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Engineering of ecological niches to create stable artificial consortia for complex biotransformations

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The design of controllable artificial microbial consortia has attracted considerable interest in recent years to capitalize on the inherent advantages in comparison to monocultures such as the distribution of the metabolic burden by division of labor, the modularity and the ability to convert complex substrates. One promising approach to control the consortia composition, function and stability is the provision of defined ecological niches fitted to the specific needs of the consortium members. In this review, we discuss recent examples for the creation of metabolic niches by biological engineering of resource partitioning and syntrophic interactions. Moreover, we introduce a complementing process engineering approach to provide defined spatial niches with differing abiotic conditions (e.g. O_2, T, light) in stirred tank reactors harboring biofilms. This enables the co-cultivation of microorganisms with non-overlapping abiotic requirements and the control of the strain ratio in consortia characterized by substrate competition.

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Introduction

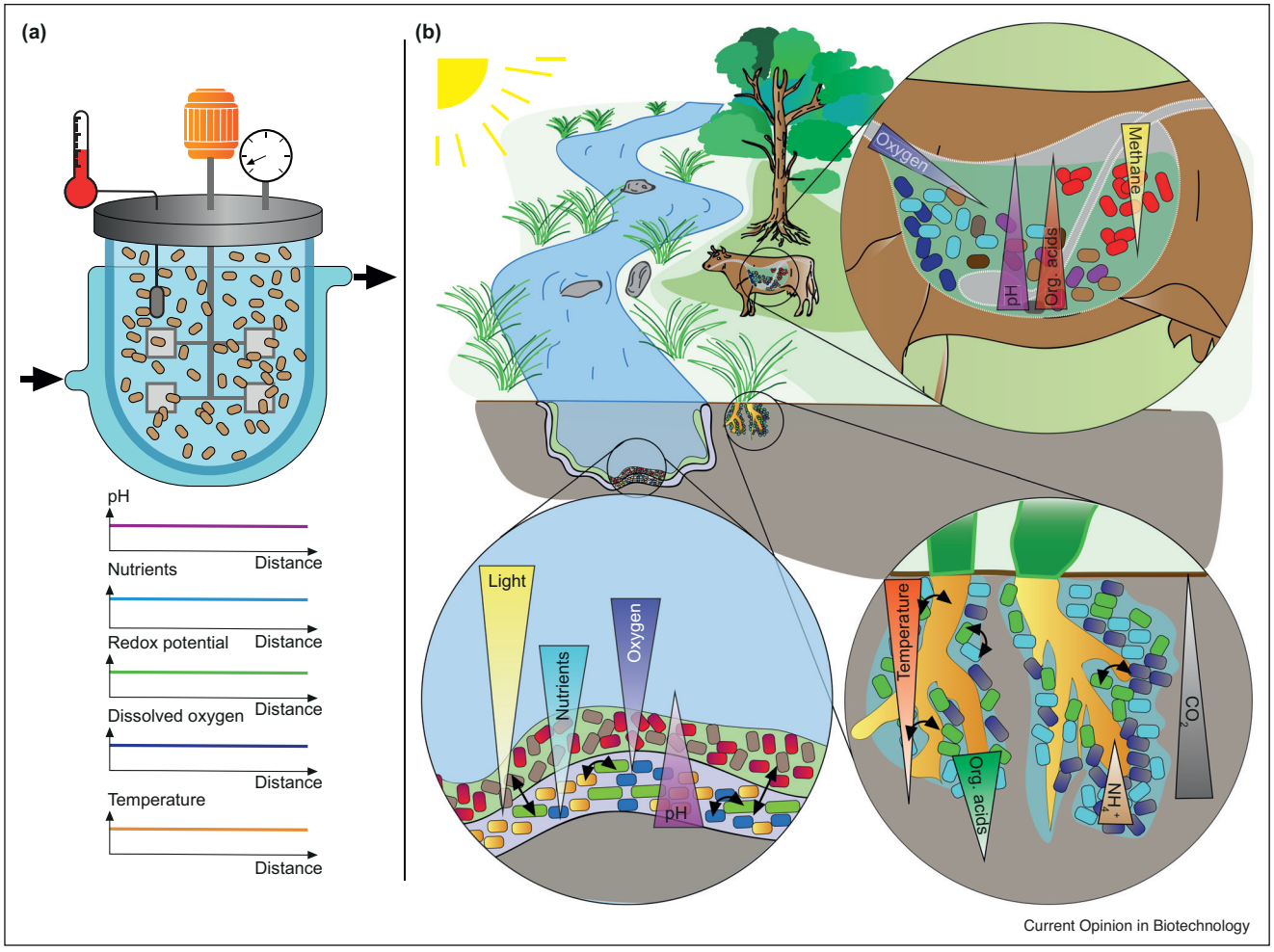
Over the last decades, monocultures of genetically engineered microorganisms growing in the homogeneous environment of well mixed stirred tank reactors have been predominately used as biomanufacturing systems to successfully produce a wide range of chemicals in an industrial setting [1] (Figure 1a). However, this approach faces limitations when it comes to more complex biotransformations. The number of new properties that can be implemented into one microorganism is limited due to the imposed metabolic burden, cytosolic or periplasmic space limitations, competing biochemical reactions and toxic intermediates [2,3] which leads to poor fermentation performance. In nature, complex tasks are thus distributed either within subcellular compartments [4,5] or between different microorganisms [6,7]. Natural ecosystems found for example, in soils, sediments or digestive organs (Figure 1b) are almost exclusively organized as mixed communities of up to several thousand species forming complex ecological interaction webs. The growth environment is of heterogenous nature and characterized by several gradients of abiotic factors such as temperature, pH, light intensity and the concentrations of dissolved oxygen, carbon dioxide, nitrogen and various metabolites. Thus, a multitude of ecological niches exist that are occupied by specifically adapted microorganisms. Such niches allow for a high microbial diversity and lead to outstanding metabolic capabilities and the high robustness of microbial consortia [8].

