Master of Science in Natural Resource Sciences- Biotechnology

Bioethanol:

production of ethanol with anaerobic thermophilc mutant strains

Sigríður Helga Sigurðardóttir

Supervisors: Ólafur Héðinn Friðjónsson, Guðmundur Óli Hreggviðsson and Jóhann Örlygsson.

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You do not really understand something unless you can explain it to your grandmother
- Albert Einstein

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Declaration

I hereby declare that I am the only author of this thesis and it is the product of my own research.

Sigríður Helga Sigurðardóttir

It is hereby confirmed that this master thesis is satisfactory to M.Sc.-degree from the Faculty of Business and Science, department of Natural Resource Science.

Dr. Ólafur Héðinn Friðjónsson

Dr. Guðmundur Óli Hreggviðsson

Abstract

Thermophilic microorganisms have been considered promising ethanol producers for decades, especially for their potential to ferment variety of carbohydrates. Species of the genera *Thermoanaerobacter* and *Thermoanaerobacterium* have gained a special interest in this respect, not only due to their efficient utilization of potentially inexpensive substrates for industrial fermentation but also because of their capacities to be genetically engineered.

In this project mutant strain from *Thermoanerobacterium islandicus* (strain AK_{17}) was designed and developed. The objective was to make a mutant strain that produces more ethanol and less of other by-products compared to the wild type strain during fermentation of glucose. Lactate dehydrogenase knockout strains, Δ*ldh*, were designed and constructed. Also, alcohol dehydrogenase deletions and an *adh* insertion cassette were designed and constructed. Th *adh* insertion cassette consisted of *adh* gene and kanamycin gene with the flanking sequences of *ldh,* enabling replacement of the *ldh* with the *adh*. The knockout of *ldh* gene in the mutant Δ*ldh* strain was confirmed with a PCR and a Southern blot. HPLC analysis of by-products showed that the mutant Δ*ldh* strain produces almost no lactic acid and more ethanol (10%) than the wild type strain. Attempt to knockout the *adh* gene for creating a mutant Δ*adh* strain was not successful, which may indicate that the *adh* gene in AK_{17} is vital. The results show that it is possible to engineer the pathway of glucose fermentation to ethanol with genetic transformation. Accordingly, future work will involve deletion of other genes such as acetate kinase and phosphate acetyltransferase and increasing copy number of *adh* with already constructed insertion cassette, in order to improve ethanol production further.

Keywords: *Thermoanerobacterium islandicus*, ethanol production, lactate dehydrogenase knockout mutant, insertion/deletion cassettes and alcohol dehydrogenase.

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I would like to thank Dr. Ólafur Héðinn Friðjónsson and Dr. Guðmundur Óli Hreggviðsson for giving me the opportunity to work on this project and for their valuable help and guidance. I would also like to thank the staff at Matís, especially Sólveig Ólafsdóttir and Jón Óskar Jónsson for all their help on the research part of this thesis. In addition I would also like to thank my fiancé Sigurður Ari Ómarsson and my parents, Sigurður Guðni Sigurðsson and Margrét A. Jakobsdóttir for all the support.

Akranes

Sigríður Helga Sigurðardóttir

29. May 2009

Útdráttur

Hitakærar loftfælnar bakteríur hafa um árabil verið rannsakaðar með tilliti til notkunar til etanól framleiðslu. *Thermoanaerobacter* og *Thermoanaerobacterium* tegundir þykja sérstaklega áhugaverðar þar sem þær geta nýtt sér fjölbreyttar gerðir sykra og sýnt hefur verið fram á að unnt er að erfðabreyta þeim og hafa þannig áhrif á efnaskipti gerjunar.

Meginmarkmið rannsóknarinnar sem þessi meistararitgerð byggir á var að erfðabreyta *Thermoanerobacterium islandicus* stofni AK_{17} þannig að framleiðsla etanóls ykist og dregið yrði úr framleiðslu á öðrum lokaafurðum við gerjun stofnsins á glúkósa. Hannaðar og smíðaðar voru laktat dehydrogenase og alkohol dehydrogenase úrfellingar kassettur (Δ*ldh* og Δ*adh*) auk innsetningar kassettu með *adh* geni og kanamycin geni með aðliggjandi röðum *ldh.* Stofn AK_{17} var ummyndaður með *ldh* úrfellingar kassettunni og úrfelling á *ldh* geninu var staðfest með PCR mögnun og Southern blot. Mæling með HPLC súlu á afurðum gerjunar sýndi að Δ*ldh* stofninn framleiðir nær enga laktik sýru og meira etanól (10%) en villta týpan. Ekki tókst að fella út *adh* genið í stofni AK_{17}. Líkleg ástæða er að genið er stofninum lífsnauðsynlegt. Niðurstöðurnar sýna fram á að unnt er að hafa áhrif á efnaskipti AK_{17} með erfðabreytingum. Stefnt er því að fella út fleiri gen sbr. acetat kinasa, fosfat acetyl transferasa og auka eintakafjölda *adh* gensins með fyrirliggjandi kassettu, til að auka ethanól framleiðslu stofnsins ennfrekar.

Lykilorð: *Thermoanerobacterium islandicus*, etanól framleiðsla, lakat dehydrogenase erfðabreytur stofn, innsetingar/úrfellingar kassetta og alcohol dehydrogenase.

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1 Research objective

This thesis describes an attempt to improve ethanol production in the anaerobic thermophilic bacterial species *Thermoanerobacterium islandicus* or strain AK_{17} with genetic modifications. The prerequisite for this work was an access to the genomic sequence, information about the corresponding metabolic pathway and further the capability of the strain to be genetically transformed. A low coverage sequencing of the genome was performed. The metabolic pathway could be deduced from related bacteria, and genes encoding enzymes participating in the pathway of glucose fermentation were identified. Transformation protocol was established and transformation was achieved. Thermophilic microorganisms have been considered promising ethanol producers for decades, especially for their potential to ferment variety of carbohydrates. Species of the genera *Thermoanaerobacter* and *Thermoanaerobacterium* have gained a special interest in this respect, not only due to their efficient utilization of potentially inexpensive substrates for industrial fermentation but also because of their capacities to be genetically engineered.

The production of an undesirable by product was abolished and the ethanol production was improved in the resulting mutant. The results show that this bacterium can be genetically modified, with resulting impact on the pathway of glucose fermentation to ethanol.

The results described in an early master's thesis (BioHydrogen: Bioprospecting: Thermophilic hydrogen producing anaerobes in Icelandic hot-springs edited by Steinar Rafn Back Baldursson) showed that the strain AK_{17} later designated *Thermoanerobacterum islandicus*, produced more ethanol than other thermophilic anaerobic bacteria investigated in the project. Accordingly, strain AK_{17} was selected for the research described in this master's thesis.

By analysing metabolic pathways in related bacteria, candidate genes involved in ethanol metabolicm were identified and selected for genetic modification in *T.* anaerobacter. Lactate dehydrogenasi (1.1.1.27) and phosphate acetyl transferase

(6.2.1.1.) were selected for gene knockout and it was decided in increase the copy number of alcohol dehydrogenase gene with insertion chromosomal insertion. Accordingly, the work described in this thesis involved isolating the DNA, genome sequence analysis, finding specific gene, design and developing knockout mutants and insertion mutants. The genotype of the new mutant strains was verified with PCR and Southern blots. The phenotype was analysed with HPLC.

2 Introduction

This chapter is about theoretical review on ethanol production in thermophilic anaerobic bacteria

2.1 Ethanol

Ethanol (figure 1) which is also called; ethyl alcohol, grain alcohol, abbreviated EtOH, and has the chemical formula CH_3CH_2OH. The chemical is made up of couple of chemical compounds where the hydroxyl group bonded to a carbon atom with so-called chemistry bond. It is a liquid that is clear and colourless. Bio ethanol is defined as an ethanol that is made from cellulosic biomass through fermentation[1].

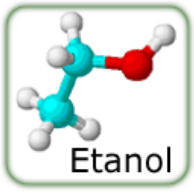


Figure 1 Ethanol[2]

The melt point for ethanol is -114.1°C, the boiling point is 78.5°C and the density at 20°C is 0,789 g/mL[3]. Ethanol is both drinkable and can be used as a fuel[4].

[1] U.S. Department of Energy (4/8 2007)
[2] U.S. Department of Energy (4/8 2007)

One of the most abundant organic molecules in the biosphere is D-glucose and gives energy to most of organisms. The first step in bio ethanol production from glucose is glycolysis is when the glucose is converted to pyruvate. There are 10 steps in the glycolysis and ATP and NADH are produced. The net reaction of glycolysis can be seen in figure 2. The whole glycolysis can be seen in figure 3[5].

$$\textbf{D-glucose} + 2HPO_4^{-2} + 2\ ADP + 2NAD \rightarrow$$

$$CH_3C{=}OCH_2 + 2\ ATP + 2NADH + 2H^+$$

Figure 2 Net reaction of glycolysis[6]

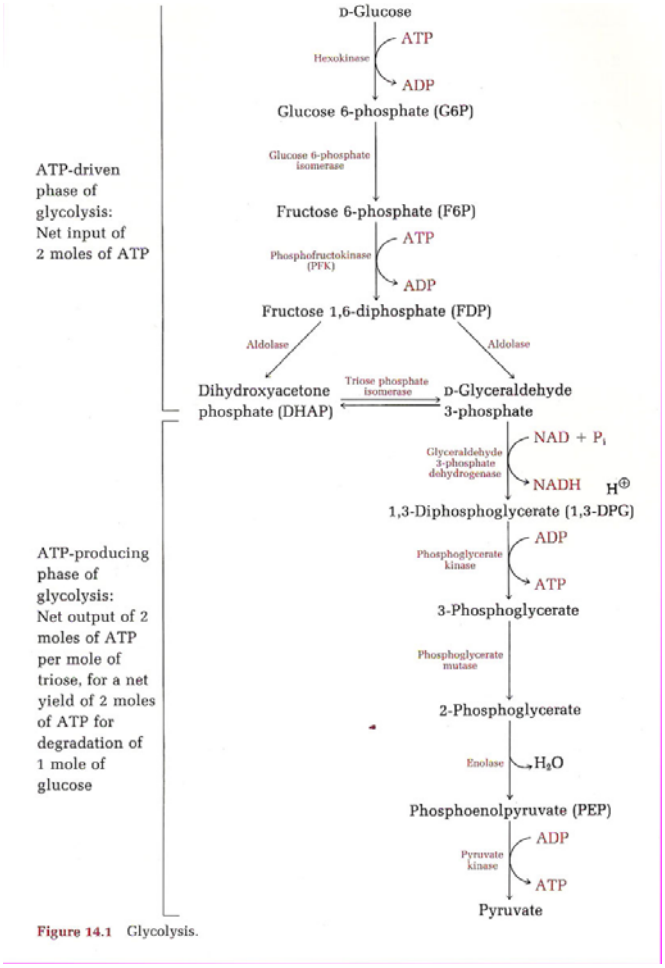


Figure 3 Glycolysis[7]

[3] Shakhashiri (2009)
[4] Keith Addison (e.d)
[5] Rawn, J. David. 1983:554
[6] Rawn, J. David. 1983:554

The glycolysis is done with enzymes that converts glucose to pyruvate and the free energy from glycolysis is –28.5 kJ/mol and the enzymes standard free-energy changes at 298 K. Table 1 shows how must free energy is gotten from each step it is table 14.1 pages 556 in Biochemistry after J. David Rawn[8].

Table 1 Free energy from glycolysis, al 10 step[9]

Enzyme	**Reaction**	**ΔG° (kJ/mol)**
Hexokinase	D-glucose + ATP ↔glucose 6-phosphate + ADP	- 16.74
Glucsose 6-phospate isomerase	Glucose 6-phosphate ↔ fructose 6-phosphate	+ 1.67
Phosphofrutokinase	Fructose 6-phosphate +ATP ↔ fructose 1,6-diphosphate +ADP	- 14.2
Aldolase	Fructose 1,6-disphosphate ↔D-glyceraldehyde 3-phosphate + dihydroxy acetone phospate	+ 23.8
Triose phosphate isomerase	Glyceraldehyde 3-phospate ↔ dihydroxyacetone phosphate	+ 14.6
Glyceraldehydes 3-phosphate dehydrogenise	Glyceraldehydes 3-phospate + P_i + NAD ↔ 1,3-diphosphoglycerate + NADH + H^+	+ 6.3
Phosphoglycerate kinase	1,3-diphosphoglycerate + ADP ↔ 3-phosphoglycerate + ATP	- 18.8
Phosphoglyceratemutase	3-phosphoglycerate ↔ 2-phosphoglycerate	+ 4.6
Enolase	2-phosphoglycerate ↔ phosphoenolpyruvate + H_2O	+1.7
Pyruvate Kinase	Phoshoenolpyruvate +ADP ↔ pyruvate + ATP	-31.8

[7] Rawn, J. David. 1983:555
[8] Rawn, J. David. 1983:554-556
[9] Rawn, J. David. 1983:556

The pyruvate can be utilized under anaerobic or aerobic conditions. When pyruvate is converted under anaerobic conditions many end products are made, one of the end products is ethanol. When ethanol is produced in yeast cells under anaerobic conditions it is done in two steps, the first one is when the thiamine pyrophosphatase converts pyruvate to acetaldehyde and carbon dioxide. The second step is when acetaldehyde is reduced to ethanol with alcohol dehydrogenase, which is a NAD-dependent enzyme. The NADH that is used in the second step comes from the glycolysis when glyceraldehydes 3-phosphoate dehydrogenase converts glyceraldehydes 3-phospate to 1, 3-diphosphoglycerate. The net oxidation of glucose to ethanol can be seen in figure 4 and the energy is –166 kJ/mol[10]. Strain AK_{17} produces ethanol from glucose and it is done anaerobically.

Glucose + $2HPO_4$ + 2 ADP → 2 ethanol + 2 ATP +$2CO_2$

Figure 4 Glucose oxidation to ethanol[11]

2.2 Energy

Energy is one of the most imported things in world that we live in today. Most of energy that is used today is un-renewable and the primary energy is fossil fuels like coal, oil and natural gases. The un-renewable energy affects that the global climate change which leads to a health and natural problems that are the most difficult environment problems today[12].

With the current state of the world, energy problems will cause finance troubles and other problems for many families. Today we have started to realise these problems and the syndromes from them. Most of the population in the world does not have enough energy to meet the basic needs of the community and in most places in

[10] Rawn, J. David. 1983:575-576
[11] Rawn, J. David. 1983:576
[12] Levin et al. 2004:173

the world the energy cost are rising. The energy uses of the past have cost global environmental problems like air pollution, water pollution, ocean pollution and climate change (greenhouse effects). There are risks of uncertainty with energy supplies in the world. Since 1850, the energy use has almost 20-folded when the population has only grown about 52% of the energy growth; this problem is assumed to grow in the next years. Most of the energy that has been used in the past was from burning oil and gas. The search is on for energy sources that are less expensive and/or more enviromentally friendly that oil and gas whish are causing environmental problems with increased global warming and polution[13].

2.2.1 Ethanol as a energy

Ethanol is one of the chemicals that could possibly be used, mixed with gasoline or inset of gasoline as the main source of energy in the world.

The designer of Model T Ford, Henry Ford did design the car to run on alcohol and said that the alcohol was "the fuel of the future". The oil companies did not agree and nobody thought of alcohol as a fuel until the oil crisis in the early 1970s[14].

Petroleum accounts for 97% of energy consumption for transportation usage worldwide today. In the last 20 years both industry and governments all over the world have been searching for alternative transportation fuel sources. In the United States and Brazil have already started to use fermentation –derived ethanol production instead of petroleum. Fuel ethanol is mainly produced by fermentation of corn in the United States but in Brazil it is produced by sucrose. Every country in the world could produce ethanol in similar ways because over the last two decades, the technology for ethanol production from non-food-plant and also in large-scale production has been develped that will hopefully be reality in the next years. In this research corn stover, sugar cane waste, wheat or rice straw, forestry and paper mill discards, the paper potion of municipal waste, and dedicated energy crops (biomass) have been tested for

[13] Holdren, John T. 1991:231
[14] Keith Addison (e.d)

degradation and conversion to ethanol and all these studies have shown that it is possible. The main focus has been on utilizing monomeric sugars from biomass carbohydrates for bio-ethanol production[15].

After 1990 ethanol has been used as oxygenate to mix with gasoline. In every year, the United States uses 2 billion gallons of ethanol to blend it to gasoline[16]. In United States, in 2006, use of ethanol and biodiesel was 4 billion gallons but the plan for 2012 is that the country will be using 7.5 billion gallons[17]. In Brazil ethanol producing from cane sugar is about 12.5 billion litres, which is used in 22% blend fuel or 100% ethanol fuel. About 5 billion litres of ethanol is produced from starch crops, mainly corn, in the United States. These 5 billion litres gives 111 fuelling station ethanol blends[18]. The United States of America is the country that produced 7 billion gallons of cleaner, ethanol-blended gasoline or around 12 present of the total fuel used in the United States, which was the most production in the world in 2007. In the United States, most of the ethanol blends that are used as fuel are E85, which is 85% ethanol and 15% gasoline, or E10 which is 10% ethanol and 90% gasoline. The E10 ethanol bland can be used in most gasoline cars without engine conversion. Brazil also uses ethanol as a fuel and ethanol that is used is at least 24% of total fuel usage or around 4 billion gallons of ethanol each year[19]. In Brazil ethanol fuel is either used as 22% blend or 100% ethanol[20]. In South Africa the use of ethanol blends is increasing. Now many others countries are starting or using an ethanol fuel programs[21].

Ethanol is not only blended with gasoline; it has also been blended with metyl tertiary butyl ether (MTBE). Metyl tertiary butyl ether is oxygenate. Comparing to gasoline this fuel does reduce carbon monoxide levels, by improving overall combustion (oxidation) of the fuel. Although this kind of fuel blend is not allowed in all states of America for example in California because it can contaminate around 1 % of the domestic wells in the state. Ethanol alone has been chosen instead of the blended oxygenates as a fuel that could replace gasoline. The California's environmental consciousness has accepted ethanol as a fuel because of their very

[15] Mielenz, J. R. 2001:324
[16] U.S. Department of Energy (4/8 2005)
[17] U.S. Department of Energy (8/9 2006)
[18] Mielenz, J. R. 2001:324
[19] Keith Addison (e.d)
[20] Mielenz, J. R. 2001:324
[21] Keith Addison (e.d)

different agricultural economy and the lack of any clear fuel alternative. The problem in ethanol production is that this fuel only gives 65-69% of the energy that we can get from same density of hydrocarbon fuels (petroleum). However, anxieties of global warming will continue and using ethanol is better for the environment than gasoline. Ethanol fuel is better because it reduces net carbon dioxide; the ethanol fermentation is already part of the global carbon cycle when petroleum puts extra carbon in the global carbon cycle. The OPEC (oil producing and exporting countries) has also raised the price of petroleum[22].

Today ethanol is made by fermenting and distilling starch or sugar crops like sugar-cane, maize, sorghum, wheat and other and even other part for plats like cornstalks, fruit and vegetable waste[23]. This has caused questions concerning the ethics of utilizing biomass for producing ethanol, which could be used as a food. Some people even say that this biomass could stop the hunger in the world. If the food and ethanol production in Brazil is viewed to see how must area is use for ethanol production in comparison to food production, the ethanol production covers only a very small part of the Brazil area. In Brazil primary food crops are in about 55 million ha and only 4.1 million ha are used for sugarcane. Only 1.7 million ha of these 4.1 million ha were used for ethanol producing that is only about 3 per cent of the total area. Recent studies show that the shortages and increasing prices for food in Brazil is not resulting from ethanol production. The policies of farming export crops and selling out of the country is also a big case for the shortages and price on the food[24].

The benefits that come from using an ethanol with gasoline are many, first to a mention is reduced gasoline usage. Ethanol is better for the atmosphere than the gasoline because ethanol emits less carbon monoxide, so mixing ethanol (or any other oxygenates) to gasoline decrease the emission on carbon monoxide. Ethanol is more expensive in production than gasoline, like it is produced today. In the United States a special tax on the gasoline put the price of ethanol on a competitive level[25]. The use of ethanol as a fuel increases the greenhouse effects in much less extent than the gasoline. Furthermore, ethanol is biodegradable and therefore, has no harmful effects on the environment. The ethanol is a renewable fuel and gives high octane at low

[22] Mielenz, J. R. 2001:324.
[23] Keith Addison. (e.d)
[24] Emil Bedi. (e.d)
[25] U.S. Department of Energy (8/9 2006)

cost. Ethanol mixed with gasoline can be used on all engines without changing them[26]. If it is possible to produce bio ethanol as intended in this project then it will be at least on the same price as gasoline. Then the government will not have to subsidize ethanol so the ethanol is competitive to other source of energy.

2.3 Ethanol production with microorganisms

Ethanol production with microorganisms, which is the topic of the project described in this thesis, is different from when ethanol is made by fermenting and distilling starch or sugar crops like it is done today.

The main problem of producing ethanol by fermentation is the high production cost compared to the local cost of gasoline, even though prices for crude oil have increased which has helped in closing the cost gap. When fermenting microorganisms on sugar to produce ethanol, most of the cost is due to the loss of half of the carbon during the fermentation. Producing ethanol from cane sugar with microorganisms is rather simple but when ethanol is produced from corn or wheat starch the process gets more complicated, these processes need enzymes to hydrolyze starch to glucose prior to fermentation. Using biomass to produce ethanol, the process gets even more complicated because it has to get polymeric sugar from cellulose and hemicellose that is about 23-53% and 20-35 % of plants. Cellulose is beta-linked glucose but most of the sugars in hemicellose are xylose and arabinose including traces of glucose, mannose and galactose. Figure 5 on the next page shows two ways to hydrolyse these carbohydrate polymers. The bottom part of figure 5 shows the conventional hydrolysis, which is done with acid and enzymes. This hydrolysis is called SSF/SSCF process, where the biomass is both ethanol fermented and enzymes produced. In the top part of figure 5, sulfuric acid is used to hydrolyse the biomass carbohydrates.

[26] Keith Addison (e.d)

After hydrolysis the acid is separated from the sugar which is then fermented to ethanol[27].

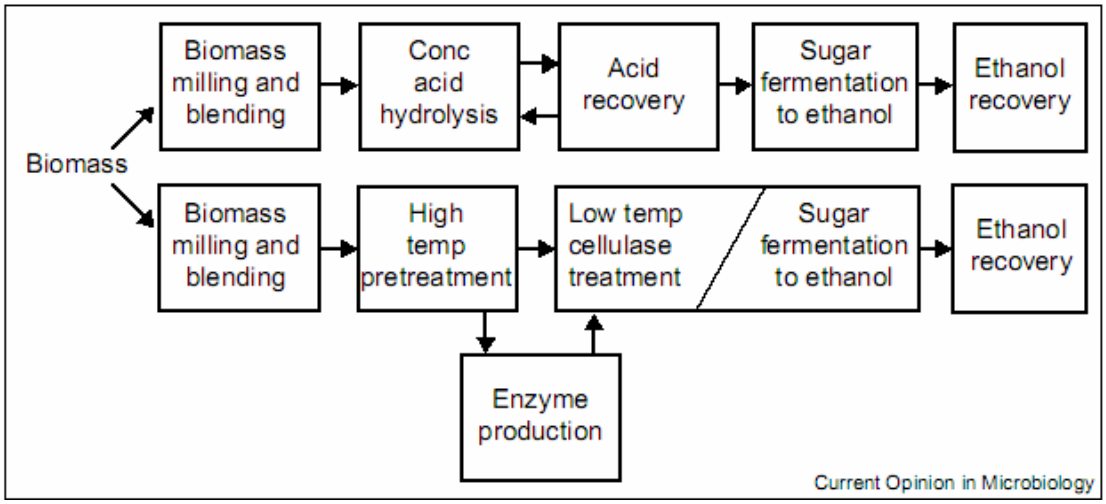


Figure 5 Hydrolyzis on biomass[28]

The SSCF step to ferment a biomass to ethanol can be a challenge since this step involves converting pentose and multiple hexose sugars. A progress has though been made on the process with applying fermentation microorganism; a progress is especially made with the aid of genetic engineering. Fermentation pathways for sugars like glucose, xylose and arabinose have been genetically engineered both in yeats and bacteria. *E. coli* has been genetically engineered for producing ethanol form biomass sugars and *Zymomonas* has been evaluated for its potential in concentrated-acid process[29].

Bio-ethanol can be produced with microorganisms by converting biomass. Numbers of microorganisms have been studied for producing ethanol in the last two decades. Results from different studies were gathered and summarized together in table 2 on the next page, which shows the characteristics and traits for bio ethanol production with microorganisms[30].

[27] Mielenz, J. R. 2001:324-325.
[28] Mielenz, J. R. 2001:325
[29] Mielenz, J. R. 2001:326
[30] Dien, B.S. et al. 2003:258

Table 2 Important traits for ethanol production[31]

Trait	Requirement
Ethanol yield	>90% of theoretical
Ethanol tolerance	>40 g l^{-1}
Ethanol productivity	>1 g l^{-1} h^{-1}
Robust grower and simple growth requirements	Inexpensive medium formulation
Able to grow in undiluted hydrolysates	Resistance to inhibitors
Culture growth conditions retard contaminants	Acidic pH or higher temperatures

Ethanol yield is the trait that has had most attention of all the traits that are mentioned in Table 2. Ethanol yield has been most studied because more than one-third of production cost is used for feedstock, which is important for maximizing ethanol yield. The reason for these studies is to find strains that have a high ethanol yield; these strains then have to produce few side products and metabolize all major sugars. These major sugars are glucose, xylose, arabinose, galactose and mannose[32].

The enzymatic digestion of cellulose produces the most ethanol (highest ethanol yields). Before biomass is enzymatically digested, it is pretreated by heating the biomass in the presence of a mineral acid or bas catalyst. The pre-treatment is performed to hydrolyze the hemicellolse completely or partially, lignin is removed and the cellulose is de-crystallized which gives the enzyme access to the cellulose fibers. The cellulose can be enzymatically digested (saccharification) with following sequenctial fermentation of products or saccharification and fermentation can take place simultaneously (SSF). SSF produces more ethanol and needs fewer enzymes because the end products inhibition is relieved by the fermentation yeasts. SSF needs however the enzymes and culture to have the same temperature and pH. The most active preparations, *Trihoderma reesei* cellulases, have the optimal activity at pH 4.5 and 55°C. When *Saccharomyces* cultures are used to ferment, the SSF is controlled at pH 4.5 and 37°C[33].

