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INTRACELLULAR LIPID PRODUCTION FROM LIGNIN MODEL MONOMERS BY ACINETOBACTER BAYLYI ADP1

Master of Science thesis

Examiner: Adj. Prof. Ville Santala and Dr. Suvi Santala
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ABSTRACT

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Lignin is a complex aromatic polymer consisting of phenylpropanoid units (C9 units), which, together with cellulose and hemicellulose, is the major component of lignocellulosic biomass. The cellulose and hemicellulose are usually separated from lignocellulosic biomass and converted into fuels and chemicals, whereas the lignin is usually regarded as unwelcome by-product due to its recalcitrance and inherent heterogeneity. Thus it is necessary to further exploit the value of lignin. The aim of this study is to explore the conversion of lignin model monomers into wax ester, an important industrial raw material, through microbial conversion.

The present work shows that *Acinetobacter baylyi* ADP1 can accumulate wax ester by using lignin monomers as carbon sources under nitrogen-limited condition. To improve the consumption of lignin monomers, the *crc* gene, which is involved in catabolite repression under the presence of acetate, was knocked out, and the resulting strain was designated as crc strain. The wax ester production was first conducted with wild type ADP1 using coumaric acid and ferulic acid as sole carbon source respectively. Wax ester productions of 16.9 and 14.4 μmol/100 mg dried biomass (0.169 and 0.144 mmol/g freeze-dried biomass) were obtained respectively. Co-utilization of acetate, coumaric acid and ferulic acid was conducted with both wild type and crc strain. Wild type ADP1 accumulated wax ester up to 29.2 μmol/100 mg dried biomass (0.292 mmol/g freeze-dried biomass), which was much higher than crc strain (0.1125 mmol/g freeze-dried biomass). In crc strain, the catabolite repression caused by acetate seemed to be relieved but the consumption rate of carbon source was very low. The reason still needs to be further explored.

PREFACE

The thesis is based on the experiments conducted from November 2015 to June 2016 at the Department of Chemistry and Bioengineering, Tampere University of Technology, Finland.

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I would like to express my gratitude to my parents for their endless kindness and love. Finally, I want to sincerely thank Jiaxue Hao who always gives me encouragement when I meet difficulty.

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Jin Luo

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LIST OF ABBREVIATIONS

AC	adenlylate cyclase
ACP	Acyl carrier protein
ADP1	*Acinetobacter baylyi* ADP1
ATP	Adenosine 5’ triphosphate
cAMP	Cyclic adenosine monophosphate
CAP	Catabolite activator protein
CCR	Carbon catabolite repression
CDSs	Coding DNA squences
CHPs	Conserved hypothetical proteins
CMD	γ-carboxymuconolactonedecarboxylase
CMLE	ß-carboxy-cis, cis-muconatelactonizingenzyme;
CoA	Coenzyme A
CRC	Catabolite repression control
C1, 2O	Catechol 1, 2-dioxygenase
ED pathway	Entner–Doudoroff pathway
ELH	Enol-lactone hydrolase
EMP pathway	Embden–Meyerhof–Parnas pathway
EIIA	Enzyme II A
Fe	Iron
HPLC	High performance liquid chromatography
LA medium	LB medium with agar
LB medium	Lysogeny broth medium
LCC	Lignin-carbohydrate complex
LiP	Lignin peroxidase
MA/9 medium	Minimal salt medium
MI	Muconalactone isomerase
MLE	Cis, cis-muconatelactonizing enzyme
MnP	Manganese peroxidase
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NMR	Nuclear magnetic resonance
OD	Optical density
OD_{600}	Optical density at 600 nm
PCR	Polymerase chain reaction
PHAs	Polyhydroxyalkanoic acids
PQQ	Pyrrolo-quinoline quinone
P3,4O	Protocatechuate 3,4-dioxygenase
TAG	Triacylglycerol
TCA cycle	Tricarboxylic acid cycle
TLC	Thin Layer chromatography
TH	ß-ketoadipyl-CoA thiolase
TR	ß-ketoadipate:succunyl-CoA transferase
VP	Versatile peroxidase
WS/DGAT	Wax ester synthase/acyl-CoA:diacylglycerol acyltransferase
WE	Wax ester
4-HBA	4-Hydroxybenzoic acid

1. INTRODUCTION

As energy and environment issues are becoming increasingly outstanding, the need of the transition from nonrenewable carbon sources to renewable carbon sources is more and more urgent [1]. Lignocellulosic biomass provides abundant renewable carbon sources and has the potential to be the alternative for the productions of fuels and industrial chemicals. Currently, some technologies have been developed and applied for the bioconversion of lignocellulosic biomass, in which cellulose is usually regarded as the main fraction of great interest for bioconversion. However, lignin, the second most abundant component in lignocellulosic biomass after cellulose, is underutilized and its utilization is usually limited to combustion for heat and power production due to its recalcitrance and heterogeneity [2]. In order to make best use of lignin, the development of new technology to convert lignin into high value-added products is necessary.

Lignin is an aromatic polymer whose composition varies from species to species. In nature, the initial degradation of lignin is accomplished by several enzymes, such as laccases and ligninolytic peroxidases, produced mainly by white-rot fungi, releasing different kinds of low molecular weight aromatic compounds [3]. These lignin monomers can be further catabolized by some soil bacteria through β - ketoadipate pathway, wherein different types of aromatic compounds are “funneled” to β - ketoadipate which subsequently enters into central carbon metabolism[4]. *Acinetobacter spp.* is one of the bacteria which have the ability of catabolizing a wide range of aromatic compounds, including some lignin monomers [5]. Moreover, it has been also reported that some valuable compounds such as wax esters (WEs) can be produced by *Acinetobacter spp.* as storage compound [6]. The combination of lignin monomer catabolism and lipid accumulation offers a potential approach to convert lignin into high value products.

Acinetobacter baylyi ADP1 is the laboratory strain whose genome has been completely sequenced. Many of its gene functions, metabolic pathways and physiological properties have been well characterized, which provides solid base for genetic engineering. It shares most advantages that make *Escherichia coli* an excellent model organism. In addition, it is naturally transformable and has natural tendency of homologous recombination, which substantially simplifies genetic manipulation [7]. Therefore, *A. baylyi* ADP1 can be an excellent model organism to study lipid production from lignin monomers.

The aim of this work is to explore the WE production by *A. baylyi* ADP1, using lignin monomers as carbon sources. To achieve this, several experiments were conducted. The knock-out of *crc* gene was conducted to optimize the lignin monomer catabolism pathway, and the resulting strain was designated as crc strain. The growth of wild type ADP1 on different lignin monomers was tested, using 4-hydroxybenzoic acid (4-HBA), coumaric acid, ferulic acid and vanillic acid at different concentrations as sole carbon source. The differences of the growths and carbon source consumptions between wild

type ADP1 and crc strain were compared. Finally, the cultivations for WE production were carried out under nitrogen-limited condition, and the growths, carbon source consumptions and WE productions were compared between wild type ADP1 and crc strain. In addition, the effect of iron (Fe) on WE production was also explored with wild type ADP1 using glucose as carbon source.

2. THEORETICAL BACKGROUND

2.1 Lignin

2.1.1 Composition and structure of lignin

Lignin is an aromatic polymer in which phenylpropanoid as the basic structural unit [8]. It is one of the most abundant natural polymers on the earth. Together with cellulose and hemicellulose, lignin serves as an important component in all vascular plants. In industry, lignin is usually regarded as unwelcome by-products due to its recalcitrance to degradation and heterogeneity in composition [8]. In this section, some backgrounds of lignin will be introduced, including its functions in plants, composition and structure.

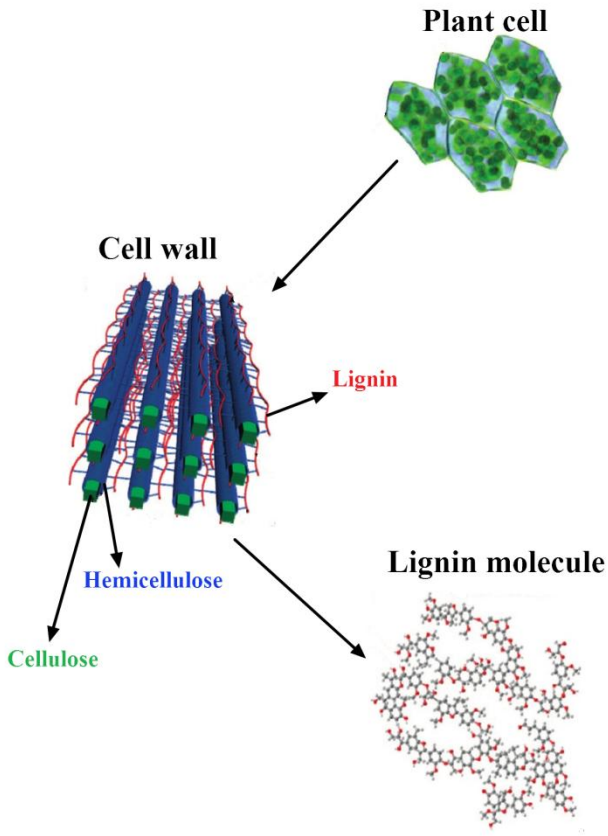


Figure 1. *Crosslink between lignin, cellulose and hemicellulose. Figure modified from [10].*

In nature, lignin mainly derives from vascular plants, and also the presence of lignin in algae has been reported in recent years [9]. Lignin is crucial to vascular plants and exists both in cell walls and between cells. It binds to cellulose or crosslinks other components (Figure 1), providing plants with firm structure and strength, and protecting plants from outside influences. As one of the components of xylem vessels which function as transport tissue in vascular plants, lignin provides extra strength to xylem and protects it from being collapsed by pressure. Therefore, the formation of lignin is thought to be related to the evolution from aquatic plants to terrestrial plants [9].

Lignin is a complex aromatic polymer which consists of phenylpropanoid units (C9 units). The formation lignin derives from the oxidative polymerization mainly of three monolignol precursors, courmaryl, coniferyl and sinapyl alcohols (Figure 2). The polymerization is mainly accomplished through alkylaryl ether linkages which occur at both alcoholic and phenolic hydroxyl groups [11]. As shown in figure 2, the three monoligols can result in

three types of lignin structures, para-hydroxyphenyl, guaiacyl and syringyl structures respectively. Different combinations of the three structures as well as the side chains lead to the diversity of lignin in composition and structure [8]. The ratio of para-hydroxyphenyl, guaiacyl and syringyl structures in lignin is flexible, depending on the types of tissue and species. For example, para-hydroxyphenyl structure is dominant in the middle lamella while the secondary wall of xylem has a high content of guaiacyl lignin [11]. The content of lignin also varies in different tissues and species. Donaldson and his co-workers analyzed the concentrations of lignin in wheat and poplar, and the results show that wheat had a lignin concentration of 31% in the middle lamella and 9% in the secondary wall of fiber while poplar had the corresponding values of 63% and 6% [12]. In addition, the content and composition of lignin in woods are regarded as essential considerations in pulp and paper industry, due to the fact that lignin composition has a significant effect on the hydrolysis of lignin, for example, the woods with high content of para-hydroxyphenyl lignin are difficult to hydrolyze.

Lignin is commonly thought to be a three-dimensional polymer with an amorphous and cross-linked structure. But it has been also reported that, in some species, the frequencies of cross-link are quite low or even none and some linear behaviors of lignin were observed [11]. It is worth noting that lignin can covalently bind to some polysaccharides, mainly hemicellulose, forming lignin-carbohydrate complex (LCC) [13]. In hemicellulose, different sugar monomers, including arabinose, xylose, galactose, glucose, manose, can covalently link to lignin through ester, phenyl glycoside and benzyl ether linkages. Almost all kinds of lignin have covalent links to polysaccharides through these linkages but they only exist in a small amount.

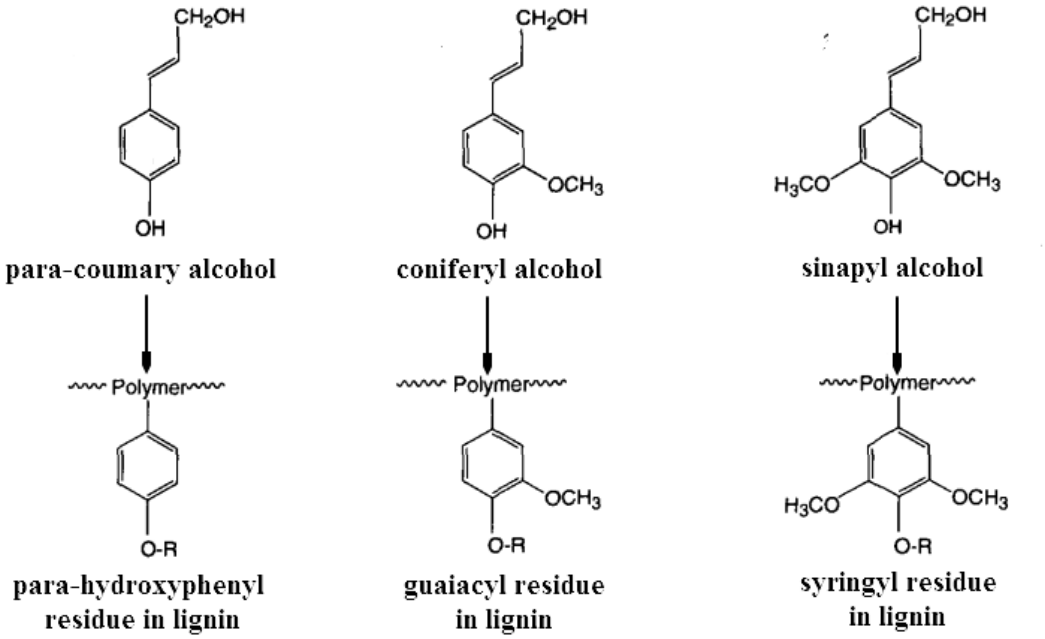


Figure 2. *The three main structures of lignin. Figure modified from [9].*

Normally, the individual polymer chains do not have exact molecular weight due to the different degree of polymerization, and lignin is no exception. Thus, different types of average values, such as number average molar mass and mass average molar mass, are usually applied to describe the molecular weight of polymers. The molecular weight of kraft lignin, the lignin separated during pulping process, ranges from 200 to 200000

g/mol, depending on the source of the wood, the method of analysis and the way of separation. The molecular weight of lignin is important for its application, because molecular weight will affect the mechanical properties of lignin, thereby affecting the properties of lignin-derived products [14].

2.1.2 Degradation of lignin in nature

In lignocellulosic biomass, lignin covalently links to hemicellulose and embeds cellulose inside, forming a natural barrier to prevent cellulose from being degraded. Therefore, lignin is regarded as an unwelcome component in some industries in which cellulose is the component of great interest. For example, in paper making and bioethanol production processes, pretreatment of lignocellulosic biomass is an essential step, whose purpose is to break down lignin structure so that cellulose can be separate from lignin for further processes [15, 16]. Currently, some methods have been developed for lignocellulosic biomass pretreatment and lignin depolymerization, including pyrolysis, alkaline catalysis, acid catalysis, biological catalysis and so on according to different needs [16]. In nature, the degradation of lignin is accomplished mainly by fungi and bacteria through a series of enzymatic reactions. These processes will be summarized in this section.

The ether and C-C linkages in lignin are highly stable, which make lignin recalcitrant to degradation [17]. Since Boruff and Buswell reported the degradation of lignin by microorganisms in 1934 [18], many researches regarding lignin degradation have been done. In nature, basidiomycetes are the main contributors of lignin degradation, of which white-rot basidiomycete and brown-rot basidiomycete are the two most frequent lignin decomposers [3]. Brown-rot basidiomycete can only partly modify lignin, after which degrades cellulose and hemicellulose, leaving behind a brown, dry and crumbly substance comprising oxidized lignin. Different from brown-rot basidiomycete, white –rot basidiomycete can selectively degrade lignin to a great extent or simultaneously degrade lignin and cellulose, leaving behind white, moist, cellulose-rich residues [3]. Apart from basidiomycete, some *Pseudomonas* species and actinomycetes were also reported to be able to degrade lignin [19]. However, bacteria have a weaker lignin-degrading ability compared with fungi, and it is also reported that some bacteria can only significantly degrade low molecular weight oligomers or modified lignin [20].

White-rot basidiomycete can produce a series of enzymes which are involved in lignin-degrading process. These enzymes mainly include laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP). Since laccase was first identified in *Rhusvernicifera* by Yoshida in 1883, a large number of studies about laccase have been conducted. It is reported that laccase is a multiple function enzyme which widely exists in plants, insects, bacteria and fungi [21]. Depending on its origin, laccase serves different functions. In plants, laccase is related to fruit body development, plant wounding responese, lignin synthesis and so on. In fungi, laccase is though to be related to fungal morphgenisis, pigment synthesis, pathogenisis as well as lignin degradation [21]. The structure of laccase has been extensively explored in bacteria and fungi. Generally, laccase thought to be monomer comprising three cupredoxin-like domains. It is worth noting that laccase contains four copper atoms, of which one copper is located in one of of the domains (domain 3), and the remaining three coppers are embedded between domain 2 and domain 3, forming a trinuclear cluster. These coppers are essential to the catalytical behaviour of laccase. Compared with LiP and MnP, laccase has a low redox

potential and can only oxidize the phenolic units of lignin which account for less than 10% of the total lignin polymer. Only under the presence of some synthetic small mediators, such as 1-hydroxybenzotriazole, can laccase catalyse the oxidization of non-phenolic components [17]. In the catalytical reaction , laccase first removes one electron and proton from the phenolic structure, forming phenoxy radicals, after which the reduced laccase is oxidized by oxygen. This process is accomplished by the coppers transferring electrons coordinatively [22]. The formation of phenoxy radicals can subsequently lead to a series of reactions, including the cleavage of C-C and C-O bonds between and within the phenylpropanoid units, thereby causing the degradation of lignin.