However, the range of final metabolic products that are produced in natural ecosystems is limited, and the high complexity of natural communities hampers the targeted adaption to the desired product. For this reason, the design of stable, less complex and better controllable artificial co-cultures has attracted considerable interest in recent years in order to capitalize on the several advantages of microbial communities such as the distribution of the metabolic burden by division of labor, the modularity and the ability to convert complex substrates. To this end, several strategies including the engineering of chemical symbiosis, of quorum sensing and of ecological niches have been proposed and tested [8–10]. In this review, we will discuss the most recent progress in the engineering of ecological niches by biological as well as process engineering which are used as tools to enable, control and stabilize synthetic microbial communities with unprecedented abilities.

Biological approaches to engineer metabolic niches

An ecological niche is the set of biotic and abiotic factors which allow a species to exist [10] whereby recent definitions include reciprocal relationships between the species and the environmental factors [11]. Using measures that enable niche partitioning, that is, the occupation of one specific niche by only one consortium partner, a stable co-occurrence of several species can be fostered [12]. Below,

Figure 1



Schematic overview of the concentration profiles of abiotic parameters **(a)** in an industrially used stirred tank bioreactor for axenic cultivations and **(b)** in natural ecosystems with undefined microbial communities. While abiotic parameters in the natural ecosystem (b) show a multitude of gradients which allow the formation of spatial niches and microecosystems, the bioreactor in (a) is ideally mixed to provide homogenous conditions.

we discuss biological approaches to create such niches that employ the engineering of resource partitioning and of syntrophic interactions.

Engineering resource specific niches

Resource partitioning is a common mechanism of niche differentiation, wherein each community member metabolizes a different set of nutrients in order to avoid the direct competition for available substrates [8]. Consequently, the prerequisite for the creation of such a resource specific niche is that the target product be producible from a mixture of substrates, for example, a chemical or enzymatic hydrolysate of lignocellulosic carbohydrates. In the example of lignocellulosic hydrolysates the substrates consist of — depending on the carbohydrate source — varying proportions of the C_6 sugars glucose, mannose and galactose and the C_5 sugars xylose and arabinose. Many microorganisms can principally utilize mixed sugars, but a diauxic growth pattern is often observed, where one sugar (usually glucose) is preferentially consumed in a first phase, followed by a second phase in which the less-preferred sugars are metabolized. This phenomenon is caused by a complex regulatory network known as carbon catabolite repression (CCR) and is an undesired trait in industrial fermentations, as it leads to increased culture durations and incomplete sugar utilization thereby limiting achievable yields and productivities [13–15]. A widely reported approach to enable simultaneous sugar utilization without CCR is the engineering and optimization of several strains specialized in the fermentation of only one specific sugar and their combination in artificial consortia that are stabilized by resource partitioning. Typically, a combination of rational strain design, for example, by knocking out genes that are essential for assimilation of non-target sugars, and

