

Metabolic engineering of non-pathogenic microorganisms for 2,3-butanediol production

Jae Won Lee^{a,b,c}, Ye-Gi Lee^{b,c}, Yong-Su Jin^{a,b,c}, Christopher V. Rao^{b,c,d†}

^aDepartment of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

^bCarl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

^cDOE Center for Advanced Bioenergy and Bioproducts Innovation, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

^dDepartment of Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

[†]Corresponding author.

E-mail address: cvrao@illinois.edu (C. V. Rao)

ABSTRACT

2,3-butanediol (2,3-BDO) is a promising commodity chemical with various industrial applications. While petroleum-based chemical processes currently dominate the industrial production of 2,3-BDO, fermentation-based production of 2,3-BDO provides an attractive alternative to chemical-based processes with regards to economic and environmental sustainability. The achievement of high 2,3-BDO titer, yield, and productivity in microbial fermentation is a prerequisite for the production of 2,3-BDO at large scales. Also, enantiopure production of 2,3-BDO production is desirable because 2,3-BDO stereoisomers have unique physicochemical properties. Pursuant to these goals, many metabolic engineering strategies to improve 2,3-BDO production from inexpensive sugars by *Klebsiella oxytoca*, *Bacillus* species, and *Saccharomyces cerevisiae* have been developed. This review summarizes the recent advances in metabolic engineering of non-pathogenic microorganisms to enable efficient and enantiopure production of 2,3-BDO.

Keywords 2,3-butanediol (2,3-BDO), *Klebsiella oxytoca*, *Bacillus* species, *Saccharomyces cerevisiae*, 2,3-BDO stereoisomers

Key points

- *K. oxytoca*, *Bacillus* species, *S. cerevisiae* has been engineered to achieve efficient 2,3-BDO production
- Metabolic engineering of non-pathogenic microorganisms enabled enantiopure production of 2,3-BDO
- Cost-effective 2,3-BDO production can be feasible by using renewable biomass

1. Introduction

2,3-butanediol (2,3-BDO) is a promising platform chemical with diverse industrial applications. 2,3-BDO can be used as a starting material for chemical conversion. In particular, dehydration of 2,3-BDO leads to 1,3-butadiene, which is a precursor of synthetic rubber (Liu et al. 2016). Methyl ethyl ketone (MEK), an effective fuel additive, can also be produced from 2,3-BDO via pinacol rearrangement (Haveren et al. 2008). In addition, 2,3-BDO can be used as a preservative in cosmetics because of its antibacterial properties (Baek et al 2016). Moreover, 2,3-BDO can be used as a biostimulant in agriculture. The efficacy of 2,3-BDO for the protection of plant species against bacterial (Ryu et al. 2003), fungal (Cortes-Barco et al. 2010), and viral infection (Kong et al. 2018) has been validated.

Currently, 2,3-BDO is commercially produced by petroleum-based chemical processes that mainly depend on the non-specific hydrolysis of 2,3-butane oxide under harsh conditions (160 – 220 °C and 50 bar) (Gräffe et al. 2021). However, these chemical processes generate several byproducts, which causes additional cost for the downstream purification of 2,3-BDO (Ge et al. 2016). Therefore, microbial production has been developed as an alternative method for cost-effective and environmentally friendly 2,3-BDO production (Song et al. 2019).

Most studies for microbial 2,3-BDO production had been limited to bacteria such as *Klebsiella*, *Enterobacter*, and *Bacillus* species. These bacterial species are able to natively produce 2,3-BDO to prevent intracellular acidification (Tsau et al. 1992), regulate the NADH/NAD⁺ balance (Johansen et al. 1975; Magee and Kosaric 1987), and store energy (Xiao and Xu 2007). However, many native 2,3-BDO producers are classified as Risk Group 2 pathogen (pathogenic to human), which hinders their applicability in industrial-scale 2,3-BDO production (Celińska and Grajek 2009; Ji et al. 2011a). Exceptions are *Klebsiella oxytoca* and *Bacillus* spp. known to be generally

recognized as safe (GRAS) microorganisms (de Boer Sietske and Diderichsen 1991; Park et al. 2013a), which can produce 2,3-BDO from various substrates (Cho et al. 2015b; Li et al. 2014; Meng et al. 2020; Yang et al. 2013). As such, *K. oxytoca* and *Bacillus* spp. are regarded as promising hosts for industrial 2,3-BDO production due to their safe characteristics and native 2,3-BDO producing capability.

Substantial research for efficient 2,3-BDO production by non-native 2,3-BDO as well as native 2,3-BDO producers have been performed. In particular, industrially relevant host strains such as *Escherichia coli* (Erian et al. 2018), *Saccharomyces cerevisiae* (Lee and Seo 2019), and *Lactococcus lactis* (Kandasamy et al. 2016) have been utilized as host strains for 2,3-BDO production. Among them, *S. cerevisiae* has been intensively engineered for 2,3-BDO production because of its robustness against various environmental stresses, genetic tractability, and well-elucidated physiology (Hong and Nielsen 2012; Ostergaard et al. 2000). Besides, *S. cerevisiae* is also a GRAS microorganism, so that it has been used as a cell factory for various bio-based products via industrial-scale fermentation (Lee et al. 2012; Weber et al. 2010).

2,3-BDO stereoisomers – (2R,3R)-BDO, *meso*-BDO, and (2S,3S)-BDO – can be produced during microbial 2,3-BDO production. Enantiopure stereoisomers of 2,3-BDO have distinctive physicochemical properties (Celińska and Grajek 2009; Knowlton et al. 1946; Ji et al. 2011a), providing unique efficacies especially in the cosmetic (Baek et al 2016) and agricultural industries (Cho et al. 2008; Cortes-Barco et al. 2010; Kong et al. 2018; Ryu et al. 2003). The optical purity of 2,3-BDO is determined by the stereospecificity of 2,3-butanediol dehydrogenase (BDH) that catalyzes the reversible conversion between acetoin and 2,3-BDO. Recently, microbial production of enantiopure 2,3-BDO has been developed by modulating the stereospecificity of BDH in a host strain (Ge et al. 2016; Park et al. 2015; Qiu et al. 2016; Song et al. 2020).

To achieve cost-effective 2,3-BDO production, researchers have sought less expensive renewable biomass than sugar substrates. In the past decades, the relatively high cost of sugar substrates has limited the economic viability of microbial 2,3-BDO production (Ji et al. 2011a). Recent development in metabolic engineering have enabled microorganisms to produce 2,3-BDO using abundant and inexpensive feedstocks including lignocellulosic biomass (Cha et al. 2020), byproducts of food processing (Meng et al. 2020), industrial wastes (Cho et al. 2015a), and agricultural wastes (Li et al. 2014).

This review summarizes the metabolic engineering strategies employed in *K. oxytoca*, *Bacillus* spp., and *S. cerevisiae* to achieve efficient 2,3-BDO production. In addition, this review also covers the recent progress of 2,3-BDO stereoisomers production, and cost-effective 2,3-BDO production using abundant and inexpensive feedstocks.

2. Strain improvement for 2,3-BDO production

2.1. 2,3-BDO synthetic pathways in bacteria and yeast

2,3-BDO biosynthesis is physiologically important to bacteria for preventing acidification, regulating the intracellular NADH/NAD⁺ balance, and storing carbon for cell growth. By switching the metabolism from acid to 2,3-BDO (neutral compound) production, bacteria can prevent intracellular acidification (Tsau et al. 1992). Moreover, the reversible conversion between acetoin and 2,3-BDO with concomitant NADH/NAD⁺ transformation plays a role in regulating intracellular ratio of NADH/NAD⁺ (Johansen et al. 1975; Magee and Kosaric 1987). Finally, bacteria may reuse 2,3-BDO as a carbon source for cell growth when other carbon sources are depleted (Xiao and Xu 2007).

In native 2,3-BDO producing bacteria, pyruvate is converted into α -acetolactate by α -acetolactate synthase (ALS). Then, α -acetolactate is anaerobically transformed into acetoin by α -acetolactate decarboxylase (ALDC). In aerobic conditions, α -acetolactate can spontaneously be decarboxylated to diacetyl and then is transformed into acetoin by diacetyl reductase (DAR). Finally, acetoin is reduced to 2,3-BDO by 2,3-butanediol dehydrogenase (BDH) with concomitant oxidation of NADH to NAD⁺. However, 2,3-BDO production from glucose is not a redox-neutral reaction. One mole of surplus NADH is generated via 2,3-BDO production because two moles of NADH are generated from one mole of glucose via glycolysis but only one mole of NADH is re-oxidized to NAD⁺ in the 2,3-BDO pathway (Syu 2001). Thus, pyruvate is channeled not only into acetoin and 2,3-BDO but also into mixtures of ethanol, acetate, lactate, and formate to regulate the NADH/NAD⁺ ratio in bacteria, depending on culture conditions (Maddox 2001) (Fig. 1). As a result, 2,3-BDO production often involves the production of undesirable byproducts, so it is important to minimize byproducts formation for efficient 2,3-BDO production.

