Genetic and environmental factors determining heterogeneity in preservation stress resistance of Aspergillus niger conidia

Seekles, S.J.

CHAPTER 1

Introduction

Introduction

Food and the preservation of food are a crucial part of human existence and a central part of many human societies. Humans have been preserving food since pre-historic times. The oldest evidence of food preservation dates back to the Middle Pleistocene; archeological data suggests that prehistoric humans saved the bones of animals inside a cave (Israel) for delayed consumption of the bone marrow and grease safely stored inside [1]. Food preservation is necessary to protect food from microbial food spoilage, thereby securing food products for human consumption. Although food preservation has become more sophisticated since pre-historic times, still around 25% of the global food supply is lost, post-harvest, due to microbial spoilage [2]. A significant portion of this post-harvest food spoilage is due to filamentous fungi, commonly referred to as “moulds” [3,4]. Most isolated food spoilage moulds are part of the phylum Ascomycota and include several species belong to the genus *Penicillium*, *Paecilomyces* and *Aspergillus* [5]. In general preservation strategies are aimed at preventing the outgrowth of fungal spores. Both sexual (ascospores) and asexual (conidiospores) spores from these fungi are relevant for spoilage (see a recent review on food spoilage by heat-resistant molds [6]). In this thesis we focus on the asexual spores (conidia) of these food spoiling fungi.

Filamentous fungi produce asexually spores (conidia) that are everywhere

Filamentous fungi produce conidia as a means of reproduction. Conidia have general characteristics that are shared among all species, six of these characteristics are listed here. First, conidia are dormant structures, meaning they are thought to be metabolically inactive, although this has been topic of debate [7]. Second, the conidia are the offspring of the fungus, meant to germinate and thrive after arriving at a place where the proper conditions are met [8]. Third, conidia are per definition the asexual (clonal) offspring of the fungus, meaning that they are genetically identical to the parent. Fourth, to increase the chance of successful reproduction, large quantities of these asexual spores are spread into the environment. Conidia tend to be easily dispersed in these large quanti-

ties through water or wind [8]. Fifth, conidia, meant for reproduction and long-distance travel through water and air, are heavily protected cells. They are meant to survive harsh conditions (drought, heat) and long periods of time before the proper conditions are met that support germination [9]. Sixth, the large airborne population of conidia is reported to be heterogenous with regards to their resistance against environmental stressors. Especially these last two characteristics make conidia well suited as reproductive structures for survival in nature, an therefore challenging structures to inactivate by commonly used preservation techniques.

Modern techniques of food preservation

In modern food preservation, various techniques are used to protect foods against spoilage by fungal spores. The most commonly used food preservation methods belong to one of three groups [10]. First, inactivation methods (for example inactivation by heating or applying radiation) are meant to kill the fungal spore and thereby protecting the food from spoilage. Second, growth inhibition methods (for example inhibition by adding preservatives or storing foods in cold environments) are meant to delay or prevent outgrowth of the fungus. Third, prevention methods (for example prevention by packaging) are meant to prevent recontamination after inactivation methods have been applied. In practice, these three methods are often combined where food is treated, packaged and subsequently stored in a way that inhibits fungal growth [11]. Studies have shown that 10 – 1000 conidia from genus *Aspergillus* are present in every cubic meter of air [12] and that these amounts vary with the season [13–15]. This makes preventing contamination nearly impossible. Even the air inside food factories contains many conidia that can potentially contaminate the food product throughout the production line. There are, however, ways to mitigate this challenge. Air filters are often installed inside clean rooms being part of these production lines [16]. Also, inactivation of contaminants present on the food product is commonly followed by instant packaging, thereby preventing contact with new potential contaminators from the air [17]. In this thesis, I will focus on the conidia of the

food spoiler *Aspergillus niger* and I will specifically explore the mechanisms that make these conidia resilient against commonly used preservation techniques such as UV-C inactivation, heat inactivation and inhibition by weak acid preservatives.

