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纖維酒精關鍵技術精進開發及工程放大可行性研究

Development of core technology and its scale-up feasibility for cellulosic ethanol

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目　錄

中文摘要

為了因應氣候變化和自然資源遞減的全球性問題，各國於生質能源與生質化學品的開發都投入了顯著努力，以減低我們對石化的依賴。經由代謝工程的策略運用，許多以可再生原料為基礎的生產系統已被成功建立，並有效的使用於各種高價值化學品的生物合成上，如生物塑料單體和燃料替代品。本計畫的目標為運用代謝工程方法，改造模型微生物大腸桿菌，使其生產 1,3 -丁二烯的前驅物 2,3 -丁二醇和腐胺 (putrescine)。除了應用在合成橡膠工業，1,3 -丁二烯和其前驅物 2,3 -丁二醇與腐胺在多種生物塑料的製程上，也是非常重要的單體。在本研究中，我們將繞過天然代謝途徑，設計新穎的合成路線來進行腐胺的生產。我們將探討大腸桿菌合成 2,3 -丁二醇作為唯一發酵物的先天能力。我們也將調查在微生物系統內直接生物合成 1,3 -丁二烯的可能性。在本年度中，我們進行了完整的文獻及資料收尋，並有系統地在大腸桿菌中組裝異源的 2,3 -丁二醇合成途徑。另外，我們也利用合理的設計來重新佈線細胞的自然代謝，以提高生產平台的效率。在腐胺方面，我們已成功地將大腸桿菌中天然的腐胺代謝途徑移除。此菌株將有助於我們測試和優化之後所設計的非天然腐胺合成途徑。

Abstract

Development of biobased chemical production system has been an emerging thrust to address the global concerns over climate change and diminishing natural resources. Utilization of metabolic engineering strategies has enabled the biosynthesis of a diverse pool of commodity and specialty chemicals, such as bioplastic monomers and fuel substitutes, from renewable feedstock. This work aims to biologically produce the 1,3-butadiene precursors 2,3-butanediol and putrescine by metabolically engineering the model microorganism *Escherichia coli*. With important application in the synthetic rubber industry, 1,3-butadiene along with 2,3-butanediol and putrescine are essential monomers in a wide variety of bioplastics. Design of novel synthetic pathways for the generation of putrescine, which bypasses its native metabolic route, will be performed in this study. We will explore the innate capability of *E. coli* to synthesize 2,3-butanediol as the sole fermentation product. We will also investigate the possibility of directly biosynthesize 1,3-butadiene in the microbial system. In this year, we conducted comprehensive literature search on the natural and recombinant production of 2,3-butanediol. We systematically assembled the heterologous 2,3-butanediol pathway in *E. coli* and rationally re-wired cell's natural metabolism to improve production efficiency. On the end of engineering synthetic putrescine production, we successfully deleted the native putrescine biosynthetic pathway in *E. coli* to obtain the putrescine auxotroph.

This strain can be applied to test the efficiency of our non-natural putrescine biosynthesis based on the designed pathway and to improve the synthetic pathway via random mutagenesis and rational-design approaches.

壹、計畫緣起與目的

Concerns over environmental health and limited fossil fuel supply have sparked numerous efforts towards the biosynthesis of valuable chemicals from renewable resources. This includes the microbial production of many naturally-occurring or non-natural bio-monomer building blocks such as succinic acid, lactic acid, cadaverine, 1,3-propanediol and isoprene, which play essential role in the industrial synthesis of polyamide, polyester and polyvinyl material [1-5]. Currently, a huge portion of conventional plastics are derived from non-renewable petroleum and natural gas feedstock. Approximately 8% of the world's total oil consumption has been applied towards the global plastic synthesis [6], with the annual production capacity exceeding 265 million tons in 2010 and an expected growth surpassing 297 million tons by 2015 [7]. Nevertheless, fluctuation of fossil fuel supply has led to increasing interest in the biosynthesis of plastic monomers from sustainable raw material. Development of bioplastics production using engineered microorganism offers an alternative to the traditional petroleum-based industry and the possibility of constructing a carbon-neutral process. The global demand for bioplastics is predicted to reach 1 million tons per year, which represents a $3 billion dollar market [7]. Thus, it is of great interest for us to establish an efficient microbial platform for the biosynthesis of important bio-monomers 2,3-butanediol (2,3-BDO) and putrescine, which can be applied to the industrial production of 1,3-butadiene and other commodity polymers.

1,3-Butadiene, an important industrial chemical used as a monomer in the production of synthetic rubber, has been synthesized largely by steam cracking processes. Recent advancement in the renewable generation of short chain alcohols and diols has coupled 1,3-butadiene synthesis to biological systems via dehydration of ethanol or 1,4-butanediol produced from biomass-derived feedstock. Direct biosynthesis of 1,3-butadiene by recombinant microorganism via the formation of 5-hydroxypent-3-enoate or crotonol intermediate has been described very recently in patent applications by the advanced biochemical company Codexis [8, 9]. On the other hand, 2,3-BDO and putrescine can also act as chemical precursor towards the synthesis of 1,3-butadiene [10]. This work will focus primarily on the biosynthesis of 2,3-BDO and putrescine in modified *Escherichia coli* and the pathway optimization via mutagenesis and metabolic engineering approaches. In addition to being 1,3-butadiene precursor, 2,3-BDO can be used as antifreeze agent, and its derivatives such as 2-butanone can be applied as an effective liquid fuel additive and lacquer solvent [11, 12]. Industrial biosynthesis of 2,3-BDO by the native microbial producers *Bacillus* and *Klebsiella* (Table 1) could be dated back as far as the World War II era [11]. In contrast, no natural over-producer of putrescine has been isolated to date. Large-scale production of putrescine in engineered hosts has only been recently reported in *E. coli* [13] and *Corynebacterium glutamicum* [14]. Currently, putrescine is used in the formation of polyamide Nylon-4,6 by reacting with adipic acid, commercialized under the

trade name Stanyl by the plastics company DSM [7]. Development of efficient biosynthetic system for the production of putrescine and 2,3-BDO from renewable feedstock should add economic and sustainability benefits to the existing process.

