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PRODUCTION OF ALPHA-OLEFIN IN ACINETOBACTER BAYLYI ADP1

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ABSTRACT

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As energy and environmental issues become more and more serious, bioproduction of valuable chemicals, e.g. medium-chain alpha-olefins, as an alternative source become important increasingly. Medium-chain alpha-olefins, e.g. 1-undecene, are important hydrocarbon chemicals for various industrial applications. They can be used for industrial intermediates of detergents, cosmetic products, greases, lubricants, plasticizers, and “drop-in” next generation biofuel. Currently, medium-chain alpha-olefins are derived from fossil sources.

This study demonstrated *Acinetobacter baylyi* ADP1 as an excellent biological engineering platform for the bioproduction of medium-chain alpha-olefins. An iron-dependent desaturase/decarboxylase UndA and a membrane-bound desaturase-like enzyme UndB can convert fatty acids to corresponding olefins. Glyoxylate shunt blocking was applied for *A. baylyi* ADP1 to optimize biosynthesis of 1-undecene, the 1-undecene production titer of 350 ng/ml was obtained by the expression of UndA. Biosynthesis system based on UndB produced 331 ng/ml of 1-undecene. Expression of UndB is feasible in *A. baylyi* ADP1, which can make ADP1 to produce wider range of olefins (including olefins with even number of carbon atoms: C12 and C14). This study demonstrated the potential of UndB in *A. baylyi* ADP1 and a possible 1-undecene biosynthesis rate limiting factor. The bioproduction of 1-undecene in *A. baylyi* ADP1 should be improved and optimized in further researches.

Keywords: medium-chain alpha-olefins, 1-undecene, *Acinetobacter baylyi* ADP1, glyoxylate shunt, desaturase, biosynthesis

The originality of this thesis has been checked using the Turnitin Originality Check service.

PREFACE

The research and laboratory work of this master's thesis were conducted in Synbio research team in the Faculty of Engineering and Natural Sciences in Tampere University (Finland), from November 2018 to August 2019.

I would like to express my deepest gratitude to my supervisor Assistant Professor (tenure track) Ville Santala, for his instructive advice and helpful suggestions on my thesis works. Ville Santala gave me this opportunity to research this frontier field of synthetic biology and metabolic engineering. In addition, I want to thank Ville's funding support from his Sbio Project to my research from July 2019 to August 2019. I would like to express my thanks to another supervisor M.Sc. Jin Luo, his guidance and help of my laboratory works and design of my experiment made me complete this thesis smoothly.

Specially, I want to thank Ph.D. Elena Efimova for her help in chemical analysis and instructions of instruments, she helped me solve several difficult problems during my experiments. I also want to give my thanks to all those who helped me for my laboratory works and gave me valuable ideas for my thesis work: Ph.D. Suvi Santala, Ph.D. Tapio Lehtinen, Ph.D. Rahul Mangayil, M.Sc. Milla Salmela, and other group members from the Synbio team.

I want to thank all my friends who helped me throughout my master's thesis work. Finally, I am grateful to my parents: Professor Heling Qu and Associate Professor Taidong Liu, thanks for your love, encouragement, and unlimited support.

Tampere, 01 September 2019

Changshuo Liu

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LIST OF SYMBOLS AND ABBREVIATIONS

ACP	Acyl carrier protein
ADP1	*Acinetobacter baylyi* ADP1
ATP	Adenosine 5’ triphosphate
ED pathway	Entner–Doudoroff pathway
EMP pathway	Embden-Meyerhof-Parnas pathway
FAME	Fatty acid methyl ester
FAS	Fatty acid synthesis
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
GM	Genetically modified
GMO	Genetically modified organism
LA	Lysogeny broth-agar
LB	Lysogeny broth
MCAO	Medium-chain alpha-olefin
NADPH	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
OD_{600}	Optical density at 600 nm wavelength
PCR	Polymerase chain reaction
TCA cycle	Tricarboxylic acid cycle
TLC	Thin layer chromatography
UIAP	Unidentified alkene product
WE	Wax ester
WS/DGAT	Wax ester synthase/acyl-CoA: diacylglycerol acyltransferase

1. INTRODUCTION

In future bioproduction will become an increasingly important following the foreseeable depletion of petroleum and consideration of environmental issues. Bioproduction based on microorganisms is able to utilize renewable starting materials and recyclable wastes to produce various chemicals with high commercial value, including bio-fuels, fine chemicals, and other commodities [1][2][3]. For some products, e.g. isobutanol [4], production from microorganisms is more reliable and effective than production from fossil source. Bioproduction technologies are rapidly developed by the supports of metabolic engineering and synthetic biology. Metabolic engineering can optimize metabolic pathways by adding or deleting of metabolic steps, and synthetic biology can construct a new metabolic pathway by biological elements [5]. Metabolic engineering and synthetic biology enable the bioproduction of various chemicals and increased production efficiency.

Medium-chain alpha-olefins (MCAOs) are important industrial compounds with high value and wide usage, for example, they can be used for “drop-in” chemicals in next generation biofuel, and precursors of commodities [6]. Nowadays, the main source of MCAOs is petroleum. MCAOs are derived from petrochemical processes, which is an efficient way to produce MCAOs, but with many environmental problems. Hence, an alternative production of MCAOs is necessary. Microbial biosynthesis with the powers of metabolic engineering and synthetic biology is a potential method for the production of MCAOs, since some MACOs-production enzymes are found and applied to microorganism hosts [7][8][9][10]. Previous studies have demonstrated the production of one MCAO 1-undecene in *Escherichia coli* and *Acinetobacter baylyi* ADP1, with titers of about 55 mg/l and 700 µg/l respectively [10][11].

A. baylyi ADP1 has many interesting properties in the context of MACO production. The growth properties of *A. baylyi* ADP1 are similar to the ones of *E. coli* such as simple cultivation requirements and rapid growth. In addition, *A. baylyi* ADP1 has two properties that can overcome shortages of *Escherichia coli* and make genetically modification simpler: natural transformation and homologous recombination [12][13]. Storage lipid production is quite rare property among microorganisms, but *A. baylyi* ADP1 is able to produce storage lipids. Thus, it can efficiently produce precursors for MACOs biosynthesis.

The aim of the thesis was to increase the production of 1-undecene and explore the potential of alpha-olefins production in *A. baylyi* ADP1 by different strategies of metabolic engineering and synthetic biology. In this thesis, three stages of experiments were conducted for achieving the aim. The first stage was the characterization of native alkene synthesis system. Thioesterase 'TesA can produce fatty acids and iron-dependent desaturase/decarboxylase UndA can produce 1-undecene [9][14]. Intracellular 1-undecene and fatty acids from *A*. *baylyi* ADP1 were measured by GC-MS. Additionally, the methods of fatty acids measurement and analysis were optimized in this stage. The second stage was exploring the effect of glyoxylate shunt deletion on 1-undecene production in *A*. *baylyi* ADP1. To block the glyoxylate shunt, the gene encoding for isocitrate lyase, *aceA*, was deleted. *A*. *baylyi* ADP1 Δ*aceA* strain was constructed to show the effect of glyoxylate shunt deletion. The third stage was exploring the olefins production of heterologous membrane-bound desaturase-like enzyme UndB in A. *baylyi* ADP1. Two *undB* strains were constructed and measured their capacity of production of 1-undecene, and other products of olefin were also analyzed by GC-MS.

2. THEORETICAL BACKGROUND

2.1 Metabolic engineering and synthetic biology

Since Neolithic age, microbes have been used to produce foods and beverages for humankind, for example, lactic acid for pickled food and alcoholic beverages. With the help of biotechnological development, not only foods and beverages were produced, but also many other products and chemicals were started to produce using natural pathways of microorganism. For example, penicillin is an antibiotic, which is a secondary metabolite produced by *Penicillium*. The genetic engineering was developed since Berg et al. created the first recombinant DNA molecules in 1972 [15]. Metabolic engineering was defined as a sub-discipline of genetic engineering in 1991 [16]. Because some of the organic chemical compounds are hard to synthesize with chemical methods, genetically engineered microbes provide an alternative method for the productions.

Metabolic engineering refers to the optimization of genetic and regulatory processes to increase carbon flow to target compounds via production pathway. The aim of metabolic engineering is to produce useful chemical compounds in industrial scale to solve many environmental problems and natural source limits, not only that, metabolically engineered organisms can utilize some worthless wastes as carbon source to grow and produce valuable compounds, for example, lignocellulose that normally will be burned can be used as carbon source for some microbes. Lignin has rich sources, which is from agricultural industries, and it is by-product from pulp and paper-making industries, and second generation biorefineries [17][18]. Such renewable biomass could be utilized by metabolically engineered organisms to produce bio-fuel, which can be alternative fossil fuels. Those bio-fuels include alcohols, alkanes, and fatty acid-based compounds [19].

Traditionally, to increase the target productions, the organisms are induced by chemicals or physical methods to obtain mutants. With the development of metabolic engineering, the metabolic pathways of microbes are analyzed, and the functions of related enzymes could be characterized. Nowadays, to improve the target production, there are several strategies for metabolic engineering: i) overexpression of related gene; ii) inhibition of competing metabolic reactions; iii) expression and production in the heterologous host; iv) design and create enzymes by protein engineering [20]. The metabolic engineering was developed on the basis of genetic engineering. Metabolic engineering can be used to optimize the carbon flow by modification of metabolic pathway. The modification can increase the production because it adjusts the carbon flow from the cell growth to the

production. For instance, reversed β-oxidation pathway was engineered in *E.coli*, in which the production of longer-chain linear n-alcohols and extracellular long-chain fatty acids is more efficient than the native pathway [21].

However, traditional genetic engineering only allows scientists to use native elements to modify genes of those metabolically engineered organisms. Thus, some of the natural limits (e.g. complexity and versatility of biological system) restrict the development of metabolic engineering to obtain higher rate and yield of production. Synthetic biology is a solution to overcome those natural limitations. Synthetic biology allows to design and construct novel biological basic elements, gene networks, and pathway engineering, and it is a mature technology with many useful applications [22][23][24]. The standardization is the key point of synthetic biology, as it provides a faster and easier approach to design and construct novel components of biology [25]. The novel constructed biological components by synthetic biology can be inserted to host microbes, which solves many limits of metabolic engineering, and those novel biological components can be optimized by metabolic engineering. DNA elements such as promoters, repressors genes, and operons can be designed and built by synthetic biology with a reasonable cost.

In the last few decades, with the rapid development of synthetic biology, valuable applications are developed increasingly. One valuable application is biosensor that can for example contain engineered strains producing measurable signal in special condition. Lux operon of *Aliivibrio fischeri* [26] is an excellent gene expression system, which is applied to many biosensors. Green fluorescent protein (GFP) also can be used as a fluorescence reporter for biosensors [27]. For example, a twin-layer biosensor was designed to monitor alkane production in *A. baylyi* ADP1 [28]. An opioid biosynthesis pathway was designed and expressed in yeast [30], this research provides an alternative opioid production for farming of opium poppies. In addition, synthetic biology supports protein engineering: the applications include the construction of novel protein, optimization of protein structure and function. As an example from gene-level protein fusion, production of muconic acid was improved in engineered *E. coli* by overexpression of a fusion protein (AroC and MenF). A special gene sequence was inserted between AroC and MenF, which linked the two protein together [29].

The five challenges that restrict the development of synthetic biology are listed as follows. i) Many biological parts are not characterized [30]. For example, many standard biological parts are created and registered. However, some parts, such as promotors, are still not characterized. They may be not suitable for some laboratory strains. ii) The circuitry is unpredictable [30]. In other words, the combination of characterized parts may not work. iii) The complexity is unwieldy [30]. Following the increasing of circuits, the

processes of tests and constructions become more and more complex. For instance, over 10 genes are used to produce a precursor of artemisinin [31]. The processes of this work cost a lot of time. iv) Variability crashes the system [30]. Many factors e.g. growth condition can cause random noises to the cells. Those noises could make genes mutate, then break the function of circuits. v) Many biological parts are incompatible [30]. The previous research shows that a simple circuit may cause complex behaviors in the host cells [32]. The behaviors include the negative effect on cell growth and slow-down the production of protein. Those challenges have had a negative impact on the development of synthetic biology, some of those are hard to solve. However, with the development of biotechnology and bioengineering, those problems could be solved soon in the future. Some possible solutions for those challenges have already found out: computer modeling [33] can be used to solve the unpredictable circuitry; physical isolation of synthetic network [30] can solve the incompatible parts; the accurate DNA-replication machinery [30] can be applied to make circuits more stable.

Since cell factories that are based on metabolic engineering and synthetic biology can produce valuable chemical compounds with a fast cycle and reasonable cost, recently, more and more industries are showing an interest for those platform organisms [4]. The goal of those cell factories is to utilize renewable sources and produce valuable productions that are hard to produce by chemical synthesis and extraction from nature. Hence, the target of those cell factories is commercialization. Several companies have already utilized those cell factories engineered by such advanced biotechnology for the production of valuable chemicals. The main products include fuels, chemicals, foods and pharmaceuticals [4]. There are many commercial-scale productions by cell factories, which include lactic acid and farnesene (as chemicals), biodiesel and cellulosic bioethanol (as biofuels), valencene and vanillin (as foods), artemisinin and antibiotic cephalexin (as pharms) [4].

An excellent cell factory not only requires high production titer, rate, and yield (TRY), but also good adaptability. Those adaptability involves many fields, such as osmotic tolerance and pH tolerance. As known cell factories, *Saccharomyces cerevisiae* and *Escherichia coli* have good properties and they are both well-studied strains. Engineered *S. cerevisiae* and *E. coli* are used widely in many factories [4]. A concept of the iterative loop can be applied for design an industrial strain [34]. The iterative loop (Figure 1) includes requirements and goals of the cell factory, for example, the metabolic pathway should be optimized, and the physiology of the strain should be characterized [35], and the goals can be for example high titer, rate, yield, and robustness[4].

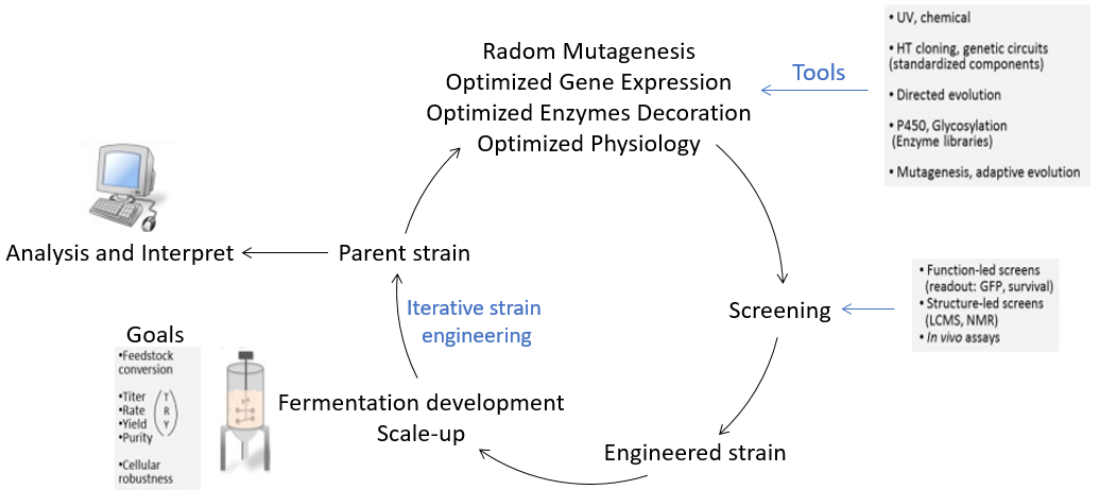

Figure 1. *Schematic of iterative loop of cell factory development process, requirements, approaches, tools, and goals. Picture modified from [4].*

2.2 Production of fatty acid-derived chemicals

In natural microbes, there are two main pathways for the production of fatty acid-derived chemicals: fatty acid pathway and CoA-dependent reverse β-oxidation pathway [36]. Several long alkyl chain molecules can be synthesized from fatty acid metabolism, such as fatty aldehydes, fatty alcohols, fatty acyl ethyl esters, wax esters, and alkanes. Those fatty acid-derived chemicals are components of membrane lipid, or they can be also used as storage components in natural organisms. Such chemicals can be used not only for industry as biofuels, but also as intermediate compounds for synthesis. For example, fatty acid-derived biofuels and chemicals have been produced by engineered *E.coli and S.cerevisiae* [37][38]. Another example is that because fatty aldehyde is the intermediate compounds of wax ester biosynthesis, improvement of fatty aldehyde production also improved the production of wax ester by overexpression a native fatty acyl-CoA/ACP reductase Acr1 in *A. baylyi* ADP1 [39]. Fatty acid pathway starts from a common intermediate acetyl-CoA. ATP and NADPH are the main cofactors needed for fatty acid biosynthesis. The final product of fatty acid biosynthesis is fatty acyl-ACP. Fatty acyl-ACP can cause inhibition to related enzymes in fatty acid biosynthesis pathway, resulting in feedback regulation to control the biosynthesis process in *E.coli* [40][41].

Fatty acids can be synthesized from fatty acyl-ACP by thioesterase. Fatty acyl-CoA ligase catalyzes the conversion of free fatty acid to corresponding carbon atoms fatty acyl-

CoA. Those compounds are important intermediates of fatty acid-derived chemicals, various fatty acid-derived chemicals can be synthesized by esterification, reduction, decarbonylation, etc. with different enzymes (figure 2).

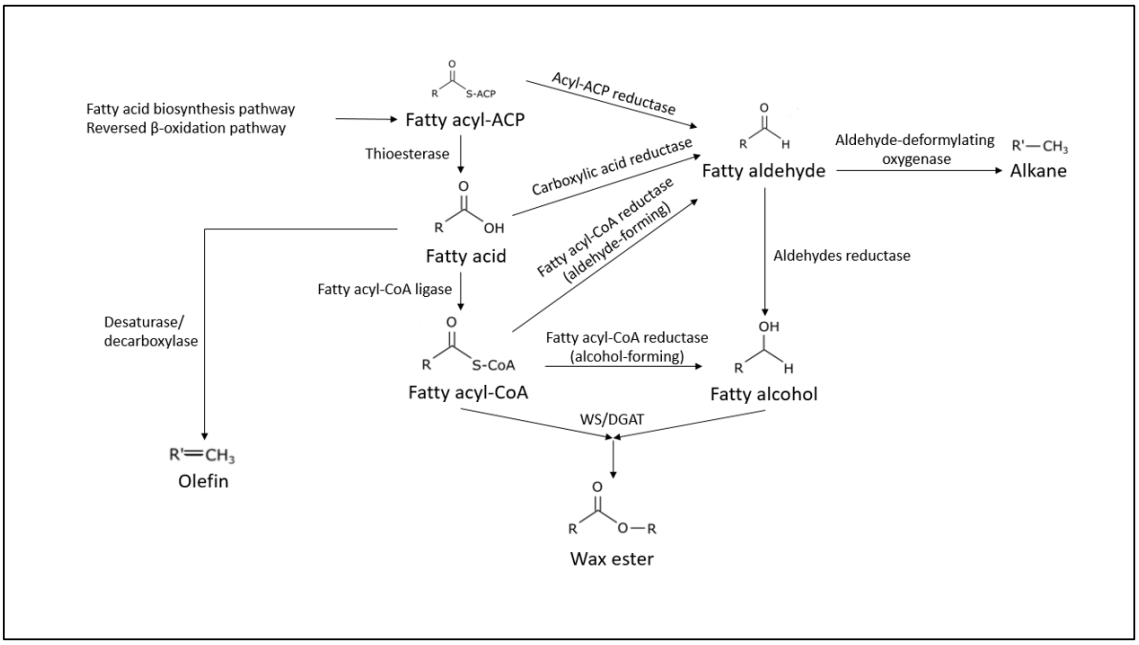

Figure 2. *Example of production of fatty acid-derived chemicals and enzymes for the synthesis reactions by fatty acid biosynthesis pathway and reverse β-oxidation pathway. ACP - Acyl carrier protein; CoA - Coenzyme A; WS/DGAT – wax esters synthase/acyl-CoA:diacylglycerol acyltransferase; R - acyl chain with n carbon atoms; R' - acyl chain with n-1 carbon atoms.*

Fatty aldehydes are the important key intermediate compounds for the production of many fatty acid-derived chemicals, which is also a fatty acid-derived chemical. There are multiple synthesis routes for the production of fatty aldehydes, they can obtained by reductase from fatty acyl-ACP, free fatty acid, and fatty acyl-CoA [42][43]. Additionally, previous research has shown that fatty aldehydes can be synthesized directly from acyl-CoA by reductase Acr1 in *A. baylyi* ADP1 [44]. Fatty aldehydes are key compounds in its natural metabolic pathway of the production of wax ester. In other word, *A. baylyi* ADP1 has natural ability to produce fatty aldehydes, which means that *A. baylyi* ADP1 has the potential to produce other valuable fatty acid-derived chemicals by the heterologous expression [45].

Fatty alcohols are another kind of fatty acid-derived chemical intermediate which can be synthesized directly from fatty acyl-CoA by fatty acyl-CoA reductase or synthesized from

fatty aldehydes by aldehydes reductase. Fatty alcohols have many commercial applications. Short-chain (C11-C14) fatty alcohols can be used to make detergents, lubricants, surfactants, etc. Long-chain (C16-C22) fatty alcohols are the potential intermediate compounds in cosmetics or food industries [46]. The main source of fatty alcohols is chemical synthesis by petrochemical feedstocks, and fatty acids from natural organisms (e.g. coconut, palm, tallow) [47]. A one possible way to obtain fatty alcohols is biosynthesis by microbes. For example, a recent research presents that engineered strains produced 12.5 g/L fatty alcohol in bioreactors [48].

