Microbial cell factories for the sustainable manufacturing of B vitamins
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Vitamins are essential compounds in human and animal diets. Their demand is increasing globally in food, feed, cosmetics, chemical and pharmaceutical industries. Most current production methods are unsustainable because they use non-renewable sources and often generate hazardous waste. Many microorganisms produce vitamins naturally, but their corresponding metabolic pathways are tightly regulated since vitamins are needed only in catalytic amounts. Metabolic engineering is accelerating the development of microbial cell factories for vitamins that could compete with chemical methods that have been optimized over decades, but scientific hurdles remain. Additional technological and regulatory issues need to be overcome for innovative bioprocesses to reach the market. Here, we review the current state of development and challenges for fermentative processes for the B vitamin group.

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Introduction
Vitamins are essential organic compounds functioning mainly as coenzymes of metabolic reactions in all organisms. The 13 vitamins are classified as fat-soluble (A, D, E, K) or water-soluble (C, B group). Plants and microorganisms naturally produce vitamins, while humans and animals need to consume them [1⁴]. Vitamin demand is increased by pathological conditions, malnutrition, poor diet, high exercise, pregnancy, stress and drug abuse [1⁴].

Moreover, food processing and preservation methods can decrease vitamin content, as vitamins are sensitive to light, heat, oxygen and pH [1⁴]. Consequently, vitamins are produced for the feed, food, cosmetics, chemical and pharmaceutical industry.

Vitamins are currently produced by chemical synthesis and/or biotechnology [1⁴]. Organic synthesis often employs non-renewable chemicals and produces hazardous waste. Besides concerns of sustainability, economics has been the major driving factor for the development of bioprocesses during the last decades. Vitamin B₂ is a remarkable example. Its organic synthesis employs toxic compounds [2⁵], yet iterative rounds of mutagenesis and screening of Ashbya gossypii and Bacillus subtilis strains resulted in significant vitamin production. The global market volume of fermented vitamin increased from 5 to 75% from 1999 to 2012 [3⁶]. Overall, the bioprocess reduced the environmental footprint and production costs by 43% [4⁷].

Bioprocesses can have a positive impact on both the economics and the environment, but the question is whether other vitamins will follow. Thanks to an improved understanding of the vitamin biosynthetic steps and regulation (mainly in microbes), and novel metabolic engineering tools [5⁸], it should be possible to engineer high-yielding vitamin-producing strains. Additionally, high yield and purity vitamin purification from the fermentation broth must be achieved at an industrially acceptable cost. Here, we review how metabolic engineering challenges could be addressed for all B vitamins, and briefly discuss the importance of technical, environmental and regulatory issues. Only peer-reviewed work is considered. We do not cover B vitamin history, discovery, sources, detailed chemical synthesis, physiological role or deficiency [1⁴,2⁵].

Current biotechnological status on B vitamins
The global B vitamin market is large and growing by >4% annually.³ Market size and cost depends on the vitamin and its application. By volume, the largest consumption is in feed, followed by the food, pharmaceutical and chemical industries, with most vitamins currently being manufactured by chemical synthesis. B vitamin fermentations
Table 1

