Engineering nonphosphorylative metabolism for the biosynthesis of sustainable chemicals

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Dedication

To my family

Abstract

Lignocellulosic biomass is one of the largest sources of organic carbon on Earth with the potential to replace fossil fuels for the production of transportation fuels and chemicals. The two biggest challenges facing biosynthesis is the limited natural metabolic capacity of microorganisms and the effective utilization of lignocellulosic biomass. To overcome the first obstacle, over the past several decades researchers have successfully expanded the natural metabolic pathways of microorganisms to allow biosynthesis of a wide array of compounds with applications as advanced biofuels, industrial chemicals, and pharmaceuticals. Most industrial fermentations convert glucose, the major sugar present in biomass, into a value added chemical but are unable to utilize pentose sugars which make up ~30% of a typical biomass feedstock. To improve the overall economics of fermentation process, it is important to ensure that all major sugars present in the feedstock are efficiently converted to target chemicals. This work addresses both these challenges by establishing a novel alterative pathway called nonphosphorylative pathway in *Escherichia coli* which enables the utilization of underutilized pentose sugars such as D-xylose and L-arabinose using fewer steps and with higher theoretical yields than conventional glycolysis and pentose phosphate pathways (PPP). This nonphosphorylative pathway can convert D-xylose and L-arabinose to 2-ketoglutarate (2-KG), an important TCA cycle intermediate, using less than 6 steps. A growth selection platform based on 2-ketoglutarate (2-KG) auxotrophy was designed in *E. coli* to confirm the functionality of nonphosphorylative metabolism in host organism. The growth selection platform was also used to mine nonphosphorylative gene clusters from other organisms

with improved activity. The pathway was then expanded to allow biosynthesis of two commercially important chemicals, 1,4-butanediol (BDO) and γ-aminobutyric acid (GABA). To improve production titers and yields of the process, protein engineering was used to reduce by-product formation and metabolic engineering was used to eliminate competing pathways and increase carbon flux towards the target compound. Furthermore, to improve uptake of pentoses by *E. coli*, pentose transporter was overexpressed to allow better carbon utilization. This nonphosphorylative metabolism serves as an efficient platform for biosynthesis and can be extended to produce a variety of compounds derived from TCA cycle including, but not limited to, L-glutamate, mesaconate, 5-aminolevulinic acid, and glutaconate. While the nonphosphorylative pathway has been successfully used for conversion of simple pentose sugars into important chemicals like BDO and GABA, the breakdown of biomass into these pentoses is the bigger challenge. This work also briefly addresses this challenge by comparing different acid hydrolysis treatment conditions to breakdown arabinoxylans in wheat bran into sugars - glucose, D-xylose, and L-arabinose - which can then be used in fermentation via nonphosphorylative metabolism.

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Chapter 1

Introduction

1.1 Motivation

Energy is essential in our daily lives and most of our energy comes from burning fossil fuels like petroleum, coal, and natural gas. According to the US Energy Information Administration (EIA), fossil fuels meet 81% of the U.S. energy demand.[1] Fossil fuels take millions of years to form within the Earth and scientists around the world estimate we will run out of fossil fuels within the next 50 to 120 years.[2] Although the United States is the world's largest producer of natural gas today, these resources are limited in supply and with the current explosion in global population and energy demand, we will soon run out of these non-renewable sources of energy. We currently consume 90 million barrels of oil each day and this will keep rising with the growing population. Our dependence on fossil fuels has led to many environmental problems including climate

change, air pollution, oil spills, and acid rain. The World Bank estimates that 5.3 trillion cubic feet of natural gas, making up 25% of US total consumption, is flared annually worldwide, generating 400 million tons of unnecessary carbon dioxide emissions.[3] Of the many environmental damages, the most serious and potentially irreversible consequence of burning fossil fuels is global warming. In 2014, approximately 78% of US global warming emissions were energy-related emissions of carbon dioxide.[4] Furthermore, while US was once self-sufficient in oil, in 2015 24% of the oil consumed in the US was imported from foreign countries.[1] These rising environmental and national security concerns are the major drivers for the search for alternative, renewable and green energy sources.

One of the most promising alternatives to fossil fuels is lignocellulosic biomass, generated from atmospheric CO_2, water, and sunlight through photosynthesis. It is considered to be the only sustainable source of organic carbon on Earth with potential to replace fossil fuels for the production of transportation fuels and fine chemicals with net zero carbon emission. The worldwide production of lignocellulosic biomass is estimated to exceed 220 billion tons, which can support global oil consumption for more than 15 years.[5] While lignocellulosic biomass is an inedible feedstock, there has been a lot of debate on the use of edible biomass such as corn, sugarcane and other starch and sugar crops for the production of fuels and chemicals. The limited supply of these crops could lead to competition with food production resulting in increasing food prices. This “food versus fuel” debate has moved the focus of researchers towards using agricultural waste products as feedstocks for biosynthesis of sustainable chemicals and fuels. Disposal of

these forestry and agricultural residues causes severe environmental problems and from an economic point of view, lignocellulosic biomass is produced more quickly and is cheaper than food crops such as corn starch and sugar cane.

The biggest challenge that limits the applicability of biosynthesis for production of chemicals and fuels is that most of the target compounds are not part of the natural metabolism of microorganisms. To address this challenge, researchers use metabolic engineering, systems and synthetic biology strategies to expand the natural metabolic capacity of microorganisms by introducing artificial pathways and heterologous enzymes. My thesis work is aimed at designing novel biosynthetic pathways in *E. coli* for the synthesis of sustainable chemicals and polymers using artificial pathway design, metabolic engineering, and protein engineering techniques to improve production titers and yields.

1.2 Engineering artificial biosynthetic pathways

Efficient and sustainable microbial production is a promising alternative to help the transition from a fossil-based economy to an economy based on renewable feedstocks. By 2020, biomanufacturing is predicted to provide cheaper and more sustainable synthetic routes for a wide array of diverse products in sectors such as cosmetics, polymers, flavors, and pharmaceuticals. According to the goals set by the U.S. Department of Energy, by 2025 30% of the transportation fuels should be replaced with biofuels and 25% of industrial chemicals will be derived from biomass.[6] Recent advances in metabolic engineering, systems and synthetic biology has enabled the production of advanced biofuels with properties similar to petroleum-derived fuels in industrial host

organisms such as *Escherichia coli* and *Saccharomyces cerevisiae*. There has also been a tremendous increase in the number of key building block chemicals and fine chemicals produced microbially from biomass feedstocks.

Biosynthesis, in the form of fermentation, has been around for centuries and in fact natural fermentation precedes human history. The earliest evidence of an alcoholic drink made from fruit, rice, dates back to 7000 B.C. in the village of Jiahu, China. Over the centuries, scientists have understood the fermentation process, identified the enzyme responsible for ethanol production, and have expanded the application of fermentation for the production of bread, cheese, vinegar, organic acids, amino acids, and pharmaceutical compounds such as penicillin and other drugs and antibodies. Traditionally, biosynthesis was limited to compounds found in nature and metabolic engineering efforts were used to manipulate the natural metabolic pathways of host organisms to improve production titers of metabolites such as ethanol[7-9], lactic acid[10-13], citric acid, and amino acids including lysine[14-15] and glutamic acid.[16] With progress in recombinant DNA technology, today the focus of metabolic engineering has shifted to produce almost any desired compound ranging from a small molecule drug such as artemisinin[17-19] to large complex polymers like spider silk.[20] This vast potential of metabolic engineering has resulted in an explosion in the diversity of molecules produced by engineered microbes and has enabled sustainable production of polymers, fuels, pharmaceuticals, and industrial chemicals.

In this section, some of the most widely used biosynthetic pathways for advanced biofuels and chemical production has been discussed. The four main pathways are 2-ketoacid pathway, fatty acid biosynthesis pathway, reverse β-oxidation pathway, and

isoprenoid pathway. All of these pathways allow carbon chain elongation and have been engineered to produce various commercially relevant compounds and several studies have also compared the efficacy of these different pathways for the production of same compound.

1.2.1 2-keto acid pathways

Amino acid biosynthesis is vital for every living organism and L-amino acids have an annual market volume of over 5 million tons. 2-ketoacids are key intermediates of the amino acid biosynthetic pathways organic compounds with a ketone group adjacent to the carboxyl group. The successes in fermentative production of natural amino acids such as glutamic acid and lysine[14, 21-22] , opened up the possibility to artificially expand these pathways for the production of higher-chain alcohols, carboxylic acids, pharmaceuticals, and other chemicals.

Higher alcohols are great substitutes for gasoline but natural metabolic pathways for higher alcohol production are not optimum. To improve production, a synthetic approach was developed where 2-ketoacid intermediates of branched aliphatic amino acids were first decarboxylated to aldehydes by ketoacid decarboxylase (KDC) and then reduced to alcohols by the action of alcohol dehydrogenase (ADH). Alcohols with naturally existing 2-ketoacid precursors such as isobutanol and C5 alcohols such as 2-methyl-1-butanol and 3-methyl-1-butanol were overproduced by expressing appropriate KDC and ADH enzymes (**Figure 1.1**). After screening several different KDCs and ADHs, KivD from *Lactococcus lactis* and Adh2 from *Saccharomyces cerevisiae* were chosen as the best combination of enzymes for the synthesis of 1-propanol, 1-butanol,

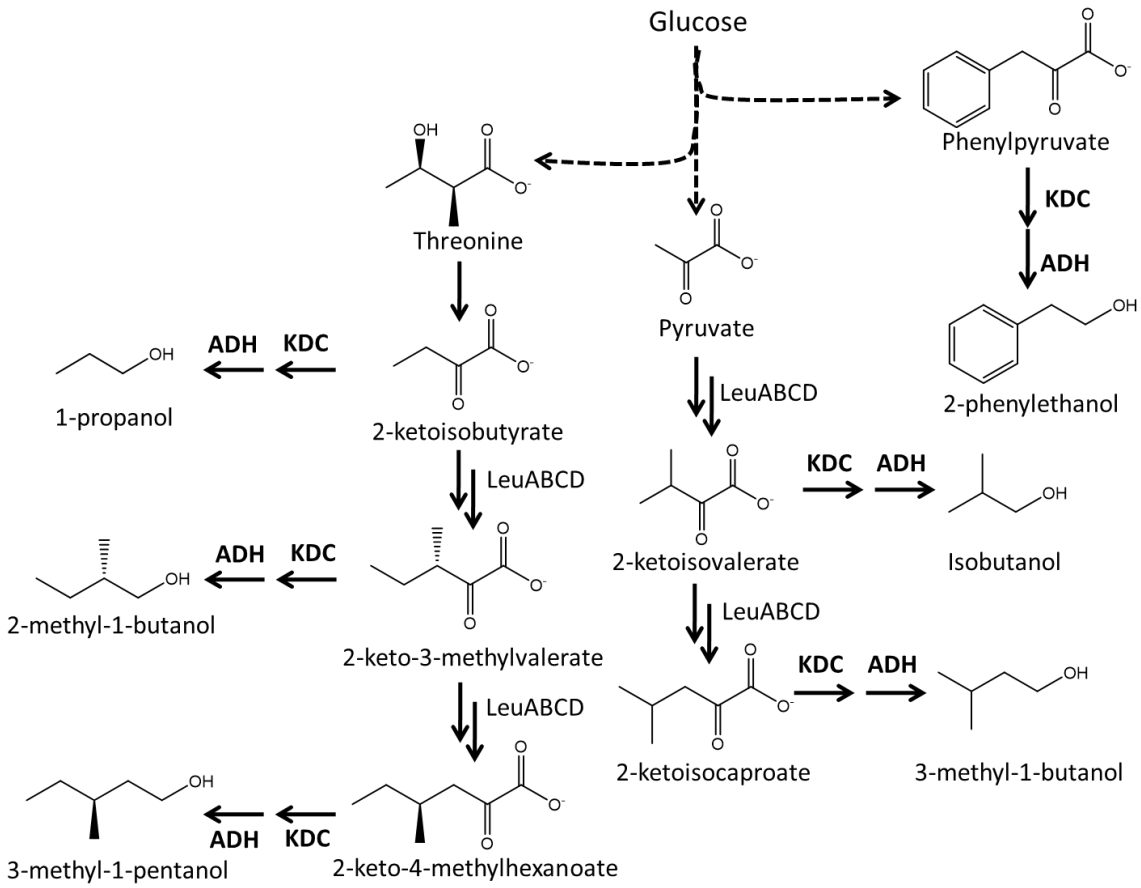

Figure 1.1 Expanding 2-ketoacid pathways for the production of alcohols. Deacrboxylation of 2-ketoacid is catalyzed by 2-ketoacid decarboxylase (KDC) followed by reduction of aldehyde by aldehyde dehydrogenase (ADH).

isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol.[23-26] Overexpression of 2-ketoisovalerate biosynthetic genes, deletion of pyruvate consumption pathways, and the use of *B. subtilis* acetolactate synthase enzyme with higher specificity for pyruvate resulted in 22 g/L of isobutanol in *E.coli*.[23] Higher alcohols with more than 5 carbon atoms do not have naturally occurring 2-ketoacid intermediates since the amino acid biosynthetic pathway is limited by carbon length. To enable the production of these compounds, a "+1" elongation pathway was developed to extend the carbon chain of existing 2-ketoacid intermediates and produce non-natural C6-

C9 2-ketoacids.[27-28] The LeuABCD gene cluster was employed for elongation of the carbon backbone and protein engineering was used to enlarge the binding pocket of 2-isopropylmalatesynthase (LeuA) to expand its substrate range allowing the production of alcohols such as 3-methyl-1-pentanol, 4-methyl-1-pentanol, 4-methyl-1-hexanol and 5-methyl-1-hexanol.[27-28] These studies demonstrate the importance of protein engineering and pathway optimization for the successful biosynthesis of commodity chemicals and biofuels.

In addition to alcohols, the 2-ketoacid pathway has also been used for the production of carboxylic acids where aldehydes produced by decarboxylation of 2-ketoacids are oxidized to acids. The *E.coli* enzyme, phenylacetaldehyde dehydrogenase (PadA)[29], was used to produce isobutyrate[30] and isovalerate[31-32] at high titers and *Burkholderia ambifaria* α-ketoglutaric semialdehyde dehydrogenase was used for isocaproate production.[32]

Apart from these aliphatic compounds, aromatic 2-ketoacids, phenylpyruvate (PP) and 4-hydroxyphenylpyruvate (HPP), have been used for biosynthesis of polymer building blocks[33], flavonoids[34], coumarins[35], stilbenes[36], and aromatic polyketides[37], using transamination chemistry.

1.2.2 Fatty acid biosynthetic pathway

Fatty acids are the basic components of cell membranes and constitute a major portion of cell mass. Free fatty acids (FFAs) are non-esterified carboxylic acids containing acyl chains from 4 to 18 carbons and are produced in cells through enzymatic cleavage of

lipids and acyl-thioesters.[38] FFAs can be converted to petroleum-derived diesel compounds using a single step acid-catalyzed esterification step and can also be catalytically decarboxylated to linear alkanes in the diesel range.[39] Fatty acid metabolic pathway is an attractive route for the production of high-energy density liquid transportation fuels and higher value oleochemicals. In the oleochemical industry, free fatty acids (FFAs) are precursors for agrochemicals, biocidal agents, soaps, surfactants, and polymer additives.[38] Overproduction of fatty acids in engineered microorganisms such as *E. coli* has been demonstrated for several decades and the use of this pathway for production of a plethora of microbial fuels and fatty acid derivatives including fatty acid esters, fatty alcohols, alkanes, alkenes, and long-chain dicarboxylic acids have been elucidated in many reviews.

The first step in type II fatty acid biosynthesis pathway (**Figure 1.2a**) in *E. coli* is the conversion of acetyl CoA to malonyl CoA catalyzed by acetyl-CoA carboxylase enzyme (AccABCD). Acetyl-CoA carboxylase, AccABCD is a 4-subunit enzyme that requires biotin as a co-factor and uses ATP for transfer of bicarbonate to the substrate. Malonyl CoA, the donor for two carbon extender units for chain elongation, is subsequently transferred to ACP (acyl carrier protein) by malonyl-CoA: ACP transacylase (FabD). Malonyl-ACP and acetyl-CoA are condensed in the first cycle of the pathway by β-ketoacyl-ACP synthase III (FabH) in a Claisen condensation reaction to form a β-ketoacyl-ACP. For successive iterations of the cycle, malonyl-ACP is condensed with acyl-ACP using different condensing enzymes, FabB and FabF. The second step of pathway is reduction of the β-keto group in β-ketoacyl-ACP to produce β-

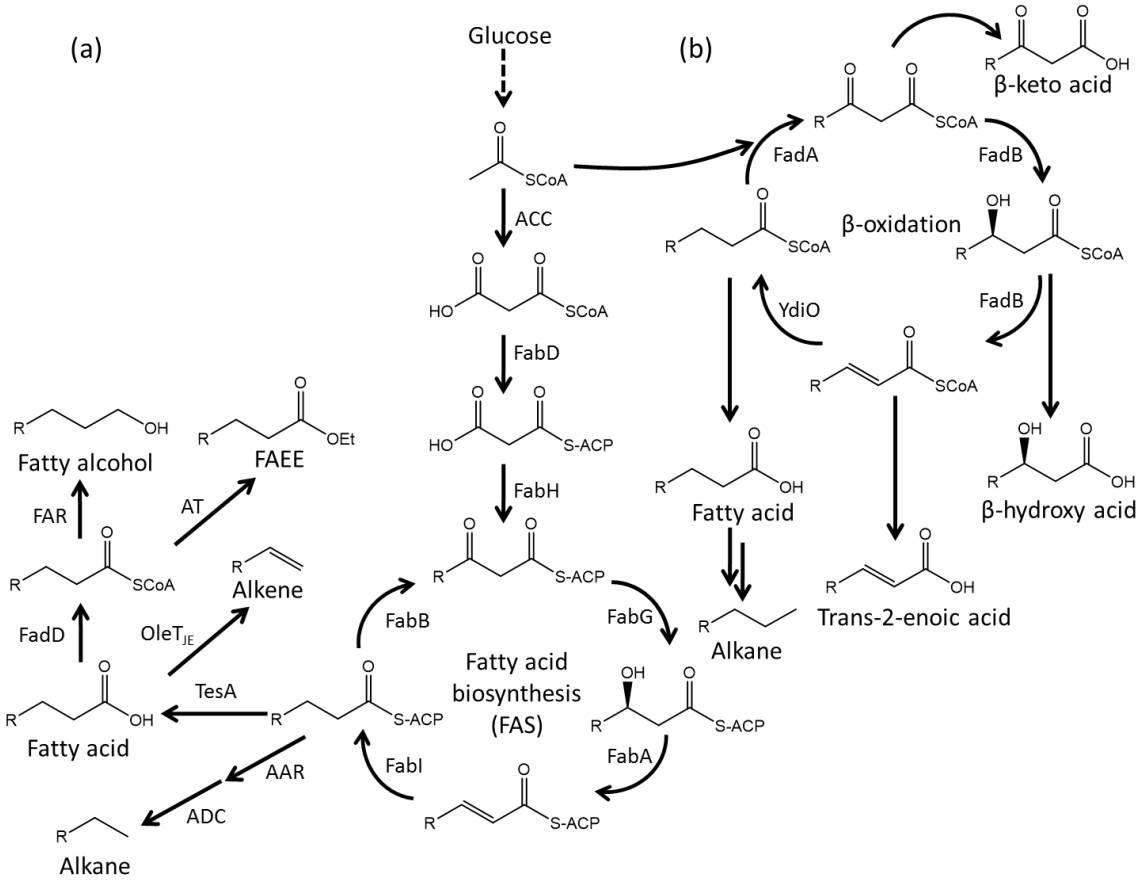

Figure 1.2 (a) Fatty acid biosynthesis pathway (FAB) for the production of fatty acids, fatty alcohols, fatty acid ethyl esters (FAEE), alkanes, and alkenes. (b) Reverse β-oxidation pathway for the biosynthesis of fatty acids, β-ketoacids, β-hydroxyacids, trans-2- enoic acids, and alkanes.

hydroxyacyl-ACP using the NADPH-dependent β-ketoacyl-ACP reductase (FabG). This β-hydroxyacyl-ACP is then dehydrated by β-hydroxyacyl-ACP hydratase (FabA or FabZ) to enoyl-ACP which is finally reduced to a saturated acyl-ACP by action of NADH-dependent enoyl-ACP reductase (FabI). This cycle continues until long-chain acyl ACPs (C16, C18) are incorporated into the phospholipids by acyltransferases or converted to other useful metabolites. Thioesterases (TesA and TesB) present in *E. coli* can convert acyl-ACPs to free fatty acids.[38-40]

The two critical steps for overproduction of FFAs is blocking FFA consumption by eliminating the β-oxidation pathway and optimizing thioesterase expression to eliminate feedback inhibition of key FAB pathway enzymes by accumulation of long chain acyl ACPs. Overexpression of acetyl-CoA carboxylase in *E. coli* resulted in a 5-fold improvement in FFA titer.[41] Using four distinct genetic changes, *E.coli* was engineered to produce 2.5 g/L total fatty acids with 50% being in the free fatty acid form and <10% excreted into the fermentation medium. Glycerol, a waste-product of plant-derived biodiesel, was used as the carbon source for fatty acid production and a conversion efficiency of 4.8% and a linear productivity of 0.024 g/h/g of dry cell mass was achieved.[42] The choice of thioesterase is critical in fine-tuning the FAB pathway for a specific carbon chain length. By overexpressing *E. coli* thioesterase TesA, which exhibits a preference for C14 fatty acids, and by eliminating key enzymes of the β-oxidation pathway, ~1.2 g/L free fatty acids was obtained corresponding to 14% of the theoretical yield.[43] In the same study, ~400 mg/L of fatty acid ethyl esters (FAEE), a major component of biodiesel, was produced with a composition ranging from C12-C18 by overexpressing wax-ester synthase (*atfA*). In addition to FFAs and FAEEs, fatty alcohols, a potential biofuel has been produced at 60 mg/L by overexpressing an acyl-CoA synthase to activate fatty acids to acyl-CoAs followed by reduction by an acyl-CoA reductase.[43] The FAB pathway can also be used to synthesize alkanes by cleaving the thioester bond of acyl-ACP using an acyl-ACP reductase to generate an aldehyde. This fatty acid aldehyde is converted to an alkane by the action of an aldehyde deformylase which uses oxygen and NADPH to generate the alkane, formate, and water. The

overexpression of these two enzymes in *E. coli* allowed production of ~300 mg/L pentadecane, pentadecene, and heptadecene.[44-45] Apart from E. coli, *S. cerevisiae* has also been used for fatty acid-derived oleochemical and biofuel production[46-47] resulting in the accumulation of 10.4 g/L FFA in a fed-batch cultivation. The strain was also engineered to produce 0.8 mg/l alkanes and 1.5 g/L fatty alcohols by screening pathway enzymes, alcohol dehydrogenases and aldehyde reductases.[46] The FAB pathway has also been used for synthesis of terminal alkenes in *E. coli* by expressing a functionally promiscuous P450 enzyme from *Jeotgalicoccus* spp. (OleT) to decarboxylate free fatty acids to alkenes.[48] An industrial yeast strain, *Candida tropicalis*, was engineered for the production of long-chain dicarboxylic acids and ω-hydroxyfatty acids by extending the FAB pathway using a P450 enzyme capable of hydroxylating fatty acids at the ω-position. For ω-hydroxyfatty acids, the β-oxidation pathway and the enzymes capable of oxidizing the alcohol group were eliminated and for the production of α,ω-hydroxyfatty acids, a fatty alcohol oxidase was used to catalyze the further oxidation of ω-hydroxyfatty acids.[49]

1.2.3 Reverse β-oxidation pathways

While both 2-ketoacid pathway and fatty acid biosynthesis pathway have been used for the production of long chain compounds, both of these pathways have low theoretical yields and are not very efficient. Due to additional need for energy and cofactors in chain elongation pathways there is a need to develop pathways that are carbon and energy efficient to improve overall product yields. The engineered reverse β-oxidation pathway shows great promise as a platform for the production of long chain alcohols, acids, ω-

hydroxy acids, alkenes, and alkanes at high yields. It is much more efficient than the FAB pathway since it does not involve any ATP consuming reaction and it uses acetyl-CoA for acyl-CoA elongation enabling product synthesis at very high carbon and energy efficiency. Furthermore, the cyclic and modular nature of this pathway allows flexibility to produce compounds with different structural and chemical functionalities. Engineering this pathway as a platform for biosynthesis of advanced fuels and chemicals requires the reversal of β-oxidation in the presence of a non-fatty acid substrate such as glucose. The reversal of β-oxidation for efficient fuel and chemical synthesis was first demonstrated in *E. coli* and has been recently extended to eukaryotic systems such as *S. cerevisiae*.[50-51]

The pathway involves four main steps - (i) condensation of acetyl-CoA with an acyl-CoA molecule catalyzed by a thiolase enzyme to yield a ketoacyl-CoA; (ii) a hydroxyacyl-CoA dehydrogenase (HCADHs) that reduces ketoacyl-CoA to hydroxyacyl-CoA; (iii) an enoyl-CoA hydratase (ECHs) that dehydrates hydroxyacyl-CoA to generate trans-enoyl-CoA; and (iv) an acyl-CoA dehydrogenase/trans-enoyl-CoA reductase that reduces trans-enoyl-CoA to an acyl-CoA with 2 more carbon atoms than the initial acyl-CoA (**Figure 1.2b**).[52] Finally, product synthesis is obtained by the action of termination enzymes such as acyl-CoA thioesterases (ACTs) or aldehyde-forming acyl-CoA reductases (ACRs) and alcohol dehydrogenases (ADHs).[53] The simplest operation of this pathway involves condensation of two acetyl-CoA molecules to butyryl-CoA and the operation of multiple cycles of this pathway using a long-chain thiolase allows the synthesis of longer (C>4) chain length compounds. After screening several candidate enzymes for each step and eliminating competing pathways, 500 mg/L 3-ketobutyric

acid, ~150 mg/L of 3-hydroxybutyric acid and ~200 mg/L of trans-2-butenoic acid was obtained.[50] A bioreactor experiment using high glucose concentration and an engineered *E. coli* strain allowed the production of ~7 g/L extracellular long chain fatty acids which is higher than titers reported using FAB pathway.[50] Since this pathway only requires acetyl-CoA for its initiation, it is feedstock independent and this was demonstrated by using glycerol as the carbon source for biosynthesis.[54-55] The modular feature of this pathway has enabled multi-entry and multi-exit biosynthesis of various odd-chain compounds such as propionate, trans-2-pentenoate, valerate, and pentanol at high efficiencies.[54] In another study, a ten step *de novo* pathway was developed using enzymes from nine different organisms to biosynthesize 4-methyl pentanol using CoA-dependent chemistry and the highest titer observed was 192 mg/L.[56] The reverse β-oxidation pathway has also been used for the biosynthesis of alkanes by converting free fatty acids using a broad specificity carboxylic acid reductase and a cyanobacterial aldehyde dehydrogenase (AD).[57] The product distribution of different alkanes are controlled by using upstream (thiolase) and intermediate (thioesterase) enzymes as control points for chain-length specificity. In a more recent study, the β-oxidation cycle was engineered to accept a wide variety of ω- and ω-1- functionalized primers and α-functionalized extender units to allow biosynthesis of ω-phenylalkanoic, α,ω-dicarboxylic, ω-hydroxy, ω-1-oxo, ω-1-methyl, 2-methyl, 2-methyl-2-enolic and 2,3-dihydroxy acids, β-hydroxy-ω-lactones, and ω-1-methyl alcohols.[53]

The engineered β-oxidation pathway has the potential to achieve a maximum theoretical yield of 66.7% (C-mole basis) of n-alcohols on glucose with generation of 1

ATP molecule per two-carbon unit incorporated into the n-alcohol molecule. The high efficiency of this pathway is because it directly uses acetyl-CoA as the donor of two-carbon units during elongation. On the other hand, fatty acid biosynthesis pathway has a net consumption of 1 ATP per n-alcohol molecule due to the consumption of ATP in the synthesis of malonyl-ACP, the donor of two-carbon unit for chain elongation. The acyl-ACP intermediates in the fatty acid biosynthesis pathway also need to be converted to free acids and acylated in an ATP-consuming step before their reduction to alcohols. The third pathway used for alcohol synthesis is the 2-keto acid pathway which is also less efficient than β-oxidation pathway with a maximum theoretical yield of n-hexanol (the highest chain linear alcohol reported with 2-ketoacid pathway) of 50% C-mole (versus 66.7% for β-oxidation pathway).[50]

1.2.4 Isoprenoid pathways

Isoprenoids are the largest class of natural products with >50,000 compounds used widely as flavors and pharmaceuticals.[58-60] They also have the potential to serve as advanced biofuels due to the branches and rings in their hydrocarbon chains. The isoprenoid-based alcohol, isopentanol, is a potential gasoline alternative, C15 isoprenoids, farnesane and bisabolane, are great substitutes for diesel, and pinene dimers with constrained ring structures are great for jet-fuel replacements given their high energy density.[60]

Isoprenoid pathway is another pathway used for carbon chain elongation, in which the carbon backbone increase by multiples of five through the addition of pyrophosphate-activated isopentenyl pyrophosphate (IPP) to dimethylallyl pyrophosphate

(DMAPP) (**Figure 1.3**). Both IPP and DMAPP are derived either from the mevalonate pathway (MEV) or the methylerythritol pathway (MEP). DMAPP primes the sequential head-to-tail condensations of IPP molecules by prenyltransferases to generate prenyl diphosphate precursors geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15), and geranyl geranyl diphosphate (GGPP, C20). These precursors are then modified through cyclization or rearrangement catalyzed by terpene synthases to generate the final carbon skeletons found in isoprenoid natural products.

Isopentenol production was developed in *E. coli* through over-expression of key enzymes of the MEP pathway, improving precursor and NADPH supply by activating the pentose phosphate pathway (PPP), and the over-expression of codon-optimized genes *nudF* and *yhfR* from *Bacillus subtilis* resulting in 61.9 mg/L isopentenol from 20 g/L glucose.[61] The MVA pathway has also been engineered for the production of isoprenoid based C5 alcohols producing 2.23 g/L of 3-methyl-3-buten-1-ol, 150 mg/L of 3-methyl-2-buten-1-ol, and 300 mg/L of 3-methyl-1-butanol, in *E. coli*.[62] Bisabolene, with properties similar to D2 diesel fuel, has been produced in both *E. coli* and *S. cerevisiae* at titers higher than 900 mg/L by screening for bisabolene synthase.[63] The jet fuel replacement, pinene ($C_{10}H_{16}$) has been produced in *E. coli* at 28 g/L using GPP as an intermediate.[64] Farnesane is the only isoprenoid-based biofuel being commercialized and it has been produced in *S. cerevisiae* by overexpressing the native yeast MVA pathway and the farnesene synthase from *Artemisia annua*.[59, 65] This olefin is then chemically hydrogenated to produce farnesane, a high-performance advanced biofuel.[60] Apart from

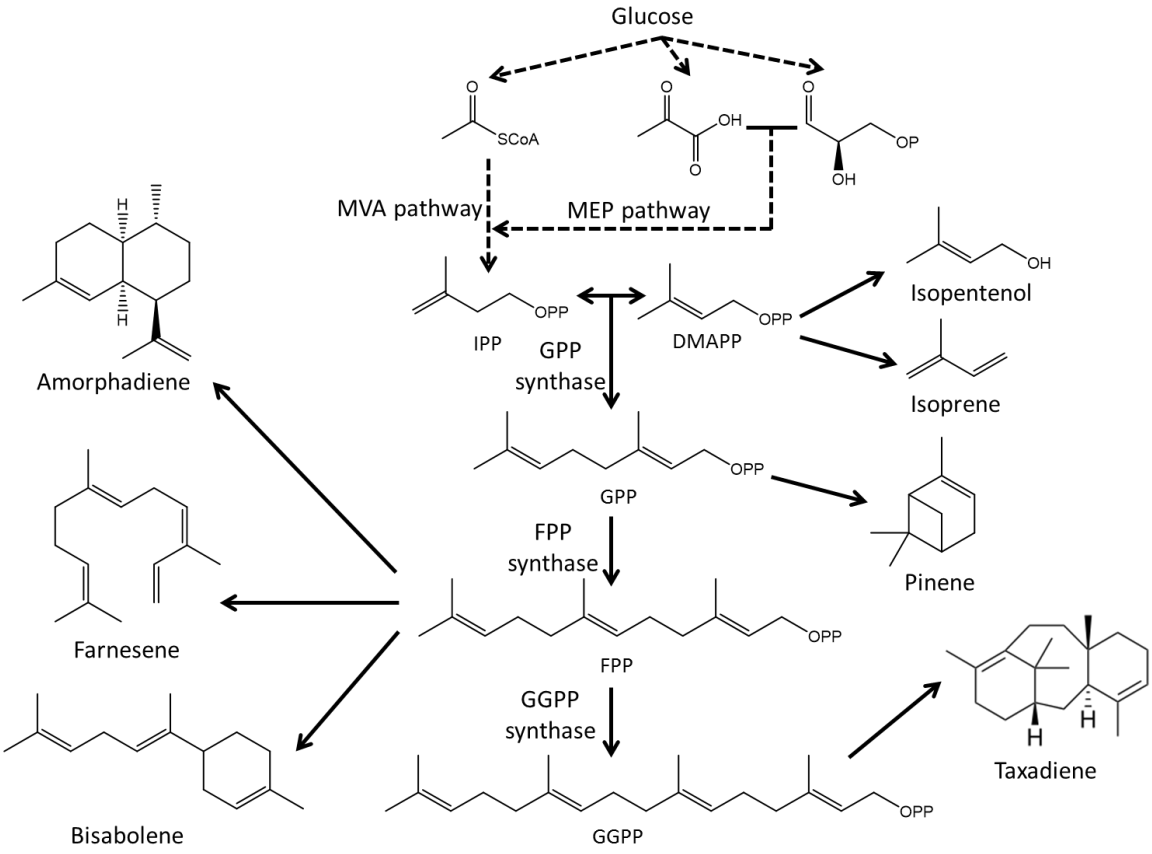

Figure 1.3 Isoprenoid pathway for the biosynthesis of advanced biofuels and pharmaceutical precursor molecules. MVA: Mevalonate; MEP: Methylerythritol pathway; IPP:Isopentenyl pyrophosphate; DMAPP: Dimethylallyl pyrophosphate; GPP: Geranyl diphosphate; FPP: Farnesyl diphosphate; GGPP: Geranyl geranyl diphosphate

biofuels, the isoprenoid pathway has also been exploited for the production of two important drug precursors – taxadiene, the first committed intermediate to Taxol, an anticancer drug, and amorphadiene, precursor to artemisinin, an anti-malarial drug. Engineering the MVA pathway by replacing yeast HMG-CoA synthase and HMG-CoA reductase with genes from *Staphylococcus aureus* has resulted in 27 g/L amorphadiene in *E. coli* [18] and overexpressing every enzyme of the MVA pathway produced 40 g/L amorphadiene in *S. cerevisiae*.[19] For taxadiene production, an *E. coli* strain was engineered using a multivariate modular approach to balance the two pathway modules –

upstream MEP pathway generating IPP and a heterologous downstream terpenoid-forming pathway, allowing production of 1 g/L taxadiene.[66]

1.3 Thesis overview

This thesis details the design and engineering of an alternate nonphosphorylative metabolic pathway in *E. coli* to convert underutilized lignocellulosic sugars – D-xylose, L-arabinose, and D-galacturonate – into sustainable chemicals and polymers such as 1, 4-butanediol (BDO) and γ-aminobutyric acid (GABA). Several strategies including gene knockouts, protein engineering, gene mining and transporter engineering have been used to improve production titers and yields of the target compounds. The goal of this work is to establish a novel metabolic platform for biosynthesis that is orthogonal to the intrinsic metabolism of *E. coli*. This nonphosphorylative pathway has fewer steps, lesser feedback regulation and is capable of converting pentoses and sugar acids present in biomass into valuable chemicals with potentially higher theoretical yields. The pathway can be used for the biosynthesis of a wide array of chemicals and polymers derived from the TCA cycle including, but not limited to, 1,4-butanediol (BDO), mesaconate, L-glutamate, γ-aminobutyric acid (GABA), and 5-mainolevulinic acid (ALA).

Chapter 2 is a literature review describing the use of 2-ketoacid pathway for the biosynthesis of a wide array of industrially relevant compounds including aliphatic alcohols and acids, polar compounds such as diols, diamines, and diacids, and aromatic compounds such as styrene, phenolic acids, and polyphenols. Chapter 3 discusses semi-synthetic production of three complex pharmaceuticals – simvastatin, artemisinin, and warfarin – and two commodity chemicals, β-methyl-δ-valerolactone and butadiene, using

a combination of chemical and biological approaches. Chapter 4 presents a broad review on the recent advances made in the production of three promising bio-based and biodegradable polymers – polylactic acid (PLA), polyhydroxyalkanoate (PHA), and polybutylene succinate (PBS).

Chapter 5 demonstrates the design and successful engineering of nonphosphorylative metabolism in *E. coli* and expansion of the pathway for the biosynthesis of 1, 4-butanediol (BDO). This chapter also describes the design of a growth selection platform to establish the functionality of this heterologous pathway in *E.coli* and to mine more active nonphosphorylative gene clusters from different organisms. Chapter 6 shows the expansion of the nonphosphorylative pathway for the biosynthesis of γ-aminobutyric acid (GABA), an important chemical in food and pharmaceutical industry, from pentoses D-xylose and L-arabinose. To improve titers of GABA several competing pathways were eliminated, different nonphosphorylative operons were screened, and pentose transporter was overexpressed to improve sugar uptake by cells. Finally, chapter 7 focuses on the acid hydrolysis of lignocellulosic biomass specifically wheat bran for direct fermentation into chemicals such as BDO or GABA. In this chapter, different acid hydrolysis conditions are compared for breakdown of the polymers like arabinoxylans into simple sugars. These sugars derived from hydrolysis of biomass polymers are then used directly for fermentation.

Chapter 2

Novel pathways and products from 2-keto acids*

2.1 Introduction

Crude oil is the world's primary energy source and also a major source for the production of important chemicals. However, it is a non-renewable and the usage of fossil resources has been causing irreparable harm to the environment. To address the environment and sustainability issue of petroleum, biosynthesis emerges as a promising alternative to fuels and chemicals. Metabolic engineering of micro-organisms has enabled the production of various fuels[23, 43, 50, 67-68] and chemicals[69-72] from renewable resources. However, one of the major challenges of biosynthesis is that majority of the useful industrial chemicals are

* Adapted with permission from Jambunathan, P., & Zhang, K. (2014). Novel pathways and products from 2-keto acids. *Current opinion in biotechnology*, *29*, 1-7.

not metabolic intermediates in a production organism such as *E. coli*. This necessitates the introduction of biosynthetic production pathway that is compatible with the host organism in order to achieve high yields and rates of target compounds.

Amino acid biosynthetic pathway is one such universal pathway. L- Amino acids are one of the most important industrial products derived from fermentation of microorganisms and their annual market volume is over 5 million tons.[73] In particular, fermentation of L-glutamate, L-lysine and L-threonine has been engineered to reach titers over 100g/L and yields close to theoretical maximum.[73] With successes in natural amino acid production[74-75], it opens the possibility to expand amino acid pathways for production of higher-chain alcohols, carboxylic acids, pharmaceuticals and other chemicals.

The key intermediates of the amino acid biosynthetic pathways are 2-ketoacids that can be converted to a wide range of chemically diverse compounds. 2-keto acids are organic compounds that have a ketone group adjacent to the carboxylic acid group. This review will focus on the various biochemical reactions of 2-ketoacids – (i) decarboxylation, (ii) reduction, (iii) chain elongation, and (iv) transamination – and the wide range of products obtained from this diverse chemistry. The compounds have been divided into three classes – aliphatic, polar and aromatic – depending on the amino acid pathway from which they are derived.

2.2 Aliphatic compounds

Aliphatic amino acids such as isoleucine, leucine and valine contain hydrophobic side chains. If their corresponding ketoacids undergo decarboxylation, they can be converted to aldehydes that can further be either reduced to alcohols or oxidized to acids. These reactions are utilized in the last two steps of the natural Erhlich pathway for fusel alcohol production.[76] The decarboxylation chemistry of aliphatic 2-keto acid intermediates has been expanded to synthesize higher chain alcohols and industrially important carboxylic acids (**Figure 2.1**).

