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REVIEW ARTICLE

Co-culturing microbial consortia: approaches for applications in biomanufacturing and bioprocessing

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ABSTRACT

The application of microbial co-cultures is now recognized in the fields of biotechnology, ecology, and medicine. Understanding the biological interactions that govern the association of microorganisms would shape the way in which artificial/synthetic co-cultures or consortia are developed. The ability to accurately predict and control cell-to-cell interactions fully would be a significant enabler in synthetic biology. Co-culturing method development holds the key to strategically engineer environments in which the co-cultured microorganism can be monitored. Various approaches have been employed which aim to emulate the natural environment and gain access to the untapped natural resources emerging from cross-talk between partners. Amongst these methods are the use of a communal liquid medium for growth, use of a solid–liquid interface, membrane separation, spatial separation, and use of microfluidics systems. Maximizing the information content of interactions monitored is one of the major challenges that needs to be addressed by these designs. This review critically evaluates the significance and drawbacks of the co-culturing approaches used to this day in biotechnological applications, relevant to biomanufacturing. It is recommended that experimental results for a co-cultured species should be validated with different co-culture approaches due to variations in interactions that could exist as a result of the culturing method selected.

Introduction

Microbial communities have evolved and shaped the face of the Earth from the beginning of time [1–3]. Humans have co-evolved with microbes, assimilating them within their bodies to carry out complex tasks, and one can say the first examples of biotechnology used combinations (consortia) of microbes for the fermentation and production of food and drinks [4,5]. Learning from the past, the study of co-cultures, in which two or more populations of cells are grown with some degree of contact between them [6] in symbiosis, has been seen today as a method to enhance current biotechnological processes [7].

Co-culturing microorganisms have further evolved, finding their way into biomanufacturing, for the production of pharmaceuticals, nutraceutical, food, and drinks on a large scale [8,9], and plays a prominent role in the bioremediation and bioenergy sectors [10,11]. Successful co-culture systems have shown great

potential for biotechnological application due to their versatility, robustness, and ability to undertake sophisticated tasks [12]. The synthetic/artificial co-culture systems surpass the limitations of monocultures or consortia in nature with the added advantages in exploring allelopathic interactions [13] in food industries involving fermentation [4] and natural product/drug discovery [14]. However, a full understanding of microbial molecular networks is still largely needed [9]. To date, fully deciphering the communication networks has been the focus of co-culture research. A deeper understanding of microbial interactions can benefit biotechnological and synthetic biology advancements, and provide a more sustainable and economical method for bio-productions [5].

Microbial networks involve macromolecules and small molecules, such as metabolites, used in communication during intra or inter-species microbial interactions [15]. The symbiotic/antagonistic/allelopathic

interaction between microorganisms can be a combination of physical interactions [16], info-chemicals [17], special signaling molecules (quorum sensing), adhesion factors (biofilms), and metabolites [18]. Info-chemicals include both hormones (conveys information within an individual) and semio-chemicals (mediates information between individuals), collectively known to influence the behavior, physiology, and structure of individuals of another species [19]. Alternatively, one partner can induce the production of *de novo* products or induction of *de novo* cryptic biosynthetic pathways in others [14,20]. A better understanding of these interacting cues or functions of particular microorganisms can enable the construction of high-performance consortia to accomplish the desired tasks [21]. Elucidation of the interplay at the molecular level can benefit applications in the field of synthetic engineering, allowing for the creation of engineered synthetic communities for ecological, industrial, and medical applications [22,23].

Co-culturing techniques are designed with a few goals in mind (biomass generation, bio-production, or clean-up systems), which will shape the choice of microorganisms and growth parameters. A better understanding of the trigger-response mechanisms [7] will shape the way in which to improve a bioprocess. However, detecting and interpreting microbial cues has proven to be difficult, due to the dynamic nature of the system and the complexity of microbial communities. As the synergistic interaction that exists between co-cultured microorganisms is species-specific, the same effects will not be obtained by species from similar genera, indicating that each partnership has to be evaluated singularly [24]. Additionally, microbial communities are highly susceptible to abiotic and biotic stresses [6], changes that will be reflected in their intra and extracellular metabolomes. Moreover, high turnover rates, physicochemical diversity, and low concentrations (due to poor co-culture designs) present additional analytical challenges which often lead to poor coverage, detection, and quantification of these info-chemicals [25,26].

Various co-culturing methods have been developed to address these challenges. Small co-culturing vessels and targeted metabolite profiling are deemed to be ideal for trapping metabolic dependencies at a high resolution [27]. Finding a balance between various strategic propositions would allow for better resolution and coverage of the untapped/novel natural product resources. By evaluating each co-culturing method, it is possible to address the shortcomings that need to be overcome in future designs. The availability of this information in a concise review helps to visualize the best designs for a given context that presents the potential for being taken further.

In this review, we provide an overview of the current co-culturing techniques for microbial consortia and explore the associated advantages and challenges with a specific focus on biotechnology applications, in particular biomanufacturing and bioprocessing. The overview, potential, and challenges of the co-culturing techniques for biomedical engineering applications have been extensively reviewed elsewhere in recent times [28–32] and hence is not covered here. The techniques evaluated include methods such as communal liquid medium growth (microorganisms come into direct physical contact); solid–liquid interface systems (involves encapsulation of microorganisms which are co-cultured in a liquid media); membrane separation (microorganisms are separated using permeable substances/membranes); spatial separations (involves no direct physical contact, instead monocultures are inoculated separately and are allowed to interact in space) and microfluidic systems (commonly employed in mammalian research with better control over fluids and microenvironments).

Current techniques for co-culture biotechnology

This section will provide a compendium of techniques currently used to study co-cultures. Broadly, these methods are classified as communal liquid medium growth, solid–liquid interface, membrane separation, spatial separation, and microfluidics systems. An overview of key co-culturing techniques used currently in biotechnology is given in Table 1.

Communal liquid medium growth

Microorganisms co-cultured in a communal liquid medium (CLM) allow for a better understanding of the underlying effects that govern microbial interactions. With this method, the changes in biochemical components and overall growth of the interacting species can be investigated thoroughly. For example, it can be used to identify over-yielding (higher biomass compared to its component monoculture) or under-yielding (lower biomass compared to its component monoculture) effects between the co-cultured partners at the different time frames and phases [57]. CLM systems, to an extent, emulate conditions in the real world, if microorganisms from the same niche are isolated and grown together, or in the case of artificial co-cultures, it provides a way to attest a relationship if these organisms were to find themselves in a shared environment. For this to succeed, various parameters such as

Table 1. A survey of key co-culturing techniques used in biotechnology.

Co-cultured microorganisms	Co-culturing technique	Info-chemicals of interest	Field of study	Ref.
Colletotrichum lagenarium, Bacillus amyloliquefaciens	Agar System	Antifungal proteins	Food Technology	[33]
Botrytis cinerea, Pseudomonas sp.	Agar System	x	Agriculture	[34]
E. coli, Salmonella typhimurium	Agar System	x	Food Technology	[35]
Fusarium sp., *Aspergillus* strain	Agar System	*de novo* production of 18 metabolites	Biotechnology (natural products)	[14]
Sarocladium strictum, Fusarium oxysporum	Agar System	Fusaric acid	Medical	[20]
Streptomycetes from rhizosphere of Araucariaceae, *Neofusicoccum parvum*	Agar System	24 anti-fungal compounds	Ecology	[36]
Shewanella putrfaciens, Brochothrix thermosphacta, Pseudomonas sp.	Agar Systems	Formic acid and 2 unidentified organic acids	Food Technology	[37]
Candida albicans, Clostridium perfringens, K. pneumoniae, E. coli, E. faecalis	Biofilms	Upregulation of WOR1	Medical	[38]
Aspergillus nidulans, actinomycetes	Dialysis tube membrane	Lecanoric acid, orsellinic acid, polyketides	Ecology	[16]
Chlorella vulgaris, Microcystis aeruginosa	Dialysis tube membrane	Linoleic acid and nitrous oxide	Ecology	[39]
Denitrifying anaerobic methane oxidation (DAMO) and anaerobic ammonium oxidation (Anammox)	Direct mixing	Nitrate and nitrite	Ecology	[40]
Chlorella vulgaris, Pseudokirchneriella subcapitata	Direct mixing	Chlorellin	Ecology	[13]
E. coli, Bacillus megaterium	Direct mixing	Peptide-based signaling: auto-inducing peptides	Biotechnology	[41]
Fusarium tricinctum, Bacillus subtilis	Direct mixing	Inducing secondary metabolites production (78 fold increase)	Biotechnology	[18]
Ignicoccus hospitalis, Nanoarchaeum equitans	Direct mixing	Increase in CO_2 fixation and nitrogen assimilation enzymes (Glutamine and asparagine synthase)	Ecology	[42]
C. sorokiniana, A. brasilense	Encapsulation	x	Bioremediation	[43]
Delftia acidovorans, Arthrobacter sp.	Encapsulation	x	Bioremediation	[44]
Klebsiella oxytoca, Bacillus subtilis, Rizoctonia solani	Encapsulation	x	Ecology	[45]
Rhodosporidium toruloides, Saccharomycopsis fibuligera	Encapsulation	x	Biofuels	[46]
Zymomonas mobilis, Pichia stipitis	Encapsulation	Suggest metabolites present (not investigated) for efficient ethanol production	Biofuels	[47]
Chlorella protothecoides (Heterotrophic and autotrophic)	Gas separation	CO_2 and O_2 exchange	Biofuels	[48]
Oocystis marsonii, Microcystis aeruginosa	Membrane separation	Allelopathic metabolites not identified	Biotechnology	[49]
Microcystis aeruginosa (mycrocystins producing and non-producing)	Membrane separation	Bioactives, toxins (microcystins) and peptides	Biotechnology	[50]
Rhodotorula glutinis, Chlorella vulgaris	Membrane Separation	Propionic acid, pyruvic acid, acetic acids	Biofuels	[51]
Lactobacillus brevis subsp. *lindneri* or *L. plantarum* with *S. cerevisiae* or *S. exiguus*	Membrane Separation	Amino acids such as valine and isoleucine	Food technology	[52]
Lactobacillus, S. cerevisiae or *Z. florentina*	Membrane Separation	Amino acids	Food technology	[53]
P. aeruginosa, A. fumigatus *R. solanacearum, A. flavus*	Microfluidics	x Chlamydospores (*A. flavus*)	Biotechnology (natural products)	[54]
Sphingobium chlorophenolicum, Ralstonia metallidurans	Microfluidics	x	Bioremediation	[55]
Chlorella protothecoides, Tetraselmis suecica.	Pelletization and flocculation	Bio-flocculating compounds	Bioremediation	[56]

priority effects, inoculation ratio and the timing at which one monoculture is seeded into the other do play an important role in establishing a balance with the co-culture [7].

