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Synthetic microbial consortia for biosynthesis and biodegradation: promises and challenges

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Abstract
Functional differentiation and metabolite exchange enable microbial consortia to perform complex metabolic tasks and efficiently cycle the nutrients. Inspired by the cooperative relationships in environmental microbial consortia, synthetic microbial consortia have great promise for studying the microbial interactions in nature and more importantly for various engineering applications. However, challenges coexist with promises, and the potential of consortium-based technologies is far from being fully harnessed. Thorough understanding of the underlying molecular mechanisms of microbial interactions is greatly needed for the rational design and optimization of defined consortia. These knowledge gaps could be potentially filled with the assistance of the ongoing revolution in systems biology and synthetic biology tools. As current fundamental and technical obstacles down the road being removed, we would expect new avenues with synthetic microbial consortia playing important roles in biological and environmental engineering processes such as bioproduction of desired chemicals and fuels, as well as biodegradation of persistent contaminants.

Keywords Microbial consortia · Systems biology · Synthetic biology · Biosynthesis · Biodegradation

Introduction

Microorganisms ubiquitously exist in nature and lie at the heart of biogeochemical cycling [34], most of which stay together with others to survive and thrive in complex microbial communities. Ecological interactions among species shape the structure and functions of the community [66]. The diversity of functions and division of labor enable microbial communities to cycle the nutrients and to perform complicated functions more efficiently than individual populations. Moreover, growing in mixed cultures also exhibits stronger resistance and resilience for individual members to environmental changes [63]. Inspired by these distinct properties of environmental microbial communities, the consortium-based concept has become promising for resilient and cost-effective biotechnologies, in which synthetic microbial consortia containing 2 or more key species carry out desired functions cooperatively based on the microbial interaction principles in nature.

We have dealt with undefined microbial consortia for centuries in different fields such as wastewater treatment, biogas production, as well as biodegradation and bioremediation. However, the enormous potential of microbial consortia is far from fully harnessed. In recent years, the understanding and application of microbial consortia have attracted broad interest in biosynthesis and bioprocessing [12, 17, 30, 117]. For example, biorefinery using biomass as feedstock is a sustainable solution for producing fuels and chemicals, mitigating climate change caused by traditional petroleum refineries [22]. Lignocellulose is a low-cost feedstock for biorefineries due to its abundance in nature. However, it is still challenging to genetically engineer complex pathways such as cellulolytic pathways in model strains for efficient and stable biosynthetic performance from cellulose-based feedstock [17]. Bioconversion of cellulosic biomass using synthetic microbial consortia is thus holding promise as an alternative approach for lignocellulosic biorefineries [64].

Some synthetic microbial consortia have already revealed superior capabilities in biosynthesis and biodegradation

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[122, 129, 145]. However, the lack of rational design of microbial consortia is the bottleneck of utilizing the vast potential of microbial consortia [60]. The current design of defined consortia mostly comes from the assembly of genetically engineerable model microorganisms such as *Escherichia coli* and yeast strains [61, 90, 145], which might genetically lack cooperative and communicative bases as they are not commonly found growing together in nature. Despite extensive research on microbial interactions, elucidating the underlying molecular mechanisms among co-existing microorganisms remains a major challenge to achieve a rational design of synthetic microbial consortia. The rapid development of omics technology and genome-editing tools in recent years opens opportunities and challenges in understanding and applying synthetic microbial consortia. Omics tools arm researchers with holistic views of metabolic fluxes, growth dynamics, and regulations in defined consortia [23, 85, 104], but pinpointing specific genes/pathways and connecting them between consortia members to achieve certain phenotypes of the consortium still needs to be further tackled. Moreover, the CRISPR/Cas-based toolkits enable rapid and efficient genome editing, transcriptional control, as well as high-throughput and trackable mutagenesis [28, 39, 132], but optimization of the machinery is needed particularly for non-model microorganisms. The integration of fundamental and technical gears will facilitate synthetic microbial consortia toward a stable and cost-effective approach for engineering applications such as biofuel/bioproducts synthesis and target biodegradation (Fig. 1).

In this review, we start from macro-scale microbial consortia identified and characterized from natural environments. Microbial interactions that may sustain a functional consortium are discussed with their potential applications in engineering fields and the implications for constructing synthetic microbial consortia. We then introduce the concept of synthetic microbial consortia and the synthetic and systems biology tools essential for the design and optimization of synthetic microbial consortia. The examples of using synthetic consortia for biosynthesis and biodegradation are given. In the end, we bring up current challenges that limit the application of synthetic microbial consortia together with some suggestions on future research directions to make the consortium-based biotechnologies more competitive in industrial and environmental applications.

Environmental microbial consortia

In nature, microorganisms typically occur in complex communities containing multiple populations that may metabolically interact with each other. In some cases, cells from a single population survive and thrive in complex communities where the interactions with cells from other populations determine the fitness of that population [133]. More importantly, functional differentiation and metabolite exchange during ecological interactions, particularly in cooperative relationships, enable co-existing species to cycle the nutrients efficiently and to gain strong resistance and resilience to environmental perturbations [63].

