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Aerobic Utilization of Methanol for Microbial Growth and Production

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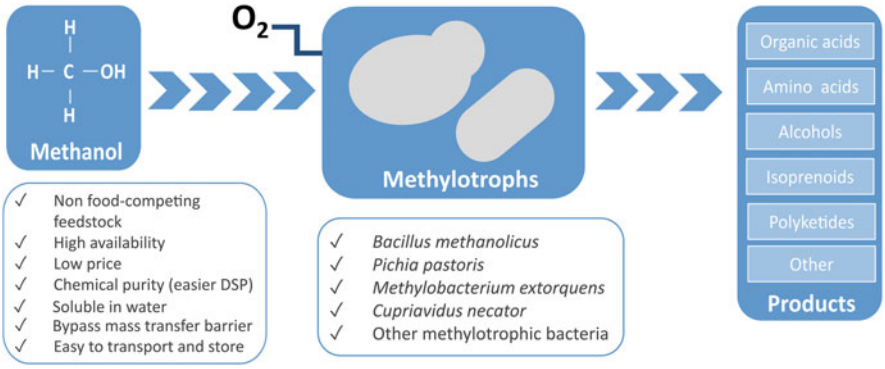
Abstract Methanol is a reduced one-carbon (C1) compound. It supports growth of aerobic methylotrophs that gain ATP from reduced redox equivalents by respiratory phosphorylation in their electron transport chains. Notably, linear oxidation of methanol to carbon dioxide may yield three reduced redox equivalents if methanol oxidation is NAD-dependent as, e.g., in *Bacillus methanolicus*. Methanol has a higher degree of reduction per carbon than glucose (6 vs. 4), and thus, lends itself as an ideal carbon source for microbial production of reduced target compounds. However, C–C bond formation in the RuMP or serine cycle, a prerequisite for production of larger molecules, requires ATP and/or reduced redox equivalents. Moreover, heat dissipation and a high demand for oxygen during catabolic oxidation of methanol may pose challenges for fermentation processes. In this chapter, we summarize metabolic pathways for aerobic methanol utilization, aerobic methylotrophs as industrial production hosts, strain engineering, and methanol bioreactor processes. In addition, we provide technological and market outlooks.

Graphical Abstract



Keywords Biochemical networks, Methanol, Methylotrophy, Process engineering, Strain engineering

1 Methanol Assimilatory Pathways Compatible with Aerobic Growth

Aerobic growth on methanol is naturally supported by four different metabolic pathways found among methylotrophic bacteria and yeast: the Ribulose Monophosphate (RuMP) pathway, the Dihydroxyacetone (DHA) pathway, the Calvin-Benson-Bassham (CBB) cycle, and the Serine cycle as illustrated in Fig. 1. All of them require first the oxidation of methanol to formaldehyde and eventually the oxidation of formaldehyde to CO_2. Indeed, the assimilation of one-carbon (C1) compounds takes place either at the level of formaldehyde for the RuMP and the DHA pathways, or CO_2 for the CBB cycle or a combination thereof (i.e., methylenetetrahydrofolate (methylene-H_4F) and CO_2) for the Serine cycle. All these pathways are cyclic and require a C1 acceptor (i.e., ribulose-5-phosphate, xylose-5-phosphate, ribulose-1, 5-bisphosphate and glycine, respectively) to enable the formation of a C–C bond. The efficiency of methanol assimilation is then determined by the capability of the cells to produce and regenerate the C1 acceptor and especially when C1 is assimilated as a toxic compound such as formaldehyde. This has to be carefully regulated. We will below describe each of these four assimilatory pathways in some more detail.

1.1 The Ribulose Monophosphate Pathway

The ribulose monophosphate (RuMP) pathway operates in Beta- and Gammaproteobacteria as well as in Gram-positive bacteria (e.g., *B. methanolicus*). In thermophilic Gram-positive methylotrophs of the genus *Bacillus*, methanol is first converted to formaldehyde using a cytoplasmic NAD-dependent methanol dehydrogenase (NAD-Mdh) or a nicotinoprotein methanol dehydrogenase (Mdo) with a bound NAD(P) cofactor that uses an unknown electron acceptor for reduction [1]. Up to date, the NAD-Mdhs from methylotrophic *B. methanolicus* and non-methylotrophic *B. stearothermophilus* are the best characterized [2, 3]. Genomic analyses of thermophilic Bacilli strains revealed that in total 3 different NAD-Mdhs are encoded, i.e., two Mdh genes are chromosomal and one is plasmid-borne [4, 5]. These NAD-Mdhs are transcribed at different levels depending on substrate conditions (methylotrophic vs. non-methylotrophic growth) and revealed a broad substrate spectrum [2]. NAD-Mdhs are decameric proteins and in each of the subunit, one molecule of NAD(H), one Zn2+, and two Mg2+ cofactors are found. NAD-Mdhs interact with an activator protein (ACT, encoded by the chromosomal *act* gene) which facilitates the oxidation of NADH [6]. In vitro, the activity of NAD-Mdhs can be enhanced by ACT [2] suggesting that these enzymes also play roles in formaldehyde detoxification of the cells. It was shown in vitro that the three Mdhs from *B. methanolicus* have higher affinity for formaldehyde ($K_m \leq 7$ mM) than for methanol ($K_m \geq 170$ mM [2]. Interestingly, the NAD-Mdh of

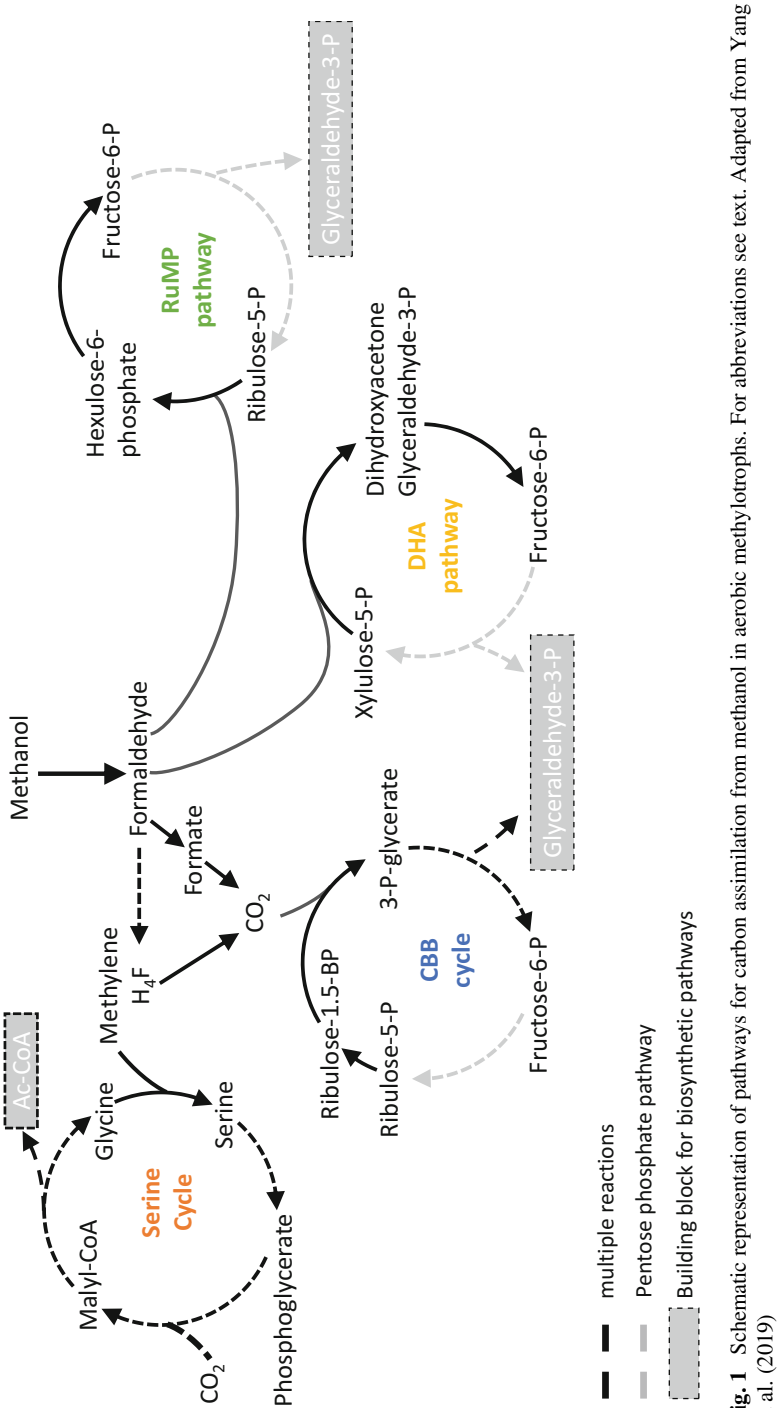


Fig. 1 Schematic representation of pathways for carbon assimilation from methanol in aerobic methylotrophs. For abbreviations see text. Adapted from Yang et al. (2019)

non-methylotrophic *B. stearothermophilus* has a higher affinity for methanol ($K_m = 20$ mM) [3] compared to the *B. methanolicus* enzymes.