In these studies, no strain has been found that meets all the requirements that are mentioned above that are listed in table 2, many interesting discoveries have been

[31] Dien, B.S. et al. 2003:258
[32] Dien, B.S. et al. 2003:258
[33] Dien, B.S. et al. 2003:258

made in the development of ethanol production with microorganisms. Of all the strains that have been tested *Escherichia coli*, *Klebsiella oxytoca* and *Zymomonas mobilis* were considered the best strains to produce ethanol. The fact that all these bacteria are Gram-negative bacteria, indcates that Gram-negative bacteria are better at producing ethanol than Gram-positive. Even though researches on Gram positive and thermophilic bacteria for ethanol fermentation have been performed but the metabolic engineering of these strains is still in its early stages[34].

One of the first metabolic engineering was made on *E. coli* to selectively produce ethanol. *E. coli* has many qualities that are good for producing ethanol; the bacteria can ferment a wide spectrum of sugars, no requirements for complex growth factors and a prior industrial use. The problems that follow using *E.coli* as a producer is that culture are of narrow and neutral pH growth range, culture is also not as hardy as culture of yeast and *E. coli* strains can cause public danger[35].

Figure 6 shows on the next page how *E.coli* produces ethanol and other organic acid from ferments sugars. Fermentation from pyruvat to ethanol involves pyruvate formate lyase (PFL). This kind of fermentation uses two NADH, H^+ but when sugar is converted to pyruvat only one NADH,H^+ is created and this makes the fermentation unbalanced. The fermentation is balanced in *E. coli* by producing acetic and succine acids[36].

[34] Dien, B.S. et al. 2003:258-259
[35] Dien, B.S. et al. 2003:259
[36] Dien, B.S. et al. 2003:259

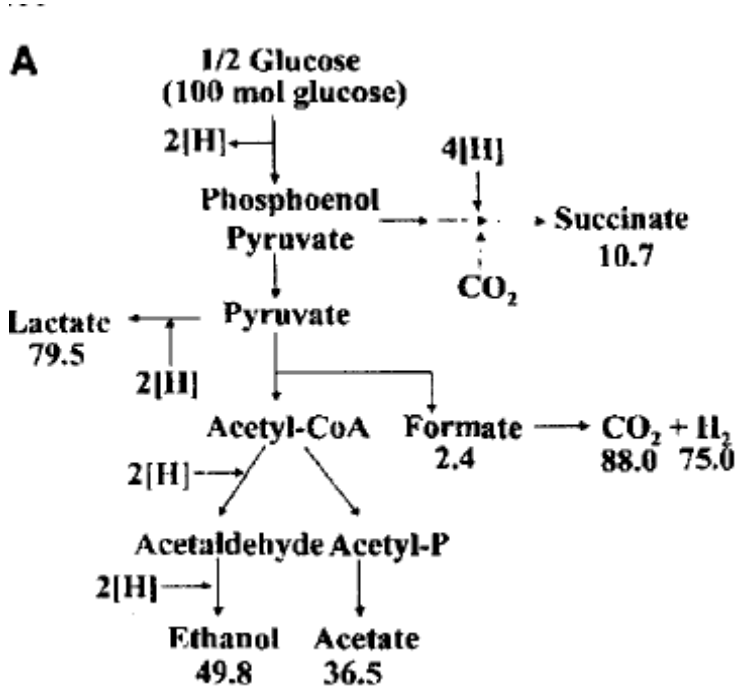


Figure 6 E.coli *fermentation on glucose*[37]

When *Z. mobilis* and yeasts produce ethanol from pyruvat they are defined homo-ethanol fermentative because when they ferment pyruvat into ethanol they utilize pyruvat decarboxylase (PDC) which only uses one $NADH,H^+$ for each ethanol produced. When *pdc* was put into *E. coli* it was expected that the bacteria would only produce ethanol. This experiment did not go as expected because the native alcohol dehydrogenase (ADH) activity of *E.coli* is not sufficient to produce high ethanol yield. Therefore *adhII* was isolated from *Z. mobilis*, it was then added along with *pdc* to a plasmid. The plasmid is used to transform *E.coli* into a mutated strain that almost exclusively produces ethanol. Figure 7 on the next page shows ethanol production in the mutant strain of *E.coli*. Ethanol is produced instead of succinate, production on lactate has decreased from 79.5 to 5.7 and production on acetate has also decreased from 36.5 to 0.7. The genes (*pdc* and *adhII*) were co-expressed under the control of the native *lac* promoter. This growth was called PET (production of ethanol operon)[38].

[37] Dien, B.S. et al. 2003:260
[38] Dien, B.S. et al. 2003:259

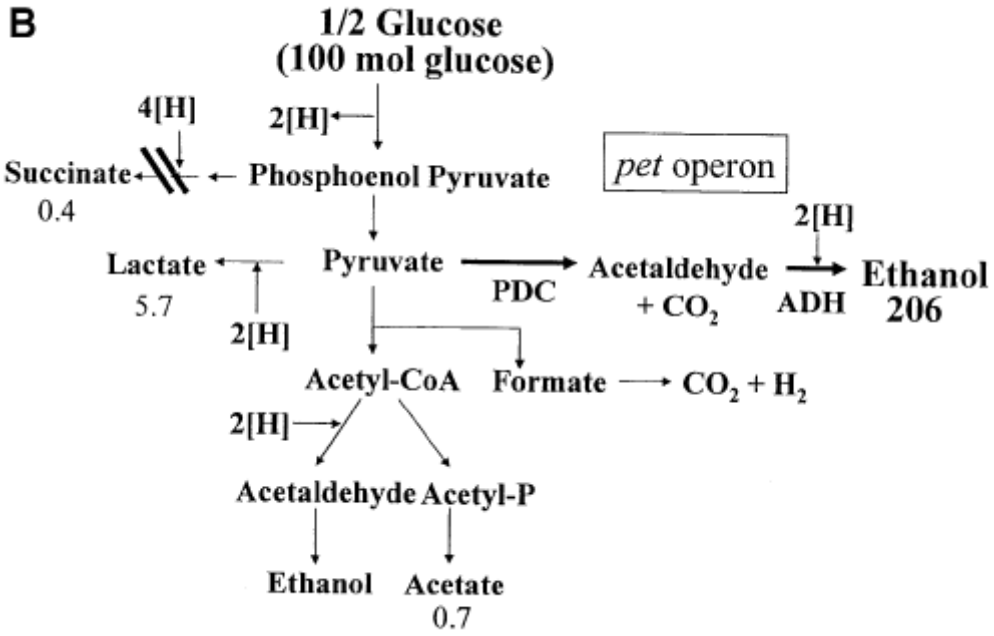


Figure 7 Transformed E.coli with pet *fermentation on glucose*[39]

The unusual Gram-negative microorganism *Z. mobilis* has several appealing properties as a biocatalyst for ethanol production and as mentioned earlier has homo-ethanol fermentation. The ethanol tolerance is up to 120 g/l ethanol in addition to higher ethanol yield (it produces 5-10% more ethanol per fermented glucose than *Saccharomyces*). *Z. mobilis* had also much higher specific ethanol productivity than *Saccharomyces* sp. (2.5 x). Unlike *E. coli*, *Z. mobilis* is a rather safe bacterium (GRAS). In the 1970s and 1980s some researchers stated that ethanol production with *Z. mobilis* would be better than producing ethanol with *S. cerevisiae.* In both cases starch is converted to ethanol. Today the industry prefers to produce ethanol with *S. cerevisiae* because of the yeast hardiness[40].

The *Zymomonas* high ethanol production and yield are because of unique physiology. This bacterium is the only one that uses Entner-Dudoroff (ED) pathway in metabolized glucose anaerobically instead of Embden-Meyerhoff-Parnas (EMP) or glycolytic pathways. ED pathway does not cost as must ATP as EMP pathway, or only half of ATP per mole glucose that are used in EMP. This different pathway causes *Zymomonas* to produce less biomass than yeasts and funnel more carbon to the

[39] Dien, B.S. et al. 2003:260
[40] Dien, B.S. et al. 2003:261

end products. As a direct result from the low ATP yield *Zymomonas* maintains a high glucose flux through the ED pathway.

About 50% of the cell proteins are enzymes that are involved in fermentation and they are constitutively expressed. *Zymomonas* have also a simple nutritional nature; some strains can even live on only pantothenate and biotin. The problem with using *Zymomonas* to produce ethanol is that the strain only ferments glucose, fructose and sucrose but later on strains were found that could ferment also xylose and arabinose. Figure 8 shows how *Zymomonas* ferments xylose to ethanol[41].

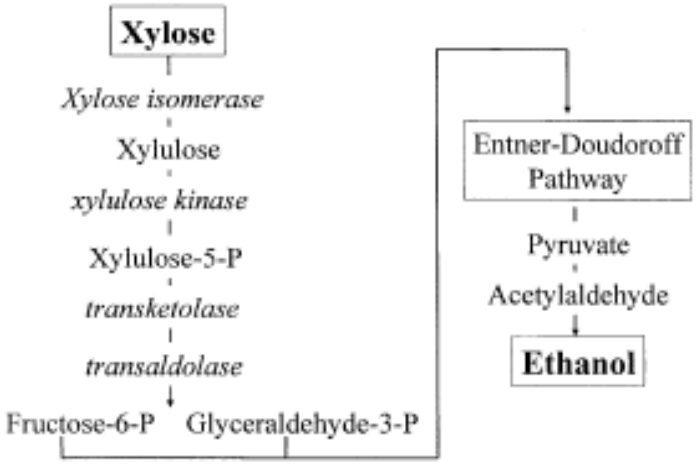


Figure 8 Fermentation Z.mobilis *on xylose*[42]

K. oxytoca and *Erwina chrysanthemi* were also transformed with *pet*. Even though these strains do not produce as much ethanol as *E.coli*, production in the *pet* transformed *K. oxytoca* increased especially for converting cellulose to ethanol. Ethanol production increased by 90% in *K. oxytoca* strain M5A1, that had been transformed with the *pet* operon. The wild strain (of *K. oxytoca*) produced organic acids and neutral products for ferment glucose[43].

Table 3 on the next page shows the ethanol production for fermentation xylose and sugar mixture with ethanolgenic bacteria. These bacteria are three strains of *E.coli*, KO11 that is mutant with *pcdc* and *adhII*, FBR5 that is mutant with *pfl* and *ldh*

[41] Dien, B.S. et al. 2003:261
[42] Dien, B.S. et al. 2003:262
[43] Dien, B.S. et al. 2003:263

and LY01 is a strain of KO11 with extra ethanol tolerance. Two strains of *K. oyxoca* are also in this comparison, M5A1 that is a mutant strain with *pet* and P2 that is a mutant strain M5A1 with *cat* and *pfl* gene. And finally two strains of *Z. mobilis*, AX101 that is a mutant strain that ferments both arbinose and xylose and CP4:pZB5 that is a mutant strain that grows on xylose and has ethanol yield 86%. FBR5 mutant strain of *E. coli* is the one that produces most ethanol when production in g/l per hour is compared.

Table 3 Comparison of fermentation with xylose and sugar mixture for ethanolgenic bacteria strains[44]

Strain	Host	Sugars[a]	Maximum ethanol (g l^{-1})	Ethanol yield (%)	Ethanol production (g l^{-1} h^{-1})	Citation
E. coli	KO11[b]	Xyl 90	41.0	89	0.85	Yomano et al. 1998
		Ara:Gal:Glc:Xyl, 23:11:27:39	41.7	90	0.62	Asghari et al. 1996
	FBR5	Xyl95	41.5	90	0.59	Dien et al. 2000
		Ara:Xyl:Glc 15:30:30	34.0	90	0.92	Dien at al. 2000
	LY01	Xyl 140	63.2	88	0.66	Yomano et al. 1998
K. oxytoca	M5A1(*pLOI555*)	Xyl 100	46.0	95	0.96	Ohta et al. 1991b
	P2	Ara:Xyl:Glc 20:40:20	34.2	84[c]	0.35	Bothast et al. 1994
Z. mobilis	*AX101*	Ara:Glc:Xyl 20:40:40	42[d]	84	0.61	Mohagheghi et al. 2002
	CP4:pZB5	Xyl 60	23.0	94	0.32	Lawford and Rousseau 1999

Thermophilic microorganisms have been considered promising ethanol producers for decades, especially for their potential to ferment variety of carbohydrates. Ethanol production through growth and fermentation at high temperature has several advantages. Higher temperature results in lower viscosity of polysaccharides in solutions and growth temperature at temperatures higher than 70°C leads to "self-distillation" of ethanol. This potentially alleviates the problem of ethanol intolerance and should prolong the fermenting life of the culture. The latter also opens up the possibility of continuous or fed-batch fermentation for ethanol production. Furthermore fermenting at higher temperatures decrease energy costs because of less need for cooling (fermentation generates quite a lot of heat) and distillation and the risk of contamination[45].

Ethanol production is in the natural metabolism of the thermophilic anaerobic bacteria and they can produce it from very broad range of carbohydrates, including

[44] Dien, B.S. et al. 2003:264
[45] Dien, B.S. et al. 2003:258

pentose sugars and their polymers. The ability of bacteria to grow on broad range of carbohydrates is one of the important traits to produce ethanol on industrial scale. The only problem that follows producing ethanol with thermophilic anaerobic bacteria is that these bacteria have rather low ethanol tolerance, less than 2 % (v/v) ethanol. This problem is the main limiting factor for not seriously considering producing ethanol on an industrial scale. This low ethanol tolerance is a problem because the ethanol concentration must be below 5% (v/v) in the fermentation broth when distillation is performed[46].

The ethanol tolerance in thermophilic bacteria has been investigated with *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. These researches show that ethanol tolerance decrease with increasing temperature and it is because high temperature has physical effects on membranes, which are caused by decreased membrane organization and increased membrane fluidity[47].

Earlier research on bacteria and yeast on ethanol tolerance in microorganisms shows a difference of toxicity between ethanol that is produced and added. The microorganisms had higher tolerance to added ethanol than the ethanol they produces themselves. Resent research on *Thermoanerobacertium thermosaccharoolyticum* HG-8 shows different results, the ethanol tolerance were the same for added ethanol and produced ethanol. This discrepancy was a result from salt inhibition caused by the base that was added to control pH[48].

Only a few wild type and mutant thermophilic anaerobic bacteria have been found in previous researches that have ethanol tolerance of 5% at their optimum temperature for growth. One report shows that mutant strain of *Thermoanaerobacter ethanolicu* (39E-H8) has an ethanol tolerance of 8% (v/v) at 60°C[49].

These researches on ethanol tolerance of thermophilic bacteria were conducted with continuous cultures but not with high cell density. That is important because research on mesophilic and thermophilic yeasts in immobilization increase tolerance to ethanol and other toxic[50].

[46] Georgieva, T. I., Mikkelsen, M. J. and Ahring, B. K. 2007:365
[47] Georgieva, et.al. 2007:365
[48] Georgieva, et al. 2007:365
[49] Georgieva, et al. 2007:365
[50] Georgieva, et al. 2007:365

Ethanol fermentation from lignocelluloses is based on that the microorganisms have an ethanol tolerance higher than 5% (v/v) to help fermenting lignocelluloses. Tolerance to ethanol is a very important characteristic for thermophilic anaerobic bacteria to produce bio ethanol[51].

2.4 Thermophilic microorganisms

Thermophilic prokariotes include *Eubacteria* and *Archaea*[52]. Thermophilic microorganisms live at a high temperature, above 45°C or 113°F and some of the archaea live even above 100°C (boiling point of water). Most of thermophilic prokariotes have optimum temperature between 55°C and 105°C. Figure 9 on the next page shows how the microorganisms genus are dicided according to different grow temperature. The group, including thermophilic bacteria, live in areas like, hot spring, tropical soils, compost heaps, excrement, in hot water heaters and in your garbage[53]. Other places that thermophilic bacteria live in, are oil wells, thermal springs, volcanic mud and manures[54]. The thermophiles live all around the world, they have been found in hot springs in western United States, New Zealand, Iceland, Japan, Italy, Indonesia, Central America and central Africa. The largest single concentration region of hot springs, with thermophilic bacteria, is Yellowstone National Park in Wyoming USA[55]. Thermophilic bacteria can live in compost or manure and then the soil contains 1-10 % thermophilic types of microorgansms while processed fertilized contains only 0.25% or less of thermophilic microorganisms[56].

[51] Georgieva, et al. 2007:365-366
[52] Carlier J-P, Bonne I and Bedora-Faure M. 2006:812
[53] Jenkins. 2000
[54] Carlier J-P, Bonne I and Bedora-Faure M. 2006:812
[55] Madigan, M. T., Martinko, J. M. and Parker, J. 2003:155
[56] Jenkins. 2000

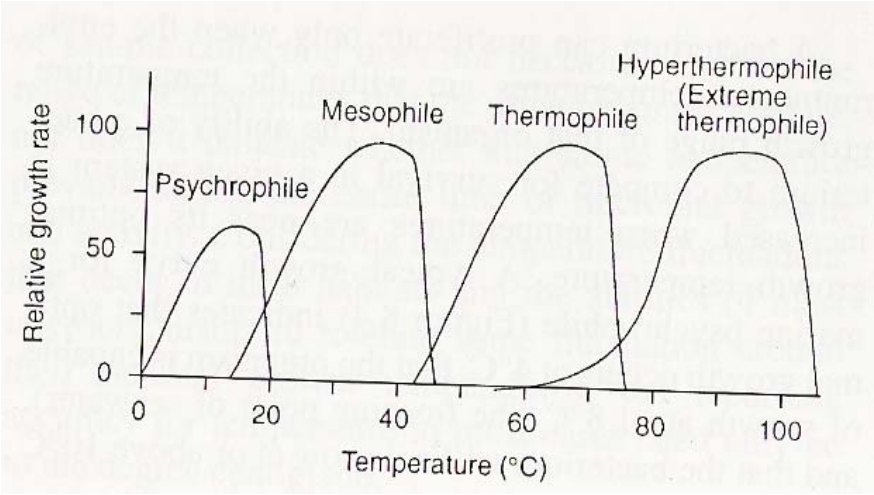


Figure 9 Devision of organisms according to growth temperature[57]

Thermophilic microorganisms are divided into two groups. Thermophiles which optimum growth temperature is 45-80°C, and hyperthermophiles which optimum growth temperature is above 80°. Thermophilic bacteria live in hot springs up to 80-85°C, while hyperthermophile bacteria lives in hot springs that are close to the boiling point[58].

Thermophilic microorganisms produce enzymes are not readily denatured at high temperatures. Many different structural reasons are for increased thermostability. Thermostable proteins have usually better atom packing compared to mesophilic counterparts. Also, they contain higher number of salt bridges. Thermostable proteins show in comparison to thermolable proteins greater hydrophobicity, deletion or shortening of lopps, smaller and fewer cavities and increased surface area buried upon oligomerzation, residue substitution with and outside the secondary structures, increased occurrence of proline reiduses in loops, decreased occurrence of thermolabile residues, increased helical content, increased polar surface area and a better hydrogen bonding. Proteins form hyperthermophilic bacteria are shorter and have increased proportion of charged residues[59]. The ribosome heat stability coincides with the maximum growth temperatures of thermophilic microorganisms. The ribosomes melt when the temperature gets higher than the maximum growth temperature and protein synthesis stops. Many thermophiles have high proportions of

[57] Atlas, R. M. and Bartha, R. 1998:294
[58] Madigan, M. T., Martinko, J. M. and Parker, J. 2003:155-156
[59] Kumar, S. and Nussinov.R. 2001:1217

guanine and cytosine in their DNA. High GC proportions raise the melting point and increases stability of the DNA molecules. However, some thermophilic prokariotes have rather low proportions of these bases (<40% G+C), especially some hyperthermophilic Achaea[60]. In the thermally stabilized DNA is polyvalent action even more effective as reflected by the variety of polyamines in hyperthermophilies. Hyperthermophilic microorganisms contain small, basic proteins that bind duplex DNA which increase T_m in vitro, several of these proteins compact or bend double-stranded DNA upon binding. In *Methanothermus* a protein with nucleosome-like structures are used to cover the chromosome. In hyperthermophilies the effects of histones and histone-like proteins have to be demonstrated on DNA secondary structure[61].

Disintegration of the cell membranes is another problem that thermophilic prokariotes have to overcome. With higher growth temperatures the fatty acids in the cell membranes changes, the fatty acids chain length increases and their degree of unsaturation decreases[62]. High proportion of saturated lipids prevents the cells from melting at high temperature[63].

Miquel isolated the first thermophilic bacteria in 1879; this bacterium was found in the sands of the Sahara Desert but is not found in soil of cool forests. The temperature was 72°C were the bacterium was found[64].

The subject of this work, *T. islandicum* belongs to *Thermoanerobiaceae* family, which includes genera like *Thermoanerobacter, Moorella* and *Thermoanerobacterium*. The genera are grouped in three clusters (V, VI and VII)[65]. Lee et al (1993) published results of taxonomic distinction of saccharolytic thermophilic anaerobes. In this study three different groups were identified for thermophilic anaerobic bacteria. In group I was only one bacterium, *Clostridium termocellum* LQRI, which was least closely related to the other strains. Group I contained bacteria that could ferment cellulose. In group II were three *Thermoanaerobacterium* strains which are closely related. Bacteria in group II can reduce thiosulfate to elemental sulfur but cannot ferment cellulose. In group III were

[60] Atlas, R. M. and Bartha R. 1998:295
[61] Grogan, D. W. 1998: 1044
[62] Atlas, R. M. and Bartha R. 1998:296
[63] Atlas, R. M and Bartha, R. 1998:294-295
[64] Jenkins. 2000.
[65] Carlier, J-P., Bonne, I. and Bedora-Faure M. 2006

also three *Thermoanaerobacterium* strains which are closely related. Bacteria in group III reduces thiosulfate to H_2S, these bacteria cannot ferment cellulose[66].

Colins et al (1994) published results of phylogeny of the genus *Clostridium*. In this study, *Clostridium* species were studied further and indentified into 19 clusters. Bacteria were indentified after how related they were and after their biological qualities. In this result *Thermoanerobacter* species, *Acetogenium kivui* and *Clostridium thermocopriae* are in cluster V. The members in cluster V have a high sequence similarity values, from 93 to 99% even though they have some different phenotypes. This result shows that *A. kivui* and *C. thermocopriae* should be genus in *Thermoanaerobacter*. Cluster V is cluster A in Rainey et al reaches[67]. In cluster A there are five species of *Thermoanaerobacter, Thermobacteroides acetoethylicus, Acetogenium kivui* and *Clostridium thermocopriae.* These genus have a similar DNA base composition, 31 to 38 mol% G+C, high similarity in their rDNA base composition of 56.7 to 59.6 mol% G+C and they have be defined by unique 16S rDNA signature nucleotides, polysaccharolytic activity and spore formation are similar[68]. In cluster VI are *Colstridium thermoaceticum* and *Clostridium thermoautorohicum*. Their difference from other clostridia is their high DNA base compositions, 53-55 mol % G+C, and they have $_{LL}$-diaminopimelic acid in their cell wall. Later *Desulfotomaculum australicum* was also added to the cluster. Cluster VI is cluster B according to Rainey et al. [69]. In cluster B are *Clostridium thermoaceticum* and *Clostridium thermoautorohicum* which are highly related, 99.2%, their 16S rRNA sequences is 99.8 % same, they have a similarly DNA hybridization, 50%, and they share some signature 16S rRNA nucleotides. Members of cluster B have a DNA base composition of 53-55 mol% which is significantly higher than of other thermophilic and mesophilic clostridia. Cluster B is equidistantly related to cluster A (88.4%) and C (90.7%)[70]. In cluster VII are genuses of *Thermoanaerobacterium* and *Clostridium thermoamylolyticum*, *C. thermoamyloyticum* that have high sequence similarity values relatedness with *Thermoanaerobacterium* species, from 98-99%. Cluster VII is cluster

[66] Lee, Y-E., Jain, M. K., Lee, C, Lowe, S. E. and Zeikus, J. G. 1993: 41-47
[67] Collins, M. D., Lawson, P.A., Willems, A., Cordoba, J. J., Frenandez-Garayabal, J., Gracia, P., Cai, J., Hipper, H. and Farrow, J. A. E. 1994:812-819
[68] Rainey, F.A., N. L. Ward, H. W. Morgan, R. Toalster, and E. Stackebrand. 1993:4776
[69] Collins, M. D et al. 1994:819
[70] Rainey, F.A. et al. 1993:4777

C of Rainey et al results[71]. Cluster C consists of genera of *Clostridium, Thermoanaerobium* and *Thermoanaerobacterium*. The members of cluster C have a DNA base composition of 29-36 mol% G+C and their rDNA base composition is 53.2 to 54.2 and 50-54 mol% G+C which is a slightly higher than mesophilic clostridia[72].

2.4.1 Thermoanerobacterium

Thermoanerobacterium and *Thermoanaerobacter* are diverse including members that have been isolated from many different places like deep surface oil wells[73], geothermal water outlets[74], hot springs[75] and leach ate of waste pile from a canning factory[76]. The difference between *Thermoanaerobacterium* and *Thermoanaerobacter* is that *Thermoanareobacterium* is able to reduce thiosulfate to hydrogen sulphide but not to sulphur like *Thermoanaerobacter* is able to do[77].

Thermoanerobacterium species are Gram-positive bacteria. *Thermoanerobacterium* can growth up to almost 80°C but the optimal growth temperatures are 60-70°C in most cases[78].