This type of co-culturing is useful to enhance biomass yield [58], in a process such as fermentation [4], biofuels, nutraceutical, and chemicals production, where enhancing the growth of the main partner would give higher bioproduct yields [8]. Moreover, synergistic or antagonistic partnerships could be exploited for various biotechnological applications, without the need to use gene modifications. Systems such as direct mixing, pelletization, flocculation, and biofilms, fall into this category (Figure 1).
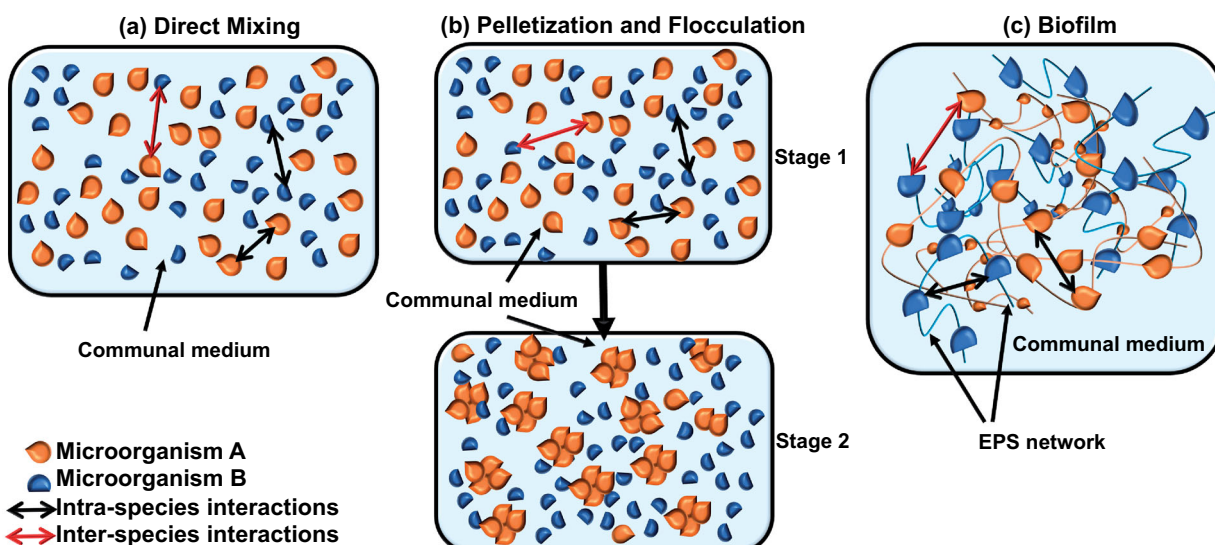
Figure 1. Communal liquid medium growth co-culture system. (a) Direct Mixing: Microorganism A and B come into direct contact allowing them to exchange info-chemicals at a close proximity. (b) Pelletization and Flocculation: Microorganism B releases bio-flocculants, which induce Microorganism A to form aggregates. This process is not 100% efficient, as shown by the non-flocculated cells. (c) Biofilm: both microorganisms secrete EPS compounds creating an intertwined network (filaments, orange-Microorganism A, blue-Microorganism B).

Direct mixing

Direct mixing (Figure 1(a)) refers to co-cultures grown in the same environment, where microorganisms come into physical contact with each other. These microorganisms interact in close proximity, exchanging signaling molecules and metabolites. Co-culturing experiments involving the direct mixing of microorganisms have been shown to have enhanced functions and accomplished tasks difficult to be achieved with monocultures [15]. These include processes such as bioremediation [59,60], hydrogen production [61], acetone-butanol-ethanol production via fermentation [62], the production of nondairy probiotic [4], and bioactive compounds with antifungal properties superior to those obtained with monocultures [63].

Direct mixing co-culturing methods have been used to study the interactions between fungi and bacteria [33,64], yeast and algae [65], algae and bacteria [66], and between algae species [13]. Compared to its monoculture, the marine fungus, *Emericella* sp. secreted emericellamides A and B (a secondary metabolite of marine cyclic depsipeptide with antimicrobial properties) in much higher concentrations when co-cultured with the bacterium *Salinispora arenicola* [64]. Similarly, *Bacillus amyloliquefaciens*, when co-cultured with *Colletotrichum lagenarium* (plant pathogenic fungus), secreted an antifungal protein, as a result of being exposed to the fungus. This secreted protein by bacteria exhibits β-1,3-glucanase activity on fungi (decomposition of fungal hyphal walls), thereby acting as an effective biocontrol candidate and antagonist against

the plant pathogen [33]. A symbiotic interaction or cross-talk between *Chlorella* sp. (algae) and *Saccharomyces cerevisiae* (yeast) in a bioreactor, showed enhanced CO_2 bio-fixation with a simultaneous increase in biomass and lipid productivity with co-culture compared to microalgal monoculture [67]. Similarly, *Rhodotorula glutinis* (yeast) and *Scenedesmus obliquus* (algae) grown in communal media showed synergistic interactions where higher biomass and lipid productivity was observed compared to each monoculture. These results indicated that a combination of gas exchange (O_2 and CO_2) and a source of trace elements from naturally lysed cells played a vital part in the synergism [65]. A combination of both synergistic and antagonistic interactions between *Prorocentrum minimum* (algae) and *Dinoroseobacter shibae* (bacteria) was illustrated with this method [66], backing up the proposed "Jekyll and Hyde" lifestyle [68]. Briefly, the authors investigated the population dynamics of co-culture and demonstrated that co-culture reproducibly went from mutualistic phase (where both bacteria and algae profit) to pathogenic phase (where bacteria-induced algal death). With respect to the inter-species interactions, the co-culture of two microalgae *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* resulted in higher levels of extracellular chlorellin (a mixture of fatty acids and hydrocarbons), responsible for inhibitory effects on both species. This investigation showed the application of direct mixing as a tool to analyze the evolution of allelopathic chemicals [13]. Furthermore, the population density of the starting inoculum

(inoculation ratio) needs to be assessed prior to setting up the co-culture. This has been true for studies conducted using *Spirulina platensis* and *Rhodotorula glutinis* [69] and *Scenedesmus obliquus* and *Candida tropicalis* [70], where the growth rate of the yeast/bacteria exceeded that of the alga. By adjusting the population density to alga:bacteria (3:1) and alga:yeast (2:1) it was possible to construct a balanced co-culture with enhanced alga biomass output. Later, a study with co-cultures of *Chlorella pyrenoidosa* and *Rhodotorula glutinis*, confirmed the importance of inoculation ratios/population density, where a ratio of alga:yeast (3:1) is identified as optimal for achieving the highest biomass concentration and the lipid productivity [71] and to improve nutrient removal from wastewater and protein productivity [72].

Direct mixing co-culture can be used to identify and understand the effects of secreted metabolites by microorganisms on each other. However, as shown by Oh and coworkers [64], when analyzing the supernatant of *Emericella* sp. for emericellamides A and B, the concentration of these depsipeptides in the media can be very low for their isolation, structural elucidation, and detection by LC-MS. This finding suggested that direct mixing is not an ideal way to trap extracellular metabolites. Similarly, the various extraction and concentration steps of the compound could result in loss or degradations of compounds. This method is, therefore, limited to the analysis and production of larger molecules such as exopolymeric substances (EPS) and/or info-chemicals with higher extracellular concentration. In addition, directly mixed cultures in the same communal media are not suitable for microorganisms that have slightly different demands in culturing conditions or in circumstances where microorganisms cannot exist in direct contact [43], necessitating other approaches, as discussed below.

Pelletization and flocculation

Alternative methods of co-culturing such as pelletization and flocculation (Figure 1(b)) involve a naturally close association of microorganisms. During co-culture, flocculating compounds (bio-flocculants) released by one partner cause the other microorganism to agglomerate and form pellets. The mechanism for aggregation has been attributed to cell surface charge and/or filaments of the bacteria/fungus [70,73–75]. This method has several added advantages such as improved settling ability and optimized symbiosis within the microbial community through mutually beneficial associations. Key parameters that govern the bio-aggregation/bio-flocculation are surface charge, hydrophobicity, pH, salinity, temperature, divalent cations concentration (calcium and magnesium ions), population density, the initial ratio of co-cultured partners, timing for triggering flocculant formation, and the concentration of the flocculant releasing microorganisms. The use of synthetic flocculants on a commercial scale is being widely criticized due to their toxicity to humans and the environment. In contrast, bio-flocculants produced by a variety of microorganisms are considered as good alternatives. However, their large-scale production is limited by factors such as lower concentration, lower flocculating efficiency, and associated high production costs. The overall yield and flocculation efficiency of bio-flocculants can be substantially improved by co-culturing optimal strains. This method has been successfully used to decrease the capital costs associated with microbial harvesting and dewatering [56,75,76], for screening of optimal strains for co-culturing and in bioremediation [73].

Harvesting microalgae biomass that contains products of value has been achieved with the aid of natural pelletization and flocculation, by co-culturing microalgae with fungi or bacteria. In the case of fungi-assisted algae harvesting, the co-culturing of *Chlorella protothecoides* and *Tetraselmis suecica* with fungal strains resulted in higher biomass, lipid productivity, and bioremediation efficacy compared to monocultures [56]. Similar trends were observed with co-cultures of *Chlorella vulgaris* and two species of *Aspergillus* sp. [73]. The influence of rotation speed, culture time of *Pleurotus ostreatus* (an edible fungi) pellets and pH on harvesting efficiency of *Chlorella* sp. was recently investigated, where authors reported 100 rpm rotation speed with lower pH values resulted in a maximum harvesting efficiency of 65% in 150 min [77]. In the case of bacteria-assisted algae harvesting, *Bacillus* sp. (bacterium) at pH above 9 showed a flocculation efficiency of up to 95% with *Nannochloropsis oceanica* (algae) in a liquid medium [74]. Similarly, co-culturing of *C. vulgaris* with bacteria (with direct physical contact) caused the microalgae to flocculate, a phenomenon not seen in either axenic *C. vulgaris* culture or even when grown in the bacterial culture supernatant [78], suggesting that the presence of the bacterium is essential for microalgal flocculation. However, the effects of the bacteria on the growth and biochemical composition of the microalgae were not explored in this study. In the case of bacterial co-cultures, the consortium of *Halomonas* sp. and *Micrococcus* sp. [79] and *Staphylococcus* sp. and *Pseudomonas* sp. [80] triggered the production of the novel bioflocculant, CBF-F26 and MMF1 respectively.

The screening involving the individual co-cultures of *Aspergillus fumigatus* (fungi) with eleven different strains of microalgae showed variations in bio-flocculation efficiencies. Furthermore, the biochemical analysis showed that synergistic interactions with *A. fumigatus* were evident only with few microalgal strains out of eleven. This was indicated by the increase in lipid production that was similar or higher than the sum of the monoculture of the microalgae and fungus [81]. However, these observations were only limited to cells grown using glucose as the carbon source, and not in cells grown using pretreated wheat straws as the alternate carbon source. Hence, the benefits of this co-culture were shown to depend on both the microorganisms being co-cultured and the carbon source provided. This was also evident in results found during the co-culture of *Aspergillus niger* (fungi) and *C. vulgaris* (microalgae) [75], where the heterotrophic co-culture conditions lowered the flocculation efficiency when compared to autotrophic conditions. This demonstrated that co-culture conditions are important to reap the full benefits of the synergistic interaction. Similarly, the co-culturing of *C. vulgaris* and *A. niger* [76] highlighted the importance of population density, inoculum size, and timing during pelletization. In this case, the concentration of the flocculant and its binding strength was proven not to be effective at very high microalgae biomass concentrations, resulting in variations in pellet morphology, however, a co-culturing ratio of 1:300 (fungi:microalgae) yielded >90% cell harvest efficiency.

The trigger-response mechanism can be manipulated by variations in the growth environment and by selecting the optimal organisms with varying degrees of bio-flocculant producing capacity [79,81]. The use of pelletization and flocculation, however, is limited only to microbial co-cultures where the mechanism of bio-aggregation/bio-flocculation can be triggered and maintained. The nature of the bio-flocculant and its binding capacity would also be a limiting factor, as the duration of this would need to factored in when harvesting the biomass. However, using bio-flocculants would decrease the costs of centrifugation and the environmental impact of synthetic chemicals. Overall, the strategy of using palletisation/flocculation for co-culturing has been shown to be effective not only for microbial harvesting and downstream processing but also to improve biomass productivity and product yield in such processes compared to monocultures.