The life cycle of *Aspergillus niger*

Most filamentous fungi belonging the Ascomycota are heterothallic, meaning that a haploid strain has a single mating type, commonly addressed as mating type MAT1-1 or MAT1-2 [18]. It requires fusion of two haploid strains of opposite mating type to form a diploid, which in turn, after going through meiosis, is able to form ascospores to complete the sexual cycle [19,20]. However, a sexual cycle of *A. niger* has not been observed yet and this organism is thought to be a truly asexual species, thereby only reproducing through conidia. Besides asexual and sexual reproduction, a third means of 'reproduction' has been explored under laboratory conditions in this fungus: the para-sexual cycle [21]. In this life cycle, the offspring is not a perfect genetical clone, as in the asexual cycle, but is also not the result of meiosis as seen in the sexual cycle. In the parasexual cycle the diploid phase undergoes chromosomal reshuffling resulting in haploid offspring with chromosomes of either parent. An overview of the three life cycles is given in Figure 1.1.

Molecular genetics in *Aspergillus niger*

The filamentous fungus *A. niger* is most commonly known as a biotechnologically relevant producer of citric acid [24]. The production of almost all commercially available citric acid is produced by fermentation of *A. niger*, and its usefulness as a cell factory has since then been expanded to the production of other organic acids and proteins [25]. However, *A. niger* is also a common food contaminant, found for example in yoghurt, on grapes, coffee beans or the onion in your kitchen drawer [26–28]. The filamentous fungus *A. niger* has been used as a cell factory for decades, therefore our knowledge on the

organism is relatively large compared to other food spoiling moulds [25]. This includes our understanding of the genetics of *A. niger* and the genetic toolbox available for this organism, including CRISPR-Cas9 mediated genome editing [29–31], making it a prime candidate to use molecular genetic tools to study biological interesting aspects such as the resistance of food spoilage fungi towards food preservation techniques. In this thesis, we expand the genetic toolbox of *A. niger* to *Penicillium roqueforti* and *Paecilomyces variotii*, two other filamentous fungi that are common food spoilers. The research described in this thesis is part of a TIFN funded project entitled "Heterogeneity in spores of food spoilage fungi" in which the molecular mechanisms and heterogeneity of spores against food preservation strategies in *A. niger*, *P.roqueforti* and *P. variotii* are studied (https://topsectoragrifood.nl/project/heterogeneity-in-spores-of-food-spoilage-fungi/).

Compatible solutes are part of the fungal response to deal with harsh conditions

Finding the optimal preservation strategies against fungi can be challenging due to the large array of molecular stress resistance mechanisms present in these organisms. As mentioned above, conidia are relatively stress resistant cells and are able to survive common preservation techniques such as inactivation by heat. One of the major contributors to the high (preservation) stress resistance of fungal spores is considered to be the high concentrations of internal compatible solutes [32]. These molecules are defined as 'compatible' with the cytoplasm, as they accumulate to high concentrations without becoming toxic for the cell. These compatible solutes are thought to be quickly accumulated in vegetative cells upon encountering a stressful environment such as drought or heat. Different kind of molecules have been reported to act as compatible solutes, including trehalose, polyols, betaines and specific amino acids such as proline [33]. There are many synonyms to 'compatible solute' that are commonly used throughout the literature, such as osmoprotectant, osmolyte, chemical chaperone, co-solvent or kosmotropic solute. The most important characteristic of these compounds is that they protect the cell against stressors by stabilizing macromolecules (mainly proteins, but also structures

such as the plasma membrane). There are three main theories proposed on how these molecules protect macro-molecules.