Through the RuMP pathway essentially all the carbon required for biomass formation is assimilated from formaldehyde in a reaction catalyzed by 3-hexulose-phosphate synthase (Hps) that condenses formaldehyde and ribulose-5-phosphate (Ru5P) into hexulose-6-phosphate (H6P), which is subsequently isomerized to fructose-6-phosphate (F6P) by 6-phospho-3-hexuloisomerase (Phi) [7]. F6P is then further cleaved either to glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone-phosphate (DHAP) by the glycolytic fructose-bisphosphate aldolase (FBA variant) or to GAP and pyruvate by the keto-hydroxyglutarate aldolase (KDPG aldolase variant), an enzyme from the Entner-Doudoroff pathway. Regeneration of the initial C1 acceptor Ru5P is achieved via a sequence of enzymatic reactions through the pentose phosphate pathway (PPP) and for which several variants exist. Ru5P can be regenerated via the sedoheptulose-1,7-biphosphatase (SBPase) variant or via the transaldolase (TA) variant. Ribulose-phosphate 3-epimerase (Rpe) or ribose-5-phosphate isomerase (Rpi) ultimately produces Ru5P, respectively, from xylulose-5-phosphate and from ribose-5-phosphate. The GAP generated in these pathways is further converted though the glycolysis for biosynthetic purpose. Finally, this fixation process allows the formation of one molecule of GAP from 3 molecules of formaldehyde [7]. Overall, the RuMP pathway converts methanol into biomass with a high energetic efficiency of 40–50% [8].

1.2 The Dihydroxyacetone Pathway

The dihydroxyacetone (DHA) pathway, which is also called the xylulose monophosphate (XuMP) pathway, occurs in methylotrophic yeasts (e.g., *Hansenula polymorpha* (also known as *Pichia angusta*) and *Candida boidinii*) growing on methanol [9]. In these organisms, methanol is first oxidized by alcohol oxidase (AOX) in a reaction that uses oxygen as an electron acceptor and produces not only formaldehyde and hydrogen peroxide [9]. AOX is located in the peroxisome. This enzyme is a homo-octameric protein: each inactive AOX monomer is synthetized in the cytoplasm and then assembled together to form the active octameric AOX protein in the peroxisome. During growth on methanol, AOX can account for up to 30% of the total cellular protein [10]. Hydrogen peroxide produced by AOX during methanol oxidation is detoxified into oxygen and water by a catalase (CAT) also located in the peroxisome.

The DHA pathway is similar to the RuMP pathway in principle. The formaldehyde is condensed with a phosphorylated pentose, xylulose 5-phosphate (Xu5P) catalyzed by dihydroxyacetone synthase (Das). The products of the reaction are GAP and DHA [7]. Once produced, DHA is phosphorylated to DHAP by the dihydroxyacetone kinase (Dak) which is essential to allow a growth on methanol [11]. For every molecule of GAP that is incorporated into biomass, three molecules of formaldehyde are fixed, generating three molecules of DHA, and consuming three

molecules of Xu5P. Recycling Xu5P is completed through molecular rearrangements similar to the reactions of PPP [7, 12]. Compared to the RuMP pathway, DHA pathway operates at a lower efficiency of 30–35% and this is explained by the energetically wasteful oxidation of methanol with O_2 [8].

In yeast, methylotrophy takes place in the peroxisome and a peroxisomal targeting signal (PTS) sequence enables the proteins to cross the peroxisomal membrane [13]. AOX, Das, and CAT contain a PTS1 sequence on the C-terminus. This sequence was also found on isoforms of PPP enzymes involved in the recycling of the Xu5P indicating that recycling of the C1 acceptor is also completed in the peroxisome [12]. A second PTS exists in yeast to address proteins to the peroxisome, PTS2, which is found on the N-terminus of proteins but is rarer than PTS1. It was shown that a *Pichia angusta* strain lacking peroxisome was not able to grow on methanol despite AO, DAS, and CAT were present in the cytosol. This means that this spatial organization is crucial for the survival of methylotrophic yeasts [14].

1.3 The Calvin-Benson-Bassham Cycle

The Calvin-Benson-Bassham (CBB) cycle, also known as the ribulose bisphosphate (RuBP) pathway is the most widespread carbon fixation pathway found in the biosphere. The CBB cycle is well known for autotrophic microorganisms assimilating carbon at the level of CO_2. This assimilatory pathway is not a unique feature of methylotrophs and some methylotrophs possess other carbon assimilation pathways besides the CBB cycle [15]. The CBB cycle can be found in all autotrophic methylotrophs such as autotrophic alphaproteobacterial methylotrophs, e.g., *Paracoccus denitrificans,* or chemolithoautotrophic bacteria, e.g., *Cupriavidus necator* (known as *Ralstonia eutropha*) [15, 16]. However, the use of the CBB cycle for growth on methanol is characterized by a low energetic efficiency of only 20–35% [8].

First methanol is oxidized to CO_2, which is further assimilated in the CBB cycle, as in classical autotrophic organisms. Those methylotrophs use pyrrolo-quinoline-quinone (PQQ) dependent methanol dehydrogenases to oxidize methanol into formaldehyde. PQQ is a prosthetic group capturing electrons from methanol oxidation to transfer them specifically to cytochrome c [17]. PQQ-Mdhs have a higher affinity for methanol than for higher alcohols [18]. Most of the PQQ-Mdhs are composed by two subunits: one large and one small, respectively, encoded by the genes *mxaF* and *mxaI* [17]. However, some bacteria such as *Burkholderia* spp. do not possess these genes but have instead an *mdh2* gene encoding for a monomeric PQQ-Mdh [18]. So far, MxaFI are the best characterized PQQ-Mdhs but recently, a third type of PQQ-Mdh encoded by *xoxF* gene was described. While MxaF uses Ca^{2+} as a cofactor, XoxF binds to rare earth elements such as lanthanides (Ln^{3+}) [19].

A number of paralogous pathways exist for the efficient oxidation of formaldehyde to CO_2, sometimes even within one organism [20]. They comprise linear cofactor-dependent pathways, such as the tetrahydromethanopterin (H_4MPT) or

the tetrahydrofolate (H_4F)-dependent pathway, which are widespread among methylotrophic proteobacteria [20]. H_4MPT pathway is one of the most elaborated pathways to oxidize formaldehyde to formate. It is a pathway commonly found in obligate anaerobic archaea, methanogens, and acetogens [21]. However, this pathway was first described in the aerobic mesophilic bacterium *Methylobacterium extorquens* AM1 [21]. Formaldehyde condensation with H_4MPT to methylene-H_4MPT (CH_2H_4MPT) can occur spontaneously but is catalyzed by a formaldehyde-activating enzyme (Fae). Then, CH_2H_4MPT is converted to formate via a series of enzymatic reactions. H_4F reacts non-enzymatically with formaldehyde to generate methylene-H_4F which can either be used in the Serine cycle for biosynthesis or converted into formate via a series of enzymatic reactions [20]. Other linear oxidation pathways are dependent on thiol-compounds, such as glutathione (GSH, *Paracoccus denitrificans*), mycothiol (MySH, Gram-positive methylotrophs), and bacillithiol (BSH, *B. methanolicus*) [20, 22]. GSH-dependent pathway is the simplest pathway to convert formaldehyde to formate in only two reactions vs four for the other pathways. In addition, it is found in many organisms from all kingdoms (i.e., plants, mammals, bacteria). Overall, all these pathways use formate as an intermediate which is then oxidized to CO_2 via formate dehydrogenases (Fdh). A large number of Fdhs exist that differ by the cofactor they use (i.e., NAD, NADP, cytochrome, menaquinone, H_2) [21].

Finally, CO_2 enters the CBB cycle by a carboxylation of the RuBP to 3 phosphoglycerate (3PG) in a reaction catalyzed by ribulose bisphosphate carboxylase. 3PG is either used as precursors of cell biomass or subsequently converted into GAP. GAP is then used by a mechanism of carbon skeleton recombination involving the PPP enzymes to refill the RuBP pool. In total three molecules of CO_2 are processed into a single molecule of 3PG [23].

1.4 The Serine Cycle

The Serine cycle for carbon assimilation belong to Alphaproteobacteria (e.g., *M. extorquens*). Within this pathway, methanol is oxidized to formaldehyde by a PQQ-dependent enzyme, while formaldehyde is assimilated as methylene-H_4F after spontaneous reactions with H_4F [21]. Methylene-H_4F enters the Serine cycle in a reaction catalyzed by serine transhydroxymethylase yielding serine from glycine. After several reaction steps yielding to phosphoenolpyruvate (PEP), it is condensed with CO_2 to form oxaloacetate (OAA). OAA is transformed into malate which is further converted to malyl-CoA. Malyl-CoA is subsequently cleaved into two molecules: glyoxylate and acetyl-CoA. The assimilation of C1 units via the Serine cycle requires regeneration of glyoxylate from acetyl-CoA. In many methylotrophs, glyoxylate regeneration is done via the ethylmalonyl-CoA (EMC) pathway that has been described in *M. extorquens* [24, 25]. In the cyclic EMC pathway, two molecules of CO_2 are needed to ensure glyoxylate regeneration. However, some methylotrophs lack the EMC pathway and regenerate glyoxylate using the

traditional glyoxylate shunt [26]. The EMC pathway is not specific of C1 assimilation since it has been described to be functional during *M. extorquens* growth on acetate [27].

2 Aerobic Methylotrophic Microorganisms

Aerobic methylotrophy is found within various clades of bacteria (i.e., Alpha-, Beta-, Gamma-proteobacteria, Actinobacteria, Firmicutes, Verrucomicrobia) and within fungi (ascomycoteous yeasts and mold fungi) [9, 28, 29]. Many of these are facultative methylotrophs, i.e. capable of utilizing multi-carbon compounds. Here the focus will be on the most studied aerobic methylotrophs used for bioproduction.

