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Metabolic Engineering of *Acinetobacter baylyi* ADP1 for Improved Growth and Wax Ester Production Using Components of Lignocellulosic Hydrolysates as Carbon Sources

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Abstract

Microorganisms can be used in bioprocesses to produce various chemicals, such as fuels, cosmetics and medical products, as an environmentally friendly alternative for chemical synthesis. In these bioprocesses the raw materials (*e.g.* lignocellulose) can be converted to compounds with high complexity with a minimum energy input and waste material production. The metabolic capabilities and robustness of the bioprocess host organism limit the yield and purity of the product. Molecules that currently cannot be produced efficiently with robust microbial host organisms include wax esters, which have several industrial applications and are currently produced with the Jojoba plant.

Acinetobacter baylyi ADP1 is a non-pathogenic soil bacterium that produces wax esters that can readily incorporate foreign DNA into their genome and utilize various plant-derived molecules as a carbon source. For these reasons, *A. baylyi* ADP1 has become a model organism of bacterial genetics and metabolism, which has led to the accumulation of a vast amount of information about its biology. This Doctor of Science thesis describes experiments where the metabolism of *A. baylyi* ADP1 was engineered for improved growth and wax ester production using lignocellulose-derived molecules as raw material.

With a gene knockout (*rmlA*) and expression of a foreign gene (*pykF*), it was possible to double the growth rate of *A. baylyi* APD1 on glucose, double the molar wax ester yield from glucose while improving product purity and collection of the cells. An additional gene knockout (*poxB*) improved growth and wax ester production in the presence of acetate, a common growth inhibitor found in lignocellulosic hydrolysates. A combination of these modifications led to a strain that produced 0.45 g/l of wax esters in a medium containing glucose, amino acids from casein hydrolysate and acetate as carbon sources. In addition, a biodetoxification strain for the removal of inhibitors produced in the pretreatment of lignocellulosic biomass was produced with a single gene knockout.

While significant improvements in growth and wax ester production of *A. baylyi* ADP1 from components of lignocellulosic hydrolysates were obtained with metabolic engineering, the wax ester production needs to be further improved if this strain is going to be used in industrial applications. The strains that were produced here can be used as platform for further improvements of wax ester production by *A. baylyi* ADP1.

II

Tiivistelmä

Mikro-organismeja voidaan käyttää erilaisten kemikaalien, esim. polttoaineiden, kosmetiikkatuotteiden ja lääkeaineiden, tuottamisessa bioprosesseissa, jotka tarjoavat ympäristöystävällisemmän vaihtoehdon kemialliselle synteesille. Tällaisissa bioprosesseissa raaka-aineet (esim. lignoselluloosa) voidaan muuttaa monimutkaisiksi yhdisteiksi siten, että energiankulutus ja jätevirrat ovat minimaalisia. Bioprosesseissa tuotettujen yhdisteiden saantoa ja puhtausastetta rajoittaa käytetyn tuotto-organismin aineenvaihdunta ja kasvuominaisuudet. Yhdisteisiin, joita hyvät kasvuominaisuudet omaavilla tuotto-organismilla ei voida tällä hetkellä tuottaa tehokkaasti, lukeutuu monia teollisia käyttökohteita omaavat vahaesterit, joita tällä hetkellä tuotetaan Jojoba-nimisellä kasvilla.

Acinetobacter baylyi ADP1 on ihmiselle vaaraton maaperäbakteeri, joka tuottaa vahaesteitä, kykenee liittämään vierasperäistä DNA:a genomiinsa ja pystyy kasvamaan useilla kasviperäisillä hiilenlähteillä. Näistä syistä *A. baylyi* ADP1:stä on tullut yksi bakteerigenetiikan ja –aineenvaihdunnan malliorganismeista, minkä vuoksi on kertynyt erittäin paljon tietoa sen biologiasta. Tässä tekniikan tohtorin väitöskirjassa esitellään tulokset kokeista, joissa on muokattu *A. baylyi* ADP1:n aineenvaihduntaa tavoilla, jotka parantavat sen kasvuominaisuuksia ja vahaesterintuottoa, kun lignoselluloosaperäisiä yhdisteitä käytetään raaka-aineena.

Yhdellä geenipoistolla (*rmlA*) ja yhtä vierasgeeniä (*pykF*) ilmentämällä pystyttiin kaksinkertaistamaan *A. baylyi* ADP1:n kasvunopeus ja vahaesterisaanto glukoosilla, parantamaan tuotteen puhtausastetta ja sen keräämisen helppoutta. Yhden geenipoiston (*poxB*) lisääminen paransi kasvua ja vahaesterintuottoa asetaatin, mikä on yleinen lignoselluloosahydrolysaattien kasvuinhibiittori, läsnäollessa. Nämä muokkaukset yhdistävällä kannalla pystyttiin tuottamaan 0,45 g/l vahaestereitä kasvuliemessä, mikä sisälsi glukoosia, kaseiinihydrolysaatin aminohappoja ja asetaattia hiilenlähteinä. Tuotettiin myös erillinen kanta lignoselluloosahydrolysaatin inhibiittorien poistamista varten yhdellä geenipoistolla.

Vaikka merkittäviä parannuksia pystyttiin tekemään *A. baylyi* ADP1:n kasvuominaisuuksiin ja vahaesterientuottoon lignoselluloosahydrolysaatin komponenteista, vahaesterintuottoa pitää kuitenkin vielä parantaa, jotta tätä kantaa voitaisiin käyttää teollisissa sovelluksissa. Kehitetyt kannat luovat pohjan sille, että *A. baylyi* ADP1:stä voidaan kehittää tällainen riittävän tehokas vahaesterientuotto-organismi.

Preface

The research for the publications of this Doctor of Science thesis was carried out at the Laboratory of Chemistry and Bioengineering at Tampere University of Technology (TUT) in Finland. A one-month research period for Publication IV was carried out at the Department of Food, Environmental and Nutritional Sciences (DeFENS) at the University of Milan in Italy. The research was funded by the Jenny and Antti Wihuri Foundation, the Finnish Cultural Foundation, the Finnish Foundation for Technology Promotion, and the doctoral programme in Engineering and Natural Sciences of TUT. The City of Tampere provided financial support for printing this thesis.

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Tampere, January 2018

Matti Kannisto

Contents

List of Symbols and Abbreviations

4-HB	4-Hydroxybenzoate
5-HMF	5-Hydroxymethylfurfural
ATP	Adenosine triphosphate
CAA	Amino acids from casein hydrolysate
Cas	CRISPR-associated proteins
Cm	Chloramphenicol
CoA	Coenzyme A
CRISPR	Clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
DCW	Dry cell weight
ED	Entner-Doudoroff
EMP	Embden-Meyerhoff-Parnass
EPS	Exopolysaccharide
EtOH	Ethanol
FAEE	Fatty acid ethyl esters
GC	Gas chromatography
GDH	Glucose dehydrogenase
HPLC	High performance liquid chromatography
Km	Kanamycin
KO	Knockout
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
PEP	Phosphoenolpyruvate
PPP	Pentose phosphate pathway
PQQ	Pyrrolo-quinoline quinone
Spec	Spectinomycin
TCA	Tricarboxylic acid
TLC	Thin layer chromatography
WE	Wax ester
WS/DGAT	Wax ester synthase/acyl-CoA:diacylglycerol acyltranferase
WT	Wild type

List of Publications

I. Kannisto M, Aho T, Karp M, Santala V. 2014. Metabolic engineering of *Acinetobacter baylyi* ADP1 for improved growth on gluconate and glucose. Appl Environ Microbiol 80:7021-7027

II. Kannisto MS, Mangayil RK, Shrivastava-Bhattacharya A, Pletschke BI, Karp MT, Santala VP. 2015. Metabolic engineering of *Acinetobacter baylyi* ADP1 for removal of *Clostridium butyricum* growth inhibitors produced from lignocellulosic hydrolysates. Biotechnol Biofuels 8:198

III. Kannisto M, Efimova E, Karp M, Santala V. 2017. Growth and wax ester production of an *Acinetobacter baylyi* ADP1 mutant deficient in exopolysaccharide capsule synthesis. J Ind Microbiol Biotechnol 44:99-105

IV. Kannisto M, Efimova E, Lehtinen T, Salmela M, Fiumara T, Santala S, Karp M, Guglielmetti S, Romano D, Santala V. 2017. Metabolic engineering of *Acinetobacter baylyi* ADP1 for improved growth and wax ester production on mixtures of glucose and acetate. Submitted for publication

Author's Contribution

I. Matti Kannisto designed and carried out the experiments, interpreted the results, drafted the manuscript and is the corresponding author. Tommi Aho participated in planning of the experiment for *pykF* expression optimization and carried out all the computational work. Matti Karp and Ville Santala participated in design of the experiments and interpretation of the results. All co-authors contributed to the manuscript.

II. Matti Kannisto designed and carried out the experiments (except design of the H_2 production experiment), interpreted the results, drafted the manuscript and is the corresponding author. The H_2 production experiment was designed by Ville Santala and Rahul Mangayil and was carried out by Matti Kannisto. Rahul Mangayil carried out the anaerobic cultivations and the GC analyses. Ankita Shrivastava-Bhattacharya carried out preparation of the rice straw hydrolysate. Matti Karp, Brett Pletschke and Ville Santala participated in design of the experiments and interpretation of the results. All co-authors contributed to the manuscript.

III. Matti Kannisto designed and carried out the experiments, interpreted the results, drafted the manuscript and is the corresponding author. Elena Efimova carried out the NMR analyses. Matti Karp and Ville Santala participated in design of the experiments and interpretation of the results. All co-authors contributed to the manuscript.

IV. Matti Kannisto designed and carried out the experiments, interpreted the results, drafted the manuscript and is the corresponding author. Elena Efimova carried out the NMR analyses. Teodoro Fiumara constructed the *poxB* knockout strain under supervision of Tapio Lehtinen and Suvi Santala. Tapio Lehtinen, Milla Salmela, Suvi Santala, Matti Karp, Simone Guglielmetti, Diego Romano and Ville Santala participated in design of the experiments and interpretation of the results. All co-authors contributed to the manuscript.

The experimental work was carried out under the supervision of Professor Brett Pletschke (Publication II), Associate Professor Simone Guglielmetti (Publication IV), Doctor Diego Romano (Publication IV), Professor Emeritus Matti Karp (Publications I-IV) and Assistant Professor Ville Santala (Publications I-IV).

1 Introduction

The production of chemicals with microorganisms offers an environmentally friendly alternative to chemical synthesis. During the 20th century, bioprocesses were developed for the production of various chemicals, such as solvents like acetone and butanol, amino acids, vitamins, and antibiotics [31]. After the development of recombinant DNA technology, the range of products that can be produced in bioprocesses increased dramatically [31]. Not only did genetic engineering allow production of new products in well-studied organisms, like insulin production in *Escherichia coli* [10], it enabled improving existing host organisms and thus allowed greater yields of natural products to be achieved. Drugs produced with genetically engineered microorganisms can be produced more reliably than those extracted from natural sources [143]. For example, a precursor to the malaria drug, artemisinin, can be produced with genetically engineered yeast, which is expected to even out fluctuations in the availability of the drug [143]. Although high-value products, such as drugs, can be produced in an economically feasible way using refined chemicals as a raw material, bulk chemicals need to be produced from less expensive raw materials. One such raw material is lignocellulosic biomass which includes wood and the inedible parts of food crops. The utilization of lignocellulosic biomass requires that its components are liberated in pretreatments, which also release compounds that can be inhibitory to the bioprocesses.

Wax esters (WE) are lipids that can be used in many applications including cosmetics, biofuel production and in medical products [158]. WEs are currently produced from the Jojoba plant, which grows primarily in deserts [158]. If WEs could be produced efficiently with microorganisms, these lipids could be produced anywhere from inexpensive raw materials such as lignocellulose. *Acinetobacter baylyi* ADP1 (ADP1) is a soil bacterium that naturally accumulates WEs for energy and carbon storage [56]. It grows on various components of lignocellulosic hydrolysates and has a metabolism that is easy to engineer [204]. Currently, the WE yields obtained with ADP1 are not sufficiently high for economically feasible production of these storage lipids. In this thesis, it

was attempted to improve the growth and WE production of ADP1 using various components of lignocellulosic hydrolysates as a carbon source by means of metabolic engineering.

In the Background section of this thesis, the composition and utilization of lignocellulosic biomass, the use of metabolic engineering to improve host organisms for bioprocesses and the biology of ADP1 are explained. Several excellent review articles have recently been published about lignocellulosic hydrolysates [84], [85] metabolic engineering [93], [103], [131], [148], [203] and metabolism and genetics of ADP1 [29], [45], [204], and so they are covered here only briefly. The parts of the metabolism of ADP1 studied in the Publications I-IV are discussed more thoroughly. The metabolism of ADP1 has some peculiar aspects and it was attempted in the experiments to engineer those metabolic steps to improve growth and WE production. While many aspects of the metabolism of ADP1 have been studied extensively, there is no information available about certain parts of its metabolism. Those aspects of its metabolism which are relevant to this thesis are discussed with respect to the metabolism of *E. coli*, which is a fairly close relative of ADP1. Since *E. coli* is probably the most studied model bacterium, the differences between ADP1 and *E. coli* are also discussed. The hypotheses on which the experiments are based are described in the Research Objectives and Questions section. Materials and Methods briefly explains how the metabolic engineering and the experiments to test the engineered strains were performed. In the Results and Discussion section, the results of the experiments carried out for Publications I – IV are discussed and compared with the growth characteristics and WE yields of other microorganisms. In the Conclusions section, the overall success of the attempt to engineer the metabolism of ADP1 for improved growth and WE production is discussed. This section also includes an evaluation of whether the work carried out here has taken ADP1 closer to being a realistic choice as a host organism in bioprocesses where components of lignocellulosic hydrolysates are converted to WEs. Finally, suggestions for further improvements that need to be carried out in order to engineer ADP1 into an efficient WE producer are given.

2 Background

2.1 Lignocellulosic hydrolysates

Sugars and starch have been used in production of the first-generation biofuels, but since these raw materials could be also used as human food, and as a feedstock for animals, lignocellulosic biomass, which consists of the inedible parts of plants, has been considered as a better alternative [64]. Lignocellulosic biomass is also less expensive than starch, and can be cultivated with less use of fertilizers and on different land areas than food crops [64]. The composition of lignocellulosic biomass varies greatly depending on the plant it is produced from [21]. In general, it can be said that it is preferable that the biomass should have a small and easily degradable lignin content. The composition of the plant from which the lignocellulosic biomass is derived can also be subjected to modification by means of genetic engineering [149]. The main sugar constituents of lignocellulosic biomass (Figure 1) are glucose and xylose, while arabinose, galactose and mannose are also present in smaller quantities.

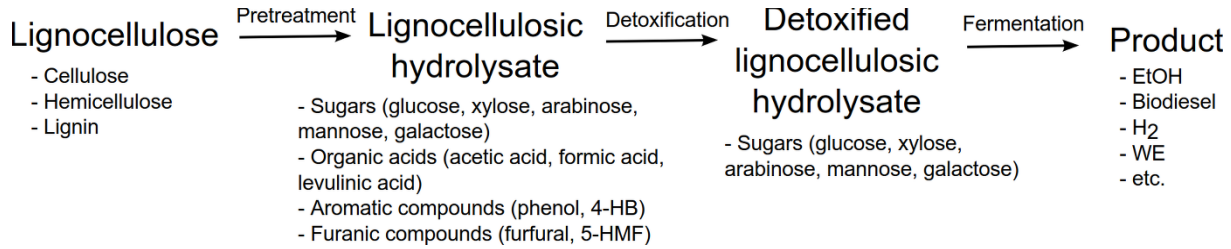

FIGURE 1 A schematic presentation of lignocellulose utilization in bioprocesses. Examples of the products and lignocellulose components (in parentheses**)** are given below each phase.