| Vitamin | Coenzyme or cofactor | Main commercial form | Chemical synthesis | Fermentation |
|---------|----------------------|----------------------|-------------------|-------------|
|         | Name                 | WS\(^a\) | Application        | Volume\(^b\) | Price\(^c\) | CMP\(^d\) Steps | Ref. | Status | Main challenges |
| B\(_1\) | TPP (thiamine pyrophosphate) | Thiamine hydrochloride | 100 | Feed, supplements, pharma | Medium | Medium | C | ~3 | [2**] | Very early | Low cost of chemical process. Complex pathway and enzyme bottleneck (ThiC) |
| B\(_2\) | FMN (flavin mononucleotide), FAD (flavin adenine dinucleotide) | Riboflavin | 0.05 | Feed, supplement, pigment | Medium | Medium | F | 7 | [2**] | Commercial | Phosphorylation of precursors, product reactivity and export |
| B\(_3\) | NAD\(^+\) (nicotinamide adenine dinucleotide), NADP\(^+\) (nicotinamide adenine dinucleotide phosphate) | Nicotinic acid (niacin) | 18 | Feed, supplement, pharma | Large | Low | C/B | 2 | [6,7] | Very early | Very low cost of chemical and biocatalytic process |
| B\(_5\) | Coenzyme A | Calcium D(+)-pantothenate | 350 | Feed, supplements, cosmetics | Medium-large | Medium | C/B | 5 | [2**] | Advanced | Limited cofactor supply (Met-THF) and enzyme inhibition (PanB) |
| B\(_6\) | Pyridoxal phosphate | Pyridoxine hydrochloride | 200 | Feed, food, cosmetics | Medium | Medium | C | 3 or 5 | [2**] | Early | Enzyme bottleneck (PdxJ), accumulation of toxic intermediates and product export |
| B\(_7\) | Biocytin | Biotin | 0.22 | Feed, supplements, cosmetics | Small | High | C | >10 | [2**] | Early | High cofactor demand (SAM) and enzyme bottleneck (BioB) |
| B\(_9\) | THF (tetrahydrofolic acid) | Folic acid | 1.6 | Feed, supplements, pharma | Medium | Low-medium | C | 1 | [8] | Very early | Low cost of chemical process |
| B\(_{12}\) | | Adenosylcobalamin | 1.25 | Feed, supplements, pharma | Small | High | F | ~70 | [9] | Commercial | Enzyme inhibition (HemA, HemE, CysG, CobA), riboswitches (cysG, cbi), long fermentation cycles, expensive media and lack of tools for improving current production hosts |

\(^{a}\) WS: Water solubility (approximate) in mg/mL at room temperature (20–25°C).
\(^{b}\) A low, medium and large volume is about <1000, 1000–20 000 and >20 000 tons per year, respectively. (Data are based on our own estimations.)
\(^{c}\) A low, medium and large cost is about <10, 10–200 and >200 USD per kg, respectively. (Data are based on our own estimations.)
\(^{d}\) CMP: current manufacturing process: Chemical organic synthesis (C), fermentation (F), reactions involving enzymes and/or whole cell biocatalysts (B).
Biosynthesis pathways of B vitamins. (a) Thiamine biosynthesis pathway in *E. coli*. Gray dotted lines show *Saccharomyces cerevisiae* pathway (THI5), *Bacillus subtilis* pathway (thiO), or poorly understood steps (TMP phosphatase). TMP (thiamine monophosphate); TPP (thiamine pyrophosphate). (b) Purine metabolism. (c) Thiamine Vitamin B1 complex. (d) O-Enolase-phosphate. (e) Pyruvate + glyceraldehyde-3P. (f) Panthothenate Vitamin B5. (g) PEP + E4P. (h) Glutamate. (i) Glycine + succinyl-CoA.
should address different challenges to reach commercial status (Table 1).

**Vitamin B$_1$**

Thiamine pyrophosphate (TPP), an essential cofactor in carbohydrate and amino acid metabolism [2**,3**], is synthesized via thiamine monophosphate (TMP) by separate construction of the thiazole (THZ-P) and pyrimidine (HMP-P) heterocycles, followed by their coupling (Figure 1a) [10,11]. Distinct routes exist. In bacteria, THZ-P is synthesized by >5 steps from 1-deoxy-D-xylulose-5-phosphate (DXP), cysteine and tyrosine or glycine, while in yeast [12] and archaea [13] the thiazole ring is synthesized by Thi4 from NAD and glycine. HMP-P is generated from 5-aminoimidazole ribotide by ThiC in bacteria/plants and from PLP/histidine by Thi5 in yeast. Thiamine biosynthesis is heavily regulated by TPP riboswitches in bacteria/eukaryotes, and a transcriptional repressor in archaea [14].

Microbial fermentations have met limited success [15]. Before the pathway was fully elucidated, *B. subtilis* production strains with mutations in three genes were reported [16] (Table 2). Knocking out thiN increases thiamine production over TMP/TPP. Recently described TMP phosphatases [17] could further increase the dephosphorylated product. *Aspergillus oryzae* was engineered to produce 4-fold intracellular TPP by overexpressing three genes [18] (Table 2).

