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Development of a microbial process for the conversion of carbon dioxide and electricity to
higher alcohols as biofuels

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular Biology

by

Han Li

2013

ABSTRACT OF THE DISSERTATION

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higher alcohols as biofuels

by

Han Li

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2013

Professor James C. Liao, Chair

Man-made photovoltaic device is relatively efficient in converting sunlight to electricity, but the electrical energy generated is difficult to store. Current methods, such as chemical batteries, hydraulic pumping, and water splitting, suffer from low energy density or incompatibility with current transportation infrastructure. The biological systems, on the other hand, can store sunlight in high energy-density carbon-carbon bonds. However, the photosynthesis has low solar energy harvesting efficiency, for which no near-term improvements are in sight. One way to solve both problems is to combine man-made solar cells to biological Carbon dioxide (CO_2) fixation and fuel production. Therefore, a microbial process to produce biofuels from CO_2 and electricity is needed. In this work, we first used metabolic engineering methods to achieve the production of isobutanol, n-butanol, and 3-methyl-1-butanol (3MB) in a lithoautotrophic bacterium *Ralstonia eutropha* H16, which can utilize formate as the sole carbon and energy source. We then demonstrated electrochemical production of formate from CO_2 and finally

integrated the electrochemical reactions with the microbial fuel production. Besides achieving the goals in engineering, this work explored important design principles of metabolic pathway and developed new tools in synthetic biology.

To produce higher alcoholos, two different carbon chain building routes were constructed. First, Coenzyme A (CoA)-dependent pathway was used to synthesize isobutanol and n-butanol in *R. eutropha* H16. We demonstrated for the first time the production of isobutanol, a branched-chain alcohol, using the CoA-dependent pathway in recombinant *R. eutropha* H16. The isobutanol production pathway deviates from the CoA-dependent n-butanol production pathway in that it contains an extra carbon chain rearrangement step catalyzed by the isobutyryl-CoA mutase in *R. eutropha*, which has not been characterized previously. Metabolic engineering methods such as heterologous gene expression, codon optimization, and promoter strength altering were applied to first achieve the production of ~200mg/L n-butanol from fructose or ~30mg/L from formate by engineered *R. eutropha*. The isobutyryl-CoA mutase was then added to the pathway to achieve the production of ~30mg/L isobutanol from fructose. The carbon skeleton rearrangement chemistry explored here might be used to expand the repertoire of the chemicals accessible with the CoA-dependent pathway.

Next, keto acid-dependent pathway was used to synthesize isobutanol and 3MB in *R. eutropha* H16. We integrated the set of genes for isobutanol and 3MB production into *R. eutropha* H16 genome, namely *alsS* from *Bacillus subtilis*, and *ilvC* and *ilvD* from *Escherichia coli*. The genes *kivd* from *Lactococcus lactis* and *yqhD* from *E.coli* were then introduced using a plasmid. *R. entropha* uses poly[R-(−)-3-hydroxybutyrate] (PHB) as a storage compound and as the metabolic sink for carbon and reducing equivalents. We disrupted the PHB synthesis and used the synthetic

isobutanol and 3MB production pathway as the new metabolic sink. In a pH-coupled formic acid feeding fermentor, the engineered strain LH74D produced fuels with the final titer of over 1.4 g/l (~846mg/l isobutanol and ~570mg/l 3MB) and peak productivity of 25 mg/l/h.

Most natural metabolic pathways are regulated on transcriptional level or on protein level by allosteric effectors. In this work, non-native regulatory mechanisms were introduced to the synthetic pathways. In particular, on transcriptional level, a synthetic anhydrotetracycline (aTc)-controllable gene expression system in *R. eutropha* H16 was developed and applied in regulating the biofuel production gene. The system is composed of a controllable promoter containing the operator tetO, the repressor tetR, and the inducer aTc. The active hybrids between the *tetO* operators and the native P_{rrsC} were first identified and shown to be repressable by tetR. Next, two mutants of the native P_{phaC1} promoter were obtained from a high-throughput screening of 300 candidates to tune the tetR expression. The optimized system, which contains the $P_{rrsC-O1-O1}$ hybrid promoter and the $P_{phaC1-G3}::tetR$ cassette, has decreased leaky expression level and can be regulated gradually by different aTc concentration with a ~11 fold dynamic range. The system was used to alleviate cellular toxicity caused by AlsS overexpression, which impeded our metabolic engineering work on isobutanol production in *R. eutropha* H16. The system reported in this study may be a useful tool for future research and engineering work in this organism.

Finally, we combined the electrochemical formate production with the microbial fuel synthesis by the engineered host in an integrated process. The challenge was that the growth of the microbial host was inhibited by the electrochemical reaction in a transient manner, suggesting that unstable compounds such as reactive oxygen or nitrogen species might be responsible for the growth inhibition. Reporter assays in *R. eutropha* H16 showed that *sodC* and *norA* promoters,

which drives the genes for O_2^- and NO defense pathway, respectively, were induced when electricity was on. To circumvent this toxicity problem, a porous ceramic cup was used to shield the anode. This inexpensive shield provides a tortuous diffusion path for chemicals. Therefore, the reactive compounds produced by the anode may be quenched before reaching the cells growing outside the cup. Using this approach, healthy growth of *Ralstonia* strains and production of over 140mg/l biofuels were achieved with the electricity and CO_2 as the sole source of energy and carbon, respectively.

The dissertation of Han Li is approved.

<div align="center">

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DEDICATION

This dissertation is dedicated to my parents and my husband.

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ACKNOWLEDGEMENT

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Finally, I would like to thank my parents and my husband for their unconditional love and support. They are the most important role models, mentors, and friends in my life.

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1. Introduction

1.1 Objective

The objective of this work is to develop a microbial process to convert CO_2 and electricity into transportation fuels. To achieve the objective, this work also aims to explore the design principles in metabolic engineering and develop new tools in synthetic biology.

1.2 Significance

Compared to the current-day biofuel production schemes which involve formation and subsequent conversion of terrestrial plant biomass, the process developed here has the following advantages:

1) It has higher sun-to-fuel energy efficiency potentially. To achieve sustainability, the energy in the fuels must ultimately come from the Sun. Solar energy harvesting using man-made devices has higher efficiency (10-45%) than the "light reaction" of biological photosynthesis (less than 1%). The energy output of the man-made devices is in the form of electricity. Therefore, if electricity can be used to power the CO_2-fixing "dark reaction" in place of the biological "light reaction", higher overall efficiency may be achieved. Furthermore, since the microbial host is engineered to directly convert CO_2 to fuels, the energy lost in forming the biomass intermediate can be minimized.

2) By decoupling the "light reaction" and "dark reaction", the CO_2-fixing microbial cells can be cultured in a conventional three-dimensional (3D) way, for which established scaling-up strategies exists. Previously, photosynthetic microorganisms have been used to produce fuels from CO_2 using light as the energy source, which also bypasses the formation of biomass

intermediates. However, the major bottleneck for the large scale application of this technology is that the microbes need to be cultured in a two-dimensional (2D) way to ensure adequate light exposure, which represents technical and economical difficulties.

3) It represents a novel way to store the electricity generated from renewable sources such as solar, hydro, and wind energy. The electricity generated from renewable sources is intermittent and not evenly distributed. Therefore it needs to be stored efficiently. The current method of electricity storage via batteries suffers from the low energy density, which generally ranges between 0.1-0.7MJ/kg (or 0.5-2.0 MJ/L). In contrast, the energy density of gasoline is around 45MJ/kg. Energy density is particularly important for transportation application given the limited on board space in the vehicles. The process developed in this work provides a promising approach for storing intermittent electricity in the form of liquid fuel that can be used for transportation directly.

1.3 General principles and approaches

In this work, the facultative lithoautotrophic bacterium *Ralstonia eutropha* was used as the microbial catalyst in the process. And the higher alcohols such as isobutanol, n-butanol, and 3-methyl-1-butanol were chosen as the target products, which can be used as gasoline substitutes.

This work tackles the objective from four different levels:

1) Designing of the metabolic pathways. Since the goal is to knit the C4-C5 carbon chains from CO_2, common carbon-carbon bond formation chemistries in biological system form the basis of the pathway designing. To this end, two different carbon-chain elongation routes were designed in the host organism *R. eutropha*: The Coenzyme A-dependent chain elongation route, and the

keto acid-dependent chain elongation route. Besides reaching the desired carbon number, each route also requires additional steps to rearrange or functionalize the carbon chain to yield the final product. These steps were designed based on the versatile biochemical reactions with the carbonyl group, which include decarboxylation, reduction, and isomerization.

2) Installation of the designed pathways in the host. Once the pathways were designed, the enzymes that catalyze each step of the pathways were identified based on previous biochemical studies or using bioinformatic methods. The corresponding genes were then introduced into the host using recombinant DNA. Special attention was given to the following aspects: Firstly, the genes were often from other organisms and their functional overexpression in the heterologous host needs to be checked using biochemical assays. Second, the pathways were tailored to incorporate the suitable driving force in host metabolism, e.g. the abundant cofactor. Lastly, the expression levels of different genes within the pathways were tuned by altering promoter strength and gene dosage to achieve optimal performance.

3) Engineering the regulatory mechanisms of the synthetic pathways. Most natural metabolic pathways are regulated on transcriptional level or on protein level by allosteric effectors. In this work, non-native regulatory mechanisms were introduced to the synthetic pathways to better fit them into the host metabolic network. On transcriptional level, a chemical controllable gene expression system was developed. On protein level, the feedback regulation behavior of the key enzymes was engineered.

4) Integration of the microbial catalyst to the overall process. The goal of this work is to use electricity to power the biofuel production. However, electric energy is not an energy source that can be directly used by the host. Therefore, electricity was first used to produce formate

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electrochemically, which then delivers the energy once enters the cells. The challenge in developing this integrated process was that the electrochemical reaction had toxicity effect to the microbial cells. The cellular response to the electrochemical reaction was characterized and the cause of the toxicity effect was revealed, which led to the solution in process engineering to overcome the problem.

1.4 Specific aims

The specific aims of this work are:

1) Synthesis of isobutanol and n-butanol using engineered Coenzyme A-dependent pathways in *Ralstonia eutropha* H16.

2) Synthesis of isobutanol and 3-methyl-1-butano using engineered keto acid-dependent pathway in *Ralstonia eutropha* H16.

3) Development of a synthetic anhydrotetracycline-controllable gene expression system in *Ralstonia eutropha* H16 and its application in regulating the engineered pathways.

4) Development of an integrated microbial process for one-pot biofuel synthesis directly from CO_2 and electricity

The following chapters will discuss each of the specific aims.

2. Background: principles and biomolecular fundamentals of biofuel production

2.1 Introduction

The 21st century started with the resurrection of biofuels as a potential fossil fuel substitute. Petroleum, which powered the sustained economic growth of the last century, has begun to reach or has reached its peak. The rapid increase in demand has outpaced the production in the past few decades. The energy shortage situation is further complicated by political uncertainty and environmental impact associated with petroleum import and usage. In particular, CO_2 produced from fossil fuels has been implicated as a significant cause of climate change.

The current concept of biofuel life cycle starts from recycling CO_2 with the help of solar energy and water to produce biomass via a well-known metabolic process, photosynthesis. Distinct from man-made solar energy harvesting systems which mainly generate electrical power, biological systems utilize photosynthesis to capture and store solar energy into the form of chemical bonds in biomass. This naturally evolved process provides a unique opportunity to access and exploit solar energy via biological or thermochemical [1-3] conversion of biomass to produce liquid fuels. Biomass can be defined as the collection of all organic matter composing biological organisms, but the main components utilized for biofuel production are sugars (starch, simple sugars, and lignocelluloses) and lipids [4].

Sugars are the most abundant raw material for biofuel production. Bioethanol produced from plant starch and simple sugars has been the most successful biofuel to date. Traditionally, ethanol is produced in the yeast *Saccharomyces cerevisiae* or the proteobacteria *Zymomonas mobilis* from hexoses through a well-studied pathway known as glycolysis, followed by decarboxylation of puruvate and further reduction [5]. Lipids serve as another energy storage material in living organisms, which can be readily extracted from oil plants such as soybean and palm and

converted to biodiesel via transesterification. Because of the high C/O ratio in lipids, biodiesel generated from transesterification of biomass lipids enjoys the advantage of high energy density. In addition, the close resemblance between biodiesel and its counterpart derived from petroleum makes it compatible with existing petroleum-based infrastructure with only minor modifications [3].

Although ethanol can be produced by natural hosts with high yields, starch and simple sugars represent only a small fraction of the total plant biomass. Thus the utilization of corn or sugar cane as feedstock becomes economically challenging [4], in addition to the food-versus-fuel issue. Utilization of non-food lignocelluloses therefore presents a necessary direction for large-scale biofuel production. On the other hand, biodiesel has yet to contribute significantly to substitute for petroleum-based diesel fuel due to the limited availability of oil plant feedstocks [6]. As such, a complete solution to the biofuel problem requires integrative consideration of agricultural practice, land use policy, water resource distribution, infrastructure of fuel distribution and usage, and environmental evaluation, in addition to the technical aspects of biological conversion. Nevertheless, this chapter will focus only on the biomolecular aspects of biofuel production, as improvement in the overall fuel production efficiency by biomolecular engineering for either the current or more desirable fuel molecules will certainly impact each step in the whole biofuel life cycle.

2.2. General issues

2.2.1 Desirable fuel properties

Biofuels are designed to substitute liquid fuels currently used in internal combustion engines, diesel engines, and jet engines. Depending on the specific applications, each category has unique

requirements to meet both performance and regulatory standards. All of these petroleum-based fuels consist primarily of alkanes of various lengths and branching patterns. The gasoline currently used for internal combustion engines consists of smaller alkanes containing from 6 to 9 carbons on average and must meet specifications for vapor pressure and octane number. In general, increasing carbon chain length lowers the octane number while increasing chain branching increases octane number. Diesel fuels contain the largest alkanes of 12 to 20 carbons in length and must meet cetane number requirements. Higher cetane number is desirable, however it increases with chain length and decreases with branching. The freezing point of diesel, and the related cloud point, is also an important consideration because the long-chain alkanes can begin solidifying at temperatures as high as 10 ℃, and this causes obvious complications for a liquid fuel engine. Jet fuels lie in between the other fuels, containing alkanes of length 10 to 15, and the most important specification for jet fuel is low freezing point to maintain fuel liquidity at the low temperatures encountered at high altitudes.

Although ethanol represents an initial success as a biofuel because of its high production efficiency, it does not compare favorably to gasoline. It provides much less energy per volume, a low vapor pressure, and is hygroscopic which can lead to corrosion in pipelines and engine ducts (Table 2.1). Furthermore, when added to the current gasoline blends ethanol raises the vapor pressure of the mixture, ultimately increasing the price by forcing extraction of other light components in the gasoline, though this is partially offset by an increase in octane number. Meanwhile, advanced biofuels such as n-butanol and other higher alcohols with longer carbon backbones have better properties as fuels than ethanol, including a higher heating value and low hygroscopicity. Higher alcohols also lower the vapor pressure of current gasoline blends, and while the octane number of n-butanol is slightly less than standard gasoline, branched-chain

isomers such as isobutanol have a higher octane number allowing for more flexibility in fuel design.

Table 2.1 Comparison of chemical properties of automobile fuels

Fuel	Ethanol	n-Butanol	Isobutanol	Alkanes (Gasoline)	Alkanes (Diesel)	Fatty acid methyl esters (Biodiesel)
Heating Value (MJ/L)	21	29	29	32	39	37
Vapor Pressure (psi)	1.1	0.077	0.17	0.1 - 30	< 0.01	< 0.01
Blended VP (psi)[a]	20[b]	6.4[b]	6.8*[b]	7.8 - 15		
Avg Octane Number[c]	116	87	110	90		
Cetane Number					45	49 - 58
Freezing Point (°C)					-30 - 9.9	7.5 - 16
Hygroscopicity	High	Low	Low	Low	Very Low	Very Low
Fits Current Infrastruture?	No	Yes	Yes	Yes	Yes	No

a Represents vapor pressure of fuel mixture

b Alcohol blended at 10% with gasoline

c Average of Research octane number (RON) and Motor octane number (MON)

2.2.2 Common choices

Regardless of the fuel molecules of interest, research and development in this area involve some common issues and choices that include the selection of host organism, metabolic pathways, and enzyme origins. These steps are followed by system optimization to improve the metabolic process for the particular production condition of interest. The choice of host organisms can be either native producers or user-friendly but non-native hosts. The advantage of native producers include higher production efficiency, at least initially, and higher tolerance of product toxicity. However, many native producers are not readily amenable to genetic engineering, and their physiological regulations are either poorly understood or not easily tractable. Therefore, non-native but well characterized hosts such as *Escherichia coli* and *S. cerevisiae* may offer advantages for long-term success, provided that other shortcomings can be overcome. With the help of genetic tools, biosynthetic pathways can be transferred from one organism to the host of choice. The availability of genome sequencing and bioinformatic tools has greatly accelerated the discovery of candidate genes and pathways [7]. And the development of evolutionary techniques has enabled the rapid alteration and improvement of enzyme activities [8]. Finally, optimization of whole cell performance is carried out using a combination of biochemical, genetic, and modeling techniques.

2.2.3 Performance criteria

A major challenge in biofuel production is the efficiency of the metabolic process. In addition to demonstrating scientific feasibility, the titer, yield, and productivity of the process need to be considered as the performance criteria for biofuel production. These quantities are somewhat related but not necessarily interdependent and present different challenges in research and

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development. Therefore, when considering the performance of a process, all three criteria need to be evaluated. Product titer is the concentration (e.g. g/L) of product accumulated in the bioreactor. It is perhaps the first performance index of interest in the early stage of research. In the industrial process, it determines the cost of product recovery. Yield (g product/g substrate) is defined as the amount of product produced per unit of substrate consumed. This quantity is perhaps the most important performance index for industrial scale production, since it directly determines the raw material cost, which is a dominating factor in biofuel production. For a given metabolic pathway, the theoretical maximum yield can be calculated based on the stoichiometry. The practical yield, however, depends on the physiology and the regulation of the whole cell. Productivity (e.g. g/L/hr) refers to the rate of production per unit volume of reactor. It determines the cost of operation. Occasionally, productivity per cell (rate of production per cell mass) has been used to judge the performance of cells in the research stage.

2.2.4 Cell growth versus product formation

It has been well recognized that product formation and cell growth are not necessarily correlated. According to mass balance, when cell growth increases, the product yield decreases, when the same amount of materials is consumed. Thus, the ideal process minimizes the percentage of the cell mass formation from the substrate while maximizing the percentage of product formation. One way to achieve this goal is to grow the cells before induction of product formation pathways. If the product formation pathways can be kept active for a long period of time without cell growth, the product yield can be increased. This type of operation is fed-batch in nature. Given the separation of product formation and growth phases, optimization of process with respect to growth does not necessarily lead to increased production, and tolerance to product toxicity

evaluated based on cell growth is not necessarily informative. More sophisticated considerations are in order.

2.3. Metabolic networks for fuel production

2.3.1 Ethanol

Ethanol production by fermentation has a long history dating back several thousand years. The natural pathways for ethanol production from sugars in *S. cerevisiae* and *Z. mobilis* have led to yields exceeding 95% of theoretical maximum, which is 0.51 g of ethanol per g of glucose. With such an efficient metabolic process, further improvement mainly resides in broadening the substrate range, enhancing resistance to product toxicity, and increasing robustness in various process conditions.

Natural hosts *S. cerevisiae* and *Z. mobilis* lack the ability to ferment pentoses, which are significant hydrolysis products of lignocellulosic biomass. To tackle this problem, one possibility is to introduce pentose metabolizing pathways into ethanologenic hosts *S. cerevisiae* [9-11] and *Z. mobilis* [12]. On the other hand, one can express the ethanologenic pathways into *E. coli*, whose broad range of carbohydrate metabolizing capacity makes it a top candidate for biocatalyst engineering [13]. Thus, the homoethanologenic pathway from *Z. mobilis* has been packed into a portable cassette (PET operon) and integrated into the *E. coli* chromosome at the *pfl* locus, while the *frd* gene was deleted to eliminate succinate production and thus prevent carbon loss. The resulting recombinant strain KO11 was capable of producing ethanol at a yield as high as 95% in a complex medium [13, 14]. In addition, biomass-derived feedstock such as rice hulls or sugar cane bagasses have been tested for fermentation, and yields above 95% of theoretical yield were achieved [13]. However, unlike natural ethanol producers, *E. coli* has a

much lower inherent ethanol tolerance. To address this issue, metabolic evolution was used to generate better ethanol tolerance strains [15]. More recently, to meet the need of lignocellulosic ethanol production, a strain with higher tolerance to toxic side products (e.g. furfural) generated in the acid hydrolysis of hemicellulose has also been isolated [16]. Interestingly, although current work has been focusing on introducing heterologous pathways to combine both pentose utilization and ethanologenesis traits in one biocatalyst organism, ethanol fermentation in *E. coli* has been achieved without foreign gene expression. This strain has been reported to harbor mutations in the pyruvate dehydrogenase (PDH) operon that result in a mutant PDH that functions in anaerobic conditions and thus allows the balanced production of ethanol at a yield of 82% from glucose or xylose under anaerobic condition [17]. This discovery demonstrated the surprisingly high malleability of natural pathways, as well as the potential power of evolutionary methods to generate novel metabolic network not existing in nature [13].

2.3.2 Isopropanol and 1-butanol production by the CoA-dependent pathway

Both isopropanol and 1-butanol have long been known to be produced in various strains of *Clostridium* via the Coenzyme A (CoA)-dependent acetone–butanol–ethanol (ABE) fermentation pathway (Figure 2.1) [18-20]. The recent call for longer chain alcohols as renewable fuels has rekindled the enthusiasm to investigate and optimize these natural hosts. For example, the hyper-amylolytic and hyper-butanologenic strain *Clostridium beijerinckii* BA101 was isolated after chemical mutagenesis and selection and can produce total solvent titers as high as 33 g/L [21-23]. Furthermore, global transcriptomic studies of physiological regulation in *Clostridium acetobutylicum* have provided significant guidance into additional strain improvement [24], such as widening the solvent production window by manipulating the sporulation program [23, 24].

Besides upstream optimization of the *Clostridium* host, improvement of downstream fermentation techniques, such as utilization of a fibrous bed bioreactor that immobilizes cells during continuous production [25], represents another avenue towards economically competitive production. More recently, these natural pathways have also been transplanted into user friendly hosts for further engineering. For example, acetone production pathways from *C. acetobutylicum* were introduced into *E. coli* with the combination of a secondary alcohol dehydrogenase (SADH) to convert acetone to isopropanol. This led to a maximum production of 4.9 g/L isopropanol, which out-competed that of its natural host [26]. Similarly, 1-butanol production pathways from *Clostridium* species have also been imported into *E. coli* [27] and yeast [28] resulting in the production of 550 mg/L and 2.5 mg/L of 1-butanol, respectively, which are much lower than those produced by *Clostridia*. While these results demonstrated scientific feasibility, technical difficulty in engineering these platform hosts for the synthesis of higher alcohols remains.
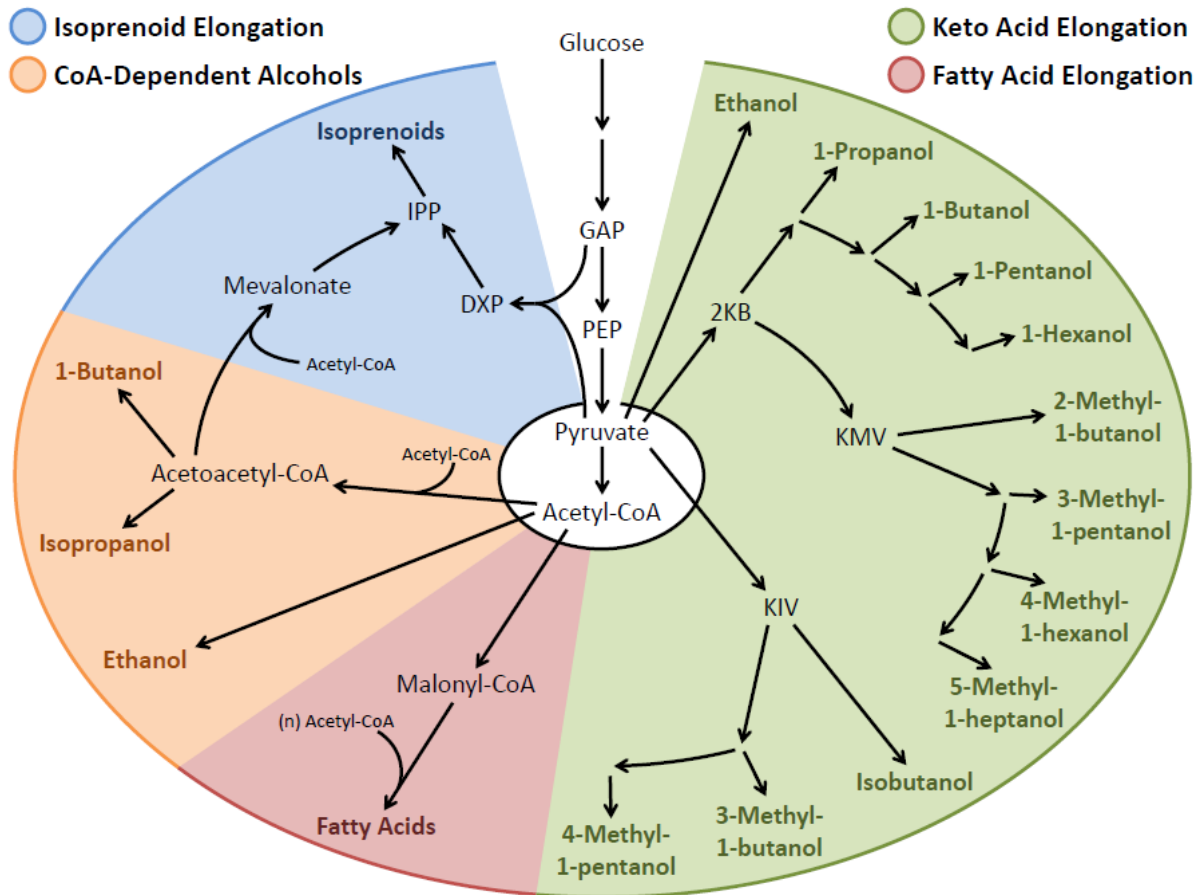
Figure 2.1 Schematic of biosynthetic pathways used in the production of biofuels. Metabolic pathways currently being explored for biofuel production can be grouped into four categories: 1) Natural alcohol production pathways, which reside in natural hosts to produce ethanol, isopropanol, and 1-butanol (orange). 2) Fatty acid elongation pathways for production of fatty acids which can then be converted to biodiesels (red). 3) Isoprenoid elongation pathways for the production of isoprenoid-derived hydrocarbon fuels (blue). 4) Keto acid elongation pathways, which elongate the simple keto acid, pyruvate, to form keto acids with chain length ranging from 4-9 for the production of higher alcohols with 3-8 carbons (green). GAP – Glyceraldehyde-3-phosphate. PEP– Phosphoenol pyruvate. AceCoA – Acetyl Coenzyme A. 2KB – 2-Ketobutyrate. KMV – 2-Keto-3-methylvalerate. KIV – 2-Ketoisovalerate. DXP – 1-Deoxy-D-xylulose-5-phosphate. IPP – Isopentenyl diphosphate.

