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Synthetic microbial consortia for small molecule production

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Microbial consortia were designed for the production of small molecules with 'labor' being divided between two or more microorganisms. Examples of linear designs are substrate conversion preceding target molecule production or subdivision of two consecutive steps of target molecule production. Here, we review synthetic biology design approaches for microbial consortia based on ecological principles and microbial interactions that is, mutualism, and commensalism. Besides highlighting the technical challenges regarding industrial application of synthetic microbial consortia, we forecast the extension of the concept from binary linear to ternary linear and more complex microbial consortia in biotechnological applications. Microbial consortia are here reviewed and proposed as a rational solution toward feedstock accessibility as it has been shown for production of L-lysine, L-pipecolic acid and cadaverine from starch or production of fumarate from microcrystalline cellulose and alkaline pre-treated corn, or alternatively to establish new multi-step pathway for the production of rosmarinic acid from xylose and glucose.

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Introduction

Microbes populate Earth, and microbial communities are involved in many processes which affect human health directly (the microbiome) or indirectly (in natural environments or via biotechnological applications as in fermented foods). Some of the better described natural microbial communities include the anaerobic food chain, phototrophic consortia, acidophilic communities, communities of the rhizosphere and phyllosphere as well microfloras of the intestine or the oral cavity [1]. These natural microbial communities may involve species from a wide range of phylogenetic groups [2], and their composition is shaped by external cues such as availability of growth substrates and trading of metabolites. Those molecular signals are exchanged or detected by a subpopulation of the ecological network which in return responds to them accordingly to its specific role in that network [3]. Thus, nutrient availability and signal transduction coordinate the subpopulations and drive *labor division* within the ecological network [4].

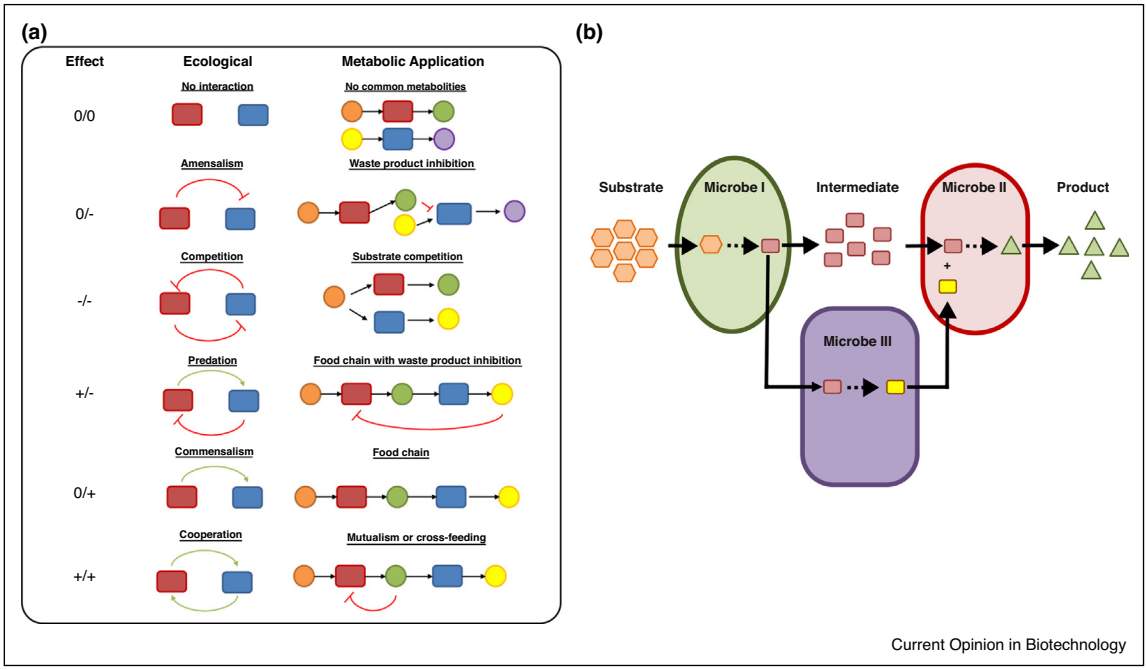
Degradation of complex biological materials is governed by microbial consortia. In a biofilm, microbes show higher robustness against environmental perturbations and microbial invasion than a single species [4]. Division of labor allows reducing biosynthetic load and metabolic stress on the single microbe [5] in unstructured or structured environments (as recreated in artificial microbial arenas [6]). Degradation of the polymeric chitin in the water habitat involves partitioning of natural populations into subpopulations resulting in a linear food web with the first supplying hydrolysis products to the second [7].

