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Yeast as C1 cell factory: Transforming methanol and Formate into high-value compounds

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ABSTRACT

Microbial transformation of greenhouse gases, such as carbon dioxide and methane, into valuable biochemicals appears as a key strategy to sustainably decarbonize manufacturing industries. Numerous unresolved technological constraints still hamper the industrial adoption of these single-carbon (C1) gas-based bioprocesses. Conversion of these gases into liquid C1 compounds like methanol and formate helps to reduce emissions and close the carbon loop. Certain industrial yeasts possess intrinsic capabilities to tolerate and assimilate methanol and formate, which opens an attractive route to eco-efficiently valorise these compounds. To increase the C1-based biomanufacturing potential of yeasts, synthetic methylotrophy has been developed in versatile non-methylotrophic chassis. Strategic non-rational genome engineering and strain evolutions combined with rational designs brings to light hidden C1-pathways and mechanisms of substrate tolerance. Developments in methanol-based bioproduction include simple organic acids with clear promise for industrial scale-up as well as proof-of-concept investigations of complex polyketides with intricate pathways. Recent advances in bioproduction have demonstrated encouraging results from techniques such as modular co-culture engineering and peroxisomal coupling of biosynthetic pathways with C1 metabolism. Formate-based growth and biosynthesis in yeasts is in its early stages but holds the potential to be transformative in the coming decade. This review discusses the advances, challenges, and future perspectives in methanol-based biomanufacturing and innovative initiatives in formatotrophy in yeasts. Although it is a long way off, developments in synthetic biology assisted evolutionary engineering and artificial pathways will fill up the gaps in the scalability of C1-based bioprocesses, transforming yeasts into a reliable, climate-neutral, and resource-efficient platform for the green bioeconomy of the future.

1. Introduction

Methylotrophy is regarded as an exceptional capability of some microbes to use single carbon (C1) methylated substrates (such as methane and methanol) as the sole source of carbon and energy (Chistoserdova and Kalyuzhnaya, 2018). Some methylotrophic strains were also reported to assimilate other C1 molecules, including CO_2, CO and formate (Chistoserdova and Kalyuzhnaya, 2018). This challenging phenomenon of building all carbon-carbon bonds required for cellular processes from reduced carbon compounds has always captivated the research community. Hence, from the 19th to the 20th centuries, most of the methylotrophy-derived research was focused on the discovery of C1 compounds consumers, their unique metabolic pathways, and elucidating key enzymes in C1 assimilation (Chistoserdova et al., 2009). Recently, C1 compounds have emerged as the next generation feedstock for a circular bioeconomy, and methylotrophy has gained prominence in biomanufacturing (Orsi et al., 2023; Vasudevan et al., 2023). Valorising gaseous C1 compounds, e.g., methane (CH_4) and carbon dioxide (CO_2),

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for biomanufacturing carbon-negative chemicals have the additional benefit of contributing to climate change mitigation. But the vision of establishing a greenhouse gas-based biorefinery is a long-standing challenge due to the technological challenges (Hu et al., 2022).

Liquid C1 substrates such as methanol and formate can be sustainably produced from methane and CO_2 (Álvarez et al., 2017; Baldo et al., 2024; Cotton et al., 2020; Pfeifenschneider et al., 2017; Ruiz-Valencia et al., 2024; Zhang et al., 2021; Zhai et al., 2025). Hence, these substrates can indirectly support a sustainable bioeconomy by acting as intermediaries in sequestration of greenhouse gases (Cotton et al., 2020). Unlike C1-gases, methanol and formate are highly soluble and they are easier to store and transport. Methanol is additionally considered safer than methane despite its flammability, due to its higher flash point and lower vapor pressure (Parris et al., 2024). This C1 compound has a higher degree of reduction per carbon than canonical carbon sources, which can increase the titre and yield of biofuels and chemicals (Antoniewicz, 2019; Villa et al., 2023). Formate offers an advantage over other C1-compounds, as a carbon source because it has a low reduction potential to effectively transfer electrons to NAD(*P*)H, giving the cell reducing power directly (Yishai et al., 2016). Native formate-using microorganisms (formatotrophs) can grow on formate as the sole carbon source through two strategies. They can oxidize formate to CO_2 generating reducing power, which support the carbon fixation through the Wood-Ljungdahl pathway (reductive acetyl-CoA pathway) or the Calvin-Benson-Bassham (CBB) cycle. In the second strategy, direct incorporation of formate into carbon metabolism occurs through formyl-THF, an active intermediate that feeds into pathways like the serine cycle (Bar-Even, 2016). Among these pathways, the most energy-efficient is the Wood–Ljungdahl pathway, which is anaerobic and found in acetogens and methanogens—organisms that are difficult to genetically manipulate (Turlin et al., 2022). Although aerobic methanotrophs were previously regarded as an industrially viable option, they assimilate formate via serine pathway which is ATP-inefficient and thus result in poor biomass yield (Baldo et al., 2024; Bar-Even et al., 2013).

More research has been done on native and synthetic methylotrophy in bacteria than in yeasts. *Komagataella phaffii* (previously known as *Pichia pastoris*) and *Ogataea polymorpha* have recently recognized attention for their unique methylotrophic capabilities, which hold promise for future C1 biorefineries (Fabarius et al., 2021). Developing yeast-based methylotrophy is particularly significant due to their advantages over bacterial industrial chassis, including higher tolerance to formate (Cotton et al., 2020), methanol (Mota et al., 2021), extreme pH conditions and temperature fluctuations (Palma et al., 2025). Furthermore, their excellent protein expression capacity and ability to perform eukaryotic post-translational modifications make them ideal hosts for reconstituting metabolic pathways from diverse sources (Porro et al., 2005). The potential for organelle-targeted expression in yeasts presents an additional option for enhancing the production of specific metabolites that depend on organelle-specific metabolic processes (Avalos et al., 2013; Bayer et al., 2009; Wu et al., 2025).

One of the key distinguishing features of yeast methylotrophy is the crucial role of peroxisome in compartmentalizing and neutralizing the toxic intermediates (formaldehyde and hydrogen peroxide (H_2O_2)) formed during methanol oxidation (Fabarius et al., 2021). However, bacteria do not possess such special isolation and bacterial cytosolic methanol oxidation results in circulation of highly toxic formaldehyde around the cell (Kelso et al., 2022). In yeasts, dihydroxy acetone synthase (DAS1) catalyzes the fixation of formaldehyde to xylulose-5-phosphate to detoxify and incorporate formaldehyde into central metabolism. Yeasts also employ a strategic earlier induction of *DAS1* gene expression compared to alcohol oxidase (*AOX1*) to prevent formaldehyde accumulation in peroxisome (Sakai et al., 1996). The peroxisomal compartmentalization also promotes methanol utilization by ensuring proper partitioning of formaldehyde flux between carbon fixation and energy generation (van der Klei et al., 2006). In the context of bioproduction, local concentration of the pathway enzymes in the peroxisome can strengthen the metabolic flux and enhance product yields by coupling this flux with specific bioproduct synthetic pathways (Zhai et al., 2023; Zhan et al., 2023).

Although *Saccharomyces cerevisiae* and *Yarrowia lipolytica* are not classified as methylotrophs, they possess latent pathways for methanol and formate assimilation (Chen et al., 2024; Espinosa et al., 2020). *S. cerevisiae* is widely regarded as the prominent chassis for industrial biomanufacturing due to its ease of genetic manipulation than methylotrophic yeasts (Parapouli et al., 2020) and its versatility in producing a wide range of metabolites, including complex natural products (Gao et al., 2022). *Y. lipolytica* exhibits notable metabolic flexibility (Egermeier et al., 2017; Park and Ledesma-Amaro, 2023) and demonstrates higher tolerance to methanol, formaldehyde and formate compared to other yeasts (Fabarius et al., 2021; Konzock et al., 2021; Kuang et al., 2025). Given their favourable industrial properties, these two yeasts are key candidates for implementing 'artificial/synthetic' methylotrophy by transferring native C1 pathways or developing chimeric pathways (Wang G et al., 2021; Wang J et al., 2021; Zhu et al., 2024).

Native methylotrophic yeasts can also potentially be manipulated into 'synthetic methanotrophs' by heterologous expression of methane-oxidizing enzymes from methanotrophic bacteria (Yu et al., 2024). Methanotrophs are native methane-oxidizing microbes; however, their industrial application faces significant challenges, including a lack of sufficient genetic tools and slow growth rates (Baldo et al., 2024; Strong et al., 2015). Particulate methane monooxygenase (pMMO) is regarded as the only true catalyst existing in nature for highly selective methane oxidation (Choi et al., 2003). However, its expression in *E. coli* poses challenges due to its membrane-bound nature and the inability of *E. coli* to manage the toxicity of methanol, the immediate product of methane oxidation (Lu et al., 2019a). Given the native methylotrophic nature of yeasts and their demonstrated capacity to express challenging membrane proteins (Ayub et al., 2022), synthetic methanotrophy in yeasts is poised for substantial innovation.

Recent advances in metabolic engineering have demonstrated the ability of both native and synthetic yeast methylotrophs to produce a variety of platform chemicals, including organic acids, fatty alcohols, bioplastic precursors, and natural products (NPs) from methanol (Cai et al., 2022; Duan et al., 2018; Gan et al., 2023; Guo et al., 2023; Wefelmeier et al., 2024; Yu et al., 2023; Zhai et al., 2023). Formate-based biomanufacturing in yeasts is, however, still in its infancy. Recently, by overexpressing endogenous genes and using strain evolution, the reductive glycine (rGly) pathway was implemented in yeast and demonstrated the biosynthesis of the amino acids glycine and serine from formate (Bysani et al., 2024).

Adaptive laboratory evolution (ALE) has become an indispensable tool in yeast methylotrophy (Chen et al., 2024; Sarikaya et al., 2024; Wang S et al., 2023; Wang K et al., 2023). Recent ALE innovations have contributed immensely to resolving various challenges in C1-based biomanufacturing, including improving methanol and formate tolerance and assimilation, stimulating latent C1 pathways and stable integration of C1 assimilation pathways with host's core metabolism. This review discusses recent advances in yeast methylotrophy focusing on bioproduction of various metabolites from both native and synthetic methylotrophs. This review also addresses the various challenges and future perspectives on developing yeast platforms as next generation C1 chassis.

2. Native and synthetic methanol assimilation in yeast

2.1. Native methanol utilization capability in yeast

2.1.1. XuMP pathway

The XuMP pathway is the predominant methanol assimilation pathway in native yeast methylotrophs. The initial step in yeast

methylotrophy (Ozimek et al., 2005) is the oxidation of methanol to formaldehyde and H_2O_2 catalyzed by AOX1 (Zhu et al., 2020). As described in Fig. 1, H_2O_2 is detoxified to water and oxygen by a peroxisomal enzyme catalase (CAT). Toxicity of formaldehyde is tackled by maintaining a low intracellular formaldehyde concentration via assimilation or dissimilation pathway. In the assimilatory branch, which is localized entirely within the peroxisome, formaldehyde is funnelled into central carbon metabolism. Formaldehyde is first combined with xylulose-5-phosphate (Xu5P) by DAS to form dihydroxyacetone (DHA) and glyceraldehyde-3-phosphate (GAP). Dihydroxyacetone kinase (DAK1) then converts DHA to dihydroxyacetone phosphate (DHAP) which is subsequently condensed with GAP to form fructose 1,6-bisphosphate (FBP) by fructose 1,6-bisphosphate aldolase (FBA). Finally, FBP is converted to fructose-6-phosphate (F6P) by fructose 1,6-bisphosphatase (FBP). Fructose-6-phosphate is used both for the regeneration of Xu5P and synthesis of cellular metabolites via glycolysis or pentose phosphate pathway (Fig. 1). In the dissimilatory branch, which operates both in the peroxisome and the cytosol, formaldehyde is detoxified into CO_2 via a glutathione-dependent pathway through the action of formaldehyde dehydrogenase (FLD) and formate dehydrogenase (FDH), producing energy in the form of NADH (Yurimoto et al., 2011).

2.1.2. *Methanol assimilation pathways that co-assimilate formate and CO_2*

The oxygen-tolerant reductive glycine (rGly) pathway: The canonical methanol assimilation (XuMP) pathway is a formaldehyde fixation pathway which produces GAP, which is subsequently used for biomass formation. Recently, an alternative pathway, the oxygen-tolerant reductive glycine (rGly) pathway, was identified in *K. phaffiii* (Mitic et al., 2023). This pathway functions as a formate fixation route capable of co-assimilating methanol, formate, and CO_2. In this pathway, formate fixation occurs via the tetrahydrofolate cycle, ultimately leading to pyruvate and subsequently to oxaloacetate. Most enzymatic components of this rGly pathway are widely distributed among microorganisms, and different variations of this pathway have been proposed to exist in both aerobic and anaerobic microbes (Figueroa et al., 2018; Sánchez-Andrea et al., 2020; Sánchez-Andrea et al., 2020). By incorporating native genes from other organisms, rGly pathway was integrated and overexpressed as a synthetic route in the model organism *E. coli* (Kim et al., 2023), enabling growth on formate and methanol in combination with CO_2. As recent C1 cell factory research focuses on developing microbes capable of co-utilizing multiple C1 liquid substrates alongside CO_2 gas (Guo et al., 2024a, 2024b), the discovery of this native rGly pathway in *K. phaffiii* holds significant industrial potential.

Adh2-Sfa1-rGly (ASrG) pathway: The native methylotrophic capacity of *S. cerevisiae* was previously unknown until a study uncovered a growth and transcriptional response of these yeasts to methanol (Espinosa et al., 2019). *S. cerevisiae* was notably shown to possess alcohol dehydrogenase 2 (ADH2) which is capable of oxidizing methanol (Zavec et al., 2021). Subsequent ALE experiments were conducted to improve methanol-dependent growth and further elucidate the pathway (Espinosa et al., 2020). However, the exact methanol assimilation pathway remains unidentified. Interestingly, in a recent study conducted by Guo et al., 2024a, SCRaMbLE (Synthetic chromosome rearrangement and modification by LoxP-mediated evolution) mediated genome rearrangement and ALE was used in *S. cerevisiae*. This strategy revealed a new pathway referred as ASrG which is capable of co-assimilating formate and CO_2. In this study, the initial strain used for genomic reconstruction and ALE, possessed a recombinant XuMP pathway. However, after evolution, this recombinant pathway was deleted and yeast started assimilating methanol as sole carbon source at a μ of 0.009 h^{-1} via native ASrG pathway. When the XuMP pathway was reintroduced into the evolved strain, there was a reduction in lag phase and a 10.2 % and a 35.5 % increase in biomass and methanol consumption, respectively. However, this strain showed slow growth rate reflecting the poor efficiency of the ASrG pathway.