2,3-BDO is synthesized as a natural fermentation product by a few different bacteria, such as *Klebsiella pneumoniae*, *Bacillus polymyxa*, *Enterobacter aerogenes*, and *Serratia marcescens* [11]. Biosynthesis of 2,3-BDO initiates with the condensation of two pyruvate molecules to yield acetolactate, followed by the formation of acetoin or diacetyl upon decarboxylation of acetolactate (Figure 1). In the case of ALDC-catalyzed decarboxylation, the *R*-enantiomer of acetoin is generated. On the other hand, when the decarboxylation of acetolactate occurs spontaneously, diacetyl is formed, which then can be converted into (*S*)-acetoin by DAR (diacetyl reductase). When the keto-group of acetoin is reduced to a hydroxyl by stereospecific sADH (secondary alcohol dehydrogenase), the reaction generates a second stereo center with either the (*S*) or the (*R*)-configuration depending on the enzyme stereospecificity (Figure 1). As a result, the pathway can form three different stereo isomers of 2,3-BDO – the (*R,R*)-, *meso*-, and (*S,S*)-forms. In general, 2,3-BDO is naturally produced as a mixture of two stereo isomers by the native microorganisms (Table 1), in which the isomer ratio can vary significantly depending on the microbial species and fermentation condition [11, 12, 15]. Biosynthesis of enantiomerically pure 2,3-BDO from the native bacteria is difficult due to the existence of multiple pathways and stereospecific sADH that can lead to the formation of (*R,R*)-, *meso*-,

and (*S*,*S*)-2,3-BDO. Recent advances in genomic sequencing and expansion of genetic tool box has allowed metabolic engineering of heterologous hosts to produce enantiomerically pure 2,3-BDO (Table 1). Greater than 99% purity of (*R*,*R*)-2,3-BDO and *meso*-2,3-BDO was achieved in engineered *E. coli* strains by characterizing stereospecificity of various sADH and eliminating competing pathways [16, 17]. Production of enantiomerically pure (*S*,*S*)-2,3-BDO was also established in recombinant *E. coli* by extracellular feeding of the precursor diacetyl [18]. Recently, biosynthesis of (*R*,*R*)- and *meso*-2,3-BDO was developed in cyanobacterium *Synechococcus elongatus* PCC7942 upon introduction of various stereospecific and NADPH-dependent ALDC and sADH [19]. In this work, we will investigate the possibility of producing enantiomerically pure 2,3-BDO by recruiting endogenous enzyme homologues in *E. coli* and exploring novel functionality via random mutagenesis and anaerobic growth selection.

Putrescine, on the other hand, is usually synthesized in minute quantity by microorganisms as an amino acid degradation product. Biosynthesis of putrescine can be achieved via two different routes, one involves L-arginine as the intermediate and another one stems from L-ornithine (Figure 2). Both metabolic routes initiate from L-glutamate, which through a series of reaction and transformation generates L-ornithine via enzymes encoded by the *arg* operon. The L-ornithine is then converted into putrescine by its direct decarboxylation or via the formation and subsequent degradation of L-arginine. Enhancement of metabolic flux towards

putrescine production has been achieved in engineered *E. coli* by deregulating L-ornithine biosynthesis on the transcriptional, translational, and allosteric level and overexpressing specific transporter [13]. Coupled with deletion of competing pathways, the final strain accumulated significant level of putrescine from glucose in fed-batch cultures. Metabolic engineering of *Corynebacterium glutamicum* was also performed to over-produce putrescine from the L-ornithine and L-arginine pathway [14]. In both heterologous production systems, the L-ornithine pathway demonstrated dramatically better performance in putrescine biosynthesis compared to the L-arginine pathway, which could be attributed to the inhibitory effect of the byproduct urea. Since both L-ornithine and L-arginine pathways are heavily regulated in the systems level, construction of a novel synthetic pathway which bypasses the native metabolic routes may be beneficial for putrescine overproduction and will offer an alternative to the existing method. Here, we aim to design and assemble a non-natural pathway for putrescine biosynthesis using *E. coli* as the platform host. Mutagenesis-based evolution strategy will be employed to engineer the pathway enzymes and streamline the carbon flux.

In this work, anaerobic growth selection scheme based on NADH consumption will be used to characterize endogenous production of 2,3-BDO and engineer novel enzyme functionality for putrescine biosynthesis via non-natural pathway. Evolution of enzymes and pathways based on growth selection has been well-developed and widely-employed towards the optimization of production systems. It offers the advantage of mutant enrichment

by serial cultivation and the ability to select for a large pool of mutant library, which is generally not possible in color screenings. In order for the evolution strategy to be successful, a stable and efficient selection scheme must be established according to the intrinsic characteristics of the system. There exist a few widely-used selection platforms for metabolic engineering purposes, including essential metabolite auxotroph [20], amino acid analogues [21], and growth retardation caused by redox imbalance [22]. All of them operate under the identical principle of which cell growth restores when the imposed inhibitory stress is alleviated or removed by beneficial mutations on the target. Anaerobic growth selection based on redox balance is often used in directed evolution when no reporter system is readily available and when the pathway is non-related to essential metabolites required for growth therefore cannot use nutrient auxotroph as selection background. It operates under the principle where recycling of NADH by mixed-acid fermentation is essential for cell growth anaerobically (Figure 3). Under anaerobic condition, respiration is hindered by the lack of electron acceptor and glycolysis becomes the only route for ATP production, which is necessary for cell survival. Regeneration of the essential glycolytic cofactor NAD^+ by the production of lactate, ethanol and succinate is thus important to maintain cell growth (Figure 3). When the mixed-acid fermentation pathways are deleted, NADH accumulates and glycolysis stalls anaerobically due to the lack of available NAD^+. As a result, ATP synthesis stops and cell dies. Growth of such strain can only be rescued when a native or synthetic pathway which consumes NADH is introduced to

alleviate the NAD^+ bottleneck (Figure 3). Coupling of the desired reaction(s) as the primary NAD^+ regeneration route to the growth rescue phenotype allows the evolution of pathway efficiency, alternative substrate utilization, change of cofactor specificity, and greater robustness. This anaerobic growth selection scheme based on NADH consumption has been successfully applied to the optimization of various production systems such as succinate [22], lactate [23], ethanol [24], and 1-butanol [25] in *E. coli*. Here, we will couple the NADH consumption to the reactions found in the 2,3-BDO and putrescine biosynthetic pathway. Random mutagenesis will then be performed to increase productivity and engineer desired traits by growth enrichment.

