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Metabolic Engineering and Synthetic Biology – Review

Rewiring the microbial metabolic network for efficient utilization of mixed carbon sources

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Abstract: Carbon sources represent the most dominant cost factor in the industrial biomanufacturing of products. Thus, it has attracted much attention to seek cheap and renewable feedstocks, such as lignocellulose, crude glycerol, methanol, and carbon dioxide, for biosynthesis of value-added compounds. Co-utilization of these carbon sources by microorganisms not only can reduce the production cost but also serves as a promising approach to improve the carbon yield. However, co-utilization of mixed carbon sources usually suffers from a low utilization rate. In the past few years, the development of metabolic engineering strategies to enhance carbon source co-utilization efficiency by inactivation of carbon catabolite repression has made significant progress. In this article, we provide informative and comprehensive insights into the co-utilization of two or more carbon sources including glucose, xylose, arabinose, glycerol, and C1 compounds, and we put our focus on parallel utilization, synergetic utilization, and complementary utilization of different carbon sources. Our goal is not only to summarize strategies of co-utilization of carbon sources, but also to discuss how to improve the carbon yield and the titer of target products.

Keywords: Co-utilization, Carbon source, Lignocellulosic hydrolysates, Crude glycerol, Methanol

Introduction

Metabolic engineering of microorganisms for converting simple carbon sources into value-added products has made great progress over the past decade. To make these biological processes outcompete chemical synthesis, the research focus now is shifting into the utilization of renewable, abundant, and cheap feedstocks to produce target products at higher titer, yield, and productivity. Lignocellulose is the most abundant renewable feedstock on earth (Alio et al., 2019; Kumar et al., 2017; Rol et al., 2019). Employment of lignocellulosic biomass as a carbon source to produce value-added compounds does not compete with the global food supply (Heo et al., 2019; Nanda et al., 2015). Lignocellulose hydrolysates mainly consist of glucose, xylose, and arabinose (Yu et al., 2018). For cost-effective generation of target products using lignocellulose hydrolysates, construction of efficient microbial cell factories for utilization of one or more of these monosaccharides has gained much attention. However, glucose serves as the preferred carbon source for most microorganisms, the presence of glucose severely impairs metabolism of other carbon sources such as xylose, arabinose, and glycerol, due to carbon catabolite repression (CCR) (Fujiwara et al., 2020). In recent years, various strategies including adaptive evolution of host strains, deletion of phosphotransferase system (PTS), evolution of transporter proteins, overexpression of sugar transporters, and replacement of the promoters of genes encoding pentose catabolic pathways have been developed to achieve efficient carbon sources' parallel utilization by removal of the CCR (Chiang et al., 2013; Gonzalez and Antoniewicz, 2017; Kim et al., 2015; Reider Apel et al., 2016; Wang et al., 2018; Young et al., 2012). Moreover, in some cases, the mixed sugar co-utilization efficiency can be further improved by construction of a synergetic utilization mechanism based on the unique metabolic characteristic for each carbon source. For instance, in order to enhance the carbon yield, the glycolysis pathway was blocked to achieve noncatabolic utilization of glucose as a skeleton molecule for production of polysaccharides and glycosylated compounds (Wu et al., 2017). However, efficient transportation of glucose into the cells requires phosphoenolpyruvate (PEP) as the cofactor (Choe et al., 2017; Long et al., 2017). Owing to the high efficiency for generating PEP, glycerol was selected as the second carbon source to support cell growth and drive glucose uptake (Tang et al., 2020; Wu et al., 2017; Wu et al., 2018). By designing and establishing such a SynCar, the titer and yield of glucose-derived products can be significantly increased.

In addition to lignocellulose, several cheap and accessible C1 carbon sources including methanol and carbon dioxide (CO_2) also serve as promising substrates, moreover, methanol can offer extra reducing power in industrial biomanufacturing. However, C1 compounds are difficult to use as the sole carbon source for cell growth and product formation by most of microbes, due to their limited metabolic efficiencies in heterotrophic microorganisms (Dai et al., 2017; Lesmeier et al., 2015; Muller et al., 2015). As an alternative, the introduction of another sugar to support cell growth and drive methanol or CO_2 uptake serves as an attractive strategy. In recent years, the development of mixed feedstock co-utilization mechanisms for efficient production of desired chemicals has made great progress. Our intent with this review is to summarize recent advances in co-utilization of carbon sources for high-performance generation of value-added compounds, with emphasis on the applied metabolic engineering strategies for co-utilization of two or more carbon sources, including glucose, xylose, arabinose,

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glycerol, methanol, and CO_2 (Table 1). We focus on rational utilization of these carbon sources, especially parallel utilization, synergetic utilization, and complementary utilization (Fig. 1), for improving titer, yield, and productivity of target production. We also discuss the current challenges confronted in this research area, and intend to provide future perspectives for economical biosynthesis of target products using cheap and renewable feedstocks.

Metabolic Engineering Strategies for Co-utilization of Glucose, Xylose, and Arabinose

