行政院原子能委員會

委託研究計畫研究報告

纖維水解液之生物精煉菌株開發及其放大可行性研究

Development of biorefinery strains and its scale-up feasibility for cellulosic hydrolysates

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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。在本年度中，由於腐胺在產量潛力目前遠不及2,3 -丁二醇，我們決定將本計畫全力投入於2,3 -丁二醇在大腸桿菌中的產量提升和創新此二大研究方向。在提升大腸桿菌生產2,3 -丁二醇方面，我們將2,3 -丁二醇設計為唯一的發酵途徑，並結合不同異源合成酵素，進行生產 2,3-丁二醇同分異構物。後續經優化整體代謝途徑，及大量表現不同種類醇脫氫酵素，使得2,3-丁二醇產量更進一步提升,在未優化發酵條件和使用小型搖瓶的狀態下，兩天內可轉化葡萄糖或木糖進入約 8-12 g/L的(R,R)-2,3-丁二醇。另外，在創新和建立專利位置方面，我們聚焦於找尋潛在大腸桿菌中合成2,3 -丁二醇代謝途徑的天然內源基因。我們利用並改良已建立之『NADH 再循環之厭氧培養』篩選平台，進行可能存在大腸桿菌中合成2,3 -丁二醇內源酵素的選別，並成功篩選出一個基因。最後，大腸桿菌可

藉由表現自身所含基因，在沒有任何異源酵素的作用下，天然生產 2,3-丁二醇。這是大腸桿菌利用自身酵素天然生產 2,3-丁二醇的第一個示範。

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. This year, due to the higher potential of 2,3-butanediol in terms of attainable titer compared to putrescine, we focused entirely on the development of novel *E. coli* strains for 2,3-butanediol biosynthesis with two major aims: further improve production titer and create patent position. Towards the aspect of increasing productivity, we established a recombinant pathway for 2,3-butanediol biosynthesis under anaerobic condition in *E. coli* with deletion of mixed acid fermentation pathway and over-expression of exogenous genes for the production of different 2,3-butanediol isoforms. Based on our results, we successfully improved 2,3-butanediol production efficiency to 8-12 g/L in 2 days using diverse substrates such as glucose or xylose. In terms of novelty for creating patent position, we

focused on the exploration of the innate capability of *E. coli* to synthesize 2,3-butanediol. By using anaerobic selection platform based on NADH recycling system, we successfully identified an endogenous E. coli enzyme capable of catalyzing 2,3-butanediol synthesis reactions naturally. At the end, we successfully achieved endogenous production of 2,3-butanediol in *E. coli* without expression of any heterologous enzyme. This is the first demonstration of 2,3-BDO production from glucose in *E. coli* using its native enzymes.

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

1. Identify the synthetic 2,3-BDO pathway bottleneck and improve its productivity

- **Construct heterologous biosynthetic pathway to produce 2,3-BDO isoforms**

We have surveyed the literature about producing 2,3-BDO by recombinant micro- organisms in recent years. Scientists attempted to improve 2,3-BDO productivity of these non-native species by metabolic engineering technology. They often knock out genes of mixed acid pathway to direct carbon flux into 2,3-BDO synthesis pathway and improve titer and productivity. They also overexpress heterologous genes of 2,3-BDO synthetic pathway in non-native

species and inducing these genes expression. In this project, we created strains that can only secret 2,3-BDO as its fermentation product in the first year tasks. The strains are deletion mutants (Δ*ldhA* Δ*adhE* Δ*frdBC* and Δ*ldhA* Δ*adhE* Δ*frdBC* Δ*pta*) with mixed-acid fermentation reactions removed and lost their ability to synthesize the natural fermentation products lactate, ethanol and succinate. Then, we overexpressed heterologous genes of 2,3-BDO synthetic pathway from *Bacillus subtilis* and some other microorganisms in the deletion mutants. After production test and sample analysis by GC, identity (R,R or meso) of the 2,3-BDO isoforms were confirmed.

We found some interesting results from production of the transformed strains which overexpressed the essential enzymes AlsS, AlsD, and sADH. These transformed strains accumulated lots of acetoin in the broth (Figure 4). One possible reason is that the enzyme responsible for the conversion of acetoin to 2,3-BDO in the last step is not strong enough, which might be a result of poor expression from our current plasmid construct (last gene in the synthetic operon). To test if increasing expression of the alcohol dehydrogenase will enhance production titer, we redesigned the plasmids: one contains the synthase AlsS and the decarboxylase AlsD while the other one harbors two sADH with different cofactor preference. Then, we transformed these two plasmids into the *E. coli* deletion mutant Δ*ldhA* Δ*adhE* Δ*frdBC* to check if this strategy can push more acetoin into 2,3-BDO.