貳、研究方法與過程

Literature survey of recombinant 2,3-BDO production

First, we set out to survey the literature about producing 2,3-BDO by recombinant microorganisms in recent years. Scientists attempted to improve 2,3-BDO productivity of these non-native species by metabolic engineering technology. There are usually three strategies: (1) substrate alternation: the relatively high cost of conventional starch or sugar substrates has been identified as a major factor affecting the economic viability of 2,3-BDO fermentation. Therefore, using cheaper alternative biomass-derived sugars for 2,3-BDO production was pursued; (2) knock-out genes: knock-out genes of mixed acid pathway can focus carbon flux into 2,3-BDO synthetic pathway and improve

titer and productivity; (3) overexpress genes: put heterologous genes of 2,3-BDO synthetic pathway in non-native species and inducing these genes expression. A list of the recombinant 2,3-BDO production in *E. coli*, yeast, cyanobacteria, and other non-native hosts is shown in Table 1.

For pathogenic reason, benign microbes such as *E. coli*, *S. cerevisiae* and cyanobacteria have frequently been used as a heterologous host engineered for 2,3-BDO production. In 2014, Nakashima et al. describes a novel method, BICES (biomass-inducible chromosome-based expression system), for producing 2,3-BDO from glucose and xylose in *E. coli*. Foreign genes of 2,3-BDO pathway are knocked into the chromosome, and their expression is induced with xylose that is present in most biomass feed stock. They also deleted *pflB*, *ldhA*, *adhE* and *Pta-AckA* genes to reduce production of lactate, acetate and ethanol in *E. coli*. Metabolic engineering stain was cultured in the M9+YE medium containing 70 g/L glucose and 10 g/L xylose. After 72h, 32 g/L glucose and 8 g/L xylose was added. The maximal concentrations of R,S-form 2,3-BDO reached 54 g/L .

S. cerevisiae is a popular stain to use in consolidated bioprocess (CBP, hydrolysis and ethanol fermentation into one step) for bioethanol production. In 2,3-BDO production, Kim et al. (2014) wanted *S. cerevisiae* to produce 2,3-BDO from xylose that is abundant in lignocellulosic hydrolysate and make the production of 2,3-BDO more sustainable and economical. The XYL1, XYL2, and XYL3 genes coding for xylose assimilating enzymes derived from *S. stipitis* were introduced into the strain to

enable xylose utilization. Additionally, the *alsS* and *alsD* genes from *B. subtilis* and endogenous BDH1 gene were overexpressed to increase 2,3-BDO production from xylose. As a result, the resulting strain produced 20.7 g/L of (R, R) 2,3-BDO from xylose with a yield of 0.27g 2,3-BDO/g xylose. The titer of 2,3-BDO from xylose increased up to 43.6 g/L under a fed-batch fermentation.

Atmospheric CO_2 levels are increasing since the beginning of the industrial revolution. This increase adds to the greenhouse effect and thus to climate change. Cyanobacteria fix CO_2 more efficiently than plants, and can be engineered to produce carbon feed stocks useful for making plastics, solvents, and medicines. Oliver et al. (2013) reported a 2,3-BDO synthetic pathway coupled with cofactor NADPH in *S. elongatus* sp. strain PCC7942. The 2,3-BDO production was observed with the genes encoding three enzymes (*als*S from *B. subtilis*, *als*D from *E. aerogenes*, and *adh* from *C. beijerinckii*) integrated into the cyanobacterial genome. The titer reached 2.38 g/L in BG-11 medium.

Establish 2,3-BDO biosynthetic pathway based on existing knowledge

We first established the 2,3-BDO biosynthetic pathways in *E. coli* using glucose. Existing knowledge on the natural and synthetic pathways leading to the production of (*R,R*)-, *meso*-, and (*S,S*)-2,3-BDO is the initial basis for constructing the microbial production system. We bioprospected for ALS, ALDC, DAR and

sADH homologues based on literature survey and protein blast, and assessed their catalytic efficiency and substrate specificity towards our desired precursor, which is essential for achieving high efficiency and purity of target product formation. To suit the cofactor abundance in *E. coli* under anaerobic condition, NADH-dependent DAR and sADH was preferentially recruited in the pathway assembly. Optimized expression of heterologous proteins is another important aspect in pathway engineering, and was achieved by modifying codon usage of the foreign genes. In some cases, *in vivo* feeding of pathway intermediates was used to further determine the most well-expressed and active enzyme combinations. The coding genes corresponding to each selected enzyme was then assembled into synthetic operon and cloned into *E. coli* by plasmid transformation or genomic integration.

Characterize 2,3-BDO production in *E. coli* and the resulting growth restoration

Production of 2,3-BDO was performed in the *E. coli* strain of which the native fermentation pathways were deleted (Figure 3). Previously, it was shown that when the mixed-acid fermentation reactions (lactate, ethanol, and succinate) are removed by disrupting the corresponding genes *ldhA*, *adhE*, and *frdBC* in the genome, the resulting *E. coli* strain lost its ability to grow under anaerobic condition due to the accumulation of NADH intracellularly. Growth of such strain can only be restored by the introduction of a NADH-consuming pathway, which regenerates the NAD^+ required in glycolysis. In this case, the synthetic 2,3-BDO pathway was

introduced as the only NADH outlet. A bulk of carbon flux should be directed to the 2,3-BDO biosynthesis as it is the only way for cells to survive anaerobically. This system can therefore be applied to identify and select for the best-performing enzyme combination. Production titer of 2,3-BDO and cell growth under anaerobic condition was measured to assess the efficiency of each assembled pathway and determine the most suitable one for further use. The extent of growth restoration will also be analyzed to see if there exists room for improvement.