Lignocellulosic biomass has to be pretreated prior to fermentation in order to release the fermentable sugars [68]. The pretreatment methods can be divided into acid-based methods, mild alkaline methods, oxidative methods, hydrothermal processing, chemical pulping processes, and

the use of alternative solvents [85]. These methods aim at hydrolysing or solubilizing hemicellulose or removing lignin. Acid-based methods include a promising method, acid hydrolysis. In this process, the lignocellulosic biomass is treated with mineral acids, organic acids or sulfur dioxide. In hydrothermal processing, liquid water or water vapor at high pressure are used. Alkaline pretreatments with mild alkali are less efficient but also produce less inhibitors than acid pretreatment or hydrothermal processing. The oxidative methods aim at using oxidants to separate the cellulose and hemicellulose from the lignin and to decrystallize the cellulose. In chemical pulping, cellulose can be separated from hemicellulose and lignin, which are converted to liquors that can be used for energy production or fermentation by, for example,e *S. cerevisiae*. In an alternative solvent pretreatment, the lignocellulose components can be separated by disrupting their non-covalent interactions without significant inhibitor formation. However, the collection of hemicellulose and lignin fractions must be improved before this method can be used industrially.

The hydrolysis of the lignocellulosic biomass yields inhibitors in varying amounts and depends on the source of the biomass and the pretreatment methods [179]. Different inhibitors are derived from different lignocellulose components [85]. Hexose sugars can be degraded to 5-HMF and pentose sugars to furfural. 5-HMF and furfural can be further degraded into levulinate and formic acid, respectively. The aromatic inhibitors like 4-HB are derived from lignin, while acetate is derived from hemicellulose. The presence of the inhibitors can usually be considered harmful to the bioprocesses [84], but they can also be beneficial. For example, 3.3 g/l of a protonated form of acetate was shown to increase the ethanol (EtOH) yield of *S. cerevisiae* by 20 % [180]. However, when the protonated form of acetic acid was higher than 5 g/l the growth of *S. cerevisiae* was inhibited [180]. When cultivated on xylose the acetate tolerance of *S. cerevisiae* has been shown to be lower than when cultivated on glucose, which was considered to be due to the lower fermentation rate of xylose [66]. The presence of acetate in the growth medium increases toxicity of furfural to *S. cerevisiae* [138] and *E. coli* [206]. Also, acetate appears to be more toxic to *E. coli* growing on glucose in a minimal medium [126] than in a rich medium [102]. Thus it can be said that, while acetate is not very inhibitory in itself, it might be worthwhile to remove it from the lignocellulosic hydrolysates with techniques like biodetoxification (see below), if inhibitors like furfural are also present. Alternatively, tolerance of the host organism towards this organic acid might be improved by adaptation or metabolic engineering.

The presence of lignocellulosic inhibitors and the sequential use of the sugars have been considered as one of the biggest problems in the production of second generation biofuels [191]. Most of the experiments carried out for this thesis were done using individual components of the lignocellulosic hydrolysates, or a combination of these components, as carbon source. While experiments carried out with actual lignocellulosic hydrolysates would have unarguably produced data more relevant to real-life applications, using individual components in a synthetic medium produces results that can be generalized more easily. One experiment was, however, carried out

with enzymatically hydrolysated rice straw (Publication II), in order to show that the genetic modifications work with actual lignocellulosic hydrolysates. Glucose was used in all publications of this thesis because it is often the most abundant carbon source found in lignocellulosic hydrolysates. Although not the most severe inhibitor of lignocellulosic hydrolysates, 4-hydroxybenzoate (4-HB) was used as a model compound of aromatic inhibitors due to its abundance in many lignocellulosic hydrolysates [139].

The host organism using lignocellulosic hydrolysates should be capable of catabolizing all the carbon sources present in this raw material in order to maximize the product yield. This is especially important in bioprocesses where products like biofuels, which are of lower value, are produced. If the host organism is capable of catabolizing multiple carbon sources present in the raw material, it would be of advantage if these would be consumed simultaneously because this shortens the bioprocess running time [94]. However, in their natural environment, microorganisms often benefit from sequential usage of the carbon sources. This allows them to consume the best carbon source first, so that the competing microorganisms do not consume it before them. The carbon catabolite repression of xylose and arabinose consumption by *E. coli* has been particularly well characterized [32]. In the presence of glucose, arabinose and xylose, *E. coli* consumes first glucose, then arabinose and only then xylose [32]. In this example, glucose prevents the catabolism of both arabinose and xylose, and arabinose prevents the catabolism of xylose. When catabolite repression occurs in this way in multiple layers, the metabolic engineering of a strain that consumes the sugars simultaneously becomes more difficult. An alternative to engineering the metabolism of the host organism for simultaneous consumption of all carbon sources is to engineer different strains of the host organism for consumption of only one carbon source, and then use them in a mixed culture. This approach has been shown to be successful in the consumption of glucose, arabinose and xylose simultaneously with three different strains of *E. coli* C [201]. The simultaneous use of glucose and xylose, which would have otherwise been consumed sequentially, has been achieved by encapsulating *S. cerevisiae* [191]. The simultaneous utilization of the carbon sources was caused by a gradient of glucose in the encapsulated cell mass, and the low glucose concentration in the middle of the cell mass allowed xylose consumption [191].

2.1.1 Biodetoxification of lignocellulosic hydrolysates

There are a large variety of techniques for removing the inhibitors produced in hydrolysis of lignocellulosic biomass. These techniques differ greatly in their abilities to remove the inhibitors without decreasing the sugar content of the hydrolysate, their physical characteristics, and in their cost. These techniques can be divided into physical, chemical and biological methods [84]. Biodetoxification, which is one of the biological methods, is generally slower than the physical or chemical methods but requires less energy and does not produce so much waste water [35]. Another advantage of using biodetoxification is that, unlike with physico-chemical methods, the carbon of the inhibitory molecules is not lost in the process but can be used in the synthesis of

other valuable molecules as a side-stream of the actual fermentation where the detoxified lignocellulosic hydrolysate is used as raw material. As biodetoxification competes with other, more robust detoxification methods, it might be necessary to produce these valuable compounds with the biodetoxification strain in order to make biodetoxification economically feasible. If the biodetoxification organism could also perform other functions, such as the production of cellulases or the removal of oxygen for anaerobic host organisms, this detoxification method might become a more attractive alternative.

Biodetoxification can be carried out by isolating microorganisms capable of using the lignocellulosic inhibitors as a carbon source but which do not consume the lignocellulosic sugars [195], or engineering such organisms from model organisms by knocking out the genes for sugar catabolism [100]. The tolerance of the host organism towards the inhibitors can also be achieved by engineering the metabolic steps involved in inhibitor tolerance [194]. Many organisms in nature are capable of growing on lignocellulosic inhibitors [58], [76], [196] and the genes for these functions can be cloned from them. ADP1 can grow on many organic acids but cannot grow on any other sugar than glucose as a sole carbon source. This creates an opportunity to convert ADP1 into a detoxification strain that consumes only the organic acids, which can be growth-inhibitory to the bioprocess host microorganisms.

2.2 Growth rate

Bioprocesses benefit from a high growth rate in the host organism as long as the increased growth rate does not decrease product formation, as this reduces the time needed to run the bioprocess. This applies especially when lower value chemicals are produced. Thus, if other parameters remain unchanged, an increase in the growth rate of the host organism is a goal worth striving for. Bacterial growth can be divided into a lag phase, an acceleration phase, an exponential phase, a retardation phase, a stationary phase, and a phase of decline [122]. The most important phase with respect to WE production by *A. baylyi* is the stationary phase (when growth is not limited by carbon) as WEs are synthesized most efficiently then [56]. Thus, in order to increase the WE productivity of ADP1, shortening the lag and exponential growth phases can be considered important. The lag phase has traditionally been considered to be due to the adaptation of the metabolism of all the cells in the population to the presence of the carbon source by synthesis of catabolic enzymes [122]. More recently, it has become evident that the lag phase is also affected by the fact that only a part of the population of the cells present initially in the growth medium start to grow, while the rest of the cells do not grow at all during the cultivation. This has been shown to occur with *E. coli* K12 BW25113 after the carbon source is changed from glycolytic to gluconeogenic [98]. The proportion of the cells starting to grow on a gluconeogenic carbon source depends on the gluconeogenic flux after the change in the carbon source and could be affected by increasing the uptake of the gluconeogenic substrate [98]. If this is the

case, the lag phase could be reduced through metabolic engineering by increasing the carbon source uptake rate. When ADP1 is cultivated on glucose as a sole carbon source, a significant lag phase is caused by the fact that ADP1 has to convert glucose to gluconate prior to further catabolism [188]. This lag phase could be shortened by increasing the activity or availability of glucose dehydrogenase (GDH), which is responsible for this oxidation reaction [188] or by increasing the amount of the inoculate (see Figure 4 in Publication I and Figure S5 in Publication III). However, it can be considered that the exponential growth phase is the most important determinant of the total cultivation time in bioprocesses using ADP1 in WE production since it cannot be increased by increasing the amount of inoculate. The exponential growth rate (μ) can be calculated as follows:

$$\mu = \frac{lnX_2 - lnX_1}{t_2 - t_1}$$

where X_1 is the number of cells at timepoint t_1 and X_2 the number of cells at timepoint t_2. The exponential growth phase is the only phase of growth that can be reproducibly quantified [167]. Since the metabolic state of the bacterial cell, and thus the amount of the product formed from the substrate, is highly dependent on the growth state of the cell, it has been considered highly important to report exponential growth rates in the research articles [127]. This is especially true for experiments measuring WE production by ADP1, since it produces WEs at different rates in the exponential growth phase and in the stationary phase [56] and a small difference in the stage of growth might have dramatic effects on the WE yields. Because of this, the experiments presented in this thesis that involve comparison of WE production by metabolically engineered strains with wild type (WT) ADP1 have been carried out in the exponential growth phase and the samples have been taken at time points where similar amounts of the substrate have been consumed.

The growth rate of a bacterium depends on the carbon source, and some carbon sources allow faster growth than others [111]. A generalization has been made that the metabolism of ADP1 is geared towards the catabolism of carbon sources that enter its metabolism as intermediates of the tricarboxylic acid (TCA) cycle, but it works less well on carbon sources that are processed via glycolysis [12], [204]. This is in contrast to *E. coli,* which grows faster on carbon sources that are processed via glycolysis than carbon sources that enter directly from the TCA cycle [7]. These characteristics can be influenced by means of metabolic engineering. The growth rate of *E. coli* on pyruvate and succinate has been improved by increasing the expression levels of phosphoenolpyruvate (PEP) synthase (*pps*) and PEP carboxykinase (*pck*) genes, respectively [23]. The authors concluded that at least in the improvement of growth rate on pyruvate by overexpression of *pps*, the improved growth has to be due to the enhanced synthesis of key biosynthetic metabolites, rather than improved energy production [23]. Another example is the improved growth rate of *Gluconobacter oxydans* on glucose after knocking out the genes responsible for the formation of gluconate from glucose [99]. This improvement, however, resulted in acetate

formation [99]. The growth rate of ADP1 on glucose has already been improved by supplementing the cells with extra pyrrolo-quinoline quinone (PQQ) (see Sugar metabolism) [188].

2.3 Biomass yield

The amount of bacterial cells in a growth medium can be determined by estimating the number of the cells or measuring the dry weight of the bacterial biomass [122]. However, it has been known for a century that the size and shape of a bacterial cell may change during different phases of growth [67] and thus the measured biomass values do not necessarily reflect the number of cells present in the cultivation. Since the titer and purity of the intracellularly accumulating WEs depend on the final biomass obtained, not on the number of the cells, determination of the bacterial numbers in the cultivations is not discussed here.

In order to be economically feasible, bioprocesses usually have to allow the cells to grow to a high cell density [155]. This is especially true in the case of products that have lower economic value [192], but bioprocesses where more valuable products are produced also benefit if the cells grow to a high density [174]. This is because the increased cell density allows the use of smaller fermentors and downstream processing equipment like centrifuges, and less materials such as purified water [174]. The density of bacterial cells in a growth medium is limited by the fact that at a certain point the medium loses its fluidity [104]. The viscosity of *E. coli* B increases rapidly when cell densities above 200 g/l are reached, and the medium loses fluidity at 220 g/l [124]. Thus, it can be said that the maximum cell density that can be obtained in bioreactors with this bacterium is around 220 g/l [124]. Often, however, other problems start to limit the growth of bacteria before this upper limit has been achieved. Some nutrient might start to limit the growth, or the production of inhibiting metabolites might be triggered [174]. Many nutrients inhibit growth at high concentrations, which is why batch cultivations are not for the best approach to achieving high cell density. A major problem encountered when growing microorganisms to high cell density is the accumulation of growth-inhibiting side products, for example acetate with *E. coli* [174]. This problem can be prevented by modifying the growth conditions (*e.g.* by limiting the amount of the carbon source in the growth medium) or by metabolic engineering [174].

Some bacteria secrete signalling molecules that, when accumulated to a threshold concentration, induce a physiological change in the cell population. This phenomenon, which is often observed at elevated cell densities, is referred to as quorum sensing [110]. Since WE-producing bioprocesses using ADP1 as a host organism should preferably produce high cell density, the possible effects of quorum sensing on the metabolism of ADP1 might become relevant. ADP1 has been shown to produce four quorum-sensing molecules in a minimal medium, produced mainly in the stationary phase [63]. It has been suggested that quorum sensing controls the efficient utilization of extracellular molecules produced by the bacteria, and that the cell density does not affect how

beneficial it is to produce intracellular molecules [28]. Quorum sensing has also been thought to improve stress tolerance in high cell density cultivations [192]. However, since the functions of these molecules are unknown, it cannot be speculated how they might affect WE production or the biomass accumulation by ADP1.

2.4 Metabolic engineering

Since 1991, the sub-discipline of genetic engineering that involves the manipulation of metabolic pathways has been referred to as metabolic engineering [11]. Systems metabolic engineering uses the methods of systems biology and 'omics' technologies to achieve more complete control over metabolism [103]. The Meta-Council on Emerging Technologies of the World Economic Forum acknowledged systems metabolic engineering as one of the top 10 emerging technologies of 2016 [198]. As an environmentally friendly alternative to fossil fuels, metabolically engineered organisms are used in biofuel production from renewable sources [148]. However, as noted in the report [198], production by metabolically engineered organisms might be more suitable than chemical synthesis from petrochemicals when the products are complex organic molecules. One group of complex organic compounds that might more feasibly be produced with metabolically engineered organisms, rather than with synthetic chemistry, are active pharmaceutical ingredients, which have been divided by Keasling into three categories: alkaloids, polyketides and isoprenoids [93]. Such compounds are currently extracted from natural sources because their chemical synthesis would be very complicated and expensive. Furthermore, unlike in chemical synthesis where waste material is produced, bioprocesses using metabolically engineered microorganisms can use waste materials from other industries as a raw material. These waste materials include lignocellulosic biomass from, for example, agricultural industries. However, metabolically engineered organisms are not suitable for the production of chemicals that are toxic to the host organism, and the metabolism of the host organisms limits the range of chemicals that can be produced [93].

Metabolic engineering is often used to optimize the flow of carbon from the growth substrates to the product formation. This means that the optimal bioprocess host organism has a metabolism that converts the raw material as fast as possible, with as high a yield as possible, to as high a titer as possible. Product yield and purity can be improved by eliminating pathways that result in the formation of side-products, such as acetate with *E. coli* [140] or exopolysaccharides (EPS) with ADP1 (see Results and Discussion). On the other hand, the addition of anabolic steps or complete pathways allows the formation of products that the organism does not naturally produce, and the incorporation of catabolic pathways allows broadening of the carbon source range used. The addition of anabolic pathways can be used to tailor the product to better meet the requirements of a given application, or for the production of compounds that do not occur naturally in the microorganisms. The addition of catabolic pathways can be used to allow more complete

utilization of complex raw materials such as lignocellulosic hydrolysates. Metabolic engineering can also be used in improving the tolerance of the host organism towards toxic products, such as solvents.