S. cerevisiae has an endogenous 2,3-BDO biosynthetic pathway. Pyruvate is converted into α -acetolactate by ALS (Ilv2p) in the mitochondria. In contrast to the bacterial 2,3-BDO pathway, α -acetolactate cannot be enzymatically converted into acetoin because an endogenous ALDC is not present in *S. cerevisiae*. Instead, α -acetolactate is spontaneously decarboxylated to diacetyl under aerobic conditions. Then, diacetyl is reduced into 2,3-BDO via acetoin by BDH (Bdh1p) in *S. cerevisiae* (Fig. 1). However, the activities of the 2,3-BDO biosynthetic enzymes in *S. cerevisiae* are not strong enough to drive efficient 2,3-BDO production as compared to those of bacterial enzymes. Particularly, the Ilv2p is located in the mitochondria while other enzymes in the 2,3-BDO biosynthetic pathway are present in the cytosol (Brat et al. 2012). This location mismatch of the enzymes cannot support efficient 2,3-BDO production. Moreover, as diacetyl is formed by

non-enzymatic decarboxylation of α -acetolactate (Dulieu and Poncelet 1999), this slow decarboxylation step also impedes efficient 2,3-BDO production by *S. cerevisiae*. Therefore, it is necessary to introduce heterologous 2,3-BDO biosynthetic enzymes into *S. cerevisiae* and amplify the metabolic fluxes toward 2,3-BDO production. Also, *S. cerevisiae* is known to display the Crabtree effect: a situation where other metabolic pathways are repressed in the presence of glucose and ethanol is produced as a major product (DE DEKEN 1966). As such, efficient 2,3-BDO production by *S. cerevisiae* requires elimination of ethanol production along with amplification of the 2,3-BDO biosynthetic enzymes. However, *S. cerevisiae* produces ethanol with coupled NAD^+ regeneration to maintain redox balance, the elimination of ethanol production leads to growth defects due to cytosolic NADH accumulation. Thus, alleviation of redox imbalance caused by the elimination of ethanol production should be also considered for efficient 2,3-BDO production in yeast. Taken together, based on the understanding of metabolic pathways, several approaches including mutant screening, genetic engineering, *in silico* simulations based on genome-scale metabolic models, and environmental perturbations have been conducted to improve 2,3-BDO production in bacteria and yeast (Table 1).

Meanwhile, different stereoisomers of 2,3-BDO – (2R,3R)-BDO, *meso*-BDO, and (2S,3S)-BDO – are produced from different forms of acetoin (Fig. 2). In both bacteria and yeast, the diacetyl can be further reduced to (R)-acetoin or (S)-acetoin via BDH or DAR. From two different forms of acetoin, 2,3-BDO stereoisomers can be produced by the BDH enzymatic reaction. The BDH stereo-specificities can determine 2,3-BDO stereoisomers produced by microorganisms. The BDH can be divided into three classes: (2R,3R)-BDH, (2S,3S)-BDH, and *meso*-BDH. The (2R,3R)-BDH converts (R)-acetoin to (2R,3R)-BDO and (S)-acetoin to *meso*-BDO, while the (2S,3S)-BDH converts (S)-acetoin to (2S,3S)-BDO. Besides, the *meso*-BDH converts R-acetoin

to *meso*-BDO and S-acetoin to *meso*-BDO and (2S,3S)-BDO. Thus, the ratio of 2,3-BDO stereoisomers production can be variant depending on the stereospecificity of the BDH enzyme in a host strain. Besides, a single strain may have either multiple BDH enzymes or a single BDH enzyme with activity for both (R)-acetoin and (S)-acetoin. Therefore, several metabolic engineering approaches have been made to produce enantiopure 2,3-BDO. The different BDHs and metabolic engineering strategies employed in microorganisms for enantiopure 2,3-BDO production are summarized in Table 2.

2.2. *Klebsiella oxytoca*

Wild-type *K. oxytoca* produces 2,3-BDO along with several byproducts, including lactate, formate, acetate, succinate, ethanol, and acetoin during fermentation (Fig. 1). Thus, various genetic and environmental perturbations have been attempted to enhance 2,3-BDO production by minimizing byproducts. The perturbations can be summarized into three primary categories; 1) blocking of competing byproducts pathways to increase 2,3-BDO yield, 2) restoring the redox imbalance caused by the elimination of byproducts production via aeration, and 3) overexpression of rate-limiting 2,3-BDO biosynthetic enzymes.

Ji et al. (2008) isolated *K. oxytoca* mutants from a wild-type *K. oxytoca* after UV mutagenesis and obtained a mutant that has deficiency in the activities of lactate dehydrogenase and phosphotransacetylase. The obtained mutant produced 88% and 92% less lactate and acetate, respectively, as compared to the wild-type *K. oxytoca*, resulting in an 7.8% increased production of 2,3-BDO. Nonetheless, acetoin and ethanol were still produced as byproducts. To reduce acetoin and ethanol formations, different aeration levels were applied to batch fermentations by controlling agitation speeds because intracellular ratio of NADH/NAD⁺ can be also regulated by respiration

via regeneration of NAD^+ under aerobic condition (Ji et al. 2009). Interestingly, the patterns of byproducts production were quite different depending on dissolved oxygen levels. Specifically, ethanol production decreased and acetoin production increased under high aeration conditions, and an opposite pattern was observed under low aeration conditions. Therefore, to enhance 2,3-BDO production by minimizing acetoin and ethanol accumulation, a two-stage agitation speed control strategy – combining the advantages of ethanol reduction at high agitation speed and acetoin reduction at low agitation speed – was employed. As a result, the final 2,3-BDO titer reached 95.5 g/L with a yield of 0.478 g/g and a productivity of 1.71 g/L·h, which were 6.2%, 6.2%, and 22.1% higher than the batch fermentation results using constant agitation speeds. Ji et al. (2010) also attempted to eliminate acetoin and ethanol production by blocking the ethanol-producing pathway instead of controlling agitation speeds. A *K. oxytoca aldA* knockout mutant was constructed by replacing the *aldA* gene encoding aldehyde dehydrogenase with a tetracycline resistance cassette. The knockout of *aldA* gene increased the intracellular ratio of NADH/NAD^+ , which drove the forward reaction from acetoin into 2,3-BDO to fulfill the redox imbalance *in vivo*. As a result, the 2,3-BDO yield of the *aldA* knockout mutant increased by 6.8 %, while ethanol and acetoin concentrations decreased by 92.2% and 64.1%, respectively, as compared with a parental strain. The final 2,3-BDO titer by the *aldA* mutant reached 130 g/L with a productivity of 1.63 g/L·h and a yield of 0.48 g/g glucose in fed-batch fermentation.

Considering a target metabolic pathway only without realizing global and complex metabolic network interactions often hampers the development of an ideal engineered strain. Thus, *in silico* genome-scale metabolic models have been employed as a useful tool to provide systematic strategies for improving 2,3-BDO production. Park et al. (2013a) performed *in silico* single gene knockout simulation using flux balance analysis (FBA) to reduce the formation of byproducts and

enhance the metabolic flux toward 2,3-BDO biosynthesis. As a result, the *ldhA* gene encoding lactate dehydrogenase was targeted as a single gene knockout candidate based on the criteria of maximizing 2,3-BDO yield and productivity. The *ldhA* knockout mutant exhibited 94% less lactate production and 76% more 2,3-BDO production as compared with the wild-type strain. However, the mutant strain produced large amounts of byproducts: 8.0 g/L of formic acid and 3.5 g/L of ethanol from 100 g/L of glucose. To further enhance 2,3-BDO production, *in silico* simulation was performed to select an additional knockout candidate. As a result, the *pflB* gene encoding pyruvate formate lyase (PFL), which converts pyruvate into acetyl-CoA and formic acid, was selected and inactivated in the *ldhA* knockout mutant (Park et al. 2013b). The 2,3-BDO yield of the *ldhA pflB* double-knockout mutant was much higher (0.44 g/g glucose) than those (0.18 g/g glucose and 0.32 g/g glucose) of the wild type and the *ldhA* knockout mutant. However, the volumetric 2,3-BDO productivity (0.51 g/L·h) of the *ldhA pflB* double-knockout mutant was lower than those (0.58 g/L·h and 1.07 g/L·h) of the wild type and the *ldhA* knockout mutant. This is due to the growth inhibition caused by insufficient acetyl-CoA supply from the inactivation of PFL. In particular, acetyl-CoA is essential to energy generation and cell growth as it is a key substrate of the TCA cycle. Another way to convert pyruvate into acetyl-CoA is oxidation by pyruvate dehydrogenase (PDH) (Fig. 1a). However, PDH activity was insufficient to substitute the PFL activity under oxygen-limiting conditions, as compared to aerobic conditions. To restore cell growth defects by supplying sufficient acetyl-CoA, a two-stage aeration control was applied to fed-batch fermentation. As a result, the cell growth was restored and final 2,3-BDO titer reached 113 g/L with a yield of 0.45 g/g glucose and a productivity of 2.1 g/L·h in the fed-batch fermentation.

