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GENETICALLY ENGINEERED *THERMOSYNECHOCOCCUS ELONGATUS* BP1:

ASSESSMENT OF POTENTIAL BIORISKS AND BIOFUEL PRODUCTION

by

Thu Ho Anh Nguyen-Jones
B.S. December 2013, Old Dominion University

A Dissertation Submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
Requirements for the Degree of

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OLD DOMINION UNIVERSITY
December 2021

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ABSTRACT

GENETICALLY ENGINEERED *THERMOSYNECHOCOCCUS ELONGATUS* BP1: ASSESSMENT OF POTENTIAL BIORISKS AND BIOFUEL PRODUCTION

Thu Ho Anh Nguyen-Jones
Old Dominion University, 2021
Director: Dr. James W. Lee

According to the International Energy Outlook 2019, released by the U.S. Energy Information Administration, it is projected that the energy consumption will increase up to 50% between 2018 and 2050 worldwide. As fossil fuel being a finite source of energy with the risk of depletion, many countries are now facing an energy security crisis. Therefore, it is important to develop other renewable and sustainable energy sources that will allow countries to shift away from depending on fossil fuels. Among several types of renewable energy, biofuel production using genetically engineered cyanobacteria is capturing much interest due to its many advantages. Different forms of biofuels such as ethanol, butanol, isobutanol, biodiesel have been successfully produced using cyanobacteria. Though this is a promising approach in achieving a renewable and sustainable energy source, research is still in its early stage and there are multiple aspects yet to be done.

Here, the first aim of this work seeks to answer the question of whether genetically engineered cyanobacteria could pose a biosafety risk by transferring their gene(s) into other bacteria upon contact. This was done via a horizontal gene transfer study which included co-culturing of genetically engineered cyanobacteria carrying kanamycin resistant gene and wild-type *E. coli* DH5α. *E. coli* cells were then screened for kanamycin resistant gene after being in

the same environment with the genetically engineered cyanobacteria. By doing so, it would provide a better understanding of the risk this approach might have.

In addition, this work discussed the use of a thermophilic strain of cyanobacteria, *Thermosynechococcus elongatus* BP1, for isobutanol production. It is expected that the thermophilic property of this strain would provide some biosafety features which could eliminate some environmental as well as ecological risks. A cassette carrying a set of genes is inserted into *Thermosynechococcus elongatus* BP1 cells and isobutanol production was detected and quantified by GC-MS. Lastly, this dissertation also investigated the tolerance of *Thermosynechococcus elongatus* BP1 toward different alcohols by supplementing cell cultures with different concentrations of ethanol, isobutanol, and 1-butanol to help understand the effect of these alcohols have on cell growth for industrial scaled up.

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This dissertation is dedicated to my Lord, Jesus Christ, for His never-ending blessings.

To my late grandfather and father, Giang Bui and Duc Nguyen, who encouraged me to pursue my education when it was not the norm for girls where I grew up.

To my mother, Huong Ho, who sacrificed everything she had to give me the emotional and financial support for my study and to take the role of my father since he passed away.

To my beloved husband, Justin Paul Jones, for his unconditional love and support as I completed this dissertation.

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NOMENCLATURE

T. elongatus BP1 *Thermosynechococcus elongatus* BP1

E. coli *Escherichia coli*

WT Wild-type

HGT Horizontal gene transfer

DNA Deoxyribonucleic acid

RNA Ribonucleic acid

RS Recombination site

PnirA Promoter nirA

Pcpc Promoter cpc

PslpA Promoter slpA

Ptrc Promoter trc

Kan Kanamycin

Term Terminator

KDC Ketoacid decarboxylase

BDH Butanol dehydrogenase

KIVD Ketoisovalerate decarboxylase

yqhD Alcohol dehydrogenase

2-KIV 2-ketoisovalerate

PCR Polymerase chain reaction

RT-PCR Reverse transcription polymerase chain reaction

cDNA Complimentary deoxyribonucleic acid

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CHAPTER I

INTRODUCTION

CURRENT STATE OF GLOBAL ENERGY

Energy security, generally defined as the sufficient energy available to support economic activity and social welfare (Matsumoto and Andriosopoulos, 2016), is an urgent topic for many countries due to the current state of energy supply and demand. According to the Southeast Asia Energy Outlook 2019 Report published by the International Energy Agency (IEA), energy demand in Southeast Asia countries has increased by 80% since 2000. A substantial portion of this demand is met by the doubling of fossil fuel usage ((IEA)). This report also mentioned that only 15% of the current energy demand is met by using renewable energy in the region. Oil demand in the region is projected to go up to 9 million barrels per day instead of the current use of 6.5 million barrels per day ((IEA)). With the rapid increase in energy demand, countries in Southeast Asia have become dependent on energy imports. India, China, Thailand, Singapore, and Pakistan started to import liquefied natural gas over the years of 2004 to 2015 (Vivoda, 2019). IEA projected that in 2040, the cost for energy imports in Southeast Asia could be as high as $300 billion. Worldwide, oil imports have increased by 80% between 1990 and 2015 (Mohsin et al., 2018).

Figure 1 reflects the fluctuation of oil prices between 1990 and 2015 as well as oil production in the same period. It is important to point out that during this period, while oil prices fluctuate, they are trending upward along with oil production. The OPEC spare capacity, however, shows a descending trend. OPEC spare capacity serves as an “indicator of the world’s oil market to response to potential crises that reduce oil supplies” (U.S Energy Information

Administration, EIA). It is the additional volume of oil that can be produced by OPEC members within 30 days (Azadi et al., 2017). As fossil fuels being non-renewable, there is a risk of depletion in the future. This leads to worldwide energy crisis as well as political conflicts between regions for the ownership of this finite energy source.

To address this issue of energy security not only in Asian countries but also worldwide, there is a need to generate more renewable and sustainable energy source that can compete with the convenient and prevalence of fossil fuels while also meeting the increasing demand of energy globally. Different approaches for energy generation have been assessed such as solar, wind, geothermal, or tidal energy. While each of these approaches has its own merit, they still have their shortcomings. For example, while solar energy is considered to have a high potential to become the leading renewable energy due to abundancy of sunlight, it still poses an environmental risk of pollution during photovoltaic cell production and the competition of cultivable land. In addition, production of these cells is dependent on the availability of certain rare metals (Grandell and Höök, 2015; Hayat et al., 2019). Most of these types of renewable energy while beneficial in certain areas, still have the limitation of location dependence.

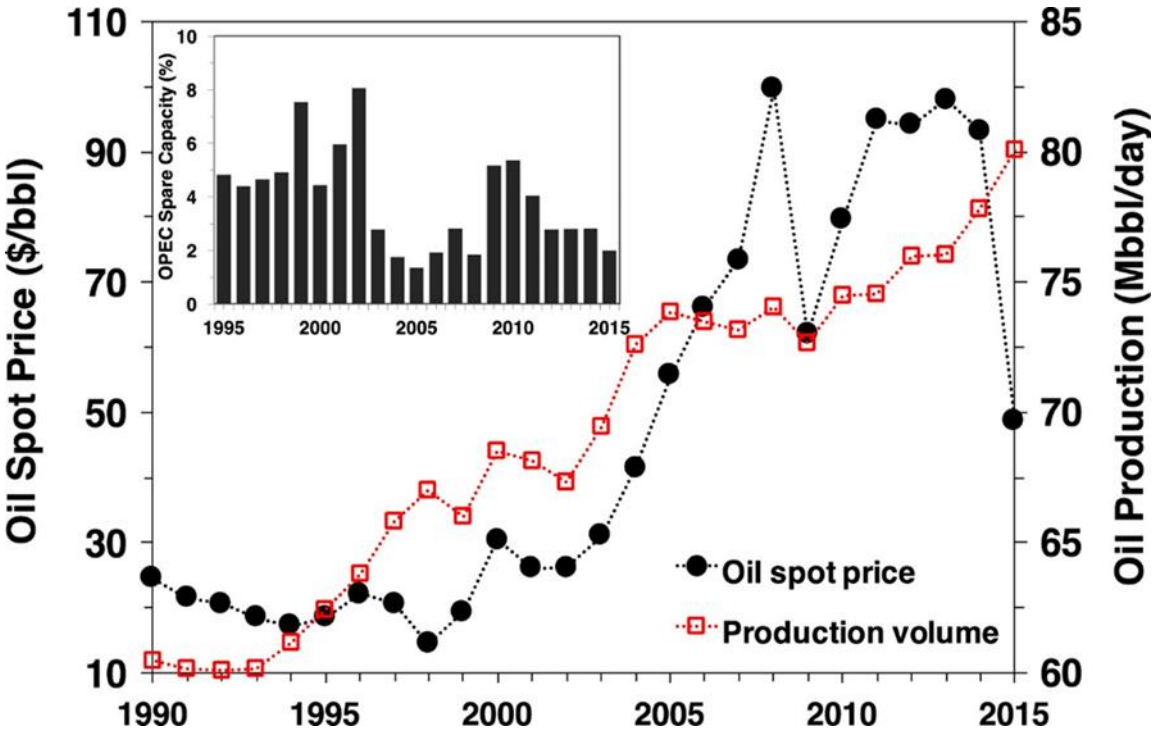

Fig. 1. Trend in oil production and price over the years. Reproduced from (Azadi et al., 2017).

DEVELOPMENT OF BIOFUELS

Because of the risk of fossil fuel depletion along with the remaining disadvantages of solar, wind, geothermal, or tidal energy, another approach in generating energy needs should be investigated. Over the years, biofuel has emerged as a potential renewable energy source. Biofuel is a form of renewable fuel that is produced using different biology sources (Mathimani and Pugazhendhi, 2019). Over the years, four different generations of biofuel have been developed. The first generation of biofuel, which is also called conventional biofuel, depends on the use of edible feedstock such as corn and sugarcane (Vassilev and Vassileva, 2016; Araújo et al., 2017; Alalwan et al., 2019; Mathimani and Pugazhendhi, 2019) to produce ethanol and biodiesel via fermentation and esterification/trans-esterification respectively (Araújo et al., 2017). Between

2000 and 2008, ethanol was produced using corn in the U.S and sugarcane in Brazil to meet the quadruple energy demand globally in this period (Sims et al., 2010) . Though this has opened a new door to meet the energy demand, this first generation of biofuel is unfavorable as it comes with a competition of land uses between agriculture and fuel production. In addition, since edible crops are used for ethanol and biodiesel production rather than food, there are also a conflict of crop use for food versus fuel production (Sims et al., 2010). Because of these disadvantages, The second generation of biofuel research aims to utilize the use of non-edible crops and lignocellulosic biomass material such as sugarcane bagasse, cereal straw, agricultural residues (Sims et al., 2010). This generation of biofuel tackles the issue of edible crop use competition while reducing the use of agriculture land. However, due to the nature of the lignocellulosic biomass being resistant to degradation, pretreatment step is often needed (Bhatia et al., 2017; Binod et al., 2019). Pretreatment of these materials lead to the production of toxic by-products. To remove these toxic by-products, another detoxification step is also needed which is not cost effective for large scale production (Bhatia et al., 2017; Binod et al., 2019). In recent years, research has focused on developing the third generation of biofuel by using microalgae biomass as the feedstock for biofuel production (Leong et al., 2018). Microalgae are unicellular organisms that can be found in a wide range of environments such as freshwater, saline lake, salt water, and can tolerate different pH, temperatures, light conditions, etc. (Khan et al., 2018) Being photosynthetic organisms, microalgae can capture CO_2 and convert it into desired biofuel products and as a result, carbon net neutral can be achieved (Zeng et al., 2011). In addition, the fast growth rate of microalgae allows short harvesting cycle and production of biooil from microalgae is significantly higher than other feedstock and requires less cultivating area (Leong et al., 2018; Chowdhury and Loganathan, 2019). Currently, oil production from microalgae is

10,000 L/hectare/year which significantly exceeds oil production rate from other feedstock such as canola, soybeans, sunflower, jatropha, and palm (Chowdhury and Loganathan, 2019). By using microalgae, the third generation of biofuel has provided answers for some of the questions facing the first and second generations of biofuel. However, there is still room to enhance the production rate of biofuel from microalgae (Figure 2). Therefore, another generation of biofuel research has arrived. The latest generation of biofuel seeks to genetically engineer the genome of certain microbes to increase productivity to achieve better product yield as well as enhance CO_2 capture (Dutta et al., 2014; Liew et al., 2014).

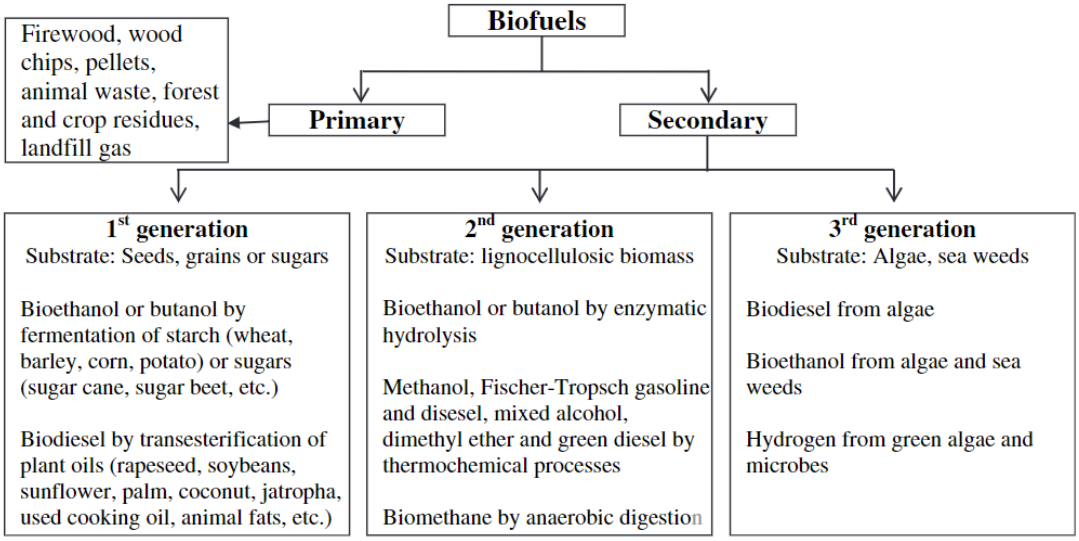

Fig. 2. Summary of the first three generations of biofuel. Reproduced from (Nigam and Singh, 2011).

CHEMICAL CONVERSION IN MICROORGANISMS

For thousands of years, humans have been using microorganisms in wine and beer brewing without the knowledge of the exact process. In the mid-1830s, it was pointed out by Schwann and Cagniard-Latour that yeast was an organism that was responsible for alcohol production during fermentation for alcohol production (Buchholz and Collins, 2013). However, not until Pasteur's work in 1850s that fermentation was better understood as a process carried out by activities of living yeast and different microorganisms were responsible for different fermentation process, e.g., alcohol fermentation caused by yeast vs. lactic acid fermentation by bacteria (Alba-Lois and Segal-Kischinevzky, 2010; Buchholz and Collins, 2013). Years later, Eduard Buchner showed that glucose could be converted into ethanol in a cell-free lysate after grinding yeast cells with pestle and mortar (Alba-Lois and Segal-Kischinevzky, 2010; Zhang, 2015). He also discovered that the conversion of sugar to alcohol occurred inside the cells (Alba-Lois and Segal-Kischinevzky, 2010). In his Nobel Prize lecture, Buchner stated that cells were chemical factories and different products were produced in different workshops. (Lacroix et al., 2008). Since Buchner's work, many scientists have discovered other pathways in living cells, each including different biochemical reactions.

Glycolysis was the first pathway studied by scientists after the discovery of the relationship between microorganisms and fermentation (Kresge et al., 2005). Otto Meyerhof played a significant role in solving more than a third of the glycolysis pathway. Starting in 1932 with the discovery of phosphate uptake during carbohydrate breakdown and splitting of ATP (adenosine triphosphate), Meyerhof along with other scientists had worked out the steps of the glycolysis pathway over the next few years (Kresge et al., 2005). Glycolysis consists of 10 conversion steps that convert glucose into pyruvate while producing ATP. This multi-step

pathway provides important intermediates for other pathways. For example, pyruvate from glycolysis is used to make acetyl-CoA from which fatty acids are made. Pyruvate is also used for the synthesis of the amino acid alanine, etc (Li et al., 2015).

In photosynthesis organisms such as cyanobacteria, algae, and higher plants, scientists have discovered the light reaction and Calvin-Benson cycle that allow the conversion of CO_2 into energy using sunlight and water (Sharkey, 2019). In the first step of photosynthesis, light reactions, energy from light is used to generate high energy electrons in the reaction center of photosystem II (PS2) (Johnson, 2016; Lea-Smith et al., 2016). These electrons are transported through the electron transport chain from PS2 to cytochrome complex *b6f* and then to photosystem I (PSI) where the second light reaction happens. These electrons are then used for the synthesis of NADPH from NADP+ by feeredoxin-NADP+ reductase (Johnson, 2016; Lea-Smith et al., 2016). Back in PS2, water splitting provides electrons to replace the transported electrons. In addition, each water splitting also generates 2 protons and ½ O2 (Lea-Smith et al., 2016). During the electron transport process and water splitting, a proton gradient is generated due to the difference in proton concentration between the two sides of the membrane. This proton gradient is used for ATP synthesis from ADP (adenosine diphosphate) by ATP synthase as the protons are transferred down the gradient (Lea-Smith et al., 2016). Overall, the light reactions produce NADPH and ATP needed for the second part of photosynthesis, the Calvin cycle, which involves CO_2 fixation. The Calvin cycle consists of three steps: carbon fixation, phosphoglyceraldehyde (PGAL), and lastly regeneration of ribulose biphosphate (RuBP) (Hoefnagels, 2009). In the first step, an enzyme called rubisco combines CO_2 and RuBP to form an unstable six-carbon product which then breakdowns to two phosphoglyceric acid (PGA). ATP and NADPH synthesized from the light reaction provide the energy needed to convert PGA to

PGAL. While some of the PGAL molecules are used for the last step of the Calvin cycle, regeneration of RuBP, other PGAL molecules are combined to form glucose (Hoefnagels, 2009).

Besides the pathways mentioned above, many other metabolic pathways have been solved by scientists over the years. Some of the other major pathways often discussed are the citric acid cycle and oxidative phosphorylation that provide intermediates for fatty acid synthesis, the pentose phosphate pathway produces NADPH and ribose-5-phosphate. Ribose-5-phosphate is used for nucleotides synthesis. The gluconeogenesis that breakdowns lactate, glycerol, and amino acids to synthesize glucose (Berge et al., 2002). Each of these pathways does not work separately. Instead, they are interconnected to one another to create a metabolic network.

SYNTHETIC BIOLOGY

Synthetic biology is an emerging field of study that combines science and engineering to manipulate biological processes which involve designing, developing, and modification genetic materials of organisms (Shapira et al., 2017). The work of Francois Jacob and Jacques Monod in 1961 is considered the starting point of synthetic biology (Figure 3). Their study of the lac operon in *E. coli* gave insights into the presence of regulatory circuits within a cell that controlled how the cell responded to environment stimuli (Cameron et al., 2014). With the advancing of technologies such as the success of polymerase chain reaction (PCR) and automatic DNA sequencing, researchers had gained better understanding of the molecular networks within a cell (Cameron et al., 2014). This led to the first successful engineered genetic circuits that was able to carry out the desired functions in 2000 (Gardner et al., 2000; Cameron et al., 2014). Since then, synthetic biology has been growing rapidly and been applied in multiple areas.

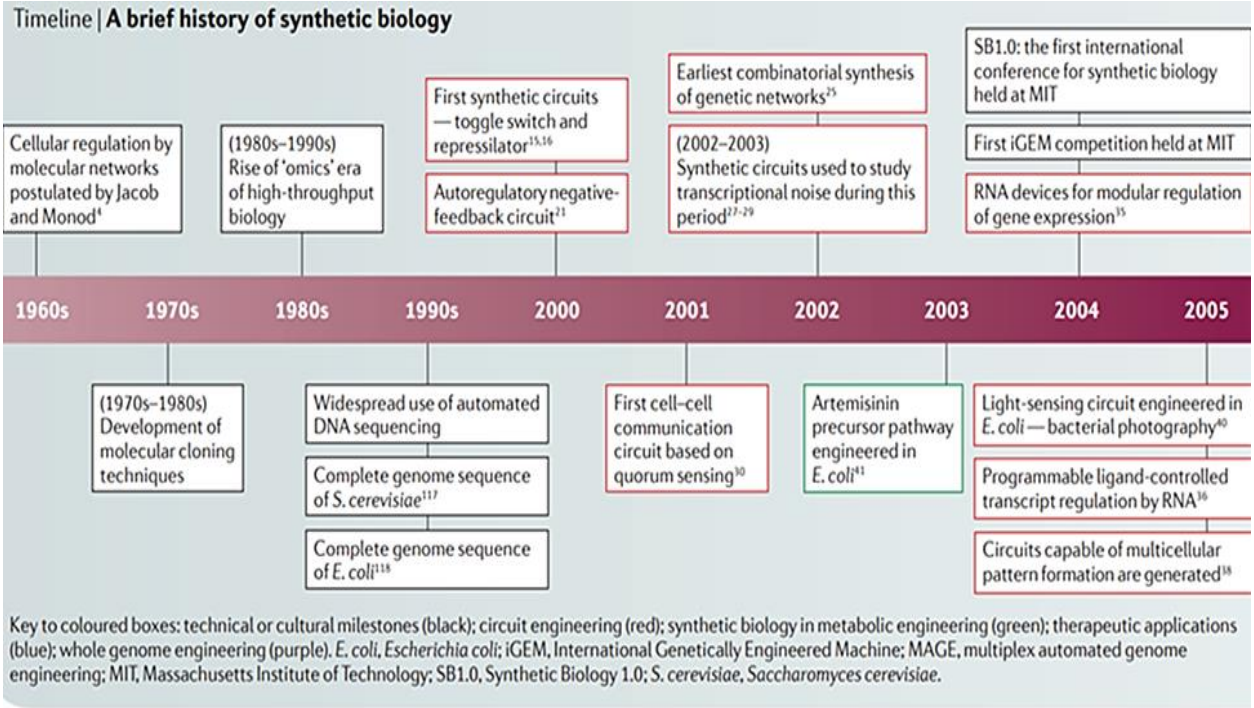

Fig. 3. Timeline for the development of synthetic biology over the years. Reproduced from (Cameron et al., 2014).

Synthetic biology has been used in studying different disease mechanisms. By reconstructing the signaling pathway of the human B cell antigen receptor (BCR), researchers were able to identify a mutation in the immunoglobulin-β-encoding gene which caused agammaglobulinemia, an immunodeficiency, in patients (Ferrari et al., 2007; Khalil and Collins, 2010). In this study, PCR and sequencing were used to analyze the patient's genomic DNA. Results obtained showed a mutation that Gln80 was replaced by a stop codon within the extracellular immunoglobulin domain of Igβ which prevented the expression of functional transmembrane proteins and affected the assembly of the preBCR (Ferrari et al., 2007). The

effects of this mutation were studied by a reconstitution experiment in which wild-type Igβ and mutant Igβ were cloned into *D. melanogaster* S2 Schneider cells (Ferrari et al., 2007).

Besides being used for disease mechanism study, synthetic biology also plays a key role in disease treatments. Many studies have attempted to use genetically modified microbes for drug development as well as a method of drug delivery. With the recent advances in synthetic biology that have given a better look at human's microbiome, the collective of organisms that present in and all a human body, multiple studies have investigated using different microbes to deliver treatment to the target sites of certain diseases. For example, *Salmonella typhimurium*, a gram-negative bacterium, has been experimentally used as anti-cancer treatments. In one study, *Salmonella typhimurium* was genetically engineered to secret interferon-gamma (IFN-γ) which induce host cells' defense against tumors (Yoon et al., 2017). Currently, hundreds of microbiota-based therapeutics are being assessed (Reardon, 2014).

In addition, synthetic biology is also widely used in different industrial areas such as biofuel production. *Zymomonas mobilis*, same as yeast *Saccharomyces cerevisiae*, is an ethanol producer from sugar. However, compared to yeast, *Zymomonas mobilis* uses the Entner-Doudoroff (ED) glycolysis pathway that is more efficient and consumes less ATP while breaking down sugars instead of the common Embden-Meyerhof-Parnas (EMP) pathway found in yeast (Majidian et al., 2018). However, *Z. mobili*s can only utilize a few sugars such as glucose, fructose, and sucrose (Agrawal et al., 2017; Majidian et al., 2018). By introducing plasmids carrying the α-galactosidase and lactose permease genes from *E. coli*, Yanase was able to make *Z. mobilis* ferment melibiose to ethanol which expanded the substrate range for *Z. mobilis* (Yanase et al., 1990). Besides *Z. mobilis*, *Escherichia coli* is also widely used in biofuel production research. Introduction of an alcohol dehydrogenase II gene and a pyruvate

decarboxylase gene from *Z. mobilis* resulted in an increase in *E. coli* cell growth and ethanol production from glucose (Ingram et al., 1987). In addition to ethanol, *E. coli* was also genetically engineered to produce 1-butanol as another fuel source. In a 2008 study, Atsumi and the group were able to achieve 1-butanol production using *E. coli* by inserting a set of genes that convert 2-acetyl-coA into 1-butanol. In addition, by deleting other pathways that compete with the 1-butanol production pathways, they were able to double the production of 1-butanol (Atsumi et al., 2008).

Overall, with the expanding knowledge of biochemical synthesis within different organisms and the growing of synthetic biology, researchers now have the tools to study and utilize different organisms to solve different issues in today's world. This includes but does not limit to disease mechanisms, drug deliveries, biofuel research, agriculture, biotechnologies, etc. Though synthetic biology has many promising advantages, there are concerns that come with this approach. Most importantly, it is the question about biosafety and biosecurity aspects of synthetic biology. As synthetic biology is still in its early stage, few studies have been done to assess the long-term impacts of this approach on the environment as well as human health. The list of risks includes allergies, antibiotic resistance, carcinogens, pathogenicity, or toxicity regarding human health (Hewett et al., 2016). For the environment, the risks include the change or depletion of the environment, competition with native species, horizontal gene transfer, and pathogenicity or toxicity (Hewett et al., 2016).