These ALE and non-rational engineering studies suggests the presence of latent methanol assimilation pathway in many yeast strains and their self-evolving ability hinting at the possibility to design chimeric pathways and expand the product spectrum in future. These pathways which can co-utilize CO_2 with methanol has added advantage of compensating for the carbon loss when highly reduced methanol is used as the sole substrate to produce highly oxidized compounds eg. lactic

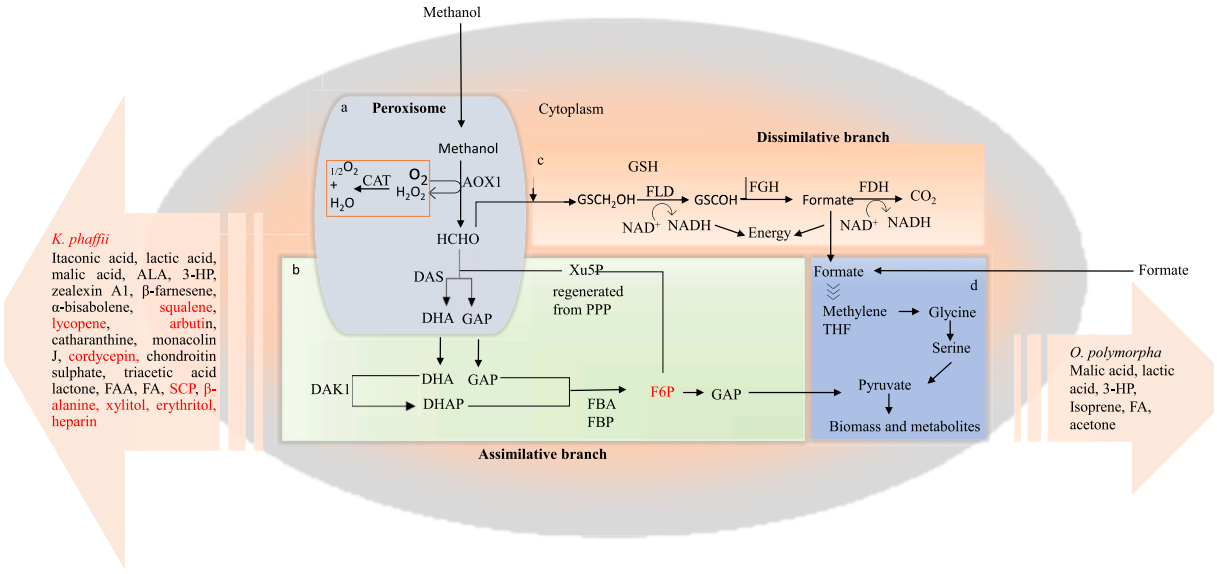

Fig. 1. The XuMP pathway showing the compartmentalization of methanol oxidation in the peroxisome (**a**), formaldehyde metabolism and entry into central carbon metabolism via assimilative branch (**b**), formaldehyde metabolism via dissimilative branch (**c**), where it is further oxidized for energy generation. The O_2-tolerant rGly pathway (Mitic et al., 2023) represents an alternative methanol assimilation pathway in *K. phaffiii* co-assimilating formate and CO_2. Formate fixation occurs via the tetrahydrofolate cycle leads to the formation of pyruvate (**d**). The spectrum of bioproducts already produced using methanol as a carbon source by native methylotrophs, such as *K. phaffiii* and *O. polymorpha*, is also represented. AOX1: alcohol oxidase, CAT: catalase, HCHO: formaldehyde, FLD: formaldehyde dehydrogenase, FGH: S-formylglutathione hydrolase, FDH: formate dehydrogenase, DAS: dihydroxyacetone synthase, TPI: triosephosphate isomerase, DAK: dihydroxyacetone kinase, FBA: fructose 1,6-bisphosphate aldolase, FBP: fructose 1,6-bisphosphatase, DHA: dihydroxyacetone, GAP: glyceraldehyde 3-phosphate, DHAP: dihydroxyacetone phosphate, F6P: fructose 6-phosphate, Xu_5P: xylulose 5-phosphate, GSH: glutathione,; PPP: pentose phosphate pathway.

acid (Claassens et al., 2022; Löwe and Kremling, 2021).

2.2. Strategies to develop synthetic yeast methylotrophs

To establish synthetic methylotrophy in yeasts, efforts have focused on the heterologous expression of the XuMP pathway (Dai et al., 2017) and the bacterial ribulose monophosphate (RuMP) pathway (Espinosa et al., 2020), as well as the development of a hybrid XuMP/RuMP pathway (Wang G et al., 2021) in non-methylotrophic yeast (Fig. 2). Designing computationally aided new-to-nature C1 pathways (Table 1, Fehér et al., 2014; Fernández-castané et al., 2014; Guo et al., 2024b; Lu et al., 2019b) represents a promising avenue in synthetic methylotrophy; this approach remains one of the least explored strategies in yeast-based methylotrophy.

2.2.1. Heterologous XuMP pathway

Previous ALE studies indicated *S. cerevisiae* innate methanol assimilation capacity (Espinosa et al., 2020; Espinosa et al., 2019). The lack of understanding of this inherent methanol utilization pathway hinders open avenues to harness its potential for shifting into a methylotrophic yeast growth style through rational engineering. Expression of the XuMP pathway enable synthetic methylotrophy in *S. cerevisiae*, but it requires supplementation with yeast extract as a co-substrate (Dai et al., 2017). In this study, the researchers initially expressed the prokaryotic methanol assimilation pathway in *S. cerevisiae*. Regrettably, this strain was not able to grow in a defined medium supplemented with methanol as the sole carbon source. Speculating that the cause might be the variation in codon usage between *S. cerevisiae* and *B. methanolicus* (from which the genes for the methanol utilization pathway were derived). Another study was conducted using methanol assimilation pathway genes derived from *K. phaffii*. In a fermentation study in the presence of 10 g. L^{-1} of methanol, the engineered strains showed a 3 % increase in biomass consuming 1.04 g. L^{-1} methanol and producing 0.26 g. L^{-1} of pyruvate. Although this confirmed the functional expression of the XuMP pathway in *S. cerevisiae*, methanol consumption remained very low. To further enhance the methanol uptake rate, 1 g. L^{-1} of yeast extract was supplemented, leading to an increase in biomass and methanol consumption by 11.7 % and 2.35 g. L^{-1}, respectively. The supplementation of yeast extract is suggested to enhance the XuMP pathway flux. Recent studies have indicated that the synthesis of amino acids from methanol is challenging in synthetic methylotrophy (Zhang et al., 2023). Since yeast extract is primarily composed of amino acids, its positive effect on growth enhancement in methanol-based systems is evident (Gonzalez et al., 2018). In addition, an enhancement in TCA cycle flux was also suggested as a reason to facilitating cell growth.

Overexpression of methanol assimilation pathway enzymes in non-methylotrophic yeasts alone do not significantly enhance methanol utilization; the efficiency of the pathway critically depends on the constant recycling of Xu5P, an essential formaldehyde acceptor (Kelso et al., 2022; Zhu et al., 2020). Native methylotrophs efficiently recycle Xu5P via a peroxisomal non-oxidative phase of the pentose phosphate pathway (PPP) (Rußmayer et al., 2015). In synthetic methylotrophs, when the XuMP pathway is introduced, the peroxisomal Xu5P recycling pathway must be constructed to balance the metabolic flux between

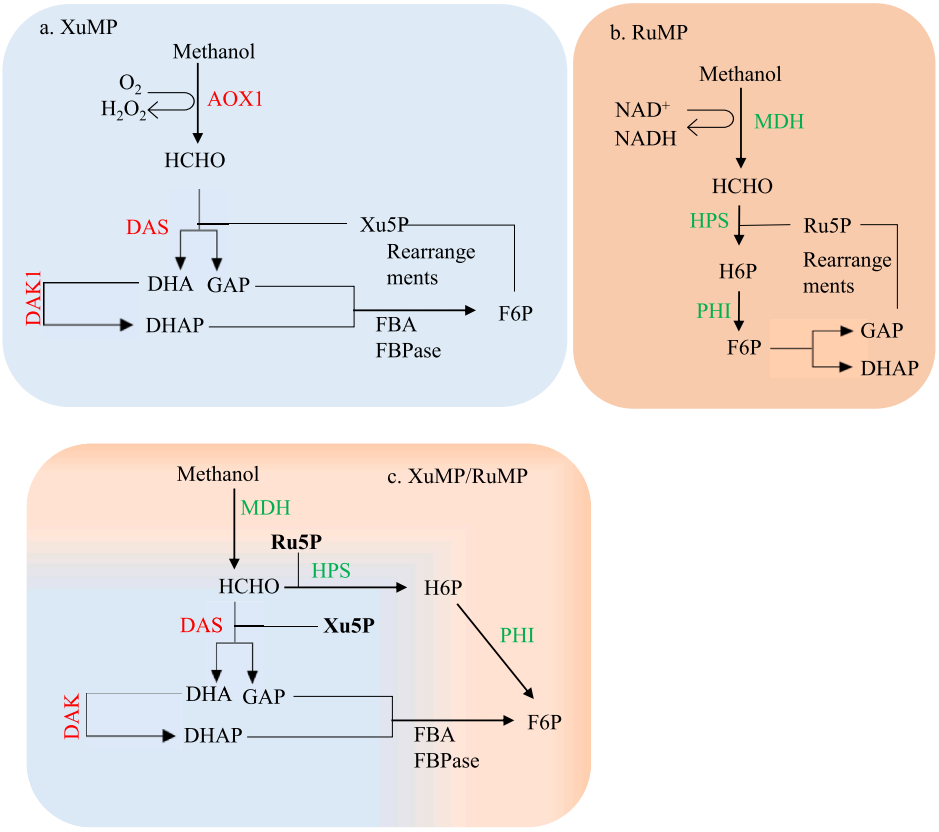

Fig. 2. Strategies for developing synthetic methylotrophy, synthetic methylotrophy involves various approaches, including the expression of XuMP pathway genes from *K. phaffiii* in non-methylotrophic yeasts such as *S. cerevisiae* or *Y. lipolytica (a)*. Another strategy involves the expression of RuMP pathway genes from bacteria in yeast *(b)*. Additionally, a rationally designed chimeric pathway has been developed, combining elements of the yeast XuMP pathway, such as *DAS* and *DAK*, with bacterial RuMP pathway genes, including *MDH, HPS,* and *PHI* (Wang G et al., 2021) *(c)*. Genes shown in red and green represent recombinantly introduced XuMP and RuMP pathway genes. AOX1: alcohol oxidase, HCHO: formaldehyde, DAS: dihydroxyacetone synthase, DAK: dihydroxyacetone kinase, F6P: fructose 6-phosphate, FBA: fructose 1,6-bisphosphate aldolase, FBP: fructose 1,6-bisphosphatase, DHA: dihydroxyacetone, GAP: glyceraldehyde 3-phosphate, DHAP: dihydroxyacetone phosphate, Xu_5P: xylulose 5-phosphate, MDH: NAD^+-dependent methanol dehydrogenase, HPS: 3-hexulose-6-phosphate synthase, PHI: 6-phospho-3-hexuloisomerase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Modified and artificial methylotrophic pathways.

Pathway	Descriptions	Engineered (in vitro or in vivo)	Advantages	Disadvantage	References
New pathways based on native pathways					
XuMP/RuMP hybrid pathway	Mix-and-match approach	*S. cerevisiae, Y. lipolytica*	Helps to overcome the limitations of pathway enzymes	Growth is not industrially relevant, need optimization	(Wang G et al., 2021; Zhan et al., 2023)
Methanol condensation (MCC) pathway	RuMP coupled with a nonnative pathway (non-oxidative glycolysis pathway)	Cell free system	Circumvents carbon loss in the native RuMP cycle, high carbon efficiency and ATP independent	Not yet demonstrated in vivo	(Bogorad et al., 2014)
Homoserine cycle	Variant of serine pathway	*E. coli*	Simplified pathway, outperform the serine cycle, support higher yields acetyl-CoA derived bioproducts	Need protein engineering to enhance kinetics of pathway enzymes	(He et al., 2020)
Modified serine pathway	Variant of serine pathway	*E. coli*	Simplified pathway, support *E. coli* to grow on formate (or methanol) alone	Requires further optimization	(Yu and Liao, 2018)
Serine–threonine cycle	Variant of serine pathway	*E. coli*	Low interference with central metabolism, bypasses the formation of the toxic intermediate hydroxypyruvate in serine pathway	Requires further optimization	(Klein et al., 2022; Yishai et al., 2017)
rGly pathway	Modified anaerobic C1 fixation pathway (Reductive acetyl-CoA pathway)	*E. coli,*	Linear, limited overlap with central metabolism, most efficient theoretical route—in terms of energy utilization, resource consumption and biomass yield, support the highest yields of acetyl-CoA and pyruvate	Need long term evolution or engineering to bypass limiting reactions and increase growth rate	(Kim et al., 2020; Yishai et al., 2018)
Dihydroxyacetone synthase (DAS) pathway	Mix and match approach/in silico designed pathway	*E. coli*	Strains performed like *E. coli* harbouring RuMP pathway	Requires further optimization	(De Simone et al., 2020)
Synthetic pathways based on artificial, promiscuous or repurposed enzymes					
Formolase pathway	Based on computationally designed enzyme formolase (FLS)	In vitro, *E. coli*	Linear, generate metabolite that can directly enter central carbon metabolism	Catalytic efficiency of FLS is very low, exhibits one-third carbon loss during acetyl-CoA synthesis	(Siegel et al., 2015; Wang et al., 2017)
2-hydroxyacyl-CoA-lyase (HACL) pathway	Based on newly identified activity of enzyme RuHACL from *Rhodospiralleles bacterium*	*E. coli* (whole cell catalysis)	Linear pathway, achieved production of glycolate (from formaldehyde)	HACL poorly expressed in *E. coli*	(Chou et al., 2019)
Formyl-CoA elongation (FORCE) pathway	Orthogonal synthetic pathway, variant of HACL pathway	In vitro (cell free system), *E. coli* co-culture system	Suitable for synthetic methylotrophy, facile flux regulation between C1 based growth and product formation	Formation of byproduct (formate)	(Chou et al., 2021)
Synthetic acetyl-CoA (SACA) pathway	Repurposed and engineered GALS (glycoaldehyde synthase)	In vitro, *E. coli*	Linear pathway	Low substrate affinities of enzyme, no growth in minimal media	(Lu et al., 2019a, 2019b, 2019c)
Erythrulose monophosphate (EuMP) cycle	Promiscuous activity of dihydroxyacetone phosphate dependent aldolase	In vitro, *E. coli*	Similar energetic efficiency as the RuMP cycle	In vivo establishment need more metabolic engineering and long-term ALE	(Wu et al., 2023a, 2023b)
Glycoaldehyde assimilation (GAA) pathway	Catalyzed by engineered enzymes Gals and TalB^{F178Y}	In vitro, *E. coli* (whole cell catalysis)	Linear pathway, 88 % carbon yield	No proof of in vivo functionality, low substrate affinities of enzyme	(Jo et al., 2022; Yang et al., 2019)
Glycolaldehyde-allose 6-phosphate (GAPA) pathway	Synthetic pathway based on artificial aldolase reaction	In vitro	94 % carbon yield	No proof of in vivo functionality promiscuities and kinetics problems of enzymes	(Mao et al., 2021)

downstream metabolism for product synthesis and the regeneration of Xu5P, ensuring continuous formaldehyde assimilation and methanol utilization. (Gan et al., 2023; Zhang et al., 2023). Another strategy to enhance the Xu5P precursor pool is to engineer the strain to utilize the co-substrate xylose. When xylose is metabolized, metabolic flux via PPP pathway is elevated which, in turn, improves Xu5P recycling and methanol utilization (Kelso et al., 2022). Introducing the xylose metabolic pathway also prevents the accumulation of formaldehyde by promoting the XuMP pathway. Addition of xylose is more beneficial than adding yeast extract and effectively supports the growth of synthetic methylotrophs. However, the goal of synthetic methylotrophy is to engineer strains that can grow solely on methanol (Gan et al., 2023). A xylose-assisted ALE approach is promising for gradually decreasing xylose dependence and improving methanol adaptation. In synthetic methylotrophy using *S. cerevisiae*, the strains are initially grown in minimal medium containing methanol and xylose (1 % *v*/v, each), and later, xylose supplementation is reduced to 0.2 % (Zhan et al., 2023). This synthetic methylotroph achieved a growth rate ($\mu = 0.051\ h^{-1}$) in minimal medium containing sole methanol that can be further improved to align with the growth rate of native methylotrophic yeast ($\mu = 0.085\ h^{-1}$). This strategy demonstrates that by integrating alternative detoxification pathways—in this case, the xylose utilization pathway, which detoxifies formaldehyde by supplying Xu5P—and then gradually weaning the strain off this pathway, the microbe can evolve its native detoxification system for formaldehyde neutralization.