2.3.3 Keto acid chain elongation pathways

As discussed above, although ethanol represents the predominant portion of biofuels produced currently, it suffers from non-ideal physicochemical properties as fuel (Table 2.1). Higher alcohols with more favorable fuel properties such as 1-butanol can only be produced naturally in some *Clostridium* species [29]. Recombinant organisms [26, 30] expressing the Coenzyme A (CoA)-dependent pathways only achieved relatively low titers. This CoA-dependent pathway is an extension of the oxidative decarboxylation of pyruvate to produce acetyl-CoA, which is also a precursor to ethanol (Fig. 2.2).
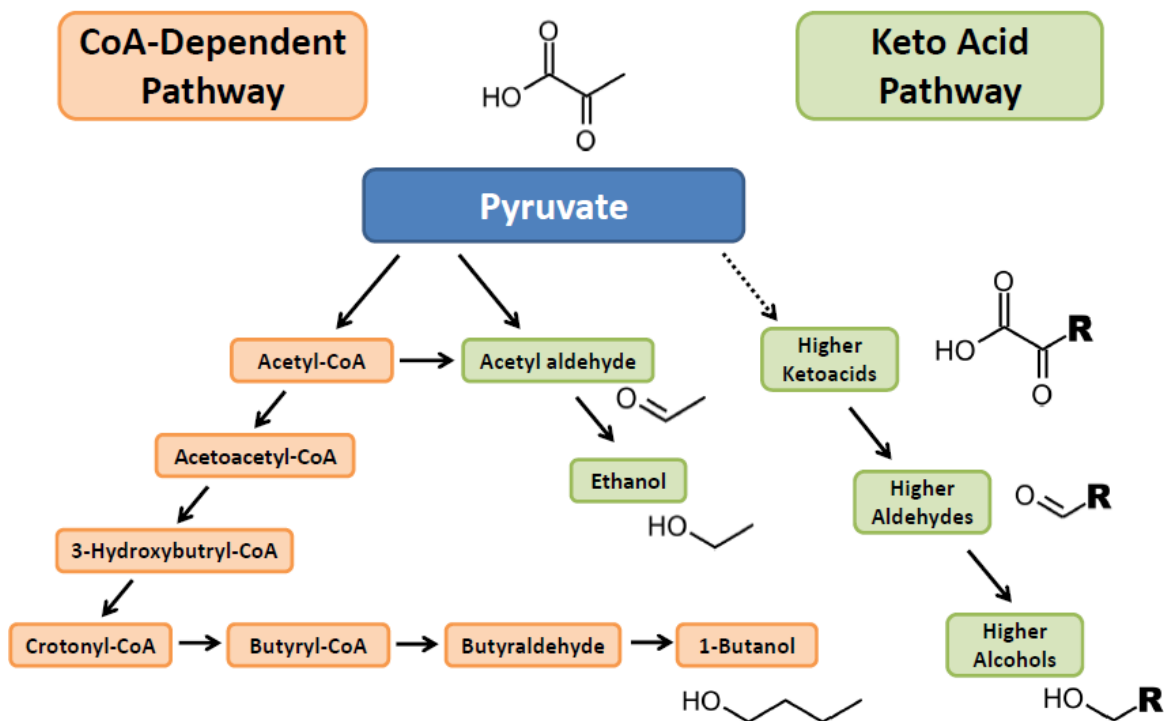
Figure 2.2: Pathway diversity for alcohol production from pyruvate. Higher alcohols such as 1-butanol can be produced in natural hosts by a series of the Coenzyme A-dependent pathway from pyruvate. Alternatively, the highly efficient ethanol producing pathway in natural producers *S. cerevisiae* and *Z. mobilis* proceeds via decarboxylation of pyruvate (a keto acid) to form acetyl aldehyde, which is then reduced to ethanol by an alcohol dehydrogenase. Based on the comparison above, if higher keto acids with longer chains can be synthesized, decarboxylated, and reduced, higher alcohol may be produced efficiently.

However, this CoA-dependent pathway for ethanol production does not produce ethanol efficiently. On the other hand, the natural ethanol producers *S. cerevisiae* and *Z. mobilis* use a non-oxidative decarboxylation pathway that is independent of CoA (Fig. 2.2). This pathway starts by decarboxylation of pyruvate (a keto acid) to form acetyl aldehyde, which is then reduced to ethanol by an alcohol dehydrogenase. The above comparison implies that the keto acid decarboxylation pathway is a more efficient route for alcohol production.

This observation suggests that if a long-chain keto acid can be synthesized and decarboxylated and reduced, the corresponding long-chain alcohol may be produced efficiently. Such pathways represent an extension of the efficient ethanol production pathway via non-oxidative decarboxylation of pyruvate. Luckily, nature provides metabolic engineers with toolkits for keto acid chain elongation and decarboxylation.

In amino acid biosynthesis, two types of keto acid chain elongation are involved (Fig. 2.3): the 2-isopropylmalate synthase (IPMS or LeuA) chain elongation which adds one net carbon while retaining branching number, and the acetohydroxy acid synthase (AHAS) chain elongation which increases the carbon number by two with a branch in the main chain. In these two pathways acetyl-CoA and pyruvate, respectively, are utilized as elongation units. Reducing power is also applied to convert the added carbonyl carbon into an alkane carbon so that the functional nature of the carbon chain is reset after each cycle of elongation. Moreover, thanks to the promiscuity and potential evolvability of the key enzymes catalyzing the carbon condensation [31], these two types of chain elongation modules can be applied repetitively in tandem or hybrid fashion to generate a broad panel of 2-ketoacids with different carbon number and structures.
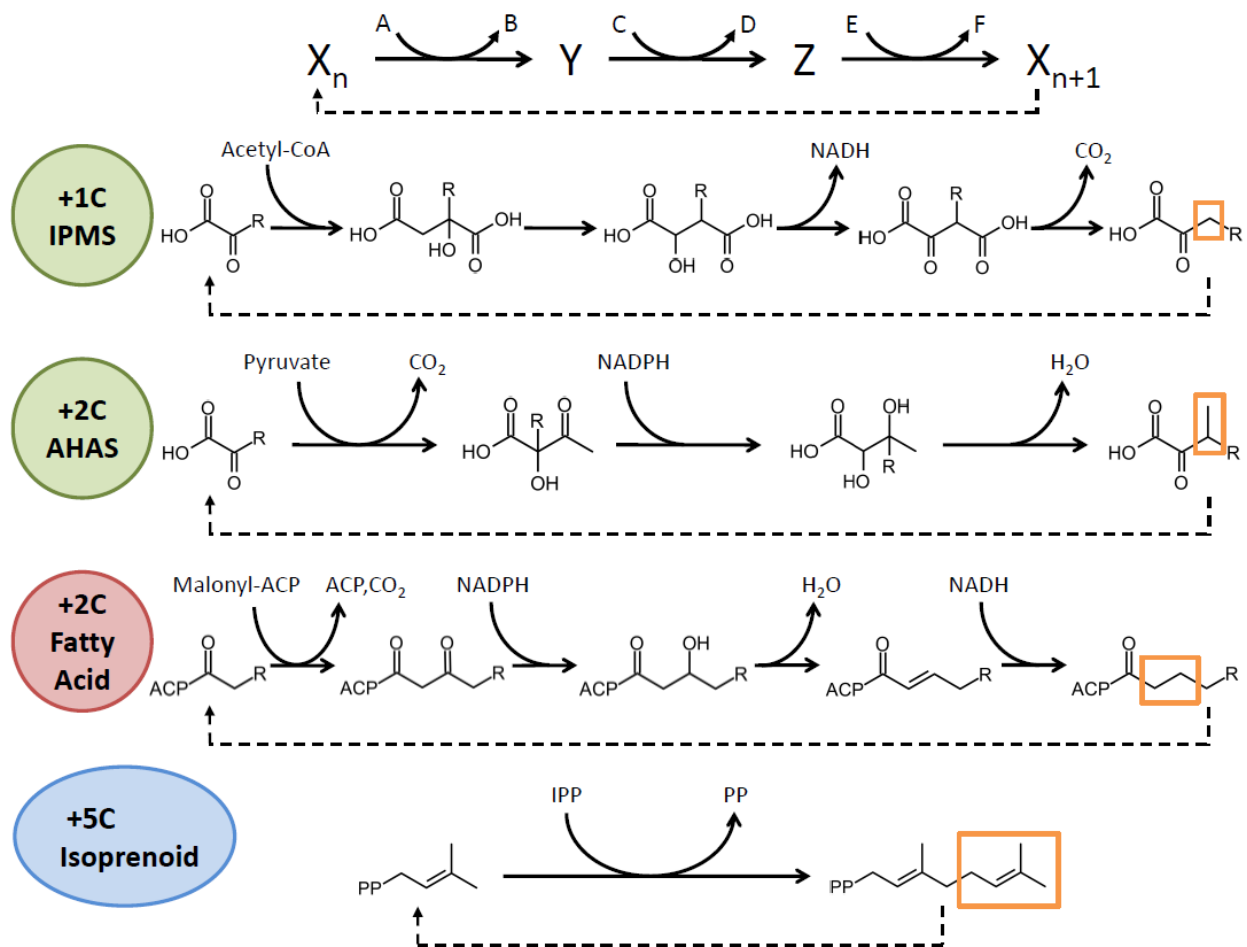
Figure 2.3: Biosynthetic pathways for carbon chain elongation. Four chain elongation pathways are currently used to compose desirable hydrocarbon chain for biofuel molecules: 1) IPMS chain elongation, which elongates a keto acid carbon chain by 1 carbon in each cycle. 2) AHAS chain elongation, which increases carbon number of a keto acid by 2 and generates a branch point. 3) Fatty acid elongation, which adds two carbons linearly to a fatty acyl-ACP. 4) Isoprenoid elongation, which adds IPP monomers to elongate the carbon chain by 5 in each cycle. The larger subunits can also be used as monomers for additions of 10 carbons, 15, etc. IPMS – Isopropylmalate synthase (leucine biosynthesis). AHAS – Aceto-hydroxy acid synthase (valine/isoleucine synthesis). IPP – Isopentenyl diphosphate. ACP –acyl carrier protein.

Under the keto acid elongation scheme, pyruvate (a 3-carbon keto acid), which is a common central metabolite, can be converted to 2-ketoisovalerate (a 5-carbon keto acid) via the AHAS chain elongation (Figure 2.3). 2-Ketoisovalerate is the precursor for valine and leucine

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biosynthesis, and this pathway is used in almost all microorganisms. Once the 5-carbon keto acid is formed, it can be decarboxylated by a keto acid decarboxylase (KDC), such as KIVD from *Lactococcus lactis* [32, 33]. This enzyme is a homologue of pyruvate decarboxylase, but was found to have a larger active site cavity to accommodate larger substrates [31]. The decarboxylation of a keto acid generates an aldehyde, which can be reduced to the corresponding alcohol by various alcohol dehydrogenases (ADHs) such as Adh2 in *S. cerevisiae* [33, 34], AdhA in *L. lactis* [35], and YqhD in *E. coli* [35]. Thus, 2-ketoisovalerate produced from the AHAS elongation pathway is converted to isobutanol [33]. This reaction scheme is very efficient and produces more than 20 g/L of isobutanol from glucose with a yield reaching 85% of the theoretical maximum [33]. Note that the final concentration far exceeded the toxicity level that inhibits cell growth, and the cells continued to produce isobutanol in the non-growing phase for a long period of time. This is an example of separation between cell growth and product formation resulting in high-yield production of isobutanol.

2-Ketoisovalerate produced from the AHAS elongation pathway can be further elongated via the IPMS elongation pathway to produce 2-keto-4-methylvalerate (a 6-carbon keto acid) (Figure 2.4). This compound can then be decarboxylated by a KDC and reduced by an ADH to produce 3-methyl-1-butanol [33, 36].
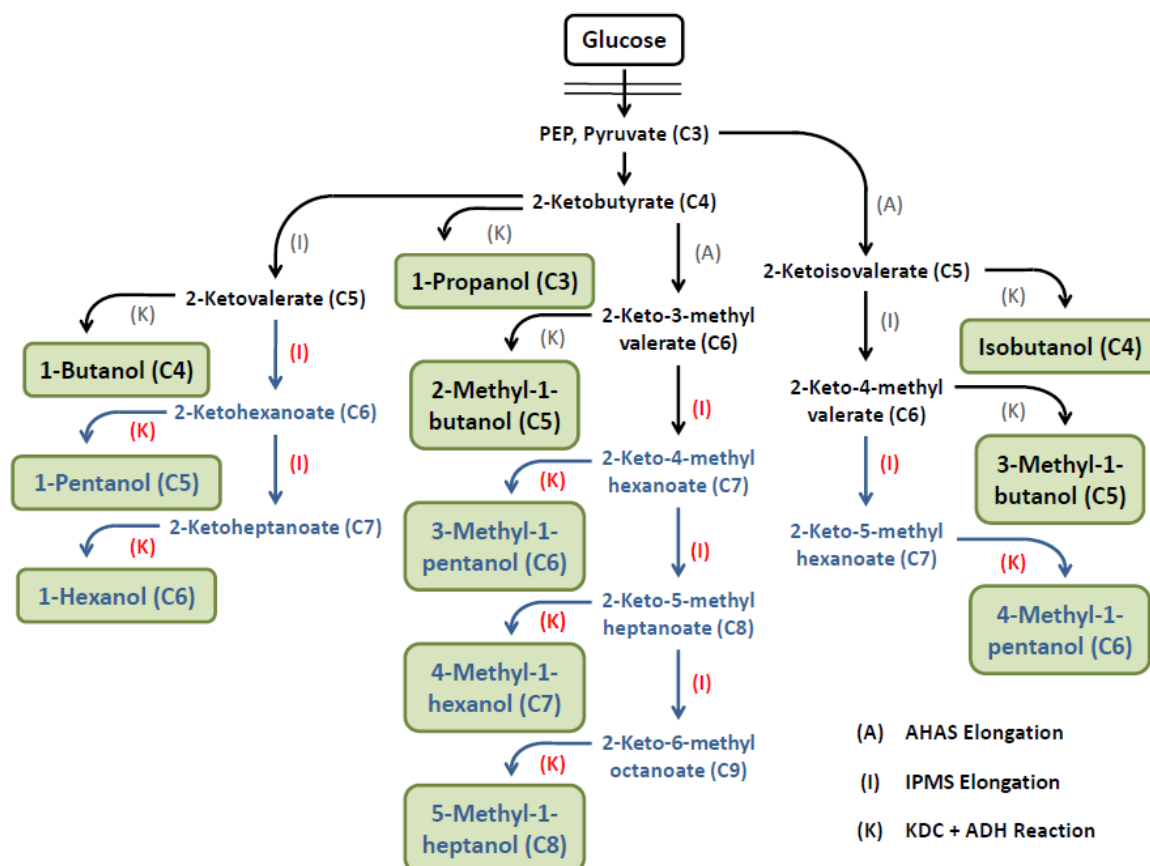
Figure 2.4: Non-natural synthetic pathway for longer chain alcohol production. Natural keto acids such as 2-ketoisovalerate (valine) and 2-keto-3-methylvalerate (isoleucine) are produced by AHAS elongation (A) from pyruvate and 2-ketobutyrate, respectively. Some natural keto acids such as 2-ketovalerate (norvaline), and 2-keto-4-methylvalerate (leucine), as well as 2-keto-3-methylvalerate, can be elongated by the engineered IPMS elongation pathway (I), which are then decarboxylated by the redesigned KDC and reduced by ADH (K) to become non-natural C5–C8 alcohols. All non-natural reactions are represented with blue arrows and red letters.

2.3.4 Fatty acid chain elongation pathways

A significant amount of energy captured by photosynthesis is stored in the form of lipids in plants and algae [37]. The mechanisms for fatty acid biosynthesis have been well documented in biochemistry textbooks. The universal fatty acid biosynthetic pathway starts with the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, which is then charged with the acyl carrier protein (ACP) and serves as the repetitive unit to be added in fatty acid chain elongation through decarboxylative condensation (Figure 2.3). Within each cycle of chain

elongation, two carbons in the form of an acetyl group are added to the growing fatty acyl-ACP carbon chain, followed by sequential input of reducing equivalents to remove the oxygen and saturate the carbon bond. This restores the original acyl-carbon chain structure, which is then poised for the next round of elongation. While development in molecular biology and metabolic engineering in oil plants and algae have revealed potential feasibility to enhance fatty acid yield as well as tailor this fatty acid chain elongation pathway [38-40], much work has been done recently in industry-friendly microorganisms to optimize fatty acid chain elongation towards production of carbon chains of specific length [41-43]. For example, to increase fatty acid synthesis in microorganisms, the enzyme catalyzing the first committed step of fatty acid chain elongation, acetyl-CoA carboxylase (ACC), was overexpressed and fatty acid degradation genes (e.g. *fadD* in *E. coli*) were deleted [41]. To alter final elongation products from predominantly 16-18 carbon chains found in most organisms to shorter carbon chains for higher quality fuel production [41], a thioesterase from plants has been heterologously expressed in *E. coli*. Since this enzyme releases fatty acids from ACP to terminate elongation, thus determining the carbon chain length, 12-14 carbon fatty acids were able to be produced in significant quantities [41-43]. While the direct products of fatty acid biosynthesis have a high energy density, they make very poor fuels. Therefore modifications are needed to transform those long chain acids into liquid transportation fuels. The predominant portion of fatty acids in living organisms is esterified with glycerol, other polyols, and fatty alcohols. Current biodiesel production utilizes chemical transesterification reactions between triacylglycerols from oil plant and algal feedstock and short chain alcohols to form fatty acid methyl esters (FAME) and fatty acid ethyl esters (FAEE) [44]. Intracellular fatty acid esterification for FAEE production has been demonstrated in *E. coli* by coupling ethanol production pathways from *Z. mobilis* with the broad substrate range wax ester

synthase/acyl-CoA–diacylglycerol acyltransferase (WS/DGAT) from *Acinetobacter baylyi* [45].

Similarly, production of FAEEs and fatty acid isoamyl esters (FAIEs) has also been achieved in

recombinant *S. cerevisiae* [46]. In addition to esterification, other naturally occurring

mechanisms for fatty acid chain reduction and/or defunctionalization are under investigation,

such as fatty alcohol formation [47] and decarboxylative alkane formation [48], though

corresponding pathways have yet to be applied to biofuel production. Thanks to the

exponentially increasing genomic sequencing data and ever-advancing DNA recombination

techonologies, more diverse and efficient fatty acid conversion systems may be discovered to

provide knowledge and raw materials for future engineering purposes.

2.3.5 Isoprenoid chain elongation pathways

Isoprenoids are another category of hydrocarbons synthesized in a broad range of organisms and

are used as pigments, antioxidants, and organic solvents [49]. The products, intermediates, and

derivatives from isoprenoid biosynthetic pathways are also attractive targets as nutraceuticals

and pharmaceuticals [20], as well as for jet fuel alternatives and gasoline additives [37, 50].

Regardless of their structural diversity, all isoprenoids are synthesized through a universal chain

elongation pathway shared by numerous species. The isomers isopentenyl diphosphate (IPP) and

dimethylallyl diphosphate (DMAPP) are the basic units to be added consecutively to the growing

carbon chain coupled by the release of a diphosphate (Figure 2.3). Thus, five carbons are added

in each cycle of chain elongation. IPP or DMAPP can be synthesized either through the

mevalonate pathway or 1-deoxy-D-xylulose-5-phosphate (DXP) pathway. The former uses

acetyl-CoA as the starting metabolite, while the latter starts from glyceraldehyde-3-phosphate

(GAP) and pyruvate. In contrast to the fatty acid chain elongation and the keto acid chain

elongation, no CO_2 is released and no reducing equivalents are input after carbon condensation. As a result, double bonds in the building blocks are preserved thus causing the periodic occurrence of carbon double bonds all throughout the hydrocarbon chain. Due to the potential of isoprenoid compounds to deliver commercially attractive chemicals, much work has been published on engineering isoprenoid biosynthetic pathways, especially in microorganisms [51-57]. One major strategy is to enhance carbon flux for building block supply by manipulating DXP pathways in *E. coli*. Specifically, genes *dxs* and *idi*, encoding the 1-deoxy-D-xylulose-5-phosphate synthase and IPP isomerase, respectively, were overexpressed to enhance carbon flux for IPP supply [51-54, 58-61]. Moreover, both combinatorial and rational methods have been used to fine-tune gene expression within this pathway as well as to discover and engineer its regulatory mechanisms [55-57, 61]. For example, in a recent study 24 endogenous genes shown to affect lycopene production in recombinant *E. coli* harboring the *crtEBI* operon were subjected to a recombination-based combinatorial genome engineering approach in search of the optimal expression pattern. The mutant that delivered the highest lycopene yield of 9000 ppm had four genes (*dxs, idi, dxr, rpoS*) overexpressed and one gene (*ytjc*) knocked out simultaneously [61]. On the other hand, mevalonate pathways from yeast have also been transplanted into *E. coli*, providing an efficient precursor supplying platform for further engineering of downstream pathways [58, 59]. Similarly, combinatorial approaches have also been used to manipulate activity of different genes (such as *atoB, HMGS, HMGR*) in these pathways by controlling their post-translational processing [60].

In addition, to determine the chain length in isoprenoid synthesis pathways, isoprenyl pyrophosphate synthases (IPPSs) that yield final products of different lengths have been identified and the mechanisms underlying chain length determination have been studied [62].

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This provided knowledge and materials for protein engineers to tailor isoprenoid synthesis pathways. For example, by introducing and engineering geranyl-geranyl diphosphate (GGPP) synthase from *Archaeoglobus fulgidus*, the production of desired 20-carbon product GGPP was enhanced [51, 52].

Isoprenoid biosynthesis pathways generate a large family of branched and cyclic hydrocarbons that may possess the properties favorable for jet fuels and diesel fuels [37, 44, 50]. Although most efforts in isoprenoid biosynthesis engineering has been targeting nutraceuticals and pharmaceuticals (such as lycopene) as final products, the IPP/DMAPP overproducing systems and elongation pathways mentioned above may be readily adaptable for potential biofuel production. For instance, isopentenol, a proposed gasoline additive or substitute, was produced by overexpressing *nudF* from *B. subtilis* in combination with the mevalonate pathway in *E. coli* and reached a titer of 112 mg/L [63].

2.4 Cell as a system

2.4.1 Strain optimization

The above pathways explored for production of next generation biofuels including higher alcohols, fatty acid derivatives, and isoprenoid derivatives, can all be divided into two parts: chain elongation and functional group modification. The first part synthesizes molecules of the desired chain length. These keto acids, fatty acids, or isoprenoids are then turned into fuel-quality molecules such as alcohols, esters, and alkanes. Nonetheless, construction of desired biosynthesis pathways is only the first step towards economically viable biofuel production. Increasing the titer, yield and productivity to the economically viable level is a major challenge, and will ultimately determine the feasibility of each approach.

Once the target pathway is selected, maximum theoretical yield can be calculated based on the stoichiometry of the pathway (Table 2.2). This is done by either simple hand calculation or using linear optimization to maximize the yield. In such calculations, assumptions such as inter-conversion between NADH and NADPH and the existence of recycling pathways will affect the result. Since the ideal production occurs after cell growth, maximum theoretical yield calculation assumes no growth during production phase. However, the maximum theoretical yields are difficult to achieve in practice due to several reasons: 1) other endogenous pathways may compete with the target pathway for carbon metabolites, cofactors, or energy [50]; 2) the desired pathway may cause imbalance of cofactors such as NADH and NADPH [5, 20]; 3) final products or intermediates generated by the target pathway may disturb host metabolism in a specific or non-specific way [5]. In order to effectively incorporate synthetic pathways into host metabolic networks, as well as optimize host physiology for efficient production, both rational and combinatorial approaches have been applied in many different schemes [5].

Rational strategies take advantage of accumulated knowledge on genetic and biochemical metabolic regulations and propose focused and usually small-scale alterations to existing systems. Competing pathways are readily identifiable by inspecting the pathways or stoichiometric models, and a remedy is usually straightforward. Similarly, possibilities of $NADH/NAD^+$ or $NADPH/NADP^+$ limitations can be suggested relatively easily by inspecting the pathways or by stoichiometric modeling. However, proof or remedy of this problem is non-trivial. In general, various mathematical modeling techniques may help to identify potential targets for gene knockouts [64-67] and enzyme overexpression [68-71].