As microorganisms such as bacteria are used in synthetic biology, there are concerns regarding the problem of horizontal gene transfer between microbes. It is well known that bacteria can transfer genes across species from one cell line to another. As different genes are inserted into bacteria during genetical engineering process, these genes can potentially be

transferred into other organisms upon contact. This poses a greater concern if antibiotic resistant genes are used in the process as these genes can be transferred among different organisms and make their way to human. This could add to the “super bug” or multidrug resistant bacteria crisis. It is now considered the “post-antibiotic era” by the CDC as multidrug resistant bacteria have become a substantial threat to the U.S. public health (Ventola, 2015). Because of these potential risks from genetically engineered organisms, it is important to develop a form of containment so that these organisms cannot escape into the environment and be a potential threat. In addition, a biosafety mechanism should also be considered during genetical engineering such as programmed cell death so that the cells cannot proliferate if they do get out of containment.

CYANOBACTERIA

Cyanobacteria are prokaryotes that perform oxygenic photosynthesis. Evidence has pointed to the roles of cyanobacteria in the oxygenation of the ocean and terrestrial over the years especially during the Great Oxidation Event which led to the diversification of complex life (Demoulin et al., 2019). Before the 20th century, cyanobacteria were considered blue-green algae instead of bacteria and followed the International Code of Botanical Nomenclature (Demoulin et al., 2019). However, in 1978, Stainer proposed that cyanobacteria should follow the International Code of Nomenclature of Bacteria due to their prokaryotic properties (Stainer et al., 1978). Over years of evolution, cyanobacteria have adapted to a wide range of living habitats such as hot springs, freshwater, marine, desert, etc.

Cyanobacteria can be either unicellular or multicellular. Following Stainer’s bacteriological approach, cyanobacteria is divided into five different subsections based on their

cell reproduction, differentiation, and molecular or biochemical attributes (Dvořák et al., 2015; Demoulin et al., 2019). These subsections include Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales, and Stigonematales. Section I and II, Chroococcales and Pleurocapsales, are unicellular cells. Chroococcales reproduce via binary fission or budding while Pleurocapsales reproduce by multiple fission with small daughter cells, or both binary and multiple fission (Rippka et al., 1979). Section III, IV, and V, Oscillatoriales, Nostocales, and Stigonematales, are filamentous cells that reproduce via intercalary cell division. Oscillatoriales only contain vegetative cells in the cell filaments and only divide in one plane. Nostocales and Stigonematales can produce heterocyst which allows nitrogen fixation when there is an absence of combined nitrogen (Rippka et al., 1979). They can also produce akinetes under environmental stress (Rippka et al., 1979; Demoulin et al., 2019). Section V, Stigonematales, however, can divide in more than one plane while Nostocales of section IV can only divide in one plane that is at right angles to the long axis of the filament (Rippka et al., 1979).

Like other prokaryotes, cyanobacteria do not have membrane bound organelles. The outside of a cyanobacteria cell contains a slime coat, a capsule, and a sheath that help with gliding as well as protect the cell under extreme conditions (Noreña-Caro and Benton, 2018). Inside the sheath, the cell wall of a cyanobacteria cell is made of a lipopolysaccharide rich outer membrane and a peptidoglycan inner layer (Noreña-Caro and Benton, 2018). Though being gram-negative, cyanobacteria have a thicker peptidoglycan compared to other gram-negative bacteria. The thickness of the peptidoglycan layer in cyanobacteria has a range of 10 nm in unicellular strains to 15 – 35 nm in filamentous strain and more than 700 nm in other large cyanobacteria (Hoiczyk and Hansel, 2000). This layer is 2 – 6 nm thick in other gram-negative bacteria while being 20 – 40 nm thick in gram-positive bacteria (Hoiczyk and Hansel, 2000).

Inside the cell membrane, the thylakoids containing the photosynthesis light reaction centers are arranged along the long axis of the cell in the cytoplasm (Noreña-Caro and Benton, 2018). In the cytoplasm of cyanobacteria are the circular chromosomes along with other components such as RuBisCo housing carboxysomes that play a part in the CO_2-concentrating mechanism, polymer granules, and gas vesicles (Rae et al., 2013; Noreña-Caro and Benton, 2018) (Figure 4).

Cyanobacteria are considered a valuable resource as they play important roles in different areas. In agriculture, dried cyanobacteria have been added into soil since the 1950s. This process is called "algalisation." Algalisaion have shown to improve crop productivity by 15 – 20% with no extra economic inputs (Garlapati et al., 2019). Nitrogen fixation process in the heterocysts of cyanobacteria helps convert atmospheric dinitrogen N_2 into usable form such as ammonia for plant growth. Field studies have shown an increase in biomass productivity as well as reduction in the use of chemical fertilizers when cyanobacteria are used as bio-fertilizers (Garlapati et al., 2019). Cyanobacteria also help solubilize the insoluble organic phosphate in the soil for plant use (Singh et al., 2016). In addition, cyanobacteria are also used in plant disease treatment. Cyanobacteria can produce different compounds such as polyketides, amides, alkaloids, fatty acids, indoles, and lipopeptides that can act as antibacterial and antifungal agents (Singh et al., 2016). Protein rich cyanobacteria such as Arthrospira platensis (Spirulina platensis) are used as food supplements as they provide a high amount of nutrients such as vitamin B12, beta-carotene, thiamine, riboflavin, etc (Singh et al., 2016).

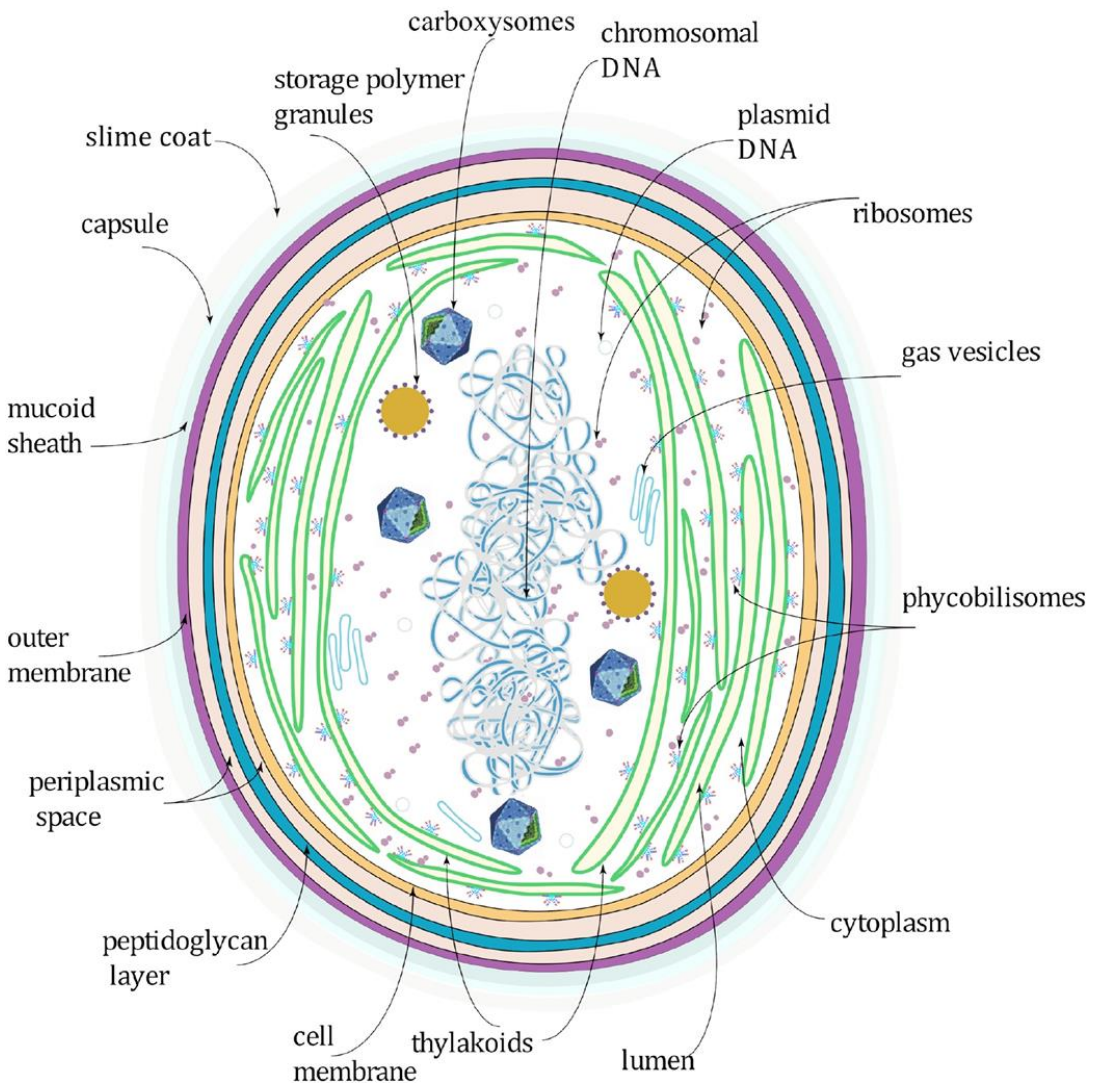

Fig. 4. Cellular structure of a cyanobacteria cell. Reproduced from (Noreña-Caro and Benton, 2018).

Another big use of cyanobacteria lies in the biofuel industry. Due to their fast cell growth and photosynthesis property along with the growing of synthetic biology, scientists have been looking into modifying the metabolic pathways of cyanobacteria to produce different biofuels such as ethanol, butanol, isobutanol, etc. The most common strains of cyanobacteria used for

biofuel studies include *Synechocystis* PCC 6803 and *Synechococcus elongatus* PCC 7942. By inserting a pyruvate decarboxylase (pdc) gene and an alcohol dehydrogenase II (adh) gene *from Z. mobilis* into *Synechococcus elongatus* sp. PCC 7942, Deng's group was able to achieve about 450 nmol/L of ethanol production over 7-day period (Deng and Coleman, 1999). Using the same approach with *Synechocystis sp*. PCC 6803, Dexter and his colleague saw 10 mM of ethanol produced after 6 days of culturing (Dexter and Fu, 2009). 1-butanol was also produced using genetically engineered *Synechococcus elongatus* sp. PCC 7942 with a set of five different genes that convert 2-acetyl-CoA into 1-butanol (Lan and Liao, 2011). *Synechococcus elongatus* sp. PCC 7942 also produced isobutanol from 2-ketoisovalerate, an intermediate of valine synthesis pathway, after a 2-ketoisovalerate decarboxylase gene and an alcohol dehydrogenase gene were introduced into the cells (Atsumi et al., 2009).

Harboring many beneficial properties, cyanobacteria are indeed important bio-resources that can be utilized in many areas from increasing crop yield and soil enhancement to food supplement to achieving sustainable energy sources. While some of this research is still in the early stage, the outlook of solving some of the current world crisis using cyanobacteria is hopeful.

***THERMOSYNECHOCOCCUS ELONGATUS* BP1**

Thermosynechococcus elongatus BP1 (*T. elongatus* BP1) is a thermophilic rod-shaped strain of cyanobacteria isolated from hot springs. Unlike other mesophilic cyanobacteria such as *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* sp. PCC 7942 that grow at ambient temperature, *T. elongatus* BP1 has the optimal growth temperature of 55 -57°C (Nakamura et al., 2002). The circular chromosome of *T. elongatus* BP1 contains 2,593,857 bp without any plasmid detected (Nakamura et al., 2002). Genome study of *T. elongatus* BP1 showed that there are three

extra genes found for the heat-shock proteins in *T. elongatus* BP1 genome compared to the mesophilic *Synechocystis*. The extra gene for heat shock proteins are thought to be responsible for the thermophilic property of *T. elongatus* BP1 (Nakamura et al., 2002). *T. elongatus* BP1 can uptake foreign DNA via natural transformation, electroporation, or conjugation (Iwai et al., 2004; Onai et al., 2004a). Natural transformation is the uptake of free-floating DNA from the environment by the cells while electroporation is the introduction of DNA into the cells using an electric field and conjugation involves DNA transfer from a donor cell to a recipient cell. Though being thermophilic, *T. elongatus* BP1 can tolerate a wide range of temperature from 30°C to 60°C. At temperature below 30°C, however, cells are viable but are not able to proliferate (Onai et al., 2004b).

The thermophilic property of *T. elongatus* BP1 makes this strain an ideal candidate for biofuel study as it provides a built-in biosafety mechanism. A study was carried out to compare the growth of *T. elongatus* BP1 under laboratory condition with temperature controlled at 42°C and under greenhouse condition where temperature was not controlled (Sacko et al., 2020). The temperature in the greenhouse ranged from 15.44°C – 25.30°C in the cool season and 31.42°C – 36.27°C in the warm season. Results from this study showed that while *T. elongatus* BP1 cells, both wild-type and genetically engineered, were actively growing under the laboratory condition, both *T. elongatus* BP1 cells in the greenhouse were not actively replicating (Sacko et al., 2020). This indicated that *T. elongatus* BP1 cells provide some biosafe features if genetically engineered cells get out of the elevated temperature controlled growing condition, they are not likely to replicate and compete with other native microbes or pose an environmental bio risk. In addition, the risk of mesophilic bacteria contamination in the cell culture can also be greatly reduced if a thermophilic strain of cell is used.

RESEARCH AIMS

This dissertation contained three aims. The first aim was to assess whether genetically engineered cyanobacteria, *T. elongatus* BP1, could transfer gene horizontally into other bacteria (*E. Coli* DH5α). *T. elongatus* BP1 was modified by the introduction of a cassette carrying a set of alcohol production genes including kanamycin resistant gene into the cells' genome. These GE *T. elongatus* BP1 cells were co-cultured with wild-type *E. coli* DH5α followed by the spreading of the co-cultures onto agar LB medium containing kanamycin. This would allow the selection of *E. coli* cells with kanamycin resistant gene obtained from the GE *T. elongatus* BP1. PCR was used to verify the presence of kanamycin resistant gene within *E. coli* DH5α cells. Secondly, *T. elongatus* BP1 cells were genetically engineered to produce isobutanol as biofuel. A cassette carried an isoketovalerate decarboxylase (KIVD) and an alcohol dehydrogenase gene (yqhD) was designed using CLC Genomic Workbench 12 software. After the cassette was inserted into *T. elongatus* BP1 cells, gene expression study was done via RNA extraction and reverse transcription PCR while isobutanol production detection was done using GCMS. Lastly, the potential of *T. elongatus* BP1 cells being a biofuel production host was studied. The cells were challenged with different concentrations of three representative alcohol which were ethanol, isobutanol, and 1-butanol. Cell growth was monitored using OD730 for a period of 7 days to assess the tolerance of *T. elongatus* BP1 to these alcohols which could give insight into the maximum productivity possible. Together, this dissertation would provide more understanding about using *T. elongatus* BP1 as a future biofuel production host from the biorisk aspect to the production limit of different alcohols.

CHAPTER 2

STUDYING OF HORIZONTAL GENE TRANSFER FROM GENETICALLY ENGINEERED *THERMOSYNECHOCOCCUS ELONGATUS* BP1 TO WILD-TYPE *E. COLI* DH5α

PREFACE

The content of this chapter is reprinted with permission from Nguyen, T. H.; Barnes, C. L.; Agola, J. P.; Sherazi, S.; Greene, L. H.; Lee, J. W., Demonstration of Horizontal Gene Transfer from Genetically Engineered Thermosynechococcus elongatus BP1 to Wild-type E. coli Dh5alpha. Gene 2019, 704, 49-58.

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INTRODUCTION

While biofuel production from photosynthetic cyanobacteria seems to be a promising solution for energy crisis, the biosafety aspect of this approach remains a concern. The long-term effect of genetically engineered cyanobacteria usage on the ecosystem and human health is yet to be fully understood as it is still a relatively new area. One of the main concerns is the potential risk of horizontal gene transfer from genetically engineered cyanobacteria into other organisms. Horizontal gene transfer describes the process where genetic materials are transferred across different organisms and not from parental to daughter cells (Soucy et al., 2015; Daubin and Szollosi, 2016; Sieber et al., 2017; Sand and Jelavic, 2018). Horizontal gene transfer mechanisms are done via conjugation and transduction. Conjugation refers to a process of genetic material

sharing via cell to cell contact while bacteriophages play a role in transduction (Huddleston, 2014). One of the most serious issues due to gene sharing between microorganisms is the wide spread of antibiotic resistance among different bacteria (Lerminiaux and Cameron, 2019).

With the current state of genetically engineered biofuel production cyanobacteria gaining attraction, it is necessary to investigate their ability to transfer genes to other organisms. While gene transfer from bacteria such as *E. coli* to cyanobacteria has been well known, not much work has been done to look at the reverse direction. This chapter focuses on assessing whether genetically engineered *T. elongatus* BP1 cells can transfer genes horizontally into *E. coli* DH5α.

MATERIALS AND METHODS

Strains and culture conditions

Cells of *T. elongatus* BP1 (BA000039.2) which was isolated originally from the Beppu hot springs in Japan and subsequently used in many research laboratories throughout the world including the Lee laboratory at Old Dominion University were grown photoautotrophically at 45°C under constant light illumination (30 µE m^{-2} s^{-1}) provided by daylight fluorescent lamps. BG-11_0SA medium used in this study to grow *T. elongatus* BP1 was a special modified BG-11 culture medium, in which 4 mM of $(NH_4)_2SO_4$ was used as the nitrogen source instead of KNO_3 as described previously (Suzuki et al., 1996). The BG-11_0SA growth medium for *T. elongatus* BP1 transformants was supplemented with kanamycin (40µg/ml). *E. coli* DH5α cells (NCBI_ CP025520.1) from ABClonal (Woburn, MA 01801, USA) were growth at 37°C in LB broth. *E. coli* cells containing plasmids were grown in LB medium supplemented with either 100 µg/ml ampicillin or 40 µg/ml kanamycin.

Construction of pUC57-based pKB plasmid

A pUC57-based plasmid carrying the Lee-laboratory-designed DNA cassette comprising a nirA promoter (1405963..1406190) of *T. elongatus* BP1, a keto-acid decarboxylase gene (NCBI_AAS49166.1) modified from *Lactococus lactis* branched-chain alpha-ketoacid decarboxylase (AAS49166), a butanol dehydrogenase gene (NCBI_BA000016.3) modified from the *Clostrium perfringens* NADPH-dependent butanol dehydrogenase (NP_562172), a heat-tolerant kanamycin resistant synthetic gene, and a 120-bp rubisco rbcS 3' UTR terminator selected from *T. elongatus* BP1 (1573695..1573814) was synthesized by GenScript and is hereby referred to as pKB. The plasmid also carried two additional promoters, a continuous promoter from the cpc gene of *T. elongatus* BP1 (2042749..2042864) and another continuous promoter from the slpA gene (HE611334) for *Thermus thermophilus*, preceding to the kanamycin gene to ensure the expression of antibiotic property in thermophilic *T. elongatus* BP1. Genomic DNA of *T. elongatus* BP1 was extracted using Promega Wizard Genomic DNA Purification Kit. A DNA fragment coding for psbA2, upstream homologous sequence of the target site in *T. elongatus* BP1, was amplified from genomic DNA of wild-type *T. elongatus* BP1 by PCR using primers CRSI_fwd (TTGGCCGGCCTAACGACTATGACTACAGTTCTG) and CRSI_rev (TTCCTGCAGGTTAAACTCGCCATAAAAAAGCCCCCCTGTG). This resulted in a 1.2 kb fragment which was cloned into the pKB plasmid between FseI and SbfI sites to serve as the first homologous recommendation site sequence (RSI). The second homologous fragment coding for tll1845 was also amplified using primers CRSII_fwd (TTATTTAAATTTACTGGGCGGGGAGTTGATACTG) and CRSII_rev (TTGGCGCGCCAGTGCTACTTTGGCGTTCACCAGG). The resultant 1kb fragment was

cloned into the same plasmid between SwaI and AscI to serve as the second homologous recommendation site sequence (RSII).

Genetic transformation of pKB plasmid into *T. elongatus BP1*

Genetic transformation of pKB plasmid into *T. elongatus* BP1 cells was done via electroporation. Wild-type *T. elongatus* BP1 cells grown in liquid BG-11_0SA medium were collected by centrifugation. Cells were then resuspended in water to reach OD_{730} =20.0. 400 µL of cell suspension was mixed with 1µg of pKB plasmid. Electroporation was done at 5 kV/cm using BioRad PowerPac Basic. After pulsing, cells were immediately transferred into flasks containing 5 ml of pre-warmed (45°C) BG-11_0SA and then incubated at 45°C without shaking for 24 hours. After 24 hours, cells were spread on agar BG-11_0SA medium containing kanamycin (40µg/ml). After 10-13 days of photoautotrophic incubation under a photosynthetic light intensity of 30 µE m^{-2} s^{-1} at 45°C, the transformed cells containing the kanamycin-resistant selectable marker DNA construct grew on antibiotic-containing culture plates as green colonies. Individual colonies were streaked onto new plates as well as grown photoautotraphically in BG-11_0SA liquid culture medium with kanamycin.

Verifying the presence of cassette in *T. elongatus* BP1

After green colonies were seen on BG-11_0SA agar plates containing kanamycin, colonies PCR assays were done using primers CRSI_fwd (TTGGCCGGCCTAACGACTATGACTACAGTTCTG) and BP1_tll1845_rev (CCTCTGCGACACCTACTACATCCAC) to verify the presence of the cassette within the genomic DNA of GE *T. elongatus* BP1. In addition, we also verified the presence of pKB

plasmid within the GE *T. elongatus* BP1 besides the integrated cassette using primers Amp_fwd (TTACCAATGCTTAATCAGTGAGGCAC) and bdh0318_rev (CCTCCATTTCCTTTGCACCCT).

Horizontal gene transfer study

T. elongatus BP1 transformant cells were photoautotrophically grown at 45°C in a Percival growth chamber in BG-11_0SA medium containing kanamycin (40µg/ml), and wild-type *E. coli* DH5α cells were grown in LB broth at 37°C in a shaking incubator. *T. elongatus* BP1 transformant cells were collected by centrifugation. The supernatant was discarded, and the cells were resuspended in fresh BG-11_0SA medium without antibiotic. Both *T. elongatus* BP1 transformants and wild-type *E. coli* cells were resuspended to the final concentration of 10^7 cells/mL.

Using the cells resuspension at the final concentration of 10^7 cells/mL, 15 mL cells resuspension of *T. elongatus* BP1 transformants in BG-11_0SA were mixed with 15 mL cells resuspension of wild-type *E. coli* DH5α in LB. The "15 mL+15 mL" GE *T. elongatus* and wild-type *E. coli* liquid co-cultures were incubated in a shaker at 37°C under continuous photosynthetic light intensity of about 8 µE m^{-2} s^{-1} provided by daylight fluorescent lamps. In addition to the liquid co-cultures, two controls were also set up using these *T. elongatus* BP1 transformants and wild-type *E. coli* DH5α. Control 1 consisted of 15 mL of *T. elongatus* BP1 transformants and 15 mL of LB broth without wild-type *E. coli* DH5α cells. Control 2 consisted of 15 mL of wild-type *E. coli* DH5α and 15 mL of BG-11_0SA medium without *T. elongatus* BP1 transformants (Figure 5). These controls were also incubated in a shaking incubator at 37°C under the same lighting conditions. Samples from each liquid co-culture and control were

collected after 1, 2, and 3 days and were then spread on control LB agar plates and selection LB plates containing kanamycin. Typically, about 100 µl of liquid co-culture sample was used to spread onto the surface of a 45 mL antibiotic containing LB agar medium per petri dish (100 mm diameter, 15 mm deep) plate. The LB plates were incubated at 37°C under the same continuous photosynthetic lighting condition of 8 µE m^{-2} s^{-1}. PCR was used to verify the presence of the cassette DNA within these colonies.

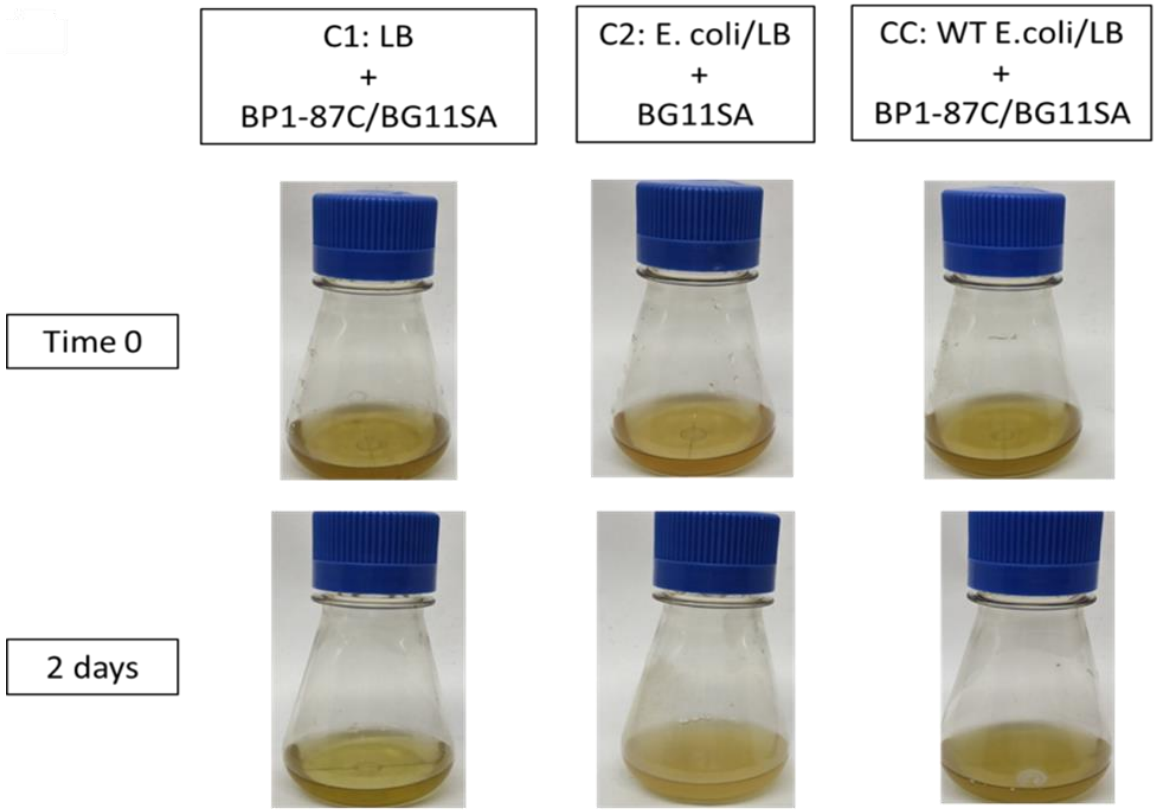

Fig. 5. Experimental set up of horizontal gene transfer study of *T. elongatus* BP1 and *E. coli* DH5α.

