Chapter 2

Literature review

2.1 The *Aspergillus* genus

2.1.1 Description

Moulds of the genus *Aspergillus* are ubiquitous in our environment and have a multi-faceted relationship with human societies. Some of these fungi can be pathogenic to animals and plants, colonising lung tissues, secreting mycotoxins and suppressing immune response by masking detectable components of their cell wall [1, 2], thus causing e.g. variants of aspergillosis in immunocompromised people. Additionally, the abundance and resilience of *Aspergillus* spores contribute to food and feed spoilage, which is of critical importance for the food industry [3, 4]. On the other hand, the metabolic processes of *Aspergilli* spp., most notably in *A. niger*, *A. oryzae* and *A. tereus* have long been utilised for the industrial production of useful substances for the biochemical and food industry: organic acids such as citric acid, itaconate and galactarate [5], enzymes like oxidases, phytases, glucoamylases [6], chitinases [7] and other secondary metabolites such as malate and lovastatin [5]. Due to their enzymatic production, species of the genus are furthermore used in fermentation processes. For example *A. oryzae* plays a vital role in the production of soy sauce and miso [8]. The use of *A. nidulans* as a model organism in genetic research and cell biology has elucidated phenomena significant for the understanding not only of *Aspergilli* and related filamentous fungi, but of eukariotic organisms as a whole [9].

The signature characteristic of *Aspergillus* fungi is the structure of their conidiophore — the specialised hyphal stalk carrying the asexual spores of the fungus, the conidia. Its resemblance to an asperges, a device used for sprinkling holy water during Catholic liturgies, designates the name of the genus [9]. With about ten thousand spores carried

by a conidiophore [10], an *A. niger* colony can produce more than a billion conidia [11], which are dispersed in the environment with varying efficiency through three primary vectors: air currents, water droplets and animal carriers, in particular the hydrophobic surfaces of insect bodies [10]. Once the conidium settles into a suitable medium, it can germinate.

2.1.2 The role of heterogeneity

Like many other filamentous fungi, *Aspergilli* have acquired their ecological niche by evolving various survival mechanisms that increase their stress resistance and chance of propagation. These are generally rooted in a significant heterogeneity expressed at multiple stages of their development. As a most trivial example, differences can be observed in the sizes of conidia — $5.3 \pm 0.6\,\mu m$ for *A. niger* [12], from 2.4 to 2.7 µm for *A. nidulans* [13], from 2 to 3.5 µm for *A. fumigatus* and from 3 to 6 µm for *A. flavus* [14].

As the fungus grows into a more complex morphology, a rich variety in form and function is revealed. A functional diversity in spatially differentiated zones of the vegetative mycelium has been confirmed by gene expression analysis [15–17] and attributed to epigenetic adaptations [18]. This is accompanied by a morphological heterogeneity in the sizes and shapes of mycelial microcolonies, ranging from dispersed mycelia to loose clumps and structured pellets [12, 19]. The causes for these differently self-organised structures can be traced back to the density and the composition of the spore inoculum, with lower spore densities leading to ~~more structured~~, compact hyphal pellets and higher densities resulting in dispersed mycelia [12, 20]. A primary mechanism in this heterogeneous morphological development is the aggregation of spores and hyphae through coagulative interactions [12, 19, 21], accompanied by an asynchronicity in the timing of the conidial germination [22], partly explained by the varying degrees of maturation of the spores as they disperse from the conidiophores [10]. At the same time, the germination percentage of the spore ensemble varies depending on environmental stimuli, with different germination rates exhibited upon changing the chemical components of the medium [11, 22]. It is suggested that the emergent regulation of the germination incidence through a heterogeneous environmental response is part of a bet-hedging mechanism, a finely tuned evolutionary strategy that secures a reserve of resistant dormant spores in case of unfavourable environmental changes [22, 23].

All of these adaptation techniques have a direct impact on the biochemical productivity or virulence of *Aspergillus* fungi and can be exploited in their industrial utilisation [12, 19, 20] or for targeted prevention of their harmful effects [24]. As the inoculation stage both allows for external control and largely determines the later growth of the fungus, a

research focussed on the early conidial development can capture essential principles before the physiological variation of the spores causes a greater divergence. Thus, the inherent heterogeneity of the spores can be combined with deterministic models to explain the experimentally observed germination statistics.

