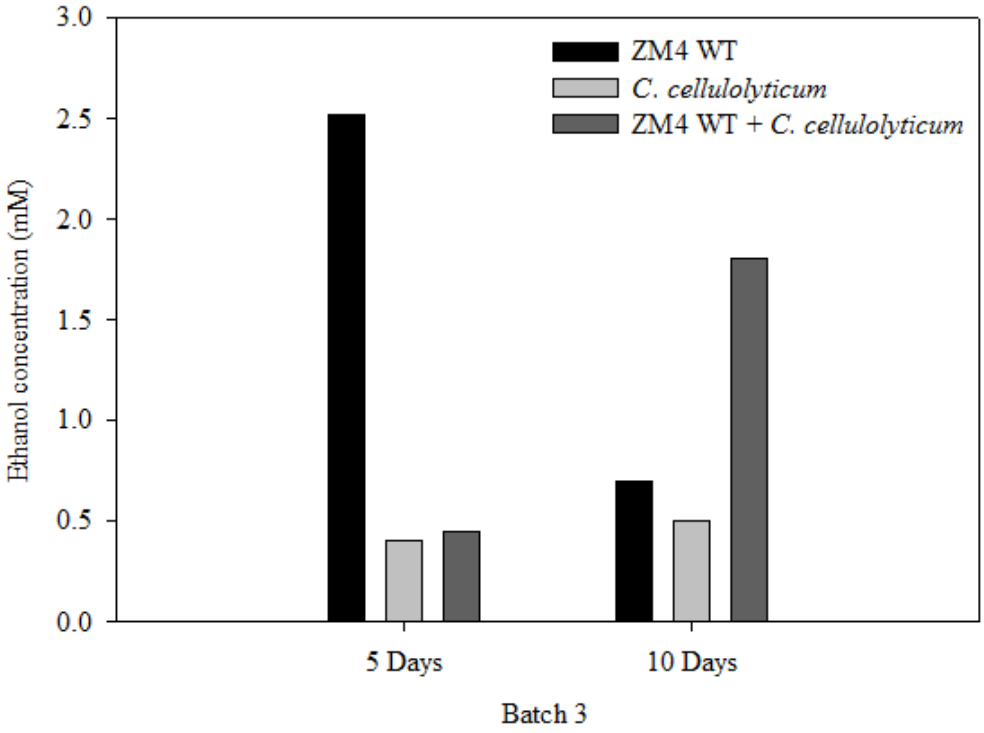


Figure 5.4: Ethanol production from microcrystalline cellulose.

From the above results the culture with wild type ZM4 alone produced ethanol but obviously not from microcrystalline cellulose as there has not been any reported cellulase gene associated with *Zymomonas mobilis.* It is likely that the microcrystalline cellulose substrate contained some free glucose monosaccharides, which the wild type ZM4 utilized to produce the ethanol concentrations detected, or the ethanol detected is coming from the offshoot of initially growing the wild type ZM4 in glucose before using it to inoculate the cellulose medium. The highest ethanol concentration detected was 2.52 mM from batch 3 after 5 days. Ethanol production was observed in *Clostridium cellulolyticum*, which had the least amount of ethanol with the maximum of 0.7 mM concentration after 10 days. Clearly as reported by several researchers, *Clostridium cellulolyticum* is efficient cellulose degrader but inefficient in producing significant amount of ethanol. It is noteworthy to point out that whereas the ethanol "fortune" of wild type ZM4 decreased across the three batches for the 10 days determination, *Clostridium cellulolyticum* had an improvement across the three batches at the 10 days determination. In addition to the function of breaking down cellulose, some loose sugars and degraded cellulose where converted to ethanol by *Clostridium cellulolyticum.* For the co-culture, there was no consistency in ethanol production in the three batches and for both the 5 day and 10 day cultures. Whereas batches 1 and 2 saw a decrease in ethanol concentration from 5 days to 10 days, batch 3 was the exact opposite in which ethanol production increased in 10 days. It is suspected that these inconsistencies are due to failure to wash the cells from their initial medium, failure to wash the cellulose free of soluble sugars, failure to adequately grow and properly dilute the *C. cellulolyticum*.

From the data presented, it does not appear that there is any nartural synergy between wild type ZM4 and *Clostridium cellulolyticum* for concommitant cellulose degradation and ethanol production from microcrystalline cellulose. Further experiments will be required using a different

carbon source that does not yield loose monosaccharides that could support the growth of the wild type ZM4.

Fresh batches of cultures were grown on cellulose filter paper under the same conditions as described above. The use of cellulose filter paper was also to determine the amount of reducing sugars released according to the methods of Xiao et al. [197] and to see whether there would be any synergy between wild type ZM4 and *C. cellulolyticum* in the co-culture using an alternative source of cellulose. Ethanol production was quantified as described previously. Ethanol was originally detected in the wild type ZM4 cultures using microcrystalline cellulose as the substrate and the switch to cellulose filter paper was in part to establish if free contaminating monosaccharides exist in the medium with microcrystalline cellulose. Figure 5.5 below is the standard curve of reducing sugar activity for estimation of the amount of reducing sugars released by the wild type ZM4, *C. cellulolyticum* and ZM4 + *Clostridium.*

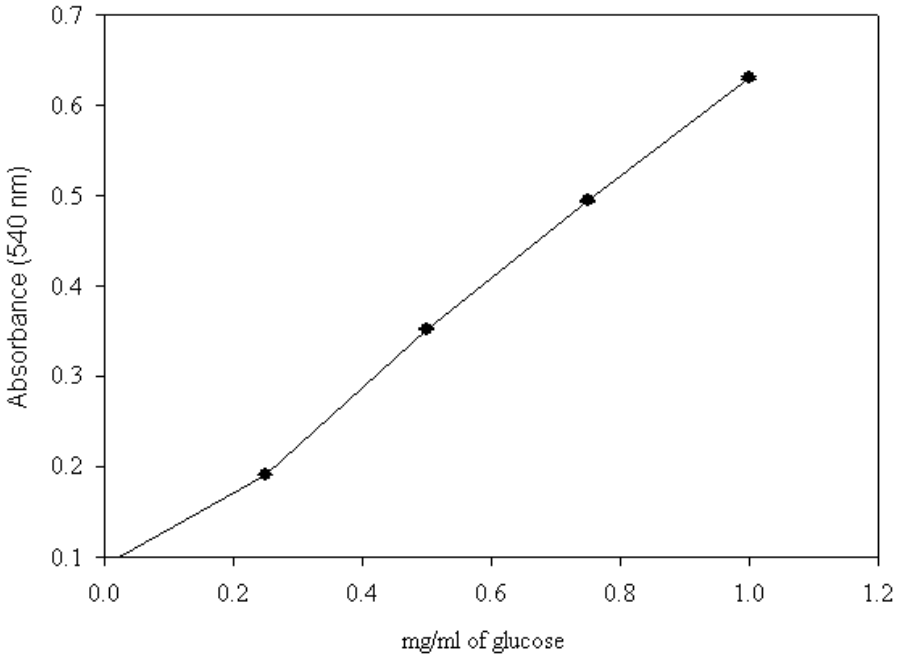


Figure 5.5: Standard curve of reducing sugar activity.

Cultures were incubated under similar conditions as described previously and the reducing sugars released calculated after 5 and 10 days, respectively. Figures 5.6 and 5.7 show the amount of reducing sugars released from batch 1 samples and from a repeat experiment designated as batch 2.

