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REVIEW

BIOTECHNOLOGY AND BIOENGINEERING WILEY

The transition of *Rhodobacter sphaeroides* into a microbial cell factory

Enrico Orsi[1,2] | Jules Beekwilder[3] | Gerrit Eggink[1,4] | Servé W. M. Kengen[5] | Ruud A. Weusthuis[1]

[1]Bioprocess Engineering, Wageningen University, Wageningen, The Netherlands

[2]Max Planck Institute of Molecular Plant Physiology, Potsdam-Golm, Germany

[3]Wageningen Plant Research, Wageningen, The Netherlands

[4]Wageningen Food and Biobased Research, Wageningen, The Netherlands

[5]Laboratory of Microbiology, Wageningen University, Wageningen, The Netherlands

Correspondence

Ruud A. Weusthuis, Bioprocess Engineering, Wageningen University, 6708PB Wageningen, The Netherlands.
Email: ruud.weusthuis@wur.nl

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Abstract

Microbial cell factories are the workhorses of industrial biotechnology and improving their performances can significantly optimize industrial bioprocesses. Microbial strain engineering is often employed for increasing the competitiveness of bio-based product synthesis over more classical petroleum-based synthesis. Recently, efforts for strain optimization have been standardized within the iterative concept of "design-build-test-learn" (DBTL). This approach has been successfully employed for the improvement of traditional cell factories like *Escherichia coli* and *Saccharomyces cerevisiae*. Within the past decade, several new-to-industry microorganisms have been investigated as novel cell factories, including the versatile α-proteobacterium *Rhodobacter sphaeroides*. Despite its history as a laboratory strain for fundamental studies, there is a growing interest in this bacterium for its ability to synthesize relevant compounds for the bioeconomy, such as isoprenoids, poly-β-hydroxybutyrate, and hydrogen. In this study, we reflect on the reasons for establishing *R. sphaeroides* as a cell factory from the perspective of the DBTL concept. Moreover, we discuss current and future opportunities for extending the use of this microorganism for the bio-based economy. We believe that applying the DBTL pipeline for *R. sphaeroides* will further strengthen its relevance as a microbial cell factory. Moreover, the proposed use of strain engineering via the DBTL approach may be extended to other microorganisms that have not been critically investigated yet for industrial applications.

KEYWORDS

DBTL cycles, industrial biotechnology, metabolic engineering, microbial cell factory, *Rhodobacter sphaeroides*, strain engineering, synthetic biology

1 | INTRODUCTION

Bioeconomy aims to reform economic systems via sustainable use of renewable resources (Aguilar et al., 2019). Implementation of such an economy requires the transition from fossil-based to bio-based products (Bugge et al., 2016; Carlson, 2007). These compounds can be allocated within diverse market sectors, which differ in their scopes, volumes, and prices (Figure 1a). Their production can be obtained through biotechnological processes (Lopes, 2015). In industrial biotechnology, large improvements have been obtained by