Verifying the Presence of Cassette DNA in *E. coli* DH5α

The presence of the cassette DNA in selected *E. coli* colonies from kanamycin LB plates were verified by colony PCR using primers CpcPKan_fwd (TAATAGGCGTTTCCCTTCGTTGCC) and CpcPKan_rev (CAAAATGGTATGCGTTTTGACACATCC). Plasmid extraction was also done for the same selected *E. coli* colonies obtained on LB agar plates containing kanamycin. Plasmid PCR using primers Amp_fwd (TTACCAATGCTTAATCAGTGAGGCAC) and bdh0318_rev (CCTCCATTTCCTTTGCACCCT) was performed for selected *E. coli* cells from HGT experiment with *T. elongatus* BP1 transformants carrying pKB plasmids.

Testing *E. coli* DH5α's Ability to Uptake DNA from Supernatant

To test whether wild-type *E. coli* DH5α has the ability to take in plasmid DNA from the liquid medium, a control experiment was carried out as follows. 15 mL of wild-type *E. coli* DH5α cell resuspension at the concentration of 10^7cells/mL in LB broth was added to 15 mL of BG-11_0SA medium followed by the addition of a known amount of pKB plasmid DNA (10 ng, 100 ng, 1000 ng) (Figure 6). The cultures were incubated at 37°C under continuous photosynthetic light intensity of about 8 µE m^{-2} s^{-1} for 2 days. The cells were then plated on to LB agar control plates and LB agar plates with kanamycin.

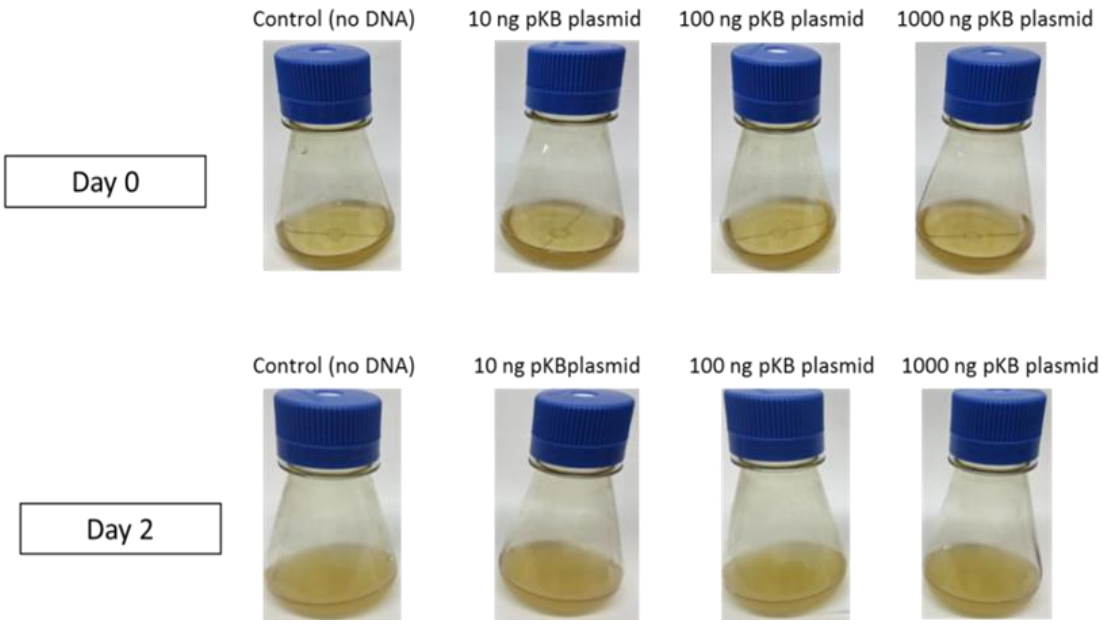

Fig. 6. Experimental set up for testing of *E. coli* DH5α's ability to uptake free floating DNA.

PCR and DNA sequencing

PCR assays were performed using Phusion High-Fidelity PCR Master Mix in a BioRad C1000 Thermal Cycler. All samples were run for 35-40 cycles. Gel electrophoresis was conducted using 0.8-1% agarose in 1X TAE buffer. The 2-Log DNA Ladder (0.1-10.0 kb) was obtained from New England BioLabs. The agarose gels were imaged using UVP PhotoDoc-It Imaging System. DNA sequencing was conducted by the Eastern Virginia Medical School Bioinformatics Analytics Core.

RESULTS AND DISCUSSION

Genetic transformation of pKB plasmids into *T. elongatus* BP1

To create genetically engineered *T. elongatus* BP1, pKB plasmids were introduced into the cyanobacteria cells via electroporation. After green colonies of GE *T. elongatus* BP1

transformants were selected from agar BG-11_0SA plates containing kanamycin, colony PCR was carried out to verify the presence of the cassette DNA within these cells. The results showed the expected 6.7 kb PCR product band for most of the selected colonies indicated the integration of the cassette into the genomic DNA of *T. elongatus* BP1 transformants (Figures 7 - 9). Wild-type *T. elongatus* BP1 used as a control did not show the 6.7 kb band.

In addition to verifying the integration of the cassette in genomic DNA, we also tested the presence of cassette in the form of plasmids within GE *T. elongatus* BP1 cells. Plasmid extraction was carried out for GE *T. elongatus* BP1 cells followed by PCR using Amp_fwd and Bdh0318_rev. The results showed the expected band at 4.5 kb indicating that *T. elongatus* BP1 transformants indeed still retained pKB plasmids after a few months of culturing (Figure 10). The control PCR using wild-type *T. elongatus* BP1 did not produce the same band. It is worth noting that previous research indicates that a plasmid would be able to replicate in both cyanobacteria and *E. coli* if it were a hybrid plasmid containing replicons from both organisms and if it were a broad-host-range plasmid such as RSF1010 (Huang et al., 2010; Taton et al., 2014). Here, we used pUC57 plasmids as the backbone for pKB with an amplicon from *E. coli*.

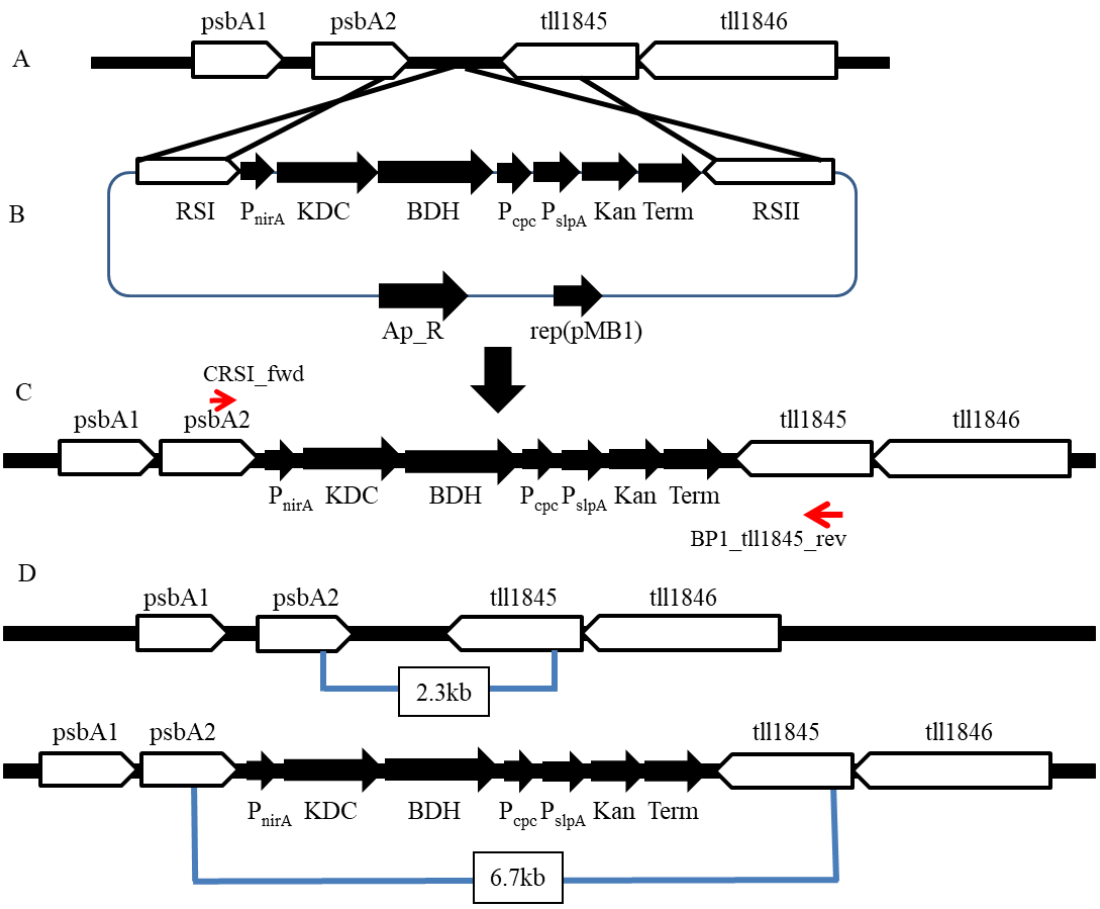

Fig. 7. Schematic of cloning the pKB cassettes into the genomic DNA of T. elongatus BP1. (A) Schematic of the genome at the site of integration. (B) A plasmid carrying a set of genes of interest (filled arrows) including PnirA - nirA promoter, KDC - keto-acid decarboxylase, BDH – butanol dehydrogenase (pKB), Pcpc - cpc promoter, PslpA - slpA promoter, Kan - kanamycin resistant gene, and Term - rubisco terminator. The cassette was flanked by upstream (RSI) and downstream (RSII) homologous recombination site sequences which are used to guide the integration of the cassette into the desired insertion site between psbA2 and tll1845 genes within the genomic DNA of *T. elongatus* BP1. (C) Schematic of integrated cassette in genomic DNA. Red arrows indicated primers used for verifying the integration of cassette within genomic DNA of T. elongatus BP1 transformants. (D) Schematic for size of expected PCR bands (2.3 kb and 6.7 kb) without and with the cassette integration, respectively.

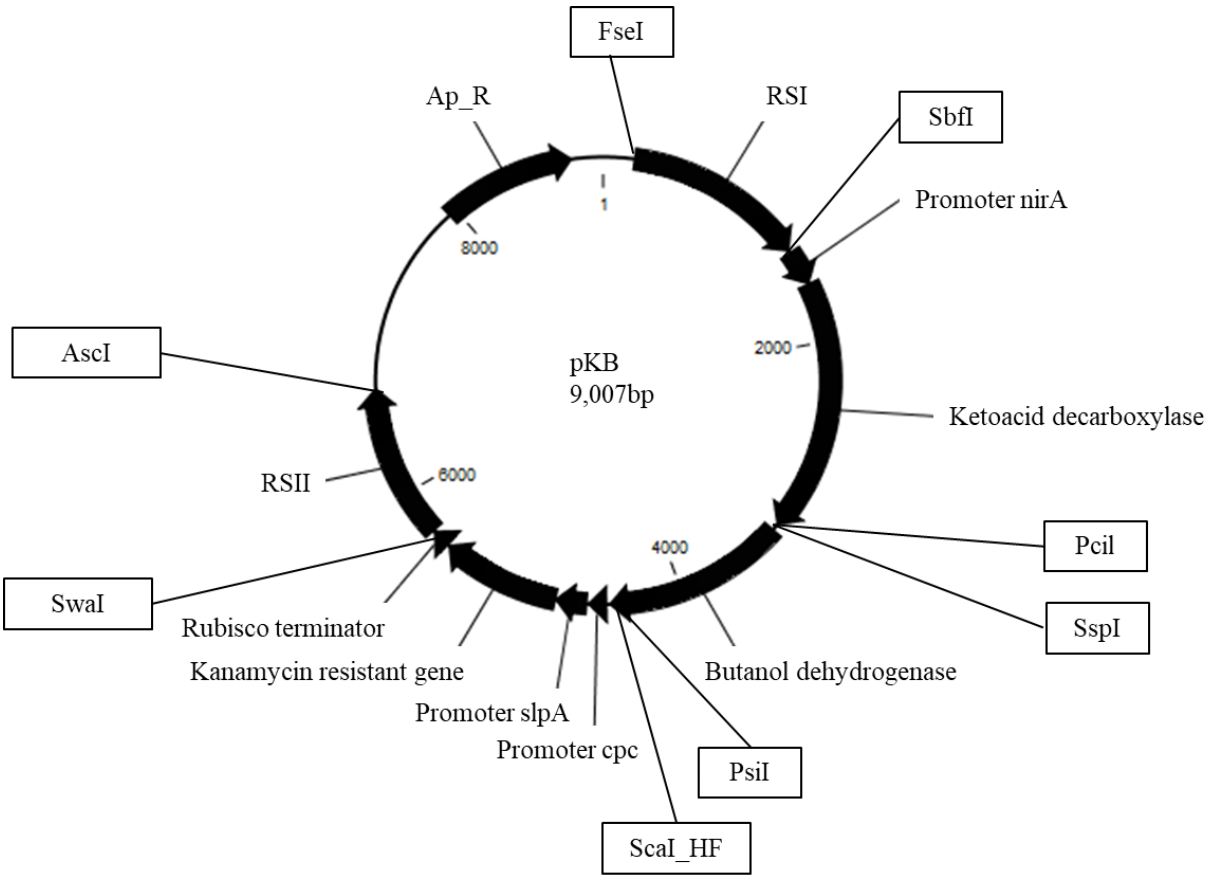

Fig. 8. Schematic of pUC57-based pKB plasmid.

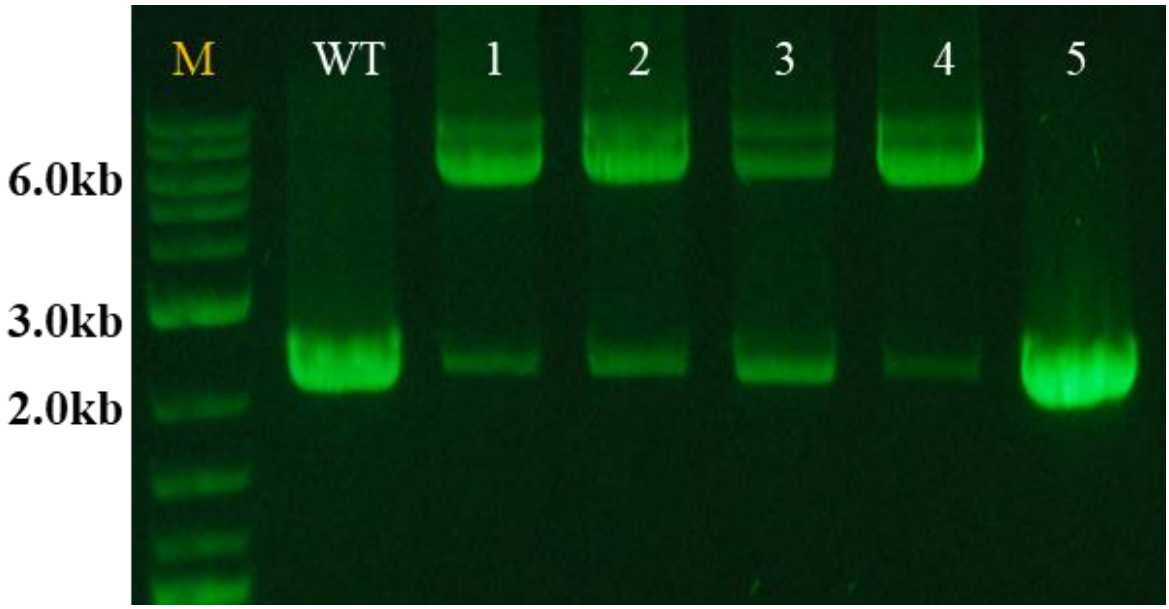

Fig. 9. PCR analysis of *T. elongatus* BP1 colonies carrying pKB plasmids. For most of the selected colonies, a band at 6.7 kb was detected which indicated the integration of the cassette into genomic DNA of these GE *T. elongatus* BP1 cells. Because cyanobacteria are polyploid, the band at 2.3 kb indicated that there were some copies of the chromosome in each selected colony still had not integrated the cassette. CRSI_fwd and BP1_tll1845_rev primers used are indicated by red arrows in Figure 7. The lanes are marked as follows: M = molecular weight marker, WT = wild-type *T. elongatus*, Lanes 1-5 are selected *T. elongatus* colonies from BG-11_0SA agar plates containing kanamycin.

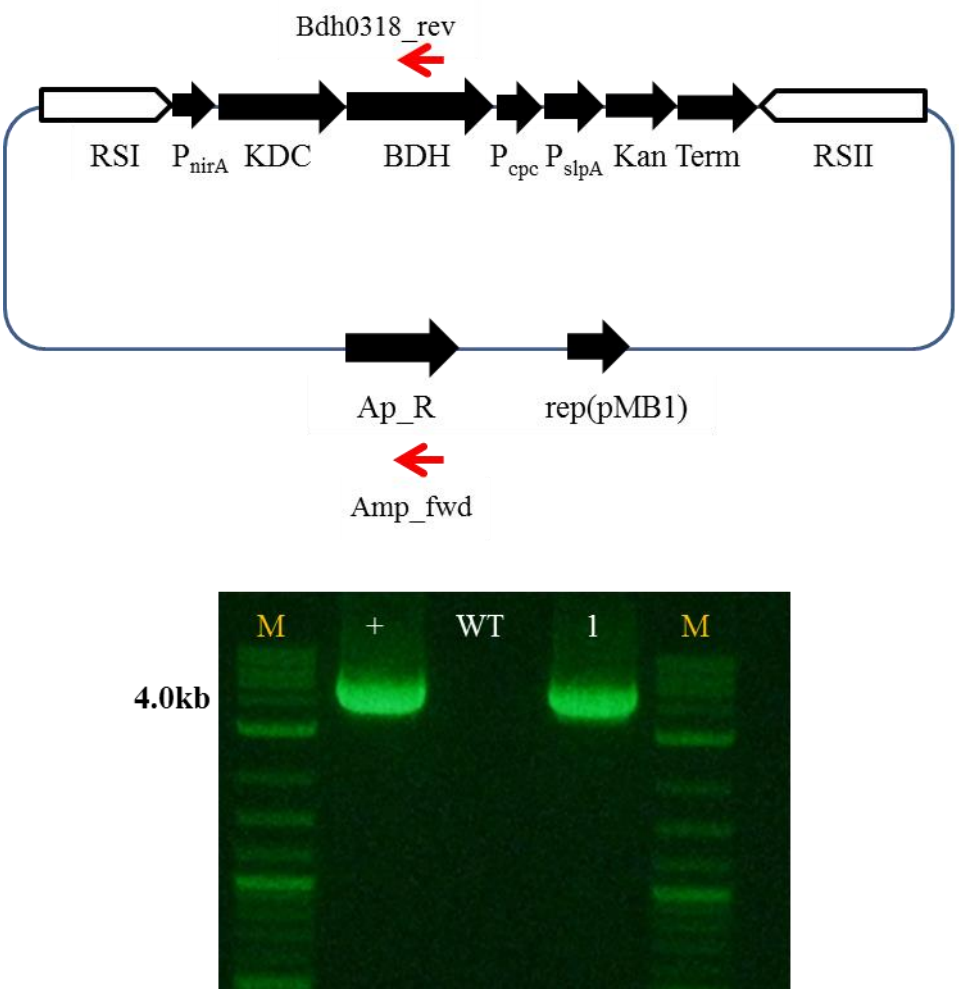

Fig. 10. Verification of pKB plasmid presence within GE *T. elongatus* BP1 cells by PCR. Only GE *T. elongatus* BP1 cells (lane 1) showed the expected band at 4.5 kb indicating the presence of the pKB plasmid. Primers used are indicated by red arrows. This band was not seen in the wild-type *T. elongatus.*

Horizontal gene transfer study

To study whether GE *T. elongatus* BP1 has ability to transfer pUC plasmids into wild-type *E. coli* DH5α, the two organisms were co-incubated at 1:1 population ratio for a designated period. Then, the co-incubated liquid cell culture was sampled and plated on LB agar medium. The results for the control 1, which only contained *T. elongatus* BP1 transformants, showed no colony growth on LB plates with and without kanamycin after 48 hours of plate incubation at 37°C under lighting. This indicated that *T. elongatus* BP1 could not grow on LB plates. Therefore, any colony formed on LB agar plates from this experiment would be *E. coli*. Bacterial growth was seen on LB plates without antibiotic for control 2 which contained wild-type *E. coli* only. However, control 2 did not have any colony growth on LB plates with kanamycin. The only colony growth observed on LB agar plates containing kanamycin was from co-cultured samples (the "15 mL+15 mL" GE *T. elongatus* and wild-type *E. coli* liquid co-cultures) plated after 2 days of liquid co-incubation. Typically, the observed number of HGT *E. coli* colonies ranged from 20 to 75 per plate. Because control 1 eliminated the possibility of *T. elongatus* BP1 growing on LB agar plates within this period, the colonies on LB plates with kanamycin were indeed *E. coli* (Figure 11). It is worth noting that these *E. coli* colonies grew much slower on LB agar plates containing kanamycin compared to the control wild-type *E. coli* on plates without antibiotic which only took 24 hours to form observable colonies instead of 48 hours. Randomly selected colonies on kanamycin plates were further analyzed by PCR to confirm the presence of the target plasmid within the cells.

The colony PCR studies of selected *E. coli* colonies from LB agar plates containing kanamycin showed that the kanamycin-resistant transgene and its promoters within the pKB plasmid were successfully amplified for four of the selected colonies with the presence of the

band at 1.1 kb for colonies 3, 6, 7 and 9 (Figure 12). This indicated that the kanamycin-resistant genes from pKB cassettes were transferred from *T. elongatus* BP1 transformants into *E. coli* DH5α. For further analysis, plasmid extraction was carried out for these colonies followed by PCR using Amp_fwd and Bdh0318_rev which were the same pair of primer used for checking the respective plasmids in *T. elongatus* BP1 transformants. Interestingly, our PCR results showed that the majority of the selected colonies did not have the expected band from the plasmid PCR (Figure 13). This same pattern of the plasmid PCR results was also observed in multiple replications of the HGT experiment (five replications).

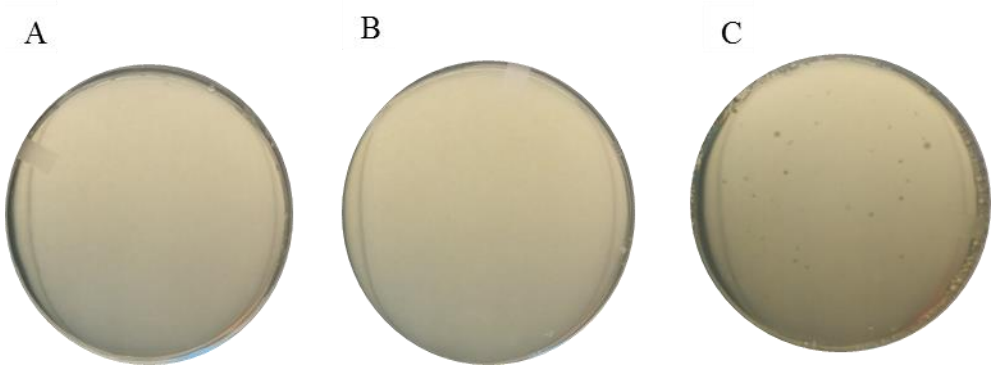

Fig. 11. Detection of *E. coli* growth on kanamycin LB agar plates after horizontal gene transfer. (A) No *E. coli* colonies were observed on a LB agar plate containing kanamycin 40 µg/mL from control 1. (B) No *E. coli* colonies were observed on a LB agar plate containing kanamycin 40 µg/mL from control 2. (C) *E. coli* colonies observed on a LB agar plate containing kanamycin 40 µg/mL after co-culturing with *T. elongatus* BP1 transformants carrying pKB plasmids. Co-cultures were grown for 2 days and additional 48 hours of incubation on the plates.

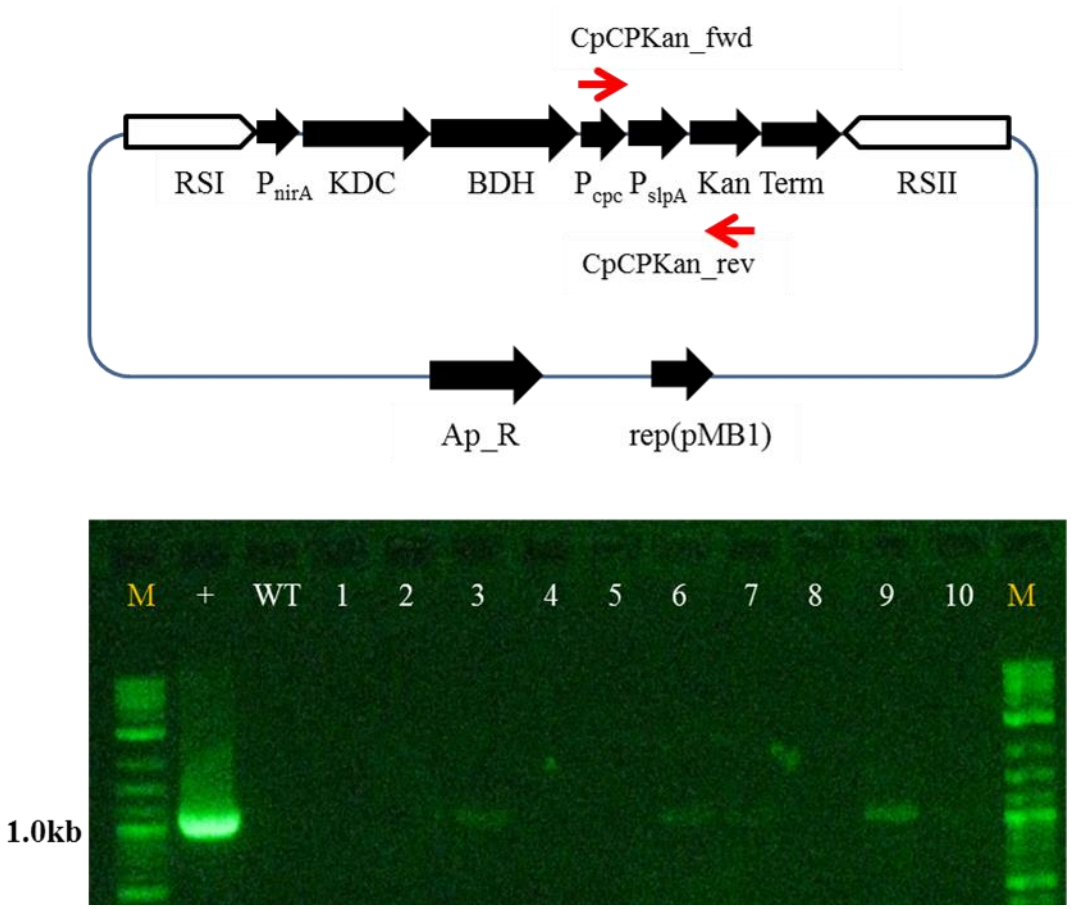

Fig. 12. Colony PCR results of selected *E. coli* colonies from horizontal gene transfer study with the pKB transformants. Promoter cpc (P_{cpc}), promoter slpA (P_{slpA}), and kanamycin (Kan) were amplified which indicated by the presence of the 1.1kb bands in colony PCR. This confirmed the transfer of the cassette into *E. coli* from *T. elongatus* BP1 transformants. The lanes are marked as follows: M = molecular weight marker, + = pKB plasmid, WT = wild-type *E. coli* plasmid DNA, Lanes 1-10 are selected *E. coli* colonies from LB agar plates containing kanamycin.