Although a lot of knowledge has been gained about engineering the metabolism of microorganisms over recent decades, not much about the engineering principles has been drawn from this research. As Yadav et al. have noted, most research has merely produced demonstrations of how the technology could be applied [203]. A lot of research has concentrated on engineering the central carbon metabolism, which refers to the uptake and oxidation of the metabolites and includes pathways such as glycolysis, gluconeogenesis, the pentose phosphate pathway (PPP), and the TCA cycle [142]. These pathways produce precursors for the anabolic pathways involved in the synthesis of most of the products produced in bioprocesses. It is generally accepted that all of the metabolites produced by microorganisms can be formed from 12 precursor metabolites [132]. Since these precursors are part of multiple metabolic pathways, modifications that affect one pathway inevitably also affect other pathways [129]. Thus, by modifying these pathways, it is possible to optimize the capability of the host organism to produce the wanted product. All of the metabolic engineering carried out in this thesis involves the central carbon metabolism by affecting glycolysis (Publication II), PEP-pyruvate-oxaloacetate node (Publication I), acetate production (Publication IV), or gluconeogenesis (Publication III). The modifications carried out in Publications I, III and IV aim to maximize the amount of the WE synthesis precursor, acetyl-coenzyme A (acetyl-CoA), by modifying the central carbon metabolism of ADP1, while the modification carried out in Publication II completely eliminates the sugar metabolism.

The PEP-pyruvate-oxaloacetate node of *E. coli* has been much studied [165] and engineered [107], but the PEP-pyruvate-oxaloacetate node of ADP1 has been studied only by Elbahloul & Steinbüchel, who overexpressed its gene for PEP carboxylase, although this did not affect the growth rate on gluconate [43]. As noted by Nielsen & Keasling, once the central carbon metabolism has been optimized for production of a precursor metabolite for some product, this strain can be easily engineered to produce other metabolites using the same precursor [131]. Thus, the metabolic engineering work carried out for this thesis, that is engineering central carbon metabolism of ADP1 for WE synthesis, might also be of use when other metabolites are produced from the precursor molecule acetyl-CoA. Engineering the central carbon metabolism might also be used to improve the growth characteristics of the host organism, which is especially important when producing WEs with ADP1, since this bacterium produces WEs mainly in the lag phase and the final WE titer is limited by the dry cell weight (DCW) obtained (see Wax ester synthesis). The growth rate can be improved by, for example, adding new metabolic steps, as is shown in this thesis (Publication I), or by affecting the transcription factors that control the metabolic fluxes [130]. The presence of a plasmid burdens the cells [170] and thus higher product formation might be obtained by integrating heterologous genes to the host chromosome [203]. In this respect, ADP1 is an ideal host organism for metabolic engineering, since it readily incorporates foreign

DNA into its genome (see Natural transformation). Computational models for the metabolism of ADP1 have also been developed [39], which further makes this bacterium an attractive host organism for metabolic engineering.

The host organism for metabolic engineering should have a suitable metabolism and physiology for production of the product molecule, and there should also be tools available for engineering the genome of the organism [93]. For example, the host organism used in this thesis, ADP1, is particularly suitable as a chassis in synthetic biology, as has been discussed by Santala [164]. ADP1 has a broad growth substrate range, is easy to cultivate and naturally produces large amounts of EPSs and WEs. Furthermore, ADP1 is naturally transformable, which makes genetic engineering of this organism very easy and not very time-consuming. Most biotechnological applications using ADP1 as a host organism harness it either as a host organism in bioprocesses or as a whole cell biosensor (see Biotechnological applications). When developing an efficient host organism in bioprocesses with metabolic engineering, it is important to be able to measure consumption of the raw materials and formation of the product (and possibly the side products). Traditionally, the concentrations of these metabolites have been measured with the methods of analytical chemistry, but more recently it has become possible to assess the efficiency of the host organisms using biosensors [123]. Biosensors can be used to measure concentrations of metabolic intermediates like long-chain aldehydes by producing light as an output [106]. This allows real-time measurement of the intracellular metabolite, which is infeasible with the methods of analytical chemistry. The analytical chemistry methods are often more laborious and time-consuming than methods based on biosensors, and biosensors also tend to have higher throughput.

Synthetic biology is the most advanced form of genetic engineering and differs from the other sub-disciplines mainly due to the high level of standardization, which makes it much easier to design and build new genetic constructs [46]. Synthetic biology is considered to be able to produce solutions to the problems encountered in bioprocesses using more recalcitrant raw materials [59]. Synthetic biology and metabolic engineering partially overlap, but while metabolic engineering is involved mostly in improving existing metabolic pathways or adding few metabolic steps or pathways to the host organism, synthetic biology allows the construction of completely new pathways in microorganisms devoted to the formation of the product [131]. Furthermore, the production of efficient host organisms for bioprocesses benefits from both disciplines, since the novel metabolic pathways constructed with synthetic biology can be optimized with metabolic engineering [131]. In addition, the central carbon metabolism might not be geared towards synthesis of a novel compound whose production is possible with synthetic biology, and metabolic engineering can be used to fix this [131]. Synthetic biology has experienced rapid progress in the last few decades and is currently moving towards increasingly complex applications, such as *E. coli* capable of reacting to light with different wavelengths [51], and to higher organisms [144]. Synthetic biology has also allowed the engineering of metabolic pathways for opioid production

in yeast [61] which has raised, in addition to hopes, concerns about easier access to the drug by the public [40]. Traditionally, genetic engineering has been performed using multiple cloning sites containing restriction digestion sites for the numerous restriction endonucleases. In synthetic biology, construction of the genetic constructs is standardized, so that only a limited number of restriction enzymes are used, which greatly simplifies the design of the constructs. Also, the need to eliminate restriction enzyme recognition sites from the inserts is reduced by the fact that not so many enzymes are used in the construction. Synthetic biology allows automatization of the engineering of microorganisms, which makes producing applications more economically feasible [65].

Some prokaryotic species possess an adaptive immune system called clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins (Cas), which afford them protection against foreign DNA from viruses, for instance [25]. The immunological memory is stored in CRISPR arrays as spacers (~25-40 bp) which are located between the palindromic repeats (~21-40 bp). CRISPR/Cas systems-mediated immunity starts with an adaptation phase during which the foreign DNA is sampled by Cas proteins and inserted into a CRISPR array as a new spacer. In the expression phase, the whole CRISPR array is transcribed into precursor CRISPR RNA (pre-crRNA), which is then processed by Cas proteins and ribonucleases into crRNAs, which consist of a spacer and a single repeat. Effector CRISPR ribonucleoprotein complexes (crRNP) are then formed, in which the crRNAs are then bound by Cas proteins and other CRISPR/Cas components. In the interference phase, the cRNP complexes use the crRNAs to identify the target DNA, which will be degraded.

In addition to being a breakthrough discovery in microbiology, CRISPR/Cas systems have also been harnessed in genetic engineering [25]. CRISPR/Cas systems can be used to accurately modify and study the genomes of prokaryotic and eukaryotic species more cheaply and more quickly than be done with traditional methods. While most research on the applications of CRISPR/Cas has been carried out with eukaryotes, some applications on prokaryotic organisms have also been developed. The existence of numerous efficient tools for genetic engineering in many prokaryotes might be the reason for the relatively greater interest in applying CRISPR/Cas on eukaryotes. CRISPR/Cas, however, allows prokaryotes to be engineered even if there are no efficient genetic engineering tools available. Most bacteria cannot repair the double-stranded breaks generated by CRISPR/Cas by non-homologous end joining, so they require a template for homology-directed repair. This template can be used to generate desired modifications to the genome. While the genome of APD1 can be engineered with great ease and there are good tools for this, the KOs carried out in Publications II-IV were produced with a KO cassette containing an antibiotic resistance selection marker. Since there are only a limited number of such selection markers for any given host organism, the generation of a large number of gene KOs would require the use of markerless deletion of the genes, which could be achieved with, for example,

CRISPR/Cas techniques. This would also allow more sophisticated integration of foreign genes into the genome of ADP1.

2.5 *Acinetobacter baylyi* ADP1

Research on *A. baylyi* ADP1 (Table 1) began in 1960 when Taylor & Juni isolated its parental strain, BD4, from soil by using meso-2,3-butanediol as a sole carbon source in a minimal medium [181]. The strain was characterized as a Gram-negative aerobe, which grows in liquid media either alone or as pairs or short chains of rods which are heavily encapsulated by EPSs [181]. BD4 was characterized as non-motile [181] but later research has shown that the strain ADP1 can move on surfaces by twitching, and that it is inhibited by exposure to blue light [15]. In subsequent research, the central metabolism [182] and EPS capsule synthesis [183], [81] were studied. Juni & Janik used BD4 in an experiment to elucidate the intermediates of the EPS capsule synthesis, where unencapsulated mutants, which had different EPS capsule synthesis genes disrupted by mutations, were grown together [82]. The authors noticed that this resulted in the formation of stably-encapsulated strains which was shown to be due to the genetic transformation of the intact gene from one mutant strain into another, which had a different EPS capsule synthesis gene disrupted than the donor strain. In this study BD4 was subjected to UV mutagenesis and a strain was isolated, along with many unencapsulated strains, that produced a small EPS capsule. This strain was referred to as BD413, and is currently known as ADP1. This strain has been referred to as *A. calcoaceticus* (see for example [91]) but has later been shown to belong to the species *A. baylyi* [189]. Another less-frequently used name for the strain is *A. calcoaceticus* LMD 82.3 [188]. The genetic difference between BD4 and ADP1 has not been elucidated, and phenotypic differences between the strains have been studied only by comparing their EPS production [91]. The main difference between the strains in this respect is that, while most of the EPS produced by BD4 remain on the cell surface as a large capsule, ADP1 excretes half of the EPS it produces to the growth medium and also produces less EPS than the parental strain BD4 [91]. This difference causes ADP1 to form much denser pellets in centrifugation, which makes it easier to study this bacterium [80]. Since the initial studies by Juni & Janik [82], ADP1 has become an important model organism for transformation [204] and metabolism studies [204], [29], while most of the studies about the EPS capsule have been carried out with the parental strain BD4 [17], [91], [90], [92], [135]. While most of the early research with ADP1 focused on natural transformation, more recently its versatile metabolism has also attracted a lot of attention. In particular, the catabolism of aromatic compounds, and the complex regulatory networks governing these pathways, have been studied extensively [204]. These studies have elucidated sophisticated mechanisms of gene expression and biochemistry of aromatic compounds [16]. However, glycolysis, pyruvate metabolism and EPS metabolism, the metabolic pathways studied in

this thesis, have not been studied so extensively with ADP1. The development of metabolic engineering and synthetic biology have enabled the engineering of ADP1 as a host organism for various biotechnological applications [45]. These applications include production of valuable chemicals [43] and biosensing [106].

TABLE 1 Taxonomy of *A. baylyi* ADP1.

Domain	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Pseudomonadales
Family	Moraxellaceae
Genus	Acinetobacter
Species	Baylyi
Strain	ADP1

2.5.1 Genetics

The genome of ADP1 is relatively small (3 598 621 bp and 3325 predicted coding sequences) and has a G+C content of 40.3 % [12]. The single-gene KO mutant study by de Berardinis et al. indicated that 499 of these 3325 genes might be essential [30]. Approximately one fifth of the genome has been estimated to code for genes involved in catabolism, and these genes are arranged in five clusters referred to as "islands of catabolic diversity" [12]. ADP1, however, lacks genes for the following enzymes that are part of the central metabolism in most organisms: glucokinase (and hexokinase and a glucose transporter phosphotransferase system), 6-phosphofructokinase, pyruvate kinase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconolactonase [12]. The lack of, for example, a pyruvate kinase gene is also shared with closely related *A. baumannii* strains [185]. ADP1, however, does have genes for the catabolism of glucose via the Entner-Doudoroff (ED) pathway [12]. The lack of several genes for enzymes of Embden-Meyerhoff-Parnass (EMP) glycolysis complicates metabolic engineering efforts to allow the ADP1 to catabolise sugars other than glucose. For example, the phosphorylative catabolism of xylose or arabinose results in the production of fructose-6P and glyceraldehyde-3P, both of which would need to be processed via EMP glycolysis in order to be completely oxidized.

The genome of ADP1 contains two prophages of 53 kb and 9 kb [12]. The 9-kb prophage can be activated in cultivations of ADP1 and its emergence reduces the growth and natural transformation capability of ADP1 [153]. This phage uses the natural transformation machinery of ADP1 to infect the cells and therefore cannot infect cells whose natural-transformation genes have been knocked out [153]. The authors suggested that the reduced transformability was due to the selection of phage-resistant cells [153]. In the laboratory evolution experiment by Renda et al. 49 kb of the larger prophage region was lost in all sequenced clones under conditions allowing adaptive evolution, but none was lost in clones of the mutation accumulation cultivations [154].

This suggests that deletion of this region from the genome of ADP1 increases the fitness of the strain [154]. Suárez et al. have removed all six transposon IS*1236* sequences from the genome of ADP1 and found out that this strain accumulated mutations that inactivated the reporter gene 7 to 21 times less often [178]. This strain also had an improved ability for natural transformation, underwent autolysis to a smaller degree, had a shorter lag phase and grew to a higher optical density [178]. As the authors of the above-mentioned study suggested [178], this strain is much more suitable as a host organism in metabolic engineering than the WT ADP1.

2.5.1.1 Natural transformation

Bacteria can acquire novel genes in a process called natural transformation [77]. In natural transformation the bacterium actively takes up and integrates foreign DNA as a part of its physiology [77]. Thus, natural transformation differs from the other mechanisms of lateral gene transfer, conjugation and transduction, in the sense that the transfer of DNA is initiated by the recipient cell, not extrachromosomal genetic elements of the donor cell [77]. The ability of ADP1 to undergo natural transformation was first described by Juni & Janik [82] and has since then been studied intensively [9], [113]. The exceptional ability of ADP1 to undergo natural transformation is exemplified by the experiment of Palmen et al. where 25 % of a bacterial population was transformed with plasmid DNA [137]. The competence of ADP1 to undergo natural transformation has been shown to decrease in laboratory evolution experiments [9], [154]. Bacher et al. suggested that this might be due to the fact that the mutant alleles that do not allow the cells to be competent for transformation can be transferred to the competent cells, but the alleles allowing competence for transformation cannot be transferred to the noncompetent cells [9]. However, Renda et al. have suggested that the activation of the prophage CRAϕ, which resides in the genome of ADP1, could drive the evolution of diminished transformability, since non-transformable mutants of ADP1 are completely resistant to this phage [153]. McLeman et al. have tested whether an ADP1 strain that is incapable of recombination of foreign DNA into its chromosome would have decreased evolvability to resistance to different phages. They found out that there was no difference between the recombigenic WT strain and the non-recombigenic *drpA* mutant strain [113].

The competence of ADP1 for natural transformation is induced at the beginning of the exponential growth phase and lasts until the early stationary phase [137]. The competence induction does not depend on the carbon source used, nor on whether a minimal or rich medium is used, but the medium must contain divalent cations (Mg^{2+}, Mn^{2+}, Ca^{2+}) in order for transformation to occur [137]. Competence induction is also not affected by the acidity of the growth medium, although the transformation frequency is lower in an acidic (pH below 6.5) medium [137]. Transformation frequency is also dependent on the DNA homology, the genomic location of the insertion [150] and the type of DNA and selection marker used [137]. Plasmid DNA that contains an insertion cassette can integrate into the chromosome of ADP1 by replacement recombination, where the cassette replaces the DNA sequence corresponding to the flanking regions used in the cassette,

or by integration of the whole plasmid into the chromosome [137]. While the former has been shown to occur much more frequently [137], the KO cassette amplified with PCR was used in transformations carried out for the publications of this thesis in order to eliminate the possibility of obtaining the latter type of transformants. Transformation frequency increases with increasing concentrations of DNA up to a limit, after which a maximum frequency is reached [137]. Increasing the cultivation time in the presence of the transforming DNA also increases the transformation frequency, but a plateau at which the transformation frequency stops increasing is reached after a few hours of cultivation [137]. Culture supernatants of ADP1 contain extracellular DNA, but it has been shown that this DNA is not actively excreted but is liberated by lysis of the cells [136]. Bacher et al. have shown that ADP1 and its non-competent *com* mutant grow slower in the presence of extracellular DNA [9].