Alkanes are another valuable fatty acid-derived chemical, which is the predominant components of liquid fuels. Biologically produced alkanes are considering for replacing fossil fuels in diesel or jet fuels [49][50]. In nature, various species are found with the capacity of alkanes production, such as bacteria, algae, plants [43][51][52]. The first research described the metabolic pathway of alkanes. The cyanobacterial pathway relies on acyl-ACP produced from fatty acid metabolic pathway. Acyl-ACP is first reduced by acyl-ACP reductase to fatty aldehydes and then fatty aldehydes are catalyzed to alkanes by aldehyde-deformylating oxygenase[43]. The alkane biosynthesis pathway also can be heterologously expressed in other host microbes [43]. Additionally, an enzyme, CER1, was explored from *Arabidopsis thaliana*, which can be used to replace aldehyde aldehyde-deformylating oxygenase from cyanobacteria [53][54].

Wax esters are a kind of storage lipid, which also has many industrial applications, including cosmetics, candles, lubricants, medicines, printing, coatings, polishes, food industries, etc. [55][56]. Wax esters as storage lipids can be found from various natural organisms, including plant jojoba (*Simmondsia chinensis*) [57], bacteria Acinetobacter and Marinobacter [58][59]. The wax esters in the market are mainly from jojoba oil, which is obtained from the desert plan jojoba. However, the production of wax esters is a slow process due to the growth of jojoba plant. The production via cell factory can be faster process than that from plants. An essential enzyme for the wax ester production in microbes is synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT) [60]. The mechanism of WS/DGAT is catalytic esterification reaction: fatty alcohols and fatty acyl-CoAs can be catalyzed to form wax esters by WS/DGAT.

A. baylyi ADP1 has been previously engineered to produce fatty acid-derived chemicals, such as wax esters [61], alkanes [62], alpha-olefins [11]. According to previous research, *A. baylyi* ADP1 produced 1-undecene by heterologous expression of a fatty acid decarboxylase UndA [11], which is the cornerstone for this thesis to research alpha-olefins production.

2.3 *Acinetobacter baylyi* ADP1

Acinetobacter species is a gram-negative coccobacilli, which can be isolated from water, soil and living organisms. There are several characteristical properties of genus *Acinetobacter* bacterium: oxidase-negative, strictly aerobic, catalase positive and non-motile ("Acinetobacter" originates from "akinetos", this Greek word means "unable to move"). *Acinetobacter* species strain can be cultivated between 20°C to 30°C with simple media, and there are no growth factor requirements [12][63]. *Acinetobacter* species were defined to belong genus *Moraxella* at first in 1968 according to their cell shape, absence of flagella, G+C content of DNA and nutritional properties [64]. Nowadays, many strains of *Acinetobacter* species are characterized (table 1) [65]. Recently, *Acinetobacter* species give deep interest to researchers and medical clinicians, because of their pathogenic potential and biotechnical potential [65][66]. As a common nosocomial pathogen, *Acinetobacter baumanni* and its close relatives could infect those patients who are with underlying disease and poor immunity [65]. However, not every *Acinetobacter* species is pathogenic bacteria, some of them have the capacity to degrade wastes and environmental pollutants, and produce useful chemical productions with economical value [66]. Additional, some of *Acinetobacter* species became excellent genetical platform because of its special properties, such as *Acinetobacter baylyi* ADP1 [13].

Table 1. *Some of Acinetobacter species strains and their major source. Modified from* [65].

Species name	Major source
A. baumannii	Human clinical specimens
A. baylyi	Activated sludge, soil
A. beijerinckii	Soil, water
A. bereziniae	Human specimens, soil
A. bouvetii	Activated sludge
A. calcoaceticus	Soil, water
A. gerneri	Activated sludge
A. grimontii	Activated sludge
A. guillouiae	Human faeces, water, soil
A. gyllenbergii	Human specimens

Acinetobacter baylyi ADP1 originates from a mutant BD413. BD413 was obtained by UV-irradiation from BD4 (the meaning of BD is butanediol) isolation with mineral salts me-

dium (0.5% meso 2,3-butanediol as carbon source) by Taylor and Juni [67]. *Acinetobacter baylyi* ADP1 is non-pathogenic, nutritionally versatile soil bacterium, the major source from nature is soil and activated sludge [12][65]. The genome of *A. baylyi* ADP1 was totally sequenced, the length of *A. baylyi* ADP1's circular chromosome is 3598621 bp, which C+G content is 40.3% [12]. *A. baylyi* ADP1 has 3325 identified protein coding genes, 499 coding genes are defined as essential genes for growth, and catabolism related genes are about one fifth of the whole genome [12]. Comparing with *P. aeruginosa* and *P. putida* protein coding genes, protein coding genes of *A. baylyi* ADP1 is close to *P. aeruginosa* and *P. putida* (figure 3). However, there still different of their gene contents (table 3), for example, *A. baylyi* ADP1 has a smaller genome, and the G+C contents are different (*A. baylyi* ADP1: 40.3%, *P. aeruginosa:* 66.6%, *P. putida:* 61.6%) [12].

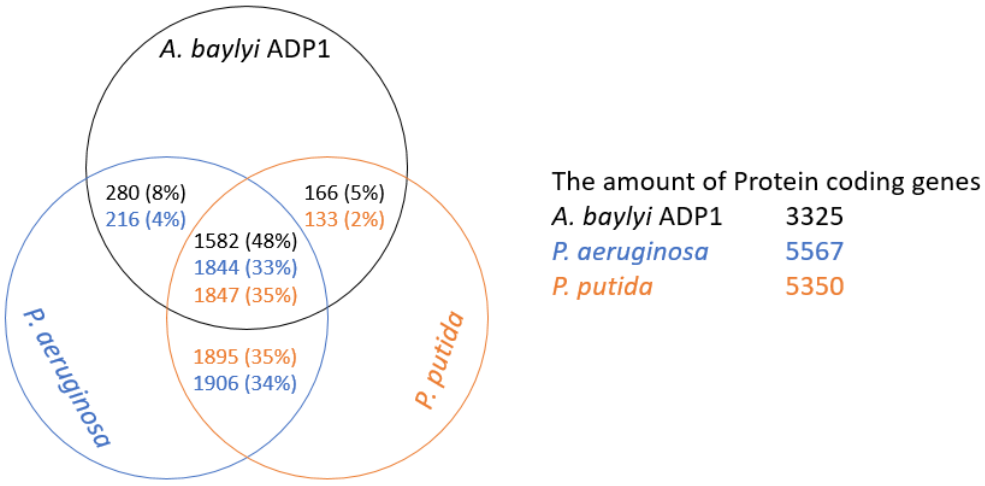

Figure 3. *The similarity of genes between A. baylyi ADP1, P. aeruginosa, and P. putida. The black, blue, and orange circle shows the genes of A. baylyi ADP1, P. aeruginosa, and P. putida respectively. The intersections between circles show the number and percentage of the same genes in different strains. Picture modified from [12].*

Table 2. *Comparison of General features between A. baylyi ADP1, P. aeruginosa, P. putida, and E. coli. Table modified from [12].*

General features	*ADP1*	*P.aeruginosa*	*P.putida*	*E.Coli*
Genome Size (Mb)	3.6	6.3	6.4	4.6
GC%	40.3	66.6	61.6	50.8
Nb CDS	3325	5567	5420	4273
% Coding	88.8	89	87.8	92
rRNA operon	7	4	7	7
tRNA	76	63	63	82
Known and putative protein (%)	62.6	65.9	62.0	80.5
CHP (%)	20.3	22.0	23.0	12.3
No homology (%)	13.9	11.1	15.0	7.2

Different from other non-motile *Acinetobacter* species, *A. baylyi* ADP1 has the capacity of motile. This motile capacity is based on its thin and thick pili on the cell surface. The motile capacity is called twitching motility. Those pilis are constructed by pilin (protein subunits) [68]. A research showed that a pilin-like competence factor, ComP, was related to natural transformation of *Acinetobacter* species [69]. For *A. baylyi* ADP1 and many other species, type-IV pili plays an important role in natural transformation and twitching motility [70]. There are two kinds of different situations for natural transformation and twitching: when pili catch DNA molecule, pili will pull the DNA molecule to the cell; when pili attach a surface for twitching motility, pili will pull the cell to the surface. Those weights require different forces from pili. The power source is from pili's ATPase retraction motor proteins reel [70][71]. A previous research presented that *A. baylyi* ADP1 employs pili during the process of natural transformation[70]. The natural transformation is an essential ability to bring the opportunity of gene manipulation for *A. baylyi* ADP1.

2.3.1 *A. baylyi* ADP1 as platform of genetic engineering

As an ideal organism for genetic engineering and biotechnology, *A. baylyi* ADP1 has its own excellent properties to outstand from other strains. Some properties of *A. baylyi* ADP1 are similar to that of another usual model organism *E. coli*. *A. baylyi* ADP1 shares many advantages with *E.coli*, those advantages make *E.coli* become an excellent genetic engineering platform [13]. First, both of *A. baylyi* ADP1 and *E. coli* have been already sequenced [12][72], which means that gene functions already have been determined. Thus, basic of metabolic pathway and organism properties also have been confirmed. In addition, they are same at some characteristical properties: aerobic, prototrophic, simple cultivation condition, rapid growth. However, *A. baylyi* ADP1 has several characteristics that are useful for genetic engineering. Comparing with *A. baylyi* ADP1 and *E. coli* (table 2), the length of the genome of *A. baylyi* ADP1 is smaller than that of *E. coli*. It means that *A. baylyi* ADP1 is easier and more suitable for genetic manipulation. Two characteristics of *A. baylyi* ADP1 overcome the weak points of *E. coli*, and they are important for genetic manipulation.

First, *A. baylyi* ADP1 has the competence of natural transformation [73], which means the cell could accept and acquire new genes [74]. Juni & Janik described the competence of natural transformation of *A. baylyi* ADP1 [75]. The mechanism of natural transformation of *A. baylyi* ADP1 is based on pili, and the pilin-like competence factor [69][71]. *A. baylyi* ADP1 can absorb the extracellular DNA molecule (both circular and linear DNA) during the beginning of the exponential growth phase to the early stationary phase[76]. There is no requirement of carbon source in media for natural transformation. However,

the essential condition of media is divalent cations (Mg2+, Mn2+, Ca2+). Several conditions could affect the frequency during the culture. To utilize this competence of *A. baylyi* ADP1 for genetic engineering efficiently, following conditions of DNA need to be considered in the cultivation of strains. pH of media affects the frequency of natural transformation: alkaline media can increase the frequency; the frequency will be limited in acidic (lower than 6.5) media [76]. Additionally, the type of target DNA and selection marker [76], DNA homology and insertion location in the genome [77] also can affect the frequency of natural transformation of *A. baylyi* ADP1. Some methods such as electroporation are used in laboratory for transforming novel genes into *E. coli* because *E. coli* has no ability of natural transformation. Those methods could cause much more complex operation and potential wrong results.

Another advantage of *A. baylyi* ADP1 is the natural tendency of homologous recombination [78]. Homologous recombination is a process that two DNA molecules with similar sequence exchange. It is a major DNA repairment ability in bacteria. With this competence, modification to the genome, such as gene knocking-out and replacement, becomes much more easier [13]. About *E. coli*, the modification of genome must use more complex tools. For example, some phage tools are used to modify the genome of *E. coli*.

Moreover, *A. baylyi* ADP1 can produce fatty aldehydes naturally. Thus, *A. baylyi* ADP1 has the potential capacity to produce many oleochemicals with few heterologous enzyme expressions. In contrast to *A. baylyi* ADP1's natural fatty aldehydes production, *E.coli* cannot produce fatty aldehydes naturally. Therefore, one or more heterologous enzymes should be expressed if *E.coli* will be used for some oleochemicals production. For example, the production of wax ester in *E.coli* was conducted by heterologous co-expression with acyl-CoA reductase gene and WS/DGAT gene [79].

2.3.2 Metabolism of *A. baylyi* ADP1

Nutritionally versatile bacterium *A. baylyi* ADP1 can utilize various carbon sources for its metabolism, such as aliphatic acids, aromatic acids, and ferulate (lignin-derived compounds) [11][80][81]. However, due to the lack of Embden-Meyerhof-Parnas (EMP) pathway enzymes, the only sugar that *A. baylyi* ADP1 can metabolize for cell growth is glucose [67]. Entner–Doudoroff (ED) pathway is the reaction pathway from glucose to pyruvate, which is found widely in bacteria. ED pathway was first reported in 1952, a modified ED glycolysis can utilize and metabolize glucose in *Pseudomonas saccharophila* [82].

ED pathway in *A. baylyi* ADP1 (figure 4) starts on an enzyme that binds with the cell membrane, glucose dehydrogenase, which oxidizes glucose to gluconolactone. Then,

gluconolactone is catalysed to gluconate by gluconolactonase, and release H^+. The production of H^+ in this step causes pH increasing of media. The above steps are conducted in outside of cells (extracellular steps). After the formation of gluconate, gluconate is transported into cells (intracellular steps), which is phosphorylated to 6-phosphogluconate. 2-keto-3-deoxy-6-phosphoglucanate is formed from 6-phosphogluconate by 6-phosphogluconate dehydratase. And then 2-keto-3-deoxy-6-phosphoglucanate is converted to pyruvate and glyceraldehyde-3-phosphate by aldolase. Pyruvate is converted to acetyl-CoA and is used by *A. baylyi* ADP1 for TCA cycle or fatty acid metabolic pathway. Glyceraldehyde-3-phosphate is converted to phosphoenolpyruvate. Due to the lack of pyruvate kinase, phosphoenolpyruvate cannot be converted to pyruvate directly in *A. baylyi* ADP1. A pathway was discovered to offset the impact of the lack of pyruvate kinase: phosphoenolpyruvate can be converted to oxaloacetate, then to malate, and malate can be converted to pyruvate [83]. However, a research shows that all glyceraldehyde-3-phosphate from ED pathway is used for exopolysaccharide synthesis by the radiolabelling method [84].

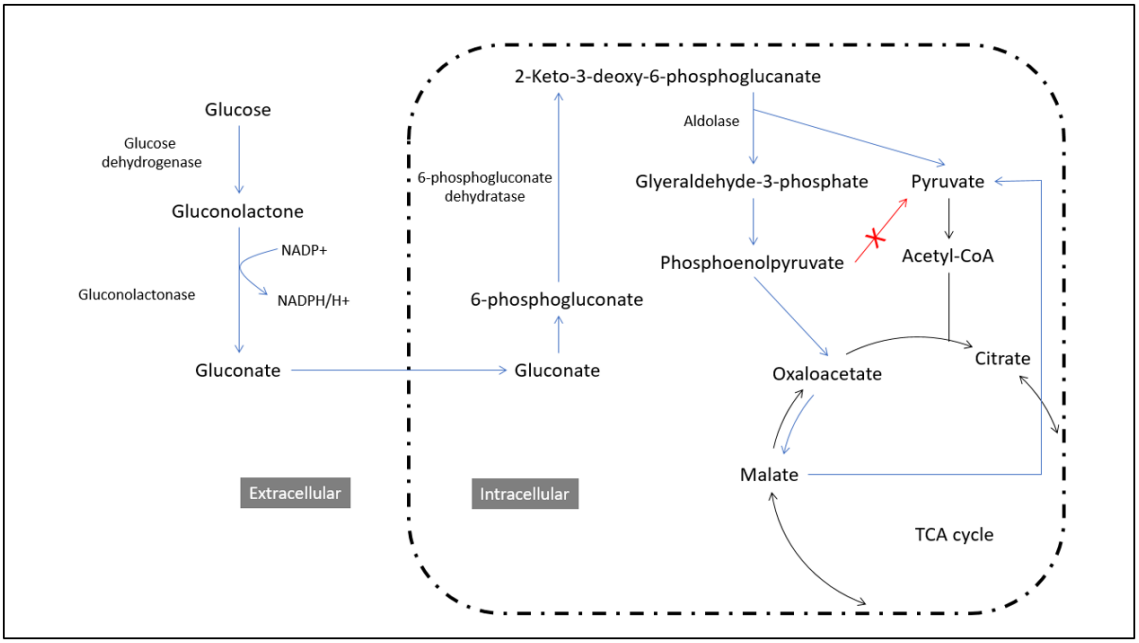

Figure 4. *Diagram of Entner–Doudoroff (ED) pathway in A. baylyi ADP1. The ED pathway is illustrated by blue arrows and the lack of pyruvate kinase is highlighted with a red arrow.*

2.3.3 Applications of *A. baylyi* ADP1 in bioengineering

As an excellent platform for genetic engineering, *A. baylyi* ADP1 has many applications of bioengineering and biotechnologies, such as the production of chemicals, bioremediation, and biosensors [66]. Bioengineering studies in *A. baylyi* ADP1 solved many problems, and the practicality of *A. baylyi* ADP1 on industrial purpose is rapid increasing [85].

One of the important applications of *A. baylyi* ADP1 is the production of was esters. *Acinetobacter* species are known as a microbe that produces wax esters as storage lipids [58]. Hence, *A. baylyi* ADP1 can be used for the bioproduction of wax esters. Santala et al. reconstructed the pathway of wax ester production in *A. baylyi* ADP1 in 2014 [61]. The main product of native *A. baylyi* ADP1 fatty acyl-CoA reductase acr1 is C16-C18 fatty aldehydes, while the wax esters will be C16-C18. Since shorter carbon-chain (C12-C16) wax esters are better than longer-chain wax esters in industrial uses, Santala et al. replaced to LuxCDE complex. The LuxCDE complex can increase shorter carbon-chain (C12-C16) wax esters production. In 2017, Lehtinen et al. reported a two-step method to produce wax esters from carbon dioxide [86]. The first step is that *Sporomusa ovata* converted carbon dioxide to acetate by microbial electrosynthesis technology. Then acetate is utilized by *A. baylyi* ADP1 as the carbon source to produce long-chain alkyl ester (second step), such as wax ester. This research demonstrated carbon dioxide and electricity can be utilized by bacteria as sole carbon source to produce long-chain chemical compounds.

Triacylglycerols can be produced by *A. baylyi* ADP1. Santala et al. increased triacylglycerols production 5.6-fold more than wild type of *A. baylyi* ADP1 by the construction of an engineered *A. baylyi* ADP1 strain with four genes deletion [87]. Silico predictions were used to simulate the effect of gene deletion. This research demonstrated genes deletion can increase triacylglycerols production, and *A. baylyi* ADP1 can be used for the metabolic engineering model system.

A. baylyi ADP1 has been studied to produce alka(e)nes. Lehtinen et al. reported an alkane production system with *Acetobacterium woodii* and *A. baylyi* ADP1 [62]. *Acetobacterium woodii* converts hydrogen and carbon dioxide to acetate. Then engineered *A. baylyi* ADP1 with expression cassette converts acetate to alkanes. Since natural *A. baylyi* ADP1 is not an alkane producer, heterologous expression of acyl-ACP reductase gene and aldehyde deformylating oxygenase gene was applied in the engineered *A. baylyi* ADP1. Those enzymes are efficient in alkane production in *A. baylyi* ADP1. 1-Alkene, e.g. 1-undecene, can be produced by *A. baylyi* ADP1 [11]. Luo et al. inserted

the genes of two heterologous enzymes to *A. baylyi* ADP1 with high ferulate toxic resistance after adaptive laboratory evolution. The engineered *A. baylyi* ADP1 can utilize a lignin-derived model compound ferulate to produce 1-alkene. This is the first research that demonstrated the lignin-derived model compound can be used as a sustainable resource for alkene production.

A. baylyi ADP1 also can be used as a sentinel. Horizontal gene transfer (HGT) is a potential risk from genetically modified organisms (GMO). HGT could contaminate other non-GMO in gene level. For example, antibiotic resistance could be transferred from GMO bacteria to other non-GMO bacteria. *A. baylyi* ADP1, as a sentinel, improved the researches of HGT because of its properties: *A. baylyi* ADP1 has natural transformation capacity. The competence of natural transformation causes that native DNA of *A. baylyi* ADP1 cannot be discriminated with foreign DNA. The transformation assay system based on *A. baylyi* ADP1 was developed for detecting HGT. One of HGT sentinel system is based on green fluorescent protein (GFP) expression in *A. baylyi* ADP1, which indicate natural transformation by fluorescent detection [88]. Moreover, *A. baylyi* ADP1 can be used for GHT detection from commercialized GMO. For example, kanamycin-resistance gene (nptII) from genetically modified plants e.g. potato and papaya can be detected by *A. baylyi* ADP1 [89].

2.4 Alpha-olefins

Olefin, also known as alkene, is a kind of organic chemical compound with carbon and hydrogen atoms. It is an unsaturated hydrocarbon. The difference between olefins and alkanes is that olefins have at least one carbon-carbon double bond. The general formula of olefin is C_nH_{2n}. Lower monoolefins, e.g. ethylene and propylene, are the key chemicals of the petrochemical industry. The lower monoolefins can be used to produce indispensable commodities. For example, industrial polymerization can convert ethylene to polyethylene (plastics). Longer-chain olefins also have important industrial applications such as biofuel or precursors of value chemicals. Based on the chemical structures, olefin has several formations. According to the number of carbon-carbon double bond, olefin can be divided to diene (2 carbon-carbon double bond), triene (3 carbon-carbon double bond), and so on. According to its molecular structure, olefins can be divided to cyclic or linear (acyclic or aliphatic) olefins. Alpha-olefin (1-alkene) is linear olefin that containing one carbon-carbon double bond located at position 1. As terminal alkene hydrocarbons and platform chemicals, alpha-olefins play an important role on purpose of industries. Due to the terminal double bond, alpha-olefins can be readily reacted for derivatization and polymerization.

