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Review

New approaches in bioprocess-control: Consortium guidance by synthetic cell-cell communication based on fungal pheromones

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Bioconversions in industrial processes are currently dominated by single-strain approaches. With the growing complexity of tasks to be carried out, microbial consortia become increasingly advantageous and eventually may outperform single-strain fermentations. Consortium approaches benefit from the combined metabolic capabilities of highly specialized strains and species, and the inherent division of labor reduces the metabolic burden for each strain while increasing product yields and reaction specificities. However, consortium-based designs still suffer from a lack of available tools to control the behavior and performance of the individual subpopulations and of the entire consortium. Here, we propose to implement novel control elements for microbial consortia based on artificial cell–cell communication *via* fungal mating pheromones. Coupling to the desired output is mediated by pheromone-responsive gene expression, thereby creating pheromone-dependent communication channels between different subpopulations of the consortia. We highlight the benefits of artificial communication to specifically target individual subpopulations of microbial consortia and to control *e.g.* their metabolic profile or proliferation rate in a predefined and customized manner. Due to the steadily increasing knowledge of sexual cycles of industrially relevant fungi, a growing number of strains and species can be integrated into pheromone-controlled sensor-actor systems, exploiting their unique metabolic properties for microbial consortia approaches.

Keywords: Bioprocess-control / Cell-cell communication / Fungal pheromone / Sensor-actor systems

Received: November 2, 2017; *revised:* February 8, 2018; *accepted:* March 13, 2018

DOI: 10.1002/elsc.201700181

1 Introduction

Currently, the vast majority of microbial bioconversions in biotechnological processes is carried out by a single strain, isolated from environmental samples or genetically engineered to perform the desired reactions with high yield and specificity [1–3]. While being beneficial in terms of predictability and reliability, the palette of biochemical reactions is restricted to the

inherent and engineered traits of this specific strain. With the increasing complexity of biotechnological processes, *e.g.* for the bioconversion of low-cost substrates into high-value products, the application of a single strain may no longer be the best choice. Frequently, single-strain strategies are outperformed by cellular consortia [1–7]. The benefits of cellular consortia are manifold: by combining multiple strains into a single consortium, the metabolic burden for each strain is significantly reduced (*via* division of labor). In the case of genetically engineered strains, the number of genetic elements to be introduced and stably maintained in a single cell decreases, thereby increasing the long-term stability and limiting the risk of circuit crosstalk. Furthermore, compartmentalization of specific reactions by using different host cells might reduce the risk of side-product formation and unwanted cross-reactions [3]. Integrating strains that are highly specialized for certain reactions (*e.g.* wood-degrading fungi for lignocellulose degradation) into “specialist communities” might be more straightforward than engineering the required traits in

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Abbreviations: *C. albicans*, *Candida albicans*; *C. cinereus*, *Coprinus cinereus*; GPCR, G-protein coupled receptor; *L. kluyveri*, *Lachancea kluyveri*; MAPK, Mitogen-activated protein kinase; *P. rhodozyma*, *Phaffia rhodozyma*; RNAi, RNA interference; *S. cerevisiae*, *Saccharomyces cerevisiae*; *S. commune*, *Schizophyllum commune*; *S. macrospora*, *Sordaria macrospora*; *S. pombe*, *Schizosaccharomyces pombe*; *T. reesei*, *Trichoderma reesei*

heterologous host cells. Consequently, consortium approaches are rapidly emerging, e.g. for applications in biofuel production, bioremediation, biomineral and food industry [6–8].

Currently, consortium-based approaches are often limited by stability issues and a lack of suitable control elements to ensure that individual subpopulations as well as the entire community behave precisely as intended. Even minor fluctuations in the composition and activity of the consortium can have detrimental effects on product yields and process times [3, 4, 7]. Despite its enormous benefits, the consortium-based strategy must not come at the costs of limited process reliability due to changes in community composition during the bioconversion process. It is challenging to control the composition of microbial communities consisting of two or three species, let alone consortia encompassing even more strains or species. It has been proposed that engineering cellular communication or metabolic interdependence (synthetic mutualism) might serve to stabilize the composition of cellular consortia and to address specific subpopulations, providing control elements for both cellular metabolism and proliferation [2, 4, 6, 7, 9]. Cooperative cross-feeding strategies of essential nutrients, however, only promote stable consortia at intermediate cell densities [10] and are prone to the evolution of cheaters [5]. Here, we suggest that engineering artificial cell-cell communication, coupled to well-characterized synthetic biology modules, could serve as a novel control element for microbial consortia, allowing for an advanced form of community control.

Fungal species are an extremely valuable source for highly active enzymes as well as for numerous secondary metabolites with high market values, including antibiotics, pharmaceuticals, organic acids and vitamins [11, 12]. Hundreds of fungal species are currently under investigation or in use for commercial production of these compounds [11], although the fraction of employed fungi is still surprisingly small, considering that 1.5–5 million fungal species may exist [13]. In several applications and fermentation strategies, these fungi might clearly outperform the commonly employed strains of the model yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), due to elevated temperature tolerance, resistance against osmotic and ethanol stress, fermentation efficiency and yield as well as tolerance against fermentation inhibitors [14–17]. Consequently, numerous consortium-based approaches consisting of multiple fungal species [18–20], fungi and bacteria [21–28] as well as fungi and algae [29], covering a wide range of applications, have been reported. In this review, we will focus on synthetic consortia composed of yeasts and fungi, and propose consortium control by pheromone-based cell–cell communication. Similar strategies might also be applied to bacterial consortia (employing quorum sensing molecules for controlled cell–cell communication), as numerous synthetic biology circuits encompassing quorum sensing devices have been reported previously [30–32].