Biofilms

Biofilms (superficial microbial colonies) (Figure 1(c)) can be naturally formed on solid surfaces at the solid–liquid interface by a single species or a combination of species [82]. An extracellular matrix in biofilms, where the microbiome resides and communicates, is composed of hydrated EPS. EPS are mainly comprised of proteins, polysaccharides, amino acids, nucleic acids, lipids, and other biopolymers (humic substances). These EPS, immobilize biofilm cells by providing mechanical stability and keeping them in close proximity, thereby forming an inter-connected cohesive three-dimensional polymer network where cross-talk between cells results in the formation of synergistic micro-consortia [83]. The secretion and uptake of substances within a biofilm may be analyzed by gene activation or inactivation to deduce how they influence each other, however, their molecular level interactions are yet to be sufficiently defined [38,83]. Appropriate co-culturing methods are required for a better understanding of regulatory factors for EPS production and assessing molecular level interactions between different partners in multispecies biofilms. Biofilms have found application in biomedical, bioremediation, and bioenergy-related fields [84].

As has been emphasized by other investigators in the medical context [85], knowledge of interspecies interaction within the biofilm is vital for an understanding of biofilm physiology and the treatment of biofilm-related co-cultivation strategies in biomanufacturing. An illustration of biofilm-associated induction has been shown, where microorganisms within the biofilm can cause activation of genes for biofilm production in another strain, therefore enabling them to survive in environmentally challenging conditions [38]. Briefly, the interactions between the bacteria and *Candida albicans* within the gut microbiome have been shown to support each other's growth and survival via modulation of the local chemistry of their environments in multiple ways. Bacteria-induced biofilm formation in yeast has also been investigated, where co-culture of *S. cerevisiae* and LAB (lactic acid bacteria) or monoculture of *S. cerevisiae* exposed to bacterial supernatant resulted in biofilm formation [82]. Recently, mycoalgae biofilms (lichen type) on a supporting polymer matrix have been investigated for various bioremediation and bioprocessing applications such as biomass harvesting [84,86], which stemmed from previous knowledge of fungi and algae interactions [87]. Plastic composite support biofilm reactor was used for simultaneous saccharification and fermentation of ethanol in a potato waste-based medium by co-cultures of *A. niger* and *S. cerevisiae*, where the influence of temperature, pH, and aeration rates on ethanol production was investigated. Maximum ethanol production was reported at pH 5.8, 35 °C with no aeration [88]. The advantage of using this

co-culture method in this instance is due to the induction of biofilm formation on a support matrix, with the attachment efficiency dependent on the species of co-cultivation and the material of the matrix. In summary, the potential usefulness of this co-culture method is evident but requires a further understanding of how these microorganisms interact, which will facilitate future couplings of synergistic microorganisms for their intended applications as biofilms.

However, it is also evident that similar to co-culturing by pelletization and flocculation, biofilm formation is limited to microorganisms that can form biofilms and/or those that can induce biofilm production. For example, monocultures of yeast or LAB were unable to form biofilms [82]. This could be due to the inability to form the required components for biofilms such as EPS or the requirement for other regulatory signals. Likewise, the trigger-response stimulus that will be established between the biofilm-forming microorganisms will vary the outcome of the assemblage, therefore, each biofilm is unique to itself making reproducibility a challenge. Additionally, since metabolites and signaling molecules are not secreted only through the biofilm, other methods of co-culture are required to investigate other means of communication.

Solid–liquid interface

The solid–liquid interface systems involve trapping a monoculture or a co-culture within a porous vessel, usually in soft beads or cell droplets. The bead/droplet is then suspended in a liquid or a gaseous medium. The medium composition of the bead or capsule can differ from the suspension fluids. Extra-cellular metabolites interaction is facilitated through the porous membrane. Amongst these methods are encapsulation and cell droplet formation techniques (Figure 2), useful for co-culturing microorganisms that require protection against environmental stresses, have dissimilar growth characteristics, nutritional requirements, and hinder substrate competition [43], for which direct mixing or membrane separation methods are not suitable. Solid–liquid interface systems have been used to produce nondairy probiotic drinks, such as during the fermentation of peanut-soy milk using *P. acidilactici* and *S. cerevisiae* [4], and in increasing lipid content in microalga *Chlorella* sp. by entrapping it with *Trichosporonoides spathulata* in glass beads [89]. These methods are useful for co-culturing microorganisms that require an uninterrupted supply of nutrients with relatively low competition, especially when co-culturing
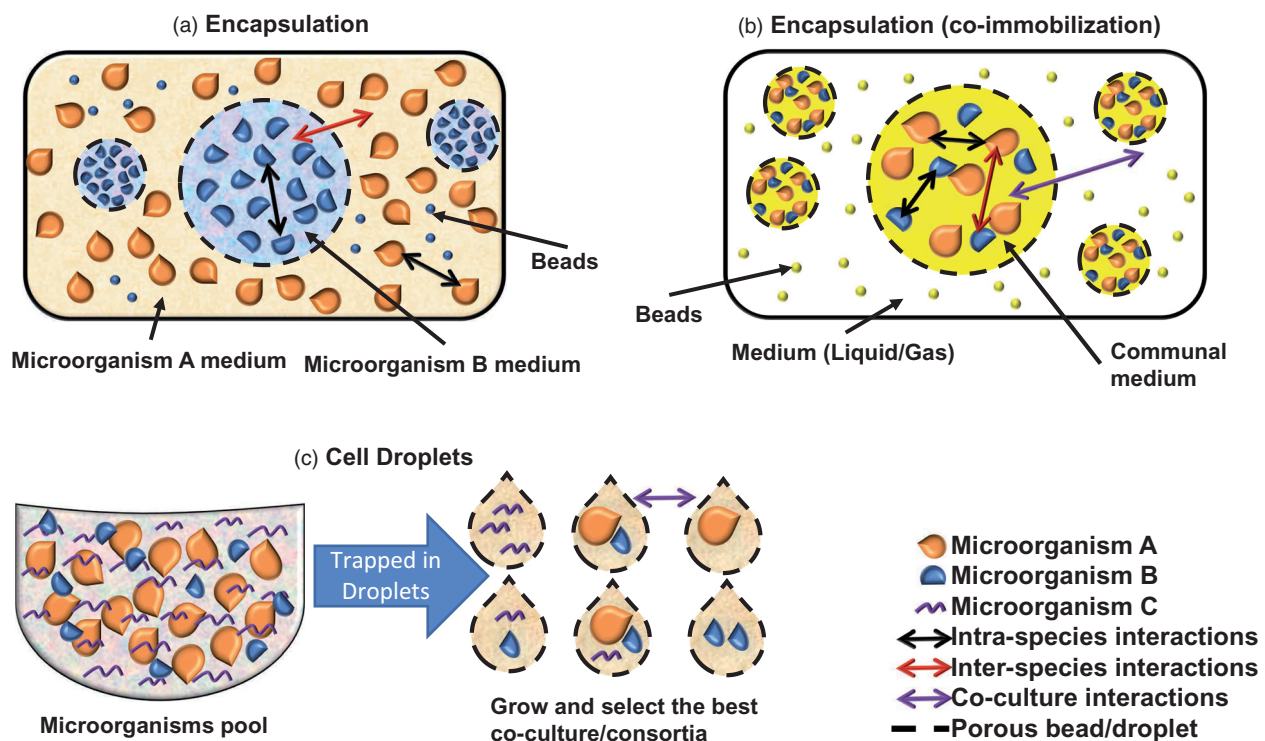
Figure 2. Solid–liquid interface co-culture system. (a) Encapsulation: Microorganism A is grown in liquid culture, whilst Microorganisms B is trapped within beads. The info-chemicals diffusing from the beads aid Microorganism A (for example in growth). (b) Encapsulation (co-immobilization): Microorganism A and B are both trapped within the beads. The info-chemicals diffusing into the growth chamber can affect the outer media (e.g. fermentation or compound digestion). (c) Cell droplets: droplets are used to isolate sub-cultures of species from within a microorganism pool. The best performing/surviving microorganism co-culture/consortia is chosen for further application.

microorganisms with very dissimilar nutritional requirements, as there still may be competition for gaseous compounds diluted within the media/flowing across the capsule membrane.

Encapsulation

Encapsulation is a method of co-culture that can overcome the challenges posed by variations in the growth environment. This method involves the immobilization of microorganisms in substances such as alginate, agar, and κ-carrageenan structures [43,45,89,90]. Often, one of the two microorganisms is trapped in beads and co-cultured with the other microorganism in the liquid medium (Figure 2(a)). This method does not allow them to come into physical contact with one another [43,89,91]. Alternatively, co-immobilization (Figure 2(b)), where both microorganisms are encapsulated within the same bead is used to facilitate biomass harvesting and promote closer interactions [89,92]. It enables a more effective transfer of info-chemicals and metabolites between interacting species with minimal loss in the bulk medium due to diffusion. This isolation from the environment also makes them less affected by the culturing conditions outside the bead. This has been demonstrated to be beneficial for co-cultures that have the potential to replace sequential processes such as fermentation [47], direct oil conversion from starch [46] and bioremediation [43,44].

The immobilization of *Zymomonas mobilis* (bacterium) in beads and its co-culture with free-flowing cells of *Pichia stipitis* (yeast) yielded 96% more bioethanol than the theoretical value [47]. The immobilization relieved oxygen competition between the two microorganisms whilst mitigating the inhibition of the bacteria caused by the yeast when directly mixed. Observations of their interactions confirmed some level of inhibition, however, evidence shows that *Z. mobilis* was also utilizing an additional source of nutrient/or carbon, other than glucose when co-cultured with *P. stipitis*. Another example is the immobilization of *Aureobasidium pullulans* (yeast link fungus) to polyurethane foam with encapsulated *S. cerevisiae* in calcium-alginate beads, in co-culture, where an improved purity and yield of fructo-oligosaccharides was demonstrated, compared to monocultures [93]. Similarly, yeasts *Rhodosporidium toruloides* and a mutant version of *Saccharomycopsis fibuligera* were co-immobilized in polyvinyl alcohol (PVA) and alginate beads that allowed for the conversion of cassava starch to cell lipids in a single process [46]. Additionally, Magdouli and coworkers [94] highlighted the possibility of recycling *Synechococcus* sp. (cyanobacterium) beads during co-culturing with *C.*

reinhardtii (microalgae) to improve the growth and lipid production of the microalgae. In the case of co-immobilization, co-encapsulation of algae and bacteria has great potential in bioremediation applications, such as reduction of ammonium and phosphorous from the wastewater, however, a realization of this potential is limited by growth suppression by native wastewater bacterial community. This limitation can be overcome by immobilization of algae and bacteria in alginate beads [43], where beads inhibit both liberation of immobilized microorganisms into wastewater and penetration of outside microbiome into the beads. Similarly, co-encapsulation of yeast and microalgae has been shown to result in similar lipid productivity compared to their directly mixed co-culture, however, the added advantage of this method is reduced cost and simplification of downstream harvesting process [89].

