Microbial communities for bioprocessing: lessons learned from nature

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Abstract

Microbial bioprocessing has evolved from the use of undefined natural consortia to the construction of synthetic communities tailored to specific processes and bioproducts. This evolution is enabled by recent advances in biotechnology, including cultivation of non-model microbes, metabolic engineering, bioinformatics, and numerical modeling. Equipped with these powerful tools, engineers have designed co-cultures and consortia with an expanded set of capabilities, mainly via "bottom-up" approaches that tether isolates together in culture. Here, we present a brief review of the opportunities, challenges, and recent developments in consortia-based bioprocessing with a focus on lignocellulosic biomass conversion. With improved understanding of microbial community composition and function, we further present a vision to harness defined consortia down-selected from nature via "top down" approaches.

Keywords

Microbial consortia; non-model microbes; synthetic biology; fungi; bacteria; bioprocessing

Introduction

Microbial bioprocessing is a powerful tool that has transformed nearly every aspect of our lives, from the production of food, fuel, solvents, and drugs, to waste water treatment and remediation. From an engineer's standpoint, we typically think of these products as the outcome of one microbial workhorse, where a single strain has been isolated and manipulated in the laboratory to tackle a specific task. However, history reminds us that undefined mixtures of microbes have been used for millennia to advance society. Africans brewed wine as early as 690 BC [1]; yeasts in the environment have been used to make bread for millennia [2]. Biogas

generated from mixed microbial metabolism was even used for heating bath water in Assyria in the 10th century BC, and gave rise to modern anaerobic digestion technologies [3]. In the 1970s, oil spills were first remediated by stimulating the growth of naturally occurring microorganisms in the ocean [4]. Rather than building multiple functions into a single microbe, these "top-down" approaches utilize undefined microbial communities that divide-and-conquer difficult metabolic tasks to enable or accelerate outcomes.

The advent of synthetic biology in the past century, and particularly in the past decade, has taken bioprocessing to the next stage, where "bottom-up" approaches based on defined or targeted microbial communities have become viable approaches for bioprocessing. Examples include the production of antibiotics [5], wastewater treatment plants based on anaerobic ammonia oxidation [6], and biofuel production [7]. Among these applications, the production of clean and renewable biofuels is of particular interest in both developing and developed parts of the world, because biofuels generate less environmental impact compared to traditional fossil fuels and hold promise as cost-efficient alternatives [8]. This manuscript reviews recent advances in bioprocessing based on microbial consortia, with a focus on highlighting gaps in knowledge and opportunities for development to convert lignocellulosic (non-food) biomass to biofuels. Both "top-down" and "bottom-up" approaches to biofuel production have been extensively investigated in the past twenty years. Nevertheless, several outstanding questions remain, including which approach (or combination of approaches) are best to enhance conversion, how stability of the consortia influences degradation, and how advances in bioinformatics and metabolic modeling should be incorporated to aid in the design of stable consortia.

Consortia-Enabled Biofuels: An Alternative to CBP

Conventional methods of biofuel production are usually energy intensive, requiring physical and/or chemical pre-treatment of biomass before enzymatic hydrolysis and microbial fermentation [9,10]. Consolidated bioprocessing (CBP) has been proposed as an alternative to conventional methods, and it combines cellulase production, biomass hydrolysis, and sugar fermentation into one step [11]. Classical CBP aims to bypass pretreatment and convert lignocellulosic feedstock into desired products by using one microorganism, such as the yeast *Saccharomyces cerevisiae* [12–14]. However, it has become clear in the past decade that a multitude of challenges make it difficult to engineer a "superbug" microbe that both breaks down

and converts biomass into fuels efficiently at industrial scale [15]. Major issues include the difficulty of establishing genetic systems in novel organisms, relatively low specific activity of recombinantly expressed enzymes (including activity against crude substrates), and limited tolerance to concentrated byproducts such as ethanol [15].

A promising alternative to classical CBP is consortia-based bioprocessing, a field of research that has made significant progress recently [16,17]. A microbial consortium consists of more than one strain of microorganism, often with complementary metabolic functions, where difficult tasks can be divided across a diverse subset of microbes. A defined consortium is usually constructed with via a "bottom-up" approach, co-culturing two (or more) isolated strains that have been well characterized (Figure 1). At the other extreme, undefined consortia originate from environmental microbial communities with an unknown number of constituents (Figure 1). However, defined consortia can also be constructed by a "top-down" approach that enriches a stable microbial community, where each member is later isolated and subsequently characterized (Figure 1).