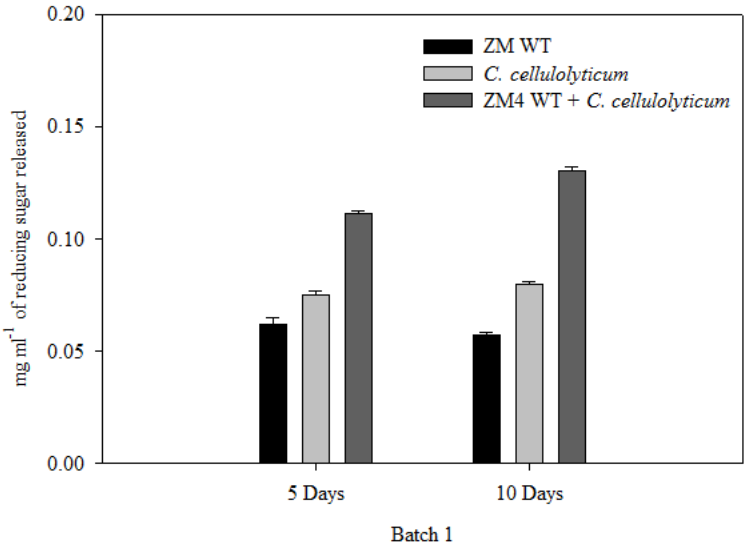


Figure 5.6: Amount of reducing sugars released in mg ml^{-1}. Error bars represent standard deviations among three replicates.

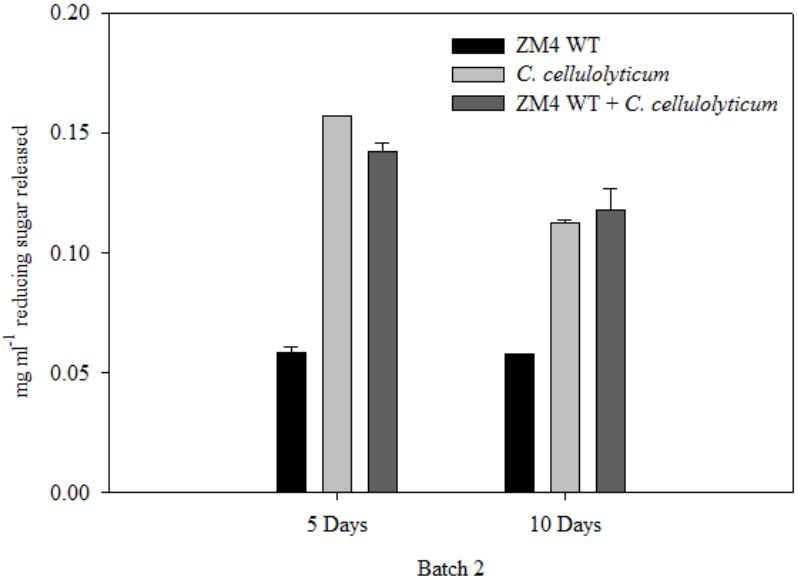


Figure 5.7: Amount of reducing sugars released in mg ml^{-1}. Error bars represent standard deviations among three replicates.

From the batch 1 samples, the co-culture suggests a synergy between wild type ZM4 and *Clostridium* and had the highest amount of reducing sugars released for both 5-day and 10-day cultures with a slight improvement in the amount of sugar released after 10 days. The *Clostridium* culture alone showed far less amounts of reducing sugar released and the wild type ZM4 alone sample even less. Batch 1 samples suggest an evidence of synergy between the two microorganisms hence the remarkable improvement in the amount of sugar released; however, ZM4 is not known to be able to utilize any carbon source outside of glucose, fructose, sucrose and occasionally mannose. It is therefore concluded that free monosaccharides are not present in the medium and any reducing sugar released is solely as a result of the activity of *Clostridium* in the co-culture. Lack of reducing sugars in batch 1 when *Clostridium* was cultured alone suggests a failure of that batch to grow—a recurring problem with this organism. The reducing sugar detected in the wild type ZM4 alone samples are more likely to be carry over from the RM medium based inoculum with glucose as a carbon source and was not produced from the degradation of the cellulose filter paper. Additionally, the same condition may have influenced the amount of reducing sugars detected from the co-culture. A repeat of the same experiment designated as batch 2 gave a remarkably different result. The amount of reducing sugar released by *Clostridium* alone was higher than earlier observed in batch 1 and also higher than that of the co-culture in the 5-day determination, decreasing slightly after 10 days. A lower amount of reducing sugar was determined for the wild type ZM4 samples alone and would seem to correspond to the lower amount of reducing sugar determined for the co-culture. As suggested before, the data support the conclusion that there is no evidence of synergy between the wild type ZM4 and *Clostridium* in co-culture. Batch 1 showed a greater amount from the 10-days samples over the 5-days samples whereas batch 2 showed the opposite. These inconsistencies are likely the result of experimental heterogeneity

including carry over of free sugars from RM medium used to cultivate *Z. mobilis* and difficulties in growing *C. cellulolyticum*, which cannot be measure by optical density since they majority of biomass is bound to insoluble cellulosic material.

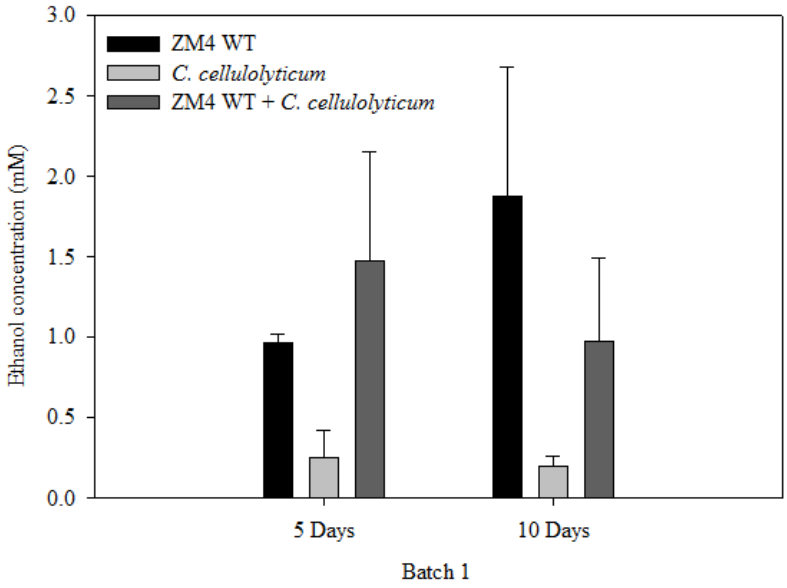


Figure 5.8: Ethanol production from cellulose filter paper. Error bars represent standard deviations among three replicates.

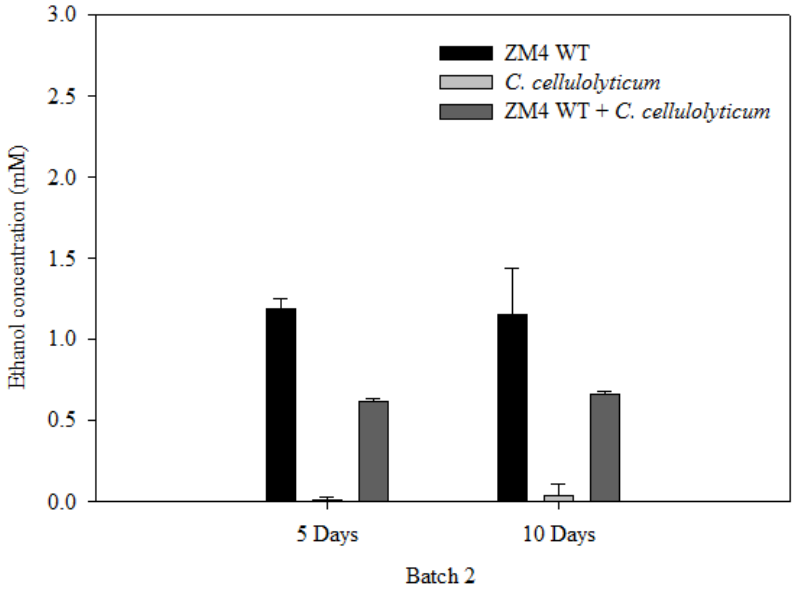


Figure 5.9: Ethanol production from cellulose filter paper. Error bars represent standard deviations among three replicates.