Table 1

Examples for the use of microbial consortia to convert non-edible low-value gaseous substrates, mixed sugars, or lignocellulose to different target products

Substrate	Product	Microorganisms	Titer	Main consortia feature	Ref.
Different mixtures of glucose, galactose and mannose, 7.5 g/L total sugars	Cell biomass	Three different *E. coli* strains each engineered to preferentially metabolize one of the hexoses (by rational strain design and adaptive evolution)	n.a.	Simultaneous consumption of all sugars, no carbon catabolite repression up to 51 % higher growth rate than the wild type strain able to consume all sugars up to 29% higher cell density	[14]
20 g/L glucose, 10 g/L xylose, 5 g/L arabinose	Ethanol	Three different *S. cerevisiae* strains each engineered to preferentially metabolize one of the sugars	12.5 g/L	Simultaneous consumption of all sugars, no carbon catabolite repression Stable fermentation kinetics during prolonged repeated batch cultivation in contrast to generalist strain.	[15]
67 g/L glucose, 33 g/L xylose	Ethanol	Two different ethanologenic *E. coli* each engineered to metabolize only one of the sugars	46 g/L	Simultaneous consumption of both sugars, no carbon catabolite repression 0.49 gL^{-1} h^{-1} productivity 28 % higher titer than the monoculture.	[16••]
Hydrolyzed pretreated sugarcane bagasse, 100 g/L solids	Ethanol	Xylose fermenting, glucose negative ethanologenic *E. coli*, *S. cerevisiae* (turbo yeast)	24.9 g/L	Simultaneous consumption of both sugars, no carbon catabolite repression shortened fermentation time (< 30 hour)	[17]
Glucose	Anthocyanins	Four *E. coli* strains collectively expressing 15 heterologous enzymes	< 10 mg/L pelargonidin 3-*O*-glucoside	Enables for the first time the synthesis of anthocynins from glucose outside of plants Reduced metabolic burden by division of labor	[35••]
Glucose	Rosmarinic acid	Three *E. coli* strains	172 mg/L	Reduced metabolic burden by division of labor 38-fold higher titer than for monoculture.	[36]
Methanol	Monacolin J and lovastatin	Two *Pichia pastoris* strains	594 mg/L monocolin J and 251 mg/L lovastatin	Avoidance of metabolic pathway imbalances found in the monoculture Reduced metabolic burden by division of labor	[37]
Syngas	Butanol, hexanol	*C. autoethanogenum*, *C. kluyveri*	0.14 mmol/h butanol, 0.04 mmol/h hexanol	Syntrophic interactions Extension of the product spectrum that can be produced from syngas	[38•]
CO_2		*S. elongatus*, *R. glutinis*		Artificial lichen co-culture: Phototroph provides sucrose as carbon source for heterotroph, heterotroph limits generation of toxic reactive oxygen species.	[39]
Alkali-extracted deshelled corn cobs	Acetone, butanol, ethanol	*C. cellulovorans, C. beijerinckii*	22.1 g/L solvents	Engineered mesophilic *C. cellulovorans* as cellulolytic strain provided soluble sugars and butyric acid Engineered mesophilic *C. beijerinckii* is solventogenic and converted hexose and pentose sugars and butyric acid to the final products	[20•]
Delignified rice straw	Butyric acid	*C. thermocellum*, *C. thermobutyricum*	33.9 g/L	Thermophilic, cellulolytic *C. thermocellum* provided soluble sugars, acetic acid and ethanol Thermophilic *C. thermobutyricum* converted sugars and by-products *C. thermocellum* to butyric acid	[40]

Table 1 (*Continued*)

