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REVIEW

Cell factories for biosynthesis of D-glucaric acid: a fusion of static and dynamic strategies

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Abstract

D-glucaric acid is an important organic acid with numerous applications in therapy, food, and materials, contributing significantly to its substantial market value. The biosynthesis of D-glucaric acid (GA) from renewable sources such as glucose has garnered significant attention due to its potential for sustainable and cost-effective production. This review summarizes the current understanding of the cell factories for GA production in different chassis strains, from static to dynamic control strategies for regulating their metabolic networks. We highlight recent advances in the optimization of D-glucaric acid biosynthesis, including metabolic dynamic control, alternative feedstocks, metabolic compartments, and so on. Additionally, we compare the differences between different chassis strains and discuss the challenges that each chassis strain must overcome to achieve highly efficient GA productions. In this review, the processes of engineering a desirable cell factory for highly efficient GA production are just like an epitome of metabolic engineering of strains for chemical biosynthesis, inferring general trends for industrial chassis strain developments.

Keywords Cell factory · D-glucaric acid · Dynamic control · Metabolic engineering

Introduction

D-glucaric acid (GA) is a natural chemical derived from glucose in fruits, vegetables, and a small range of mammals (Walaszek et al. 1996). The biorefinery of glucose conversion into GA is in great demand, for GA and its derivatives could be widely used in various fields, including therapy, food, and materials. Specifically, GA derivative D-glucaro-1,4-lactone showed the potential to prevent diethylnitrosamine-induced liver cancer (Walaszek 1990; Walaszek et al. 1997; Yang et al. 2018), as GA was proven to support liver detoxification *via* downregulating hepatocyte apoptosis, reducing the generation of ROS, reducing deconjugation of glucuronide, and inhibiting the reabsorption of toxins in hepatocytes by β-glucuronidase (Ayyadurai et al. 2023). Additionally, serving as the precursor of adipic acid, GA was a desirable bio-renewable source for nylon-66 synthesis and supplement in gelatins and desserts as a food ingredient (Polen et al. 2013). Given its importance, GA is deemed a top value-added chemical from biomass by the U.S. Department of Energy in 2004 (Werpy and Petersen 2004). The global GA market size attained a value of nearly USD 1.20 billion in 2023, and it was anticipated that the GA market will experience healthy growth with an estimated value of approximately USD 2.82 billion by 2032 (https://www.expertmarketresearch.com/reports/glucaric-acid-market).

The current methods to produce GA include chemical synthesis and biosynthesis. Initially, nitric acid oxidation was employed to chemically synthesize GA from glucose. However, its low conversion rate of glucose to GA (< 50% yield) and the rapid oxidation exothermic stage impeded large-scale industrial production (Smith et al. 2012). With the development of chemical methods, various catalysts such as mono-metal, bi-metal, and bifunction/composite catalysts flourished. The applications of assisted technologies, including ultrasound, microwave, and photocatalysis, were also developed to improve oxidation efficiencies (Zhang et al. 2021). Using the 2,2,6,6-tetramethyl-1-piperidinyloxy

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free radical (TEMPO) as the medium for electrochemical oxidation, glucose conversion rate to GA could achieve 85% yield under optimal conditions (Ibert et al. 2010). By introducing enzymes in vitro as catalysts, the conversion rate of sucrose to GA could achieve a yield of 75% (Su et al. 2019). However, the abovementioned methods encountered challenges due to the high costs associated with highly efficient metal catalysts and enzyme purification procedures. Additionally, chemical synthesis methods bring environmental concerns and resource limitations. In comparison, establishing cell factories for GA biosynthesis offers a sustainable and economically viable alternative. In nature, GA is the end product of the D-glucuronic acid pathway, which involves numerous steps (Fig. 1) (Marselos et al. 1976). The potential of cell factories for biosynthesizing GA was demonstrated in *Escherichia coli* by expressing three heterologous enzymes (Fig. 1), producing 1.13 g/L GA via three steps from glucose (Moon et al. 2009). The first enzyme, *myo*-inositol-1-phosphate synthase (Ino1, also known as MIPS), converts glucose-6-phosphate (G6P) to *myo*-inositol-1-phosphate, which is then hydrolytically dephosphorylated to produce *myo*-inositol (MI) by an endogenous phosphatase. The following enzyme, *myo*-inositol oxygenase (MIOX), converts MI to D-glucuronic acid using oxygen. Finally, uronate dehydrogenase (Udh) facilitates the final step, simplifying the conversion of D-glucuronic acid to GA compared to the natural pathway (Fig. 1) (Moon et al. 2009).

For this GA biosynthesis chassis strain, multiple challenges had arisen: (a) by-products from the overflow of the GA pathway carbon flux, (b) the low catalytic activity of the key biosynthetic enzyme MIOX, and (c) an imbalance between the self-growth and GA production of the chassis strains (Su et al. 2020). Thus, further studies focused on the construction of different chassis strains, including *E. coli*, *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Bacillus subtilis*, to find out which chassis strain would be suitable for the industrialization of GA biosynthesis (Table 1). This review summarizes the multiple strategies applied in GA production, showing the metabolic engineering trend for cell factory construction from static strategies to complex pathway reprogramming with dynamic regulation.

