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Microbial Engineering Community (MCE): Selecting Key Players in Microbiomes

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Microbial Community Engineering (MCE) is an emerging paradigm in biotechnology, which focuses the attention on microbial consortia or communities, sometimes addressed as microbiomes. Microbial communities are ubiquitous in nature and useful in many areas. Earth's microbial ecosystems are important in the production of foods, nitrogen fixation and carbon cycles, recycling of micronutrients, bioremediation and in maintaining the health of humans, animals, and plants, among many others. MCE is a growing field that can be exploited to produce bulk and fine chemicals, bioenergy as well as pharmaceuticals by enhancing the effect of natural microbiomes. Adapted/selected microbial consortia have the potential to advance specialized tasks by resorting to high-order communities' mechanisms, which are difficult to accomplish with monocultures. Notwithstanding the interest in MCE, biotechnological applications remain rudimentary; mixed cultures of partially known composition govern the processes of wastewater treatment and the anaerobic digestion of organic refuses, yet the vast potential of "*microbial ecological power*", observed in most natural environments, remains largely underemployed. After a brief overview of key mechanisms that govern microbiomes, this work is aimed to suggest experimental approaches for the separation of constituents of complex microbiomes and to use MCE to reproduce the natural biological order by designing interlinked modular bioreactors where MCE would overcome limitations of natural systems.

1. Introduction

The growing interest in MCE arises, in part, due to the limitations inherent to engineer a single cellular chassis and incorporating complex pathways within it as well as the obstacles to transfer long DNA efficiently, and the need of large precursors and cofactors in the engineered cells. Monocultures are often more sensitive to environmental changes or contaminations, requiring highly controlled culture conditions and specialized sterilization protocols. MCE can facilitate maintaining different thresholds of metabolic plasticity, which can contribute to buffer external pressures on microbiomes. While the current approach in systems biology uses and integrates multilevel data, such as multi-omics techniques that provide key insights into physiological information at the single-cell level, the understanding, and control of microbial interactions in mixed consortia are at infancy state. As a matter of fact, it is widely recognized that microbial communities exhibit a higher-level of complexity than monocultures and therefore multidisciplinary and time-optimized techniques are required (Singh et al., 2019). Mixed consortia can accomplish tasks that are difficult or potentially impossible to achieve using monocultures; despite their potential, the mechanisms underlying microbial community maintenance and function are poorly known. This limited understanding is in part due to the greater challenges associated with the increased complexity when dealing with multi-population interactions. However, communities dominate the microbial world; coexisting organisms cannot help but interact (Hays et al., 2015). These interactions include touching using dedicated signals, gene transfers and competitive or cooperative scenarios (competition for and exchange of resources), including alteration of environmental conditions to influence the growth of neighbours.

Microbial cultures that consist of multiple microbial species, by definition, contain an increased range of genes and metabolic capabilities in comparison to monocultures. This diversity allows for the emergence of communal properties such as robustness and division of labour. Besides the engineering of strains by synthetic biology, scientists should address a more ecological perspective by taking into consideration the

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environment by manipulating macro parameters in bioreactors to improve community function (e.g. changing substrate composition, aeration, pH, temperature, and shear-stress). This approach can enable the application of microbial communities to actual-world problems by enhancing the natural attributes of microbial consortia.
Microbial communities have long been recognized for their important impact on human health, agriculture, and industry. Beyond human health, microbial activity is essential for a wide range of industrial applications including microbially mediated denitrification in wastewater treatment, biofuels production (Cortes-Tolalpa et al., 2017) and Microbial Fuel Cells aimed to simultaneously treat wastewater and generate electrical energy (Finkelstein et al., 2006). This myriad of important functions suggest that MCE can serve in medical, agricultural, and industrial applications and have sparked recent interest in developing microbiome-based biotechnologies. MCE refers to the use of naturally occurring microbial communities to select a specific function or to enhance an existing one. Such attempts on a given community can be done by various approaches. One microbiome engineering technique is the modulation of environmental conditions to effect changes in community functions, which is frequently used to optimize bioreactor performance. Another approach is to modify the community composition by adding some beneficial species or removing undesirable ones. Antibiotics development is the most emblematic example of this approach in therapeutics, acting as a tool for removing pathogenic species. Probiotics use, in contrast, represents an additive tool, aiming to improve community function by introducing beneficial species. Moreover, the separation of differently sized species can be performed by immobilization or entrapment in different matrices such as molecular sieves. Other approaches can benefit of interactions within microbiomes, which can pertain either exclusively to the biotic phase (species–species) or can be influenced by environmental factors, (species–environment) (Gómez Camacho et al., 2019). One of the main challenges is the separation of different vital constituents in different bioreactors, without loss of species-species interactions. Ecological stable communities, with defined niche differentiation, offer the possibility to avoid competitive exclusion of species for a specific nutrient or set of nutrients. Distinctive pools of nutrients can be segregated, for biotechnological applications, via physical barriers as occurs in biofilm formation. Spatial organization is an important feature which can be used not only for nutrients/product segregation but also to promote stable coexistence of microbiome players and can be studied using micro-fluidic devices, immobilization, bioprinting, and entrapment in natural or artificial matrixes.This paper is aimed to highlight different approaches which can be used to separate groups of microbial communities to increase their biotechnological performance. Experimental methods, at different scales, will be analysed and commented.