Table 2.2 Energy yield of various fuels

Fuel	Mass Energy Density (MJ/kg)	Volumetric Energy Density (MJ/L)	Max Biochemical Yield (g/g)[a]	Pathway Stoichiometric Yield (g/g)	Energy Yield from Glucose (%)[b]
Gasoline	42.7	32.0	-	-	-
Jet Fuel	43.8	34.8	-	-	-
Diesel	45.5	38.7	-	-	-
Ethanol	29.7	20.8	0.511	0.511	97.6
Propanol	33.6	27.0	0.444	0.444	95.9
1-Butanol	36.1	29.2	0.411	0.411	95.4
Isobutanol	36.1	29.0	0.411	0.411	95.4
1-Pentanol	37.7	30.8	0.391	0.326	79.0
3-Methylbutanol	37.7	30.5	0.391	0.326	79.0
2-Methylbutanol	37.7	30.5	0.391	0.391	94.8
Fatty Acids (C12-C22)	37 - 41	33 - 35	0.35 - 0.39	0.34 - 0.37	89 - 90
Isoprene - Mev[c]	43.8	29.8	0.324	0.252	71.0
Isoprene - DXP[d]	43.8	29.8	0.324	0.300	84.5

a Theoretical yield based on best available cell metabolic pathways
b Based on an energy density for glucose of 15.6 MJ/kg, using pathway yield
c Using Mevalonate pathway for isoprenoids
d Using 1-Deoxy-D-xylulose-5-phosphate pathway for isoprenoids

Some illustrative examples of rational metabolic engineering involve alleviation of a

reduction/oxidation (redox) imbalance or cofactor imbalance caused by heterologous pathway

importing. For instance, in order to enable the most efficient ethanologenic yeast *S. cerevisiae* to utilize xylose for bioethanol fermentation, xylose reductase (XR) and xylitol dehydrogenase (XDH) genes from *Pichia stipitis* were over-expressed [9]. However, this two-enzyme pathway was not redox balanced by itself due to NADPH dependence of XR and NAD^+-dependence of XDH, causing a significant accumulation of the intermediate xylitol [72-74]. To transform this pathway into a closed redox loop, protein engineering has been carried out to switch coenzyme specificity of either one of the two enzymes and resulted in significant yield improvement [8, 72, 73, 75]. To solve similar problems from a different angle, some host genes from outside the production pathways such as the ones encoding malate dehyrogenase, formate dehydrogenase, and pyridine nucleotide transhydrogenase have also been manipulated to compensate cofactor need for product synthesis [76-78].

By contrast, combinatorial strategies are especially suitable to solve metabolic engineering problems where little information is known, such as product toxicity [5]. Almost all biofuel products have solvent-like properties and may be harmful for microbial membranes. Although traditional mutagenesis and stress adaptation methods have enjoyed significant success in developing desirable phenotypes including product tolerance[5, 15, 79, 80], mechanisms involved in those traits may be complicated and elusive. In addition, classic mutagenesis methods such as chemical or UV treatment introduce multiple mutations which are not readily identifiable traditionally and may contain both beneficial and detrimental mutations. However, thanks to the development of rapid genome sequencing technologies, mutations can now be detected and their individual phenotypes can also be potentially characterized and transferred.

In contrast to traditional mutagenesis, transcription machinery [81-84] and transcription factors [85] can be targeted for mutagenesis to alter global transcriptome profiles and thus the phenotype.

In combination with high-throughput screening methods, these combinatorial approaches have enjoyed promising success to improve complex cellular phenotypes such as glucose and ethanol tolerance in yeast [81], butanol tolerance [84], heat-shock resistance [85], and production yield of chemicals in isoprenoid biosynthesis pathways in *E. coli* [60, 61, 86].

2.4.2 Synthetic host

Besides optimizing naturally existing host and pathways, the possibility has been proposed to create production microorganisms from scratch with a more defined metabolism based on the concept that standardized and interchangeable gene network modules can be assembled into a larger system with predictable behavior [85, 87, 88]. To advance toward this ultimate goal, advanced molecular tools are required. In particular, DNA assembly technologies capable of integrating large DNA fragments have enabled quick assembly of complex pathways and, more strikingly, a whole genome of a microorganism [89-91]. Regulatable protein expression platforms and devices have also been developed to sustain the proper functioning of heterologous proteins (especially those from organisms living in extreme environments) [8, 50, 92], as well as to fine tune expression levels of multiple pathway components simultaneously to achieve global metabolic optimization [57, 60, 93].

2.5 CO_2 as a feedstock

To achieve sustainability, the energy must ultimately come from the Sun, and the carbon skeletons of the liquid fuels must be derived from CO_2. In general, solar energy harvesting and CO_2 reduction can be accomplished using either man-made devices or biological systems (Fig 2.5). Each has its pros and cons. Solar energy harvesting using man-made devices has achieved

reasonably high efficiency (10-45%), but the energy is output in the form of electricity, whose storage remains problematic for transportation applications. Also, CO_2 reduction to liquid fuels by man-made systems is heretofore inefficient and non-specific. On the other hand, biological systems have utilized photosynthesis to capture solar energy and reduce CO_2 to biomass for millions of years. The typical photosynthesis efficiency from solar to biomass energy is less than 1% for plants, although the opportunity for improvement is enormous thanks to the advances in genomic and molecular biology tools. In particular, biological reduction of CO_2 to make long-chain reduced carbon compounds is effective and specific for CO_2 re-utilization. With metabolic engineering tools, biological systems can be tailored to make compounds of interest with specific structures and conformations. This capability cannot be easily achieved using non-biological methods.

Typically, biological fuel production processes depend on plant biomass. In particular, gasoline and diesel substitutes have been produced using engineered microbial catalysts from biomass-derived substrates, including simple sugars [31, 33, 94-98], cellulose [99, 100], and minimally treated biomass [101]. However, the harvesting, processing, and decomposing of recalcitrant biomass are still challenging [102].
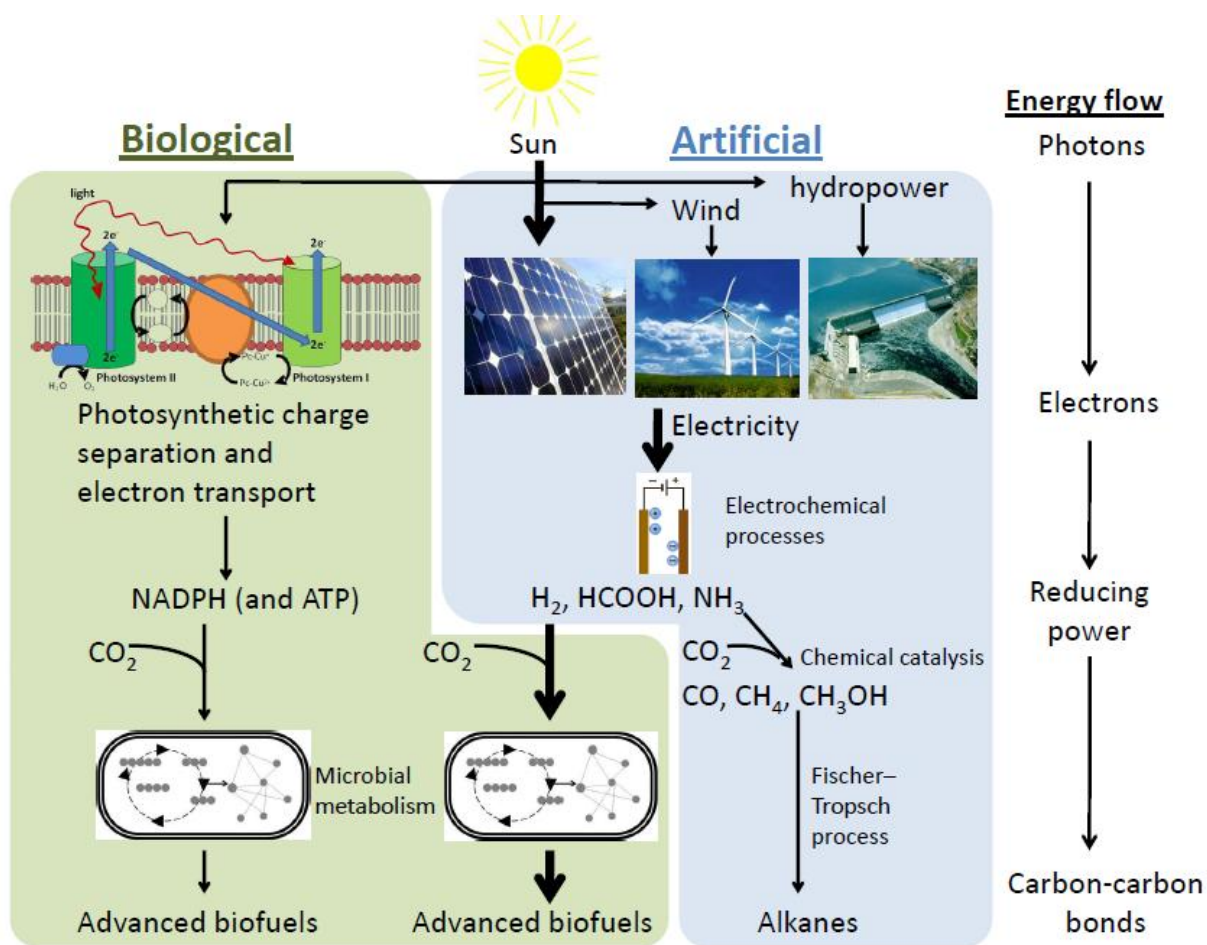
Figure 2.5 General schemes of solar energy harvesting and carbon fixation by biological and artificial approaches. Man-made devices are relatively efficient for solar energy harvesting and reducing power generation; while biological metabolic pathways are more versatile and specific for synthesis of carbon-based fuels and chemicals from carbon dioxide. A hybrid process of artificial "light reaction" and biological "dark reaction" is desirable.

The capability of biological systems to catalyze fuel production from biomass can be extended to CO_2. Thus, instead of plants, CO_2-fixing microorganisms can be metabolically engineered to produce liquid fuels directly, bypassing the lignocellulose processing issues. In this scenario, CO_2 fixation does not strictly depend on light. The CO_2-fixing "dark reactions" in autotrophic microorganisms can be powered by energy and reducing equivalence derived from a variety of

sources in addition to solar energy. However, the energy needed for CO_2 fixation must ultimately come from the Sun. The conversion of solar energy to drive biological CO_2 fixation and fuel production can again be accomplished using two approaches (Fig. 2.5): biological or non-biological. In the former case, the cell utilizes the complete system of photosynthesis, including both the light and dark reactions, and directly produces liquid fuel. In the latter approach, a man-made device is used to harvest sunlight in the form of electricity, which then powers the biological production of fuel from CO_2. Such processes have been dubbed "electrofuel" production. Either approach does not compete with food crops for farmland and bypasses the recalcitrance problem of lignocellulose, while fully capitalizing on the biological capability to synthesize liquid fuels with high specificity and efficiency.

2.6 Electrofuel: Separation of light and dark reactions

Although suggested to be scalable, these biofuel production processes using photosynthetic microorganisms still need to overcome substantial challenges to be economically viable. First, the efficiency of biological photosystems is constrained by the nature of the biological molecules used, which can only utilize radiation within a limited spectrum. As a consequence, about 50% of solar energy cannot be used [103]. Second, the biological photosystems have not been optimized for the maximal energy conversion when light is abundant. Therefore the efficiency of solar energy capture is also limited by the saturation effect and other complex cellular regulations.[103, 104] In addition, large-scale culturing of photosynthetic organisms represents a new bio-production paradigm that requires large two-dimensional (2D) light exposing surface areas, since sufficient light exposure is only available within a layer of around 20 centimeters from the surface. Compared to the conventional three-dimensional (3D) microbial fermentation

processes, which have been used in industrial scale for centuries, the 2D process still requires substantial breakthroughs to be economically competitive. For example, the open-pond culture faces the difficulties in nutrient delivery, product collection, culture maintenance, and water lost [105]. Alternatively, closed photo bioreactors may be needed to maximize sunlight utilization and cell growth,[106-108] particularly for genetically modified organisms. However, the cost is inevitably increased.

On the other hand, the traditional 3D microbial bioreactors that can hold large amount of cultures in the bulk of the bioreactor are relatively inexpensive, but cannot distribute light to the bulk. One way to circumvent the need for photo-bioreactors is to separate the "light" and "dark" reactions of the photosynthesis process, such that the light reactions can be substituted by man-made photovoltaic solar panels or wind turbines. Both devices generate intermittent electricity, which faces a major problem in storage. If the intermittent electricity can be utilized to drive the "dark reactions" in a bulk bioreactor, electricity storage problem can be solved and the need for 2D photo-bioreactor can be avoided. In addition, this approach could have better sun-to-fuel efficiency. For example, plants growing at the current average growth rate of 1kg biomass dry weight m^{-2} $year^{-1}$ in the United States capture only about 0.28% of the incident solar energy [109], whereas man-made solar cells collect energy from sunlight and generate electricity with relatively high efficiencies ranging from 10 to 40%[110, 111]. The high sunlight harvesting efficiencies will greatly reduce the land usage [109]. Furthermore, it expands the boundaries of biofuels by exploring the vast repertoire of lithoautotrophic microorganisms which can fix CO_2 in the dark and have diverse metabolic and physiological features. For example, while the photosynthetic plants, cyanobacteria, and algae used for traditional biofuel production utilize Calve-Benson-Bassham (CBB) cycle exclusively for CO_2 fixation, a number of other CO_2

fixation pathways, such as the Wood-Ljungdahl pathway, exist in lithoautotrophic microorganisms which have higher energy efficiencies [112].

The Electrofuel production method can also serve as a mean for electricity storage. The current method of electricity storage via batteries suffers from the low energy density, which generally ranges between 0.1-0.7MJ/kg (or 0.5-2.0 MJ/L) [113]. In contrast, the energy density of gasoline is around 45MJ/kg. Given the limited on board space in the vehicles, the low energy density of batteries greatly hampered their usage in transportation sector. Although major innovations in lithium-ion battery technology have been made recently [114-117], energy densities of five times greater are required for the future all-electric vehicles to have a 300–400 mile driving range [117]. To match the performance of internal combustion engines in global scale, batteries with orders of magnitude higher energy densities may be necessary. Alternatively, electrolytic water splitting can store electrical energy in chemical bonds in H_2 molecules with efficiencies higher than 50%. However, the volumetric energy density of H_2 is low (5.6 MJ/L at 700 bar) and H_2 utilization in the transportation sector remains difficult. The development of electrofuel provides a promising approach for storing intermittent electricity, such as solar and wind power-generated electricity, in the form of liquid fuel that can be used for transportation directly.

2.7 Reference

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3. Synthesis of isobutanol and n-butanol using engineered Coenzyme A-dependent pathways in *Ralstonia eutropha* H16

3. 1 Introduction

As discussed in chapter 2, Coenzyme A (CoA) thioesters are often used for the forming, breaking, and rearranging of carbon-carbon bonds. As a result, a variety of CoA-dependent pathways have been explored for the bioproduction of fuels and chemicals with vastly diverse carbon skeletons [1] [2] [3] [4] [5] [6] [7]. Analysis of the genome sequence of the bacterium *Ralstonia eutropha* H16 suggested that this organism may possess a highly versatile CoA-dependent metabolic network, which may include new tools in CoA-dependent chemistry for biotechnology application. For example, an extremely broad range of organic acids can be CoA-acylated by *R. eutropha* H16 and incorporated in polyhydroxyalkanoates (PHAs). Consistently, over 30 isologs of the β-ketothiolase, a key enzyme in PHA synthesis, have been predicted in the genome with potentially different substrate spectra [8, 9]. These β-ketothiolases are useful catalysts for carbon-carbon bond formation by Claisen condensation. *R. eutropha* has also been studied as a model organism for biodegradation of organic compounds in the environment [9]. It has been shown to degrade a variety of aromatic compounds using CoA-dependent mechanisms [10]. Recent enzymology and bioinformatic studies on *R. eutropha* H16 continue to identify CoA-dependent enzymes with novel chemistry, which possibly function in uncommon organic compound degradation [11, 12]. *R. eutropha* H16 can utilize many different types of biomass-derived feedstocks including fructose, organic acids, glycerol [13], and plant oil [14]. It can also fix CO_2 using non-photosynthetic energy source such as H_2 or formate, which makes it a desirable electrofuel production host. With these metabolic features, *R. eutropha* serves as a

good host organism to device new CoA-dependent pathways for the production of desired chemicals from renewable sources.

3.2 Methods and materials

3.2.1 Bacterium strain, medium, and production condition

Ralstonia eutropha H16 strain was purchased from American Type Culture Collection (ATCC). *R. eutropha* strains were regularly cultured in rich medium (16g/L nutrient broth, 10g/L Yeast extract, 5g/L $(NH_4)SO_4$) at 30 ℃. If the strains contain plasmids, 200mg/L kanamycin was added. For biofuel production from fructose, strains were cultured in German minimal medium [15] with 10g/L fructose in rubber-capped test tubes at 30 ℃. Culture samples were taken daily to assay for biofuel levels by gas chromatography. For biofuel production from formate, German minimal medium [15] was used with 20mM sodium formate initially. 20% formic acid was continuously added to the production medium according to the pH changes caused by bacterial formate consumption. Air was bubbled throught the medium for aeration and for constant removal of alcohol product. Evaporated alcohols in venting gas were condensed with a Graham condenser and collected. Daily, samples of culture broth and condensation liquid were taken and alcohols were quantified using gas chromatography. Introduction of the plasmid into *R. eutropha* cells was performed using previously reported electroporation method [16].

3.2.2 Plasmid construction

All cloning and plasmid preparation were done using *E. coli* XL1-blue cells (Stratagene, La Jolla, CA). LB medium was used to culture *E. coli.* Detailed information about plasmids and primers used in this chapter can be found in Table 3.1 and Table 3.2.

Table 3.1 strains and plasmids

Strain	Comments	Reference
Ralstonia eutropha H16	wild type strain	ATCC
LH201	*R. eutropha* H16 transformed with pLH201, Kan[R]	This study
LH202	*R. eutropha* H16 transformed with pLH 202, Kan[R]	This study
LH204	*R. eutropha* H16 transformed with pLH 204, Kan[R]	This study
LH205	*R. eutropha* H16 transformed with pLH 205, Kan[R]	This study
LH206	*R. eutropha* H16 transformed with pLH 206, Kan[R]	This study

Plasmid	Comments	Reference
pBHR1	broad host-range multiple copy plasmid	MoBiTec, Göttingen, Germany
pLH 201	pBHR1 with P_{cat}::*phaJ-ter-Bldh-yqhD*, for n-butanol production	This study
pLH 202	pBHR1 with P_{cat}::*phaJ-phaA-phaB-terOP-bldh-yqhD*, for n-butanol production	This study
CAT-lacZ	pBHR1 with P_{cat}::*lacZ*, for promoter activity assay	This study
pepck-lacZ	pBHR1 with P_{pepck}::*lacZ*, for promoter activity assay	This study
rrsC-lacZ	pBHR1 with P_{rrsC}::*lacZ*, for promoter activity assay	This study
pdh-lacZ	pBHR1 with P_{pdh}::*lacZ*, for promoter activity assay	This study
phaC1-lacZ	pBHR1 with P_{phaC1}::*lacZ*, for promoter activity assay	This study
pLH 204	pBHR1 with P_{pdh}::*phaJ-phaA-phaB-terOP-bldh-yqhD*, for n-butanol production	This study
pLH 205	pBHR1 with P_{phaC1}::*phaJ-phaA-phaB-terOP-bldh-yqhD*, for n-butanol production	This study
pLH 206	pBHR1 with P_{phaC1}::*phaJ-sbm1-phaA-phaB-terOP-bldh-yqhD*, for n-butanol and isobutanol production	This study

To construct pLH 201, the isothermal DNA assembly method [17] was used. Primers YCAT ad up Rev/YCAT ad down fwd were used to amplify the vector backbone using pBHR1 as the template. Primers YCAT ad up_phaJ fwd/phaJ_ter rev were used to amplify the *phaJ* (*Aeromonas caviae*) gene from pEL70 [18]. Primers phaJ_ter fwd/ter_bldh rev were used to amplify *ter* (*Treponema denticola*) gene from pEL30 [19]. *Bldh* (*Clostridium saccharoperbutylacetonicum* N1-4) and *YqhD* (*Escherichi coli*) genes were synthesized by DNA2.0 (Menlo Park, CA, USA) with codon optimized for *R. eutropha* H16. The synthesized genes *Bldh* and *yqhD* were then used as templates for amplification using primer pairs ter_bldh fwd/ bldh_yqhD rev, and bldh_yqhD fwd/ yqhD_YCAT ad down rev, respectively. All the

fragments from amplification including the vector, *phaJ*, *ter*, *bldh*, and *yqhD*, were then assembled.

pLH202 is constructed in a similar fashion. The genes *phaA* and *phaB1* are next to each other in *R. eutropha* H16 genome. They were amplified as one fragment using the primer pair phaJ_phaAB fwd/ phaAB_terOP rev. The *terOP* was synthesized by DNA2.0 with codon optimized for *R. eutropha* H16, which was amplified using primers phaAB_terOP fwd/ terOP_bldh rev. The *bldh* and *yqhD* were amplified together as one fragment from pLH201 using primers terOP_bldh fwd/yqhD_YCAT ad down rev. These fragments were then assembled together to form pLH202.

The plasmids with a panel of promoters driving the reporter gene *lacZ* were constructed as follows: All the plasmids used a vector backbone amplified from pBHR1 using primers YCAT ad up rev/YCAT ad down fwd. The promoter P_{cat} was amplified from pBHR1 using primers Pcat fwd/Pcat rev. Promoter fragments of P_{pepck}, P_{rrsC}, P_{pdh}, and P_{phaC1} were amplified from *R. eutropha* H16 genomic DNA using primers Ppepck fwd/Ppepck rev, PrrsC fwd/PrrsC rev, Ppdh fwd/Ppdh rev, and PphaC1 fwd/PphaC1 rev, respectively. The β-galactosidase reporter gene *lacZ* was amplified using lacZ fwd/lacZ rev. The vector backbone and the *lacZ* fragment were assembled with each promoter fragment, individually. The resulted plasmids have *promoter::lacZ* cassette inserted in pBHR1 in the opposite direction of the original *CAT* cassette. The constructs were designed so that the same ribosomal binding site (RBS) of the sequence 5'-AGGAG-3' was used for every promoter tested.

To construct pLH204 and pLH205, a restriction digestion method was used to switch the promoter of pLH202. The P_{pdh} and P_{phaC1} promoters were amplified using primers vector_Ppdh

fwd/Ppdh Acc65I rev, and vector_PphaC1 fwd/PphaC1 Acc65I rev, respectively. Part of the pBHR1 was amplified using primers vector XhoI fwd/vector rev. The vector fragment was linked with each promoter fragment by splicing by overlap extension (SOE) PCR, respectively. The resulted fragments were inserted between XhoI/Acc65I sites of pLH202.

To construct pLH206, a two-step restriction digestion method was used. First, sbm1 gene was amplified from *R. eutropha* H16 genomic DNA using primers sbm1 KpnI SacI fwd/sbm1 SbfI rev. The product was inserted into KpnI/SbfI sites of pLH202, which has the *phaJ* gene replaced by *sbm1*. Next, the *phaJ* gene was added back by inserting into the KpnI/SacI sites (The SacI site was introduced in the last step by including it in the primer.). The fragment for insertion was amplified using primers YCAT ad up_phaJ fwd/phaJ SacI rev.

The sequences of primers are shown in Table 3.2.

