BACTERIAL WAX ESTER PRODUCTION FROM VOLATILE FATTY ACIDS



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This thesis is dedicated to my dad, Paul, and my grandad, Louis,
who took great pride in everything I achieved.

I hope this would have made you proudest of all.

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Declaration

I declare that the work contained within this thesis is entirely my own, except for the contributions stated below, which I would like to acknowledge.

Chapter 3

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Chapter 6

The application of zeolite for removal of ammonium from acidogenic fermentate was conceptualised and developed by Nicholas Tenci.

Abstract

Waxes are industrially important commodity chemicals that have applications in a variety of sectors, ranging from cosmetics and additives to industrial lubricants. However, at present they are overwhelmingly fossil fuel derived. In order to reach global targets on greenhouse gas emissions and tackle climate change, fossil fuel usage must rapidly decrease and the source of carbon in key chemicals must shift from fossil to biological.

One biological source of waxes for potential exploitation is micro-organisms, which accumulate lipids, such as waxes, as carbon storage molecules in response to stress. However, currently the process is not economically viable, due in part to the high cost of sugar substrates, and hence alternative low-cost substrates are of great interest. Volatile fatty acids (VFAs) sourced from the acidogenic fermentation of organic waste, including sewage, agricultural and municipal wastes, could provide one such low-cost carbon source for affordable bioproduction.

The research in this thesis focused on bacterial wax production by *Acinetobacter baylyi* ADP1 from acidogenic fermentate, the raw mixture of VFAs derived from the anaerobic degradation of organic waste. Growth of the strain on the abundant VFAs was characterised, and gene overexpression was attempted to improve VFA utilisation. A mutant strain of *A. baylyi* ADP1 with the glyoxylate shunt knocked out was shown to produce waxes from synthetic VFAs and their mixtures, as well as from fermentate. In the course of this study, the highest titres obtained to date from VFA substrates of 163 ± 5 mg/L were achieved. However, the high nitrogen content of the acidogenic fermentate was identified as a significant limitation.

The impact of growth conditions on wax ester production was also investigated thoroughly, identifying the optimal C:N ratio for wax production from acetate, which was employed as a proxy for mixed VFA-rich fermentate. The effects of phosphorus and other micronutrient limitations were also explored, and, for the first time in bacteria, the potentially compounding effects of multiple limitations were methodically and extensively investigated. Attempts were then made to replicate the optimal C:N ratio in acidogenic fermentate by removing ammonium ions from the fermentate by two distinct methods: gas stripping of ammonia and ion exchange, employing zeolites as a novel binding matrix. Overall, the work in this thesis aims to improve our genetic and physiological understanding of bacterial wax production, and contribute towards the realisation of sustainable microbial waxes for commercial and consumer use.

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List of Abbreviations

ACC	acetyl coenzyme A carboxylase
ACS	acetyl coenzyme A synthetase
AckA-Pta	acetate kinase – phosphate acetyltransferase
AD	anaerobic digestion
ADP1	*Acinetobacter baylyi* ADP1
AF	acidogenic fermentation
CAM	crassulacean acid metabolism
CDW	cell dry weight
C:N	carbon: nitrogen
FAS	fatty acid synthase
FID	flame ionisation detector
GC	gas chromatography
GFP	green fluorescent protein
NMR	nuclear magnetic resonance
OD_{600}	optical density at 600 nm
PHA	polyhydroxyalkanoate
RBS	ribosome binding site
RpoN	RNA polymerase, nitrogen limitation N
SDG	sustainable development goal
TAG	triacylglycerol
VFA	volatile fatty acid
WE	wax ester
WT	wild type

Chapter 1 – Introduction

Anthropogenic carbon emissions in the past 150 years have caused unprecedented changes in the Earth's climate. Indeed, 2023 is predicted to have been the hottest year on record, at an estimated 1.1 °C above pre-industrial levels (1), and is known to have contained both the warmest global month (July) and day (6th July) ever recorded (2). In fact, it has been almost 50 years since a year of below average global temperature occurred, based on the 1950-1980 average. With rising temperatures, our world has become increasingly unpredictable and dangerous. The number of 'weather-related disasters' has risen five-fold since 1970 (3), including storms, wildfires, droughts, floods and extreme temperatures. The rate of sea-level rise in the past decade was more than 2.5 times the average rate over the past century (4, 5), driven largely by thermal expansion due to rising temperatures, increasing the rate and intensity of many natural disasters. Climate change is thus no longer a problem for the future; it is already having far-reaching and devastating effects. The political and technological response must increase rapidly and unanimously to prevent further irreversible damage to the delicate ecological balance of the planet.

In response to mounting pressure and worsening planetary vital signs, the United Nations Sustainable Development Goals (SDGs) were developed in 2015 as 'an urgent call to action by all countries in a global partnership' (6). The 17 goals were designed to recognise that sustainability is both social and environmental, and that many of the challenges facing humanity today are inherently interlinked. In the same year, the Paris Agreement was signed by 198 parties with an overarching goal to limit average global temperature rise to 'well below 2 °C above pre-industrial levels' and to 'pursue efforts to limit the temperature increase to 1.5 °C' (7). This legally binding treaty aimed to jumpstart political action on climate change by forcing countries to commit to individual emissions reductions targets, which must increase in ambition every five years. While it has had some successes, including encouraging major global economies and companies to set net zero emissions goals and supporting legal challenges against governments and multinational corporations for insufficient action on climate change (8, 9), many consider the Paris Agreement to be unambitious and inadequate. Even if all the pledges and targets were met, the estimated average warming would be 2.1 °C (10), which is outside the self-agreed range. Beyond this, many countries do not have policies that reflect their targets, and those that do are largely considered off-track in meeting them

without being held to account. Although estimates vary, the current 'Business as Usual' scenario, used to describe the projected emissions and associated temperature increase if behavioural patterns and rates of change continue in line with the recent past, suggest a global temperature rise of approximately 0.3 °C per decade (11). This would result in over 3 °C of temperature increase by 2100 (12), well outside the limits of the Paris Agreement and what climate scientists consider safe boundaries.

To meet the Paris Agreement targets and achieve the SDGs, societal dependence on fossil fuels must be reduced and, ultimately, ended. At present, two of the biggest areas targeted for emissions reductions are the energy and transport sectors, both of which are heavily fossil fuel reliant. Energy generation, specifically electricity and heating, accounted for 43% of global CO_2 emissions in 2019 (13), the largest single contributor by more than 20%. However, a transition to low-carbon alternatives is rapidly approaching. In 2025 renewable energy is expected to contribute 35% to global electricity generation and the figure is rising year on year (14). At the same time, electricity generation often now costs less from renewables, predominantly solar and on-shore wind, than from fossil fuel sources like coal and natural gas (15), and prices continue to fall (16). Meanwhile, transport currently accounts for 21% of global CO_2 emissions, with nearly 75% of these from road vehicles (17). With a rise in renewable energy generation, and a foreseeable future of 100% renewable electricity, electric vehicles are a logical step to eliminating another large portion of global emissions. Although there are limitations, including battery costs, metals availability and infrastructure deployment (18, 19), the trends are upwards. Electric vehicles comprised just 4% of total global vehicle sales in 2020, but this rose rapidly to 14% by 2022 and 18% in 2023 (20). Similarly, the total number of electric vehicle charging stations in the UK increased 33% from April 2022 to 2023 (21), indicating continued growth is expected in the area. These changes are continually reducing reliance on carbon-based fuels.

Another major use of fossil fuels is in chemical and materials. In 2022, 2.3 billion tonnes of non-fuel petrochemicals and materials were produced globally (22), to manufacture everything from clothes and personal care items to packaging and building materials. Despite efforts to move towards more reusable materials and reduce unnecessary consumption, in line with number 12 of the UN SDGs, demand for these carbon-based products is forecast to grow until at least 2050 (23), by which time petrochemicals will account for almost half the total growth in oil demand as demand in energy and transport decreases due to electrification. The International Energy Agency recently identified petrochemicals as 'one of the key blind spots

in the global energy debate' (23), with current policies largely failing to address the dependence of the chemical industry on fossil fuels. Unlike many other sectors, decarbonisation of petrochemicals is not an option because decarbonisation of carbon-based materials is a fundamental contradiction.

The source of the carbon within such products is, however, within our control and is one of the keys to making them sustainable. With fossil-derived carbon off limits for achieving climate and sustainability targets, two major sources remain – atmospheric CO_2 and biomass. Utilisation of the former for chemical and materials production is a very attractive proposition, as there are around 3.2 trillion tonnes available (24) (over 1 trillion tonnes of which have been added since the 19th century), distributed evenly across all geographic locations. Furthermore, consumption of CO_2 from the atmosphere would not only prevent future emissions but (at least temporarily) actively reduce the present concentration of the greenhouse gas, offering the potential for carbon negative products. Understandably there has been great interest in this concept, which is widely referred to as carbon capture and utilisation (CCU). Unfortunately, to date the deployment of such technology has been hampered by high costs, large energy requirements, a lack of economic incentives and weak policy (25, 26), and lags well behind what would be required to reach the emissions targets required by the Paris Agreement (7, 27). Where successful, the carbon capture has been performed almost exclusively from large CO_2 point sources, such as fossil fuel power stations, where a waste stream with very high CO_2 concentration is available. Direct air capture is more technologically challenging, hugely energy intensive and expensive because CO_2 makes up just 420 ppm (or 0.04 %) of molecules in the air.

While human technology is currently woefully inadequate at capturing CO_2 from the atmosphere, an enormous variety of photosynthetic organisms have been doing exactly that for approximately three billion years (28). In this sense, biomass can be considered as captured atmospheric CO_2 and, while not as geographically ubiquitous, it offers equivalent benefits on CO_2 levels and emissions. This makes it the obvious choice of carbon source for replacing many petroleum derived chemicals and materials. However, while it is inherently renewable, biomass is still a limited resource. The capacity of the Earth to produce biomass is already being tested: biodiversity is being eroded by monocultures, soil quality is deteriorating due to intensive farming and almost every ecosystem is threatened by the need to produce food for an ever-growing human population. It may seem hard to imagine then, where the additional resources needed to replace fossil fuels in chemical production could be found. This concern

is very real. Poor biofuels policies in the past few decades have incentivised farmers to grow fuel crops instead of food and resulted in significant deforestation (29) while potentially matching or exceeding the greenhouse gas emissions of the equivalent petroleum fuel (30). Such examples have brought to light the problems that can arise if this situation is managed incorrectly.

However, this does not mean it is unachievable. Billions of tonnes of organic waste are generated every year globally as the waste products of agriculture or municipal waste management (31, 32). Whether through burning or fermentation processes, this material typically ends up as CO_2, but contains more than sufficient carbon to replace fossil carbon in the chemical industry. With a recognised need for better management of organic materials, 6 of the 17 SDGs are directly related to the production and utilisation of biomass, highlighting the importance of making well informed, conscientious decisions about the exploitation of this vital resource.

The concept of a biorefinery is often loosely defined as 'the sustainable processing of biomass into a spectrum of bio-based products (food, feed, chemicals, materials) and bioenergy (biofuels, power and/or heat)' (33). It is intended as a bio-based, renewable alternative to a traditional oil refinery, in which a range of fuels and synthetic chemicals are produced from crude oil and raw natural gas. For chemical production, long chain hydrocarbons, many of which are themselves of low value, are transformed by cracking into a limited number of platform chemicals, with the five major products being ethylene, propylene, benzene, butadiene or xylene. The platform chemicals obtained are controlled by the conditions under which cracking occurs, and from these a plethora of chemically diverse, high value products can be synthesised, including agrochemicals, polymers, pharmaceuticals, adhesives, cosmetics, flavourings and more.

Crucially, the majority of 'petrochemicals' are not naturally present in petroleum: they are synthesised from it. In a similar way, such chemicals could instead be synthesised from biomass, if an appropriate and reliable pool of platform chemicals can be obtained. This describes a more complete picture of a biorefinery, in which structurally complex and heterogenous biomass, potentially from a wide number of sources, is first broken down into a small pool of platform chemicals, which can then be used to synthesise a wide range of desired products. However, the choice of the initial degradation step, and thus the platform chemicals obtained, is still for debate. One proposed option for this homogenisation process is anaerobic digestion (AD) (32). AD is used for the degradation of a wide range of organic material,

including sewage sludge, agricultural residues, animal waste and municipal waste, yielding a mixture of biomethane, biohydrogen, CO_2 and volatile fatty acids as products. The exact product composition is variable depending on reactor conditions, but can be reliably controlled, resulting in a comparable 'platform chemical pool' to petrochemical cracking.

Once platform chemicals are obtained, the final stage in the biorefinery model is upgrading to more complex, higher value chemicals. To produce a diverse range of chemical products, a similarly diverse range of reactions and processes will be necessary. This will most likely include a mixture of chemical processes (such as oxidation, reduction, polymerisation, dehydration, dehydrogenation to name but a few) and biological ones (enzymatic reactions and microbial fermentations). While chemical reactions will likely be more efficient for well-established reactions and products, microbial biosynthesis can offer some key advantages in chemical production (34). Metabolic pathways to many complex molecules are already naturally established, or can be easily engineered, in micro-organisms compared to the complexity of a chemical process. Enzymes are often highly specific, limiting side product generation (35), and microbes can often co-utilise several feedstocks, so can be fed a mixed input stream while a chemical reactor might need a pure one (36). Finally, reactions that chemically require organic solvents, extreme temperatures and pressures, or hazardous reagents (acids, bases, heavy metal catalysts) can be performed under benign, biological conditions (34, 37).

Based on the principles discussed here, the research detailed in this thesis focuses on the production of a commercially important petrochemical product, waxes, by bacterial biosynthesis from a widely available waste stream; mixed volatile fatty acids generated from anaerobic digestion. While the precise results are specific to the bacterial species and product chosen, it is hoped the overall findings and general approach will be transferable to a range of bioproducts that can be generated in micro-organisms. The purpose of this is to contribute in some small way towards the realisation of a complete biorefinery model for sustainable chemical production, and to eventually replace all petrochemicals with biochemicals.

1.1. Chapter breakdown

The next chapter, Chapter 2, presents an introduction to the interest in, and production of, bacterial wax esters (WEs) from renewable, sustainable feedstocks. It justifies the choice of organism used in this work, the bacterium *Acinetobacter baylyi* ADP1, and the use of acidogenic fermentation (AF) to produce volatile fatty acids (VFAs) as a feedstock. It also contains an in-depth, published review article on WE production in bacteria, examining the range of bacterial species, feedstocks, and genetic modifications used so far, and highlighting both successful approaches and key areas for future development.

Chapter 3 describes initial experiments exploring the behaviour of *A. baylyi* ADP1 on the three main volatile fatty acid substrates found in acidogenic fermentate: acetate, propionate, and butyrate. While growth on acetate and butyrate has been reported in the literature, their use as sole substrates without the addition of a glycolytic or amino acid rich carbon source is not well documented and the tolerance to and growth rate on these substrates is not known. This chapter also details some early-stage research on a gene overexpression plasmid developed to increase acetate uptake and utilisation, with the aim of improving WE accumulation. While improvements were observed, the magnitude of these improvements was low, and hence this system was not expanded on further.

In Chapter 4, a high-wax production mutant strain of *A. baylyi* ADP1 engineered by the Santala group at Tampere University, Finland was compared to the wild type bacterium. Tolerance to and wax production on minimal synthetic media with VFAs was explored and optimised, demonstrating the success of this strain without the need for a glycolytic substrate. Attempts were made to translate these findings to the VFA-rich liquid generated from AF, highlighting both the potential of engineered strains and the additional complications and limitations of working with raw, mixed substrates and waste streams. This chapter is presented as a pre-print journal article, prepared for submission.

Chapter 5 focuses on investigating the effects of nutrient limitation of WE accumulation in *A. baylyi* ADP1 under a range of nutrient limitations and forms the basis of a manuscript published in Bioresource Technology Reports in 2023. The study is the first to take a systematic approach to examine the effect of nitrogen limitation on wax titres and yields, revealing an optimal carbon: nitrogen (C:N) ratio range for production. A similar approach was taken for phosphate limitation, which was previously found to improve triglyceride accumulation but remained to date untested for wax production. The combination of nutrient limitation parameters was also explored to detect compounding effects.

Based on the observations detailed in Chapter 5, research in Chapter 6 attempted to replicate these results by removing nitrogen from acidogenic fermentate to achieve the ideal C:N ratio using a raw mixed culture medium. Employing a nitrogen removal technique developed in our lab, based on ion-exchange in the porous, alumina-silicate zeolite, a high nitrogen content synthetic medium was treated to the optimal C:N ratios, resulting in consistent, improved wax production. Based on these promising results, the technique was applied to fermentate, but the benefit observed previously was not achieved. To gain further understanding, gas stripping of ammonia as a method of nitrogen removal was also explored, with mixed results.

Chapter 7 outlines recommendations for the direction future work should take to build on the findings of this thesis. This includes short-term plans to complete the work of Chapter 6, identifying the cause of the complications observed in the zeolite treatment of the fermentate that prevent wax accumulation. Long-term research goals which build upon, but are not directly linked to, the findings of this thesis are also suggested.

Chapter 2 - Literature review

This chapter mainly comprises a literature review, which is divided into four parts. The first presents a background on waxes as a materials class, their industrial relevance and how they are categorised based on their origin and chemical composition. In particular, the advantages and most common sources of natural waxes are discussed, along with the limitations of these current production methods. This leads to the introduction of microbial WEs as a potential biochemical of interest in the future.

The second and third parts introduce and explore the specific bacterial species and feedstock that were chosen for this project, respectively; *Acinetobacter baylyi* ADP1 and VFAs generated by acidogenic fermentation of crassulacean acid metabolism (CAM) plants. Part 2 comprises an overview of the rise of *A. baylyi* ADP1 as a model organism for synthetic biology and briefly summarises its application and relevance for bacterial WE production. Part 3 provides an overview of anaerobic digestion and acidogenic fermentation as methods of generating economic value and renewable resources from waste, as well as a summary of suitable feedstocks and an overview of CAM plants. The advantages of acidogenic fermentation for generating VFAs for chemical production over traditional anaerobic digestion for biomethane are discussed and the current state of the art in utilising these VFAs as feedstocks for the generation of microbial lipids is summarised.

The fourth section of this chapter provides a summary of literature on bacterial WE synthesis in the form of a review article published in Biotechnology Advances in 2021, which details a variety of strains of relevance, feedstocks that have been investigated and genetic engineering approaches. A discussion of the current limitations and areas for future focus is also included.

The final sections of this chapter summarise the identified research gap and give the aims and objectives of the research detailed in this thesis.

2.1. Why we want waxes

Waxes are lipophilic, organic substances exploited in a range of applications, including coatings, lubricants, packaging, cosmetics and as food additives (38). Industrially, waxes are divided into 3 distinct categories: (i) mineral waxes such as paraffin wax, which are direct crude oil derivatives; (ii) synthetic waxes, which are chemically synthesised from fossil fuels such as natural gas; and (iii) natural waxes, derived from plants, including jojoba and carnauba waxes, or animals, such as beeswax and spermaceti. In 2023 the global wax market was valued at over $10 billion, and mineral waxes accounted for 68% of this, with synthetic waxes accounting for the majority of the remaining share (39). Natural waxes made up just 12% of the total industrial wax production by market share (40), and since natural waxes have higher value this number likely over-represents the contribution of natural wax to global wax production - in 2020 natural waxes were just 5% of all waxes sold (by mass) (41).

The market as a whole is forecast to grow to between $14.2-16.7 billion by 2030 (39, 40). This growth is driven by the desirable properties of waxes and their derivatives, including water and chemical resistance and non-toxicity. Currently the vast majority of this growth is projected to be in mineral and synthetic waxes, with markets for both expected to increase around 70% from 2020 to 2030 (40). Over the same period, the predicted growth in the natural wax market is much smaller, at just 20%, due to their higher cost and lower availability.

Natural waxes

Natural waxes are produced in or by living organisms to serve a biological function, with specific compositions beneficial for particular applications. They possess material advantages over their mineral and synthetic counterparts. Being biologically derived, they have much lower embedded and associated carbon emissions, with the potential to be fully renewable. Their composition is also environmentally favourable: while petroleum-based waxes are almost exclusively hydrocarbons, animal and plant waxes are a mixture of long chain organic molecules, including WEs, fatty acids and alcohols, and triacylglycerols (TAGs). The additional chemical functionalities in these molecules make them better suited to biological processes, improving biocompatibility and biodegradability.

Current applications of plant waxes include jojoba wax in personal care products, lubricants and pharmaceuticals (42), candelilla wax as a common a food additive and varnish (43), and carnauba wax in vehicle, shoe and furniture polishes (44). Transgenic plants have also been specifically designed to produce waxes with tuneable compositions (45, 46).

However, since plant waxes rely on crop yields, production is land-limited and seasonal, with availability and price susceptible to low yields or crop failures (47, 48). Sourcing waxes directly from plants can also lead to the environmental and social issues of large-scale agriculture, making their sustainability a highly complex matter. Beeswax is the most common animal wax, which is used in an enormous range of applications, from candles and cosmetics to food additives and preservatives (49-51). Whilst renewable and bioderived, the sustainability of industrial scale production of beeswax, like plant waxes, is highly debated. Farmed bees may pose risks to wild insect species due to competition and the spread of disease (52), damaging already threatened natural pollinator populations.

Typically, natural wax containing products are relatively high-value and produced at small-scale, as the high cost and low productivity of natural waxes prohibits their use in low value, high through-put processes. Crucially, due to current limitations on production, demand for many of these waxes outstrips supply (47), and if costs decreased, uptake would likely increase in markets currently serviced by petrochemical waxes, due to growing regulatory pressures and consumer preferences for renewable, sustainable products (53).

Wax esters

Wax esters, a major component of natural waxes, are the product of esterification between a fatty acid and a long chain alcohol. They display a wide range of properties, depending on the length of the carbon chain and degree of saturation in both the alcohol and acid, which vary greatly depending on the source and biological purpose (54, 55). WEs are the primary component in a number of high value, highly desirable natural waxes including beeswax, carnauba wax, jojoba oil and spermaceti wax, a high value wax used in industrial lubricants, cosmetics and textiles until the ban on commercial whale hunting in 1982 (55, 56). With all of this in mind, a sustainable method to produce low cost natural WEs at large scale is of great interest to meet demand in growing wax markets.

A potential new source of wax esters

Micro-organisms are known to accumulate neutral lipids, such as WEs and TAGs, as a way to capitalise on an excess of available carbon in their environment when other essential nutrients needed for growth are in short supply (57-59). These carbon stores can subsequently be used by the cell should the extracellular carbon supply run-out. This behaviour allows carbon-storing species to survive future periods of starvation, and to outcompete other species in environments where nutrient supplies are variable. The production of WEs specifically as neutral lipid storage

molecules has been observed in a number of species of bacteria (60-64), as well as in several species of yeast and algae (64-70).

2.2. Choice of microbial species

The rise of *Acinetobacter baylyi* ADP1 as a model organism

Escherichia coli has dominated the fields of microbiology and genetics for the past century. After its discovery in 1884 (71), the microbe was studied extensively through-out the early 1900s due to its non-pathogenic nature, ease of isolation from numerous sources, and rapid, reliable growth on many different nutrients. By the 1940s, during the dawn of molecular biology (72), *E. coli* was already well established as a model organism. This early dominance resulted in many fundamental concepts and principles being discovered and established within the species (72, 73), and with them a host of molecular methods were developed that further enhanced the bacterium's position as biologists' organism of choice. The inception of genetic engineering resulted in an explosion of genetic tools that reinforced this positive feedback loop, and *E. coli*'s place as the most studied micro-organism in history was cemented (74).

However, as the breadth and complexity of genetic circuits and modifications have increased, the desire for alternative model organisms for genetic engineering has grown (75). For many applications, alternative species may offer more attractive metabolic features or endogenous pathways (76). While not as widely studied as *E. coli*, tools have recently been developed for several species suggested as next-generation chasses.

One such proposed chassis is the soil bacterium *Acinetobacter baylyi* ADP1 (77, 78). Like *E. coli*, it is Gram negative and possesses many of the same desirable features, including ease of cultivation and rapid growth on a diverse range of substrates, including long-chain lipid molecules and aromatics. However, the unique advantage of *A. baylyi* ADP1 is that the strain is naturally competent during the exponential growth phase, meaning it can efficiently uptake DNA in both plasmid and linear form (79, 80). While plasmid uptake allows for easy transformation, linear fragments can be directly incorporated into the genome through homologous recombination (81). This ability also extends to the assembly of plasmids *in vivo* via the addition of suitably chosen linear DNA fragments, further simplifying and streamlining DNA cloning (82). In this regard, *A. baylyi* ADP1 is naturally superior to *E. coli* for efficient, high-throughput genetic manipulation (83).

With the recognition of these unique benefits, several genetic studies have been conducted and tools developed to bring forward the realisation of the potential of *A baylyi* ADP1. The first

significant breakthrough was the publication of the complete genome sequence (84). This facilitated the generation of a complete collection of single gene deletion mutants, elucidating gene essentiality data at a genome-wide level (85), and a simple metabolic model for *in silico* experimentation (86). From this, a set of expression vectors was established, containing both plasmids and circuits for efficient genome integration, and libraries of promoters and ribosome binding sites were curated to allow a greater degree of flexibility and control in gene expression (83, 87). The recent development of a generalisable platform for gene repression in *A. baylyi* ADP1 exemplifies the benefits and potential future impacts of the strain (88, 89).

A. baylyi ADP1 for wax ester production

For wax production studies, *A. baylyi* ADP1 possesses two key benefits which distinguish it from other microbial species. As mentioned, it is reasonably well studied due to the interest in its natural competence and ease of genetic manipulation, resulting in a significant number of resources for its use and behaviour. However, even more crucial and attractive than this is its natural ability to accumulate WEs as the major component of its carbon storage molecules. Numerous studies have exploited these traits to study wax production in *A. baylyi* ADP1, thus far focusing predominantly on ideal glycolytic substrates such as glucose and gluconate as feedstocks (90-93), as their high energy content results in excellent cell growth and hence good production. Growth on long chain hydrocarbon and fatty acid substrates also obtains efficient WE accumulation with high yields due to structural similarity of the substrate to the product and the small number of metabolic steps required for conversion (94-96).

A. baylyi ADP1 has also been used to produce WEs from a number of waste streams, including waste cooking oil (94), aromatic compounds from lignin hydrolysate (97-99) and acetate from CO_2 via microbial electrosynthesis (100). The strain has been demonstrated to utilize butyrate for growth and lipid production (101), indicating that VFAs in general could be a suitable, low cost feedstock for the strain, although no other VFAs have been explored for their toxicity or suitability as a carbon source. For more details on wax production in *A. baylyi* ADP1 and the key results obtained in the literature so far, please see Part 4 for an extensive review.

2.3. Acidogenic fermentation for volatile fatty acids production

Anaerobic digestion

Anaerobic digestion (AD) is the process by which organic material is broken down by micro-organisms in the absence of oxygen. This natural process occurs in many environments, including soils and ocean sediments, and is an essential part of the carbon and nitrogen cycles,

converting waste carbohydrates and other plant/animal material into simple products carbon dioxide, methane, hydrogen and ammonia. AD has been harnessed by humans for thousands of years, to manage organic waste, including agricultural waste and sewage sludge, and to produce fuel and fermented food and drinks products (102).

AD consists of four stages; hydrolysis, acidogenesis, acetogenesis and methanogenesis, each of which is completed by a unique set of micro-organisms (see Figure 2.1). Initially biomass, a complex mixture of large, insoluble, organic polymers, is broken down into simpler amino and fatty acids, and sugars by hydrolytic bacteria (103). Hydrolysis is often the rate-limiting step of AD (104), so physical and chemical pre-treatments are common to improve this, particularly in lignin-rich biomass (105). Next, the larger molecules produced in hydrolysis are fermented into VFAs, predominantly acetate, propionate and butyrate, by acidogenesis. Acidogenic bacteria grow rapidly, making this step the fastest in the process (102). Acetogenesis further oxidises longer chain acid products of acidogenesis into acetate, as well as hydrogen and carbon dioxide (106). Finally, methanogens use the small molecule products from the previous stages to produce methane (107). At the end of this process biogas, a methane-carbon dioxide mixture, is produced, along with the indigestible material and any dead cell matter remaining in the reactor. This forms a final sludge rich in ammonium, phosphate and other inorganic nutrients that can be used as fertiliser. Biogas generation for energy is attractive because it can be generated cheaply from waste and the carbon released when burning biogas is biogenic rather than fossil fuel derived, so does not contribute to total net carbon emissions.

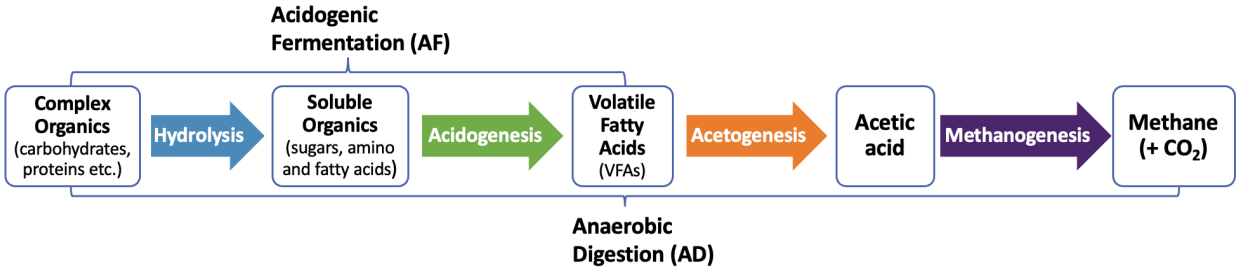


Figure 2.1. A process diagram for anaerobic digestion and acidogenic fermentation

Suitable feedstocks for anaerobic digestion

An enormous variety of organic material is suitable for AD, with the conditions of the process and the product composition dependent on the initial feedstock (102). Wastewater, manure and sewage sludge can be utilised as feedstock (106, 108), although these materials are already depleted in energy content and nutrients and so productivity from them is low (109). However,

chemically richer feedstocks, such as farm and agricultural waste, waste fats and oils, and food waste, can be used alone or in combination with depleted waste streams, resulting in large-scale production of energy and useful chemicals (110, 111).

Specific crops, such as maize, can achieve very high productivities in anaerobic digestion (112), though the growth of dedicated energy crops on arable land leads to a number of environmental and social sustainability problems (113, 114). More recently, crassulacean acid metabolism (CAM) plants have attracted attention as potential energy crops (115). CAM plants have evolved to grow efficiently in hot, arid conditions due to their adapted stomata, which minimise water loss associated with photosynthesis and respiration by only opening at night (116). This allows such crops to thrive in environments and conditions where other plants cannot: semi-arid regions with minimal water and fertilisers. As such, they do not compete with traditional agriculture for land and avoid destruction of naturally biodiverse ecosystems. Importantly, the anaerobic digestion of CAM plants can yield similar methane productivities to maize (117), and their use is economically desirable, generating energy and additional revenue streams for remote, rural communities (118).

Limitations of anaerobic digestion for energy generation

The production and utilisation of biogas as an energy source is not without problems. While pure methane is energy dense, the mixture produced from AD is typically 30-50 % CO_2 (119, 120), which is incombustible and hence greatly reduces the calorific value of the gas. For some applications, biogas requires upgrading to enhance methane content, which can be expensive and energy intensive (120), reducing the net benefits of its generation. Furthermore, it is widely argued that using biomass for energy generation is not viable on the scales required globally, and for a truly sustainable future, energy generation must be decarbonised. The total global energy consumption from fossil fuels in 2022 was nearly 140,000 TWh (121), while the total energy production of food for human consumption was less than 10,000 TWh (122) and total global food and crop production, which includes crops for animal feed and biofuels is around 25,000 TWh (123). Even assuming 100% energy conversion (when less than 50 % is realistic), global crop production would need to increase over 600 % to fully replace fossil fuels. When converted into masses, the numbers are similarly staggering; based on an estimated energy content of 2.2 kWh/kg for dried biomass, the annual requirement for energy would be 64 billion tonnes. At present, the total production of agriculture and forestry sectors worldwide is 11.9 billion tonnes, 41% of which is food (124). Sewage and manure could contribute a further 3-4 billion tonnes of dry biomass (125), but even then a several fold increase in total global

production would be required. This is theoretically achievable, since the global net primary production of land-based biomass is estimated to be 48-69 billion tonnes of carbon per year (126), which can be roughly approximated as 100-140 billion tonnes of biomass annually (127). However, realising this as a utilisable resource would require an enormous scale-up of the agriculture and forestry industries, which in itself would require additional energy and pose other sustainability challenges around habitat and biodiversity loss. These numbers highlight that the available biomass sources, predominantly energy crops and waste streams, are largely insufficient to meet the energy needs of the population, and carbon-free technologies, such as solar and wind power, must be central to the renewable energy revolution.

Acidogenic fermentation for biochemical production

The phrase 'decarbonisation' is associated with the reduction of CO_2 emissions associated with a given product or process, which in the energy sector can be achieved by switching to 'low carbon sources'. However, unlike energy, organic chemicals are inherently carbon based: by these definitions they can never truly be 'decarbonised' because the consumption and degradation of such chemicals will always release CO_2. Yet, if these chemicals are produced from biogenic carbon sources, such as biomass or atmospheric CO_2, the carbon released is equal to that which was utilised to make the product. In this case there is no net increase in atmospheric CO_2 and the materials have potential to be considered sustainable and renewable.

In the production of biochemicals instead of bioenergy, the total decomposition of organics to single carbon molecules is highly inefficient, as micro-organisms break high-energy carbon-carbon bonds, only for them to be reassembled in whatever chemical or biological process is used to upgrade the treated waste into useful chemicals. An alternative degradation process called acidogenic fermentation can be used, in which methanogenesis is inhibited. In this case, the final product is a fermentate that is not only rich in inorganics but also in volatile fatty acids acetate, propionate, butyrate, valerate and hexanoate (C2-C6 chains) (128-130). While less widely studied and deployed than anaerobic digestion, acidogenic fermentation has been used to generate volatile fatty acids from a similarly diverse range of organic waste streams, including sewage (131), agricultural (129) and food wastes (128), as well as designated energy crops (117, 132).

The deployment of acidogenic fermentation for biochemical production may seem counterintuitive. By degrading biological material, bonds are broken, and the energy contained in the material lost, so if the molecule of interest is a complex organic, minimal amounts of

degradation would be most efficient for biochemical production. In cases where a chemical can be produced from a specific, consistent and homogenous biomass source this method can be very effective, as particular molecules can be isolated from the biomass and then chemically or biologically transformed as required in a minimal number of steps (133-135). However, this scenario is rare. Typically, waste streams are complex mixtures of a wide number of different materials, each of which is composed of a mixture of proteins, lipids, and carbohydrates, and separating out individual molecules would be highly complex and costly. The situation is complicated further by the variation that can occur within waste streams – almost all organic waste is seasonally and geographically heterogeneous in composition and quantity due to variations in crop cycles, diets and socio-economic development (136, 137). Between waste streams of different kinds, e.g. forestry residues, food waste and sewage, variation is greater still.

The conversion of these biomass sources into VFA-rich acidogenic fermentate (herein referred to as fermentate) can therefore be thought of as a practical compromise. The process simplifies the vast possible pool of complex organics into a smaller set of simple molecules, whilst retaining some complexity relative to complete digestion to biogas. This homogenisation limits the variability and improves consistency between different waste streams, or different batches of the same stream, although some variation persists (138, 139). This allows industrial processes which utilise this material as a feedstock to be more widely applicable across different sites and industries.

Microbial bioproduction from fermentation derived volatile fatty acids

The major class of biochemicals which has been investigated thus far for microbial production from VFAs is lipids. VFAs are a logical and favourable feedstock for lipid synthesis because acetyl-CoA, which can be derived from acetate via a single step conversion, is the first molecule in the fatty acid biosynthesis pathway. Since longer chain VFAs can be catabolised into their constituent acetyl-CoA sub-units, almost the entire carbon source is metabolically positioned for efficient lipid synthesis.

A range of micro-organisms, including algae, bacteria and yeast, have been demonstrated to be capable of producing lipids from VFAs, with polyhydroxyalkanoates (PHAs) (140-142) and TAGs for biodiesel generation (143, 144) the major target molecules. Synthetic VFA mixtures are commonly used to optimise the growth and production of these microbial hosts, which can then be deployed to fermentation-generated VFA mixtures from a range of sources, including

waste paper (142, 144), food waste, and sewage sludge (145). Mixed microbial cultures can be used for lipid synthesis (146), with lipid accumulating bacteria selected for through feed and famine cycles. However, this selects for all lipid accumulating bacteria, the majority of which use a mixture of TAGs as carbon storage molecules. If a different lipid class, such as WEs, or a specific chain length profile is desired, a pure culture is preferable (142).

Although VFA composition from AF varies based on the feedstock and conditions used, relatively little work has attempted to harness this compositional tunability for lipid production. As in synthetic culture media, a high C:N ratio has been found to stimulate PHA production (141-143), although this requires a fermentate with very limited nitrogen content, which is rare due to the natural protein content of most biomass sources (130, 132, 145). The relative ratios of the different VFAs present can also impact cellular lipid content and composition (140, 142, 144-146). However, the translation of these findings from synthetic VFAs to fermentates is relatively poorly understood, with only Gong *et al*. and Annamalai *et al*. studying lipid production on fermentates of multiple compositions (144, 145). No general trends were observed across these two studies to indicate an optimal VFA composition for lipid accumulation, although utilising a higher total VFA concentration did increase both lipid titre and lipid content per cell (144), likely by facilitated better cell growth.

2.4. Bacterial wax synthesis – a review

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Abstract

Biological wax esters offer a sustainable, renewable and biodegradable alternative to many fossil fuel derived chemicals including plastics and paraffins. Many species of bacteria accumulate waxes with similar structure and properties to highly desirable animal and plant waxes such as Spermaceti and Jojoba oils, the use of which is limited by resource requirements, high cost and ethical concerns. While bacterial fermentations overcome these issues, a commercially viable bacterial wax production process would require high yields and renewable, affordable feedstock to make it economically competitive and environmentally beneficial. This review describes recent progress in wax ester generation in both wild type and genetically engineered bacteria, with a focus on comparing substrates and quantifying obtained waxes. The full breadth of wax accumulating species is discussed, with emphasis on species generating high yields and utilising renewable substrates. Key areas of the field that have, thus far, received limited attention are highlighted, such as waste stream valorisation, mixed microbial cultures, and efficient wax extraction, as, until effectively addressed, these will slow progress in creating commercially viable wax production methods.

Keywords – Wax ester synthesis, bacteria, sustainable, biotechnology, *Acinetobacter*, environment, economic

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1. Introduction

Waxes are lipophilic, organic substances with an enormous range of applications ranging from coatings and lubricants to cosmetics and food additives (Santala et al., 2011). Interest in waxes has increased recently as an alternative to plastics, with many of the same desirable characteristics, including water and chemical resistance and non-toxicity, but with the added

advantage of their biodegradability. In 2019 the global wax market was valued at under $10 billion, but non-petroleum, natural waxes from plants and animals constituted just 5% of this (Grand Research View, 2020). With a forecast increase in market size to almost $12.5 billion by 2027, and the finitude and harmful emissions of petrochemical resources becoming ever more of a concern, a shift towards natural waxes is both desirable and inevitable. However, natural plant and animal waxes are currently restricted to small volume, high value products due to high production costs, land limitations (Vanhercke, 2014), intermittent production and prices susceptible to low yields or crop failures (Research Nester, 2019; Rontani, 2010).

Wax esters (WEs), the major component of biological waxes, are esters of fatty acids and alcohols, with typical chain lengths of 20 carbons or greater (Bart et al., 2013). As well as plants and animals, some species of micro-organism produce WEs directly as storage lipids, synthesised and accumulated intracellularly under nutrient, typically nitrogen, limited conditions to preserve excess carbon (Fixter et al., 1986; Santala et al., 2018). The advantages of bacterial WEs over current natural and petroleum waxes are numerous. Oleaginous bacteria grow on a wide range of feedstocks (Salcedo-Vite et al., 2019), including many considered waste streams, thus contributing to closing the carbon cycle. The WE profile of bacteria varies by species (Manilla-Pérez et al., 2011), growth conditions (Russell, 1978; Russell and Volkman, 1980) and feedstock (Salcedo-Vite et al., 2019), offering the opportunity to produce WEs with specific and diverse chemical profiles. Furthermore, employing bacterial WE production can reduce pressure on land usage if secondary biomass is utilised, mitigating 'food-vs-fuel' type debates, whilst decreasing product variability and seasonality and offering potential for greater scalability and cost reduction. Similar benefits can also be achieved in water utilisation and efficiency. Growing additional crops for WE production requires mass irrigation and consumes precious drinking water: in the biofuels area this has sparked a 'drink or drive' discussion comparable to 'food-vs-fuel' (Dominguez-Faus et al., 2009). However, microbial systems utilising organic waste as a feedstock avoid extra irrigation, requiring only fermentation water in reactors which can be more easily managed. Fermentation water can also be recycled using low-cost water treatment methods such as constructed wetlands, which themselves can provide biomass for bioproduction (Rasool et al., 2023).

As an area of undoubted growing industrial and environmental interest, it is important that researchers are aware of the suitability of species and substrates, as well as the current limits in terms of yields, to make informed decisions and optimise production. However, it is equally important that areas of the field which are thus far under-explored, but of vital importance to

improving process scalability and viability, are identified and highlighted to encourage further exploration.

This review aims to provide a broad assessment of the successes and progress thus far in WE production by both wild type (WT) and genetically engineered bacteria. WE producing species are discussed in the context of the substrates from which they produce WEs, with substrates classified as structurally-related substrates, renewables and waste streams. Genetic modifications to improve WE synthesis is divided into modifications of organisms which naturally synthesise WEs and those which do not. The limited reported attempts to utilise waste streams as feedstocks and to employ mixed microbial cultures for WE production are also highlighted, before discussing the three major areas of the field we identified for future research focus; novel substrates, efficient WE extraction and process optimisation and quantification.

2. Bacterial wax ester production pathway

Neutral lipids, including WEs and triacylglycerols (TAGs), are accumulated by bacteria for storage when carbon is plentiful but other essential nutrients, usually nitrogen, are limited (Alvarez, 2016; Ishige et al., 2003; Rontani, 2010). Stored carbon is subsequently made available for metabolism by the cell during periods of carbon shortage. This behaviour is believed to be an adaptation to extreme environments in which nutrient supplies are variable, with those species able to store carbon better equipped to survive periods of starvation. The use of storage lipids as a store of metabolic water, which is produced during fatty acid oxidation, has also been suggested (Alvarez et al., 2004). WE accumulation, and other responses to nutrient limitation, are triggered by universal regulation within cells at the molecular level by a wide range of regulatory systems, such as transcription and sigma factors (Shimizu, 2014). In this way, WE production is inherently linked to a number of environmental stress responses.

Bacterial WE production occurs in three stages; production of the fatty acid, the fatty alcohol and their esterification (Kalscheuer et al., 2003). Where the carbon source is a small organic molecule, it is taken up into the tricarboxylic acid (TCA) cycle, and converted into acetyl-CoA, then acetyl-acyl carrier protein (ACP). From this, long chain fatty acids are constructed through sequential elongation steps, each adding two carbons to the ACP-bound acyl chain through reaction with malonyl-ACP and elimination of CO_2 (see Figure 2.2) (Cronan and Thomas, 2009). This results in acyl-ACPs of even carbon chains, commonly C4-C18. Odd chains are achieved if the starting molecule is propionyl-CoA (Stillwell, 2016).

Long chain fatty acids, typically C14 and above (Ishige et al., 2003; Kannisto et al., 2014; Stillwell, 2016), are released from the ACP and form fatty acyl-CoAs, which can be reduced to primary fatty alcohols if a species possesses reductase enzymes. Reduction is usually catalysed by two enzymes, a fatty acyl-CoA reductase (FAR) and a fatty aldehyde reductase (FAldR), sequentially reducing a fatty acyl-CoA to an aldehyde then a fatty alcohol, although in some species a single reductase completes both steps (Hofvander et al., 2011). While fatty acid biosynthesis occurs in all bacteria, fatty alcohol production is less common, hence WE synthesis is limited to a small number of species. A wax synthase (WS) then catalyses the alcohol condensation with a second acyl-CoA, producing the WE (Bart et al., 2013). The WS is usually a bifunctional wax-synthase/diglyceride acyltransferase (WS/DGAT), also responsible for TAG synthesis (Kalscheuer and Steinbüchel, 2003). Fatty acid and WE biosynthesis pathways from simple carbon substrates via acetyl-CoA are shown in orange and blue in Figure 2.2.

If the substrate is a long-chain alkane, the WE synthesis pathway is different. The alkane is oxidised to a fatty alcohol by an alkane hydroxylase (AlkM) (Ratajczak et al., 1998), as shown in green in Figure 2.2. Further oxidations by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) enzymes lead to the fatty acid, which is then converted to an acyl-CoA and condensed with another alcohol molecule to form a WE. Alkanes, and long-chain acids and alcohols, are therefore converted to WEs without entering the central carbon metabolism, with the majority of these WEs exhibiting acid and alcohol moieties of the same chain length as the substrate.

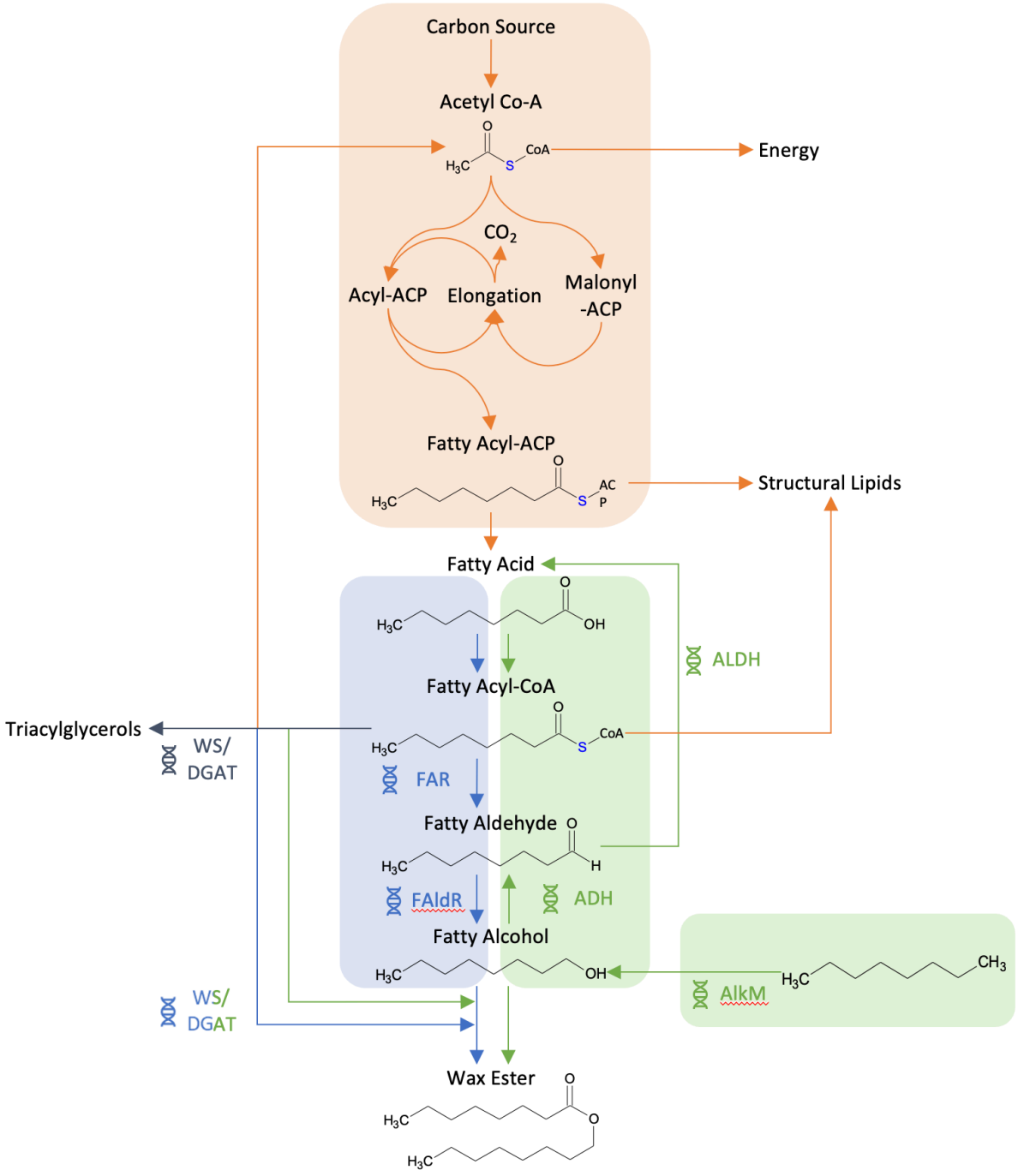


Figure 2.2. The import and synthesis pathways relating to bacterial wax ester formation and the associated enzymes: fatty acid and structural lipid biosynthesis pathways (orange), fatty alcohol production from fatty acids (blue), alkane uptake and oxidation (green) and triacylglycerol production (dark blue) (Cronan and Thomas, 2009; Lehtinen et al., 2018).