2. Macro analysis of microbial interactions

2.1 Division of labour

Division of labour (DL) is a key feature of co-cultures and mixed consortia, where different populations execute different tasks at a higher efficiency than monocultures. In ecological contexts, DL encompasses both features: specialisation of different cells and cooperation between them to provide an inclusive benefit to the community. From an engineering perspective, the DL can be seen as a simple separation of tasks, regardless of the ecological and evolutionary consequences. However, when properly constructed or engineered, DL can facilitate consortia functions in multiple ways. DL can enable the rational organization of the metabolic pathways by compartmentalizing different steps into different members of the microbial community. This separation is necessary when multiple processes cannot coexist in the same cell. For example, complex feedstocks such as lignocellulosic biomass, which contain many C forms such as simple C_5 and C_6 sugars and lignin, have garnered significant interest for renewable microbial biosynthesis of chemicals and energy carriers. Single populations typically cannot utilize multiple sugars simultaneously due to carbon catabolite repression (i.e. a cell strategy to optimize uptake of C-sources requiring less or simpler hydrolytic enzymes), instead of consuming one sugar at a time in order of preference. In contrast, microbial consortia can more quickly and efficiently utilize mixtures of different C-sources type by metabolizing one C-source per each population, improving the efficiency of each process and hence that of the whole. DL can reduce the metabolic burden experienced by the cells by decreasing the number of expressed enzymes, taking advantage of heterologous parts produced or provided by other community members (Roell et al., 2019). In addition, it can make the systems more modular, facilitating easier manipulation and engineering each separate component. DL also plays an important role in anabolic pathways, where multiple subpopulations can divide the biosynthesis labour or divert towards secondary pathways, for example, sequestering toxic intermediates. The extension of these concepts to bioprocesses presents new opportunities for engineering control mechanisms in cell populations to maintain stability and other self-regulating processes by which biological systems tend to adjust the conditions that are optimal for their survival. Additionally, competition and conflict between different metabolic processes could be resolved by DL. For instance, in a hypothetical pathway, an enzyme (E)

transforms a substrate (S) into an intermediate (I), whereas a second enzyme (E1) transforms the intermediate (I) into a product (P). These two enzymes (E and E1) may compete for the same intracellular resource, such as cellular space, co-factors for correct enzyme activity or building blocks for biosynthesis. If both enzymes are contained within the same cell and competition is asymmetric, (E) would have preference over (E1) and hence, accumulation of (I) occurs. Vice versa, if each enzyme is contained within different cells, competition is no longer present. In addition, MCE is believed to have expanded functional and metabolic capacities which can allow for DL across organisms, even though the precise mechanisms are not always well defined. Recent developments of omics techniques have enabled the study of metagenomics of microbial communities and elucidate the functions of those microbes. An interesting example regarding the roles of different organisms in mixed consortia is the methanogenic microbiome, where fermentative bacteria and archaea demonstrate how the DL within the community is well organized. Several experiments have shown the importance of diversity in microbiomes; while this supports microbiome ecological theory, they have also highlighted the need to gain deeper insights. Using DL approaches can result in microbial consortia which are simpler to manipulate than monocultures, since each sub-population would contain only a subset of the overall complexity. This level of controllability is a fundamental principle and feature of developing innovative biotechnological process, even in biorefinery contexts, as units are simply specific assemblies of discrete parts that can be changed without reconstructing the entire system. This occurs in natural bioremediation attenuations where microbiome selection is carried out by biogeochemical differences in different points of the same contaminated matrix (Anantharaman et al., 2016).