Table 3.2 Primers and sequences

Primer name	Sequence
YCAT ad up Rev	ggtacctttctcctctttaatgaattcttattccggatgagcattcatca
YCAT ad down fwd	tctagaccatgggcaaatattatacgcaaggcgacaaggtgctgatgccg
YCAT ad up_phaJ fwd	gaattcattaaagaggagaaaggtaccatgtctgcgcaatctctcgaagt
phaJ_ter fwd	gttgttaagctgccttaaaggagaaacctaggatgggcagcagccatcac
phaJ_ter rev	gtgatggctgctgcccatcctaggtttctcctttaaggcagcttaacaac
ter_bldh fwd	aggttcgacaggatttaaaggagtaacttaagatgatcaaggacaccctg
ter_bldh rev	cagggtgtccttgatcatcttaagttactcctttaaatcctgtcgaacct
bldh_yqhD fwd	tgcgtcctggccgggtgaaggagataacgcgtatgaacaatttcaatctg
bldh_yqhD rev	cagattgaaattgttcatacgcgttatctccttcacccggccaggacgca
yqhD_YCAT ad down rev	ccttgcgtataatatttgcccatggtctagatcagcgggctgcctcgtag
phaJ_phaAB fwd	gcgaagcggttgttaagctgccttaacgcttgcatgagtgccggcgtgcg
phaAB_terOP rev	ccatcggcttcacgatcatcctaggtttctccttcagcccatatgcaggccg
phaAB_terOP fwd	cggcctgcatatgggctgaaggagaaacctaggatgatcgtgaagccgatgg
terOP_bldh rev	cagggtgtccttgatcatcttaagttactccttcagatccggtcgaagcg
terOP_bldh fwd	cgcttcgaccggatctgaaggagtaacttaagatgatcaaggacaccctg
Pcat fwd	gcccatggcatggctctagagaataaatacctgtgacggaagatc
Pcat rev	ccgtaatcatggtcatggtacctttctcctttttagcttccttagctcctg
Ppepck fwd	gcccatggcatggctctagagtctgacaggcgtgcggccagctggcccac
Ppepck rev	ccgtaatcatggtcatggtacctttctcctactgcagactccagattcgt
PrrsC fwd	gcccatggcatggctctagattcaactgctctgcttggcattcgacgtct
PrrsC rev	ccgtaatcatggtcatggtacctttctccttgcagagctttcttcgcgag
Pphd fwd	gcccatggcatggctctagagcgcggtgcggccacggggggcgccgtcgtc
Pphd rev	ccgtaatcatggtcatggtacctttctcctgactgtctcctgggtgaatt
PphaC1 fwd	gcccatggcatggctctagaccgggcaagtaccttgccgacatctatgcg
PphaC1 rev	ccgtaatcatggtcatggtacctttctcctgatttgattgtctctctgcc
lacZ fwd	aggagaaaggtaccatgaccatgattacggattcac
lacZ rev	agaattcattaaagaggagaaaggtaccttattttgacaccagaccaac
vector_Ppdh fwd	ctaagaaaccattattatcatgagcgcggtgcggccacggggggcgc
Ppdh Acc65I rev	agacatggtacctttctcctgactgtctcctgggtgaattc
vector_PphaC1 fwd	ctaagaaaccattattatcatgaccgggcaagtaccttgccgacatc
PphaC1 Acc65I rev	agacatggtacctttctcctgatttgattgtctctctgccgtc
Vector XhoI fwd	gtcttgctcgaggccgcgattaaattccaac
Vector rev	tcatgataataatggtttcttagacgtcc
sbm1 KpnI SacI fwd	gagaaaggtaccaccgcattgagctcatgaccgacctttccgatgtgcat
sbm1 SbfI rev	agggaacctgcaggctacatattgcgccggtactgcccgcccac
phaJ SacI rev	ggtcatgagctctgactcctttaaggcagcttaacaaccgcttcgccgg

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3.2.3 Enzyme assays

R. eutropha crude cell extract for phaA, phaB, and TER assays was prepared by Qiagen Tissuelyser using lysis buffer (50mM Tris-HCl pH=7.5, DTT 1mM). The enzyme activities were measured using previsouly reported method [20]. Briefly, phaA activity was monitored by decrease OD_{303nm} which corresponds to the disappearance of the substrate acetoacetyl-CoA. PhaB activity was monitored by decrease in OD_{340nm} which corresponds to the disappearance of the substrate NADPH. And TER activity was monitored by decrease in OD_{340nm} which corresponds to the disappearance of the substrate NADH.

The β-galactosidase reporter assays were performed using previously reported method [16].

The *R. eutropha* crude cell extract for Bldh assay was prepared anaerobically in sealed vials by Qiagen Tissuelyser II. The lysis buffer contained 50mM potassium phosphate buffer pH=7.5, 10mM 2-Mercaptoethanol, and 1mM $MgSO_4$. The cell extract was kept on ice in anaerobic environment at all times. The activity was measured in the oxidation direction. The assay system contains 50mM Tris-HCl pH=9.0, 10mM DTT, 2mM $NADP^+$, 0.2mM CoA, and 10mM butyraldehyde or isobutyraldehyde. Activity was monitored by the increase of OD_{340nm}. CoA was omitted to assay the endogenous non CoA-acylating aldehyde dehydrogenase activity, which convert aldehyde to acid independent of CoA. CoA-acylating activity of Bldh was calculated by subtracting activity level without CoA addition from the activity level with CoA.

The *R. eutropha* crude cell extract for Bldh assay was prepared by Qiagen Tissuelyser II with the buffer A containing 50mM potassium phosphate pH=7.4, 50mM KCl, 10mM $MgSO_4$, and 50μM Vitamin B12. Reaction was performed in 200μl volume with buffer A, appropriate amount of crude cell extract, and 1mM of butyryl-CoA. Reaction was incubated at 30°C. 100μl 2N KOH

was used to stop the reaction followed by addition of 100ul 15% H_2SO_4. Next, 500mg of NaCl

was added to saturate the solution and then 250μl Ethyl acetate was used to extract the product.

The samples were then assayed by gas chromatography.

3.3 Results and discussion

3.3.1 Designing of the pathway
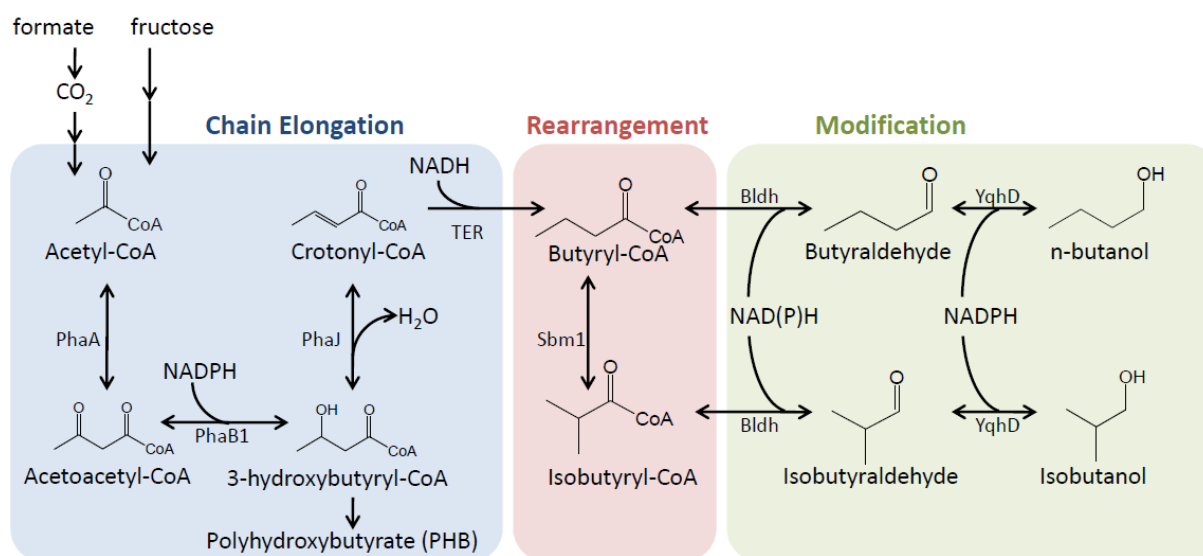
Figure 3.1 Pathway design for n-butanol and isobutanol biosynthesis using CoA-dependent pathway in *Ralstonia eutropha*. The pathway for n-butanol biosynthesis is composed of a chain elongation phase and a CoA group modification phase. If an acyl-CoA rearrangement phase is added, the pathway can be adapted to produce isobutanol.

CoA-dependent pathway for production of isobutanol, a gasoline substitute, has not been realized

experimentally before (Figure 3.1). The pathway shares the carbon chain elongation steps with

the n-butanol production pathway: a β-ketothiolase (encoded by *phaA* in *R. eutropha*) and a

NADPH-dependent acetoacetyl-CoA reductase (encoded by *phaB1* in *R. eutropha*) convert two

acetyl-CoA into 3-hydroxybutyryl-CoA, which is also the building block of the

polyhydroxybutyrate (PHB) (Figure 3.1). Further dehydration and reduction convert the 3-hydroxybutyryl-CoA into butyryl-CoA via Crotonyl-CoA. Through these reactions, the CoA-dependent carbon chain elongation chemistry linearly adds two carbons at a time to the carbon chain (Figure 3.1). The high level of PHB accumulation up to 90% of cell dry weight [21] suggests that the metabolic network in this organism can supply a large amount of acetyl-CoA, which makes this pathway an attractive choice for biofuel production. After the elongation, the acyl-CoA compound can be reduced to form n-butanol (Figure 3.1). If the carbon chain elongation reiterates for more cycles, C6, C8, and C10 n-alcohols can be produced in a similar manner [2-4]. However, only straight chain products have been accessible so far.

Previously it has been suggested that if an acyl-CoA isomerization step can be added to rearrange the carbon chain, branched chain products could also be produced [22, 23] (Figure 3.1). In this study, we took advantage of *R. eutropha*'s large CoA chemistry tool kit and explored its native acyl-CoA isomerization enzyme. Recently, a novel class of isobutyryl-CoA mutase has been identified and characterized in *Geobacillus kaustophilus*, *Nocardia farcinica*, and *Burkholderia xenovorans* [12]. Based on protein homology analysis, *R. eutropha* has also been predicted to have the isobutyryl-CoA mutase enzyme [12], although it was previsously misannotated as methylmalonyl-CoA mutase and has not been experimentally characterized. In this chapter, the CoA-dependent n-butanol production was first achieved in *R. eutropha* by overexpressing both native and heterologous genes. Next, the n-butanol production pathway was optimized by altering the promoter strength. Finally, the predicted *R. eutropha* isobutyryl-CoA mutase was added to achieve isobutanol production.

3.3.2 CoA-dependent n-butanol production in *R. eutropha*

We first focused on effectively introduction of the CoA-dependent n-butanol production pathway into *R. eutropha*. The pathway naturally exists in *Clostridium* species and has been heterologously introduced into various organisms such as *Escherichi coli* [1, 20] and cyanobacteria [18, 19] for biofuel production. Based on these studies, we first attempt to overexpress the key enzymes in the pathway (Figure 3.1, 3.2A). As mentioned above, *R. eutropha* naturally synthesizes (*R*)-stereoisomer of the 3-hydroxybutyryl-CoA as an intermediate in PHB synthesis [24]. To channel the carbon from the PHB biosynthetic pathway, a previously characterized (*R*)-specific enoyl-CoA hydratase (encoded by *PhaJ* in *Aeromonas caviae*) [25] was chosen (Figure 3.2A). After dehydration, the recently identified NADH-dependent trans-2-Enoyl-CoA Reductase (TER) from *Treponema denticola* was used to reduce the double bond thanks to its favorable catalytic features for driving the carbon chain extension [20, 26, 27]. The subsequent reduction of butyryl-CoA can be carried out by a CoA-acylating aldehyde dehydrogenase (encoded by *bldh* from *Clostridium saccharoperbutylacetonicum* N1-4) and a broad-substrate range alcohol dehydrogenase (encoded by *yqhD* from *E.coli*) [18]. These four genes formed a synthetic operon in the multiple-copy plasmid, which was transformed into *R. eutropha* H16 to create strain LH201 (Figure 3.2A). LH201 produced ~30mg/L n-butanol in minimal medium with fructose as the sole carbon source (Figure 3.2B), which suggested that the proposed pathway is functional *in vivo*. To improve the pathway flux, the first two steps of the pathway which relied on the endogenous activity of the PHB biosynthesis pathway in LH201 were strengthened by overexpression the *R. eutropha phaA* and *phaB1* genes. In addition, since the TER activity was relatively low in LH201 (Figure 3.2E), codon optimization was performed for *T. denticola Ter* and the codon optimized *ter* (*terOP*) was added to the synthetic operon.

These two changes resulted in strain LH202 (Figure 3.2A). Compared with LH201, LH202 has enhanced activities for phaA, phaB1, and TER (Figure 3.2C, D, E) based on enzyme assays using crude cell extract. LH202 also produced around ~2.5 fold higher n-butanol (~80ml/L) from fructose (Figure 3.2B).
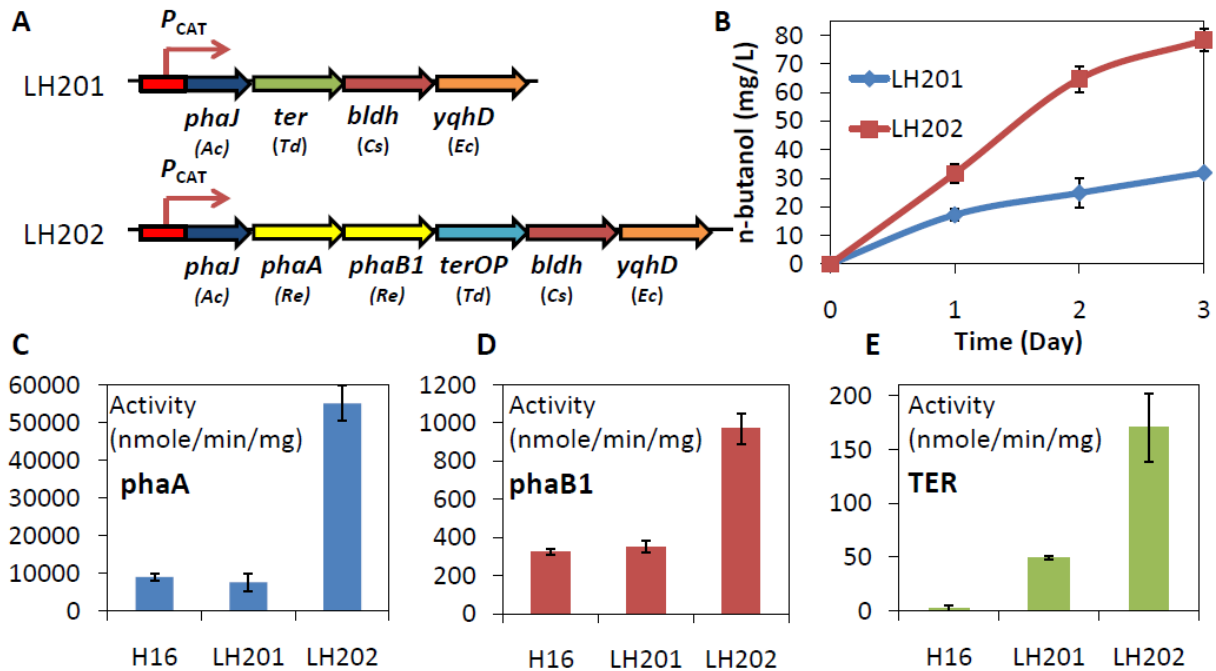
Figure 3.2 Construction of the n-butanol pathway in *Ralstonia eutropha*. A) Synthetic operons for n-butanol production in stain LH201 and LH202. *Ac, Aeromonas caviae , Td, Treponema denticola*, *Cs, Clostridium saccharoperbutylacetonicum* N1-4 , *Ec, Escherichi coli* , *Re, Ralstonia eutropha* H16. *terOP, ter* gene codon optimized for R. eutropha. B) n-butanol production by LH201 and LH202 in minimal medium with fructose as the sole carbon source. C), D), E) β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase, and NADH-dependent trans-2-Enoyl-CoA Reductase (TER) activities , repectively, in wild type H16, LH201, and LH202 strains as measured using enzyme assays with crude cell extract. Error bars represent standard deviation of 3 replicate experiments (n=3).

3.3.3 Improvement of n-butanol production by tuning promoter strength

The synthetic operons in strain LH201 and LH202 are driven by a pre-existing promoter, the chloramphenicol acetyl transferase (CAT) promoter, in a multiple-copy plasmid. Though this plasmid and the promoter are currently widely used in *R. eutropha*, it may not provide the suitable strength of gene expression for biofuel production. To this end, we characterized promoters of several *R. eutropha* genes using β-galactosidase reporter assays (Figure 3.3A), including the promoters of *pepck* (encodes the phosphoenolpyruvate carboxykinase), *pdh* (encodes the pyruvate dehydrogenase), *rrsC* (produces the 16S ribosomal RNA), and *phaC1* (encodes the Polyhydroxybutyrate polymerase) [28]. The results showed that the *CAT* promoter in the broad-host range plasmid has low activity in *R. eutropha* compared with all of the native promoters tested. And the activities of *rrsC*, *phaC1*, and *pdh* promoters are relatively high. Thus, the *phd* and *phaC1* promoters were used in place of P_{cat} in LH202 to construct strains LH204 and LH205, respectively. These two strains produced more than two fold n-butanol compared to LH202 (Figure 3.3B), probably because of the enhanced pathway activity. Especially, the best producer LH205 produced ~200mg/L n-butanol in 3 days from fructose. In autotrophic condition using formate as the sole carbon and energy source, LH205 produced ~30mg/L n-butanol in 3 days (Figure 3.3C), which suggested that this strain has the potential to be used in the "electrofuel" production scheme where formate is generated using CO_2 and solar or wind electricity [16].
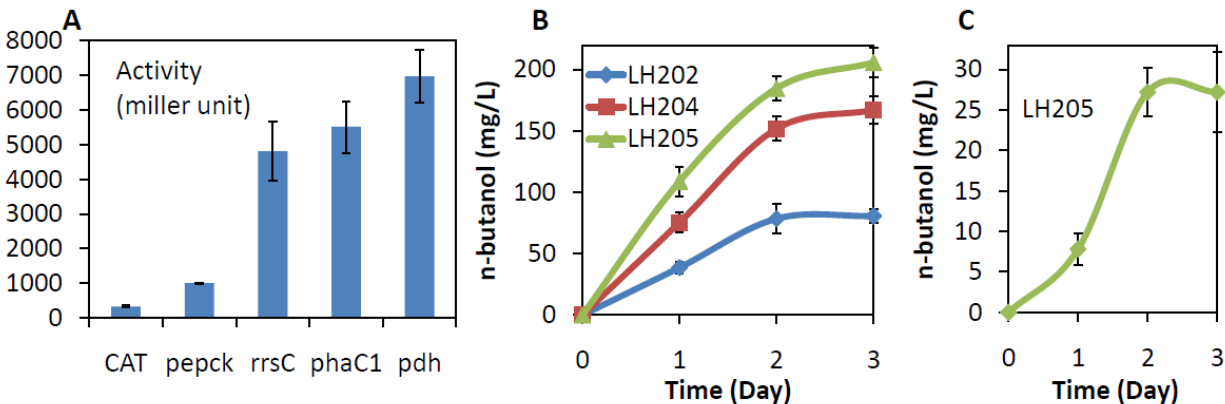
Figure 3.3 Improvement of n-butanol production by altering promoter strength of the synthetic operons and autotrophic n-butanol production from formate. A) Expression strength of the broad host-range promoter P*cat* and several native promoters of *Ralstonia eutropha* H16 as measured by the β-galactosidase reporter assays. *pepck* encodes the phosphoenolpyruvate carboxykinase, *pdh* encodes the pyruvate dehydrogenase, *rrsC* produces the 16S ribosomal RNA, and *phaC1* encodes the Polyhydroxybutyrate polymerase. B) Replacement of the P*cat* in LH202 with P*pdh* in LH204 and P*phaC1* in LH205, repectively, increased the n-butanol production from fructose. C) The strain LH205 produced n-butanol using formate as the sole carbon and energy source. Formate can be produced from CO_2 using electrochemical method. Error bars represent standard deviation of 3 replicate experiments (n=3).

3.3.4 Charaterization of the novel isobutyryl-CoA mutase and CoA-dependent n-butanol production in *R. eutropha*

Next we sought to explore the CoA-dependent pathway for the production of isobutanol. Note that the enzymes which reduce the acyl-CoA to alcohol have to be promiscuous to accommodate the rearranged carbon skeleton (Figure 3.1). The NADPH-dependent alcohol dehydrogenase yqhD from *E.coli* has been previously used to reduce isobutyraldehyde to isobutanol and showed high activity [16, 29, 30]. However, the CoA-acylating aldehyde dehydrogenase has not been tested for branched-chain substrates. We performed enzyme assays using crude cell extract of *R. eutropha* strain that overexpresses *C. saccharoperbutylacetonicum* N1-4 Bldh. The results

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showed that Bldh has comparable activity for the branched C4 substrate isobutyraldehyde as for the linear substrate butyraldehyde (Figure 3.4A).

Finally, the carbon chain rearrangement step was added (Figure 3.1). The *R. eutropha* gene *sbm1* (H16_A0280) has been previously annotated as methylmalonyl-CoA mutase (MCM). However, bioinformatic study has recently suggested that it may instead encode a novel family of isobutyryl-CoA mutase (ICM) which is a fusion protein of the enzyme subunits and the G-protein Chaperone [12]. We overespressed *sbm1* in LH205 to construct strain LH206. The reactivity of the putative ICM, sbm1, was characterized using crude cell extract with butyryl-CoA as the substrate (Figure 3.4B, C, F). The results showed that sbm1 can indeed convert butyryl-CoA to isobutyryl-CoA (Figure 3.4C). The enzyme activity in LH206 was estimated to be ~21.4 mmol/mg/min (Figure 3.4F); whereas LH205 has minimal level of ICM activity (Figure 3.4B), suggesting that the endogenous *sbm1* gene may not be expressed in this condition. After confirming the functionality of sbm1 *in vitro* using enzyme assays, we tested LH206 for biofuel production. The results showed that LH206 produced isobutanol in minimal medium with fructose as the sole carbon source (Figure 3.4D, E). Since the ICM family enzymes have been characterized previously to be vitamin B12 dependent [12], supplementation of the vitamin B12 improved the isobutanol titer produced by LH206 (Figure 3.4G) to ~30mg/L.
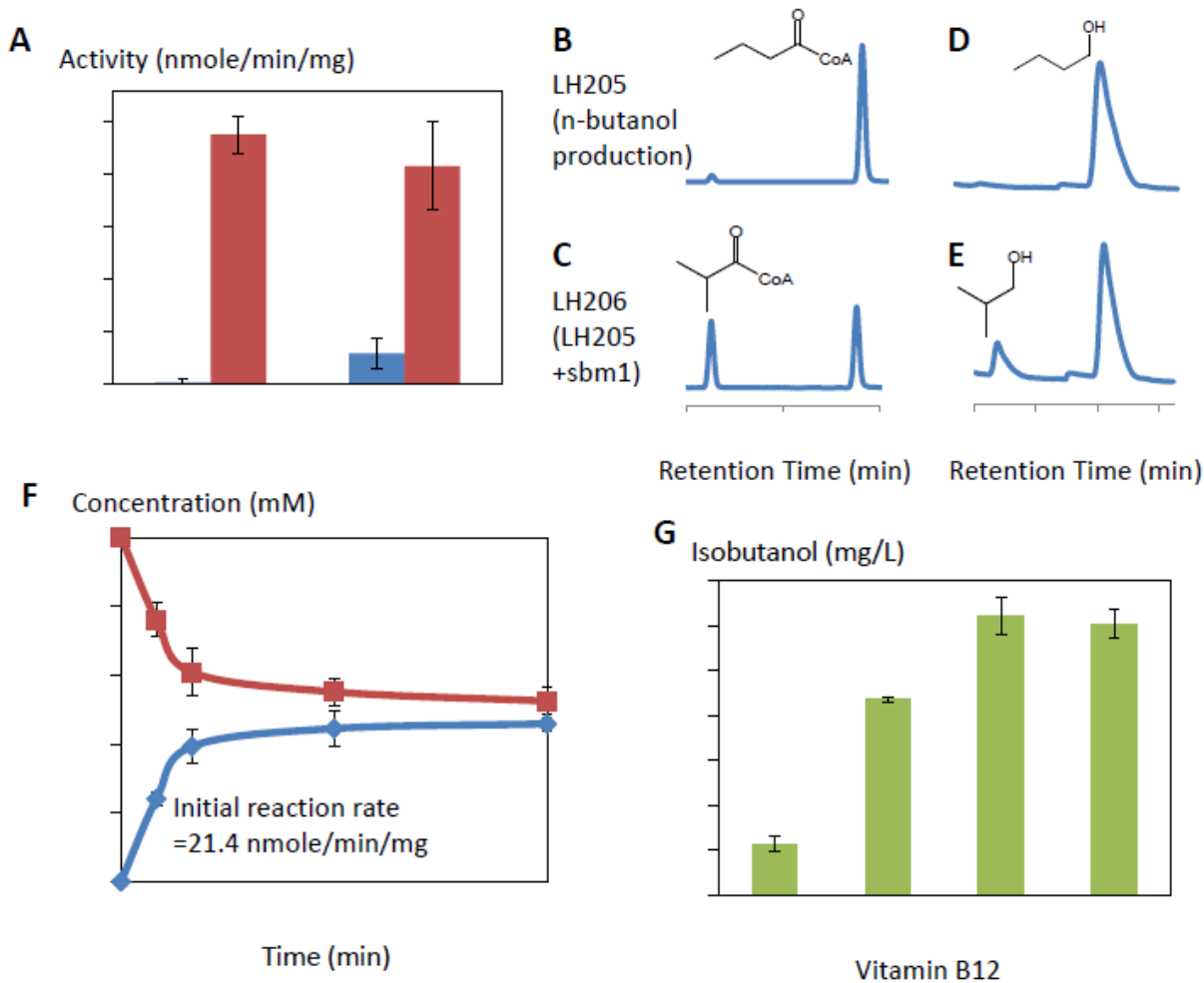
Figure 3.4 Isobutanol production using CoA-dependent pathway. A) CoA-acylating aldehyde dehydrogenase activity for isobutyraldehyde and butyraldehyde assayed using wild type H16 and LH206 crude cell extract. In LH206 the CoA-acylating aldehyde dehydrogenase (encoded by *bldh* from *Clostridium saccharoperbutylacetonicum* N1-4) is overexpressed. B) Typical gas chromatography trace of isobutyryl-CoA mutase enzyme assay end point sample using LH205 crude cell extract with butyryl-CoA as substrate. C) Typical gas chromatography trace of isobutyryl-CoA mutase enzyme assay end point sample using LH206 crude cell extract with butyryl-CoA as substrate. Compared with LH205, which has the CoA-dependent n-butanol production pathway, LH206 has an extra *gene sbm1* from *Ralstonia eutropha* H16 overexpressed. *Sbm1* encodes the isobutyryl-CoA mutase. D) Typical Gas chromatography trace of biofuel production samples of LH205 from fructose, showing the production of n-butanol. E) Typical Gas chromatography trace of biofuel production samples of LH205 from fructose, showing the production of n-butanol and isobutanol. F) Time course of isobutyryl-CoA mutase enzyme assay using LH206 crude cell extract with 1mM butyryl-CoA as substrate. The reaction reached equilibrium in around 60min. The initial reaction rate was calculated using the slope of the first 5min. G) Supplementing vitamin B12 increased the isobutanol titer produced by LH206 from fructose. Error bars represent standard deviation of 3 replicate experiments (n=3).