Chain lengths of 8 to 10 bring many excellent properties to olefins. The medium chain length olefins can be used as "drop-in" biofuel with outstanding properties. Comparing with long-chain biofuel, medium-chain biofuel has a low freezing point, which could make medium-chain biofuels suitable for cold weather. In addition, the energy density of medium-chain biofuel is higher than that of short-chain biofuel, which is high efficiency as a fuel.

Particularly, medium-chain alpha-olefins (MCAOs) are attractive chemicals because MCAOs have many advantages to become optimal platform chemicals. MCAOs can be converted to other valuable chemicals or fine chemicals, such as detergents, cosmetic products, greases, lubricants, and plasticizers. In addition to the above commodities, “drop-in” chemicals in next generation biofuel is an attractive application of MCAOs. MCAOs as biofuel have many advantages to replace petroleum-based fuels. For example, defined chain length olefins-based biofuel has high energy density than petroleum-based fuels, and olefins-based biofuel can be applied to existing fuel equipment [36]. 1-Undecene is a 11-carbon alpha-olefin, unsaturated aliphatic hydrocarbon. 1-undecene attracts the most attention in the industrial field, because of the excellent properties and potential industrial applications of MCAOs. Under room temperature, 1-undecene is a colorless liquid with mild odor, the density is lower than water. Like other MCAOs, 1-undecene insoluble in water, while soluble in ether, chloroform, ethyl acetate, etc. Those organic chemicals can be used as the solvent for measurement and analysis of 1-undecene. The chemical category of 1-undecene is fatty acyls.

Alpha-olefins can be found in many organisms in nature, such as plants and microorganisms. Alpha-olefins play the important metabolic role in microorganisms that related to plants, and it may provide survival assistance for hosts that produce alpha-olefins [90]. Alpha-olefins, e.g.1-undecene, can be found from plants. Usually, the location of 1-undecene in plants is cell membrane, which can be extracted from the oil of the plants, e.g. oil from *Petasites japonicus*. In addition to plants, alpha-olefins also can be found from microorganisms, the main native producer is prokaryotes. Alpha-olefins are not common in microorganisms. Most of the microorganisms are not the natural producer of alpha-olefins, and other natural alpha-olefins prokaryotes producers only produce trace amounts of alpha-olefins. According to previous reports, alpha-olefins can be found from some strains of *Jeotgalicoccus* [7], *Pseudomonas* [9], and *Synechococcus* [8]. 1-Undecene is an important biological marker of *P. aeruginosa*, which can be clinic verification of *Pseudomonas* strains infection [91]. The strains of Genu *Pseudomonas* that can produce 1-undecene include *P. aeruginosa*, *Pseudomonas fluorescens*, etc. *A. baylyi* ADP1

is also a native producer of 1-undecene, which can produce trace amounts of 1-undecene ([11] and this thesis Results and Discussion). The enzyme that produces 1-undecene is not identified, but that UndA homologs exist in *Acinetobacter spp*. was reported [9].

Due to high value of alpha-olefins, the industrial demands of alpha-olefins are increasing. Currently, alpha-olefins e.g. 1-undecene were derived from petroleum. For example, they can be produced from petroleum by thermal cracking, fluid catalytic cracking, and hydrocracking. The petroleum limits the olefin industries because it is an expensive and non-renewable source. Additionally, petrochemical industries can cause unavoidable environmental issue. Hence, following the foreseeable shortage of fossil fuel and the importance of environmental protection, an alternative source of alpha-olefins must be found. The production of alpha-olefins from plants has many limitations, because plant oils containing alpha-olefins are always used as food. It is a method with high cost to produce alpha-olefins. In addition, long growth periods of plants are also a limiting factor. Therefore, plants as the source are suitable of the production of alpha-olefins. On the other hand, microbial biosynthesis based on cell factories is a potential alternative supply route of alpha-olefins. Some platform strains can utilize renewable materials as feedstock. Long-chain olefins biosynthesis pathways and the key enzymes of olefins production in microbes are reported in previous research [6][7][8][9][10], which support microbial biosynthesis of alpha-olefins. However, the challenges also exist because native producer of alpha-olefins only produces trace amounts of alpha-olefins. Hence, a suitable cell factory must be explored. Additionally, based on the optimal cell factory, the biosynthesis pathway, and key enzyme should be optimized to increase the production of alpha-olefins.

2.4.1 Alpha-olefins production enzymes

Few enzymes that produce long-chain alpha-olefins were found from its natural producer. So far, four enzymes were determined to produce long-chain alpha-olefins (or terminal olefins) according to previous reports: i) OleTJE; ii) multidomain polyketide synthase (PKS); iii) UndA; iv) UndB.

$OleT_{JE}$ is a terminal olefin-forming fatty acid decarboxylase that was isolated from *Jeotgalicoccus* spp. ATCC 8456. $OleT_{JE}$ is a P450 enzyme of cyp152 family [7]. This enzyme can convert long-chain fatty acids to corresponding alpha-olefins. Except for alpha-olefins, $OleT_{JE}$ can produce α- and β-hydroxy fatty acids also. For *Jeotgalicoccus* spp., 18-methyl-1-nonadecene and 17-methyl-1-nonadecene are the main terminal olefin productions. The carbon length of the main terminal olefin products of $OleT_{JE}$ is from 12 to 20.

$OleT_{JE}$ is peroxygenase because it belongs to cyp152 family, and H_2O_2 is the main source of electron for $OleT_{JE}$ [92]. A possible reaction of $OleT_{JE}$ is shown in figure 5 A. $OleT_{JE}$ was expressed in other excellent laboratory strains e.g. *E. coli* and *S. cerevisiae*, which obtained high production of olefins by metabolic engineering strategies [93][94].

Multidomain polyketide synthase (PKS) was found from cyanobacterium *Synechococcus* spp. strain PCC 7002. PKS is similar to type I polyketide synthase. This enzyme produces terminal olefins by an elongation decarboxylation mechanism. The possible substrate of PKS is fatty acyl-ACP. The carbon length of main olefin products is 18. Figure 5 B shows the proposed reaction of KPS. KPS has the potential of terminal olefins production because of its combinatorial nature. KPS can be designed to produce shorter-chain diesel constituents-analogous olefins by modification of substrate specificity [8].

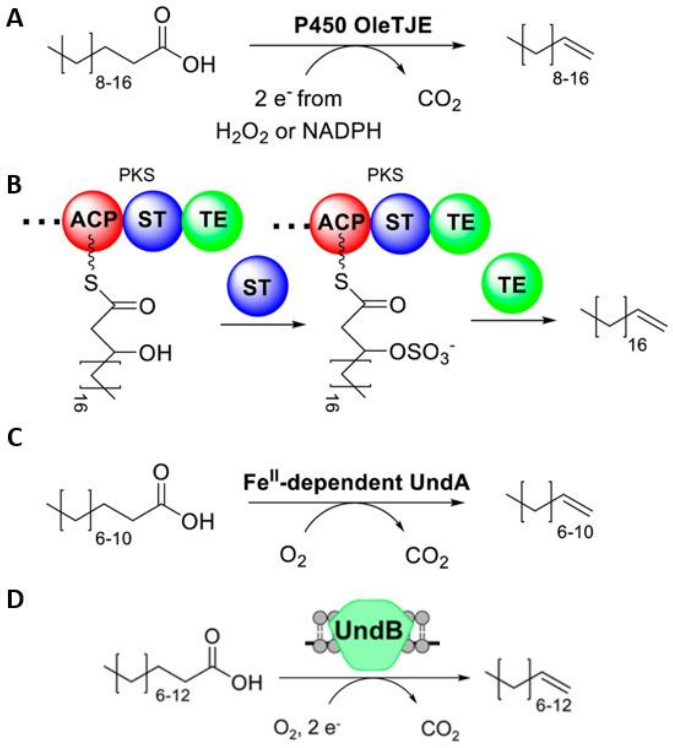

Figure 5. *Proposed enzymatic reactions of alpha-olefins production by* ***(A)*** *OleTJE* ***(B)*** *multidomain polyketide synthase (PKS)* ***(C)*** *UndA* ***(D)*** *UndB. ACP - acyl carrier protein, ST - sulfotransferase, TE - thioesterase. Modified from [10].*

Production of MCAO 1-undecene from *P. aeruginosa* relies on iron-dependent desaturase/decarboxylase UndA. UndA belongs to nonheme Fe(II) enzyme family. UndA can be activated by oxygen, its nonheme Fe(II)-dependent mechanism can transfer fatty acids to corresponding terminal olefins. The reaction of UndA is shown in figure 5 C. UndA

has a narrow substrate spectrum, the carbon length of its substrate is from 10 to 14. Thus, the products of UndA are medium-chain olefins. According to the structure of UndA, the depth of its substrate binding pocket limits the substrate length [9]. UndA has the potential for metabolic engineering to produce MCAOs. Rui et al. applied UndA to *E. coli*, 6 mg/l extracellular 1-undecene was obtained from *E. coli* with UndA homologs overexpression [9]. In addition to *E. coli*, Luo et al. proved the possibility of UndA expression in *A. baylyi* ADP1. A UndA homolog was expressed in *A. baylyi* ADP1, which obtain about 418 µg/l without thioesterase 'TesA expression, and about 694 µg/l with thioesterase 'TesA expression [11].

A membrane-bound desaturase-like enzyme UndB was reported as olefin production enzyme, which can convert lauric acid to 1-undecene [10]. Unlike UndA, UndB has a larger substrate spectrum. UndB can accept C6-C18 free fatty acids as its substrate to produce corresponding alpha-olefins [10]. The reaction mechanism is oxidative decarboxylation, which is shown in figure 5 D. Rui et al. tested three enzymes that can produce 1-undecene in *E. coli*: $OleT_{JE}$, a UndA homolog, and UndB. The study showed that the highest titer of 1-undecene was obtain from UndB in three enzymes, the 1-undecene titer of UndB was 3-fold than that of others at least [10]. Hence, UndB has the potential to increase production of 1-undecene in microbial biosynthesis system.

2.4.2 Microbial biosynthesis of precursors for olefins production

The natural producers of olefins produce only trace amounts of olefins. Heterologous expression of those olefins-production enzymes in other excellent cell factories that can produce precursors (substrates) for olefins-production enzymes can increase the production of olefins. Four native and modified pathways in microorganisms were reported to produce precursors (including medium-chain precursors): amino acid biosynthesis, terpene biosynthesis, reversed β-oxidation pathway, and fatty acid biosynthesis pathway [6]. Generally, amino acid biosynthesis and terpene biosynthesis can produce medium-chain chemical precursors, but they do not produce precursors for alpha-olefins production. Reversed β-oxidation pathway and fatty acid biosynthesis pathway usually are used to produce precursors that can be converted to MCAOs by heterologous enzyme expression. The products of fatty acyl-CoAs from reversed β-oxidation pathway and fatty acid biosynthesis pathway can be converted to corresponding free fatty acids by intracellular thioesterase. Then those free fatty acids can be converted to corresponding olefins by olefins-production enzymes e.g. $OleT_{JE}$, UndA, and UndB.

β-oxidation pathway converts fatty acyl-CoA to acetyl-CoA. Thus, reversal of β-oxidation pathway is the synthesis of fatty acyl-CoA. Comparing with fatty acid synthesis pathway, both pathways contain the same intermediate compounds. However, the enzymes and co-factors are different, which can avoid cross-talk in different pathways. Reversed β-oxidation pathway is a good metabolic platform pathway, which can produce precursors for downstream reactions with different carbon lengths. However, this pathway has some limits in metabolic engineering because the reversed β-oxidation pathway is a modified engineered pathway. To make β-oxidation pathway reverse, several genetic engineering operations should be applied to target strains such as genes removing. Additionally, another limitation of reversed β-oxidation pathway is its short turn of the cycle: normally, the cycle of reversed β-oxidation pathway only runs once, and the carbon length of the main product is 4 [6]. That engineered reversed β-oxidation pathway was applied to *S. cerevisiae* and *E. coli* to produce free fatty acids has been reported [21] [95].

Fatty acid synthesis includes two types: type I and II fatty acid synthesis (FAS). Type I FAS exists in eukaryotes, and type II FAS exists in bacteria. Type II FAS is one of the biosynthesis pathways that can produce long carbon chain fatty acyl-CoAs and fatty acyl-ACPs. Fatty acid biosynthesis pathway in Figure 6 shows the process of fatty acid biosynthesis pathway. In the beginning, acyl-CoA (C2) is transformed to malonyl-CoA (C3) by acyl-CoA carboxylase, which is called carboxylation. After that, there are four steps to elongate carbon chain: (1) condensation, (2) reduction, (3) dehydration, and (4) reduction. Firstly, condensation is that acyl-CoA and malonyl-CoA are catalyzed by 3-ketoacyl synthase to 3-ketoacyl-ACP/CoA, and a carbon dioxide molecule is released. Secondly, 3-ketoacyl-ACP/CoA is reduced to 3-hydroxyacyl-ACP/CoA by 3-ketoacyl reductase, NADPH as a reducing agent. Then, dehydration is water removing, one water molecule is removed by enoyl dehydratase, and 2,3-trans-enoyl-ACP/CoA is formed. Finally, acyl-ACP/CoA (C4) is produced from 2,3-trans-enoyl-ACP/CoA by enoyl reductase, NADPH is also reductant for this step. To continue the elongation, fatty acyl-ACP/CoA will be synthesized with malonyl-CoA to continue the elongation four processes. The elongation cycle will terminate when C16 acyl-ACP/CoA is produced.

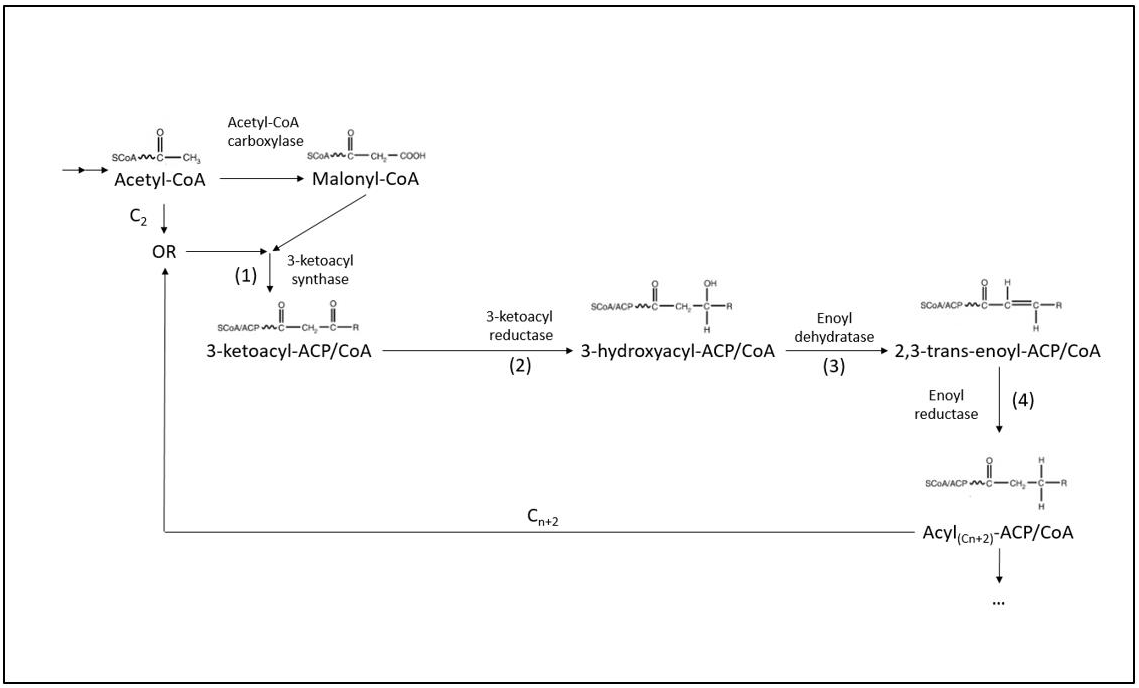

Figure 6. *Schematic representation of fatty acid metabolic pathway and carbon chain elongation. The elongation includes four processes, (1) condensation; (2) reduction; (3) dehydration; (4) reduction. ACP - Acyl carrier protein; CoA - Coenzyme A. Modified from* [96].

FAS is an important metabolic pathway for most organism, because the products from FAS play a critical metabolic role. Type II FAS is widely used for metabolic engineering to provide fatty acyl intermediate chemicals. Comparing to type I FAS, type II FAS can produce various fatty acids with different carbon lengths. Additionally, ACP intermediates in type II FAS are diffusible, which can be applied for other metabolic pathways.

2.4.3 Synthetic biology and metabolic engineering for microbial biosynthesis of medium-chain alpha-olefins

In this work, *A. baylyi* ADP1 was used to produce MCAO 1-undecene. Therefore, to produce 1-undecene, two factors are essential: i) 1-undecene production enzyme; ii) a pathway to provide precursors for 1-undecene production enzyme. $OleT_{JE}$, UndA, and UndB are the enzymes that can produce 1-undecene. $OleT_{JE}$ has a large substrate spectrum, which could produce various olefins, and the main production of $OleT_{JE}$ is C19 compounds [92]. Hence, $OleT_{JE}$ is not suitable for the production of 1-undecene in *A. baylyi* ADP1. UndA is the best choice for 1-undecene production in those three enzymes, because of its narrow substrate spectrum and the main product of 1-undecene. Expression of UndB in *A. baylyi* ADP1 is also attractive. UndB has a large substrate spectrum, which causes various non-target olefins production. Additionally, UndB is a membrane-

bound enzyme, which could inhibit cell growth [97]. On the other hand, according to pervious report, UndB has outstanding performance of 1-undecene production in *E. coli* [10]. Hence, it is worth to test the expression of UndB in *A. baylyi* ADP1. The substrates of UndA and UndB are free fatty acids. To produce 1-undecene, the substrate should be lauric acid. Native thioesterase in *A. baylyi* ADP1 can convert fatty acyl-ACPs and fatty acyl-CoAs to the corresponding free fatty acids. The type II FAS in *A. baylyi* ADP1 can produce medium-chain fatty acyl compounds as precursors. Because *A. baylyi* ADP1 has native fatty acid synthesis pathway (type II FAS), fatty acid synthesis pathway is the suitable precursors-provide pathway in *A. baylyi* ADP1.

To increase the production of 1-undecene in *A. baylyi* ADP1, increasing of 1-undecene precursors production is a potential method. ‘TesA was selected to co-express with UndA or UndB in *A. baylyi* ADP1. In metabolic engineering, one thioesterase is used to produce free fatty acids, which is TesA periplasmic form (called ‘TesA). TesA was found from *E.coli*, and it is engineered to remove its leader sequence of the periplasmic form [14]. The substrate of ‘TesA is fatty acyl-ACP and fatty acyl-CoA, main carbon length is 12 and 14. The expression of ‘TesA can provide more lauric acid to UndA and UndB, which is a strategy to increase the production of 1-undecene.

Another strategy of increasing 1-undecene is glyoxylate shunt deletion (figure 7). The key enzyme of glyoxylate shunt is AceA (ACIAD3383), which encoding for isocitrate lyase. To block glyoxylate shunt, the common operation is knock-out the gene *aceA* (Δ*aceA*). Glyoxylate shunt plays an important role in the growth of strains utilizing acetate as carbon source and non-glycolytic carbon sources. Without glyoxylate shunt, the strain cannot grow with acetate or non-glycolytic carbon sources as a single carbon source. A method to verify glyoxylate shunt deletion is to cultivate *aceA* deletion strain with acetate as a single carbon source. No cell growth means that glyoxylate shunt is deleted successfully. Glyoxylate shunt is a competitive pathway with fatty acid synthesis pathway. After blocking of glyoxylate shunt, more acetyl-CoA may flow to fatty acid synthesis pathway from cell growth substrate. Santala et al. increased the production of wax esters in *A. baylyi* ADP1 by inhibition of glyoxylate shunt [98]. In this work, glucose was used as a single carbon source for *A. baylyi* ADP1. The glyoxylate shunt is not essential because the alternative pathways can provide carbons for cell growth. Hence, theoretically, glyoxylate shunt blocking can increase production of 1-undecene without inhibition of cell growth.