Higher alcohols are considered better gasoline substitutes, but they are not produced in significant quantities by natural microorganisms. A synthetic approach was developed to produce these alcohols using the 2-ketoacid intermediates of branched-chain amino acid biosynthetic pathways.[23] C4 alcohol isobutanol pathway was successfully designed by engineering the valine pathway. First, the natural Erhlich pathway 2-ketoacid decarboxylase (KDC), KIVD from *L. lactis*[77], was introduced into *E. coli* to convert 2-ketoisovalerate to isobutyraldehyde. Then an alcohol dehydrogenase (ADH), ADH2 from *S. cerevisiae*, was introduced to convert isobutyraldehyde to isobutanol. The fermentation titer of isobutanol reached 22 g/L in shake flask experiments.[23] In a scale-up study, isobutanol titer was raised to 50 g/L in a bioreactor.[78] The isobutanol pathway has also been successfully implemented in other organisms such as cyanobacteria[79] and yeast.[80]

Besides isobutanol, longer chain C5-C8 alcohol pathways are designed by employing protein engineering and pathway engineering strategies. C5 alcohols such as 2-methyl-1-butanol and 3-methyl-1-butanol have naturally existing 2-keto acid

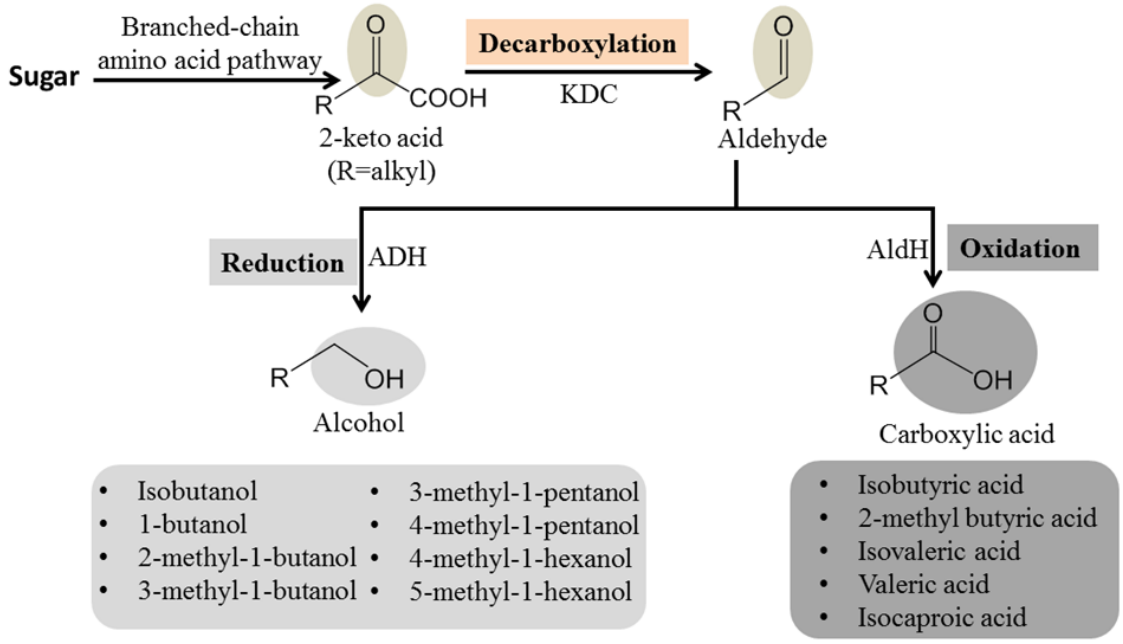

Figure 2.1 2-Ketoacid pathways for the production of aliphatic alcohols and carboxylic acids. KDC: 2-ketoacid decarboxylase; ADH: alcohol dehydrogenase; AldH: aldehyde dehydrogenase.

precursors, 2-keto-3-methylvalerate (isoleucine pathway) and 2-ketoisocaproate (leucine pathway) respectively, and have been produced by over-expressing KIVD and ADH2 in *E. coli*.[81-83] Higher-chain alcohols with more than 5 carbon atoms do not have naturally occurring 2-ketoacid intermediates since the amino acid biosynthetic pathway is limited by carbon length. To overcome this problem, the *leuABCD* gene products were used to extend the carbon chain of existing keto-acid intermediates.[84-85] To synthesize 2-ketoacid precursors for higher alcohols (C5-C8), protein engineering strategies were used to enlarge the binding pocket of LeuA by directed mutagenesis and consequently expand its substrate range. These precursors were then converted to respective alcohols by the action of two heterologous enzymes - a 2-ketoacid decarboxylase KIVD (from *L. lactis*) followed by an alcohol dehydrogenase ADH6 (from *S. cerevisiae*) to synthesize a variety

of alcohols, including 3-methyl-1-pentanol, 4-methyl-1-pentanol, 4-methyl-1-hexanol and 5-methyl-1-hexanol.[84]

Aliphatic carboxylic acids have broad industrial applications. Isobutyric acid, a C4 acid, was metabolically produced via the 2-keto acid pathway from 2-ketoisovalerate. Similar to isobutanol production, 2-ketoisovalerate was first decarboxylated into isobutyraldehyde by the ketoacid decarboxylase KIVD from *L. lactis.*[77] To oxidize isobutyraldehyde, seven aldehyde dehydrogenases from different organisms were chosen and screened for their activities. Phenylacetaldehyde dehydrogenase (PadA[86]) from *E. coli* produced a high titer of isobutyrate at 4.8 g/L.[30] By eliminating the competing reduction reaction from the alcohol dehydrogenase, YqhD in *E. coli*, isobutyrate titer in shake flask fermentation was increased to 12 g/L from 40 g/L glucose. Based on the successful production of isobutyrate via keto acid pathway, various C5-C6 acids were synthesized using similar metabolic engineering approaches. C5 acids such as valeric acid and 2-methylbutyric acid have been produced from a common 2-ketoacid intermediate, 2-ketobutyrate.[87] For increasing the specificity of KIVD towards larger substrates such as 2-ketomethylvalerate, mutations were performed to replace bulky residues with small hydrophobic residues in key locations.[84] Another C5 acid, isovalerate was produced in *E. coli* from the 2-ketoacid intermediate, 2-ketoisocaproate, which was obtained from 2-ketoisovalerate by the action of *leuABCD* gene products through the carbon chain elongation process.[88] Isocaproate, a C6 acid, was also produced via the 2-ketoacid pathway from 2-ketohomoleucine that is derived from 2-ketoisocaproate by the 2-ketoacid elongation process using a mutant LeuA enzyme.[88]

2.3 Polar bifunctional compounds

Amino acids such as lysine and glutamate contain polar groups (amino and carboxyl) and the conversion of the 2-ketoacid precursors of these amino acids can generate chemicals with bifunctional groups such as diamines, dicarboxylic acids and diols (**Figure 2.2**). These compounds can be used as building blocks for synthesis of important polymers such as Nylon 5 or as monomers for biodegradable polyesters.[89-91] They are high-volume industrial chemicals: for example, both 1,4-Butanediol and adipic acid have an annual market of over 5 billion pounds.[92]

Dicarboxylic acids such as glutaconate and adipate have been produced from 2-ketoglutarate, the 2-ketoacid precursor of the glutamate. The pathway to glutaconate utilized the reduction chemistry of 2-ketoacids.[91] First, 2-ketoglutarate was reduced to 2-hydroxyglutarate in a NADH-dependent reaction catalyzed by a dehydrogenase enzyme (HgdH). Then this hydroxy-acid underwent a series of biochemical reactions, including activation by CoA, dehydration and isomerization catalyzed by GctAB and HgdCAB. The designed synthetic pathway enabled the production of 350 mg/L glutaconate in *E. coli*. Adipate, on the other hand, was produced using the ketoacid elongation enzymes AksADEF involved in coenzyme B synthesis. These "+1" chain elongation reactions are similar to the LeuABCD catalyzed elongation used for production of long chain alcohols and acids.[85, 93] 2-Ketoglutarate was elongated by 2 units to produce 2-oxopimelate (C7) which could be decarboxylated and further oxidized to adipate.[94]

Apart from dicarboxylic acids, diols including 1,4-butanediol has been produced in *E. coli* from 2-ketoglutarate.[92] In this work, 2-ketoglutarate was first decarboxylated to

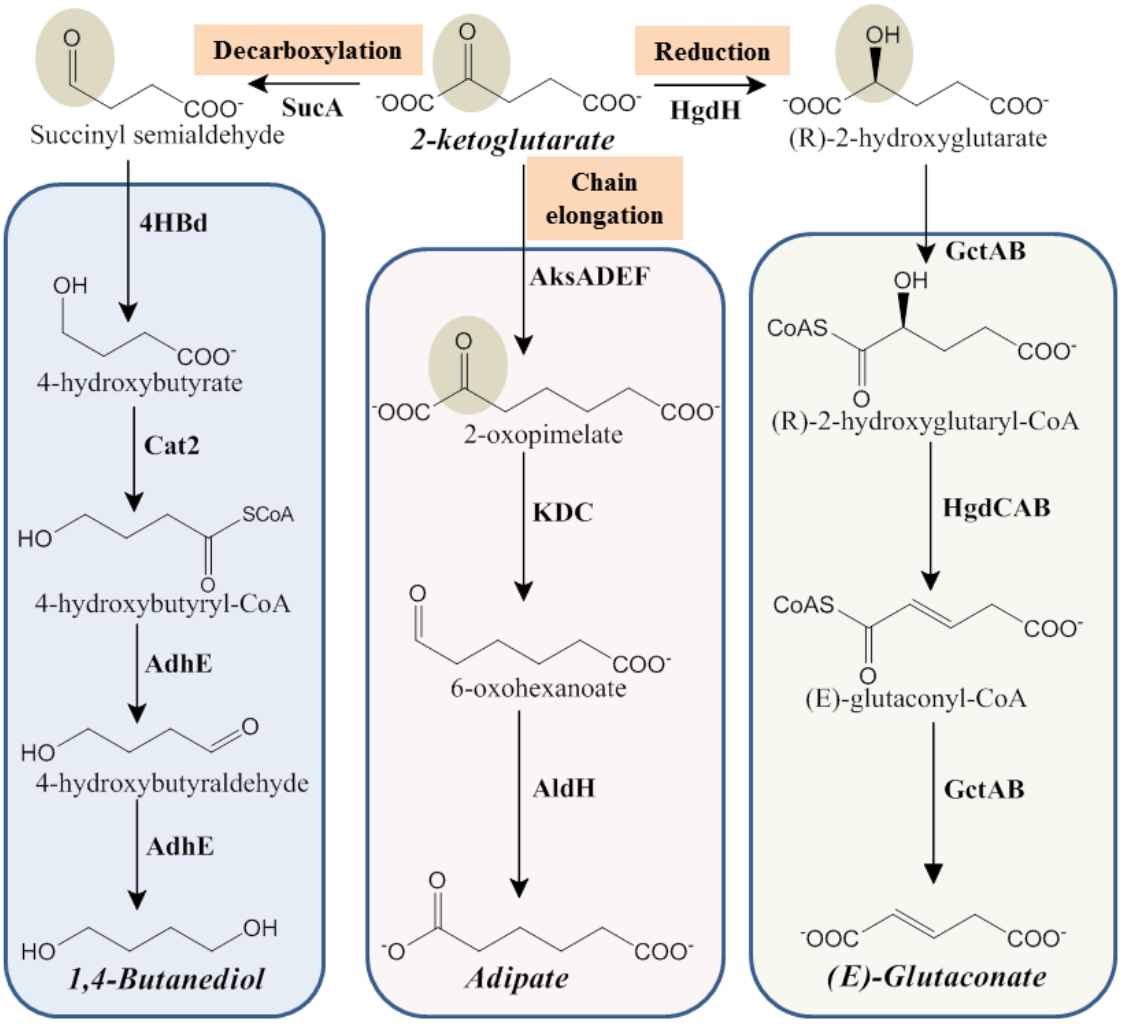

Figure 2.2 2-Ketoacid pathway for the production of polar bifunctional compounds – 1,4-butanediol, adipic acid and glutaconic acid. I: Butanediol pathway; II: Adipate pathway; III: Glutaconate pathway. SucA: a-ketoglutarate decarboxylase; 4HBd: 4-hydroxybutyrate dehydrogenase; Cat2: 4-hydroxybutyryl-CoA transferase; AdhE: Alcohol dehydrogenase; AksA: homocitrate synthase; AksD: Homoaconitase a subunit; AksE: homoaconitase b subunit; AksF: threo-isohomocitrate dehydrogenase; KDC: ketoacid decarboxylase; AldH: aldehyde dehydrogenase; HgdH: 2-hydroxyglutarate dehydrogenase; GctAB: glutaconate CoA transferase; HgdCAB: 2-hydroxyglutaryl-CoA dehydratase.

succinyl semialdehyde by a decarboxylase (SucA). Then 4-hydroxybutyate was produced by reduction of succinyl semialdehyde enabled by 4-HBd from *P. gingivalis*. 4-Hydroxybutyate was activated to 4-hydroxybutyryl-CoA by a CoA transferase (Cat2) from *P. gingivalis*. Finally, 4-hydroxybutyryl-CoA was converted to 1,4-butanediol after

two reduction steps catalyzed by *C. acetobutylicum A*dhE dehydrogenase. Further metabolic engineering on this designed pathway enabled the production of 18 g/L of 1,4-butanediol from carbohydrate feedstocks.[92]

2.4 Aromatic compounds

Amino acids such as tyrosine, tryptophan and phenylalanine have aromatic side chains and these can form the backbone of several pharmaceuticals, dyes, flavor compounds, organic solvents and polymers that are currently derived from petroleum-based sources. The two aromatic 2-ketoacids, phenylpyruvate (PP) and 4-hydroxyphenylpyruvate (HPP) are converted to amino acids, phenylalanine and tyrosine, respectively by the action of an endogenous transaminase TyrB (**Figure 2.3**). Recently, the transamination chemistry of 2-ketoacids has been used to develop novel pathways for the production of polymer building blocks[95], flavonoids[96-98], coumarins[99-100], stilbenes[101-103], pharmaceuticals[104-107] and aromatic polyketides.[108]

An ammonia lyase enzyme (PAL/TAL) could catalyze the conversion of aromatic amino acids to respective phenylpropanoic acids including cinnamic acid and *p*-coumaric acid.[109] These acids are key intermediates of the phenylpropanoid pathway and are used in the synthesis of various aromatic compounds. For example, Niessen *et al.* developed a pathway to produce styrene from glucose.[95] Phenylalanine was converted to cinnamic acid by action of ammonia lyase which was further decarboxylated to styrene. Over-expression of PAL2 from *A. thaliana* and ferulate decarboxylase (FDC1) from *S. cerevisiae* in an L-phenylalanine overproducing strain led to the production of 0.26 g/L of styrene.[95] The hydroxylation and methylation chemistry of phenylpropanoic acids have

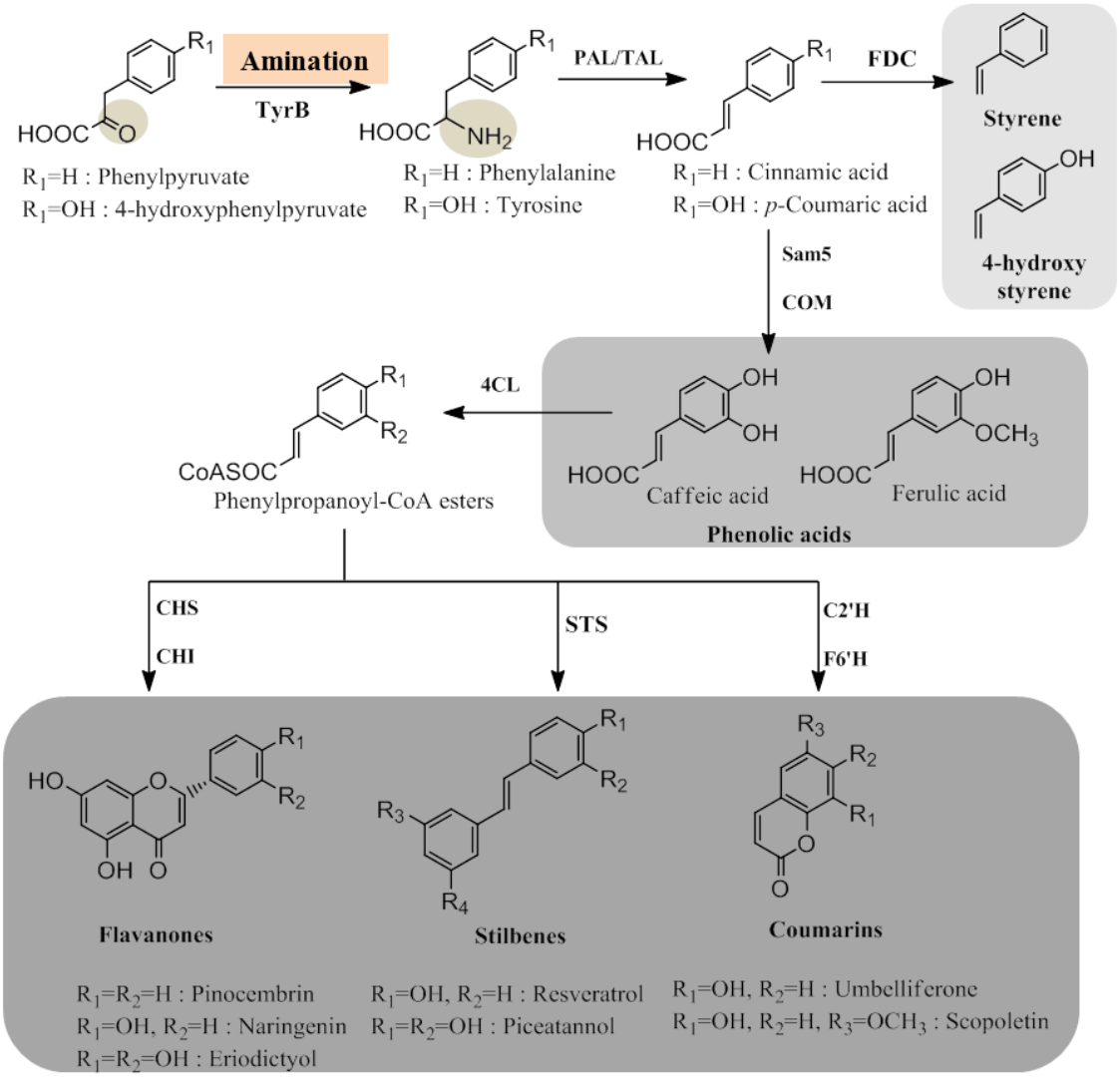

Figure 2.3 2-Ketoacid biosynthetic pathway for aromatic products. TyrB: tyrosine aminotransferase; PAL: phenylalanine ammonia lyase; TAL: tyrosine ammonia lyase; FDC: ferulate decarboxylase; C3H: p-Coumarate-3-hydroxylase; COM: caffeate O-methyl transferase; 4CL: 4-coumarate ligase; CHS: chalcone synthase; CHI: chalcone isomerase; STS: stilbene synthase; C20H: p-coumaroyl-CoA o-hydroxylase; F60H: feruloyl-CoA o-hydroxylase.

been used to produce caffeic acid and ferulic acid respectively.[97, 106] Endogenous 4-hydroxyphenylacetate 3-hydroxylases (4HPA3Hs) and TAL from R. capsulatus were co-expressed in E. coli to produce 12 mg/L of caffeic acid from simple sugars.[106] Hong *et al.* over-expressed Sam5 from *S. espanaensis* that is a bacteria-compatible *p*-coumarate 3-hydroxylase (C3H) enzyme to produce 150 mg/L of caffeic acid from glucose.[109] In the

same paper, ferulic acid was produced via the 3-O-methylation of caffeic acid catalyzed by O-methyltransferase (OMT). Co-expression of Sam5 from *S. espanaensis* and OMT from *Arabidopsis thaliana* resulted in 196 mg/L of ferulic acid.[109]

The phenylpropanoic acids can also be acylated by acetyl CoA to produce phenylpropanoyl-CoA esters. The hydroxylation of these CoA esters followed by spontaneous lactonization has resulted in the *de novo* biosynthesis of simple coumarins including umbelliferone and scopoletin in *E. coli.*[99-100] These CoA esters can also be used to produce flavonoids (naringenin, pinocembrin, eriodictyol) and stilbenes (reseveratrol, piceatannol) by condensation with malonyl CoA molecules in a Claisen cyclization reaction catalyzed by a polyketide synthase[97-98, 101-103, 110-111]. The details of these compounds have been reviewed by Yan *et al.*.[112]

2.5 Conclusion

Recent advances in metabolic engineering have resulted in the development of natural amino acid over-producing strains. 2-keto acid are the key intermediates of the amino acid biosynthetic pathway and the chemically diverse nature of 2-ketoacid compounds has been exploited to produce fuels, chemicals and pharmaceuticals in metabolically engineered organisms. One of the biggest challenges in using the 2-ketoacid pathway is to search for enzymes with high activities towards the designed metabolic routes. To address this challenge, both bioprospecting on naturally existing enzymes in different organisms or protein engineering have been performed to discover biocatalysts with the desired activity. So far, some 2-ketoacid derivatives are still at low production titers and there is a need to make these 2-ketoacid processes industrially feasible. Several metabolic

engineering strategies including elimination of competing pathways[30], re-directing carbon and energy flux through desired pathway[83], use of feedback resistant enzymes[84], increasing precursor pool[23], and coupling complementary driving forces between two pathways[113] are suitable to increase the titers and production rates of various 2-ketoacid derived compounds.

Chapter 3

Combining biological and chemical approaches for green synthesis of chemicals*

3.1 Introduction

Over the past several years, there have been numerous reports of semi-synthesis of important pharmaceuticals and other commodity chemicals that combines biological and chemical pathways to achieve the final products.[114-118] In most of these cases, biological fermentation allows the production of an important precursor/intermediate using renewable biomass-derived sugars, following which the precursor is transformed using single or multi-step chemical reactions to yield the final compound. This hybrid process

* Adapted with permission from Jambunathan, P., & Zhang, K. (2015). Combining biological and chemical approaches for green synthesis of chemicals. *Current Opinion in Chemical Engineering*, *10*, 35-41.

can convert bio-derived precursors into useful commodity chemicals, thus establishing a more sustainable and greener route for the production of these high-volume compounds. This approach is also useful for the synthesis of chiral drug precursors since biological enzymes offer much better stereoselectivity as compared to chemical catalysts at milder reaction conditions.[119]

In this review, we have divided target compounds into two categories: pharmaceuticals and commodity chemicals. In the first section, we discuss the most recent advancements in the semi-synthesis of three widely prescribed drugs – simvastatin, artemisinin and warfarin. In the second section, we focus on chembiosynthesis of commodity chemicals including monomers for industrially relevant polymers such as β-methyl-δ-valerolactone and butadiene. For few of the compounds discussed in this review, a direct total biosynthetic[120] or chemical synthetic[121-122] pathway has been established, but the titers obtained are very low for industrial relevance, making semi-synthesis an attractive option at this stage.

3.2 Synthesis of Pharmaceuticals

In the past, there have been several successful stories in the pharmaceutical industry where biological route has been used to synthesize an optically pure precursor which is subsequently subjected to chemical reactions to yield the target drug. Examples include the biosynthesis of the taxol precursor, taxadiene[114], and Tamiflu precursor, shikimic acid[123], in engineered *E. coli*. This semi-synthetic approach reduces dependence on isolation of relevant metabolites from natural resources and also significantly improves process economics and sustainability of drug production. In case of drugs such as

Lipitor[124] and Sitagliptin[125-126], while pure chemical synthetic routes exist, biosynthesis has been used to replace some of the chemical reactions with the objective of reducing waste and eliminating use of hazardous catalysts. Over the past few years there have been significant advancements in the synthesis of other drugs, some of which are covered in detail in this section. We have reviewed the recent progress made in the field for semi-synthesis of three widely used drugs – (i) simvastatin, a cholesterol-lowering drug, (ii) artemisinin, an antimalarial drug, and (iii) warfarin, an anticoagulant used for prevention of thrombosis.

3.2.1 Simvastatin

As a derivative of lovastatin, simvastatin has a 2, 2-dimethylbutyroloxy side chain at C8 position as against a 2-methylbutyroloxy side chain in its natural counterpart. Traditionally, the semi-synthetic process for producing simvastatin involves isolation of lovastatin from *A. terreus* fermentation, hydrolysis to yield monacolin J, protection of free alcohol to allow subsequent regioselective esterification of C8 alcohol with dimethylbutyryl chloride.[127-129] In an effort to improve the overall efficiency of the process, Xie et al. demonstrated the ability to use the acyl transferase homolog, LovD, which catalyzes the last step of lovastatin biosynthesis, to selectively acylate monacolin J for the single-step synthesis of simvastatin using chemically synthesized α-dimethylbutyryl-S-methyl mercaptopropionate (DMB-S-MMP) as the acyl donor[130-131] as shown in **Figure 3.1**. This one-step process significantly reduces the number of chemical transformations needed, improves process efficiency and also reduced the cost of manufacturing of simvastatin. In a more recent report, a variant of LovD with 29

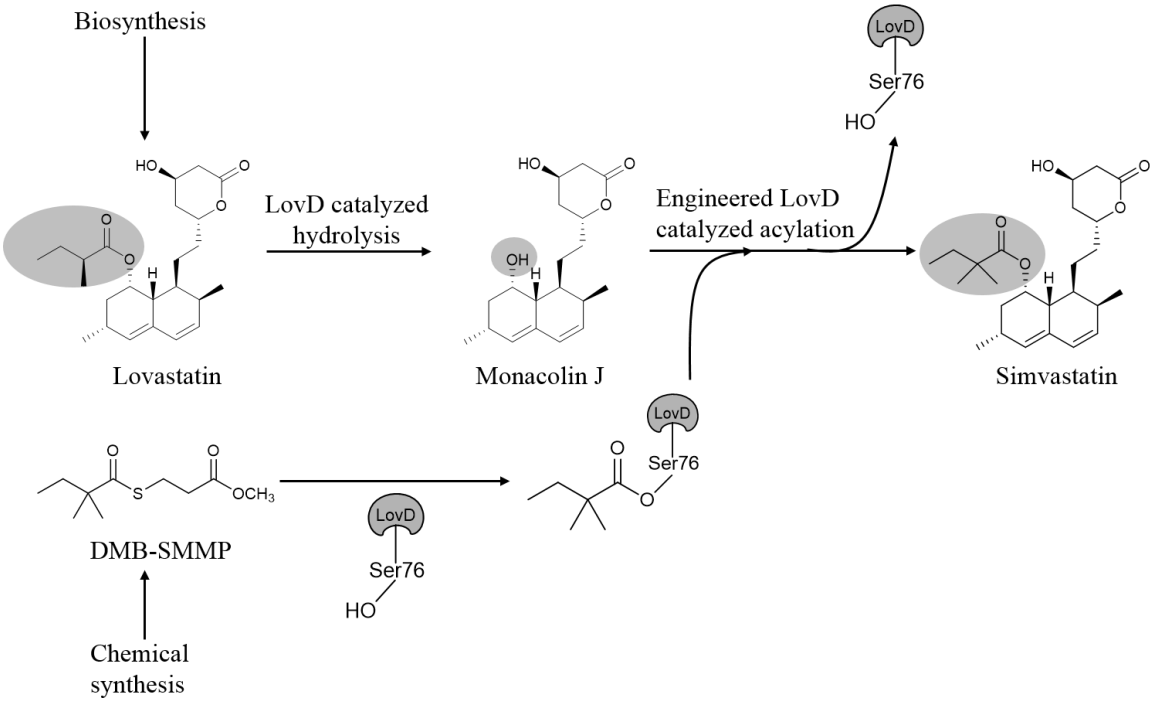

Figure 3.1 Conversion of lovastatin to simvastatin using LovD. Biologically produced lovastatin is first hydrolyzed in a reaction catalyzed by LovD, followed by an acylation reaction catalyzed by LovD mutant (LovD9 obtained after nine rounds of evolution). Chemically synthesized α-dimethylbutyryl-S-methylmercaptopropionate (DMB-SMMP) acts as an acyl donor for the reaction.

mutations was identified by directed evolution, which is 1000-fold more efficient in synthesizing simvastatin than the wild type enzyme. The authors used microsecond molecular dynamics (MD) in solution to explain how distant mutations could improve catalytic efficiency of the active site by lowering the free energy of catalytic conformation of active site.[115]

3.2.2 Artemisinin

Artemisinin is a potent antimalarial drug which is naturally produced by the plant *Artemisia annua* and has a long history of use in Chinese medicine. Due to tremendous fluctuations in the price and supply of this drug as a consequence of inconsistent weather[132-133], the semi-synthetic artemisinin project was started which involved

microbial production of artemisinic acid, a chemical precursor of artemisinin, followed by a chemical transformation step to produce artemisinin. After studying the artemisinin pathway in *A. annua*[134], *E. coli* was originally chosen as the chassis organism to produce artemisinic acid[135], but due to the problem of expression of eukaryotic enzymes in *E. coli* [136], the pathway was transferred into a *S. cerevisiae* CEN.PK2 strain[137]. Over-expression of mevalonate pathway genes along with expression of the P450 enzyme (CYP71AV1) and its cognate reductase (CPR1) allowed the production of 40 g/L of amorphadiene, but artemisinic acid production was still very low.[138-139] Expression of cytochrome *b5*[140] and the aldehyde and alcohol dehydrogenase (ADH1 and ALDH1)[141] from *A. annua* improved P450 activity and increased artemisinic acid titer to 25 g/L as shown in **Figure 3.2**, which was the starting goal of the semi-synthetic artemisinin project.[142] Artemisinic acid was extracted from the fermentation medium with isopropyl myristate (IPM) at high purities and was subsequently used as a substrate for chemical transformation to artemisinin.[142]

The chemical process for converting artemisinic acid to artemisinin involves the following steps: 1) hydrogenation of artemisinic acid (AA) to dihydroartemisinic acid (DHAA); 2) esterification of DHAA to avoid formation of by-products; and 3) generation of a singlet oxygen by chemical or photochemical means to convert DHAA methyl ester to artemisinin.[142] For stereoselective conversion of AA to DHAA, several catalysts have been screened[142-143] to achieve high diastereoselectivities, and recently, work performed by researchers at Sanofi provided $RuCl_2$ [(R)-DTBM-Segphos] $(DMF)_n$ catalyst which yielded 95:5 selectivity.[144-145] The conversion of DHAA ester to artemisinin involves

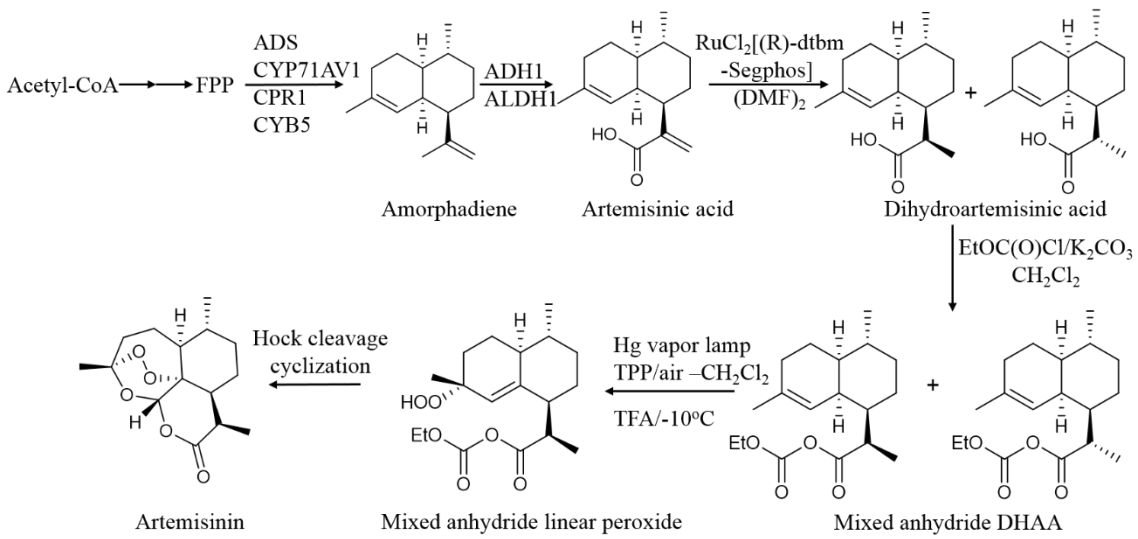

Figure 3.2 Semi-synthetic pathway for production of artemisinin. Biological route in S. cerevisiae for synthesis of the precursor, artemisinic acid using the mevalonate pathway. Chemical conversion of artemisinic acid to artemisinin developed by Sanofi which includes diastereoselective hydrogenation of artemisinic acid to dihydroartemisinic acid, followed by its esterification to mixed anhydrides and finally a Schenck ene reaction and Hock cleavage cyclization to produce artemisinin.

regioselective Schenck ene reaction between a singlet oxygen (either derived by chemical reaction or photochemically) and the double bond of DHAA, followed by a Hock cleavage catalyzed by a strong Lewis acid and a subsequent addition of triplet oxygen and cyclization.[146] Sanofi designed a one-pot synthesis route to convert a DHAA derivative (mixed anhydride) to artemisinin and they obtained an overall yield of 55% of artemisinin starting with artemisinic acid. This semi-synthetic route has capacity to produce 60 tons of artemisinin annually, which corresponds to a third of the global annual need for the drug.[146]

3.2.3 Warfarin

Warfarin is one of the most commonly prescribed 4-hydroxycoumarin (4HC) type anticoagulant used in the prevention of thrombosis or thromboembolism, which is one of the leading causes of morbidity and mortality worldwide. Recently, a *de novo*

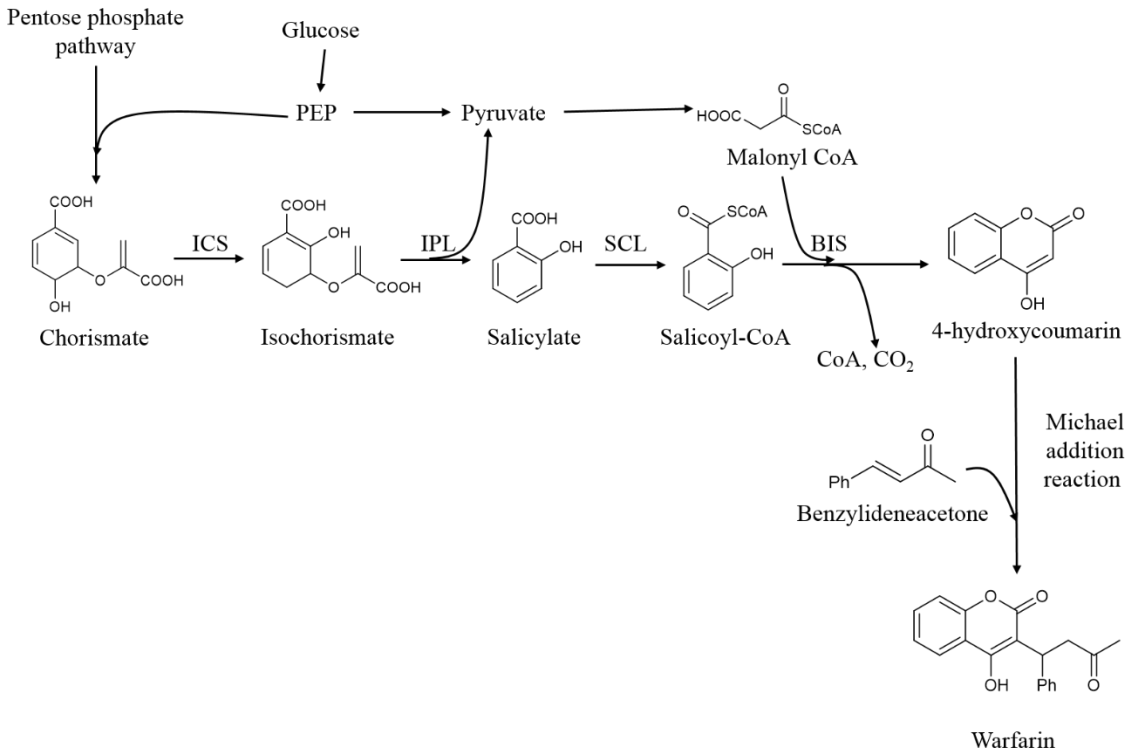

Figure 3.3 Chemoenzymatic pathway for warfarin production. 4-hydroxycoumarin (4HC) is first biologically produced in E. coli from chorismate, derived via the shikimic acid pathway. 4HC is then converted to warfarin by a Michael addition reaction with benzylideneacetone.

biosynthetic pathway was designed in *E. coli* for the production of 4HC[147] as shown in **Figure 3.3** by employing a biphenyl synthases (BIS) to catalyze the decarboxylative condensation of salicoyl-CoA with malonyl-CoA to form a diketide intermediate which undergoes intracellular cyclization and enolization to form 4HC.[148] The pathway used EntC from *E. coli* and PfPchB from *P. fluorescence* as isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL) respectively, to convert chorismate to salicylate and a salicylate: CoA ligase (SCL) SdgA from Streptomyces sp. to convert salicylate to salicoyl-CoA, the substrate for BIS enzyme.[147] After identifying BIS catalyzed step as the bottleneck of the pathway, they identified 4-hydroxy-2 (1H)-quinolone synthase (PqsD) by function-based bioprospecting and this enzyme allowed > 99% yield for 4HC

synthesis and with further engineering they achieved ~483 mg/L 4HC, a ~11 fold increase compared to their parent strain.[147]

The synthesis of warfarin proceeds via Michael addition reaction of 4HC and benzylideneacetone[149] and while most of the studies involve the use of harmful organic solvents , Rogozinska et al. carried out the reaction on water using commercially available amines ((S,S)-diphenylethylenediamine) and achieved 70% *ee* with reasonable yield (~30%).[150] When ultrasound bath was applied to the reaction yields were significantly improved and this method was later used by Lin et al. to demonstrate *in situ* semi-synthesis of warfarin using biologically derived 4HC.[147] In more recent reports, a novel chiral porous metal organic framework (MOF)[151] and a novel polystyrene bound 1,5,7- triazabicyclo[4.4.0]dec-5-ene (TBD)[152] , was used for Michael addition of 4HC to α,β-unsaturated ketones to synthesize (S)-warfarin and its analogues.

3.3 Synthesis of commodity polymers

Apart from pharmaceuticals, semi-synthesis has also been used successfully for the industrial production of high-volume commodity chemicals such as polyethylene, acrylic acid and butanediene, by adopting a biological route for synthesis of their precursors – ethanol[117], 3-hydroxypropionic acid[153] and 1,4-butanediol[154-155] respectively from biomass-derived sugar. This approach addresses the growing concern associated with use of fossil-based feedstocks and helps to establish a sustainable and more environment-friendly route for the production of these compounds. In this section we will discuss the recent work done in establishing a semi-synthetic pathway for two monomers - β-methyl-

δ-valerolactone (βMδVL) and butadiene – both of which can be polymerized to produce bio-based high-performance polymers.

3.3.1 β-methyl-δ-valerolactone (βMδVL)

Polymer industry is the third largest manufacturing industry in US with an annual market of nearly $400 billion. The stiff nature of current biodegradable polyesters such as polylactic acid (PLA) and polyhydroxybutyrate (PHB) have limited their applications in the polymer industry. It is envisioned that this challenge can be addressed by developing ABA type triblock polymers with a rigid, glassy end block A (such as PLA) and a soft, rubbery block B in center with low glass transition temperature. However, there is no biobased soft block available, and to this end, Xiong et al. recently developed a biosynthetic route to produce β-methyl-δ-valerolactone followed by block copolymerization of βMδVL with lactide (LA) to yield P(L)LA-PβMδVL-P(L)LA with mechanically tunable properties[120] (**Figure 3.4**). They designed a semi-synthetic approach to βMδVL, which included a biological pathway to synthesize the key intermediate, mevalonate, followed by its chemical conversion to βMδVL. The authors employed the endogenous enzyme AtoB of *E. coli* to produce acetoacetyl-CoA and the HMG-CoA synthase (MvaS) and HMG-CoA reductase (MvaE) from *Lactobacillus casei* to produce 88 g/L mevalonate from acetyl-CoA with a productivity of 2 g/L/h in a 1.3 L fermentor.[120] Mevalonate was dehydrated to anhydromevalonolactone using sulfuric acid and the unsaturated lactone was hydrogenated to βMδVL using Pd/C as catalyst. The polymerization of βMδVL was carried out in bulk monomer at room temperature using triazabicyclodecene (TBD) as organocatalyst and subsequently chain extension with

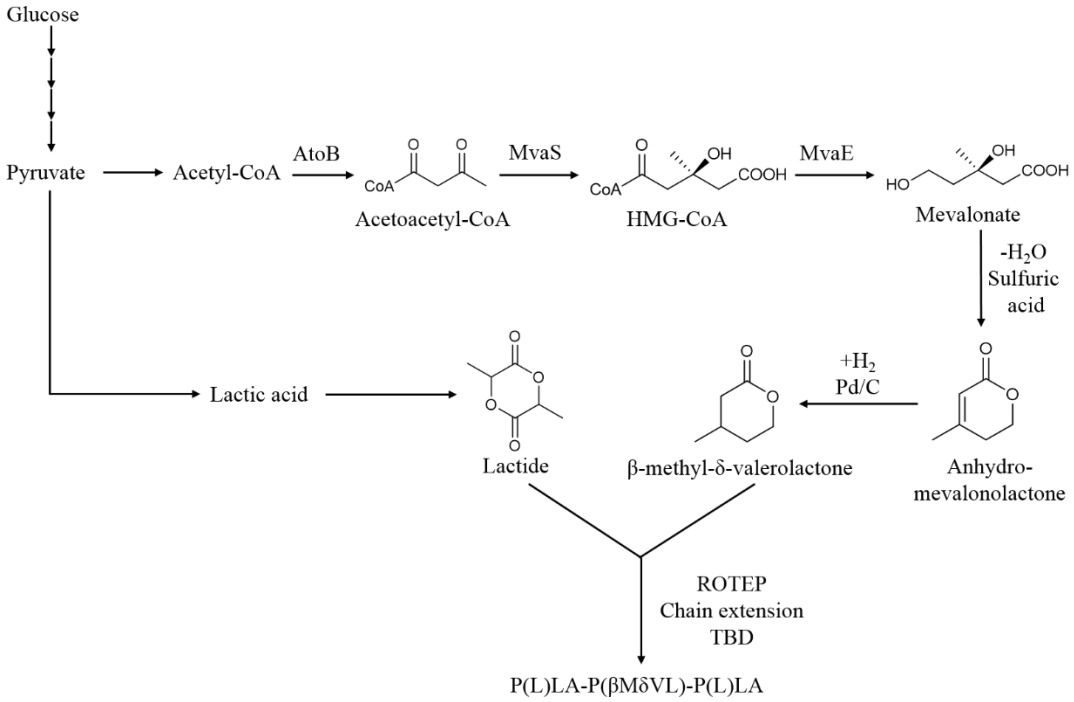

Figure 3.4 Semi-synthetic pathway for production of branched lactone, β-methyl-δ-valerolactone (βMδVL), and its copolymerization with lactide. Mevalonate was first produced in E. coli, followed by sulfuric acid catalyzed dehydration and hydrogenation to βMδVL. βMδVL and lactide were then copolymerized using ring-opening transesterification polymerization (ROTEP) to produce a triblock polymer P(L)LA-P(βMδVL)-P(L)LA with mechanically tunable properties.

lactide yielded triblock P(L)LA-P(βMδVL)-P(L)LA whose mechanical and thermal properties could be tuned by controlling molar mass, architecture and end block tacticity.[120]

3.3.2 Butadiene

1, 3-butadiene is used as feedstock for synthetic rubbers and for Nylon production, making it one of the most important conjugated dienes in the petrochemical industry.[118] Due to the recent shale gas revolution, there has been lightening of the feedstock[156] and this has resulted in an increased interest in exploring catalytic conversion of bio-derived ethanol and C4 alcohols and diols to butadiene. Recently there have been several studies

investigating the use of different metal catalysts to carry out the conversion of ethanol to BD[157-158] and in 2013, Axens, IFPEN and Michelin launched a joint research program to develop an economically competitive process for bio-synthetic rubber from bioethanol.[118] Apart from ethanol, bio-derived C4 alcohols could also be used for sustainable BD production. The biological production of n-butanol is via ABE fermentation (acetone-butanol-ethanol) of biomass-derived sugars in *Clostridia* species[159-160], and subsequent dehydration using acid-catalyzed gas phase reaction produces 1-butene which can further be dehydrogenated to yield BD.[161-162] Due to prior commercialization of ABE fermentation[159] and dehydrogenation of butenes[163], this route shows great potential for production of bio-based BD. Butanediols (1, 4-BDO, 2, 3-BDO and 1, 3-BDO) could also be used as substrate to produce BD through double dehydration reactions. Recently, Genomatica developed a bio-based route for the synthesis of 1, 4-BDO from biomass-derived sugars[154] and this process has been tested to produce 5 million pounds of BDO in 2012. Additonally, 2, 3-BDO production has also been reported in *Clostridia* species from CO-containing industrial waste gas or syngas via Wood-Ljungdahl pathway[164-165] and this has been commercialized by LanzaTech and INVISTA. The final BDO isomer, 1, 3-BDO is an intermediate of the old BD synthesis pathway based on acetaldehyde. Although there are several groups focusing on fermentative production of this diol from biomass sugars, none of the pathways have been commercialized or licensed for industrial production. All three BDOs undergo double dehydration reactions to produce BD, but they have different by-products owing to different dehydration mechanisms.[118]

3.4 Conclusion

Merging chemical and biological methods has improved overall efficiency and allowed production of much higher yields of certain compounds by reducing the total number of steps involved in synthesis.[115] In some cases, it has also made some processes more environment friendly by reducing waste and use of hazardous chemicals.[147] Furthermore, when biosynthesis is used for the production of a chiral precursor from biomass-derived sugar, it allows high enantioselectivities and regioselectivities, important in case of drugs, and also eliminates dependence on fossil-based feedstocks. In this review, we have covered the recent developments in the semi-synthesis of three widely prescribed pharmaceutical drugs – simvastatin, artemisinin and warfarin- and two commodity chemicals - βMδVL, which shows great potential in production of high-performance ABA-type bio-based polyesters and butadiene with wide applications in synthetic rubber industry.