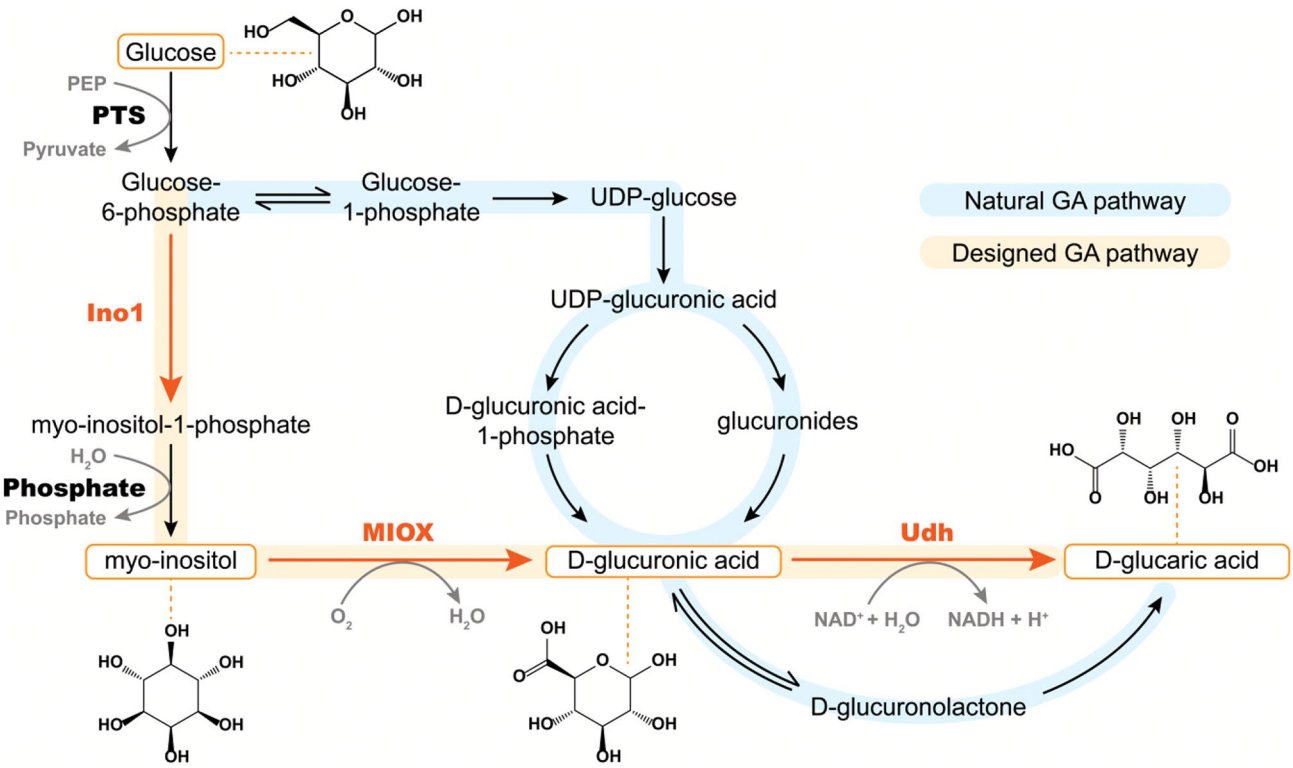

Fig. 1 Natural pathway and designed pathway for the GA biosynthesis: in the designed GA pathway, three heterologous steps are shown in orange (modified from reference (Moon et al. 2009); in the natural GA pathway, enzymes are not shown, (modified from reference (Marselos et al. 1976). Abbreviations: PTS, phosphoenolpyruvate-dependent phosphotransferase system; Phosphatase, endogen ous enzymes, SuhB in *E. coli*, Inm1/2 in *S. cerevisiae*; PEP, Phosphoenolpyruvate

Table 1 Summary of GA production in cell factories

Year	Host	Genes	Engineering strategies (partly displayed)	Substrate[a]	Boost [b](-fold)	Titer (g/l)	Yield[c](g/g)	Fermentation Condition[d](bioreactor; batch or fed-batch; time)
2009 (Moon et al. 2009)	*E. coli* BL21 Star (DE3)	*INO1*, *udh*, *MmMiox*	First realized in *E. coli*	Glc	/	1.13	0.15 (G)	250-mL baffled flasks; batch; 72 h
2010 (Moon et al. 2010)	*E. coli* BL21 Star (DE3)	*INO1*, *udh*, *MmMiox*	Static-protein scaffold	Glc	5	2.50	0.25 (G)	250-mL baffled flasks; batch; 48 h
2014 (Shiue and Prather 2014)	*E. coli* MG1655 (DE3) derivative	*SUMO-MmMiox*, *udh*	Static-fusion tags	MI	1.75	4.85	0.45 (MI)	250-mL baffled flasks; batch; 72 h
			Static-increase MI uptake	MI	1.65	4.58	0.42 (MI)	
2015 (Shiue et al. 2015)	*E. coli* MG1655 (DE3) derivative	*INO1*, *udh*, *SUMO-MmMiox*	Static-metabolic flux remodeling	Glc, Ara	18[y]	0.50	0.76 (G)	250-mL baffled flasks; batch; 72 h
			Static-metabolic flux remodeling	Glc, Xyl	18[y]	1.19	0.73 (G)	
			Static-metabolic flux remodeling	Glc, Gl	9[y]	0.81	0.44 (G)	
2015 (Reizman et al. 2015)	*E. coli* MG1655 (DE3) derivative	*INO1*, *udh*, *MmMiox*	Dynamic-degradation tag	Glc	1.42	1.56	0.12 (G)	250-mL baffled flasks; fed-batch; 24–32 h
2016 (Gupta et al. 2016)	*S. cerevisiae* CEN.PK2-1D derivative	*INO1*, *inm1*, *AtMiox4*, *udh*	First realized in *S. cerevisiae*	Glc	/	0.98	0.03 (G)	250-mL baffled flasks; fed-batch; 72 h
				Glc, MI	/	1.60	/	250-mL baffled flasks; batch; 72 h
2016 (Liu et al. 2016)	*P. pastoris* GS115	*MmMiox*, *udh*	a. First realized in *P. pastoris* b. Static-fusion protein	Glc, MI	/	6.61	/	3-L fermenter; fed-batch; 96 h
2017 (Gupta et al. 2017)	*E. coli* MG1655 (DE3) derivative	*INO1*, *udh*, *MmMiox*	Dynamic-QS system	Glc	/	0.85	0.09 (G)	250-mL baffled flasks; batch
2018 (Doong et al. 2018)	*E. coli* MG1655 (DE3) derivative	*INO1*, *udh*, *MmMiox*	a. Dynamic-MI biosensor b. Dynamic-QS system	Glc	4	1.98	0.20 (G)	3-L fermenter; batch; 72 h
2018 (Qu et al. 2018)	*E. coli* BL21 (DE3) derivative	*cscB*, *cscA*, *cscK*, *INO1*, *udh*, *suhB*, *mMiox*	a. Static-expand the substrate pool b. Dynamic-riboswitch	Suc	/	1.42	0.14 (S)	shake flasks; batch; 84 h
2018 (Chen et al. 2018)	*S. cerevisiae* BY4741 derivative	*AtMiox4*, *udh*	Static-overexpression	Glc, MI	/	6.00	/	5-L fermenter; fed-batch; 216 h
2020 (Su et al. 2020)	*E. coli* BL21 (DE3)	*INO1*, *udh*, *AtMiox4*, *suhB*	Static-metabolic flux remodeling	Glc, Gl	7.67[y]	5.35	0.54 (G)	1-L shake flasks; batch; 72 h
2020 (Marques et al. 2020)	*S. cerevisiae* CEN.PK2-1D derivative	*INO1*, *udh*, *FjMiox*, *inm1*	Static-homologous protein	Glc, MI	1.24	1.85	0.08 (G + MI)	500-mL shake flasks; batch; 96 h
		INO1, *udh*, *TmMiox*, *inm1*	Static-homologous protein	Glc, MI	1.18	1.76	0.08 (G + MI)	500-mL shake flasks; batch; 96 h
2020 (Zhang et al. 2020)	*S. cerevisiae* BY4741 derivative	*AtMiox4*, *udh*, vgb	Static-chassis strain optimization	Glc	1.29	6.38	/	5-L fermenter; fed-batch; 228 h
2020 (Xu et al. 2020)	*B. subtilis* 168 derivative	*INO1*, *udh*, *MmMiox* mutant	a. Static-metabolic flux remodeling b. Dynamic-QS system	Glc	/	0.80	/	250-mL baffled flasks; batch