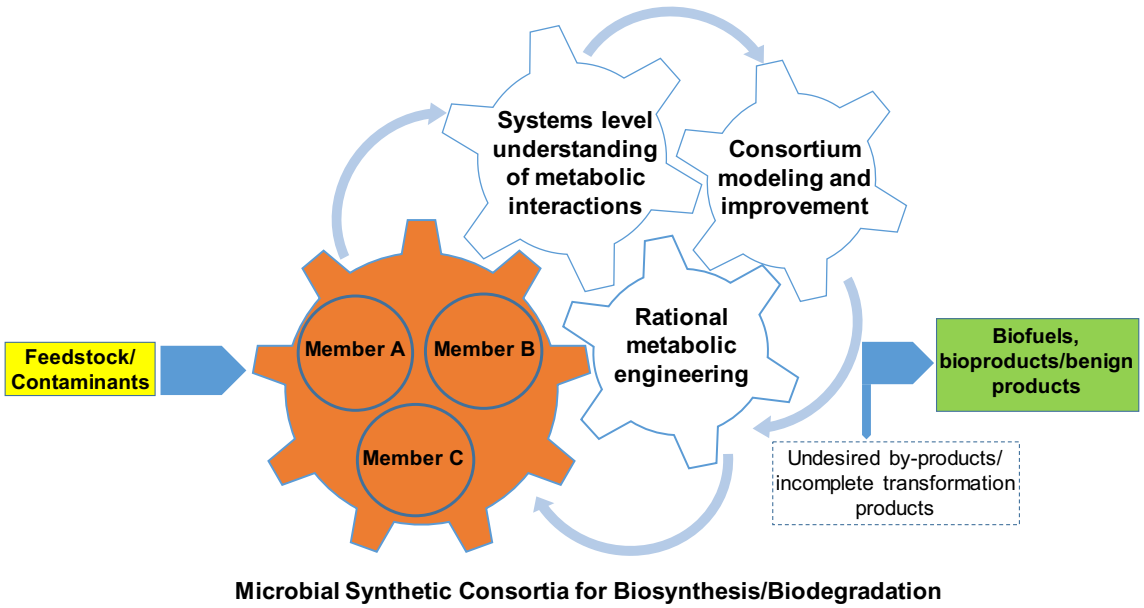

Fig. 1 Design, characterization, and optimization of synthetic microbial consortia for biosynthesis/biodegradation

Microbe–microbe interactions

Consortia are co-existing groups of two or more microbial species [127]. Microbial interactions determine the stability and functions of consortia. With combinations of positive, negative, and neutral effects between two species, there are six basic interaction modes: mutualism (e.g., syntrophy), commensalism, parasitism or predation, competition, amensalism, and neutralism [35]. Stable and robust growth is more likely achieved in consortia based on cooperative relationships, such as mutualism and commensalism. Mutualism refers to the cross-feeding between two species occurring via the exchange of metabolic products, which is beneficial to both partners [134]. In the case of commensalism, one community member benefits from the other (e.g., growing on the metabolites of the other), while the other community member is neither positively nor negatively affected [35].

Communicating by the exchange of metabolites or signals, members of a consortium coordinate their activities and benefit each other through the division of labor, which enables microbial consortia to have the capacity to perform complex functions and allows parallel or sequential processes for resource utilization. Diversity of biochemical reactions in consortia boosts the overall resource utilization efficiency and reduces the formation of byproducts [8]. Moreover, natural consortia may contain members that metabolize inhibitory and/or toxic byproducts of primary substrates, such as acetic acid, which if not being further consumed will waste the energy and carbon in it and inhibit the biomass production due to acidification and anion accumulation [15, 29, 107, 108]. Additionally, microbial consortia may have stronger resistance and resilience to fluctuations of environmental factors (e.g., pH, temperature, nutrient levels, and the presence of toxic compounds) [20]. The diversity of metabolic pathways possessed in different members can facilitate the survival of consortia in sub-optimal environments, which lack readily available substrates and/or have toxic compounds present [72]. The environmental resilience and metabolic diversity of microbial consortia are important to maintain the desired functions in bioremediation and bioproduction processes.

Microbial interactions that sustain environmental microbial consortia

One important microbial interaction in nature is cross-feeding (usually corresponds to mutualism, and sometimes commensalism, Fig. 2), which can lead to the more sustainable and robust growth of both partners than each in isolation [92]. One specific example of environmental consortia based on cross-feeding is syntrophy, an obligately mutualistic interaction. Syntrophy typically refers to a cooperative relationship in which the continuous utilization of one

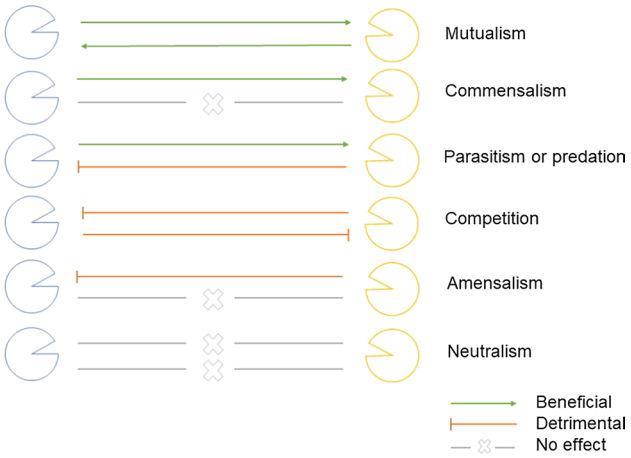

Fig. 2 Six basic interaction modes between two species in microbial consortia

compound by organism A for growth is dependent on organism B that lives on the metabolic intermediate of A. More specifically, organism A carries out the degradation of specific compounds, which only becomes thermodynamically favorable to sustain the growth of A when the metabolites are kept at lower levels by organism B via exergonic consumption [84].

Syntrophy is widely occurring in natural environments and to some extent, contributing to shaping the biogeochemical cycles of carbon, sulfur, and nitrogen. The most common example is the syntrophy between syntrophic bacteria and methanogens in various anaerobic environments, which is essential for anaerobic global carbon cycles converting organic matter to methane and carbon dioxide [112]. The anaerobic degradation of a wide range of carbon compounds, including the complex polymers like lipids, proteins, and polysaccharides by syntrophic consortia, usually takes several steps under anaerobic condition. The compounds are initially utilized by hydrolytic bacteria to form monomers, then by fermentative microorganisms to produce intermediates such as short-chain fatty acids (e.g., butyrate, propionate, acetate, and formate), CO_2, and H_2. Under standard conditions (i.e., 273 K, 1 atm, and 1 M of reactants), the fermentation of certain organic carbon intermediates is thermodynamically unfavorable (i.e., endergonic). The consumption of fermentation end products (e.g., H_2, CO_2, and acetate) by partners such as methanogenic archaea and homoacetogens makes the fermentation reactions of the initial organic compounds thermodynamically favorable (exergonic) [119]. Such syntrophy between fermenters and methanogens is widely distributed in terrestrial and aquatic microbiomes, as well as in microbiomes in the gut of high-level organisms.

Another example of syntrophy with metabolic cooperation is the co-existence of sulfate-reducing bacteria (SRB)

and anaerobic methanotrophic (ANME) archaea as an obligate co-culture discovered recently from the ocean floor. The ANME archaea oxidize methane to CO_2 by reverse methanogenic pathway. SRB take the electrons originally donated from methane and reduce sulfate to provide energy for growth of both partners [14, 31, 67]. The syntrophy between SRB and ANME uncovers an important route in the sulfur and carbon cycles coupling sulfate reduction to methane oxidation. Recent studies show that the interspecies electron transfer couples the two species by passing electrons from methane oxidation by ANME archaeon to SRB rather than using traditional diffusible syntrophic substrates such as hydrogen, formate, and acetate mentioned above [110, 120]. However, the detailed electron transfer mechanism remains to be further explored.