2.4.1.1 Strain AK_{17}

Strain AK_{17} was isolated from a hot spring in the area of Viti that is at the Krafla area in the north-eastern part of Iceland. The hot spring temperature was 73°C and the pH was 6.5[79]. The 16S rRNA studies showed that strain AK_{17} is closely related to *Thermoanerobacter* and AK_{17} is closest relatied to *Thermoanaerobacterium*

[71] Collins, M. D et al. 1994:819
[72] Rainey, F.A. et al. 1993:4777
[73] Cayol et al. 1995
[74] Cook et al. 1996
[75] Wiegel and Ljungdahl. 1981
[76] Cann et al. 2001
[77] Cann et al. 2001
[78] Mai, V. and Wiegel, J. 2000
[79] Steinar Rafn Beck Baldursson. 2006:50-51

aotearonese (97.7%). Other closly related bakterium are the *T. thermosulfuricum* (96.2%) and *T. thermohydrosulfuricum* (96.1%)[80]. Strain AK is called *T. islandicus*[81].

Strain AK_{17} is a Gram-positive thermophilic anaerobic bacterium[82]. Strain AK_{17} grows in the range of 50-72°C but the optimal temperature is 58-65. The pH range is 4.5-6.5 and the G + C ratio was 35.5 mol%. Strain AK_{17} can grow on carbohydrates such as arabinose, fructose, galactose, glucose, mannose, ribose, xylose, lactose and sucrose and also on the amino acids serine and threonine[83]. The optimum growth is on glucose, the temperature is between 55 and 60°C and at pH 6.0[84]. Acetate, ethanol, carbon dioxide and hydrogen are the main end products of glucose fermentation in strain AK_{17}, the fermentation is showed in figure 10[85]. Figure 11 on next page shows the fermentation products and glucose degradation for strain AK_{17}[86].

$$\mathbf{C_6H_{12}O_6 + H_2O \rightarrow 1.56\ C_2H_5OH +}$$

$$\mathbf{0.57\ CH_3COOH + 2.13\ CO_2 + 0.38\ H_2}$$

Figure 10 The end products from fermentation of glucose

[80] Steinar Rafn Beck Baldursson. 2006:57
[81] Johann Orlyggsson (verbal refernece, 20. may 2007)
[82] Steinar Rafn Beck Baldursson. 2006:50
[83] Steinar Rafn Beck Baldursson. 2006:78
[84] Steinar Rafn Beck Baldursson. 2006:50
[85] Johann Orlygsson and Steinar Rafn beck Baldursson. 2007: 98
[86] Steinar Rafn Beck Baldursson. 2006:75

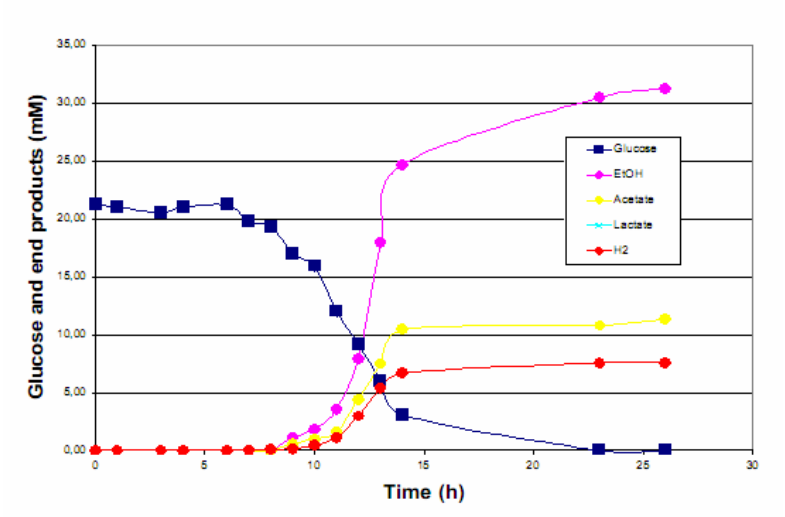


Figure 11 Fermentation products and glucose degradation for strain AK_{17}

2.4.2 *Thermoanerobacterium saccharolyticum*

Potential sustainability, security and rural economic benefits are the reasons for metabolic engineering of microorganisms and genetic modification in microorganisms. For the last two decades has the main focus been on microorganisms that produce ethanol by fermenting xylose and other non-glucose sugar and these studies have given information of the metabolic engieering of microorganisms that produce ethanol with fermenting cellulose.

Thermoanerobacterium saccharolyticum is a thermophilic, anaerobic Gram-positive bacteria. Researches on *T. saccharolyticum*, which is a hemiclluolytic thermophilic bacteria, shows lactic acids production from cellulose and this bacteria can hydrolyze xylose and ferment the majority of biomass-derive sugars but not cellulose [87]. The end products *T. saccharolyticum* for fermentation sugar are ethanol, acetate, lactate, CO_2 and H_2 [88].

Before the project described in this thesis started other results had been published that were in same professional line as this project.

Thermanaerobacter strains have been studied with regard to genetic modifications for several years. Mai et al (1996) described transformation of

[87] Shaw, A. et al. 2008:13769.
[88] Desai, S.G. 2004:600-602

Thermoanaerobacterium sp. strain JW/SL-YS485 with plasmid PIKM1 conferring *kan* resistans. The new mutant strain was tested in mineral medium with kanamycin, 50 and 100 µg/ml, and without kanamycin. Table 4 shows the results from this kanamycin test. Both samples of wild type do not grow on kanamycin but does grow on agar without kanamycin. Both samples of the mutant strains do grow both on kanamycin and on agar without kanamycin[89].

Table 4 Growth of Thermoanerobacterium wild type and mutant strain on min with/wtihout kanamycin[90]

Growth of *Thermoanaerobacterium* wild-type JW/SL-YS485 and transformant YS485 pIKM1 in the presence and absence of kanamycin

Strain	Temperature (°C)	Doubling times (min) in mineral medium+kanamycin (µg ml^{-1})		
		0	50	100
YS 485 (control	60	52	no growth	no growth
YS 485 (control)	48	240	no growth	no growth
YS 485 pIKM1	60	55	56	68
YS 485 pIKM1	48	240	240	240

Average of three measurements with values within a variation of ± 5%.

Deasi et al. (2004) published results about cloning L-lactate dehydrogenase and knockout lactic acid gene in *Thermoanaerobacterium saccharolyticum* JW/SL-YS485. Lactat dehydrogenase deletion cassette was constructed, figure 12 shows the sequenced of $_L$-lactate dehydrogenase When this sequence was compared to $_L$-*ldh* in other bacteria it was similar to the other sequence (69-82%). The strain was tranformed with the cassette[91].

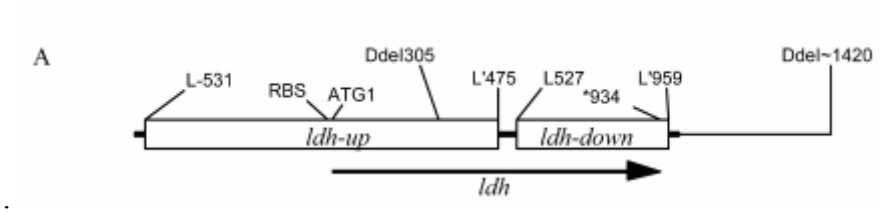


Figure 12 $_L$- lactat dehydrogenase sequence[92].

[89] Mai, V., Lorenz, W. W. and Wiegel, J. 1997:163-166
[90] Mai, V., Lorenz, W. W. and Wiegel, J. 1997:165
[91] Desai, S.G. 2004:600-603
[92] Desai, S.G. 2004:603

The *ldh* deletion by homologous recombination (double crossover) was verified with PCR and Southern blots and none of the strains produced lactic acid[93].

The wild type and strain TD1 were batch grown on glucose and xylose. The growth rates in exponential phase were 0.4±0.03 per hour for the wild type both for glucose and xylose. The same rates applied for the mutant strain TD1 on glucose but on xylose the growth rate was only 0.3±0.02 per hour. In the mutant strain TD1, both for glucose and xylose, lactic acid was at 0.3 M during the growth time, which is much lower than the regular strain produces. In the wild type the lactic acid increased during the growth time both on glucose and xylose with final concentration of 8.1 and 1.8 mM. Table 5 shows the end product analysis of glucose and xylose fermentation by wild type and mutant strain of *T.saccharolyticum*. In table 5 on the next page ND signifies not detected[94].

Table 5 End product from the wild type and strain TD1 of glucose and xylose[95]

	Glucose		Xylose	
Strain	Wild type	TD1	Wild-type	TD1
Substrate (S) (mM)	27.8	27.8	33.4	33.4
Lactate (L) (mM)	8.1 (0.49)[b]	ND	1.8 (0.22)	ND
Acetate (A) (mM)	15.5 (0.42)	16.8 (1.06)	15.8 (0.21)	16.4 (0.14)
Ethanol (E) (mM)	36.7 (1.34)	38.6 (1.20)	38.9 (0.85)	40.8 (1.76)
$Y_{L/S}$	0.29	-	0.06	-
$Y_{A/S}$	0.56	0.60	0.47	0.49
$Y_{E/S}$	1.32	1.39	1.16	1.22
C and available electronrecovery (%)	108(2.3)	100(4.1)	101(1.5)	103(3.4)
Cell yield[a]	0.053 (0.003)	0.058 (0.001)	0.036 (0.003)	0.040 (0.004)

Thus, summarised, the PCR and Southern blot results indicated that the mutant strain has a knockout instead of double-crossover event. The mutant strain of *T. saccharolyticum* did not produce lactic acid on glucose and xylose, although it produced more of ethanol and acetate and did not produce any extra end products compared to the wild type[96].

[93] Desai, S.G. 2004:602-603
[94] Desai, S.G. 2004:603
[95] Desai, S.G. 2004:604
[96] Desai, S.G. 2004:604

Shortly after the project described in this thesis started, Shaw et al (2008) published results on furhter metabolic engineering of a thermophilic bacterium *T. saccharolyticum* to produce ethanol at high yield. *T. saccharolyticum* is well studied bacterium in terms of metabolism. Furthermore, genetic tools have been developed for this bacterium[97]. The approach described in the paper of Shaw et al is basically very similar to the approach described in this thesis. Three different mutant strains were constructed where the lactate dehyrogenase, acetate kinase and phosphate acetyltransferase genes were deleted in two separate mutants and together in the third mutant. The resulting knockout strains with genotypes L-*ldh*-, *ack*-*taa*- and *ack*-*pta*-L-*ldh*- (strain ALK1) showed less acetic or/and less lactic acid and more ethanol production compared to the wild type strain ferments glucose or xylose. Figure 13 on the next page shows the production on the mutant strains from 0.5 mol glucose or 0.6 mol xylose. Figure 13 shows how pyruvate is demolished under anaerobic conditions and which end products are made, L-lactic acid, CO_2, acetic acid, H_2, NAD(P)H and ethanol. The figure also shows were NAD(P)H is used in the process. When the mutant strains are compared to the wild type, the mutant L-*ldh*- strain did produce no lactic acid and a little bit more ethanol. The mutant *pat/ack*- strain didn't produce any acetic acid, less hydrogen (over 25-fold) and more ethanol. The mutant L-*ldh*-*pat/ack*- strain (ALK1) did produce less hydrogen, little bit more of CO_2 and NAD(P)H but ethanol was the only organic end product[98].

[97] Shaw, A. et al. 2008:13769-13770.
[98] Shaw, A. et al. 2008:13769-13774.

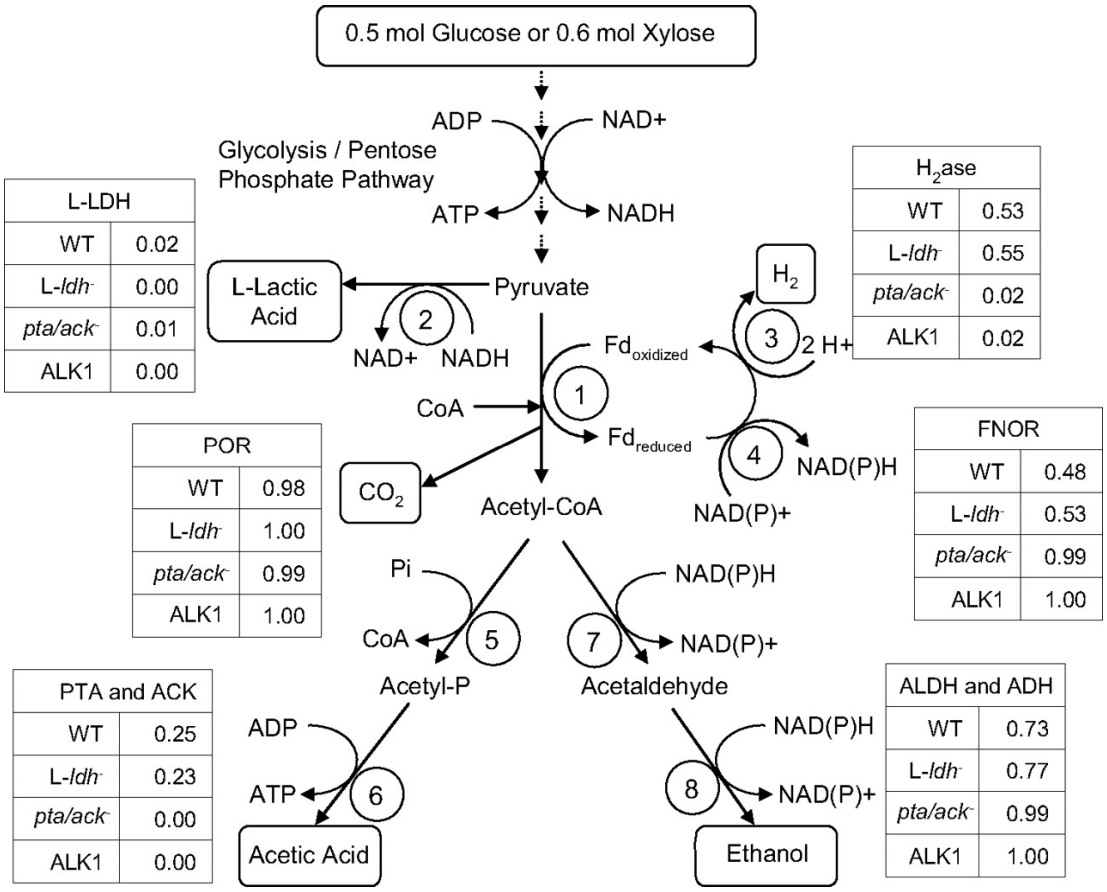


Figure 13 Fermentative pathway in T.saccharolyticum. Enzyme 1 is pyruvate/ferredoxin oxidoreductase (POR), enzyme 2 is L-lactate dehydrogenase (L-LDH), enzyme 3 is hydrogenase, H_2ase, enzyme 4 is ferrdoxin/NAD(P)H oxidoreductase (FNOR), enzyme 5 is phosphate acetyltransferase (PTA), enzyme 6 is acetate kinase (ACK), enzyme 7 is acetaldhyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH)[99].

Strain ALK2, which was derived from strain ALK1 after continuous culture for roughly 3,000 hours with progressively higher feed xylose concentrations, both batch and continuous culture showed more capacity for xylose than the wild type. Almost all xylose feed (over 99%) was used at concentrations up to 70 g/L with a main ethanol yield of 0.46 g of ethanol/xylose in continuous culture at a pH from 5.2 to 5.4 without base addition. The maximum ethanol concentration and volumetric

[99] Shaw, A. et al. 2008:13770.

productivity was 33 g/L and 2.2 g/L per hour. In culture without antibiotic selection there was no decrease in ethanol production[100].

When the growth of strain ALK2 is compared to the growth of the wild type, with fermentation of around 80mM xylose, it shows that after 10 hours of growth the xylose has all been used, figure 14. Figure 14 shows the wild type of *T. saccharolyticum* to the left (A) and strain ALK2 to the right (B). *T. saccharolyticum* took less than 10 hours to finish 80 mM xylose and and produced around 70 mM of ethanol, around 40 mM of acetic acid and around 10 mM of lactic acid and the cells dry weight is around 1,5 g/L. Strain ALK2 took less than 10 hours to finish 80 mM of xylose and produce around 120 mM ethanol and none lactic acid and acetic acid and the cells dry weight is around 1,5 g/L[101].

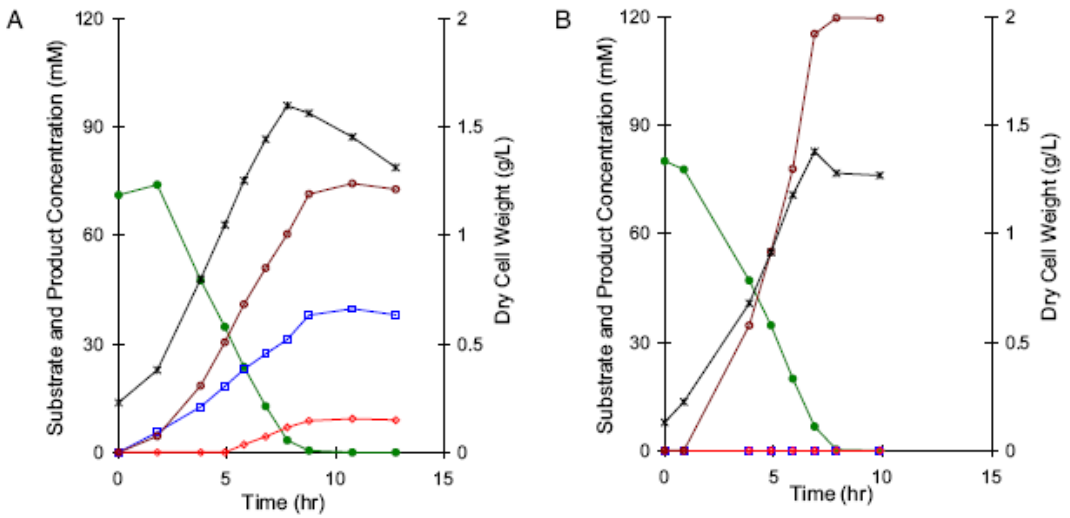


Figure 14 Comparing growth wild type of T.saccharolyticum to of strain ALK2[102]

The maximum cell concentration was 0.15g/g xylose for the wild strain and 0.12 g/g xylose for strain ALK2. This 20% lower cells yield are similar to lower ATP gain per mole xylose fermentation, which decrease by 23%, this decrease is mostly because of loss of acetate kinase activity in the mutant strain. The specific ethanol production from fermentation xylose is 1.4-ethanol/cell hour for strain ALK2[103].

[100] Shaw, A. et al. 2008:13769-13770.
[101] Shaw, A. et al. 2008:13770-13771.
[102] Shaw, A. et al. 2008:13771.
[103] Shaw, A. et al. 2008:13770-13771.

Table 6 shows which enzymes have specific activities when pyruvat is converted to ethanol. The enzymes that have activities are pyruvate/ferredoxin oxidoreductase (POR), ferrdoxin/NAD(P)H oxidoreductase (FNOR), acetaldehyde dehydrongenase (ALDH) and alcohol dehydrogenase (ADH). FNOR, ALDH and ADH all have activities in the wild type but in strain ALK2 these enzymes have more specific activities with cofactor NADPH than with NADH; this can be seen in table 6.

Table 6 Specific acticities of enzymes in the pyruvat to ethanol pathway[104].

	Specific activities (μmol·min⁻¹·mg⁻¹ protein)			
Enzyme	ALK2	SD	WT*	SD*†
POR	1.9	0.1	3.7	0.3
FNOR–NADH	0.73	0.08	1.08	0.01
FNOR–NADPH	2.3	0.5	0.55	0.09
Aldh–NADH	0.005	0.001	0.031	0.015
Aldh–NADPH	0.019	0.005	0.11	0.05
Adh–NADH	0.012	0.004	1.05	0.16
Adh–NADPH	0.23	0.04	0.11	0.06

*Data from ref 12.
†SD, standard deviation.

Strain ALK2 was put on sugar mixtures; the culture was put in 1 l fed-bacth fermented with glucose, xylose, galactose and mannose. The sugars were each 12.5g/L and the total concentration was 50g/L. Figure 15 on the next page shows the fermentation, strain ALK2 began by using glucose (purple) and xylose (green) with the glucose fermenting faster. When glucose and xylose were around 5 g/L the growth started to consume mannose (red) and when mannose got down to around 5 g/L it began to consume galactose (blue). When the concentration for glucose and xylose had dropped to 0% the concentration for mannose had reduced by 98% and the concentration for galactose had reduced by 92%. When ethanol production from strain ALK2 on this sugar mixture was tested; the final concentration was 37 g/L, maximum ethanol productivity was 2.7 g/L per hour and average ethanol productivity was 1.5 g/L per hour. The ethanol production can be seen in figure 15.

[104] Shaw, A. et al. 2008:13771

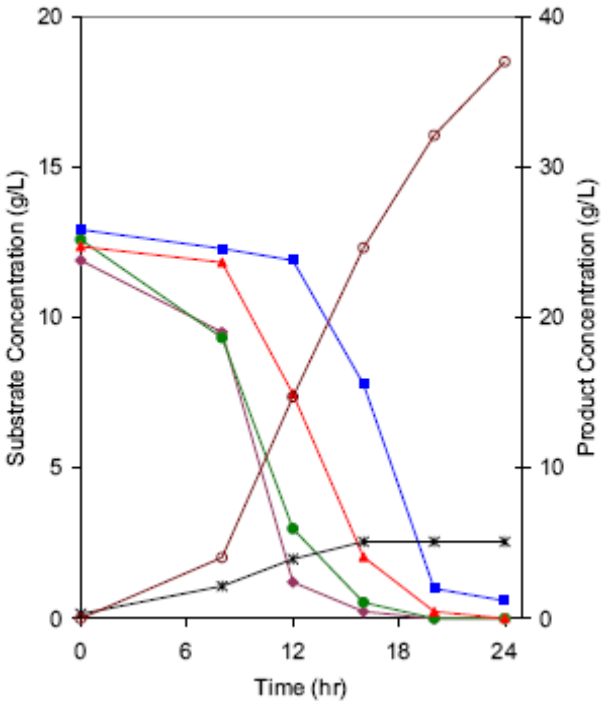


Figure 15 Mixed sugar fed-batch fermentation on strain ALK2 at 55°C without pH control[105].

As seen from the detailed report above, *Thermanaerobacterium saccharoliticum* has been studied in depth in terms of genetic and metabolic engineering. No reports of metabolic engineering of other *Thermoanaerbacter* species have been reported. In this work, genetic methods and metaobolic engineering was applied to a new *Thermoanaerobacter* species, i.e. *T. islandicum* or strain AK17.

[105] Shaw, A. et al. 2008:13771.

3 Materials and methods

In this chapter, the materials and methods that are used in this project are described. First, cultivation conditions are described. Then the methods, which were used in the project, are described in details.

3.1 Medium and agar/culture

Both liquid- and agar medium was used to cultivate the bacteria in this project. First were the bacteria cultivated in liquid medium and then on agar. The anaerobic bacteria were cultivated under anaerobic condition because they die if they come in touch with oxygen.

3.1.1 Medium

A special medium established for anaerobic thermophilic bacteria was used. The medium is made in following way.

Many solutions were used in the medium. These solutions are designated as follows: A, B, C, D, E, F, G, H, C_1, C_2 and 1 M glucose. The content of each solution is described in tables 7-20, respectively.

Table 7 Solution A

Solution A	
NaH_2PO_4	276g/L
dH_2O	1 L

Table 8 Solution B

Solution B	
Na_2HPO_4	284 g/L
dH_2O	1 L

Solutions A and B were incubated at 60° C to prevent preciptation.

Table 9 Solution C

Solution C	
Rezazurin	0.1 g/L
dH_2O	1 L

Table 12 Solution D

Solution D	
NaCl	24 g/L
NH_4Cl	24 g/L
$CaCl_2$-$2H_2O$	8.8 g/L
$MgCl_2$-$6H_2O$	8.0 g/L
dH_2O	1 L

Table 10 Solution E

Solution E	
$NaHCO_3$	80 g/L
dH_2O	1 L

Table 13 Solution H

Solution H	
Na_2S- $9H_2O$	240.2 g/L
dH_2O	1 L

Table 11 AB-buffer solution

AB-buffer solution	
Solution A	180 ml
Solution B	20 ml
dH_2O	200 ml

This AB-buffer solution is for pH 6, the pH 6 is use because the bacteria grow best on pH 6.

Medium is made according to the description in table 14.

Table 14 Medium

Medium	
AB-buffer solution	50 ml
Solution C	5 ml
dH_2O	885 ml
YE	2 g

After adjusting the pH at pH 6 the medium was boiled and cooled down on ice with N_2. Then, 18 ml of medium were poured in each bottle. Subsequently, the bottles were flushed and autoclaved.

Before the inoculation of the bacteria solutions C_1 and C_2 and 1 M glucose are injected in bottles. This has to be done before inoculation of the bacteria, because C_2 solution is made medium oxygen free. Solution F is a trace element solution. Solution G is a vitamin solution, made after DSMZ medium nr. 141. 1 M glucose is also injected into the bottles before inoculation of the bacteria.

Table 15 Solution F

Solution F	
$FeCl\text{-}4H_2O$	2.0 g
EDTA	0.5 g
$CuCl_2$	0.03 g
H_3BO_3	0.05 g
$ZnCl_2$	0.05 g
$MnCl_2\text{-}4H_2O$	0.05 g
$(NH_4)_6Mo_7O_{24}\text{-}4H_2O$	0.05 g
$AlCl_2$	0.05 g
$CoCl_2\text{-}6H_2O$	0.05 g
Na_2WO_4	0.01 g
Na_2SeO_4	0.3 mg

Table 16 Solution C_1

Solution C_1	
Solution G	1 ml
Solution F	1 ml
Solution D	12.5 ml
dH_2O	35.5 ml

Table 17 Solution C_2

Solution C_2	
Solution E	9 ml
Cystein Chloride	0.5 g
Solution H	1 ml

Table 18 Solution G

Solution G	
Pyridoxamine ($C_8H_{12}N_2O_2$*2HCl)	0.25 g
Nicotinic acid ($C_6H_5NO_2$)	0.1 g
Nicotinamide ($C_6H_5N_2O$)	0.1 g
DL-panthothenic acid ($C_9H_{16}NO_5$*1/2Ca)	0.05 g
Vitamin B12 ($C_{63}H_{88}CoN_{14}O_{14}P$)	0.05 g
p-aminobenzoic acid ($C_7H_7NO_2$)	0.05 g
Pyridoxine * HCl (B6; $C_8H_{11}NO_3$*HCl)	0.1 g
D Biotin ($C_{10}H_{16}N_2O_3S$)	0.02 g
Thioctic acid (Lipoic acid) ($C_8H_{14}O_2S_2$)	0.05 g
Folic acid ($C_{19}H_{19}N_7O_6$)	0.02 g
Riboflavin (B2; $C_{17}H_{20}N_4O_6$)	0.05 g
Thiamine * HCl (B1; $C_{12}H_{17}ClN_4OS$ * HCl)	0.1 g

Table 19 Glucose 1 M

Glucose 1M	
Glucose	18.016 g
H_2O	100 ml

Solutions C_1, C_2 and the glucose were injected into the bottles, before inoculation of the bacteria. That was done to prevent oxygen in the system C_2 solution and 1 M glucose and C_1 solution is added so the bacteria can grow. The bacteria were not inoculated until the liquid was clear and colourless (it is pink before).