A riboswitch-based biosensor enabled the discovery [19] and engineering [20] of thiamine transporters, and an increased thiamine production in *E. coli* overexpressing thiFSGHCE and thiD or thiM combined with transposon mutagenesis [21]. This identified genes involved in iron/sulfur metabolism, consistent with the fact that ThiC/ThiH are Fe–S cluster-dependent enzymes catalyzing complex rearrangements. These enzymes are inhibited by S-adenosyl-methionine (SAM) metabolites and represent potential bottlenecks, with ThiC having very low activity (k$_{\text{cat}}$ = 0.14 min$^{-1}$) [22,23**,24]. Another hurdle is the branched nature of the pathway that requires balanced expression of many genes. Since thiamine fermentation will require high yields due to its low cost, major metabolic engineering breakthroughs will be needed.

**Vitamin B$_2$**

By 2018, riboflavin is exclusively produced by fermentation using *A. gossypii* and *B. subtilis* (Table 2). A comprehensive compilation of production strains is available [3*] as well as systems biology data for *B. subtilis* [25**].

The bacterial, archaean and fungal biosynthesis pathway starts with one molecule of guanosine triphosphate (GTP) and two of ribulose 5-phosphate (R5P) (Figure 1b). GTP and phosphorylated pentoses are abundant in cells, and the biosynthetic enzymes for these riboflavin precursors are efficient. Conversely, the riboflavin metabolic enzymes are very slow [26*], and it is thus necessary to strongly and stably overexpress the corresponding genes. Mutations in the flavokinase/flavin adenine dinucleotide (FAD) synthetase gene and mRNA stabilization by modified flavin mononucleotide (FMN) riboswitches are important features of modern production strains.

However, there are still unresolved issues: (1) A superordinate regulator protein (RibR) was recently discovered in *B. subtilis* [27**]. RibR affects the activity of FMN riboswitches and could limit production. (2) The phosphatase(s) of the riboflavin pathway has not been identified yet (early intermediates are phosphorylated) [28]. (3) Flavins are highly reactive molecules [29*]. Intracellular flavins (and corresponding flavoproteins) can be a major source for reactive oxygen species (ROS), thus lowering yields by creating oxidative damage to cells. A way to reduce ROS-related cellular stress could be to introduce a riboflavin binding dodecin [30].

(4) The hydrophobic dimethylbenzene portion of the isoalloxazine ring system of riboflavin probably supports diffusion of riboflavin over the cytoplasmic membrane in the absence of a dedicated transport system. The *B. subtilis* cell appears not to be able to actively export flavins (in contrast to *A. gossypii*) [31] and introduction of the riboflavin transporter gene ribM from *Streptomyces daccaonensis* enhanced riboflavin production [32]. Roseoflavin from *S. daccaonensis* negatively affects FMN riboswitches and flavoenzymes [33], and it is used to select riboflavin overproducing bacteria [34]. This antimetabolite could be used to further improve vitamin production.