Although most microbes can use xylose or arabinose as the sole carbon source to support cell growth, the biomass formation rate is much lower than using glucose (Martínez et al., 2008; Xia et al., 2012). Moreover, simultaneous utilization of glucose, xylose, and arabinose causes CCR (Fig. 2), resulting in diauxic growth with a long lag duration occurring between growth phases (Ammar et al., 2018; Fujiwara et al., 2020). These issues have limited the commercial assimilation of lignocellulosic biomass through biological processes. To release CCR and promote efficient co-utilization of these sugars, inactivation of genes involved in PTS including *ptsG* (encoding enzyme $IIBC^{Glc}$), *crr* (encoding enzyme IIA^{Glc}), evolution of transporter proteins, and pentose transporter regulators such as *xylR* (encoding xylose transcriptional activator) and *araC* (encoding L-arabinose transcriptional regulator) are commonly used strategies (Ammar et al., 2018; Desai and Rao, 2010; Jarmander et al., 2014; Koirala et al., 2016; Liang et al., 2015; Luo et al., 2014; Young et al., 2012). Zhu et al. deleted gene *ptsG* in *Escherichia coli* to generate a recombinant strain that is able to co-utilize glucose and xylose as carbon sources for the production of succinate. When fermenting in Luria-Bertani (LB) medium with 27 g/l glucose and 14 g/l xylose, the strain FZ560/pHL413KF1 consumed 92% of sugars and accumulated 43 g/l succinate within 48 h. Then the ratio of glucose and xylose was optimized in the medium, leading to more than 93% of xylose and 97% of glucose being consumed in 24 h of fermentation, and succinate titer was further increased to 107.0 g/l (Zhu et al., 2020). Similarly, Li et al. produced 4-hydroxymandelic acid (4-HMA) in glucose–xylose mixed cultures by using *ptsG*-deactivated *E. coli*. Remarkably, this mutated strain generated 1.11 g/l 4-HMA in medium containing mixed carbon sources, representing 48.6% and 35.3% improvement, respectively, compared with that using glucose or xylose as the sole carbon source. For scale-up production of 4-HMA, the final titer reached 15.8 g/l in a 5-l fermenter after 60 h (Li et al., 2016a). In another example, Saini et al. inactivated *ptsG* and overexpressed *Zmglf* (encoding glucose facilitator from *Zymomonas mobilis*) to produce n-butanol in glucose and xylose mixed cultures. The recombinant *E. coli* strain produced 2.6 g/l n-butanol, and the carbon yield reached 32% of the theoretical yield. Furthermore, a coculture system containing a glucose-selective strain and a xylose-selective strain was constructed to simultaneously consume glucose and xylose. When using a mixed glucose and xylose medium, n-butanol titer reached 5.2 g/l with a yield of 63% of the theoretical yield (Saini et al., 2017). These studies serve as successful examples of cofermentation of glucose and xylose by disrupting PTS. The combination use of mixed carbon sources is shown to be beneficial for productivity and/or yield. However, inactivation of PTS usually limits the glucose transportation rate and causes slower cell growth and low productivity. This can be improved by overexpression of the non-PTS glucose transportation system encoded by *galP* and *Zmglf* (Luo et al., 2014; Saini et al., 2017; Zhao et al., 2019). Alternatively, pentose uptake rate and utilization efficiency can be enhanced by expression of pentose transporters and their catabolic pathways (Fig. 2) (Fujiwara et al., 2020; Horazdovsky and Hogg, 1989; Wang et al., 2018). For example, introduction of *E. coli* native xylose (or arabinose) metabolic pathway (containing genes *xylAB* or *araBAD*) into *Pseudomonas putida* KT2440 enabled this strain to grow well on xylose (or arabinose). In a glucose and xylose (or arabinose) mixed medium, the cell density of the engineered strain was comparable to that using glucose as the sole carbon source (Wang et al., 2019a). Kogure et al. used a glucose/pentose mixed-sugar–assimilating *Corynebacterium glutamicum* strain to produce shikimate. Genes *xylAB* and *araBAD* were overexpressed for metabolism of xylose and arabinose in the engineered strain, leading to 136.9 g/l shikimate produced in a glucose, xylose, and arabinose mixture. This titer is comparable to that of using glucose as the sole carbon source (141 g/l) (Kogure et al., 2016). Likewise, to avoid CCR and boost xylose consumption, D-xylose-proton symporter (XylT), xylose isomerase (XylA) and xylulokinase (XylB) from *Clostridium acetobutylicum* were introduced into *Clostridium tyrobutyricum*. The recombinant strain generated 42.6 g/l butyrate with a yield of 0.35 g/g via fermenting in glucose and xylose mixed cultures (Fu et al., 2017). In another example, to further enhance the arabinose consumption rate, an arabinose transporter named PcaraT was identifed from *Penicillium chrysogenum*.. Employment of PcaraT to replace the endogenous arabinose transporter GAL2 in *Saccharomyces cerevisiae* resulted in a 450-fold increase in arabinose consumption rate in *S. cerevisiae*. The kinetic parameters of these two transporters toward arabinose were also evaluated. The results showed that the Km value of PcaraT (0.13 mmol/L) was much lower than that of GAL2 (335 mmol/L). Additionally, in the presence of glucose, the binding affinity of PcaraT towards arabinose decreased by 63%, while it was reduced to 85% for GAL2 (Bracher et al., 2018). Besides deletion of PTS and overexpression of pentose transporters, other strategies like adaptive evolution and development of coculture systems also displayed great potential in combinatorial co-utilization of glucose, xylose, and arabinose (Saini et al., 2017; Zhang et al., 2015). For instance, two xylose transporters GXS1 from *Candida intermedia* and XUT3 from *Scheffersomyces stipites* were engineered via directed evolution. Overexpression of the mutant transporter in yeast enabled to improve the growth rate on xylose by 70%. Further analysis of the mutant transporters revealed that the amino acids F40 of GXS1 and E538 of XUT3 are key residues for transporter function. Although overexpression of the mutant transporter released the CCR, these xylose transporters are still inhibited by glucose (Young et al., 2012). In order to overcome this issue, further directed evolution of CiGXS1 FIM mutant strain was performed. Then, a glucose/xylose co-transporter was identified from this mutant strain. This co-transporter enable to co-transport glucose/xylose and had higher affinity towards xylose. The xylose transportation rate even outcompeted that of glucose when equal concentrations of glucose and xylose in the medium (Li et al., 2016b).

In some cases, the carbon sources' co-utilization efficiency can be further improved by separation of the production phase and cell growth phase via using two different carbon sources. For example, when using glucose as the building block for synthesis of polysaccharides and glycosylated compounds, noncatabolic utilization of glucose serves as a promising approach to improve the carbon yield. The native glucose metabolic pathway was blocked in a glucaric acid-producing *E. coli* strain by deletion of *pgi* and *zwf* (encoding phosphoglucose isomerase and glucose 6-phosphate dehydrogenase, respectively). When adding glucose into the

Table 1. The Strategies for Co-utilization of Different Carbon Sources in Microbes.

Target compound	Substrate	Titer (g/L)	Yield (g/g)	Productivity (g/L/h)	Strategies	Host	Reference
Succinate	Glucose and xylose	107.0	0.75	0.60	Deletion of gene *ptsG*	*Escherichia coli*	(Zhu et al., 2020)
4-Hydroxymandelic acid	Glucose and xylose	15.8	0.19	0.26	Disruption of gene *ptsG*	*E. coli*	(Li et al., 2016)
n-Butanol	Glucose and xylose	5.2	0.42	0.17	Disruption of gene *ptsG*; overexpression of gene *Zmglf*; construction of a coculture system containing glucose-selective strain and xylose-selective strain	*E. coli*	(Saini et al., 2017)
Shikimate	Glucose, xylose, and arabinose	136.9	0.46	2.85	Overexpression of genes of *xylAB* and *araBAD* for pentoses transportation and metabolism	*Corynebacterium glutamicum*	(Kogure et al., 2016)
Butyrate	Glucose and xylose	42.6	0.35	0.56	Overexpression of genes of *xylT*, *xylA*, and xylB for pentoses transportation and metabolism	*Clostridium tyrobutyricum*	(Fu et al., 2017)
Glucaric acid	Glucose and arabinose	0.50	0.76	0.01	Deletion of genes *pgi* and *zwf*, separation of the production process and cell-growth process	*E. coli*	(Shiue et al., 2015)
Glucaric acid	Glucose and xylose	1.19	0.73	0.02	Deletion of genes *pgi* and *zwf*, separation of the production process and cell-growth process	*E. coli*	(Shiue et al., 2015)
Trehalose	Glucose and xylose	5.55	0.26	0.05	Deletion of genes *pgi* and *zwf*, separation of the production process and cell-growth process	*E. coli*	(Wu et al., 2018)
cis, *cis*-Muconic acid	Glucose and xylose	4.09	0.31	0.06	PTS was replaced with the galactose permease/glucokinase system, separation of the production process and cell-growth process	*E. coli*	(Fujiwara et al., 2020)
L-Tyrosine	Glucose and xylose	1.34	0.32	0.01	Replace PTS system with the galactose permease/glucokinase system, separation of the production process and cell-growth process	*E. coli*	(Fujiwara et al., 2020)
Ethanol	Glucose and xylose	48.72	0.49	4.06	Control expression of *ptsG*, separation of the production process and cell-growth process	*E. coli*	(Sun et al., 2018)
1,4-Butanediol	Glucose and xylose	12.0	0.26 (on xylose)	0.40	Overexpression of genes of *xylABCDX* for xylose assimilation	*E. coli*	(Tai et al., 2016)
1,4-Butanediol	Glucose and arabinose	15.6	0.22 (on arabinose)	0.22	Overexpression of genes of *araCDABE* for arabinose assimilation	*E. coli*	(Tai et al., 2016)
1,4-Butanediol	Glucose and galacturonate	16.5	0.33 (on galacturonate)	0.18	Overexpression of genes of *udh, garD, and ycbC* for galacturonate assimilation	*E. coli*	(Tai et al., 2016)
Mesaconate	Glucose, xylose and arabinose	14.7	0.74 (on xylose and arabinose)	0.31	Overexpression of genes of *araE* for arabinose assimilation	*E. coli*	(Bai et al., 2016)
Poly (lactate-co-glycolate)	Glucose and xylose	12.6 wt%	NA	NA	Deletion of gene *ptsG*	*E. coli*	(Choi et al., 2016)