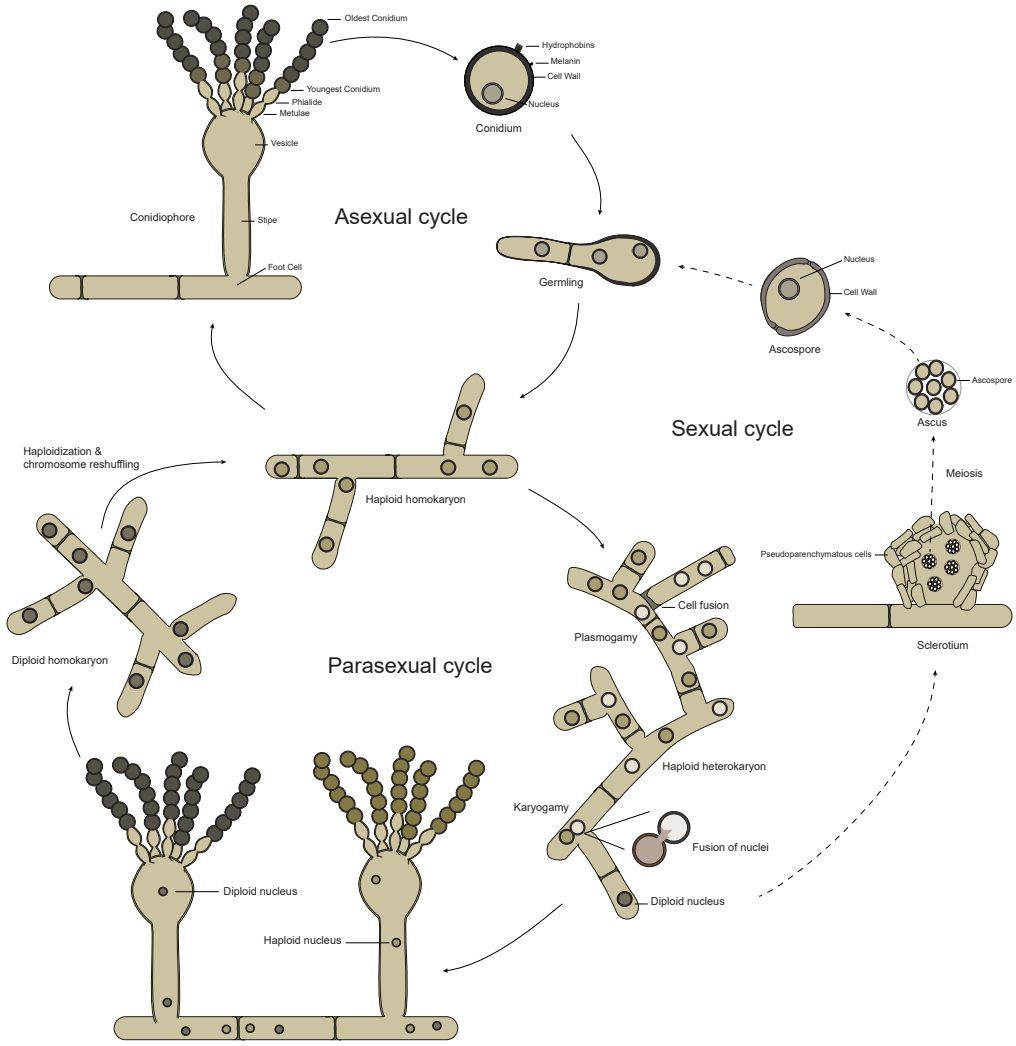

Figure 1.1. Life cycle of *Aspergillus niger*. The sexual cycle depicted here has not yet been proven to exist in *A. niger,* therefore this depiction was based on the sexual cycle found in closely related species *Aspergillus tubingensis* [22,23]. Offspring of the fungus is either a genetic copy of the parent (asexual cycle), has undergone meiosis (sexual cycle), or has undergone haploidization and chromosome reshuffling (parasexual cycle).

One theory is the so-called ‘water replacement theory’ [34,35]. This theory states that the compatible solutes effectively replace water on a molecular level, creating water

bridges and thereby stabilizing proteins and membranes [36–38]. Another theory is the vitrification theory, also known as the 'glassy state theory' [39–41]. This theory proposes that compatible solutes in high concentrations establish a so-called glassy state inside the cell. This glassy state is a chemically steady state that looks like a glass, in which the non-covalent intramolecular bonds (such as water bridges) are preserved, thereby preventing denaturation of proteins. Intramolecular bonds in this state become extremely rigid, and therefore stable, even when the cell is treated with stressors such as extreme heat or cold. This is one of the main arguments raised as to why the compatible solute glycerol is so essential in keeping bacterial and fungal stocks alive in freezers kept at -80 °C [42]. A less often discussed theory is the preferential exclusion theory [43]. This theory stems from thermodynamics and has to do with the so-called 'folding equilibrium' of proteins in liquid. Depending on the amount of compatible solute present, the folding equilibrium will shift, making it thermodynamically more favorable for the protein to stay in the folded form. This has to do with the interaction between protein, water and compatible solute, where the water molecules are favored to interact with the compatible solute, thereby 'drying' or depriving the proteins from water molecules. This 'dried' and strongly folded protein would in turn be better protected against most forms molecular damage. Some adjusted versions of each theory exist, but these three form the basis for most theories currently proposed on the mode of action of compatible solutes [44]. It is also good to note that the three theories are not thought to be mutually exclusive [45].