2 Fungal mating systems and the yeast pheromone response

The majority of biotechnologically relevant yeasts and fungi are ascomycetes, which also represent the largest phylum within the

Figure 1. Sexual cycle of the budding yeast *S. cerevisiae*. Haploid cells of the mating types **a** and α communicate via short peptide pheromones (**a**-factor and α -factor) released by cells of the corresponding mating type. Presence of the mating pheromone of the opposite mating type is detected by specific GPCRs located in the cellular membrane. Pheromone signaling triggers a wide variety of cellular reactions, including cell-cycle arrest and polarized growth, ultimately resulting in cell fusion to form a diploid cell. Under certain environmental conditions, diploid cells can undergo meiosis and sporulation to give rise to four haploid ascospores, thereby completing the sexual cycle. Modified from [36].

fungal kingdom [33]. Despite their long evolutionary history, all ascomycetes studied so far show bipolar mating systems, i.e. the sexual identity of the cell is determined by a single mating (*MAT*) locus containing one out of two different alleles [34]. In fact, both alleles are typically highly divergent at the DNA level and are hence frequently referred to as “idiomorphs” rather than alleles. In contrast, mating systems in basidiomycetes can be bipolar or tetrapolar (i.e. two unlinked mating loci control sexual identity), and these loci are often multiallelic, resulting in thousands of different mating types [13, 35].

Mating has been studied in a number of fungal species. The most detailed picture has been unraveled in *S. cerevisiae*. Several excellent reviews have covered this subject [33, 36, 37], so we will only outline the core design principles. Haploid cells of *S. cerevisiae* exist in one of two mating types, termed **a** or α , determined by the idiomorph at the *MAT* locus (*MATa* and *MAT α* , respectively). Cells of opposite mating types communicate with each other via diffusible peptide pheromones, with **a**-type cells secreting **a**-factor (a farnesylated, C-terminally methylated dodecapeptide) and α -type cells releasing α -factor (an unmodified peptide consisting of 13 amino acids, Fig. 1). Both cell types carry mating type-specific G-protein coupled receptors (GPCRs) embedded in the plasma membrane. These receptors are activated upon binding of the pheromone of the opposite mating type. Downstream of the receptor, the pheromone signaling cascade proceeds via G-protein signaling and a mitogen-activated protein kinase (MAPK) module to key effectors such as Ste12p (the master transcription factor for pheromone-induced genes) and Far1p (linking the MAPK module to the cell cycle

Figure 2. Coupling of artificial cell-cell communication to the desired cellular behavior (exemplified for the budding yeast *S. cerevisiae*). (A) The authentic pheromone signaling cascade encompasses a pheromone receptor, typically interacting with a trimeric G-protein composed of a $G\alpha$ subunit and a $G\beta\gamma$ dimer. Binding of a cognate pheromone to the GPCR triggers G-protein dissociation, with the released $G\beta\gamma$ dimer forwarding the signal to the MAPK signaling cascade assembled at a specific scaffold protein. Phosphorylation of the MAPK (indicated by the red circles) results in interference with the cell-cycle, causing cell-cycle arrest in G1 phase, as well as activation of specific transcription factors (TF) binding to pheromone-responsive promoter elements to trigger transcriptional reprogramming in the course of the pheromone response. Taking use of the authentic signaling circuit, the pheromone signal can therefore be linked to the expression of user-defined target gene(s) placed downstream of pheromone-responsive promoters. (B) Engineering of the MAPK scaffold protein to include additional binding sites for positive (red) or negative (blue) regulators that either enhance MAPK phosphorylation or accelerate MAPK dephosphorylation. Modifying the MAPK phosphorylation status allows for fine-tuning of the kinetics as well as the dose-response curve of the pheromone response [72]. (C) Heterologous GPCRs can efficiently be coupled to the yeast pheromone response. Replacing the pheromone receptor by a heterologous GPCR and engineering of the $G\alpha$ subunit to provide efficient coupling to both the GPCR and the $G\beta\gamma$ dimer can be used to transform the presence of cognate GPCR ligands into the desired cellular behavior.

machinery, Fig. 2A). This leads to pheromone-induced cell cycle arrest, polarized growth (known as “shmoo” effect) and transcriptional reprogramming. Ultimately, cells of both mating types undergo plasmogamy and karyogamy to form a diploid zygote that can undergo meiosis and sporulation to give rise to haploid spores of both mating types, thereby completing the sexual cycle (Fig. 1).

Essentially similar sexual cycles were also identified in phylogenetically distant ascomycetes such as *Schizosaccharomyces pombe* (*S. pombe*), *Candida albicans* (*C. albicans*) and *Neurospora crassa*, among others [33, 35, 37]. Although their sexual programs differ in many details, the core concepts of ascomycete

mating such as pheromone dimorphism (one lipid-modified α -like pheromone and one unmodified α -like pheromone) seem to be highly conserved. In contrast, Basidiomycota and Mucoromycota utilize exclusively lipid-modified peptide pheromones and trisporic acid (terpenoid compound), respectively, as signaling molecules. This is considered to be one of the main distinguishing features between the phyla [13, 35, 38].