Chapter 4

Engineered biosynthesis of biodegradable polymers*

4.1 Introduction

The worldwide annual production of plastics was 311 million tonnes in 2014 which is expected to triple by 2050, when it would account for 20% of global annual oil consumption.[166] The production of these conventional petrochemical plastics involves consumption of large amounts of fossil fuel resources and releases hundreds of millions of tons of CO_2 into the atmosphere. Due to the non-renewability and non-biodegradability of petrochemical feedstocks and the environmental concerns of plastic

* Adapted with permission from Jambunathan, P., & Zhang, K. (2016). Engineered biosynthesis of biodegradable polymers. *Journal of industrial microbiology & biotechnology*, 1-22.

Table 4.1 Summary of different microorganisms and fermentation modes used for production of different sustainable monomers

Product	Substrate	Microorganism	Fermentation mode	Lactic acid yield/titer /productivity	Reference
L-lactic acid	Glucose	*C. glutamicum*	Cell-recycle continuous reactor	43 g/L/h	[167]
	Xylose	*B. coagulans*	Fed-batch	216 g/L 4 g/L/h	[168]
	Cellobiose	*S. cerevisiae*	Batch	2.8 g/L/h	[169]
	Liquid stillage from ethanol plant	*L. rhamnosus*	Cell immobilization	42 g/L 1.69 g/L/h	[170]
	Glucose	*L. rhamnosus*	Membrane cell-recycle bioreactors	92 g/L 57 g/L/h	[171]
	Wheat straw hydrolysates	*B. coagulans*	Membrane integrated repeated batch	2.4 g/L/h	[172]
	Wood hydrolysate	*E. facecalis*	Batch	24 - 93 g/L 1.7 – 3.2 g/L/h	[173]
	Cassava starch hydrolysate	*L. casei*	Solid state fermentation	0.97 g/g of reducing sugar	[174]
	Cellulose	*L. bulgaricus*	Simultaneous saccharification and fermentation	0.45 g/L/h	[175]
	Glucose	*R. oryzae*	Cell immobilization	93.2 g/L	[176]
PHB	Glucose	*R. eutropha*	Fed-batch	121 g/L 2.42 g/L/h	[177]

	Glucose	*E. coli*	Fed-batch	80 g/L 2 g/L/h	[178]
	Saccharified potato starch	*R. eutropha*	Fed-batch	1.47 g/L/h	[179]
	Beet molasses	*A. vinelandii*	Two-stage fed-batch	36 g/L 1 g/L/h	[180]
	Waste glycerol	*C. necator*	Fed-batch	1.1 g/L/h	[181]
Succinic acid	Glucose	*A. succiniciproduce ns*	Continuous	83 g/L 10.4 g/L/h	[182]
	Glucose	*S. cerevisiae*	Batch	12.97 g/L	[183]
	Cane molasses	*A. succinogenes*	Fed-batch	55.2 g/L 1.15 g/L/h	[184]
	Corn straw hydrolysates	*A. succinogenes*	Fed-batch	53.2 g/L 121 g/L/h	[185]
	Sugarcane bagasse hydrolysates	*E. coli*	Repetitive fermentations	83 g/L	[186]
	Wheat flour	*A. succiniciproduce ns*	Batch	16 g/L	[187]

pollution, bioplastics are fast emerging as a promising alternative. Bioplastics include bio-based plastics (derived from biological resources) and biodegradable plastics (derived from fossil resources but degradable by microorganisms in nature). The global production of bioplastics is expected to grow at an annual rate of 30% in the coming decade, and expected to reach 3.45 million metric tonnes in 2020.[188] Some of the important biobased polymers include polyhydroxyalkanoates (PHA), polylactic acid (PLA), polybutylene succinate (PBS), polyethylene (PE), and polytrimethylene terephthalate (PTT), all of which contain at least one monomer synthesized via bacterial fermentation.

This review focuses on the recent advances in biotechnological production of three major biodegradable polymers – polylactic acid (PLA), polyhydroxyalkanoate (PHA) and polybutylene succinate (PBS). While PHA is produced completely by a biosynthetic process in microbes, industrial production of PLA and PBS involves microbial production of its monomer precursors, lactic acid and succinic acid and butanediol, respectively, followed by chemical transformation and polymerization. In addition to the biosynthetic pathways involved in the production of these compounds, the article covers the different lignocellulosic substrates used to lower raw material cost, various fermentation technologies and downstream recovery operations used to obtain pure monomer precursors and polymers. A summary of different microorganisms, fermentation modes and product titers for different monomers is given in **Table 1**.

4.2 Polylactic acid (PLA)

Polylactic acid (PLA) is a thermoplastic polyester derived from renewable resources such as corn starch, sugarcane, wheat and tapioca roots. The global PLA market is projected to reach $5.2 billion by 2020 and it is one of the largest bioplastics in terms of consumption volume.[189] Currently, Nature Works LLC is the leader in PLA technology and market with an annual capacity of 150,000 tons in 2013, holding a market share of 45.2%. The company has developed two lactic acid based products – a) polydilactide-based resins (Nature-Works PLA®), used for packaging and b) Ingeo™ polydilactide-based fibers that are used in textile applications. Growing environmental concerns and limited fossil fuel resources are the major factors that drive the utilization of PLA by both consumers as well as manufacturers. Conventionally, PLA is synthesized using a two-step process

which includes fermentative production of lactic acid followed by a chemical process to polymerize the lactic acid monomer. Industrially, companies such as Natureworks, use ring opening polymerization of the lactide intermediate to synthesize PLA biopolymer.[175, 190]

4.2.1 Fermentative production by lactic acid bacteria (LAB)

An optically pure L- or D- lactic acid is preferred over a racemic DL-lactic acid to synthesize highly crystalline PLA that can be used commercially.[12] Since chemical synthesis of lactic acid from petrochemical sources always produces the racemic mixture, industrial production of lactic acid is predominantly carried out by microbial fermentation process.

LAB are one of the possible hosts for commercial lactic acid production since they produce lactic acid as the main fermentation product thus yielding maximum productivity. LAB are anaerobic and have two major pathways for assimilation of glucose and xylose – Embden-Meyerhof-Parnas (EMP) pathway and the pentose phosphoketolase (PK) pathway. Based on the nature of fermentation and the assimilation pathway used, LAB can be homofermentative or heterofermentative. Homofermentative bacteria produce lactic acid as the only fermentation product via EMP pathway whereas heterofermentative bacteria use PK pathway to produce a mixture of products including lactic acid, ethanol, diacetyl formate, acetic acid and carbon dioxide.[191]

Although a high lactic acid concentration is desired, most organisms cannot grow and produce lactic acid at a pH below 4 due to their low acid tolerance.[192] Several studies

have focused on engineering the acid tolerance of LAB and other microorganisms to prevent product inhibition. One of the successful approaches is genome shuffling in which classical methods such as chemostat adaptation, UV radiation and nitrosoguanidine (NTG) mutations are used to generate improved populations and genome shuffling of these generates a new strain with improved acid tolerance. Stemmer et al. used this approach to generate a genome shuffled *Lactobacillus* strain that grew faster and produced two times more lactic acid than the wild type, lowering the pH of broth to 3.5.[193] At such low pH, most of the product exists as free acid (pK_a of lactic acid 3.78) and it can be purified by direct extraction of the fermentation broth, thus avoiding a wasteful and expensive purification. Although this is very promising, realistic sugar concentrations were not used in this study. Similar methods were also used to improve the glucose tolerance of *Lactobacillus rhamnosus* to avoid substrate inhibition at high glucose concentrations resulting in a dramatically enhanced lactic acid production.[194-195]

4.2.2 Alternative cheaper substrates for lactic acid production

One of the other challenges in large-scale fermentative production of lactic acid is the cost of raw materials. Substrate cost accounts for almost 30-40% of the total production costs.[196] Although the use of refined carbohydrates or pure sugars such as glucose, sucrose, lactose etc. would reduce downstream product purification cost, they would result in an increased overall production cost given the high cost of pure sugars. Approximately 3.5 billion tonnes of agricultural residues are produced per annum globally[197], and some alternate cheaper agricultural residues that have been used for lactic acid production include lignocellulose/hemicellulose hydrolysates[198], wood

hydrolysates[199], corncob, corn stalks[200], cassava bagasse[201-203], cellulose[204-205], paper sludge[206], defatted rice bran[207], waste cardboard[208], unpolished rice[209], carrot processing waste[197], corn fiber hydrolysates[174] and wheat bran.[203, 210]

In order to achieve maximum yields and productivity, it is important that the mixed sugars present in lignocellulosic hydrolysates be utilized simultaneously without carbon catabolite repression. But in many LAB, sugars are sequentially metabolized and the utilization of glucose represses the utilization of other sugars.[178] A few LAB strains have demonstrated simultaneous consumption of lignocellulose-derived sugars e.g. *Lactobacillus brevis*[211-212], *L. plantarum*[211] and novel isolated LAB strain *Enterococcus mundtii*.[213] Thus, it is essential to isolate novel strains or develop engineered microorganisms that are capable of using lignocellulose directly for the production of high yields of lactic acid with high productivity.

4.2.3 Lactic acid production by other engineered microorganisms

Due to the low-pH tolerance and complex nutritional requirements of LAB, several other micro-organisms have been studied for their ability to ferment different sugars to lactic acid in a cost-efficient manner. A competitive commercial process requires robust, fast-growing, acid tolerant, and high yielding strains that have simple nutritional requirements.[214]

4.2.3.1 Filamentous fungi

Filamentous fungi such as *Rhizopus sp.* have shown great potential as suitable candidates for the production of lactic acid using simple, low-cost nutrients.[215-217] They have several

advantages over LAB including use of chemically defined medium simplifying product separation, their ability to effectively use pentose as well as hexose sugars, low-cost downstream separation of biomass due to their filamentous and pellet forms, and the production of L-lactic acid as the sole isomer.[216] *Rhizopus oryzae* is the best known fungal source of lactic acid and there have been several studies using submerged fermentation, immobilized cells or pellets and different reactor configurations including pneumatic and stirred tank reactors for the production of enantiomerically pure L-lactic acid. However, since they are aerobic in nature, fermentation requires significant agitation and aeration which increases energy cost. Also, due to the production of by-products such as ethanol and fumaric acid, lactic acid production using *Rhizopus sp*. suffers from low yields and productivity. Production of lactic acid using filamentous fungi has been covered extensively by Zhang et al..[216]

4.2.3.2 Bacteria

Escherichia coli, the workhorse of biotechnology industry, can easily metabolize hexose and pentose sugars using a simple mineral salt medium. But under anaerobic conditions, it produces a mixture of organic acids including D-lactic acid, acetic acid, succinic acid, formic acid and ethanol which reduces the yield of lactic acid and also makes product separation difficult. Several combinations of gene knockouts have been attempted to avoid formation of these by-products but most of them result in very long fermentation times due to significantly slower growth of microorganism.[185, 218-219] Zhou et al. successfully engineered *E. coli* to produce 48.6 g/L of D-lactic acid with high yield of 0.98 g/g of xylose by knocking out *pflB*, *frdBC*, *adhE* and *ackA* genes involved in the

production of fumaric acid, succinic acid, ethanol and acetic acid respectively. Thus, the resultant strain SZ63 produced negligible by-products but had a long fermentation time of 168 hours resulting in low productivity.[218] Additionally, the same strain, SZ63 was also used for the production of L-lactic acid from xylose in mineral salt medium, by replacing a part of D-LDH gene of *E. coli* (*ldhA*) with L-LDH gene of *Pediococcus acidilactici* (*ldhL*) and afforded 40 g/L of L-lactic acid with yield of 0.93 g/g xylose in 312 h.[185] Despite the advantages of being able to use simple mineral salt medium to achieve high yields of optically pure L-and D-lactic acid from hexoses and pentoses, lactic acid fermentation using *E. coli* suffers from low productivity and low acid tolerance requiring fermentation to be carried out at pH~7.[220]

Corynebacterium glutamicum is another aerobic bacterium that has been genetically engineered to produce lactic acid from hexose and pentose sugars. Under oxygen-limited conditions, cell growth is arrested but it retains its ability to produce mixed organic acids such as L-lactic acid, acetic acid and succinic acid from glucose using mineral salt medium.[221] Cells are first grown aerobically to a very high density and this high density culture is used for the anaerobic production of lactic acid resulting in a high-throughput process.[222] *C. glutamicum* has been used for the production of L-lactic acid with high volumetric productivity of 42.9 g/l/h along with significant succinic acid production.[223] The same strain was also used for the production of D-lactic acid by expressing D-LDH gene from *Lactobacillus delbrueckii* in the *C. glutamicum* Δ*ldhA* strain.[167] Due to the inability of the bacterium to use pentoses such as xylose and arabinose, the corresponding genes for xylose and arabinose metabolism from *E. coli*

were expressed under a constitutive promoter *trc* in *C. glutamicum* allowing production of L-lactic acid from mixture of glucose and xylose and glucose and arabinose respectively.[224-226] Sasaki et al. developed a *C. glutamicum* strain capable of utilizing glucose, xylose and cellobiose simultaneously to produce lactic acid.[226] Although very high volumetric productivities using simple mineral salt medium can be obtained by using *C. glutamicum* strain, the bacterium has extremely low acid tolerance and the lactic acid fermentation operates at pH of 7.0 and it produces a mixture of lactic acid, succinic acid and acetic acid giving low yields of lactic acid.

More recently, a thermophilic lactic acid producer, *Bacillus coagulans*, has been isolated and identified as an efficient lactic acid producer capable of using a wide variety of substrates. This organism has shown a remarkable capability of fermenting pentoses, hexoses and cellobioses and is also resistant to inhibitors present in lignocellulosic hydrolyzates. Dilute acid biomass pretreatment followed by simultaneous saccharification and co-fermentation (SSCF) of *B. coagulans* IPE22 allowed production of 46 g of lactic acid from 100 g wheat straw.[227] In a different study, cost-effective lactic acid production with high optical purity was obtained when excess sludge was used as a nutrient source instead of yeast extract in a repeated batch fermentation using *B. coagulans* strain.[228] Another strain, *B. coagulans* C106, was isolated from the environment and was used for lactic acid production from xylose at 50 ^{o}C and pH of 6 in a mineral salts medium containing 1-2% (w/v) of yeast extract. A fed batch fermentation using this strain resulted in lactic acid titer and productivity of 215.7 g/L and 4 g/L/h, respectively, which are one of the highest values on xylose reported so far.[229]

4.2.3.3 Yeast

Yeasts such as *Saccharomyces cerevisiae* and *Kluyveromyces* sp. are much more tolerant to low-pH conditions compared to bacterial species; this significantly reduces overall lactic acid production cost by simplifying the product recovery and purification stage. But yeasts do not natively produce significant amounts of lactic acid. Under anaerobic conditions, yeasts produce ethanol from pyruvic acid but they can be metabolically engineered to produce lactic acid by expressing heterologous LDH gene from *Lactobacillus sp.*.[230-231] Several pyruvate decarboxylase (*pdc*) mutants were generated to inhibit ethanol production during anaerobic fermentation and genome integration of heterologous LDH gene into PDC1 locus was used for improved lactic acid production.[192, 232-236] Tokuhiro et al. developed a *pdc1 adh1* double mutant that had much better growth rates allowing production of 71.8 g/L of lactic acid with yield of 0.74 g/g of glucose in 63 hours.[237] Similar studies were done using *Kluyveromyces lactis* for the production of L-lactic acid by using a strain lacking pyruvate decarboxylase (KlPDC1) and pyruvate dehydrogenase (KlPDA1) genes and expressing bovine LDH gene under KlPDC1 promoter, but fermentation time to produce 60 g/L L-lactic acid was 500 hours.[238] *S. cerevisiae* was also engineered to produce lactic acid from cellobiose by integrating eight copies of bovine LDH genes and two copies of BGL1 gene from *Aspergillus aculeatus* into its genome, resulting in 2.8 g/l/h of lactic acid from 95 g/L of cellobiose with yield of 0.7 g/g of Cellobiose.[239] Cargill screened 1200 yeast strains and developed a novel yeast strain CB1 capable of producing lactic acid at pH of 3.[240] Replacing lactic acid bacteria with a genetically engineered yeast strain and the low pH

fermentation technology made the process more cost competitive and also significantly reduced environmental footprint.

4.2.4 PLA copolymers with improved properties

Despite several advantages of PLA, one of the biggest disadvantages of PLA is that it is very stiff and brittle with high glass transition temperature which impedes its applications in high mechanical strength fields. One of the approaches to achieve a good toughness-stiffness balance is to copolymerize PLA with rubbery polymers. Plasticizers such as polyethylene glycol (PEG) can be copolymerized with PLA to improve polymer process-ability.[241-242] One strategy that has been used to expand the applicability of PLA is the use of PLA-containing block copolymers, particularly ABA triblock thermoplastic elastomers (TPE) with rigid PLA as end blocks and soft, rubbery midblocks. Although several PLA-containing block polymers with favorable properties have been reported, the starting materials used for the synthesis of these polymers are either derived from fossil fuels or prohibitively expensive natural products. Recently, Xiong et al. developed an efficient biosynthetic route for the production of a branched lactone, β-methyl-δ-valerolactone (βMδVL), which can be transformed into a rubbery polymer with low glass transition temperature.[243] The artificial pathway expands the mevalonate pathway in *E. coli*, to convert mevalonate to βMδVL (**Figure 4.1**). For the production of the mevalonate precursor, the *E. coli* endogenous acetyl-CoA acetyltransferase (AtoB) enzyme was over-expressed and the heterologous enzymes, HMG-CoA synthase (MvaS) and HMG-CoA reductase (MvaE), from *Lactobacillus casei* were cloned into *E. coli*. To biosynthesize anhydromevalonolactone, mevalonate was first converted to mevalonyl-CoA using acyl-

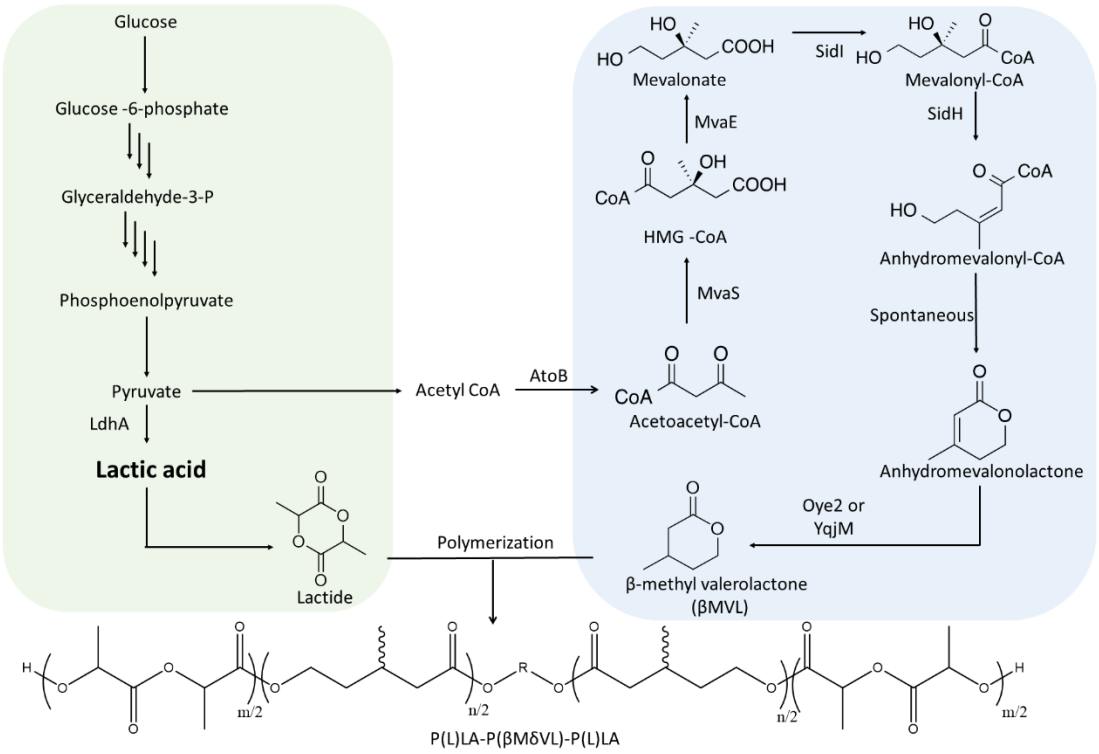

Figure 4.1 Biosynthetic pathway in *E. coli* for the production of lactic acid and β-methyl-δ-valerolactone (βMδVL). LdhA: lactate dehydrogenase; AtoB: acetyl-CoA acetyltransferase; MvaS: HMG-CoA synthase; MvaE: HMG-CoA reductase; SidI: acyl-CoA ligase; SidH: enoyl-CoA hydratase; Oye2: enoate reductase; YqjM: enoate reductase.

CoA ligase (SidI) of *Aspergillus fumigatus*, which was further transformed to anhydromevalonyl-CoA by enoyl-CoA hydratase (SidH) from *A. fumigatus*, and finally spontaneous cyclization produced anhydromevalonolactone. In the last step, enoate reductases, Oye2 from *S. cerevisiae* and YqjM mutant from *B. subtilis*, were used to convert the unsaturated lactone to βMδVL.[243] This bio-derived monomer, βMδVL, was converted to a rubbery polymer using controlled polymerization techniques at ambient temperature, and the addition of lactide to poly (β-methyl-δ-valerolactone) midblocks resulted in the first scalable biobased soft polyester block with mechanically tunable properties and a low glass transition temperature of -50 ºC.[243]

4.2.5 Fermentation technologies used for lactic acid production

While batch fermentation is the most common mode used for lactic acid production, numerous other studies have used fed-batch, repeated batch, and continuous fermentation. Batch and fed-batch cultures allow higher lactic acid concentrations and yields as compared to continuous cultures due to complete utilization of substrate, whereas the productivities are generally much higher in continuous fermentation due to operation at high dilution rates.[244] These fermentation modes have been reviewed by Abdel-Rahman et al..[12] To further improve lactic acid production in batch fermentation mode, it has been observed that use of mixed cultures of LAB may be more effective than single cultures. Garde et al. showed that use of mixed cultures of *Lactobacillus brevis* and *Lactobacillus pentosus* for lactic acid production from hemicellulose hydrolysate allowed almost complete utilization of substrate components and a lactic acid yield of 95%, which was higher than yields obtained by pure *Lb. pentosus* culture (88%) and pure *Lb. brevis* culture (51%).[245] Mixed cultures of different microorganisms have also been used where one organism breaks down the polymeric substrate while the other carries out the fermentation. One such study involved the use of mixed cultures of *Aspergillus niger* and *Lactobacillus sp.* to produce lactic acid directly from Jerusalem artichoke tubers in a simultaneous saccharification and fermentation (SSF) process.[246] *Aspergillus* produces the enzymes, inulinase and invertase, required to break down inulin present in artichoke tubers, which cannot be metabolized by *Lactobacillus sp.*. These studies look promising for the use of mixed cultures for industrial lactic acid production from cheap polymeric substrates or under nutrient-limiting conditions, without compromising on yield and

productivity. Using high cell densities in fermentative production systems is another way to improve lactic acid production by allowing high productivities and reduced contamination problems. Two methods used for achieving high cell densities, which will be discussed in this paper, are cell immobilization in continuous cultures and membrane cell recycling.

4.2.5.1 Cell immobilization

Immobilization of cells allows increased cell concentrations in continuous fermentors by preventing cell washout at high dilution rates, thus resulting in higher lactic acid productivity. One of the most common methods used for immobilization is adsorption (or attachment) on solid carrier surfaces where the cells are held to the surface by physical forces (van der Waals forces) or electrostatic forces or covalent binding between the cell membrane and the carrier.[12] The advantage of this method is its simplicity but the relative weakness of adsorptive binding force is one of the biggest disadvantages. Several supports have been used for lactic acid production including activated carbon[247], aluminum beads[248], glass and ceramics[249] and zeolites[170] amongst others. In a recent study, powdered zeolite molecular sieves 13X were used for immobilization of *L. rhamnosus* for lactic acid production from liquid stillage from bioethanol production. The study was performed without mineral or nitrogen supplementation and the maximal process productivity was 1.7 g/L/h with maximum lactic acid concentration of 42.2 g/L.[170] Another method used for cell immobilization is physical entrapment of cells in the core of beads. One of the most common materials used for entrapment is polysaccharide gels like calcium alginate gel beads.[179] Some of the limitations of this method include

slow leakage of cells during long operations and diffusional resistance of the gel matrix resulting in insufficient oxygen supply and reduced fermentation efficiencies. Tanyildizi et al. used this method to immobilize *R. oryzae* cells in open pore matrix of polyurethane foam to reduce diffusional resistance to substrate transfers.[215] Lactic acid production was 55% higher when immobilized *R. oryzae* was used as compared to free cells. Other methods of immobilization include containment[250], where cells are entrapped behind a barrier such as membrane filters, and self-aggregation[251], which a natural immobilization technique observed in molds and fungi.

4.2.5.2 Cell recycle

Membrane cell-recycle bioreactors (MCRB) are also used to achieve high cell densities and can enhance volumetric productivity of lactic acid up to 160 g/L/h, which is twenty times higher than that obtained in batch fermentations.[252] But the lactic acid concentration obtained in MCRB is significantly low (less than 60 g/L in most studies) when compared to batch processes which can easily achieve lactic acid concentrations above 120 g/L, thus increasing downstream energy cost of water removal.[253] To improve the economic advantage of MCRB by increasing lactic acid concentration, Kwon et al. used two MCRBs in series to produce 92 g/L lactic acid with a productivity of 57 g/L/h by *Lb. rhamnosus.*[253] One of the types of membrane used for MCRBs is polymeric membranes that have very low tolerance to high temperatures, and undergo membrane fouling which necessitates frequent cleaning procedures, thus weakening the membrane. Ramchandran et al. investigated the use of submerged polymeric membranes for lactic acid production and used fresh nutrient-rich medium as backwash to reduce membrane

fouling.[254] This not only improved performance of membrane module but also increased lactic acid production by more than 2-fold by replacing growth medium containing inhibitory fermentation products with fresh medium. Ceramic membranes offer several advantages over polymeric membranes including thermal stability, easier cleaning and higher abrasive and mechanical resistance. Lu et al. used a pilot scale bioreactor for lactic acid production comprising of 3000 L fermentor and an external ceramic microfiltration membrane to perform cell recycle. With repeated feeding medium used to alleviate substrate inhibition, pilot system with cell recycle was able to achieve lactic acid yield of 157 g/L and productivity of 8.8 g/L/h.[184] Recently, Zhang et al. used *Bacillus coagulans* IPE22 to produce lactic acid from wheat hydrolysates and to eliminate sequential utilization of sugars and product inhibition, they used membrane integrated repeated batch fermentation (MIRB). Using MIRB system, the lactic acid productivity was increased from 1 g/L (batch 1) to 2.4 g/L (batch 6) by repeated batch fermentation.[255]

4.2.6 Downstream processing of lactic acid

Conventional lactic acid fermentation produces calcium lactate, due to pH neutralization, which is treated with concentrated sulphuric acid to give free lactic acid and calcium sulphate (or gypsum). This traditional recovery method is a major economic hurdle in the lactic acid production process due to the use of large quantities of expensive chemicals which account for 50% of the production cost and generation of gypsum waste. Some alternative technologies used for the recovery of lactic acid, which will be discussed later in more detail , include adsorption[256], reactive distillation[257], solvent extraction[183], electrodialysis with bipolar membranes[258], nanofiltration[259-260] and ion exchange[261-262], all

of which avoid the formation of large quantities of insoluble salts and are more cost and energy efficient compared to traditional chemical separation processes.[191, 260] In bipolar membrane electrodialysis, water splitting reaction occurs at the bipolar membrane which generates protons for conversion of lactate salt to lactic acid and hydroxide ion for sodium cation to form sodium hydroxide which can be recycled back to the fermentor. Li et al. used this method for lactic acid recovery and pH control and improved the lactic acid yield from 0.46 g/g glucose (without electrodialysis) to 0.61 g/g glucose.[260] Furthermore, to improve the efficacy and capacity of electrodialysis, nanofiltration has been used as a pretreatment method to remove Mg-, Ca- and sulphate ions from lactate fermentation broth.[191] In one such study, cross-flow nanofiltration was used to retain 94% of sugar and this membrane module was integrated with a downstream bipolar electrodialysis unit that allowed continuous lactic acid production with an optical purity of 85.6%.[263] Although electrodialysis is an expensive technology owing to large energy consumption, the recycling of unconverted sugars can significantly reduce raw material consumption making it economically feasible. Resin adsorption has also been used to recover lactic acid from fermentation broth in a study by Wang et al., where microfiltration membrane integrated with fermenter was used to relieve product inhibition and to extend cell growth period from 4 h to 120 h.[264] Reactive liquid-liquid extraction is another promising technology that has been studied for lactic acid recovery and recently, a new extractant , N,N- didodecylpyridin-4-amine, was developed that has the highest distribution coefficient of lactic acid and back extraction was feasible at

elevated temperatures with single stage recoveries up to 80% using heptane as an anti-solvent.[265]

4.3 Polyhydroxybutyrate (PHB)

Polyhydroxyalkanoates (PHAs) are microbial polyesters containing 3-, 4-, 5- and 6-hydroxycarboxylic acids that accumulate as intracellular carbon/energy storage granules in a wide variety of microorganisms usually when there is a growth limiting nutrient such as O, N, P, S or trace elements in the presence of excess carbon source. They are completely biosynthetic and biodegradable with zero toxic waste since microorganisms present in the soil, sea and sewage degrade them into carbon dioxide and water under aerobic conditions and into methane under anaerobic conditions.[186, 266-267] In addition to their biodegradability, these polymers are biocompatible and they have properties similar to thermoplastics such as polypropylene, making them an ideal substitute for conventional petrochemical plastics.[268-269] Depending on the number of carbon atoms, PHAs are divided into two groups – short chain length (SCL), which consist of 3-5 carbon atoms and have thermoplastic properties similar to polypropylene, and medium chain length (MCL), which consist of 6-14 carbon atoms and have elastomer like properties.[270] In this review, we will mainly focus polyhydroxybutyrate (PHB) and its copolymers with higher acyl CoAs. The biosynthetic pathway for production of short chain PHAs from sugars is shown in **Figure 4.2**.

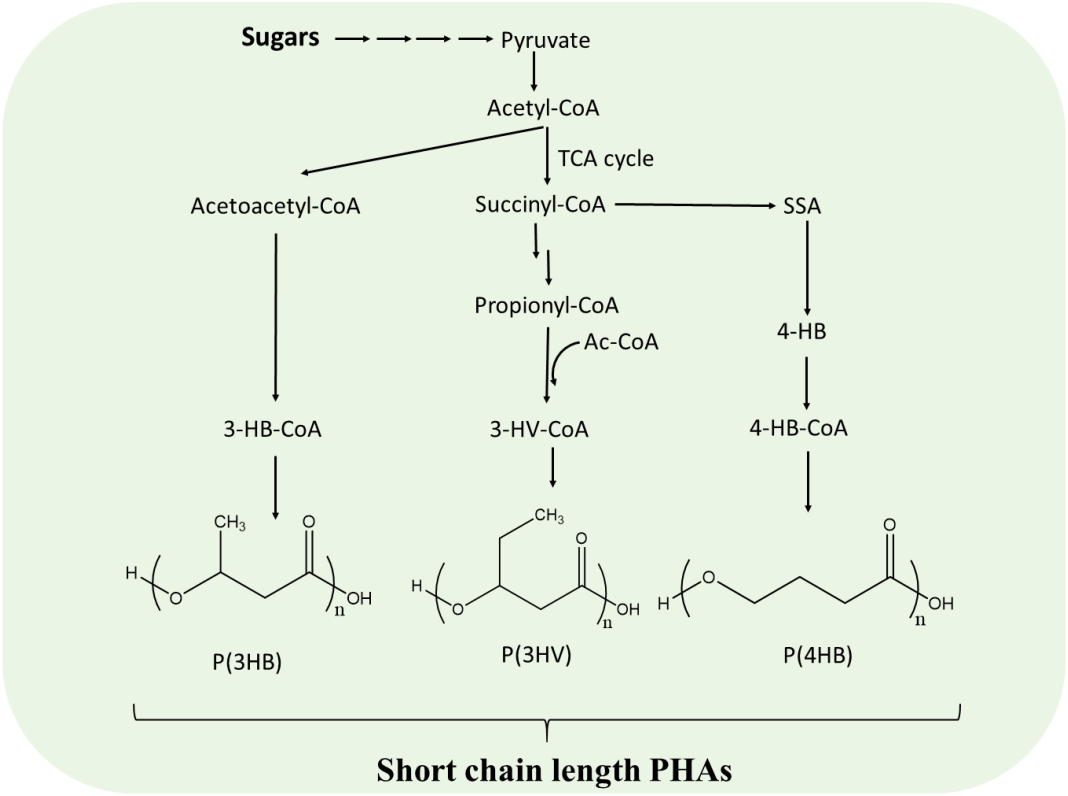

Figure 4.2 Biosynthetic pathway for the production of short chain polyhydroxyalkanoates (PHAs). SSA: succinic semialdehyde; 3-HB-CoA: 3-hydroxybutyryl-CoA; 3-HV-CoA: 3-hydroxyvaleryl-CoA; 4-HB-CoA: 4-hydroxybutyryl-CoA; Ac-CoA: acetyl CoA; P (3HB): poly (3-hydroxybutyrate); P (3HV): poly (3-hydroxyvalerate); P (4-HB): poly (4-hydroxybutyrate)

PHB was the first PHA to be discovered.[271] It is accumulated in bacteria at up to 80% of the dry cell weight and it has material properties very similar to conventional plastics like polyethylene and polypropylene. But, PHB is a brittle and rigid polymer with low flexibility and it has high melting temperature (170 ^{o}C) making polymer processing difficult.[272-273] On the other hand, medium chain length-PHAs made up of longer monomers, are typically elastomers having high flexibility. Thus, copolymerization of 3HB with longer monomers such as HV (hydroxyvalerate), HH (hydroxyhexanoate) or HO (hydroxyoctanoate) can result in more flexible and tougher plastics with reduced

melting point such as poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)], poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)], poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) [P(3HB-co-3HHx)], and poly(3-hydroxybutyrate-co-3-hydroxyalkanoate) [P(3HB-co-3HA)].[274-277] PHB was first industrially produced by Imperial Chemical Industries ltd. (ICI/Zeneca Bioproducts, Bellingham, UK) in 1970 under the trade name Biopol™. In 1996, the technology was sold to Monsanto and then to Metabolix in 2001. In 2010, Telles, a joint venture company between Archer Daniel Midlands Company (ADM) and Metabolix, Inc. opened the first commercial plant to produce corn-syrup based PHA resin, Mirel™, in Clinton, Iowa, U.S.A at a capacity of 50,000 tons per year. Another example of a successful copolymer is P(3HB-co-3HHx) that is produced on an industrial scale.[278] Furthermore, U.S.A. based Procter & Gamble has trademarked scl and mcl PA copolymers of C_4 and C_6-C_{12} as Nodax™.[176]

4.3.1 PHA production by bacteria

The two most widely studied bacteria for PHB production are *Ralstonia eutropha* and *Alcaligenes latus*. *R. eutropha* accumulates PHB when nitrogen and phosphorous is completely depleted in the medium. ICI used fed-batch culture of *R. eutropha* for the industrial production of PHB from glucose and P (3HB-co-3HV) from a mixture of glucose and propionic acid under phosphate limiting conditions. The strain produced 121 g/L of PHB with 76% polymer content in 50 hours resulting in a high productivity of 2.4 g/L/h using an automatic fed-batch culture technique where glucose concentration was maintained at 10-20 g/L.[279] Unlike *R. eutropha*, *A. latus* can accumulate PHA during

growth and does not require nutrient limitation thus allowing use of complex nitrogen sources such as corn steep liquor and yeast extract to support cell growth as well as PHA synthesis.

