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UTILIZATION OF CELLULOSIC VINASSE WITH ACINETOBACTER BAYLYI ADP1

Master of Science Thesis
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ABSTRACT

Ilmari Laaksonen: Utilization of cellulosic vinasse with *Acinetobacter baylyi* ADP1
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Ethanol is industrially produced through a fermentation reaction where different feedstocks are used to provide sugars for the yeast cells to ferment into ethanol. The fermentation product is then distilled and sieved, separating the product into ethanol and stillage factions. The stillage, which is also known as the vinasse contains everything in the fermentation product other than most of the ethanol and the live yeast cells. As the production of ethanol and thus vinasse increases annually, new methods to utilize the vinasse besides using it as a fertilizer have become a necessity. Cellulosic vinasse produced from cellulosic feedstocks is especially dangerous for soils, as it contains both aromatic and organic acid compounds that are toxic to most organisms. By utilizing the new trend of bioprocessing, these compounds can be converted into more valuable products through microbial metabolic pathways.

In this study it is demonstrated that *A. baylyi* ADP1 can grow in diluted cellulosic vinasses and be able to deplete most of them from the medium. It was also discovered that *A. baylyi* ADP1 cells consumed glycerol even though neither the synthetic nor real vinasse contained gluconate. A gene knockout of ACIAD2924 transcribing an acidic transcription factor A (*atfA*) was found to increase the cell volumes of *A. baylyi* ADP1 by 87% for 24 hours but returning to normal dimensions afterwards. This increase of cell volumes effect on wax ester was investigated in this study while also demonstrating that the lack of *atfA* possibly affects parts of the lipid synthesis pathways of *A. baylyi* ADP1. The *ΔatfA* strain was shown to synthesise wax esters in the real vinasse, but not in mediums with glucose as the sole carbon source. Wild-type cells were on the other hand able to synthesise wax esters in pure glucose cultures, but not in vinasse cultures. Discoveries in this study promote further studies into increasing the cell viability of the *A. baylyi ΔatfA* to increase the duration of the cell volume increase and investigating the effect of the *atfA* protein on the lipid synthesis pathway.

Keywords: Vinasse, Wax ester production, Aerobic digestion, Homologous recombination, Acidic transcription factor A, Increased cell volume, *Acinetobacter baylyi* ADP1

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TIIVISTELMÄ

Ilmari Laaksonen: Lignoselluloosallisen vinassin hyödyntäminen *Acinetobacter baylyi* ADP1:llä
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Etanolia tuotetaan teollisesti fermentaatioreaktiossa, jossa hiivasoluja ruokitaan erillaisilla sokeri- tai ravinnerikkailla raaka-aineilla etanolin tuottamiseksi. Fermentaatioreaktio tuotteesta erotetaan seulomalla ja tislaamalla puhdas etanoli ja tislausjäte toisistaan. Syntyvä tislausjäte, joka tunnetaan myös vinassina, sisältää kaiken muun materiaalin fermentaatiotuotteesta paitsi etanolin ja aktiiviset hiivasolut. Vinassi sisältää runsaan määrän orgaanisia yhdisteitä, mitä hiivasolut tuottivat tai eivät käsitelleet raaka-aineesta. Etanolin tuotannon kasvaessa vuosittain, sen tislausjätteenä syntyvälle vinansille pitää kehittää uusia käyttökohteita lannoitteena käyttämisen lisäksi. Vaihtoehtoisten käyttökohteiden löytäminen tämänkaltaisille jätevirroille on maailmalla noussut tärkeäksi tutkimuskohteeksi ja jopa vaatimukseksi. Erityisesti puusta valmistettu etanoli eli lignoselluloosallinen vinassi on ympäristölle vaarallista sen sisältämien aromaattisten yhdisteiden ja orgaanisten happojen takia. Näitä yhdisteitä kuitenkin pystyttäisiin hyödyntämään bioprosesseissa, jossa mikrobit katabolisoivat niistä arvokkaampia yhdisteitä.

Tässä työssä osoitettiin että *A. baylyi* ADP1 pystyy kasvamaan lignoselluloosallisessa vinassissa ja pystyy kuluttamaan tästä useimmat yhdisteet, joita itse etanolia tuottavat sienet eivät pysty hyödyntämään. Sekä synteettisessä, että oikeassa vinassissa *A. baylyi* ADP1 kulutti glyserolia ilman glukonaattia, mitä ei aiemmissa tutkimuksissa ole todettu solujen tekevän. Vinassissa kasvattamisen lisäksi työssä testattiin geeninpoisto ACIAD2924 geenille, joka on vastuussa happoisen transkriptio tekijä A:n (*atfA)* geenieksprессiosta. Tämän proteiinin puuttuminen solun genomista oletettiin kasvattavan *A. baylyi* ADP1n solukokoa edellisen tutkimuksen perusteelta. Työssä todettiin ADP1 solujen kasvavan noin 87% tilavuudeltansa 24 tunnin ajaksi, minkä jälkeen solut palautuivat normaaliin kokoonsa. Tämän lisäksi *ΔatfA* solujen todettiin tuottavan vahaestereitä vain vinassi kasvatuksissa, eikä ollenkaan kasvatuksissa joissa glukoosi toimi ainoana hiilen lähteenä. Villityypin solut eivät tuottaneet vahaestereitä vinassissa. Työn päätöksenä ehdotettiin että tulevissa tutkimuksissa tulisi etsiä keinoja pidentää *ΔatfA* solujen koon kasvun kestoa ja sen vaikutusta solujen lipidi synteesin uusien metabolisten muokkausten avulla.

Avainsanat: Vinassi, Vahaesterien tuotanto, Aerobinen digestio, Homologinen rekombinaatio, Hapollinen transkriptio tekijä A, Solukoon kasvattaminen, *Acinetobacter baylyi* ADP1

Tämän julkaisun alkuperäisyys on tarkastettu Turnitin OriginalityCheck –ohjelmalla.

PREFACE

The research and laboratory work of this master's thesis were conducted in Synbio research team in the Faculty of Engineering and Natural Sciences in Tampere University, Finland, between September 2020 and December 2020.

I would like to express my sincere gratitude to my supervisor Associate Professor Ville Santala, for this subject and assistance on my thesis works. I would like to thank Academy Doctoral Researcher Rahul Mangayil, Doctoral Researcher Jin Luo, Doctoral Researcher Changshuo Liu and the rest of the SynBio group members for assisting me in the chemical and microscopy analyses. They greatly helped me learn new things and made my experiments go smoothly.

I would also thank my family for supporting and helping in overcoming challenges and writing throughout my master's thesis work.

Tampere, 31 January 2022

Ilmari Laaksonen

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LIST OF SYMBOLS AND ABBREVIATIONS

atfA	Acidic transcription factor A
Da	Daltons, atomic mass unit which is approximately 1 g/mol
WS/DGAT	Wax ester synthase/acyl coenzyme A: diacylglycerol acyltransferase
TAG	Triacylglycerol
WE	Wax ester
DNA	Deoxyribose nucleic acid
RNA	Ribonucleic acid
tRNA	Transfer Ribonucleic acid
mRNA	Messenger Ribonucleic acid
FAS	Fatty acid synthesis
Wt	Wild type
LB	Lysogeny broth
LA	Lysogeny broth-agar
OD_{600}	Optical density at 600 nm
PCR	Polymerase chain reaction
TCA	Tricarboxylic acid cycle
TLC	Thin layer chromatography
1G & 2G	First & second-generation ethanol process
LAB	Lactic acid bacteria
w/v %	Weight per volume % (g/100 ml)
CamR	Chloramphenicol resistance

1. INTRODUCTION

The steadily increasing production of different materials and the subsequent depletion of natural resources has significantly increased the demand to recycle previously undermined waste products into resources. A prominent method of realising this is microbial bioproduction, where microorganisms are used to convert these waste material streams into for example biomass, recycled resources like biofuels or even into value added products or chemicals like vanillate. Where the traditional processes require large energy intensive machinery and chemicals to produce more refined chemicals, microorganisms can achieve this in fermentation tanks with the help of metabolic engineering. In this branch of synthetic biology, the microorganisms' metabolic pathways can be altered and optimized to cause them to accumulate products by adding, deleting, or enhancing genes related to these pathways.

Vinasse, also known as ethanol stillage, is a side product of ethanol distillation process which contains all the contents of the fermentation broth excluding most of the fermenting microbes and ethanol. In 2019 and 2020 approximately 110 and 98 billion litres of ethanol were produced globally [1, 2]. Vinasse is generated at the rate of 15 l of vinasse for every 1 l of ethanol, equalling to 1650 and 1480 billion l of vinasse in produced 2019 and 2020 respectively. As the production of ethanol is forecasted to start steadily increasing again in 2021 [1], the increase of valorization of the subsequently produced vinasse is needed. In the recent years a significant amount of research into new methods of vinasse valorization has sprouted, diversifying the available methods of utilization away from primarily using it as a fertilizer. Vinasse is rich in organic acids and aromatic compounds leftover by the fermenting microbes which would allow it to be used in culturing microbes capable of utilizing these compounds like *Acinetobacter baylyi* ADP1.

A. baylyi ADP1 has been receiving significant attention and fame as the next model organism in the bioengineering field due to similar medium requirements and rapid growth properties as the current model organism *Escherichia coli*. It trumps the *E. coli* by having higher natural transformation frequency and being susceptible to homologous recombination, simplifying and allowing more intricate genetic modifications of its metabolic functions. As *A. baylyi* ADP1 can also metabolize aromatic compounds, it is suitable for applications utilizing plant-based lignin rich mediums normally toxic to most

bacteria. In addition, it can naturally produce storage lipids or wax esters unlike for example *E. coli*. Through metabolic engineering it can also be easily modified to boost the production of these compounds.

Lipids, or triacylglycerols (TAG) and wax esters (WE), are energy storage molecules for living organisms which are accumulated when their need for energy is satiated. Wax esters are especially high-valued as they can be used in multiple different applications including cosmetics, food-industry, pharmaceuticals, biofuels, etc. [3]. The wax esters produced by *A. baylyi* are composed of saturated 32 to 36 carbons long (C_{32}–C_{36}) and unsaturated C_{16}–C_{18} wax esters where the ratio between different length and saturated esters are mostly determined by the available nutrients [4, 5, 6]. The unsaturated fatty acids and -alcohols are more commonly sought after as they are useful in producing biofuels and other more valuable products. This has sparked significant amount of research into enhancing their production in *A. baylyi* [7, 8, 9, 10] through metabolic engineering.

In this thesis vinasse was evaluated as a feedstock for A. baylyi ADP1 and for its prospective use for wax ester production. Four different goals were established for the evaluation in this work. The first goal was to prove that A. baylyi ADP1 can grow in both synthetic and real vinasse, and that it can utilize the nutrients in them effectively. Second goal was to investigate whether A. baylyi ADP1 can use the nutrients in the vinasse to produce wax esters in addition to surviving in it. The third goal was to confirm that a gene knockout of an acidic transcription factor A (atfA) expressing gene caused an increase in A. baylyi ADP1 cells volumes, initially discovered by Withers et al. [11]. The final goal was to test whether the lack of *atfA* in *A. baylyi* ADP1 effects its ability to grow and produce wax esters in vinasse

2. THEORETICAL BACKGROUND

2.1 Bioengineering of prokaryotic cells

Deoxyribonucleic acid (DNA) is an organic polymer consisting of two coiled polynucleotide chains that together form a double helix structure [12, 13]. The repeating monomers that the DNA polymer is made from are called nucleotides. Nucleotide monomers consist of a phosphate group, a sugar group, and a variable nitrogenous group. The phosphate group is always bound to the 5th carbon in the deoxyribose sugar molecule and the nitrogenous base to the 1st carbon. Nucleotide monomers polymerize through esterification between the phosphate group and the sugar molecule of another monomer forming a chain known as sugar phosphate backbone. The structure of nucleotides and the sugar phosphate backbone are illustrated in Figure 1 A.

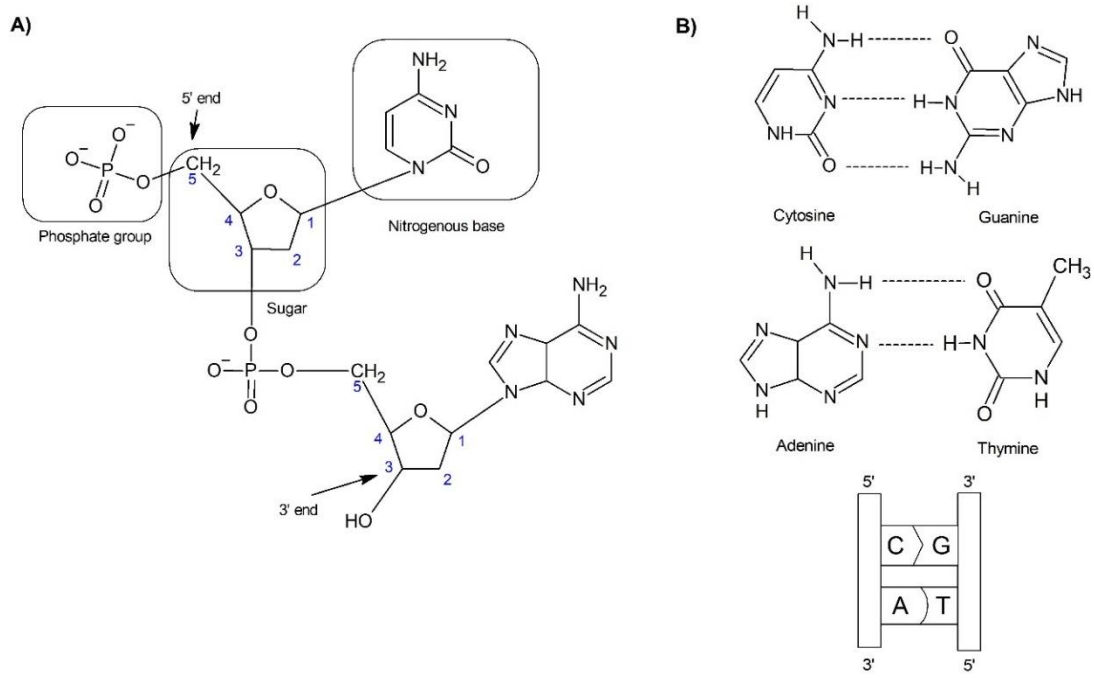


Figure 1. *A) Basic chemical structure of DNA. A single nucleotide consists of three parts: Phosphate group, sugar, and nitrogenous base. B) The nucleotides can only bind to a complementary nucleotide [13]. Complementary nucleotide pairs are adenine and thymine (A-T) and cysteine and guanine (C-G).*

The variable nitrogenous bases in DNA can be adenosine (A), thymine (T), guanine (G) or cytosine (C). These nitrogenous bases can only bind to one other specific nitrogenous base, called its complementary base, established by the amount of hydrogen bonding available to the two nitrogenous bases [14]. Adenosine binds only with thymine through two hydrogen bonds and guanine with cytosine through three hydrogen bonds. Two strands of polynucleotide chains bind together through the complementary hydrogen bonds between the nitrogenous bases. These two strands run in opposite directions or antiparallel to each other due to the conformity of the nitrogenous bases as illustrated in Figure 1 B. Once bound, the strands typically orient into a helix formation. The two antiparallel strands of DNA are named according to their direction of propagation allowing them to be easily distinguished from each other. The strand that propagates from the 5' to 3' direction is named the coding strand and the one that propagates from 3' to 5' direction is the template strand.

Another form of nucleic acids is ribonucleic acid (RNA) which is also present in the prokaryotic cells [14, 15]. Unlike DNA that stores information and exists as a double-strand, RNA is a single-strand molecule that moves information. RNA is similar to the structure of DNA, but instead of being built from A, T, G, and C bases it is built from A, U, G and C bases where the thymine is replaced by uracil (U), and instead of a deoxyribose sugar it has a ribose sugar group.

The DNA in a prokaryotic cell is generally organised in a supercoiled circular looping formation but can also exist as smaller individual loops called plasmids. The long supercoiled or highly twisted and writhed loop contains the hereditary DNA that is always passed to the next generation of cells and is called the genome. Plasmids exists outside the genome and are also known as extrachromosomal DNA that encode genes that help the cell to survive in different environmental stresses. A typical example is an antibiotic resistance plasmid, used commonly in biological engineering applications. The genetic data of the DNA can be partitioned into multiple unique batches of sequences called genes that each encode a different function or process within the cell. Information from the genes can be used or expressed by turning the data into proteins and enzymes that interact with various reactions inside and outside the cell. This process is known as protein synthesis, and it consists of two steps: transcription and translation.

2.1.1 RNA transcription in prokaryotic cells

In the transcription process the supercoiled DNA is first unwound and opened so that the two strands can be separated. RNA polymerase (RNAP) protein complex or holoenzyme finds a promoter sequence from the template strand and binds to it. The RNAP holoenzyme then starts to transcribe the template strand, creating complementary messenger RNA (mRNA) that is identical to the sequence on the coding strand, hence the naming of the strands. The holoenzyme is built of a RNAP core enzyme, which itself is built of several different proteins, and of a sigma factor (σ-factor), that is specific for a set of different genes. For example, σ^{32} with a molecular mass of 32 kDa is universally responsible for genes expressing heat shock responses in prokaryotes. There are seven different σ-factors that each allows the RNAP to bind to the correct factor specific site [16, 17].

Each gene has a recognition sequence known as a promoter or regulatory sequence that defines the area where the transcription process should start to express that specific gene. The core promoter is the minimal stretch of DNA sequence required for the initiating of transcription and it contains an anchor region, Pribnow box and the transcription start site in that respective order [14, 15]. The anchor region and Pribnow box sequences are typically –35 and –10 nucleotides away from the transcription start site. The –35 region contains a sequence close to TTGACA which acts as anchors for the σ-factor. The –10 region contains a sequence close to TATAAT which together with the –35 region act as the site for the promoter to bind to.

Once the RNAP holoenzyme has bound to the promoter site, it forms a structure termed as a closed promoter complex [16]. This closed promoter complex binds more tightly to the DNA sequence near the –10 region with the help of the sigma factor and begins to denature the DNA complex forming an open promoter complex. The sigma factor then leaves the holoenzyme complex, allowing the elongation process to begin. The RNAP begins to read the complementary strand of the DNA and then begins to match the nucleotides and fuse them together to form mRNA.

At the end of a gene is a termination sequence which can either be a direct termination sequence or a factor binding sequence. The direct termination sequence contains a long stretch of GC-rich region. The G and C nucleotides in the region are capable of arranging themselves to bind with each other causing a physical hairpin structure to form as the strand folds in on itself. This hairpin structure physically stops the RNAP from advancing, thus terminating the transcription event. A secondary type of termination is achieved by a protein factor rho, which binds to a specific sequence on the created

RNA termed as *rut* [16]. When rho binds to the rut, it uses ATP to twist the RNA strand around itself, causing the strand to be pulled out of the RNA polymerase, thus terminating the transcription event [16].

Genes in prokaryotes tend to appear as operons or clusters of genes, controlled and regulated as a unit by a single regulatory region [18]. The genes in the operon are usually related to a similar function like expressing several different enzymes for same biochemical pathway. An example of this is the lactose (*lac*) operon which contains three genes *lacZ*, *lacY* and *lacA* that encode lactose uptake and metabolism in multiple different bacteria.

Genes and operons are regulated by at least one regulatory region that may contain several binding sites for different types of transcription factors (TF). The TFs can cause either activating or repressing functions to the expression of the genes they regulate [17, 19]. Activating transcription factors in the operons helps the RNAP holoenzyme to bind to the site thus increasing the efficiency of the transcription. These transcription factors illustrated in Figure 2 A can generally be categorized by the method of activation into class I activation, class II activation or conformational change activation [20]. Class I activators bind upstream of the –35 region and from there they interact directly with the α-subunit C-terminal domain (αCTD) of RNAP. The αCTD is a protein that acts as the RNAP holoenzymes tail which interacts with various regulators. The Class I activators attract the RNAP to the promoter region by binding to the smaller αCTD and thus drawing the large RNAP to the site. Class II activators bind adjacent to the –35 region and contacts with the σ-factors or the α-subunit N-terminal domain of the RNAP, thus recruiting the RNAP holoenzyme to the gene. The conformational changing activators bind on to or near to the core promoter region causing conformational change in the region. These activators do not contact the RNAP directly, but conformally allow the RNAP to bind to the promoter region.

Repressors inhibit or block the RNAP holoenzyme to bind to the site, causing the transcription process to stop or slow down. The simple methods of transcription factor induced repression are steric hindrance, promoter looping, and modulation of an activator illustrated in Figure 2 B. Steric hindrance repressors compete with the RNAP in binding to the promoter sequence blocking the RNAP from binding to the promoter. Like conformational changing activators, repressors can cause conformational changes to the promoters in form of looping to RNAP from binding to the promoter. In most genes there exist a complex mixture of different activators and repressors where for example some repressors only bind to the promoter sequence if it is activated by a

separate activator. These repressors modulate the activation of the RNAP and can be called as anti-activators.