The wild-type *Y. lipolytica* strain has demonstrated the ability to tolerate methanol (Vartiainen et al., 2019). This yeast possesses a native ADH2 enzyme capable of oxidizing methanol into formaldehyde and a homologous FLD enzyme responsible for formaldehyde detoxification. However, this yeast does not have a downstream pathway for formaldehyde fixation (Vartiainen et al., 2019). A recent study demonstrated that the XuMP pathway expression in peroxisomes of *Y. lypolitica* enabled growth in methanol with the assistance of yeast extract (Zhang et al., 2023). With the heterologous XuMP pathway alone, the yeast

failed to grow on methanol. However, in a whole-cell catalysis study, when the cells were transferred to a medium containing 10 g. L^{-1} methanol, the OD_{600} of the engineered strains slightly increased after 48 h, while the OD_{600} of wild-type cells decreased by 50 %. The addition of yeast extract (1 g. L^{-1}) along with methanol (10 g. L^{-1}) resulted in the OD_{600} of the engineered strain increasing to its highest value of 0.96 at 72 h. Further engineering for enhanced xylose uptake, peroxisomal Xu5P recycling, and overexpression of heat shock protein 70 (HSP70) enabled the cells to better respond to stress during methanol oxidation. Following these modifications, this synthetic methylotroph achieved an OD_{600} of 10.5 and consumed 5.2 g. L^{-1} of methanol within 72 h, utilizing 20 g. L^{-1} xylose as co-substrate in test tube batch fermentation study (Zhang et al., 2023).

2.2.2. Bacterial RuMP in yeast

During methanol oxidation via the XuMP pathway, AOX1 generates toxic H_2O_2, while methanol dehydrogenase (MDH) enzyme associated with the RuMP pathway provides NADH as a byproduct, thus conserving energy (Wendisch et al., 2021). Introducing a high-energy-efficient RuMP pathway in yeast is therefore considered a promising strategy for efficient biomanufacturing from methanol (Kelso et al., 2022). In a previous study with *E. coli*, it was shown that 40 % of methanol consumed was fixed into central carbon metabolites when the RuMP cycle from *Bacillus methanolicus* was introduced (Müller et al., 2015). In silico modelling studies suggest that this bacterial C1 assimilation pathway is ideal for *S. cerevisiae* (Comer et al., 2017). Previously, the RuMP pathway was introduced in *S. cerevisiae* by expressing genes *MDH* (from *Bacillus stearothermophilus*), 3-hexulose-6-phosphate synthase (*HPS*), and 6-phospho-3-hexuloisomerase (*PHI*) (from *B. methanolicus*) (Espinosa et al., 2020). When the RuMP pathway was compared with a hybrid XuMP pathway (where *AOX1* was replaced with *MDH* gene), *S. cerevisiae* strains harbouring the RuMP pathway showed superior growth on minimal solid medium containing methanol as a carbon source compared to the hybrid pathway (Espinosa et al., 2020). However, these strains did not grow in liquid cultures containing methanol, even with the addition of yeast extract.

A major challenge of the RuMP pathway is that, as all enzymes are expressed in the yeast cytosol, this leads to toxic formaldehyde accumulation. To address this, the glutathione-dependent formaldehyde dehydrogenase protein (SFA1P) from the native formaldehyde dissimilation pathway was overexpressed in *S. cerevisiae* to enhance formaldehyde detoxification. This enabled methanol assimilation in liquid cultures containing yeast extract as a co-substrate (Espinosa et al., 2020). However, in liquid cultures, no growth advantage was observed for strains harbouring the RuMP pathway compared to the hybrid-XuMP pathway. Like an earlier study conducted by Dai et al. (2017), this study also indicates that the overexpression of bacterial-derived pathways in yeast needs to be more strategically executed to achieve a growth advantage in *S. cerevisiae* (Kelso et al., 2022). The primary reason restricting methanol utilization via MDH in the RuMP route is the enzyme's poor methanol oxidation efficiency (Zhan et al., 2023). In addition, MDH catalyzes the reverse reaction—the reduction of formaldehyde to methanol—which has been reported to be up to 1000 times more favourable than methanol oxidation (Kim et al., 2025; Price et al., 2016). This reversibility limits methanol bioconversion and constrains flux through the RuMP pathway. In this context, AOX1 is advantageous, as it catalyzes an irreversible methanol oxidation reaction, thereby driving metabolic flux more efficiently. Hence, chimeric pathway designs incorporating AOX1 or engineered MDHs are preferred to overcome the kinetic and thermodynamic limitations of MDH.

2.2.3. XuMP/RuMP hybrid

Different combinations of RuMP and XuMP cycle genes from *B. methanolicus* and *K. phaffi* respectively (Zhan et al., 2023) were used to generate 16 combinatorial methanol assimilation pathways in *S. cerevisiae*. These combinations were designed by combining four methanol oxidation enzymes (AOX1, MDH1, MDH2 and MDH3) and four formaldehyde assimilation enzymes (DAS1-DAK, DAS2-DAK, HPS-PHI and HPS-PHI). Recombinant strains were analyzed for difference in biomass yield after growing them in in minimal medium containing methanol (0.5 % *v*/v) and yeast extract (1 % *w/v*). Compared to the wild-type strain, two recombinant strains containing *DAS1-DAK* genes with either *AOX1* or *MDH3* showed increase in OD_{600} by 65 % and 52 %, respectively. Among the three *MDH* paralogues (*MDH1, MDH2,* and *MDH3*) from *B. methanolicus* used in this study, MDH3 performed 2-fold better than other MDHs while activity of AOX1 was 2.3- fold higher than MDH3. These results showed that the efficiency of methanol utilization is heavily influenced by the source and type of methanol-oxidizing enzyme.

A hybrid pathway combining bacterial RuMP pathway genes (*MDH*, *HPS*, and *PHI*) with partial yeast XuMP pathway genes (*DAS1* and *DAK*) was designed in *Y. lipolytica* (Wang G et al., 2021; Wang J et al., 2021). In initial experiments, strains harbouring cytosolic or peroxisomal chimeric pathways consumed only a very small amount of methanol, which was barely detectable. Further strain engineering, including *FLD* deletion and overexpression of key genes associated with glycolysis and PPP, enhanced Ru5P and Xu5P pools, leading to improved methanol consumption. A subsequent ALE experiment produced the evolved strain Iso4, which exhibited methanol assimilation of 1.1 g. L^{-1} over 72 h which is 4.7 % higher than the non-evolved engineered strain. In minimal medium containing methanol as the sole carbon source, Iso4 maintained biomass at its initial level throughout a prolonged cultivation period (229 h), whereas the parental and non-evolved engineered strains experienced a rapid biomass decline to near zero. This supports the conclusion that the final evolved strain enabled cellular maintenance in the presence of methanol as the sole carbon source. ^{13}C labeling confirmed that the assimilated methanol was converted into proteinogenic amino acids. Interestingly, this evolved strain lacked XuMP pathway gene expression but instead exhibited transcriptional upregulation of the RuMP pathway and Ru5P regeneration pathway, following a gene expression profile like native bacterial methylotrophs.

These chimeric pathways demonstrate a strategic approach to overcoming the limitations of methanol-oxidizing enzymes through a mix-and-match strategy. A key limitation of the XuMP pathway is the involvement of AOX1, which does not produce NADH, impacting cellular redox balance. The chimeric pathway designed by Wang G et al. (2021) incorporated *MDH* gene from *B. stearothermophilus*. It is suggested that rapid formaldehyde assimilation via the yeast XuMP pathway helps mitigate the thermodynamically unfavorable nature of MDH-catalyzed methanol oxidation. Zhan et al. (2023) developed a dual catalytic system incorporating both MDH3 and AOX1 as a methanol oxidation module. This engineered *S. cerevisiae* strain exhibited final biomass and methanol utilization increases of 28 % and 152 %, respectively, compared to the parental strain. Although the implementation of chimeric pathways and methylotrophy in *S. cerevisiae* and *Y. lipolytica* is challenging, requiring systematic metabolic engineering and ALE, the final strains achieved moderate methanol assimilation in these non-methylotrophic yeasts. ALE studies further highlighted the importance of coordinated fine-tuning of both the C1 assimilation pathway and central metabolism in yeast to establish stable methylotrophy. These findings lay the foundation for future efforts to develop methanol-based bioproduction of a broad spectrum of bioproducts.

2.2.4. Synthetic pathway for co-assimilation of methanol, formate and CO_2

A recent study demonstrated the growth of engineered *K. phaffii* and *S. cerevisiae* solely on methanol without extra metabolic modifications for bioproduction from methanol and CO_2 (Guo et al., 2024b). A - synthetic C1 assimilation pathway referred as methanol and formic acid oxidation module with the reductive glycine pathway (MFORG pathway) was introduced into *K. phaffii* after blocking the native XuMP pathway enabling mixotrophic growth on methanol, CO_2 and formate. This pathway was introduced into *S. cerevisiae* to demonstrate the co-

assimilation of C1 substrates in this non-methylotroph. Using this pathway the CO_2 fixation rate of *K. phaffii* and *S. cerevisiae* was 0.044 g. L^{-1}. h^{-1} and 0.014 g. L^{-1}. h^{-1} respectively with methanol co-feeding. Further, both strains were engineered to synthesise 5-aminolevulinic acid (ALA) and lactic acid from methanol and CO_2. This pathway is a promising strategy to compensate for the carbon loss (greater than 70 %) via the dissimilatory branch when methanol is used as a substrate in *K. phaffii*, which leads to significant reductions in growth and bioproduct yield (Jorda et al., 2014). Through this synthetic pathway, formate and CO_2 can be reassimilated, enhancing the carbon conversion efficiency of methanol and improving biomass yield in *K. phaffii*.

3. Bioproduction from methanol

Use of food-or-feed-derived sugar is not suitable for sustainable-development of biobased products (Usmani et al., 2021). Relative to conventionally used renewable substrates, methanol has a high degree of reduction (6 versus 4 per carbon atom over glucose) and can be of high-purity depending on its origin, permitting byproduct-free bioproduction (Zhang et al., 2018). The utilization of green methanol—methanol derived from either biomass (bio-methanol) or greenhouse gases (e-methanol)—for bioproduction is considered a key future approach for sustainable biorefinery development worldwide (IRENA, 2021; Reiter et al., 2024). Notably, the annual production capacity of commercial green methanol plants is expected to increase to approximately eight million metric tons by 2027 (Methanol Institute, 2023). Consequently, in the last decade, several studies have been conducted to demonstrate the feasibility of employing methanol as a carbon source for the biosynthesis of different groups of industrially relevant compounds from both native (Fig. 1) and synthetic methylotrophic yeasts (Table 2) (Duan et al., 2025).

3.1. Organic acids

The global market for organic acids has reached $28.6 billion in 2024 and is projected to reach $53 billion by 2033 (IMARC, 2025). But market expansion of organic acids is limited by the environmental toxicity of current production strategies (Ali et al., 2024). Methylotrophy and synthetic methylotrophy is regarded as a new mode of green and socially responsible production of organic acids and have the potential to replace conventionally used unsustainable production processes in the future. Though there are challenges to reaching industrial scale implementation, methanol-based production titres of organic acids such as D-Lactic acid (Inoue et al., 2025), 3-HP (Àvila-Cabré et al., 2024) and itaconic acid (Severinsen et al., 2024) are promising.

Lactic acid: Both D- and L-lactic acids are industrially valuable compounds, particularly for food, pharmaceutical, and cosmetic applications, while polylactic acid is a significant bioplastic. Since only a limited number of bacteria are capable of selectively producing the D-enantiomer over the L-form, microbial production of D-lactic acid is in high demand in industrial applications. A test tube-level fermentation study provided D-lactic acid enantiomer production of 3.48 g. L^{-1}(0.22 g. g^{-1} methanol) form *K. phaffii* (Yamada et al., 2019). This yield is very low when compared to the yield from glucose however, comparable to lactic acid yield (0.36 g. g^{-1}) reported from lignocellulosic biomass in a previous study (Laopaiboon et al., 2010). In this study, the copy number of lactate dehydrogenase (LDH) was increased to a maximum of 9 to enhance product yield, and a comparative analysis of different recombinant strains revealed a correlation between copy number and lactate titre. In a time-course study evaluating cell growth and D-lactic acid production from methanol, both wild-type and recombinant strains showed similar growth through the fermentation study and one of the strains showed an enhanced growth after 192 h than parent strain. Methanol consumption rates during the early phase of fermentation ($0-120h$) was also similar in all the strains. The engineered strains steadily produced lactic acid up to 96 h. After 96 h, while D-lactic acid production and cell growth in the recombinant strains plateaued, methanol consumption continued steadily. Upon depletion of methanol, some engineered strains also consumed lactic acid. This study reaffirms the high capacity for NADH production in *K. phaffii* during methanol metabolism. Exploiting this property is beneficial for the biosynthesis of lactic acid and other similar products that depend on NADH for enzymatic activity, as previously demonstrated by Schroer et al. (2010).