Although natural producers, such as *R. eutropha* and *A. latus*, are well adapted to PHB accumulation and can store up to 90% of its weight in PHA granules, they show very poor growth during fermentation, they can depolymerize PHB and use it as a secondary energy source and the extraction of PHA polymers from these cells is very difficult. To address this issue, the PHA biosynthetic pathway can be expressed in non-PHA producers with more robust central metabolic pathway for more efficient production of PHA using inexpensive carbon sources. An example of one such host organism is *E. coli* which offers several advantages including fast growth, accumulation of large amounts of PHA due to the absence of intracellular depolymerases, ability to use several inexpensive carbon sources and easy recovery of PHA granules.[280] Synthesis of PHB by recombinant *E. coli* is dependent on the amount of acetyl-CoA available and does not require nutrient limitation. Slater et al. was the first to introduce *pha* genes into *E. coli* in 1988 and after several efforts to improve PHB production using recombinant *E. coli*, a PHB concentration higher than 80 g/L with productivity greater than 2 g/L/h was obtained using pH-stat fed-batch culture.[177] A recombinant *E. coli* strain with *R. eutropha* PHB biosynthetic genes was used to produce 80% (w/w) of PHB after 35 hours of fermentation using molasses as carbon source.[281] In addition to P (3HB), recombinant *E. coli* was also used to produce 4.4 g/L of P (4HB) after 60 hours of pH-controlled fed-batch fermentation from glucose and 4-hydroxybutyric acid as carbon sources.[282]

Recombinant *E. coli* strain was also used to synthesize copolymers such as terpolymer P (3HB-co-3HV-co-3HHx) from dodecanoic acid plus odd carbon number fatty acid using *Aeromonas* PHA biosynthetic genes.[283]

Several studies have attempted to evolve PHA synthases to broaden their substrate specificities and to enable them to accept both scl- and mcl- monomers into the growing polymer chain to efficiently produce PHB copolymers. One of the earliest studies was to evolve a PHA synthase capable of accepting both 3HB-CoA and 3HHx-CoA to efficiently produce P (3HB-co-3HHx), a tough and flexible polymer. *In vitro* and *in vivo* evolution of PHA synthases from *Aeromonas* sp. was performed by random mutagenesis to screen for an enzyme with enhanced activity.[284-285] In one of the studies, random mutagenesis of *A. caviae* PHA synthase gene ($PhaC_{Ac}$) resulted in two single mutants (N149S and D171G), both of which had increased *in vitro* activities resulting in a 6.5 fold increase in PHA accumulation and a concomitant increase in the 3HHx fraction from 10% to 18%.[285] The double mutant of *A. caviae* PHA synthase (N149S and D171G), was expressed in recombinant *R. eutropha* and it resulted in incorporation of 0.4 mol% of 3-hydroxyocatnoate (3HO) and 18 mol% of 3HHx in the PHA copolymer from octanoate as carbon source.[169, 286] The PHA synthase of *Pseudomonas* sp. 61-3 accepts both scl- and mcl- monomers but has very weak activity towards scl-monomers. *In vitro* evolution of this PHA synthase by PCR-mediated random mutagenesis resulted in a quadruple mutant with increased substrate specificity towards 3-HB without lowering its activity towards MCL-HA-CoAs resulting in 340-400 times higher production of P (3HB). This has also allowed the production of P (3HB-co-3HA) copolymer with over 95

mol% 3HB and a small amount of MCL-3HA.[287-294] Several other studies of evolution of PHA synthases using site-saturated mutagenesis (allowing the substitution of predetermined protein sites against all twenty possible amino acids at once) and random mutagenesis has been reviewed in detail by Park et al..[295]

4.3.2 PHA production in yeast

In order to develop more cost-effective systems for PHA synthesis, eukaryotic cells including yeast and insect cells and transgenic plants have been studied for their ability to produce PHA. Synthesis of PHB has been demonstrated in eukaryotic cells such as *Saccharomyces cerevisiae* by expression of PHB synthase gene from *R. eutropha*, but this resulted in very low PHB accumulation of 0.5% of dry cell weight possibly due to low activities of endogenous β-ketoacyl-CoA-thiolase and acetoacetyl-CoA reductase enzymes.[296] Kocharin et al. engineered the acetyl-CoA supply in *S. cerevisiae* by over-expressing the genes of ethanol degradation pathway along with PHB pathway genes. This increased acetyl-CoA supply improved the productivity of PHB by 16 times indicating that availability of acetyl-CoA precursor has an effect on PHB production.[297] In a different study, the same group over-expressed the phosphoketolase pathway of *Aspergillus nidulans* to increase acetyl-CoA supply and improved PHB production in *S. cerevisiae.*[298]

4.3.3 PHA production in plants

PHA production in plants is considerably less expensive than bacterial and yeast systems as they do not require an external energy source such as electricity to carry out

fermentation. In addition to being cost-effective, plant production systems are environmentally friendly since they only require photosynthetically fixed CO_2 and water to produce PHA which is degraded back to CO_2 and water and they also provide a useful tool to study plant metabolism.[299] PHA production in plants can be achieved in different subcellular compartments. Acetyl-CoA, required for PHB synthesis, is present in the cytosol, plastid, mitochondrion and peroxisome of the plant, and thus PHB production can theoretically be achieved in any of these compartments. PHB production in plants was first demonstrated in 1992 in the cytoplasm of cells of *Arabidopsis thaliana* by over-expressing the genes – *phaB*, encoding acetoacetyl-CoA reductase and *phaC*, encoding PHB synthase – from *R. eutropha* under a constitutive cauliflower mosaic virus 35S (CaMV35S) promoter.[300] In order to use transgenic plants for commercial production of PHAs, there is an urgent need to improve the yields of PHA obtained using these plants. One of the reasons for low productivity may be attributed to the adverse effects of *phaB* or *phaA* genes on plant growth.[300-301] Constitutive expression of PHA synthesis genes (*phaA*) significantly reduced the transformation efficiency in potato and tobacco.[302] To solve this problem, an inducible promoter was used to express *phaA* gene and, although this resulted in two-fold increase in PHB production in *Arabidopsis* lines, there was no increase in PHB amount (<1% dwt.) in potato and tobacco.[303] One of the strategies used to improve PHB synthesis in plants was to increase the acetyl-CoA pool for PHB synthesis. This was achieved by using specific enzyme inhibitors to suppress the competing anabolic pathways involved in acetyl-CoA consumption. Use of Quizalofop

(herbicide), which inhibits the conversion of acetyl-CoA to malonyl-CoA, increased PHB content in cytosol by 170% and in the cytosol by 150%.[304]

4.3.4 PHA production on alternate carbon source

Despite its several advantages of being biodegradable and biocompatible, high production cost of PHA makes it 5-10 times more expensive than petroleum-derived polymers such as polypropylene and polyethylene (US \$0.25-0.5 kg^{-1}).[305] One of the biggest factors contributing to high production cost of PHA is the cost of substrate (mainly carbon source) which accounts for 30-40% of total production cost. Apart from glucose, several other carbon sources including lactic acid[306], acetic acid[307], oleic acid[187], carbon dioxide, plant oils[308] and waste glycerol[309] have been used as the sole carbon source to produce PHB using fed-batch cultures of *R. eutropha*. *R. eutropha* was engineered to produce 94 g/L of PHB with productivity of 1.5 g/L/h from saccharified potato starch when grown under phosphate limitation.[310] Industrial by-products such as beet molasses and sugarcane molasses have been used for PHB production using different microorganisms.[180, 281, 311-312] A two-stage fed-batch culture of *A. vinelandii* UWD mutant was used to produce 36 g/L of PHB with productivity of 1 g/L/h from beet molasses.[180] In order to reduce raw material cost, methanol was used as carbon source for PHB production using an automatic fed-batch culture of *Methylobacterium extorquens* resulting in a high PHB concentration (149 g/L) but a very low productivity of 0.88 g/L/h.[313] Methylotrophic bacteria have been used to synthesize PHBV copolymer with 3HV content up to 91.5 mol% using methanol and n-amyl alcohol as carbon sources under nitrogen limiting conditions.[314] The advantage of using alcohols as carbon sources

is the reduced chance of contamination given that they are sterile carbon sources. Fatty acids and vegetable oils are also promising substrates for PHA production since the theoretical yield of PHA from fatty acids is 0.65 g g^{-1} [173] whereas that from glucose is 0.3-0.4g g^{-1}.[315] *C. necator* H16 was used for the production of PHA from soybean oil and yield obtained was 0.72 - 0.76 g g^{-1}.[316] A recombinant strain of *R. eutropha* transformed with the PHA synthase of *Aeromonas caviae* was used to produce terpolymers of poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyheptanoate) using odd numbered alkanoic acids.[317] Recently, waste glycerol was used for PHB production using *C. necator* strain and the productivity of the process was 1.1 g/L/h with PHB content of 50%.[181] Tanaka et al. recently reviewed the production of PHB using C1 carbon substrates such as methanol, methane and carbon dioxide.[273]

4.3.5 Recovery of PHA

High production costs of PHB and other PHAs have adversely affected their market penetration and one of the major cost drivers is the downstream recovery and purification of PHA. Since the polymer is produced intracellularly, the recovery methods focus on either solubilizing the PHA granules or dissolution of the non-PHA biomass. Different recovery methods used for PHA have been reviewed in great detail by Kunasundari and Sudesh.[318]

Solvent extraction is the most widely used technology for recovery of PHA from cell biomass which involves solubilization of PHA followed by non-solvent precipitation. The most commonly used solvents include chlorinated hydrocarbons and cyclic carbonates and typical non-solvents used for precipitation include methanol and

ethanol.[319] Although these methods give the best recovery yields and purity, the solvents used are very toxic to the environment and the process becomes lengthy when PHB concentration exceeds 5% (w/v) due to high viscosity of the polymer solution. 1,2-propylene carbonate is relatively less toxic than chlorinated solvents and maximum PHA yield of 95% and purity of 84% was reported during recovery of PHA from *Cupriavidus necator* cells which is comparable to the values obtained from chloroform extraction.[320] Another recovery method for PHA involves solubilization of non-PHA biomass using chemical or enzymatic digestion. In the case of chemical digestion, sodium hypochlorite and surfactants are the two most commonly used chemicals. Surfactant-chelate digestion was also studied using Triton X-100 and EDTA system which isolated PHA with 90% purity from enzymatically hydrolyzed *Sinorhizobium meliloti* cells.[171] Another method involves selective dissolution of non-PHA cell mass by protons to enhance PHA recovery. The PHB granules recovered using this method were highly crystalline and it also lowered recovery cost by using cheaper chemicals with higher recovery efficiencies.[168] In case of enzymatic digestion, proteases have been used to lyse cells followed by filtration of PHA granules using chloroform extraction resulting in 94% purity as against 66% purity obtained using undigested cells.[171]

Recently, two new solvents –dimethyl carbonate (DMC) and ammonium laurate - were investigated as novel green alternatives for recovery of PHA from *C. necator cells* and both methods were directly applied to concentrated microbial slurries without any pre-treatment to allow high recovery yields and purity of PHB and other copolymers.[321]

4.4 Polybutylene succinate (PBS)

Polybutylene succinate (PBS) is a biodegradable thermoplastic polymer synthesized by polycondensation of succinic acid and butanediol. These monomers can be either derived from fossil fuels or from renewable resources and currently commercially available PBS is synthesized from chemically derived monomers. Butanediol (BDO) can be derived from glucose using a total biosynthetic route[154] which was further optimized using a rational approach to strain engineering[322] and a computational framework ORACLE (Optimization and Risk Analysis of Complex Living Entities) to identify metabolic engineering targets for improved BDO production[323], leading to commercial-scale production by Genomatica within 5 years of project start-up. The GENO BDO™ process by Genomatica has been commercial since 2012 and has been licensed for commercial plants by both BASF and Novamont. In a more recent study, a novel, nonphosphorylative pathway was used to convert biomass sugars – D-xylose, L-arabinose and D-galacturonate - to BDO with a 100% theoretical maximum molar yield.[324] The pathway allowed assimilation of sugars into the TCA cycle in less than 6 steps and further built artificial biosynthetic pathways to BDO using downstream enzymes, 2-ketoacid decarboxylases and alcohol dehydrogenases. The titers, yields and rates reported were higher than those previously reported for BDO production and the nonphosphorylative platform could also be extended for other TCA-cycle derivatives.[324] For bio-based succinic acid, Bioamber built the first plant in 2008 with an initial annual capacity of 2000 metric tons. The plant uses *Escherichia coli* as host microorganism with wheat-derived glucose as a substrate for succinic acid production. In 2015, Bioamber will

start production of 30,000 tonnes/year of succinic acid in its Sarnia facility, which is predicted to result in 100% reduction in greenhouse gas emissions and 60% reduction in total energy consumption as compared to petroleum process. In December 2012, Reverdia, a joint venture between DSM and Roquette, started a 10,000 tonnes/year plant for production of bio-based succinic acid from starch using low-pH yeast technology. Myriant technologies received $50 million grant from the US Department of Energy (DOE) and set up a succinic acid plant with production capacity of 30 million pounds from unrefined sugars as feedstock using *E. coli* as host organism.[325]

4.4.1 Natural producers of succinic acid

Succinic acid is an important intermediate of TCA cycle and is produced by several microorganisms as a fermentation product under anaerobic conditions (**Figure 4.3**). Some natural producers of succinic acid include *Anaerobospirillum succiniciproducens*, *Actinobacillus succinogenes*, *Mannheimia succiniciproducens* and *Basfia succiniciproducens*.

4.4.1.1 *A. succiniciproducens*

A. succiniciproducens is a microorganism which produces succinic acid and acetic acid as major fermentation products, and ethanol and lactic acid as minor by-products under strictly anaerobic conditions.[326-327] In spite of being one of the most extensively studied microorganisms for succinate production, the genome sequence of *A. succiniciproducens* is not available. Thus metabolic engineering of this strain is difficult and researchers have instead focused on optimizing process conditions to improve succinate yields. This

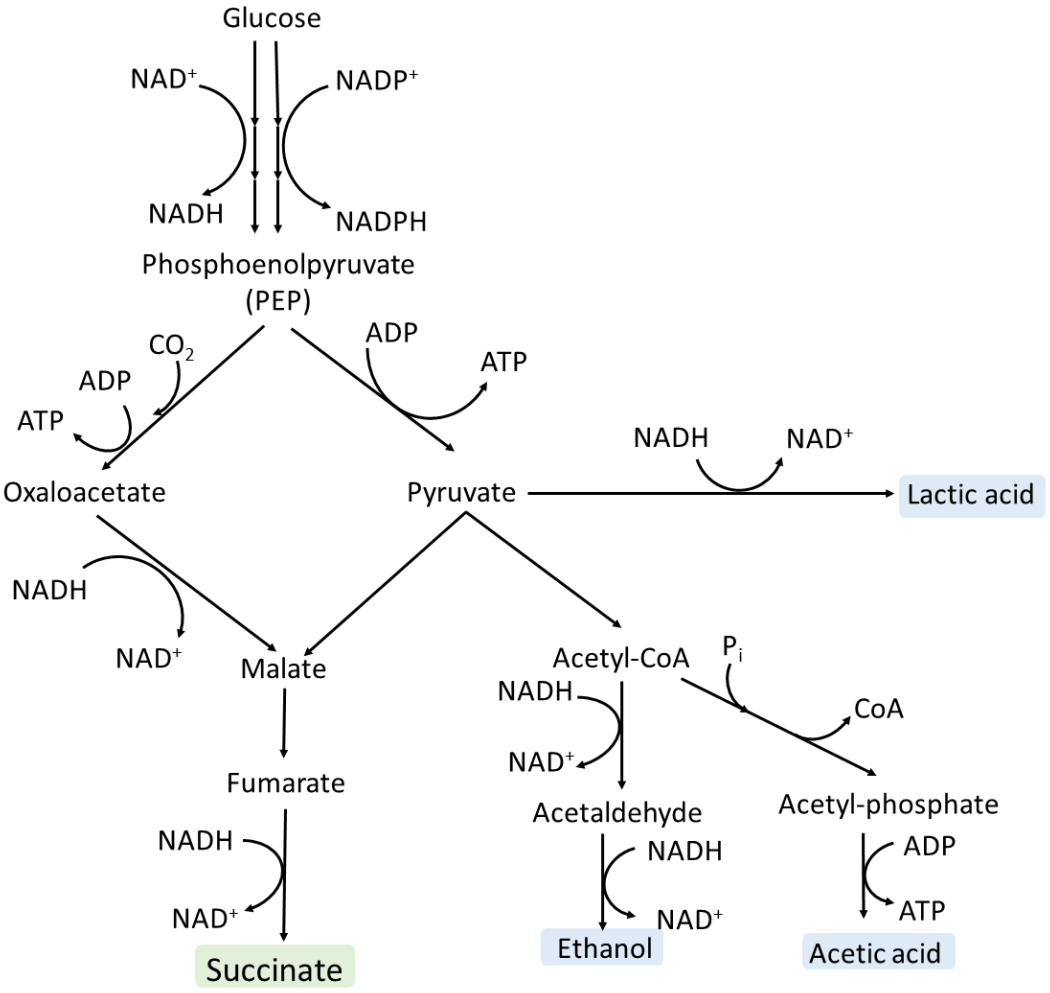

Figure 4.3 Biosynthetic pathway for production of succinic acid from glucose under anaerobic conditions

microorganism uses the phosphoenolpyruvate (PEP) carboxylation pathway for succinic acid production[327] and the final succinic acid yield is limited by the availability of reducing equivalents. With the addition of an external electron donor such as H_2 (H_2/CO_2 at 5:95 v/v), an increased succinic acid yield of 0.91 g/g and a volumetric productivity of 1.8 g/L/h was obtained, which could be attributed to the increased NADH availability in the cell.[327] The optimal pH range for succinic acid production using *A.*

succiniciproducens is 5.8 to 6.4 and the pH is maintained by the addition of alkaline carbonates or alkaline earth hydroxides.[328]

4.4.1.2 *A. succinogenes*

A. succinogenes produces succinate, acetate, formate and ethanol as major fermentation products when glucose is used as the substrate.[329] Similar to *A. succiniciproducens,* this microorganism also uses PEP carboxylation pathway for succinic acid production, and increasing CO_2 concentration enhances cell growth as well as succinic acid production. When electrically reduced neutral red, a redox dye which serves as an electron donor for fumarate reductase, was used in glucose medium, glucose consumption, cell growth and succinic acid production were all increased by 20% and acetate production was reduced by 50%.[330] Only a few metabolic engineering studies for *A. succinogenes* have been reported due to limited genetic information and lack of appropriate genetic tools. One such study to improve succinate production used directed evolution to isolate several variants of *A. succinogenes* 130Z strain (FZ 6, 9, 21, 45 and 53), all of which were resistant to 1-8 g/L of fluroacetate.[331-332] These strains produced more succinic acid and were more tolerant to high succinic acid concentrations, with one of the mutants producing 106 g/L succinate, which is the one of the highest reported titers. These mutants also produced less acetate and formic acid compared to the parent 130Z strain. There have been several efforts focused on replacing expensive nitrogen sources such as yeast extract with cheaper sources such as corn steep liquor.[333] McKinlay et al. used chemically defined medium for succinic acid production and since this eliminates the use

of complex medium, downstream separation and purification processes are much cheaper and simpler.[334]

4.4.1.3 *M. succiniciproducens*

Another promising succinic acid producing bacterium is *M. succiniciproducens*, which produces succinic acid, acetic acid and formic acid in the ratio of 2:1:1 under 100% CO_2 saturation and in the pH range of 6-7.5.[335-336] Unlike *A. succinogenes*, this microorganism has a complete TCA cycle and can efficiently grow in both aerobic as well as anaerobic conditions.[336] Similar to the aforementioned natural succinate producers, *M. succiniciproducens* can also metabolize a wide variety of substrates and hydrolysates but exhibits many auxotrophies. In order to reduce cost of the medium, whey and corn steep liquor were used and anaerobic batch cultures resulted in a succinic acid yield of 71% and a productivity of 1.2 g/L/h while continuous culture using the same medium resulted in yields of 69% and a maximum productivity of 3.9 g/L/h.[337] According to a study, focused on optimizing CO_2 concentrations, a medium containing 141 mM of dissolved carbon dioxide resulted in a succinic acid yield that was 1.5 times higher than that achieved by a medium containing 8.74 mM of dissolved CO_2.[338-339] The compete genome sequence of this bacterium was determined by Hong et al.[336], and based on the genome sequence, gene knockout studies were performed and PEP carboxykinase (PEPCK) was identified as the major succinate producing pathway under anaerobic conditions. Lee et al. developed a gene knockout method to delete the genes *ldhA*, *pflB*, *pta* and *ackA* involved in by-product formation and the resulting strain designated LPK7 was able to produce 52.4 g/L of succinic acid in 29 h, although cell growth stopped after 19.5 h when

the succinic acid titer reached 36 g/L.[340] In order to reduce pyruvate and malate production, malic enzyme was over-expressed in LPK7 strain and although malate excretion was reduced by 37%, pyruvate excretion increased. Furthermore, after glucose depletion, pyruvate was used to produce acetate in the cells.[341] Availability of genome sequence of this bacterium also helped to develop a chemically defined medium (CDM) and use of this medium allowed 17% increase in final succinic acid concentration, 36% increase in productivity and 15% increase in succinic acid yield as compared to complex medium. Additionally, by-product formation was reduced by 30%.[342]

4.4.2 Recombinant engineered succinic acid producers

Although all the natural producers produce succinic acid as a major fermentation product, none of them can tolerate high succinic acid concentrations and they all require complex media for their growth due to their numerous auxotrophies. Additionally, most of these natural producers require anaerobic conditions for succinic acid production which has several disadvantages including poor cell growth, slow carbon throughput and limited NADH availability. In order to be cost-competitive with the current chemical process, fermentative production should produce 150 g/L succinic acid with a productivity of 5 g/L/h.[343] To achieve these targets it is important to develop more efficient producers that can produce succinic acid at high titers and tolerate high substrate and product concentrations while utilizing simple media.

4.4.2.1 *C. glutamicum*

C. glutamicum, under an oxygen-deprived condition, produces lactic and succinic acids as major products and acetate as minor fermentation product. Addition of bicarbonate to the medium resulted in 3.6 fold increase in succinic acid production rate, implying that bicarbonate was used for succinic acid synthesis.[222] Over-expression of pyruvate carboxylase (*pyc*) gene in lactate dehydrogenase (*ldhA*) deficient strain resulted in the production of 146 g/L of succinate in 46 hours with a molar yield of 1.4 mol/mol.[344] This is one of the highest concentration of succinic acid achieved on a laboratory scale. Succinate production under aerobic conditions using *C. glutamicum* was explored due to advantage of faster cell growth in presence of oxygen and the capability to use minimal media for succinic acid production, which significantly reduces downstream purification costs.[345-346] Despite its several advantages, *C. glutamicum* cannot metabolize pentose sugars, and to overcome this challenge, *E. coli* xylose-catabolizing enzymes - xylose isomerase and xylulokinase – were over-expressed in *C. glutamicum* which allowed concomitant use of glucose and xylose and the titer and productivity of succinate obtained with mixed sugars was comparable to that obtained with glucose alone.[224]

4.4.2.2 *Escherichia coli*

Under anaerobic conditions, *E. coli* converts glucose to ethanol, formic, lactic and acetic acids and only a trace amount of succinic acid is produced. One approach to improve succinic acid production in *E. coli* is to eliminate competing pathways to reduce by-product formation. One of the earliest efforts in this direction was to develop *E. coli* NZN111 strain with lactate dehydrogenase (*ldhA*) and pyruvate formate lyase (*pflB*)

genes knocked out.[8, 347] Although this strain increased succinic acid yield at the expense of ethanol and acetic, formic and lactic acids, large amounts of pyruvic acid was excreted and cell growth was severely inhibited resulting in very low succinic acid productivity. A spontaneous chromosomal mutation in NZN111, mapped to the *ptsG* gene, resulted in a mutant, AFP111, which increased PEP pool and restored cell growth on glucose, was found to increase the yield and productivity of succinic acid. Furthermore, inactivation of *ptsG* gene allows simultaneous utilization of sugars.[348] Dual phase fermentations using *E. coli* AFP11, in which an aerobic growth phase is followed by an anaerobic succinic acid production phase, demonstrated the activation of glyoxylate shunt as a succinate producing pathway under aerobic conditions.[349] In one study, the glyoxylate shunt was activated by disrupting the *iclR* gene, which codes for transcriptional repressor of the glyoxylate shunt, and competing fermentative pathways were eliminated by knocking out *ldhA*, *adhE* and *ack-pta*, resulting in a metabolically engineered *E. coli* strain SBS550MG. SBS550MG was transformed with the pyruvate carboxylase (*pyc*) gene from *L. lactis* to divert flux from pyruvate to OAA and the resulting strain, SBS550MG/pHL413, increased succinic acid yield from glucose to 1.6 mol/mol.[350] Although glyoxylate pathway reduced NADH requirement, a major drawback of this pathway is that it wastes carbon through CO_2 or formate production. Balzer et al. reduced formate production by over-expressing NAD+- dependent formate dehydrogenase (*fdh*) gene of *Candida boidinii* in SBS550MG/pHL413. This new pathway produced 1 mole NADH from 1 mole of formate, thus retaining the reducing power of formate and resulting in enhanced succinic acid production and reduced formate concentration (0-3

mM).[351] Jantama et al. developed a strain KJ134 (*ldhA*, *adhE*, *pta-ackA*, *focA-pflB*, *mgsA*, *poxB*, *tdcDE*, *citF*, *aspC*, *sfcA*) which produced nearly theoretical maximum yields of succinic acid during anaerobic batch fermentation using mineral salt medium. This strain may be useful for cost-effective succinic acid production at a commercial scale due to significantly lower cost of medium required for fermentation.[352]

4.4.2.3 *Saccharomyces cerevisiae*

All of the above mentioned prokaryotic microorganisms have very low acid tolerance and exhibit poor cell growth under high glucose concentrations. *S. cerevisiae*, on the other hand, is a well-characterized industrial production organism which exhibits good growth characteristics and has an extraordinarily high acid and osmo-tolerance. Succinate is one of the major components produced during sake fermentation by yeast and thus, most of the early studies focus on increasing succinic acid production in sake yeast strains. Disruption of succinate dehydrogenase subunits (SDH1 and SDH2) and isocitrate dehydrogenase isoenzymes (IDH1 and IDP1) of *S. cerevisiae* resulted in succinic acid titer of 3.6 g/L which is 4.8 fold higher than the titer obtained using wild type *S. cerevisiae.*[353] Another huge advantage of using yeast for succinic acid production is that these eukaryotic organisms quantitatively export succinic acid into the culture broth, thus reducing end-product inhibition and eliminating the need of disrupting the cells, simplifying downstream processing. Since *S. cerevisiae* is a well-known glycerol and ethanol producer, the main by-products were ethanol, glycerol and acetate. In a later study, a *S. cerevisiae* strain was developed which produced 8.5 g/L succinic acid with no glycerol formation and it used all ethanol for acetate production.[354] *In silico* metabolic

engineering strategy was used to develop a multiple deletion *S. cerevisiae* strain 8D, that required glycine supplementation to grow. By using directed evolution, a mutant 8D strain was isolated that did not require glycine supplementation and also exhibited 60-fold improvement in biomass-coupled succinic acid production (0.6 vs 0.01 g succinic acid/ g biomass) and 20-fold improvement in succinic acid titer (0.6 vs 0.03 g/L) with respect to reference strain under aerobic conditions.[355]

4.4.3 Alternative cheaper substrates for succinic acid production

In order to reduce overall fermentation costs, it is important to look into alternative inexpensive substrates including agricultural residues and industrial by-products instead of refined carbohydrates as a carbon source and corn steep liquor instead of yeast extract as nitrogen source in the medium. Studies have used untreated wood hydrolysate[356], NaOH treated wood hydrolysate, glycerol[357], and non-treated whey[358] as substrates for economical succinic acid production. Straw hydrolysates (corn, rice and wheat) were used as substrates for succinic acid production in *A. succinogenes* and at substrate concentrations greater than 60 g/L, both cell growth and succinic acid production were inhibited.[172] To address this problem, simultaneous saccharification and fermentation (SSF) technique was used for succinic acid production from corn stover in a 5L bioreactor and the maximum succinic acid concentration and yield achieved was 47.4 g/L and 0.72 g/g substrate, respectively.[359] SSF could eliminate both end-product and substrate inhibition since the reducing sugars formed by cellulose hydrolysis were quickly utilized by *A. succinogenes* maintaining very low glucose and xylose concentration throughout fermentation period. In another study using glycerol as

substrate, an *E. coli* strain (*pck**, Δ*ptsI*, Δ*pflB*) achieved succinic acid yield of 0.8 mol/mol glycerol, which is 80% of the maximum theoretical yield for glycerol, in anaerobic fermentation using mineral salts medium.[360] To utilize sucrose or sucrose-containing substrates, sucrose-utilizing genes (*cscKB* and *cscA*) from *E. coli* KO11 were expressed in an engineered *E. coli* KJ122 strain, followed by growth-based selection, to enable high succinic acid production and reduced by-product accumulation using a low-cost simple medium. succinic acid concentrations of 47 g/L and 56 g/L were obtained from sucrose and sugarcane molasses respectively, in simple batch fermentation in 10L bioreactor using simple low-cost medium.[361] In a different study, sugarcane bagasse hydrolysate was used as a substrate for succinic acid production using *E. coli* strain BA305 (Δ*pflB*, Δ*ldhA*, Δ*ppc*, Δ*ptsG*), over-expressing PEP carboxykinase from *B. subtilis* 168, and produced 39.3 g/L succinic acid in a fed-batch fermentation after 120 h.[362] The same strain, *E. coli* BA305 over-expressing PEPCK from *B. subtilis*, was used to efficiently ferment lignocellulose hydrolysate by employing repetitive anaerobic fermentations. This method of fermentation enhanced ATP supply with every stage and allowed production of 83 g/L succinic acid with a high yield of 0.87 g/g in 36 h of three repetitive anaerobic fermentations.[363]

4.4.4 Fermentation technologies

Many different fermentation strategies have been investigated for the large scale fermentative production of succinic acid. In addition to the common batch and continuous cultivations, Meynial-Salles et al. used a continuous cell recycle bioreactor for anaerobic fermentation of *A. succiniciproducens* which resulted in a high succinate

volumetric productivity of 14.8 g/L/h which is 20 times higher than that obtained using batch culture under same fermentation conditions.[364] Urbance et al. carried out a continuous and repeat-batch biofilm fermentation of *A. succinogenes* to increase succinic acid productivities through high cell densities and biofilm formation. Although a high succinic acid productivity of 8.8 g/L/h was reported, yield of succinic acid was less than 50% and specific productivity was also very poor.[365-366] Recently, Yan et al. determined the optimum operating conditions for succinic acid production using continuous fermentation in fibrous bed bioreactor employing *A. succinogenes* and achieved succinic acid concentration of 55.3 g/L with a productivity of 2.8 g/L/h.[367] A novel external-recycle, biofilm reactor was used recently to carry out continuous anaerobic fermentation using *A. succinogenes* and glucose and CO_2 as carbon source. The highest succinic acid titer obtained was 48.5 g/L and succinic acid yield on glucose was 0.91g/g.[368] Apart from continuous fermentations, an immobilized fermentation system was studied by Shi et al. using *C. glutamicum* strain immobilized in porous polyurethane filler and using cassava bagasse hydrolysate (CBH) as substrate for succinic acid production. To regulate pH of fermentation medium, mixed alkalis (NaOH and $Mg(OH)_2$) were used instead of $NaHCO_3$ and a succinic acid productivity of 0.4 g/L/h was achieved from 35 g/L glucose of CBH.[369]

4.4.5 Recovery and purification of succinic acid

Condensation polymerization of PBS requires succinic acid of high purity (above 98%) and this is the biggest obstacle facing use of bio-succinic acid in industrial PBS synthesis. Downstream processing remains a major challenge for cost-effective microbial

production of succinic acid and purification costs account for 60% of the total production costs.[370]

4.4.5.1 Precipitation

The traditional method for isolation of carboxylic acids including succinic acid from aqueous fermentation broth is precipitation with calcium hydroxide or calcium oxide, resulting in generation of large quantities of low-quality gypsum which cannot be used commercially. Alternatively, ammonia[371-372] and magnesium hydroxide[373] have also been used recently as a titrant for recovery of succinic acid from fermentation broth and in both cases, reagents can be fully recycled and salt produced can be sold commercially.

4.4.5.2 Membrane separation

Membrane filtration (including microfiltration, ultrafiltration and nanofiltration) has been used widely for the separation of solids from liquids and has several advantages including low operating cost and low energy consumption. Recently, a study showed that while ultrafiltration can remove 100% cells and 92% proteins from fermentation broth, centrifugation could only remove 92% cells and 53% protein.[374] In a different study, to overcome product inhibition, a mono-polar electrodialysis pilot was coupled to the cell recycle reactor to continuously remove succinate and acetate from the permeate and recycle an organic acid free solution back into the fermentation medium. Use of this integrated membrane-bioreactor-electrodialysis enhanced the cell growth, productivity and final concentration, allowing a maximum productivity of 10.4 g/L/h, a molar yield of 1.35 and a final succinic acid concentration of 83 g/L.[364] In another study, ultrafiltration

membrane was integrated with fermentation to produce 99.4% pure succinic acid which was recovered from broth and directly used for the synthesis of PBS.[375]

4.4.5.3 Liquid-liquid extraction

Liquid-liquid extraction (LLE) is used extensively due to its simplicity, ease of scale-up, high output and low energy consumption. In order to improve the yield and selectivity of liquid-liquid extraction of organic acids from aqueous phase, extractants including aliphatic amines have been used in reactive extraction.[376-378] In a recent study, a hollow fiber membrane contactor (HFMC) was operated in liquid-liquid extraction (LLE) mode for extracting succinic acid from an aqueous feed.[182] Two different extractant solutions were used – a) a 30% (v/v) tripropylamine (TPA) dissolved in 1-octanol and b) 30% trioctylamine (TOA)-TPA mixture in a 1:4 weight ratio dissolved in 1-octanol. Operating conditions such as feed flow rate, organic phase flow rate, and initial succinic acid-water concentration were varied and removal efficiencies of more than 95% were obtained in most cases.

4.5 Conclusion

Recent advances in metabolic engineering have allowed commercial production of some biobased polymers and monomers from renewable feedstocks using engineered microorganisms. The growing environmental concerns over use of non-biodegradable plastics and the limited fossil fuel resources are the major drivers for the emerging bioplastics industry. Many biopolymers are already in industrial production including PHA, PLA, PBS, PE and PPC. In this paper, we reviewed the recent developments in the

biotechnological production of three bio-based polymers –PLA, PHB and PBS. Some of the common problems of fermentative production of these monomers include substrate inhibition, end-product inhibition, inability of microorganisms to metabolize pentose sugars, and low pH tolerance of host organism. To overcome these limitations, recombinant microorganisms were used and several metabolic engineering strategies such as over-expression of heterologous genes, deletion of competing pathways, enhancing pool of precursors, were employed to improve product titers. Apart from batch, fed-batch and continuous fermentation, other fermentation technologies that have been investigated recently include cell immobilization and cell membrane recycling, both of which employ high cell densities to enhance productivities. Although the bioplastics industry is growing rapidly, there are several challenges that need to be addressed in the coming years to make them competitive with their petrochemical counterparts which include the low performance of some biobased plastics, low efficiency of microbial fermentation processes and their relatively high cost of production and downstream processing.[188, 379]

Chapter 5

Engineering Nonphosphorylative Metabolism of Biomass Sugars to TCA Cycle Derivatives

5.1 Introduction

The use of edible biomass such as corn or sugarcane for biomanufacturing has affected food supply on a global scale and elevated food prices[380]. In an effort to circumvent the contention of resources for "food versus chemical" purposes, lignocellulosic feedstock presents a promising solution. Lignocellulosic feedstock is the most abundant inedible biomass with an annual output of around 2×10^{11} metric tons[381]. Common sources of lignocellulose include corn stover, switchgrass, sugar beet pulp, and citrus peel.

* Adapted with permission from Tai, Y. S.[‡], Xiong, M.[‡], Jambunathan, P.[‡], Wang, J., Wang, J., Stapleton, C., & Zhang, K. (2016). Engineering nonphosphorylative metabolism to generate lignocellulose-derived products. *Nature chemical biology*, *12*(4), 247-253. ([‡] Contributed equally)

Utilization of D-xylose, L-arabinose, and D-galacturonate is critical for the economic viability of lignocellulosic fermentation, as they constitute more than 1/3 of the sugars in lignocellulose[382-383]. Over the past several years, there has been remarkable progress in lignocellulosic ethanol production[384-387]. However, the low price of ethanol has led other studies to focus on producing more valuable compounds, such as xylitol and biodiesel[388-390]. The development of new metabolic platforms with an expanded chemical repertoire will promote the applications of cellulosic processes.

As the metabolic hub of the cell, the TCA cycle leads to a variety of high value bioproducts, including amino acids and industrial chemicals (**Figure 5.1**). The conventional metabolic routes for carbon feedstocks to enter the TCA cycle are glycolysis and pentose phosphate pathways (PPP). These traditional metabolisms, however, involve lengthy reaction steps (>10 steps to TCA cycle) and complex regulations that limit the production yield and rate. For example, one reported pathway to produce 1,4-butanediol (BDO) requires 21 reaction steps, imposing significant difficulty on feasible metabolic engineering[92]. Moreover, after several decades of industrial practices, the fermentation yields of amino acids are still much lower than their theoretical maxima[391].

An attractive alternative exists in an unconventional metabolism that converts lignocellulosic materials directly into 2-ketoglutarate (2-KG) in less than 6 steps (**Figure 5.1**). In this proposed mechanism, D-xylose is first converted into D-xylonolactone by D-xylose dehydrogenase (XDH), followed by hydrolysis to D-xylonate by D-xylonolactonase (XL). D-xylonate is subsequently dehydrated to 2-keto-3-*deoxy*-D-

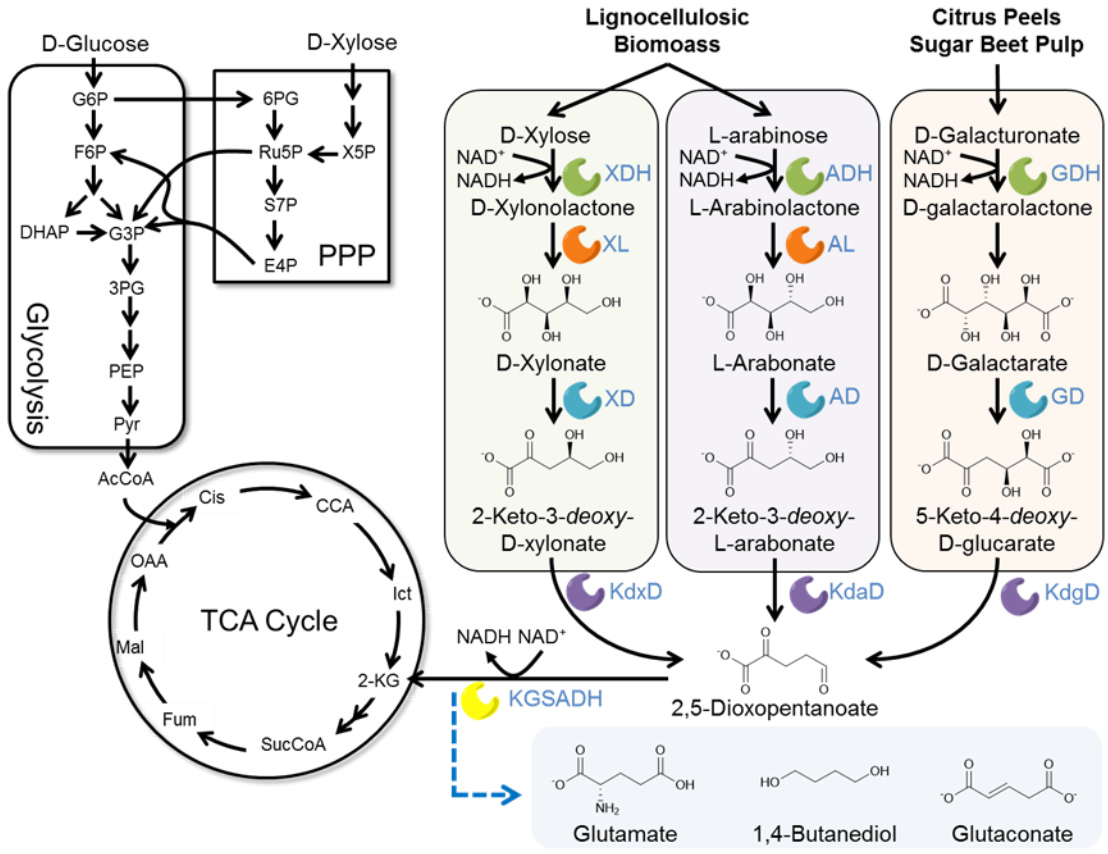

Figure 5.1 Assimilation pathways of lignocellulosic sugars through the nonphosphorylative metabolism. The pathway for D-xylose metabolism consists of D-xylose dehydrogenase (XDH), D-xylonolactonase (XL), D-xylonate dehydratase (XD), and 2-keto-3-deoxy-D-xylonate dehydratase (KdxD). The L-arabinose assimilation pathway is comprised of L-arabinose dehydrogenase (ADH), L-arabinolactonase (AL), L-arabonate dehydratase (AD), and 2-keto-3-deoxy-L-arabonate dehydratase (KdaD). The pathway for D-galacturonate metabolism was designed by using uronate dehydrogenase (UDH), D-galactarate dehydratase (GD), and 5-keto-4-deoxy-D-glucarate dehydratase (KdgD). The DOP produced from these feedstocks is then converted into 2-KG by 2-ketoglutarate semialdehyde dehydrogenase (KGSADH) which is a key intermediate of the TCA cycle.

xylonate by D-xylonate dehydratase (XD), which is then converted to 2,5-dioxopentanoate (DOP) by 2-keto-3-*deoxy*-D-xylonate dehydratase (KdxD). This D-xylose oxidation pathway was first discovered in 1960[392] and the xylose-inducible *xylXABCD* operon (CC0823—CC0819) was later annotated in *Caulobacter crescentus*[393]. Through a similar metabolism, L-arabinose can be converted to DOP by L-arabinose dehydrogenase (ADH), L-arabinolactonase (AL), L-arabonate dehydratase (AD), and 2-

keto-3-*deoxy*-L-arabonate dehydratase (KdaD). The intermediate, DOP, produced from D-xylose and L-arabinose can be further oxidized to 2-KG, a key intermediate of the TCA cycle, by 2-ketoglutarate semialdehyde dehydrogenase (KGSADH)[394]. This L-arabinose degradation pathway has been discovered in *Pseudomonas fragi*[392], and later demonstrated in *Azospirillum brasilense*[395].

A comparable metabolism for the assimilation of uronic acids, such as D-galacturonate, has also been identified. Uronate dehydrogenase (UDH) can catalyze the transformation of D-galacturonate into D-galactaro-1,4-lactone[396]; the lactone ring is then hydrolyzed either spontaneously or with the aid of a lactonase to form D-galactarate[397]. D-Galactarate can be converted to 5-keto-4-*deoxy*-D-glucarate by D-galactarate dehydratase (GD) and then to DOP by 5-keto-4-*deoxy*-D-glucarate dehydratase (KdgD)[398] . The DOP produced can again be further oxidized to 2-KG using a KGSADH described earlier. This alternative metabolism does not involve any phosphorylating reactions, making it more energy-efficient than the conventional pathways such as glycolysis and PPP. This nonphosphorylative pathway can be utilized as a shortcut to the TCA cycle, potentially accelerating the production of TCA cycle derivatives. In addition, the theoretical yield of 2-KG from these pentoses and uronic acids is 100 mol% through this metabolism, which is notably higher than that from pentose phosphate pathway (83 mol%).

Nonphosphorylative metabolism has been known for over fifty years[392]; however, the full reconstitution of this pathway from sugars to 2-KG has not been demonstrated in the workhorse microorganism, *E. coli*. Recently, several reports have partially

reconstituted and applied the nonphosphorylative pathway from D-xylose[399-402], but the utilization of L-arabinose and D-galacturonate for chemical synthesis via this pathway has not been explored. Here we describe our selection platform to discover nonphosphorylative gene clusters that are functional in *E. coli*. We first tested the platform using a previously identified gene cluster from *C. crescentus*[393-394], and then subsequently used the platform for gene mining to assemble novel, putative gene clusters from various microorganisms that allow the nonphosphorylative assimilation of D-xylose, L-arabinose, and D-galacturonate. We purified the corresponding enzymes and determined their kinetic parameters to validate their *in vivo* activities. The establishment of this alternative metabolism in *E. coli* provides a novel metabolic platform for biosynthesis of a variety of chemicals such as succinate, glutaconate, and the "glutamate family" of amino acids. Furthermore, it enables biotransformation of pharmaceutically important natural products catalyzed by 2-ketoglutarate-dependent dioxygenases[91, 403].

In particular, we designed a new synthetic pathway to 1,4-butanediol (BDO)[404], a raw material for many commercial products such as Spandex. This pathway uses a 2-ketoacid decarboxylase (KDC) to convert DOP into butanedial. Butanedial is then transformed into BDO by an alcohol dehydrogenase (ADH). The total biosynthetic pathway starting from the pentoses to BDO requires only 6 steps, which is less than 1/3 of the previously reported pathway[92]. Additionally, we utilized protein engineering techniques to reduce the accumulation of the byproduct, 1,2,4-butanetriol (BTO), by improving the selectivity of KDC towards DOP. The engineered KDC improved BDO titer from 1.83 to 3.8 g/l at a yield of 63% of the theoretical maximum.