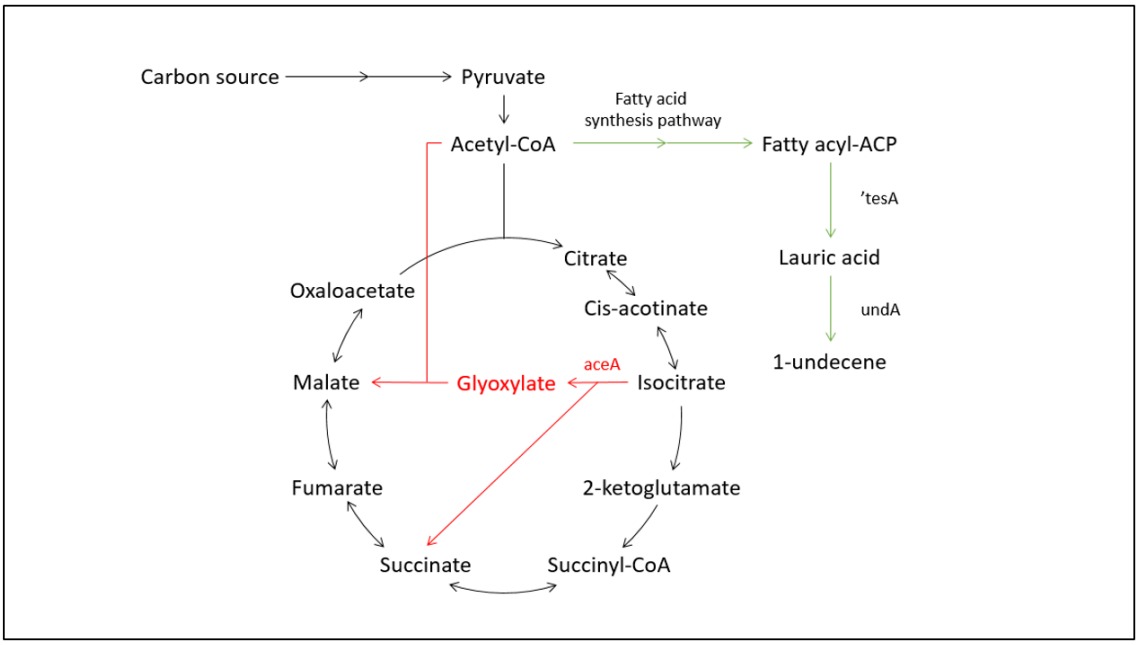

Figure 7. *Schematic representation of glyoxylate shunt deletion. The isocitrate lyase is encoded by aceA gene. Red arrows show the blocking of pathway due to aceA deletion and green arrows represent the carbon flow to the fatty acid synthesis pathway when glyoxylate shunt is blocked.*

3. RESEARCH METHODOLOGY AND MATERIALS

3.1 Strains

In this thesis, *E. coli* XL-1 (Stratagene, USA) strain was used for the storage of plasmids and the construction of plasmids. *A. baylyi* ADP1 strains were used for the production of olefins, which are listed in table 3. The wild type of *A. baylyi* ADP1 and *A. baylyi* ADP1 pBAV1C (empty plasmid) were used for controls.

Table 3. *A. baylyi ADP1 strains used in this thesis.*

Strain	Genotype	antibiotic resistance	Source
ADP1 WT	Wild type (DSM 24193), wt	N/A	
ADP1 p-E	wt + pBAV1C plasmid	chloramphenicol	[11]
ADP1 p-UA	wt + pBAV1C-chn-*undA*	chloramphenicol	[11]
ADP1 p-TAUA	wt + pBAV1C-chn-*'tesA -undA*	chloramphenicol	[11]
ADP1 Δ*aceA'*	Δ*aceA*::t5-*undA*-Spec^R, pBAV1K-ara-*'tesA*	spectinomycin, kanamycin	This work
ADP1 Δ*aceA*	Δ*aceA*::t5-*undA*-histag-Spec^R, pBAV1K-ara-*'tesA*	spectinomycin, kanamycin	This work
ADP1 p-UB	wt + pBAV1C-chn-*undB*	chloramphenicol	This work
ADP1 p-TAUB	wt + pBAV1C-chn-*'tesA -undB*	chloramphenicol	This work

3.2 Construction of strains

Several plasmids were constructed in this thesis, which included pUC57-*aceA* flanking-t5-*undA*-Spec^R-*aceA* flanking-Amp^R (pUC57-t5-*undA*), pBAV1C-chn-*undB*, and pBAV1C-chn-*'tesA-undB.* Those new constructed plasmids were transformed into wild type of *A. baylyi* ADP1. The method of plasmid constructions and strain constructions are described in this section.

3.2.1 Genetic engineering

PCR amplification

Target gene sequences, e.g. *undA* and *undB,* were amplified by PCR. PCR amplification also was also used for genome or plasmid verification. The compositions of PCR reaction included 0.5 µM forward primer, 0.5 µM reverse primer, 200 µM d (C/A/T/G)NTPs (Thermo-Scientific, USA), 1X Phusion Hot buffer (Thermo-Scientific, USA), 250 ng DNA

template (1-5 µl, depends on DNA concentration), and 1U Phusion Hot Start II (Thermo-Scientific, USA). The final volume of PCR reaction is 50 µl, which is filled by double-distilled sterile water. A control for PCR amplification is the same PCR reaction without DNA template. The PCR program is listed in table 4, and the primers that are used in this thesis were listed in table 5.

Table 4. *The program of PCR for amplification.*

Program	Temperature (°C)	Time (s)	Cycle
Initial denaturation	98	120	1
Denaturation	98	10	
Annealing	50	30/kb*	30
Elongation	72	45	
Final elongation	72	5	1
Pause	4	-	-

* The annealing time is 30 kb/s according to the instructions of manufacturer of Phusion Hot Start II.

Table 5. *Primers used in this thesis. Primers were from Invitrogen, Thermo Fisher Scientific, USA.*

Primer	Sequence (5'-3')	Desciption
CSL_F_19	TAAGTCTAGAGAAAGAGGAGAAACATATGATTGACGCATTTGTTCGTATCG	Upstream base pairing region of *undA*
CSL_R_19	TAAGCTCGAGTCAGCCCGCAGCCAAC	Downstream base pairing region of *undA*
CSR R 19_2	TAAGCTCGAGTTAGTGGTGATGGTGATGATGACCTGAACCGCCCGCAGCCAACGCC	Downstream base pairing region of *undA*
VS_15_168	TAATGAATTCGCGGCGGCCGCTTCTAGAGAAAGAGGAGAAATACTAGATGATTGACGCATTTGTTTATC	Upstream base pairing region of *undA*
1084V1	TTTTTCTATCATTCATTTTTAAGTC	For verification of *aceA* knocking out
1084V2	CTCAACATGATATGCACACTGC	For verification of *aceA* knocking out
tl15	TGGAATTCGCGGCCGCTTCTAGAGAAAGAGGGAAATACTAGATGGCGGACACGTTATTGATTCTGGG	Upstream base pairing region of *'tesA*
tl16	GTTTCTTCCTGCAGCGGCCGCTACTATATTATTATGAGTCATGATTTACTAAAGGCTGC	Downstream base pairing region of '*tesA*

After PCR amplification, the PCR product was verified by gel electrophoresis. The product was purified using PCR purification kit (Thermo-Scientific, USA) according to the instructions of manufacturer for downstream application. The DNA concentration was

quantified by NanoDrop2000 (Thermo-Scientific, USA) according to the instructions of manufacturer.

Restriction digestions

Traditional gene cloning method including restriction digestions and ligations were used for the construction of plasmids. In addition, the constructed plasmids were verified by restriction digestion analysis. The digestion reaction is shown in table 6, the reagents of digestion are from Thermo-Scientific, USA. Fast AP was not added when the digestion was done for verification. Double-distilled sterile water was used to fill digestion reaction to total volume. Digestion was done at 37°C incubation for 20 minutes. Control of digestion is the digestion reaction without restriction enzymes.

Table 6. *The components of digesting reactions.*

Components	PCR product	Plasmid DNA
10X FastDigest Green Buffer	2 µl	2 µl
DNA	0.2 µg	1 µg
Restriction enzyme (FastDigest enzyme)	1 µl each	1 µl each
Fast AP	-	1 ul
Total volume	30 µl	20 µl

The Digestion products were verified by gel electrophoresis. For downstream application, digestion products were purified by gel extraction with corresponding bands, GeneJET gel extraction kit (Thermo scientific, USA) was applied for gel extraction according to the instructions of manufacturer. After purification of digestion products, DNA concentration was quantified by NanoDrop2000.

Ligation

Insert DNA and linear vector DNA (from digestion products after purifications) were ligated by DNA ligase. The ligation reactions were consisting of 2.5U T4 DNA ligase (Thermo scientific, USA), 1x ligation buffer (Thermo scientific, USA), 50-400 ng linear vector DNA, and 50-500 ng insert DNA. The mass of insert DNA to vector DNA ratio was about 3 to 1. Control without insert DNA was prepared for ligation. The same volume double-distilled sterile water substituted insert DNA in the control. The ligation reactions were incubated overnight at room temperature. When ligation was done, the ligation reaction was incubated for 20 minutes at 65°C, which is for ligase inactivation. The ligation product was stored in 4°C until it was used for transformation.

Transformation in *E. coli*

New constructed plasmids were transformed into *E. coli* XL-1 competent cells. Ligation reaction was used for transformation directly. The part of ligation reaction (2 µl) was added to 40 µl *E. coli* XL-1 competent cells and the mixture was transferred to a pre-cooled electroporation cuvette. The above steps were done on ice. The transformation was done by electroporator BioRad Micropulser (Bio-Rad, USA) with Eco-1 program. After electroporation, 1 ml pre-warmed (37°C) sterile low salt LB medium (compositions see 3.3) was added to the electroporation cuvette, which can provide an optimal growth condition for surviving cells. The mixture from electroporation cuvette (100µL and 900µL) was plated to LA plates (compositions see 3.3) with corresponding antibiotics respectively following incubation at 37°C. The control ligation reaction was transformed with the same steps. A single colony from the plate was picked and inoculated to 5mL low salt LB medium supplemented with 0.4% (w/v) glucose and corresponding antibiotic at 37°C and 300 rpm, cultivating for overnight. Then a glycerol stock (total 1mL, 25% v/v sterile glycerol) was prepared and stored at -86°C.

Transformation in *A. baylyi* ADP1

The methodology of transformation in *A. baylyi* ADP1 is easier than *E. coli*, which does not need extra instruments and methods. The transformation of *A. baylyi* ADP1 is that adding DNA to the exponential phase of *A. baylyi* ADP1 cultivation. Wild type *A. baylyi* ADP1 was pre-cultured in 5 ml low salt LB medium supplemented with 1% (w/v) glucose at 30°C and 300 rpm overnight. A part of pre-culture (50 µl) was inoculated to 1 ml low salt LB medium supplemented with 1% (w/v) glucose. The cultivation was incubated at 30°C and 300 rpm for 2 hours, which the cell growth reaches exponential phase. Cultivation of the exponential phase was divided to two 0.5 ml aliquots in 14 ml cultivation tubes, and 1 µg DNA was added to one of the aliquots, another one was used as a control without DNA addition. Both aliquots were incubated at 30°C and 300 rpm for 3 hours, then 100 µl and 400 µl cultivation were plated to LA plates with corresponding antibiotic respectively following incubation at 30°C. A single colony from the plate was picked and inoculated to 5mL low salt LB medium supplemented with 0.4% (w/v) glucose and corresponding antibiotic at 30°C and 300 rpm following incubating for overnight. Then a glycerol stock (total 1 ml, 25% v/v sterile glycerol) was prepared and stored at -86°C.

DNA extractions

Extraction of plasmid DNA and genome DNA was done using Plasmid Miniprep Kit (Thermo-Scientific, USA) and GeneJET genomic DNA purification kit (Thermo-Scientific, USA) respectively according to the instructions of manufacturer.

3.2.2 Construction of Δ*aceA* strains

Two Δ*aceA* strains were constructed for this thesis, ADP1 Δ*aceA'* (no 1-undecene production) and ADP1 Δ*aceA.* The gene expression of *undA* was controlled under strong promoter T5, and *'tesA* gene was controlled under arabinose promoter. The construction steps were the same for both strains, while the primers for PCR amplification of *undA* sequence were different. The processes of the construction of Δ*aceA* strains was as follows.

Firstly, a plasmid of pUC57-t5-*undA* was constructed. Gene fragment of *undA* was amplified from pBAV1C-chn-*undA* (source from [11]) by PCR. For the construction of ADP1 Δ*aceA'* strain, the primers were CSL_F_19 and CSL_R_19, and the primers for ADP1 Δ*aceA* strain were VS_15_168 and CSR R 19_2 (primers details are shown in table 5).

Verified and purified PCR product was used for insert DNA, and plasmid pUC57-*aceA* flanking-t5-*acr1*-Spec^R-*aceA* flanking-Amp^R (source from [99]) was used for vector DNA. That restriction enzymes were used for digestion were FastDigest XhoI and XbaI. Digestion products were purified by gel extraction after the selection of correct fragments. After ligation, new constructed plasmid pUC57-t5-*undA* was transformed into *E. coli* XL-1. Plasmids were extracted from *E. coli* XL-1 pUC57-t5-*undA*, which was verified by digestion with FastDigest enzyme XhoI and XbaI. Verified plasmids were transformed into wild type *A. baylyi* ADP1. Due to the natural tendency of homologous recombination of *A. baylyi* ADP1, *aceA* in the genome of *A. baylyi* ADP1 was removed, meanwhile, t5-*undA*-Spec^R was replaced into its genome. The genome of *A. baylyi* ADP1 Δ*aceA*::t5-*undA*-Spec^R was extracted using GeneJET genomic DNA purification kit and the integration was verified by PCR amplification. The primers for verification were 1084 V1 and 1084 V2. A control sample was the genome from the wild type of *A. baylyi* ADP1. The length of PCR product from genome of *A. baylyi* ADP1 Δ*aceA*::t5-*undA*-Spec^R (2959 bp) should be longer than that of wild type (2490 bp).

After correct *A. baylyi* ADP1 Δ*aceA*::t5-*undA*-Spec^R strain was constructed, plasmid pBAV1K-ara-*'tesA* was transformed into *A. baylyi* ADP1 Δ*aceA*::t5-*undA*-Spec^R strain to construct *A. baylyi* ADP1 Δ*aceA*::t5-*undA*-Spec^R, pBAV1K-ara-*'tesA*. Plasmids pBAV1K-ara-*'tesA* were extracted from *A. baylyi* ADP1 Δ*aceA*::t5-*undA*-Spec^R, pBAV1K-ara-*'tesA*, which were verified by digestion with FastDigest enzyme EcoRI and PstI.

The ADP1 Δ*aceA'* strain was constructed first, after 1-undecene tests, there was no detected 1-undecene production from this strain. Hence, another strain ADP1 Δ*aceA* was constructed. Different with ADP1 Δ*aceA'*, the genotype of ADP1 Δ*aceA* is Δ*aceA*::t5-

undA-histag-Spec^R, pBAV1K-ara-*'tesA*. The primers provided a histag for *undA* gene, which can be used for potential protein extraction purposes.

3.2.3 Construction of *undB* strains

ADP1 pBAV1C-chn-*undB* and ADP1 pBAV1C-chn-*'tesA -undB* were constructed for exploring the effect of enzyme UndB. The UndB (Pmen_4370) is encoded by *undB* gene, which was on plasmid pUC57-*undB*-Amp^R (GenScript, USA). The plasmid contains suitable restriction enzyme sites; thus, PCR amplification was not necessary. The plasmid pUC57-*undB*-Amp^R was digested by FastDigest enzyme XbaI and PstI as insert DNA. And plasmid pBAV1C-chn and pBAV1C-chn-*'tesA* (source from [11]) were digested by FastDigest enzyme SpeI (BcuI) and PstI as vector DNA. The digestion products with correct bands were purified by gel extraction. After ligation, plasmid pBAV1C-chn-*undB* and pBAV1C-chn-*'tesA -undB* were transformed into *E. coli* XL-1. The plasmids from *E. coli* XL-1 was extracted and then the plasmids were verified by digestion with FastDigest enzyme XbaI and PstI. Verified plasmids were transformed into the wild type of *A. baylyi* ADP1. The verifications of ADP1 pBAV1C-chn-*undB* and ADP1 pBAV1C-chn-*'tesA -undB* were same with digestion verification of their corresponding *E. coli* XL-1 strains.

3.3 Media and cultivation

The composition of modified lysogeny broth (LB) medium was 10 g/l tryptone, 5 g/l yeast extract, 1 g/l NaCl. The composition of lysogeny broth-agar (LA) plate is 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, and 15 g/l agar, supplemented with 1% (w/v) glucose and corresponding antibiotic. MA/9 low salt medium contained 4.40 g/l disodium hydrogen phosphate, 3.40 g/l potassium dihydrogen phosphate, 1.00 g/l NH_4Cl, 0.008 g/l nitrilotriacetic acid, 1.00 g/l NaCl, 240.70 mg/l $MgSO_4$, 11.10 mg/l $CaCl_2$, and 0.50 mg/l $FeCl_3$. All mediums were sterilized by autoclaving.

The antibiotics were as follows: 25 µg/ml chloramphenicol, 25 µg/ml spectinomycin, 100 µg/ml kanamycin. The inducers were as follows: cyclohexanone (ACROS Organics, United Kingdom), and arabinose (ACROS Organics, China).

Biomass from cultivations was measured as optical density at absorbance 600nm using spectrophotometer Ultrospec 500pro (Amersham Biosciences, GE Healthcare Life Sciences, USA).

3.3.1 Cultivation of expression of *'tesA* and *undA* in *A. baylyi* ADP1

A small-scale cultivation and a large-scale cultivation were applied for the tests of expression of *'tesA* and *undA* in *A. baylyi* ADP1. The large-scale cultivation provided a large amount of biomass because methylation lipid analytics require the amount of biomass. The strains were used for this section as follows: ADP1 p-E (control), ADP1 p-UA, ADP1 p-TAUA. Pre-culture was that strains were inoculated to LB medium with 0.4% (w/v) glucose and chloramphenicol. The pre-cultivation was incubated at 30°C and 300 rpm for overnight.

Small-scale cultivations contained 5 ml cultivation volume in 14 ml cultivation tube at 25°C and 300 rpm. The cells from preculture were inoculated to MA/9 low salt medium supplemented with 5.0% (w/v) glucose, 0.2% (w/v) casein amino acid, and chloramphenicol (initial OD was 0.1). Cyclohexanone for inducer was added to cultivation except for cultivations of ADP1 p-E. The concentrations of cyclohexanone were 0mM, 0.0001mM, 0.0005mM, 0.005mM, and 0.1mM. The total cultivation time of small-scale cultivation was 17 hours. Parallel groups were used for each cultivation. Optical density was measured at the end-point of cultivation.

For large-scale cultivation, cells from pre-culture were inoculated to MA/9 low salt medium supplemented with 5.0% (w/v) glucose, 0.2% (w/v) casein amino acid, and 25ug/ml chloramphenicol (solve in ethanol) in 250 mL flask, the cultivation volume was 50mL. The initial OD_{600} is 0.1. Cyclohexanone as inducer was added to cultivation except for cultivations of ADP1 p-E, the concentrations of cyclohexanone were 0mM, 0.0001mM, 0.0005mM, and 0.1mM. The total cultivation time of small-scale cultivation was 3 hours and 15 minutes. Parallel groups were used for each cultivation. Optical density was measured at the end-point of cultivation.

The expression of *'tesA* and *undA* was determined by the production of fatty acids and 1-undecene, intracellular fatty acids were analyzed by TLC and GC-MS, and intracellular 1-undecene was analyzed by GC-MS.

3.3.2 Cultivation for *aceA* knock-out strain

1-Undecene production was compared by cultivations with ADP1 Δ*aceA* and wild type ADP1 strain in MA/9 low salt medium, cultivations of ADP1 Δ*aceA* were induced by different concentrations of arabinose for *'tesA* expression. The concentrations of arabinose (w/v) are as follows: 0%, 0.1%, 0.2%, 0.5%, 1.0%. The strains were inoculated and pre-

cultivated in 5mL low salt LB medium with 0.4% (w/v) glucose and 25 µg/mL spectinomycin, 100 µg/mL kanamycin at 30°C and 300 rpm for overnight. No antibiotic was added into the cultivations of wild type ADP1 (both pre-cultivation and cultivation follows). Cells were inoculated from pre-cultivations (initial OD_{600} was 0.1) into MA/9 low salt medium supplemented with 5.0% (w/v) glucose, 0.2% (w/v) casein amino acid, and100 µg/mL kanamycin, cultivation volume was 5mL in headspace screwtop 20mL clear vials (Agilent Technologies, Germany), at 25°C and 300 rpm. Arabinose as an inducer was added to cultivations of ADP1 Δ*aceA* when OD_{600} reached about 1.0. After induction, all cultivations were incubated for 2 hours, OD_{600} was measured, and a stir bar was put and a headspace cap (Agilent Technologies, USA) was sealed to each vial. And all cap-sealed vials were incubated at 25°C for overnight. Cultivation was performed in duplicate. Production of 1-undecene was analyzed using GC-MS from the headspace sample.

3.3.3 Cultivation for *undB* strains

The cultivation for exploring olefins production from *undB* strains was carried out with the two constructed strains, which are as follows: ADP1 p-UB, ADP1 p-TAUB. Wild type ADP1 strain was used as a control that was not added antibiotic and inducer. Additionally, ADP1 p-UA and ADP1 p-TAUA as positive control were applied for the cultivation to compare the production of 1-undecene with *undB* strains. The strains were pre-cultivated in 5 ml low salt LB medium with 0.4% (w/v) glucose and chloramphenicol at 30°C and 300 rpm for overnight. Cells were transferred from pre-cultivations (initial OD_{600} was 0.1) into 5 ml MA/9 low salt medium supplemented with 5.0% (w/v) glucose, 0.2% (w/v) casein amino acid, and chloramphenicol in headspace screwtop 20 ml clear vials and cultivated at 25°C and 300 rpm. 0.1 mM cyclohexanone for inducer was added to cultivations when the OD_{600} reached around 1.0. Cultivations were incubated for 2 hours after induction. Then optical density was measured, a stir bar was put, and a headspace cap was sealed to each vial. And all cap-sealed vials were incubated at 25°C for overnight. Cultivation was performed in duplicate. Production of 1-undecene was analyzed using GC-MS from the headspace sample.

3.4 Measurement and analysis of fatty acids and olefins

3.4.1 GC-MS and GC-FID programs

Gas chromatography-mass spectrometry (GC-MS) and gas chromatography-flame ionization detector (GC-FID) were applied for measurement and analysis of fatty acids and

olefins. GC–MS instrument was Agilent Technologies 6890N GC system with 5975B inert XL MSD, column HP-5MS 30 m × 0.25 mm with 0.25 μm film thickness, GC-FID instrument is ThermoFinningan, column DB-1 100% polydimethylsiloxane 30m x 0,32mm x 0,25um film. The programs are as follows:

GC-MS alkene program: injection temperature 250°C, Helium flow 4.7 mL/min, 1 μl splitless injection, oven program: 55°C hold 5 min, 55-280°C 20°/min ramp, 280°C hold 3 min.

