academicJournals

Vol. 11(3), pp. 75-83, 21 January, 2017
DOI: 10.5897/AJMR2016.8339
Article Number: 7E24D0A62424
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Engineered microbial consortium for the efficient conversion of biomass to biofuels: A preliminary study

Ugochukwu Anieto

Department of Biological Sciences, University of North Texas, Denton 76203, TX, USA.

Received 15 October, 2016; Accepted 5 January, 2017

This work showed ethanol production by a microbial consortium of *Clostridium cellulolyticum* and a recombinant *Zymomonas mobilis* (ZM4 pAA1). The ZM4 pAA1 and wild type ZM4 (ZM4 WT) were first tested on RM medium (ATCC 1341) containing 2% cellobiose as the carbon source. Ethanol production from *Z*M4 pAA1 was three times higher than that observed from the *Z*M4 WT. Concomitant with ethanol production was the reduction in OD from 2.00 to 1.580. The ZM4 pAA1 was then co-cultured with *C. cellulolyticum* using cellobiose and microcrystalline cellulose, respectively, as carbon sources. Results indicate that the ZM4 pAA1 with *C. cellulolyticum* utilized 2.0 g/L cellobiose, producing as much as 0.40 mM of ethanol, whereas only 0.20 mM ethanol was detected for the ZM4 WT co-cultured with *C. cellulolyticum* under similar conditions. A consortium of the ZM4 pAA1 and *C. cellulolyticum* using 7.5 g/L microcrystalline cellulose gave a far lower ethanol yield than when using cellobiose. In the latter case, ethanol production was detected within 5 days, whereas it took about 10 days for ethanol to be detectable for the ZM4 WT and *C. cellulolyticum*. Future efforts will concentrate on identifying suitable partners for the ZM4 pAA1, the correct concentration of feedstocks at which synergy will be observed, as well as optimize medium formulations and inoculation techniques.

Key words: Biofuel, ethanol, cellulosome, consortium, *Zymomonas mobilis, Clostridium cellulolyticum.*

INTRODUCTION

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 × 4.0-5.0 µm, motile, does not sporulate, does not produce capsules, intracellular lipids or glycogen, optimal pH range for growth is 6 to 7.0, optimal temperature range is 25 to 31°C, the G + C content of the cellular DNA is about 47.5 to 49.5% with an average *T*m of 89.3 to 89.5°C (Gunasekeran et al., 1990). *Z. mobilis* uses the Entner-Doudoroff (ED) pathway which is found in microrganisms that are strictly aerobic, conducts fermentation with 50% less ATP produced relative to the Embden-Meyerhof-Parnas (EMP) pathway, which leads to improved ethanol yield (Yang et al., 2016).

E-mail: ugochukwuanieto@my.unt.edu.

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 rods that are 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 3 or more days old (Petitdemange et al., 1984). *C. cellulolyticum* produces several cellulases, which are re-grouped into an extracellular enzymatic complex called cellulosomes and cellulolytic activities allow the release of soluble cellodextrins from cellulose, which in return permits microbial growth (Desvaux, 2005).

The wild-type *Z. mobilis* was primarily isolated from alcoholic liquids in natural environments containing fermentable sugars such as plant saps, and can only utilize a limited carbon source, including glucose, fructose and sucrose (Weir, 2016). This drawback is a major reason why it has not been used extensively for bioethanol production on an industrial scale. *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 due to its inability to utilize higher concentrations of pure cellulosic substrates. 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) (Senthikumar and Gunasekaran, 2005) in *C. cellulolyticum*.

Engineered microbial consortia and co-culturing of wild type bacteria have become pivotal tools in biotechnology and have been utilized in the production of a wide range of products from biofuels to pharmaceuticals. For examples, Abate et al. (1996) 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. Shin et al. (2010) genetically engineered two *E. coli* strains for xylan utilization, with one strain expressing two hemicellulases to hydrolyze xylan into xylooligosaccharides and another one importing the xylooligosaccharides to produce ethanol, with a 55% theoretical yield. Similarly, Shou et al. (2007) demonstrated a slightly more ideal cooperation using two engineered *Saccharomyces cerevisiae* strains. One strain required adenine and overproduced lysine while the other strain required lysine and overproduced adenine. Singh et al. (2014) co-cultured *Pichia stipitis* and *Z. mobilis* for bioethanol production from kans grass biomass with significant yields and Zhang et al. (2016a) employed *C. cellulolyticum* and hydrogen fermentation bacteria for enhanced biohydrogen production from corn stover with significant differences seen in the metabolites of the co-culture system over the mono-cultures. Other reports of successful bio-catalysis based on microbial consortia have equally been reported (Fu et al., 2009; He et al., 2011; Ho et al., 2011; Li et al., 2011; Quinn et al., 2016; Reddy and Basappa, 1996; Yaun et al., 2016; Zhang et al., 2016b; Zhong et al., 2016).

In order to improve its industrial appeal in substrate utilization and sugar transport, *Z. mobilis* has been genetically modified extensively with significant improvements over the wild type, for example Luo and Bao (2015), expressed a heterologous β-D-glucosidase from *Bacillus polymyxa* in *Z. mobilis,* where the signal peptide ZMO 1086 facilitated its secretion. Other reported efforts include the works of Deanda et al. (1996), Dunn and Rao (2014), Yanase et al. (2005) and Yanase et al. (2012). To the best of the author's knowledge, there has not been any reported microbial consortium involving *Z. mobilis and C. cellulolyticum* whether as wild types or engineered clones for the production of bioethanol. Given the immense potentials as previously outlined of these two bacteria, it became necessary to establish a testbed from which further research would be conducted in order to improve the process of bioethanol production. The present study aimed to study the effects of using a consortium of a recombinant *Z. mobilis* and *C. cellulolyticum* on the conversion of biomass to bioethanol and using this study as a Launchpad for further experimental studies and process improvement.