Sexual development has also been observed in the filamentous ascomycete *Trichoderma reesei* (*T. reesei*) [39], which secretes cellulases and hemicellulases at high levels, rendering it an attractive host for the conversion of lignocellulosic feedstocks into bioethanol and biofuels [17, 18, 24]. *T. reesei* was found to

Figure 3. Pheromone-controlled sensor-actor systems for advanced control of consortium performance. Microbial consortia can be engineered to carry out highly complex tasks and bioconversions, and utilizing artificial cell-cell communication to wire individual subpopulations might serve to implement novel control elements for guidance of the biochemical reactions as well as for the control of consortium behavior and maintenance in general. For example, substrate availability or accumulation of intermediate products might be utilized as trigger elements to switch on/off cell-cell communication, thereby adapting the cellular behavior to optimize process flow and product yields.

be heterothallic, but crossing of industrially relevant strains was perturbed by the female sterility of the strain QM6a, the ancestor strain of all *T. reesei* strains currently employed [39]. Since the first report on sexual development in this fungus, a number of follow-up studies have been conducted, leading to the identification and characterization of the mating pheromones [40], the pheromone receptors [41] as well as positive and negative regulators of sexual development in response to varying environmental cues [42–44]. Recently, the genetic basis for female sterility in QM6a derivatives was uncovered and female fertile strains were created, paving the way for strain improvement *via* standard crossing techniques [45] and, potentially, for integration into communication-controlled consortia. These studies might serve as a blueprint for research on additional fungi with economic importance, eventually leading to the detailed characterization of their sexual cycles.

It has been estimated that 20% of all fungal species lack sexual cycles and proliferate exclusively by asexual means. However, this number is likely to be reduced during the ongoing “fungal sexual revolution” that has led to the identification of sex-associated properties in a huge number of species spanning all branches of the fungal kingdom [33, 38, 46]. Recent sequencing efforts and functional studies have uncovered the presence of the genetic equipment for mating (genes for *MAT* loci, pheromones and pheromone receptors [46]), molecular mechanisms for mating type switches [47, 48] as well as full or cryptic sexual cycles in numerous fungi, including phytopathogens [49–52], opportunistic human pathogens [53, 54] as well as fungal species with high economic impact [55]. Recent examples of the latter include *Aspergillus terreus* (lovastatin synthesis) [56], *Penicillium roquefortii* (blue-veined cheese production) [57], *Penicillium chrysogenum* (penicillin synthesis) [58] and *Phaffia rhodozyma* (*P. rhodozyma*; astaxanthin carotenoid synthesis) [59]. Identification of sexual programs will facilitate strain improvement by crossing and selection techniques, allowing for the combination of favorable traits [55, 60]. The existence of sexual cycles in these species might also favor their integration into pheromone-controlled sensor-actor systems.

3 Applying the yeast pheromone response for artificial sensor-actor systems

Engineering artificial cell-cell communication might be a key step towards the development of advanced consortium control elements, with a tremendous potential for applications in biosensors, bioprocess engineering, environmental monitoring and bioremediation [2–4, 6–9]. By combining pheromone-secreting and pheromone-responding cells, we have recently created an intra-species cell-cell communication system for signal detection and amplification in *S. cerevisiae* [61, 62]. To this end, sensor cells were engineered to respond to a user-defined environmental cue by expression of the *MF α 1* gene (encoding the α -factor pheromone), resulting in analyte-dependent pheromone secretion. The latter acts as an inducer molecule for the expression of a target gene under the control of a pheromone-responsive promoter in specifically engineered actor cells, wiring sensor and actor cells *via* a diffusible signal. This system provides an opportunity for controlled cell-cell communication as well as for signal amplification in biosensor devices [61, 62]. Due to the low molecular weight of the yeast pheromone, sensor and actor cells can also be immobilized in adjacent compartments, *e.g.* in hydrogel matrices. As the pheromones diffuse across the compartment boundary, artificial patterns were created, and competition of both cell types for essential nutrients was prevented [63].

This approach might serve as a blueprint for the development of cellular consortia performing complex tasks (Fig. 3). A variety of specifically functionalized cell populations, characterized by particular sensor and/or actor performances (*e.g.* expressing a set of specific enzymes), can be implemented into such systems. For example, Schofield *et al.* (2007) reported a set of engineered yeast cells capable of either detecting or degrading the hazardous organophosphate paraoxon [64]. However, due to the low stability of multiple episomal plasmids within a single cell, both functionalities (detection and degradation) could not be implemented within a single cell. This limitation might be overcome by sensor-actor systems relying on artificial cell-cell communication to develop bioremediation devices with high economic

impact. Upon combining specifically engineered strains into a single consortium, pheromone-based cell-cell communication could be utilized to control the performance of individual cells as well as the entire community (Fig. 3).

The vast majority of pheromone-based cell-cell communication modules utilizes pheromone-inducible promoter elements in combination with well-established reporter genes, e.g. to visualize pheromone response in the engineered receiver cells (Fig. 2A). Pheromone-responsive promoter elements have been identified and characterized in *S. cerevisiae* (such as *FIG1*, *FUS1* and *PRM1* [61, 62, 65, 66]), *C. albicans* (*FIG1* [67]) as well as *S. pombe* (*rep1*⁺ and *sxa2*⁺ [68–70]), among others, and microarray analyses are expected to unravel numerous of these genetic elements in other fungi. Hence, the pheromone response of many species might be integrated into sensor-actor systems to yield pheromone-dependent expression of a set of target proteins, thereby controlling and fueling the intended bioprocess (Fig. 3). Recently, a pheromone-induced RNA interference (RNAi) pathway was created in *S. cerevisiae* [71]. Since wild-type *S. cerevisiae* cells do not possess RNAi pathways, core components of the RNAi pathway were introduced into yeast. The pheromone-induced expression of the designed hairpin RNAs allowed for silencing of a specific set of target genes. In that way, modulation of the carbon flux was established to improve product yields (parahydroxybenzoic acid in this study). Furthermore, pheromone response in *S. cerevisiae* could be modulated by Ste5p engineering, a scaffold protein with a key function in the pheromone signaling cascade (Fig. 2B). By including additional binding domains for positive and/or negative regulators into the scaffold protein, both the kinetics and the dose-response curves of the pheromone signal cascade could be tuned [72]. Thus, at least in *S. cerevisiae*, a set of functionalities is now available to couple the pheromone signal to various output responses, enabling the development and fine-tuning of advanced circuits to control the system's performance.