Syntrophy also occurs to another type of ANME archaea that links the nitrogen cycle to the carbon cycle, nitrate-dependent denitrifying anaerobic methane oxidation (DAMO) archaea. In anoxic environments where organic carbons are limiting, DAMO archaea and anaerobic ammonium oxidation (Anammox) bacteria may co-exist via syntrophic interactions [50, 78]. Nitrate-dependent DAMO archaea anaerobically oxidize methane coupled to the reduction of nitrate to nitrite [33]. Anammox bacteria then consume the produced nitrite to oxidize ammonium and produce dinitrogen gas. As anammox bacteria also utilize nitrite as their reducing power to fix CO_2 for their autotrophic growth, forming nitrate [70]. The formed nitrate can, in turn, be used by DAMO archaea. Such interactions are not only important to the biogeochemical cycles of nitrogen and carbon, but also to the engineering applications for nutrient removal. Theoretically, DAMO–anammox can result in a complete reduction of nitrate. It has been demonstrated in a recent enrichment study, where the consortium containing anammox bacteria and nitrate-dependent DAMO archaea is capable of complete removal of nitrate and ammonium through their syntrophic interactions with provided methane gas [54].

Besides the metabolically syntrophic cross-feeding between organisms A and B, detoxification is another important benefit that allows microbial consortia to sustain cell growth and functions. In this relationship, organism B is fed on the metabolites of organism A, while the sustainable growth of A benefits from the removal of its toxic metabolites by the other organism. One example is sulfur utilizing consortia, facilitating the conversion of organic sulfur to inorganic forms and promotes the biogeological cycling. The growth of SRB in pure culture is significantly inhibited by the accumulation of metabolic sulfide produced from elemental sulfur [98, 105]. In the consortium of green sulfur bacteria and SRB, sulfide produced by SRB is oxidized to elemental sulfur by green sulfur bacteria. Elemental sulfur is then reduced by SRB resulting in regeneration of sulfide. The concentrations of sulfide and elemental sulfur are kept at non-inhibitory levels allowing both partners to thrive [10].

Such one-way cross-feeding plus detoxification relationships have also been found in aerobic environments. Methanotrophic bacteria oxidize methane to methanol and methanol to formaldehyde in methane oxidation [49]. Co-existing methylotrophic organisms in laboratory enrichment are capable of oxidizing methanol relieving its inhibition on the growth of methanotroph [91]. It is also reported that the highly toxic formaldehyde is removed in the consortium of methanotrophs and methylotrophs [111]. Another example is the co-existence of ammonia-oxidizing bacteria (AOB) or archaea (AOA) and nitrite-oxidizing bacteria (NOB) found in both natural and built environments [42, 65, 93, 137]. During the nitrification process, ammonia is oxidized by AOB or AOA leading to the production of toxic metabolites nitrite, which is then removed by NOB through oxidation of nitrite to nitrate [80, 100, 121].

Microbial consortia could benefit from syntrophy via the third approach: organism A provides B essential substrates, while organism B offers protective habitats such as biofilms. *Pseudomonas* species are one group of important biofilm-forming microorganisms found in environmental consortia [47]. Cyanolichen contains phototrophic cyanobacteria and heterotrophic fungi forming a symbiotic relationship, where the phototrophic bacteria provide organic carbons to the heterotrophic fungi, and the filaments of fungi create a protective habitat that also traps moisture and nutrients for the cyanobacteria [2].

Systems-level understanding of microbial interactions in consortia

Thanks to the rapid development of high-throughput sequencing and analytical chemistry, omics approaches ((meta)transcriptomics, (meta)proteomics, and metabolomics) have been broadly employed to analyze monoculture, defined consortia, enrichment culture, as well as environmental microbial communities to fill the knowledge gap of the underlying molecular mechanisms of microbial interactions [6, 21, 79, 143, 146]. Omics tools enlighten the understanding of microbial interactions by providing valuable information on functional diversity, gene expression levels, regulatory networks, and metabolite profiles [7, 103, 106, 127]. Different types of meta-omics analyses can complement and support each other, leading to integrated omics, a more comprehensive approach to decipher microbial interactions in detail (Fig. 3). With known genomic information of each consortium member, the current technology can generate informative metatranscriptomics and metaproteomics data to compare the temporal gene expression (mRNA and protein) between monocultures and consortia. For example, the temporal proteomes complemented with the

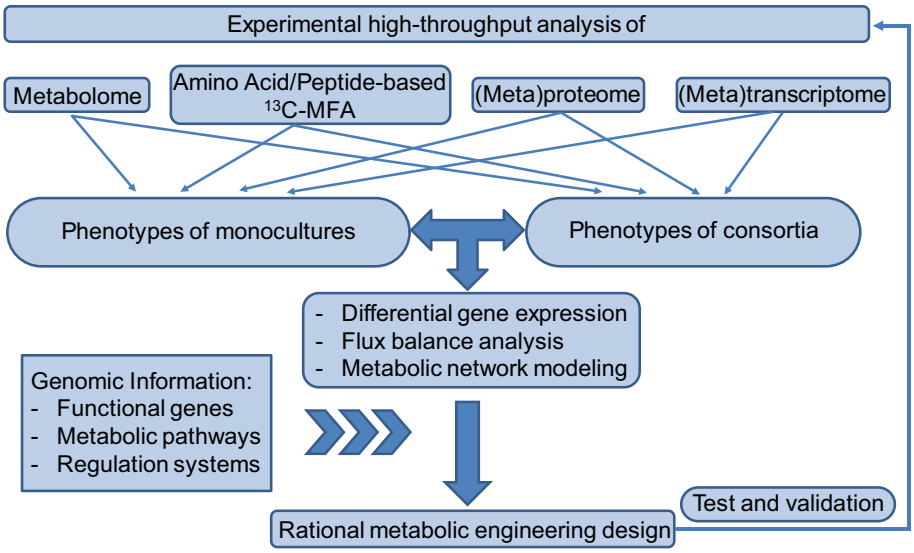

Fig. 3 Schematic workflow using omics tools for metabolic network elucidation and rational metabolic engineering design

metabolomic analysis elucidated the possible interactions between *Ketogulonicigenium vulgare* and *Bacillus megaterium*, which are artificially assembled together to produce 2-keto-gulonic acid (2-KGA), the precursor of vitamin C. The profiling of proteins and metabolites revealed that *B. megaterium* helped *K. vulgare* to resist reactive oxygen stress, and after sporulation and lysis *B. megaterium* also provided necessary nutrients, such as purine, for *K. vulgare* to grow better and produce more 2-KGA [79].