Substrate	Product	Microorganisms	Titer	Main consortia feature	Ref.
Delignified rice straw	Butanol	*C. thermocellum*, *C. saccharoperbutylacetonicum*	5.5 g/L	Thermophilic, cellulolytic *C. thermocellum* provided soluble sugars Mesophilic *C. saccharoperbutylacetonicum* is solventogenic and converted sugars to butanol Delayed inoculation of *C. saccharoperbutylacetonicum* after temperature shift from 55 to 30°C	[41]
Pretreated corn stover	Isobutanol	*T. reesei, E. coli*	1.88 g/L	Cellulolytic *T. reesei* provided soluble sugars Engineered *E. coli* converted the sugars to isobutanol	[23]
40 g/L microcrystalline cellulose	Fumaric acid	*T. reesei*, *Rhizopus delemar*	6.9 g/L	Cellulolytic *T. reesei* provided soluble sugars *Rhizopus delemar* converted the sugars to fumaric acid Fumaric acid production occurs only under N limitation	[22]
17.5 g/L cellulose, 9 g/L xylose from pretreated wheat straw	Ethanol	*T. reesei*, *S. cerevisiae* and *Scheffersomyces stipitis*	9.8 g/L	Cellulolytic *T. reesei* provided soluble sugars *S. cerevisiae* converted glucose to ethanol *S. stipitis* converted xylose to ethanol Consortium operated in a membrane aerated biofilm reactor allowing for concomitant aerobic and anaerobic conditions	[25]
50 g/L microcrystalline cellulose	Lactic acid	*T. reesei*, *Lactobacillus pentosus*		Cellulolytic *T. reesei* provided soluble sugars *L. pentosus* converted glucose to lactic acid Consortium operated in a membrane aerated biofilm reactor allowing for concomitant aerobic and anaerobic conditions	[26••]

adaptive evolution is applied to engineer the sugar specialist strains [13,15,16••]. As detailed in Table 1, different consortia of such sugar specialists were shown to simultaneously consume different sugars without CCR, while also outperforming the respective generalist strains in terms of growth rate, final cell densities, productivity and yield [14,16••,17]. Employing sugar specialized *Saccharomyces cerevisiae* strains, Verhoeven *et al*. demonstrated, that such a consortium showed stable fermentation kinetics in prolonged repeated batch cultivations on a sugar mixture in contrast to an engineered generalist strain whose performance deteriorated over time [15].

Engineering consortia with syntrophic interactions

Syntrophy is one type of interaction found in consortia that is defined as a one-way or two-way metabolic interaction between consortium members, in which one partner utilizes intermediate products that are released by the other [18]. If the consuming partner is not able to feed on the substrate of the intermediates-producing partner, both occupy a unique metabolic niche in a microbial food chain. As described below, such food chains are engineered to distribute long synthetic pathways over several strains or to extend the product range of microorganisms able to grow on gaseous substrates such as syngas or CO_2.

Heterologous enzyme expression in genetically modified microbial hosts constitutes a metabolic burden on the host that increases steadily with the number of overexpressed enzymes. An increased burden results in decreased metabolic fluxes and lower availability of precursors and cofactors [2]. One way to address this problem is based on the principle of division of labor and divides the desired long metabolic pathway among multiple members of a community. This approach offers several advantages over the monoculture approach, including the individual genetic optimization of each host, the selection of the most suitable organism for the respective partial transformation, as well as the simplified reusability of the individual partial pathways due to the modularity of the system. Recent examples of employing artificial food chains in communities to catalyze complex biotransformations include the synthesis of anthocynanin achieved by the expression of 15 recombinant enzymes in four

Escherichia coli hosts [18], of rosmarinic acid [19] and of monacolin J and lovastatin [20•].

Artificial food chains are also used to extend the product spectrum of gas fermentations. Syngas (a mixture of CO, H_2 and CO_2) can be metabolized by few anaerobic microorganisms such as *Clostridium autoethanogenum*, which produces acetic acid and ethanol in axenic cultures. When combined with *Clostridium kluyveri*, hexanol and butanol could be produced as final products [21]. CO_2 can be fixated by autotrophic organisms such as the cyanobacterium *Synechococcus elongatus*, which secretes sucrose that can then further be converted for example, by heterotrophic yeast strains to unsaturated fatty acids, a system which mimics naturally occurring lichens [22].

