Metabolic engineering of *Thermoanaerobacterium* strains AK17 and HG-8 for production of 1,2-propanediol

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Metabolic engineering of Thermoanaerobacterium strains AK17 and HG-8 for production of 1,2-propanediol

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Abstract

The aim of this study is to genetically engineer the metabolic pathways of two thermophilic bacteria, *Thermoanaerobacterium thermosaccharolyticum* HG-8 and *Thermoanaerobacterium* AK17, for 1,2-propanediol production.

Not many bacteria produce 1,2-propanediol naturally, but HG-8 is one of the best known natural 1,2-propanediol producer. For HG-8 the idea was to use homologous recombination to knock out genes that contribute to production of fermentation byproducts. Ethanol and lactate are produced in HG-8 in significant amounts. Lactate dehydrogenase catalyzes lactate formation and aldehyde-alcohol dehydrogenase catalyzes ethanol formation. By knocking out the *ldh* gene that codes for lactate dehydrogenase and the *adhE* gene that codes for aldehyde-alcohol dehydrogenase it might be possible to increase 1,2-propanediol production in the bacteria. The *ldh* transformation was successful and lactate production of the engineered strain did decrease. There was, however, no increase in 1,2-propanediol production. The *adhE* transformation was partly successful as it resulted in single crossover, expressing both the *adhE* gene but also the antibiotic resistance marker. Promising results were that this engineered strain produced more 1,2-propanediol than the wild type.

AK17 cannot produce 1,2-propanediol naturally. The idea was to engineer the strain by cloning from HG-8 the genes that catalyze the missing steps of 1,2-propanediol production. The genes of interest were the genes that encode methylglyoxal synthase (MGS), methylglyoxal reductase (MGR) and glycerol dehydrogenase (GDH). This transformation was unsuccessful. Possibly because the plasmids have mutated or there is something in the transformed genes, or expression of them in AK17, that is having lethal effect on the bacteria.

Útdráttur

Markmið rannsóknarinnar var að erfðabreyta efnaskiptaferli tveggja hitakærra baktería, *Thermoanaerobacterium thermosaccharolyticum* HG-8 og *Thermoanaerobacterium* AK17, með það að markmiði að ná fram 1,2-própandíól framleiðslu.

Ekki er algengt að bakteríur framleiði 1,2-própandíól en HG-8 er ein þekktasta bakterían sem framleiðir 1,2-própandíól. Lagt var upp með að nota samstæða endurröðun til að slá út genum í HG-8 sem að taka þátt í framleiðslu á öðrum afurðum en 1,2-própandíól. HG-8 framleiðir etanól og laktat í miklu magni. Laktat dehýdrógenasi hvatar laktat framleiðslu og aldehýð-alkóhól dehýdrógenasi hvatar etanól framleiðslu. Með því að slá út *ldh* geninu sem kóðar fyrir laktat dehýdrógenasa og *adhE* sem kóðar fyrir aldehýð-alkóhól dehýdrógenasa, gæti verið mögulegt að auka framleiðslu 1,2-própandíól í HG-8. *Ldh* ummyndunin bar árangur og var minni laktat framleiðsla í erfðabreytta stofninum. Það var hins vegar ekki aukning í 1,2-própandíól framleiðslu. *AdhE* ummyndunin var að hluta til árangursrík þar sem einföld endurröðun náðist. Erfðabreytti stofninn innihélt bæði *adhE* genið og genið sem var notað sem valmerki. Áhugavert var að þessi erfðabreytti stofn framleiddi meira 1,2-própandíól en villigerðin.

AK17 getur ekki framleitt 1,2-própandíól náttúrulega. Því var lagt upp með að erfðabreyta stofninum með því að klóna ferlana úr HG-8 sem hvata skrefin sem vanta uppá fyrir 1,2-própandíól framleiðslu. Um er að ræða genin fyrir methylglyoxal synthase (MGS), methylglyoxal reductase (MGR) og glycerol dehydrogenase (GDH). Þessi erfðabreyting bar ekki árangur. Það gæti stafað af því að plasmíðið hefur stökkbreyst eða það er eitthvað við genin eða genasamsetninguna sem er ekki lífvænlegt fyrir AK17.

Preface

In the fall of 2017, my work with *Thermoanaerobacterium* AK17 started in the Introduction to Systems Biology course taught by Steinn Guðmundsson. With me on this project was Arnar Kári Sigurðarson Sandholt. We started to build a metabolic model of the bacterium, using genomic data from Matís. The model was originally put together by comparing the genome to a model of a related bacterium, *Thermoanaerobacterium saccharolyticum* (Currie *et al.*, 2015). Arnar Kári continued the work with AK17 and finished building the model in 2019.

The experiments done for this thesis focused on engineering the metabolic pathway of AK17 and *Thermoanaerobacterium thermosaccharolyticum* HG-8 for 1,2-propanediol production. Previous experiments with the AK17 model suggested that AK17 has the potential to produce 1,2-propanediol if the pathway that converts dihydroxyacetone phosphate to 1,2-propanediol via methylglyoxal and acetol is added to its genome. This pathway can be found in HG-8.

Table of Contents

Abstract v
Útdráttur vii
Preface ix
Table of Contents x
List of Figures xii
List of Tables xiii
Acknowledgements xv
1. Introduction 17
2. Background 21
2.1. Biotechnology 21
2.1.1. Biomass on earth 22
2.1.2. Fermentation of carbohydrates 23
2.2. Genetic engineering 23
2.2.1. Bacterial transformation 24
2.2.2. Metabolic engineering 25
2.3. Life in the most extreme places 26
2.3.1. Thermophiles 27
2.3.2. *Thermoanaerobacterium thermosaccharolyticum* HG8 27
2.3.3. *Thermoanaerobacterium* AK17 28
2.4. 1,2-propanediol 30
2.5. Genetic engineering bacteria for 1,2-propanediol production 30
2.5.1. Methylglyoxal 31
2.6. High-performance liquid chromatography 31
3. Materials and Methods 33
3.1. Culture conditions for AK17 and HG-8 33
3.1.1. Preparation of liquid medium 33
3.1.2. Preparation of agarose plates 33
3.1.3. Cultivation of HG-8 and AK17 34
3.2. Genetic engineering of HG-8 knockout strain 34
3.2.1. HG-8 lactate knockout strain 34
3.2.2. HG-8 ethanol knockout strain 34
3.2.3. Transformation of HG-8 35
3.3. Genetic engineering AK17 for 1,2-propanediol production 36
3.3.1. Transformation of AK17 36

3.4. Cloning of plasmids for AK17 tranformation ... 36
3.4.1. Transformation of *E. coli* ... 37
3.5. Verification of experimental results ... 38
3.5.1. Colony PCR ... 38
3.5.2. Gel electrophoresis ... 40
3.5.3. High-performance liquid chromatography ... 41
4. Results and discussion ... 43
4.1. Lactate and ethanol knockouts in HG-8 ... 43
4.1.1. HG-8 lactate knockouts ... 43
4.1.2. HG-8 ethanol knockouts ... 44
4.1.3. 1,2-propanediol production in HG-8 in phosphate limited medium ... 45
4.2. Expressing the 1,2-propanediol pathway in AK17 ... 47
4.2.1. Transforming the plasmid backbone into AK17 ... 49
5. Conclusions ... 51
References ... 53
Appendix A ... 59
Appendix B ... 60

List of Figures

Figure 1. Metabolic pathway for 1,2-propanediol production. 17

Figure 2. Phylogenetic position of strain AK17. 29

Figure 3. Metabolic pathway of HG-8 showing 1,2-propanediol, acetate, lactate and ethanol production. 35

Figure 4. pAM26, pAM27, pAM28 plasmides used for AK17 transformation. 37

Figure 5. PCR verification test of the *ldh* gene knockouts in HG-8. 44

Figure 6. PCR verification test of the *adhE* gene knockouts in HG-8. 45

Figure 7. PCR verification test of the pMU131 transformation on AK17. 49

List of Tables

Table 1. Components used for a 20 µL colony PCR reaction. 38

Table 2. Thermocycler program for colony PCR 39

Table 3. Primers used for PCR amplification of lactate (*ldh*) knockout......................... 39

Table 4. Primers used for PCR amplification of ethanol (*adhE*) knockout. 40

Table 5. Primers used for PCR amplification of AK17 transformation 40

Table 6. The yield of lactate, acetate, 1,2-propanediol and ethanol in the HG-8 wild type and the engineered strains 43

Table 7. The yield of 1,2-propanediol, ethanol, lactate and acetate in the HG-8 wild type and engineered strains in phosphate limited medium. 46

Table 8. Effects of kanamycin concentration on transformants 48

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1. Introduction

Biotechnology is a field that combines biological studies and technological approaches. It uses organisms, biological systems or their processes for renewable, sustainable production. One way to utilize biological processes is to alter the genome of important organisms, like crops in agriculture, to improve them. Another possibility is to utilize the fermentation processes of microorganisms to break down renewable carbon sources to produce energy, like food, feed or fuels. The renewable carbon sources come from the biodegradable portion (biomass) of organic matter in agriculture, forestry, aquaculture, and other industrial waste. In some occasions there can be competition for the biomass, for example with the food industry. There are, however, great potentials in waste products and other unused biomass for renewable energy production (Rosillo-Calle, 2016). Fermentation is the metabolic process that organisms use to anaerobically break down carbohydrates, manly into acids and alcohols. Using fermentation processes to produce valuable products is a viable and environmentally friendly alternative to synthetic processes that use non-renewable materials.

1,2-propanediol is a valuable organic compound that is widely used in cosmetics, food, and as an antifreeze. 1,2-propanediol is mainly synthesized chemically from non-renewable petrochemical derivatives (Martin and Murphy, 2000), but can be produced in an economical, more environmentally friendly way with fermentation (Cameron *et al.*, 1998; Altaras and Cameron, 1999; Niimi *et al.*, 2011). For anaerobic fermenting microorganism, carbohydrates from biomass are first broken down by glycolysis. An intermediate step of glycolysis is the production of Dihydroxyacetone phosphate (DHAP). One of the metabolic pathways for 1,2-propanediol production is the conversion of DHAP to 1,2-propanediol via methylglyoxal (MG) and acetol (Figure 1). There are, however, not many microbes known that can produce 1,2-propanediol naturally (Saxena *et al.*, 2010).

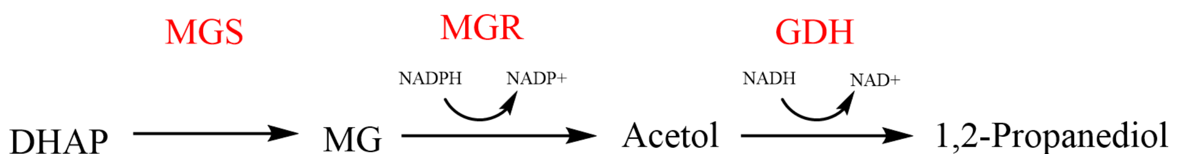

Figure 1: Metabolic pathway for 1,2-propanediol production from Dihydroxyacetone phosphate (DHAP). Catalyzed by methylglyoxal synthase (MGS), methylglyoxal reductase (MGR) and glycerol dehydrogenase (GDH).