Table 20 Solution and glucose in the medium

In the bottles	
Glucose	0.4 ml
Solution C_1	1 ml
Solution C_2	0.2 ml
Bacteria	0.2 ml

3.1.2 Agar

Agar medium was made in the same way except 15 g/L of agar was added to the liquid medium. Both agar and gelrite medium solutions (15 g/L) were prepared. The agar was also boiled, and cooled down on ice with N_2. After that C_1, C_2 and glucose was added 1 L bottle of agar medium.

Table 21 Agar

Agar	
Glucose	20 ml
Solution C_1	50 ml
Solution C_2	10 ml

The cultures medium was cooled down to 5-8°C in the cool-room before adding cultures on plates. 0.2 ml of culture medium was added on the plates and then placed in a special anaerobic growth box (from GasPark). GasPark™ EZ Anaerobe Container System is used to make the box anaerobic and BBL Dry Anaerobic Indicator Strips to

see when the box is anaerobic. The agar cultures grew at 60°C like the liquid medium cultures.

3.2 Methods

Some of the methods used in this project are described in detail in this thesis, but for the others, references are cited.

3.2.1 DNA isolation

DNA isolation was made in two different ways in this project. A phenol based method was applied which was time consuming but give a lot of DNA. Also a MasterPure^{TM} DNA Purificarion Kit was used which gave lower yield but took must shorter time.

3.2.1.1 DNA isolation with phenol method

Before the phenol method was carried out, 10% SDS, 10% sarcocyl and 3 M sodium acetate pH 5.75 solutions were made. Then, following protocol was used for DNA isolation with phenol

1. The bottles are cooled down to ca. 8°C and opened in a cooling compartment (8°C).
2. The culture is centrifuged at 3.500 rpm for 10 min. The water phase ispoured of, the cells are in the precipitate.

3. The cells are suspended in TE buffer pH 7.8. In this protocul usually 1.5 ml. of TE buffer per 18 ml growth was used. RNase in added in the solution in this step.
4. Mix to solution:
    a. 0.2 mg/ml lysozyme (final concentrations)

    The solution is incubated in a water bath at 37°C for 30 min.
5. Then following is added to the solution:
    a. 1% SDS (final concentrations),
    b. 1% sarcocyl (final concentrations)
    c. 1 mg/ml proteinase K (final concentrations)

    The solution is incubated for 2 hours at 50°C in every 30 minutes is the solution mix gently.
6. Mix to solution:
    a. Phenol pH 8.0 was added; same volume of the organic phase was added as the water phase was (The pH is checked, the upper phase must be at least pH 7.8).
    b. Solution mixed gently for 5 min.
7. Centrifuge the solution at 5000 rpm for five minutes. Take the water phase (upper phase) to a new sterile tube. The water phase is used on.
8. Mix to the water phase:
    a. Phenol pH 8.0 same volume of organic phase as of water phase.
    b. Solution mixed gently for 5 min.
9. Centrifuge the solution at 5000 rpm for five minutes. Take the water phase (upper phase) to a new sterile tube (be careful just to take only take water phase no dirt).

    Mix to the water phase:
    a. Chloroform: isoamylalchol (24:1) were added, same volume of the organic phase as of the water phase.

b. Solution mixed gently for 5 min.

10. Centrifuge the solution at 5000 rpm for five minutes. Take the water phase (upper phase) to a new sterile tube. The water phase is use further on. In this step, do not mix the organic phase (chloroform) to the water phase.
11. Add to the solution:
    a. 3 M sodium acetate to a final concentration of 0.3 M. Be sure the sodium acetate is well dissolved.
    b. Stir gently.
12. Add slowly to solution ice-cold ethanol (at least 96%) two times the volume of the solution.
13. If DNA precipitates in the tube it will be like white wool. Then it will be possible to wind it slowly around sterile glass stick. Subsequently, it can be air dried.

    Centrifuge the ethanol solution at 13.000 rpm at 4°C for 30 min.
14. Take the ethanol of the precipitate (DNA).
15. Wash gently the DNA with 70% ethanol.
16. Dissolve DNA in TE buffer (very small volume).
17. Be sure that DNA is well dissolve before analysing the DNA on agarose gel.
18. Agarose gel is made.
19. Mix is made for the agarose gel
    a. 5 µl DNA solution
    b. 5 µl loading buffer
20. The agarose gel on
    a. 88 V
    b. 400 mA
    c. 40 min.

3.2.1.2 DNA isolation with MasterPure™ DNA Purificarion Kit

The DNA was also isolated with MasterPure™ DNA Purificarion Kit – DNA from Epicentre[106]. The DNA purification protocol for cell samples was used; this protocol is in two parts, part A and B.

3.2.2 16S rRNA analysis

Isolated genomic DNA was used as template for 16S rRNA amplification. The 16S rRNA analysis was done prior to genomic sequencing to confirm that this was stain AK_{17}. Primers that are complmentary to conserved bacterial sequences in the16S RNA gene were used, i.e., F9 and R1544. Only one universal 16S rRNA primer was used to sequence the PCR products, that is R805[107]. Big Dye Terminator Cycle Sequencing Ready Reaction K (Applied Biosystems) was used to sequence the PCR products.

3.2.3 Zymographs - activity staining in a native protein gel

Activity staining in a native protein gel was used to analyse alcohol dehydrogense (ADH) activity in strain AK_{17}. Five different alcohols were used for the activity staining.

First experiments were done to find away to open the bacteria so it would be possible to analyze ADH activity in strain AK_{17}. A few of different ways were tried but only one was found that worked. In the method that worked the cultures (19.8 ml) were centrifuged in 10 min at 3500 rpm on 4°C. The supernatant (medium) was taken away and the cells dissolved in 1.5 ml sterile water. This sediment (cells) was put in liquid nitrogen for a few seconds and then put in a 61.0°C for few minutes. This was repeated two times. The cells were put on ice and water and sonicated for 10 min, one tube (sample) was centrifuged but the other one not. First, it was centrifuged for 5 min

[106] Epicentre biotechnologies. (e.d)
[107] Skirnisdottir et al. (2000)

at 4,000 rpm, then 5 min at 7,000 rpm and finally 20 min at 13,200 rpm. All steps were done at 4°C. Protein concentration of these samples was analysed with Bradford reagents.

Bradford was done to see how must proteins were in the cells (enzyme measure). Bradford was done on the samples before they were put on both native protein gel and SDS-gel. Bradford is shown in table 22 shows how.

Table 22 Bradford

Bradford	
10.0 µl	Sample
40.0 µl	Bradford
160.0 µl	dH_2O

The samples did have enough enzymes/proteins so they were put on a big protein gel.

Protein gel was made consisting of two different type of gels. The gel on the bottom is 10% resolving gel. The 10% resolving gel covered with butanol and cleaned of with water following polymerization. The 4% stacking gel on top of the resolving gel was prepared.

Table 23 10% resolving gel

10% resolving gel	
8.375 ml	40% acryl amid
8.375 ml	Tris HCl pH 8.8
16.75 ml	dH_2O
0.175 ml	10% AMPS
17.5 µl	TEMED

Table 24 4% resolving gel or stacking gel

75% resolving gel/ stacking gel	
0.67 ml	40% acryl amid
1.25 ml	Buffer B
3.05 ml	dH_2O
25 µl	10% AMPS
30 µl	TEMED

Table 25 Loading buffer for protein gel

Loading buffer	
Tris/HCl pH 6.88	4.0 ml
EDTA 0.5 M pH 8.0	800.0 µl
87 % glycerol	7.0 ml (51.61% final)
Bromo phenol blue	1.0 µl

After the 4% resolving gel had polymerized the samples with loading buffer were put on the gel. The loading buffer did not contain SDS, (Table 25). In each well, 65 µl of sample and 35 µl loading buffer were loaded. The gel was run at 40 mA over night. Following the run, the gel was cut to strips corresponding to the lanes and each piece put in a small bag. Components of the activity assay were added into the bag, MTT, PMS, NAD NADP and alcohol, table 26-31 as following.

Table 26 MTT solution

MTT	
0.25 g	MTT
50.0 ml	DMSO

Table 28 NAD solution

NAD	
0.50 g	NAD
50.0 ml	dH_2O

Table 27 PMS solution

PMS	
0.25 g	PMS
50.0 ml	dH_2O

Table 29 PMS solution

PMS	
0.25 g	PMS
50.0 ml	dH_2O

Table 30 NADP solution

NADP	
25.3 mg	NADP
10.12 ml	dH_2O

Table 31 ADH reaction solution (without alcohol)

ADH substrate solution without alcohol	
10.0 ml	1M Tris/HCl pH 8.3
40.0 ml	dH_2O

1.0 ml	MTT
1.0 ml	PMS
1.0 ml	NAD
1.0 ml	NADP

9 ml of ADH reaction solution without alcohol was put into each bag. 1.5 ml of alcohol was added to the solution in the bag. Yet, a control sample did not contain any alcohol. Following alcohols were tested: ethanol; butanol; 2-propanol; ethyl lactate and 2-hexanol. 10% glucose was also tested. The bags with the gel, reaction solution and the corresponding alcohols were covered with aluminium foil and put on shaking board at 37°C for 45 min and at 60°C for 30 min.

3.2.4 Sequence analysis

The genome of stain AK_{17} was sequenced with FLX 454 pyrosequencing technology. The sequencing was carried out at Eurofins MWG, Germany. 20 µg of pure and undegraded DNA was prepared and delivered. Sequencing was carried out using quarter of standard FLX plat giving an estimated ~6 fold coverage.

3.2.5 Construction of Lactate dehydrogenase gene deletion cassettes

A lactate dehydrogenase gene deletion cassette consists of a kanamycin gene within lactate dehydrogenase gene flanking sequences. The lactate dehydrogenase gene has been replaced with a kanamycin marker gene. One of the end products from strain AK_{17}, by fermenting glucose, is lactic acid. This cassette can be used to transform strain AK_{17} and delete the gene responsible for formation of lactic acid. Consequently, the mutant strain resulting from transformation of strain AK_{17} with lactate dehydrogenase deletion cassettes should produce little or no lactate.

The sequence analyses revealed the complete lactate dehydrogenase gene along with flanking seqences. Special primers were designed to amplify the flanking

regions of the gene. The primers for amplifiaction of the 5' flanking sequence were: LDH5'-eco-f containing *Eco*RI restriction site and LDH5'-SOEkan-r containing 5' kanamycin complimentary sequence.

The PCR was performed according to conventional protocols using the Eppendorf Triple Multi High Fidelity system (polymerase and buffer). The PCR temperatur profile is shown in figure 16. The expected product was 547 bp in size.

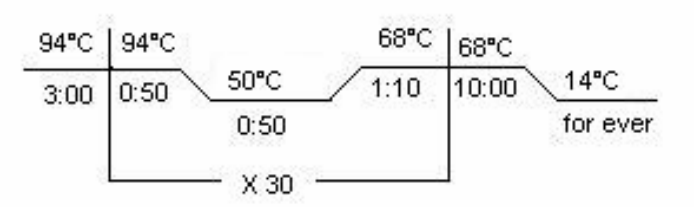


Figure 16 PCR to get 5'flank LDH sequnece

The PCR product was run on an agarosagel and purified with GFX. GFX is used to purify DNA from an agarosagel. A normal GFX manual was used (GFX manual from GE).

A kanmycin marker gene (759 bp) encoding thermostable kanamycin nucleotidyl transferase[108] was amplified as described before, using the pAsp. 1.2.2SE D4 (*Thermus* plasmid with kanamycin) was used as a template. The primers were kan-SOEldh-f containing upstream 5'ldh flanking sequence and kan-bam-r containing *Bam*HI restriction site (see appendix 4). The temperature and time were the same as before. The PCR product was run on agarosagel and purified with GFX.

The third PCR in the SOE process was done to splice the kanamycin gen with the upstream *ldh* flanking sequence. The splicing is based on pairing of complimentary sequences in LDH5'- SOEkan-r and kan-SOEldh-f sequences in each template. The primers used to amplify the spliced sequence were the LDH5'-eco-f and Kan-bam-r. The PCR products from the two previous reactions, the 5'flank LDH and the Kan gene were used as template. The PCR profile is shown in figure 17. The expected product was 1306 bp in size.

[108] Hoseki, J., Yano, T., Koyama, Y., Kuramitsu, S. and Kagamiyama, H. 1999:951.

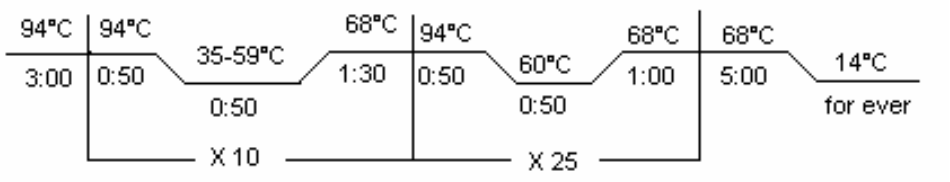


Figure 17 PCR profile of the splicing PCR reaction

The PCR product was run on agarosegel and purified with GFX.

The fourth PCR reaction was done to isolate the 3' flanking sequence of the *ldh* gene. Two PCR reactions were carried out with different reverse primers. The expected products were 485 bp in size and 501 bp, respectively. The PCR procedure was as previously described. The primers were LDH3'- hind III-R, LDH3'-hind-R2 and LDH3'-bam-f. The PCR profile was as described for the 5' flanking sequence. The 3' flanking PCR product was run on agarosagel and purfied with GFX.

Two separate PCR reactions were performed with two different reverse primers. This was done as a precaution in case of failure in amplification as the primer binding sites were very close to the contig.

To construct the deletion cassettes few restriction/ligation steps were carried out. First, the ldh3'flanking site was cut with *Bam*HI and *Hind*III and ligated into *Bam*HI and *Hind*III cut vector pOF1154[109]. T4 ligase was used and the ligation was carried out overnight at 16°C. *E.coli* Top10 cells were transformed with the ligation mix. Transformants on plates were screened for inserts with LDH3'-bam-f and LDH3'hindIII'r or LDH3'-hind-r2 primers in a PCR reaction. Positvie clones/plasmids were designated pSS1. Plasmid DNA was isolated from clones number B3 and B4 containing the ldh3' flanking sequence. The SOE splice product was cut with *Eco*RI and *Bam*HI and ligated into pSS1 following *Eco*RI/*Bam*HI digestion. The resulting ligation product was transformed in to TOP10 cells and the clones was screened for correct insert with LDH5' eco-f and Kan-bam-r primers. Plasmids DNA was isolated from clones that give positive signal.

[109] Olafur Fridjonsson and Mattes, R. 2001:4193

3.2.6 Alcohol dehydrogenase gene, retrieval and construction of deletion cassette

An alcohol dehydrogenase gene deletion cassette is a DNA sequence consisting of the kanmycin gene marker between the alcohol dehydrogenase flanking sequences. Thus the alcohol dehydrogenase gene is replaced by the kanamycin marker. One of the end products from strain AK_{17}, by fermenting glucose, is ethanol. The purpose of deleting the gene (if possible) is to study the impact on the ethanol production. Continued ethanol production would indicate presence of other unidentified *adh*.

The genome sequence was screened for alcohol dehydrogenase (*adh*) genes. Thereby, enzymes belonging to different groups, short chain, medium chain and long chain *adh* were taken into account. Only one gene was identified belonging to the group of long chain alcohol dehydrogenases. Partial sequences of this gene were found on three separate contigs, two covering each end and one very small contig with sequence from the middle of the gene. To get the whole gene, bio-primers were used and the gene was amplified PCR with adhS17-nde-f and adhS17-bam-r primers. The sequence was cloned into Topo vector and TOP 10 *E.coli* cells from Invitrogen.

An *Δadh: kan* knockout cassette was constructed in a similar way as for ldhT17 gene. First was the 3' flank sequence was amplified with adh3'-bam-f and adh3'-hind-r and the PCR product was cut with *Bam*HI and *Hin*dIII (appendix 5). Plasmid pOF 1154 was cut with *Bam*HI and *Hin*dIII and ligated with flank 3' sequence of the alcohol dehydrogenise gene. The resulting plasmid was designated pSS3.

Then the flank 5' sequence was amplified with adhS17-5'mfe-f and adh5'flank-kan-soe-r primers and kanamycin gene was amplified with kan-adh5'-SOE-f and kan bam-r. Subsequently, the products were spliced in a SOE PCR reaction with primers adhS17-5'mfe-f and kan bam-r.

The spliced product was cut with *Mfe*I and *Bam*HI and ligated into pSS3 following *Eco*RI and *Bam*HI digestion, the resulting plasmid was designated pSS4 (see in appendix 5).

3.2.7 Design and construction of alcohol dehydrogenase insertion cassette

In order to investigate the effect of increased *adh* gene dosage on ethanol formation, an insertion cassette was constructed where the lactate dehydrogenise gene was replaced with the alcohol dehydrogenase gene. Thus, the insertion cassette consisted of the *adh* gene and kanamycin gene within *ldh* flanking sequences. The construction of the cassette was as following. First the LDH 5'flank sequence was amplified with LDH5-mfe-f and LDH-5adh-soe-r and the 5' part of the ADH5' gene was amplified with adhS17-5ldh-soe-f and adh-1500-eco-r (appendix 6).

The LDH 5'flank sequence product and ADH5'gene product were spliced together in a SOE-PCR reaction with LDH5-mfe-f and adh-1500-eco-r primers. The splice product was cut with *Mfe*I and *Eco*RI and ligated into plasmid pSS1 containing the LDH'3 flanking sequence following *Eco*RI digestion to make pSS5.

Then ADH'3 gene was obtained with PCR using adhS17-nde-f and adhS17-bam-r primers. The PCR product was digested with *Eco*RI and *Bam*HI. Plasmid pSS5 was also cut with *Eco*RI and *Bam*HI and these products were ligated together to make pSS6 (see construction schema in appendix 6).

In order to select insertion clones on agarplates, the kanamycin selection marker was inserted into plasmid pSS6 as following. First kanamycin gene was amplified with kan-T17SD-bam-f and kan-mfe-r primers and the PCR product was cut with BamHI and MfeI and plasmid pSS6 also cut with *Bam*HI and *Mfe*I. These products were ligated together to make pSS10 (see construction schem in appendix 6).

3.2.8 Transformation of *T. islandicum* strain 17

A protocol was developed for the transformation of *T. islandicum* strain 17. It was based on the protocol described in: Volker Mai, W. Walter Lorenz and Juergen Wiegel 1997. *Transformation of Thermoanerobacterium sp. Strain JW/SL-YS485 with plasmid PIKM1 conferring kanamycin resistance.* FEMS microbiology. Letters 148,

163-167. First, the strain 17 was growhn at 60°C in the normal medium pH6, yet considerably modified.

The transformation was performed multiple times with the knockout cassettes to find conditions that worked for this bacterium.

1. The cells were grown in two bottels with 19.8 ml of the normal medium pH6 at 60°C, anaerobic.
2. Isonicotinc acid hyrazide (niacin) was added into part of the cultures when the OD was 0.500 - 0.700. Niacin weakens the cell wall and makes it leakier. Cultures with niacin served as control. The growth was continued at 60°C but when the OD was reashed 0.800 – 0.900 (normaly the growth with niacin was lower), the electrophoration was done.
3. The cultures were poured into Hungate tubes, one tube for each culture. The tubes were centrifuged at 3500 rpm for 20 min. at 4°C. The supernatant was taken of with syringes. Then the rest of the culture was put into the Hungate tubes and the centrifugation was repeated. The supernatnat was taken of with a syringe.
4. The cells were washed in 8 ml of 10% glyserol (flushed) and centrifuged again at 3500 rpm for 20 min at 4°C. This was done two times.
5. The cells were dissolved in 2.0 ml water (flushed and with solution C_2) and 2.0 ml sucrose (flushed and with solution C_2).
6. The cells were incubated at 48°C in waterbath for 10-15 min or until spheroplast (autoplast) formation was observed using light microscopy.
7. The cell were centrifuged in the Hungate tubes in 3500 rpm for 10 min at 4°C and re-suspended in 0.1 ml of water.
8. The electroporation was done with BioRad Gene Pulser. 0.1 ml of cells was transferred into prechilled electrohoresis cuvettes. ~1 μg of plasmid DNA (deletion/insertion cassettes) was added to the cuvette. No plasmid was added in one cuvette and that is the negative kontrol. The BioRade Gene Pulser was adjusted at 1.25 kV and a pulse given with time constant of 4 ms.

The time constant were cheaked at this point and it should be 2.3 to 3.6 ms. The time constant that obtained by the transformation of *T.islandicum* was rather low copaired to other reference research.

9. The mixture was transferred immediately into a 5 ml medium in hungate tubes. The mixture growth in 55°C over-night.
10. After the cultures had grown ower nigth, 2* 1.5 ml of easch cultuere was trasferred in to a empty eppentop bootels and centririfuged at 13,200 rpm for 30 s at 4°C. Almost all medium were taken away but the rest of the medium and the cell were put on agar with 40 μg/ml kanamycin. This was done at a tempature of 4°C and the box were the agar basin were placed in to be anerobic (oxygen-free) were also preserved at 4°C until in were anerobic. The box was placed in 60°C after it was anerobic.

3.2.9 Southern blot-protocol

Southern blot was done to verify the deletion with homologous recombination in the mutant strains and thus confirm the resulting genotype. Southern blot was done with using both flanking sequences, the kanamycin gene and the respective knockout gene as probes. DNA was isolated, with the MasterPure™ DNA Purification Kit – DNA from Epicentre, from the mutant strains. The DNA purification protocol for cell samples was used for the isolation. Five positive cultures from the Δ*adh* were used in the southern blot analysis and four positive cultures from the Δ*ldh*. Plasmids that were used in the transformation were also used in the southern blot as control and DNA from strain AK_{17} was used as a negative control.

In southern blot for mutant Δ*ldh* a DNA from all the positive cultures, the plasmid E2 and the wild type were cut with *Hind*III and *Bam*HI. In southern blot for mutant Δ*adh* a DNA from all the positive cultures, the plasmid E2 and the wild type were cut with *Hind*III. These cut products were placed on agarosegel at 50V for about 3 hours.

The gels were washed, first with 0.25 M HCl for 5 min and then washed in 1.5 M NaCl/ 0.5 NaOH for 15 min. This was performed twice. The gels were placed on a glass plate with a Hybond™ blotting filter paper. Hybond™- N+ membrane

optimised for nucleic acid transfer was placed on the gels and a Hybond^{TM} blotting paper was placed over the membrane. All this was placed in a tray and 1 M NH_4 – Acetat/20mM NaOH added in the tray, kept over-night.

The membranes were washed in 2x SSC for 2 min. The membranes were stored at room temperture for 30 min to dry the membranes and then placed in UV for 5 min.

Then the probes were made with a Dig High Prime DNA Labeling and Detection Starter Kit I and rest of the Southern blot was done with this kit. First the PCR was used to get fragments from 3'flank sequences, 5'flank sequences, kanamycin gene and knockout genes. The PCR produces were added on agarosegel, the fragments were cut out and purified with GFX. 2µl of each GFX product was added on agarosegel to see the concentration before the probes were made. The concentration of the template in the reaction mix was ~ 1 ng/µl. Sterile water was added with the GFX produces up to 16µl and the probes were placed in eppendorf thermomixer at 99°C for 10 min. A λ probe was also made to color the marker. 4µl Dig High Prime DNA (mix) was added in each probe. The reaction mix was placed at 37°C for 20 hours. 1µl of EDTA was added to the probes to stop the reaction.

The membranes were placed in bags with 8 ml of Dig Easy Hub Granules (with dH_2O). The bags were put in a tray with a little bit of sterile water and placed in 42°C washbath for 1 hour. 7µl of probe and 50 µl sterile water were mixed together and then placed in thermomixer at 99°C for 5 min and then on ice.

3 ml of Dig Easy Hub for each sample was placed in 42°C washbath for 15 min. The probes were added into Dig Easy Hub, one sample probe in each and λ probe in all. The Dig Easy Hub was removed from the bags with the members and Dig Easy Hub with probes placed instead. The bags were put again in a tray with a little bit of sterile water and placed in 42°C washbath over-night.

The membranes were washed with 2x SSC 0.1% SDS and 0.5xSSC 0.1% SDS. First the membranes were washed twice with 2xSSC 0.1% SDS at 15-25°C for 15 min. under constant agitation and then washed twice with 0.5%SSC, 0.1%SDS at 65°C for 15 min and agitation every 5 min. After the wash the immunological detection step was made. First the membranes were placed in washing buffer for 2 min on shaking, then in blocking solution for 30 min on shaking and then in antibody solution for 30 min on shaking. After this the membranes were washed twice with

washing buffer for 15 min on shaking. The membranes were added to a bag with detection buffer without shaking. Finally color substrate solution was added in the bags with the membranes, an aluminium foil put over the bag and then the bag container placed in a dark place. The membranes were in a dark place until bands came forward. After the southern blot was finished the membranes were washed with a TE-buffer for 5 min and let dry on a paper.