Table 1 (continued)

Year	Host	Genes	Engineering strategies (partly displayed)	Substrate[a]	Boost [b](-fold)	Titer (g/l)	Yield[c](g/g)	Fermentation Condition[d](bioreactor; batch or fed-batch; time)
2021 (Li et al. 2021)	*S. cerevisiae* INVSc1 derivative	*AtMiox4*, *udh*	Same as ref. (Chen et al. 2018)	Glc, MI	/	11.21	/	250-mL baffled flasks; fed-batch; 168 h
			Static-expand the substrate pool	Avicel	/	0.54	0.04 (A)	250-mL Erlenmeyer flasks; consolidated bioprocessing; 168 h
			Static-expand the substrate pool	SECS	/	0.45	0.03 (SECS)	250-mL Erlenmeyer flasks; consolidated bioprocessing; 168 h
2021 (Zhao et al. 2021)	*S. cerevisiae* BY4741 derivative	*AtMiox4*, *udh*	Static-chassis strain optimization	Glc, MI	1.76	10.60	/	5-L fermenter; fed-batch fermentation; 240 h
2021 (Cheah et al. 2021)	*S. cerevisiae* CEN. PK2-1 C	*MmMiox*, *udh*	Static-metabolic compartment	MI	1.2	/	/	50-mL unbaffled flasks; batch
2022 (Li et al. 2022)	*S. cerevisiae* BY4741 derivative	*AtMiox4*, *udh*	a. Static-metabolic flux remodeling b. Static-fusion protein	Glc, MI	1.6	10.85	/	5-L fermenter; fed-batch fermentation; 264 h
2022 (Guo et al. 2022)	*S. cerevisiae* BY4741 derivative	*Miox*, *udh*	Static-chassis strain optimization	Glc, MI	1.5	9.50	0.22 (G)	7-L fermenter; fed-batch fermentation; 168 h
2023 (Zhao et al. 2023)	*S. cerevisiae* BY4471	*AtMiox*, *udh*	a. Static-fusion protein b.Static-metabolic flux remodeling	Glc, MI	/	15.60	1.17(MI) 0.24 (G)	5-L fermenter; fed-batch fermentation; 168 h

[a, c] *Abbreviations* in substrate and yield. *Glc/G* glucose, *MI myo*-inositol, *Ara* arabinose, *Xyl* xylose, *Gl* glycerol, *Suc/S* sucrose, *A* avicel, *SECS/S* steam-exploded corn stover

[b] Relative boosts of titers compared to the control strains in the same work. Those with superscript "y" denote the relative enhancement in GA yields

[d] Factors not shown were not found in the reference. Except for the *B. subtilis*, which were cultured at 37°C, the others were cultured at 30°C

The construction of the GA pathway in different chassis strains

The chassis strains used for the biosynthesis of GA include *E. coli*, *B. subtilis*, *S. cerevisiae*, and *P. pastoris*. *E. coli*, widely preferred due to its short growth cycle and well-defined metabolic pathway, is ideal for expressing heterologous pathways. However, the inevitable production of endotoxin limits *E. coli*'s use in producing food-grade chemicals. Conversely, due to its non-toxic properties, *B. subtilis* is generally regarded as safe (GRAS) strain, making it an excellent choice as a safe cell factory for protein expression and commodity chemicals production (Widner et al. 2005). The biosynthesis of GA was first achieved in *E. coli* in 2009, with a GA production of 1.13 g/L (Moon et al. 2009). This biosynthesis involved a heterologous GA pathway comprising genes encoding Ino1 from *S. cerevisiae*, *Mm*MIOX from *Mus musculus*, and Udh from *Pseudomonas syringae*, along with the endogenous phosphatase suhB in *E. coli*. In 2020, the GA pathway was introduced to *B. subtilis* with the help of a pyruvate-responsive genetic circuit, which increased GA production to 0.802 g/L compared to the control group (Xu et al. 2020). Metabolic analysis in *E. coli* for GA biosynthesis indicated that MIOX, the least active enzyme, exhibited improved expression solubilities with high substrate concentrations during the exponential phase while low expression solubilities during the stationary phase (Moon et al. 2009, 2010). Strategies such as balancing the expressions of key enzymes, exploring new enzyme sources, modifying rate-limiting enzymes, and enhancing metabolite transfer efficiencies could be employed to enhance GA production. Additionally, subsequent studies indicated that when glucose was used as the sole carbon and energy source, it primarily entered endogenous cellular metabolism rather than contributing to GA production (Moon et al. 2009; Gupta et al. 2016; Liu et al. 2016; Qu et al. 2018). Therefore, using a medium with various carbon sources may improve GA production. Despite different strategies being used in *E. coli* for enhancing GA production, the GA titer in *E. coli* could not be achieved

above 5.35 g/L (Shiue and Prather 2014; Su et al. 2020). It was hypothesized that the acidic environment generated by increased GA concentration showed toxicity to *E. coli*, resulting in a ceiling GA titer (Shiue and Prather 2014).

In contrast, *S. cerevisiae* is resilient to low pH (Parapouli et al. 2020) and possesses post-translational modifications that can aid in the correct folding of heterologous enzymes like MIOX. Additionally, *S. cerevisiae* harbors a multi-copy delta sequence in genomes, allowing heterologous genes to be integrated with high copies, which leads to high expression levels (Tschumper and Carbon 1982). Similar to *S. cerevisiae*, *P. pastoris* has significant potential for producing chemicals like GA as well (Peña et al. 2018). Ino1 and Udh exhibit higher activities at optimal growth temperatures between 28–30°C, making yeast such as *S. cerevisiae* and *P. pastoris* good chassis choices (Moon et al. 2009; Wagschal et al. 2015). Furthermore, *P. pastoris* harbors a strong promoter P_{AOX1} for high expression levels of enzymes and can grow to high cell densities in simple media (Karbalaei et al. 2020). While porting the GA pathway into *S. cerevisiae* and *P. pastoris*, the GA production reached titers of over 5.35 g/L, but the rate-limiting conversion catalyzed by MIOX was also observed (Gupta et al. 2016; Liu et al. 2016). In *S. cerevisiae*, it was found that MI inhibited the activity of Ino1, and its availability was limited during rapid growth phases, as it was the native component of the phospholipid cell membrane (Gupta et al. 2016). The highest GA production in *P. pastoris* and *S. cerevisiae* reached 6.61 g/L (Liu et al. 2016) and 15.6 g/L (Zhao et al. 2023), respectively, with engineering strategies including metabolic remodeling, Udh-MIOX fusion, supplement of MI in culture, etc. These high GA titers demonstrated the potential of yeast as the chassis strains for highly efficient GA production.

