

Exploiting unconventional prokaryotic hosts for industrial biotechnology

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Abstract

The development of cost-efficient biotechnological processes is a major challenge in the substitution of fossil-based industrial production processes. The remarkable progress in genetic engineering ensures efficient and fast tailoring of microbial metabolism for a wide range of bioconversions. However, the improvement of intrinsic properties such as tolerance, handling, growth, and substrate consumption rates is still challenging. At the same time, synthetic biology tools are becoming easier applicable and transferable to non-model organisms. These trends resulted in the exploitation of new and unconventional microbial systems with sophisticated properties which render them promising hosts for the bio-based industry. Here, we highlight the metabolic and cellular capabilities of representative prokaryotic newcomers and discuss the potential and drawbacks of these hosts for industrial application.

A paradigm change – exploiting novel microbial hosts

The bioeconomy relies on the use of biogenic resources and waste streams such as lignocellulosic hydrolysates, biorefinery or industrial side streams, or off-gases for the production of energy and materials (Figure 1A). This must happen in a cost-efficient way if it is to replace fossil-based processes and thus enable a circular economy. Highly efficient biotechnological conversions are thus required, which places severe requirements to meet on the microbial systems used in industrial biotechnology. Ideally, boundary conditions such as a defined substrate, product, and **bioprocess window** should guide the selection of a suitable microbial system up front (Figure 1B) before performing iterative rounds of strain development to eventually yield an optimized industrial process [1] (Figure 1C).

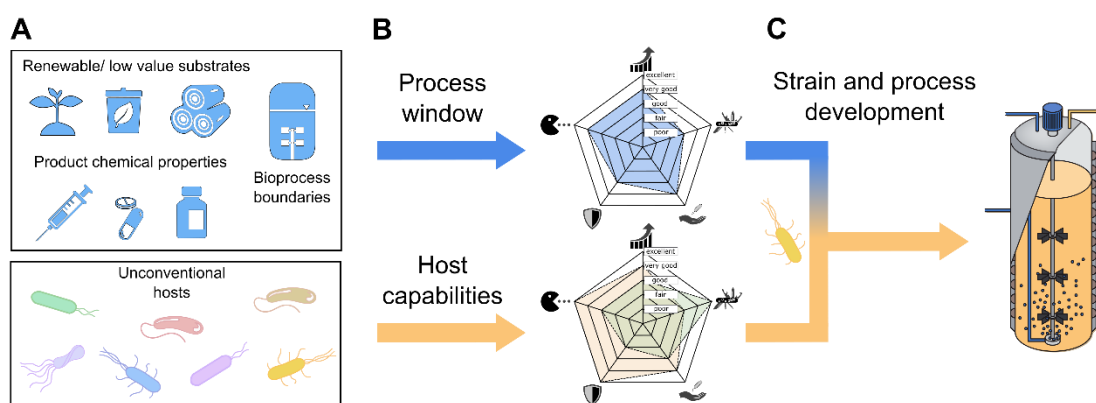


Fig. 1. Exploiting unconventional prokaryotic hosts for industrial biotechnology. (A) Based on the available substrate, process boundaries, and desired product properties, natural strains can be selected that (B) best match the envisioned process window in terms of growth efficiency, tool availability, handling, tolerance, and metabolic capability. This *ab initio* selection ensures the highest chance of success of subsequent strain and process development (C) for envisioned industrial bioprocesses.

Highly robust and tolerant strains towards non-natural or even toxic substrates or products are required which are able to utilize so far untapped carbon and energy sources. Moreover, growth in low-cost media such as prepared with sea-water [2] or a high resistance against phage infections are of growing interest. Flexible use of (seasonally available) feedstocks further relies on versatile microbes which are easy to handle at lab scale as well as in the industrial environment. Ideally, the metabolism of such microbes can efficiently be engineered to further expand the substrate and product spectra. For heterotrophic organisms this approach is generally feasible [3][4], but the metabolic engineering of autotrophic organisms, and the engineering of growth on C1 substrates in heterotrophs, is often much more challenging [5]. In addition, a high growth efficiency

enables the development of more productive processes which are competitive with classical chemical approaches.

Metabolic engineering of microbial strains is a rapidly evolving field that, in combination with synthetic biology approaches and metabolic modeling, ensures fast tailoring of the metabolism [6,7]. This currently leads to an impressive flood of publications describing approaches for the production of valuable natural and non-natural products [8]. However, recent progress also clearly points to obstacles in this field which cannot be easily overcome. The optimization of basic cellular properties such as tolerance to chemical and physical stresses, high growth and substrate consumption rates, or the ability to efficiently utilize C1 carbon sources, are still challenging (See **outstanding questions**). This is mainly due to the complexity of these traits and the lack of underlying biochemical knowledge.