2.5.2 Metabolism

ADP1 is known as a nutritionally versatile bacterium and it can catabolize a wide variety of plant-derived carbon sources, such as aliphatic and aromatic acids [204], [53], a trait found also in other *A. baylyi* strains [52]. Utilization of glucose as a source of carbon and energy is relatively rare among *Acinetobacter* strains [83]. Glucose is metabolized via modified ED glycolysis [48]. ED glycolysis produces one molecule of adenosine triphosphate (ATP) per glucose catabolized, while EMP glycolysis produces two molecules of ATP per catabolized glucose [57]. In ADP1, no ATP is produced due to lack of pyruvate kinase (see Sugar metabolism). ED glycolysis, however, requires less protein and might thus be a more optimal glycolytic strategy for aerobic organisms like ADP1, *i.e.* those that do not live under energy-deprived conditions [57]. ED glycolysis is thermodynamically more favourable than EMP glycolysis, if the total change in the Gibbs energies of all the reactions are compared [57].

2.5.2.1 Sugar metabolism

Glucose is the only sugar that is known to be able to support the growth of ADP1 as a sole carbon source [181]. Other sugars present in cellulosic biomass, such as xylose and galactose, can be oxidized partially to their corresponding lactones by GDH, which then hydrolyze to sugar acids at an elevated pH [187]. This process provides the bacterium with energy and allows the cells to grow to higher biomass in cultivations that are limited by carbon [187]. Enzymatic activities for glucose and gluconate oxidation can only be detected in cells grown on glucose, and not in cells grown on acetate or EtOH [182]. However, when acetate-grown cells are transferred to a medium containing glucose, they are able to produce gluconate but do not further catabolize it [188]. The data presented in Publication IV also indicate that the presence of 2.7 g/l of acetate, but not 1.1 g/l of EtOH, represses glucose catabolism. Thus, it seems that the genes for the production of gluconate from glucose are constitutively expressed, but the genes for gluconate catabolism are only expressed when glucose, but not acetate, is present in the growth medium.

The catabolism of glucose (Figure 2) begins with its oxidation to gluconolactone by GDH. *A. baylyi* has GDH in both soluble and membrane-bound forms [37] but it appears that only the membrane-bound form of GDH is active in glucose oxidation [13]. GDH has PQQ as a prosthetic group [38]. The electrons derived from glucose oxidation are funneled from GDH to cytochrome b via ubiquinone [13] and can be used in the production of ATP and the transport of amino acids into the cell [186]. Gluconolactone is converted by gluconolactonase to gluconate, but this reaction also occurs also spontaneously at pH 7.0 [187]. Gluconate formation results in the production of H^+, and thus a decrease in the pH of the growth medium will always occur when glucose is used as the sole carbon source in cultivations of ADP1. Gluconate formation from glucose appears to be pH-dependent, at least in *A. calcoaceticus* LMD 79.41, and does not proceed at pH 5.0 [187]. Thus, if the pH is not controlled, the decrease in pH caused by gluconate formation might start to limit the glucose catabolism. Gluconate is imported to the cytoplasm where its carbon 6 is phosphorylated, after which it is dehydrated and cleaved to pyruvate and glyceraldehyde-3P. ADP1 can use pyruvate for the production of ATP, biomass precursors in the TCA cycle or in gluconeogenesis. Glyceraldehyde-3P, on the other hand, cannot be funneled directly into TCA, but has to be converted first to PEP, which is then carboxylated to oxaloacetate. Oxaloacetate can then be converted to malate, which can be decarboxylated to pyruvate [34]. Dolin and Juni have proposed that ADP1 could use this pathway to circumvent the lack of pyruvate kinase [34]. The direct formation of pyruvate from PEP by pyruvate kinase results in the formation of one molecule of ATP, while pyruvate formation via oxaloacetate and malate does not result in any synthesis of ATP, or of the other energy carrier molecules (orthophosphate is produced instead of ATP). Glyceraldehyde-3P can be used in gluconeogenesis, which produces precursors for EPS synthesis [183].

It has been suggested that glucose catabolism in ADP1 might involve recycling the glyceraldehyde-3P to 6P-gluconate via a 6P-glucose intermediate [204]. However, BD4 (the parental strain of ADP1) does not have glucose-6P dehydrogenase activity [182]. Furthermore, a radiolabelling study has shown that practically all glyceraldehyde-3P (but not pyruvate) formed from glucose is used in EPS synthesis in the strain BD4 [183]. If there is carbon recycling to 6-phosphogluconate, the carbon flux through the recycling pathway is probably relatively small. The unencapsulated mutants of *A. baylyi* lyse when grown on glucose as a sole carbon source, and it has been suggested that this is due to the lack of pyruvate kinase activity in the cell [182]. The EPS negative mutant would not be able to funnel all the glyceraldehyde-3P produced from glucose to pyruvate via the TCA cycle, because CO_2 might be present in a limiting concentration [182].

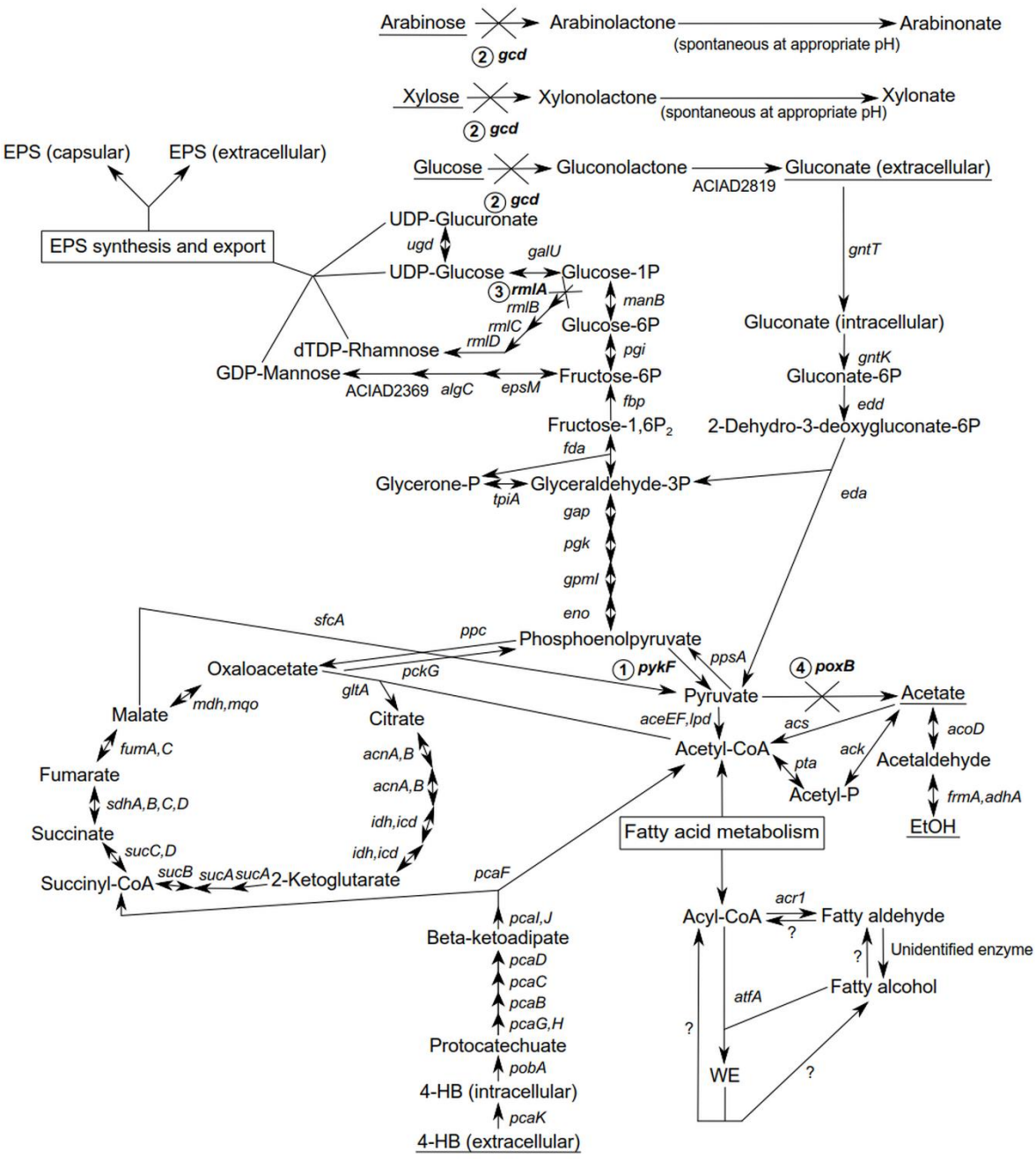

FIGURE 2 Metabolic reactions and pathways of *A. baylyi* ADP1 relevant to this thesis. Irreversible reactions are marked with single-headed arrows and reversible reactions are marked with two-headed arrows. The crossed arrows indicate reactions which were eliminated by gene KOs. The arrows going through boxes indicate that the product is formed in the pathway written in the box. The genes (or their identifiers if names are unavailable) coding for the enzymes catalyzing the reactions are shown next to the arrows. The WE catabolic pathway has not been characterized and the arrows marked with question marks indicate reactions potentially involved in WE catabolism.

2.5.2.2 Exopolysaccharide metabolism

A. baylyi strains produce EPS (Figure 3) capsules which protect the cells from desiccation [135] and prevent them from forming aggregates [81]. The EPS of BD4 and ADP1 also acts as an emulsifier, but requires a protein component for this function [92]. It has been shown that the outer membrane protein OmpA is secreted in ADP1, and that this protein possesses emulsifying activity [193].

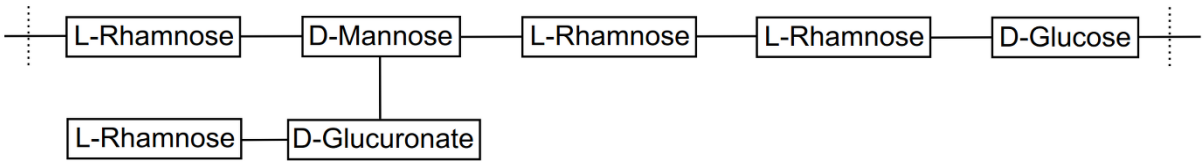

FIGURE 3 A simplified structure of the repeating unit of the EPS produced by *A. baylyi* BD4 and ADP1. Adapted from the more detailed structure determined by Kaplan et al. [90].

BD4 produces EPS at a constant rate (4 mg/h/g of DCW) throughout its growth on succinate [17]. The composition of the EPS produced in this way (rhamnose/glucose/mannose, 4:2:1) does not change much during growth on this carbon source [17]. However, when the carbon source is changed to glutamate or EtOH, the composition changes slightly (rhamnose/glucose/mannose, 10:5:1) [17]. When BD4 is cultivated on glucose, the composition differs from the above-mentioned values (rhamnose/glucose/glucuronic acid/mannose, 4:1:1:1) [90]. In the article by Kaplan et al. [90], it was shown that the glucuronic acid can be converted to glucose, and it could be that the glucose fraction in the studies by Bryan et al. [17] also contains glucuronic acid. Regardless of the carbon source or the analysis methods used, rhamnose is the most abundant EPS component, glucose (and glucoronic acid) is the second most abundant, and the least abundant is mannose. The synthesis of EPS starts with activation of the gluconeogenically produced sugars. The rhamnose, glucose and the glucuronic acid components of EPS are produced from glucose-1P, while the mannose component is produced from fructose-6P (Figure 2). All of these activated sugars are thus produced from metabolites that cannot be converted to metabolites of the "lower" part of the gluconeogenic pathway, since ADP1 cannot phosphorylate fructose-6P to fructose-1,6P (Figure 2).

A laboratory evolution experiment by Renda et al. showed that increased cellular aggregation due to deletion of EPS synthesis-related genes occurred more frequently under high selective pressure than under reduced selective pressure [154]. Thus, it appears that, at least when grown in a rich medium, the presence of an EPS capsule (and/or excreted EPS) does not confer selective advantage to the cells. Because of this, and also for the reasons explained below, it might be worthwhile to test how EPS-negative mutants of ADP1 behave in WE-producing bioprocesses. The mutations that eliminated EPS capsule synthesis in the experiment by Renda et al. [154] occurred in the genes-coding for perosamine synthase (*per*) or glucose-6P isomerase (*pgi*). The

latter of these genes codes for an enzyme that catalyzes the conversion of fructose-6P to glucose-6P (Figure 2), which can then be converted to glucose-1P, a starting point for synthesis of three EPS components: glucose, glucuronic acid, and rhamnose. Thus, it seems that metabolites produced from glucose-6P are not essential to growth, at least in a rich medium. The fourth EPS component is synthesized from fructose-6P, which is also a metabolite used in PPP. Thus, it might be possible to prevent synthesis of all four EPS components with two gene KOs. However, it is uncertain whether glucose-1P is also used by ADP1 for other purposes, and thus it was considered a safer alternative to knock out the gene for first step in the synthesis of rhamnose, the main component of the EPS of ADP1, in order to produce the EPS negative mutant while only having a minimal effect on other metabolic functions.

In a bioprocess where the components of lignocellulosic biomass are converted to WEs, the EPS capsule can be considered as an unwanted by-product, since the carbon used for its synthesis could have been used in WE synthesis. The EPS capsule might also lower the purity of the WE-containing biomass and make it more difficult to collect the cells from the growth medium. Furthermore, under the conditions encountered in the bioprocesses, protection against desiccation, a biological function of the EPS capsule, is not needed. Thus, it is likely that WE production might benefit from elimination of this trait from ADP1. It is, however, unclear how this would affect the survival of the cells and the metabolic flux toward WE synthesis. Another aspect to consider when studying the suitability of using an EPS synthesis-deficient strain of ADP1 in the above-mentioned bioprocess is whether the capsule protects the cells from the growth inhibitors formed during hydrolysis of the lignocellulosic biomass.

2.5.2.3 Acetate metabolism

ADP1 can catabolize acetate via two pathways (Figure 2). Acetate can be phosphorylated by acetate kinase into acetyl-P, which is then converted to acetyl-CoA by phosphotransacetylase. Acetyl-CoA can also be formed directly from acetate by acetyl-CoA synthetase. The former pathway converts ATP to ADP and P, and is reversible, while the latter pathway converts ATP to AMP and PP, and is irreversible.

E. coli TG1 starts to accumulate acetate to the growth medium in a fed-batch cultivation when the specific growth rate exceeds 0.17 h^{-1} [97]. ADP1, on the other hand, does not produce acetate, even at elevated growth rates when cultivated on glucose (see Results and Discussion). *A. baylyi* strains, however, produce acetate as an intermediate of EtOH catabolism and this organic acid can accumulate in the growth medium when grown on EtOH [1], [145]. Acetate production from EtOH also occurs with ADP1 (see Results and Discussion). The lack of acetate production is a beneficial trait because acetate accumulation can harm the bioprocess in many ways. Acetate formation reduces recombinant protein yields with *E. coli* [41]. Acetate can also inhibit the growth of *E. coli* K12 at low (0.5 g/l) concentrations [126]. The growth-inhibitory effect of acetate varies widely between different bacterial strains [102]. The carbon sources and the medium composition

also affect the toxicity of acetate. For example, acetate is more toxic to *E. coli* in the presence of glucose than glycerol in a rich medium [102], but the opposite occurs in a minimal medium [126]. Overexpression of phosphoenolpyruvate carboxylase, *ppc*, has been shown to reduce acetate formation from glucose by *E. coli* [49]. Acetate formation from glucose by *Bacillus subtilis* has been reduced by knocking out the gene for pyruvate kinase, which also improved the yield of the biomass and reduced growth rate [60]. Similar results have been obtained with *E. coli* with both genes for pyruvate kinase knocked out, which had lowered the growth rate and reduced acetate production [146]. The elimination of acetate formation by knocking out genes involved in its synthesis has been used to improve the production of pyruvate [22] and citramalate [140] by *E. coli*. Among these genes is *poxB*, the product of which funnels carbon away from product formation in these strains. Recently, acetate excretion by *E. coli* has been reduced by a synthetic biology application where the expression of *pta*, *ackA* and *poxB* were controlled by light at three different wavelengths [51]. *E. coli* produces acetate primarily through the Pta-AckA pathway [197]. The Pta-AckA pathway simultaneously produces and consumes acetate, while the direction of the carbon flow is determined by thermodynamic factors, *i.e.* the concentrations of reactants and products [47]. Thus, acetate excretion by *E. coli* K-12 MG1655 during growth on glucose is not due to repression of acetate catabolism, but due rather to the higher flux of the acetate production than the acetate consumption.