This method has several drawbacks, nevertheless, one of which includes the reduced growth shown by a decrease in biomass production during co-culture compared to the direct mixing method [89]. The fragility of the beads is also an issue that leads to leakages of the trapped microorganism (in a period of few days) into the culture environment [47,89]. The economic feasibility of this method is another challenge, as for industrial applications, mass production of uniform alginate beads is required which is costly.

Cell droplets

Monocultures and co-cultures can be isolated in droplets, micro- or macro-droplets, where the info-chemicals are exchanged between the isolated droplets via diffusion [95,96]. Droplets can be made using a microfluidic device that could encapsulate and co-cultivate subsets of a community by dispersing aqueous droplets in a continuous oil phase [97] or by encapsulating microorganisms within microdroplets composed of agar and single cells, forming microcolonies that could still exchange substances between each other [95]. Alternatively, an aqueous two-phase system environment can be used where microcolonies can be relocated by using magnetic remote control [96]. The cell droplets technique (Figure 2(c)) has been highlighted for its ability to enable the culturing of microorganisms that often cannot be easily cultured under laboratory conditions.

Microdroplets were used as a method to isolate symbiotic interactions from within a microbial community [97]. Later separation of the microorganism's assemblage into smaller portions will facilitate a better understanding of the subset communications that govern complex systems. Microdroplets were achieved by dispersing aqueous droplets in a continuous oil phase

within a microfluidic device. This method allowed for the isolation of symbiotic microorganisms only as these would keep generating with time. This work presents itself as a method used to isolate natural symbionts from complex ecological systems to be studied for biotechnological applications. Encapsulating cells in gel microdroplets (made up of agarose) was recently described as an alternative to surrounding cells with oil [98]. This method described high-throughput screening of cell to cell interactions (HiSCI) in isolating the algae growth supporting bacteria. The porous nature of the gel matrix allowed the free flow of nutrients, metabolites, and gases to and from the encapsulated cells. Byun and coworkers [96] designed an aqueous two-phase system that trapped bacterial colonies within magnetic dextran phases (DEX). This DEX phase was then suspended as cell droplets and patterned within a polyethylene glycol (PEG) phase. With such magnetized droplets, it was possible to observe how the microorganisms interacted over varying distances by relocating the cell droplets at different time intervals compared to a stationary location. Their results indicate that relocation can enhance communication between the droplet colonies. This method proved to be advantageous for microorganisms subjected to changing environmental conditions. As opposed to other co-culture methods, where microorganisms remain in one environment, this method enabled tracking changes that can occur when the microorganisms were exposed to slightly different surroundings. A limitation of this technique is that not all bacterial species partitioned well in the cell droplets. In addition, the phases pose limitations for different types of microorganisms that can be negatively affected by the substances constituting the phases. This methodology was further developed by Han and coworkers [99], where authors used a density adjusted PEG/DEX aqueous two-phase system which can generate various size-controlled spheroids in a conventional multi-well plate. This method offers the added advantage of simple culture mode switching from spheroid to a surface-attached adhesion culture with the addition of few drops of the polymer-free medium, thereby avoiding conventional laborious spheroid manipulation steps and errors associated with it. Nevertheless, the approach is more suited to studying interactions in co-cultures more than employing it as a strategy for large-scale manufacturing, given the limitations of the volumes employed.
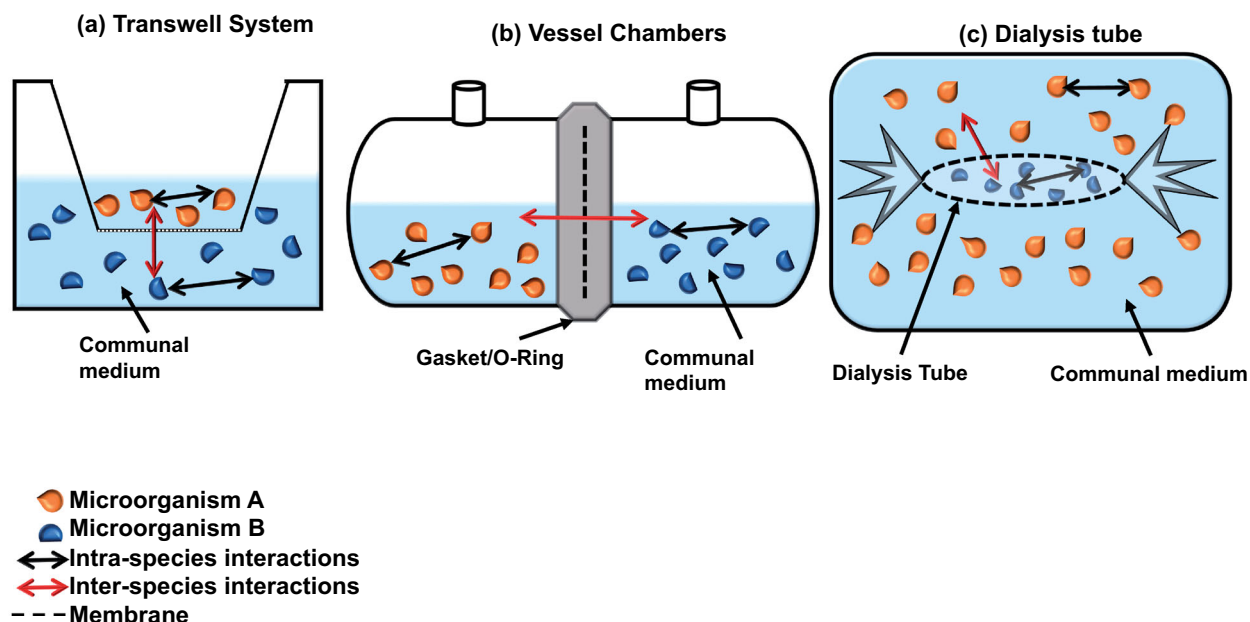
Membrane separation

A membrane can form a separation barrier between microorganisms during co-culture. It has the added benefit of easing the task associated with monitoring the population density of each microorganism and their allelopathic interactions. Several types of signaling molecules have been identified so far using different types of membrane separation co-culture systems (Table 1). This technique is primarily employed to investigate diffusible molecular mechanisms used for interactions within co-culture and their ultimate effects. These include the use of a dialysis tube membrane [39,100]; vessel chambers [49,50,101,102] and a Transwell® system [53]. Amongst the biomolecules identified are amino acids [52], fatty acids [39], and sugar derivatives [51] (Table 1). Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are common analytical techniques employed to identify metabolites secreted within the growth medium [16,39].

Transwell® system

The isolation and identification of extracellular molecules can also be achieved using a Transwell® system (Figure 3(a)). This system comprises a six-well plate with two separated parts, and a lower compartment (reservoir), and an upper compartment (insert) each of which can hold different co-culture partners separated by a permeable membrane (polycarbonate) which only allows the diffusion of metabolites [53,103]. A small-scale Transwell® system has been developed within the assay plates, required for low cell and media volumes, and enabling replication and multiple studies to be conducted simultaneously [53]. As used in biomedical research, this method can be used to understand secretion factor profiles and their levels as a physiological response during cross-talk between different cell types, in particular mammalian cell lines [104,105].

In the past, this method of co-culture has been used to understand the trophic relations between LAB and yeast co-culture (Table 1) that occur during sourdough leavening, which are otherwise difficult to understand due to the complex proteolytic activity taking place in sourdough [52]. Briefly, higher growth rates and final yields were demonstrated for both LABs compared to their monocultures, where yeasts were unaffected and were found to compete partially with LAB for nitrogen sources and are also responsible for the synthesis and secretion of amino acids (valine and isoleucine). These secreted amino acids are responsible for enhancing the growth of LAB. In contrast, the lower diffusion rates and accessibility in the Transwell system were highlighted in reducing the overall toxicologic impact and improving growth profiles as demonstrated with co-cultures of A. niger and Nostoc sp. (cyanobacteria), where Nostoc sp. grown on wastewater was known to produce

(a) Transswell System

(b) Vessel Chambers

(c) Dialysis tube

Communal medium

Gasket/O-Ring

Communal medium

Dialysis Tube

Communal medium

🟠 Microorganism A
🔵 Microorganism B
⟷ Intra-species interactions
⟷ Inter-species interactions
– – – Membrane

Figure 3. Membrane separation co-culture system. The co-culture microorganisms grown in communal media can only mediate through info-chemicals. Direct contact is not possible. (a) Transswell® systems: a horizontal oriented permeable membrane is placed between the two microorganisms allowing for an exchange of info-chemicals. This method only allows the cultivation of low concentration of cultures. (b) Vessel chambers: a vessel is sectioned into half by placing a vertically oriented permeable membrane which allows the diffusion of info-chemicals. (c) Dialysis tube: Microorganism A is grown in liquid culture, whilst Microorganisms B is trapped with a dialysis tube. The info-chemicals diffuse through the permeable membrane of the dialysis tubing.

signaling molecules toxic to *A. niger* [106]. More recently, the role of amino acid metabolism in synergistic interactions between LAB and yeasts (Table 1) isolated from water kefir has been described, where higher biomass yields were obtained compared to their monocultures [53].

This method of co-culture is very easy to set-up and is convenient for studies requiring small culture volumes (up to 5 ml). Moreover, the porosity of the polycarbonate membrane can be selected depending on the ultimate aim of the study. For example, 8 µm porosity has been selected as the main aim of the model was to assess the invasion of metastatic cancer cells through the structural blood-brain barrier [104], whereas 0.25 to 0.4 µm porosity has been used to study the cross-talk between bacteria and yeasts [52,53]. The set-up can be ideal in screening co-culture partners. However, this setup is not suitable for larger culture volumes thereby limiting its application to planktonic research (due to low cell abundance) and studies involving time-course sampling for metabolomic and proteomic investigations (due to low biomass availability). Additionally, this method requires pre-optimization of overall setup with respect to compartment suitability for each co-culture partner as demonstrated by Stadie and coworkers [53], where best effects were only obtained when yeasts

were cultivated in the reservoir and lactobacilli in the insert.

Vessel chambers

Vessel chambers (Figure 3(b)) consist of two vessels connected through an O-ring junction (made up of silicone for leak-proof sealing) equipped with a permeable membrane filter (a 0.22 µm hydrophilic polyvinylidene fluoride (PVDF) or 0.45 µm cellulose nitrate). Each microorganism is cultured in its own half of the vessel. The membrane allows for the diffusion of the metabolites from one chamber to another. This method has been used to assess ecological systems such as the predator-prey interaction [101] and interactions within phytoplankton communities [102].

In case of predator-prey interactions, co-culturing of *Pseudomonas fluorescens* (bacterium) with *Dictyostelium discoideum* (ameba) resulted in high levels of the bacterial alkaloids, pyreudiones A–D being produced by *P. fluorescens* to protect itself from the ameba. The permeable membrane allowed predator-prey signaling molecules to diffuse between the chambers, activating the self-defense mechanism of the bacterium, thereby decreasing growth rates or causing cell lysis of ameba [101]. In planktonic research, vessel chambers were used to study the effect of *Dinoroseobacter shibae* (bacterium) on the metabolic profile of the diatom,

Thalassiosira pseudonana [102]. The study showed that the intracellular amino acid levels of *T. pseudonana* were upregulated when in co-culture with no improvements in microalgal growth rates. The authors highlighted the application of this co-culturing technique for the investigation of various plankton interactions and understanding the metabolic fluxes within plankton communities. In the case of bacterial interactions, co-cultures of *Streptomyces* sp. and *Pseudomonas* sp. in a glass vessel separated by a 0.22 µm PVDF led to upregulation of several metabolites. Such a co-fermentation approach induced the expression of cryptic indole alkaloid BGC in *Streptomyces* sp. and later characterization of indolocarbazole alkaloid, a phenomenon not observed with their monocultures [107]. With respect to intraspecific interactions, Briand and coworkers [50] studied the effects of three types of *Microcystis aeruginosa* on each other. The aim of the study was to elucidate the factors that regulate the production of secondary metabolites and toxins (during co-culture) essential for cyanobacterial blooms. With this co-culture technique, the authors demonstrated quantitative changes in the production of major extracellular peptides (regulatory factor) as a physiological response to co-culturing when compared to that of monocultures.