The major contributors in the degradation of lignin are lignin peroxidase (LiP) and manganese peroxidase (MnP), which were discovered in the middle of 1980s in *Phanerochaete chrysosporium*, a type of white rot basidiomycete. Their structures and catalytic behaviors have been extensively studied [23]. LiP can catalyze the oxidation of the non-phenolic units which account for more than 90% of the lignin polymer, while MnP can only catalyze phenolic compounds in most cases. These ligninolytic peroxides share the general structures containing ferric heme and porphyrin which confer high redox potential to the enzymes and play an important role in the catalytic behaviors. As shown below, these enzymes have similar catalytic cycles which mainly include three reactions. In reaction 1, hydrogen peroxide, the natural electron acceptor, oxidizes the resting peroxidase, forming compound I. After that, compound I reacts with its reducing substrate, forming compound II which will subsequently oxidize another substrate.

$$\text{Peroxidase } [Fe^{3+}\bullet\text{porphyrin}] + H_2O_2 \rightarrow \text{Compound I } [Fe^{4+}{=}O\bullet\text{porphyrin}^{\cdot+}] + H_2O \quad (1)$$

$$\text{Compound I } [Fe^{4+}{=}O\bullet\text{porphyrin}^{\cdot+}] + S \rightarrow \text{Compound II } [Fe^{4+}{=}O\bullet\text{porphyrin}] + S^{\cdot} \quad (2)$$

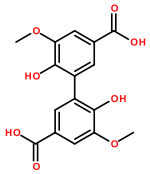
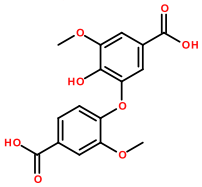
$$\text{Compound II}[Fe^{4+}{=}O\bullet\text{porphyrin}] + S \rightarrow \text{Peroxidase } [Fe^{3+}\bullet\text{porphyrin}] + S^{\cdot} + H2O \quad (3)$$

These processes apply to most ligninolytic peroxides, but they differ in the reducing substrates oxidized in reaction 2 and 3. Compared with MnP, MiP has a relatively unspecific substrate, that is, both phenolic and non-phenolic compounds can be the reducing substrates in reaction 2 and 3. Similar to laccase, the formation of aromatic radicals is the first step of the catalytic process, after which a series of reactions are initiated, including the cleavage of C-C, C-O and the break-down of aromatic rings. Veratryl alcohol is thought to be an important mediator in the catalytic reaction undertaken by LiP. Veratryl alcohol can reduce compound II which has a lower redox potential than compound I, accelerating the regeneration of LiP. Different from LiP, MnP does not catalyze the oxidation of lignin directly, though some report has shown that phenolic compounds can be oxidized by the compound I of MnP [24]. $Mn^{2+}$is the only substrate which can efficiently reduce the compound I and compound II of MnP, after which $Mn^{3+}$is formed and MnP regenerates again. The formed Mn^{3+} is thought to be a diffusive oxidant which can oxidize not only phenolic compounds but also the secondary substrates which are far away from the catalytic site.

Apart from laccase, LiP and MnP, some other enzymes are also involved in the process of lignin degradation, such as versatile peroxidase (VP) which can catalyze the substrates of both LiP and MnP. The degradation of lignin results from the concerted action of different enzymes, leading to the formation of different types of low molecular weight compounds, including monomers, dimers, and oligomeric fragments. Some products from the treatment of lignin by bacteria and fungi are shown in table 1. All the

aromatic acid are benzoic acid, indicating the C-C cleavage of lignin polymer. Other products include biphenyl dicarboxylic acid, dipnenyl ether and so on [17]. These aromatic compounds can be further catabolize by bacteria throughβ - ketoadipate pathway. This will be discussed in the following section.

Table 1. *The common products of lignin degradation by bacteria. Information is from [17]*

Compound		Fungal lignin degrader	Bacterial lignin degrader
Benzoic acid	4-hydroxy	*P. chrysosporium*	*A. aneurinilyticus*
	4-hydroxy-3-methoxy	*P. chrysosporium*	*A. aneurinilyticus*
	4-hydroxy-3-methoxy-6-carboxy	*P. chrysosporium*	
	4-hydroxy-3-methoxy-5-carboxy		*P. putida*, *R. jostii* RHA1
	3,4-dimethoxy	*P. chrysosporium*	
	3,4-dimethoxy-2-carboxy	*P. chrysosporium*	
	2-hydroxy-3-methoxy		*A. aneurinilyticus*, *P. putida*
	2,3-dihydroxy		
	2,3,4-trihydroxy		*Bacillus* sp.
Benzaldehyde	4-hydroxy-3-methoxy		*S. paucimobilis*
	3,4,5- trihydroxy		*Bacillus* sp.
Cinnamic acid	4-hydroxy		*Bacillus* sp.
	4-hydroxy-3-methoxy		*Bacillus* sp., *P. putida*, *R. jostii* RHA1
Biphenyl-5,5'-dicarboxylic acid, 2,2'dihydroxy, 3,3'-dimethoxy		*P. chrysosporium*	
Diphenyl ether		*P. chrysosporium*	
Propiophenone-3'-hydroxy	4-hydroxy-3-methoxy		*S. paucimobilis*, *P. putida*, *R. jostii* RHA1
Acetophenone	4-hydroxy-3-methoxy		Soil metabolite
Phenol	2-methoxy		Soil metabolite
	2-methoxy-4-vinyl		Soil metabolite

2.1.3 Utilization of lignin

Lignin represents an abundant renewable source which is underutilized. Several methods have been developed to specifically isolate lignin from lignocellulosic biomass [8]. However, in practice, lignin is usually generated from timber enterprises or

co-generated in paper making industry as by-product or waste [25]. Due to the growing up of industrial biorefinery, in which mainly carbohydrate is converted into fuel or chemical, more and more lignin will be generated. Currently, only insignificant proportion of the generated lignin are utilized to produce valuable products, and a substantial amount of lignin are burned for in-house energy needs or dumped to water bodies.

Although a large amount of lignin derived from pulping and pretreatment processes are limited to combustion, lignin has a wide range of applications, some have already been implemented and some are still in research stage. Here, some applications are briefly introduced. Lignin used as water reducer for concrete can improve concrete strength and reduce damage caused by moisture and acid rain. Lignin can be also used for the preparation of different polymers, foams and plastics. For example, compared with conventional polyurethane, lignin-based polyurethane foams present some advantages in response to temperature and humidity test [26]. By introducing hydrophilic or lipophilic groups and altering molecular weight, lignin can be used to produce various surfactants. Other applications of lignin include adhesive, antioxidant, rubber intensifier and so on. In addition, novel polymers synthesis from lignin degradation products was proposed [27]. Some polymers, such as polyhydroxy styrene derivatives, polyethers, polyesters and polybenzalazine, having hydroxyphenyl, guaiacyl, and syringyl groups can be synthesized from lignin monomers. Bioconversion provides another way for lignin utilization [28].Lignin is first obtained from the pretreatment of lignocellulosic biomass, after which the lignin is depolymerized into various aromatic compounds. A subset of bacteria has the ability to catabolize these compounds and generate intracellular carbon storage compounds which can be used as chemicals, fuels and precursors of materials.

Pretreatment is an essential step for efficient biomass utilization, one of whose purposes is to separate different components for specific uses. However, current pretreatment of biomass is focused on cellulose, and selective isolation of lignin from pretreatment becomes one of the barriers that limit the utilization of lignin. Therefore, it is necessary to develop a pathway to obtain sufficient lignin from pretreatment without affecting the separation of cellulose.

2.2 *Acinetobacter baylyi* ADP1

2.2.1 Description of *A. baylyi* ADP1

Acinetobacter species are Gram-negative encapsulated coccobacilli which widely spread in nature, and can be found in soil, water and the body of living organisms. They are oxidase-negative, strictly aerobic, non-fermentative and non-motile, and often found in pair or cluster under microscope. They can be cultured in most laboratory mediums, including both nutrient rich medium and minimal medium. *Acinetobacter* species was first isolated from soil in 1911. The genus *Acinetobacter* was created in 1954, which recently has been classified in the order *Pseudomonadales* and the family *Moraxellaceae* [29]. Due to the high similarity, it is hard to differentiate *Acinetobacter* species through phenotypic characteristics, such as cell morphology and nutrient utilization. A more reliable classification was obtained by the analysis of the combination of phenotypic characteristics and DNA-DNA hybridization [29], and so far

62 species have been recognized, of which 49 of them have validly published names, 6 have effectively published names and 5 refer to DNA groups.

Acinetobacter has been paid attention to due to its unique properties, which is mainly reflected in four aspects. First, some *Acinetobacter* species, such as *Acinetobacter rbaumannii*, *Acinetobacter pittii* and *Acinetobacter rnosocomialis*, are important nosocomial pathogens which often lead to infection in humans, especially those with low immunity [30]. These infections include pulmonary infection, wound and skin infection, urinary system infection, bacteriemia and cephalomeningitis. It is worth noting that these species have a strong adhesion to the surface of most material and ability of antibiotic acquiring, which significantly increases the frequency of causing nosocomial infection. Second, *Acinetobacter* has a great potential in the application of waste treatment, Some *Acinetobacter* species have the ability of degrading different aromatic compounds, such as benzene, phenol, toluene, styrene and some halogenated compounds, which are regarded as highly toxic pollutants and difficult to remove. This ability has made them attracting microorganisms in the treatment of wastewater [30]. In addition, some *Acinetobacter* strains were confirmed to be able to accumulate polyphosphate, providing an alternative to chemical precipitation for the treatment of phosphate contaminated water [31].Third, *Acinetobacter* species can produce different types of biopolymers as carbon storage compounds, with a wide range of substrates as carbon source, including some aromatic compounds. Some of these polymers, such as glycolipids, lipases, polyesters and polysaccharides, have great values and can be used to produce fuel and chemicals [30]. Fourth, recently a specific strain *A.baylyi* ADP1was isolated and showed significant advantages to be an ideal model organism for genetic study [32].

E. coli have become a widely used model organism for genetic study due to its rapid growth, simple substrate requirement, well-studied genetic background and cultivation condition. However, *E. coli* has two shortages which make genetic manipulation tedious and error prone. The first shortage is the lack of natural competence. *E. coli* must be processed prior to transformation, and electroporation or other methods are needed for successful transformation. The second shortage is the lack of natural recombination ability. Therefore, additional recombination system is needed to achieve successful gene recombination. For example, λ prophage containing recombination genes *exo*, *bet* and *gam* are usually transferred into *E. coli* before the transformation of linear DNA [33]. Gene *exo* and *bet* are responsible for the recombination activity while gene *gam* expresses the protein which inhibits the degradation of linear DNA by nuclease. These processes are time consuming and sometimes cause undesirable results.

A. baylyi ADP1 shares the most properties that make *E. coli* an excellent laboratory model organism [7]. Moreover, ADP1 possesses two advantages that can overcome the aforementioned problems. First, *A. baylyi* ADP1 is naturally transformable [34]. Both linear DNA and plasmid DNA can be taken up by ADP1 during exponential growth. The induction of natural transformation is achieved by the inoculation of stationary phase culture into fresh medium, after which the cells are in competent state. The genes expressing the proteins which mediate the transformation show a similarity with the genes related to type–IV pili biogenesis. In ADP1, a pilin-like protein was identified and thought to be an important component for its natural competence [35]. The second advantage is the inherent recombination ability of foreign DNA. After transformation, foreign DNA containing homologous sequence to the target DNA can be efficiently

integrated into the recipient genome of ADP1 without addition of recombination system [35]. The recombination efficiency will be increased with the increase of the sequence similarity. This is so-called homologous recombination which is RecA protein-dependent. These two advantages simplify the process of genetic modification, allowing the manipulation simply by direct addition of linear or plasmid DNA into exponential phase culture. And different types of genetic modifications, including gene deletion, replacement, complementation, operon insertion and chromosomal protein tag, have been successfully and effectively implemented with ADP1 [7]. Furthermore, ADP1 lacks the genes associated with pathogenesis, which make it an attractive organism for industrial use [5].

Table 2. *The comparison of the genomic features between A. baylyi ADP1 and the selected species which have close proximity with ADP1 in terms of lineage. Data is from [5]*

	A. baylyi ADP1	PSAE	PSPU	PSSY	ESCO
General features					
Size (Mb)	3.6	6.3	6.4	5.8	4.6
GC%	40.3	66.6	61.6	58.4	50.8
Nb CDS	3325	5567	5420	5615	4273
% Coding	88.8	89	87.7	86.8	92
rRNA	7	4	7	5	7
tRNA	76	63	63	63	82
Known and putative proteins (%)	62.6	65.9	62.0	61.0	80.5
CHP (%)	20.3	22.0	23.0	28.0	12.3
No homology (%)	13.9	11.1	15.0	11.0	7.2
TIGR categories (%)					
amino acid biosynthesis	3.52	1.65	2.16	1.81	2.09
Biosynthesis of cofactors, prosthetic groups and carriers	3.61	2.02	2.57	2.17	1.85
Cell envelope	3.97	2.54	5.58	6.55	3.16
Cellular process	6.16	3.49	6.14	8.01	3.47
Central inmediary metabolism	3.49	2.08	1.34	1.49	1.34
DNA metabolism	2.16	1.33	2.01	2.27	1.87
Energy metabolism	2.97	5.29	7.81	5.47	6.78
Fatty acid and phospholipid metabolism	1.47	1.27	1.91	1.51	1.23
Protein fate	2.25	2.17	3.07	3.40	2.13
protein synthesis	4.03	1.94	2.25	2.04	2.24
Purines, pyrimidines, nucleosides and nucleotides	0.96	0.95	1.10	0.97	1.41
Regulatory functions	6.97	4.69	9.13	7.45	3.23
Transcription	2.19	0.73	1.12	0.85	0.75
Transport and binding proteins	10.43	4.68	11.20	10.03	5.79

PSAE, *Pseudomonas aeruginosa*; PSPU, *Pseudomonas putida*; PSSY, *Pseudomonas syringae*; ESCO, *Escherichia coli*.

The genome of ADP1 has been completely sequenced [5]. It has a circular chromosome with a very small length of 3.6 Mb while the chromosome of *E. coli* is 4.6 Mb in length, which makes genetic manipulation more amenable. Based on the analysis of 16sRNA gene sequence, *Acinetobacter* species has the closest proximity with *Pseudomonas* species. However, their GC contents are much different, which are 40% and 62% respectively. In ADP1, about 88.8% of the chromosome are coding DNA sequences (CDSs), with 3325 identified protein coding genes. Among these protein coding genes,

62.6% of them have a definitive or putative assignment while 20.3% of them encode for conserved hypothetical proteins (CHPs) (table 2). Genes related to transporter and enzymatic functions are the most abundant in the genome of ADP1. The abundance of transporter and enzymatic function results in the fact that ADP1 can use various components as energy and carbon sources [5]. Specifically, aromatic compounds with different structures, such as 4-hydroxy benzoic acid, coumaric acid, vanillic acid, ferulic acid, can be converted into common intermediates by ADP1 (β- ketoadipate pathway), after which enter into central pathways. This is an essential pathway in terms of lignin model compounds utilization and will be discussed in the following section.

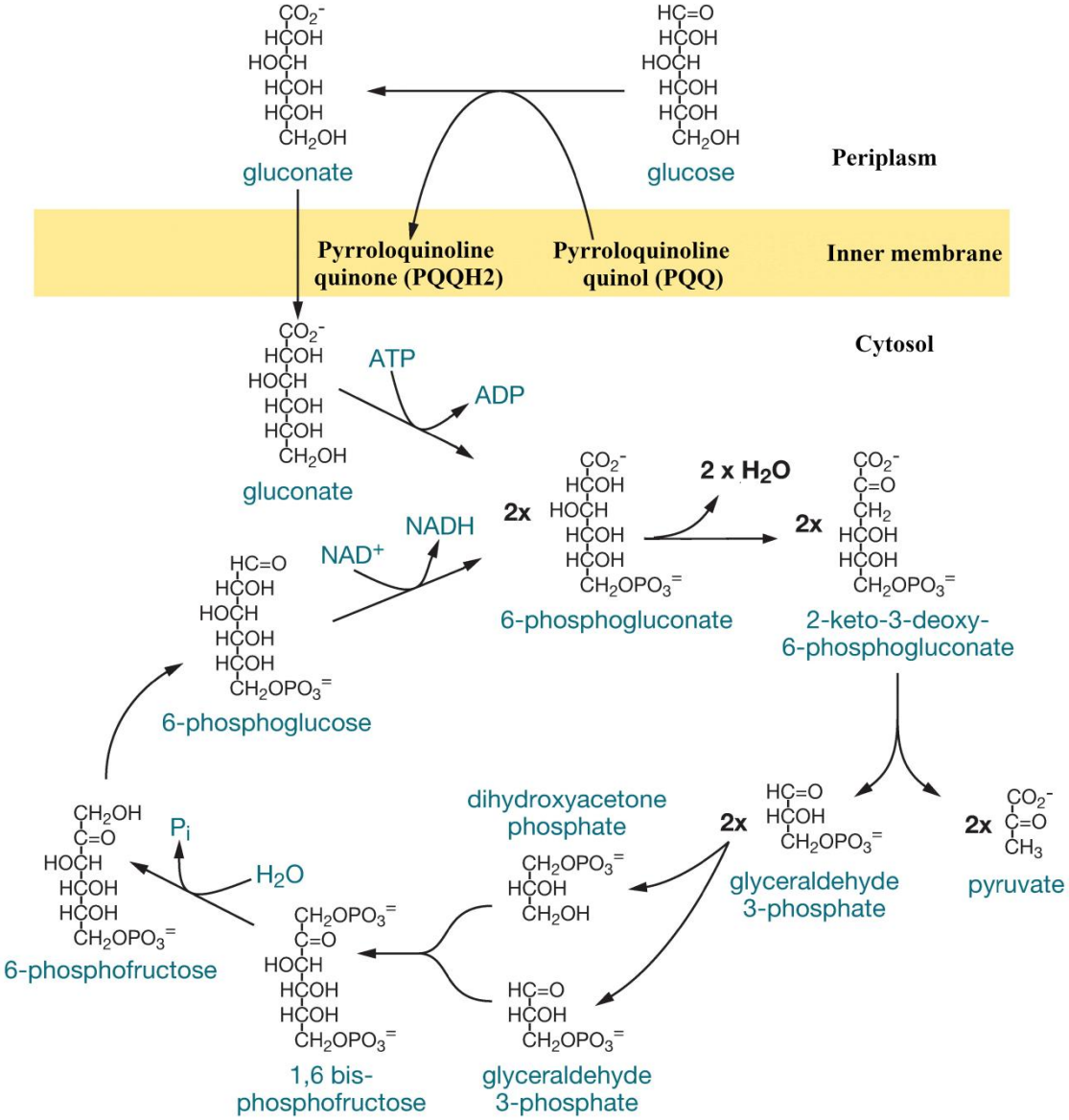


Figure 3. *A plausible mechanism of glucose-pyruvate conversion in A. baylyi ADP1. Figure modified from [37].*

Another feature of ADP1 which is worth noting is the way of glucose utilization. Embden–Meyerhof–Parnas (EMP) pathway is the first step of glucose metabolism in most organisms, in which glucose is converted into pyruvate which will subsequently enter into central pathway. However, ADP1 lacks the genes encoding for glucokinase, 6-phosphofructokinase and pyruvate kinase, which are all important components in EMP pathway. Therefore, Entner–Doudoroff (ED) pathway is thought to be used by

ADP1 for glucose utilization [5]. Figure 3 shows a plausible mechanism of glucose to pyruvate conversion in *A. baylyi* ADP1. Glucose is first converted into gluconate by glucose dehydrogenase on the surface of inner membrane. Glucose dehydrogenase is a membrane-bound aldolase dehydrogenase having pyrrolo-quinoline quinone (PQQ) as coenzyme [36]. Then, gluconate is transported into cytosol, driven by the energy derived from the oxidation of glucose. Subsequently, metabolism goes on through the modified Entner–Doudoroff (ED) pathway in which three carbon atoms are converted into pyruvate for further metabolism by tricarboxylic acid (TCA) cycle, and three carbon atoms are recycled [37]. The genes encoding for TCA cycle are complete in ADP1.