Extremophiles are microorganisms that grow in extremes in environmental conditions, such as temperature, pH or pressure. Because of their tolerance they provide benefits such as lower risk of contamination and increased biotransformation rate when cultivated in bioreactors. This ability provides advantages for biosynthetic production of valuable chemicals. Extremophiles and their enzymes do therefore show enormous biotechnological

potential (Elleuche *et al.*, 2015; Coker, 2016). One type of extremophiles are organisms that have tolerance towards heat, these organisms are called thermophiles.

Thermoanaerobacterium thermosaccharolyticum HG-8 is a thermophile that can ferment various sugars like glucose and xylose. HG-8 has been studied for biotechnical purposes and is one of relatively few microbes that can produce 1,2-propanediol naturally (Cameron and Cooney, 1986). *Thermoanaerobacterium* AK17 is a closely related strain, isolated from the Krafla area in Iceland (Orlygsson and Baldursson, 2007). AK17 has a broader substrate range than HG-8, and has been suggested as a promising host for ethanol and hydrogen production (Koskinen *et al.*, 2008). AK17 has not been known to produce 1,2-propanediol naturally. The main aim of this project was to study the feasibility of using these two thermophiles for 1,2-propanediol production. To enhance efficiency of the bacteria it is useful to study their metabolic properties and find ways to increase the 1,2-propanediol production with the tools of biotechnology.

One way to increase the production rate of a target compound is to modify the metabolic pathways of the organism. This can be done by genetically engineering the organisms, using plasmids with either heterologous genes that contribute to the desired metabolic pathway, or homologous recombination sequences that knock out genes that are involved in undesirable steps. The process of engineering the DNA of microbes with the goal of changing their metabolic pathways is referred to as metabolic engineering. The field of metabolic engineering started in the early 1990s when scientist got new insight into the metabolic properties of organisms. This insight came with the evolvement of bioinformatic tools and mathematical modeling methods that allowed for quantitative analysis (Nielsen and Keasling, 2016). Genome-scale metabolic models give insight into the metabolism of an organism and how biological systems work together in the organism (Palsson, 2015). With these models it is possible to predict specific metabolic behaviors of a given microbe, under different environmental conditions or by altering the metabolic pathways in the model. This can help to identify ways to increase or decrease the formation of certain products. These models can give idea of how the microbe might perform *in vivo* and save time-consuming lab work. Before this project started an unpublished model of the AK17 metabolism had been used to identify which genes are missing for 1,2-propanediol production in AK17 and which pathways might be knocked out to increase production.

To increase 1,2-propanediol production in HG-8 it is possible to knock out genes that contribute to formation of side products. Along with 1,2-propanediol, HG-8 also produces ethanol, acetate and lactate (Cameron and Cooney, 1986). Therefore, it is reasonable to assume that by knocking out genes that contribute to production of these compounds, it is possible to increase 1,2-propanediol production. The *ldh* gene encodes for lactate dehydrogenase which catalyzes the conversion of pyruvate to lactate and the *adhE* gene encodes for aldehyde-alcohol dehydrogenase which catalyzes the conversion of acetyl-CoA to ethanol. Experiments with the unpublished AK17 metabolic model suggested that by knocking out the *ldh* and the *adhE* gene will increase 1,2-propanediol production.

According to a previous bioinformatic analysis, AK17 only contains a partial 1,2-propanediol pathway through methylglyoxal, but by adding two or three genes to the genome, it should be able to produce 1,2-propanediol. The genes of interest in this work are i) the gene that encodes methylglyoxal synthase (MGS), which catalyzes the conversion of DHAP into methylglyoxal, ii) the gene encoding methylglyoxal reductase (MGR), which catalyzes the conversion of methylglyoxal into acetol and iii) the gene encoding glycerol

dehydrogenase (GDH), which converts acetol to 1,2-propanediol (Figure 1). Ultimately the goal is to engineer the two bacterial strains to maximize the production of 1,2-propanediol.

The growth substrate used for 1,2-propanediol production in *Thermoanaerobacterium* can come from starch, lignocellulose or even from algae, a renewable carbon source with untapped potential. The experiments described here were done with glucose medium, but the future goal is to utilize inexpensive carbon sources such as those from algae as growth substrates. Algae have many benefits such as fast growth rate, relatively easy extraction of sugars and they do not compete with agriculture for water and land (Lange *et al.*, 2020).

The experiments done in this project focused on engineering the metabolic pathways of the thermophilic bacteria, *Thermoanaerobacterium thermosaccharolyticum* HG-8 and *Thermoanaerobacterium* AK17 for 1,2-propanediol production.

2. Background

The main aim of this project was engineering the *Thermoanerobacterium* strains AK17 and HG-8 for 1,2-propanediol production. In the case of AK17 the aim was to obtain 1,2-propanediol production by heterologous gene expression and in the case of HG-8, that produces 1,2-propanediol naturally, the aim was to increase it by homologous recombination knockouts.

This chapter provides an overview of the main topics that are relevant to this thesis, starting with an introduction to biotechnology and the tools used in the experiments. This is followed by a chapter about microbes that live in extreme environments, including the two bacteria of interest for the project. Then there is an introduction on 1,2-propanediol and how the bacteria can be engineered metabolically for increased 1,2-propanediol production.

2.1. Biotechnology

Biotechnology utilizes cellular and molecular processes for economical, sustainable production of valuable products. The aim can be to create a product or process to solve a problem for human or environmental advantage. Biotechnology can be used in many areas, e.g. in medicine, biofuel production and in the food and feed industry. Although the term is relatively new, humans have utilized biotechnology for thousands of years, e.g. with selective breeding of plants and domestic animals. Selective breeding is for instance used to improve food from agriculture by mating plants with desirable phenotypes, thereby choosing practical genotypes for human benefit (Thieman and Palladino, 2019).

The term biotechnology was first used by the Hungarian engineer Karl Ereky in 1917, referring to methods used to create products from raw materials with the help of living things. Although the definition still holds ground, biotechnology has a broader definition today (Glick, Pasternak and Patten, 2010). In the last decades there has been rapid development in biotechnology. The development began with the discovery of the structure of DNA in the 1950s, that led to experiments with gene cloning and engineering. With this knowledge, scientist discovered in the 1970s a way to transfer genes from one organism to another. This process is called recombinant DNA technology and it led to production of important products in medicine, the first one being insulin used for treatment of diabetes. Recombinant DNA technology was also used in other fields, e.g. development of more nutritious and disease-free crops (Glick, Pasternak and Patten, 2010; Bhatia and Goli, 2018). Another milestone was the completion of The Human Genome Project in 2003. The project aimed to identify and sequence all the genes in human DNA. This gave the field of biotechnology a new important tool. One of the most recent breakthroughs in biotechnology was in 2013 when scientist discovered a fast and accurate way to edit genes. This technology is called CRISPR-Cas and has the potential to target, regulate and modify genomes to potentially correct genetic diseases of animals and humans (Bhatia and Goli, 2018), (Thieman and Palladino, 2019).

Microbial biotechnology is a large field within biotechnology that manipulates microorganisms for production of valuable products, one of the best-known examples is synthesis of antibiotics with microorganisms. *Aspergillus niger* is a fungus that produces citric acid naturally and has been engineered for industrial citric acid production (Tong *et*

al., 2019). *Corynebacterium glutamicum* is a gram-positive bacterium that naturally produces high levels of the amino acid L-glutamate. The bacterium is currently used to produce more than 4 million tons of different amino acids per year (Becker and Wittmann, 2016). Successful use of biotechnology requires understanding on how organisms function on a molecular level and how we can utilize these processes to produce valuable products.

2.1.1. Biomass on earth

Biomass is the mass of living organism such as trees and other plants. The biomass contains abundance of carbohydrates, the most common macromolecules in nature. Carbohydrates are initially formed by photosynthesis from carbon dioxide and water. In biotechnology the main carbon sources of interest are simple sugars, starch, lignocellulose and polysaccharides (Peters, 2006).

Biomass can by divided into 1st, 2nd and 3rd generation biomass. The 1st generation is simple sugars like the starch obtained from agriculture with sugarcane and corn being the most common sources. The drawbacks of using the raw materials from agriculture is competition with the food industry for land and water and diversion of biomass that could otherwise be used to feed an ever-growing population (Lee and Lavoie, 2013). The 2nd generation biomass consists of more complicated biomass found in trees and grass. This biomass is called lignocellulose and consists mainly of cellulose, hemicellulose and lignin, polymers that are closely linked with each other. Lignocellulose is the most abundant carbohydrate source on earth, but cellulose and hemicellulose are rich of sugars. It is, however, hard to utilize lignocellulose because hydrolyzation of cellulose is time consuming and the lignin makes degradation and extraction of carbohydrate difficult, requiring steps with costly enzymatic digestion (Mussatto and Teixeira, 2010), (Sorek *et al.*, 2014).

The 3rd generation biomass refers to polysaccharides from algae. The benefits of using 3rd generation biomass as a carbon source are that algae are relatively fast growing, compared to 1st and 2nd generation biomass and depending on species, it can contain abundance of various sugar components that can be broken down by microorganisms. Along with monosugars, such as glucose, mannose and xylose, 3rd generation biomass also contains deoxy sugars, sugar alcohols and acids, e.g. mannitol and guluronic acids. Because there is no lignin in algae, it is easy to extract the sugars, compared to lignocellulose. Another important benefit is that competition for land and water with agriculture is not a factor with algae (Lange *et al.*, 2020).

Conversion of biomass to a desirable product requires one or more of the following steps, pretreatment, enzymatic hydrolysis, and fermentation. Pretreatment is the extraction of sugar rich component and enzymatic hydrolyzation to release the monosugars, for microbe fermentation (Volynets, Ein-Mozaffari and Dahman, 2017). Microbes differ in their ability to ferment different types of carbohydrates. For biotechnological purposes it is feasible to find a microbe that has broad substrate range and can therefore utilize great a part of the biomass.

2.1.2. Fermentation of carbohydrates

In the absence of oxygen anerobic organisms use a process called fermentation to oxidize organic compounds such as carbohydrates. It is thought that the earliest living cells on earth used fermentation as the main metabolic pathway for energy production. The environment lacked oxygen but was rich in geochemically produced organic molecules (Alberts *et al.*, 2008). Humans have used the process for ages as fermentation of yeast is used in baking and brewing. During fermentation, the yeast releases alcohol for brewing and carbon dioxide that makes the bread rise.

Carbohydrate metabolism begins with glycolysis, both in aerobic and anaerobic microorganisms. A single glucose molecule is broken down by glycolysis into two pyruvate molecules, producing ATPs along the way and reducing NAD^+ to NADH. In the absence of oxygen, fermentation breaks down pyruvate into several organic end products that serve as final electron and hydrogen acceptors, this process is catalyzed by NADH. Fermentation takes place in the cytosol and regenerates the NAD^+ that is used again in the glycolysis reactions. The end products are mainly simple organic acids and alcohols such as lactic acid, acetic acid and ethanol, together with CO_2 and H_2. These products are secreted into the medium as waste metabolites. Anaerobic bacteria that rely on fermentation do not produce as much energy as aerobic bacteria which are able to utilize the citric acid cycle and the electron transport chain. This is why aerobic respiration is the preferred form of carbohydrate metabolism in nature (Jurtshuk, 1996; Alberts *et al.*, 2008).