Static strategy

GA synthesis balancing

Optimizing the synthesis steps

Three key enzymes, Ino1, MIOX, and Udh, were involved in the pathway, while MIOX was considered as the rate-limiting enzyme because of its relatively low activity (Moon et al. 2009; Shiue and Prather 2014; Liu et al. 2016), and NAD^+-dependent Udh was considered to disturbance the balance of NAD^+/NADH ratio (Zheng et al. 2018; Su et al. 2020). To work out the severe imbalance of the NAD^+/NADH ratio deduced, *nox* gene encoding NADH oxygenase from *Lactobacillus pentosus* or *Lactococcus lactis* was introduced into the cell and proved to work (Zheng et al. 2018; Su et al. 2020). As for the rate-limiting enzyme MIOX, a formally mid-valent superoxo-diiron(III/III) complex which catalyzes the ring cleavage of MI by incorporating a single atom of oxygen (Arner et al. 2001; Bollinger et al. 2009), various strategies had been used to enhance its activity, such as enhanced expressions, direct evolution (Zheng et al. 2018), and efficient homologous MIOX screening (Marques et al. 2020; Vila-Santa et al. 2021).

Overexpressing rate-limiting enzymes was a common strategy to balance the pathway flux and had been demonstrated to be effective in enhancing GA production. For instance, increasing the copy number of *Miox* copies via high-copy plasmids or integrating it into the multi-copy delta sequence in *S. cerevisiae* (Moon et al. 2009; Chen et al. 2018; Su et al. 2020), as well as screening for optimal strong ribosome binding site (RBS) with the help of RBS calculator (Su et al. 2020), all showed improved GA production titers, further indicating the limiting effect of MIOX in GA synthesis. Due to the low solubilities and poor stabilities of MIOX in *E. coli*, fusion tags for *Mm*MIOX were also studied to enhance GA production (Shiue and Prather 2014; Ki and Pack 2020). It was demonstrated that an N-terminal SUMO fusion to *Mm*MIOX resulted in 4.85 g/L GA production, nearly doubling the GA titer compared to unfused *Mm*MIOX (Shiue and Prather 2014). Interestingly, it was found that green fluorescent protein (GFP) tags could also improve the stabilities of *Mm*MIOX (Cheah et al. 2021), while superfolder GFP tags were demonstrated to enable proper folding of heterologous proteins (Zhang et al. 2017), which might be also introduced for enhancing MIOX expressions.

To further enhance MIOX activities in GA synthesis, other efficient mutants and homologs of MIOX were screened. MIOXs widely used in GA synthesis were derived from the model organism *M. musculus* and *Arabidopsis thaliana*, among which *At*MIOX4 exhibited higher catalytic efficiency (Moon et al. 2010; Gupta et al. 2016; Chen et al. 2018; Su et al. 2020). A combination of direct evolution and a real-time screening system was applied (Fig. 2b), through which variants D82Y and S173N of *Mm*MIOX were both found to show increased MIOX activities (Zheng et al. 2018). Screening of MIOX from other organisms has also been carried out by computational aid using protein sequence-based bioprospecting. *Tm*MIOX from *Talaromyces marneffei* and *Fj*MIOX from *Flavobacterium johnsoniae* were identified, both leading to a 44% increase in GA production titer compared to the *At*MIOX4 in *S. cerevisiae* (Marques et al. 2020). The endogenous MIOX in *P. pastoris*, *Pp*MIOX, was assessed but with unmeasurable enzymatic activity (Liu et al. 2016), possibly due to the strict intracellular regulation of endogenous gene expressions in the chassis strain, which was similarly observed for endogenous Ino1 repression in *S. cerevisiae* (Gupta et al. 2016).