(Figure 1 Legend Continued) pyrophosphate). (b) Riboflavin biosynthesis pathway in *B. subtilis*, GTP (guanosine triphosphate); FAD (flavin adenine dinucleotide); FMN (flavin mononucleotide). (c) Vitamin B$_6$ biosynthesis pathway in *E. coli*. Gray dotted lines show *Saccharomyces cerevisiae* pathway. DHAP (Dihydroxyacetone phosphate); PRPP (Phosphoribosyl pyrophosphate); NAD (Nicotinamide adenine dinucleotide; NADP (Nicotinamide adenine dinucleotide phosphate); NMN (nicotinamide mononucleotide); NR (nicotinamide riboside); NAM (nicotinamide); NA (nicotinic acid or niacin). (d) Pathway for de novo synthesis of vitamin B$_2$. IlvBH in *B. subtilis* (or IlvH or IlvBN and IlvG in *E. coli*; CoA (Coenzyme A). (e) *De novo* biosynthesis pathway of vitamin B$_6$. (f) Vitamin B$_6$ biosynthesis pathway in *E. coli* (black) and *B. subtilis* (gray). ACP (acyl carrier protein); CoA (Coenzyme A). (g) Vitamin B$_6$ biosynthesis pathway in *Bacillus subtilis*. PEP (2-phosphoenolpyruvate); E4P (erythrose-4-phosphate); DAHP (3-Deoxy-D-arabinohexulosonate 7-phosphate); PAB (4-amino benzoic acid); THF (tetrahydrofolate). (h) Cobalamin biosynthesis pathway. ALA (γ-aminolevulinate); DMB (5,6-dimethylbenzimidazole). The aerobic (*P. denitrificans* or *S. meliloti*) and anaerobic (*B. megaterium* or *P. shermanii*) pathways are shown in black and grey, respectively. Star (*) denotes the active co-factor of the individual vitamins. For biotin, biocytin, the active cofactor, is not shown.
| Vitamin | Host      | Strategya | Test condition | Media typeb | Fed precursor(s)c | Titerd | Ref.       |
|---------|-----------|-----------|----------------|--------------|------------------|--------|------------|
| **B1**  | *B. subtilis* | Mutations in the salvage thiamine pyrophosphokinase (*thiN*), thiamine permease (*thiT*) and thiamine ABC transporter component (*thiW*). | Test tube, 24 h | MM + 2.5% nutrient broth | Glucose | 1.3 mg/L | [16*] |
|  | **E. coli** | Overexpression of native *thiFGSHCE* and *thiD*. | 1 mL, 96 well plate, 24 h | MM | Glucose | 0.8 mg/L | TPP [21*] |
|  | A. oryzae | Overexpression of riboflavin biosynthetic genes; engineering of a non-responsive FMN riboswitch; reduction of flavokinase RibCF activity; enhancement of purine de novo synthesis and pentose supply. Detailed strain engineering efforts can be found elsewhere [47*,48*]. |  | MM | 4-fold > WT | [18] |
|  | **B2** | Overexpression of riboflavin biosynthetic genes; Overexpression of *thiA*, *nmtA* and *thiP*. | 1.5 L reactor | Corn steep liquor, yeast extract | Molasses, starch hydrolysate, thick juice, glucose feed | >26 g/L | [3*] |
|  | A. gossypii | Overexpression of riboflavin biosynthetic genes; Overexpression of *thiA*, *nmtA* and *thiP*. | Unknown | Soy flour, corn steep liquor | Plant oil | >20 g/L | [78] |
|  | **B3** | KO of NR importer *Nrt1* in salvage deficient *nrk1 urh1 pnp1* strain | 150 mL, OD 60 | 2 × YPD | 5 mM nicotinic acid | 8 mg/L | nicotinamide riboside [41*] |
|  | **B4** | Inactivation of *ilvA* gene and overexpression of native *IlvBNCD* and *PanBC* enzymes. | Unknown, 48 h | MM | β-Alanine | 1000 mg/L | [79] |
|  | C. glutamicum | Inactivation of *ilvA* gene and overexpression of native *IlvBNCD* and *PanBC* enzymes, reduced expression of the *IlvE* enzyme. | Unknown, 48 h | MM | β-Alanine, isoleucine | 1.75 g/L | [44] |
|  | **B5** | Overexpression of the *IlvBHCD*, *PanBCDE*, *GlyA* and *SerA* enzymes. | Unknown, 48 h | MM | – | 80 g/L | [48*] |