3.2.10 Measuring the end products from fermentation of glucose with column

An HPLC system was used to analyse to see the end products of the strains from fermentation of glucose. The system used was DIONEX ICS-3000. The column was from Phenomenex Rezex ROA organic acid 8% 300x7.80 mm part no 00H-0138-K0 with a 50x7.80mm guard column part no 03B-0138-K0. Running condition were as following: the column was run at 65°C and the flow rate was0,4mL/min. Running buffer was 2,5mM H2SO4 and the column was run by isocratic gradient. Detection was done with RI detector RI-101 from Shodex

This measurement was made to see the difference, between concentrations of the end products from the wild type and the mutant strains. This measurement showed if the transformation had any impact on the phenotype of the mutant strains.

Samples from the wild type and mutant Δ*ldh* strain were all measured when the OD was 0.6-1.0, the wild type had grown for around 24 hours and the mutant Δ*ldh* for about 31 hours. The wild type and mutant Δ*ldh* were also measured after about 48 hour growth.

The wild type and mutant Δ*ldh* were also measured after 0-27 hour growth and had one and the same OD.

4 Results

4.1 The growth of strain AK_{17}

The growth of strain AK_{17} was originally monitored in connection with attempts isolating chromosomal DNA. The growth stage was critical with respect to the quality of the DNA. It was best to isolate the DNA at the end of the growth-phase, as it gave the higest DNA yield and lowest proportion of degraded DNA as explained in chapter 2.2.

The anaerobic growth of strain AK_{17} was monitored as follows. Glucose medium, pH7 (described in materials and methods page 33-37) was incubated with cells, which had been cultivated for 4 days. The cells were diluted 1:50 in a 20 ml sealed bottle and incubated at 60°C. The absorbance at 600 nm was measured every one hour for two days. Figure 18 on next page shows the growth curve of the strain. A long lag-phase is seen, as no growth appears the first 24 hours. Then after about 24 hours too about 27 hours the bacteria grow very fast until the OD is about 0.75. The growth is very little the next 12.5 hours, the OD reaches about 0.8 and after that no growth is detected because the glucose is finished. In short, the growth takes about 39.5 hours and after that the bacteria start to die. The growth behaviour of the strain was verified with repeated cultivation experiments applying the same conditions.

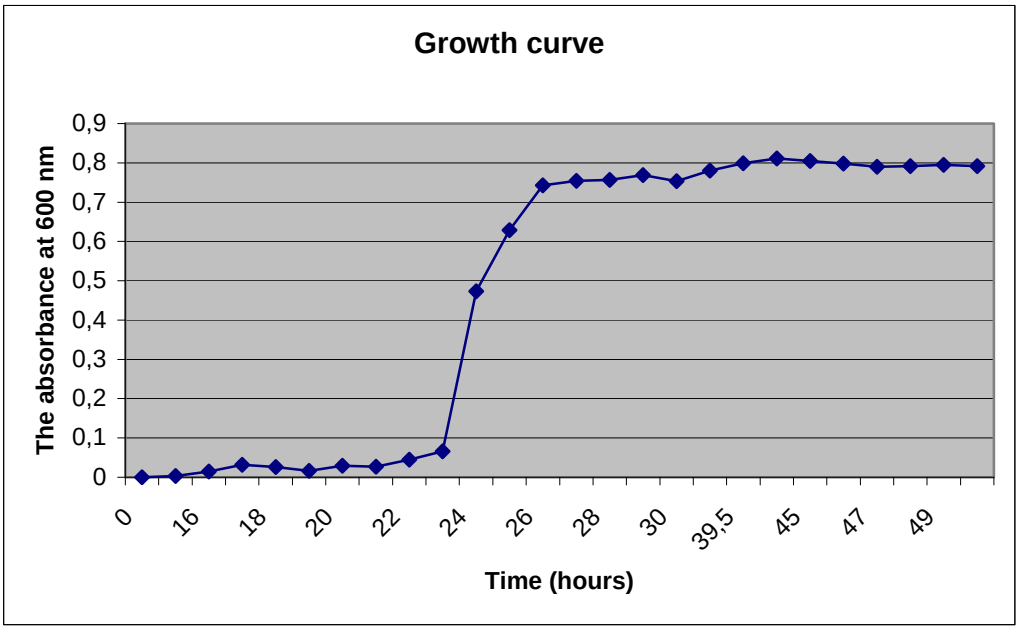


Figure 18 Growth curve of strain AK_{17} on time

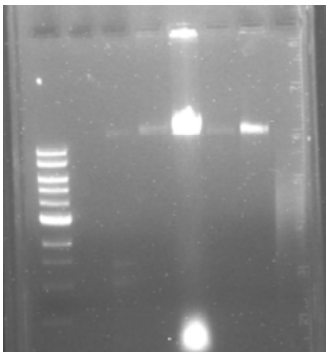
It was a problem to grow strain AK_{17} on plates in earlier studies. In this study growth on agarose plates was achieved in four days but not on the so-called Norwegian agar (Difco) commonly used for thermophilic bacteria.

4.2 Isolated DNA

~20 µg of genomic DNA was needed to sequence the genome of AK_{17} with FLX high throughput sequencing platform. Therefore, the strain was cultivated and the genomic DNA isolated and purified. The work showed that the growth phase of the cells is critical with respect to yield quality of the DNA, as previously mentioned.

The DNA was isolated from bacteria, when the bacteria had been grown for different time length. Figure 19 on the next page shows different yield of DNA isolation following different time-length of incubation. Optimal yield was obtained after growth for 24-39.5 hours. Too long incubation resulted in degraded DNA; to

short incubation time resulted in little or no DNA, which comes of low concentration of cells.



1 2 3 4 5 6 7 8

Figure 19 Agarose gel with genomic DNA isolated from cultures grown from differently long time. Lane1: Marker, lane 2: DNA isolated following growth for 15 hours, lane3: DNA following growth for 19 hours, lane 4: DNA following growth for 23 hours, lane 5: DNA following growth for 24 hours, lane 6: DNA following growth for 28 hours, DNA following growth for 39,5 hours and lane 8: DNA following 60 hours. Discrepancy in DNA yield in lanes 5,6 and 7 may be because of pipetting imprecision due to unsolved DNA in samples.

The DNA that was used for the genomic sequencing was from cultivation of the bacteria for about 39.5 hours or when the OD is about 0.8 (lane 7 in figure 19 and OD is from figure 18).

4.3 Genome sequencing

The results of the genome sequencing in terms of number of reads, bases, contigs etc. are indicated in table 32 on the next page. Assuming the genome size of *T. islandicus* or strain AK_{17} is similar to the genome size of *Thermoanaerobacter tengcongensis* MB4, about 2,6 mb, the coverage is about 5,6 fold. This should be enough to give use

approx. 95% of the genome sequences and thus, likely at least partial sequences of all genes[110].

Table 32 Results of the genome sequencing in terms of number of reads, bases, contigs etc.

Large Run Results	
Total Number of Reads	*74431*
Total Number of Bases	*14563191*
Assembler Results	
Number Assembled	*39185*
Number Partial	*9820*
Number Singleton	*5421*
Number Repeat	*1053*
Number Duplicate	*18570*
Number Outlier	*382*
Sum of Large Contigs	
Total Number of Reads	*35024*
Number of Large Contigs	*1235*
Total Number of Bases	*1231588*
Sum of All Contigs	
Total Number of Reads	*50255*
Number of All Contigs	*4741*
Total Number of Bases	*2109003*

A local database was created with the program BioEdit[111]. The lactate dehydrogenease (*ldh)* gene from *T. ethanolicus* was blasted on the database using blastN. The results revealed the whole lactate dehydrogenase gene with the flanking sequence. The sequences could then be used in making plasmid deletion cassettes with the *ldh* flanking sequence, flanking kanamycin marker instead of the gene. The intension was to use this plasmid for transformation of strain AK_{17}. Partial sequence of the alcohol dehydrogenase gene (*adh*) was also retreaved following blasting the database with various *adh* nucleotide sequences of different families using blastN. Gen retrieval was used to get the rest of the gene and also the flanking sequences.

[110] Bouck J, Miller W, Gorrell JH, Muzny D, Gibbs RA. 1998:1074-1084.
[111] Hall, T.A. 1999.

After the whole alcohol dehydrogenase gene with the flanking sequences were retrieved; it was possible to make a plasmid deletion cassette and an insertion cassette that was used in subsequent experiments. Also, acetyl kinase gene was identified, genes. This gene along with phosphate acetyl transferase encode enzymes responsible for formation of acetic acid by-products. The corresponding work creating deletion mutants will be a subject of another study.

4.4 Kanamycin test

A selection marker is needed in order to carry out knockout or insertional mutagenesis. Only few selection markers have been described for thermophilic bacteria. Thereof, a gene encoding thermostable kanamycin nucleoidyl transferase is commonly used[112]. As we intended to use this marker for *Thermanaerobacter islandicus* transformation. Kanamycin test was done to evaluate the minimal inhibitory concentration (MIC) for kanamycin.

The test was carried out in duplicates. That is two bottles for each kanamycin concentration. Figure 20 on next page shows cultures with different kanamycin concentration. The inhibitory effect is clear. The bacteria only grow in media with no kanamycin or in bottles with 7.58 μg/ml kanamycin. Accordingly, the MIC was estimated to be ~10 μg/ml in liquid medium.

[112] Hoseki, J., Yano, T., Koyama, Y., Kuramitsu, S. and Kagamiyama, H. 1999:951.

Figure 20 Kanamycin test

The kanamycin test was also done on agarose because it is known that MIC may be different on agar/agarose medium compared to liquid medium. This appeared in the first transformation experiment, when the cells without DNA (negative control) grew on plates with 13 μg/ml kanamycin. The test showed that stain AK_{17} can grow on kanamycin agarose plates up to 30 μg/ml and even 40 μg/ml following prolonged incubation (table 33). Therefore, the agarose plates used in transformation experiments contained 50 μg/ml kanamycin.

Table 33 Kanamycin test on agar

Kanamycin	**Growth**	**The growth look**
0	+	A lot of growth, very small colonies
13	+	A lot of growth, very small colonies
20	+	Colonies are different in sized, no very smal colonies
30	+	Big colonies (11 and 16)
40	-/+	Growth under prolonged incubation
50	-	No growth
100	-	No growth
150	-	No growth
200	-	No growth

4.5 Niacin test

Niacin is used in the transformation experiments to make the cell wall weaker (leakier) and facilitate DNA uptake. As the effect of niacin on strain AK_{17} was unknown, cultivation with different concentration of niacin was tested. After culivating strain Ak $_{17}$ for 22 hours, the absorption was measured and niacin added to the bottles. After the cultures had grown for 3 more hours the absorption was measured again. Table 34 shows the results from this test. According to the results 1 μg/ml of niacin was then used subsequent transformation experiments. The growth is very close to the growth in cultures without niacin but it was assumed that the niacin of this concentration would make the cell wall weaker.

Table 34 Niacin test on stain AK_{17}

	Absorbtion at 600 nm (before niacin)	Niacin	Absorbition at 600 nm (after niacin)	Difference
Bottle 1	0.333	0	0.543	0.21
Bottle 2	0.450	4 μg/ml	0.580	0.13
Bottle 3	0.320	3 μg/ml	0.498	0.108
Bottle 4	0.388	2 μg/ml	0.508	0.12
Bottle 5	0.519	1 μg/ml	0.666	0.147

4.6 Zymographs for analysing *adh* activity in strain AK_{17}

Strain 17 produces ethanol. It uses alcohol dehydrogenase (ADH) to convert acetaldehyde or acetyl-CoA to ethanol. Many bacteria possess many different types of ADHs for various metabolic reactions. In order to investigate if *T. islandicus* or strain AK_{17} possessed more than one ADH, the AHD activity in strain AK_{17} was analysed with zymographs. The results should give us information on numbers; size and reactivity of different ADHs in the cell extract and possibly indicate which enzyme participates in the reduction of acetaldehyde to ethanol (short chain, medium chain, long chain).

As described in the material and methods chapter, Bradford was used to analyse proteins/enzymes in the solution. The Bradford results indicated successful disruption of the cells and it was possible to use native protein gel for analysing *adh* activity in strain AK_{17}.

Figures 21 shows the results of the gel-staining experiments. Only two gels are shown, the control gel without alcohol and the gel immerged in ethanol. Other alcohols were also tested, i.e. butanol, 2-propanol, 2-hexanol, ethyl lactate and 10% glucose. The first lane in each gel contained insoluble fraction, the second lane the soluble fraction. The control gel without the alcohol was also run because the enzyme may mediate MMT/PMS reduction /oxidation yet of low magnitude. Few alcohols were tested in case if different enzymes show different specificity. The same pattern appears on all the gels. The ethanol has the strongest band intensity of the alcohols, then the butanol, iso-propanol, ethyl lactate and iso-hexanol (butanol was a little bit better than the other alcohols) and 10% glucose showed little reactivity and the control very little (figure 21 on next page).

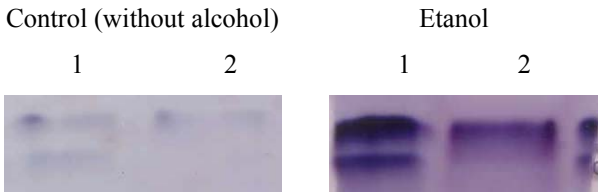


Figure 21 Control gel without alcohol and gel immerged in ethanol. Lane 1 contains enzymes from insoluble fraction and lane 2 contains enzymes from soluble fraction.

Two bands of large size appear in the insoluble fraction and one in the soluble. This indicates that the bacterium possesses only one soluble ADH enzyme of large size as it ran relatively short distance into the gel. Thus it may belong to the group of large chain alcohol dehydrogenase. The size of the soluble band corresponds to the size of one of the two bands in the insoluble fraction. The larger band in the soluble fraction might therefore be the co-precipitated. The other ADHs is probably membrane bound.

4.7 Lactate dehydrogenase gene

One of the undesirable by product in the metabolic pathway leading to production of ethanol in Thermanaerobacter sp. is lactic acid[113]. One objective of this work was to create an *ldh* knockout mutant to prevent formation of this undesirable product and consequently conduce towards enhanced production of ethanol. To achieve this an *ldh*-deletion cassette was designed and constructed as described in materials and methods on page 44-47.

The plasmid with the lactate dehydrogenase deletion cassette was named pSS2 and is 4416 bp. Figure 22 on next page shows the plasmid map of pSS2. The *ldh*

[113] Talabardon, M., Schwitzuébel, J.- P. and Péringer, P. 2000:85

flanking sequence were approx. 500 bp. and the kanamycin gene approx. 750 bp. This plasmid was used to transform stain AK_{17} to a Δ*lac* mutant.

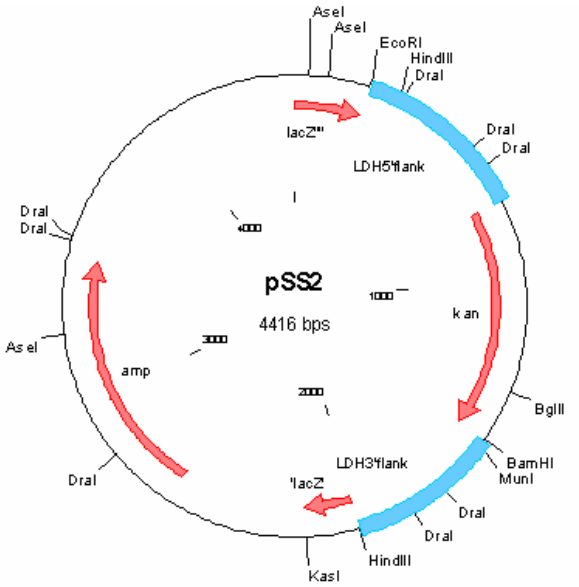


Figure 22 , ldh deletion cassette in *plasmid pSS2, pOF1154 with LDH flank sequences and the kanamycin gene. The construction of the plasmid was verified with restriction analysis*

4.7.1 Transformation with Δ*ldh*: *kan* plasmid

Attempts to transform of strain AK_{17} with the lactate dehydrogenase deletion cassette done few times before transformant colonies were isolated.

The transformation was successful when the cells were dissolved in 10% glycerol and water and time constant showed 2.8-3.6 ms., which is rather low compared to reference work of *T. saccharoliticus*. Following the electrophoration part the cells were placed on agar with 40 µg/ml kanamycin. Colonies appeared on plates following incubation at 60°C in 4 days. Majority of the colonies were very small but several large colonies appeared as well. These large colonies showed positive results in PCR tests were the kanamycin gene was amplified with kan-SOEldh-f and kan-bam-r primers.

Result from an experiment, which was a typical transformation experiments, is shown in table 35 on next page. The time constant showed 2.3-2.6 ms., the negative control and sample 1 had 2.6 ms but the sample 2 had 2.3 ms.

Table 35 Growth on agar after transformation with Δldh

Sample	**Kan**	**Growth**
Without plasmid	-	Very many colonies
Without plasmid	+	No growth
Sample 1	-	Biofilm all over the plate, very must growth
Sample 1	+	Many large colonies

Selected positive colonies were inoculated in a medium and following growth under anaerobic conditions, the DNA was isolated with MasterPure^{TM} DNA Purificarion Kit from Epicenter[114]. Figure 23 on the next page shows results of PCR screening for on the isolated DNA with the 5'flank LDH (f) – kanamycin(r) primers, kanamycin (f) – 3'flank LDH(r) primers, 5'flank LDH (f) – 3'flank LDH (r) primers and kanamycin gene (f-r) primers (appendix 3). DNA from the wild strain (AK_{17}) was screened as well as control. The 5'flank LDH to kanamycin is about 1200 bp., the kanamycin to 3'flank LDH is about 1300 bp., 5'flank LDH to 3'flank LDH is round 1800 bp. and kanamycin is round 700 bp., the wild type does not have these fragments. The results indicate that the mutant strains were without the lactate dehydrogenise gene but had the kanamycin gene instead. However, to verify the results Southern blot was carried out which results are explained below.

[114] Epicentre biotechnologies. (e.d)

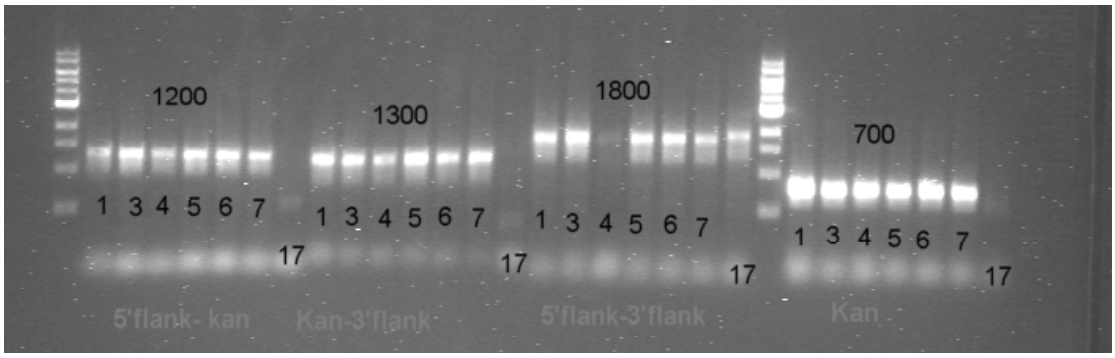


Figure 23 LDH isolated colonies were screed with 5'flank primers, kanamycin primers and 3'flank primers. PCR products of expected size appear. Lanes are designated according to number of strains, 1, 3, 4, 5, 6 and 7 are mutant strains. Strain 17 is the wild type.

4.7.2 Southern blot

According to PCR screening, all the six colonies were potential lactate dehydrogenase-gene knockout mutants. To verify the genotype of the transformed strains, Southern blot was carried out.

Figure 24 shows a map of the *ldh* gene and flanking regions in the wild type strain and figure 25 on the next page shows a map of the *kan* gene in the *ldh* locus in the mutant strains.

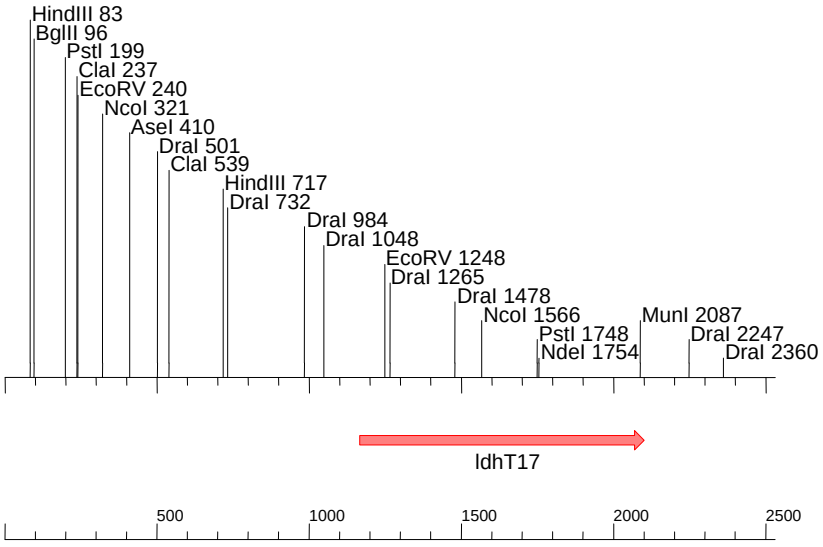


Figure 24 Map of the ldh gene and flanking regions in the wild type

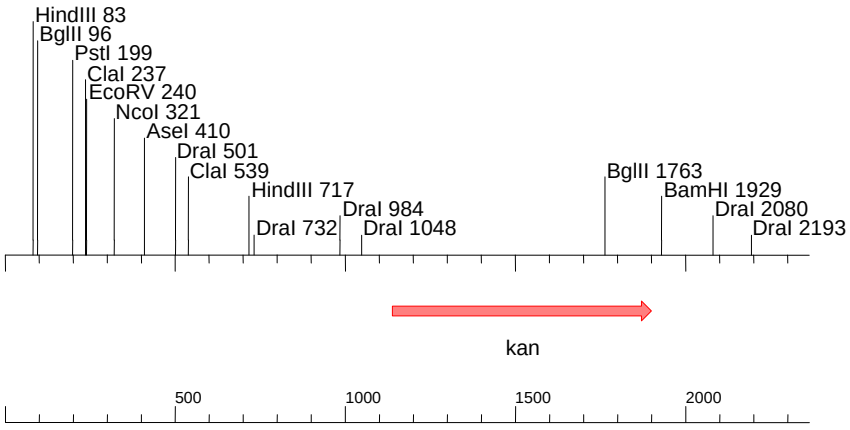


Figure 25 Map of the kan gene in the ldh locus in mutant strains

The chromosomal DNA was cut with *Hin*dIII and *Bam*HI. The wild type and the mutant have the *Hin*dIII site in common but *Bam*HI was installed through the *kan* insertionin the mutant. Figures 26-28 show the results from Southern blot analysis. A figure 26 shows the Southern blot using LDH5' flank sequencing as a probe. In lane 1 is plasmid E2, which was used in transformation. In lane 2 is the DNA from strain $AK_{17.}$ The respective band is unclear but it is a bit larger than in the mutant strains. The mutant strains are in lanes 3, 4, 5 and 6 and do have the LDH 5'flank sequence which presumably about 1200 bp. In lane 7 is the λ marker.

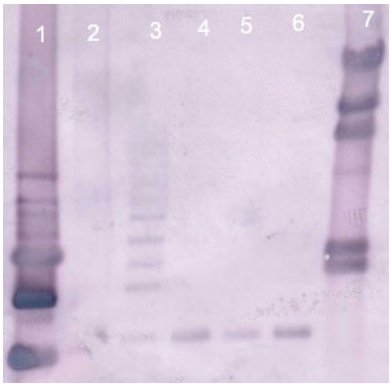


Figure 26 LDH5'flank southern blot

Figure 27 on next page shows the Southern blot using kanamycin gen in the Δ*ldh* mutant strains as a probe. In lane 1 is plasmid E2, which was used in transformation. In lane 2 is the DNA from strain AK_{17}. A clear kan band is not

detected. The mutant strains are in lanes 3, 4, 5 and 6 and do have the fragment with the kan-marker which is about 1200 bp. In lane 7 is the λ marker. In lane 8 is 1 Kb marker.

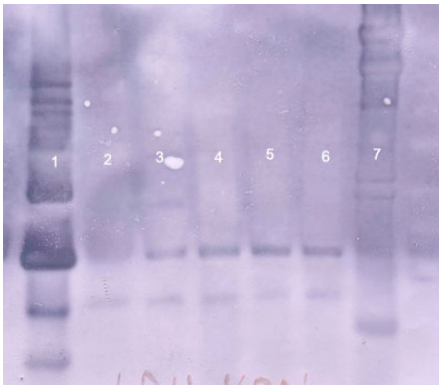


Figure 27 Kan gene probe in Δldh southern blot

Figure 28 shows the Southern blot using lactate dehydrogenase gene as a probe. In lane 1 is plasmid E2, which was used in transformation. In lane 2 is the DNA from strain AK_{17} and it has ldh band on fragment of >2000 bp. band according to the map. The mutant strains are in lanes 3, 4, 5 and 6 and do not have LDH gene band. Unspecific binding of the probe can be detected in 4 and5. In lane 7 is the λ marker.

Figure 28 LDH gene southern blot

The results from southern blot on LDH mutant strains show that the mutant strains had the lactate dehydrogenise flank sequences and kanamycin gene but strain AK_{17} has lactate dehydrogenise gene and no kanamycin gene.

4.8 Alcohol dehydrogenase gene

4.8.1 Cloning and sequencing

The FLX genome sequencing yielded many contigs due to low sequence coverage. Consequently, many genes observed in the genome through blasting, including the *adh* gene, were only partial. As precise map of the gene was preferable for intended deletion and insertion experiments the *adh* gene was fully retrieved as explained in Materials and Methods and cloned and sequenced. The net sequence with the deduced amino acid sequence is showed in appendix. The Adh belongs to the group of long chain ADHs[115]. The enzyme is 94.7 kDa and shows the highest identity with (98%) from *Thermoanaerobacterium saccharolyticum*[116]

The results of genome sequencing and *adh* zymographs indicated the existence of only one soluble alcohol dehydrogenase in *Thermanaerobacter* strain AK_{17}. In order to investigate the role of the detected ADH in the metabolic pathway of the glucose consumption, *adh*-deletion cassettes were designed and constructed as described in M and M. The objective was to study if knockout of this gene was possible and if so, the impact on ethanol production.