This insight has led to a paradigm shift towards the development and application of **unconventional prokaryotic hosts**. Certainly, many eukaryotic hosts like *Saccharomyces cerevisiae* also have highly advantageous traits, but these will not be in the scope of this opinion article. Historically grown microbial systems such as *Escherichia coli* are established in industrial biotechnology and therefore have the “first mover advantage”, but they are not per se superior catalysts. In fact, wildtype *E. coli* has very few outstanding natural properties, and in our opinion its central position in the biotechnology community is mainly due to the long history of tool development and ease of handling. Nature’s diversity provides an enormous resource to meet the abovementioned challenges with organisms that possess superior trophic levels, growth efficiency, tolerance or handling, and such microbes should be selected already during the first stages of process design [9,10]. Many of these organisms are increasingly coming into focus, often occupying specific industrial and scientific “niches” where established hosts simply cannot perform [1]. Some of these novel microbial hosts will be highlighted here as exemplary case studies, such as the solvent-tolerant *Pseudomonas taiwanensis* (Box 1), the fast-growing *Vibrio natriegens* (Box 2), the metabolically versatile *Paenibacillus polymyxa* (Box 3) or the methylotrophic *Bacillus methanolicus* (Box 4). In our opinion, these and other unconventional hosts possess superior properties that, with the current knowledge, cannot be easily engineered into established biotechnological workhorses.

Requirements for novel microbial hosts to reach industrial maturity

Microbial biotechnology is highly diverse and it is almost impossible to directly compare for example the production of a secondary metabolite from glucose to the conversion of carbon monoxide into acetate. That said, certain universal parameters such as growth efficiency, tool availability, handling, tolerance, and metabolic capacity, can be defined that are relevant for almost all processes. These parameters can help to guide

the evaluation and selection of a microbial host for a specific industrial application (Figure 2A):

Growth efficiency of the applied microbial host is an essential factor for reducing production costs of a biotechnological process. For this, exploitation of the exceptionally fast-growing *Vibrio natriegens* as novel host for molecular biology and industrial biotechnology is targeted massively (Box 2; [11–13]. Since a high growth rate (μ) goes hand in hand with a high specific substrate consumption rate (q_s), *V. natriegens* shows q_s values which are significantly higher than those of established microbial systems under both aerobic and anaerobic conditions. This property is an excellent prerequisite to reach outstanding productivities as recently shown for alanine production with *V. natriegens* (Box 2; [13]). Growth efficiency also applies to high biomass yields, low maintenance demand, long-term viability, and growth-uncoupled product formation, which all reduce substrate costs for biomass production, especially for high cell density processes. Some of these traits can be enhanced by the engineering of streamlined chassis through genome reduction [14], provided that the required tools are available.

Tool availability is also an important prerequisite to fully exploit a novel microbial system. Model organisms such as *E. coli* particularly excel in this department, nowadays even enabling full-scale genomic engineering [15,16]. Genetic engineering can be a challenge for non-conventional microbes. However, the courage to use novel microbial systems with superior intrinsic properties is promoted by the steadily increasing development of widely applicable genetic engineering toolboxes. This has been impressively demonstrated for example for the fast-growing *V. natriegens*. Within less than five years *V. natriegens* has been made accessible for genetic manipulation, and a variety of tools to manipulate its metabolism were established and have already been applied to engineer **chassis** and production strains [17]. For *Pseudomonas sp.* an impressive amount of synthetic biology tools for the model organism *P. putida* KT2440 were developed within the recent years [6], which are also applicable for the deep engineering of unconventional hosts such as *P. taiwanensis* (Box 1, [14]. Also, for *P. polymyxa* (Box 3), which was regarded for a long time as resistant towards genetic manipulation, tools for genetic and metabolic engineering were rapidly developed by the design of an efficient CRISPR-Cas tool [18], ending up in the first described Cpf1 based multiplexing CRISPRi/a system for prokaryotes within only 3 years [19,20].

Convenient *handling* is not only restricted to lab scale and must also be considered for the industrial environment. Efficient cultivation in simple mineral media lowers production costs and widens the application range of microbes. The use of robust strains or extremophiles as well as recalcitrant substrates enables (semi-) autosterile process development, for example in sea water [2]. Morphology also plays an important role in the handling of microbes. Unicellular growth is usually preferred, but biofilm catalysis can also

provide major advantages in certain niche applications [21,22]. Engineered production strains have to prove their robustness in industrial scale where gradients (e.g., O₂, CO₂) can significantly impact the performance [23]. This issue is often not sufficiently considered but should be addressed already in the early stage of process development especially for non-conventional hosts [24]. Further, unconventional hosts should be safe to handle with a biosafety level 1 classification. A unified biosafety classification is sorely needed in this context, especially in the European Union where large differences exist between member countries. Strains should ideally be Generally Regarded as Safe, although this is rare and often confused for microbes [25].