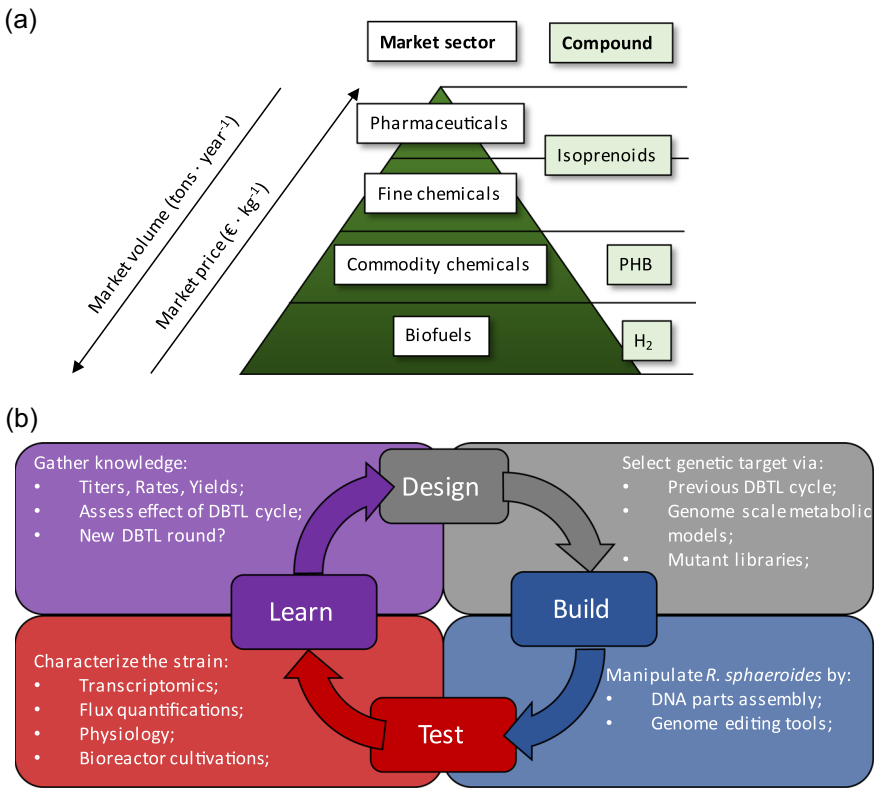

FIGURE 1 (a) Overview of the bioeconomy pyramid. This is divided into different sectors based in the market volume and price per kilo of the compounds produced. In the green boxes, compounds produced by *Rhodobacter sphaeroides* are shown. They are allocated to the respective target market sector. (b) Schematic representation of the Design-Build-Test-Learn (DBTL) cycle for rational strain engineering. Within each module are listed the key aspects discussed in this manuscript. PHB, poly-β-hydroxybutyrate [Color figure can be viewed at wileyonlinelibrary.com]

the optimization of microbial cell factories (S. Y. Lee et al., 2012; Park et al., 2018; Xu, Ban, et al., 2013). Historically, the bacterium *Escherichia coli* and the yeast *Saccharomyces cerevisiae* were the first organisms used as platforms for industrial bioproduction (Adams, 2016; Chen et al., 2013; Hong & Nielsen, 2012; Xu, Gu, et al., 2013).

Efforts for improving cell factories have been standardized within the "design-build-test-learn" (DBTL) concept (Nielsen & Keasling, 2016). The four DBTL modules are highly interdependent (Figure 1b), and each of them has a specific goal. In the "design" phase, the modification to implement is rationally planned. Then, in the "build" phase, the output from the previous step is translated into DNA host's manipulation. Subsequently, within the "test" phase, integrative approaches are used for assessing the effects of such modification on the cellular phenotype. Finally, in the "learn" phase, the generated experimental data are compared with the available literature. Then, two scenarios are possible. In the first one, the engineered strain reaches the expected levels of titers, rates, and yields (TRY) for the target product, therefore concluding the strain improvement process. In the second one, the TRY values generated are still below the expected threshold. In this case, the data obtained can be implemented for a new "design" step of a novel DBTL round. Usually, iterative DBTL cycles successfully concur with the improvement of TRY values for a compound of interest (Nielsen & Keasling, 2016).

Within this decade, the booming of genome editing technologies allowed to investigate nontraditional microorganisms as novel platforms for bioprocesses (Calero & Nikel, 2019; Moses et al., 2017). Microbial candidates are generally selected due to favorable phenotypic properties for a proposed bioprocess. In this regard, the α-proteobacterium *Rhodobacter sphaeroides* is an example of a nontraditional platform with high potential for industrial applications due to its metabolic versatility. Hitherto, the genetic toolkit for improving *R. sphaeroides* is limited compared with traditional industrial platforms. Nevertheless, recent advancements expanded the available technologies for studying and engineering this species, resulting in an improvement of its DBTL modules.

In this study, we propose that insights from all fields of investigation involving *R. sphaeroides* contributed to the foundation of its DBTL approach. Moreover, we reason that recent improvements in the DBTL pipeline can be employed for further optimizing this cell factory for industrial applications.

2 | *R. SPHAEROIDES* A LABORATORY ORGANISM WITH POTENTIAL AS CELL FACTORY FOR INDUSTRIAL BIOTECHNOLOGY

There are several reasons justifying the interest in *R. sphaeroides* as *chassis* for biotechnological productions.

1. This mesophilic prokaryote has been serving for long time as model organism for studying anoxygenic photosynthesis, but also chemotaxis and quorum sensing (Mackenzie et al., 2007). For this reason, a lot of fundamental metabolic knowledge is available.

2. It displays high metabolic versatility (Madigan & Gest, 1978), which can be exploited for different process conditions. It can thrive by aerobic or anaerobic respiration and anoxygenic photosynthesis (Mackenzie et al., 2007). This allows its use in photo-, chemo-, auto-, and heterotrophic bioprocesses. As a facultative anaerobe, it can grow without oxygen if an alternative electron acceptor is provided, such as dimethyl sulfoxide. Moreover, it accepts a wide range of organic substrates (Figure 2), ranging from C_1 compounds to fatty acids (Tabita, 1995). Therefore, a variety of feedstocks, including waste streams, can support microbial growth within a bioreactor.
3. Furthermore, *R. sphaeroides* is a natural producer of relevant bio-based compounds, such as (Figures 1a and 2): isoprenoids (Qiang et al., 2019; Zhang et al., 2018), poly-β-hydroxybutyrate (PHB; Kobayashi & Kondo, 2019), and hydrogen (H_2; Akroum-Amrouche et al., 2019; Shimizu et al., 2019a). These molecules range through different market sectors, thereby allowing different applications of this microorganism within the biotechnological industry.