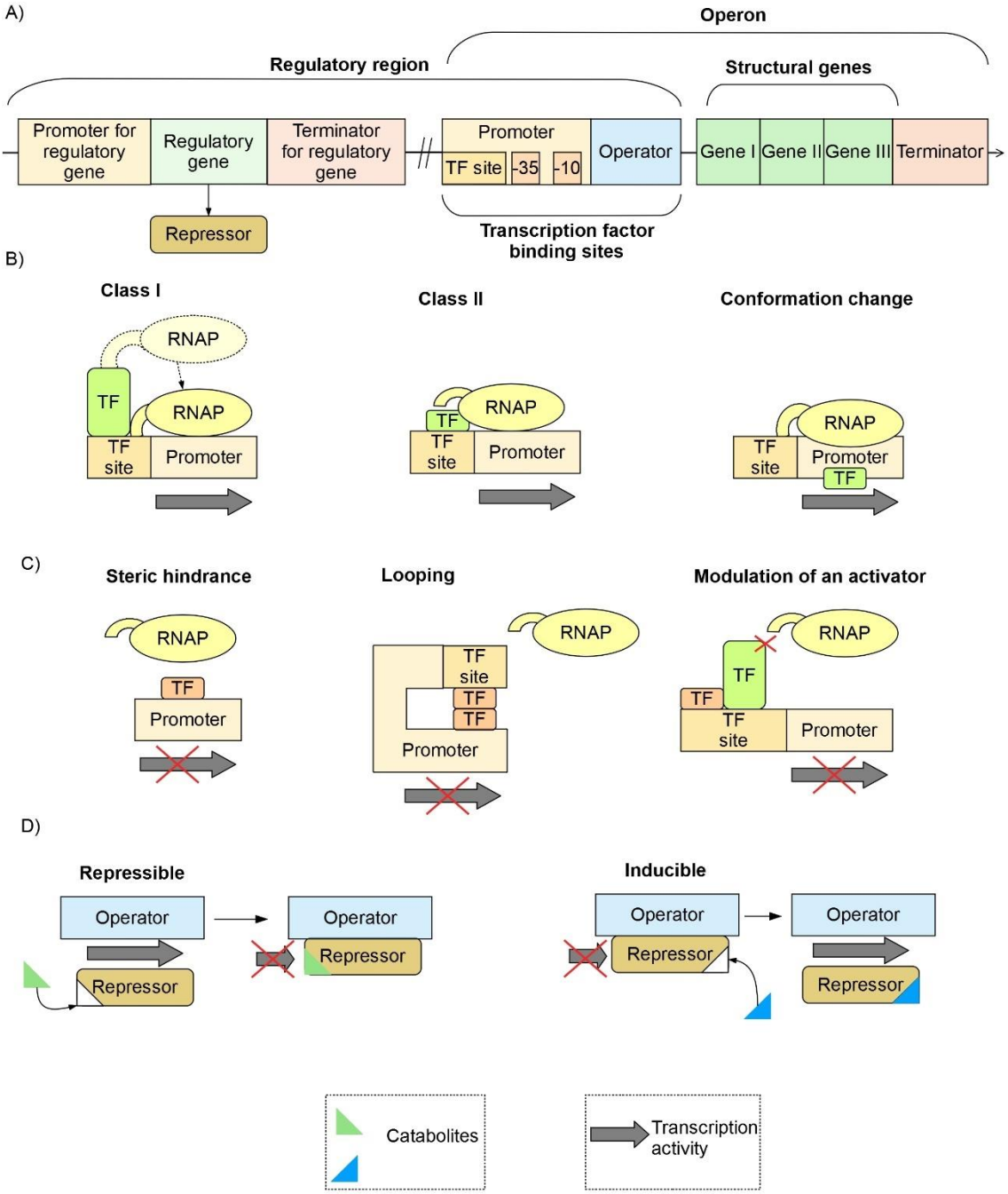


Figure 2. *A) The structure of an operon. Transcription factor (TF) site is a common location in promoter for TF binding. B) Three mechanisms of activation by TFs. C) Three mechanisms of repression at promoter site by TFs. D) TF regulation by activating catabolite ligands in operator.*

In many cases the genes or operons have activatable or inducible repressors that bind to the regions downstream of promoter or to the operator site. These repressors are generally either repressible or inducible by specific catabolites that are related to the genes they regulate. Repressible repressors are proteins that only bind to the operator site when they first bind with a ligand catabolite which activates the protein. Inducible repressors on the other hand bind normally to the operator site and then unbind when a ligand catabolite activates the protein. Similarly, the activators and repressors can require other TFs to function and are typically called co-dependent TFs. Combining these systems, the regulation of the genes can form complex regulatory systems capable of adjusting their expression to various situations. To reduce the need for large numbers of catabolites and to increase the regulatory reliability, some genes or operons have built-in regulatory genes. These genes are expressed before the regulated gene and have their own promoter. Expression of the regulatory genes creates repressive proteins that are used to regulate the following gene.

2.1.2 DNA cloning

Living organisms have multiple ways of recognizing, repairing, and deleting parts of their genomic data. In genetic engineering these natural methods are exploited for intentional genetical changes in the cells. The first technique used in genetical engineering was made possible by the discovery of restriction sites and their respective enzymes. Enzymes are biological catalysts that either alter the speed of a biological reaction or lower their activation energy allowing the reaction to happen more easily. The enzymes have specific binding locations in their structure that usually allow only specific molecules that are called substrates to bind. Once substrates have bound to the enzyme, they are oriented in a certain way that allows other substrates to bind to them more easily. The enzyme can also temporarily react with the substrate forming an intermediate complex that reduces the required energy for a reaction to proceed. Restriction enzymes are catalysts that only recognize specific patterns of nucleotide sequences within the genome and cut the pattern in half when it binds to it as illustrated in Figure 3.

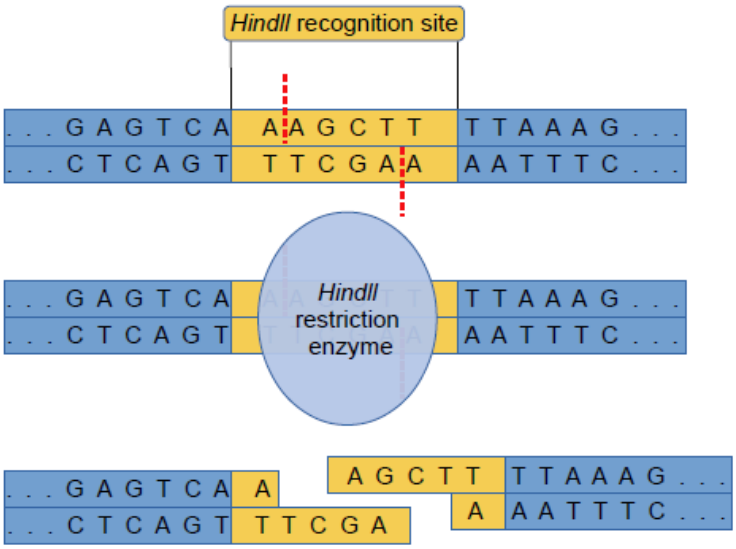


Figure 3. *Restriction enzyme recognizes and cleaves a specific sequence causing the sequence to be cut into two compatible parts. Cleaving can cause the forming ends to be overhangs as HindII does or blunts where the recognition sequence is cut through halfway forming two equal length sequences.*

The use of restriction enzymes requires the knowledge of the whole genome of the organism as there are multiple identical restriction sites within the genome in different locations. This can cause multiple different sized pieces of genome to be formed as the restriction enzyme cuts all the appropriate sites that it can find. The different sized pieces of genome can be isolated using gel electrophoresis method in which DNA is separated based on its size and length. In this process the DNA is forced to move through an agarose gel using an electrical field. The speed of the moving DNA is dependent mostly on its length where the shorter pieces move faster than the longer ones. Too long or short pieces however can either stick too much or not at all to the agarose gel which can cause them to move at irregular speeds. Usually a DNA ladder mix, containing a mixture of DNA pieces of specific and known lengths, is used to make identifying of the fragments possible.

By cleaving fragments of DNA from the genome, it becomes possible to glue or ligate these fragments to a different genome that has been cleaved from identical restriction sites. In molecular cloning this is an essential step in the making of recombinant DNA where an altered fragment of DNA is ligated back onto a plasmid. At molecular level this is achieved by an enzyme group known as DNA ligases that force the two fragments 3’ hydroxyl end (acceptor) and 5’ phosphate end (donor) to form a phosphodiester bond in three steps [21]. Both the acceptor and donor strands nucleobases must match so that the separate strands are simultaneously accepted by the enzyme.

First the ligase enzyme self-adenylylates meaning that it uses an ATP molecule to release energy and then covalently bind to the remaining AMP molecule, thus activating the enzyme [21]. The AMP, or adenyl group, is then transferred onto the 5’ phosphate end of the donor strand forming a pyrophosphate bond between the phosphates of the AMP and the donor strand [21]. Finally, this pyrophosphate bond is cleaved by the 3’ hydroxyl end, binding the 3’ hydroxyl end to the 5’ phosphate end and releasing the AMP [21]. A genetic fragment that contains DNA from two or more sources is called a recombinant DNA.

The *in vitro* recombinant DNA can be integrated into the host cell through a plasmid transformation, a homologous recombination process or a viral transfection. Plasmids are small circular DNA molecules that mostly store information like antibiotic resistances and other selective advantages that can easily and quickly be activated only when they are needed. As they are not part of the primary or genomic DNA of the cell, they can also be easily discarded when deemed useless by the cell. This makes them inherently unstable to modify a cell lines function permanently but offer a simple and quick method of introducing temporary features to the cell. Plasmid transformation is a process, where a cell absorbs exogeneous genetic material from the environment through its cell membrane. This natural transformation process promotes evolution by providing a source of genetic diversity and giving the bacteria a chance to find beneficial traits to survive in the environment.

The transformation of the recombinant DNA into the host cells require more genetic material than is used in creating the recombinant DNA *in vitro*. The recombinant DNA or any of the required genetic material can be replicated to increase their quantity. Replication in laboratory environment is carried out by using cells with high competency meaning the ability to intake foreign DNA from the environment. The current model organism or the most commonly used for this and other common biological methods is *E. coli*. The intake of DNA into the competent cells is forced by for example exposure to calcium and heat, or with short pulses of high voltage electric shocks that increase the permeability of the cell membrane.

Competent cells are most commonly used to replicate and test *in vitro* constructed recombinant DNA. To verify that the cells have successfully transformed with the DNA, the plasmid is typically engineered to have a sequence expressing a specific antibiotic resistance. When the bacteria culture that has been transformed with the DNA is grown in a medium with that antibiotic, only the cells that have successfully integrated the recombinant DNA can grow in it. The grown cells are then cultivated to another plate con-

taining the same medium and antibiotic to remove the cells without the antibiotic resistance gene that were growing with the assistance of the ones that had the gene.

Modification to the target genome using a piece of recombinant DNA can be performed generally by either targeted double strand break or homologous recombination explored in Figure 4. The more commonly used method is to cleave a precise part of the DNA causing a double strand break (DSB) to occur at the site. The DSB can then allow the insertion of foreign DNA into the genome or to simply delete that part of the DNA. The precise cleaving of the DNA can be accomplished by for example using zinc finger proteins or transcription activator-like effector nucleases (TALEN) to guide a cleaving endonuclease *FokI* to the target [22, 23, 24]. A more modern technique utilizes a bacterial antiviral defence protein CRISPR/Cas9 to cleave a specific sequence specified by constructed single guide RNA (sgRNA) [24].

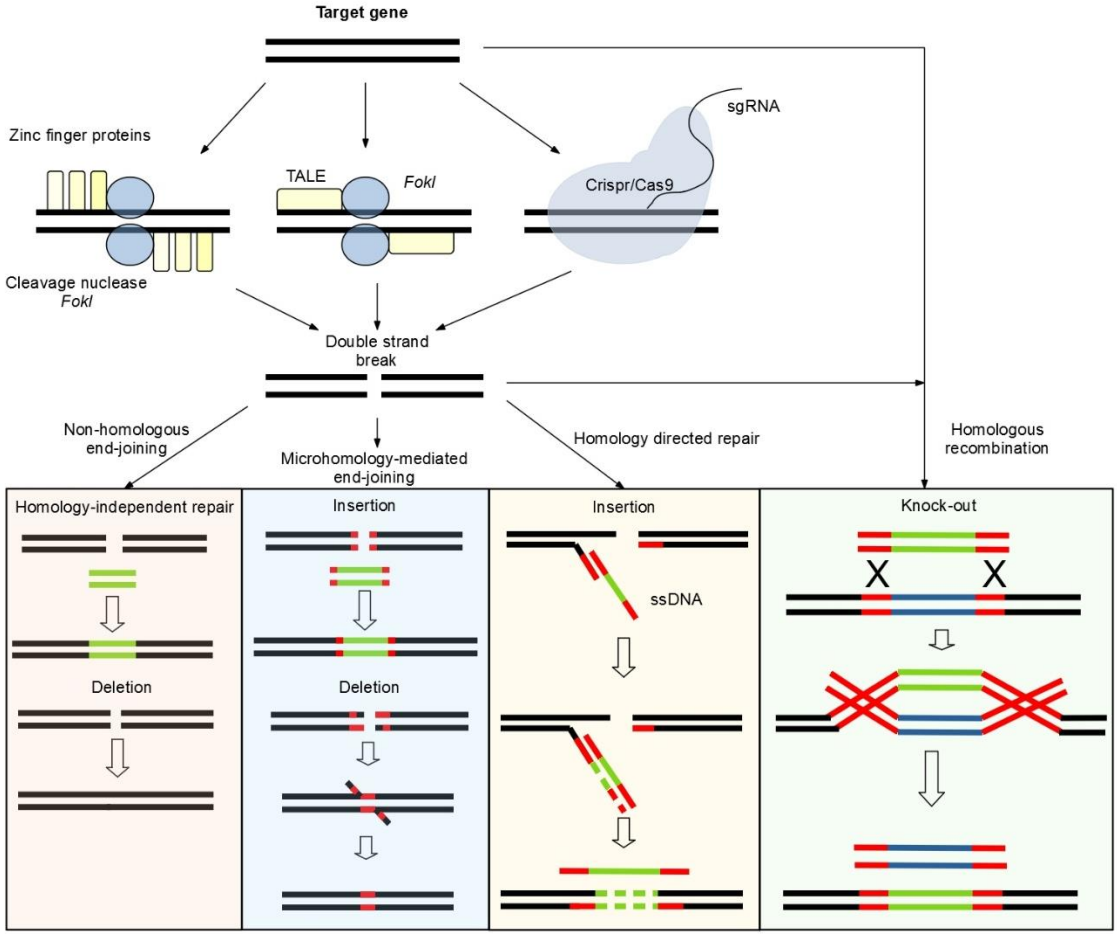


Figure 4. *Applications of genome editing technology for targeted gene editing. Green lines indicate a vector or template DNA, red lines indicate homologous regions and blue lines indicate original target sequence. Dashed lines indicate newly synthesised sequence based on the given template DNA. Homologous recombination (fourth box) can function with either intact or broken target sequences.*

When a strand of DNA is broken in the cell by for example radiation, the cell can repair the break by utilizing either homologous or non-homologous methods. Homology means similarity of structure and in the case of DNA this means similarity of batches of DNA in sequences. When there is no template DNA that could be used to mirror the broken sequence, the cell utilizes either non-homologous end-joining (NHEJ) or microhomology-mediated end-joining (MMEJ) [24, 25, 26].

In NHEJ the cells ATP-dependent DNA ligase LigD can fill the gaps with new nucleic acids if the damage creates an overhang structure [25]. If there however are blunt ends, it can create untemplated addition of nucleic acids to ligate the gaps. Alternatively, it can delete parts of the damaged region to create overhangs to the sequence that it can then ligate together. In MMEJ there are very small homologous regions of 6–10

base pairs that act as reference points for the LigD [25, 26]. If the homologous regions near the DSB are complementary, the LigD can ligate them together without altering the DNA. If these regions are not completely complementary, the LigD will match the available complementary regions. This causes parts of the non-complementary DNA to dislocate and be deleted from the genome. If there are either pieces of single stranded DNA (ssDNA) or double stranded DNA (dsDNA) with matching homologous regions to the regions around the DSB, the LigD will ligate it into to the DSB. Both NHEJ and MMEJ methods are very error prone as they cause significant amount of DNA alteration through either deletion or addition of random sequences.

Homology directed repair (HDR) is a precise repair pathway that utilizes pieces of homologous DNA as a template to repair the damage on the DNA [23, 27]. A ssDNA that has homologous regions with the target DSB will invade the target homologous region. The invasion of the ssDNA allows DNA synthesis to occur, creating a new strand based on the invading template strand. When the template ssDNA has been mirrored to the damaged strand, it gets released back into the cell to be recycled. The repaired strand is then ligated to the matching homologous region thus restoring the strand. The still broken strand is then complemented to the newly fixed strand.

Unlike in HDR where a damage is repaired using a template, in homologous recombination (HR) either ssDNA or dsDNA template strand with homologous regions replaces the target sequence. Essentially HR is an exchange between two DNA sequences that share aligned homologous end regions. This property of the HR is a commonly and historically utilized strategy of introducing precise gene knockouts and replacements to the genomes of eukaryotic and prokaryotic cells. The recombination in bacteria is machinated by a homologous recombinase protein RecA that requires at least 12 bp long homologous regions but longer regions significantly increase RecA dependent recombination [28]. In archaea and eukaryotes HR is machinated by RadA and Rad51 proteins respectively [25].

The actual recombination of the two strands also referred to as a crossover, initiates through the matching homologous regions that cross over the other duplex. In the crossover event the strands exchange their shoulders as illustrated in Figure 5. The shoulder crossover subsequently ferry the strands respective down- and upstream sequences to the other strand [28, 29]. The homologous strands initially invade each other by first separating and then exchanging strands between the two duplexes as they find identical complementary region to bind to. The exchanging strands create a junction of strands known as the Holliday junction between the two duplexes [28]. The Hol-

liday junction propagates along the duplexes leaving the invading strands sequence into the invaded strands DNA like a zipper.

To allow the junction to be resolved a resolvase protein RuvC binds to the junction and orientates the two duplexes by twisting one of the strands by 180° [27, 28]. This twist orientates the two strands into a X formation where each strand is sterically separated but still connected. The junction can now be resolved by cleaving the strands either horizontally or vertically when they are in the RuvC protein. If the cut is imagined as a wall, the strands on the same side of the wall are ligated together causing two possible outcomes for each junction. For each homologous region there are at least two Holliday junctions formed resulting in four possible outcomes. Two of the outcomes achieve the transferring of the invading strands on to the other duplex. The other two outcomes result in strand recombinations where the strands have created recombinant shoulders that ferry either the downstream or upstream sequence to the other duplex. A matching outcome must happen on the other homologous region for the whole insert DNA to be integrated to the target DNA [28].

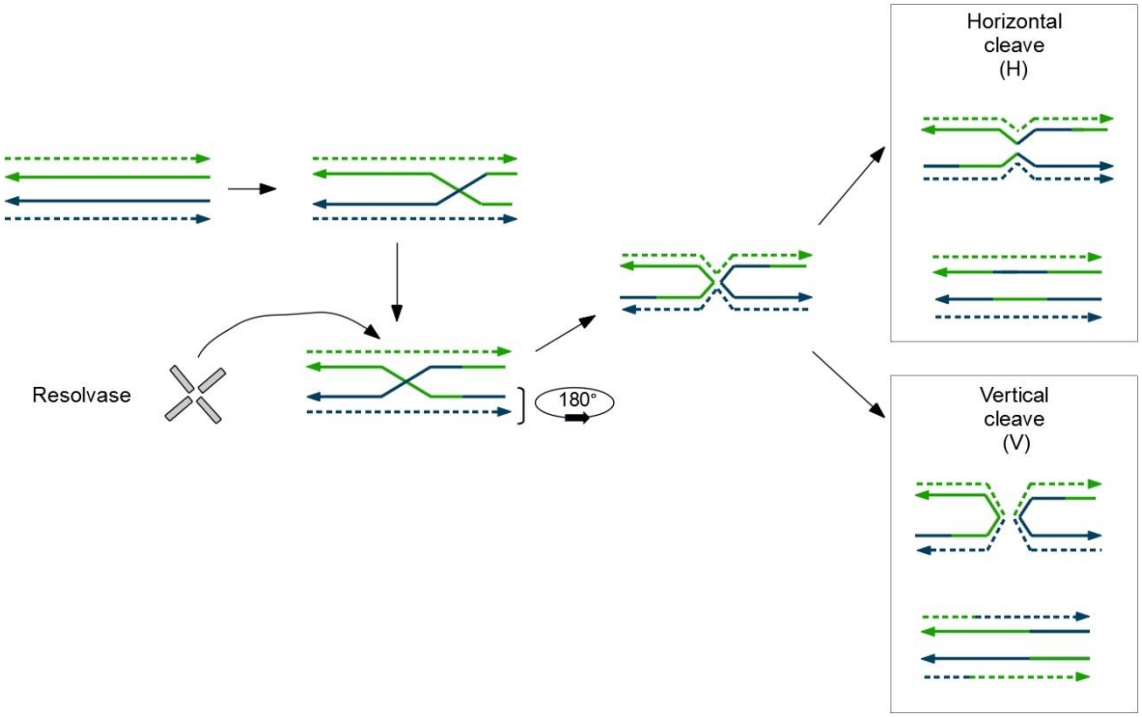


Figure 5. *Homologous recombination and the resolution of the Holliday junction. The vertical cleave resolution of Holliday junction produces crossover of the two dsDNA. Green and blue dashed and continuous lines are used to distinguish the two dsDNAs.*

2.2 Vinasse

In ethanol production traditionally sugar-rich plant matter is used as a feedstock in a process known as alcoholic fermentation. The fermentation reaction occurs in anaerobic conditions where microbes chemically convert energetic carbohydrate sugars into alcohol and carbon dioxide. Any material with high enough sugar concentration can be used as a feedstock in the fermentation process as long as the sugar is in an available form for the microbes. Typically, the sugar-rich plant matter is first juiced where the soluble sugars are separated from lignocellulosic plant husks to achieve this.

The different sources of saccharides or glucose polymers used in fermentation are classified as sugars, starches, and celluloses [30]. Sugar beets, sugarcanes and different molasses contain monomeric sugars which can be immediately converted by the yeast into ethanol. Starch is a polymeric carbohydrate consisting of numerous glucose monomers covalently bound by α–1,4 carbon linkages. For the microbes to utilize it, the covalent chains must first be cleaved in pre-treatment. A typical pre-treatment is enzymatic hydrolysis which breaks the starch polymer into single glucose monomers. Cellulose is also a polymeric carbohydrate with identical composition and structure to starch, but every other glucose monomer in it is rotated by 180°. This is due to the monomers binding through β–1,4 carbons allowing the cellulose polymer to crystallize more and become stronger than starch. It is a very durable and water-insoluble material that has an integral role in the rigid structure of cell walls.

In nature cellulose is always found in lignocellulosic form, where it is tightly intertwined with polymers of lignin and hemicellulose. Hemicellulose is an amorphous heteropolymer that consists of multiple different types of polysaccharides such as xylose, arabinose, mannose, and galactose [31, 32]. Due to the amorphous structure of the hemicellulose, it is responsible for supporting and linking the three parts of the lignocellulosic matrix. Lignin is a non-amorphic complex heteropolymer. It is built of three phenylic monolignols coniferyl-, sinapyl- and *p*-coumaryl alcohols and their derivatives. Lignin is responsible for the structural integrity and water insolubility of the lignocellulosic matrix. To access the sugars in cellulose and hemicellulose, the lignocellulosic matrix structure must first be broken down. The hemicellulose and cellulose must be separated from the lignin and then lysed into glucose units. An example of a novel solution to achieve the same end result is a process known as gasification. In this process the lignocellulosic mass is thermally turned into a mixture of methane (CH_4), hydrogen (H_2), nitrogen (N_2), carbon monoxide (CO) and carbon dioxide (CO_2), also known as syngas. This syngas is then either chemically or biochemically synthesised into ethanol. The chemical synthesis process involves separation using catalyses to separate and liquefy the

gases into alcohol and purified hydrogen [33]. The biochemical synthesis process on the other hand uses bacteria from the genus *Clostridium* to synthesize acetate and ethanol in a fermentation reaction using the H_2, CO, and CO_2 gases from the syngas [34].