Comparative transcriptomics and proteomics have also been applied to study interspecies relationships in various syntrophic consortia, including interspecies hydrogen transfer in consortia containing a hydrogenic fermenter and a hydrogenotrophic microorganism (methanogenic archaea or dechlorinating bacteria) [85, 88], nutrient cross-feeding between corrinoid-auxotrophic and corrinoid-producing bacteria [86, 87], and stress-related in-contact interactions resulting in exclusive Mn oxidation in a co-culture [76]. Multiple comparative omics analyses also provided insights into interspecies interactions in the archaeal consortium of marine hyperthermophiles *Ignicoccus hospitalis* and *Nanoarchaeum equitans*: compared with monocultures, the metabolism (e.g., ribosome protein synthesis and amino acid metabolism) of *I. hospitalis* was redirected to alternative pathways to sustain the growth of *N. equitans*, resulting in the reduction of metabolic diversity in the consortium [104].

The integrative omics analysis would be even more powerful if complemented with metabolic flux analyses (MFA). However, even for ^{13}C-labeling metabolic flux analysis (^{13}C-MFA) in a consortium, it is difficult to distinguish labeling fingerprints of different species using the typical amino acid-based isotopic tracing experiments for monocultures, as amino acids produced by different populations cannot be easily distinguished [40]. To overcome this, a peptide-based ^{13}C-MFA together with protein-based stable isotope probing (SIP) and transcriptomic analyses have been developed, in which the unique sequences of amino acids in peptide allow the assignment of peptides to specific species in consortia [41]. Experimental peptide labeling patterns can be obtained by mass spectrometry via procedures similar to proteomics and protein-based SIP [13, 16, 57], and metabolic fluxes to each consortium member can be inferred from the peptide labeling patterns [101]. The extensive data generated from meta-omics can then be used to carry out flux balance analysis and to establish predictive models for microbial consortia [48, 101]. In addition, the above-mentioned systems biology tools can be combined with single-cell technologies such as fluorescence-activated cell sorting (FACS), Raman-activated cell sorting, and NanoSIMS to interpret the metabolic roles played by individual members in microbial consortia [32, 51, 74].

Engineering applications of environmental microbial consortia

The discovery of microorganisms growing syntrophically in nature has directed the design of engineering applications in pollution control and renewable energy production. One example of macroscopic microbial consortia used in environmental engineering is the production of biogas through anaerobic digestion of excess activated sludge from wastewater treatment and combined with food wastes. Anaerobic digestion is considered as an energy-efficient and environment-friendly approach for bioenergy production [77], where syntrophic interactions between fermentative microorganisms and methanogens play crucial roles in methane production. The loss of activity of one partner may severely affect the activity of the other, causing acid accumulation and a significant decrease in methane content in the biogas [45, 53, 126]. More recently, the concept of

complete nitrogen removal by membrane biofilm reactor has been tested in the laboratory taking advantage of the syntrophic interactions between DAMO microorganisms and anammox bacteria [138]. Algae–prokaryote consortia have also been used in wastewater treatment for energy-efficient nitrogen removal, where oxygenic phototrophs provide O_2 instead of aeration for nitrifying and heterotrophic prokaryotes while prokaryotes provide CO_2 and ammonia detoxification to algae [130].

Similar syntrophic relationship based on interspecies hydrogen transfer has been utilized in bioremediation of tetra-/tri-chloroethene (PCE and TCE)-contaminated fields with the addition of fermentable organic substrates, where fermenting bacteria supply hydrogen the sole electron donor used by the dechlorinating bacteria (i.e., *Dehalococcoides* spp.) and, in turn, hydrogen level is lowered down for the fermentation of organics to proceed further [3, 36, 82]. In addition to hydrogen, cross-feeding on another essential nutrient corrinoids has also been observed between the corrinoid-auxotrophic, dechlorinating *Dehalococcoides* and corrinoid-producing fermentative bacteria [86, 141].

Taken together, environmental microbial consortia can form tight mutualistic relationships via cross-feeding, detoxification, and biostructure formation. The microbial interactions and metabolic networks possessed in environmental microbial consortia may provide guidance for the design and optimization of stable, robust and efficient synthetic microbial consortia for a variety of engineering applications, such as biosynthesis and biodegradation.

Synthetic microbial consortia for engineering purposes

Synthetic microbial consortia refer to designed simple microbial communities with a defined composition of 2 or more (typically 2–3) species/strains. They hold great promise in a variety of engineering applications, including biosynthesis and bioremediation (bioaugmentation). Traditional medical and industrial biosynthesis processes rely on using genetically modified monoculture to create an all-in-one engineered strain capable of a broad spectrum of heterologous processes completing the bioconversion all the way to the end product. The fitness cost due to metabolic resource allocation makes it extremely challenging to engineer a single microbe to effectively and sustainably produce desired high-value products that require complex biosynthetic pathways [61, 135]. The division of labor among consortium members benefits each in terms of substrate utilization, redox balance (e.g., NAD^+/NADH cycling) and cell growth [17]. Although at its infant stage, synthetic microbial consortia have been emerging as a new paradigm as they possess the potential to overcome the limitations of using a single population. First, it can achieve higher biosynthesis efficiency with less refined substrates (e.g., pretreated beech wood) due to the capacity of microbial consortia to utilize a broader range of raw and low-cost substrates [9, 37, 115]. Second, the application of microbial consortia can also potentially simplify the multi-step process to reduce the operational cost. For example, the reorganized one-step vitamin C production by synthetic consortium eliminates the requirement of second sterilization process in conventional two-step fermentation, and notably reduces the production cost [129]. In addition, synthetic microbial consortia are advantageous to complex communities for bioremediation when the key players may compete with the other non-contributing members in the community for limited substrates. Such substrate competition would be eliminated by constructing microbial consortia containing only the contributing species. Here, we will discuss the design and optimization of synthetic microbial consortia, and consortium-based applications for biosynthesis and biodegradation.