MATERIALS AND METHODS

Construction of ZM4 pAA1

Amplification of pBBR1 MCS-3

The vector backbone, pBBR1 MCS-3 (Kovach et al., 1995) was linearized with the restriction enzyme *Kpn*I, the linearized vector verified for size correctness on electrophoresis gel to give a band size of 5.2 kb. The fragment was PCR amplified using Phusion DNA polymerase (NEB), with the PCR conditions set at 98°C for 1 min, 98°C for 30 seconds, 56°C for 30 s for annealing and 72°C for 90 s for extension. The cycle was repeated 35 times from the second to the fourth step, a final extension for 5 min at 72°C and a hold at 4°C.

Amplification of celZ and celY genes from Erwinia chrysenthemi

The *celY* endoglucanase gene and *celZ* endoglucanase gene 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 set at 98°C for 1 min, 98°C for 30 s, 72°C for 45 s (to include annealing and extension). The cycle was repeated 35 times from the second to the merged annealing and extension steps, a final extension for 5 min at 72°C and a hold at 4°C. Similar conditions were used for the *celZ* gene with the ZM4 promoter but with the annealing temperature set at 54°C for 30 s and extension time for 45 s. The amplicons were verified on the gel for size correctness and further sequenced for correctness.

Table 1. List of primers.

Primer name	Sequences
pBBR1mcs-3 (forward)	AGGGATAAGGTACCGGGCCCCCC
pBBR1mcs-3 (reverse)	GGTTGATCCAGCTTTTGTTCCCTTT
celZ with ZM4 promoter (forward)	AAAAGCTGGATCAACCGGCAATTT
celZ with ZM4 promoter (reverse)	CTCCTTCTTCAATTAGTTACAGCTACCAA
celY (forward)	CTAATTGAAGAAGGAGAATGAATGGGAAAGCC
celY (reverse)	CTCCTTCTTTATTTACCGCGCGCCAACATCAC
gfor-betaglc fusion (forward)	GTAAATAAAGAAGGAGTAAGAATGACGAACAA
gfor-betaglc fusion (reverse)	CCGGTACCTTATCCCTCTAACATGGAATTCAG

Amplification of glucose-fructose oxidoreductase (GFOR) leader sequence of Z. mobilis and B-glucosidase gene of Ruminococcus albus in Z. mobilis.

The *β-glucosidase* gene from *R. albus* was cloned and translationally fused to the glucose-fructose oxidoreductase (gfor) leader sequence of *Z. mobilis* for export; resulting in 61% secretion and 0.49 g ethanol yield per g cellobiose (Yanase et al., 2005). To amplify the 159 bp leader sequence of the glucose-fructose oxidoreductase gene, the reverse primer was designed to include 10 bp forward primer of the *β-glucosidase* gene. Similarly, the forward primer of the *β-glucosidase* gene was designed to include 10 bp of the complimentary sequence of the gfor leader reverse primer sequence. The *β-glucosidase* gene from the genomic DNA of *R. albus* (kindly provided by Professor P. Weimer, University of Wisconsin) and the leader sequence of the gfor gene of *Z. mobilis*, which was fused to the *β-glucosidase* gene, were amplified using synthetic oligonucleotide primers (Invitrogen). The PCR condition used was the same as previously described; however, the annealing temperature was 65°C. The amplicons were verified by DNA gel electrophoresis and sequenced for correctness (Table 1).

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. These three fragments, ZM4 promoter with *celZ*, *celY* and *gfor-betaglucosidase* fusion were cloned into the *Kpn*I site of the broad host range vector pBBR1MCS-3 (tc^r). The cloning was performed using the Life Technologies Gene Art Seamless Cloning and Assembly kit (Life technologies). This kit was optimized to clone up to 4 DNA fragments with a total vector and insert size of 13 kb.

Escherichia coli NEB-10 Beta competent cells were transformed with the cloned vector pAA1 as described in New England Biolabs (NEB) manual. 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 of tetracycline. To select for the right clone, the plasmid pAA1 and the vector backbone pBBR1MCS-3 were extracted from their respective host cells and subsequently digested using *Kpn*I and *Not*I HF restriction enzymes (NEB) which cut at different positions within the vector backbone. Furthermore, the vectors pAA1 and pBBR1MCS-3 were digested with the restriction enzyme *Nde*I (NEB). *Nde*I cut site CA^TATG exists within the insert that produced pAA1 but not on the vector backbone pBBRIMCS-3.

The cloned pAA1 was completely sequenced by Eurofins MWG operon using the Sanger sequencing method and verified for correctness and thereafter used for the transformation of ZM4 WT using the Gene Pulser (Bio-Rad) as described by Liang and Lee (1998) to create ZM4 pAA1. The *Z. mobilis* cultures were 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 minutes 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 re-suspended in 2 to 3 ml of 10% glycerol. The plasmid pAA1 was extracted from *E. coli* NEB-10 Beta, suspended in water and concentrated to 3000 ng/µl DNA before electroporation.