The processed feedstock is then fed into a fermentation tank, where yeasts or bacteria produce ethanol through fermentation reaction using the sugars in the feedstock. In industrial processes, additional nutrients or pH stabilizing substrates are added to the tank to achieve optimal reaction balance for ethanol production. The final fermentation product in the fermentation tank contains mostly ethyl-alcohol and water but also non-fermented fractions of the feedstock, metabolic waste products of the microbes, and the microbes themselves. After the fermentation, the microbes can be recycled from the solution by centrifuging to be used in subsequent fermentation reactions. The microbe free fermentation product is processed by rectification and distillation, where the ethanol is purified from the fermentation product. During the distillation and rectification process, everything but the ethyl-alcohol and small portion of water is diluted into a waste effluent stillage (vinasse) at an average rate of 15 l of vinasse to 1 l of distilled ethanol. In 2020 the annual production of fuel ethanol was 98 billion ($98 \cdot 10^{12}$) l [1, 2] which equals to an estimate of 1470 billion ($1.47 \cdot 10^{15}$) l based on the rate of vinasse production to distilled ethanol. The production of fuel ethanol in 2020 fell by 11% since 2019 but it has been forecasted to start increasing again, subsequently also increasing the amount of vinasse generated annually.

As stated earlier, ethanol and thus vinasse are produced from multiple different sources, where the sugary and starchy plants are the most common consisting of up to 42% and 58% of the non-cellulosic ethanol production respectively [35]. Sugarcane, rice, wheat, and corn are the most common and favourable of these plants as their ethanol yields are the greatest compared to other feedstocks [36]. The composition of the produced vinasse varies significantly between different types of feedstocks and also between the same types of feedstocks based on different locations [35, 37]. The differences in the feedstocks are caused due to the feedstocks genetical differences, use of different fertilizers, and soils resulting in different ratios of lignocellulose and cellulose in the plant matter. In addition, these factors also affect the accumulation of different organic, inorganic, and heavy metallic compounds in the plants. Common features that define sugary vinasse, explored in detail in Table 1, are its generally low pH of 3.5–4.5, high organic load due to high biological oxygen demand (BOD) and chemical oxygen demands (COD), high concentration of solids, staining brown colour, putrid smell, and corrosive properties. The variable light and dark brown colour of vinasse is

caused by mainly hydroxyl methyl furfurals, melanoidins and colloidal caramels. These compounds are formed in the Maillard reactions between reducing sugars and amino acids during fermentation process [38, 39].

Table 1. *General characteristics of sugarcane [39, 40, 41, 42, 43] and wood [43] vinasse given by the median and standard deviation (SD) of the source material.*

Parameter	Sugarcane		Wood	
	Median	SD	Median	SD
pH	4.04	0.50	5.35	0.53
BOD (g)/l)	12.0	4.058	27.6	15.2
COD (g)/l)	27.5	9.325	61.3	40
C/N (%)	20	nd	nd	nd
N (mg)/l)	276	227.22	2787	4554
P (mg)/l)	122.5	105.62	28	30
K (mg)/l)	1751	871	39	nd
SO_4^{2-} (mg/l)	1464	565.74	651	122
Phenols (mg/l)	400	248.94	8000	nd

Vinasse produced from lignocellulosic biomass on the other hand possesses notably different compositional structure to vinasse produced from sugary biomass. Lignocellulosic feedstocks consist of roughly 20% phenolic-rich lignin. Due to the presence of lignin, the feedstock requires both pre-treatment and enzymatic hydrolysis to allow microbes to efficiently utilize it. The pre-treatment of lignocellulosic biomass decomposes the lignin through biological, catalytical, physical, or solvent based methods. The enzymatic hydrolysis on the other hand utilizes peroxidases or laccases to break down the hemicellulose and cellulose into glucose monomers. These treatments can cause several changes to the overall feedstocks' organic molecule composition. The phenols brought by the lignin alone can cause lignocellulosic vinasse to be toxic to most organisms. Sugarcane vinasse can be classified into two different generations depending what parts of the initial feedstock were used in its creation. First generation (1G) vinasse contains only the sugary parts of the feedstocks meaning the juices or molasses. The second generation (2G) vinasse contains both the sugary components and the lignocellulosic bagasse components [44]. Vinasses generated from woody material or only bagasse's is referred to as cellulosic vinasse.

Due to the generation of massive volumes of vinasse it must be actively depleted to allow further production. Vinasse has traditionally been used as a fertilizer or an animal

feed like many other side products in different industrial processes. For example, sugarcane vinasse has an average NPK value of 2:1:8 which would make vinasse a good support fertilizer for potassium lacking soil. In the literature there are conflicts whether vinasse should be used as a fertilizer. For example, numerously referenced study by Rocha et al. [45] utilizing standards ISO 14.040 to 14.043 reports that long term vinasse fertigation causes damage to the soil. Other studies [35, 38, 39, 44, 46, 47] of the use of sugarcane vinasse as a fertilizer also report damaging effect to the topsoil. In these studies, the topsoil was shown to have increased amounts of substances that cause pollution into both air and the water supply. Soil deeper than 1.0 meter however experienced a positive increase in available nutrients which in turn helped plants with longer roots to survive [47]. When vinasse is able to penetrate into groundwater reservoirs or other bodies of water, it will cause harmful effects to the environment. With proper application volumes of 300 m^3 ha^{-1} [44] of vinasse fertilizer, vinasse can be used safely to increase soil porosity, potassium concentration at the expense of lowering soil pH and increase in phytotoxicity.

Lignocellulosic or woody vinasse on the other hand should not be used directly as a fertilizer due to the high phenol concentrations in the solution. The phenols in the solution cause significant degradation of soil due to their toxicity to plants. The lignocellulosic vinasse could be processed through anaerobic- or aerobic digestion by phenol resistant microbes to produce value added products or biogas. Examples of microbes that can tolerate lignocellulosic mediums are rot fungi and soil bacteria like *Staphylococcus aureus*, *Corynebacterium sp.*, *Bacillus subtilis*, *Proteus sp*. *A. calcoaceticus* PHEA-2, and *A. baylyi* ADP1 [48, 49, 50]. The *A. baylyi* ADP1 however has the weakest resistance to phenols from the aforementioned list of soil bacteria, but has been shown to be effective at catabolizing lignin-derived substrates to generate alkanes and wax esters [9, 50, 51, 52].

Current methods of using vinasse and other side streams coming from its production shown in Figure 6, include fertirrigation, animal feed production, recycling to a separate fermentation reaction or producing energy. Lignocellulosic feed can consist of bagasse's from the juice extraction process or from a variety of different woods. There exists a number of different methods of lignocellulosic pre-treatment options like alkaline hydrolysis, acid hydrolysis, steam explosion, alkaline explosion, and wet oxidation [53].

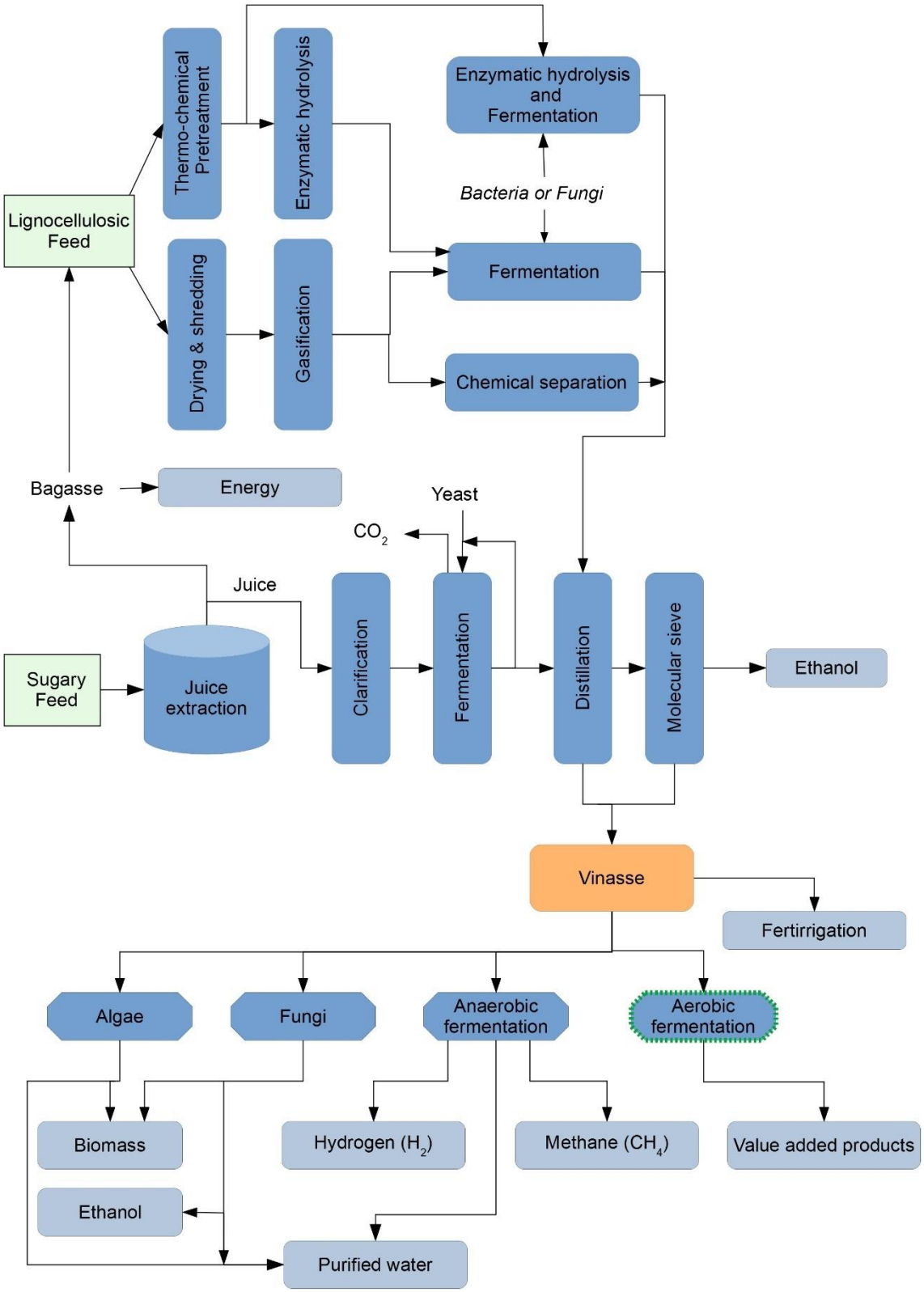


Figure 6. *The process chart of vinasse production and potential uses, where green outline indicates this works focus. Each blue box represents a single process. Light blue boxes indicate potential end products.*

Studies about utilizing vinasse as a resource have increased significantly in the recent years. In Table 2 a few studies have been listed to show the variety of products that vinasse can be converted into. Due to the high concentration of organic acids and organic matter in vinasse, the more popular valorization targets have been biomass and biogas production. As noted in Table 1 vinasse does not contain large concentrations of either nitrogen or phosphorous. The limitation of both nitrogen and phosphorus in a growth medium are detrimental for the growth of microbial cells especially for fungi cells [54].

Table 2. *Methods of utilizing vinasse as a raw resource.*

Technique	Method	Product / Application	Reference
Anaerobic digestion	Bacteria	Methane (CH_4)	[55, 56, 57]
	Bacteria	Hydrogen (H_2)	[55]
Aerobic digestion	Fungi	Biomass & Ethanol	[58]
	Fungi	Biomass & Purification	[54]
	Bacteria consort	Organic acids & ethanol	[59]
	E. coli	Biocatalysts	[60]
	Algae	Biomass & Purification	[61, 62]
Recycling	Chemical & mechanical	Lactic acid	[63]
	Fermentation	Ethanol	[64]
	Phenol oxidation	Fertilizer	[65]

Fungi are used more generally to purify vinasse and accumulate edible biomass from it. Bacteria on the other hand are more often used to generate value added products from vinasse due to their metabolic versatility and ease of genetic engineering. The most common way of using bacteria to utilize organic streams is to produce biogas through anaerobic digestion which provides a simple and effective method of *in situ* treatment of the resource stream. Digestion tanks are easy to install and connect directly to the stream output and will provide biogas to increase the efficiency of the overall process [65, 66].

2.2.1 Carbon and nutrient flow in vinasse production

When vinasse is thought of as a resource, knowing the theoretical maximum substrate concentrations in it are important factors for designing and investigating new methods of valorisation. The simplified process flow chart of vinasse production illustrated in

Figure 6 shows how the initial sugary feedstock undergo several reactions or processes that alter the molecular composition of the solution. In 1G vinasse for example, the feedstock is juiced, clarified, fermented, and then distilled. In each step the initial molecules in the feedstock are made more accessible for fermenting microbes and the rest of the molecules are distilled into the vinasse. In this subsection the nutrient flow and degradation of the feedstock in the ethanol production process are approximated for both 1G and 2G vinasses.

The first step in feedstock pre-treatment is the separation of the feedstocks juice from the husks, shells, and the dry pulpy fibrous material. All that is left after the juices have been pressed is called the bagasse. This remaining bagasse consists of 40–50% cellulose, 25–35% hemicellulose, and 15–20% lignin [67, 68]. In 2G vinasse the bagasse is also used in to generate ethanol from the sugars in the cellulose and hemicellulose. De Souza et al. [69] estimated that using the bagasse from sugarcane plants could increase the overall bioethanol yield by as much as 40%.

The next step in the juice pre-treatment is the clarification process where the extracted juice is cleared of most colloidal substrates that surround the sugar molecules. These substrates are removed by heating the liquid and optionally adding flocculating agents. The heat and the flocculation agents cause the liquid clouding colloidal material to flocculate to the bottom of the solution. The flocculating agent solubilizes substances like calcium into the solution while the heating denaturises proteins allowing them to untangle thus clearing the solution. In the case of both heating and addition of flocculation agents about 90% of the total proteins and phosphate, and 30% of the total nitrogen gets removed in the clarification process [70].

As the husks of the plants or more generally called bagasses are mostly composed of lignocellulosic materials, their pre-treatment begins with the solubilization of the lignocellulosic matrix. Most of the pre-treatments used to achieve this involve thermochemical treatments. These treatments usually partially degrade or decompose the molecules in the solution causing some un-intended by-products like organic acids, phenolics and furans to form. The amount of each of these by-products is influenced by the treatment method and the lignin content of the feedstock. For example, high pH alkaline hydrolysis creates small amounts of furan, while acid hydrolysis with low pH creates larger amounts of furan to form [53]. The furans can further degrade into levulinic, formic, and furoic acids through dehydration reactions in acidic mediums [71]. Hemicellulose and cellulose on the other hand can contain acetyl and uronic acid groups which are released in the pre-treatment as acetic and formic acids [72]. Acid hydrolysis is

considered to be the most effective and cause the least amount of degradation of the monomerized sugars [53].

The degradation of the lignocellulosic matrix produces as a side effect small quantities of phenolic compounds when lignin polymers in the matrix break. Small pockets of lactic acid are also found naturally inside the lignocellulosic matrix and are released when it is hydrolysed [53]. At this point lignin is usually removed from the medium as traditional ethanol fermentation microbes cannot utilize the monolignols in their catabolic pathways. A small amount of lignin is however usually dissolved into the medium causing slight inhibition of the fermentation reaction and darkening of the colour of the medium [53].

In the fermentation process the microbial cells feed on the clarified juice or pre-treated bagasse utilizing an anaerobic metabolism pathway also known as the fermentation reaction to produce ethanol from sugars. The microbes initially turn the different sugars into pyruvate via the Embden-Meyerhof-Parnas (EMP) pathway, explained in more detail in subsection 2.3.1. The pyruvate is then turned into acetaldehyde which in turn gets dehydrogenised into ethanol yielding two total ATP per glucose molecule [73]. Most common by-products of ethanol fermentation are glycerol and common organic acids such as acetic-, lactic-, formic-, and succinic acid. The production of the by-products is linearly proportional to the amount of available glucose in the medium. For the most common microbe used in ethanol fermentation *Saccharomyces cerevisiae*, the yields of glycerol, acetic acid, succinic acid, and total by-products are 0.023, 0.0155, 0.0054, and 0.0442 per gram of glucose respectively [74].

Lactic acid is also usually produced in industrial processes due to almost unavoidable contamination of lactic acid producing microbes (LAB) [75]. The lactic acid fermentation microbes directly compete about glucose with ethanol fermentation microbes. The lactic acid production in the ethanol fermentation process can be reduced for example by pH control, antibiotic usage or by adding substances like sulphur dioxide [75].

An example of the carbon flow and nutrient changes in the vinasse production of 2G sugarcane ethanol are illustrated in Figure 7. The model of the nutrient flow was constructed from comparing the chemical composition of the outputs of each process. The data and assumptions for this model are explained in Appendix B. Substances called other sugars in the model, contain both reducing sugars and disaccharide sugars. Essentially all of the sucrose and glucose in addition to a significant amount of other macro- and micronutrients are consumed by the yeast cells in the fermentation reaction.

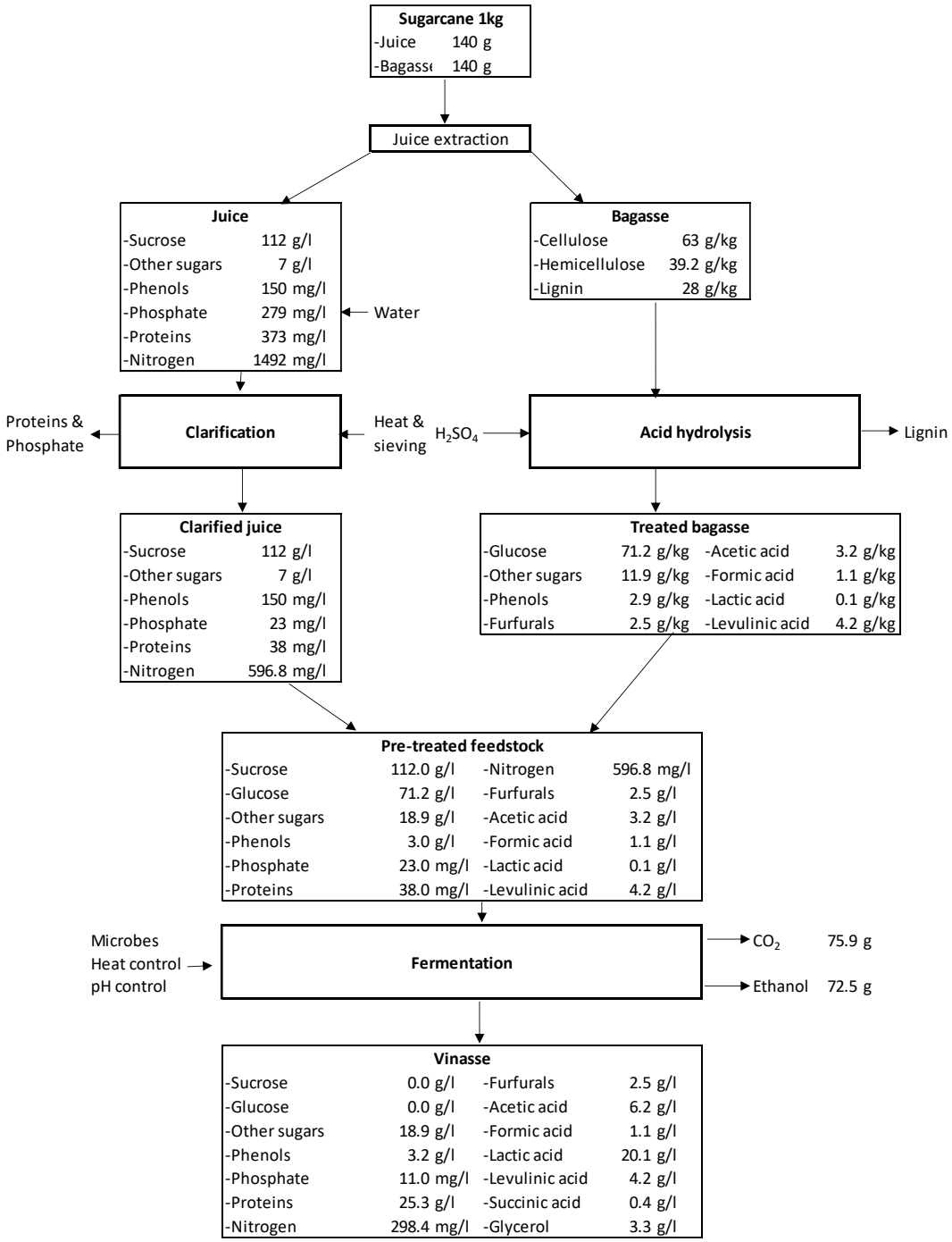


Figure 7. *The carbon and nutrient flow chart leading to the production of 2G vinasse. Data and methods are found in Appendix B.*

The advantage of the 2G ethanol process, is that the cellulosic fraction of the feedstock can add up to 63% more sugars into the fermentation reaction. However, the pre-treatment of the cellulosic biomass also releases significant amounts inhibiting compounds such as acetic, formic and levulinic acids into the fermentation reaction [76]. The compositional differences of the 1G, cellulosic and 2G ethanol processes com-

pared in Table 3, show how both 1G and cellulosic fractions influence the overall process.