Later, Inoue et al. (2025) conducted ultraviolet (UV) mutagenesis on the D-lactic acid-producing strain described above, enabling the production of 5.38 g. L^{-1} of D-lactic acid from 30 g. L^{-1} methanol after 168 h of cultivation. Cultures were inoculated at an initial OD_{600} of 10, and after 48 h, OD_{600} reached ~30, with little change observed afterward. Additionally, there was no substantial variation in the growth of the parent and mutant strains. The mutants exhibited three key changes: activation of the glyoxylate pathway, reduced expression of the *O*-glycosylated protein associated with cell wall stability, and limited formaldehyde accumulation. These strategies are worth considering when rationally constructing other yeast methylotrophs. Later, in a separate study, a combination of different D-LDH genes from various origins and eight promoters in *K. phaffiii* was used to develop 64 recombinant strains. One of the strains, integrating the D-*LDH* gene linked to *CAT1* and *FLD1* promoters, produced a maximum of 5.18 g. L^{-1} of D-lactic acid after 168 h of cultivation following gene amplification and transformation (Inoue et al., 2024). This study also used a higher methanol concentration of 30 g. L^{-1} and recombinant strains completely consumed methanol after 192 h of cultivation. The use of dual promoters enabled constant *LDH* gene expression, allowing for stable D-lactic acid production over an extended period, thereby increasing the overall titre.

A maximum L-lactate titre of 2.6 g. L^{-1} was notably achieved on day 9 of cultivation in initial studies utilizing wild type *O. polymorpha* (Wefelmeier et al., 2023). Theoretically, and based on previous reports, lactate production in yeast can be enhanced by overexpressing a lactate exporter (Branduardi et al., 2006). Since the growth pH of yeast is higher than the pKa value of lactic acid, lactate predominantly exists in its deprotonated form. As lactic acid can freely diffuse through the membrane only in its undissociated form, researchers introduced the JEN1 exporter from *S. cerevisiae* to facilitate extracellular lactate yield. However, unlike in other yeasts, this strategy did not improve lactate production. Furthermore, a two-stage cultivation strategy was employed, involving an initial biomass production phase using glucose or glycerol as the carbon source, followed by a bioproduction phase in the presence of methanol. After strain evolution, and process optimization 3.8 g. L^{-1} (0.08 g. g^{-1} methanol) of lactate was obtained in methanol fed-batch experiments in shake flasks, using urea as a nitrogen source. In initial experiments, lactate-producing strains showed severe growth impairment. When parent cells reached an OD_{600} of 3.5, the final OD_{600} of lactate-producing strains was below 1.5. However, after ALE, the evolved strain exhibited a 55 % higher growth rate, indicating a reduced adaptation time to methanol compared to the non-evolved strain. Although lactate titre was high in evolved strain, unevolved strain showed higher production yield of 0.22 g. g^{-1} indicating that in the in the evolved strain, though it assimilated higher methanol did not equivalently partitioned for bioproduction. According to this study, limiting biomass production by restraining the supply of co-substrates is a promising strategy to redirecting the flux toward organic acid production. This study highlights the critical role of medium composition, pH, and growth rates in bioproduction using yeast methylotrophy and underscores that process optimization is both strain- and product-specific.

Malic acid: Malic acid is widely used in the food, chemical, and agricultural industries. In a study conducted by Guo et al. (2021), only 0.02 g. L^{-1} of malic acid was produced when methanol was used as the sole carbon source, whereas the malic acid titre could reach 8.39 g. L^{-1} from glucose. To modulate the XuMP pathway, the key node of the pathway—glucose-6-phosphate dehydrogenase (GPI)—was knocked out. This modification increased malic acid production by 36.5-fold, but

Table 2
Bioproduction from native and synthetic methylotrophic yeast.

Yeast strain	Product	Notable strategies used for enhancing production	Mode of fermentation	Initial biomass OD_{600}/DCW or WCW (g. L^{-1})/VB (g)/BYM (g. g^{-1})	Final biomass OD_{600}/DCW or WCW (g. L^{-1})/VB (g)/ BYM (g. g^{-1})	Temperature and duration of production phase	Titre (g. L^{-1})	Yield (g. g^{-1} methanol) / productivity (g. L^{-1}. d^{-1})	Reference
1. Organic acids									
K. phaffii	Lactic acid	Blocking product consumption and engineering LDH for cofactor preference toward $NADP^+$, fermentation pH at 6.5	Fed batch fermentation in bioreactor (500 mL working volume); Batch phase in presence of glucose (20 g. L^{-1}), Production phase in methanol (10 g. L^{-1})	1 (OD_{600})	60.5 (OD_{600})	30 °C, ~350 h	4.2	0.02 g. g^{-1} 0.30 g. L^{-1}. d^{-1}	(Wu et al., 2025)
K. phaffii	Itaconic acid	Transporter expression, process optimisation	Three phase fed-batch bioreactor cultivation; batch phase for initial growth (20h), glycerol feed phase (4 h) and continuous methanol feed phase	~12 (VB)	~28 (VB) 0.14 (BYM)	32 °C, 116 h	55	0.24 g. g^{-1} 10.8 g. L^{-1}. d^{-1}	(Severinsen et al., 2024)
K. phaffii *S. cerevisiae*	L-lactic acid	Optimised the MFORG pathway	Overnight precultured cells in YPD transferred to 50 mLminimal medium with methanol (0.5 % g. L^{-1})	1 (OD_{600})	~2.3 (OD_{600}) (*K. phaffiii*) ~1.5 (OD_{600}) (*S. cerevisiae*)	30 °C, 72 h	0.21 (*K. phaffiii*) 0.07 (*S. cerevisiae*)	NM	(Guo et al., 2024a)
K. phaffii *S. cerevisiae*	ALA	Optimised the MFORG pathway	Shake flask fermentation, culture transferred to 50 mL minimal medium with methanol (0.5 % g. L^{-1}) and 0.01 M $NaHCO_3$	1 (OD_{600})	~1.8 (OD_{600}) (*K. phaffii*) ~1.25 (OD_{600}) (*S. cerevisiae*)	30 °C, 72 h	7.1×10^{-4} (*K. phaffii*) 1.67×10^{-3} (*S. cerevisiae*)	NM	(Guo et al., 2024b)
K. phaffii	3-HP	MCR fusion enzyme to restrict leakage of toxic byproducts in 3-HP pathway, strengthening of methanol assimilation, enhanced supply of malonyl-CoA and NADPH, pH control and dissolved O_2 concentration	Two phase fed-batch bioreactor cultivation; batch stage in minimal medium containing 10 g. L^{-1} methanol, during fed batch stage methanol concentration was lower than 20 g. L^{-1}	0.5 (OD_{600})	~60 (OD_{600})	30 °C, 300 h	48.2	0.23 g. g^{-1}	(Wu et al., 2023a, 2023b)
K. phaffii	Malic acid	Knocking out key node of XuMP pathway (*GPI*; glucose-6-phosphate isomerase), addition of yeast extract (1 g. L^{-1})	Shake flask fed-batch fermentation; Growth phase in buffered glycerol-complex medium, methanol feeding phase (2 % methanol was added every 24 h)	~4 (OD_{600})	~19 (OD_{600})	30 °C, 120 h	2.79	NM	(Guo et al., 2021)

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Table 2 (*continued*)

Yeast strain	Product	Notable strategies used for enhancing production	Mode of fermentation	Initial biomass OD_{600}/DCW or WCW (g. L^{-1})/VB (g)/BYM (g. g^{-1})	Final biomass OD_{600}/DCW or WCW (g. L^{-1})/VB (g)/ BYM (g. g^{-1})	Temperature and duration of production phase	Titre (g. L^{-1})	Yield (g. g^{-1} methanol) / productivity (g. L^{-1}. d^{-1})	Reference
K. phaffii	D-lactic acid	Increased copy number of D-*LDH* copy number	Test tube fermentation; Preculture in YPD (24 h), culture transferred to YPM medium with methanol (30 g. L^{-1})	5 (OD_{600})	~16 (OD_{600})	30 °C, 192 h	3.48	0.22 g. g^{-1} 0.87 g. L^{-1}. d^{-1}	(Yamada et al., 2019)
O. polymorpha	Malic acid	GSMM guided metabolic engineering, over expression of cytosolic reductive TCA cycle and malate exporter, constant substrate feeding,	Two phase fed-batch fermentation in shake flask: Preculture phase in YPD, production in minimal medium containing Yeast extract 1 g. L^{-1} using constant methanol feeding (1% daily) using a liquid injection system	~ 14 (OD_{600})	~14 (OD_{600})	37 °C, 120 h	13 (Day 4)	0.099 molmol $^{-1}$ 3.3 g. L^{-1}. d^{-1}	(Wefelmeier et al., 2024)
O. polymorpha	L-lactic acid	ALE, Nitrogen source optimization	Shake flask fed-batch fermentation in Verduyn medium containing 0.5 % methanol as carbon source, followed by additional methanol pulses of 0.5 % or 2 %, urea was used as N_2 source	0.2 (OD_{600})	~3.2 (OD_{600})	30 °C, 192 h	3.8 (Day 8)	0.08 g. g^{-1} (Day 3) 0.69 g. L^{-1}. d^{-1} (Day 4)	(Wefelmeier et al., 2023)
O. polymorpha	3-HP	Genome integration of MCR, rewiring central metabolism toward malonyl-CoA and NADPH supply, pH maintained in the range 5–6	Shake flask fed-batch fermentation; Preculture in YPD (16 h); 3-HP production in deft methanol medium, after 48 h, 10 g. L^{-1} methanol added every 24 h	0.5 (OD_{600})	18 (OD_{600})	37 °C, 168 h	7.10 (in 144 h)	0.14 g. g^{-1} 1.2 g. L^{-1}. d^{-1}	(Yu et al., 2023)
Y. lipolytica	Succinic acid	Rewiring of the peroxisomal Xu5P recycle pathway, overexpressing hsp70, supplementing yeast extract	Two phase fed-batch: preculture in synthetic defined (SD) medium containing xylose 20 g. L^{-1} (16–20 h) production phase in SD medium with methanol. 630 μL of methanol was supplemented when lower than 1 g. L^{-1}	~5 (OD_{600})	~6 (OD_{600})	30 °C, 96 h	0.35 0.92 (using yeast extract)	0.08 g. g^{-1} 0.15 g. g^{-1} (using yeast extract)	(Zhang et al., 2023)

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Table 2 (*continued*)

Yeast strain	Product	Notable strategies used for enhancing production	Mode of fermentation	Initial biomass OD_{600}/DCW or WCW (g. L^{-1})/VB (g)/BYM (g. g^{-1})	Final biomass OD_{600}/DCW or WCW (g. L^{-1})/VB (g)/ BYM (g. g^{-1})	Temperature and duration of production phase	Titre (g. L^{-1})	Yield (g. g^{-1} methanol) / productivity (g. L^{-1}. d^{-1})	Reference
2. Natural Products									
K. phaffii	Cordycepin	Improving cofactors and precursors supply	Four phase fed-batch bioreactor cultivation (5 L); Batch phase for initial growth in glycerol (20 h), Fed batch phase (14 h), Starvation phase (1 h), Methanol induction phase (110h)	244.3 (OD_{600})	470.3 (OD_{600}) 119.9 (DCW)	30 °C, 110 h	8.11	0.06 g. g^{-1} (DCW) 1.35 g. L^{-1}. d^{-1}	(Zhao et al., 2024)
K. phaffii	Astaxanthin	Increasing the NADPH supply, fusion protein for conversion efficiency	Fed-batch fermentation in bioreactor (5 L); growth phase in glycerol (40 g. L^{-1}) for 24 h followed by methanol feeding phase (maintained methanol concentration below 5 g. L^{-1}	~ 60 (OD_{600})	~ 85 (OD_{600})	25 °C, ~166 h	0.11	4.64 × 10–3 g. g^{-1}	(Wang J.N et al., 2025)
K. phaffii	Squalene	Optimizing MVA pathway, squalene transporters, adding terbinafine to prevent squalene consumption	Three phase fed-batch bioreactor cultivation (10L); Batch phase for initial growth in glycerol (18–24 h), Fed batch limited glycerol feed followed by methanol induction phase	220 (OD_{600})	~410 (OD_{600})	30 °C, ~72 h	20.80	0.076 g. g^{-1}	(Zhang et al., 2025)
K. phaffii	Zealexin A1	Enhancing the MVA pathway, enhancing formaldehyde assimilation, increasing methanol to 2 % downregulating the competitive pathway, enhancing the NADPH supply	Single phase fed-batch fermentation in bioreactor; In the early stages basal salt medium containing glycerol (20 g. L^{-1}) was used until OD_{600} reach 151, methanol (2 % *v*/v) feeding was started when glycerol was depleted	0.5 (OD_{600})	200 (OD_{600})	30 °C, 120 h	1.03 × 10^{-1}	NM	(Niu et al., 2024)
K. phaffii	β-Arbutin	Increasing shikimate pathway flux, fusion enzymes to avoid diffusion of	Three phase fed-batch bioreactor cultivation (5 L); Initial growth phase in glycerol (18. 6 h), low glycerol feed phase to slow down growth and methanol induction stage (132 h)	278 (OD_{600})	384 (OD_{600})	25 °C, 132 h	128.6	22. 9 g. L^{-1}. d^{-1}	(Yang et al., 2024)
K. phaffii	α-bisabolene	Increased methanol feeding,	Shake flask batch	0.2 (OD_{600})	~17 (OD_{600})	30 °C, 120 h	2.70 × 10^{-1}	NM	(Gao et al., 2024)

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Table 2 (*continued*)