The Gene Pulser (Bio-Rad) for generating exponential decay pulses were set at a peak voltage of 1.5 kV and 25 µF capacitance. A 200 µL aliquot of the *Z. mobilis* cultures were 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 min. 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 h. At the end of this outgrowth period, the cells were diluted with RM medium and plated on RM agar containing 15 µg/ml of tetracycline. The *Z. mobilis* cultures were also transformed with the original vector backbone pBBR1MCS-3 to create ZM4 pBBR1MCS-3 and plated on RM agar (15 µg/ml of tetracycline) to verify the expression of the plasmid with the antibiotic marker in a new host but without the inserts as seen in pAA1. ZM4 pAA1 and ZM4 pBBR1MCS-3 were inoculated into RM broth containing 15 µg/ml of tetracycline and 30 µg/ml of gentamicin and incubated at 30°C for 48 h. Gentamicin was added because *Z. mobilis* is naturally resistant to it up to 50 µg/ml, therefore most potential contaminants were not expected to survive under that condition. Thereafter, OD was determined at 600 nm using the spectrophotometer (Spectronic 20D+).

To further verify that the recombinants ZM4 pAA1 and ZM4 pBBR1MCS-3 were indeed *Z. mobilis*, the cells were once again grown in RM medium with 2% glucose as the carbon source and supplemented with 15 µg/ml of tetracycline. The ZM4 WT was also grown in RM medium with 2 percent glucose as the carbon source and supplemented with 30 µg/ml of gentamicin. Cells were harvested after 48 h and genomic DNA extraction was performed using the FastDNA spin kit (MP Biomedicals). The leader sequence of the gfor is unique to *Z. mobilis* and so this gene fragment was individually amplified from the genomic DNA extracted from ZM4 pAA1, ZM4 pBBR1MCS-3 and ZM4 WT. Band sizes of approximately 200 bp was observed for the 3 amplicons on the electrophoresis gel.

The recombinant ZM4 pAA1 (tetracycline resistant) was grown in RM medium containing 2% glucose supplemented with 15 µg/ml of tetracycline. The pAA1 was then extracted from ZM4 pAA1 using the 5 Prime fast plasmid extraction kit (5 Prime) and the inserts of

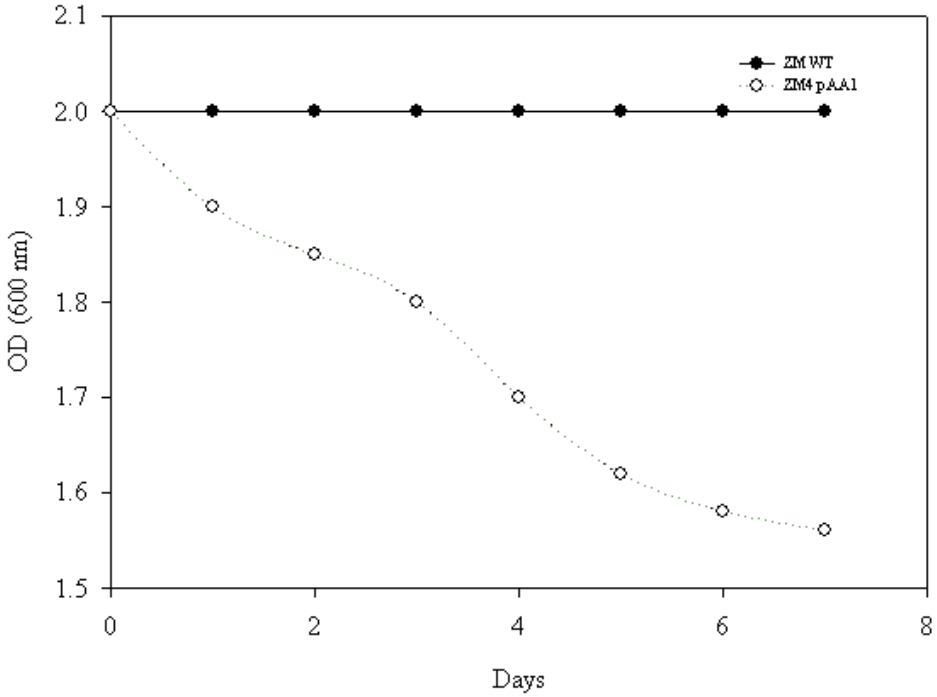

Figure 1. Optical density of the ZM4 pAA1 and ZM WT. Starting OD was 2.0 and decreased as insoluble cellobiose was consumed and ethanol production increased for the ZM4 pAA1. OD for the ZM4 WT remained the same, indicating it failed to utilize the substrate.

ZM4*celZ*, *celY* and *gfor-beta-glucosidase* individually amplified from the plasmid pAA1 using the Q5 high fidelity DNA polymerase (New England Biolabs), verified for size correctness on gel electrophoresis and subsequently sequenced for correctness.

Ethanol production test

Ethanol production from cellobiose using recombinant ZM4 pAA1 and ZM4 WT

Cultures of ZM WT were grown in RM medium containing 2% glucose and supplemented with gentamicin (30 µg/ml). The recombinant ZM4 pAA1 were similarly grown in RM medium but were supplemented with 15 µg/ml of tetracycline in order to maintain the vector (pAA1) in addition to 30 µg/ml of gentamicin. Cells were harvested after 24 h, washed thoroughly in phosphate buffered saline (PBS, pH 7.2), re-suspended in RM medium containing 2% cellobiose to give an optical density (OD) of 2.0 and the appropriate antibiotics added in each medium as previously described. The cultures were incubated at 30°C in a shaking incubator (New Brunswick) at 150 rpm. The ODs were determined every twenty-four hours for 7 days and samples taken from the cultures for ethanol quantification starting from 48 h after original inoculation and every 24 h thereafter for three days.