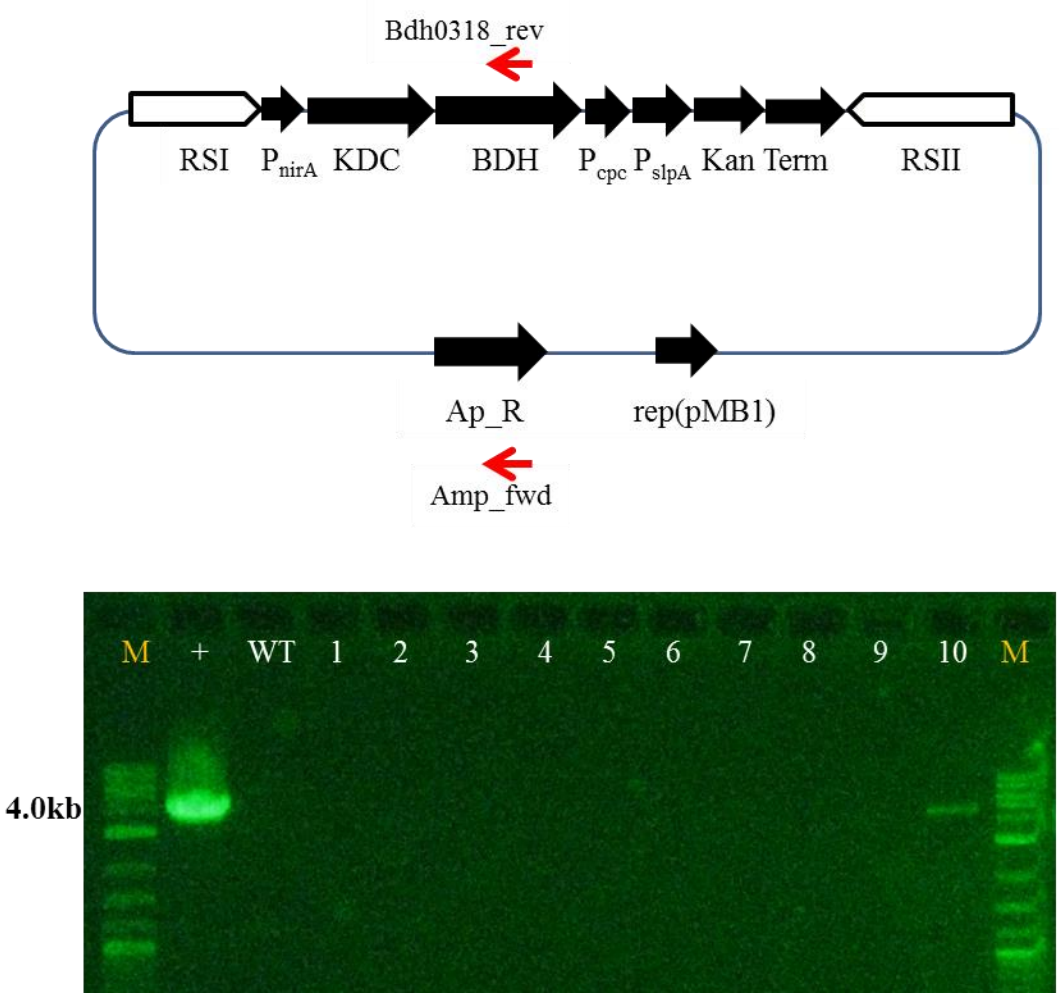

Fig. 13. Extracted plasmid PCR results of selected *E. coli* colonies from horizontal gene transfer study with the pKB transformants. Using the plasmid DNA, a 4.5 kb band was amplified to verify the presence of the pKB plasmid. The expected band was present for the pKB plasmid positive control (+) and one selected *E. coli* colony (lane 10) but not seen in the wild-type *E.coli* control (WT). Primers used are indicated by red arrows. The lanes are marked as follows: M = molecular weight marker, + = pKB plasmid, WT = wild-type *E. coli* plasmid DNA, Lanes 1-10 are selected *E. coli* colonies from LB agar plates containing kanamycin.

Based on our finding that we were only able to amplify the cassette DNA using colony and plasmid PCR for a few of the selected colonies, we expected that for some *E. coli* colonies, the cassette might have been integrated into their genomic DNA. Even though *E. coli* DH5α is a recA mutant which is deficient in homologous recombination, studies have shown that homologous recombination could still happen in *E. coli* DH5α via a recA-independent pathway (Lovett et al., 2002; Kostylev et al., 2015). Because recA-independent crossover events can happen at any homology length, although optimal at 50 bp, this could potentially happen at multiple locations on the genomic DNA of *E. coli* DH5α and the possible integration frequency as well as orientation could be different for each cell. This seemingly explains the reason for unsuccessful colony and plasmid PCR for some of the selected *E. coli* colonies. However, the main purpose of this discussion is to report the horizontal transfer of a pUC plasmid from GE *T. elongatus* BP1 into wild-type *E. coli* DH5α. The study of the location(s) of transgene integration of these plasmids in *E. coli* genomic DNA will be the focus of future work.

Testing *E. coli* DH5α's Ability to Uptake DNA from Supernatant

Even though wild-type *E. coli* DH5α is known to be not naturally competent, to ensure that the cassette was indeed horizontally transferred into *E. coli* from *T. elongatus* BP1 transformants, we also carried out a control experiment to test whether wild-type *E. coli* DH5α can naturally take up free-floating DNA from the liquid medium. *E. coli* DH5α was incubated with a known amount of pKB or pKA plasmid using the same condition and LB:BG-11_0SA medium ratio of the HGT study. However, in this experiment, *T. elongatus* BP1 transformants were omitted. Here, we used the maximum amount of 1000 ng of plasmid DNA per 30 mL (15 mL LB + 15 mL BG-11_0SA) incubation treatment because this was the amount of plasmid DNA

used in electroporating *T. elongatus* BP1. Therefore, in the event of plasmid being carried over, 1000 ng would be the maximum possible quantity of plasmid in the supernatant of co-cultures. The results from plating these *E. coli* cells on LB agar plates with and without kanamycin showed that these *E. coli* cells were only able to grow on LB plates without kanamycin. There were no colonies observed on LB plates containing kanamycin (Figures 14). This indicated that wild-type *E. coli* DH5α cells under our experimental conditions do not have the ability to take in DNA from the liquid medium environment but must be introduced into *E. coli* via other processes, requiring certain cell-to-cell interaction.

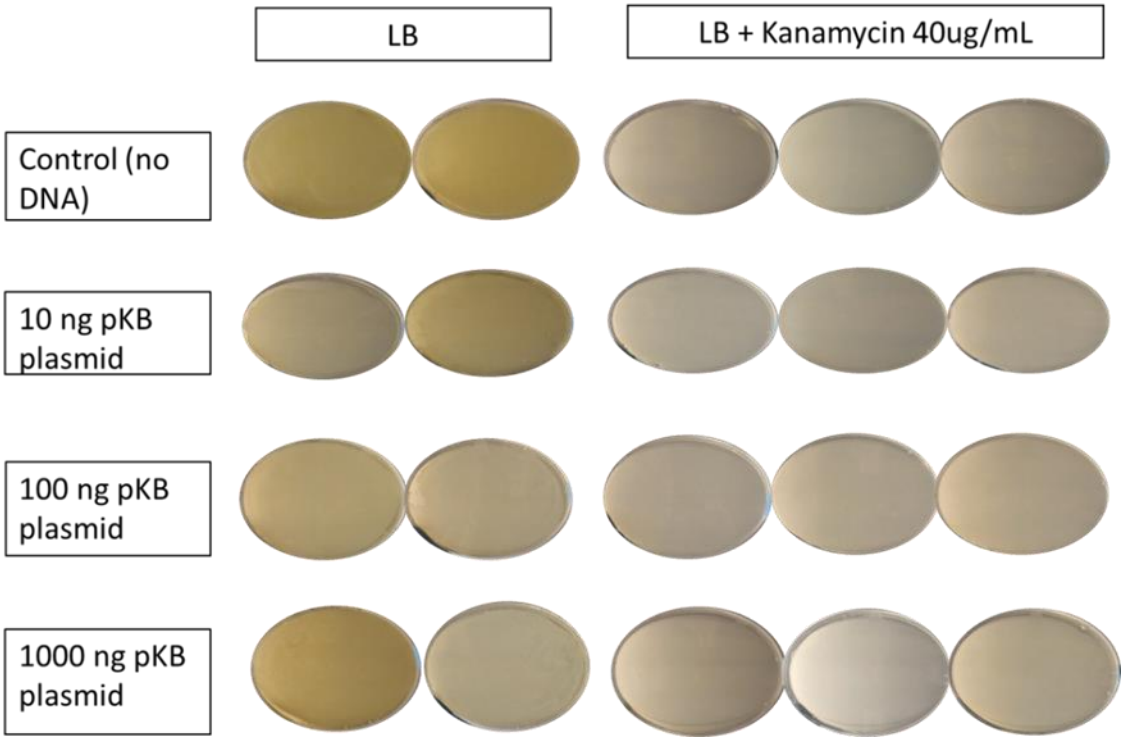

Fig. 14. Plating results of *E. coli* DH5α's ability to uptake free floating DNA. Known amounts of pKB plasmid (0, 10ng, 100ng, 1000ng) were incubated with wild-type *E. coli* DH5α for 2 days in 30 mL liquid medium (15 mL $BG11_0SA$ + 15 mL LB). Cells were then plated onto agar LB control plates and LB plates with kanamycin. No *E. coli* colonies were detected on agar LB plates while *E. coli* cells grew well on LB control plates (lawn formation). The results suggest that wild-type *E. coli* DH5α cannot take up DNA naturally from an outside aqueous environment.

CONCLUSION

Results from this study showed that GE *T. elongatus* BP1 cells were able to horizontally transfer kanamycin resistant gene into wild-type *E. coli* DH5α within 2 days of co-culturing in a mixed media of LB and BG110-SA medium. The control study showed that *E. coli* DH5α cells

were not able to uptake free floating pKB plasmid in the media. *E. coli* DH5α cells were only able to obtain kanamycin resistant gene when there were GE *T. elongatus* BP1 cells in the medium. In addition, results from this study also suggested that *E. coli* DH5α might have integrated the kanamycin resistant gene into their genomic DNA despite being recA mutant. However, further work still needs to be done to confirm. Whole genome sequencing might be needed into other to have a complete look of the genomic DNA of the *E. coli* DHα cells after co-cultured with GE *T. elongatus* BP1 to determine the insertion of kanamycin resistant gene.

CHAPTER 3

GENETICALLY ENGINEERING *THERMOSYNECHOCOCCUS ELONGATUS* BP1 FOR ISOBUTANOL PRODUCTION

INTRODUCTION

While *T. elongatus* BP1 is a thermophilic strain of cyanobacteria, it has the ability to tolerate a wide range of temperatures from 30°C - 60°C even though cells can survive but cannot proliferate at 30°C (Onai et al., 2004b). This makes *T. elongatus* BP1 a promising host for biofuel production as they can be grown where there is a fluctuation in temperature. In addition, since the cells cannot survive below 30°C, using *T. elongatus* BP1 for biofuel production will provide an innate biosafety mechanism such that if these thermophilic cells escape the cultivating environment into a cooler outside environment, they would not be able to proliferate and survive which would minimize the risk of horizontal gene transfer as well as the potential of disruption the surrounding ecology.

In this chapter, the author seeks to genetically engineer *T. elongatus* BP1 and study biofuel production from this thermophilic strain of cyanobacteria. This would allow the production of biofuel for areas that have higher temperatures in general. In addition, elevated temperature required for the growth of *T. elongatus* BP1 would also eliminate mesophilic bacteria contamination in the cultivating cultures.

MATERIALS AND METHODS

Strain and culture conditions

T. elongatus BP1 cells were grown under continuous lighting using florescent lamps in a growing chamber at 45°C. BG11 is used as the growing medium. BG11 medium was supplemented with kanamycin at a concentration of 40 µg/ml for cell selection. For the expression of the alcohol dehydrogenase (yqhD) gene, the medium is supplemented with 300mg/L isobutyraldehyde while 10 g/L of ketosiovalerate (2-KIV) and 50 mg/L of thiamine were supplemented in cell cultures for the expression of the ketoisovalerate decarboxylase (KIVD) gene. In addition, 1000 µM IPTG and 50 mM $NaHCO_3$ were added to the medium for both gene expression studies. These cultures were then placed in shaking incubators at 30°C or 42°C for expression study.

Designing of cassette

pB-BP1KY cassette is adapted and modified from a previously published study with *Synechococcus elongatus* PCC 7942 (Atsumi et al., 2009). CLC Genomic Workbench 12 software was used to design the cassette. pB-BP1KY cassette contains an inducible promoter lacIq-Ptrc, a keto-acid decarboxylase gene (NCBI_ CP006766.1) from *Lactococcus lactis* subsp. lactis KLDS4.0325, and an alcohol dehydrogenase yqhD gene (NCBI_ 947493) from *E. coli* MG1655. In addition, a continuous promoter from the cpc gene of *T. elongatus* BP1 (2042749..2042864) and another continuous promoter from the slpA gene (HE611334) of *Thermus thermophilus* are placed upstream of a heat tolerant kanamycin resistant gene. This is to ensure that *T. elongatus* BP1 cells can properly express the antibiotic resistant property. A 120 bp rbcS' UTR terminator from *T. elongatus* BP1 (1573695..1573814) is used as the terminator

sequence for the cassette. For the cassette to integrate into the genomic DNA of T. elongatus BP1, the whole cassette is flanked by two homologous sequences of *T. elongatus* BP1, the upstream recombination site I (1926542..1927714) and the downstream recombination site II (1927715..1928706). The designed cassette is synthesized by GenScript and is cloned into a pUC57-Brick plasmid (Figure 15).

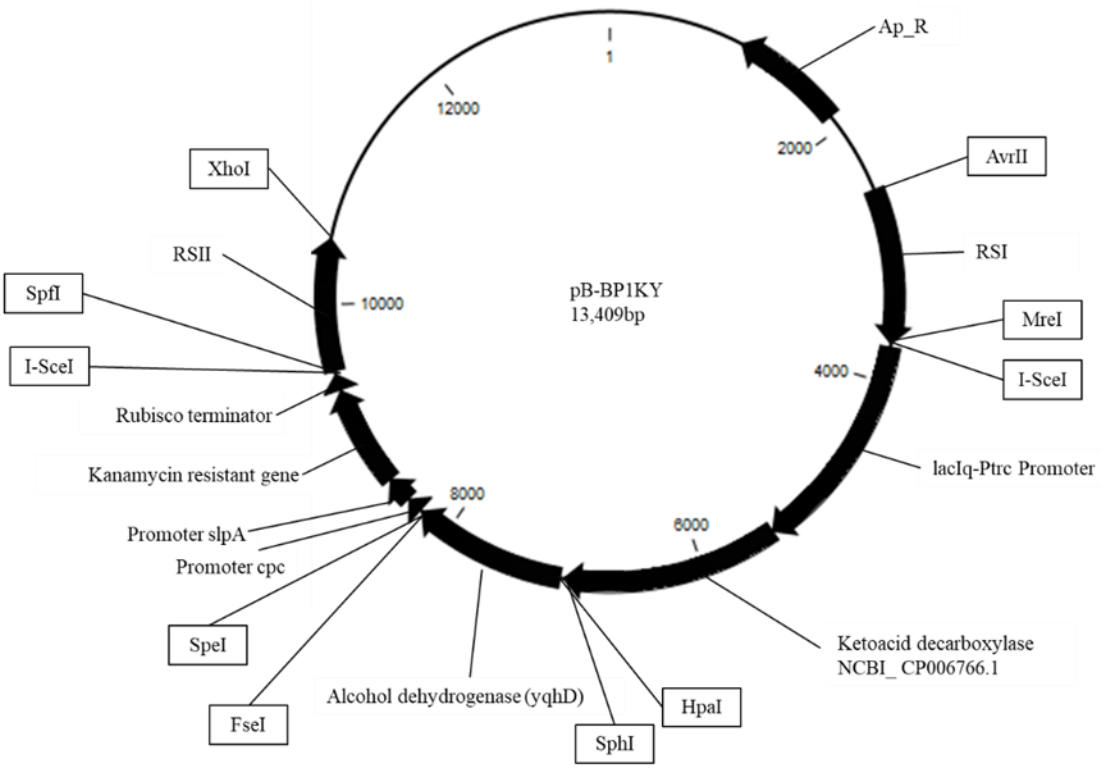

Fig. 15. Schematic of pB-BP1KY cassette within pUC57-Brick plasmid.

Transformation of *T. elongatus* BP1

After wild-type *T. elongatus* BP1 cells in BG11 medium were centrifuged and resuspended in water to the final volume of 400 µL with the final cell concentration of 10^9

cells/mL, 1 ug of pB-BP1KY plasmids is then added to the cell suspension. Transformation is done via electroporation using BioRad PowerPac Basic with the voltage of 5 kV/cm. Immediately after pulsing, *T. elongatus* BP1 cells are transferred into a flask containing 5 mL of prewarmed BG11 medium (45°C). The flask is then incubated for 24 hours in the growing chamber at 45°C without shaking. After 24 hour of incubation, *T. elongatus* BP1 cells carrying the cassette are selected by plating on BG11 agar plates containing kanamycin at the concentration of 40 µg/mL. Detection of the pB-BP1KY cassette within the cells is done via PCR using primers CRSI_fwd (TTGGCCGGCCTAACGACTATGACTACAGTTCTG) and BP1_tll1856_rev (CCTCTGCGACACCTACTACATCCAC).

Reverse transcription

RNAprotect Bacteria Reagent and the RNeasy Mini Kit was used for total RNA extraction. 1 mL of wild-type *T. elongatus* BP1 or *T. elongatus* BP1 transformants (BP1-99C) is added into 2 mL of RNAprotect Bacteria Reagent. The following steps were done according to the RNeasy Mini Kit manufacturer's quick-start protocol. For the final elution step, RNA was eluted using DEPC treated water. To remove genomic DNA contamination, the extracted RNA was then treated with DNase I amplification grade from Invitrogen. The RNA was treated at 37°C for 30 minutes and then the reaction was inactivated with 25 mM EDTA at 65°C for 10 minutes. cDNA synthesis was carried out using High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems. In addition, the treated RNA is also used as the template for PCR analysis as the negative control (- RT PCR) to ensure that there is no leftover contamination of the genomic DNA. Primers used for cDNA synthesis are rpsL_rev, Kivd0352_I_rev, yqhD_rev,

yqhD_I_rev. This is followed by PCR analysis using the synthesized cDNA as the template. The rpsL, 30S ribosomal protein is used as the housekeeping gene.

Extraction and quantification of isobutanol

To test the expression of the yqhD alcohol dehydrogenase gene, wild-type and GE *T. elongatus* BP1 cultures were grown to reach the OD730 of 1.0 and then supplemented with 300 mg/L isobutyraldehyde and cell cultures were grown for 24 hours with shaking. After 24 hours, 9 mL supernatant of the centrifuged cell culture, wild-type *T. elongatus* BP1 or BP1-99C, was added into 3 mL of DCM. For the KIVD gene expression study, cultures were grown to reach the OD730 of 1.5, then 10 g/L of 2-KIV and thiamine (10 or 50 mg/L) were added into the cultures and cultures were grown for 4 days. After 4 days, 3 mL of BP1-99C is centrifuged and the supernatant is collected then added into 3 mL of dichloromethane (DCM). All the supernatant/DCM mixtures were vortexed at high speed for 2 minutes. To help with layer separation, after vortexing, the mixture is centrifuged at 5000 x g for 10 mins. Finally, the bottom layer is transferred into a GC-MS autosampler vial which is then analyzed using a Shimadzu GCMS-QP2010 SE with a DB5-MS column. The initial GC oven temperature of 50°C is held for 2 minutes which is then increased to the final temperature of 200°C at the rate of 30°C/ min. The final temperature of 200°C is held for 3 minutes. Isobutanol detection was done using scan mode for cultures supplemented with isobutyraldehyde. For cultures supplemented with 2-KIV, selected ion monitoring (SIM) mode was used since these cultures showed lower isobutanol production and SIM mode was more sensitive compared to scan mode. The first isobutanol standard set included 15.6, 31.2, 62.5, 125, 250 mg/mL and the second isobutanol

standard set included 0.75, 1.5, 3.1, 6.2, 12, 25 mg/mL were also prepared for the scan mode and SIM mode, respectively.

RESULTS AND DISCUSSION

Transformation of *T. elongatus* BP1

After 10 – 14 days of the transformed *T. elongatus* BP1 plating, green colonies are observed on plates containing kanamycin (Figure 16). In order to verify the presence of the pB-BP1KY cassette within the cells, selected colonies are screened by colony PCR. The results from colony PCR showed the presence of the 8.0 kb band from gel electrophoresis. This indicates that the pB-BP1KY cassette has successfully integrated into *T. elongatus* BP1 cells. Wild-type *T. elongatus* BP1 cells were used as the negative control did not show the same 8.0 kb band but only showed the 2.3 kb band indicating the wild-type genomic DNA without pB-BP1KY integration (Figures 17 – 18).

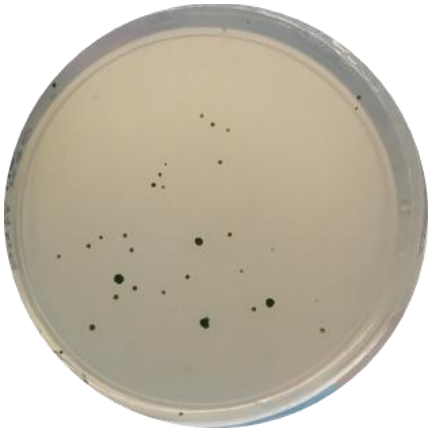

Fig. 16. Green colonies of pB-BP1KY transformed T. elongatus BP1 cells on an agar plate containing kanamycin.

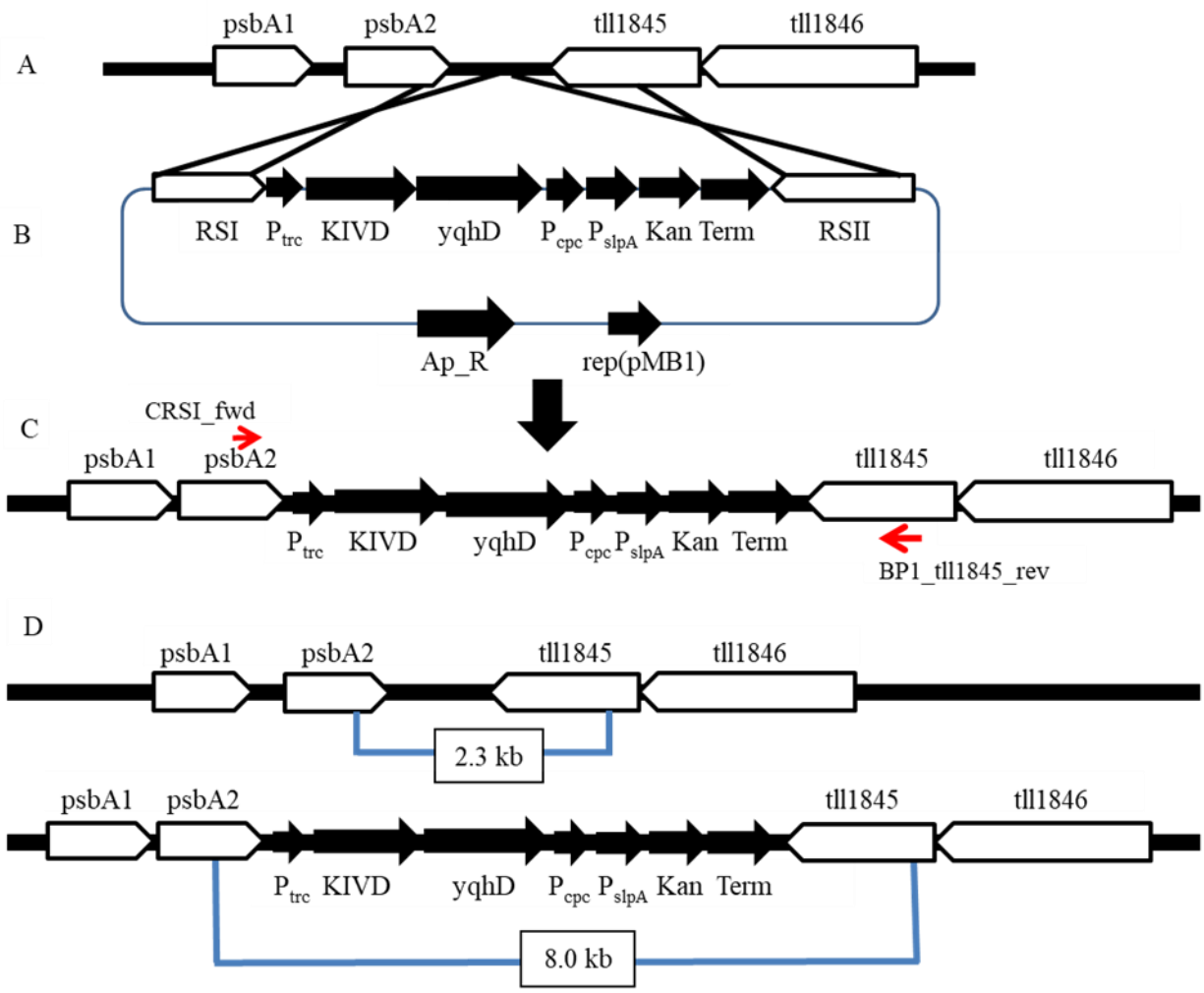

Fig. 17. Schematic of cloning the pB-BP1KY cassettes into the genomic DNA of T. elongatus BP1. (A) – genomic DNA of wild-type *T. elongatus* BP1. (B) – pB-BP1KY plasmid. (C) – integration of pB-BP1KY plasmid at the desired location. (D) – Schematic for the expected PCR results with the 2.3 kb band shows the non-integration and the 8.0 kb band shows the integration of pB-BP1KY plasmid into the gDNA of *T. elongatus* BP1.