Cho et al. (2015b) attempted to enhance 2,3-BDO production by optimizing fermentation conditions using an isolated *K. oxytoca* strain. In fed-batch fermentations, where higher agitation

speeds were used, 2,3-BDO titers increased (109.6 g/L at 300 rpm vs. 118.5 g/L at 400 rpm) along with significantly reduced formation of acids. However, the 2,3-BDO yield from glucose decreased due to acetoin accumulation (0.40 g/g at 300 rpm vs. 0.34 g/g at 400 rpm). To enhance the forward reaction from acetoin to 2,3-BDO, the endogenous *budC* encoding BDH, which exhibits 8-fold higher acetoin reduction activity than 2,3-BDO oxidation activity, was overexpressed in the isolated *K. oxytoca* strain. The 2,3-BDO titer of the resulting strain reached 142.5 g/L with a yield of 0.42 g/g and a productivity of 1.47 g/L·h in the fed-batch fermentation, while acetoin accumulation decreased 43% as compared to a parental strain.

K. oxytoca produces *meso*-BDO by *meso*-BDH as a major stereoisomer from glucose. Park et al (2015) provided evidence that metabolic engineering could change the stereoisomer selectivity from *meso*-BDO to (2R,3R)-BDO in *K. oxytoca*. A (2R,3R)-BDO producing *K. oxytoca* strain was constructed based on the *K. oxytoca* Δ *ldhA* Δ *pf1B* strain that produced *meso*-BDO with high optical purity (>98%) (Park et al. 2013b). The *budC* gene encoding *meso*-BDH in the *K. oxytoca* Δ *ldhA* Δ *pf1B* strain was replaced with the heterologous *bdh* gene encoding (2R,3R)-BDH from *Paenibacillus polymyxa*, and the expression level of the (2R,3R)-BDH was enhanced by using a multicopy plasmid. The resulting strain produced 106.7 g/L of (2R,3R)-BDO [9.3 g/L of *meso*-BDO] with a yield of 0.40 g/g glucose and a productivity of 3.1 g/L·h in fed-batch fermentation.

2.3. *Bacillus* species

Bacillus spp. produce acetoin naturally as a major byproduct along with 2,3-BDO. In the 2,3-BDO biosynthetic pathway in *Bacillus* spp., BDH catalyzes the conversion of acetoin to 2,3-BDO with concomitant oxidation of NADH to NAD⁺. As 2,3-BDO is an NADH-dependent product, NADH availability plays an important role in 2,3-BDO biosynthesis. It has been demonstrated that

the introduction of a heterologous NADH regeneration system and the inactivation of endogenous NADH oxidation pathway are effective approaches to enhance 2,3-BDO production by *Bacillus* spp. Besides, an exogenous reducing agent (vitamin C) have been used to enhance 2,3-BDO production by regulating intracellular ratio of NADH/NAD⁺.

Fu et al. (2014) introduced the *E. coli udhA* gene encoding for transhydrogenase, which converts NADPH to NADH, into *Bacillus subtilis* to increase NADH availability. As a result, the *B. subtilis* strain overexpressing the *udhA* gene had a lower NADPH/NADP⁺ ratio and exhibited a higher NADH/NAD⁺ ratio as compared with the control strain not overexpressing the *udhA* gene. With the increased NADH availability, 2,3-BDO titer by the *udh*-overexpressing *B. subtilis* strain was 13.6 % higher than that of the control strain. Finally, the *udh*-expressing *B. subtilis* strain produced 49.29 g/L of 2,3-BDO with a yield of 0.47 g/g glucose in batch fermentation. Yang et al. (2015) deleted the endogenous *yodC* gene encoding NADH oxidase and introduced the heterologous *Candida boidinii fdh* gene encoding formate dehydrogenase (FDH) into *B. subtilis* to enhance 2,3-BDO production with reduced acetoin production. The NADH oxidase (YodC) catalyzes the oxidation of NADH to NAD⁺ using molecular oxygen as the electron acceptor. As BDH competes with the NADH oxidase for NADH as a cofactor, the inactivation of YodC was beneficial for enhancing the metabolic flux toward 2,3-BDO biosynthesis. Also, as the FDH catalyzes the conversion of formate to hydrogen and carbon dioxide with reduction of NAD⁺ to NADH, the introduction of FDH can provide more NADH availability for the BDH enzymatic reaction. The *B. subtilis* strain with *yodC* deletion and *fdh* overexpression produced 19.9% more 2,3-BDO and 71.9% less acetoin than a parental strain. Yang et al. (2013) co-overexpressed the endogenous *gapA* gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and *bdh* gene encoding BDH in *Bacillus amyloliquefaciens* to enhance 2,3-BDO production and reduce

byproducts (acetoin, lactate, and succinate) accumulation. The GAPDH (GapA) catalyzes the conversion of 3-phosphate glyceraldehyde to 1,3-bisphosphoglycerate with concomitant reduction of NAD^+ to NADH. Initially, when the only GAPDH was overexpressed, the molar yield of acetoin decreased, while those of 2,3-BDO, lactate, and succinate increased as compared with a parental strain. This result suggested that all of the NADH-dependent pathways (2,3-BDO, lactate, succinate) would benefit from the improved NADH availability. However, when GAPDH and BDH were overexpressed, the molar yield of 2,3-BDO increased by 22.7%, while those of acetoin, lactate, and succinate decreased by 82.9%, 33.3%, and 39.5%, respectively, as compared with the strain overexpressing the GAPDH only. This result indicated that BDH overexpression facilitated the conversion of acetoin to 2,3-BDO by taking an advantage of the improved NADH availability from the overexpression of GAPDH. Finally, 2,3-BDO titer reached 132.9 g/L with a productivity of 2.95 g/L·h in fed-batch fermentation.

The addition of exogenous reducing agents has been proposed to enhance the production of 1,3-propanediol and citric acid, which are closely associated with the adjustment of NADH/NAD^+ ratio (Berovic 1999; Du et al. 2006). Dai et al. (2014) added vitamin C (V_c) extracellularly to enhance 2,3-BDO production by *Paenibacillus polymyxa* by regulating intracellular NADH/NAD^+ ratio. As a reductant in the enzymatic reactions, V_c functions as electron donor instead of NADH, resulting in improving intracellular NADH availability. As a result, V_c addition elevated the 2,3-BDO titer from 43.7 g/L to 71.7 g/L in fed-batch fermentation. This is the highest 2,3-BDO titer reported for *P. polymyxa* from glucose.

B. subtilis, *B. licheniformis*, and *B. amyloliquefaciens* are known to produce a mixture of (2R,3R)-BDO and *meso*-BDO (Ji et al. 2011). Enantiopure 2,3-BDO can be produced by *B. licheniformis* via simple modifications. Although, *B. licheniformis* have multiple BDH enzymes,

the cross-functional activities of BDHs in *B. licheniformis* might not be as complex as found in other bacteria. Particularly, two stereospecific BDHs, (2R,3R)-BDH (encoded by *gdh*) and *meso*-BDH (encoded by *budC*), were found to be responsible for production of (2R,3R)-BDO and *meso*-BDO in wild-type *B. licheniformis* strains. Ge et al. (2016) constructed two engineered strains, *B. licheniformis* $\Delta budC$ and *B. licheniformis* Δgdh , to produce enantiopure (2R,3R)-BDO and *meso*-BDO, respectively. In fed-batch fermentations, the *budC* knockout mutant produced 123.7 g/L of (2R,3R)-BDO with a productivity of 2.94 g/L·h, while the *gdh* knockout mutant produced 90.1 g/L of *meso*-BDO with a productivity of 2.81 g/L·h. The purity of (2R,3R)-BDO and *meso*-BDO produced by the *budC* and the *gdh* knockout mutants was 99.4% and 99.2%, respectively. Qiu et al (2016) aimed to construct an engineered *B. licheniformis* strain for efficient *meso*-2,3-BDO production. The *acoR* gene encoding acetoin dehydrogenase operon transcriptional activator, which is involved in acetoin degradation, was knocked out to provide enhanced the metabolic flux toward *meso*-2,3-BDO along with the knockout of the *gdh* gene. The *acoR gdh* double-knockout mutant produced 98.0 g/L of *meso*-2,3-BDO with a purity of >99.0% and a productivity of 0.94 g/L·h. Song et al (2020) isolated a *Bacillus licheniformis* 4071 strain from a soil sample. The strain produced 123 g/L of 2,3-BDO in fed-batch fermentation and the ratio of *meso*-BDO and (2R,3R)-BDO was about 1:1 in the 2,3-BDO production. To increase the selectivity of (2R,3R)-BDO, the *budC* gene encoding *meso*-BDH was knocked out. The *budC* knockout mutant increased the selectivity of (2R,3R)-BDO to 91% (96.3 g/L of (2R,3R)-BDO and 9.33 g/L of *meso*-BDO), which was 43% higher than that of a parental strain. These studies suggest that inactivation of a single BDH enzyme in *B. licheniformis* is sufficient for the synthesis of enantiopure 2,3-BDO with a selectivity higher than 90 %.

In contrast to *B. licheniformis*, *P. thermoglucosidasius* can produce (2R,3R)-BDO with high optical purity (>98%). High-temperature fermentation using *P. thermoglucosidasius*, which is a thermophilic microorganism, can provide a cost-effective process for industrial (2R,3R)-BDO production due to decreased hygiene and cooling costs (Zhou et al. 2020). As endogenous ALS (AlsS) has insufficient enzymatic activity, (2R,3R)-BDO biosynthetic pathway was optimized by testing different combinations of heterologous enzymes. As a result, the combination of the AlsS from *B. subtilis* and AlsD from *Streptococcus thermophilus* enzymes along with endogenous (2R,3R)-BDH (encoded by *bdhA*) exhibited the highest (2R,3R)-BDO production by engineered *P. thermoglucosidasius* strain. The resulting strain produced 7.2 g/L of (2R,3R)-BDO with ~72% theoretical yield at 55°C in batch fermentation.