A. baylyi ADP1 has captured people's attention due to its unique features. The versatility in substrate degradation, biopolymer synthesis ability and non-pathogenicity of ADP1 make it a potential microorganism for industrial use. The compatibility to heterologous genes and well-studied genetic background make it an excellent platform for the construction of new metabolic systems. Its natural competence and recombination capability provide a convenient way for genetic manipulation.

2.2.2 ß- ketoadipate pathway

ß-ketoadipate pathway widely exists in soil bacteria and fungi, through which a variety of aromatic compounds can be converted into the common intermediate, ß- ketoadipate, which will subsequently proceed to the central pathway [4]. ß- ketoadipate pathway has been observed in some gram-positive bacteria, such as genera *Bacillus*, *Rhodococuss*, *Arthrobacter*, etc, as well as some gram-negative bacteria, such as *Pseudomonas*, *Acinetobacter*, *Alcaligenes*, *Comamonas*, etc. Some eukaryotes, such as *Rhodotorula* spp., *Aspergillus* spp., etc, also possess ß- ketoadipate pathway but with some different features. ß- ketoadipate pathway is exclusively found in soil microorganisms, most of which are associated with plants [4]. This makes sense due to the fact that many aromatic compounds are released from plants. For example, some phenolic compounds are the secondary metabolites in plants, playing an important role the growth of plants, protecting plants from being invaded by pathogens and predators [39]. In addition, as lignin, a heterogeneous aromatic polymer, is one of the major components of vascular plants, various aromatic monomers can be released from the decay of plants.

ß-ketoadipate pathway is mostly chromosomally encoded and highly conserved in soil bacteria due to natural selection. But some differences are also presented in different bacteria with regard to enzyme distribution, gene regulation and organization. These differences optimize their growth in the diverse environments where they reside. As shown in figure 4, ß-ketoadipate pathway includes two branches. One is catechol branch, in which compounds such as benzoate, benzene, phenol, aniline, cinnamate, salicylate and anthranilate are converted to catechol and then ß-ketoadipate is formed through a series of enzymatic reactions. Another one is protocatechol branch, in which compounds such as 4-hydroxybenzoate, coumarate, ferulate, vanillate, coniferyl alcohol, quinate, shikimate are converted to protocatechol and subsequently ß-ketoadipate is also formed. In ß-ketoadipate pathway, the first stage is the modification of various aromatic compounds by mono- or dioxygenation steps, resulting in the formation of a dihydroxylated benzene ring, namely catechol and protocatechol [4]. The second stage is the conversion of catechol and protocatechol into ß-ketoadipate. This process has been well elucidated in *P. putida* [4, 39-42]. The first step of this process is ring

cleavage, which is catalyzed by dioxygenase. The cleavage occurs between the hydroxyl groups and is called ortho-cleavage. In protocatechol branch, the cleavage is catalyzed by protocatechuate 3,4-dioxygenase (P3,4O), in which two atoms of oxygen are introduced, forming ß-carboxy-cis, cis- muconate. The activity of P3,4O need the participation of ferric iron. And then, the formed ß-carboxy-cis, cis- muconate is lactonized by ß-carboxy-cis, cis-muconate lactonizing enzyme (CMLE), forming γ-carboxy muconolactone. γ-carboxy muconolactone is unstable and rapidly undergoes decarboxylation, catalyzed by γ-carboxymuconolactone decarboxylase (CMD) and forming ß-ketoadipate enol-lactone. This step is accompanied by the production of carbon dioxide. The formation of ß-ketoadipate enol-lactone is the convergent point of protocatecholand catechol branch. As shown in figure 4, catechol branch undergoes parallel steps catalyzed by analogous reactions: catechol 1, 2-dioxygenase catalyzes the cleavage of catechol, forming muconate; the lactonization of muconate is catalyzed by cis, cis-muconate lactonizing enzyme (MLE), forming muconolactone which is subsequently isomerized to ß-ketoadipateenol-lactone by muconalactone isomerase (MI). The ß-ketoadipateenol-lactone will be further converted into ß-ketoadipate by enol-lactone hydrolase (ELH).

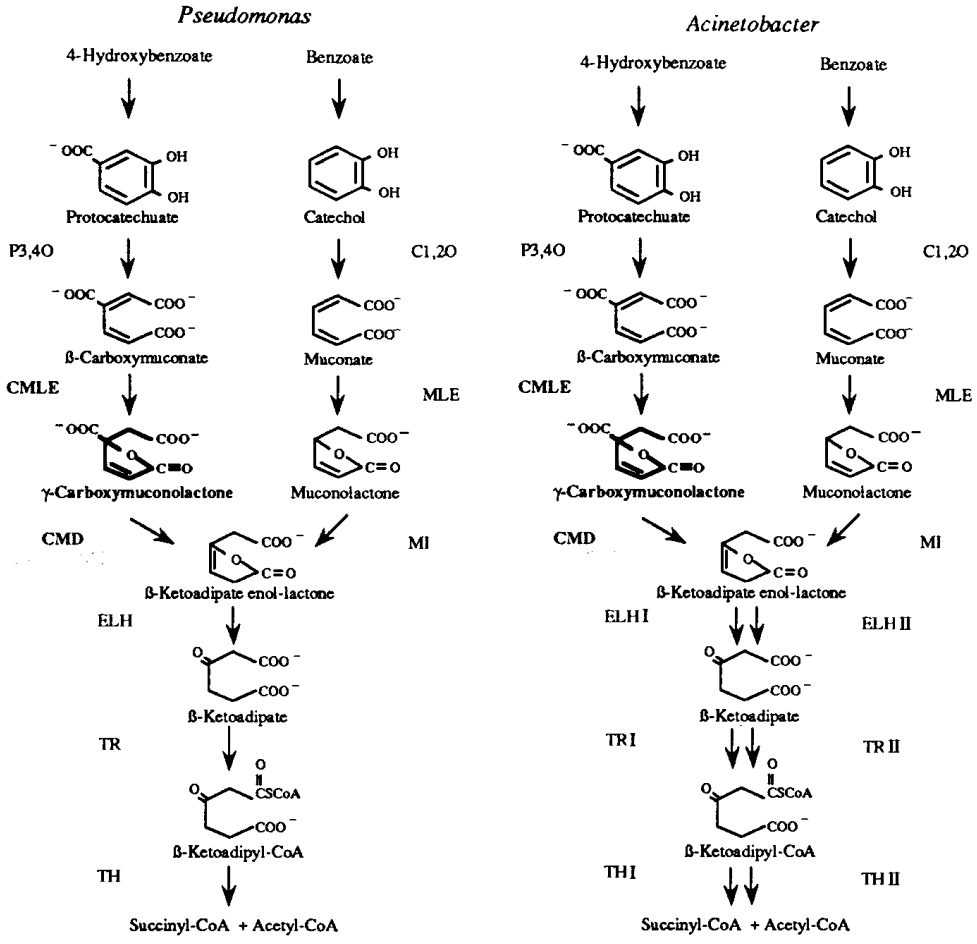


Figure 4. *ß-ketoadipate pathways in Acinetobacter and Pseudomonas. Figure modified from [4]. C1, 2O, catechol 1, 2-dioxygenase; MLE, cis, cis-muconate lactonizing enzyme; MI, muconalactone isomerase; P3,4O, protocatechuate 3,4-dioxygenase; CMLE, ß-carboxy-cis, cis-muconate lactonizing enzyme; CMD, γ-carboxymuconolactone decarboxylase; ELH, enol-lactone hydrolase; TR, ß-ketoadipate:succunyl-CoA transferase; TH, ß-ketoadipyl-CoA thiolase.*

As mentioned before, ß-ketoadipate pathway is highly conserved in different species but differs in enzyme distribution, gene regulation and organization. As shown in figure 4, in *P. putida* protocatechuate branch and catechol branch converge at the point when ß-ketoadipateenol-lactone forms while in *Acinetobacter* two branches never converge. In *Acinetobacter calcoacetica*, the last three steps of ß-ketoadipate pathway are independently catalyzed by two sets of isofunctional enzymes which are encoded by two sets of genes, *catIJFD* and *pcaIJFD* [4]. The genes *catIJF* and *pcaIJF* are almost identical in sequence and can freely exchange genetic information, while *catD* and *pcaD* are only 52% similar in nucleotide sequence. The study of enzyme regulations in *P. putida* and *A. calcoacetica* has shown that they have different inductive patterns [42, 43]. In *A. calcoacetica*, the five enzymes responsible for the conversion of protocatechuate to ß-ketoadipyl-CoA are synthesized coordinatively, induced by protocatechuate. And cis, cis-muconate induces all the enzymes responsible for the conversion of catechol to ß-ketoadipyl-CoA [43]. However, in *P. putida*, it is ß-ketoadipate that induces the two enzymes specific to protocatechuate pathway, CMLE and CMD, as well as ELH which catalyzes hydrolysis of ß-ketoadipate enol-lactone. And cis, cis-muconate only induces the three enzymes in catechol pathway [42]. The genes coding the enzymes involved in the related metabolic pathway are often contiguous, but the genes participating in the two branches of ß-ketoadipate pathway do not need to be close to each other. For example, the *pca* genes for protocatechol branch are about 290 kb from *cat* genes for catechol branch in *A. calcoacetica*, while they are 2000 kb away from each other in *P. aeruginosa*.

Many aromatic compounds, such as ferulate, coumarate, vanillate, 4-hydroxybenzoate, etc., are both good substrates and chemoattractants for most bacteria which possess ß-ketoadipate pathway. Chemotaxis and transport proteins play important roles in these processes. For example, PcaK protein functions as both 4-hydroxybenzoate chemoreceptor and transporter in *P. putida* [44], while in genus *Acinetobacter baylyi* which is non-motile, it serves as only transport protein [4].

2.2.3 Carbon catabolite repression in ß- ketoadipate pathway of *A. baylyi*

Carbon catabolite repression (CCR), simply, catabolite repression is an important regulatory system in microorganisms, which allows microorganisms to utilize the preferred carbon or energy sources first. The presence of the preferred carbon source will repress the utilization of secondary carbon source. This can be achieved by different ways, including the control of sugar transport, transcription and translation etc [45]. CCR even plays an important role in the expression of bacterial virulence factors [45].

CCR has been reported in many organisms, and the most well known ones are *E. coli* and *Bacillus subtilis*. When both glucose and lactose are present in the medium, *E. coli* will utilize glucose first and then lactose because the presence of glucose will repress the consumption of lactose. The repression is achieved by the regulation of both the catabolic gene expression and lactose transport [45]. Enzyme II A (EIIA), which is responsible for glucose transport, is the central component in CCR in *E. coli*. When glucose concentration is low, EIIA is mostly in phosphorylated form, which is in favor

for the activation of lactose catabolizing gene and the activity of lactose permease.

CCR are well understood in *E.coli* and some gram-positive bacteria but not in genus *Acinetobacter* and *Pseudomonas*. Catabolite repression control (Crc) protein plays an important role in CCR. Crc protein was first discovered in *Pseudomonas aeruginosa* by random mutagenesis. The *crc* mutant shows a loss of catabolite repression by succinate in many ways, such as the transport of glucose and mannitol, the activity of glucokinase, glucose-6-phosphate dehydrogenase, amidase etc [46]. Recently, CCR triggered by succinate and acetate has been observed in ß- ketoadipate pathway of *A. baylyi* [47, 48]. As mentioned before, in ß- ketoadipate pathway a variety of aromatic compounds can be first converted into the two starting materials, protocatechuate and catechol, which will be subsequently converted into ß- ketoadipate. Under the presence of succinate and acetate, Crc protein can repress the expression of most the genes (figure 5) involved in ß- ketoadipate pathway. It has been proved that the repression is realized at both transcriptional and post-transcriptional level [47, 48]. The repression of Crc protein on *pca-qui* operon was first reported [47]. The *pca-qui* operon is responsible for encoding protocatechuate 3,4-dioxygenase. Crc protein represses the expression of *pca-qui* operon at post-transcription level. This is reflected in the phenomenon that *crc* mutant has higher stability of *pca-qui* transcripts compared with the wild type strain [47]. It is speculated that Crc protein could be a specific nuclease, initiating the degradation of certain mRNA (*pca-qui* mRNA) but not other mRNAs. Afterwards, other operons including *pob*, *hca*, *van*, *are*, *ben*, *sal*, *ant*, *cat*, *dca* were proved to have a repression when acetate or succinate are present in the medium (figure 5) [48]. Different operons are usually repressed at different degree by acetate and succinate. It has been found that Crc protein exerts repression on most of these operons at transcription level as the promoters of most these operons in crc strain showed higher activities compared with wild type strain [48]. The In addition, there is no significant effect on gene expression caused by pyruvate, and other substrates such as lactose and gluconate have influences on the repression of genes related to ß- ketoadipate pathway at different extent. However, the mechanisms of how Crc protein behaves on gene repression remain poorly understood and need to be further explored.

Apart from CCR, cross-regulation and vertical regulation also affect the gene expression in β- ketoadipate pathway (figure 5). Cross-regulation happens between two branches. For example, the consumption of benzoate, which goes through catechol branch, is favorable for protocatechuate branch. The presence of benzoate represses the expression of *hca*, *pob* and *van* operons. The repression on *van* and *hca* operon is even higher than the repression caused by CCR. Vertical regulation happens in the same branch. For example, the formation of additional protocatechuate represses any pathway that result in the formation of protocatechuate. Only when protocatechuate concentration is lower than a certain value will this repression stop. This is thought to be a mechanism for microorganism to efficiently utilize substrate [48].

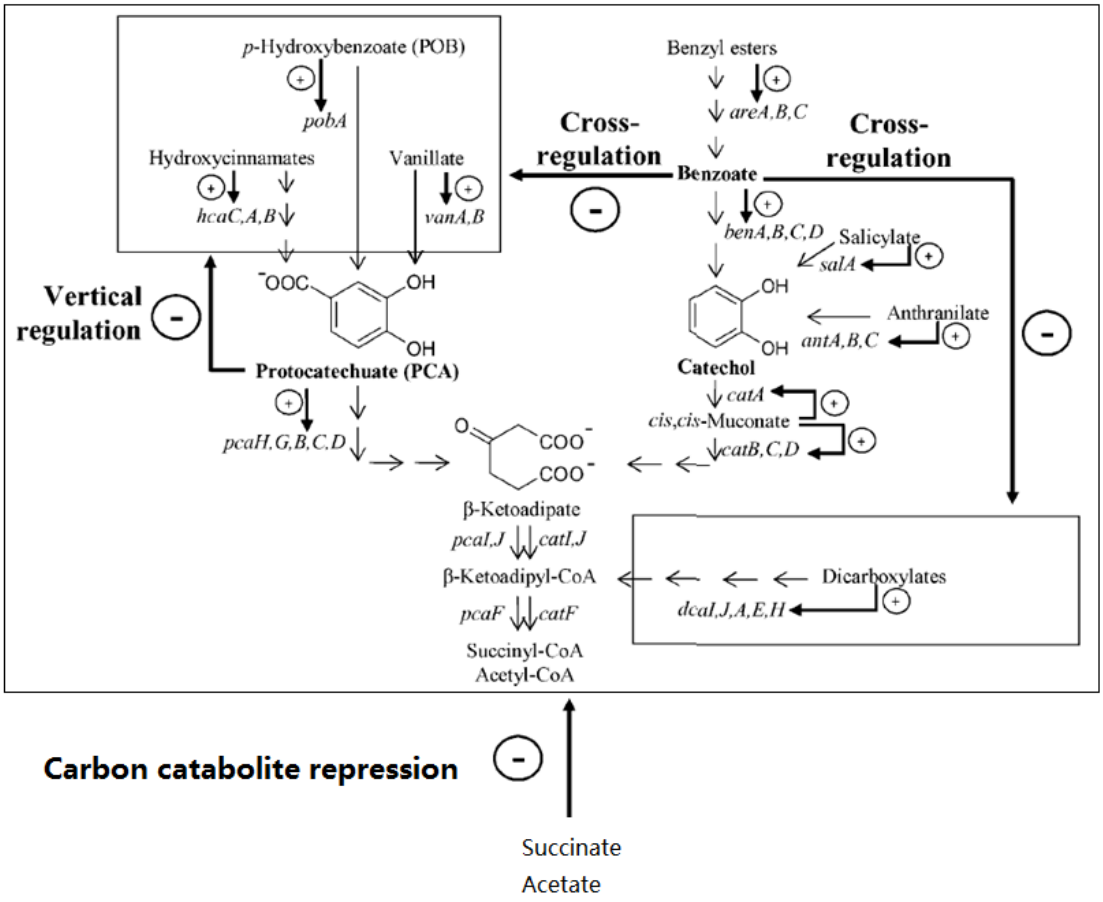


Figure 5. *Scheme of the regulatory level in β - ketoadipate pathway of A. baylyi. Figure modified from [48]. Thin arrows indicate direction of the pathway. Thick arrows indicate the regulation effects on the corresponding gene expression. All the operons are negatively influenced by carbon catabolite repression.*

2.2.4 Lipid synthesis in genus *Acinetobacter*

Some bacteria have developed a series of strategies to survive in nutrient-limited condition, one of which is the accumulation of storage compounds. When there are not enough external carbon and energy sources available to the bacteria, these storage compounds will break down and serve as energy or carbon sources to support their growth. Storage compounds are always accumulated as intracellular inclusions and exist in many different forms, depending bacterial species. For most bacteria, polyhydroxyalkanoic acids (PHAs), glycogen and starch are the primary forms [49], and some neutral lipids accumulating as storage compounds were also reported, such as triacylglycerol (TAG) and WE [50].