Considering only interactions based on nutrients, Grosskopf and Soyer [42] described major categories of binary consortia (Figure 1). This review will disclose the progress made in the last two years in terms of microbial consortia towards substrate and product accessibility with the scope of reducing the metabolic stress and genetic modifications per strain by redistributing the tasks within multiple strains, each working as an operating unit of the network.

Natural microbial consortia in biotechnology

The typical scope of a microbial biotechnological production process is the microbial conversion of a substrate into a product [8]. In nature, conversion of complex substrates is catalyzed by mixed-species microbial communities with often unknown functions [9], contrary to most biotechnological processes conducted by using pure cultures of single microbial strains. Moreover, natural microbial consortia convert the substrate not only into the desired product but also into by-products, which lower the overall process yield [4]. Historically, humankind has used natural communities for millennia, for example, for fermented foods, waste treatment, and agriculture [10]. Typically, these are black-box applications (e.g. sourdough fermentation or activated sludge processes in wastewater treatment, based of mutualistic interactions) and the principles changing or maintaining the community composition in such processes are not fully understood [11]. An

Figure 1



(a) Schematic representation of basic motifs of metabolite-based microbial interactions (adapted from Grosskopf and Soyer 2014). with positive (+), negative (−) or neutral (0) interactions: no interaction (0/0), amensalism (0/−) for inhibition of a subpopulation by a waste product of another subpopulation, competition (−/−) for a common nutrient required by both microbial subpopulations, predation/parasitism (+/−) as in linear food chains with inhibition of the first subpopulation by a waste product of the second subpopulation, commensalism (0/+) in linear food chains, and mutualism/cooperation (+/+) as in syntrophic linear food chains. Microorganisms (squares) and metabolites (circles) and stimulating (pointed arrows) and inhibitory (blunt arrows) interactions are depicted. **(b)** Schematic representation of designed microbial consortia for converting substrates into products (s. text for examples).

exemplary application in agriculture is the use of plant growth-promoting bacteria, which fix nitrogen by establishing mutualistic plant interaction, suppress plant pathogens, solubilize nutrients, or have other agriculturally beneficial features. This technology often shows limited success in-field due to unpredictable and unreliable changes in the plant root associated microbial communities [12]. This is not surprising since metabolic heterogeneity can even be found within populations of clonal microbial monocultures, as well as in several sources for metabolic divergence and phenotypic heterogeneity such as ecological factors, cell-inherent dynamics in constant environments, or molecular noise in gene expression have been identified [13].

Synthetic microbial consortia to access complex substrates

Synthetic microbial consortia are designed to reduce the heterogeneities that are typical in natural consortia by making use of engineered interdependencies. This reductionist approach is valuable to gain an understanding of for example, individual and collective ecosystem functions in rhizospheric and phyllospheric plant–microbe and microbe–microbe interactions [14] (D: https://www.doi.org/doi:10.1186/s40168-018-0445-0) as well as in the applied biotechnological contexts described here. In biorefineries, renewable and often complex feedstocks are to be converted into a multitude of value-added compounds. If the feedstock is homogenous and continuously available, specialized single synthetic microbial strains may be used. The application of defined synthetic microbial consortia may be regarded as a viable extension of biotechnological applications of metabolically engineered single strains. If feedstock supply and composition vary seasonally, application of specifically adjusted synthetic consortia matching the discrete process steps of substrate conversion and/or product formation to the current demand may be advantageous [15]. Indeed, consortia can use less refined substrates (e.g. whey, molasses) displaying a higher bioconversion efficiency than monocultures, requiring less expensive purification processes [4].