2.1 Bacillus methanolicus

Bacillus methanolicus is a Gram-positive, thermophilic, and facultative methylotrophic bacterium with a well-proven potential for conversion of methanol to value-added products at 50°C [30]. Its methylotrophic lifestyle has been well understood at the genetic, biochemical, and regulatory level and it has several novel traits such as plasmid-dependent methylotrophy, three methanol dehydrogenases and pairs of isoenzymes for most catalytic reactions of the central formaldehyde assimilating RuMP pathway [2, 6, 31–38]. Over the last two decades, genetic tools and techniques have been gradually established and taken into use [39]. Moreover, accompanied with genome sequencing of the main model strain MGA3, omics analyses and construction of a genome-scale metabolic model has opened for systems-level metabolic engineering of this bacterium [4, 5, 40–42]. One still lasting bottleneck however is the lack of any methodology for genome editing, while a technique for temporal inhibiting of targeted genes based on using CRISPRi was recently established [43]. *B. methanolicus* MGA3 has proven useful for overproduction of some amino acids and their derivatives, as well as other chemicals and recombinant proteins (see Table 1). Fed-batch cultivations have been established and demonstrated useful for evaluation of the recombinant strains. This bacterium uses the RuMP pathway for formaldehyde assimilation, and it has one cyclic and one linear pathway for formaldehyde dissimilation into CO_2.

2.2 Pichia pastoris

The methylotrophic yeasts were initially isolated in the late 1960s and early 1970s [44, 45]. These eukaryotic methylotrophs include Candida, *Pichia*, and some genera that were recently separated from *Pichia* (i.e., *Ogataea*, *Kuraishia,* and

Table 1 Aerobic methanol-based processes of secreted compounds

Product	Methylotrophic production host	Strain engineering	Fermentation conditions	Titer [g/L]	Yield [g/g]	Volumetric productivity [g/L/h]	Reference
Amino acids							
L-serine	*M. extorquens*	WT	Glycine-based minimal medium	65	–	–	[77]
L-glutamate	*B. methanolicus*	WT	Methanol fed-batch medium with vitamins & trace-metals, methionine, and a source of ammonia	55	0.36	2.78	[78]
	M. glycogenes	Classically obtained mutant	Methanol fed to 0.5% by the DO-stat method to medium with vitamins, yeast extract and peptone in 84 h in a 5-l jar bioreactor	39	–	0.46	[79]
L-lysine	*B. methanolicus*	Classically obtained homoserine dehydrogenase mutant	Methanol fed-batch, medium with glutamate, threonine, methionine and yeast extract	35	–	–	[78]
	M. glycogenes	Classically obtained threonine and lysine-co-producing mutant of expressing feedback-resistant mutant *dapA* from *E. coli*	Methanol fed to 0.5% by the DO-stat method to medium with vitamins, yeast extract, and peptone in 72 h in a 5-l jar bioreactor	8	–	0.15	[80]
	M. methylotrophus	WT expressing *E. coli* gene for dihydrodipicolinate synthase feedback-resistant to lysine and shortened lysine exporter gene from *C. glutamicum*	Methanol-controlled fed-batch cultivation in 1 L jars using mineral salts medium	11	–	0.27	[81]

(continued)

Table 1 (continued)

Product	Methylotrophic production host	Strain engineering	Fermentation conditions	Titer [g/L]	Yield [g/g]	Volumetric productivity [g/L/h]	Reference
L-threonine	*M. glycogenes*	Classically obtained mutant resistant against threonine and lysine analogs	Methanol fed to 0.5% by the DO-stat method to medium with vitamins, yeast extract, and peptone in 72 h in a 5-l jar bioreactor	11	–	0.15	[79]
γ-Aminobutyrate	*B. methanolicus*	WT, plasmid-borne expression of glutamate decarboxylase gene from *Sulfobacillus thermosulfidooxidans*	Fed-batch fermentation (~70 h) in 3-L CSTR with minimal medium; methanol concentration maintained at ~4.8 g L^{-1}; shift to acidic pH for conversion of glutamate to GABA	9	–	0.13	[73]
Cadaverine	*B. methanolicus*	WT, plasmid-borne expression of lysine decarboxylase gene *cadA* from *E. coli*	Fed-batch fermentation (~47 h) in 3-L CSTR with minimal medium; methanol concentration maintained at ~4.8 g L^{-1}	10	–	0.21	[39]
Organic acids							
Malate	*P. pastoris*	WT, overexpression of endogenous genes for pyruvate carboxylase and cytoplasmic malate dehydrogenase	Methanol medium with yeast nitrogen base, biotin, histidine and repeated feeding with 5 g/L methanol every 12 h; 96 h total	42	–	0.44	[82]
Fumarate	*P. pastoris*	WT, overexpression of endogenous genes for pyruvate carboxylase and cytoplasmic malate dehydrogenase	Methanol medium with yeast nitrogen base, biotin, histidine and repeated feeding with 5 g/L methanol every 12 h; 96 h total	0.75	–	0.01	[82]

Succinate	*P. pastoris*	WT, overexpression of endogenous genes for pyruvate carboxylase and cytoplasmic malate dehydrogenase	Methanol medium with yeast nitrogen base, biotin, histidine, and repeated feeding with 5 g/L methanol every 12 h; 96 h total	9.4	–	0.10	[82]
D-lactate	*P. pastoris*	WT, with integrated D-lactate dehydrogenase gene into the non-transcribed spacer of the ribosomal DNA locus followed by post-transformational amplification	Test-tubes with 10 g/L yeast extract, 20 g/L peptone, and 30 g/L methanol; 96 h total	3.5	–	0.04	[83]
Mesaconate	*M. extorquens*	WT, heterologous expression of *E. coli* thioesterase gene *yciA* and deletion of *phaR*	Shake flasks with 4 g L^{-1} methanol and reduced medium cobalt ion concentration (0.2 μM)	0.44	0.11	0.006	[74]
Methylsuccinate	*M. extorquens*	WT, heterologous expression of *E. coli* thioesterase gene *yciA* in WT	Shake flasks with 4 g L^{-1} methanol and reduced medium cobalt ion concentration (0.2 μM)	0.27	0.07	0.004	[74]
Itaconate	*M. extorquens*	WT, heterologous expression of codon-optimized *cis*-aconitic acid decarboxylase gene from *A. terreus* in *M. extorquens* WT	2 L jars, minimal medium with 7.7 g L^{-1} methanol followed by periodic methanol feeding for 12 days	0.03	–	0.003	[84]
2-Hydroxyisobutyrate	*M. extorquens*	WT, heterologous expression of gene for (R)-3-hydroxybutyryl coenzyme A (CoA)-specific coenzyme B12-dependent mutase from *Bacillus massiliosenegalensis*	Minimal medium; 500 g L^{-1} of methanol in nitrogen-free mineral salt medium was added to maintain 0.5 and 5 g L^{-1} methanol in the bioreactor; co-production with PHB after nitrogen starvation, 192 h total	2.1	–	0.013	[85]

(continued)

Table 1 (continued)

Product	Methylotrophic production host	Strain engineering	Fermentation conditions	Titer [g/L]	Yield [g/g]	Volumetric productivity [g/L/h]	Reference
3-Hydroxypropionate	*M. extorquens*	WT, heterologous expression of gene for malonyl-CoA reductase from *Chloroflexus aurantiacus* in a butanol tolerant mutant and deletion of endogenous acryloyl-CoA reductase gene	50 mL minimal medium with 4 g L^{-1} methanol; 62 h total	0.07	–	0.001	[86]
Alcohols							
R-acetoin	*B. methanolicus*	WT, expression of heterologous genes for acetolactate Synthase and acetolactate decarboxylase from *B. subtilis*, malic enzyme from *Geobacillus stearothermophilus* and of endogenous isocitrate lyase gene	Shake flasks with minimal medium with 6.4 g L^{-1} methanol	0.42	0.07	0.017	[87]
1-Butanol	*M. extorquens*	WT, expression of heterologous genes for *Treponema denticola* trans-enoyl-CoA reductase, *Clostridium acetobutylicum* alcohol dehydrogenase, and endogenous crotonase in a 1-butanol tolerant mutant	Shake flasks, minimal medium, but instead of methanol the C2 compound ethylamine was used, 72 h total	0.03	–	0.35	[88]

Isoprenoids and polyketides							
Mevalonate	*M. extorquens*	Mutant isolated with a mevalonate biosensor	Methanol minimal medium, 400 h total	2.7	–	0.007	[76]
α-Humulene	*M. extorquens*	Heterologous expression of genes for α-humulene synthase gene from *Zingiber zerumbet*, mevalonate pathway enzymes from *Myxococcus xanthus,* and farnesyl pyrophosphate synthase from *S. cerevisiae* in a carotenoid deficient mutant	Methanol-limited fed-batch	1.7	–	0.15	[89]
4,4′-Diaponeurosporene, 4,4′-diapolycopene	*B. methanolicus*	WT with plasmid to produce *S. aureus* dehydrosqualene synthase CrtM and dehydrosqualene desaturase CrtN	Minimal medium based on seaweed extract	–	–	–	[90]
(+)-Nootkatone	*P. pastoris*	Strain CBS7435 *his4 ku70* with plasmids to produce valencene synthase from C. nootkatensis, premnaspirodiene oxygenase from H. muticus, A. thaliana cytochrome P450 reductase, endogenous alcohol dehydrogenase and truncated hydroxy-methylglutaryl-CoA reductase		0.21	–	0.002	[91]
Monacolin	*P. pastoris*	Co-culture of 2 strains: strain 1 expressing genes for lovastatin nonaketide synthase,	Complex medium with methanol	0.59	–	0.006	[92]

(continued)

Table 1 (continued)

Product	Methylotrophic production host	Strain engineering	Fermentation conditions	Titer [g/L]	Yield [g/g]	Volumetric productivity [g/L/h]	Reference
		enoyl reductase, and thioesterase from *A. terreus* and for phosphopantetheinyl transferase from *A. nidulans*, and strain 2 expressing a codon-optimized lovA and the cytochrome P450 oxidoreductase gene from *A. terreus*					
Lovastatin	*P. pastoris*	Co-culture of 2 strains, strain 1 expressing genes for lovastatin nonaketide synthase, enoyl reductase, and thioesterase from *A. terreus* and for phosphopantetheinyl transferase from *Aspergillus nidulans*, and strain 2 expressing a codon-optimized lovA and genes for cytochrome P450 oxidoreductase, acyl-transferase, and lovastatin diketide synthase from *A. terreus* and expressed *ngpA* from *A. nidulans*	Complex medium with methanol	0.25	–	0.003	[92]