Table 3. *Compositional differences between 1G, cellulosic and 2G vinasses.*

Compound	1G vinasse (g/l)	Cellulosic vinasse (g/l)	2G vinasse (g/l)	1G vinasse (g/kg/ DW)	Cellulosic vinasse (g/kg/ DW)	2G vinasse (g/kg/ DW)
Other sugars	7.0	5.1	12.1	50.0	36.3	86.3
Glycerol	2.7	1.6	4.3	19.4	11.7	31.1
Acetic acid	1.8	4.3	6.2	13.1	30.9	43.9
Formic acid	0.0	1.1	1.1	0.0	8.0	8.0
Lactic acid	12.7	7.7	20.4	90.5	55.2	145.7
Levulinic acid	0.0	4.2	4.2	0.0	30.0	30.0
Phenols	0.3	2.9	3.2	2.1	20.5	22.7
Furfurals	0.0	2.5	2.5	0.0	17.7	17.7
Ethanol	47.3	28.6	72.5	338.1	204.2	518.1

During the fermentation reaction, the sucrose is converted into both glucose and fructose molecules that subsequently are converted into ethanol and organic acids. As the organic acids and ethanol exhibit an inhibiting effect on to growth on the fermenting yeasts [53, 74], the fermentation medium typically only reaches about 10% ethanol content before the reaction stops [30, 69]. This can cause small amounts of glucose or fructose to be left in the medium eventually accumulating into the vinasse. These sugars and the two important organic acids, acetic acid and lactic acid provide excellent growth environments for bacterial cultures.

2.3 Acinetobacter baylyi ADP1

Acinetobacter sp. strain BD134 (ATCC 33304), commonly designated as *Acinetobacter baylyi* ADP1 and formerly known as *Acinetobacter calcoaceticus* is a gram-negative, strictly aerobic, non-motile, chemoheterotroph soil bacterium. It is most commonly known for its highly versatile metabolic capabilities and its aptness to be genetically transformed. The innate aptness for genetical engineering comes from the species high natural transformation rate and competence to absorb DNA without discrimination between homologous or heterologous variants [77, 78, 79].

The genome of *A. baylyi* ADP1 has been completely sequenced to be about 3.6 Mb containing 3384 genes [50] clustered into a few genetic islands. Its genome is relatively small compared to the current model organism *Escherichia coli* K12 that has a 4.6 Mb

large genome containing 4419 genes [80]. The genome sequence of *A. baylyi* ADP1 shows that it shares significant amount of essential genes with other common water and soil dwelling bacteria like *Pseudomonas aeruginosa* PA14, *E. coli* K12, *Bacillus subtilis* and *Pseudomonas putida KT2440* [50, 81]. *A. baylyi* ADP1 shares its taxonomic roots shown in Table 4 with *P. aeruginosa* and *P. putida* from Pseudomonadales order and with *E. coli* from Gammaproteobacteria class. This shared genetic history allows the orthologous gene essentiality analysis between these three bacteria. Unlike the aforementioned bacteria and most of the other *Acinetobacters*, *A. baylyi* ADP1 is not pathogenic which encourages its use in industrial and environmental applications.

Table 4. *Taxonomy of Acinetobacter baylyi ADP1.*

Taxonomy	Classification
Domain	Bacteria
Phylum	Pseudomonadota
Class	Gammapseudomonadota
Order	Pseudomonadales
Family	Moraxellaceae
Genus	Acinetobacter
Species	*Acinetobacter baylyi*

A. baylyi ADP1 is a coccoid shaped bacteria that mostly live in either diplo- or streptococci arrangements where they bunch up into either pairs or into long chains, respectively. These arrangements can cause difficulty in isolating genetically altered cells after their genetic transformation as one of the paired cells can be transformed while the other remains untransformed. The bunching up of cells however also promotes exchanging of genetic material between the chained cells thus stimulating the strains characteristic frequency of natural transformation. The size ranges for Acinetobacter species are typically 1.5–2.5 µm in length and 0.9–1.6 µm in diameter [82, 83]. The size of a single cell varies upon the number of surrounding cells, and the amount of stored lipids and DNA within each cell. These factors are subsequently influenced by the culture's current growth phase. Bacterial culture growth or the approximate measure of cell count in a medium is measured using the absorbance or optical density (OD) of light typically at 600 nm. At OD 1 for example, the average cell length, width, and

volume are 1.46 ± 0.15 µm, 1.08 ± 0.09 µm and 1.01 µm^3 where as in OD 1.5 these amounts are 1.32 ± 0.17, 1.01 ± 0.10 and 0.79 respectively [11]. The cell volumes are calculated using the cylindrical volume formula

$$V = \frac{\pi}{4} W^2 (L - \frac{W}{3}), \quad (1)$$

where V is the cell volume, W the cell width and L the cell length. Massana et al. created multiple algorithms for calculating bacterial volume from 2D images to verify that the formula 1 is viable enough at these scales. The most accurate algorithm used perimeter and area of the image to calculate the equivalent length and width of a cell which was then used to calculate the volume. Wither et al. used the simple volume formula 1 to calculate their cell volumes and so the same formula is used in this work.

The wild-type strain of *A. baylyi* ADP1 has a quick doubling time of 35–65 minutes in rich mediums [84, 85] when grown at its optimal cultivation temperature of 30–37°C. It can be grown at these temperatures overnight on both rich and minimal salts mediums. For genetic construction applications *A. baylyi* ADP1 is an excellent model organism due to its small genome and ease of artificially expressing genes from a different species. As stated earlier, *A. baylyi* ADP1 has many matching genomes to other well studied organisms. This allows the application of already known metabolic and genetic functions from these organisms to the *A. baylyi* ADP1 genome [50, 86]. These properties of *A. baylyi* ADP1 has caused it to gain significant support to be branded a model organism for bioengineering applications.

2.3.1 Metabolism of *Acinetobacter baylyi*

The basic principle of a cell's metabolism is to convert nutrients from the environment into energy and biomass as efficiently as possible. Different nutrients like sugars and nitrates are processed in the cell through multiple chained reactions or metabolic pathways that are catalysed by various enzymes. These metabolic pathways are separated into either energy generating reactions or energy depleting reactions referred to as catabolic and anabolic reactions respectively. Each prokaryotic species has their own metabolic strategies to utilize these pathways in the most efficient way for their specific living environments. This specificity originates from the difference in the genome of the species. For example, many *Pseudomonas* species lack phosphofructokinase enzyme expressing genes required for fructose catabolism pathway to be viable [87]. The basic pathways that almost every microbe species have in their metabolic strategy are glycolysis and either an aerobic or anaerobic pyruvate utilization. Aerobic or oxygen using microbes have citric acid cycle (Krebs- or CAC- or TCA cycle) and oxidative phos-

phorylation, and anaerobic or non-oxygen using microbes have fermentation reactions to metabolise pyruvate [87]. There are three slightly different glucose-catabolizing pathways found in bacteria: Embden-Meyerhof-Parnas (EMP), Hexose monophosphate (HMS) and Enthner-Doudoroff (ED) pathways [87]. Both EMP and ED pathways convert one glucose into two pyruvate molecules while HMS produces only one pyruvate molecule. Specifically in the ED pathway, one glucose is turned into one pyruvate and one glyceraldehyde-3P which is then turned into a pyruvate through a terminal glycolytic pathway.

A. baylyi ADP1 is a nutritionally versatile bacteria that can catabolize a large spectrum of organic molecules present in soils, including a wide variety of aliphatic and aromatic acids present in plant matter. *A. baylyi* ADP1 has also been demonstrated to be able to utilize glucose as both carbon and energy source, a rare trait in Acinetobacter genus. However it cannot utilize other sugars as sole carbon sources and is only able to metabolize sugars like galactose and xylose in higher pH solutions into lactones [88, 89]. The nutritional versatility of *A. baylyi* ADP1 makes it a potential candidate to process plant-based distillery stillage containing significant amounts of acetate, glucose, xylose, galactose, varying amounts of lignocellulosic aromatic compounds and ethanol. Previous findings about glycerol, that is also produced in the fermentation reaction, indicate that *A. baylyi* ADP1 does not utilize it as a sole carbon source [7]. The strain does possess the necessary enzymes glycerol phosphate kinase and glycerol phosphate dehydrogenase to catabolize the glycerol [7]. The glycerol was consumed in mediums supplemented with gluconate only in the first culture phase and only to a minor extent [8].

Unlike *E. coli* that uses EM pathway, *A. baylyi* ADP1 metabolises glucose via a modified ED pathway illustrated in Figure 8. The modification arises due to lack of a pyruvate kinase enzyme expression in *A. baylyi ADP1*. Without this enzyme the intermediate product phosphoenolpyruvate (PEP) cannot be converted into the second pyruvate molecule directly through the terminal glycolytic pathway [90]. Instead, *A. baylyi* ADP1 could convert PEP into oxaloacetate with PEP carboxylase, placing it in the TCA cycle where it is consequentially converted into malate and then decarboxylated into pyruvate. However, this pathway does not generate any ATP from converting the PEP into pyruvate, making the pathway energetically unfavourable. Instead of turning the glyceraldehyde-3P into PEP, *A. baylyi* ADP1 could use the glyceraldehyde-3P in its extracellular polymeric substance (EPS) synthesis pathway [10, 91]. The EPS accumulates outside the cells, increasing the nutrient absorption, binding properties, salt tolerance and antimicrobial resistances of the cells [92, 93]. The production and excretion of EPS is significantly increased in mediums with high salt concentrations [92, 94]. The

increase in EPS eventually leads to the *A. baylyi* ADP1 cells clumping together and becoming more resilient against mechanical stresses [92, 93, 95].

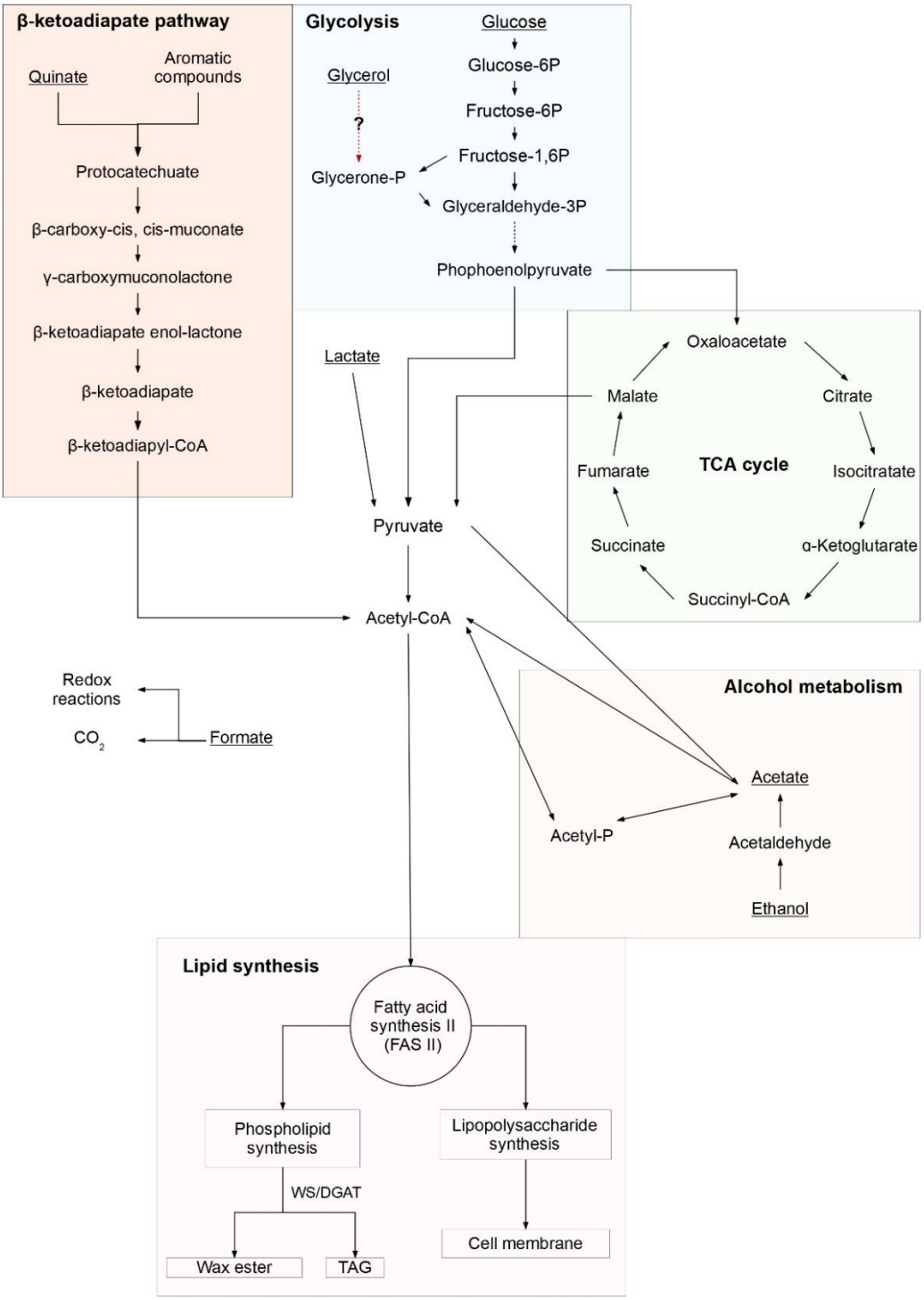


Figure 8. *Simplified metabolic pathways of A. baylyi ADP1 relevant to this study. The coloured regions separate each pathway. Dotted lines indicate that multiple reactions happen in between the two molecules. Underlined molecules are present in the synthetic medium created in this study.*

Another desirable metabolic feature of *A. baylyi* ADP1 is the ability to degrade aromatic compounds, chlorogenates, ferulates and quinates using a multistep β-ketoadipate pathway that uses a set of genes called *pca-qui* genes [77, 96]. In this pathway, molecules are initially converted into either protocatechuate or catechol and then further converted through identical enzymatic steps. In the next step they are aerobically ortho-cleaved, meaning the aromatic ring is oxidized opening the ring between the substituents, and then converted into β-ketoadipate. A coenzyme-A is then added to the β-ketoadipate, which is then reductively cleaved with CoA into acetyl-CoA and succinyl-CoA that are then used in the Krebs cycle [77]. The catabolism of aromatic compounds in *A. baylyi* ADP1 is regulated by both catabolite repression and cross-regulation mechanisms. In cross regulation, the catechol and protocatechuate pathways are regulating each other by repressing their expressions thus allowing one path to be active while other is inactive. In catabolite repression, a presence of specific catabolites or substances repress the gene expression of the aromatic catabolism. For *A. baylyi* ADP1, the presence of organic acids represses the *pca-qui* gene expression and more specifically the combination of both acetate and succinate cause the greatest repression of 95% [97, 98, 99]. The repression effect is the greatest during the exponential growth phase, but this repression stops at the stationary phase [98].

2.3.2 Growth kinetics in lignocellulosic hydrolysates

A. baylyi ADP1 depletes substances from its growth medias in a specific order dependent on the catabolic energy efficiency of these substances [7, 100]. Generally the substances in mixed media are consumed in order of simple sugars and alcohols, organic acids and finally aromatics [7, 97, 100, 101]. The order in which the different substances are depleted is controlled by the complex repression and activation based metabolic regulation system [97, 100, 102]. In this metabolic regulation system, the presence of either food molecules or their catabolic intermediates act as the repressors and activators for the metabolism expressing genes. Each species has their own regulatory system and even for organisms like *A. baylyi* ADP1 and *E. coli*, these regulatory systems are still not completely known.

The *A. baylyi* ADP1 cells consume the simple sugars and easily processable alcohols first as they are not regulated as much as they regulate other pathways. The presence of ethanol for example causes catabolite specific repression on the catabolism of acetate, which causes the cultures to initially accumulate organic acids until ethanol is depleted [7]. However, if the media does not contain ethanol, it has been shown that acetate is consumed first before the simple sugars [103].

Feedstocks derived from lignocellulosic biomass typically contain significant amounts of monomeric aromatic compounds and simple sugars like glucose, galactose, arabinose, and xylose. Most of the aromatic compounds in these solutions are created during the alkaline hydrolysis pre-treatment. In those conditions the monolignols from the lignin are deteriorated into various different phenolic compounds like ferulic acid, p-coumaric acid, vanillin, and 4-hydroxybenzoic acid (4-HBA), that are enzymatic inhibitors for most bacterial and fungal cells [104, 105]. *A. baylyi* ADP1 is able to catabolize these phenolic compounds and as such can grow in lignocellulosic hydrolysate containing mediums. However phenolic compounds tend to require more effort from the cells to catabolise, their catabolising pathways are heavily regulated. In *A. baylyi* ADP1 the β-ketoadipate pathway is partially regulated through organic acids. For example, one of the major phenols created from lignin processing, 4-HBA is directly repressed by the presence of acetic acid [97, 103]. This means that 4-HBA and many other phenolic compounds are essentially the last substances that *A. baylyi* ADP1 catabolises from lignocellulosic medias [51]. Even though *A. baylyi* ADP1 can utilize the phenolic compounds, its growth is still inhibited by them in high enough concentrations [52, 106].

2.3.3 Wax ester synthesis

Wax esters (WE) are esters of a long-aliphatic chained carboxylic acid (fatty acid) and a long-chained alcohol (fatty alcohol or n-alkane) that with triacylglycerols (TAG) serve as the energy reserves of cells. These energy reserves are created by the cell when the available energy exceeds the cells energetic requirements allowing the cell to survive even when there is no available food in the environment. Whether a cell produces primarily TAGs or WEs is specific to a species where for example *Acinetobacters* and *Marinobacters* prefer to produce WEs and *Actinobacters* TAGs [107].

The wax esters that *A. baylyi* ADP1 produces are composed of mostly C_{32}–C_{36} long esters of C_{16}–C_{18} long fatty acids and fatty alcohols [4, 5]. The majority of these wax esters are di-unsaturated [5] meaning that they have two double or triple bonds that causes a drop in the melting point of the molecule. When grown in nitrogen limited stationary phase environments, *A baylyi* ADP1 produces wax esters approximately 50 times more than in carbon limited and 10 times more than in exponential growth phase environment [4, 108]. Nitrogen limiting conditions however also directly inhibits biomass accumulation and maintenance, leading to lower amounts of cells and thus to lower overall wax ester production titers [109].

A. baylyi ADP1 has the genes that allow it to create a bifunctional wax ester synthase/acyl coenzyme A: diacylglycerol acyltransferase (WS/DGAT) enzyme which ca-

talyses the final reactions of both WE and TAG synthesises. The WS/DGAT is a versatile enzyme and can synthesise reactions for a broad range of molecules. In *A. baylyi* ADP1 for example it catalyses both long-chain length alcohols, acyl-CoA esters, and monoacylglycerols [110, 111]. However, it has been recorded that the highest activity of the WS/DGAT is expressed when utilizing medium-chain length (C_{14} to C_{18}) alcohols [110].

Preluding the wax ester synthesis, the cell creates fatty-acyl-CoA precursors through a fatty acid synthesis (FAS) reaction pathway. The FAS can be categorised into either a type I or type II FAS that both follow almost same reactions but are catalysed differently. Type I FAS is catalysed by highly integrated multifunctional enzyme known as type I fatty acid synthase. This enzyme is a dimer consisting of two identical subunits that contain multiple different functional domain centres capable of catalysing all the different reactions in FAS [112, 113, 114]. Type I FAS or FAS I is typically used by eukaryotic cells in animals but has also been seen used by *corynebacterium* and *mycobacterium* species [112]. FAS II on the other hand is typically used by prokaryotic, plant and vertebrate cells and unlike in FAS I, its catalysing enzymes are dissociated rather than integrated [113, 114, 115]. In FAS II each reaction in the pathway is catalysed by a separate enzyme and as such can be more easily influenced by mutations [115]. *A. baylyi* ADP1 like most of the other bacteria use the type II FAS.

The acetyl-CoA that is not immediately used in either biomass production or cell maintenance is siphoned into the FAS II pathway shown in Figure 9. In the FAS pathway acetyl-CoA is initially either carboxylated into malonyl-CoA with a bicarbonate molecule (HCO_3-) and bound to an acyl-carrier-protein (ACP), or immediately bound to an acyl-carrier-protein forming malonyl-ACP or acetyl-ACP respectively. In the next step acetyl-ACP and malonyl-ACP are transferased together by 3-ketoacyl-ACP synthase III. This reaction releases the malonyl-ACPs 3rd carbon as CO2 which allows an acetyl molecule to bind to its former position. The new ketone group at the C_3 is then reduced to a hydroxyl group which is then condensed, thus eliminating the water molecule. In the final step of the first elongation loop the newly formed double bond between C_2 and C_3 is reduced, effectively creating a single two carbons longer acyl-ACP molecule. This longer molecule is then used in the next elongation step that adds a new malonyl-CoA molecule starting the elongation loop over again. This elongation continues iteratively until a long enough chain is acquired, which usually is 18 carbons long hexadecanoyl-ACP for *A. baylyi* ADP1. The fatty-acyl-ACP is then released and ligated with a CoA molecule forming a fatty-acyl-CoA [115].