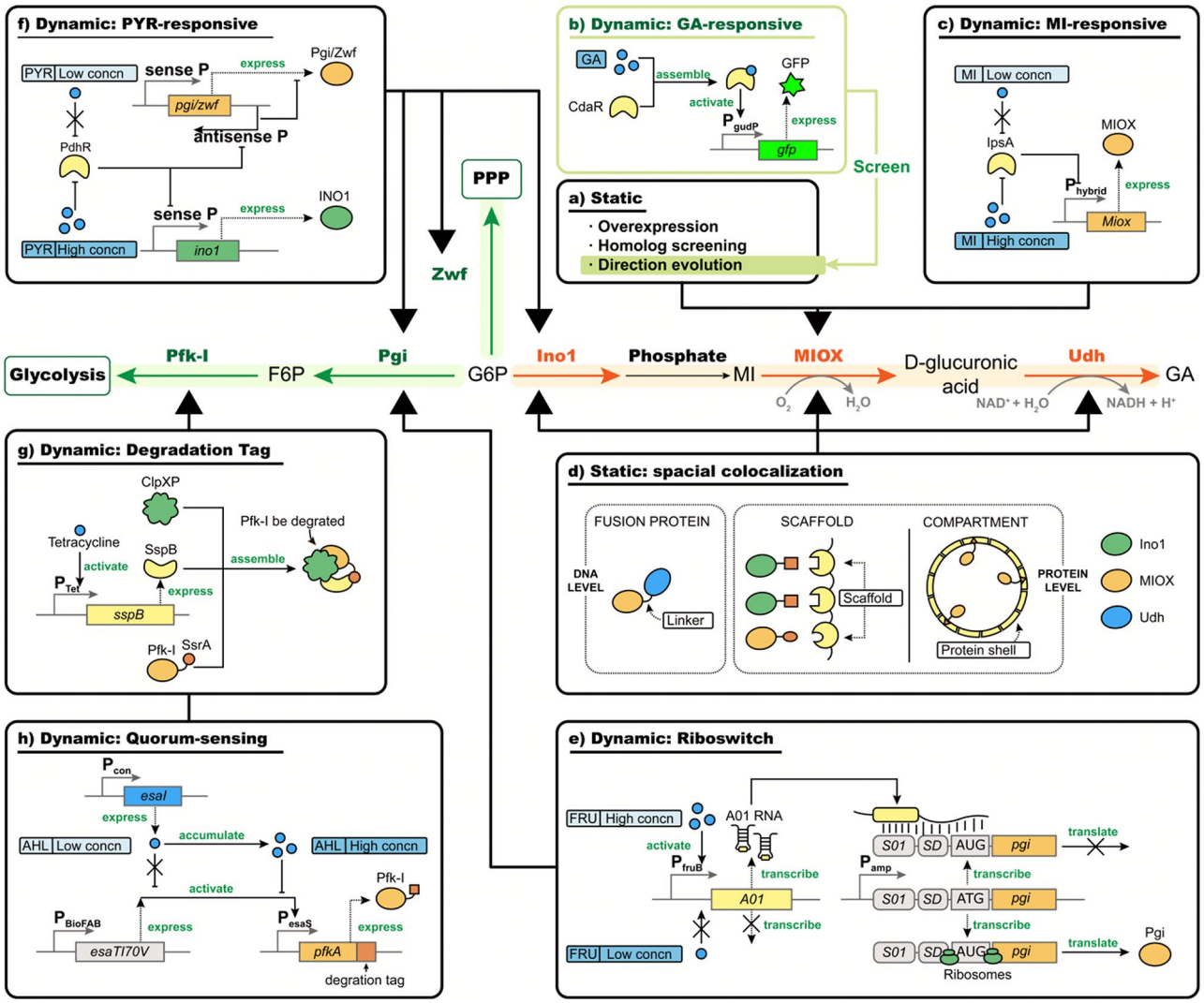

Fig. 2 Dynamic strategies and partial static strategies for GA biosynthesis: (a) optimizations for the rate-limiting step, while the direction evolution could be coupled with (b) a dynamic real-time screening system (Rogers and Church 2016), thus realizing rapid screening; (c) MI-responsive transcriptional regulator, regulating the transcription of *Miox* in answer to the MI concentrations (Doong et al. 2018); (d) spatial organ ization of pathway enzymes, fusing cascade enzymes with linkers at the DNA level(Liu et al. 2016), recruiting cascade proteins by scaffold at the protein level (Moon et al. 2010), isolating and concentrating the heterologous enzyme by metabolic compartment at the protein level (Cheah et al. 2021); (e) riboswitch, regulating Pfk-I at the translational level in response to fructose concentrations. (modified from reference (Qu et al. 2018)); f) pyruvate-responsive transcriptional regulator, regulating the transcription of *Ino1* and the translation of *Pgi* and *Zwf* in response to pyruvate concentrations (Xu et al. 2020); g) degradation tag, enabling the degradation of Pfk-I activated by tetracycline (Brockman and Prather 2015); h) quorum-sensing, regulating Pfk-I at the transcriptional level in response to AHL/cell population density (Gupta et al. 2017; Doong et al. 2018). Abbreviations: PYR, pyruvate; AHL, N-acyl-homoserine lactone; FRU, fructose

Colocalizing the enzymes

The uneven enzyme activities and scattered enzyme spatial locations also limited the efficiencies. Subsequent studies had endeavored to balance the synthesis efficiencies of each step for maximizing GA productions. Spatial colocalization of enzymes in the GA synthesis pathway was shown to increase synthesis efficiency, likely due to the enhanced concentrations of the metabolic intermediates (Dueber et al. 2009) and the improved balance of relative fluxes among adjacent enzymes. Two primary forms of spatial colocalization used in this pathway contained fusion protein (Chen et al. 2013) and scaffold (Dueber et al. 2009) (Fig. 2d). The fusion of MIOX and Udh by linkers increased the GA production by 79.7% in *P. pastoris* and 40% in *S. cerevisiae*, respectively (Liu et al. 2016; Li et al. 2022). With the fusion of signaling proteins to Ino1 and MIOX, which could be recruited by identical cognate ligands, approximately 5-fold GA production was achieved in *E. coli* (Dueber et al. 2009; Moon et al. 2010). However, the impact of pathway enzyme

fusion strategies on protein degradation and correct protein folding was also suspected to be a possible factor (DeLisa and Conrado 2009).

By fusion or domain-specific binding, spatially colocalized GA pathway enzymes could help gain more GA even with relatively low enzymatic expression levels. However, heterologous pathway enzymes such as MIOX might be toxic to cells (Cheah et al. 2021), which remain exposed in the cytoplasm. Furthermore, sometimes heterologous pathway enzymes were unstable in the cytoplasm of host strains, as the previous work had shown that overexpressed MIOX activity declined rapidly during the exponential growth (Moon et al. 2009). Therefore, metabolic compartments like a "bubble" in the cell to isolate the unstable heterologous enzyme seemed feasible to handle this issue (Fig. 2d). As a relatively independent space in the cell, metabolic compartments could help not only isolating the interactions between the heterologous pathway and cellular intrinsic metabolisms, but also spatially organizing the successive enzymes in the heterologous pathway, which could increase the local metabolite transfer efficiencies (Sheng et al. 2016; Polka et al. 2016; Grewal et al. 2021). A number of compartments were implemented in cells, such as membrane-based compartments like peroxisome (Sheng et al. 2016; Grewal et al. 2021) or protein nanocompartments like virus-like particles (VLPs) (Demchuk and Patel 2020). For GA biosynthesis, MIOX was encapsulated in self-assembled *Murine polyomavirus* (MPyV) VLP in the yeast, and 20% more GA titer was achieved with a lower MIOX expression level than that of using free-MIOX (Cheah et al. 2021). It is desirable to use the compartment to attenuate the burden of cell factories by physically encasing the heterologous enzymes, but whether it is suitable for industrialization remains to be considered.

Metabolic flux redirecting

Blocking the competitive pathways

Glucose is the main substrate for cellular life activities and is majorly imported into the cell *via* the phosphotransferase system in the form of G6P. G6P can be directed into the pentose phosphate pathway *via* glucose6-phosphate dehydrogenase (Zwf) or the glycolysis pathway *via* phosphoglucose isomerase (Pgi). These two pathways limit the pool of G6P available for GA biosynthesis. By knocking out or weakening *zwf* and *pgi*, researchers succeed in redirecting the metabolic flux for GA biosynthesis in both *E. coli* and *S. cerevisiae* (Shiue et al. 2015; Su et al. 2020; Li et al. 2022; Zhao et al. 2023), but the metabolic flux into the pentose phosphate pathway (PPP) and tricarboxylic acid cycle (TCA) were also blocked, which were two crucial systems for cell growth and intracellular $NAD(P)^+$/NAD(P)H balance (Fig. 3).

The G6P flowing into the GA synthesis would also encounter numerous issues, resulting in low GA yield. As an endogenous enzyme in yeast, Ino1 was subject to strict feedback inhibition, which could be effectively relieved by deleting the transcription repressor gene *Opi1* in *S. cerevisiae* (Fig. 3b) (Gupta et al. 2016). In yeast, metabolite leakage was another challenge for highly efficient GA biosynthesis, as MI could be transformed into constituents of membrane phospholipids and MI-containing metabolites (Gardocki and Lopes 2003). However, this could be mediated by repressing phosphatidylinositol synthase 1 (PIS1, a competitor for MI) (Fig. 3b) (Gupta et al. 2016). As for *E. coli* and *B. subtilis*, the main by-products were D-fructuronate and 5-dehudro-4-deoxy-D-glucarate (Fig. 3a), whose pathway could be blocked by deleting the gene *uxa*C (encoding uronic acid isomerase), and *gud*D (encoding glucarate dehydratase), respectively (Xu et al. 2020; Su et al. 2020). In *B. subtilis*, the deletion of the genes *yrbE* and *iolG*, both responsible for converting MI into *scyllo*-inosose (Fig. 3a), had been found to exert a regulatory effect on the central metabolic pathway as well (Xu et al. 2020).