Ethanol production from cellobiose and microcrystalline cellulose using ZM4 pAA1, ZM4 WT and Clostridium cellulolyticum

ZM4 pAA1 and ZM4 WT cultures were grown to OD of 0.4 to 0.6 in RM medium containing glucose as carbon source. The cultures were washed in PBS (pH 7.2), re-suspended, and then kept on ice until further use (maximum time <20 min). *C. cellulolyticum* was grown in Clostridium medium (ATCC 1368) containing 7.5 g/L of microcrystalline cellulose as the carbon source to an OD of 0.4 to 0.6. The cultures were subsequently used to inoculate Clostridium medium containing cellobiose and microcrystalline cellulose as carbon sources, respectively. For the mono cultures of ZM4 pAA1, ZM4 WT and *C. cellulolyticum,* the medium was inoculated with 5% inoculum size (v/v) and for the consortium, the medium was inoculated with 2.5% each of the inoculum size. For the Clostridium medium containing cellobiose, 2 g/L of the oligosaccharide cellobiose was used, whereas for the regular Clostridium medium, 7.5 g/L of cellulose was used.

RESULTS AND DISCUSSION

From Figures 1 and 2, it can be seen that the ZM4 pAA1 utilized the cellobiose and produced three times as much ethanol after 5 days, respectively, than did the ZM4 WT. The ethanol produced and analyzed using the gas chromatography technique (Shimadzu) by the ZM4 pAA1 was significantly different from that produced by the ZM4 WT ($p = 0.03761$, SigmaPlot). The ethanol detected in the wild type could have come from the residual glucose from after wash transferred into the medium at inoculation. As ethanol was being produced, the optical density (OD) of the recombinant culture declined gradually

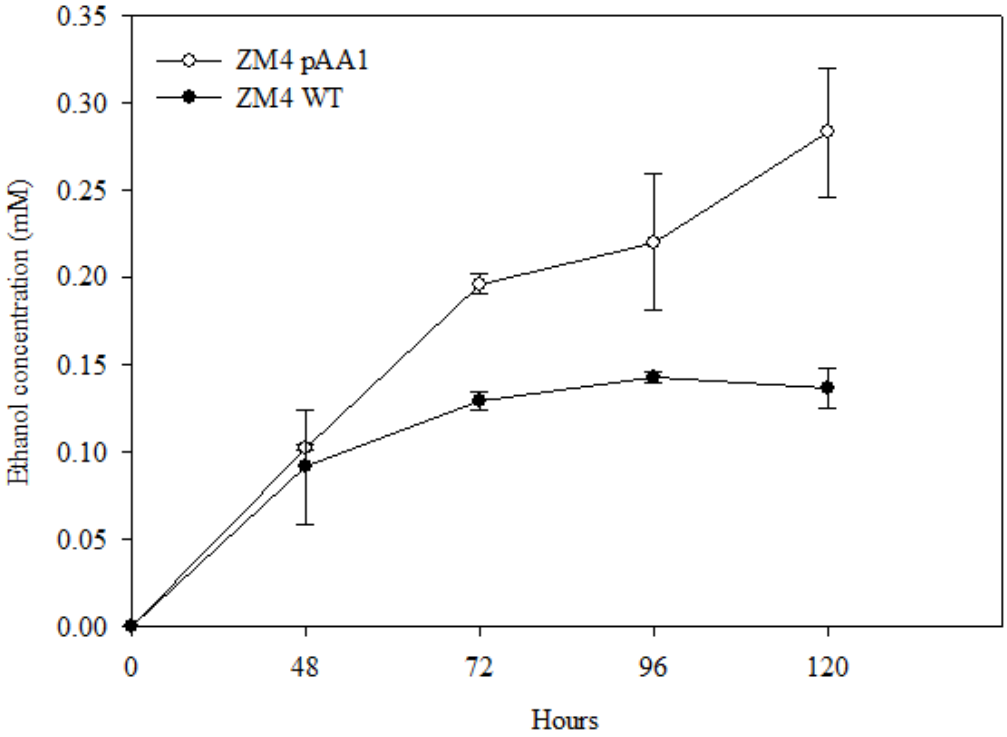

Figure 2. Ethanol production from RM medium containing 2% cellobiose as the carbon source. Error bars represent standard deviations among three replicates.

to 1.58 after 7 days. Further decline in OD was observed but with no further change after 1.44. No change was detected for the ZM4 WT for the entire duration.

As seen in Figure 3, 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. *C. cellulolyticum* produced ethanol once as can be seen from batch 1 (Figure 3) but none in subsequent batches.

In Figure 4, ethanol production can be observed from the two conditions, with ZM4 pAA1 and *C. cellulolyticum* producing more ethanol than ZM4 WT and *C. cellulolyticum*. In a study by Payot et al. (1998) detailing the metabolism of cellobiose by *C. cellulolyticum* growing in continuous culture, it was reported that *C. cellulolyticum* was able to metabolize only a small quantity of soluble carbohydrates (3 g/L), with the molar growth yield reduced when the initial cellobiose concentration exceeded (2 g/L). In this work, the concentration of cellobiose utilized was originally set at 5 g/L and the results obtained (not shown) clearly indicated that such concentration negatively impacted growth of the *C. cellulolyticum* but the recombinant ZM4 pAA1 could have aided in rescuing the situation, with subsequent tests using 2 g/L cellobiose showing an improvement. The ZM4 WT however, was unable to do same, possibly due to the lack of the *β-glucosidase* gene and also by the use of a sugar source that could not adequately support its growth. Furthermore, as described by Payot et al. (1998), *C. cellulolyticum* growth was limited due to low rate of NADH re-oxidation leading to an intracellular accumulation of the reduced nucleotide and as described by Giallo et al. (1983), acetate was the main product for the continuous cultures of *Clostridium*. 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 (Giallo et al., 1983). They further described the ability of *C. acetobutylicum* to induce metabolic shifts to produce solvents such as ethanol, butanol and acetone and this shift was associated with high intracellular ATP and NAD(P)H. It does not appear that *C. cellulolyticum* is able to induce such metabolic shifts to produce reduced compounds such as ethanol. The result obtained from the consortium of recombinant ZM4 with *C. cellulolyticum* and ZM4 WT with *C. cellulolyticum* 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 WT to convert glucose released from cellobiose to ethanol, respectively. Figure 5 shows ethanol production from microcrystalline cellulose by *C. cellulolyticum,* ZM4 pAA1 and ZM4 WT in five days.