GC-MS FAME program: injection temperature 250°C, Helium flow 4.7 mL/min, 1 μl split injection, oven program: 140°C hold 5 min,140-250°C 4°/min ramp, 250°C hold 10 min.

GC-MS headspace program: manual injection, injection temperature 250°C, Helium flow 1.0 mL/min, fiber absorbs the sample for 75S in splitless injection, oven program: 50°C hold 3 min, 50-130°C 10°/min ramp, 130-300°C 30°/min ramp 300°C hold 3 min.

GC-FID alkene program: injection temperature 220°C, Helium flow 2.0 mL/min, 1 μl split injection, oven program: 50°C hold 3 min,50-130°C 10°/min ramp, 130-200°C 30°/min ramp 200°C hold 1 min.

3.4.2 Intracellular fatty acids and olefins

Fatty acids and olefins were extracted from cells. The lipid extraction method [39] was applied for intracellular lipid extraction. The process was as follows: biomass was collected from cultures and centrifuged at 20,000g for 5 min. The cell pellet was resuspended with 500 μl methanol, then 250 μl chloroform was added suspension. Next, the mixture was vortexed at room temperature for 1 hour, then centrifuged at 20,000g for 5 min. Chloroform (250 μl) and PBS (250 μl) was added in order, slow swirling the mixture at room temperature for overnight. Then, the samples were centrifuged at 20,000g for 5 min, and the lowest phase is containing the lipids was used for the analysis.

Fatty acids were analyzed with thin layer chromatography (TLC). The lowest phase lipid was directly applied for TLC. Glass HPTLC Silica Gel 60 F_{254} plate (Merck, USA) was used for TLC, the mobile phase was hexane: diethyl ether: acetic acid, with proportions 80:20:2, respectively. The visualization reagents were as follows: iodine, $KMnO_4$, and phosphomolybdic acid solution. 1mg/ml jojoba oil, 1mg/ml lauric acid, and 5mg/ml lauric acid were used for standards.

Fatty acids were methylated to fatty acid methyl esters (FAMEs), which can be analyzed by GC-MS. A part of cultivation (7 ml, total biomass OD_{600} was 21) was collected for lipid extraction. The lowest phase was transferred to a tube, the chloroform was evaporated under nitrogen. Boron trifluoride (0.5 ml, 14% in methanol) was added to the tube and

incubated at 100°C for 1 hour. Then 0.5 ml double-distilled water and 1 ml hexane were added to the tube. The highest phase was transferred to a vial and analyzed by GC-MS with FAME program. The standard was Supelco 37 component FAME standard 47885-U (Sigma-aldrich, USA), 10 mg/ml in methylene chloride.

Intracellular 1-undecene was analyzed by GC-MS and GC-FID. The lowest phase from lipid extraction was directly used for GC-MS and GC-FID analysis. Intracellular 1-undecene was analyzed by GC-MS using GC-MS alkene program, and by GC-FID using GC-FID alkene program. The standard 1-undecene was diluted by chloroform, the range of concentrations was from 1 ng/ml to 1000 ng/ml.

3.4.3 Extracellular olefins

Two methods were applied for the collection of extracellular olefins: headspace method(modified from Rui et al. 2014 [9]), and ethyl acetate layer collection method (modified from Wang et al. 2018 [100]).

Headspace vials were used for cultivation to avoid loss of olefins during transferring from cultivation tube to headspace vials, the processes were described above in 3.3.2 and 3.3.3. After overnight incubation, the sample was heated and stirred with an aluminum block at 25°C, a Solid Phase Microextraction (SPME) fiber (d_f 30µm, needle size 24ga, polydimethylsiloxane, Supelco, Sigma-Aldrich, USA) was inserted to headspace vials and held for 12m30s, which was the process to absorb olefins. Then the SPME fiber was injected to GC-MS for analysis, GC–MS analysis was performed using GC-MS headspace program. The range of 1-undecene standard concentrations was from 1ng/mL to 500ng/mL (figure APPENDIX C 1).

Ethyl acetate layer collection was used as the method to absorb olefins by ethyl acetate. 3 ml ethyl acetate was added to a 72 hours cultivation, the mixture was incubated at 30°C and 250rpm for 4 hours. Then the mixture was centrifuged at 5000g for 10 minutes. The upper layer was directly used for GC-FID analysis with GC-FID alkene program. The range of 1-undecene standard (solve in ethyl acetate) concentrations was from 25 ng/ml to 25000 ng/ml.

4. RESULTS

4.1 Expression of '*tesA* and *undA* in *A. baylyi* ADP1

In this section, the olefins biosynthesis system in *A. baylyi* ADP1 was characterized, which includes expression of *'tesA* and *undA*. The expression of *'tesA* was characterized with the production of fatty acids, and the expression of *undA* was characterized with the production of 1-undecene. Two batches of cultivation were used for this section (see 3.3 cultivation) because methylation lipid analytics of fatty acids needs a larger amount of biomass.

Cells were cultivated in different cultivation volume, small-scale cultivations were cultivated for 17 hours totally, and large-scale cultivation was cultivated for 3 hours totally. The cell growth of cultivations was recorded as optical density at 600 nm (OD_{600}). OD_{600} of cultivations with different strains and different induction concentrations in small-scale cultivation was similar. Cultivations in large-scale shown the same cell growth situation with small-scale cultivation. However, due to the different cultivation time, OD_{600} of cultivations in small-scale cultivation is higher than that in large-scale cultivation, OD_{600} of cultivations in small-scale and large-scale cultivation is 5.5 and 3.0 approximately respectively (figure 8).

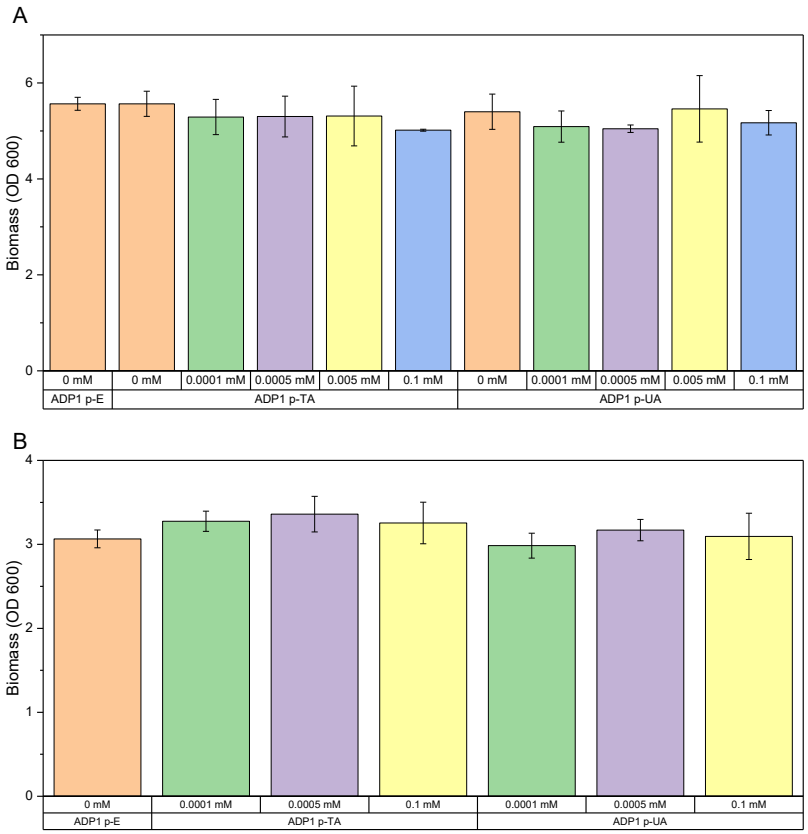

Figure 8. *Cell densities (OD_{600}) of cultivations for optimization of fatty acid analysis and characterization of the native alkene synthesis system, cultivations of ADP1 strains with different plasmids and different concentration of cyclohexanone.* ***(A)*** *Samples were taken from a small-scale culture (5ml), total cultivation hours after induction with 0 mM, 0.0001 mM, 0.0005 mM, 0.005 mM, or 0.1 mM cyclohexanone was 17 hours* ***(B)*** *Samples were taken from a large-scale culture (50 ml), total cultivation hours after induction with 0.0001 mM, 0.0005 mM, or 0.1 mM cyclohexanone was 3 hours. ADP1 p-E was used as a control without induction. The biomass data shows average value from two parallel cultivations, the error bar show standard deviations of two parallel cultivations.*

In order to measure and analyze the production of fatty acids, the method of fatty acids measurement was optimized first. There were two options for analysis of fatty acid: i) TLC; ii) GC-MS after methylation of fatty acids. The TLC method was tested first since the methylation method is more complex and could cause more error. Three reagents were prepared for TLC visualization, iodine, $KMnO_4$, and phosphomolybdic acid solution. The lipid samples were extracted from the cultivations of the small-scale cultivation. Among fatty acids, lauric acid is one of the fatty acids of interest. Thus, lauric acid was used standard. As shown in figure 9, no clear lauric acid bands from standards (red frame in figure 9) nor from the samples were detected by TLC. Hence, TLC is not the optimal analysis for analysis of lauric acid.

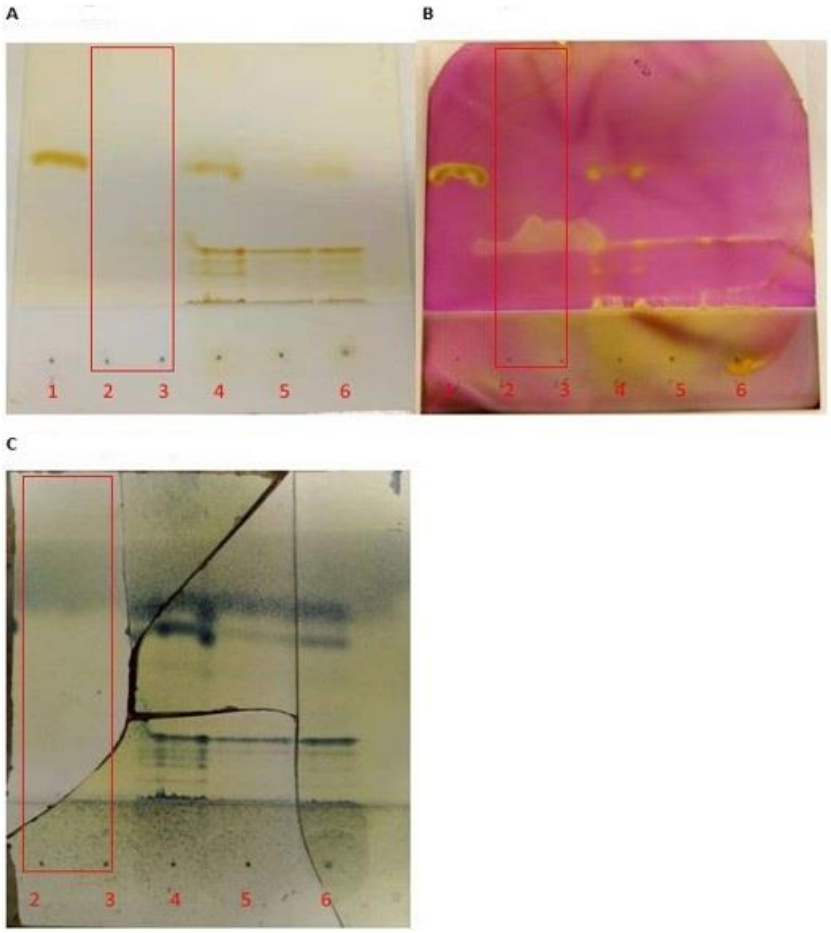

Figure 9. *TLC analysis of fatty acids from lipid samples.* ***(A)*** *visualization with Iodine;* ***(B)*** *visualization with $KMnO_4$;* ***(C)*** *visualization with Phosphomolybdic acid solution. (1) 1 mg/ml jojoba oil (2) 1 mg/ml lauric acid (3) 5 mg/ml lauric acid (4) control (ADP1 p-E) (5) ADP1 p-TA with 0.0001 mM inducer (6) ADP1 p-TA with 0.1 mM inducer. The lipid samples for TLC was taken from the cultivations of small-scale culture (5 ml), total cultivation hours after induction with 0 mM, 0.0001 mM, 0.0005 mM, 0.005 mM, or 0.1 mM cyclohexanone was 17 hours.*

GC-MS was used to analyze and measure fatty acids after methylation for characterization of *'tesA* expression. Standard for GC-MS includes 37 FAME components (from C4 to C24), methyl ester of lauric acid (C12:0), myristic acid (C14:0), palmitoleic acid (C16:1), palmitic acid (C16:0), oleic acid (C18:1), and stearic acid (C18:0) were detected by GC-MS from methylation samples. Fatty acids are converted to FAME by methylation, 1 fatty acid molecular is converted 1 FAME molecular. Thus, the concentration of FAMEs equals the concentration of fatty acids. The focus fatty acid was lauric acid. Lauric acid was detected from the control samples, and lauric acid was also detected from the *undA* samples (figure 10 A). The lauric acid concentration from control samples is similar to that from *'tesA* samples, which is approximately 7300 ng/ml, except samples of ADP1 p-TA with 0.0001mM cyclohexanone induction (5156 ± 1582 ng/ml). Additionally, the concentration of lauric acid from *undA* sample was slightly higher than that from control and

'tesA samples, which was approximately 7800 ng/ml, except samples of ADP1 p-UA with 0.0001mM cyclohexanone induction (8139 ± 1664 ng/ml).

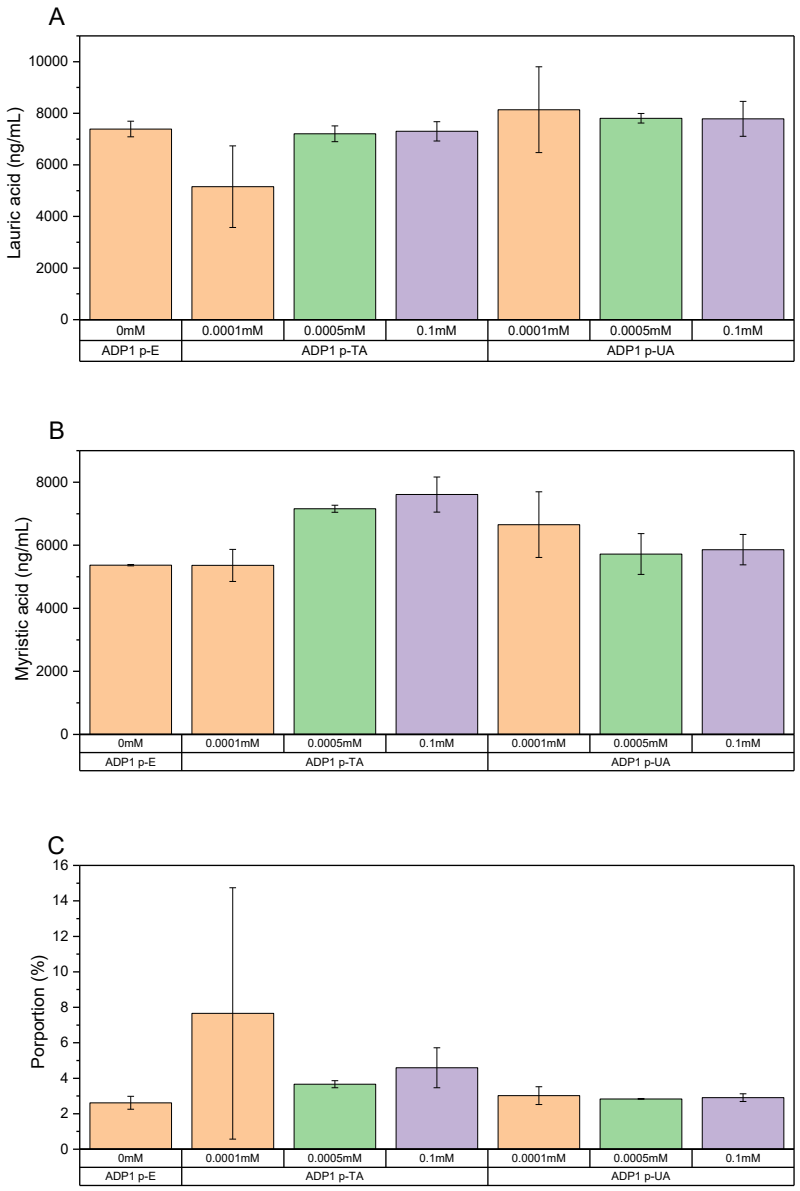

Figure 10. *Fatty acid productions from different ADP1 strains.* ***(A)*** *Lauric acid production;* ***(B)*** *Myristic acid production;* ***(C)*** *Percentage of Lauric acid and Myristic acid from a total fatty acid concentration of fatty acid. Total fatty acids include lauric acid (C12:0), myristic acid (C14:0), palmitoleic acid (C16:1), palmitic acid (C16:0), oleic acid (C18:1), and stearic acid (C18:0). The samples for fatty acid analysis were taken from a large-scale culture (50 ml), total cultivation hours after induction with 0.0001 mM, 0.0005 mM, or 0.1 mM cyclohexanone was 3 hours. ADP1 p-E was used as a control without induction. The fatty acids data shows average value from two parallel cultivations, the error bar show standard deviations of two parallel cultivations.*

Myristic acid is another production of ‘TesA, and it also was detected from all samples (figure 10 B). Myristic acid titer of the control sample is 5369 ± 22 ng/ml. Production of myristic acid from *undA* samples is similar with the control sample, but it is slightly higher. Myristic acid production in *‘tesA* samples increased with the increase of concentration of induction. The titer of myristic acid of samples with 0.0001mM cyclohexanone induction is 5360 ± 507 ng/ml, which is the same with control samples. The titer of myristic acid of samples with 0.0005 mM and 0.1 mM induction concentration is 7157 ± 111 ng/ml and 7608 ± 578 ng/ml respectively. Comparison with the production of lauric acid, production of myristic acid is lower than that of lauric acid, only *‘tesA* samples with high induction concentration reached the level of lauric production.

Figure 10 C shows the proportion of lauric acid and myristic acid in total fatty acids. There is no obvious different proportion between control samples and *undA* samples, which accounts for approximately 3% of the total fatty acid. However, the lauric acid and myristic acid proportions of *‘tesA* samples are higher than that of control samples and *undA* samples. The percentage data of *‘tesA* samples with the lowest induction concentration has a huge error, which is 7.7%, much higher than other samples. Similar to the titer of myristic acid (figure 10 B), the lauric acid and myristic acid proportions of *‘tesA* samples with 0.0005mM and 0.1mM induction concentration increased with the increase of concentration of induction.

As shown in figure 10, both of wild type of *A. baylyi* ADP1 and *A. baylyi* ADP1 with the expression of the heterologous gene *undA* can produce detectable amount of fatty acids. Additionally, expression of heterologous gene *‘tesA* can increase the proportion of lauric acid and myristic acid. The expression level of *‘tesA* is controlled by its promoter with different inducer concentrations, which increases with the increase of concentration of inducer.

Production of 1-undecene from two scales of cultivation also was measured by GC-MS, the 1-undecene samples were from obtained by lipid extraction. According to figure 11, no 1-undecene was detected from control samples and *‘tesA* samples, in other words, ADP1 not produced detectable amounts of 1-undecene in the studied condition. However, 1-undecene was produced by *A. baylyi* ADP1 when *undA* was expressed. Without induction, *undA* strains also can produce a small amount of 1-undecene, 97 ± 13 ng/ml was produced for 17 hours (figure 11 A), and 72 ± 3 ng/ml was produced for 3 hours (Figure 11 B). Both samples from large-scale cultivation and small-scale cultivation

shows the same trend, 1-undecene production increases with the increase of concentration of induction. Comparing with 1-undecene production of two scales of cultivation with same induction concentration, longer cultivation time caused higher 1-undecene production. For example, 1-undecene titer of 0.1 mM induction with 16 hours cultivation (small-scale, figure 11 A) is 580 ± 8 ng/ml, which is higher than 1-undecene titer (117 ± 26 ng/ml) of 0.1 mM induction with 3 hours cultivation (large-scale, figure 11 B). Correspondingly, the OD_{600} of 17 hours cultivation was higher than that of 3 hours cultivation (figure 8).