Table 1. Continued

Target compound	Substrate	Titer (g/L)	Yield (g/g)	Productivity (g/L/h)	Strategies	Host	Reference
Docosahexaenoic acid (DHA)	Glucose and glycerol	5.7	0.06	0.04	Glucose and glycerol as the initial and the feed carbon sources	*Schizochytrium limacinum*	(Patil and Gogate, 2015)
DHA	Glucose and glycerol	9.67	0.09	0.10	Glucose and glycerol as the initial and the feed carbon sources	*Thraustochytriidae*	(Ye et al., 2020)
DHA	Glucose and glycerol	32.36	NA	0.34	Glucose as the initial carbon source and glucose and glycerol as the feed carbon sources	*Aurantiochytrium limacinum*	(Li et al., 2015)
DHA	Glucose and fructose	20.1	0.11	0.18	Glucose and glycerol as the initial and the feed carbon sources	*Aurantiochytrium*	(Yu et al.)
1,3-Propanediol	Glucose and glycerol	13.47	0.27	0.18	Deletion of gene *ptsG*, introduction of an ATP-dependent galactose permease/glucokinase system	*E. coli*	(Yang et al., 2018a)
1,3-Propanediol	Glucose and glycerol	78.1	NA	1.63	Deletion of gene *crr*	*Klebsiella pneumoniae*	(Lu et al., 2018)
1,3-Propanediol	Glucose and glycerol	92	NA	1.70	Optimization feeding ratio of glucose and glycerol to shift redox household	*Lactobacillus diolivorans*	(Lindlbauer et al., 2017)
Trehalose	Glucose and glycerol	8.2	0.86 (on glucose)	0.33	Deletion of genes *pgi* and *zwf* to divert glucose into the biosynthetic pathway, adding glycerol to rescue cell growth	*E. coli*	(Wu et al., 2017)
D-*myo*-inositol	Glucose and glycerol	76	1.07 (on glucose)	0.79	Deletion of genes *pgi* and *zwf* to divert glucose into the biosynthetic pathway, adding glycerol to rescue cell growth	*E. coli*	(Tang et al., 2020)
Ethanol	Methanol and xylose	1.891	0.36	0.02	Introduction of the modified serine cycle, using medium supplemented with methanol and xylose	*E. coli*	(Yu and Liao, 2018)
Ethanol	Formate and xylose	1.922	0.31	0.42	Introduction of the modified serine cycle, using medium supplemented with formate and xylose	*E. coli*	(Yu and Liao, 2018)
Glutamate	Methanol and xylose	0.09	0.01	NA	Introduction of Mdh and RuMP genes, using medium supplemented with methanol and xylose, adaptive laboratory evolution	*C. glutamicum*	(Tuyishime et al., 2018)
Ethanol	Methanol and xylose	4.6	NA	1.53	Introduction of Mdh and RuMP genes, using medium supplemented with methanol and xylose, adaptive laboratory evolution	*E. coli*	(Chen et al., 2018)
1-Butanol	Methanol and xylose	2.0	0.47	0.33	Introduction of Mdh and RuMP genes, using medium supplemented with methanol and xylose, adaptive laboratory evolution	*E. coli*	(Chen et al., 2018)
Acetone	Methanol and glucose	0.755	0.01	NA	Introduction of Mdh and RuMP genes, using medium supplemented with methanol and glucose, adaptive laboratory evolution	*E. coli*	(Bennett et al., 2020)

NA = not applicable.

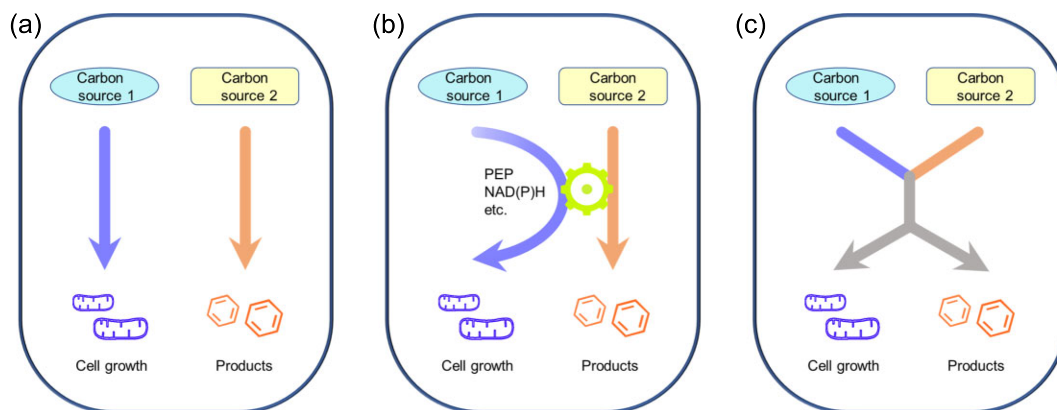

Fig. 1. Schematic representation of carbon sources' co-utilization strategies in microbes. (a) Parallel carbon utilization strategy. (b) Synergetic carbon utilization strategy. (c) Carbon-supplement co-utilization strategy.