Many microorganisms have been reported to accumulate storage compounds. For example, *Pseudomonas* spp. is able to accumulate PHA [50] while all the enterobacteria can store glycogen. TAG accumulation is found mostly in eukaryotic organisms, whereas some bacteria, such as *Rhodococcus* spp., *Mycobacterium* spp., can also accumulate TAG as storage compounds. Recently, genus *Acinetobacter* has attracted

people's attention. *Acinetobacter* spp. has been reported to accumulate WE and TAG as storage compound [49-52]. The WEs produced by *Acinetobacter* spp. are thought to have a similar chemical composition to jojoba-derived WEs which are the main natural sources of current commercialized WEs. Due to the high price of jojoba-derived WEs, *Acinetobacter* spp. might have a great potential to be an alternative for WE production.

WE is the primary component in the cellular inclusion of *Acinetobacter* spp., while some other compounds also exist in small amounts, including triacylglycerols, diacylglycerols, free fatty acids and free fatty alcohols [50]. As mentioned before, WEs can accumulate in *Acinetobacter* spp. intracellularly as storage compound under N-limited condition. They will be degraded to soluble metabolites and CO_2 when the bacteria are under C-limited condition, generating ATP for bacterial growth. The WEs produced from *Acinetobacter* spp. contains both saturated and unsaturated components with carbon chains ranging from 30 atoms to 36 atoms, among which saturated WEs with 32 and 34 atoms predominate. Accordingly, saturated fatty acids and alkanol with 16 and 18 carbon atoms are the major compositions. WEs with odd number of carbon atoms also exist sometimes but in trace amount [51]. However, the compositions of WEs can differ between substrates and strains. When cells are cultivated on substrates such as ethanol, acetate, which will be converted into acetyl- CoA before they enter into other pathway, the fatty acid will be synthesized de novo and palmitic acid are usually predominant. In contrast, when cells are cultivated on some long chain compounds such as heptadecane and hexadecanal, fatty acid is synthesized by directly using the skeleton of the substrate while de novo synthesis of fatty acid is usually inhibited.In this case, the chain length of the fatty acid is usually correlated to the chain length of the substrate, which indicates the conversion of substrate to corresponding fatty acid by monoterminal oxidation [50].

As the above said, *Acinetobacter* spp. can directly use the skeleton of the long chain compounds to synthesize storage lipids such as WEs. However, if short chain compounds, such as acetate and ethanol, are used as sole carbon source, the lipid accumulate will depend on de novo fatty acid synthesis. As shown in figure 6, carbon source will be converted into acetyl-CoA, some of which will enter into the pathway forenergy production and biomass synthesis, and some will be converted into acyl-CoA. The conversion of acetyl-CoA to acyl-CoAis conserved in bacteria, and it is achieved by a series of reactions which have been well described in *E. coli* [53]. These reactions are mainly involved in two stages, initiation and cyclic elongation. Acyl carrier protein (ACP) is thought to be an important component in the fatty acid synthesis pathway as it will covalently bind to the growing chain during fatty acid synthesis. In the termination step, the fatty acid is released from ACP and then attaches to coenzyme A (CoA), forming acyl-CoA. It is thought that either acyl-CoA or acyl-ACP is starting compound of WE synthesis pathway [54].There are three steps involved in WE synthesis pathway (figure 6). In the first step, either acyl-CoA or acyl-ACP can be reduced to corresponding aldehyde by acyl-CoA reductase. And then the fatty aldehyde is further reduced to fatty alcohol by fatty aldehyde reductase. In the final step, the fatty alcohol reacts with acyl-CoA under the catalysis of a bifunctional WE synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT), forming WE.

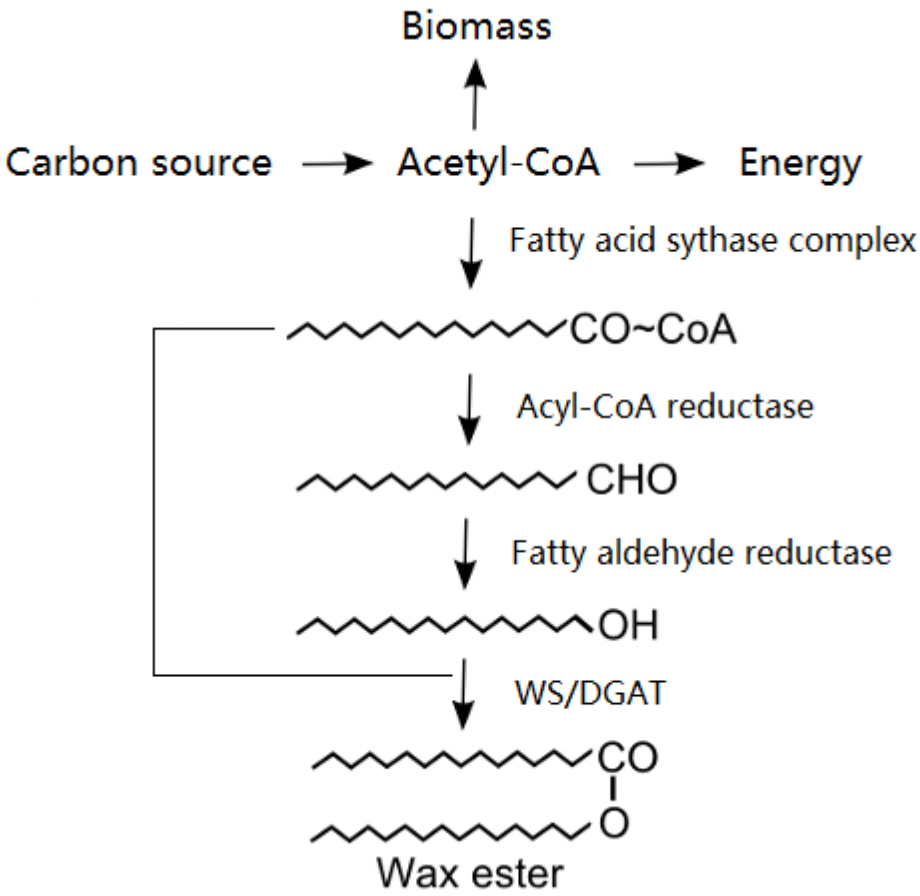


Figure 6. *The pathway of WE synthesis in Acinetobacter spp. Picture modified from [52]. WS/DGAT, WE synthase/acyl-CoA:diacylglycerol acyltransferase.*

Acyl-CoA reductase, fatty aldehyde reductase and WS/DGAT are the three enzymes which play an important role in WE synthesis pathway, among which acyl-CoA reductase and WS/DGAT have been successfully purified and well characterized in *Acinetobacter* spp.[54-56]. Acyl-CoA reductase has a molecular weight of 32.5 kDa and is encoded by acr1 gene. This enzyme is NADPH-dependent and can catalyze the reduction of acyl-CoA or acyl-ACP into corresponding aldehyde.The strain lacking acr1 gene is deficient in the accumulation of WEs when hexadecane is used as sole carbon source, but the ability to accumulate WEs can be restored by adding hexadecenal or hexadecanol in the medium.This enzyme can act on acly-CoAs with chain length from 14 to 22. But it has different preferences to the substrates with different chain length based on the rates of NADPH oxidation: C16 > C18 > C14 > C20 > C22. Fatty aldehyde reductase is another key enzyme which is responsible for the reduction of fatty aldehyde into corresponding fatty alcohol. However, this enzyme has not been characterized in *Acinetobacter* spp. but in *Marinobacter* spp. which is also known for WE accumulation [55]. The gene cer4 found in *Arabidopsis thaliana* was preliminarily regarded as fatty aldehyde reductasegene according to previous research. Based on the gene sequence, a gene with moderate similarity to *cer4* gene was obtained in *Marinobacter aquaeolei* and was proved to encode fatty aldehyde reductase finally. This enzyme is also NADPH-dependent and has a molecular weight of 57 kDa. It is active to the straight chain aldehydes with a minimum chain length of 8. The highest enzyme activity is obtained when decanal (C10) is used as substrate. A comparable activity is also obtained

when hexadecanal (C16) is used as substrate, which is expected as hexadecyl hexadecanoate is the primary WE found in *Marinobacter* spp. The third key enzyme is WS/DGAT which catalyzes the condensation between the acyl-CoA and the corresponding fatty alcohol or diacylglycerol, forming WEs and TAG. The WS/DGAT, isolated from *Acinetobacter calcoaceticus*, is thought to belong to a new type of acyltransferase, due to the fact that it shows no similarity to any other acyltransferase including the WS from jojoba, the DGAT1 and DGAT2 from yeast and plants. It is composed of 458 amino acids with a molecular weight of 52 kDa.A wide range of long chain fatty alcohols and acyl-CoAs, from C12 to C20, are accepted almost equally in the WS reaction, whereas acyl-CoA with a chain length of 20 is preferred in DGAT reaction.

2.3 Wax ester

Wax is a term derived the word “Weax” which means beeswax. Thus, in the broad sense, wax represents series of compounds which are traditionally derived from plants, animals or mineral and have similar compositions and properties to beeswax. They are usually solid under room temperature, easily liquified after heating, flammable, water-resistant and have lubricating effect. And according to different occasions, the definition of wax can vary. Waxes often exist in the form of the mixture of organic compounds with long alkyl chain, including n-alkanes, long chain ketones, fatty acids, fatty alcohol (primary or secondary), long chain aldehydes, monoesters (WEs) and so on. In narrow sense, waxes refer to the esters of fatty acids and fatty alcohol, namely monoesters (WEs). Wax is classified as a group of lipids.

Waxes have wide range of sources. They can come from animals, plants or bacteria, providing important biological functions to them. For example, various waxes, including WEs have been identified as main components in human meibum which is an important compound secreted from meibomian glands, preventing the evaporation of tear film and maintaining the surface tension or tear film [53]. Many marine animals, such as whale, synthesize considerable amount of waxes, serving as energy source and providing buoyance and echolocation. As to plants, the surfaces of leaves are coated with various waxes called cutin, which limits the diffusion of water and protects plants against diseases and pests. Waxes are not common in prokaryotes, but some species such as *Mycobacterium* spp. and aforementioned *Acinetobactor* spp. can produce waxes which can serve as storage compounds under nitrogen source-limited condition.

Due to the unique properties, waxes have been important components for industrial production. Among them, WEs are of great importance and have been widely used in many fields, including cosmetics, medicines, candles, coatings, lubricants, polishes, inks and so on. Spermaceti, which usually come the adipose tissue of sperm whale, used to be a major source of WEs. Spermaceti contains high proportion of wax ester (mainly cetyl palmitate) and small proportion of triglycerides. However, due to the recent international regulation regarding the capture of whale, whale-derived WE had been forbidden, after which jojoba oil has been the alternative of WE production. Jojoba oil is derived from the seeds of jojoba (*Simmondsia chinensis*) which is a woody, desert shrub originating from Mexico and some desert regions of America. Nowadays, this type of plant has been planted in many places such as Africa, Australia and China. About 50% of jojoba oil is WE which can be extracted by pressing the seeds. The WEs from jojoba oil have a carbon number from 38 to 44 and are mainly composed of C20:1 fatty acid

and C20:1 and C22:1 fatty alcohol. Currently, other commercial WEs mainly come from beeswax and carnauba. Beeswax is secreted in the abdominal region of honey bees (genus *Apis*), being used by the bees to build their honeycombs. Its colors can vary depending on flowers that the bees forage on. Beeswax is a complicated material and contains many different substances. Its main components include palmitate, palmitoleate, hydroxypalmitate and oleate esters of long chain fatty acid (C30-C32). The content of WEs can be more than 35%.

Currently, jojoba oil has been the main source of WE since the ban of whale capture. However, its high cost limits its application. Nowadays, WE production chemically has been developed to meet the high demand of cheap jojoba-liked WEs. A wide range of long chain esters can be synthesized by transesterification and esterification catalyzed by lipase under non-aqueous systems [58]. Lipase shows activity to a wide range of substrates, for example, from acetic acid to acids greater than C20. However, the synthesis of WEs still depends on long chain fatty alcohols which need to be chemically synthesized. Therefore, the study of WE production microbiologically is becoming the hot spot. In the following chapters, the experiments about WE production by *A. baylyi* ADP1 using lignin monomers as carbon sources will be demonstrated.

3. MATERIAL AND METHODS

3.1 Strains

The wild type strain *A. baylyi* ADP1 (DSM24193) was used for WE production experiments in this study. *E. coli* XL-1 blue (Stratagene, USA) was used to construct gene cassette for the knock-out of *crc* gene (ACIAD3526). The resulting strain ADP1△crc::Cam^r (*crc* gene was replaced with gene cassette containing chloramphenicol resistance gene) was designated as crc strain.

3.2 Medium compositions

Lysogeny broth (LB) medium was used in the cultivation during gene knock-out. The components include 10g/L tryptone, 5g/L yeast extract and 1g/L NaCl. 1% (w/v) glucose was used as carbon source. For agar plates, 15 g/L agar was added. In addition, 50 μg/ml chloramphenicol was added to the medium for the screening of ADP1 transformant, and 25 μg/ml chloramphenicol and 10 μg/ml tetracycline were added for the screening of *E. coli* transformant. MA/9 medium was used in the growth and WE production experiments. The components include 5.52 g/L $Na_2HPO_4 \cdot 2H_2O$, 3.4 g/L Na_2PO_4, 1.189 g/L $(NH_4)_2SO_4$, 0.008g/L nitrilotriacetic acid, 1g/L NaCl, 0.241 g/L, 0.241 g/L $MgSO_4$, 0.0222 g/L $CaCl_2$ and 0.02 g/L $FeCl_2$. Glucose, acetate, 4-hydroxybenzoic acid (4-HBA), p-coumaric acid, vanillic acid and ferulic acid were used as carbon source in this study (for the concentration of each lignin monomer, see the cultivation part 3.5).

The 4-HBA, p-coumaric acid, vanillic acid and ferulic acid used in this study were provided by Sigma-Aldrich (USA). The stock of each of them with a concentration of 0.1 M was prepared before being used as carbon source. Appropriate amount of each lignin model monomer was added into water. The mixture was stirred and the pH of the mixture was monitored. Meanwhile, 5 M NaOH solution was slowly dropped into the mixture until the pH reached around 8.5 at which the lignin model monomer was completely dissolved in the water, resulting the sodium salt form of each lignin model monomer.

3.3 Genetic engineering

Plasmid SM100/pIX was used as the backbone for *crc* gene (ACIAD3526) knock-out (figure 7). The plasmid contains chloramphenicol resistance gene. The primers used in this study are listed in table 3. The primers were ordered from Thermo Fisher Scientific (USA). The primers ab140, 141, 141 and 142 containing appropriate restriction sites (*KpnI*, *MfeI*, *AvrII*, *SfiI* respectively) were used to amplify the 5' flanking and 3' flanking of *crc* gene. The primers ab143 and ab144 were used for the gene knock-out confirmation.

Table 3. *List of primers used in this study*

Name	Description	Oligo sequence (5'to 3')
ab140	upstream of *crc*, sense, *KpnI*	ATATGGTACCCATAACCTTGCGCCAAAAGTG
ab141	upstream of *crc*,, antisense, *MfeI*	CAGCCAGTTGTCGAGCAGCCCTTTGGTCAC
ab142	downstream of *crc*, sense, *AvrII*	AAGCCCTAGGATTGTGTACATTTGTACACTGAATAAACG
ab143	downstream of *crc*, antisense, *SfiI*	CCTCGGCCCCCGAGGCCAGCTAAAAACATCACGCATGAAAAATAG
ab144	sense	ATGATCCAGACTTTTTTGCC
ab145	antisense,	TCACTTGTTAAGGCAAATGC

Note: ab 140, ab141, ab142, ab143 were used for flanking amplification. ab144 and ab145 were used for gene knock-out confirmation. The sequence underlined is the restriction site.