Surprisingly ethanol was detected from the wild type ZM4 monoculture in reasonable quantity, in small quantities and below detection limit from Clostridium monoculture and again detected in the co-culture. The ethanol detected in the ZM4 monoculture and in the co-culture could have come from the wild type ZM4 inoculum or fermentation of glucose carried over in the inoculum. A trend was observed; however, in which the ethanol content of the co-culture was lower when compared to that of the wild type ZM4 monoculture. *Clostridium* could have similarly utilized any simple sugars carried over from the wild type ZM4 inoculum for growth and to make its insignificant amount of ethanol typical of the species in use here. The above result is clearly random, does not have a specific pattern except for the reduction in ethanol production for *Clostridium* monoculture in batch 1 and the minimal detection of ethanol produced by the same *Clostridium* monoculture in figure 5.9.

To correct the obvious error of glucose carryover from the original inoculum of the wild type ZM4, which seemed to be giving the wild type ZM4 an unusually higher ethanol concentration over *Clostridium* and the co-culture of wild type ZM4 and *Clostridium,* wild type ZM4 cells were grown over night in RM broth, cells were washed three times in Phosphate Buffer Saline (pH 7.2) and finally re-suspended in the same buffer and kept chilled on ice. Washing the cells was to ensure that no traces of glucose or ethanol which could be carried over into the main experiment. *Clostridium cellulolyticum* was routinely grown in its medium containing microcrystalline cellulose as a carbon source. These two were then used to inoculate the tube as earlier described and incubated under the same conditions as the previous experiments. The experiments were repeated two more times to ensure consistency. The bar plots showing the amount of ethanol determined from ZM4, *C. cellulolyicum* and ZM4 + *C. cellulolyticum* are shown below in figures 5.10 and 5.11.

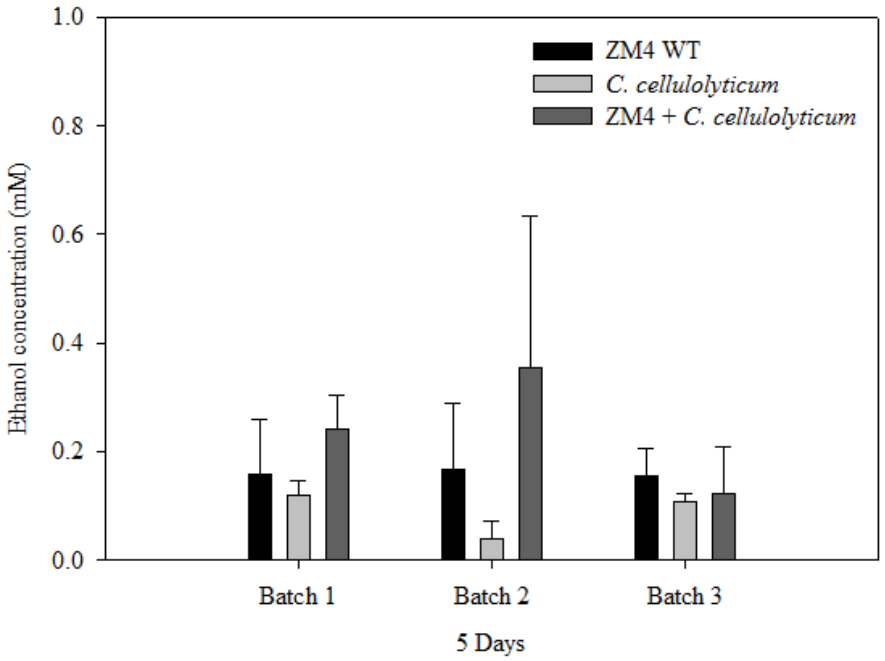


Figure 5.10: Ethanol production from microcrystalline cellulose. Error bars represent standard deviations among three replicates.

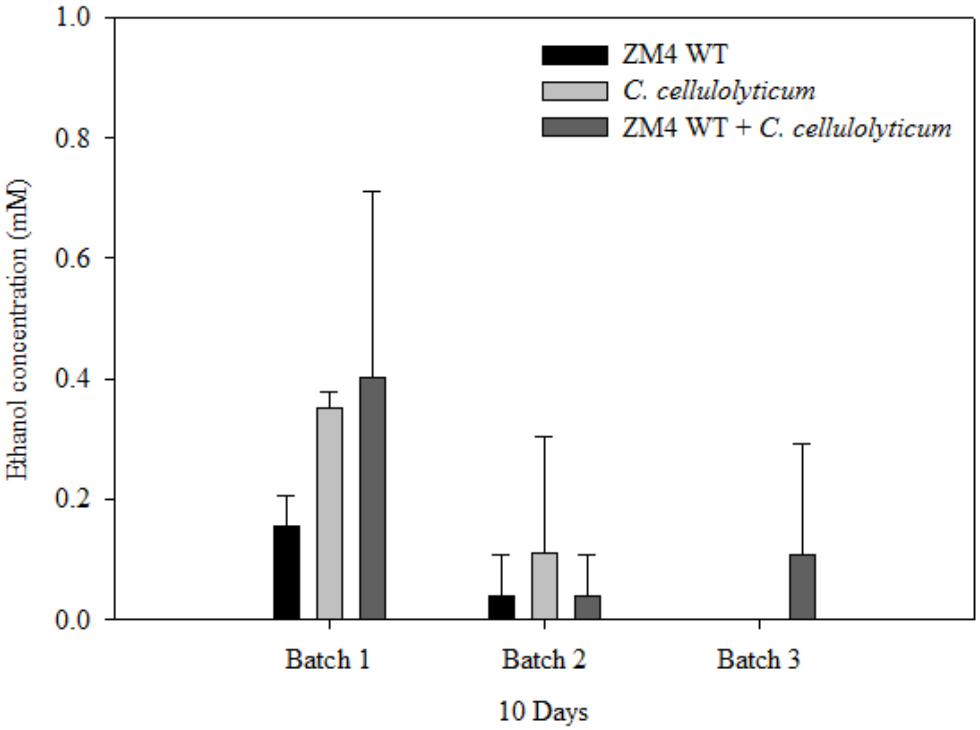


Figure 5.11: Ethanol production from microcrystalline cellulose. Error bars represent standard deviations among three replicates.

From the above bar plots, it is clear that the wild type ZM4 does not make ethanol from cellulose as earlier suggested. The ethanol content quantified from the first two experiments were carryovers from the inoculum and with the washed cells, this was no longer an issue. Traces of ethanol production continued to be seen with the wild type ZM4 alone for the five days and the ten days samples even after the thorough washing. From the above plots, the ethanol detected from wild type ZM4 alone after washing did not exceed 0.2 mM.

ZM4 is a very efficient ethanologen and the background noise seen here is likely as a result of letting the cells grow to an OD λ=600 above 1.50 in the RM medium used for the inoculation of the media containing cellulose. Though the cells were washed before use, it is likely that there was still enough residual ethanol that was carried over to be detected in these assays. One clear observation is the significant reduction in the amount of ethanol observed from wild type ZM4 cultures alone, once determined at 2.52 mM at its peak but less than 0.2 mM from the last trials that involved washing the cells before use. The five-day cultures of batches 1, 2 and 3 suggest a synergy in the co-culture, with batch 2 producing the highest amount of ethanol at slightly below 0.4 mM concentration; far less than was observed when the unwashed wild type ZM4 inoculum was used. From the 10 days cultures of the same batches, batch 1 cultures showed improved ethanol concentration across for *Clostridium* alone and for the co-culture of wild type ZM4 + *Clostridium.* Batch 2 however had no ethanol detected for the co-culture, suggesting that the *Clostridium* could have gone into the dormant/sporulation stage and the wild type ZM4 in the medium could be all dead. A similar but different pattern can be seen in batch 3, where no ethanol activity can be seen in the monocultures but still observable in the co-culture, again suggesting a synergy.