2.4. *Saccharomyces cerevisiae*

Wild-type *S. cerevisiae* produces only trace amounts of 2,3-BDO due to the low activities of the 2,3-BDO producing enzymes. To enhance 2,3-BDO biosynthesis in *S. cerevisiae*, overexpression of heterologous cytosolic ALS, ALDC, and BDH have been attempted. Ng et al. (2012) introduced *B. subtilis alsS* gene encoding ALS and *E. aerogenes budA* and *budC* genes encoding ALDC and BDH into *S. cerevisiae* for the production of 2,3-BDO. Nonetheless, the resulting strain exhibited a low 2,3-BDO yield (0.002 g/g glucose) in batch fermentation. Kim et al. (2013b) introduced *B. subtilis alsS* gene and *alsD* gene encoding ALDC and overexpressed endogenous *BDHI* gene encoding for BDH, which led to 0.04 g/g glucose of 2,3-BDO yield in the batch fermentation.

Despite many attempts to introduce heterologous 2,3-BDO biosynthetic enzymes into *S. cerevisiae*, 2,3-BDO production by engineered yeast was still limited. The reason was because

ethanol was produced as a major product instead of 2,3-BDO due to the Crabtree effect (DE DEKEN 1966). Therefore, it is necessary to reduce or eliminate ethanol production along with amplification of 2,3-BDO biosynthetic enzymes for efficient 2,3-BDO production. In fermentative metabolism of *S. cerevisiae*, pyruvate is converted into acetaldehyde by pyruvate decarboxylase (PDC) and further reduced into ethanol by alcohol dehydrogenase (ADH). Specifically, *S. cerevisiae* has three PDC isozymes (Pdc1, Pdc5, and Pdc6) (Pronk et al. 1996) and seven ADH isozymes (Adh 1~7) (de Smidt et al. 2008). To redirect carbon flux toward 2,3-BDO from ethanol production, ADH or PDC isozymes have been eliminated in engineered *S. cerevisiae* strains (Ishii et al. 2018; Kim et al. 2015; Kim and Hahn 2015; Ng et al. 2012).

Although ethanol production was significantly reduced in ADH-deficient (Adh^-) strains, retarded cell growth on glucose medium was observed due to the accumulation of toxic intermediates such as acetaldehyde and acetate (de Smidt et al. 2012). However, expression of heterologous ALS enzyme exhibiting high activities might prevent the accumulation of toxic acetaldehyde in the Adh^- strains. Kim and Hahn (2015) co-overexpressed *B. subtilis alsS*, *alsD* genes and endogenous *BDHI* gene in an Adh^- strain, and the resulting strain exhibited only 14.3% reduction in glucose consumption rate as compared with the wild-type strain overexpressing the 2,3-BDO biosynthetic enzymes. This result suggested the ALS (AlsS) activity might be high enough to compete with the PDC activity, thus avoiding acetaldehyde accumulation even in the absence of ADH isozymes.

Another strategy for minimize ethanol production is to disrupt *PDC1*, *PDC5*, and *PDC6*. A resulting PDC-deficient (Pdc^-) strain expressing *alsS*, *alsD*, *BDHI* was able to produce 2,3-BDO as a major product without ethanol accumulation but exhibited severe growth defects on glucose medium due to two major reasons. First, the Pdc^- strains cannot synthesize acetyl-CoA, which is

necessary precursor for cell growth, and thus exhibited retarded cell growth. As partial restoration of PDC activity can help to supply sufficient acetyl-CoA for cell growth, fine-tuning of PDC activity has been attempted. Kim et al. (2016) introduced the *PDC1* gene from the Crabtree-negative yeast *Candida tropicalis* (*Ct*) into a Pdc^- strain and optimized the expression levels of *CtPDC1*, thereby minimizing ethanol production while still maintaining synthesis of acetyl-CoA for cell growth. As a result, the growth was recovered and 2,3-BDO productivity increased by 2.3-fold as compared to that of the control strain not expressing the *CtPDC1* gene. Lee and Seo (2019) deleted only major isozymes of PDC and ADH ($\Delta PDC1$, $\Delta PDC6$, and $\Delta ADH1$) instead of eliminating all PDC isozymes to minimize ethanol production and prevent the growth defects. The resulting strain produced 14.9 g/L of 2,3-BDO (0.295 g 2,3-BDO/g glucose) and negligible amounts of ethanol in a batch fermentation from 50 g/L of glucose with no growth inhibition.

Second, the Pdc^- strains exhibited severe growth defects on glucose medium because of redox imbalance in the cytosol (Pronk et al. 1996). Elimination of PDC isozymes prevents re-oxidation of NADH generated in the glycolysis, so the accumulated NADH needs to be re-oxidized via respiration in mitochondria. However, as respiration is repressed by glucose in yeast, NADH accumulates in the Pdc^- strain. As a result, the Pdc^- strain exhibit slow cell growth and glucose consumption (Flikweert et al. 1996). To relieve the growth defects by redox imbalance on glucose, adaptive laboratory evolution (ALE) experiments with the Pdc^- strains identified mutations in *MTH1* (Lian et al. 2014; Oud et al. 2012). Mth1 is a transcription factor involved in glucose sensing, and it inhibits the expression of hexose transporter genes (*HXTs*). The mutations in Mth1 increased its stability and reduced the glucose consumption rate due to alleviation of glucose repression. Therefore, glycolytic fluxes can be managed not to cause severe redox imbalances in the cytosol

of the Pdc⁻ strain. Inverse engineering of the identified mutation in *MTH1* enabled decent growth of the Pdc⁻ strains on glucose.

The above-mentioned metabolic engineering strategies contributed to improve 2,3-BDO production without growth defects and minimize ethanol productions in the Adh⁻ or Pdc⁻ strains. However, substantial amounts of glycerol were produced as a byproduct in the Adh⁻ or Pdc⁻ strains expressing the 2,3-BDO biosynthetic pathway (Kim et al. 2016; Kim et al. 2015; Kim and Hahn 2015). In terms of redox balance, 1 mole of surplus NADH is generated via 2,3-BDO production from glucose (Syu 2001). *S. cerevisiae* produces ethanol and glycerol with coupled NAD⁺ regeneration to maintain redox balance in the cytosol. As ethanol production is blocked in Adh⁻ or Pdc⁻ strains, glycerol production is inevitably necessary to regenerate NAD⁺ (Bakker et al. 2001). However, glycerol production hinders efficient 2,3-BDO production as a substantial amount of carbon can be wastefully diverted to glycerol. Moreover, because the chemical properties of glycerol and 2,3-BDO are similar, downstream processing for purification can be complicated, which increases the cost. As such, reduction of glycerol accumulation during the production of 2,3-BDO in engineered yeast has been attempted through oxidizing surplus NADH. The heterologous expression of NADH oxidase that oxidizes NADH to NAD⁺ using molecular oxygen as an electron acceptor led to reduced glycerol accumulation. Kim et al. (2015) overexpressed the *Lactococcus lactis* (*Ll*) *noxE* gene encoding a water-forming NADH oxidase in a 2,3-BDO producing Pdc⁻ strain. The resulting strain exhibited a 23.8% higher yield of 2,3-BDO and a 65.3% lower yield of glycerol than the control strain not overexpressing the *noxE* gene. Kim et al. (2016) co-overexpressed the *CtPDC1* and *LlnoxE* genes in a 2,3-BDO producing Pdc⁻ strain. The glycerol yield of the resulting strain was only 12.3% of a control strain expressing the *CtPDC1* gene only. Because the *in vivo* enzymatic activity of NoxEp proportionally increased with the levels of

dissolve oxygen (DO) due to molecular oxygen acting as a substrate for the NADH oxidase reaction, glycerol accumulation significantly decreased under high aeration conditions. However, a substantial amount of acetoin also accumulated due to NADH-deficiency conditions caused by NoxEp and too much aerations. Therefore, DO levels were optimized by applying a two-stage agitation strategy to minimize glycerol and acetoin formations during 2,3-BDO production. As a result, the final 2,3-BDO titer reached 154.3 g/L with a productivity of 1.98 g/L·h and a yield of 0.40 g/g. However, 33.6 g/L of glycerol was still produced as a byproduct in fed-batch fermentation.