A high *tolerance* against relevant substrate and product concentrations is essential for industrial application. Pseudomonads such as the non-pathogenic *P. taiwanensis* VLB120 (Box 1) can withstand high oxidative and chemical stresses, enabling more efficient use of toxic substrates, production of higher titers of more toxic chemicals, and application of *in situ* solvent extraction [26,27]. Other microbes such as the pathogenic *Klebsiella* sp., or the non-pathogenic *P. polymyxa* (Box 3) can cope with high concentrations of 2,3-butandiol [28,29]. Also, a tolerance against fluctuating process parameters such as dissolved oxygen concentration, as present in large scale production environments, represents a beneficial attribute of the microbial host. Although **adaptive laboratory evolution** (ALE) is a powerful approach to increase tolerance and titers [30,31], but it often stagnates at a certain level of improvement and can even be detrimental [32]. Exemplary, ALE can increase the thermotolerance of *E. coli* up to 48°C [33]. Above this temperature most of the proteome of *E. coli* is unstable, making it unfeasible to achieve higher temperature tolerance [34]. In contrast, industrially interesting thermophiles of the (*Geo*-)*Bacillus* or *Thermus* genera grow at much higher temperatures.

The versatile *metabolic capabilities* of a (mainly heterotrophic) microbial host to utilize various substrates, is a prerequisite for the microbial funneling of complex mixtures such as hydrolysates of lignocellulose or plastics to desired products [35,36]. Pseudomonads (Box 1) are at the forefront of this development. In contrast, but equally important, are the current developments towards the valorization of alternative carbon sources such as C1 substrates, which demand the use of more specialized microbial systems. One example is *Bacillus methanolicus* (Box 4), a Gram-positive and thermophilic bacterium that is able to utilize a limited number of alternative carbon sources, however, has the ability to efficiently utilize methanol [37]. Also, the versatile utilization of terminal electron acceptors under aerobic and anaerobic conditions is an important attribute of a microbial host which impacts the ability to synthesize specific products.

A

Characteristics	Desired properties	KPIs*
 Growth efficiency	- High biomass yield and low maintenance demand - High growth, substrate uptake rate and metabolic flux	- Yield - Rate
 Tool availability	- "Engineerability" with efficient and reliable tools for genomic engineering - Knowledge base of annotated genome, metabolic models and systems analysis	- Time to market - Versatility
 Handling	- Cultivation to high cell density - Lack of overflow metabolism and by-product formation - Amenable to screening and engineering - Low impact of heterogeneities	- Rate - Scale-up
 Tolerance	- Ability to withstand high concentrations of (crude) substrate/product - Broad process windows of pH, temperature, oxygen and salt concentrations - Adaptability to physical/chemical gradients in industrial scale	- Titer - Scale-up - Substrate flexibility
 Metabolic capability	- Utilization of different, or specialized, feedstocks including, sugars C1 substrates, lignocellulosic hydrolysates, and waste streams - Production of many different types of chemicals	- Substrate and product flexibility