Stress signaling pathways, transcription factors and their target genes

How does a fungus recognize it needs to protect itself against a stressor? Several kinase dependent signal transduction pathways are known in fungi that respond to stressors, often adapted to specific stressors the cell might face from the environment. These stress signaling pathways are best-described in *Saccharomyces cerevisiae* and *Candida albicans*, but overall are preserved among fungi, and some have been described in *Aspergillus* species as well [46]. Most stressors are first recognized by a receptor, many

of which reside in the cell membrane [47]. Receptors directly activate a kinase-dependent signaling cascade machinery, belonging to either a two-component signaling (TCS) system or the mitogen-activated protein kinase (MAPK) dependent system. At the end of these, sometimes very complex, cascading pathways often resides a transcription factor (TF) which is activated. This protein activates or represses the expression of multiple genes, thereby creating a molecular response by the fungus to the observed environmental stressor. For example, upon sensing oxidative stress via the HOG pathway, the fungal cell will activate transcription factors that induce the production of catalases and superoxide dismutases [48,49], enzymes that can detoxify reactive oxygen species (ROS). In terms of conidial preservation stress resistance, transcription factor AtfA is important for the oxidative and heat stress resistance of conidia from *Aspergillus fumigatus*, *Aspergillus nidulans* and *Aspergillus oryzae* [50–53]. AtfA is known to regulate gene *tpsA*, important for trehalose accumulation in conidia. AtfA also regulates other genes encoding conidia specific proteins such as catalase *catA*, dehydrin *dprA* and "conidiation specific" gene *conJ* which are all thought to be involved in conidial stress resistance [54–56].

However, not all stress signaling pathways in fungi are fully understood, and some do not require a cell signaling cascade by kinases. The activation of the main heat shock response in *S. cerevisiae* is regulated by transcription factor "Heat shock factor 1" (Hsf1) and its homologues are conserved and active in filamentous fungi [57–59]. Upon heat shock, the Hsf1 proteins undergoes a confirmational change and forms a trimer, which is the activate form of the transcription factor, subsequently adjusting expression ~3% of all genes on the genome of *S. cerevisiae* [60], however it is not clear if additional molecules and steps are involved in this conformational change [61]. This transcription factor Hsf1 induces expression of heat shock proteins including Hsp26 and Hsp104, required for heat stress resistance [62]. Similarly, the main weak acid stress response in *S. cerevisiae* is regulated by "Weak acid resistance 1" (War1p) [63]. This transcription factor, important in the weak acid stress response, has direct interactions with the weak acid anions inside the fungal cell, leading to its activation [64,65]. Upon activation,

War1p induces the expression of Pdr12, an ABC-type efflux pump meant to extrude weak acid anions out of the cell [66].

The heterogeneity of *Aspergillus niger* conidia.

Food preservation becomes even more challenging when considering that conidia are heterogeneous in their stress resistance and germination capacity. Therefore, inactivation or inhibition of the germination of one conidium might not inactivate or inhibit another fungal conidium. The most well-described source of spore heterogeneity is caused by species and strain diversity. For example, the heat resistance of fungal conidia varies greatly depending on which species or strain is analysed [67,68]. However, a scarcely studied phenomenon is the heterogeneity in stress resistance and germination capacity of fungal spores within a single spore population, thus obtained from a single strain. Researchers have shown that conidia from a single *A. niger* strain are heterogeneous in their stress resistance towards sorbic acid [69] and heat [70]. It is remarkable that differences between individual conidia are observed when considering that these spores are clonal offspring and therefore genetically identical to each other (see Figure 1.1). Additionally, previous research has shown that cultivation temperature [71] and spore maturation [72] impact physical properties and potentially preservation stress resistance of *Aspergillus* conidia. Taken together with the fact that conidia are dispersed in large quantities, it illustrates the possibility of individual differences between conidia in terms of preservation or environmental stress resistance and germination capacity within a given spore population. These individual differences between conidia can be beneficial for survival of the fungus as a bet-hedging strategy, where individual conidia vary from one another, therein protected against specific stressors or able to germinate under specific conditions, ensuring propagation of the fungus. In this thesis, I will contribute to our understanding of the molecular mechanisms behind conidial stress resistance and heterogeneity between conidia of *A. niger*.