To eliminate glycerol accumulation, two isozymes (Gpd1, Gpd2) of glycerol-3-phosphate dehydrogenase (Gpd) converting dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P) had been eliminated in a 2,3-BDO producing Adh^- or Pdc^- strain (Kim et al. 2019; Kim and Hahn 2015). The 2,3-BDO yields increased by 54% in the Adh^- and Gpd^- strain as compared with the Adh^- strain, while the glucose consumption rate and 2,3-BDO productivity decreased by 50.0% and 24.4%, respectively (Kim and Hahn 2015). This result suggested that the redox imbalance caused by elimination of the Gpd isozymes exerted a negative effect on glucose consumption and 2,3-BDO productivity. To circumvent this problem, the *LlnoxE* was additionally overexpressed in the Adh^- and Gpd^- strain. The resulting strain exhibited 68% and 72% increases in the glucose consumption rate and 2,3-BDO productivity, respectively, as compared to a control strain not overexpressing the *LlnoxE* gene. The maximum 2,3-BDO titer by the Adh^- and Gpd^- strain expressing *LlnoxE* was 72.3 g/L with a productivity of 1.43 g/L·h and a yield of 0.41 g/g in flask fed-batch fermentation. Kim et al. (2019) also disrupted *GPD1* and *GPD2* and overexpressed the *LlnoxE* gene simultaneously in the 2,3-BDO producing Pdc^- strain. The resulting strain produced

108.6 g/L of 2,3-BDO with a yield (0.462 g/g glucose), corresponding to 92.4% of the theoretical yield, which is the highest yield from the engineered *S. cerevisiae* strains.

S. cerevisiae produces mixture of 2,3-BDO stereoisomers composing of (2R,3R)-BDO and *meso*-BDO in a ratio of 2:1 with a trace amount of (2S,3S)-BDO (Ehsani et al. 2009; Gonzalez et al. 2000). (2R,3R)-BDH (Bdh1p) converts (R)-acetoin to (2R,3R)-BDO and (S)-acetoin to *meso*-BDO in a NADH-dependent reaction, respectively. Meanwhile, D-arabinose dehydrogenase (Ara1p) can convert (R)-acetoin to *meso*-BDO and (S)-acetoin to (2S,3S)-BDO, respectively (Gonzalez et al. 2000). Besides, Bae et al. (2021) reported that Ypr1p, one of the aldo-keto reductases, can also contribute to convert (R)-acetoin to *meso*-2,3-BDO together with a previously known enzyme Ara1p, and identified that Ara1p and Ypr1p preferred NADPH to NADH as the cofactor. Interestingly, most 2,3-BDO producing Pdc⁻ *S. cerevisiae* strains can produce (2R,3R)-BDO with high optical purity (>97%) (Kim et al. 2014; Lian et al. 2014). Enantiopure (2R,3R)-BDO production by the engineered yeast might be attributed to the fact that the bacterial 2,3-BDO biosynthetic enzymes introduced in yeast have been optimized to produce (R)-acetoin (Kim et al. 2014). Moreover, even though Ara1p and Ypr1p could convert (R)-acetoin into *meso*-2,3-BDO, the (R)-acetoin might be preferentially converted into (2R,3R)-BDO due to the stereo-specificity of BDH (Bdh1p) and surplus NADH in the Pdc⁻ *S. cerevisiae* strains.

3. 2,3-BDO production from inexpensive substrates

Overall, much progress in metabolic engineering have been made for efficient microbial 2,3-BDO production. However, the production of 2,3-BDO from refined sugars such as glucose and sucrose may not be economically feasible because the cost of the sugar constitutes more than 30% of the total cost of 2,3-BDO production process (Cha et al. 2020; Li et al. 2014). Thus, inexpensive

biomasses such as crude glycerol, whey, inulin, and lignocellulose have been studied as a substrate for cost-effective 2,3-BDO production (Table 3). However, 2,3-BDO yields and productivities obtained by native microorganisms using these biomass-derived substrates are still low. As a result, there is still a need for metabolic engineering strategies to modify their endogenous biochemical pathways or to introduce heterologous pathways for achieving high 2,3-BDO yields and productivities from the biomass-derived substrates.

Crude glycerol is a by-product of biodiesel production and has been investigated as a substrate for 2,3-BDO production. *K. oxytoca* has been known to utilize glycerol through the oxidative pathway and produce 2,3-BDO (Ashok et al. 2013). However, in addition to the oxidative branch, glycerol is also metabolized through the reductive pathway, which results in the generation of 1,3-PDO as a byproduct. It may serve as an obstacle for achieving high 2,3-BDO yield and productivity from crude glycerol fermentation. Cho et al. (2015a) constructed *pudC* knockout mutant by deleting the gene encoding glycerol dehydratase (PduC), which is responsible for 1,3-PDO synthesis from glycerol. As a result, the *pudC* knockout mutant produced a negligible amount of 1,3-PDO (0.8 g/L) in comparison with the parental strain, but about 30 g/L of lactate was produced as a byproduct in fed-batch fermentation using crude glycerol. To reduce lactate production, a *pudC ldhA* double-knockout mutant by deleting the *ldhA* gene in the *pudC* knockout mutant was constructed. The resulting strain produced 131.5 g/L of 2,3-BDO with a productivity of 0.84 g/L·h and a yield of 0.44 g/g without 1,3-PDO production in fed-batch fermentation using crude glycerol, which were 78.2%, 23.5%, and 4.7% higher than the fed-batch fermentation result using wild-type *K. oxytoca* strain.

Whey is a byproduct of the dairy industry, and it usually contains about 5% lactose and 1% protein. Economic disposal of whey has become a critical problem for the dairy industry.

Utilization of the lactose in whey as a substrate for microbial 2,3-BDO production needs to be explored in aspects of transforming a potential waste into a value-added product. Meng et al. (2020) evaluated the lactose utilization capability of *K. pneumonia*, *K. oxytoca*, *Enterobacter cloacae*, *B. licheniformis*, and *E. coli* strains to identify a suitable strain for 2,3-BDO production. As a result, *K. oxytoca* exhibited the best performance in lactose utilization and BDO production among the strains. However, 2,3-BDO yield only reached 56 % of the theoretical yield (0.29 vs. 0.53 g/g lactose) because acetate, succinate, lactate, and formate were accumulated as byproducts in batch fermentation. After deleting the genes *pox*, *pta*, *frdA*, *ldhD*, and *pflB* responsible for acetate, succinate, lactate, and formate production, the resulting strain exhibited 24% higher 2,3-BDO yields than that of a parental strain without decreasing the lactose consumption rate in batch fermentation. Finally, 2,3-BDO titer reached 74.9 g/L with a productivity of 2.27 g/L·h and a yield of 0.43 g/g from lactose in fed-batch fermentation. In addition, when whey powder was used as the substrate, 65.5 g/L of 2,3-BDO was produced with a productivity of 2.73 g/L·h and a yield of 0.44 g/g.

Inulin is a storage polysaccharide present in numerous plants, such as Jerusalem artichoke and chicory. Inulin consists of linear chains of β -2,1-linked D-fructofuranose molecules terminated by a glucose residue and can be hydrolyzed by inulinase into fructose and glucose. However, most inulinases have an optimum temperature in the range of 45°C to 55°C. Utilization of a mesophilic 2,3-BDO producer may require increased dosage of inulinase when producing 2,3-BDO from inulin. Therefore, the simultaneous saccharification and fermentation (SSF) process with a thermophilic 2,3-BDO producer that has endogenous inulinases activity may decrease the dosage of the enzyme, leading to cost-effective 2,3-BDO production from inulin. Li et al (2014) evaluated the fructose utilization capability of several *B. licheniformis* strains to identify a suitable strain for

2,3-BDO production using inulin. As a result, *B. licheniformis* ATCC 14580 strain was found to produce 2,3-BDO from fructose at 50°C and exhibited highest concentration of 2,3-BDO among the strains. Besides, the *B. licheniformis* strain has an endogenous *sacC* gene encoding inulinases, which is required to produce 2,3-BDO from inulin. Finally, inulin hydrolysate has been used for 2,3-BDO production by thermophilic *B. licheniformis* ATCC 14580 strain, with a titer of 103 g/L and a high productivity of 3.4 g/L·h in fed-batch SSF process.

Lignocellulosic biomass is mainly composed of polysaccharides such as cellulose and hemicellulose. Depending on the pretreatment and hydrolysis methods used to release sugars from lignocellulosic biomass, the sugar composition of hydrolysates can be varied but generally contains mixed sugars including hexoses (glucose and galactose) and pentoses (xylose, arabinose, and ribose). However, most native microorganisms exhibit inefficient consumption of mixed sugars from hydrolysates. One major reason for the inefficient sugar consumption is carbon catabolite repression, where the utilization of xylose, galactose, or arabinose is repressed until depletion of glucose. As a result, sequential utilization or diauxic growth is observed during mixed sugar fermentation, leading to low yield and productivity for the final products (Wu et al. 2016). Cha et al. (2020) sought to engineer *K. oxytoca* to utilize glucose and other sugars (xylose, galactose, and mannose) derived from biomass hydrolysates. To facilitate efficient xylose uptake, the *xyIE* gene encoding for a xylose transporter from *E. coli* was introduced, and the *mgsA* gene encoding for methylglyoxal synthase A (MgsA) was deleted. MgsA, an enzyme initiating the methylglyoxal pathway, converts DHAP to methylglyoxal, which is known as an inhibitor of sugar metabolism. To further enhance xylose consumption rate, the resulting strain was evolved in a medium containing xylose as a sole carbon source. The evolved strain exhibited 1.5- and 1.6-fold higher xylose consumption rate (1.95 vs 1.30 g/L·h) and 2,3-BDO productivity (0.59 vs 0.37 g/L·h)

than those of a parental strain. In addition, the evolved strain showed much improved consumption rates of glucose, xylose, galactose, and mannose in the pine tree hydrolysates, and finally 2,3-BDO productivity increased by 3.2-fold (0.73 vs 0.24 g/L·h), compared to wild-type *K. oxytoca*.