2.5.2.4 Aromatic compound metabolism

The aromatic compound 4-HB, which was used in the experiments for this thesis as a representative of lignocellulosic hydrolysate's aromatic inhibitors, is catabolized by ADP1 into succinyl-CoA and acetyl-CoA (Figure 2). The catabolism of 4-HB begins with its transport into the cell via PcaK [141]. It is then converted to protocatechuate and, after four reactions, into β-ketoadipate intermediate, which is then converted by two reactions into acetyl-CoA and succinyl-CoA [53].

The catabolism of aromatic compounds in ADP1 is subject to complex control mechanisms [16], [27]. There are several carbon sources that repress the catabolism of aromatic compounds, two of the strongest of these being succinate and acetate [27]. This is unfortunate, as acetate is often found in lignocellulosic hydrolysates at high (10 g/l) concentrations [179]. In addition to repression by organic acids, some of the catabolizable aromatic compounds repress the catabolism of other, less desirable aromatic molecules [16]. The catabolite repression of aromatic compounds could be decreased by knocking out genes like *crc* [207], the products of which decrease these metabolic activities in ADP1. Glucose does not act as a catabolite repressor of aromatic compound catabolism [27]. Gluconate represses the catabolism of some aromatic compounds only to a small degree, and acts as an inducer for one (*catA*) of the pathways [16]. Amplification of the genes responsible for benzoate catabolism in a mutant lacking the two benzoate-catabolism

inducer genes has been shown to allow benzoate catabolism [151]. The combined weak expression of the genes from multiple copies allowed enzyme activities high enough to support growth on benzoate [151].

2.5.2.5 Wax ester synthesis

WEs are esters of fatty acids and fatty alcohols (Figure 4) and occur in nature as carbon storage material for plants like Jojoba (*Simmondsia chinensis*) [158], and microorganisms such as *Euglena gracilis* [70] and *A. baylyi* [56]. Jojoba is a perennial shrub that stores WEs in its seeds and grows mainly in deserts, but can also be cultivated in other environments [158]. WE synthesis has also been engineered in *Nicotiana benthamiana* in order to allow it to occur in plants which can grow in a broader range of conditions [8]. The WEs of the Jojoba plant usually contain 40 to 42 carbon atoms, and both the fatty acyl and fatty alcohol parts are mono-unsaturated [121]. *E. gracilis* synthesizes a carbohydrate paramylon as a means of carbon and energy storage under aerobic conditions, and when the growth cultivations are changed to anaerobic, converts the paramylon to WEs which also generate ATP [70]. The WEs of *E. gracilis* are mainly composed of saturated compounds containing 27 to 30 carbons [71]. *A. baylyi* produces WEs most efficiently in the presence of an excess carbon source and the simultaneous starvation of other essential nutrients such as nitrogen [56]. The WEs of ADP1 consist mainly of C16:0, C16:1 and C18:1 fatty acids and fatty alcohols [162]. The WEs of BD4, the parental strain of ADP1, also contain mainly these fatty alcohols [19]. The placement of the double bonds and their configuration are not known for the WEs of ADP1, but it has been shown that the WEs of *A. calcoaceticus* NCIB 8250, which also contain mostly fatty acids and fatty alcohols with a chain length of 16 or 18 carbons, have the double bonds between carbons 9 and 10 and have a *cis* configuration (Figure 4) [56]. Fatty acyl and fatty alcohol compositions of WEs of other *A. baylyi* species do not differ dramatically from the composition of the WEs of ADP1 mentioned above [55], but the WEs of some psychrotrophic members of *Acinetobacter* appear to contain short (C_{3v} – C_5) alcohols [62]. WE synthesis has been engineered in microorganisms like *E. coli* [89], [175] and *S. cerevisiae* [190], which do not naturally produce these compounds.

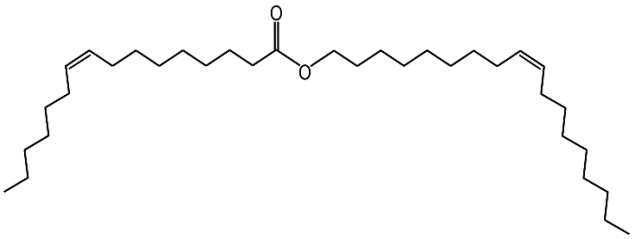

FIGURE 4 An example of a WE structure. This kind of WE is produced by for example, *A. calcoaceticus* NCIB 8250 [56].

WEs can be used in the cosmetics and pharmaceutical industries, as a lubricant, or as a raw material for biodiesel production, to name a few applications [121], [158]. Jojoba oil acts as a

moisturizer and can thus be used to improve skin care products [158]. Jojoba oil has anti-inflammatory properties and has long been used by the native peoples of America to treat inflammatory diseases [158]. Jojoba oil can also be used as an additive in diesel [6], [69] or transesterified with short-chain alcohols like methanol to produce biodiesel [20]. However, due to the high price of Jojoba oil, it is probably economically more feasible to use this lipid in other applications. The production of Jojoba oil has increased dramatically in recent years due to the increased demand for this lipid [158]. The applications where Jojoba oil is used keep increasing, which creates this increased demand [158]. It has been noted that there is a need for an inexpensive way to produce large quantities of WEs to meet the demands of various industries [87]. The WEs used in industrial applications today are produced from Jojoba, but producing WEs from microorganisms has many advantages as they are easier to engineer genetically, and can be fed on different carbon sources, thus allowing better control of the composition of the resulting lipids [4].

While WEs already have several commercial applications, they could be further improved by means of metabolic engineering. For example, the properties of Jojoba plant WEs as biofuel could be further improved by lowering the viscosity [20]. The viscosity of WEs produced by ADP1 could be lowered with WE synthases with different substrate specificities [173]. On the other hand, the composition of the WEs produced from ADP1 could be modified to more closely resemble those of the Jojoba plant by metabolic engineering, as has been done with *S. cerevisiae* engineered to produce WEs [190]. The structure of WEs produced by *A. baylyi* ADP1 could also be modified so that it contained branches, as has been done with *E. coli* [118], which improves properties of WEs as lubricants. It has been proposed that the commercialization of bioprocess products might be easier for specialised applications than it is for fuels, for example [65]. Thus, being able to tailor the structure of the WE might make the WE-producing bioprocesses using ADP1 more economically feasible.

The microbial synthesis of lipids begins with the formation of malonyl-CoA from acetyl-CoA by carboxylation, a reaction that consumes one molecule of ATP [75]. Malonyl-CoA is then transferred to the acyl carrier protein, forming malonyl-ACP. The malonyl-ACP is condensed with acetyl-CoA to produce acetoacetyl-CoA, which is converted into butyryl-CoA through two reductions and one dehydration reaction. Both of these reduction reactions consume two nicotinamide adenine dinucleotide phosphate (NADPH) molecules. Butyryl-CoA is condensed with malonyl-ACP and the cycle is repeated until a full-length acyl-ACP has been formed. Thus, elongation of the growing fatty acid chain by two carbons consumes one molecule of ATP and two molecules of NADPH. In WE synthesis by ADP1, the fatty acyl-CoA is reduced to fatty aldehyde in an NADPH-consuming reaction by Acr1, a fatty acyl-CoA reductase encoded by *acr1* [152]. Acr1 can catalyze the reduction of fatty acyl-CoAs with 14 to 22 carbons (but not with 12 or 24 carbons) and most favourably reduces hexadecanoyl-CoA [152]. The enzymatic step for the conversion of fatty aldehydes to fatty alcohols has not been elucidated. In the last step in the synthesis of WEs, a fatty acyl-CoA and a fatty alcohol are condensed to a WE molecule, liberating a CoA-SH

molecule [87]. This reaction is catalyzed by the WE synthase/acyl-CoA:diacylglycerol acyltranferase (WS/DGAT) [87], which is mainly located on the inner surface of the cell membrane and at the surface of the WE bodies, although it occurs to lesser degree in cytosol [177], [199]. WS/DGAT catalyzes, as its name suggests, and also synthesises triacylglycerols, but this activity is only about a tenth of the WE synthase activity [87]. WS/DGAT has a low substrate specificity [177], which has been utilized in engineering the production of fatty acid ethyl esters (FAEE) in *S. cerevisiae* [86], [173] and *E. coli* [88]. This *E. coli* strain has been used in a 20-liter fed-batch cultivation where a biomass of 61 g/l and a FAEE content of 18.5 % were obtained, by first cultivating a high cell density with glycerol and then producing the FAEEs from glucose and oleic acid [44]. The limitation of having to feed the microorganism with fatty acids has been recognised by Elbahloul & Steinbüchel, and they suggested [44] that this process could be carried out in bacteria naturally producing large quantities of storage lipids, such as *Acinetobacter* sp. strain 211, which produces up to 25 % of DCW lipids when cultivated on olive oil [5]. Other ester bond-forming enzymes have been characterized and used in the production of molecules such as ethyl acetate, isobutyl alcohol, and tetradecyl acetate in *E. coli* [117]. It has been suggested that ADP1 might be a suitable host organism for biodiesel production [45], but so far biodiesel production has not been engineered in ADP1.

WS/DGAT appears to be present in the ADP1 cell at a low copy number [199]. It has been proposed that the synthesis occurs at the inner cell membrane, where the WS/DGAT enzymes are docked prior to the beginning of the WE synthesis. These enzymes catalyze the formation of small lipid droplets, to which phospholipids attach, forming lipid pre-bodies. The lipid pre-bodies dissociate themselves from the membrane-bound enzymes and the small lipid droplets inside them aggregate, which results in the formation of mature lipid bodies. The mature lipid bodies of WEs are surrounded by a monolayer of phospholipids and are spherical in ADP1 [199], while in other *A. baylyi* strains WEs can be found with differently-shaped lipid bodies [200]. *Acinetobacter* sp. HO1-N has been shown to excrete WEs, free fatty acids, and mono-, di- and tri-glycerides (but not significantly free fatty alcohols) to the growth medium when cultivated on hexadecane [108]. The growth medium did not contain a significant amount of these molecules when the cells were cultivated in a rich medium [108]. The growth medium also did not contain phospholipids when cultivated in a rich medium or on hexadecane, which indicates that the cells did not liberate the lipids by lysis [108].

The accumulation and utilization of WEs by ADP1 is shown schematically in Figure 5. The time needed to reach time-points A, B and C depends on the carbon source used, and in the experiments for this thesis it took several days to reach time-point C. Prior to time-point A, the cells grow and synthesize WEs. At time-point A, the growth becomes limited by a nutrient other than the carbon source, for example nitrogen, and this is when WEs are accumulated most efficiently [56]. WEs continue to accumulate in the cells until time-point B, when the cells have depleted the growth medium of the carbon source. At this point, the cells start to consume WEs as a source

of carbon and energy. When the cells have consumed all of their WE reserves (time-point C) the number of cells starts to decrease. Therefore, the optimal point for collecting the cells in a WE-producing bioprocess is time-point B.

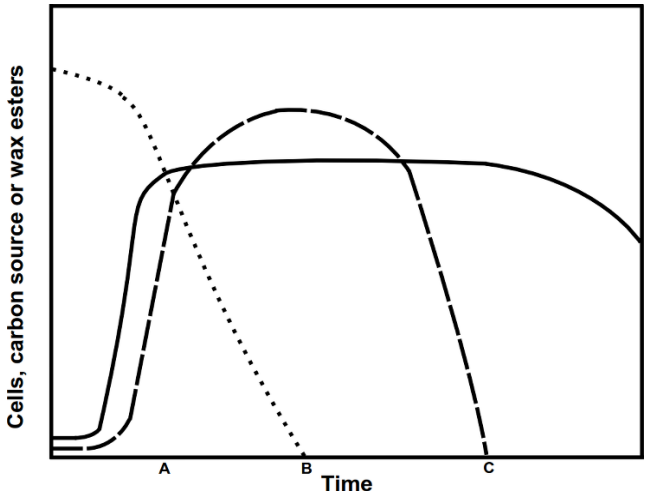

FIGURE 5 A schematic presentation of WE accumulation by ADP1. The number of cells is shown with a continuous line, the amount of WEs with a dashed line, and the amount of the carbon source with a dotted line. Adapted from [56].

The WE contents of bacteria are often only a fraction of what Jojoba seeds or *E. gracilis* contain. The highest WE content with bacteria (17 %) was obtained by feeding an *A. baylyi* strain with alkanes [72]. These carbon sources differ greatly from the ones used in this thesis since their incorporation into WEs requires only few metabolic steps [73]. Because of this, this value (17 %) cannot be compared with the WE yields obtained by cultivating the bacteria on lignocellulose-derived carbon sources. WE production has been enabled in *Rhodococcus opacus* PD630 by expressing a heterologous fatty acyl-CoA reductase [101] and in *Rhodococcus jostii* RHA1 by overexpressing a fatty acyl-CoA reductase naturally present in the organism [156]. Neither the WE percentage of DCW or the WE titer were reported for *R. opacus* PD630, but WEs constituted 46 % of the neutral lipids produced and the strain accumulated over 40 % of DCW of neutral lipids in a separate cultivation [101]. This suggests that this strain has the potential to accumulate large quantities of WEs but its suitability as a bioprocess host organism cannot be evaluated before the WE production by the strain has been properly characterized. *R. jostii* RHA1 accumulated WEs up to 13 % of DCW, but the cultivation took 72 hours to complete and the authors did not report the amount of biomass produced [156]. Thus, WE productivity and its titer might remain low with this organism unless its growth rate and final biomass obtained can be improved.

2.5.3 Biotechnological applications

Acinetobacter strains have been considered suitable for several biotechnological applications, including bioremediation, the production of biochemical, and as biosensors [2], although most of

this research involved basic microbiological science [79]. Much of the research work carried out with ADP1 has focused on genetics and metabolism, but more recently on biotechnological applications too [45]. For example, Lehtinen et al. have used ADP1 to produce WEs indirectly from electricity and CO_2 [105]. In this application *Sporomusa ovata* first converts CO_2 and electricity to organic acids, and then the ADP1 converts the organic acids into WEs [105]. Santala et al. have used a *gntT* KO mutant of ADP1 that is unable to transport gluconate into the cells. They used this in a co-cultivation to consume the acetate produced by *E. coli* as an overflow metabolite of glucose catabolism [163]. The production of triacylglycerols with metabolically-engineered ADP1 has been studied by Santala et al. [161], while Lehtinen et al. have engineered ADP1 for the production of alkanes [106]. Elbahloul et al. have studied cyanophycin production with ADP1 by optimizing growth conditions [42], and by means of metabolic engineering [43]. *A. calcoaceticus* RAG-1 has been studied with respect to its ability to produce emulsans [172] and the structure of the produced emulsan has also been engineered [78]. Even though ADP1 also produces EPSs that have emulsifying properties [91], the EPS produced by ADP1 has not been studied extensively or subjected to engineering. The first step in the glucose catabolism of *A. baylyi* has been harnessed in the production of D-xylulose [147]. In this application, D-xylulose is formed enzymatically from D-xylose by xylose isomerase. Then *A. baylyi* cells are used to convert the residual D-xylose to D-xylonic acid, which is then removed with methanol precipitation and ion exchange. ADP1 has also been used as a host for biosensor applications where the synthesis of WEs [160] or the synthesis or degradation of alkanes [106] have been monitored in real time.