Scope and outline of this thesis

The conidia produced by filamentous fungi pose a constant threat to food preservation. Two major treatments to prevent food spoilage fungi from colonizing food are (1) the heat treatment of food, in order to heat inactivate the conidia, and (2) to include weak acid preservatives (especially sorbic acid) in the food, in order to inactivate the conidia or inhibit fungal growth. Fungi have evolved several mechanisms to survive heat stress and sorbic acid stress in order to survive and proliferate. In addition, earlier research has already indicated that different strains of the same species and spore populations from a single strain are highly heterogenic in their resistance to these stressors. The aim of the thesis is to study the heterogeneity in stress resistance of conidia between strains and between the spore populations of the same strain, and subsequently to identify important factors determining stress resistance.

In **Chapter 2** we investigate the strain diversity in stress resistance of *A. niger* against sorbic acid, a common chemical preservative used by food industries. The sorbic acid stress resistance of 100 *A. niger* strains isolated from various sources, including contaminated foods, was investigated. Additionally, a total of 240 transcription factor knock-out strains were screened for their weak acid stress resistance, revealing transcription factors that are involved in the weak acid stress response of *A. niger*. A novel transcription factor involved in weak acid stress resistance, named *warB*, was identified and further investigated.

In **Chapter 3** we investigate the role of strain diversity in the stress resistance of *P. variotii*, *P. roqueforti* and *A. niger* against heat. The heat stress resistance of ~20 strains of each species was investigated. The degree of variation in conidial heat resistance due to factor “strain” was quantified, and results were compared to previous reports in other organisms. The strain variability observed between conidia of the three food spoiling fungi were in the same order of magnitude as spores from bacterial species.

Genomes of 24 *Aspergillus niger* strains displaying various sensitivities towards heat and weak acids were sequenced with the aim to perform genome wide association studies (GWAS) and bulk-segregant analyses in order to find genetic elements involved in conidial heat resistance and weak acid stress resistance. However, GWAS studies were inconclusive, providing too many target genes putatively involved in both heat resistance and weak acid stress resistance. Additionally, the parasexual crossing of *A. niger* strains, which is required for bulk segregant analysis, turned out to be highly challenging due to widespread heterokaryon incompatibility (described in **Chapter 5**). However, the 24 genomes of *A. niger* revealed an equal distribution of mating types in this presumably asexual species. As a first step to further explore the possible sexual state of *A. niger*, which would be interesting for many future genetic studies, including studies on conidial stress resistance, a high-quality genome of the *A. niger* neotype strain CBS 554.65 containing the MAT1-2 mating type locus was analysed and presented in **Chapter 4**.

In **Chapter 5** the analysis and comparison of the 24 genomes of *A. niger sensu stricto* strains is described in more detail. Together with publicly available genomes, we show that the 32 *A. niger* genome sequenced strains cluster in three distinct phylogenetic groups. A successful parasexual cross between two *A. niger sensu stricto* strains with two different mating types is described, creating the first diploid *A. niger* strain containing both mating types.

In **Chapter 6** the CRISPR/Cas9 genome editing tool for *A. niger* was adopted for genetically less well characterised food spoiling fungi *P. roqueforti* and *P. variotii*. CRISPR/Cas9 was successfully implemented to create targeted mutations in a *pks* gene required for DHN-melanin synthesis. The role of melanin in mediating conidial heat stress and UV stress was studied in more detail using the generated melanin mutants in all three species.