Expanding the substrate pool

Glucose was usually used as the sole carbon source for the GA biosynthesis, while adding MI to the medium could improve the production of GA in all kinds of chassis strains (Moon et al. 2009; Gupta et al. 2016; Liu et al. 2016). The reason might be related to the fact that MIOX activity seemed positively correlated with the concentration of substrate MI (Arner et al. 2001; Moon et al. 2009). Furthermore, as the native component of the phospholipid cell membrane in yeast, additional MI was possibly biased to enhance cell growth, which boosted GA production. The intracellular MI could be divided into endogenous and exogenous sources. In yeast, MI could be synthesized from G6P *via* Ino1 and Inm1/2, or imported from the extracellular *via* ITR1 and other transporters (Fig. 3b) (Nikawa et al. 1991). In *E. coli*, endogenous synthesis of MI could be realized by introducing the Ino1 from *S. cerevisiae*, and the PtsG on the cell membrane possibly functioned as an MI transporter in *E. coli* (Fig. 3a) (Shiue and Prather 2014). While adding MI into the medium, it was feasible to increase the concentration of intracellular MI by regulating the transporter proteins, which had been implemented by overexpressing the ITR1 in *S. cerevisiae* (Li et al. 2022; Zhao et al. 2023) and relieving the inhibition towards PtsG in *E. coli* (Shiue and Prather 2014). In addition to importing additional MI, utilizing alternative carbon sources such as xylose, arabinose, and glycerol for cell growth could also help divert

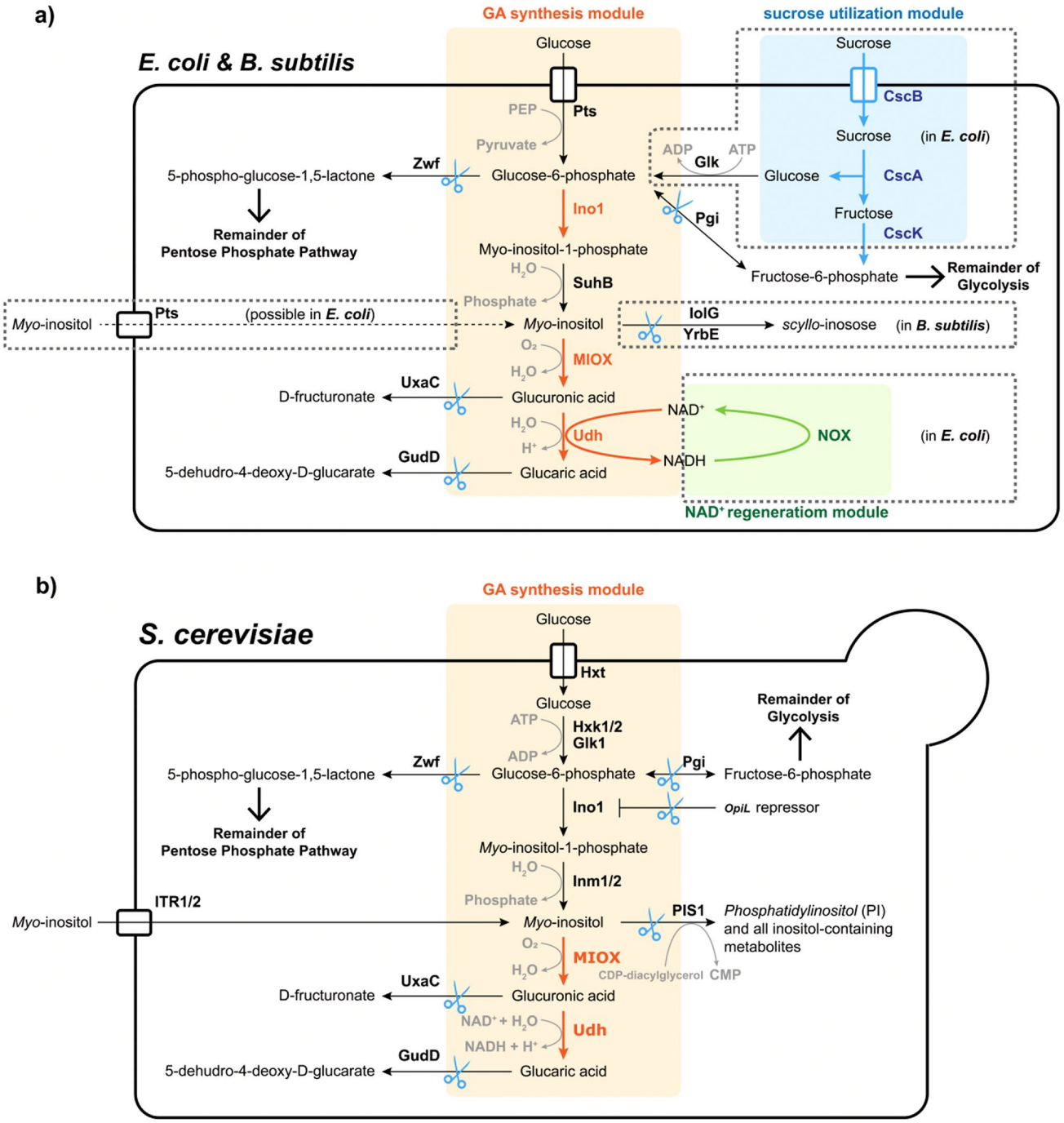

Fig. 3 Metabolic flux remodeling for GA biosynthesis in *E. coli*, *B. subtilis*, and *S. cerevisiae*: (**a**) metabolic pathway manipulation towards *E. coli* and *B. subtilis*; (**b**) metabolic pathway manipulation towards *S. cerevisiae.* (modified from reference (Gupta et al. 2016)). Elements: arrows and enzymes that are neither black nor grey indicate reactions and enzymes that require exogenous expression; orange blocks indicate GA synthesis module; blue block indicates sucrose utilization module; green block indicates NAD regeneration module; reactions labeled with blue scissors indicate knockout or downregulation; dashed boxes indicate features specific to *E. coli* or *B. subtilis*

glucose solely for GA biosynthesis (Shiue et al. 2015; Su et al. 2020).

Considering the economic and environmental benefits of various substrates (Choi et al. 2020), several studies also switched to using substrates such as sucrose and cellulose. In order to use sucrose as a carbon source, a sucrose utilization pathway was introduced into the GA-producing strain (Fig. 3a) while blocking the cellular glucose import system and the utilization of glucose for cell biomass (Qu et al. 2018). As a result, the fructose from sucrose was used for

cell growth, and the glucose from sucrose flowed into the GA pathway (Qu et al. 2018). The cellulose utilization for GA biosynthesis was achieved through the microbial consortium system, which used another cellulose-degrading bacterium—*Trichoderma reesei* (Li et al. 2021). Although the yields were still low, the potential for GA production from cellulose was worth exploring because of its environmentally friendly and cost-effective nature. Besides these substrates, producing GA from CO_2 could also represent a novel, economically viable, and environmentally friendly approach based on the established synthetic autotrophy model for CO_2 utilization (Gleizer et al. 2019).