2.2 Conidial germination

2.2.1 Germination phases

The transition of *Aspergillus* conidia to vegetative mycelium is characterised by several distinct phases. A dormant spore, as it inoculates the new environment, has a rigid, hydrophobic, multi-layered cell wall [1] which confers the spore its ability to withstand high thermal and oxidative stress, resist osmotic pressure, dehydration, UV light and fluctuations in pH levels [25]. This dormant state can preserve the fungus in unfavourable conditions for over a year [26], before germination marks its transition from a fermentative to a respiratory metabolism and exchanges its high resistance for a more vulnerable but vegetatively active regime [25].

The activation of dormant conidia becomes evident through the onset of isotropic growth (the swelling phase), during which the diameter of the spore doubles its size, as water is taken up and the cell wall engages in a restructuring procedure. Compatible solutes like trehalose and mannitol, vital for the long-term resistance of dormant conidia, become degraded, along with the dense protective outer layer of the cell wall, consisting of rodlet proteins [25]. This prominent structural change in the conidium has been observed between 2 and 6 hours from inoculation in *A. niger* conidia [12, 27]. After the isotropic swelling phase, a polarity axis is established through the deposition of cortical markers, leading to the targeted transport of vesicles and key proteins and resulting in the protrusion of a germ tube [25]. Thus, the fungus enters its polarised growth phase.

2.2.2 Factors for germination

While the metabolic pathways leading to the break of conidial dormancy have yet to be mapped in satisfactory detail, it is known that specific sensing mechanisms enable conidia to adjust their germination response to the presence of nutrients in the medium. The specific combination of carbon, nitrogen and inorganic salts has been observed to elicit different responses in terms of germination percentage [11]. Glucose and amino acids are typical nutrition components consumed by filamentous fungi in their natural environments as part of their saprotrophic lifestyle. As different mould species occupy distinct

environmental niches, their susceptibility to specific amino acids varies, with alanine and proline being the highest-inducing carbon source shared among most *Aspergillus* species [22], leading to a nearly complete germination of the inoculum.

The mechanism through which carbon signals trigger the break of dormancy has only been partly understood so far. One carbon source sensor identified in conidia is the heterotrimeric G-protein signalling system, which activates the cAMP-PKA pathway in response to glucose and sets early processes of germination in motion [25, 28, 29]. Other components of environmental sensing include Ras proteins [30] and the Calcineurin pathway [25]. The functioning and coordination of these pathways is a topic of ongoing research, but current knowledge outlines a complex network of dependencies.

It has been found that the germination of a spore is not merely a consequence of an activating trigger, but rather an interplay between environmental stimuli and inhibitory signals. The mutual germination blocking of conidia from different or identical species becomes evident when observing how the inoculum density affects the percentage of germinated spores. In prior experiments with *A. niger* conidia, an increase of the spore count from 5000 to 40000 per 150 μL suspension has led to a decrease of the germination incidence of about 20% in media using alanine, proline and arginine as carbon sources [3]. In general, a reduction of the germination percentage has been documented at conidial densities higher than 10^5 spores/mL [31, 32], the consequences of which reflect in the subsequent morphological development and productivity of the mycelium [20]. The decrease in germination occurrences cannot be explained by a competition for nutrients, since the carbon sources primarily have a signalling role and are not consumed during the dormant conidium phase [3]. While there may exist other germination-determining scarcities at crowded inoculi, e.g. of O_2 and CO_2 [33], a likely factor is the increased concentration of an auto-inhibitor produced by the spores themselves.

2.3 Germination inhibitor

2.3.1 Volatile organic compounds

Volatile organic compounds (VOCs) are a broad class of small, heat-stable molecules known to enable communication between organisms and across kingdoms, e.g. as means of attraction, warning or repulsion [34, 35]. Their low molecular weight (50-300 Da) and high vapour pressure at ambient conditions make them highly diffusible, conferring their potential to reach molecular receptors at considerable distances within a short time. If a spore times its own germination through the slow release of a such a self-inhibitory compound, then it would also inhibit the germination of nearby spores at high

densities, producing a so-called "crowding effect". It has been proposed that such a mechanism prevents premature germination within the conidiophores, where spores are packed densely, delaying the break of dormancy until dispersal [36].