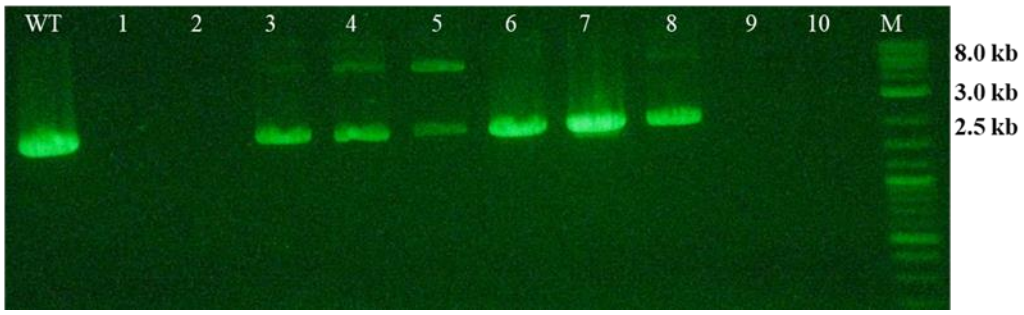

Fig. 18. PCR results from the selected pB-BP1KY *T. elongatus* BP1 transformant colonies from the BG11 plates with kanamycin. The wild-type (WT) *T. elongatus* BP1 control shows the band at 2.3 kb while some of the selected colonies show the band at 8.0 kb indicating the integration of pB-BP1KY construct in their gDNA. (M) – DNA marker, (WT) – wild-type cells, (1-10) selected green colonies of *T. elongatus* BP1.

Reverse transcription PCR (RT – PCR) analysis

In order to study the transcription of the KIVD and yqhD genes within the GE *T. elongatus* BP1 cells, reverse transcription PCR using the extracted RNA were carried out. After being treated with Dnase I to eliminate possible genomic DNA contamination, RNA was used for cDNA synthesis which was then used as the templates for PCR. The results of the reverse transcription PCR showed the expression of both KIVD and yqhD genes of GE *T. elongatus* BP1 with the presence of all four bands at the expected sizes of 0.4, 0.38, 1.01, and 0.49 kb (Figure 19, table 1). These bands represent the housekeeping gene, the KIVD gene, and two different fragments of the yqhD gene, respectively. The same PCR was also carried out for the wild-type *T. elongatus* BP1 cells. For the wild type, there was only one band at 0.4 kb which was the housekeeping gene used to ensure that the steps were carried out successfully. In addition, to ensure that cDNA was indeed the PCR template and not from genomic DNA contamination, a

negative control PCR was also carried out using the Dnase I treated RNA. The results showed no band presence which indicated that the RNA samples were cleaned from genomic DNA residues (Figure 19).

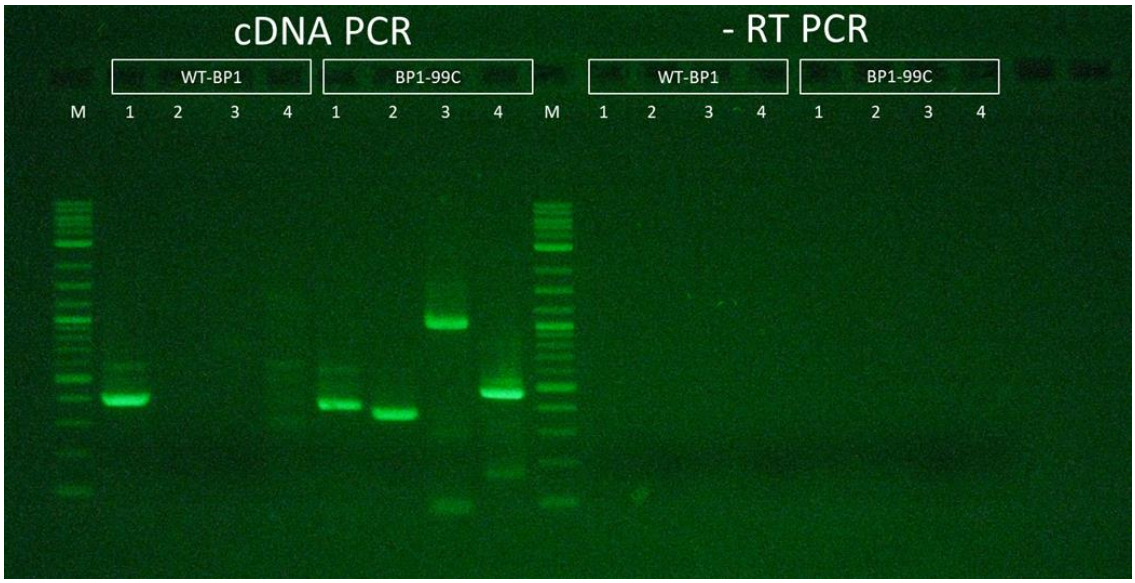

Fig. 19. Reverse transcription analysis of KIVD and yqhD gene expression by GE *T. elongatus* BP1 cells. The negative control PCR (-RT PCR) does not have any band indicating that there is no genomic DNA contamination in the RNA samples. The PCR results from complimentary DNA (cDNA PCR) show that there was only one positive result for wild-type *T. elongatus* BP1 sample indicating the housekeeping gene rpsL (1). For the GE *T. elongatus* BP1 cells, BP1-99C, all the expected bands are seen which include the housekeeping gene rpsL (1), the KIVD gene (2), and the yqhD gene (3,4).

Table 1. Primers used for reverse transcription study along with the expected band for each PCR.

PCR	Forward primer	Reverse primer	Expected band
1	rpsL_fwd	rpsL_rev	0.40 kb
2	Kivd_I_fwd	Kivd_I_rev	0.38 kb
3	yqhD_fwd	yqhD_rev	1.01 kb
4	yqhD_I_fwd	yqhD_I_rev	0.49 kb

Quantification of isobutanol production from GE *T. elongatus* BP1

The isobutanol production from GE *T. elongatus* BP1 was carried out at 30°C and 42°C as the yqhD gene was from *E. coli* and the KIVD gene was from a *Lactococcus lactis* which are both mesophiles but have been shown to be stable at 42°C (Lin et al., 2014). In addition, while *T. elongatus* BP1 is a thermophilic strain of cyanobacteria with the optimal growth temperature at 55 - 57°C, they can tolerate a wide range of temperature from 30 - 60°C. Therefore, these two temperatures were selected as they would provide a good baseline to access the isobutanol productivity without causing damages to the genes due to high temperature (> 45°C) or harming *T. elongatus BP1* cells with low temperature (< 30°C). In order to increase the activity of KIVD gene, additional thiamine was added into the cultures to provide enough co-factor for KIVD gene which would enhance the production of the KIVD gene product, isobutyraldehyde, which was then converted into isobutanol.

For the yqhD gene expression study, *T. elongatus* BP1 cultures were supplemented with 300 mg/L isobutyraldehyde and isobutanol detection was carried out using GC-MS scan mode. Based on the isobutanol standards, the isobutanol peak was identified as to be the one with retention time of 2.116 (Figure 20). Since the negative control standard, DCM only without isobutanol showed a small peak with the same retention time of 2.116, which was shown to be not isobutanol when compared against the NIST database, all the spectra were corrected by subtracting the DCM background from the spectra. This peak could be due to impurity present in the DCM. For wild-type *T. elongatus* BP1 cultured at 30°C, while there was a small peak with Rt of 2.116, NIST database search did not show any isobutanol match which was the same with the DCM impurity background peak. Therefore, it is safe to assumed that no isobutanol was produced by wild-type *T. elongatus* BP1 at 30°C. For the GE *T. elongatus* BP1, BP1-99C, there was a significant increasing in intensity for the Rt 2.116 peak which was identified as isobutanol peak via matching with NIST database. This indicate that the yqhD gene was expressed by GE *T. elongatus* BP1 cells at 30°C and the cells were able to produce isobutanol from the supplemented isobutyraldehyde. For the 42°C samples, both the wild-type *T. elongatus* BP1 and GE *T. elongatus* BP1-99C showed strong Rt 2.116 peaks (Figure 21). This indicated that at higher temperature, wild-type *T. elongatus* BP1 cells were more active and the endogenous alcohol dehydrogenase gene naturally existing within the cells were able to convert isobutyraldehyde to isobutanol. While there was production of isobutanol from wild-type *T. elongatus* BP1 cells, isobutanol production from GE *T. elongatus* BP1-99C was about 30% higher compared to wild-type. This suggested that the presence of the extra alcohol dehydrogenase yqhD gene enhanced productivity. It is also important to note that the Rt 2.116 peak from GE *T. elongatus* BP1-99C at 42°C was less intense compared to GE *T. elongatus* BP1-99C at 30°C. Further investigation is

needed to see whether it was due to isobutanol evaporation at higher temperature or if the yqhD gene started to lose some activity. Isobutanol productivity was calculated via peak integration of the Rt 2.116 peak. The integrated areas were also corrected by subtracting the area of the same impurity peak from the DCM control standard to remove the background peak. Overall, based on the calibration curve, the isobutanol produced from these samples were 95 mg/mL for GE *T. elongatus* BP1-99C at 30°C, 25 mg/mL for wild-type *T. elongatus* BP1 at 42°C, and 74 mg/mL GE *T. elongatus* BP1-99C at 42°C (Figure 22).

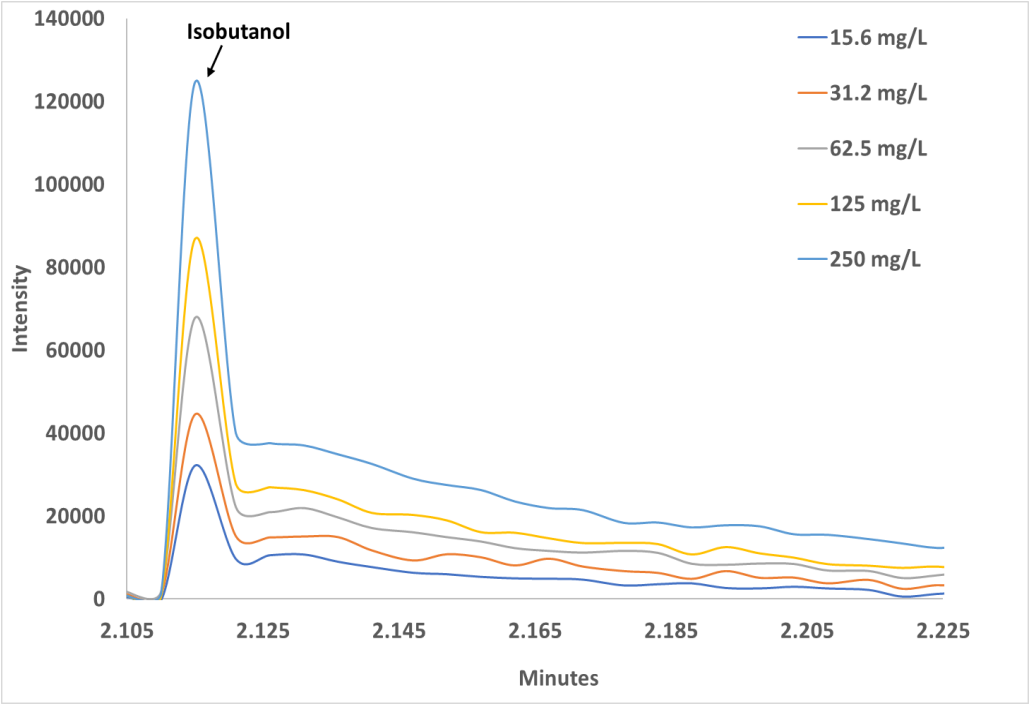

Fig. 20. GC-MS results for isobutanol standards at different concentrations using scan mode. The increasing intensity of the peak with Rt of 2.114 reflects the increasing concentration of isobutanol in the standards.

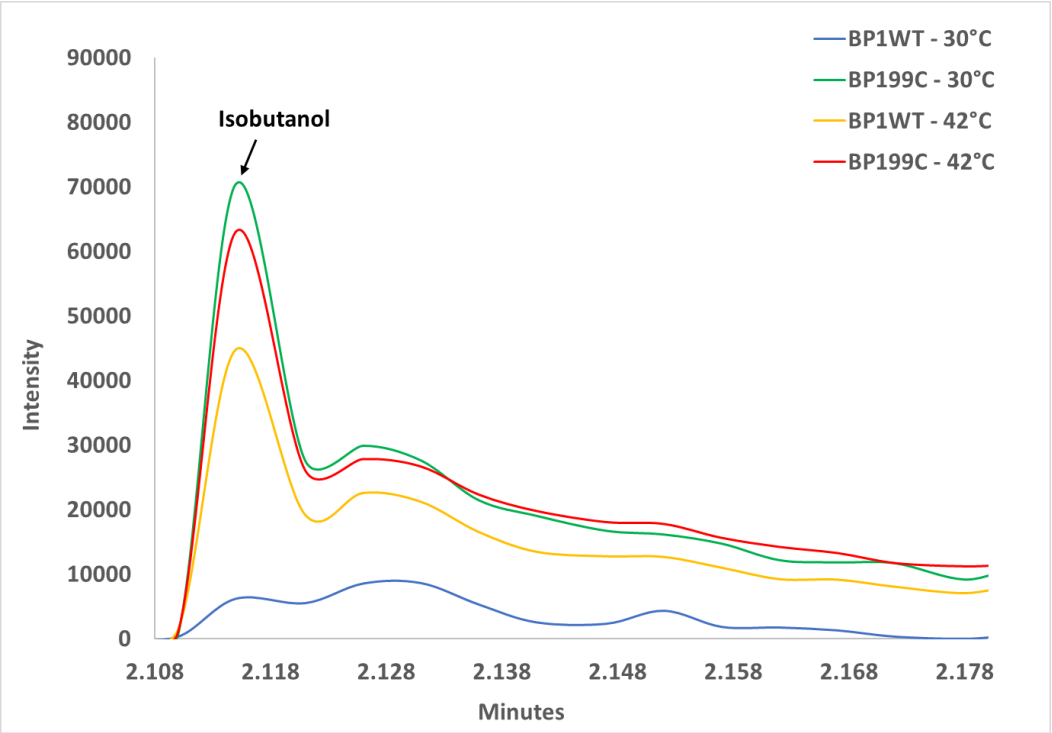

Fig. 21. GC-MS results of isobutanol production from cultures supplemented with isobutyraldehyde at 30 and 42°C. BP1WT – Wild-type *T. elongatus* BP1, BP199C – GE *T. elongatus BP1*.

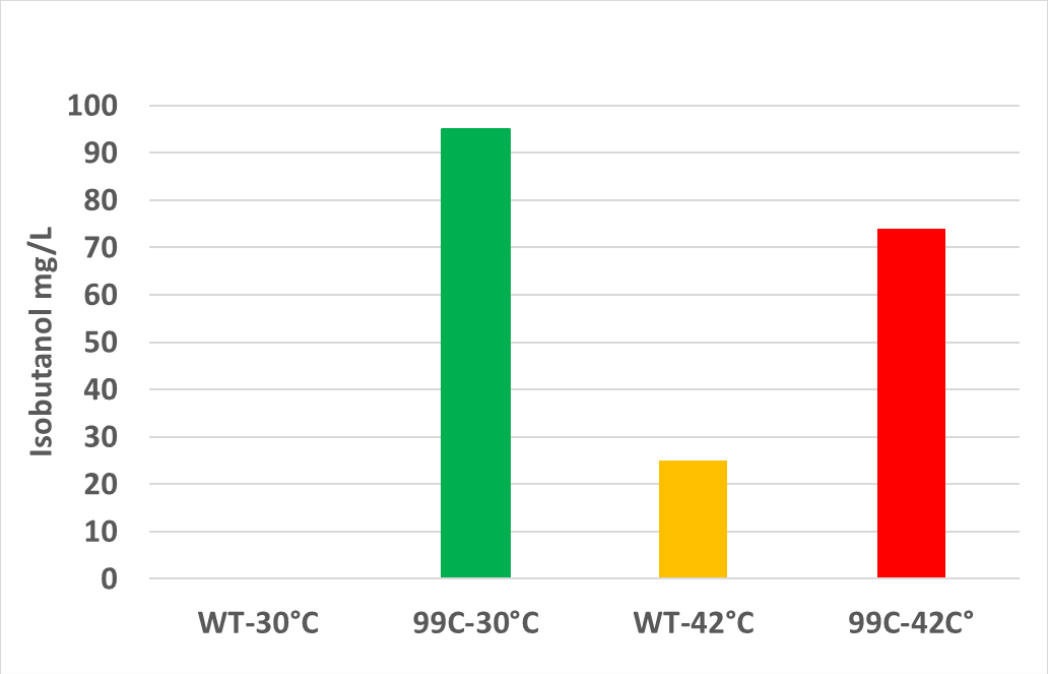

Fig. 22. Quantification of isobutanol produced from *T. elongatus* BP1 cells supplemented with 300 mg/L isobutyraldehyde at 30 and 42°C. BP1WT – Wild-type *T. elongatus* BP1, BP199C – GE *T. elongatus* BP1.

For the KIVD study, two different concentrations of thiamine used were 10 and 50 mg/L. As mentioned earlier, SIM mode was used for this study since the isobutanol production level was low and SIM mode was more sensitive. The low isobutanol productivity from cultures supplemented with 2-KIV could be due to the presence of other competitive pathways. 2-KIV is also a substrate used in other metabolic pathways such as L-valine and L-leucin synthesis. Therefore, it was expected that not all of the supplemented 2-KIV would be used by the KIVD gene for the conversion into isobutyraldehyde which would be then converted into isobutanol by the yqhD gene. The standards identified the isobutanol peak to be the one with the retention time of 2.114 which increases with higher concentration of isobutanol. GC-MS results using selected

ion monitoring showed that only the GE *T. elongatus* BP1 culture supplemented with higher thiamine concentration (50 mg/L) had a significant increase in the Rt 2.114 peak. These results showed that with 50 mg/L thiamine supplemented, GE *T. elongatus* BP1 cells were able to produce isobutanol from 2-KIV. This could be explained by the fact that thiamine is a co-factor of the KIVD gene. With an increasing in thiamine concentration, the activity of the KIVD gene was enhanced and compete with other pathways. Therefore, more of the 2-KIV was converted into isobutyraldehyde. With the low concentration of thiamine (10 mg/L), the activity of the KIVD gene might have been not enough to compete with other naturally exist pathways in the cells. Based on the calibration curve, the estimated amount of isobutanol produced from this culture was 13 mg/L (Figures 23 – 25). Since the DCM control did not have the same Rt 2.114 peak, this data set was not corrected with DCM background subtraction.

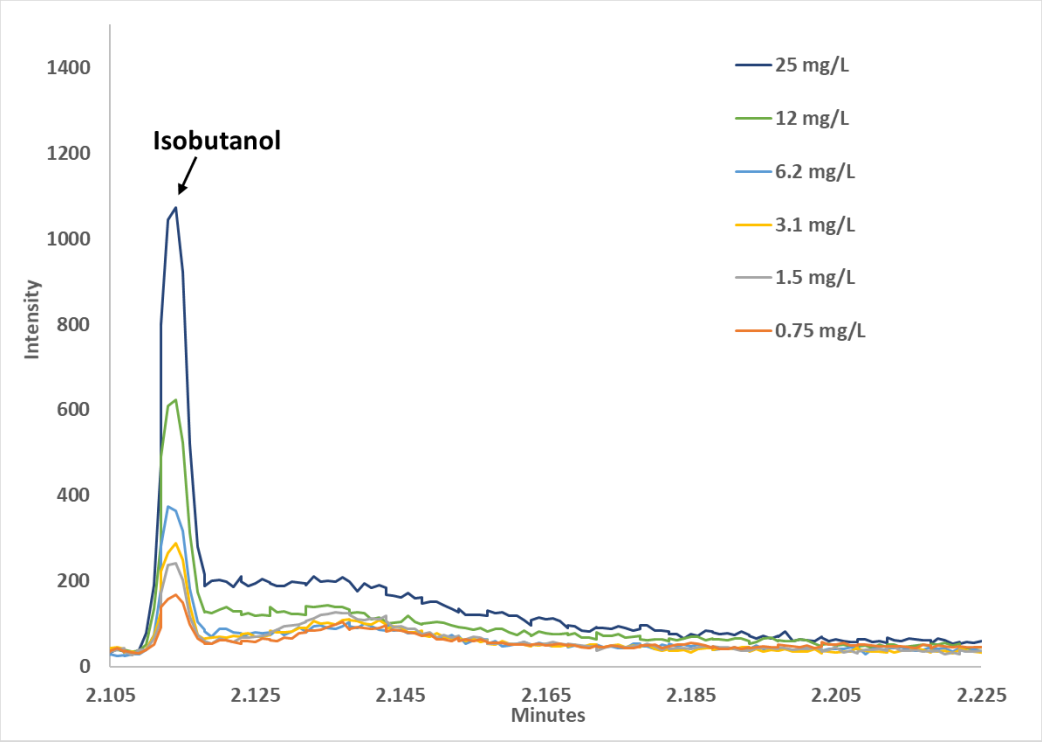

Fig. 23. GC-MS results for isobutanol standards at different concentrations using SIM mode. The increasing intensity of the peak with Rt of 2.114 reflected the increasing concentration of isobutanol in the sample.

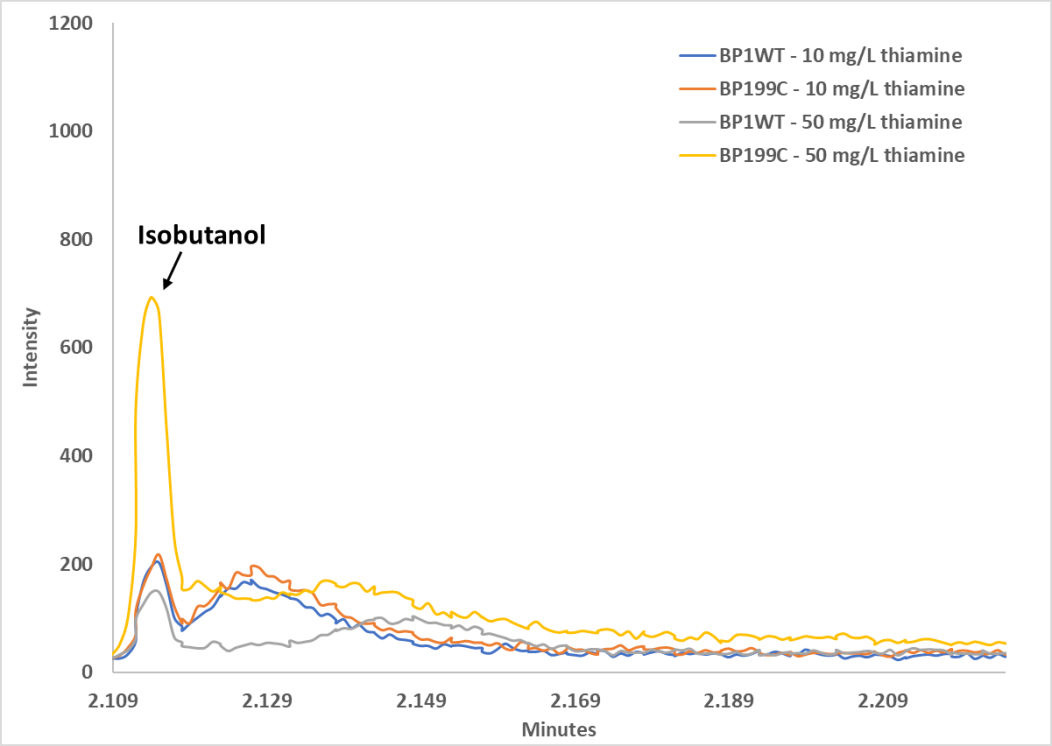

Fig. 24. GC-MS results for isobutanol detection in different cultures of GE *T. elongatus* BP1 supplemented with 2-KIV and thiamine. Sample with high thiamine supplement showed a significant increase in the peak with Rt of 2.114 indicating the presence of isobutanol in the sample. (WTBP1) – wild-*type T. elongatus* BP1, (BP1-99C) – GE *T. elongatus* BP1 carrying pB-BP1KY construct.

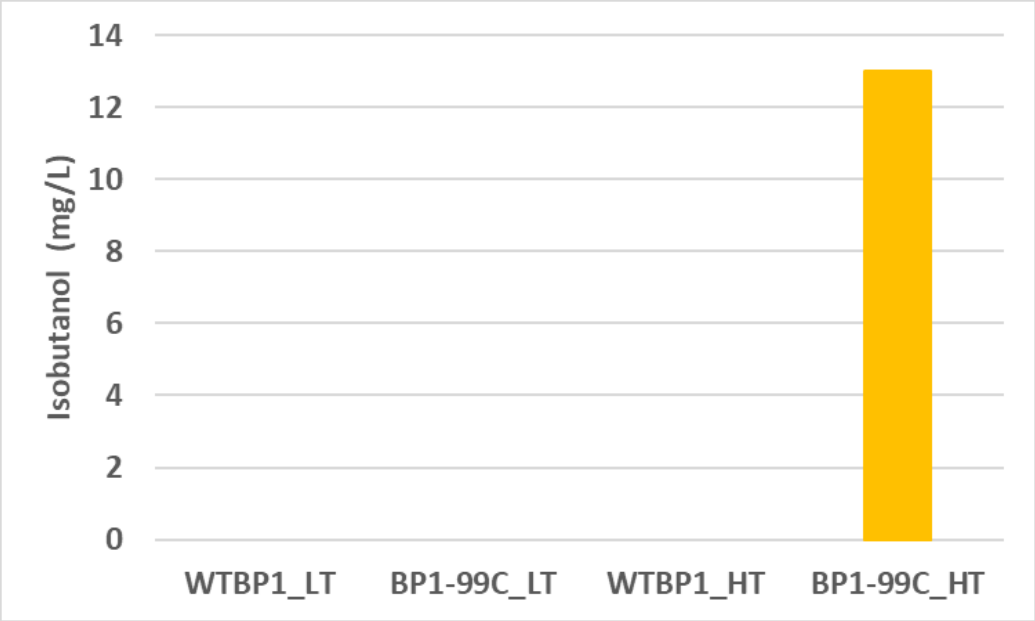

Fig. 25. Quantification of isobutanol produced from *T. elongatus* BP1 cells supplemented with 2-KIV and thiamine. GE *T. elongatus* BP1 culture with high thiamine supplement showed a production rate of 13 mg/L of isobutanol over a 4-day period. No isobutanol was detected for any other samples. (WTBP1) – wild-type *T. elongatus* BP1, (BP1-99C) – GE *T. elongatus* BP1 carrying pB-BP1KY construct, (LT) – low thiamine supplement of 10 mg/L, (HT)- high thiamine supplement of 50 mg/L.