3. Wax ester production in wild type strains

With bacterial WE production a matter of growing industrial and environmental interest, research in this area is increasingly important. While genetic engineering and synthetic biology offer useful tools to improve production, it is necessary to recognise the advantages of optimising and exploiting WT organisms, to assess what is achievable without genetic modification and to identify desirable hosts and pathways for modification. For these reasons, this section will focus on WE production in WT bacteria.

A summary of all WT species in which WE accumulation has been characterised is given in Table 2.1, along with the substrates from which WE production has (or, conversely, has not) been detected. The most extensively studied bacterial genus, with six strains that accumulated WEs when grown on a wide range of substrates, including alkanes (Sakai et al., 2014), fatty acids and alcohols (Ishige et al., 2002), sugars (Salcedo-Vite et al., 2019; Santala et al., 2011), simple organic acids (Salmela et al., 2018) and even lignin derivatives (Kannisto et al., 2017), is *Acinetobacter*. However, many other species including *Alcanivorax* (Kalscheuer et al., 2007; Manilla-Pérez et al., 2010), *Marinobacter* (Holtzapple and Schmidt-Dannert, 2007; Lenneman et al., 2013; Nakano et al., 2012), *Mycobacterium* (Sirakova et al., 2012), *Rhodococcus* (Hernandez et al., 2008; Shields-Menard et al., 2015) and *Streptomyces* (Kaddor et al., 2009) have also been demonstrated to accumulate WEs as at least a component of their storage lipids on some substrates. The composition of WEs produced varies depending on species, although saturated and unsaturated C16 and C18 components are most common, especially for the alcohol moiety (Barney et al., 2012). For a given species, carbon source may also affect both the overall lipid and WE profile accumulated (Salcedo-Vite et al., 2019).

Table 2.1. Bacterial strains producing WEs and the substrates utilised for production

Genus	Species	WE produced on	WE not produced on	Ref
GRAM-POSITIVE				
Mycobacterium	*ratisbonense* SD4	phytane	gluconate, hexadecane	(Silva et al., 2007)
	tuberculosis	cholesterol		(Holert et al., 2020)
		glycerol		(Deb et al., 2009)
		oleic acid/oleate		(Sirakova et al., 2012)
	smegmatis mc^{2}155	hexadecanol	glucose	(Kalscheuer and Steinbüchel, 2003)
Rhodococcus	A27	fructose (traces)		(Rottig et al., 2016a)
	jostii RHA1	cholesterol, phytosterol, pyruvate (traces)	glucose, succinate	(Holert et al., 2020)
		hexadecane, hexadecanol	3HB, acetate, gluconate	(Hernandez et al., 2008)
		glucose (traces)		(Round et al., 2017)
	opacus PD630	phenyldecane		(Alvarez et al., 2002)
	rhodochrous	glucose		(Shields-Menard et al., 2015)
Streptomyces	*avermitilis* MA-4680	glucose+hexadecanol	glucose, hexadecane, hexadecanol	(Kaddor et al., 2009)
	coelicolor		glucose, hexadecanol	(Arabolaza et al., 2008)
	sp. strain G25		glucose	(Rottig et al., 2016b)
GRAM-NEGATIVE				
Acinetobacter	*baylyi* ADP1	1,16-hexadecandiol		(Kalscheuer et al., 2003)
		4HB		(Kannisto et al., 2017; Singh et al., 2019)
		acetate		(Kannisto et al., 2017; Lehtinen et al., 2017; Salcedo-Vite et al., 2019; Santala et al., 2018)
		acetate and butyrate mix	glycerol	(Mangayil et al., 2019; Salmela et al., 2018)
		acetate and coumerate mix		(Salmela et al., 2019)
		glucose (with casamino acids)		(Lehtinen et al., 2018; Santala et al., 2011; Santala et al., 2014)
		gluconate		(Kannisto et al., 2014)
		pyruvate	ethanol, glycerol, xylose	(Manilla-Pérez et al., 2011; Salcedo-Vite et al., 2019)
	calcoaceticus (various strains)	succinate		(Fixter et al., 1986; Reiser and Somerville, 1997)
	HO1-N	hexadecane, icosane, octadecane		(Dewitt et al., 1982)
	M1	hexadecane, hexadecanol, cis-11-hexadecanal, hexadecenoic acid, octadecane		(Ishige et al., 2002; Sakai et al., 2014)
	NRRLB-14920	oleic acid, oleic alcohol		(Kaneshiro et al., 1996)
	sp. strain PHY9	6,10,14-trimethylpentadecan-2-one	phytol	(Rontani et al., 1999)
Alcanivorax	*borkumensis* SK2	hexadecane, octadecane, pyruvate	acetate	(Kalscheuer et al., 2007; Manilla-Pérez et al., 2010; Manilla-Pérez et al., 2011)
	jadensis T9	hexadecane, pyruvate	acetate	(Manilla-Pérez et al., 2010; Manilla-Pérez et al., 2011)
		hexadecane, tetradecane		(Bredemeier et al., 2003)
		pyruvate		(Kalscheuer et al., 2007)
Marinobacter	*aquaeolei* VT8	citrate, pentadecanol, succinate		(Lenneman et al., 2013; Wahlen et al., 2009)
	atlanticus CP1	succinate		(Bird et al., 2018)
	hydrocarbonoclasticus SP17	hexadecane	acetate	(Klein et al., 2008)
		hexadecane, pyruvate		(Manilla-Pérez et al., 2011)
	hydrocarbonoclasticus DSM 8798	phytol, 6,10,14-trimethylpentadecan-2-one		(Rontani et al., 1999)
	PAD-2	hexadecane, succinate		(Nakano et al., 2012)
	sp. strain CAB	phytol, 6,10,14-trimethylpentadecan-2-one		(Rontani et al., 1999)
	squalenivorans	squalene		(Rontani et al., 2003)
Micrococcus	*cryophilus*	casamino acids		(Russell, 1974, 1978; Russell and Volkman, 1980)
Nocardia	-	fructose		(Rottig et al., 2016a)
	corallina No 724	glucose, hexadecane, valerate (traces)		(Alvarez et al., 1997)
Thalassolitus	*oleivorans*	pyruvate		(Kalscheuer et al., 2007)
		hexadecane	pyruvate	(Manilla-Pérez et al., 2011)

3.1. Utilisable substrates

Structurally related substrates closely resemble the alcohol and acid residue of the WE, such as alkanes, alcohols or fatty acids of the same chain length. Most common in the literature is hexadecane, which has been identified to yield WEs, predominantly palmityl palmitate, in ten species, with hexadecanol and hexadecenoic acid also widely tested. In contrast, when non-lipid substrates are used, many bacteria will preferentially accumulate WEs with stearyl acid or alcohol moieties (Barney et al., 2012). Bacterial WE production from such substrates was first reported for an unidentified Gram-negative *coccus* (Stewart and Kallio, 1959); since then many bacterial species have been observed to exhibit this trait. More novel alkanes and alcohols, including phytane (Silva et al., 2007) cholesterol, (Holbert et al., 2020), 6,10,14-trimethylpentadecan-2-one (Rontani et al. 1999) and squalene (Rontani et al., 2003) have also been explored as substrates for WE production, although many were comprised of fragmented derivatives rather than the whole substrate. It is, however, important to note that currently the majority of long chain hydrocarbons and their derivatives are almost entirely derived from petroleum (Rahman et al., 2018). With a growing ambition to transition chemical processes away from fossil-fuel dependence, the exploitation of renewable feedstocks for WE production will inevitably grow.

A broad range of sugars and organic acids can be sourced renewably and employed as feedstock for WE production by microbial processes (Li et al., 2001; Maleki and Eiteman, 2017; McKinlay et al., 2007; Meng et al., 2016). Production on renewable substrates is largely dominated by *A. baylyi* ADP1, with WEs produced from acetate, butyrate, glucose, gluconate and pyruvate (see Table 2.1). *Marinobacter* species *aquaeolei* VT8, atlanticus CP1 and *hydrocarbonoclasticus* SP17 have also demonstrated WEs as major components of storage lipids, with succinate, citrate and pyruvate all suitable feedstocks. Conversely, many strains, particularly of the *Alcanivorax*, *Rhodococcus* and *Streptomyces* genii which can produce WEs from structurally-related alkanes and fatty alcohols and acids, fail to do so from short chain acids and simple sugars. This indicates a lack of FAR and/or FAldR enzymes (see Figure 2.2), with such strains instead accumulating solely TAGs under such conditions. However, despite this reduced selection of suitable chasses, the value of these substrates as renewable feedstocks should not be overlooked.

However, sugars and sugar derived feedstocks can be expensive and problematic in terms of sustainability. High sugar crops must be grown on arable land with fertilisers and plentiful water to attain good yields and are limited by growing seasons (Marzo et al., 2019). The land

use raises similar ethical concerns as plant wax production, while large-scale agriculture causes water and land contamination and biodiversity loss (Mohr and Raman, 2013). These issues, along with the massive substrate flexibility of bacteria, has led to growing interest in other sources of renewable carbon sources, including the valorisation of waste streams (see Section 5).

3.2. Wax ester quantification

WEs have been detected in a small number of bacterial species and have been characterised and quantified in even fewer. Quantification is extremely important for progress towards commercial technologies, as processes must be economically viable at scale and competitive with established petrochemical alternatives. Three measures of quantification relevant to industrial production are given (Table 2.2). WE per biomass expresses the quantity of wax each gram of biomass produces. Wax titre expresses the extractable WEs per litre of broth. This is dependent upon both WE per biomass and growth, since in some conditions (high C, low N) WE per biomass is high but biomass production is poor, so the absolute amount of WE is low. Titre is also important in industrial scale reactors, specifically: 1) more concentrated streams require less energy for downstream processing and 2) higher product concentrations allow smaller, lower cost reactors. Finally, yield, how much substrate is converted into the desired product, is vital as feedstock contributes heavily to operational costs. The higher the substrate cost, the more crucial the yield; if waste-streams can be used as feedstock, some yield can be sacrificed.

The maximum achieved WE titre and yield for a WT strain at flask scale was reported to be with *Acinetobacter* NRRL B-14920 grown on soybean oil (comprised of mixed TAGs). A titre of 2.61 g/L WEs accumulated at a yield of 32.2% from the oil, though this figure may be misleading as 4 g/L of glucose and 5 g/L yeast extract were also present in the culture medium (Kaneshiro et al., 1996). *Acinetobacter* M1 achieved the highest WE content, at 17%, when grown on hexadecane, but no other metrics for this production were provided (Ishige et al., 2002). On a non-lipid substrate, *A. baylyi* ADP1 on glucose and casamino acids produced 0.58 g/L of WEs at a yield of 3% g/g, and to a WE content of 9% CDW, the highest achieved across all strains and substrates tested (Luo et al., 2020).

Table 2.2. The maximum wax content, titre and yield achieved for bacterial strains on different substrates

Genus	Species	Substrate	Maximum WE per biomass (g/g)	Maximum WE titre (g/L)	Maximum yield (g WEs/ g substrate)	Reference
Acinetobacter	*baylyi* ADP1	acetate	0.077	0.094	0.023	(Lehtinen et al., 2017)
		courmerate	0.058	0.202	0.037	(Salmela et al., 2019)
		glucose + casamino acids	0.09[a]	0.58	0.03	(Luo et al., 2020)
	M1	hexadecane	0.17	-	-	(Ishige et al., 2002)
		oleic acid + oleyl alcohol	-	-	0.095	(Kaneshiro et al., 1996)
	calcoaceticus NCIB 10487	succinate	0.141	-	-	(Fixter et al., 1986)
Alcarnivorax	*borkumensis* SK2[b]	hexadecane	0.027	0.005	0.0011	(Manilla-Pérez et al., 2011)
		wastewater (fed-batch)	-	8.45[c]	-	
	jadensis T9[b]	hexadecane	0.014	0.021	0.004	(Manilla-Pérez et al., 2011)
		hexadecane	-	-	0.03	(Bredemeier et al., 2003)
Marinobacter	*atlanticus* CP1	succinate	0.035	0.025	-	(Bird et al., 2018)
	aquaeolei VT8	citrate	0.035	-	-	(Lenneman et al., 2013)
	hydrocarbonoclasticus[b]	hexadecane	0.040	0.004	0.0008	(Manilla-Pérez et al., 2011)
		pyruvate	0.010	0.012	0.0015	
	squalenivorans	squalene	0.011	0.01	-	(Rontani et al., 2003)
Micrococcus	*cryophilus*	casamino acids	0.013	-	-	(Russell, 1974)
Rhodococcus	*josti* RHA1	glucose	0.000002	-	-	(Round et al., 2017)

[a] Higher wax content was obtained in a different paper (Lehtinen et al., 2018) but with lower wax titre and yield
[b] Combination of intracellular and extracellular waxes
[c] Fed-batch reactor rather than batch flask experiment

Outside the *Acinetobacter* genus, WE accumulation is generally much lower, with maximum WE content of 4 % CDW (Manilla-Pérez et al., 2011), titre of 0.025 g/L (Bird et al., 2018) and yield of 3% (Bredemeier et al., 2003) for flask grown cultures. *Alcanivorax borkumensis* SK2 was able to produce 8.45 g/L of WEs in a fed batch reactor, the highest WE titre yet recorded, although accumulation of this WE took almost 11 days.

Several strains, including *A. borkumensis* SK2 and *A. jadensis* T9, have been demonstrated to excrete WEs as well as accumulating them intracellularly (Klein et al., 2008; Manilla-Pérez et al., 2010; Manilla-Pérez et al., 2011; Nakano et al., 2012). In such cases the values presented

for quantification are a total of all produced lipids. Both of these strains produced both WEs and TAGs, but the extra-cellular lipids were predominantly WEs (Manilla-Pérez et al., 2011). The ability to export WEs, especially selectively over TAGs, would be advantageous for industrial down-stream processing by reducing extraction costs and increasing efficiency. However, the genes and mechanism responsible have not yet been identified, preventing expression of this phenotype in other species.

4. Wax ester production in modified organisms

WE production has also been engineered into bacteria and can be divided into two approaches: 1) modifications to improve the breadth of utilisable substrates and 2) alterations to the metabolic pathways through which WEs and their precursors are produced. Attempts at the former have been minimal, largely due to many WE accumulating bacteria already exhibiting high substrate flexibility. Conversely, engineering of the carbon metabolism to divert flux through alternate or non-native pathways to generate WEs has been successful, with many different approaches being taken. Expression of non-native genes can impart new pathways between metabolites not previously accessible, improving efficiency or enabling synthesis of new products (e.g. WEs or fatty alcohols). Gene knock-outs can prevent or limit the flux directed into non-WE products, improving yields and conversion efficiency. In contrast, overexpression of key genes in WE production (see Figure 2.2) is an effective way to increase flux through a desired pathway, particularly where one reaction is the bottleneck of the process or if there are multiple, competing pathways.

For the purpose of this review, genetic modifications have been divided into two categories: native producing strains with modifications to improve or alter WE production and non-native producing strains with modifications which impart WE production ability. Here 'native producing strains' includes all strains in which WEs have been observed to accumulate.

4.1. Modifications in native producing strains

4.1.1. *Acinetobacter baylyi* ADP1

Given the success of WE production in WT ADP1, it is unsurprising various engineering approaches have been attempted in this strain. Initial rational engineering involved overexpression of the FAR enzyme acr1, increasing flux through the WE synthesis pathway by increasing fatty alcohol synthesis. WE content increased by 2.7-times, while titre and productivity both doubled (see Table 2.3) (Lehtinen et al., 2018). When published, this was the highest bacterial WE titre without hydrocarbon supplementation.

Another approach was deletion of *aceA*, the isocitrate lyase in the glyoxylate cycle necessary for growth on non-glycolytic substrates but not on glucose, which directs additional flux towards acetyl-CoA for WE synthesis. Inducible *aceA* mutants were grown on acetate, with high arabinose inducer concentration allowing growth. Gradual arabinose oxidation to non-inducing derivatives caused *aceA* expression, and hence growth, to decrease, redirecting flux to WE production (Santala et al., 2018). WE content and yield increased compared to the WT, although titre was limited by restricted growth. Acetate was suggested as a more feasible substrate than glucose due to its lower cost and high abundance. However, the set-up used 50 mM acetate and 0.5% arabinose, or 4.1 g/L of sodium acetate and 5 g/L of arabinose, with respective costs of £0.21 and £24.20 per litre of broth (prices from Sigma, most cost-effective quantity). The significant inducer cost makes the process prohibitively expensive, regardless of the improved yield.

Most recently these two modifications have been combined into a single strain with complementary effects. While *aceA* deletion reduced biomass production even in glucose-cultured cells, in combination with *acr1* overexpression it gave similar CDW to the WT but with improved WE content and titre. The yield, whilst lower than just *aceA* deletion, was higher than both WT and *acr1* overexpression and no inducer was required, making the process more economical (Luo et al., 2020). The modified strain also produced WEs on nitrogen rich feedstocks, with amino acids, casamino acids, yeast extract and bakers' yeast hydrolysate all giving greater WE content than the WT.

Table 2.3. The maximum wax content, titre and yield achieved for a range of strains and genetic modifications

Species	Feedstock	Modification	Maximum WE content (%)	Maximum wax titre (g/L)	Maximum yield (g WE/ g substrate)	Ref.
Native producing strains						
Acinetobacter baylyi ADP1	Glucose + casamino acids	*aceA* deletion and *acr1* (FAR) overexpression	0.27	1.82	0.075	(Luo et al., 2020)
	Glucose + casamino acids	*acr1* (FAR) overexpression	0.125	0.45	0.04	(Lehtinen et al., 2018)
	Acetate + arabinose	*aceA* deletion	0.19	0.184	0.08	(Santala et al., 2018)
	Glucose, casamino acids + arabinose	*acr1* replaced by *LuxCDE*	0.014	0.091	-	(Santala et al., 2014)
Marinobacter atlanticus CP1	Succinate	Deletion of one FAR(acrB) or FAldR(farA)	0.15	0.035	-	(Bird et al., 2018)
Rhodococcus jostii RHA1	Glucose	*fcrA* and *ws2* (*M.* DSM 8798) expressed constituitively from chromosome	0.157 0.10[a]	- 5.0 (scale up)[a]	0.047 0.025[a]	(Round et al., 2019)
	Glucose	native *fcrA* overexpression on plasmids	0.13	-	-	(Round et al., 2017)
Rhodococcus opacus PD630	Gluconate	Expression of fatty acyl-CoA reductase from *M. hydrocarbonoclausticus*	Total neutral lipids changed from 100% TAG to 46% WE and 54% TAG			(Lanfranconi and Alvarez, 2017)
Non-native producing strains						
Escherichia Coli	Oleate	Co-expression of jojoba plant alcohol-producing, bifunctional FAR/FAldR and ADP1 WS/DGAT	0.01	*De novo* WE synthesis minimal, 1.5% CDW were butyl esters		(Kalscheuer et al., 2006)
	LB Medium + arabinose	Expression of *lpp*β (phosphatidic acid phosphatase gene) and *atfA* (ADP1 WS/DGAT)	WEs and TAGs accumulated, while no WEs present in control strain			(Comba et al., 2014)
`	Glucose + Oleic acid + Octanol + Pyruvate	FadD28 and Mas from *M. Tuberculosis* for methyl-branched fatty acid synthesis, PapA5 for esterification	- -	0.138 0.79[a] (scale up)	- 0.002[a]	(Menendez-Bravo et al., 2016)
Pseudomonas citronellolis	Hexadecanol	Heterologous expression of *atfA* (ADP1 WS/DGAT)	WEs accumulated on hexadecanol, no TAGs detected. No accumulation on hexadecane, hexadecenoic acid or gluconate			(Kalscheuer and Steinbüchel, 2003)

[a] fed batch process

Modification to alter the WE profile of ADP1 has also been attempted in a mutant possessing an alternative FAR complex LuxCDE in place of the native *acr1*, with hope the new FAR would express different chain length preferences (Santala et al., 2014). A maximum titre of 91 mg/L

WE was achieved, a reduction from the WT 162 mg/L. The effect was partly observed, with changes in both chain length and saturation profiles of the acid and alcohol components. However, the average chain length reduced by 2 just carbons, not achieving the 'wider spectrum of alkanol chain lengths' the authors claim is required.

4.1.2. *Rhodococcus* strains

While successful in producing WEs from structurally-related substrates, *Rhodococcus* strains have been limited in WE production from simple substrates (see Table 2.1), with WEs from glucose in WT *R. jostii* RHA1 contributing just 0.0002% of CDW. Inducible overexpression of the native FAR enzyme FcrA, which had very low levels of transcription and expression in the WT, increased the carbon flux into fatty alcohol production, with WE production of up to 13% of CDW (Round et al., 2017). The FcrA performed both stages of the fatty acyl to alcohol reduction, meaning overexpression of a specific FAldR could therefore further increase WE production. The group also tested a WS/DGAT with increased WS activity (Round et al., 2019), utilising both the FcrA and a WS from *Marinobacter hydrocarbonoclasticus* DSM 8798. The resulting species accumulated up to 15% CDW as WEs at yields of 0.05 g/g glucose substrate. A fed batch fermenter was also explored, in which the strain produced 5 g/L (1 g/L per day), with yield of 0.025 g/g.

The *Rhodococcus* strain *R. opacus* PD630, which did not naturally produce WEs on simple substrates, was also engineered for *de novo* WE synthesis by expression of a fatty acyl-CoA reductase from *M. hydrocarbobonclausticus*, which reduced acyl chains to alcohols (Lanfranconi and Alvarez, 2017). Whereas the WT on gluconate produced 100% TAGs, storage lipids in the modified strain were 46% WEs. This strain can also grow and accumulate TAGs on various model and industrial hydrolysates (Wang et al., 2014), hence this modification could facilitate WE production from lignocellulosic biomass waste streams.

4.2. Modifications in non-native producing strains

In the first study to isolate and characterise the WS/DGAT in *Acinetobacter baylyi* ADP1, the enzyme was expressed heterologously in *E. coli* S17-1 and *Pseudomonas citronellolis* (Kalscheuer and Steinbüchel, 2003). WS activity in the WT strains was negligible, whilst modified cells demonstrated activities comparable to ADP1. Recombinant *E. Coli* XL-1 Blue producing the WS/DGAT enzyme from *M. smegmatis* mc^2155 was also engineered, but WS activity in this strain was only slightly higher than the WT. The production of jojoba-like WEs from *E. coli* was subsequently attempted by co-expression of a fatty-alcohol producing, acyl-

CoA reductase from the jojoba plant and the WS/DGAT from ADP1 (Kalscheuer et al., 2006). The mutant produced saturated C_{32} ester palmityl palmitate when grown on oleate, a monosaturated C_{18} fatty acid. WE content in cells was 1%, three times that of cells grown on LB medium, suggesting *de novo* WE synthesis was minimal.

To diversify the chemical characteristics of microbial WEs, *E. coli* was engineered to produce methyl-branched esters (MBEs) using propionate as a substrate (Menendez-Bravo et al., 2014). The group achieved up to 98 mg/L on propionate and octanol, the MBE alcohol component (Murli et al., 2003; Pfeifer et al., 2001). To avoid alcohol supplementation, further engineering with three fatty alcohol biosynthesis pathways was attempted, achieving a maximum titre of 37 mg/L of MBEs from propionate alone. Further exploration using different promoters and plasmid copy numbers achieved maximum titres of 138 mg/L in batch and 790 mg/L in a fed-batch process using an octanol, oleic acid, glucose and propionate mixture as feedstock (Menendez-Bravo et al., 2016). However, the authors' calculated yield of 2 mg MBE/g glucose is low and misleading, as it does not account for the other substrates.

Other *E. coli* modifications have largely focussed on TAG production (Janssen and Steinbuchel, 2014) or short chain esters, e.g. fatty acid ethyl esters, for use in biofuels (Liu and Khosla, 2010; Rahman et al., 2019; Rottig et al., 2015), which will not be discussed in detail here.

5. Production from waste streams

Many waste streams are energetically and chemically rich with a diverse range of molecules and functionalities, and their valorisation improves process efficiency. They are economically attractive due to their inherent low value; since waste processing otherwise incurs significant costs, their price may be negligible, or even negative. However, utilising waste streams presents several challenges for bacterial degradation; 1) in many waste streams few bacterial species are able to process the major components, 2) components or contaminants may be toxic to bacterial cultures and 3) mixtures are complex and can be highly variable, even if taken from the same source or process. Nonetheless, WE production has been achieved using waste stream feedstocks, and the largest future expansion is likely to come from this area (see Section 7.1 for further discussion).

5.1. Lignin derivatives

Lignocellulosic biomass, the rigid organic material which gives plants their structure resilience, is a common agricultural waste in forms such as wheat stems, corn stover, wood shavings and

sugarcane bagasse. It is comprised of three major components; cellulose, the polymer form of glucose, hemicellulose, a polymeric mixture of five and six carbon sugars, and lignin, an irregular aromatic polymer (Sanderson, 2011). Valorisation of this biomass is of interest for fuel, gas and platform chemical production employing the biorefinery concept, the biomass-based equivalent of a fossil-fuel refinery for chemical production. Pretreatment and hydrolysis of the biomass in the refinery hydrolyse the cellulose and hemicellulose into a mixture of C_5 and C_6 sugars, which are directly suitable for many digestion and fermentation processes (Galbe and Wallberg, 2019). However, the complex structure of lignin makes processing this component difficult (Xie et al., 2016), and, once separated from the soluble sugars, it is typically burnt for energy. However, direct utilisation of lignin, which can be depolymerised into a range of aromatic monomers, as a feedstock would provide greater flexibility and potential for higher efficiency.

Lignin can be hydrolysed and depolymerised into a range of aromatic acid and alcohol monomers which often cannot be utilised for fermentation and may act as microbial inhibitors. However, the utilisation of two of these monomers for bacterial WE production has been demonstrated in *A. baylyi* ADP1. Coumarate, a common lignin-related aromatic acid, was reported to be co-consumed with acetate, with maximum combined tolerance of 55 mM acetate and 30 mM coumarate respectively (Salmela et al., 2019). A fed-batch bioreactor provided sequential coumarate additions to avoid exceeding tolerance limits and produced WE titres of up to 202 mg/L, with a yield of 3.7% w/w and cell WE content of 5.8% w/w.

Aromatic acid 4-hydroxy-benzoate (4HB), which is derived from lignocellulosic biomass pre-treatment, also inhibits many bacterial species, but could be utilised by ADP1 as a sole carbon source for growth and WE production for concentrations of up to 4 g/L (Reiser and Somerville, 1997). Genetic modification of ADP1 has since provided an ADP1 mutant which preferentially utilised 4HB and benzoate even in the presence of better substrates such as glucose and arabinose (Singh et al., 2019).

5.2. Other waste stream utilisation

Another effluent demonstrated to sustain WE production is alkane-rich wastewater from the oil and gas industry. Given its success on hydrocarbons, *A. borkumensis* SK2 was investigated for WE production in a continuous bioreactor using wastewater, achieving a titre of 8.45 g/L, with 6.70 g/L produced extracellularly, over 10 days (Sudmalis et al., 2018). Despite the claim that the process also removed 99% of organics, only 80% of organics were removed at the

maximum WE titre. Whilst the long-term viability of the process can also be questioned, since it relies on the oil and gas industry, the progress in converting a classically energy and resource intensive purification into a source of valuable chemical production is nevertheless an impressive landmark, with opportunity for further development and scale-up.

Carbon dioxide, a large and environmentally relevant waste stream, can be reduced to industrially relevant molecules including hydrogen, methane and organic acids and alcohols by microbial electrosynthesis (MES) (Kong et al., 2020). A two-step WE synthesis from CO_2 has been proposed, with acetogenic bacteria producing acetate by MES, then *A. baylyi* ADP1 converting this to WEs (Lehtinen et al., 2017). Acetate concentrations of 38 mM were achieved over 10 days of MES, 13 mM of which was used to produce 18 mg/L of WEs (4.6% C/C yield). While the titre was reduced compared to pure acetate, due to the low substrate concentration, the yield doubled and the authors suggest significant improvements on the system should be possible (Lehtinen et al., 2017).

6. Wax ester production by mixed microbial communities

An area of research attracting a lot of recent interest is the use of mixed natural or synthetic microbial communities for biotechnology (Altermann and Hickey, 2020), as the deployment of monocultures for the generation of products can be problematic and limiting. For instance, the probability of monocultures suffering contamination increases with operation time, meaning processes must be undertaken in a non-continuous mode, which may be impractical for large scale production. Furthermore, production capacities of monocultures, particularly modified strains, can decrease over 10s-100s of generations because cells evolve to increase their growth rate and reduce metabolic burden as a survival advantage (Rugbjerg and Sommer, 2019). For instance, plasmids or chromosomal DNA encoding for product generation may be lost over many generations.

In continuous fermentations, mixed communities offer several advantages over single strain production, including generating greater yields, higher growth rates and a wider range of biological transformations (Hesseltine, 1992). Processes can be operated continuously without risk of strain degeneration: 1) conditions are chosen such that product generation, requiring DNA retention, provides an evolutionary advantage (Temudo et al., 2008) and 2) if multiple strains within the culture facilitate the same reaction the metabolic burden on each is reduced, so the driving force to lose genes decreases (Jawed et al., 2019). In addition, multiple products can be obtained simultaneously from the range of species present. Mixed communities

potentially process a broader range of substrates, with mixed cultures utilising complex, impure and typically cheaper feedstocks such lignocellulosic biomass and waste streams (Jawed et al., 2019; Sabra and Zeng, 2014). Microbial communities can also offer protection from contamination, so processes can be carried out in open cultures, making continuous culture processes even cheaper to perform (Sabra and Zeng, 2014).

However, mixed communities, particularly artificial ones, also pose problems, mainly regarding population stability. If conditions change, even slightly, any species with a specific growth advantage will rapidly outcompete other members and the balance may be lost. Storing mixed cultures also causes issues, as traditional methods such as lyophilisation will not preserve all strains proportionally, so maintaining the balance of species is very difficult (Hesseltine, 1992). Studying and understanding mixed communities is much more challenging than a single species, hence many traditional processes are still carried out with uncharacterised mixed communities.

The simplest form of a bacterial community is a co-culture, with two species cultured simultaneously, which can be used to simultaneously generate WEs and other products. An *A. baylyi* ADP1, *Clostridium butyricum* co-culture was investigated for WE and 1,3-propanediol production from crude glycerol, a by-product of biodiesel production (Mangayil et al., 2019). ADP1 is unable to grow on glycerol as a sole carbon source (Salcedo-Vite et al., 2019). However, *C. butyricum* fermented it anaerobically to produce 1,3-propanediol, with the acetate and butyrate byproducts then utilised aerobically by ADP1. Under optimal conditions, a WE content of 3.4% CDW was achieved, but with titre of just 0.034 g/L, although 1,3-propanediol at 5.5 g/L was also achieved. A modified ADP1 strain deficient in glucose consumption, ADP1g, has also been exploited in co-cultures with *Clostridium butyricum* for WEs and H_2 production from glucose (Salmela et al., 2018). *C. butyricum* consumed glucose in an anaerobic fermentation, producing H_2 gas, acetate and butyrate, the latter two again acting as substrates for ADP1. This approach yielded 1.1% w/w of WE from glucose, at 0.028 g/L, as well as 1.7% mol/mol of H_2. In both cases ADP1 benefits *C. butyricum* growth by removing inhibitors and increases process efficiency by utilising otherwise waste metabolites. These mixed cultures still required sterile growth conditions since they are vulnerable to external contamination and likely to suffer from similar heterogeneity problems as monocultures, leading over time to lower production rates.

Alternatively, an open mixed bacterial community has been used to investigate neutral lipid production from organic-rich wastewater, which was inoculated with sludge from a wastewater

treatment plant. Selection for hydrocarbonoclastic strains was applied by alternating periods of excess carbon and starvation at negligible nitrogen content, with high accumulation rate also encouraged by shortening the 'feast' periods (Castro et al., 2018). The final culture was unsurprisingly dominated by lipid-accumulating species of *Rhodococcus*, *Acinetobacter* and *Pseudomonas.* A factorial experimental design determined the optimal lipid-accumulating conditions as minimal nitrogen, high carbon: nitrogen ratio and the shortest time point (29 hours), and neutral lipids were produced up to 33% of CDW, although just 7% of this was WEs due to the low prevalence of WE-producing bacteria (*Acinetobacter*). Selecting for WE over TAG production would be challenging due to their similar structure and function, although using substrates on which *Acinetobacter* species, but few others, can grow, such as 4HB, could increase the *Acinetobacter* popularity.

7. Future perspectives

With the long-term goal to produce WEs as bulk commodity chemicals, greater focus on scalable and affordable technologies must be a priority. As well as improving yields, the focus of most work discussed here, downstream processing and substrate purchase are known to contribute significantly to the cost of a biotechnological process (Straathof, 2011), so these areas will undoubtedly be key targets in readying this process for commercialisation. With this in mind, the current challenges and opportunities for progress in these areas are discussed in more detail below.

7.1. Novel substrates

With a maximum yield for bacterial processes thus far at 7.5 % per gram of substrate on glucose (Luo et al., 2020), and global sugar prices currently around €240 tonne^{-1} (IndexMundi, 2020), 1 tonne of bacterial WE would require 13 tonnes of glucose to produce, a cost of over €3,100 from substrate alone. By comparison, paraffin wax prices in Europe are around €1,100 per tonne (Trinder, 2018), while beeswax can cost between €3,400-8,500 per tonne depending on quality and origin (Eshete and Eshetie, 2018). Despite this price disparity indicating that, in certain markets, consumers are willing to pay a premium for natural, non-fossil products, the substrate cost alone for bacterial WE production is near treble the refined paraffin wax price and approaching the lower end cost of beeswax. It is therefore obvious this cost must be decreased in order to make prices competitive with either existing product.

Focus on waste streams as low-cost feedstocks is growing, but the streams of interest must be expanded to determine an optimum balance between price and suitability, both of the sourcing

method and substrate success. As very few waste streams have been investigated for WE production thus far, it is appropriate to consider that waste streams explored as feedstocks in other biotechnological processes may be suitable for WE production, particularly streams which have previously been utilised for microbial lipid and biofuel production. As with alkane-rich wastewater, agro-industrial effluent from dairy, olive and confectionary industries have been demonstrated to be appropriate feedstocks for biofuel production by oleaginous yeast *Wickerhamomyces anomalus* EC28, as well *as* waste cooking oils from meat, fish and potato frying (Arous et al., 2017). These effluent streams have greater longevity than oil and gas wastewater, the availability of which will decrease in the future as fossil fuel use declines. Anaerobic digestate, the nutrient and lignocellulosic-rich residual material after anaerobic degradation of organic matter, has also previously been demonstrated to be suitable feedstock for fungal production of lipid-based biofuels (Zhong et al., 2016). Since digestion can be performed on a wide variety of organic material, including maize and other specific energy crops, sewage and agricultural, animal and food waste, digestate is a widely available and adaptable resource, although the composition can vary greatly, complicating the production.

Raw food-waste has been identified as a feedstock for microbial fermentation and conversion to useful products. Orange peel has been highlighted as a particularly rich in utilisable substrates, with mixed sugars in the peel hydrolysate previously converted to lactic acid by several strains of *Lactobacillus delbrueckii* (Bustamante et al., 2020). Pectin can also be isolated from orange peel and converted to ethanol by microbial consortia (Ojewumi et al., 2018). Although this waste stream has not yet been demonstrated for lipid production, it would likely show less chemical diversity and variation than those above, and hence achieving a reliable WE production pathway may be easier.

These examples highlight the diversity and availability of rich and utilisable waste streams which remain largely unexplored for bacterial WE production. Another approach to increase the breadth of substrates utilisable for this purpose is adaptive laboratory evolution (ALE) (Dragosits and Mattanovich, 2013), which can select for certain behaviours by applying gradually changing external pressures. This technique has previously been used to select for higher lipid accumulation (Castro et al., 2018; Daskalaki et al., 2019), but ALE can also be used to improve utilisation and tolerance of substrates which can act as inhibitors at moderate concentrations, e.g. organic acids (Fernández-Sandoval et al., 2012), alcohols (Horinouchi et al., 2018) and aromatics. This can facilitate bioprocesses with higher concentrations of mixed and unconventional substrates, meaning greater WE titres can be achieved, which is necessary

for processes to be viable at industrial scale. If improvements to the desired metrics are observed, sequencing can detect the affected genes, offering insight into approaches subsequent genetic engineering should take to further improve tolerances.

As highlighted in the discussion of Section 6, mixed cultures will undoubtedly play a part in processing complex carbon sources, although their success for WE production specifically may be limited by the pref

erence of many species for TAG accumulation over WEs (Castro et al., 2018). Synthetic consortia have yet to be explored for this purpose beyond two strain co-cultures but could improve this situation.

7.2. Wax ester extraction

While not discussed at length here, one area for improvement is extraction of WEs from cells, as downstream processing is a large contributor to the costs of biotechnological products. Regardless of yield and substrate cost, this step is critical to process viability. In recent literature (Holert et al., 2020; Luo et al., 2020), the Bligh and Dyer, and Folch methods first reported in the 1950s are still common for extraction (Bligh and Dyer, 1959; Folch et al., 1957), despite both methods employing toxic solvents chloroform and methanol which are not scalable due to costs and regulations. The limited options in terms of extraction methods, as well as the high cost and inefficiency of many, has been highlighted in a recent review of lipid extraction from bacteria (Hwangbo and Chu, 2020). A method which is both efficient and viable at scale, whilst sufficiently cheap to keep product prices competitive is essential if the area is to have industrial potential. Greener solvent extraction methods employing renewable solvents have been proposed (Breil et al., 2017), but alternatives to centrifugation for cell disruption (e.g. milling, induced lysis etc) should also be investigated to reduce energy consumption and costs (Patel et al., 2018; Peternel, 2013).

Improvement to extraction could also be achieved by exploiting strains that naturally excrete lipids, as demonstrated by several species via a so far uncharacterised mechanism (Bredemeier et al., 2003; Manilla-Pérez et al., 2010; Manilla-Pérez et al., 2011; Nakano et al., 2012). Species with this trait produce WEs primarily when grown on alkanes and complex alcohols. Research should therefore be undertaken to confirm findings of extracellular WE production on pyruvate (Manilla-Pérez et al., 2011). If reproducible, studies should be expanded to other simple substrates. Alternatively, genetic modification of these species to produce FAR or FAldR enzymes and thus synthesise WEs *de novo* could be explored. A second field of future work

around these species would be to identify the genes associated with, and hence better understand, lipid export. These could be expressed in other species already demonstrating high WE yields and employing renewable substrates, e.g. *A. baylyi* ADP1. In yeast *Yarrowia lipolytica,* such an approach combining genetic engineering and *in situ* solvent extraction produced fatty acids above 100% CDW for the first time (Ledesma-Amaro et al., 2016).

7.3. Quantification and optimisation

Maximisation of WE production by increasing yields, WE content and titres, through either condition optimisation and/or genetic modification, has been the major focus of the literature thus far, particularly in species of *Acinetobacter*, *Marinobacter* and *Rhodococcus*, as emphasised by Table 2.2 and Table 2.3. Undoubtedly genetic engineering, directed evolution and ALE will be the key factors in improving these metrics, as has been demonstrated by the near 3-fold increase in all three in *A. baylyi* ADP1 on glucose (Luo et al., 2020).

Alongside this, identification of optimal conditions for different strains and mutants will be essential. To streamline this process, more researchers should consider performing and publishing robust, multi-factor experimental designs for process optimisation (Castro et al., 2018; Rottig et al., 2016a). This would reveal more accurate values of maximum WE output, which are necessary to decide whether pursuing a particular strain, modification and/or feedstock is viable.

A final, yet important factor highlighted by this review as lacking in terms of research effort is quantification of WE production across all species. As evidenced by the small fraction of strains from Table 2.1 present in Table 2.2, in many species WEs have been confirmed by TLC and gas chromatography/mass spectrometry, yet no data has been published on the quantity produced. Similarly, several authors publish only limited data (e.g. the WE content, but not CDW, titre or yield) which is unhelpful and potentially misleading. Quantification is fundamental to assess whether optimisation and scale-up are viable, and by omitting this data, key species or substrates may be overlooked by the research community and commercially.

8. Conclusion

This review has assessed a wide range of bacteria for WE production on various substrates. While great success has historically been achieved with respect to alkanes and their derivatives (Ishige et al., 2003), many strains have since been shown to produce WEs on a range of simple, renewable substrates. The main factor differentiating these behaviours is the presence of fatty acyl reduction enzymes that facilitate *de novo* fatty alcohol synthesis. *A. baylyi* ADP1 has

received the most attention in the literature, which is arguably reasonable given the broad range of suitable substrates and high WE yields achieved, although similar achievements in other strains may be made given comparable research attention. Nonetheless, ADP1's natural competence has made it a particularly desirable strain for genetic modifications, and this shows no sign of relenting.

Genetic modification can either impart or improve WE synthesis ability in various bacterial strains. Overexpression of FAR-coding genes has been successfully employed to improve production in both ADP1 (Lehtinen et al., 2018) and *Rhodococcus* strains (Lanfranconi and Alvarez, 2017; Round et al., 2017; Round et al., 2019), suggesting a likely biological bottleneck, while redirection of carbon flux can enhance efficiency and yield (Luo et al., 2020). The WE synthesis pathway has also been constructed in *E. coli* but, while interesting as a proof of concept, native producing strains remain more desirable chasses. The few authors to investigate scale up processes found fed-batch reactors to drastically improve WE content and titre over flask scale (Round et al., 2019; Sudmalis et al., 2018), indicating good prospects for future development at pilot and potentially commercial scales.

Future developments in bacterial WE production are likely to focus on increasing efficiency, scalability and economic viability. The deployment of model and real-life waste streams as substrates has already demonstrated the possibility of microbial WE production as a method of waste valorisation, and interest in this area is only expected to grow, accompanied by an expansion of the waste streams identified as suitable substrates. The importance of appropriate substrate choice and efficient downstream processing in the future commercial viability of bacterial WEs are highlighted to be as important as the optimisation of WE production and yields in the bacteria. With sufficient research into these, thus far under-explored, areas of the field, the case for and prospect bacterial WEs becomes ever stronger.

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The statement shall describe the candidate's and co-authors' independent research contributions in the thesis publications. For each publication there should exist a complete statement that is to be filled out and signed by the candidate and supervisor **(only required where there isn't already a statement of contribution within the paper itself).**

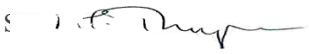
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Student Confirmation

Student Name:	Laura Martin		
Contribution to the Paper	Planned and conducted literature search, collated the findings, and wrote the paper.		
Signature		Date	05.12.2023

Supervisor Confirmation

By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

Supervisor name and title: Professor Ian Thompson		
Supervisor comments I agree with summary		
	Date	08.12.2023

This completed form should be included in the thesis, at the end of the relevant chapter.

2.5. Research gap

The examination of the literature presented here highlights a clear research gap. Although research into bacterial WE production is ongoing, one of the biggest limitations to date is feedstock cost, which hampers the commercial viability of production compared to fossil-derived waxes. At the same time, fermentation-derived VFAs have been identified as a suitable substrate for bioproduction, while their potential to be derived from a huge variety of waste streams makes their use environmentally and economically attractive.

A handful of studies have investigated WE production from acetate (60, 90, 100, 147), but the application of other VFAs, their mixtures or acid-rich waste streams in WE biosynthesis remains, to date, unexplored. The work in this thesis addresses this research gap in the following ways:

1. Synthetic VFAs acetate, propionate, butyrate and valerate, which comprise the majority of acidogenic fermentate, were explored as potential growth substrates for the WE producing bacterium *Acinetobacter baylyi* ADP1.
2. Results from investigations on synthetic substrates were extrapolated to acidogenic fermentate, a complex mixed substrate for bioproduction. Fermentate was employed with minimal treatment and preparation. The effect of fermentate composition, controlled by varying the feedstock employed, was also examined.
3. The ability of minor genetic edits to improve tolerance, growth and WE production on these fatty acid substrates was investigated.
4. Based on the observed limitations of genetic engineering, control of key nutrient ratios was examined as an alternative method of increasing bioproduction. Nutrient concentrations in fermentate were then manipulated, by the deployment of low-cost ammonium removal methods, to replicate the optimal conditions identified in synthetic media.

2.6. Aims and objectives

The aim of this project was to investigate bacterial production of industrially important WE by the bacterium *Acinetobacter baylyi* ADP1 from the low-cost, renewable feedstock VFAs. Within this overarching aim were a series of smaller objectives:

- **Characterise the growth and tolerance of ADP1** on a range of pure VFAs and on acidogenic fermentate.
- **Quantify wax production** in the wild type ADP1 bacterium and a mutant strain engineered for wax overproduction in nitrogen-rich conditions. Compare the two strains in both synthetic media conditions and in fermentate.
- **Alter the VFA composition of fermentate** by utilising different fermentation conditions and compare growth and WE synthesis in the new fermentate by the wild type and mutant. Identify the desired composition through experiments with synthetic culture media.
- **Investigate optimal nutrient conditions** for wax ester production by ADP1 in synthetic media on acetate, as a simplified model for fermentate. Identify significant parameters for improving wax production.
- **Re-create these optimal nutrient conditions in fermentate** by altering the available nutrient levels.

Chapter 3 – Growth and wax production on volatile fatty acids

3.1. Introduction

Cost is one of the largest barriers to commercial microbial bioproduction; the production of low value, large scale commodity chemicals such as lipids and biofuels is often not economically feasible (148) unless feedstocks are abundant, conveniently located and easily refined. Several obvious targets to reduce costs include increasing yields to reduce substrate costs, process intensification to decrease operating volumes and capital expenditure, and improved separation and purification to decrease energy and solvent requirements (149-151).