It is possible to utilize fermentation of microorganisms to break down carbohydrates from biomass to a specific valuable product. The usage of microbial fermentation for chemical production is a promising option for many reasons. One of the benefits is the use of renewable feedstock rather than nonrenewable resources that will at some point become scarce. There are, however, still some limitations to fermentation compared to synthetic processes, e.g. is that the production range is relatively small. Productivity is usually not high and recovery and purification of the product from the fermented solution can be challenging. There are no simple solutions to these limitations but they can be reduced significantly with the help of metabolic engineering (Cameron *et al.*, 1998). To increase production of a certain fermentation product for an industrial purpose, it is possible to engineer genes coding for enzymes in the metabolic pathway. The aim of many genetic engineering strategies is to stop or change the secretion of specific end products to increase the yield of others.

2.2. Genetic engineering

Genetic engineering is the use of recombinant DNA technology to directly manipulate the genetic material of an organism to achieve a desired trait. It is the process of transporting genes from one organism to another, usually from a different species, with the goal of expressing the encoded proteins in the new organism.

The first step of genetic engineering an organism is to extract the gene of interest from the cells of a donor organism. There are few ways to extract DNA from a cell, usually this is done by breaking up the cell and using enzymes to destroy everything except the DNA. The region of interest can then be multiplied from the extracted DNA to a concentration that will

allow isolation and insertion into a plasmid. This is done with the polymerase chain reaction (PCR) that utilizes DNA polymerase to exponentially amplify the DNA fragment by using designed primers that are unique to the region of interest (Godbey, 2014). After the gene has been isolated the next step is to clone it, i.e. create multiple copies of the gene. The most common way for gene cloning is to use plasmid vectors, a circular piece of DNA that is replicated independently of chromosomal DNA in bacteria. The construction of plasmids is done by the help of restriction enzymes that cut the DNA at a specific site, and a DNA ligase that is able to stitch together two DNA pieces. These enzymes make it possible to insert the gene of interest into the plasmid at a specific location (Lodish *et al.*, 2000). Gibson Assembly is a cloning method that was used to assemble the plasmids for this study.

It is possible to construct plasmids from scratch with the help of enzymes. Many plasmids are, however, commercially available and can therefore speed up the engineering process. These plasmids have bacterial origin of replication that allows them to be amplified by *Escherichia coli*. They also have one or more antibiotic resistant markers, bacterial genes that are used as selection markers later in the process. Such markers code for proteins that give the bacteria resistance to a specific antibiotic, such as kanamycin or ampicillin (Godbey, 2014).

The constructed plasmid containing the gene of interest is transferred into *E. coli* to produce multiple copies of it, in a process referred to as amplification. The *E. coli* cells are then grown in a liquid medium until a specific density is reached. Thereafter they are plated on a petri dish containing LB or SOC agar, nutritionally rich medium for bacterial growth. The plates also contain the antibiotic that the plasmids genes carry resistance to, enabling selection of transformed cells. The next step is to extract the plasmid from the *E. coli* cells. The extracted plasmid is digested with restriction enzymes and analyzed with gel electrophoresis, a process where agarose gel and electrical current are used to separate DNA fragments by size. By using a positive control and DNA ladder that contains DNA fragments of known size, it is possible to establish whether the amplification was successful or not (Godbey, 2014). If the correct size of DNA fragment has been amplified, it can be introduced into the host cell.

In nature, genes can be transferred horizontally from one bacterium to another by three different parasexual processes. By direct contact of two cells, a process referred to as conjugation. Transduction is when a bacteriophage vector transfers DNA from host cell to a new bacterium. Finally genes can transferred by transformation, in a process where competent cells take up and express naked DNA from the environment (Adrio and Demain, 2010). In the lab when a cloned plasmid is introduced into a host cell, it can be taken up from the surroundings with transformation.

2.2.1. Bacterial transformation

Transformation of bacteria takes place between exogenous DNA and the recipient cell. Unlike other horizontal gene transfer processes, transformation is entirely directed by the recipient cell. Proteins of the recipient cell allow extracellular exogenous DNA to be integrated into its genome. The integrated DNA can allow the cell to adapt to changes in the environment and provide resistance to antibiotics (Johnston *et al.*, 2014).

For the bacterium to be able to take up extracellular DNA and express its genes, the bacterium needs to have competence for genetic transformation, i.e. the capability of taking up exogenous DNA. Natural competence is regulated at a specific growth phase in bacteria. The phase varies among transformable species, for some it is during the early exponential phase and for other it is in the stationary phase (Johnston *et al.*, 2014). Relatively few species have the natural ability for transformation.

The transformation process of bacteria can be harnessed in biotechnology in several ways. First, by natural competence where the cell alters its genome to take up extracellular DNA. Transformation can also be induced by changes in the physical or chemical environment, with cold calcium chloride treatment, protoplast fusion, electroporation, or heat shock (Adrio and Demain, 2010). Electroporation refers to the process of subjecting cells to an external electrical field for a short period. The electrical shock causes the cell membrane to become temporarily permeable, enabling extracellular DNA to flow through the membrane (Dower, 1990). Along with natural competence and heat shock, electroporation was the main transformation approach used in this project.

2.2.2. Metabolic engineering

The process of engineering the DNA of microbes with the goal of changing their metabolic pathways is referred to as metabolic engineering. Metabolic engineering is frequently used to optimize the production of a specific valuable product. This includes fuels, chemicals, pharmaceuticals, foods, and feeds. Altering cells into an efficient metabolic factory is, however, a challenging task because cells have evolved a robust metabolic network with well-coordinated regulation and complex interaction between pathways. This regulation system of the cell helps them to maintain homeostasis, even when they are exposed to various environmental conditions (Nielsen and Keasling, 2016).

The engineering process requires a detailed understanding of organism cellular system. This includes analysis of the biochemical reactions in question, information on the fermented products and the metabolic flux. Metabolic flux of an organism refers to the rate at which material is processed through a metabolic pathway and is determined by the activity of the enzymes in a that particular pathway (Stephanopoulos, 1999). When the relevant information has been obtained it is possible to start constructing the new recombinant strain. To be able to change the metabolic flux it is necessary to redirect the line of communication between endogenous metabolic pathways or disrupt the signals that regulates the mechanism. Metabolic engineering can, therefore, involve de-regulation of the metabolic pathway or insertion of heterologous pathways that take over cellular regulation (Nielsen and Keasling, 2016). This might involve introduction of new enzymes or removal of enzymes that contribute to the production of byproducts. The aim can also be to alleviate reaction bottlenecks by over-expressing specific enzymes (Cameron *et al.*, 1998).

Microbial products made during the exponential phase of growth are called primary metabolites and belong to the normal growth process of the microbe. It is possible to utilize this growth process in the production of valuable compounds. However, it is also possible to engineer the microbe to turn on expression of the relevant enzymes in stationary phase and with that maximize the product formation of the microbe. This transcription initiation can be done by engineering promoters that regulate transcription in response to various

signals. Regulation can be achieved by changing environmental or physiological signals (temperature, pH or chemicals) known to be connected to gene expression through an inducible promoter (Brautaset, Lale and Valla, 2009). It is also possible to engineer these promotors directly for transcriptional control. There are, however, limited numbers of available promoters that can be fine-tuned with engineering or external signals (Xu, Wei and Liu, 2019). For thermophilic bacteria, these promotors have not been studied much.

Over the last decade there have been great advantages in genetic engineering tools. Tools like CRISPR-Cas and multiplex genome engineering, where multiple changes are induced across the genome simultaneously, have the potential to revolutionize the biotechnology industry (Ryan *et al.*, 2014). These new tools require optimization for each microbe. For thermophilic microbes these genetic tools are still very limited despite the microbes industrial and scientific relevance (Mougiakos *et al.*, 2017).

2.3. Life in the most extreme places

Microbial live can be found in the most extreme conditions. Microbes that live in extreme conditions are called extremophiles. These conditions can be extreme temperature, heat and cold, or extreme pH, salinity, or pressure. Extremophiles have adapted to live in extreme environments that are fatal to most other lifeforms, and most often extremophiles cannot survive in conditions habitable for humans. The proteins from extremophiles can keep their structure and activity under conditions that would unfold and inactivate the protein structure of other organisms (Bonch-Osmolovskaya and Atomi, 2015).

It is often necessary to use enzymes in extreme conditions where the use of mesophilic enzymes is not optimal, because the enzymes are being used in unfavorable conditions. It is possible to make some improvements on these enzymes e.g. with protein engineering. This can, however, be a difficult and expensive processes that can sometimes be avoided by using enzymes from extremophiles that are already adapted to these harsh conditions. Despite their enormous potential, there are still some obstacles to overcome. The biggest obstacle is the ability to produce the enzymes on industrial scale (Coker, 2016).

An example of an industrial made extremophile enzyme is DNA polymerase, that is used in the PCR to synthesize complementary DNA strands from the template DNA (Ishino and Ishino, 2014). DNA polymerase I (*Taq* polymerase) was originally identified in the extreme thermophile *Thermus aquaticus* (Chien, Edgar and Trela, 1976). Extremophiles have also been used in biofuel synthesis with good results, e.g. hydrogen production in *Thermoanaerobacterium thermosaccharolyticum* W16 from xylose and glucose (Ren *et al.*, 2008) or alcohol production of the hyperthermophilic archaeon *Pyrococcus furiosus* with single gene insertion (Basen *et al.*, 2014).

2.3.1. Thermophiles

A thermophile is a type of extremophile that thrives in high temperature environments, usually above 45°C. They are a part of various ecological niches in hot springs and other geothermal sites (Panda *et al.*, 2019). In 1966, Thomas Brock discovered that microorganisms live in the hot springs of Yellowstone National Park (Brock, 1967). Since then, thermophiles have been discovered and isolated from geothermal areas and other places around the world, e.g. in Iceland.

Thermophiles have been less studied than many other microbes. This is mainly because of the difficulty with isolation and cultivation (Panda *et al.*, 2019). Thermophiles have, however, some advantages in biotechnology compared to mesophiles. They have rapid growth rates at high temperatures, and the temperature prevents contamination from mesophilic bacteria (Sveinsdottir, Sigurbjornsdottir and Orlygsson, 2011). Generally, thermophiles have the ability to tolerate environmental fluctuations, like in pH and temperature, as well as having low nutritional requirements (Taylor *et al.*, 2009; Scully and Orlygsson, 2014). Thermophiles have wide range of polysaccharide degrading enzymes that are stable at these extreme conditions. Therefore, they are able to hydrolyze and ferment a wide subrange of complex natural carbohydrates, like starch and lignocellulose (Chang and Yao, 2011; Elleuche *et al.*, 2015).

The genus *Thermoanaerobacterium* is a thermophilic Gram positive, saccharolytic, spore forming anaerobe (Orlygsson and Baldursson, 2007). Bacteria of this genus produce primarily L-lactic acid, acetic acid, ethanol, CO_2 and H_2, and can be distinguished from other thermophiles by the ability to reduce thiosulfate to hydrogen sulfide or elemental sulfur (Lee *et al.*, 1993). *Thermoanaerobacterium* have been studied for biotechnological applications, such as for ethanol production from lignocellulosic biomass (Lynd *et al.*, 2002; Scully and Orlygsson, 2014). But because of its branched fermentation pathway, there is need for metabolic engineering to be able to increase the yield of a single end product to an industrial scale. Genetic transformation was considered one of the biggest obstacles for engineering these strains, because of challenges in introducing them to foreign DNA. It has, however, been showed that under a specific growth conditions several *Thermoanaerobacterium* strains, like *T. saccharolyticum* and *T. thermosaccharolyticum* stains, are naturally competent. This makes genetic manipulation, such as plasmid transformation easier for these strains (Shaw, Hogsett and Lynd, 2010).