6-Methylsalicylate	*P. pastoris*	Expressing expression genes for *A. nidulans* phosphopantetheinyl transferase and for *A. terreus* 6-methylsalicylic acid synthase	Steel bioreactor culture with glycerol minimal medium operated at pH 5 in glycerol fed-batch mode	2.2	–	0.1	[93]

Komagataella) [9]. In these yeasts, methanol is assimilated via AOX and the DHA pathway within the peroxisomes. During growth on methanol, this organelle can expand and occupy up to 80% of the volume of the cell. Without peroxisome, methylotrophic yeasts are unable to grow on methanol [14]. Of the methylotrophic yeasts, *Pichia pastoris* (syn. *Komagataella* spp.) and *Ogataea* (syn. *Hansenula*) *polymorpha* have been most intensely investigated for heterologous protein production [46]. *P. pastoris* has been the first yeast to be used at industrial scale, more than 40 years ago, for the commercial production of single-cell proteins (SCP) using methanol [47]. *P. pastoris* is a chassis of great interest for the protein production, thanks to its high secretory capacity and its strong methanol inducible promoters, like AOX1 promoter, that are used to express heterologous proteins [48, 49]. During protein production, mixing methanol with another carbon source as glycerol [50], sorbitol [51], or glucose [52] has been a strategy commonly used to improve the amounts of protein secreted. So far, most efforts have been focused on mixed cultures because methanol bioconversion still remains difficult. Indeed, methylotrophic yeasts such as *P. pastoris* typically shows a relatively slow specific growth rate on pure methanol ($\mu = 0.10–0.15\ h^{-1}$) [53]. Moreover, during the first step of methanol assimilation (methanol oxidation to formaldehyde), the energy is released in the form of heat and is not conserved as reducing power (NADH). Finally, ^{13}C-MFA studies revealed that at least 50% of methanol is directly dissimilated to CO_2, hence the C1 assimilation ratio is low [50, 52]. With the development of synthetic and systems biology tools (e.g., CRISPR/Cas9, genome-scale metabolic models, omics analysis), the knowledge of methanol metabolism and its regulation is improving [12, 54–57]. Traditionally, *P. pastoris* has mainly been used as host for recombinant protein production growing non-methylotrophically and with methanol solely used as an inducer of the AOX1 promoter. The use of *P. pastoris* is now expanding towards the production of value-added products from methanol as illustrated by the recent examples listed in Table 1.

2.3 Methylobacterium extorquens

Methylobacterium spp. are ubiquitously present on plant leaves of almost all plants, on decaying plant material, in polluted soils, in buds of scotch pine and in legumes [58]. One of the most intensively studied species of the genus is *Methylobacterium extorquens* and in particular strain AM1 which has been investigated as a model organism for methylotrophy for more than 50 years [59]. *M. extorquens* (recently renamed *Methylorubrum extorquens*) is a pink-pigmented Gram-negative facultative methylotrophic bacterium able to use methanol as well as succinate, oxalate, and acetate as carbon sources and can co-consume some of them [60]. Methanol is assimilated via a PQQ-dependent Mdh associated with the Serine cycle at a growth rate around 0.18 h^{-1} [61]. *M. extorquens* AM1 strain is a model organism for enzyme and pathway discovery that enabled the elucidation of the H_4MPT dissimilation pathway and of the EMCP. *M. extorquens* is a natural producer of poly-3-hydroxybutyrate (PHB), whose production from methanol has been investigated for

more than 25 years [61]. A suite of omics & genetic engineering tools has been developed [61]. In particular a fully annotated genome sequence, a genome-scale metabolic model as well as transcriptomics, proteomics, and metabolomics data are available. Furthermore, genetic tools for gene deletions and overexpression, as well as random mutagenesis and chromosomal gene integration exist [61]. All this system level knowledge and tools are now used to turn *M. extorquens* AM1 into a platform host for producing value-added products from methanol. The different compounds that *M. extorquens* can produce from methanol are listed in Table 1.

2.4 Cupriavidus necator

Methylotrophic capabilities exist in some autotrophs. Among them *Cupriavidus necator*, an aerobic facultative autotrophic bacterium, is the most widely studied due to its ability to accumulate large amount of PHB (up to 80% of intracellular content) [62, 63]. This Gram-negative soil bacterium can grow with CO_2 and H_2 as sole source of carbon and energy but also on formate and methanol [8, 64, 65]. Genomic analyses of *C. necator* revealed the presence of two putative NAD-Mdhs and two putative PQQ-Mdhs encoded, respectively, by genes *mdh1*, *mdh2*, DDK22_23240, and DDK22_31350 (gene names designated as in UniProt). In vitro analysis of the two NAD-Mdhs showed that only the *mdh2* encoded NAD–Mdh exhibited a methanol oxidation activity [66]. *C. necator* possesses the genes encoding a glutathione-dependent pathway needed to convert formaldehyde to formate. In addition, it has been demonstrated that *C. necator* could be adapted for the degradation formaldehyde into formate [67]. Finally, oxidation of formate catalyzed by a formate dehydrogenase delivers one NADH and one CO_2, which can be assimilated via the CBB cycle. While most efforts to engineer *C. necator* have focused on the bioplastics production recently, it has been shown that *C. necator* can also be used for producing short-chain alcohols and fatty acids related compounds from CO_2 or formate [8, 68]. Finally, the complete sequencing and annotation of the *C. necator* genome, as well as the development of genetic tools [69, 70] make it suitable for C1 based bioeconomy [71].

2.5 Other Methylotrophic Bacteria

Methylophilus methylotrophus and *Methylobacillus glycogenes* are two Gram-negative obligate methylotrophic and mesophilic bacterial species that have to some extent been explored as production hosts for methanol-based industrial biotechnology. Both organisms have been used as hosts for production of L-lysine with titers up to 11 g/L from methanol at 37°C by construction of recombinant strains overexpressing key genes of the aspartate pathway (Table 1). Beside of these

organisms, few other aerobic methylotrophic bacteria have been used as host for methanol-based chemicals production [72].

3 Strain Engineering of Methylotrophs

Metabolic engineering of aerobic methylotrophic production hosts has focused primarily on the Gram-negative *M. extorquens,* the Gram-positive *B. methanolicus,* and the yeast *P. pastoris.* The prevailing metabolic engineering strategy relies on extension of endogenous biosynthesis pathways. Extension of L-glutamate by a glutamate decarboxylase yielded GABA, an example typical for *B. methanolicus* engineering (Fig. 2) since there are no tools for gene deletion or disruption available for this bacterium [73]. On the other hand, pathway interruption has been applied as metabolic engineering strategy, e.g., by interception of the EMCP in *M. extorquens* via an exogenous thioesterase for production of methylsuccinate from the EMCP intermediate methylsuccinyl-CoA [74, 75]. Systems metabolic engineering addressing multiple targets such as precursor supply, redox cofactor balance, regulatory or transport engineering in addition to enabling the dedicated terminal product pathway has been reported rarely, e.g., for methanol-based production of mevalonate by *M. extorquens* [76].

Strain engineering will here be described for different product classes: secreted solutes such as amino acids, organic acids, alcohols, isoprenoids, polyketides (Table 1) as well as polymeric compounds such as PHB and proteins (Table 2). Finally, usage of biomass as non-animal protein source in the form of single-cell protein for feed or vegan purposes is briefly discussed.

3.1 Amino Acids

Wild-type strains of *M. extorquens* and *B. methanolicus* are known for the overproduction and secretion of the amino acids L-serine and L-glutamate, respectively. *M. extorquens* produces up to 55 g/L L-serine [77], the key intermediate of its eponymous formaldehyde fixation cycle. However, this required the addition of the C2 compound glycine as carbon substrate to methanol minimal medium, thus, L-serine production was not based solely on methanol as carbon substrate. Under magnesium limiting conditions, the *B. methanolicus* wild type produced up to 55 g/L L-glutamate [78] from methanol as sole carbon substrate (Table 1). A phenylalanine auxotrophic mutant of *M. glycogenes* secreted up to about 39 g/L L-glutamate [79].

The L-glutamate biosynthesis pathway of *B. methanolicus* MGA3 was extended to the ω-amino acid γ-aminobutyrate (GABA) by glutamate decarboxylase (expression of the heterologous gene from *Sulfobacillus thermosulfidooxidans* [73] (Fig. 2)). The driving force in this extended pathway leading to GABA is the decarboxylation step. Since the exogenous glutamate decarboxylase has a low pH