Based on this nonphosphorylative platform, we also expanded the sugar repertoire to produce 5.6 g/l BDO from L-arabinose and 2.3 g/l BDO from D-galacturonate, which has not been reported before. We then examined the scale-up feasibility for each substrate in 1.3 L bioreactors, where engineered strains were able to produce 12 g/l of BDO from D-xylose in 30 hours, 15.6 g/l BDO from L-arabinose in 75 hours, and 16.5 g/l of BDO from D-galacturonate in 90 hours.

5.2 Results

5.2.1. Establishing the nonphosphorylative metabolism in *E. coli*

To facilitate the discovery and engineering of nonphosphorylative gene clusters, we developed a selection platform based on cell growth (**Figure 5.2a**). Here, we knocked out the *E. coli* isocitrate dehydrogenase gene, *icd*, so the oxidation of isocitrate to 2-KG was interrupted. Thus, the cells required an exogenous supply of 2-KG to grow. Since the alternate nonphosphorylative pathway can convert pentoses (such as D-xylose and L-arabinose) and uronic acids (such as D-galacturonate) to 2-KG, the activity of the pathway was coupled to cell growth. This platform can thus be used to screen for active gene clusters in *E. coli*. Gene clusters can also be further optimized by using directed evolution on the introduced pathway and identifying cells with improved growth.

To build the selection platform, we cloned the gene cluster *xylBCDX* (**Figure 5.2a**) from *C. crescentus* into plasmid pBDO-1 (**Table 5.S1**) where *xylB* encodes the XDH; *xylC* encodes the XL; *xylD* encodes the XD and *xylX* encodes the KdxD (**Figure 5.1**). We cloned the gene *xylA* of the *C. crescentus* xylose operon, annotated as the

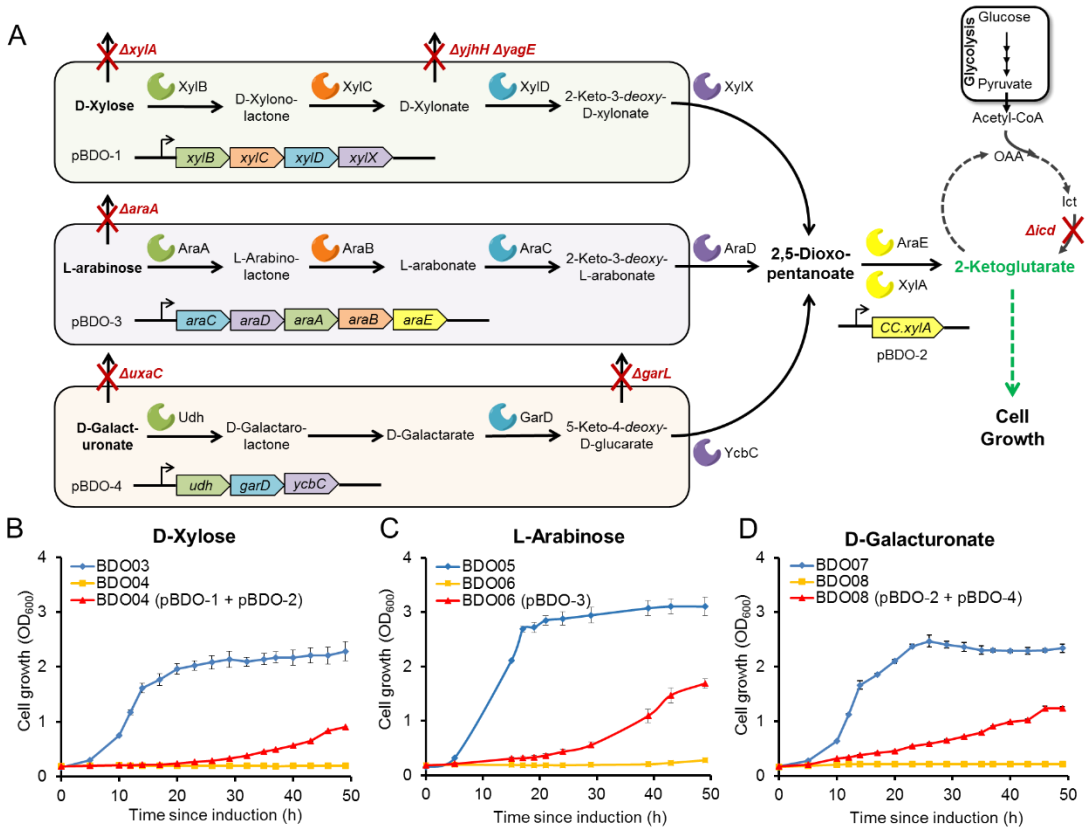

Figure 5.2 The growth platform to test functional nonphosphorylative gene clusters in E. coli. (a) The growth assay was designed based on the supply of 2-KG, a TCA cycle intermediate. Isocitrate dehydrogenase gene (icd) was knocked out to cut off 2-KG production through glycolysis/TCA cycle. The nonphosphorylative pathway plasmids (pBDO-1 and pBDO-2 for the D-xylose pathway, pBDO-3 for the L-arabinose pathway, pBDO-4 (a synthetic operon) and pBDO-2 for the D-galacturonate pathway) were then transformed into cells to compensate the production of 2-KG. (b) Strains BDO03 (BW25113 ΔxylA ΔyjhH ΔyagE), BDO04 (BW25113 ΔxylA ΔyjhH ΔyagE Δicd), and BDO04 transformed with plasmids pBDO-1 and pBDO-2 were grown in M9 minimum media supplemented with 5 g/l glucose and 5 g/l D-xylose. (c) Strains BDO05 (BW25113), BDO06 (BW25113 Δicd), and BDO06 transformed with plasmid pBDO-3 were grown in M9 minimum media supplemented with 5 g/l glucose and 5 g/l L-arabinose. (d) Strains BDO07 (BW25113 ΔuxaC ΔgarL), BDO08 (BW25113 ΔuxaC ΔgarL Δicd), and BDO08 transformed with plasmids pBDO-4 and pBDO-2 were grown in M9 minimum media supplemented with 5 g/l glucose and 5 g/l D-galacturonate. All error bars shown in (b), (c), and (d) represent SD (n=3).

KGSADH, into a separate plasmid pBDO-2. Furthermore, to maximize the flux of D-xylose through the nonphosphorylative pathway, we knocked out the endogenous D-xylose (*xylA*) and D-xylonate (*yagE, yjhH*) consuming pathways in *E. coli*, generating strain BDO03. We also deleted *icd* gene to generate strain BDO04, a 2-KG auxotroph that is incapable of producing 2-KG. While strain BDO03 showed exponential growth in

minimal media containing glucose and D-xylose, strain BDO04 showed almost no growth due to the disruption of the TCA cycle. We then transformed BDO04 with plasmids pBDO-1 and pBDO-2, and the resulting strain showed growth on mixed sugars with OD reaching ~1.0 after 50 hours (**Figure 5.2b**).This could be attributed to the 2-KG produced from D-xylose via the nonphosphorylative pathway (pBDO-1 and pBDO-2).

Similar gene clusters encoding the L-arabinose assimilation pathway have been identified in several species of soil bacteria including *Burkholderia spp.*[405], *Pseudomonas saccharophilia*[406] and *Rhizobium spp*[407]. In this work, we discovered a novel L-arabinose assimilation gene cluster from *Burkholderia multivorans* using BLAST based on the previously identified L-arabinose gene cluster of *Burkholderia thailandensis*[394]. To demonstrate the platform, we cloned this putative L-arabinose gene cluster from *B. multivorans araCDABE* (*BmulJ 5323-5321-5320-5316-5314*), responsible for L-arabinose degradation to 2-KG, into plasmid pBDO-3 (**Table 5.S1**). The gene *araA* encodes the ADH; *araB* codes the AL; *araC* codes the AD; *araD* was annotated as the KdaD and *araE* encodes the KGSADH (**Figure 5.1**). To eliminate the L-arabinose consumption pathways in *E. coli*, we used strain BDO05 with *araA* gene knocked out, which served as the positive control for the growth assay (**Table 5.S1**). Strain BDO05 showed exponential growth in media containing both glucose and L-arabinose, but when *icd* gene was knocked out (strain BDO06), cells could not grow in the same media due to the disruption of the TCA cycle. When BDO06 strain was transformed with plasmid pBDO-3 containing the L-arabinose assimilation gene cluster, growth on glucose and L-arabinose media was restored due to the supplementation of 2-KG through the

nonphosphorylative pathway. The BDO06 strain transformed with pBDO-3 grew to an OD of ~1.7 in 50 hours (**Figure 5.2c**), thus establishing the *in vivo* activity of the novel *B. multivorans* L-arabinose gene cluster in *E. coli*.

Gene clusters with an analogous function for the hexuronic acid degradation have been found in *Bacillus* species[398] and *Pseudomonas putida*[408]. To establish D-galacturonate pathway, we designed a synthetic operon consisting of the following genes: *udh* from *P. putida* encoding the GDH, *garD* from *E. coli* encoding the GD, and *ycbC* from *Bacillus subtilis* encoding the putative KdgD (**Figure 5.1**). We cloned this operon into plasmid pBDO-4 (**Table 5.S1**). We used the plasmid pBDO-2 with *xylA* gene of *C. crescentus* to convert DOP to 2-KG. In order to maximize the flux of galacturonate via the heterologous pathway, we knocked out the genes encoding the pathways involved in consumption of either the substrate (Δ*uxaC*) or intermediates (Δ*garL*) resulting in strain BDO07 (**Table 5.S1**). BDO07 grew exponentially after induction in the media containing both glucose and D-galacturonate. Similar to the D-xylose and L-arabinose pathways, when *icd* was knocked out (strain BDO08), cells could not grow since the strain is a 2-KG auxotroph. When we transformed strain BDO08 with plasmids pBDO-2 and pBDO-4, 2-KG was produced from D-galacturonate using the nonphosphorylative pathway, thus allowing cells to grow to an OD of ~1.25 in 50 hours (**Figure 5.2d**).

5.2.2. Validating the enzymatic activities by *in vitro* assays

After demonstrating that the putative gene clusters could function *in vivo* using a growth-based selection platform, we sought to further confirm key enzymatic functions and identify *in vitro* activities of the nonphosphorylative pathways. All the kinetic parameters

Table 5.1 Kinetic parameters of key pathway enzymes. Data is presented as mean ± SD (n=3).

Enzyme	Substrate	K_m (mM) [b]	k_{cat} (s^{-1}) [b]	k_{cat}/K_m (mM^{-1} s^{-1})
XylB	D-Xylose	0.85±0.08	12.1±2.2	14
XylD	D-Xylonate	1.18±0.05	7.60±1.1	6.4
XylX[a]	2-Keto-3-*deoxy*-D-xylonate	1.90±0.08	0.53±0.1	0.26
AraA	L-Arabinose	3.14±0.12	101.4±5.2	32.3
AraC	L-Arabonate	2.05±0.05	0.17±0.01	0.083
AraD[a]	2-Keto-3-*deoxy*-L-arabonate	9.69±0.2	0.2±0.02	0.023
Udh	D-Galacturonate	0.15±0.05	24.1±3.6	160
GarD	D-Galactarate	0.76±0.02	18.9±0.8	25
YcbC[a]	5-Keto-4-*deoxy*-D-glucarate	0.43±0.06	2.17±0.2	5.1
YqhD[a]	Butanedial	1.9±0.1	45.0±6.2	23
Kivd[a]	2,5-Dioxopentanoate	2.8±0.6	4.8±0.4	1.7
Kivd (V461I)[a]	2,5-Dioxopentanoate	2.2±0.4	5.6±0.7	2.5
Kivd[a]	2-Keto-3-*deoxy*-D-xylonate	1.6±0.7	12.3±3.5	7.7
Kivd (V461I)[a]	2-Keto-3-*deoxy*-D-xylonate	4.7±1.3	2.4±0.5	0.51

[a] Enzyme activity was determined using a coupled assay
[b] Data represent mean ± SD (n=3)

are shown in **Table 5.1**. In the D-xylose pathway from *C. crescentus*, D-xylose dehydrogenase (XylB) and xylonate dehydratase (XylD) have k_{cat} values of 12.1 s^{-1} and 7.6 s^{-1}, respectively. However, the enzyme 2-keto-3-*deoxy*-D-xylonate dehydratase (XylX) has a relatively low k_{cat} of 0.53 s^{-1}. Since XylX is also the enzyme with the highest K_m (1.9 mM) in the pathway, its specific constant (k_{cat}/K_m) is therefore the lowest (0.26 $s^{-1}mM^{-1}$) among the three enzymes. This indicates that XylX is the bottleneck enzyme in the D-xylose degradation pathway.

In the L-arabinose pathway from *B. multivorans*, the first enzyme, L-arabinose dehydrogenase (AraA), has the highest k_{cat} value of 101.4 s^{-1} while the downstream enzymes, L-arabonate dehydratase (AraC) and 2-keto-3-*deoxy*-L-arabonate dehydratase

(AraD) have relatively low k_{cat} values of 0.17 s^{-1} and 0.23 s^{-1}, respectively. The enzyme AraD has the highest K_m (9.7 mM) and a low k_{cat}, making it the rate-limiting step of the L-arabinose pathway.

For the D-galacturonate pathway, uronate dehydrogenase (Udh) from *P. putida* has the highest k_{cat} (24.1 s^{-1}) and lowest K_m (0.15 mM) among the three enzymes. Galactarate dehydratase (GarD) from *E. coli* has the highest K_m (0.76 mM) in the pathway but a much higher k_{cat} (18.9 s^{-1}) than the 5-keto-4-*deoxy*-D-glucarate dehydratase (YcbC) from *B. subtilis*. Similar to D-xylose and L-arabinose pathway, the last enzyme (YcbC) in the D-galacturonate pathway that produces DOP has the lowest specific constant and is considered the bottleneck enzyme in the D-galacturonate degradation. These bottleneck enzymes from the three substrates could explain why transformants harboring these nonphosphorylative pathways did not grow as well as the wild type cells.

5.2.3. Identification of enzymes to convert DOP into BDO

With the establishment of the nonphosphorylative metabolism, we explored its biosynthetic applications by designing new synthetic pathways to BDO. We hypothesized that DOP can be converted to BDO by a 2-ketoacid decarboxylase (KDC) and an alcohol dehydrogenase (ADH) (**Figure 5.3a**). We designed the BDO producing pathways using the following steps: (1) introducing the nonphosphorylative metabolism to convert the pentoses and hexuronic acid into a pool of DOP, (2) screening for the best KDC that can convert DOP to butanedial, and (3) screening for the best ADH that can reduce butanedial into BDO.

Since the D-xylose pathway of *C. crescentus* has been partially established in *E. coli*[399], we selected it for enzyme screening. First, to convert DOP to butanedial, we screened three KDCs, 2-ketoacid decarboxylase (Kivd) from *Lactococcus lactis*[409], indolepyruvate decarboxylase (IpdC) from *Salmonella typhimurium*[410], and benzoylformate decarboxylase (BFD) from *P. putida*[411] and cloned them into plasmids pBDO-5, pBDO-6, pBDO-7, respectively (**Table 5.S1**). These plasmids also carried a promiscuous alcohol dehydrogenase YqhD from *E. coli*, for screening based on BDO production. To maximize the carbon flux towards the desired pathway, we transformed strain BDO03 with these plasmids individually along with the DOP producing plasmid, pBDO-1. It is important to note that all the strains used for BDO production do not have *icd* inactivation, and can thus utilize glucose for growth. In the shake flask experiment, we used 20 g/l of D-xylose and the strains carrying Kivd, IpdC and BFD produced 1.83, 1.20 and 0.63 g/l BDO, along with 3.56, 1.06 and 0.03 g/l BTO, respectively (**Figure 5.3b**). It is notable that all of these enzymes are promiscuous enough to catalyze the decarboxylation of DOP to BDO. Overall, the data (**Figure 5.3b**) indicated that Kivd, amongst the three set of investigated enzymes, was the best KDC towards DOP.

Besides KDC, we also investigated ADHs from different organisms to see which combination would produce maximal titer of BDO. Other than YqhD, we chose the following ADHs as candidate enzymes: *L. lactis* alcohol dehydrogenase AdhA[412]; *Saccharomyces cerevisiae* alcohol dehydrogenase Adh6[413]; *E. coli* putative alcohol dehydrogenase YahK (PDB ID: 1UUF); and *E. coli* putative alcohol dehydrogenase YgjB[414]. We cloned these enzymes individually after Kivd to build an expression cassette

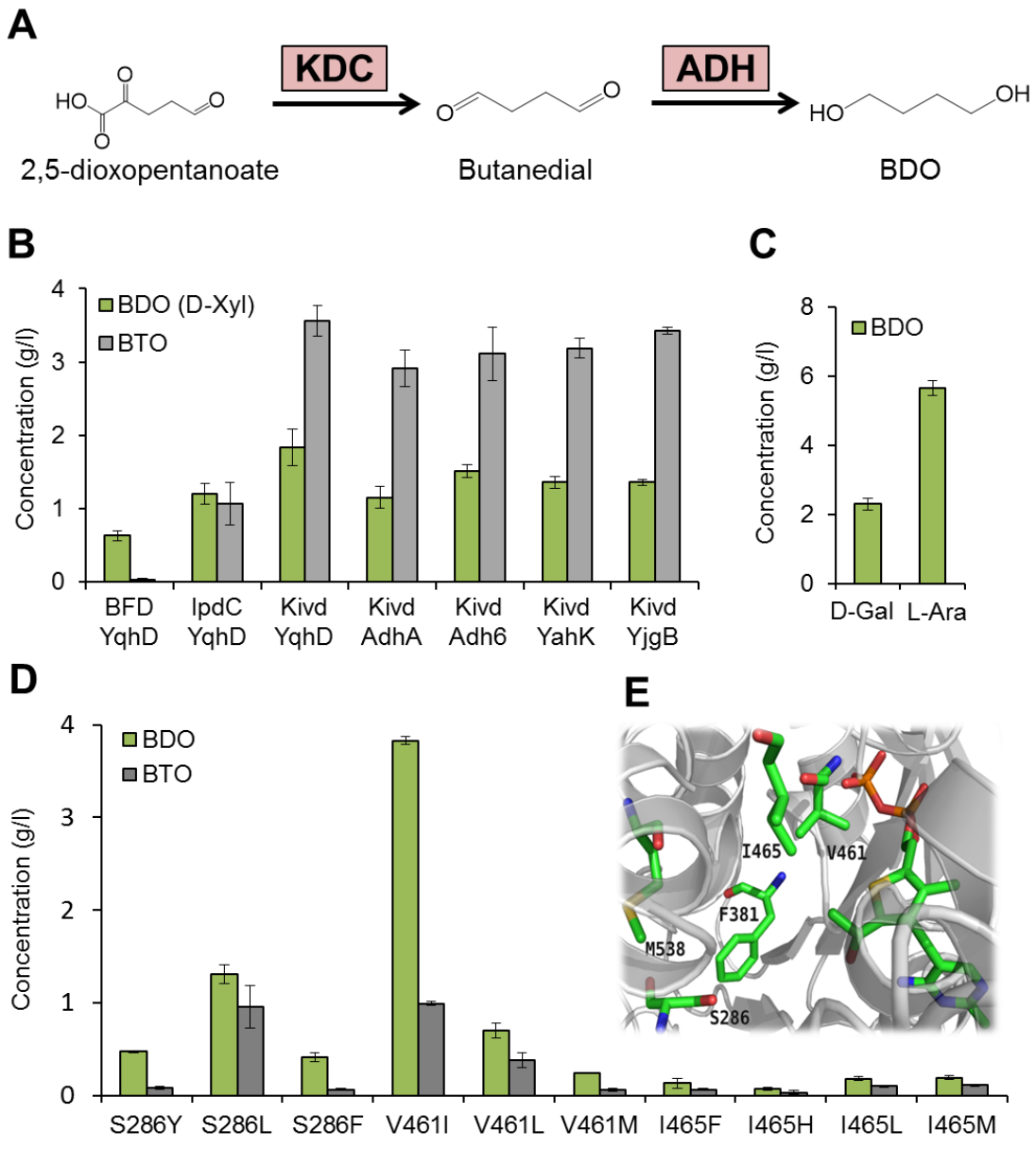

Figure 5.3 BDO production using different combinations of 2-ketoacid decarboxylase (KDC) and alcohol dehydrogenase (ADH) and different Kivd mutants. (a) BDO production pathway from DOP catalyzed by KDC and ADH. (b) Shake-flask production of BDO from D-xylose. Three KDC (Kivd, IpdC, and BFD) and five ADH (YqhD, AdhA, Adh6, YahK, and Yjgb) were examined. (c) Shake-flask production of BDO from L-arabinose and D-galacturonate using Kivd + YqhD. There was no BTO production using these substrates. (d) Shake-flask production of BDO using Kivd mutants from D-xylose. Ten Kivd mutants, S286Y, S286L, S286F, V461I, V461L, V461M, I465F, I465H, I465L, and I465M, were tested for BDO production. (e) The binding pocket of Kivd (PDB ID: 2VBG). Residues S286, V461, and I465 were mutated to larger residues to improve the substrate specificity. Error bars in (b), (c) and (d) represent SD (n=3).

on a high copy plasmid as pBDO-8, pBDO-9, pBDO-10, and pBDO-11, respectively (**Table 5.S1**). Strains carrying AdhA, Adh6, YahK, and YgjB could produce 1.15, 1.51, 1.36 and 1.36 g/l BDO together with 3.11, 2.92, 3.19 and 3.43 g/l BTO, respectively (**Figure 5.3b**). Overall, the best combination was Kivd with YqhD allowing a yield of 0.15 g/g, which is only 25% of the theoretical maximum. However, the byproduct BTO yield from D-xylose was 0.28 g/g, which was around two times higher than BDO. The apparent K_m and k_{cat} of Kivd towards DOP is 4.8 mM and 4.8 s^{-1}, respectively, and the apparent kinetic parameters of YqhD towards butanedial is 1.9 mM (K_m) and 45.0 s^{-1} (k_{cat}) (**Table 5.1)**.

Based on the screening results of the D-xylose pathway, we applied Kivd and YqhD (pBDO-5) for the L-arabinose and D-galacturonate pathways. For L-arabinose, we cloned the putative *B. multivorans* cluster *araCDAB* (*BmulJ 5323-5321-5320-5316*) that can convert L-arabinose to DOP, into plasmid pBDO-12. The strain BDO05 transformed with plasmids pBDO-12 and pBDO-5, was able to produce 5.65 g/l of BDO from 20 g/l L-arabinose in production experiments. For D-galacturonate, we transformed strain BDO07 with plasmids pBDO-4 and pBDO-5 and the engineered strain was capable of producing 2.34 g/l of BDO from 20 g/l D-galacturonate (**Figure 5.3c**). Both L-arabinose and D-galacturonate pathways did not result in BTO production.

5.2.4. Optimization of BDO production by protein engineering

While the discovery of Kivd and YqhD allowed for the production of BDO, the promiscuous nature of Kivd did not provide a good selectivity for the decarboxylation step in the D-xylose pathway. BTO is produced by decarboxylation of the D-xylose

intermediate, 2-keto-3-*deoxy*-D-xylonate **(Figure 5.S7)**. This suggests that compared to DOP, Kivd prefers to bind to the intermediate 2-keto-3-*deoxy*-D-xylonate as a substrate leading to a much higher titer of BTO than our target product. Conversely, there was no accumulation of BTO using L-arabinose as the substrate, indicating that Kivd is not active on the stereoisomer, 2-keto-3-*deoxy*-L-arabonate. To increase the production of BDO, we examined the effect of protein engineering on improving Kivd selectivity towards DOP. According to the crystal structure[415] (PDB ID: 2VBG), amino acid residues S286, V461 and I465, in combination with the cofactor thiamine diphosphate (ThDP), delineate the active site of Kivd (**Figure 5.3d&e**) . Since 2-keto-3-*deoxy*-D-xylonate, with its extra hydroxyl group, is a bulkier substrate than DOP, we attempted to shrink the binding site of Kivd to enhance its selectivity towards the smaller substrate. We constructed ten mutants of Kivd, including S286Y, S286L, S286F, V461I, V461L, V461M, I465F, I465H, I465L and I465M, and tested them in shake flask experiments.

The fermentation results indicated that the best mutant, V461I, produced 3.83 g/l BDO with only 0.99 g/l BTO, which represents a yield of 0.37 g/g D-xylose which is 63% of the theoretical maximum. Compared with wild type Kivd, BDO production titer with V461I mutant increased over 2-fold. This can be attributed to the extra methyl group in isoleucine compared to valine, which shrinks the Kivd binding pocket, making it more selective towards DOP. Enzymatic assays also showed that the mutant V461I on Kivd notably reduces the specific constant (k_{cat}/K_m) towards BTO substrate, 2-keto-3-*deoxy*-D-xylonate, from 7.7 to 0.5 $mM^{-1}s^{-1}$ (**Table 5.1**), while improving the activity

towards BDO substrate (DOP) from 1.7 to 2.5 $mM^{-1}s^{-1}$. Therefore, the enzyme characterization data was consistent with the fermentation results (**Figure 5.3d**).

5.2.5. Fermentation scale-up

We tested the scale-up feasibility of these BDO biosynthetic pathways by fed-batch fermentations in 1.3-L bioreactors. For the D-xylose pathway, we used the recombinant strain BDO03 transformed with plasmids pBDO-1 and pBDO-16 and fed a mixture of glucose and D-xylose as substrates during the fermentation process. The engineered strain produced 9.21 g/l BDO in 36 hours and consumed 42.1 g/l of D-xylose (**Figure 5.4a**). Glucose was fed to support cell growth. To further exploit glucose for the production of value-added chemicals, we introduced another plasmid pMEV-1[416] into the engineered strain for the co-production of BDO and mevalonate (MEV). MEV is an important intermediate in the production of the branched lactone, β-methyl-δ-valerolactone, which could be used as building blocks for high-performing biobased polymers[416]. We used the BDO03 strain transformed with pBDO-1, pBDO-16, and pMEV-1 for the fed-batch fermentation and the engineered strain produced 12.0 g/l BDO by consuming 46 g/l of D-xylose in 30 hours after induction (**Figure 5.4b**). Not only was glucose efficiently utilized (20.2 mol% of glucose was converted into MEV), but the yield of BDO from D-xylose was also improved from 36% to 43% of the theoretical maximum by introducing MEV production pathway. Acetate started to accumulate to a final concentration of 11 g/l when cells entered stationary phase and inhibited further production of both BDO and MEV.

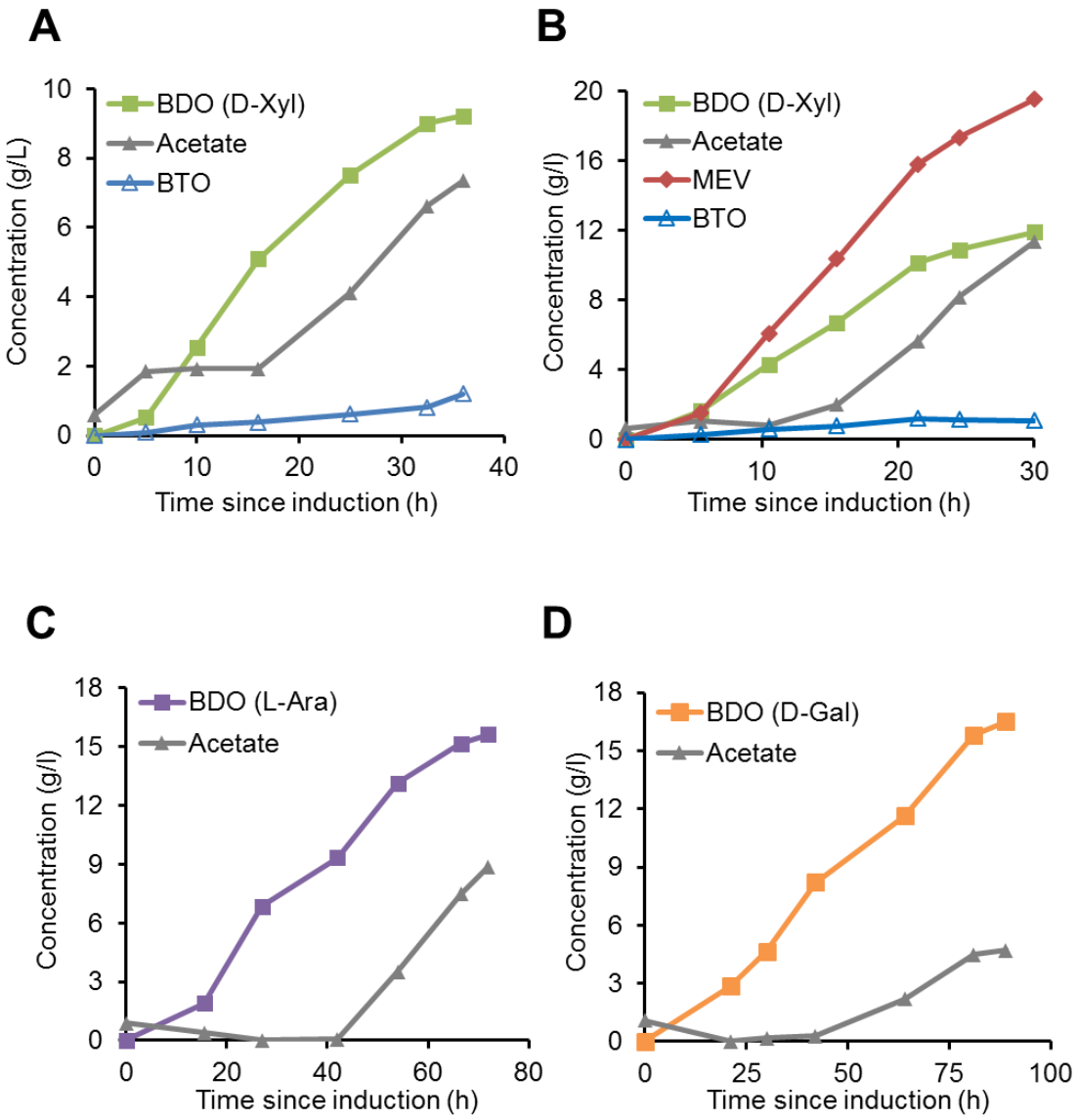

Figure 5.4 Production of BDO from D-xylose, L-arabinose, and D-galacturonate in 1.3-l bioreactors. (a) Production of BDO from D-xylose. (b) Co-production of BDO and mevalonate from D-xylose and glucose. (c) Production of BDO from L-arabinose. (d) Production of BDO from D-galacturonate. Abbreviations: D-Xyl, D-xylose; MEV, mevalonate; L-Ara, L-arabinose; D-Gal, D-galacturonate. Bioreactor experiments were performed in at least triplicates for each substrate and results for one representative experiment are shown.

We used the L-arabinose recombinant strain, BDO05 transformed with pBDO-5 and pBDO-12, in the fed-batch fermentation with a mixture of glucose and L-arabinose as the feed. The engineered strain produced 15.6 g/l BDO in 72 hours and consumed 70.5

g/l of L-arabinose which resulted in a yield of 37% of the theoretical maximum. The final acetate concentration was 8.9 g/l, inhibiting further production of BDO (**Figure 5.4c**). Similarly, we tested the D-galacturonate strain BDO07 transformed with pBDO-4 and pBDO-5 in a 1.3-L bioreactor. We fed a mixture of glucose and D-galacturonate to the bioreactor and the engineered strain produced 16.5 g/L of BDO from 50.5 g/L of D-galacturonate (70% of the theoretical maximum) in 90 hours (**Figure 5.4d**).

5.2.6. Identification of new gene clusters

After successfully demonstrating the use of the nonphosphorylative metabolism of D-xylose, L-arabinose, and D-galacturonate for BDO production, we wanted to identify putative nonphosphorylative operons from other organisms that may show higher activities in *E. coli*. Therefore, we used the growth based selection platform that employs a 2-KG auxotroph to perform gene mining. Using BLAST, we identified a putative operon from *Burkholderia xenovorans* LB400 (DR64_8447—DR64_8450, DR64_8452) for nonphosphorylative assimilation of D-xylose to 2-KG. (**Figure 5.S8**). We cloned genes for converting D-xylose to DOP (DR64_8447—DR64_8450) into plasmid pBDO-23 and cloned DR64_8452 to convert DOP to 2-KG into plasmid pBDO-24. To test the *in vivo* activity of this gene cluster in *E. coli*, we transformed the 2-KG auxotroph BDO04 with plasmids pBDO-23 and pBDO-24. The *B. xenovorans* D-xylose gene

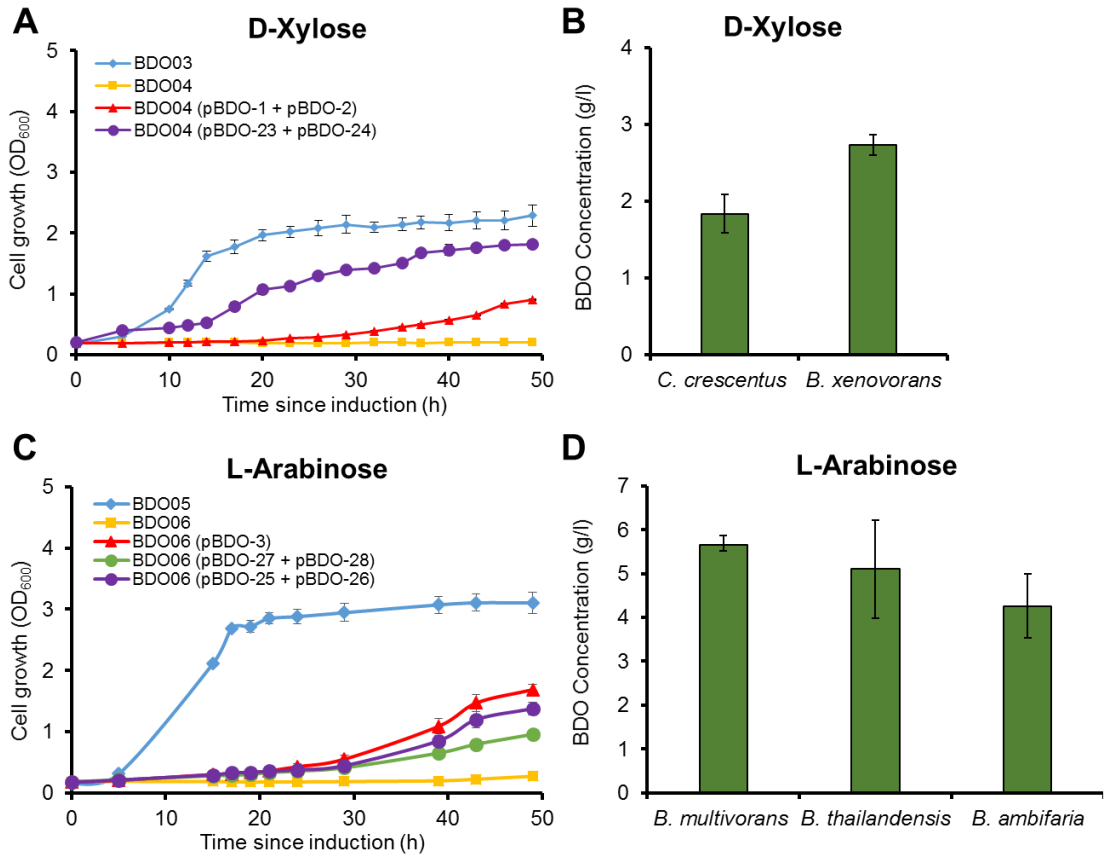

Figure 5.5 Growth platform to mine putative nonphosphorylative clusters in E. coli and BDO production using these novel operons. (a) Strains BDO03 (BW25113 ΔxylA ΔyjhH ΔyagE), BDO04 (BW25113 ΔxylA ΔyjhH ΔyagE Δicd), BDO04 transformed with C. crescentus operon (pBDO-1 and pBDO-2) and BDO04 transformed with *B. xenovorans* operon (pBDO-23 and pBDO-24) were grown in M9 minimum media supplemented with 5 g/l glucose and 5 g/l D-xylose. (b) BDO production using newly identified *B. xenovorans* D-xylose operon and previous *C. crescentus* operon with Kivd and YqhD as downstream enzymes. (c) Strains BDO05 (BW25113), BDO06 (BW25113 Δ*icd*), BDO06 transformed with *B. multivorans* operon (pBDO-3), BDO06 transformed with *B. ambifaria* operon (pBDO-25 and pBDO-26) and BDO06 transformed with *B. thailandensis* operon (pBDO-27 and pBDO-28) were grown in M9 minimum media supplemented with 5 g/l glucose and 5 g/l L-arabinose. (d) BDO production using previously identified *B. thailandensis* and novel *B. multivorans* and *B. ambifaria* L-arabinose operons with Kivd and YqhD as downstream enzymes. All error bars shown in (b) and (d) represent SD (n=3).

cluster rescued the growth of *E. coli* with OD reaching ~1.8 in 50 hours (**Figure 5.5a**). We further investigated the production of BDO using the newly identified *B. xenovorans* operon. The recombinant strain, BDO03 transformed with plasmids pBDO-23 and pBDO-5, was able to produce 2.73 g/l BDO with no BTO accumulation (**Figure 5.5b**).

To further corroborate this production profile, we characterized the *in vitro* enzyme activities of the new operon (**Table 5.S2**). The 2-keto-3-*deoxy*-D-xylonate dehydratase of *B. xenovorans* (DR64_8450) has a 9-fold higher k_{cat} (4.7 s^{-1}) compared to the corresponding dehydratase (XylX) of *C. crescentus* (0.53 s^{-1}). This can explain why there was no BTO accumulation using *B. xenovorans* operon. Thus the selection strategy could be used to discover highly active enzymes from different microorganisms. These enzymes could be combinatorially assembled into synthetic operons for potential biosynthesis.

For L-arabinose, we tested operons from two other *Burkholderia* species—an uncharacterized, putative *Burkholderia ambifaria* gene cluster (Bamb_4925—4918, Bamb_4915) and a previously identified, uncharacterized *Burkholderia thailandensis* gene cluster (BTH_II1632—1625)[394], both of which had high sequence similarity (**Figure 5.S8**) to *B. multivorans* L-arabinose operon. We cloned the putative *B. ambifaria* genes Bamb_4918, Bamb_4922, Bamb_4923, and Bamb_4925 converting L-arabinose to DOP into plasmid pBDO-25. We cloned gene Bamb_4915 that converts DOP to 2-KG into plasmid pBDO-26. Similarly, we cloned the *B. thailandensis* genes responsible for DOP production—BTH_II1625, BTH_II1629, BTH_II1630, and BTH_II1632—into plasmid pBDO-27, and BTH_II1631, that converts DOP to 2-KG, into plasmid pBDO-28. Both gene clusters rescued the growth of the 2-KG auxotroph, BDO06, to an OD of ~1.5 in 50 hours (**Figure 5.5c**) via the nonphosphorylative pathway. After establishing the *in vivo* activities of both L-arabinose gene clusters, we used both clusters for BDO production. The strain carrying *B. ambifaria* genes (BDO05 with plasmids pBDO-25 and

pBDO-5) produced 4.3 g/l BDO; and the *B. thailandensis* gene overexpression strain (BDO05 with plasmids pBDO-27 and pBDO-5) produced 5.0 g/l BDO in production experiments (**Figure 5.5d**). Similar to *B. multivorans* operon, both *B. ambifaria* and *B. thailandensis* gene clusters did not produce any BTO.

5.3. Discussion

The nonphosphorylative metabolism allows assimilation of lignocellulosic sugars or sugar acids into the important TCA cycle intermediate, 2-KG, in fewer than 6 steps. This work is the first to demonstrate the complete nonphosphorylative metabolism of D-xylose, L-arabinose, and D-galacturonate to 2-KG in the workhorse microorganism, *E. coli*. To discover gene clusters that are functional in *E. coli*, we developed a selection platform utilizing a 2-KG auxotroph. In particular, we applied the platform to identify a new nonphosphorylative D-xylose operon from *B. xenovorans* that has a more active 2-keto-3-*deoxy*-D-xylonate dehydratase than the previously reported one from *C. crescentus*. The discovery of more active enzymes/operons will enable further optimization of these pathways. The establishment of these pathways can serve as a new biosynthetic platform for TCA cycle derivatives which have extensive applications. Here BDO production is used as an example.

To establish the downstream pathway to BDO, we screened several different decarboxylases and dehydrogenases. The best enzyme combination for BDO production was the 2-ketoacid decarboxylase (Kivd) from *L. lactis* and the endogenous alcohol dehydrogenase (YqhD). We identified a Kivd mutant V461I by protein engineering which successfully improved BDO titer from D-xylose by more than 100% and reduced

BTO accumulation. In a previous report, a different Kivd mutant V461A has been shown to improve 3-methyl-1-pentanol production[417] by expanding the binding pocket to accommodate a bulkier substrate. Conversely, in this work we shrank the binding pocket of Kivd to decrease the selectivity towards the bulkier and undesired substrate, 2-keto-3-*deoxy*-D-xylonate. In the future, directed evolution strategy can be combined with this rational design to further improve the selectivity. In a recent report, the nonphosphorylative D-xylose operon from *C. crescentus* has been used to produce 0.44 g/l BDO, utilizing a different downstream pathway. We validated the distinction and efficacy of our pathway by extensive enzymatic assays (**Table 5.1**) and *in vitro* production experiments (**Figure 5.S1**). In addition, the higher production titer and yield (3.88 g/l BDO with a yield of 0.37 g/g D-xylose) indicates that our pathway has a higher *in vivo* efficiency.

Furthermore, we tested the *C. crescentus* D-xylose gene cluster, *B. multivorans* L-arabinose operon and D-galacturonate synthetic operon, in a 1.3-L bioreactor to study the scale-up feasibility. Acetate accumulation and inefficient co-utilization of sugars caused by carbon catabolite repression[418] were two important limiting factors in the processes. The strains could thus be further improved by knocking out acetate producing pathways or relieving carbon catabolite repression with the overexpression of D-xylose, L-arabinose, or D-galacturonate transporters[418]. Fermentation process engineering or strain evolution can also be applied for optimization.