2.2 Possible communication mechanisms in microbiome

Chemical-based signals within mixed microbial populations are believed to be the predominant form of interactions; they can incentivize and stabilize coexistence. The chemicals molecules exchanged are typically metabolites, which can directly influence growth via cellular metabolism, or small molecules, which can induce gene expressions to promote survival via cell-to-cell signalling. For example, one of the most common symbiotic interactions in natural microbial communities is commensalism. In this type of symbiosis, one population benefits from another population but receives no harm nor gives benefits in return. Commensalism promotes mutual coexistence in a situation where a larger population supports the growth of a lower fitness one, that would otherwise be unable to survive. Another form of commensalism can be reached via quorum sensing (QS). QS is a density-dependent form of communication, which also serves as a mean to program population interactions. An interesting attribute of QS systems is the molecular mechanisms of this process, as well as the chemical nature of self-inducers. While acyl-homoserine lactones (AHL) systems are typically associated with Gram-negative species, QS in Gram-positive bacteria involves the production of small linear or cyclic peptides (larger than AHL), probably due to different diffusion resistances across the membrane structures of each bacterial group. The QS can participate (Tsoia et al., 2019), in combination with other regulatory mechanisms, in secondary metabolic processes by activating genes for the production of molecules with a wide variety of functions, such as toxic compounds, antimicrobials, antioxidants, virulence factors, among others. QS effects are believed to act at high density, for instance, in similar conditions to that occurring in microcolonies or biofilms, natural or induced by immobilization and/or entrapment in bioreactors. This is just one example of the potentiality of QS: one population is unable to synthesize key metabolites for survival unless a second population is present at a sufficiently high density which can stabilize the microbial population by activating QS mechanisms. Another symbiotic relationship that can maintain coexistence is mutualism, in which subpopulations depend on each other to survive. One of the most common methods for achieving mutualism is cross-feeding, in which each population is fed by the metabolites produced by other members for the collective survival. In addition, microbial consortia exhibit robust proliferation with balanced growth rates when low numbers of toxin producers were included at inoculation or the toxin range was short (Doekes Id et al., 2019). Hence, a major challenge for ecological research is to understand how competing species survive in rapidly changing environments to elucidated basic principles that may help to understand more complex ecosystems.

2.3 Horizontal gene transfer

The non-genealogical transfer of genetic material between microbial species encompasses different mechanisms, which are termed horizontal gene transfer (HGT). It has been suggested as a primary driving force of microbial evolution involving mobile genes migrating among different microbial groups, which encode functions such as new metabolic capabilities that are advantageous for microorganisms under certain conditions. These genes can promote diversification and specialization in a microbial community by forming various phenotypes that can coexist in di□erent ecological niches. HGT in mixed populations induce specialization of different members into di□erent categories as for example scavengers, harvesters and

pioneers, that metabolize di□erent polysaccharides and coexist symbiotically. For example, pioneers can degrade insoluble polymers into smaller oligomers that can be consumed by harvesters, while scavengers that are less e□cient at degrading the polymer and cannot not use the polymer at all. This level of diversification is possible because HGT can drive acquisition or loss of physiological traits that may not be possible alone through mutations. In MCE, HGT is a valuable trait for maintaining robust coexistence between populations.

3. Experimental approaches to study microbial consortium

3.1 Microfluidics approach

Physical and chemical environments play critical roles in community function. Several technologies are available which allow the testing of various environmental parameters, such as the chemical concentration, timescale, volume and spatial distribution, and their combined effects on microbial consortia. Microfluidic devices (Figure 1) could provide the means to study microbial co-cultures or consortia through the fabrication of microscale environments conditions. Microbiomes can be studied using microfluidics by generating chemical gradients, via entrapment of cells to focus the study on the interactions or by creating micro-habitats to study their dynamics by constructing and dispersing biofilms. These devices have the advantage of being parallelizable, which also allows high-throughput experimentation, but are limited from the analysis point of view. However, the recent development of analytical techniques, such as flow cytometry, mass spectrometry, liquid chromatography coupled to microfluidics devices could enable also high-throughput collection physiological data (i.e. avoiding off-line tests). Microfluidics is used to create specific environments, by a single or combination of the following characteristic properties: (i) cell density and chemical gradient generators, (ii) droplet encapsulating of single or multispecies cells and (iii) selective microbiome entrapment to capture cells of different species.