3 Research Objectives and Questions

The main goal of this work was to improve the growth and WE production by ADP1 on the main components of lignocellulosic biomass, *i.e.* sugars, aliphatic acids and aromatic compounds. As discussed above, ADP1 can use glucose, aliphatic acids like acetate, and certain aromatic compounds like 4-HB as carbon sources. Since ADP1 grows relatively poorly on glucose (low growth rate and biomass yield), and glucose is the most abundant component of lignocellulosic hydrolysates, much research has concentrated on glucose utilization. The unique characteristics of the metabolism of ADP1, such as the lack of certain glycolytic enzymes and production of EPSs, made it possible to test whether modification of its main metabolic pathways would improve the growth characteristics or the WE production. The objectives of this thesis are summarized below (the publications where these aspects were studied are shown in parentheses):

- Improvement of growth rate (Publications I & IV)
- Improvement of biomass yield (Publications III & IV)
- Improvement of WE yield, titer and purity (Publications III & IV)
- Improvement of tolerance towards a lignocellulosic inhibitor acetate (Publication IV)
- Production of a lignocellulosic hydrolysate biodetoxification strain (Publication II)

The selective advantage of the lack of pyruvate kinase in ADP1 is not known. However, the unencapsulated mutants of BD4 lyse when cultivated on glucose, which has been considered to be due to the lack of pyruvate kinase activity [82]. Thus, it could be hypothesized that the expression of a gene for this enzyme might affect the growth characteristics of the mini-encapsulated WT ADP1.

The EPS capsule is the major by-product in WE production with ADP1. Although unencapsulated mutants of BD4 have been produced before, the effects of these mutations on growth, or on macro-molecular composition, have not been characterized. It is hypothesized that by knocking

out the gene for the first step in the synthesis of the most abundant component of the EPS of ADP1, the growth and WE production might be affected on three components of lignocellulosic hydrolysates: glucose, acetate and 4-HB. The established hypothesis that the lysis of the unencapsulated cells grown on glucose is due to the lack of pyruvate kinase activity was also tested.

Lignocellulosic hydrolysates often contain large amounts of acetate, thus making it a major carbon source in WE-producing bioprocesses that use ADP1 as a host organism and lignocellulosic hydrolysates as a raw material. While ADP1 does not excrete acetate when cultivated on glucose, the strain has a gene for the production of this organic acid from pyruvate. The formation of acetyl-CoA from acetate requires ATP, and thus the presence of this enzymatic activity might waste energy, and thus affect the growth and WE production in growth media containing glucose and/or acetate.

The presence of multiple pathways for sugar catabolism has been shown to be a problem in engineering *E. coli* for biodetoxification [100]. Furthermore, *S. cerevisiae* engineered for selective acetate removal also consumes the sugars present in the cultivation medium to some degree [169]. This problem has also been encountered with bacterial strains used in biodetoxification which have not been subjected to metabolic engineering [58]. ADP1 has a single enzymatic step for oxidation of all lignocellulosic sugars: the first step in its glucose catabolism, catalyzed by GDH. It was hypothesized that ADP1 could outperform the above-mentioned strains if the gene for this enzymatic step was knocked out.

4 Materials and Methods

4.1 Metabolic engineering

All metabolically engineered strains (Table 2) were produced from *A. baylyi* ADP1 (DSM 24193) or strains derived from it by KO mutations (Figure 6). Construction of the plasmids and gene KO cassettes was performed according to well-established methods [157]. The KO cassettes used to produce KO mutations of *gcd*, *rmlA* and *poxB* were constructed from the KO cassettes of Santala et al. [161] by changing the flanking sites and the antibiotic resistance marker when necessary. The plasmid backbone used was pBAV1C [162], which is a chloramphenicol (Cm) resistant version of the pBAV1K plasmid [18]. The gene for pyruvate kinase, *pykF*, was cloned from *E. coli* K12 MG1655 and was expressed under control of the arabinose promoter, which has been shown to work well in ADP1 [125]. The primers used to clone *pykF* contained standard BioBrick restriction sites [95]. Metabolic modelling of the effects of the *pykF* expression on the metabolism of ADP1 was done with metabolic flux balance analysis [50]. The control plasmid, pBAV1C, was produced by removing the promoter from pBAV1C-pBAD with *XbaI* and *SpeI* and ligating the plasmid backbone with itself. All the transformations of ADP1 were carried out as explained by Metzgar et al. [119]. Successful construction of the metabolically engineered strains was verified by amplifying the modified regions with PCR and analyzing the sizes of the PCR products with agarose gel electrophoresis.

TABLE 2 The metabolically engineered *Acinetobacter baylyi* strains used in the experiments.

Strain designation used in this thesis[a]	KO mutation(s)/antibiotic marker(s)	Plasmid/antibiotic marker	Publication(s)
ADP1	-	-	I, II, III
ADP1_ctrl	-	pBAV1C/Cm	I
ADP1_pykF	-	pBAV1C-pBAD-*pykF*/Cm	I, III
ADP1Δgcd	*gcd* (ACIAD2983)/Cm	-	II
ADP1ΔrmlA	*rmlA* (ACIAD0079)/Cm	-	III
ADP1ΔrmlA_pykF	*rmlA* (ACIAD0079)/Km	pBAV1C-pBAD-*pykF*/Cm	III, IV
ADP1ΔrmlAΔpoxB_pykF	*rmlA* (ACIAD0079)/Km, *poxB* (ACIAD3381)/Spec	pBAV1C-pBAD-*pykF*/Cm	IV

[a]Different designations may have been used in the Publications I-IV

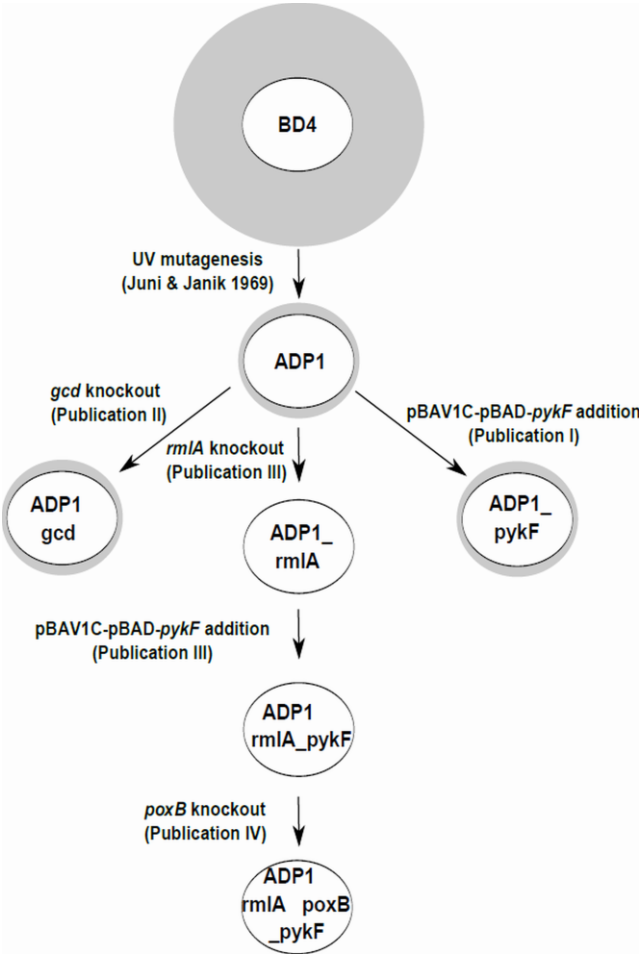

FIGURE 6 Lineage of the *Acinetobacter baylyi* strains used in the experiments. The strain names are shown inside the representations of the cells and the presence of the EPS capsule is shown in grey.

4.2 Cultivation of the cells

The cultivations were carried out at 30 °C at 300 rpm in 250 ml Erlenmeyer flasks, which were closed with a loose aluminium foil and contained 50 ml of medium. Co-cultivations with *Clostridium butyricum* were carried out in sealed 25 ml glass tubes containing 10 ml of the medium. Cultivation of the bacteria for transformations was carried out in a slightly modified version of the LB medium [14] which had the following composition: 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl. The platings of the bacteria were carried out on a solid medium, which had a similar composition to the LB medium described above but also contained 15 g/l of agar. The minimal medium used in the experiments had the following composition: 5.1 g/l K_2HPO_4, 3.4 g/l KH_2PO_4, 1.0 g/l NH_4Cl, 0.3 g/l $MgSO_4$, 22.2 mg/l $CaCl_2$, 4.2 mg/l $FeCl_3$ (and 2.0 g/l CAA when appropriate). The co-cultivations with *C. butyricum* were carried out in the medium used by Seppälä et al. [171] but with slight modifications: 1.5 g/l K_2HPO_4, 2.0 g/l $(NH_4)_2SO_4$, 0.2 g/l $MgSO_4*7H_2O$, 0.015 g/l $CaCl_2*2H_2O$, 0.005 g/l $FeSO_4*7H_2O$, 0.3 g/l yeast extract, 2 ml/l of trace element solution (1.0 ml/l 25 % HCl, 70 mg/l $ZnCl_2$, 100 mg/l $MnCl_2*4H_2O$, 60 mg/l H_3BO_3, 200 mg/l $CoCl_2*6H_2O$, 20 mg/l $CuCl_2*2H_2O$, 20 mg/l $NiCl_2*6H_2O$ and 40 mg/l $Na_2MoO_4*2H_2O$). The antibiotics used in the transformations were added to the growth media from stock solutions to achieve concentrations of 25 – 50 mg/l (Cm), 50 mg/l (Km), or 100 mg/l (Spec). In the cultivation experiments, 25 – 50 mg/l of Cm was used. For experiments where it was intended to cultivate the bacteria using a substrate as a sole carbon source, the stock solution was not used and the Cm was dissolved directly into the growth medium. This was done in order to prevent the EtOH from the Cm stock solution (prepared in 70 % EtOH) from interfering with the experiment. The carbon sources used in the publications of this thesis are shown in Table 3. The calculations for the optimization of the substrate and *pykF* inducer concentrations in Publication I were done with MATLAB [112].

TABLE 3 Carbon sources used in the experiments.

Carbon source	Publication
Glucose	I, II, III, IV
Gluconate	I
Arabinose[a]	II
Xylose	II
Levulinate	II
Acetate	II, III, IV
Formate	II
4-HB	II, III
EtOH	III, IV
Rice straw hydrolysate	II

[a]The experiments where arabinose was used as an inducer of *pykF* expression are not listed here.

4.3 Measurement of growth and metabolites

The measurements carried out in the experiments are summarized in Table 4. The growth of the bacterial strains was measured spectrophotometrically (growth rate) or gravimetrically (DCW). Concentrations of the small metabolites were determined spectrophotometrically or by chromatographic methods, and the pH was measured with a pocket pH meter. In the preparation of the rice straw hydrolysate used in Publication II, a dinitrosalisylic acid assay was used instead of HPLC. The lipids were solvents extracted from the cells and the total lipid content was measured gravimetrically. The number of WEs was determined either semi-quantitatively (TLC) or quantitatively (NMR) from the extracted lipids. In Publication I, the expression level of *pykF* in ADP1 was determined with an enzymatic assay. In Publication III, the aggregation of the *rmlA* KO strains was shown to occur by inspecting the cells with a light microscope.

TABLE 4 The methods used to measure growth and metabolite concentrations in the experiments.

Measured parameter	Method	Publication
OD_{600}	Spectrophotometer (600 nm)	I, II, III, IV
DCW (or weight of the NMR sample)	Gravimetric analysis (after lyophilization)	III, IV
$c_{4\text{-HB}}$	Spectrophotometer (280 nm) as in Dal et al. [27]	II, III
c_{H2}	GC as in Mangayil et al. [109]	II
$c_{\text{wax esters}}$ (quantitative)	NMR (after solvent extraction)	III, IV
$c_{\text{wax esters}}$ (semiquantitative)	TLC (after solvent extraction)	I, III, IV
c_{glucose}	HPLC	I, II, III, IV
c_{glucose}	Dinitrosalicylic adic assay [120]	II
$c_{\text{other metabolites}}$	HPLC	I, II, III, IV
Total lipid content	Gravimetric analysis (after solvent extraction)	III, IV
Pyruvate kinase activity	Enzymatic assay as in Netzer et al. [128]	I
pH	pH meter	III, IV
Cellular aggregation	Light microscopy	III

5 Results and Discussion

5.1 Improvements in growth

5.1.1 Growth rate

The specific growth rates for *A. baylyi* strains on various carbon sources are shown in Table 5. The highest growth rates were obtained with malate and oxaloacetate, which are TCA cycle intermediates. Pyruvate and acetate produce intermediary growth rates when used as carbon sources, while only low growth rates can be obtained when using glucose as the carbon source. 4-HB, which is catabolized to succinyl-CoA and acetyl-CoA, also supports the intermediary growth rate. It has been hypothesized that the lack of pyruvate kinase activity in BD4, the parental strain of ADP1, causes some of the carbon from the glucose catabolism to get stuck in the glycolysis of the unencapsulated mutants, causing cells to lyse [82]. One way to reduce the concentrations of glycolytic intermediates in ADP1 is to improve the flux from glycolysis to the TCA cycle by expressing *pykF*. The expression of *pykF* would also reduce the number of enzymatic steps needed to produce pyruvate from PEP. Metabolic pathways usually involve a minimum number of enzymatic steps to produce one essential metabolite from another [132], so it could be expected that this simplification of metabolism might improve the growth of ADP1. The use of pyruvate kinase also yields one molecule of ATP per conversion of PEP to pyruvate, while pyruvate formation from PEP via oxaloacetate and malate intermediates, the pathway used by WT ADP1, yields none. It was considered worthwhile to test whether the growth rate of ADP1 on glucose could be improved by expressing *pykF*, a gene for pyruvate kinase from *E. coli*.

TABLE 5 Growth rates in the exponential growth phase of *A. baylyi* strains on different carbon sources.

Strain	Carbon source	Concentration (mM)	μ (h^{-1})	Reference
ADP1	Glucose	28	0.15	[91]
BD4	Glucose	28	0.26	[91]
ADP1	Glucose	10	0.25	[188]
ADP1	Glucose	10	0.19	Publication III
ADP1_ctrl	Glucose	82	0.18	Publication I
ADP1_pykF	Glucose	82	0.42	Publication I
ADP1_pykF	Glucose	10	0.30	Publication III
ADP1ΔrmlA_pykF	Glucose	10	0.28	Publication III
ADP1ΔrmlA_pykF	Glucose	22	0.36	Publication IV
ADP1ΔrmlAΔpoxB_pykF	Glucose	21	0.25	Publication IV
ADP1_ctrl	Gluconate	76	0.12	Publication I
ADP1_pykF	Gluconate	75	0.44	Publication I
ADP1	Malate	38	1.09	[34]
ADP1	Pyruvate	57	0.67	[34]
ADP1	Oxaloacetate	38	1.04	[34]
ADP1	Acetate	10	0.69	Publication III
ADP1ΔrmlA	Acetate	10	0.52	Publication III
ADP1	4-HB	10	0.55	Publication III
ADP1ΔrmlA	4-HB	10	0.56	Publication III

It was shown that there is a correlation between added inducer concentration, pyruvate kinase activity, and the specific growth rate of ADP1_pykF on gluconate (Publication I). With ADP1_ctrl there was no increase in pyruvate kinase activity with increasing inducer concentration, but its specific growth rate increased slightly. This is most likely due to the fact that the cells were cultivated on gluconate and the GDH oxidation of sugars (here the inducer, arabinose) allows the cells to grow faster [187]. If glucose had been used as a carbon source, this effect would probably have been smaller, or even non-existent, because glucose is more preferred substrate than arabinose for the GDH of *A. baylyi* LMD79.41 [33] and ADP1 (see Figure 1a in Publication II). Because the growth rate was found to be dependent on the expression level of *pykF*, it was decided to attempt to optimize the growth rate by varying the glucose and arabinose concentrations. This was done in order to find out the maximum effect of *pykF* expression so that its other possible effects on the metabolism of the bacterium could be determined in subsequent experiments.