The plasmid with the alcohol dehydrogenase deletion cassette was named pSS4 and is 4543 bps. Figure 29 shows map of the plasmid. The *adh*5' and 3' flanking sequence were approx. 500 bp. and the kanamycin gene approx. 750 bp. The plasmid pSS4 was used in transformation experiments were knockout of *adh* gene of strain AK_{17} was attempted.

[115] Ludwig, B., Akundi, A. and Kendall, K. 1995:3729
[116] Shaw, A. et al. 2008:13769.

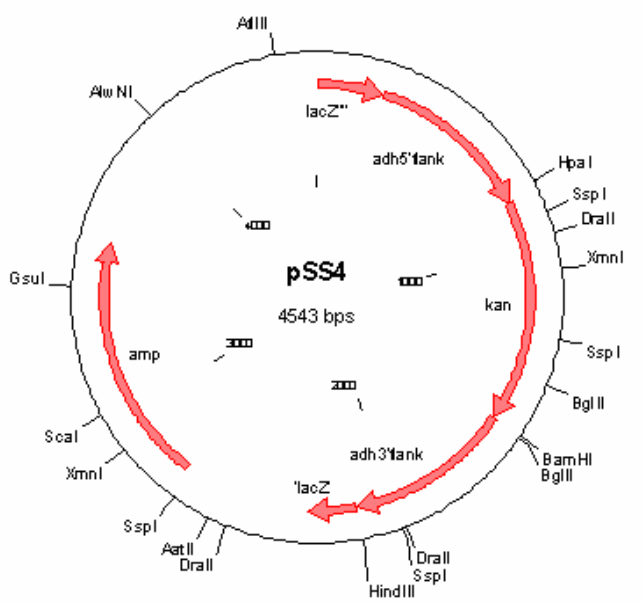


Figure 29 Plamsid pSS4. adh deletion cassette containing the kan gene between flanking sequences of adhT17. The construction of the plasmid was verified with restriction analysis.

4.8.2 Transformation with Δ*adh* : *kan* plasmid pSS4

Transformation of strain AK_{17} with alcohol dehydrogenase gene was done only once to get the Δ*adh* mutant strain. Plasmid pSS4 DNA, from-clones 16 and 17 were used in the transformation. The time constant showed 2.6- 2.8 ms. that is normally too low. The transformation experiment on the agar was as it should be; table 36 on next page shows the results.

Table 36 Growth on agar after transformation with Δadh

Sample	**Kan**	**Growth**
Without plasmid	-	Very many colonies
Without plasmid	+	No growth
Sample 1	-	Biofilm all over the plate
Sample 1	+	No growth
Sample 2	-	Biofilm all over the plate, very much growth
Sample 2	+	Many small colonies, no large colonies

Selected colonies were inoculated in a medium and following growth under anaerobic conditions; the DNA was isolated with MasterPure^{TM} DNA Purificarion Kit from Epicenter. Figure 30 shows results of PCR screening with the kanamycin gene (f-r) primers. DNA from the wild strain (AK_{17}) was screened as well as control. The results indicated insertion of the *kan* gene into the chromosome. Yet, other PCR results (not shown) were unclear and did not indicate deletion of the *adh* gene by homologous recombination with double crossover. Therefore, for getting clear results, Southern blot was performed.

Figure 30 ADH isolated colonies were screed with kanamycin primers. PCR products of expected size appear. Lanes are designated according to number of strains, 1, 2, 4, 7, 9 and 12 are mutant strains. The wild type does not have number.

4.8.3 Southern blot

According to PCR screening with kan-primers, all the six colonies were potentially alcohol dehyrogenase-gene knockout mutant. To verify the genotype of the

transforming strains Southern blot was carried out. New cultures of the clones were prepared and chromosomal DNA was isolated again. The genomic DNA was cut with *Hin*dIII restriction enzyme and run on agarose gel. Following blotting, the nylon membranes were incubated separately with ADH5'flank probe, *adh*-probe, ADH3'flank probe and *kan*-probe, all labelled with dioxygen antibody from Roche. Following immunological detection with dioxygen enzyme conjugated antigens and colour reagents NBT and BCIP, (Dig detection protocols, Roche), bands appeared on the membrane where hybridization had taken place.

Figure 31 shows map of the *adh* locus in the wild type strain and figure 32 shows a map of the *kan* in the *adh l*ocus in a potential mutant strain.

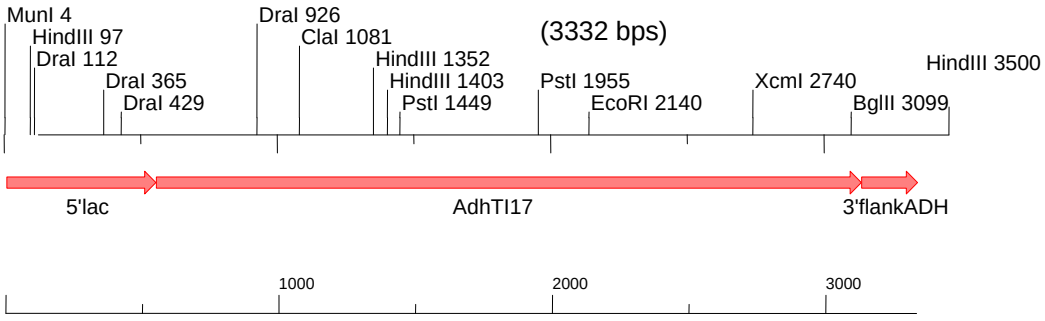


Figure 31 Map of the adh locus in the wild type strain

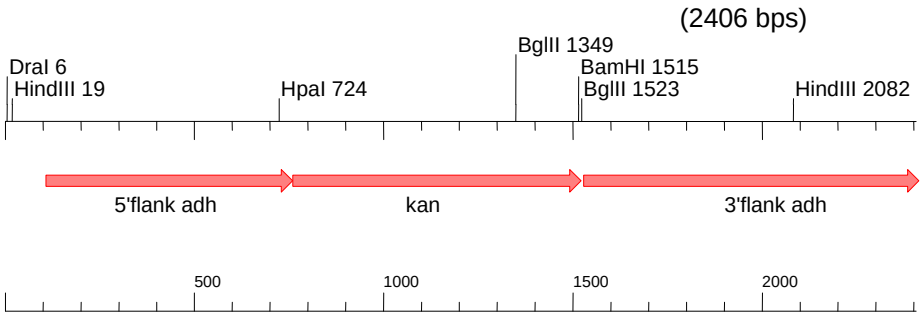


Figure 32 Map of the kan within adh flanking sequences in a potential mutant strain

Figures 33-35 show the results from Southern blot analysis. A figure 33 on next page shows the Southern blot with ADH5' flank sequence as a probe. In lane 1 plasmid 17 was run but it has apparently not blotted. . In lane 2 is the DNA from strain AK_{17}. The mutant strains are in lanes 3, 4, 5, 6 and 7 show the same size of band as the wild

type. Expected size of band by the mutants was ~1500. bp. λ marker is in lane 8. This result shows that the transformation did not work.

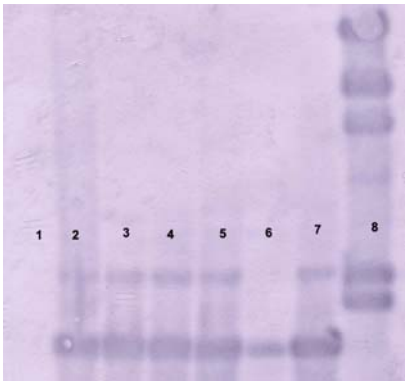


Figure 33 ADH5' flank southern blot. Lambda marker in lane 8 was detected with dig-labled lambda DNA probe.

Figure 34 shows the Southern blot following hybridization with ADH3' flank probe. In lane 1 is plasmid 17 (apparently not blotted). In lane 2 is the DNA from strain AK_{17} (also not blotted). The mutant strains are in lanes 3, 4, 5, 6 and 7 and have a band about 2200bp. It is difficult to say if the band is right or not because the band for the wild type should have 2200 bp but for the mutant 2100 bp. In lane 8 is the λ marker.

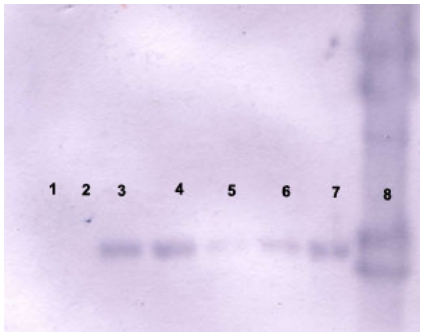


Figure 34 ADH3' flank southern blot

Figure 35 shows the Southern blot with kanamycin gen in the Δ*ldh* mutant strains as a probe. The only lane that is shown is lane 8 and the λ marker is there. Plasmid 17, DNA from the wild type and the mutant strains didn't show any bands. The wild type shouldn't have shown any bands but the mutant strains and the plasmid

should have shown a 2000 bp. band. This result indicates that the kanamycin gene didn't insert into the wild type. These results do not corrispond to the result of the PCR with the kanamycin primers.

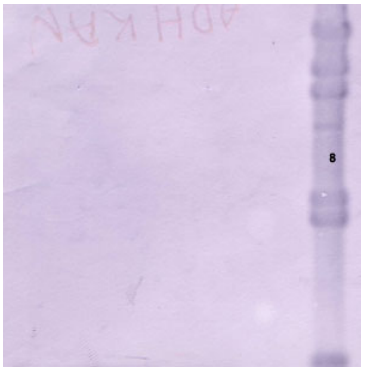


Figure 35 Kan. gene in Δadh southern blot

Southern blot was not done on the ADH gene since earlier results showed that the transformation didn't work.

4.9 Design and construction of alcohol dehydrogenase insertion cassette

Alcohol dehydrogenase participates in the conversion of acetaldehyde to ethanol. Previous results in this project including genome sequencing, activity staining and attempts to inactive the soluble ADH indicated existence of only one soluble ADH participating in key metabolic pathways of the bacteria.

The objective of this experiment was to study if additional ADH gene in the genome of strain AK_{17} would have a positive impact on the ethanol production. I.e. increased copy number results in increased production of ADH enzymes participating the metabolic pathway of ethanol production, consequently improving the production. To investigate this, an insertion cassette containing the *adh* gene between flanking

sequences of the *ldh* gene was designed and constructed. The construction schema is shown in the appendix 6.

The plasmid with the *Δldh: adh*T17 was named pSS10 and is 6987 bp. (figure 36 on next page). Due to the size of the *adh* gene the construction of the plasmid took place in three streps. It was verified with restriction analysis. A kanamycin gene for selection of transformants was inserted downstream the LDH 3' flanking sequence. The *kan* gene included a upstream SD (Shine Delgarno) sequence but its expression was assumed to be dependent on upstream promoter sequences, in the' LDH'5 flanking sequences. Plasmid pSS10 was used in attempts to transform stain AK_{17}.

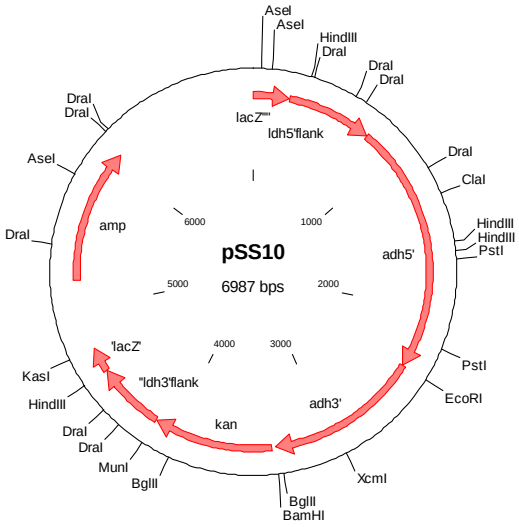


Figure 36 Plasmid pSS10- with adh gene between ldh flank sequences, and the kanamycin selection marker downstream of the adh3' sequence. The construction of the plasmid was verified with restriction analysis.

4.9.1 Transforming with alcohol dehydrogenase insertion cassette

Transformation on strain AK_{17} with alcohol dehydrogenase insertion cassette with plasmid pSS10 was done few times, in an attempt to get a mutant strain. This transforming did not work. The time constant showed 1.5- 2.1 ms. that is normally too low.

4.10 Measuring the end products from fermentation of glucose with column

The analysis of the end products shows the difference between the wild type and the mutant strains.

The glucose was finished after 48 hours of growth both in the wild type and mutant Δ*ldh* strain. The lactic acid was less in the mutant strain than in the wild type but the acetic acid and ethanol productions were similar in the strains.

Table 37 on this and next page shows results from measuring the wild type and mutant Δ*ldh* strain at different growth times. Table 38 on the next page shows the concentration of glucose and the end products lactic acid, acetic acid and ethanol from the wild type and mutant Δ*ldh* strain at different growth times.

Table 37 Measuring wild type and the mutant Δldh on time

Wild type					
	Area				
	Area µRIU*min				
time	Glucose	Lactic Acid	Acetic Acid	EtOH	OD
0	16,0841	0,0626	0,039	0,047	0.038
21,5	12,8215	0,1472	0,3719	0,5612	0.364
22,5	10,3052	0,1992	0,4668	0,7084	0.494
23,5	8,902	0,3337	0,7168	1,163	0.651
25,5	1,3397	0,5645	1,3495	2,0428	0.897

Lactate mutant strain					
	Area				
	Area µRIU*min				
time	Glucose	Lactic Acid	Acetic Acid	EtOH	OD
0	14,4818	0,0482	0,0243	0,0414	0.031
23	2,3674	0,0646	1,3989	2,2151	0.835
24	1,1114	0,0668	1,3998	2,2473	0.897
25	0,29	0,0671	1,6857	2,7824	0.904
27	0,0809	0,0645	1,5303	2,5864	0.832

Table 38 Concentration of end products from the wild type and the mutant Δldh on time

Villti					
time	Glucose	Lactic Acid	Acetic Acid	EtOH	OD
	mg/mL	promil	mg/mL	%	
0	33,3	1,78	0,017	0,003	0.038
21,5	26,5	4,20	0,159	0,040	0.364
22,5	21,3	5,68	0,199	0,051	0.494
23,5	18,4	9,51	0,306	0,084	0.651
25,5	2,8	16,09	0,577	0,147	0.897

Lactate					
time	Glucose	Lactic Acid	Acetic Acid	EtOH	OD
	mg/mL	promil	mg/mL	%	
0	30,0	1,37	0,010	0,003	0.031
23	4,9	1,84	0,598	0,159	0.835
24	2,3	1,90	0,598	0,162	0.897
25	0,6	1,91	0,720	0,200	0.904
27	0,2	1,84	0,654	0,186	0.832

When the OD is 0.897 the glucose is similar (2.8 and 2.3), the acetic acid production is similar (0.577 and 0.598). While the lactic acid production is different, in the wild type it is 16.09 promil when it is only 1.90 promil in the mutant Δ*ldh* and the ethanol production is increase in the mutant strain of about 10% (0.147 to 0.162). This result shows that the transformation had an impact on the phenotype. Figure 37 shows the information from the column when the end products are measured and other chemicals in the samples.

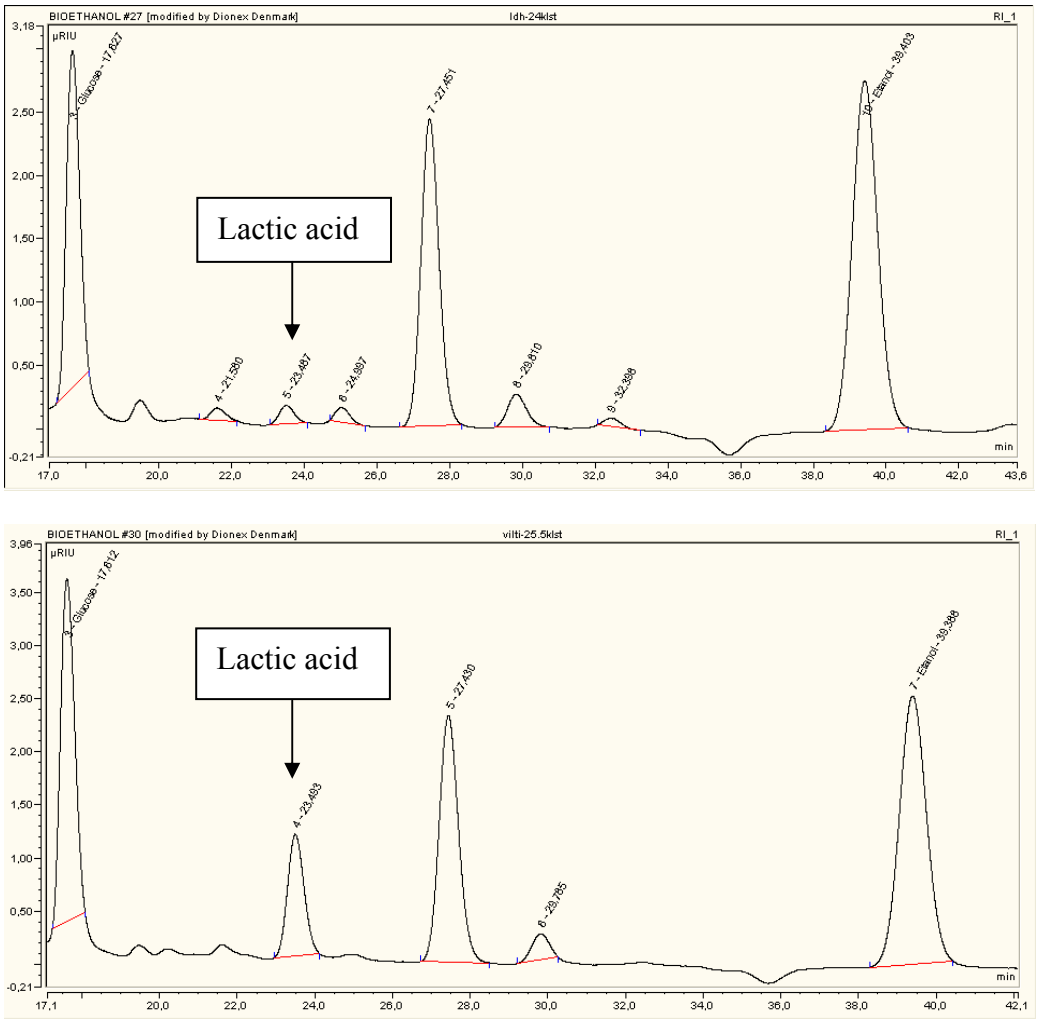


Figure 37 Measuring wild type and the mutant Δldh with OD 0.897. A clear difference between the WT and the mutant is seen. Only a very small lactic acid peak is seen by the mutant (above) compared to the wild type (below)

5 Discussion

Ethanol production by fermentation sugar with thermophilic microorganisms has been studied a lot for over 20 years[117] but only recently such biotechnology has been exploited to lower the cost of production and increase the production yield[118]. The metabolism of thermophilic microorganisms has been well studied and is the prerequisite for the possibility to use gene technology to transform wild types and make new "better" mutant strains. The research on metabolic engineering of these microorganisms and the knowledge of mesophilic recombinant microorganisms which produce ethanol in high yield are also important for the possibilities to produce ethanol with mutant strains of thermophilic bacteria[119].

Using bio-ethanol as a fuel is possible and countries all over the world have started to use it instead of gasoline[120]. Petroleum adds extra carbon in the global carbon cycle, which increases environmental problems (ex. global warming). Using bio-ethanol instead of petroleum is better for the environment since the fermentation is already a part of the global carbon cycle[121]. It is very important to find new fuel instead of petroleum and bio-ethanol is a good alternative.

This project took about two years and its main goal was making mutant strains that produce less lactate acid or acetic acid and more ethanol than the wild type of strain AK_{17}. In this project, one lactate dehydrogenase knockout mutant was made and an attempt was made to make an alcohol dehydrogenase knockout mutant. Deletion of genes responsible for acetic acid formation will be a subject of another study. Additionally one alcohol dehydrogenase insertion cassette was made which is ready to be used in transformation of strain AK_{17} to a mutant strain. The discussion below will be divided into six subchapters. In the first subchapter growth of strain AK_{17} will

[117] Dien, B.S. et al. 2003:258
[118] Mai, V., Lorenz, W. W. and Wiegel, J. 1997:163-167
[119] Shaw, A. et al. 2008:13769.
[120] Mielenz, J. R. 2001:324
[121] Mielenz, J. R. 2001:324.

be discussed, in subchapters 2 and 3 ways to find the target genes are discussed and in subchapters 4-6 the target genes themselves will be discussed.

5.1 Growth on strain AK_{17}

Comparing the growth curve of strain AK_{17} (on page 55) in this thesis to the growth from earlier master's thesis when the strain was isolated, figure 38, shows a different growth time. The growth curve of strain AK_{17} has a long lag-phase, which covers the first 24 hours, but in the earlier master thesis the lag-phase is much shorter, only around 10 hours. In the earlier study the OD grows very fast until the OD is about 1.2 but in this project the OD only goes to about 0.8 during the growth-phase. In the earlier study the OD fell down to about 1.00 in the stationary phase but in this study the OD stayed at around 0.8 in the stationary phase[122].

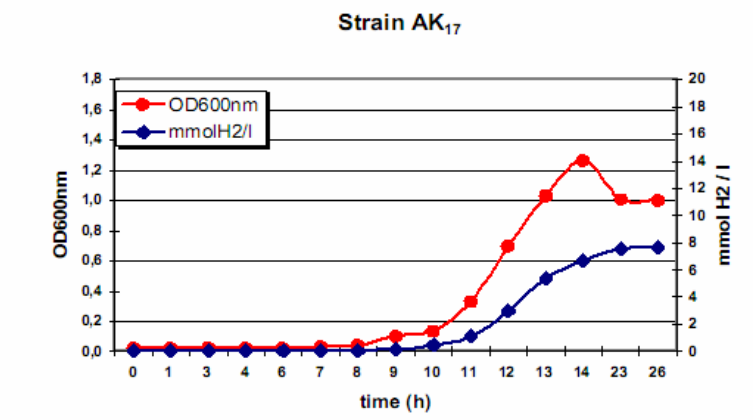


Figure 38 Growth curve of strain AK_{17} from earlier master[123]

This different growth times in the growth curves on the same strain is possibly because the cultures that were used in this study were older than the cultures used in

[122] Steinar Rafn Beck Baldursson. 2006:70
[123] Steinar Rafn Beck Baldursson. 2006:70

the earlier master study. The difference in OD cannot be due to glucose concentration since glucose concentration that was around 20 mM in both studies, which is too little of a difference to explain the gap in OD (0.4)[124].

5.2 GeneMining

The first plan was to use technology called GeneMining to find the target gene that encodes the enzyme (phospahate acetate transferase, lactate dehydrogenize, acetate kinase and two alcohol dehydrogenase genes). The first outcome showed too much variation between the enzymes in the family and some of them were even in more than one family. These inconsistencies along with new emerging sequencing technology are the reasons that this technology was not used more in this project.

5.3 Genome sequencing

The genome sequencing revealed the lactate dehydrogenase gene with the flanking sequences and partial sequences of the alcohol dehydrogenase gene. The genome DNA was searched and blasted for another alcohol dehydrogenase gene (similar to short chain, medium chain and long chain ADHs), but only one gene was found. The reason for searching for another alcohol dehydrogenase gene is that closely related strains, like *T. ethanolicus* and *Thermoanaerobacterium brockii*[125], have more than one alcohol dehydrogenase gene. In addition there was searched for a phosphatetransacetylase gene, no significant sequence similarity was found. However, a gene encoding acetate kinase, the other enzyme participating in formation of acetic acid, was observed. (This gene will be a subject of another study). The next step in

[124] Steinar Rafn Beck Baldursson. 2006: 70
[125] Burdette, D. S., Tchernajencko, V. and Zeikus, J.G. 1999: 11

this further study is to make a knockout mutant for the gene. Knocking out the acetate kinase gene is important because strain AK_{17} produces a lot of acetic acid. Since Δ*ldh* mutant did produce more ethanol than the wild type it is very likely that an Δ*AcCoA* mutant would produce even more ethanol than the Δ*ldh* mutant. Developing a mutant with phosphatetransacetylasee gene knockout is also important since the enzyme is used to produce acetic acid, which strain AK_{17} produces more of than lactic acid. When an insertion cassette will be used to transform strain AK_{17}, it will be interesting to see how much the ethanol production will change. Is the *adh* gene the only gene that effects the ethanol production or are some other gene/genes involved?

5.4 Lactate dehydrogenase gene

Transformation experiments were done with the lactate dehydrogenase deletion plasmid pSS2. The plasmid does not replicate in *T. islandicus* or strain AK_{17}, but due to the sequences homologous to the *ldh* flanking sequences in the chromosome, homologous recombination can take place and the marker can incorporate into the chromosome. Thereby, through a double crossover event, the *ldh* gene is deleted and replaced with the *kan* gene. The first experiments were performed in the similar way as in *Transformation of Thermoanerobacterium sp. Strain JW/SL-YS485 with plasmid PIKM1 conferring kanamycin resistance* written by Volker Mai, W. Walter Lorenz and Juergen Wiegel. In that protocol sterile pre-reduced water (flushed and Na_2S added) is used to wash the cells. AB- buffer, from the medium, with 270 mM sucrose (both solution with C_2- solution) was used to dissolve the cells and this solution was incubated at 48°C to induce the cell spheroplast (autoplast) formation[126]. This protocol did not work for the transformation of *ldh* plasmid pSS2 with strain AK_{17}, which was confirmed with results from trying electroporation on the cells with BioRad Gene pulser. After discussions with other colleagues we came to the conclusion that it is better to wash the cells with 10% glycerol rather than sterile water

[126] Mai, V., Lorenz, W. W. and Wiegel, J. 1997: 163-167

since, it is less likely that 10% glycerol damages the cells. Another conclusion was that water would be better in the incubation step (electrophoresis) than the AB-buffer due to the salt concentration in the AB buffer. After these changes, electroporation with BioRad Gene pulser did show positive results.