* KPIs = Key performance indicators

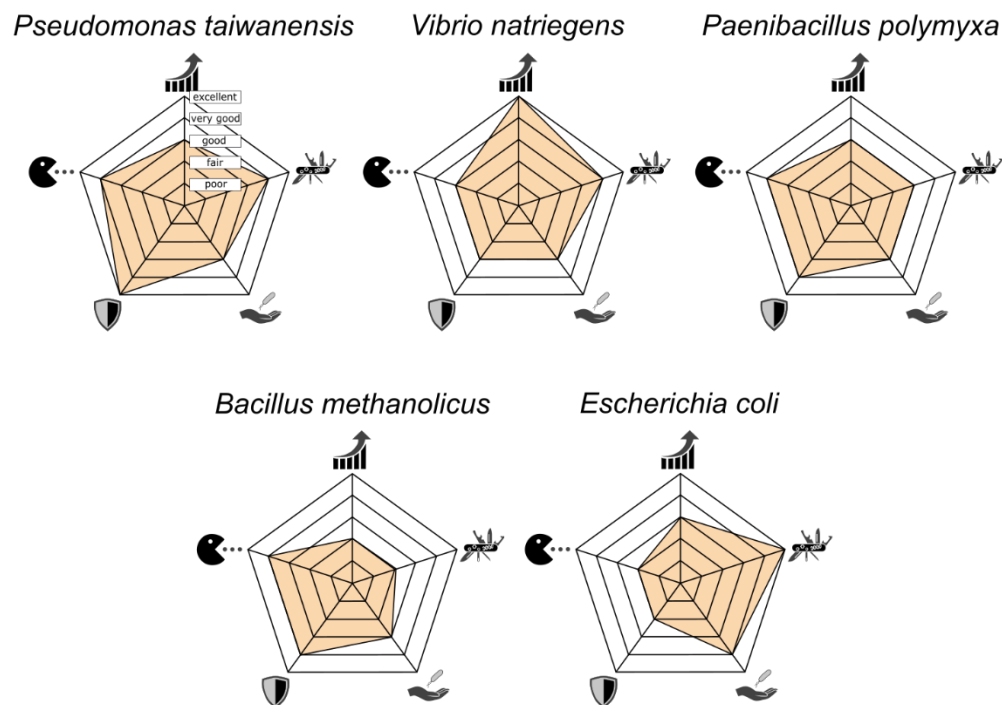
B

Fig. 2. (A) Characteristics, desired properties, and key performance indicators (KPIs) of novel microbial hosts to reach industrial maturity. (B) Estimation of strain characteristics of emerging production hosts regarding defined key requirements, compared to wildtype *E. coli*.

Economic and environmental constraints impact host selection

Currently, most biotechnological production processes are carbohydrate-based (e.g. glucose or sucrose; [38]) applying heterotrophs such as *E. coli* or *C. glutamicum* which utilize organic compounds as carbon and energy source. Carbohydrate-based processes generally possess a high technology readiness level and therefore will be preferentially further applied in industry. However, the establishment of more sugar-based processes will increase the demand for this substrate, and the consequent price increase is a major risk for industrial development of bulk processes which have a long return of investment time [39]. The currently applied carbohydrate-based substrates, which derive from starch or sugar-containing plant material, are often viewed as a dilemma for the envisioned bioeconomy since these feedstocks, when used at very large scale, may compete with food production [38]. Even if this point is controversially discussed, at least for ethical reasons the fuel versus food debate also applies for the biotechnological production of chemicals. Perspectively, bulk bio-based production processes should therefore preferentially be based on non-food feedstocks such as methane, methanol, formate, syngas, and CO₂ (Figure 3; [40]) – carbon sources which do not belong to the natural substrate spectrum of industrially established heterotrophs. Alternatively, more complex and crude substrates such as lignocellulosic or plastic hydrolysates could be used, requiring a higher chemical tolerance and a versatile metabolism of the applied microbial system [41]. Consequently, either heterotrophs are engineered to efficiently utilize such carbon sources [42,43] or novel hosts which are naturally able to assimilate these substrates or show an intrinsic high tolerance towards crude substrates such as *P. taiwanensis* (Box 1) or *B. methanolicus* (Box 4) must be exploited for industrial processes.

The recently approved **Green Deal** by the European Union aims at the reduction of net greenhouse gas emissions to zero by 2050, which will also impact future biotechnological processes. The biotechnological production of chemicals and fuels from biomass is considered atmospheric carbon neutral because as the biomass grows, it absorbs CO₂ which is eventually incorporated in the product. However, biomass-based fermentation processes or anaerobic digesters produce significant amounts of CO₂ as byproduct, which are currently released into the environment. Moreover, taking the whole value chain into account (including e.g. energy for culturing and harvesting of the biomass or reactor cooling and downstream processing), significant net amounts of CO₂ might be emitted. Consequently, to minimize CO₂ emissions, future biotechnological processes have to rely on integrated carbon capture and utilization technologies as well as biotechnological **cascade processes** where a (by-)product (e.g., CO₂, methane, acetate) of one process stage is the substrate for a subsequent (Figure 3).