In addition to authentic pheromone-based communication systems, several synthetic cell-cell communication modules (intra-species and inter-species) have been investigated (see Section 4). We envisage that these modules can be interfaced with the sensor-actor system approach. Hence, it might be feasible to scale-up sensor-actor systems to more complex consortia consisting of multiple species and/or multiple communication channels between different subpopulations of the consortium, thus providing novel options for customized population control and consortium behavior in industrially relevant settings.

4 Artificial cell-cell communication in fungi

Synthetic cell-cell communication utilizing fungal cells has been implemented in several studies, the majority of them manipulating the authentic pheromone system of *S. cerevisiae* to achieve directed cell-cell communication modes. In a recent report, Youk and Lim (2014) demonstrated that *a*-type cells of *S. cerevisiae* secreting and sensing the α -factor pheromone can be engineered to exhibit a variety of social ecosystem behaviors [73]. This provided valuable insights into the molecular basis of signaling modes that can determine the fate and behavior of multicellular systems. Likewise, synthetic quorum sensing circuits based

on the *S. cerevisiae* α -factor pheromone have been implemented in yeast [65]. In these circuits, synthesis of the pheromone itself is induced by the pheromone response cascade, thereby creating a positive feedback loop that drives elevated pheromone secretion with increasing population densities. Upon reaching a threshold value, a sustained pheromone response of the entire culture is triggered. This effect could be further tuned by modulating pheromone secretion rates and engineering the dose-response profile of the pheromone-inducible promoter. This circuit behavior essentially resembled bacterial quorum sensing systems and might be adopted for large-scale fermentation approaches, providing autonomous systems that obviate the need for adding complex and expensive inducer molecules to trigger the expression of target genes [65]. Similar circuits – based on artificial expression of plant hormones and cognate receptors [74] or on bacterial quorum sensing molecules [75] – have previously been developed for use in *S. cerevisiae* and *Escherichia coli*, respectively.

Furthermore, synthetic cell-cell communication based on authentic yeast pheromones has also been utilized to implement logic gates [76, 77] or flip-flop memory devices [78]. A recent study created novel yeast consortia to integrate six different input signals into a multiplexer circuit [76]. Implementing cellular logic gates and memory might enhance the long-term stability and reliability of consortium-based approaches as these consortia could be programmed to respond to a wide variety of environmental conditions in a predefined and customized manner, unlocking the full potential of these approaches.

However, exploitation of pheromone-based communication systems to couple cell-cell communication to the desired cellular behavior is not restricted to manipulating authentic communication systems. Several previous reports demonstrated functional expression of fungal pheromones and cognate receptors in *S. cerevisiae* to study mating processes in ascomycetes and basidiomycetes, thereby creating artificial inter-species communication modes. To this end, mating pheromones of *C. albicans* [77, 78], *Sordaria macrospora* (*S. macrospora*) [79, 80], *Schizophyllum commune* (*S. commune*) [79, 81] and *Coprinus cinereus* (*C. cinereus*) [82] have been functionally expressed in yeast (Tables 1 and 2). In addition, we recently showed that the α -factor mating pheromone of *S. cerevisiae* can be secreted by engineered cells of the fission yeast *S. pombe*, using either the authentic *S. cerevisiae* *MF α 1* or a chimeric gene (*map2/MF α 1*). The latter was based on the *S. pombe* P-factor pheromone precursor gene with two of the four P-factor repeats being replaced by *S. cerevisiae* α -factor coding sequences [68, 69]. Interestingly, pheromone quantification and activity assessment revealed that expression of the authentic gene did not give rise to the fully processed pheromone, whereas α -factor derived from the chimeric gene was fully active [68, 69, 83]. In contrast, the *S. pombe* P-factor pheromone could be secreted by engineered *S. cerevisiae* cells, either by use of authentic or chimeric genes. Since the chimeric *map2/MF α 1* gene encoded both *S. pombe* P-factor and *S. cerevisiae* α -factor, different populations of actor cells could be controlled by its expression in *S. pombe* host cells [68, 69]. This might provide an option to include nodes and branching points into synthetic consortia wired by cell-cell communication.