"Bottom-up" Approaches: Advancements and Opportunities

Most progress in consortia-based bioprocessing to date is based on a "bottom-up" methodology, where synthetic communities of microorganisms have been constructed to accomplish specific goals (Figure 1). Members in these microbial consortia often have a commensal or syntrophic relationship, i.e. the products made by one member are beneficial for and/or used as substrates by another member. A number of synthetic consortia have proven to be more efficient in bioprocessing than mono-cultures [7]. For example, Xu and Tschirner [18] demonstrated improved efficiency (up to two-fold) of ethanol production by a co-culture of two strains of fermentative *Clostridium* compared to mono-cultures. They hypothesized that the observed synergy was a result of *Clostridium thermolacticum* utilizing the degraded substrates from *Clostridium thermocellum*, which are less favorable for *C. thermocellum*. Similarly, Zuroff *et al.* [19] established symbiosis between *C. phytofermentans* and a yeast (either *S. cerevisiae* or *Candida molishiana*) stable for 50 days. While *C. phytofermentans* is sensitive to oxygen, both yeasts removed oxygen from the co-culture in return for soluble sugars released by *C. phytofermentans* hydrolysis. The ethanol yield from the co-culture of *C. phytofermentans* and *S. cerevisiae* was more than two-fold higher than either of the mono-cultures. One example of a

cross-kingdom consortium divided the tasks of hydrolysis and fermentation to the fungus *Trichoderma reesei* and the bacterium *Escherichia coli*, respectively [20]. This stable co-culture converted microcrystalline cellulose and pretreated corn stover to isobutanol, yielding 62% of the theoretical maximum.

While mixed microbial consortia generally outperform a single microorganism, their full potential for bioprocessing has yet to be realized for cost-efficient industrial scale applications. To optimize synthetic microbial consortia, numerical modeling has become an important tool for prediction of metabolic compatibility and consortia design [20–22]. For example, a genome-scale metabolic model of *C. cellulolyticum* can successfully predict chemostat growth and byproduct secretion, and it can be used to develop a dynamic model of metabolic interactions in the co-culture with *C. acetobutylicum* [23]. Experimental data have confirmed that modelling frameworks incorporating the interspecies exchange of metabolites predicts the species ratio in microbial consortia [20,24], but these models usually make simplistic assumptions such as maximizing total biomass, which sometimes lead to inaccurate predictions [25]. Zomorrodi [26] showed that it is important to include species- and community- level fitness functions when modeling microbial communities. In addition to genome-scale metabolic modeling, a simpler coarse-grained model, which is tailored to solve the inference problem from the experimental data, has been proposed as a key to understanding microbial communities [27].

In addition to numerical modeling, the incorporation of metabolic engineering promises to offer greater flexibility to control the behavior of synthetic microbial consortia. For example, Shin et al. [28] engineered an *E. coli* strain to co-express and secrete xylanase and acetylxylan esterase, hydrolyzing xylan in growth media into xylooligosaccharides. A second engineered *E. coli* strain assimilated xylooligosaccharides and converted them into ethanol. This modularly designed co-culture reached 55% of the theoretical maximum for ethanol yield. Another application of metabolic engineering split the signal production and sensing components originally from *Staphylococcus aureus* between two strains of *Bacillus megaterium* [29]. Successfully engineered cell-cell communications in both Gram-positive and Gram-negative hosts [29–31] could be extended to achieve dynamic control of biomass-degrading consortia. Additionally, such a strategy provides an inherent advantage of avoiding the need to monitor cell growth and add exogenous molecules at a specific cell density, and the modular design allows for tuning of each member of the synthetic co-culture [29]. Compared to classical CBP, which

usually attempts to constitutively overexpress or knock out select genes in one organism, consortia-based metabolic engineering could increase process efficiency and productivity by dividing metabolic burdens between consortia members.

Although synthetic microbial consortia constructed from "bottom-up" indubitably represent a step forward, this approach still faces a number of major challenges. First and foremost is the inability to co-culture microorganisms that are known to form stable and functional communities *a priori*. More than 99% of all microorganisms have not been cultivated or genomically characterized, thus the "bottom-up" construction of synthetic consortia is inherently limited by knowledge of characterized strains. Additionally, considering that most model organisms are well domesticated to thrive independently under laboratory conditions, they may not be the best candidates to form robust, stable consortia with other microorganisms. The underlying mechanisms for synergistic effects of synthetic consortia are primarily hypothesized based on the knowledge about their metabolic pathways. In addition to the example of a co-culture of *T. reesei* and *E. coli* mentioned above [20], such limitations were apparent in the study of a *Clostridia* co-culture that was constructed to convert filter paper to hydrogen [32]. In this co-culture, *C. thermopalmarium* depends on the hydrolytic products from *C. thermocellum*, and enhances the overall yield of hydrogen compared to the mono-culture of *C. thermocellum*. However, lack of knowledge regarding the underlying mechanisms of the interactions in this microbial consortium creates obstacles for further engineering efforts such as enhancing stability and scaling up. Moreover, besides the division of labor, it is equally critical to consider the spatial and temporal organization and cumulative behavior as a function of interactions when constructing and evaluating microbial consortia [33].