|  | **B6** | Overexpression of native *Epd*, *PdxJ* and *Dxs* enzymes. | Shake flask (50 mL) | MM + YE | – | 78 mg/L | [52] |
|  | E. coli | Overexpression of *E. coli* *Epd* and native *PdxJ* enzyme. | Shake flask | MM + YE | – | 1300 mg/L | [52] |
|  | S. mellioti | Overexpression of *E. coli* *Epd* and *S. mellioti* *PdxR*, *SerC*, *PdxA* and *PdxJ* enzymes. | 24 well plate (3 mL) | MM + YE | – | 41 mg/L | [55] |
|  | **B7** | Overexpression of *E. coli* *PdxA* and *S. mellioti* *PdxJ* enzymes. | 24 well plate (3 mL) | MM + AAAs | 4-Hydroxy-L-threonine | 65 mg/L | [51] |
|  | S. marcescens | Use of chemical mutagenesis (methylnitrotrisoguanidine) with selection on ethionine and S-2-aminoethylcysteine; overexpression of biotin native operon with biosynthetic genes and ParB (plasmid stabilization system). | Unknown, 9 days | MM + 3 % urea + YE + L-cysteine | 30% sucrose | 500 mg/L | [61*] |
|  | Agrobacterium/Rhizobium | Overexpression of a modified biotin operon from *E. coli*; use of a strong artificial tac promoter and introduction of an improved RBS in front of BioB. | 2-L fed-batch fermenter, 20 days | MM + glutamine + betaine | Glucose + diaminononanoic acid | 110 mg/L | [61*] |
| Vitamin | Host | Strategy | Test condition | Media type | Fed precursor(s) | Titer | Ref. |
|---------|------|----------|----------------|------------|----------------|-------|-----|
| B. subtilis | Overexpression of native biotin operon and selection on S-2-aminoethyl-L-cysteine. | 300 mL, 20 h | MM | 3.5% glucose and 7% maltose + 6 7.5 g/L lysine | 21 mg/L | [65] |
| E. coli | Use of chemical mutagenesis with selection on acidomycine and 5-(2-thienyl)valeric acid; overexpression of native biotin operon from a high-copy number plasmid. | Shake flask (20 mL), 24 h | Complex | – | 11 mg/L | [67] |
| B9 | A. gossypii | Overexpression of fol genes and deletion of met7 and competing genes AgADE12 and AgRIB1 | Shake flask, 120 h | MA2 complex medium | 4-Aminobenzoic acid | 7 mg/L | [70] |
| B12 | Pseudomonas denitrificans | Random mutagenesis using raditional (UV light) and chemicals (ethyleneimine and nitrosomethylurea), overexpression of cobF-cobM gene cluster as well as cogA and cobE genes; optimization of promoters, RBSs, terminators. Detailed strain engineering efforts can be found elsewhere [76]. | 120 m³ fermenter, 7 days | Beet molasses, corn steep liquor | Sucrose, betaine, DMBI | 214 mg/L | [80] |
| Propionibacterium shermanii | Overexpression of biosynthetic genes. | Unknown | MM | Glucose, DMBI | 206 mg/L | [72] |
| Bacillus megaterium | Overexpression of hemACDBL, sirA, cbiX/CDETLFGA, cysGA, cbiY, btuR, glmS, metH, rpmR with xylose-inducible promoter; antisense RNA for hemE, hemZ, sirB. Bypassing of the B12 riboswitch. | Shake flask (50 mL) | MM | Glucose | 0.220 mg/L | [73] |
| S. meliloti | Random mutagenesis biased on atmospheric and room-temperature plasma (ARTP); overexpression of hemE; deletion of cobI, and usage of a riboswitch based on butB element from Salmonella typhimurium in front of a gfp reporter gene driven by the constitutive promoter PmelA. | Shake flask (30 mL), 7 days | MM, corn steep liquor | Sucrose, betaine, DMBI | 156 mg/L | [75] |
| E. coli | The 22 native cob genes located in six operons from P. denitrificans were PCR-amplified and cloned in three compatible plasmids under the strona dhn inducible T7 promoter. | Shake flask (50 mL) | LB media | Aminolevulinic acid | 0.65 μg/g CDW | [74] |