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3.3.5 Identification of the competing reaction catalyzed by the CoA-independent aldehyde dehydrogenases

We observed consumption of n-butanol by *R. eutropha*, while isobutanol cannot be consumed. Consistently, we observed high endogenous activity in wild type *R. eutropha* which oxidizes butyraldehyde, but not isobutyraldehyde, in the presence of $NADP^+$ (Figure 3.5), suggesting that some native enzymes *R. eutropha* may convert aldehydes to acids. The CoA-acylating aldehyde dehydrogenases oxidize aldehyde to form acyl-CoA, while the non-CoA-acylating aldehyde dehydrogenases convert aldehyde to acid (Figure 3.5A). Both types of enzymes can use $NAD(P)^+$ as electron acceptor, but CoA-acylating enzymes require an additional substrate Coenzyme A. When measuring the aldehyde dehydrogenase activity using crude cell extract of LH206, reactions both with and without CoA added were performed. CoA-acylating activity of Bldh was calculated by subtracting activity level without CoA addition from the activity level with CoA (Figure 3.4A). The non CoA-acylating aldehyde dehydrogenase activity for butyraldehyde and isobutyraldehyde was observed (Figure 3.5B), which might be conferred by unidentified endogenous enzymes. Similar level of non CoA-acylating aldehyde dehydrogenase activity was also seen in wild type cell crude extract. The activity for butyraldehyde is much higher than that for isobutyraldehyde. The non-CoA-acylating aldehyde dehydrogenases enzymes may need to be identified and disrupted to preserve the aldehyde intermediates and block the back consumption of biofuel products.
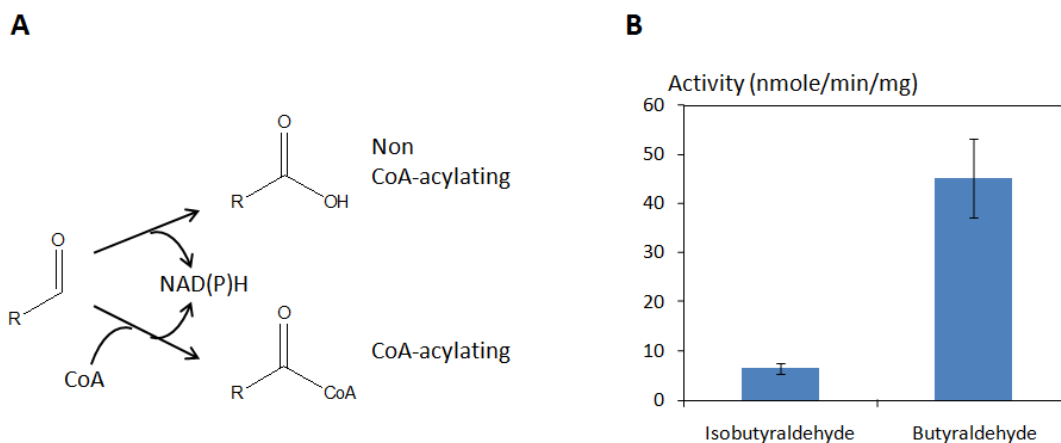
Figure 3.5 CoA-independent aldehyde dehydrogenase activity in LH206

3.4 Conclusions

In conclusion, work in this chapter demonstrated the production of n-butanol and isobutanol using CoA-dependent pathway in *R. eutropha* H16 from renewable source. In particular, the radical-mediated carbon chain rearrangement chemistry is for the first time explored for biotechnology application, which enabled the isobutanol production using CoA-dependent pathway. Both n-butanol and isobutanol were produced from fructose. n-Butanol production was also achieved from formate, which can serve as a feedstock for "electrofuel" production with *R. eutropha* [16]. Both the n-butanol and isobutanol production levels in *R. eutropha* need to be improved. One of the major limitations would be the oxygen-sensitivity of the CoA-acylating aldehyde dehydrogenase from *Clostridium* species. Replacement with this enzyme by oxygen-tolerant counterparts may improve the productivity [31].

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4. Synthesis of isobutanol and 3-methyl-1-butano using engineered keto acid-dependent pathway in *Ralstonia eutropha* H16

4.1 Introduction

The pathway described in chapter 3 resulted in isobutanol production with relatively low titer. To improve the productivity, we sought to explore alternative pathways. As mentioned in chapter 2, keto acid carbon chain elongation, which includes the IPMS elongation and the AHAS elongation, is another way to build the carbon chain besides the CoA-dependent pathways.

In this chapter, production of isobutanol and 3-methyl-1-butanol (3MB) directly from CO_2 with electrolytic H_2, or formate, as the sole energy source in the facultative lithoautotrophic microorganism *R. eutropha* H16 was described, which was achieved using the engineered keto acid chain elongation pathways.

4.2 Method and materials

4.2.1 Construction of the *Ralstonia* higher alcohol production strain LH74D

Escherichia coli XL-1 Blue (Stratagene, La Jolla, CA) was used in all the cloning work. Detailed information about strains and plasmids used in this chapter can be found in Table 4.1.

The genes *alsS* (*Bacillus subtilis*), *ilvC* (*E.coli*), and *ilvD* (*E.coli*) were introduced by chromosomal integration into the *Ralstonia eutropha* H16 genome. The -200bp to +230bp DNA fragments relative to *Ralstonia eutropha* H16 *phaB2* gene start codon and the last 639bp of *phaC2* gene open reading frame were amplified from genomic DNA and assembled with splicing by overlap extension PCR (SOE-PCR) via a linker region containing the SacI restriction site between the two fragments. The SOE product was digested with MluI and XbaI and inserted into

the conjugation vector pNHG1 [1] to form pLH50. The artificial operon containing *alsS*, *ilvC*, and *ilvD* was amplified from plasmid pSA69 [2] and assembled by SOE-PCR with the 836bp *phaC1* promoter region amplified from *R. eutropha* H16 genomic DNA. This fragment was then inserted into the SacI site of pLH50 to form plasmid pLH63. The pLH63 was used to perform conjugation by the reported method [3]. After double-crossover selection on sucrose, the strain with *alsS*, *ilvC*, and *ilvD* overexpression was confirmed by PCR of genomic DNA and enzyme assays using cell lysate.

The PHB biosynthesis genes were knocked out by chromosomal replacement with a chloramphenicol acetyltransferase (CAT) cassette. The -448bp to +146bp DNA fragment relative to *R. eutropha phaC1*gene start codon and 500bp downstream of *phaB1* gene were amplified from genomic DNA. The PCR products were assembled by SOE with the chloramphenicol acetyltransferase (CAT) gene with an added ribosome binding site sequence of AGGAG. The assembly product was inserted into MluI and XbaI sites of pNHG1, resulting plasmid pLH51. The plasmid was then introduced into the above-mentioned *alsS*, *ilvC* and *ilvD* overexpression strain by conjugation. After double-crossover selection, the resulting strain was confirmed by PCR and named LH67.

The genes *kivd* (*Lactococcus lactis*) and *yqhD* (*E. coli*) were introduced by a multiple-copy plasmid. The genes were amplified using genomic DNA of appropriate organisms. The *kivd-yqhD* artificial operon was then made by SOE PCR with the ribosome binding site sequence AGGAG in front of each gene. The operon was assembled with the backbone of the broad-host-range vector pBHR1 (MoBiTec, Göttingen, Germany) using isothermal DNA assembly methods [4] to form plasmid YL22. The *kivd-yqhD* operon was placed between the BspEI and NcoI

restriction sites to disrupt the CAT gene in the plasmid. The promoter of the original CAT gene drives the expression of *kivd-yqhD* operon. The plasmid was then used to transform LH67 strain by electroporation. Briefly, over night culture of *R. eutropha* in rich medium (16g/L nutrient broth, 10g/L Yeast extract, 5g/L $(NH_4)SO_4$) was inoculated into 20ml rich medium and allowed to grow to OD_{600}=0.8 in 30 °C. The cells were harvested by centrifugation, washed twice with ice-cold 0.3M sucrose solution, and then resuspended in 2ml of ice-cold 0.3M sucrose solution. 0.1ml of this resuspended cells were mixed with ~50ng plasmid DNA and electroporated with 11.5kV/cm, 5.0ms, followed by rescuing with 0.2ml rich medium in 30 °C for 2 hours and plated on rich medium plates containing 200mg/l kanamycin. Colonies from the transformation were confirmed by PCR. The strain was named LH74D.

Table 4.1 Plasmids and Strains

Plasmid	Description	Reference or Source
pSA69	*P_LLacO1:alS-ilvC-ilvD*	[2]
pBHR1	broad-host-range vector	MoBiTec, Göttingen, Germany
pNHG1	suicide vector containing *sucB*	[1]
pLH50	pNHG1 with homologous regions for making knockout *ΔphaB2C2*	this study
pLH51	pNHG1 with *ΔphaC1AB1::CAT*	this study
pLH63	pNHG1 with *ΔphaB2C2::PphaC1:alsS-ilvC-ilvD*	this study
pYL22	pBHR1 with *ΔCAT::kivd-yqhD*	
pLH129	pBHR1 with *P_{katG}:lacZ*	this study
pLH130	pBHR1 with *P_{norA}:lacZ*	this study
pLH131	pBHR1 with *P_{sodC}:lacZ*	this study
Strain	**Description**	**Reference or Source**
XL-1 Blue	*Escherichia coli* strain used in cloning and growth study	Stratagene, La Jolla, CA
S17-1	*E. coli* strain used in conjugation	ATCC
H16	*Ralstonia eutropha* wild type	A gift from Dr. Botho Bowien
LH67	H16 with *ΔphaB2C2::P_{phaC1}:alsS-ilvC-ilvD*, *ΔphaC1AB1::CAT*	this study
LH74D	LH67 transformed with pYL22	this study
LH118	H16 transformed with pLH129	this study
LH119	H16 transformed with pLH130	this study
LH120	H16 transformed with pLH131	this study

4.2.2 Enzyme assays of *alsS*, *ilvC*, *ilvD*

R. eutropha H16 and LH67 were cultured under autotrophic condition in German minimal medium [5] with $H_2:CO_2:O_2=8:1:1$ as the gas phase for 48 hours in 30 °C. 20ml of culture was harvested by centrifugation, washed twice with ice-cold lysis buffer (5mM $MgSO_4$, 50 mM Tris-Cl, pH 8.0), and resuspended with 1ml lysis buffer. After bead beating, the lysate was then centrifuged at 13,200 rpm for 20 minutes at 4 °C. The supernatant was then retrieved for enzyme assays. Acetohydroxy-acid synthase (AHAS), ilvC, and ilvD assays were performed as described previously [6].

4.2.3 Autotrophic fermentation

To perform the H_2 based autotrophic biofuel production, *R. eutropha* LH74D was cultured in 1.8L German minimal medium in a 5L fermentor. The gas flow rates were as follows: H_2 200ml/min, O_2/CO_2 mixture (1:1 ratio) 50 ml/min. The initial OD_{600} was around 1.0. H_2 was provided by an electricity-powered hydrogen generator (No-Maintenace H_2 Generator 500, PerkinElmer Inc., CA) and fed directly to the fermentor without purification or compression. Evaporated alcohols in venting gas were condensed with a Graham condenser and collected. Daily, samples of culture broth and condensation liquid were taken and alcohols were quantified using gas chromatography (GC).

For the formate-based fermentation, *R. eutropha* LH74D cells were cultured in 1.8L J minimal medium in a 5L fermentor. J minimal medium was prepared by autoclaving 1 g/l $(NH_4)_2SO_4$, 0.5 g/l KH_2PO_4, and 6.8 g/l $NaHPO_4$ in ddH2O and aseptically adding 0.2 g/l $MgSO_4$-$7H_2O$, 20 mg/l $FeSO_4$-$7H_2O$, 4mg/l $CaSO_4$-$2H_2O$, 100 ug/l thiamine hydrochloride, and 1ml/L SL7 metals solution (SL7 metal solution contains 1%v/v 5M HCl (aq), 1.5 g/l $FeCl_2$-$4H_2O$, 0.19 g/l $CoCl_2$-

$6H_2O$, 0.1 g/l $MnCl_2\text{-}4H_2O$, 0.07 g/l $ZnCl_2$, 0.062 g/l H_3BO_3, 0.036 g/l $Na_2MoO_4\text{-}2H_2O$, 0.025 g/l $NiCl_2\text{-}6H_2O$, and 0.017 g/l $CuCl_2\text{-}2H_2O$). Control set points for agitation, temperature, pH, dissolved oxygen content (DO), air flow % and O_2 flow % were 300 rpm, 30^0C, 7.2, 5%, 100%, and 0%, respectively. Gas flow was controlled by a dynamic-control cascade driven by DO with a gas flow of 0.5 SLPM at 0% out and 2.5 SLPM at 100% out. Formic acid, or formate, is toxic to microbial cells at high concentrations because the protonated acid molecules penetrate the cell membrane and acidify the cytoplasm upon proton dissociation. As a result, the proton motive force across the membrane is reduced. To keep a constant flow of formate concentration in cell culture, pH-coupled formic acid feeding (pH-stat fermentation) was used to add formic acid in small increments. Briefly, 50% v/v formic acid with 2 g/l KH_2PO_4 was fed following a pH-driven control cascade set to no flow with 0% out and 1 second pulses every 10 seconds at -100% out by the controller. This feed thereby serves to lower the pH and replenish the carbon supply as formate is consumed by the cells. Evaporated alcohols in venting gas were condensed with a Graham condenser and collected. Samples of culture broth and condensation liquid were taken and alcohols were quantified using gas chromatography (GC) [2].

4.3 Results and discussion

4.3.1 Designing of the pathway

During lithotrophic growth, molecular H_2 is oxidized by a membrane-bound hydrogenase (MBH) and a soluble hydrogenase (SH) to provide *R. eutropha* with both the energy and the reducing power [7-9], which then drives the CBB cycle and other metabolic pathways (Figure 4.1A). Owing to their high activities and the unique oxygen-tolerance [10-12], these hydrogenases are

well suited for use as energy-input channels bridging artificial H_2 generation and biological carbon-carbon bond synthesis. Formate is utilized by the formate dehydrogenase (Fdh) to yield NADH and CO_2 (Figure 4.1A).

One special metabolic feature of *R. entropha* is that it is one of the best natural polyhydroxyalkanoate (PHA) hyper-producers known. Polymers such as poly[R-(–)-3-hydroxybutyrate] (PHB) can accumulate up to ~80% of dry cell weight and are not only used as the storage compound but also as the metabolic sink for carbon and reducing equivalents [13-15]. Biosynthesis of PHB starts from condensation of two molecules of acetyl-CoA to acetoacetyl-CoA, followed by NADPH dependent reduction and polymerization [9] (Figrue 4.1B). Once formed, the insoluble polymers are stored in specialized granules whose decomposition is strictly regulated. Although the chain elongation step (i.e. joining of two acetyl-CoAs) is highly endergonic, the irreversibility of the final polymerization step contributes greatly to drive the continuous electron and carbon flow into the sink. When the PHB synthesis is disrupted, large amount of pyruvate (the precursor of acetyl-CoA) is secreted out of the cells along with the fermentation products [16], suggesting that the overall metabolic network is well-tuned for pushing carbon and reducing power through this pathway at the pyruvate node. These previous studies lead to a possibility: if the biofuel production pathway also originates from pyruvate and utilizes NADPH to drive a highly irreversible pathway, it may serve as an engineered substitute of the native PHB biosynthesis pathway as a new "metabolic sink" [17]. To achieve this objective, the keto acid pathways for isobutanol and 3MB production were chosen [2]. The biosynthetic pathways involve several irreversible decarboxylation steps, which provide a strong thermodynamic driving force for chain elongation (Figure 4.1B) and serve as a sink for both pyruvate and NADPH.

A

B

Figure 4.1 Designing *Ralstonia eutropha* cells as the biocatalyst in the process of electricity storage. (a) Schematic presentation of the energy conversion and carbon flow route of the overall process. CBB cycle, Calvin-Benson-Bassham cycle;ETC, electron transportation chain;MBH, membrane-bound hydrogenase;SH, soluble hydrogenase; FDH, formate dehydrogenase.(b)Engineered metabolic pathways from CO_2 to fuels in the context of the host's metabolic network. RuBP, Ribulose-1,5-bisphosphate;3PGA, 3-phospho-D-glycerate;2PGA, 2-phospho-D-glycerate; PEP, phosphoenolpyruvate; PHB, poly[R-(−)-3-hydroxybutyrate];AHAS, acetohydroxy-acid synthase;KDC, 2-keto-acid decarboxylase;ADH, alcohol dehydrogenase.

4.3.2 Functional overexpression of the keto acid decarboxylase (KDC) and the alcohol dehydrogenase (ADH)

The isobutanol and 3MB production pathway converts the keto acid intermediates of amino acid biosynthesis, 2-ketoisovalerate (KIV) and 2-Ketoisocaproate (KIC), into biofuels through two non-native steps borrowed from the Ehrlich pathway: decarboxylation and reduction [2] (Figure 4.1B). Thus, effectively introducing these two steps is of critical importance for creating the synthetic pathway. To this end, we first overexpressed the keto acid decarboxylase (KDC) *kivd* and the alcohol dehydrogenase (ADH) *adhA* from *Lactococcus lactis* using a multicopy plasmid under the control a constitutive promoter [2, 18]. When 3g/L KIV was supplemented in the minimal medium that also containing 5g/L fructose, the resulting strain produced ~118mg/L isobutanol (Figure 4.2A), suggesting functional introduction of the two-step Ehrlich reactions. However, comparable amount of isobutyraldehyde (~79mg/L) was also detected, indicating that the activity of the alcohol dehydrogenase did not match with that of the keto acid decarboxylase. When replacing the *adhA* with a different alcohol dehydrogenase *adh2* from the yeast *Saccharomyces cerevisiae*, we observed similar results in KIV feeding experiments with substantial accumulation of isobutyraldehyde intermediate (Figure 4.2A).

Adh2 and AdhA are NADH dependent and function in the fermentative pathways of their native hosts to drain the electrons in excess. However, *R. eutropha* is a strictly respiratory bacterium that does not normally utilize fermentative pathways. Although *R. eutropha* can also induce the expression of a full set of fermentative enzymes when in severe oxygen deficiency [19], it is still unclear why this organism cannot rely on fermentation to support growth. These lines of evidence suggest that NADH may preferentially be diverted to the electron transportation chain through complex and unknown regulatory mechanisms. On the other hand, the highly efficient PHA production pathway is a NADPH dependent biosynthetic pathway, suggesting the presence of abundant NADPH supply in the cell. Therefore, we replaced *adh2* or *adhA* in the plasmid with a NADPH dependent alcohol dehydrogenase *yqhD* from *Escherichia. coli* [18]. The resulting strain showed significantly improved aldehyde reductase activity and was able to produce ~1.2g/L isobutanol from 3g/L of KIV with low isobutyraldehyde accumulation (~28mg/L) (Figuer 4.2A).

These results pinpointed the levels of different reducing cofactors available in the cell under heterotrophic growth. In lithoautotrophic biofuel production scenario, the oxidation of H_2 directly yields NADH. But *R. eutropha* is equipped with unusually high number of transhydrogenase isoenzymes that actively convert NADH to NADPH [8] (Figure 4.1A). Indeed, previous studies have shown that NADPH/NADP$^+$ ratio is much higher than that of NADH/NAD$^+$ under autotrophic condition [16], suggesting that the NADPH dependent aldehyde reaction catalyzed by YqhD may also be favorable for the purpose of biofuel production from CO_2.
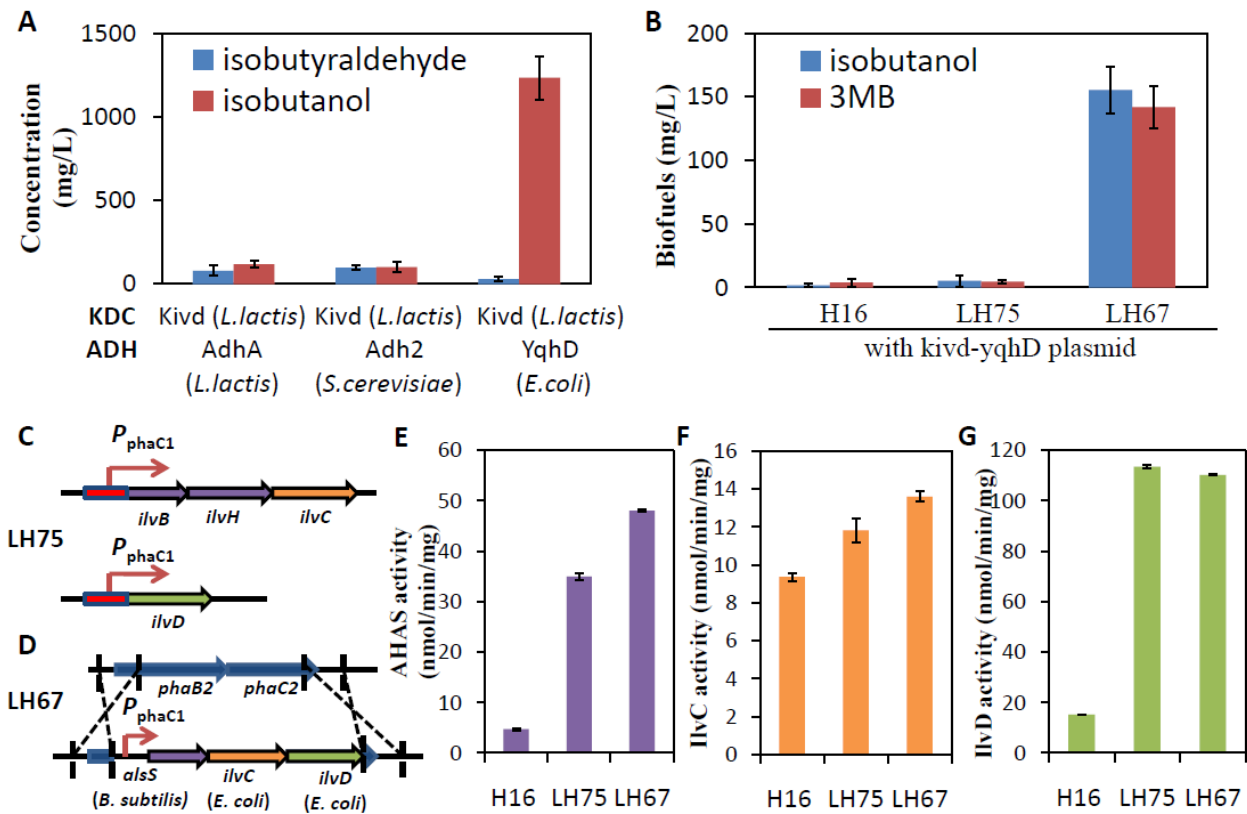
Figure 4.2 Construction of the synthetic isobutanol and 3-methyl-1-butanol production pathway in *Ralstonia eutropha*. (**a**) isobutanol and isobutyraldehyde formation by the synthetic Ehrlich cassette. The 2-keto-acid decarboxylase (KDC) encoded by *kivd* of *Lactococcus lactis* was overexpressed in combination with different acohol dehydrogenases (ADHs) encoded by *adhA* (*L.lactis), adh2* (*Saccharomyces cerevisiae*), and *yqhD* (*Escherichia.coli*), respectively. 3g of 2-ketoisovalerate was added in the culture as the immediate precursor of the synthetic pathway. The culture medium is German minimal medium (supplementary information) with 4g/L fructose. (**b**) Heterotrophic isobutanol and 3-methyl-1-butanol (3MB) production from 4g/L fructose in German minimal medium using H16, LH75, and LH67 strains transformed with a plasmid harboring the *kivd* and *yqhD* overexpression cassette. LH106 is the strain resulted from LH75 transformed with the *kivd* and *yqhD* plasmid. LH74 is the strain resulted from LH67 transformed with the *kivd* and *yqhD* plasmid.(**c**) Construction of LH75 strain. Integration of the *phaC1* promoter in front of the *R.eutropha ilvBHC* operon and *ilvD* gene to enhance branched-chaineamino acid biosynthesis. (**d**) Construction of LH67 strain. Integration of alsS (*Bacillus subtilis*), ilvC *(E.coli)*, and ilvD (*E.coli*) in *R.eutropha* genome. The AHAS (acetohydroxy-acid synthase, encoded by *ilvBH* or *alsS*) (**e**), IlvC (**f**), and IlvD (**g**) specific activities *in vitro* as measured using cell extract of wildtype H16, LH75 and LH67. Error bars indicate standard deviation (n=3).

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4.3.3 Enhancing the upstream amino acid biosynthesis pathway

Without keto acids added, biofuel production from fructose by the wildtype strain H16 with *kivd*

and *yqhD* overexpression reached only ~1.7 mg/L of isobutanol and ~3.8mg/L of 3MB (Figure

4.2B). These data pointed towards the necessity for the enhancement of the native keto acid

chain elongation pathway. To do so, the strong *phaC1* promoter that drives the expression of the

host's PHA synthesis operon (*phaC1AB1*) was knocked-in in front of both the *ilvBHC* operon

and the *ilvD* gene in *R. eutropha* genome, which encode the enzymes responsible for the

branched-chain amino acid biosynthesis (Figure 4.2C). The resulting strain LH75 showed

significantly higher levels of acetohydroxy-acid synthase (AHAS), IlvC, and IlvD enzyme

activities compared to the wildtype when assayed *in vitro* using cell lysate (Figure 4.2 E,F,G).