Improving the cell viability

The imbalance between cell viability and product biosynthesis usually seemed inevitable. The yields of the products were usually positively correlated with the expression of the pathway enzymes, which would raise the competition between cell viability and product biosynthesis, destroy the cell homeostasis (Guo et al. 2022), and cause the undesirable titer of the target product (Lu et al. 2019). Enhancing cell viability was confirmed to be beneficial for GA biosynthesis, as magnesium ions, which could promote the growth rate of yeast cells, had improved GA production by promoting the utilization of glucose and MI (Zhao et al. 2021). Additionally, reinforcing the cellular oxygen supplementation by transforming *vitreoscilla* hemoglobin (VHb) into the *S. cerevisiae* resulted in higher glucose uptake, cell growth, and ethanol reassimilation rates (Zhang et al. 2020). Furthermore, decreasing ROS to boost the viability of microbial cell factories was also demonstrated as a potential strategy to lighten the metabolic burden for the chassis strain (Guo et al. 2022). While focusing on enhancing cell viability was a worthwhile endeavor, exploring the feasibility of implementing these methods together or coupling them with other strategies would also be significant for highly efficient GA productions.

Prospect for static strategies

Conventional static strategies such as enzyme engineering, and metabolic engineering have significantly advanced the construction of efficient GA cell factories. Nowadays, interdisciplinary collaboration with computational biology and bioinformatics has led to new breakthroughs in these strategies, thereby advancing biosynthesis technology. Emerging learning-based models for example AlphaFold (Callaway 2024), MutCompute (Shroff et al. 2020), and so on offer more precise and rational approaches to modifying enzymes or redirecting metabolic fluxer while conserving resources (Goshisht 2024).

Despite cutting-edge technologies providing numerous tools and insights to enhance GA production in cell factories, they couldn't encompass all possibilities in wet experiments, necessitating continuous updates and expansion of experimental data. Moreover, achieving a balance between GA production and cell growth remains a significant challenge, prompting the need for dynamic strategies to effectively address this issue.

Dynamic strategy

Real-time screening of high-yield strains for GA biosynthesis

High-yield strains that are to be applied in the industrialization of biosynthetic chemicals require a mass of screenings through constant design-build-test-learn cycles. Thus, reasonable high-throughput screening (HTS) methods could effectively help obtain the high-yield strains. The most frequently used HTS methods nowadays include biochemical and cell-based assays (Blay et al. 2020). Conventional screening of GA high-yield strains prefers biochemical assays such as high-performance liquid chromatography (HPLC). Cell-based assays aimed at achieving real-time screening of GA engineering strains mainly rely on fluorescence quantification (Rogers and Church 2016). In 2015, CdaR, a transcriptional activator in *E. coli*, in response to several diacids, such as GA, galactarate, and glycerate(Monterrubio et al. 2000), was applied as a GA detector (Rogers and Church 2016). By expressing fluorescent proteins under the CdaR inducible system, the GA biosensor was constructed, and positively correlated the concentration of GA with the visible intensity of fluorescence, which could be used for the real-time screening to identify different MIOX mutants in *E. coli* (Fig. 2b) (Rogers et al. 2015). However, the CdaR inducible system in *E. coli* could not be easily applied in yeast, and there was a lack of GA-related biosensors used in yeast, which raised the challenge of realizing real-time screening in yeast.

Integrating the GA biosynthesis module and the GA biosensor module in one strain, as mentioned above, brought challenges, specifically including uncertainty mutations (as the biosensor module might also be affected by mutations that led to false positives), weak reproducibility between parallel samples, omission of extracellular GA, and increased burden on the chassis strains (Zheng et al. 2018). Thus, isolating the biosensor module from the cell factory was taken into consideration. In 2018, the GA biosensor module was constructed in one *E. coli* strain, acting

as a precise extracellular GA indicator, used to detect the produced GA from the other engineering strains harboring the GA biosynthesis module (Zheng et al. 2018). It had also been proved that real-time monitoring of GA production in yeast could be achieved using the same two-strain GA indicator system, with the help of the *E. coli* strain harboring the GA biosensor module (Zheng et al. 2018; Zhao et al. 2023). Although HPLC, with its universal applicability, was suitable for low-throughput quantitative screening, the newly developed real-time screening method above provided a high-throughput approach for highly efficient GA productions.

Dynamic regulation of the intracellular metabolism

Dynamic regulations in cell factories could help optimize production processes by responding to changes in environmental conditions, metabolites, and other factors that possibly affect cellular metabolism. These systems enabled cellular real-time adjustments to the production process, improving the efficiency of yielding desired products (Doong et al. 2018). Dynamic control systems could also be applied to optimize substrate utilization and waste minimization, reducing the overall cost. Emerging online dynamic regulation strategies for GA biosynthesis mainly contained four types: degradation tag, quorum-sensing, riboswitch, and transcriptional regulator.

GA-pathway-independent dynamic control

The initial dynamic control applied in GA production involved the gene encoding a key regulatory glycolytic enzyme, phosphofructokinase-I (Pfk-I). Manipulation of Pfk-I was successfully proven to mediate cell growth and metabolism *via* redirecting central carbon flux (Yi et al. 2012). Thus, it was reasonable to establish correlations between cellular growth and the steady-state levels of Pfk-I. Pgi and Zwf were also used as dynamic tunable objects (Qu et al. 2018; Xu et al. 2020). As mentioned in static strategies, knocking down or weakening *pgi* and *zwf* could maximize the utilization efficiency of mixed carbon sources by redirecting glucose to the desired GA biosynthesis pathway and using other carbon sources for cell growth. All the enzymes above were used for constructing GA-pathway-independent dynamic control in diverse forms. The detailed processes are mentioned as follows.

1) Degradation tag. In 2015, a degradation tag known as SsrA was appended to the coding sequence of Pfk-I (Brockman and Prather 2015). This modification allowed for the regulation of Pfk-I's half-life through the inducible expressions of SspB, which facilitated the degradation of the SsrA-tagged proteins by binding to both ClpXP-the protein degradation machinery (Baker and Sauer 2012) and the SsrA tag itself (Fig. 2G) (Jh et al. 2011). When reaching the ideal growth density, the level of Pfk-I could be tuned down through the induction of SspB, thereby redirecting the carbon flux into the heterologous pathway for GA production. This led to the accumulation of G6P, resulting in a 42% improvement in GA production compared to strains lacking this system (Reizman et al. 2015). However, while the dynamic approach showcased promising outcomes, the inducers involved had limited applicability in industries. Thus, switches that could function without needing external inducers were required to fulfill industrial requirements.