In contrast to the Transwell® setup, vessel chambers allow larger culture volumes (up to 500 ml), thereby permitting sampling for omics investigations even in cases with limited biomass availability. Also, this method supported equal growth conditions for both partners in contrast to the dialysis tube system (discussed in Section "Dialysis tube system"), where unequal growth conditions were used. Vessel chambers are a good method for assessing predator-prey interactions and in assessing allelopathic activities. However, the success of this method in illustrating the allelopathic interactions strongly depends on the nature of the molecules exchanged (as these need to be able to diffuse readily through the membrane) and cannot be applied to the microorganisms which require physical contact to elicit the response. An example of hindered interaction, when using vessel chambers was witnessed when associating green algae *Oocystis marsonii* with *Microcystis aeruginosa*. These microorganisms were investigated with respect to algae blooms and eutrophication of waters. The use of membrane-diffusion, however, hindered the allelopathic activity of the cyanobacteria on the green algae, when compared to the direct mixing method, where direct cell-to-cell contact was necessary for the toxic effects of the cyanobacteria to play a part [49]. This highlights the importance of the use of the right co-culturing system to study

natural habitats within the laboratory setting. Using comparative methods of co-culturing, in this case, direct mixing and membrane, demonstrated that other factors come into play in microbial communication, opening the door to more avenues to explore!

Dialysis tube system
Dialysis tube systems (Figure 3(c)) involve the use of semi-permeable dialysis membrane/bags to separate microorganisms in co-culture. One microorganism (a guest strain) is inoculated within the dialysis bag, usually held together with a mechanical spring, to prevent it from collapsing. The bag is then re-suspended in a large vessel containing the other microorganism (the host strain) in free liquid media [100]. Both microorganisms are in liquid media, however, the composition of the media can differ. The porous membrane of the dialysis tube is biocompatible and made up of polycarbonate/cellulose (molecular weight ranging 8–14 kDa, enough to separate fungi and bacteria), allowing for info-chemical interactions but preventing direct cell-to-cell contact.

A novel methanotrophic process was described with this method for the consortia of *Methylocystis* sp. M6 and *Hyphomicrobium* sp. NM3. Such a membrane system allowed the cross-feeding of methane-derived carbon species from *Methylocystis* sp., thereby improving the methanotrophic performance and the biomass yield of *Hyphomicrobium* sp. [108]. This method of co-culture along with biochemical analysis and -omic approaches (proteomics and metabolomics) has been successfully implemented to elucidate novel interspecies allelopathic interactions. An underlying interspecies molecular mechanism was briefly described, where *Microcystis aeruginosa* mediated negative allelopathic effects (inhibits growth) on *Chlorella vulgaris*, via the release of linoleic acid [39]. Moreover, the role of nitric oxide (cell signaling compound produced by *C. vulgaris*) in stimulating the positive feedback mechanism of linoleic acid released by *M. aeruginosa* and its toxicity was demonstrated. Similarly, Shi and coworkers [100] employed this method with LC-MS based metabolomics platform to illustrate and define chemically mediated interactions (mainly secondary metabolites) between not only fungal-bacterial (*Cladosporium* sp. and *B. subtilis*) community but also between two microbial strains of the same background (*Streptomyces* sp. WU20 and *Streptomyces* sp. WU63). LC-MS analysis of the fungal-bacterial community revealed production of diphenyl ether with polyhydroxy side chains including six novel antibiotics as a result of defense mechanism (of *Cladosporium*) against the growth inhibition resulting

from surfactins (antifungal cyclopeptides) secreted by *B. subtilis*.

Another type of encapsulation involves the entrapment of microbes in a hydrogel within a dialysis tube [109]. An example of such a co-culture technique involves the co-culturing of *Synechococcus elongatus* and *Azotobacter vinelandii*, where *S. elongatus* was trapped within a polyacrylate hydrogel matrix, which facilitated the secretion of sucrose to be consumed by *A. vinelandii*. This method allowed to cater to the growth and nutritional requirements of each microorganism. The advantage of this method is the ability to optimize environmental conditions of the two different species that have different environmental requirements for their particular functions. In this instance, the *S. elongatus* was subjected to osmotic stress by the hydrogel, causing the release of compounds that enhanced the growth of the co-cultured species. Although this stress response of *S. elongatus* was species-specific, the further use of the dialysis tubing prevented direct physical contact between the two microorganisms.

This method of co-culture offers faster diffusion rates (for secondary metabolites/info-chemicals), quick equilibrium conditions, easy set-up, and larger culture volumes (1.5 to 5 L) that allows sampling for omics investigations and the further isolation of target compounds. Furthermore, this method allows different growth spaces for both partners in contrast to vessel chambers, where equal growth conditions were used. This added advantage minimizes the impact of guest strain signaling molecules while discriminating the interactions of co-culture from that of monocultures. In contrast, Paul and coworkers [102] criticized this method for not allowing identical growth conditions of the interacting partners or sufficient diffusion between both culturing chambers. In summary, this method in combination with systems biology approaches has a great potential in understanding the functioning of a microbial ecosystem, allelopathic interactions and in the discovery of novel drugs/natural biomarkers within the co-culture community.

Spatial separation

Spatial separation consists of methods where the partners are spatially separated not allowing direct exchange of materials as seen in the co-culturing methods discussed earlier, but allows the indirect exchange of chemicals, through contact of different phases, for example, gas-liquid and liquid-solid phases. This method provi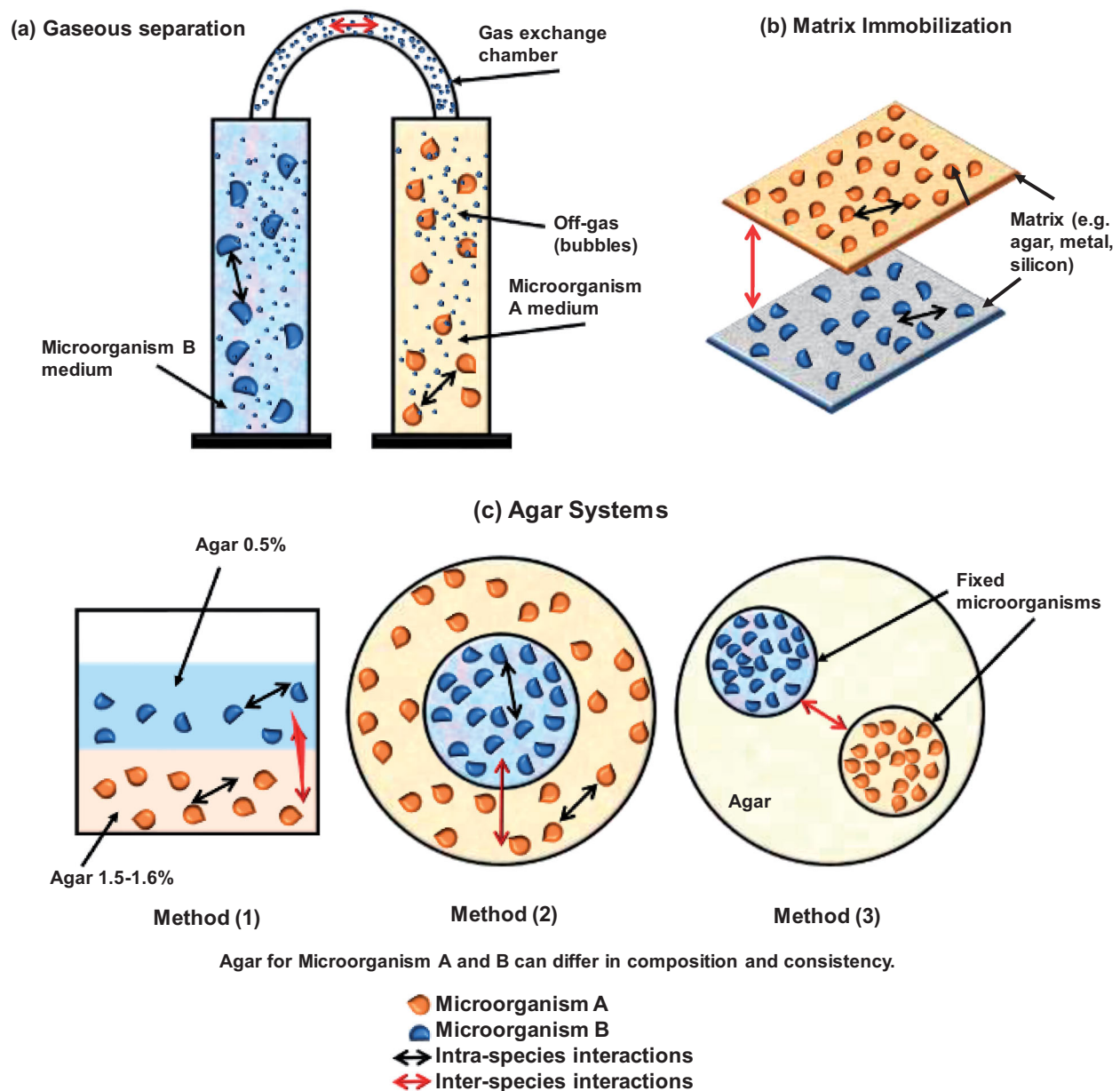des an effective way for eliminating competition for nutrients as the cells are inoculated in separate vessels, as in gaseous separation, or attached on solid matrices as seen in matrix immobilization and agar systems (Figure 4).

Gaseous separation

In contrast to direct mixing and membrane separation, gaseous separation (Figure 4(a)) allows only for the exchange of gases between the co-cultured microorganisms. Here, the microorganisms are grown in separate vessels connected via a port. The two species in co-culture are not exposed to the nonvolatile metabolites present within the culturing liquid or solid media produced by either of the species, thereby reducing competition for nutrients. The exchange of gases, resulting for example from respiration, facilitated by the connection port, can, however, affect the growth mechanism and consequently the intercellular and/or extracellular metabolome of the receiving organism. Therefore, this method can be used to only assess the effect of volatile metabolites on microorganisms.

Santos and coworkers [48] demonstrated the symbiotic association via a gaseous exchange between the heterotrophic and photoautotrophic cultures of *Chlorella protothecoides*. The heterotrophic *C. protothecoides* cultured in a photo-bioreactor were fed off-gas from the outlet autotrophic reactor, and vice-versa. The symbiotic bioreactor demonstrated that the enriched air with off-gas from the other bioreactor increased both the biomass and oil productivity of the microalgae. Similarly, autotroph *C. protothecoides* (microalgae) was co-cultured with heterotroph *R. toruloides* (yeast) in a vertical-alveolar-panel (VAP) photobioreactor, thereby taking advantage of their symbiotic association via complementary nutritional metabolism, that is, respiration and photosynthesis [110]. The VAP facilitated the exchange of carbon dioxide and oxygen between the two microorganisms, resulting in greater microalgal biomass and lipid production.