The time constants in these experiments were lower than in the research of *Thermoanaerobacterium* sp. Strain JW/SL-YS485 where the time constants showed 4-8 ms.[127]. The time constant reflects the resistance. Too many ions in the solution lower the resistant and the constant becomes low. High time constant indicates that the cells conduct the current. The time constants in the lactate dehydrogenase transformation experiments were 2.1-3.6 ms., which are even lower than the lower limit from the *Thermoanaerobacterium* sp. Strain JW/SL-YS485 results. This can be because the cells were not washed enough with 10% glycerol, there was a little bit of salt left. It would be interesting to try washing the cells three times with 10% glycerol instead of two times like in the protocol and see if the time constant would increases.

Although the mutant Δ*ldh* strain in this research did not produce any lactic acid there is a trace of lactic acid in the starting culture. This does compare to the results from Shaw et al. of *T. saccharolyticum* were the L-*ldh*$^-$ strain did not produce detectable lactic acid during the growth time. The mutant Δ*ldh* strain in this project did also produce more ethanol, of 10%, like the L-*ldh*$^-$ mutant in earlier research did[128]. However, only one measurement of the end products was carried out and the results have to be verified. Before the growth on mutant Δ*ldh* strain starts, a little bit of lactic acid is detected in the medium, 0.0482 promil and it increases almost nothing. This little production does compare to the results from Deasi et al. study about cloning L-lactate dehydrogenase and knockout lactic acid gene in *Thermoanaerobacterium saccharolyticum* JW/SL-YS485. Deasi et al. study showed also a little lactic acid production; 0.3 M of lactic acid was produced during the growth time both on glucose and xylose that is much lower than the wild type strain produce. In the wild type strain the lactic acid increased during the growth time both glucose and xylose with a final concentration of 8.1 and 1.8 mM[129].

[127] Mai, V., Lorenz, W. W. and Wiegel, J. 1997: 163-167
[128] Shaw et. al. 2008: 13769
[129] Desai, S.G. 2004:603

5.5 Alcohol dehydrogenase gene

In the beginning native protein gels were used to estimate the alcohol dehydrogenase activity in the strain.

Measuring the activity with native protein gel was also a problem. Yet, with new loading buffer that didn't include SDS, activity could be detected.

An ethanol tolerance test for Ak_{17} was carried out. According to the results ethanol concentration >2% affected the cell growth. The strain has to be adopted or engineered to tolerate higher ethanol concentration for potential industrial exploitation.

Closely related strains like *Clostiridum thermocellum* and *Thermoanaerobacter ethanolicus* have a low ethanol tolerance for growth, <2% [vol/vol] compared to genus like *Saccharomyces* and *Zymomonas*, which have a high alcohol tolerance for growth of > 6% [vol/vol]. Mutant strain of *T. ethanolicus* has been made with an extra ethanol tolerance and the strain has a tolerance of >4% [vol/vol] [130].

Alcohol dehydrogenase enzyme is used to produce ethanol by reducing acetaldhyde with NADH or NADPH[131]. Two alcohol dehydrogenases from *Thermoanaerobacter ethanolicus* have been isolated. Bryant and Wiegel (1983) isolated one that is NADP dependent and is a primary alcohol dehydrogenase[132]. Bryant, Wiegel and Ljungdahl (1987) isolated another one which is also NADP dependent but it has preferentially oxidizes secondary alcohols[133].

Substrates from cell extract of cells of *T. ethanolicus* obtained during growth cycle at 55, 60 and 68°C for 36 hours were used to analyze the activity of alcohol dehydrogenase. These substrates were ethanol, 2-propanol and 1-butanol. The results from 60°C and 68°C showed that in early part of the fermentation secondary alcohol dehydrogenase appears but in the end of the pathway the secondary alcohol

[130] Burdette, D.S., Jung, S.-H., Shen, G.-J., Hollingsworth, R. I. and Zeikus, J.G. 2002:1914.
[131] Rawn, J. David. 1983:575-576
[132] Bryant, F.O., Wiegel, J. and Ljungdahl, L.G. 1988:461
[133] Bryant, F.and Ljungdahl, L.G. 1981: 793

dehydrogenase decreases. During the fermentation and in the beginning of the stationary phase the primary alcohol dehydrogenase slowly increases. Results from 55°C show that both alcohol dehydrogenases raised at the same time but the secondary alcohol dehydrogenase does decrease before the primary alcohol dehydrogenase does. Ethanol was analyzed for activity with both secondary and primary; 2-propanol was analyzed for specific activity with secondary and 1-butanol had a high activity with the primary and a very low activity with the secondary alcohol dehydrogenase. The specific activity with ethanol for primary alcohol dehydrogenase were 25.7 and for secondary alcohol dehydrogenase 11.4[134].

The protein gel showed two bands, one that is probably a membrane-bound enzyme and another one that is soluble. These enzymes are very large since the bands ran only a short distance down into the gel. When the genome DNA from strain AK_{17} was sequenced and blasted only one alcohol dehydrogenase was found. Screening and blasting was attempted for medium and short alcohol dehydrogenases, which yielded no results. The primary alcohol dehydrogenase gene is active late in the growth cycle but the secondary gene is active early in the growth cycle. The affinity of the secondary alcohol dehydrogenase gene for acetaldehyde is relatively high according to a rather low K_m (44.8 mM) compared to the affinity of the primary alcohol dehydrogenase with K_m 210 mM and the activity is increased with pyruvate. These results support the idea that the secondary alcohol dehydrogenase is the enzyme that is responsible for ethanol production. The primary alcohol dehydrogenase enzymes have probably a role late in the fermentation, perhaps of converting alcohols to aldhydes[135]. Translating this knowledge to strain AK_{17} is not possible because all the experiments in this project indicate only one soluble alcohol dehydrogenase. The protein gel showed a two alcohol dehydrogenase enzymes but one was member-bounded. It is likely that the membrane-bound enzyme has nothing to do with the metabolic of producing ethanol but it cannot be precluded. Yet, it is likely that the long chain alcohol dehydrogenase observed through the genomic sequencing is the enzyme responsible for conversion of acetaldehyde into ethanol.

[134] Bryant, F.O., Wiegel, J. and Ljungdahl, L.G. 1988:461- 462
[135] Bryant, F.O., Wiegel, J. and Ljungdahl, L.G. 1988:464

5.6 Design and construction of alcohol dehydrogenase insertion cassette

The transformation on strain AK_{17} with alcohol dehydrogenase insertion cassette with plasmid pSS10 did not work. Plasmid pSS10 is much larger than the other plasmids that are used to transform strain AK_{17}, so it is probably more difficult to transform the bacteria. The transformation did probably not work because the time constants were low in all the experiments that were made. As were discussed in the lactate dehydrogenase gene chapter it is possible to try to wash the cells more with the 10% glycerol to get a higher time constant. In the third experiment an AcCoA knock out plasmid was used as a control to see if the transformation process or plasmid pSS10 was the problem. In this experiment the time constant was 1.5 ms. in all transformations and colonies only grew on the plates without kanamycin.

Adding transformed cultures on plates can be another possible problem in this process. In the second transformation experiment no colonies grew on the plates, not even on the plates without kanamycin. The problem with this experiment is probably with adding transformed cultures on plates. Due to sensitivity to oxygen the cultures probably died before they were placed on the plate or died on the plates. Transformation of the themophilic anaerobic bacteria is generally a difficult task where many critical factors have to be considered.

In pSS10 the flanking sequences are relatively short compared to the sequences between, i.e. the *adh* gen and the *kan* gen. The chances of homolog recombination correlate with the size of the homologous sequence. Therefore there is higher chance of recombination between the *adh* sequence in the plasmid and in the chromosome rather than between the *ldh* flanking sequences. Homologous recombination in the *adh* gen does not have any effect in increasing the *adh* copy number. Furthermore, the *kan* gene is downstream of the *adh* gen, far away from the promoter. Although the *kan* gene contains an upstream *T. islandicus* ribosomal binding site, long transcripts with the *kan* gen may be too infrequent to promote prober expression. Thus, expression from the *ldh* promoter may be too low to mediate kanamycin resistance. This may be linked to the growth phase, i.e. expression of *ldh* is low in the beginning but increases during the growth phase. For the cell to survive,

it is important that the marker gene is well expressed immediately in the beginning of the growth phase. Inserting promoter up stream of the *kan* gene may improve pSS10 insertion cassette. Other locus may be chosen for *adh* insertion. This will be a subject of a prolonged study.

6 Conclusion

Fossil fuels, petroleum and natural gases are not a part of the global carbon cycle but ethanol fermentation is already a part of the cycle. Therefore it is possible to consider ethanol as a fuel of the future even though ethanol gives less energy than those fossil fuels and is more expensive. Producing bio-ethanol with microorganisms is a good option because it will not be in competition with the food industry like ethanol production is today. If bio-ethanol production will be possible, hopefully it will cost less than ethanol production does today since ethanol production costs a lot more than producing fossil fuels. Fermentation on biomass with microorganism to produce bio-ethanol will hopefully lower the cost even more.

The Δ*ldh* mutant strain that was made in this project is very interesting even though the lactic acid production in the wild type is very low in the beginning. This mutant strain shows that the lactic acid production did not only stop, the knockout had also effect on the ethanol production. That the ethanol production did increase gives hope of that knocking out acetate kinase will increase the ethanol production even more since production on acetic acid is higher than on lactic acid in the wild type. An alcohol dehydrogenase insertion cassette was designed and constructed where lactate dehydrogenase gene was replaced with the alcohol dehydrogenase, and the next step is to transform the wild type with the plasmid. Designing a strain with an insertion cassette where the acetate kinase gene will be replaced with the alcohol dehydrogenase gene is possible since this project has already shown the possibility of genetic transformation. Although the transformation with the insertion cassette didn't work in this project the results open an opportunity to design and construct a mutant strain with insertion cassette.

7 References

Atlas Ronald M., Bartha Richard (1998). *Microbial ecology fundamentals and applications.* Addison Wesley Longman.

Bouck, J., Miller W., Gorrell, J. H., Muzny, D. and Gibbs, R. A. (1998). *Analysis of the quality and utility of random shotgun sequencing at low redundancies.* Genome Res. 8, 1074-1084.

Bryant, F. and Ljungdahl, L.G. (1981). *Characterization of an alcohol dehydrogenase from Thermoanaerobacter ethanolicus active with ethanol and secondary alcohol.* Biochem. Biophys. Res.commun. 100,793- 799.

Bryant, F.O, Wiegel, J. and Ljungdahl, L.G. (1988). *Pruification and properties of primary and secondary alcohol dehydrogenases from Thermoanaerobacter ethanolicus.* Appl Environ Microbiol. 54,460- 465.

Burdette, D. S., Jung, S.-H., Shen, G.-J., Hollingsworth, I. and Zeikus, J. G. (2002). *Physiological function of alcohol dehydrogenases and long-chain (C_{30}) fatty acids in alcohol tolerance of Thermoanaerobacter ethanolicus.* Appl. Environ. Microbiol. 68, 1914-1918.

Burdette, D.S., Tchernajencko, V and Zeikus, G. J. (1999). *Effect of themal and chemical denaturants on Thermoanaerobacter ethanolicus secondary-alcohol dehydrogenase stability and activity.* Enz Microbiol Technol. 27, 11-18

Cann ISKO, Stroot PG, Mackie KR, White BA and Mackie RI (2001). *Characterization of tow novel saccharolytic, anaerobic thermophiles, Thermoanaerobacterium polysaccharolyticum sp. nov. and Thermoanaerobacterium zeae sp. nov., and emendation of the genus Thermoanaerobacterium.* Int. J. Syst. Evolutionary Microbiology. 51, 293-302

Carlier J-P, Bonne I and Bedora-Faure M (2006). *Isolation from canned foods of a novel Thermoanaerobacter species phylogenetically related to Thermoanaerobacter mathranii (Larsen 1997): Emendation of the species description and proposal of Thermoanaerobacter mathranii subsp. Alimentarius subsp. Nov.* SeienceDirect. 12 , 153-159.

Cayol J-L, Ollivier B, Patel BKC, Ravot G, Magot M, Ageron E, Grimont PAD and Garcia J-L (1995). *Description of Thermoanaerobacter brockii subsp. lactietylicus supsp. nov., isolated from deep subsurface Franch oil well, a proposal to reclassify Thermoanaerobacter finnii as Thermoanaerobacter brockii subsp. finnii comb. nov., and an emended description of Thermoanaerobacter brokii.* Int. J. Syst. Bacteriol. 45, 783-789. Int. J. Syst. Bacteriol. 44, 812-826

Collins, M. D., Lawson, P.A., Willems, A., Cordoba, J. J., Frenandez-Garayabal, J., Gracia, P., Cai, J., Hipper, H. and Farrow, J. A. E. (1994). *The phylogeny of the Genus Clostridium: Proposal of five newgenera and elven new species combination.*

Cook GM, Rainey FA, Patel BKC and Morgan HW (1996). *Characterization of a new obligately anaerobic thermophile, Thermoanaerobacter wiegelii sp. nov*. Int J. Syst Bacteriol. 46, 123-127.

Dien, S. G., Cotta, M. A. and Jeffries, T.W. (2003). *Bacteria engineered for fuel ethanol production: current status.* Appl Microbiol Biotechnol. 63, 258-266.

Deasi, S. G., Guerinot, M. L. and Lynd, L.R. (2004). *Cloning of $_L$-lactate dehydrogenase and elimination of lactic acid production via gene knockout in Thermoanaerobacterium saccharolyticum JW/SL-YS485.* Appl Microbiol Biotechnol. 65, 600-605.

Emil Bedi. (e.d). *Journey to Forever: Ethanol fuel*. April 13 2009 from http://journeytoforever.org/biofuel_food.html

Epicentre biotechnologies. (e.d). *MasterPure^{TM} Purification Kit.* 29. may 2009 from: http://www.epibio.com/pdftechlit/113pl027.pdf

Georgieva, T. I., Mikkelsen, M. J. and Ahring, B. K. (2007). *High ethanol tolerance of the thermophilic anaerobic ethanol producer Thermoanaerobacter BG1L1*. CEJB. 2, 364-377.

Holdren, John P. (1991). *Population and the Energy Problem.* Population & Environment. 12, 231-255.

Grogan, D. W. (1998). *Hyperthermophiles and the problem of DNA instability*. Molecular Microbilology. 28, 1043-1049.

Hall, T.A. 1999. *BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT.* Nucl. Acids. Symp. Ser. 41:95-98.

Hoseki J, Yano T, Koyama Y, Kuramitsu S, Kagamiyama H. (1999). *Directed evolution of thermostable kanamycin-resistance gene: a convenient selection marker for Thermus thermophilus.* J Biochem. 126, 954-956.

Jenkins, Joseph (2000). *The Humanure Handbook (2nd Edition).* Jenkins Publishing. 20. september 2008 from http://weblife.org/humanure/index.html

Johann Orlyggson and Steinar Rafn Beck Baldursson. (2007). *Phylogenetic and physioloical studies of four hydrogen-producing thermoanareobes from Icelandic geothermal ares.* ICEL. AGRIC. SCI. 20:93-105.

Keith Addison (e.d). *Journey to Forver: Ethanol fuel.* April 8 2009 from http://journeytoforever.org/ethanol.html

Kumar, S. and Nussinov, R. (2001). *How do thermophilic proteins deal with heat?* CMLS, Cell. Mol. Life Sci. 58, 1216-1233.

Lee, Y-E., Jain, M. K., Lee, C., Lowe, S. E. and Zeikus, J. G. (1993). *Taxonomic Distinction of Saccharolytic Thermophilic Anaerobes: Description of Thermoanaerobacterium xylanoyticum gen. nov., sp. nov., and Thermoanaerobacterium saccharolyticum gen. nov., sp. nov.; Reclassification of Thermoanaerobium brockii, Clostridium thermosulfurogenes, and Clostridium thermohydrosulfuricum E100-69 as Thermoanaerobacter brockii comb. Nov., Thermoanaerobacterium thermosulfurigenes com. Nov., and Thermoanaerobacter thermohydrosulfuricus com. Nov., Respectively; and Transfer of Clostridium thermohydrosulfuricum 39E to Thermoanaerobacter ethanolicus.* Int. J. syst Bacteriol. 43, 41-51.

Levin, D.B, Pitt, L and Love, M. (2004). *Biohydrogen production technologies: Prospects and limitation for practical application.* Int. J. Hydrogen Energy 29, 173-185.

Ludwig, B., Akundi, A. and Kendall, K. 1995. *A long-chain. Secondary alcohol dehydrogenase from Rhodococcus erythropolis ATCC 4277.* App. Enciron. Microbiol. 61, 3729-3733.

Madigan MT, Martinko J, parker J (2003). *Brock biology of microorganisms.* Prentice Hall.

Mai, V., and Wiegel, J. (2000). *Advamces in Development of a Genetic System for Thermoanaerobacterium spp.: Expression of Genes Encoding and Integration of Genes into the Chromosome.* Georgia: Department of Microbiology and Center for Biological Resource Recovery, University of Georgia, Athens.

Mai, V., Lorenz, W. and Wiegel, J. (1997). *Transformation of Thermoanaerobacterium sp. Strain JW/SL-YS485 with plasmid pIKM! Conferring kanamycin resistance.* FEMS microbiology Letters. 148, 163-167.

Mielenz, J. R. (2001). *Ethanol production for biomass: technology and commercialization status.* Current Opinion in Microbiology. 4, 324-329

Olafur Fridjonsson and Mattes, R. (2001). *Production of recombinant α-galactosidases in Thermus thermophilus.* Appl. Enciron. Microbiol. 67, 4192-4198.

Rawn, J. David. (1983). *Biochemistry.* Harper&Row, Publishers, Inc

Rainey, F.A., N. L. Ward, H. W. Morgan, R. Toalster, and E. Stackebrand. 1993. *Phylogenetic analysis of anaerobic thermophilic bacteria: aid for their reclassification*. J. Bacteriol. 175:4772-4779.

Saffarini, D. A., Schultz, R. and Beliaev, A. (2003). *Involvvement of cycilc AMP (cAMP) and cAMP Receptor Protein in Anaerobic Respiration of Shewanella oneidensis.* J. Bacteriol. 185, 3668-3671.

Shakhashiri. (February 5. 2009). *Ethanol (chemical of the week)*. April 15 2009 from http://scifun.chem.wisc.edu/CHEMWEEK/PDF/Ethanol.pdf

Shaw, A. Joe, Podkaminer, Kara K., Desai, Sunil G., Bardsley, Joh S., Rogers, Stephen R., Thorne, Philip G., Hogsett, David A. and Lynd, Lee R. (2008). *Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield.* PNAS. 105, 13769-13774.

Skirnisdottir S, Hreggvidsson GO, Hjorleifsdottir S, Marteinsson VT, Petursdottir SK, Holst O and Kristjansson JK. (2000). *The infuence of sulfide and temperatur on the species composition and community structure of hot spring microbial mats.* Appl. Environ. Microbiol. 66, 2835-2841.

Steinar Rafn Beck Baldursson. 2006. *BioHydrogen. Bioprospecting: Thermophilic hydrogen producing anaerobes in Icelandic hot-springs.* MS-thesis. University of Akureyri.

Talabardon, M., Schwitzguébel, J.-P. and Péringer, P. (2000). *Anaerobic thermophilic fermentation for acetic acid production from milk permeate.* Journal of Biotechnology. 76, 83-92.

U.S. Department of Energy. (Last uppdate 8/9 2006). *Clean Cities- Fuel Blends.* July 2 2007 from http://www.eere.energy.gov/cleancities/blends/ethanol.html

U.S. Department of Energy. (Last uppdate 4/8 2007). *Alternative Fuels Data Center.* July 2 2007 from http://www.eere.energy.gov/afdc/altfuel/whatis_eth.html

Wiegel J and Ljungdahl LG (1981). *Thermoanerobacter ethanolicus gen. Nov. and sp. Nov. a new extreme thermophilic, anaerobic bacterium*. Arch. Microbiol. 128, 343-348.

8 Appendixes

8.1 The growth of stain AK_{17}

Time	Day	Hours in gorw	Growth
16:30	13/8 ´07	0	0
07:30	14/8 ´07	15	0,003
08:30	14/8 ´07	16	0,015
09:30	14/8 ´07	17	0,032
10:30	14/8 ´07	18	0,026
11:30	14/8 ´07	19	0,016
12:30	14/8 ´07	20	0,029
13:30	14/8 ´07	21	0,027
14:30	14/8 ´07	22	0,045
15:30	14/8 ´07	23	0,066
08:30	24/8 ´07	24	0,473
09:30	24/8 ´07	25	0,629
10:30	24/8 ´07	26	0,743
11:30	24/8 ´07	27	0,754
12:30	24/8 ´07	28	0,757
13:30	24/8 ´07	29	0,769
14:30	24/8 ´07	30	0,753
15:30	24/8 ´07	31	0,78
08:00	15/8 ´07	39,5	0,799
10:00	5/9 ´07	44	0,811
11:00	5/9 ´07	45	0,805
12:00	5/9 ´07	46	0,798
13:00	5/9 ´07	47	0,79
14:00	5/9 ´07	48	0,792
15:00	5/9 ´07	49	0,795
16:00	5/9 ´07	50	0,792

8.2 Kanamycin test

Kanamycin stength	Growth
0 µg/ml	+/+
7,58 µg/ml	+/-
26,52 µg/ml	-/-
75,38 µg/ml	-/-
150 µg/ml	-/-
223,88 µg/ml	-/-

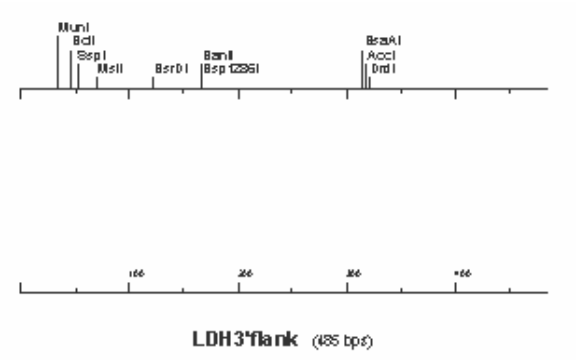
8.3 Primer in this project

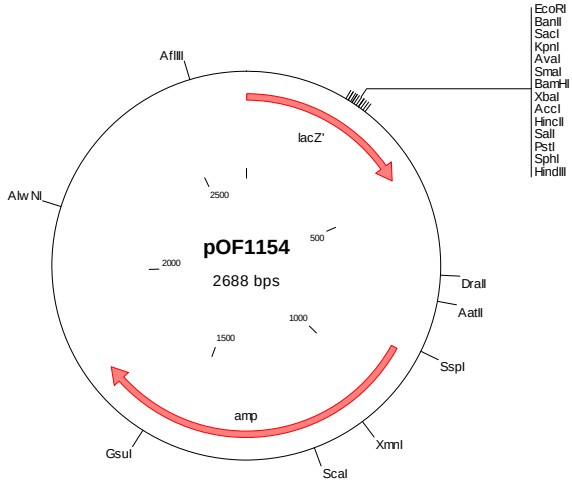
Name of the primer	**Sequence**	**Where the primer was used**
LDH5'-eco-f	CGG GAA TTC GAG AGT GTC GTA GCT CAT GTA GTC	Ldh 5'flank for in Δ*ldh* knockout cassett
LDH5'-SOEkan-r	TATTATTGGTCCATTCATTAC ATCGCCTCCTAA ATAATATATATTTTC	Ldh 5'flank for in Δ*ldh* knockout cassett
kan-SOEldh-f	TTATTTAGGAGGCGATGTAATGAATGGAC CAATAATAATGAC	Kan. gene for in Δ*ldh* knockout cassett
kan-bam-r	CGCGGATCCTCAAAATGGTATGCGTTTTG ACAC	Kan. gene for in Δ*ldh* knockout cassett
LDH3'- hind III-R	CCCCAAGCTTCGGATTAGTTATGTTTATGA GATTTT	Ldh 3'flank for in Δ*ldh* knockout cassett
LDH3'-hind-R2	CCCCAAGCTTAGAACATCTGCTGATTTATC AAATCC	Ldh 3'flank for in Δ*ldh* knockout cassett
LDH3'-bam-f	CCC CAA GCT TAG AAC ATC TGC TGA TTT ATC AAA TCC	Ldh 3'flank for in Δ*ldh* knockout cassett
ADH_S17-2bio	GGGACAAATCCGAGAATGCCTCTTAC	To get the whole *adh* gene
ADH_s17-4	CGGAGATCTACAGGAAGGCATACAG	To get the whole *adh* gene
Adh_S17-6 bio	AGCGCCGACATTTATTATTAAAAATTATG	To get the whole *adh* gene
adh_s17-8	GTAGAACTCCCCCTTTATTTACAAC	To get the whole *adh* gene
adhS17-nde-f	CGCCAATTGCATATGGCAACGACAAAAAC GGAATTA G	To get the whole *adh* gene
adhS17-bam-r	CGCGGATCCTTATGCACTGTATGCCTTCCT GTAG	To get the whole *adh* gene
adhS17-seq1800-f	CTTGCTACACTGTCAAAGATTAC	To sequenced *adh* coloines
adhS17-seq2300-r	TTGATGCAGGCAGTCCTAAG	To sequenced *adh* coloines
adhS17-1600-r	ATTGCAGAACCGCCGCCTAC	To sequenced *adh* coloines
adhS17-seq1600-f	AGGCGGCGGTTCTGCAATAG	To sequenced *adh* coloines
adh3'-bam-f	CGGGGATCCGGAGATCTACAGGAAGGCAT ACAG	Adh 3'flank for in Δ*adh* knockout cassett
adh3'-hind-r	GTATTCATTGGCCGTTATAGCTCTG	Adh 3'flank for in Δ*adh* knockout cassett
adhS17-5'-mfe-f	CGCCAATTGTCAACATCACTTTGCTGCCTT TC	Adh 5'flank for in Δ*adh* knockout cassett
adh5'flank-kan-soe-r	CATTATTATTGGTCCATTCATGATAAACAC CTCCGTGTTAATTTG	Adh 5'flank for in Δ*adh* knockout cassett
kan-adh5'-SOE-f	ATTAACACGGAGGTGTTTATCATGAATGG ACCAATAATAATGAC	Kan gene for in Δ*adh* knockout cassett
kan-bam-r	CGCGGATCCTCAAAATGGTATGCGTTTTG ACAC	Kan gene for in Δ*adh* knockout cassett
LDH5-mfe-f	GCGCAATTGGAGAGTGTCGTAGCTCATGT AGTC	Ldh 5'flank for insertion cassette
LDH-5adh-soe-r	CGTTTTTGTCGTTGCCATTACATCGCCTCC TAAATAATA	Ldh 5'flank for insertion cassette
adhS17-5ldh-soe-f	TTATTTAGGAGGCGATGTAATGGCAACGA CAAAAACGGAATTAG	Adh5' gene for insertion cassette/adh gene for insertion cassette
adh-1500-eco-r	GCCGCCTACAGCGATTATTAAG	Adh5' gene for insertion cassette
adhS17-nde-f	CGCCAATTGCATATGGCAACGACAAAAAC GGAATTAG	Adh3' gene for insertion cassette
adhS17-bam-r	CGCGGATCCTTATGCACTGTATGCCTTCCT GTAG	Adh3' gene for insertion cassette/ adh gene for insertion cassette