Another area for potential for cost saving is the employment of lower cost substrates. Typical bioproduction processes have employed sugar substrates for their high energy density, high microbial tolerance and ease of metabolism, but such feedstocks are relatively expensive, with a typical cost of glucose in 2022 of $600-700/tonne (152). This makes bioproducts uncompetitive with their traditional, fossil-derived counterparts: at a time when fossil diesel costs in the USA were $1.25/kg, glucose costs of $400/tonne resulted in a biodiesel cost of $5.9/kg (153). The same is true for WEs; the current maximum conversion of 0.075 g/g of glucose to WEs (93) would lead to bacterial WEs costing $8/kg from substrate alone, while paraffin wax costs $1.7-2.3/kg and beeswax is around $6.4/kg (154). Furthermore, sugar crops for bioproduction compete with traditional agriculture, which has environmental and social ramifications. Decades of research has attempted to obtain sugars from lignocellulosic biomass to avoid these problems (155), but this remains technologically and economically challenging (156, 157).

Volatile fatty acids (VFAs) have potential as a carbon source for bioproduction (143). They can be sourced renewably at low cost due to their ease of generation from organic waste via acidogenic fermentation (AF) (130, 139, 158), which is currently used in biological remediation of waste streams including sewage, animal manure, food waste and industrial wastewater. However, in many cases the behaviour of microbial strains of interest for bioproduction on VFAs, including growth rates and tolerances, is not well known. Of the available VFAs, acetate is of particular interest because it is the major product in most AF processes and it has potential alternative renewable sources from microbial electrosynthesis and gas fermentation (159, 160). For these reasons, the work described in this chapter explored

the growth and tolerance limits of *Acinetobacter baylyi* ADP1 on sodium acetate, propionate and butyrate, and subsequently focused on WE production studies from acetate as a carbon source.

Since this thesis will focus on the production of WEs, ensuring a reproducible and reliable method for the identification and quantification of WEs is essential. Within the literature, gas chromatography-mass spectrometry (GC-MS) is widely considered as the gold standard for quantifying neutral lipids and is employed for quantification and detailed structural and compositional analysis (161). However, GC-MS requires lipid derivatisation to improve volatility (162, 163), a preparation process which is time consuming and destructive. Where WEs have been analysed by GC without derivatisation, a single sample could take 30-40 minutes to run (164, 165), resulting in very long analysis times and high equipment operating costs.

As an alternative, proton nuclear magnetic resonance (^{1}H-NMR) has been used for WE quantification in a number of studies (38, 93, 100, 162). NMR is used to study atomic nuclei with unpaired protons or neutrons (collectively nucleons), in this case hydrogen atoms. Nucleons each have an inherent spin, and an unpaired nucleon gives its nucleus an overall nuclear spin, which can be randomly oriented in any direction. NMR works by applying a strong external magnetic field to nuclei with spin, forcing them to align either with or against the magnetic field, and creating an energy difference between these two states (166). Radio waves at a specific frequency are then applied to temporarily 'flip' the low energy spins into the higher energy state. The decay of these nuclei back to the low energy state is then measured and converted into a spectrum, with the exact decay response of each atom influenced by its chemical environment – the atoms and functional groups it is bonded to. In this way, molecules can be identified by the combination of peaks that make up their spectra, with variations in functional groups causing spectra to differ. In particular, WEs can be identified separately to TAGs and other lipid classes due to the unique environment of the α-alkoxymethylene protons after the ester bond, which result in a specific and easily identifiable peak (see Figure 3.1 for details) (38, 167). Unlike GC-MS, ^{1}H-NMR does not require derivatisation, it is non-destructive, and has short measuring times (168), resulting in accurate lipid analysis in a fraction of the time of GC. Internal standards can be used to calculate absolute concentrations from the spectra obtained, and the use of digital standards can further reduce sample preparation time (169). While this method is widely utilised, independent verification of its

reliability, accuracy and sensitivity is important before it is employed to draw conclusions about factors that influence WE production.

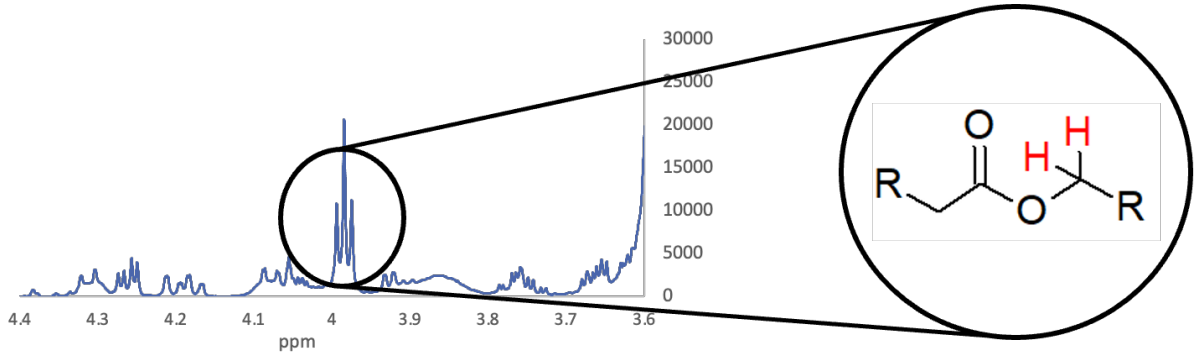


Figure 3.1. The unique methylene (CH_2) peak in the ^{1}H-NMR spectrum of WEs which was integrated for quantification, and the 2-α-alkoxy-methylene protons from WEs which are responsible for it.

Using this quantification method, suitable culture conditions for the bulk of the experiments in this thesis were identified. In particular, the effect of initial pH on WE production from acetate was of interest, as previous studies have found that pH can significantly affect growth, VFA tolerance and lipid accumulation in a range of micro-organisms (170-172). However, to date the effect of pH on WE production has not be investigated.

With acetate identified as a carbon source of interest, methods for improving growth, tolerance and WE production on acetate are also important considerations. One such method is the overexpression of enzymes required for lipid synthesis (173-175), especially known bottlenecks which limit substrate availability for and the rate of subsequent steps. As the first molecule in the fatty acid biosynthesis pathway (176), increasing the available pool of acetyl-CoA would be expected to increase WE production. This approach has been effective in increasing lipid biosynthesis in algae (177).

When acetate is used as a carbon source, two ATP-consuming pathways can be used to convert it into acetyl-CoA. One is a two-step, reversible reaction which goes via acetyl phosphate and is catalysed by acetate kinase and phosphate acetyltransferase (AckA-Pta) in the first and second steps, respectively. The other is a one-step reaction, catalysed by the enzyme acetyl-CoA synthetase (ACS), which directly combines acetate and co-enzyme A, is irreversible, and has high acetate affinity. Moreover, the overexpression of *acs* has been successful at increasing the production of neutral lipids in the microalgae *Chlamydomonas reinhardtii*, where overexpression of the endogenous enzyme resulted in a 2.4-fold increase in accumulation of TAGs, the dominant storage lipid in this strain (178). Since the pathways for WE and TAG synthesis are very similar, diverging only due to the esterification of a fatty acid and fatty

alcohol in WEs compared to fatty acid and glycerol in TAGs, overexpression of *acs* in bacteria may increase WE production.

3.2. Aims and objectives

The aim of the research described in this chapter was to validate reported methods for the culture, growth and lipid extraction of *Acinetobacter baylyi* ADP1. This was to ensure they were sufficiently reproducible and reliable to be used to investigate the more complex growth behaviours which would be investigated in the rest of the thesis. A brief investigation into the benefits of overexpression of a particular enzyme, acetyl-CoA synthetase, was also conducted as a potential avenue for exploration.

This work was broken down into the following objectives:

- To characterise the tolerance, growth rate and behaviour of ADP1 grown on volatile fatty acids, specifically the sodium salts of acetate, propionate and butyrate.
- To validate methods reported in previous studies for the extraction and quantification of WEs from ADP1 cells. This includes testing the detection limits of the ^{1}H-NMR technique to ensure adequate cell samples were collected for wax analysis.
- To explore the effect of initial culture pH on growth and WE production for ADP1 grown on acetate in batch cultures.
- To create an overexpression plasmid for the native ADP1 *acs*, transform the plasmid into ADP1 and grow the transformed strain on acetate.
- To evaluate the acetate tolerance and WE production of the ADP1 strain overexpressing *acs*.

3.3. Materials and methods

3.3.1. Strain

Wild type *Acinetobacter baylyi* ADP1 was obtained from Zhang *et* al (179). Bacterial stocks were stored at -80 °C in 20% v/v glycerol/water.

3.3.2. Chemicals

All chemicals were sourced from SigmaAldrich (Merck, Rahway, New Jersey, USA). HiFi Assembly Mastermix and high-fidelity Q5 polymerase were obtained from New England BioLabs (NEB, Ipswich, MA, USA). Polymerase chain reaction (PCR) purification and QIAprep spin miniprep kit were obtained from Qiagen (Hilden, Germany).

3.3.3. Media recipe

Mineral medium contained 2.5 g/L KH_2PO_4, 2.5 g/L Na_2HPO_4, 1 g/L NH_4Cl, 0.1 g/L $MgSO_4.7H_2O$, 0.01 g/L $CaCl_2.2H_2O$ and 1 mL/L of 1000x Bauchop and Elsden trace elements solution (180). Medium was prepared, adjusted to pH 6.8 as standard and autoclaved. 300 g/L sodium acetate, 300 g/L sodium propionate and 100 g/L sodium butyrate stocks were prepared and autoclaved separately and added as required immediately prior to inoculation.

3.3.4. Preculture conditions

Glycerol stocks were streaked on LB agar plates in the absence (wild type) or presence of 50 ug/mL kanamycin (plasmid containing strains). Single colonies were randomly picked and inoculated into LB broth, cultured for 4-8 hours, then transferred twice in minimal media with the carbon source of interest, each time cultured overnight. All precultures were grown at 30 °C and 150 rpm.

3.3.5. Growth experiments

Growth and tolerance experiments were conducted in 25 mL of culture in 250 mL conical flasks. Inoculum was transferred directly from the preculture to the experimental flasks. All cultures were grown at 30 °C and 150 rpm and experiments were performed in biological triplicate. 0.5-1 mL samples were taken for OD_{600} measurements by UV-1800 UV/Vis spectrophotometer (Shimadzu, Kyoto, Japan). Samples were diluted with ultrapure water to fall in the OD_{600} range 0.1-0.8. Growth rates were calculated by evaluating the slope of the linear plot of $\ln(OD_{600})$ against time from at least 4 data points, with all linear fits having an R^2 value of at least 0.98. Lag phases were calculated as the intercept between this linear plot and the horizontal line at the initial OD_{600}. Sigmoid curves were fitted in Origin for visualisation.

3.3.6. Wax production experiments

Wax production experiments were conducted in 200 mL of culture in 1 L conical flasks. Inoculum was centrifuged at 6000 g for 6 minutes (Eppendorf 5810 R, Eppendorf, Hamburg, Germany), supernatant discarded and cells resuspended in media prior to inoculation. All cultures were grown at 30 °C and 150 rpm and experiments were performed in triplicate.

3.3.7. Wax extraction and analysis

Samples of 15-30 mL were taken at regular intervals for OD_{600} assessment. Samples were centrifuged at 10,000 g for 8 minutes, the supernatant discarded, the pellet resuspended in ultrapure water and transferred to a pre-weighed Eppendorf tube. Samples were centrifuged again at 13,000 g, the supernatant discarded, and the pellet lyophilised overnight. Cell dry weight was determined gravimetrically.

Lipids were extracted following an adapted Bligh and Dyer method (Santala et al., 2011b). Cell pellets were resuspended in 2 mL of PBS, then 2.5 mL chloroform and 5 mL of methanol were added, and the mixture shaken for 4 h at 150 rpm. A further 2.5 mL of methanol, 2.5 mL PBS and 2.5 mL of chloroform were then added, and the sample was mixed again and stored overnight at 4 °C. The suspension was centrifuged at 10,000 g for 8 min, and the lower organic phase was transferred to a glass vial to evaporate. The aqueous phase was extracted again by addition of 5 mL of chloroform, mixed and incubated overnight at 4 ◦C, then centrifuged and separated as before.

NMR analyses were performed using a Bruker Avance III spectrometer (700 MHz) with a TCI cryoprobe (Bruker, MA, USA). Lipid samples were dissolved in 600 µL of chloroform-d and ^{1}H-NMR spectra were recorded at ambient temperature. Spectra were processed and analysed using MestreNova 14.1.1. with an artificial reference peak at δ −1 ppm, corresponding to a single hydrogen atom with concentration of 10 mM. Global Spectrum Deconvolution was used to accurately isolate and integrate the triplet peak at δ 4.05 ppm, corresponding to the 2 α-alkoxy methylene protons from the alcohol component of WEs. From this the WE concentration was determined and used to calculate the mass of WEs produced, assuming an average Mw for WEs of 506 g/mol (Lehtinen et al., 2018).

3.3.8. Acetate quantification by GC-FID

The remaining acetate content at each time point was measured from the supernatant of the extracted cells for CDW and WE quantification using gas chromatography-flame ionisation detection (GC-FID) (Shimadzu GC-2010, Shimadzu, Tokyo, Japan) with a 30 m × 0.25 mm ×

0.25 µm fused-silica capillary column (ZB-FFAP). The temperatures of the detector and injector were set at 350 °C and 250 °C, respectively. The oven temperatures were set at 100 °C for 2 min and then increased at a rate of 16 °C/min. Calibration was performed with standards of sodium acetate at concentrations from 0.31 to 20.00 g/L. All samples and standards (450 µL) were acidified with 10 % v/v of formic acid (50 µL) prior to analysis. C-conversion efficiency was calculated from the total acetate consumption for each sample.

3.3.9. Plasmid construction

The native *acs* gene in *A. baylyi* ADP1 and its accompanying ribosome binding site (RBS) were amplified from WT-ADP1 gDNA and cloned into a pBAV1k expression vector using NEBuilder® HiFi DNA Assembly (NEB, Ipswich, MA, USA). Polymerase chain reaction (PCR) was performed using the primers and templates given in Table 3.1 with high-fidelity Q5 polymerase at an annealing temperature of 58 °C, as determined by NEBuilder HiFi DNA Assembly tool.

Table 3.1. The primer sequences and reaction details for the PCR reactions performed to make plasmid pLM001. Capitalised sections of the primer sequence were to anneal directly to the fragment being cloned, lowercase sections were overlap or spacer regions.

Reaction	Function	Template	T_{ext} (s)	Primer Name	Sequence
1	PCR of pBAV1k with overhangs to bond to ACS + RBS fragment.	pBAV1k-T5-gfp (87)	73	pBAV1k BB_fwd	cacatcatagTTACTAGAG AAAGAGGAGAAATAC
				pBAV1k BB_rev	ttgatttttTTGTTATCCGC TCACAATTG
2	PCR of ACS + RBS fragment with overhangs to bond to pBAV1k backbone.	WT ADP1	40	ACS + RBS_fwd	cggataacaaAAAAAATCA AGGAAGTGATACTATG
				ACS + RBS_rev	tctctagtaaCTATGATGTG GAATTTTGCG
3	Sequencing pLM001	pLM001	-	ACS2 seq_F	GATGATGTGTACTGGTGTACGG
				ACS2 seq_R	CCGTACACCAGTACACATCATC

PCR products were analysed by gel electrophoresis on 0.8% w/w agarose gels, with the GeneRuler 1 kB DNA ladder from ThermoFisher (Waltham, MA, USA) used for reference. Following the successful amplification, PCR products were digested with Dpn1 for 15 minutes

at 37 °C to degrade the template DNA, purified with Qiagen QIAquick purification kit and assembled using the NEBuilder HiFi DNA Assembly Reaction Protocol (181).

A. baylyi ADP1 was transformed with the assembled plasmid, pBAV1k-T5-*acs*-*gfp* (referred to as pLM001), by the addition of approximately 1 μg of purified plasmid DNA and 70 μL of liquid cell culture (grown overnight in LB) to 1 mL of fresh LB broth. The transformation mix was incubated overnight at 30°C and 150 rpm, then plated on LB agar plates supplemented with 30 μg/mL kanamycin. To confirm successful assembly, pLM001 was also transformed into *E. coli* DH5α and transformants were selected on LB agar plates supplemented with 50 μg/mL kanamycin. A single colony was picked and grown overnight in LB broth supplemented with 50 μg/mL kanamycin. The cells were collected by centrifugation at 1,500 *g* for 10 minutes, and the plasmid was extracted by miniprep, purified and sent for sequencing with primers ACS2 seq_F and ACS2 seq_R, as given in Table 3.1. To construct the necessary control strain, ADP1 wild type (WT) was transformed with pBAV1k-T5-*gfp*. Transformation was verified by visual examination of the LB-kanamycin plates, with GFP expression giving colonies a distinct yellow/green colour. Plates were also analysed by fluorescent imaging using a VersaDoc imaging system (Bio-Rad, Hercules, CA, USA) with an FITC filter, with excitation wavelength of 475 nm and emission wavelength of 530 nm, to capture the fluorescence of the GFP.

3.4. Results

3.4.1. Growth rate and tolerance determination

3.4.1.1. Acetate

Growth and tolerance of *A. baylyi* ADP1 on sodium acetate as a sole carbon source was initially explored. Cultures were grown under aerobic conditions in flasks, as initial tests found the strain struggled to grow on acetate at increased concentrations in tubes due to poor aeration.

The growth curves demonstrated a clear exponential phase of growth, with most featuring a lag phase due to cell adaptation to the substrate and its concentration (Figure 3.2). A stationary phase was identified at high substrate concentrations (Figure 3.2, blue line), while at low concentrations death phase began almost immediately following the exponential phase (Figure 3.2, red line). Maximum growth was limited by substrate concentration up to at least 100 mM, beyond which carbon was no longer the limiting nutrient, as evidenced by comparable maximum OD_{600} at 150, 200 and 250 mM of acetate. A maximum concentration of 300 mM sodium acetate was also tested, but no growth was observed indicating this exceeded the tolerance limit. Growth rate decreased with increasing acetate concentration, with a maximum growth rate of 0.69 hr^{-1} achieved on 25 mM sodium acetate, decreasing to 0.24 hr^{-1} by 250 mM (Table 3.2). Moreover, lag phase increased with acid concentration: at 25 mM a lag phase of less than 1 hr was observed, while at 250 mM the lag phase was almost 20 hours. This indicated a trade-off between achieving high cell densities and rapid growth.

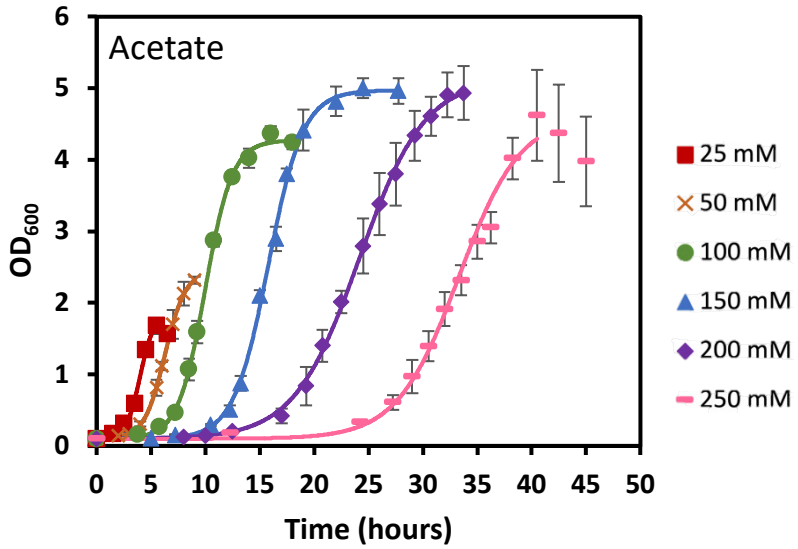


Figure 3.2. Growth curves for ADP1 grown on sodium acetate concentrations from 25-250 mM acetate; 25 mM (red squares), 50 mM (orange crosses), 100 mM (green circles), 150 mM (blue triangles), 200 mM (purple diamonds) and 250 mM (pink dashes). Data points and error bars represent the means and standard deviations, respectively, of three biological replicates. Solid lines represent sigmoid fits to the raw data.

3.4.1.2. Propionate and butyrate

Growth on sodium propionate and butyrate was also measured, and both substrates were suitable sole carbon sources for ADP1, with growth in the range of 2-6 OD_{600} achieved, depending on the carbon source and concentration used (Figure 3.3 A and B). In both cases, growth was achieved up to a maximum of 100 mM, while at 150 mM no growth was observed on either substrate. A decrease in growth rate and a longer lag period were observed with increasing substrate concentration, as observed for acetate. Growth was substantially slower than at the equivalent concentration of acetate, both when measured as acid and total carbon concentration (Table 3.2).

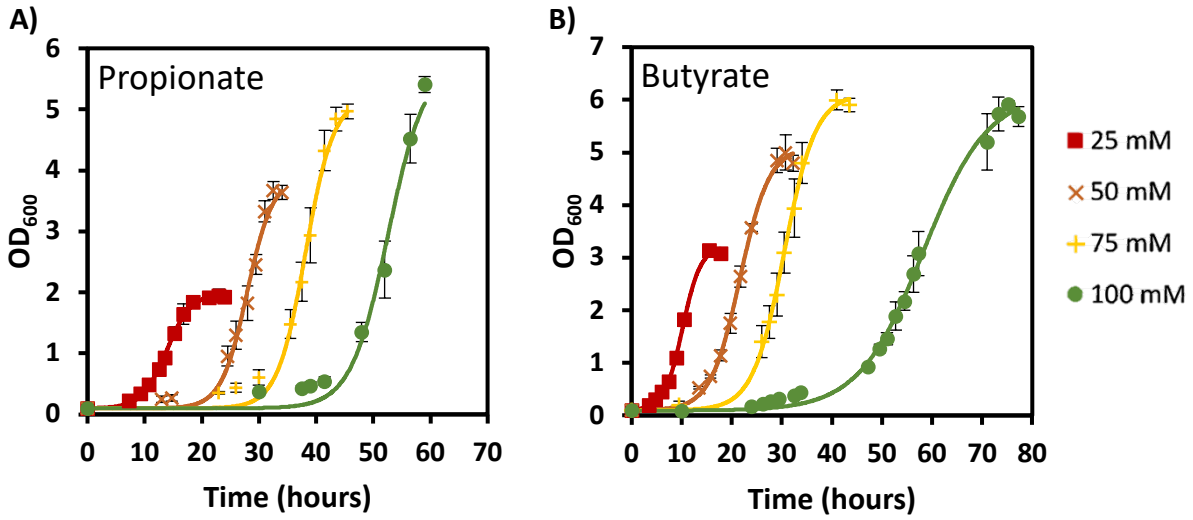


Figure 3.3. Growth curves for ADP1 grown on A) sodium propionate and B) sodium butyrate at different concentrations; 25 mM (red squares), 50 mM (orange crosses), 75 mM (yellow pluses) and 100 mM (green circles). Data points and error bars represent the means and standard deviations, respectively, of three biological replicates. Solid lines represent sigmoid fits to the raw data.

Table 3.2. The growth rate (μ), generation time (T_g) and lag time (T_L) of ADP1 on sodium salts of acetate, propionate and butyrate as sole carbon sources at different concentrations. Blank rows were not tested, dashes indicate no growth was observed during the measured time windows.

Conc	Sodium Acetate			Sodium Propionate			Sodium Butyrate		
(mM)	μ (hr^{-1})	T_g (hr)	T_L (hr)	μ (hr^{-1})	T_g (hr)	T_L (hr)	μ (hr^{-1})	T_g (hr)	T_L (hr)
25	0.69 ± 0.02	1.01 ± 0.03	0.8 ± 0.1	0.23 ± 0.02	3.0 ± 0.2	4.0 ± 0.2	0.31 ± 0.01	2.25 ± 0.05	1.21 ± 0.06
50	0.64 ± 0.02	1.09 ± 0.04	2.22 ± 0.04	0.19 ± 0.01	3.6 ± 0.2	12.7 ± 0.4	0.21 ± 0.01	3.3 ± 0.2	6.3 ± 0.2
75				0.17 ± 0.01	4.0 ± 0.2	19.8 ± 0.4	0.18 ± 0.03	3.9 ± 0.6	11.0 ± 0.8
100	0.52 ± 0.03	1.34 ± 0.07	4.0 ± 0.4	0.14 ± 0.01	4.9 ± 0.2	29.5 ± 0.3	0.13 ± 0.01	5.5 ± 0.5	29.4 ± 0.5
150	0.44 ± 0.03	1.6 ± 0.1	8.3 ± 0.5	-	-		-	-	
200	0.29 ± 0.03	2.4 ± 0.2	12.1 ± 0.4						
250	0.24 ± 0.03	2.9 ± 0.3	19.7 ± 0.3						

Due to its prevalence in acidogenic fermentate and the favourable tolerance and growth rates of ADP1 demonstrated, acetate was chosen as the primary carbon source of interest. A concentration of 150 mM was selected as standard for future experiments as a compromise between growth rate, lag phase and maximum cell density within the culture.

3.4.2. Wax quantification validation

To confirm the suitability of the WE extraction and quantification method for the rest of this thesis, multiple liquid samples from the same culture were quantified by ^{1}H-NMR. *A. baylyi* ADP1 was grown on 150 mM acetate in 200 mL of liquid culture in a 1L flask. A C:N ratio of 50:1 was used, as this had previously been demonstrated to enhance WE production in the strain (61). The culture was grown overnight to an OD_{600} of approximately 1, ensuring acetate had not been fully consumed. Samples of 40 mL and 15 mL in volume were taken, in triplicate, to measure the variation caused by the extraction procedure, as well as the effect of using a smaller sample on quantification variability. Samples were freeze dried, extracted individually, and quantified.

Samples of 40 mL gave an average titre of 7.9 ± 0.5 mg/L, a 7% variation across the triplicate, while 15 mL aliquots gave an average titre of 7.71 ± 0.08 mg/L, only a 1% variation (Figure 3.4A). Importantly, titre was not affected by the sample volume used, indicating that ^{1}H-NMR was sufficiently sensitive to accurately quantify WEs even in relatively small sample volumes. However, the WE content per cell dry weight was also calculated and was significantly lower in the 15 mL samples than in 40 mL samples (Figure 3.4B). In this case, 40 mL samples gave a WE content of 1.87 ± 0.06 %, while 15 mL samples gave a content of 1.61 ± 0.02 %. The determination of WE content was therefore affected by the culture volume, highlighting a limitation of the quantification method.

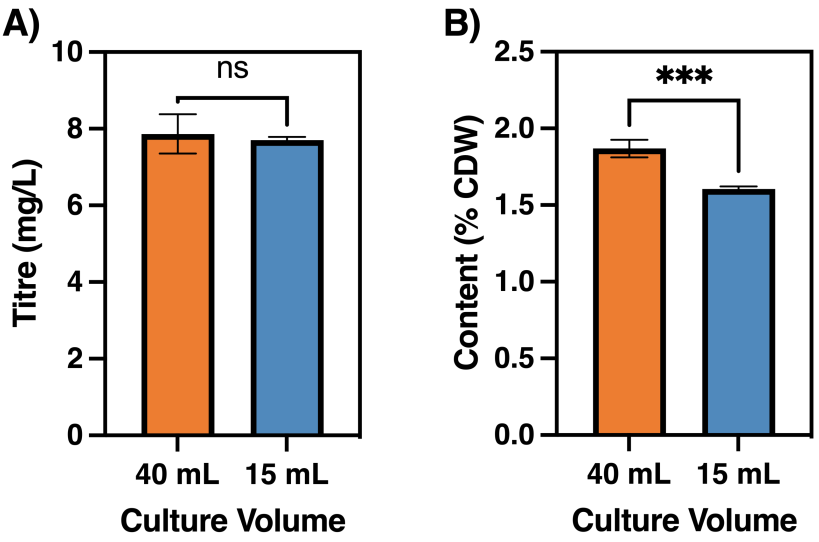


Figure 3.4. Comparison of WE A) titre and B) content per cell from technical replicates taken from a single ADP1 culture with 15 mL and 40 mL sample volumes. Data and error bars represent the means and standard deviations, respectively, of three technical replicates. Significance of difference between strains analysed by t-test; $p > 0.05$ (ns), $p < 0.001$ (***).

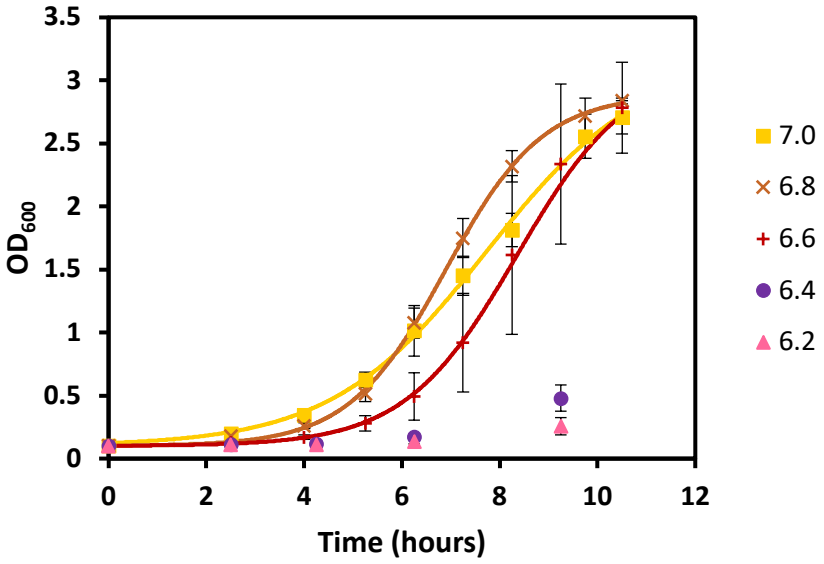
The accuracy of the digital internal standard was also verified. Chemical standards of cetyl palmitate were prepared in triplicate in deuterated chloroform at concentrations of 1 mM and 5 mM in triplicate. The samples were quantified by ^{1}H-NMR, with experimentally determined concentrations of 1.01 ± 0.03 mM and 5.1 ± 0.3 mM, respectively, showing very good agreement with the expected concentrations and validating the digital internal standard for quantification of WEs.

3.4.3. Effect of initial pH

3.4.3.1. Growth

Another key parameter for growth cultures which is not well documented in the literature is the initial culture pH, which is likely to affect growth rate and lag phase. The effect of this parameter on wax production is also unknown.

Initial growth experiments were conducted in 25 mL cultures in 250 mL flasks with 50 mM acetate and starting pH between 6.2 and 7.0 (Figure 3.5). In the range of pH 6.6 – 7.0 maximum growth was observed after 10 hours, while at pH 6.4 and 6.2 the lag phase was much longer, although full growth was observed after 24 hours (not shown). The calculated growth rates were 0.53 ± 0.09, 0.60 ± 0.03 and 0.47 ± 0.04 hr^{-1} at pH 6.6, 6.8 and 7.0 respectively, indicating a slight preference for pH 6.8 as the optimal condition, but with a slightly shorter lag phase at



pH 7.0. During this experiment, pH was found to steadily increase with culture growth as the acetate was consumed, up to a maximum of around 9 during death phase.

Figure 3.5. Growth curves for ADP1 grown on 50 mM acetate with initial pHs of 6.20 (pink triangles), 6.40 (purple circles), 6.60 (red pluses), 6.80 (orange crosses) and 7.0 (yellow squares). Data points and error bars represent the means and standard deviations, respectively, of three biological replicates. Solid lines represent sigmoid fits to the raw data.

To measure the effect of pH on WE production, the experiment was replicated with 200 mL cultures of *A. baylyi* ADP1 grown in 1L flasks. An initial concentration of 150 mM acetate was employed to obtain higher maximum cell densities, and thus increase the WE titre. Based on the previous experiment and consensus that low pHs reduce acid tolerance (Gao 2020 and Orr 2019), it was assumed that if impaired growth was observed at 50 mM at a given pH, this negative effect would amplified at 150 mM acetate. As such, pH 6.8 was chosen as the minimum pH. Similarly, as pH increases during bacterial growth, starting at a high pH could limit the growth window and hence the maximum growth obtained. A narrow window of initial pH from 6.80-7.40 was therefore chosen for investigation (Figure 3.6).

The maximum growth under all four conditions was equivalent, with a maximum OD_{600} between 5.3-5.7 depending on the exact condition. For pH 7.20 and 7.40 the growth rate and lag phase were also very similar, with growth curves almost entirely overlaying. At pH 7.00 the rate of growth was similar, but the lag phase was slightly longer and hence growth was slightly delayed. At pH 6.8 a much longer lag phase was initially visible, with an OD_{600} after 16 hours of just 1.05 ± 0.08, compared to a maximum of 2.4 ± 0.1 at pH 7.20. However, growth

under this condition was faster, and after 40 hours the gap in growth had almost fully closed, meaning maximum growth was achieved after a similar time period.

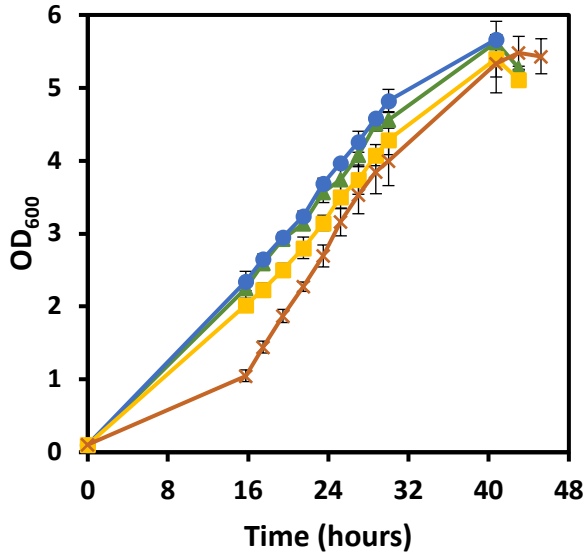


Figure 3.6. Growth curves for ADP1 grown on 150 mM acetate with initial pHs of 6.80 (orange crosses), 7.00 (yellow squares), 7.20 (blue circles) and 7.40 (green triangles). Data points and error bars represent the means and standard deviations, respectively, of three biological replicates.

3.4.3.2. Wax production

WE production under the different initial pH's was also explored. Samples were collected over 40 hours, with the results shown in Figure 3.7. WE production was measured by three metrics: titre – the mass of WE per litre of culture broth, content – the percentage by mass of biomass that was comprised of WEs, and carbon conversion (C-conversion) efficiency – the percentage of the carbon from the consumed acetate feedstock that was present as WEs. In all conditions, the titre increased over time for the majority of the time period due to increasing cell biomass, while content per cell and C-conversion efficiency either increased then decreased or decreased continually. Samples from pH 7.0, 7.2 and 7.4 all showed consistent production, with no significant differences by any metric. However, pH 6.8 gave significantly higher WE production, with maximum titre, content per cell and carbon conversion efficiency of 25 mg/L, 3.0 % CDW and 1.5 %, respectively. This pH also showed the greatest degree of variability between replicates, although for all three metrics each individual replicate was above the 95% confidence interval for pH 7.0 and 7.2, and only one of the three replicates was within the confidence interval of pH 7.4. For this reason, pH 6.8 was chosen as the initial media pH for all subsequent studies.

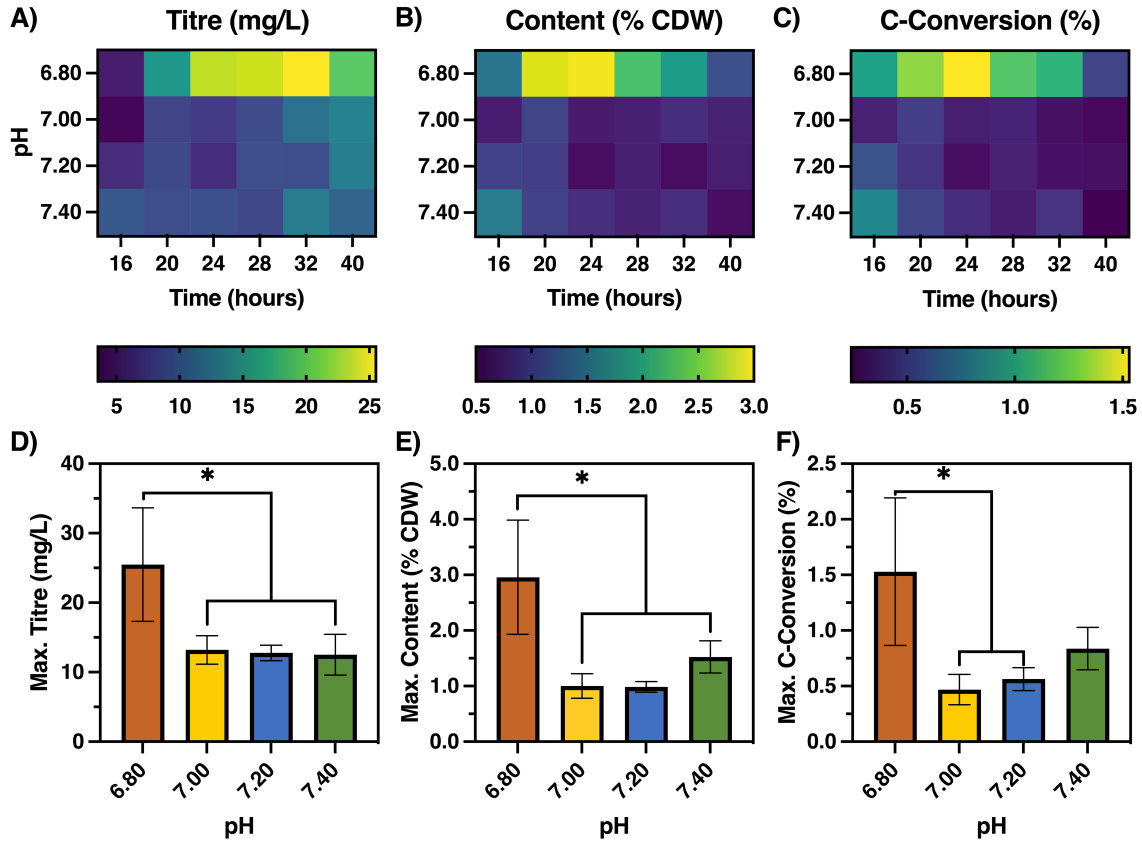


Figure 3.7. Heat maps showing A) WE titre (mg/L), B) WE content per biomass (% CDW) and C) Carbon conversion efficiency from acetate to WEs (%), over time under different initial pHs. Data represents means of three biological replicates, values from blue (low) to yellow (high). Maximum achieved D) WE titre (mg/L), E) WE content per biomass (% CDW) and F) Carbon conversion efficiency from acetate to WEs (%) for each pH. Data and error bars represent the means and standard deviations, respectively, of three biological replicates. Pairwise statistical significance testing performed by ANOVA between different initial pH's, $p \geq 0.05$ (not shown), $p < 0.05$ (*)

3.4.4. Overexpression of acetyl-CoA synthetase

3.4.4.1. Plasmid construction

Successful construction of plasmid pLM001 (Figure 3.8A) was confirmed by gel electrophoresis and Sanger sequencing. Transformation of pLM001 and pBAV1k-T5-gfp (Figure 3.8B) into wild type ADP1 was confirmed by growth on LB agar with 50 µg/mL of kanamycin, as the pBAV1k backbone contains a kanamycin resistance cassette. Transformation was further verified by visual examination of the LB-kanamycin plates (Figure 3.8C) and by imaging using an FITC filter to capture the fluorescence of the GFP (Figure 3.8D).

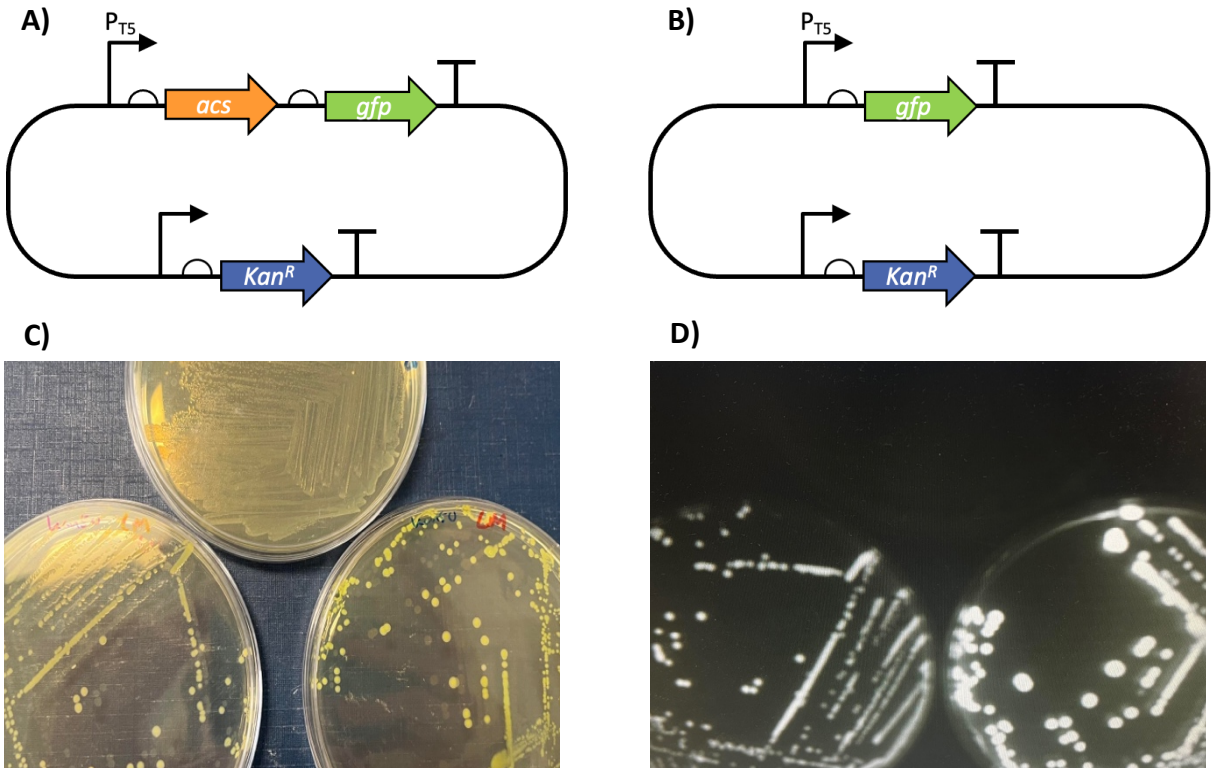


Figure 3.8. Construct diagrams of A) the *acs* overexpression plasmid pLM001 and B) the control plasmid pBAV1k-T5-*gfp*. LB agar plates with (from top left, anticlockwise) ADP1, ADP1-pLM001 (ADP1-*acs*) and ADP1- pBAV1k-T5-*gfp* (ADP1-control): C) photograph of the plates in the laboratory, D) plates imaged using FITC filter.

3.4.4.2. Acetate tolerance

The acetate tolerance of the strain overexpressing acs was tested in flask cultures comparable to those in section 3.4.1. *A. baylyi* ADP1-pLM001 (herein referred to as ADP1-*acs*) grew in the presence of acetate up to 250 mM, but at 300 mM no growth was observed, even after 120 hours, in agreement with the growth kinetics observed for the wild type strain. In contrast, *A. baylyi* ADP1-pBAV1k-T5-*gfp* (herein ADP1-control) had a reduced acetate tolerance, with growth observed at 200 mM acetate but not at 250 mM. Growth of both strains at 150 mM acetate was consistent and reasonably quick, and so WE production was attempted at this concentration.

3.4.4.3. Wax production

Plasmid-containing strains were also analysed for WE production under the same conditions tested for ADP1 WT: 150 mM acetate, initial pH of 6.80 and 200 mL of culture in a 1L shake flask. Growth of the two strains was very comparable to the wild type, with a relative difference in maximum OD_{600} of less than 10% between the three strains. ADP1-*acs* showed the highest maximum WE titre of 80 ± 10 mg/L, a 2.6-fold and 3-fold increase over the ADP1 WT (30 ±

10 mg/L) and ADP1-control (28 ± 2 mg/L), respectively (Figure 3.9 D). This difference was statistically significant at the 1% level. The ADP1-*acs* strain also demonstrated the highest maximum content per cell and carbon conversion efficiency (Figure 3.9 E and F), at 5.5 ± 0.8 % CDW and 2.1 ± 0.5 %. However, in both cases the variation across all conditions meant that the differences observed were not statistically significant.

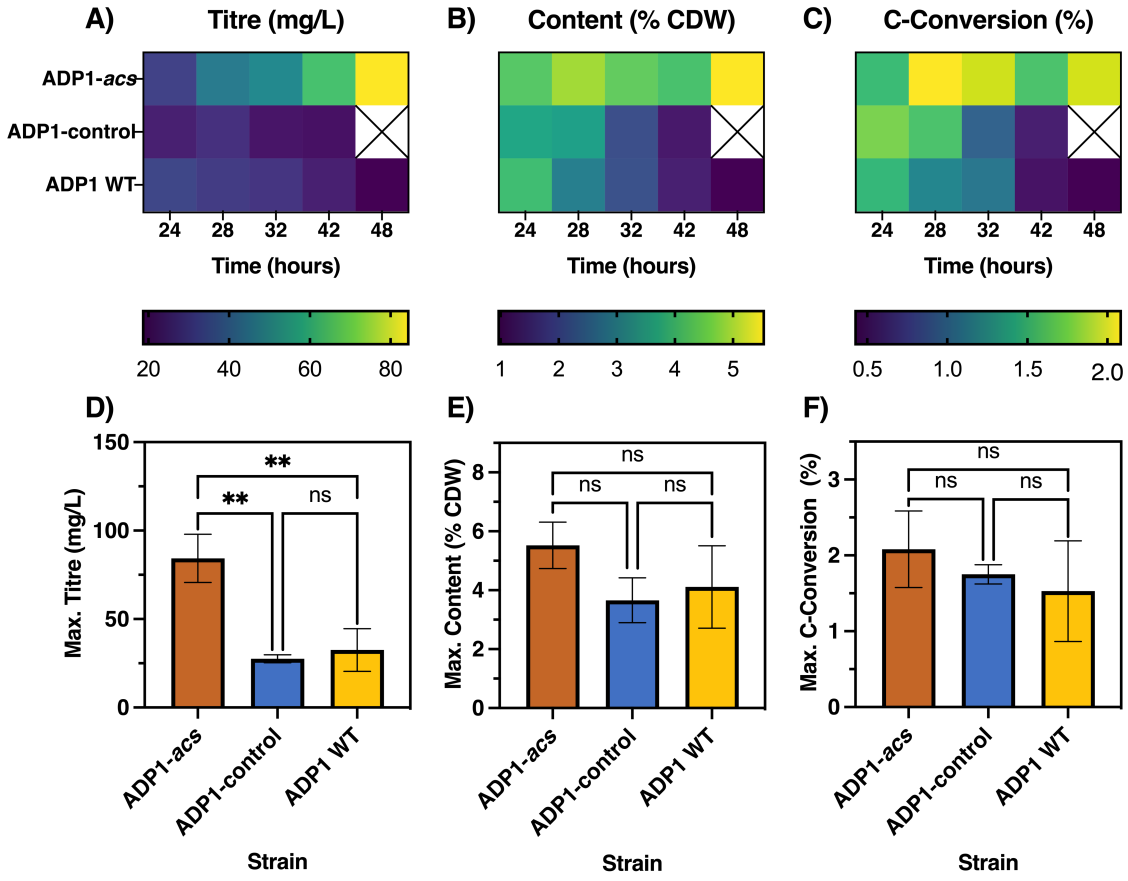


Figure 3.9. Heat maps showing A) WE titre (mg/L), B) WE content per biomass (% CDW) and C) Carbon conversion efficiency from acetate to WEs (%), over time by ADP1-*acs*, ADP1-control and ADP1 WT. Data represents the means of three biological replicates, values from blue (low) to yellow (high). Maximum achieved D) WE titre (mg/L), E) WE content per biomass (% CDW) and F) Carbon conversion efficiency from acetate to WEs for each strain. Data and error bars represent the means and standard deviations, respectively, of three biological replicates. Pairwise statistical significance testing performed by ANOVA between different initial pH's, $p \geq 0.05$ (ns), $p < 0.01$ (**).