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**Table 2 (Continued)**

| Vitamin | Host | Strategy | Test condition | Media type | Fed precursor(s) | Titer | Ref. |
|---------|------|----------|----------------|------------|----------------|-------|-----|

**Notes:**

- **RBS:** ribosome binding sequence.
- **MM:** minimal media. **YPD:** yeast extract peptone dextrose, **YE:** yeast extract. **AA:** amino acids.
- **DMBI:** 5,6-dimethylbenimidazole.
- If the titer is not reported in mg/L, it is given in the units described in the respective publication or review. **CDW:** Cell dry weight.
Vitamin B₃
There are three forms of vitamin B₃, all of which are converted to the essential cofactors enzymatically [35]. Although the commercial forms are niacin (NA) and the corresponding niacinamide (NAM), nicotinamide riboside (NR) has gained attention recently in treatment of age-related diseases [35,36].

Figure 1c illustrates the biosynthetic pathway to NAD⁺ in E. coli and Saccharomyces cerevisiae. NA and NAM are breakdown products or intermediates in salvage pathways. NR can also be synthesized from NAD⁺ using salvage enzymes [37,38].

There is no commercial fermentation process for NA or NAM [6] but biocatalytic routes exist that use 3-cyanopyridine as a starting material that is hydrolysed to NA by a nitrilase or hydrated to NAM by a nitrile hydratase [7]. The enzymatic hydrolysis is catalyzed by whole-cell biocatalysts of engineered Rhodococcus rhodochrous [39]. Recent reports describe the use of recombinant E. coli expressing R. rhodochrous nitrile hydratase for NAM production [40]. A yeast strain producing extracellular NR by the native NAD⁺ breakdown process (>8 mg/L NR with media supplementation of NA) was constructed by knocking out the NR importer Nrt1 [41] (Table 2). With the high-yielding biocatalytic process in place there is little motivation for a fermentative process to NA or NAM, while fermentation of NR could be appealing if a market demand emerges.

Vitamin B₅
Pantothenic acid (pantothenate) is a precursor of coenzyme A (CoA) and the acyl carrier protein (ACP) that play essential roles in fatty acid metabolism [42,43]. The active form is pantothenate (panthol and pantetheine are two more vitamers), and it is stable as a calcium salt, which is the commercial form [43].

De novo synthesis of vitamin B₅ occurs in microorganisms, plants and fungi. In B. subtilis and E. coli, it involves seven enzymatic reactions starting from pyruvate and aspartate [42]. Pantoate is generated from two molecules of pyruvate by the acetalactate synthase complex and 4 enzymes (Figure 1d). Pantoate and β-alanine (generated via decarboxylation of aspartate by PanD) are condensed to pantothenate by PanC.

Most microbial engineering efforts target genes involved in the metabolic pathway and cofactor generation, for example, inactivating threonine dehydratase (ileA) and overexpressing ileBCND/panBC enhance biosynthesis of ketoisovalerate/pantothenate, resulting in 18 mg/L/h vitamin B₅ using C. glutamicum [44] (Table 2). Glycine accumulation was suggested to affect regeneration of 5,10-methylenetetrahydrofolate (Met-THF) [45], a cofactor of PanB. Hence, Met-THF supply seems to be an important bottleneck. Production could be further enhanced to <35 mg/L/h by maintaining the ketoisovalerate-consuming aminotransferase IlvE at low levels with β-alanine [44] (Table 2). The highest titre was reported in B. subtilis (Table 2), and it could be improved by manipulating pantothenate kinase (coaA) to reduce pantothenate consumption [47]. In some bacteria, PanB is inhibited by pantoate, pantothenate and CoA [43], but protein engineering can partially remove this inhibition [48*].

Vitamin B₆
Pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and their respective 5'-phosphate derivatives PNP, PLP, PMP compose this vitamin. Pyridoxine hydrochloride, the commercial form, is synthesized from PN. In vivo, PLP is the most important vitamer, serving as a cofactor for a plethora of enzymes mostly involved in amino acid metabolism [49].

Two de novo synthesis routes are known [50]. The DXP-dependent pathway involves six enzymatic steps starting from erythrose 4-phosphate and DXP. Epd, PdxB and SerC generate the toxic intermediate 4-phosphohydroxy-L-threonine (4HTP) [51], which is converted to PNP by PdxA and PdxJ. PdxH converts PNP to PLP (Figure 1e). The DXP-independent pathway, widely distributed in archaea, bacteria, fungi and plants [52], involves only the PdxST enzymatic complex. Non-producer organisms are endowed with a salvage pathway allowing interconversion of B₆ vitamers [50]. E. coli and B. subtilis possess promiscuous enzymes feeding precursors into a truncated DXP-dependent pathway consisting of SerC and E. coli PdxA/PdxJ [53*,54*].

Since the PdxST complex is catalytically inefficient, some organisms have been genetically engineered for producing PN/PL via the DXP-dependent pathway (Table 2). A B. subtilis strain carrying the respective enzymes PdxA and PdxJ from E. coli and Sinorhizobium meliloti produced 900 μg/L/h PN from 4-hydroxy-L-threonine (4HT) (Table 2) [51]. 4HT can be converted to 4HTP by homoserine kinase (ThrB) [51]. It seems that PdxJ is a bottleneck in the DXP-dependent pathway because it is an inefficient enzyme [52] that more efficient orthologs or protein engineering might improve. Little is known about PNP and PLP phosphatases, which may facilitate vitamers export and prevent accumulation of toxic intermediates like 4HTP [55]. Unfortunately, production levels should exceed 10 g/L in 48 h for being commercial relevant [55], so major metabolic engineering efforts are needed.