Design of synthetic microbial consortia

Successful synthetic microbial consortia not only carry out the desired functions but also sustain cell growth in a stable and robust way. More stable relationships among consortium members are formed when they highly depend on each other. Microbial interactions that lead to the interdependence and stable relationships include cross-feeding, detoxification, and biofilm formation, which are important consortium design principles [56, 85, 86, 115]. There are typically two strategies to select consortium members: (1) top-down (from complex to simple): the consortium members are the identified keystone players from one specific complex microbial community [109, 147] (Fig. 4a), and (2) bottom-up (from simple to complex): the consortium members are selected from an inclusive pool of isolated and/or engineered microorganisms, which may possess the desired traits but not necessarily have common environmental origins [58, 68] (Fig. 4b). Although the bottom-up approach facilitated by a variety of synthetic biology tools is a simple and common method to construct synthetic microbial consortia, the top-down strategy offers naturally occurring microbial interdependence that might be missing from an artificial combination of engineered microorganisms using the bottom-up strategy. The most efficient and stable macroscopic microbial consortia for cellulose utilization exist in nature, such as rumen microbiome. However, it is typically non-model species that are involved in those environmental microbial communities. The unavailability of isolates of unconventional microorganisms as well as the lack of their genomic information, metabolic pathways, and suitable engineering tools are the major obstacles that prevent the top-down approach from being widely applied in synthetic

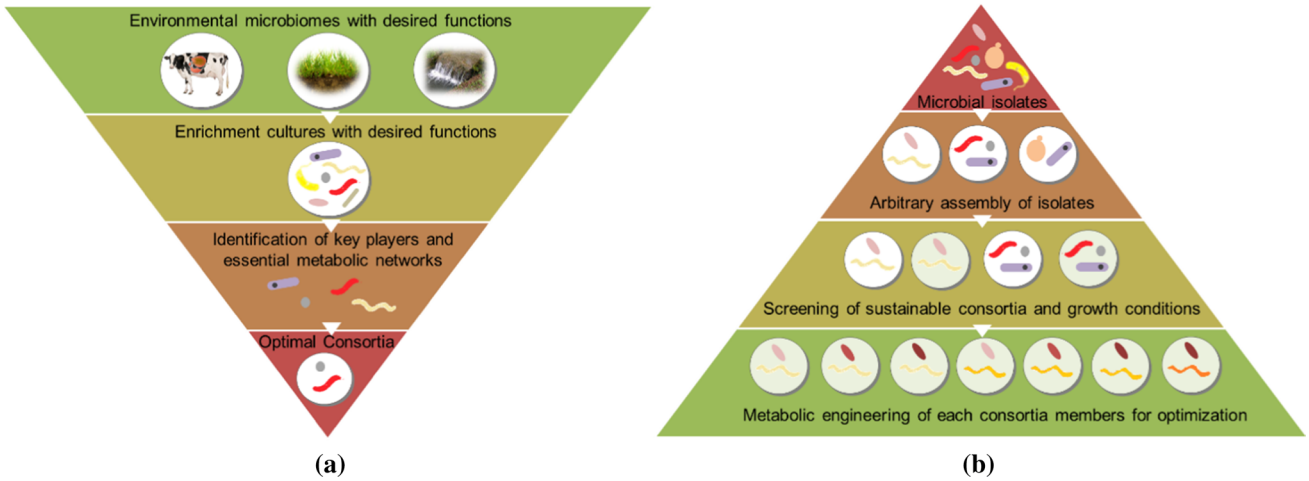

Fig. 4 Top-down (**a**) and bottom-up (**b**) approaches for synthetic consortia construction

consortia construction. It is challenging particularly for biosynthesis purposes as they require more accurate control on the metabolic fluxes and the output products. As single-cell technologies and systems biology tools for community studies are being advanced [83, 144], one may expect a better understanding of the environmental microbial consortia, which will benefit a more stable and robust design of synthetic consortia using environmental microorganisms.

Optimization of synthetic microbial consortia

Although promising, synthetic microbial consortia need to be optimized for the desired performance. One trade-off of using synthetic consortia for biosynthesis is the introduction of redundant metabolic pathways, rendering diluted flux toward the desired product. In addition, for defined consortia used as a bioaugmentation seed in bioremediation/biodegradation, they may need to carry out the reactions at sub-optimal growth conditions (e.g., low temperatures and low/high pH values) and low substrate levels (e.g., treating groundwater contaminations). To make the synthetic consortia cost-effective, stable, and robust, we need to modify the consortia or consortia members individually. The optimization methods include directed evolution, genomic and metabolic engineering, and artificial cell-to-cell communications.

Directed evolution

Directed evolution is a process simulating Darwinian selection to identify mutants with desired traits through iterative cycles of mutagenesis and enrichment of selected mutants. Different from natural evolutionary adaptation [128], directed evolution uses accelerated mutagenesis induced by a chemical mutagen or realized by molecular regulation and genetic/genomic engineering [26]. Random or targeted mutagenesis libraries are constructed, and the mutants are selected by monitoring the emergence of desired traits. Such mutagenesis and selection cycles drive the consortium system toward the desired phenotypes circumventing a thorough understanding of the metabolic networks and the underlying regulation mechanisms [5, 27]. The feedback-regulated evolution of phenotype has also been achieved in an adaptive control system, where the mutagenesis rate is maximized when no desired product is present and decreased when the desired product is in high concentration [24]. It has been successfully applied to select mutants with higher production of tyrosine and isoprenoid in *E. coli* [24]. Although directed evolution is typically based on single cells or proteins, theoretically it can also be applied to microbial consortia by evolving all members as growing together or generating mutant strains of individual consortium members for new consortia construction. It is a tremendous amount of work to construct mutant libraries and conduct selection experiments. Thus, more targeted approaches are needed. Directed evolution can be combined with genetic/genomic engineering to obtain desired traits when the genes/pathways essential for acquiring/losing the desired trait are known [73].