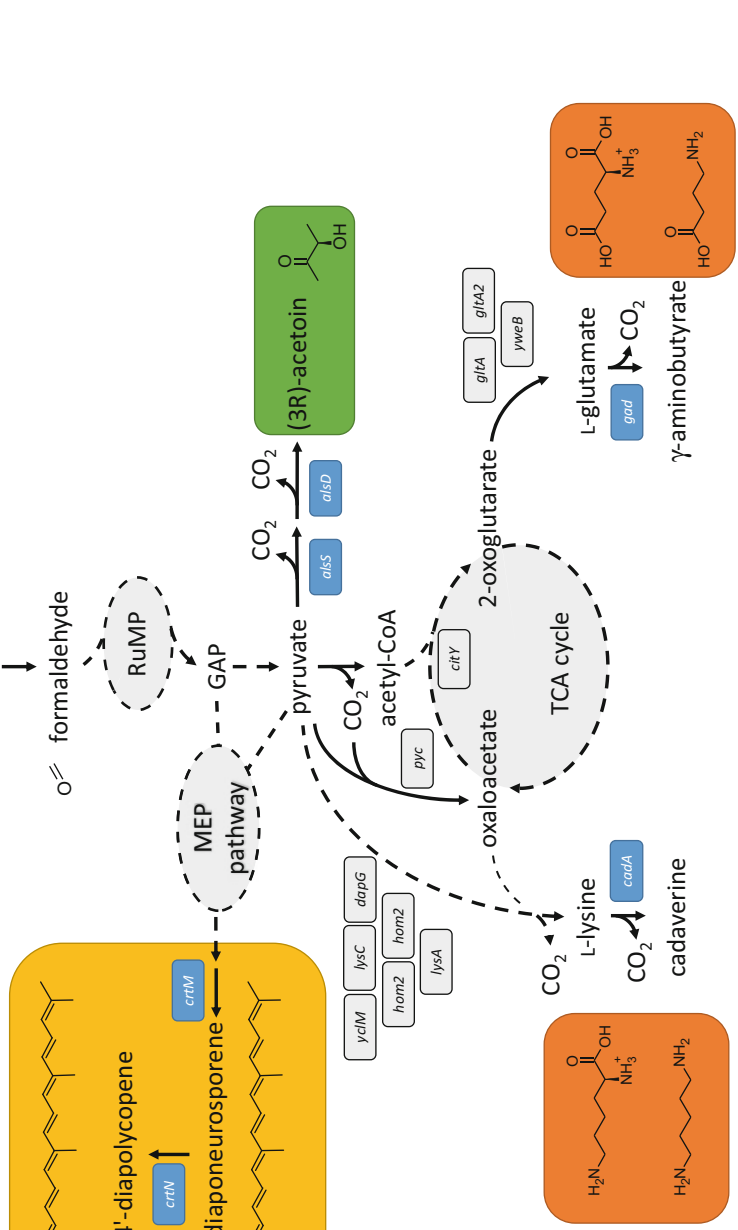


Fig. 2 Metabolic engineering of *B. methanolicus* for methanol-based production. Shading is used to highlight products: alcohols (green), isoprenoids (yellow), diamines and amino acids (orange). Endogenous genes (gray boxes) and heterologous genes (blue boxes) are shown next to the reactions they catalyze. Straight arrows represent single reactions, dashed arrows several reactions such as those in the ribulose monophosphate pathway (RuMP), the methylerythritol phosphate

Fig. 2 (continued) pathway (MEP) and the tricarboxylic acid (TCA) cycle. Endogenous genes code for pyruvate carboxylase (*pyc*), glutamate dehydrogenase (*yweB*), glutamate synthase (*gltA, gltA2*), aspartokinase (*dapG, lysC, yclM*), homoserine dehydrogenase (*hom-1, hom-2*), *meso*-diaminopimelate decarboxylase (*lysA*). Heterologous genes code for acetolactate synthase (*alsS*), acetolactate dehydrogenase (*alsD*), glutamate decarboxylase (*gad*) lysine decarboxylase (*cadA*), diapophytoene synthase (*crtM*), dehydrosqualene desaturase (*crtN*)

Table 2 Aerobic methanol-based processes of polymeric compounds

Polymeric product	Species	Conditions	Titer [g/L]	Reference
PHB-*co*-HV	*Methylocystis* sp., wild type	Methane + valerate medium	2	[103]
PHB	*M. extorquens*, wild type	Methanol-controlled (1.4 g/L) high-cell density fed-batch fermentation	53	[101]
GFP	*M. extorquens*, carrying plasmid with Gfp gene	Methanol-controlled (1.4 g/L) high-cell density fed-batch fermentation	4	[58]
Enterocin P	*M. extorquens*, carrying plasmid with enterocin P gene from *Enterococcus faecium*	Methanol-controlled (1.4 g/L) high-cell density fed-batch fermentation	0.0001	[110]
Cry1Aa	*M. extorquens* carrying plasmid with gene for insecticidal crystal protein from *Bacillus thuringiensis*	Methanol-controlled (1.4 g/L) high-cell density fed-batch fermentation	–	[111]
Proteases, α-amylases and sfGFP	*B. methanolicus* carrying plasmid with gene for proteases, α-amylases or sfGFP	Baffled flasks, minimal medium	–	[106]

optimum, reduction of the pH medium to was required for maximize GABA production, however, still more L-glutamate than GABA was secreted indicating a potential for increased GABA production in this host [73].

The amino acid L-lysine is used as feed additive. Classically obtained *B. methanolicus* mutants were isolated and shown to produce up to 65 g/L L-lysine [78]. This titer exceeds those obtained with metabolically engineered *M. methylotrophus* and *B. methanolicus* strains. Upon heterologous expression of the genes encoding a lysine-insensitive dihydrodipicolinate synthase variant from *E. coli* [94] and the lysine/arginine/citrulline exporter from *Corynebacterium glutamicum* [95, 96] 11 g/L L-lysine accumulated. The same titer was obtained when *B. methanolicus* was overexpressing endogenous aspartokinase III (encoded by *yclM*) [33]. Transport engineering using the heterologous lysine/arginine/citrulline exporter from *C. glutamicum* also improved L-lysine secretion by *B. methanolicus* [97]. Genome sequencing of classically obtained L-lysine producing *B. methanolicus* mutants, biochemical analysis of key enzymes, transcriptional analysis of their genes, and gene overexpression allowed to understand the key genetic and metabolic changes supporting high L-lysine production [32, 35, 97]. However, exploring their full potential by gene-directed mutagenesis has not yet been possible due to the lack of genome editing methods for this bacterium. A decarboxylation step was also used to extend L-lysine biosynthesis for production of the diamine cadaverine (Fig. 2), and improved expression of the lysine

decarboxylase gene *cadA* from *E. coli* using a theta-type replicating plasmid led to a cadaverine titer of about 10 g/L in fed-batch methanol fermentations [39, 98].

An L-threonine producing *M. glycogenes* mutant that reached 11 g/L as L-threonine titer was obtained by classical mutagenesis and screening of amino acid auxotrophs that were resistant to analogs of L-threonine and L-lysine [79]. A mutant isolated in parallel co-produced about 3 g/L L-lysine with about 6 g/L L-threonine, an often observed problem when screening undefined mutants.

3.2 Organic Acids

P. pastoris was used to produce a mixture of malate, fumarate, and succinate from methanol (Table 1). To this end, the endogenous pyruvate carboxylase and cytoplasmic malate dehydrogenase genes were overexpressed and the recombinant was cultured in MMYH medium (10 g/L methanol, 13 4 g/L YNB, 0.1 g/L biotin, 0.05 g/L histidine) with feeding 5 g/L methanol every 12 h [82]. Production of D-lactate was achieved by integration of the gene for D-lactate dehydrogenase gene from *Leuconostoc mesenteroides* into the non-transcribed spacer of the ribosomal DNA locus of *P. pastoris* followed by post-transformational gene amplification. The recombinant produced about 3.5 g/L of D-lactate from methanol in a 96-h test tube fermentation [83].

M. extorquens was used to produce mesaconate and (2S)-methylsuccinate. These organic acids were derived from the respective CoA esters that are intermediates of the methylotrophic EMCP pathway by thioesterase YciA from *E. coli* [75]. Upon abolishment of PHB biosynthesis via deletion of *phaA* and balancing of medium cobalt ion concentration for the two cobalt-dependent mutases in the EMCP yielded a combined mesaconate and (2S)-methylsuccinate concentration of 0.65 g/L [74]. Expression of the codon-optimized gene encoding *cis*-aconitic acid decarboxylase from *Aspergillus terreus* in wild-type *M. extorquens* led to higher itaconate production than in the PHB-negative *phaR* mutant [84]. By use of heterologous (R)-3-hydroxybutyryl coenzyme A (CoA)-specific coenzyme B12-dependent mutase from *Bacillus massiliosenegalensis* JC6, 2-hydroxyisobutyrate could be co-produced with PHB after complete nitrogen consumption [85]. Conversion of the central intermediate of fatty acid biosynthesis, malonyl-CoA, by malonyl-CoA reductase from *Chloroflexus aurantiacus* yielded 3-hydroxypropionate [86]. Improving promoter strength and copy number for heterologous gene expression in a strain evolved for higher tolerance to butanol combined with deletion of the acrylyl-CoA reductase gene for endogenous 3-hydroxypropionate catabolism finally led to a titer of 0.07 g/L [86].

3.3 Alcohols

Since 1-butanol is growth inhibiting to *M. extorquens*, a more tolerant mutant was isolated [88]. Genome sequencing revealed an SNP leading to the amino acid exchange L171R in the K^+/H^+ antiporter and introduction of the mutant gene *kefB* into the parent strain improved tolerance to 1-butanol. Upon plasmid-borne expression of the genes for *Treponema denticola* trans-enoyl-CoA reductase, *Clostridium acetobutylicum* alcohol dehydrogenase, and native crotonase, about 0.02 g/L 1-butanol was produced (Table 1) [88]. Thus, the EMCP intermediate crotonyl-CoA was the central precursor for 1-butanol biosynthesis. Importantly, 1-butanol production was dependent on the EMCP, but was not based on methanol. Instead, the process required the C2 compound ethylamine as carbon substrate [99]. This phenomenon is not understood [99], however, it has to be noted that methanol-based production of the dicarboxylic acids mesaconate and methylsuccinate was possible by deriving them via thioesterase from their respective CoA esters that are intermediates of the EMCP as crotonyl-CoA is [74, 75].

Methanol-based production of the alcohol (3*R*)-acetoin was achieved with recombinant *B. methanolicus* [87]. First, the genes for acetolactate synthase and acetolactate decarboxylase from *B. subtilis* were functionally expressed to convert two molecules of the central intermediate pyruvate to (3*R*)-acetoin and two molecules of carbon dioxide. While this conversion was driven by two decarboxylating reactions, improving anaplerotic precursor supply by plasmid-borne expression of the endogenous isocitrate lyase gene and the malic enzyme gene from *Geobacillus stearothermophilus* increased acetoin titers up to 0.42 ± 0.01 g/L (Table 1) [87].