Altogether, these characteristics render *R. sphaeroides* an attractive and versatile *chassis* to explore for applications within the bio-based economy. For efficiently improving this microorganism as cell factory, a DBTL approach is needed.

3 | DIFFERENT RESEARCH FIELDS CONTRIBUTED TO THE DBTL METHOD IN *R. SPHAEROIDES*

Establishment of a DBTL pipeline in a *chassis* requires contribution from different research fields, including -omics techniques, genome engineering, and phenotypic screening methods (Gill et al., 2016; Y. Liu & Nielsen, 2019). These are all integrated in the DBTL method, allowing the further improvement of a microorganism into a cell factory.

As mentioned above, *R. sphaeroides* has been studied in a wide range of fundamental and applied research areas, which mutually contributed to developing its DBTL method. In addition,

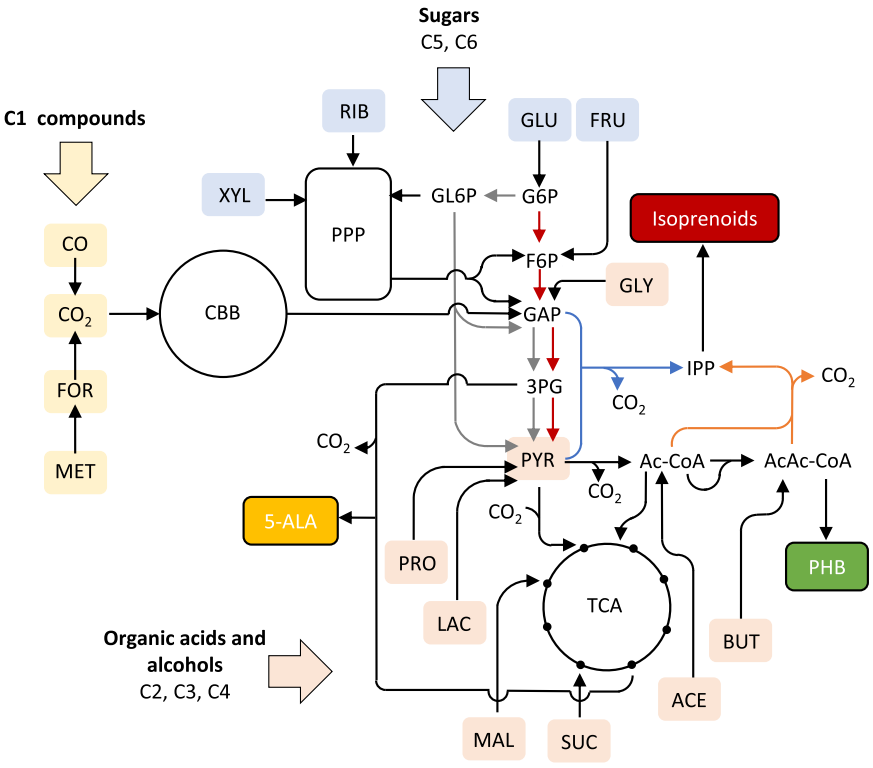

FIGURE 2 Lumped network of the carbon metabolism of *Rhodobacter sphaeroides*, including pathways for substrate uptake and product formation. Substrates are highlighted in different colors, each describing a different growth mode (light blue: chemoheterotrophic; light orange: photoheterotrophic; light yellow: photo- or chemolitho-autotrophic). The three carbon products described in this review are highlighted: 5-aminolevulinic acid (5-ALA, yellow); isoprenoids (red) and poly-β-hydroxybutyrate (PHB, green). Some pathways with parallel flux are highlighted, for example, glycolysis: Emden–Meyerhof–Parnas (red); Entner–Doudoroff (gray); isoprenoid synthesis: 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (blue); mevalonate pathway (orange). AcAc-CoA, acetoacetyl-CoA; Ac-CoA, acetyl-CoA; ACE, acetate; BUT, butyrate; CBB, Calvin–Benson–Bassham cycle; CO, carbon monoxide; F6P, fructose-6 phosphate; FOR, formate; FRU, fructose; G6P, glucose-6 phosphate; GAP, glyceraldehyde-3 phosphate; GL6P, 6-phosphoglucanolactone; GLU, glucose; GLY, glycerol; IPP, isopentenyl pyrophosphate; LAC, lactate; MAL, malate; MET, methanol; PPP, pentose phosphate pathway; PRO, propionate; PYR, pyruvate; RIB, ribose; SUC, succinate; TCA, Krebs cycle; XYL, xylose. The figure has been adapted from (Imam et al., 2013; Orsi, Mougiakos, et al., 2020; Tabita, 1995) [Color figure can be viewed at wileyonlinelibrary.com]

investigations in genetic engineering and synthetic biology expanded the toolboxes available for genomic modifications in this organism (Huo, 2011; Ind et al., 2009; Inui et al., 2003; Jaschke et al., 2011; Luo et al., 2020; Mougiakos et al., 2019; Tikh et al., 2014).