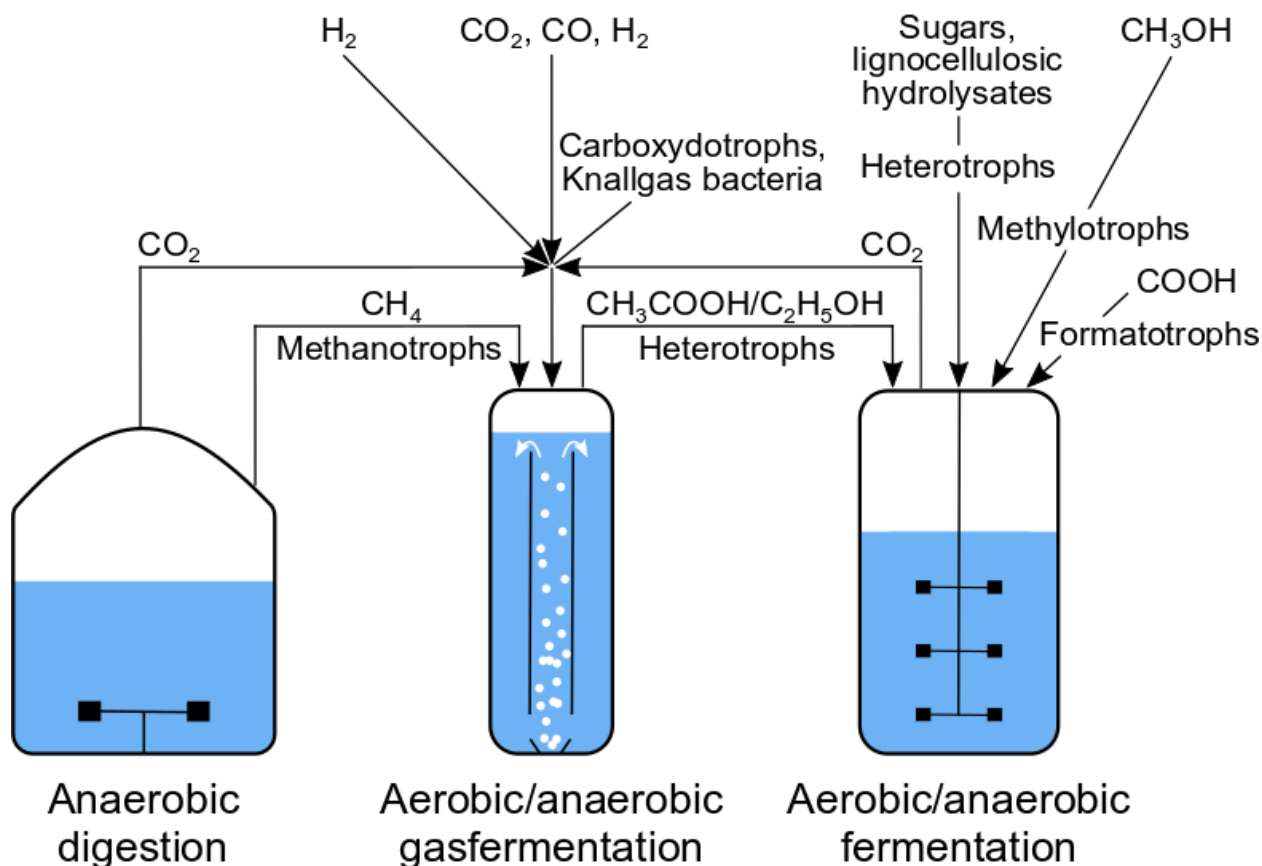


Fig. 3. Valorization of abundant feedstocks and its proposed cascading utilization to reduce greenhouse gas emissions.

Gas fermentation will play an important role in this scenario to convert (waste) gases such as CO_2 , CO , H_2 , methane and mixtures thereof into chemicals and fuels [44]. Carboxydutrophic bacteria generate energy by aerobic or anaerobic oxidation of CO . Aerobic carboxydutrophs use the Calvin-cycle, whereas strictly anaerobic carboxydutrophs, also called acetogenic bacteria or acetogens, harness the Wood–Ljungdahl (WL) pathway for CO_2 -fixation. In anaerobic gas fermentations acetogens such as *Acetobacterium woodii*, *Clostridium ljungdahlii*, *Moorella thermoacetica*, and *Clostridium autoethanogenum* convert $\text{CO}/\text{CO}_2/\text{H}_2$ -containing gases efficiently to ethanol and acetate [45]. Anaerobic gas fermentation has become a technology of industrial maturity and first production plants have been successfully installed by companies such as LanzaTech [44]. Among the known pathways for CO_2 -fixation, the WL is the most efficient one with regard to ATP and H_2 /electron requirements [46]. However, due to the anaerobic life style, acetogenic bacteria are energy limited and the production of longer-

chain or ATP-demanding molecules is challenging [46]. To overcome this limitation, recently aerobic carboxydrotrophs such as *Oligotropha carboxydovorans* and *Hydrogenophaga pseudoflava* have been proposed for industrial gas fermentation since the aerobic oxidation of CO provides a higher ATP-yield compared to the anaerobic route [45,47,48]. Also, Knallgas bacteria such *Ralstonia eutropha* which are naturally unable to oxidize CO have a high potential to convert CO₂ and H₂ containing gases to chemicals and microbial upgrading of methane with methanotrophs, methanol with methylotrophs or formate with formatotrophs to value-added products might represent another piece of the puzzle in the envisioned circular economy (Figure 3). In this refinery concept, products derived from **C1 substrates**, such as acetate or ethanol, can be further valorized by applying these substrates as feedstock for heterotrophic hosts to produce more complex chemicals (Figure 3; [38]). With these considerations in mind, economic and ecological constraints will help make greater use of microbial diversity for industrial biotechnology.