The genomic DNA was extracted from wild type ADP1 using GeneJET genomic DNA purification kit (Thermo scientific, USA) and following the provider's instruction. The extracted genomic DNA was used as template to amplify the flanking regions of *crc* gene (for PCR instruction, see 3.4.1). Primer ab140 and ab141were used to amplify the 5' flanking region (upstream) of *crc* gene. Primer ab142 and ab143 were used to amplify the 3' flanking region (downstream) of crc gene. The PCR products were confirmed using gel electrophoresis. The cloning of 5' flanking and 3' flanking to the plasmid was done sequentially. 5' flanking and plasmid SM100/pIX were digested with restriction enzyme *KpnI* and *MfeI* (for digestion instruction see 3.4.2). The digestion products were purified with gel electrophoresis and extracted with GeneJET gel extraction kit (Thermo scientific, USA). The digested 5' flanking was ligated to the plasmid using the restriction site *KpnI* and *MfeI* (for the ligation reaction, see 3.4.2). The ligation without insert was used as control. The resulting ligation products were transformed into *E. coli XL-1* competent cells (for transformation in *E. coli*, see 3.4.3). The transformed cells were plated to LA plates containing tetracycline and chloramphenicol and cultivated at 37 degree over night. Several colonies were selected from the transformant plate (the number of colonies in transformant plate should be more than 10 times of the number from the control plate) and then cultivated in LB medium. Plasmid was extracted from the selected transformant, after which digestion with the same enzymes was applied for cloning verification. Similarly, the confirmed plasmid containing 5' flanking and 3' flanking were digested respectively with restriction enzyme *AvrII* and *SfiI*, purified using gel electrophoresis again, and the digested 3' flanking was ligated with the plasmid. Again, the ligation product was transformed to *E. coli* competent cells and plasmid was extracted from the selected transformant. As there was a *XbaI* site in the 3 flanking and another one in the plasmid backbone, the clone verification of 3 flanking

was done by digestion with enzyme *XbaI*. After the verification, the plasmid containing 5' and 3' flanking was transformed to ADP1 (for the transformation in ADP1, see 3.4.4). The ADP1 transformant was plated on the medium containing chloramphenicol. As ADP1 has natural tendency of homologous recombination, crc gene will be replaced by the gene cassette flanked with homologous sequence after transforming the plasmid to the cells. The whole genomic DNA was extracted from the selected transformant. Gene knock-out confirmation was done using PCR with primer ab144 and ab145 (see 3.4.4).

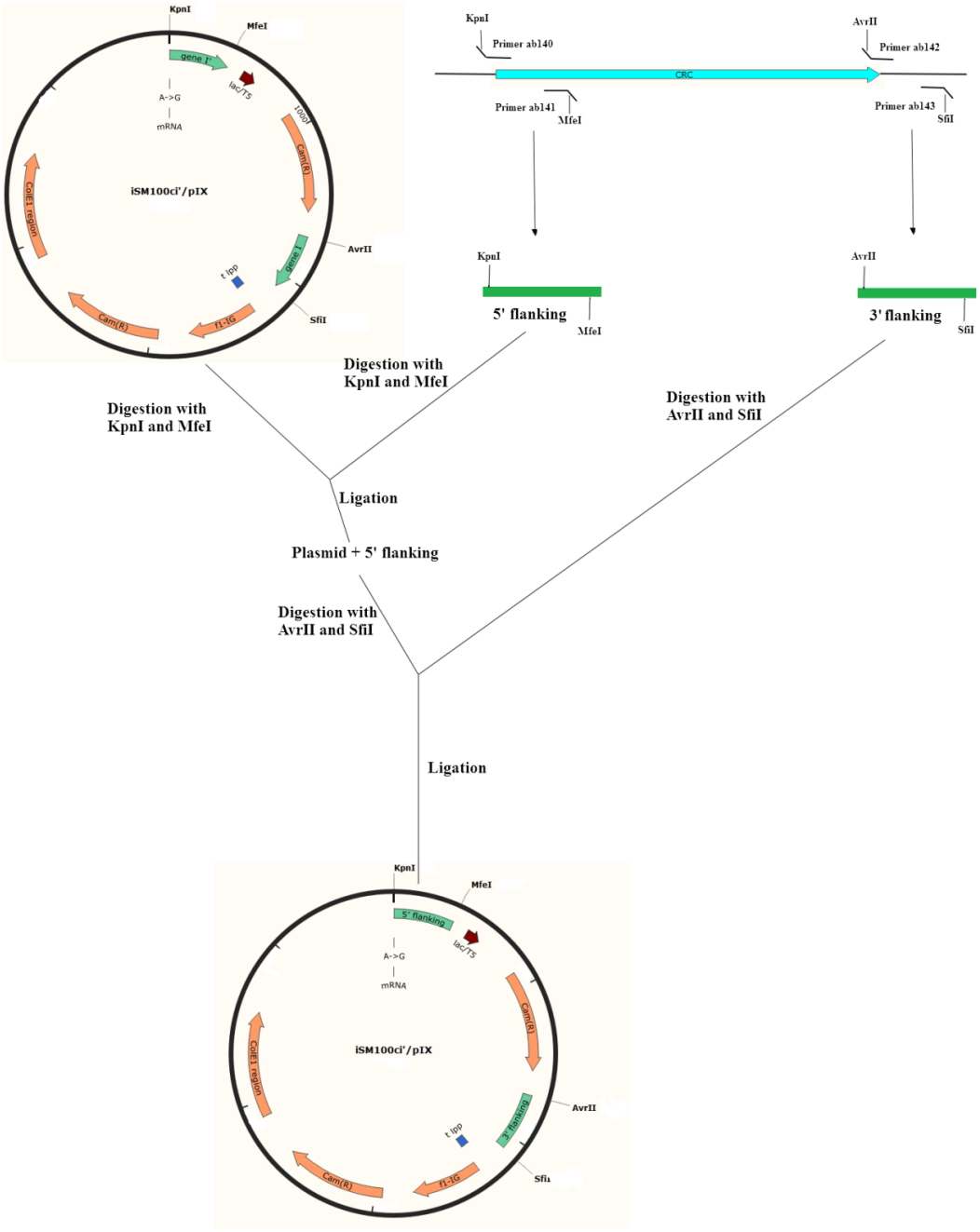


Figure 7. *Schematic of the gene cassette construction for crc gene knock-out*

3.4 General information about the genetic engineering

3.4.1 PCR amplification

Amplification of 5' and 3' flanking was done by PCR. The genomic DNA of ADP1 was used as the template. Primer ab140 and ab141 were used to amplify the 5' flanking of *crc* gene, and primer ab142 and ab143 were used to amplify 3' flanking. The PCR was done in 50µl reaction system, with 1xPhusion HF buffer, 200uM dNTP, 0.5 µM each primer, 250 ng template DNA and 0.02U/µl Phusion Hot start II DNA polymerase. Sterile Milli Q water was added up to 50 µl. The reaction parameters are listed in table 4. After PCR reaction, the PCR products were confirmed using gel electrophoresis with 1-2.5% (w/v) agarose (Sigma, USA).

Table 4. *The parameters of PCR for flanking amplification.*

Step	Temperature	Time	Cycles
Initial denaturation	98℃	30s	1
Denaturation	98℃	10s	30
Annealing	50℃	20s	
Extension	72℃	11s	
Final extension	72℃	10min	1
Hold	4℃	-	-

3.4.2 Digestion and ligation reaction

Double digestion was done for 5' and 3' flanking as well as plasmid. For the digestion of 5' flanking, restriction enzyme *KpnI* and *MfeI* were used and *KpnI* buffer was applied. Both enzymes are active at 37℃. Restriction enzyme SfiI and *AvrII* were used for the digestion of 3' flanking and Tango buffer was used for this digestion. Enzyme *SfiI* is active at 50℃ and *AvrII* is active at 37℃. Thus, a sequential incubation was applied. The components of digestion reaction are shown in table 5. The digestion products were purified using gel electrophoresis and the purified products were extracted from the corresponding bands with GeneJET gel extraction kit (Thermo scientific, USA)

After transformation of the ligation products (recombinant plasmids), the cells containing the recombinant plasmids were screened out by plating them on the plates containing antibiotic. After that, digestion was also applied to the plasmids extracted from the selected transformants to verify the cloning. For the verification of 5' flanking cloning, the same enzymes used for the digestion of 5' flanking was used. For the verification of 3' flanking cloning, enzyme *XbaI* was used.

Table 5. *The components of the digestion reaction*

5’ flanking (insert)		SM100C/pIX (vector)	
Components	30 µl reaction	Components	30 µl reaction
Insert	200ng	Vector	500ng
Enzyme *KpnI*	1 µl	Enzyme *KpnI*	1 µl
Enzyme *MfeI*	2 µl	Enzyme *MfeI*	2 µl
10xKpnI buffer	3 µl	10xKpnI buffer	3 µl
Sterile MQ water	up to 30 µl	Sterile MQ water	up to 30 µl
Incubate at 37℃ for 2 h			
3’ flanking (insert)		SM100C/pIX containing 5 ’flanking (vector)	
Components	30 µl reaction	Components	30 µl reaction
Insert	200ng	Vector	500ng
Enzyme *SfiI*	1 µl	Enzyme *SfiI*	1 µl
Enzyme *AvrII*	1 µl	Enzyme *AvrII*	1 µl
10xTango buffer	3 µl	10xTango buffer	3 µl
Sterile MQ water	up to 30 µl	Sterile MQ water	up to 30 µl
Incubate at 50℃ for 5h, and then incubate at 37℃ for 2h			

The purified digested inserts and vectors were ligated using T4 DNA ligase and 10x ligation buffer. To avoid self ligation of vectors, dephosphorylation step was applied to vectors before the ligation between inserts and vectors. Dephosphorylation was conducted in 20 µl reaction system with 2 µl buffer, 1 µl phosphatase and 17µl vector. The mixture was incubated at 37 ℃ for 10 min and then inactivated at 75℃ for 5 min. The dephosphorylated vector was directly used for ligation reaction. The components for ligation reaction are listed in table 6. The mixture was incubated for 2 h at room temperature and then inactivated at 65℃ for 20min. The mixture was directly used for transformation.

Table 6. *The components of the ligation reaction*

Treatment		Control	
Components	20µl reaction	Components	20µl reaction
Insert	5ng	Insert	-
Vector	25ng	Vector	25ng
T4 ligase	0.5 µl	T4 ligase	0.5 µl
10x ligation buffer	2 µl	10x ligation buffer	2 µl
sterile MQ water	up to 20 µl	sterile MQ water	up to 20 µl
Incubate at 37℃ for 2h, inactivate at 65℃ for 20min			

3.4.3 Competent cell preparation and tranformation in *E. coli*

After each ligation, the recombinant plasmids were transformed to *E. coli* electrocompetent cells. Electrocompetent cells were prepared beforehand. Simply, *E. coli* was first inoculated from the overnight culture into 2 flasks of 50ml fresh LB medium and cultivated until the OD600 reached 0.8. And then, the cells were harvested into centrifuge tubes and centrifuged at 14000 rpm for 5 min. The supernatant was discarded and two tubes of cells were combined into one tube by suspension with 20ml

cold sterile MQ water. Cells were pelleted again and washed with 40ml, 20ml and 5ml cold sterile 10% glycerol in sequence. Finally, the cells were resuspended with 500 µl 10% glycerol and then divided into 40 µl aliquots for use. All the processes were done with an ice box.

All the steps for transformation were also done with an ice box. 2 µl of ligation product was pipetted into the 40 µl electrocompetent. The mixture was transferred to pre-cooled electroporation cuvette. The cells were transfromed with electropotor (Bio-Rad, USA) using Eco-1 program. Then, 1 ml of prewarmed (37℃) LB medium was added into the cuvette. Cells were plated to LA medium containing 25 µg/ml chloramphenicol and 10 ug/ml tetracycline respectively and incubated at 37 ℃ overnight. The same processes were done with the control in which the ligation without insert was used. Single colony was picked from the plate and cultivated in LB medium with antibiotic. Plasmid was extracted from the cells and restriction analysis was used to verify the cloning.

3.4.4 Transformation in ADP1

As ADP1 is naturally transformable, competent cell preparation is not needed for transformation. The processes of transformation in ADP1 are as follows. 50 µl cells were inoculated from the overnight preculture into 2ml fresh LB medium containing 1% glucose and incubated at 30 ℃, 300 rpm, for 2-3 h, so that the cells reached exponential phase. Then the cells were divided into 0.5 ml aliquots. Two microgram of plasmid was added into the 0.5 ml culture and a control without plasmid was needed. The cells were incubated in the shaker (30℃, 300rpm) for 6 h, after which the whole 0.5 ml culture was spread to 3 LA plates containing 50 µg/ml chloramphenicol and the plates were kept at 30 ℃ until some colonies appeared. Single colony was picked and cultivated in LB medium containing antibiotic overnight. The genomic DNA was extracted from the cells for gene knock-out confirmation. To confirm the gene knock-out, primer ab144 and ab145 were used to amplify the genomic DNA from both wild type ADP1 and the transformant. The PCR products were run on the gel. As *crc* gene and the replacement had different length, the gene knock-out could be confirmed by analyzing the length of the PCR products which were amplified with the same primers.

3.5 Cultivations

3.5.1 Growth tests in 5 ml cultivation using different carbon sources

Wild type ADP1

Different concentration of carbon sources (glucose, 4-hydroxybenzoic acid (4-HBA), p-coumaric acid, vanillic acid and ferulic acid) were used to test the growth of wild type ADP1 respectively. The tests were conducted in 5 ml/10ml test tube and MA/9 supplemented with appropriate concentration of each carbon source was used as the medium. The effect of Fe on the growth was also tested with glucose as carbon source in the medium with and without $FeCl_2$. The concentrations of different carbon sources

used for the growth tests were as follows: 1% and 5% glucose with and without $FeCl_2$; 25 mM and 50 mM 4-HBA with and without $FeCl_2$; 15 mM, 25 mM and 50 mM p-coumaric acid, vallinic acid and ferulic acid respectively. Duplicates were prepared for each sample. Cells cultivated in MA/9 medium supplemented with 1% glucose were used as inoculant. Appropriate amount of cells were inoculated from the precultures to make the initial OD600 to 0.05. Cells were cultivated at 30 ℃, 300 rpm and OD600 measurement was conducted every 24 h. The growth curves of the cells cultivated with different concentration of carbon sources were drawn and compared.

Wild type ADP1 vs. crc strain

The growths of wild type ADP1 and crc strain were compared by the cultivations with different carbon sources in MA/9 medium. The carbon sources with certain concentrations used in this experiment are shown as follows: 2% glucose; 25 mM acetate; 0.2% casamino acids; 2 mM 4-HBA; 2% glucose+25 mM acetate; 25mM acetate+2 mM 4-HBA; 2% glucose+2 mM 4-HBA. The cultivation was carried out in 5 ml/10 ml test tube in two parallel tubes. Cells cultivated in MA/9 medium supplemented with 1% glucose were used as inoculant. The initial OD_{600} was 0.05 and the strains were cultivated at 30 ℃, 300 rpm, for 96 h. OD measurement was carried out daily and the growth curves were compared between wild type and crc strain.

3.5.2 Co-utilization of acetate and 4-HBA between wild type ADP1 and crc strain

The cultivation to explore the co-utilization of acetate and 4-HBA between wild type ADP1 and crc strain was carried out in 50 ml/250 ml flasks containing MA/9 medium supplemented with 25mM acetate and 25 mM 4-HBA. Preculture was carried out using the same medium and the initial OD of the experiment was 0.05. Cells were cultivated for 12 h. OD measurement and sampling for HPLC analysis were carried out every 2 h. After 12 h cultivation, same amount of biomass were taken for TLC analysis. The growths between wild type ADP1 and crc strain were compared by drawing the growth curve and the consumptions of acetate and 4-HBA were compared based on the HPLC results.

3.5.3 WE production in 50ml cultivation (wild type ADP1)

The WE production experiment was carried out in 50 ml/250 mL flasks containing MA/9 medium. 5% glucose and 50 mM 4-HBA were used as sole carbon sources respectively. Effect of Fe on WE production was also tested in the medium with and without $FeCl_2$. Preculture was carried out with 1% glucose and the initial OD for the WE production experiment was 0.05. Cells were cultivated at 30 ℃, 300 rpm, for 48 h when glucose was used as carbon source and for 72 h when 4-HBA was used as carbon source. After cultivation, OD600 was measured. 1ml samples were taken and centrifuged at 10000 g for 3 min. The supernatant was taken for HPLC analysis to analyze the consumption of the carbon sources. 3ml sample was taken for TLC analysis (for the TLC samples in other experiments, appropriate amount of samples were taken to make sure the biomass from different groups of samples were the same). Forty milliliter of each sample was taken and centrifuged at 30000 g for 30 min, after which

the supernatant was removed from the centrifuge tubes. And then, the centrifuge tubes were covered with poked parafilm and put into glass jar, after which the jar was installed to the freeze-dryer (Christ ALPHA 1-4LD plus, Germany). The freeze-drying was conducted from 24 h, after which the dried cells were sent for NMR analysis to quantify the WE production.

3.5.4 The effect of C: N ratio on WE production

Wild type ADP1 (coumaric acid and ferulic acid as sole carbon source respectively)

The cultivations to explore the effect of C: N ration on WE production were carried out in 50 ml/250 mL flasks in both normal condition and nitrogen-limiting condition. In nitrogen-limiting condition, MA/9 medium was still used, but the $(NH_4)_2SO_4$ concentration was 0.119 g/L, which is one tenth of the normal concentration. The other conditions for the two cultivations were the same. 10mM coumaric acid and 10mM ferulic acid were used as sole carbon source. The precuture was carried out in normal nitrogen concentration with the same medium used in the cultivation and the initial OD was 0.05. Cells were cultivated at 30 ℃, 300 rpm, for 72 h. OD measurement, sampling for TLC and HPLC were conducted every 24h. After 24h and 48 h, 10 mM corresponding carbon source (coumaric acid and ferulic acid) were supplemented to the cultivation. After 72h, 40ml sample was centrifuged at 10000 g for 30 min and THE pellet was freeze-dried, after which NMR was applied for WE quantification.

Wild type ADP1 vs. crc strain (co-utilization of acetate, coumaric acid and ferulic acid)

The cultivation to compare the WE productions between wild type ADP1 and crc strain in different C: N ratio was also carried out in 50 ml/250 mL flasks. Cultivations in normal nitrogen concentration and nitrogen-limiting condition (same as previous experiment) were applied to each strain. Co-utilization of 5 mM coumaric acid, 5 mM ferulic aicd and 10 mM acetate was applied in this experiment. Preculture was carried out in normal nitrogen concentration with the same medium used in the cultivation. Cells were cultivated at 30 ℃, 300 rpm, for 72 h. Again, after every 24 h, OD was measured, and appropriate amount of sample was taken for TLC and HPLC. After 24 h and 48h, 5mM coumaric acid, 5mM ferulic aicd and 10mM acetate were supplemented to the cultivations. After 72 h, 40ml sample was taken and cells was collected at 10000 g for 30 min for freeze-dry, after which NMR was applied for WE quantification.