2. <u>Explore endogenous 2,3-BDO biosynthetic activity in E. coli</u>

- **Modification of the anaerobic NADH selection platform**

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- **Selection of endogenous sADH for 2,3-BDO biosynthesis**

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參、主要發現與結論

1. Establish heterologous biosynthetic pathway to produce 2,3-BDO isoforms

To biosynthesize 2,3-BDO, three enzymes are involved: Als (acetolactate synthase), AldC (acetolactate decarboxylase) and sADH (secondary alcohol dehydrogenase). If we want non-native strain to produce 2,3-BDO, it must be transformed with these genes. We constructed two plasmids with sADH from two different organism and transformed them into deletion mutant (Δ*ldhA* Δ*adhE* Δ*frdBC* Δ*pta*). The *E. coli* strain overexpressing *B. subtilis als*S, *alsD* and gene C produced (R, R)-form of 2,3-BDO, reaching about 2.35 ± 0.38 g/L after 48 hours. The other *E. coli* strain overexpressing *alsS*, *alsD* from *B. subtilis* and gene A secreted meso-form of 2,3-BDO, reaching about 1 ± 0.5g/L after 48 hours. Figure 4 shows the production titer of the recombinant *E. coli* strains. It is worth to note that the production stability was quite low; 2,3-BDO titer variation among different colonies were large and sometime even undetected. Also, high level of acetoin was accumulated in the culture broth.

2. Improving production titer of 2,3-BDO

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3. Production of 2,3-BDO using diverse carbon source

As shown from previous section, *E. coli* deletion mutant (Δ*ldhA* Δ*adhE* Δ*frdBC*) harboring plasmids pKM5 and pKM9 accumulated the highest titer of 2,3-BDO, reaching about 7~10 g/L after 48 hours. Since plasmid pKM9 contains A-sADH and B-sADH with different cofactor preference, we tested 2,3-BDO production using different common carbon source such as glucose (hexose), xylose (pentose), or mixture of both. The goal was to analyze the strain's potential and ability to utilize lignocellulosic hydrolysates directly for growth and 2,3-BDO synthesis. As shown by the production data in Figure 6, the highest production of (R, R) 2,3-BDO was observed from the strain cultured in the sugar mixture of glucose and xylose, with a titer around 11.5 ± 1.3 g/L in 2 days. As for the xylose only medium, the strain grew slowly and had low cell density overall, however, the cells still secreted about 6.4 ± 1.3 g/L of 2,3-BDO. This result demonstrated the potential of this engineered *E. coli* strain to utilize different carbon source and transform them into 2,3-BDO with similar efficiency.

4. Identification of endogenous sADH for 2,3-BDO production

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5. 2,3-BDO production using native enzymes from *E. coli*