Vitamin B₇
D-(+)-Biotin (or biotin) is an essential cofactor for carboxylation reactions. Biotin intermediate pimelic acid is synthesized by two alternative routes [56]. In E. coli, fatty acid biosynthesis is hijacked by O-methylation of the
malonyl-ACP by BioC, elongation by the fatty acid cycle and hydrolysis of the ester by BioH to form pimeloyl-ACP [57**]. In *B. subtilis* a cytochrome P450-dependent enzyme (BioI) oxidatively cleaves a long-chain acyl-ACP to pimeloyl-ACP [58]. Pimeloyl-ACP feeds into the last four-enzyme pathway part (Figure 1f). Variations in the methylase/esterase (BioCH) pair and downstream (BioFADB) genes have been identified [59]. Biotin production is mainly regulated by a transcription factor that can function as a biotin ligase [60**].

In the past, efforts for engineering biotin production strains using random mutagenesis and antimetabolites met limited success [61*]. The highest biotin titer reported is 500 mg/L with *Serratia marcescens* in a ~10-day fermentation (Table 2). One challenge seems to be a high demand of the co-factor SAM by BioC, BioB and BioA, although these enzymes are strongly inhibited by SAM byproducts [62]. SAM levels can be increased via classical [63*] or emergent [64*] strain engineering methods, while non-feedback inhibited homologs could be found in metagenomic screens [48*]. In *B. subtilis*, BioA is replaced by BioK, which uses lysine as amino donor instead of SAM. Lysine feeding to an engineered *B. subtilis* strain produced >600 mg/L desthiobiotin (direct biotin precursor) but only up to 21 mg/L biotin, indicating that the last step catalyzed by BioB is a bottleneck [65*] (Table 2).

BioB is a very slow enzyme (κcat = 0.12 min⁻¹) and one of its Fe–S cluster needs to be regenerated and the other one repaired for each catalytic turnover [66**]. Overexpressing BioB can result in growth inhibition [67], so a better understanding in the regeneration and repair of Fe–S clusters might be worthwhile [68*]. To develop a biotin bioprocess, major metabolic (SAM supply) and protein (BioB) engineering efforts are needed.

Vitamin B₁₂
Cobalamins are composed of adenosyl- (AdoB₁₂), methyl- (CH₃B₁₂), hydroxy- (OHB₁₂) and cyanocobalamin (CNB₁₂). AdoB₁₂/CNB₁₂ are the active forms, while OHB₁₂ can be an intermediate (or co-factor of dehalogenases) and CNB₁₂ is the most stable and commercial form due to its longer shelf life. Cobalamin chemical synthesis involves ~70 reactions (Table 1), whereas microbes can produce it using fewer steps by a de novo or salvage pathway. The former is composed of ~30 genes starting from glutamate and can be aerobic or anaerobic (Figure 1h), whereas the latter pathway has ~12 genes (e.g., *E. coli*) but it needs dedicated ATP-driven ABC transporters for importing cobinamide, a cobalamin analog [71*].

High titers of ~200 mg/L were reported for *Propionibacterium shermanii*, but *Pseudomonas denitrificans*, which produces similar titers is the current industrial host (Table 2) [72*]. The engineering of the de novo *Bacillus megaterium* pathway [73] or the transfer of 22 upstream genes from *P. denitrificans* into *E. coli* enabled de novo *B₁₂* production [74], while a riboswitch-based screening system was developed to screen for high producers in *S. meliloti* [75*] but the yields are very low (Table 2).

Since both pathways are highly regulated at different levels [71*,72*,73,76*], the main challenges are to remove the inhibition of mRNAs of *cysG* and the *chi* operon by the cobalamin riboswitch as well as enzymes including HemA by protoheme, HemE by coproporphyrinogen III, CysG by cobalamin and CobA by its own substrate [71*,77]. Other issues are the need to supply sufficient cobalt to the broth and generate enough SAM co-factor for methylation reactions (e.g., CysG).