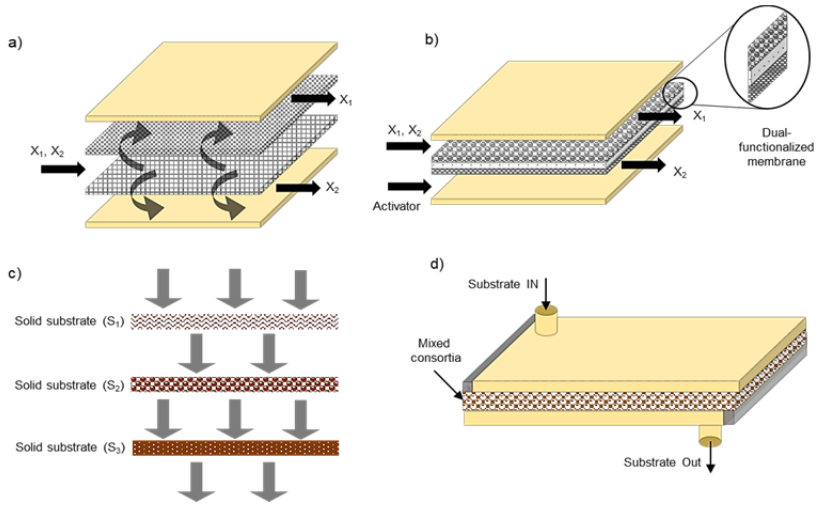

Figure 1. Microfluidics scalable devices (nL, µL and mL) approaches to select key players in mixed consortia

Additionally, the use of membrane systems could be useful in the separation of species; transwell membranes or in-flow devices can be used to culture species which are very difficult to grow in submerged systems. Figure 1a) shows a planar conformation of two differently functionalised membranes which are "transversally" crossed by the cell suspension, collecting different permeates. Figure 1b) shows a bilayer membrane differently functionalized on each side, one side is in contact with the microbial suspension (e.g. µg/L or mg/L) while the other is in contact with a chemical or a biochemical "activator" capable of causing a selective action on the microbiome. Figure 1c) represents a "percolation" arrangement in which the microbial suspension crosses solid layers of agar-media containing different compounds. Figure 1d) shows a micro system obtained with a 2D printing system.

3.2 Spatial patterning

Different approaches have been proposed to study the spatial distribution either in two or three dimensions and the following represents a short list. For instance, moulding aims at embedding cells into solid media, while inkjet printing can pattern droplets of microbes at picolitre-scale at high resolution. Moreover, recent developments in 3D printing can be used to design microscale geometries around bacteria to create spatially

separated communities or compartments. Advanced multispecies entrapment can also be performed using coaxial spheres to create calcium alginate artefacts of different diameters, hence promoting the selection of key microbial groups due to the different mass transfer of certain compounds.

3.3 Regime Analysis

All the above techniques, however, present a common problem: they are hard or not at all scalable, so the provided information on the microbiome behaviour is limited, and cannot directly be applied to larger scales. In order to get broader information at bioreactor scales, scale-up and scale-down techniques can be used. These approaches originate from the observation of the selection effects which are introduced by spatial non-uniformity on bioreactors created, mainly, by non-homogeneous mixing conditions. Regime analysis is a practical approach for predicting the existence of gradients in bioreactors. It comprises the identification of the limiting (i.e. the slowest) step by comparing characteristic times of relevant cellular and operational mechanisms. Accordingly, when the bioreactor mixing time is longer than a relevant cellular process (e.g. biomass growth, metabolites production), then gradients are likely to occur. Mixing times and circulation times in microbial and cell culturing bioreactors can be difficult to determine experimentally, hence modelling approaches can be employed. Mixing times are significantly higher for eukaryotic cell cultures than bacterial, due to the higher shear-stress sensitivity; as a consequence, moderate to low power inputs are used in the former cultivations to prevent hydrodynamic damage of the cells. Regime analysis considers the dynamics of a high-order system as the result of different lower-order contributions; each contribution exhibits a different characteristic time and can be of interest in order to regulate intraspecies, species-species and species-environment interactions (Maity et al., 2015). Engineering parameters that can serve as a selective pressure, and adequately applied to controlled microbiomes, can promote an effective microbial selection. For instance, mass transfer phenomena, biokinetic selection due to different microorganism's retention times in the vessel and local different mixing intensities or aeration can be used to control microbial populations and, to a certain extent, the morphology of the biotic phase inducing a "quasi-natural" selection.