Similarly, the design and proof-of-concept of a synthetic microbial consortium for the conversion of cellulose to butanol and/or isobutanol have been reviewed recently [16]. Co-culturing, the cellulase-secreting fungus *Trichoderma reesei* with an isobutanol producing *Escherichia coli* strain for production of isobutanol from the lignocellulosic feedstock, and an *E. coli*-*E. coli* consortium for optimal output of 4-hydroxybutyric acid and malic acid from a

sugar mixture [17] or ethanol production from an *Saccharomyces cerevisiae-E. coli* consortium from sugarcane bagasse slurry [18] have been described. These and additional examples are listed in Table 1.

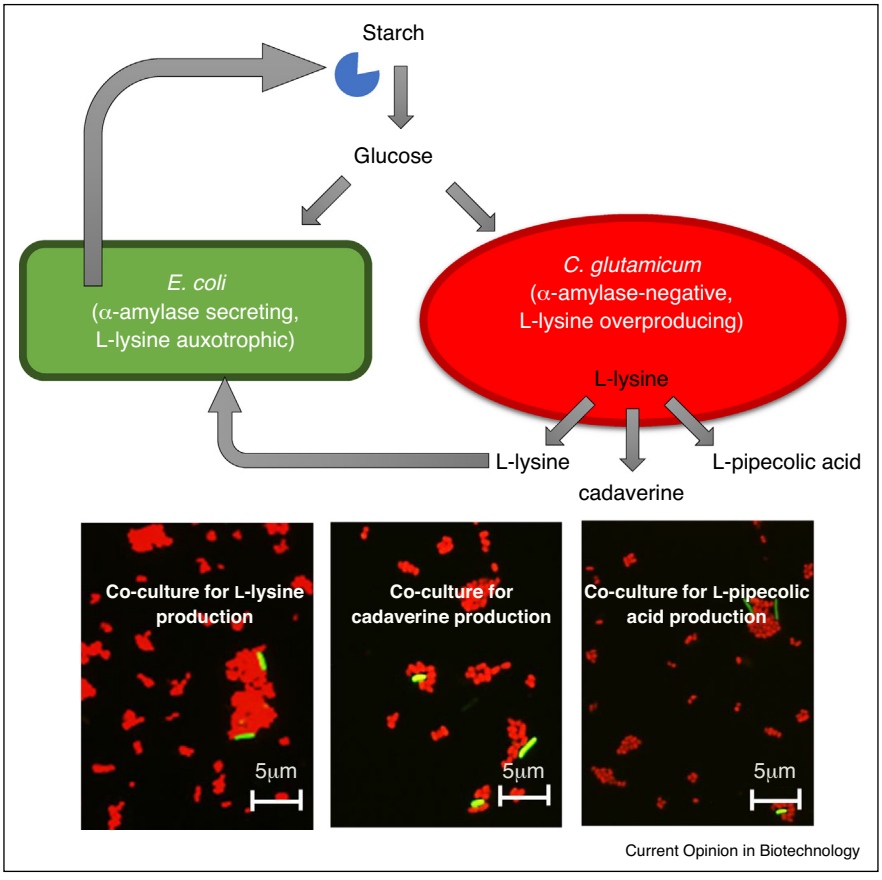
Corynebacterium glutamicum is used in monoculture for many production purposes, in particular for the million ton-scale amino acid production [19]. Recently, the concept of dividing labor between a substrate-converting *E. coli* strain and a value-added compound producing *C. glutamicum* strain [20•] has been exemplified for starch-based and sucrose-based production of L-lysine, cadaverine, and L-pipecolic acid (Figure 2). Proof-of-concept examples of different levels of interdependence, namely commensal (0/+ interaction; Figure 1) and mutualistic (+/+ interaction; Figure 1) consortia have been developed. As a proof-of- concept of a commensal consortium, an L-lysine auxotrophic, sucrose negative *E. coli* strain was co-cultivated with an L-lysine producing *C. glutamicum* that secretes fructose to feed the *E. coli* strain when grown with sucrose as a consequence of deletion of the fructose importer gene *ptsF* [20•]. With minimal sucrose medium, neither strain grew as a