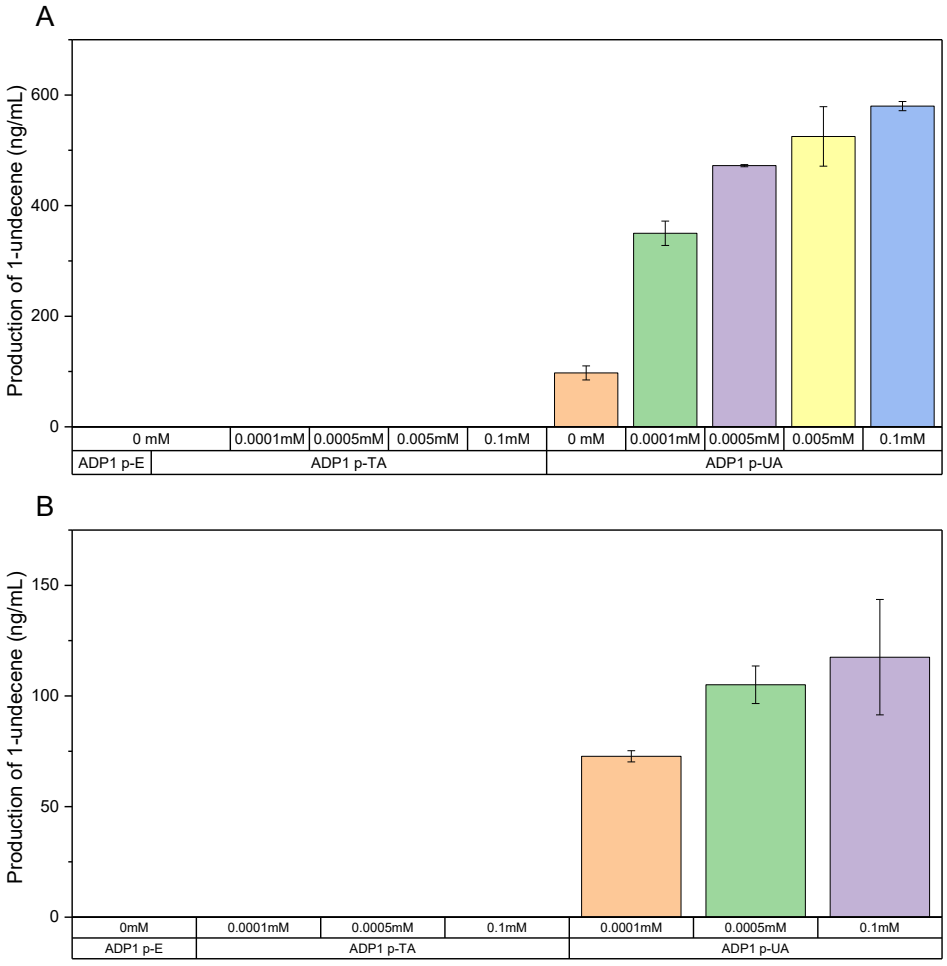

Figure 11. *1-Undecene production of different ADP1 strains.* ***(A)*** *Samples were taken from a small-scale culture (5 ml), total cultivation hours after induction with 0 mM, 0.0001 mM, 0.0005 mM, 0.005 mM, or 0.1 mM cyclohexanone was 17 hours* ***(B)*** *Samples were taken from a large-scale culture (50 ml), total cultivation hours after induction with 0.0001 mM, 0.0005 mM, or 0.1 mM cyclohexanone was 3 hours. ADP1 p-E was used as a control without induction (0 mM). 1-Undecene production from ADP1 p-E and ADP1 p-TA was not detected by GC-MS, the data is marked as 0 in this figure. The 1-undecene data shows average value from two parallel cultivations, the error bar shows standard deviations of two parallel cultivations.*

4.2 1-Undecene production in *aceA* knock-out strain

In order to explore the effect of glyoxylate shunt deletion on 1-undecene production, a strain of ADP1 Δ*aceA* (genotype see table 3) was constructed. To test the new constructed strain ADP1 Δ*aceA'*, the strain was cultivated by different cultivation conditions (APPENDIX A), and intracellular (lipid extraction) and extracellular (ethyl acetate layer extraction) 1-undecene was detected by GC-FID. The results from GC-FID show that no 1-undecene was detected from samples. To confirm the above results, headspace 1-undecene also was analysed by GC-MS, the samples include also related intermediate strains and positive control strains (APPENDIX A). The same results were obtained from GC-MS, in which no 1-undecene was detected from ADP1 Δ*aceA'* and its related intermediate strains. The results illustrate that the construction of ADP1 Δ*aceA'* failed, as the strain cannot produce detectable 1-undecene. Hence, the strain was re-constructed, and the construction of strain ADP1 Δ*aceA* was successful: the strain produced detectable 1-undecene measured by GC-MS. Both ADP1 Δ*aceA'* and ADP1 Δ*aceA* was verified by restriction enzyme analysis and PCR amplification of target sequence. According to the verification, the both strains are as planned (APPENDIX B 1 and 2).

The re-constructed strain ADP1 Δ*aceA* was used for exploring the effect of glyoxylate shunt deletion. Cell growth (OD_{600}) of the cultivations is no significant different (figure 12 A). That OD_{600} of cultivations reached about 1.0 took 9 hours, cell growth of wild type ADP1 was slightly faster than that of other samples, which reached approximately 1.5. Samples were incubated for 2 hours after induction, OD_{600} increased to about 1.8. The cell growth of wild type samples was still higher than that of other samples (OD about 2). In the end-point, OD_{600} of cultivations was about 3.8. Different from other samples, ADP1 Δ*aceA* samples with 1.0% arabinose induction shown a different growth situation, OD_{600} of one parallel sample is 5.6, and OD_{600} of another parallel sample is 3.5.

From the samples collected from headspace, 1-undecene was using GC-MS, as shown in figure 12 B. All ADP1 Δ*aceA* samples produced 1-undecene. The highest titer of 1-undecene was 362 ± 8 ng/ml, which is from ADP1 Δ*aceA* samples without induction. The lowest titer of 1-undecene was 318 ± 5 ng/ml. The production of 1-undecene shows a slightly decreasing trend, the titer of 1-undecene decreases with increasing induction concentration. Generally, there is no significant difference of 1-undecene production between ADP1 Δ*aceA* samples with different induction. On the other hand, the trace amount of 1-undecene was detected from ADP1 wild type samples.

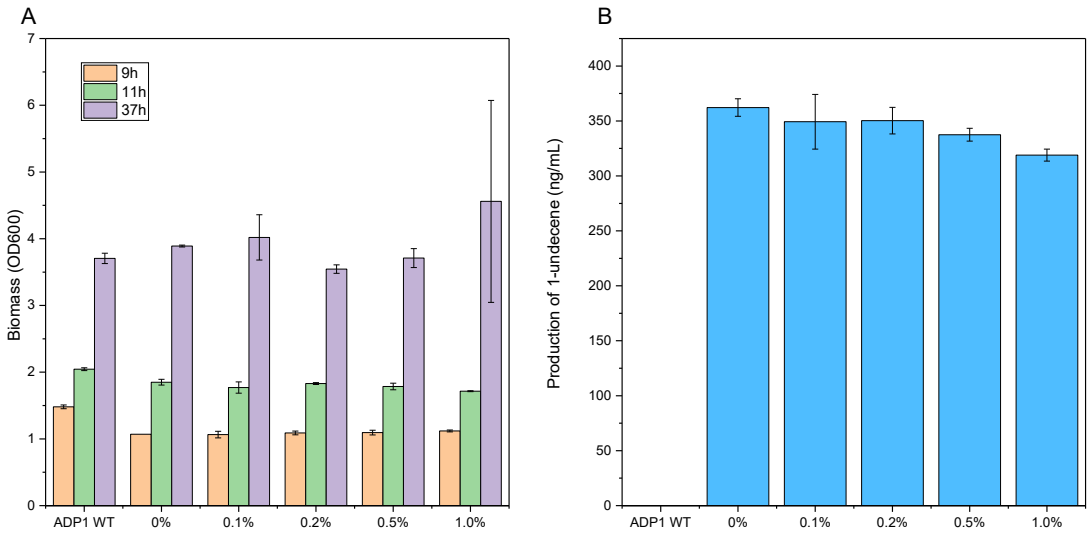

Figure 12. *Cell biomass and 1-undecene production from ADP1 ΔaceA with different concentration (w/v) of arabinose as inducer, and ADP1 WT cultivations.* ***(A)*** *Cell density (OD_{600}), the optical density of cultivations was measured at 9h (OD was about 1, induction point), 11h (the point of sealing caps to headspace vials), and 37h (end-point, GC-MS measurement was done)* ***(B)*** *1-undecene production, the undecane was collected from cultivations headspace. ADP1 WT was used as a control without induction. The concentration of 1-undecene from ADP1 WT is negative number after calculation by standards calibration, thus data of ADP1 WT was recorded as 0 here, but small amount of 1-undecene was detected from this cultivation (the original GCMS data is shown in table APPENDIX C 1). The biomass and 1-undecene data show average value from two parallel cultivations, the error bar shows standard deviations of two parallel cultivations.*

4.3 Olefins synthesis system with *undB* in *A. baylyi* ADP1

Two *undB* strains (ADP1 p-TAUB, ADP1 p-UB) were constructed to explore the olefins production potential of *undB* expression in A. *baylyi* ADP1 (genotypes see table 3, verification see figure APPENDIX B 3). To compare 1-undecene production between UndA and UndB, two *undA* strains were cultivated in the same condition with *undB* strains. The optical density of cultivations was measured at three time points. OD_{600} of cultivations reached about 1.0 which took 7 hours. Cyclohexanone (0.1 mM) was added to cultivations (expect control cultivation ADP1 wild type). After induction (2 hours), the cultivation with the lowest cell growth was the control cultivation, which OD_{600} was1.5. OD_{600} of other cultivation was over 1.5. In the end-point (all GC-MS measurement was done), OD_{600} of cultivations was over 3.0. The sample with the lowest cell growth was the control sample (OD_{600} was approximately 3.1), and the sample with the highest cell growth wasADP1 p-TAUB sample (OD_{600} was approximately 3.6). However, ADP1 p-TAUB samples have a bigger standard deviation than other samples. Basically, the cell growth of *undA* and

undB samples did not show an obvious difference, their growth can be considered as in the same level (figure 13 A).

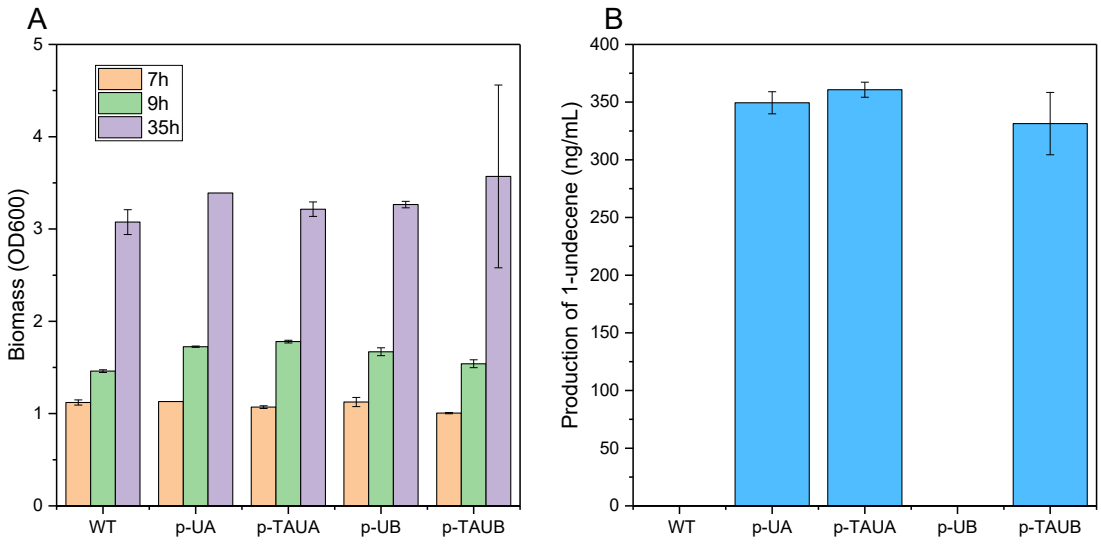

Figure 13. *Cell biomass and 1-undecene production from different ADP1 strains induced with 0.1 mM cyclohexanone cultivations.* ***(A)*** *Cell density (OD_{600}), the optical density of cultivations was measured at 7h (OD was about 1, induction point), 9h (the point of sealing caps to headspace vials), and 35h (end-point, GC-MS measurement was done)* ***(B)*** *1-undecene production, the undecane was collected from cultivations headspace. ADP1 WT was used as a control without induction. The concentration of 1-Undecene from ADP1 WT and ADP1 p-UB is negative number after calculation by standards calibration, thus data of ADP1 WT and ADP1 p-UB was recorded as 0 here, but small amount of 1-Undecene was detected from those cultivations (The original GC-MS data is shown in table APPENDIX C 2). The biomass and 1-Undecene data show average value from two parallel cultivations, the error bar shows standard deviations of two parallel cultivations.*

Olefins from the headspace sample were measured and analysed by GC-MS. As shown in figure 13 B, 1-undecene productions of *undA* samples were similar, the titer of 1-undecene of ADP1 p-TAUA sample was slightly higher than that of ADP1 p-UA sample, which was 361 ± 7 ng/ml and 349 ± 10 ng/ml respectively. Sample of ADP1 p-UB produced a tiny amount of 1-undecene, which was just a little higher than 1-undecene production of ADP1 wild type (figure APPENDIX C 2). However, the titer of 1-undecene increased to 331 ± 27 ng/ml with *'tesA* expression, 1-undecene production from ADP1 p-TAUB reaches the level of *undA* samples.

Diverse olefin productions were detected from *undB* samples. Those olefin productions are unidentified alkene(olefin) products (UIAP), because the only 1-undecene standard was used for GC-MS measurement. Total of 11 kinds of UIAPs were detected, the possible identification of those UIAPs was defined by MS results (APPENDIX D). UIAPs from *undB* samples may include C12-17 alpha-olefins and other olefins (more than one

carbon-carbon double bonds) compounds, the possible results of UIAPs were listed in table 7.

Table 7. *Possible identification and details of unidentified olefin production from undB samples. The data from GC-MS results (APPENDIX D).*

UIAP number	Producer	Molecular weight (MW)	Component[1]	Possible olefin[2]
UIAP 1	TAUB	164	C12:3	-
UIAP 2	TAUB	166	C12:2	-
UIAP 3	TAUB	168	C12:1	1-dodecene
UIAP 4	UB, TAUB	180	C13:2	-
UIAP 5	UB, TAUB	182	C13:1	1-tridecene
UIAP 6	UB, TAUB	194	C14:2	-
UIAP 7	UB, TAUB	196	C14:1	1-tetradecene
UIAP 8	UB, TAUB	208	C15:2	-
UIAP 9	UB, TAUB	210	C15:1	1-pentadecene
UIAP 10	UB, TAUB	236	C17:2	-
UIAP 11	UB, TAUB	238	C17:1	1-heptadecene

UIAP: Unidentified alkene(olefin) product.

[1] Cn:m, n is chain length, m is number of carbon-carbon double bond.

[2] Some productions cannot be given a possible identification, because the location of carbon-carbon double bond is unknown.

Comparing with *undA* samples, ADP1 p-UB can produce other long-chain olefins. Eight olefins (UIAP4 – UIAP11) were detected from ADP1 p-UB sample, which includes C13, C14, C15, and C17. Each chain length contains an alpha-olefin (e.g. C13:1), and a di-olefin (e.g. C13:2). According to the peak area from GC results (APPENDIX D), the concentration of those olefins can be estimated. The concentration of C14, C15, and C17 alpha-olefin was higher than that of its diolefin. However, in contrast, the concentration of C13 alpha-olefin was higher than that of its diolefin.

ADP1 p-TAUB produced extra fatty acids by expression of *'tesA*, and it also produced long-chain olefins. According to the retention time and molecular weight from GC-MS results (APPENDIX D), like ADP1 p-UB strain, UIAP4 – UIAP11 were produced also from ADP1 p-TAUB strain. Additionally, the concentration of UIAP4 – UIAP11 was increased by extra fatty acids production in ADP1 p-TAUB strain. Except of 1-undecene production, the concentration of UIAP5 (could be 1-tridecene) was rapidly increased when *'tesA* expression. On the other hand, the expression of *'tesA* can make UndB produce extra C12 olefins, which were C12 alpha-olefin, diolefin, and triene. According to peak area from GC results (APPENDIX D), the comparison of C12 olefins concentration was: C12:2 > C12:1 > C12:3. The C12 olefins were not detected from *undA* samples and ADP1 p-UB sample.

5. DISCUSSION

1-Undecene has been previously produced by iron-dependent desaturase/decarboxylase UndA in *E. coli* and *A. baylyi* ADP1 [9][11], and membrane-bound desaturase-like enzyme UndB in E.coli [10]. In this thesis, 1-undecene was produced by UndA and UndB in *A. baylyi* ADP1. The aim of this thesis is to increase the production of 1-undecene and explore the potential of UndB in *A. baylyi* ADP1. Firstly, UndA and 'tesA were expressed in *A. baylyi* ADP1, which was to explore the native free fatty acids and 1-undecene biosynthesis mechanism of *A. baylyi* ADP1. In addition, glyoxylate shunt blocking has been proved to increase carbon flowing to the fatty acid synthesis pathway in *A. baylyi* ADP1 [98]. Thus, *aceA* was knocked out from the wild type of *A. baylyi* ADP1 for the construction of ADP1 Δ*aceA* strains. Moreover, UndB was the first time expressed in *A. baylyi* ADP1.

5.1 Optimization of measurement and analysis of free fatty acids and 1-undecene

In order to measure and analyse free fatty acids that were produced by cells, the analysis method was optimized to be optimized. Due to the high polarity of fatty acids, fatty acids cannot be analyzed using GC system directly. TLC was tested to compare the production of free fatty acids. TLC is a simple analysis method, which lipid samples from cells can be measured using TLC without extra treatment. Different compounds can be separated on TLC plate, the colour of the band for each compound can be compared with the corresponding standard. An issue of TLC for fatty acids was that TLC cannot separate different carbon lengths of fatty acids. Thus, TLC only is used for comparing total fatty acids and cannot be used for quantitative analysis of fatty acids and analysis of single definite carbon length fatty acid. Another challenge was the visualization. If fatty acids can be efficiently visualized on TLC plate, the TLC will be suitable for this kind of study. The production of fatty acids can be determined through comparing the intensity of the band of the band on TLC plate, the quantitative analysis is not necessary.

However, according to the result of TLC analysis (figure 9), fatty acids cannot be visualized on TLC plate. Three reagents, iodine, $KMnO_4$, and phosphomolybdic acid solution, were tried for visualization of fatty acids, but all of them did not work for fatty acids.

An alternative analysis method is that fatty acids are analyzed by GC-MS where fatty acids are analyzed in the form of FAMEs after methylation reaction as the methylation

decrease the polarity of fatty acids. The suitability of the method for fatty acid analysis was seen also in this thesis. In addition, GC-MS analysis after methylation allows quantitative analysis of different carbon length fatty acids. 1 mole of FAME equals 1 mole of corresponding fatty acid. Thus, the concentration of FAME is the concentration of its corresponding fatty acid. On the other hand, methylation contains complex treatments of fatty acids sample, loss of fatty acids may be caused during the treatment, which may lead to higher errors for the measurements.

In this study, two methods of extracellular 1-undecene analysis were applied, which were the headspace method [9] and ethyl acetate extraction method [100]. 1-Undecene is able to escape from the cells after biosynthesis. It is semi-volatile metabolites and float on the water because of its density [101]. In comparison, GC-MS analysis of headspace 1-undecene was detected to be more reliable and accurate than ethyl acetate extraction method. In this thesis, headspace vials were applied for cultivations directly, which can decrease the loss of 1-undecene during transferring cultivations from cultivation tubes to headspace vials. For the minimal detectable concentration of 1-undecene, GC-MS can detect 1 ng/ml 1-undecene from headspace (figure APPENDIX C 1). With the ethyl acetate extraction method based on GC-FID, so low concentrations of 1-undecene were not detected. However, GC-MS analysis of 1-undecene from headspace samples was more complex, and the time consuming due to the manual injection for GC-MS. Intracellular 1-undecene was detected by GC-MS after lipid extraction, but the trace amount of 1-undecene from extracted lipid was not detected by GC-MS (figure 11). Thus analysis and quantification of MCAOs e.g. 1-undecene are the challenge when the bioproductions are improved by engineering means. [9]. Hence, it is necessary to develop a simple MCAOs detection method.

5.2 Expression of *undA* and *'tesA* in *A. baylyi* ADP1

According to the results (figure 10), a natural biosynthesis mechanism of 1-undecene exists in *A. baylyi* ADP1. *A. baylyi* ADP1 has a completed fatty acid synthesis pathway to provide precursors for the synthesis of storage lipids. Therefore, the production of fatty acids shows at least one native thioesterase in *A. baylyi* ADP1. Free fatty acids in high concentrations are toxic to the cells, normally fatty acids are present in the cell as esters, but they are important compounds of cell precursor compounds. Thus, it is reasonable that *A. baylyi* ADP1 has native thioesterase activity. However, the thioesterase in *A. baylyi* ADP1 is still not identified.

Since GC-MS is not able to detect trace amounts of intracellular 1-undecene, there was no detectable 1-undecene from wild type *A. baylyi* ADP1 and ADP1 p-TA (figure 11).

However, 1-undecene detection from headspace supplied the results of natural 1-undecene production from wild type *A. baylyi* ADP1 (figure 12 and 13, table APPENDIX C 1 and C 2). Trace amount of 1-undecene was detected from the wild type of *A. baylyi* ADP1*,* which demonstrates *A. baylyi* ADP1 has a natural biosynthesis mechanism of 1-undecene. This result is in a line with previous report from Luo et al. [11]. Thus at least one 1-undecene production enzyme exists in *A. baylyi* ADP1, but it is not characterized. 1-Undecene is naturally produced by plant-related microorganisms and 1-undecene may play the role of surviving mechanisms. However, the role of 1-undecene in *A. baylyi* ADP1 is not known.

Expression of *'tesA* and *undA* did not affect cell growth (figure 10), neither inducer cyclohexanone adding. It was shown here that the overexpression of *'tesA* and *undA* is not harmful for the *A. baylyi* ADP1, there was no negative effect on cell growth with extra production of free fatty acids and 1-undecene. Production of lauric acid from ADP1 p-TA strains did not show a clear increasing trend following increasing concentration of inducer, but the production of myristic acid and lauric acid and myristic acid proportion in total fatty acid showed an increasing trend. This can be due to the fact that 'TesA produces also myristic acid [14]. Production of fatty acids from ADP1 p-UA strains was similar to wild type ADP1. However, theoretically, the concentration of lauric acid and myristic acid should decrease following expression of *undA*, because UndA can accept lauric acid and myristic acid to produce corresponding alpha-olefin [9].