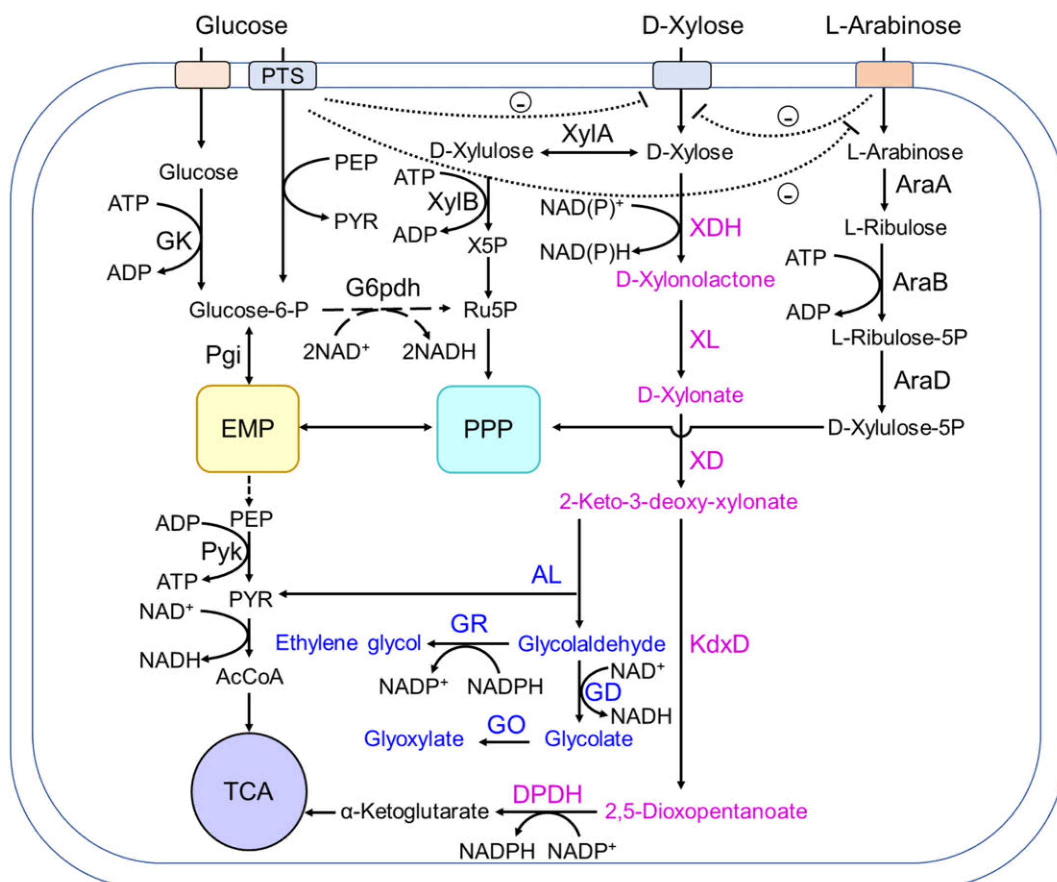

Fig. 2. Metabolic pathways of xylose, arabinose, and glucose co-utilization in microbes. Blue-colored characters indicate Dahms pathway; purple-colored characters indicate Weimberg pathway; and black-colored dash lines indicate the CCR. EMP, Embden–Meyerhof–Parnas pathway; PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle; PTS, phosphotransferase system; GK, glucose kinase; Pgi, glucose-6-phosphate isomerase; Pyk, pyruvate kinase; G6pdh, glucose 6-phosphate dehydrogenase; XylA, xylose isomerase; XylB, xylulokinase; XDH, xylose dehydrogenase; XL, xylonolactonase; XD, xylonate dehydratase; KdxD, 2-keto-3-deoxy-D-xylonate dehydratase; DPDH, 2,5-dioxopentanoate dehydrogenase; AL, aldolase; GR, glycolaldehyde reductase; GD, glycolaldehyde dehydrogenase; GO, glycolate oxidase; AraA, L-arabinose isomerase; AraB, ribulokinase; AraD, L-ribulose 5-phosphate epimerase. Metabolites: PEP, phosphoenolpyruvate; PYR, pyruvate; AcCoA, acetyl coenzyme A; X5P, D-xylulose 5-phosphate; Ru5P, ribulose 5-phosphate.

cultures, glucose can only be diverted into the glucaric acid biosynthetic pathway rather than glycolysis. In order to rescue cell growth, xylose or arabinose can be used as the second carbon source for biomass formation (Fig. 2). By employing this strategy, the production yield of glucaric acid was increased by 18-fold compared with that using glucose as the sole carbon source (Shiue et al., 2015).These results demonstrate that the carbon yield could be greatly improved by co-utilization of two carbon sources to separate the cell growth and product synthesis via rewiring the intracellular metabolic. In another example, Wu et al. engineered an *E. coli* strain for producing trehalose in glucose and xylose mixed medium by developing a SynCar. In this study,

xylose was responsible for biomass formation and PEP generation while glucose was specifically diverted for trehalose synthesis. By coupling PEP generation with PTS to improve glucose uptake, trehalose titer was enhanced to 5.55 g/l in shake flask experiments. Trehalose yield significantly increased from 0.19 g/g to 0.71 g/g (Wu et al., 2018). These results demonstrate an attractive strategy for co-utilization of lignocellulosic hydrolysates. Recently, novel pentose metabolic pathways including Weimberg pathway and Dahms pathway found in Archaea have gained much research interest (Fig. 2) (Wang et al., 2017a; Wang et al., 2017b; Wang et al., 2017c) because these pathways are nonphosphorylative and more straightforward to assimilate pentose into the tricarboxylic acid (TCA) cycle with a higher carbon yield. On this basis, Fujiwara et al. proposed a strategy to parallel-utilize glucose and xylose for production of shikimate pathway derivatives, *cis*, *cis*-muconic acid (MA), and L-tyrosine (Fujiwara et al., 2020). The *E. coli* native Embden–Meyerhof–Parnas pathway (EMP) and pentose phosphate pathway (PP pathway) were completely inactivated by disruption of 2-keto-3-deoxygluconate-6-phosphate aldolase, PEP synthase, PEP carboxylase, PEP carboxykinase, and pyruvate kinase. Thus, glucose was mainly diverted into the shikimate pathway and used for target product synthesis. Meanwhile, the xylose Dahms pathway was introduced into the host strain to provide energy and support cell growth. By cultivating the engineered strains in glucose and xylose mixed medium, the titers of MA and L-tyrosine reached 4.09 g/l and 1.34 g/l, with higher yields of 0.31 g/g glucose and 0.35 g/g glucose, respectively (Fujiwara et al., 2020). In order to further improve the co-utilization efficiency of mixed sugars, a smart dynamic control system was constructed to separate the production phase and growth phase using glucose and xylose as the co-substrates. In this system, a temperature-sensitive promoter P_R/P_L was employed to control the expression of *ptsG*. When the temperature was lower than 34°C, the regulator protein CI857 was activated and combined with the promoter P_R/P_L to repress the expression of *ptsG*. Meanwhile, xylose was assimilated by cells to support cell growth. When the temperature was higher than 37°C, CI857 was inactivated to initiate the transcription of *ptsG*. Thus, glucose was transported into cells to produce ethanol. By employing such a dynamic control system, the yield of ethanol was improved by 3.8 times than the titer produced by control strain (Sun et al., 2018).