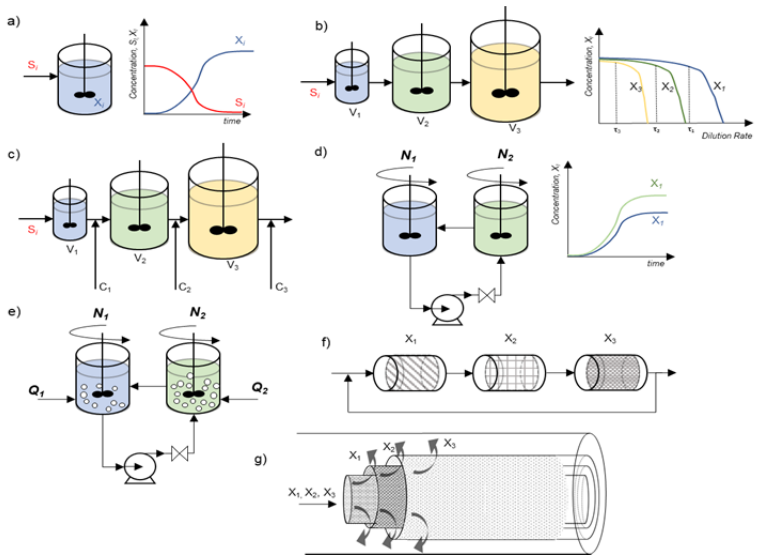

Figure 2. Large bioreactors (mL and higher volumes) configurations to select key players in mixed consortia

Figure 2 shows some of the possible experimental arrangements of laboratory bioreactors which can be useful to induce microbial separations in co-cultures or mixed consortia systems. Figure 2a) shows a bioreactor operated in batch conditions that allows selecting a microbial subgroup from a mixed system (used as an inoculum) by exposing to a particular C-source or a particular substance (acid, base, surfactant, antibiotic, bio-stimulants etc.). This configuration is also useful in the case of selection with *repeated fed-batch* technique. Figure 2b) shows an arrangement able to select microbial species using the principle of biokinetic selection. In this configuration, passing from V1 to V2 and then to V3, the residence time increases in the vessels, hence V3 there will be enriched in slow-growing populations, while in V1 there will be an enrichment of the fastest-growing and in V2, an intermediate condition will be found. A variant of this system is shown in Figure 2c), in which a different carbon source (e.g. lignin, cellulose, carbohydrates) is added to each bioreactor. Figure 2d) shows an arrangement able to operate the selection based on the resistance to shear-stress induced by stirrers operated at different revolution numbers as N1 and N2 due to microcirculation. The configuration, in

the hypothesis of inducing a circulation between V1 and V2, allows to evaluate also the effects induced by the macro-circulation. While Figure 2e) allows to evaluate the effects induced in the microbial population of a different degree of aeration (oxygenation) when Q1≠Q2 and N1=N2; when Q1 = Q2 and N1≠ N2, the effect of different k_la (oxygen mass transfer coefficients) could be investigated within the microbial systems. Finally, the arrangement shown in Figure 2f) and g) show a very effective and easily scalable solution using the dimensional "separation" of microorganisms. The first shows an arrangement where the microbial suspension cross different cartridges filled with different material at different degree of porosity, or different materials (e.g. activated carbon, zeolite, celite); while the second shows an example of microbial selection obtained by using tubes (i.e. celite) with different porosities assembled in a *tube-and-tube* type of bioreactors configuration where the mixed microbial suspension enters in the central tube and then is forced to move radially through tubes having different porosity. All the above solutions can be more or less effective depending on the type of consortia that is intended to be separated, it is not possible to exclude the use of several methods simultaneously, as happens for the selection from anaerobic digestion (AD) consortia of dark fermentation (DF) bacterial species (Gómez Camacho et al., 2019).

4. Conclusions

Microbes existing in consortia provide robustness and broader metabolic capacities which are attractive for applications in energy, environment, and potentially healthcare. There are many complex tasks that consortia are well suited to address, which can reduce the cost of biotechnological processes which are primarily based on single microbial strains. The applications in bioremediation and bioenergy (AD and DF) are the most consolidated, whereas in wastewater treatment (e.g. anaerobic ammonium oxidation - Anammox) to save nitrification operational costs, as well as in soil nitrogen fixation, are progressing. Generally, the gap towards a bioeconomy can be reduced by resorting to the microbial diversity which is contained in microbiomes, in particular, the areas of renewable feedstocks, biochemicals production, and biofuels. The efforts to use cellulosic feedstocks for energy production must be further investigated; besides being the most abundant raw material on earth, there are costly to process but the burden could be lessened using different specialized microbiomes. The stability of the long-term microbial consortia remains the determining aspect that must be verified with different experimental campaigns, which is not yet widespread in the literature. Despite the actual progress, the road ahead is very long; time could be shortened if the scientific community is able to better understand microbiome consortia dynamics, address higher complexity systems, especially to increase robustness and longevity, which fundamentally requires experimental devices and omics analytics.

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