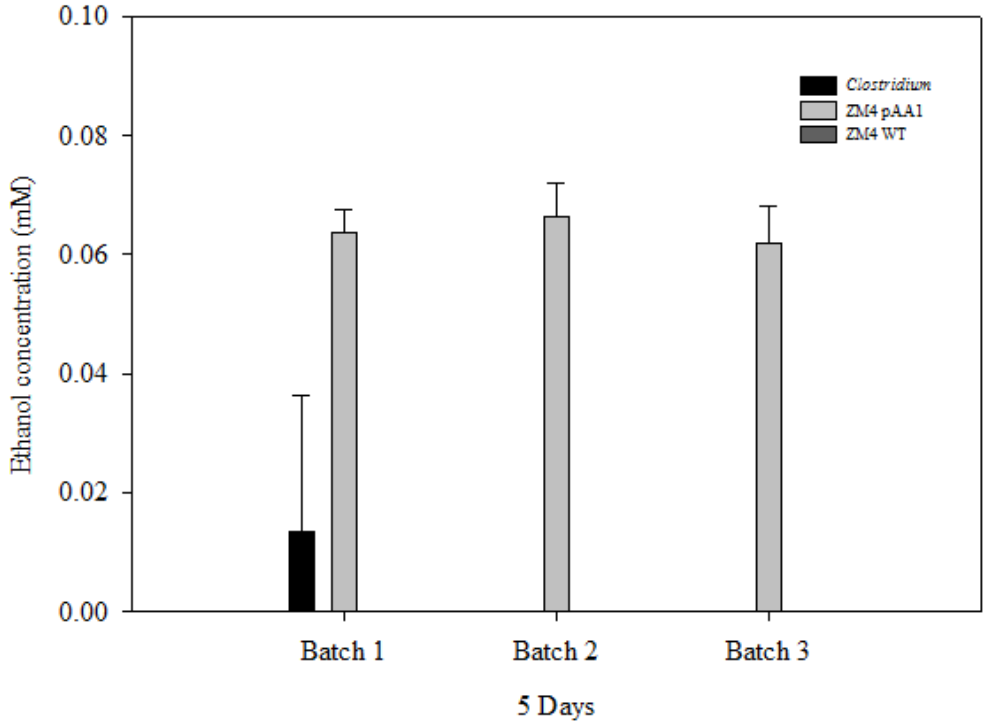

Figure 3. Ethanol production from cellobiose. Error bars represent standard deviations among three replicates.

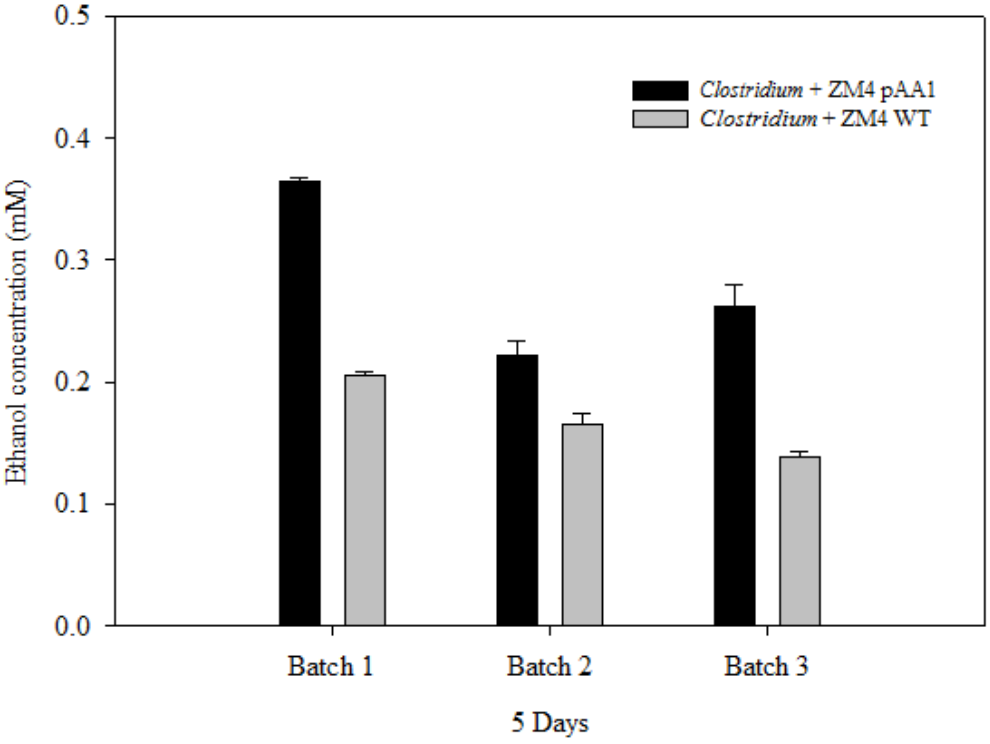

Figure 4. Ethanol production from cellobioise. Error bars represent standard deviations among triplicate samples.