3.6 Lipid analytics

3.6.1 Lipid extraction

Lipid was extracted from the biomass for further TLC analysis. Same amount of biomass was taken from each sample for lipid extraction (except the one described in 3.5). The extraction was conducted in 1.5ml system. First, the cells were collected by centrifugation at 20000g for 5 min. The cell pellet was suspended in 500 ul Methanol and mixed by vortex until the mixture became uniform (about 30 min). And then 250 ul chloroform was added to the tube and mixed by vortex at room temperature for 1 h. The

mixture was then centrifuged at 20000 g for 5 min, after which 250 ul chloroform and 250 ul PBS buffer were added to the mixture. The suspension was swirled slowly at room temperature for 1 h to overnight. Finally, the suspension was centrifuged at 20000 g for 5 min again. Two phases should be seen after centrifuge and white cell debris can be seen at the interface between the two phases. The lipid will be in the lower phase which is the chloroform phase.

3.6.2 TLC analysis

Thin Layer chromatography (TLC) was carried out to visualize the composition of the lipids produced from each cultivation, using HPTLC Silica Gel 60 F_{254} glass plates (Merck, USA). The mobile phase used for TLC was n-hexane: diethyl ether: acetic acid 90: 15: 1. For the cultivation described in 3.5, 30 µl sample and 10 µl standard were applied on the plates. For other cultivations, 100 µl sample and 30 µl standard were applied. 1 µg/ml of jojoba oil was used as the standard. The samples and standard were dropped slowly onto the plate using pipette. After all the samples were dried up, the plate was transferred to the TLC developing tank in which there was the mobile phase. Then, the TLC chromatogram was developed with mobile phase until the mobile phase approached the top of the plate. When finished, plate was dried up and dyed with Iodine to visualize the lipid bands.

3.6.3 Analysis of carbon source consumption

The consumption of carbon sources during cultivations was measured by high performance liquid chromatography (HPLC). The carbon sources analyzed include glucose, acetate, 4-HBA, coumaric acid, and ferulic acid. The samples from the culture were first centrifuged at 20000 g for 5 min, after which the supernatant was collected and filtered through 0.2 um filters. The standard solutions of each compound to be analyzed were also prepared with a concentration of 0.5 mM, 1 mM, 5 mM, 7.5 mM and 10 mM. The dilution of sample was applied before running HPLC so that the concentration of sample was in the range of the standard concentration. The analysis was conducted with LC- 20AC prominence liquid chromatograph equipped with LC-20AD solvent delivery module, DGU-$20A_3$ prominence degasser, SIL-20AC HT prominence auto sampler and RID-10A refractive Index detector (Shimadzu, Japan). Phenomenex Rezex RHM-monosaccharide H^+ (8%) column was used and 0.01 N sulfuric acid was used as mobile phase with a pumping rate of 0.6 ml/min. The identification and quantification of each compound were based on the retention time and standard curve obtained from the standards.

3.6.4 WE quantification

Wes ester quantification was carried out through ^1H nuclear magnetic resonance (NMR) [48]. The cells were first collected by centrifugation and then freeze-dried with Christ APLHA1-4 LD plus freeze dryer (Germany) for 24 h. The total lipid fraction was extracted from the dry cells and analyzed using Varian Mercury spectrometer (300 MHz). The different functional groups were identified by the chemical shifts (parts per million, ppm) relative to tetramethylsilane. The spectra were processed using ACD NMR processor program. The areas of the peaks were proportional to the amount of each functional group. The chemical shifts from δ 4.05-4.10 are corresponding to the

CH_2OR group in WE. The integral values of the peak at δ 4.05-4.10 and the peak of the standard which concentration is known can be used to determine the concentration of WE.

4. RESULTS

4.1 Strain construction

In order to knock out crc gene in wild type *A. baylyi* ADP1, a gene cassette containing selection marker, chloramphenicol resistance gene, and homologous sequences (5' flanking and 3' flanking of *crc* gene, ACIAD3526) was constructed. The two segments of homologous sequences were cloned to plasmid SM100/pIX one after another using *E .coli* XL-1 as host. The constructed plasmid was transformed into wild type ADP1 strain and the strain was selected on LA plate containing 50 μg/ml chloramphenicol. The resulting ADP1 strain was designated as crc strain in which *crc* gene was replaced with chloramphenicol resistance gene. PCR was carried out to confirm the knock-out of *crc* gene. Because *crc* gene and the insert (chloramphenicol resistance gene) have different lengths, the confirmation could be achieved by observing the lengths of the PCR products amplified with the same primers. Firstly, the genomes of wild type APD1 and crc strain were extracted using genomic purification kit. The extracted genomes were amplified with primer ab144 and ab145, after which gel electrophoresis was carried out to identify the lengths of the PCR products. As shown in figure 8, the PCR product from wild type strain had a length of 2 kb while the one from crc strain had a length of more than 2 kb, which indicates that the *crc* gene in crc strain has been replaced.

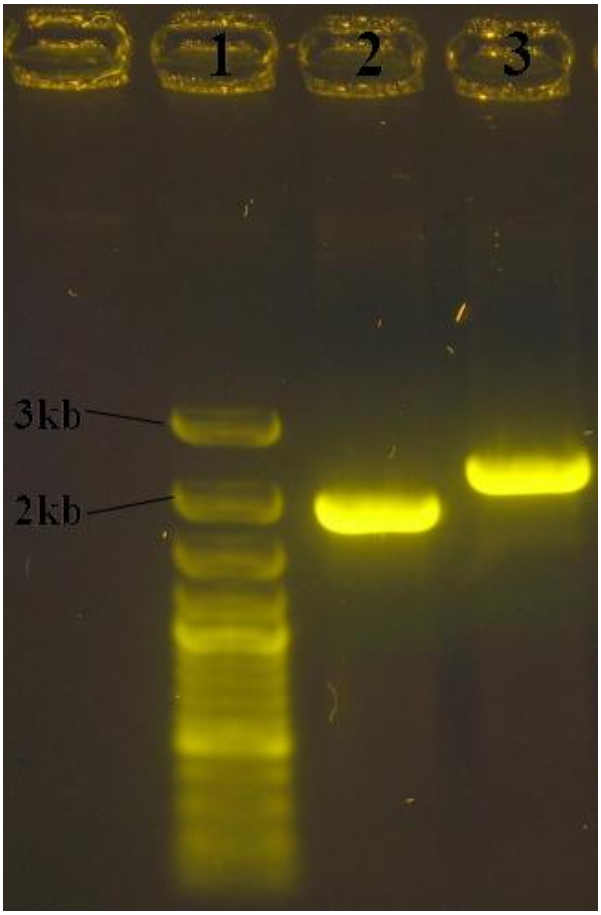


Figure 8. *Gene knock-out confirmation. 1: 100bp DNA ladder; 2: PCR product from wild type ADP1; 3: PRC product from crc strain.*

However, it is worth noting that there was another MfeI restriction site inside the amplified 5' flanking. After the 5' flanking was digested with enzyme MfeI and KpnI, two segments of DNA were obtained (one is about 200 bp with KpnI and MfeI site, another one is 120 bp with two MfeI sites). The 200 bp segment, which was about 120 bp shorter than it should be, was used as 5' flanking to knock out *crc* gene. This might affect the function of another gene, *pyrE* (ACIAD 3525), which has some overlapped sequence with *crc* gene and has an opposite encoding direction.

4.2 The growth of cell in the cultivations using different concentrations and combinations of carbon sources

Wild type ADP1

Before using the lignin model monomers as carbon sources to cultivate ADP1 for WE production experiment, the growths of ADP1 with these lignin model monomers (4-HBA, coumaric acid, ferulic acid and vanillic acid) as well as glucose as sole carbon source were tested. In addition, to explore the influence of carbon source concentration on cell growth, cultivations with different concentrations of these carbon sources were carried out respectively. The effect of Fe on cell growth was also tested with glucose and 4-HBA as sole carbon source. These tests were conducted with MA/9 medium in 5 ml cultivation.

According to figure 9, Fe had a positive influence on the growth of ADP1 when glucose and 4-HBA were used as sole carbon source. The cells cultivated without $FeCl_2$ added had a lower growth rate, and the OD_{600} was significantly lower than the cells cultivated with $FeCl_2$ added (figure 9 A and B). The influence of Fe on cell growth was more significant when glucose was used as carbon source than when 4-HBA was used. As the limits of sampling point and cultivation time, the cell growth couldn't be well characterized. However, the influence of carbon source concentration on cell growth can be seen in the cultivations with 4-HBA, coumaric acid, ferulic acid and vanillic acid used as sole carbon sources (figure 9 B, C, D and E)). When the concentration of carbon source increased, the lag phase of cells increased (figure 9 B, D and E). When the concentration increased to a certain point, the cell growth was inhibited (figure 9 C and D). But for different carbon sources, the concentrations that caused the inhibition of cell growth were different. ADP1 couldn't grow in 25 mM coumaric acid but could grow in 25 mM ferulic acid, and 50 mM ferulic acid was able to inhibit the growth. It seems that ADP1 can grow well in all the four lignin model monomers when the concentration is less than or equal to 15 mM, though different growth statuses might present in different lignin model monomers.

Wild type ADP1 vs. crc strain

To compare the growths between wild type ADP1 and crc strain, different carbon sources (2% glucose, 25 mM acetate, 2 mM 4-HBA, 0.2% casamino acid and their combinations) were used to cultivate them respectively.

As shown in figure 10, although there were some differences of growths between wild type and crc stain for some carbon sources, the differences were not significant basically. When 2% glucose was used as sole carbon source, crc strain grew faster than wild before about 24 h. After 24 h, the differences between the replicates of each strain became bigger, and thus it was hard to characterize the growths of both wild type and crc strain. A faster growth of crc strain before 24 h was also observed in the co-utilization of glucose and acetate as well as the co-utilization of glucose and 4-HBA. When grown on casamino acid, wild type reached a slightly higher optical density than crc strain. Both strains had similar growth in 2 mM 4-HBA. When 25 mM acetate was used as sole carbon source, a faster growth and higher optical density were observed in wild type, compared with crc strain. A similar phenomenon was also observed in the co-utilization of acetate and 4- HBA. This was unexpected as I thought crc strain would

have a better growth when both acetate and 4-HBA were present in the medium.

It is worth noting that it took a long time to get crc adapted to MA/9 medium. After crc strain was screened out from the LA plate containing chloramphenicol, it could grow very well in LB medium containing chloramphenicol. However, in the beginning, it almost didn't grow in MA/9 medium containing chloramphenicol, even growing very slowly without adding antibiotic. It took more than one week for it to adapt MA/9 medium.

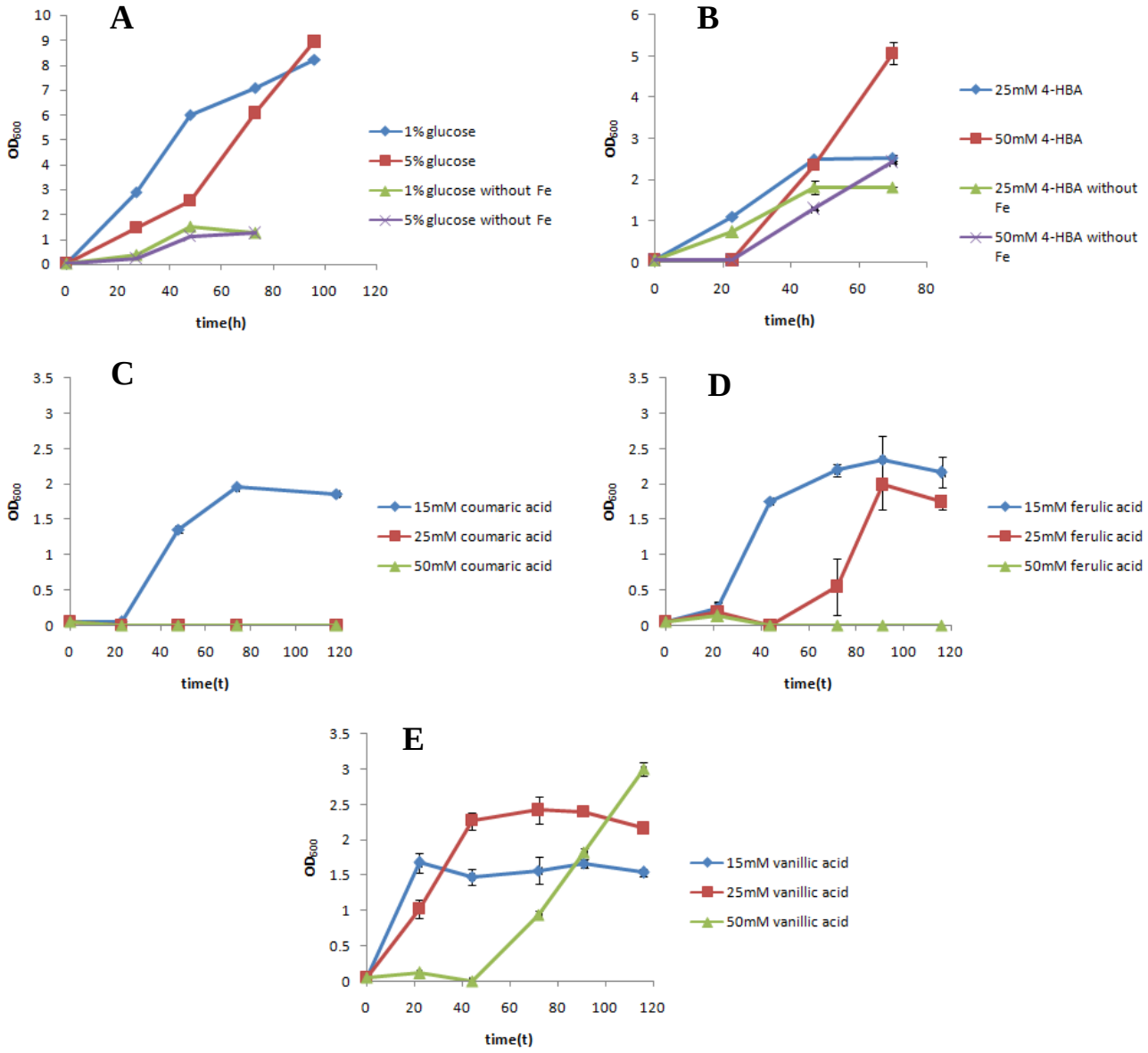


Figure 9. *The growth curves of wild type ADP1 cultivated with different concentrations of carbon sources. A: 1% and 5% glucose as carbon source with and without the addition of $FeCl_2$; B: 25 mM and 50 mM 4-HBA as carbon source with and without the addition of $FeCl_2$; C: 15 mM, 25 mM and 50 mM coumaric acid as carbon source; D: 25 mM and 50 mM ferulic acid as carbon source: E: 25 mM and 50 mM vanillic acid as carbon source.*

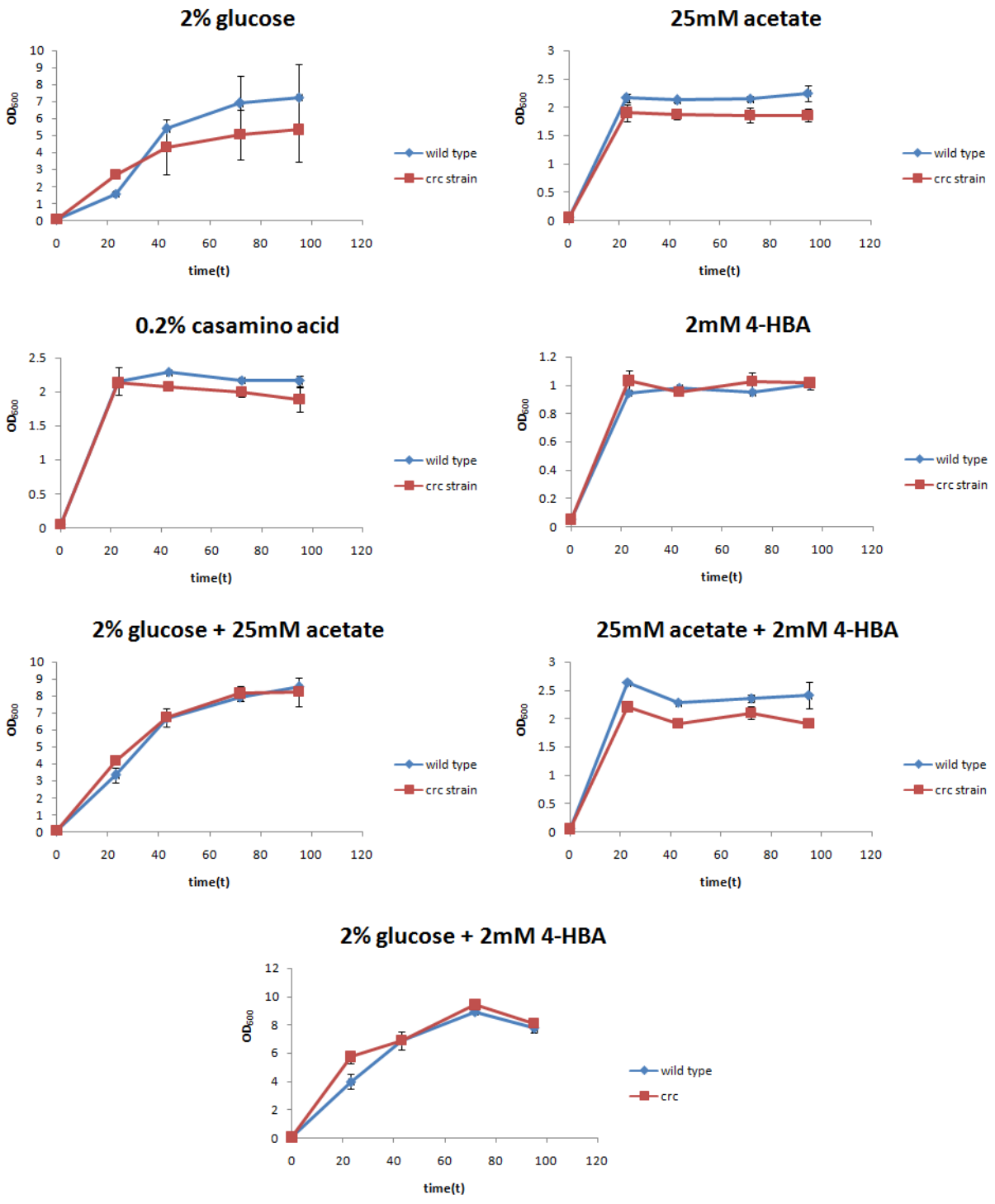


Figure 10. *Comparison of the growths between wild type ADP1 and crc strain with different carbon sources.*

4.3 Co-utilization of acetate and 4-HBA in 50 ml cultivation with wild type ADP1 and crc strain

To further explore the behaviors of catabolite repression to ß- ketoadipate pathway in wild type and crc strain, cultivations using both 25 mM acetate and 25 mM 4-HBA as carbon sources were carried out for 12 h. The samples were taken every 2 h to monitor the consumption of carbon sources and the growth of cell.