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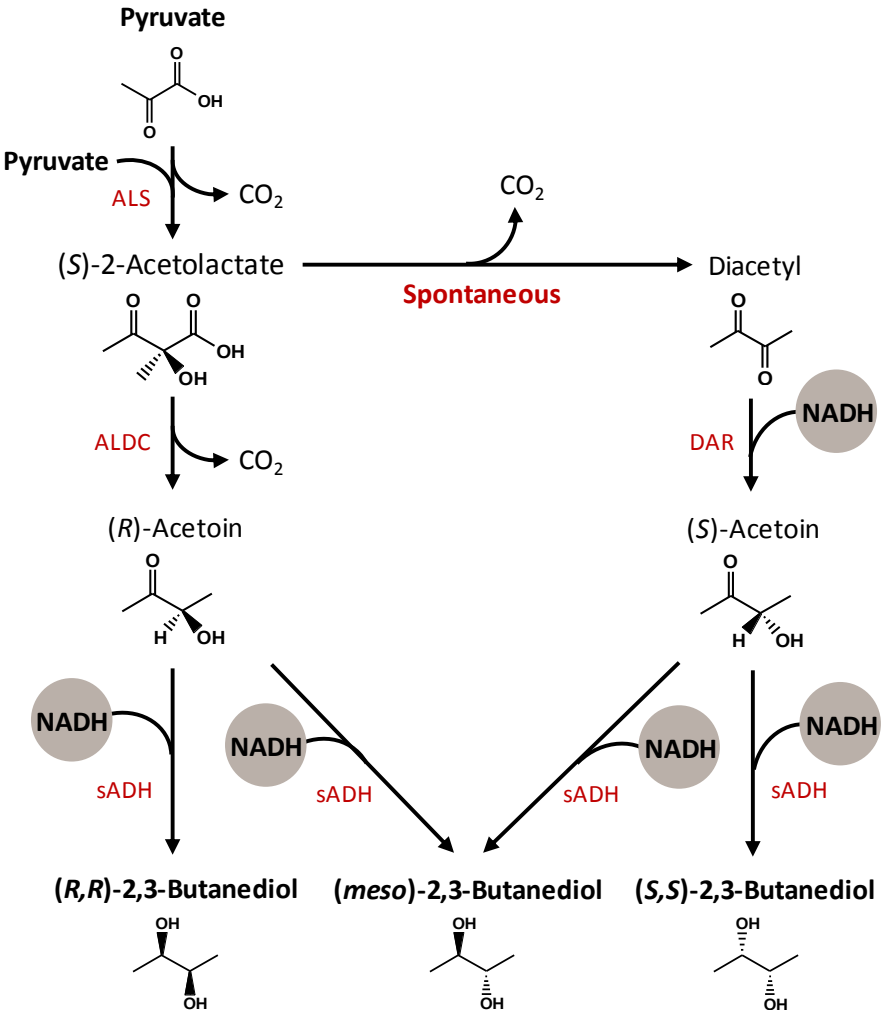

Figure 1. Biosynthetic pathway for the production of various isomeric forms of 2,3-BDO. ALS, acetolactate synthase; ALDC, acetolactate decarboxylase; DAR, diacetyl reductase; sADH, secondary alcohol dehydrogenase