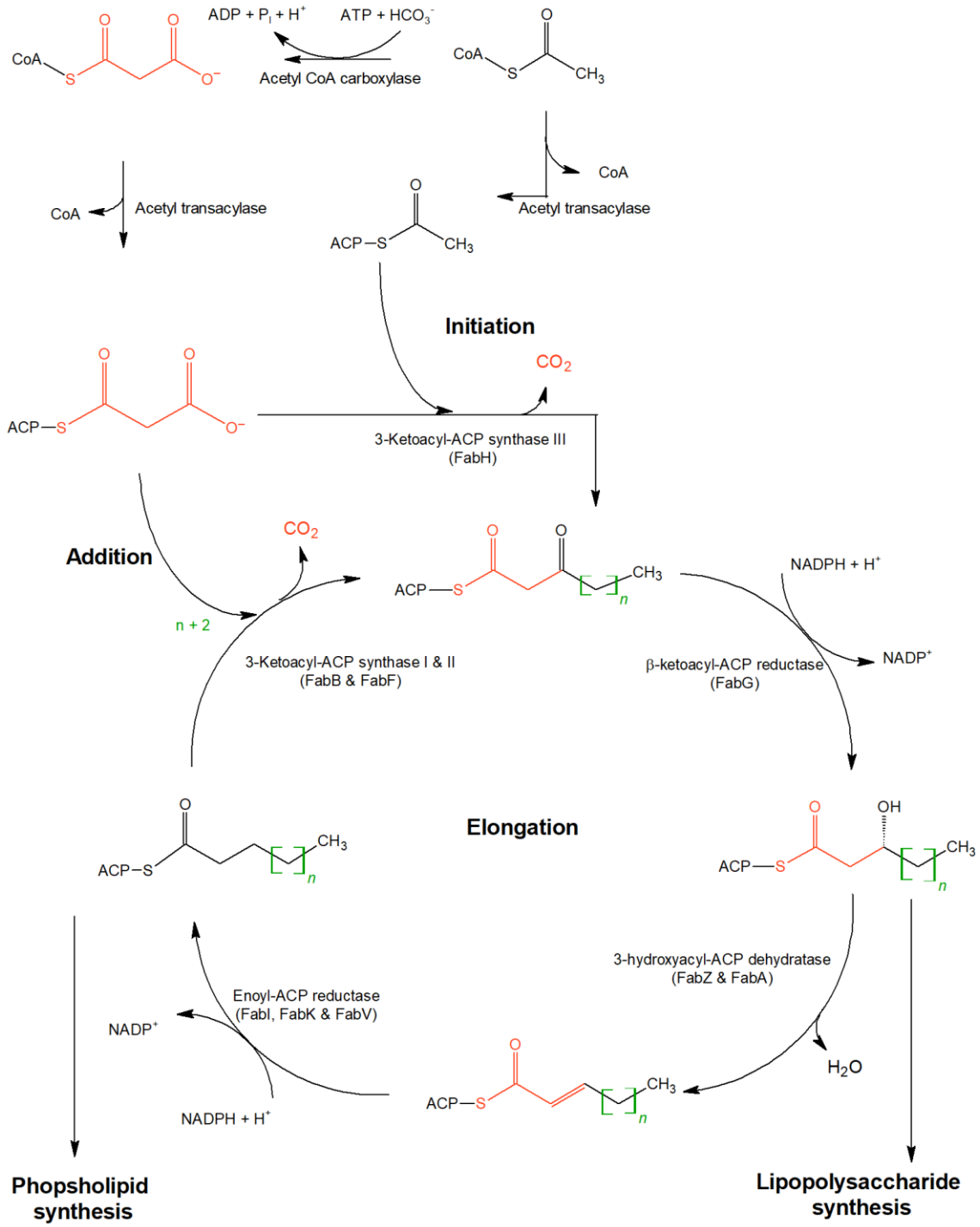


Figure 9. *Type II fatty-acid synthesis (FAS-II) pathway predicted to be used by A. baylyi ADP1 to produce precursors for wax ester synthesis from acetyl-CoA [114, 115]. The red formulas track the original malonyl molecule through the pathway while the green track the increasing chain number n.*

The fatty-acyl-CoA molecules from the FAS are then converted into wax esters in three general steps as shown in Figure 10. First the fatty-acyl-CoA is reduced by NADPH-dependent long-chain acyl-CoA reductase to the respective fatty aldehyde [115]. In the

second step these aldehydes are reduced again by NADPH-dependent fatty-aldehyde reductase into their respective fatty-alcohols [115, 116]. In the final step the WS/DGAT enzyme is used to esterify a second fatty-acyl-CoA and the synthesised fatty-alcohol into a single wax ester. In addition to using fatty-acyls, fatty-alkanes can also be used in the wax ester synthesis. In this case the fatty-alkane would first be hydroxylated into alcohol, then oxidized into aldehyde, and further oxidized to acid, and finally be synthesized into fatty-acyl-CoA [107].

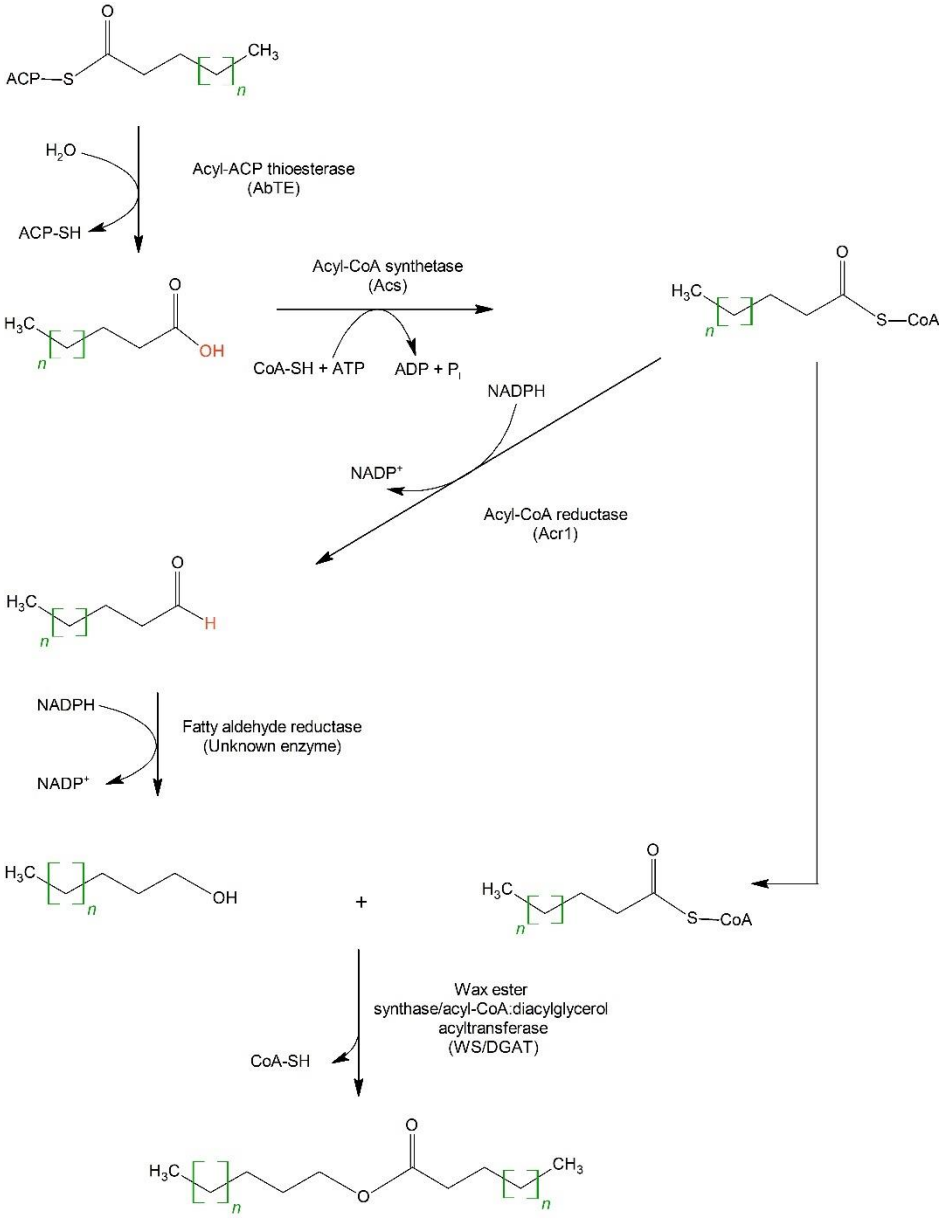


Figure 10. *The wax ester synthesis pathway used by A. baylyi ADP1 which utilizes the fatty-acyl-ACP molecules produced by the FAS II pathway [9, 107, 117]. The green parenthesis and n indicate n-amount of additional carbon chains.*

Both fatty acid and wax ester synthesises in *A. baylyi* ADP1 occur in multiple locations throughout the cell's different membranes. Initially the lipids occupy the peripheral areas of the cells but in more significant amounts they begin to accumulate into the middle of the cells [118, 119]. The created lipids initially form inconsistent lipid-prebodies, without any clear boundary layers between each other, that later form into large lipid inclusions with clear boundary layers [118]. These large lipid inclusions eventually separate from the cell and organelle membranes into the cytoplasm [118, 119, 120]. In *A. baylyi* ADP1 cells there are generally only a few wax ester inclusions in total, that can normally comprise of up to 4–20% of the total dry cell weight depending on the used substrates [4, 7, 111, 117, 121, 122].

There might be unknown reaction pathways that use a non-WS/DGAT enzymes to synthesise small amounts of lipids indicated by small amounts of TAG found in ΔWS/DGAT *A. baylyi* mutants [107].

2.3.4 Bioengineering of A. baylyi ADP1

In metabolic engineering the existing metabolic pathways of an organism are modified to either improve, stop, or alter the production of specific metabolites. For *A. baylyi* ADP1 the metabolic engineering research has been focused on the improvement of WE production [8, 117, 121] and the catabolism of aromatic compounds [9, 52, 103]. Although WE in any form are already commercially viable, it is possible to further improve their composition or production volume by metabolic engineering. As an industry standard the Jojoba plants WEs are widely used as additive in biofuels or also turned directly into biofuels through transesterification with short chain alcohols [3, 120, 123]. The structural composition of WEs produced by *E. coli* and *S. cerevisiae* have already been shown to be controllable through heterologous gene expressions [120, 124]. More economically viable strategy is to first increase the volume of WE synthesis or to find abundant resource streams to be converted into WEs. In general, there are five primary methods in metabolic engineering to improve a substrates or WEs production:
1) silencing competing pathways,
2) overexpressing genes that produce fatty-acid pre-cursors and intermediates to increase flux towards wax ester synthesis,
3) overexpressing enzymes and proteins that by-pass natural regulation fatty-acid and wax ester synthesis or silence repressing enzymes,
4) expressing genes that alleviate stresses and problems caused by overproduction of wax esters.

5) balancing the cellular requirements for survival and synthesis of fatty-acids and wax esters [125].

Most of the genetic engineering studies involved in wax ester production in *A. baylyi* ADP1 utilize at least one of these methods summarised in Table 5. In some of the referred studies there are multiple methods used in which case it is listed to utilize only the first method it fulfils from the aforementioned list.

Table 5. *The five different methods of improving the production of wax esters and the representative studies for each method.*

Method of improvement	Specific target	Reference studies
1) Silencing competing pathways	*poxB*, *dgkA* & *lip1*	[8]
	rmlA	[126]
	Lip1, lip2, fadE, acr1,	[127]
2) Overexpressing genes to push the flux	*Acr1*, *Acr1*-homolog & *Aar*	[117]
	pykF	[128]
3) Controlling regulation and repression	*pobA* 4-HB catabolism repression	[98]
4) Stress alleviation	EPS Controlled production	[93]
5) Balancing and optimizing cell survival to production	*aceA* Switch between biomass and lipid synthesis	[121]
	Preferential carbon source utilization demonstration	[100]
	Growth and lipid accumulation performance on different carbon sources	[7]

In the study by Withers et al. [11] they deleted gene called ACIAD2924 that is responsible for the production of a protein they named **acidic transcription factor A (*atfA*)**. This is not to be misunderstood to the *A. baylyi* ADP1 enzyme **acyltransferase (*atfA*)** also known as WS/DGAT. In this work the protein acidic transcription factor A will be referred to as "*atfA"*. The *atfA* is a highly abundant protein in both *A. baylyi* ADP1 and *A. baumannii* and it is responsible for assisting the RNA polymerase in the transcription of DNA into RNA. For each molecule for RNA polymerase there are 0.37 *atfA* proteins

in a single cell. The *atfA* was however not confirmed to be part of the transcriptional complex in the study. In a genome-wide survey study of single-gene deletions by Berardinis et al. [81] found that the ACIAD2924 gene was not essential indicating that the *atfA* is not the sole or dependant transcription factor in the *A. baylyi* ADP1 RNA transcription. The *atfA* gene was analysed to have affected at least 500 expressions and transcriptions within the *A. baylyi* ADP1 genome [11]. The clear effect of these changes has not been discovered yet.

Most important and novel property of both *A. baylyi* ADP1 and *A. baumannii* strain that had the *atfA* deleted (*ΔatfA)* was that the cells volumes almost doubled in size. The length, width, and volume of the *ΔatfA* were reported to be 2.09±0.24 μm, 1.40±0.10 μm and 2.49 μm^3 respectively at OD_{600} value 0.1 compared to wild type dimensions of 1.46±0.15 μm, 1.08±0.09 μm and 1.01 μm^3 respectively. The increased volume was also reflected in the total DNA content in the cells. This means that the cells had to deplete extra resources to build and maintain the increased DNA in themselves. This had also lowered their fitness level causing more than 98% of the *ΔatfA* strain to be replaced by wild type cells after 28 generations. A single generation of *A. baylyi* ADP1 wild type typically lasts for 4–5 hours in glucose mediums. In the Withers et al. study it was also concluded that there was no difference in the general growth kinetics of the *ΔatfA* strain and the wild strain.

Generally, the increase in the cells size is governed by the availability of nutrients in the media [129, 130, 131]. The high availability of nutrients allows the cell to reproduce more quickly, which achieved by the replication of the cell's DNA and the following cell division. To increase the speed of the DNA replication the cell needs to initiate multiple simultaneous DNA replication processes to occur, causing an increase of total DNA concentration in the cell [132]. As the cell creates more DNA, it also begins to synthesise more lipids to accommodate the extra DNA and to prepare for the cell division.

The model of increasing cell size correlates with the findings of Withers et al. study where they discovered that *ΔatfA* strain had overexpressed genes related to chromosomal partition and DNA synthesis. Similarly, they also concluded that several acyl-CoA and acetyl catabolism genes were downregulated that. Genes ACIAD0541 and -0540 are related to turning acetyl-CoA to acetyl-phosphate and acetyl-phosphate to acetate respectively, whereas ACIAD3390 expresses the succinyl-CoA: acetate-CoA transferase enzyme that transfers the CoA to the acetate [115]. Withers et al. found that the downregulation of these genes caused both *A. baylyi* ADP1 *ΔatfA* and *A. baumannii ΔatfA* to be unable to catabolize ethanol.

The effect of increased lipid production or storage has been observed to also increase the size of the corresponding cells [118, 131]. The causality relation of this effect is usually that the increase in cell size is due to the increase in lipid production overexpressed by the increase in DNA concentration. The increase in lipid production allows the cell to upkeep the lipid membrane containing the cells inner organelles. After the initial expansion of the lipid membrane, the nutrients provided to the lipopolysaccharide synthesis could be siphoned to the phospholipid synthesis.

As the WS/DGAT enzyme expresses the highest activity in the endoplasmic and the inner cell membrane, increasing the surface area of the cell membrane could allow more of the wax ester synthesis reactions to occur. In addition to theoretically increasing the intra- and extracellular wax ester production, the larger volume of the cell could allow more wax ester storage which in wild type is limited to only a few inclusions. In this work it is investigated whether the silencing of the *atfA* gene affects the cell size and whether this affects the cells' ability to produce wax esters.

3. MATERIALS AND METHODS

3.1 Bacterial strains

Wild-type *Acinetobacter baylyi* ADP1 (DSM24193) was both used and modified with gene knockout of *atfA* to measure its ability to metabolize vinasse into wax esters. The control cultures for the gene constructions used the wild-type cells. Both wild-type and *ΔatfA* strains were stored as glycerol stocks in −80 °C freezer and fresh batches were prepared separately for each experiment. All of the *A. baylyi* ADP1 cultures were incubated at 30 °C in aerobic environment.

Escherichia coli XL1-Blue was used as a host to construct and replicate the plasmid with a pre-built gene knockout cassette used in previous studies [103]. Competent *E. coli* cells were prepared beforehand in glycerol stocks and stored in −80 °C freezer. All of the *E. coli* growths were incubated at 37 °C in aerobic environments. All the strains used in this study are listed in Table 6.

Table 6. *Bacterial strains used in this study.*

Strains	Characteristics
Acinetobacter baylyi ADP1	Wild type (DSM 24193), wt
A. baylyi ADP1:*ΔatfA*	wt + ACIAD2924 knockout cassette
Escherichia coli	Wild type
E. coli:gkc:2924UFS	wt + construction plasmid with ACIAD2924 upstream flank
E. coli:2924DFS: gkc:2924UFS	wt + construction plasmid with ACIAD2924 up- and downstream flank

3.2 Mediums

Low salt LB medium was used in the *E. coli* growths and to make all of the LB agar plates. The medium was composed of the following: 10 g/l tryptone, 5 g/l yeast extract and 0.5 g/l NaCl. For LB agar plates, 15 g/l agar was added to this recipe. Sterilization of the medium was done in autoclave. In selective LB mediums, the antibiotic was added to slightly cooled autoclaved medium to avoid degradation.

A MA/9 medium was used in *A. baylyi* ADP1 and *A. baylyi* ADP1 *ΔatfA* precultures. The MA/9 5x salts stock was composed of the following: 22 g/l Na_2HPO_4, 17 g/l KH_2PO_4, 5 g/l NH_4Cl, 0.04 g/l Nitrilotriacetic acid and 5 g/l NaCl. The salt stock was used to make the actual MA/9 medium that contained the following: 9.8 g/l MA/9 salts 5x stock, 9,0 g/l glucose, 3.0 g/l acetate, 240 mg/l $MgSO_4$, 11 mg/l $CaCl_2$, 0.5 mg/l $FeCl_3$ and 2 g/l casein amino acids. Minimal salts medium (MSM) and Minimal salts buffer ingredient list and method is listed in Appendix A.

Agarose gel for the electrophoresis procedure was prepared by adding 3.5 g of agarose to 350 ml of 1x TAE and was warmed in microwave for 3 minutes, mixing every 30 seconds. After cooling the solution to 37 °C, 35 µl of SYBR was added to the solution and then immediately poured into a gel tray with well combs. Tray was covered and left to solidify after which the gel was cut and stored submerged in TAE in a fridge.

Vinasse stock solution was received fresh from ST1. The solution was stored in a cool fridge in airtight containers. The solution was transferred to −20 °C freezer after one month in the fridge for long-term storage. Composition of the vinasse was analysed beforehand by the provider of the sample (data not shown).

Synthetic vinasse medium was designed by comparing multiple sources of vinasses from literature [39, 43, 62, 133, 134, 135] and the real vinasse sample composition. This synthetic medium does not contain aromatic compounds found in wood vinasse as their presence in the real vinasse is low. Quinic acid is used as a substitute for all of the aromatic compounds as it is a common aromatic in plant materials. Due to the substitution and simplification of the substrates, the synthetic medias composition is closer the more abundant sugarcane and -beet vinasses. The composition of the synthetic vinasse shown in Table 7 contained both organic acids and aromatic compounds comprising of larger than 1% weight fraction in the real vinasses.

Table 7. *Composition of a synthetic vinasse created for this study.*

Substrate	Concentration (g/l)
Acetic acid	20
Ethanol	35
Formic acid	17
Glucose	50
Glycerol	80
Lactic acid	103
Levulinic acid	26
Quinic acid	40

The only sugar included in the synthetic vinasse was glucose as it is present in almost every type of vinasse and will boost the exponential growth of the culture. As has been found in other studies, *A. baylyi* ADP1 is not able to efficiently utilize mixed sugars in the presence of organic acids or in low pH [9, 103, 128].

3.3 Gene construction

A pre-made knockout cassette shown in Table 11, initially constructed by Santala et al. [8] was modified to cause *atfA* knockout on the gene ACIAD2924 in *A. baylyi* ADP1. The knockout cassettes flanking regions were replaced with flanking regions corresponding to the ACIAD2924 gene obtained from work done by de Berardinis et al. [81].

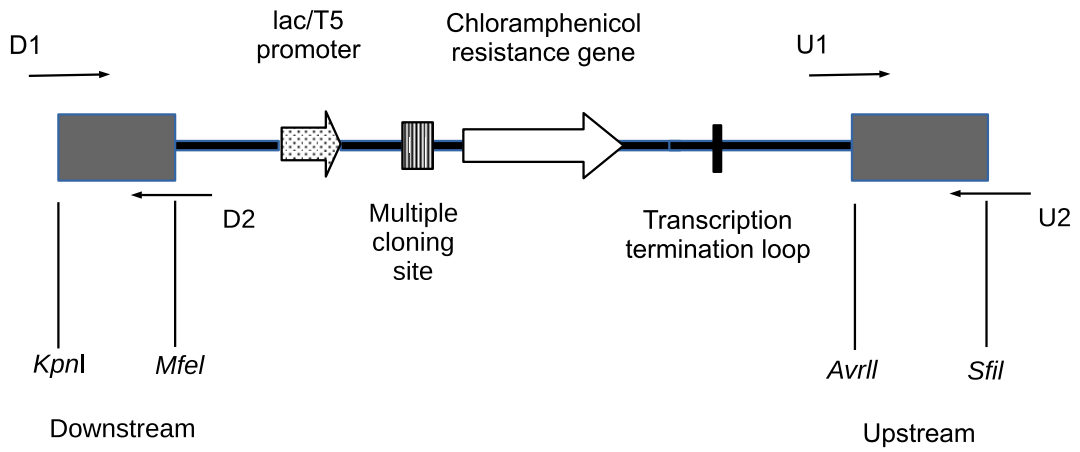


Figure 11. *The atfA gene knockout cassette's structure. Adapted from Santala et al. [8].*

The primers (Table 8) that were used to constrict the knockout cassette to ACIAD2924 were designed by placing 4 bases as dummies to 5' end, the desired flanking region, and additional bases to increase the specific recognition sequence length of the flanking region. In the designing process the primer length, dummy length, and both sequence and starting sequence were optimized to achieve functional melting temperatures between the primer pairs. The T_m of the primer pairs P3-P4 and P5-P6 were calculated with Thermo Fisher T_m calculator to have differences of 4.1°C and 4.8°C respectively.

Table 8. *Primers used to construct A. baylyi ADP1 ΔatfA strain. Underscores separate the dummy bases, restriction sites, flanking regions, and specificity enhancing bases.*

Primer	Sequence (5'–3')	Description
P3	ATAA_GGTACC_GGCAGTAAAAAAC CGTGCATG_ATAG	The primer for ACIAD2924 downstream forward strand (D1) containing *KpnI*
P4	ATAA_CAATTG_TAAAACTACCTCAT AATGAAAAAATG_TGAATGG	The primer for ACIAD2924 downstream reverse strand (D2) containing *MfeI*
P5	ACGG_CCTAGG_CGAAAATCAGATG TAGCATAAC_ATTTGAAAATG	The primer for ACIAD2924 upstream forward strand (U1) containing *AvrIII*
P6	ATAA_GGCCCCCGAGGCC_AGCCA TGCTGAAATCTGTTC_AATAATTG	The primer for ACIAD2924 upstream reverse strand (U2) containing *SfiI*

The modification of the gene cassette was started by acquiring the flanking regions of wild-type *A. baylyi* ADP1 from its genomic DNA. The genome mixture was used as a template DNA in the PCR with either the forward (upstream) or reverse (downstream) primers to replicate the corresponding flanking regions. Reagents used for the PCR are shown in Table 9 and the PCR program is shown in Table 10. The reactions for the PCR were prepared on ice and a negative control was prepared without the Phusion II hotstart polymerase (ThermoScientific, USA). Results of the PCR were visualized and verified using electrophoresis by running 5 µl of the PCR products with 1 µl of DNA loading dye in TAE gel at 80 V for 30 minutes. The verified products were purified using GeneJET PCR Purification KIT (ThermoScientific, USA) and stored in −20 °C freezer.