2.3.2. *Thermoanaerobacterium thermosaccharolyticum* HG8

Thermoanaerobacterium (formerly *Clostridium*) *thermosaccharolyticum* are thermophilic bacteria that ferment sugars to ethanol, lactate, acetate and butyrate along with CO_2 and H_2. Few strains, e.g. HG-6 and HG-8, can also produce significant amounts of acetol and 1,2-propanediol (Cameron and Cooney, 1986).

HG-8 has been studied for biotechnical purposes, manly for ethanol production (Lynd *et al.*, 1991; Ahring *et al.*, 1996). It has been used in co-culture with *Clostridium thermocellum* for direct conversion of cellulose to ethanol. HG-8 can utilize pentose sugars like xylose that *C. thermocellum* is unable to ferment. HG-8 is also less sensitive to inhibition by acetate and

ethanol and utilizes cellobiose faster than *C. thermocellum* (Venkateswaran and Demain, 1986).

T. thermosaccharolyticum HG-8 is one of relatively few microbes that are known to produce 1,2-propanediol naturally. HG-8 can convert wide range of sugars into 1,2-propanediol (Altaras, Etzel and Cameron, 2001), where the product is enantiomerically pure (R)-1,2-propanediol with yield up to 0.27 g/g glucose (Cameron and Cooney, 1986). HG-8 has been studied for 1,2-propanediol production (Altaras, Etzel and Cameron, 2001) and is a a good candidate for metabolic engineering, e.g. removal of competing pathways or over-expression of enzymes, to increase the 1,2-propanediol production, possibly to an industrial scale.

2.3.3. *Thermoanaerobacterium* AK17

Thermoanaerobacterium AK17 was isolated in NE Iceland, from the geothermal area Krafla, in 2004. Temperature measured at the area was 70°C and pH was 6.5. According to analysis of the 16s rRNA of the AK17 strain, the most closely related strain is *Thermoanerobacterium aotearoense* (Figure 2) (Orlygsson and Baldursson, 2007).

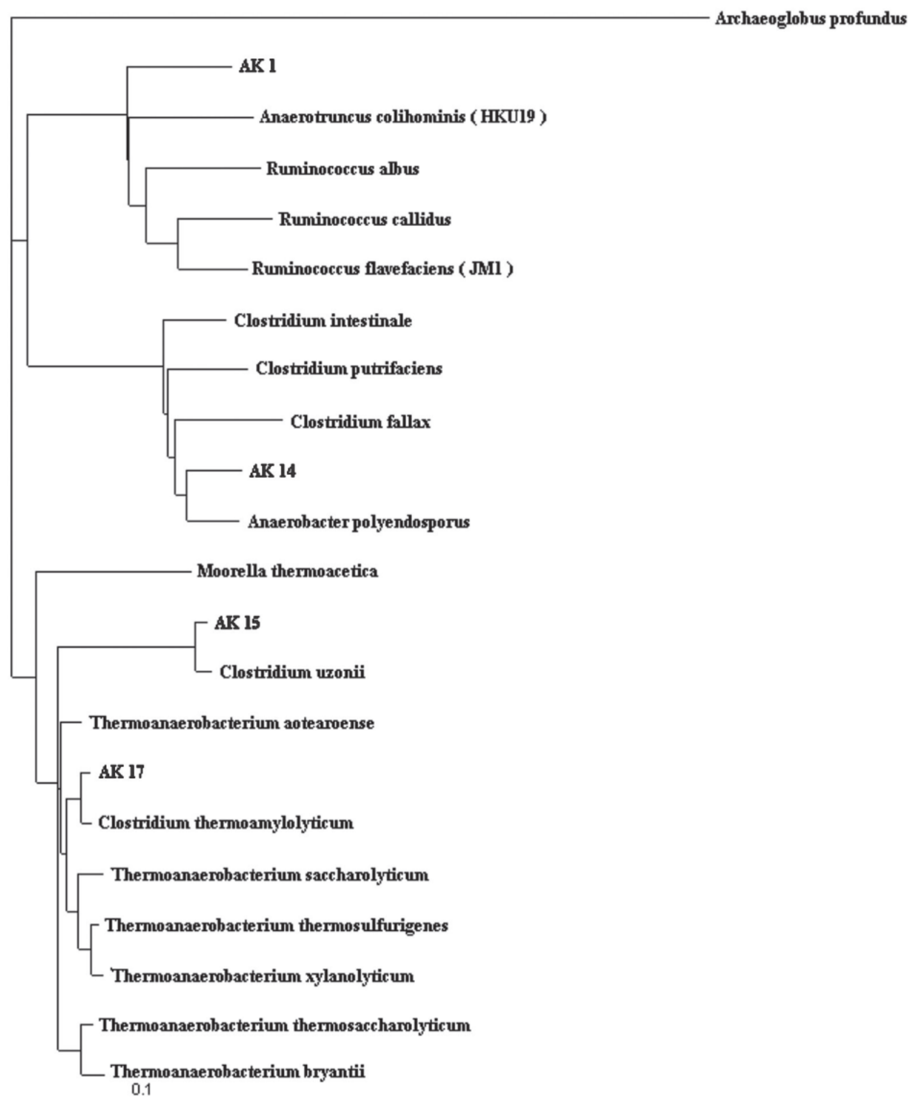

Figure 2: Phylogenetic position of strain AK17 among closely related strains (Orlygsson and Baldursson, 2007).

AK17 has high carbon recovery, fast growth rate (doubling time about 1.24 hours from glucose) and can break down a wide range of carbohydrates. It can for example utilize most of the monosugars from lignocellulose, the biomass of green plants (Orlygsson and Baldursson, 2007; Almarsdottir, Sigurbjornsdottir and Orlygsson, 2011; Sveinsdottir, Sigurbjornsdottir and Orlygsson, 2011). Ethanol tolerance of the strain is relatively high and ethanol production is high for a thermoanaerobe, about 1.6 mol/mol glucose (theoretical yield is 2 mol/mol glucose) (Koskinen *et al.*, 2008). Other advantages of the AK17 strain are less sensitivity to fluctuations in pH and a higher threshold to hydrogen pressure than many related strains. This strain can also degrade the amino acids serine and threonine but it is not common for strains of *Thermoanerobacterium* to be able to break down amino acids (Orlygsson and Baldursson, 2007). The optimum growth conditions for AK17 are 58°C and pH 6.0. These properties make the strain a good candidate for ethanol and hydrogen production (Koskinen *et al.*, 2008).

It might also be possible to utilize the broad substrate range of AK17 to produce other valuable compounds, like 1,2-propanediol. Since the strain does not produce 1,2-propanediol naturally it is necessary to add in genes that catalyze the missing steps in the 1,2-propanediol metabolic pathway.

2.4. 1,2-propanediol

1,2-propanediol (propylene glycol or 1,2-PD) is an industrially important compound that is produced globally on a large scale. 1,2-propanediol has the molecular formula $C_3H_8O_2$ with the molecular weight 76.09 g/mol. 1,2-propanediol is a clear and colorless liquid compound that absorbs water and is often used as a base for production of polyester compounds (National Center for Biotechnology Information, 2020). 1,2-propanediol is used in various industries such as the cosmetic, food and pharmaceutical industries (Saxena *et al.*, 2010). It is also used as synthetic antifreeze and as a de-icer (Bennett and San, 2001).

1,2-propanediol is mostly synthesized chemically from propylene oxide, a nonrenewable petrochemicals derivative in a 1:1 ratio of *R*- and *S*-stereoisomer forms (Martin and Murphy, 2000; Saxena *et al.*, 2010). 1,2-propanediol can, however, be produced in a more environmentally friendly way using biomass and the biochemical pathways of microbes. There are three known biochemical pathways that produce 1,2-propanediol. The first one involves the use of deoxyhexoses like fucose and rhamnose as carbon source. The sugars are cleaved to produce L-lactaldehyde that is then reduced to 1,2-propanediol. This route is not commercially feasible because of the high cost of fucose and rhamnose. The second pathway can be found in lactic acid bacteria and involves lactic acid degradation under anoxic conditions. The third pathway utilizes DHAP, which is formed during glycolysis. DHAP is converted to 1,2-propanediol via methylglyoxal (MG) through reduction of either lactaldehyde or acetol (hydroxyacetone) (Bennett and San, 2001; Saxena *et al.*, 2010). The third pathway, with the production of 1,2-propanediol through acetol, is the one that is focused on in this study.

Relatively few microbes have been studied with respect to 1,2-propanediol production, there among is *E. coli* (Altaras and Cameron, 1999; Clomburg and Gonzalez, 2011), *Saccharomyces cerevisiae* (Jung, Choi and Oh, 2008; Jung *et al.*, 2011) and *Corynebacterium glutamicum* (Niimi *et al.*, 2011). The production of 1,2-propanediol by these strains has been improved by genetic engineering.

2.5. Genetic engineering bacteria for 1,2-propanediol production

Anaerobic, fermenting microorganism break down carbohydrates from biomass by glycolysis. An intermediate step of glycolysis is the production of DHAP. There are three enzymes responsible for the conversion of DHAP to 1,2-propanediol, methylglyoxal

synthase, methylglyoxal reductase and glycerol dehydrogenase (Figure 3). These enzymes are of interest in genetic engineering for 1,2-propanediol production. One intermediate step of the 1,2-Production is the creation of methylglyoxal.

2.5.1. Methylglyoxal

Methylglyoxal (MG) is a ketoaldehyde that is produced during glycolysis in small amounts. The known enzymes that synthesize MG are methylglyoxal synthase (MGS), cytochrome P450 and amine oxidase. The compound is highly toxic and known to accumulate in many cells and reacts with biological macromolecules such as DNA, RNA and proteins (Kalapos, 1999). There are a few known enzymes that can break down MG into non-toxic compounds. There among is the glyoxalase that converts methylglyoxal to lactate. Methylglyoxal reductase (MGS) converts methylglyoxal to lactaldehyde and an aldo-keto reductase converts it into acetol (Ko *et al.*, 2005).

2.6. High-performance liquid chromatography

High performance liquid chromatography (HPLC) is a column chromatography used to separate, identify, and quantify components in a complex mixture. The mixture is mixed with liquid solvent that creates mobile phase and pumped at high pressure through a column. The components separate differently based on their interactions with specific chemical or physical interactions with the stationary phase in the column, causing separation by the different flow rate of various components. At the end of the column there is a detector that registers the various components present in the mixture (Malviya *et al.*, 2010).

3. Materials and Methods

The AK17 and HG-8 strains used in the research were provided by Matís and the lab work was conducted in Matís, Reykjavík. The HG-8 stains number is ATCC 31960 and it was bought from ATCC. This chapter introduces the main protocols and tools used for the engineering of AK17 and HG-8. Protocols used in the experiments have been used in Matís.

3.1. Culture conditions for AK17 and HG-8

AK17 and HG-8 were grown in a liquid medium in gas tight hungate culture tubes, that are designed to maintain anaerobic conditions. Transformed bacteria were transferred to agarose plates after growing in liquid medium overnight.