3.5. Discussion

3.5.1. Volatile fatty acid tolerance

The maximum previously reported growth rate of *A. baylyi* ADP1 on acetate was 0.93 ± 0.06 hr^{-1}, obtained between 5-35 mM (60), which was higher than the value obtained here, although a different media composition and higher shaking rate were used. However, the decrease in growth rate with increasing acid concentration, observed here for all three acids, is consistent with previous findings (60). This is expected, as VFAs are known to be inhibitory to bacterial growth. Specifically, the growth rates on sodium propionate were substantially lower than on acetate or butyrate, and increasing concentration caused a rapid decrease in growth rate. In *E. coli*, propionate metabolism occurs through the methylcitrate pathway (182), which is different to that for metabolising acetate and butyrate. Furthermore, the expression of the genes in this pathway is induced by propionate (183), while acetate is metabolised through the citric acid (TCA) cycle, which is constitutively expressed as part of respiration. Although preculturing on the chosen carbon source should help to alleviate the delay in substrate induced gene expression (184), this may explain the much lower growth rates and longer lag times observed at low concentrations of sodium propionate compared to the other carbon sources.

The tolerance of *A. baylyi* ADP1 to sodium propionate and butyrate was reported here for the first time. Previous studies have employed acetate (60, 90, 101), and in one case butyrate (101), as carbon sources for this strain, but only acetate has been used as a sole carbon source. Tolerances followed a similar trend to growth rates, with highest tolerance to sodium acetate and a significant decrease for propionate and butyrate. Tolerance of cells to acetate was demonstrated up to 250 mM (approximately 15 g/L), growth on which has been reported previously (60), while at 300 mM acetate, no growth was observed. The maximum concentration at which growth was achieved for propionate and butyrate was 100 mM, indicating a much lower tolerance for these substrates than acetate.

3.5.2. Validity and reliability of wax quantification

The accuracy and reliability of both the NMR quantification method and the WE extraction procedure were determined to ensure their suitability for the rest of the experiments in this thesis. The NMR method was found to be very accurate in determining the concentrations of standards at 1 mM and 5 mM of cetyl palmitate. Within triplicated samples, variability was ± 3% and ± 5% at 1 mM and 5 mM concentrations, respectively, showing the method also measured WE concentration reliably. In comparison, the variability in the full extraction

procedure was ± 1% in the 15 mL volume and ± 7% in the 40 mL volume. Both variabilities were deemed acceptable for the quantification and comparison between conditions in future studies in this thesis. These variabilities demonstrated that the extraction procedure did not introduce additional variation to the measurement of WE titre.

The effect of different culture sample volumes on the measured WE production was also examined. While the WE titres calculated were identical for 15 mL and 40 mL sample volumes, a significant difference was observed between the calculated WE contents. This difference can be attributed to the mass determination of the cell pellets, with any variability in this measurement carried forwards to the WE content. Cell pellets from 40 mL culture samples had an average mass of 16.8 ± 0.5 mg, giving a total cell density of 420 ± 10 mg/L, while all three pellets from 15 mL samples had a mass of 7.2 mg, resulting in a cell density of 480 ± 0 mg/L. This problem was likely caused by the low mass of the 15 mL samples resulting in inaccurate measurement. To avoid this problem, sample volumes were selected for future experiments to give cell pellet dry weights of at least 10 mg/L to improve the accuracy and reliability of the measured WE content per cell.

3.5.3. Initial pH

Initial pH was found to have a small, but significant, effect on the maximum WE production, with pH 6.80 the preferred initial pH from the range tested. No effect on WE production has been documented previously, whether an initial or maintained pH, although studies have observed a pH preference for the accumulation of other intracellular lipids. Mildly alkaline pHs have been reported to provide a beneficial stress on microalgae, increasing their lipid accumulation both during the growth (170) and starvation phase (185) of a two phase production regime. Similarly, in the yeast *Yarrowia lipolytica*, the use of alkaline pH improved growth on high concentrations of acetate, resulting in higher lipid titres (171). However, other researchers have observed that acidic pHs can increase lipid yields in *Y. lipolytica* grown on glucose (186), while PHA production in mixed culture was found to be relatively unaffected by initial pH (187). These results indicate that the effect of pH is complex and can be both strain and substrate specific.

In the case of growth on acetate, and other VFAs, the optimal initial pH is likely to be a compromise. As explained in several other studies, alkaline pH's decrease acetate toxicity and improve tolerance in many microorganisms because the dissociated acids must be taken up by active transport, while protonated acids can travel passively through the cell membrane and

reach toxic intracellular concentrations (188). At higher pH the ratio of protonated: dissociated acids is lower, and hence intracellular acid concentration can be better regulated. However, if the culture pH is not controlled it will rise as the VFAs are consumed, and potentially detrimental levels of alkalinity will be reached more quickly if the initial pH is higher. Because of this, if this study was extrapolated into continuous or batch-fed culture conditions with a constant pH, a reassessment of the optimal pH would likely be necessary.

3.5.4. Improvements from *acs* overexpression

In this study, overexpression of the native *acs* gene resulted in a 2.6-fold increase in the WE titre. The maximum WE content per cell in ADP1-*acs* also increased 50 % and 34 % over the ADP1-control and ADP1 WT respectively, although these increases were non-significant and much lower than the 2.4-fold increase in TAG content per CDW reported in the literature in microalgae (178).

After completion of this section of the thesis, a study by another group was published detailing the overexpression of *acs*, alongside several other lipid synthesis genes, for enhanced lipid production from acetate in the yeast *Yarrowia lipolytica* (189). The authors observed an increase in lipid content, which is also comprised mostly of TAGs (190), of just under 2-fold from the overexpression of *acs* alone. When co-expressed with acetyl-CoA carboxylase (*acc*), this lipid content per cell more than doubled again, and a further 25% increase was achieved by the triple overexpression of *acs*, *acc* and fatty acid synthase (*fas*). The enzyme ACC converts acetyl-CoA into malonyl-CoA, which is added to the fatty acyl-ACP chain for each chain elongation step in fatty acid synthesis, while FAS is a multi-enzyme protein that catalyses all 4-steps in each chain elongation cycle. In this case, the maximum growth and acetate utilisation rate were also increased, with a more than 3-fold increase in maximum OD_{600} from acetate achieved by the combined overexpression of *acs* and *acc*. These findings highlight that, while *acs* overexpression does improve lipid production, including WE production, other enzymatic steps also cause bottlenecks in microbial lipid biosynthesis pathways. To further build on the work in this study, future investigations should focus on the overexpression of multiple genes in the WE biosynthesis pathway to achieve optimised results.

3.6. Conclusions

In this chapter, the growth and tolerance of *A. baylyi* ADP1 on sodium acetate, propionate and butyrate was explored, as a preliminary experiment to probing WE production from these acids. All three VFAs were demonstrated to be suitable as sole carbon sources, although maximum tolerances were around 250 mM for acetate and 100 mM for propionate and butyrate. Growth rates were also variable, with acetate the highest and propionate generally the lowest at equivalent acid concentrations. Growth rates decreased and lag phases increased with increasing acid concentrations for all substrates.

A reported method (191) for simple and rapid quantification of WEs produced by ADP1 was tested for accuracy and reliability. The ^{1}H-NMR quantification method was highly accurate and reliable at both 1 mM and 5 mM concentrations of WE. The extraction method for WEs was also found to be reliable, with no increase in variability for extracted samples compared to the quantified standards. The use of smaller sample volumes did not affect WE titre but did affect the calculated WE content due to difficulty accurately weighing cell pellets. To address this, sample volumes for future experiments were chosen to keep dried cell pellets above 10 mg.

Initial culture pH was varied over a small range close to neutral, and it was observed that pH 6.8 gave the optimal growth rate and WE production from acetate, with the latter significantly higher by all three key metrics than the other conditions tested. The optimal pH was hypothesised to be a compromise between acid toxicity and pH rises during growth due to acetate consumption. This pH (6.8) was chosen for future experiments in this thesis.

The overexpression of *acs* on a high copy number plasmid under a strong constitutive promoter was found to significantly increase WE titre from acetate by 2.6-fold and 3.1-fold compared to wild type ADP1 and a specifically designed control strain, respectively. However, growth on acetate and tolerance were not changed, and while WE content per cell and carbon conversion efficiency were increased, the changes were not significant due to high variability between replicates. This overexpression was not sufficiently successful to be explored further at the time of experimentation, and so other methods for improving WE synthesis from VFAs were explored. However, based on more recent publications, simultaneous overexpression of multiple genes in the fatty acid synthesis pathway was identified as a potentially effective method to improve WE production more substantially.

Chapter 4 - Mixed waste streams for bioproduction: optimising bacterial wax ester production in nitrogen-rich acidogenic fermentate

In this chapter, the application of acidogenic fermentate as a media and substrate for bacterial WE production was explored for the first time. A mutant strain of *A. baylyi* ADP1 (93) which was unable to utilise acetate as a sole carbon source and able to produce WEs under nitrogen rich conditions was employed. Initially growth of the mutant and wild type ADP1 on individual even and odd chain VFAs of length C2-C6 was compared, confirming the pathways for short chain fatty acid metabolism in the wild type strain. Growth and WE production were then demonstrated on a mixture of sodium acetate and propionate, demonstrating the benefits of a mixture of fatty acids for WE production in a metabolically partitioned strain. To translate this finding to a mixed substrate system, the two *A. baylyi* ADP1 strains were then grown on VFA-rich acidogenic fermentate generated from *Opuntia ficus indica*, a desert crop of interest as a bioenergy crop for marginal, arid regions (118). WE production was achieved, with a 5.6-fold and 9-fold increase in WE titre and yield, respectively, in the mutant compared to the wild type.

It was observed that the acid tolerance of the mutant strain was reduced compared to wild type ADP1. To increase the carbon density of the culture medium without increasing the acid concentration, the use of longer chain VFAs (specifically butyrate and valerate) was explored. Although the tolerance of the mutant to these acids was lower than to acetate and propionate, in synthetic culture medium the total carbon density of the medium could be increased up to 260 mM, compared to 190 mM. The maximum WE titre also increased to 190 ± 10 mg/L, a 27 %, increase compared to equivalent conditions with acetate and propionate.

Finally, in an attempt to replicate this finding on fermentate, as was achieved for propionate and acetate, AF was performed on a nitrogen-rich substrate with a known protocol (132), yielding a VFA mixture with a higher proportion of butyric and valeric acids. While growth of both the wild type and mutant ADP1 was achieved on this second fermentate, WE production was severely reduced. These findings reflect the potential limitation of genetic engineering in overcoming natural metabolic regulation and the complexities of translating synthetic system findings to complex mixed media and waste streams.

This chapter is submitted as a draft manuscript which has been prepared for publication.

Mixed waste streams for bioproduction: optimising bacterial wax ester production in nitrogen-rich acidogenic fermentate

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Abstract

Microbial lipids offer a promising alternative to petrochemicals, but high associated costs and low conversion efficiencies pose barriers to their commercialisation. In particular, sugar-based feedstocks are too expensive for the production of commodity chemicals, and recently attention has turned to volatile fatty acids (VFAs) as a cheaper, more widely available carbon source. Acidogenic fermentation (AF) can be used to produce high concentrations of VFAs from municipal and agricultural waste. By harnessing metabolically engineered *Acinetobacter baylyi* ADP1, the suitability of VFAs as sole carbon sources for wax ester (WE) production was investigated. These studies resulted in the highest WE accumulation in ADP1 achieved to date, at 37 % of cell dry weight, and the first reported production of bacterial WEs from a raw, mixed waste stream, utilising fermentate as the sole carbon source. Wax titres of over 160 mg/L from VFAs were achieved, highlighting the unique benefits of mixed feedstocks typically considered problematic for bioproduction. Finally, the potential advantages of employing fermentates rich in longer chain VFAs are explored. In synthetic media, WE titres up to 190 mg/L were achieved, but translation to fermentate was challenging, emphasising the need for continued research in this area.

4.1. Introduction

Microbial storage lipids have been identified as an attractive, renewable alternative to a variety of petrochemical compounds, including polymers (Rodriguez-Perez et al., 2018), fuels (Koreti et al., 2022; Shields-Menard et al., 2018) and waxes (Martin, Huang, and Thompson, 2021). However, the production of storage lipids is inherently in competition with growth at the metabolic level (Luo et al., 2020). Nutrient limitation can overcome a cell's natural tendency to direct resources to growth, instead shifting carbon flux towards storage lipids (Castro et al., 2018; Manilla-Pérez et al., 2011; Martin, Huang, and Thompson, 2023). However, this comes at a cost; growth is restricted and, ultimately, production is limited.

To circumvent this problem, recent advances in metabolic engineering have attempted to balance growth and bioproduction by controlled co-feeding of two distinct substrates to concurrently activate both pathways. This can be achieved by exploiting multiple naturally-occurring metabolic routes (Park et al., 2019), or alternatively by engineering a partition of central carbon metabolism such that the pathways for growth and bioproduction are decoupled (Luo et al., 2020; Santala, Efimova, and Santala, 2018). In both cases, careful selection of substrates allows each to be predominantly directed to a single metabolic fate. Such an approach is particularly attractive if a cheap carbon source can be used as the major production substrate, reducing reliance on expensive sugar-based feedstocks. This substrate is then 'doped' with small amounts of a rich growth substrate, resulting in moderate, controlled growth and efficient lipid production from a cost-effective feedstock pool. Using this approach, an engineered strain of *A. baylyi* ADP1 recently achieved a total C/C yield for wax ester (WE) production of 18%, the highest ever reported, when cultured on acetate and co-fed small amounts of glucose. (Santala et al., 2021)

Acetate is a popular low-cost substrate due to its wide availability and its potential to be sourced from waste streams or directly from CO_2 (David, Tripathi, and Sani, 2020; Lehtinen et al., 2017; May, Evans, and LaBelle, 2016). The direct conversion of acetate to acetyl-CoA, which is directly upstream of the free fatty acid synthesis pathway, also makes it a logical choice of carbon source for efficient lipid production (Gong et al., 2015). An abundant, sustainable source of acetate and other volatile fatty acids (VFAs) is acidogenic fermentation (AF), an anaerobic degradation process widely used to process a variety of waste streams, including post-consumer food (Dahiya et al., 2015; David, Tripathi, and Sani, 2020; Srisowmeya, Chakravarthy, and Nandhini Devi, 2020), agricultural (Iglesias-Iglesias et al., 2021) and sewage wastes (Ramos-Suarez, Zhang, and Outram, 2021; Tayou et al., 2022). By varying a

number of fermentation factors, including carbon: nitrogen (C:N) ratio of the substrate, pH, temperature, organic loading and initial inoculum, the composition of the fermentate can be significantly altered (Gong et al., 2021; Moretto et al., 2019; Tenci et al., 2023).

Acidogenic fermentate, which typically contains acetate, propionate, butyrate, valerate and hexanoate, has been utilised by a number of researchers as a suitable source of VFAs for the production of microbial storage lipids, predominantly polyhydroxyalkanoates (PHAs) (Al Battashi et al., 2021; Aremu, Ishola, and Taherzadeh, 2021; Cerrone et al., 2012; Kumar, Ray, and Kalia, 2016; Szacherska et al., 2021) and fatty acids for biodiesel (Annamalai et al., 2020; Patel et al., 2021). Thus far, promising product titres and yields have been achieved in both pure and mixed culture fermentations. However, only a handful of studies have explored the tunability of the VFA composition in fermentate (Al Battashi et al., 2021), which has previously been found to influence both microbial lipid yield and composition (Albuquerque et al., 2011; Gong et al., 2021; Kumar, Ray, and Kalia, 2016).

The scope of this study was to demonstrate that an untreated, mixed waste stream deployed as a complete growth medium could intrinsically provide co-feeding of multiple substrates. The multiple components of waste streams commonly considered a hinderance to biological and chemical processes, yield advantages for controlling individual metabolic pathways within a cell, namely growth and lipid production. Using *A. baylyi* ADP1 wild type (WT) and a previously engineered glyoxylate shunt knock-out ADP1 M+ as the platform strains for bioproduction, the VFAs present in fermentate were individually investigated for their potential as sole carbon sources, due to the different metabolic pathways for odd and even chain length fatty acids. In doing so, this work showcases the utilisation of several substrates not previously demonstrated in ADP1. The co-feeding strategy developed previously (Santala et al., 2021) was then employed with these feedstocks, utilising odd chain VFAs as the growth feedstocks and even chain VFAs for WE production. The effect of the genetic engineering in M+ on the tolerance to these substrates was explored, and WE production was detected near the tolerance limit. To investigate the effect of supplying carbon to growth and production pathways at different rates, the relative proportions of growth and production substrates in the culture medium were varied. Given the success of WE production on these short chain VFAs, the first example of WE production from VFA rich fermentate was attempted, with production significantly increased in the M+ strain over the WT bacteria.

Due to the decreased acid tolerance of M+, utilisation of VFAs with longer chain lengths was also explored. This was of interest because: i) longer chain VFAs can increase carbon density

in the culture medium without increasing total acid concentration, ii) longer carbon chains (C4 and above) are higher energy substrates that offer greater energy generation and reducing power, and iii) as VFA mixtures have been found to improve lipid productivity compared to acetate alone (Moon et al., 2013). Following successful results with pure butyrate and valerate, ADP1 WT and M+ were grown on fermentate from a fermentation under conditions designed to produce higher proportions of these acids in the fermentate. The ability to generate such diverse fermentate compositions exemplifies the benefits of fermentate tuneability for microbial growth and bioproduction. However, translation of the results under this condition was not trivial, emphasising the difficulty of replicating results from synthetic laboratory media on complex, mixed substrates.

The model AF processes used in this study utilised *Opuntia ficus indica* (*opuntia*) and dried pig blood as biomass sources. *Opuntia* is a crassulacean acid metabolism (CAM) plant, a class of plants specially adapted for growth in arid conditions due to an adapted photosynthesis pathway which minimises water loss (Snyman, 2013). CAM plants are of interest as bioenergy and bioproduction crops, avoiding competition with agriculture for arable land and growing well in poor soils with erratic rainfall (Yadav et al., 2019), and are a good model for the fermentation of energy crops and agricultural residues. In contrast, pig blood has been used previously as a model substrate for high protein biomass (Kovács et al., 2013; Tenci et al., 2023) a common waste stream from animal agriculture which is of interest for anaerobic digestion (Afazeli et al., 2014; Hejnfelt & Angelidaki, 2009). Overall, our findings here are applicable to the valorisation of a wide range of organic waste streams.

4.2. Materials and methods

4.2.1. Bacterial strains, chemicals and culture media

A. baylyi ADP1 (DSM 24193) wild type and *A. baylyi* Δ*acr1*::*Kan*^{r}/*tdk* Δ*aceA*::P$_{T5}$-*acr1*-*spec*^{r} (ADP1 M+) (Luo et al., 2020) (kindly donated by V Santala, Tampere University, Finland). Bacterial stocks were stored at -80 °C in 20% v/v glycerol/water. All chemicals were sourced from SigmaAldrich (Merck).

4.2.2. Culture conditions

Glycerol stocks were streaked on LB agar plates (with antibiotics for ADP1 M+) which were incubated at 30 °C for 24 hours. Single colonies were picked and inoculated into LB broth, grown to mid-exponential phase and transferred to precultures in flasks either LB broth or minimal media.

Minimal media contained 2.5 g/L KH_2PO_4, 2.5 g/L Na_2HPO_4, 1 g/L NH_4Cl, 0.1 g/L $MgSO_4.7H_2O$, 0.01 g/L $CaCl_2.2H_2O$ and 1 mL/L of 1000x Bauchop and Elsden trace elements solution (Bauchop & Elsden, 1960). Medium was prepared, adjusted to pH 6.8 as standard and autoclaved. 300 g/L sodium acetate, 300 g/L sodium propionate, 100 g/L sodium butyrate, 40 g/L valeric acid and 10 g/L hexanoic acid stocks were prepared, adjusted to pH 6.8 and autoclaved separately and added as required immediately prior to inoculation. All ADP1 M+ cultures contained kanamycin and spectinomycin at 50 µg mL^{-1}.

Immediately prior to each experiment, precultures were spun down at 6000 g for 6 minutes, resuspended in phosphate buffer and experiments were inoculated. All liquid cultures and precultures were cultivated at 30 °C and 150 rpm.

4.2.2.1. Plate reader experiments

VFA tolerance experiments were performed in clear bottomed, black 96 well plates on a Tecan Spark (Tecan Group Ltd, Männedorf, Switzerland). Each well contained 200 µL of culture. Plates were sealed with breathable film to maintain sterility and incubated in a Tecan humidity cassette to prevent evaporation. Plates were incubated at 30 °C at 180 rpm orbital shaking with 3 mm amplitude and 15-minute sampling interval time. All conditions were measured in triplicate.

4.2.2.2. Wax production experiments

Wax production experiments on all feedstocks were conducted in either 50 mL of culture in 250 mL flasks or 200 mL of culture in 1L flasks. Growth was measured by OD_{600} with UV-

1800 UV/Vis spectrophotometer (Shimadzu, Kyoto, Japan). 10-25 mL of cultures were extracted as detailed previously. (Martin, Huang, and Thompson, 2023)

4.2.3. Acidogenic fermentation

4.2.3.1. Feedstock and inoculum

Opuntia ficus indica cladodes were obtained from Pepe Aromas (Azaruja, Portugal). Cladodes were diced, dried at 60 °C until a constant mass (around 5 days), ground in a blender (Waring, USA) and passed through a 1 mm screen. For butyrate and valeric acid enriched digestion, food-grade dried pig blood was obtained from Tongmaster Seasonings (Coatbridge, UK).

Anaerobic digestion sludge from a local mesophilic food waste processing facility (Severn Trent Green Power, Cassington, UK) was used as an inoculum. Sludge was stored under anaerobic conditions at 30 °C until use and deployed within 2 days of collection.

4.2.3.2. Feedstock and inoculum

For the *Opuntia ficus indica* (*Opuntia*) digestion, fermentations were performed in 500 mL glass bottles at a working volume of 300 mL. Dried *Opuntia* was weighed into each bottle, with a total of 200 mL/L of sludge and 39 g/L of volatile solids (VS) equivalent of opuntia were used, giving a substrate: inoculum ratio of 8:1 on a VS basis. Fermentations were incubated for 20 days.

Additional fermentation conditions were chosen and replicated from the literature (Tenci et al., 2023) to enrich the concentration of butyrate and valerate in the fermentate. Fermentations were performed in 2L bottles with 1 L working volume, with a total of 330 mL/L of sludge and 60 g/L VS equivalent of dried blood.

Synthetic basic medium was used to provide the necessary micronutrients, trace metals and vitamins, and was prepared according to the literature recommendations, with the omission of sodium sulphide. The liquid reaction mixture (containing the basic medium, deionised water and the appropriate volume of sludge) was prepared in bulk and adjusted to pH 8.0 before addition to bottles containing the dried substrate. All bottles were incubated at 37 °C with a constant agitation of 125 rpm. Before use, fermentate was stored at – 80 °C to prevent degradation.

4.2.4. Acidogenic fermentate growth and wax production

Prior to use as a medium, fermentate was defrosted overnight at 4 °C, centrifuged for 15 minutes at 5,800 *g* and for 10 minutes at 10,000 *g*, pH adjusted to 6.8 with 3M NaOH, then

autoclaved. Post-autoclaving, fermentate was re-clarified by centrifugation for 10 minutes at 10,000 *g*, then stored at 4 °C for up to 3 days before use. Fermentate was diluted to concentrations of 4-12 g/L of total VFA with deionised water, as required, and the final culture medium was autoclaved in flask prior to inoculation.

4.2.5. Lipid extraction and quantification

For NMR quantification, lipids were extracted following an adapted Bligh and Dyer method (Martin, Huang, and Thompson, 2023; Santala et al., 2011). Cell pellets were resuspended in 2 mL of PBS, 2.5 mL chloroform and 5 mL of methanol and the mixture shaken for approx. 4 hours at 150 rpm. A further 2.5 mL of each of methanol, PBS and chloroform were added, the sample mixed again and stored overnight at +4 °C. The suspension was centrifuged at 10,000 g for 8 min, and the lower organic phase was transferred to a glass vial to evaporate. The aqueous phase was extracted again by addition of 5 mL of chloroform, mixed and incubated overnight at +4 °C, then centrifuged and separated as before.

NMR analysis was performed using Bruker Avance III spectrometer (700 MHz) with a TCI cryoprobe (Bruker, MA, US). Extracted lipids were dissolved in 600 µL of chloroform-d_3 and ^{1}H spectra recorded at ambient temperature. Spectra were processed and analysed using MestreNova 14.1.1. with an artificial reference peak at δ -1 ppm. Global Spectrum Deconvolution was used to accurately isolate and integrate the triplet peak at δ 4.05 ppm, corresponding to the 2 α-alkoxy methylene protons from the alcohol component of WEs. From this the WE concentration was determined and used to calculate the mass of WEs produced, assuming an average Mw for WEs of 506 g/mol (Lehtinen et al., 2018).

4.2.6. Volatile fatty acid quantification by GC-FID

VFA concentration at each time point was measured from the supernatant of the extracted cells for cell dry weight (CDW) and WE quantification using gas chromatography-flame ionisation detection (GC-FID) (Shimadzu GC-2010) with a 30 m × 0.25 mm × 0.25 µm fused-silica capillary column (ZB-FFAP). The detector and injector were set at 350 ◦C and 250 ◦C, respectively. The oven temperature was set at 100 °C for 2 min and then increased at a rate of 16 °C/min. Calibration was performed with standards of acetic, propionic, isobutyric, butyric, isovaleric, valeric and hexanoic acids at concentrations from 0.31 to 20.00 g/L. All samples and standards (250 µL) were acidified by 1:1 dilution with 20% v/v formic acid to a final concentration of 10 % v/v prior to analysis. C-conversion efficiency and theoretical maximum yield were calculated based on the VFA consumption for each sample at the given time point,

accounting for both the concentration and number of carbons in each acid. Theoretical maximum yield was calculated by balancing the consumed VFA carbon with the direct carbon incorporation and the indirect carbon utilisation to generate the necessary reducing equivalents. An average chain length of 34 carbons, requiring 32 molecules of NADPH per molecule of WE as produced from acetyl-CoA, was assumed, with 2, 3, 4, 5 and 6 carbon fatty acids able to generate 2, 3, 6, 7 and 10 molecules of NADH, respectively, in their complete oxidation.

4.2.7. Statistical analysis

Results were analysed for statistical significance in GraphPad Prism (GraphPad, San Diego, CA, United States). The maximum titre, content and C-conversion was identified for each condition over the whole time-course, and each set of maxima were analysed by One-Way ANOVA, with multiple pairwise comparisons. The Šidák correction was applied to control the false positive rate, and the Brown-Forsythe test was performed to confirm that group variances were not significantly different (and hence that the data was suitable for ANOVA). Asterisks indicate significance at different levels as follows: * = 5%, ** = 1%, *** = 0.1% and **** = 0.01%.

4.3. Results

4.3.1. Volatile fatty acids as sole carbon sources

The ability of ADP1 to grow on VFAs as single carbon sources was tested for straight chain fatty acids with chain length C2-C6. VFA concentrations were used such that the total carbon concentration present was 60 mM in all cases. A negative control containing no carbon source was also measured.

Figure 4.1 shows the growth curves for WT ADP1 (Figure 4.1A and C) and the M+ strain (Figure 4.1B and D). All VFAs tested were suitable as sole carbon sources for the WT, with maximum growth increasing for longer chain VFAs than short ones. In contrast, propionate and valerate were used readily by M+ and in both cases good growth was detected, with maximum OD_{600} of 2.0 ± 0.2 and 1.41 ± 0.04 achieved, respectively. However, cultures on acetate, butyrate and hexanoate showed negligible growth over the period measured.

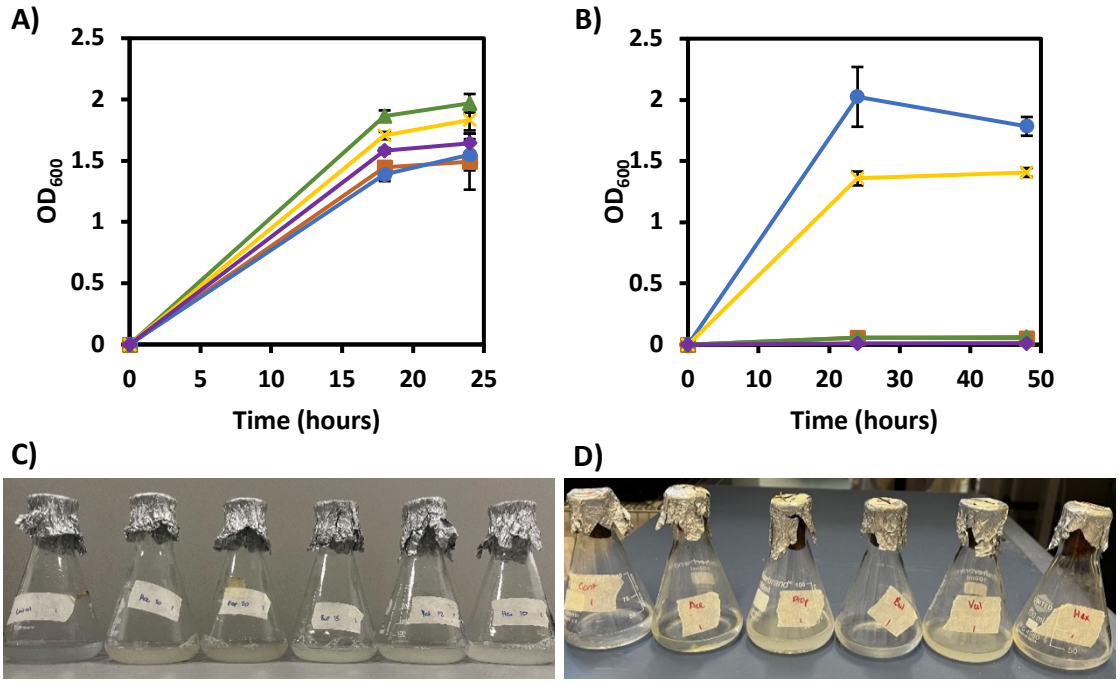


Figure 4.1. Growth curves for ADP1 grown in minimal media containing acetate (orange), propionate (blue), butyrate (green), valerate (yellow) and hexanoate (purple) over 96 hours. A) WT and B) M+ mutant strains. Cultures were grown in biological triplicate, data points show mean and standard deviation, normalised by subtracting the negative control with no carbon source. C) and D) Photograph of 1 flask from each condition for the WT and mutant strains, after 24 and 48 hours respectively. Data and error bars represent the means and standard deviation, respectively, of three biological replicates.

4.3.2. Propionate and acetate tolerances

4.3.2.1. Propionate tolerance

After establishing propionate as a suitable substrate for both strains, growth of ADP1 WT and M+ was determined on concentrations in the range of 25-100 mM, with cells precultured on either LB broth (Figure 4.2A and E) or on 25 mM sodium propionate in minimal media (Figure 4.2B and F). In the WT strain the pre-culture method made little difference at concentrations of ≤75 mM, at which lag phase and growth rate were comparable. Above 75 mM, growth improved after pre-culturing overnight on propionate media, and at 100 mM growth was only achieved if the cells had been preadapted to the VFA. In M+, pre-culture conditions had a more pronounced effect on growth, with cells cultured on LB unable to tolerate propionate at concentrations greater than 25 mM, while cells that had been pre-adapted to the VFA demonstrated a shorter lag phase and grew at all concentrations with the exception of 100 mM. In both pre-culture conditions, propionate tolerance was lower in M+ than the WT, although pre-adapting cells in propionate significantly reduced toxicity and improved tolerance.

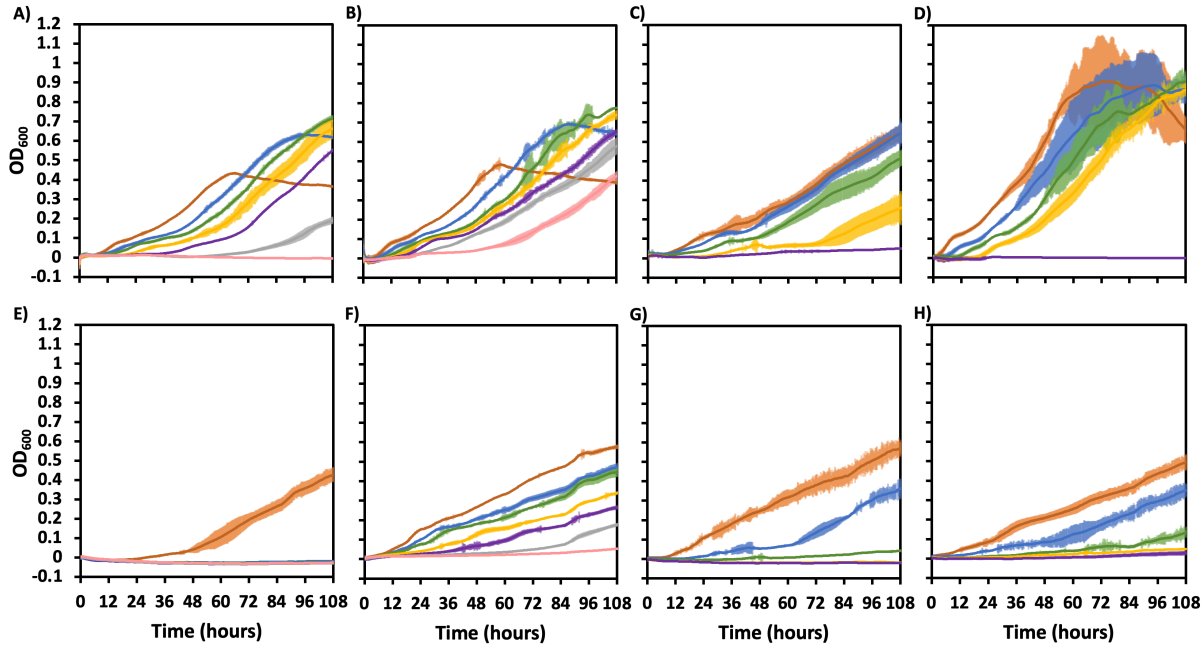


Figure 4.2. Growth curves and tolerance tests for ADP1 WT (A-D) and M+ (E-G) grown in minimal media on varying concentrations of sodium propionate and acetate. A), B), E) and F) cells cultured on propionate at 25 mM (orange), 37.5 mM (blue), 50 mM (green)l 62.5 mM (yellow), 75 mM (purple), 87.5 mM (grey) and 100 mM (pink). C), D), G) and H) cells cultured on 25 mM propionate with acetate at 25 mM (orange), 50 mM (blue), 75 mM (green), 100 mM (yellow) and 150 mM (purple). A), C), E) and G) were precultured on LB, while B), D), F) and H) were precultured on 25 mM propionate in minimal media. Data and error bars represent the means and

standard deviations, respectively, of three biological replicates. Means were smoothed by adjacent averaging over 20 points. Negative controls of precultured cells with no carbon source were used as a blank.

4.3.2.2. Combined acetate and propionate tolerance

The combined tolerance of both strains to acetate and propionate in a co-fed culture was also explored. Since propionate tolerance in the mutant strain was only slightly lower than that of ADP1 WT, a range of acetate concentrations within the tolerance of the wild type bacterium were tested (25-150 mM), combined with a constant propionate concentration of 25 mM. Again, cells were precultured on either LB (Figure 4.2C and G) or minimal media with 25 mM propionate (Figure 4.2D and H). In the WT strain, growth occurred at 25 mM propionate in the presence of up to 100 mM sodium acetate, but the combination with 150 mM acetate proved toxic. For M+, growth was observed up to 25 mM propionate and 50 mM acetate (75 mM total acid), with a small amount of growth observed with 75 mM acetate and cell death at higher acetate concentrations. In contrast to the propionate-only cultures, under these conditions the preculture method made little difference to the tolerance in either strain and in all cases growth on 25 mM propionate with 25 mM acetate began after just a few hours. The lag phase increased with increasing acetate concentration for both strains, but in the WT was decreased by preculturing on propionate.

4.3.3. Effect of acetate: propionate ratio on growth and wax production

The effect of acetate: propionate ratio was explored, measuring the effects on both growth and WE production. A total acid concentration of 75 mM was investigated, based on the tolerance tests, with compositions ranging from 68 mM acetate and 7 mM propionate (A68P7) to 75 mM propionate only (A0P75). This resulted in total carbon concentrations from 160 mM to 225 mM. Cultures were sampled regularly (time course data for all metrics is available in the Supplementary Information, Figure 4.S1), and the maximum of each metric for each condition was compared (Figure 4.3). The effect of acetate: propionate ratio on all metrics was statistically significant, as calculated by One-Way ANOVA, and sequential pairwise significances are marked on Figure 4.3A-C.

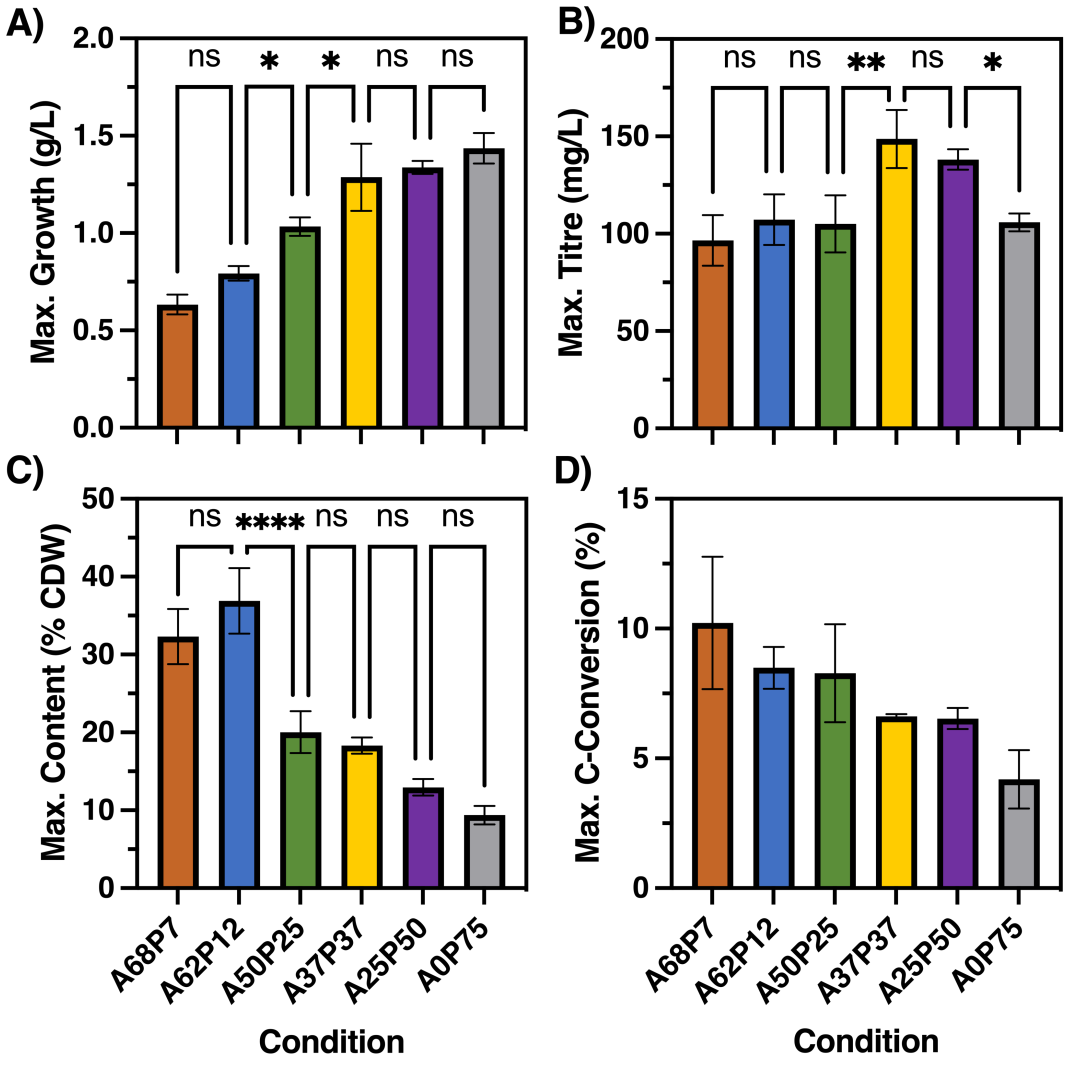


Figure 4.3. Comparisons of the maximum A) growth, B) WE titre, C) WE content per cell and D) C-conversion efficiency for ADP1 M+ mutant at different concentrations of acetate (A) and propionate (P), with total acid concentration of 75 mM in all conditions. Numbers denote concentration of the given acid (so A68P7 = 68 mM acetate, 7 mM propionate). Data and error bars represent the means and standard deviations, respectively, of three biological replicates. Pairwise statistical significance testing performed by ANOVA between sequential conditions, $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.0001$ (****).

As expected, maximum growth increased with increasing propionate proportion (Figure 4.3A). Wax titre was comparable in at A68P7, A62P12, A50P25 and A0P75, but significantly higher at A37P37 and A25P50 (Figure 4.3B), with a maximum of 150 ± 10 mg/L, indicating an optimal range for balancing growth and production. WE content per cell was maximised at low propionate proportion, with a maximum of 37 ± 4 % (Figure 4.3C), the highest ever reported in any bacterial species, although poor growth in these conditions limited titre. Maximum carbon conversion efficiency also decreased with increasing propionate concentration (Figure

4.3D), ranging from 10 ± 3 % to 4 ± 1 %, although pairwise comparisons between adjacent conditions were not significant.

4.3.4. Growth and wax production on acidogenic fermentate

After confirmation that acetate-propionate mixtures were suitable substrates for growth and WE production, the suitability of fermentate as a complete growth medium was tested. Table 4.1 shows the concentration of total VFAs, nitrogen and phosphate present in the fermentate, as produced. Fermentate was diluted with deionised water to maintain a constant carbon: nitrogen: phosphate ratio, which has previously been found to impact WE production (Martin, Huang, and Thompson, 2023).

Table 4.1. The composition of the as digested fermentate from *Opuntia ficus indica* used for growth and WE production.

Component	Concentration (g/L)
VFA	20.3 ± 0.1
- Acetate	10.2 ± 0.1
- Propionate	4.20 ± 0.01
- Isobutyrate	0.10 ± 0.02
- Butyrate	2.49 ± 0.02
- Isovalerate	0.279 ± 0.006
- Valerate	1.85 ± 0.01
- Hexanoate	1.22 ± 0.04
Total Nitrogen	1.15 ± 0.02
Total Phosphate	0.191 ± 0.007

Suitable VFA concentrations were selected for the growth of the M+ and WT strains at 6 g/L VFA and 10 g/L VFA, respectively (see Figure 4.S1). Figures 4.4A and B show the growth curves and VFA consumption of both strains, measured over a 7-day period. Growth in both strains was good, with maximum OD_{600} of 8.0 ± 0.4 and 6.5 ± 0.4 for the WT and M+, respectively. Total VFA consumption was also observed for both strains.

The WE production in these strains was at regular intervals, and the maxima achieved for each strain were compared (Figure 4.4C-F). All metrics were significantly higher in the mutant strain: titre increased from 29 ± 5 mg/L in the WT to 163 ± 5 mg/L in M1, maximum content from 3 ± 1 % to 16.1 ± 0.6%, and C-Conversion efficiency from 1.4 ± 0.6 % to 12 ± 2 %, respectively. Maximum titre and content per cell were obtained at 36 and 24 hours respectively in the WT bacteria, and subsequently both decreased rapidly to near zero. In M+ both were

obtained at 48 hours, with titre remaining consistent until at least 64 hours, while content per cell decreased with increase growth.

The total wax yield from VFA consumed (Figure 4.4F) was 0.009 ± 0.003 g-WE/g-VFA in the WT, which increased to 0.08 ± 0.01 g-WE/g-VFA in M+, calculated from the VFA profiles for the different strains at their maximum wax titre. The total VFA yield per volatile solids (VS) of 27.7 ± 0.2 % for the AF, in good agreement with literature values for CAM plants (Tenci et al., 2023), resulted in WE yields from VS of 0.24 ± 0.08 % and 2.2 ± 0.3 % for WT and M+, respectively.

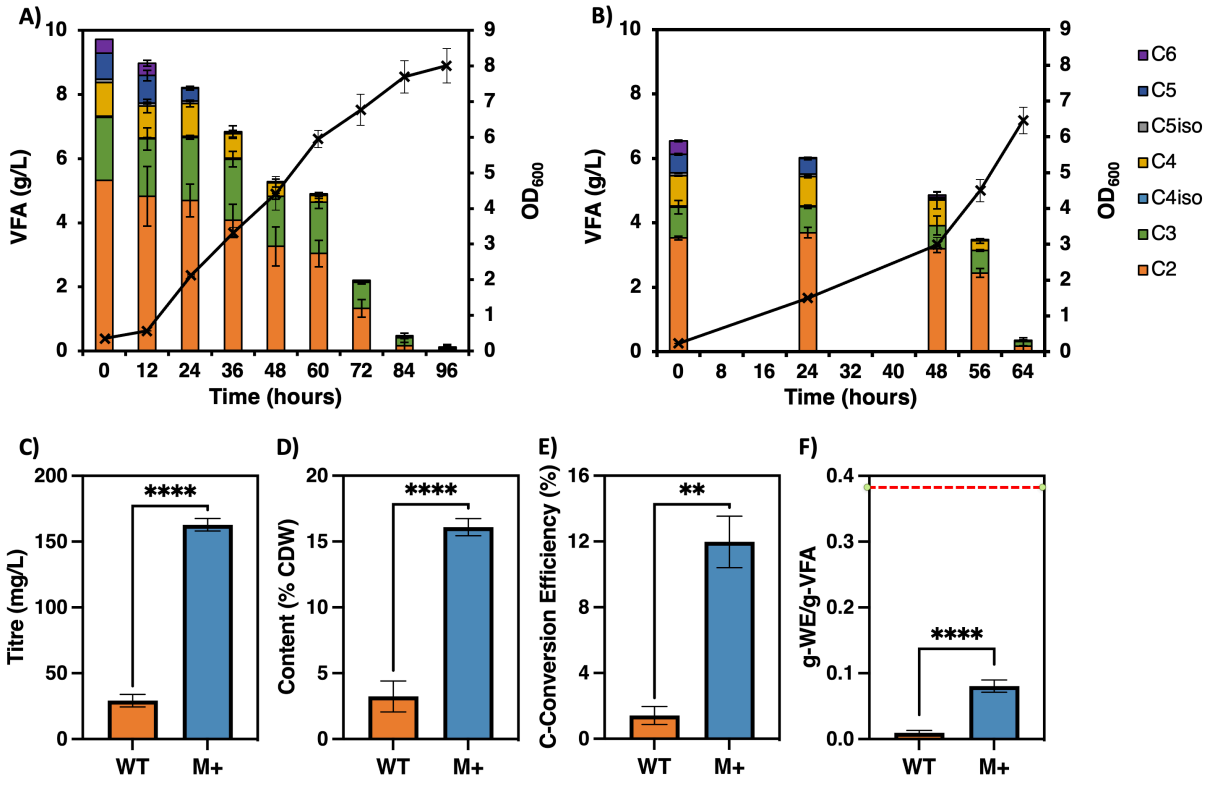


Figure 4.4. Growth curves and VFA consumption from fermentate for A) ADP1 WT on 10 g/L total VFA and B) ADP1 M+ on 6 g/L total VFA. Comparisons of the maximum C) WE titre, D) WE content per cell, E) C-Conversion efficiency to WE and F) Mass yield of WE from VFA. The red line indicates the theoretical maximum mass yield from the VFA mixture present in the fermentate. Data and error bars represent the means and standard deviations, respectively, with nine biological replicates for the WT and three biological replicates for M+. Significance of difference between strains analysed by t-test; $p < 0.01$ (**), $p < 0.0001$ (****).