While the results reported in this work demonstrate the production of a commodity chemical, BDO, the nonphosphorylative platform can also be extended to

produce several TCA cycle derivatives including glutamate, glutaconate and 1-butanol, among others. The growth selection platform provides an effective and robust tool to screen better enzymes or identify nonphosphorylative pathways for other substrates. Compared to conventional metabolism such as glycolysis and PPP, these fewer-step and higher theoretical yield nonphosphorylative pathways are of critical importance to make lignocellulosic bioproducts more economically feasible.

5.4. Materials and Methods

5.4.1 Bacterial strains and growth conditions

The *E. coli* strains used in this study are listed in **Table 5.S1**. XL10-Gold was used for cloning and BL21 was used for protein expression and purification. Most of the other strains were derived from the wild-type *E. coli* K-12 strain BW2511370. P1 phages of *xylA*, *yjhH*, *yagE*, *icd*, *uxaC*, and *garL* were obtained from the Keio collection. The phages were used to transfect the corresponding strain for the construction of targeted knockout strains. All the knockout strains were then transformed with pCP20 plasmid to remove the kanamycin marker. The correct knockouts were verified by colony PCR. Unless otherwise stated, these *E. coli* strains were grown in test tubes at 37 °C in 2×YT rich medium (16 g/l Bacto-tryptone, 10 g/l yeast extract and 5 g/l NaCl) supplemented with appropriate antibiotics (ampicillin 100 mg/l and kanamycin 50 mg/l).

5.4.2 Plasmids construction

All the primers used in this study were ordered from Eurofins MWG Operon and are listed in **Table 5.S3** in supporting information. PCR reactions were carried out with

Phusion High-Fidelity DNA polymerase (New England Biolabs) according to the manufacturer's instructions. The sequences of all the plasmids produced were verified by restriction mapping and DNA sequencing.

To construct plasmid pBDO-1, five fragments of *xylB*, *xylC*, *xylD*, and *xylX* were amplified from *C. crescentus* genomic DNA by using primer pairs of xylBAcc-F/xylBBamHI-R, xylCBamHI-F/xylCNhe-R, xylDNheI-F/xylDHind-R, xylXHind-F/xylXBlpRem-R and xylXBlpRem-F/xylXBlpI-R, and then the fragment of *xylX* was amplified with primer pairs of xylXHind-F/xylXBlpI-R by using xylX-1 and xylX-2 as template. The four fragments of *xylB*, *xylC*, *xylD*, and *xylX* were digested with Acc65I/BamHI, BamHI/NheI, NheI/HindIII and HindIII/BlpI, and then ligated with linearized pZAlac vector23 digested with Acc65I and BlpI to form the plasmid, pBDO-1. To make the plasmid pBDO-2, the coding region of *xylA* was PCR amplified by oligos of CC0822Acc-F and CC0822Xba-R using genomic DNA of *C. crescentus* as template, and then this coding region was inserted into the site between Acc65I and XbaI of vector pZElac23 after digestion. To construct the plasmids pBDO-3 and pBDO-12, the gene fragments of *araC (BmulJ 5323), araD (BmulJ 5321), araA (BmulJ 5320), araB (BmulJ 5316)* and *araE (BmulJ 5314)* were amplified from *Burkholderia multivorans* genomic DNA using primer pairs of araC-Acc65I-F/araC-NheI-R, araD-NheI-F/araD-remBlpI-R, araD-remBlpI-F/araA-HindIII-R, araA-HindIII-F/araB-NdeI-R and araB-NdeI-F/araE-BlpI-R respectively. The two fragments of araD-araA were then used as templates for overlap PCR using primer pair araD-NheI-F/araA-HindIII-R. The fragments *araC*, *araD-araA*, *araB* and *araE* were double-digested with enzymes Acc65I/NheI, NheI/HindIII,

HindIII/NdeI and NdeI/BlpI respectively and these were ligated with linearized pZA-lac vector digested with Acc65I/BlpI to form the plasmid pBDO-3. To construct pBDO-12, fragment *araB* was amplified from *B. multivorans* genomic DNA using different primer pair araB-HindIII-F/araB-BlpI-R and the resulting PCR product was digested with HindIII/BlpI. The fragments *araC* Acc65I/NheI digest, *araD-araA* NheI/HindIII digest and *araB* HindIII/BlpI digest were ligated with linearized pZA-lac vector digested with Acc65I and BlpI to construct pBDO-12. To make plasmid pBDO-4, one fragment of vector from pBDO-1 plasmid, one fragment of *ycbC* from *B. subtilis*, two fragments of garD-1 and garD-2 from *E. coli*, and two more fragments from *P. putida KT2440***,** were amplified using primer pairs of pZA-F/pZAAcc-R, KdaBS-F/KdaBS-R, GarD-F/GarD-Acc-R, GarD-Acc-F/GarD-R, udh-F/udh-Bsa-R, and udh-Bsa-F/udh-R, respectively. These six fragments were assembled by the golden gate method130 to form plasmid pBDO-4. Four fragments of *BFD, kivd, ipdC* and *yqhD* were amplified from genomic DNA of *P. putida*, *L. lactis, S. typhimurium* and *E. coli*, respectively, by using primer pairs of BFDAcc-F/BFDSphI-R, KIVDAcc-F/KIVDSphI-R, IPDCAcc-F/IPDCSphI-R and YqhDSphI-F/YqhDXbaI-R, respectively. Kivd and yqhD were digested with Acc65I/SphI and SphI/XbaI, and then inserted into the corresponding site of pZElac to form plasmid pBDO-5. *Kivd* in pBDO-5 was replaced by *ipdC* and *BFD* to build plasmids, pBDO-6 and pBDO-7. Two fragments of *adhA* and *adh6* were amplified from *L. lactis* and *S. cerevisiae* genomic DNA, respectively by using primer pairs adhA-SphI-F/adhA-XbaI-R and Adh6-SphI-F/Adh6-XbaI-R, another two fragments of *yahK* and *yjgB* were amplified from *E. coli* genomic DNA with primer pairs of yahK-SphI-F/yahK-

XbaI-R and yjgB-SphI-F/yjgB-XbaI-R. These four fragments were used to replace *yqhD* in plasmid pBDO-5 to make plasmids of pBDO-8, pBDO-9, pBDO-10 and pBDO-11.

Twenty Kivd mutant fragments of S286Y-1, S286Y-2, S286L-1, S286L-2, S286F-1, S286F-2, V461I-1, V461I-2, V461L-1, V461L-2, V461M-1, V461M-2, I465F-1, I465F-2, I465H-1, I465H-2, I465L-1, I465L-2, I465M-1 and I465M-2 were amplified from plasmid pBDO-5 by using primer pairs of KIVDAcc-F/S286Y-R, S286Y-F/KIVDSphI-R, KIVDAcc-F/S286L-R, S286L-F/KIVDSphI-R, KIVDAcc-F/S286F-R, S286F-F/KIVDSphI-R, KIVDAcc-F/V461I-R, V461I-F/KIVDSphI-R, KIVDAcc-F/V461L-R, V461L-F/KIVDSphI-R, KIVDAcc-F/V461M-R, V461M-F/KIVDSphI-R, KIVDAcc-F/I465F-R, I465F-F/KIVDSphI-R, KIVDAcc-F/I465H-R, I465H-F/KIVDSphI-R, KIVDAcc-F/I465L-R, I465L-F/KIVDSphI-R, KIVDAcc-F/I465M-R and I465M-F/KIVDSphI-R, respectively. Ten fragments of S286Y, S286L, S286F, V461I, V461L, V461M, I465F, I465H, I465L and I465M amplified with primers KIVDAcc-F, KIVDSphI-R by using PCR templates of S286Y-1 and S286Y-2; S286L-1 and S286L-2; S286F-1, and S286F-2; V461I-1 and V461I-2; V461L-1 and V461L-2; V461M-1 and V461M-2; I465F-1 and I465F-2; I465H-1 and I465H-2; I465L-1 and I465L-2; and I465M-1 and I465M-2; replaced the wild type *kivd* of pBDO-5 to form plasmids of pBDO-13, pBDO-14, pBDO-15, pBDO-16, pBDO-17, pBDO-18, pBDO-19, pBDO-20, pBDO-21, and pBDO-22.

To construct the plasmid pBDO-23, the gene fragments of *DR64-8447, DR64-8448, DR64-8449 and DR64-8450* were amplified from *B. xenovorans* LB400 genomic DNA using primer pairs of DR64-8447-F/DR64-8447-R, DR64-8448-F/DR64-8448-R,

DR64-8449-F/DR64-8449-R and DR64-8450-F/DR64-8450-R respectively. The fragments *DR64-8447, DR64-8448, DR64-8449* and *DR64-8450* were double-digested with enzymes Acc65I/NheI, NheI/HindIII, HindIII/NdeI and NdeI/BlpI respectively and these were ligated with linearized pZA-lac vector digested with Acc65I/BlpI to form the plasmid pBDO-23. To make the plasmid pBDO-24, the *DR64-8452* gene was PCR amplified by oligos DR64-8452-F and DR64-8452-R using genomic DNA of *B. xenovorans* as template, and then this coding region was inserted into the site between Acc65I and XbaI of vector pZElac23 after digestion. To construct the plasmid pBDO-25, the gene fragments of *Bamb4925, Bamb4923, Bamb4922* and *Bamb4918* were amplified from *B. ambifaria* genomic DNA using primer pairs of Bamb4925-Acc-F/Bamb4925-Nhe-R, Bamb4923-Nhe-F/Bamb4922-HindR, and Bamb4918-Hind-F/Bamb4918-Nde-R, respectively. The fragments *Bamb4925, Bamb4923-4922* and *Bamb4918* were double-digested with enzymes Acc65I/NheI, NheI/HindIII and HindIII/NdeI respectively and these were ligated with linearized pZA-lac vector digested with Acc65I/NdeI to form the plasmid pBDO-25. To make the plasmid pBDO-26, the *Bamb4915* gene was PCR amplified by oligos Bamb4915-Acc-F/Bamb4915-Xba-R using genomic DNA of *B. ambifaria* as template, and then this coding region was inserted into the site between Acc65I and XbaI of vector pZElac23 after digestion. To construct the plasmid pBDO-27, the gene fragments of *BTH_II1632, BTH_II1630, BTH_II1629* and *BTH_II1625* were amplified from *B. thailandensis* genomic DNA using primer pairs of BTH1632-Acc-F/BTH1632-BamHR, BTH1630BamHF/BTH1629-Hind-R, and BTH1625-Hind-F/BTH1625-Blp-R respectively. The fragments *BTH_II1632, BTH_II1630-1629* and

BTH_II1625 were double-digested with enzymes Acc65I/BamHI, BamHI/HindIII and HindIII/BlpI respectively and these were ligated with linearized pZA-lac vector digested with Acc65I/BlpI to form the plasmid pBDO-27. To make the plasmid pBDO-28, the *BTH_II1631* gene was PCR amplified by oligos BTH1631-Acc-F/BTH1631-Xba-R using genomic DNA of *B. thailandensis* as template, and then this coding region was inserted into the site between Acc65I and XbaI of vector pZElac23 after digestion.

To characterize enzyme activities, hexahistidine (His6)-tagged xylB, xylD, xylX, araA, araC, araD, udh, garD, ycbC, DR64_8447, DR64_8449, and DR64_8450 were amplified from pBDO-1, pBDO-3, pBDO-4, and pBDO-23 using primers His-xylB-F and His-xylB-R; His-xylD-F and His-xylD-R; His-xylX-F and His-xylX-R; His-araA-F and His-araA-R; His-araC-F and His-araC-R; His-araD-F and His-araD-R; His-udh-F and His-udh-R; His-garD-F and His-garD-R; His-ycbC-F and His-ycbC-R; His-DR64_8447-F and His-DR64_8447-R; His-DR64_8449-F and His-DR64_8449-R; and His-DR64_8450-F and His-DR64_8450-R, respectively. These fragments were then ligated with the ColE1 *ori* backbone to create pBDO-29, pBDO-30, pBDO-31, pBDO-32, pBDO-33, pBDO-34, pBDO-35, pBDO-36, pBDO-37, pBDO-38, pBDO-39, and pBDO-40. All plasmids in this work were sequenced using appropriate primers to confirm sequence fidelity.

5.4.3. Growth assay

For the D-xylose, L-arabinose and D-galacturonate growth assays, the Δ*icd* strains (BDO04 for D-xylose, BDO06 for L-arabinose and BDO08 for galacturonate) were transformed with 2-ketoglutarate producing plasmids (pBDO-1 and pBDO-2 for *C.*

crescentus D-xylose; pBDO-3 for *B. multivorans* L-arabinose; pBDO-2 and pBDO-4 for D-galacturonate, pBDO-23 and pBDO-24 for *B. xenovorans* D-xylose, pBDO-25 and pBDO-26 for *B. ambifaria* L-arabinose; and pBDO-27 and pBDO-28 for *B. thailandensis* L-arabinose). Three freshly transformed colonies were inoculated overnight in 2 ml 2×YT containing appropriate antibiotics. The optical density (OD) of all strains were measured using a spectrophotometer at 600 nm and the cell densities were normalized before starting the assays. M9 minimal media containing 5 g/l of each carbon source (glucose and D-xylose/L-arabinose/D-galacturonate), appropriate antibiotics and 0.2 mM IPTG was used for all assays. Optical density was measured every few h using a spectrophotometer.

5.4.4. Protein expression and purification

His-tagged proteins were transformed into BL21 strain. The transformed cells were inoculated from an overnight pre-culture at 1/100 dilution and grown in 200 ml of 2×YT medium containing 100 mg/l ampicillin. When the OD600 reached 0.6, 0.5 mM IPTG was added to induce protein expression, followed by incubation at 30°C overnight. Then the cells were pelleted by centrifuging at 3,220 rcf for 15 minutes. The supernatants were discarded and the pellets were stored at -80 °C. All the following steps were carried out at 4 °C to prevent protein degradation. For lysis, the cell pellets were first thawed on ice-water mixture and re-suspended in 15 ml lysis buffer. The lysis buffer (pH=7.6) contained 50 mM Tris-HCl, 100 mM NaCl, 10 mM imidazole, 5% glycerol, 1 mM DTT. Cell lysis was performed by sonication using the Heat Systems Ultrasonics W-225 Sonicator in a continuous mode set at 50% duty cycle and output control 5. Each sample

was sonicated for 6 cycles of one-minute sonication with intermittent one-minute cooling on ice-water mixture. The cell lysates were centrifuged at 10,733 rcf for 15 minutes. The supernatant was collected for purification. 4 ml of HisPur Ni-NTA resin solution (Thermo Scientific) was loaded in a column and the storage buffer was allowed to pass through by gravity to get a 2 ml final resin bed volume. The resin was equilibrated with 10 ml of lysis buffer and drained. The supernatant was then loaded in the column and allowed to pass through by gravity. The column was then washed twice with 10 ml of wash buffer (50 mM Tris-HCl, 100 mM NaCl, and 25mM imidazole, pH=7.6). The bound protein was eluted with 15 ml of elution buffer (pH=8.0) which contained 50 mM Tris-HCl, 250 mMNaCl, and 250 mM imidazole. The final protein sample was then buffer-exchanged using Amicon Ultra centrifugal filters (Millipore) with the storage buffer (50M Tris-HCl, 2mM MgSO4, 20% glycerol, pH=8.0). The concentrated protein were aliquoted (50 □l) into PCR tubes, flash frozen with dry ice and ethanol mixture and stored at -80°C. Purified protein concentration was determined by Quick Start Bradford protein assay kit purchased from Bio-Rad Laboratories.

5.4.5. Enzymatic assays

D-Xylose dehydrogenase (XylB/DR64-8447), L-arabinose dehydrogenase (AraA), and uronate dehydrogenase (Udh): Enzyme activities of XylB/DR64-8447, AraA and Udh were assayed by monitoring initial NADH generation at 340 nm at 30 °C using D-xylose, L-arabinose, and D-galacturonate as substrates, respectively120. Kinetic assays were carried out using 0 to 10 mM D-xylose/L-arabinose/D-galacturonate and 1 mM NAD+ in 100 mM Tris-HCl and 5 mM MgCl2, pH 7.5. A series of enzymatic assays were

conducted to estimate the initial activity as a function of starting substrate concentration. This data was used to fit the parameters of the Michaelis-Menten kinetic model, *kcat* and *KM*, by nonlinear least-squares regression using the intrinsic *nlinfit* function of the Matlab software program. Kinetic constants (*kcat*/*KM*) for following enzymes were calculated with the same method.

Xylonate dehydratase (XylD/DR64-8449), L-arabonate dehydratase (AraC), and D-galactarate dehydratase (GarD): Enzymatic activities of the three dehydratases were assayed according to a modified procedure of MacGee and Doudoroff using the semicarbazide method131. Kinetic assays were carried out using 100 nM of D-xylonate dehydratase/L-arabonate dehydratase/D-galactarate dehydratase in 100 mM Tris-HCl and 5 mM MgSO4, pH 7.5. The reaction was initiated by the addition of D-xylonate/L-arabonate/D-galactarate and stopped after 0, 1, 2, 3, 5, and 10 min with 2% (v/v) trifluoroacetic acid. The samples were then mixed with 100 □l of 0.1 M semicarbazide hydrochloride (containing 1.5% sodium acetate trihydrate) and incubated at room temperature for 30 min. Finally, the 2-ketoacids produced were quantified by detection of their semicarbazone absorbance at 250 nm.

2-Keto-3-deoxy-D-xylonate dehydratase (XylX/DR64-8450), 2-keto-3-deoxy-L-arabonate dehydratase (AraD), and 5-keto-4-deoxy-D-glucarate dehydratase (YcbC): Enzymatic activities of XylX, DR64-8450, AraD, and YcbC were assayed spectrophotometrically in a coupled assay with the corresponding previous dehydratase and 2-ketoglutaric semialdehyde dehydrogenase (KGSADH)131. The assay was performed in 100 mM Tris-HCl buffer (pH=7.5) with 5mM MgSO4 containing 0-20 mM

D-xylonate/L-arabonate/D-galactarate and 1mM NAD+. After the addition of 100 nM D-xylonate dehydratase (XylD/DR64-8449)/L-arabonate dehydratase (AraC)/D-galactarate dehydratase (GarD) and 100 nM of the KGSADH, the mixture was incubated at 25 °C for 15 min. No change in absorbance at 340 nm was observed in this stage. The reaction was initiated by the addition of an appropriate amount of 2-keto-3-deoxy-D-xylonate dehydratase (XylX/DR64-8450)/2-keto-3-deoxy-L-arabonate dehydratase (AraD)/5-keto-4-deoxy-D-glucarate dehydratase (YcbC), and the increasing absorbance at 340 nm caused by NADH produced in the reaction was monitored.

2-Ketoacid decarboxylase (Kivd) and alcohol dehydrogenase (YqhD): The decarboxylase activity of Kivd was measured by a coupled enzymatic assay with AraC, AraD, and YqhD132. Excess AraC, AraD, and YqhD was used and the oxidation of NADPH was monitored at 340 nm. The assay mixture contained 1 mM NADPH, 1 □M AraC, 1 □M AraD, and 100 nM YqhD and 0.1-10 mM L-arabonate in assay buffer (100 mM Tris-HCl buffer, pH=7.5, 5 mM MgSO4, 0.5 mM ThDP) with a total volume of 0.1 ml. The mixture was incubated at 30 °C for 1 h and 10 nM Kivd was added. The dehydrogenase activity of YqhD was assayed according to NADPH initial consumption rates in a coupled assay. The assay mixture contained 1mM NADPH, 100 nM Kivd, 1 □M AraC, 1 □M AraD, and 0.1-10 mM L-arabonate in 100 □l of 100 mM Tris-HCl buffer (pH=7.5) with 5 mM MgSO4. The mixture was first incubated at 30 °C for 1 h. Afterwards, 10 nM YqhD was added and the NADPH consumption rate was monitored.

5.4.6. Shake flask batch fermentation

125 ml conical flasks with 0.2 g CaCO3 were autoclaved and dried to perform all small-scale fermentations. The flasks were filled with 5 ml M9 medium supplemented with 5 g/l yeast extract, 20 g/l glucose, 20 g/l D-xylose/L-arabinose/D-galacturonate and the corresponding antibiotics. 200 µl of overnight cultures incubated in 2×YT medium were transferred into the flasks and placed in a shaker at a speed of 250 rpm. After adding 0.1 mM isopropyl-β-D-thiogalactoside (IPTG), the flasks were screw-capped and sealed by parafilm and the fermentation was performed for 48 h at 30 °C. The fermentation products were measured by HPLC.

5.4.7. Fed-batch fermentation in bioreactors

Fermentation media for bioreactor cultures contained the following composition, in grams per liter: glucose, 10; yeast extract, 10; K2HPO4, 7.5; citric acid monohydrate, 2.0; MgSO4·7H2O, 2.0, ferric ammonium citrate, 0.3; thiamine hydrochloride, 0.008; D-(+)-biotin, 0.008; nicotinic acid, 0.008; pyridoxine, 0.032; ampicillin, 0.1; kanamycin, 0.05; spectinomycin, 0.1 (for BDO and MEV co-production only); 95—98% H2SO4, 0.8 mL; and 1 ml trace metal solution. Trace metal solution contained, in grams per liter: NaCl, 10; citric acid, 40; ZnSO4·7H2O, 1.0; MnSO4·H2O, 30; CuSO4·5H2O, 0.1; H3BO3, 0.1; Na2MoO4·2H2O, 0.1; FeSO4·7H2O, 1.0; CoCl2·6H2O, 1.0. The feed solution contained, in grams per liter: glucose, 600; K2HPO4, 7.4; antifoam, 10 ml.

Fermentation experiments were performed in 1.3-l Bioflo 115 Bioreactors (Eppendorf) using an initial working volume of 0.5 l. The bioreactor was inoculated with

10% of overnight pre-culture with 2×YT medium. The culture condition was set at 37 °C, 20% dissolved oxygen level (DO), and pH 6.8. After OD600 reached 6.0, 0.2 mM IPTG and 20 g/l D-xylose/L-arabinose/D-galacturonate was added. Temperature was changed to 30 °C and DO was set to 10%. The pH was controlled at 6.8 by automatic addition of 26% ammonium hydroxide solution. Air flow rate was maintained at 1 vvm during the whole process and DO was controlled by the agitation rate (from 300 to 800 rpm). The feeding rate of glucose was manually adjusted according to the glucose consumption rate of cells to meet metabolic balance. D-Xylose, L-arabinose, or D-galacturonate was added in batches. Fermentation culture was sampled every few h to determine cell density and production level.

5.4.8. Metabolite analysis

Fermentation products were analyzed using an Agilent 1260 Infinity HPLC equipped with an Aminex HPX87H column and a refractive-index detector (RID). The mobile phase was 0.01 N H2SO4 with a flow rate of 0.6 ml/min. The column temperature and RID temperature were 35 °C and 50 °C, respectively.

5.5. Supplementary Information

Table 5.S1. Strains and plasmids used in this study.

Name	Relevant genotype	Reference
Strains		
BW25113	*rrnB*$_{T14}$ ΔlacZ$_{WJ16}$*hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	[419]
XL10-Gold	Tet^{R}Δ (*mcrA*)183 Δ (*mcrCB-hsaSMR-mrr*)173 *endA1supE*44 *thi-1 recA1*	Stratagene
BL21	*E. coli* B F- *dcmompThsdS*(r_B- m_B-) gal [malB$^+$]$_{K-12}$(λ^S)	[420]
BDO01	BW25113 Δ*xylA*	This work
BDO02	BW25113 Δ*xylA*Δ*yjhH*	This work
BDO03	BW25113 Δ*xylA*Δ*yjhH*Δ*yagE*	This work
BDO04	BW25113 Δ*xylA*Δ*yjhH*Δ*yagE*Δ*icd*	This work
BDO05	BW25113	[419]
BDO06	BW25113 Δ*icd*	This work
BDO07	BW25113 Δ*garL*Δ*uxaC*	This work
BDO08	BW25113 Δ*garL*Δ*uxaC*Δ*icd*	This work
Plasmids		
pBDO-1	P15A origin,Kan^R, P$_L$*lacO*$_1$: *xylB-xylC-xylD-xylX*	This work
pBDO-2	ColE1 origin, Amp^R, P$_L$*lacO*$_1$:*xylA*(CC)	This work
pBDO-3	P15A origin, Kan^R, P$_L$*lacO*$_1$:*araC-araD-araA-araB-araE*	This work
pBDO-4	P15A origin, Kan^R, P$_L$*lacO*$_1$:*udh-garD-ycbC*	This work
pBDO-5	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: *kivd -yqhD*	This work
pBDO-6	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: *ipdC -yqhD*	This work
pBDO-7	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: *BFD-yqhD*	This work
pBDO-8	ColE1 origin, Amp^R, P$_L$*lacO*$_1$:*kivd-adhA*	This work
pBDO-9	ColE1 origin, Amp^R, P$_L$*lacO*$_1$:*kivd-adh6*	This work

pBDO-10	ColE1 origin, Amp^R, P$_L$*lacO*$_1$:*kivd-yahK*	This work
pBDO-11	ColE1 origin, Amp^R, P$_L$*lacO*$_1$:*kivd-yjgB*	This work
pBDO-12	P15A origin, Kan^R, P$_L$*lacO*$_1$:*araC-araD-araA-araB*	This work
pBDO-13	ColE1 origin, Amp^R, P$_L$*lacO*$_1$:*kivd(S286Y)-yqhD*	This work
pBDO-14	ColE1 origin, Amp^R, P$_L$*lacO*$_1$:*kivd(S286L)-yqhD*	This work
pBDO-15	ColE1 origin, Amp^R, P$_L$*lacO*$_1$:*kivd(S286F)-yqhD*	This work
pBDO-16	ColE1 origin, Amp^R, P$_L$*lacO*$_1$:*kivd(V461I)-yqhD*	This work
pBDO-17	ColE1 origin, Amp^R, P$_L$*lacO*$_1$:*kivd(V461L)-yqhD*	This work
pBDO-18	ColE1 origin, Amp^R, P$_L$*lacO*$_1$:*kivd(V461M)-yqhD*	This work
pBDO-19	ColE1 origin, Amp^R, P$_L$*lacO*$_1$:*kivd(I465F)-yqhD*	This work
pBDO-20	ColE1 origin, Amp^R, P$_L$*lacO*$_1$:*kivd(I465H)-yqhD*	This work
pBDO-21	ColE1 origin, Amp^R, P$_L$*lacO*$_1$:*kivd(I465L)-yqhD*	This work
pBDO-22	ColE1 origin, Amp^R, P$_L$*lacO*$_1$:*kivd(I465M)-yqhD*	This work
pBDO-23	P15A origin,Kan^R, P$_L$*lacO*$_1$: *DR64_8447-8448-8449-8450*	This work
pBDO-24	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: *DR64_8452*	This work
pBDO-25	P15A origin,Kan^R, P$_L$*lacO*$_1$: *Bamb_4925-4923-4922-4918*	This work
pBDO-26	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: *Bamb_4915*	This work
pBDO-27	P15A origin,Kan^R, P$_L$*lacO*$_1$: *BTH_II1632-1630-1629-1625*	This work
pBDO-28	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: *BTH_II1631*	This work
pBDO-29	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: 6xhis-xylB	This work
pBDO-30	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: 6xhis-xylD	This work
pBDO-31	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: 6xhis-xylX	This work
pBDO-32	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: 6xhis-araA	This work
pBDO-33	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: 6xhis-araC	This work
pBDO-34	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: 6xhis-araD	This work
pBDO-35	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: 6xhis-udh	This work

pBDO-36	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: 6xhis-garD	This work
pBDO-37	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: 6xhis-ycbC	This work
pBDO-38	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: 6xhis-DR64_8447	This work
pBDO-39	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: 6xhis- DR64_8449	This work
pBDO-40	ColE1 origin, Amp^R, P$_L$*lacO*$_1$: 6xhis- DR64_8450	This work
pMEV-1	pUC origin, Spec^R, P$_L$*lacO*$_1$: *atoB-mvaS-mvaE*	[416]

Table 5.S2. *In vitro* enzymatic activities of *B. xenovorans* and *C. crescentus* D-xylose operon

Enzyme name	*B. xenovorans*			*C. crescentus*		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
D-xylose dehydrogenase	1.97±0.12	49.9±4.2	25.3	0.85±0.08	12.1±2.2	14
D-xylonate dehydratase	1.52±0.08	1.73±0.3	1.14	1.18±0.05	7.6±1.1	6.4
2-keto-3-deoxy-D-xylonate dehydratase[a]	8.96±0.2	4.72±0.5	0.53	1.9±0.08	0.53±0.1	0.26

[a] Enzyme activity was determined using a coupled assay

Table 5.S3. Primers used in this study

Primer Name	Sequence
xylBAcc-F	GGGCCC GGTACC ATGTCCTCAGCCATCTATCCCAGCCT
xylBBamHI-R	GGGCCCGGATCC TTAACGCCAGCCGGCGTCGATCCAGT
xylCBamHI-F	GGGCCC GGATCC AGGAGAAATTAACTATGACCGCTCAAGTCACTTGCGTATG
xylCNhe-R	GGGCCC GCTAGC TTAGACAAGGCGGACCTCATGCTGGG
xylDNheI-F	GGGCCC GCTAGC AGGAGAAATTAACTATGAGGTCCGCCTTGTCTAACCGCAC
xylDHind-R	GGGCCC AAGCTT TTAGTGGTTGTGGCGGGGCAGCTTGG

xylXHind-F	GGGCCC AAGCTT AGGAGAAATTAACTATGGTTTGTCGGCGGCTTCTAGCATG
xylXBlpRem-R	GCGCAGCTGGCGTTGTTGTCCTTGGCCTTTCTGAGCAGCAGGGCCGAACGAC CTTCGAA
xylXBlpRem-F	TTCGAAGGTCGTTCGGCCCTGCTGCTCAGAAAGGCCAAGGACAACAACGCC AGCTGCGC
xylXBlpI-R	GGGCCC GCTCAGC TTAGAGGAGGCCGCGGCCGGCCAGGT
CC0822Acc-F	GGGCCC GGTACC ATGACCGACACCCTGCGCCATTACAT
CC0822Xba-R	GGGCCC TCTAGA TTACGACCACGAGTAGGAGGTTTTGG
BFDAcc-F	GGGCCC GGTACC ATGGCTTCGGTACACGGCACCACATA
BFDSphI-R	GGGCCC GCATGC TTACTTCACCGGGCTTACGGTGCTTA
KIVDAcc-F	GGGCCC GGTACC ATGTATACAGTAGGAGATTACCTATT
KIVDSphI-R	GGGCCC GCATGC TTATGATTTATTTTGTTCAGCAAATA
IPDCAcc-F	GGGCCC GGTACC ATGCAAAACCCCTATACCGTGGCCGA
IPDCSphI-R	GGGCCC GCATGC TTATCCCCCGTTGCGGGCTTCCAGCG
YqhDSphI-F	GGGCCC GCATGC AGGAGAAATTAACTATGAACAACTTTAATCTGCACACCCC
YqhDXbaI-R	GGGCCC TCTAGA TTAGCGGGCGGCTTCGTATATACGGC
Adh6-SphI-F	GGGCCC GCATGC AGGAGATATACCATGTCTTATCCTGAGAAATTTGAAGG
Adh6-XbaI-R	GGGCCC TCTAGA CTAGTCTGAAAATTCTTTGTCGTAGC
yahK-SphI-F	GGGCCC GCATGC AAGGAGATATACC ATGAAGATCAAAGCTGTTGGTGCATA
yahK-XbaI-R	GGGCCC TCTAGA TTAGTCTGTTAGTGTGCGATTATCGA
yjgB-SphI-F	GGGCCC GCATGC AAGGAGATATACC ATGTCGATGATAAAAAGCTATGCCGC
yjgB-XbaI-R	GGGCCC TCTAGA TTAAAAATCGGCTTTCAACACCACGC
adhA-SphI-F	GGGCCC GCATGC AAGGAGATATACC ATGAAAGCAGCAGTAGTAAGACACAA
adhA-XbaI-R	GGGCCC TCTAGA TTATTTAGTAAAATCAATGACCATTC

pZAAcc-R	GGGCCC GGTCTCA ATAG TTTCTCCTCTTTAATGAATTCGGTCA
KdaBS-F	GGGCCC GGTCTCA CTAT GGTACC ATGAGCCGTATCAGAAAAGCACCCGC
KdaBS-R	GGGCCC GGTCTCA TTAC TTAAACCGTCGCGGCTTTTTTCGGAA
GarD-F	GGGCCC GGTCTCA GTAA GCTAGC AGGAGAAATTAACTATGGCCAACATCGAAATCAGACA
GarD-Acc-R	GGGCCC GGTCTCA ACCG CCATCAGGCCGTACGGCGTACC
GarD-Acc-F	GGGCCC GGTCTCA CGGT GCCCGTCATTAAAATGGCAACCCG
GarD-R	GGGCCC GGTCTCA CAGG TTAGGTCACCGGTGCCGGGTTAAACA
udh-F	GGGCCC GGTCTCA CCTG AAGCTT AGGAGAAATTAACTATGACCACTACCCCCTTCAATCG
udh-Bsa-R	GGGCCC GGTCTCA TGTC TCGATGCCGTAGCGGTCAAAGTAG
udh-Bsa-F	GGGCCC GGTCTCA GACA GTCAGCATTCGCATCGGCTCGTCG
udh-R	GGGCCC GGTCTCA GCGG TTAGTTGAACGGGCCGGCCACGGCGA
pZA-F	GGGCCC GGTCTCA CCGC GCTGAGCTCTAGAGGCATCAAATAAAACGAAAG
KivdS286Y-R	TTTAAATGATGAGTGAAGGCTCCTGTTGAGTAGTCTGTGAGTTTAACTCCAA GCATCA
KivdS286Y-F	TGATGCTTGGAGTTAAACTCACAGACTACTCAACAGGAGCCTTCACTCATCAT TTAAA
KivdS286L-R	TAAATGATGAGTGAAGGCTCCTGTTGAGAGGTCTGTGAGTTTAACTCCAAGC ATCAGG
KivdS286L-F	CCTGATGCTTGGAGTTAAACTCACAGACCTCTCAACAGGAGCCTTCACTCATC ATTTA
KivdS286F-R	TTAAATGATGAGTGAAGGCTCCTGTTGAGAAGTCTGTGAGTTTAACTCCAAG CATCAG
KivdS286F-F	CTGATGCTTGGAGTTAAACTCACAGACTTCTCAACAGGAGCCTTCACTCATCA TTTAA
KivdV461I-R	TTGATTTGGTCCATGAATTTCTCTTTCGATTGTATAACCATCATTATTGATAAT AAAGC
KivdV461I-F	GCTTTATTATCAATAATGATGGTTATACAATCGAAAGAGAAATTCATGGACC AAATCAA

KivdV461L-R	TTGATTTGGTCCATGAATTTCTCTTTCGAGTGTATAACCATCATTATTGATAATAAAGC
KivdV461L-F	GCTTTATTATCAATAATGATGGTTATACACTCGAAAGAGAAATTCATGGACCAAATCAA
KivdV461M-R	TGATTTGGTCCATGAATTTCTCTTTCCATTGTATAACCATCATTATTGATAATAA
KivdV461M-F	TTATTATCAATAATGATGGTTATACAATGGAAAGAGAAATTCATGGACCAAATCA
KivdI465F-R	TCATTGTAGCTTTGATTTGGTCCATGGAATTCTCTTTCGACTGTATAACCATCAT
KivdI465F-F	ATGATGGTTATACAGTCGAAAGAGAATTCCATGGACCAAATCAAAGCTACAATGA
KivdI465H-R	TCATTGTAGCTTTGATTTGGTCCATGGTGTTCTCTTTCGACTGTATAACCATCAT
KivdI465H-F	ATGATGGTTATACAGTCGAAAGAGAACACCATGGACCAAATCAAAGCTACAATGA
KivdI465L-R	TCATTGTAGCTTTGATTTGGTCCATGCAGTTCTCTTTCGACTGTATAACCATCAT
KivdI465L-F	ATGATGGTTATACAGTCGAAAGAGAACTGCATGGACCAAATCAAAGCTACAATGA
KivdI465M-R	TCATTGTAGCTTTGATTTGGTCCATGCATTTCTCTTTCGACTGTATAACCATCAT
KivdI465M-F	ATGATGGTTATACAGTCGAAAGAGAAATGCATGGACCAAATCAAAGCTACAATGA
Kivd-BamHI-R	GGGCCC GGATCC ATGTATACAGTAGGAGATTACCTATT
Kivd-Xba-R	GGGCCC TCTAGA TTATGATTTATTTTGTTCAGCAAATA
YqhD-BamHI-R	GGGCCC GGATCC ATGAACAACTTTAATCTGCACACCCC
YqhD-XbaI-R	GGGCCC TCTAGA TTAGCGGGCGGCTTCGTATATACGGC
XylA-BamHI-F	GGGCCC GGATCC ATGACCGACACCCTGCGCCATTACAT
XylA-XbaI-R	GGGCCC TCTAGA TTACGACCACGAGTAGGAGGTTTTGG
XylX-BamHI-F	GGGCCC GGATCC ATGGTTTGTCGGCGGCTTCTAGCATG

XylX-XbaI-R	GGGCCC TCTAGA TTAGAGGAGGCCGCGGCCGGCCAGGT
AraC-Acc65I-F	CCGAATTCATTAAAGAGGAGAAAGGTACCATGTCGGCAACGAAACCCAGGC TGCGCTCC
AraC-NheI-R	GATCCTGCGTCAGTCAAACGGCGGGCTAGCTCAGTGCGAGTGGCTCGGCAC CTCCGCGCC
AraD-NheI-F	GAGGTGCCGAGCCACTCGCACTGAGCTAGCCCGCCGTTTGACTGACGCAGG ATCCGAACC
AraD-remBlpI-R	GGCTCATCGTGCGCTCCTTGGTTCGTTGCTCACCGTGCCCAGCGCAGCACGA GCGGATCG
AraD-remBlpI-F	CGATCCGCTCGTGCTGCGCTGGGCACGGTGAGCAACGAACCAAGGAGCGCA CGATGAGCC
AraA-HindIII-R	TCGATGCTCAGGCGGCGCGCACGCAAGCTTTCAGCGGCCGAACGCTTCGGT GTCGACGCG
AraA-HindIII-F	GACACCGAAGCGTTCGGCCGCTGAAAGCTTGCGTGCGCGCCGCCTGAGCAT CGATTATCG
AraB-NdeI-R	TTGCGCCGCGTCGCCGCCATATGTCAGGTTCCGACGCCGCGCTTCAGTGCGA ATCGCGCG
AraB-NdeI-F	CTGAAGCGCGGCGTCGGAACCTGACATATGGCGGCGACGCGGCGCAACCCG ACCTGGGCC
AraE-BlpI-R	TCGTTTTATTTGATGCCTCTAGAGCTCAGCTCAGATCGGGTAATGCCGCGGC GCGGTCTG
AraB-BlpI-R	CCAGGTCGGGTTGCGCCGCGTCGCCGCGCTCAGCTCAGGTTCCGACGCCGC GCTTCAGTG
His-xylB-F	GGGCCCGGATCCATGTCCTCAGCCATCTATCCCAGCCT
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His-xylD-F	GAGAGGATCGCATCACCATCACCATCACGGATCCATGAGGTCCGCCTTGTCT AACCGCAC
His-xylD-R	GACTGAGCCTTTCGTTTTATTTGATGCCTCTAGATTAGTGGTTGTGGCGGGG CAGCTTGG
His-xylX-F	GGGCCCGGATCCATGGTTTGTCGGCGGCTTCTAGCATG
His-xylX-R	GGGCCCTCTAGATTAGAGGAGGCCGCGGCCGGCCAGGT

His-udh-F	GGGCCCGGATCCATGACCACTACCCCCTTCAATCGCCT
His-udh-R	GGGCCCTCTAGATTAGTTGAACGGGCCGGCCACGGCGA
His-garD-F	GGGCCCGGATCCATGGCCAACATCGAAATCAGACAAGA
His-garD-R	GGGCCCTCTAGATTAGGTCACCGGTGCCGGGTTAAACA
His-ycbC-F	GGGCCCGGATCCATGAGCCGTATCAGAAAAGCACCCGC
His-ycbC-R	GGGCCCTCTAGATTAAACCGTCGCGGCTTTTTTCGGAA
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His-araC-F	GGATCGCATCACCATCACCATCACGGATCCATGTCGGCAACGAAACCCAGGC TGCGCTCC
His-araC-R	GAGCCTTTCGTTTTATTTGATGCCTCTAGATTAGTGCGAGTGGCTCGGCACCT CCGCGCC
His-araD-F	GGATCGCATCACCATCACCATCACGGATCCATGACATCGAGCCGTACGCCGC GTTACCGC
His-araD-R	GAGCCTTTCGTTTTATTTGATGCCTCTAGATTAGCGTGCCCAGCGCAGCACGA GCGGATC
DR64-8447-F	AATTCATTAAAGAGGAGAAAGGTACCATGTCGTACGCAATCTATCCCAGCCT
DR64-8447-R	ACAGGGGGATGAATTTTCATAGTTAATTTCTCCTGGATCCTTATTCTCCGTAC CACCCGG
DR64-8448-F	CCGGGTGGTACGGAGAATAAGGATCCAGGAGAAATTAACTATGAAAATTCA TCCCCCTGT
DR64-8448-R	CGCGGTGTGGATGCTGACATAGTTAATTTCTCCTGCTAGCTTATTGCGCGAA GCCCCATT
DR64-8449-F	AATGGGGCTTCGCGCAATAAGCTAGCAGGAGAAATTAACTATGTCAGCATCC ACACCGCG
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DR64-8450-F	ATCCGAGGCATTCGCACTAAAAGCTTAGGAGAAATTAACTATGTCCGCAACT TCTCCATC

DR64-8450-R	CGTTTTATTTGATGCCTCTAGACATATGTTAGGCCGACGCAAGCAGCCCGCGTGCG
DR64-8452-F	TTAAAGAGGAGAAAGGTACCATGAGCCAGTTTGCGAACTA
DR64-8452-R	TTTTATTTGATGCCTCTAGATTAAACCGCGCCCGGACTCA
HisDR64-8447-F	GCATCACCATCACCATCACGGATCCATGTCGTACGCAATCTATCCCAGCC
HisDR64-8447-R	TTTCGTTTTATTTGATGCCTCTAGATTATTCTCCGTACCACCCGGCGTCG
HisDR64-8449-F	GCATCACCATCACCATCACGGATCCATGTCAGCATCCACACCGCGCCGGC
HisDR64-8449-R	TTTCGTTTTATTTGATGCCTCTAGATTAGTGCGAATGCCTCGGATTGCCG
HisDR64-8450-F	GCATCACCATCACCATCACGGATCCATGTCCGCAACTTCTCCATCCAGTT
HisDR64-8450-R	TTTCGTTTTATTTGATGCCTCTAGATTAGGCCGACGCAAGCAGCCCGCGT
Bamb4925-Acc-F	ACTGACCGAATTCATTAAAGAGGAGAAAGGTACCATGTCGGCAACAAAACCCAGGCTGCG
Bamb4925-Nhe-R	CTGCTCGATGTCATAGTTAATTTCTCCTGCTAGCTCAATGCGAATGGCTCGGCACGTCCG
Bamb4923-Nhe-F	GCCATTCGCATTGAGCTAGCAGGAGAAATTAACTATGACATCGAGCAGCACACCGCGCTA
Bamb4922-Hind-R	CAATCTGTTGCATGGGTTTTTTCTCCTGAAGCTTTCAGCGGCCGAACGCGTCGGTCCCGA
Bamb4918-Hind-F	GTTCGGCCGCTGAAAGCTTCAGGAGAAAAAACCCATGCAACAGATTGATCCGGCCGCGTC
Bamb4918-Nde-R	TCGTTTTATTTGATGCCTCTAGAGCTCACATATGTCAGCCGCGCGGCGCGCCCATGAATC
Bamb4915-Acc-F	ACTGACCGAATTCATTAAAGAGGAGAAAGGTACCATGACCGACAGACGGATGCTGATCGC
Bamb4915-Xba-R	GACTGAGCCTTTCGTTTTATTTGATGCCTCTAGATCATATCGGGTAATGCCGCGGCGTGG
BTH1632-Acc-F	ACCGAATTCATTAAAGAGGAGAAACCACGGTACCATGTCGGCATCGAAACCCAAGCTGCG
BTH1632-BamHR	GGCTCGTATTCATGGGTTTTTTCTCCTGGGATCCTCAGTGCGAATGCCGCGGCACCGCGG

BTH1630-BamHF	GCATTCGCACTGAGGATCCCAGGAGAAAAAACCCATGAATACGAGCCGTTC GCCGCGCTA
BTH1629-Hind-R	TTCGATGATTCCATAGTTAATTTCTCCTAAGCTTTCAGCGCTGAAACGGGTCG GCCGCGA
BTH1625-Hind-F	CGTTTCAGCGCTGAAAGCTTAGGAGAAATTAACTATGGAATCATCGAATCGG CCGGCGCG
BTH1625-Blp-R	GTTTTATTTGATGCCTCTAGAGCTCAGCCATATGTCACGCGTTGCGCGCGAG CGCGAACC
BTH1631-Acc-F	ACTGACCGAATTCATTAAAGAGGAGAAAGGTACCATGAACGGGCCCACGGG CGAACTCCT
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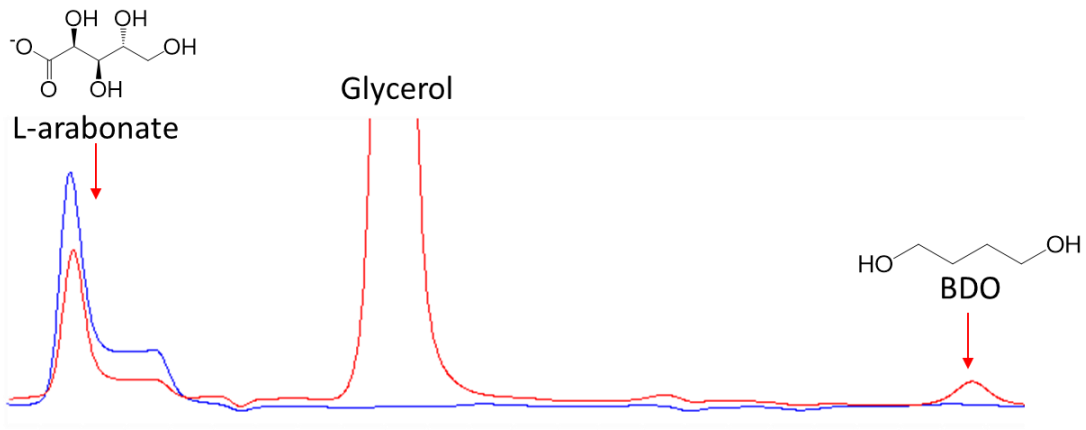


Figure 5.S1 HPLC peak of in vitro BDO production using purified enzymes. Reaction was carried out in 100 mM Tris-HCl buffer (pH ~ 7.5) with 5 mM MgSO4 using 5 mM L-arabonate, 1 µM purified L-arabonate dehydratase (AraC), 1 µM purified L-KDA dehydratase (AraD), 1 µM purified Kivd, 1 µM purified YqhD, 5 mM thiamine diphosphate (ThDP) and 1 mM NADPH as co-factor. This reaction mixture was allowed to stand at room temperature for 30 mins and HPLC was performed on the resulting mixture. HPLC results showed that 2.5 mM L-arabonate was consumed and 1.7 mM BDO was produced after half hour. This validates the *in vitro* functionality of our proposed BDO pathway.