There have also been efforts to produce 2,3-BDO from lignocellulosic sugars by yeast. Kim et al. (2014) engineered *S. cerevisiae* capable of producing 2,3-BDO from xylose. The heterologous xylose oxidoreductase pathway composed of xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Scheffersomyces stipitis* and the endogenous xylulose kinase (XK) were overexpressed in an evolved Pdc⁻ strain of *S. cerevisiae*. Additionally, the heterologous 2,3-BDO biosynthetic pathway from *B. subtilis* was introduced into the Pdc⁻ *S. cerevisiae* capable of metabolizing xylose. The resulting strain produced 20.7 g/L of 2,3-BDO from xylose with a yield of 0.27 g/g and a productivity of 0.18 g/L·h in batch fermentation. In the following study, Kim et al. (2017b) customized the XR/XDH pathway by expressing heterologous transaldolase and the NADH-preferring XR from *Scheffersomyces stipitis* to further improve xylose consumption efficiency, resulting in improved 2,3-BDO production from xylose. The resulting strain showed 2.1-fold and 1.8-fold higher xylose consumption rate and 2,3-BDO production as compared to the parental strain. To alleviate redox imbalance and acetyl-CoA deficiency in the resulting strain, *LlnoxE* and *CtPDC1* genes were additionally introduced. In fed-batch fermentation, the final engineered strain produced 96.8 g/L of 2,3-BDO from xylose with a productivity of 0.58 g/L·h.

As another component in cellulosic biomass, cellobiose consisting of two units of glucose link with a β -1,4-glucosidase bond does not exert catabolite repression on the metabolism of other sugars. Engineering *S. cerevisiae* able to utilize cellobiose could be a promising strategy for simultaneous and efficient conversion of mixed sugars in lignocellulosic biomass. Nan et al. (2014) engineered *S. cerevisiae* capable of producing 2,3-BDO from cellobiose. The genes encoding a

cellodextrin transporter (*cdt-1*) and intracellular β -glucosidase (*ghl-1*) from the cellulolytic fungus *Neurospora crassa* were introduced into an evolved Pdc⁻ *S. cerevisiae* along with overexpression of the genes for 2,3-BDO biosynthesis from *B. subtilis*. The resulting strain produced 5.29 g/L of 2,3-BDO from cellobiose with a productivity of 0.22 g/L·h and a yield of 0.29 g/g, which suggested the possibility of sustainable and efficient 2,3-BDO production from cellulosic hydrolysates.

4. Conclusions and future perspectives

Microbial production of 2,3-BDO is an attractive option to substitute petroleum-based chemical processes considering economic and environmental sustainability. In the past decade, metabolic engineering approaches have facilitated efficient 2,3-BDO production by native and non-native 2,3-BDO producing microorganisms. However, for safe and cost-effective production of bio-based 2,3-BDO at large scales, the host strain needs to exhibit non-pathogenicity as well as high production efficiency. For these reasons, *K. oxytoca*, *Bacillus* spp., and *S. cerevisiae* have been employed as a host strain and extensively engineered to produce 2,3-BDO with the following metabolic designs: 1) overexpression of rate-limiting 2,3-BDO biosynthetic enzymes, 2) blocking of competing byproduct pathways to increase 2,3-BDO yield, 3) restoring the redox imbalance caused by the elimination of byproducts production via aeration or supplementary pathway, and 4) coordination of cofactor production that redistributes carbon flux toward 2,3-BDO pathway.

Fascinating metabolic engineering strategies have been also established to produce enantiopure 2,3-BDO stereoisomers in addition to using renewable biomass such as crude glycerol, whey, inulin, and lignocellulose as a substrate for 2,3-BDO production. Besides, advanced metabolic engineering tools such as CRISPR-Cas9 have allowed genome editing of genetically intractable industrial host strain, resulting in improvement of 2,3-BDO production from renewable

biomass. For instance, an industrial polyploid *S. cerevisiae* has various advantages for large-scale fermentation with a biomass hydrolysate due to its fast growth rate, sugar metabolism, and high tolerance against fermentation inhibitors, but its genetic manipulation has been limited. Lee and Seo (2019) manipulated the genome of industrial polyploid *S. cerevisiae* using the CRISPR-Cas9 genome editing tool and obtained comparable 2,3-BDO production from cassava hydrolysate with 2,3-BDO producing bacteria such as *K. oxytoca*.

As only few reports are available on techno-economic assessment (TEA) of bio-based 2,3-BDO production, it is difficult to evaluate the feasibility of industrial production of 2,3-BDO. Although previous metabolic engineering endeavors enabled efficient 2,3-BDO production via microbial fermentation, insufficient 2,3-BDO productivity as compared to 2,3-BDO titer and yield might be one of the hurdles for establishment of industrial 2,3-BDO plant (Fig. 3) (Van Dien 2013). As volumetric productivity determines the overall fermentation volume needed for a given plant output, low productivity requires high volume fermenters, which are a significant portion of the plant capital investment, to achieve the given output. Depending on the selling price of the target chemical, the minimum productivity required for commercialization can be varied. However, productivities below 2.0 g/L·h are generally not considered economically viable due to their high capital cost (Van Dien 2013), while a productivity of 3.5 g/L·h has been reported for a commercial 1,3-propanediol (Nakamura and Whited 2003). As such, continued research is required to improve 2,3-BDO productivity. Exploring an additional metabolic or supplementary pathway in addition to NADH oxidase and environmental perturbations that can solve the redox imbalance caused by elimination of byproducts production will be necessary. When the surplus NADH can be efficiently re-oxidized via metabolic rewiring, the 2,3-BDO productivity as well as 2,3-BDO yield will be further improved.

Overall, the recent advances in metabolic engineering of the microorganisms to enable efficient and enantiopure production of 2,3-BDO endow the fermentation-based production of 2,3-BDO as an attractive option to substitute the chemical processes.

List of abbreviations

2,3-BDO, 2,3-butanediol; **BDH**, 2,3-butanediol dehydrogenase; **ALS**, α -acetolactate synthase; **ALDC**, α -acetolactate decarboxylase; **DAR**, diacetyl reductase; **FBA**, flux balance analysis; **PFL**, pyruvate formate lyase; **PDH**, pyruvate dehydrogenase; **FDH**, formate dehydrogenase; **V_c**, vitamin C; **ADH**, alcohol dehydrogenase; **Adh⁻**, ADH-deficient; **Pdc⁻**, PDC-deficient; **ALE**, adaptive laboratory evolution; **DHAP**, dihydroxyacetone phosphate; **G3P**, glycerol-3-phosphate; **PEPC**, phosphoenolpyruvate carboxylase; **MDH**, malate dehydrogenase; **FH**, fumarase; **FRD**, fumarate reductase; **LDH**, lactate dehydrogenase; **ALD**, aldehyde dehydrogenase; **PTA**, phosphate acetyltransferase; **ACK**, acetate kinase; **AR**, aldose reductase; **TPI**, triose phosphate isomerase; **GPDH**, glycerol-3-phosphate dehydrogenase; **SSF**, simultaneous saccharification and fermentation; **XR**, xylose reductase; **XDH**, xylitol dehydrogenase; **XK**, xylulose kinase; **TEA**, Techno-economic assessment.

Author Contributions

J.W.L reviewed the literature and wrote the manuscript. Y.-G.L and Y.-S.J. critically read, revised, and improved the manuscript. C.V.R. conceived the idea, reviewed, and supervised the study. All authors read and approved the manuscript.

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Figure captions

Fig. 1. Overview of 2,3-BDO biosynthetic pathways in (A) bacteria and (B) yeast. The dashed line represents a spontaneous reaction which is activated under aerobic conditions. PEPC, phosphoenolpyruvate carboxylase; MDH, malate dehydrogenase; FH, fumarase; FRD, fumarate reductase; LDH, lactate dehydrogenase; PFL, pyruvate formate-lyase; PDH, pyruvate dehydrogenase; ALD, aldehyde dehydrogenase; ADH, alcohol dehydrogenase; PTA, phosphate acetyltransferase; ACK, acetate kinase; ALS, α -acetolactate synthase; ALDC, α -acetolactate decarboxylase; BDH, 2,3- butanediol dehydrogenase; DAR, diacetyl reductase; AR, aldose reductase; TPI, triose phosphate isomerase; GPDH, glycerol-3-phosphate dehydrogenase.

Fig. 2. Biosynthetic pathway for the production of three 2,3-BDO stereoisomers. The dashed line represents a spontaneous reaction which is activated under aerobic conditions. DAR, diacetyl reductase; BDH, 2,3-butanediol dehydrogenase.