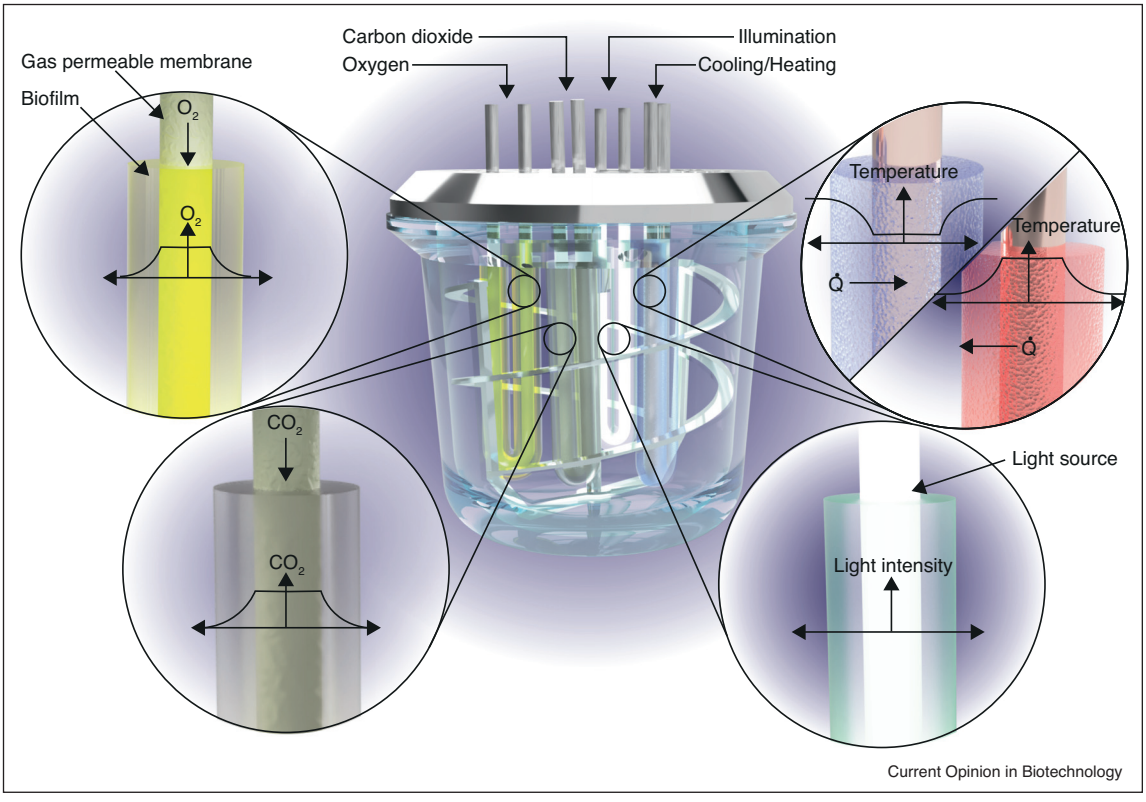
Process engineering approaches to engineer ecological niches

The case for further ecological niches — direct fermentation of lignocellulose

Besides the above presented examples of the biological engineering of metabolic niches to enable stable consortia, there are however applications where these two approaches are not feasible, for example, if a common good is produced by a cooperator strain. This is the case for instance in consortia where one strain produces enzymes that hydrolyze lignocellulosic polymeric carbohydrates to soluble sugars which can be metabolized by both the fermentation specialist and the cellulolytic specialist.

The two dominating cellulolytic specialist types are either anaerobic bacteria such as certain thermophilic or mesophilic *Clostridia*, or mesophilic aerobic fungi such as *Trichoderma reesei* [19]. The cellulolytic bacteria have mainly been combined with other anaerobic *Clostridia*, for example, for production of acetone butanol ethanol mixtures [20•] or butyric acid [25] (see Table 1). To allow the combination of the thermophilic *Clostridium thermocellum*, one of the most efficient cellulose degraders [21], with the mesophilic *Clostridium saccharoperbutylacetonicum*, a sequential fermentation strategy involving a temperature shift from 55°C to 30°C after 24 hour had to be applied to produce butanol [27].

Figure 2



Schematic representation of a stirred tank bioreactor providing multiple niches of abiotic parameters for the formation of microbial biofilms. The bioreactor has a variety of inlets. For instance, gas permeable dense membranes allow the formation of gradient of gases such as oxygen or CO_2 and the locally restricted provision of substrates. Microorganisms can form a biofilm on the surface of the membrane which allows the co-cultivation of aerobes and anaerobes in the same vessel. Furthermore, it is possible to provide light for phototrophic microorganisms by integrating light fibers in the reactor. Flushing the membranes with a temperature-controlled fluid can also allow the local charging or discharging of heat to or from the biofilm.

Alternatively, also aerobic cellulolytic fungi can be utilized in co-cultures with different microbial partners to produce a variety of products. Minty *et al.* established a consortium of *T. reesei* and a genetically engineered *E. coli* to convert pretreated corn stover to isobutanol [28]. Recently, the fermenting strain was exchanged to *Rhizopus delemar* to produce fumaric acid, which is induced by a nitrogen limitation [22].