"Top-down" Approaches: Exploiting Natural Partnerships

Natural consortia of microorganisms have been applied in multiple contexts, including the conversion of biomass to fuels. Biogas produced from anaerobic digestion is one of the most broadly adopted biofuels in developing countries, with 27 million biogas plants in China, and 4 million in India [34]. Despite the significant economic and environmental benefits offered by biogas plants, there is limited knowledge about the parameters controlling their efficiency and reliability, because they are usually regarded as a "black box" of unknown microbes [35].

The first step towards cracking open and understanding these complex systems is to investigate the natural consortia of microorganisms inside. Recent advances in sequencing technology have enabled us to start filling the gaps of our knowledge about the composition and diversity of natural microbial communities. In an agricultural biogas plant, 454-pyrosequencing of the total microbial community found that *Firmicutes*, followed by *Bacteroidetes* and *Proteobacteria* were the dominant bacterial members responsible for polysaccharide degradation, while *Methanomicrobiales* represent the most abundant group of methanogenic archaea [36]. Similar findings were reported in a biogas-producing anaerobic digestion experiment, where the most abundant members of microbial community were the species *Methanoculleus marisnigri* and from the class *Clostridia*, as determined by short-read next generation DNA sequencing [37]. Additionally, an extensive temporal pyrosequencing dataset from anaerobic digestion facilities demonstrated the unprecedented level of stability maintained by the resilience of syntrophic bacterial communities [38].

The remarkable stability and resilience observed in these systems stems from a few key factors. First, diversity of a microbial consortium usually translates into functional redundancy. In the event of environmental perturbation, some members of the microbial consortium may experience lowered activity or a reduction in population size. Redundant members may respond positively to make up for the loss of function for the entire consortium [39]. Another stabilizing mechanism is the network of interactions between organisms [39]. Therefore, it is highly attractive to extract a defined microbial consortium from an existing complex consortium. This should retain part of the network of interactions between the members and the corollary stability selected by nature. On the other hand, from an engineering perspective, a microbial consortium with a small number of members should be easier to control than a complex consortium.

Compared to earlier efforts aimed at constructing synthetic microbial consortia from well-studied model organisms, extracting a defined consortium from nature offers a number of advantages. The classic approach to synthesize a consortium for biomass conversion is to select a strain that is capable of breaking down biomass into simple compounds that can be converted by another strain into desired products. However, it is generally impractical to test all possible combinations considering the sheer number of theoretically viable pairs. A better approach is to enrich for naturally occurring microbial consortia from environmental samples, such as the rumen and fecal material of herbivores, under culture conditions that will eventually be used for

production (e.g. [40–42]). The enrichment approach generally ends up with a minimal set of microbes that are highly stable, each of which could be isolated for genomic and transcriptomic analysis, as well as physiological characterization (Figure 1). On the other hand, this "top-down" approach is limited by the lack of established genetic engineering tools for microbes obtained through enrichment. Therefore, the development of a genetic system for these isolated members should set a foundation for later metabolic engineering, enabling another level of control on the performance of and interactions within the consortium.

The Case for Exploiting the Herbivore Gut Microbiota

One of the best examples of natural consortia that hydrolyze and ferment biomass is found in the herbivore gut. Microorganisms living in the digestive systems of natural grazers have co-evolved with their host for millions of years to utilize lignocellulosic hydrolysates. Microbes span a range of bacteria, fungi, protozoa, and methanogenic archaea [43–45], and the plethora of biomass-degrading genes and genomes discovered from cow rumen indicate that herbivorous grazers' digestive systems should be an ideal source for down-selecting lignocellulolytic consortia [46,47]. Indeed, members from all four types of microorganisms mentioned above have been isolated and characterized from the cow rumen [48–51], and there is a body of literature on mixed cultures of bacteria and archaea (e.g. [52–55]). However, studies exploring the potential of directly down-selected co-cultures including eukaryotic microorganisms remain limited.

The mutualistic paring of fungi and methanogens is one of the consortia achieved by enrichment techniques [41,42]. The primary metabolites of anaerobic fungi include carbon dioxide, hydrogen, and volatile fatty acids, which in turn are utilized by methanogens for growth and methane production. Although anaerobic fungi are outnumbered by their bacterial counterparts in the rumen, there is evidence that fungal populations play a major role in the breakdown of cell walls [56–59]. Flagellated fungal zoospores attach themselves and develop hyphae that penetrate into plant tissues [60]. The physical breakdown of biomass coupled to the array of lignocellulolytic enzymes produced by these fungi [61] ensures efficient degradation of biomass. For example, anaerobic fungal enzymes are rich in xylan-degrading enzymes and hence unbiased in substrate preference [61]. Therefore, a wide variety of feedstocks that are already produced and often considered as "waste" such as corn stover can be used for methane