Challenges to establish novel microbial hosts for industrial biotechnology

Classically, novel microbial hosts were implemented in biotechnological processes for their ability to produce a certain chemical, for example, antibiotics with *Streptomyces* sp., or vitamin B12 with *Pseudomonas denitrificans* [49]. The advent of modern metabolic engineering and synthetic biology has greatly decreased this reliance on novel hosts by allowing the insertion of production pathways in microbial workhorses such as *E. coli* [50]. Since then, the selection of a novel microbial host is more often driven by other limitations, such as the utilization of a specific substrate (as elucidated above). However, besides the obvious advantage, the abovementioned requirements should be carefully considered. Especially, the transfer of a novel host into industrial scale comes with greater risk compared to established microbes, because long-term experience with regard to for example genetic stability or cellular heterogeneity is simply missing (**see Outstanding questions**). As mentioned above, the ability to grow very fast, to utilize a special carbon or energy source, or to tolerate a toxic product or extreme process conditions, can be enormously advantageous and these beneficial traits might even be utilized in combination for syntrophic cultures [51]. However, driving the fitness of a certain trait to extremes often comes with drawbacks in other areas. Examples for this are the limited substrate and product flexibility of C1 metabolizing strains, the high metabolic energy demand imposed by solvent-tolerance [52], or the relatively poor genetic accessibility of halophilic or thermophilic microbes [53]. These trade-offs can affect the application range or production parameters (yield, titer, rate) of the envisioned biocatalyst, and should therefore be carefully weighed against the advantage of the unconventional host.

On the road from the discovery and development of unconventional hosts endemic to natural habitats into their industrial applications, *in silico* methods can support and guide the process at various stages in a **systems biotechnology** approach [54]. It has become common to reconstruct so-called metagenome-assembled genomes (MAGs)

from environmental samples [55], providing a first glimpse at the metabolism of uncultured organisms and informing isolation strategies [56]. On successful isolation and based on the sequenced genome, several pipelines are available to reconstruct genome-scale metabolic network models (e.g., [57–59]. Using constraint-based modeling techniques [60], such models enable a first assessment of the host's metabolic capabilities [61], including theoretically achievable product yields. Furthermore, feasible product and substrate ranges can be explored, including the effort in terms of genetic modifications required to redirect carbon flow from a chosen substrate to a desired product. This assessment of the metabolism's flexibility and versatility comes together with concrete genetic engineering strategies for given substrate-product pairs. Once the novel host driven process is established at lab-scale, process optimization and upscaling can be supported by *in silico* optimization strategies such as evolutionary algorithms (e.g., [62]. More recently, due to their ability to easily integrate heterogeneous multiomics data, machine learning techniques have been used to directly predict phenotypic behavior based on such data [63,64]. Especially for novel hosts, for which underlying biochemical mechanisms might be unknown, this might be a worthwhile approach which can also be combined with metabolic modeling [65,66] and employed for metabolic engineering [67].

Conclusion

Industrial biotechnology is considered a key technology for the transition to a bio-based circular economy, which is constantly influenced by rapid scientific developments and changing ecological and socio-economic framework conditions. As discussed here, unconventional microbial hosts with superior intrinsic properties have the potential to meet challenges that cannot easily be overcome with established hosts. Rapid advances in genetic and metabolic engineering further lower the threshold to select such novel hosts, and their characterization will also enable the rational engineering of complex traits into more established microbes. The here discussed examples possess promising features but ultimately still have to prove their suitability for industrial application. Undoubtedly, nature can offer many more so far unexplored microbial candidates with high potential to occupy industrial niches. However, many questions concerning efficient strategies for establishing (the complex traits of) unconventional hosts for industrial applications still remain (see **outstanding questions**). Future studies essentially have to provide an improved genetic engineering and synthetic biology toolbox as available for established microbial systems. This tool development not only fosters expansion of the hosts' application range, it also speeds up the characterization of promising complex features, supported by systematic analysis with readily available omics-technologies. Gas fermentation will also be an important piece of the puzzle in the envisioned circular

economy but economic feasibility strongly relies on efficient mass transfer in large scale bioreactors. Other beneficial traits, e.g., a high growth rate or exceptional tolerance, come along with challenges like a high oxygen demand or low biomass yield. These examples emphasize the need for an integrated approach of strain- and process design, taking the whole process chain into account, when considering an unconventional microbial system for industrial biotechnology.

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Glossary:

Adaptive laboratory evolution: Method to enhance and understand specific microbial traits by long-term selection under specific growth conditions.

Bioprocess window: the boundary conditions for a bioprocess, determined by the technical restriction of the equipment and the biological restrictions of the microbial host.

C1 substrates: Biotechnological feedstocks that contain one carbon atom such as carbon dioxide, carbon monoxide, formate, methanol, and methane

Cascade processes: Stepwise utilisation of different carbon sources or intermediates as well as process variants in a cascading manner.

Chassis: Genetically engineered organism with enhanced basic properties such as growth rate, tolerance, or stability.

Green Deal: Initiative by the European Commission with the overarching aim of making Europe climate neutral in 2050.