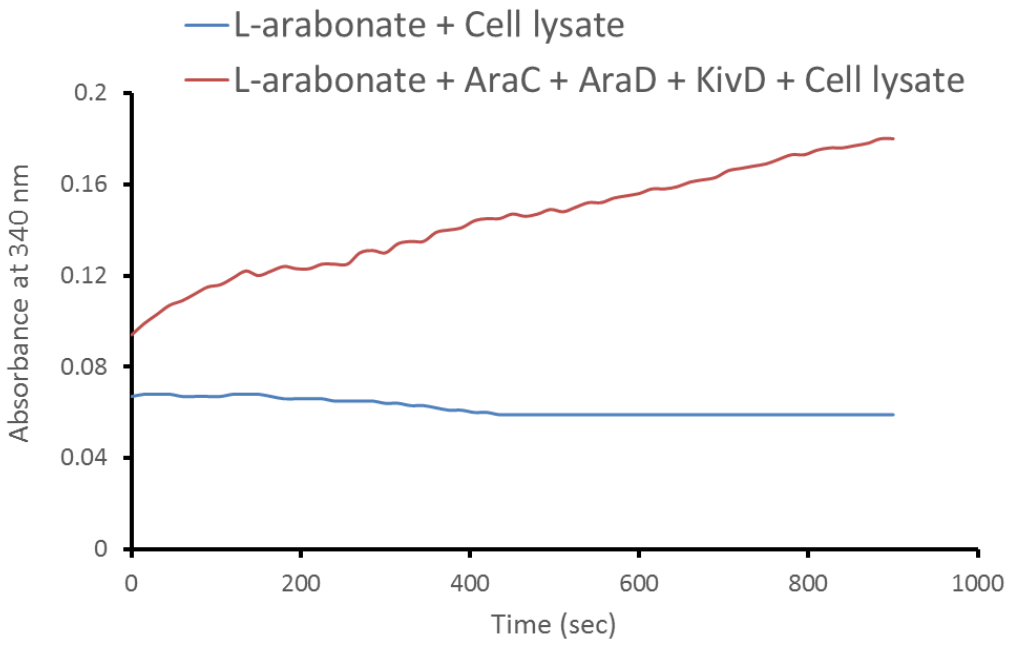


Figure 5.S2 Butanedial oxidation by endogenous *E. coli* enzymes. In vitro enzyme assay was performed using cell extract. The reaction was carried out in 100 mM Tris-HCl buffer (pH ~ 7.5) with 5 mM MgSO4 containing 1 µM L-arabonate dehydratase (AraC), 1 µM L-KDA dehydratase (AraD), 1 µM Kivd with 5 mM thiamine diphosphate (ThDP) and 1mM NAD+ as co-factor. The reaction was initiated by adding 1 mM L-arabonate and 0.1 mg/ml of cell lysate and the absorbance was immediately measured at 340 nm using a spectrophotometer. A steady rise in absorbance was observed at 340 nm indicating oxidation of butanedial using NAD^+ by endogenous E. coli enzymes. A reaction mixture containing 1 mM L-arabonate, 0.1 mg/ml cell lysate and 1mM NAD^+ without purified AraC, AraD and Kivd was used as negative control. This sample did not show any increase in absorbance at 340 nm.

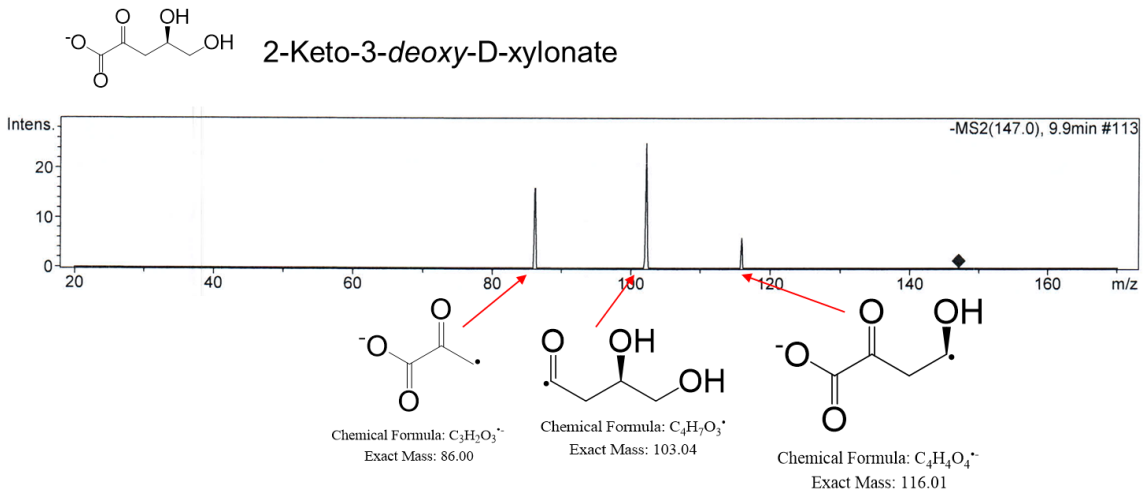


Figure 5.S3 LC-MS data for 2-keto-3-deoxy-D-xylonate

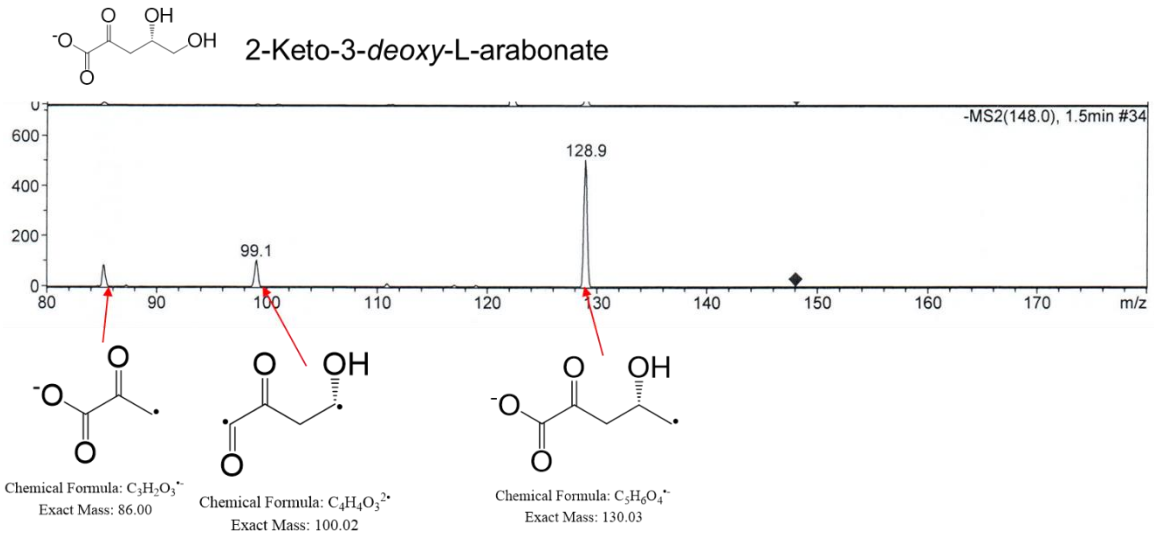


Figure 5.S4 LC-MS data for 2-keto-3-deoxy-L-arabonate

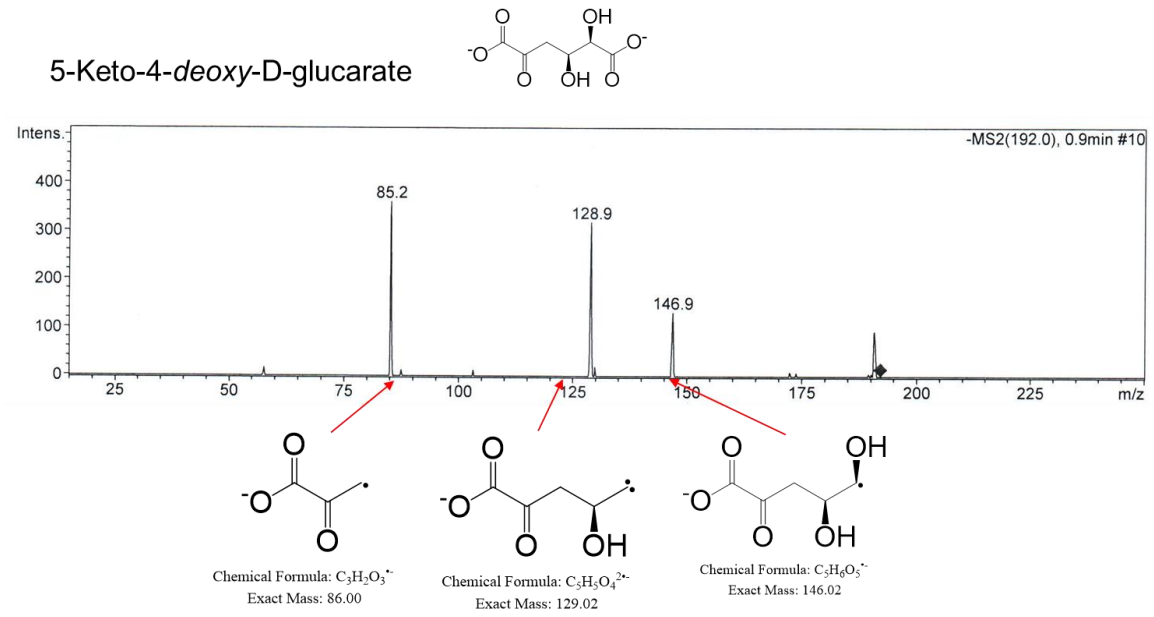


Figure 5.S5 LC-MS data for 5-keto-4-deoxy-D-glucarate

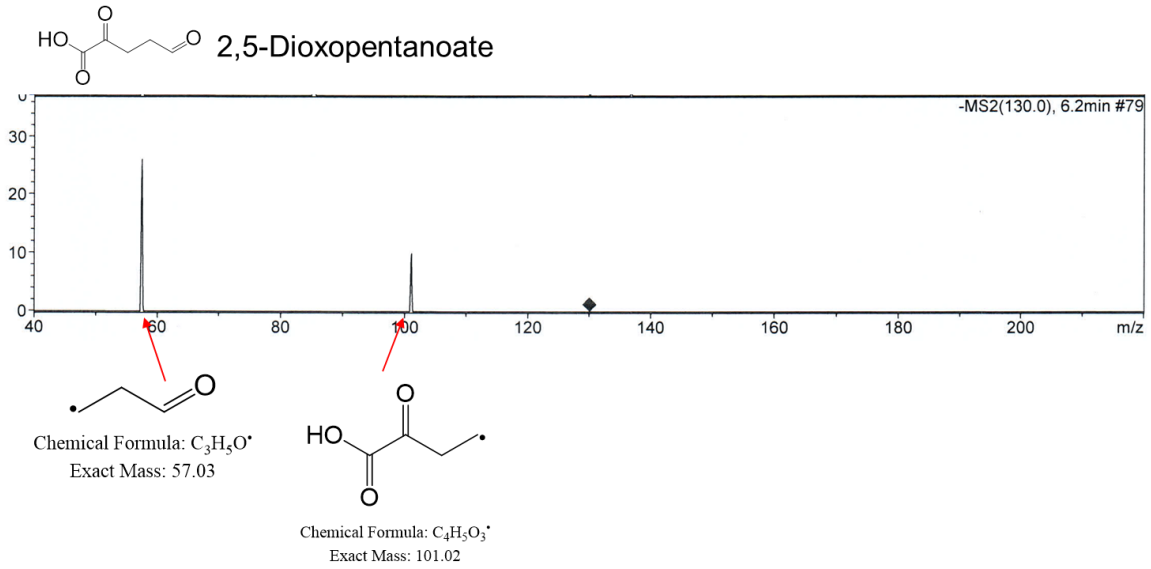
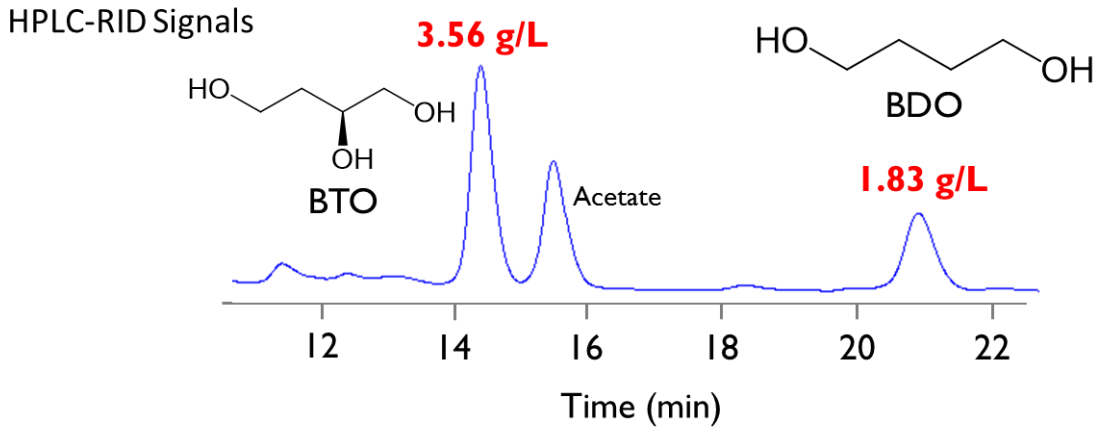


Figure 5.S6 LC-MS data for 2, 5-dioxopentanoate.

a)



b)

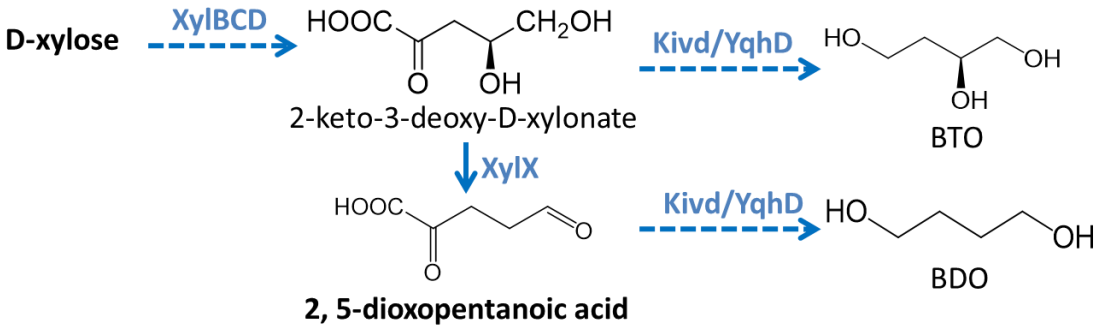


Figure 5.S7 Accumulation of 1,2,4-butanedtriol (BTO). a) HPLC signal showing BTO accumulation with C. crescentus D-xylose operon. b) Mechanism showing BTO formation due to promiscous nature of 2-ketoacid decarboxylase (Kivd).

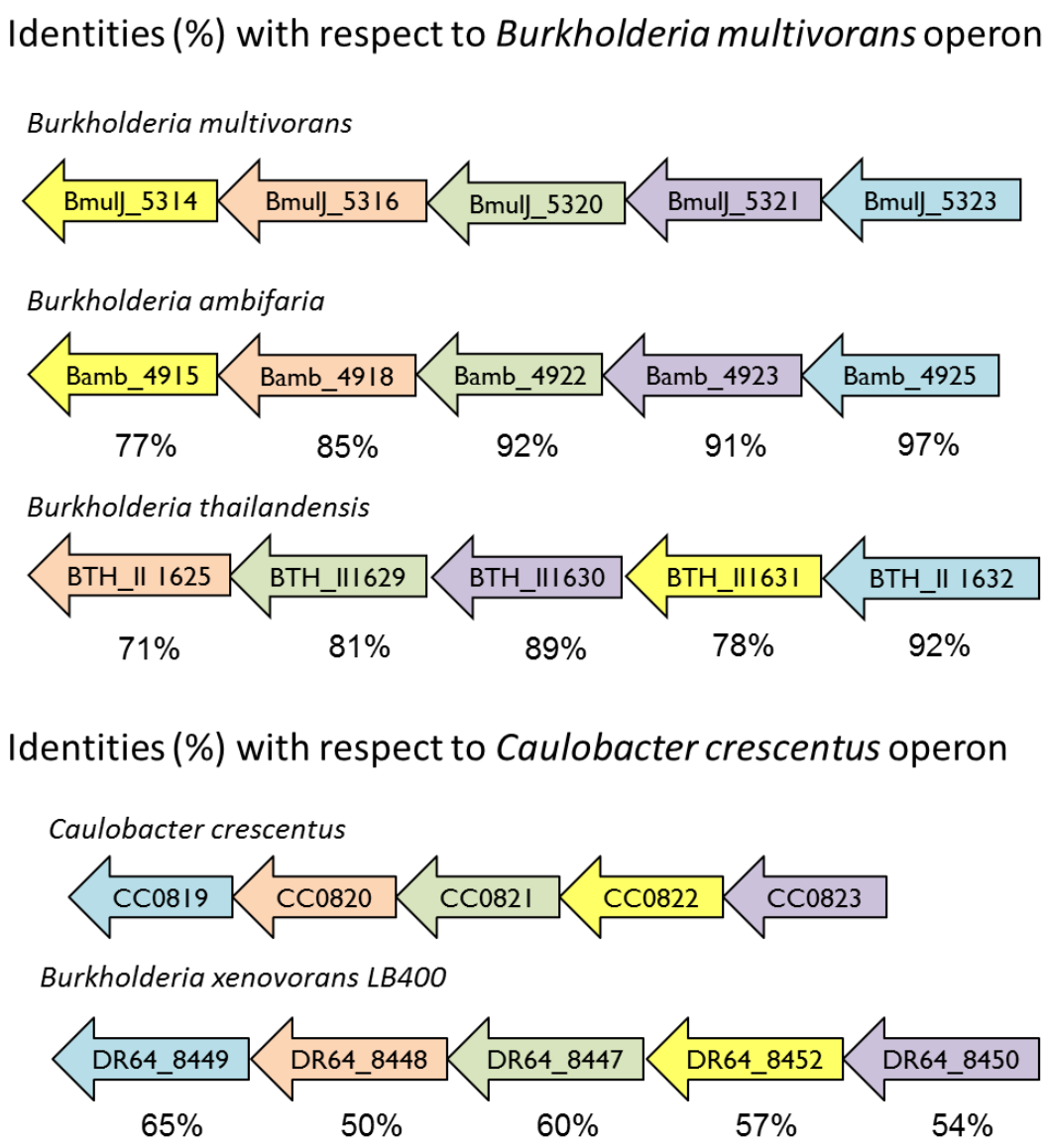


Figure 5.S8 Sequence identities of different D-xylose and L-arabinose operons. Sequence identities of B. ambifaria and B. thailandensis L-arabinose operons with respect to B. multivorans L-arabinose operon. Sequence identity of B. xenovorans D-xylose operon with respect to C. crescentus D-xylose operon. Enzymes are color coded- green: D-xylose/L-arabinose dehydrogenase; orange: D-xylonolactonase/L-arabinolactonase; blue: D-xylonate/L-arabonate dehydratase; purple: 2-keto-3-deoxy-D-xylonate/2-keto-3-deoxy-L-arabonate dehydratase; yellow: 2-ketoglutarate semialdehyde dehydrogenase.

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Author contributions

Y.T., M.X., P.J., and K.Z. designed experiments. Y.T., M.X., P.J., J.W., J.W. and C.S. performed experiments. Y.T., M.X., P.J., and K.Z. analyzed data. Y.T., M.X., P.J., J.W., J.W., C.S., and K. Z. wrote and edited the paper.

Competing financial interests

M.X. and K.Z. are co-inventors on a patent application entitled: Biosynthetic pathways and methods (Patent Application Number WO2014100173).

Chapter 6

Engineering Nonphosphorylative Metabolism for the production of γ-amino butyric acid (GABA)

6.1 Introduction

γ-amino butyric acid (GABA) is a 4-carbon non-protein amino acid widely found in microorganisms, plants, and animals.[421] Its potential to serve as a major inhibitory neurotransmitter in mammalian central nervous system along with its hypotensive, antidiabetic, and diuretic effects has found wide applications in the pharmaceutical and food industry.[422-424] Furthermore, it is used in the chemical industry as a precursor for the synthesis of 2-pyrrolidone and a promising novel biodegradable polymer, Nylon-4.[425-427] Nylon-4 is a linear polyamide with repeating GABA units having excellent physical properties due its high melting point of 260^{o}C and it is biodegradable in soil [428] and

activated sludge.[429] GABA represents a new building block for bio-plastics and the synthesis of GABA from abundantly available lignocellulosic biomass can enable the production of new bio-plastic materials at a low cost. Thus, the development of an economic biosynthetic process for sustainable production of GABA is important to meet its increasing demand in pharmaceutical, food, and bioplastics industry.

GABA is produced by the irreversible decarboxylation of glutamate catalyzed by glutamate decarboxylase (GAD), a pyridoxal-5'-phosphate-dependent enzyme.[430-431] The glutamate/GABA antiporter pumps out GABA into the extracellular environment while bringing in more glutamate. The decarboxylation reaction converts an acidic substrate (glutamate) into a neutral compound (GABA) via the incorporation of free protons (H^+), thus controlling the acidification in the cytosolic environment.[432] When the transporter pumps out GABA, it results in a local alkalization of the extracellular medium, and thus GAD and the GABA antiporter constitute the GAD acid resistance system. Three main strategies have been employed to enable the biological production of GABA. The first process involves the use of purified GAD enzyme for the conversion of glutamate monosodium salt (MSG) to GABA. While this approach has advantages of high conversion yield and purity, use of a purified enzyme makes the downstream processing difficult and expensive.[433-435] To address these challenges, some studies have attempted reversible immobilization of GAD enzyme to allow catalyst recycle and to ease downstream separation.[436] The second approach for GABA production is to use whole cells as catalyst for the conversion of glutamate to GABA using natural GABA producers such as lactic acid bacteria (LAB)[437-438] or by overexpressing GAD enzymes in

recombinant hosts such as *Escherichia coli*.[426] The overexpression of glutamate decarboxylase and glutamate/GABA antiporter in a GABA aminotransferase mutant of *E. coli* resulted in 5.46 g/L of GABA from 10 g/L MSG.[439] In a subsequent study by the same group, the use of a synthetic protein scaffold to connect the relevant enzymes increased the titer to 5.65 g/L GABA, which corresponds to a GABA yield of 93%.[440] Although both these approaches have shown great success in producing GABA, use of MSG as a substrate necessitates the need for additional processes to link MSG and GABA production processes in the industry. This leads to the third process for GABA production which uses biomass-derived sugars like glucose as starting material making it more economical than other approaches that use MSG. Being a major glutamate producing microorganism, recombinant *C. glutamicum* has been used widely for producing GABA directly from glucose.[441-442] In one such study, recombinant *C. glutamicum* expressing *E. coli* glutamate decarboxylase (GadB) was cultivated in medium with 50 g/L glucose and 0.1 mM pyridoxal 5'-phosphate (PLP), a co-factor for GadB, resulting in production of over 12 g/L GABA in 72 h.[441] *E. coli* has also been engineered for direct GABA production from glucose by using synthetic protein scaffolds to co-localize the GABA pathway enzymes and GABA shunt enzymes resulting in ~1.1-1.3 g/L GABA from 10-20 g/L glucose.[443]

With growing concern of the environmental damage of using fossil-based feedstocks and the debate of "food versus fuel" with edible feedstocks like corn, there is a pressing need to search for renewable and low-cost feedstocks. Lignocellulosic biomass, with an annual output of 2×10^{11} tons, presents a promising solution. Most industrial

fermentations convert glucose to value-added chemicals but other pentoses like D-xylose and L-arabinose, which are the most abundant sugars in hemicellulose, are being wasted. Thus, efficient utilization of these pentoses, which make up 30 % of plant biomass, is important to ensure an economically viable biosynthetic process.[444] While wild-type *E. coli* can utilize these sugars, simultaneous utilization of D-xylose or L-arabinose in the presence of glucose is inhibited due to carbon catabolite repression (CCR).[445] Furthermore, it was recently reported that both D-xylose and L-arabinose can repress the utilization and metabolism of the other sugar through the action of regulatory proteins XylR and AraC, respectively.[446-448] Apart from this challenge of simultaneous utilization of pentoses, the conventional pathways for pentose metabolism (pentose phosphate pathway and glycolysis) requires at least 10 steps to enter the TCA cycle. To avoid these numerous tightly regulated steps and the problem of simultaneous sugar utilization, it is important to look for alternative metabolic pathways for biosynthesis which are orthogonal to intrinsic metabolism. One such pathway, the nonphosphorylative pathway, was recently studied for the biosynthesis of 1,4-butanediol and mesaconate and it only involves 5 steps to convert pentoses such as D-xylose and L-arabinose into 2-ketoglutarate (2-KG), an important TCA cycle intermediate.[324, 449]

In this work, we demonstrate the production of GABA from under-utilized biomass sugars, D-xylose and L-arabinose, using the nonphosphorylative pathway. Considering the importance of glutamate in *E. coli* metabolism, we knocked out the *sucA* gene encoding 2-KG decarboxylase to increase intracellular glutamate pools. We also knocked out GABA aminotransferase (*gabT*) which redirects GABA back into the TCA

cycle. Apart from gene knockouts, we also screened different nonphosphorylative operons to choose the best performing nonphosphorylative gene cluster for D-xylose and L-arabinose metabolism. Lastly, we overexpressed the glutamate dehydrogenase (GDH) enzyme and the arabinose proton symporter (AraE) in order to improve sugar uptake and increase GABA titers. The final D-xylose strain produced 1.52 g/L GABA from 20 g/L D-xylose in 48 hours with a yield of 0.15 g GABA/g of D-xylose, corresponding to 22% of the theoretical maximum yield. The L-arabinose strain produced 1.4 g/L GABA from 20 g/L L-arabinose in 48 hours with a yield of 0.34 g of GABA/g of L-arabinose, corresponding to 50% of the theoretical maximum yield. This work reports the successful production of an important commercially relevant chemical GABA from D-xylose and L-arabinose using nonphosphorylative metabolism.

6.2 Results

6.2.1 GABA production from nonphosphorylative metabolism

To efficiently use pentoses (D-xylose and L-arabinose) as feedstocks for GABA production, biosynthetic pathways were designed by expanding the nonphosphorylative metabolism (**Figure 6.1**). In the D-xylose pathway, D-xylose is converted into 2-KG in five enzymatic steps enabled by D-xylose dehydrogenase (XDH), D-xylonolactonase (XL), D-xylonate dehydratase (XD), 2-keto-3-deoxy-D-xylonate dehydratase (KdxD), and 2-ketoglutarate semialdehyde dehydrogenase (KGSADH).[450] Similarly, L-arabinose is transformed into 2-KG by the action of enzymes L-arabinose dehydrogenase (ADH), L-arabinolactonase (AL), L-arabonate dehydratase (AD), 2-keto-3-deoxy-L-arabonate dehydratase (KdaD), and 2-ketoglutarate semialdehyde dehydrogenase (KGSADH). 2-

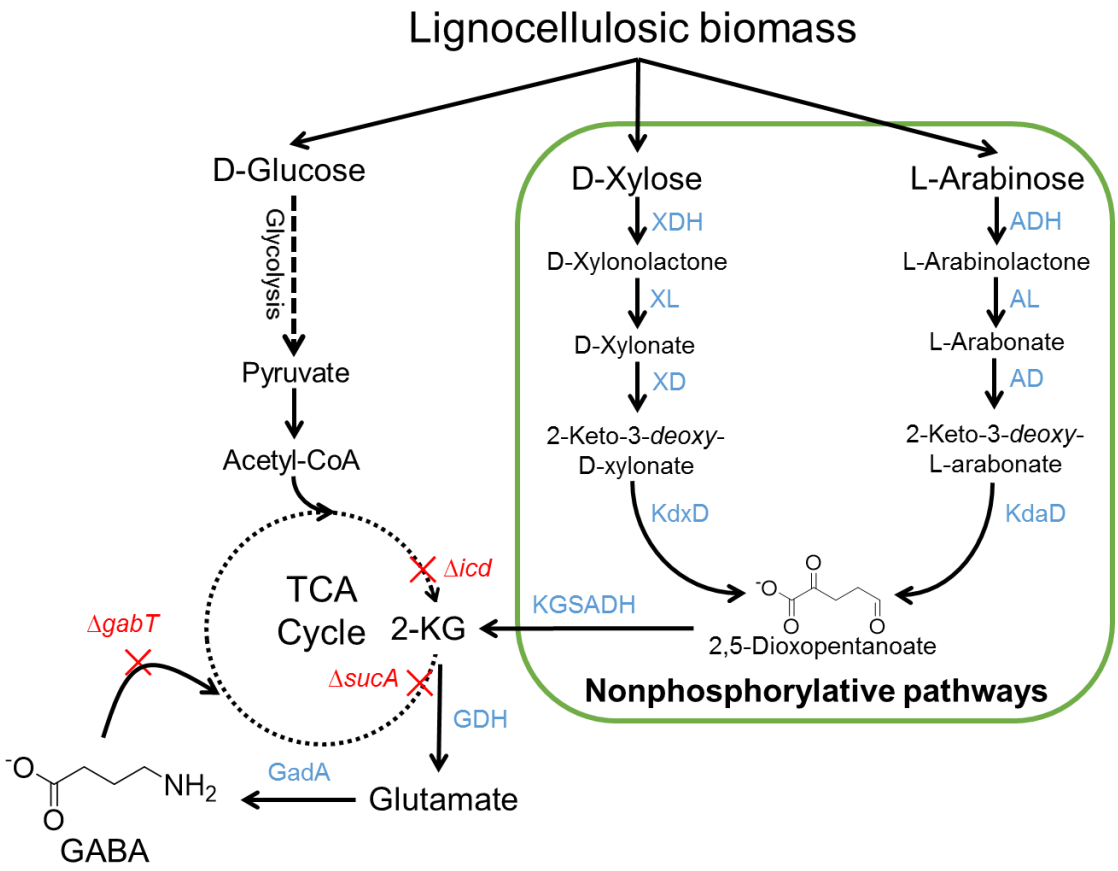

Figure 6.1 Metabolic pathways from lignocellulosic sugars to GABA via nonphosphorylative metabolism. The pathway for D-xylose consists of D-xylose dehydrogenase (XDH), D-xylonolactonase (XL), D-xylonate dehydratase (XD), and 2-keto-3-deoxy-D-xylonate dehydratase (XD), and 2-keto-3-deoxy-D-xylonate dehydratase (KdxD). The L-arabinose assimilation pathway is composed of L-arabinose dehydrogenase (ADH), L-arabinolactonase (AL), L-arabonate dehydratase (AD), and 2-keto-3-deoxy-L-arabonate dehydratase (KdaD). The produced DOP is then converted into 2- ketoglutarate (2-KG) by 2-ketoglutarate semialdehyde dehydrogenase (KGSADH). 2-KG is then transformed to GABA by glutamate dehydrogenase (GDH) and glutamate decarboxylase (GadA).

KG produced from the nonphosphorylative metabolism is converted into glutamate by glutamate dehydrogenase (GDH) which is decarboxylated by glutamate decarboxylase (GadA) in a pyridoxal-5'-phosphate (PLP)-dependent reaction to produce GABA.

Based on the pathway design, we constructed two strains, strain GX-1 (BW25113 Δ*xylA*Δ*yihH*Δ*yagE*Δ*icd*) and strain GA-1 (BW25113 Δ*icd*) (**Table 6.1**), to synthesize

Table 6.1 Strains and plasmids used in this study

Name	Relevant genotype	Reference
Strains		
BW25113	*rrnB*$_{T14}$ ΔlacZ$_{WJ16}$*hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	[451]
XL10-Gold	Tet^{R}Δ (*mcrA*)183 Δ (*mcrCB-hsaSMR-mrr*)173 *endA1supE*44 *thi-1 recA1*	Stratagene
GX-1	BW25113 Δ*xylA*Δ*yjhH*Δ*yagE*Δ*icd*	[452]
GA-1	BW25113 Δ*icd*	[452]
GX-2	BW25113 Δ*xylA*Δ*yjhH*Δ*yagE*Δ*icd*Δ*sucA*	[449]
GA-2	BW25113 Δ*icd*Δ*sucA*	[449]
GX-3	BW25113 Δ*xylA*Δ*yjhH*Δ*yagE*Δ*icd*Δ*sucA*Δ*gabT*	This work
GA-3	BW25113 Δ*icd*Δ*sucA*Δ*gabT*	This work
Plasmids		
pGABA-1	P15A origin, KanR, PL*lacO*1: *xylB-xylC-xylD-xylX*	[452]
pGABA-2	ColE1 origin, AmpR, PL*lac*O1:*xylA*$_{CC}$*-gadA*	This work
pGABA-3	P15A origin, KanR, PL*lacO*1: *araC-araD-araA-araB*	[453]
pGABA-4	P15A origin, KanR, PL*lacO*1: *DR64_8447-8448-8449-8450*	[452]
pGABA-5	P15A origin, KanR, PL*lacO*1: *xylB-xylC-xylD-DR64_8450*	[449]
pGABA-6	P15A origin, KanR, PL*lacO*1: *Bamb_4925-4923-4922-4918*	[452]
pGABA-7	P15A origin, KanR, PL*lacO*1: *BTH_II1632-1630-1629-1625*	[454]
pGABA-8	pUC origin, SpecR, PL*lacO*1: *gdhA*	[449]
pGABA-9	pUC origin, SpecR, PL*lacO*1: *gdhA-araE*	This work

GABA from D-xylose and L-arabinose, respectively. To maximize the flux of D-xylose and L-arabinose towards GABA, for strain GX-1, the D-xylose (Δ*xylA*) and D-xylonate (Δ*yihH*Δ*yagE*) consumption pathways were knocked out and the strain GA-1 was derived from the wild-type *E. coli* K-12 BW25113 which has L-arabinose (*ΔaraA*) gene already knocked out.[451] Furthermore, both strains GX-1 and GA-1 had isocitrate dehydrogenase (*icd*) gene of TCA cycle knocked out to ensure that the glucose supplemented in fermentation medium was only used for growth and not for the production of GABA. For

GABA production from D-xylose, strain GX-1 was transformed with two plasmids: a medium-copy plasmid expressing the D-xylose non-phosphorylative operon from *Caulobacter crescentus* pGABA-1 (pZA-*xylBCDX*) to produce 2,5-dioxopentanoate (DOP)[324], and a high-copy plasmid expressing the $xylA_{CC}$ gene to convert DOP to 2-KG and *gadA* gene to convert glutamate to GABA, pGABA-2 (pZE- $xylA_{CC}$-*gadA*). The conversion of 2-KG to glutamate was catalyzed by the endogenous glutamate dehydrogenase (GDH) of *E. coli*. Similarly for L-arabinose to GABA conversion, the strain GA-1 was transformed with L-arabinose gene cluster from *Burkholderia multivorans araCDAB* (*BmulJ 5323-5321-5320-5316*)[453] over-expressed on a medium copy plasmid pGABA-3 (pZA- *araCDAB*) and the same high-copy plasmid pGABA-2 to convert DOP to 2-KG and glutamate to GABA. The two strains were then examined for the production of GABA using shake flask fermentations. After 48 hours, strain GX-1 only produced 0.11 g/l GABA from 20 g/l of D-xylose and strain GA-1 produced 0.05 g/l of GABA from 20 g/l of L-arabinose (**Figure 6.2**). Glucose (20 g/l) was supplemented for cell growth, but did not contribute to the production of GABA due to the *icd* deletion. Although these preliminary results demonstrate the feasibility of using nonphosphorylative metabolism for production of GABA from the two pentoses, D-xylose and L-arabinose, the titers are extremely low for practical purposes.

6.2.2 Improving GABA biosynthesis by metabolic engineering

To improve GABA production from D-xylose and L-arabinose via this nonphosphorylative metabolism, we applied metabolic engineering strategies to drive the carbon flux towards GABA. The *E. coli* gene *sucA* encodes a subunit of 2-KG

decarboxylase[455] which catalyzes the conversion of 2-KG to succinyl-CoA in the TCA cycle and diverts the carbon flux from glutamate production. Therefore, we knocked out *sucA* gene to increase glutamate pools in the production strains GX-1 and GA-1 generating strains GX-2 (GX-1 Δ*sucA*) and GA-2 (GA-1 Δ*sucA*), respectively. The new strains GX-2 and GA-2 were transformed with plasmids pGABA-1 and pGABA-3, respectively to convert D-xylose and L-arabinose to DOP and plasmid pGABA-2 to convert DOP to GABA. After 48 hour shake flask fermentations, the Δ*sucA* knockout improved the GABA titer more than 10-fold resulting in 0.36 g/L GABA from D-xylose and 0.40 g/L GABA from L-arabinose (**Figure 6.2**).