2) Quorum-sensing (QS) systems. Several QS systems and their associated signal molecules, such as the LuxI/R QS system (Kim et al. 2017; Wu et al. 2020), EsaI/R QS system (Gupta et al. 2017), PrgX QS system (Wu et al. 2020), and autoinducer-2 (AI-2) QS signal (Tsao et al. 2010), had been harnessed to implement self-regulation within chassis strains. For the production of compounds like MI or GA, leveraging the principles of bacterial quorum sensing systems had been proven effective (Gupta et al. 2017; Doong et al. 2018). A notable example was the utilization of the Esa QS system derived from *Pantoea stewartia* (Minogue et al. 2002; Gupta et al. 2017; Doong et al. 2018). Within this framework, the accumulation of N-acyl-homoserine lactone (AHL), a signal molecule synthesized by AHL synthase EsaI (Minogue et al. 2002), served as the signal for cell population densities (Steindler and Venturi 2007). When AHL reached a certain concentration, it continuously impeded the binding of the P_{esaS} activator EsaRI70V, causing it to release from P_{esaS} and subsequently deactivate Pfk-I expression controlled by P_{esaS}, thereby shifting the metabolic flux from cell growth to GA production (Fig. 2h) (Gupta et al. 2017; Doong et al. 2018). Through regulating the expression level of AHL synthase EsaI, the expression of Pfk-I could be downregulated once cell growth reaches a desired level (Gupta et al. 2017; Doong et al. 2018).

3) Riboswitch. As mentioned previously, the chassis strain using sucrose as the sole carbon source could utilize the decomposed fructose and glucose as the substrate for cell growth and GA production, respectively (Qu et al. 2018). One problem emerged as fructose was depleted more rapidly, meaning an advanced growth arrest, which would be fatal to cellular viabilities (Qu et al. 2018). Thus, *A01* RNA controlled by a fructose-induced promoter was used as a riboswitch, whose transcript products could bind to the complementary sequence putting at the upstream of the mRNA *pgi*, as well as binding its Shine-Dalgarno (SD) sequence and AUG start codon. This riboswitch directed glucose flux into the GA biosynthesis pathway exclusively when a 'sufficient-fructose signal' was input, while it could also allow the entrance of glucose flux into the PPP pathway

for cell growth when a 'deficient-fructose signal' was input (Fig. 2e) (Qu et al. 2018).

4) Pyruvate-responsive transcriptional regulator. The PdhR, a pyruvate-sensing transcription regulator (Ogasawara et al. 2007), represses transcription of the pyruvate dehydrogenase multienzyme complex, and the repression could be relieved when pyruvate was present (Ogasawara et al. 2007). Thus, the expression of the target genes could be regulated based on the pyruvate concentration by leveraging the 'bio-bricks' within the above operon. Since the PdhR solely functioned to repress the transcription downstream of the PdhR-binding site, researchers introduced the PdhR-mediated antisense transcription system, which could degrade sense transcripts, as a 'NOT' gate to facilitate signal conversion (Xu et al. 2020). Subsequently, a parallel genetic circuit mediated by pyruvate was constructed. When intracellular pyruvate reached specific concentrations, the pyruvate-activated genetic circuit in one arm was used to enhance GA biosynthesis, while the antisense transcription-based pyruvate-inhibited genetic circuit in other arm suppressed competitive pathways (Fig. 2f) (Xu et al. 2020). Harboring this pyruvate-responsive genetic circuit, the engineered *B. subtilis* could enhance GA production titer by 2.5-fold (Xu et al. 2020).

GA-pathway-dependent dynamic control

In addition to using metabolites produced during cell growth as signals, metabolites produced in the GA pathway could also be utilized as signals for dynamic control. By integrating the IpsA-binding region upstream of *Miox*, the transcription of *Miox* could be dynamically regulated by IpsA, an MI-responsive transcriptional regulator from *Corynebacterium glutamicum* (Baumgart et al. 2013), in response to MI concentration[46]. IpsA could repress the transcription of *Miox* by binding to the engineered upstream region of the *Miox* (Doong et al. 2018). When sufficient MI was present and bound to IpsA, the MI-bound IpsA would be released from the DNA binding positions, allowing the transcription of *Miox* to continue, driving a 2.5-fold increase in GA titer (Fig. 2c) (Doong et al. 2018). Coupled with the Esa QS system mentioned above, the strain harboring the MI-responsive transcriptional regulator showed an improved GA titer of 4-fold (Doong et al. 2018).

Prospect for dynamic strategies

Efforts toward dynamic regulation for highly efficient GA production strains give us a glimpse of the abundant dynamic strategies nowadays. Increasingly, more and more related studies are emerging to develop optimal dynamic systems (Xu et al. 2020; Verma et al. 2022). However, dynamic control for chemical biosynthesis is still in its early stages and has a long way to go for industrial applications. One notable limitation is the scarcity of regulatory promoters in eukaryotic cells compared to prokaryotic cells, hindering dynamic regulations that could maximize GA production limitations. Nevertheless, numerous studies on dynamic modules (Hornby et al. 2001; Williams et al. 2013; Zhang et al. 2022; Xu et al. 2023) and related screening methods (L et al. 2022) in eukaryotic cells are emerging, offering infinite possibilities for autonomous dynamic modules in various chassis strains. Another notable limitation concerns the leakage of biosensor expressions and the uncertain range of dynamic regulations. With the development of artificial intelligence (AI), the dynamic range of transcription factor-based biosensors for GA sensing could be fine-tuned, which would help overcome the obstacles of imprecise dynamic control strategies and maximize GA production (Ding et al. 2020).

Conclusion and future directions

As a valuable chemical with a global market size expected to reach USD 2.82 billion by 2032, GA shows widespread applications in therapy, food, and other industries. Thus, successive studies occurred to try to get highly efficient GA production to an ideal scale, whether by chemical or biological methods. In this review, we discuss different strategies for GA biosynthesis in cell factories constructed upon chassis strain *E. coli*, *S. cerevisiae*, and *P. pastoris* (Moon et al. 2009; Gupta et al. 2016; Liu et al. 2016), from static strategies to complex pathway reprogramming, including dynamic regulation strategies.

The static approaches involve rate-limiting step optimizations, enzyme colocalizations, metabolic flux redirections, and cell viability improvements, modifying the cellular metabolic network to boost GA production. On the other hand, dynamic approaches utilize degradation tags, QS systems, riboswitch, and transcriptional regulators to decouple GA production from cell growth in specific phases, achieving highly efficient GA production. We also emphasize the advantages and disadvantages of these strategies, as well as future directions and challenges. Although static approaches are widely applied for engineering strains, their scopes and efficiencies are still limited by the complexities of metabolic networks to control. Dynamic approaches could overcome these limitations and decouple the chemical bio-productions from cell growth to maximize GA production. However, the stability of dynamic approaches still requires further studies to be applied in industries.

In summary, synthesizing GA in cell factories is a complex process that requires a combination of different

strategies and technologies for constructing highly efficient chassis strains. Future studies should focus on the integration of these strategies to achieve more efficient and stable industrial engineering strains with the help of a better understanding of cell factories' metabolic networks.

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Declarations

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