Systems biotechnology: The application of omics driven systems biology methods to gain a holistic understanding of, and to engineer, a microbial production host.

Unconventional prokaryotic hosts: Bacteria with specific beneficial features that are not yet established in industrial biotechnology.

Boxes

(Box 1) *Pseudomonas taiwanensis*

Growth efficiency Routine cultures of *P. taiwanensis* VLB120 in mineral glucose medium grow at a rate of 0.6-0.7 h⁻¹ [14]. With related Pseudomonads, this can increase above 1 h⁻¹ on succinate [68]. The maintenance demand of Pseudomonads is relatively low [69], especially when compared to other oxidative biocatalysts such as *Gluconobacter oxydans*. Biomass yields around 0.5 g_{CDW}/g_{glucose} are generally achieved, but can drop dramatically under solvent-stress conditions [52].

Tool availability A wide and rapidly expanding range of systems- and synthetic biology tools are developed by the *Pseudomonas* community [6]. These tools enable deep genetic engineering aimed at product formation [70] and the generation of streamlined chassis strains [14]. These developments are fundamentally supported by the availability of detailed metabolic models [71,72], and the outstanding genome database www.pseudomonas.com [73].

Handling As an obligate aerobe, *P. taiwanensis* doesn't produce typical overflow metabolites such as acetate or lactate. Pseudomonads are also robust against oxygen heterogeneities associated with large scale cultivation [23]. One drawback is posed by a highly active periplasmic glucose dehydrogenase (GCD), which rapidly converts glucose to gluconate [74]. Although this doesn't affect biomass yield or growth rate much, it does require titration and back-titration in batch cultures. This can be avoided by disruption of the *gcd* gene, but depending on the strain this can significantly affect the growth rate [75]. The use of glycerol as an alternative substrate is advantageous in this respect, and it has the further benefit of enabling higher yields in engineered aromatics-producing strains [76].

Tolerance *P. taiwanensis* VLB120 and related non-pathogenic strains such as *P. putida* S12 and *P. putida* DOT-T1E are best known for their high tolerance to organic solvents with a logP_{o/w} between 2.5 and 4 [26,27]. Toxicity of more hydrophilic chemicals such as phenol or (S)-styrene epoxide can be alleviated through *in situ* product removal in biphasic cultivations [77,78], where solvent-tolerance enables a wider degree of freedom in the selection of the optimal extractant [79,80]. Pseudomonads are also well equipped to withstand oxidative stress [81].

Metabolic capability Based on their broad carbohydrate substrate spectrum, Pseudomonads have been identified as key hosts for the biological funneling of complex mixtures of lignin aromatics [35,82], and plastic-derived monomers [83] into useful products. Their versatile metabolism towards aromatics also provides a wealth of biocatalytic potential that can be exploited for metabolic engineering [84,85] and for whole-cell biotransformations [86,87]. However, this versatility also has a downside, and often degradation pathways of intended products and precursors have to be identified and disrupted [70].

(Box 2) *Vibrio natriegens*

Growth efficiency Among the non-pathogenic organisms, *V. natriegens* shows currently the highest growth rate on our planet [13]. In complex medium this Gram-negative bacterium grows with a generation time of 9.4 – 9.8 min. Under aerobic batch conditions in minimal medium with glucose, *V. natriegens* grows with a μ of 1.48 – 1.70 h⁻¹, exhibits a biomass yield ($Y_{X/S}$) of 0.5 g_{CDW} g_{Glc}⁻¹ and a biomass specific glucose consumption rate (q_s) of 3.5 – 3.9 g_{Glc} g_{CDW}⁻¹ h⁻¹ [13,88]. Even in the absence of oxygen, this facultatively anaerobic bacterium shows a μ of 0.92 h⁻¹ and a q_s of 7.81 g_{Glc} g_{CDW}⁻¹ h⁻¹ and ferments glucose mainly to acetate, succinate, formate, lactate, and ethanol [13]. μ and q_s under both conditions are at least two times higher compared to established microbial systems [13].

Tool availability The genome of *V. natriegens* is sequenced and genetic, synthetic biology, and metabolic engineering tools such as synthetic promoters, expression and (multiplex) genome editing systems as well as metabolic flux analysis have been developed. These tools have already been applied to engineer first genome-reduced derivatives and production strains [17].

Handling *V. natriegens* grows rapidly on common liquid and solid complex media when supplemented with sufficient sodium ions [11,13]. However, storage for a prolonged time at 4 °C negatively impacts the viability and should be omitted [11]. Due to its easy handling in the lab, the Weinstock group [11] and Lee and colleagues [12] proposed to utilize the fast-growing *V. natriegens* as novel host for routine applications in molecular biology and biotechnology.