4.3.5. Increasing carbon density within volatile fatty acid tolerance limits

4.3.5.1. Tolerance tests using longer chain volatile fatty acids

Growth and production in the systems shown so far were limited by relatively low substrate concentrations used due to the inherent tolerance limits of bacteria to VFAs. However, in the

M+ strain the tolerance limits of propionate and acetate in a wide range of ratios were similar and it became evident that total acid concentration was a factor. Based on this observation, the decision was made to address this problem by investigating longer chain fatty acids as carbon sources. In particular, butyrate and valerate, which are also significant components of fermentate, were chosen as carbon sources to explore.

Figure 4.5 shows growth data for ADP1 M+ grown on combinations of acetate, propionate, butyrate and valerate at a range of different concentrations and compositions. Total acid concentrations between 25-100 mM were used based on M+ tolerance data for propionate/acetate mixtures, and in each case, cultures were grown on either a growth substrate (odd chain VFA) or a mixture of one growth and one production substrate (even chain VFA). Generally, lag phase increased with increasing total acid concentration. Cultures containing more than one carbon source showed distinct growth phases, indicating substrate co-utilisation was not necessarily simultaneous.

Employing propionate as the carbon source for growth, tolerance and growth kinetics were similar regardless of whether acetate (Figure 4.5A) or butyrate (Figure 4.5B) was used as the co-substrate. Total acid concentration was still the key factor determining lag phase, and growth was detected in all conditions in both cases. However, the effect of substituting valerate for propionate was more pronounced. In 25 mM valerate cells grew to OD > 0.5, whilst those in 25 mM propionate reached a maximum OD of just 0.3, indicating that the additional carbon in valerate was beneficial to growth. Conversely, maximum valerate tolerance was just 50 mM (Figure 4.5C and D), substantially lower than propionate, although below this threshold growth on the two substrates was comparable. While low concentrations of acetate (25 mM and 50 mM) were either beneficial or insignificant to growth (Figure 4.5C), the addition of butyrate proved more challenging (Figure 4.5D), with samples containing 50 mM valerate and any butyrate struggling to grow.

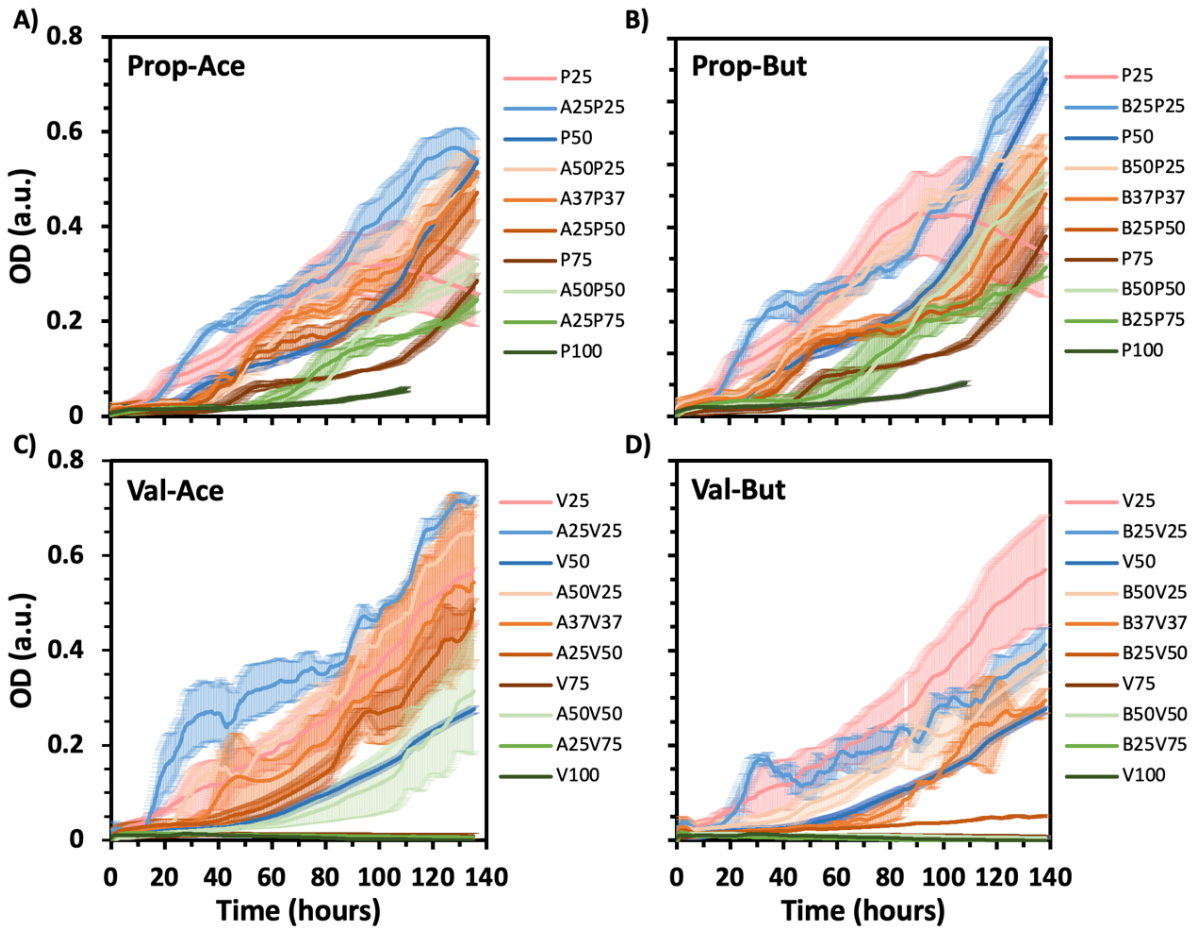


Figure 4.5. Growth curves obtained in 96 well plates for ADP1 M+ grown in minimal media on varying concentrations and combinations of acetate, propionate, butyrate and valerate. A) Propionate-Acetate, B) Propionate-Butyrate, C) Valerate-Acetate and D) Valerate-Butyrate. Data and error bars represent the means and standard deviations, respectively, of three biological replicates.

4.3.5.2. Wax production using longer chain volatile fatty acids

Based on these results, WE production experiments were performed on similar combinations of growth and production VFAs. For each combination, two ratios of production: growth substrates were tested based on the results of the acetate: propionate experiment (Figure 4.3): 1:1, which gave the highest WE titre and 5:1 which resulted in the highest content per cell. Multiple total acid concentrations were also tested to account for the reductions in tolerance to butyrate and valerate, as observed in the growth study (Figure 4.5). Table 4.2 contains the details of all conditions tested.

Table 4.2. Summary table of the different combinations and concentrations of acetate (A), propionate (P), butyrate (B) and valerate (V) tested for WE production, as well as the total carbon concentration under each condition. Samples containing high concentrations of valerate (37.5 mM) or butyrate (above 62.5 mM), shown in bold, and were unable to grow so were not analysed further (also see Figure 4.6A). Conditions underlined were close to the tolerance limits, and as such one of the three replicates failed to grow. In these cases, growth and WE production data was calculated from the two grown replicates.

VFA Ratio	**1:1**			**5:1**		
Total VFA (mM)	**50**	**60**	**75**	**50**	**60**	**75**
VFAs	**Total Carbon Concentration (mM)**					
A-P	-	**-**	187.5	-	**-**	162.5
A-V	-	210	**262.5**	-	150	187.5
B-P	-	210	262.5	191.7	230	**287.5**
B-V	225	**270**	**337.5**	208.3	250	**312.5**

With a 1:1 production: growth substrate ratio, the increased carbon concentration afforded by utilising longer chain VFAs enabled improved growth, up to tolerance limits (Figure 4.6A). The highest carbon concentration at which growth was achieved was 262.5 mM, with 37.5 mM butyrate and 37.5 mM propionate (B37P37), a 40 % increase from the equivalent acetate-propionate experiment. At this concentration, biomass production of 1.6 ± 0.1 g/L was also maximised, an increase of 23 % compared to 1.3 ± 0.2 g/L achieved on A37P37, although resulting in a lower conversion efficiency of VFA carbon to biomass.

Under 5:1 conditions, growth on 62.5 mM acetate and 12.5 mM valerate (A62V12) was better than A62P12, but growth in butyrate containing cultures with high production substrate concentration was severely limited. In particular maximum growth with 50 mM butyrate and 10 mM propionate (B50P10) was restricted to 0.36 ± 0.01 g/L, less than half the maximum on A62P12, despite an increase of 40 % in total carbon concentration. Even when the total carbon concentration was increased by more than 50%, such as on B50V10, growth still decreased compared to the acetate-propionate equivalent.

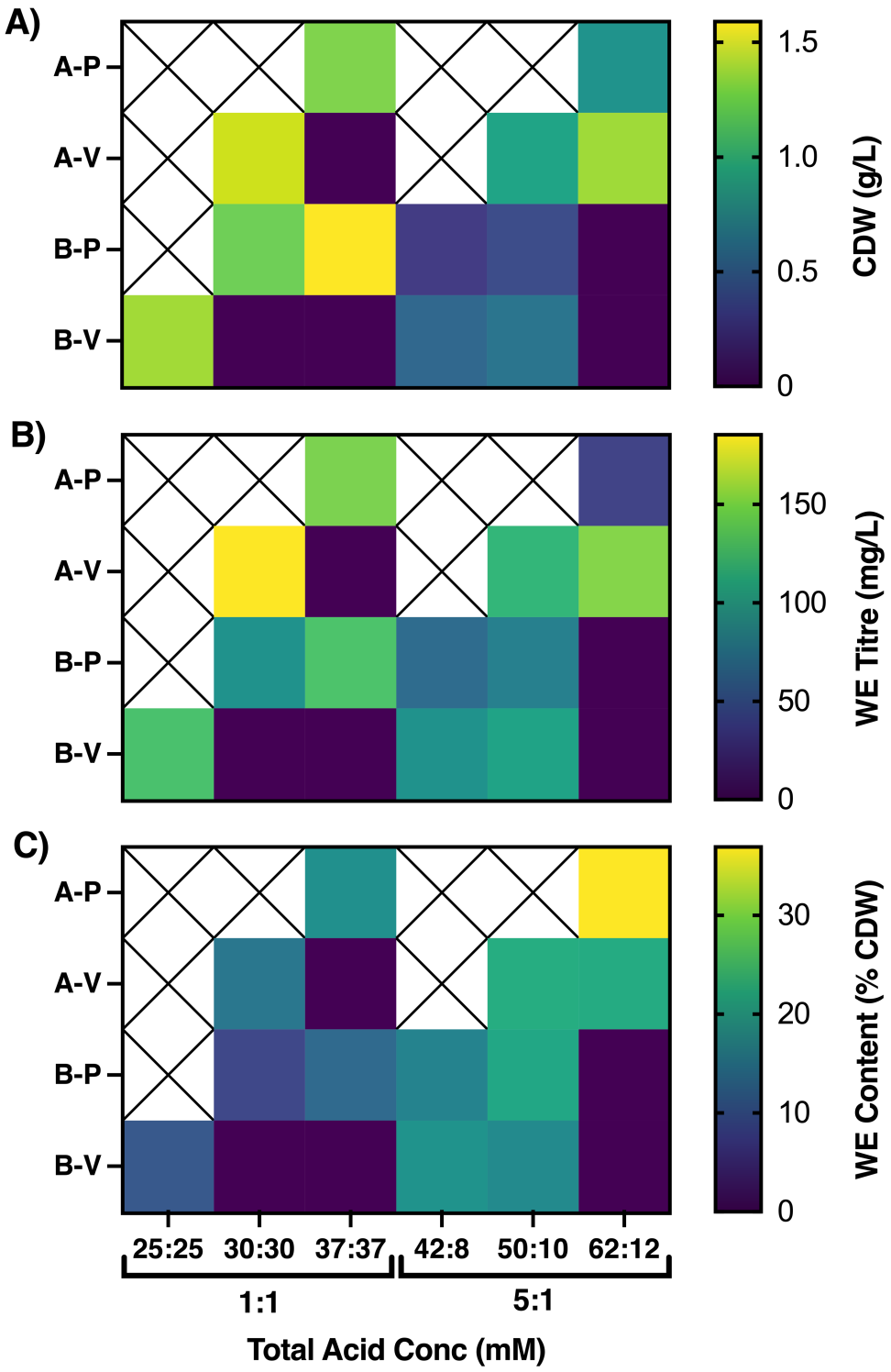


Figure 4.6. Heat maps of the maximum A) growth (g/L), B) titre (mg/L) and C) content per cell (% CDW) achieved under each condition. Numbers denote concentration of the given acid (A68P7 = 68 mM acetate, 7 mM propionate). Crossed out squares indicate conditions not measured due to a low total carbon concentration. Purple squares show samples which did not grow, so all metrics were zero. Data shows mean of three replicates.

Overall, the trends in WE titre and content were similar to those observed in acetate and propionate cultures (Figure 4.6B and C). Samples with 1:1 production: growth substrate ratio grew better than those with a ratio of 5:1, and thus WE titres were higher. Similarly, higher

total acid concentrations (up to the tolerance limit) also yielded better growth and higher titres. The A30V30 condition gave the greatest WE titre of 190 ± 10 mg/L, a 27 % increase on the maximum achieved in the acetate-propionate experiment. Conversely, WE contents were higher in samples with 5:1 ratio, although the maximum content observed was 23 ± 3 %, below the acetate-propionate maximum.

4.3.6. Tuning of fermentate to replicate optimal conditions

Well-defined, synthetic media conditions are an over-simplification of the composition of fermentate, which contains a mixture of VFAs and regularly includes branched VFAs. To validate our findings of potential growth and WE titre improvements from longer chain VFAs, AF of dried pig blood was used to generate fermentate with an increased proportion of longer chain VFAs (Tenci et al., 2023). Replicating reported conditions (Tenci et al., 2023), a total VFA concentration of 29.8 ± 0.8 g/L was achieved, with a relative composition (% in g/L) of 37:12:30:19:2 of C2:C3:C4:C5:C6, respectively, and absolute composition given in Table 4.3. C4 and C5 acids, both linear and branched, constituted 50 % of the total VFA by mass, a substantial increase from the 23 % in the *Opuntia*-derived fermentate, while nitrogen concentration increased 6.4-fold due to the protein rich feedstock.

Table 4.3. The VFA composition of the fermentate obtained by digesting dried pig blood.

Component	Concentration (g/L)
VFA	29.8 ± 0.8
- Acetate	11.1 ± 0.6
- Propionate	3.5 ± 0.2
- Isobutyrate	3.4 ± 0.2
- Butyrate	5.8 ± 0.3
- Isovalerate	4.8 ± 0.3
- Valerate	1.1 ± 0.2
- Hexanoate	0.07 ± 0.03
Total Organic Carbon	17 ± 1
Total Nitrogen	7.4 ± 0.4

ADP1 WT and M+ were both grown on this C4/C5-rich fermentate, at a concentration of 6 g/L total VFA. VFA consumption and growth curves are shown in Figure 4.7A and B for WT and M+ respectively, and behaviour in both was similar, reaching maximum OD_{600} of 2. In both cases, just over 2 g/L of VFA remained even after cells entered death phase, including around 1 g/L of iso-valerate, suggesting neither strain was able to utilise this substrate as a carbon or energy source.

WE measurements revealed that production by all metrics was higher in the M+ strain than the WT (Figure 4.7C-F). Maximum titre, content and C-conversion efficiency were 14 ± 2 mg/L, 2.9 ± 0.7 % CDW and 2.5 % in M+, compared to 2 ± 1 mg/L, 0.2 ± 0.1 % CDW and 0.2 ± 0.1 %. in the WT, increases of 9, 15 and 12-fold respectively. WE yield from consumed VFA was also much greater, with 0.014 ± 0.004 in the M1 strain compared to 0.0004 ± 0.0003, a 35-fold increase.

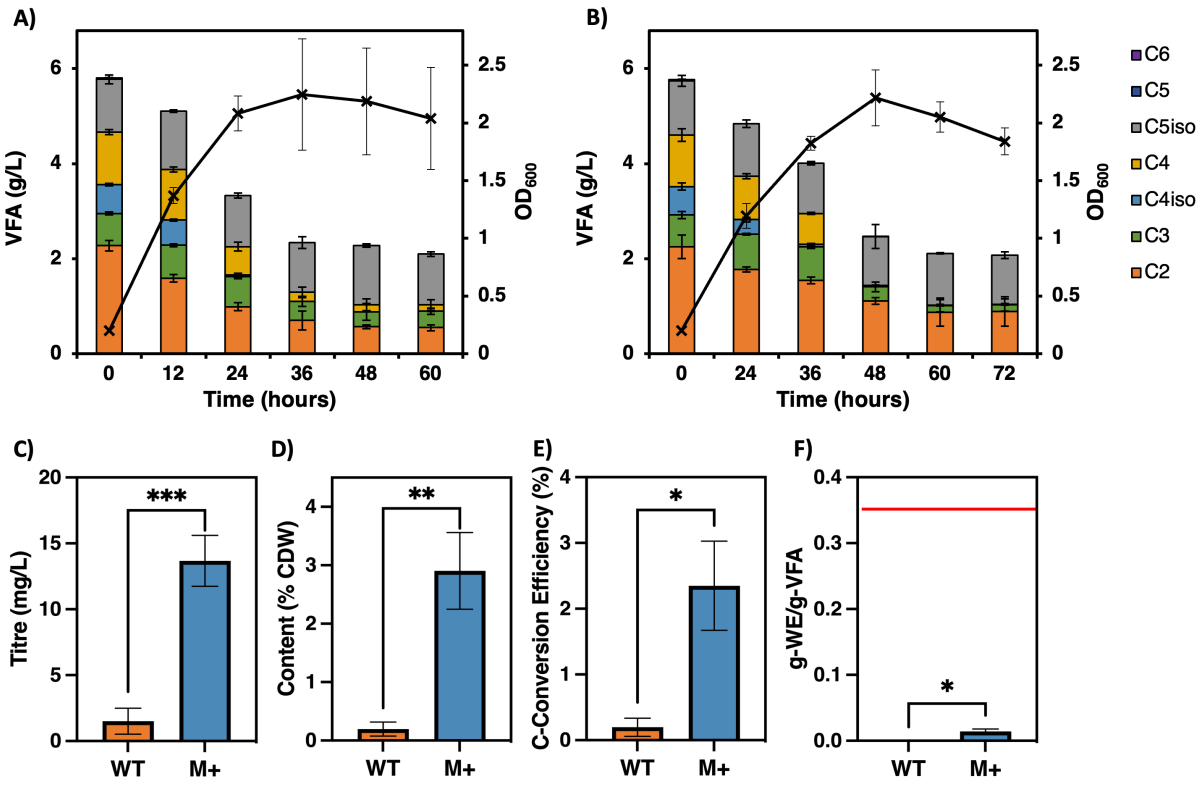


Figure 4.7. Growth curves and VFA consumption from C4/C5-rich fermentate for A) ADP1 WT and B) ADP1 M+ on 6 g/L total VFA. Comparisons of the maximum C) WE titre, D) WE content per cell, E) C-Conversion efficiency to WE and F) Mass yield of WE from VFA. The red line indicates the theoretical maximum mass yield from the VFA mixture present in the C4/C5-rich fermentate. Data and error bars represent the means and standard deviations, respectively, of three biological replicates. Significance of difference between strains analysed by t-test; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

4.4. Discussion

4.4.1. Towards theoretical maximum yields

The exploitation of VFAs for lipid production has inherently low yields due to the contrast between the highly reduced products and the highly oxidised substrate. Cells must generate reducing agents, as well as other necessary co-factors, by the complete oxidation of feedstock to CO_2, reducing the theoretical maximum efficiency. This is evidenced by the reduction in WE titre demonstrated when using propionate as a growth substrate (150 ± 10 mg/L) in comparison to gluconate (340 mg/L (Santala et al., 2021)). While 1 mol of gluconate produces 2 mol of acetyl-CoA as well as 3 mol of NADH and 1 mol of ATP as cofactors, 2 mol of propionate (which has the equivalent carbon content) also produces 2 mol of acetyl CoA, but only 2 mol of NADH. When yield is considered in terms of mass, this effect is compounded by the mass of oxygen present in VFAs that is lost in their conversion to WEs.

An estimate of the theoretical maximum yield in fermentate was calculated from the VFA consumption profile of the M+ strain at maximum WE production (see section 4.2.6 in Methods for more details). This maximum of 0.39 ± 0.03 g-WE/g-VFA (Figure 4.4F) accounted for the conservation of carbon and electrons only, and did not consider biomass production or energy generation, both of which would further reduce this value. The experimental maximum achieved in M+ of 0.08 ± 0.01 g-WE/g-VFA was already 21% of this calculated theoretical maximum, and hence at least as much of the true maximum yield.

4.4.2. Production without a production substrate?

Growth: production substrate ratio has been shown previously to affect both growth and WE production efficiency (Santala et al., 2021). This study demonstrated that by altering the ratio of propionate: acetate (a simplified version of the available substrates in fermentate), a 4-fold increase in WE content per cell, a 2.5-fold increase C-conversion efficiency or a 50% increase in titre could be achieved, in agreement with literature observations (Santala et al., 2021).

Unlike previous studies however, up to 100 mg/L of WE accumulated even without the addition of the production substrate (acetate). It has previously been observed that propionate inhibits multiple enzymes in the TCA cycle (Brock, 2010; Stumpf et al., 1980) whilst simultaneously increasing acetyl-CoA production (Wang et al., 2018), leading to an accumulation of acetyl-CoA in propionate grown cells. Such an effect may not have been present when gluconate was used as the growth substrate in previous works (Santala et al., 2021). Acetyl-CoA is the first molecule in the fatty acid synthesis pathway (Millard et al., 2021) and has also been found to

relieve the self-inhibition of translation of acetyl-CoA carboxylase (ACC), the enzyme which catalyses the first step in the pathway (Meades et al., 2010). More acetyl-CoA would therefore increase ACC concentration and WE production, especially because flux through the TCA cycle is limited and the glyoxylate shunt is not available. This may explain why propionate as a growth substrate resulted in moderately high WE accumulation, while gluconate did not. In addition to this, the utilisation of the methyl-citrate pathway to metabolise propionate results in higher flux through and accumulation of methyl-citrate in propionate grown cells. Methyl-citrate has also previously been found to activate ACC (Cheema-Dhadli, Leznoff, and Halperin, 1975) and thus increase fatty acid and WE production compared to gluconate grown cells.

Citric acid cycle equivalents, acetate and citrate, have similarly been shown to downregulate the TCA cycle (Millard et al., 2021) and upregulate ACC (Icard et al., 2021), respectively. The increase in WE production when combining acetate and gluconate substrates compared to gluconate alone, as reported previously (Santala et al., 2021), is likely linked to these regulatory changes. In contrast, a smaller relative increase in WE production (Figure 4.3A) was detected when co-utilising propionate and acetate compared to solely propionate, as the relative change in TCA cycle inhibition and ACC activation was lower.

4.4.3. Potential benefits of longer chain volatile fatty acids

The utilisation of longer chain VFAs (C4-C6) as carbon sources is relatively under-reported, and yet is of interest for several reasons. Longer fatty acids are catabolised by beta-oxidation, generating an additional 2 equivalents of NADH per acetyl-CoA unit cleaved from the backbone. The benefits of this in butyrate and valerate were demonstrated in the initial ADP1 growth test (Figure 4.1), where maximum OD_{600} achieved by the WT was approximately 30% higher than those achieved on acetate and propionate, despite equimolar carbon concentration in all cultures. In addition, greater reducing power and a lower proportion of molecular weight comprised of oxygen mean the maximum theoretical yields of WEs also increase for longer chains: on acetate the maximum is just 0.26 g-WE/g-VFA, while on butyrate it is 0.42 g-WE/g-VFA and on hexanoate it is 0.49 g-WE/g-VFA. Utilising longer chain VFAs also increases the total carbon concentration in a culture without increasing the acid molarity, thus partially circumventing the issue of substrate inhibition and enabling greater cell densities to be achieved. This would be particularly beneficial for the M+ strain, in which the glyoxylate shunt knock-out causes decreased acid tolerance compared to the WT bacteria (Park, Shin, and Park, 2019). Finally, it was observed that both the WT and M+ strain preferentially consumed longer chain VFAs over short ones (Figure 4.4), adding further cause to investigate their use.

As hoped, by increasing chain length, higher total carbon concentrations were reached without exceeding acid tolerance, but tolerances were not equal at a molar level. The metabolism of long chains into multiple short ones caused the concentration of acetate and propionate, and hence the total acid concentration, to initially increase, and hence tolerance limits were still exceeded. This was confirmed by the detection of acetate and propionate in cultures where they were not provided as substrates.

In cultures with equimolar amounts of growth and production substrates, culturing M+ on longer chain VFAs improved growth, yet when production substrate was in excess, many cultures showed severely limited growth despite an increase in total available carbon concentration. This is partially explained by the metabolism of valerate into acetate and propionate, and hence only 60 % of the carbon was directly available for growth in M+. Under two of the 5:1 conditions, at total acid concentrations of 50 and 60 mM, only 25 and 30 mM of carbon were available for growth, respectively, regardless of whether propionate or valerate was used, whereas in the equivalent acetate-propionate experiment 37.5 mM was available. High concentrations of butyrate also reduced growth even when total carbon increased. Replacing acetate with butyrate in VFA mixtures has previously been observed to decrease growth in algal strains, even if total carbon concentration increases (Fei et al., 2015). Similarly, increasing VFA concentration can reduce biomass production, with C4 acids hypothesised to cause acute stress (Moon et al., 2013).

Aside from substituting acetate for butyrate at high concentrations, generally increasing acetyl-CoA concentration (both by directly increasing production substrate concentration or by chain elongation of either substrate) increased biomass production. This confirms previous observations that even with the glyoxylate shunt knocked out, acetate can contribute carbon to the central carbon metabolism (Santala et al., 2021).

4.4.4. Suitability of engineered strains for waste stream valorisation

This study is the first to report the production of WEs from a non-lipid, mixed waste-stream. Like many organic waste streams (Abbas et al., 2011; Prandi et al., 2019), the abundance of nitrogen in fermentate makes it largely unsuitable for microbial lipid production, which is typically induced by nitrogen-limitation (Luo et al., 2020; Martin, Huang, and Thompson, 2023; Wang et al., 2019; Wierzchowska et al., 2021). It is therefore an ideal candidate for growth and bioproduction of metabolically engineered bacteria, in which natural regulatory restrictions can be relaxed. The M+ mutant was specifically chosen because its suitability for wax production from nitrogen-rich substrates has been previously demonstrated (Luo et al.,

2020). However, fermentate does not contain a significant source of glycolytic substrates or amino acids, which were previously required by this strain for growth, and hence its utilisation represents a significant step forward in the biological upgrading of low value waste streams to useful chemicals.

The translation of results on propionate/acetate to *Opuntia* fermentate was promising, with high maximum WE titres achieved comparable to those on synthetic media. However, in the C4/C5 rich fermentate this was not the case: although M+ still significantly outperformed the WT, the absolute values were much lower than in the closest equivalent synthetic media. This difference is likely due to the high ammonium concentration in this fermentate resulting from the degradation of proteins in pig blood. The C:N ratio was therefore just 2.7:1, compared to the synthetic system where it was between 9:1 and 11:1. In contrast, the *Opuntia* fermentate had a ratio of 9.8:1, much more comparable to the ratio range of 7:1 to 10:1 in the propionate/acetate synthetic media, hence the better translation of these results.

The decrease in storage lipid accumulation with increasing nitrogen concentration in wild type strains is well documented in the literature (Martin, Huang, and Thompson, 2023; Wang et al., 2019; Wierzchowska et al., 2021), as high C:N ratios are known to cause neutral lipid accumulation as a stress response. While the M+ strain was specifically designed to produce higher WE titres and yields in nitrogen-rich conditions (Luo et al., 2020), it is clear this capability is still limited. Although its maximum WE titre, 1.82 g/L from glucose, is the highest recorded to date, this was achieved at a high C:N ratio of 64:1, conditions under which titre in the WT was still 580 mg/L. When non-optimal, high nitrogen substrates were used, such as baker's yeast hydrolysate (typical C:N ratio around 5:1 (Yu et al., 2020)), maximum WE titre dropped to just 70 mg/L in the M+ strain and less than 40 mg/L in the WT. This lower absolute production demonstrates that the natural regulation in ADP1 which prevents WE accumulation in nutrient rich environments still dominates cellular metabolism at low C:N ratios. It is therefore unsurprising that with a C:N ratio below 3, C4/C5 rich fermentate greatly impeded WE production. High nitrogen concentrations may still pose an obstacle to efficient microbial bioproduction of lipids, even in engineered production strains, and tackling nitrogen-rich waste streams may require an alternative solution.

4.4.5. Broader metabolic findings and implications

As well as advances in sustainable bioproduction of WEs and other microbial lipids and bioproducts, this work has revealed several important genetic and metabolic traits of ADP1 not previously explored. We established that the M+ strain can utilise odd chain VFAs as sole

carbon sources (Figure 4.1), suggesting that short chain fatty acid metabolism in ADP1 is similar to that of *E. coli*, with propionate metabolised through the methyl-citrate pathway (Textor et al., 1997). In particular, all even chain VFAs are broken down into acetyl-CoA units, and hence the glyoxylate shunt knockout inhibits their utilisation for growth, while odd chain VFAs are catabolised into acetyl-CoA and propionyl-CoA units (Figure 4.8), so remain viable carbon sources (Jaremko & Yu, 2011; Rand et al., 2017).

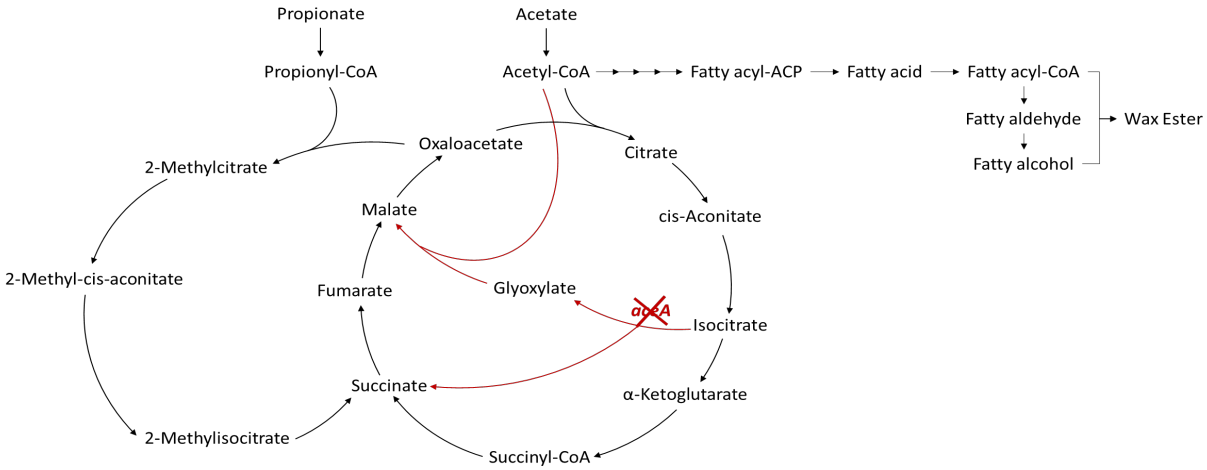


Figure 4.8. Map of the interconnected tricarboxylic acid and methylcitrate cycles in ADP1, the latter of which is used to metabolise propionate and other odd-chain fatty acids. The *aceA* knock-out in the glyoxylate shunt is shown: *aceA* is essential for bacteria to utilise two-carbon feedstocks, such as acetate, for the production of carbohydrates and for growth. Without *aceA* cells can still consume acetate, and other even chain fatty acids which are metabolised via acetyl-CoA, to generate energy, but it cannot be incorporated into biomass.

Propionate growth and tolerance tests of cells precultured on LB or propionate and grown with and without acetate revealed a stark contrast in behaviour. As expected, WT and M+ cells pre-adapted to low concentrations of propionate (25 mM) displayed higher propionate tolerance and grew more quickly with shorter lag phase (Kim, Jeong, and Lee, 2021), but this benefit was lost with the addition of acetate, and lag phase was reduced further. The ability of low acetate concentration to improve growth under these conditions was unexpected and not previously reported for this strain or for glyoxylate shunt knockouts in other bacterial species. This likely contributes to the ability of cells to grow in fermentate without a separate pre-adaptation phase, which would be desirable in large scale processes as it minimises the number of stages, and therefore the number of reactors and the cost, required for cultivation.

4.5. Conclusions

The results obtained here represent a significant step towards the utilisation of VFAs, in particular VFA-rich waste streams, as sole carbon and energy sources for WE production. By utilising an engineered strain of ADP1, WE titres of up to 190 mg/L were achieved on synthetic VFAs and up to 160 mg/L on fermentate, with a 5.6-fold increase over the WT. The highest ever WE content per cell in ADP1 of 37 % CDW was also recorded.

The challenges of translating a process from synthetic media conditions to a complex, mixed substrate were also highlighted in this study. While the initial investigation with fermentate obtained from anaerobic digestion of *Opuntia* proved highly successful, this was found not to be reproducible across all fermentate systems. In particular, the difficulty of obtaining a waste stream with optimal composition is highlighted. Controlling the feedstock to manipulate one parameter, here the VFA composition, affected other parameters, such as nutrient concentrations, with unforeseen consequences on bioproduction as a result.

To build upon these findings, future work should explore the combination of waste streams containing higher energy, more reduced carbon sources, such as waste cooking oils or crude glycerol, in combination with fermentate. The C:N ratio of waste streams remains a critical factor; whilst genetic engineering can improve the nitrogen tolerance of lipid production, this improvement is not universal and the effective C:N range is still limited. Concentrated waste streams, such as the fermentate from digestions with high substrate loading, as investigated here, also present opportunities for fed-batch and continuous culture processes, both of which can facilitate greater biomass accumulation and hence higher WE titres. Advances in product intensification are essential in improving process scalability and viability for industrial bioproduction of microbial lipids.

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4.7. Supplementary information

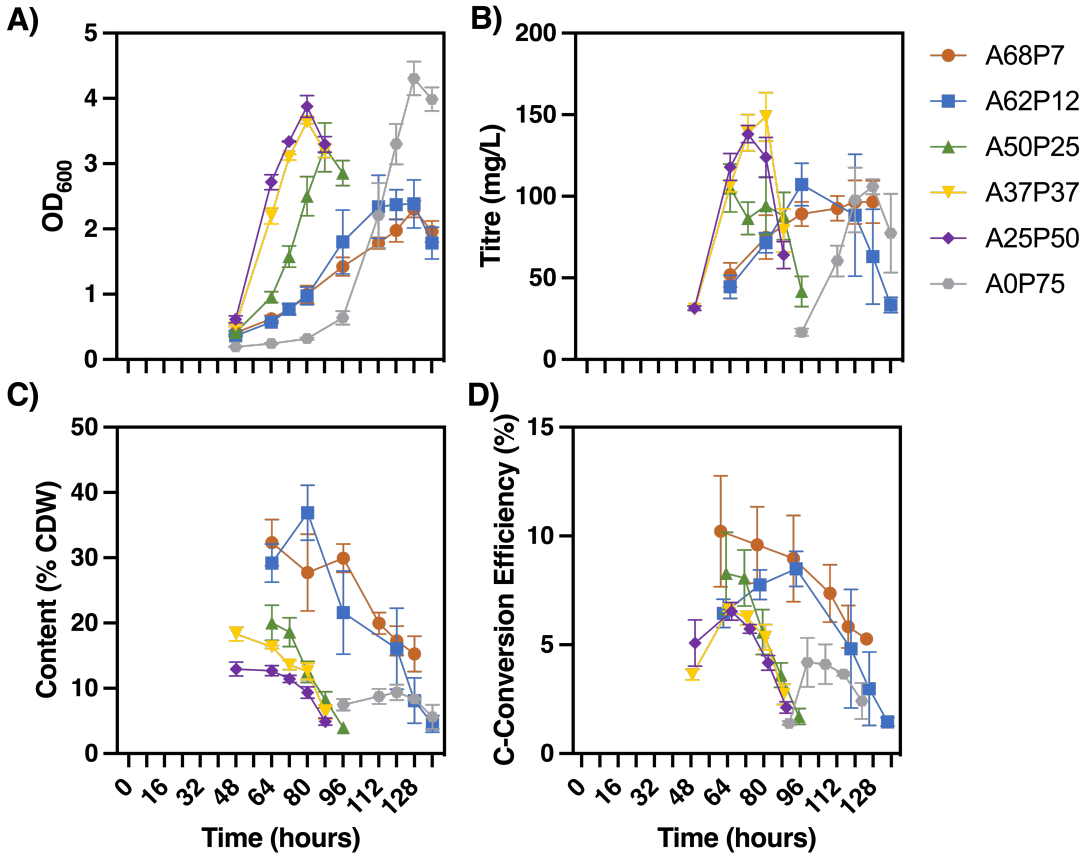


Figure 4.S1. Comparisons of the A) growth, B) WE titre, C) WE content per cell and D) C-conversion efficiency for ADP1 M+ mutant over time at different concentrations of acetate (A) and propionate (P), with total acid concentration of 75 mM in all conditions. Numbers denote concentration of the given acid (A68P7 = 68 mM acetate, 7 mM propionate). Data and error bars represent the means and standard deviations, respectively, of three biological replicates. Conditions were A68P7 (orange circles), A62P12 (blue squares), A50P25 (green triangles), A37P37 (yellow inverse triangles), A25P50 (purple diamonds), A0P75 (grey circles).

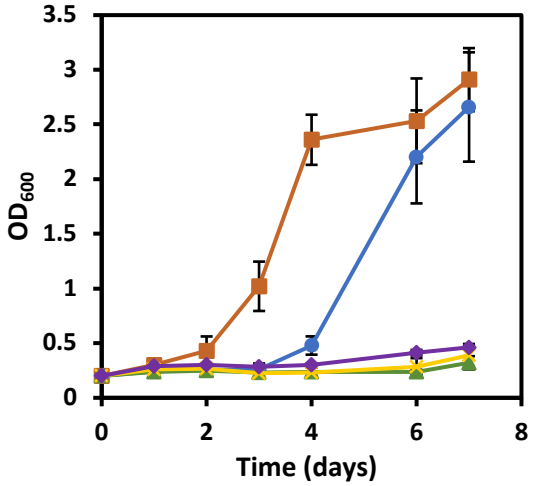


Figure 4.S2. Growth curves for *A. baylyi* ADP1 M+ on VFA rich digestate at a range of dilutions. VFA concentrations of 4 (orange), 6 (blue), 8 (green), 10 (yellow) and 12 g/L (purple) were tested.

Table 4.S1. Maximum growth (g/L of cell dry weight) of ADP1 M+ on different concentrations and combinations of acetate, propionate, butyrate and valerate.

VFAs	**Total Carbon Concentration (mM)**					
	1:1			**5:1**		
	50	**60**	**75**	**50**	**60**	**75**
A-P	-	-	1.3 ± 0.2	-	-	0.8 ± 0.2
A-V	-	1.47 ± 0.05	0	-	0.9 ± 0.1	1.4 ± 0.3
B-P	-	1.24 ± 0.07	1.6 ± 0.1	0.28 ± 0.04	0.36 ± 0.01	0
B-V	1.4 ± 0.1	0	0	0.52 ± 0.04	0.61 ± 0.04	0

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Student Confirmation

Student Name:	Laura Martin		
Contribution to the Paper	Planned and conducted experiments, analysed the results and wrote the paper.		
Signature		Date	05.12.2023

Supervisor Confirmation

By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

Supervisor name and title: Professor Ian Thompson		
Supervisor comments I agree with this summary		
	Date	08.12.2023

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Chapter 5 - The effect of nutrient limitation on bacterial wax ester production

In this chapter, the effect of limitation of several key nutrients on WE production in wild type *A. baylyi* ADP1 is explored. Bacterial growth and WE production is considered on acetate due to the low cost and wide availability, as previously discussed in this thesis. This investigation was undertaken to due to the negative effects of high nitrogen concentration on WE production that was demonstrated in the blood-derived fermentate in the previous chapter. The aim of this study was to identify optimal concentrations and ratios of key growth nutrients, with the aim of then applying these findings to WE production in fermentate.

Nitrogen and phosphorus limitation are initially considered separately, with a systematic approach to identify the optimal range of limitation for WE production of each element. Nitrogen limitation is found to be more effective in increasing WE titres, content per cell and the efficiency of carbon conversion from acetate to WEs, as reported previously. Although phosphorus starvation had been previously identified to increase TAGs production in several micro-organisms (192-194), this work demonstrates for the first time that the equivalent benefit is not demonstrated for WE production. However, in another previously unreported behaviour, the preferred class of storage lipid accumulated by *A. baylyi* ADP1 changed from WEs to TAGs with decreasing phosphorus availability.

The limitation of micronutrients magnesium, calcium and potassium is also investigated, with increased magnesium and calcium concentrations found to improve WE titres significantly, in agreement with previous literature. Finally, to investigate any potential compounding effects, nitrogen and phosphorus were co-limited simultaneously, with an interaction effect observed on both WE titre and content per cell.

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THE EFFECT OF NUTRIENT LIMITATION ON BACTERIAL WAX ESTER PRODUCTION

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Abstract

Nutrient limitation is widely employed to alter the behaviour of micro-organisms. Here, the impact of nitrogen and, for the first time, phosphorus limitation is investigated on the production of bacterial storage lipids; specifically wax esters, a class of storage lipids of industrial interest, by the bacterium *Acinetobacter baylyi* ADP1 grown on the low-cost substrate acetate. Studies determined the absolute and temporal effects of nutrient limitation and identified a maximum wax titre of 132 mg/L and content of 17 % of biomass. A 4-fold increase in wax production was achievable by manipulating carbon: phosphorus ratio. Multivariable analysis identified a novel interaction effect between carbon: nitrogen and carbon: phosphorus ratios on wax production. Extreme phosphorus starvation shifted the dominant lipid class from wax esters to triacylglycerols, the first report of the potential of phosphorus limitation to alter the type of lipid generated. These findings offer valuable insights for future microbial bioproduction studies.

Key words

Wax Ester; Lipid Accumulation; Nutrient Limitation; Nitrogen; Phosphorus; *Acinetobacter baylyi* ADP1; Microbiology; Biotechnology; Bioproduction

5.1. Introduction

Nutrient limitations are among the most common stresses experienced by micro-organisms and are known to have significant and varied effects on growth and physiology. When faced with essential nutrient depletion, growth is typically limited, and carbon and energy fluxes are redirected towards increasing the bioavailability of the desired nutrient (Gupta & Laxman, 2021; Liu et al., 2016; Zhang et al., 2016). Whether by upregulating proteins and pathways for extracellular scavenging or degrading non-essential intracellular metabolites (Peterson et al., 2005), the need to source a scarce element significantly alters the metabolism of a micro-organism (Ikaran et al., 2015; Wang et al., 2018). However, assuming that the period of limitation will be brief, in some microbes nutrient starvation also activates additional metabolic pathways to monopolise resources for the future.

Carbon storage molecules are a common example of such microbial 'future-proofing'. These molecules, typically in the form of carbon-dense lipids, are accumulated intracellularly as an alternative to growth when essential nutrients are limited, but carbon is plentiful. One specific lipid class of interest is wax esters (WEs). These long chain esters are hydrophobic and chemically inert, making them highly desirable for a wide range of applications, from cosmetics and food additives to industrial lubricants (Bart et al., 2013; Santala et al., 2011a). They comprise the major component of many highly desirable biologically derived waxes, including jojoba and spermaceti oils, widespread usage of which is restricted by expensive and unethical sourcing, respectively. An alternative source of WEs is therefore of great industrial interest.

Although a range of microbial species are capable of accumulating high concentrations of storage lipids (Lennen & Pfleger, 2013; Martin et al., 2021), these are typically a mixture of triacylglycerols (TAGs), diacylglycerols (DAGs) and fatty acids. WEs are less prevalent due to the scarcity of endogenous long chain fatty acid reductase enzymes in micro-organisms, which are essential to generate the fatty alcohol required for esterification (Cronan & Thomas, 2009). Such enzymes are, however, present in the bacterial species *Acinetobacter baylyi* ADP1, which is widely known for its ability to accumulate WEs (Ishige et al., 2003; Santala & Santala, 2021). While storage lipid composition in this species has been shown to be substrate-dependent (Salcedo-Vite et al., 2019), WEs always comprise the major component. Finally, ADP1 is well characterised, with a fully sequenced genome, and is naturally competent for transformation of DNA (Santala & Santala, 2021). While this study focuses on the effects of

nutrient limitation in the wild type (WT) bacteria, findings here can be easily translated to engineered and mutant strains, potentially further improving the yields and titres achieved.

Previously, most researchers have focused on nitrogen as the limiting nutrient, which has been shown to increase lipid accumulation in a wide range of bacteria (Manilla-Pérez et al., 2011), yeast (Pomraning et al., 2016), algae (Ikaran et al., 2015) and microalgae (Wang et al., 2019), although the optimal degree of N-limitation is rarely determined. However, many economical and sustainable substrates are mixed waste streams inherently high in nitrogen (Luo et al., 2020). For this reason, alternative nutrient limitation strategies to induce lipid accumulation are of interest. Fortunately, starvation of phosphorus, another essential nutrient, has also been found to result in lipid production in yeast (Huang et al., 2018; Wang et al., 2018), algae (Shrestha et al., 2020) and micro-algae (Yang et al., 2018a), although its effect on bacterial storage lipid accumulation has not been investigated.

The effect of combined limitation of both nitrogen and phosphorus, and any interaction between these factors, has also been largely ignored in the literature; where tested, results have been inconsistent. In microalgae, Yang *et al* found excess phosphorus with N limitation gave the highest lipid titres and content (% CDW), followed by P and N limitation, then N-limitation with sufficient P, with sufficient N and P resulting in the least lipid production (Yang et al., 2018b). Conversely, Wierzchowska *et al* took two different approaches to lipid accumulation in yeast; initially detecting no significant effect of N on lipid content, then observing the optimal combination was simultaneous N and P limitation (Wierzchowska et al., 2021). This limited and conflicting data on such key growth parameters highlights the need for further investigation in this area.

As well as macronutrients, micronutrients are also known to affect both microbial growth and lipid accumulation. Divalent cations magnesium (Mg^{2+}) and calcium (Ca^{2+}) are of particular importance to cell function, with the former used for metabolic enzymes, nucleic acid stabilisation and DNA replication (Walker, 1994), and the latter in structure, transport and gene expression (Dominguez, 2004). Limitation of both has been demonstrated to reduce bacterial growth (Chen et al., 2019; Lusk et al., 1968). Furthermore, increasing the concentration of both ions has also been demonstrated to improve lipid production in freshwater and marine microalgae (Huang et al., 2014; Singh et al., 2016). Increasing the concentration of potassium (K^{+}), the main bacterial cation, used as a messenger and for maintaining pH and membrane potential (Deshpande et al., 2013), has also been shown to increase lipid accumulation, particularly of fatty acids, in microalgae (Janchot et al., 2019). However, despite the

demonstrated benefits on a range of other micro-organisms for lipid accumulation, there has been little exploration of the effects of these key micronutrients on bacterial lipid production.