219 production. This saves arable land for cultivation of food rather than dedicating them for biofuel
220 production as in the case of corn to ethanol, which is one of the two largest bio-ethanol programs
221 in the world [62]. The other major bio-ethanol program extracts juice from sugar cane as a sugar
222 source for fermentation, and the bagasse is either burnt for additional energy or simply discarded
223 [63]. It has been estimated that if 50% of the bagasse after juice extraction is converted to
224 ethanol, potentially using a microbial consortium, ethanol yield per hectare of land can increase
225 from 6,000 L/ha to 10,000 L/ha [64]. Moreover, the efficient conversion of lignocellulosic
226 biomass to biofuels should reduce the burden of disposing the agricultural "waste" products and
227 lessens their environmental impact, serving as a solution to the "food, energy, and environment
228 trilemma" [62].
229
230 **Conclusions and Perspectives**
231 Humanity has, knowingly or unknowingly, harnessed the power of microbes and
232 microbial communities for bioprocessing since ancient times. More recently, we have come to
233 recognize that microbial processing ranges between two extremes: from microbial communities
234 that are "black boxes" to single microbe mediated fermentations. While the use of "top-down"
235 approaches exploit nature to accomplish difficult tasks, control of these communities is limited
236 due to lack of understanding of the microbial community composition, metabolism, and
237 dynamics. The advance of new tools, including metabolic engineering of non-model hosts,
238 sequencing and bioinformatics, and numerical modeling, have enabled engineers to parse the
239 roles of community members like never before to understand their function. With these tools in
240 hand, synthetic co-cultures can be constructed from the "bottom-up" to capture the behavior of
241 natural microbial communities. Considering the strengths and weakness of both "top-down" and
242 "bottom-up" approaches, the "top-down" approach is likely advantageous when the goal focuses
243 on the output and stability of microbial processing and the ability to control each member of the
244 microbial consortia is less of a concern. On the other hand, the "bottom-up" approach is likely
245 favorable when the ability to manipulate each member of the consortia is necessary.
246 Along these lines, defined consortia down-selected from environmental samples are a
247 highly attractive compromise to synthetic co-cultures – combining the advantages of both "top-
248 down" and "bottom-up" approaches. However, this methodology still faces a number of major
249 challenges, including the resistance to cultivation by many microorganisms, the difficulty in

developing novel genetic systems for metabolic engineering, and deciphering the genetic codes and interwoven metabolism of the microbial consortia. To move the field forward, it is critical to integrate all tools at hand to make informed decisions, with a focus on selecting stable consortia that have the ability to thrive at industrial scale. Genomic and transcriptomic characterization of each member of a defined consortium allows for the discovery of metabolic pathways and potentially their controls, which lays the foundation for system optimization, especially when combined with numerical modeling. Insights gained through metagenomic and metatranscriptomic analysis of natural microbial communities should further guide the enrichment strategy when down-selecting microbial consortia, particularly for applications relevant to biomass hydrolysis and product fermentation.

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(*) Papers of special interest

Solomon et al. 2016

By analyzing the transcriptomes of previously uncharacterized anaerobic gut fungi, this study discovered a large, comprehensive array of unbiased, biomass-degrading enzymes that synergistically degrade crude, untreated plant biomass, and are competitive with optimized commercial preparations from *Aspergillus* and *Trichoderma*.

Solomon et al. 2014

This paper summarizes the practice of using next-generation sequencing, proteomics, and bioinformatics to derive biological insight from complex microbial communities, including their composition and function.

Haitjema et al. 2014

This paper details recent methodological progress in the study of anaerobic gut fungi. Specifically, advances in isolation, culture, and cellulolytic enzyme discovery should promote bioengineering efforts to adapt these non-model organisms for biofuel production.

Marchand and Collins 2015

This study was the first to engineer synthetic quorum sensing and cell-cell communication system in a Gram-positive host, *Bacillus megaterium*. It also split the signal production and sensing components between two strains of *B. megaterium*. It has the potential to enable the generation of dynamic gene regulatory networks in *B. megaterium* and other Gram-positive strains.

Hoffner and Barton 2014

This study outlines a roadmap towards the quantitative design and optimization of low cost resilient artificial ecologies based on microbial consortia, using algal production of fuels and chemicals as an example. The proposed numerical model integrates metabolic information with the ecological scale of the inter-species interactions and the process scale of bioreactors.

Konopka et al. 2015

This paper identified principles behind the functional stability and robustness in microbial communities. The authors pointed out that the network of interactions between organisms provides a buffer against disturbance beyond the effect of functional redundancy, as alternative pathways with different combinations of microbes can be recruited to fulfill specific functions.

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