Tolerance *V. natriegens* is a marine bacterium, requires sodium ions (Na⁺) for growth and consequently tolerates sea salts. It has an exceptionally high tolerance towards selenium, an attribute which was already utilized for efficient production of selenium nanoparticles [89].

Metabolic capability *V. natriegens* utilizes several industrially relevant substrates including carbohydrates, amino, carboxylic and aromatic acids, polyamines, alcohols and the chitin monomers glucosamine and N-acetylglucosamine as well as starch. This nutritional flexibility is an important prerequisite for the flexible feedstock concept in industrial biotechnology [17]. Besides the facultatively anaerobic lifestyle, *V. natriegens* can fix N₂ [90] and is able to perform extracellular electron transfer [91].

(Box 3) *Paenibacillus polymyxa*

Growth efficiency Cultures of *P. polymyxa* DSM 365 in glucose or sucrose medium grow at a rate of 0.7-0.76 h⁻¹ [28] under aerobic conditions whereas growth rates of 0.3 – 0.6 h⁻¹ are described for microaerophilic conditions [92]. *Paenibacillus* as most Bacilli is a mesophilic Gram-positive spore forming bacterium with an ubiquitarian appearance.

Tool availability For a long time, Paenibacilli were not really genetically accessible, since only a limited number of genetic engineering tools were available. Just recently, a rapidly expanding range of synthetic biology tools were developed. These tools enable in-depth genetic engineering for product formation [18,92] as well as targeted pathway modelling via multiplexed pathway perturbation towards streamlined chassis strains [93]. These developments are fundamentally supported by the availability of initial metabolic models which can be optimized in the near future [94].

Handling As a facultative anaerobe, *P. polymyxa* tends to produce typical overflow metabolites such as acetate or lactate, which can be reduced via metabolic engineering [92]. Based on their native production of the antibiotic polymyxin [95] as well as further antimicrobial peptides such as for example fucariscidin [96], Paenibacilli are very robust against contaminants and non-sterile complex media.

Tolerance *P. polymyxa* DSM 365 and related strains such as *Bacillus licheniformis*, or *B. ameloquefaciens* are known for their high tolerance to green organic solvents such as 2,3-butandiol, with concentration tolerances up to 115 g/L [97–99].

Metabolic capability Based on their broad carbohydrate substrate spectrum, Paenibacilli can be applied as production host of a great variety of carbohydrate polymeric substrates in biorefinery approaches [95,96], including lignin degradation [100,101]. Their versatile metabolism towards valuable products such as exopolysaccharides and for example enantiomeric pure R,R-2,3-butandiol or secondary metabolites such as antibiotics or antimicrobial peptides makes them highly interesting for metabolic engineering as well as agricultural applications in its native form [96]. Especially its ability to fix nitrogen and to convey growth promoting properties to plants makes *P. polymyxa* highly interesting for commercial applications.

(Box 4) *Bacillus methanolicus*

Growth efficiency *Bacillus methanolicus* MGA3 is a Gram-positive, aerobic, thermotolerant and relatively fast growing facultatively methylotroph which shows a μ of 0.65 and 0.53 h⁻¹ and q_s values of 30 and 36 C-mmol g_{CDW}⁻¹ h⁻¹ on methanol and D-mannitol as sole carbon and energy source, respectively [102,103]. The optimal growth temperature for *B. methanolicus* was determined to be around 50 – 55 °C [104,105].

Tool availability In contrast to other Bacilli, the genetic engineering toolbox for *B. methanolicus* is quite limited and thus restricts its efficient engineering [106]. Recently, a CRISPR interference (CRISPRi) system based on a catalytically deactivated Cas9 protein (dCas9) has been developed [107]. However, an efficient and robust gene deletion system is still pending. The whole genome sequence of *B. methanolicus* is available [108] and transcriptomics as well as metabolomics have already been applied to study the physiology of this promising bacterium [102,109].

Handling As a thermotolerant, restricted methylotroph *B. methanolicus* can be cultivated at elevated temperatures on methanol. Toxicity of methanol in combination with elevated temperature on the one hand favors auto sterility of the processes, and on the other hand complicates handling by necessary consideration of safety issues [110].

Tolerance *B. methanolicus* can cope with methanol concentrations of up to 2 M and grows at temperatures from 35 up to 60 °C which makes this bacterium highly interesting for industrial production [111].

Metabolic capability *B. methanolicus* is a so-called restricted methylotroph, which means that it can utilize beside methanol only D-mannitol, D-glucose, and D-arabitol as sources for energy and growth. In contrast to other strict methylotrophs the genome of *B. methanolicus* is equipped with the whole set of genes for the citric acid as well as the glyoxylate cycle, which are only weakly transcribed during growth on methanol [102]. Furthermore, *B. methanolicus* can grow in seawater [112] and has the ability to efficiently produce L-lysine and L-glutamate from methanol at elevated temperatures [104].

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