Create an *E.coli* putrescine auxotroph for the selection of non-natural pathway

In this project, we will attempt at designing a non-natural synthetic pathway to produce putrescine in *E. coli* with chemistry and reaction sequence different from existing methods. We will survey the literature and pathway database, and identify unique reaction chemistry and enzyme combinations which can be applied to produce our desired chemical or its metabolic precursor. Protein engineering via random mutagenesis will be performed to modify the substrate specificity of selected enzymes and enable them to catalyze unique reactions that are part of the novel synthetic pathway.

In order to test and improve the efficiency of the non-natural putrescine pathway, an *E. coli* putrescine auxotroph was first created by deletion of the *speA* and *speC* gene from the host's genome. The resulting double mutant will lose its ability to synthesize putrescine from L-ornithine or L-arginine (Figure 2),

thus opening the possibility of evolving any non-natural putrescine biosynthesis pathway using this strain. In this case, pathway engineering on the enzyme or systems level will be coupled to the putrescine auxotroph, in which growth restoration should be observed if the synthetic putrescine pathway functions well. Before the complete pathway can be engineered and assembled, we will also try feeding of pathway intermediates to debug bottleneck enzymatic step and optimize the target enzymes one by one with a bottom-up approach. Once desired activity for each step of the synthetic pathway is verified, we will examine growth restoration of the putrescine auxotroph upon introduction of the complete non-natural pathway and further improve its efficiency by directed evolution. Success of this work should ensure solid patent position on putrescine biosynthesis based on the designed non-natural pathway.

參、主要發現與結論

Created an *E. coli* strain with 2,3-BDO as its only fermentation route

In order to create an *E. coli* strain that can only secret 2,3-BDO as its fermentation product, we first transformed the two knock-out mutants with plasmids harboring the 2,3-BDO biosynthetic genes. The two deletion mutants (Δ*ldhA* Δ*adhE* Δ*frdBC* and Δ*ldhA* Δ*adhE* Δ*frdBC* Δ*pta*), which had their mixed-acid fermentation reactions removed, lost their ability to synthesize the natural fermentation products lactate, ethanol and

succinate, and cannot grow under anaerobic condition due to the accumulation of NADH. The inability to grow anaerobically of the two mutants was confirmed in Luria Broth (LB) with glucose supplementation as we can see from the clear culture tube after 24 h incubation in the anaerobic bag (Figure 4). Plasmids harboring the gene *alsS* (encoding acetolactate synthase) from *Bacillus subtilis*, *alsD* (encoding acetolactate decarboxylase) from *Bacillus subtiluis*, and *sADH* (encoding secondary alcohol dehydrogenase) from *Bacillus subtilis* or *Klebsiella pneumonia* were transformed into the mixed-acid deletion mutants. The NADH-dependent sADH from *B. subtilis* and *K. pneumonia* were chosen because NADH, rather than NADPH, is the cofactor synthesized by glycolysis and the cofactor accumulating anaerobically without the mixed-acid fermentation pathways. By over-expressing the NADH-dependent sADH along with AlsS and AlsD, the pathway should help recycle the excess NADH in the mutant strains and restore their anaerobic growth.

Production test of 2,3-BDO in *E. coli*

Upon transformation of the *E. coli* mutants with 2,3-BDO biosynthetic enzymes AlsS, AlsD, and the NADH-dependent sADH, we performed anaerobic growth test and production test on the selected transformed colonies. Overnight cultures of the transformants were used to inoculate (0.2%) fresh LB glucose media with addition of IPTG and antibiotics in the anaerobic growth test. The inoculated culture was then placed in the anaerobic bag system with O_2-absorbing catalyst pouch purchased from BD and allowed to grow at 37^{o}C. On the other hand, production tests were

performed using similar procedure. Overnight cultures of the transformants were used to inoculate (1%) fresh TB media and M9 (with 0.5% yeast extract) media supplemented with 2% glucose. The inoculated cultures were allowed to grow at 37^{o}C to mid-log phase then induced with 0.5mM IPTG and placed back to the incubator. Production tests were performed under both anaerobic and micro-aerobic conditions. The anaerobic growth test and the production test cultures were allowed to grow/produce at 37^{o}C for 48 h before sampling. Overexpression of the essential enzymes AlsS, AlsD, and sADH from *Bacillus subtilis* resulted in 2,3-BDO production in both wild type *E. coli* and the mutant strain, reaching above 3 g/L in 2 days with a yield around 0.3 g/g of glucose as shown in [16]. This productivity and yield sets a benchmark for our further testing using the same set of enzyme combination.

Deleted the putrescine biosynthetic gene *speC*

In order to test the efficiency of the non-natural putrescine pathway, we first aim to create an *E. coli* putrescine auxotroph by deleting its biosynthetic gene *spec* and *speA*. The knock-out was performed using Datsenko and Wanner's method [26] where two primers with homologous sequence flanking the upstream and downstream of the target gene were used to amplify the antibiotics gene-cassette using polymerase chain reaction (PCR). The resulting PCR fragment was then transformed into the recipient strain where homologous recombination occurs to replace the target chromosome gene upon overexpression of the λ-red recombinase. The antibiotics gene-cassette can then be removed by expressing the

FLP recombinase, leaving a FLP scar on the genome. Using the method, we successfully deleted the *speA* gene from the chromosome of wild type *E. coli* strain BW25113. The verification PCR was performed on different colonies using primer sets binding to the upstream and downstream of *speA* gene and primer sets binding inside of the kanamycin cassette. As shown in Figure 5, the size of the colony PCR products confirmed the deletion of *speA* and also the removal of the kanamycin gene from the genome.

As one can see from Figure 2, *E. coli* can synthesize putrescine natively upon conversion of L-ornithine or L-arginine by the enzyme products of *speC* or *speA*. Therefore, in order to create an *E. coli* putrescine auxotroph, we must delete both *speC* and *speA* together. We are currently working on creating this double mutant by carrying one more round of gene knock-out to remove *speC* based on the Δ*speA* strain which we created.