CONCLUSION

Results from the reverse transcription study showed that GE *T. elongatus* BP1 cells carrying pB-BP1KY construct were able to express both KIVD and yqhD genes. When the cultures were supplemented with isobutyraldehyde, isobutanol was detected in GE *T. elongatus* BP1 cultures at both temperatures of 30°C and 42°C. For the wild-type *T. elongatus* BP1 cultures at 42°C, isobutanol was also detected when isobutyraldehyde was supplemented. However, the production of GE *T. elongatus* BP1 cultures at 42°C was about 3 times higher than wild-type *T.*

elongatus BP1. This indicated that the presence of the heterologous alcohol dehydrogenase yqhD gene led to an increase in isobutanol production in *T. elongatus* BP1. When the cultures were supplemented with 2-ketoisovalerate and 50 mg/L of thiamine at 30°C, isobutanol was detected in GE *T. elongatus* BP1 cultures but not wild-type *T. elongatus* BP1. This showed that GE *T. elongatus* BP1 cells can produce isobutanol at 30°C from both KIVD and yqhD gene. Unfortunately, due to the current COVID-19 pandemic, the 2-KIV has been backordered for an extended time. Therefore, isobutanol production of KIVD gene at 42°C could not be done within the time frame of this dissertation.

CHAPTER 4

STUDYING OF *THERMOSYNECHOCOCCUS ELONGATUS* BP1 ALCOHOL TOLERANCE

INTRODUCTION

While the results from the previous work showed that GE *T. elongatus* BP1 were able to produce isobutanol and it would be a potential host for biofuel production, it is important know the tolerance of *T. elongatus* BP1 toward different alcohol. This would determine whether *T. elongatus* BP1 can be used for production of other alcohols such as ethanol, isobutanol, and butanol on the industrial scale and be economically feasible. Currently, distillation has been used as a method for recovery bioethanol and to be economically feasible, the bioethanol contains need to be at least 40 g/L.

Therefore, in this chapter, wild-type *T. elongatus* BP1 and GE *T. elongatus* BP1 carrying pB-BP1KY construct are challenged to different concentrations of three representative alcohol which were ethanol, isobutanol, and 1-butanol to examine the effect of these alcohol on cell growth. This study was also carried out at 2 different temperatures 30°C and 42°C. If cell death occurred earlier in *T. elongatus* BP1 cultures with alcohols at 30°C compared to control cultures without alcohol supplemented, it would act as another layer of biosafety when thermophilic cyanobacteria used as biofuel production host.

MATERIALS AND METHODS

Wild-type and GE *T. elongatus* BP1 cells were grown in BG11 medium at 45°C in a growth chamber under continuous lighting provided by fluorescent lamps. Kanamycin (40

µg/mL) is supplemented for GE *T. elongatus* BP1 selection. The genetic modification process of the GE *T. elongatus* BP1 (BP1-99C) used in this study was described in detail in chapter III. The GE *T. elongatus* BP1 cells used in this chapter carried the pB-BP1KY cassette.

For the alcohol tolerance study, two sets of wild-type *T. elongatus* BP1 and GE *T. elongatus* BP1-99C cell cultures were used. One set was incubated at room temperature (25 - 30°C) facing a glass window for natural light. This was to mimic the uncontrolled environment that the cells face if they escaped containment. Under this condition, the natural light would be the light source for cell growth. The second set were incubated at 42°C under continuous lighting provided by fluorescent lamps (50 µmol m^{-2} s^{-1}) as the normal biofuel production condition. Ethanol, isobutanol, and 1-butanol were added on the first day of the experiment at the concentrations of 10, 20, and 40 g/L for each alcohol. Cell growth was also monitored using OD730 for 7 days.

RESULTS AND DISCUSSION

Alcohol tolerance study

For *T. elongatus* BP1 to be used as a potential host to produce different biofuels, cell cultures should be able to withstand a certain amount of alcohol that is economically reasonable when the production is scaled up to industrial level. Therefore, wild-type *T. elongatus* BP1 and GE *T. elongatus* BP1 cells were challenged to three representative alcohols ethanol, isobutanol, and 1-butanol at three different concentrations of 10, 20, and 40 g/L. The maximum concentration of 40 g/L alcohol was selected based on the knowledge that distillation, a preferred method of alcohol recovery, requires a minimal amount of 40 g/L (4 %wt) of alcohol to be economically feasible (Vane, 2008) . The other two lower concentrations were chosen as most

microorganisms have low tolerance to isobutanol and 1-butanol due to their toxicity (Gao et al., 2020). This study was carried out under two different culturing conditions. One set of cultures were carried out at 42°C with continuous lighting to replicate biofuel production environment where lighting and temperature would be controlled. The second set of cultures were incubated without continuous lighting but with natural light by placing in front of a glass window. In addition, the second set of cultures were also incubated at room temperature (25 – 30°C). This was to examine whether cell death would occur earlier as the cells were challenged to both cooler environment and the alcohol compared to cooler temperature only. In a previous study where wild-type and GE *T. elongatus* BP1 cells were grown in a greenhouse with temperature ranged from 15.44 – 25.30°C in the cool season and 31.42 – 36.27°C in the warm season, both wild-type and GE *T. elongatus* BP1 cells were not actively growing (Sacko et al., 2020). In this study, the cells were not only challenged with cooler temperature but also with the presence of alcohols.

The results showed that with the presence of ethanol, both wild-type and GE *T. elongatus* BP1 at 42°C were able to grow even with the highest amount of ethanol of 40 g/L. However, compared to the control cultures without any ethanol, the growth of both wild-type and GE *T. elongatus* BP1 cells decreased as the control culture OD730 was about 1.4 on the last day while the OD730 of the cultures with ethanol was about 1.2 (Figures 26 – 27). These results indicated that *T. elongatus* BP1 could be used for producing up to 40 g/L of ethanol. This met the preferred minimal amount for distillation and be economically beneficial. For the cells that were incubated at room temperature, OD730 remains relatively the same for 7 days indicated there was no cell growth (OD730 ~0.1). In addition, some cultures started to turn clear at day 3 indicating cell death (Figures 28 – 29). Cell death for cultures incubated at room tempt were most likely not due

to the presence of ethanol but only due to the cooler environment as one of the cultures turned clear early was the control wild-type *T. elongatus* BP1 which was not subjected to ethanol supplemented.

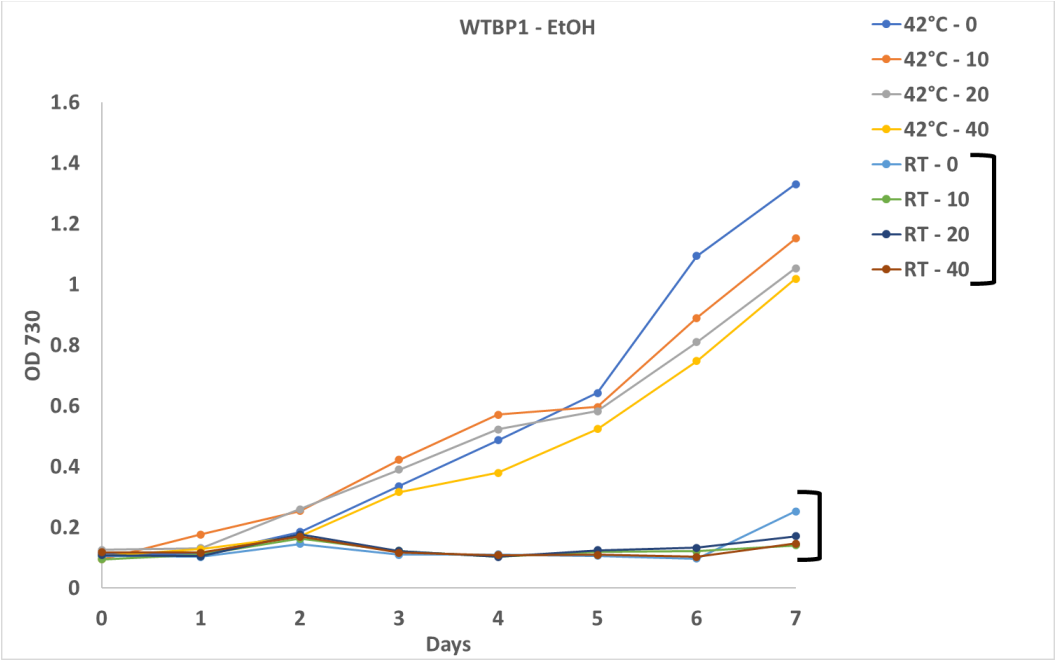

Fig. 26. Ethanol tolerance of wild-type *T. elongatus* BP1 at different concentrations. 42C – Cultures grown at 42°C. RT – Cultures grown at room temperature. 0, 10, 20, 40 – concentration in g/L.

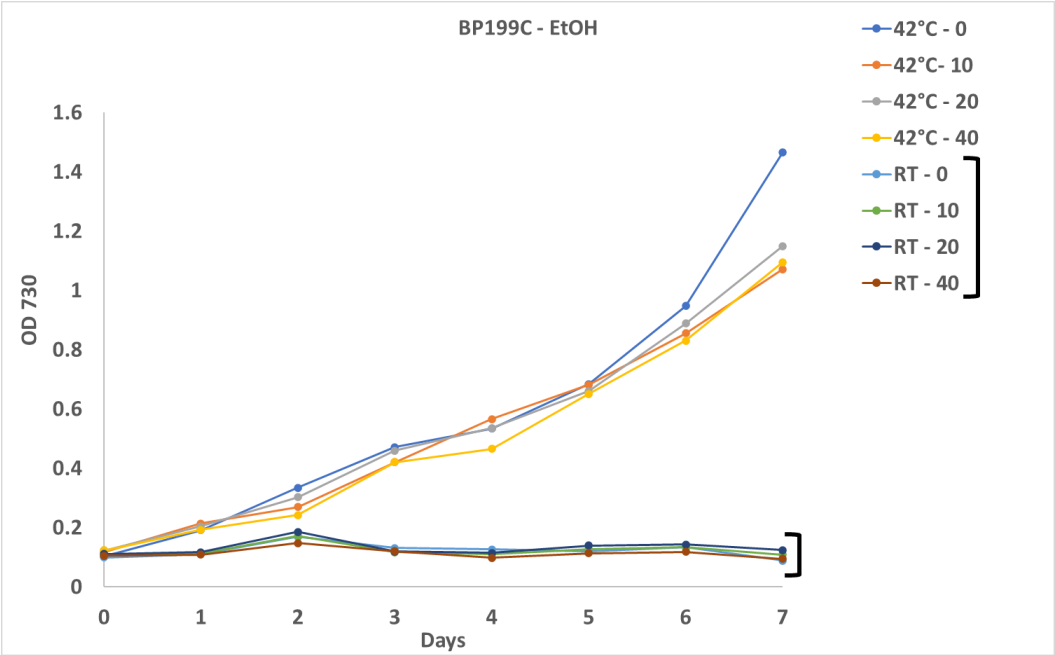

Fig. 27. Ethanol tolerance of GE *T. elongatus* BP1 at different concentrations. 42C – Cultures grown at 42°C. RT – Cultures grown at room temperature. 0, 10, 20, 40 – concentration in g/L.

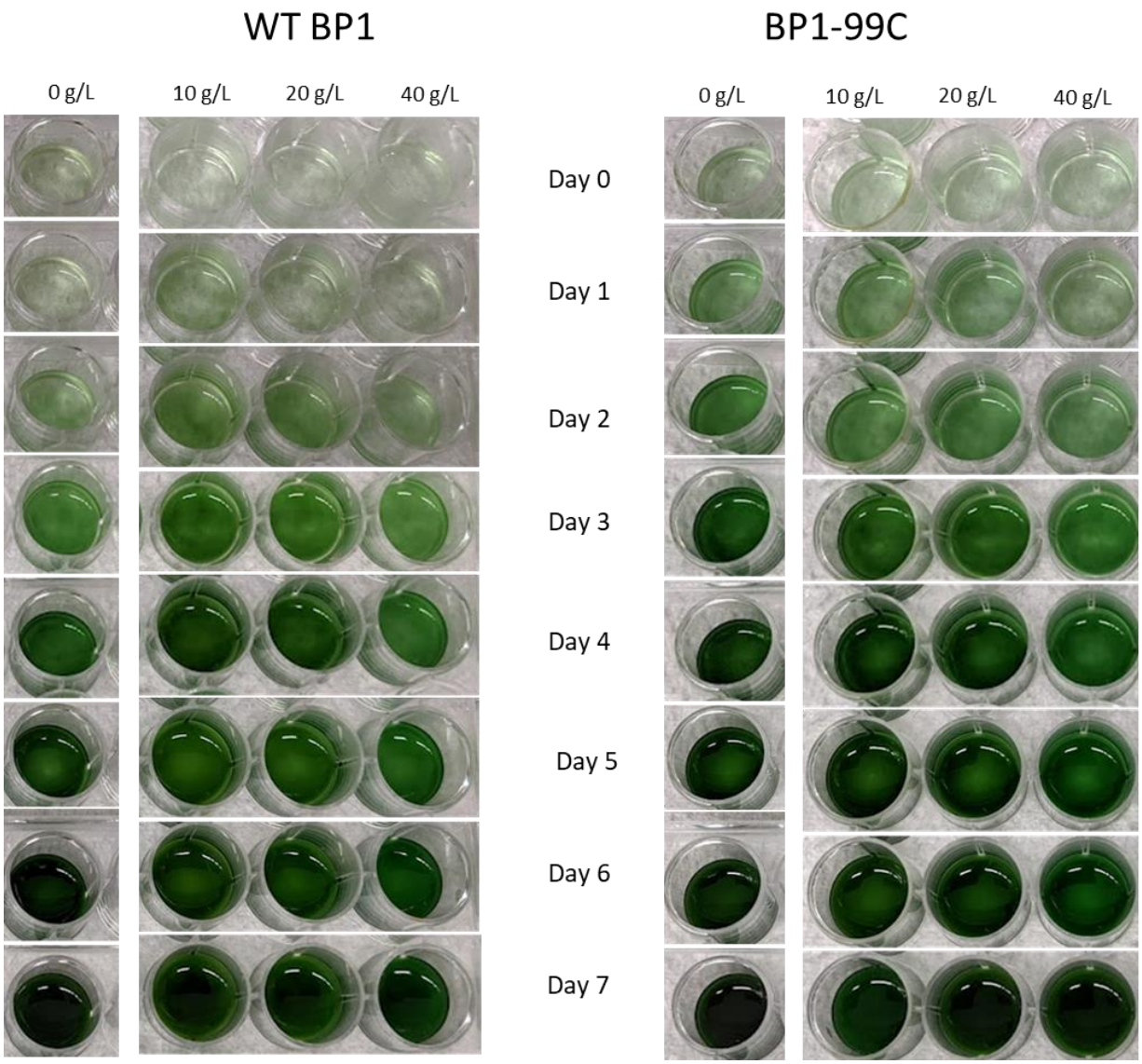

Fig. 28. Growth of wild-type and GE *T. elongatus* BP1 at 42°C with ethanol supplemented.

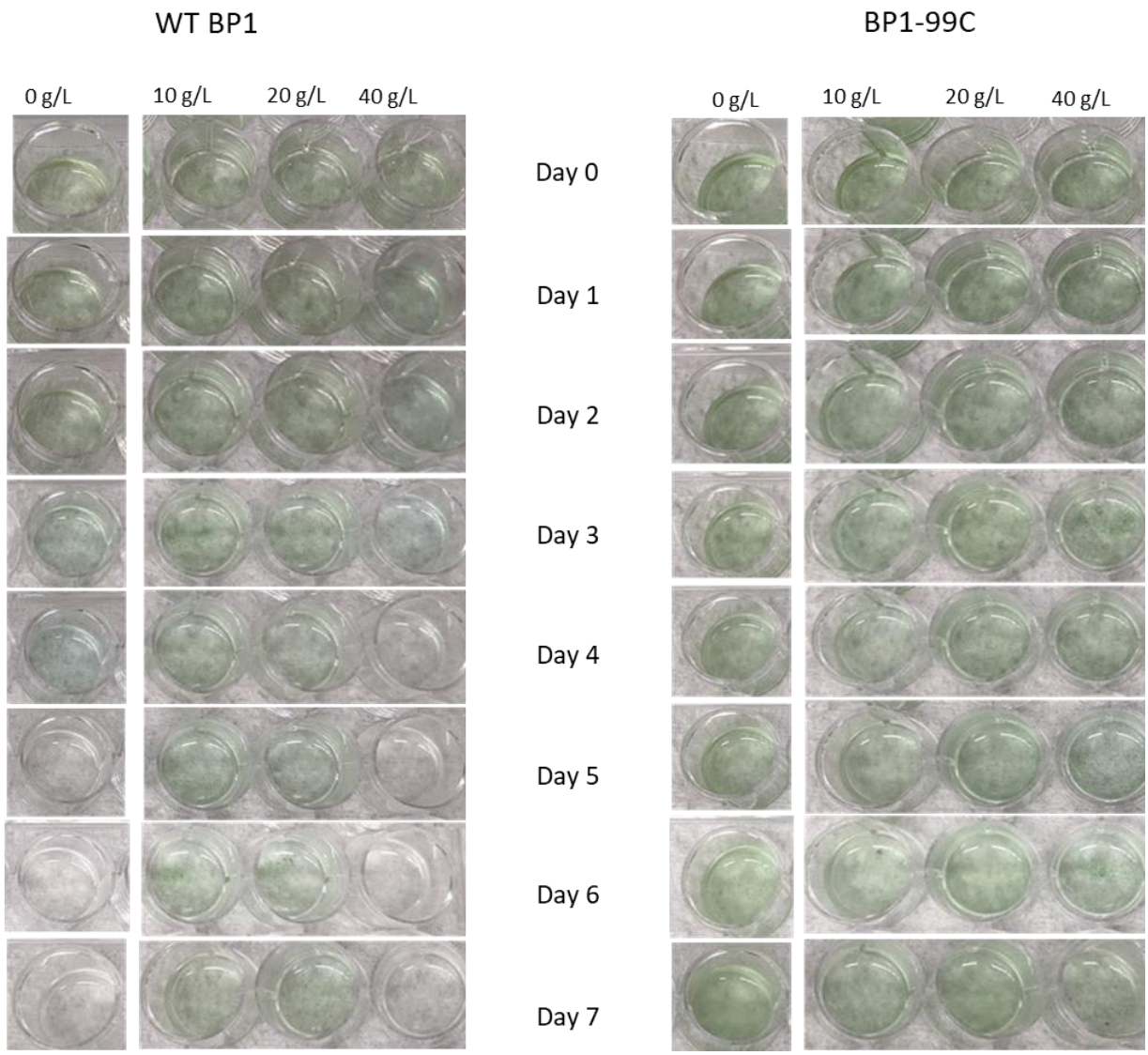

Fig. 29. Growth of wild-type and GE *T. elongatus* BP1 at RT with ethanol supplemented.

While all the *T. elongatus* BP1 cultures showed good tolerance to ethanol, the story was different when it came to isobutanol and 1-butanol. It is well known that isobutanol and 1-butanol are more toxic to microorganism and only a few strains of microorganisms can tolerance more than 2 % wt isobutanol or butanol (Kanno et al., 2013; Gao et al., 2020). This could be because longer chain alcohol can cause membrane damage and break the hydrogen bonds

between lipid tails (Huffer et al., 2011). This was found to be true for *T. elongatus* BP1 cells. When being challenged to isobutanol and 1-butanol, only cultures contained 10 g/L of isobutanol, or 1-butanol showed cell growth at 42°C while cell death occurred within 24 hours for cultures contained 20 and 40 g/L as they turned completely clear. However, it seemed that for the culture of GE *T. elongatus* BP1 with 20 g/L isobutanol, slight cell growth was observed on day 5 as the cultures started to turn green again. This could mean that isobutanol being less toxic than 1-butanol did not cause complete cell death in the cultures and an exceedingly small population of cells were able to recuperate after a few days.

For cultures supplemented with isobutanol and 1-butanol incubated at room temperature, the results were like ethanol supplemented ones that no cell growth was observed for any cultures. However, unlike isobutanol and 1-butanol cultures that were incubated at 42°C, cell death seemed to happen randomly for the same cultures at room temperature. If all cultures with higher isobutanol and 1-butanol content (20 and 40 g/L) turned clear within 24 hours at 42°C, some of the same cultures were able to sustain for a few more days at room temperature before turning completely clear (Figures 30 -37).

Since *T. elongatus* BP1 was able to grow at 10 g/L of all three alcohol, it would be interesting to compare the growth of the cells at this same alcohol concentration. Results showed that for all three alcohols, *T. elongatus* BP1 cells experienced slower growth rate compared to the control without any alcohol. This was true with both wild-type and GE *T. elongatus* BP1. However, for all three alcohols, the growth rate relatively the same. This suggested that the effect of 10 g/L of ethanol, isobutanol, and 1-butanol on *T. elongatus* BP1 cells were not distinctively different (Figures 38 – 39). Overall, results from this study suggested that *T. elongatus* BP1 could tolerate up to 40 g/L of ethanol and 10 g/L of isobutanol and 1- butanol. The tolerance

limit of *T. elongatus* BP1 for isobutanol and 1-butanol could be in the range of 10 – 20 g/L. In addition, while the presence of alcohols did not seem to change how the cells behave at cooler temperature, speed up or slow down cell death, this study still confirmed that *T. elongatus* BP1 cells at least have one layer of biosafety feature that is if the cells escaped high temperature cultivation environment to the cooler outside environment, these cells would not be able to survive. This would prevent the threat of GE *T. elongatus* BP1 compete with other wild-type organisms as well as lower the risk of GE *T. elongatus* BP1 transferring their genes to other microorganisms which could be potentially harmful especially if antibiotic resistant gene involved.

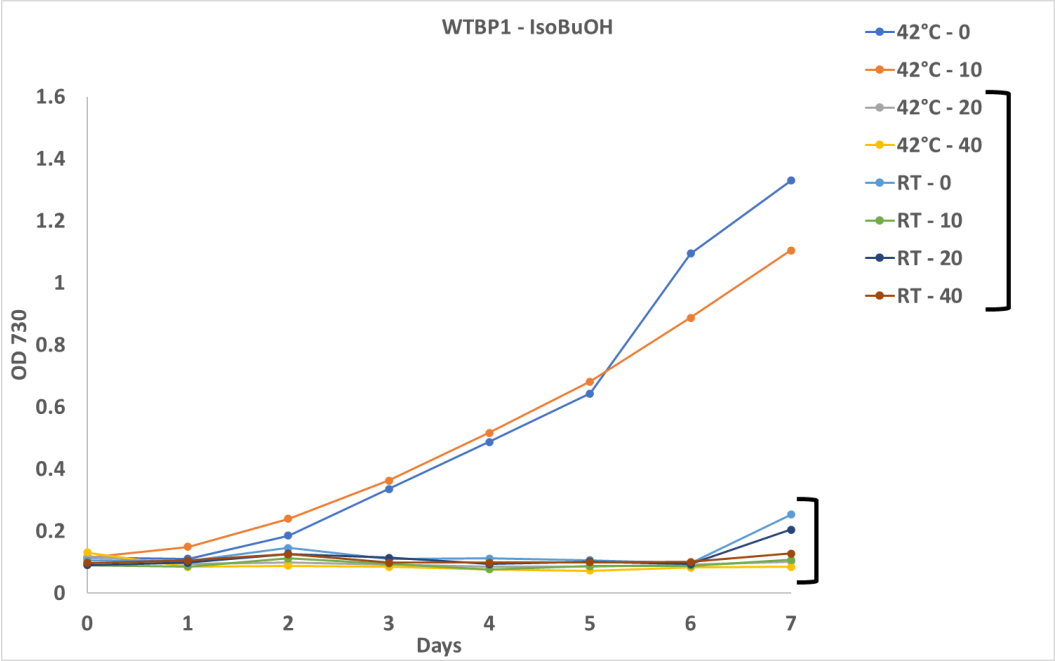

Fig. 30. Isobutanol tolerance of wild-type *T. elongatus* BP1 at different concentrations. 42C – Cultures grown at 42°C. RT – Cultures grown at room temperature. 0, 10, 20, 40 – concentration in g/L.