Fig. 3. Summary of 2,3-BDO titers, yields, and productivities reported in previous studies. [1] Ishii et al (2018), [2] Kim et al (2013b), [3] Dai et al (2014), [4] Qiu et al (2016), [5] Jantama et al (2015), [6] Kim and Hahn (2015), [7] Cho et al (2015b), [8] Song et al (2020), [9] Kim et al (2019), [10] Ji et al (2010), [11] Ji et al (2009), [12] Lee and Seo (2019), [13] Kim et al (2016), [14] Park et al (2013b), [15] Kim et al (2013), [16] Li et al (2013), [17] Ge et al (2016), and [18] Park et al (2015). The direction of the arrow means the desired 2,3-BDO titer, yield, and productivity for industrial 2,3-BDO production.

Table 1. Summarized results of 2,3-BDO production in engineered microorganisms

Host strain	Engineering strategy		Culture method		2,3-BDO production			References
	Genome modification	Overexpressed genes	Substrate	Type	Conc. (g/L)	Yield (g/g)	Productivity (g/L·h)	
Engineered 2,3-BDO producing bacteria								
<i>K. oxytoca</i> ME-UD-3	<i>^mldhA, ^mpta</i>	–	Glucose	Batch	42.0	N.M	N.M.	(Ji et al. 2008)
<i>K. oxytoca</i> ME-UD-3	<i>^mldhA, ^mpta</i>	–	Glucose	Batch ^a	95.5	0.48	1.71	(Ji et al. 2009)
<i>K. oxytoca</i> ME-XJ-8	<i>^mldhA, ^mpta, ΔaldA</i>	–	Glucose	Fed-batch	130	0.48	1.63	(Ji et al. 2010)
<i>K. oxytoca</i>	<i>ΔldhA</i>	–	Glucose	Batch	29.9	0.33	1.07	(Park et al. 2013a)
<i>K. oxytoca</i>	<i>ΔldhA, ΔpflB</i>	–	Glucose	Fed-batch ^a	113	0.45	2.10	(Park et al. 2013b)
<i>K. oxytoca</i> GSC 12206	<i>ΔldhA</i>	–	Glucose	Fed-batch	115	0.41	2.27	(Kim et al. 2013)
<i>K. oxytoca</i> KMS005-73T	<i>ΔadhE, ΔackA-pta ΔldhA</i>	–	Glucose	Fed-batch ^a	117.4	0.49	1.20	(Jantama et al. 2015)
<i>K. oxytoca</i> M1	–	<i>budC</i>	Glucose	Fed-batch	142.5	0.42	1.47	(Cho et al. 2015b)
<i>B. subtilis</i> BSF20	–	<i>EcudhA</i>	Glucose	Batch	49.3	0.47	0.22	(Fu et al. 2014)
<i>B. subtilis</i> AFYL	<i>ΔyodC</i>	<i>^{Cb}fdh</i>	Glucose	Batch	44.0	N.M	N.M	(Yang et al. 2015)
<i>B. amyloliquefaciens</i> B10-127	–	<i>bdh, gapA</i>	Glucose	Fed-batch	132.9	N.M.	2.95	(Yang et al. 2013)
<i>B. licheniformis</i> 10-1-A	–	–	Glucose	Fed-batch	115.7	0.47	2.40	(Li et al. 2013)

Table 1. Summarized results of 2,3-BDO production in engineered microorganisms (Continued)

Host strain	Engineering strategy		Culture method		2,3-BDO production			References
	Genome modification	Overexpressed genes	Substrate	Type	Conc. (g/L)	Yield (g/g)	Productivity (g/L·h)	
Engineered 2,3-BDO producing bacteria								
<i>Paenibacillus polymyxa</i> CJX518	–	–	Glucose	Fed-batch	71.7	0.39	0.81	(Dai et al. 2014)
Engineered 2,3-BDO producing yeasts								
<i>S. cerevisiae</i> D452-2	$\Delta PDC1, \Delta PDC5, \Delta PDC6, ^tMTH1$	–	Glucose	Fed-batch	81.0	0.27	0.16	(Ishii et al. 2018)
<i>S. cerevisiae</i> D452-2	$\Delta PDC1, \Delta PDC5, MTH1A81P$	^{Bs} <i>alsS</i> , ^{Bs} <i>alsD</i> , ^{Sc} <i>BDH1</i>	Glucose	Fed-batch	96.2	0.28	0.39	(Kim et al. 2013b)
<i>S. cerevisiae</i> D452-2	$\Delta PDC1, \Delta PDC5, \Delta PDC6$	^{Bs} <i>alsS</i> , ^{Bs} <i>alsD</i> , ^{Sc} <i>BDH1</i> , ^{Ll} <i>noxE</i>	Glucose	Batch	29.4	0.37	0.40	(Kim et al. 2015)
<i>S. cerevisiae</i> D452-2	$\Delta PDC1, \Delta PDC5, \Delta PDC6$	^{Ct} <i>PDC1</i> , ^{Bs} <i>alsS</i> , ^{Bs} <i>alsD</i> , ^{Sc} <i>BDH1</i> , ^{Ll} <i>noxE</i>	Glucose	Fed-batch ^a	154.3	0.40	1.97	(Kim et al. 2016)
<i>S. cerevisiae</i> CEN.PK2-1	$\Delta ADH1\sim5, \Delta GPD1, \Delta GPD2$	^{Bs} <i>alsS</i> , ^{Bs} <i>alsD</i> , ^{Sc} <i>BDH1</i> , ^{Ll} <i>noxE</i>	Glucose	Fed-batch	72.9	0.41	1.43	(Kim and Hahn 2015)
<i>S. cerevisiae</i> D452-2	$\Delta PDC1, \Delta PDC5, \Delta PDC6, \Delta GPD1, \Delta GPD2$	^{Ct} <i>PDC1</i> , ^{Bs} <i>alsS</i> , ^{Bs} <i>alsD</i> , ^{Sc} <i>BDH1</i> , ^{Ll} <i>noxE</i>	Glucose	Fed-batch	108.6	0.46	1.55	(Kim et al. 2019)
<i>S. cerevisiae</i> 4-JHS200	$\Delta PDC1, \Delta PDC6, \Delta ADH1$	^{Bs} <i>alsS</i> , ^{Bs} <i>alsD</i> , ^{Sc} <i>BDH1</i> , ^{Ll} <i>noxE</i>	Glucose	Fed-batch ^a	178	0.34	1.88	(Lee and Seo 2019)

Genes coding for enzymes as follows: *ldhA*, lactate dehydrogenase; *pta*, phosphotransacetylase; *aldA*, aldehyde dehydrogenase; *pflB*, pyruvate formate dehydrogenase; *adhE* and *ADH1*, alcohol dehydrogenase E; *ack-pta*, acetate kinase A phosphotransacetylase; *budC*, acetoin reductase; *udhA*, transhydrogenase; *yodC* and *noxE*, NADH oxidase; *fdh*, formate dehydrogenase; *bdh* and *BDH1*, 2,3-BDO dehydrogenase; *gapA*, glyceraldehyde-3-phosphate dehydrogenase; *alsS* and *ILV2*, α -acetolactate synthase; *alsD*, α -acetolactate decarboxylase; *PDC1*, *PDC5*, and *PDC6*; pyruvate decarboxylase; *MTH1*, transcriptional regulator involved in the glucose-sensing signal transduction pathway; *GPD1*, *GPD2*, glyceraldehyde-3-phosphate dehydrogenase; The superscripts in the gene names present as follows: *Ec*, *Escherichia coli*; *Cb*, *Candida boidinii*; *Bs*, *Bacillus subtilis*; *Sc*, *Saccharomyces cerevisiae*; *Ll*, *Lactococcus lactis*; *Ct*, *Candida tropicalis*; t, truncated. m, UV mutagenesis.

^aTwo-stage agitation speed control strategy. N.M. not mentioned.

Table 2. Summarized results of enantiopure 2,3-BDO production in engineered microorganisms

Host strain	Engineering strategy	BDH ^a	Substrate (Culture type)	2,3-BDO production				Reference
				Stereo-specificity	Conc. (g/L)	Yield (g/g)	Productivity (g/L·h)	
<i>K. oxytoca</i>	$\Delta ldhA, \Delta pflB, \Delta budC$	<i>ppbdh</i>	Glucose (Fed-batch)	2R,3R-BDO (M ^b)	106.7	0.40	3.10	(Park et al. 2015)
				<i>meso</i> -BDO (m ^c)	9.3	0.04	0.27	
<i>B. licheniformis</i> MW3	$\Delta budC$	<i>gdh</i>	Glucose (Fed-batch)	2R,3R-BDO (specificity > 99%)	123.7	N.M	2.94	(Ge et al. 2016)
<i>B. licheniformis</i> MW3	Δgdh	<i>budC</i>	Glucose (Fed-batch)	<i>meso</i> -BDO (specificity > 99%)	90.1	0.49	2.81	(Ge et al. 2016)
<i>B. licheniformis</i> WX-02	$\Delta budC$	<i>gdh</i>	Glucose (Batch)	2R,3R-BDO	30.8	N.M	1.28	(Qi et al. 2014)
<i>B. licheniformis</i> WX-02	$\Delta gdh, \Delta acoR$	<i>budC</i>	Glucose (Fed-batch)	<i>meso</i> -BDO (specificity > 99%)	98.0	0.40	0.94	(Qiu et al. 2016)
<i>B. licheniformis</i> 4071	$\Delta budC$	<i>gdh</i>	Glucose (Fed-batch)	2R,3R-BDO (M ^b)	96.3	0.39	1.34	(Song et al. 2020)
				<i>meso</i> -BDO (m ^c)	9.33	0.04	0.13	
<i>Parageobacillus Thermoglucosidasius</i> DSM2542 ^T	$^{Bs}alsS, ^{St}alsD,$	<i>bdhA</i>	Glucose (Batch)	2R,3R-BDO	7.2	0.36	0.15	(Zhou et al. 2020)
<i>K. pneumoniae</i> CICC 10011 and <i>B. subtilis</i> 168			Glucose (Batch)	2S,3S-BDO	12.5	0.36	N.M.	(Liu et al. 2011)
<i>S. cerevisiae</i> D452-2	$\Delta PDC1, \Delta PDC5, ^{Bs}alsS, ^{Bs}alsD, ^{Ss}XYL1, ^{Ss}XYL2, ^{Ss}XYL3$	<i>BDH1</i>	Xylose (Fed-batch)	2R,3R-BDO (specificity > 97%)	43.6	0.27	0.20	(Kim et al. 2014)