肆、參考文獻

1. Lee JW, Kim HU, Choi S, Yi J, Lee SY: **Microbial production of building block chemicals and polymers**. *Current opinion in biotechnology* 2011, **22**(6):758-767.
2. Lee JW, Kim TY, Jang YS, Choi S, Lee SY: **Systems metabolic engineering for chemicals and materials**. *Trends in biotechnology* 2011, **29**(8):370-378.
3. Lee JW, Na D, Park JM, Lee J, Choi S, Lee SY: **Systems metabolic engineering of microorganisms for natural and non-natural chemicals**. *Nature chemical biology* 2012, **8**(6):536-546.
4. Erickson B, Nelson, Winters P: **Perspective on opportunities in industrial biotechnology in renewable chemicals**. *Biotechnology journal* 2012, **7**(2):176-185.
5. Curran KA, Alper HS: **Expanding the chemical palate of cells by combining systems biology and metabolic engineering**. *Metabolic engineering* 2012, **14**(4):289-297.
6. Thompson RC, Swan SH, Moore CJ, vom Saal FS: **Our plastic age**. *Philos T R Soc B* 2009, **364**(1526):1973-1976.
7. Adkins J, Pugh S, McKenna R, Nielsen DR: **Engineering microbial chemical factories to produce renewable "biomonomers"**. *Frontiers in microbiology* 2012, **3**:313.
8. COPE GA, Clark L: **Recombinant host cells and processes for producing 1,3-butadiene through a 5-hydroxypent-3-enoate intermediate**. In. U.S.A.; 2013.
9. Davis SC, Agard NJ, Grate JH: **Recombinant host cells**

and processes for producing 1,3-butadiene through a crotonol intermediate. In. U.S.A.; 2013.

10. Winfield ME: **The Catalytic Dehydration of 2,3-Butanediol to Butadiene .2. Adsorption Equilibria**. *Aust J Sci Res Ser A* 1950, **3**(2):290-305.
11. Ji XJ, Huang H, Ouyang PK: **Microbial 2,3-butanediol production: A state-of-the-art review**. *Biotechnol Adv* 2011, **29**(3):351-364.
12. Celinska E, Grajek W: **Biotechnological production of 2,3-butanediol-Current state and prospects**. *Biotechnol Adv* 2009, **27**(6):715-725.
13. Qian ZG, Xia XX, Lee SY: **Metabolic Engineering of Escherichia coli for the Production of Putrescine: A Four Carbon Diamine**. *Biotechnol Bioeng* 2009, **104**(4):651-662.
14. Schneider J, Wendisch VF: **Putrescine production by engineered Corynebacterium glutamicum**. *Appl Microbiol Biot* 2010, **88**(4):859-868.
15. Syu MJ: **Biological production of 2,3-butanediol**. *Appl Microbiol Biot* 2001, **55**(1):10-18.
16. Yan YJ, Lee CC, Liao JC: **Enantioselective synthesis of pure (R,R)-2,3-butanediol in Escherichia coli with stereospecific secondary alcohol dehydrogenases**. *Org Biomol Chem* 2009, **7**(19):3914-3917.
17. Nielsen DR, Yoon SH, Yuan CJ, Prather KLJ: **Metabolic engineering of acetoin and meso-2,3-butanediol biosynthesis in E. coli**. *Biotechnology journal* 2010,

5(3):274-284.

18. Ui S, Takusagawa Y, Sato T, Ohtsuki T, Mimura A, Ohkuma M, Kudo T: **Production of L-2,3-butanediol by a new pathway constructed in Escherichia coli**. *Lett Appl Microbiol* 2004, **39**(6):533-537.
19. Oliver JWK, Machado IMP, Yoneda H, Atsumi S: **Cyanobacterial conversion of carbon dioxide to 2,3-butanediol**. *P Natl Acad Sci USA* 2013, **110**(4):1249-1254.
20. Atsumi S, Liao JC: **Directed evolution of Methanococcus jannaschii citramalate synthase for biosynthesis of 1-propanol and 1-butanol by Escherichia coli**. *Appl Environ Microbiol* 2008, **74**(24):7802-7808.
21. Connor MR, Cann AF, Liao JC: **3-Methyl-1-butanol production in Escherichia coli: random mutagenesis and two-phase fermentation**. *Appl Microbiol Biot* 2010, **86**(4):1155-1164.
22. Jantama K, Haupt MJ, Svoronos SA, Zhang X, Moore JC, Shanmugam KT, Ingram LO: **Combining metabolic engineering and metabolic evolution to develop nonrecombinant strains of Escherichia coli C that produce succinate and malate**. *Biotechnol Bioeng* 2008, **99**(5):1140-1153.
23. Zhou S, Shanmugam KT, Yomano LP, Grabar TB, Ingram LO: **Fermentation of 12% (w/v) glucose to 1.2 M lactate by Escherichia coli strain SZ194 using mineral salts medium**. *Biotechnol Lett* 2006, **28**(9):663-670.

24. Yomano LP, York SW, Zhou S, Shanmugam KT, Ingram LO: **Re-engineering Escherichia coli for ethanol production**. *Biotechnol Lett* 2008, **30**(12):2097-2103.

25. Shen CR, Lan EI, Dekishima Y, Baez A, Cho KM, Liao JC: **Driving Forces Enable High-Titer Anaerobic 1-Butanol Synthesis in Escherichia coli**. *Applied and Environmental Microbiology* 2011, **77**(9):2905-2915.

26. Datsenko KA, Wanner BL: **One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products**. *Proc Natl Acad Sci U S A* 2000, **97**(12):6640-6645.