3.1.1. Preparation of liquid medium

Medium for 10 mL hungate culture tubes includes 9 mL AB medium (pH 5.6), 0.5 mL C1 solution, 0.2 mL glucose and 0.1 mL C2. The contents of each solution can be found in appendix A. After preparing AB medium stock solution, the pH was adjusted, and the medium was autoclaved. Medium was stored at room temperature. Stock solutions for C1, glucose and C2 were filtered (0,45 μm) and stored in 4°C.

Medium for the hungate tubes was prepared in sterile environment, starting by mixing AB medium, C1 and glucose together in a beaker and 9.7 mL were transferred to each tube. After adding rubber cap and plastic screw to the tube, the medium was flushed with nitrogen for 1-2 minutes at 2 bars. After the nitrogen flushing, C2 was added with a syringe to each tube and the tubes incubated for 20 minutes at 50-65°C. The same protocol was applied to 5 mL tubes. When the medium was ready it was stored at room temperature.

3.1.2. Preparation of agarose plates

Preparation of 20 mL agarose plates with nutrients was done in a sterile environment, by flame or in the hood. The agarose AB medium (AB medium mixed with 15 g/L agarose) was melted in microwave and cooled down to 50°C. 1 mL C1, 0.4 mL glucose, 0.2 mL C2 and 18 mL of AB medium + agarose were prepped for each plate. Antibiotics (kanamycin, erythromycin, or ampicillin) were added in the amount needed for each cultivation, and the medium incubated at 55-65°C for 10 minutes before it was pipetted to the plates. The plates were kept in a sterile environment until the medium had hardened completely.

3.1.3. Cultivation of HG-8 and AK17

Before inoculation, the tubes were heated at 55-65°C for about 20 minutes. The stock culture was maintained in 10 or 5 mL hungate tubes at 4°C and kept on ice before the inoculation. 200 µL of the stock culture was inoculated into 10 mL tube with syringe via the tube septum. The new culture was incubated at 60°C overnight or until there was some visible growth. The cultures were stored at 4°C until they were transferred to a new liquid medium or agarose plates.

To prepare the liquid bacteria culture for the plates, 1 mL of culture was transferred into 1.5 mL Eppendorf and centrifuged at 13.0 rpm in 4°C for 5 minutes. Supernatant was removed, cells resuspended in 100 µL of dH_2O and kept on ice. 150 µL of the resuspended cells were inoculated on the plate with glass beads to spread out the cells. GasPack was prepared by dipping a plastic indicator in water that was inserted into the bag. Plates were placed in the bag, along with a reducing agent. The bag was closed tightly and incubated at room temperature until the plastic indicator was white (for 2-4 hours). The GasPack was incubated at 50-65°C for 2-4 days or until growth was observed.

3.2. Genetic engineering of HG-8 knockout strain

HG-8 produces 1,2-propanediol naturally, but by knocking out production of other fermentation products with genetic engineering, it might be possible to increase 1,2-propanediol production. HG-8 secretes lactate, acetate, and ethanol naturally, therefore it might be worthwhile to knock out enzymes that are involved in the production of these compounds.

3.2.1. HG-8 lactate knockout strain

Lactate Dehydrogenase gene (*ldh*) is responsible for pyruvate breaking down to lactate. The AK17 metabolic model (unpublished, Matís) suggested that by knocking out the *ldh* gene to decrease lactate production, 1,2-propanediol production could increase (Figure 3).

Plasmid pAM15 containing the antibiotic resistant marker for kanamycin had already been constructed at Matís. Transforming HG-8 with this plasmid was expected to knock out the *ldh* gene and insert the kanamycin resistance gene. This would give the bacteria resistance to kanamycin and the *ldh* knockout should eliminate or decrease the production of lactate.

3.2.2. HG-8 ethanol knockout strain

Aldehyde-dehydrogenase gene (*adhE*) is a bifunctional enzyme that codes for aldehyde-alcohol dehydrogenase and catalyzes both the reduction of acetyl-CoA to acetaldehyde and reduction of the acetaldehyde to ethanol (Kim *et al.*, 2019). Experiments with the AK17

metabolic model (unpublished, Matís) suggested that knocking out the *adhE* gene to decrease ethanol production, will increase 1,2-propanediol production (Figure 3).

Plasmid pAM14 containing the antibiotic resistant marker for erythromycin had already been constructed at Matís. Transforming HG-8 with this plasmid should give the bacteria resistance to erythromycin and knockout the *adhE* gene and thereby decrease ethanol production.

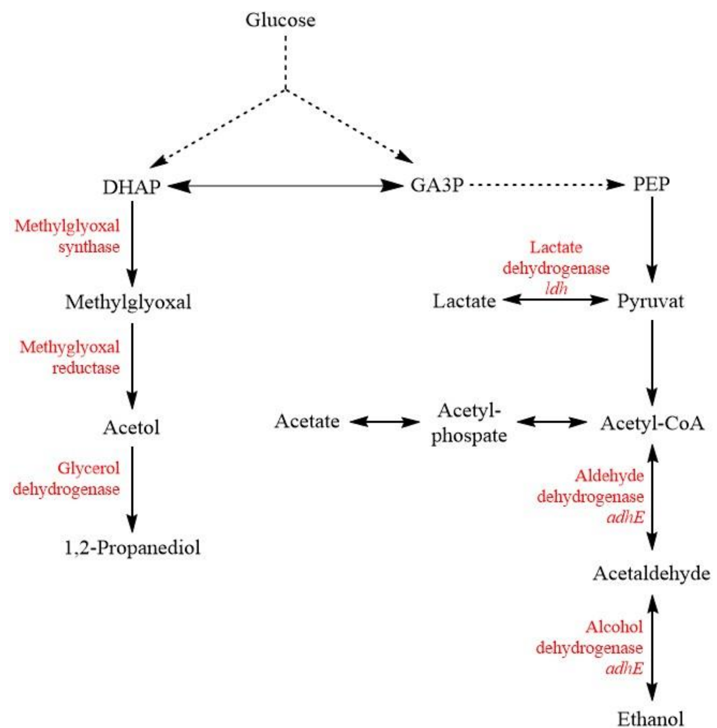

Figure 3: Metabolic pathway of HG-8 showing 1,2-propanediol production along with acetate, lactate and ethanol end products. Knockout of the ldh and adhE was predicted to increase 1,2-propanediol production.

3.2.3. Transformation of HG-8

Knockouts of the lactate and ethanol genes from HG-8 were done via electroporation. 10 mL of overnight culture was centrifuged for 10 minutes at 4000 rcf in a falcon tube, the cells were kept on ice and centrifuged at 4°C. The supernatant was removed, and the cells washed with 10 mL of 10% glycerol. After mixing thoroughly, the mixture was centrifuged again for 10 minutes. This step was then repeated one more time. The cells were resuspended in 100 µL dH_20. Then 45 µL of the resuspended cells were mixed with 5 µL (or maximum 1 µg) of plasmid. The plasmid-cell mixture was carefully transferred to a cooled cuvette (it is important to exclude air) and placed in the electroporation socket where the cells are subjected to a high-voltage electrical pulse of defined magnitude and length (1250 V / 25 µF / 400 Ω / 1 mm). The plasmid-cell mixture was then transferred quickly to a new pre-heated liquid medium, with a syringe. The transformed cells were then cultivated in a liquid medium

overnight and the day after transferred to plates containing antibiotics (see section 3.1.3). Strains were tested for successful transformation using colony PCR (see section 3.5.1).

3.3. Genetic engineering AK17 for 1,2-propanediol production

Three enzymes are involved in 1,2-propanediol production from DHAP through acetol, i) methylglyoxal synthase (MGS); ii) methylglyoxal reductase (MGR); and iii) glycerol dehydrogenase (GDH) (Figure 3). The gene for MGS is already presented in AK17 based on bioinformatics analysis, but its activity is unknown. The genes, for MGR and GDH, have not been identified in AK17. According to a previous bioinformatic analysis, AK17 should be able to produce 1,2-propanediol if the genes that catalyze these missing steps are added into the bacteria (unpublished, Matís)

The genes were cloned from the 1,2-propanediol producer, HG-8. Three candidate genes had been identified that could produce MGR. It was therefore decided to test all three. MGR1, MGR2 and MGR3 are abbreviations for the three different genes that were inserted into plasmids, along with the genes for MGS and GDH (see section 3.4). For the transformation, a shuttle vector that was specially designed for AK17 was used. The vector can be replicated in both *E. coli* and in AK17.

3.3.1. Transformation of AK17

Transformation of AK17 was done both with electroporation and via natural competence, but the bacteria had previously shown positive results with natural competence. The electroporation was done the same way as HG-8 transformation (see section 3.2.3).

Natural transformation was done by mixing the plasmids with the cells directly. AK17 cells were grown in hungate tubes, in liquid medium overnight, until they were in the exponential phase. New tubes of 5 mL medium were incubated at 50–65°C. Then 100 µL of AK17 liquid culture was taken from the hungate tube with a syringe. 20 µL of water was mixed with 300 ng of plasmid. This plasmid mixture was drawn into the syringe and the plasmid-cell mixture placed into the new 5 mL medium tubes. The cells were then incubated in a liquid medium overnight and transferred to plates containing antibiotics the day after (see section 3.1.3).

3.4. Cloning of plasmids for AK17 tranformation

A cloned plasmid with MGS-GDH-MGR gene combination for the AK17 transformation was available before this study started. This transformation did not work and therefore three additional plasmids were constructed where the MGS was placed at the end of the gene combination in order to reduce its expression, thereby reducing the toxic effects of methyl

glyoxal accumulation. These plasmids were denoted pAM23, pAM24 and pAM25 and contained GDH-MGRx-MGS, with x = 1,2,3. Finally, new plasmids were constructed, where the MGS was excluded, only GDH and the three different MGRs (Figure 4). These plasmids had already been constructed at Matís. Each gene was amplified with PCR and then purified from the agarose gel, before being inserted into the plasmid with Gibson assembly. The amplification of plasmids pAM27 and pAM28 in *E. coli* was a part of this project.

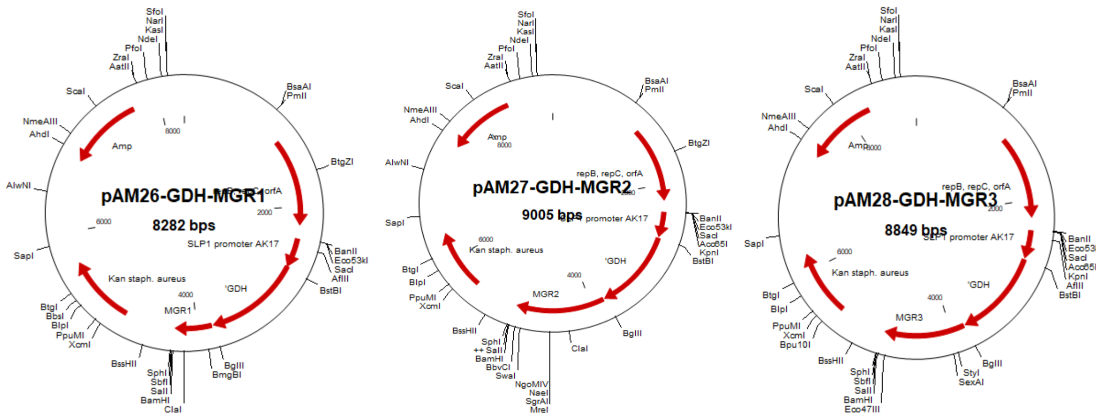

Figure 4: pAM26, pAM27, pAM28 plasmides, containing GDH and MGR genes, used for AK17 transformation (Figure courtesy of Antoine Moenaert).