The three batches under this condition showed different results but one thing does stand out from this last experiment, the co-culture at any point it made ethanol, was more than that of the monocultures, suggesting a small but present synergy between cellulose degrading *C. cellulolyticum* and wild type ZM4. Furthermore the quantity of ethanol determined was far lower than those seen from the previous experiments in which the wild type ZM4 was directly inoculated from glucose containing medium. Therefore the higher values from the previous experiments were likely from the pre-formed ethanol in the wild type ZM4 inoculum. To cultivate *Clostridium cellulolyticum* under strict anaerobic conditions and produce detectable quantities of ethanol could give varying results. Sometimes ethanol is detected within the first five days and by the tenth day, there could either be an increase in the amount of ethanol detected, suggesting continuous conversion of the substrate. Alternatively, there could be no ethanol detected, which suggests that the cells may have gone into the dormant/sporulation stage, as is common for *Clostridium* species. This condition is not only triggered by nutrient limitations but likely here by the earlier suggested pyruvate overflow and heavy carbon flux through glycolysis that is higher than the rate of procession of pyruvate ferridoxin oxidoreductase (PFO) and lactate dehydrogenase (LDH). The difficulty in growing *Clostridium* and its unpredictable growth pattern makes it difficult to track its performance in co-culture using synthetic medium.

5.1 Ethanol Production from Cellobiose Using Recombinant ZM4 (pAA1) AND ZM4 (Wild Type)

Cultures of *Z. mobilis* were grown in RM medium containing 2% glucose and supplemented with gentamicin (30 μg ml^{-1}.). The recombinant *Z. mobilis* were similarly grown in RM plus glucose but were supplemented with 15 μg ml^{-1} of tetracycline in order to maintain the vector. Cells were harvested after 24 hours, washed thoroughly in PBS (pH 7.2) and re-suspended

in RM medium containing 2% cellobiose to give an OD of 2.0. Appropriate antibiotics were included in each medium. The cultures were incubated at 30ºC in a shaking incubator at 150 revolutions per minute (rpm). OD was determined every twenty-four hours for 7 days and samples taken from the cultures for ethanol determination starting from 48 hours after original inoculation and every 24 hours thereafter for three days.

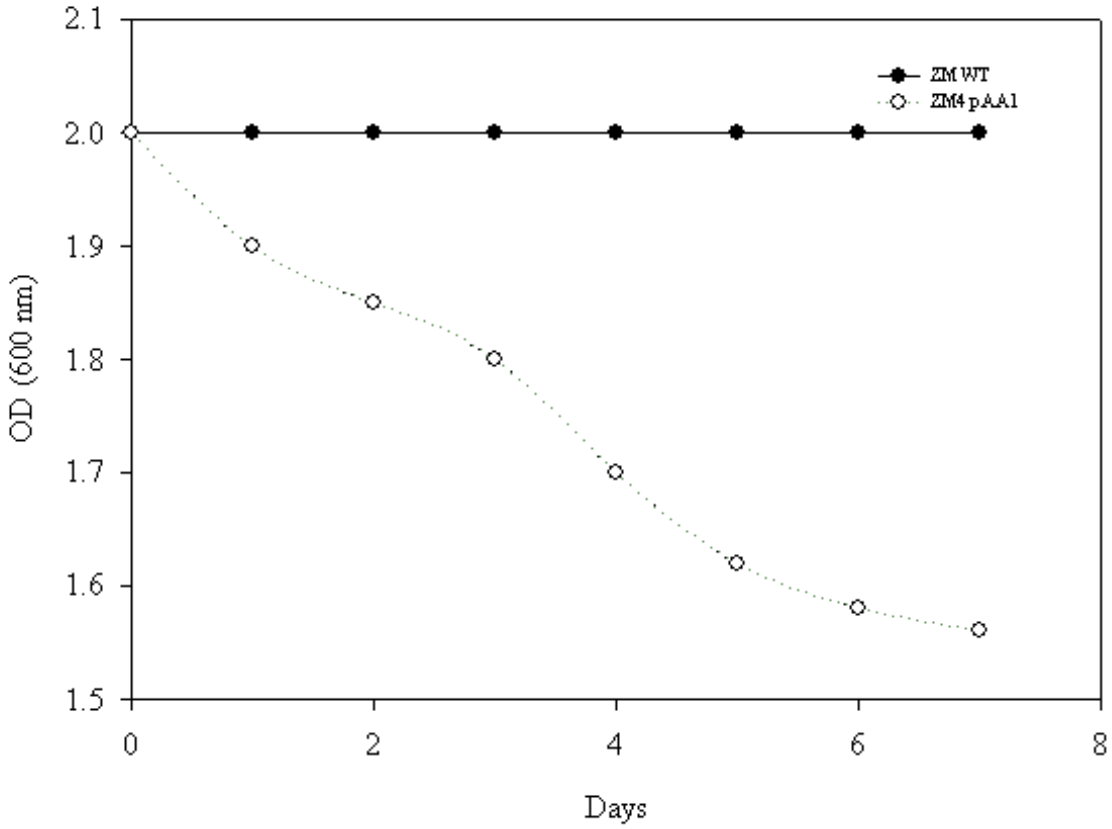


Figure 5.12: Optical density of the ZM4 pAA1 and ZM WT.

Starting OD was 2.0 and decreased as insoluble cellobiose was consumed and ethanol production increased for the ZM4 pAA1. OD for the ZM4 WT stayed the same indicating it failed to utilize the substrate.

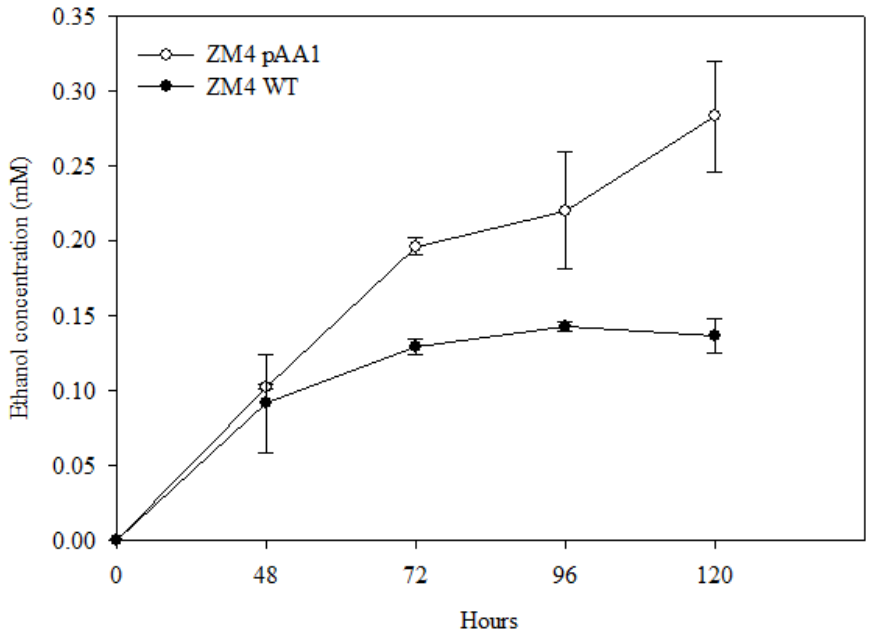


Figure 5.13: Ethanol production from RM medium containing 2% cellobiose as the carbon source. Error bars represent standard deviations among three replicates.

From figures 5.12 and 5.13 it can be seen that the recombinant ZM4 actively degraded the insoluble cellobiose substrate and concomitantly produced three times as much ethanol after 5 days than did the wild type ZM4 ($p= 0.03761$). The ethanol detected in the wild type could again have come from the presence of glucose transferred into the medium at inoculation. As ethanol is produced, the OD of the recombinant culture reduced and was 1.58 after 7 days. Further reduction was recorded subsequently but did not change any further after 1.44. No change was detected for the wild type ZM4 for the entire duration.

5.2 Ethanol Production from Cellobiose and Microcrystalline Cellulose Using *Z. mobilis* ZM4 pAA1, ZM4 WT and *Clostridium cellulolyticum*

Z. mobilis ZM4 pAA1 and ZM4 WT cultures were grown to OD of 0.4-0.6 in RM medium containing glucose. The cultures were washed in PBS (pH 7.2), re-suspended, and then kept on ice

until further use (maximum time < 20 minutes). *Clostridium cellulolyticum* was grown in Clostridial medium containing 7.5 g L^{-1} of cellulose as the carbon source to an OD of 0.4-0.6. The cultures were then used to inoculate Clostridial medium containing cellobiose and microcrystalline cellulose as carbon sources respectively.