Yeast strain	Product	Notable strategies used for enhancing production	Mode of fermentation	Initial biomass OD_{600}/DCW or WCW (g. L^{-1})/VB (g)/BYM (g. g^{-1})	Final biomass OD_{600}/DCW or WCW (g. L^{-1})/VB (g)/ BYM (g. g^{-1})	Temperature and duration of production phase	Titre (g. L^{-1})	Yield (g. g^{-1} methanol) / productivity (g. L^{-1}. d^{-1})	Reference
		additional copies of α-bisabolene synthase, optimizing the MVA pathway, increased methanol feeding	fermentation; preculture in YPD (16 h) followed with culturing in minimal medium containing in methanol (20 g. L^{-1})						
K. phaffii	β-farnesene	Increased methanol feeding, choosing strong promoter for biosynthetic pathway, MVA pathway enhancement	Shake flask batch fermentation; preculture in YPD (16 h) followed with culturing in minimal medium containing in methanol (20 g. L^{-1})	0.2 (OD_{600})	NM	30 °C, 120 h	3.38×10^{-1}	NM	(Gao et al., 2024)
K. phaffii	Triacetic acid lactone	Boost supply of acetyl-CoA via PK pathway	Shake flask batch fermentation; preculture in YPD (48 h) followed with culturing in minimal medium containing in methanol (2 % *v*/v)	NM	NM	30 °C, 72 h	5.71×10^{-2}	1.0 × 10–3 g. g^{-1}	(Feng et al., 2023)
K. phaffii	Lycopene	improving the NADPH supply, inhibition of competitive pathway (farnesol production)	Fed-batch bioreactor cultivation (5 L); Initial growth phase in glycerol (18–24 h), 30 min starvation period followed by methanol induction phase (125 h)	440 (OD_{600})	~700 (OD_{600})	30 °C, 125 h	7.24	0.07 g. g^{-1} (DCW)	(Zhang X. Y et al., 2023)
K. phaffii	Catharanthine	Used CRISPR-Cas9 editing for genomic integration of expression cassette, supplementing mannitol, Weakening competitive pathways, enhancing cofactor and precursors	Fed-batch fermentation in bioreactor using methanol (2 % *v*/v) and mannitol (1%)	140 (OD_{600})	230 (OD_{600})	30 °C, 72 h	2.5×10^{-3} 5.0×10^{-4} (methanol as carbon source)	NM	(Gao et al., 2023; Kim et al., 2025)
K. phaffii	Monacolin J	Codon optimization, pH optimization, pathway splitting, co-culture system	Three phase fed-batch bioreactor cultivation; batch phase for initial growth in glycerol 40 g. L^{-1} (16 h), glycerol feed-batch phase (12h), non-feeding period (0.5 h),	~300 (WCW) Co-culture inoculation ratio-1:2	475 (WCW)	30 °C, 68 h	0.59	2×10^{-3} g. g^{-1}	(Liu et al., 2018)

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Table 2 (*continued*)

Yeast strain	Product	Notable strategies used for enhancing production	Mode of fermentation	Initial biomass OD_{600}/DCW or WCW (g. L^{-1})/VB (g)/BYM (g. g^{-1})	Final biomass OD_{600}/DCW or WCW (g. L^{-1})/VB (g)/ BYM (g. g^{-1})	Temperature and duration of production phase	Titre (g. L^{-1})	Yield (g. g^{-1} methanol) / productivity (g. L^{-1}. d^{-1})	Reference
			methanol feed phase (methanol feeding rate is gradually increased from 2.4 g. h^{-1} to 24 g. h^{-1} over the first 7.5 h then a constant feeding rate of 24 g. h^{-1} (68 h)						
K. phaffii	Lovastatin	Codon optimization, pH optimization, pathway splitting, co-culture system	Three phase fed-batch bioreactor cultivation; batch phase for initial growth in glycerol 40 g. L^{-1} (16 h), glycerol feed-batch phase (12 h), non-feeding period (0.5 h), methanol feed phase involving 100 % methanol feed for continuous feeding with a gradual increase in methanol feed rate, lasting until the end of the fermentation.	~300 (WCW) Co-culture inoculation ratio-1:1	416 (WCW)	30 °C, 68 h	0.25	1×10^{-3} g. g^{-1}	(Liu et al., 2018)
K. phaffii	6-Methylsalicylic acid	Balancing growth and methanol induction phases	Three phase fed-batch bioreactor cultivation; batch phase for initial growth in glycerol 40 g. L^{-1} (32 h), glycerol feed-batch phase (14 h), methanol feed phase (20 h)	252 (WCW)	~225 (WCW)	30 °C, 20 h	2.2	9×10^{-3} g. g^{-1}	(Gao et al., 2013)
O. polymorpha	Isoprene	GSMM, used isoprene synthase from *Populus alba,* enhancing flux through mevalonate pathway	Fermentation in serum bottles in minimal medium containing methanol (0.5 %), 50 % of the headspace of the culture was replaced with pure oxygen.	0 (OD_{600})	~3.5 (OD_{600})	37 °C, NM	4.40×10^{-3}	NM	(Wefelmeier et al., 2024)
S. cerevisiae	Flaviolin	Engineered module circuit strategy, ALE	Biomass and bioproduction in minimal medium containing methanol (1% *v*/v)	0.15 (OD_{600})	~0.8 (OD_{600})	30 °C, 96 h	NM, measured absorbance at 340 nm	NM	(Zhan et al., 2023)
Fatty acid derived products									
K. phaffii	FFA	Enhancing acetyl CoA pool, central metabolism	Fed-batch fermentation in bioreactor;	1 (OD_{600})	~200 (OD_{600})	30 °C, ~230 h	23.4	0.078 g. g^{-1}	(Cai et al., 2022)

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Table 2 (*continued*)

Yeast strain	Product	Notable strategies used for enhancing production	Mode of fermentation	Initial biomass OD_{600}/DCW or WCW (g. L^{-1})/VB (g)/BYM (g. g^{-1})	Final biomass OD_{600}/DCW or WCW (g. L^{-1})/VB (g)/ BYM (g. g^{-1})	Temperature and duration of production phase	Titre (g. L^{-1})	Yield (g. g^{-1} methanol) / productivity (g. L^{-1}. d^{-1})	Reference
		rewiring and enhancing formaldehyde assimilation for cellular robustness,	batch stage in minimal medium containing 10 g. L^{-1} methanol, fed batch stage kept the methanol concentration below 20 g. L^{-1}						
K. phaffii	FA	Deletion of the aldehyde dehydrogenase gene HFD1 preventing fatty aldehyde consumption, introducing an extra copy of *FaCoAR* gene	Fed-batch fermentation in bioreactor; batch stage in minimal medium containing 10 g. L^{-1} methanol, fed batch stage kept the methanol concentration below 20 g. L^{-1}	1 (OD_{600})	163 (OD_{600})	30 °C, ~230 h	2.0	8×10^{-3} g. g^{-1}	(Cai et al., 2022)
O. polymorpha	FA	Coupling of methanol utilization and product synthesis in peroxisome, supply of peroxisomal fatty acyl-CoA and NADPH	Two phase fed-batch bioreactor cultivation; preculture in minimal media containing 20 g. L^{-1} glucose (24 h), methanol feeding phase (methanol kept below 5 g. L^{-1})	0.5 (OD_{600})	186 (OD_{600})	37 °C, 255 h	3.6	0.016 g. g^{-1} 0.309 g. L^{-1}. d^{-1}	(Zhai et al., 2023)
Other products									
K. phaffii	Xylitol	Engineered NADPH-dependent *XYL2* mutants and deleted the endogenously characterized xylitol dehydrogenase	Preculture in YPD, Transferred to 20 mL YP medium supplemented with methanol (2 % *v*/v) in shake flask	0.2 (OD_{600})	NM	30 °C, 96 h	0.25	0.015 g. g^{-1}	(Lu et al., 2025)
K. phaffii	Erythritol	Hybrid RuMP/ XuMP pathway, sedoheptulose-1,7-biphosphatase disruption, triosephosphate isomerase overexpression	Fed batch fermentation in 1 L bioreactor; Seed culture in YPD, Glycerol batch phase (20 g. L^{-1}), Methanol feeding phase in which methanol was maintained at 3 g. L^{-1}	~40 (OD_{600})	~250 (OD_{600})	25 °C, 2 days	31.5	NM	(Wang S et al., 2025)
K. phaffii	SCP	ALE for thermotolerance at 33 °C, engineering for decreasing cell wall thickness and enhance nitrogen metabolism	Fermentation in pilot-scale fed-batch bioreactor (500 L) Initial batch stage using 2 % glycerol followed by methanol induction phase	30 (DCW)	63.37 (DCW)	33 °C, 39 h	0.43 g_{DCW}. $g^{-1}_{methanol}$	0.506 $g_{protein}$. g^{-1}_{DCW}	(Meng et al., 2023)
K. phaffii	Heparin	Optimizing expression of rate	Two phase fed batch in 3 L	~50 (DCW)	125 (DCW)	28 °C, 132 h	2.08	NM	(Zhang et al., 2022)

(*continued on next page*)

Table 2 (*continued*)

Yeast strain	Product	Notable strategies used for enhancing production	Mode of fermentation	Initial biomass OD_{600}/DCW or WCW (g. L^{-1})/VB (g)/BYM (g. g^{-1})	Final biomass OD_{600}/DCW or WCW (g. L^{-1})/VB (g)/ BYM (g. g^{-1})	Temperature and duration of production phase	Titre (g. L^{-1})	Yield (g. g^{-1} methanol) / productivity (g. L^{-1}. d^{-1})	Reference
		limiting enzyme NDST (*N*-deacetylase/ N-sulfotransferase) using N-terminal engineering and fusion strategies	fermenter; Seed culture in YPD medium for 16-24 h, during production phase methanol concentration was maintained at 2 % (/v)						
K. phaffii	β-alanine	Multiple copy of *ADC* gene and over expression of ASPDH to enhance aspartate pool	Two phase 1 L fed-batch fermentation; initial growth in presence of 40 g. L^{-1} glycerol (20 h), methanol concentration maintained at 3 g. L^{-1} during production phase	80 (OD_{600})	123 (OD_{600})	30 °C, ~86 h	5.6	NM	(Miao et al., 2021)

NM = Not mentioned, BYM = Biomass yield per g methanol, WCW = Wet Cell Weight, DCW = Dry Cell Weight, VB = Viable Biomass,

also resulted in a significant defect in methanol assimilation and growth. Later, supplementing 1 g. L^{-1} of yeast extract restored cell growth and methanol utilization, leading to a final titre of 2.79 g. L^{-1}.

Genome-scale metabolic modelling (GSMM) in *O. polymorpha* predicted a high-efficiency malate production with a theoretical yield of 0.25 mol malate. Mol methanol^{-1} by restricting growth and directing all carbon toward product synthesis (Wefelmeier et al., 2024). Hence, a two-phase production strategy was adopted: an initial biomass accumulation phase followed by malate production using methanol as the sole carbon source. The recombinant strain, containing malate dehydrogenase from *Rhizopus oryzae*, an overexpressed cytosolic reductive TCA cycle, and a malate exporter, was used for malate production. To reduce methanol toxicity, the initial methanol concentration was set to 4 g. L^{-1}, followed by two 16 g. L^{-1} methanol pulses once the initial supply was depleted. No malate production was observed during the preculture phase. After 144 h, the recombinant strain reached a malate concentration of 7.7 g. L^{-1} but showed almost no growth with methanol as the carbon source. Growth on methanol likely triggers an increase in the flux toward malate, as the cell's energy requirements are elevated. This is further supported by the heightened activity of the malate-aspartate shuttle in the presence of methanol, which increases the malate pool but limits ATP generation due to the export of malate from the cytosol. Consequently, this high flux toward malate production occurs at the expense of cellular growth, explaining the observed high malate concentrations and low growth rates during the methanol-based cultivation. A pH below 4 enables the cell to import malate, and in the absence of methanol in the medium, the cell utilized malate as a carbon source. Using a bioreactor that permit maintaining a constant methanol concentration through daily feeding of 1 % and controlling the pH of the cultivation medium, this study achieved a final titre of 13.2 g. L^{-1} and productivities of 3.4 g. L^{-1}. d^{-1}. So far, a similar titre has not been achieved with native methylotrophic bacteria (Wefelmeier et al., 2024). Hence, this study emphasizes the future potential of using yeast methylotrophy for C1-substrate-based carboxylic acid production and provides guidelines for optimization strategies.

Succinic acid: The production of succinic acid, a high-value dicarboxylic acid, was demonstrated in *Y. lipolytica* to showcase the feasibility of biotransforming methanol into valuable products via synthetic methylotrophy (Zhang et al., 2023). After introducing methylotrophy into *Y. lipolytica*, the rewiring of the peroxisomal Xu5P recycling pathway significantly enhanced methanol assimilation in this strain. To promote succinic acid accumulation via the TCA cycle, the fifth subunit of succinate dehydrogenase was deleted. A two-stage production method was employed for methanol bioconversion to succinic acid. Engineered cells were first grown on xylose 20 g. L^{-1} as a carbon source until an OD_{600} of 5 was reached, after which they were transferred to a minimal medium containing methanol (20 g. L^{-1}). After consuming 4.2 g. L^{-1} of methanol, a low product yield of 0.35 g. L^{-1} was detected after 96 h. In a subsequent experiment, supplementing the medium with yeast extract 2 g. L^{-1} increased the final titre to 0.92 g. L^{-1}. Despite the low yield, this study highlights the feasibility of using the oleaginous yeast *Y. lipolytica* as a promising candidate for methylotrophy (Arnesen et al., 2020). This strain is particularly envisioned as a future host for lipid-derived metabolites derived from C1 substrates (Fabarius et al., 2021).

3-Hydroxypropionic acid (3-HP): 3-HP is one of the most important bulk chemicals with a market value of USD 10 billion year^{-1} (Bhagwat et al., 2021). Polymerised 3-HP (P-3HP) is a major substitute for petrochemicals, and 3-HP is also used in the production of acrylic acid (Bhagwat et al., 2021). Production of 3-HP from native methylotrophs was achieved either via engineering the β-alanine pathway (Àvila-Cabré et al., 2024) or malonyl-CoA pathway (Wu et al., 2023a, 2023b; Yu et al., 2023). To engineer *K. phaffiii* for 3-HP production, GSMM was first evaluated to determine the maximum theoretical yield through both pathways, assuming the entire carbon source was used for 3-HP synthesis instead of growth (Àvila-Cabré et al., 2024). Based on this analysis, the β-alanine pathway was selected as the optimal route due to its lower ATP requirement, producing 1 mol 3-HP per 3 mol methanol. Following an initial study to confirm pathway functionality, *panD* was overexpressed to optimize metabolic flux through the β-alanine route. In buffered minimal methanol medium, recombinant strains reached OD_{600}; 2.5 in 48 h, with 3-HP titre; 1.24 g. L^{-1}. As the β-alanine pathway consumes 1 mol NADPH per mol 3-HP, it negatively affected anabolic processes, reducing growth rate (μ_{max}); 0.11 h^{-1} compared to the wild type; 0.14 h^{-1}. Additionally, redirection of β-alanine, which is required for vitamin B5 synthesis (a cofactor for several enzymes), may have contributed to growth reduction. In the final fed-batch experiment, 3-HP

titre; 21.4 g. L^{-1} (yield; 0.15 g. $g_{methanol}{}^{-1}$) was achieved. However, a 3-HP yield <0.5 g. g^{-1} from methanol is not industrially viable. Further metabolic engineering should focus on optimizing carbon flux partitioning between assimilatory and dissimilatory pathways, as >50 % carbon from methanol is lost as CO_2 via dissimilation. Additionally, redirecting pyruvate flux into the β-alanine pathway is suggested as a future strategy to enhance 3-HP production (Àvila-Cabré et al., 2024).