The precise control of such consortia, that involve the competition for a common good by microorganisms that not all synthesize the final product, is necessary to achieve optimal yields and fermentation kinetics. In the case of lignocellulose conversion, the cellulolytic specialists need to get enough substrate to produce a sufficient amount of hydrolytic enzymes, while not consuming excessive quantities of the carbon source, because, once consumed, this fraction of the substrate is not converted to the desired target product. Thus, the ratio of the strains has to be adjusted, which in homogenous batch co-culture systems is roughly influenced by varying the inoculation density of the strains [23,24]. However, inoculation density is not a suitable approach for example, continuous or repeated batch fermentations and thus requires the development of new tools to control these strain ratios. To this end, we proposed to employ biofilm reactors that allow for the formation of defined spatial niches where other abiotic conditions (e.g. O_2, T, light) prevail than in the otherwise homogeneous reactor environment (Figure 2). This approach turns the often-stated difficulty to find matching fermentation conditions for all strains (which is required in completely homogeneous reactors) to an advantageous feature as it allows to control the ratio of the strains by adjusting the size of the niche.

Application of engineered spatial niches

To demonstrate the feasibility of the proposed reactor concept, we exemplarily developed a biofilm system for the direct fermentation of cellulose, that enables concomitant aerobic and anaerobic conditions using a locally controlled aeration through a dense oxygen permeable membrane. Directly on the membrane, an oxygen saturated niche is present where *T. reesei* forms a biofilm (Box 1) and secretes cellulolytic enzymes while the upper part of the biofilm as well as the growth medium is oxygen depleted and forms a suitable environment for anaerobic product formation. The system was suitable to produce ethanol from pretreated wheat straw by the combined action of *T. reesei*, *S. cerevisiae* and *Scheffersomyces stipites* [25]. By replacing the yeast strains with the facultative anaerobe *Lactobacillus pentosus*, lactic acid could be produced in high yields and titres from cellulosic substrates [26••]. Based on the latter, we developed the lactate platform concept, where the heterogenous lignocellulosic carbohydrates are funnelled to lactic acid as central intermediate which is then further converted to the final product. This minimizes the required metabolic capabilities of product forming microorganisms and facilitates their integration into an artificial community utilizing all biomass fractions. By employing the strict anaerobes *Clostridium tyrobutyricum*, *Veillonella criceti* or *Megasphaera elsdenii* as lactate consuming product forming strains, single or targeted mixtures of C2 to C6 carboxylic acids could be produced from cellulosic feedstocks [27].

Box 1 Properties of biofilms

A biofilm is a three-dimensional aggregation of microorganisms which are embedded in a self-produced matrix of hydrated extracellular polymeric substances [28]. Biofilms are formed by prokaryotic and eukaryotic microorganisms and are estimated to account for around 80 % of bacterial and archaeal cells [29]. Natural environments are often deficient in nutrients and exhibit spatial gradients of different abiotic parameters such as light, carbon and oxygen [30]. The firm binding to beneficial habitats enables homeostasis and the sessile growth modus manifests in a high level of spatial organization promoting stable and complex trophic interactions [31]. Biofilms may also protect the microorganisms therein from harsh and life-hostile conditions such as biological attacks and toxins. The robustness of biofilms, the enhanced productivity and the straightforward possibility to use them in continuous processes renders them useful also for biocatalytic applications [32–34].

Beyond oxygen niches

The above presented membrane bioreactor has the potential to provide a variety of controllable, artificial habitats based on defined spatial inhomogeneities in one vessel and offers the possibility to co-cultivate microorganisms with highly diverse requirements for abiotic parameters thereby extending the flexibility in community construction. Temperature gradients can be created by installing local heat sinks or sources in the reactor, which for example, allows the co-cultivation of thermophilic anaerobe *C. thermocellum* with mesophilic strains to avoid temperature shifts. Furthermore, the provision of CO_2 through the membrane offers the possibility to locally decrease the pH and could in combination with a light niche foster the growth of autotrophic microorganisms in consortia.

Conclusion

The current literature reveals the striking capabilities of artificial microbial consortia ranging from the conversion of non-edible substrates such as lignocellulose or syngas to the formation of highly functionalized organic molecules. The provision of ecological niches — which are typical for natural ecosystems — is a successful strategy to create stable consortia. In this context, the engineering of spatial inhomogeneities in scalable adapted conventional stirred tank reactor is a valuable tool to co-cultivate microorganisms with non-matching abiotic requirement and to control the strain ratio in consortia characterized by substrate competition. The combination of biological and process engineering tools for the creation of ecological niches offers unique opportunities to further develop

sophisticated artificial consortia and outperform traditional homogenous monoculture systems.

Conflict of interest statement

Nothing declared.

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