Genome and metabolic engineering

Advances in synthetic biology toolkits, particularly the rapid development of genome-editing techniques open up more possibilities of optimizing synthetic microbial consortia through metabolic engineering. Engineered nucleases coupled with sequence-specific DNA-binding domain enable the site-directed genome editing via the generation of double-strand break (DSB) followed by nonhomologous end joining (NHEJ) or homology-directed repair (HDR) in diverse cell types and organisms [46]. The site-specific DSB

can be generated by (1) Zinc-finger nucleases (ZFNs) [124], (2) transcription activator-like effector nucleases (TALENs) [89], and (3) clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) systems. CRISPR/Cas genome-editing systems emerge rapidly in recent years due to their advantages over the other two genome-editing approaches, including higher efficiency, easier procedure, and the availability for multiplex genomic modification [46, 96]. The type II CRISPR/Cas system (i.e., CRISPR/Cas9) originated from *Streptococcus pyogenes* is the most applied CRISPR/Cas-based genome-editing machinery. It includes a Cas-encoding gene (*cas9*), a single guide RNA (sgRNA), and an editing template, which are carried by a plasmid to be introduced to the target cells. The sgRNA is composed of crRNA, tracrRNA, and a ~ 20 bp target sequence (protospacer) in the to-be-edited genome plus a protospacer-adjacent motif (PAM, a three-nucleotide sequence of NGG) (Fig. 5). Besides numerous applications in plants and animals, high selectivity and efficiency of genome editing by CRISPR/Cas9 have also been demonstrated in microorganisms, particularly those model strains like yeast and *E. coli* involved in biosynthesis [1, 4, 28, 59, 75, 94, 140]. Recently, there is an emerging need for CRISPR/Cas-based toolkits tailored to non-model strains of specific interest for bioenergy and bioproduct synthesis [113]. One challenge to edit non-conventional microbial genomes is that some bacteria such as *Clostridium* species are lack of NHEJ and active HDR. The mutated Cas9, Cas9 nickase has been successfully applied to carry out DNA deletion and insertion via the single-nick-triggered homologous recombination (SNHR) strategy for the cellulolytic *Clostridium cellulolyticum*, resulting in editing efficiency as high as 95% [140]. Other challenges for CRISPR/Cas9 application to non-model strains include the selection of effective promoter and compatible carrying vectors, as well as the optimization of sgRNA. The editing efficiency by the CRISPR/Cas9 machinery can be enhanced by selecting promoters and vectors most compatible with the target cells [95, 102]. Furthermore, researchers have recently developed a high-throughput method screening high-efficiency sgRNAs in a non-conventional yeast strain, which can be potentially applied to other non-conventional microbial strains [113]. Efficient CRISPR/Cas-based genome editing can be applied for knocking out and/or knocking in genes that are essential in microbe–microbe interactions, thus enhancing the stability and performance of synthetic consortia. For example, the enhancement of cellulolytic activity of *C. cellulolyticum* converting cellulose into fermentable intermediates will open the possibility of constructing highly efficient cellulose-based synthetic consortia with specific fermenting bacteria converting the intermediates into biofuels and/or bioproducts.

Besides genomic manipulation, transcriptional control can also be achieved by CRISPR-assisted systems, CRISPR interference (CRISPRi) with deactivated or "dead" Cas9 (dCas9) for transcriptional repression and CRISPR activation (CRISPRa) with a transcriptional activator protein fused with dCas9 for upregulation of gene expression [11, 19, 139]. The CRISPR-enabled transcriptional control has been applied to a twin-clostridial consortium. The consortium was able to produce 22.1 g/L acetone–butanol–ethanol (ABE) from alkali-extracted, deshelled corn cobs, which matches the titer of ABE produced from starchy feedstock and is evident to be a promising platform for ABE production

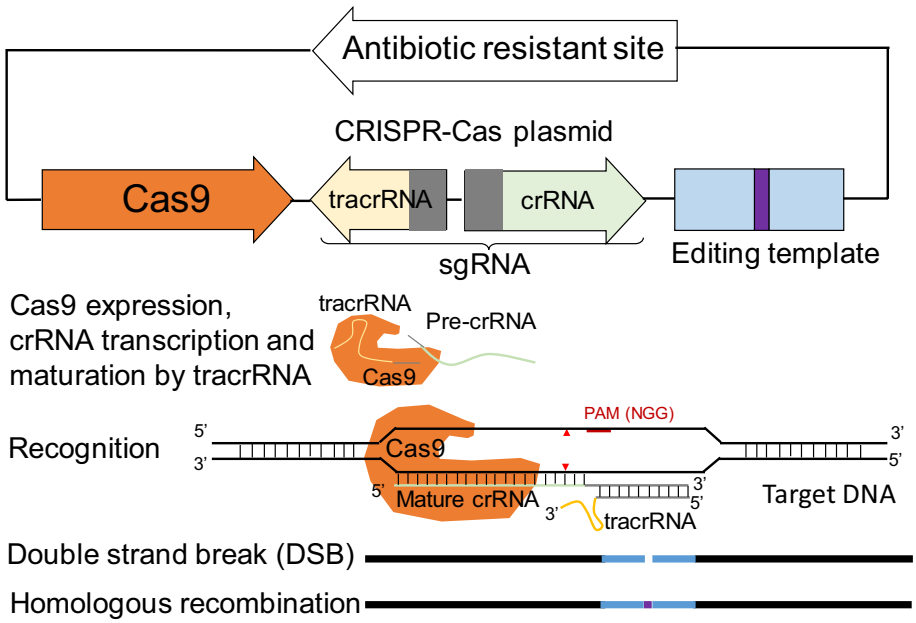

Fig. 5 CRISPR–Cas9 system

from lignocellulose. The desired metabolic pathway is divided into four modules and shared between *Clostridium cellulovorans* and *Clostridium beijerinckii* via metabolic engineering. CRISPRi system was designed to decrease the transcription level of the target hydrogenase gene in *C. cellulovorans*. The production of ethanol was increased by tenfold, indicating that the NADH generated was redirected to the synthesis of ethanol rather than hydrogen gas [132]. Moreover, a recently developed CRISPR-enabled trackable genome engineering (CREATE) technique enables (1) the high-throughput construction of site-specific mutagenesis libraries (including site saturation mutagenesis of a given protein), which can link genotypes to phenotypes, and (2) the reconstruction of adaptive evolution libraries containing mutants with all evolved genotypes, which can pinpoint which mutation leads to the evolved phenotype [38]. The CRISPR/Cas system can also be applied to selectively remove undesired bacterial species/strains and quantitatively control the composition of consortia, which are critical to industrial synthetic consortia. Illustrated in *E. coli*, type I-E CRISPR/Cas system was found to be capable of distinguishing and removing highly similar strains in consortia, and the control of consortium composition can be achieved by varying the collection of delivered CRISPR RNAs [43].