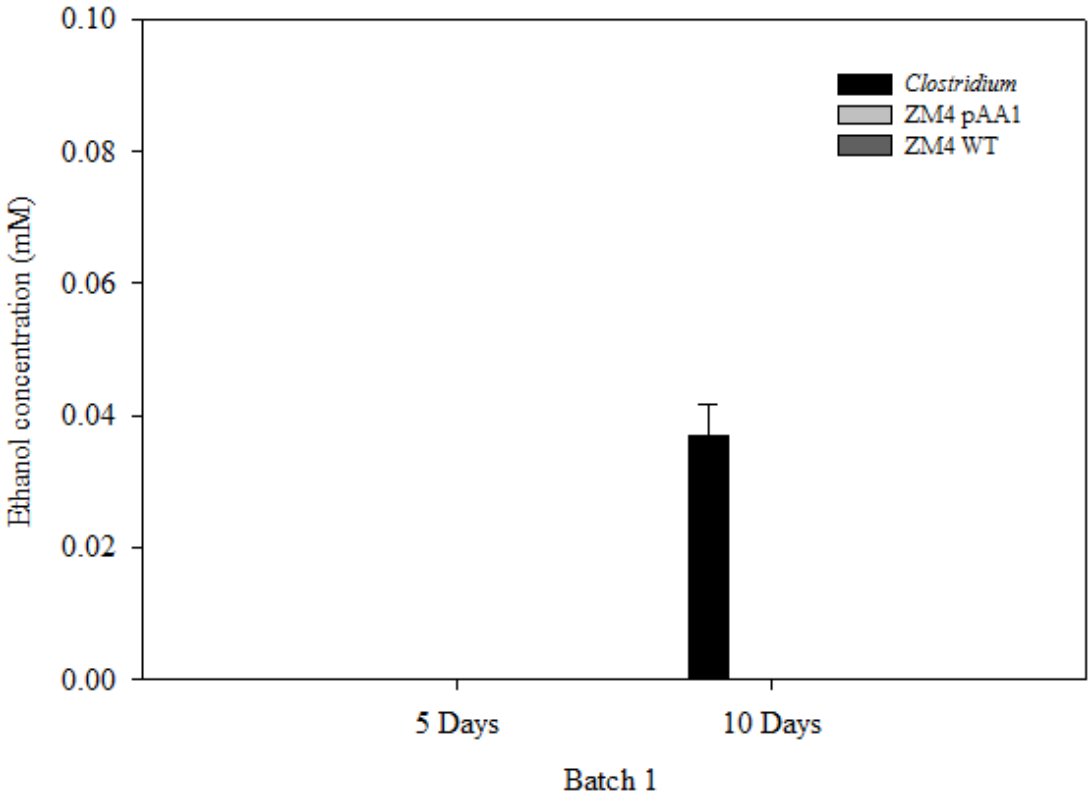

Figure 5. Ethanol production from microcrystalline cellulose. Error bars represent standard deviations among three replicates.

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

In the consortium, Figures 6a and b, ethanol production was detected for *C. cellulolyticum* with ZM4 pAA1 for the three batches after 5 days and increased after 10 days while *C. cellulolyticum* with ZM4 WT produced ethanol in batches 1 and 2 but not batch 3 and only detected after 10 days. This observation suggested that the consortium involving recombinant ZM4 was more efficient than that involving the wild type. This pattern could 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 consortium involving the recombinant ZM4 pAA1 from the 10-day culture as compared to that produced after 5 days, suggesting that maximum ethanol yield could be possible in slightly over 5 days. The consortium involving the ZM4 WT produced less ethanol and that was detected only after 10 days, suggesting less efficiency in synergy.

Conclusion

The recombinant *Z. mobilis* bearing the plasmid vector pAA1 supported the findings of Yanase et al. (2005) 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 per gram of cellobiose. From this work, 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. Despite the tagging of 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 (Yanase et al., 2005). With the introduction of *celZ* and *celY* genes, the recombinant ZM4 with *C. cellulolyticum* was able to indicate ethanol production from microcrystalline cellulose within 5 days whereas the consortium of the ZM4 WT took twice that amount of time and produced significantly less ethanol. The recombinant ZM4 and ZM4 WT did not produce ethanol using cellulose as the carbon source as expected but *C. cellulolyticum* alone did after 10 days, twice the time it