3-HP yield was higher via the malonyl-CoA pathway in *K. phaffiii* (Wu et al., 2023a, 2023b), highlighting the role of global metabolic rewiring in enhancing precursor and cofactor availability while downregulating methanol dissimilation. 3-HP production requires an increased supply of malonyl-CoA (precursor) and NADPH (cofactor). Since free fatty acid (FFA) synthesis also depends on these same precursors, an engineered *K. phaffiii* strain overproducing FFA was utilized for 3-HP production via the malonyl-CoA pathway (Wu et al., 2023a, 2023b). Compared to the wild-type *K. phaffiii* background carrying the 3-HP biosynthetic pathway, the FFA-overproducing strain exhibited extensive metabolic rewiring, enhancing malonyl-CoA and NADPH supply, leading to 3-HP titre; 1.5 g. L^{-1}, which was 28.8 % higher than that of the strain without FFA overproduction trait. By downregulating methanol dissimilation, the highest 3-HP titre; 2.2 g. L^{-1} was achieved in the engineered strain. In fed-batch fermentation conducted in shake flasks, 3-HP titre; 13 g. L^{-1} from 65 g. L^{-1} methanol (yield; 0.20 g. $g^{-1}_{methanol}$) after 236 h.

Itaconic acid (*IA*): IA is a monounsaturated dicarboxylic acid used widely in polymer and plastic synthesis (Severinsen et al., 2024). Expression of three genes: *CADA, MTTA*, and *MFSA* from *Aspergillus tereus* in *K. phaffii*, combined with systematic strain and process engineering enabled the production of 28 g L^{-1} itaconic acid in 5 days of fermentation. In this study, cytotoxicity of itaconic acid accumulation was prevented by regulation of expression level of MFSA - a plasma membrane transporter - for its efficient export from the cell. Later, optimisation of nitrogen limitation and growth temperature enhanced production to 50 and 55 g. L^{-1}. After initial shake flask culture studies, a four-phase fed-batch fermentation was implemented for IA production. This process included a 27 h glycerol batch phase followed by an 8-h glycerol feed phase to support biomass production. Next, a 17 h methanol co-feed phase was introduced to activate the biosynthetic pathway gene, followed by a final pure methanol feed phase for IA production. During the IA production phase, biomass increased from 85 g. L^{-1} to 140 g. L^{-1}, yielding 28.2 g. L^{-1} of IA. However, this high biomass accumulation resulted in a low yield of 0.1 g. $g^{-1}_{methanol}$. To address this, a 4 h glycerol feed phase was implemented for biomass production, along with nitrogen limitation, elevated incubation temperature (30 °C), and an increased gene copy number to enhance IA production. These modifications led to a 13 % reduction in biomass accumulation over 64 h, shifting the metabolic balance toward methanol conversion into IA rather than biomass growth. Consequently, the IA titre increased to 55 g. L^{-1}, and the yield doubled to 0.26 g. $g^{-1}_{methanol}$. Considering the carbon source, this yield is promising, as the industrially relevant IA yield from *Aspergillus terreus* was 0.58 g. $g^{-1}_{glucose}$ (Krull et al., 2017).

3.2. Natural products (NPs)

Natural products from plants and microbes are used widely as drugs, flavours, and fragrances (Vasudevan and Lee, 2020). These secondary metabolites are produced in low amounts in natural systems. Hence, microbial biosynthesis of these compounds is crucial to meet the rising industrial demand (Nielsen and Keasling, 2016). Yeasts are versatile hosts to produce diverse NPs, as they can tackle the toxicity of hydrophobic NPs and their pathway intermediates (Naseri, 2023). As many industrially relevant NPs are originated from plants, yeasts are particularly significant as a host when considering the gene expression level of these eukaryotic enzymes and subcellular-organelle specific localization (eg. cytochrome *p*450) (Chen et al., 2020; Gao et al., 2023). Although glucose-based production of various NPs has been reported from yeast, biosynthesis of these complex metabolites from methanol is rather more complicated.

Production of some NPs directly from methanol, e.g., triacetic acid lactone (6-methyl-4-hydroxy-2-pyrone, or TAL) is proven difficult as the yield is very low (0.001 g. $g^{-1}_{methanol}$), when compared to its yield from other renewable carbon source eg. 0.041 g. g^{-1}_{xylose} (Feng et al., 2023). Acetyl-CoA serves as a key precursor for most of the NPs (Feng et al., 2023). Unlike in glucose metabolism, when methanol is used as the sole carbon source, energy derives primarily from methanol dissimilation rather than the TCA cycle (Cai et al., 2022), leading to low availability of acetyl-CoA. Similar strategies used to increase the acetyl-CoA pool from glucose do not work when methanol is used as the carbon source (Cai et al., 2022). Implementing more efficient synthetic C1-based acetyl-CoA biosynthetic pathways, such as the SACA (synthetic acetyl-CoA) pathway, which is superior to natural pathways in minimizing carbon loss and increasing carbon flux toward acetyl-CoA, could be a promising strategy for the future production of acetyl-CoA-derived NPs (Feng et al., 2023).

6-methylsalicylic acid (6-MSA) production in *K. phaffiii* was demonstrated previously using methanol and glycerol as carbon sources. The antimicrobial nature of 6-MSA poses toxicity to cells, resulting in low product yield (Gao et al., 2013). Sequential methanol feeding to control production rates, or in situ product recovery methods can help increase the yield of 6-MSA. An earlier study on citrinin production from *K. phaffiii* demonstrated that synthesizing polyketides with a large gene cluster and a complex synthetic pathway is feasible from methanol (Xue et al., 2017). This study points out that low pH levels during *K. phaffiii* culturing and the intense aerobic environment resulting from methanol oxidation can generate strong redox environments in vivo, leading to NPs degradation. Deploying a non-extreme pH culture system for *K. phaffiii* and dynamic regulation of medium pH can resolve these challenges and stabilisation of NPs.

Constructing NPs pathways in a pure culture has limitations such as metabolic stress resulting from the overexpression of long and complex pathways, along with stress induced by methanol metabolism. Strategies like a dual-cell co-culture approaches are promising for integrating complex NPs synthesis in methylotrophic yeasts, increasing cell density and enhancing yield. Biosynthesis of drugs monacolin J and lovastatin from methanol induced a metabolic burden in *K. phaffiii*. When this pathway was split as two separate modules in different *K. phaffiii* strains in a co-culture system, there was a 55 and 71 % increase in monacolin J and lovastatin production, respectively (Liu et al., 2018).

Microbial production of terpenoid vinblastine is challenging due to the complicated 31-step biosynthetic pathway (Qu et al., 2019). Its extraction from plant result in very low yield (0.0002 % fresh weight) (Miettinen et al., 2014). Previously, the pathway for its precursors, catharanthine and vindoline, was elucidated and reconstituted in *S. cerevisiae* (Gao et al., 2022b). Recently, catharanthine was synthesized from methanol in *K. phaffii*, achieving a titre of 2.57 mg. L^{-1} (Gao et al., 2022a; Kim, 2023). Engineered *K. phaffii* showed remarkable strain stability and consistency in catharanthine production from methanol. Reconstitution of this complicated pathway, involving the integration of 30 heterologous gene expression cassettes, represents the one of the most complex biosynthetic pathways ever reconstituted in *K. phaffii* (Gao et al., 2022a). A multitier modular metabolic pathway engineering strategy was employed. When methanol was used as the sole carbon source in 1 L fermenters with 2 % methanol added 4 h after induction, the OD_{600} reached 170, and catharanthine production was 0.62 mg. L^{-1}. Since using methanol as the sole carbon source can lead to low biosynthesis, this study incorporated the co-substrate mannitol to maximize methanol utilization for gene expression induction and the production of NP precursors, such as acetyl-CoA and geranyl pyrophosphate (GPP). Later, in a fed-batch fermentation experiment over 70 h, a mixture of methanol and mannitol (1 % *v*/v) was fed every 4 h after induction. As a result, cell densities and catharanthine titres reached 230 and 2.57 mg. L^{-1}, respectively.

Heparin is a polysaccharide widely used as an anticoagulant drug, and microbial heparin biosynthesis is a sustainable alternative to animal-derived heparin production. However, the biosynthesis of heparin in microbial cells requires co-expression of multiple enzymes, including both heparosan synthesis enzymes and modification enzymes. Engineered strains were used for high-cell-density fermentation in a 3 L fermenter to evaluate heparin biosynthesis in the recombinant strain. Methanol was supplied as both a carbon source and inducer at 36 h, and at 168 h, the production of bioengineered heparin reached 2.08 g·L^{-1}, indicating the potential for further scale-up of non-animal sourced polymer in future for commercial application. This study is another example of synthesizing a complex natural product from methanol, inspiring the synthesis of more NPs (Zhang et al., 2022).

Production of NPs from microbial chassis requires extensive metabolic engineering and process optimization. Beyond the complexities of using methanol as a carbon source, intrinsic challenges—such as the difficulty of expressing membrane-bound enzymes in the pathway and the low availability of precursor pools—further complicate NP biosynthesis. However, these studies provide a framework for identifying key limiting steps and bottlenecks associated with using C1 substrates, expanding our understanding beyond previous NP production chassis based on glucose.

3.3. Free fatty acid (FFA) and fatty alcohol (FA)

Fatty acids and their derivatives are promising raw materials for manufacturing advanced biofuels, detergents, lubricants, surfactants, etc. Wild type yeasts can accumulate only ~50 mg. L^{-1} FFA using methanol or glucose as the carbon source (Cai et al., 2022). Free fatty acid production requires enhanced acetyl CoA and malonyl CoA pool and the NADPH cofactor (Tang et al., 2015). Previously, systematic central carbon metabolism rewiring was conducted to improve the flux to acetyl-CoA, redirect metabolic flux to biosynthesis, and decrease the accumulation of toxic formaldehyde to obtain a FFA titre of 23.4 g. L^{-1} (0.078 g. $g^{-1}_{methanol}$) from *K. phaffiii* (Cai et al., 2022). Engineering to enhance malonyl-CoA pool was not successful which is suggested to be due to the toxicity of malonyl-CoA to the cell. Similarly, as *K. phaffiii* grown in methanol are rich in NADPH, and additional engineering was not successful in enhancing NADPH. Further engineering was carried out to produce fatty alcohols (FA) from methanol to a titre up to 2.0 g. L^{-1}(yield, 0.008 g. $g^{-1}_{methanol}$). Construction of a cytosolic FA biosynthesis pathway in *O. polymorpha* resulted in low production, possibly due to spatial isolation with peroxisomal methanol metabolism. Hence, by targeting the FA biosynthesis pathway to the peroxisome, where methanol utilization is localized, FA synthesis enhanced by 3.9-fold in *O. polymorpha* (Zhai et al., 2023). Further supply enhancement of fatty acyl-CoA precursor and NADPH in the peroxisomes by global metabolic rewiring improved FA production by 2.5-fold, achieving a titre of 3.6 g. L^{-1} (yield, 0.016 g. $g^{-1}_{methanol}$).

The production of FFA from yeast using methanol as the sole carbon source can lead to decreased phospholipid levels and subsequent cell death, as observed in *O. polymorpha* (Gao et al., 2022a). This indicates a link between lipid metabolism and methanol tolerance. After ALE, the evolved strains showed inactivation of a putative lipase (LPL1), and a membrane protein (IZH3) linked to zinc metabolism—both associated with lipid metabolism. This inactivation subsequently repaired cell damage by restoring phospholipid metabolism.

3.4. SCP and other bioproducts

Single-cell protein (SCP) offers a sustainable solution to meet the rising global food demand driven by population growth (Meng et al., 2023). Dietary protein is often expensive, and SCP can serve as an affordable alternative supplement. Methanol has long been used as a substrate for SCP production using methylotrophic yeasts; however, the sensitivity of these yeasts to high fermentation temperatures (around 33 °C) has limited large-scale industrial applications. Recently, ALE was performed to develop a thermotolerant *K. phaffii* strain. This evolved strain exhibited increased cell wall thickness, which was linked to the upregulation of the gene *PAS_chr4_0305*, involved in *O*-glycosylation associated with cell wall integrity. To improve protein content, the strain was further engineered by deleting *PAS_chr4_0305* to reduce cell wall thickness, and by overexpressing glutamine synthetase (GLN1) to enhance nitrogen metabolism to increase protein content of biomass. In pilot-scale fed-batch fermentation at 33 °C, this engineered strain achieved a methanol conversion rate of 0.43 g_{DCW}. $g^{-1}_{methanol}$ and a protein content of 0.506 g. g^{-1}_{DCW}. The thermotolerant SCP production strain developed here shows strong potential for repurposing in other methanol-based bioproduction processes, especially for synthesizing amino acids or amine-based products (Meng et al., 2023).

3-Aminopropionic acid (β-alanine) is one of the few naturally occurring β-amino acids, found in animals, plants, and microorganisms. It serves as a precursor in the biosynthesis of various nitrogen-containing compounds. β-Alanine can be synthesized from L-aspartic acid via decarboxylation catalyzed by L-aspartate-α-decarboxylase (ADC). For β-alanine production from methanol in *K. phaffii*, a combined metabolic engineering strategy was employed, involving multicopy expression of ADC and overexpression of aspartate dehydrogenase (ASPDH) to enhance the intracellular supply of aspartate from oxaloacetate via reductive amination.

During the methanol-dependent production phase, starting from an OD_{600} of 0.3, the engineered cells exhibited a growth rate approximately 10 times lower than that of the wild-type strain. This reduced growth is speculated to result from the metabolic burden imposed by the overexpression of ADC and the diversion of aspartate, a key metabolic intermediate involved in several essential cellular pathways. Despite these challenges, the achieved β-alanine titre of 5.6 g. L^{-1} is considered satisfactory, especially when compared to other methanol-based bioproduction systems, which typically yield less than 1 g. L^{-1} (Miao et al., 2021).