All findings and advancements generated in these research fields can be integrated into a DBTL pipeline for *R. sphaeroides* (Table 1). In the following sections, key technologies and advancements for each DBTL module are presented.

3.1 | Design

This phase focuses on the genome design of the cell factory. It is worth mentioning that optimal design of a cell factory should be tailored to the process conditions. Because *R. sphaeroides* is a metabolically versatile microorganism, different substrates can in principle support product formation (Figure 2). Therefore, this species holds potential for investigating bioproduction using relevant substrates for the bioeconomy, such as C1 compounds (Dürre & Eikmanns, 2015). Design for optimizing substrate uptake and further conversion into product should be introduced at this step.

For rationally modifying a cell factory, genetic targets need to be identified. This is possible when the host's genome is sequenced and annotated. The first sequenced genome of *R. sphaeroides* was obtained two decades ago (Mackenzie et al., 2001), and was followed by several revisions (Kontur et al., 2012; Ribeiro et al., 2012). Because of the high GC content of this microorganism (69%; Porter et al., 2011), heterologous gene expression often requires adaptation of the DNA sequence to meet the codon usage of *R. sphaeroides*.

Knowledge of essential genes and the effect of single-gene inactivations can provide a useful starting point for predicting the effect of their deletions. To our knowledge, two mutant collections are available for this microorganism (Hwang & Lee, 2008; Lang et al., 1995), and were used for investigating the carotenoid synthesis and quorum sensing, respectively.

In addition, use of genome-scale metabolic models allows predicting the effect of gene manipulations on metabolic pathways. A genome-scale model exists for *R. sphaeroides* (Imam et al., 2011), and was followed by a more recent expansion (Imam et al., 2013). These are stoichiometric models. Despite being descriptive of the overall metabolic network of the microorganism, they do not provide global information on the kinetic parameters of its metabolism.

On the other hand, use of kinetic models would increase the comprehension of the physiology of the microorganism, aiding the design phase of the DBTL pipeline. This type of model was already implemented in this species for describing H_2 synthesis in respect to NH_4^+ ions concentration in the medium (Waligórska et al., 2009). For other industrially relevant compounds, kinetic models were developed in *E. coli*, in particular for studying the biosynthesis isoprenoids (Weaver et al., 2015) and PHB (Van Wegen et al., 2001). Therefore, the collection of different kinetic parameters and their organization in genome-scale kinetic models is desirable for improving the design phase of the DBTL pipeline.

Ultimately, important knowledge has been obtained from fundamental studies on photosynthetic gene regulation and physiology (Gomelsky et al., 2008; Moskvin et al., 2005; Pappas et al., 2004; Zeilstra-Ryalls & Kaplan, 2004). This was further combined in a model for describing the effect of oxygen availability on the expression of photosynthetic genes (Pandey et al., 2017). All this knowledge can be applied for, for example, improving isoprenoid or H_2 production under different growth modes. Although these pieces of information were generated for different research purposes, their integration can aid in predicting the effect of genetic and environmental manipulations on *R. sphaeroides*.

3.2 | Build

In this phase, the designed genome modifications are implemented. The main system for delivering genetic information within *R. sphaeroides* is conjugation, although evidence of direct DNA transition via electroporation has been reported (Jun et al., 2014; Luo et al., 2020; Serdyuk et al., 2013). While the latter is more rapid in its execution, the presence of active restriction endonucleases that cleave exogenous DNA can hinder its realization. Therefore, the implementation of an efficient electroporation protocol might require prior inactivation of such restriction endonuclease systems. Conjugation, which is not affected by endonucleases, is the most used transformation technique for *R. sphaeroides*. Despite being less easy to operate (it involves the presence of a donor *E. coli* strain), this technique allows to reach high numbers of colony-forming units (CFUs) on a plate (Mougiakos et al., 2019). It is worth noting that high CFU values are beneficial when looking for rare mutations, which occur at low frequencies.