Unfortunately, when the *kivd* and *yqhD* cassette was transformed to this strain, the isobutanol

and 3MB production with 4g/L fructose as the carbon source in minimal medium was similar to

the strain H16 transformed with the same Ehrlich cassette but without enhancement of the amino

acid pathway (Figure 4.2B)

The high enzymatic activity *in vitro* and low productivity *in vivo* suggests that post-translational

regulations on the native enzymes may control the flux. In fact, the anabolic AHAS enzymes that

catalyzed the first-committed step of the keto acid chain elongation are well-known for their

strict feedback inhibition by pathway end products and intermediates in order to partition carbon

flux appropriately. To disrupt the post-translational regulation, a catabolic AHAS encoded by

alsS from *Bacillus subtilis* was used [2], which has high specificity to pyruvate and is not

subjected to feedback inhibition. Initial attempt to introduce *alsS* by multiple-copy plasmid

overexpression yielded no viable transformants, indicating cellular toxicity caused by foreign

gene expression. Chromosomal integration strategy was then used to reduce the dosage of the

alsS gene. The *alsS* gene together with *ilvC* and *ilvD* genes from *E.coli* were cloned to form a synthetic operon driven by the Ralstonial *phaC1* promoter, which was then integrated into the *R. eutropha* genome to replace the native *phaB2C2* operon (Figure 4.2D). The resulting strain LH67, although only showed marginally elevated enzymatic activities *in vitro* (Figure 4.2E,F,G) compared with LH75, was able to provide much more keto acid intermediates for biofuel production *in vivo*. When *kivd* and *yqhD* were introduced to LH67, the resulting strain LH74 produced ~155mg/L isobutanol and ~142mg/L 3MB under the same conditions as described above (Figure 4.2B). The biofuel titer was about 30-fold higher for both isobutanol and 3MB than that of LH106 featuring transcriptional enhancement of native enzymes. To integrate the fuel production pathways with host metabolism, the PHB biosynthesis genes *phaC1AB1* in strain LH74 were disrupted by a chloramphenicol acetyltransferase (CAT) cassette to give rise to the production strain LH74D (Figure 4.3A), which produced isobutanol and 3MB to ~176mg/L and ~160mg/L from fructose.
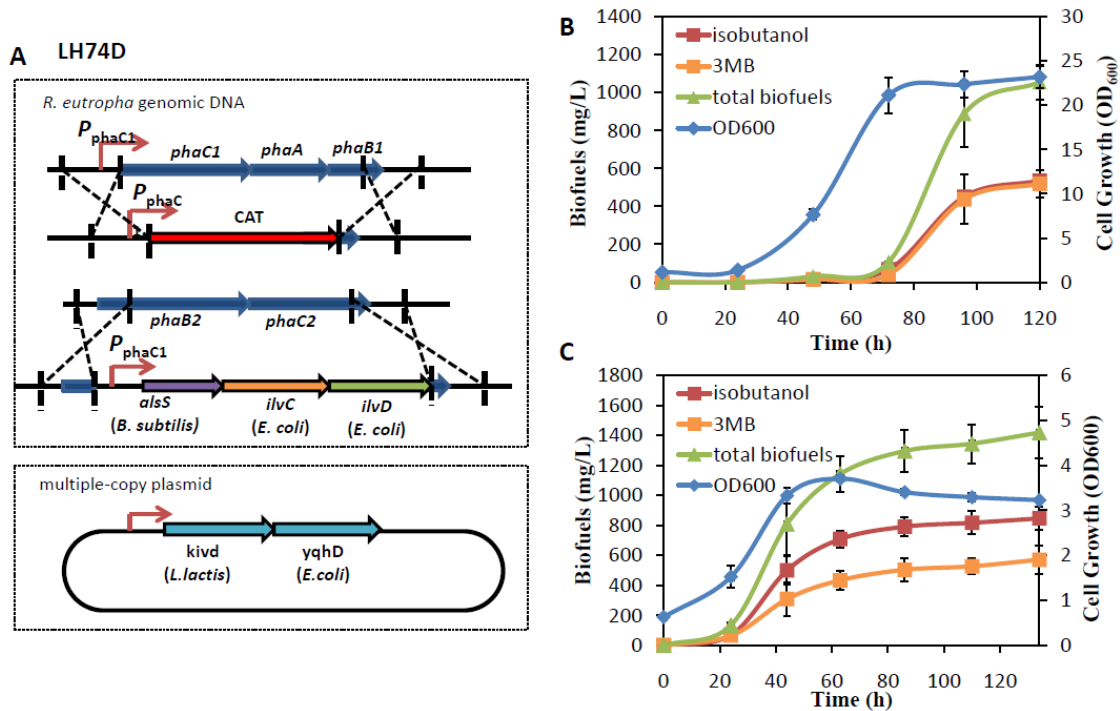
Figure 4.3 Autotrophic higher alcohol production by the engineered Ralstonia strain. **(a)** Construction of the production strain LH74D. **(b)** Biofuel production performance by LH74D from CO_2 using electrolysis-generated H_2 as the sole energy source. **(c)** Biofuel production performance by LH74D using formic acid as the sole carbon and energy source. Detailed conditions and methods are described in supplementary information. Error bars indicate standard deviation (n=3).

4.3.4 Autotrophic higher alcohol production by the engineered *R. eutropha* strain

After demonstrating its isobutanol and 3MB productivity heterotrophically, LH74D was tested

for autotrophic biofuel production on CO_2 and H_2. The O_2/CO_2 flow rate was adjusted

accordingly to keep the ratio of $H_2:CO_2:O_2=8:1:1$. Under these conditions, the strain LH74D was

able to produce a final titer exceeding 1 g/L of fuels (~536mg/L isobutanol and ~520mg/L 3MB)

in 5 days in the J minimal medium (Figure 4.3B). Notably, the maximal production rate was

reached at ~380mg $L^{-1}day^{-1}$ and ~400mg $L^{-1}day^{-1}$ for isobutanol and 3MB, respectively, when

the cells entered the stationary phase, indicating high metabolic flux through the engineered

biofuel production pathway. This result demonstrates the feasibility of using hydrogen to drive

CO_2 reduction to isobutanol and 3MB. However, the low solubility and mass transfer of

hydrogen limits the efficiency of its utilization by the cells.

We then tested the feasibility of using formic acid as the diffusible and soluble reducing power.

Formic acid, or formate, is toxic to microbial cells at high concentrations because the protonated

acid molecules penetrate the cell membrane and acidify the cytoplasm upon proton dissociation.

As a result, the proton motive force across the membrane is reduced [20]. To keep a constant low

formate concentration in cell culture, pH-coupled formic acid feeding was used to add formic

acid in small increments. These conditions enabled normal cell growth and relatively high

biofuel productivity (Figure 4.3C) in the J minimal medium. The final titer of fuels was over 1.4

g/L (~846mg/L isobutanol and ~570mg/L 3MB) in around 5 days. Also, the specific productivity

of fuels from formate (87.9 mg L^{-1}/day/OD) was much higher than that from hydrogen and CO_2 (9.2 mg L^{-1}/day/OD). Although the peak productivity from formate to fuels (25 mg/L/h) is about 10-fold less than that demonstrated from glucose to isobutanol using *E. coli* in un-optimized shake flasks [2], further improvement in productivity can be expected using existing technologies.

4.4 Conclusions

In summary, the work described in this chapter converted H_2 and CO_2 or formate to high-energy-density liquid fuels using an engineered *R. eutropha* strain as the biocatalyst. This process can potentially complement the artificial photosynthetic "light reaction" and store intermittent renewable energy. As a biofuel production process, it also can bypass the need for agriculture land and biomass mass processing difficulty.

Besides fuels, we envision production of other chemicals using this type of platform thanks to the versatility of biological metabolic pathways.

4.5 Reference

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5. Development of a synthetic anhydrotetracycline-controllable gene expression system in *Ralstonia eutropha* H16

5.1 Introduction

As mentioned in previous chapters, *Ralstonia eutropha* H16 is a gram-negative, facultative lithoautotrophic bacterium of scientific and biotechnological importance. For example, it is one of the best studied microorganisms for biosynthesis of polyhydroxyalkanoates (PHAs) [1], which could be used as biodegradable materials. Besides a broad range of organic compounds, it can also utilize CO_2 as the carbon source through the Calvin-Benson-Bassham (CBB) cycle [2], which can be powered by the energy derived from H_2 or formate [3]. As described previously, metabolic engineering work in *R. eutropha* H16 has demonstrated the production of biofuels from sugars, CO_2 and H_2, formic acid. However, advanced metabolic engineering work requires various synthetic biology tools especially controllable gene expression systems, which are still limited in this organism. Especially, we encountered the toxicity problem caused by the constitutive *alsS* overexpression in our work on biofuel production. The gene *alsS* from *Bacillus subtilis* encodes one of the key enzymes in the biofuel production pathway, the acetolactate synthase. It caused toxicity when overexpressed in *R. eutropha* H16 especially in minimal medium. As a result, the biofuel production strain described in chapter 4 can only have one copy of *alsS* integrated in the chromosome, since strains with high level *alsS* overexpession on a multiple-copy plasmid grow extremely poorly and are not viable in autotrophic condition. Therefore, a controllable gene expression system might be needed to control the expression of this gene.

Many native genes in *R. eutropha* H16 have been found to be expressed in an inducible manner. For example, the expresssion of the *phaP1* gene, which encodes a PHA-granule associated

phasin protein, is regulated by phosphate level in the medium [4] and highly coupled to PHA accumulation [5, 6]. However, synthetic biology and metabolic engineering work require non-native gene expression systems which can be controlled independently of the host's metabolic state. To this end, some heterologous controllable gene expression systems have also been tested in *R. eutropha* H16. The Isopropyl β-D-1-thiogalactopyranoside (IPTG) controllable P_{lac} system from *Escherichia coli* could not be effectively induced [6]. And the L-arabinose controllable P_{BAD} system from *E. coli* requires more than 1g/L inducer to achieve good induction at certain conditions and may also affect the host's metabolism as indicated by the inhibited growth upon addition of the inducer [6]. These observations suggested that the widely used carbon-catabolite-repression based systems may not be suitable for implementing in *R. eutropha* H16, possibly due to the fact that the sugar uptaking and metabolism in *R. eutropha* H16 is very different from that in *E. coli* [7].

As such, we sought to develop the anhydrotetracycline (aTc)-inducible gene expression system in *R. eutropha* H16 [8]. This system is composed of a repressor protein tetR and a controllable promoter containing the tetR binding sequence (tetO operators). Upon binding of the inducer aTc to tetR, the latter disassociates from tetO, allowing expression of the target gene. The inducer aTc is freely diffusible through the cell membrane and does not require specific transporters. Furthermore, the system has not been shown have crosstalk with the host's gene regulation network. Here we report the development of the anhydrotetracycline (aTc)-inducible gene expression system in *R. eutropha* H16. We also demonstrated its application in alleviating the cellular toxicity caused by acetolactate synthase (AlsS), a key enzyme in the isobutanol production pathway [9].

5.2 Material and methods

5.2.1 Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich (St.Louis, MO) or Fisher Scientifics (Pittsburgh, PA). Restriction enzymes were purchased from New England BioLabs (Ipswich, MA). Oligonucleotides were purchased from IDT (San Diego, CA). The Rapid DNA ligation kit was from Roche (Mannheim, Germany). KOD DNA polymerase was from EMD Chemicals (San Diego, CA).

5.2.2 Strains and culture condition

Ralstonia eutropha H16 strain was purchased from American Type Culture Collection (ATCC). *R. eutropha* strains were regularly cultured in rich medium (16g/L nutrient broth, 10g/L Yeast extract, 5g/L $(NH_4)SO_4$) at 30 °C. If the strains contain plasmids, 200mg/L kanamycin was added. All promoter testing was performed using the wild type strain. Transformation of the plasmids to *R. eutropha* H16 was done using the previously described method [9].

All cloning and plasmid preparation were done using *E. coli* XL1-blue cells (Stratagene, La Jolla, CA). E. coli cells with plasmids were cultured in LB medium containing 50mg/L kanamycin.

5.2.3 Plasmid construction

The detailed information about plasmids and primers used in this study is shown in Table 1 and Table 2.

Table 5.1 Plasmids used in this study

Plasmid	Comments
pBHR1	broad host-range multiple copy plasmid (MoBiTec, Göttingen, Germany)
pLH300	pBHR1 $P_{rrsC\ wildtype}$::*CAT*
pLH 301	pBHR1 $P_{rrsC\text{-}O1}$::*CAT*, *tetO1* placed between -35 and -10 region of P_{rrsC}
pLH 302	pBHR1 $P_{rrsC\text{-}O2}$::*CAT*, *tetO2* placed between -35 and -10 region of P_{rrsC}
pLH 303	pBHR1 $P_{rrsC\text{-}O1\text{-}O1}$::*CAT*, *tetO1* placed downstream of the transcription start site in pLH301
pLH 304	pBHR1 $P_{rrsC\text{-}O1\text{-}O2}$::*CAT*, *tetO2* placed downstream of the transcription start site in pLH301
pLH 305	pBHR1 $P_{rrsC\text{-}O1}$::*CAT*, P_{cat}::*tetR*
pLH 306	pBHR1 $P_{rrsC\text{-}O1\text{-}O1}$::*CAT*, P_{cat}::*tetR*
pLH 307	pBHR1 $P_{rrsC\text{-}O1}$::*lacZ*, P_{cat}::*tetR*
pLH 308	pBHR1 $P_{rrsC\text{-}O1}$::*lacZ*, P_{phaC1}::*tetR*
pLH 309	pBHR1 P_{phaC1*}::*gfp*, contains P_{phaC1} -35 region mutagenesis library
pLH 310	pBHR1 P_{cat}::*alsS*
pLH 311	pBHR1 P_{cat}::*ilvBH* wild type
pLH 312	pBHR1 P_{cat}::*ilvBH* DDF feedback resistent mutant
pLH 313	pBHR1 $P_{rrsC\text{-}O1\text{-}O1}$::*alsS*, $P_{phaC1\text{-}G3}$::*tetR*

To survey different P_{rrsC}-*tetO* hybrid promoters (See 5.3.1), five plasmids were built: pLH300 has the wild type P_{rrsC} driving the Chloramphenicol Acetyl Transferase (*CAT*) reporter gene. Briefly, the P_{rrsC} promoter was amplified from *R. eutropha* H16 genomic DNA using primers CATP ad up_rrsC fwd/rrsC_CATP ad down rev. The primers CATP ad up rev/CATP ad down fwd were used to amplify part of the back bone of the pBHR1 vector, which contains a promoterless *CAT* gene. The two PCR products were then assembled using the isothermal DNA assembly method [10]. To place *tetO1* operator in P_{rrsC} between the -35 and -10 regions, two PCR products were obtained using primers CATP ad up_rrsC fwd/tetO1 mid rev, and tetO1 mid fwd rrsC_CATP ad down rev, respectively, with *R. eutropha* H16 genomic DNA as the template. The two fragments were then linked together using Splicing overlap extension (SOE) PCR and then assembled with the promoterless *CAT* backbone to yield pLH301. The *tetO2* operator was

placed in P_{rrsC} between the -35 and -10 regions using similar method to result in pLH302. To place an extra *tetO1* operator downstream of the transcription start site of pLH301, trunc O1 rev/trunc O1 fwd primer pair is used to amplify the pLH301 plasmid and then self-ligated using the isothermal DNA assembly method, resulting in plasmid pLH303. Similar method was used to place an extra tetO2 operator downstream of the transcription start site of pLH301 to resulte in plasmid pLH304.

The tetR expression cassette (See 5.3.2) was introduced to the system as follows: The tetR was amplified from *E. coli* genome using primers tetR fwd/BRad down_tetR rev. The PCR product was then amplified again using primers tetR fwd/BRad down rev to append the T1 terminator sequence at the end of the gene. The P_{cat} promoter was amplified from pBHR1 using primers BRad up_Pcat fwd/Pcat_tetR rev. The tetR-T1 fragment and the Pcat promoter fragment were then linked together using SOE PCR. The resulted product was restriction digested and inserted into the Bsu36I site of pLH301. The resulted plasmid was named pLH305. Similar method was used to add the P_{cat}::*tetR* cassette to pLH303, resulted in pLH306. Primers rrsC_lacZ rev/ lacZ_vector fwd were used to amplify the whole pLH306 except the *CAT* gene region. Primers rrsC_lacZ fwd/lacZ_vector rev were used to amplify the *lacZ* gene. These two fragments were then assembled to form pLH307. The primers tetR fwd/BRad up rev were used to amplify the whole pLH307 except the P_{cat} region. The primers BRad up_PphaC1 fwd/PphaC1_tetR rev were used to amplify the P_{phaC1} promoter from *R. eutropha* H16 genomic DNA. These two fragments were assembled to switch the P_{cat} to P_{phaC1}, resulting in pLH308.

Table 2 Primers used in this study

Primer name	Sequence
CATP ad up rev	ggcgcaaagggcctcgtgatacgcctattt
CATP ad down fwd	aaggagagctaacatggagaaaaaaatcactggat
CATP ad up_rrsC fwd	ggcgtatcacgaggcccttttgcgccttcaactgctctgcttggcattcga
rrsC_CATP ad down rev	gattttttttctccatgttagctctcctttgcagagctttcttcgcgaggg
tetO1 mid rev	atattactctatcaatgatagagtggcaagcgatttcgcgaaataattcg
tetO1 mid fwd	ttgccactctatcattgatagagtaatattcgccccctcgcaacacaacg
tetO2 mid rev	atattatctatcactgatagggatggcaagcgatttcgcgaaataattcg
tetO2 mid fwd	ttgccatccctatcagtgatagataatattcgccccctcgcaacacaacg
trunc O1 rev	cagagctttcttcactctatcaatgatagagtgcgaggggggcgaatatta
trunc O1 fwd	gccccctcgcactctatcattgatagagtgaagaaagctctgcaaaggag
trunc O2 rev	tgcagagctttcttctctatcactgatagggagcgaggggggcgaatatta
trunc O2 fwd	tcgcccccctcgctccctatcagtgatagagaagaaagctctgcaaaggag
tetR fwd	aggagaaaggtaccatgtccagattagataaaagtaaag
BRad down_tetR rev	tgagcctttcgtttatttgatgcctttaagacccactttcacatttaag
BRad down rev	caaatgcctgaggacaacagataaaacgaaaggcccagtctttcgactgagcctttcgtttatttgatgcct
BRad up_Pcat fwd	gagagcctgagcaaactggcctcagggaataaatacctgtgacggaagat
Pcat_tetR rev	ctaatctggacatggtacctttctccttttagcttccttagctcctg
rrsC_lacZ rev	ccagtgaatccgtaatcatggtcatgttagctctcctttgcagagctttc
rrsC_lacZ fwd	gaaagctctgcaaaggagagctaacatgaccatgattacggattcactgg
lacZ_vector fwd	ccagttggtctggtgtcaaaaataatttttttaaggcagttattggtgcc
lacZ_vector rev	ggcaccaataactgccttaaaaaaattattttttgacaccagaccaactgg
BRad up rev	cctgaggccagtttgctcaggctctcc
BRad up_PphaC1 fwd	ggagagcctgagcaaactggcctcaggccgggcaagtaccttgccgacat
PphaC1_tetR rev	ctaatctggacatggtacctttctcctgatttgattgtctctctgccgtc
YCAT ad up Rev	ggtacctttctcctctttaatgaattcttattccggatgagcattcatca
YCAT ad down fwd	tctagaccatgggcaaatattatacgcaaggcgacaaggtgctgatgccg
YCAT ad down_phaC1 fwd	aatatttgcccatggcatggctctagaccgggcaagtaccttgccgacat
phaC1_GFP rev	cctttacgcatgagctctttctcctgatttgattgtctctctgccgtcac
phaC1 -35 lib fwd	tgccgaggcggattcccgcattnnnngcgcgtgcgttgcaaggcaacaat
phaC1 -35 lib rev	attgttgccttgcaacgcacgcgcnnnnaatgcgggaatccgcctcggca
GFP fwd	aggagaaagagctcatgcgtaaaggagaagaacttttc
GFP_YCAT ad up rev	aattcattaaagaggagaaaggtaccttatttgtatagttcatccatgcc
YCAT ad up_alsS fwd	taagaattcattaaagaggagaaaggtaccatgttgacaaaagcaacaaa
alsS_YCAT ad down rev	taatatttgcccatggcatggctctagactagagagctttcgttttcatg
YCAT ad up_ilvB fwd	taagaattcattaaagaggagaaaggtaccatgcccagcgcggaattctc
YCAT ad down_ilvH rev	aatatttgcccatggcatggctctagattagaccttcaggatgcgctcgc
O1_alsS fwd	tatcattgatagagtgaagaaagctctgcaaaggagagctaacatgttgacaaaagcaacaaaag

The P_{phaC1} -35 region promoter library (see 5.3.3) was constructed as follows: vector backbone was amplified using primers YCAT ad up Rev/YCAT ad down fwd from pBHR1. The *gfp* gene was amplified using GFP fwd/GFP_YCAT ad up rev. To pool of fragments containing different P_{phaC1} mutants was generated as follows: YCAT ad down_phaC1 fwd/phaC1 -35 lib rev were used to amplify the first half of the promoter, and phaC1 -35 lib fwd/phaC1_GFP rev were used to amplify the second half of the promoter. The phaC1 -35 lib rev and phaC1 -35 lib fwd primers contain degenerate nucleotides NNNN at the last 4 positions of the -35 region. The two halves were then linked together using SOE PCR. Finally, the promoter mutant pool, and gfp fragment, and the vector backbone were assembled together, and the resulted library was named pLH309. The library has the $P_{phaC1*}::gfp$ cassette in the opposite direction of the original *CAT* gene in pBHR1. Thus the expression of *gfp* is not affected by the preexisting promoter on the vector backbone.

The plasmids for studying the toxicity of *alsS* expression (see 5.3.4) were constructed as follows: vector backbone was amplified using primers YCAT ad up Rev/YCAT ad down fwd from pBHR1. *Bacillus subtilis alsS* gene was amplified using YCAT ad up_alsS fwd/alsS_YCAT ad down rev from pSA69 [11]. The two fragments were then assembled to form plasmid pLH310. Similarly, *ilvBH* genes were amplified using primers YCAT ad up_ilvB fwd/YCAT ad down_ilvH rev from *R. eutropha* H16 genomic DNA and assembled with the vector backbone to form plasmid pLH311. The feedback resistence (fbr) ilvBH has the G14, A15, L16 (GAL) residues in *R. eutropha* H16 ilvH mutated to DDF. The resulted ilvBH fbr was placed in position of the wild type ilvBH in pLH311 to form pLH312. In pLH310, 311, and 312, the *alsS* or *ilvBH* were driven by the preexisting P_{cat} in the vector. The plasmid pLH313 contains the *alsS* gene

being regulated by the developed aTc-controllable system. Briefly, part of pLH306 was amplified using primers trunc O1 rev/YCAT ad down fwd, which was then assembled with *alsS* fragment amplified using primers O1_alsS fwd/alsS_YCAT ad down rev. And Pcat promoter which drives the tetR expression in pLH306 was replaced by the $P_{phaC1-G3}$ promoter using similar method when building pLH308.

5.2.4 Characterization of the induction profile of the controllable gene expression system

The plasmid containing the controllable gene expression system was transformed into *R. eutropha* H16. Single colonies were picked from transformation and inoculated into rich medium with 200mg/L Kanamycin and cultured overnight. The overnight culture was re-inoculated into 40mL rich medium with 200mg/L Kanamycin in shake flask to mid-log phase. Then the culture was aliquoted into 4mL each in test tubes and induced with different concentration of aTc. After ~6 hours, the cells were harvested and assayed for reporter gene activity. The CAT reporter assay was performed as follows: cell lysate was prepared from each sample using Qiagen Tissuelyser II in 100 mM Tris-HCl pH=8.0. The CAT assay system contains 100 mM Tris-HCl pH=8.0, 0.1 mM Acetyl-CoA, 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 0.25mM Chloramphenicol, and appropriate amount of cell lysate. The absorbance at 412 nm was monitored. Reactions with no Chloramphenicol served as blank. The β-galactosidase was performed using previously described method [9].

5.2.5 High-throughput screening of the promoter library

The DNA containing promoter library (pLH309) was transformed into *R. eutropha* H16. Single colonies were picked and inoculated in 96-well culture plates, which contained 300μl of rich medium with 200mg/L Kanamycin in each well. The plates were sealed with porous paper

covers and incubated for 24 hours. The culture was then diluted by 5 fold before assayed for fluorescence level in 96-well plate (Excitation: 485nm, Emission: 510nm, cutoff: 495nm). The fluorescence was normalized by cell density as measured by OD600nm.

5.2.6 AlsS toxicity test

The strains harboring *alsS* or *ilvBH* were cultivated in rich medium overnight. The cells were there harvested the resuspended in German minimal medium [12] containing 4g/L fructose and 200mg/L Kanamycin with the initial OD600nm of ~0.01. After ~36 hours, the cells reached late-log or early stationary phase. The OD600nm was measure. To test the performance of the developed controllable gene expression system for alleviating AlsS toxicity, similar method was used except the 200ng/ml aTc was used to the "with inducer" samples and OD600nm was measured after ~24 hours of culturing.