Table 9. *PCR materials for the amplification of the flanking regions. Two separate reactions were made for both set of primers to create the flanking regions.*

Reagent	Volume (µl)
Template DNA (250 ng/µl)	1
5x Phusion HF buffer	10
10 mM dNTP mix	1
Primers (10 mM)	2.5
Phusion II hotstart (2 U/µl)	0.5
dH_2O	32.5

Table 10. *PCR program for the flanking regions. The upstream reaction annealing temperature is on the left and downstream on the right.*

Cycles	Process	Temperature (°C)	Time (s)
1	Initial denaturation	98	30
30	Denaturation	98	5
	Annealing	65 / 55	30
	Extension	72	15
1	Final extension	72	300

The pre-constructed gene cassette was purified from *E. coli* XL1-Blue i/pIX [136] by using a GeneJET Plasmid Miniprep Kit (ThermoScientific, USA) and following its instructions. The purified plasmid was verified to be the correct size by visualizing with electrophoresis. The modification of the gene cassette was done one flanking region at a time starting with the upstream region. Digestion of the purified plasmid and the flanking regions was done by mixing the reagents listed in Table 11 in ice. After a gentle mixing, the upstream double digestion reaction with KpnI and MfeI was incubated at 37 °C for one hour. The downstream reaction required two consecutive incubation periods of two hours at 37 °C for AvrII and two hours at 50 °C for SfiI. For downstream digestion reaction, a second digestion was done to a separate batch with FastDigest enzymes (ThermoScientific, USA) that required only 15 minutes of incubation at both temperatures.

Table 11. *Gene knockout cassettes digestion reaction reagents. Restriction enzymes for upstream digestion are on the left and for downstream on the right.*

Reagent	Amount
10X Fast digest buffer	3 µl
KpnI / AvrIII (10 U/µl)	10 U
MfeI / SfiI (10 U/µl)	10 U
Purified plasmid	1 µg
dH_2O	Up to 30 µl

Double digestion reaction produces two linear fragments of DNA, a large fragment containing the plasmid backbone and the gene cassette and a small fragment containing the sequence between the two restriction areas. The larger fragment was purified from the digestion products by visualizing them with electrophoresis at 80V for one hour and then processed with a GeneJET Gel Extraction Kit (ThermoScientific, USA).

After digestion of both the flanking region and the purified plasmid, they were ligated together by combining reagents shown in Table 12. The reaction was gently mixed with a pipette at ice and then incubated at room temperature for two hours. After incubation, the reaction was heat inactivated by heating the reaction tube to 65 °C for 10 minutes and then chilled on ice and stored in −20 °C freezer.

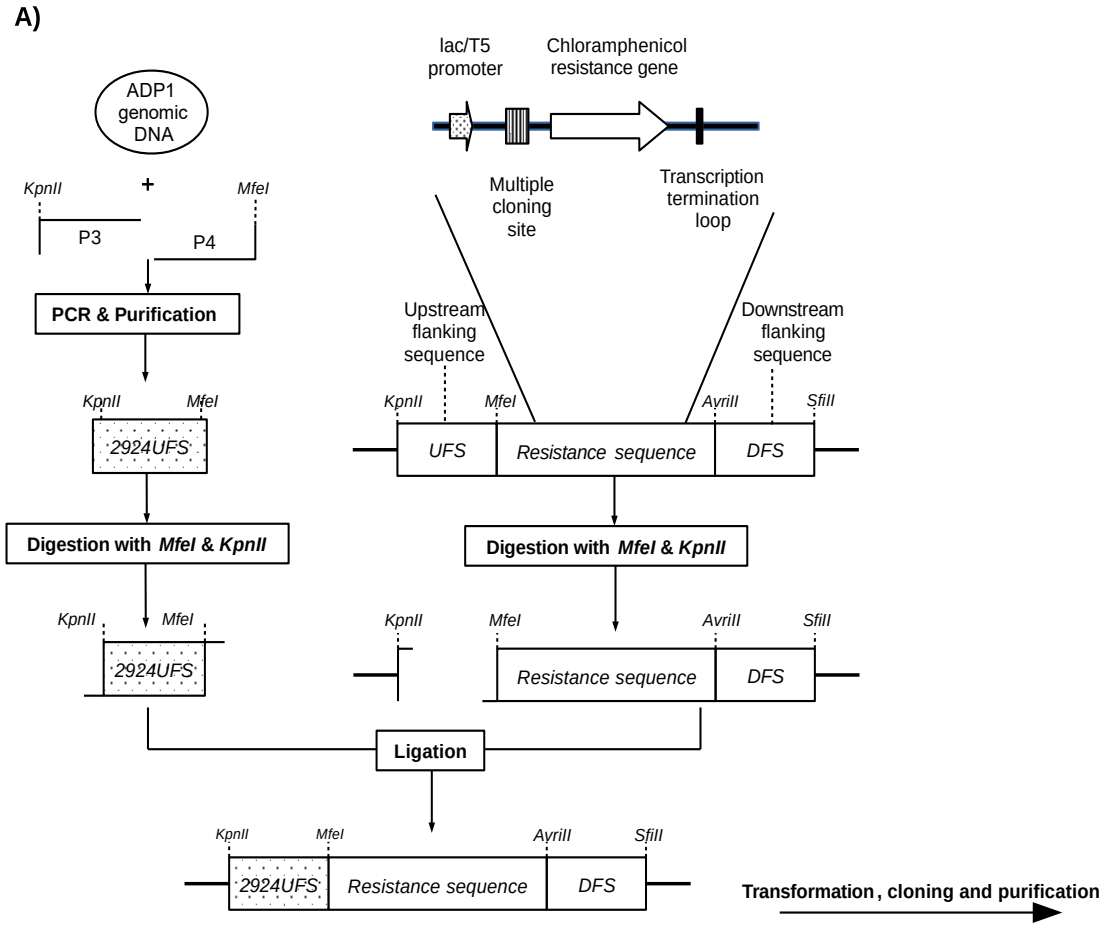
Table 12. *Ligation reaction for a digested flanking region (insert) and the purified plasmid (vector).*

Reagent	Amount
Vector DNA	100 ng
Insert DNA	20 ng
T4 DNA Ligase (5 U/µl)	2.5 U
T4 DNA Ligase Buffer 10X	3 µl
dH_2O	Up to 20 µl

The ligated plasmid was transformed into *E. coli* to be replicated in order to increase the available concentration of the plasmid for the next modification step. All the reagents and cuvettes used in electroporation were pre-chilled and kept on cold containers. A pre-chilled electroporation cuvette was filled with an aliquot of competent cells to which 2 µl of the ligation product was added quickly. Before inserting into the electroporation machine, bubbles were removed from the cuvettes. The cells were trans-

formed using Eco-1 program and then 1 ml of pre-warmed 37 °C LB medium was added to the cuvette and allowed to incubate at 37 °C for 30 minutes.

Two plates containing LB medium supplemented with chloramphenicol (CamR) were filled with 100 and 900 µl of the inoculated LB medium and allowed to incubate overnight. Next day 5 different cultures of different sizes were inoculated into 5 ml of LB medium with CamR and incubated at 37 °C and at a shaking of 300 revolutions per minute (rpm) overnight. The plasmid was purified from the culture and used to finish the modification of switching the downstream flanking region. The gene cassettes construction is visualized summarily in Figure 12.



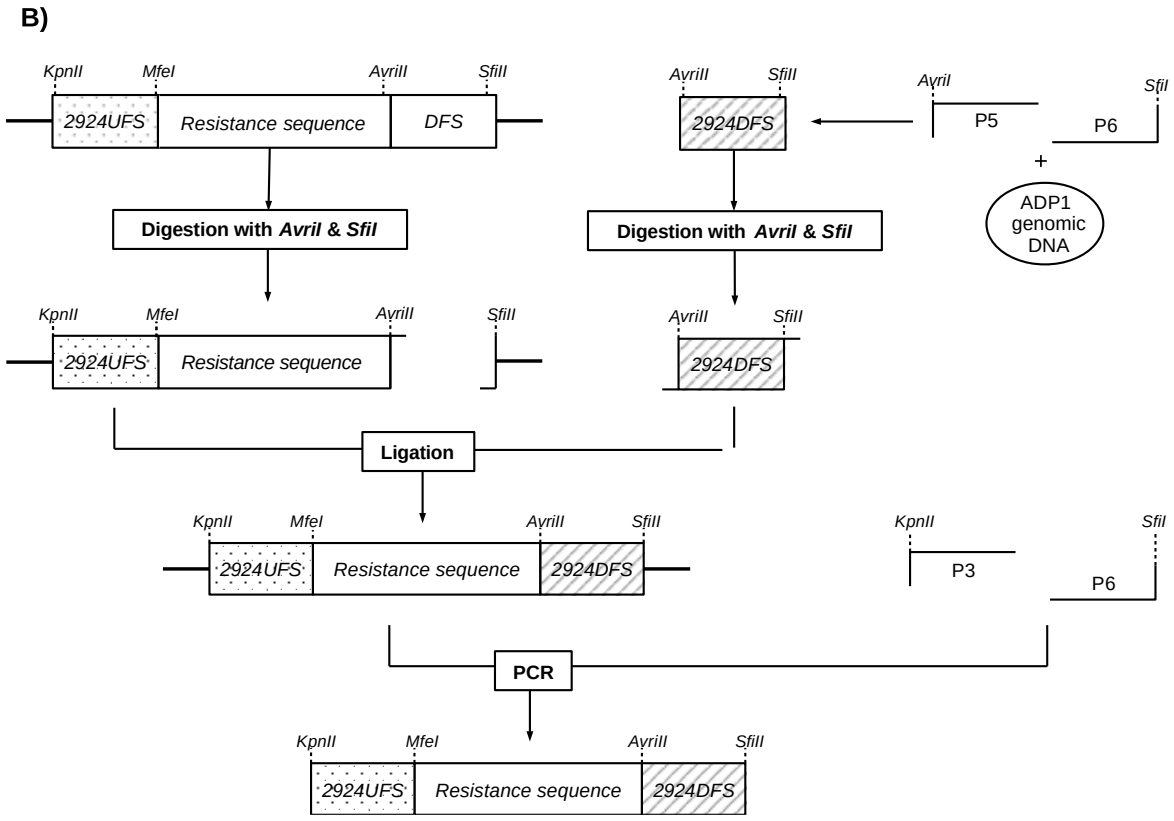


Figure 12. *The construction of the gene knockout cassette. A) The initial pre-constructed gene cassette was ligated with an upstream flanking region of the target site ACIAD 2924. B) The construct was ligated with a downstream flanking region of the target site. The used primers P3, P4, P5 and P6 produce the respective flanking regions for the site.*

Transformation of the modified gene cassette into *A. baylyi* ADP1 shown in Figure 13 was done by natural plasmid transformation. *A. baylyi* ADP1 was grown overnight in a preculture of low salt LB medium with 1% glucose. In the morning 50 µl of the preculture was used to inoculate 2 ml of low salt LB medium with 1% glucose and allowed to reach exponential growth phase. The culture was divided into two 0.5 ml aliquots, one was left as a control, and one had 1 µg of PCR product added to it. Both of the aliquots were incubated for 20 hours at 37 °C. Each aliquot was plated onto two plates of LA medium with 1% glucose and 50 µg/ml of CamR and then incubated overnight. The next day colonies from the transformed plate are transferred to secondary plates containing the same medium and incubated for overnight, repeating this process once more the next day. After the colonies appeared on the 3rd plates, they were inoculated onto LB + 1% glucose + CamR medium and stored in glycerol preps.

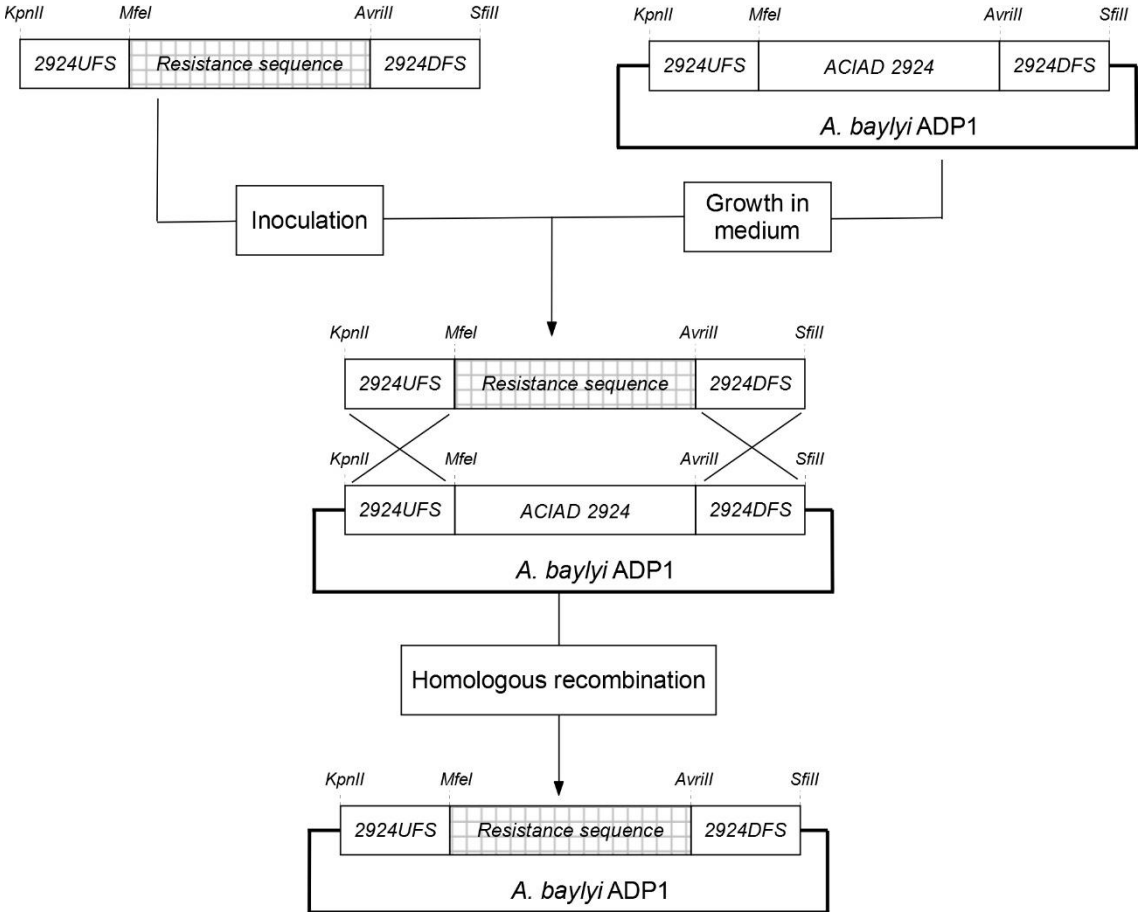


Figure 13. *Process chart of the process of homologous recombination with the constructed gene cassette. The wild-type A. baylyi ADP1 was grown in a LB medium and was inoculated with the purified gene cassette.*

3.4 Cultivation of *A. baylyi* ADP1

All the *A. baylyi* ADP1 and its mutant *ΔatfA* strains were cultivated in shaker incubators at 30 °C at 300 rpm. Pre-cultures of the strains were cultivated in 15 ml cell culture tubes containing roughly 5 ml of MA/9 medium for 18–24 hours. Cells from the pre-cultures were inoculated directly to the pre-filled experimental flasks to an OD of 0.1.

The large-scale cultivations were prepared in 500 ml Erlenmeyer flasks containing roughly 50 ml of MA/9 low salt medium supplemented with 5% (w/v) glucose, 0.4 % (w/v) casein amino acid and the variable amount of synthetic vinasse or real vinasse. In addition to the growth medium, 2 ml of the MSM buffer solution was added to each growth to stabilize the pH levels to approximately 7 during the growth process. The Δ*atfA* cultures were supplemented with 25 µg/ml of CamR. The optical density was measured after mixing the flasks. The flasks were capped with an aluminium foil used during the autoclave sterilization allowing them to be aerobic environments.

The sodium (Na+) tolerance test cultures were cultivated in 250 ml flasks containing 50 ml of MA/9 medium supplemented with 5% (w/v) glucose, 0.2% (w/v) casein amino acids. The experiments were performed by adding 0.5, 1, 1.5, 2, 2.5 or 3.5 ml of MSM 100x buffer into the cultivations. The OD600 was measured after 24 and 48 hours of inoculation.

Wax ester synthesis cultivations were prepared in 500 ml Erlenmeyer flasks containing 50 ml of MA/9 medium supplemented with 10% (w/v) glucose, 0.4% (w/v) casamino acid. The Δ*atfA* culture was additionally supplemented with 25 µg/ml of CamR.

3.5 HPLC conditions

The nutrient depletion of the medium was measured using Shimadzu HPLC machine setup, consisting of an autosampler SIL-20AC, Refractive index detector RUD-10A, degasser DGU-20As, pump LC-20AD and system controller CBM-20A. The samples were injected into a Rezex^{tm} RHM-Monosaccharide H+ OOH-0132-K0 300x 7.8 mm column equipped with a guard column. The compounds were eluted at 70 °C with an isocratic flow rate of 0.5 ml/min with a mobile phase of 0.01 normality (N) of H_2SO_4.

Organic acids and aromatic compounds were measured using the same column. HPLC samples were prepared by centrifuging 2 ml of culture at 20000 *g* for 5 minutes and filtering the supernatant into Eppendorf tubes then stored in −20 °C freezer. The remaining cell mass from centrifuging was stored and used as TLC samples.

3.6 Analyses

The production of wax esters was tested with TLC analysis. The stored cell masses were suspended in 500 µl of methanol (MeOH) and vortexed for 30 minutes. To the suspended cells 250 µl of chloroform ($CHCl_3$) were added and vortexed for 1 hour. The mixture was then centrifuged at 20000 *g* for 5 minutes and without disturbing the layers, 250 µl of both $CHCl_3$ and PBS were added. This mixture was then slowly swirled overnight at 4 °C. Next day the samples were centrifuged at 20000 *g* for 5 minutes and stored at 4 °C before used the same day.

The mobile phase for the TLC was a solution of 90:15:1 of hexane, diethyl ether and acetic acid, and the staining agent used was iodine. From the prepared samples 30 µl of the lowest phase of each sample was transferred onto a glass-backed plate in regular intervals that were compared to a control sample of jojoba oil control sample. The plates were placed in a sealed container containing the mobile phase for roughly 10 minutes so that the mobile phase had reached the top of the plate. Soaked plates were

immediately transferred to another sealed container containing iodine fragments until stains were visible.

The OD_{600} of the cultures were tested every 24 hours with a Shimadzu UV-Vis spectrometer. Some cultures were cultivated in a CQG OD measurement device from Aquila biolabs that measured the OD_{600} of the cultures in real time. The OD_{600} could be measured from clear solutions, but due to the dark colour and viscosity of vinasse, the OD could not be measured accurately from samples cultivated in more than 5% concentrated vinasses. The cell growth was measured by using a Colonies Forming Units (CFU) standard method [137]. Samples from the cultures were serially diluted using LB medium to a known factor and 0.1 ml of the dilution was plated onto a LB agar plate. The plates were incubated for 24 hours and then the colonies were counted.

To compare the size differences of the two strains, three samples of each strain were taken immediately after transformation, and after 24- and 48-hour cultivations. The cultivation samples were taken from the same cultivation as the one used in TLC analysis. The samples were prepared by pipetting 2 µl of fixation agent on to the analysis slide and then pipetting 1 µl of diluted sample on to the agent. The slide was then sealed with a second plate.

4. RESULTS

Due to the high concentration of organic acids in both synthetic and real vinasse mediums, a large concentration of buffer solution was required. Cultures grown in mediums with high concentration of the used of K_2HPO_4 and NaH_2PO_4 produced smaller growths. The tolerance limit for *A. baylyi* ADP1 of K_2HPO_4 and NaH_2PO_4 buffer was tested to be 88 and 56 mM respectively or 2 ml of MSM 100x solution. Cultures containing more buffer than 2 ml showed reduced OD_{600} during the 48-hour cultivation period. Cultures supplemented with 0.5 to 2.0 ml of MSM buffer retained an OD_{600} of 8.5 ± 0.3. The 2.5 ml supplementation of the buffer caused to OD_{600} to fall to 7.6 and respectively a 3.5 ml supplemented medium reached an OD_{600} of 6.1.

4.1 A. baylyi ADP1 *ΔatfA* phenotype changes

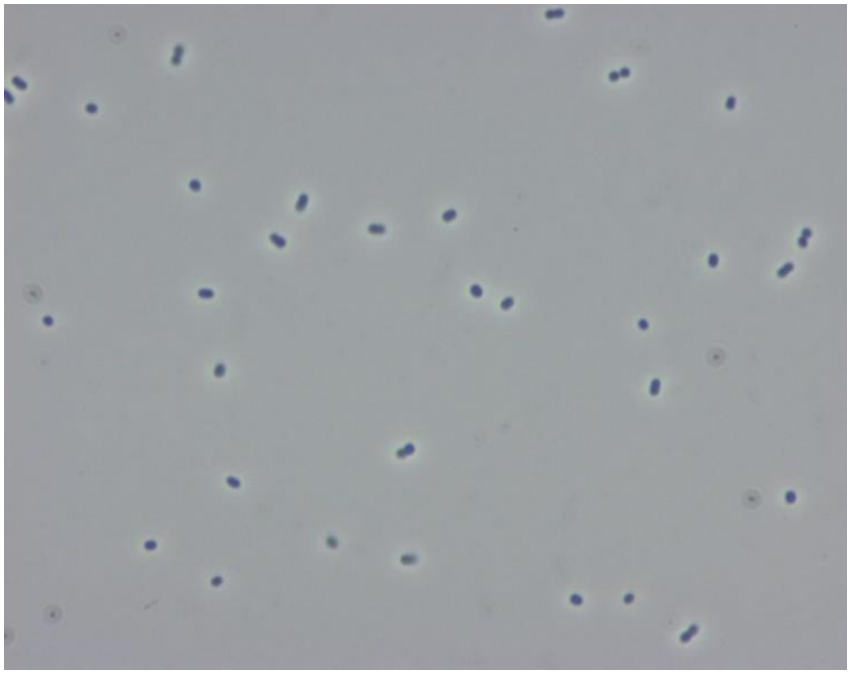
The deletion of the gene *atfA* in *A. baylyi* ADP1 was verified by a PCR method. In the method the genome of the mutant was extracted, and PCR was run with it using primers P3 and P6. A negative control was run alongside this PCR with a wild-type strain. The reaction created a faint band with a size of approximately 2 kb and the negative control a 3 kb product which matches the previous results of verifying the presence of the gene cassette by Kannisto et al. [103].