Additionally, the carbon sources' co-utilization systems can be constructed by using xylose or arabinose to generate value-added products and using glucose to support cell growth. Bai et al. designed and constructed a novel 1,4-butanediol (1,4-BDO) biosynthetic pathway in *E. coli* by recruiting xylose or arabinose Weimberg pathway, the theoretical molar yield of 1,4-BDO generated from xylose or arabinose reached 100%. When using glucose as the carbon source to support cell growth, the engineered *E. coli* strains produced 12.0 g/l or 15.6 g/l of 1,4-BDO from 46 g/l xylose or 70.5 g/l arabinose, respectively (Tai et al., 2016). On this basis, an *E. coli* strain harboring optimized xylose and arabinose Weimberg pathway was constructed and engineered to synthesize mesaconate by simultaneous utilization of glucose, xylose, and arabinose. This engineered strain exhibited significant productivity and produced 14.7 g/l mesaconate from glucose, xylose, and arabinose mixed medium. The yield of mesaconate reached 85% of the theoretical maximum (Bai et al., 2016). Poly(lactate-co-glycolate) (PLGA) is a biodegradable, biocompatible, and nontoxic synthetic polyester that is widely used in biomedical and therapeutic industries (Makadia and Siegel, 2011). Choi et al. constructed a biosynthetic pathway which condensed D-lactyl-CoA and glycolyl-CoA to form PLGA in *E. coli*. Part of the Dahms pathway was employed to generate glycolyl-CoA and D-lactyl-CoA. Glucose was consumed simultaneously to support cell growth and D-lactyl-CoA formation by inactivating PTS. The host strain yielded 12.6 wt% of PLGA in shake flask experiments. Furthermore, this engineered strain also can be used to convert glucose into 2-hydroxybutyryl-CoA, 3-hydroxybutyryl-CoA, and 4-hydroxybutyryl-CoA. These chemicals can be polymerized with the xylose generated glycolyl-CoA to produce corresponding copolymers (Choi et al., 2016).

Metabolic Engineering Strategies for Glucose and Glycerol Co-utilization

Except combinatorial co-utilization of glucose, xylose, and arabinose, glucose–glycerol mixed fermentation also attracted extensive attention. As an inevitable by-product in the biodiesel industry, crude glycerol becomes a budget feedstock and causes environmental issues (Güngörmüşler-Yilmaz and Azbar, 2015). The price of crude glycerol is much cheaper (~77$/T) than that of glucose (~881$/T) (Trondle et al., 2018). Therefore, simultaneous utilization of glycerol and glucose serves as a promising method to reduce the processing cost in the biomanufacturing industry. For instance, economical biosynthesis of docosahexaenoic acid (DHA) was achieved by using glucose and glycerol as co-substrates. When using *Aurantiochytrium limacinum* SR21 as the host strain, up to 5.7 g/l of DHA was produced (Patil and Gogate, 2015). To improve DHA yield, 10 fed-batch cofermentation strategies using different ratios of glucose/glycerol were employed. The engineered *A. limacinum* SR21 strain generated 32.36 g/l of DHA, which was 15.24% higher than that of using glucose as the sole carbon source (Li et al., 2015). During this fermentation process, glucose was mainly assimilated to support cell growth in the early stage of fermentation, while glycerol was consumed for fatty acid accumulation in the late stage of fermentation. Optimization of the feeding strategies further boosted the DHA titer to 9.67 g/l in *Thraustochytriidae* PKU#Mn16 strain in glucose–glycerol mixed medium. It was also observed that several genes involved in fatty acid biosynthetic pathway were upregulated under co-substrate fermentation conditions (Ye et al., 2020). Similarly, it was also found that co-utilization of glucose and glycerol significantly improved the ε-poly-L-lysine titer and productivity, probably due to the enhanced transcriptional intensity of genes involved in central carbon metabolism, lysine biosynthetic pathway, and cell respiration system (Chen et al., 2012; Zeng et al., 2019).

For efficient production of target products, increasing efforts have been made to construct smart glucose and glycerol co-utilization system based on their metabolic properties in microbes. Compared with glucose, glycerol has a higher reduced state and is suitable for producing redox-demanding compounds such as 1,3-propanediol (1,3-PDO) (Dharmadi et al., 2006). In order to achieve high productivity and yield of 1,3-PDO, the commonly used strategy is employing glucose to support cell growth and using glycerol as substrate to yield target products (Fig. 3a). Yang et al. constructed an engineered *E. coli* to co-utilize glucose and glycerol for the production of 1,3-PDO. To avoid CCR and promote efficient glycerol utilization, *ptsG* which encodes a glucose-specific transporter was deleted and an ATP-dependent transport system (encoded by *galP* and *glk*) was introduced into this strain. The produced *E. coli* strain generated 13.47 g/l of 1,3-PDO after the introduction of a NADPH-regeneration system and optimization of fermentation conditions. The final 1,3-PDO titer was increased by 325% compared with the original *E. coli* producer (Yang