Table 1: Literature survey on the current art of 2,3-BDO production by recombinant microorganisms

Strain	Engineering strategy			Culture condition		Titer(g/L)	Reference
	Knockout gene	Expression gene	Cofactor	Carbon sources	O_2 supply		
E. coli MG1655	PflB LdhA Pta-Ack AdhE	alsSD (*B. subtilis*) butAB (L.l) BDH1 (S.c) bdhA (*B. subtilis*)	NADH	glucose+xylose	microaerobic	54 g/L (meso)	Nakashima et al. (2014)
E. coli BL21(DE3)		*B. subtilis* *B.licheniformis* *K. pneumonia* *S. marcescens* *E. cloacae*	NADH	M9+ glucose		2.86 g/L (R,R) 5.86g/L (R,R+meso) 0.26 g/L (S,S) 6.42g/L (S,S) 12.77g/L (meso)	Xu et al. (2014)
E. coli		gdh (*B. subtilis*) fdh (*C. boidinii*) bdh(*E. cloacae*)	NADH	diacetyl		17.8g/L (S,S) fermentor	Wang et al. (2013)
E. coli K12 MG1655	frdA, ldhA, pta, adhE,	bud ABC operon (*E. aerogenes*)	NADH	M9+ mannitol or mannitol/glucose algal hydrolysate		6.5 g/L (meso)	Mazumdar et al.(2013)
E. coli	ackA poxB	alsS (*B. subtilis*) alsD (*B. subtilis*) adh (*C. beijerinckii*)	NADPH	M9+ glycerol+YE	microaerobic	9.54g/l (R,R)	Shen et al. (2012)

Table 1 continue

Strain	Engineering strategy			Culture condition		Titer(g/L)	Reference
	Knockout gene	Expression gene	Cofactor	Carbon sources	O_2 supply		
E. coli	poxB, ldhA, ackA, pta	alsS, alsD(*B. subtilis*) budC(*K. peumoniae*) ced3A(*S. degradans*)	NADH	cellodextrin		4.2 g/L (meso)	Shin et al. (2012)
E. coli W3110		budA, budC, (*K. pneumonia*)		minimal med.+ glucose	aerobic anaerobic	14.3 g/L (meso) 1.72 g/L	Lee et al. (2012)
E. coli		bdh (*E. cloacae*)	NADH	glucose:DA=1:1		12g/L(S,S)	Wang et al. (2012)
E. coli	ldhA, pta, adhE, poxB	alsS, alsD, (*B. subtilis*) budC (*K. pneumonia*)	NADH	glucose	microaerobic	14.50g/L(meso)	Li et al. (2010)
E. coli	ldhA, ilvC poxB, pps, aceEF, lpdA, pflB, tdcE	ilvBN, ilvIH(*E. coli*) ilvGM(*S.typhimurium*) aldBC (*L. lactis*) adc(*C. acetobuylicum*) bdh1(*S. cerevisiae*) butBC(*L. lactis*)	NADH	LB+ glucose	aerobic+ anaerobic	1.12g/L (meso)	Nielsen et al. (2010)
E. coli	adhE, ldhA, frdBC, fnr, pta, pflB	bdhA (*B. subtilis*) budC (*K. pneumonia*) adh (*T. brockii*) adh (*C. beijerinckii*)	NADH NADH NADPH NADPH	M9+ glucose	microaerobic	5.8 g/L(R,R) <0.06g/L(R,R) 6.1 g/L(R,R) 5.1 g/L(R,R)	Yan et al. (2009)

Table 1 continue

Strain	Engineering strategy			Culture condition		Titer(g/L)	Reference
	Knockout gene	Expression gene	Cofactor	Carbon sources	O_2 supply		
S. cerevisiae	PDC	XYL1, XYL2, XYL3 alsS , alsD (*B. subtilis*) BDH1(S.c)	NADH	YP+xylose	microaerobic	8.9 g/L (R, R)	Kim et al. (2014)
S. cerevisiae	PDC	MTH1(point mutation) alsS, alsD(*B. subtilis*) BDH1 (S.c)		YP med.+glucose	microaerobic aerobic	6.4 g/L	Kim et al. (2013)
S. cerevisiae	ADH1 ADH3 ADH5	alsS (*B. subtilis*) budA (*E. aerogenes*) budC(*E. aerogenes*)	NADH	glucose	microaerobic	2.29 g/L	Ng et al. (2012)

Table 1 continue

Strain	Engineering strategy			Culture condition		Titer(g/L)	Reference
	Knockout gene	Expression gene	Cofactor	Carbon sources	O_2 supply		
S. elongates PCC7942		alsS (*B. subtilis*) alsD (*E. aerogenes*) adh (*C. beijerinckii*)	NADPH	BG-11	aerobic	0.496 g/L	Oliver et al. (2013)
Synechocystis PCC6803		1.als(*E. faecalis*) aldc(*L. lactis*) butA(*L. lactis*) 2. als(*L. lactis*) aldc (*B.brevis*) ar(*Enterobacter*) 3. als(*L. lactis*) aldc (*B. brevis*) ar(*L. lactis*NCW1)	1.NADH 2.NADH 3.NADPH	BG11+CAPSO	aerobic	0.43 g/L (meso)	Savakis et al. (2013)
S. elongatus		alsS (*B. subtilis*) alsD(6 spices) adh (sADH 3 spices)	NADPH	BG-11		2.38 g/L(R,R)	Oliver et al. (2013)
Clostridium sp	pta, ack spo0A, spo0J	fdh, pfl als, ald, adh		Syngas(CO/H_2) or (CO_2/H_2)		9.19 g/L (meso)	Tyurin and Kiriukhin(2013)
B. Subtilis RB03		alsSD (*B. subtilis*) bdhA		glucose	microaerobic	6.1 g/L (meso)	Biswas et al. (2012)
L. lactis	ldhB-ldhX adhE, mtlF	mtlD+mtlP als+butA(*L. lactis*)	NADH	glucose	anaerobic	0.15mol.mol of glucose^{-1}	Gaspar et al. (2011)