In **Chapter 7** the impact of both the age of conidia and the internal compatible solute compositions of conidia on heat resistance and germination kinetics in *A. niger*

is described. Young conidia contained limited compatible solutes and were found heat stress sensitive. Knock-out strains deleted in trehalose and/or mannitol biosynthesis genes were made using CRISPR/Cas9 genome editing to investigate the role of compatible solutes in heat resistance and germination. The results indicate that trehalose and mannitol levels are important parameters in relation to mediating heat resistance and that the higher sensitivity to heat stress observed in relatively young conidia is due to low levels of compatible solutes.

In **Chapter 8** the impact of cultivation temperature on the resulting heat resistance of conidia was studied at transcriptome, proteome and physiology level. The analyses suggest that apart from increased trehalose levels also the induced expression of heat shock proteins could be responsible for the observation that conidia were more heat resistant when the mycelium was cultivated at 37 °C compared to 28 °C.

In **Chapter 9** the results are discussed and reflected upon. The genetic factors (species and strain) as well as the environmental factors (age, cultivation temperature) all contribute to the observed heterogeneity in preservation stress resistance of *A. niger* conidia. Internal compatible solutes, as well as protein levels from protective proteins such as heat shock proteins, potentially contribute to the individual differences observed between conidia.

The work described in this thesis may contribute in a further understanding of the genetic and environmental factors determining the heterogeneity observed in preservation stress resistance of *A. niger* conidia.

1

References

1. Blasco R, Rosell J, Arilla M, Margalida A, Villalba D, Gopher A, et al. Bone marrow storage and delayed consumption at Middle Pleistocene Qesem Cave, Israel (420 to 200 ka). Sci Adv. 2019;5:eaav9822.

2. Gram L, Ravn L, Rasch M, Bruhn JB, Christensen AB, Givskov M. Food spoilage - Interactions between food spoilage bacteria. Int J Food Microbiol. 2002;78:79–97.

3. Sperber WH. Introduction to the microbiological spoilage of foods and beverages. In: Sperber WH, Doyle MP, editors. Compend Microbiol spoilage foods beverages. New York: Springer; 2009. p. 1–40.

4. Snyder AB, Worobo RW. Fungal spoilage in food processing. J Food Prot. International Association for Food Protection; 2018;81:1035–40.

5. Snyder AB, Churey JJ, Worobo RW. Association of fungal genera from spoiled processed foods with physicochemical food properties and processing conditions. Food Microbiol. 2019;83:211–8.

6. Rico-Munoz E. Heat resistant molds in foods and beverages: recent advances on assessment and prevention. Curr Opin Food Sci. 2017;17:75–83.

7. Novodvorska M, Stratford M, Blythe MJ, Wilson R, Beniston RG, Archer DB. Metabolic activity in dormant conidia of *Aspergillus niger* and developmental changes during conidial outgrowth. Fungal Genet Biol. 2016;94:23–31.

8. Wyatt TT, Wösten HAB, Dijksterhuis J. Fungal spores for dispersion in space and time. Adv Appl Microbiol. 2013;85:43–91.

9. Dijksterhuis J. Fungal spores : Highly variable and stress-resistant vehicles for distribution and spoilage. J Food Microbiol. 2019;81:2–11.

10. Garnier L, Valence F, Mounier J. Diversity and control of spoilage fungi in dairy products: an update. Microorganisms. 2017;5:42.

11. Davies CR, Wohlgemuth F, Young T, Violet J, Dickinson M, Sanders JW, et al. Evolving challenges and strategies for fungal control in the food supply chain. Fungal Biol Rev. 2021;36:15–26.

12. Jara D, Portnoy J, Dhar M, Barnes C. Relation of indoor and outdoor airborne fungal spore levels in the Kansas City metropolitan area. Allergy asthma Proc. 2017;38:130–5.

13. Guinea J, Peláez T, Alcalá L, Bouza E. Outdoor environmental levels of Aspergillus spp. conidia over a wide geographical area. Med Mycol. 2006;44:349–56.

14. Abu-Dieyeh MH, Barham R, Abu-Elteen K, Al-Rashidi R, Shaheen I. Seasonal variation of fungal spore populations in the atmosphere of Zarqa area, Jordan. Aerobiologia (Bologna). 2010;26:263–76.

15. De Antoni Zoppas BC, Valencia-Barrera RM, Vergamini Duso SM, Fernández-González D. Fungal spores prevalent in the aerosol of the city of Caxias do Sul, Rio Grande do Sul, Brazil, over a 2-year period (2001-2002). Aerobiologia (Bologna). 2006;22:119–26.