Traditionally, a suicide plasmid system was the preferred method for homologous recombination (HR) based chromosomal deletions or insertions, alongside with traditional mutagenesis (Jaschke et al., 2011). A CRISPR/Cas9 system was recently developed for the same purpose (Mougiakos et al., 2019), improving the efficiency of HR-based genome editing. An expansion of the Cas9-toolkit promptly followed, proving the capability of performing base-editing in this species (Luo et al., 2020). Moreover, integration of a large DNA fragment (>8 kb) was realized via transposon insertion (Orsi, Beekwlider, van Gelder, et al., 2020). Heterologous gene expression is possible via an inducible plasmid (Ind et al., 2009). Also, BioBrick™ systems have been developed (Huo, 2011; Tikh et al., 2014).

This building phase largely benefited from recent advancements, rendering genome editing in *R. sphaeroides* easier to implement. In particular, they allowed successful genomic manipulations ranging from single nucleotide substitutions (Luo et al., 2020; Mougiakos et al., 2019) to entire pathway integration (Orsi, Beekwilder, Peek, et al., 2020). Nevertheless, while implementation of cas9-mediated gene deletion resulted in efficient mutation rates, its use for gene integration is still rather inefficient (Luo et al., 2020; Mougiakos et al., 2019). Recently, template-independent genome editing was

TABLE 1 Overview of significant contributions from different research fields to the Design-Build-Test-Learn cycle of *Rhodobacter sphaeroides*

	Modules			
Field of study	**Design**	**Build**	**Test**	**Learn**
Photosynthesis	Genome-scale (Imam et al., 2011, 2013), Oxygen-response model (Pandey et al., 2017)	Electroporation (Jun et al., 2014; Luo et al., 2020; Serdyuk et al., 2013), suicide plasmid-mediated genome editing (Jaschke et al., 2011), and BioBricks™ (Tikh et al., 2014)	Transcriptomics and growth modes (Arai et al., 2008; Imam et al., 2014; Pappas et al., 2004)	Genes regulation and physiological behavior (Arai et al., 2008; Moskvin et al., 2005; Pandey et al., 2017; Pappas et al., 2004; Zeilstra-Ryalls & Kaplan 1995, 2004)
Quorum sensing	Mutant library (Hwang & Lee, 2008)			Genes regulation and physiological behavior (Hwang & Lee, 2008)
Tools development	Genome-scale metabolic model (Imam et al., 2011)	Electroporation (Serdyuk et al., 2013), replicative plasmid (Ind et al., 2009; Inui et al., 2003), BioBricks™ (Huo, 2011), and Cas9-toolkit (Luo et al., 2020; Mougiakos et al., 2019)		Expansion and acceleration of the toolkit for R. sphaeroides investigation and manipulation
Isoprenoid synthesis	Mutant library (Lang et al., 1995)	Pathway integration via transposon-insertion (Orsi, Beekwlider, van Gelder, et al., 2020), and Cas9-toolkit (Luo et al., 2020)	Transcriptomics (Zhang et al., 2019), media composition (Orsi, Folch, et al., 2019), growth modes (Yen & Chiu, 2007), and 13C flux ratio analysis (Orsi, Beekwilder, Peek, et al., 2020)	Improved TRY via: Adaptation of cultivation conditions (S. Liu et al., 2015; Orsi, Folch, et al., 2019; Yen & Chiu, 2007; Zhang et al., 2019), engineering of transcriptional regulators (Zhu, Lu, et al., 2017), pathways enzymes engineering (Lu et al., 2013, 2014, 2015; Qiang et al., 2019; Su et al., 2018), complete pathway replacement (Orsi, Beekwlider, van Gelder, et al., 2020), engineering of redox metabolism (Xu, Wu, et al., 2020; Zhu, Ye, et al., 2017), prevention of by-product formation (Zhu, Lu et al., 2017), and growth uncoupled isoprenoid production (Orsi, Mougiakos, et al., 2020)
PHB synthesis		Cas9-toolkit (Mougiakos et al., 2019)	Media composition (M. Kim et al., 2012)	Improved TRY via: Adaptation of cultivation conditions (M. Kim et al., 2012) and pathways enzymes engineering (Kobayashi & Kondo, 2019)
H_2 synthesis	Genome-scale metabolic model (Imam et al., 2011), Kinetic model (Waligórska et al., 2009)		Media composition (M. Kim et al., 2012; T. Liu et al., 2014; Waligórska et al., 2009), growth modes (E. J. Kim, Kim, et al., 2008), flux balance analysis (Golomysova et al., 2010), and 13C flux ratio analysis (Tao et al., 2012)	Improved TRY via: Engineering of transcriptional regulators Ryu et al. (2014); Shimizu et al. (2019b), Adaptation of cultivation conditions M. Kim, Baek, et al. (2006); M. Kim et al. (2012); Waligórska et al. (2009), Prevention of by-product formation Ryu et al. (2014); Tao et al. (2012)

Abbreviation: TRY, titers, rates, and yields.

described as an emerging technique for prokaryotic strain engineering (Finger-Bou et al., 2020). Although genes encoding for the nonhomologous end-joining repair proteins Ku and LigD are present in this microorganism, no evidence of their activity has been reported yet in *R. sphaeroides* (Luo et al., 2020). Meanwhile, a BioBrick™ system composed of seven promoters, seven ribosome-binding-sites (RBSs), and five terminators have been characterized in this species (Huo, 2011; Tikh et al., 2014). These numbers are considerably lower than those of the BioBrick™ parts available for *E. coli* (e.g., >300 promoters), and might limit the potential for fine-tuning of gene expression. Nonetheless, BioBrick™ components for *R. sphaeroides* were developed, which permitted modulation of gene expression, as already reported for, for example, isoprenoid and membrane-protein synthesis (Lu et al., 2014; Tikh et al., 2014).

Rapid exploration of this microorganism for industrial applications would benefit from a high-throughput and automated build phase, where a combination of genetic parts can be efficiently assembled and transferred within the microbial host. Such an automated pipeline can in principle be obtained using integrated microfluidic technologies (Shih & Moraes, 2016). These could support automated steps from DNA synthesis and assembly to transfer and selection within the microbial host.

3.3 | Test

Here, the effects of genomic manipulations are tested in respect to product formation. Because such experimentation should be reproducible through different DBTL cycles, standard experimental conditions are required.

Defined media have been designed for standardizing physiological studies under both photoheterotrophic (Imam et al., 2011; E. J. Kim, Kim, et al., 2008) and chemoheterotrophic cultivation conditions (Orsi, Folch, et al., 2019).

Different growth modes (E. J. Kim, Kim, et al., 2008; I. H. Lee et al., 2002; Yen & Chiu, 2007) and media compositions (M. Kim et al., 2012; Orsi, Folch, et al., 2019; Shimizu et al., 2019a) were tested to assess their effect on bioproduction. Often, an adaptation of the bacterium to different conditions was analyzed by transcriptomic studies (Arai et al., 2008; Imam et al., 2014; Pappas et al., 2004; Zhang et al., 2019).

Metabolic studies such as flux balance analysis (Golomysova et al., 2010) and ^{13}C-metabolic flux analysis (Fuhrer & Sauer, 2009; Fuhrer et al., 2005) have been used to study flux distributions in this microorganism. Moreover, ^{13}C-cultivations have been applied for determining flux partitioning during the synthesis of H_2 (Tao et al., 2012) and isoprenoids (Orsi, Beekwilder, Peek, et al., 2020).

In summary, this microorganism can be cultivated using defined media in both photo- and chemotrophic modes. The regulation of gene expressions for such growth conditions is largely known. This has been obtained via transcriptome analyses and has been exploited for improving bioproduction. Moreover, the intracellular fluxes controlling product formation have been characterized, also via the use of labeled isotopes. Therefore, a set of research techniques can be employed to thoroughly study *R. sphaeroides* phenotypes during synthesis of economically relevant compounds.

3.4 | Learn

3.4.1 | Improving TRY values and expanding the bioproduction portfolio

In the last step of the cycle, the effects of the modifications implemented in the microbial host are critically evaluated in terms of TRY values. Many studies improved such values for all the endogenous compounds synthesized by *R. sphaeroides* (E. J. Kim, Kim, et al., 2006; Kobayashi & Kondo, 2019; Lu et al., 2013, 2014, 2015; Ryu et al., 2014; Shimizu et al., 2019a; Zhang et al., 2018).

Moreover, *R. sphaeroides* revealed to be a versatile platform for producing heterologous isoprenoids like flavors and fragrances (Beekwilder et al., 2014; Chen et al., 2019; Schempp et al., 2018), as well as carotenoids like lycopene (Su et al., 2018) or β-carotene (Qiang et al., 2019). All these studies drive the improvement of *R. sphaeroides* towards a cell factory capable of producing an increasing range of different products.

3.4.2 | Examples of "learn" phase as input for a new DTBL cycle

As described above, the final "learn" step can be used as input for a new DBTL cycle. Fundamental studies provided knowledge that could be used for designing strain optimization strategies. An example comes from studies on transcriptional regulation of photosynthetic genes, whose knowledge was exploited for overexpressing the *ppsR* regulator for increasing coenzyme Q_{10} production while decreasing competition from carotenoid synthesis (Zhu, Lu, et al., 2017). Similarly, insights from fundamental research on the function of the transcriptional activator NifA was combined with the effect of adapting the cultivation conditions for investigating the competition between light-harvesting complex synthesis, nitrogenase activity, and H_2 production (E. J. Kim, Kim, et al., 2006; Ryu et al., 2014; Shimizu et al., 2019b).

Other application-oriented studies improved *R. sphaeroides* via iterative DBTL cycles. By thoroughly controlling rate-limiting enzymes expression via a library of RBSs, isoprenoid flux was increased via the endogenous 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Lu et al., 2014). Research on *R. sphaeroides* physiology demonstrated that the endogenous MEP pathway exclusively supports growth-coupled production, while expression of the heterologous mevalonate pathway allows synthesis also during nongrowth conditions (Orsi, Folch, et al., 2019). A strain was developed, in which the MEP pathway was functionally replaced by the mevalonate pathway (Orsi, Beekwlider, van Gelder, et al., 2020). Use of such a strain allowed to design a ^{13}C labeling-based

flux ratio analysis to study MEP and mevalonate split ratios (Orsi, Beekwilder, Peek, et al., 2020). Based on this knowledge, a strategy for the growth of uncoupled isoprenoid production was implemented, which consisted in exclusive exploitation of the mevalonate pathway in combination with inactivation of PHB synthesis (Orsi, Mougiakos, et al., 2020).

We predict that the application of DBTL cycles in *R. sphaeroides* will result in rapid improvements of this microbial platform for the existing bioprocesses. Moreover, it will allow exploring its versatile metabolism for bioproduction under new growth modes, where traditional cell factories cannot be used. Automated DBTL pipelines can be performed in infrastructures called "biofoundries" (Chao et al., 2017), where rapid prototyping and optimization of cell factories is implemented (Carbonell et al., 2018). Such an accelerated and high-throughput strain engineering approach is desirable for developing competitive cell factories for existing and future industrial applications of *R. sphaeroides*.

4 | AN OUTLOOK ON THE INDUSTRIAL APPLICATIONS OF *R. SPHAEROIDES*: CURRENT SITUATION AND FUTURE PERSPECTIVES

In the previous sections, we highlighted how technical advancements in the DBTL cycle can accelerate *R. sphaeroides* optimization for industrial applications. Here, we provide an outlook on the state of the art of industrial processes involving this microorganism. Moreover, in light of the recent developments, we propose to further extend its applications within the bio-based economy.

This microorganism is considered nonpathogenic and generally regarded as safe (GRAS). Nevertheless, a recent correspondence proved that the platform *Pseudomonas putida* (a consolidated cell factory) was erroneously described as GRAS (Kampers et al., 2019). Therefore, for supporting the wide use of *R. sphaeroides* for human applications, an accurate assessment of its biological safety and security in a laboratory and industrial settings is desirable. It is important to note that built-in safety mechanisms can also be implemented at the genetic level by the designer (Asin-Garcia et al., 2020).

To our knowledge, *R. sphaeroides* is being employed in few companies worldwide. The Dutch flavors and fragrances company Isobionics BV (www.isobionics.com) produces sesquiterpenes as aromas and ingredient compounds using this microorganism as a platform. The Chinese company CN Lab Nutrition (www.cnlabnutrition.com) focuses on nutraceuticals, and provides coenzyme Q_{10} with a rate of up to 30 tons per month. Moreover, a portfolio of nonnative isoprenoids has been synthesized using this bacterium in academic research (Beekwilder et al., 2014; Chen et al., 2019; Orsi, Folch, et al., 2019; Qiang et al., 2019; Su et al., 2018).

Another application of this bacterium is within the feed industry for livestock. The Chinese company Hebei Shixiang Biological Technology Co., Ltd. (www.hbshixiang.en.china.cn) commercializes *R. sphaeroides* biomass as poultry feed. Similarly, the Indian company Prions Biotech (www.prionsbiotech.com) provides fish feed solutions containing this bacterium. Although use of genetically modified *R. sphaeroides* might be discouraged for this application, cultivation parameters could be optimized thanks to available knowledge. This could allow to enrich biomass composition with high-value endogenous products such as carotenoids, cobalamin, coenzyme Q_{10}, or 5-aminolevulinic acid (S. Liu et al., 2016).

Although *R. sphaeroides*-derived products are commercialized in the feed and isoprenoid markets, no industrial application exists yet for PHB and H_2 production. The main reason is due to the low market price of these compounds (Figure 1a), which renders their cost-competitive production challenging. Because feedstocks constitute approximatively 40% of the total operating cost for the synthesis of such compounds (Choi & Lee, 1997; Khosravi-Darani et al., 2013), diverse range of substrates should be evaluated for reducing production costs. Waste streams can be used as substrates for microbial synthesis of PHB (Van Loosdrecht et al., 1997) or H_2 (Chandrasekhar et al., 2020). Several studies were performed on PHB and H_2 production from waste streams in *R. sphaeroides* (Ghimire et al., 2016; Gu et al., 1999; Luongo et al., 2017). Nevertheless, they did not result yet in the coupling of waste streams treatment to industrial production of these compounds. A drawback associated with this type of feedstocks is the variability of their elemental composition, which makes scaling-up of the processes cumbersome (Rodriguez-Perez et al., 2018).

Still, the use of cheap and renewable feedstocks could improve both commercial- and experimental-processes where *R. sphaeroides* is involved. This microorganism could be exploited for evaluating alternative feedstocks for the bio-based economy because of (i) its versatile metabolism, (ii) its wide substrate acceptance range, and (iii) recent advancements in its DBTL method. A closely related species, *R. capsulatus*, demonstrated efficient isoprenoid synthesis via aerobic-chemolithoautotrophic growth (Khan et al., 2015). This growth mode is becoming of particular interest due to the rising attention towards C1-carbon sources as feedstocks (Choi et al., 2020; Claassens et al., 2016; Cotton et al., 2020; Dürre & Eikmanns, 2015; Gleizer et al., 2019; Satanowski & Bar-Even, 2020; Yishai et al., 2016). In this perspective, state of the art technologies in the DBTL pipeline could be used in *R. sphaeroides* to optimize this platform for assimilation of C1 substrates, while coupling it to synthesis to a range of bio-based compounds.

5 | DISCUSSION

In this study, we summarized the state-of-the-art technologies composing the DBTL pipeline in *R. sphaeroides*. Moreover, we proposed that the use of such a streamlined method for strain engineering will consolidate this species as a valuable microbial cell factory for the bio-based economy. Ultimately, we presented an outlook on the industrial applications of this platform, which can be further expanded by means of DBTL cycles.

The DBTL concept was firstly introduced for model organisms like *E. coli* and *S. cerevisiae* (Nielsen & Keasling, 2016), which are considered traditional cell factories for prokaryotes and eukaryotes, respectively (Chen et al., 2013; Hong & Nielsen, 2012). In fact, these organisms were already model species in biology when genetic engineering developed in the 1970s (Chen et al., 2013; Hong & Nielsen, 2012). Ever since they have been investigated as cell factories (S. Y. Lee et al., 2012), their DBTL pipelines included cutting edge technologies for strain engineering.

The revolution in genetic engineering techniques that occurred within this decade allowed to investigate new-to-industry *chassis* for bioproduction (Calero & Nikel, 2019). The α-proteobacterium *R. sphaeroides* is included among these novel industrial platforms. Despite its original role as laboratory species for fundamental studies, this organism presents high versatility in its growth modes and substrate acceptance ranges (Mackenzie et al., 2007; Tabita, 1995). This metabolic flexibility reflected in employment of *R. sphaeroides* for many research topics, including photosynthesis, quorum sensing, chemotaxis, and production of H_2, PHB, and isoprenoids.

We reasoned that, while providing useful insights on the biology of the microorganism, these research areas developed critical technologies for establishing a DBTL pipeline in this species. Therefore, different fundamental and applied studies in *R. sphaeroides* concurred in developing this organism as a promising cell factory.

Moreover, recent advancements in the four modules of the DBTL method allowed to further consolidate *R. sphaeroides* as versatile platform for the bioeconomy. Of particular importance are the availability of a genome-scale metabolic model (Imam et al., 2011, 2013), a CRISPR/Cas9 toolkit (Luo et al., 2020; Mougiakos et al., 2019), and tools for flux ratio analysis in the central carbon and isoprenoid metabolisms (Orsi, Beekwilder, et al., 2020; Tao et al., 2012). We envision that practising DBTL cycles in *R. sphaeroides* will facilitate the study of this bacterium for industry-oriented applications. Moreover, use of automated biofoundries might further accelerate improvements in this species. Possibly, its versatile metabolism can be optimized for the use of cheap and renewable feedstocks, either consolidating existing bioprocesses or exploring new ones.

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CONFLICTS OF INTEREST

Jules Beekwilder is currently employed by Isobionics BV (The Netherlands).

AUTHOR CONTRIBUTIONS

Enrico Orsi conceptualized and wrote the whole manuscript under the supervision of Servé W. M. Kengen and Ruud A. Weusthuis. Jules Beekwilder, Gerrit Eggink, Servé W. M. Kengen, and Ruud A. Weusthuis read and reviewed the manuscript. All authors approved the submitted version.

ORCID

Enrico Orsi http://orcid.org/0000-0002-4741-0344
Jules Beekwilder https://orcid.org/0000-0003-3238-4427
Gerrit Eggink https://orcid.org/0000-0002-9867-6468
Servé W. M. Kengen https://orcid.org/0000-0001-6139-8946
Ruud A. Weusthuis https://orcid.org/0000-0001-6452-5701

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