Furthermore, heterologous pheromone receptors have been functionally expressed in *S. cerevisiae*, although efficient coupling to the pheromone response signaling cascade occasionally

Table 1. Artificial intra-species communication

Sender/receiver	Sender module	Receptor	Reference
<i>S. cerevisiae</i>	<i>S. cerevisiae</i> MF α 1 (α -factor pheromone)	<i>S. cerevisiae</i> Ste2p (pheromone receptor)	[61–63, 73, 76–78]
<i>S. cerevisiae</i>	<i>S. commune</i> Bbp2(4) (a-like pheromone)	<i>S. commune</i> Bbr1 (pheromone receptor)	[79, 81]
<i>S. cerevisiae</i>	<i>S. commune</i> Bbp1(1) (a-like pheromone)	<i>S. commune</i> Bbr2 (pheromone receptor)	[81]
<i>S. cerevisiae</i>	<i>S. macrospora</i> ppg1 (α -like pheromone)	<i>S. macrospora</i> PRE2 (pheromone receptor)	[79, 80]
<i>S. cerevisiae</i>	<i>C. cinereus</i> phb2.2 ⁴² (a-like pheromone)	<i>C. cinereus</i> rcb2 ⁶ (pheromone receptor)	[82]
<i>S. cerevisiae</i>	<i>C. albicans</i> MF α 1 (α -factor pheromone)	<i>C. albicans</i> Ste2p (pheromone receptor)	[77, 78]
<i>S. cerevisiae</i>	<i>Arabidopsis thaliana</i> AtIPT4 (isopentenyl-adenine synthesis)	<i>Arabidopsis thaliana</i> AtCRE1 (isopentenyl-adenine receptor)	[74]
<i>S. cerevisiae</i>	<i>Agrobacterium tumefaciens</i> iaaH (auxin synthesis)	synthetic ADCTF (auxin-degradable CRISPR transcription factor)	[87]
<i>S. pombe</i>	<i>S. pombe</i> mfm1-V5H	<i>S. pombe</i> Map3-F204G	[131]
	<i>S. pombe</i> mfm1-V5G	<i>S. pombe</i> Map3-F214H	
	<i>S. pombe</i> mfm1-V5H (M-factor variants)	<i>S. pombe</i> Map3-F214H (pheromone receptor variants)	

Table 2. Artificial inter-species communication

Sender	Receiver	Sender module	Receptor	Reference
<i>S. cerevisiae</i>	<i>S. pombe</i>	<i>S. pombe</i> map2 chimeric MF α 1/map2 (P-factor pheromone)	<i>S. pombe</i> Mam2 (pheromone receptor)	[68, 69]
<i>S. pombe</i>	<i>S. cerevisiae</i>	<i>S. cerevisiae</i> MF α 1 chimeric map2/MF α 1 (α -factor pheromone)	<i>S. cerevisiae</i> Ste2p (pheromone receptor)	[68, 69]
<i>S. macrospora</i>	<i>S. cerevisiae</i>	<i>S. macrospora</i> ppg1 (α -like pheromone)	<i>S. macrospora</i> PRE2 (pheromone receptor)	[80]
<i>S. commune</i>	<i>S. cerevisiae</i>	<i>S. commune</i> Bap2(1) (a-like pheromone)	<i>S. commune</i> bar1 chimeric bar1y (pheromone receptor)	[85]
<i>L. kluyveri</i>	<i>S. cerevisiae</i>	<i>L. kluyveri</i> MF α (α -factor pheromone)	<i>L. kluyveri</i> Ste2p (pheromone receptor)	[86]

required the generation of chimeric pheromone receptors [84, 85] or heterologous G α subunits [82] (see also Section 5). Pheromone receptors from numerous asco- and basidiomycetes were functionally expressed in *S. cerevisiae*, including *Lachancea kluyveri* (*L. kluyveri*) [86], *C. albicans* [77, 78], *S. macrospora* [79, 80], *S. commune* [79, 81, 85], *C. cinereus* [82] as well as *Penicillium chrysogenum* [58], and induction of pheromone signaling was demonstrated by the cellular response to authentic or synthetic pheromones (Tables 1 and 2). Likewise, authentic and chimeric pheromone receptors from *Candida tropicalis*, *Candida parapsilosis* and *Lodderomyces elongisporus* were successfully expressed in *C. albicans* to study ligand binding and inter-species crosstalk [84]. However, it should be noted that most of the inter-species communication modes established in these approaches (Table 2) were tested upon physical separation of both species, i.e. media of densely grown sender cells were used to trigger a pheromone response in the receiver cells (temporal separation) [68, 69, 80, 85], or cells were streaked next to each other on agar plates (spatial separation) [86]. Thus, the functionality of these modules remains to be demonstrated in true co-cultures and cellular consortia of communicating species.

As pointed out above, systems integrating multiple different species into a single device would provide the opportunity to exploit the diverse metabolic capacities of different species, and wiring them by pheromone-based cell-cell communication would allow for the implementation of sensor-actor systems that fulfill increasingly complex tasks. Additionally, purely synthetic communication modules – based on plant signaling components

such as isopentenyladenine [74] or auxin [87] – have been utilized for artificial cell-cell communication in *S. cerevisiae* (Table 1), representing fully orthogonal communication modes that might also be transferrable to alternative host cells to yield inter-species communication. Furthermore, several fungal species such as *S. cerevisiae* and *C. albicans* are known to utilize quorum sensing systems, based on farnesol or aromatic alcohols, to regulate a set of metabolic traits as well as filament and biofilm formation [88]. If cognate receptors and signaling pathways can be unraveled, these quorum sensing molecules could also be exploited, in addition to pheromone-based communication systems, to create control elements in consortium-based approaches.

5 Heterologous expression of GPCRs in yeast

Controlling differentially functionalized cell types by artificial pheromone signaling crucially depends on the capacity of the engineered cell to respond to the diffusible signal molecules in an appropriate manner. Designing and implementing sensor-actor systems of growing complexity will certainly be based not only on authentic fungal pheromones, but require the use of additional communication modes. Both signal perception and coupling to the intracellular signaling components remain challenging. In this regard, expression of heterologous GPCRs might be of high relevance.