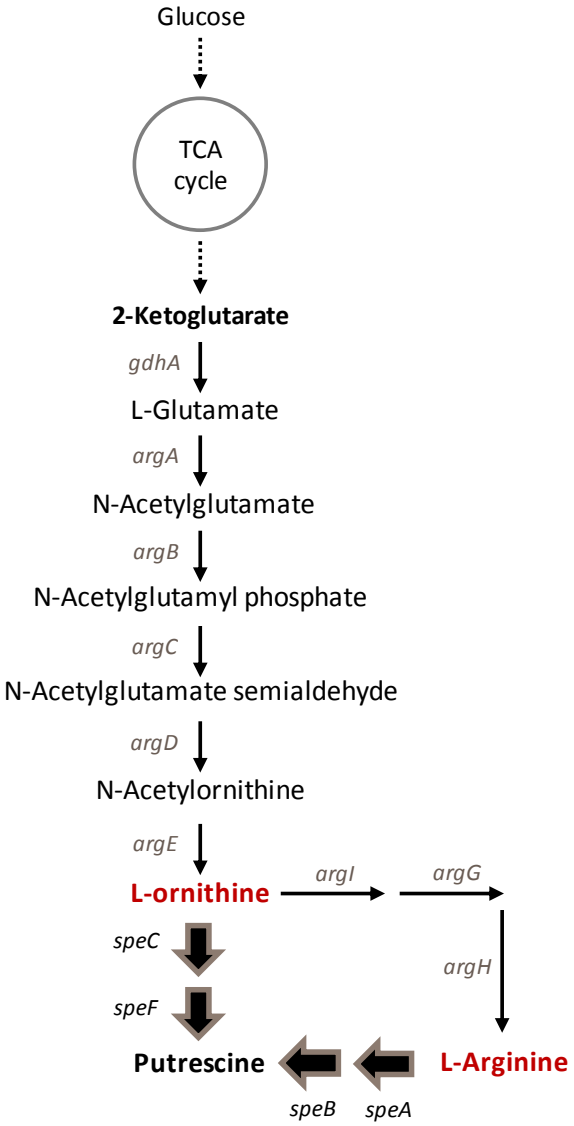

Figure 2. Natural biosynthetic pathway for putrescine

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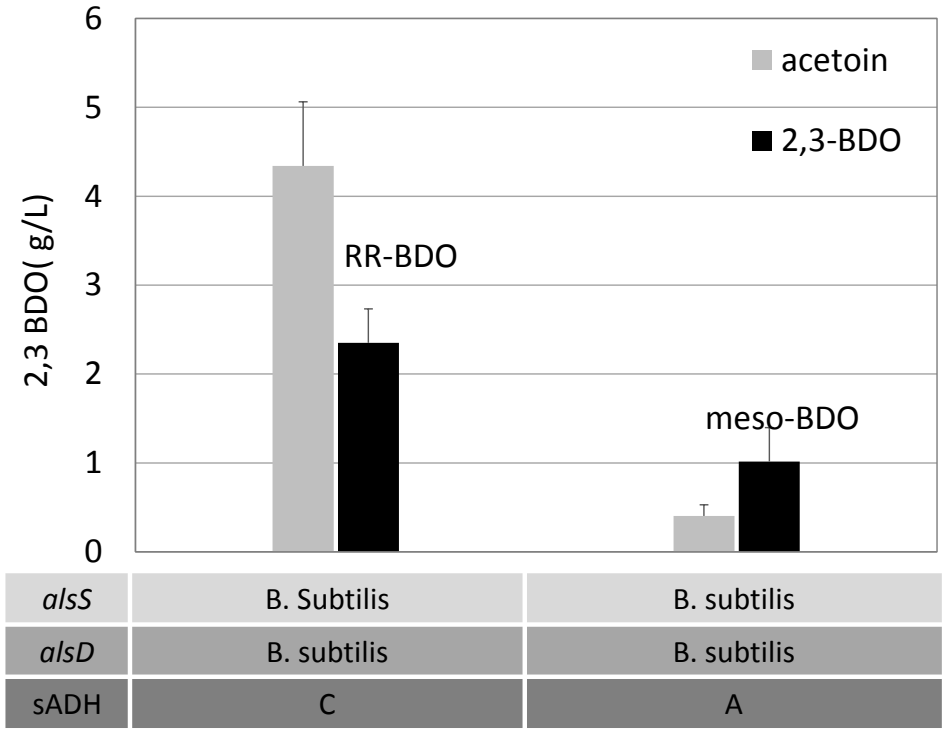

Figure 4. Production of 2,3-BDO isoforms in recombinant E. coli using different heterologous sADH. Gene B and C: undisclosed information

Figure 5. Contents removed for public upload

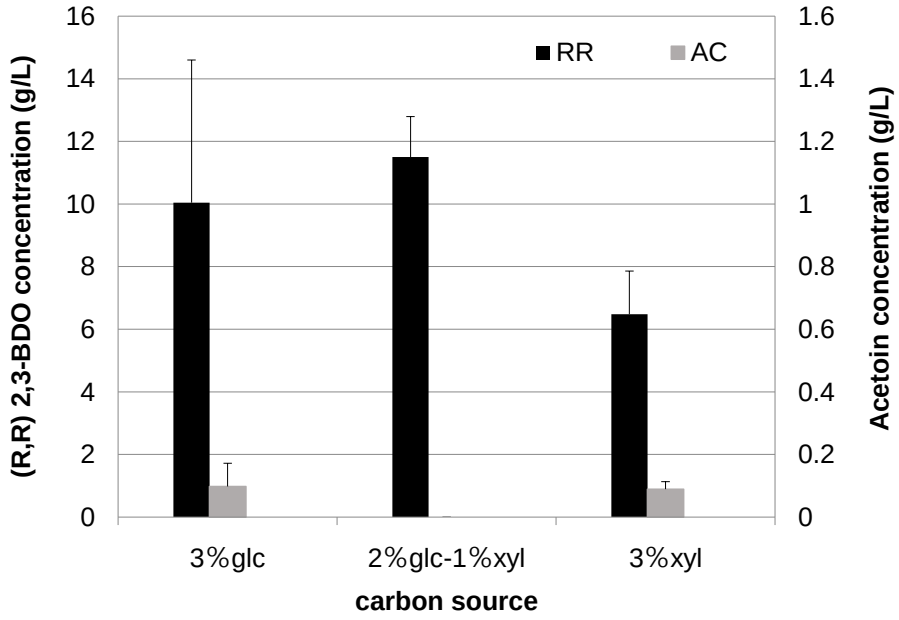

Figure 6. Production of 2,3-BDO by our engineered E. coli using different carbon source

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