4. Formatotrophy in yeast

4.1. Formate assimilation pathways

Formate is regarded as a sustainable C1 feedstock for microbial bioprocessing due to its lower toxicity than methanol, its ability to exist as a liquid at room temperature and non-flammability. Formate can be directly produced from renewable carbon sources such as CO_2 electrochemically with high energy efficiency (Turlin et al., 2022; Claassens et al., 2022). Recently reported microbial bioconversion technologies for converting CO_2 to formate using *Stenotrophomonas maltophilia* are highly promising, as they operate without the need for H_2, cofactors, or photon addition (Ruiz-Valencia et al., 2024). As economic and process efficiency of carbon capture from CO_2 to formate technologies is increasing, formate may compete with glucose as feedstock in the near future (Kurt et al., 2023). Yeasts possess native genetic and metabolic ability to assimilate formate (Mitic et al., 2023; Chen et al., 2024). Various formate assimilation routes identified in yeasts are as follows:

4.1.1. rGly pathway

A native rGly pathway variant was reported for formate utilization in *K. phaffii* (which also facilitated the co-assimilation of methanol and CO_2 (section 2.1; Mitic et al., 2023). To establish aerobic formatotrophy in heterologous hosts, synthetic version of rGly pathway is suggested as the most energy efficient route (Yishai et al., 2017). This pathway can be introduced conveniently in heterologous hosts due to its linearity and simple network architecture with relatively low interference with the host's core metabolism (Claassens et al., 2022) (Fig. 3a). In *S. cerevisiae*, all the genetic components of a native rGly pathway are present, but this strain is unable to naturally assimilate formate. Instead, this pathway generates reducing equivalents and energy and drive the net flux in the

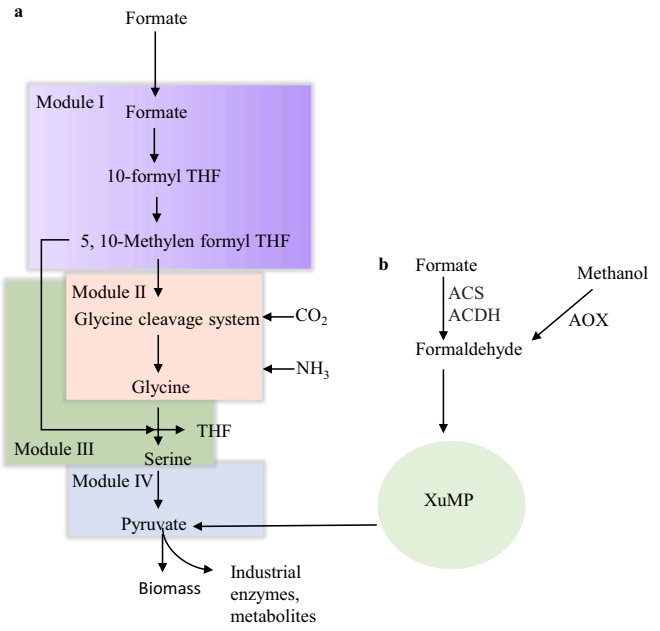

Fig. 3. rGly pathway consists of distinct modules: A module for the biosynthesis of glycine, where formate attached to tetrahydrofolate (THF) and is reduced to methylene-THF, then condensed with ammonium and CO_2 to produce glycine by the reversed activity of the glycine cleavage system. In the glycine-to-serine conversion module methylene-THF condenses glycine and hydroxymethyl to create serine. Lastly, in the pyruvate synthesis module, serine is converted to pyruvate, which plays a major role in cellular metabolism (Claassens et al., 2022) (**a**). In *K. phaffiii*, a synthetic formate assimilation pathway was developed by co-expressing acetyl-CoA synthase (ACS) and acetaldehyde dehydrogenase (ACDH) for industrial enzyme production. Formate is reduced to formaldehyde through the coupled activity of ACS and ACDH, which then integrates into the native methanol assimilation pathway, facilitating enzyme biosynthesis (**b**).

reverse direction toward the oxidation of serine and glycine to formate and CO_2 (Bysani et al., 2024). Feasibility of redirecting of this metabolic flux for assimilating formate for biosynthesis of serine and glycine has been demonstrated by manipulating the expression level of endogenous rGly pathway enzymes and in presence of methanol (Bysani et al., 2024). In a previous study, the glycine biosynthesis module of the rGly pathway was successfully implemented in *S. cerevisiae* by overexpressing native enzymes using strong endogenous promoters (Gonzalez de la Cruz et al., 2019). Engineered *S. cerevisiae* strains showed a stable formate-dependent growth over a wide range of formate concentrations (1–500 mM). In a subsequent study, complete serine synthesis from formate and CO_2 was demonstrated using a growth-coupled selection strategy combined with ALE (Orsi et al., 2023). In the study by Bysani et al. (2024), the successful implementation of the complete rGly pathway in *S. cerevisiae* was demonstrated. This was evidenced by the incorporation of ^{13}C from formate into pyruvate, coupled with CO_2 assimilation.

4.1.2. Threonine-based serine pathway

Combining non-rational genome engineering and ALE had remarkable effect in elucidating new formate assimilation pathways (Chen et al., 2024; Guo et al., 2024a). ALE, combined with 'Helicase coupled with activation-induced cytidine deaminase' (Helicase-AID) technology (Wang J et al., 2021), was used to stimulate and enhance the natural formate assimilation pathway in *Y. lipolytica* W29. Evolution resulted in activation of a native formate and CO_2 co-assimilation pathway referred to threonine-based serine pathway (gSer-tSer bicycle). Evolved mutants showed upregulation of multiple formate dehydrogenases and enhanced formate tolerance up to 2 M (Chen et al., 2024).

4.1.3. ASrG pathway

Directed genome evolution strategy SCRaMbLE was used in combination with ALE to develop synthetic methylotrophy in *S. cerevisiae* revealing concurrent assimilation of methanol, formate, and CO_2 via a newly identified ASrG pathway (section 2.1.2.2, Guo et al., 2024a). Formate dependent growth (0.010 h^{-1}) was slower than methanol dependent growth (μ = 0.014 h^{-1}). Later, formate-dependent growth was improved (μ =0.013 $h^{-1)}$ by co-feeding $NaHCO_3$. Though these pathways are low energy efficient, they have the potential to be evolved or tactically improved to regulate metabolic fluxes by rational strategies to enhance formate assimilation in future.

4.2. Recent advances in bioproduction using formate

Implementing stable formatotrophy in yeast requires rigorous research to attain an industrially relevant growth rate for bioproduction. A low degree of reduction of formate is a major hurdle in assimilating formate into biomass, which requires an additional energy source (Guo et al., 2024a). This also necessitates high substrate concentrations in the culture medium or frequent supplementation of formate for microbial growth and product synthesis, which may inhibit cell growth (Collas et al., 2023). Industrial enzyme xylanase production has been reported from engineered *K. phaffii* using formate as carbon source and inducer (Liu et al., 2022) employing a synthetic formate assimilation pathway containing acetyl-CoA synthase gene (*ACS*) from *E. coli* and acetaldehyde dehydrogenase gene (*ACDH*) from the non-pathogenic *L. innocua* (Fig. 3b).

Formate has not yet been used as a sole substrate to produce biochemicals in yeast. But a recent study demonstrated free fatty acids (FFAs) production from evolved *S. cerevisiae* using glucose (20 gL^{-1}) and high concentration of formate (50 g. L^{-1}) (Wang K et al., 2023). In another study, co-feeding formic acid significantly increased the biomass yield on glucose in the industrially relevant yeast *Y. lipolytica*. When glucose and formate were supplied together, the wild strain *Y. lipolytica* W29 exhibited the capability to consume formic acid at molar ratios exceeding 10:1 relative to glucose (van Winden et al., 2022a, 2022b). Most recently, positive effect of formate co-feeding for lipid production and biomass formation in *Y. lypolytica* has been demonstrated and suggested as a viable strategy to reduce the cost of microbial lipid production (Kuang et al., 2025).

Rising atmospheric CO_2 and its impact on global climate change are pressing concerns of recent times. To mitigate climate change and achieve the 'net zero' target, negative emission technologies (CO_2 removal) play a significant role (Zahed et al., 2021). Due to the limitations in the efficiency of one-step CO_2 fixation, a two-step fixation technology—such as the conversion of CO_2 to formate, followed by bioconversion of this C1 compound into high-value products—is envisioned as a key approach for a carbon-neutral economy (Kurt et al., 2023; Nattermann et al., 2023). This integrated process of converting CO_2 to bioproducts via formic acid provides a unique platform for CO_2 capture by fixing both atmospheric CO_2 and reutilizing CO_2 generated during microbial metabolism through formate assimilation pathways. The recycling of carbon in microbial bioprocesses further enhances carbon flux for bioproduction and reduces the carbon footprint of biorefineries (Chen and Xia, 2024).

5. Challenges and future perspectives

Balancing formaldehyde assimilation, dissimilation and bioproduction: Proof-of-concept studies indicate that to enable C1-based bioproduction to be industrially reliable, precise balancing of carbon flux between C1 substrate utilization, and bioproduction is key beyond mere integration of product biosynthetic pathways with C1 assimilation pathways (Gan et al., 2023; Guo et al., 2021; Guo et al., 2023; Wefelmeier et al., 2024). The accumulation of toxic formaldehyde during methylotrophic growth is mitigated by the parallel dissimilation pathway in yeasts (Fig. 1)

(Yurimoto et al., 2005). This pathway, although it provides energy and reducing power for cell growth in *K. phaffii*, only converts approximately 50–80 % of methanol to CO_2, resulting in considerable carbon loss (Jorda et al., 2014; Jordà et al., 2012). Consequently, methanol-based growth is marked by low carbon flux to pyruvate and acetyl-CoA (33 % and 13 % of methanol uptake, respectively) (Berrios et al., 2022), subsequently limiting growth and product yield. The current understanding of balancing formaldehyde assimilation and dissimilation in the context of native and synthetic methylotrophy remains incomplete (Yamada et al., 2019; Yurimoto et al., 2011). Filling this knowledge gap and rationally implementing intrinsic regulatory mechanisms that exist in other native methylotrophic microbes can further reinforce yeast methylotrophy (Klein et al., 2022). In the future, incorporating formaldehyde-sensing systems (Woolston et al., 2018) as deployed in *E. coli* could also help balance both assimilation and dissimilation pathways. An earlier approach to enhance malic acid production from methanol by the deletion of FDH to weaken the formaldehyde dissimilation pathway did not significantly increase production (titre increased from 0.02 to 0.08 g. L^{-1}) (Guo et al., 2021). Since the dissimilation pathway is a primary source of reducing power and energy, simply knocking out or weakening one or more genes in this pathway without compensating for the loss of NADH and ATP can lead to formic acid accumulation, metabolic imbalance, and growth defects.

Another reason for low bioproduction is the confinement of metabolic flux in the XuMP pathway that resides in the peroxisomes. Rational redirection of this flux toward the product generation pathway by modifying key metabolic node has been proven successful to enhance malic acid production from methanol (Fukuoka et al., 2019; Guo et al., 2021).

Scope of native methylotrophy: As illustrated in Table 2, native methylotrophy has made significant progress in the last decade. Isoprene production from *O. polymorpha* is of note, as the titre obtained in this study was higher than that reported from other yeasts using glucose as a carbon source. *O. polymorpha* is thermotolerant, and hence its application in industry is beneficial, as it can reduce the cooling cost associated with biomanufacturing from methanol in industrial settings (Cotton et al., 2020). Advantages of using this strain for isoprene production is that *O. polymorpha* can be cultivated at a temperature closer to the melting point of isoprene, facilitating product purification and avoiding product inhibition (Wefelmeier et al., 2024). Introducing NAD^+-dependent MDH from *B. methanolicus* is another strategy proposed to enhance the efficiency of methanol assimilation and methanol-based bioproduction in yeast. *O. polymorpha* can act as an ideal host than other yeasts for this MDH that exhibit an activity optimum temperature above 45 °C (Krog et al., 2013; Wefelmeier et al., 2024).

Despite this progress, challenges in genetic manipulation of this strain restricts its use in methylotrophy. To enhance methanol assimilation and the functional expression of multigene biosynthetic pathways, extensive pathway-specific engineering and rewiring of core metabolism are required. Unlike *S. cerevisiae*, advanced metabolic engineering tools for *K. phaffiii* and *O. polymorpha* are limited. Although various plasmids are available, they are low-copy-number, and in these non-conventional yeasts, plasmid partitioning during cell division is often imperfect. Genomic integration of expression cassettes is the preferred strategy for developing industrial strains. However, targeted genome integration is challenging in these yeasts due to their reliance on non-homologous end-joining (NHEJ) for DNA repair. As a result, random integration of transformed expression cassettes can lead to variable gene expression among transformants and unintended disruptions of other genetic elements. This limitation restricts the development of in vivo DNA assembly tools for extensive genome engineering (Löbs et al., 2017). Hopefully, new interventions in synthetic biology and CRISPR/Cas9 systems (Gao et al., 2022b; Pan et al., 2022) will upgrade this microbial chassis as stable methylotrophic platforms.

ALE and non-rational genome engineering designs: Given the intricate and interconnected metabolic networks, achieving beneficial phenotypes in yeast methylotrophy using rational metabolic engineering is tiresome. Innovative high-throughput ALE strategies, e.g., iterative ALE (microbial microdroplet culture (MMC) and shake flask culture (SFC)), parallel shaken pH-auxostat (CPA) system, Helicase-AID-assisted ALE have made remarkable progress in a short period of time (Chen et al., 2024; Sarikaya et al., 2024; Wang S et al., 2023; Wang K et al., 2023). These strategies unfolded mechanistic insights on methanol and formate tolerance, improved growth kinetics, and metabolic fluxes. Yeast C1 pathway activation was also achieved by ALE providing prospects on designing chimeric pathways and expanding product spectrum (Espinosa et al., 2020; Zhan et al., 2023). However, these advances are insufficient for immediate industrial deployment of evolved strains. Recently, methanol tolerance of up to 10–13 % has been attained in *K. phaffii* (Wang S et al., 2023). This study and previous studies (Lairón-Peris et al., 2021; Linney et al., 2023) have emphasized the interplay of membrane lipid composition in solvent tolerance. Hence, engineering membrane lipids in methylotrophs could also provide a more stable base strain to subsequently develop methylotrophy.

ALE has also revealed many genetic elements contributing to methanol tolerance (Table 3). These findings provide a roadmap for rationally engineering of synthetic methylotrophic yeast. Hence, by combining the strengths of rational engineering with ALE, researchers can significantly reduce the time and increase the likelihood of success in engineering yeast for methanol utilization. A recent study emphasizes the unforeseen capability of non-rational strategies of genome-scale chromosome rearrangement along with ALE to harness the inherent and hidden methylotrophic traits in yeast systems. Adopting these innovative strategies, like SCRaMbLE, that are used in *S. cerevisiae* can also be adapted to other non-methylotrophic industrial yeasts (Guo et al., 2024a).

Innovative rational engineering and synthetic biology approaches: Combining Genome-scale metabolic modelling (GSMM) is an essential tool in rational system biology for expanding our knowledge of C1 metabolism and engineering methylotrophic strains to increase bioproduction to biomass accumulation by redirecting carbon flux to product synthesis (Kabimoldayev et al., 2018). The role of GSMM in designing and reconstructing the methanol utilization pathway, as well as in adopting rational methanol feeding strategies for increasing malic acid production in *O. polymorpha* and 3-HP production in *K. phaffiii* has already been demonstrated (Àvila-Cabré et al., 2024; Wefelmeier et al., 2024).