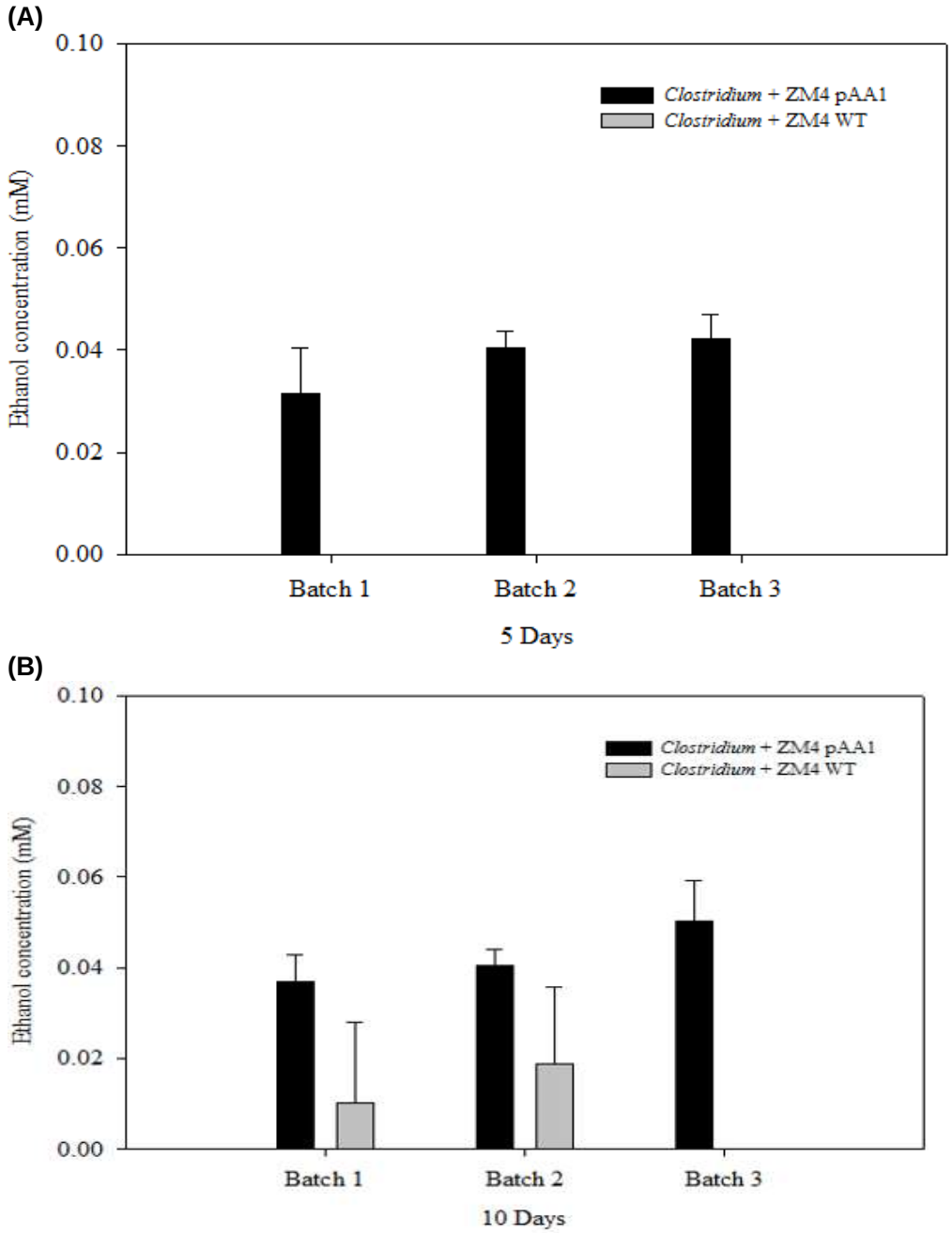

Figure 6. Ethanol production after 5 (A) and 10 (B) days using microcrystalline cellulose as a carbon source. Error bars represent standard deviations among three replicates.

took to make ethanol for the consortium.

This preliminary study was initiated to study the effect of the microbial consortium on ethanol production. It is far from exhaustive, did not establish a convincing case for the consortium using the two microorganisms and more investigation is required in order to optimize the desired synergy between the ZM4 pAA1 and *C. cellulolyticum.* The concentration of microcrystalline cellulose used was 7.5 g/L, but further investigation is needed to determine the best concentration at which a synergy can be clearly observed. Furthermore, the culture growth conditions and incubation times chosen could also be a major factor in contributing to the lack of a clear evidence of synergy; therefore, different culture conditions and time points

would be tested to better investigate the consortium.

Based on the wide differences in the genetics and physiology of the two bacteria used, the low yields could simply have been as a result of metabolic bottlenecks, which were not investigated in this preliminary study. Batch culture conditions were investigated and the need to investigate same conditions using the continuous culture conditions cannot be overemphasized. Using pure cellobiose and pure microcrystalline cellulose only, were insufficient, other carbon sources to reflect natural conditions would be investigated

Finally, the recombinant ZM4 pAA1 would be partnered with other known cellulose degraders other than the one used here to further understand its efficiency in a consortium.

Conflict of interest

There is no conflict of interest in preparation of the manuscript.

REFERENCES

Abate C, Callieri D, Rodriguez E, Garro O (1996). Ethanol production by a mixed culture of flocculent strains of *Zymomonas mobilis* and *Saccharomyces* sp. Appl. Microbiol. Biol. 45(5):580-583.

Bayer TS, Widmaier DM, Temme K, Mirsky EA, Santi DV, Voigt CA (2009). Synthesis of Methyl Halides from Biomass Using Engineered Microbes. J. Am. Chem. Soc. 131(18):6508-6515.

Deanda K, Zhang M, Eddy C, Picataggio S (1996). Development of an arabinose-fermenting *Zymomonas mobilis* strain by metabolic pathway engineering. Appl. Environ. Microbiol. 62(12):4465-4470.

Desvaux M (2005). The cellulosome of *Clostridium cellulolyticum.* Enzym. Microb. Technol. 37(4):373-385.

Dunn KL, Rao CV (2014). Expression of a xylose-specific transporter improves ethanol production by metabolically engineered *Zymomonas mobilis.* Appl. Microbiol. Biotechnol. 98(15):6897-6905.

Fu N, Peiris P, Markham J, Bavor J (2009). A novel co-culture process with *Zymomonas mobilis* and *Pichia stipitis* for efficient ethanol production on glucose/xylose mixtures. Enzym. Microbiol. Technol. 45(3):210-217.

Giallo J, Gaudin C, Belaich JP, Petitdemange H, Caillet-Mangin F (1983). Metabolism of glucose and cellobiose by cellulolytic mesophilic *Clostridium* sp. strain H10. Appl. Environ. Microbiol. 45(3):843-849.