In Figure 14 A and B the wild-type and mutant *A. baylyi* ADP1 strains after 24 h cultivations are shown respectively. The circumference of the cells of both wild- and *ΔatfA* strain are visually observably the same. In several samples that were imaged, several cells were seen moving in the microscope feed. This action was not observed in any of the wild-type samples. The OD_{600} of the culture samples that were analysed with the microscope were initially 5, but for the analysis they were diluted into OD_{600} of 1. The analysis of the samples was done within 30 minutes of sampling and diluting except for the sample taken directly from the glycerol prep of the first transformed colonies.

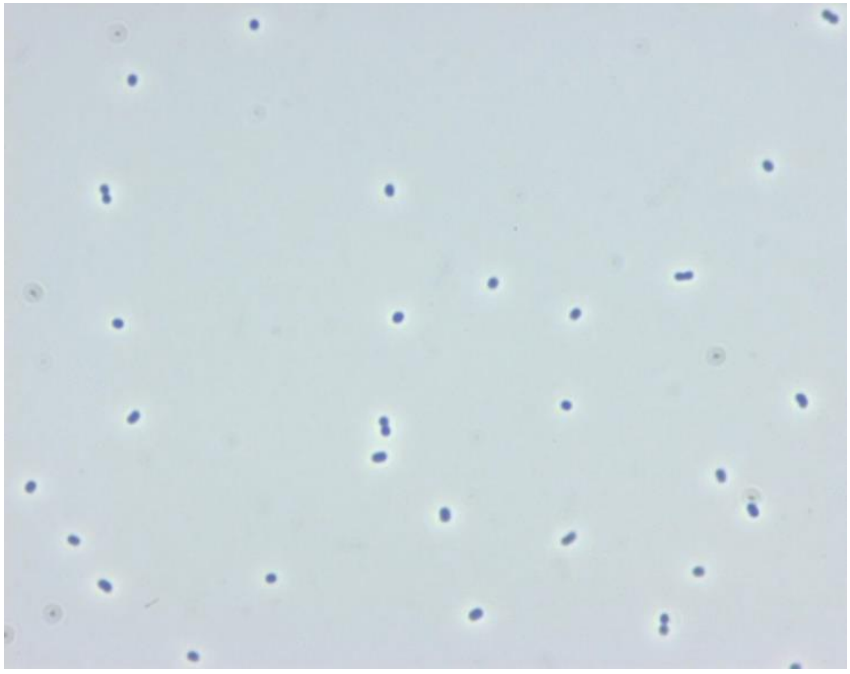
The sample of the *ΔatfA* strain that was cultivated into glycerol prep illustrated in Figure 15 C showed that the cells were larger than the wild type before or after the cultivations. The cells length and width were measured to be 1.70 µm and 1.40 µm respectively and the volume was thus calculated to be 1.89 μm^3 using formula 1. Cells in this sample were also observed to make chains of 3 or more cells more often than the culti-

vated wild type and mutant cells. It can be observed that the enlarged cells from the glycerol prep samples have retorted to normal *A. baylyi* ADP1 cell sizes within 24 hours of cultivation.

A)



B)



C) 

Figure 14. *Microscopic image of (A) wild type A. baylyi ADP1 after 24 hours, (B) A. baylyi ADP1 ΔatfA mutant after 24 hours, and (C) A. baylyi ADP1 ΔatfA mutant in the inoculant at 0h.*

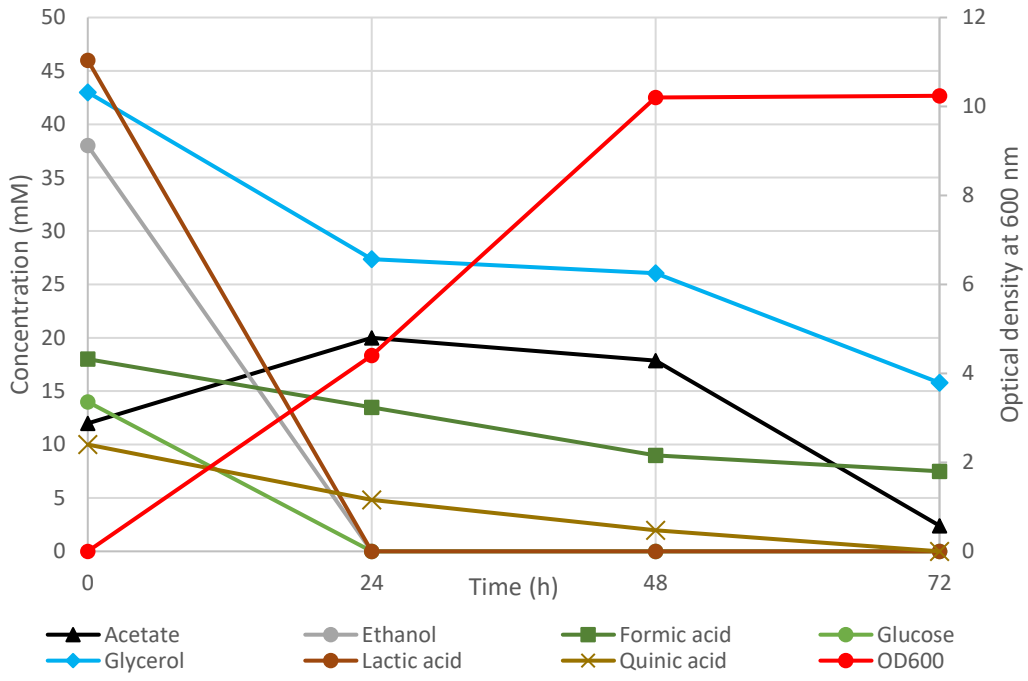
4.2 Growth kinetics on vinasse

The growth performance of the *A. baylyi* ADP1 was dependent on the pH of the medium where the largest growth was observed in pH 7. The high acidity of both synthetic and real vinasse mediums was initially set to pH 7 to which the buffer was added. Initial growth tests using a 5% synthetic medium showed that the pH dropped rapidly causing the growth of the culture and depletion of the nutrients to slow down. The buffer amount was increased to two times the original concentration which alleviated the pH drop but was not enough to keep the pH in the 6–8 range completely. The variable amounts of buffer were tested on quick growths utilizing a simple glucose medium, which resulted in significantly lower growth rates when the buffer was four times more concentrated than originally estimated.

As *A. baylyi* ADP1 does not catabolize levulinic acid it is used as a control substance in the HPLC data and is not shown in the following results. The substrate depletion order was observed initially in the synthetic vinasse cultivations. In the synthetic vinasse mediums *A. baylyi* ADP1 initially consumed ethanol, glucose, and lactic acid from the medium in the first 24 hours. In the 5% synthetic vinasse cultivation shown in Figure 15 A, the pH of the cultivation dropped to 5.73 and it was noted that the cells accumulated

acetic acid instead of catabolizing it. Both quinic acid and glycerol are catabolized steadily instead of the acetic acid in the lower pH medium. After 24 hours the pH is fixed to 7.0 with KOH which caused the acetate once again to be catabolized, while halting the glycerol catabolism. After 48 hours the pH of the solution had increased to 8.35 restarting the quinic acid and glycerol catabolism. The 5% synthetic vinasse cultivation with initial acetic acid concentration at 50 mM, also initially consumed all the ethanol in the medium as shown in Figure 15 B. As the ethanol was turned into acetic acid, the culture acetic acid concentration grew to 94 mM which lowered the pH of the culture into 5.13. The pH was adjusted back to 7 after 24 hours, allowing the acetic acid catabolism to continue.

A)



B)

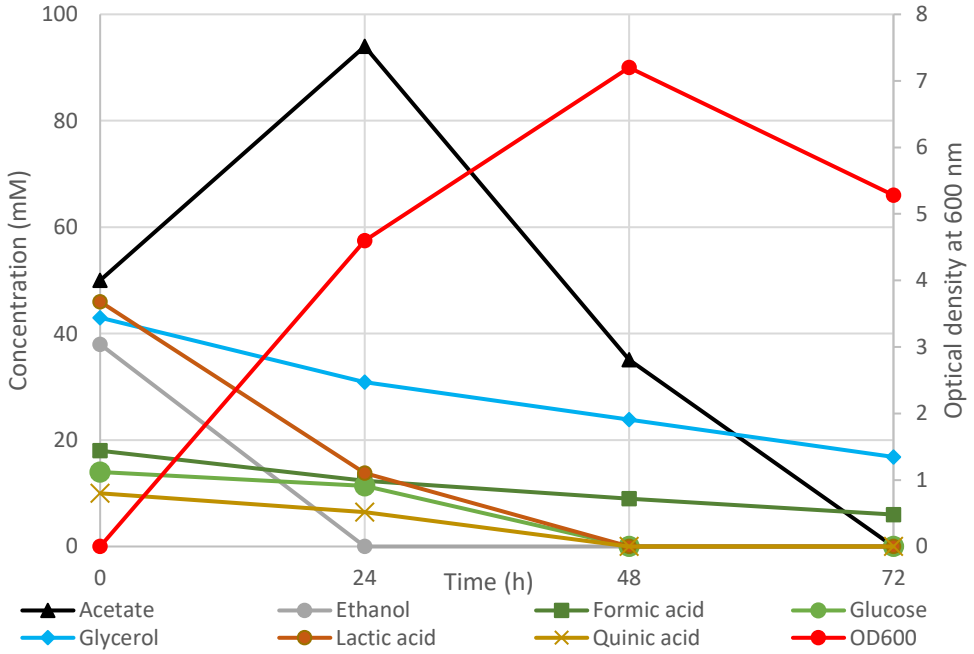


Figure 15. *Growth and substrate consumption of wild-type A. baylyi ADP1 in A) 5% synthetic vinasse and B) 5% synthetic vinasse supplemented to 50 mM acetic acid.*

The 10% synthetic vinasse cultivations pH immediately increased to 8.13 unlike in the 5% cultivations where the pH dropped to 5.7. In these cultivations acetic acid did not increase during the first 24 hours, but the was only depleted by roughly 40%. Due to the large time gap of 24 hours between samplings, it is possible that the acetic acid has initially increased and then begun its depletion. However, in the 10% synthetic vinasse with no ethanol cultivations the acetate was also shown to only decrease by 30% as can be observed in Figure 16. In all of the 10% synthetic vinasse cultivations, the quinic acid had been depleted in the first 24 hours. Glycerol and formic acid have been shown to in all the cultivation to be depleted slowly but steadily in synchronization with each other even in non-optimal growth conditions.

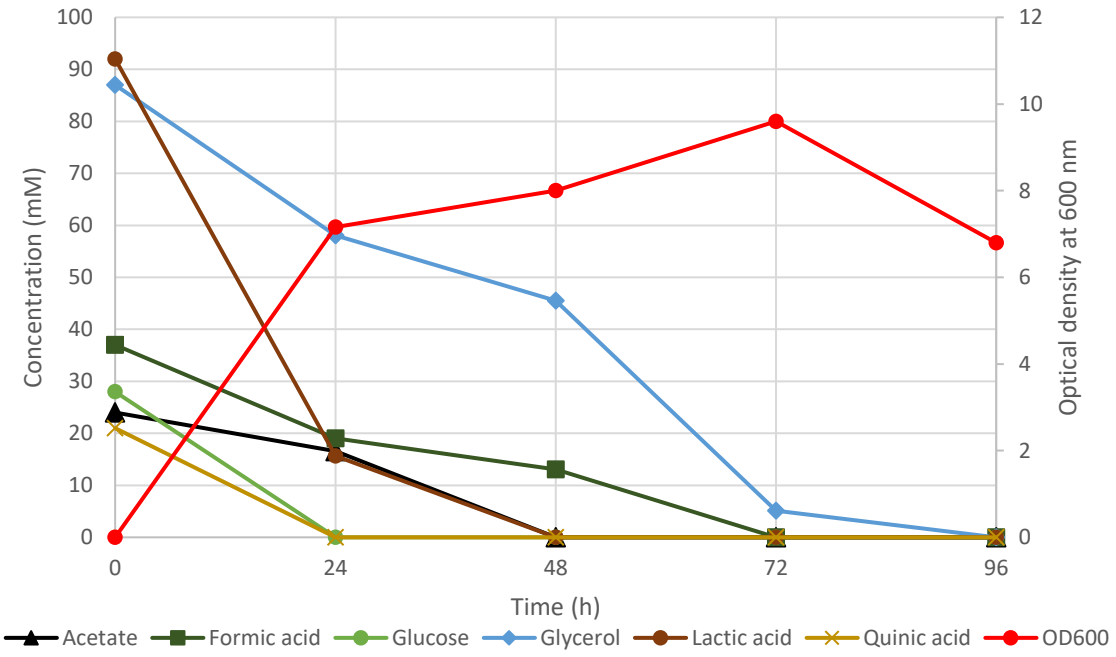
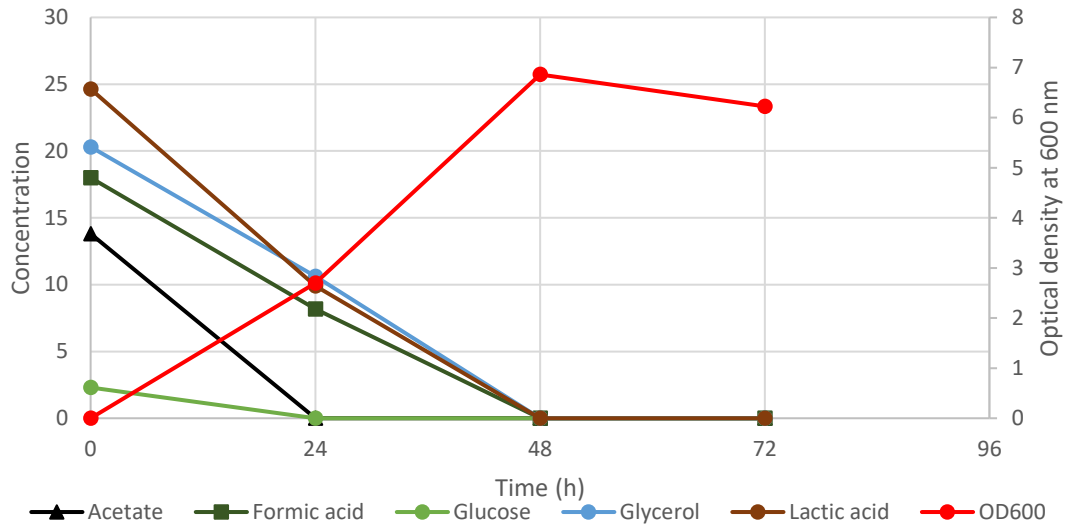


Figure 16. *Growth and substrate consumption of wild-type A. baylyi ADP1 in 10% synthetic vinasse with no ethanol.*

Cultivations done with 5% real vinasse (5R) using *A. baylyi* ADP1 wild type exhibit better growth performance when compared to the synthetic vinasse cultivations as shown in Figure 17 A. The pH of the cultivation remained in range of 6–8.5 allowing the cells to survive in the medium unlike in the synthetic medium cultivations. The OD_{600} of the initial medium was 3.3 and within 24 hours of starting the cultivation, it had reached 6.2 continuing to increase in a steady pace. Substrate depletion in the medium showed that unlike in other cultivations, glycerol and acetic acid were depleted first before lactic acid or the aromatic compounds. Lactic acid and glucose concentrations in the medium were reduced by roughly 50% in the first 24 hours. After 48 hours all of the observed substrates were depleted in the medium leading to the cell decline due to lack of nutrients. The vinasse had been stored in the dark fridge for 1 month between the cultivations with wild-type and *ΔatfA* strain during which time the OD_{600} of the vinasse had increased by 3.

A)



B)

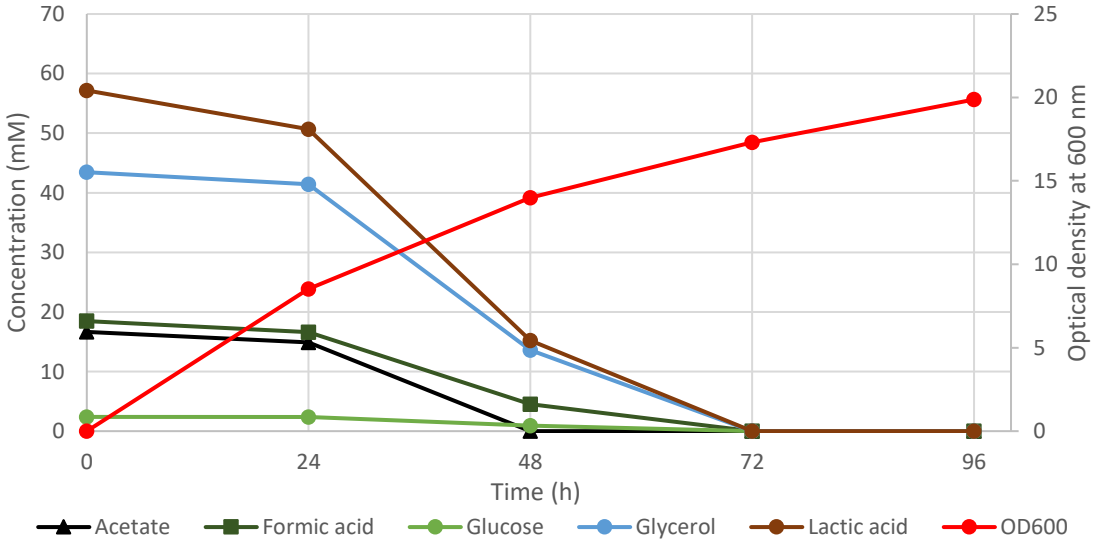


Figure 17. *Growth and substrate consumption of 5% real vinasse medium by A) A. baylyi wt. and B) A. baylyi ADP1 ΔatfA strain.*

The growth of *A. baylyi* ADP1 *ΔatfA* in real 5% vinasses (5RM) behaved slightly differently than in the synthetic mediums as shown in Figure 17. Depletion of the observed substrates was not observed in the first 24 hours, but a single unidentified substrate was depleted completely during this time with a retention time of 17.4 minutes in the HPLC analysis. After 24 hours the culture begun to rapidly deplete all of the other substrates leaving only levulinic acid and the varied sugars into the medium.

Cultivations in 5% and 10% filtered vinasse resulted in 50% reduced initial growth in the first 24 hours compared to non-filtered vinasse. After 24 hours the cultivations

OD_{600} did not increase, and the pH had stabilized in 8.5. Substrate depletion analysis of both 5% and 10% mediums showed that the cells had used 75% of the available lactic acid and 50% of glycerol and formic acid but had not depleted aromatics or sugars at all. Due to this property, no further cultivations were performed with filtered vinasse. The comparison of the three cultivations in 5% real vinasse are compared in Figure 18.

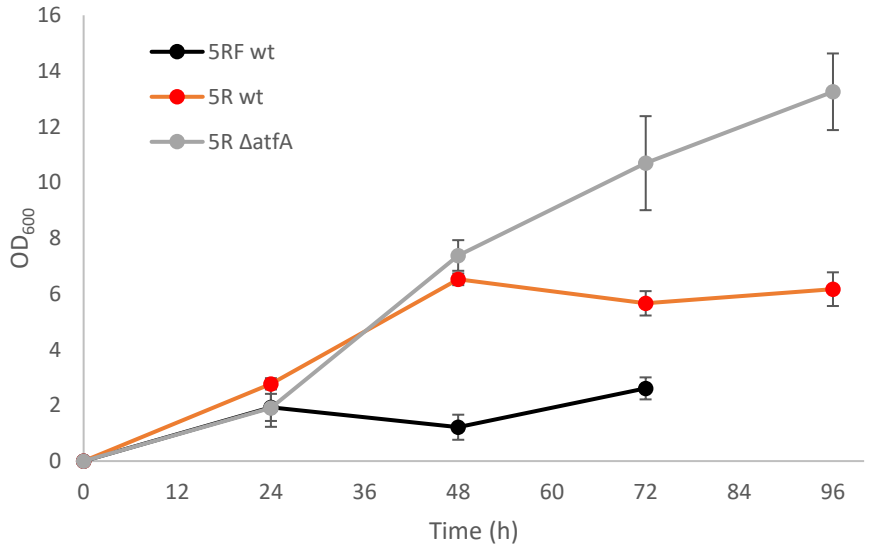


Figure 18. *Comparison of growth curves of wild-type (wt) and mutant ΔatfA cultures in 5% real vinasse (5R) and 5% real filtered vinasse (5RF). The OD_{600} values were set to begin from 0.*

The 10% diluted real vinasse cultivations were performed twice during this work, although no growth was detected in the first cultivation. However, the pH of both the inoculated and the control cultivation reduced at a steady rate of 0.15 per 24 hours. The same decrease in pH was also recorded during the second cultivation attempt. In the second 10% diluted vinasse cultivation, two replicate cultures were contaminated with an unknown strain. Initially only one culture was contaminated but the other active culture was contaminated within one day. The control flask with no *A. baylyi* ADP1 was also contaminated after 144 hours. The genome of the strain was analysed by colony PCR sequencing using the universal 16s RNA gene primers 27F and 1492R. The fragments were then sequenced by Macrogen and the results are shown in Appendix C. Analysis of the sequences using BLAST revealed them to be most likely from the *Bacillus subtilis group*. The results provided 103 hits for *Bacillus* genus of which 97 were part of *Bacillus subtillis* group and 58 of those hits for *Bacillus velezensis [138]*. Upon visual inspection, the colonies morphologies were clearly distinguishable from the *A. baylyi* ADP1 colonies as they appeared as jagged rather than smooth colonies when grown on agar plates. The contaminated cultures OD_{600} and CFU growth curves are

displayed in Figure 19 and were the only cultures capable of growing in a 10% vinasse medium.