The replicating shuttle vector pMU131 was used for the plasmid construction. The plasmid is 6403 bp and contains Gram-positive origin of replication, isolated from a native plasmid of *Thermoanaerobacterium saccharolyticum*. It also has thermostable kanamycin resistance marker, the pUC origin of replication and ampicillin resistance (Shaw, Hogsett and Lynd, 2010).

3.4.1. Transformation of *E. coli*

The plasmids were transformed into *E. coli* for amplification. Transformation was done via heat shock with stable, competent, *E. coli* cells. The cells were taken from the -80°C freezer and thawed on ice for 10 minutes. 25 µL of cells were mixed with 1 µL of 20 ng plasmid DNA in Eppendorf tubes by flickering gently. The mixture was kept on ice for 30 minutes. Heat shock was done at exactly 42°C for exactly 30 seconds. This should induce cell membrane permeability, enabling the plasmids to get into the cell. Then the *E. coli*-plasmid-DNA mixture was placed on ice for 5 minutes. 950 µL of SOC medium was added and the mixture was placed in thermomixer at 30°C at 700 rpm for 60 minutes. The agar plates containing antibiotics (ampicillin and kanamycin) were warmed to 30°C and 50, 200 and 700 µL cell mixture were placed on three different plates and spread out with glass beads. Plates were incubated at 30°C for 24 hours. Colonies were picked for colony PCR. Positive colonies were cultivated on new plates for about 24 hours. The transformed *E. coli* was cultivated in a liquid culture of LB medium and antibiotics (ampicillin and kanamycin) at 30°C, overnight for amplification of the plasmids. The plasmids were then extracted from

the *E. coli* cells with plasmid miniprep and digested with restriction enzyme (*Xba*I) before they were analyzed with PCR and gel electrophoresis.

Cells with positive plasmids according to PCR results, were stored in glycerol stock solution (LB-medium with 10% glycerol) at -80°C. When plasmids were used, they were first isolated and purified using Monarch Plasmid Miniprep Kit according to manufactures protocol (New England Biolabs, 2020).

3.5. Verification of experimental results

The experimental results need to be tested to find out if the metabolic engineering worked and to select cells that carry the target gene insertion. The transformed bacteria were analyzed with PCR and gel electrophoresis to verify that the transformation had indeed worked as planned. The fermentation products from positive cultures were quantified to see if the transformation had any effects on production.

3.5.1. Colony PCR

After incubation of plated cultures, single colonies were analyzed to find out if the E. coli, AK17 or HG-8 transformation had been successful. The first step was to amplify the DNA fragment of interest from the cells, this was done with colony PCR. Colonies from the plates were suspended in 30 µL dH2O and master mix 1 was prepared for all colonies. Standard Taq DNA polymerase (from New England Biolabs) was added to master mix 2 just before it was added to the master mix 1 for each sample. Table 1 shows the amount of each component used for the PCR.

Table 1: Components used for a 20 µL colony PCR reaction.

Master mix 1	Master mix 2
1 µL Template DNA	0.1 µL Standard *Taq* Polymerase
0.5 µL 10 µM Forward Primer	0.5 µL dNTP
0.5 µL 10 µM Reverse Primer	2.0 µL Standard *Taq* buffer
8.0 µL dH_2O	7.5 µL dH_2O

The samples were prepared on ice and colonies partly diluted in 30-50 µL dH2O. Remains of PCR positive colonies were transferred to a liquid medium or inoculated onto a plate. The plasmid used for transformation was used as a positive PCR control and dH2O as a negative control. The thermocycler was programmed for the PCR reaction according to table 2.

Table 2: Thermocycler program for colony PCR.

Step	Temperature	Time	Cycles
1. Initial Denaturation	95◦C	10 minutes.	
2. Denaturation	95◦C	20 sec.	35 cycles
3. Annealing	55◦C	20 sec.	
4. Extension	68◦C	1 minutes / kb	
5. Final Extension	68◦C	5 minutes.	
6. Cool down	4◦C	∞	

Tables 3 and 4 show the expected size of the amplified DNA fragments for HG-8 transformation. For comparison, the *adhE* gene is 2600 bp and *ldh* gene 930 bp. The whole amplification of 5´region + *ldh* + 3' region would be about **1996 bp** for non-transformed HG-8 tested for *ldh* knock out. The non-transformed HG-8 tested for *adhE* knock out would be **3713 bp** for 5´region + *adhE* + 3' region. Table 5 shows the expected size of the heterologous genes inserted into AK17.

Table 3: Forward and reverse primers used for the PCR amplification of pAM15 – Lactate (ldh) knockout and size of the amplified DNA fragments with the kanamycin gene. The 5´region is 555 bp, 3´region 511 bp and the kanamycin gene 812 bp.

Ldh KO for HG-8	Forward Primer	Reverse Primer	Base Pair Size
PCR 2: kanamycin gene	*Fwd kan 5´ldh-HG8*	*Rev kan 3´ldh-HG8*	**812 bp**
PCR 1+2: 5´region and kanamycin gene	*Fwd 5´ldh-HG8*	*Rev kan 3´ldh-HG8*	**1367 bp**
PRC 2+3: kanamycin gene and 3´region	*Fwd kan 5´ldh-HG8*	*Rev 3´ldh-HG8*	**1323 bp**
PCR 1+2+3: 5´region, kanamycin gene and 3´region	*Fwd 5´ldh-HG8*	*Rev 3´ldh-HG8*	**1878 bp**

Table 4: Forward and reverse primers used for the PCR amplification of pAM14 – Ethanol (adhE) knockout and size of the amplified DNA fragments with the kanamycin gene. The 5´region is 556 bp, 3´region 557 bp and the erythromycin gene 777 bp.

AdhE KO for HG-8	Forward Primer	Reverse Primer	Base Pair Size
PCR 2: erythromycin gene	*Fwd erm 5´adhE-HG8*	*Rev erm 3´adhE-HG8*	**777 bp**
PCR 1+2: 5´region and erythromycin gene	*Fwd 5´adhE-HG8*	*Rev erm 3´adhE-HG8*	**1333 bp**
PRC 2+3: erythromycin gene and 3´region	*Fwd erm 5´adhE-HG8*	*Rev 3´adhE-HG8*	**1334 bp**
PCR 1+2+3: 5´region, erythromycin gene and 3´region	*Fwd 5´adhE-HG8*	*Rev 3´adhE-HG8*	**1890 bp**

Table 5: Forward and reverse primers used for the PCR amplification of AK17 transformation for each plasmid and size of the amplified DNA fragments. The SLP1 promoter is 376 bp, GDH gene 1151 bp, MGS gene 440 bp, MGR1 gene 465 bp, MGR2 gene 1190 bp and the MGR3 gene 1032 bp.

Plasmid	Genes	Fwd. Primer	Rev. Primer	Size
pAM23	GDH+MGR1+MGS	SLP1	MGS-pmu	**2432 bp**
pAM24	GDH+MGR2+MGS	SLP1	MGS-pmu	**3157 bp**
pAM25	GDH+MGR3+MGS	SLP1	MGS-pmu	**2999 bp**
pAM26	GDH+MGR1	SLP1	MGR1-pmu	**1992 bp**
pAM27	GDH+MGR2	SLP1	MGR2-pmu	**2717 bp**
pAM28	GDH+MGR3	SLP1	MGR2-pmu	**2559 bp**

3.5.2. Gel electrophoresis

PCR products were analyzed with agarose gel electrophoresis and SYBR safe DNA gel stain in 10x TAE buffer 5 µL of loading dye was added to each tube and the samples were loaded to the gel with 1 kb ladder as a size reference. Small gel was to run at 90 V for 40 minutes, medium gel at 100 V for 40 minutes and large gel at 150 V for 40 minutes. The DNA fragments were then visualized in UV-light.

3.5.3. High-performance liquid chromatography

HPLC analysis was performed on the HG-8 knock-out strains. Colonies positive according to PCR analysis where cultured in 5 mL tubes overnight and then inoculated in 10 mL tubes and incubated again. 1 mL samples were taken from the 10 mL tubes before inoculation (T0) and then again about 16 hours later or until the cultures are fully grown (T1). The samples were analyzed with ion chromatography, a modified HPLC. The output is g/l for each substrate. Given the amount of glucose in the medium at the beginning of cultivation, the yield can be calculated for each substrate as g/g glucose.

4. Results and discussion

The main results of the experiments are discussed in this chapter. The results from the experiments with lactate and ethanol knockouts in HG-8 are described in section 4.1 and the experiments involving AK17 and expression of the 1,2-propanediol pathway are described in section 4.2.

4.1. Lactate and ethanol knockouts in HG-8

Transformation experiments were done on HG-8 to knock out the lactate dehydrogenase gene (*ldh*) and aldehyde-dehydrogenase gene (*adhE*), with the aim to increase 1,2-propanediol production. Yield for each fermentation compound produced in different HG-8 strains was calculated from the HPLC results (Table 6).

Table 6: The yield of lactate, acetate, 1,2-propanediol and ethanol in the HG-8 wild type and the engineered strains. Table includes cultural results from two different lactate mutant (Δldh) clones, and three different ethanol mutant clones. The yield for each fermentation product is calculated from the HPLC measurements as the proportion between the compound and glucose used (g/g).

HG-8 strain	Lactate/ glucose (g/g)	Acetate/ glucose (g/g)	1,2-PD/ glucose (g/g)	Ethanol/ glucose (g/g)
Wild Type	0.038	0.200	0.029	0.249
Lactate mutant (Δldh)	0	0.221	0	0.28
Lactate mutant (Δldh)	0.002	0.215	0	0.269
Lactate mutant (Δldh)	0.004	0.21	0.022	0.266
Ethanol mutant	0.028	0.196	0.032	0.240
Ethanol mutant	0.026	0.186	0.019	0.236

4.1.1. HG-8 lactate knockouts

The knockout of lactate dehydrogenase (the *ldh* gene) in HG-8 should stop lactate production in the new strain. The knockout was confirmed with PCR (Figure 5). The strain contained the kanamycin resistance gene, which was used as a selective marker, but not the *ldh* gene. Analysis with HPLC showed that two of the lactate mutant (Δldh) clones were still producing lactate but, in less quantity, than the wild type (Table 6). This indicates that there is another pathway in HG-8 that produces lactate or there is an isozyme of lactate dehydrogenase in HG-8 that has yet to be identified. The production of 1,2-propanediol did not increase. On

the contrary, two Δldh clones produced no 1,2-propanediol, contrary to prediction by the AK17 model. There was, however, a small increase in ethanol production (Table 6). Thus, the lactate mutant (Δldh) prefers ethanol production over 1,2-propanediol.