Table 1

Recent examples of small molecule production processes based on synthetic microbial communities. Genetically engineered strains are given in bold

Biotechnological process	Synthetic microbial consortia	Reference
Production of ethanol, isobutanol and butanol from cellulose	***Clostridium*** sp. modules displaying cellulosomes	[16]
Production of isobutanol from the lignocellulosic feedstock	Cellulase-secreting fungus ***Trichoderma reesei*** and an isobutanol producing *E. coli*	[17]
Production of hydroxybutyric acid and malic acid from a sugar mixture	*E. coli* for optimal output of 4-hydroxybutyric acid and *E. coli* consortium for malic acid from a sugar mixture	[17]
Production of ethanol from pre-treated sugarcane bagasse	*S. cerevisiae* ferments glucose to ethanol and glucose- negative ethanologenic ***E. coli*** ferments xylose to ethanol	[18]
Production of fumarate from microcrystalline cellulose and alkaline pre-treated corn stover	*Trichoderma reseeii* secreting cellulolytic enzymes and *Rhizopus delemar* producing fumaric acid	[24]
Production of L-lysine from sucrose	Sucrose negative and L-lysine auxotrophic ***E. coli*** and sucrose positive ***C. glutamicum*** producing L-lysine	[20•]
Production of L-lysine, L- pipecolic acid and cadaverine from starch	L-lysine auxotrophic ***E. coli*** secreting α-amylase and starch negative ***C. glutamicum*** overproducing eitherL-lysine or L- lysine + L-pipecolic acid or L-lysine + cadaverine	[20•]
Production of L-lysine from glucose	L-lysine overproducing ***C. glutamicum*** andL-lysine auxotrophic ***C. glutamicum***	[21•]
Production of cadaverine from glucose and glycerol	Glucose positive ***E. coli*** overproducing L-lysine, glucose negative and glycerol positive ***E. coli*** converting L- lysine into cadaverine	[28]
Production of caffeyl alcohol from glucose/ glycerol	***E. coli*** producing *p*-coumaryl alcohol and ***E. coli*** converting *p*-coumaryl alcohol into caffeyl alcohol	[29]
Production of resveratroloside and polydatin from *p*-coumaric acid	***E. coli*** converting *p*- coumaric acid into resveratrol and ***E. coli*** glycosylating resveratrol glucoside to resveratroloside and polydatin	[31]
Production of naringenin from glucose	***E. coli*** producing L-tyrosine and *p*-coumaric acid and ***E. coli*** converting L- tyrosine and *p*-coumaric acid into naringenin	[32]
Production of apigetrin from *p*-coumaric acid	***E. coli*** producing apigenin from *p*-coumaric acid and ***E. coli*** glycosylating apigenin to apigetrin	[31]
Production of bisdemethoxycurcumin from glucose	***E. coli*** producing *p*-coumaric acid and ***E. coli*** converting *p*-coumaric acid into bisdemethoxycurcumin	[34]
Production of anthocyanins from glucose, glycerol and xylose	Glucose, glycerol and xylose utilizing ***E. coli*** producing phenylpropanoic acids and ***E. coli*** converting phenylpropanoic acids and malonate into flavanones and ***E. coli*** converting flavanones into flavan-3-ols and ***E. coli*** converting flavan-3-ols into anthocyanins	[36••]
Production of di-methylated and tri- methylated phenylpropanoids from glucose	***E. coli*** producing producing pterostilbene/naringenin/apigenin and ***S. venezuelae*** methylating pterostilbene/naringenin/ apigenin to di-methylated and tri-methylated phenylpropanoids	[27]
Production of monacolin/lovastatin from methanol	***Pichia pastoris*** producing dihydromonacolin L from methanol and ***P. pastoris*** converting dihydromonacolin L into lovastatin	[25]
Production of rosmarinic acid from xylose and glucose	Glucose-negative ***E.coli*** producing caffeic acid and glucose-negative ***E.coli*** producing salvianic acid A and xylose-negative ***E. coli*** converting caffeic acid and salvianic acid A into rosmarinic acid	[37••]
Production of salidroside from glucose and xylose	Glucose-negative, L-phenylalanine auxotrophic ***E.coli*** producing L- tyrosine and tyrosol and xylose-negative, L-tyrosine auxotrophic ***E.coli*** producing L-phenylalanine and salidroside	[35•]
Production of berkeleylactone from an axenic environment	Mixture of the thermophilic fungi *Penicillium fuscum* and *P. camembertii/clavigerum* producing the macrolide antibotics berkeleylactones only in co-culture	[38]