As outlined above, a number of fungal pheromone receptors has been functionally expressed and coupled to the MAPK signaling cascade in *S. cerevisiae* and *C. albicans*. Forwarding the signal (ligand perception by the heterologous GPCR) to downstream signaling components (MAPK cascade) allows for expressing target genes placed downstream of pheromone-inducible promoter elements in a ligand-dependent manner (Fig. 2C). Even mammalian homologues can be functionally expressed in yeast cells, providing an opportunity to engineer cellular responses to novel signaling molecules. Since the yeast mating pheromone response pathway shares many similarities with mammalian GPCR signaling systems, yeast also became a valuable model organism to investigate the signal transduction mechanisms of GPCRs [89]. The pheromone-induced MAPK cascade of haploid yeast cells is activated by only one specific receptor (α -factor receptor or α -factor receptor, respectively), which can be replaced by a heterologous GPCR, thus excluding primary activation of the pathway by the endogenous receptors [90]. This would not only be of interest for uncovering GPCR function and ligand specificity (since GPCRs comprise the largest group of drug targets in mammals [91, 92]). It may also serve to create orthogonal communication channels in sensor-actor systems. For example, the human somatostatin receptor SSTR5 has been functionally expressed in yeast [93], and somatostatin synthesis and secretion in *S. cerevisiae* have also been reported [94]. Hence, it is conceivable to combine these two modules into novel, fully orthogonal communication channels for directed cell-cell communication. Similarly, one can envisage to express a common GPCR in several subpopulations of microbial consortia while coupling the respective signaling cascades to different output genes, implementing a system in which multiple cell types of the community can respond differently to a single one input signal, providing a predefined response at the individual (single cells) as well as at the global (consortium) scale.

Using yeast-based whole-cell assays, GPCRs were pharmacologically characterized and a plethora of ligands could be identified. However, some modifications of the yeast MAPK signaling cascade and regulatory components proved to be helpful or even essential [89, 90, 95]. In most cases, this focused on the modification of the $G\alpha$ subunit to ensure fruitful interaction with both the heterologous GPCR and the $G\beta\gamma$ dimer [90, 96–99]. So-called “transplants” (five C-terminal amino acids of the yeast $G\alpha$ subunit substituted by the corresponding stretch of mammalian homologs) emerged as the most efficient variants so far (Fig. 2C) [100]. Likewise, co-expression of accessory proteins might improve ligand affinity and specificity [101, 102].

6 Application potential in biotechnology

The use of artificial cell-cell communication modules might be of particularly high relevance in biotechnology applications, serving as advanced consortium control elements to wire specialized strains and species in fermentation approaches. Recently, a novel approach for lignocellulose degradation by engineered *S. cerevisiae* consortia was demonstrated. One strain displayed a scaffold protein at its surface, while each of the three additional strains secreted one type of cellulose-hydrolyzing enzymes that attached to the scaffold protein *via* specific anchor domains,

thereby forming a mini-cellulosome [19, 20]. The consortium approach allowed for individual strain optimization and division of labor, significantly reducing the metabolic burden of cellulase expression by individual cells. Optimum consortium performance required a well-defined ratio of the four yeast strains to be adjusted and maintained, which might critically limit its long-term stability. In a follow-up study, the authors developed yeast strains displaying even more sophisticated cellulosomes (based on anchor and adaptor scaffolds), but assembly at the yeast surface had to be carried out *ex vivo* since integration of all yeast strains into a single consortium would have been an extremely challenging task in terms of community coordination and maintenance [103]. We propose the utilization of sensor-actor systems relying on cellular communication and metabolic interdependence to engineer beneficial interactions or feedback-controlled circuits, thus ensuring stable community composition and target behavior.

In the process of wine making, several non-*Saccharomyces* yeasts are known to significantly contribute to the desirable product properties, whereas *S. cerevisiae* is required to complete the fermentation [104]. It remains challenging to control this process, since non-*Saccharomyces* yeasts undergo early cell death in mixed fermentations with *S. cerevisiae*. Numerous studies revealed the existence of contact-based mechanisms as well as peptides secreted by *S. cerevisiae* to trigger cell death in competing yeasts [105–108]. We propose that pheromone-based communication might delay the death of non-*Saccharomyces* strains in grape must fermentations for wine making, allowing for the accumulation of desirable compounds while maintaining the dominance of *S. cerevisiae* in later stages of the fermentation [104].

Likewise, some fungi secrete small molecules to combat potentially competing species. *C. albicans* is known to secrete farnesol as a quorum sensing molecule, which was reported to induce cell death in several yeasts and fungi, thereby favoring survival and spread of *C. albicans* [109–113]. Introducing detoxification mechanisms (*e.g. via* antioxidants) in pheromone-responding strains might provide an opportunity to protect relevant subpopulations in microbial consortia, overriding the authentic cell death mechanism to create a higher-level control element.

Pheromone-responsive target gene expression induced by pheromones that are secreted by the cells themselves [65] or by another consortium partner [61, 62, 68, 69] also has some advantages over commonly used promoter/inductor systems in yeasts. Promoter elements responding to the presence of carbohydrates such as maltose or galactose [114] interfere with glucose consumption and are not suitable for fermentation strategies based on complex carbon sources such as molasses. Induction *via* pheromone signaling is independent of the utilized carbon sources, their concentrations and catabolite repression effects. Additionally, the use of toxic compounds like methanol (commonly employed to induce the *AOX1* promoter in *Pichia pastoris* [115]) or other expensive agents (*e.g.* doxycycline for Tet-based systems in *S. cerevisiae* [114]) can be avoided.