Guedon E, Dexvaux M, Payot S, Petitdemange H (1999). Growth inhibition of *Clostridium cellulolyticum* by an inefficiently regulated carbon flow. Microbiology 145(8):1831-1838.

Gunasekeran P, Karunakaran T, Nellaiah H, Kamini N.R, Mukundan A.G (1990). Current status and prospective of an ethanol producer, *Zymomonas mobilis.* Indian J. Microbiol 30: 107-133.

He Q, Hemme C.L, Jiang H, He Z, Zhou J (2011). Mechanisms of enhanced cellulosic bioethanol fermentation by co-cultivation of *Clostridium* and *Thermoanaerobacter* spp. Bioresour. Technol. 102(20):9586-9592.

Ho Q, Chang JJ, Lin JJ, Chin TY, Matthew GM, Huang CC (2011). Establishment of functional rumen bacterial consortia (FRBC) for simultaneous biohydrogen and bioethanol production from lignocellulose. Int. J. Hydrogen Energ. 36(19):12168-12176.

Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Loop RM, Peterson KM (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166(1):175-176.

Li Y, Park J-Y, Shiroma R, Tokuyasu K (2011). Bioethanol production from rice straw by a sequential use of *Saccharomyces cerevisiae* and *Pichia stipitis* with heat inactivation of *Saccharomyces cerevisiae* cells prior to xylose fermentation. J. Biosci. Bioeng. 111(6): 682-686.

Liang CC, Lee WC (1998). Characteristics and transformation of *Zymomonas mobilis* with plasmid pKT230 by electroporation. Bioprocess Eng. 19(2):81-85.

Luo Z, Bao J (2015). Secretive expression of heterologous β-glucosidase in *Zymomonas mobilis.* Bioresour. Bioprocess. 2:1-6.

Payot S, Guedon E, Cailliez C, Gelhaye E, Petitdemange H (1998). Metabolism of cellobiose by cellulolytic mesophilic *Clostridium* sp. strain H10. Appl. Environ. Microbiol. 45(3):843-849.

Petitdemange E, Caillet F, Giallo J, Gaudin C (1984). *Clostridium cellulolyticum* sp. nov, a Cellulolytic, Mesophilic Species from Decayed Grass. Int. J. Syst. Bacteriol. 34:155-159.

Quinn BM, Apolinario EA, Gross A, Sowers KR (2016). Characterization of a microbial consortium that converts mariculture fish waste to biomethane. Aquaculture 453:154-162.

Reddy OV, Basappa SC (1996). Direct fermentation of cassava starch to ethanol by mixed cultures of *Endomycopsis fibuligera* and *Zymomonas mobilis;* Synergism and limitation. Biotechnol. Lett. 18(11):1315-1318.

Senthikumar V, Gunasekaran P (2005). Bioethanol production from cellulosic substrates: Engineered bacteria and process integration challenges. J. Sci. Ind. Res. India 64(11):845-853.

Shin HD, McClendon S, Vo T, Chen R.R (2010). *Escherichia coli* Binary Cultured Engineered for Direct Fermentation of Hemicellulose to a Biofuel. Appl. Environ. Microbiol. 76(24):8150-8159.

Shou W, Ram S, Vilar J.M.G (2007). Synthetic cooperation in engineered yeast populations. Proc. Natl. Acad. Sci. U.S.A 104(6): 1877-1882.

Singh LK, Majumder CB, Ghosh S (2014). Development of sequential co-culture system (*Pichia stipitis* and *Zymomonas mobilis*) for ethanol production from Kans grass biomass. Biochem. Eng. J. 82: 150-157.

Weir PM (2016). The ecology of *Zymomonas:* a review. Folia Microbiol (Praha) 61:385-392.

Yanase H, Nozaki K, Okamoto K (2005). Ethanol production from cellulosic materials by genetically engineered *Zymomonas mobilis.* Biotechnol. Lett. 27(4):259-263.

Yanase H, Miyawaki H, Sakurai M, Kawakami A, Matsumoto M, Haga K, Kojima M, Okamoto K (2012). Ethanol production from wood hydrolysate using genetically engineered *Zymomonas mobilis.* Appl. Microbiol. Biotechnol. 94(6):1667-1678.

Yang S, Fei Q, Zhang Y, Contreras L.M, Utturkar S.D, Himmel M.E, Zhang M (2016). *Zymomonas mobilis* as a model system for production of biofuels and biochemical. Microbiol. Biotechnol. 9(6):699-717.

Yaun X, Ma L, Wen B, Zhou D, Kuang M, Yang W, Cui Z (2016). Enhancing anaerobic digestion of cotton stalk by pretreatment with a microbial consortium (MC1). Bioresour. Technol. 207:293-301.

Zhang SC, Lai QH, Lu Y, Liu ZD, Wang TM, Zhang C, Xing XH (2016a). Enhanced biohydrogen production from corn stover by the combination of *Clostridium cellulolyticum* and hydrogen fermentation bacteria. J. Biosci. Bioeng. 122:482-487.

Zhang H, Zhang P, Ye J, Wu Y, Fang W, Gou X, Zeng G (2016b). Improvement of methane production from rice straw with rumen fluid pretreatment. A feasibility study. Int. J. Biodeterior. Biodegr. 113:9-16.

Zhong C, Wang C, Wang F, Jia H, Wei P, Zhao W (2016). Enhanced biogas production from wheat straw with the application of synergistic microbial consortium pretreatment. RSC Adv. 6:60187-60195.