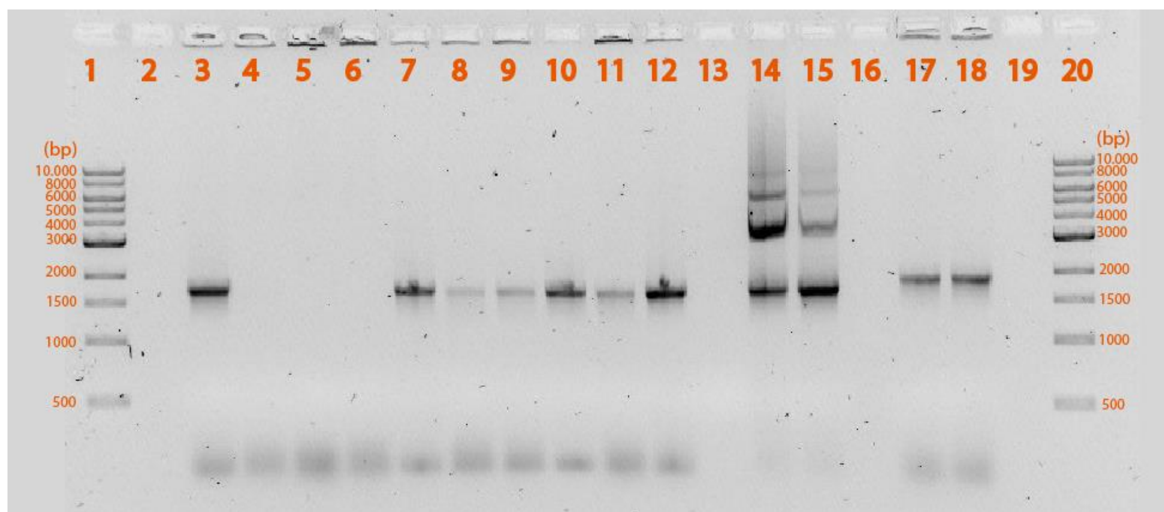

Figure 5: Gel electrophoresis of PCR (PCR 1+2+3) verification test of the ldh gene knockouts (plasmid pAM15) in HG-8. Lanes 1 and 20 show 1 kb ladder. Lanes 3 to 12 are different clones of the lactate knockout transformation. Lanes 14 and 15 are the pAM15 positive control and lanes 17 and 18 are HG-8 control. The expected size of lactate knockouts containing the kanamycin gene is 1878 bp and expected size of the HG-8 control, still containing the ldh gene, is 1996 bp. Lanes 3,7,8,9,10,11 and 12 show clones containing the ldh knockout.

4.1.2. HG-8 ethanol knockouts

For the ethanol knockout transformation, only a single crossover was obtained, which was confirmed with PCR. The obtained clones contained both the *adhE* gene and the erythromycin gene, which was used as a selective marker (Figure 6). HPLC results showed that the amount of fermentation products (there among ethanol) in the engineered strain was similar to the wild type (Table 6). This indicates that the gene is still expressed in the strain and that single crossover is not sufficient to change the metabolic pathway.

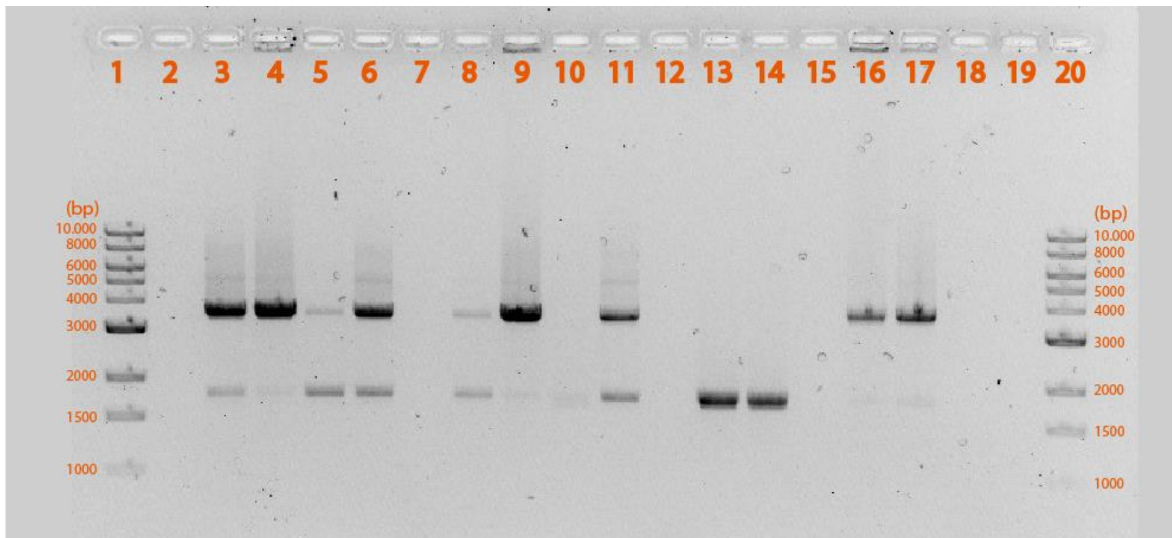

Figure 6: Gel electrophoresis of PCR (PCR 1+2+3) verification test of the adhE gene knockouts (plasmid pAM14) in HG-8. Lanes 1 and 20 show 1 kb ladder. Lanes 3 to 11 are different clones of the ethanol knockout transformation. Lanes 13 and 14 are the pAM14 positive control and lanes 16 and 17 are HG-8 control. The expected size of ethanol knockouts containing the erythromycin gene is 1890 bp and expected size of the HG-8 control, still containing the adhE gene, is 3713 bp. Lanes 3,5,6,8 and 11 show clones containing the erythromycin gene but all of them also have the adhE gene.

Serial passage was done on the clone obtained from the *adhE* knockout transformation, with the aim to get a double crossover mutant. The original transformed strain (ethanol mutant (A)) was inoculated into a medium containing erythromycin and cultivated overnight. The erythromycin culture was then inoculated into a new tube without erythromycin and cultivated. These two steps were repeated one more time. This experiment was unsuccessful, the serial passaged strain (ethanol mutant (B)) did still contain the *adhE* gene according to PCR results (not shown here).

4.1.3. 1,2-propanediol production in HG-8 in phosphate limited medium

The culture medium can affect the growth and metabolic flux. Studies have shown that methylglyoxal synthase activity can be inhibited by phosphate (Tran-Din and Gottschalk, 1985). Therefore, we decided to test if a phosphate limited medium would increase 1,2-propanediol production of the engineered strains.

The HG-8 wild type and the engineered strains, lactate mutant (Δldh), ethanol mutant(A) and (B), were grown on a phosphate limited medium, in an attempt to increase the 1,2-propanediol production. Two liquid cultures were made for each HG-8 strain. T0 and T1 samples were taken from each culture to get analyzed by HPLC (results can be found in appendix B). The average yield was calculated from the HPLC results (Table 7).

Table 7: The yield of 1,2-propanediol, ethanol, lactate and acetate in the HG-8 wild type and engineered strains, lactate mutant (Δldh), ethanol mutant (A) and (B). The yield for each fermentation product is calculated from the HPLC measurements as the proportion between the fermentation compound and glucose used (g/g).

HG-8 strain	1,2-PD / glucose (g/g)	Ethanol / glucose (g/g)	Lactate / glucose (g/g)	Acetate / glucose (g/g)
Wild Type	0.224	0.136	0.564	0.263
Lactate mutant (Δldh)	0.124	0.214	0.379	0.240
Ethanol mutant (A)	0.256	0.080	0.593	0.167
Ethanol mutant (B)	0.207	0.149	0.499	0.254

1,2-propanediol production was observed in all HG-8 strains. The lactate mutant (Δldh) still produced lactate, but in lower amount than the wild type. The lactate mutant (Δldh) also produced less 1,2-propanediol than the wild type but almost double amount of ethanol. From these results it appears that by knocking out the *ldh* gene from the HG-8 genome the 1,2-propanediol production does not increase. The preferred end product for glucose utilization is in this case ethanol. One possible explanation is that the pyruvate production does not change in the engineered strain, and when lactate dehydrogenase is not available the ethanol pathway is chosen. This does, however, not explain why the strain produces less 1,2-propanediol than the wild type. Another possible explanation is NADH / NADPH balancing. Lactate dehydrogenase requires one NADH that is not used when the lactate dehydrogenase pathway is not active. Glycerol dehydrogenase is NADH dependent but the methylglyoxal reductase NADPH dependent. Alcohol dehydrogenase does however require two NADH but no NADPH.

The ethanol mutant(A) produced less ethanol than the wild type and has higher 1,2-propanediol yield. The serial passaged strain, ethanol mutant(B) did, however, not decrease its ethanol production and it produced a little bit less 1,2-propanediol than the wild type. Both the ethanol mutants were growing slower than the wild type and lactate mutant (Δldh), and even though they were cultivated for a longer time they did not utilize all the glucose. So, the cultivation time for the ethanol mutant is noticeably longer than the wild type.

The experiments with the phosphate limited medium were also obtained with the goal of getting a double crossover for the ethanol mutant. This change in the medium did not have the desired effect on the genome.

4.2. Expressing the 1,2-propanediol pathway in AK17

The construction of pAM27 and pAM28 plasmids containing GDH and MGR was verified with PCR and positive plasmids were transformed into *E. coli* for amplification. Plasmids were extracted from the *E. coli* cells with plasmid miniprep and cells containing the plasmid according to PCR results were stored in glycerol stock solution in -80°C.

The pAM23, pAM24, pAM25 (GDH-MGR-MGS) plasmids were introduced to strains AK17 wild type, AK17 ethanol mutant and HG-8 wild type with natural competence and electroporation. After cultivation on plates containing 100 µg/mL kanamycin, several clones were obtained. However, PCR results showed that the clones did not contain the GDH-MGR-MGS genes. The transformation was repeated with 200 µg/mL kanamycin, but then no growth was observed. This indicates that the bacteria did not survive the transformation, or 200 µg/mL kanamycin is to high concentration.

Since the transformation with the pAM23-25 plasmids was not successful, the focus shifted to the plasmids without the MGS gene. These plasmids were constructed because there is already an MGS gene present in AK17, with unknown activity. The hypothesis was that the MGS gene is active in AK17, and when the MGS gene from HG-8 is also present, the strain might be overexpressing MGS. This might cause an increased production of the toxic compound methylglyoxal, that can have lethal effect on the bacterium.

To identify a suitable concentration of kanamycin, natural competence transformations were performed on HG-8 and AK17 WT with pAM26 (GDH-MGR1), positive control (PMU171 and PMU131 respectively) and negative control. With 0, 100 and 200 µg/mL kanamycin (Table 8).

Table 8: Effects of kanamycin concentration on transformants. Growth of HG-8 and AK17 WT with pAM26, positive control (PMU171/PMU131) and negative control (C-). Kanamycin concentrations were 0, 100 and 200 µg/ml. Growth on the petri dishes corresponded to fully covered plates or countable number of colonies.

		no kan	100 µg/mL kan	200 µg/mL kan
AK17	pAM26	0	0	~10
	pMU131	covered	covered	~100
	C-	covered	covered +	0
HG-8	pAM26	covered	covered	>10
	pMU171	covered	covered	covered
	C-	covered	covered	0

Cultures with the pAM26 plasmid showed weak growth on a medium with 200 µg/mL kanamycin, but the positive controls were growing better at that concentration. This indicates that the colonies have the plasmid and are resistant to kanamycin up to 200 µg/ml. For some reason, the AK17+pAM26 cultures did not grow with 100 µg/mL or no kanamycin, but there we would have expected to have fully grown plates. The most likely explanation for this is that we made some mistake transferring bacteria to the plates. Positive colonies from pAM26 on 200 µg/mL kanamycin culture, both AK17 and HG-8, were placed in 5 mL liquid cultures. PCR from these cultures were, however, negative for the GDH-MGR1 genes, so the transformation was not successful.