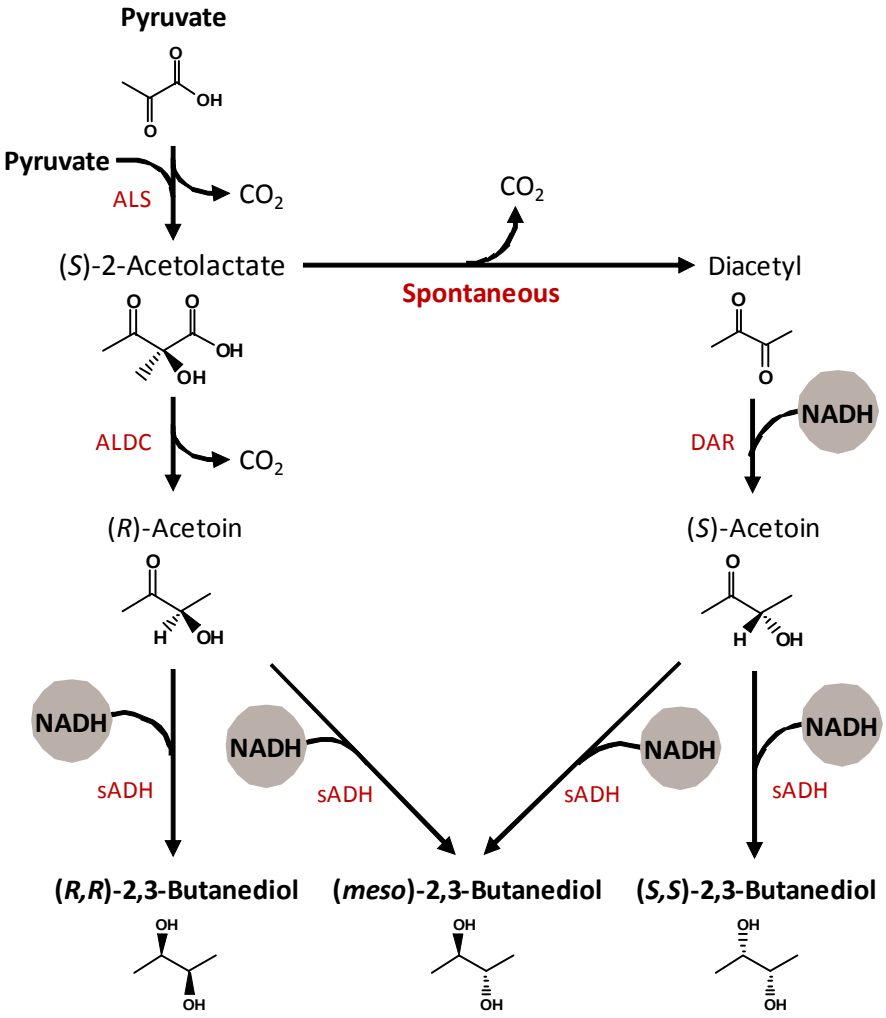

Figure 1: Biosynthetic pathway for the production of various isomeric forms of 2,3-BDO. ALS, acetolactate synthase; ALDC, acetolactate decarboxylase; DAR, diacetyl reductase; sADH, secondary alcohol dehydrogenase