The set-up for this experiment was similar to the previously described set-ups but now with the inclusion of the recombinant ZM4 pAA1 to test the activity of the plasmid-encoded genes in the breakdown of cellobiose and to check whether this clone does in anyway enhance the production of ethanol in the co-culture of ZM4 pAA1 with *Clostridium.* For the mono cultures of ZM4 pAA1, ZM4 WT and *C. cellulolyticum,* the medium was inoculated with 5% inoculum size (v v^{-1}) and for the co-culture medium was inoculated with 2.5% each of the inoculum size. For the Clostridial medium containing cellobiose, 2 g L^{-1} of the oligosaccharide was used whereas for the regular Clostridial medium, 7.5 g L^{-1} of cellulose was used.

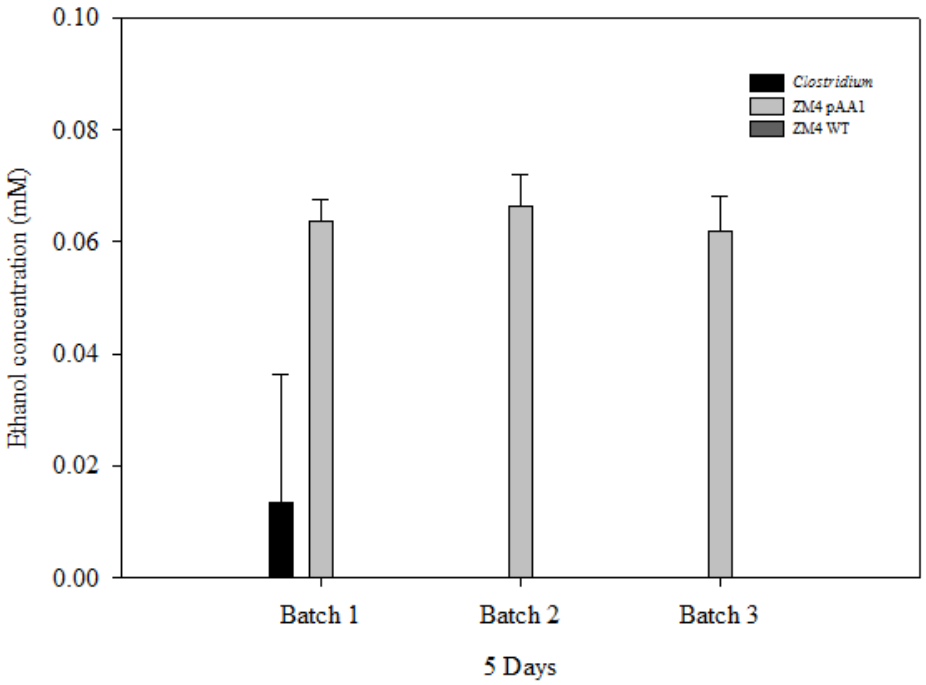


Figure 5.14: Ethanol production from cellobiose. Error bars represent standard deviations among three replicates.

As seen in figure 5.14, ZM4 pAA1 produced the most ethanol and was consistent across the three batches whereas ethanol production was not observed at all for ZM4 WT across the three batches. *Clostridium* produced ethanol once as can be seen from batch 1 (figure 5.14) but none in subsequent batches. The 2 g L^{-1} of cellobiose utilized was to enable the growth of *Clostridium,* which seem to be adversely affected at any concentration above 2 grams (data not shown).

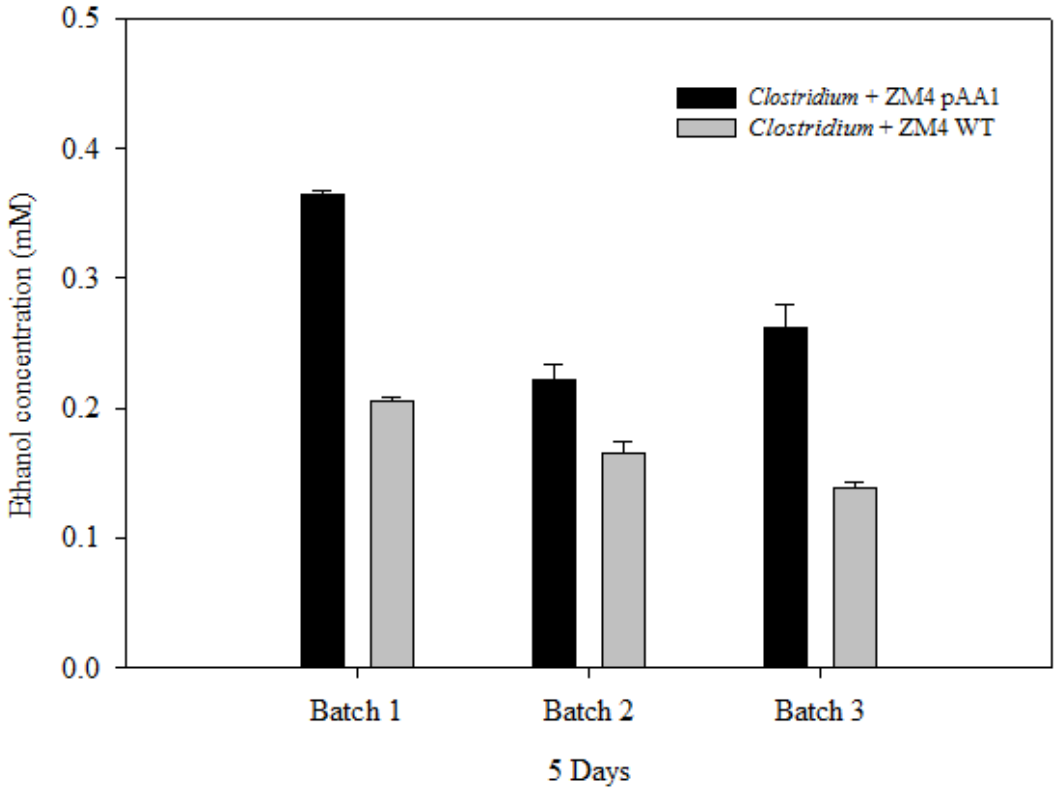


Figure 5.15: Ethanol production from cellobioise. Error bars represent standard deviations among triplicate samples.

In 5.15 above, ethanol production can be observed from the two conditions, with *Z. mobilis* ZM4 pAA1 and *Clostridium* producing more ethanol than ZM4 wild type and *Clostridium.* In a study by Payot et al. [198] detailing the metabolism of cellobiose by *Clostridium cellulolyticum* growing in continuous culture, they reported that *C. cellulolyticum* is able to metabolize only a small quantity of soluble carbohydrates (3 g L^{-1}), with the molar growth yield reduced when the

initial cellobiose concentration exceeded (2 g L^{-1}). In my experiment, the concentration of cellobiose utilized was originally set at 5 g L^{-1} and the results obtained (not shown) clearly showed that such concentration negatively impacts growth of the *Clostridium* but the recombinant ZM4 pAA1 acted to partially rescue this phenotype. Subsequent tests with 2 g L^{-1} cellobiose showed a slight improvement. The ZM4 WT however, was unable to do same due to the absence of the β-glucosidase gene and also by the use of a medium that may not adequately support its growth. Furthermore as described by Payot et al. [198], *Clostridium cellulolyticum* is limited due to low rate of NADH reoxidation leading to an intracellular accumulation of the reduced nucleotide. As previously described by Giallo et al. [199] for batch cultures, acetate is the main product for the continuous culture seen in the work of Payot and coworkers. The acetate formation was found to increase with increasing carbon flow, leading to a high ATP production and to an insufficient rate of NADH regeneration. They further described the ability of *Clostridium acetobutylicum* to induce metabolic shifts to produce solvents such as ethanol, butanol and acetone and this shift is associated with high intracellular ATP and NAD(P)H contents. Solvent production in *C. acetobutylicum* is induced under conditions of low ATP requirement, high ATP availability and high intracellular NADH content but as determined by Payot et al. [198], *C. cellulolyticm* is not able to induce metabolic shift to produced reduced compounds such as ethanol. The result obtained from the co-culturing of recombinant ZM4 with *C. cellulolyticum* and wild type ZM4 with *C. celluloyticum* is consistent with previously observed findings, therefore the ethanol produced in the medium is likely as a result of the secretion of the fused β-glucosidase into the medium by ZM4 pAA1 and the ability of ZM4 wild type to convert glucose released from cellobiose to ethanol, respectively.