5.3. Results and discussion

5.3.1 Designing and construction of the *P_{rrsC}-tetO* hybrid promoters

It has been shown that controllable hybrid promoters can be created by placing the operator sequences in a constitutive parent promoter [13]. In *R. eutropha* H16, we chose the promoter of the *rrsC* gene as the parent promoter (Figure 5.1A), which drives the transcription of an operon containing 16s, 23s, and 5s ribosomal RNAs and other translation-related genes. Using bioinformatic tools, we identified the putative -10 and -35 elements of the *P_{rrsC}* and the transcriptional start site (Figure 5.1A).
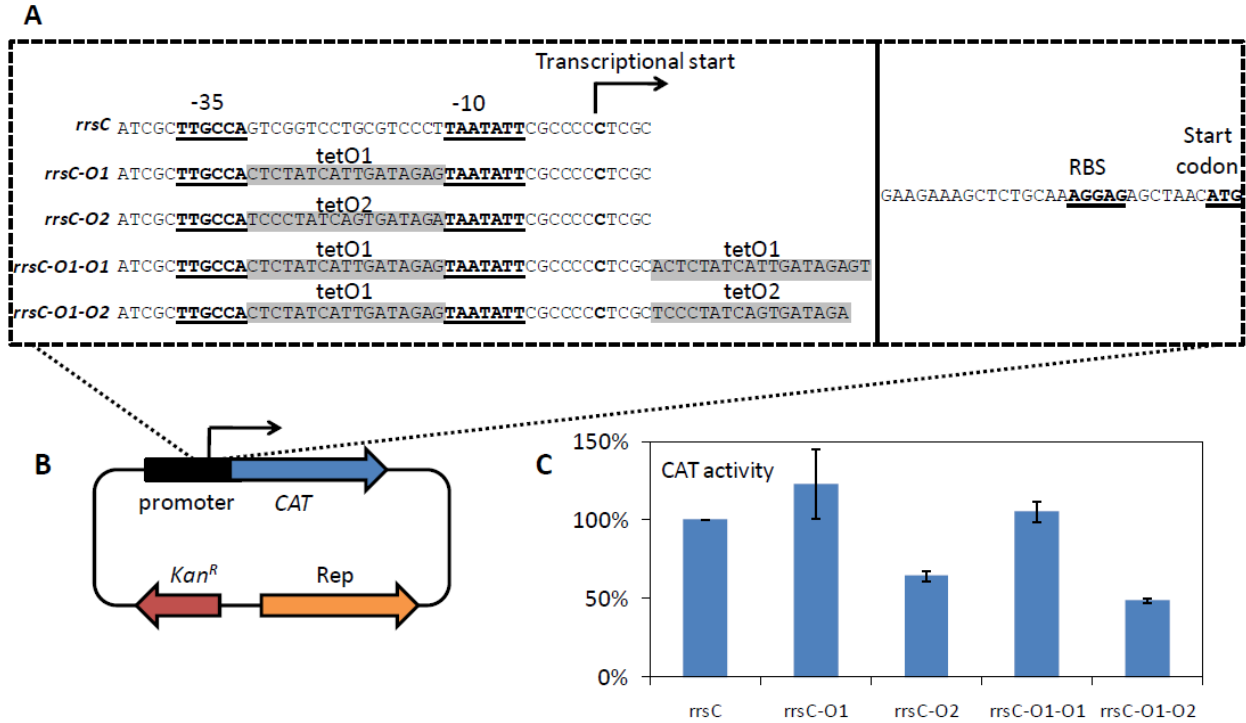
Figure 5.1 Characterization of the P_{rrsC} *tetO* hybrid promoters. A) Sequence of the cloned wild type P_{rrsC} promoter from *R. eutropha* H16 and 4 hybrid promoters with *tetO1* or *tetO2* operators placed at different positions of PrrsC. B) Plasmid map showing the chloramphenicol acetyltransferase (CAT) reporter cassette for promoter characterization. C) Activity of different promoters as measured by CAT activity level. Error bars stand for the standard deviation of 3 independent repeats. (n=3)

There are two different *tetO* operators (*tetO1* and *tetO2*) which can both be recognized by the tetR repressor [14]. We took a two-step approach to survey their compatibility with the parent promoter. Firstly, when placed between the -35 and -10 regions of P_{rrsC}, the *tetO2* lowered the promoter's strength as measured by Chloramphenicol Acetyl Transferase (CAT) reporter assays in *R. eutropha* H16 (Figure 5.1A, B, C). Thus, *tetO1* was chosen in this position, resulting in the hybrid promoter $P_{rrsC-O1}$ (Figure 5.1A). Next, a second operator was inserted downstream of transcriptional start site, which can potentially confer more stringent repression [15, 16] (Figure 5.1A). The insertion of *tetO2*, but not *tetO1*, at this position decreased the activity of $P_{rrsC-O1}$

(Figure 5.1A, C). Therefore, the hybrid promoter the $P_{rrsC-O1}$ and $P_{rrsC-O1-O1}$ (Figure 5.1A) were chosen for subsequent development, which maintained the strength of the wild type P_{rrsC} (Figure 5.1C).
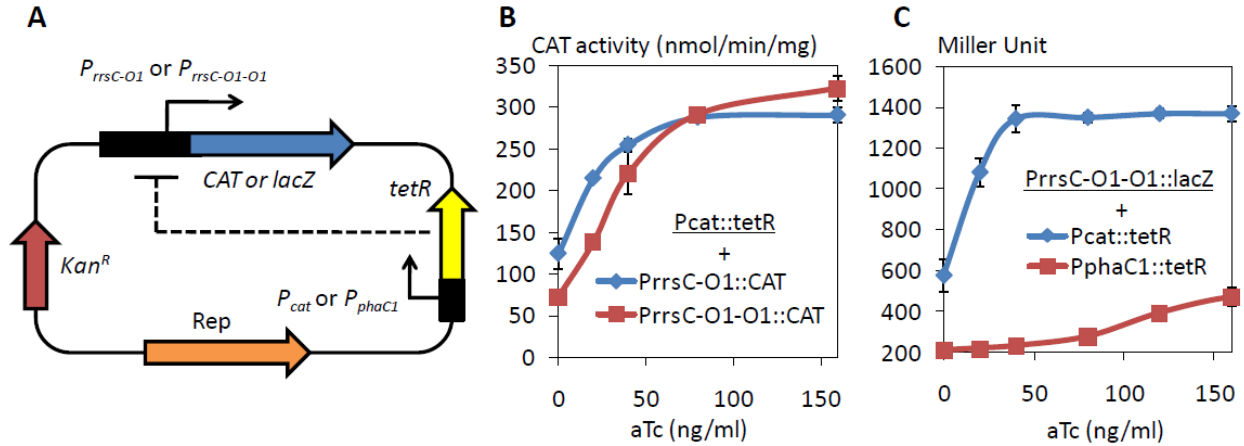

Figure 5.2 Repression of the P_{rrsC} *tetO* hybrid promoters by tetR. A) Plasmid map showing the *CAT* or *lacZ* reporter cassette for promoter characterization and the *tetR* expression cassette driven by P_{cat} or P_{phaC1}. The two cassettes were placed in opposite direction to avoid interference. B) Induction profile of the $P_{rrsC-O1}$ and $P_{rrsC-O1-O1}$ promoters in combination with P_{cat}::*tetR* with different concentration of the inducer aTc. C) Induction profile of the $P_{rrsC-O1-O1}$ promoter in combination with P_{cat}::*tetR* or P_{phaC1}::*tetR* with different concentration of the inducer aTc. Error bars stand for the standard deviation of 3 independent repeats. (n=3)

5.3.2 Repression of the hybrid promoters by tetR

Next, we introduced the repressor protein tetR to the system by overexpressing it on the same plasmid that also contains the reporter gene cassette driven by $P_{rrsC-O1}$ or $P_{rrsC-O1-O1}$ (Figure 5.2A). We first employed the promoter P_{cat}, which drives the expression of the CAT antibiotics marker in the widely used broad host-range plasmids pBBR122 and pBHR1 (MoBiTec, Göttingen, Germany) [17] and has been used for heterologous gene expression in *R. eutropha* H16 [18].

When $P_{cat}::tetR$ cassette was added (Figure 5.2A), the $P_{rrsC-O1}$ and $P_{rrsC-O1-O1}$ promoters can be repressed (Figure 5.2B). The gene expression level can be regulated with different concentration of the inducer as measured by the CAT reporter assays (Figure 5.2B). Noticeably, the $P_{rrsC-O1-O1}$ promoter had lower leaky expression (the basal expression when no aTc was added) compared to $P_{rrsC-O1}$ (Figure 5.2B), which is possibly attributed to its extra tetR binding site (Figure 5.1A). Thus, further development was focused on the $P_{rrsC-O1-O1}$ promoter.

To further improve the stringency of the gene expression system, we tried to increase the expression level of the tetR repressor. We switched the promoter of the tetR expression cassette to P_{phaC1} (Figure 5.2A), which has been shown to be a relatively constitutive and strong promoter in *R. eutropha* H16 [6, 19]. Using β-galactosidase reporter assays, we showed that $P_{phaC1}::tetR$ cassette indeed lowered the leaky expression activity of the $P_{rrsC-O1-O1}$ promoter (Figure 5.2C). However, the strength of the resulted system plateaued at a very low level even with high concentration of aTc (160ng/ml) (Figure 5.2C), which suggested an overly tight repression caused by the high tetR level.

5.3.3 Identification of the suitable promoter to drive tetR expression using high-throughput promoter library screening

As shown above, the P_{cat} and P_{phaC1} promoters delivered too low or too high tetR level to match with $P_{rrsC-O1-O1}$ promoter, respectively. Therefore, it might be helpful to fine tone the tetR level with promoters of intermediate strength [20]. To identify such promoters, we constructed a promoter library by mutating the last four nucleotides of the -35 region in P_{phaC1} promoter (Figure 5.3A). The promoter library was used to drive the green fluorescent protein (GFP)

reporter gene, which enabled the high-throughput screening of promoter activity *in vivo* (Figure 5.3A). The *R. eutropha* H16 P_{phaC1} promoter has a -35 region sequence that is identical to the -35 sequence of the *E. coli* consensus σ70 promoters (5'-TTGACA-3') (Figure 5.3A). Previous studies have shown that *R. eutropha* H16 promoters with higher similarity to *E. coli* σ70 promoter consensus sequence tend to have higher activities [21]. Thus, mutations in the -35 region of P_{phaC1} were hypothesized to generate promoters of lower activity.
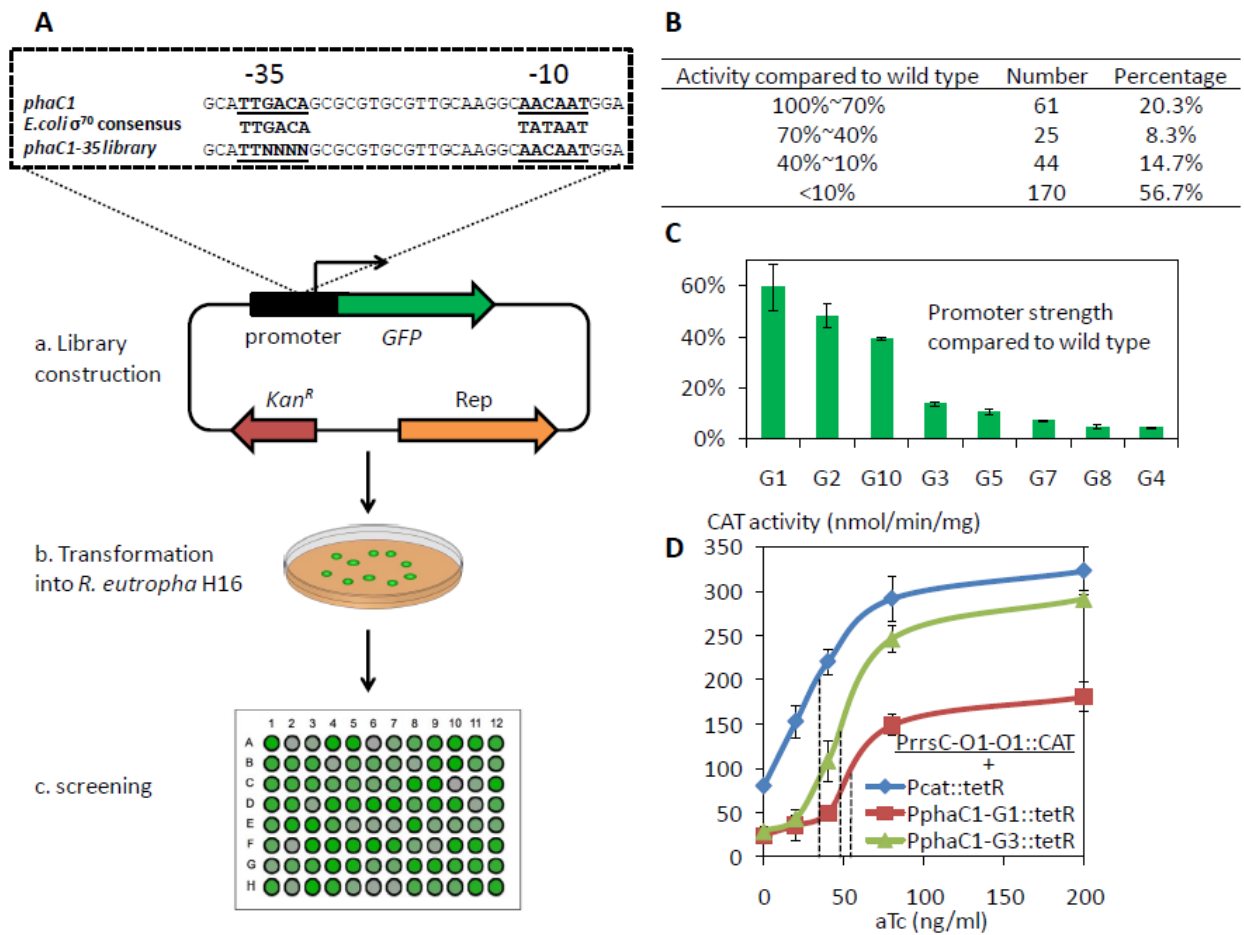
Figure 5.3 Identification of promoters of different strength to drive *tetR* expression using high-throughput promoter library screening. A) Illustration of the high-throughput promoter library screening method. a. To construct the library, the last 4 nucleotides of the -35 region of P_{phaC1} were randamized and inserted in front of the gfp reporter gene. b. The library was transformed into *R. eutropha* H16. c. Single colonies were picked and cultured in 96-well plates. Fluorescent of each sample was then measured using 96-well plate reader. B) Distribution of the promoter activities of 300 candidates measured. C) Characterization of 8 promoter variants from the screen with step-wise decreased activities as measured by GFP level. D) Induction profile of the $P_{rrsC-O1-O1}$ promoter in combination with $P_{cat}::tetR$, $P_{phaC1-G1}::tetR$ or $P_{phaC1-G3}::tetR$ with different concentration of the inducer aTc. The dash lines indicate the Ind50 (concentration of aTc required to achieve 50% induction). Error bars stand for the standard deviation of 3 independent repeats. (n=3)

A total of 300 candidates from the library were screened (Figure 5.3B). More than 50% of the candidates showed severely reduced activity (<10% compared to the wild type promoter) as measured by GFP level. ~20% of the candidates have slightly reduced activity (100%-70%). And the candidates with intermediate activities (70%-10%) were relatively hard to obtain. This all-or-non distribution of the promoter activities suggested that promoter activity is very sensitive to changes in the -35 region. Eight P_{phaC1} mutants were characterized, which have step-wise reduced activities compared to the wild type promoter (Figure 5.3C). Promoters $P_{phaC1-G1}$ (with -35 sequence of 5'-TTGACT-3') and $P_{phaC1-G3}$ (with the -35 sequence of 5'-TTCGGC-3') have ~60% and ~15% activity compared to the wild type P_{phaC1}, respectively. These two promoters have strength between that of P_{cat} and P_{phaC1} and were tested to drive the expression of tetR.

The induction profiles of the gene expression system containing $P_{phaC1-G1}::tetR$ or $P_{phaC1-G3}::tetR$ in combination with $P_{rrsC-O1-O1}::CAT$ were shown in Figure 5.3D. These two systems indeed had significantly lower leaky expression compared to the system with $P_{cat}::tetR$. The Ind50 (inducer concentration needed to yield 50% induction) of the three systems were Ind50(P_{cat})< Ind50($P_{phaC1-G3}$)< Ind50($P_{phaC1-G1}$) (Figure 5.3D, indicated by dash lines), which corresponds to

their levels of tetR expression. The system with the best dynamic profile contains $P_{phaC1-G3}::tetR$ in combination with the $P_{rrsC-O1-O1}$, which has relatively low leaky expression and a ~11 fold induction. The dynamic range is comparable with the L-arabinose controllable P_{BAD} system (~12 fold induction) [6] in *R. eutropha* H16, which has been the only non-native controllable gene expression system available in this organism.

5.3.4 Application of the aTc-controllable gene expression system

We next sought to demonstrate the application of the synthetic aTc-controllable gene expression system in metabolic engineering. In our previous work, production of biofuels isobutanol and 3-methyl-1-butanol has been achieved autotrophically in engineered *R. eutropha* H16 [9]. However, one of the key enzymes in the biofuel production pathway, the acetolactate synthase (encoded by *alsS* from *Bacillus subtilis*), caused toxicity when overexpressed in *R. eutropha* H16 especially in minimal medium (Figure 5.4A). As a result, the biofuel production strain reported previously can only have one copy of *alsS* integrated in the chromosome [9], since strains with high level *alsS* overexpession on a multiple-copy plasmid grow extremely poorly and are not viable in autotrophic condition. AlsS catalyzes the formation of acetolactate from pyruvate, which can also be catalyzed by the acetohydroxy acid synthase (AHAS) encoded by *ilvBH* in *R. eutropha* H16. Although ilvBH did not cause significant growth retardation (Figure 5.4A), much less biofuel was produced when ilvBH was used in place of AlsS (Figure 4.2). Thus, a dilemma exists that on one hand, AlsS cannot be replaced by ilvBH and needs to be highly overexpressed to achieve high biofuel production; on the other hand, AlsS's toxicity effect needs to be overcome.
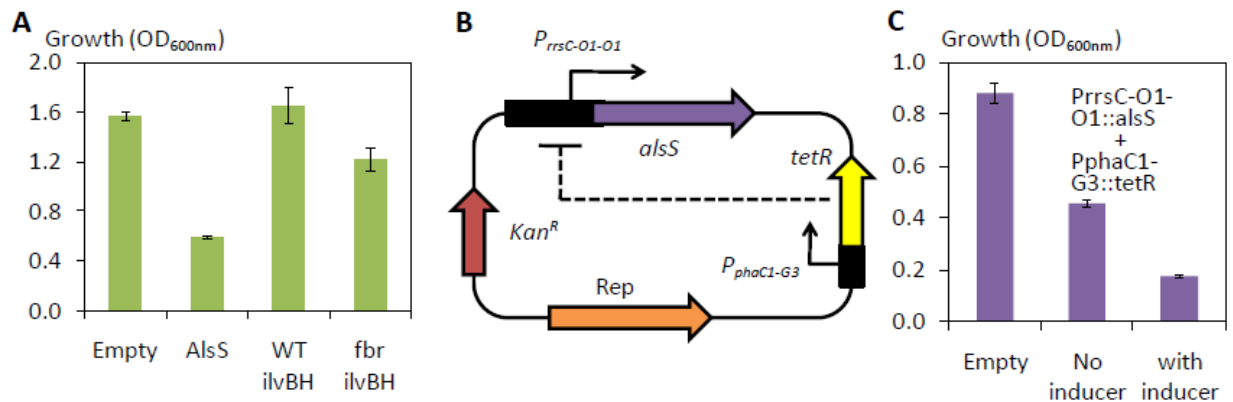
Figure 5.4 Application of the developed aTc-controllable gene expression system in alleviating the AlsS toxicity. A) Overexpression of the *alsS* from *Bacillus subtilis* or the feedback resistant *ilvBH* (*fbr ilvBH*) from *R. eutropha* H16 on multiple-copy plasmid caused growth retardation, while the wild type *ilvBH* has no toxicity effect. B) Plasmid map showing the *alsS* under the control of $P_{rrsC-O1-O1}$ promoter in combination $P_{phaC1-G3}::tetR$. C) With no inducer added, the aTc-controllable gene expression system was repressed and the AlsS toxicity was alleviated. Error bars stand for the standard deviation of 3 independent repeats. (n=3)
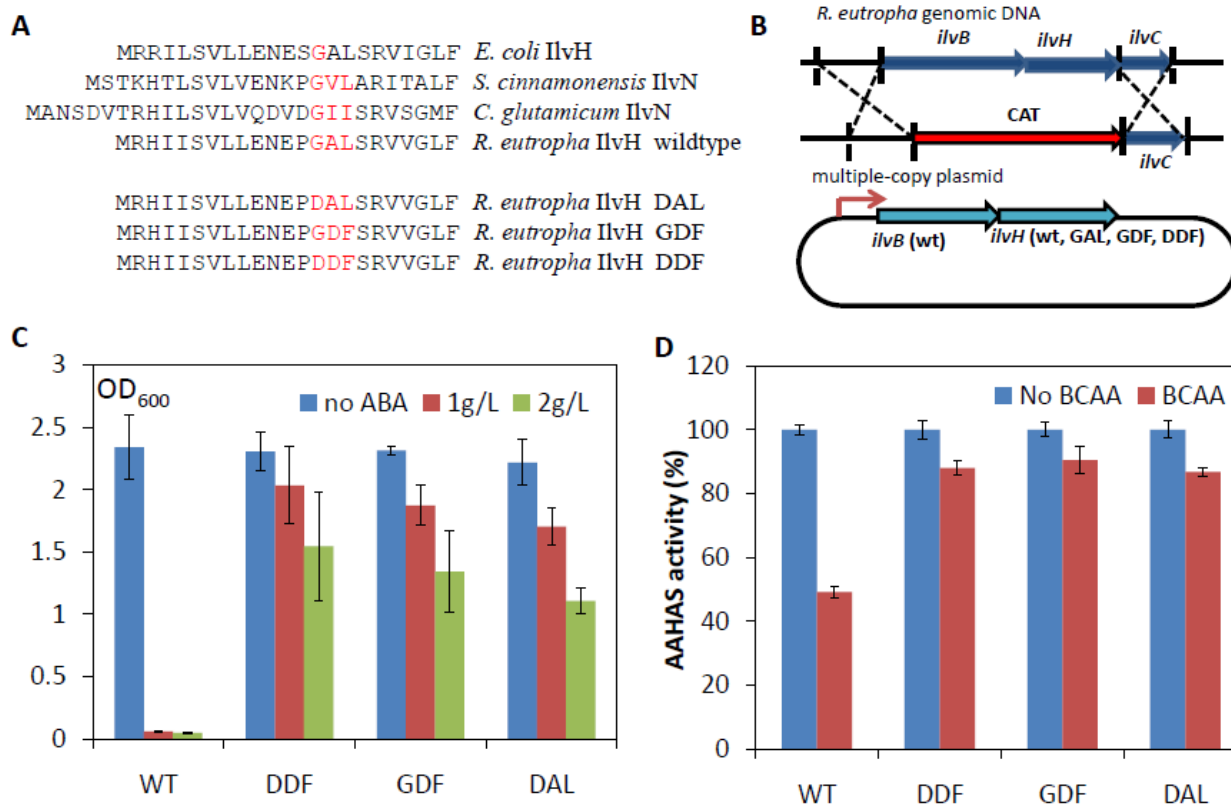

Figure 5.5 Construction and characterization of the feedback resistant ilvBH

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It is well known that AHAS enzymes such as ilvBH catalyze the first committed step of the branched-chain amino acid biosynthesis pathways and are subjected to feedback inhibition *in vivo* by downstream metabolites such as valine and leucine. On the other hand, the alsS in *B. subtilis* functions in the acetoin fermentation pathway and is not feedback regulated. Therefore, one hypothesis is that the unregulated activity of AlsS caused imbalance of the metabolic pathways *in vivo*. To test this hypothesis, feedback-resistant *R. eutropha* H16 ilvBH (fbr ilvBH) was constructed by mutating the allosteric regulator binding site in the ilvH protein (Figure 5.5).

Ralstonia eutropha H16 native acetohydroxy acid synthase (AHAS) is composed of the large catalytic subunit ilvB and the small regulatory subunit ilvH. Previous studies have shown that conserved amino acid residues at the N-terminus of the small subunit play important role in binding the feedback regulators [22]. By homologous sequence alignment, we identified G14, A15, L16 (GAL) residues in *R. eutropha* H16 ilvH as the potential feedback regulatory sites, and mutated GAL to DAL, GDF, and DDF, respectively, according to previous studies (Figure 5.5A)[22]. To characterize these mutant AHASs in *R. eutropha* H16 without the interference from the endogenous wild type ilvBH, we performed in-frame knockout to disrupt the *ilvBH* genes. Next, the *ilvB* gene together with the wild type, DAL, GDF, and DDF *ilvH* gene, respectively, were introduced to the knockout strain using a multiple-copy plasmid (Figure 5.5B). The valine analog 2-aminobutyrate (ABA) can inhibit cell growth by mimicking valine to inhibit AHAS activity by feedback inhibition. Therefore, feedback resistant AHAS is not inhibited by ABA and thus can rescue growth. Our results showed DAL, GDF, and DDF *ilvH* mutants, but not the wild type *ilvH*, rescued cell growth when challenged with 1g/L or 2g/L ABA in minimal medium with fructose as the sole carbon source (Figure 5.5C). Using crude cell extract, AHAS activity was also assayed in the presence of branched-chain amino acids (BCAA) valine, leucine,

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and isoleucin, which are common feedback inhibitor of AHAS. The results showed that all three mutants maintained relatively high activity even with the presence of feedback inhibitor (Figure 5.5D). In summary, these results suggest that all three AHAS variants are feedback resistant and functional both *in vivo* and *in vitro*. The DDF ilvBH was used to conduct further study.

Consistent with the hypothesis, the fbr DDF ilvBH also caused growth retardation (Figure 5.4A). These results indicate that the activity of AlsS needs to be delivered in a controllable manner. In fact, the expression of the *alsS* operon is tightly controlled in its native host *B. subtilis* on transcriptional level by an inducible promoter [23].

We placed the *B. subtilis alsS* under the control of the $P_{rrsC\text{-}O1\text{-}O1}$ promoter on a multiple-copy plasmid, which also contains the $P_{phaC1\text{-}G3}::tetR$ repressor cassette (Figure 5.4B). When the strain is cultivated in minimal medium with the inducer aTc, growth retardation was observed (Figure 5.4C), suggesting that *alsS* was expressed with relatively high level. When inducer was not added, the cell reached higher OD compared to the induced condition (Figure 5.4C), suggesting that the expression level was repressed and the toxicity effect was alleviated.