VOCs, in particular 8-carbon (C-8) oxylipins, have been found to accumulate in the headspace of *A. oryzae* [37] and *A. flavus* [38]. These include 1,3-octadiene, 1,5-octadien-3-ol, 1-octen-3-ol, 3-octanone, octanal, 2-octenal, 1-octanol; and 2,4-octadienal. Among these, 1-octen-3-ol has proven to have the strongest inhibitory effect on germination in *A. nidulans*, followed by 3-octanone and 3-octanol [31]. These are also the three primary compounds identified in solvent extractions from the medium of the fungus. In *A. oryzae*, the dominant headspace VOCs are 1-octen-3-ol, 2-octenal and 3-octanone [38]. Using a freeze-thaw cycle to destroy the integrity of the cell membrane, a substantial release of 1-octen-3-ol has been observed in *A. flavus* conidia as well, followed by 3-octanone, 2-octen-1-ol and 1-octen-3-one [39].

2.3.2 1-octen-3-ol

1-octen-3-ol is a secondary alcohol and a flavour compound in mushrooms. Along with 3-octanone and 3-octanol, it is produced by the oxygenation and breakdown of linoleic acid [31]. 1-octen-3-ol was first recognised as a primary germination auto-inhibitor in *Penicillum paneum*, a mould related to *Aspergilli* via the order *Eurotiales* [36]. When exposed to exogenous concentrations of 1-octen-3-ol (4 mM), dormant *P. paneum* conidia do not swell and swollen conidia do not germinate [36]. Evidently, the auto-inhibitor mildly increases the cell membrane permeability and transiently lowers the internal pH level of the conidia, which influences respiration and protein synthesis [40, 41]. At an inoculum density of 10^6 spores/mL, the germination rate for 1, 2.5, 4 and 5 mM added 1-octen-3-ol is measured at $83 \pm 8.1\%$, $35 \pm 6.3\%$, $13 \pm 2.1\%$ and $1 \pm 0.6\%$, respectively, against a no-inhibitor control experiment with $88\% \pm 5.3\%$ germination success.

Experiments with *A. nidulans* have shown that dormant conidia exposed to 4 mM also do not reach a swelling phase at the expected 4-hour mark and remain in their passive state until the end of the 16-hour experiment [31]. At an inoculum density of 10^5 spores/mL, added 1-octen-3-ol concentrations of 1, 2.5, 4 and 5 mM yield $87 \pm 6.1\%$, $36 \pm 4.3\%$, $14 \pm 2.1\%$ and $1 \pm 0.3\%$ germinated spores, respectively, against a no-inhibitor control experiment with $95\% \pm 5.3\%$ germination incidence. These results are quantitatively similar to the outcome of the experiments with *P. paneum*, suggesting that analogical mechanisms may be at play during the inhibition of *Aspergillus* spores.

The effects of 1-octen-3-ol on filamentous fungi are known to be reversible [31, 38, 40]. Thus, conidia blocked from germination can resume their break of dormancy once the

ambient inhibitor concentration is lowered, e.g. when the spore is transferred to a less crowded environment.

2.3.3 Open questions

Although 1-octen-3-ol is recognised as an appropriate candidate for auto-inhibition in *Aspergillus* conidia, there is inconclusive evidence that it is as a sole primary endogenous factor in this process for all *Aspergillus* species. For instance, *A. niger* spores washed of superficial compounds also exhibit reduced germination under crowding [3]. A possible explanation for this is that the VOC is constantly produced or very tightly bound to the conidium [40]. Furthermore, experiments with *A. flavus* conidia have indicated that the threshold concentration for inhibiting germination is not less than 10 mM, while the quantity of 1-octen-3-ol released by spores, even after freeze-dry forcing, is below 1 μM [39]. A concerted interaction with another potential inhibitor may paint a more complete picture. This could be one or several of the less prominent VOCs found in the headspace (such as 3-octanone). Alternatively, some studies point at the role of a heat-labile peptide, indicated by the loss of auto-inhibition after a heat-treatment of the conidia [39]. However, a thermal instability would imply a considerable molecular size, which has implications on the diffusion rate and permeability through the cell wall of the dormant conidium.

A lightweight, volatile and thermodynamically stable molecule such as 1-octen-3-ol would clearly have radically different dynamics compared to a protein large enough to denaturate or break down under heat. This not only has implications for the diffusion of the inhibitor through the surrounding medium, but also affects its permeation through the layers of the cell wall. Therefore, to estimate the inhibitor release timing, the properties of the conidial envelope need to be carefully studied.