Based on the results it seems that *A. baylyi* ADP1 has natural thioesterase activity and 1-undecene biosynthesis mechanism. Additionally, the production of free fatty acids (especially myristic acid) can be slightly increased by expression of *'tesA*, and expression of *undA* in *A. baylyi* ADP1 can rapidly increase the production of 1-undecene.

5.3 Effect of glyoxylate shunt blocking on production of 1-undecene

The first construction of ADP1 *aceA* knocking out strain (ADP1 Δ*aceA'* in this thesis) was failed, because of no detectable 1-undecene production was seen from the ADP1 Δ*aceA'*. There can be several possible reasons, for example: i) comparing with expression of *undA* on plasmids, expression of *undA* on genome has lower copy number. Thus, low expression level of UndA may cause trace amount of 1-undecene production (titer is lower than minimal standard concentration 1 ng/ml); ii) T5 promoter for *undA* was mutated because high pressure of overexpression during construction process; iii) the distance between ribosome binding site (RBS) and start codon was too close, only 3 bp. *E.*

coli XL-1 strain contains pUC57-t5-*undA* was tested, but there was no detectable 1-undecene also. Therefore, the possible reason i was excluded. ADP1 *aceA* knocking out strain (ADP1 Δ*aceA* in this thesis) was re-constructed. Comparing with the first construction, the distance between RBS and start codon was added from 3 bp to optimal distance 6 bp, and a His-tag was added after *undA* sequence. The His-tag is for possible UndA protein extraction, if the new constructed strain still does not produce 1-undecene, protein extraction can be used for verification of *undA* expression. Re-constructed ADP1 Δ*aceA* strain produced 1-undecene after testing (figure 12). Thus, the most possible reason is the short distance between RBS and start codon. However, the exact reason needs to be found out by sequencing and other possible reasons cannot be excluded (including manual mistakes during construction).

In theory, when glyoxylate shunt was deleted, *undA* overexpressed with help of the strong promoter (T5), and extra free fatty acids were provided by *'tesA* expression in ADP1 Δ*aceA*, the production of 1-undecene should be increased. However, the production of 1-undecene did not increase with those strategies (figure 12). This was a surprising result. According to the activity of enzymes, the activity of UndA is much lower than the activity of 'TesA [9][102], which can be a possible reason. Hence, UndA is a possible rate limit factor for this 1-undecene biosynthesis system in *A. baylyi* ADP1. The rate of UndA reached the upper limit, even if more free fatty acids are provided to UndA, the production of 1-undecene will not increase. This possible reason should be studied by further experiments.

5.4 Effect of *undB* expression on production of olefins

That the overexpression of UndB has toxic effect on the cells because it is a membrane-bound enzyme was reported in previous research [97]. However, the cells were not affected with the expression of UndB in this study (figure 13). ADP1 p-UA and ADP1 p-TAUA were used for positive controls, compared to the previous research [11], the titer of *undA* strains was lower than *undA* stains in previous research. It is may because the cultivation method was different, the cultivations in this study used headspace vials, the aeration may be not good like cultivation tubes. Another difference with the previous research [11] is that ADP1 p-UA and ADP1 p-TAUA did not produce that much 1-tridecene (figure APPENDIX D 1 B).

ADP1 p-UB only produced the trace amount of 1-undecene, which is similar to the production from control strain (the wild type of *A. baylyi* ADP1) (figure 13). UndB (*Pmen_4370*) was not tested in the previous study, other UndB homolog *PFL_0203* was tested with the feedstock of lauric acid, and the production of 1-undecene from UndB

(*PFL_0203*) was about 10mg/L [10]. Thus, this might be due to the properties of different UndB homologs. UndB (*Pmen_4370*) may be more inclined to produce other olefins in low substrate concentration of lauric acid. Comparing with *undA* strains, ADP1 p-UB produced eight extra olefins, the carbon length is from 13 to 17 (table 7). It is because UndB has a wild substrate spectrum [10].

Following expression of 'TesA, production of 1-undecene from UndB significantly increased, which reached a similar level of UndA production level. However, compared to the previous study [10], in which UndB was used for 1-undecene production with co-expression of thioesterase *UcFatB2* in *E. coli,* the titer of 1-undecene from *A. baylyi* ADP1 was lower. The possible reason of difference of 1-undcene from UndB in this study may be similar with the reason of the difference of 1-undecene production for UndA between *E. coli* and *A. baylyi* ADP1 in pervious study [11], which is due to difference of metabolism between *E. coli* and *A. baylyi* ADP1, and the difference of cultivation and genetic constructs..

Olefins with two unusual carbon chain lengths were produced by UndB, which is C12 and C14 olefins, and C12 olefins were produced with co-expression of 'TesA and C14 olefins were increase by co-expression of 'TesA. UndB converts fatty acids to corresponding olefins, the number of carbon atoms are always minus one. In other word, to produce C12 and C14 olefins, the substrates for UndB should be C13 and C14 fatty acids. However, normally, the fatty acids exist with even number of carbon atoms [103]. Additionally, no previous studies reported that UndB can produce C12 and C14 olefins. According to this study and previous study of UndA [11], UndA cannot produce C12 olefins with co-expression of 'TesA. Therefore, the olefins with even number of carbon atoms mechanism of UndB are unknown, which should be explored in the future.

5.5 Proposal for future work

In this thesis, *A. baylyi* ADP1 was explored as host for UndA and UndB to produce 1-undecene. Production of 1-undecene was not increased in this study. However, the studies about *A. baylyi* ADP1, UndB, and several metabolic engineering strategies establish a foundation of MCAOs production in *A. baylyi* ADP1. Many challenges still exist. Rate limit enzymes should be explored in this 1-undecene bioproduction system based on *A. baylyi* ADP1. UndB expression system is still not optimized in *A. baylyi* ADP1, there were only a few previous researches for UndB. Analysis and quantification of MACOs are still a challenge for the bioproduction of MACOs, thus, a simple and accurate analysis method should be explored.

The production of 1-undecene was not optimized, there are several proposals for optimization of bioproduction 1-undecene in *A. baylyi* ADP1: i) rate limit enzyme (maybe UndA) should be explored, which could balance the enzyme activities. For example, ADP1 Δ*aceA*::t5-*'tesA*-Spec^{R}, pBAV1K-chn-*undA* can be constructed to test the production of 1-undecene; ii) production of olefins by UndB in *A. baylyi* ADP1 should be explored deeply, some metabolic engineering strategies can be applied for UndB, e.g. glyoxylate shunt blocking; iii) the reason of unsatisfactory 1-undecene production with UndA and UndB in *A. baylyi* ADP1 should be found; and other metabolic engineering and synthetic biology strategies can be applied for bioproduction of MACOs in *A. baylyi* ADP1, for example, construction optimal MACOs biosynthesis pathway with blocking of competing pathways.

6. CONCLUSION

In this thesis, *A. baylyi* ADP1 was applied for bioproduction of 1-undecene. The aim of this study was to optimize microbial biosynthesis system of medium-chain alpha-olefins, thereby to increase the production of 1-undecene. Two enzymes that can produce medium-chain alpha-olefins, UndA and UndB, were expressed in *A. baylyi* ADP1. In addition, glyoxylate shunt blocking based on gene *aceA* knock-out was applied to optimize biosynthesis pathways of medium-chain alpha-olefins.

The biosynthesis system of 1-undecene with UndA, co-expression of 'TesA, and glyoxylate shunt blocking obtained approximately 350 ng/ml as the titer of 1-undecene. The titer of 1-undecene from the biosynthesis system of 1-undecene with UndB, co-expression of 'TesA was 331 ± 27ng/ml. Compared to the previous studies [9][10][11], the titers of 1-undecene in this study were still lower. In other word, the biosynthesis system of 1-undecene was not improved in this study. However, many valuable implications and results for bioproduction of 1-undecene were explored, which can inspire for future work: i) natural biosynthesis mechanism of free fatty acids and 1-undecene exists in *A. baylyi* ADP1; ii) UndA is a possible rate limit factor of 1-undecene bioproduction; iii) expression of UndB is feasible in *A. baylyi* ADP1, which can make ADP1 to produce wider range of alkenes. Additionally, for 1-undecene production with UndB, extra lauric acid is needed; iv) UndB can produce olefins with even number of carbon atoms in *A. baylyi* ADP1 with co-expression of 'TesA.

This thesis demonstrated *A. baylyi* ADP1 can be applied for bioproduction of medium-chain alpha-olefins. However, the titer of 1-undecene did not increase due to many complex reasons, including rate limit enzyme, cultivation method, and selection of efficient enzymes. Hence, for the future work of bioproduction of medium-chain alpha-olefins, the focus should be solving rate limit factors, pathway optimization based on metabolic engineering and synthetic biology with *A. baylyi* ADP1, and optimization of substrates (e.g. free fatty acids and co-factors) for UndA and UndB.

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APPENDIX A: CULTIVATION AND STRAINS FOR TESTING OF ADP1 Δ*ACEA'*

Table APPENDIX A 1. *Cultivation and strains for testing of ADP1 ΔaceA'. kan - kanamycin, spec - spectinomycin, casaa - casein amino acid, ara – arabinose.*

Measurement	Strains	Cultivation
Ethyl acetate extraction GC-FID	ADP1 Δ*aceA*'	Strain was inoculated from glycerol stock to LA plate (1% glucose + kan + spec). A single colony from plate was inoculated to LB + 0.4% glucose + kan overnight (pre-culture). Cultivations were conducted (initial OD of 0.1) in MA/9 (4 x Fe) + 5% glucose + 0.2% casaa + kan at 25/100 in 12/100 ml flasks. Three cultivations (+ duplicates) were cultivated for 6h, 10h, and 24h.
Lipid extraction GC-FID	ADP1 Δ*aceA*'	Strain was inoculated from glycerol stock to LA plate (1% glucose + kan + spec). A single colony from plate was inoculated to LB + 0.4% glucose + kan + spec overnight (pre-culture). Cultivations were conducted (initial OD of 0.1) in MA/9 (4 x Fe) + 5% glucose + 0.2% casaa + kan at 25/100 in 5/14ml tubes. Five cultivations (duplicate) were cultivated for ~13h, 17h, 20h, 24h, 40h.
	ADP1 Δ*aceA*' ADP1 p-UA ADP1 WT	Strain was inoculated from glycerol stock to LA plate (1% glucose + kan + spec). A single colony from plate was inoculated to LB + 0.4% glucose + kan + spec overnight (pre-culture). Cultivations were conducted (initial OD of 0.1) in MA/9 (4 x Fe) + 5% glucose + 0.2% casaa + kan at 25/100 in 5/14ml tubes for 20h. Duplicate: ADP1 p-UA (0.1mM cyclohexanone), ADP1 WT (0% ara), ADP1 *ΔaceA*' (0 % ara), ADP1 *ΔaceA*' (0.1% ara), ADP1 *ΔaceA*' (0.2% ara), ADP1 *ΔaceA*' (0.5% ara), ADP1 *ΔaceA*' (1.0% ara).

Headspace, GC-MS	ADP1 Δ*aceA*' ADP1 p-UA ADP1 WT ADP1 *ΔaceA*::T5-*undA*-Spec^R	Strain was inoculated from glycerol stock to LB + 0.4% glucose + antibiotics overnight (1084upt strain 24h+overnight). Cultivations were conducted (initial OD of 0.1) in MA/9 (4 x Fe) + 5% glucose + 0.2% casaa + antibiotics at 25/100 in 5/20 ml headspace vials. (ADP1 *ΔaceA*', preculture added kan and spec, and culture only added kan, WT without antibiotics) Cultivated for 5 hours, 0.1mM cyclohexanone was added to cultivations of ADP1 pBAV1C-chn-*undA* (OD was ~1.0), then cultivated for 5 hours, sealed the cap to headspace vials, then incubated for 20h at 25/300.
	XL-1 WT: wild type of E. coli XL-1 XL-1 T5U: E. coli XL-1 pUC57-t5-*undA* XL-1 U: E. coli XL-1 pBAV1C-chnR-*undA* XL-1 TU: E. coli XL-1 pBAV1C-chnR-*'tesA*-*undA*	Strain was inoculated from glycerol stock to LB + 0.4% glucose + antibiotics overnight. Cultivations were conducted (initial OD of 0.1) in MA/9 (4 x Fe) + 5% glucose + 0.2% casaa + antibiotics at 30/100 in 5/20ml headspace vials. (XL-1 T5U, preculture added spec and amp, and culture only added spec, XL-1 WT without antibiotics) Cultivated for 5 hours, 0.1mM cyclohexanone was added to cultivations of XL-1 U and XL-1 TU, then culture for 2 hours, sealed the cap to headspace vials, then incubate for 13h at 25/300.

APPENDIX B: RESULTS OF VERIFICATION

Verification of ADP1 *ΔaceA*'

According to figure APPENDIX B 1, new constructed strain ADP1 *ΔaceA*' was verified by restriction enzyme digestion and PCR amplification. The gene cassette pUC57-t5-*undA* was extracted from *E. coli* XL-1 and was digested by XhoI and XbaI. The length of fragments from both of samples was matched. However, gene cassette from sample 2 was not completely digested, which caused an extra band (the gene cassette without digestion or just one enzyme worked). Gene cassette from sample 1 was correct, thus, extracted gene cassette from sample 1 was selected for further steps to the transformed into *A. baylyi* ADP1.

After the construction of *A. baylyi* ADP1 *ΔaceA*::*T5-undA-Spec*^{r}, the genome of the strain was extracted and amplified by PCR, the strains could be verified by the length of PCR product. Strains for genome extraction and PCR: ADP1 WT, sample 2 (ADP1 *ΔaceA*::*T5-undA-Spec*^{r}), sample 3 (ADP1 *ΔaceA*::*T5-undA-Spec*^{r}), ADP1 *ΔaceA*::*acr1-Spec*^{r}. Primers were: 1084 V1, 1084 V2. Correct length of PCR products: WT: 2490 bp, Sample 2: 2959 bp, Sample 3: 2959 bp, ADP1 *ΔaceA::acr1*: 3094 bp. The length of WT was about 2500 bp, and length of sample 2, sample 3, and ADP1 *ΔaceA::acr1* was about 3000 bp. The lengths were matched.

The plasmid pBAV1K-ara-*'tesA* was extracted from new constructed strain *A. baylyi* ADP1 *ΔaceA*'. Restriction enzymes were: EcoRI and PstI. There should be two fragments with 2000 bp and 2600 bp after digestion. The length of bands after digestion was matched.

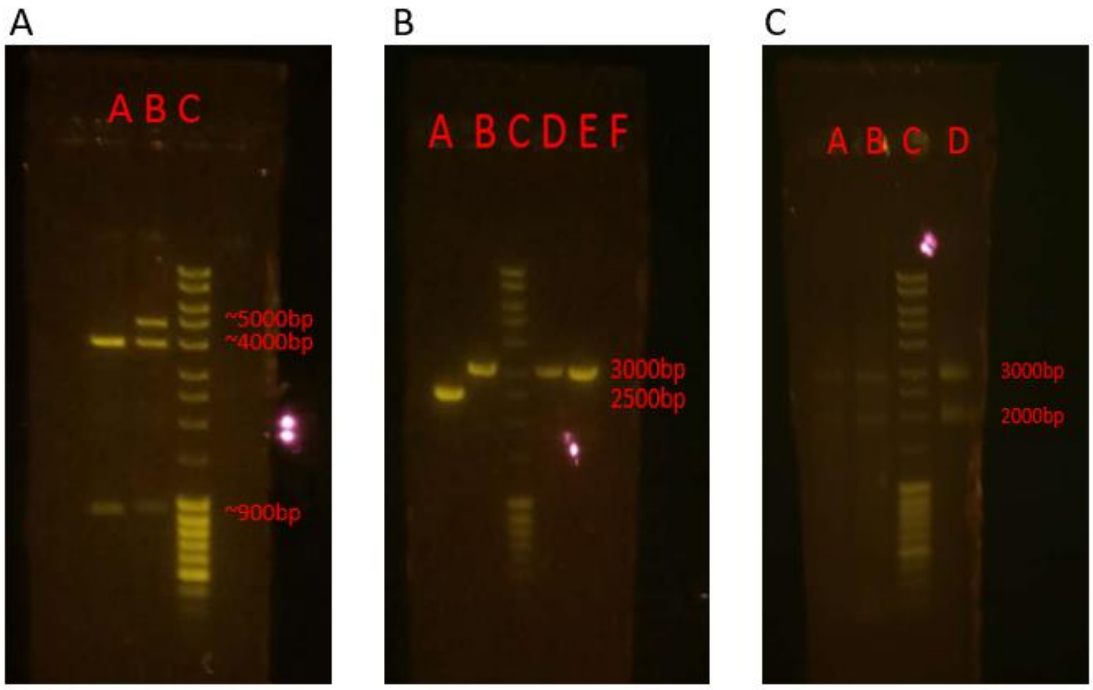


Figure APPENDIX B 1. *Verification of ADP1 ΔaceA'.* ***(A)*** *Electrophoresis result of gene cassette pUC57-t5-undA verification by digestion. A: sample 1; B: sample 2; C: Massruler SM0403 Ladder (Thermo-Scientific, USA).* ***(B)*** *Electrophoresis result of verification aceA knock-out in genome by PCR. A: WT; B: sample 2; C: Massruler SM0403 Ladder; D: sample 3; E: ADP1 ΔaceA::acr1; F: control.* ***(C)*** *Electrophoresis result of verification by digestion for plasmid pBAV1K-ara-'tesA from ΔaceA'. A: sample 3; B: sample 4; C: Massruler SM0403 Ladder; D: control (original plasmids).*

Verification of ADP1 *ΔaceA*

According to figure APPENDIX B 2, new constructed strain ADP1 *ΔaceA* was verified by digestion and PCR amplification. The gene cassette pUC57-t5-*undA* was extracted from *E. coli* XL-1 and was digested by XhoI and XbaI. The correct length of fragments after digestion is about 4000bp and about 900bp. The length of bands from both samples was matched.

The genome of *A. baylyi* ADP1 *ΔaceA*::*T5-undA-Spec*^{r} was extracted and verified by PCR amplification, primers were: 1084 V1, 1084 V2. The correct length of PCR product was 2925 bp, the correct length of PCR product from control (ADP1 WT) genome was 2490 bp. The length of bands from both of samples and control was matched.

The plasmid pBAV1K-ara-*'tesA* was extracted from new constructed strain *A. baylyi* ADP1 *ΔaceA*'. Restriction enzymes were: EcoRI and PstI. There should be two fragments with 2000 bp and 2600 bp after digestion. The length of bands after digestion was matched.

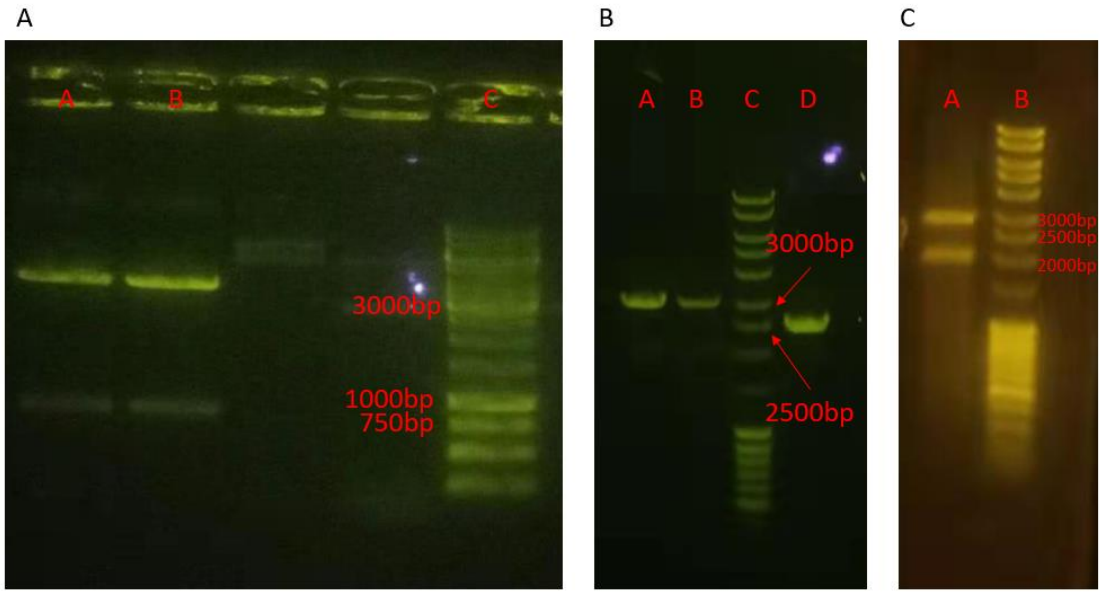


Figure APPENDIX B 2. *Verification of ADP1 ΔaceA.* ***(A)*** *Electrophoresis result of gene cassette pUC57-t5-undA verification by digestion. A: sample 1; B: sample 2; C: GeneRuler 1kb DNA ladder SM0313 (Thermo-Scientific, USA).* ***(B)*** *Electrophoresis result of verification aceA knock-out in genome by PCR. A: sample 1; B: sample 2; C: Massruler SM0403 Ladder; D: ADP1 WT* ***(C)*** *Electrophoresis result of verification by digestion for plasmid pBAV1K-ara-'tesA from ΔaceA. A: sample; B: Massruler SM0403 Ladder.*

Verification of ADP1 p-UB and ADP1 p-TAUB

After the construction of ADP1 p-UB and ADP1 p-TAUB, the plasmids pBAV1C-chnR-*undB* and pBAV1C-chnR-*'tesA-undB* were extracted and verified by digestion with XbaI and PstI. The correct length of fragments from pBAV1C-chnR-*undB* was 2874 bp and 2676 bp, the correct length of fragments from pBAV1C-chnR-*'tesA-undB* was 3455 bp and 2676 bp. According to figure APPENDIX B 3, the length of bands from pBAV1C-chnR-*undB* and pBAV1C-chnR-*'tesA-undB* after digestion was matched with the correct length.