Recent efforts in biotechnology also aim at microbial biofilms, owing to their superior stability, and the cellular immobilization might allow for biofilm reuse. Brethauer *et al.* (2014) developed a membrane-aerated bioreactor to convert lignocellulosic

feedstocks into bioethanol by the combined metabolic capacities of three fungal species [18]. The obligate aerobe *T. reesei* formed a biofilm directly on top of the oxygen permeable membrane, secreting cellulases and hemicellulases as a first step towards lignocellulose breakdown while depleting the oxygen to provide an anaerobic milieu in the bulk reactor. The yeast species *S. cerevisiae* and *Scheffersomyces stipites* formed a second biofilm on top of the *T. reesei* layer, converting the released hexoses and pentoses into bioethanol in oxygen-depleted conditions [18]. While the immobilization of multiple strains and species might overcome stability issues associated with microbial consortia, cell-cell communication could be engineered to control and fine-tune the activity and behavior of individual subpopulations in this design. Within the confined space of microbial biofilms, cellular communication might be even more promising, given the short distances between sender and receiver cells and the locally high signal molecule concentrations that can be anticipated. Likewise, communicating cells could be embedded into hydrogel matrices, thereby creating artificial biofilm-like structures. We previously demonstrated that small peptide pheromones can freely diffuse within hydrogel matrices to allow for cell-cell communication between sender and actor cells immobilized in adjacent compartments [63]. Immobilizing the cells in structured hydrogels might serve to maintain stable community composition and prevent competition for essential nutrients, while synthetic cell-cell communication could be utilized to create the regulatory circuits that determine cellular behavior in the individual hydrogel layers/compartments.

7 Future challenges and guidelines

The applicability of pheromone-based cell-cell communication systems might be challenged by a number of potential pitfalls arising from inherent traits of the mating systems of the fungal species involved. However, most of these issues could be overcome *via* genetic engineering, thereby broadening the application potential of communicating consortia. For example, unlike *S. cerevisiae*, numerous yeasts and fungi do not undergo sexual differentiation in rich media, and some species even require highly specific conditions regarding the growth medium and supplements, incubation temperature, illumination etc. to develop sexual structures [33, 46]. In particular, sexual differentiation in *S. pombe* [33, 37] and *Neurospora crassa* [116, 117] is crucially dependent on nitrogen starvation, whereas sexual development of *T. reesei* is triggered by light [39]. Some yeasts, such as *C. albicans* and *Candida tropicalis*, even require a phenotypic switch from the white to the opaque state for sexual differentiation, since only opaque cells are competent for mating [118–120]. This might pose severe hurdles for the integration of these organisms into pheromone-based communication systems, given that they are generally unresponsive to their mating pheromones under standard fermentation conditions. Unraveling the molecular basis of these environmental prerequisites might pave the way to circumvent their requirement by genetic means. In *S. pombe*, nitrogen starvation results in reduction of the intracellular concentration of cyclic adenosine monophosphate which can be mimicked by deletion of the gene encoding adenylate cyclase. This leads to the uncoupling of sexual differentiation (*i.e.*

expression of pheromones and receptors) and nitrogen availability, rendering these cells responsive to the mating pheromones even in rich media [121]. Likewise, deletion of the gene encoding the ENVOY regulator overcomes the light requirements for sexual development in *T. reesei* [44], and ectopic expression of *WOR1*, the master regulator for white-opaque switching in *C. albicans*, forces these cells to stay in the mating competent opaque state, preventing the phenotypic switch to the mating incompetent white state by environmental cues [122]. These examples illustrate that more intensive in-depth research on sexual processes in industrially relevant fungi species might be a key to facilitate their integration into communication-controlled consortia.

Pheromone response is typically accompanied by a sustained growth arrest for synchronizing the cell cycle of both putative mating partners prior to karyogamy. Pheromone-induced cell cycle arrest has been studied in most detail in *S. cerevisiae* [33, 36, 37], but was also observed in *S. pombe* [123] and *Fusarium oxysporum* [124], among others. Growth arrest might be a major obstacle in bioprocess engineering when high cell densities and/or synchronized growth behavior are intended. The core components responsible for pheromone-induced growth arrest have been identified, and the cell cycle can now be uncoupled from the pheromone response in respective deletion mutants such as $\Delta far1$ in *S. cerevisiae* [125] and $\Delta rum1$ in *S. pombe* [126]. However, a recent study highlighted the potential of the pheromone-induced cell cycle arrest as a metabolically active phase for the production of valuable compounds [127]. Due to the cell cycle arrest, carbon utilization was directed towards product synthesis (para-hydroxybenzoic acid) instead of biomass accumulation, significantly increasing the glucose-specific product yields [127]. In addition, pheromone-driven cell cycle arrest might represent a suitable control element for maintaining community composition, possibly serving to prevent one species from dominating the consortium while others become extinct. Thus, modulating the cell cycle arrest in response to mating pheromones might provide another element for advanced consortium control.