Technological, environmental and regulatory issues
To develop efficient bioprocesses, genetic tools must be available [5*] and the host must be suitable for fermentation [81*]. The current *B₁₂* bioprocess is sub-optimal: it involves long fermentation cycles, expensive media and lack of genetic tools in commercial hosts [71*]. Additionally, down-stream processing (DSP) is an important step. B vitamins have different water-solubility (Table 1). Riboflavin’s low solubility enabled an efficient DSP: a large fraction of the product crystallizes during fermentation; the crystals are washed and separated [3*]. Similar DSPs could be developed for vitamins B₁ and B₉. Cobalamin fermentation produces a mixture of AdoB₁₂, McB₁₂ and OHB₁₂. During DSP, the vitamins are converted to CNB₁₂ with potassium cyanide at high pressure/temperature [72*]. The biocatalytic
Box 1. Sustainability and regulatory issues

Aside from cost, sustainability is a motivating factor for switching to bioprocesses. However, predicting environmental impact is challenging. In the case of riboflavin, a life-cycle assessment (LCA) determined that the fermented process scored better than the chemical one [47]: Reduction of potential of global warming by 30% (CO2), acidification by 50% (SO2), ozone creation by 60% (NOx) and cumulative energy consumption by 40%; the eutrophication potential, however, increased by 40% (PO4). The LCA of vitamin B12 can be a guide, but the exact specification of the chemical and fermentative processes are important to calculate their actual impact. Similar to vitamin B12, the conventional chemical routes to B1, B5, B6 and B7 rely on non-renewable sources, produce toxic waste and have high-energy demands. The synthesis of vitamin B12 has switched to a more sustainable process. While vitamin B12 is currently produced by fermentation, the conversion of the product mixture to CNB12 using potassium cyanide is not sustainable. Performing this step biologically could result in a greener bioprocess.

Additives of food and feed produced with natural hosts or genetically modified microorganisms (GMM) in closed systems are not evaluated under the same regulations as genetically modified food and feed in the European Union (EU) [83]. In most cases, the biologically produced vitamin can be identical to the chemically produced vitamin. However, the production method will affect the presence of impurities. For example, aniline is a non-desired impurity in the chemically produced riboflavin that is absent in the fermented version [15]. Conversely, it is unlikely to find DNA in chemically produced additives, which could be an issue in fermented products. The European Food Safety Authority (EFSA) states that it is mandatory that neither GMM nor recombinant DNA can be present in the final product that is placed on the EU market as non-GMO food or feed additive [83,84]. Further, several regulatory steps need to be taken to replace a chemical production process of a vitamin with a biological process, and these steps differ depending on the vitamin application, the host organism, the purity level obtained, the stability of the product, and the specific impurities present in the product. One step involves showing that the new product is safe — generally done by toxicity studies and/or by proving that it is near-identical to the previously approved product, for example, by demonstrating that it conforms to the specifications of the relevant pharmacopoeia monographs (e.g. European pharmacopoeia in the EU). In the US, it is standard practice to notify the FDA that an ingredient is Generally Recognized As Safe (GRAS), for example, riboflavin biosynthesized by Eremothecium ashbyii has been designated as GRAS [11].

Conversion of cyanopyridine to NAM is quantitatively, thus simplifying its purification [7]. DSPs for vitamins B1, B2 and B3 are unknown. The DSP of vitamin B2 is well-established [82]. Finally, there are also environmental and regulatory issues (Box 1).

Conclusion

Relatively small cellular requirements for vitamins mean that their biosynthesis has not evolved to be high yielding in nature. Yet, industrial bioprocesses for vitamin B2 and B12 have successfully been established through rational and classical metabolic engineering, and the question is whether the six other B vitamins will follow. While low current manufacturing cost combined with highly complex biosynthetic pathways and sloppy enzymes makes industrially relevant cell factories extremely challenging, several factors points towards a future transition towards bio-based processes: (i) our fundamental understanding of vitamin biosynthesis increases; (ii) as the synthetic biology toolbox expands, our ability to engineer microbes is rapidly advancing; (iii) environmental restrictions are already today increasing cost of polluting chemical processes; and (iv) in certain segments, for example, food supplements and cosmetics, we observe a consumer demand for naturally produced biochemicals.

Conflict of interest statement
LSG, MM and HJG have filed patent applications that are related to the fermentation of some B vitamins.

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