Substrate choice is vital to the feasibility of microbial biosynthesis processes. Currently, glucose and other sugars dominate studies of microbial lipid synthesis (Kannisto et al., 2014; Luo et al., 2020; Martin et al., 2021; Rottig et al., 2016; Round et al., 2019), and, with WE titres of up to 0.58 g/L achieved from growth on glucose employing WT ADP1 (Luo et al., 2020), the attractiveness of such feedstocks is evident. Unfortunately, sugars are expensive, and their production requires arable land to grow high-sugar fuel crops, igniting land-use conflicts with food production, housing and natural ecosystems (Mohr & Raman, 2013). With this in mind, the utilisation of waste-stream derived substrates is becoming increasingly attractive. Acetate (or acetic acid) can be produced from a wide range of waste streams. Anaerobic digestion, commonly used to degrade mixed biological waste into biogas for fuel, can be tuned to produce high yields of acetic acid, as well as other volatile fatty acids also suitable for fermentation (Patel et al., 2021). Alternatively, microbial electrosynthesis can fix CO_2 from the atmosphere into platform chemicals, such as acetate (Kong et al., 2020). Moreover, several researchers have demonstrated the potential of acetate as a substrate for WE production in ADP1, which is able to utilise the substrate either in a co-feeding system with another, higher energy, feedstock (Santala et al., 2021) or as a sole carbon and energy source (Lehtinen et al., 2017; Santala et al., 2018).

In this study, the effect of several micro and macro nutrients on the production of wax esters from acetate as a sole carbon source by ADP1 was explored. Thorough single factor studies were performed by limiting the macro-nutrients nitrogen and phosphorus, with a range of conditions explored to identify any trends or significant behavioural changes over the experimental space and identify a narrower range of interest. The temporal variation of wax production was also monitored to explore how production and consumption of WEs varied under different conditions and to accurately compare maximum wax production under different conditions, accounting for differences in growth behaviour. Total wax titre, intracellular concentration and percentage yield of waxes were all calculated to provide a comprehensive account of the different benefits of wax production under different conditions. Binary studies of the effects of micronutrients calcium, potassium, magnesium, and a common trace elements mix were also conducted. From this initial data, an optimised, multi-factor experiment was conducted, to identify any interaction effects of nitrogen and phosphorus limitation under the most optimal micronutrient conditions.

5.2. Materials and methods

5.2.1. Chemicals

All chemicals were sourced from SigmaAldrich (Merck).

5.2.2. Media

The standard mineral medium contained 4.18 g/L MOPS buffer and 0.1 g/L $MgSO_4.7H_2O$, adjusted to pH 6.8 with NaOH and sterilised by autoclave. Concentrated solutions of 300 g/L sodium acetate, 100 g/L NH_4Cl, 200 g/L KH_2PO_4, 85 g/L K_sSO_4, 1000g/L $CaCl_2.2H_2O$, 1000x solution Bauchop & Elsden (TE) (Bauchop & Elsden, 1960) and saturated $FeSO_4$ were prepared and sterilised separately and added to the media immediately prior to starting the experiment. The standard media recipe contained 10 µL/L of $CaCl_2$ and $FeSO_4$ stocks, 1 mL/L of TE solution and 150 mM (or 41 mL/L) of sodium acetate stock. In each condition, pH was readjusted aseptically to 6.8 before inoculation.

5.2.3. Culture conditions

Bacterial stocks were stored at -80°C in 20 % glycerol/water. Stocks were plated on LB agar and incubated at 30 °C for 24 hours, with a single colony transferred in LB liquid media and grown until mid-exponential phase. Bacteria were pre-cultured twice in conical flasks in mineral medium with acetate and excess N and P and transferred during mid-exponential phase. Immediately prior to starting each experiment, precultures were spun down at 6000 g for 6 minutes, resuspended in MOPS buffer and flasks were inoculated at an initial OD of 0.1. Experiments were performed in 200 mL cultures in 1L conical flasks, in triplicate. All liquid cultures, and pre-cultures, were cultivated at 30 °C and 150 rpm.

5.2.4. Nitrogen, phosphorus and micronutrient limitation studies

NH_4Cl and KH_2PO_4 stocks were added as required to achieve the desired C:N or C:P molar ratios for each experiment. K_2SO_4 was used to maintain a constant K^+ concentration of 15 mM under all conditions. Under N limitation, excess P at C:P of 20:1 was used, while under phosphorus limitation excess N at C:N of 10:1 was used. Nitrogen and phosphorus concentrations were quantified experimentally using spectrophotometric kit-based assays (Hach Lange: LCK 303 for ammonia and LCK 349/LCK 350 for phosphorus, results in supplementary information). Where micronutrients were varied, the concentration of $CaCl_2$, $MgSO_4$, K_2SO_4 or TE solutions were varied accordingly.

5.2.5. Growth curves and cell dry weight measurements

Growth was measured by absorbance at 600 nm with UV-1800 UV/Vis spectrophotometer (Shimadzu, Kyoto, Japan). For cell dry weight and lipid extractions, 15-40 mL of cultures (depending on OD) were extracted and centrifuged for 8 min at 10,000 g. Cell pellets were resuspended in 1.5 mL of DI water, transferred to an Eppendorf tube and centrifuged for 5 min at 13,000 g. Washed cell pellets were lyophilised overnight and the biomass determined gravimetrically.

5.2.6. Lipid extraction and quantification

For NMR quantification, lipids were extracted following an adapted Bligh and Dyer method (Santala et al., 2011b). Each cell pellet was resuspended in 2 mL of PBS, then 2.5 mL chloroform and 5 mL of methanol were added, and the mixture shaken for approx. 5 hours at 150 rpm. 2.5 mL of methanol, 2.5 mL PBS and 2.5 mL of chloroform were then added, the sample mixed again and stored overnight at +4 °C. The suspension was centrifuged at 10,000 g for 8 min, and the lower organic phase was transferred to a pre-weighed glass vial to evaporate. The aqueous phase was extracted again by addition of 5 mL of chloroform, mixed and incubated overnight at +4 °C, then centrifuged and separated as before.

NMR analysis was performed using Bruker Avance III spectrometer (700 MHz) with a TCI cryoprobe (Bruker, MA, US). Lipid samples were dissolved in 600 µL of chloroform-d_3 and ^{1}H spectra recorded at ambient temperature. Spectra were processed and analysed using MestreNova 14.1.1. with an artificial reference peak at δ -1 ppm, corresponding to a single hydrogen atom with concentration of 10 mM. Global Spectrum Deconvolution was used to accurately isolate and integrate the triplet peak at δ 4.05 ppm, corresponding to the 2 α-alkoxy methylene protons from the alcohol component of WEs. From this the WE concentration was determined and used to calculate the mass of WEs produced, assuming an average Mw for WEs of 506 g/mol (Lehtinen et al., 2018). Where present, TAGs were quantified from the same NMR spectra from the doublet of doublet peak at δ 4.29 ppm (Nieva-Echevarría et al., 2014), corresponding to 2 of the ROC**H$_2$**-CH(OR')-C**H$_2$**OR'' protons. An average Mw of 847.4 g/mol was used to calculate mas of TAGs, assuming an approximately equal likelihood of C16 or C18 fatty acids esterified to each glycerol alcohol, as these are the most prevalent fatty acids in acetate-grown ADP1, and an average of 1 unsaturated C=C bond per TAG (Salcedo-Vite et al., 2019).

5.2.7. Acetate consumption and C-conversion

Remaining acetate content at each time point was measured from the supernatant of the extracted cells for CDW and WE quantification using gas chromatography-flame ionisation detection (GC-FID) (Shimadzu GC-2010) with a 30m x 0.25 mm x 0.25 µm fused-silica capillary column (ZB-FFAP). The temperatures of the detector and injector were set at 350°C and 250°C, respectively. The oven temperatures were set at 100°C for 2 minutes and then increased at a rate of 16°C/min. Calibration was performed with standards of sodium acetate at concentrations from 0.31 – 20.00 g/L. All samples and standards (450 µL) were acidified with 10 % v/v of formic acid (50 µL) prior to analysis. C-conversion efficiency was calculated from the total acetate consumption for each sample.

5.2.8. Multivariable experiment

Based on the optimal C:N and C:P ranges and the optimal micronutrient condition identified, a multivariable experiment was conducted. A 2-factor, central composite designed experiment was constructed and analysed using OriginPro 2022b 'Design of Experiments' application (OriginLab, Northampton, MA, United States). Experimental C:N and C:P ratios were quantified and confirmed to agree with intended values. Each condition was replicated in biological duplicate, with four replicates of the centre point, and data was blocked by axial/equatorial group and replicate. Samples for WE analysis were taken every 8 hours from 24-56 hours. The maximum value, for titre and content, was taken from each replicate individually, regardless of time point, and maximum average was calculated from this. Data analysis was performed in the application, resulting in a fitted response surface for both titre and content of WE, TAG and combined lipids.

5.2.9. Statistical analysis

All results were analysed for statistical significance in GraphPad Prism (GraphPad, San Diego, CA, United States). For single variable experiments, time-dependent data across all conditions was analysed by a mixed-effects model to determine the effect of Time, condition and the interactions between the two. The maximum of each metric was identified for each condition over the whole time-course, and each set of maxima were analysed by One-Way ANOVA, with multiple pairwise comparisons. The Šidák correction was applied to control the false positive rate, and the Brown-Forsythe test was performed to confirm that group variances were not significantly different (and hence that the data was suitable for ANOVA).

For the multivariable experiment, statistical analysis was performed in OriginPro 2022b. The significance of each variable, and their interaction, on the model was analysed by a t-test on the coded co-efficients and analysis of variance (ANOVA) on the significance of the model components. P-values from both tests are reported.

Asterisks indicate significance at different levels as follows: * = 5%, ** = 1%, *** = 0.1% and **** = 0.01%.

5.3. Results and discussion

5.3.1. Nitrogen limitation

The effect of nitrogen limitation was explored by varying the C:N molar ratio at a constant carbon concentration over the range of 10:1 (found by preliminary experiments to be in excess and produce maximum growth) to 160:1, similar to values trialed in literature which reported substantial lipid accumulation (Manilla-Pérez et al., 2011). As expected, growth was severely impaired by nitrogen limitation at C:N 40:1 and above, and became more variable with greater nitrogen depletion (Figure 5.1A). However, despite nitrogen concentration halving sequentially between 20:1, 40:1, 80:1 and 160:1, in each case the maximal growth of the lower-nitrogen culture exceeded half that of the one above it, in agreement with previous literature (Manilla-Pérez et al., 2011). Cultures at 10:1 and 20:1 C:N consumed almost all the acetate present in 48 hours, while in all other conditions acetate consumption plateaued and significant quantities of acetate remained even after 48 hours. Cultures containing less nitrogen consumed less acetate, in agreement with the reduced growth, though acetate consumption was still observed after growth had plateaued.

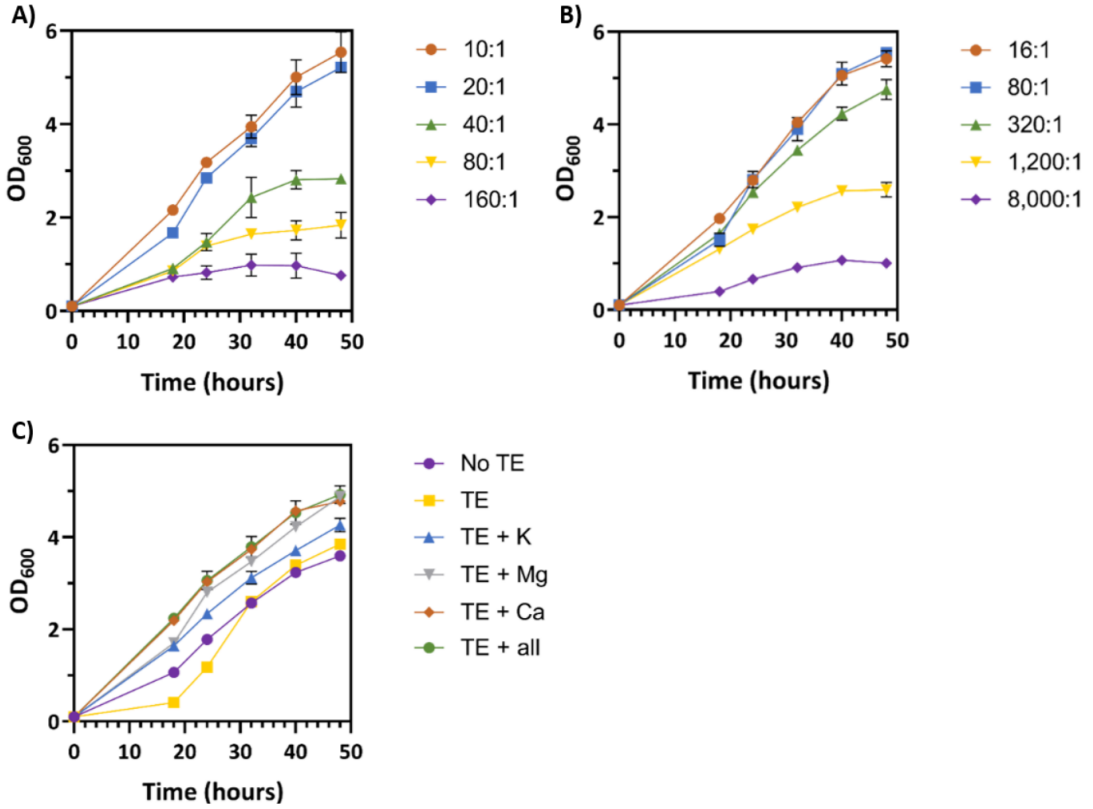


Figure 5.1. Growth curves for ADP1 grown on 150 mM acetate under A) different C:N ratios, B) different C:P ratios and C) under different micronutrient conditions. Data points and error bars represent the means and standard deviations, respectively, of three biological replicates.

Figure 5.2 shows WE titre, content and carbon conversion efficiency over 48 hours and statistical comparisons of the maximum achieved for each C:N condition. A final time point was taken at 64 hours, but in all cases OD, CDW, wax titre and content had decreased, indicating cells had entered death phase, and hence this was not included. Wax titre and content per cell in 10:1 samples was very low across all time points, with a maximum titre of 8 ± 2 mg/L after 24 hours, or content of 1.1 ± 0.4 % CDW after 18 hours, both of which decreased over time. At 20:1, both content and titre increased across the whole time-course, with a comparable trend to 10:1, but with maxima of 40 ± 10 mg/L and 4 ± 1 % CDW, after 40 and 18 hours respectively.

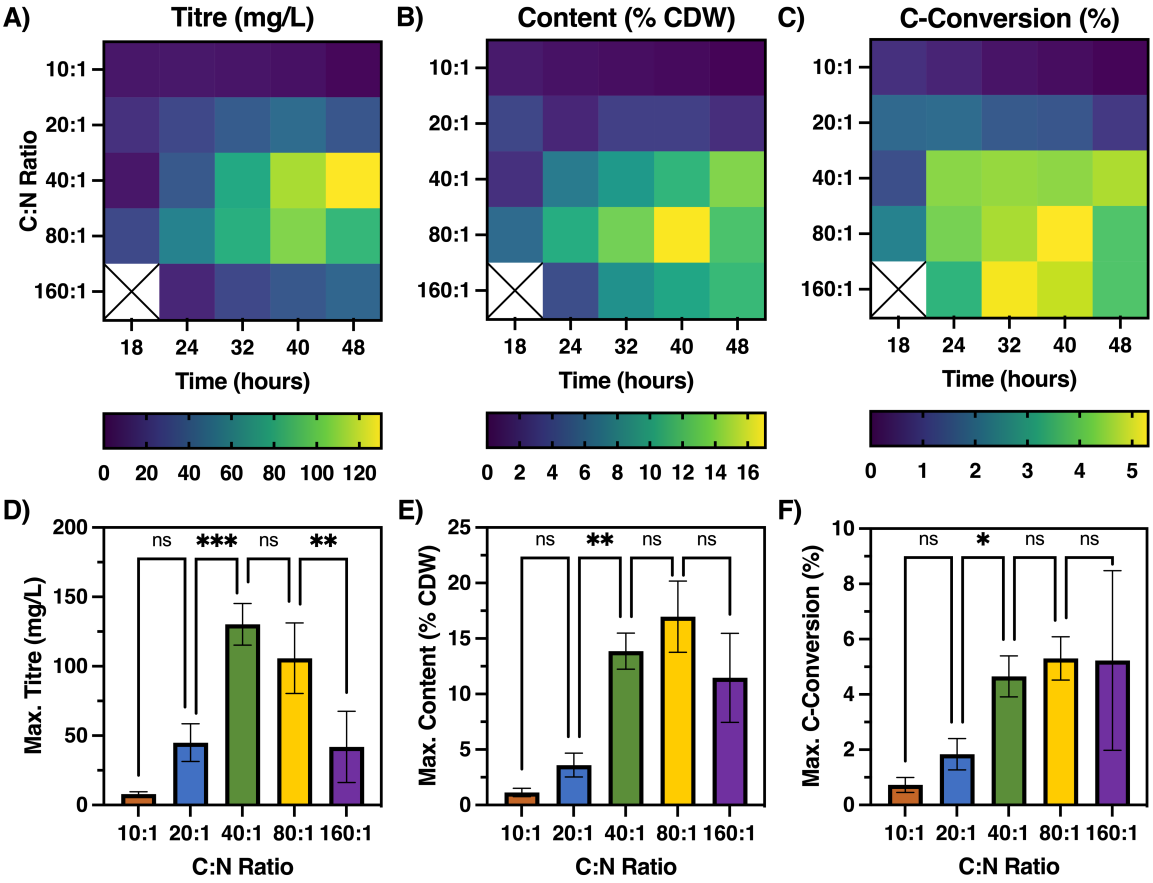


Figure 5.2. Heat maps showing **A)** WE titre (mg/L), **B)** WE content per biomass (% CDW) and **C)** Carbon conversion efficiency from acetate to WEs (%), over time under different C:N ratios from 10:1 to 160:1. Data shows means of 3 biological replications (except for 10:1, where n=2), values from blue (low) to yellow (high). Maximum achieved **D)** WE titre (mg/L), **E)** WE content per biomass (% CDW) and **F)** Carbon conversion efficiency to WEs for each condition. Data shows means and error bars represent standard deviation (n=3 for all but 10:1, where n=2). Pairwise statistical significance testing performed by ANOVA between sequential conditions, ns = >0.05, * = 0.05, ** = 0.01, *** = 0.001, **** = 0.0001.

WE generation was massively increased at 40:1 and 80:1 C:N, and increased with time, reaching maximum titres and contents of 130 ± 10 mg/L and 14 ± 2 % for 40:1 after 48 hours and 110 ± 30 and 17 ± 3% for 80:1 after 40 hours, respectively. By 160:1 WE content reduced slightly and became more variable, at 11 ± 4 %, while titre was hindered by poor growth, with a maximum of 40 ± 30 mg/L. The extreme uncertainty in titre was a consequence of substantial variability in both growth and WE production due to extreme N starvation. Maximum carbon conversion efficiency increased with C:N ratio, most notably between 20:1 and 40:1, to a maximum of 5.3 ± 0.8 % at 80:1 after 40 hours. At 160:1 carbon conversion efficiency remained high; however the maximum was much earlier (24 hours) and showed more variability (5 ± 3 %).

A mixed effects statistical model identified C:N ratio had a significant effect on WE titre ($p < 0.01$), content ($p < 0.001$) and conversion efficiency ($p < 0.05$). By the same analysis, time had a significant impact on titre ($p < 0.01$), content ($p < 0.01$) and conversion efficiency ($p < 0.05$), respectively. This is consistent with 40:1, 80:1 and 160:1 showing increasing titre and content with time, while 10:1 and 20:1 showed little change. Conversely, at 10:1 and 20:1 conversion efficiency decreased with time, while at 40:1 – 160:1 it increased initially, but the effect then varied by condition.

The maximum of each metric was identified for each C:N ratio over the whole time-course. Pairwise comparisons were performed by One-Way ANOVA between the maxima for sequential C:N ratios, as shown in Figures 5.2D, 5.2E and 5.2F. No significant difference in titre or content was detected between 10:1 and 20:1, but at 40:1 both were higher ($p < 0.001$ and $p < 0.01$, respectively). From 40:1 to 80:1 there was again no significant difference in either metric, but titre showed a significant decrease from 80:1 to 160:1 ($p < 0.05$) although the change in content was not significant due to the high variation. For C-conversion efficiency (Figure 5.2F), Welch's ANOVA was performed, which removes the assumption of equal variance between samples given the large relative variation in 160:1. In this case, the overall effect of C:N on C-conversion efficiency was significant ($p < 0.01$), and sequential comparisons showed a significant increase in efficiency between 20:1 and 40:1 ($p < 0.05$).

While the effect of nitrogen limitation on accumulation of WEs is well documented (Luo et al., 2020; Shrestha et al., 2020), there have been relatively few systematic studies to identify the degree of nitrogen limitation which affords optimal results, especially in bacteria. The trade-off between growth limitation and lipid accumulation is crucial, as demonstrated here; if limitation is too extreme (C:N 160:1) then, regardless of the efficiency, poor growth limits

absolute WE concentration. A moderate limitation (in the range of 40:1-80:1) is therefore more effective for high WE titres.

It is often assumed that greater N limitation yields a higher WE content per cell, even if growth is poor. Many two-stage WE production studies, in which cells are initially grown in media replete in nutrients and then starved of nitrogen to induce lipid accumulation, therefore employ minimal nitrogen in the second stage (Manilla-Pérez et al., 2011; Poontawee & Limtong, 2020; Salcedo-Vite et al., 2019). However, maximum WE content per cell was actually achieved in the range of 40:1-160:1 C:N, indicating that this assumption may be false. Furthermore, there was no significant loss in conversion efficiency by employing moderate rather than extreme nitrogen limitation, suggesting more moderate limitations could be more effective across all metrics and for multiple process designs.

As well as indicating the optimal C:N range for further exploration (see section 3.4), these findings are of particular interest for the valorisation of waste streams as industrial bacterial feedstocks. Most waste streams contain significant amounts of nitrogen due to relatively high protein contents (Luo et al., 2020), and concentration typically varies between batches due to changes in composition. While both concentration and variability can be reduced through industrial treatments, strict requirements of very low, or specific, N concentrations are difficult and costly to achieve. The identification of a broad range of values within which production is high and consistent therefore makes utilisation of such feedstocks more feasible.

5.3.2. phosphorus limitation

ADP1 was also grown at a range of C:P molar ratios with excess nitrogen (C:N of 10:1). Based on previous literature (Wu et al., 2010), a range of 16:1 to 8,000:1 was initially explored, with results shown in Figure 5.3. The lowest ratio of these conditions (16:1) represented an excess of phosphorus, while at ratios greater than 320:1 phosphorus starvation occurred, and growth was limited (Figure 5.1B). As in the nitrogen limitation study, excess phosphorus resulted in consistently low WE titres and contents per cell over the entire period, with a maximum of just 11 ± 2 mg/L and 0.9 ± 0.3 % CDW. Ratios of 80:1 and 320:1 both initially showed slight WE accumulation, but at 80:1 WEs were subsequently depleted, while at 320:1 titre continued to increase with time. At 1,200:1, the first condition under which growth was severely impaired, WE titre and content were initially high, reaching a maximum of 39 ± 3 mg/L with a content of 6.4 ± 0.2 % CDW, then decreased rapidly to near zero. Extreme phosphorus limitation demonstrated negative effects on WE accumulation; at 8,000:1 poor growth resulted in very low WE production, with a maximum titre of just 4 ± 1 mg/L.

A mixed effects statistical model identified C:P ratio had significant effects on WE titre, % CDW and conversion efficiency, with $p < 0.0001$ for all. By the same analysis, time had a significant impact on content ($p < 0.001$) and conversion efficiency ($p < 0.01$) but was not a significant factor in titre. This is consistent with all conditions showing higher contents and efficiencies at earlier timepoints, while for titre, 16:1 and 320:1 increased with time while 1,200:1 and 8,000:1 decreased.

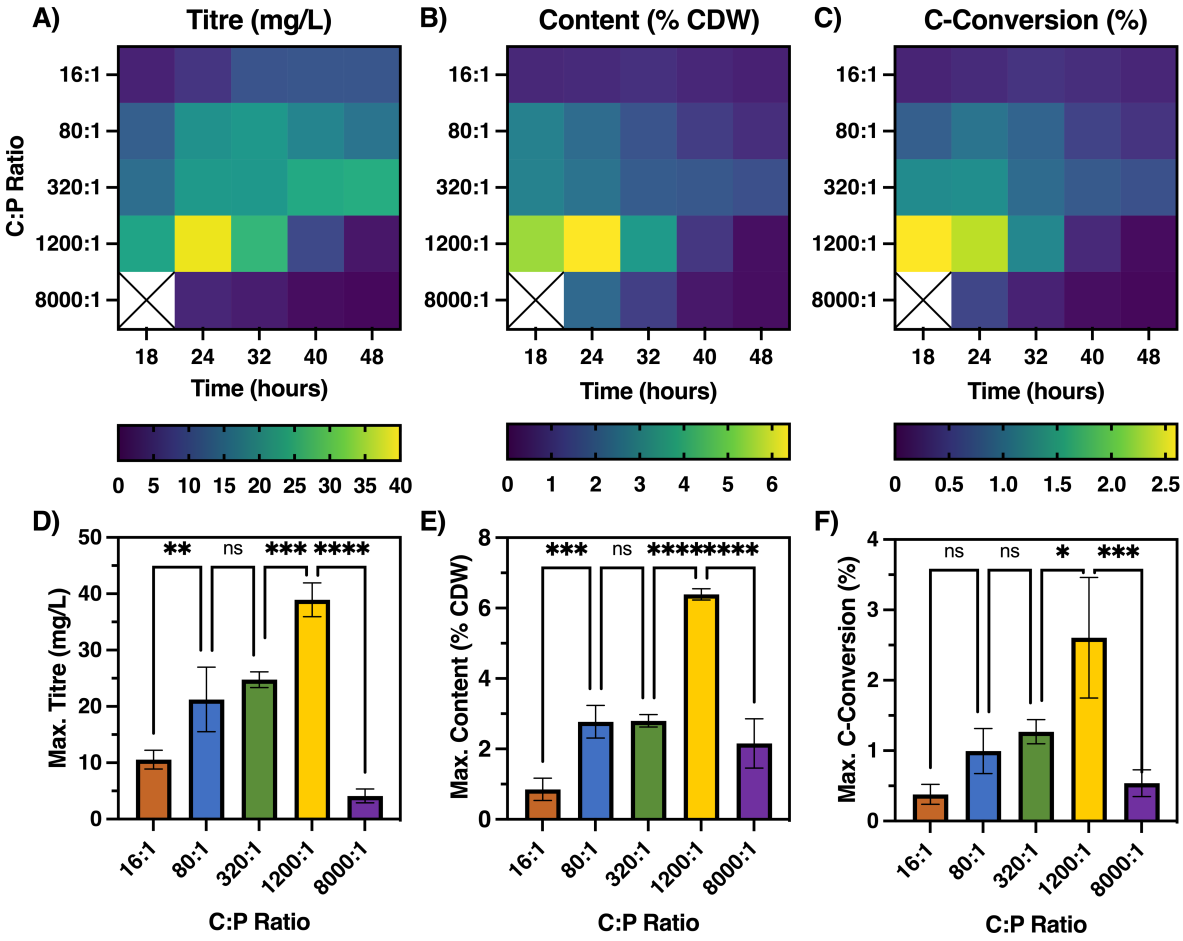


Figure 5.3. Heat maps showing **A)** WE titre (mg/L), **B)** WE content per biomass (% CDW) and **C)** Carbon conversion efficiency from acetate to WEs, over time under different C:P ratios from 16:1 to 8,000:1. Data shows means of three biological replicates, values from blue (low) to yellow (high). Maximum achieved **D)** WE titre (mg/L), **E)** WE content per biomass (% CDW) and **F)** Carbon conversion efficiency to WEs for each condition. Data and error bars represent the means and standard deviations, respectively, of three biological replicates. Pairwise statistical significance testing performed by ANOVA between sequential conditions, ns = >0.05, * = 0.05, ** = 0.01, *** = 0.001, **** = 0.0001.

Pairwise comparison between maximum values of C:P ratios, as shown in Figures 5.3D-F, showed that titre ($p < 0.01$) and wax content ($p < 0.001$) increased significantly from 16:1 to 80:1, although the difference in conversion efficiency was not significant. At 80:1 and 320:1

production was comparable, but, by all metrics, the greatest and most efficient WE production was achieved at 1,200:1. Above this, production decreased significantly due to extreme phosphorus starvation.

Under phosphorus starvation conditions (1,200:1 and 8,000:1), significant TAG accumulation was also detected. Figure 5.4 shows the TAG production relative to the WE production over time, both in titre and content per cell, for all C:P ratios tested, and the maximum total lipid production (TAGs+WEs) compared to just WEs. Up to 320:1, negligible TAGs were detected at all times, while at 1,200:1 a maximum of 59 ± 1 mg/L of TAG was produced after 40 hours, before decreasing at 48 hours as cells entered death phase. This was greater than the maximum WE titre under this condition, and the maximum across all conditions, of 39 ± 3 mg/L, which occurred after 24 hours. At 8,000:1, the maximum TAG titre of 19 ± 5 mg/L was achieved at 40 hours, which accounted for 6 ± 1 % of CDW, almost 5-fold the maximum WE accumulation of 4 ± 1 mg/L at 24 hours, accounting for 2.2 ± 0.7 % CDW.

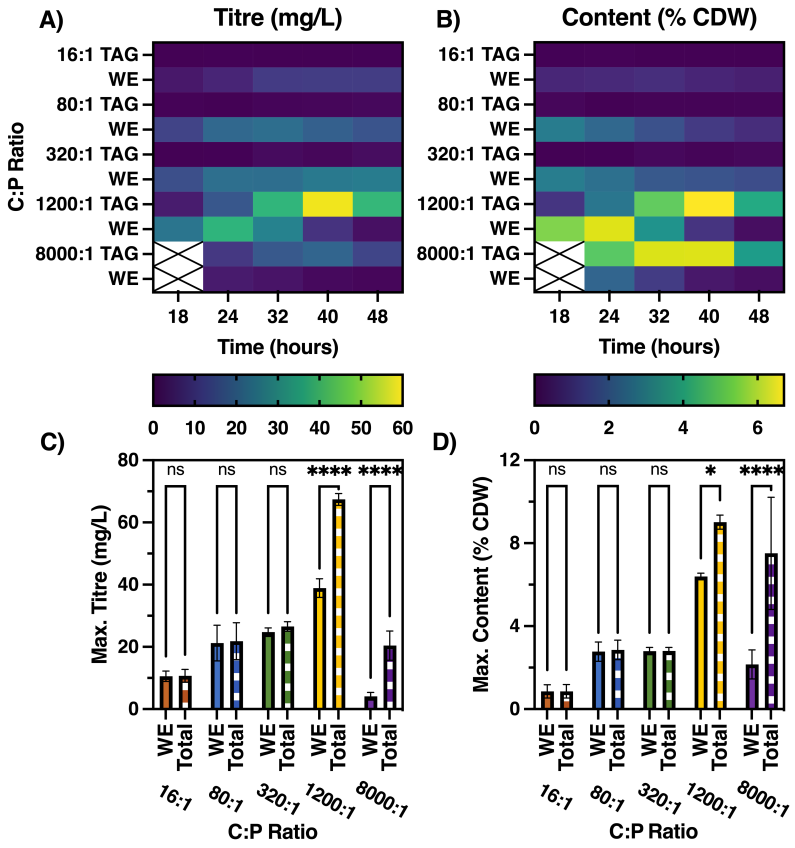


Figure 5.4. Heat maps comparing TAG and WE accumulation over time for different C:P ratios, showing **A)** titre (mg/L) and **B)** content (% CDW). Rows show TAG and WE accumulation for each condition, respectively, on the same colour gradient. Comparisons of the maximum **C)** titre and **D)** content for just WE accumulation compared to total lipid (TAG and WE combined). Pairwise comparison between WE and WE + TAG performed by 2-way ANOVA, ns = >0.05, * = 0.05, ** = 0.01, *** = 0.001, **** = 0.0001.

The transition from accumulation of WEs to TAGs at high C:P ratios is previously unreported in the literature. Work by Salcedo-Vite *et al* has previously shown that ADP1 accumulates different ratios of TAGs:WEs when grown on different carbon sources, though on acetate the authors observed no TAG production (Salcedo-Vite et al., 2019), in agreement with the other conditions tested here.

Cellular responses to phosphorus limitation tend to involve gene expression changes to produce proteins for increased extracellular phosphorus uptake and to release stored intracellular phosphorus. Previous literature has observed the accumulation of lipids, particularly TAGs, due to phosphate starvation in micro-organisms (Wu et al., 2010; Yang et al., 2018a), in agreement with the results observed here. Based on this, TAGs should be considered as a phosphate *source*, metabolites whose synthesis results in the release of phosphate, and thus synthesis of these molecules increases as a response to phosphorus limitation (Gupta & Laxman, 2021). Such molecules do not contain phosphate, but instead have phosphate-containing precursors; TAGs are produced from diacylglycerol phosphates, a class of phospholipids, in a two-step process, the first of which releases phosphate and facilitates conversion of ADP to ATP. Under phosphate rich conditions, glycerol-based phospholipids are used for membranes and cell walls, but under extreme starvation, phosphate must be released for other essential metabolic uses, including ATP generation. TAG production thus allows phosphate release, whilst maintaining efficient carbon storage.

Whilst explaining the increase in TAG production under phosphorus starvation, this does not directly explain the rapid accumulation then depletion of WEs observed at 1,200:1 C:P. Upregulation of fatty acid synthesis is a known response to phosphorus limitation (Huang et al., 2019; Wang et al., 2018), and, with sufficient nutrient concentrations, in ADP1 this accumulation would naturally lead to WE production. However, fatty acid anabolism requires many equivalents of reducing agents NADH and NADPH, both of which have been observed to be broken down under phosphorus depletion (Wang et al., 2018). With severe phosphorus starvation, as observed at 8,000:1 and in later timepoints at 1,200:1, the intracellular concentration of NAD(P)H is expected to decrease, limiting fatty acid production. It is therefore hypothesised that accumulated WEs would be degraded under these conditions to produce fatty acids for a plethora of cellular functions, potentially including TAG synthesis to release further phosphorus. Such a hypothesis could be examined by feeding carbon-labelled WEs to phosphorus-starved ADP1, then testing extracted TAGs for the appropriate labelling. However, such experimentation is beyond the scope of this work.

5.3.3. Micronutrient limitation

The minimal media recipe employed here (Kennerley, 2022) contained significantly lower concentrations of micronutrients than media reported in other WE accumulation studies in ADP1 (Kannisto et al., 2014; Lehtinen et al., 2018). To test whether this influenced WE production, the concentrations of 3 key micronutrients were increased individually, based on the maximum concentrations used in literature recipes (Kannisto et al., 2014). Mg^{2+}, Ca^{2+} and K^{+} ions were chosen specifically due to their known cellular functions, links to lipid production and having the greatest concentration difference between recipes. The exact concentrations of each ion tested are given in Table 5.1. The effect of culturing ADP1 without a trace elements mix was also explored, since in many reports they are not required for high wax production (Kannisto et al., 2014; Luo et al., 2020). Cultures were grown at C:N and C:P ratios of 20:1 and 320:1, respectively.

Table 5.1: Concentrations of Mg^{2+}, Ca^{2+} and K^{+} in the standard media, and with the supplementation of additional mineral ions.

Nutrient	Regular Conc (mM)	+ Conc (mM)
Mg^{2+}	0.95	4.42
Ca^{2+}	0.09	0.21
K^{+}	15	50

Maximal growth was reduced by approximately 10% when TEs were omitted (Figure 5.1C), but the effect on WE production was minimal (Figure 5.5). Conversely, increasing Mg^{2+} and Ca^{2+} concentration, independently, improved maximum growth by around 25%. An increase in WE production was also observed, from a maximum of 43 ± 5 mg/L in TE media to 65 ± 7 mg/L and 70 ± 8 mg/L in media with TEs plus additional Mg^{2+} and Ca^{2+} respectively. Similar trends were observed in WE content, with increased Ca^{2+} concentration resulting in the greatest WE content per cell of 4.8 ± 0.5 % CDW, compared to 2.9 ± 0.3 % CDW with the standard media with TEs. Conversely, increased K^{+} concentration restricted WE production, with a maximum titre of just 23 ± 2 mg/L, despite improving maximum growth by around 10% compared to TE media. Maximum WE content per cell was 3.0 ± 0.2 % CDW, equal to that of the standard media with TEs, but it occurred earlier in time when growth was lower. The combination of increasing all 3 nutrients was also tested to account for any interaction between their effects. In this case, the behaviour observed was very similar to the addition of either Mg^{2+} or Ca^{2+} individually, with improved growth and maximal WE titre and content of 67 ± 5 mg/L and 4.1 ± 0.3 % CDW respectively.

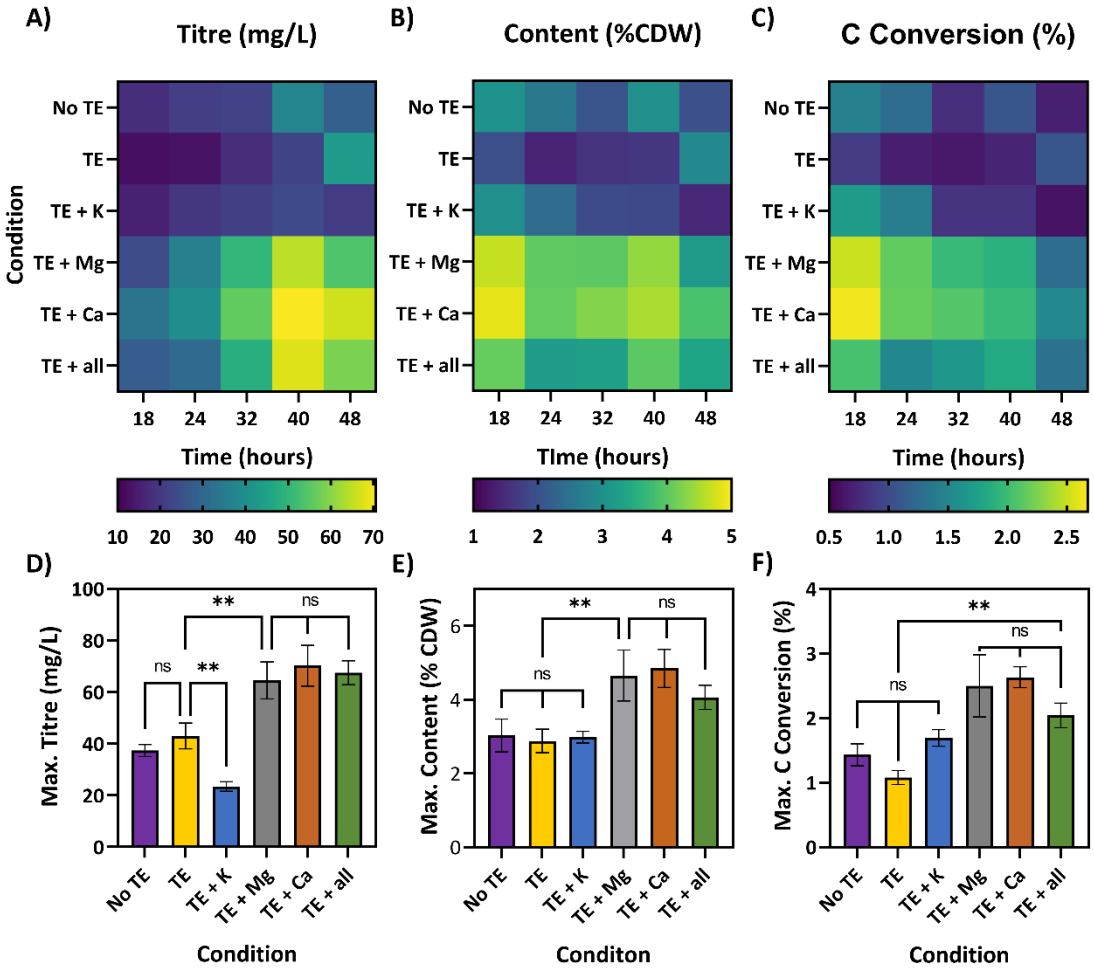


Figure 5.5. Heat maps showing A**)** WE titre (mg/L), **B)** WE content per biomass (% CDW) and C**)** Carbon conversion efficiency from acetate to WEs (%), over time under different micronutrient conditions. Data shows means of three biological replicates, values from blue (low) to yellow (high). Maximum achieved D**)** WE titre (mg/L), **E)** WE content per biomass (% CDW) and **F)** Carbon conversion efficiency to WEs (%) for each condition. Cultures were at C:N ratio of 20:1 and C:P ratio of 320:1 Data and error bars represent the means and standard deviations, respectively, of three biological replicates. Multiple comparison tests were performed between all conditions, ns = >0.05, * = 0.05, ** = 0.01. All p-values given in supplementary information.

A mixed effects statistical model identified time, condition and the interaction of the two to be significant ($p < 0.0001$ for all metrics), with overall trends that titre increased with time for most conditions, while content and C-conversion decreased with time. Pairwise comparison between maxima showed no statistical difference between with or without TEs on any metric. Similarly, there was no pairwise difference between any of TE + Mg^{2+}, TE + Ca^{2+} and TE + all, on any metric. However, WE production under each of these three conditions was significantly greater than both with and without TEs ($p < 0.01$ across all metrics). The maximum WE titre achieved with TE + K^{+} was significantly lower than for TE, but maximum content and conversion were comparable.

The negligible effect of TEs on WE production suggests ADP1 cells may accumulate and store sufficient TEs for multiple generations. Several studies have utilised media without TEs for WE production in ADP1 (Kannisto et al., 2014; Luo et al., 2020), including the highest reported WE titre from the wild type bacteria (Luo et al., 2020), supporting these findings. To observe the true effect of TE limitation, sequential culturing in the absence of TEs may be needed prior to growth and production experiments. However, this was beyond the scope of this study. The growth improvement in the presence of TEs may, however, be beneficial under growth-limiting conditions, such as nutrient limitations.

Increasing the concentration of Ca^{2+} has previously been shown to increase growth rate and maximum OD in other species of *Acinetobacter* (Chen et al., 2019), in agreement with the observed growth behaviour for the TE + Ca^{2+} and TE + all samples. Similarly, both Ca^{2+} and Mg^{2+} deficiency have been shown to limit maximum growth in other bacteria (Vincent, 1962). However, in all cases in which Ca^{2+}, Mg^{2+} or both were increased in concentration, the WE content per cell also increased, indicating an additional effect beyond just growth improvement, and suggesting the benefits to lipid production previously observed in microalgae may translate to bacteria (Huang et al., 2014; Singh et al., 2016). Conversely though, in this work increasing K^{+} concentration did not yield equivalent lipid-accumulation benefits as previously reported in other micro-organisms (Janchot et al., 2019).

The benefits of additional Ca^{2+} and Mg^{2+} were similar in magnitude and were not compounding, suggesting the improved growth and lipid production achieved at higher nutrient concentrations originated from a metabolic function which can be fulfilled by either ion. Previously, it was observed that *E. coli* mutants with a higher minimum Mg^{2+} requirement could utilise Ca^{2+} ions as a substitute, and, when Mg^{2+} was replaced by Ca^{2+}, the necessary concentration was much lower (Lusk et al., 1968). Although the same was not observed for WT *E. coli*, in that additional Ca^{2+} did not affect growth regardless of Mg^{2+} concentration, other bacterial species have been shown to benefit from additional, non-specific divalent ions. Specifically, *Rhizobium trifolii* has been demonstrated to require a minimum total divalent ion concentration which was met by either Ca^{2+} or Mg^{2+}, as well as individual minimum concentrations of both, in order to avoid signs of deficiency, including reduced growth (Vincent, 1962).

Given the results of using additional Mg^{2+}, Ca^{2+} and all 3 ions (Mg^{2+}, Ca^{2+} and K^{+}) were comparable across all metrics, the optimal nutrient was chosen by efficiency, as a combination of quantity and cost. To achieve these effects, an additional 0.9 g/L of $MgSO_4.7H_2O$ was used,

compared to just 18 mg/L of $CaCl_2$. Based on a bulk-cost of approximately $100/tonne for both $CaCl_2$ and $MgSO_4.7H_2O$ (typical price on Alibaba at time of writing), this results in a 50-fold price difference between the required quantities of the two reagents, making additional Ca^{2+} supplementation the obvious choice.

5.3.4. Multifactor experiment

Based upon the findings of the previous 3 experiments, a multi-factor experiment was designed to explore the WE production landscape within a more targeted range of C:N and C:P concentrations, with the aim to investigate possible combined effects of N and P limitation on WE production. A 2-factor, central composite designed experiment was constructed, with parameters for each variable as given in Table 5.2.

A C:N range of 40:1 – 80:1 was chosen, which encompassed the maxima in both WE content and titre. For C:P, 1,200:1 was shown to yield the greatest WE titre and content, but this occurred at early time points and subsequently depleted over time. Conversely, at 320:1, WEs accumulated over time, suggesting the optimal C:P ratio for WE accumulation lies between these two ratios. Furthermore, WE accumulation behaviour at 320:1 was more similar, and therefore perhaps would be more complimentary, to that due to N limitation. Since N limitation also showed a greater relative and absolute effect on WE production, a C:P range of 450:1 – 1,350:1 was selected, with an aim of maximising WE production and the possible interaction between N and P limitation. TEs were included in the media based on their benefit to growth and the maximum concentration of Ca^{2+} was also included for the growth and WE production benefits observed.

Table 5.2: The parameter space of conditions explored in the multifactor C:N and C:P experiment.

Condition	N Level	P Level	C:N Ratio	C:P Ratio	N:P ratio	Number of replicates
1	-1	-1	40:1	450:1	11.25:1	2
2	-1	1	40:1	1,350:1	33.75:1	2
3	1	-1	80:1	450:1	5.63:1	2
4	1	1	80:1	1,350:1	16.88:1	2
5	$-\sqrt{2}$	0	32:1	900:1	28.13:1	2
6	$\sqrt{2}$	0	88:1	900:1	10.23:1	2
7	0	$-\sqrt{2}$	60:1	264:1	4.39:1	2
8	0	$\sqrt{2}$	60:1	1,536:1	25.61:1	2
9	0	0	60:1	900:1	15.00:1	4

5.3.4.1. Wax ester production

Figure 5.6A and B show surface response plots for WE titre and content, while Table 5.3 contains statistical information about the fits and significances calculated. Local maxima in titre were achieved at the minimum and maximum N:P ratios, which occur at the maximum level of one nutrient and the minimum level of the other, while, where both nutrients were present at the same level (either 40:1, 450:1 or 80:1, 1,350:1), titre decreased to a local minimum. Across the whole range, a minimum and maximum titre of 110 ± 10 mg/L and 150 ± 30 mg/L were observed, respectively. WE content varied from 7.8 ± 0.8% to 17 ± 3%, with higher C:N ratios consistently resulting in higher WE content over the range analysed. In both titre and content, the absolute maximum was achieved at the higher C:N ratio (80:1) and lower C:P ratio (450:1), which corresponds to the lowest N:P ratio.