Gaseous separation methods are ideal for assessing the role of volatile molecules within co-culture systems which can be used as a tool to untangle and validate the possible effects of microorganisms on each other. The upscaling or perhaps expansion of this concept to validate gaseous exchanges within a consortium is feasible [111]. However, spatial separation methods are not a true reflection of how the microorganisms interact in nature. For example, if yeast and algae were co-cultured together in the same medium, the number of gases produced may be lower than in monoculture. Also, the composition of the gases may differ. In nature, as the microorganisms come into contact, other interactions

(a) Gaseous separation

Gas exchange chamber

Off-gas (bubbles)

Microorganism A medium

Microorganism B medium

(b) Matrix Immobilization

Matrix (e.g. agar, metal, silicon)

(c) Agar Systems

Agar 0.5%

Agar 1.5-1.6%

Fixed microorganisms

Agar

Method (1)   Method (2)   Method (3)

Agar for Microorganism A and B can differ in composition and consistency.

🟠 Microorganism A
🔵 Microorganism B
⬌ Intra-species interactions
⬌ Inter-species interactions

Figure 4. Spatial separation co-culture systems. The co-culture microorganisms can only mediate through info-chemicals. Direct contact is not possible. (a) Gaseous separation: each microorganism is grown in its own vessel. The vessels are connected through a chamber that allows for volatile info-chemicals to be exchanged. (b) Matrix immobilization: microorganisms are trapped within a porous matrix. Overlapping the matrixes allows for info-chemical exchange. (c) Agar Systems. Microorganisms A and B can be co-culture on agar plates. The composition of the media can be different. The agar diffusible info-chemicals allow for the species to communicate.

may take place, perhaps also at the expense of gaseous exchange. This represents the limitation of this co-culturing method in terms of info-chemical analysis.

Matrix immobilization

In this method, microorganisms are secured or attached to a surface/matrix (Figure 4(b)). Unlike the encapsulation method discussed in Section "Encapsulation," this approach allows a greater degree of separation between partners and hence potentially a higher degree of control over interactions. The matrix composition will vary according to the nature of the microorganisms in co-culture. The microorganisms attach themselves to the support because of stress (producing EPS) or within crevices that facilitate binding, as in the case of hollow-fiber membranes [112]. Additionally, the microorganisms can be trapped between thin layers of different solidifying agents such as agar [113], hydrogels, κ-carrageenan, and gelatin or combinations of these [35,114]. These layers can be superimposed onto

each other to facilitate interaction [115]. Matrix immobilization is widely used in tissue engineering applications [116] and has also been developed to investigate the cross-talk between microorganisms in co-culture systems. In contrast to the use of shakers and bioreactors, the use of this system enabled the creation of models, which were used to simulate microbial interactions in their local environments [113]. This made this method invaluable for the investigation of microbial interactions in solid matrices such as food [114].

A hollow fiber matrix bioreactor (HfMBR) was used to enrich denitrifying anaerobic methane oxidation (DAMO) microbes and anammox bacteria consortium for flue gas denitrification purposes [112]. The use of a direct mixing method for the same consortia resulted in a limited mass transfer of methane due to the formation of microbial clusters. In contrast to direct mixing, HfMBR allows molecular diffusion of methane through the biofilm's substratum directly to the biofilm without any bubble formation. Moreover, compared to direct mixing, the activity of DAMO archaea in the ternary biofilm built by HfMBR was found to be three times higher [112]. Therefore, attaching an environmental inoculum within the hollow fiber allows for quick recovery of the system as the matrix facilitated methane gas diffusion through the reactor.

Some matrix systems do suffer from mass transfer limitations. However, Smet and coworkers [117] showed that matrix immobilized cells of *S. typhimurium* and *E. coli* growth dynamics were similar to those grown in static communal liquid media. However, growth profiles were lower when compared to shaken liquid cultures, where the mass transfer is facilitated. Therefore, better nutrient and gas distribution methods should be incorporated into this method. Additionally, the methods employed for metabolite extraction are more complex compared to liquid cultures. Difficulties were encountered when extracting metabolites embedded or bound to the matrix, where a stomacher was used to homogenize the samples [37]. Therefore, these metabolites may not be detected or accurate levels of the secreted compounds cannot be determined.

Matrix immobilization can be used quite flexibly in a co-culture system to analyze secreted substances by microorganisms and to act as a supporting matrix. However, unlike mixed cultures, the use of such matrices cannot provide a native environment in which the microorganisms can interact physically. With such matrix or spatial separation techniques, the potential of consortia partners to produce the secondary metabolites during cross-talk is greatly underestimated under laboratory conditions, as indicated by the genomic sequence of fungi. This is demonstrated by the lack of response when *Aspergillus nidulans* and 58 soil-dwelling actinomycetes were co-cultured using a dialysis tube membrane [16]. Besides, using qRT-PCR analyses, the authors demonstrated no fungal response was initiated when the fungal culture was treated with the supernatant of the bacterial culture and when treated with the supernatant of co-culture (of bacterium and fungus lacking the PKS gene) [16]. It is evident, therefore, that unlike the use of matrices, the physical interaction that may exist naturally between two microorganisms was enabled by the directly mixed culture to elicit the fungal gene expression. This was further validated by the authors with scanning electron microscopy (SEM) and metabolomics platform [16]. On a positive note, membranes can also be used as a deduction tool to the mode of interaction in the co-culture experiments. On the other hand, 3D bioprinting technology is obtaining a wider interest in research communities for studying microbial interactions [118–120]. A recent investigation highlighted several advantages of hydrogel-based immobilization for on-demand bioproduction and preservation when compared to direct mixing techniques [119]. Briefly, this method involves 3D printing of microbe-laden hydrogels that spatially compartmentalize each organism (yeast and bacteria in this case). This minimizes or removes competition for nutrients, where authors have reported identical growth rates as that of monoculture for both partners, partners do not impede cell growth of other and overall technique offers more control over a consortium controlling population dynamics. More importantly, this technique was demonstrated for the production of both small molecules and active peptides with the ability to repeated re-use and preservation of the consortia for up to 1 year via lyophilization, thereby offering unique advantages over direct mixing techniques.

Agar systems

Agar systems are another example of spatial separation co-culturing. This technique uses agar of various compositions such as potato dextrose [34] and LB-agar to create porous solid support, onto which microorganisms can be inoculated. Unlike matrix immobilization, the cultures here are not trapped in a matrix but rather allowed to grow on the surface. The configuration of the agar system may vary according to the purpose of the study (as shown (Figure 4(c)).

In Figure 4(c), Method (1) shows superimposed agar of different compositions, which allow a transversal exchange of molecules with a degree of physical contact. In Method (2), longitudinal communication across

the agar is obtained on the boundaries between the two agar phases. The microorganisms at the boundary may come into physical contact and secrete different molecules to those away from the boundary. Whereas, in Method (3), the microorganisms are placed far apart. This design intends to elicit a response/exchange by relying on traveling-released cues between the species over a distance.

The porosity of the agar allows for the exchange of info-chemical between the microorganisms. This method has been extensively used to elucidate the interaction between fungi and bacteria co-culture [34,36,121] and as a valuable tool in studying the co-culture cross-talk in ecology, agriculture, medicine, and biotechnological applications [121].

Agar systems have been used to study the allelopathic interactions between *Botrytis cinerea* (fungus) and the rhizobacterium *Pseudomonas* sp. [34]. *Botrytis cinerea* is responsible for gray mold syndrome on leaves, whereas rhizobacterium was shown to promote plant growth and antagonistic effects on *in vitro* fungal growth. Co-culturing of fungi and bacteria on the potato-based agar plate allowed the area of contact between the two species to be observed microscopically. This revealed a growth disruption of fungi around *Pseudomonas* sp., where *Pseudomonas* sp. did not affect the polygalacturonase activity of *B. cinerea* but inhibited its growth by causing coagulation, and leakage of protoplasm. Similarly, other studies using agar systems have revealed the secretion of compounds such as antifungal, antibacterial substances as well as *de novo* metabolites during co-culturing [121]. Toxicological studies using potato dextrose agar were used to understand the mechanisms of food poisoning caused by *Burkholderia gladioli* (bacterium), when *Rhizopus microspores* (fungus) cultures contaminated with *B. gladioli* were used for the fermentation/production of Asian food dish tempe bongkrek [121]. This study not only identified that the fungus aided the bacterial growth which in turn increased the production of a lethal toxin (bongkrekic acid), but also showed that the bacteria produced antibiotics of the enacyloxin family. In the case of ecological studies, Dalmas and coworkers [36] used this method along with the LC-MS platform and demonstrated that *Streptomycetes* (from the rhizosphere of Araucariaceae) produce exudates (twenty-four compounds), some of which suppress the growth/activity of the fungus *Neofusicoccum parvum*. Under laboratory conditions, many genes for secondary metabolite synthesis are presumably silent as revealed by transcriptomic analysis on cultured fungi. Activation of such silent genes will enable us to discover novel

secondary metabolites and to uncover the mechanism of silent gene activation. Yao and coworkers [122] used the agar co-culture method and metabolomics platform to develop an interactive model (using co-culture of *Trametes versicolor* and *Ganoderma applanatum*) for activating the silent genes. This work led to the identification of 62 novel features that were either newly synthesized or highly produced in the co-culture compared to their monocultures.

The use of agar plates was criticized by Mouget and coworkers [123], pointing out that only agar diffusible molecules are permitted to be exchanged. This was shown by the null-effect when *Pseudomonas diminuta* and *P. vesicularis* were co-cultured on agar plates with *Scenedesmus bicellularis* and *Chlorella* sp. Furthermore, the volume, porosity and composition of the agar can also lead to a varying rate of diffusion for info-chemicals. More importantly, the very low concentration of extracellular metabolites in a large pool of culture medium makes their isolation, identification and quantification difficult with poor reproducibility. The use of small plates/petri dishes (2 cm) instead of conventional plates/petri dishes (9 or 15 cm) may solve the above problems. Bertrand and coworkers [14] used 2 cm multi-well plates inoculated with pre-cultured agar plugs of Fusarium and Aspergillus fungi. The limited nutrient supply due to smaller size wells increased the competition between co-culture partners resulting in stronger and faster stimuli (increased concentration of *de novo* metabolites).

Ideally, any co-culturing strategy should aim at providing the platform that will mimic the naturally occurring ecology. With the use of agar co-cultures, it is important to note that the microorganisms that are directly below the spot inoculated area could become anoxic. Therefore, the compounds released may not reflect the true ecological exchange between the co-cultured partners. Hence, this method may work better when co-culturing anaerobic microorganisms. Therefore, the ultimate method of co-culture using agar would depend on the type of microorganisms being co-cultured and may have to be validated by other co-culture methods if the most number of molecules being secreted are aimed to be detected.