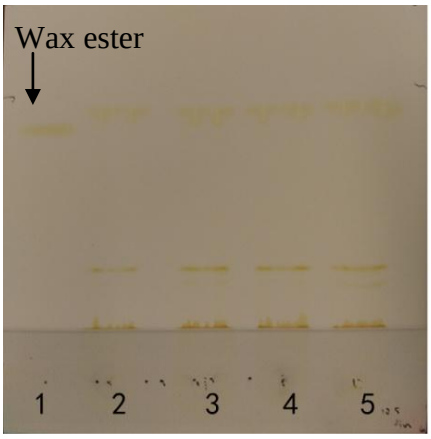


Figure 11. *TLC analysis of wax ester. 1:10 ul 1mg/ml jojoba oil as standard; 2 and 3: samples from the cultivations of wild type ADP1; 4 and 5: samples from the cultivations of crc strain*

There was a huge difference of the consumption of carbon sources between wild type and crc strain (figure 12 A). Wild type utilized acetate much faster than crc strain. At 6 h, wild type consumed almost all the acetate while there was still about 21 mM 4-HBA left in the culture. The consumption rate of 4-HBA was low during the first 6 h. However, once the acetate was depleted after 6 h, the consumption rate of 4-HBA started increasing. The concentration of 4-HBA decreased from 21 mM to 4 mM. In contrast, crc strain consumed acetate much more slowly than wild type. At 6 h, there was still 17 mM acetate left, and until 10 h almost all acetate was consumed. Different from wild type ADP1, the consumption of 4-HBA went on even under the presence of acetate, but it seemed that the consumption rate was lower than that of wild type after acetate was all depleted. After 12 h cultivation, almost all carbon sources were consumed in the cultivations of both wild type and crc strain. As to the growth, wild type grew faster than crc strain (figure 12 B). After 12 h, although almost all carbon sources were consumed by both strains, wild type reached a higher optical density than crc strain. After 12 h cultivation, the biomass from the cultivation of two strains was collected for lipid extraction. However, no WE was detected by TLC from the extraction products of both cultivations (figure 11).

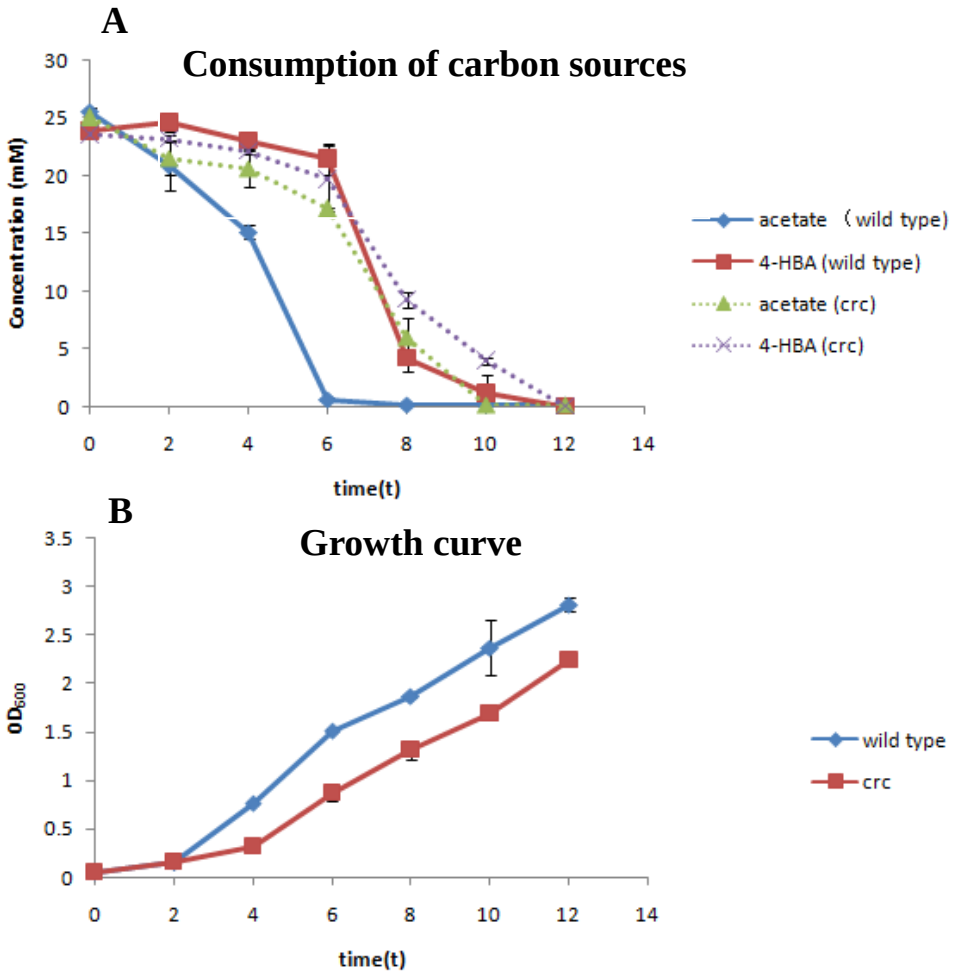


Figure 12. *The consumption of acetate and 4- HBA (A) and the growth curve of wild type ADP1 and crc strain (B).*

4.4 Thin layer chromatography (TLC) in 50ml cultivations with and without iron (wild type ADP1)

The previous results have shown that cells couldn't grow to a high optical density in the medium without Fe. To explore the effect of Fe on WE production by wild type ADP1, the cultivations with and without $FeCl_2$ added were conducted. The cells were cultivated for 48 h, with 5% glucose used as sole carbon source. After 48 h, the glucose consumption, optical density and WE production were compared.

When Fe was present in the medium, the concentration of glucose decreased from 49.94 g/L to 35.45 g/L after 48 h. In contrast, when no Fe was present in the medium, much less glucose was consumed and the glucose concentration only decreased by about 0.39 g/L (figure 13 A). Correspondingly, the OD_{600} was much higher when Fe was present than the OD_{600} when no Fe was present, which were 7.54 and 1.36 respectively (figure 13 B). According to the TLC result, WE accumulated in both cultivations (figure 13 D, the biomass in different lanes were different); however, Nuclear magnetic spectroscopy (NMR) analysis showed that more WE was produced per unit biomass in the cultivation without Fe (30.55 μmol/100 mg freeze-dried biomass) compared with that in the

cultivation with Fe (14.65 µmol/100 mg freeze-dried biomass), though a low optical density was obtained.

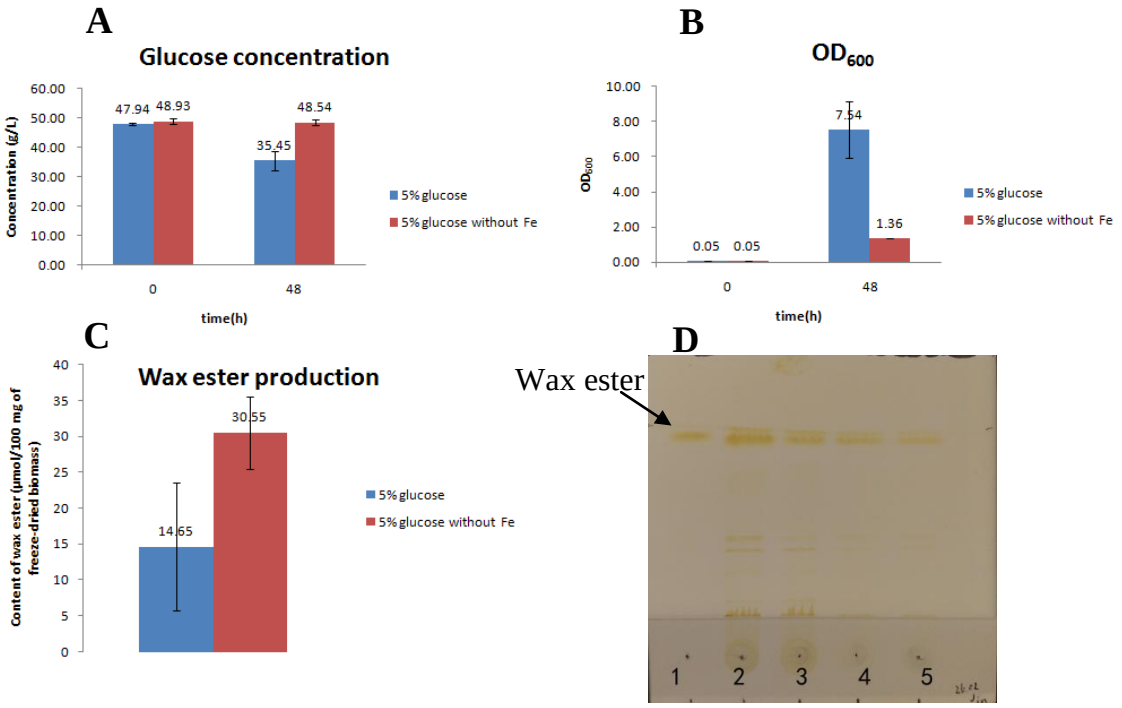


Figure 13. *The glucose concentrations (A), OD_{600} (B) and WE productions(C) of cultivations with and without $FeCl_2$ added after 48 h respectively. TLC analysis of WE (D); 1:10 ul 1mg/ml jojoba oil as standard; 2 and 3: samples from the cultivation with $FeCl_2$ added; 4 and 5: samples from the cultivation without $FeCl_2$ added. The biomass in lane 1 and 2 are higher than the one in lane 4 and 5.*

4.5 The effect of C: N ratio on WE production using lignin monomers as carbon sources

Wild type ADP1

To explore the effect of C: N ratio on wax ester production by wild type ADP1, the cultivations with high nitrogen concentration (1.19 g/L $(NH_4)_2SO_4$, normal concentration) and low nitrogen concentration (0.119 g/L $(NH_4)_2SO_4$, one tenth of the normal concentration) were carried out. 10 mM lignin monomers, coumaric acid and ferulic acid, were used sole carbon source respectively. Cells were cultivated for 72 h and sampling was conducted every 24 h. After each sampling, 10 mM carbon source was supplemented to the culture.

The cells had similar growth trends when grown on coumaric acid and ferulic acid in 72 h cultivation (figure 14 A). A little higher OD_{600} was observed when ferulic acid was used as carbon source in high N condition. A huge difference between the cultivations in

high N and low N condition was observed. In high N condition, the OD_{600} constantly increased over time and the OD_{600} of the cells grown on coumaric acid and ferulic acid reached 2.63 and 3.10 respectively. In contrast, in low N condition, the OD_{600} began to stabilize before 24 h and kept less than 1 over time. After 72 h cultivation, no WE was detected in the cultivations in high N condition while 16.9 µmol/100 mg biomass (coumaric acid as carbon source) and 14.4 µmol/100 mg biomass (ferulic acid as carbon source) WE were detected respectively in the cultivations in low N condition (figure 14 B). According to the TLC analysis, WE had started accumulating at 24 h in low N condition (figure 14 C and D). As the bands of WE became darker over time, WE seemed to accumulate constantly in the 72 h cultivation.

Wild type ADP1 vs. crc strain

To compare the WE productions and the co-utilizations of acetate and lignin monomers (coumaric acid and ferulic acid) between wild type and crc strain, 10 mM acetate, 5 mM coumaric acid and ferulic acid were used as carbon sources to cultivate the cells. High N and low N condition were also applied to the cultivations. Sampling was conducted every 24 h. After sampling, each carbon source with the same concentration as before was supplemented to the culture. The consumption of carbon sources was monitored.

Similar as the previous results, cells reached higher OD_{600} in high N condition than in low N condition (figure 15 C). In low N condition, the OD_{600} of cells began to stabilize before 24 h and was kept at around 1 afterwards (the OD_{600} for crc even decreased from 0.94 to 0.78). Compared with wild type, crc had a lower OD_{600} after 72 h cultivation. In both high N and low N condition, wild type consumed all the carbon sources (acetate, coumaric acid and ferulic acid) in 24 h (figure 15 A). After supplementation of carbon sources at 24 h and 48 h, carbon sources began to accumulate in low N condition while in high N condition no carbon sources accumulated. At 24 h and 48 h, 10 mM acetate, 5 mM of coumaric acid and ferulic acid were supplemented. In low N condition, coumaric acid and ferulic acid accumulated to 4 mM and 4.8 mM respectively while a strong accumulation of acetate was observed, which accumulated to 21 mM after 72 h. In contrast, crc strain also consumed all the acetate in both high N and low N condition before 24 h, but there were still some coumaric acid and ferulic acid remaining, which were 1.8 mM and 1.2 mM in high N condition and 2.7 mM and 2.9 mM in low N condition respectively. After supplementation of carbon sources, similar to wild type, carbon sources accumulated in low N condition while no carbon sources accumulated in high N condition. Compared with wild type, crc strain had a stronger accumulation of coumaric acid and ferulic acid, which accumulated to 7.3 mM and 8 mM respectively, and had a much less accumulation of acetate, which accumulated to 6 mM finally.

After 72 h cultivation, the net consumptions of acetate, coumaric acid and ferulic acid by wild type in low N condition were 0.45 mM, 0.55 mM, and 0.51 mM respectively while the consumptions by crc strain were 1.2 mM, 0.35 mM and 0.385 mM. As to WE production, no WE was detected in the cultivations in high N condition, and in low N condition wild type produced more WE than crc after 72 h cultivation, which were 29.2 and 11.25 µmol/100 mg biomass (figure 15 D). According to TLC results, both wild type and crc strain began to accumulate WE in 24 h (figure 15 E and F).

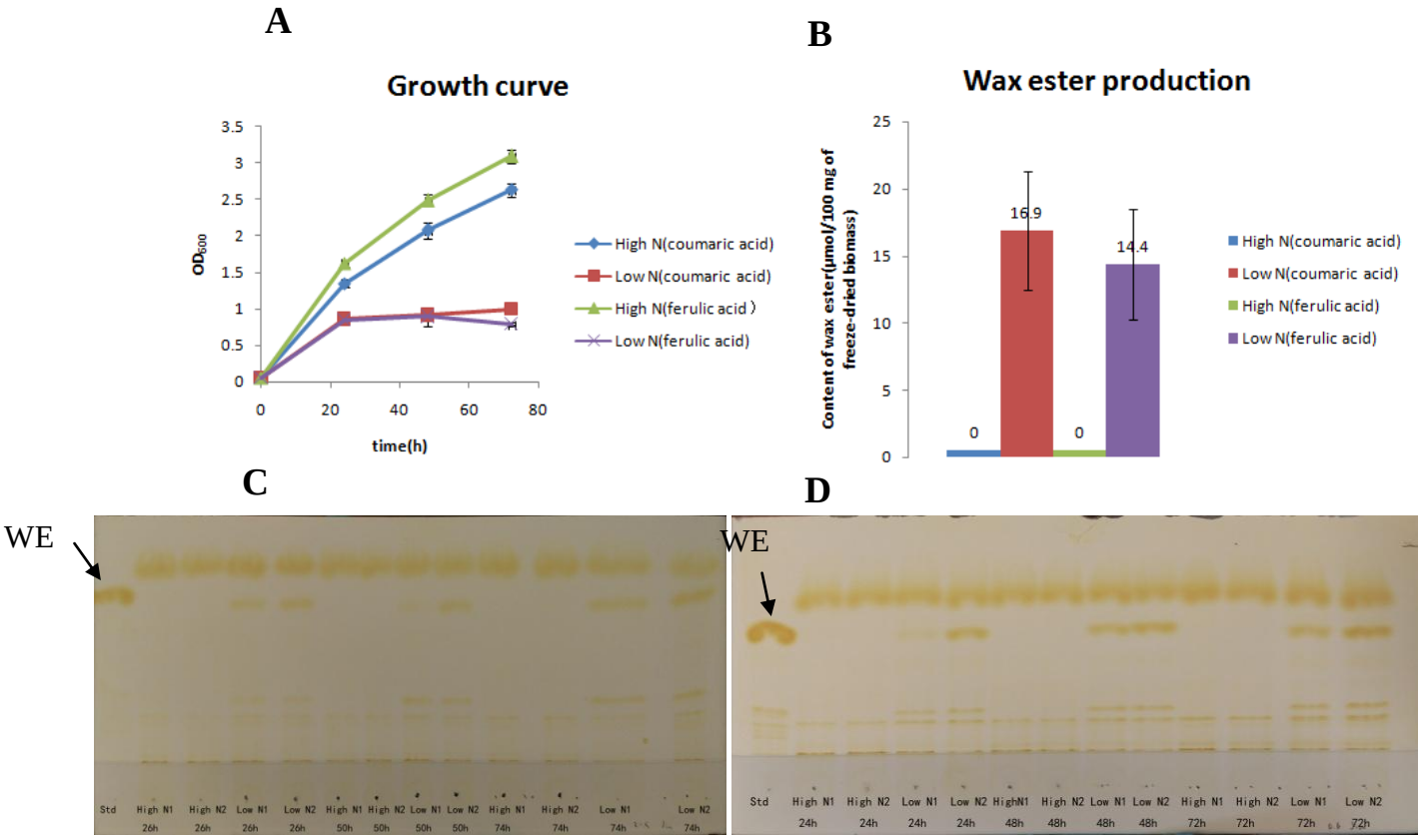


Figure 14. *The growth curves (A) and WE production (B) of wild type ADP1 cultivated in high N and low N condition, with coumaric acid and ferulic acid as sole carbon source respectively. TLC analysis of the wax ester over time from the cultivations with coumaric acid (C) and ferulic acid (D) as sole carbon source respectively; 30 µl 1 µg/ml jojoba oil as standard.*