In the process of developing the aTc-controllable gene expression system, tetO operators were placed in the P_{rrsC} promoter between -35 and -10 region or downstream of the transcriptional start site (Figure 5.1A, B, C). The activity of the resulted promoters varied depending on the position and the sequence of the operators. Specifically, the tetO2 operator affected the promoter activity adversely at both positions. Similar phenomenon was also seen during the construction of synthetic aTc-controllable promoters in *Clostridium acetobutylicum* [16], but not in *E. coli* [13] or *Synechocystis sp* [24]. Thus, a screen is needed to survey different hybrid promoter configurations when adapting the aTc-controllable system to a new host. More understanding on

transcription machinery of the host might be useful for predicting the favorable promoter configurations and thus narrowing down the candidates to be screened.

The dynamic range of the aTc-controllable system developed in this study is not optimal compared to similar systems in *E. coli* [13] or *Synechocystis sp* [24], which is largely due to the relatively high leaky expression. The leaky expression may be responsible for the incomplete rescue of AlsS toxicity (Figure 5.4C). In this system, the relatively strong promoter P_{rrsC} was chosen as the backbone with the rationale that it may deliver high expression when fully induced. However, strong promoters may also be difficult to repress completely. In this study, we improved the stringency of the system by introducing two tetO operators to P_{rrsC} and tuning the tetR expression level. Previous studies have shown that small changes in the promoters involving only a few nucleotides can cause critical changes in the regulation profile of the system [24]. Further optimizations are possible by systematically mutagenizing other regions in both the working promoter and the promoter driving tetR.

Controllable gene expression systems, especially the non-native ones, are extremely useful in metabolic engineering studies. In the case of the AlsS toxicity issue, high level isobutanol production has been achieved with the inducible P_{LlacO1} system in recombinant *E. coli* [11]; while in recombinant *Clostridium cellulolyticum*, constitutive *alsS* expression inhibited growth and hampered biofuel production [25]. In this study, AlsS toxicity was alleviated using the developed aTc-controllable system. Further studies are needed to test the biofuel production performance using this system in *R. eutropha* H16.

5.4 Conclusions

In conclusion, we developed an aTc-controllable gene expression system in *R. eutropha* H16 which can be gradually regulated with different aTc concentration with a ~11 fold dynamic range. A tetR repressable promoter was first constructed by hybridizing the tetO operator with the *R. eutropha* H16 P_{rrsC} promoter. The regulation profile of the system was then improved by fine tuning the expression level of the repressor tetR using suitable mutant promoters of P_{phaC1}, which were identified from a high-throughput promoter library screening. The AlsS toxicity issue, which impeded our metabolic engineering work on isobutanol production, was alleviated using the developed system. This aTc-controllable gene expression system is a useful synthetic biology tool for future scientific research and metabolic engineering in *R. eutropha* H16.

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6. Development of an integrated microbial process for one-pot biofuel synthesis directly from CO_2 and electricity

6.1 Introduction

Compared to H_2, formic acid would be a favorable energy carrier at the interface between electrolysis and microbial cells. Electrochemical production of formic acid from CO_2 and H_2O has been extensively studied and can achieve relatively high current efficiencies [1-3]. Formate is highly soluble and is readily converted to both carbon dioxide and NADH in a stoichiometric ratio by formate dehydrogenase in the cells, circumventing the poor mass transfer issue of both CO_2 and H_2 as gas substrates. However, the high solubility of formic acid increases the cost of product separation from electrochemical process. If not separated effectively, accumulated formate can be decomposed at the anode, reducing the yield of the process [1]. As such, an integrated process featuring simultaneous electrochemical formate production and biological formate utilization is desirable, since the costly product separation could be circumvented and no formate accumulation would occur. When producing compounds more reduced than formate, such as higher alcohols, more reducing power than CO_2 is required. Thus, excess CO_2 will be released by the microbes, which provide dissolved CO_2 in the vicinity of the working electrode to be reduced electrochemically. However, the adverse effect of the electrochemical process on microbial cells [4-6] needs to be addressed. As such, an integrated process for production of liquid fuel from electricity requires 1) metabolic engineering of a lithoautotrophic organism to produce liquid fuels, 2) electrochemical production of formate from CO_2, and 3) eliminating the adverse effect of electrolysis on microbial cells.

6.2 Method and materials

6.2.1 Electrochemical formate production

The setup of the electrolysis device is illustrated in Figure 6.1. Electrolysis was performed in a 500ml glass vessel containing 350ml German minimal medium [7] with the following modifications: the medium was supplemented with 1.6g/l $NaHCO_3$ and 10g/l Na_2SO_4 and the Hoagland solution was omitted. The reactor was placed in a 30^0C water bath and agitated by a magnetic stir bar. The reactor was capped with an air-tight silicon stopper. Holes were made in the stopper to allow electric wires and gas tubes to pass through.

A 5cm$\times$3cm platinum mesh (52 mesh woven from 0.1mm [0.004in] dia wire, 99.9% [metals basis], Alfa Aesar, MA, USA) was used as the anode and a 6cm$\times$10cm Indium foil (0.127mm [0.005in] thick, 99.99% [metals basis], Alfa Aesar, MA, USA) was used as the cathode. The anode was rolled and inserted in the porous ceramic cup (round bottom with straight wall, 2.1 μm pore size, 7/8" outer diameter, 0.563" inner diameter, 0.156" wall thickness, 3-1/8" length. Small Parts, USA). The CO_2 gas was bubbled with a glass gas sparger with the flow rate of 30-40ml/min. The air was bubbled with a thin rubber tube with the flow rate of 200mL/min. Electricity was provided by a direct-current power supply. The voltage between two electrodes was around 4V and current was around 250mA. The potential of the cathode was around -1.6V against the Ag/AgCl reference electrode. Electrochemical production of formate was detected by Agilent 1200 HPLC with a BioRad (Biorad Laboratories, Hercules, CA) Aminex HPX87 column (0.5mM H_2SO4, 0.6 ml/min, column temperature at 35^0C).

6.2.2 Growth Study using *E. coli* in electrolytic conditions

The effect of electricity exposure to microorganisms has been studied [4] [5]. And the production of the reactive oxygen and nitrogen species by Pt anode has been detected [8] [9]. In this study, the effect of electrochemical formate production on bacteria growth was studied using *E.coli* as a model.

E.coli XL-1 Blue cells were grown in LB medium overnight at 37^0C and harvested by centrifugation. The cells were washed twice with the minimal medium used in electrolysis (see above) and inoculated in the electrolysis reactor. 4g/l glucose was added as the carbon source. Electrolysis was performed as mentioned above except that no porous ceramic cup was used. And the anode was fixed to the CO_2 gas sparger in the center of the reactor to avoid touching the cathode. The control was performed in the same conditions except that the electricity was off all through the experiments.

6.2.3 β-galactosidase assays

The β-galactosidase assays were performed as follows: After growing overnight in rich medium (10 g/l peptone, 10 g/l yeast extract, 5 g/l beef extract, and 5 g/l $(NH_4)_2SO_4$), *Ralstonia* LH118, LH119, and LH120 cells were harvested and inoculated into the electro-microbial bioreactors Electrolysis was performed with the above-mentioned conditions except that 4g/l fructose was added as the carbon source. After electrolytic exposure for 3 hours, cells were harvested by centrifugation and concentrated 100 fold in minimal medium. OD_{600} of the concentrated cells was measured. For the control, electricity was off during the experiments.

The β-galactosidase reactions were started by adding 20-100μl of concentrated cells into a reaction mixture containing 100 μl chloroform, 50 μl 0.1% SDS, 200 μl ortho-nitrophenyl-β-galactoside (ONPG, 4mg/ml) , 950 μl Z buffer (Z buffer per 50 ml contains 0.80g $Na_2HPO_4 \cdot 7H_2O$, 0.28g $NaH_2PO_4 \cdot H_2O$, 0.5ml 1M KCl, 0.05 ml 1M MgSO4, and 0.135 ml β – mercaptoethanol). The assay tubes were vortexed for 10-15 seconds. The reactions were allowed to proceed for appropriate time (ranging from 30 to 200min depending on the strength of the promoters that drive *lacZ* expression). The reactions were stopped by addition of 500μl 1M Na_2CO_3. The tubes were centrifuged at 13,200 rpm for 1 min to separate chloroform. 1 ml solution of the aqueous layer was used to measure A_{420} and A_{550} using 1cm path-length cuvette. β-galactosidase activity was calculated as follows:

β-galactosidase activity (Miller unit ΔA_{420} min^{-1} ml^{-1}) =

$1000 \times (A_{420} - 1.75 \times A_{550}) / (reaction\ time\ (min) \times amount\ of\ the\ cells\ added\ (ml) \times OD_{600})$

6.2.4 Biofuel production by the integrated electro-microbial process

Ralstonia LH74D cells were grown in German minimal medium with 4g/l fructose to late log phase. Cells were then harvested and washed three times with minimal medium containing no carbon source. The washed cells were then inoculated into the electro-microbial bioreactors. The electrolysis was performed using the above-mentioned conditions. A porous ceramic cup was used to shield the anode. Evaporated alcohols in venting gas were condensed with a Graham condenser and collected. Daily, samples of culture broth and condensation liquid were taken and alcohols were quantified using gas chromatography (GC) [10].

In this process, H_2 was produced electrochemically as a by-product. Both formate and hydrogen can serve as the energy source to support cell growth and biofuel production. Since electrolysis

produces fine H_2 bubbles, mass transfer rate can be increased without mechanically dispersing large volume of gas [11], which is a significant energy cost in the conventional fermentation processes. Thus, hydrogen by-product will not be wasted [12].

6.3 Results and discussion

As discussed previously, supplying formate by in-situ electrochemical CO_2 reduction in culture medium may eventually increase efficiency and avoid product purification (Figure 6.1A). To test the feasibility of an integrated electro-microbial process, we tested Pb, In, Zn and other metals [2] as a cathode to reduce CO_2 to formic acid with H_2O as the proton source. At the anode (Pt mesh), O_2 is produced from H_2O, and is conveniently utilized by *Ralstonia* in the integrated process. By voltammetry study and the Faradaic yield measurement, we determined that the optimal potential is around -1.6V against the Ag/AgCl reference electrode for the formate production reaction using an In plate cathode in the German minimal medium [7] bubbled with air containing 15% CO_2 (data not shown). Under these conditions, formate can be generated at a relatively high rate, with hydrogen generated as a by-product. Both formate and hydrogen can serve as the energy source to support cell growth and biofuel production (Figures. 6.1B, C). Since electrolysis produces fine H_2 bubbles, mass transfer rate can be increased without mechanically dispersing large volume of gas substrate[11], which is a significant energy cost in the conventional fermentation processes. Thus, hydrogen by-product will not be wasted.

However, when *Ralstonia* cells were inoculated in the electrochemical reactor, no growth was observed. Growth study using the fast-growing microorganism *E. coli* showed transient inhibition of electrolysis on cell growth (Figure 6.1B). One possibility is that unstable toxic

compounds might be produced in the electrolysis reaction. When electricity is turned off, the inhibitory compounds decay quickly and the cell growth is resumed. We hypothesized that reactive oxygen species and reactive nitrogen species may be generated by the anode, thus causing growth inhibition. To test this hypothesis, three plasmid-based reporter construct were assembled. Each of the plasmids contain a *lacZ* gene driven by the promoter of the *Ralstonia* genes *katG* (encoding a catalase), *sodC* (encoding a copper-zinc superoxide dismutase), or *norA* (encoding an iron-sulfur cluster repair di-iron protein). The promoters of *katG*, *sodC* and *norA* have been shown to be activated by hydrogen peroxide (H_2O_2), superoxide free radicals (O_2^-) and nitric oxide (NO), respectively [13-15]. The plasmids were then transformed into the wild type *Ralstonia* strain H16. When the plasmid-bearing strains were exposed to electrolysis, expression of β-galactosidase from both *sodC* and *norA* promoters were greatly induced, but not for *katG* promoter (Figure 6.1C). These results were consistent with the arguments that O_2^- and NO might be generated on the Pt anode [8, 9], and suggested that these unstable reactive compounds trigger stress responses in *Ralstonia* cells and may be responsible for the transient growth inhibition.

To circumvent this toxicity problem, a porous ceramic cup was used to separate the cathode and the anode (Figure 6.1D). The porous ceramic material provides a tortuous diffusion path for chemicals. Therefore, the reactive compounds produced on the anode inside the cup may be decomposed before reaching the cells growing outside the cup. This strategy is more economical compared to the use of ion-exchange membranes to separate the electrodes. Using this approach, healthy growth of *Ralstonia* biofuel production strain LH74D on electricity and CO_2 was achieved. Over 140mg/L biofuels were produced in 4 days (Figure 6.1E). Further optimization of the culture condition is needed to achieve high productivity over a prolonged time period.
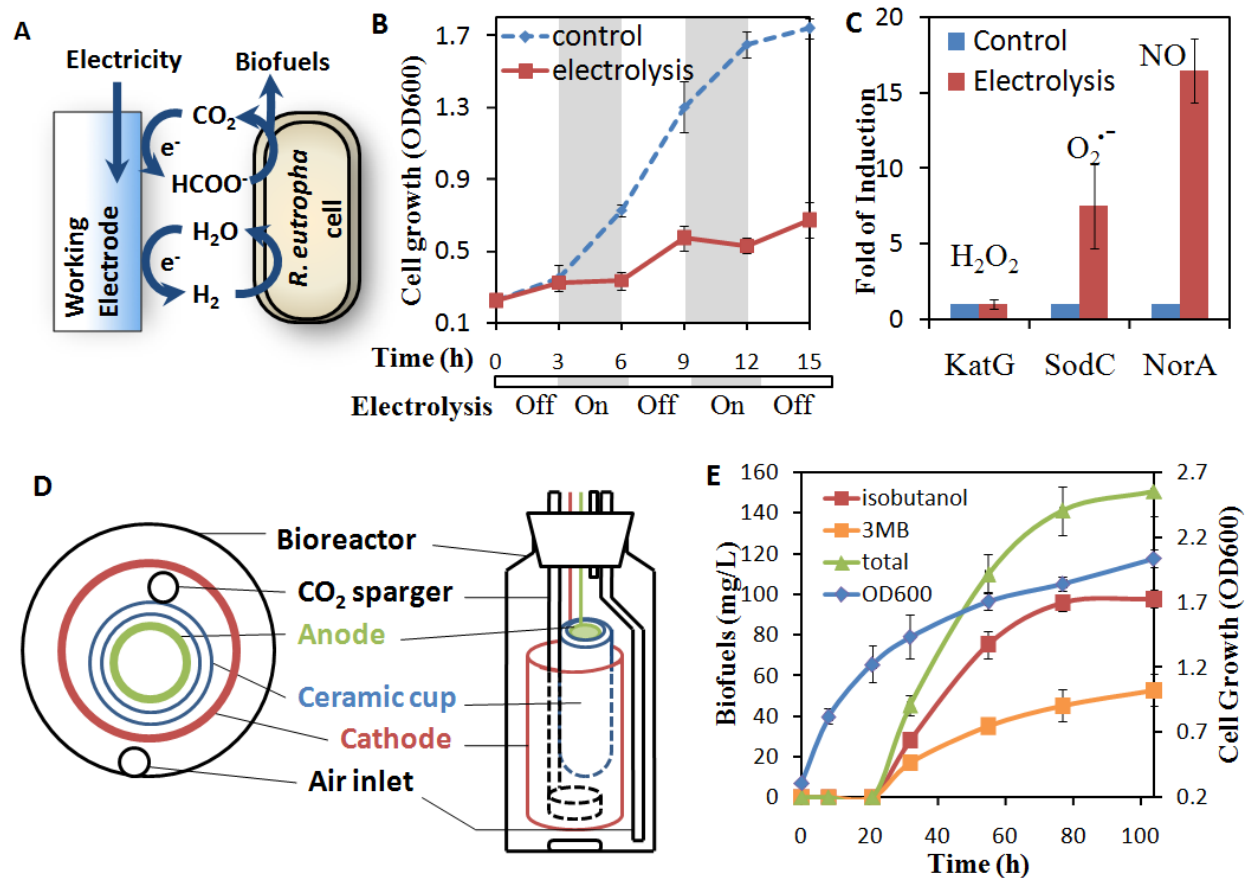
Figure 6.1 The integrated electro-microbial process for biofuel production from electricity and CO_2 (**a**) Schematic presentation showing the in-situ electrochemical CO_2 reduction (and H_2O splitting) coupled with biofuel production by the engineered *Ralstonia eutropha* strain. (**b**) Transient inhibitory effect of in-situ electrolysis on the growth of *E.coli* cells. (**c**) The induction of Ralstonia katG, sodC, and NorA promoters in electrolysis conditions. The katG, sodC, and NorA promoters are induced by hydrogen peroxide (H_2O_2), superoxide free radicals (O_2^-) and nitric oxide (NO), respectively. The promoters are used to drive the expression of the *lacZ* reporter gene. And the promoter activities are measured by the β-galactosidase assay. Error bars indicate standard deviation (n=3). (**d**) The configuration of the electro-microbial bioreactor. The cathode and the anode form concentric cylinders. The porous ceramic cup seperates the two electrodes. (**e**) Biofuel production by the LH74 strain (described in the text) in the integrated electro-microbial process. Error bars indicate standard deviation (n=3).

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Transfer of electrons to the microbes can occur either directly from an electrode or indirectly through an electron mediator (Figure 6.2). Early studies have suggested that some species such as *Geobacter metallireducens* [16] are able to accept electrons directly from electrodes as respiration electron donor. It has also been suggested that electricity current can directly drive methane production from CO_2 by a microbial biofilm containing predominantly a methanogen, *Methanobacterium palustre* [17]. Recently, a broad range of microorganisms including *Sporomusa* species, *Clostridium* species, and *Moorella thermoacetica* have been demonstrated to be able to accept electrons directly from electrode and produce native fermentation products such as acetate and 2-oxobutyrate from CO_2 [18, 19](Figure 6.2). These processes achieved high current efficiencies with low over potential. Moreover, one of the organisms that are shown to be capable of accepting electrons directly from electrodes, *Clostridium ljungdahlii* [20], has recently been engineered to produce 1-butanol, a gasoline substitute, from fructose. The results indicated the possibility that the direct electron transfer approach may be used to make liquid fuels using CO_2 and electricity.

However, several biological and engineering difficulties have to be considered. First, fundamental metabolic engineering barriers need to be overcome for the production of non-native liquid fuel. These anaerobic acetogenic microbes do not perform respiration and rely largely on fermentative pathways to generate ATP. If the carbon flux is diverted away from the native fermentation product formation, the overall fuel yield may be drastically reduced due to energy deficiency [21]. To solve this problem, basic energy conversion mechanism and metabolic regulations in these organisms need to be understood. Alternatively, acetate produced by acetogens from CO_2 can be fed to an aerobic organism in a separate reactor to produce long carbon chain products [22]. Second, direct electron transfer involves a 2D biofilm-based

production process [23], which enjoys the favorable features of long-term stability and robustness in lab scales but has not been used in industrial scales [24, 25]. For the microbes to accept electrons, they need to be constrained to the biofilms on the electrodes. As such, a large electrode surface area is needed to support large scale production, which represents higher cost and requires specially designed bioreactors.

Alternatively, electrons can be delivered to the microbes via chemicals (Figure 6.2). To be sustainable in large scale, these carriers need to be derived from electrochemical reactions with minimal cost and without adverse environmental effects. Hydrogen and formic acid are two top choices for this purpose (Figure 6.2).

Other alternative electron carriers have also been reported (Figure 6.2). Growth of the lithoautotroph *Nitrosomonas europaea* using CO_2 and electricity-generated ammonia is one example [23]. In this case, *N. europaea* cells utilized the reducing energy in ammonia and secreted the oxidized end product nitrite, which was continuously recycled to a separated electrochemical module and reduced back to ammonia with around 100% current efficiencies. The challenge of this system is the low growth rate and the evaporation of ammonia from the system, causing drop in efficiency. Another chemolithoautotroph *Acidithiobacillus ferrooxidans* can utilize Fe^{2+} as the energy source to power carbon fixation [26-28], which makes it another attractive host for eletrofuel production. Some artificial redox carriers such as neutral red can also be used to introduce reducing energy to biological systems to drive metabolism [29], which suggest the possibilities that novel artificial electron carriers with different chemical properties could be designed to deliver electrons to desired microorganisms. However, the cost for their large scale production and environmental impacts need to be addressed.
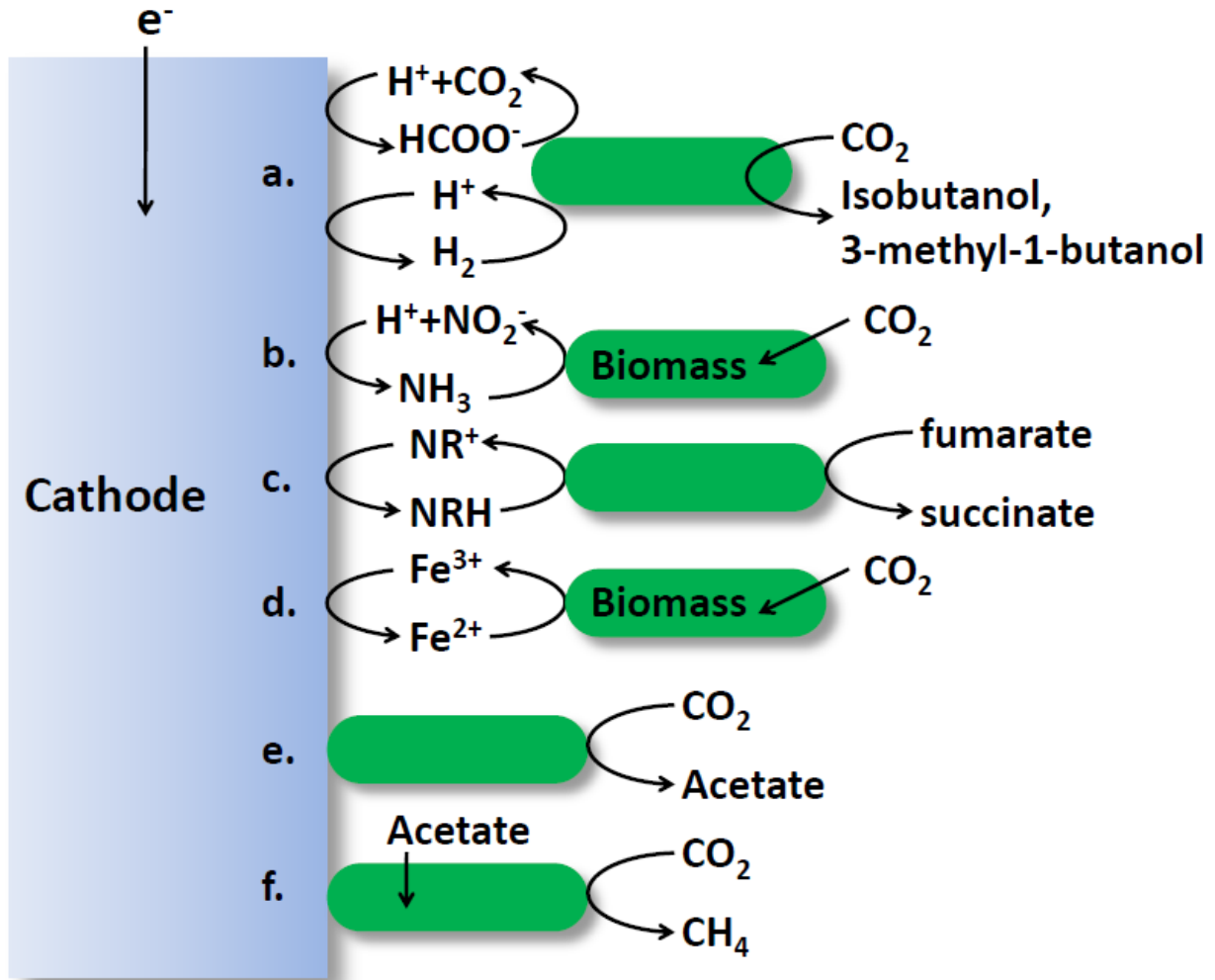
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Figure 6.2 Direct and indirect methods to transfer electrons to the microorganisms. a) Hydrogen and formate as the mediators to deliver electrons to engineered *Ralstonia eutropha* cells for higher alcohols production from carbon dioxide. b) Ammonia as the electron donor for the autotrophic growth of *Nitrosomonas europaea* cells. c) Reduced Neutral Red (NRH) delivers electrons to support growth and fumarate reduction in *Actinobacillus succinogenes*. d) *Acidithiobacillus ferrooxidans* can utilize Fe^{2+} as the energy source to power carbon fixation and support growth. e) A broad range of microorganisms including *Sporomusa* species, *Clostridium* species, and *Moorella thermoacetica* accept electrons directly from electrode and produce native fermentation products such as acetate from CO_2. f) A microbial biofilm containing predominantly a methanogen, *Methanobacterium palustre,* directly obtains electrons from cathode to drive methane production from CO_2. Acetate was also present to support the growth of the biofilm.

6.4 Conclusions

In summary, we demonstrate the feasibility of conversion of electricity to high-energy-density liquid fuels in an integrated process using an engineered *R. eutropha* strain as the biocatalyst and CO_2 as the carbon source. The electro-microbial process first generates formate or hydrogen as the diffusible reducing intermediates, which then drive the microbial reduction of CO_2 to isobutanol and 3MB. This process does not depend on the biological "light reaction"; and the electricity generated from photovoltaic cells or wind turbines, or off-peak grid power can be used to drive CO_2 fixation and fuel production. Thus, it provides a way to store intermittent renewable energy in liquid transportation fuel with high energy density.

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