Figure 5.16 shows ethanol production from microcrystalline cellulose by *C. cellulolyticum,* ZM4 pAA1 and ZM4 (WT) in five days.

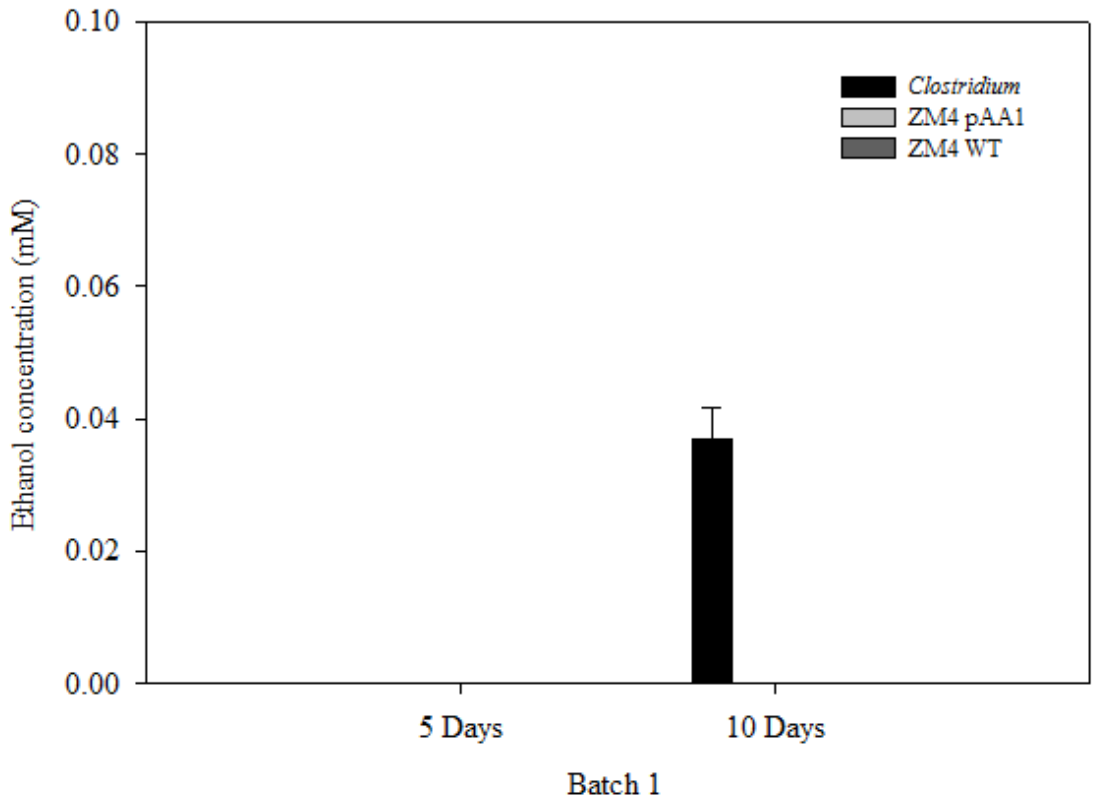
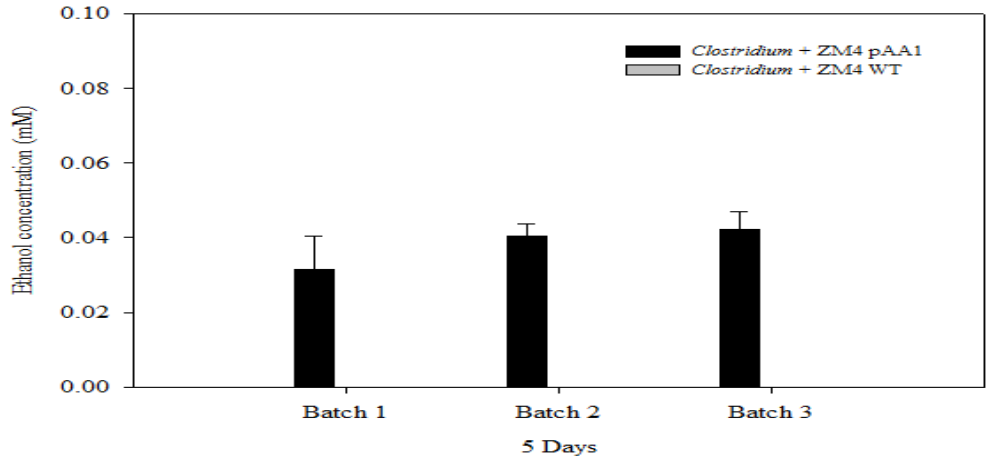
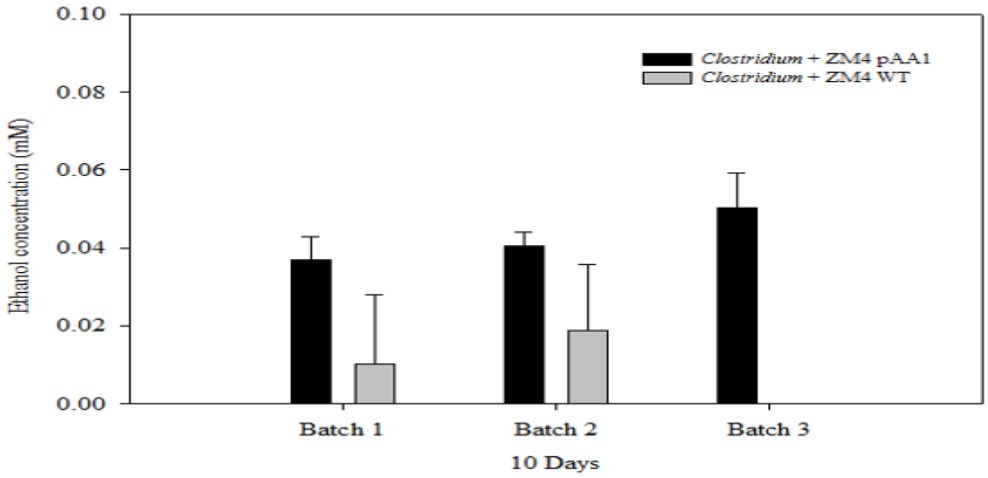


Figure 5.16: Ethanol production from microcrystalline cellulose. Error bars represent standard deviations among three replicates.

C. cellulolyticum showed ethanol production for only one batch but no ethanol detected from ZM4 pAA1 and ZM4 WT respectively. No ethanol was detected in the first 5 days but was seen in 10 days for *Clostridium.* The amount of ethanol produced by *Clostridium* only is far lower than seen when *Clostridium* was co-cultured with ZM4 pAA1 using cellobiose as the carbon source.



(A)



(B)

Figure 5.17: Ethanol production after 5 (A) and 10 (B) days using microcrystalline cellulose as a carbon source. Error bars represent standard deviations among three replicates.