Student's t-test performed on the coded coefficients determined that the interaction coefficient of C:N and C:P, equivalent to N:P, and the C:N squared term were significant for fitting the contour plots of both the WE titre and content ($p < 0.01$ for all; see Table 5.3), and that the C:N ratio coefficient was highly significant in determining WE content ($p < 0.0001$). This interaction can clearly be seen in the contour plots (Figure 5.6A and B). For titre, the plot has a distinct saddle shape; when one nutrient is fixed at the lower level, increasing the ratio of the other nutrient increased WE titre, yet when either nutrient was fixed at the higher level, increasing the other nutrient decreased WE titre. For WE content, at all C:P ratios, a higher C:N ratio resulted in higher WE content per cell. However, at C:P of 450:1 this effect was much more pronounced than at 1,350:1. This result led to behaviour whereby at C:N of 40:1, increasing C:P ratio increased WE content per cell, yet at C:N 60:1 there was a negligible effect and by C:N 80:1 this was reversed and a lower C:P ratio (more P) was favourable for higher WE content. Statistical analysis of the data by ANOVA confirmed the effect of C:N ratio on content was highly significant ($p = 3.53 \times 10^{-7}$). C:N squared was also significant on both titre and content, and the interaction of C:N x C:P significantly affected content ($p < 0.05$ for all).

The observed significance of C:N ratio on WE content per cell is in agreement with previous studies for other lipid accumulating species (Castro et al., 2018), though this is the first time such a comprehensive study has been performed for ADP1. Similarly, no study has previously explored the relationship between C:N and C:P ratio in bacteria. This relationship was found to be significant on WE content over the ranges sampled here, and a significant factor in the regression model of both content and titre.

The maximum WE titre and content recorded in this experiment were 150 ± 30 mg/L and 17 ± 3 % CDW respectively and were achieved simultaneously. These are just short of the literature maxima from acetate of 184 mg/L and 19 % CDW (Santala et al., 2018), achieved by a genetically engineered strain of ADP1. In that instance, an additional 5 g/L of arabinose inducer was required, which has previously been highlighted as inefficient and economically infeasible (Martin et al., 2021). This highlights the significance and potential of nutrient limitation as a factor in bacterial lipid production.

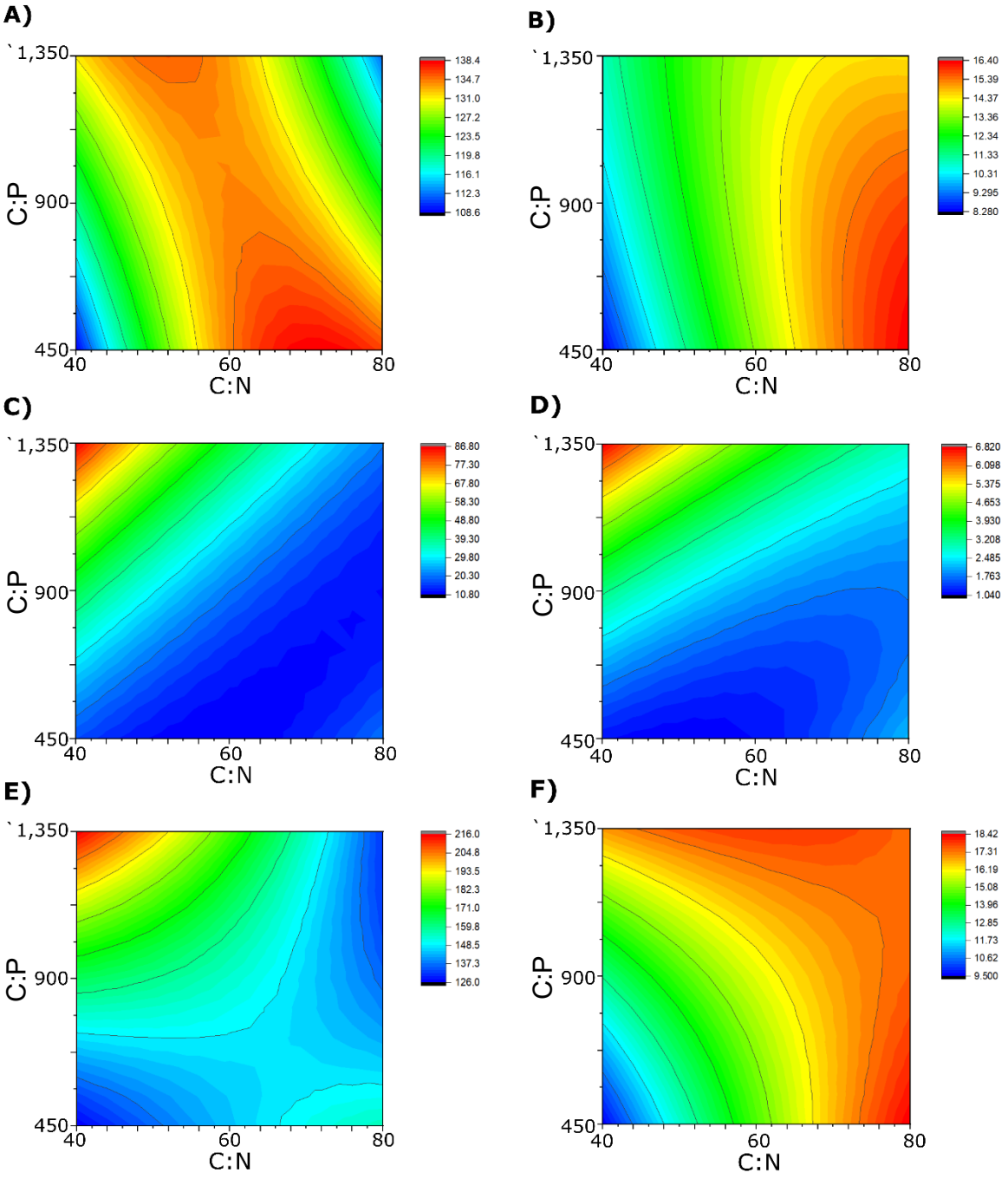


Figure 5.6. Contour plots showing the maximum titre and content of the different lipids detected when C:N and C:P were co-varied. **A)** titre and **B)** content per cell of WEs; maximum **C)** titre and D) content per cell of TAGs and the maximum **E)** titre and **F)** content per cell of combined lipids (WEs+TAGs).

Table 5.3: Key statistical values determined for each linear factor, quadratic factor and the interaction factor from full quadratic models fitted to the multivariable experiment data for WE production.

Titre	**Coefficient Value [LCI, UCI]**	**P value (t-statistic)**	**DF (x,y)**	**F Value**	**P value (F-statistic)**
C:N	2.10 [-6.49, 10.7]	0.519	(1,14)	0.27	0.608
C:P	-0.46 [-9.05, 8.12]	0.886	(1,14)	0.01	0.910
C:N x C:N	-11.9 [-23.2, -0.52]	0.003 **	(1,14)	6.02	0.028 *
C:P x C:P	-0.33 [-11.7, 11.0]	0.919	(1,14)	0.00	0.952
C:N x C:P	-11.7 [-23.8, 0.45]	0.003 **	(1,14)	4.26	0.058
Content	**Coefficient Value [LCI, UCI]**	**P value (t-statistic)**	**DF (x,y)**	**F Value**	**P value (F-statistic)**
C:N	2.92 [2.22, 3.61]	6.82×10^{-7} ****	(1,14)	80.5	3.53×10^{-7} ****
C:P	0.19 [-0.51, 0.89]	0.525	(1,14)	0.34	0.568
C:N x C:N	-1.07 [-1.99, -0.15]	0.004 **	(1,14)	5.65	0.032 *
C:P x C:P	-0.34 [-1.26, 0.58]	0.263	(1,14)	0.63	0.441
C:N x C:P	-1.13 [-2.11, -0.15]	0.002 **	(1,14)	6.09	0.027 *

Asterisks indicate significance at levels as follows: * = 5%, ** = 1%, *** = 0.1% and **** = 0.01%.

5.3.4.2. Triacylglycerol production

Since significant TAG production was detected in the C:P ratio experiment, neutral lipid extracts from the multivariable experiment were also analysed for the presence of TAGs. TAG production was observed under all conditions tested. The trend of increasing TAG with increasing C:P ratio was strong at C:N 40:1, although by C:N 80:1 the change was negligible (Figure 5.6C and 5.6D).

The optimal condition for TAG production was found to be at the maximum N:P ratio (the minimum C:N of 40:1 and maximum C:P of 1,350:1), where the titre was 91.6 ± 0.6 mg/L and content 7.29 ± 0.09 % CDW. Under this same condition the WE titre was 120 ± 10 mg/L and content was 10 ± 1 % CDW, resulting in a WE:TAG mass ratio of 1.3:1. The minimum TAG accumulation also occurred at C:N 40:1, but with C:P of 450:1, under which the TAG titre was

15.0 ± 0.3 mg/L and content was 1.09 ± 0.03 %. This coincided with the minimum WE accumulation with a titre of 110 ± 10 mg/L and content of 7.8 ± 0.8 % CDW, with a WE:TAG mass ratio of 7:1. When analysed statistically (see Table 5.4), all factors were highly significant on titre ($p < 0.001$ for both statistics), with the exception of C:N squared, the significance of which was found by ANOVA to be much smaller ($p < 0.05$). The effects on content were similar, with all factors significant ($p < 0.01$) except C:N squared ($p = 0.248$).

Table 5.4: Key statistical values determined for each linear factor, quadratic factor and the interaction factor from full quadratic models fitted to the multivariable experiment data for TAG production.

Titre	**Coefficient Value [95% LCI, 95% UCI]**	**P value (t-statistic)**	**DF (x,y)**	**F Value**	**P value (F-statistic)**
C:N	-15.8 [-18.9, -12.7]	1.22×10^{-8} ****	(1,14)	119	3.23×10^{-8} ****
C:P	17.5 [14.4, 20.6]	4.15×10^{-9} ****	(1,14)	146	8.72×10^{-9} ****
C:N x C:N	7.78 [3.66, 11.9]	1.48×10^{-5} ****	(1,14)	6.20	0.026 *
C:P x C:P	8.08 [3.96, 12.2]	$1.05\ 10^{-5}$ ****	(1,14)	17.7	8.83×10^{-4} ***
C:N x C:P	-17.3 [-21.7, -12.9]	4.86×10^{-9} ****	(1,14)	70.6	7.68×10^{-7} ***
Content	**Coefficient Value [95% LCI, 95% UCI]**	**P value (t-statistic)**	**DF (x,y)**	**F Value**	**P value (F-statistic)**
C:N	-0.75 [-1.03, -0.46]	1.56×10^{-5} ****	(1,14)	30.6	7.37×10^{-5} ****
C:P	1.58 [1.29, 1.87]	8.30×10^{-9} ****	(1,14)	138	1.24×10^{-8} ****
C:N x C:N	0.51 [0.13, 0.89]	4.11×10^{-4} ***	(1,14)	1.46	0.248
C:P x C:P	0.74 [0.35, 1.12]	$1.74\ 10^{-5}$ ****	(1,14)	17.1	0.001 **
C:N x C:P	-1.23 [-1.64, -0.83]	1.10×10^{-7} ****	(1,14)	42.0	1.45×10^{-5} ***

Asterisks indicate significance at levels as follows: * = 5%, ** = 1%, *** = 0.1% and **** = 0.01%.

At C:P of 320:1 in the single-variable experiment, with a C:N of 10:1, the WE:TAG mass ratio was 14:1 at maximum lipid accumulation, and including TAGs did not significantly alter the maximum achieved titre or content compared to WEs alone (see Figure 5.4C and 5.4D). In this experiment, a C:P ratio of 450:1 resulted in WE:TAG mass ratios of 7:1 and 9:1 at C:N of 40:1 and 80:1, respectively, representing a 2-fold change in the relative abundances of the different lipid classes. WE and combined lipid accumulation increased approximately 4 and 6-fold at the two C:N ratios, respectively. This further supports the evidence that phosphorus starvation results in a greater TAG production and TAGs representing a greater proportion of the cellular storage lipids.

Crucially, as no TAGs were detected in the C:N single-variable experiment, despite nitrogen starvation and lipid accumulation, the conclusion that TAG production is induced by phosphorus starvation is still supported, and the increasing TAG accumulation with increasing phosphorus limitation at C:N 40:1 strengthens this conclusion. The interaction of the C:N and C:P is highlighted by the fact that both the minimum and maximum TAG accumulation were achieved at 40:1 C:N, yet at 80:1 C:N the maximum TAG titre was minimal and did not change significantly with C:P ratio.

5.3.4.3. Combined lipid production

Conditions under which maximum combined lipid production was achieved were also modeled (Figure 5.6E and 5.6F). For titre, the distribution of minima and maxima remained largely the same as that for WEs, which made up between 56 % and 89 % of lipids for all samples. However, when both WEs and TAGs were considered, the overall maximum moved from the minimum N:P ratio to the maximum N:P ratio, with a maximum combined lipids of 210 ± 10 mg/L.

For combined lipid content, the plot resembled that for WEs, with the minimum and maximum still occurring at the same points, and a maximum content of 19 ± 3 % CDW. However, the effect of C:P ratio on content was more pronounced at low compared to high C:N ratios, while for the WE plot the effect was more prominent at high C:N ratios. The lowest N:P ratio was preserved as the optimal WE accumulation condition, resulting in both the greatest quantity and purity of WEs, due to the highest WE:TAG mass ratio of 9:1. Statistically, combined lipid titre and content were not significantly affected by C:N squared or C:P squared, but were affected by C:N, C:P and N:P ratio, as determined by both ANOVA and t-test of the coefficient effect on the model ($p < 0.01$ for all, see Table 5.5).

Table 5.4: Key statistical values determined for each linear factor, quadratic factor and the interaction factor from full quadratic models fitted to the multivariable experiment data for combined lipid production (WEs+TAGs).

Titre	**Coefficient Value [95% LCI, 95% UCI]**	**P value (t-statistic)**	**DF (x,y)**	**F Value**	**P value (F-statistic)**
C:N	-13.9 [-22.2, -5.68]	2.47×10^{-5} ****	(1,14)	13.1	1.43×10^{-5} ****
C:P	16.45 [8.20, 24.7]	5.35×10^{-4} ***	(1,14)	18.3	4.90×10^{-4} ***
C:N x C:N	-3.50 [-14.4, 7.42]	0.068	(1,14)	2.02	0.069
C:P x C:P	7.08 [-3.83, 18.0]	0.270	(1,14)	1.94	0.426
C:N x C:P	-28.4 [-40.0, -16.7]	5.22×10^{-5} ****	(1,14)	27.2	8.56×10^{-4} ***
Content	**Coefficient Value [95% LCI, 95% UCI]**	**P value (t-statistic)**	**DF (x,y)**	**F Value**	**P value (F-statistic)**
C:N	2.32 [1.55, 3.08]	0.002 **	(1,14)	42.1	0.003 **
C:P	1.61 [0.85, 2.38]	5.78×10^{-4} ***	(1,14)	20.3	7.68×10^{-4} ***
C:N x C:N	-0.68 [-1.69, 0.34]	0.332	(1,14)	3.89	0.177
C:P x C:P	0.39 [-0.63, 1.40]	0.064	(1,14)	0.67	0.186
C:N x C:P	-2.13 [-3.21, -1.05]	5.00×10^{-6} ****	(1,14)	17.8	1.32×10^{-4} ***

Asterisks indicate significance at levels as follows: * = 5%, ** = 1%, *** = 0.1% and **** = 0.01%.

5.4. Conclusion

In this study the significance of nutrient limitation on controlling and improving WE accumulation in *A. baylyi* ADP1 was investigated. By controlling carbon: nitrogen ratio, a 16-fold increase in WE titre and a 15-fold increase in WE content per cell were achieved. Furthermore, more extreme nitrogen starvation did not always result in greater WE accumulation, highlighting the importance of such investigations in optimising future production processes.

For the first time, the effect of carbon: phosphorus ratio on WE accumulation was demonstrated, with a maximum 4-fold increase in titre and 7-fold increase in content. The preferred storage lipid class changed from WEs to TAGs under extreme starvation, a previously unreported phenomenon. Divalent cations Mg^{2+} and Ca^{2+} were also shown to contribute to lipid production, with a greater than 50% increase in both WE titre and content when these were supplemented at higher concentrations.

By a multivariable analysis within an optimal subset of carbon: nitrogen and carbon: phosphorus ratios, it was demonstrated that, as well as nitrogen having a significant effect on WE content, the interaction of the two variables was also significant in WE accumulation. Nitrogen: phosphorus ratio was also observed to affect TAG accumulation, which occurred at more moderate carbon: phosphorus ratios under lipid accumulating conditions (moderate-high C:N ratios). These findings highlight the importance of condition exploration in attempts to optimise the production of WEs and other biochemicals, and guide future decisions on bioreactor conditions and optimisation.

5.5. Acknowledgements

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5.7. Supplementary information

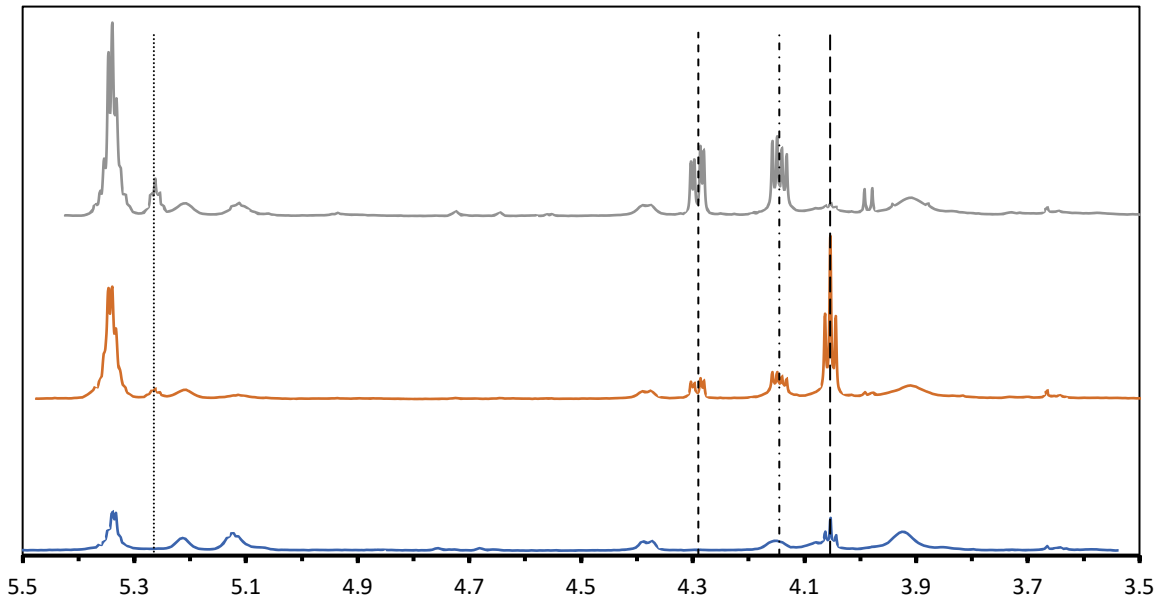


Figure 5.S1: Raw NMR spectrum data in the range δ 3.5-5.5 ppm. Three spectra are shown to exemplify the different features of spectra for samples with minimal lipid production (C:P 16:1 after 18 hours, blue), with predominantly wax accumulation (C:P 1,200:1 after 24 hours, orange) and predominantly TAG accumulation (C:P 1,200:1 after 48 hours, grey). Vertical lines added mark characteristic peaks of TAGs at δ 5.27 (dot), 4.29 (dash) and 4.15 (dot-dash) and of WEs at δ 4.05 (long dash). Spectra are deliberately offset and are unscaled.

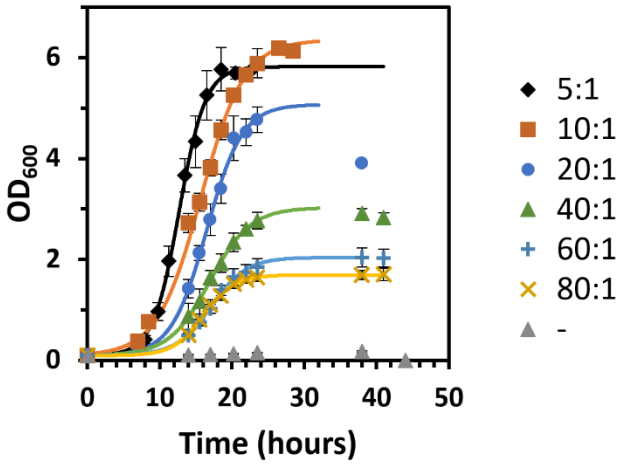


Figure 5.S2: Preliminary growth curves for ADP1 grown on 150 mM acetate at small scale (20 mL cultures in 250 mL flasks). Sigmoidal growth curves were fitted to data using OriginPro 2022b. Data points and error bars represent the means and standard deviations, respectively, of three biological replicates.

Table 5.S1: Accurate C:N and C:P molar ratios for single and multivariable experiments. Acetate concentration measured by GC-FID. N concentration measured by Hach LCK 303 Ammonium cuvette test. P concentration measured by Hach LCK 350 Phosphate (ortho/total) cuvette test for C:P 16:1, 80:1 and 320:1 and multivariable conditions 1,3,7 and LCK 349 Phosphate (ortho/total) cuvette test for C:P 1,200:1 and 8,000:1 and multivariable conditions 2,4,5,6,8,9.

	Theoretical C:N	**1**	**2**	**3**	**4**	**Average**	**S.D.**
	10:1	8.99	9.67	9.79	-	9.5	0.4
	20:1	24.5	18.7	19.2	-	21	3
	40:1	41.9	40.7	36.8	-	40	3
	80:1	82.5	90.6	87.0	-	87	4
	160:1	138	151	132	-	140	10
	Theoretical C:P	**1**	**2**	**3**	**4**	**Average**	**S.D.**
	16	16.5	16.9	15.4	-	16.3	0.8
	80	90.0	83.3	85.5	-	86	3
	320	332	324	309	-	320	10
	1200	1234	1260	1237	-	1240	10
	8000	7575	8743	7372	-	7900	700
Condition	**Theoretical C:N**	**1**	**2**	**3**	**4**	**Average**	**S.D.**
1	40	39.4	38.8	-	-	39.1	0.5
2	40	41.8	36.3	-	-	39	4
3	80	88.6	86.3	-	-	87	2
4	80	84.7	75.4	-	-	80	7
5	32	29.4	36.9	-	-	33	5
6	88	90.9	87.3	-	-	89	3
7	60	54.6	57.8	-	-	56	2
8	60	55.5	55.8	-	-	55.6	0.2
9	60	63.4	60.8	60.1	64.8	62	2
Condition	**Theoretical C:P**	**1**	**2**	**3**	**4**	**Average**	**S.D.**
1	450	419	443	-	-	430	20
2	1350	1427	1318	-	-	1370	80
3	450	464	478	-	-	470	10
4	1350	1410	1214	-	-	1300	100
5	900	899	964	-	-	930	50
6	900	883	948	-	-	920	50
7	264	202	229	-	-	220	20
8	1536	1503	1579	-	-	1540	50
9	900	938	912	930	893	920	20

Table 5.S2: Pairwise significance tests conducted on maximum WE titres, contents and C-Conversion efficiencies of different conditions for micronutrients study.

Titres					
P-values	**TE**	**TE + Mg^{2+}**	**TE + Ca^{2+}**	**TE + K^{+}**	**TE + all**
No TE	0.7517	0.0006	0.0001	0.0833	0.0002
TE		0.0052	0.0007	0.0086	0.0015
TE + Mg^{2+}			0.7946	<0.0001	0.9646
TE + Ca^{2+}				<0.0001	0.9959
TE + K^{+}					<0.0001
TE + all					
Content					
P-values	**TE**	**TE + Mg^{2+}**	**TE + Ca^{2+}**	**TE + K^{+}**	**TE + all**
No TE	0.9982	0.0072	0.0030	>0.999	0.1107
TE		0.0037	0.0016	0.9997	0.0569
TE + Mg^{2+}			0.9934	0.0059	0.5929
TE + Ca^{2+}				0.0025	0.3162
TE + K^{+}					0.0909
TE + all					
C-Conversion Efficiency					
P-values	**TE**	**TE + Mg^{2+}**	**TE + Ca^{2+}**	**TE + K^{+}**	**TE + all**
No TE	0.5072	0.7662	0.0017	0.0006	0.0774
TE		0.0752	0.0001	<0.0001	0.0039
TE + Mg^{2+}			0.0147	0.0048	0.5172
TE + Ca^{2+}				0.9815	0.2591
TE + K^{+}					0.0917
TE + all					

Statement of Authorship for joint/multi-authored papers for PGR thesis

To appear at the end of each thesis chapter submitted as an article/paper

The statement shall describe the candidate's and co-authors' independent research contributions in the thesis publications. For each publication there should exist a complete statement that is to be filled out and signed by the candidate and supervisor **(only required where there isn't already a statement of contribution within the paper itself).**

Title of Paper	The effect of nutrient limitation on bacterial wax ester production
Publication Status	☒Published □ Accepted for Publication □Submitted for Publication □Unpublished and unsubmitted work written in a manuscript style
Publication Details	Martin, Laura & Huang, Wei & Thompson, Ian. (2023). The effect of nutrient limitation on bacterial wax ester production. Bioresource Technology Reports. 22. 101423. 10.1016/j.biteb.2023.101423.

Student Confirmation

Student Name:	Laura Martin		
Contribution to the Paper	Planned and conducted experiments, analysed the results and wrote the paper.		
Signature		Date	05.12.2023

Supervisor Confirmation

By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

Supervisor name and title: Professor Ian Thompson		
Supervisor comments I agree with this summary		
Signature 	Date	08.12.2023

This completed form should be included in the thesis, at the end of the relevant chapter.

Chapter 6 – Manipulation of carbon: nitrogen ratio of acidogenic fermentate for improved wax ester production

6.1. Introduction

Whilst the C:N ratio has been identified as a significant parameter in lipid production in numerous bacterial strains, including *A. baylyi* ADP1, it poses a challenge for bioproduction from waste streams and raw materials (196). Unlike laboratory media, which can be prepared to desired nutritional requirements, waste streams have an intrinsic carbon and nitrogen content due to their origin, and hence their C:N ratio is fixed. Since the ideal production ratios have high carbon and low nitrogen concentrations (197), there are two options to increase this ratio: include an additional source of carbon or remove the nitrogen from the waste stream.

Including an additional carbon source is challenging, as many waste streams are relatively rich in nitrogen. A small number of sources, such as waste cooking oil, wastepaper and forestry byproducts (142, 198, 199), are carbon dense with low nitrogen contents. However, these waste streams make up a limited amount of total organic waste (200) and are often in very high demand (201). Furthermore, this approach sometimes leads to the employment of sugars or other pure substrates to provide the additional carbon (202-204), undermining many of the benefits of valorising waste streams.

The alternative is to remove nitrogen, most of which is present as ammonium, from the acidogenic fermentate. If that ammonium is removed, the C:N ratio of the fermentate would be significantly increased, providing a mixed waste stream substrate with ideal lipid production conditions (144, 205, 206). In addition, ammonium is a valuable commodity, with global production capacity set to increase by around 25% by 2030 (207), and its recovery is an environmentally important endeavour. Nearly 3 tonnes of CO_2 are emitted per tonne of ammonia produced industrially (208), the vast majority of which is utilised in fertilisers. Isolating ammonium from fermentate reduces reliance on this energy intensive process whilst also providing a more versatile ammonium source than typical bioderived sources of ammonia (209), such as the residual liquid digestate remaining after biogas production. In this way, removing the nitrogen from fermentate allows the separation of a single, mixed waste stream into two higher-value and more desirable products.

Research in our group by fellow DPhil candidate Nicholas Tenci has established that zeolites can be used to remove ammonium ions directly from fermentate. Zeolites are an abundant, low-cost class of alumina silicates with a highly porous structure. They are widely employed in separation and purification processes, as well as wastewater treatment and other forms of bioremediation, because of their high capacity for cation exchange from dilute aqueous media (210, 211). Typical target cations for removal are potentially polluting inorganics, such as ammonium and heavy metals, with ammonium adsorption capacities of over 25 mg/g identified for natural zeolites (212), and higher still achievable through physical and chemical modification (210). Zeolites can also be regenerated efficiently and effectively, allowing their re-use for multiple treatment cycles and improving their economic and environmental credentials (213). Previously, zeolites have also been used to remove ammonium from anaerobic digestion reactors fed with high nitrogen substrates in order to decrease the inhibition of methanogenesis and increase biogas productivity and calorific value (214). However, their application to fermentate for bioproduction is novel.

An alternative method for ammonium removal and recovery is degassing. If the pH of the fermentate is high, the majority of ammonium ions will become dissolved ammonia gas due to the acid-base equilibrium. Under the right conditions this gas can be removed from the fermentate directly, a highly efficient process with minimal solvent requirements or waste generation that can generate a number of high value products (209). Again, many studies have investigated degassing in anaerobic digesters *in-situ* to increase methane yields (209, 215-217), with promising results. This method therefore also holds potential as an effective way to remove ammonium from fermentate prior to its utilisation in bioproduction.

6.2. Aims and objectives

The aim of the work in this chapter was to validate whether methods of removing nitrogen from fermentate were compatible with microbial lipid production, specifically the production of WEs by *A. baylyi* ADP1.

This work was broken down into the following objectives:

- To identify the required treatment time and zeolite loading for zeolite treatment of synthetic minimal media and fermentate to achieve C:N ratios of approximately 40:1 – 80:1, which was identified as the optimal range in Chapter 5.
- To test the effect of zeolite treatment of minimal media on the growth and WE production of ADP1. Since the ion exchange method is non-selective, it was unclear how the potential removal of other cations would affect the bacterial growth. Similarly, the exact composition and the effect of ions released by the zeolite during the exchange process was also unknown. Treatment of different media compositions was tested to help isolate any potential negative effects or problems caused by these unknown parameters.
- To investigate the effect of zeolite treatment of fermentate on growth and WE production in *A. baylyi* ADP1, once any problems identified by the synthetic media tests had been rectified.
- To adapt a literature-derived method of degassing fermentate to remove ammonia gas using the equipment available, and to validate the protocol and parameters were suitable to achieve the desired results with our modified set up.
- To investigate the effect of degassing fermentate on the growth and WE production in *A. baylyi* ADP1.

6.3. Materials and methods

6.3.1. Materials

Zeolite was purchased from Finest Filters (Liverpool, UK), and was characterised by semi-quantitative X-Ray diffraction to be majority clinoptilolite. Chemicals for synthetic minimal media (KH_2PO_4, Na_2HPO_4, $MgSO_4.7H_2O$ and $CaCl_2.2H_2O$) were purchased from Sigma. Spectrophotometric test kits for total organic carbon, total nitrogen and ammonium nitrogen were obtained from Hach. Fermentate was produced by the anaerobic fermentation of *Opuntia* as detailed in Chapter 4, but with fermentations prepared with substrate and inoculum concentrations of 30 g/L and 5 g/L volatile solids, respectively, and incubated for 12 days.

6.3.2. Zeolite methods

6.3.2.1. Preparation

The batch was graded by particle size, and particles between 2-4 mm in diameter were used. After grading, zeolite was washed with excess deionised water, then soaked in excess 1 M sodium chloride overnight to exchange any currently bound cations for sodium ions. The zeolite was then washed with 3 times in excess deionised water to remove any unbound sodium chloride, then dried at 80 °C overnight to a constant mass.

6.3.2.2. Treatment of synthetic media

Synthetic media was prepared as detailed in Chapter 3, with 150 mM of sodium acetate as the sole carbon source and ammonium chloride as the sole nitrogen source in a C:N ratio of 10:1. Treatment was performed in 1L conical flasks incubated at 30 °C and shaking at 200 rpm. 500 mL volume of media was treated per flask with 30 wt% of zeolite for 2 hours, resulting in a final C:N ratio of 200:1. This was readjusted to approximately 40:1 by the addition of concentrated ammonium chloride (100 g/L) solution. As detailed, three alternative media treatments were tested: 1) treatment of the phosphate buffer with ammonium chloride, prior to the addition of carbon source or trace elements, 2) treatment of the complete media (including carbon source and trace elements) with no further additions and 3) treatment of the complete media, followed by the re-addition of trace elements.

6.3.2.3. Treatment of fermentate

Zeolite treatment for fermentate was performed under identical conditions to the synthetic media. Treatment was performed for 3 hours and resulted in a C:N ratio of 55:1. This ratio was considered sufficiently within the ideal C:N range identified in Chapter 5, and so was not adjusted further.

6.3.3. Degassing of fermentate

Based on a protocol from a previous PhD thesis (218), fermentate was degassed to remove ammonia gas, decreasing the nitrogen content. Initially the pH of digestate was raised to pH 11 by addition of 0.1M sodium hydroxide to push the ammonium – ammonia equilibrium in the direction of ammonia gas. The basic fermentate was then heated to 80 °C in a water bath to decrease the solubility of the gas, and an air pump with flow rate 5.5 L/min was used to bubble air through the fermentate, removing the ammonia. 500 mL of fermentate was degassed in a 2L bottle, ensuring the entire volume remained below the water line in the water bath for the entire time.

6.3.4. Growth experiments

Growth experiments were conducted in 200 mL of culture media in 1L conical flasks and were performed in biological triplicate. In zeolite treated fermentate, dilutions were performed in water to a total VFA concentration of 10 g/L, maintaining the previously determined and controlled C:N ratio. In degassed fermentate, fermentate was diluted to 3 g/L of total organic carbon, corresponding to around 6 g/L of total VFA concentration. 0.5-1 mL samples were taken for OD_{600} measurements by UV-1800 UV/Vis spectrophotometer (Shimadzu, Kyoto, Japan). Samples were diluted with ultrapure water to fall in the range 0.1-0.8.

6.3.5. Analytical methods

The concentration of ammonium ions, total nitrogen and total organic carbon present in fermentate were determined by Hach kits LCK303, LCK138 and LCK386, respectively. Where appropriate, samples were degassed on a TOC-X5, incubated with a HT 200 S heating block and measured on a DR2800 spectrophotometer, all from Hach Lange. VFA quantification was performed via GC-FID, as detailed in Chapter 4. Lipid extraction by an adapted Bligh and Dyer method and wax ester quantification by ^{1}H-NMR were performed as described in Chapter 3.

6.4. Results

6.4.1. Nitrogen removal from synthetic media by zeolite

Initially, the effect of zeolite treatment on bacterial growth was tested using synthetic growth media based on a phosphate buffer. Synthetic medium with 150 mM acetate as the carbon source was initially prepared with around 1.6 g/L of ammonium chloride, representing a 10:1 C:N ratio, equivalent to that in fermentate. A 30 wt% loading was applied for a treatment time of 1 hour. After this, ammonium concentration was determined to have decreased to 26 ± 1 mg/L while the acetate concentration was unchanged, resulting in a C:N ratio of 200:1. This was adjusted to 40:1 by the addition of concentrated ammonium chloride, the original and sole nitrogen source in the media.

After establishing a protocol for media treatment with zeolite, growth and production studies were conducted in the treated media. To separate the different possible effects of the zeolite on growth and WE accumulation, three different treatment conditions were investigated, alongside a positive and negative control (Figure 6.1). Synthetic growth media was prepared accordingly, and final C:N ratios were confirmed experimentally to be between 38-45 for all zeolite treated conditions. In all cases, the 150 mM acetate was added after zeolite treatment (if applicable) and autoclaving:

A. **10:1 C:N media (Negative control):** The standard minimal growth medium, as employed in previous chapters, made with a C:N ratio of 10:1.

B. **40:1 C:N media (Positive control):** The standard minimal growth medium, made with a C:N ratio of 40:1, achieved by decreasing the concentration of ammonium chloride used.

C. **Buffer treatment:** A phosphate buffer made with 2.5 g/L KH_2PO_4, 2.5 g/L Na_2HPO_4, and a C:N ratio of 10:1. Zeolite treatment was performed and ammonium concentration was checked and adjusted as required to a final C:N ratio of approximately 40:1. Additional components required for the standard minimal media, such as the additional metal cations (magnesium, calcium and iron) and trace elements solutions were then added after zeolite treatment. By comparing this treatment to the positive control, the effect of any inhibitory compounds or ions being released by the zeolite during treatment would be demonstrated.

D. **Total treatment:** The standard minimal growth medium was prepared with a C:N ratio of 10:1, as in the negative control. Then, zeolite treatment was performed and

ammonium concentration was checked and adjusted as required to a final C:N ratio of approximately 40:1. Comparison of this result with the buffer treatment and the positive control would identify whether the zeolite removed any essential trace elements that could limit growth or WE production.

E. **Additional trace metals:** The standard minimal growth medium was prepared and treated as above for Total treatment. After treatment, essential growth elements magnesium, calcium, iron and the trace elements mixture were added back into the medium, at the same concentration as they were initially added. If adverse effects of the total treatment were detected but not the buffer treatment, this would confirm if this was caused by the removal of essential cations and establish whether the re-addition of these could reverse such effects.

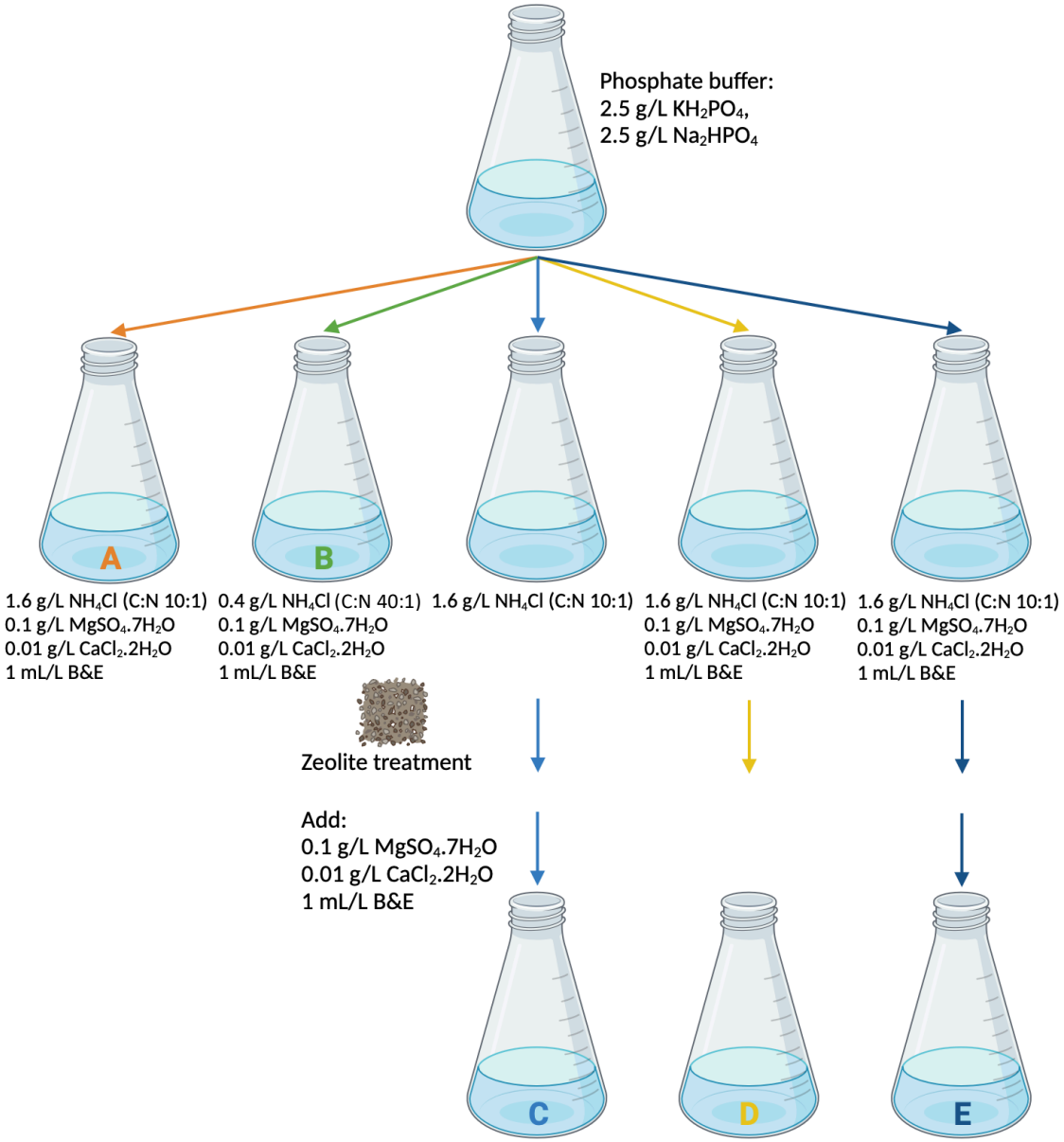


Figure 6.1. Graphic representing the preparation methods for the different media conditions A-E, as described in section 6.4.1: A – negative control, B – positive control, C – buffer treatment, D – total treatment and E – additional trace metals.

6.4.2. Effect of zeolite nitrogen removal on growth and wax production

The growth and WE production of ADP1 in the five conditions outline above was investigated by growth experiments performed over 48 hours. Lag phases in between conditions varied as the data for the negative and positive control conditions was taken from Chapter 5, resulting in growth curves that were temporally shifted. However, the maximum growth in each condition is shown in Figure 6.2A. As previously, C:N ratio of 10:1 gave significantly higher growth than

at 40:1. Between the conditions with C:N of 40:1, no significant difference in growth was detected, with OD_{600} values ranging from 2.5 to 2.92.

WE production was also measured, and the maxima of each metric are shown in Figure 6.2B and C. At C:N 40:1, a maximum of 130 ± 10 mg/L was obtained, with maximum content of 14 ± 2 %, values which proved to be the highest across all conditions. These metrics showed greater variation than the growth, with the lowest titre of 100 ± 20 mg/L in the condition E and minimum content of 8.7 ± 0.8 % in condition D. These values were significantly different to the positive control, at the 5% and 1% levels, respectively, corresponding to a 23% and 37% decrease in WE titre and content. However, these metrics were still 12 and 8-fold higher than the negative control, differences which were both significant at the 0.01% level. Overall, this experiment demonstrated that zeolite treatment can effectively remove nitrogen from growth medium for ADP1 without major negative effects on the WE production.

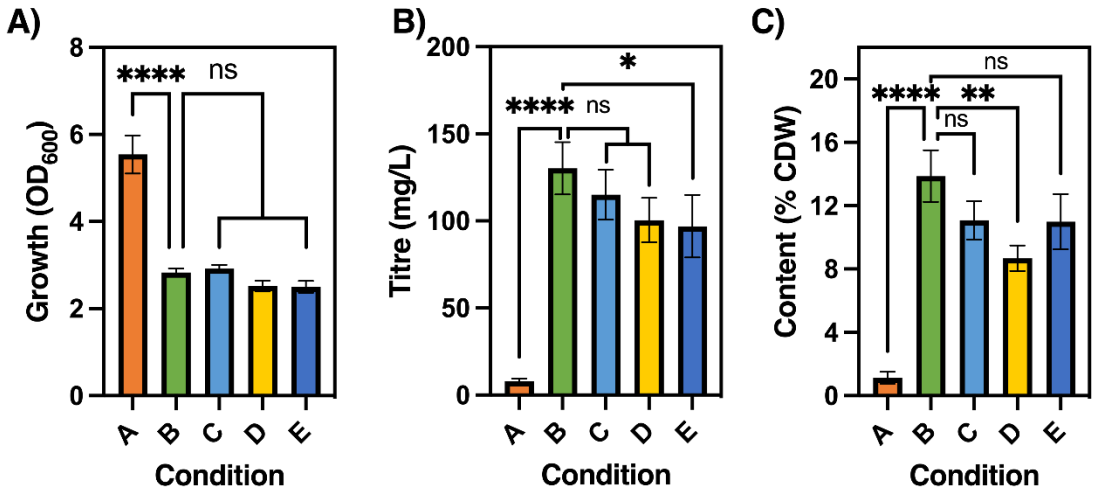


Figure 6.2. Maximum A) growth, B) WE titre and C) WE content per cell measured under different zeolite treatment conditions: A – negative control, B – positive control, C – buffer treatment, D – total treatment and E – additional trace metals. In all conditions, apart from the negative control, C:N ratio was 40:1. Data and error bars represent the means and standard deviations, respectively, of three biological replicates. Pairwise statistical significance testing performed by ANOVA between condition 2 (positive control) and each condition, $p > 0.05$ (not shown), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.0001$ (****). For conditions A, B and C maximum growth, WE titre and content were all achieved after 48 hours and for D after 70 hours. For E maximum titre and content were achieved after 40 hours, but maximum growth was achieved after 70 hours.

6.4.3. Translation of zeolite nitrogen removal for wax production on fermentate

Since employment of zeolite treatment successfully resulted in the desired increase in WE production from an initially high nitrogen culture media, this technique was subsequently

applied to fermentate. As before, 30 wt% loading of zeolite was used to treat fermentate with an initial C:N ratio of 9:1. Ammonium removal in fermentate was less efficient than in synthetic culture media likely due to the higher total cation concentration of fermentate and potential fouling of the zeolite by suspended solids; after three hours, the final C:N ratio was 55:1. Since this ratio was determined in Chapter 5 to be in the optimal range for wax production, it was not adjusted further.

Growth and WE production by ADP1 was measured in the untreated and treated fermentate from the same AF batch, with both diluted to a total initial VFA concentration of 10 g/L (Figure 6.3A and B). After 72 hours, growth, as measured by OD_{600}, was 74 % higher in the untreated fermentate, with almost all VFAs consumed, while in treated fermentate residual VFA remained even after growth had plateaued. The differences in the maximum growth (Figure 6.4A) indicate that nitrogen removal was effective in restricting nutrient availability, and hence stunting growth, which is in agreement with the results in the treated synthetic media (Figure 6.2A). However, when WE production was analysed, there was no detected difference in WE titre or content per cell between the two conditions, as shown in Figure 6.4B and C. This indicated another factor relating to the zeolite treatment must be preventing WE accumulation.

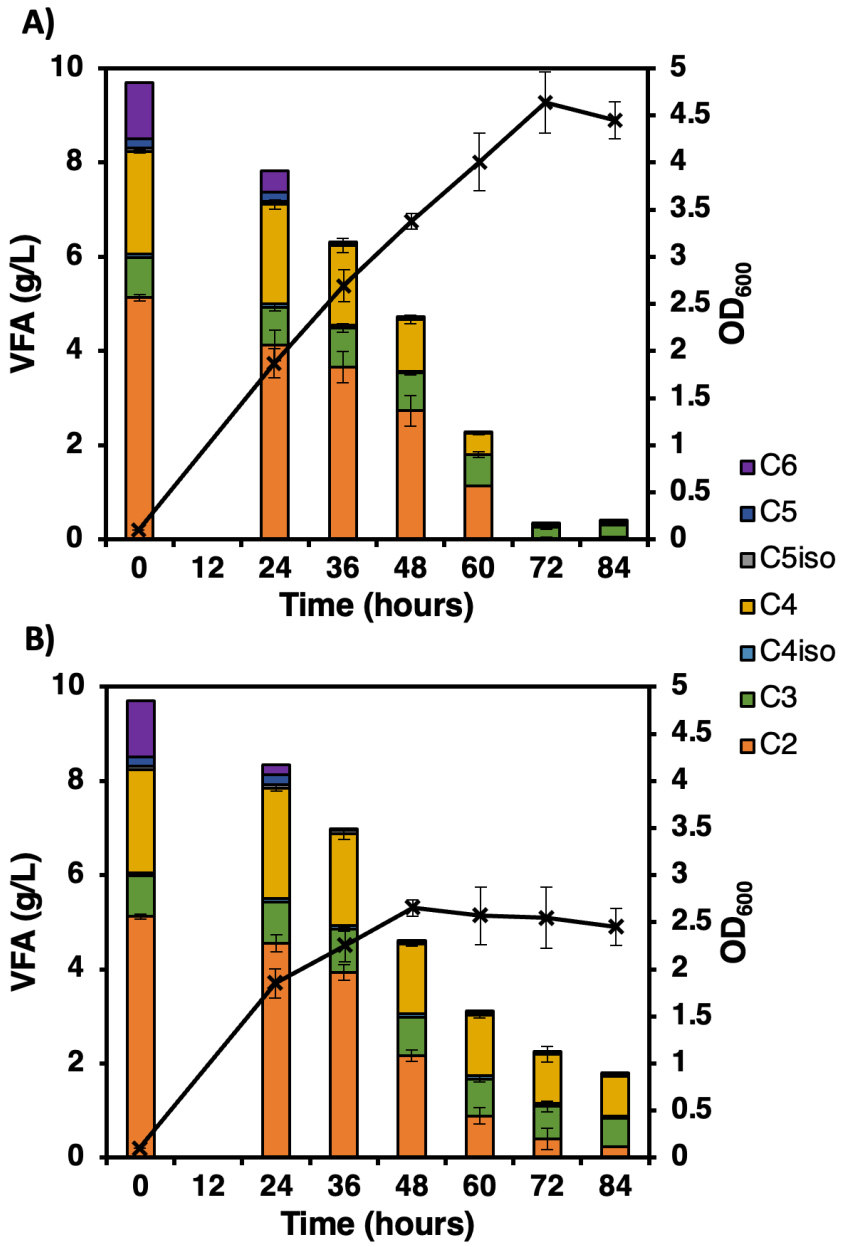


Figure 6.3. Growth (OD_{600}) and VFA consumption (g/L) of ADP1 on A) untreated and B) zeolite treated fermentate, with C:N ratios of 9:1 and 55:1, respectively. Data and error bars represent the means and standard deviations, respectively, of three biological replicates.