2.4 The conidial cell wall

2.4.1 Function and dimensions

The multi-layered, rigid cell wall of an *Aspergillus* conidium ensures its long-term viability, protects it against various stresses, enables its adhesion to surfaces, including those of other spores, masks it from immune response and forms a substantial screening barrier for signalling molecules such as nutrients or inhibitors [42]. While an exhaustive comparison of the spore wall thickness and consistency in different *Aspergillus* species is difficult to obtain, documented measurements hint on a significant variance within and

across species, strains and stages of development. For instance, transmission electron microscope (TEM) studies of *A. fumigatus* indicate a cell wall thickness of about 25 nm in 0.5-day old spores and 50 nm in 1-day old spores [43]. Meanwhile, coherent anti-Stokes Raman spectroscopy (CARS) of dormant *A. nidulans* conidia outlines a thickness range of 273-490 nm [44]. Apart from the likely physiological difference between the species, an underestimation of the true thickness by the TEM procedure is possible due to the dehydration of the samples, while the large variance in the CARS procedure can be traced to the indirect inference of the cell wall extent by employing spectral variations. A consensual assumption would be that the cell wall's thickness is in the order of a hundred nanometers in dormant conidia [45].

2.4.2 The inner cell wall layers

The cell wall structure is characterised by several distinct layers. The innermost zone remains mostly conserved through the vegetative transformations of the conidium and consists of glucans (50-60% of the cell wall dry weight [2]), chitin (7-15% of the cell wall dry weight in *A. fumigatus* [46]) and glycoproteins (20-30% of the cell wall dry weight in filamentous fungi [2]). A common structural molecule, chitin comprises folded molecules, forming a microfibril matrix that glucans and glycoproteins are cross-linked with [42]. Glucans are polysaccharides of different molecular species found in great abundance in the cell wall. A general categorisation distinguishes these as α- and β-glucans. The β-(1,3)-glucan forms long molecules that extend throughout the cell wall and covalently bind to other components. It thus forms a rigid structural network that provides mechanical strength and a scaffold for galactomannan, melanin and other compounds [1, 42, 47]. Being a characteristic molecule in fungi, β-(1,3)-glucan is recognised by receptors such as Dectin-1 in humans, necessitating the evolutionary adaptation of some fungi to mask it via α-(1,3)-glucans [42, 48] — molecules constituting about 19% of the cell wall polysaccharides in *A. fumigatus* [48]. The biosynthesis, organisation and maintenance of the polysaccharides and other connected structures occurs to a great extent within the cell wall itself, enabled by transglycosidases and yapsin orthologs [42, 49]. These are an example of the various GPI-anchored proteins tethered to the cell membrane, which along with the glycoproteins freely embedded in the cell wall matrix serve re-structuring, environmental sensing and transportation functions.

2.4.3 The outer cell wall layers

The outer layer of the cell wall is the region that undergoes the most significant transformation during the break of dormancy and the consequent onset of hyphal growth. One

of its major components during the dormant conidial stage is the mechanically resistant and highly insoluble hydrophobin layer, a tightly packed structure of RodA proteins that group in parallel fascicles to form long amyloid-like structures of about 10 nm thickness [50, 51]. The amphipathic behaviour of Class-I hydrophobin molecules enables them to self-assemble at hydrophilic-hydrophobic interfaces [52] and form bilayers when in isolation [51]. The resulting rigid hydrophobic barrier between the spore and its environment ensures its long-lasting protection against stresses and immune system response [50, 52], while selectively permitting molecular transport through its structure. As the outermost component of the cell wall, hydrophobins also contribute to the adhesion of spores and thus to their agglomeration and virulence [42, 53].

Interacting with the hydrophobin layer and cross-linking with the inner regions of the cell wall is a layer of dihydroxynaphthalene (DHN) melanin, a negatively charged and hydrophobic pigment that plays an integral role in the structural assembly of the conidial wall layers [54] and confers the spore its resilience against UV radiation, heat stress and toxins, further enhancing its viability [2, 55].

While being highly insoluble and chemically resistant to outside influences, the outer layer undergoes a programmed degradation by aspartic proteases upon the break of conidial dormancy [25, 56], removing the rigid rodlet structure to give way to the vegetative expansion of the spore. Thus, the the protective coating of the spore has its most substantial barrier function in the early period prior to germination.