Another enzyme that diverts carbon flux away from our designed GABA production pathway is the GABA aminotransferase enzyme (GabT) in *E. coli* which catalyzes the conversion of GABA to succinyl semialdehyde and directs it back into the TCA cycle. We knocked out this gene to improve accumulation of GABA and generated strains GX-3 (GX-2, Δ*gabT*) and GA-3 (GA-2, Δ*gabT*). The D-xylose strain GX-3 transformed with plasmids pGABA-1 and pGABA-2 produced 0.59 g/L GABA and L-arabinose strain GA-3 with plasmids pGABA-3 and pGABA-2 produced 0.50 g/L GABA after 48 hours in shake flask fermentations (**Figure 6.2**). Both Δ*sucA* and Δ*gabT* knockouts improved GABA titer from D-xylose by 5-fold and from L-arabinose by 10-fold.

6.2.3 Screening operons of nonphosphorylative metabolism

After identifying gene knockouts to improve GABA production from both D-xylose and L-arabinose, various operons of the nonphosphorylative metabolism were screened to

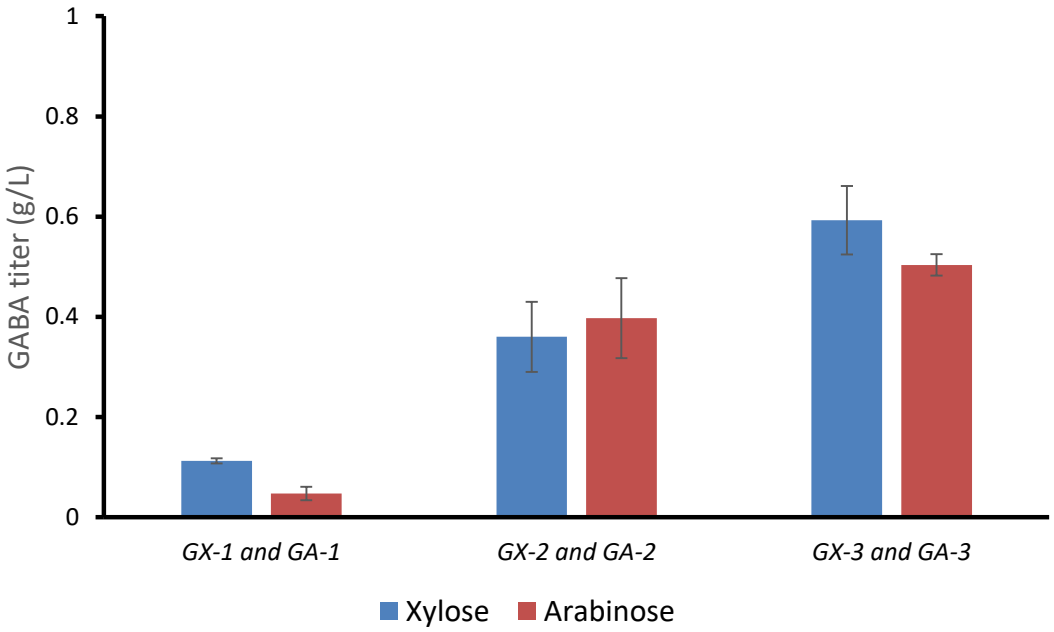

Figure 6.2 Metabolic engineering strategies to increase GABA titers. GX-1: BW25113 Δ*xylA* Δ*yjhH* Δ*yagE* Δ*icd*; GA-1: BW25113 Δ*icd*; GX-2: GX-1Δ*sucA*; GA-2: GA-1Δ*sucA*; GX-3: GX-2 Δ*gabT*; GA-3: GA-2 Δ*gabT*

further improve GABA production. For D-xylose pathway, three operons were tested for the activity of their nonphosphorylative enzymes. Apart from the *xylBCDX* operon from *C. crescentus* used in the previous section for metabolic engineering studies, the D-xylose operon from *B. xenovorans LB400 DR64_8447-8448-8449-8450* (pGABA-4) and a synthetic operon *xylB-xylC-xylD-DR64_8250* (pGABA-5) with the first three genes from *C. crescentus* (*xylB-xylC-xylD*) and the last gene (*DR64_8450*) from *B. xenovorans LB400* were screened for GABA production. The synthetic operon was constructed by combining the genes from two organisms since the 2-keto-3-*deoxy*-D-xylonate dehydratase (KdxD) enzyme from the *B. xenovorans LB400* operon was reported to have a higher *in vitro* activity (DR64_8250, k_{cat}/K_m=0.53 $s^{-1}mM^{-1}$) than the corresponding enzyme from the *C. crescentus* operon (XylX, k_{cat}/K_m=0.26 $s^{-1}mM^{-1}$).[452] On the other

hand, *C. crescentus* operon has more active D-xylose dehydrogenase (XDH) and D-xylonate dehydratase (XD) enzymes than the *B. xenovorans* operon. The D-xylose strain GX-3 was transformed with the corresponding plasmids pGABA-1 and pGABA-2 for *C. crescentus*, pGABA-4 and pGABA-2 for *B. xenovorans*, and pGABA-5 and pGABA-2 for the synthetic operon and shake flask fermentations was performed. The GX-3 strain harboring the synthetic operon (pGABA-5 and pGABA-2) had highest GABA titer of 0.78 g/L from 20 g/L D-xylose (**Figure 6.3**).

Three L-arabinose operons were also tested for GABA production via the nonphosphorylative metabolism: the *B. multivorans* operon *araCDAB* (pGABA-3) used in the previous metabolic engineering section, the *Burkholderia ambifaria* L-arabinose operon *Bamb_4925-4923-4922-4918* (pGABA-6), and the L-arabinose operon *BTH_II1632-1630-1629-1625* from *Burkholderia thailandensis* (pGABA-7). The L-arabinose strain GA-3 was transformed with plasmids pGABA-3 and pGABA-2 for *B. multivorans*, pGABA-6 and pGABA-2 for *B. ambifaria*, and pGABA-7 and pGABA-2 for *B. thailandensis* and 48 hour shake flask fermentations were carried out. The GX-3 strain harboring the *B. ambifaria* operon produced highest GABA titer of 0.7 g/L from 20 g/L L-arabinose (**Figure 6.3**). *B. thailandensis* operon was the least active and only produced 0.21 g/l GABA (**Figure 6.3**).

The trends for both D-xylose and L-arabinose operons are consistent with previous studies using different nonphosphorylative operons for the production of BDO and mesaconate from pentose sugars.[324, 449]

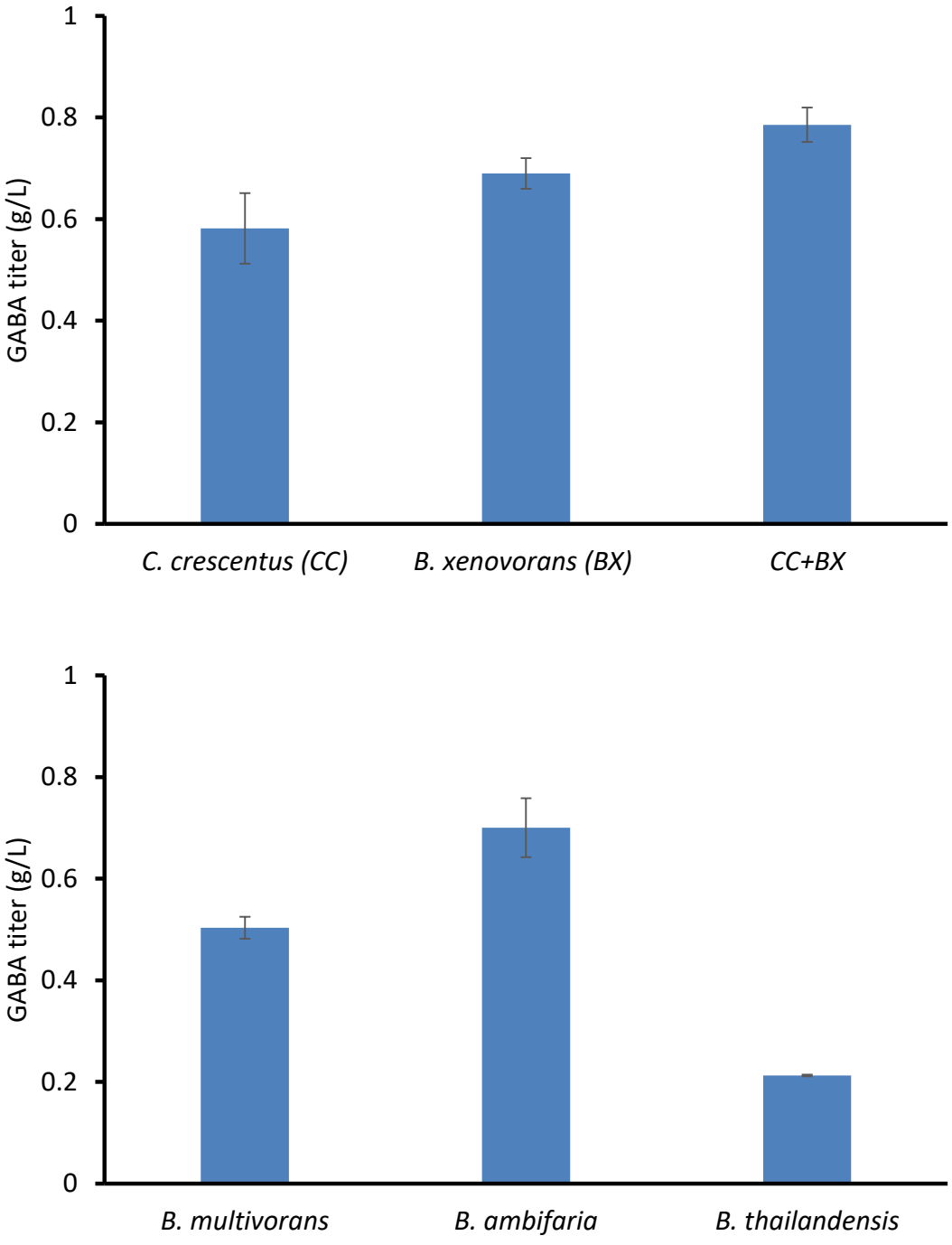

Figure 6.3 Screening nonphosphorylative operons to improve GABA production for a) D-xylose, and b) L-arabinose.

6.2.4 Overexpression of glutamate dehydrogenase and pentose transporter

All the experiments mentioned in above sections used the *E. coli* endogenous glutamate dehydrogenase (GDH) enzyme for converting 2-KG to glutamate. To further increase intracellular glutamate pool and GABA titers, we overexpressed the *E. coli* GDH enzyme on a low copy plasmid, pGABA-8 (pZS-GDH). When xylose strain GX-3 was transformed with plasmids pGABA-5, pGABA-2, and pGABA-8, and subjected to shake flask fermentation for 48 hours, it produced 1.3 g/L GABA from 20 g/L D-xylose (**Figure 6.4).** This corresponds to 0.18 g of GABA/g of D-xylose consumed which is 27% of the theoretical maximum yield. Similarly when L-arabinose strain GA-3 was transformed with plasmids pGABA-6, pGABA-2, and GABA-4, it produced 0.96 g/L GABA after 48 hours from 20 g/L L-arabinose with a yield of 0.46 g of GABA/g of L-arabinose corresponding to 67% of the theoretical maximum (**Figure 6.4**). Thus, overexpression of GDH significantly improved GABA titers by increasing glutamate pool.

The above mentioned strains GX-3 (with pGABA-5, pGABA-2 and pGABA-8) and GA-3 (with pGABA-6, pGABA-2 and pGABA-8) only consumed 7.2 g/L D-xylose and 2.1 g/L L-arabinose respectively after 48 hour fermentations. Glucose was also not completely consumed with GX-3 D-xylose strain consuming 6 g/L glucose and GA-3 L-arabinose strain consuming 7.5 g/L glucose in 48 hours. Since the nonphosphorylative metabolism does not involve the endogenous *E. coli* xylose and arabinose metabolic genes which are subjected to glucose repression or reciprocal regulation, we believe that pentose sugar consumption can be improved by overexpressing the pentose transporters.

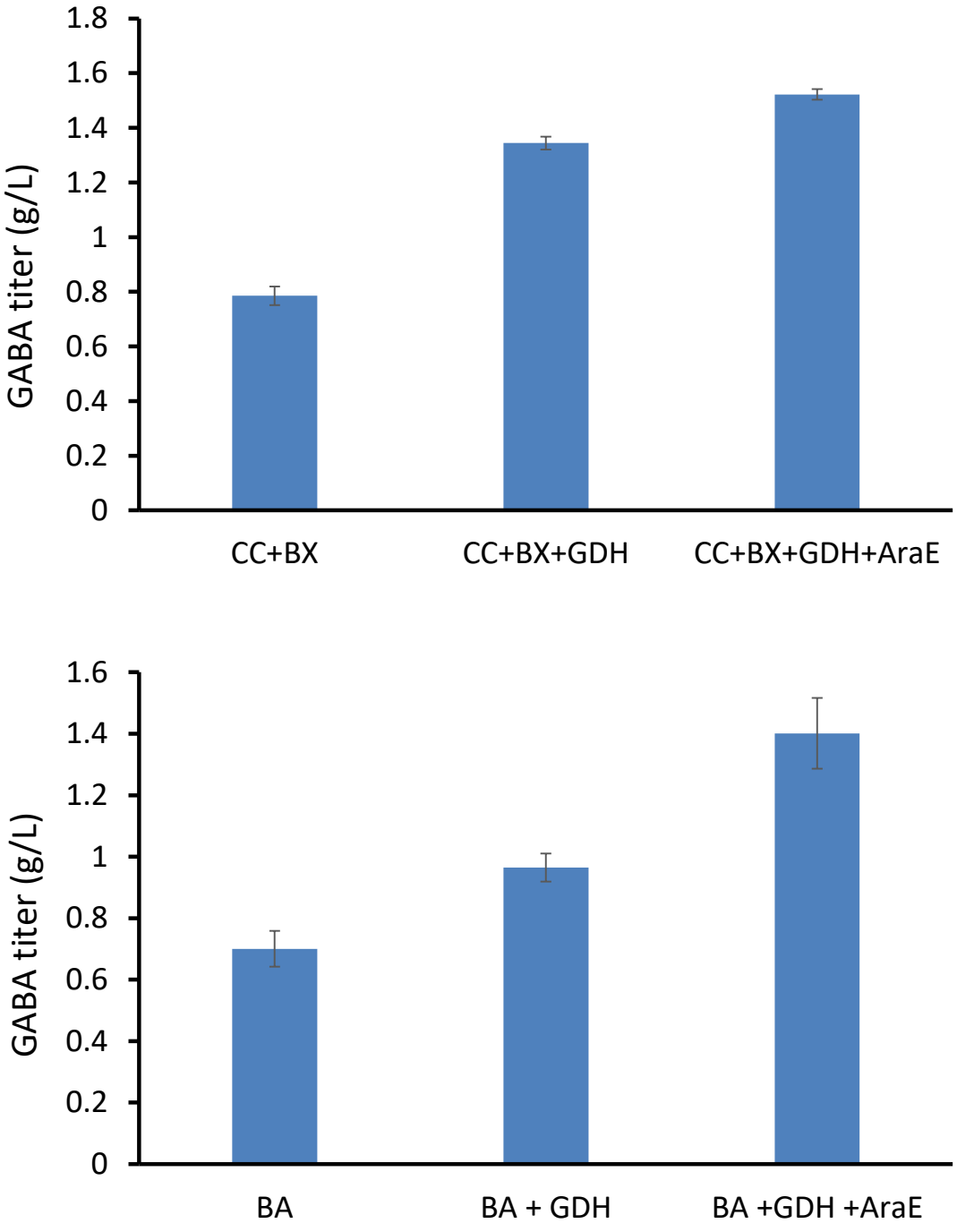

Figure 6.4 Overexpression of glutamate dehydrogenase (GDH) and arabinose symporter (AraE) for a) D-xylose and b) L-arabinose. CC+BX is the synthetic D-xylose operon combining C. crescentus and B. xenovorans and BA is B. ambifaria operon for L-arabinose.

There are two pentose transporter systems in *E. coli*. One system contains ATP-binding cassette (ABC) transporters (XylFGH and AraFGH) which have higher affinity but lower capacity. The other system contains pentose/proton symporters (XylE and AraE) which shows lower affinity but higher capacity.[456-457] It is well known that the low affinity symporters are promiscuous and can transport heterologous sugars.[458-459] A recent study comparing the ATP-binding cassette transporters and pentose/proton symporters of D-xylose and L-arabinose showed that the arabinose symporter (AraE) was the most effective transporter for both D-xylose as well as L-arabinose.[449] Therefore, we overexpressed the *E. coli* arabinose symporter (AraE) on a low-copy plasmid, pGABA-9 (pZS-GDH-AraE). When D-xylose strain GX-3 was transformed with three plasmids – pGABA-5, pGABA-2 and pGABA-9 – it produced 1.5 g/L GABA by consuming 10 g/L D-xylose after 48 hours (**Figure 6.4**). Although it improved xylose consumption the GABA yield reduced to 0.15 g of GABA/g of D-xylose. Similarly, L-arabinose strain GA-3 with plasmids pGABA-6, pGABA-2 and pGABA-9 produced 1.4 g/L GABA by consuming 4.1 g/L of L-arabinose but yield reduced to 0.34 g of GABA/g of L-arabinose (**Figure 6.4**). Overexpression of transporter AraE improved sugar uptake and subsequently increased GABA titers but reduced the yields of GABA from both sugars. We also did a time course experiment to compare strains with and without AraE overexpression to understand how sugar uptake changes with time. From **Figure 6.5** we can see that while the overexpression of AraE improves both D-xylose and L-arabinose consumption and GABA production, it reduces the consumption of glucose. Furthermore, the rate of consumption of pentose sugar is highest in first 16 hours for both D-xylose and

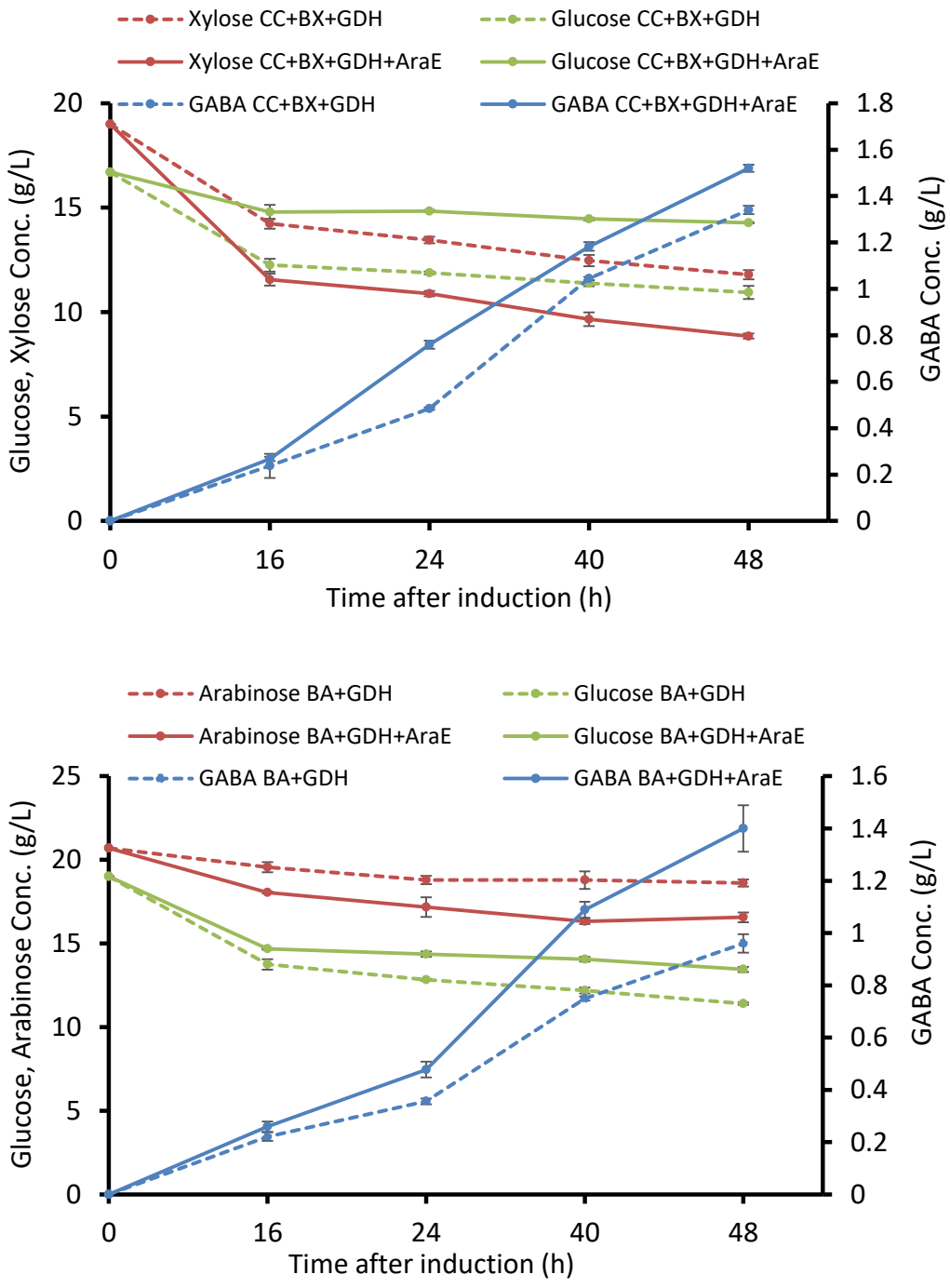

Figure 6.5 Time-course experiment to study sugar uptake of a) D-xylose and b) L-arabinose with and without transporter (AraE) overexpression. Dotted lines is without AraE overexpression and solid lines is with overexpression of AraE.

L-arabinose after which the rate of uptake decreases significantly as seen from the slope of the curve (**Figure 6.5**). Both the strains consume less than half of the sugars in fermentation medium after 48 hours.

6.3 Discussion

GABA is an important chemical with applications in the pharmaceutical and food industry[422-424] and with increasing demand as a building block for biodegradable plastics like Nylon-4.[425-427] Biosynthesis of GABA has been extensively studied but most of these efforts have focused on using MSG as a starting material. To make it more economical and viable for industrial production, it is important to explore the biosynthesis of GABA from biomass-derived sugars such as glucose, D-xylose, and L-arabinose. While there have been recent studies on producing GABA from glucose using protein scaffolds [443, 460] and other metabolic engineering strategies[441-442], there have been no reports using pentose sugars for GABA production. This is the first study to successfully demonstrate the biosynthesis of GABA from under-utilized pentoses, D-xylose and L-arabinose, derived from lignocellulosic biomass. To avoid carbon catabolite repression (CCR) and reciprocal regulation, the nonphosphorylative pathway which is orthogonal to *E. coli* intrinsic metabolism was used to convert D-xylose and L-arabinose to GABA.

To improve GABA titers, two endogenous *E. coli* genes – 2-KG dehydrogenase (*sucA*) and GABA aminotransferase (*gabT*) – were knocked out. The *sucA* deletion helped to significantly improve GABA titers by increasing the intracellular glutamate pool and the *gabT* deletion improved GABA production by eliminating GABA consuming pathway in *E. coli*. To improve upstream nonphosphorylative enzyme

activity, different nonphosphorylative operons for D-xylose and L-arabinose were screened to choose the operon with best enzymatic activity. For D-xylose, the synthetic operon combining *C. crescentus* and *B. xenovorans* enzymes performed the best and for L-arabinose the *B. ambifaria* operon gave highest GABA titers. These two operons were subsequently used for all future experiments. To further increase the conversion of glutamate to GABA, the *E. coli* glutamate dehydrogenase (GDH) enzyme was overexpressed on a low-copy plasmid which had a significant impact on GABA titer. Finally, since the strains only used up 7 g/L D-xylose and 2 g/L L-arabinose after 48 hours, the arabinose symporter (AraE) was overexpressed to increase sugar uptake and GABA titers. The overexpression of AraE increased sugar consumption as well as GABA titers but after 48 hours, 10 g/L D-xylose and 16 g/L L-arabinose was still left in the fermentation medium. Glucose was also not completely consumed after 48 hours and the overexpression of transporter AraE reduced the glucose consumption. This low sugar uptake by the strains could be attributed to low pH ~5 of fermentation media (optimum pH for glutamate decarboxylase) which inhibits cell growth and sugar metabolism.

The wild-type glutamate decarboxylases have optimal pH around 4.5 for the decarboxylation of glutamate while *E. coli* has optimum cell growth at pH ~ 7.[432] Thus, to allow GABA production, calcium carbonate was not added to fermentation flasks to maintain lower pH (pH~5) and improve GAD enzyme activity. Due to this discrepancy of optimal pH between cell growth and GAD activity, production of GABA is challenging. One way to tackle this challenge is to use GAD mutant which exhibits activity over a wider pH range (pH~ 6-7) and carry out fermentation at neutral pH to allow optimal cell

growth.[442] Neutral-pH fermentation could also ensure complete utilization of sugars thus improving GABA production significantly.

6.4 Conclusion

This work demonstrated the successful conversion of D-xylose and L-arabinose, important sugars in hemicellulose, to an important bioplastic monomer GABA via efficient nonphosphorylative metabolism. To eliminate competing pathways and improve intracellular glutamate pool, metabolic engineering was used to knockout *sucA* and *gabT* genes. Three nonphosphorylative operons were screened for both D-xylose and L-arabinose to improve upstream enzyme activity and the best operons – synthetic D-xylose operon (*xylB-xylC-xylD-DR64_8250*) and *B. ambifaria* L-arabinose operon (*Bamb_4925-4923-4922-4918*) – were used for further experiments. Overexpression of glutamate dehydrogenase (GDH) enzyme to improve conversion of glutamate to GABA significantly improved GABA titers from both D-xylose and L-arabinose. Finally, to improve sugar uptake, L-arabinose symporter AraE was overexpressed which increased both pentose uptake as well as GABA titer but lowered the yield of GABA from both pentoses. The final D-xylose strain produced 1.5 g/L GABA with a yield of 0.15 g of GABA/g of D-xylose and the final L-arabinose strain produced 1.4 g/L GABA with a yield of 0.34 g of GABA/ g of L-arabinose. Further efforts to screen glutamate decarboxylases with broad pH range can allow fermentations to be carried out at pH 6-7 which can improve cell growth and sugar metabolism and subsequently could increase GABA titers and yields. Simultaneous co-utilization of glucose, D-xylose, and L-

arabinose for GABA production could also be attempted after optimizing fermentation conditions.

6.5 Materials and Methods

6.5.1 Bacterial and growth conditions

The *E. coli* strains used in this study are listed in **Table 6.1**. XL10-Gold was used for cloning and the other strains were derived from the wild-type *E. coli* K-12 strain BW25113.[451] The P1 phage of *sucA* was obtained from the Keio collection.[461] Phage was used to transfect the corresponding strains for construction of targeted knockout strains.[462] All the knockout strains were then transformed with pCP20 plasmid to remove the kanamycin marker. The correct knockouts were verified by colony PCR. Unless otherwise stated, these *E. coli* strains were grown in test tubes at 37 °C in 2×YT rich medium (16 g/l tryptone, 10 g/l yeast extract, and 5 g/l NaCl) supplemented with appropriate antibiotics (100 mg/l ampicillin, 50 mg/l kanamycin, and 100 mg/l spectinomycin). Chemicals used in the study were purchased from Sigma-Aldrich unless otherwise specified.

6.5.2 Plasmids construction

All plasmids used in the study are listed in **Table 6.1**. All primers used in this study were ordered from Eurofins MWG Operon and are listed in **Table 6.2**. PCR reactions were carried out with Q5 High-Fidelity DNA polymerase (*New England Biolabs*) according to the manufacturer's instructions. FastDigest restriction enzymes were purchased from

Thermo Scientific. Sequences of all the plasmids constructed were verified by restriction mappings and DNA sequencing.

The recombinant plasmid pZE-xylA-gadA (pGABA-2) was constructed as follows: *gadA* gene was amplified from *E. coli* BW25113 genomic DNA by PCR using gadA-F and gadA-R as primers. The backbone vector for pZE plasmid and *xylA* fragment were amplified from pZE-xylA plasmid using primers pZE-Xba-F and pZE-Acc-R and xylA-F and xylA-R, respectively. All three fragments were then assembled using NEBuiderHiFi DNA Assembly Master Mix. For construction of pZS-GDH-AraE plasmid (pGABA-9), the fragment pZS-AraE and GDH were obtained from plasmids pM-12 [449] and pGABA-8 respectively by digestion using Acc65I and SphI enzymes and ligated using Quick ligase.

6.5.3 Shake flask fermentation

125-ml conical flasks were autoclaved and dried to perform all small-scale fermentations. The flasks were filled with 5 ml fermentation medium (M9 minimal media supplemented with 5 g/l yeast extract, 20 g/l glucose, 20 g/l D-xylose (or L-arabinose), 5μM coenzyme B12, 100 mg/l ampicillin, 50 mg/l kanamycin, and 100 mg/l spectinomycin). For *sucA* deletion strains GX2, GX-3, GA2, and GA-3, 5 mM succinic acid was supplemented. To start fermentation, 200 μl of overnight cultures incubated in 2×YT medium were transferred into the flasks. After adding 0.5 mM isopropyl-β-D-thiogalactoside (IPTG), the flasks were put into a shaker at 250 rpm and 30 °C, and the fermentation was performed for 48 h. The fermentation products were analyzed by HPLC. Error bars

indicated the SD of the results obtained from three independent experiments (n=3) by picking three different colonies for fermentation.

6.5.4 Metabolite Analysis

Fermentation samples were centrifuged at 13000 rpm for 5 minutes and supernatants were analyzed using an Agilent 1260 Infinity HPLC instrument (Agilent Technology, Palo Alto, CA, USA). Concentration of sugars and acetate were measured using an Aminex HPX-87H ion exclusion column (300 x 7.8 mm) (Bio-Rad, Hercules, California, USA) and a refractive-index detector (RID) maintained at temperatures 35°C and 50°C, respectively. The mobile phase was 5 mM H_2SO_4, with a flow rate of 0.6 ml/min. For the detection of GABA, supernatants were mixed with o-pthaldialdehyde (OPA) (1:10) and analyzed by HPLC using the Zorbax Eclipse plus C18 column (4.6 x 1.50 mm) (Agilent Technology, Palo Alto, CA, USA) and a diode array detector (DAD), operating at 40 °C. The mobile phase for GABA detection consisted of two solvents: solvent A (10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, 5 mM NaN_3, pH 8.2) and solvent B (45% acetonitrile: 45% methanol: 10% water; v: v: v) and a flow rate of 1.5 ml/min. HPLC gradient was set as follows: 0.35 min, 98% A and 2% B; 13.4 min, 43% A and 57% B; 13.5-15.7min, 100% B; 15.8-18 min, 98% A and 2% B. All chemical reagents and GABA standard were purchased from Sigma Aldrich (St. Louis, MO, USA) for determination of metabolite analysis.

Chapter 7

Ongoing and Future Work

7.1 Introduction

The research presented in this dissertation demonstrates the successful use of nonphosphorylative metabolism for the conversion of under-utilized pentose sugars, D-xylose and L-arabinose, into value-added chemicals, 1, 4-butanediol (BDO) and γ-aminobutyric acid (GABA). The work uses several molecular biology techniques for process optimization including metabolic engineering, protein engineering, transporter engineering, growth-based assays, and gene mining. While these results provide a new alternative platform for biosynthesis, they also incite new questions and motivate further research into using different feedstocks for fermentation. Although there has been extensive research and tremendous progress in biosynthesis of both existing as well as

novel chemicals and pharmaceuticals, the feedstocks used in most studies are simple sugars such as glucose, xylose, arabinose, and others. With the surge in global population, there is an urgent need to switch to agricultural and forest residues for the production of bioethanol, other advanced fuels, and commodity chemicals. The advantages of using lignocellulosic biomass include value-added utilization of agricultural residues, reduced greenhouse gas emissions, and improved security of national energy. However, hydrolyzing the cellulose and hemicellulose polymers into hexose and pentose sugars is the biggest bottleneck is production of fuels from lignocellulosic biomass and this limits its industrial applications. In this chapter, we look at one potential feedstock, wheat bran (section 7.2) which has very high content of arabinoxylans and its pretreatment methods, specifically acid hydrolysis. We also present some preliminary results of hydrolysis of wheat bran feedstock for breakdown into sugars (section 7.3).

7.2 Wheat bran: A promising feedstock

Lignocellulosic feedstock is the most abundant inedible biomass with an annual output of around 2×10^{11} metric tons.[381] Lignocellulose is mainly composed of cellulose, hemicellulose, and lignin where cellulose and hemicellulose can be hydrolyzed into fermentable sugars and lignin can be used as combined heat and power source. Common sources of lignocellulose include corn stover, switchgrass, sugar beet pulp, citrus peel, and wheat straw. In order to improve overall economics of fermentation processes, there is increasing pressure to ensure complete utilization of these feedstocks. One such promising feedstock is industrial wheat bran which makes up 14-19% of the wheat grain

and is generated in enormous quantities as a side product of white wheat flour production.[463-464] Currently, wheat bran is used as an animal feed but the emergence of cheaper and more nutritious feed materials makes it necessary to find better uses for wheat bran.[465] It is a great source of polysaccharides such as hemicellulose and cellulose which makes it a very promising low-cost feedstock for the production of renewable fuels and chemicals. The United States Department of Agriculture (USDA) estimates the worldwide wheat production for 2016-17 will be ~745 million metric tons, which corresponds to 100-150 million metric tons of wheat bran.[466]

7.2.1 Composition of wheat bran

Wheat bran consists of the outer coat of the wheat grain and can be separated from the other parts of the wheat kernel by milling. It mainly comprises of non-starch polysaccharides (NSP) (~38%), starch (~19%), protein (~18%), and lignin (~6%), with NSP consisting mainly of ~70% arabinoxylans, ~19% cellulose and ~6% β-(1,3)/β-(1,4)-glucan.[463] Industrial wheat bran is one of the most hemicellulose rich products with arabinoxylan being the main hemicellulose component, which consists of two pentose sugars: arabinose and xylose. The arabinoxylan structure is highly branched with β-(1→4)-xylan backbone with branching α-L-arabinofuranosyl groups. The physical and structural properties of these arabinoxylans is highly dependent on the degree and type of branching as well as on the distribution pattern of substitutions along the xylan backbone.[465, 467]

7.2.2 Hydrolysis of wheat bran

While starch can be hydrolyzed by amylase, enzymatic hydrolysis of bran using hemicellulolytic and cellulolytic enzymes is not sufficient to degrade them to simple sugars which necessitates the use of chemical and physical pretreatment methods. In comparison to enzymatic hydrolysis, chemical hydrolysis [468-470] has lower efficiency, but requires fewer steps and is often quicker, taking hours rather than days for saccharification. In particular, acid hydrolysis can be done directly on raw lignocellulose. In most studies, wheat bran is first subjected to liquefaction and saccharification to hydrolyze the starch fraction and the remaining solid material, called destarched bran or starch-free bran is hydrolyzed in different ways to release simple sugars. Arabinoxylans can be fractionated from destarched bran using several methods including chromatography [467], alkaline extraction/fractionation using hydrogen peroxide[471-472] and potassium hydroxide [473-474], acid hydrolysis [9], enzymatic hydrolysis [9], delignification followed by extraction [475], and hydrothermal treatments.[463] **Table 7.1** summarizes the sugar composition of different arabinoxylans obtained using different fractionation or hydrolysis methods.

Thus, the high percentages of pentose sugars, xylose and arabinose, in wheat bran and the low-cost of feedstock ($100/ton) make it a really great candidate for use in nonphosphorylative pathways we have established.[324] The successful conversion of pentose sugars present in arabinoxylans to value-added chemicals such as 1,4-butanediol (BDO), glutamate, 5-aminolevulinic acid, 4-aminobutyic acid (GABA), can significantly improve process economics and can address environmental concerns. In this chapter,

Table 7.1 Sugar composition and pretreatment methods for different lignocellulosic feedstocks

Starting material	Glucose (%)	Xylose (%)	Arabinose (%)	Pretreatment method	Reference
Destarched wheat bran	24.7	25.9	18.7	Hydrolyzed using 72% sulfuric acid at 30°C for 1 hour followed by dilution to 1M at 100°C for 2 hours	475
Water-soluble hemicellulose (WSH)	17.3	50.4	28.7	Two different arabinoxylans were obtained by fractionation of WSH using chromatography. Acid hydrolysis yielded sugars.	467
	2.4	42.4	45.5		
Starch-free bran (SFB)[a]	1.8	31.3	17.3	Acid hydrolysis of SFB at 130°C using 1% H_2SO_4 for 40 mins.	9
	17.7	19.4	8.1	Non-acid catalyzed pretreatment of SFB at 170°C for 20 min followed by enzymatic hydrolysis at 50°C for 72 h	
	16.4	23.3	13.3	Acid catalyzed pretreatment with 0.2% H_2SO_4 at 160°C for 20 min followed by enzymatic hydrolysis for 72 h	
	4.8	13.4	3.8	Enzymatic hydrolysis	

different acid hydrolysis conditions have been compared to break down the arabinoxylans into xylose and arabinose.

7.3 Materials and methods

Wheat bran was obtained from a local mill company. Concentrated acid hydrolysis was used to hydrolyze the lignocellulosic biomass, following the procedure published by Liu

et al[468]. A measured amount of wheat bran was mixed thoroughly with 40 mL of 75% sulfuric acid and stirred using a stir bar at 50 ^{o}C. After 30 mins, 60 mL of double distilled water (ddH_2O) was added to dilute the sulfuric acid concentration to 30%. This mixture was then stirred on a magnetic stirrer for fixed amount of time at 75 ^{o}C. To reduce the viscosity of suspension, an additional 40 mL of ddH_2O was added to the solution.

The resulting slurry was vacuum filtered using a vacuum pump and a 0.45 µm filter to remove any undissolved residue. The remaining slurry was washed with ddH_2O and filtered through to collect additional sugars left in the residual biomass. To analyze sugar concentration in the hydrolysate, 500 µL of filtrate was neutralized with 1M NaOH until pH reached ~7. This neutralized solution was analyzed with the Agilent 1260 Infinity system (Agilent Technologies, Santa Clara, CA), using the Aminex HPX-87H column (Bio-Rad Laboratories, Inc, Philadelphia, PA) and a refractive-index detector. The sugar standards used were 1 g/L glucose, 1 g/L xylose, and 1 g/L arabinose. The column was kept at 35 ^{o}C, while the detector was kept at 50 ^{o}C. Samples of hydrolysates in the amount of 20.0 µL were injected into the HPLC column for analysis. A mobile phase of 5mM H_2SO_4 was used with a flow rate of 0.6 mL/min.

7.4 Preliminary Results

Wheat bran, a feedstock rich in arabinoxylans, was used as substrate for hydrolysis into fermentable sugars. When 25 g wheat bran was used in the experiment, the suspension was very thick indicating incomplete hydrolysis due to very high feedstock: acid (F: A) ratio. The mass fraction of sugars (g/g of wheat bran) obtained was very low (**Table 7.2**)

Table 7.2 Mass fraction of sugars obtained using different acid hydrolysis conditions for wheat bran

Mass of wheat bran (g)	Hydrolysis time @ 75^{o}C (h)	Mass fraction of sugars (g of sugar/ g of wheat bran)		
		Glucose	Xylose	Arabinose
25	1.5	0.016	0.014	0.009
15	5	0.02	0.009	0.007
5	2	0.03	0.015	0.013

suggesting that lowering the feed: acid (F: A) ratio could help improve the amount of sugars obtained.

To ensure complete hydrolysis and efficient mixing, the mass of feedstock was reduced and F: A ratio was lowered. Both 5 and 15 g of wheat bran was treated using same volume and concentration of sulfuric acid. Hydrolysis was carried out for different times (2h and 5h) to compare the effect of hydrolysis time on sugar concentration and subsequently identify optimal F: A ratio and hydrolysis time. The best condition which gave the highest mass fraction of sugars was 5 g of wheat bran subjected to acid hydrolysis for 2 hours. However, the mass fraction of pentoses, xylose and arabinose, was still very low for purposes of subsequent fermentation (**Table 7.2**).

7.5 Future Work

Wheat bran is produced globally as a by-product of the wheat milling industry and it is an underutilized source of pentose sugars. It is an excellent source of hemicellulosic arabinoxylan which mainly consists of two pentose sugars, xylose and arabinose. In this dissertation, effective utilization of pentose sugars, D-xylose and L-arabinose, has been demonstrated via nonphosphorylative metabolism and these sugars have been efficiently converted to commercially relevant commodity chemicals, 1, 4-butanediol (BDO) and γ-

aminobutyric acid (GABA). However, in order to make this process economic and competitive with other synthesis routes, it is important to use low-cost and more abundant feedstocks such as wheat bran as starting material for nonphosphorylative biosynthesis.

Pretreatment of lignocellulosic biomass is the most challenging step in its utilization for sustainable production of fuels and chemicals. Pretreatment is essential to make the cellulose and hemicellulose polymers more accessible for hydrolysis. It is important to utilize both starch and hemicellulose/cellulose part to increase the production titers and yields of downstream fermentation processes. Starch can be hydrolyzed by amylases but enzymatic hydrolysis of wheat bran using cellulolytic and hemicellulolytic enzymes is not enough and may need the use of chemical and physical methods. The three types of hydrolysis processes used for the breakdown of polymers into fermentable sugars are enzymatic, dilute acid, and concentrated acid hydrolysis. The type of pretreatment and hydrolysis method depends largely on the type of lignocellulosic biomass, specifically the composition of the feedstock.

Future efforts focusing on optimizing pre-treatment of wheat bran, extraction of arabinoxylans, and hydrolysis of arabinoxylans into pentoses are crucial to making it a promising source for pentose sugars. After hydrolysis, the neutralized hydrolysate can be used as a substrate for fermentation to convert the sugars into sustainable chemicals using artificial biosynthesis pathways such as nonphosphorylative pathway. This overall process including hydrolysis of wheat bran and fermentation of the resulting sugars into value-added chemicals could be a very economic process for biosynthesis.

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