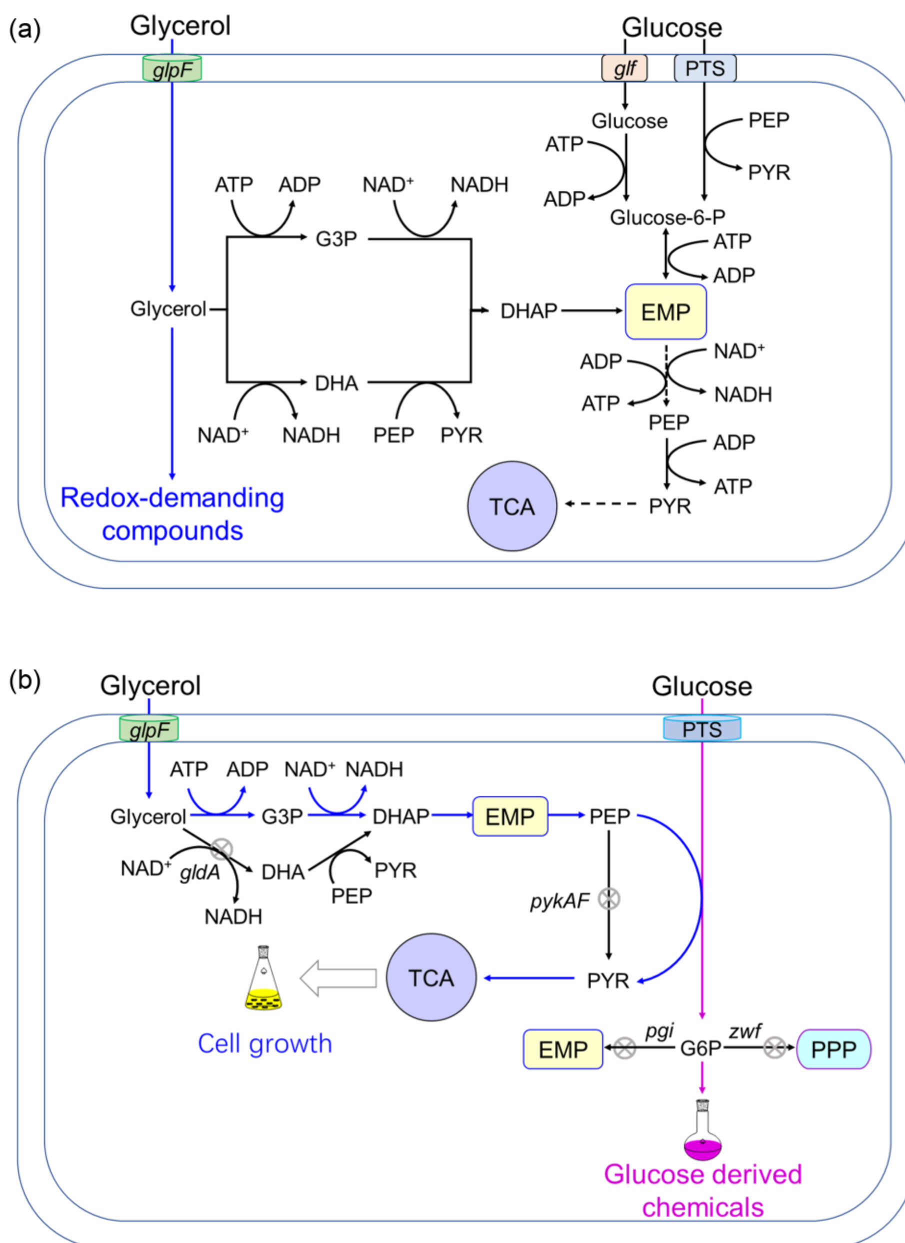

Fig. 3. Glucose and glycerol co-utilization strategies in microbes. (a) Glucose and glycerol co-utilization strategy for the production of redox-demanding products. Black-colored arrows indicate the native metabolic pathways. Blue-colored arrows indicate the chemical biosynthesis pathways of glycerol. (b) Glucose and glycerol synergetic utilization strategy for the production of glucose derived products. Black-colored arrows indicate the native metabolic pathways; blue-colored arrows indicate the main metabolic pathways of glycerol for cell growth; and purple-colored arrows indicate the chemical biosynthesis pathways of glucose. G3P, glyceraldehyde 3-phosphate; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; G6P, glucose 6-phosphate; EMP, Embden–Meyerhof–Parnas pathway; PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle; *glpF*, glycerol facilitator; *glpK*, glycerol kinase; *gldA*, glycerol dehydrogenase; *glf*, glucose facilitator; PTS, phosphotransferase system; *pgi*, encoding phosphoglucose isomerase; *zwf*, encoding glucose 6-phosphate dehydrogenase; *pykAF*, encoding pyruvate kinase.

et al., 2018a). Lu et al. deleted the *crr* gene encoding Enzyme IIA^{Glc} to further weaken the CCR in *Klebsiella pneumoniae*. This strategy combined with enhancement of the TCA cycle transcriptional level and reduction of lactate accumulation resulted in 78.1 g/l of 1,3-PDO in a bioreactor (Lu et al., 2018). In another example, Lindlbauer et al. optimized feeding a ratio of glucose and glycerol to shift redox household of *Lactobacillus diolivorans*, thereby redirecting the carbon flux into 1,3-PDO biosynthesis rather than the by-product 3-hydroxypropionic acid formation. The 1,3-PDO titer was dramatically improved to 92 g/L with a higher productivity of 1.7 g/L/h in an optimized fed-batch cultivation (Lindlbauer et al., 2017). This strategy was also proved to be effective to significantly improve the production of propanol and 1,2-propanediol when using glucose and glycerol mixed carbon sources (Chen et al., 2017).

Glucose possesses advantages for cell growth and proliferation; however, there is a trade-off between biomass formation and target product synthesis, especially when using glucose to generate polysaccharides and glycosylated molecules. To address this dilemma, a routine method is to disrupt or down-regulate genes involved in central metabolic pathways, which however greatly

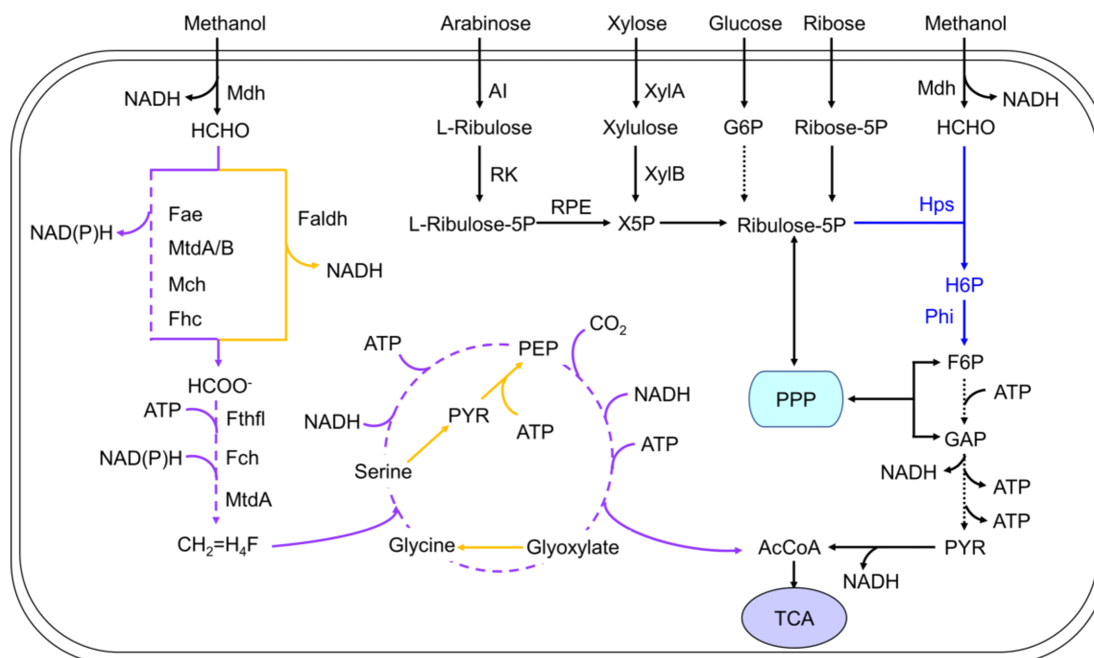

Fig. 4. Metabolic pathways of methanol assimilation with other carbon sources. Black-colored arrows indicate the native metabolic pathways in most microbes; blue-colored arrows with blue-colored enzymes and metabolites indicate the ribulose monophosphate (RuMP) pathway; purple-colored arrows indicate the natural serine cycle; and yellow-colored arrows indicate the modified serine cycle. PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle; Mdh, methanol dehydrogenase; Hps, 3-hexulose-6-phosphate synthase; Phi, 6-phospho-3-hexuloisomerase; XylA, xylose isomerase; XylB, xylulokinase; AI, L-arabinose isomerase; RK, ribulokinase; RPE, ribulose 5-phosphate epimerase; Faldh, formaldehyde dehydrogenase; Fae, formaldehyde-activating enzyme; Mtd, NADP-dependent methylene-tetrahydromethanopterin/methylene-tetrahydrofolate dehydrogenase; Mch, N(5), N(10)-methenyltetrahydromethanopterin cyclohydrolase; Fhc, formyltransferase/hydrolase complex; Fthfl, formate THF ligase; Fch, methenyltetrahydrofolate cyclohydrolase. Metabolites: G6P, glucose 6-phosphate; H6P, hexulose 6-phosphate; F6P, fructose 6-phosphate; GAP, 3-phosphoglyceraldehyde; PEP, phosphoenolpyruvate; PYR, pyruvate; X5P, xylulose 5-phosphate.