2.5 Conidial aggregation

The outer cell wall layer of *Aspergillus* conidia has adhesive properties, enabling e.g. spores of *A. fumigatus* to attach to epithelial host cells [57]. Furthermore, electrostatic and salt bridging between spore surface polysaccharides and hydrophobic interactions of surface proteins give rise to spore agglomeration and a subsequent coagulative pellet formation in *A. niger* [12]. This process appears correlated with the onset of conidial swelling in germination-inducing media [58]. The conidial aggregates are heterogeneous in size, possibly as a way to diversify survival strategies [19]. An inquiry into the distributions of spore counts within aggregated clusters yields varying results depending on the inoculum density, strain used, method of cultivation and measurement technique [12, 21, 59]. Nonetheless, the observations show that spore agglomeration consistently occurs within the first hour from inoculation, followed by a slight breakage of the clusters, possibly from the stress induced by swelling spores and growing germ tubes [12].

2.6 Diffusion

2.6.1 The diffusion constant

Diffusion is a process of fundamental importance in nature, arising from the spatially and temporally uncorrelated Brownian motion of particles driven by thermal kicks [60]. Fick's laws of diffusion [61] lay the physical framework for the statistical description of this phenomenon and give rise to mathematical relationships fundamental to scientific modelling. According to these, the flux of particles follows a direction from a higher to a lower concentration, during which their total mass in the system is conserved [60]. The mathematical formulation of these principles can be used to quantify the relationship between time and the mean squared displacement of a diffusing molecule, expressed through the diffusion constant D, measured in units of area per time. This measure can be obtained experimentally or inferred through other characteristic properties of the diffusing substance and its surrounding medium.

2.6.2 The permeation constant

The permeation of molecules through a barrier is closely related to diffusion and is a highly relevant phenomenon in biological systems, where interfaces like membranes define distinct spatial compartments. Most generally, semi-permeable barriers represent heterogeneities in the medium of diffusion, where the diffusion constant is altered locally due to a less accessible region [62]. The relationship between the diffusion constant of the particle-barrier system and the specific geometry and interface type of the barrier is captured by the permeation constant P_s, measured in units of speed [60]. This relationship includes different parameters depending on the choice of barrier model.

In the pore-flow model [63], permeation can be viewed as one-dimensional migration of particles along capillary channels of the barrier due to molecular interactions [60]. This approximation is representative of porous barriers, the passage through which is limited by the size and tortuosity of its permeable cavities. In this case, P_s is dependent on the fraction of the surface area occupied by the pores [60, 64].

If the cavities of the barrier are within the molecular scale, as is the case of biological membranes, the diffusing substance can still permeate through its hydrophobic-hydrophilic interaction at the barrier interface [65]. In this so-called solution-diffusion model, the diffusing substance is assumed to partition into the barrier as a solute [63]. Thus, the permeation constant is dependent on the partition coefficient for the given

system: an equilibrium constant denoting the stable-state ratio of the compound concentrations in a mixture of the barrier and non-barrier medium. Most commonly, this is the octanol-water partition coefficient K_p, applicable for lipid membranes in an aqueous environment [66], but also recognised as useful in approximating other types of membranes [67]. A further extension of this theory differentiates the permeabilities of the different structural layers of the lipid membrane [68].

2.6.3 Estimating the diffusion and permeation constants

The quantification of diffusion and permeability is essential in studying how a molecular signal diffuses through the cell wall into and out of the spore. In the case of dormant conidia, this includes the passage through an aqueous medium, the hydrophobin-melanin layer of the cell wall, the inner polysaccharide-rich cell wall and the cell membrane.

While the volatility of 1-octen-3-ol makes it difficult to study its diffusion coefficient experimentally, one can approximate it via the Stokes-Einstein relation,

$$D = \frac{k_B T}{\zeta}, \tag{2.1}$$

using the Stokes radius a of the diffusing particle and the viscosity η of the medium, which collectively determine the friction coefficient $\zeta = 6\pi\eta a$ for spherical approximations of the molecule [60]. Alternatively, available data on molecules of a similar size can be used. Glucose is a widely studied compound with only a slightly larger molecular mass (180.16 g/mol, compared to 128.21 g/mol of 1-octen-3-ol), which diffuses in water with a constant $D = 6 \times 10^{-6}\,\mathrm{cm^2\,s^{-1}}$ [69]. Experiments with various proteins indicate diffusion coefficients similarly in the order of $10^{-7} - 10^{-6}\,\mathrm{cm^2\,s^{-1}}$ [70, 71].