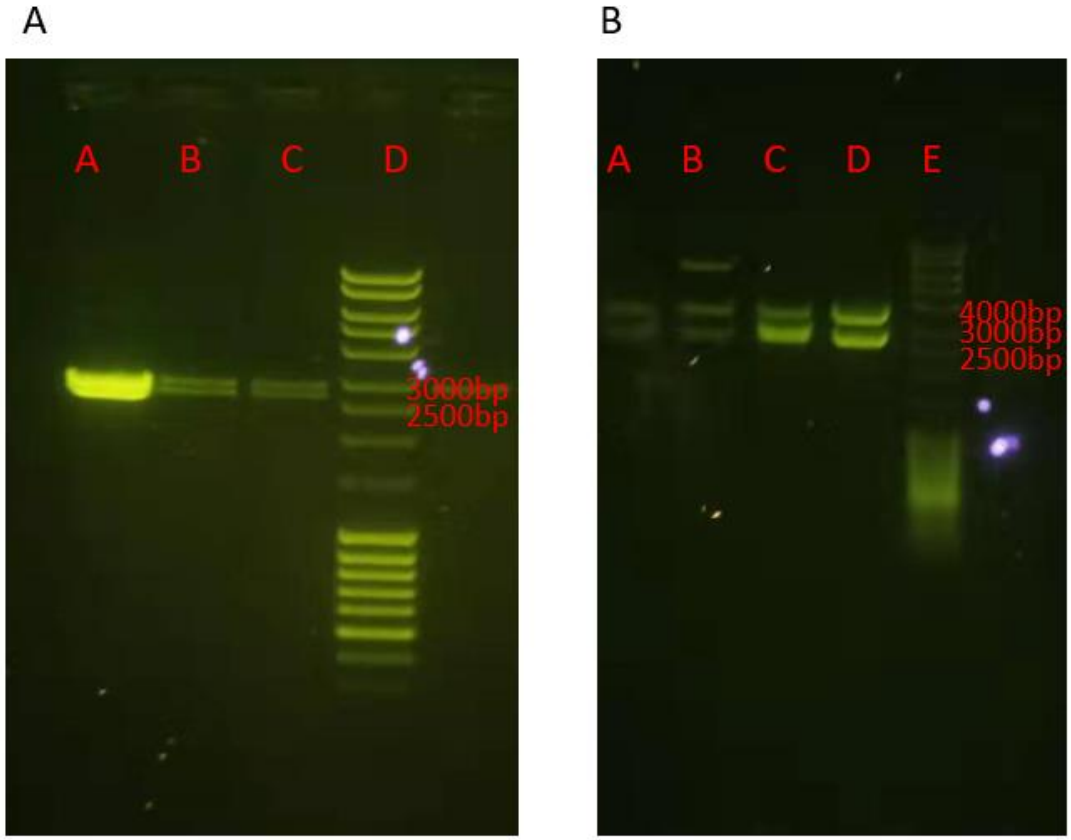


Figure APPENDIX B 3. *Verification of ADP1 p-UB and ADP1 p-TAUB.* ***(A)*** *Electrophoresis result of pBAV1C-chnR-undB verification by digestion. A: positive control (original plasmid); B: sample 1; C: sample2; D: Massruler SM0403 Ladder.* ***(B)*** *(A) Electrophoresis result of pBAV1C-chnR-'tesA-undB verification by digestion. A: sample 1; B: sample 2; C: positive control 1 (original plasmid); D: positive control 2 (original plasmid); E: Massruler SM0403 Ladder.*

APPENDIX C: GC RESULTS OF 1-UNDECENE PRODUCTION

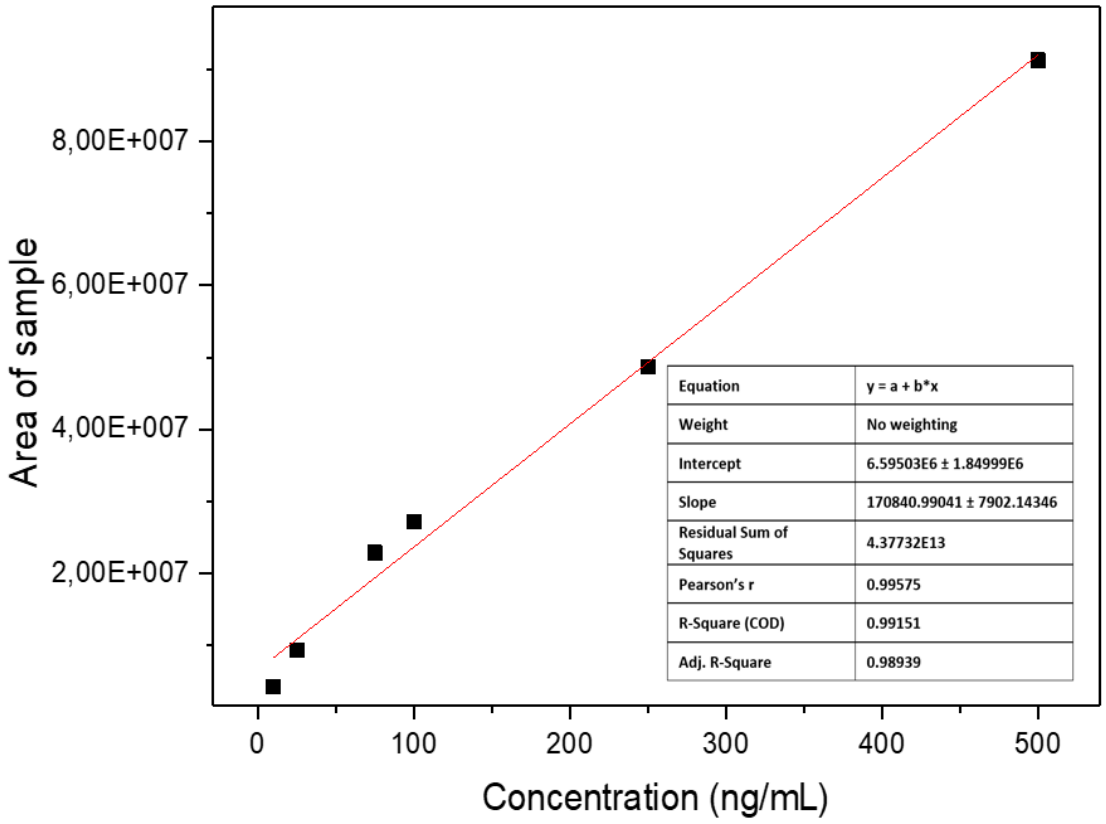


Figure ADDPENDIX C 1. *Calibration of 1-undecene standards, measured by GC-MS. The equation for the slope is y=170840.9904x+6595030.867.*

Table APPENDIX C 1. *GC data of 1-undecene production from ADP1 ΔaceA samples.*

	Area	Concentration[1] (ng/ml)	Standard deviation[2]
ADP1 WT	341197	-36.6062	0.051812
ADP1 *ΔaceA* 0%ara	68477550	362.2229	7.932306
ADP1 *ΔaceA* 0.1%ara	66272370.5	349.3151	24.87473
ADP1 *ΔaceA* 0.2%ara	66440882	350.3015	12.12506
ADP1 *ΔaceA* 0.5%ara	64253207.5	337.4962	5.803279
ADP1 *ΔaceA* 1.0%ara	61075407.5	318.8952	5.469057

[1]Calculated according to the standards (figure appendix C 1).

[2]Calculated according to the concentration data of two parallel cultivations.

Table APPENDIX C 2. *GC data of 1-undecene production from ADP1 undB samples.*

	Area	Concentration[1] (ng/ml)	Standard deviation[2]
ADP1 WT	376016	-36.4024	0.5231
ADP1 p-UA	66288473.5	349.4094	9.616981
ADP1 p-TAUA	68227404.5	360.7587	6.544222
ADP1 p-UB	2130353	-26.1335	0.166263
ADP1 p-TAUB	63205022	331.3607	27.01009

[1]Calculated according to the standards (figure appendix C 1).

[2]Calculated according to the concentration data of two parallel cultivations.

APPENDIX D: GC-MS RESULTS OF OLEFINS

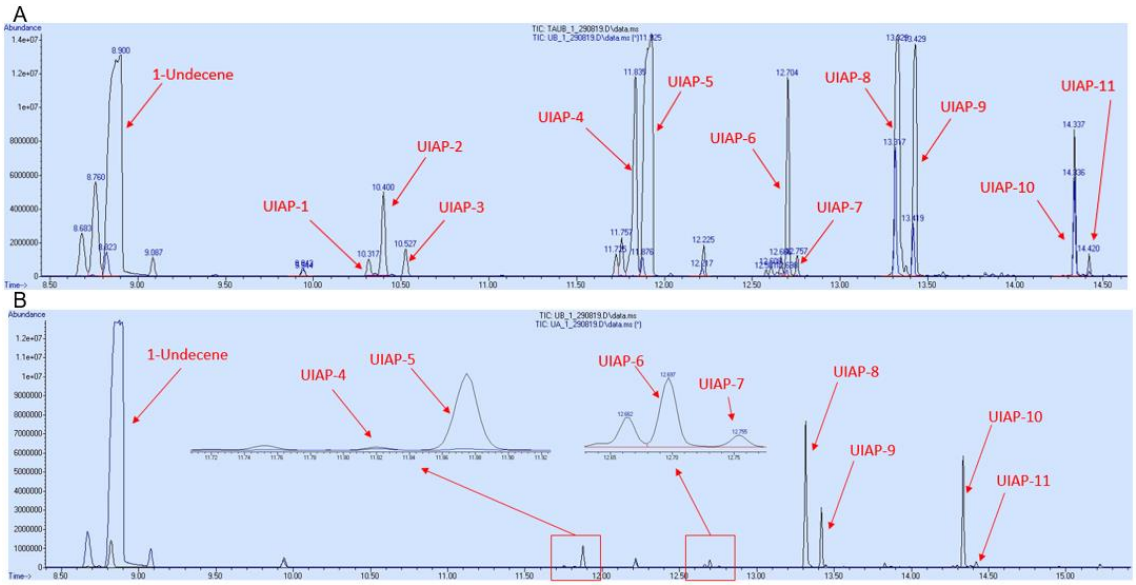
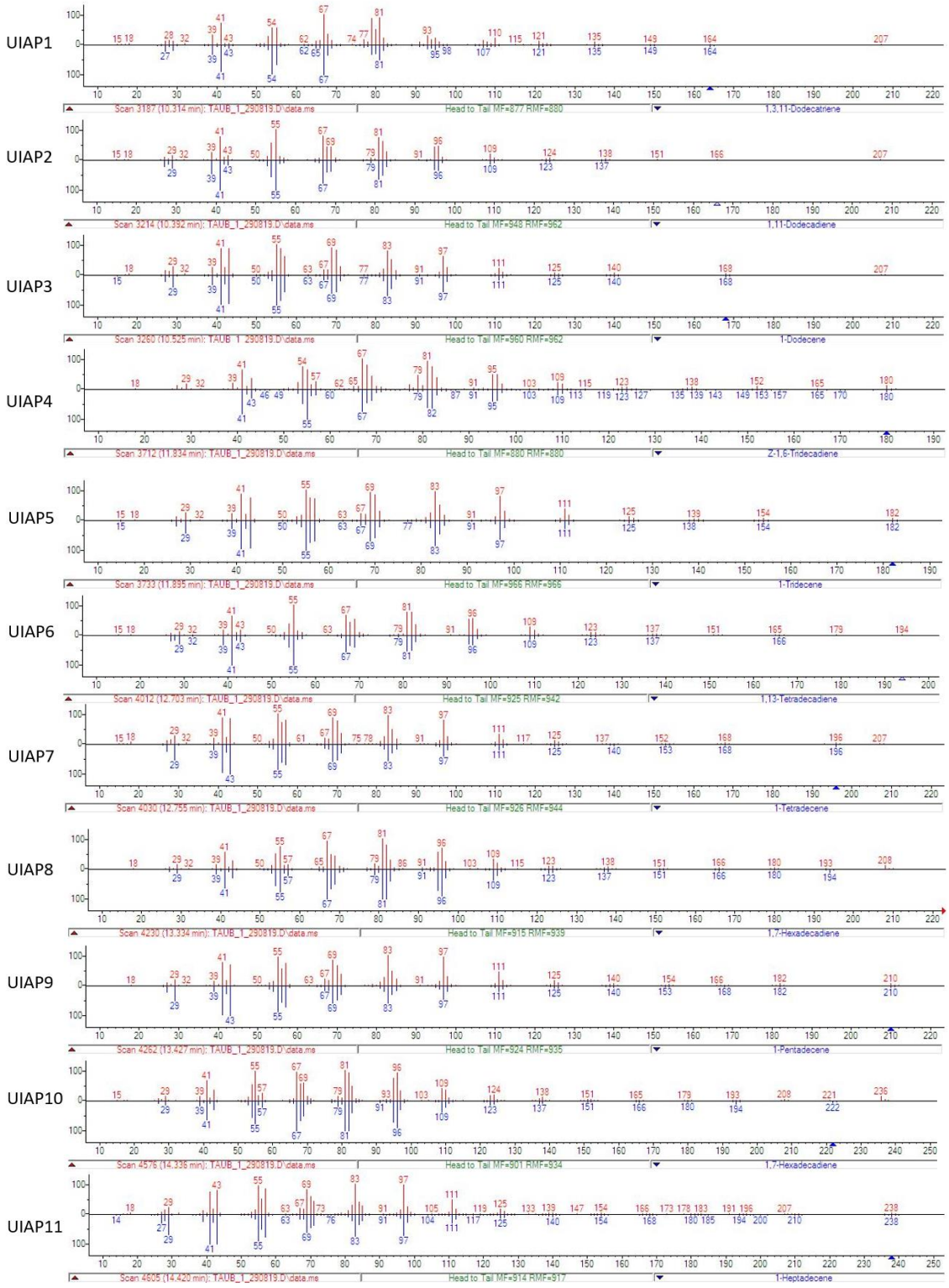
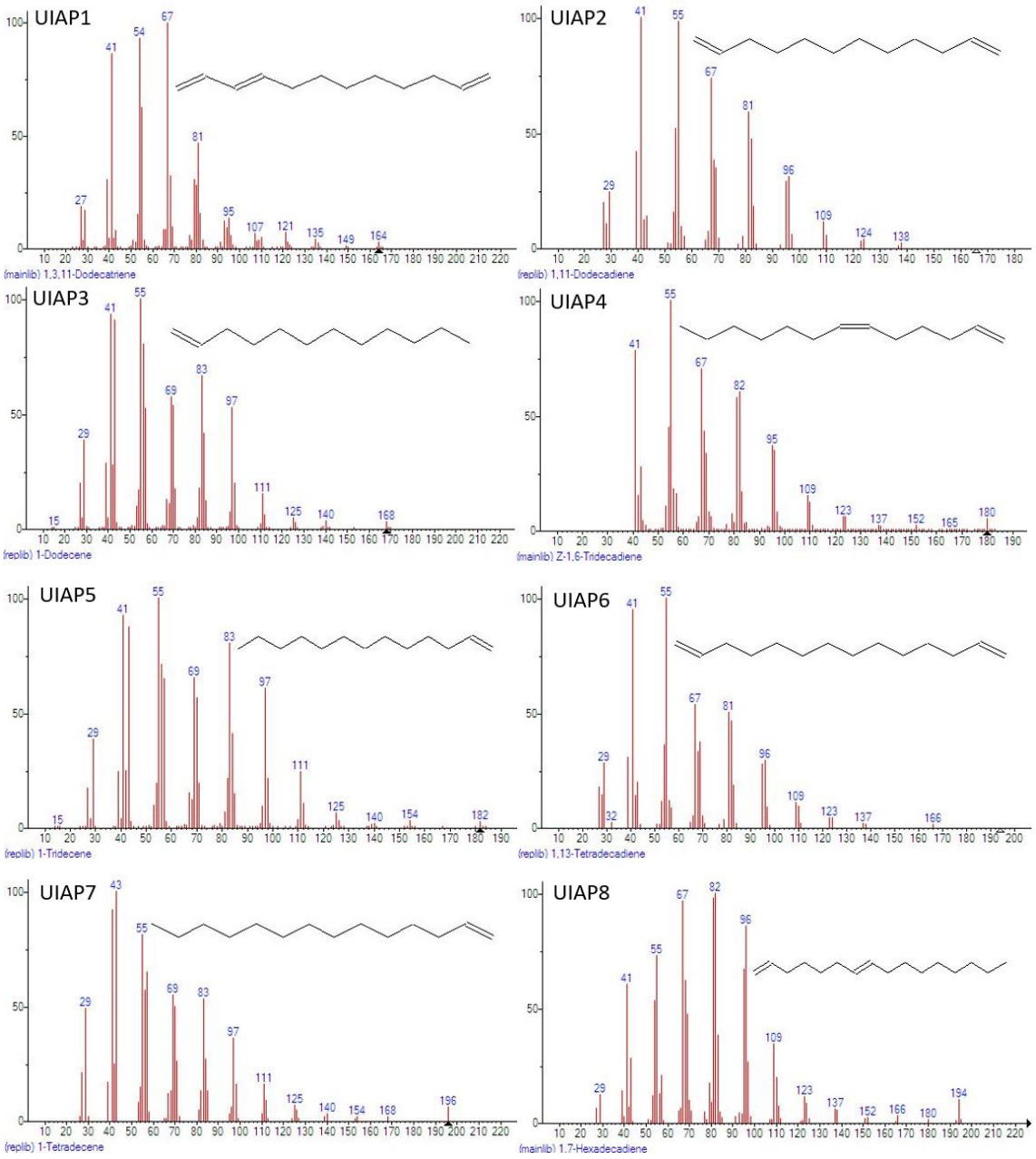


Figure APPENDIX D 1. *GC results of olefins production from ADP1 undB samples. UIAP – unidentified alkene (olefin) product.* ***(A)*** *comparison of olefins production between ADP1 p-TAUB and ADP1 p-UB.* ***(B)*** *comparison of olefins production between ADP1 p-UA and ADP1 p-UB.*

A


UIAP1
UIAP2
UIAP3
UIAP4
UIAP5
UIAP6
UIAP7
UIAP8
UIAP9
UIAP10
UIAP11

B


UIAP1
(mainlib) 1,3,11-Dodecatriene
UIAP2
(replib) 1,11-Dodecadiene
UIAP3
(replib) 1-Dodecene
UIAP4
(mainlib) Z-1,6-Tridecadiene
UIAP5
(replib) 1-Tridecene
UIAP6
(replib) 1,13-Tetradecadiene
UIAP7
(replib) 1-Tetradecene
UIAP8
(mainlib) 1,7-Hexadecadiene

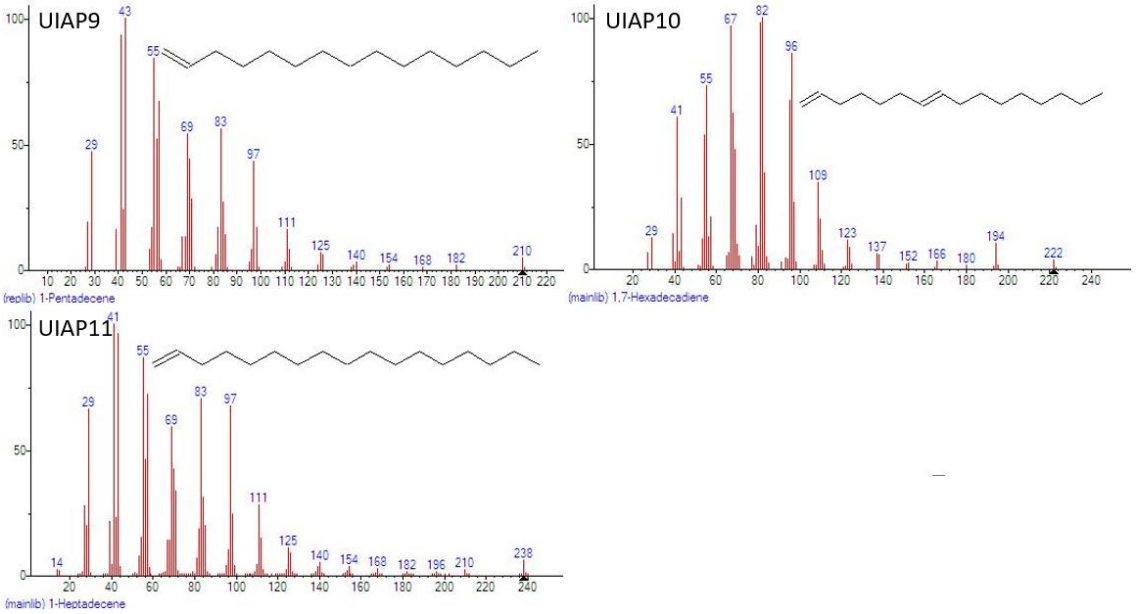


Figure APPENDIX D 2. *MS results of olefins production from ADP1 undB samples. UIAP – unidentified alkene (olefin) product.* ***(A)*** *production data matching with database.* ***(B)*** *details and possible identification by database matching results. UIAP 8 is shown in this figure as hexadecadiene (C16:2), same as UIAP 11, UIAP 8 and UIAP 11 were not match perfectly with hexadecadiene, according their retention time and mole weight, UIAP 8 is closer to C15:2 and UIAP 11 is closer to C17:2.*