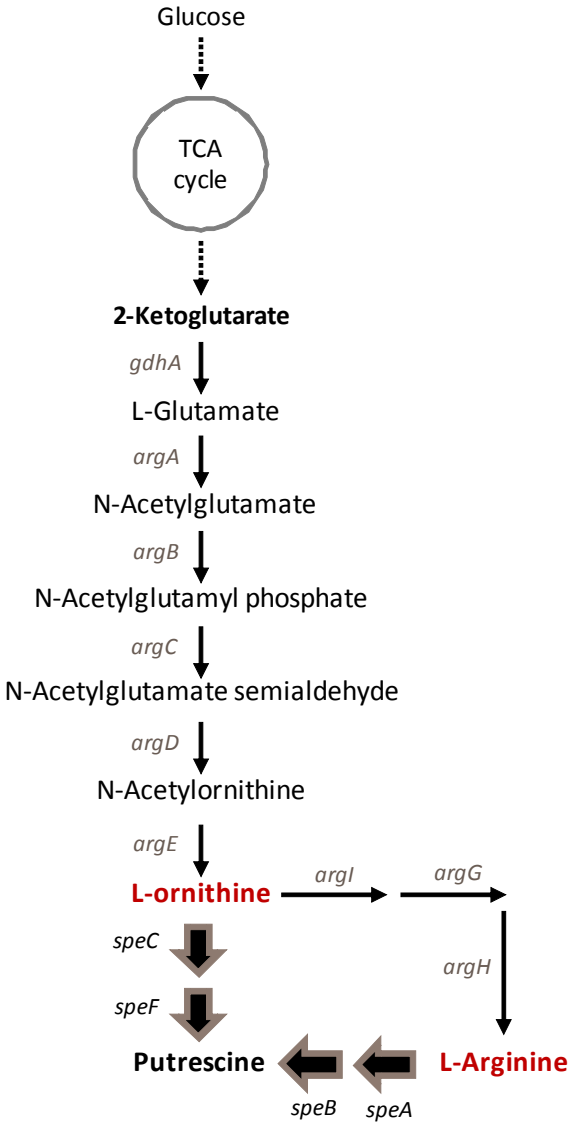

Figure 2: Natural biosynthetic pathway for putrescine

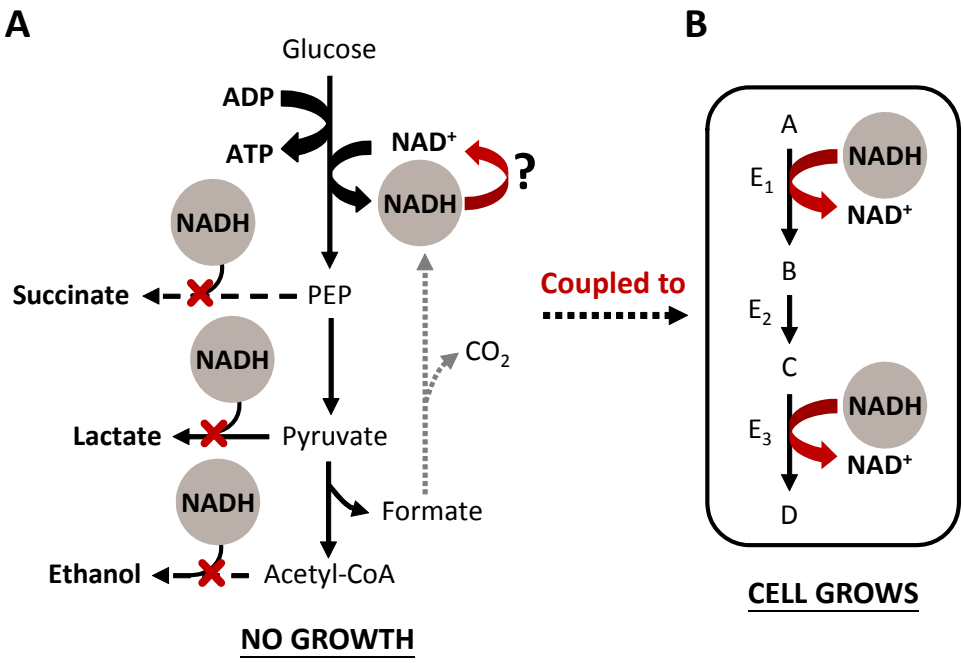

Figure 3: **(A)** Illustration of the anaerobic growth selection scheme based on redox imbalance. Mixed-acid fermentation pathways (succinate, lactate, and ethanol) are deleted, leading to accumulation of NADH and the cessation of glycolysis. The resulting strain cannot grow anaerobically. **(B)** Growth of such strain is rescued when a pathway which consumes NADH is introduced. The consumption of NADH can be linked directly to central metabolism (i.e. substrate A is derived from glucose) as shown on the left panel. PEP, phosphoenolpyruvate

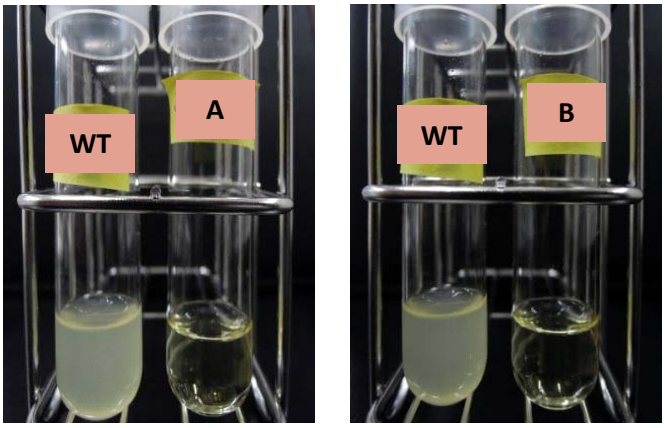

Figure 4: Growth retardation achieved anaerobically by deleting the native fermentation reactions in *E. coli*. WT: wild type, A: Δ*ldhA* Δ*adhE* Δ*frdBC*, B: Δ*ldhA* Δ*adhE* Δ*frdBC* Δ*pta*. Strain A and B can no longer grow under anaerobic condition.

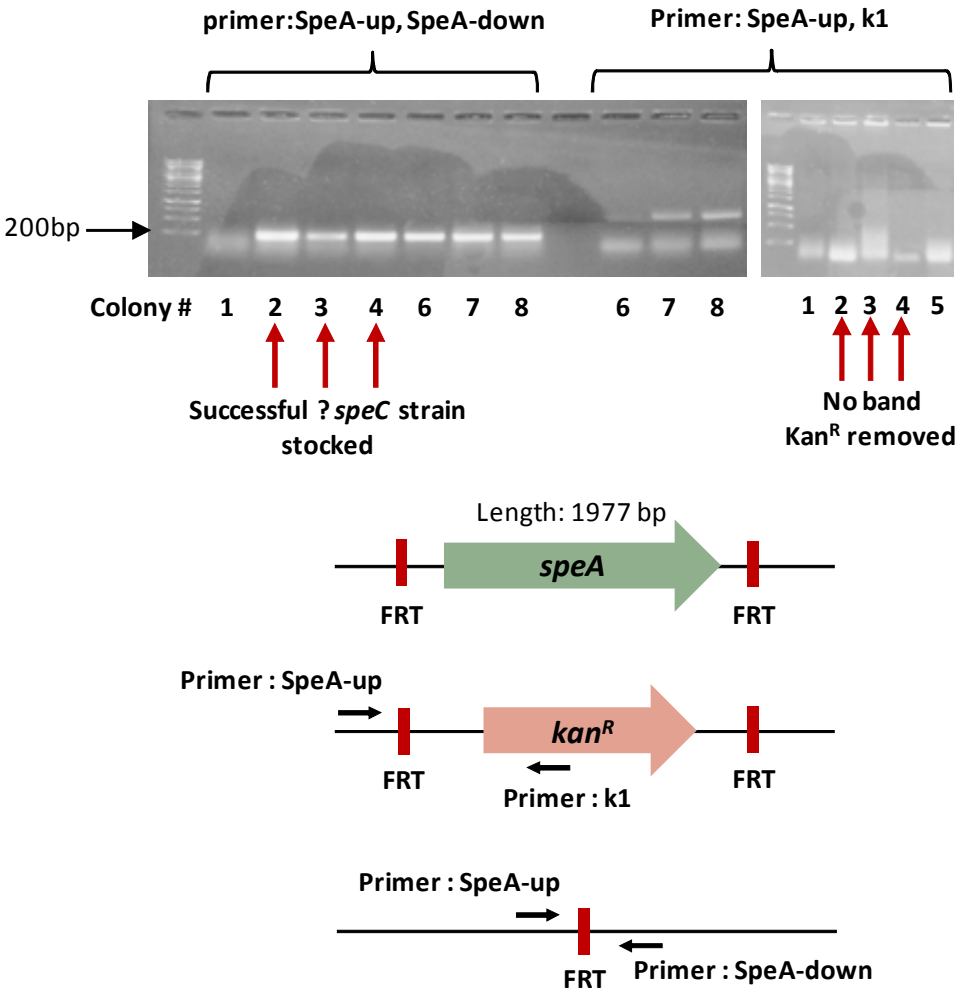

Figure 5: Successful deletion of *speA* from the genome of *E. coli* wild type. The DNA gel picture shows the verification of *speA* removal using colony PCR (PCR product less than 200bp using primers binding upstream and downstream of the gene).