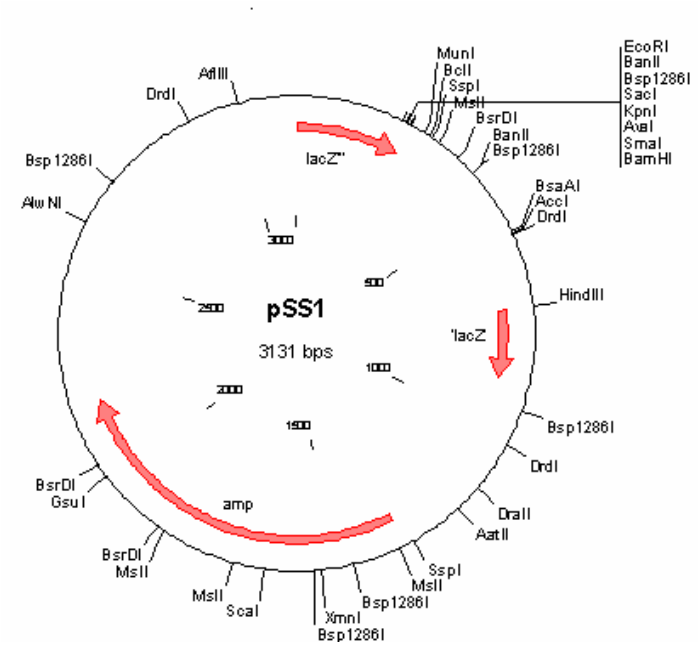
kan-T17SD-bam-f	CCCgGATCcTTAGGAGGCGATGTAATGAATGGACCAATAATAATGACTAGAG	Kan. gene for insertion cassette
kan-mfe-r	CGCAATTGTTCAAAATGGTATGCGTTTTGACAC	Kan. gene for insertion cassette

8.4 Making a knockout mutant : Δ*ldh::kan*

1. **Cloning adh3'flank sequence anplified with:**
   adh3'-bam-f :: adh3'-hind-r.
   Cut with með BamHI and HindIII ligate into pOF1154 (pUC18) which were cut with BamHI og HindIII





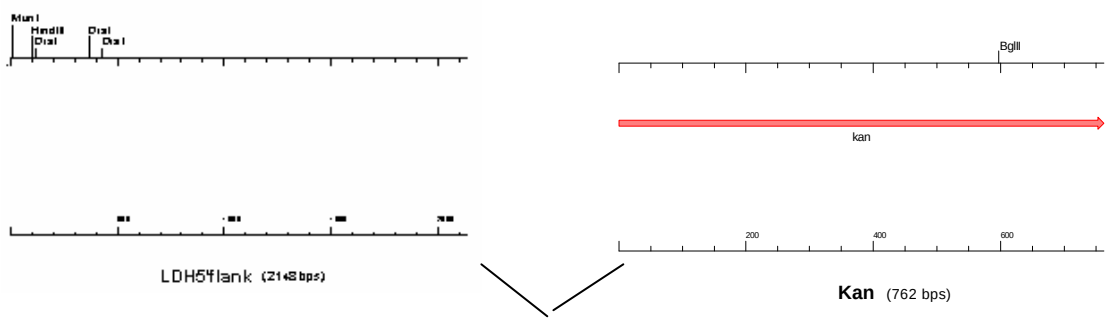


2. SOE PCR ldh5'flank – kan gene

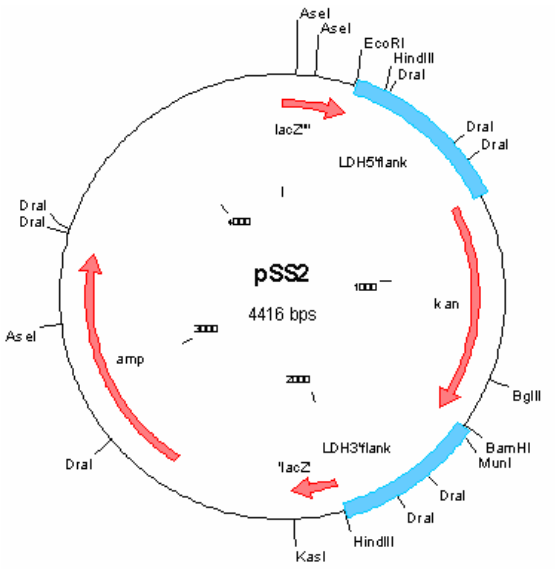
(i) Amplify ldh'5 flank seq with
LDH 5' –eco-f :: LDH5'-SOEkan-r
Template: Strain 17 chromosomal DNA

(ii) Amplify *kan* gene with
kan-SOEldh-f :: kan-bam-r
Template: pOF5712 plasmid DNA

(iii) Amliplify and splice adh'5 seqeunce and kan gene with
LDH5'-eco-f :: kan-bam-r
Template: product from PCR1 and 2.

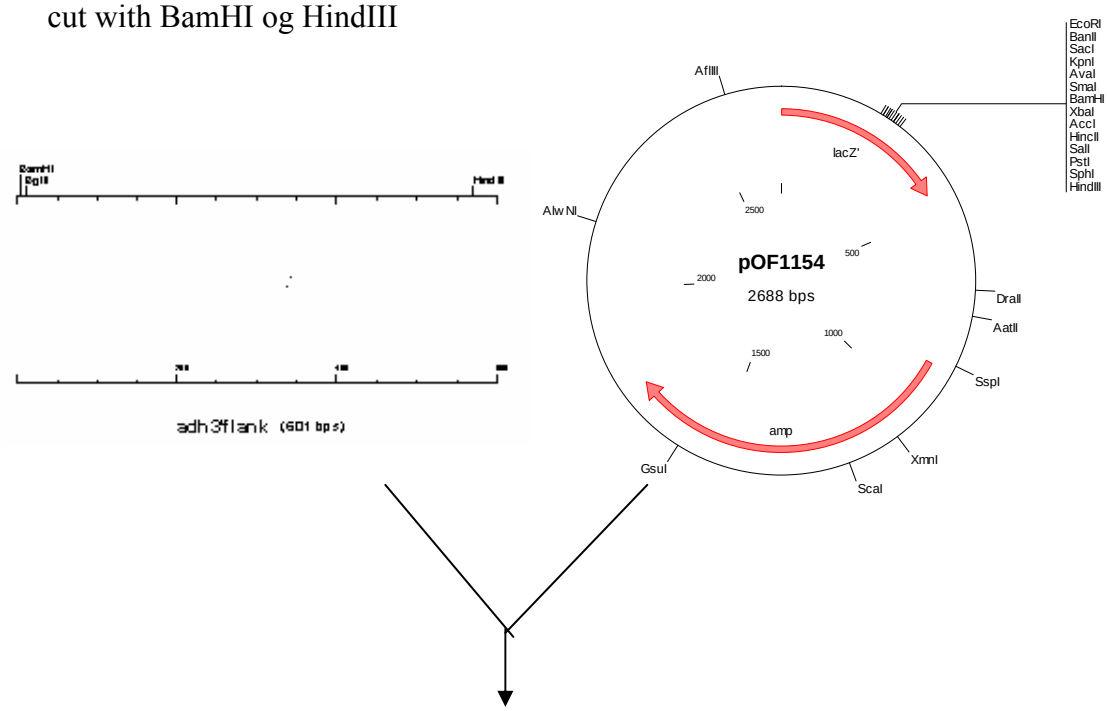


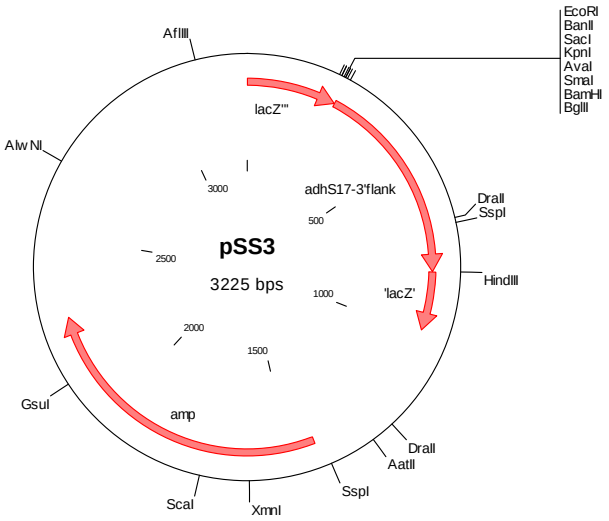
Cloning: Cut PCR-SOE produkt with EcoRI and BamHI
Ligate intoEcoRI cut plasmid pSS1.



8.5 Making a knockout mutant: Δ*adh::kan*

2. **Cloning adh3'flank sequence anplified with:**
   adh3'-bam-f :: adh3'-hind-r.
   Cut with með BamHI and HindIII ligate into pOF1154 (pUC18) which were cut with BamHI og HindIII



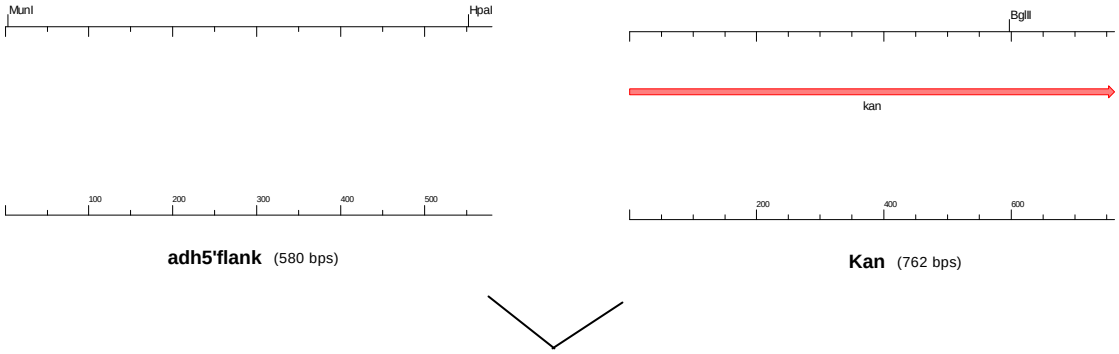


2. SOE PCR adh5'flank – kan gene

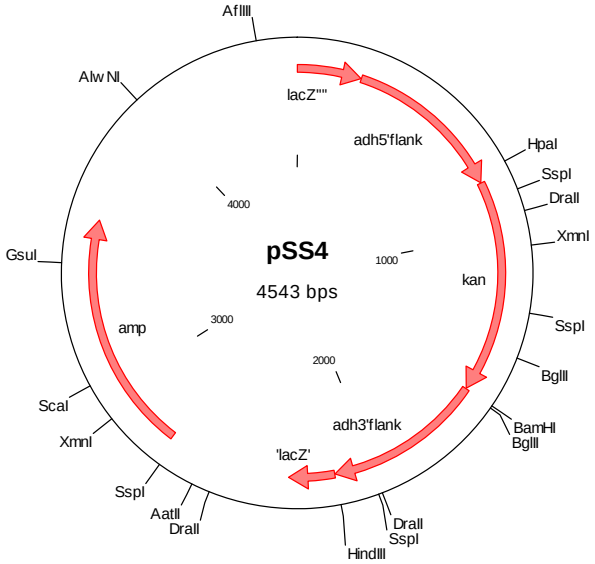
(iv) Amplify adh'5 flank seq with
adhS17-5'-mfe-f :: adh5'flank-kan-soe-r
Template: Strain 17 chromosomal DNA

(v) Amplify *kan* gene with
kan-adh5'-SOE-f :: kan-bam-r
Template: pOF5712 plasmid DNA

(vi) Amliplify and splice adh'5 seqeunce and kan gene with
adhS17-5'-mfe-f :: kan-bam-r
Template: product from PCR1 and 2.



Cloning: Cut PCR-SOE produkt with MfeI and BamHI
Ligate intoEcoRI – BamHI cut plasmid pSS3.

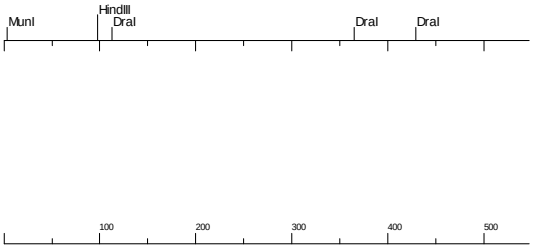


8.6 Construction of adh –Δldh insertion cassette: Δldh::kan

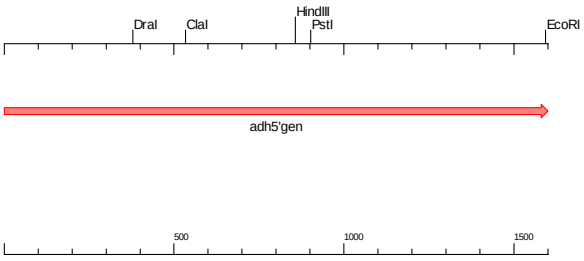
Adh gene spliced in two part because it is so big

SOE – PCR: ldh5'flank spliceds with adh5'gen

1. PCR ldh 5'flank region with LDH5-mfe-f and LDH-5adh-soe-r
2. PCR adh5'gen with adhS17-5ldh-soe-f and adh-1500-eco-r
3. Splice the PRC products with LDH5-mfe-f and adh-1500-eco-r

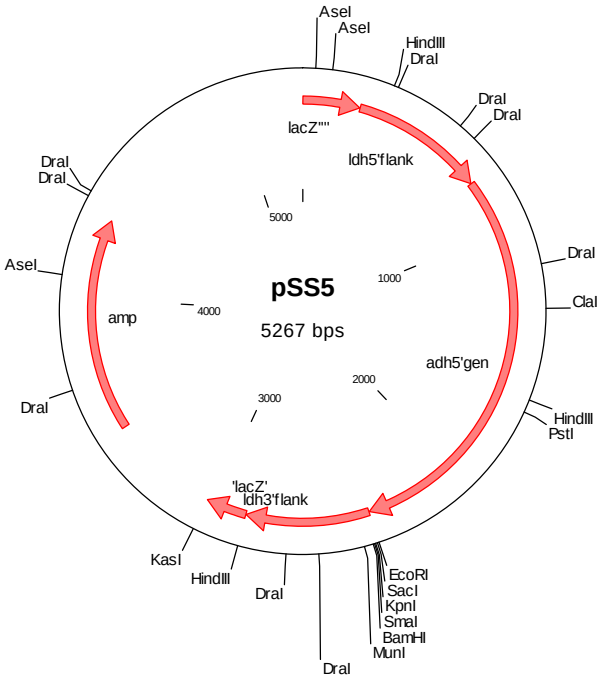


LDH5'flank (547 bps)



Adh5'gen (1601 bps)

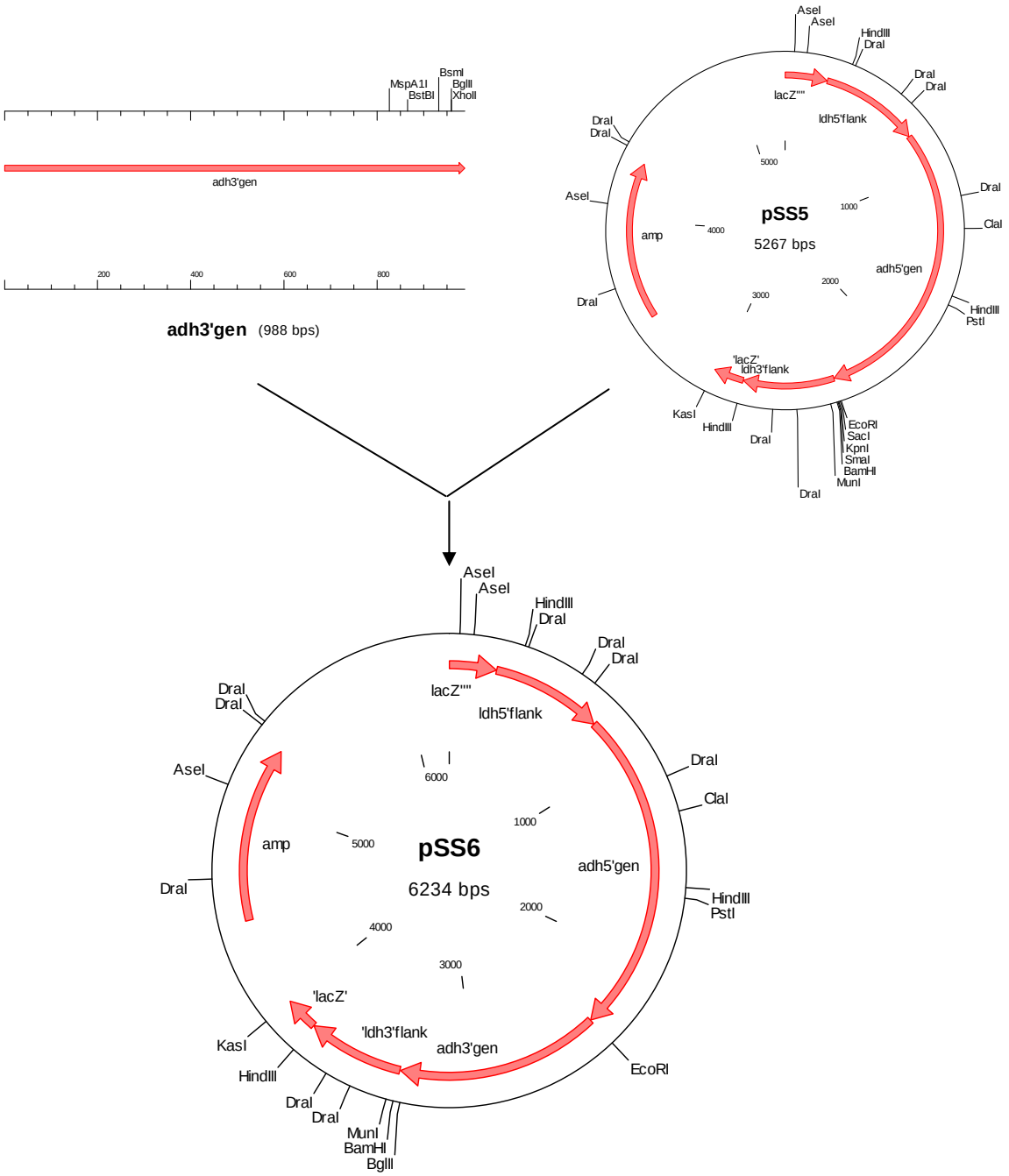
Cloning SOE produktu to plasmid pSS1:
Plasmid pSS1 cut with EcoRI
SOE product cut with MfeI (MunI) and EcoRI
Ligate in pSS1



It may be possible to amplify the whole adh gene.
2b. PCR: adhS17-5ldh-soe-f :: adhS17-bam-r

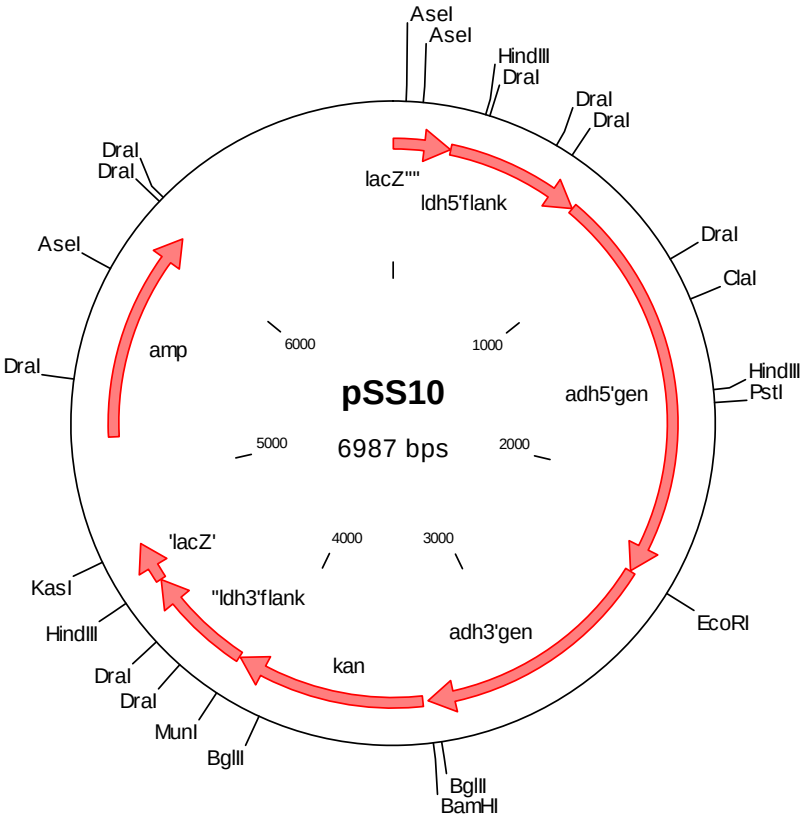
3b: SOE PCR: LDH5-mfe-f :: adhS17-bam-r and ligate the PCR product into pSS1 (EcoRI-BamHI cut)

adhS17-3'gene spliced into pSS5
PCR: adh3'- gene with adhS17-nde-f and adhS17-bam-r
Cloning:
Cut the PCR product adh3' gene with EcoRI and BamHI.
Cut pSS5 with EcoRI and BamHI
Ligate the PCR product into cut pSS5 = pSS6



Cloning kan gene into pSS6

1. PCR kan gene with kan-T17SD-bam-f and kan-mfe-r
2. Cut PCR – kan gene with BamHI / MfeI
3. Cut pSS6 with BamHI / MfeI
4. Ligate kan into cut pSS6.

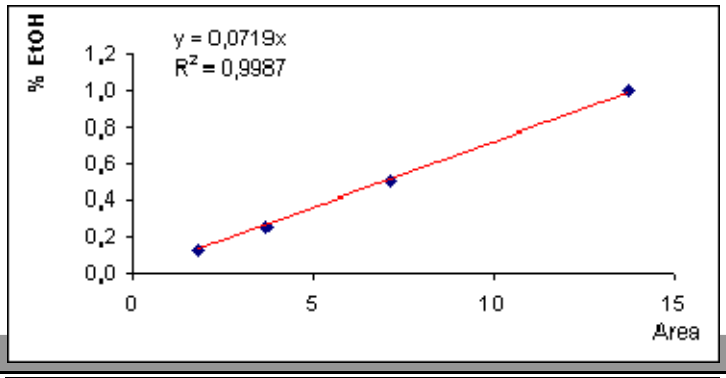


8.7 Strandard for HPCL column

EtOH

%	Area µRIU*min
1	13,7451
0,5	7,1203
0,25	3,7112
0,125	1,8276

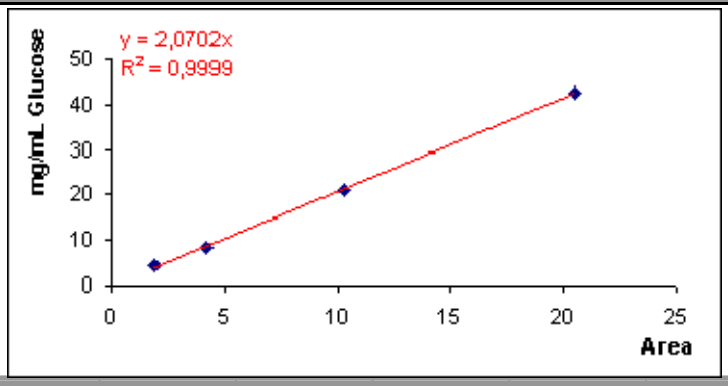
0,0719



Glucose

mg/mL	Area µRIU*min
42,5	20,5281
21,3	10,2644
8,5	4,1817
4,3	1,9052

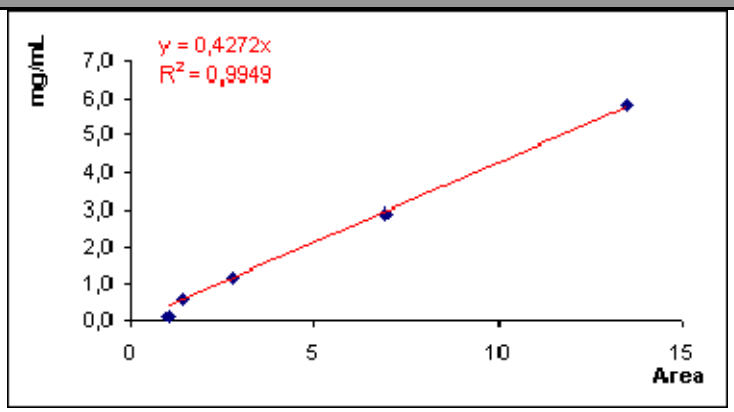
2,1



Acetic Acid

vigtað 970mg
leyst upp 50mL
styrkur 19,4mg/mL

styrkur mg/mL	Area
5,8	13,4818
2,9	6,9531
1,2	2,7489
0,6	1,418
0,1	1,0191



Lactic Acid

styrkur %	Area µRIU*min
1	34,3188
0,5	18,6546
0,25	9,2457
0,1	3,6056
0,02	0,9616
0,01	0,3564

0,0285