The development of cellular consortia relying on pheromone-based cell-cell communication might be challenged by self-fertile (homothallic) organisms that respond to their own pheromones. These cells overcome the need to find a suitable mating partner (which is mandatory in heterothallic organisms) and undergo sexual development even when grown in isolation [13, 33]. Some fungi with high economic impact are regarded as homothallic, including *Pichia pastoris* (a common host for heterologous expression of proteins) [47] and *P. rhodozyma* [59]. A recent study on the sexual development in *P. rhodozyma* might illustrate how this issue can be circumvented. By creating two triple deletion strains (each lacking a pheromone, a pheromone receptor and a homeodomain transcription factor), it was possible to transform the homothallic *P. rhodozyma* into a heterothallic (obligatory outbreeding) organism with two “artificial mating types” [59]. These strains might be ideally suited for integration into pheromone-based communication modules. However, it should be noted that some homothallic species – such as *Fusarium graminearum* – can undergo sexual development in the absence of functional pheromone and receptor genes, indicating that they do not rely on pheromone-based cell-cell communication

for the formation of sexual structures [51, 52]. In contrast, the homothallic fungi *S. macrospora* and *P. rhodozyma* still require at least one functional pheromone-receptor pair for sexual development [59, 128].

Occasionally, pheromone-based cell-cell communication might suffer from unwanted crosstalk due to non-specific receptor activation by non-cognate pheromones. Crosstalk is also considered as a severe issue in bacterial cell-cell communication modes centered on quorum-sensing molecules, given that channel crosstalk is frequently observed in these circuits [129]. Similarly, pheromone response in *C. albicans* can be triggered by pheromones of related species such as *Candida parapsilosis* and *Lodderomyces elongisporus* [67]. Crosstalk between the pheromone receptors of *C. albicans*, *Candida tropicalis* and *Candida lusitanae* [84] as well as between the *S. cerevisiae* α -factor pheromone and pheromone receptors of *L. kluyveri* [86] and *Fusarium oxysporum* [130] has also been reported. Thus, designing artificial intra- and inter-species communication for use in cellular consortia must include rigorous testing of crosstalk and, if necessary, switch to fully orthogonal communication modes. To this end, plant-derived signaling molecules [74, 87] as well as pheromones from distantly related species might be considered (see Section 4). In addition, directed evolution of pheromones and pheromone receptors might be a valuable approach to limit crosstalk and to open up new communication channels. By individual amino acid substitutions of the *S. pombe* M-factor pheromone and its cognate receptor, Seike *et al.* (2015) recently identified matching combinations of mutant pheromones and receptors that could promote efficient mating but did not allow for crosstalk with their wild-type counterparts [131]. Hence, cells expressing the mutant M-factor could mate exclusively with cells expressing the mutant pheromone receptor and *vice versa*. While providing valuable insights into speciation and reproductive isolation, this study also pointed at a method for establishing novel, fully orthogonal communication modes [131]. Functional studies on fungal pheromones as well as their interactions with cognate receptors have been conducted in various species [67, 84, 124, 132–134], which might facilitate the straightforward design and testing of “artificial pheromones”.

Numerous fungi with high economic potential have poorly characterized sexual cycles and/or are considered to proliferate exclusively by asexual means, potentially impeding their integration into pheromone-based communication devices. However, a recent example illustrates that pheromone-controlled cell-cell communication might be implementable even in the absence of full sexual development. Although a functional sexual cycle has not been observed in the plant pathogen *Fusarium oxysporum* (a promising host for lignocellulosic ethanol production [14]), this fungus encodes a functional pheromone receptor responsive to both mating pheromones and plant-derived signal molecules [130]. Hence, if ligands can be identified and secreted by engineered sender cells in a controlled manner, the presence of functional receptors and downstream signaling compounds might be fully sufficient for interfacing these fungi with pheromone-based communication devices.

8 Outlook

The last years have witnessed the rise of microbial consortia for novel applications, aiming to overcome the limitations posed by single-culture approaches. With the growing complexity of the tasks carried out by consortia, and the trend towards integrating more and more “specialists” into these communities, successful implementation and maintenance call for novel consortium control elements. Artificial cell-cell communication, coupled to the desired output in engineered receiver cells, is a promising strategy to develop autonomous systems that do not require external control or supervision [2–4, 6, 7, 9]. Mating pheromones, a rather ubiquitous communication mode in fungal species, might be of particular relevance for consortia encompassing fungal hosts.

The utilization of nonconventional yeasts and fungi is of great interest in biotechnological applications, taking use of their beneficial traits without the need for transfer into heterologous hosts. More and more (industrially important) fungi are being regarded as sexual species, and the respective functional pheromones and receptors are progressively identified [46]. We assume that further research on these components and the sexual events they trigger might promote the integration of such species into pheromone-controlled sensor-actor systems. In particular, in-depth characterization of pheromone signaling cascades and their downstream elements (such as pheromone-inducible promoters) in various fungal species would facilitate the implementation of pheromone-controlled consortia, paving the way for rational engineering of the desired cellular behavior in response to cellular communication. Similarly, expression of heterologous GPCRs will open up new communication channels in fungal consortia, provided that functional coupling to authentic signaling cascades can be achieved. Most of the studies employing heterologous GPCRs were carried out in *S. cerevisiae* host cells, and further research should focus on the heterologous expression of foreign GPCRs in a wide variety of yeast and fungal hosts to broaden the applicability of these communication channels in cellular consortia. Likewise, quantitative description and molecular modelling of the pheromone secretion profile as well as the pheromone response (including dose-response curves and kinetic analyses) in different fungi will certainly enhance the predictability and straightforward engineering of communicating systems, and rigorous testing for orthogonality and channel cross-talk will be essential to prevent undesired interactions. Ultimately, we envisage that control elements based on engineered cell-cell communication could be implemented into a wide variety of consortium-based approaches, and transferring these communities from laboratory to industrial scales will unlock the full potential of communication-controlled fungal consortia.

This work was funded by a grant to Kai Ostermann (OS 176/5-1) by the German Research Foundation.

The authors have declared no conflict of interest.

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