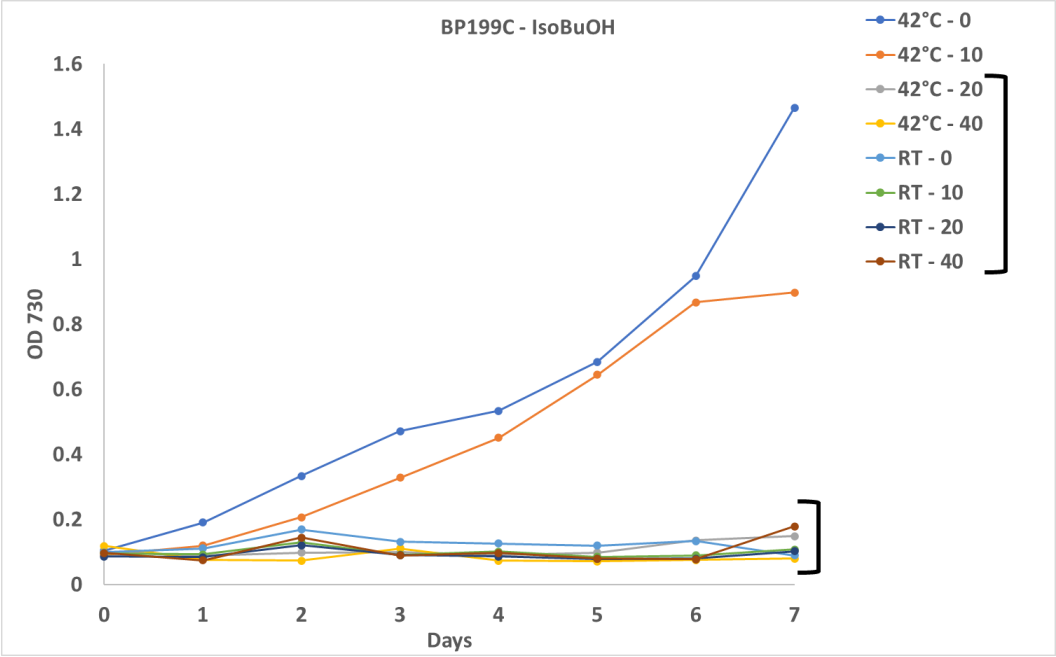

Fig. 31. Isobutanol tolerance of GE *T. elongatus* BP1 at different concentrations. 42C – Cultures grown at 42°C. RT – Cultures grown at room temperature. 0, 10, 20, 40 – concentration in g/L.

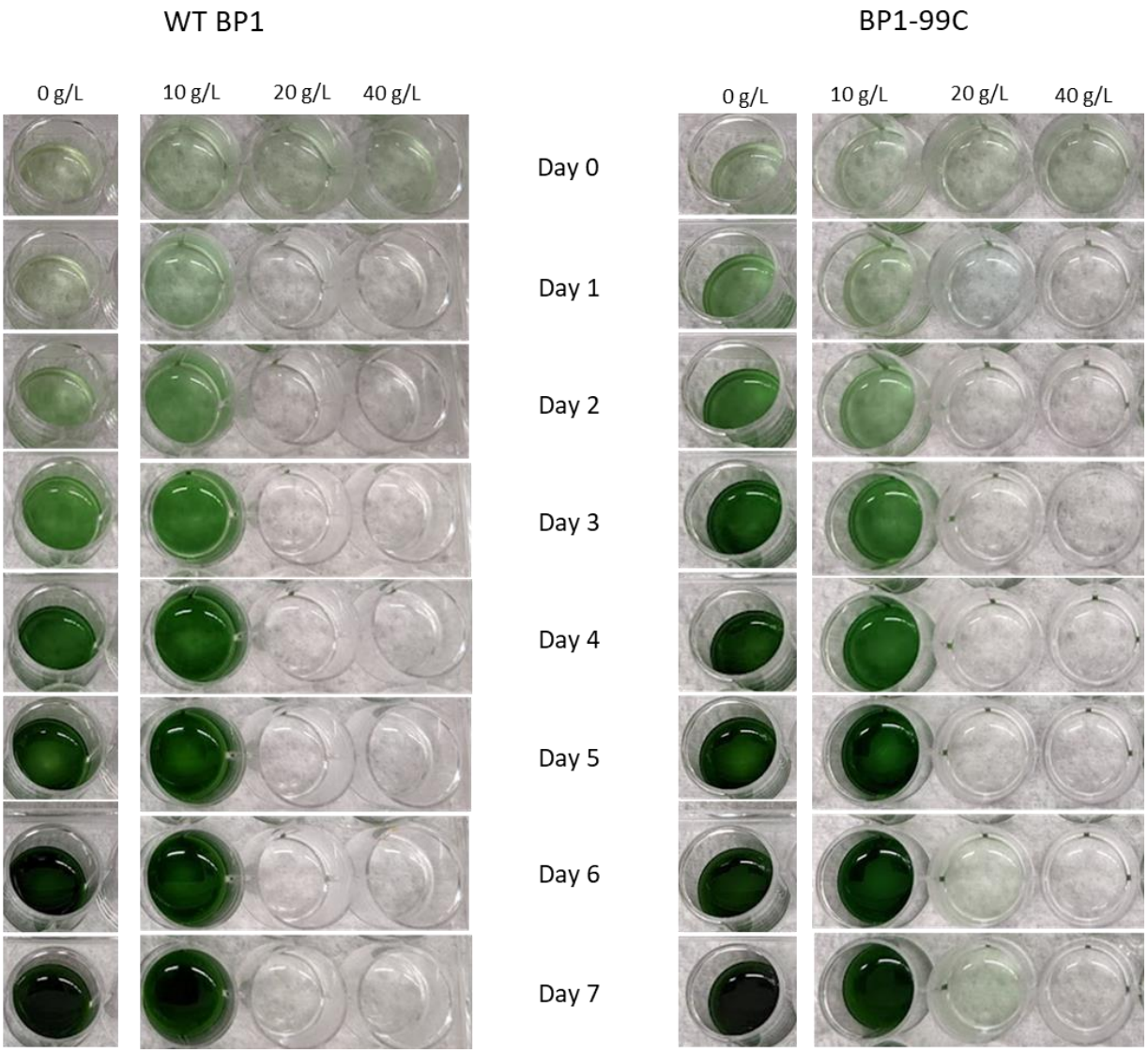

Fig. 32. Growth of wild-type and GE *T. elongatus* BP1 at 42°C with isobutanol supplemented.

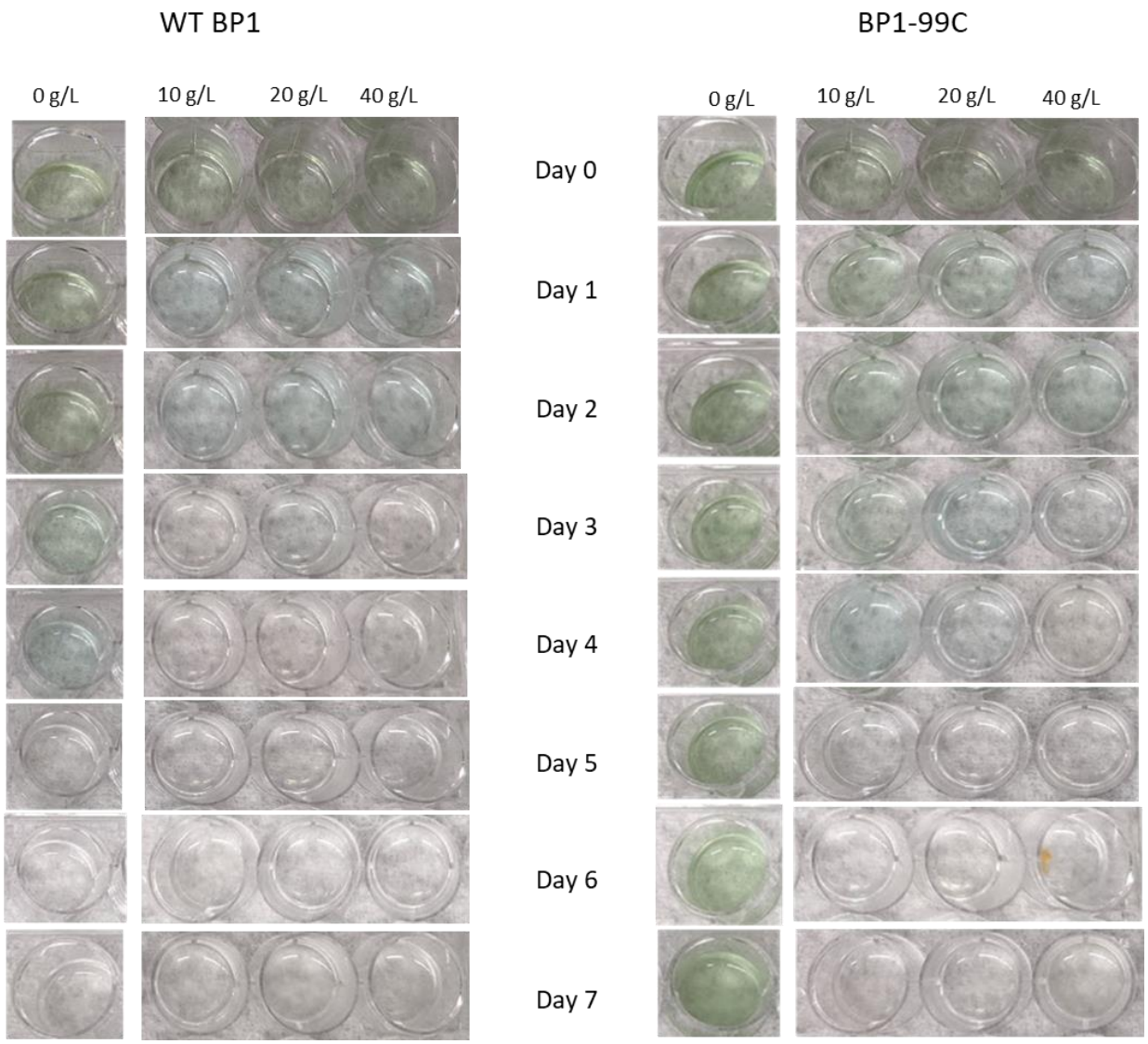

Fig. 33. Growth of wild-type and GE *T. elongatus* BP1 at RT with isobutanol supplemented.

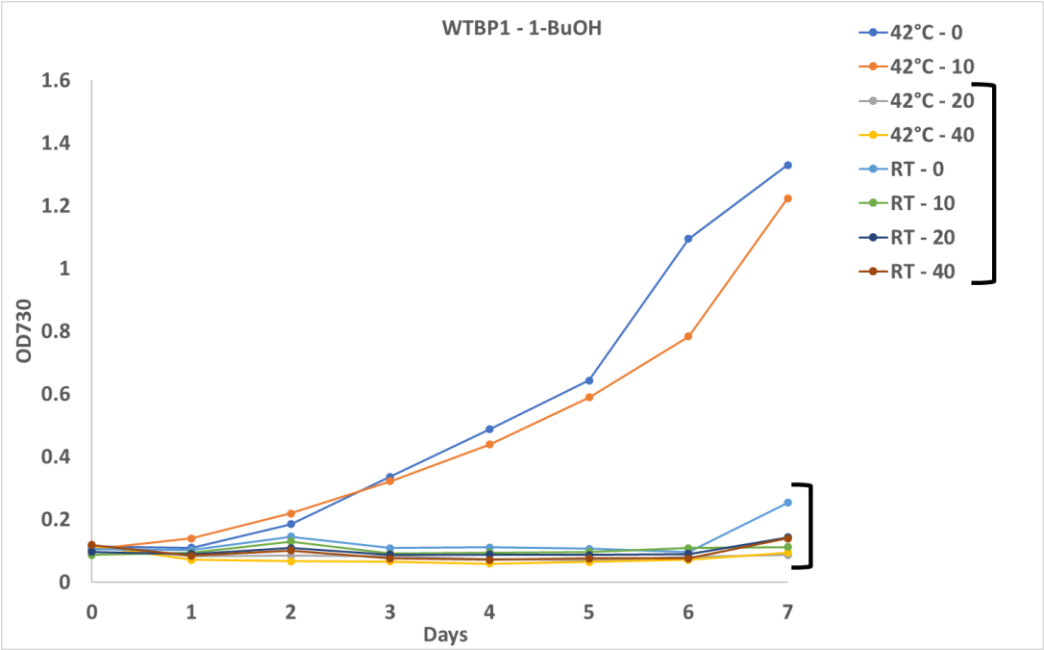

Fig. 34. 1-Butanol tolerance of wild-type *T. elongatus* BP1 at different concentrations. 42C – Cultures grown at 42°C. RT – Cultures grown at room temperature. 0, 10, 20, 40 – concentration g/L.

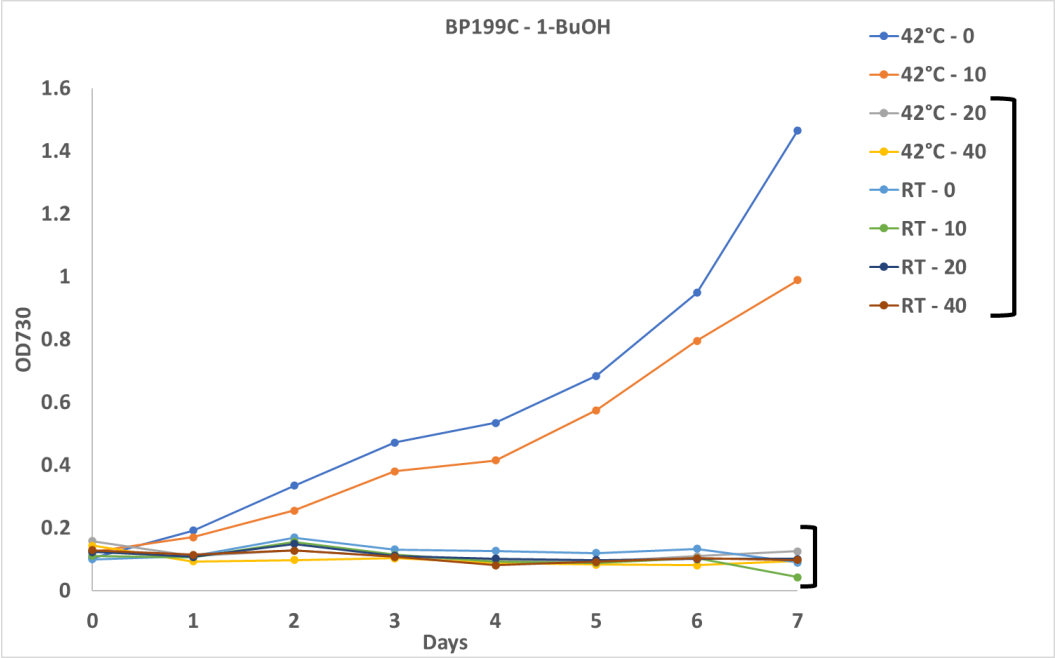

Fig. 35. 1-Butanol tolerance of wild-type *T. elongatus* BP1 at different concentrations. 42C – Cultures grown at 42°C. RT – Cultures grown at room temperature. 0, 10, 20, 40 – concentration in g/L.

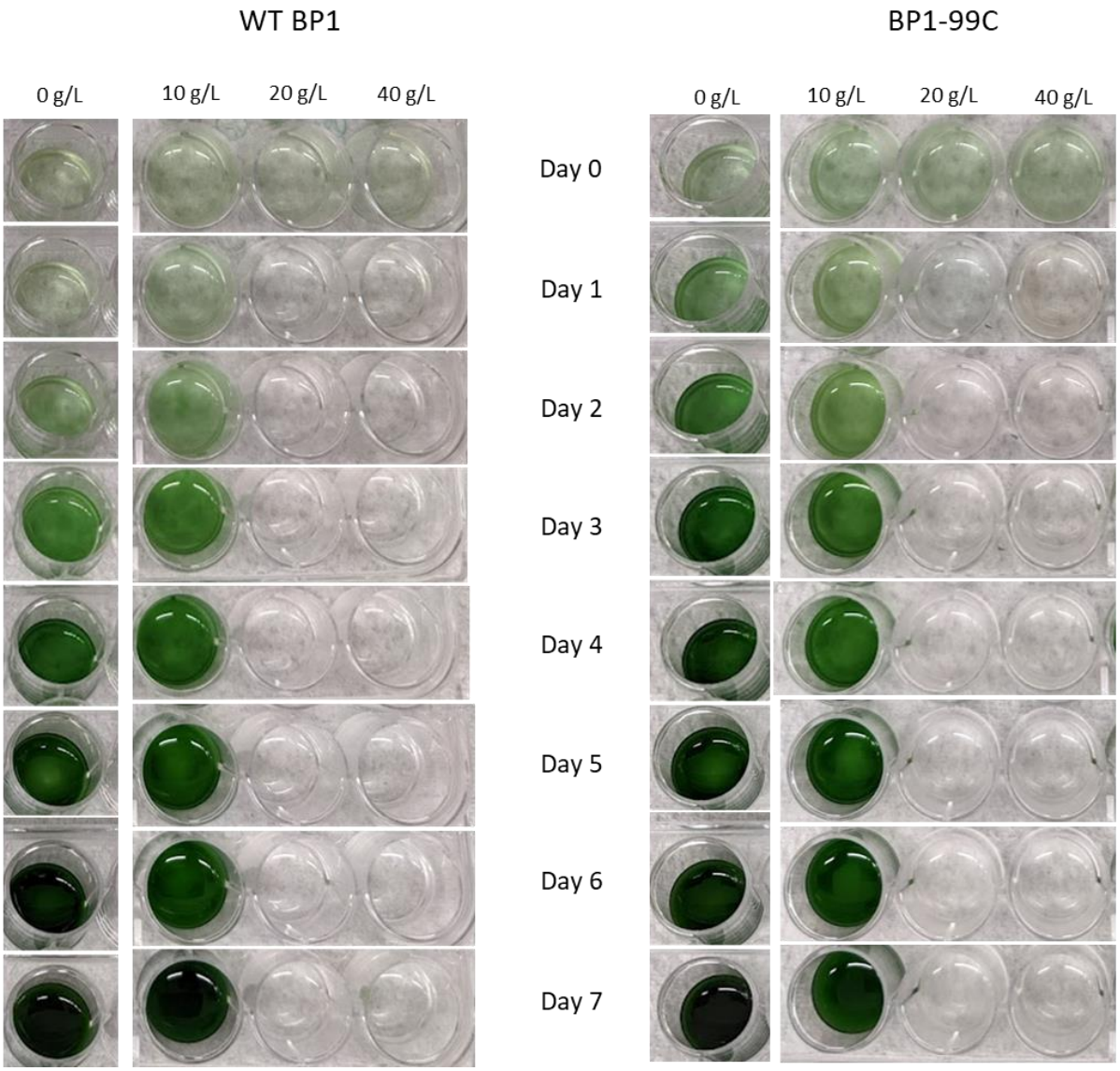

Fig. 36. Growth of wild-type and GE *T. elongatus* BP1 at 42°C with 1-Butanol supplemented.

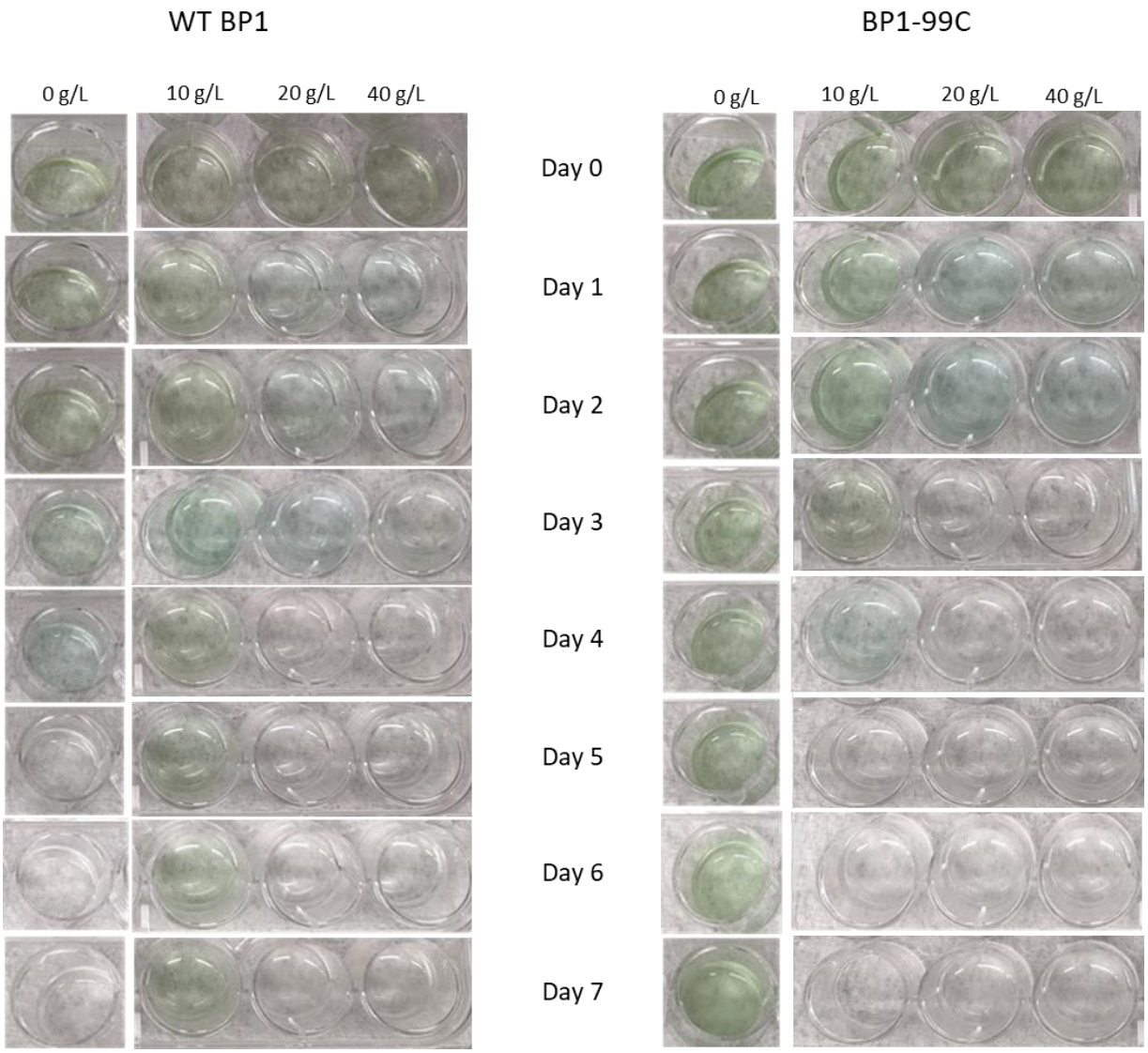

Fig. 37. Growth of wild-type and GE *T. elongatus* BP1 at RT with 1-Butanol supplemented.

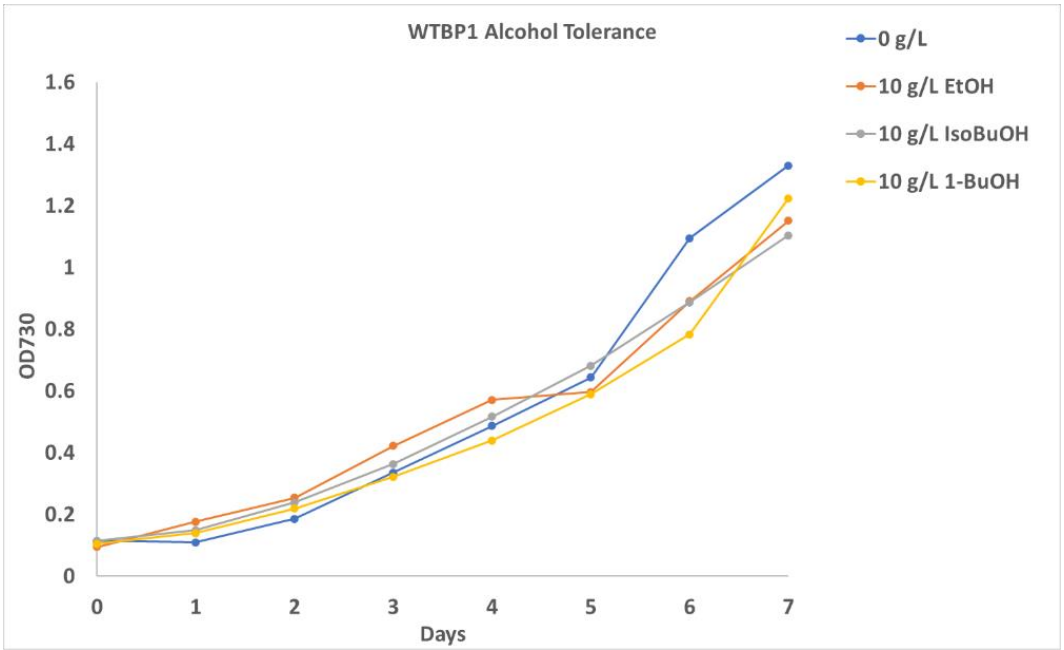

Fig. 38. Comparison of wild-type *T. elongatus* BP1 tolerance for 10 g/L of ethanol, isobutanol, 1-butanol.

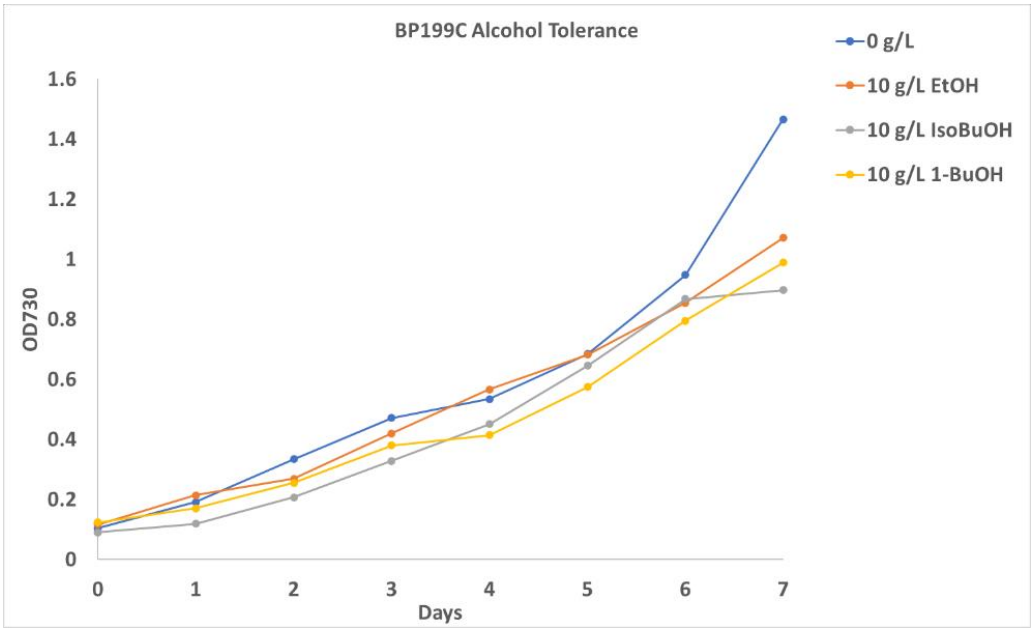

Fig. 39. Comparison of GE *T. elongatus* BP1 tolerance for 10 g/L of ethanol, isobutanol, 1-butanol.