In the co-culture, figure 5.17, ethanol production was detected for *Clostridium* with ZM4 pAA1 for the three batches after 5 days and increased after 10 days while *Clostridium* with ZM4 wild type produced ethanol in batch 1 and batch 2 and not batch 3 and only detected after 10 days. This observation clearly suggests that the co-culture involving recombinant ZM4 is more efficient than the wild type. This can be seen from both the time it took for ethanol to be detected and the quantity of ethanol detected. There appear to be only slight increases in ethanol production from the co-culture involving the recombinant ZM4 from the the 10-day culture compared to that

produced after five days, suggesting that maximum ethanol yield could be possible in slightly over 5 days. The co-culture involving the wild type ZM4 produced less ethanol and that was detected only after 10 days suggesting less efficiency in synergy.

CHAPTER 6

CONCLUSIONS

Ethanol production by ZM4 has been extensively studied and well documented. Furthermore, there exists a litany of articles describing the bioengineering efforts of ZM4 in order to improve ethanol yield and expand substrate utilization range. The work described herein is unique and hinged on two main points:

1) Determination of ethanol tolerance of the cellulosome of *Clostridium cellulolyticum* for determination of a possible limiting step in biomass to ethanol conversion, and
2) Ethanol production by a *consortium* of engineered ZM4 with *C. cellulolyticum.*

From the results described, the cellulosome of *C. cellulolyticum* can clearly withstand high concentrations of ethanol even though *C. celluloltyticum* does not make significant amounts of ethanol itself. The reason for this has been extensively studied and described by several workers cited in this project. However, the importance to this study is that cellulosome stability at elevated ethanol concentrations is not likely to be a major impediment to biomass to ethanol conversion in the co-culture described here.

On the second objective, the recombinant *Z. mobilis* ZM4 bearing the plasmid vector pAA1 clearly supports the findings of Yanase et al. [56] in which ZM4 re-engineered with β-glucosidase gene from *Ruminococcus albus* was able to secrete 61% through the cytoplasmic membrane which resulted in the production of 0.49 g ethanol g^{-1} cellobiose. From this project, the highest ethanol concentration determined for recombinant ZM4 pAA1 was approximately 0.06 mM whereas the wild type showed no evidence of ethanol production after 5 days on *C. cellulolyticum* medium containing cellobiose. The celZ, celY and gFOR-betaglucosidase genes are all capable of breaking down cellooligosachharides including cellobiose [56]. Even with tagging the β-glucosidase gene to gFOR, only about 36% of the total activity was reported to be located on the cell surface fraction,

with 20% of the activity on the periplasmic fraction [56]. To determine ethanol production and optical density of ZM4 pAA1 and of ZM4 WT on RM medium containing cellobiose, ZM4 pAA1 and ZM4 WT cultures were grown in RM medium containing glucose for 24 hours. Cells were washed and re-suspended in RM medium with cellobiose as the carbon source, to give an OD of 2.0 (about 10^7 cells ml^{-1}) before incubation as described by Yanase et al. [56]. With the recombinant ZM4, increase in ethanol production was matched by a steady decrease in OD whereas the wild type ZM4 had no change in OD and a slight increase in ethanol production, which remained the same from 72 hours to 120 hours. Furthermore the recombinant ZM4 produced three times as much ethanol than the wild type ZM4 in RM medium containing cellobiose. Ethanol detected in the wild type is likely from the presence of glucose residue in the medium.

With the introduction of celZ and celY genes, the recombinant ZM4 with *Clostridium* was able to make ethanol from microcrystalline cellulose in 5 days whereas the co-culture with wild type ZM4 was able to make ethanol after 10 days. The recombinant ZM4 and wild type ZM4 did not produce ethanol as expected but *Clostridium* did after 10 days—twice the time it took to make ethanol in a consortium of the recombinant ZM4 pAA1 and *Clostridium.* The consortium was tested with Clostridial medium containing 2 g L^{-1} microcrystalline cellulose but this produced no result and so was determined to be unsuitable and not pursued further.

An attempt was made to describe the various experiments detailing the bioengineering of ZM4 in the literature review and such included the expansion of the substrate range of ZM4 by the inclusion of genes from other microorganisms. The result of these complex bioengineering efforts has been encouraging but more work is required in order to achieve higher titers of ethanol production from different carbon sources. The failure of *C. cellulolyticum* to make ethanol on cellobiose has been described previously by Payot et al. [198] who demonstrated that the main

product of *C. cellulolyticum* cellobiose catabolism was acetate, whereas the production of reduced compounds such as ethanol or lactate was low. This was accompanied by the accumulation of intracellular NADH leading to an NADH/NAD^+ ratio as high as 57 [198]. This high value of NADH/NAD^+ ratio inhibited glyceraldehyde-3-phosphate dehydrogenase activity which in turn limited cellobiose catabolism and cell growth. Another study by Guedon et al. [200] supports the earlier claim by Payot et al. [198] but added that growth on synthetic medium showed a 10-fold increase in the production rates of ethanol and lactate over growth in a complex medium.

The entire experiment for ethanol production using microcrystalline cellulose was extended up to 10 days to ensure maximum ethanol production, which was seen in both the co-culture and the single cultures. More investigation is required in order to effectively optimize synergy between the ZM4 pAA1 and *Clostridium.* The concentration of microcrystalline cellulose used was 7.5 g L^{-1}, but further investigation is needed to determine the best concentration at which a synergy can be clearly observed. Furthermore, the incubation times chosen may also be a factor; therefore, different time points should be tested in order to better investigate the synergy.

Fan et al. [201] decribed the surface assembly of cellusomes on *S. cerevisiae* for cellulosic ethanol production. They reported that the engineered *S. cerevisiae* showed a significant hydrolytic activity towards microcrystalline cellulose with an ethanol titre of 1412 mg $L^{-1.}$ Additionally Anderson et al. [202] reported of the engineering of *Bacillus subtilis* with multicellulase-containing minicellulosome. An analysis of the sugars released from acid-pretreated corn stover indicates that the cells have stable cellulolytic activity that enables them to break down 62.3% ± 2.6% of biomass, It is unknown if the failure of the final cloning assembly pAA2 involving inaZ-cipC-phoZ significantly influenced the final outcome of these experiments. The construct may have given the co-culture of *C. cellulolyticum* and recombinant ZM4 a better chance at utilizing

the microcrystalline cellulose. The experiment on the ethanol tolerance of *C. cellulolyticum* proved that cellulosomes can tolerate ethanol concentrations as high as 25%, a concentration unlikely to be reached using complex carbon sources. Further studies are required to determine the behavior of cellulosomes under stress conditions other than ethanol.

Finally, the recombinant ZM4 pAA1 could be partnered with other known cellulose degraders than the one used herein to further understand its behavior in a consortium. This study could also be expanded beyond the use of two microorganisms to include others as seen in natural systems.