Table 2. Summarized results of enantiopure 2,3-BDO production in engineered microorganisms (Continued)

Host strain	Engineering strategy	BDH ^a	Substrate (Culture type)	2,3-BDO production				Reference
				Stereo-specificity	Conc. (g/L)	Yield (g/g)	Productivity (g/L·h)	
<i>S. cerevisiae</i> D452-2	Δ <i>PDC1</i> , Δ <i>PDC5</i> , Δ <i>PDC6</i> , ^{Scyto} <i>ILV2</i> , ^{Bs} <i>alsD</i> , ^{MTHI}	<i>BDHI</i>	Glucose, Galactose (Fed-batch)	2R,3R-BDO (specificity > 97%)	100	N.M.	N.M.	(Lian et al. 2014)

Genes coding for enzymes as follows: *ldhA*, lactate dehydrogenase; *pflB*, pyruvate formate dehydrogenase; *budC*, acetoin reductase; *bdh*, 2,3-BDO dehydrogenase; *gdh*, glycerol dehydrogenase; *acoR*, transcriptional regulator of acetoin metabolism; *alsS*, α -acetolactate synthase; *alsD*, α -acetolactate decarboxylase. The superscripts in the gene names present as follows: *Pp*, *Paenibacillus polymyxa*; *St*, *Streptococcus thermophilus*.

^abutanediol dehydrogenase. ^bMajor product. ^cminor product. N.M. not mentioned.

Table 3. Summarized results of enantiopure 2,3-BDO production from abundant and cheap biomass.

Host strain	Engineering strategy	Culture method		2,3-BDO production			References	
	Genome modification/ Overexpressed genes	Substrate	Type	Stereo-specificity	Conc. (g/L)	Yield (g/g)		Productivity (g/L·h)
Engineered 2,3-BDO producing bacteria								
<i>K. oxytoca</i> ME-XJ-8	<i>crp</i>	Glucose-Xylose (G ^a :40, X ^b :20 g/L)	Batch	<i>meso</i> -2,3- BDO	23.9	0.44	0.70	(Ji et al. 2011b)
<i>K. oxytoca</i> M3	$\Delta pduC, \Delta ldhA$	Glycerol	Fed-batch	<i>meso</i> -2,3- BDO	131.5	0.44	0.84	(Cho et al. 2015a)
<i>K. oxytoca</i> UM2-17	–	Agave baggasse (G ^a :19.3, X ^b :6.3 g/L)	Batch	<i>meso</i> -2,3- BDO	10.3	0.40	0.43	(Pasaye-Anaya et al. 2019)
<i>K. oxytoca</i> CHA006	<i>xylE</i>	Sunflower	Batch	<i>meso</i> -2,3- BDO	6.11	0.34	0.76	(Cha et al. 2020)
		Pine tree (G ^a :12.5, X ^b :2, M ^c :5 g/L)	Batch	<i>meso</i> -2,3- BDO	5.82	0.30	0.73	
<i>K. oxytoca</i> PDL-K5	$\Delta pox\Delta pta\Delta frdA\Delta ldhD$ $\Delta pflB$	Lactose	Fed-batch	<i>meso</i> -2,3- BDO	74.9	0.43	2.27	(Meng et al. 2020)
		Whey power	Fed-batch	<i>meso</i> -2,3- BDO	65.5	0.44	2.73	
<i>K. oxytoca</i> BRC1	<i>bdh</i>	Empty palm Fruit bunches (G ^a :39.1, X ^b :17.3 g/L)	Batch	<i>meso</i> -2,3- BDO	N.M.	N.M.	N.M.	(Kang et al. 2015)
<i>B.</i> <i>licheniformis</i> ATCC 14580	–	Inulin	Fed-batch	<i>meso</i> -2,3- BDO (2R,3R)- BDO	103.0	N.M.	3.4	(Li et al. 2014)
<i>P. polymyxa</i> ZJ-9	–	Inulin (76.27 g/L)	Batch	(2R,3R)- BDO	36.86	0.48	0.88	(Gao et al. 2010)

Table 3. Summarized results of enantiopure 2,3-BDO production from abundant and cheap biomass (Continued).

Host strain	Engineering strategy	Culture method		2,3-BDO production			References	
	Genome modification Overexpressed genes	Substrate	Type	Stereo-specificity	Conc. (g/L)	Yield (g/g)		Productivity (g/L·h)
Engineered 2,3-BDO producing yeasts								
<i>S. cerevisiae</i> D452-2	$\Delta PDC1$, $\Delta PDC5$, ^{Bs} <i>alsS</i> , ^{Bs} <i>alsD</i> , ^{Sc} <i>BDH1</i> , ^{Ss} <i>XYL1</i> , ^{Ss} <i>XYL2</i> , ^{Ss} <i>XYL3</i>	Xylose	Fed-batch	(2R,3R)- BDO	43.6	0.27	0.20	(Kim et al. 2014)
<i>S. cerevisiae</i> D452-2	$\Delta PDC1$, $\Delta PDC5$, $\Delta PDC6$, ^{Ci} <i>PDC1</i> , ^{Bs} <i>alsS</i> , ^{Bs} <i>alsD</i> , ^{Sc} <i>BDH1</i> , ^{Ss} <i>XYL1m</i> , ^{Ss} <i>XYL2</i> , ^{Ss} <i>XYL3</i> , ^{Ss} <i>TAL</i> , ^{L1} <i>noxE</i>	Xylose	Fed-batch	(2R,3R)- BDO	96.8	N.M.	0.58	(Kim et al. 2017b)
<i>S. cerevisiae</i> D452-2	$\Delta PDC1$, $\Delta PDC5$, ^{Bs} <i>alsS</i> , ^{Bs} <i>alsD</i> , ^{NC} <i>cdt1</i> , ^{NC} <i>gh1-1</i>	Cellobiose (20 g/L)	Batch	(2R,3R)- BDO	5.3	0.29	0.22	(Nan et al. 2014)
<i>S. cerevisiae</i> CEN.PK2-1	$\Delta PDC1$, $\Delta PDC5$, $\Delta PDC6$, ^{Sscyto} <i>ILV2</i> , ^{Bs} <i>alsD</i> , ^{Sc} <i>BDH1</i> , ⁱ <i>MTH1</i>	Glucose, galactose	Fed-batch	(2R,3R)- BDO	100	N.M.	N.M.	(Lian et al. 2014)
<i>S. cerevisiae</i> D452-2	$\Delta PDC1$, $\Delta PDC5$, $\Delta PDC6$, $\Delta GPD1$, $\Delta GPD2$, ^{Ci} <i>PDC1</i> , ^{Bs} <i>alsS</i> , ^{Bs} <i>alsD</i> , ^{Sc} <i>BDH1</i> , ^{L1} <i>noxE</i>	Cassava hydrolysate (G ^a : 121.2 g/L)	Batch	(2R,3R)- BDO	47.4	0.39	1.03	(Kim et al. 2019)
<i>S. cerevisiae</i> 4-JHS200	$\Delta PDC1$, $\Delta PDC6$, $\Delta ADH1$, ^{Bs} <i>alsS</i> , ^{Bs} <i>alsD</i> , ^{Sc} <i>BDH1</i> , ^{L1} <i>noxE</i>	Cassava hydrolysate	Fed-batch	(2R,3R)- BDO	132	0.32	1.92	(Lee and Seo 2019)

Genes coding for enzymes as follows: *crp*, the mutant cyclic adenosine monophosphate (cAMP) receptor protein CRP; *pduC*, glycerol dehydratase large subunit; *xylE*, xylose transporter; *pox*, pyruvate dehydrogenase; *frdA*, fumarate reductase flavoprotein subunit; *XYL1*, xylose reductase; *XYL2*, xylitol dehydrogenase; *XYL3*, xylulose kinase; *TAL*, transaldolase; *cdt1*, cellodextrin transporter; *gh1-1*, intracellular β -glucosidase; The superscripts in the gene names present as follows: ^{Sscyto}, *S. cerevisiae* cytosolic.

^aG: Glucose, ^bX: Xylose. ^cM: Mannose. N.M. not mentioned.