CRISPR/Cas-based techniques are powerful tools to genetically and/or metabolically engineer each member of microbial consortia. Ideally, an optimized synthetic consortium should have (1) enhanced interdependency and stability, (2) alleviated substrate competition among members, and (3) increased flux toward the desired products. However, different from monocultures, it is even more challenging to optimize synthetic consortia. To achieve a rational metabolic engineering design and promotes the stability and efficiency of the consortium, it is critical to have a thorough understanding of metabolic networks among consortium members. Systems biology tools discussed in Sect. 2.3 can be employed to disentangle complex microbial interactions in undefined and defined consortia. Due to the complexity of microbial networks, although with a tremendous amount of information obtained by systems biology, it is still challenging to disentangle the key components that directly contribute to the cooperative interactions. High-throughput experimental screening tools and model simulation or machine learning are needed to complement with the traditional systems biology, for further identification and validation.

Artificial cell-to-cell communication

Another possible way to manipulate synthetic microbial consortia is introducing artificial cell-to-cell communication circuits. Cell-to-cell communication plays a crucial role in the organization and regulation of multicellular traits such as division of labor, inter- and intracellular communications, and coordination of cellular activities. Design and incorporation of cell-to-cell communications enable researchers to engineer population-level behaviors and functions [25]. Quorum sensing (QS) is one of the most common mechanisms of intra- and interspecies communication. The large diversity and availability in synthetic biology make QS an attractive approach for coordinating complex behavior in synthetic consortia [109, 118]. The population-level behavior is coordinated by bacteria which produce and respond to specific signaling molecules (acyl-homoserine lactones, AHLs) in a density-dependent way. The gene transcription is controlled by certain QS regulating proteins (either activators or repressors), which AHLs can bind to. There are a number of QS systems found in environmental microorganisms, including lux, esa, las, tra, rpa, rhl, and cin [71, 118]. To develop QS-based genetic circuits for regulating system behaviors in synthetic consortia, variants of the QS regulator EsaR were obtained from directed evolution, with more than 70-fold higher signal sensitivity than the wild-type EsaR. Thus, the variants can be used at low signal molecule concentrations ranging from 5 to 10,000 nM [118]. By engineering *esaR* promoters with a second EasR binding site, researchers were able to modulate QS-dependent gene expression, opening possibilities of using a single QS signal to tune the regulation of multiple genes, which can be used to control microbial behaviors in synthetic consortia [116]. For consortia constructed based on multiple QS systems, system pairs that exhibit orthogonality are more useful as they do not form interactions (i.e., signal crosstalk, promoter crosstalk, or both) that may interfere different regulation systems in the consortium. Two engineered QS systems, rpa and tra, were examined to be completely orthogonal. The orthogonality of QS systems can also be predicted by a software tool [71]. Three in silico identified orthogonal QS communication channels were simultaneously applied to a consortium of three *E. coli* populations, and they successfully controlled each population level in response to the specific AHL signal as predicted [71]. Thus, the QS-based cell–cell communication systems provide potential modules for versatile control of synthetic consortia [114].

Synthetic microbial consortia for biosynthesis

Lignocellulosic biomass, the most abundant renewable carbon source, is an ideal feedstock for the production of biofuel and chemicals [44]. Biosynthesis of fuels and value-added chemicals from lignocellulosic biomass has been regarded as a sustainable alternative of current petroleum feedstock platforms. However, challenges remain in improving the bioconversion efficiency and lowering processing costs [97]. Consolidate bioprocessing (CBP) is thought to be a promising scheme for biorefinery due to its low cost and simple operation. However, monoculture typically has

limited productivity, yield, and titer. Harnessing consortia is an attractive alternative for CBP, although there are challenges such as maintaining stability and boosting the productivity of consortia [136].

Biofuels

A synthetic consortium of three epiphytic strains of *Enterococcus* was reported to produce 79.5 mL H_2 per gram of added wheat straw xylose which is many folds more than the monoculture of those species. Another consortium consisting *C. beijerinckii* with *C. cellulovorans* was designed to enhance the production of acetone–butanol–ethanol from previous leftover wheat straw; however, the interactions among the above species remain elusive [125]. Cross-kingdom interactions also guide the design of synthetic consortium for biofuel production: a consortium containing a cellulolytic fungus *Trichoderma reesei* producing soluble saccharides and an engineered *E. coli* strain metabolizing the saccharides to isobutanol, achieved up to 1.88 g/L production of isobutanol from cellulosic biomass. This study also demonstrates that the cooperator–cheater interaction could be applied to the design of stable consortia with tuning capacity [90].

Moreover, physical compartmentation enables the growth of consortium consisting of both aerobic and anaerobic microorganisms, broadening the spectrum of potential applications of microbial consortia. A recent study demonstrates this concept with bioproduction of ethanol from wheat straw in single biofilm membrane reactor featuring both aerobic conditions for the cellulase-producing fungal strain *T. reesei* Rut C30 and anaerobic conditions for an ethanol-producing yeast strain *Saccharomyces cerevisiae*. The oxygen permeable membrane at the bottom of the reactor enables the formation of fungi biofilm, in which oxygen is depleted, and cellulolytic enzymes are synthesized and released. Sugars from cellulose hydrolysis are then fermented to ethanol in the upper anaerobic yeast biofilm [18].

Bioproducts

Up to 19.8 g/L lactic acid was produced by a fungal–bacterial consortium of the aerobic fungus *T. reesei* and facultative anaerobic lactic acid bacterium *Lactobacillus pentosus* from non-detoxified steam-pretreated beech wood in a spatially structured biofilm, similar as described above. On dense oxygen permeable membrane, the oxygen is depleted in the biofilm of *T. reesei,* producing cellulases that hydrolyze cellulose to sugars including cellobiose, which may inhibit the fungal growth at high concentrations. In the anaerobic bulk liquid, *L. pentosus* fed with released sugars consumes cellobiose, which alleviates the inhibitory effect of cellobiose on *T. reesei*. The self-inhibitory by-product acetic acid produced by *L. pentosus* can, in turn, be consumed by *T. reesei* via cross-feeding, thus promoting a stable mutualistic relationship between the two species [115].