Repeated pAM26-28 transformation experiments on 200 µg/mL kanamycin, with fresh medium and a new kanamycin batch showed either no growth or when there was growth the colonies did not have the target genes according to PCR results.

4.2.1. Transforming the plasmid backbone into AK17

To verify that the backbone can be transformed into AK17, a natural competence transformation was performed with pMU131, using different kanamycin concentrations (0, 120, 140, 160, 180, 200 and 220 µg/ml). Similar growth was observed on all the plates containing kanamycin whereas the expectation was that growth would be reduced with increased concentration. This indicates that the kanamycin resistance gene was inserted, and the mutant tolerates concentrations up to 220 µg/mL kanamycin and possibly higher. PCR was done on colonies from 180, 200 and 220 µg/mL kanamycin plates, with all of them showing that the kanamycin resistant gene was present, indicating that the transformation was successful (Figure 7).

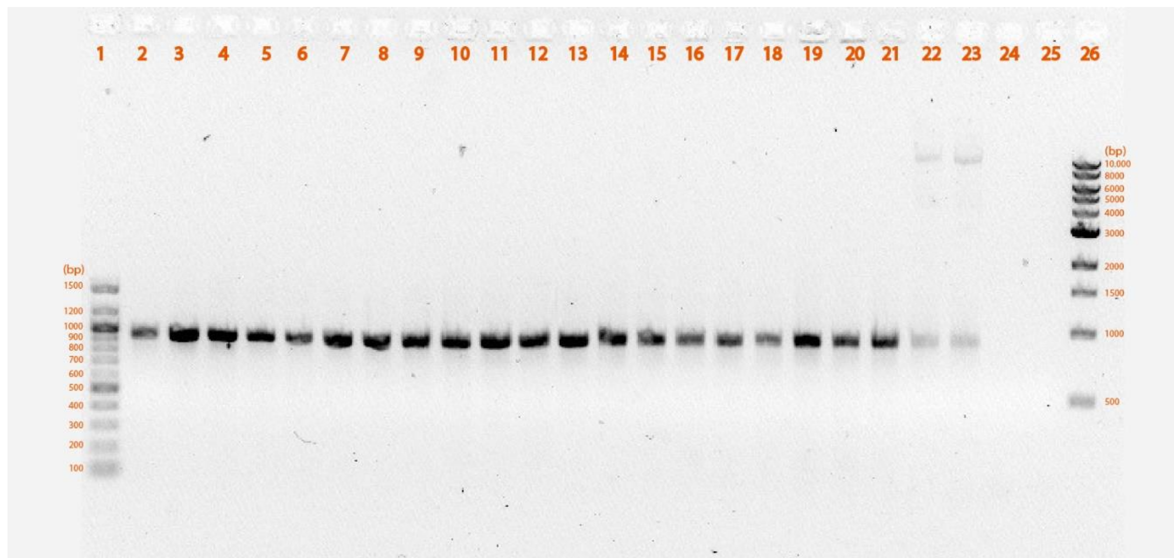

Figure 7: Gel electrophoresis of PCR verification test of the pMU131 transformation on AK17. Lane 1 shows 100 bp ladder and lane 26 shows 1 kb ladder. Lanes 2 to 21 are different colonies of the pMU131 transformation. Lanes 22 and 23 are the pMU131 positive control and lanes 24 and 25 negative control. The clones were tested for the kanamycin gene, expected size of transformed clones containing the kanamycin gene is 795 bp. All the lanes show clones containing the gene.

These results indicate that there is something in the constructed plasmids that is lethal to AK17. Possibly because the plasmids have mutated or there is something in the transformed genes, or expression of the genes in AK17, that is having lethal effect on the bacteria. It is also possible that the mutants need less kanamycin to grow, something between 100 and 200 µg/ml. Even though the pmu131 transformed AK17 can grow at up to 220 µg/mL kanamycin concentration.

5. Conclusions

Metabolic engineering is a challenging task. Metabolism of bacteria has evolved to optimize cell growth and maintenance. When we try to interfere with this process with metabolic engineering the regulation of the bacteria will try to counteract the changes to keep homeostasis. The main aim of this project was engineering the *Thermoanerobacterium* strains AK17 and HG-8, by heterologous gene expression (AK17) and homologous recombination knockouts (HG-8), to increase 1,2-propanediol production. In the case of AK17 the aim was to obtain 1,2-propanediol production and in the case of HG-8 the aim was to increase it.

For HG-8, the focus was on knocking out genes that contribute to the production of fermentation byproducts, other than 1,2-propanediol. HG-8 produces ethanol and lactate in significant amounts. The *ldh* gene codes for lactate dehydrogenase that catalyzes lactate production. HG-8 was transformed with the goal to knock out the *ldh* gene. This transformation was successful. Lactate production of the newly constructed strain did decrease. However, there was no increase in 1,2-propanediol production.

The *adhE* gene codes for aldehyde-alcohol dehydrogenase that catalyzes ethanol formation in HG-8. Another transformation was made on HG-8 with the goal to knock out the *adhE* gene. This transformation was partly successful, the strain did still express the *adhE* gene but also the antibiotic resistance marker, the erythromycin gene. The transformation did therefore only result in a single crossover. This engineered strain did show promising results because it produced more 1,2-propanediol than the wild type. Experiments to obtain a double crossover were unsuccessful.

For AK17 the aim was to insert genes from HG-8, that catalyze the missing steps of 1,2-propanediol production. The genes code for methylglyoxal synthase that catalyzes the conversion of DHAP into methylglyoxal, methylglyoxal reductase that catalyzes the conversion of methylglyoxal into acetol and glycerol dehydrogenase that converts acetol to 1,2-propanediol. These genes were transformed into a plasmid containing the thermostable kanamycin resistance marker. This transformation was unsuccessful. Possible reasons are that there is something in the genes, or the genes composition, that has a lethal effect on AK17. The plasmids could also be mutated.

To get a better picture of the effects that the transformed genes have on AK17, further experiments are needed. One option would be to do a control experiment on the pAM26-28 plasmids (GDH + MGR). This can be done by transforming HG-8 with the plasmids. If the bacteria do not grow, it indicates that overexpression of the genes might have toxic effects on HG-8, or that the plasmid has mutated. If the bacteria grow with the transformed plasmids, we might increase 1,2-propanediol production in HG-8, but the genes are most likely toxic for AK17.

To obtain a better understanding of the 1,2-propanediol metabolic microbiology in AK17, it can be tested for enzyme activity. By testing whether the AK17 produces acetol natively, it is possible to find out if only glycerol dehydrogenase is needed for 1,2-propanediol production. After that methylglyoxal can be added to the medium to see if there is a methylglyoxal reductase activity in AK17. Acetol production would indicate that there is only need to express glycerol dehydrogenase and methylglyoxal synthase. These

experiments need to be done on phosphate free medium because phosphate can inhibit methylglyoxal synthesis activity. If a viable transformant is obtained from further experiments, it would be interesting to measure transcriptomics or proteomics information, to better understand the transformation bottlenecks.

Obtaining a double crossover ethanol mutant for HG-8 is important. One experiment could be serial passage on glycerol with phosphate free medium. Glycerol is more reduced than glucose and needs to be oxidized (by NAD+) before entering the glycolysis. Using glycerol might therefore help with NADH / NADPH balancing. Another future HG-8 experiment is to obtain a double lactate/ethanol knockout mutant. This could potentially shift the metabolic flux towards more 1,2-propanediol production.

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Appendix A

The content of each solution in the liquid and agarose medium for HG-8 and AK17.

- AB-medium, 900 mL includes: 50 mL AB -Buffer solution, 2 g YE, 850 mL dH_2O and 0.5 mL Solution C (Resazurin).
- Glucose, 100 mL includes: 18.016 g glucose and 100 mL dH_2O
- C1, 50 mL includes: 12.5 mL solution D, 1 mL solution F, 1 mL solution G, 35.5 mL dH_2O.
- C2, 10.5 mL includes: 9 mL solution E, 0.5 mL Cysteine chloride, 1 mL solution H.
- AB -Buffer solution, 274 mL includes: 108 mL Solution A, 29 mL Solution B, 137 mL dH_2O.
- Solution A, 500 mL includes: 138 g NaH_2PO_4 and 500 mL dH_2O.
- Solution B, 500 mL includes: 138 g Na_2HPO_4 and 500 mL dH_2O.
- D, 1 l includes; 24 g NaCl, 24 g NH_4Cl, 8.8 g $CaCl_2$ x 2 H_2O, 8 g $MgCl_2$ x 6 H_2O, 1 l dH_2O.
- E, 250 mL includes; 20 g $NaHCO_3$, 250 mL dH_2O.
- F, 1 l includes; 10 mL HCl 25% (7.7 M), 2 g $FeCl_2$ x 4 H_2O, 0.5 g EDTA, 0.03 g $CuCl_2$, 0.05 g H_3BO_3, 0.05 g $ZnCl_2$, 0.05 g $MnCl_2$ x 4 H_2O, 0.05 g $(NH_4)_6Mo_7O_{24}$ x 4 H_2O, 0.05 g $CoCl_3$ x 6 H_2O, 0.03 g Na_2WO_4, 0.03 g Na_2SeO_3, 990 mL dH_2O.
- G, 1 l includes; 2 mg D-Biotin, 2 mg Folic acid, 10 mg Pyridoxine-HCl (B6), 5 mg Thiamine-HCl (B1) x 2 H_2O, 5 mg Riboflavin (B2), 5 mg Nicotinic acid, 5 mg D-Ca-Pantothenate (B5), 0.1 mg Vitamin B12, 5 mg P-Aminobenzoic acid, 5 mg Lipoic acid/Thioctic acid, 1 l dH_2O.
- H, 50 mL includes; 12.01 g Na_2S x 9 H_2O, dH_2O 50 ml.

Appendix B

HPLC results from the HG-8 wild type (WT), lactate mutant (Δldh) and the ethanol mutants (-adhE (A) and (B)) cultivated on a medium without phosphate.

	Lactose (g/L)	Glucose (g/L)	Manni-tol (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	1,2-PD (g/L)	Ethanol (g/L)
T0 A.	0	3.791	0	0.411	0	0	0
T0 B.	0	3.69	0	0.399	0	0	0
T1 HG8 WT A.	0	0	0	0.718	0.985	0.8521	0.495
T1 HG8 WT B.	0	0	0	0.627	0.979	0.8236	0.519
T1 HG8 Δldh A.	0	0	0	0.501	0.908	0.4651	0.816
T1 HG8 Δldh B.	0	0.276	0	0.482	0.824	0.4308	0.728
T1 HG8 ethanol mutant(A). A.	0	0.912	0	0.717	0.461	0.7083	0.225
T1 HG8 ethanol mutant(A). B.	0	1.047	0	0.763	0.458	0.7024	0.215
T1 HG8 ethanol mutant(B). A.	0	0	0	0.64	0.932	0.7538	0.557
T1 HG8 ethanol mutant(B). B.	0	0	0	0.574	0.967	0.7952	0.554