Hydrophobins, of which Class I form stable rodlet structures and Class II aggregate into semi-soluble assemblages [52, 72], are found in various species of fungi, with Class I being characteristic for *Aspergillus* members (RodA in *A. nidulans* and HYP1 in *A. fumigatus*) [72]. In-vitro, SC3 hydrophobins, members of Class I and characteristic for the *Schizophyllum commune* fungi, have been observed to form membranes with very selective permeability, blocking the passage of particles heavier than 200 Da (roughly equivalent to 0.8 nm) [73]. Artificial bilayers from HFBI hydrophobins (members of Class II), commonly found in *Trichoderma reesei*, have exhibited a very low permeability for water [74]. Thus, assuming this permeability is preserved in the outer cell wall complex, both classes can impose a considerable barrier for molecules entering and exiting the spore. This implies that the passive diffusion of large proteins would effectively be blocked. While self-assembled hydrophobin bilayers may obey the solution-diffusion

principle of lipid bilayers, their structural configuration in the outer cell wall may exhibit discontinuities and be more akin to a porous mesh, as some microscopic images suggest [75].

The lack of data on the diffusivity of the separate cell wall components also requires the use of molecular analogues to estimate the range of the cell wall permeability. For example, agarose, commonly used as a growth substrate, can be regarded as a more loosely-structured representative of the polysaccharides. The diffusivity of glucose in an agarose film is very similar to its diffusivity in water, with an estimated $D = 6.38 \times 10^{-6}\,\mathrm{cm^2\,s^{-1}}$ [76]. A much more rigid and structured polysaccharide, cellulose, yields a low diffusivity in comparison: finite-element studies of glucose diffusing in cellulose nanofibril peripheral nerve conduits indicate $D \approx 1.7 \times 10^{-7}\,\mathrm{cm^2\,s^{-1}}$ [77]. A study related to the food industry has obtained a diffusion coefficient of 1-octen-3-ol in deuterium water ($\mathsf{D_2O}$) of roughly $8 \times 10^{-6}\,\mathrm{cm^2\,s^{-1}}$ and in agar gel of 2×10^{-6} to $6 \times 10^{-6}\,\mathrm{cm^2\,s^{-1}}$, depending on the measurement method. Due to its heavier molecule, $\mathsf{D_2O}$ has about 125% the viscosity of normal water [78], which implies that the Stokes-Einstein relation (Equation 2.1) would lead to a diffusion coefficient of 1-octen-3-ol in water of about 2.5×10^{-6} to $7.5 \times 10^{-6}\,\mathrm{cm^2\,s^{-1}}$, a result aligning closely with the diffusivity of glucose in water.

The permeability of lipid bilayers is an extensively studied subject. Water molecules have been found to permeate through cell membranes with a P_s of 3×10^{-10} to $7 \times 10^{-3}\,\mathrm{cm\,s^{-1}}$ [79]. The permeation constant of glucose through a synthetic membrane of L-α-dipalmitoylphosphatidylcholine (DPPC) has been estimated at $P_s \approx 1.4 \times 10^{-10}\,\mathrm{cm\,s^{-1}}$ [80]. Another estimate indicates $P_s = 2.4 \times 10^{-10}\,\mathrm{cm\,s^{-1}}$ for glucose permeating a synthetic lipid bilayer [81]. The same source also notes that natural lipid membranes exhibit a significantly higher permeability for glucose, facilitated by glucose transporters (GLUT1). A similar mechanism, however, has not been described for VOCs.

The complex structure of the conidial cell wall and its transformation during the germination phases make the quantification of an effective cell-wall encompassing permeation coefficient difficult. An analytical approximation can be made by viewing all N cell wall components as layered shell-like barriers and combining their individual permeation constants P_i with the formula [82]

$$\frac{1}{P_{\mathrm{eff}}} = \sum_{i}^{N} \frac{1}{P_i}. \tag{2.2}$$

Alternatively, insights can be gained from experimental studies. Results from fluorescent staining of conidia of filamentous fungi like *A. niger* and *A. nidulans* with fluorescein

isothiocyanate (FITC) dextran indicate that a spore in the process of germination can take up particles of weight 150 kDa (Stokes radius of about 8 nm) [83], a very high upper limit that well encompasses potential heat-labile peptides. However, in their dormant phase, the conidia are heterogeneously stained, indicating that not all FITC-dextran molecules can pass through the cell wall barrier. Since the smallest mentioned molecule of the viability dye in the study is 10 kDa, the maximum permissible molecular size for cell walls of dormant conidia could fall anywhere within the range of 10 to 70 kDa (Stokes radius of 2.3 nm and 6.0 nm, respectively).