impairs the cell growth rate and product titers. In order to solve this issue, the carbon metabolic network can be reconstituted by employing glycerol to support cell growth and using glucose as the substrate to yield polysaccharides and glycosylated molecules (Fig. 3b). For example, Shiue et al. deleted genes *pgi* and *zwf* to divert glucose into the D-glucaric acid biosynthetic pathway and added glycerol into the cultures to rescue cell growth. The results showed that D-glucaric acid yield was greatly increased by 9-fold in glucose and glycerol mixed cultures (Shiue et al., 2015). On this basis, Wu et al. designed a SynCar in *E. coli*. This SynCar system recruited glucose as building block for noncatabolic production of trehalose and using glycerol only to support cell growth. In this system, glucose catabolic pathways were completely disrupted, and the PEP generated from glycerol provided a driving force to promote glucose uptake via PTS. As a result, the final engineered strain produced 8.2 g/l trehalose with a yield of 0.86 g/g (trehalose/glucose) at the end of 96 h, indicating a 5.2-fold increase in titer compared with that of control strain (Wu et al., 2017). Similarly, Tang et al. employed this SynCar system to produce D-myo-inositol. They overexpressed *glpK* encoding glycerol kinase to weaken glucose inhibition effect on glycerol catabolism, and the titer of D-myo-inositol reached 76 g/L in a fed-batch fermentation condition using glucose and glycerol mixed carbon sources (Tang et al., 2020). These studies demonstrate a novel glucose–glycerol co-utilization approach and provide a series of reliable, high-yield production systems for generation of value-added compounds. Additionally, the developed synergistic carbon source co-utilization mechanism can also be employed to produce other glucose derived chemicals such as *N*-acetylglucosamine, chondroitin, hyaluronic acid, and heparin.

Metabolic Engineering Strategies for Co-utilization of C1 Compounds With Other Carbon Sources

It has been estimated that the cost of carbon sources represents about 40% of the total bioprocessing cost (Khosravi-Darani et al., 2013). Employment of several C1 carbon sources such as methanol and CO_2 to reduce the production cost has attracted much attention, due to their lower price and serious role in greenhouse effect (Clomburg et al., 2017; Kim et al., 2020; Yang et al., 2018b). Some methylotrophic and autotrophic bacteria exhibit the ability to assimilate C1 carbon sources, but most of these microbes cannot be employed for biomanufacturing due to their slower growth rate (Bennett et al., 2020; Kim et al., 2020) and lack of corresponding genetic tools for DNA manipulations (Wang et al., 2019b; Whitaker et al., 2017). Recently, engineering of model microorganisms for co-utilization of C1 carbon sources with C5 or C6 sugar to generate value-added chemicals has made significant progress. For example, serine cycle and ribulose monophosphate (RuMP) cycle are the two main methanol metabolic pathways which naturally exist in methylotrophic organisms (Fig. 4). The serine cycle is oxygen-insensitive and is able to synthesize acetyl-CoA without carbon loss (Smejkalová et al., 2010). In this cycle, methanol is converted into formaldehyde, which is sequentially condensed with 5,10-methylene-tetrahydrofolate (H_4F) to form 5,10-methylene-H_4F via 7 enzymatic steps. Then, 5,10-methylene-H_4F is reacted with glycine to generate serine catalyzed by serine hydroxymethyltransferase (SHMT) (Fig. 4). Yu et al. modified this natural serine cycle and introduced it into *E. coli*. The engineered *E. coli* strain was able to convert methanol to ethanol by

cofermenting methanol and xylose (Fig. 4). 36.3 mM of ethanol was produced in the engineered *E. coli* strain by simultaneously consuming 30 mM xylose and 22.6 mM methanol (Yu and Liao, 2018). The serine cycle consumes three molecules of ATP and four molecules of NAD(P)H to form one molecule of pyruvate; while another methanol catabolic pathway, the RuMP cycle is able to synthesize one molecule of pyruvate along with generation of one molecule of ATP and three molecules of NAD(P)H (Zhang et al., 2019). Thus, the RuMP cycle serves as a promising methanol utilization pathway to economically produce target products. In the RuMP cycle, methanol is first converted into formaldehyde by NAD-dependent methanol dehydrogenase (Mdh). Then formaldehyde is condensed with ribulose 5-phosphate (Ru5P) to form hexulose-6-phosphate (H6P) by H6P synthase (HPS), which is sequentially isomerized into fructose-6-phosphate (F6P) catalyzed by phosphohexuloisomerase (PHI). The generated F6P can be catabolized via the glycolysis pathway or PP pathway for cell growth or product formation (Fig. 4). In order to employ the RuMP cycle for methanol utilization in *E. coli*, a series of Mdh, HPS, and PHI were identified and characterized. It was found that the enzymes from *Bacillus methanolicus* are the most-efficient candidates. The engineered *E. coli* strain harboring *mdh2*, *hps,* and *phi* was cultivated in a methanol and ribose mixed M9 medium, the results of isotopic labelling experiments demonstrated that up to 40% of labeled methanol was incorporated into H6P (Muller et al., 2015). Similarly, another model microorganism *C. glutamicum* was also engineered to assimilate methanol. Mdh from *B. methanolicus*, HPS, and PHI from *Bacillus subtilis* were introduced into *C. glutamicum*. When using methanol and glucose as the co-substrates, the methanol consumption rate of this engineered strain was up to 1.7 mM/h. The cell density was increased by 18% compared with the strain without *mdh, hps,* and *phi* overexpression (Witthoff et al., 2015). To maximize the methanol assimilation rate in the presence of another carbon source, Bennett et al. knocked out *pgi* gene to drive more glucose into Ru5P formation. The results showed that up to 99% of glucose was diverted into Ru5P synthesis and the methanol assimilation rate was improved from 0.06 mmol/gDW/h to 0.28 mmol/gDW/h (Bennett et al., 2018). Meyer et al. constructed an *E. coli* strain that was able to use methanol as the essential carbon source for cell growth by blocking Entner–Doudoroff pathway (ED pathway), PP pathway, and TCA cycle. When using gluconate as the carbon source and introducing the RuMP cycle into this strain, the cell growth only can be rescued by condensing gluconate generated R5P with methanol to form F6P. After laboratory evolution using cell growth as a driving force, methanol consumption rate in the engineered strain reached 13 mmol/gDW/h, which was comparable with natural methylotrophs (15 mmol/gDW/h) (Meyer et al., 2018). In addition, Gonzalez et al. found that the cell biomass in methanol and yeast extract (1 g/l) mixed medium was 33% higher than that in methanol and glucose (1 g/l) mixed medium. Further research revealed that threonine acted in a primary beneficial role of improving methanol assimilation in the presence of yeast extract (Gonzalez et al., 2018).