16. Price DL, Simmons RB, Crow Jr SA, Ahearn DG. Mold colonization during use of preservative-treated and untreated air filters, including HEPA filters from hospitals and commercial locations over an 8-year period (1996–2003). J Ind Microbiol Biotechnol. 2005;32:319–21.

17. Scaramuzza N, Cigarini M, Mutti P, Berni E. Sanitization of packaging and machineries in the food industry: Effect of hydrogen peroxide on ascospores and conidia of filamentous fungi. Int J Food Microbiol. 2020;316:108421.

18. Coppin E, Debuchy R, Arnaise S, Picard M. Mating types and sexual development in filamentous ascomycetes. Microbiol Mol Biol Rev. 1997;61:411–28.

19. Ojeda-López M, Chen W, Eagle CE, Gutiérrez G, Jia WL, Swilaiman SS, et al. Evolution of asexual and sexual reproduction in the aspergilli. Stud Mycol. 2018;91:37–59.

20. Glass NL, Nelson MA. Mating-type genes in mycelial ascomycetes. In: Wessels JGH, Meinhardt F, editors. Growth, Differ Sex. Heidelberg: Springer, Berlin; 1994. p. 295–306.

21. Pontecorvo G. The parasexual cycle in fungi. Annu Rev Microbiol. 1956;10:393–400.

22. Horn BW, Olarte RA, Peterson SW, Carbone I. Sexual reproduction in *Aspergillus tubingensis* from section *Nigri*. Mycologia. 2013;105:1153–63.

23. Olarte RA, Horn BW, Singh R, Carbone I. Sexual recombination in *Aspergillus tubingensis*. Mycologia. 2015;107:307–12.

24. Currie JN. The citric acid fermentation of *Aspergillus niger*. J Biol Chem. 1917;31:15–37.

25. Cairns TC, Nai C, Meyer V. How a fungus shapes biotechnology: 100 years of *Aspergillus niger* research. Fungal Biol Biotechnol. 2018;5:1–14.

26. Noonim P, Mahakarnchanakul W, Nielsen KF, Frisvad JC, Samson RA. Fumonisin B2 production by *Aspergillus niger* in Thai coffee beans. Food Addit Contam Part A. 2009;26:94–100.

27. Gougouli M, Koutsoumanis KP. Risk assessment of fungal spoilage: A case study of *Aspergillus niger* on yogurt. Food Microbiol. 2017;65:264–73.

28. Perrone G, Susca A, Cozzi G, Ehrlich K, Varga J, Frisvad JC, et al. Biodiversity of Aspergillus species in some important agricultural products. Stud Mycol. 2007;59:53–66.

29. Nødvig CS, Nielsen JB, Kogle ME, Mortensen UH. A CRISPR-Cas9 system for genetic engineering of filamentous fungi. PLoS One. 2015;10:1–18.

30. van Leeuwe TM, Arentshorst M, Ernst T, Alazi E, Punt PJ, Ram AFJ. Efficient marker free CRISPR/Cas9 genome editing for functional analysis of gene families in filamentous fungi. Fungal Biol Biotechnol. 2019;6:1–13.

31. Zheng X, Zheng P, Zhang K, Cairns TC, Meyer V, Sun J, et al. 5S rRNA Promoter for guide RNA expression enabled highly efficient CRISPR/Cas9 genome editing in *Aspergillus niger*. ACS Synth Biol. 2018;8:1568–74.

32. Dijksterhuis J, de Vries RP. Compatible solutes and fungal development. Biochem J. 2006;399:e3-5.

33. Welsh DT. Ecological significance of compatible solute accumulation by micro-organisms: from single cells to global climate. FEMS Microbiol Rev. 2000;24:263–90.

34. Mensink MA, Frijlink HW, van der Voort Maarschalk K, Hinrichs WLJ. How sugars protect proteins in the solid state and during drying (review): Mechanisms of stabilization in relation to stress conditions. Eur J Pharm Biopharm. 2017;114:288–95.