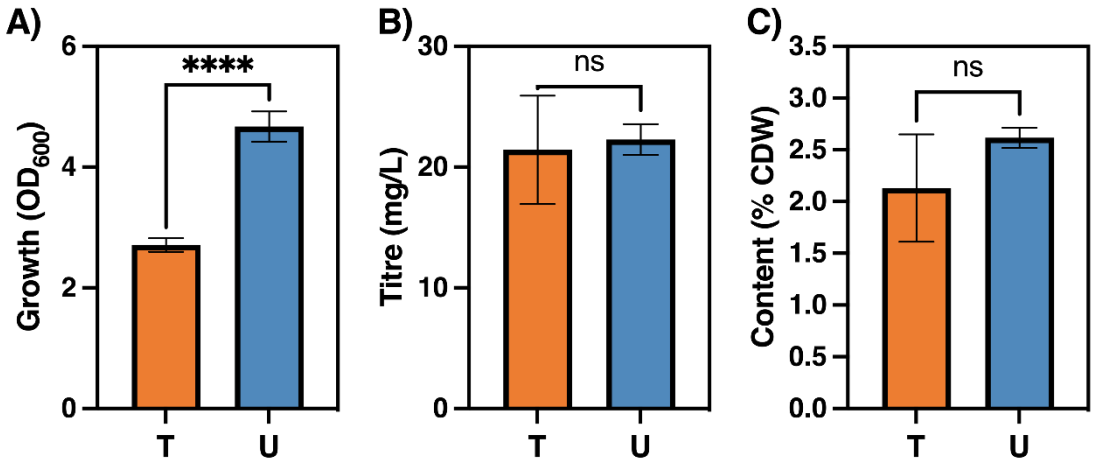


Figure 6.4. The maximum A) growth, B) WE titre and C) WE content per cell for T – treated and U - untreated across the time course. Data points and error bars represent the means and standard deviations, respectively, of three biological replicates. Significance of difference between strains analysed by t-test; $p > 0.05$ (ns), $p < 0.0001$ (****). For untreated samples, maximum growth was reached after 72 hours, with maximum WE titre and conent were achieved after 36 hours. For treated samples, maximum growth was achieved at 60 hours, with maximum WE titre at 48 hours and content after 24 hours.

6.4.4. Degassing of fermentate as an alternative nitrogen removal method

To identify and also avoid potential causes of the decreased WE production capability of ADP1 in zeolite treated fermentate, degassing was investigated as an alternative method of nitrogen removal. Based on previously identified parameters (218), degassing was performed with the fermentate at 80 °C using a water bath. A pump with a flow rate of 5.5 L/min was used to bubble gas through the fermentate. Prior to degassing, the fermentate pH was adjusted to near pH 11, which was found to give the most efficient ammonium removal in the previous study. This was achieved by the addition of 1 mL per 100 mL of 10 M sodium hydroxide, resulting in a final concentration of 0.1 M. During pH adjustment, a colour change in the fermentate was observed and flocculation of the suspended solids occurred (see Figure 6.5), resulting in the gravity separation of almost all solid particles. To take advantage of this, centrifugation was performed at pH 11 prior to degassing.



Figure 6.5. Fermentate at pH 5.6 (the initial pH), 9.5 (after partial pH adjustment) and 11, showing the colour change, flocculation and gravity sedimentation caused by the pH adjustment.

The effectiveness of degassing for ammonia removal is shown in Figure 6.6. Over the three hours of treatment the total nitrogen concentration in the fermentate decreased from 768 ± 2 mg/ to 58 ± 2 mg/L, a 92 ± 4 % reduction. The ammonium concentration at the initial and final timepoint were also determined, and from this the initial and final non-ammonium nitrogen were calculated, then plotted as a dotted line showing the theoretical limit of nitrogen removal by degassing. Based on these numbers, degassing for 3 hours removed 99 ± 1 % of the ammonium nitrogen. An exponential decay model was fitted to the total nitrogen data, with an R^2 of the fit of 0.998. The half-life of nitrogen removal by degassing under these conditions was 26 minutes, and the plateau value of total nitrogen was 53 mg/L, in perfect agreement with the final non-ammonium nitrogen concentration of 53 ± 2 mg/L.

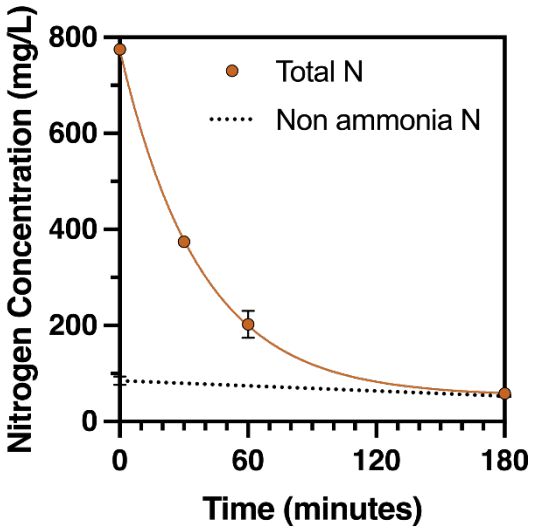


Figure 6.6. The total nitrogen concentration over time during the degassing process. The data points show the experimentally determined concentration, and the solid line orange line shows an exponential decay model fit of the data. The black dotted line represents the total concentration of non-ammonia nitrogen in the fermentate. Data points and error bars represent the means and standard deviations, respectively, of three technical replicates.

6.4.5. Growth and precipitation in fermentate

After establishing that degassing was effective in nitrogen removal from the fermentate, growth and WE production was attempted in degassed fermentate. ADP1 was grown under four different conditions; the untreated, pH adjusted, mid C:N degassed and high C:N degassed fermentate. The pH adjusted fermentate was adjusted to pH 11 by the addition of sodium hydroxide, then returned to pH 7 with hydrochloric acid without degassing. This was performed as a control to observe any effects of the pH adjustment process. The mid C:N and high C:N degassed fermentate were degassed for 1 hour and 2 hours, respectively, and then adjusted to pH 7 with hydrochloric acid prior to growth experiments. All fermentates were centrifuged to remove suspended solids, with all except the untreated centrifuged at pH 11 due to the additional flocculation and sedimentation observed. Each condition was then diluted to approximately 3 g/L total organic carbon ahead of utilisation as growth media. The concentrations of key growth nutrients in each media are given in Table 6.1.

Table 6.1. The total organic carbon, nitrogen and C:N ratio of the four different fermentate conditions tested for growth and WE production.

Component	Total Organic Carbon (g/L)	Total Nitrogen (g/L)	C:N Ratio
Untreated	3.3 ± 0.2	0.37 ± 0.03	10.3 ± 0.3
pH adjusted	2.8 ± 0.3	0.39 ± 0.03	8 ± 1
Mid C:N Degassed	2.9 ± 0.1	0.10 ± 0.01	32 ± 3
High C:N Degassed	3.3 ± 0.2	0.041 ± 0.004	94 ± 3

ADP1 was grown on the four different culture media for 66 hours. Growth was measured on each fermentate condition by OD_{600}, as shown in Figure 6.7A. Decreasing nitrogen concentration was once again found to limit maximum growth, with maximum OD_{600} in the high C:N degassed fermentate just under half that in the mid C:N fermentate. However, despite very similar carbon concentrations and C:N ratios, the maximum OD_{600} in untreated fermentate was 67 % higher than in the pH adjusted fermentate.

Aliquots of liquid culture were taken, centrifuged and freeze-dried to obtain CDW measurements and WE production data. Upon centrifugation, a significant, likely inorganic, precipitate was detected in the cell pellet of the untreated sample which was not present in the other samples (Figure 6.7C). After freeze-drying, the untreated pellets visibly separated into what will be subsequently referred to as the precipitate pellet and cell pellet (Figure 6.7D and E). To address this, the two parts were separated, then weighed and extracted individually. This behaviour was not observed in the other conditions, under which only a cell pellet was recovered. The cell dry weight, as calculated from the freeze-dried cell pellets only, was plotted over time as an alternative measure for growth, as shown in Figure 6.7B. In this case, the growth was very comparable between the untreated and pH adjusted fermentates, with maximum CDWs of 1.4 ± 0.4 and 1.5 ± 0.1 g/L. Again, growth under nitrogen limitation was restricted, as expected, with maximum CDWs of 1.2 ± 0.1 g/L and 0.60 ± 0.05 g/L for the mid C:N and high C:N degassed conditions, respectively.

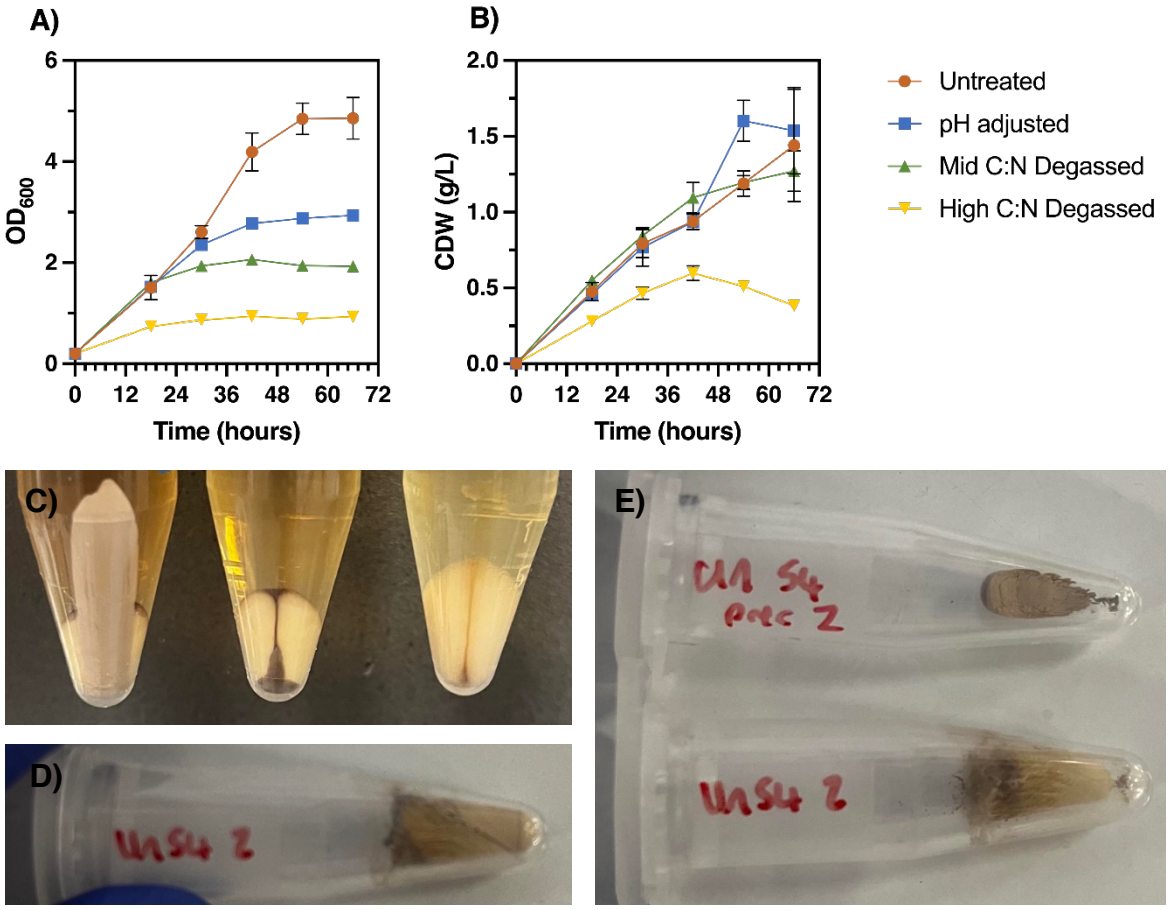


Figure 6.7. Growth of ADP1 as measured by A) OD_{600} and B) cell dry weight (g/L) on fermentate which is untreated (orange circles), pH adjusted (blue squares), partially degassed (green triangles) and fully degassed (yellow inverted triangles). Data points and error bars represent the means and standard deviations, respectively, of three biological replicates. C) Pellets after centrifugation for ADP1 grown on untreated, pH adjusted and mid C:N degassed fermentate (from left to right). D) Freeze dried pellet from ADP1 grown on untreated fermentate, showing the precipitate separating from the cell pellet and E) the same pellet after separation into the precipitate and cell pellets.

6.4.6. Effect of degassing on wax production

As well as affecting cell growth, the different fermentate conditions were analysed for their effect on WE production. Figure 6.8 shows the titre, content per cell and C-conversion efficiency for each condition from 18 to 54 hours, and a statistical comparison of the maxima under each condition for each metric. As is clear, the production in untreated, pH adjusted and mid C:N degassed fermentate was low across all time points, and when analysed by one way ANOVA, the maxima of these three conditions were not significantly different. However, in high C:N degassed fermentate significantly more wax was produced, with a maximum titre of 40 ± 4 mg/L and maximum content of 7 ± 1 %, a 10-fold and 14-fold increase, respectively, on the next highest values under any of the other three conditions. In the untreated condition,

precipitate pellets were also extracted for WEs, but none were detected, confirming the conclusion that these were not cell pellets and justifying their separation.

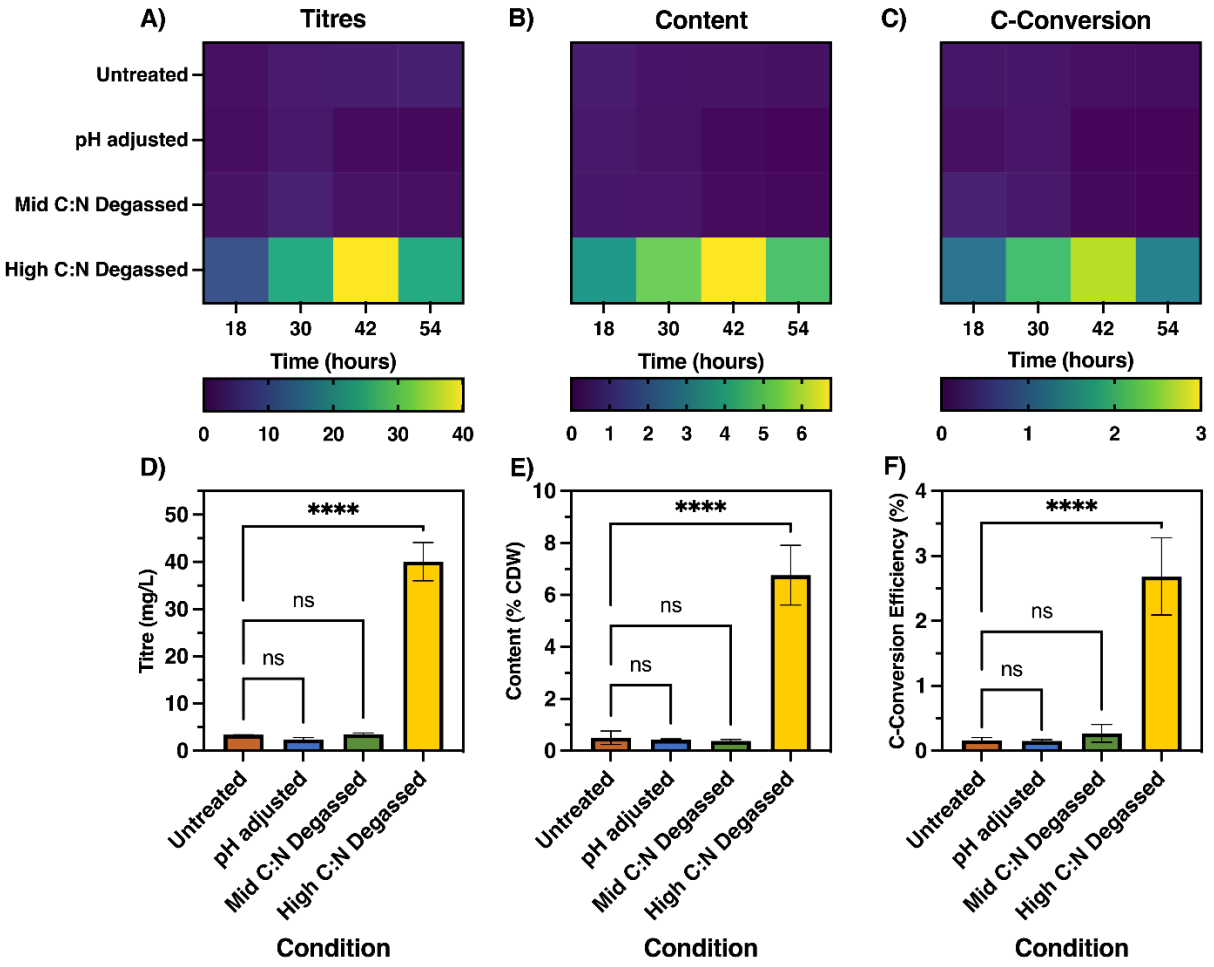


Figure 6.8. Heat maps showing A) WE titre (mg/L), B) WE content per biomass (% CDW) and C) Carbon conversion efficiency from VFAs to WEs (%), over time under different conditions. Data shows means of three biological replicates, values from blue (low) to yellow (high). Maximum achieved D) WE titre (mg/L), E) WE content per biomass (% CDW) and F) Carbon conversion efficiency from VFAs to WEs for each condition. Data and error bars represent the means and standard deviations, respectively, of three biological replicates. Pairwise statistical significance testing performed by ANOVA between untreated and different treatment conditions, $p \geq 0.05$ (ns), $p < 0.0001$ (****).

6.5. Discussion

Two methods of ammonium ion removal, ion exchange using zeolite to bind ammonium ions and degassing by stripping ammonia gas, were attempted to decrease the nitrogen content of fermentate, and hence increase the C:N ratio. Both techniques proved highly effective in reducing the nitrogen concentration, with almost complete ammonium removal by both processes. Fermentate treated by either method was able to support good bacterial growth and, in both cases, maximum growth decreased after treatment due to the reduced nitrogen availability. This reduction was in good agreement with what would be expected for the available nitrogen concentration, as was demonstrated in synthetic minimal media after zeolite treatment (Figure 6.2A).

Considerable precipitation was observed in the degassing experiment during growth of ADP1 on untreated fermentate. This was identified by the difference in growth behaviour when growth was measured by OD_{600} compared to by CDW of isolated cell pellet, with the precipitate removed. As was observed during the pH adjustment of the pH adjusted and degassed samples, pH increases can cause precipitation in fermentate. This likely explains why precipitation was not observed during bacterial growth in samples where pH adjustment had already been performed, as the precipitate had already formed and been removed in the pH adjustment and associated flocculation and centrifugation steps. pH increases also occurred during bacterial growth in batch due to the consumption of the VFA substrate (219). Prior to this experiment, attempts were made to mitigate the effects of this on growth. The pH of the fermentate was measured at each time point, and a second, sterile AF sample was diluted, pH adjusted and used to blank OD_{600} measurements. However, this experiment highlighted this correction was insufficient to account for the full extent of precipitation occurring, as large differences in OD_{600} were still observed at near identical cell dry weights, as calculated from the cell pellets. Therefore, an additional factor in the cell growth must contribute to precipitation aside from just pH change. This highlights that CDW may be a more accurate measure of growth for cultures grown on fermentate.

Unfortunately, while growth behaviour in these treated fermentates was as anticipated, the WE production was not. For zeolite treatment, there was no significant difference between the WEs produced on the untreated and treated fermentates. After degassing, an increase in WE production was achieved at a C:N ratio of 94:1, with a 10-fold increase in WE titre compared to the untreated fermentate. However, this titre was still less than half of the expected titre for this C:N ratio, based on the findings in Chapter 5. When the WE content per cell was

considered, the maximum achieved here was 7 ± 1 % CDW, again less than half what would be expected based on findings in synthetic minimal media systems. In addition, degassed fermentate with a C:N ratio of 32:1 did not produce significantly more wax, by any metric, than the untreated or pH adjusted fermentates, which had C:N ratios of 10.3 ± 0.3 and 8 ± 1, respectively. This was also surprising, given the high productivities obtained under a C:N ratio of 40:1, both in Chapter 5 and earlier in this chapter.

One possible explanation for the reduced WE production is the increased sodium concentrations of the fermentate after undergoing either ammonium removal procedure. As the zeolite treatment is an ion exchange process, for every ammonium ion that is removed, a sodium ion is released into the medium. Similarly, in order to achieve efficient degassing, the medium was adjusted to pH 11, requiring the addition of substantial quantities of sodium hydroxide. In both cases, this sodium concentration was not sufficient to cause toxicity and prevent cell growth. Previous studies have reported *Acinetobacter* species to be reasonably salt tolerant, with impacts on maximum OD and growth rate above 0.3 M NaCl, but good growth still achieved at 0.5 M (220). However, the effect of salt concentration on lipid biosynthesis in this strain has not been investigated. By degassing, approximately 0.1 M NaOH was added to the fermentate in the pH adjustment: after zeolite treatment the added concentration was harder to calculate, and would need to be quantified experimentally.

Interestingly, zeolite treatment did have a significant effect of WE production in synthetic media, although both titre and content did decrease slightly in zeolite treated samples compared to the positive control. However, the decreased ammonium removal efficiency of zeolite in the fermentate as compared to the synthetic minimal media suggests the presence of a higher concentration of cations, which bind to the zeolite in place of ammonium during the ion exchange treatment. Removal of a fixed quantity of ammonium from fermentate would therefore require the exchange of more sodium ions from the zeolite than in the synthetic minimal media, resulting in a greater increase in sodium concentration when treating this medium. If the initial concentration of cations is also higher in fermentate, these effects would compound and result in a media with a higher sodium (and total cation) concentration. It is possible that total cation concentration post treatment may have been inhibitory to WE accumulation, although sodium is specifically suspected as the inhibitor, as it has previously been shown to inhibit ADP1 growth more than potassium (220). In addition, increasing the concentration of magnesium and calcium was actually found to increase WE accumulation in Chapter 5, although the effect of high concentrations of these ions was not studied.

If future advances were to render these methods successful then the economic feasibility of each method would need to be considered. In the zeolite treatment method, the major costs would be the zeolite itself and the salt required to regenerate the binding sites. Zeolite associated costs would vary depending on the recyclability of the zeolites, which in literature have been shown to work effectively over at least 5 cycles (221), and the minimum loading required, which was not optimised here. Clinoptilolite was chosen in this work as a cheap and widely available, naturally occurring zeolite, but other zeolites, including synthetic zeolites, have previously been shown to have much higher ammonium binding capacities (221). This would need to be balanced with regeneration efficiency, both in terms of cost and environmental footprint due to resource consumption. Salt costs would depend on the minimum concentration of salt needed to regenerate the zeolite, which was not optimised here. If sodium were found to pose a problem, and another salt such as KCl, $CaCl_2$ or $MgCl_2$ were used instead, this could increase the cost of the salt up to 10-fold (222).

Conversely, as well as the cost of reagents required for the pH adjustments, the degassing process would also involve significant energy costs. Heating and maintaining the fermentate at sufficiently high temperatures to decrease the ammonia solubility would have significant associated energy costs, as would powering the gas spargers needed to strip the ammonia gas from the liquid. An optimisation of this process for wastewater was performed previously (218), finding pH 9.5 and 70 °C sufficient to remove around 80 % of ammonia under the correct sparging conditions. Alternatively, using a slightly higher pH of 10 and higher gas flow rate allowed for approaching 90% of ammonia removal at just 50 °C. The compounding relationship of these three factors allows treatment conditions to be balanced depending on which is most cost effective, with potential to change conditions while maintaining a consistent removal rate to account for volatile energy or chemical reagent costs.

However, this process would facilitate energy saving over the zeolite (and untreated) processes in another step of the treatment. The observation that high pH can cause flocculation of the solids in fermentate allows for more efficient clarification of the fermentate by reducing the centrifugation requirements, which is a highly energy intensive process (223). Based on the exact specifications required and the optimised parameters for each process, the potential benefits and savings of each would have to be balanced against the energy and reagent uses. For completeness this should be done in economic terms and also in terms of environmental impacts, such as resource consumption and waste generation, ideally through a life cycle assessment of each process.

6.6. Conclusion

In this chapter, the suitability of nitrogen removal methods was tested on fermentate to improve WE production in ADP1. This was intended to translate the findings of Chapter 5, specifically the optimal C:N ratio for WE production in ADP1, to a complex, mixed media with fixed initial concentrations of carbon and nitrogen. Zeolite treatment and degassing were both tested and shown to be highly effective in removing ammonium from the fermentate, and hence could effectively increase C:N ratios from approximately 10:1 (the typical values obtained for fermentate from *Opuntia*) to within the optimal range for WE production identified in Chapter 5. Furthermore, bacterial growth was not impaired by either method of nitrogen removal.

Preliminary tests on synthetic growth medium replicated optimal conditions obtained in Chapter 5, with a C:N ratio of 40:1 achieved through zeolite treatment. WE production was also in good agreement with the previous chapter, with titres of over 100 mg/L obtained and significant difference in behaviour from the negative control.

However, in fermentate, WE production after either nitrogen removal treatment was found to be much lower than expected, given the results on synthetic medium in both this chapter and Chapter 5. The cause of this decrease in production remains unknown, although relation to salinity of the growth medium was hypothesised, as both treatment methods result in the addition of considerable amounts of sodium to the medium.

Chapter 7 – Conclusions

This thesis aimed to improve the understanding of bacterial WE production from a mixture of unrefined, waste-derived VFAs. The research focused on the production of WEs by *Acinetobacter baylyi* ADP1 from individual and mixed synthetic VFAs to elucidate growth and production behaviours on these substrates, many of which had not been previously explored. These findings were then transferred, with varying degrees of success, to acidogenic fermentate, the raw mixture of VFAs derived from anaerobic digestion.

Based on the work completed in this thesis, the major findings can be summarised as follows:

- Synthetic and fermentation-derived VFAs with chain lengths C2-C6 are suitable substrates for bacterial growth and WE production. Specifically, acidogenic fermentate can be used as a complete growth medium with minimal purification.
- Genetic engineering can be used to improve WE titres, content per cell and carbon conversion efficiency from substrate. Specifically, overexpression of *acs* was shown to increase WE production from acetate. Knocking out the glyoxylate shunt created a semi-partitioned metabolism, which could use odd chain VFAs for cell growth while even chain VFAs were used for energy generation and WE production. Fermentate, a low-cost substrate, can simultaneously deliver a mixture of odd and even chain VFAs.
- Longer chain VFAs are more carbon and energy dense as substrates, and can facilitate greater bacterial growth, but also show greater toxicity and inhibition.
- The optimal degree of nitrogen and phosphate limitation in batch cultures were identified, and a compounding effect on WE production of co-limiting these two essential growth nutrients was observed for the first time.
- Nitrogen limitation conditions in synthetic culture media could be replicated in fermentate by removing ammonium ions through zeolite-based ion exchange or ammonia gas by gas stripping. High C:N ratios were achieved, but WE production was not significantly improved, suggesting another aspect of the treatment interfered with cellular WE accumulation pathways.

Overall, this thesis highlights the potential of fermentation-derived VFAs as a feedstock for the production of WEs, and other microbial lipids, in bacteria. However, it emphasises the challenges of working with complex, mixed substrates and the research necessary for their effective and reliable utilisation in the future bioeconomy.

Chapter 8 – Future outlook

In carrying out these investigations and in the preparation of this thesis, a number of additional areas of interest for future exploration were identified. These fall into two distinct categories: 1) those which follow-on directly from the results of studies conducted here and 2) those which do not relate directly to the work in this thesis, but which have been recognised as limitations, either of the particular experimental set-ups used here or in the field as a whole.

8.1. Short term and follow-on strategies

8.1.1. Improving on *acs* overexpression

In Chapter 3, it was identified that simultaneous overexpression of a number of genes in the fatty acid synthesis pathway could prove more beneficial than overexpressing *acs* alone, and that genes identified in recently published work may be a greatly improved combination (189). Non-native *acs* genes may also result in greater acetate utilisation rate or higher WE production (224). The alternative conversion pathway from acetate to acetyl-CoA, via the AckA-Pta enzymes, could also be overexpressed. This pathway is known to be the primary route by which acetate is exported and imported when bacteria grow on glucose, and is usually responsible for the behaviour of high concentrations of acetate (225), such as those used here. Previously, overexpression of this pathway in *E. coli* has improved PHA production more significantly than *acs* overexpression (226).

Should gene overexpression significantly increase WE production, these changes could also be integrated onto the genome to improve strain stability, reduce burden and remove the need for antibiotic use. This could be achieved by knocking out the appropriate endogenous gene (including the accompanying promoter region) from the genome using a Tdk/Kan^R cassette (81), which contains both a positive and negative selection marker. This cassette can be used to generate unmarked deletions (85), which simplifies further engineering, such as deletions or insertions. Gibson assembly could then be used to construct a cassette consisting of the desired gene with a synthetic promoter and RBS, which would be used to knock-out Tdk/Kan^R by natural transformation, homologous recombination and counter-selection. This method also provides the option to test a promoter and RBS library to optimise expression strength.

8.1.2. Combining waste streams

The study detailed in Chapter 4 focussed on the utilisation of acidogenic fermentate directly as a source of VFAs for WE production. It was discussed that the low energy content and highly oxidised state of VFAs limited the maximum potential yields of highly reduced lipids. This

could be addressed by employing waste streams containing higher energy, more reduced carbon sources in combination with fermentate. One potential source is waste cooking oils, which are very energy dense but are in limited supply due to their suitability for biofuel generation (227). Crude glycerol is a desirable alternative, as it is widely available in the EU as a by-product of biofuel production from TAGs (228). While it is not naturally utilisable by ADP1 (60, 229), its utilisation via glycolysis can be achieved with the expression of a single exogenous gene, glycerate 3-kinase. Compared to acetate, glycerol is also less oxidised, so can generate greater reducing power per mole, and could also be co-utilised as the growth substrate for the M+ knock-out strain.

8.1.3. Validating nitrogen removal from fermentate

8.1.3.1. Investigating salt tolerance

The majority of direct follow-on studies from this investigation relate to the content of Chapter 6, in which the inhibition of WE production in ADP1 was observed and was hypothesised to be caused by high concentrations of salts in the treated fermentates. To investigate this hypothesis, WE production experiments under conditions known to produce high WE titres, such as those reported in Chapter 5, should be replicated with the addition of sodium chloride at a range of concentrations to identify whether the addition of salt reduces WE production in synthetic culture media. The concentration of sodium and other major cations, such as potassium, magnesium and calcium, should also be quantified in the synthetic media and the different fermentate conditions, and any correlation between WE production inhibition and specific ion concentrations should be identified. The most suitable technique for this quantification would be Inductively Coupled Plasma (ICP) spectroscopy, which is employed to analyse and quantify metal ions in a wide range of samples, such as soil, wastewater, medical samples and more (230, 231).

If sodium were found to be inhibitory from these tests, addition of other salts (potassium chloride, magnesium chloride and calcium chloride) under the optimal WE producing conditions should be investigated, as both zeolite equilibration and pH adjustment could be performed using salts and bases with alternative metal ions, if needed. Potassium is the obvious choice of replacement, as both the chloride and hydroxide forms are widely available and can be exchanged for the sodium equivalents in current protocols. Furthermore, moderate to high concentrations of potassium chloride have been found to affect growth less than equivalent concentrations of sodium chloride, with a higher maximum tolerance (220), suggesting it may impact cellular metabolism less. Experiments in Chapter 5 found that high potassium

concentrations also reduced WE accumulation, although it appeared this effect could be overcome by the addition of small amounts of calcium and magnesium. These divalent cations would also be of interest, but solubility of magnesium and calcium hydroxides in water is very low, so they are unsuitable for pH adjustment in degassing. While the chloride salts could be used to equilibrate the zeolite, a previous work found that regenerating a range of zeolites with calcium greatly reduced their ammonium binding, so this would also need to be considered (221). It is possible that the higher ionic charge results in stronger binding to the zeolite, reducing the efficiency of ion exchange for ammonium.

8.1.3.2. Improving gas stripping

The utilisation of a more efficient gas stripping or vacuum stripping set-up could also help overcome the problem of high salt concentration. While pH 11 was required for efficient degassing under the conditions used here, as well as heating to 80 °C, efficient gas stripping has been demonstrated in anaerobic digesters with pH 9.5 (215, 216), with vacuum stripping in particular able to remove more than 2 g/L of ammonium nitrogen in 6 hours (215). If a lower pH were employed, the amount of base required would be reduced, thereby reducing the ion concentration present in the medium. The greatest improvements compared to the protocol employed here could be achieved by changing the gas flow rate, employing a gas sparger with a smaller bubble size (232), applying a vacuum to remove the dissolved gas (215) or by utilising gas permeable membranes (233).

8.1.3.3. Life cycle assessment

If ammonium ion removal from fermentate can be developed as an efficient method to reduce the C:N ratio of this low-cost feedstock, the door is opened to opportunities for bioproduction of several microbial lipids, including WEs, TAGs and PHAs. The efficiency of the ammonium ion removal methods would then need to be analysed from a full life cycle perspective. If employing zeolite, regeneration after treatment would need to be considered. This would include quantifying the salt(s) needed for regeneration and testing the effectiveness of regenerated zeolite over multiple treatment cycles, as well as accounting for the generation of brine as a by-product and the recovery of ammonium in a utilisable form from the liquid effluent. Conversely, for degassing the primary concern would be energy utilisation for the gas sparger and heating. Both processes have been deployed at pilot scale for removal and recovery of ammonium (234-236), so data should be available to indicate potentially optimal process parameters, though the specifics will vary between systems. Quantification of acids and bases required for pH adjustment would also be necessary.

8.1.4. Combining findings

Efforts should also be made to combine the key findings of different chapters of this thesis, specifically applying the findings for engineered ADP1 strains in Chapters 3 and 4 to the optimally nutrient limited systems from Chapters 5 and 6. In particular, if ammonium ion removal from fermentate without inhibiting WE production is possible, the effectiveness of *A. baylyi* ADP1 M1 and ADP1-*acs* in WE production from low nitrogen fermentate should be investigated. Ammonium removal could also be used to valorise the nitrogen rich blood digestate produced in Chapter 4 for bioproduction, increasing the substrate pool for AF as a precursor to bioproduction to include ammonium rich sources like sewage and animal agriculture wastes.

8.2. Longer term strategies

As well as building directly upon the findings of this thesis, a number of other avenues could be explored to translate these findings into more broadly and directly applicable results with further increased WE production.

8.2.1. Deployment of bioreactors

The deployment of continuous or fed batch reactors can offer several advantages in bioproduction efficiency over batch cultures (147, 189). A significant contributor to this improvement is that higher cell densities can be obtained, which in turn lead to increased product titres. Higher titres then also improve the efficiency and economic viability of product extraction and downstream processing through process intensification. Continuous or pulsed feeding is particularly beneficial for inhibitory substrates such as VFAs, enabling a greater total carbon density to be transferred to cells without resulting in toxicity issues (60). Since the VFA-rich fermentate obtained from AF can be highly concentrated, a continuous or fed-batch system could be envisaged with this fermentate employed as the carbon source feed solution. Parameters including optimal feeding rate and concentration, retention time, effluent rate, pH and nutrient ratios would all need to be explored to optimise performance. Once established, scale up could also be investigated, with potential for further efficiency gains as well as recycling of key components, all of which are essential considerations in any potential industrial application.

In a similar vein, a continuous reactor could also be deployed for the AF step, again bringing the process closer to an industrially feasible set-up. Conditions within the reactor could be tuned to alter the VFA composition of the fermentate, and the long-term stability of such changes would need to be assessed. The use of *in situ* or in-line filtration methods to remove particulates and clarify fermentate for bioproduction would make the process more efficient and scalable. For example, membrane filtration could be used to simultaneously clarify and sterilise the fermentate (237), removing the need for both centrifuging and autoclaving, which are inherently limited to laboratory scale work. If successful, either gas stripping (216) or zeolite treatment (238) for ammonium removal could be combined with the AF process, either within the reactor itself or as a flow-through, post-treatment (239).

8.2.2. Efficient extraction and green solvents

One area where significant improvement is required for the scale-up and commercialisation of any microbial lipid production process is in the extraction stages, as laboratory scale studies are heavily reliant on chloroform for extractions. Less hazardous, more sustainable solvent alternatives need to be found and investigated as a priority if the generation of bioplastics or biofuels from microorganisms is to become a realisable technology, as chloroform is highly toxic, a suspected carcinogen, tightly regulated (240, 241) and currently almost entirely fossil fuel derived (242). Mid-chain alkanes, such as dodecane and ethyl acetate have been demonstrated to be suitable extraction solvents for fatty acids (243) and alkanes (244), respectively, and may be effective for other lipids including WEs. Other widely employed substitutions for chloroform include methyl tert-butyl ether and isopropyl acetate, both of which have lower health and waste impacts than chloroform (245).

As well as the solvent, the extraction procedure itself is not practical or economical at scale, requiring centrifugation, lyophilisation and batch liquid-liquid extraction. Several studies have proposed improved methods to extract lipids from microorganisms which are efficient and can be applied to continuous systems. Studies by Ledesma-Amaro *et al.* previously combined genetic engineering with *in situ* solvent-solvent extraction to create a strain of *Y. lipolytica* capable of overproducing and exporting fatty acids, removing almost all down-stream processing steps and achieving a fatty acid content of more than 120 % CDW (243). In this way, changing a lipid product from being intracellular to extracellular by secretion can be hugely beneficial for improving the extraction process; however, since WE molecules are significantly larger than fatty acids, this poses a challenge. Notably, a relatively under-studied bacterial strain, *Alcanivorax borkumensis* SK2, is capable of secreting WEs into the culture medium, with almost 40 % of WEs detected in the media rather than in the cell pellet (61). This strain produces primarily TAGs and is not capable of producing WEs on non-lipid substrates (246), but if the secretion genes could be identified then expressed in other chasses such as ADP1, extraction would be hugely simplified. So far, mutants not capable of excreting WEs and TAGs have been obtained and sequenced (247, 248), suggesting the identity of the export genes, but these are yet to be expressed in other strains.

8.2.3. Investigating nitrogen limitation regulation

Since genetic regulation, especially related to nitrogen concentration, has proven difficult to overcome when trying to increase WE production, a final area of potential future investigation is the regulatory system that controls lipid accumulation under nitrogen starvation. One method

in which regulation can be altered on a genomic level rather than on a single gene level is through the manipulation of sigma factors. Sigma factors are proteins necessary for the initiation of gene transcription in bacteria; they enable RNA polymerases to bind to gene promoters. Transcription of specific genes is controlled by different sigma factors, many of which are responsive to environmental signals. Nitrogen starvation is linked to sigma-54 (also referred to as RpoN), which is necessary for transcription of genes involved in nitrogen metabolism and assimilation (249).

Previously it has been reported that RpoN negatively regulates the glyoxylate shunt, meaning that when nitrogen limitation is detected, the glyoxylate shunt is downregulated (250). This could explain why when the M1 mutant, which had the glyoxylate shunt completely knocked out, was grown in non-nitrogen limited conditions it accumulated WE similarly to the WT under nitrogen limitation. However, as was discovered in Chapter 4, this effect was reduced in nitrogen rich conditions, and WE production still decreased, suggesting other parts of the WE accumulation pathway are also regulated by nitrogen concentration, and possibly by RpoN.

Given this, investigations could be made into the role of RpoN in regulating the fatty acid and WE biosynthesis pathways. Typically, sigma factors bind to specific nucleotide sequences of DNA, called consensus sequences, which are present a fixed distance upstream of the regulated gene. In ADP1, an example of such sequence was previously identified ahead of *areCBA* gene, which is important for growth on benzyl alkanoates (251). This sequence was in reasonable agreement with consensus sequences identified for RpoN in other bacterial strains (252).

To confirm this sequence is an RpoN consensus sequence, the known RpoN-dependent promoter in ADP1 (251) could be cloned into a *gfp* containing plasmid, and ADP1 containing this plasmid grown under nitrogen limitation. If the promoter is correct, the strain would be expected to fluoresce due to the natural expression of RpoN under low nitrogen conditions. The *rpoN* gene, which has been identified in *A. baylyi* ADP1 (84), could also be cloned from the genome of ADP1 and these two cassettes could be combined on a single plasmid. If *gfp* expression is still induced in this system when grown in nitrogen rich conditions, this would demonstrate that synthetic RpoN overexpression can successfully induce translation of RpoN regulated genes. If this was successful, WE production could be quantified under nitrogen-replete conditions with the overexpression of RpoN to confirm whether any benefit to WE production can be achieved by altering nitrogen limitation regulation. To understand these effects, potential RpoN regulated genes could be identified on the ADP1 genome through chromatin immunoprecipitation coupled with sequencing to identify RpoN consensus

sequences. Alternatively, transcriptomics data can be compared between strains of ADP1 with *rpoN* knocked out and *rpoN* overexpression (253). Particular attention should be paid to any consensus sequences upstream of or the upregulation of genes known to be involved in lipid or WE biosynthesis.

Chapter 9 – General concluding remarks

The replacement of petrochemicals with molecules generated from biogenic carbon is an essential element in the reduction of anthropogenic carbon emissions needed to prevent catastrophic climate change. In particular, microbial biosynthesis of complex chemicals can offer some key advantages over traditional chemical reactions, including high product specificity, feedstock co-utilisation, and the employment of gentle, biological conditions and non-hazardous reagents.

In this study, WEs were identified as a molecule of interest with relatively limited research attention compared to other microbial lipids, such as TAGs and PHAs, although many of the findings in this thesis will be transferable across neutral lipid classes. *Acinetobacter baylyi* ADP1 was the obvious choice for microbial chassis because it has been sequenced with a well characterised genome, is naturally competent for genetic engineering and is able to produce WEs. Since most studies to date on WE production in ADP1 have utilised sugar derived feedstocks, which are expensive and usually in direct competition with food production, a less conventional carbon and energy source for bacterial growth was chosen. Specifically, generation of VFAs from waste streams has enormous economic and environmental advantages. VFAs offer an excellent solution, as they can be generated by AF of an enormous range of organic materials, from traditional energy crops such as maize and corn to agricultural residues, municipal solid waste and sewage. They are metabolised by a wide range of microorganisms including ADP1, as demonstrated in Chapters 3 and 4, and can be generated cheaply and reliably with minimal geographic limitations.

For the research in this study, a combination of pure, synthetic VFAs and fermentation generated VFAs were tested. Synthetic acids were employed initially because this was the first study to attempt WE production from VFAs other than acetate, and hence exploitation of a simplified system was desirable. Many of the variables had not been investigated to date and significant parameter exploration and optimisation was required, including the concentrations of individual VFAs (Chapter 3); mixed VFAs (Chapter 4); and nitrogen, phosphate and micronutrients (Chapter 5).

Where fermentation generated VFAs were employed, the CAM plant *Opuntia ficus indica* and dried pig blood were used as AF feedstocks. The former is suitable for growth in semi-arid regions with poor soil and has been proposed as an economic crop for remote rural

communities, while the latter is a model for high protein waste streams from animal agriculture, which are often difficult to process. These homogenous feedstocks were also selected in the interest of simplification because real waste streams can be highly variable in composition. As such, their employment would have introduced additional variation to the AF process, and hence to the VFAs produced, rendering observations and findings harder to explain. Access to a real, relatively representative waste stream, such as food waste or sewage, for laboratory use was also restricted due to health and safety limitations. However, extending the work undertaken here to include such waste streams would be an essential part of taking these findings forwards.

While testing hypotheses and developing protocols in model culture media is a widely used method and has the benefits outlined above, this study has demonstrated that the success of this method is mixed. In the first part of Chapter 4, the WE production results from ADP1 M1 and WT on sodium acetate and propionate translated almost perfectly from synthetic VFAs to *Opuntia* fermentate, highlighting the potential benefits of this approach. However, when the same technique was attempted with fermentate from pig blood this success was not repeated, emphasising the importance of using a synthetic model that accurately accounts for all the parameters of the true system. Similarly, in Chapter 5 the optimal C:N ratio was identified and in Chapter 6 an ammonium removal method was shown to replicate the results of this optimal ratio effectively in synthetic media. However, when repeated in fermentate the behaviour observed was totally unpredicted and thus far unexplained, although hypotheses were presented for the most likely cause. These complications highlight a major limitation of this approach: the accuracy and reliability of the model system is limited both by our knowledge of the waste stream being replicated and our understanding of the effects this will have on bacterial metabolism. For these reasons, any process employing actual waste streams as substrates for bioproduction must assess the stream itself, the effect of natural variation within the stream, and the presence of potential inhibitors on microbial growth and production. When employing fermentate as the substrate, this principal also extends to testing the robustness of the AF process to feedstock variability.

Aside from controlling the culture parameters, genetic engineering also proved to be highly effective in producing greater lipid yields by manipulating carbon flux through the central carbon metabolism. This was demonstrated in Chapter 4, where the ADP1 M1 mutant strain produced 5.6 times as much wax as the WT and with an a 9-fold improvement in efficiency. However, as was discovered when attempting to utilise nitrogen-rich fermentate from blood,

the metabolic system is still under inherent regulatory limits which cannot be fully overcome by the overexpression or deletion of a small number of genes. As a consequence, even though WE accumulation under all conditions was improved compared to WT ADP1, it was still highly dependent on the C:N ratio. Without fully understanding such metabolic regulation, the potential for genetic engineering to improve product yields of lipids, and other bioproducts, is limited. A similar barrier was faced in the overexpression of *acs*. WE titre was increased by 2.6-fold compared to the WT ADP1, but content per cell and production efficiency were not significantly different. To achieve highly improved productivities, it is essential to consider the entire metabolic pathway and identify numerous potential bottlenecks.

This study has demonstrated the suitability of VFAs and VFA-rich fermentate for the microbial bioproduction of WEs, achieving the highest WE titres to date from short chain fatty acid substrates, both synthetic and from fermentation. In doing so, it has expanded the potential substrate pool for microbial WE production to include an abundant and low-cost waste stream and simultaneously increased the scope of microbial bioproducts that can be generated from the valorisation of fermentate. Through this process, several unknown metabolic features of ADP1 were also identified and explored, improving the knowledge and understanding of a strain which has great potential for industrial biotechnology and genetic engineering. It is hoped that the work presented in this thesis will be a useful resource to future researchers and contribute to advancing the bacterial bioproduction of lipids and other sustainable chemicals.

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