Figure 2



Division of labor between substrate conversion (starch hydrolysis) and production (L-lysine, cadaverine and L-pipecolic acid from glucose) in three different synthetic mutualistic *E. coli–C. glutamicum* consortia (adapted from Sgobba *et al.* [20•]). The fluorescence microscopy pictures showed three *E. coli–C. glutamicum* consortia in which *C. glutamicum* cells express the red fluorescence protein Crimson, and *E. coli* cells express green GfpUV. Each consortium was designed to produce either L-lysine, L- pipecolic acid or cadaverine.

monoculture. However, the commensal consortium grew and produced L-lysine. Thus, net production of L-lysine only resulted when the L-lysine producing *C. glutamicum* strain produced more L-lysine than required to support the growth of the L-lysine auxotrophic *E. coli* strain. In this commensal consortium, the *E. coli* strain is not beneficial for the *C. glutamicum* strain (0/+ interaction, Figure 1). In similar approaches isobutanol and fumarate, respectively, were produced from lignocellulosic feedstock using a consortium consisting of cellulase-secreting *T. reesei* and either an isobutanol producing *E. coli* [17] or a fumaric acid producing *Rhizopus delemar* [24]. Moreover, production of ethanol from pre-treated sugarcane bagasse by a consortium with *S. cerevisiae* fermenting glucose to ethanol and an ethanologenic *E. coli* fermenting xylose to ethanol has been shown [18]. Additional examples can be found in Table 1.

A mutualistic *E. coli–C. glutamicum* consortium was developed, that is, both bacteria depended on the metabolic activity of the other (+/+ interaction, Figure 1). One strain was designed for degrading the polymeric feedstock starch to glucose, the other for the conversion of glucose to L-lysine and metabolic dependency was realized by rendering the first strain auxotrophic for lysine [20•]. An L- lysine auxotrophic, α-amylase secreting *E. coli* strain and a naturally amylase-negative, L-lysine overproducing *C. glutamicum* strain were co-cultured in the starch minimal medium [20•]. While the *C. glutamicum* strain depended on α-amylase secretion by the *E. coli* consortium partner to access the carbon source starch, growth (and, thus, α-amylase secretion) of the L-lysine-auxotrophic *E. coli* strain depended on the provision of L- lysine by the *C. glutamicum* consortium partner. Neither strain grew with starch in a single culture, whereas the consortium catabolized starch and produced L-lysine [20•]. The concept could be extended to starch-based production of lysine-derived products cadaverine and L-pipecolic acid, respectively, by variants of this consortium. To this end, the L-lysine auxotrophic, α-amylase secreting *E. coli* strain was combined with amylase-negative *C. glutamicum* strains overproducing either cadaverine or L-pipecolic acid [20•]. For each combination, starch-based production

of the desired product could be shown only for the designed consortium, but not for the individual strains alone. However, this extension of the original concept was only possible, because cadaverine and L-pipecolic acid are derived from L-lysine and the amylase-negative *C. glutamicum* strains produced enough L-lysine to maintain the growth of the *E. coli* strain in addition to the targeted products cadaverine and L-pipecolic acid, respectively.