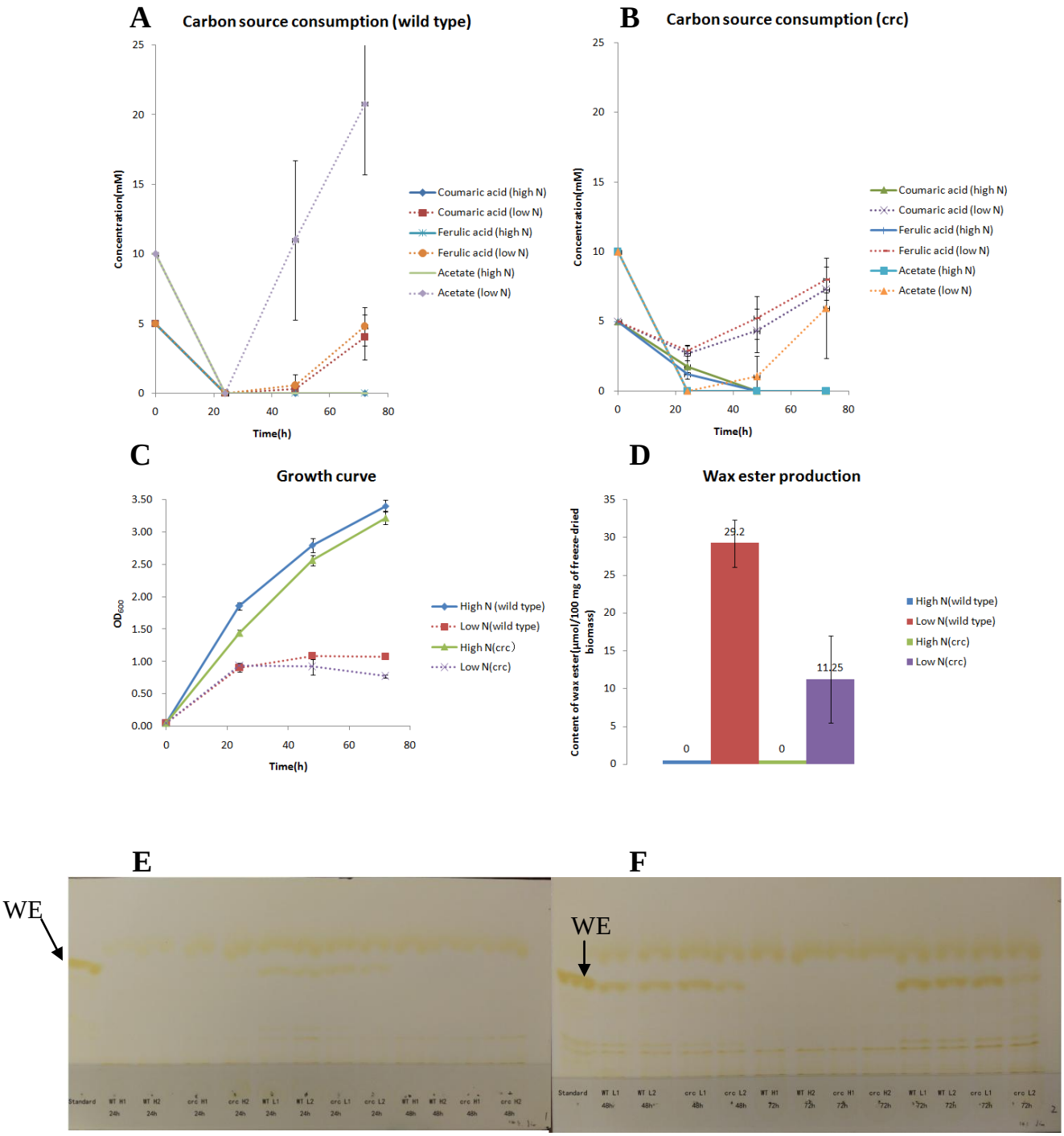


Figure 15. *Carbon source consumptions (A and B), growth curves (C) and WE production(D) of wild type and crc strain cultivated in high N and low N condition, with the co-utilization of acetate, coumaric acid and ferulic acid. TLC analysis of the WE produced from the co-utilization in high N and low N condition (E and F); 30 μl 1 μg/ml jojoba oil as standard.*

5. DISSCUSION

The purpose of this study was to explore the WE production by *A. baylyi* ADP1, using lignin model monomers as carbon sources. Also, *crc* gene, which has been proved to be involved in the catabolite repression to β-ketoadipate pathway under the presence of acetate [57, 58], was knocked out from wild type ADP1, and the resulting strain was designated as crc strain. A series of experiments were conducted in this study. First, the wild ADP1 was cultivated with different lignin monomers at different concentrations, aiming to find out the appropriate concentration of each lignin monomer that could be used for WE production. In addition, the effect of Fe on cell growth was explored. Second, the growths and carbon source consumptions of wild type and crc strain were compared to explore the effect of the knock-out of *crc* gene. Finally, the WE productions were carried out in nitrogen-limiting condition for wild type ADP1and crc strain respectively.

5.1 Growth of wild type ADP1 on lignin monomers

Monod equation, which is based on empirical consideration, is usually used to describe the specific growth rate of bacteria. However, in many cases, the growths of bacterial do not behave in this conventional way. When substrate concentration increases to a certain degree, the growth of bacteria is sometimes inhibited. In this study, inhibitions caused by high concentration of lignin monomers were observed. The inhibitions were manifested in two aspects: the increase of lag phase and the reduction of growth rate. The results of lignin monomer degradations were shown in figure 2. As the initial concentration of lignin monomer increased, the lag phase was prolonged. This is evident when 4-HBA, ferulic acid and vanillic acid were used as carbon sources (figure 2 B, D and E). For example, cells cultivated on 15 mM and 25 mM vanillic acid started growing at the beginning while cells cultivated on 50 mM vanillic acid started growing after 48 h (figure 2 E). From the cultivation with 25 mM ferulic acid (figure 2 D), it can also be roughly seen that growth rate was increased as time went on according to the slope of the growth curve. However, the effect of high substrate concentration on cell growth rate could not be well described in this study due to the limit of sampling points. When the concentration of lignin monomers were increased to a certain value, the growth was completely inhibited. This phenomenon was obvious in the cultivations with coumaric acid and ferulic acid, in which the cells were not able to grow in 25 mM coumaric acid and 50 mM ferulic acid. The four lignin monomers used in this test, 4-HBA, vanillic acid, ferulic acid and coumaric acid, had different concentration values at which the growth was completely inhibited. According to the duration of lag phase and the concentrations that cells could tolerate, ADP1 utilized 4-HBA the best, followed by vanillic acid, ferulic acid and coumaric acid in sequence. Good growth of ADP1 was achieved in all the four lignin monomers when the concentration was 15 mM and cells could reach stationary phase in 72 h.

The growth inhibition of ADP1 can also be caused by high concentration of glucose, but the concentration that causes a significant inhibition is much higher than lignin monomers. For the degradation of most toxic xenobiotics, a relatively low substrate concentration can also cause a growth inhibition which may prolong the lag phase [59]. This would be an obstacle for effective degradation of these types of compounds as the initial concentration should be kept below the value which may cause a significant growth inhibition. The substrate inhibition has been well studied in enzyme-catalyzed reaction, but the inhibitions in bacterial growth are not exactly the same [60] and can be attributed to many mechanisms [61].

The effect of Fe on the growth was also explored. As shown in figure 2 A and B, cells cultivated without the addition of Fe couldn't reach a high optical density, which indicates that Fe might have a positive effect on the growth of ADP1. Generally, Fe has diverse functions in bacteria; for example, many enzymes necessary in tricarboxylic acid cycle, such as aconitase, need the participation of Fe for their activities [62]. However, the growth was not completely inhibited without the addition of Fe.

5.2 Comparison of growths and carbon source consumptions between wild type and crc strain

First, it is worth noting that crc strain was not able to grow in minimal salt medium initially after being screened out from the LA plate containing chloramphenicol, but could grow very well in LB medium. It took a very long time (more than 1 week) to get crc strain adapted in minimal salt medium. Considering LB medium is nutrient-rich medium, it might take longer time for the cells to adapt to minimal salt medium. However, wild type took a much shorter time to adapt to minimal salt medium compared with crc strain. After crc strain adapted to minimal salt medium, it still had a longer lag phase than wild type ADP1 when inoculating the precultures for both strains from the glycerol stocks. As mentioned in chapter 4.1, the function of *pyrE* gene might be affected when knocking out the *crc* gene. The *pyrE* gene is involved in the biosynthesis of pyrimidine which is the substrate of nucleic acid synthesis [63]. If this is the case, this might account for the long adaption time of crc strain in minimal salt medium.

As shown in figure 10, in general, the growths of wild type and crc strain on different carbon sources showed some differences, but not too big. Difference was also observed when glucose was used as carbon source, but the errors between the duplicates was big (one of the wild type duplicate had a very high OD while the other one had a similar OD as the duplicates of crc strain). Also, according to the growths in the cultivations using both glucose and 4- HBA as carbon sources, there was almost no difference in the growths between wild type and crc strain. Thus, I think the growths of crc strain and wild type on glucose don't differ too much. An interesting phenomenon worth noting is that in all the cultivations in which there were glucose as carbon source crc strain seemed grew a little faster than wild type in the first 24 h. Significant differences were mainly observed in the cultivation with acetate as sole carbon source and the cultivation with acetate and 4-HBA as carbon sources. In those cultivations, both wild type and crc strain reached stationary phase in 24 h. However, wild type reached higher OD than crc strain though the substrate concentrations were the same for both strains. This was unexpected as crc strain even showed a slower growth than wild type. Therefore, to

further explore the difference between them, co-utilization of acetate and 4-HBA was conducted with both wild type and crc strain, and carbon source consumption was monitored.

According to the results of the co-utilization (figure 12), the catabolite repression by acetate was obvious in wild type ADP1; 4-HBA was consumed at a very low rate when acetate was present, and once acetate was depleted (or below the detection limit) the consumption rate increased drastically. The acetate was consumed very fast by wild type ADP1. This repression was reported to be caused by Crc protein acting on the transcripts involved in the ß- ketoadipate pathway, decreasing the stability of the transcripts [57]. The subsequence research also reported that Crc protein also acts at transcriptional level, affecting the promoter activity under the presence of acetate or succinate [58]. In *A. baylyi*, most genes related to ß- ketoadipate pathway undergo the catabolite repression under the presence of acetate or succinate. In contrast, crc strain, which *crc* gene has been knocked out, could consume 4-HBA under the presence of acetate, but the consumption rate seemed to be very low. In addition, acetate was also consumed by crc strain at a much lower rate than it was consumed by wild type stain. All carbon sources were consumed by both strains in 12 h. However, wild type had a higher OD than crc strain and also no WE was produced by both strains after the cultivation according to the TLC result, which indicates that some carbon sources were consumed by crc strain for other purposes.

The reason why crc strain had lower carbon source consumption rate and OD than wild type needs to be further studied. However, if the function of pyrE gene was affected during the process of the *crc* gene knock-out, this could be a possible reason. The pyrE gene is responsible for the synthesis of pyrimidine which is the substrate of nucleic acid synthesis. It has been reported that pyrE mutant has uracil auxotrophy in the medium without pyrimidine compounds, such as uracil, cytosine, thymine or there derivatives [64]. It might account for the phenomena that crc strain didn’t grow in minimal salt medium in the beginning. But the strain was able to grow in minimal salt medium finally, which indicates that there might be an alternative pathway for pyrimidine compounds synthesis or the pyrimidine compounds might come from other sources. But this remains to be proved by further experiment, because whether pyrE gene was affected is not confirmed and its function is also not studied very well in *Acinetobacter* spp. .

5.3 WE production using lignin monomers as carbon sources

WE was produced successfully by using lignin monomers as carbon sources in this study. As mentioned in previous chapter, the synthesis of WE starts from acetyl-CoA which will also go into TCA cycle and other pathways for biomass production. Thus, the production of WE is subject the competition of the biomass production. The production of biomass depends on carbon source and nitrogen source. When nitrogen is limited and carbon source is excessive, the synthesis of lipid will take place. This theory was proved in this study in which no WE was produced in high N condition while certain content of WE was detected in low N condition.

The WE production experiments showed that wild type ADP1 successfully accumulated WE by using coumaric acid and ferulic acid as sole carbon source under low N condition. The difference of WE yield was not significant between the cultivations with

coumaric acid and ferulic acid (16.9 and 14.4 µmol/100mg freeze-dried biomass respectively). Both compounds follow protocatechuate branch of ß- ketoadipate pathway. As the common intermediate of ß- ketoadipate pathway, ß- ketoadipate will be further converted into succinly-CoA and acetyl-CoA, which will both enter into TCA cycle for energy production. Some of acetyl-CoA will be used for WE synthesis and some will be used for biomass production.

In the co-utilization of acetate, coumaric acid and ferulic acid, WE with a yield of 29.2 µmol/100mg freeze-dried biomass (0.292mmol/g freeze-dried biomass) was obtained from wild type and 11.25 µmol/100mg freeze-dried biomass (0.1125mmol/g freeze-dried biomass) from crc strain under low N condition. According to the growth curve, in low N condition, the OD of both wild type and crc strain began to stabilize in 24 h, which indicates that nitrogen source was depleted and the biomass production was inhibited. WE stared to accumulate when the OD began to stabilize. Carbon sources with the same concentration as initial point (10 mM acetate, 5 mM coumaric acid and ferulic) were supplemented at 24 h and 48 h to promote WE accumulation. According to the carbon source consumption analysis, wild type consumed all the carbon sources in the first 24 h. A strong accumulation of acetate and small amount of coumaric acid and ferulic acid were observed at 48h. I speculate that most of the supplemented acetate was consumed first, and the accumulated acetate was probably from the conversion of acetyl-CoA which derived from the catabolism of coumaric acid and ferulic acid. Because the catabolism of coumaric acid and ferulic acid can be repressed under the presence of acetate, but here the supplemented coumaric acid and ferulic acid were consumed mostly. After 48 h, only a little carbon source was consumed, which indicates that the accumulation of WE probably approached maximum. In contrast, crc strain consumed coumaric acid and ferulic acid constantly during the whole cultivation but the consumption rate was low, thus a moderate accumulation of coumaric acid and ferulic acid was observed. The acetate seemed to be consumed relatively fast initially, and the consumption rate decreased as time went on, which indicates the accumulation of WE, but accumulation still continued. According to the analysis of total carbon source consumption, wild type consumed more coumaric acid and ferulic acid than crc strain while crc strain consumed more acetate, but overall more carbon source seemed to be used by crc strain. However, crc strain accumulated much less WE than wild type while both strains had similar OD, which indicates that crc strain didn’t convert substrate into WE as efficiently as wild type ADP1.

Although crc strain is able to convert lignin monomers into WE, its conversion efficiency and carbon source consumption rate are relatively low compared with wild type ADP1. This is unexpected as it has been reported that the knock-out of *crc* gene will inactivate the catabolite repression caused by acetate but will not affect the growth and carbon source utilization of the strain [57, 58]. And if the *pyrE* gene was affected in crc strain and ADP1 has another alternative mechanism for pyrimidine compounds synthesis, this mechanism might be more energy-consuming, which might account for the fact that crc strain accumulated less WE and had a lower OD than the wild type. And the mechanism might also be less efficient for pyrimidine synthesis, which might account for the low carbon source consumption rate. Because low efficiency of pyrimidine synthesis might affect the synthesis of nucleic acid, which might might affect the whole metabolic system. Certainly, this speculation need to be further discussed and explored.

It is also worth noting that the cells cultivated without the addition of Fe couldn’t reach a high OD but had higher WE content, according to the results from Figure 13, which indicates that addition of Fe might have a positive effect on cell growth but negative effect on wax ester accumulation. As mentioned before, Fe participates in many reactions which are essential for energy production and cell growth [62]. The lack of Fe might inhibit the cell growth, which led to the result that more carbon source was converted into WE.

6. CONCLUSION

Lignin is one of the most abundant natural polymers on the earth. However, the use of lignin is usually limited combustion due to its inherent heterogeneity and recalcitrance to degradation. The idea of this research is to convert lignin into more valuable product, WE, through microbial conversion. Before the conversion, lignin should be degraded into some low molecular weight compounds, such as some lignin monomers.

Acinetobacter spp. has been reported to be able to use different types of lignin monomers as carbon sources [4] and accumulate WE as storage compound [45-48]. In this study, I demonstrated the growth of ADP1 on different lignin monomers. And I also demonstrated that it is possible to produce WE by ADP1 using lignin monomers as carbon sources in nitrogen-limited condition. The knock-out of *crc* gene was also conducted, which proved that ADP1 can be a convenient model system to study metabolic engineering. Also, in this study, the catabolite repression caused by acetate was observed in wild type ADP1 and seemed to be inactivated in crc strain. However, the performance of crc strain was not as optimistic as expected, in terms of growth and carbon source consumption and WE production. Another gene (*pyrE* gene) was suspected to be affected in crc strain, which needs to be further explored.

This study has proved that *A. baylyi* ADP1 can accumulate WE by using lignin monomers as carbon sources. However, the degradation products of lignin are very complicated, including not only the monomers used in this study but also many other compounds such as diphenyl ether, phenol, acetophenone etc [17]. The composition also varies depending on the sources of lignin and the methods of pretreatment. Therefore, the next step should be focused on the conversion of the pretreated lignin, for example, alkaline pretreated lignin. In addition, developing efficient methods for lignin recovery and depolymerization is of great importance for the utilization of lignin. As *A. baylyi* ADP1 is an excellent model organism, some genetic modifications can be applied to optimize the pathways for substrate utilization and WE accumulation.

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