The conventional industrial bioproduction of 2-keto-L-gulonic acid (2-KGA), the precursor of vitamin C, is limited by a long incubation period and the additional sterilization process. Synthetic microbial consortia have been successfully applied for one-step vitamin C production, in which the yield of 2-KGA is comparable to the original two-step fermentation process. Consortia optimization via metabolic engineering was carried out to produce 2-KGA from D-sorbitol. The consortium of *Gluconobacter oxydans* and *Ketogulonicigenium vulgare* was reorganized with alleviated competition for substrate and enhancement of symbiotic relationship by deleting genes involved in sorbose metabolism of *G. oxydans* [129].

Traditional engineered strains typically hold long reconstituted metabolic pathways to produce high-value metabolites; however, parts of the pathway may require specialized environments for optimal performance, and the host may be metabolically overburdened. A consortium of engineered *E. coli* and *S. cerevisiae* is reported to successfully produce 33 mg/L oxygenated taxanes, precursors of the anti-cancer drug paclitaxel, through the distribution of a heterologous pathway into two engineered bacteria. In this division of labor, *S. cerevisiae* utilizes metabolic intermediates produced by *E. coli*. The fast growth of *E. coli* and the complete protein expression system of *S. cerevisiae* are integrated for the biosynthesis of taxanes. To avoid the competition between the two species, researchers engineered mutualistic relationships in the consortium, where *S. cerevisiae* grew solely on acetate, a self-inhibitory product of *E. coli* [145].

Engineered *E. coli* consortia containing two functionally different strains have been applied to synthesize high-value natural products. Flavonoid was produced by an *E. coli* co-culture. The malonyl-CoA dependent upstream *E. coli* strain converts phenylpropanoic acids to flavanones, and the NADPH-dependent downstream *E. coli* strain transforms flavanones to flavan-3-ols. These two *E. coli* strains were individually optimized by improving flux to essential substrates and cofactors, and the combined consortia were screened based on titer. With the aid of empirical modeling, the optimization of carbon source, strain compatibility, temperature, and inoculation ratio leads to a 970-fold improvement in titer over the published production by single populations [62]. A more complex consortium containing four *E. coli* strains has been recently designed to conduct complete biosynthesis of anthocyanins from sugar. The division of metabolic burden and genetic optimization of individual strain enabled cooperative overexpression of 15 exogenous or modified enzymes from various plants and microbes, achieving milligram-per-liter production

titer, which is several orders of magnitude higher than previous studies using enzymes from eukaryotic organisms [61].

Synthetic microbial consortia for biodegradation/bioremediation

Synthetic microbial consortia may play important roles in bioremediation, as the division of labor in consortia is important for the degradation of persistent pollutants, which usually requires multiple steps, and cultures must be robust to the complex environment [52]. The constructed and optimized synthetic consortia can serve as the seed culture for bioaugmentation of in situ bioremediation practice and for biodegradation in more confined reactors as ex situ remediation approaches. It is worth noting that the application of engineered microbial consortia currently is, to the most extent, restrained in well-controlled bioprocesses to avoid genetic contamination from environmental microorganisms.

In a batch study, the inhibitory effect of acetylene, a product of tetra- and trichloroethene biodegradation on the dechlorinating bacterium *Dehalococcoides* sp., was eliminated by co-cultivating an acetylene-fermenting bacterium, *Pelobacter*. The acetylene fermentation products also sustained the growth of the dechlorinating bacteria as energy and carbon source [81]. In addition, a consortium consisting of *Bacillus clausii* T and *Bacillus clausii* O/C, both isolated from human probiotics, alleviated the toxicity of antibiotics and showed a higher removal efficiency of select antibiotics than pure cultures [69]. A defined consortium isolated from petrochemical landfarm site (containing *Mycobacterium fortuitum*, *Bacillus cereus*, *Microbacterium* sp., *Gordonia polyisoprenivorans*, *Microbacteriaceae bacterium*, the Naphthalene-utilizing bacterium, and a fungus *Fusarium oxysporum*) was tested to degrade polycyclic aromatic hydrocarbons (PAHs) including anthracene, phenanthrene, and pyrene in soil. On average, 78% of three PAHs with different concentrations were mineralized by the consortium in 70 days. And the consortium showed more effective anthracene degradation than any of the isolates [55]. Similarly, the aliphatic and aromatic hydrocarbons of crude oil were efficiently degraded by a defined microalgal–bacterial consortium containing four bacterial species *Sphingomonas* sp. GY2B, *Burkholderia cepacia* GS3C, *Pseudomonas* sp. GP3A, and *Pandoraea pnomenusa* GP3B and one oil-tolerant microalga *Scenedesmus obliquus* GH2. Almost all alkanes, alkylcycloalkanes, alkylbenzenes naphthalene, fluorene, and phenanthrene were removed by this consortium [122].

Challenges and future research directions

Challenges remain to be overcome before fully harnessing the potential of synthetic microbial consortia [117, 147]. First, the available number of orthogonal cell–cell interaction channels is limited [123]. Second, the underlying mechanisms and regulations of microbial interactions and their functional flexibility are poorly understood [142]. Although ubiquitously occurring in nature, inter- and intra-kingdom communications have not been well studied, as well [131]. Third, long-term homostasis of consortia could be difficult to maintain, as the long-term behavior of engineered organisms is unpredictable [17]. Fourth, some useful functions such as cellulolysis are possessed by non-model microorganisms such as *Caldicellulosiruptor saccharolyticus* [99], for which efficient genome-editing tools are lacking due to the limited knowledge of organism-specific biochemical pathways and regulatory mechanisms. In addition, fine tuning the behavior of multiple populations in consortia is still challenging. Expanding directed evolution used for a single population to multiple populations under varying environments is also needed.

Future research is needed to address the above challenges. Potential directions include (1) obtaining a systems-level understanding of microbial interactions and metabolic networks in synthetic consortia for rational metabolic engineering design; (2) developing the state-of-the-art high-efficiency genome-editing toolkits for non-model microorganisms; (3) developing high-throughput screening tools and inexpensive gene-chip assay for directed evolution in multiple populations. The success of synthetic microbial consortia is, to a large extent, dependent on the advancement of systems biology, synthetic biology, analytical, and modeling tools. Only when we decipher the codes of microorganisms given by the mother nature to form strong and stable relationships among each other and know how to re-code them, synthetic microbial consortia will fully show their power in biosynthesis and biodegradation, as well as many other engineering applications.

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