What are the technical hurdles? A 25L batch-fermentation with this consortium demonstrated that the process was scalable. As the *E. coli* and *C. glutamicum* strains expressed different fluorescent proteins, the composition of the consortium could be followed. Single cell analysis by fluorescence microscopy revealed that the *C. glutamicum* strain outnumbered the *E. coli* strain by far (Figure 2). Likely, few α-amylase secreting *E. coli* cells suffice to maintain starch hydrolysis in the consortium. When comparing lysine productivity, the consortium's performance falls short of that of α-amylase secreting, L-lysine overproducing *C. glutamicum* strains [20•]. In addition, starch-based production of cadaverine and L-pipecolic acid by the designed consortia suffered from overall lower titers, yields, and productivities than observed for glucose-based production by *C. glutamicum* monocultures [20•]. This may exemplify that several technical hurdles have to be taken before synthetic microbial consortia find widespread application. Besides the obvious need to improve production parameters of the consortia, process parameters including protocols for reliable storage of the consortia and for maintaining their stability during bioreactor production have to be developed for technical realization. Accordingly, a microfluidic cocultivation system has been designed and characterized for the analysis of growth and interactions within microbial consortia at single-cell resolution [21•]. Two strains were co-cultivated, but were physically separated to isolate interactions by diffusible compounds from cell–cell contact interactions. A simple commensal consortium was used: a L-lysine-producing *C. glutamicum* strain and an L-lysine auxotrophic mutant *C. glutamicum* strain. This approach allowed to characterize the interactions due to the diffusible compound L-lysine without the interference of communications requiring cell–cell contact [21•]. Once the technical hurdles are overcome, synthetic consortia consisting of substrate converter and producer strains may find broader application within the biorefinery concept to support flexible conversion of renewable feedstocks to a multitude of value-added compounds irrespective of seasonal or other variations of the complex biomass substrates.

Synthetic microbial consortia for complex biosynthesis

Synthetic microbial consortia have also been developed to partition individual biosynthetic steps of complex metabolic pathways to two or more microbial strains or species. This pathway modularization approach lowers the metabolic burden on the single microorganism [22,23]. If low product concentrations and yields are observed with monocultures and if this is due to the high metabolic burden, synthetic microbial consortia may overcome the bottleneck. In this way, the sum of genetic modifications that can be implemented is higher and particular features of different strains or species can be combined in a coordinated fashion [27,28]. Moreover, microbial consortia overcome metabolic burden for example, by circumventing accumulation of inhibitory intermediates [4]. Uptake and export of substrates and products by transport proteins constitute a prerequisite for any process of small molecule production [23], but are more relevant for consortia since transport processes of intermediates come into play in addition.

Proof-of-concept studies employing microbial consortia designed to divide the labor of a complex biosynthetic pathway have been described for a number of applications targeting various product classes (Table 1) including classical and modern approaches to vitamin C production [23]. Several species have been used in microbial consortia for small molecule production including fungi and yeasts such as *Trichoderma reesii* [24], *Pichia pastoris* [25] or *S. cerevisiae* [26] and bacteria such as clostridia [16], streptomycetes [27], and *C. glutamicum* (Table 1). However, most synthetic consortia rely on *E. coli* strains; this is particularly obvious when complex biosynthetic routes are subdivided to be realized by a consortium (Table 1). To divide labor between different partners in a consortium, rationales for subdivision of a complex biosynthetic route have to be developed. Amongst others, this subdivision can be guided by how that pathway evolved in the donor host (biosynthesis of a common precursor from primary metabolism such as an amino acid, followed by conversion to an aglycon that subsequently is glycosylated and finally decorated by e.g. methylation), by the availability of transport proteins for exchange of pathway intermediates, by distinct biochemical requirements of subpathways regarding NADPH or ATP provision or by tolerance to accumulating intermediates or co-products.