Two batch cultivations were carried out at optimized substrate and inducer concentrations, using gluconate or glucose as carbon sources. The metabolism of the former starts at a later point in the ED glycolysis of ADP1 (Figure 2). The gluconate allows the cell to start growing earlier than it would when cultivated on glucose since the oxidation of glucose to gluconate causes the cells to endure a longer lag phase [188]. Glucose, on the other hand, yields more energy through reactions by GDH and lactonase, and thus gluconate has a lower energy content than glucose.

The presence of glucose also prevents oxidation of the inducer, arabinose, to arabinolactone, which allows the expression of *pykF* to be maintained at an appropriate level. When cultivated on gluconate, it took approximately twice as long for ADP1_ctrl to grow to the stationary phase than ADP1_pykF, while both strains had a lag phase of similar length. Furthermore, when cultivated on glucose the cells also experienced lag phases of approximately the same length, but ADP1_pykF grew faster. The difference between the growth rates of the strains was more pronounced on glucose than when gluconate was used as a carbon source. This could be due to the fact that more energy can be obtained from glucose than gluconate, and the use of gluconate allows arabinose oxidation by GDH, which decreases *pykF* expression and thereby the growth rate. The oxidation of arabinose when ADP1_pykF is grown on gluconate resulted in a more linear growth than when glucose was used as a carbon source (Publication I). The growth of ADP1_pykF on glucose ceased to be exponential after approximately half of the carbon source had been depleted. There could be several reasons for this. One explanation for this behaviour is that some other medium component was depleted at this point, for example nitrogen, after which the cells merely accumulated WEs. On the other hand, since conversion of glucose to gluconate by GDH and gluconolactonase decreases the pH of the medium, it might be that the pH of the medium started to decrease at this point, if the buffering capacity of the used buffer ran out.

The *rmlA* KO eliminated EPS capsule formation and decreased the growth rate on acetate as a sole carbon source, but did not affect the growth rate when 4-HB was used as a sole carbon source (Publication III). The *rmlA* KO diminished the growth rate of ADP1 on glucose, as could be expected. However, the expression of *pykF* restored the growth rate of ADP1ΔrmlA to a similar level to that of ADP1_pykF. Thus, the *rmlA* KO did not affect the growth rate of ADP1_pykF on glucose. When *poxB* was knocked out from ADP1ΔrmlA_pykF, the growth rate on glucose decreased (Publication IV). The decrease in growth rate (and also biomass yield) on glucose has also been shown to occur with the *poxB* KO strain of *E. coli* [3]. The growth rate of ADP1ΔrmlAΔpoxB_pykF in the exponential growth phase was also lower than that of ADP1ΔrmlA_pykF when the medium contained glucose, CAA and EtOH, but was unaffected in a medium containing either acetate alone, or glucose, CAA and acetate (Publication IV). When the cells grew linearly, the growth rate was similar for both strains on glucose alone but when cultivated on glucose, CAA and acetate, the ADP1ΔrmlAΔpoxB_pykF grew significantly faster than the ADP1ΔrmlA_pykF. This indicates that whether *poxB* KO increases or decreases the growth rate of ADP1ΔrmlA_pykF depends on the medium composition. However, since lignocellulosic hydrolysates often contain large amounts of both glucose and acetate, *poxB* KO might be beneficial with respect to growth rate in bioprocesses where WEs are produced with ADP1ΔrmlA_pykF from lignocellulosic hydrolysates.

5.1.2 Biomass yield and final biomass of the cultivation

The biomass yield (grams of biomass per gram of substrate) from glucose with ADP1_pykF (0.37 ± 0.04 g_{Cells} /$g_{Glucose}$) (Publication III) is significantly lower than what is usually obtained with *E. coli* (0.5 g_{Cells} /$g_{Glucose}$) [174], although it is in the same range as what has been obtained with the *E. coli* strain HB101 (0.35 g_{Cells} /$g_{Glucose}$) [170]. The value obtained with ADP1_pykF might be slightly lowered by the presence of pBAV1C-pBAD-*pykF* in the cells, since plasmid maintenance reduces biomass yield [170]. This effect is larger with high-copy-number plasmids [170]. Indeed, pBAV1K-T5-luxABCDE, the plasmid containing the backbone from which pBAV1C-pBAD-*pykF* has been derived, has a copy number of ~60 in ADP1 [18]. The plasmid RSF1050, which also has a copy number of 60 and is approximately same size (7.4 kb [36]) as pBAV1K-T5-luxABCDE (8.6 kb) [18]), decreases the biomass yield of *E. coli* HB101 from 0.35 g_{Cells} /$g_{Glucose}$ (μ = 0.42 h^{-1}) to 0.30 g_{Cells} /$g_{Glucose}$ (μ = 0.36 h^{-1}) in a minimal medium on glucose [170]. Thus, it could be expected that the presence of pBAV1C-pBAD-*pykF* (5 - 6 kb (Publication IV)) might, at least partly, explain the relatively low biomass yield from glucose of ADP1_pykF. The expression of *pykF*, which increased the growth rate, did not affect biomass formation based on OD_{600} and glucose consumption values (Publication I).

Since EPS constitutes a large portion of the biomass of ADP1 (see Exopolysaccharide metabolism), an *rmlA* KO mutant was constructed and its growth on glucose, acetate and 4-HB was characterized to see if biomass yield could be improved (Publication III). The strain grew poorly on glucose, but the expression of *pykF* allowed proper growth. When ADP1ΔrmlA was cultivated on glucose, the absorbance of the growth medium at a wavelength of 280 nm increased significantly (1.55 ± 0.02). This was assumed to be caused by lysis of the cells. In the WT ADP1 cultivation, absorbance at 280 nm was also slightly increased (0.05 ± 0.01), but this could be expected since it has been shown that ADP1 undergoes lysis to a small degree during exponential growth [136]. However, although ADP1ΔrmlA_pykF had an increased WE yield (see below), the biomass yield from glucose was lowered to 0.23 ± 0.07 g_{Cells} /$g_{Glucose}$ (Publication III). On acetate, the biomass yield of 0.52 ± 0.09 g_{Cells} /$g_{Acetate}$ obtained with WT ADP1 in these experiments is significantly higher than what has been obtained by O´Beirne & Hamer with *E. coli* W3310, who reported a biomass yield of 0.25 g_{Cells} /$g_{Acetate}$ [133]. The *rmlA* KO did not significantly affect the biomass yield from acetate (0.58 ± 0.12 g_{Cells} /$g_{Acetate}$). The biomass yield from 4-HB was also quite high for both ADP1 (0.52 ± 0.02 g_{Cells} /$g_{4\text{-}HB}$) and ADP1ΔrmlA (0.49 ± 0.06 g_{Cells} /$g_{4\text{-}HB}$). Thus it seems that the effects of the *rmlA* KO mutation are specific to cultivations carried out on glucose, and cannot be applied to cultivations on carbon sources such as acetate and 4-HB. This could be due to the peculiarities of glucose catabolism by ADP1 (see Figure 2). Nevertheless, the high biomass yields of ADP1 from the common inhibitors found in lignocellulosic hydrolysates, acetate and 4-HB, indicate that this strain might be suitable for efficient WE production from these compounds.

ADP1 cannot grow on sugars other than glucose [181], which limits the biomass obtained when cultivated on lignocellulosic hydrolysates. Since the GDH of ADP1 also oxidizes pentoses, the catabolic pathways for pentose utilization would have to be oxidative. An oxidative xylose degradation pathway of *Caulobacter crescentus* [176] has been successfully used in enabling the oxidative catabolism of xylose in *Pseudomonas putida* S12, which also possesses a gene for GDH [116]. *P. putida* S12 has also been engineered to catabolize xylose and arabinose via the phosphorylative pathway [115]. However, efficient pentose catabolism required inactivation of GDH, which did not affect glucose consumption of *P. putida* S12 [115]. ADP1 has only ED glycolysis and the inactivation GDH eliminates glucose catabolism (Publication II), so this strategy cannot be used with ADP1.

The highest final biomass values obtained were 9.7 ± 1.4 g/l for ADP1_pykF (Publication III), 5.0 ± 0.1 g/l for ADP1ΔrmlA_pykF (Publication IV) and 5.2 ± 0.1 g/l for ADP1ΔrmlAΔpoxB_pykF (Publication IV). It should be noted, however, that these values were determined from samples that were not washed prior to lyophilization. The samples were not washed in order to ensure that no cells, and thus WEs, are lost during this phase, and also because the WE content of the cells might have changed during this time-consuming step. Thus, these samples contained extra mass from medium components that are not removed during lyophilization. Because of this, they do not represent the DCW of the cultivation, but rather the masses of the NMR samples. Also, the biomass value of ADP1_pykF probably contains more of these impurities since it does not form so dense pellet as the EPS capsule negative *rmlA* KO strains, and thus less medium is removed from the sample by removal of the supernatant after centrifugation.

5.1.3 Biomass composition and aggregation of the cells

The *rmlA* KO improved the purity of the produced WEs (see below) and made it easier to collect the cells by centrifugation, an effect caused by aggregation of the cells in the absence of the EPS capsule [81]. The lack of EPS production can be also considered an advantage because the excretion of EPS in the growth medium increases its viscosity, which in turn makes it more difficult to maintain good mixing and oxygenation of the growth medium [114]. However, it should be noted that it was not shown in the research article that the *rmlA* KO completely eliminates the synthesis of EPS, and so it cannot be ruled out that the ADP1ΔrmlA_pykF might produce smaller fragments of EPS in the growth medium. Rhamnose is part of the backbone of the EPS molecule produced by ADP1 (see Figure 4). Thus, it could be assumed that if the *rmlA* KO strain still produces some extracellular carbohydrates or EPS, they are either compounds of glucuronic acid, mannose and free glucose, or they are larger molecules with structures that do not contain rhamnose at all and are unable to form the EPS capsule around ADP1 cells. However, unidentified compounds were not observed in the HPLC analyses, indicating that at least free glucose or mannose were not produced. It should be also noted that ADP1 produces mannitol as a compatible solute during salt stress and produces mannitol-1P from fructose-6 (and probably mannitol

from mannitol-1P) [159]. Thus, ADP1ΔrmlA might have altered salt stress tolerance due to the increased concentration of fructose-6P, the starting metabolite for mannitol synthesis.

It has been shown that *Acinetobacter* sp. strain GJ12 grows as bacilli in a rich medium but as cocci in a poor medium [74]. It was suggested that this might be due to changes in the growth rate; under conditions allowing a slow growth rate, the cells would grow as small cocci, while under conditions allowing fast growth rate, they would grow as bacilli. The same kind of behaviour was observed with ADP1 (Figure S4 in Publication III). When grown on acetate or 4-HB, which allow faster growth rates, the cells were clearly larger and had a bacillar shape, but when grown on glucose, on which ADP1 grows slowly, there were also small cells with a coccal shape. The coccal shape of BD4 cultivated on glucose has been also observed by Taylor & Juni [181]. The phenomenon of cells being larger at high growth rates was first observed with *Salmonella typhimurium* in 1958 [166].

Although *A. baylyi* strains are usually considered to be non-pathogenic, they have been shown to be able to infect immune-compromised patients [24], and the EPSs produced by *A. baylyi* strains can harm the host macrophages and increase the pathogenicity of pathogenic bacteria [134]. In *E. coli*, adhesin Ag43 causes cells to aggregate and form biofilms [168], and biofilm formation is often the reason for persistent infections [26]. In *E. coli* ,a capsule prevents adhesin-mediated aggregation and biofilm formation [168]. Because of these characteristics, a non-flocculating, EPS-negative *A. baylyi* strain [82] might be the safest alternative for bioprocesses.

5.2 Improvements in the wax ester production

In order to be economically feasible, the bioprocess producing WEs should have high productivity, yield, titer and WE content of the biomass. For example, some cultivation conditions may result in a high WE content of the cells, but this might only have been achieved after an unacceptably long cultivation time. On the other hand, if high WE content were obtained when the cells could not be cultivated to high cell density, the WE titer would remain low.

5.2.1 Wax ester yield and productivity

The *rmlA* KO improved the WE yield on glucose as the carbon source, but not when acetate or 4-HB were used. It has been shown with the parental strain of ADP1 that the cells form approximately twice as large an EPS capsule on glucose than they do on acetate [181] and that the cells synthesize the EPS capsule much more rapidly and efficiently from glucose than from acetate [81]. Thus, it might be that the effects of the *rmlA* KO mutation on the WE synthesis of ADP1 are minimal on acetate since less carbon is directed to EPS synthesis.

The productivity in WE-producing bioprocesses can be increased (if WE-content of the cells does not change) by increasing the growth rate of the host organism. It was shown with TLC that the improved growth rate by *pykF* expression did not decrease WE accumulation by ADP1 (Publication I), thus increasing WE productivity. When the growth rate of *A. baylyi* is limited by nitrogen, the WE production increases with decreasing growth rate [56]. However, under carbon limitation, the growth rate slightly increases in WE production [56]. It is likely that the situation occurring when *pykF* is expressed in ADP1 is comparable to the latter case. As the *rmlA* KO did not affect the WE yield on acetate, but did lower the growth rate, it can be said that WE productivity was lowered by this modification (Publication III). This also applies to the *poxB* KO in ADP1ΔrmlAΔpoxB_pykF, which had unaltered WE production but a reduced growth rate on glucose as a sole carbon source (Publication IV). The WE productivity of ADP1ΔrmlA on 4-HB was unaffected, since neither the WE yield or growth rate were changed (Publication III). On glucose as a carbon source, since the WE yield was improved and the growth rate unaffected, the *rmlA* KO could be said to have increased WE productivity by ADP1_pykF. However, since the *rmlA* KO increased the length of the lag phase on glucose, acetate and 4-HB, the amount of WEs produced per cultivation time cannot be accurately estimated solely from the growth rate and WE yield values.

5.2.2 Wax ester titer

The only WE titer that has been reported with a WT ADP1 is approximately 0.16 g/l (Table 6), which was obtained in a 48-hour cultivation [162]. By expressing *pykF*, a similar titer could be obtained in 26 hours in a medium containing glucose, CAA and EtOH and the *rmlA* KO further improved the WE titer in a similar cultivation (Publication III). When ADP1ΔrmlA_pykF was cultivated in a medium that contained glucose, CAA and acetate, a 24-hour cultivation resulted in doubling of the WE titer (Publication IV). Furthermore, under these conditions, ADP1ΔrmlAΔpoxB_pykF, which was able to utilize acetate more efficiently, produced a WE titer that is approximately three times higher than that of ADP1 or ADP1_pykF. Thus, if compared to the WT ADP1, the two KO mutations and expression of *pykF* tripled the WE titer while halving the cultivation time. A WE titer that is 1.8 times higher than the one obtained with ADP1ΔrmlAΔpoxB_pykF has been obtained with *E. coli* engineered for the production of multi-methyl-branched long-chain esters (MBE) [118]. This value was, however, obtained in a high cell-density, fed-batch cultivation, where an OD_{600} of approximately 210 was achieved, and propionate, n-octanol and oleic acid were supplied to the growth medium [118]. *E. gracilis,* on the other hand, can produce a WE titer of 2.1 g/l when cultivated on glucose to a DCW of 4.2 g/l [71]. Thus, although *E. coli* can be routinely cultivated to high cell densities, it only seems to accumulate WEs to very small amount, making it difficult to increase the titers. The WE titer of ADP1 strains could be increased significantly if high cell density cultivation methods were developed for this strain. If the theoretical maximum cell density of 220 g/l [124] could be achieved with ADP1 producing a WE content of DCW (8.6 ± 0.7 %), WE titers close to 20 g/l could be obtained.