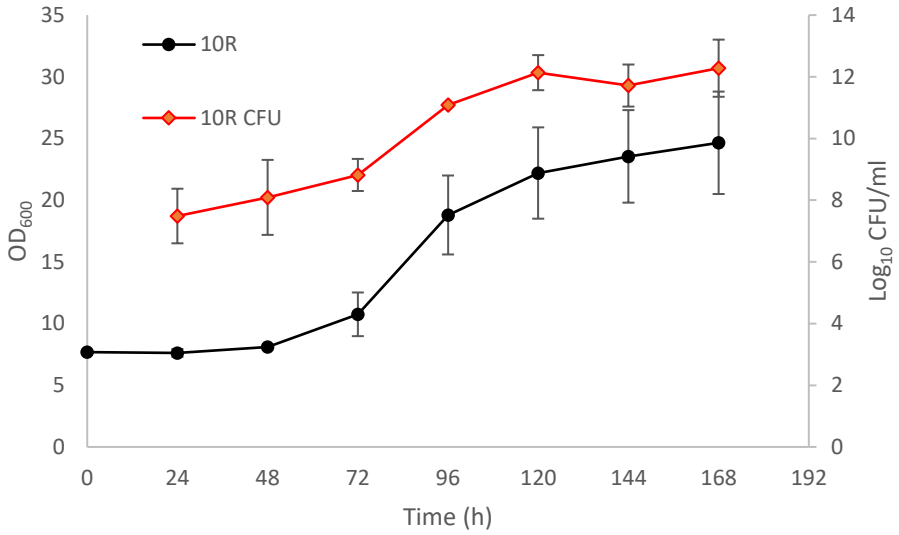


Figure 19. *OD_{600} and colony forming unit (CFU) development of the contaminated cultures containing both an unknown Bacillus strain and wild-type A. baylyi ADP1.*

Like *A. baylyi* ADP1, it could not catabolise levulinic acid or sugars other than glucose and xylose from the vinasse. But unlike *A. baylyi* ADP1, the *Bacillus* contaminant initially consumed the glycerol and formic acid in the first 24 hours of its emergence from the medium instead of the lactic acid and aromatic compounds. In the following 48 hours the culture depleted all the other substrates steadily leaving behind same various sugars and levulinic acid as the *A. baylyi* ADP1 cultures.

4.3 Wax ester production

The wax ester production of all the cultivations were tested with the TLC method to see whether any wax esters had been produced in the cultivations. The wax esters would show on the plate in deeper brown colour the more product there was, but the absolute amount cannot be calculated from this analysis. The results of the wax ester detection from the TLC analysis are summarized in Table 13.

Table 13. *The wax esters produced by different cultivations at the gathered data points. In the table the + indicates detected wax esters and the − indicates that no wax ester was detected.*

Sample	24	48
5R1	−	−
5R2	−	−
5RM1	+	−
5RM2	+	+
WaxW1	+	+
WaxW2	+	+
WaxM1	−	−
WaxM2	−	+

Wax ester is detected in variable quantities in the 5% real vinasse cultivations only in the *ΔatfA* strain. In the pure wax ester production cultivations wax ester is detected mostly only in the wild type experiments. In the real vinasse cultivations additional bands of lipids can be seen, one above and one below the wax ester bands. These lipids are most visible in the contaminated 10% real vinasse cultivations. In the 10% vinasse cultivations, the lipids appear after 72 hours of cultivation, which corresponds to the beginning of the contaminant growth.

5. DISCUSSION

5.1 Viability of using *A. baylyi* ADP1 to metabolize vinasse

The substrate flow chart shown in Figure 7 indicates how much potential organic carbon components could be used in the utilization of vinasse by *A. baylyi* ADP1. As vinasse producers can decide to concentrate their vinasse, the exact composition of the final vinasse can vary from these obtained values notably. From the approximations made here, it can be seen that especially lignocellulosic masses generate feasible amounts of organic acids that *A. baylyi* ADP1 can use very effectively. Cellulosic vinasses are however also rich in many types of reducing sugars and disaccharides that ADP1 cannot use. In addition to the sugars, the degradation of phenols and sugars in the pre-treatment process create levulinic acid that ADP1 cannot normally catabolize. The sugars and levulinic acid roughly consist of 32.4% of the total mass that is left over in cellulosic vinasses. Further complicating the use of vinasse, it contains considerable amounts of phenol and furfural compounds that are toxic to most cells in large concentrations. Although soil bacteria are traditionally more resilient to these toxic compounds, the growth of *A. baylyi* ADP1 is also limited by them. *A. baylyi* ADP1 has been shown to biotransform furfural and furfural alcohols, but only in the presence of acetate. It has been reported to be able to transform 0.2 g/l of furfurals per hour in the presence of acetate at the expense of growth rate [139]. The relatively small quantity of acetic acid in the real vinasse is not probably enough for the cells to completely remove the furfural compounds. In the same study it was reported that the presence of furfurals will delay the beginning of the growth in cultures. Since the 10% vinasse solution corresponds to 7.4 g/l of furan and 0.4 g/l of aromatic compounds, ADP1s absolute tolerance limit is slightly lower than that amount.

In both synthetic and real vinasse cultivations glycerol was seen to be consumed, which according to previous findings by Santala et. al [8] should not happen without gluconate. In the synthetic vinasse no gluconate was in the medium, but the glycerol was still consumed. In the real vinasse on the other hand, all of the glycerol was consumed in conjunction with the rest of the substances. The real vinasses did not contain gluconate, but rather a wide array of different sugar derivatives and organic acids. Based on the results from the HPLC analysis, formic acid was found to be depleted at

the same relative rate as glycerol in all cultivations. Formic acid is used in the A. baylyi ADP1 cells for redox reactions while glycerol is used for both biomass and TAG or wax ester production when supplemented with gluconate [8]. The excess amounts of formic acid could allow transport channels to import the glycerol into the cells even in growth phase II or regulate endogenous glycerol catabolism pathways. As the cells only accumulated small amounts of wax esters and other lipids, the glycerol was most likely used for biomass accumulation rather than for TAG synthesis.

The 10% vinasse cultures recorded only minimal growth in CFU counts that are within the range of error. However, the pH of the cultures including the control culture decreased at a steady rate of 0.15 per 24 hours. The vinasse was most likely oxidizing the free alcohols like dihydroconiferyl alcohol and glycerol into their respective acids. The *Bacillus* contaminant begun growing after 48–72 hours in the inoculated cultures, but also in the control culture after 168 hours. The contamination of the control culture indicates that the *A. baylyi* ADP1 did manage to catabolise some limiting substance from the vinasse. Since the control culture was also contaminated and begun growing at a later time, it is reasonable to assume that either the pH, some alcohol or some aromatic compound is a limiting factor in the vinasse.

Interestingly the OD_{600} of the pure vinasse increased significantly during its storage in the fridge. The increase in the OD_{600} of the vinasse could indicate that it slowly oxidized remaining lignocellulosic fractions creating either melanoidins or furan compounds which correspond to the darkness of the solution. Other possibility is that the nitrogen and the various sugars began to slowly crystalize and create colloidal material in the solution, also darkening the colour of it. The increase in available substrates could have resulted from the hydrolysis of substrate binding compounds, which would cause them to release the substrates into the solution. Allowing the vinasse to oxidize could thus be a potential solution to detoxify the medium for more optimal growth conditions [44].

Real vinasses contain more natural buffers created from phosphate salts and other minerals present in the solution that create a more stable growth environment. The pH of the cultivations of wild-type 5% real vinasse remained in range of 7.0 to 8.6 and within 7.0 to 8.3 with the *ΔatfA* strain. In synthetic cultivations the pH was reduced to 5.3 and as expected in low pH, the acetic acid begun to accumulate in the medium. In vinasses, the organic acids can consist of up to 60.7% of the dry mass. Additional acids that accumulate due to the catabolism of other substances can cause the buffers to be very easily overwhelmed. Even though the acetic acid reached over 5.7 g/l in the synthetic medium the cells could survive and catabolize all of the acetic acid when the pH

was returned to 7.0. With additional active pH control system, even organic acid rich vinasses can be catabolized partially by *A. baylyi* ADP1.

The results demonstrate that *A. baylyi* ADP1 is capable of catabolizing majority of the organic acids in the vinasse excluding levulinic acid. Based on previous studies ADP1 can also catabolize aromatic compounds like benzoic acid, 4-hydroxybenzoic acid, vanillic acid, and quinic acid found in real vinasse [51, 52, 106]. *A. baylyi* ADP1 is a viable option for partial vinasse valorization as a wild-type strain.

5.2 Effect of the *atfA* gene knockout

The phenotypic changes in the *A. baylyi* ADP1 *ΔatfA* caused the cells volumes increase in size that lasted from 1 to 24 hours after which the cells were observed to have retorted to their normal sizes. The cells did still retain their antibiotic resistance in the culture, indicating that the gene cassette was still in the strain's genome. It is possible that for example the cells created a separate plasmid containing the CamR sequence or generated a mutation into the genome that restored the cells size to normal. However, in this study the recovery of the cells original size was not studied, and more detailed studies should be conducted to find out why it happened.

In the Withers et al. [11] they noted that there were no growth kinetic changes in the cells after the deletion of the atfA gene. In this work however, it was discovered that the *ΔatfA* cells did behave differently than the wild-type strain in vinasse and glucose cultivations. In the wax ester production experiments where the two strains were fed a rich glucose medium, the wild type produced significant amounts of wax esters while the mutant strain did not produce almost any. In the vinasse cultivations, it could be seen that the *ΔatfA* strain did not prefer to consume the sugars in the medium before depleting other substrates. The lack of *atfA* causes at least 522 genes to express differently and this can have a major effect like cell volume increase or a minor effect like a change in substrate specificity [11]. Unlike glycerol catabolism in *A. baylyi* ADP1, where glycerol is not used when it is the sole carbon source and is only imported to the cell when gluconate is present [8], an inverse effect could have formed with glucose due to the lack of *atfA*. In the real vinasse mediums, the *ΔatfA* could produce wax esters at an early stage due to the heterogeneity of the medium as it contained significant amounts of other sugars and organic acids. Since WS/DGAT in *A. baylyi* ADP1 has a low specificity and is able to use a wide range of different molecules, the wild type cells should have also been able to create wax esters in the real vinasse [116, 120, 140]. It is thus feasible to assume that the atfA has some effect on the sugar catabolism path-

way, the FAS or WE pathways, or the prioritization of carbon and energy use in the cell's metabolism.

In many lipid analysis samples of the vinasse cultivations, two bands could be seen both above and below the expected wax ester band. These lipids are not present in control samples. Both wild-type and the *ΔatfA* strain produced these lipids only in the real vinasse cultivations, where *ΔatfA* strain also produced wax esters along these lipids. The wax ester amounts were found to decrease over time in the cultivations which can mean that the initial wax esters in 5RM1 were simply metabolised first. The unidentified compound shown in the HPLC data that was initially depleted in the first 24 hours in the 5RM cultivations match up with the TLC data, where 5RM1 depleted more of the compound than 5RM2. However, as the compound was not identified and the HPLC analysis was not designed to record fatty acids, the matching results are more likely to be a coincidence.

Since the *ΔatfA* strain has retained its antibiotic resistance it gained from the gene cassette, there is a possibility that the homologous recombination might have recombined in multiple homologous regions. The cells might have regained the ACIAD2924 gene or some other gene that alleviated the stress caused by the knockout. In this study the integration of the gene cassette was only verified through the PCR method, but its location was not verified. The results of the gene knockout in this study exhibited the same properties of A. baylyi ΔatfA as reported by Withers et al. [11] study. It is as such assumed that the gene knockout integrated into the correct location in the genome.

5.3 Concepts for improving cell viability

Increase in cell size would theoretically produce more surface area and room for wax esters to be synthesized and accumulated. However, if the cell is rendered into a state of blocked mitosis, the duplication of DNA and other organelles will cause tremendous stress on the cell. The replication of DNA requires the cell to siphon more carbon and nitrogen substrates into the essential reactions of the cell. The cells naturally attempt to create as much biomass as possible before beginning to accumulate storage lipids, but non-regulated cell growth causes the survival chance or the viability of the cell to reduce significantly. This was observed as well by Withers et al. [11] suggesting that the cells could have also been replaced by more viable cells during the analyses and experimentation.

The reduction in cell viability through increased biomass growth could be diminished through controlling the funnelling of resources to biomass or storage lipid synthesis. An

example of this kind of funnel controlling was demonstrated by Santala et al. [121] with *A. baylyi* ADP1. They created a catabolite-controlled switch utilizing an arabinose-inducible promoter for regulating between biomass and storage lipid synthesis. Controlling of the ratio between these two synthesis choices could allow the cells to initially accumulate biomass and then be regulated to accumulate storage lipids to utilize their increased volume.

Multiple genetic modification to a cell can however cause a notable reduction in cell viability especially in cases where one of the modifications already causes a large reduction in the viability. However, as stated earlier the addition of a regulating modification to a modification that inhibits regulation should neutralize the cell viability reduction.

5.4 Proposals for further studies

The *A. baylyi* ADP1 *ΔatfA* strain did create larger cells as was predicted by Withers et al [11], but the duration of this increase was temporary. Unfortunately, the very brief increase in cell volume might not have affected the wax ester production or the cells' ability to utilize vinasse in time. However, since the growth kinetics of the *ΔatfA* strain were affected in the cultures, a more focused study on the *atfA*s functions in the metabolic pathways would be advised.

The effective valorization of vinasse requires a complete conversion of all the available substrates into more valuable products. For example, levulinic acid catabolism pathways have recently been found in *P. putida* KT2440 and reconstituted into *E. coli* strains. This allowed *E. coli* to catabolize levulinic acid and as ADP1 shares many essential genes with both *E. coli* and *P. putida* KT2440, it is worth to study whether this gene also works in *A. baylyi* ADP1 [141]. Further studies with new kinds of vinasses with *A. baylyi* ADP1 using an actively controlled bioreactor to optimize the cultures growth. The discovery that glycerol was consumed in the mediums warrants further studies to find out which of the substances in the synthetic medium causes it in place of previously reported gluconate [8].

The discovery of the contaminant *Bacillus* strain that can survive in more concentrated vinasse than *A. baylyi* ADP1 could be used in subsequent studies to find a bacterium that can effectively digest cellulosic vinasse. This *Bacillus* strain was shown to prefer catabolizing the formic acid and glycerol from the vinasse whereas ADP1 preferred to catabolize the aromatic compounds and lactic acid. Although both of the strains compete for the rest of the substrates in the medium, a co-culture or consecutive culture of these two strains could be used to effectively digest the vinasse.

6. CONCLUSION

In this work, the viability of using *A. baylyi* ADP1 to metabolize cellulosic vinasse was tested. A gene knockout of a ACIAD2924 that expresses an acidic transcription factor A (*atfA*) was applied to *A. baylyi* ADP1. Lack of that gene was found to increase both the volume and the DNA concentration of the cells which was hypothesised to increase wax ester production.

ACIAD2924 gene was successfully knocked out of the genome by in-vitro constructed gene cassette with antibiotic resistance gene using the homologous recombination method. The *ΔatfA* strain was demonstrated to have increased its cell volume by 87% as expected. After 24 hours the cells had retorted back to their original dimensions but had retained their antibiotic resistance indicating that the cells had mutated to alleviate the metabolic stress caused by the gene knockout.

The cellulosic vinasse was found to have high concentrations of aliphatic alcohols, organic acids, and phenolic compounds that *A. baylyi* ADP1 could convert into wax esters. The wild type *A. baylyi* ADP1 produced wax esters when cultivated in medium with glucose as the sole carbon source. The *ΔatfA* strain produced wax esters when cultivated in cellulosic vinasse and after 48 hours in the glucose cultivation. It is hypothesised that the *atfA* caused disruptions in either glycolysis or in the fatty acid synthesis pathways. It was also discovered that some component in the synthetic and real vinasse allowed the *A. baylyi* ADP1 to consume glycerol in spite of the medium not containing gluconate.

This study demonstrated that *A. baylyi* ADP1 can be used to treat cellulosic vinasse and be applied for bioproduction of wax esters from it. The connection of increased cell volume to the production of wax esters could not be proved due to the other side effects of the lack of acidic transcription factor A. In future works the *ΔatfA* should be tested with cell growth and wax ester production regulation to combat the cells' inability to retain its increased volume.

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APPENDIX A

100x Buffer solution

Dissolve the compounds in Table APPENDIX A 1 into a 100 ml volumetric flask. Once dissolved, dilute the solution into 890 ml of MQ-water and check that pH is 7. Finally fill to 1000 ml with MQ-water, autoclave, and store at room temperature.

Table APPENDIX A 1. Components of the 100x Buffer solution.

Component	Stock (g/l)
K_2HPO_4	388
$NaH_2PO_4 \cdot 2H_2O$	212

100x $(NH_4)_2SO_4$ solution

Dissolve 200 g/l $(NH_4)_2SO_4$ in a volumetric flask. Once dissolved, dilute into desired volume. Autoclave the solution and store at room temperature.

100x MM salts solution

Add 1000 mg of ethylenediaminetetraacetic acid (EDTA) to 25 ml of MQ-water and add drops of 10M NaOH until the EDTA is completely dissolved. Add this solution to the rest of the water, bring the pH back to 4 with concentrated HCl and then add the rest of the ingredients in Table APPENDIX A 3 one by one in the order in the following table. The final solution should be clear, then green, then yellow and finally pink after auto-claving upon storage. Store in a cool and dark place.

Table APPENDIX A 3. Components of the 100x MM salts solution

Component	Stock (mg l^{-1})
EDTA	1000
$MgCl_2 \cdot 6H_2O$	10000
$ZnSO_4 \cdot 7H_2O$	200
$CaCl_2 \cdot 2H_2O$	100
$FeSO_4 \cdot 7H_2O$	500
$Na_2MoO \cdot 2H_2O$	20
$CuSO_4 \cdot 5H_2O$	20
$CoCl_2 \cdot 6H_2O$	40
$MnCl_2 \cdot 2H_2O$	100

APPENDIX B

Data and methods for feedstock-to-vinasse nutrient analysis

The data for the Figure 8 was gathered from multiple studies that measured the composition of the various intermediate products in the vinasse production chain. The sources for step of the process are summarized in Table APPENDIX B 1. It is important to note that since the data values did not originate from one source, the results of these calculations are not accurate but rather approximated and indicative.

Table APPENDIX B 1. The references used for the data in each step of the nutrient analysis and degradation. Steps with multiple references have complementing parameters from each reference.

Process step	Reference
Distribution of juice and bagasse in feedstock	[142]
Composition of sugarcane juice	[70, 143, 144]
Composition of clarified sugarcane juice	[70]
Composition of bagasse	[53, 145]
Composition of treated bagasse	[53]
Composition of vinasse	[53, 63, 74, 145, 146, 147]

The sugarcane juice and clarified juice compositions have been cited directly from the references with no calculations. In these steps it is assumed that heat and calcium are added to the system and the solution is mechanically sieved afterwards. The addition of calcium as milk of lime increased the calcium levels in the solution from 4 to 8 mg/l. Calcium is not tracked in any steps of this analysis.

The composition of bagasse has been cited from the work of Van der Pol et al. [53] and matched with the one from Rulianah et al. [145]. The treated bagasse was assumed to have been pre-treated with acid hydrolysis. At this step lignin could be separated from the bagasse solution but in this calculation, it is assumed to have been left into the solution. The composition of the treated bagasse was calculated from the degradation products of cellulose, hemicellulose, and lignin. Values for the conversion were obtained from both of the referenced materials. To simplify the calculations, only those substances that are measured and discussed in this thesis are included. Cellulose,

hemicellulose, and lignin were assumed to have been monomerized with a 95% efficiency.

The fermentation reaction was assumed to happen in optimal pH and temperature. PH control was assumed not to add anything to the substances calculated in this step. Ethanol and CO_2 production were calculated stoichiometrically and to have only depleted the sucrose and glucose from the solution. The results matched with the values approximated by the references.

APPENDIX C

The colony PCR sequencing results of the unknown strain.

The sequencing was done with the universal 16s RNA gene sequencing primers 785F and 907R.

785F sequence:

GGGAGGGTGGGTGGGGCGTCCCAGCCTTCCCGCGGGGGGTTTCGTCACCC

CCCCCCTTCCCGAAGGAACTTCGCCACCCCCCCCCCCAGTTACCCCCCCCCCCC
AATCCCCCCCCCATCCCCCCCCCCCGCCCCCCCCCCCCGCCGGGGCCGCCCTTCAAA
TTGGCCCCAGGGAGGCCAAGGACCCCCCCCCCCCCACCCATGATCCCCCATATAA
AAATACCCCCCCCTGTTCGGCCGCCGCCCGCCCGGAGATCCCCCCCCAAGGGCCC
CTTCCGCGATCAAATGATAGCGTAACCAGAGCCACCTCGCCCCCCCCCCGCTCAC
CCCTAAATCATAAATTGACTCGTTACCCCTCCCCCCTCGCCCGAAACGACCGGAT
GCCCTCGATGAGTCCCCTAACCCTGTTATGTTCGCAATGTGGCCCCCCCCCCCCA
AAGAAGGCGTGAGCAAACGGCAGTCAAAACGGCCGCCTTCGCCACTGCCCCAAA
GTTGTTCGCAGTTCCCACCGAAGGGGGGTGGGACTACCCTTCAATTTCCATTCAT
TCGGGGTGAGGTGGCTTTATGTGACATCACACCCATAACTAAACCATTTACCCCG
GGACCCCCTCACCACGATAGTTTGAAACACTCAGCGTTGCTTCACGTAAACCTTTC
CGCATGTTATCCACCGCAAAAAGGGAAAAAAAGATATAAGGTGTAGCCCGTGAAA
GGGTCTCCGGTAAGTGTCTTCCTTTCGCTCCTTGCTGGGCTTGGGAGCACAACCC
GCCCGGATGAGCACGGAGAGCTGCGTCTTATGCTTGTAACTATGGGCTTTGACTT
CTACCCGTTCAGACGCGACTTATTCCCACTGGCATGTCCCCCTGGTAATAGGAGT
AGCTCACCTAGTTTTGTGGCTGGTTCGTGAGTTCTTGAATAGGAGGTTT

907R sequence:

TAAATAGTTGGCTAGGCTAAACCATCCTTGTCGCACTGGCTACTGCGTTAGCTCTC
CCACCTAGGGGCGAAAACCCCCCTAATCCTTATCACTCATCGTCCACGGCGCGGA
CTACCCTGGTATCCAATCCCGTTCGCTCCCCCGGCTTTCGCTCCTCAGCGTCAGT
TACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTT
CACCGCTACACGTGGAATTCCCCTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCC
AATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCT
GCGAGCCCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCG
CGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGC
CGCCCTATTTGAACGGCACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAA
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GCCAGCGTTCGTCCTGAGCCAGGAATCAAACTCTAAAACTTTAATACCTTATTATC
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CTTCCCGCGATTTGGGGGGGTGGGACCCCACCATTATTATCCTTATTTTTCTATAT
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GTACTTT