Synthetic microbial consortia outperformed monocultures for example, by separation of lysine production and lysine decarboxylation [28], by avoiding accessibility of the promiscuous hydroxylase HpaBC to the early intermediate L-tyrosine for full conversion of *p*-coumaroyl-CoA to the monolignol caffeoyl alcohol [29], or by separating an engineered mevalonate pathway to provide isopentenyl pyrophosphate, which the authors speculate to diffuse to the microbial partner producing pinene from it [30]. Production of *p*-coumaric acid followed by conversion to several aglycons and their subsequent glycosylation has been separated in several synthetic consortia [31–34].

Robust processes based on microbial consortia require interdependencies of the microbial partners. This can be

realized by installing auxotrophies and cross-feeding as for example, realized in a binary synthetic consortium of two *E. coli* strains, in which one partner is auxotrophic for amino acid 1, but overproduces amino acid 2, while the other partner has opposite properties with respect to these two amino acids [35•]. In addition, the two strains differed by their potential to utilize carbon sources: one partner was able to utilize glucose, but not xylose, whereas the other partner had mutually exclusive properties concerning these carbon sources [35•].

Linear binary, ternary, and even quaternary designs have been realized for access to more complex linear biosynthetic pathways. In a landmark study, overexpression of 15 genes for enzymes and transcription factors was divided over four independent *E. coli* strains (Figure 3), reducing the metabolic burden and enabling independent strain optimization for module-specific requirements [36••]. In addition, non-linear, convergent biosynthetic pathway design is possible (Figure 4). For example, in a convergent, ternary microbial consortium two strains produce two precursors, caffeic acid and salvianic acid A, and of a third one converts those precursors into rosmarinic acid showing 38-fold higher titer as compared to a monoculture [37••].

Up to now, we have focused on synthetic consortia with two or more recombinant strains/species that have been designed rationally. However, consortia performing exquisite tasks can also be selected from the environment. For example, the thermophilic fungi *Penicillium fuscum* and *Penicillium camembertii/Penicillium clavigerum* produce a new class of macrolide antibiotics, the so-called berkeleylactones, only in co-culture which the authors attributed to cross-talk between the species [38]. It remains to be seen if such unexpected features also emerge from synthetic microbial consortia.

Challenges and adaptive laboratory evolution for the improvement of synthetic consortia

Nowadays, a big challenge in the utilization of microbial consortia would be, besides a comparable production titer with the monocultural approach, the improvement of the performance of the microbial consortium together with their stability within a wide range of growth conditions. To improve production yield over monoculture, ratios of strain I and strain II have been varied to achieve higher titer of product for example, threefold higher cadaverine production from a *E. coli–E. coli* consortium than monoculture [28].

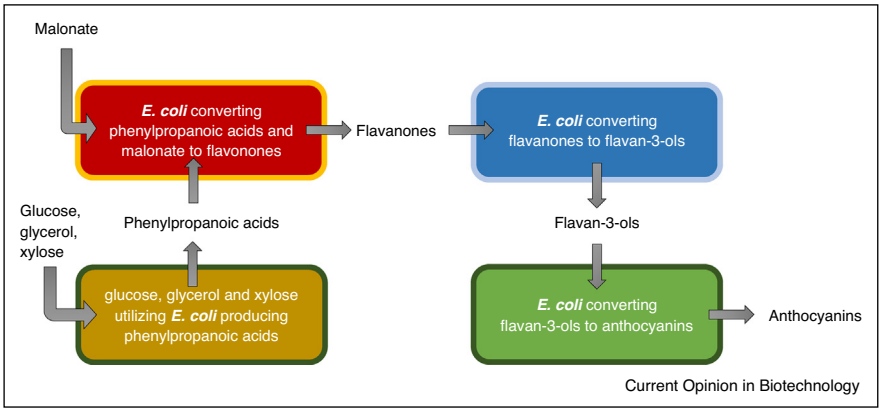
Similarly, adaptive laboratory evolution (ALE) contributes tremendously to current metabolic engineering of strains/species used in monoculture. It is conceivable that the application of ALE will yield synthetic microbial consortia with superior performance. ALE uses a selective pressure as driving force for the selection of mutants with enhanced phenotype [39]. It has been shown that ALE could enhance the bacterial mutualism performance towards biofilm formation [40], or improved commensalism interdependencies in biofilms of mouse gastrointestinal tract [41].