These cases serve as excellent examples of efficient assimilation of methanol to support cell growth. Based on this, many efforts have been made to co-utilize methanol and other carbon sources for biosynthesis of value-added compounds in microbes. For instance, Whitaker et al. engineered a methylotrophic *E. coli* strain by incorporating the heterologous RuMP cycle. The addition of methanol into yeast extract medium improved the biomass by 30%. Meanwhile, a heterologous naringenin biosynthetic pathway was transferred into this engineered strain, which demonstrated the first example for production of a target molecule by using methanol as the carbon source (Whitaker et al., 2017). Likewise, Lessmeier et al. constructed a methylotrophic *C. glutamicum* strain to achieve cadaverine production in a methanol and glucose or ribose mixed medium by heterologously expressing the RuMP cycle and deleting aldehyde dehydrogenase genes (*ald* and *fadH*). The results showed that up to 20% of produced cadaverine was generated from methanol (Lesmeier et al., 2015). It has been reported that NADH is an inhibitor for Mdh (Rohlhill et al., 2020; Woolston et al., 2018). Thus, to further enhance the catalytic efficiency of Mdh, Wang et al. exploited a NADH kinase (Pos5p) from *S. cerevisiae* to convert NADH into NADPH. The overaccumulated NADPH serves as reducing power for aspartate-semialdehyde dehydrogenase and dihydrodipicolinate reductase, which are two key enzymes involved in lysine biosynthetic pathway. By using glucose and methanol as co-substrates, the titer of lysine was increased by twofold compared with the original strain (Wang et al., 2019b). Considering that the limited availability of Ru5P is the primary rate-limiting factor for methanol assimilation (Fig. 4) (Rohlhill et al., 2020; Woolston et al., 2018), Tuyishime et al. deleted the gene *rpiB* encoding ribose phosphate isomerase to accumulate R5P in a methanol-essential *C. glutamicum* strain. After adaptive laboratory evolution experiments, this engineered strain can grow on the methanol and xylose mixed culture with a consumption mole ratio of 3.83:1. In CGXII minimal medium supplemented with 4 g/l methanol and 4 g/l xylose, the methanol-dependent strain produced 90 mg/l of glutamate (Tuyishime et al., 2018). In another example, the *E. coli* native ribose phosphate isomerase RPI was inactivated to enhance the Ru5P concentration. The engineered strain carrying the RuMP cycle was able to consume methanol and xylose at a mole ratio of 1:1. Separate introduction of ethanol or 1-butanol biosynthetic pathways into this engineered methylotrophic *E. coli* strain led to 4.6 g/l ethanol or 2.0 g/l 1-butanol being produced, respectively, in methanol and xylose mixed medium (Chen et al., 2018). The isotopic labelling experiments were further conducted using ^{13}C-labeled methanol. The results demonstrated that up to 43% of ethanol and 71% of 1-butanol generated from methanol. Recently, Bennett et al. designed an auxotrophic *E. coli* strain overexpressing the RuMP cycle for methanol and glucose co-consumption via deleting genes *pgi*, *rpiAB,* and *edd* (encoding phosphogluconate dehydratase). By integrating the acetone biosynthetic pathway, the engineered host strain produced 755 mg/l acetone in fed-batch fermentation, and up to 22% of acetone was generated from methanol (Bennett et al., 2020). In addition to the serine cycle and the RuMP cycle, a novel Synthetic Acetyl-CoA (SACA) pathway was designed and constructed to convert formaldehyde into acetyl-CoA. In this pathway, two formaldehyde are condensed by glycolaldehyde synthase to generate glycolaldehyde, which is sequentially converted into acetyl-phosphate (AcP) by AcP synthase. Then, AcP is catalyzed into acetyl-CoA under the action of phosphate acetyltransferase. The SACA pathway performs a considerable catalytic capability *in vitro*, but only supports 3% biomass from methanol. Although the methanol assimilation efficiency of the SACA pathway is lower than that of the RuMP cycle, the SACA pathway demonstrates promising feasibility for economic biosynthesis of target product due to containing less enzymatic steps and ability to metabolize methanol in an ATP-independent manner (Lu et al., 2019).

Carbon dioxide is another cheap and abundant carbon source for biosynthetic utilization. Calvin–Benson–Bassham (CBB) cycle serves as the most dominant CO_2 fixation pathway in nature. In the CBB cycle, Ru5P from PP pathway is phosphorylated to ribulose bisphosphate (RuBP) by phosphoribulokinase (PRK) using ATP

as the phosphate group donor. Then, RuBP is combined with CO_2 by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) to generate glyceraldehyde 3-phosphate (G3P), which is further diverted into central metabolic pathways. To functionally express the CBB cycle for CO_2 fixation in microbes, additional carbon sources such as glucose or xylose need to be supplemented to provide Ru5P. Recently, by introducing PRK from *Synechococcus elongatus* and RuBisCO from *Synechococcus* sp. PCC7002 into *E. coli*, the CO_2 fixation rate of the engineered stain reached 19.6 mg/L/h when using xylose as a co-substrate to provide Ru5P and support cell growth (Gong et al., 2015). Similarly, a PRK-RuBisCo containing *E. coli* strain was able to fix CO_2 at a rate of 67 mg/L/h by simultaneously consuming arabinose and glucose (Zhuang and Li, 2013). For *in situ* fixation of CO_2, Li et al. developed an engineered yeast strain harboring PRK, RuBisCO, xylose reductase, xylitol dehydrogenase, and xylulokinase for the production of ethanol by co-utilizing glucose, maltose, and xylose. The productivity of ethanol was increased from 0.9 g/l/h to 1.5 g/l/h in the maltose and xylose mixed medium (Li et al., 2017). Very recently, great progress has been made in engineering of *E. coli* strain to generate all biomass carbon from CO_2. In this study, a recombinant *E. coli* strain harboring CBB cycle from the methylotrophic bacterium *Pseudomonas* sp. 101 was constructed. It was able to grow well on xylose and CO_2 mixed medium. To enforce the carboxylation reaction catalyzed by RuBisCO, the phosphofructokinase (Pfk) and glucose-6-phosphate dehydrogenase (Zwf) were inactivated. Then, a continuous adaptive laboratory evolution strategy was applied by gradually decreasing the xylose amount in medium. After several months of cultivation, the evolved *E. coli* gained the ability to use CO_2 as the sole carbon source to support cell growth (Gleizer et al., 2019).

Challenges and Future Perspectives

Carbon sources' co-utilization in microorganisms has gained much attention with the development of metabolic engineering in recent years. Simultaneous utilization of two or more carbon sources for biomanufacturing has become an attractive approach to reduce production cost and to improve the titer and yield of target products. Lignocellulose, glycerol, and some C1 compounds including methanol and CO_2 are cheap and renewable feedstocks. Co-utilization of these carbon sources for biosynthesis of value-added chemicals represents a promising approach to achieve efficient and economical microbial production. However, the co-utilization efficiency of the mixed sugars is greatly limited by the CCR. Recently, numerous efforts have been made to overcome this issue, such as deletion of the PTS, development of coculture systems, and rewiring carbon metabolic networks. Especially, construction of synergistic carbon co-utilization mechanisms by reconstituting carbon assimilation pathways and employment of novel and orthogonal carbon metabolic pathways from archaebacteria to avoid CCR represent excellent examples to improve the carbon source co-utilization efficiency. However, the carbon yields of currently developed carbon source co-utilization systems are relatively low, which makes it challenging to use mixed sugars to achieve scaled-up production of target products at an industrial level. Thus, it is of great significance for further optimization of mixed carbon sources' metabolic networks to balance cell growth and product synthesis. Systematic optimization of the mixed carbon source ratios and fine tuning of the carbon flux between biomass formation and product synthesis using dynamic regulation elements might serve as promising strategies to overcome this obstacle. It is anticipated that the development of advanced technologies including genome-editing, dynamic regulations, genome mining, and protein engineering would facilitate the development of efficient carbon source co-utilization systems to achieve efficient and cost-effective biosynthesis of value-added chemicals in the near future.

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Conflict of Interest

The authors declare no conflict of interest.

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