APPENDIX

SEQUENCE OF PLASMID pAA1

LOCUS Final Construct of pAA1 11409 bp DNA circular SYN 31 Dec 2013

BASE COUNT 2699 a 2544 t 3182 g 2984 c

ORIGIN

1 GGTACCGGGC CCCCCCTCGA GGTCGACGGT ATCGATAAGC TTGATATCGA ATTCCTGCAG

61 CCCGGGGGAT CCACTAGTTC TAGAGCGGCC GCCACCGCGG TGGAGCTCCA ATTCGCCCTA

121 TAGTGAGTCG TATTACGCGC GCTCACTGGC CGTCGTTTTA CAACGTCGTG ACTGGGAAAA

181 CCCTGGCGTT ACCCAACTTA ATCGCCTTGC AGCACATCCC CCTTTCGCCA GCTGGCGTAA

241 TAGCGAAGAG GCCCGCACCG ATCGCCCTTC CCAACAGTTG CGCAGCCTGA ATGGCGAATG

301 GAAATTGTAA GCGTTAATAT TTTGTTAAAA TTCGCGTTAA ATTTTTGTTA AATCAGCTCA

361 TTTTTTAACC AATAGGCCGA CTGCGATGAG TGGCAGGGCG GGGCGTAATT TTTTTAAGGC

421 AGTTATTGGT GCCCTTAAAC GCCTGGTGCT ACGCCTGAAT AAGTGATAAT AAGCGGATGA

481 ATGGCAGAAA TTCGAAAGCA AATTCGACCC GGTCGTCGGT TCAGGGCAGG GTCGTTAAAT

541 AGCCGCTTAT GTCTATTGCT GGTTTACCGG TTTATTGACT ACCGGAAGCA GTGTGACCGT

601 GTGCTTCTCA AATGCCTGAG GCCAGTTTGC TCAGGCTCTC CCCGTGGAGG TAATAATTGA

661 CGATATGATC ATTTATTCTG CCTCCCAGAG CCTGATAAAA ACGGTGAATC CGTTAGCGAG

721 GTGCCGCCGG CTTCCATTCA GGTCGAGGTG GCCCGGCTCC ATGCACCGCG ACGCAACGCG

781 GGGAGGCAGA CAAGGTATAG GGCGGCGAGG CGGCTACAGC CGATAGTCTG GAACAGCGCA

841 CTTACGGGTT GCTGCGCAAC CCAAGTGCTA CCGGCGCGGC AGCGTGACCC GTGTCGGCGG

901 CTCCAACGGC TCGCCATCGT CCAGAAAACA CGGCTCATCG GGCATCGGCA GGCGCTGCTG

961 CCCGCGCCGT TCCCATTCCT CCGTTTCGGT CAAGGCTGGC AGGTCTGGTT CCATGCCCGG

1021 AATGCCGGGC TGGCTGGGCG GCTCCTCGCC GGGGCCGGTC GGTAGTTGCT GCTCGCCCGG

1081 ATACAGGGTC GGGATGCGGC GCAGGTCGCC ATGCCCCAAC AGCGATTCGT CCTGGTCGTC

1141 GTGATCAACC ACCACGGCGG CACTGAACAC CGACAGGCGC AACTGGTCGC GGGGCTGGCC

1201 CCACGCCACG CGGTCATTGA CCACGTAGGC CGACACGGTG CCGGGGCCGT TGAGCTTCAC

1261 GACGGAGATC CAGCGCTCGG CCACCAAGTC CTTGACTGCG TATTGGACCG TCCGCAAAGA

1321 ACGTCCGATG AGCTTGGAAA GTGTCTTCTG GCTGACCACC ACGGCGTTCT GGTGGCCCAT

1381 CTGCGCCACG AGGTGATGCA GCAGCATTGC CGCCGTGGGT TTCCTCGCAA TAAGCCCGGC

1441 CCACGCCTCA TGCGCTTTGC GTTCCGTTTG CACCCAGTGA CCGGGCTTGT TCTTGGCTTG

1501 AATGCCGATT TCTCTGGACT GCGTGGCCAT GCTTATCTCC ATGCGGTAGG GTGCCGCACG

1561 GTTGCGGCAC CATGCGCAAT CAGCTGCAAC TTTTCGGCAG CGCGACAACA ATTATGCGTT

1621 GCGTAAAAGT GGCAGTCAAT TACAGATTTT CTTTAACCTA CGCAATGAGC TATTGCGGGG

1681 GGTGCCGCAA TGAGCTGTTG CGTACCCCCC TTTTTTAAGT TGTTGATTTT TAAGTCTTTC

1741 GCATTTCGCC CTATATCTAG TTCTTTGGTG CCCAAAGAAG GGCACCCCTG CGGGGTTCCC

1801 CCACGCCTTC GGCGCGGCTC CCCCTCCGGC AAAAAGTGGC CCCTCCGGGG CTTGTTGATC

1861 GACTGCGCGG CCTTCGGCCT TGCCCAAGGT GGCGCTGCCC CCTTGGAACC CCCGCACTCG

1921 CCGCCGTGAG GCTCGGGGGG CAGGCGGGCG GGCTTCGCCT TCGACTGCCC CCACTCGCAT

1981 AGGCTTGGGT CGTTCCAGGC GCGTCAAGGC CAAGCCGCTG CGCGGTCGCT GCGCGAGCCT

2041 TGACCCGCCT TCCACTTGGT GTCCAACCGG CAAGCGAAGC GCGCAGGCCG CAGGCCGGAG

2101 GCTTTTCCCC AGAGAAAATT AAAAAAATTG ATGGGGCAAG GCCGCAGGCC GCGCAGTTGG

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2281 GCGAGCCAGC CGGTGGCCGC TCGCGGCCAT CGTCCACATA TCCACGGGCT GGCAAGGGAG

2341 CGCAGCGACC GCGCAGGGCG AAGCCCGGAG AGCAAGCCCG TAGGGCGCCG CAGCCGCCGT

2401 AGGCGGTCAC GACTTTGCGA AGCAAAGTCT AGTGAGTATA CTCAAGCATT GAGTGGCCCG

2461 CCGGAGGCAC CGCCTTGCGC TGCCCCCGTC GAGCCGGTTG GACACCAAAA GGGAGGGGCA

2521 GGCATGGCGG CATACGCGAT CATGCGATGC AAGAAGCTGG CGAAAATGGG CAACGTGGCG

2581 GCCAGTCTCA AGCACGCCTA CCGCGAGCGC GAGACGCCCA ACGCTGACGC CAGCAGGACG

2641 CCAGAGAACG AGCACTGGGC GGCCAGCAGC ACCGATGAAG CGATGGGCCG ACTGCGCGAG

2701 TTGCTGCCAG AGAAGCGGCG CAAGGACGCT GTGTTGGCGG TCGAGTACGT CATGACGGCC

2761 AGCCCGGAAT GGTGGAAGTC GGCCAGCCAA GAACAGCAGG CGGCGTTCTT CGAGAAGGCG

2821 CACAAGTGGC TGGCGGACAA GTACGGGGCG GATCGCATCG TGACGGCCAG CATCCACCGT

2881 GACGAAACCA GCCCGCACAT GACCGCGTTC GTGGTGCCGC TGACGCAGGA CGGCAGGCTG

2941 TCGGCCAAGG AGTTCATCGG CAACAAAGCG CAGATGACCC GCGACCAGAC CACGTTTGCG

3001 GCCGCTGTGG CCGATCTAGG GCTGCAACGG GGCATCGAGG GCAGCAAGGC ACGTCACACG

3061 CGCATTCAGG CGTTCTACGA GGCCCTGGAG CGGCCACCAG TGGGCCACGT CACCATCAGC

3121 CCGCAAGCGG TCGAGCCACG CGCCTATGCA CCGCAGGGAT TGGCCGAAAA GCTGGGAATC

3181 TCAAAGCGCG TTGAGACGCC GGAAGCCGTG GCCGACCGGC TGACAAAAGC GGTTCGGCAG

3241 GGGTATGAGC CTGCCCTACA GGCCGCCGCA GGAGCGCGTG AGATGCGCAA GAAGGCCGAT

3301 CAAGCCCAAG AGACGGCCCG AGACCTTCGG GAGCGCCTGA AGCCCGTTCT GGACGCCCTG

3361 GGGCCGTTGA ATCGGGATAT GCAGGCCAAG GCCGCCGCGA TCATCAAGGC CGTGGGCGAA

3421 AAGCTGCTGA CGGAACAGCG GGAAGTCCAG CGCCAGAAAC AGGCCCAGCG CCAGCAGGAA

3481 CGCGGGCGCG CACATTTCCC CGAAAAGTGC CACCTGACGT CTAAGAAACC ATTATTATCA

3541 TGACATTAAC CTATAAAAAT AGGCGTATCA CGAGGCCCTT TCGTCTTCAA GAATTCTCAT

3601 GTTTGACAGC TTATCATCGA TAAGCTTTAA TGCGGTAGTT TATCACAGTT AAATTGCTAA

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3721 ACCCTGGATG CTGTAGGCAT AGGCTTGGTT ATGCCGGTAC TGCCGGGCCT CTTGCGGGAT

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4321 CTTATGACTG TCTTCTTTAT CATGCAACTC GTAGGACAGG TGCCGGCAGC GCTCTGGGTC

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11341 CGTCAGGAGA TAAACAGAGT GTGGCTGGGA GAAATGCTTG ACAAGCTGAA TTCCATGTTA

11401 GAGGGATAA

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