However, high cell density cultivations would probably have to be carried out in fed-batch cultivations. Batch cultivations, such as the ones carried out in this thesis, do not provide the cells with enough nutrients to grow to high DCW or, if they do, the elevated nutrient concentrations start to inhibit growth.

TABLE 6 WE titers obtained with different microorganisms.

Strain	WE titer (g/l)	Reference
A. baylyi ADP1	0.16	[162]
A. baylyi ADP1_pykF	0.15	Publication III
A. baylyi ADP1ΔrmlA_pykF	0.19	Publication III
A. baylyi ADP1ΔrmlA_pykF	0.31	Publication IV
A. baylyi ADP1ΔrmlAΔpoxB_pykF	0.45	Publication IV
E. coli engineered for MBE production	0.79	[118]
E. gracilis	2.1	[71]

5.2.3 Wax ester content of the biomass

The wax ester content of ADP1 was increased in the experiments of this thesis to 8.6 % (Table 7). The highest yield of *de novo*-produced WEs obtained with bacteria (14 %) was obtained by cultivating *A. calcoaceticus* NCIB 10487 on succinate [56]. This value is ~1.6 times higher than the value obtained here with ADP1ΔrmlAΔpoxB_pykF (Publication IV). It might be worthwhile to better characterize the strain NCIB 10487 to find out if it is suitable for bioprocesses and metabolic engineering. *R. jostii* RHA1 has also been shown to accumulate large amounts (13 %) of WEs, but this strain had to be grown for 72 hours to achieve that value [156]. Kalscheuer and Steinbüchel have reported a WE yield of 6.9 % w/v when cultivating APD1 for 24 hours on Na-gluconate in a medium containing 0.1 g/l NH_4Cl [87]. In Publications III and IV, where similar WE contents of biomass were obtained in cultivations of a similar length, the growth media contained 1.0 g/l NH_4Cl and 2.0 g/l CAA. Thus, ADP1ΔrmlAΔpoxB_pykF might be able to produce higher WE content in either longer cultivations (where the carbon to nitrogen ratio increases due to depletion of NH_4Cl) or in cultivations in poorer media. Even though significant improvements in the WE content of ADP1 were achieved, the highest value obtained is still ~17 % of that of the Jojoba plant seeds, and the metabolism of ADP1ΔrmlAΔpoxB_pykF would have to be further engineered to improve this value.

TABLE 7 The highest WE contents of biomass obtained with different organisms.

Organism	Carbon source(s)	WEs (% of DCW)	Reference
Escherichia coli BL21(DE3)[a]	Na-oleate (in LB medium)	~1	[89]
A. calcoaceticus NCIB 8250	Succinate	2.5 – 3.0	[54]
A. baylyi ADP1	Glucose, CAA	1.8	[162]
A. baylyi ADP1	Na-gluconate	6.9	[87]
A. baylyi ADP1ΔrmlA_pykF[a]	Glucose, EtOH, CAA	7.2	Publication III
A. baylyi ADP1ΔrmlAΔpoxB_pykF[a]	Glucose, acetate, CAA	8.6	Publication IV
R. jostii RHA1[a]	Glucose	13	[156]
A. calcoaceticus ATCC 17976	Acetate	13	[56]
A. *calcoaceticus* NCIB 10487	Succinate	14	[56]
Acinetobacter sp. M-1	N-Hexadecane	17	[72]
E. gracilis SM-ZK	EtOH	57	[71]
S. chinensis (seeds)	n.a.	~50	[158]

[a]Metabolically engineered strain.

5.2.4 Metabolism of the lignocellulosic carbon sources

5.2.5 Overflow metabolism

The formation of acetate or any other overflow metabolite could not be detected with ADP1_ctrl or ADP1_pykF cultivated on glucose (Publication I). However, when gluconate was used as a carbon source and ADP1_pykF had a similar growth rate, acetate and ethanol could be detected in the HPLC samples of ADP1_pykF. Thus it seems that the overflow metabolism of ADP1 can be triggered by expression of *pykF* but this occurs only when gluconate is used as a carbon source, but not on glucose. Gluconate has lower energy content than glucose and can be directly taken up and catabolized by the cells, while glucose needs to be oxidized into gluconate prior to being taken up. Thus, although the cells grew at similar rates on both carbon sources, the carbon flux towards metabolites that can be converted to acetate, pyruvate and acetyl-CoA, could have been larger when cultivated on gluconate. However, since bioprocesses using ADP1 most likely use glucose instead of gluconate as a carbon source, the overflow metabolism leading to acetate and ethanol production is not likely to be a problem.

In Publication III it was shown that in the cultivations of ADP1ΔrmlA_pykF in a glucose medium, CAA, EtOH (from the Cm stock solution), acetate and low pH can be observed at the end of the cultivation at 26 h (Publication III). In publication IV it was shown that the acetate was formed in the medium by EtOH catabolism. In Publication III it was shown that ADP1ΔrmlA grows slower on acetate (and thus also consumed acetate slower) than ADP1. Thus the accumulation of acetate to the growth medium by ADP1ΔrmlA_pykF could be explained simply by the fact that, if both strains produced acetate from EtOH, the consumption of acetate by ADP1ΔrmlA_pykF was merely slower than with ADP1_pykF.

The acetate production from EtOH and subsequent accumulation to the growth medium by ADP1ΔrmlA_pykF could be alleviated by *poxB* KO (Publication IV). The strain ADP1ΔrmlAΔpoxB_pykF also produced acetate, but consumed it much more rapidly in the presence of glucose than ADP1ΔrmlA_pykF, which prevented the pH from decreasing to the level that no longer supports growth. ADP1ΔrmlAΔpoxB_pykF consumed EtOH more rapidly than ADP1ΔrmlA_pykF when cultivated on EtOH as a sole carbon source, but unexpectedly, it also produced more acetate than ADP1ΔrmlA_pykF. Since the only difference between the strains was the *poxB* KO in ADP1ΔrmlAΔpoxB_pykF, it is unclear why this happened. Intuitively, one might assume that more acetate would accumulate if the cells had pyruvate oxidase activity, which produces acetate from pyruvate. On the other hand, since acetate is also produced as an intermediate of EtOH catabolism, the lack of pyruvate oxidase activity might allow more rapid growth as the cell can funnel more carbon into gluconeogenesis. This could thus allow a faster growth rate, leading to more acetate production due to the increased amount of enzymes for conversion of EtOH to acetate at elevated cell densities.

5.2.6 Biodetoxification

The oxidation of the main lignocellulosic sugars, glucose, xylose and arabinose, could be completely prevented by producing a *gcd* KO in ADP1. This strain, ADP1Δgcd, could rapidly consume acetate and formate in a medium containing the aforementioned sugars. The presence of 4-HB in the growth medium allowed ADP1Δgcd to consume formate more efficiently. Formate, unlike acetate, did not repress 4-HB catabolism. This allows the smallest organic acid to be consumed faster, if acetate is depleted from the medium. Thus, although acetate represses consumption of 4-HB, it might be expected that the lignocellulosic hydrolysate still contains other compounds in addition to 4-HB, once the acetate had been consumed. Because of this, there might not be sequential use of inhibitors, if they are treated as inhibitor groups (aromatic inhibitors, aliphatic inhibitors, etc.). However, the fact that 2.7 g/l of acetate seems to inhibit glucose consumption is more troubling (Publication IV), as this would delay consumption of the most abundant component of lignocellulosic hydrolysates, glucose. While this is not a problem in the detoxification of lignocellulosic hydrolysates, it might reduce the efficiency of *gcd*-containing strains.

ADP1Δgcd tolerated glucose well up to 100 g/l, and also grew also at 150 g/l and was able to grow on an elevated concentration (10 g/l) of acetate. ADP1Δgcd did not start to consume sugars even in a seven-day cultivation where an OD_{600} value of ~5 was reached. With *E. coli* engineered not to consume lignocellulosic sugars, it has proven to be difficult to completely eliminate sugar consumption [100]. Thus, it seems that ADP1Δgcd might be more suitable for selective removal of acetate from sugar-containing solutions than the *E. coli* strain engineered for the same purpose. However, a major limitation of using ADP1Δgcd as a detoxification strain is that it cannot grow on all growth inhibitors found in lignocellulosic hydrolysates, such as furfural, 5-hydroxymethylfurfural (5-HMF) and levulinate. Furfural and 5-HMF are often present in smaller quantities in lignocellulosic hydrolysates than acetate [179], but they have been shown to inhibit

growth and ethanol production by an ethanologenic *E. coli* at these concentrations [205]. Engineering furfural degradation in ADP1 should be feasible, since this has already been achieved in *P. putida* S12 [96]. This should improve the performance of ADP1 as a biodetoxification strain and also as a host organism in other lignocellulose-utilizing bioprocesses, because this compound could serve as an additional carbon source.

ADP1Δgcd was used in the elimination of oxygen from closed cultivation tubes, which allowed H_2 production by the obligatory anaerobe *C. butyricum*. Similar experiments have been carried out previously by Tran et al. who eliminated the need for O_2 removal by N_2 flushing by cultivating *Bacillus subtilis*, which also simultaneously saccharificated the starch, allowing acetone-butanol-EtOH production by *C. butyricum* [184]. In addition to removing O_2 from the lignocellulosic hydrolysates to allow cultivation of anaerobic microorganisms, ADP1Δgcd could also be engineered to produce cellulases, to achieve further hydrolysis of cellulose, or other compounds that are beneficial to the organism fermenting the biodetoxified lignocellulosic hydrolysate.

6 Conclusions

ADP1 naturally accumulates WEs but the titers are not sufficiently high for the WE-producing bioprocesses to be economically feasible. ADP1 also grows slowly on glucose, the most common carbon source in bioprocesses, commits a large fraction of the carbon to EPS synthesis, and does not catabolize glucose in the presence of acetate. By engineering the metabolism of ADP1, it was possible to double the growth rate on glucose, improve the WE yield and purity by eliminating EPS capsule synthesis, and improve growth and WE production in the presence of acetate.

The growth rate of ADP1 on glucose was increased to 0.42 h^{-1} by expressing *pykF* from *E. coli* K-12 MG1655 (Publication I). This growth rate is still far from the values obtained, for example, with *E. coli* W3110 (~ 1.0 h^{-1}) [3] but this improvement nevertheless halves the time needed to run the bioprocesses where WEs are produced with ADP1 from glucose. On the other hand, when compared to other WE-producing microorganisms like *R. jostii* (0.19 h^{-1} (when carrying an empty plasmid pNV18)) [202], the growth rate of ADP1_pykF obtained here is relatively high. The growth rate and biomass yield from glucose might be further improved by integrating *pykF* into the genome of ADP1, since maintenance of a plasmid is known to reduce growth rate and biomass yield [170]. This would also eliminate the need of supplementing the growth medium with antibiotics. The mass of the NMR sample in the experiment yielding the highest WE titer (0.45 ± 0.05 g/l) was relatively low (5.2 ± 0.1 g/l) and WE production by ADP1 would benefit tremendously from the development of high cell-density cultivations for this strain.

By knocking out *rmlA*, the gene for the first step in the synthesis of the main component of EPS, it was possible to improve the WE yield of ADP1_pykF on glucose. This KO mutation also reduced the biomass yield from glucose and made it easier to collect the cells by centrifugation, thus improving the WE purity and reducing the cost of downstream processing. The effects of the *rmlA* KO mutation could not be extended to cultivations on acetate and 4-HB, which are also components of lignocellulosic hydrolysates. While having a doubled WE yield from glucose, ADP1ΔrmlA_pykF had a decreased tolerance towards acetate produced from EtOH in the growth medium. This drawback could

be eliminated by knocking out the gene for pyruvate oxidase, *poxB*, from the genome of ADP1ΔrmlA_pykF (Publication IV). Furthermore, the *poxB* KO improved the WE yield in a medium containing glucose and acetate as carbon sources. Since glucose and acetate are both among the most abundant carbon sources found in lignocellulosic hydrolysates, this modification should improve the properties of ADP1 as a host organism in bioprocesses using this raw material. In the presence of glucose as a sole carbon source, the growth rate of ADP1ΔrmlAΔpoxB_pykF was lower than that of ADP1ΔrmlA_pykF, indicating that, as with *E. coli* W3110 [3], pyruvate oxidase is beneficial when cultivated on this carbon source. Because of this, it might be beneficial to control expression of *poxB* in ADP1ΔrmlA_pykF so that the gene is expressed when only glucose is present, but not in the presence of both glucose and acetate.

When the gene for GDH, *gcd*, was knocked out from the genome of ADP1, the strain was rendered completely unable to catabolize lignocellulosic sugars (Publication II). These kinds of strains are suitable for the selective removal of growth inhibitors from lignocellulosic hydrolysates. The initial characterization of ADP1Δgcd indicated that it could perform efficiently as a biodetoxification strain, although the robustness of the strain should be tested with different kinds of lignocellulosic hydrolysates. Also, since the strain is unable to consume, for example, furfural and 5-HMF, the growth substrate range of this organism should be broadened to include these inhibitors.

In this Doctor of Science thesis it was shown that the properties of ADP1 as a host organism in WE-producing bioprocesses can be readily improved with metabolic engineering, and that this organism has the potential to become an efficient host organism in the biodetoxification of lignocellulosic hydrolysates. While the modifications made here improved both growth characteristics and WE-production, the catabolic capabilities of this organism should be broadened to allow consumption of other lignocellulosic sugars in addition to glucose. The robustness of ADP1 also needs to be elucidated by characterizing its growth in actual lignocellulosic hydrolysates. As biodetoxification using ADP1Δgcd is time-consuming, it might be necessary to engineer other functions like cellulase production to ADP1Δgcd to make biodetoxification using this strain more attractive. The WE structure could be tailored for specific applications, which would increase the value of this product. The development of a strain for the production of tailored WEs with better properties than those of the Jojoba plant would make it much more likely that ADP1 could be used in industrial WE production. The rapid development of the field of synthetic biology should make it increasingly easy to achieve these goals. In addition to engineering the metabolism of ADP1, cultivation techniques should be developed which allow the generation of high cell-density, since only that could allow a high titer of intracellularly accumulating WEs to be produced. The WE titers obtained in this thesis are still far from being high enough for industrial production for applications such as biofuels. Nevertheless, hopefully the progress presented in this thesis will encourage other researchers to pursue the goal of engineering ADP1 into an efficient WE producer.

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ORIGINAL PAPERS

I

METABOLIC ENGINEERING OF ACINETOBACTER BAYLYI ADP1 FOR IMPROVED GROWTH ON GLUCONATE AND GLUCOSE

by

Matti Kannisto, Tommi Aho, Matti Karp & Ville Santala, 2014

Applied and Environmental Microbiology 80:7021-7027

II

METABOLIC ENGINEERING OF ACINETOBACTER BAYLYI ADP1 FOR REMOVAL OF CLOSTRIDIUM BUTYRICUM GROWTH INHIBITORS PRODUCED FROM LIGNOCELLULOSIC HYDROLYSATES

by

Matti S. Kannisto, Rahul K. Mangayil, Ankita Shrivastava-Bhattacharya, Brett I. Pletschke, Matti T. Karp & Ville P. Santala, 2015

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III

GROWTH AND WAX ESTER PRODUCTION OF AN ACINETOBACTER BAYLYI ADP1 MUTANT DEFICIENT IN EXOPOLYSACCHARIDE CAPSULE SYNTHESIS

by

Matti Kannisto, Elena Efimova, Matti Karp & Ville Santala, 2017

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IV

METABOLIC ENGINEERING OF ACINETOBACTER BAYLYI ADP1 FOR IMPROVED GROWTH AND WAX ESTER PRODUCTION ON MIXTURES OF GLUCOSE AND ACETATE

by

Matti Kannisto, Elena Efimova, Tapio Lehtinen, Milla Salmela, Teodoro Fiumara, Suvi Santala, Matti Karp, Simone Guglielmetti, Diego Romano & Ville Santala, 2017

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