35. Crowe JH, Clegg JS, Crowe LM. Anhydrobiosis: the water replacement hypothesis. In: Reid D, editor. Prop Water Foods ISOPOW 6. Boston: Springer; 1998. p. 440–55.

36. Carpenter JF, Crowe JH. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. Biochemistry. 2002;28:3916–22.

37. Allison SD, Chang B, Randolph TW, Carpenter JF. Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. Arch Biochem Biophys. 1999;365:289–98.

38. Mensink MA, Van Bockstal PJ, Pieters S, De Meyer L, Frijlink HW, Van Der Voort Maarschalk K, et al. In-line near infrared spectroscopy during freeze-drying as a tool to measure efficiency of hydrogen bond formation between protein and sugar, predictive of protein storage stability. Int J Pharm. 2015;496:792–800.

39. Slade L, Levine H. Beyond water activity: recent advances based on an alternative approach to the assessment of food quality and safety. Crit Rev Food Sci Nutr. 1991;30:115–360.

40. Ubbink J. Structural and thermodynamic aspects of plasticization and antiplasticization in glassy encapsulation and biostabilization matrices. Adv Drug Deliv Rev. 2016;100:10–26.

41. Chang L, Shepherd D, Sun J, Ouellette D, Grant KL, Tang X, et al. Mechanism of protein stabilization by sugars during freeze-drying and storage: Native structure preservation, specific interaction, and/or immobilization in a glassy matrix? J Pharm Sci. 2005;94:1427–44.

42. Bhattacharya S. Cryoprotectants and their usage in cryopreservation process. In: Bozkurt Y, editor. Cryopreserv Biotechnol Biomed Biol Sci. London: IntechOpen; 2018. p. 7–20.

43. Canchi DR, Jayasimha P, Rau DC, Makhatadze GI, Garcia AE. Molecular mechanism for the preferential exclusion of TMAO from protein surfaces. J Phys Chem B. 2012;116:12095.

44. Jain NK, Roy I. Effect of trehalose on protein structure. Protein Sci. 2009;18:24–36.

45. Christensen D, Kirby D, Foged C, Agger EM, Andersen P, Perrie Y, et al. α,α′-trehalose 6,6′-dibehenate in non-phospholipid-based liposomes enables direct interaction with trehalose, offering stability during freeze-drying. Biochim Biophys Acta - Biomembr. 2008;1778:1365–73.

46. Hagiwara D, Sakamoto K, Abe K, Gomi K. Signaling pathways for stress responses and adaptation in Aspergillus species: stress biology in the post-genomic era. Biosci Biotechnol Biochem. 2016;80:1667–80.

47. Grice C, Bertuzzi M, Bignell E. Receptor-mediated signaling in *Aspergillus fumigatus*. Front Microbiol.

2013;4:26.

48. Angelova MB, Pashova SB, Spasova BK, Vassilev S V., Slokoska LS. Oxidative stress response of filamentous fungi induced by hydrogen peroxide and paraquat. Mycol Res. 2005;109:150–8.

49. Paris S, Wysong D, Debeaupuis J-P, Shibuya K, Philippe B, Diamond RD, et al. Catalases of *Aspergillus fumigatus*. Infect Immun. 2003;71:3551–62.

50. Hagiwara D, Suzuki S, Kamei K, Gonoi T, Kawamoto S. The role of AtfA and HOG MAPK pathway in stress tolerance in conidia of *Aspergillus fumigatus*. Fungal Genet Biol. 2014;73:138–49.

51. Lara-Rojas F, Sánchez O, Kawasaki L, Aguirre J. *Aspergillus nidulans* transcription factor AtfA interacts with the MAPK SakA to regulate general stress responses, development and spore functions. Mol Microbiol. 2011;80:436–54.

52. Sakamoto K, Iwashita K, Yamada O, Kobayashi K, Mizuno A, Akita O, et al. *Aspergillus oryzae* AtfA controls conidial germination and stress tolerance. Fungal Genet Biol. 2009;46:887–97.

53. Emri T, Szarvas V, Orosz E, Antal K, Park HS, Han KH, et al. Core oxidative stress response in *Aspergillus nidulans*. BMC Genomics. BMC Genomics; 2015;16:478.

54. Hoi JWS, Lamarre C, Beau R, Meneau I, