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Unlocking the potential of defined co-cultures for industrial biotechnology: Opportunities and challenges

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ABSTRACT

Large-scale microbial-biotechnology processes for production of chemicals almost exclusively rely on pure cultures of microbial strains. Especially for extensively engineered pure cultures, process performance can be negatively affected, which can be caused by issues such as pathway imbalance, deterioration of productivity caused by genetic instability and enzyme promiscuity. An increasing number of studies demonstrate that, under 'academic' laboratory conditions, the use of defined co-cultures (i.e. deliberate mixtures of known microbial strains) offers unique possibilities for mitigating such drawbacks. These advantages differ for dissimilatory products, whose synthesis from one or more carbon substrates provides cells with free energy, and assimilatory products, whose synthesis requires a net input of free energy. Based on advances in experimental and theoretical research, this paper highlights how defined co-cultures can address several limitations of mono-cultures for production of low-molecular-weight compounds. From this largely academic perspective, we outline the key challenges for scaling these systems to industry, which underscore the need for innovative solutions and continued research in this area.

1. Introduction

In industrial biotechnology, production processes traditionally use single microbial strains (monocultures). While in many cases effective, such monocultures can suffer from problems such as genetic instability resulting in loss of productivity, pathway imbalance and incompatibility of envisioned production strains with the functional expression of key enzymes. Defined co-cultures, i.e. mixtures of known microbial strains, have been proposed as a means to address these challenges.

A large body of knowledge generated in microbial ecology underscores the importance of microbial interactions for the functionality and stability of natural microbial communities [1–3]. Microbial co-cultures are widely applied in food fermentation processes, to enhance product quality parameters such as nutritional value and flavor [4–6]. Underlying metabolic interactions in these processes include commensalism (one species benefits and the other is neither harmed nor helped), mutualism (both species benefit) and parasitism (one species benefits and the other is not harmed) [7]. In contrast to food fermentation processes, industrial biotechnology, which uses microorganisms for production of chemicals, only rarely makes use of co-cultures of different strains or species [8,9]. Instead, it predominantly relies on monocultures of microbial strains, whose performance in industrial processes is often extended or improved by classical strain improvement and/or genetic engineering.

Products of industrial biotechnology range from fragrances and pharmaceuticals to transport fuels and chemical building blocks. Based on their connection to microbial energy metabolism, products of industrial biotechnology can be divided into two distinct categories: dissimilatory and assimilatory products. Fig. 1 depicts both categories schematically. Since the advantages of co-cultures differ for these products, this review is split into two main parts: one around dissimilatory and one around assimilatory products. Dissimilatory products are compounds whose synthesis from a carbon substrate provides the cell with ATP. Examples include fermentation products such as ethanol and lactate, whose large-scale production typically occurs in anaerobic or oxygen-limited batch cultures. Assimilatory products are compounds whose synthesis from a carbon substrate requires a net investment of ATP and biosynthetic precursors. Therefore, in contrast to dissimilatory

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product formation, assimilatory product formation competes for ATP with cellular growth and maintenance. Assimilatory products produced by microorganisms include a wide and growing range of molecules, for which the biosynthetic pathways have often been introduced and/or improved by extensive genetic engineering. Examples of assimilatory products include structurally diverse molecules such as alkaloids, terpenoids, fatty acids, flavonoids, phenylpropanoids, and polyketides. To efficiently generate the ATP required for product formation, they are typically manufactured in aerobic fed-batch or continuous fermentation processes.

The TRY acronym (titer, rate, yield) captures three key performance indicators of industrial biotechnology processes [10]. Titer refers to the final concentration of the product (c_p, kg product per m^3), which is highly relevant for efficient product purification by processes such as distillation or chromatography. Moreover, titer impacts the required bioreactor volume and, thereby, required capital investments. Rate can be expressed as productivity of the entire reactor (R_p, kg product per hour). Volumetric productivity (r_p, kg product per m^3 per hour) links productivity to installed reactor volume and is the product of the biomass concentration (c_x) and the biomass specific-production rate (q_p, kg product per kg biomass per hour). Processes with a higher volumetric productivity r_p require lower capital investments in installed bioreactor volume. The third key performance indicator is the product yield on substrate ($Y_{p/s}$, kg of product formed per kg of consumed substrate), which is equal to the ratio of the product-formation and substrate-consumption rates ($Y_{p/s} = R_p/R_s = r_p/r_s = q_p/q_s$). The $Y_{p/s}$ has a particularly strong impact on process economy for commodity products, for which feedstock costs can make up a large fraction of the overall production costs. For example, costs of the carbohydrate feedstock can account for up to 70 % of the costs of yeast-based ethanol production [11].

Genetic stability of high-performance cell factories can be an additional key performance indicator in large-scale applications. Industrial fermentation processes for assimilatory processes are operated at reactor volumes of up to several hundred m^3, while bioreactor volumes for ethanol fermentation can even run into several thousand m^3. This implies that the 'seed train' needed to scale up from milliliter-scale frozen culture samples encompasses many generations during which a strain's full production capacity needs to be maintained. Genetic stability is even more important when fermentation processes are operated continuously or as serial batch cultures with the aim to improve process economics and/or sustainability.

The aim of this paper is to review results from fundamental, application-inspired studies aimed at investigating if and how the use of defined microbial co-cultures can circumvent or mitigate limitations of monocultures as production systems. It primarily discusses results from studies that generated quantitative information on parameters such as titer, rate, yield and genetic stability. In addition, it focuses on production of low molecular weight compounds. Discussion of high molecular weight compounds (e.g. proteins and biopolymers), and their production processes (including consolidated bioprocessing, which combines enzyme production and fermentation in one step [12–16]), is therefore outside the scope of this review. The different relationships of assimilatory and dissimilatory product formation with cellular energy metabolism (Fig. 1) generate different challenges in monocultures and, consequently, provide different opportunities for the use of defined co-cultures. We therefore separately discuss co-culture strategies for dissimilatory and assimilatory product formation. These examples are summarized in Table 1. Based on this information, we subsequently outline key challenges that need to be addressed to enable broader use of defined co-cultures in industrial biotechnology. Together, these insights underline the potential of defined co-cultures to further develop and improve industrial biotechnology.

2. Dissimilatory products

The large product volumes and narrow profit margins of dissimilatory products [10] imply that small improvements of titer, rate and/or yield can already confer significant economic benefits. Improving product yield on substrate ($Y_{p/s}$), for example by reducing by-product formation, is especially important because substrate costs strongly influence process economics [10,11]. Recycling of biomass from one fermentation process to the next can help to improve volumetric productivity (r_p) [29]. When contamination by undesirable competing microorganisms is kept at bay, such biomass recycling strategies can work very well because dissimilatory product formation from a single substrate is linked to growth and survival and therefore confers a selective advantage to fast-producing mutants [30,31]. To improve process

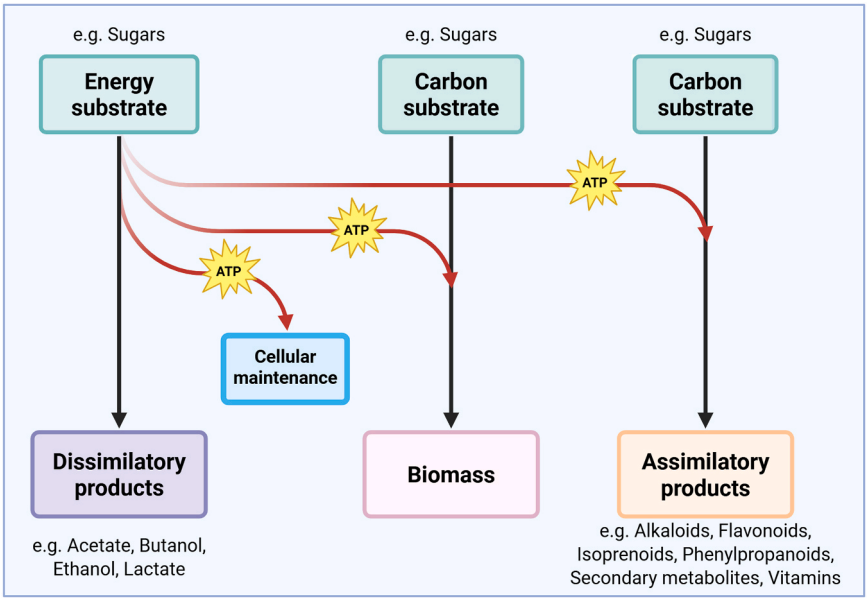

Fig. 1. Dissimilatory and assimilatory products. Dissimilatory products are compounds whose synthesis from (a) substrate(s) provides the cell factory with ATP, which can be used for growth and cellular maintenance. Synthesis of assimilatory products from (a) substrate(s) requires a net ATP input and is therefore dependent on dissimilatory pathways to generate ATP. Created in BioRender. https://BioRender.com/lf23uka.

Table 1
Selection of references on defined microbial co-cultures for industrial biotechnology, for which titer, rate, yield and/or stability of strain performance are quantitatively described.

Product	Substrate(s)	Target	Strategy	Titer/Rate/Yield of co-culture (monoculture between brackets)	Ref.
Ethanol	Glucose	Increase **product yield** on substrate through conversion of by-products to product.	Co-culture of PRK-RuBisCo based and A-ALD-based *S. cerevisiae* strains, where the A-ALD strain converts by-products from the PRK-RuBisCo strain (acetate and acetaldehyde).	1.67 (1.64, PRK-RuBisCo-based strain) g ethanol•(g glucose)$^{-1}$	[17]
1,3-propanediol	Glycerol	Increase **product yield** on substrate through conversion of by-products to product.	Co-culture of *Klebsiella spp* and *Shewanella oneidensis,* where *S. oneidensis* oxidizes the by-product lactate from *Klebsiella spp* supplying electrons for its product formation.	0.44 (0.41) g 1,3-PD •(g glycerol)$^{-1}$	[18]
Lactate	Glucose and xylose	Increase **volumetric productivity** through improved sugar utilization kinetics	Co-culture of two *E. coli* strains, each specialized in glucose and xylose consumption.	5.8 (2.6) g lactate•L^{-1}•h^{-1}	[19]
n-Butanol	Glucose and xylose	Increase **volumetric productivity** through improved sugar utilization	Co-culture of two *E. coli* strains, each specialized in glucose and xylose consumption.	0.12* (0.072*) g *n*-butanol•L^{-1}•h^{-1}	[20]
Ethanol	Arabinose, glucose and xylose	Improved **genetic stability** during long term cultivation on sugar mixture	Co-culture of three *S. cerevisiae* strains, each specialized in arabinose, glucose and xylose consumption.	Cycle length: stable at 35 h for 1000 h cultivation (increase from 25 h to 51 h)	[21]
Rosmarinic acid	Glucose	Increase **product titer** by pathway modularization	Employ engineered three-strain *E. coli* co-culture to balance complex diverging-converging biosynthetic pathway by improved metabolic flux.	74 (4.5) mg•L^{-1}	[22]
Caffeyl alcohol	Glucose	Increase **product titer** by spatial separation to avoid negative impact of enzyme promiscuity	Co-culture of *p*-CA-producing and HpaBC-expressing *E. coli* strains to prevent L-dopa by-product formation due to HpaBC promiscuity.	0.4 (0.03) g•L^{-1}	[23]
Eugenol	Glycerol and glucose	Increase **product titer** by preventing intermediate toxicity and enzyme promiscuity through pathway modularization	Tripartite *E. coli* co-culture separating coumarate, ferulic acid, and eugenol modules to avoid substrate competition and toxic build-up.	0.07 (0) g•L^{-1}	[24]
Anthocyanidin-3-*O*-glucosides	Glucose	Increase **product titer** of anthocyanin through pathway modularization	Four-strain polyculture distributing 15 pathway enzymes across modules to reduce metabolic burden and enable *de novo* anthocyanin biosynthesis.	~ 9.5* (0) mg pelargonidin-3-*O*-glucoside •L^{-1}	[25]
Oxygenated taxanes	Xylose and acetate	Extend **product range** and improve **product titer** by addressing host-specific enzyme functional expression challenge	Modular co-culture of *E. coli* producing taxadiene and *S. cerevisiae* expressing plant P450 fusion protein to support oxygenation steps in taxane biosynthesis.	0.03 (0) g•L^{-1}	[26]
Various strigolactones	Glucose	Extend **product range** and improve **product titer** by addressing host-specific enzyme functional expression challenge	Bipartite *E. coli* and *S. cerevisiae* co-culture: *E. coli* produces carlactone (3 enzyme-types), while yeast expresses downstream P450 enzymes for conversion to specific strigolactones.	Up to 47 (0) µg•L^{-1}	[27]
(S)-Norcoclaurine	Glucose and xylose	Improve **product titer** by binary modularization of two-species co-culture and module optimization.	*S. stipitis* produces shikimate from mixed sugars; *S. cerevisiae*, engineered with *Aspergillus* shikimate transporters, imports shikimate and converts it to (S)-norcoclaurine.	11.5 (0.1) mg•L^{-1}	[28]

* Values calculated based on information in figures or in text.

sustainability and reduce land use for production of fermentation feedstocks, conversion of sugar mixtures generated by hydrolysis of lignocellulosic agricultural residues is intensively explored [32,33]. Below, we will discuss examples of how the use of defined co-cultures has been explored as a means to (i) improve $Y_{p/s}$ by minimizing by-product formation (Section 2.1), (ii) improve volumetric productivity (r_p) during conversion of sugar mixtures (Section 2.2) and (iii) improve genetic stability in repeated batch cultures grown on sugar mixtures (Section 2.3). These scenarios are illustrated in Fig. 2a-c.

2.1. Minimizing by-product formation to improve yield

Ethanol production with the yeast *Saccharomyces cerevisiae* is the largest-volume process (ca. 100 Mton per year [31]) in industrial biotechnology. In anaerobic cultures of this yeast, including the large-scale batch cultures used for industrial ethanol production, glycerol formation serves as essential 'redox sink' for re-oxidation of a surplus of NADH generated in biosynthetic reactions. Without mitigating measures, glycerol formation would account for a loss of 4 % of the carbohydrate feedstock used for yeast-based ethanol production [34].

A metabolic engineering strategy to eliminate glycerol formation was based on functional expression of heterologous genes encoding the two signature enzymes of the Calvin cycle for CO_2 fixation, phosphoribulokinase (PRK) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Introduction of functional PRK and Rubisco in *S. cerevisiae* enabled a redox-cofactor-neutral conversion of glucose to 3-phosphoglycerate, whose subsequent NADH-dependent conversion to ethanol and CO_2 could replace the role of glycerol as redox sink. In fast-growing, glucose-grown anaerobic batch cultures, this strategy enabled an over 10 % higher $Y_{p/s}$ [35]. However, at suboptimal growth rates, which occur in the later stages of industrial processes due to ethanol accumulation and depletion of non-sugar nutrients, an overcapacity of the engineered PRK-Rubisco bypass led to formation of the by-products acetaldehyde and acetate [36]. An alternative metabolic engineering strategy for coupling reoxidation of 'excess' NADH to ethanol formation was based on introduction of a heterologous acetylating acetaldehyde dehydrogenase (A-ALD) [37]. Together with native yeast enzymes, A-ALD enables the NADH-dependent reduction of acetate to ethanol. However, the concentration of acetate in 'first-generation' feedstocks for ethanol production such as corn starch hydrolysates is too low to completely replace glycerol production in acetate-reducing yeast strains. Co-cultivation of an engineered PRK/Rubisco-based and an A-ALD-based strain was shown to combine the advantages of the two strains: low concentrations of acetate present in the medium, as well as acetaldehyde and acetate generated by the PRK/Rubisco strain, were efficiently converted to ethanol by the A-ALD strain, while the

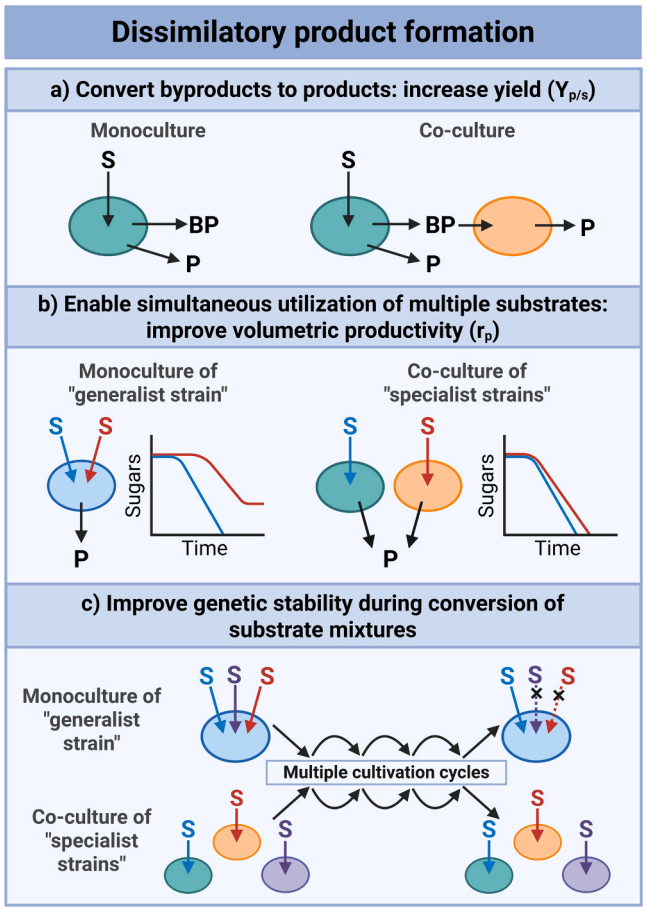

Fig. 2. Potential benefits of using defined co-cultures to improve titer, rate, yield and stability during production of low molecular weight dissimilatory products. a) In defined co-cultures, microorganisms can utilize or sequester by-products formed by other microorganisms to increase overall product yield from substrate. **b)** Defined co-cultures of specialist strains (utilizing a single substrate) could allow for a higher consumption rate of each substrate due to preferential substrate utilization by the generalist strain. **c)** Generalist strains are more likely to become genetically unstable after multiple cultivation rounds on substrate mixtures than specialist strains, resulting in diminished substrate utilization capabilities. S = substrate; P = product; BP = By-product. Created in BioRender. https://BioRender.com/zsu15iq.

PRK/Rubisco strain supported acetate- and acetaldehyde-independent, high-yield ethanol production. Optimization of the inoculation ratio of this commensalistic co-culture enabled overall fermentation times equal to those of monocultures of the PRK/Rubisco strain [17].

Wang *et al.* [18] explored a strategy for channelling by-products into product formation that involves two bacterial species. Anaerobic cultures of the *Klebsiella* strain used in this study produced 1,3-propanediol (1,3-PD) from glycerol, a conversion that requires a net input of electrons in the form of NADH. In the *Klebsiella* strain, this NADH is made available by converting part of the glycerol to more oxidized by-products such as acetate and lactate [38], which limits the maximum yield of 1,3-PD on glycerol. To mitigate by-product formation, the *Klebsiella* strain was co-cultivated with an exo-electrogenic *Shewanella oneidensis* strain. The rationale of this co-cultivation strategy was that *S. oneidensis* can oxidize lactate, generated by the *Klebsiella* strain, to acetate and CO_2 and transfer the electrons that are released during this oxidation to the *Klebsiella* strain. This interspecies electron transfer may occur either via exported $FMNH_2$ and reduced riboflavin or via direct interspecies electron transfer through pili [18,39]. The *Klebsiella* strain then uses the electrons for reducing glycerol to 1,3-PD. Consistent with this mutualistic mechanism, co-cultivation of the two bacteria led to a 7.3 % higher yield of 1,3-PD on glycerol than observed in monocultures of the *Klebsiella* strain.

Overall, these examples show that co-cultures can be used to convert by-product into product, thereby improving product yield on substrate.

2.2. Improving mixed-substrate utilization

Combined chemical and enzymatic hydrolysis can release fermentable sugars from agricultural residues such as corn stover and sugar cane bagasse, as well as from 'energy crops' such as switchgrass [32,33]. Use of such 'second-generation' feedstocks offers a large potential for improving the carbon footprint of industrial biotechnology. However, in contrast to feedstocks such as cane sugar or hydrolysed corn starch, these feedstocks contain mixtures of multiple sugars, with glucose and two pentose sugars, xylose and arabinose as main contributors. Industrial microorganisms such as *S. cerevisiae* and *Escherichia coli* have been extensively engineered to construct 'generalist' strains that convert mixtures of these three sugars. While these strains achieve high product yields on the sugar mixture under anaerobic conditions, their fermentation kinetics are often suboptimal. Engineered strains typically first consume the glucose at a high rate, while pentoses are consumed in a second, much slower sugar fermentation phase, thereby reducing the volumetric productivity r_p [21,40].

Generalist strains need to simultaneously synthesize enzymes for multiple substrate-conversion pathways. Monod pioneered research on diauxic growth, which is the sequential use of preferred and less-preferred substrates when these are provided together in batch cultures [41]. The resulting bi-phasic growth illustrates how natural evolution yielded regulation mechanisms that, in the presence of non-limiting concentrations of multiple substrates, prevent simultaneous allocation of cellular resources to multiple substrate-utilization pathways. Instead, microorganisms have evolved to maximize growth rate by allocating cellular resources to fast conversion of a single substrate at a time [42]. This principle led to multiple application-inspired studies, in which fermentation kinetics of defined co-cultures of 'substrate specialist strains' in cultures grown on sugar mixtures were compared with those of monocultures of a generalist strain.

Flores *et al.* [19] compared fermentation kinetics in aerobic cultures on a glucose-xylose mixture of an *E. coli* generalist strain capable of converting glucose and xylose, with those of a bipartite co-culture of glucose- and xylose-specialist *E. coli* strains grown on the same medium. An over 2-fold higher volumetric productivity of lactate was observed in the co-cultures than in cultures of the generalist strain (5.8 g lactate L^{-1} h^{-1} versus 2.6 g L^{-1} h^{-1}). In a conceptually similar study, Saini *et al.* [20] compared product formation in anaerobic cultures, grown on a glucose-xylose mixture, of a generalist *E. coli* strain and a co-culture of two sugar-specialist strains, all engineered for *n*-butanol production. In a 36-h growth experiment, the generalist strain produced 2.6 g L^{-1} *n*-butanol and converted only about 60 % of the available sugars. Over the same period, the co-culture produced 4.4 g L^{-1} n-butanol and achieved near-complete sugar conversion [20]. The improved productivities observed in these studies are consistent with an improved protein allocation in the co-culture and/or elimination of a pathway interference in generalist strains.

The above examples, as well as additional studies [43,44], show that the use of co-cultures of specialist strains can improve production rates during conversion of mixed substrates.

2.3. Enhancing genetic stability during sequential batch cultivation on mixed substrates

Dissimilatory product formation can, via generation of ATP (Fig. 1), be directly coupled to growth rate. This principle has been extensively used to increase product formation rates by adaptive laboratory evolution in serial-batch monocultures [30]. Selection for improved fermentation kinetics has also been documented for the Brazilian bioethanol

industry, in which ethanol production from cane sugar involves recycling of yeast biomass from one culture to the next over multi-month campaigns [31]. In contrast to these observations on single-substrate cultures, studies in which serial-batch monocultures of 'generalist' strains were grown on mixtures of carbon substrates, reported a deterioration of overall fermentation kinetics (Figs. 2**b and** 2**c**, [21,45,46]). An *S. cerevisiae* strain engineered for efficient conversion of glucose, xylose and arabinose showed a progressive increase of overall fermentation times when subjected to sequential batch cultivation cycles on mixtures of xylose and arabinose [45] or glucose, xylose and arabinose [21,46].

In a batch culture grown on equivalent concentrations of multiple substrates, the largest number of generations occurs during growth on the most preferred substrate. In serial batch cultures, selective pressure for faster utilization of a substrate is proportional to the number of generations of growth on that substrate. In cultures grown on substrate mixtures, competition for cellular resources or other interference of pathways will therefore preferentially select for faster utilization of the already favored substrate, at the expense of growth rates on the less preferred substrates. The resulting evolution towards substrate specialization rather than towards co-utilization presents a challenge in developing biomass-recycling strategies for the industrial fermentation of sugar mixtures with monocultures of generalist strains.

A co-culture of specialist strains that can each ferment only a single substrate in a mixture is not expected to show deterioration of fermentation kinetics during serial batch cultivation on substrate mixtures. Instead, in such cultures, each strain is anticipated to experience selective pressure to improve growth rate on its 'assigned' sugar. This concept was experimentally tested by Verhoeven *et al.* [21], who used metabolic engineering and adaptive laboratory evolution to obtain specialist *S. cerevisiae* strains that were able to anaerobically ferment xylose or arabinose when grown on a mixture of glucose, xylose and arabinose. A non-engineered strain of *S. cerevisiae* that cannot ferment pentose sugars was used as glucose specialist. A consortium of the three specialist strains was grown in anaerobic sequential batch cultures on glucose, xylose and arabinose, whose relative concentrations were chosen to mimic those in lignocellulosic hydrolysates. In a control experiment with a generalist strain, overall fermentation time increased from approximately 25 h in the first cycle of sequential batch fermentation to over 50 h in the 24th cycle (Fig. 2**b**). In contrast, over a similar number of cycles, fermentation kinetics of co-cultures of the three specialist strains improved rather than deteriorated [21].

While confirming that the use of specialist strains circumvents the degeneration of fermentation kinetics that was observed in monocultures of a generalist strain, the study of Verhoeven et al. [21] also identified a potential trade-off. The strong preference of the specialist strain for glucose led to a rapid build-up of biomass. Despite the lower biomass-specific conversion rates of xylose and arabinose, this biomass build-up still enabled a high volumetric conversion rate of these pentoses after glucose had been consumed. Instead, in the consortium of specialist strains, the lower conversion rate of the pentoses led to a longer overall conversion time than observed for the generalist strain. While fermentation kinetics of the consortium improved during serial batch cultivation, the overall fermentation time after the 24th cycle was still ca. 20 % slower than observed in the first cycles of serial batch cultivation of the generalist strain. This trade-off is influenced by the relative concentrations of the individual substrates, which determine the initial biomass concentration of each specialist strain upon initiation of the next growth cycle.

The above example shows that, in co-cultures, strains can be engineered to convert only one of the substrates in a substrate mixture. When producing a dissimilatory and therefore growth-coupled product, each specialist strains is under selective pressure to optimize its biomass specific productivity (q_p). This selective pressure prevents the deterioration of fermentation kinetics that is seen during prolonged batch cultivation of generalist strains on sugar mixtures.

In summary, for dissimilatory processes, defined co-cultures can improve yield, substrate range, and long-term genetic stability by distributing metabolic tasks over specialized strains.

3. Assimilatory products

In comparison with dissimilatory products, assimilatory processes (whose synthesis requires a net energy input) face different challenges. These include competition of growth and product formation for cellular resources, long product pathways, and a need to synthesize complex, non-native enzymes. In these cases, co-cultures offer alternative options to improve process performance.

Introduction of metabolic pathways for synthesis of complex, non-native assimilatory products by microbial cell factories can require expression of dozens of genes, sourced from multiple donor organisms [47,48]. Producing all the encoded enzymes in a single engineered microorganism can be challenging. The umbrella term 'metabolic burden' is often used to capture negative effects of such intensive metabolic engineering on performance and genetic stability of the resulting strains [49,50]. Most studies on the use of co-cultures for assimilatory product formation aim to mitigate metabolic burden by spatial segregation of product-pathway modules in different microbial strains [51].

Native protein synthesis is already the most ATP-intensive process in growing, wild-type microbial cells [52]. Since, moreover, cell-volume and membrane-surface limitations impose constraints on cellular protein content [53,54], expression of heterologous pathways will compete with native cell functions for amino acids, ATP and proteome space. The impact of this competition is especially large when product pathways involve enzymes that have a low catalytic turnover rate (k_{cat}) and therefore need to be expressed at high levels to sustain relevant *in vivo* fluxes. The resulting negative impact on growth rate can confer a substantial selective advantage to less- or non-producing mutants [55]. It is often assumed that even distribution of pathway enzymes over different strains can alleviate this protein burden and, thereby, enable improved productivity of co-cultures [51,56]. However, based on theoretical considerations [57], equal distribution of pathway-related protein mass over multiple strains does not in itself enable a higher biomass-specific conversion rate than is obtained in a monoculture. Instead, unless equal distribution affects intracellular concentrations of pathway intermediates and/or effectors and thus enables a higher *in-vivo* substrate saturation (V/V_{max}) of rate-controlling enzymes, it will not influence the overall protein requirement. Achieving a given rate of product formation will then, per g of biomass, require the same amount of protein in mono- and co-cultures [58]. Transport of pathway intermediates between co-culture partners can decrease efficiency due to dilution of intermediates in the extracellular space and/or costs associated with the expression of transporters [51,57]. To have a net positive impact, co-cultivation strategies should therefore address metabolic burden issues that substantially constrain monoculture performance and cannot be addressed by straightforward metabolic engineering. Below, we will discuss examples of how the use of defined co-cultures can address challenges associated with catalytic promiscuity of pathway enzymes, by-product formation caused by pathway imbalance, and sub-optimal expression of key enzymes. Fig. 3a, b and c schematically show the advantage of co-cultures for each of these challenges. In addition, we will discuss engineering of transport reactions, definition of metabolic modules and the relevance and control of the relative abundance of co-culture partners.

3.1. Addressing enzyme-related constraints in complex pathways

Enzyme promiscuity, i.e., the ability of an enzyme to perform different reactions [59], is a common challenge in the implementation of complex product pathways in industrial microorganisms [60,61]. The same holds for by-product formation caused by imbalances in

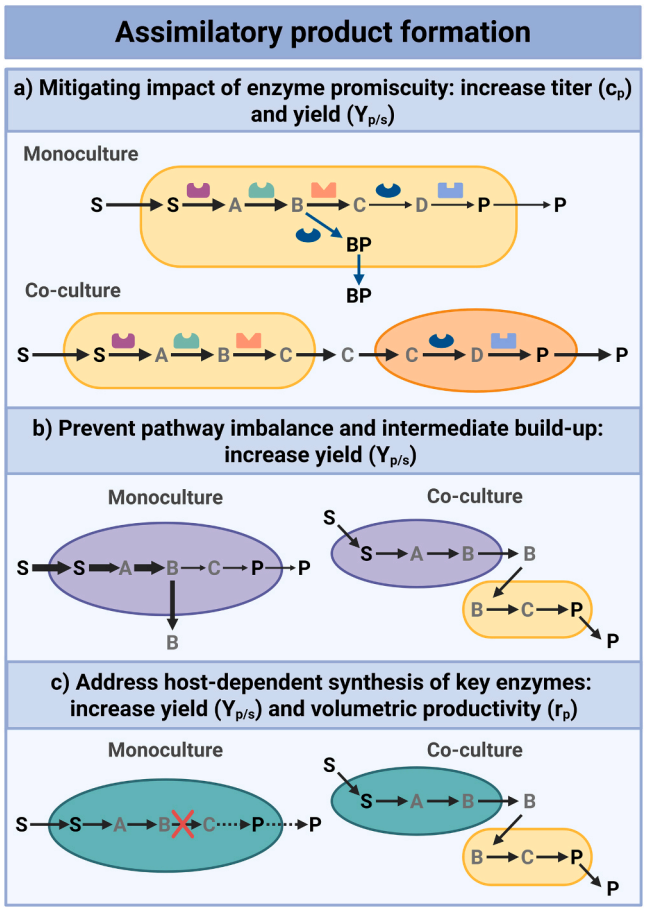

Fig. 3. Potential benefits of using co-cultures to improve titer, rate, yield and stability during production of low molecular weight assimilatory products. a) Enzyme promiscuity can cause by-product formation in long heterologously expressed product pathways. Co-cultures could mitigate this reduction in product yield by splitting pathways over separate modules, thereby spatially segregating the promiscuous enzyme from its substrate. **b)** Co-cultures can be used to reduce the accumulation of (toxic) intermediates that would otherwise inhibit growth and lower product yield. **c)** Co-cultures of different species can be used to overcome host-specific limitations of enzyme expression. S = substrate; P = product; BP = By-product. Created in BioRender. https://BioRender.com/nw7b4y7.

engineered product pathways. Research on engineering of microbial cell factories for production of phenylpropanoids stimulated multiple studies on how these challenges can be mitigated by co-cultivation strategies.

Enzyme promiscuity can lead to by-product formation, which lowers the product yield on the substrate. This is particularly problematic for long heterologous pathways, as it creates side reactions that compete with the main product path. Co-cultures can offer a solution by separating the enzyme and the metabolite for which it has promiscuous activity.

This enzyme promiscuity, for example, played a key role in a study on metabolic engineering of *E. coli* to produce monolignols [23]. Engineering of the native shikimate pathway for aromatic amino-acid synthesis, combined with introduction of a 5-step pathway for conversion of tyrosine to *p*-coumaryl alcohol (*p*-CA), enabled *p*-CA titers in shake-flask cultures of up to 0.5 g•L^{-1}. However, extension of the *p*-CA pathway by overexpression of a 4-hydroxyphenylacetate-3-hydroxylase (HpaBC) that converts *p*-CA to caffeyl alcohol (CfA), yielded a CfA titer of only 0.03 g•L^{-1}. This low titer was attributed to promiscuity of HpaBC which, in addition to the desired reaction, also converted tyrosine into the undesirable by-product L-dopa. Based on the observation that *p*-CA, but not tyrosine, easily crosses the *E. coli* cell membrane, the *p*-CA-producing strain was co-cultivated with a strain that overexpressed HpaBC in an otherwise wild-type background. After optimization of the inoculum ratio of the two strains, a CfA titer of 0.4 g•L^{-1} was achieved in shake-flask-grown commensalistic co-cultures, indicating that expression of the promiscuous HpaBC enzyme in a separate strain successfully prevented by-product formation [23].

Brooks *et al.* [24] implemented a pathway in *E. coli* for production of the plant metabolite eugenol, in which tyrosine is first converted to coumarate, whose hydroxylation then yields caffeate. In the following two reactions, caffeate is first methylated to yield ferulic acid, which is then converted into feruloyl-CoA by the enzyme 4-hydroxycinnamoyl--CoA ligase (4-CL). However, in addition to ferulic acid, 4-CL can also use coumarate as a substrate [24,62], thereby diverting this key precursor from product formation. To address promiscuity of 4-CL and other enzymes in the eugenol pathway and to prevent build-up of the toxic intermediate coumarate, a tripartite co-culture was designed and constructed. The first *E. coli* strain was engineered for efficient *de novo* production of coumarate (Module 1), a second strain for the two-step conversion of coumarate to ferulic acid (Module 2), and a third strain for the 5-step conversion of ferulic acid to eugenol (Module 3). This modular design effectively separated 4-CL, which was highly expressed in Module 3, from upstream metabolites. In addition, increasing the inoculum size of the strain carrying Module 2 relative to that of the other two strains prevented accumulation of toxic levels of coumarate. A eugenol titer of 0.07 g•L^{-1} was achieved in shake-flask cultures of the tripartite commensalistic co-culture, while no eugenol production was detected in a monoculture of an *E. coli* strain carrying all three modules. An additional advantage of the modular co-culture approach was demonstrated by further experiments, in which genetic modification or omission of Module 2 enabled production of chavicol and hydroxychavicol, respectively [24].

The above examples illustrate that by dividing the pathway between multiple strains, the promiscuous enzyme's side reaction was effectively bypassed, improving the yield of the desired product. Moreover, as also demonstrated by a study on the production of anthocyanins by tetrapartite *E. coli* co-cultures [25], the combination of strains optimized for precursor generation with 'terminal product formation' strains enables flexible 'mix and match' strategies for extending product range.

Pathway imbalances in a single strain can reduce product yields on substrate, for example by accumulation of intermediates. Balancing the activities of individual pathway enzymes is particularly challenging in the case of non-linear pathways, where optimal distribution of intermediates requires careful tuning of the *in vivo* activities of enzymes active at metabolic branchpoints. By separating pathways in different strains in a co-culture, these pathway imbalances can be relieved. Li *et al.* [22] investigated this strategy using rosmarinic acid (RA) production by engineered *E. coli* strains as the experimental model. RA is formed by condensation of two molecules derived from the phenylpropanoid pathway: caffeoyl-CoA (Caf-CoA) and salvianic acid A (SalvA). Pathways towards Caf-CoA and SalvA diverge at 4-hydroxyphenylpyruvate (4-HPP). Conversion of 4-HPP to Caf-CoA starts with a two-step conversion of HPP into coumarate, after which two enzymes, including the abovementioned HpaBC hydroxylase, convert coumarate to Caf-CoA. Conversion of 4-HPP to SalvA is catalyzed by HpaBC and a D-lactate dehydrogenase. In this two-enzyme conversion, the order of the hydroxylase and reduction reactions is interchangeable. The ability of HpaBC to catalyze three reactions involved in the synthesis of two pathway intermediates presents a major challenge for pathway balancing in monocultures. This challenge was addressed by splitting RA biosynthesis into three pathway modules allocated to three different *E. coli* strains. Module 1 was engineered for *de novo* production of coumarate, Module 2 for *de novo* SalvA production and Module 3 for conversion of coumarate (generated by Module 1) to Caf-CoA and its condensation with SalvA (generated by Module 2) to yield RA. This tripartite co-culture strategy spatially segregated the roles of HpaBC in

SalvA and Caf-CoA production and, by tuning the relative inoculum ratios of the three strains, presented a means to prevent build-up of coumarate to toxic levels. After empirically optimizing the inoculum ratios, a tripartite co-culture yielded an RA titer of 0.10 g•L^{-1} in glucose-grown shake-flask cultures, which was approximately 20-fold higher than the titer obtained with a monoculture carrying the entire RA pathway [22].

Overall, this example shows that using a commensalistic co-culture to separate different enzymes can prevent the accumulation of (toxic) intermediates, thereby enabling increased product yields on substrate.

Another challenge in mono-culture designs relates to host-dependent (dis)abilities to functionally express specific enzymes or enzyme classes. This problem can even rule out popular industrial microorganisms as production platforms. In such cases, using co-cultures with specialist strains of species that express the missing enzyme(s) can result in improved product yields and rates of product formation. The potential of defined co-cultures for circumventing challenges related to expression of specific types of enzymes is illustrated by research on microbial production of oxyfunctionalized taxanes. Already in 2010 [26], *E. coli* was successfully engineered to produce taxadiene, a key precursor for biosynthesis of oxygenated taxanes, including the anticancer drug paclitaxel. However, synthesis of oxygenated taxanes from taxadiene depends on hydroxylases whose *in vivo* activity requires a cytochrome P450 reductase. *Escherichia coli* does not naturally contain P450 enzymes [63], and heterologous expression of these proteins can be challenging. Mostly due to its ability to anchor P450 proteins to intracellular membranes, *S. cerevisiae* is a preferred host for expressing this class of enzymes [64]. This inspired Zhou *et al.* [65] to study the co-cultivation of a taxadiene-producing *E. coli* strain with an *S. cerevisiae* strain that highly expressed a fusion protein of the plant taxadiene 5α-hydroxylase and P450 reductase that together catalyse the first oxygenation step in paclitaxel biosynthesis. Co-cultures of the two species were grown on xylose. Since xylose cannot be used by wild-type *S. cerevisiae,* growth of the yeast depended on consumption of acetate generated by the *E. coli* strain. After optimization of the expression of the hydroxylase-P450 reductase fusion protein in *S. cerevisiae* and of acetate production by *E. coli*, the titer of the targeted oxygenated taxane in fed-batch co--cultures reached 0.03 g•L^{-1}. Introduction of expression cassettes encoding a second hydroxylase-P450 reductase protein and an acetylase into the *S. cerevisiae* strain enabled production of a next intermediate in the paclitaxel synthesis pathway. The versatility of this modular approach was further demonstrated by its application to the production of other compounds whose synthesis involves P450-dependent oxyfunctionalization reactions [65].

Wu *et al.* [27] demonstrated how, in bipartite co-cultures, engineered *E. coli* and *S. cerevisiae* strains could mutually complement species-specific limitations related to functional expression of key product-pathway enzymes. Their study focused on microbial production of strigolactones, an important class of plant signalling molecules. Strigolactone synthesis starts from β-carotene, whose production has been demonstrated in engineered strains of *E. coli* and *S. cerevisiae* [66]. Three plant enzymes are required for conversion of β-carotene to carlactone, the central precursor for all strigolactones: a [2Fe-2S]-containing isomerase and two enzymes that catalyse oxidative cleavage reactions, one of which is a non-heme-iron-dependent enzyme. These three enzymes could be functionally expressed in *E. coli*, which enabled construction of a carlactone-producing strain. However, in line with previously reported challenges in cytosolic expression of heterologous iron-sulfur proteins in the yeast cytosol [67], attempts to produce carlactone in *S. cerevisiae* were unsuccessful. Conversely, the subsequent conversion of carlactone to specific strigolactones, which involves P450-dependent oxidation reactions, could be established in *S. cerevisiae* but not in *E. coli*. This inspired the authors to develop a flexible, bipartite co-cultivation strategy. Co-cultivating the carlactone-producing *E. coli* strain with a range of *S. cerevisiae* strains engineered for conversion of carlactone to specific strigolactones enabled production of the targeted compounds, thereby opening the way for studies on their biological functions [27].

The above examples illustrate that, when a specific pathway enzyme cannot be functionally expressed in a preferred industrial microorganism, co-cultivation with another microorganism that does functionally express the enzyme can expand the product range.

3.2. Definition of pathway modules and transport reactions

In implementing division-of-labour strategies for assimilatory product formation, as discussed in the previous paragraph, optimal design of metabolic modules is essential. Here, 'metabolic modules' refer to segments of a production pathway that are assigned to a single co-cultivation partner. By assigning different modules to different strains, each strain handles a part of the pathway. Design of functional modules should address primary objectives, such as mitigating effects of enzyme promiscuity, circumventing host-specific protein expression challenges and preventing pathway imbalance. A second criterium, is the availability of mechanisms for transport of relevant intermediates between co-cultivation partners. This, for instance, ensures that an intermediate produced by Strain A can be exported and taken up efficiently by Strain B, either by free diffusions or via membrane transporters. To increase flexibility in the design of metabolic modules, several studies explored (over)expression of native or heterologous transporter genes, which in several cases had to be newly identified.

In a study aimed at metabolic engineering of *E. coli* for production of *cis,cis*-muconate, Zhang *et al.* [68] observed accumulation of the shikimate pathway intermediate 3-dehydroshikimate (DHS) by engineered strains. Based on this observation, a co-culture strategy was devised, in which a first *E. coli* strain converted xylose to DHS via the shikimate pathway, while a second strain, grown on glucose as carbon source, converted DHS to *cis,cis*-muconate via three heterologous enzymes. The inability of *E. coli* to import DHS under the experimental conditions was addressed by identification and overexpression, in the latter strain, of a native gene that encoded a functional DHS transporter. After optimization of strains and process conditions, this co-cultivation strategy enabled a *cis,cis*-muconate yield on sugar that corresponded to 51 % of the theoretical maximum.

Transporter engineering with the aim to improve co-culture performance can also involve export of key intermediates, as exemplified by a study on co-cultures of two *E. coli* strains [69], of which the first overexpressed and exported tyrosine, which was then converted to 4-hydroxystyrene by a second strain expressing two heterologous enzymes. Expression of a *Petunia* gene encoding a plastid amino-acid transporter improved export of tyrosine by the former strain, thereby enabling a two-fold higher 4-hydroxystyrene titer in co-cultures than observed in a co-culture with a strain lacking the heterologous transporter.

A division-of-labour strategy for producing the benzylisoquinoline alkaloid precursor (*S*)-norcoclaurine was based on the availability of a *Scheffersomyces stipitis* strain that produced high levels of the pathway intermediate shikimate [70]. Since genetic engineering tools for this non-conventional yeast were less well developed than for *S. cerevisiae*, a co-cultivation strategy was based on conversion of shikimate, produced by *Sch. stipitis*, to (*S*)-norcoclaurine by an extensively engineered *S. cerevisiae* strain. The inability of *S. cerevisiae* to efficiently import shikimate was addressed by identification of two *Aspergillus niger* shikimate transporter genes and their functional expression in the (*S*)-norcoclaurine-producing *S. cerevisiae* strain. This strategy enabled (*S*)-norcoclaurine titers in co-cultures that were two orders of magnitude higher than observed in a monoculture of the *S. cerevisiae* strain. Flexibility in modularization of the long biosynthetic pathways towards benzylisoquinoline alkaloids was further extended by a study on identification, expression in *S. cerevisiae* and functional analysis of six *Papaver somniferum* benzylisoquinoline alkaloid transporter genes [71]. Using these transporters, the opiate pathway was split into three modules assigned to three *S. cerevisiae* strains. Engineering of the strain

carrying the ultimate reactions enabled formation of different alkaloid products via a mix and match strategy.

Identification and functional expression of relevant transporters increases options to further refine optimization of modular co-cultivation strategies, for example to prevent uneven distribution of pathway proteins over co-cultivated strains, based on algorithms for calculating protein allocation such as Gecko [72,73]. In addition, it may be possible to use energy-coupled transport mechanisms to optimize substrate saturation of low-k_{cat} enzymes and/or thermodynamic driving force for *in vivo* activity of spatially segregated metabolic models. The latter concept was theoretically explored by Bekiaris *et al.* [58], who developed the ASTHERISC algorithm. This algorithm integrates genome-scale models with thermodynamic reaction parameters to predict optimal pathway module definition. Although such options to integrate systems biology into the design of co-cultivation strategies are highly interesting, we are not aware of studies in which they have been experimentally tested.

3.3. Optimizing and controlling relative abundance of co-cultivation partners

Achieving and maintaining an optimal relative abundance of co-culture partners is a key challenge in division-of-labour strategies that are based on spatial segregation of product-pathway modules. This is important, because imbalance between the co-culture partners can lead to one outperforming the other, resulting in suboptimal production. Not surprisingly, modifying inoculum ratios is a common and often successful approach for optimizing the performance of such co-cultures in laboratory studies (see e.g. [22,25,74,75]). However, specific growth rates of individual strains, and impacts of changing conditions during growth, can lead to population dynamics that negatively affect co-culture performance. In the absence of measures to stabilize relative abundance, impacts of population dynamics are likely to be even more pronounced under the intensive, dynamic cultivation conditions in large-scale industrial processes. Co-culture systems might therefore require monitoring of cell concentrations and the use of feedback control to maintain the balance. This can, for example, be done by process analytical techniques, as discussed in a recent review [76].

The use of mixtures of carbon substrates, of which only one can be used by each strain, has been successfully applied to stabilize relative abundance of strains in co-cultures (e.g. [68,77,78]). Alternatively, using consortium partners with different auxotrophic requirements enables the control of their relative abundance by supplying the required growth factors. This concept was used by Treloar *et al.* [79] to develop a deep-reinforcement-learning strategy to control relative abundance of two *E. coli* strains of which one was auxotrophic for arginine and the other for tryptophan. Simulations demonstrated the potential of this approach to control population composition and optimize productivity when supply of strain-specific substrates or growth factors can be coupled to online analysis of product concentration, for example by Raman spectroscopy or reporter proteins [80,81].

Inspired by studies in microbial ecology [82], self-stabilization of population composition can be based on mutual complementation of auxotrophies by cross-feeding of growth factors [75,83,84]. Li *et al.* [85] explored how introducing such mutual dependencies can be applied to stabilize co-cultures of an *E. coli* strain that produces caffeate with a second strain that converts caffeate to salidroside. Strains expressing one of the two pathway modules were engineered to depend on production of either glutamate-derived amino acids or TCA-cycle intermediates by the other strain. In batch co-cultures of the mutually dependent strains, salidroside titers measured at the end of fermentation were independent of the inoculation ratio and over 2-fold higher than in cultures grown without engineered auxotrophies. The same study also demonstrated how, in the same context, a synthetic regulatory circuit based on a caffeate-responsive biosensor was used to enable 'on-demand' modulation of culture composition. To this end, the biosensor was coupled to expression of a degradation-prone version of a key enzyme in glutamate metabolism, thereby tuning the abundance of the caffeate-producing strain to its consumption by the salidroside-producing strain [85].

While the approaches discussed above demonstrate how relative abundance of strains in laboratory co-cultures can be stabilized and even be subjected to 'on-demand tuning', we have not found studies in which genetic stability of such advanced co-cultures has been tested during long-term cultivation.

4. Outlook

There are few documented examples of the large-scale application of defined microbial co-cultures for production of low-molecular-weight compounds [9,86]. However, these reports either contain limited information [9], or are a bioconversion type of process [86]. This is not surprising as, from an industrial perspective, development and implementation of co-culture-based processes introduces additional levels of complexity compared to mono-culture-based processes. Inspired by rapid developments in synthetic biology and protein engineering [87,88], industrial research may therefore, especially for issues such as pathway imbalance, preferentially explore options for advanced engineering of monocultures. In this outlook, we provide suggestions on how future academic research may help to more precisely define situations in which defined co-cultures can, in an industrial context, outperform mono-cultures.

4.1. Start with the end in mind

When design, construction and analysis of co-cultures are performed with the aim to contribute to development of industrial processes, they should, just like other research in bioprocess engineering [89], start with the intended industrial application firmly in mind. The design of co-culture strategies should be based on a clear definition of which industrially relevant challenges in monocultures are addressed. Carefully designed experiments will be needed to evaluate co-culture performance under industrial conditions - for example, controlling starting strain ratios, feeding strategies, and using new bioreactor setups that can accommodate multiple organisms. This problem-based approach is illustrated by studies on the use of co-cultures for circumventing enzyme promiscuity [23,24], non-compatibility of the expression of specific enzymes with production strains [27,65] and genetic instability in generalist strains growing on sugar mixtures [21]. In contrast, problem definition is often less explicit when use of co-cultures is motivated from redistribution of metabolic burden, sometimes based on the incorrect assumption that equal distribution of product-pathway protein over co-culture partners will by definition lead to improved biomass-specific or volumetric productivity [57]. Moving beyond such intuitive design will benefit from rigorous quantitative comparison of mono- and co-cultures by integration of theoretical and experimental research on proteome allocation, bioenergetics and thermodynamics in mono- and co-cultures. Such systems biology approaches are also relevant for further optimizing the performance of co-cultures of substrate-specialists for production of dissimilatory products. In most of the academic studies cited in this review, co-culture performance was assessed based on product titer in small-scale laboratory batch cultures. We see a large potential for studies that, based on the existing and highly valuable body of knowledge, aim to quantitatively evaluate co-culture performance in terms of titer, productivity and yield [10] under simulated industrial conditions.

4.2. Compare deterioration of product formation in mono- and co-cultures

In processes aimed at dissimilatory product formation from mixed carbon substrates, co-cultures of 'carbon substrate specialists' have a clear potential to prevent the deterioration of fermentation kinetics

observed in monocultures of generalist strains [21]. The industrial relevance of this co-cultivation strategy will, in practice, depend on whether strain robustness and feedstock composition allow for biomass recycling through multiple subsequent fermentation cycles. In contrast to dissimilatory product formation, synthesis of assimilatory products competes with growth for cellular energy, proteome space and biosynthetic precursors. This competition provides a selective advantage to mutants with reduced productivity. The impact of such 'protein burden' effects on the genetic stability of co-cultures for assimilatory product formation has not yet been studied in detail. Addressing this knowledge gap is of paramount importance for assessing and improving applicability in industrial processes that, from frozen stock culture to full scale, involve many generations of growth [90].

4.3. Control the relative abundance of the strains

Controlling the relative abundance of co-culture partners under dynamic industrial process conditions can be challenging and requires robust strategies for population control. Techniques such as metabolic engineering of auxotrophic dependencies [75] and model-driven feedback control of key process parameters [79,91] are emerging solutions, but their performance requires validation under industrially relevant conditions. Further development of modeling tools (like in [92,93]) and improving techniques for monitoring and controlling population composition [76] will be crucial to unlocking the potential for industrial application.

We hope that, by inspiring readers to engage with the challenges involved in defined microbial co-cultures, this paper will contribute to the development of these scientifically fascinating systems into mature production platforms for microbial biotechnology. With ongoing advances in synthetic biology and bioprocess engineering, ever more tools become available to fine-tune interactions between co-culture partners and, thereby, optimize stable performance. In addition, quantitative analysis and optimization of co-cultures under simulated industrial conditions is essential for understanding and improving performance of co-cultures in large-scale processes. We are convinced that such research will contribute to a paradigm shift in industrial technology that will lead to much wider use of co-cultures.

5. Conclusion

Defined co-cultures offer unique opportunities to address several problems associated with the use of monoculture processes for production of assimilatory and dissimilatory products. Examples discussed in this review demonstrate, in laboratory-scale experiments, the use of well-designed co-cultivation strategies can improve yields, expand substrate ranges, prevent genetic instability, and mitigate pathway imbalances.

The next challenge is to gear research towards transitioning from laboratory-scale experiments, often with product titer as main read-out, to full-scale industrial processes. Integration of quantitative studies on co-culture performance under simulated, dynamic industrial process conditions with synthetic biology approaches for optimizing co-culture performance offers an excellent perspective to achieve this goal.

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Declaration of Competing Interest

We have nothing to declare.

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