Several other factors may obstruct the passive diffusion of molecular signals between spores. First, considering the extracellular medium of conidial clusters, the question arises whether the interiors of conidia become significant excluded volumes for the molecular paths in the medium, especially in tightly packed clusters. The presence of immobile obstacles in the spatial domain of diffusing particles is known to produce anomalous diffusion [84]. While the diffusivity within dormant spore clusters has not been studied extensively, research indicates that the pellet cores formed by such clusters are generally oxygen-depleted [85], suggesting that crowding imposes barriers for diffusion. Returning to the molecular scale, an additional diffusion-limiting factor could be the strong interaction of the permeating particle with the cell wall matrix, leading to temporary bonds that slow down the molecular passage in a non-trivial way.

2.7 Adsorption

While permeation models capture the principles of passive diffusion, they consider at most polar interactions between the permeating molecule and the barrier. However, when passing through the porous polysaccharide matrix, molecules may form covalent bonds and become transiently embedded in the cell wall. It has been shown that ions of heavy metals can be adsorbed by *Aspergillus* fungi [86], in particular through their α-glucans [87]. Such adsorption capacity has also been proven for industrial pollutant VOCs like α-pinene, n-hexane and ethyl acetate [88]. Most notably, a study has quantified the adsorption of 1-octen-3-ol by yeast glucans obtained from the cell wall [89]. A commonly used model in this case is the Langmuir isotherm, based on the binding kinetics of a molecular species to a matrix with a finite adsorption capacity [90]. While there is strong evidence for the interaction of glucans with ions and VOCs, highlighting fungi as promising biosorbents of off odours and pollutants, such behaviour has not been documented with regard to glucose or amino acids, suggesting that adsorption plays a stronger role in the inhibitor release rather than in carbon signalling.

2.8 Modelling

Computational models addressing diffusivity in fungal colonies have mostly focussed on the vegetative mycelium — a phase with macroscopically observable morphological characteristics. Techniques such as microscopic image processing [12, 91] and X-ray microcomputed tomography [92] have been used to capture spatio-temporal data against which hyphal growth models can be tested, elucidating relationships between the topology, morphology and chemical productivity of the mycelia through simulations [92–95]. While hyphal growth dynamics have been studied on a variety of scales [96], only a few models relate these to germination mechanisms. For example, the onset of polarised growth in a swelling spore is captured by a classical lattice-based model [97] that reinforced the understanding of the role of the Spitzenkörper in hyphal tip growth [98].

Attempts at modelling germination have, in contrast, had a stronger focus on statistical descriptions rather than spatial representations. For instance, the germination percentage of spores over time is commonly predicted by sigmoidal functions fitted over experimental data points [99, 100]. The benefit of these models is that they provide descriptive indicators for germination under specific environmental conditions, such as the saturation percentage of germinated spores ($p_{\max}$) and a characteristic germination time (τ_g) in the model by Dantigny et al. [100]. Nonetheless, the models are primarily based on phenomenological observations and are not necessarily derived from first principles.

Contrary to this, the mathematics of diffusion has steady foundations in statistical physics [60, 101] and has been extensively explored in computational biology models as a driving force in morphogenesis [102], growth [103] and chemical signalling [104]. Therefore, highlighting the effects of diffusion in germination inhibition can help discern universal laws and dependencies in an otherwise highly heterogeneous system.

Inhibitory kinetics have long been modelled on a molecular level using modified variants of the Michaelis-Menten equation [105]. The formulas reflect the rate and saturation levels of inhibitors binding to a substrate and can incorporate different reaction scenarios, depending on whether the inhibition is competitive, uncompetitive or non-competitive [106]. While such models have a practical use in the pharmacological industry, the pathways of inhibition in conidial germination are likely more complex and cannot be fully captured by the mass-action principles behind Michaelis-Menten models.

The quantitative modelling of conidial agglomeration, a primary factor in defining obstacles for diffusion in the dormant spore phase, is still in its infancy, with some attempts being made at describing the kinetics of spore aggregation [21]. Meanwhile, inspiration can be drawn from related stochastic phenomena such as Brownian coagulation [107] and disordered sphere packings [108].

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