3.4 Isoprenoids and Polyketides

The *B. methanolicus* MGA3 genome sequence indicated that this bacterium possesses a complete methylerythritol phosphate (MEP) pathway for the biosynthesis of the terpenoid precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) [4, 5]. This MEP pathway was later experimentally proven to be functional, as heterologous expression of the *ctrM* (encoding dehydrosqualene synthase) and *crtN* (encoding dehydrosqualene desaturase) genes of *Staphylococcus aureus* in MGA3 resulted in production of the C30 terpenoids 4,4-diaponeurosporene and 4,4-diapolycopene, respectively, from methanol (Fig. 2) [90].

M. extorquens was engineered from methanol-dependent mevalonate production, which is not an isoprenoid, but derives from the eponymous isoprenoid pyrophosphate biosynthesis pathway. The AraC-based transcriptional regulator was used to select for a mevalonate-responsive mutant. Fusion to a fluorescent reported allowed FACS screening of a library of mutants of QscR, the LysR-type transcriptional activator for most of the Serine cycle genes. Expression of the best selected QscR

mutant allowed to produce 2.7 g/L of mevalonate from methanol (Table 1) [76]. The production of α-humulene was enabled by systems metabolic engineering [89]. Precursor supply was optimized by introduction of a prokaryotic mevalonate pathway from *Myxococcus xanthus* and heterologous expression of farnesyl pyrophosphate (FPP) synthase gene from *Saccharomyces cerevisiae*. Moreover, endogenous carotenogenesis was abolished by using a carotenoid synthesis deficient mutant host strain. Upon expression of the heterologous α-humulene synthase gene from *Zingiber zerumbet* about 1.7 g/L α-humulene was produced in fed-batch cultivation (Table 1) [89].

P. pastoris was engineered to express the heterologous genes for valencene synthase from *Callitropsis nootkatensis*, premnaspirodiene oxygenase from *Hyoscyamus muticus,* and the *Arabidopsis thaliana* cytochrome P450 reductase for conversion of endogenous FPP to (+)-nootkatone [91]. Additional overexpression of endogenous genes encoding alcohol dehydrogenase and truncated hydroxy-methylglutaryl-CoA reductase allowed achieving a titer of 0.2 g/L (+)-nootkatone (Table 1) [91]. Upon heterologous expression of *Aspergillus nidulans* phosphopantetheinyl transferase gene *npgA* and *Aspergillus terreus* 6-methylsalicylic acid synthase gene *atX*, *P. pastoris* produced 2.2 g/L 6-methylsalicylate [93]. Co-cultures of two *P. pastoris* strains were developed for production of the polyketides monacolin (0.59 g/L) and lovastatin (0.25 g/L) in order to avoid accumulation of intermediates and metabolic stress [92]. One strain expressed lovastatin nonaketide synthase gene *lovB*, enoyl reductase gene *lovC*, and thioesterase gene *lovG* from *A. terreus* in combination with phosphopantetheinyl transferase gene *npgA* from *Aspergillus nidulans* to produce the intermediate dihydromonacolin L acid [92]. The second strain used in the coculture differed. For monacolin production, the second strain expressed a codon-optimized *lovA* and the cytochrome P450 oxidoreductase gene from *A. terreus*. For lovastatin production, the second strain in addition expressed *ngpA* from *A. nidulans*, and acyl-transferase gene *lovD* and lovastatin diketide synthase gene *lovF* from *A. terreus* (Table 1).

3.5 Polyhydroxybutyrate and Heterologous Proteins

Polyhydroxybutyrate (PHB) is the natural carbon storage compound of *M. extorquens*. In batch cultivation, the wild-type strain accumulated PHB to about 63% of the cell dry weight, i.e., to a concentration of 9.5 g/L PHB (Table 2) [100]. In a fed-batch fermentation of the wild-type strain in which the methanol concentration was kept at 1.4 g/L grew to biomass levels concentrations between 100 and 115 g/L with a PHB content between 40% and 46% PHB (Table 2) [101]. Upon cumate-inducible overexpression of the *phaC1* or the *phaC2* genes from *P. fluorescens* GK13 the recombinant *M. extorquens* strain accumulated PHA with some C-C double bonds [102]. Co-feeding of methanol with, e.g., valerate, as shown for PHA production by *Methylocystis* sp. with methane + valerate containing minimal medium [103], has not yet been reported.

The potential of *B. methanolicus* MGA3 as a host also for heterologous protein production has been briefly explored recent years as a consequence of the development of functional expression systems for this organism [104]. Functional recombinant expression of green fluorescent protein (GFPuv) by using the constitutive methanol dehydrogenase promoter *mdhP* was demonstrated (Table 2) [105]. Later, inducible expression systems and signal sequences for protein secretion were established for this organism, and these tools were demonstrated useful for functional expression and secretion of some heterologous α-amylases and proteases, as well as the thermostable superfolder green fluorescent protein (sfGFP) from methanol at 50°C (Table 2) [106]. None of these recombinant strains have been tested under high-cell density cultivations and whether the production levels make *B. methanolicus* an interesting host for heterologous protein production still remains unknown.

3.6 Single-Cell Protein

Biomass from methylotrophs is protein-rich (about half of the cell dry weight of an aerobic bacterium is protein) and, thus, it may be used as an alternative protein source in the feed and food industries. In animal feed it can replace fishmeal or soybean meal. Moreover, a strong market pull exists for vegan or hybrid meat, vegan dairy alternatives. The latter development gave new momentum to the story of single-cell protein from the 1970s and 1980s. As compared to soybean production, microbial protein has a much lower water demand [107]. Microbial protein is on the market already at the million-ton-scale if yeasts are considered. More than three million tons dried yeast are marketed for about 10 billion € to cater for the bakers' and brewers' needs [108] and extracts of spent brewer's yeast are used to prepare sandwich spreads (e.g., Marmite) [109]. Microbial protein from *M. extorquens* is marketed as protein source for aquaculture applications [109]. It is conceivable to make use of the cell pellet and the secreted product of a methanol-based fermentation: a vegan protein source obtained by filtration or centrifugation plus a secreted product is isolated from the culture medium. Alternatively, production of enriched microbial protein, e.g., containing carotenoids or PUFA is thinkable. As compared to animal- and plant-based food as well as to yeasts, microbial protein foods may remain niche applications.

4 Advantages and Challenges of Methanol Fermentations

From the bioprocess development point of view, using methanol as the principal source of carbon is related to some advantages but also several specific challenges, compared to standard glucose/carbohydrate-based fermentation processes. An important advantage of methanol is that it is a liquid, water-miscible, and easily transported commodity feedstock without significant fluctuations in production

batch quality. In addition, due to its properties the risk of contaminations, even in prolonged fed-batch or continuous fermentation modes, is smaller than for glucose fermentation processes. Methanol is also more chemically reduced than glucose which in principle provides more energy/reducing power for biomass growth and product biosynthesis. However, as a direct consequence, a much higher oxygen demand is expected as methanol fermentations are always conducted under fully aerobic conditions. This affects process economics due to a) high power input to enable adequate gas–liquid oxygen transfer rates, b) the need to remove the extra heat generated by the microbial metabolism, and c) suboptimal product yields due to wasteful dissipation of the generated reducing power, in the form of NADH or $PQQH_2$, from product biosynthesis to oxygen-dependent respiration. Another specific challenge is that, as the first step in assimilation into cellular metabolic pathways, methanol is converted to formaldehyde (see above), a highly reactive and cell-toxic intermediate. Therefore, methanol concentrations in the bioreactor must be tightly controlled through continuous substrate feeding and even so, methanol/formaldehyde spikes are hard to avoid and careful bioreactor design is required in large scale to prevent local growth inhibition and excess carbon dissimilation to CO_2 [8, 112].

4.1 Growth Media Composition

Methanol fermentations are in general characterized by economically and process favorable media compositions. Many aerobic methylotrophs, especially those reliant on the RuMP assimilation pathway, exhibit high growth rates ($>$0.4/h) on chemically defined media containing methanol as the main carbon source. Nitrogen can often also be supplied in the form of ammonium salts which are available as low-cost commodity chemicals. This is a big advantage as the absence of structurally more complex and insoluble media ingredients is reflected in lower cost. In addition, chemically defined media in general assure higher process reproducibility and more straightforward and cost-effective downstream/purification procedures for simple biochemicals, such as amino acids or organic acids [8, 113]. However, careful optimization of media composition is necessary when introducing a new methylotrophic strain into bioreactor scale fermentation to assure adequate C/N ratio and sufficient supply of other key elements, such as phosphorus, magnesium, sulfur, and microelements. Potential auxotrophies, e.g., deficiencies in vitamin and/or amino acid biosynthesis, must be considered and the corresponding compounds added to production media composition, particularly in the cases where producing strains were generated using random mutagenesis and selection approaches. Importantly, shake flask experiments are not always representative of the nutrient requirements in a fed-batch process in a bioreactor where extremely high growth rates and cell densities are desired. In some cases, such as in the case of glutamate production by *M. glycogenes*, small amounts of yeast extract or casamino acids can be added to further boost growth and product biosynthesis [79]. Similarly,

growth media for *B. methanolicus* must be supplied with biotin and vitamin B12 [114]. In addition to optimizing media composition, cellular pathways for assimilation of key nutrients are sometimes optimized, such as in the early example of introduction of glutamate dehydrogenase gene for more energy efficient assimilation of ammonia in *M. methylophilus*, in order to enable higher methanol to biomass conversion ratios [115].