Methylotrophic pathways are widespread among different microbial communities. This provides possibilities to in silico design better-performing chimeric C1 assimilation pathways by mixing and matching methylotrophic enzymes (De Simone et al., 2020). Natural C1 pathways are cyclic and are overlapped with native core metabolic pathways in the host. Hence, their implementation in non-methylotrophs fail to restore the delicate flux balance that exists between these pathways (Cotton et al., 2020). Innovative design of artificial C1 assimilation pathways is suggested as future solution for developing stable strains. Systematic computationally guided approaches have been used for developing several natural C1 pathway analogues, modified C1 pathways and synthetic pathways involving artificial enzymes (Table 1). Acetyl-CoA (SACA) pathway is a linear synthetic formaldehyde assimilation pathway, which are suggested to solve the constraints of cyclic native pathways and simplify biosynthesis of chemicals from C1 compounds using minimum enzymatic steps starting from formaldehyde (Lu et al., 2019b). In vivo implementation of these synthetic pathways will be possible after engineering candidate enzymes for improvement of their kinetic properties (Zhan et al., 2023).

Advancements in systems biology, synthetic biology, and in silico modelling will enable the efficient design of novel multi-component C1 cascade reactions and their facile in vivo implementation after in vitro optimization to transform methylotrophy (Qiao et al., 2023). A wide variety of synthetic biology tools have already been developed for *S. cerevisiae*. Various metabolic engineering strategies, along with

Table 3
ALE in yeast for methanol and formate tolerance.

Yeast strain	Pathway for methanol assimilation or bioproduct	Evolution method/ condition	Growth/Tolerance Enhancement	Genetic/physiological changes	Reference
K. phaffii	XuMP	• Iterative ALE (MMC and SFC) • Initial evolution in minimal medium with 1–5 % methanol • Best colonies were selected and evolved in methanol up to 10 %	• Final strain reached an OD_{600} of 20.38 after 96 h in medium containing 10 % methanol • Growth in solid medium containing 13 % methanol	• PET gene as a crucial target for altering methanol tolerance • Denser cell wall and membrane, altered cell and peroxisome membrane composition • Increasing membrane phosphatidylcholine (PC) content can improve methanol tolerance	(Wang S et al., 2023)
O. polymorpha-LDH	XuMP and pathway for lactate production	25 passages in minimal medium containing 2 % methanol using a microtiter plate system	• A 55 % increase in growth rate in 2 % methanol compared to unevolved strain (growth rate; parental strain- 0.040 h^{-1}, evolved strain-0.062 h^{-1}) • Shortened lag phase (parental strain −100 to 200 h, evolved strain- less than a day)	Mutation in methanol oxidase	(Wefelmeier et al., 2023)
O. polymorpha-LDH (lactate producing strain exhibit low specific growth rate in methanol)	XuMP and pathway for lactate production	• ALE in continuous parallel shaken pH-auxostat (CPA) equipped with real-time pH control of the culture medium • The pH-unbuffered minimal medium, containing 1% methanol, was continuously supplied as fresh feed medium	A 4.8-fold increase of the maximum specific growth rate μmax: parental strain- 0.023 h^{-1}, evolved strain- 0.13 h^{-1} Lag phase: parental strain −47 to 70 h, evolved strain- no initial lag phase	15 % increase in lactate production	(Sarikaya et al., 2024)
S. cerevisiae	XuMP pathway, glyoxylate-based serine pathway (gSerine pathway)	• Serial transfers in shake flasks in five stages • Terminated upon reaching a stable growth rate in the MM with 1% methanol (*v/v*) and 0.2 % xylose (*w/v*)	Evolved gSerine pathway promoted formaldehyde assimilation	Upregulated isoprenoid pathway alleviating damage by methanol	(Zhan et al., 2023)
S. cerevisiae	Unidentified pathway	• YNB medium with either yeast extract alone or yeast extract with 2 % methanol • Cultures were periodically switched between media with 1% (w/v) glucose and yeast extract, with or without methanol	Identified and improved native capacity for methylotrophy in 2 % methanol	Mutation in transcription factor YGR067CP directly linked to improved growth on methanol	(Espinosa et al., 2020)
Y. lipolytica	Chimeric RuMP/XuMP	30 passages in YNB media with Complete Supplement Mixture (CSM) containing essential amino acids and 2 % methanol	• Improved methanol assimilation from undetectable to a level of 1.1 g. L^{-1} per 72 h and enabled methanol-supported cellular maintenance • Evolved strains achieved higher biomass and proteinogenic amino acid production	• The combination of rational metabolic engineering (e.g., RuMP/XuMP pathway construction) and ALE fine-tuned metabolic fluxes • Reactivation of formaldehyde dissimilation pathway, glycolysis and amino acid synthesis pathway, increased tolerance to methanol and formaldehyde toxicity	(Wang G et al., 2021)
ALE in formate					
S. cerevisiae	rGly Pathway	• Synthetic minimal (SM) medium supplemented with glucose and formate under a 10 % CO_2 atmosphere, with 21 serial passages	• Initial doubling time of ~80 h reduced to ~7 h after 21 ALE cycles • ·Growth remained strictly dependent on formate and CO_2	• Modification gdh1::Δ108bp helps to balance mitochondrial C1-THF and NADPH pools, facilitating glycine synthesis and supporting rGly pathway activity.	(Bysani et al., 2024)
S. cerevisiae	rGly pathway	• Use 20 g. L^{-1} glucose as the primary carbon source with 0–50 g. L^{-1} sodium formate for evolution • At each formate concentration, the strain was cultured for 40–60 generations	• Growth at 20 g. L^{-1} glucose with 50 g. L^{-1} formate to reach a maximum OD_{600}: 6.88	• Identified MMM1p (outer mitochondrial membrane protein) as a key target to enhance the formate tolerance • Increased expression of superoxide scavenging genes, formate dehydrogenases, and genes involved in regulating cell membrane and cell wall composition	(Wang K et al., 2023)

(*continued on next page*)

Table 3 (*continued*)

Yeast strain	Pathway for methanol assimilation or bioproduct	Evolution method/ condition	Growth/Tolerance Enhancement	Genetic/physiological changes	Reference
Y. lipolytica	Uncharacterized endogenous pathway	• Helicase-AID: Combined formate pressure with genetic editing for accelerated evolution • 25 rounds of selection under increasing formate concentrations (up to 6.6 %)	• Consumed 1.77 g. L^{-1} of formate at 48 h (50 % more than wild-type) • Evolved strains adapt to 1 M formate concentration.	• Identified the role of gSer-tSer bicycle for supporting formate assimilation • Identified the role of the critical enzyme Ftl (formate-tetrahydrofolate ligase) for integrating formate into metabolic pathways	(Chen et al., 2024)

machine learning, have been employed to implement non-native pathways for the bioproduction of chemicals in these yeasts (Li and Borodina, 2015; Bao et al., 2018). Therefore, this yeast holds significant potential as a future innovative C1 cell factory over other yeast systems.

Future initiatives will also include an integrated strategy that combines systematic rational methods with random adaptive strain evolution. This tactic is specifically useful in *S. cerevisiae* as we lack a comprehensive understanding of how methanol assimilation pathways integrate into the non-methylotrophic host background. Bioproduction from yeast synthetic methylotrophy in *S. cerevisiae* is highly challenging due to insufficient growth on methanol as a sole carbon source. While supplementing with a co-substrate such as yeast extract can enhance methylotrophic growth, it significantly increases production costs, making industrial-scale application impractical. In this context, combination of advanced metabolic engineering strategies eg. modular circuit engineering in combination with random evolution is worthy of note (Zhan et al., 2023). This approach enabled fine tuning of methylotrophy via optimizing methanol oxidation, energy generation, and formaldehyde assimilation to support measurable growth on methanol while enabling bioproduction. In the future, the use of targeted genome engineering through CRISPR-Cas-based methods, eukaryotic multiplex automated genome engineering (eMAGE), and rational in vivo pathway evolution can enhance the efficiency of *S. cerevisiae* as synthetic methylotrophs (Holland and Blazeck, 2022).

Organelle engineering for specific bioproducts: Exploiting distinct biochemical environment of organelles for bioproduction can enhance product yield. Isoprenoids are natural products with high commercial value. But its commercial production in microbes is challenging due to its hydrophobicity and cellular toxicity. Interestingly, during methanol dependent growth of engineered and evolved *S. cerevisiae,* native isoprenoid biosynthetic pathway upregulated to relieve the methanol toxicity and preserve membrane stability in presence of methanol (Zhan et al., 2023). This further affirm isoprenoids as an ideal target product from yeast methylotrophy. The presence of peroxisomes in yeasts is an inherent advantage for using it as a host for isoprenoid production, as these organelles provide a lipophilic environment suitable for product accumulation (Dusséaux et al., 2020; Kang et al., 2024). Recent studies have demonstrated the feasibility of the production of α-bisabolene and zeaxanthin A1from methanol in *K. phaffii* using peroxisomal engineering (Gao et al., 2024; Niu et al., 2024). Targeting pathways to peroxisomes facilitate special coupling of biosynthesis pathways with methanol assimilation. *Y. lipolytica* is another promising host for future methanol-based bioproduction as it has a higher solvent tolerance, and this oleaginous yeast can form plenty of peroxisomes for lipid oxidation (Wang G et al., 2021). High lipid content of this host also facilitates the accumulation of hydrophobic isoprenoids (Luo et al., 2020). This characteristic is specifically advantageous for peroxisomal coupling for concurrent methanol utilization and isoprenoid bioproduction. Similarly, the production of lactic acid is directly correlated with the availability of pyruvate. To address the limited availability of pyruvate in the cytosol, lactic acid synthesis in *P. pastoris* was targeted to the mitochondria in addition to the cytosolic pathway. This organellar localization enhanced bioproduction by a 13 % increase from methanol compared to the strain containing only the cytosolic pathway (Wu et al., 2025).

Challenges and strategies for engineering yeast formatotrophy: To establish a competitive formate-based bioeconomy, several key challenges must be addressed, including low pyruvate flux from formate, metabolic burden caused by disruption of native metabolism, inefficient energy and reducing power generation, and limited formate tolerance. Engineering the rGly pathway via overexpression of native enzymes, combined with ALE, currently represents the most reliable strategy to enable formate assimilation in yeast. Although conversion of formate to pyruvate through the rGly pathway has been demonstrated, the resulting pyruvate levels are insufficient to support fully formatotrophic growth. As formate is not a natural carbon source for yeast, unregulated overexpression of native enzymes may disrupt cellular homeostasis and impose a significant metabolic load, resulting in poor growth and reduced flux through engineered pathways.

This engineered formatotrophy is not truly autotrophic but operates as a co-utilization model, with glucose serving as a critical energy and reducing power source. Under glucose-limited conditions, these energy deficits become prominent. Weak expression and suboptimal activity of native FDH is the primary reason for insufficient reducing power generation from formate. Introducing multiple copies of more robust, heterologous FDHs—such as those from methylotrophic yeasts—may significantly improve reducing power and energy yields. Additionally, employing $NADP^+$-specific FDHs and eliminating major NADPH-consuming enzymes can replenish NADPH pools, which is essential for the NADPH-dependent steps of the rGly pathway (Bysani et al., 2024).

ALE has uncovered beneficial mutations, such as those in GDH1 (glutamate dehydrogenase), which enhance NADPH availability, and PET9 (a mitochondrial ATP/ADP transporter), which regulates ATP exchange between mitochondria and cytosol—together optimizing compartmentalized C1 fluxes (Bysani et al., 2024). Reverse-engineering these mutations into other yeast strains can accelerate ALE and enhance both redox balance and formatotrophic performance. Further improvements may be achieved by evolving strains under glucose- or pyruvate-limiting conditions in continuous culture with formate. From a biosynthesis perspective using engineered formatotrophs, focusing specifically on rGly-derived chemicals—such as lactic acid, isobutanol, and cysteine (Kim et al., 2020)—can help reduce metabolic burden. Additionally, alternative synthetic formate assimilation pathways (Table 1, Bang et al., 2021), which involve fewer enzymatic steps and lower cofactor requirements than the rGly pathway, offer a promising route to reduced metabolic burden and more efficient carbon flux.

A critical factor in successful formate utilization is managing toxicity through balanced uptake and host adaptation. Yeast's inherent low-pH tolerance allows the use of protonated formate, simplifying feeding strategies and avoiding the need for external acid/base adjustments, unlike sodium formate-fed systems used for other microbes (Bysani et al., 2024). Protonated formate can passively diffuse across membranes, improving uptake, but intracellular deprotonation acidifies the cytosol, leading to stress. Engineered *S. cerevisiae* strains show maximum growth at 125 mM formate, with a sharp decline in growth observed above 300 mM (Bysani et al., 2024). While yeast can mitigate this stress

better than many organisms due to native FDH activity and pH homeostasis, further improvements in FDH expression, membrane remodeling, and transporter engineering are likely necessary. Sequential or pulsed formic acid feeding strategies, as previously described, could be worth trying to help maintain intracellular concentrations below toxic thresholds (Tejedor-Sanz et al., 2024). Although passive diffusion and anion/proton symport are described modes of formate uptake in *S. cerevisiae*, a dedicated transporter remains unidentified. Introducing bacterial transporters, such as *E. coli* FocA or other bacterial formate–nitrite transporter (FNT) family proteins and engineering them may enable regulated formate uptake (Wiechert and Beitz, 2017).

Formate exposure also induces oxidative stress by promoting reactive oxygen species (ROS) accumulation, disrupting redox balance, and damaging membranes. Targeting membrane remodeling and lipid composition (Guo et al., 2018) is therefore a viable strategy to enhance formate tolerance. Notably, yeasts increases aromatic amino acid synthesis under formate stress as a protective response (Zeng et al., 2022). Exploiting this natural tolerance trait by overexpressing the shikimate pathway could both enhance tolerance through increased aromatic amino acid production and provide a route to value-added products derived from aromatic amino acids via formatotrophy.

6. Conclusions

Methanol and formate-based biomanufacturing from yeast presents a sustainable strategy for transitioning into a sustainable future. Despite great advances achieved in yeast methylotrophy, knowledge gap in various aspects of methylotrophy still restricts establishment of methanol or formate autotrophy in yeast. With the ongoing innovation in random and rational genome engineering strategies, methanol-based bioprocessing has the potential to replace currently established sugar-based biomanufacturing platforms. Formate-based biosynthesis in yeast, though, remains in the preliminary stage. Advances in artificial formate assimilation pathway design in combination with strain evolution can unlock new possibilities in the coming decade. These breakthroughs will contribute to addressing global challenges of resource sustainability, climate change and advancing the United Nations Sustainable Development Goals (SDGs).

Declaration of generative AI in scientific writing

During the preparation of this work the author (Mrudulakumari Vasudevan Ushasree) used ChatGPT to improve the language and readability in some parts of the manuscript. After using this tool, the author reviewed and edited the content as needed and takes full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

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