Gel cassette system. An upgrade from conventional agar systems is the gel cassette system. This method was first developed by Brocklehurst and coworkers [124] for monitoring monoculture species, which was later applied to study the interactions between consortia partners [37]. Gel cassettes consisted of a gelatin matrix trapped between a gas permeable membrane

enclosed within two transparent windows made of Plexiglas and covered with a plastic film. This method is commonly used to study the behavior of bacteria in a solid structure, which emulates solid foods. Tsigaride and coworkers [37] used this method to monitor growth and metabolic activity of *Shewanella putrfaciens*, *Brochothrix thermosphacta*, and *Pseudomonas* sp. bacteria (in both mono- and co-culture) responsible for food spoilage. The cassettes allowed co-culture of various population mixes and to observation of their relationship. The findings suggested that changes in behavior were dependent on the co-cultured species. Furthermore, *Pseudomonas* sp. strains co-cultured with *B. thermosphacta* propagated, whilst the ones grown with *S. putrefaciens* perished.
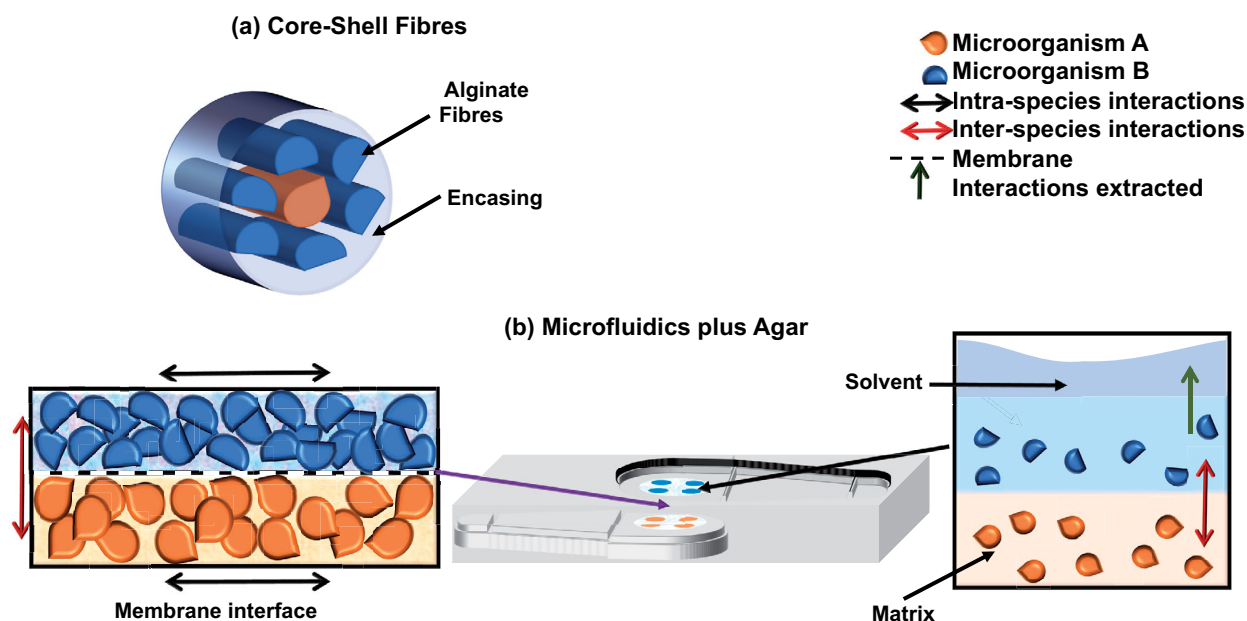
Microfluidic systems

The conventional cell models and co-culture techniques used so far in mammalian cell research do not allow for trapping paracrine communication between different cells due to poor spatial control over the cellular microenvironment and the coexistence of diffusion and convection, which makes the control of communication for monitoring difficult. In contrast, the microfluidic system offers better control over fluids and microenvironments with the use of integrated valves, where better fluid routing can be achieved along with the ability to separate the defined section of the platform isolated from the other sections. This type of culture system is commonly employed in mammalian cell research

(biomedical applications), where the cells are fragile in nature and culture volume requirements are minimal [67,125–128]. However, such systems can also be employed with other cell systems to enable better control of fluid flow, where low volume operations are preferable. Recently, the combination of microfluidic systems with co-culturing designs has gained popularity within various research fields [54,55,129–131].

Core-shell fibers

Microbial communities that interact in nature optimally and perform multifunctions usually have a specific spatially structured arrangement. Such spatial organization is crucial in modulating the degree of co-existence [132–135]. Considering this fact, a core-shell fiber method has been developed [55] in an attempt to construct a biomimetic synthetic functional community, as an alternative approach to genetic engineering (Figure 5(a)). To demonstrate this concept, the co-culture of *Sphingobium chlorophenolicum* (a pentachlorophenol (PCP) degrader) and *Ralstonia metallidurans* (a mercuric ion Hg(II) reducer) was used to remove the mixture of environmental pollutants from the soil. This system was developed by coupling microfluidics with spatially separated calcium alginate fibers to obtain a co-culture environment on the 100 μm scale. The degradation of PCP and the reduction of Hg(II) was achieved simultaneously only in a spatial arrangement, which was not achieved by directly mixed liquid cultures (unstructured communities). This investigation highlighted the

Figure 5. Microfluidics systems. (a) Micro-scale systems: coupled with agar allow for co-culturing and extraction of metabolites in the same device (micro-metabolomics platform). (b) Core-shell fibers: consists of microorganisms trapped in the filamentous alginate fibers. These microorganisms do not come into contact directly.

importance of spatial arrangements when developing co-culturing techniques. The co-culture was only successful when *S. chlorophenolicum* was placed at the center of the core-shell fiber, whereas having *S. chlorophenolicum* in the other outer cortex of the core-shell fiber decreased biodegradation efficacies by 50% [55]. The application of such techniques is gaining momentum in biomedical applications, as demonstrated recently for the proliferation of co-cultured C2C12 cells (mouse myoblasts) [136].

Microfluidic system and agar

Another novel microfluidic device (made up with transparent polydimethylsiloxane (PDMS)) developed elsewhere to study the underlying molecular mechanism in Parkinson's disease, where cross-talk between two different cell populations was monitored by soluble factors (either by perfusion or by diffusion) [129] can also be developed for biomanufacturing. The device consisted of two separate culture chambers connected by three channels and integrated pneumatic valves for isolating one cell population from another where required (Figure 5(b)). This device allows for closer replication of *in vivo* conditions where paracrine signals are effective, as the two culturing chambers are separated by a short distance of 250 μm, facilitating rapid molecular exchange and better control over the cellular microenvironment. Additionally, the chamber isolation tool encourages the concentration of the molecules in one area, facilitating isolation and detection. However, the use of external pumps in such microfluidic devices makes screening experiments nearly impossible and fabrication challenging, effectively preventing widespread integration into biology labs.

The concept of an open micro-metabolomic method was recently developed by Barkal and coworkers [54]. A device comprised of cultured micro-agar pad or liquid well within an open microfluidic channel, where organic solvents (used for metabolite extractions) can be directed to flow over the aqueous culture area. This results in the formation of biphasic interfaces, allowing for the integrated and passive extraction of metabolites over a defined period after which an organic solvent can be recovered by a simple pipetting step. Later, the micro-metabolomics platform was used to trap the chemical diversity of co-cultured fungal and bacterial secondary metabolomes in response to changing microenvironments. Here, the two micro-metabolomics platforms were placed (opposing face) between a thermoplastic layer with diffusion pores in-between to allow an exchange of metabolites.

This method offers several advantages, such as (a) ease of use; (b) one step metabolite extraction; (c) retrieval of organic phase without any aqueous media components carryover (an essential step for subsequent LC-MS platform); (d) rapid workflow with smaller extractions volumes; and (e) versatility in the choice of solvents used for metabolite extraction. As the device is coated in Parylene C (high solvent resistance) it permits for the detection of un-interrogated segments of the metabolome, unattainable with conventional extraction solvents. This system was stated to have the advantage of enabling the use of two different media for species that grow optimally in different media and to enable equidistance diffusion of metabolites, which was not the case with the use of direct mixing and agar co-culture methods.

Critical considerations/challenges

A summary of the approaches discussed in this review is presented in Table 2. Establishing a co-culture approach that will facilitate obtaining the desired information is a feat in itself. Alongside the choice of which microorganisms to be co-cultured and the method to be used, the following factors need to be taken into consideration: inoculation ratios, inoculation timing [6,7], priority effects [137], and history of the microorganism [138]. Each will have an impact in its own way on the dynamics established between the co-cultured microorganisms. This will consequently influence the availability of molecular cues to be detected. Ideally, we want a method at both laboratory and industrial scale, which will allow us to emulate the natural environment and noninvasive direct investigation of all possible forms of dynamic interactions in real-time that emerges naturally in the microbial consortia. Currently employed co-culturing methods appear to be useful in understanding only a fraction of these interactions. Moreover, this fractional knowledge obtained does not reflect true natural interactions that may be taking place, as such associations comprise numerous organisms thriving together [53]. The other critical considerations/challenges that require attention are;

i. Many genes for these interactive cues are silent under laboratory conditions, adding further limitation [14,54,122]. Furthermore, the competition for nutrients in artificial consortia disturbs the homeostasis, as partners try to out-compete one another and exhaust their available resources in a microenvironment, which is not the case with microbial communities living in nature [15].

Table 2. Key co-culturing approaches discussed in the review and their bioprocess applicability.

Co-culturing approach	Applicability	Scalability[a]	Factors to consider
Communal liquid medium growth	Industrial production/ bench scale studies		Inoculation ratio;
Direct mixing		+++	Timing of introducing co-
Pelletization and flocculation		++	culture partners.
Biofilms		++	
Solid–liquid interface			Maximal surface for effective
Encapsulation	Bench scale studies	+	contact and molecular exchange;
Cell droplets		+	Appropriate culture densities for
			maximal effect
Membrane separation			Membrane permeability to allow
Transwell® system		+	molecular exchange;
Vessel Chambers	Bench scale studies/ Screening for partners	++	Pre-optimization of set-up
Dialysis tube system		+	
Spatial separation			Matrix and medium composition to
Gaseous separation		++	enable and not restrict mass
Matrix immobilization	Industrial production/ bench scale studies	+	transfer for molecular exchange and interactions
Agar systems		+	between cultures
Gel cassette system			
Microfluidic systems			Spatial orientation and fluid flow to
Core-shell fibers	Bench scale studies	++	maximize interactions
Microfluidic system and agar		+	

[a]+++: scalable; ++: less scalable; +: least scalable.

However, such nutrient limitation might be useful as it can cause induction of de novo metabolites [14].

ii. The available techniques are not designed to trap all forms of interactive cues. For example, the use of nanospray desorption electrospray ionization imaging mass spectrometry on agar co-cultures has allowed for real-time analysis of only agar diffusible molecular signals (few forms of these interactions), with low disruption to the microbial interaction [139].

iii. Abiotic and biotic stress factors hugely affect these interactive cues, creating doubt in their reliable resemblance to that of the natural interplay. For example, stress factors arising from co-culture designs include physical restriction (cell confinement, immobilization, and limited/no molecular diffusion) and chemical restriction (nutrients), which usually results in a generation of nutrient and/or metabolite concentration gradients [140]. Biotic stress factors such as a selection of suitable co-culture partners, population dynamics, biovolume variability, media selection, nutrient source, inoculum/seeding (ratio, densities, location, and timing), pH, and salinity affect the growth kinetics [7,51,76]. To the most extent, these stress factors are interlinked, as diffusion limitation will result in difficulties in nutrient supply, thereby affecting growth. Overall, these stress factors elicit an unwanted response, thereby impairing the overall aim of the co-culture research.

iv. Inoculation ratios and timing of the monocultures need to be factored into the equation. Understanding these parameters in terms of behaviors of the monoculture vs co-culture will shed light on interactions that will govern the final co-culture [7]. Having the wrong starting ratio of two microorganisms at the co-inoculation or adding the inoculant of one to the other at the wrong time (stage of growth of the other), could lead to an unbalanced system, where one species overtakes or triggers an adverse reaction in the other.

v. A lack of appropriate sampling strategies, analytical workflows, techniques, and data analysis tools presents an additional major challenge in the detection and quantification of these interactive cues [100]. The interactive cues emerging from available small-scale spatial configuration are often having a very dilute concentration. This might be due to the poor co-culture designs offering very small sample volume for analyses, the existence of very dilute communities as in the case of phytoplankton, and contribution from the biological sample matrix such as salts, proteins, cell debris, and rich media components. Owing to the high turnover rate, dynamic nature, and diverse physicochemical properties of these interactive cues, the identification of an optimal analytical workflow (sampling, quenching, extraction, and analytical platform) represents a major challenge, as there is no single platform that is currently available, which is capable of identifying and quantifying these interactive cues, in an unbiased and reproducible way [25,26,141]. Moreover, the proposed developed and optimized

analytical workflow, for a given consortia partners are always species-specific [15,142] and might not be valid for other partners, thereby requires independent evaluation and validation.