One of the most important aspects in media composition is to determine optimal methanol concentration for the particular microorganism. Due to the inhibitory effect of methanol, specific growth rates in general decrease with increasing methanol concentrations. In addition, higher methanol concentration can lead to increased carbon loss through dissimilation mechanisms [112]. For the genus *Methylobacterium* the growth was found to be completely inhibited above 6% (v/v) of methanol while optimal growth rate was achieved at 0.5% [116]. With some variability among different methylotrophs the optimal concentration of methanol which enables fastest cell growth is between 0.5% and 2% and concentrations above 4% are avoided [8].

4.2 Basic Bioprocess Design and Setup

Due to the relatively low initial methanol concentrations which can be incorporated into the fermentation medium, compared to when using glucose or starch, batch methanol fermentations have limited applicability beyond initial trials during bioprocess setup. Fed-batch or continuous cultivation modes are therefore used to enable fastest biomass growth and highest product yields and minimize toxicity-related effects [8, 117]. The basic strategy for bioprocess control requires careful monitoring of both methanol and dissolved oxygen concentration so that enough carbon source for biomass growth is available while at the same time maintaining sufficiently high dissolved oxygen (usually at least 25% saturation). In order to balance these two constraints, methanol concentrations in fed-batch fermentations are allowed to drop significantly from initial concentrations before methanol feeding is initiated and its concentration kept constant throughout the duration of the bioprocess. In laboratory scale fermentations of *M. extorquens* methanol concentration is maintained at relatively low levels, from approx. 0.5 to 1.4 g/L however, concentrations of 10 g/L (1%) have also been used [58, 74, 101, 112, 118]. Similarly, in the case of *M. methylotrophus* fermentation for production of L-lysine an initial 2% methanol concentration was lowered to 0.2% during the bioprocess [81]. In *B. methanolicus,* methanol concentrations are usually maintained between 2.4 and 4.8 g/L [32, 33, 73, 98]. Similarly, methanol concentration in *M. glycogenes*, producing L-threonine and L-lysine was maintained at 0.5% in laboratory scale (5 L) fermentation [79]. A two-phase approach is also possible to assure adequate oxygen supply: in the first phase of the fermentation, methanol concentration is controlled by varying the methanol feed rate while the dissolved oxygen level is regulated by adjusting the agitation rate. Once the maximal oxygen transfer rate of

the fermenter is reached, methanol concentration is no longer maintained at a certain concentration; instead methanol feeding rate is adjusted according to the availability of dissolved oxygen, representing a classical carbon-limited process [101].

Another important aspect of general bioprocess design is to balance biomass growth and product biosynthesis and accumulation. For example, bioprocesses by *Methylobacterium* strains for production of PHB, a product accumulated inside the cells, are usually separated in two stages. An initial biomass-accumulation step is followed by PHB-accumulation phase, which is induced by imposing nitrogen limitation on the culture. With this approach, PHB concentrations of up to 130 g/L were obtained. PHB can be accumulated up to 60% of total biomass [113, 119, 120]. Tightly controlled low ammonium ion concentration (0.15 g/L) was also found to have a positive effect on production of the exopolysaccharide methylan in *Methylobacterium organophilum* [121].

4.3 Parameters and Approaches for Bioprocess Control

One of the most important aspects of bioprocess control is the requirement to carefully control methanol concentrations in the desired range throughout the bioprocess. As an example of how sensitive these cultures can be to spikes in methanol concentrations, a 0.04% increase in concentration caused a wash-out of a thermotolerant methylotroph methanol-limited culture growing under steady state conditions in a chemostat due to formaldehyde accumulation [122]. Therefore, several strategies have been used in the past to monitor and tightly control methanol concentrations. The approaches are sometimes based on at-line measurements of methanol content by gas chromatography however, several possibilities for online monitoring have been developed [123]. One possibility is to transfer headspace gas to a mass spectrometer for on-line monitoring of methanol content [33]. Flame ionization detectors or hydrocarbon sensors can also be used for monitoring off-gas composition [113, 114]. On the other hand, several simple methanol sensors have been developed, based on electrochemical principles and mid-infrared IR spectroscopy [123–125]. As a possible complementary solution to high sensitivity of the producing cells to changes in methanol concentration, methanol metabolism can be engineered to provide higher tolerance and consumption rates. This has been achieved by increasing the copy numbers of *hps* and *phi* genes in the facultative methylotroph *B. methanolicus* [126].

Another aspect of methanol fermentation processes is their very high demand for oxygen, originating in the highly reduced nature of methanol and the need to carry out the cultivation in fully aerobic conditions. In order to satisfy the high demand for oxygen, mixtures of compressed air and pure oxygen are often used for aeration in bioreactors in order to provide a strong electron sink and prevent growth inhibition at high-cell densities. Another option to avoid oxygen limitation during bacterial growth at high-cell densities is to carry out fermentations at high pressure to increase oxygen solubility [113]. In addition to high energy consumption, the high

consumption of oxygen during methanol fermentations is reflected in high amounts of generated heat and therefore the requirement for intense cooling of the bioreactor, which represents a significant constraint from the process economics point of view. Fermentations using the thermophilic *B. methanolicus* with optimal growth temperature at approx. 50°C are therefore an attractive alternative to mesophilic methylotrophs. For a 50°C fermentation, the cooling requirements may be reduced by 18–40% of the cooling requirements of a 30°C process. However, increasing the temperature further decreases oxygen solubility and higher pressures may therefore be required to take full advantage of thermophilic methylotrophs. Notably, some thermophilic methylotrophs may be fastidious to grow in the lab and may show non-robust behavior in the bioreactor setting [122].

As with every fermentation process, in order to achieve commercially relevant efficiency, the microbes must grow rapidly and be capable of reaching and producing the desired product at high-cell density. Several methylotrophs are able to grow on methanol at comparable rates to classical glucose-based fermentations and relatively quick process times have been achieved. RuMP pathway strains (see above) seem to enable highest growth rates among methylotrophs [8, 112]. Bioreactor scale fermentations with Serine cycle *M. extorquens* have achieved extremely high-cell densities of more than 100 g/L dry cell biomass (approx. half of that mass represented by the intracellular product PHB) in approx. 70 h bioprocess time and the processes were extended up to 186 h in lab scale fermentations [101]. RuMP-pathway *B. methanolicus* fermentations are even faster and achieved up to 66 g/L dry cell biomass in approx. 48 h fermentations [98], comparable to industrial *B. subtilis* fermentations.

4.4 Scale-Up

A very important economic advantage of methanol fermentations, compared to methane or syngas processes, is that methanol fermentations don't require any special fermenter type or peripheral equipment and existing fermentation infrastructure could be used for scale-up to industrial levels [113]. However, the key constraints of methanol fermentations, high oxygen demand, substrate toxicity, and high demand for cooling also have to be taken into account during fermentation scale-up. On industrial scale, methanol fermentations have so far been used only for production of single-cell proteins (SCPs) in the 1970s and 1980s. To a great extent this was due to the limited understanding of the metabolism of methylotrophic bacteria at the time and due to a lack of tools for their genetic modification [113]. Even though SCP technologies were largely abandoned in the 1980s due to increasing prices of fossil fuels and availability of cheaper soy proteins, the extensive research done at the time on developing and scaling up methanol fermentations is of great value today when the prospect of bio-methanol-based, carbon-negative production of chemicals is gaining increased attention.

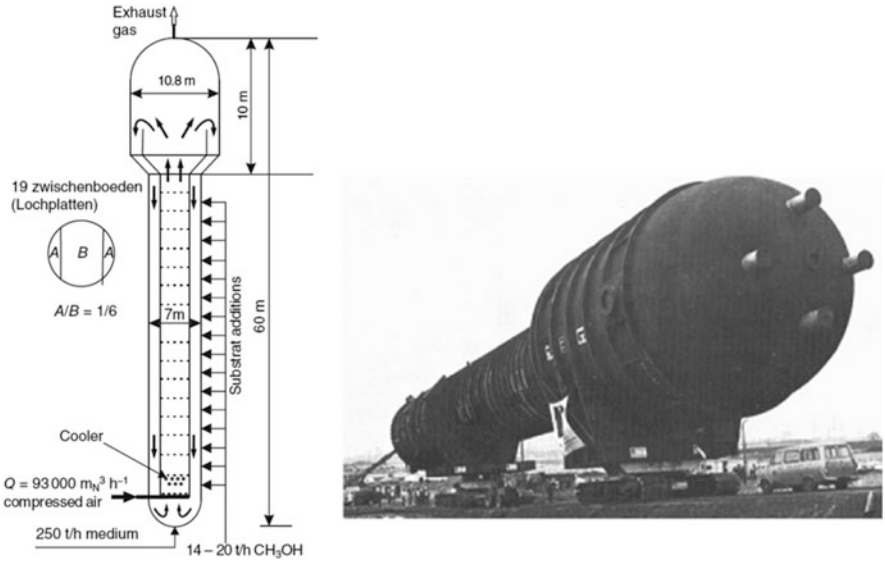


Fig. 3 Airlift reactor at the ICI factory, Billingham, UK. Originally published by Roca et al. (1995) [130]

Four industrial SCP technologies were scaled up and used commercially at the time. The best-known example is the ICI process cultivation of *M. methylotrophus*. In line with the advances of bioreactor technology of that period the fermentation was performed in a pressure airlift reactor with a working volume of 1,500 m^3 (3,000 m^3 total volume; Fig. 3) and was capable of producing up to 50,000 tons of SCP product per year. After initial infection and foaming problems continuous fermentation was successfully set up and runs of over 100 days were possible without any occurrence of contamination [127]. When using such large bioreactors, in which the culture is more difficult to mix, local high concentrations of methanol may occur, which can hamper cell growth and production. Appropriate feeding of the toxic substrate and sufficient mixing were therefore essential. ICI solved this problem by using a system of 3,000 methanol outlets in the reactor and oxygen transfer was improved by an oxygen pressure of 3 bar in the reactor head [8, 113]. The downstream process consisted of initial concentration of the bacteria by flocculation, followed by centrifugation and passing to a pneumatic hot air drier. The product had the ICI trade name Pruteen [127].