Recent studies have shown the efficiency of ALE in improving microbial consortia against acid resistance expanding the limits of thermoacidophily [39]. Therefore, ALE could be a new strategy to drive the change from monoculture to microbial consortia.

Conclusion

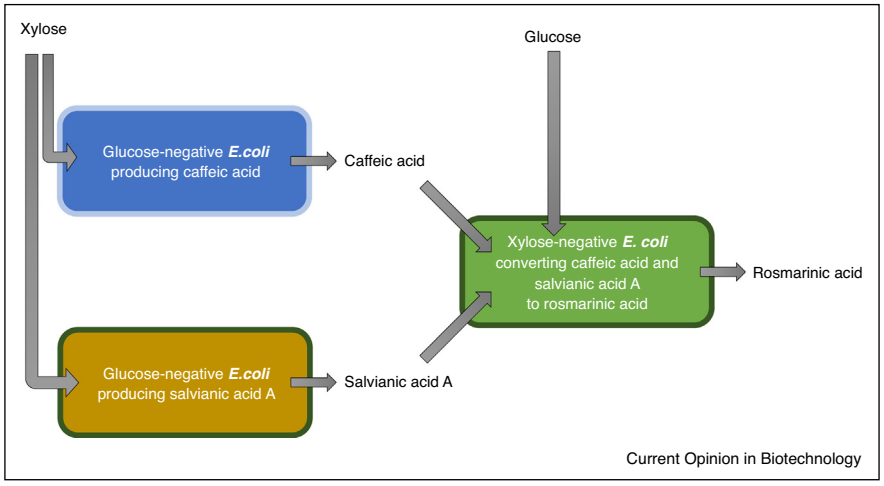
The biotechnological concept of co-culturing two or more species has long been used in fermenting foods or in classical vitamin C production. Metabolic engineering took this concept to a completely new level and many proof-of-concept examples of synthetic microbial

Figure 3



A linear biosynthetic pathway divided over four *E. coli* strains in a synthetic polyculture (adapted from Jones *et al.* [36••]). The consortium consisted of a strain (brown) producing phenylpropanoic acids from a blend of glucose, xylose, and gylcerol, a strain (red) converting malonate into flavanone, a strain (blue) converting flavanones into flavan-3-ols and a strain (green) converting flavan-3 -ols to anthocyanins.

Figure 4



A biosynthetic pathway with two converging branches divided over three *E. coli* strains in a synthetic polyculture (adapted from Li *et al.* [37••]). The consortium consisted of a strain (blue) producing caffeic acid from xylose, a strain (brown) producing salvianic acid A from xylose and a strain (green) growing in glucose and converting caffeic acid and salvianic acid A into rosmarinic acid.

consortia have been achieved. This includes dividing labor between substrate conversion and production as well as between substeps of complex linear or convergent biosynthetic routes. Metabolic interdependencies based on mutually exclusive access to growth substrates or based on amino acid auxotrophy/overproduction allow maintaining consortia with relatively fixed ratios of subpopulations. However, this has been shown at lab-scale. To apply these consortia in real life, it is required to focus on some issues:

- Requirement of a database that schedules complete information about nutrients, culture, and metabolite profiles of microbes to facilitate the selection of microbes in the process of designing consortia.
- Development of new techniques which provide a profile of interactions between microbes in consortia culture, because techniques, that is, metabolomics, proteomics, and transcriptomics are still costly.
- New methodologies to proper long-term preservation and storage of mixed microbial cultures are required.
- Upscaling requires the development of new strain development and process control strategies before commercialization can be envisioned.

Conflict of interest statement

Nothing declared.

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- • of special interest
- •• of outstanding interest

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