Future optimization strategies and co-culture design development

The current systems outlined in this review have great potential to trap the fraction of interacting cues emerging from microbial interactions, however, to make a real sense of the soup, further development to co-culture designs and analytical workflows is vital. Circumventing this problem is not an easy feat, but the following considerations hold promise for future optimization strategies, concerning co-culture designs:

i. The application of more than one technique to a particular co-culture system would indeed provide more rounded conclusions, however, it may not be a practical approach in terms of time and logistics.

ii. The environment in which the co-cultures are cultivated will inevitably affect the interactions. The analyst should consider the mode of trigger-response mechanism (either physical, diffusion, adhesion, or gaseous) intended for desired applications, as this will help in the selection of optimal co-culture technique [7]. Additionally, if microbes were affected by the media's structural make-up then it would be more logical to test trigger-response, in an environment most similar to its natural local environment. For example, it would be logical to culture fungi on agar, as it exists naturally on surfaces such as wood, rather than in liquid media. However, due to the adaptability of microorganisms, it may be possible that they are able to grow in several different media matrices.

iii. The extracellular environment in co-cultures strongly influences cell-cell interactions. This is heavily reliant on the experimental set-up, such as the bioreactor design, use of separation membranes, perturbation within the reactor, temperature, pH, and other abiotic factors. The collection of these parameters will dictate the mass transfer of volatile and nonvolatile compounds [6,101]. The development of a system that allows monitoring more than one form of interactive cues is thus necessary. For example, the development of a double system bubble column photo-bioreactor [51], allowed the exchange of both volatile and nonvolatile signals. The filter allowed for the flow of molecules and the culture parameters enhanced the dissolution rate of oxygen, for the yeast to uptake, which in turn generated the carbon dioxide necessary for the algae to grow.

iv. The main drawback of laboratory co-cultures is the fact that these are limited in the extent to which they can mimic the real world. The use of a multifunctional bioreactor that will allow a three-dimensional culture of cells, where co-cultured partners are spatially separated, is gaining popularity in tissue engineering [111,143]. Whereas few efforts have been made to adapt methods used in monoculture systems for co-cultures studies, as demonstrated with the application of gel cassettes for microbial interactions [37]. Adoption and further developments of such methods for microbial consortia hold great potential, as spatial structures will allow us to mimic the behaviors of cells as it happens in nature.

v. Similarly, the adoption of methods such as diffusion chambers (Figure 6), which are mainly developed to isolate microorganisms from the environment and to acclimatize these to laboratory conditions [144,145] may be a viable method for co-culture studies. This method is comprised of a thin film of agar that encapsulates the microorganism on a bottom base layer. Initially, the monoculture/co-cultured species could be inoculated onto the thin film of agar and left to incubate in the environment. This will allow us to capture the true representation of the interplay that exists in nature. This set-up would also allow to trap and concentrate metabolites facilitating their identification.

vi. Integration of microfluidic single-cell cultivation systems with traditional methods is emerging as a valuable tool in exploring the microbiome interactions in both natural and synthetic consortia. For example, coupling of microfluidics devices with agar systems has been fruitful [54,55]. Novel designs integrating membrane separation techniques with co-culture plates [146] have great potential for the simultaneous study of various forms of interactions and growth dynamics. A recent review highlighted the pros and cons of microfluidic systems along with an overview of different microfluidic systems and their integration with traditional methods used in environmental biotechnology [147]. With such integration, cultivations can be
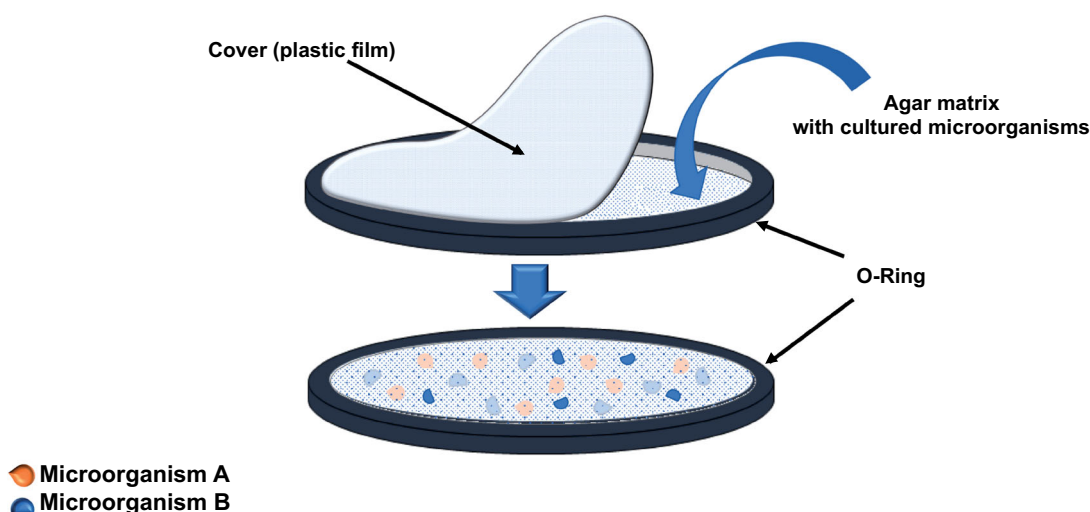
Figure 6. Diffusion Chambers – used mainly for isolation of hard to grow species from the environment and can be adapted to be used to study the behavior of artificial co-cultures in nature.

performed at population (3D) or single-cell level (2D, 1D, or 0D) with direct cell-to-cell contact or indirect contact via permeable membranes. For example, a novel microfluidic 2D co-cultivation system with spatially separated cultivation chambers was developed, allowing faster metabolite exchange due to short diffusion distances via sieve structure [148]. Such chip-based techniques allow systematic investigation of microbial interactions at a single-cell resolution. This provides a one-to-one perspective. However, it must be noted that microorganisms thrive in "families," thus the behavior of a single cell cannot be taken as a representation of the whole. The cell-to-cell effect that was observed in direct mixing experiments, where some level of separation hindered communication, is a good indicator of this. Another good example is quorum sensing in bacteria, where communication molecules are triggered by an increasing population. Furthermore, biotechnological applications look into co-cultures as tools to maximize biomass growth, thus further investigation is required into the use of "single-cell" methods, to attest if these indeed are a good way of studying microorganisms for biotechnological applications.

vii. Concerning analytical tools, so far, metagenomics in combination with transcriptomics and proteomics offers great potential as a guide for interaction discovery. For example, methods such as functional genomic responses (changes in gene expression using RNAseq, microarray analysis) have been used for a deeper understanding of interacting cues [42,66,149].

viii. For understanding the spatial distribution of interacting partners and their metabolic state, methods such as fluorescence in situ hybridization (FISH) [150] and fluorescently-tagged proteins [41,151] hold great potential. Additionally, C14-labelled sodium carbonate labeling was used to investigate biofilm formation [152].

ix. The use of metabolomics platform with high-resolution mass spectrometry (HRMS) coupled to chromatographic techniques is gaining popularity for the study of microbial interactions [9,14,54,100,102,122] However, its broad deployment to biotechnology is not yet as widespread as desired due to several challenges in the quantitative metabolomics workflow that remain [26]. Coupling of metabolomics platform with the use of stable isotope tracers could serve as a gold standard for metabolic pathway discovery and also for identifying the flow of metabolites in microbial consortia studies [27].

x. Metabolic modeling is a useful tool to study and predict the behaviors of co-cultures and provides an insight into which type or combination of techniques should be used to maximize our understanding of microbial interactions [153,154]. Metabolic modeling was used to simulate the co-culture of respiratory-deficient *S. cerevisiae* and wild-type *Scheffersomyces stipites*, to maximize the co-culture growth rate [155]. To do this, the genome-scale metabolic reconstructs of each organism were necessary. Dynamic models and substrate uptake kinetics were developed for each organism separately, to be later combined to predict the outcomes at different

microaerobic growth conditions. On the same note, the stoichiometric model-based approach was used to construct a synthetic anaerobic co-culture and integrate the metabolism of *Clostridium acetobutylicum* and *Wolinella succinogenes*. Such a model can interact via interspecies hydrogen transfer/applied different environmental conditions to infer metabolic-exchange fluxes [156]. The development of co-culture databases containing valuable experimental information on metabolites and metabolic pathways involved in co-cultures is a valuable tool for metabolic modeling [7]. This was recently demonstrated, where researchers have developed a "Metabolic Support Index" for quantifying the metabolic interactions in microbial co-cultures [157]. Modeling the interactions between the microorganisms in consortia presents many obstacles because of the complexity of the network and changes in growth parameters will further add to the complexity [6]. By dissecting the interactions into smaller manageable co-culture systems, with targeted goals, a step-by-step approach can be modeled and expanded to cover the bigger picture. For example, a novel mathematical biofilm model that can be applied to any bacterial species/environmental conditions was proposed [158]. Interactions between *Porphyromonas gingivalis* and *Streptococcus gordonii* biofilms were studied with this model, where independence between species, substrate competition, and production of toxic molecules can be explored. However, the application of the model is not universal to all systems and needs to be developed per bioprocess. However, data collection and characterization with the aim of bioprocess optimization will pose a challenge in itself, that to date needs to be overcome with the development of high-throughput methodologies and better mapping systems. The development of live-cell tracking methods may be a solution that can be extended to chemical cues tracking [159]. The potential of differential equation models, constraint-based stoichiometric models, and later integrative approaches were highlighted in exploring the complex interactions between microbial communities [160]. Authors recommended recognition of key strength of specific method first and later their integration is a key while representing multi-scale phenomena. Likewise, implementing the common language of modeling and focusing on

processes and commonalities is crucial in minimizing the barriers between scientific communities and improving our knowledge of microbial processes [161].

Conclusion

It is evident from this review that different co-culture methods are suitable for different microorganisms and for different goals that the co-culture experiment aims to achieve. Spatial separation methods are useful for the detection of metabolites and the identification of secreted molecules but would not be beneficial for co-cultured species that require physical interaction. On the other hand, encapsulation methods are more suited for microorganisms that require different environmental conditions. Furthermore, co-culture methods can also be combined such as using a combination of hydrogel matrix and a membrane for spatial separation of the co-cultured microorganisms. These co-culture methods have highlighted different advantages and challenges depending on the aim of the experiments. Therefore, it is recommended that the co-culture methods are chosen based on their advantage for the characteristics of the co-culturing species. Different co-culture methods should also be utilized to validate experimental results obtained as different environmental structures and conditions can have effects on communication between microorganisms.

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