A similar example of industrial development was the commercial process for SCP production using *Methylomonas clara*, developed by Hoechst/Uhde. This process used two 20 m^3 reactors and had an annual production capacity of up to 1,000 tons of the product called Probion [127]. Bioprocess parameters such as temperature, pH, aeration rate, dilution rate and substrate concentration were controlled automatically. Notably, methanol concentration was controlled at a very low level of 0.005%.

Similarly, Norsk-Hydro established an industrial process using *Methylomonas methanolica* for SCP production in a 45 m^3 capacity. Up to 80% of the spent medium was recycled after filtration without any negative effects on the process. The fermentation process was operated at a pressure of 4 bar. A pilot plant equipped with an airlift fermentor (20 m^3) was also constructed by Mitsubishi Gas Chemical Company (MGC) in order to produce SCPs using methylotrophic bacteria in 1974 [128, 129].

5 Outlook on Technological and Market Developments

The use of aerobic methylotrophs for biotechnological process has gained momentum in recent years. The field has benefitted from synthetic biology in many ways, but notably, also with regard to transferring the trait of methylotrophy to non-methylotrophs, which is covered in other chapters. The idea of pathway transfer with methylotrophs as donors may also be viewed from the perspective of transferring thermophilic pathways to mesophiles or for in vitro biotransformation [130] in case the donor is a thermophilic methylotroph. Another application is the integration of methanol metabolism with the *S*-adenosylmethionine (SAM)-dependent methyltransferases system in *Escherichia coli* to create chassis strains for the production of a variety of methylated compounds [131]. The application examples are manifold, however, in the following subsections we will focus on providing an outlook on developments for aerobic methanol-based bioprocesses that we expect to be driven by market pull and technology push.

5.1 CRISPR Tools for Methylotrophic Strain Engineering

As discussed here several different native methylotrophic bacterial species are currently explored as industrial cell factories via strain engineering approaches. For some of them, insufficient genetic tools are still one bottleneck for advanced metabolic engineering and in particular methods for genome editing are lacking. Recently, CRISPR interference (CRISPRi) was established as a functional tool in *B. methanolicus* [43]. Although this technique cannot be used to generate stable strains, it will serve as a valuable basis for the functional characterization of *B. methanolicus* physiology. In methylotrophic yeast *P. pastoris*, a CRISPR/Cas9 based genome editing technique for gene insertions, deletions, and replacements has been established [132], and this method was recently successfully used to re-engineer this organism into an autotroph capable of growth on CO_2 [133]. To the best of our knowledge, CRISPR-based tools are still lacking in the well-studied *M. extorquens* although some progress is being made [134], and the same holds for other methylotrophic bacteria used for strain engineering. Thus, the efforts on expanding and using the genetic toolboxes for native methylotrophs on the one

hand and progress in synthetic methylotrophy on the other hand will likely continue with the same overall long-reaching goal to develop strains for efficient conversion of methanol into value-added products.

5.2 *Adaptive Laboratory Evolution to Improve Methylotrophic Producing Strains*

Adaptive Laboratory Evolution (ALE) allows to leverage natural selection to optimize a target property of a production strain without the requirement of a priori knowledge of the genetic background [135, 136]. ALE is based on a growth advantage being positively correlated with the strain's property targeted for improvement. ALE applications to increase tolerance against a target compound are straightforward: faster growing strains are selected in the presence of increasing concentrations of the target compound. With respect to methanol, ALE has been used to select for *M. extorquens* strains with tolerance against the targeted product 1-butanol [86], to select for *C. glutamicum* strains more tolerant to methanol as co-substrate [137, 138] and to select for synthetic methylotrophs (see other chapters). Whole genome sequencing revealed an SNP mutation leading to amino acid exchange L171R in the potassium ion/proton antiporter KefB in the former case, and reduced formation of the methionine-analogue *O*-methyl-homoserine in the methanethiol pathway due to amino acid exchange S288K in *S*-adenosylmethionine synthetase MetK [137]. This approach has also been applied to select for faster growing strains under standard conditions [139], at elevated temperatures [140] or to improve consumption rates of growth substrates [137]. ALE can be coupled to flux enforcement, a metabolic engineering strategy coupling activity of a product pathway to a reaction required for growth. Thus, faster growing and by flux enforcement faster producing mutants can be selected by ALE. This has recently been applied to glutarate production by *C. glutamicum*. In this bacterium, the lysine pathway was extended to glutarate by oxidoreductase and transaminase reactions [141]. Flux was enforced by deletion of glutamate dehydrogenase gene *gdh*, thus, only the 2-oxogluatarate-dependent transaminase reactions in the glutarate biosynthesis pathway provide glutamate for growth [141, 142]. Conversion of lysine to glutarate employing only one transaminase reaction as coupling site for flux enforcement via *gdh* deletion was superior to coupling to two transaminase reactions [143]. Indeed, ALE mutants growing faster also produced more glutarate and whole genome sequencing followed by reverse genetics experiments allowed to unravel causal mutations and to gain mechanistic understanding (Prell et al. 2021). ALE can also be used to combine with biosensors as shown for methanol-based mevalonate production. An AraC-based transcriptional regulator was used to select for a mevalonate-responsive mutant, coupled to control a fluorescence reporter gene, and subsequently, a library of mutants of the transcriptional activator of most of the Serine cycle genes QscR was screened for enhanced mevalonate production [76]. Clearly, it can be forecasted that the various versions and combinations of ALE

experiments will accelerate strain development for methanol-based production of target compounds. Follow-up experiments such as whole genome sequencing, reverse genetics and physiology experiments will provide a mechanistic understanding that can be transferred to stable metabolic engineering strategies.

5.3 *Synthetic Microbial Consortia for Process Intensification*

In nature, methylotrophs often occur in microbial consortia such as in the phyllosphere [144]. This habitat is dominated by plant leaves and microbial breakdown of plant polymers such as pectin gives rise to methanol, the feedstock for the methylotrophs. The study of methylotrophic microbial consortia and their synthetic design is highly relevant to further our understanding of this ecological niche [145, 146]. Synthetic microbial consortia are also relevant for biotechnological applications [147], e.g., in biorefineries when feedstock supply and composition vary seasonally [148]. By making use of engineered interdependencies, these synthetic microbial consortia are designed to reduce the heterogeneities typically observed in natural consortia and they are adjusted to match the discrete process steps of substrate conversion and/or product formation to the current demand. For example, division of labor between substrate conversion (starch hydrolysis or chitin) and L-lysine production was achieved with *E. coli/C. glutamicum* consortia [149, 150]. The feedstock methanol, a major contamination in crude glycerol obtained from biodiesel production, supported growth and PHB production of a spontaneously obtained, uncharacterized microbial consortium, while PHB was not produced from crude glycerol itself [151]. The design, characterization, and sue of a synthetic consortium for conversion of methanol as substrate by a methylotroph to an intermediate that is used by a second microbe for growth and production of a value-added compound have not yet been reported.

A second valuable application of synthetic consortia in biotechnology are those designed to subdivide complex biosynthetic routes [147]. This pathway modularization approach lowers the metabolic burden on the single microorganism. Linear binary, ternary, and even quaternary designs as well as convergent design for pathways with up to 15 reactions have been applied [152]. The design of the synthetic consortia may be guided by how that pathway evolved in the donor host (e.g., a primary metabolite is converted in secondary metabolism to an aglycon, which, subsequently, is glycosylated, acetylated, halogenated and/or), by the availability of transport proteins for exchange of pathway intermediates, by distinct cofactor requirements of subpathways, or by tolerance regarding intermediates or co-products. The first example of methanol-based production of a consortium of two *P. pastoris* strains has been described for production of monacolin/lovastatin [92]. The linear binary consortium consisted of one *P. pastoris* strain producing dihydromonacolin L from methanol and a second *P. pastoris* strain converting dihydromonacolin L into lovastatin [92]. Likely, more applications of subdividing complex biosynthetic routes in consortia to different methylotrophs and/or to non-methylotrophs will be developed for methanol-based bioprocesses.

5.4 One Methylotrophic Production Host Yielding Two or More Products

Biorefineries have to convert feedstocks to a multitude of products and have to be adapted to varying incoming substrates and to varying market demand for products. There can be separate lines of production for specific products or for specific feedstuffs. However, the concept may also make use of microorganisms that can co-consume several feedstuffs, e.g. hexose and pentose sugars in biomass hydrolysates. Potentially, bioprocesses leading to two or more products may be interesting if downstream processing is efficient for all products. In this sense, *C. glutamicum* strains were developed to co-produce a carotenoid with an amino acid [153]. Since the carotenoid is cell-bound, whereas the amino acid is secreted, separation and purification of both products streams (cell pellet and culture supernatant) can be laid out efficiently [153]. The concept of co-production of two or more products may be relevant for methanol-based process since methylotrophic metabolism is special and may be exploited for biotechnology. In that sense, the organic acids mesaconate and methylsuccinate were co-produced by engineered *M. extorquens* since these compounds can be derived from the EMCP [74, 75], albeit downstream processing is not straightforward as both acids are secreted. By contrast, a recombinant *M. extorquens* strain co-produced cell-bound PHB with secreted hydroxyisobutyrate from methanol under nitrogen starvation conditions, thus, this process is compatible with efficient recovery of both products [85]. Another strategy could be to engineer methylotrophic hosts for co-production of single-cell protein (see Sect. 3.6), retained intracellularly, with heterologous expression of secreted enzymes. Genetic tools for recombinant expression and secretion of heterologous proteins are developed for several different some methylotrophic bacteria and yeast, which should open for such approaches in the future. Nevertheless, the concept of co-production is limited by resource allocation, i.e., a trade-off between the fraction of the substrate methanol converted either to one product or to two. Therefore, applications of this concept have to be met by the respective market demand.

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