While the tolerance limit of *T. elongatus* BP1 to isobutanol and 1-butanol tested currently was only 10 g/L which was below the preferred amount for distillation, this can be overcome by further genetically modification of these cells. Often when being exposed to butanol, cyanobacteria face the risks of cell membrane damage, metabolite leaking, and impaired photosynthetic electron transfer (Anfelt et al., 2013; Mukhopadhyay, 2015). By identifying the genes responsible for these stress response mechanisms in the cells, they can be regulated to achieve higher butanol tolerance. For example, overexpression of the heat shock proteins and heterologous expression of groESL from *Clostridium acetobytylicum* in *E. coli* showed an improvement in cell tolerance toward different alcohols such as ethanol, butanetriol, and n-, i-, 2-butanol (Zingaro et al., 2012; Abdelaal et al., 2015). In addition, in a laboratory-based evolution study, *Synechocystis* PCC 6803 grown under butanol selective pressure shown a 150%

increasing in butanol tolerance after 94 passages of 395 days (Wang et al., 2014). These studies suggested that while there are still limitations of *T. elongatus* BP1's butanol tolerance, there are potential for improvements.

CONCLUSION

The alcohol tolerance study showed that *T. elongatus* BP1 cells were able to survive up to 40g/L of ethanol even though there was a slight decrease in the growth rate. Results also showed that the extra genetic material in GE *T. elongatus* BP1 did not influence the cell alcohol tolerance compared to the wild-type cells. Currently, both wild-type and *T. elongatus* BP1 cells were only able to survive 10g/L of isobutanol and 1-butanol in the medium. However, with the tools available, genetic engineering of these cells could be done to improve cells tolerance to different alcohol.

CHAPTER 5

CONCLUSION AND DIRECTION FOR FUTURE WORK

This dissertation aimed to assess the biosafety aspect of GE *T. elongatus* BP1 and genetically engineer *T. elongatus* BP1 cells for isobutanol production. In the first study, ability of horizontal gene transfer of GE *T. elongatus* BP1 was examined by co-culturing of the GE *T. elongatus* BP1 cells and wild-type *E. coli* DH5α. Results from this first study showed that GE *T. elongatus* BP1 were able to transfer kanamycin resistant gene into *E. coli* DH5α within 2 days of co-culturing. This was an important finding as though there has been many studies done on horizontal gene transfer from *E. coli* to cyanobacteria, few studies have been done for the reverse direction. In addition, PCR results from the screening of *E. coli* DH5α after co-culturing also suggested that the kanamycin resistant gene might have integrated into the genomic genome of *E. coli* DH5α cells. If this is confirmed to be true, more serious measurement needed to be considered when using GE cyanobacteria to prevent gene transfer to other bacteria which could cause permanent genetic change.

The second study aimed to use *T. elongatus* BP1 as isobutanol production host. The cells were genetically engineered by the insertion of a cassette carrying 2 isobutanol production genes KIVD and yqhD. Results showed that the inserted yqhD gene in GE *T. elongatus* BP1 cells was able to convert the supplemented isobutyraldehyde into isobutanol at both temperature 30°C and 42°C. In addition, with 50 mg/L thiamine supplemented in the medium, GE *T. elongatus* BP1 cells were also able to produce isobutanol from the supplemented 2-ketoisovalerate at 30°C. This indicated that the KIVD gene was active at 30°C and was able to convert 2-ketoisovalerate into isobutyraldehyde which was then converted into isobutanol by the yqhD gene.

The last study looked at the tolerance of *T. elongatus* BP1 cells toward ethanol, isobutanol, and 1-butanol which were used as three representative biofuels at two different temperature 30°C and 42°C. Results from this last part showed that at 42°C both wild-type and GE *T. elongatus* BP1 cells were able to survive with up to 40 g/L ethanol in the medium even though there was a slight decrease in cell growth. Both wild-type and GE *T. elongatus* BP1 were only able to grow with 10 g/L of isobutanol and 1-butanol in cultures. Cell death occurred within 24 hours when there was 20 or 40 g/L of isobutanol/ 1-butanol in the medium. For cultures grew at 30°C with natural light, no growth was observed for any cultures.

Overall, these studies showed that there was a risk of horizontal gene transfer when GE cyanobacteria is used and *T. elongatus* BP1 can be used as potential host for biofuel production. For future work, it would be interesting carry out full genome sequencing of *E. coli* DH5α cultured with GE *T. elongatus* BP1 to determine the insertion of kanamycin resistant within the cells. It would also be interesting to study horizontal gene transfer from GE *T. elongatus* BP1 to other strains of bacteria. For biofuel study, currently either isobutyraldehyde or 2-ketoisovalerate must be supplemented into the cultures for the cells to produce isobutanol. While this is a good start, it will not be economical overall. The goal is to make GE *T. elongatus* BP1 cells produce isobutanol without the addition of isobutyraldehyde or 2-ketoisovalerate. In order to achieve this, the flux to these two genes needs to be improved by either overexpressing other genes in the pathway or blocking other competing pathways. Finally, for improving the tolerance of *T. elongatus* BP1 to different alcohols, a laboratory-based evolution study using the desired alcohol as selection pressure could be carried out as it was done for *Synechocystis* PCC 6803 or future genetically modification should be done to enhance the membrane rigidity or improve stress response in the cells when being exposed to higher concentrations of alcohol.

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APPENDIX A

FULL SEQUENCE OF PKB CASSETTE

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APPENDIX B

SEQUENCING RESULT OF THE 4.5 KB BAND OF A SELECTED PKB GE *T. ELONGATUS* BP1 TRANSFORMANT

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CACGCTCGTC GTTTGGTATG GCTTCATTCA GCTCCGGTTC CCAACGATCA AGGCGAGTTA    360
CATGATCCCC CATGTTGTGC AAAAAAGCGG TTAGCTCCTT CGGTCCTCCG ATCGTTGTCA    420
GAAGTAAGTT GGCCGCAGTG TTATCACTCA TGGTTATGGC AGCACTGCAT AATTCTCTTA    480
CTGTCATGCC ATCCGTAAGA TGCTTTTCTG TGACTGGTGA GTACTCAACC AAGTCATTCT    540
GAGAATAGTG TATGCGGCGA CCGAGTTGCT CTTGCCCGGC GTCAATACGG GATAATACCG    600
CGCCACATAG CAGAACTTTA AAAGTGCTCA TCATTGGAAA ACGTTCTTCG GGGCGAAAAC    660
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GATCTTCAGC ATCTTTTACT TTCACCAGCG TTTCTGGGTG AGCAAAAACA GGAAGGCAAA    780
ATGCCGCAAA AAAGGAATAA AGGCGACACG GAAATGTTGA ATACTCATAC TCTTTCCTTT    840
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TATTTAGAAA AAATAAACAA ATTAGGGGGG TTTCG                               935
```

APPENDIX C

SEQUENCING RESULT OF THE 1.1 KB BAND FROM *E. COLI* COLONY PCR AFTER CO-CULTURING WITH *T. ELONGATUS* BP1

```
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AGGCCCGACC GCTTGACAAG GGTGCGTGAG GTTTTTACGA TAGCGCCGGA TGCGGGGAAA    180
AAGGGCTCCT TTTGGGGGGT TTTCCCCGCA CCGGGCAGAC CTGGGCGCAG AGGAAATGCG    240
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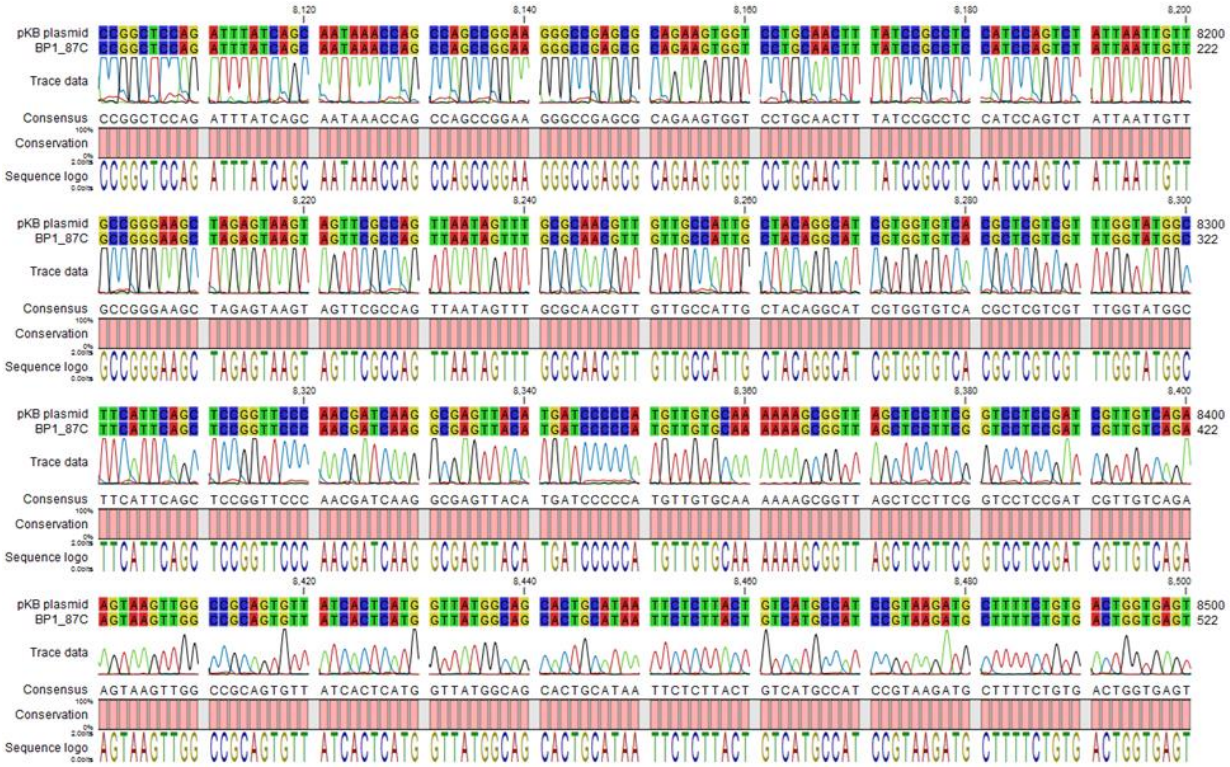
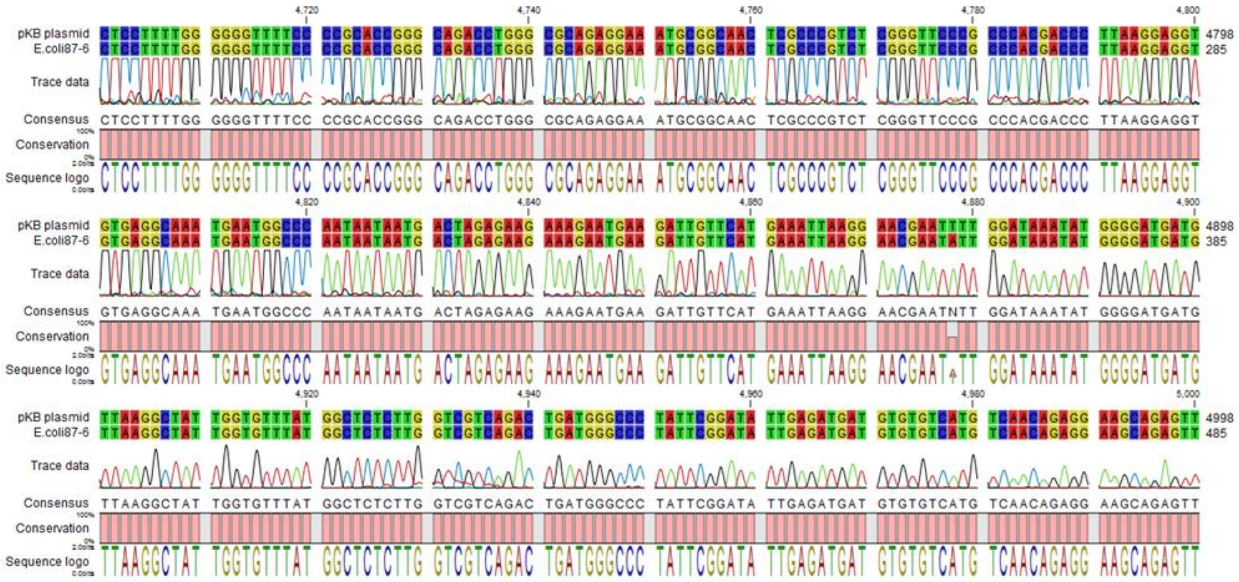
APPENDIX D

ALIGMENT OF SEQUENCING RESULTS



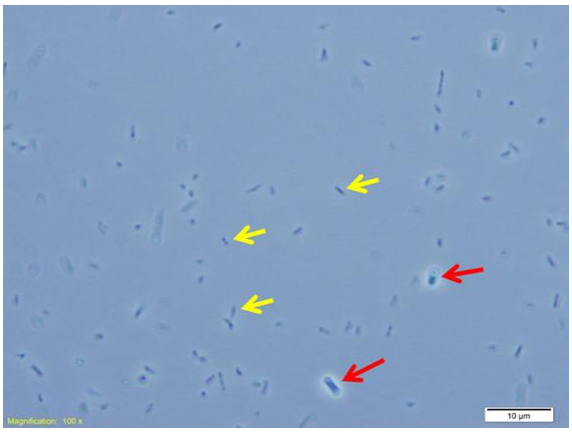
Alignment of sequencing result showing part of plasmid extracted and amplified from GE *T. elongatus* BP1 match the ampicillin gene on pKB plasmid.



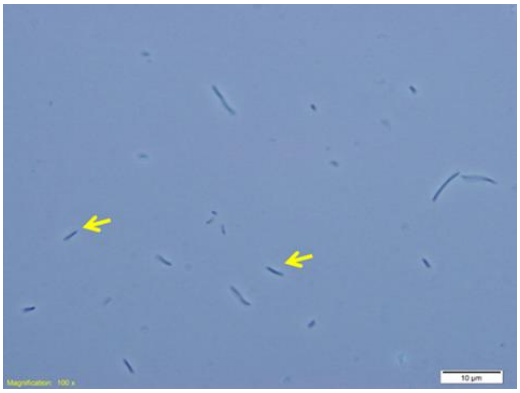
Alignment of sequencing results of colony PCR for a selected *E. coli* colony. The colony was taken from an LB agar plate containing kanamycin after co-culturing with GE *T. elongatus* BP1 transformants and the sequencing results matched the kanamycin sequence on pKB plasmid.

APPENDIX E

MICROSCOPIC IMAGE OF *T. ELONGATUS* BP1 AND *E. COLI* CO-CULTURING



Microscopic image of liquid co-cultures containing *T. elongatus* BP1 transformants and *E. coli* DH5α after 2 days of co-incubation. Red arrows indicated *T. elongatus* BP1 transformants. Yellow arrows indicate *E. coli* DH5α



Microscopic image of colony obtained from LB kanamycin plate of co-culture sample containing *T. elongatus* BP1 transformants and *E. coli* DH5α. Yellow arrows indicates *E. coli* DH5α cells

APPENDIX F

FULL SEQUENCE OF PB-BP1KY CASSETTE

ATTAATATTA ATCCTAGGTA ACGACTATGA CTACAGTTCT GCAACGTCGT CAGACAGCGA 60

ATCTGTGGGA GCGTTTTTGC GATTGGATCA CCAGCACCGA GAACCGCCTT TATATTGGCT 120

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TTATTGCCGC ACCCCCTGTG GATATTGATG GCATCCGTGA GCCTGTTTCC GGCTCCTTGC 240

TCTATGGCAA CAACATCATT ACAGCGGCAG TGGTGCCCTC CTCTAATGCC ATTGGGTTAC 300

ACCTCTACCC AATCTGGGAT GCCGCTTCCC TTGACGAGTG GCTGTACAAT GGTGGGCCCT 360

ACCAACTGAT CATCTTCCAC TTCCTGATCG GCATTTTCTG CTACATGGGT CGGGAGTGGG 420

AACTCAGCTA CCGTCTCGGT ATGCGGCCTT GGATTCCTGT GGCCTTCTCT GCCCCTGTGG 480

CAGCGGCAAC GGCTGTGCTG TTGATCTACC CCATTGGTCA AGGCAGCTTT TCCGATGGAC 540

TGATGCTGGG GATTTCCGGT ACCTTCAATT TCATGATTGT GTTCCAAGCT GAACACAATA 600

TCCTCATGCA CCCCTTCCAC ATGCTGGGGG TGGCGGGTGT CTTTGGTGGT GCTTTGTTTG 660

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TAGAACGAAG CGGCGTCGAA GCCTGTAAAG CGGCGGTGCA CAATCTTCTC GCGCAACGCG 1860

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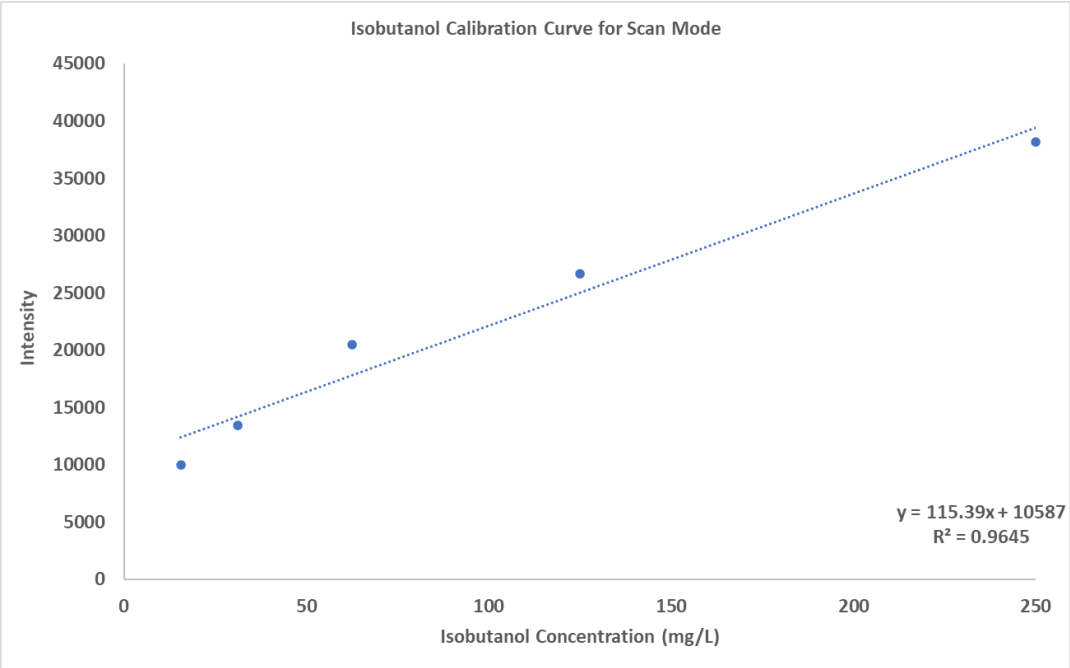
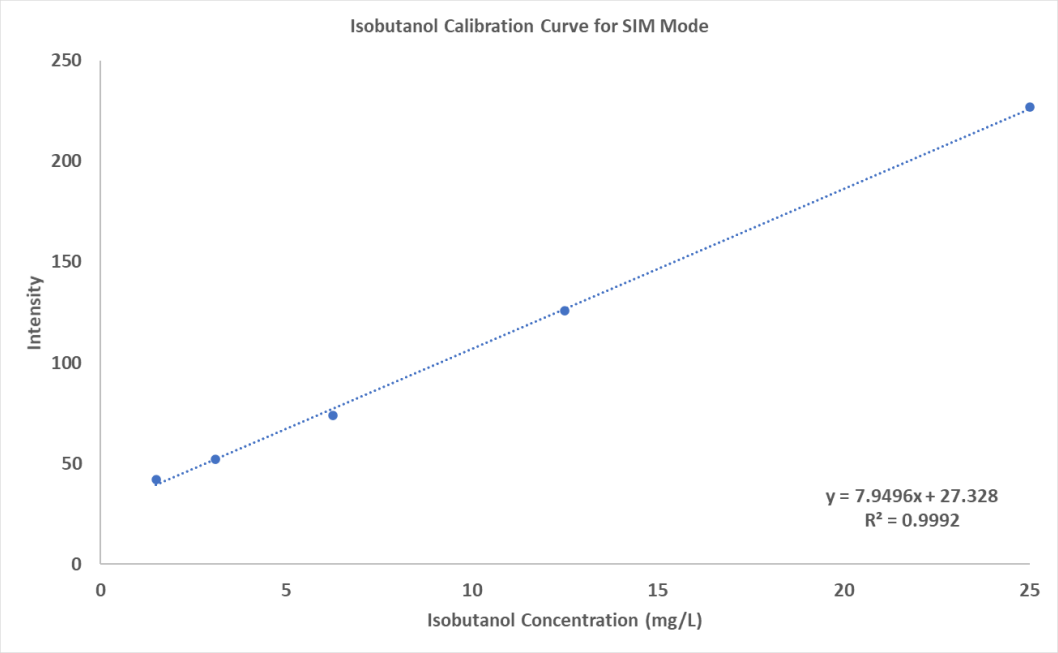
APPENDIX G

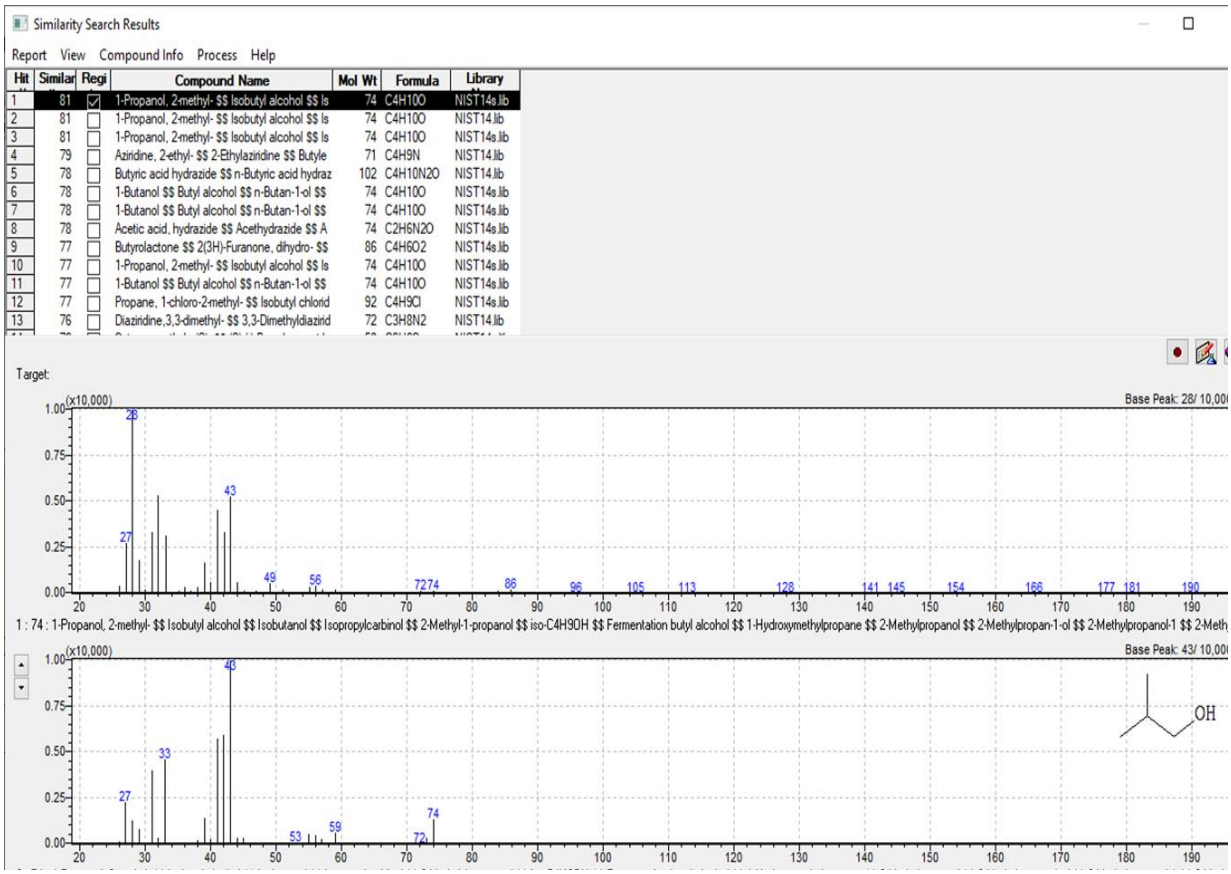
ISOBUTANOL CALIBRATION CURVE





APPENDIX H

SIMILARITY SEARCH RESULTS OF ISOBUTANOL PEAK FROM *T. ELONGATUS* BP1 CULTURE WITH ISOBUTYRALDEHYDE SUPPLEMENTED AT 42°C



APPENDIX I

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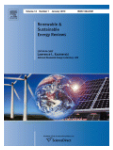

The evolution of the biofuel science

Author: Pooya Azadi,Robert Malina,Steven R.H. Barrett,Markus Kraft
Publication: Renewable and Sustainable Energy Reviews
Publisher: Elsevier
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SPRINGER NATURE

A brief history of synthetic biology

Author: D. Ewen Cameron et al
Publication: Nature Reviews Microbiology
Publisher: Springer Nature
Date: Apr 1, 2014

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Institution name	Old Dominion University
Expected presentation date	Oct 2021

Additional Data

Order reference number	Cameron 2014
Portions	figure 1

Production of liquid biofuels from renewable resources

Author: Poonam Singh Nigam,Anoop Singh
Publication: Progress in Energy and Combustion Science
Publisher: Elsevier
Date: February 2011

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Licensed Content Title	Production of liquid biofuels from renewable resources
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Licensed Content Date	Feb 1, 2011
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Portions	figure 2

Cyanobacteria as photoautotrophic biofactories of high-value chemicals

Author: Daniel Noreña-Caro,Michael G. Benton
Publication: Journal of CO2 Utilization
Publisher: Elsevier
Date: December 2018

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Expected presentation date	Oct 2021

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Demonstration of horizontal gene transfer from genetically engineered Thermosynechococcus elongatus BP1 to wild-type E. coli DH5α

Author: Thu H. Nguyen,Cherrelle L. Barnes,Jason P. Agola,Sana Sherazi,Lesley H. Greene,James W. Lee
Publication: Gene
Publisher: Elsevier
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Publications and presentations

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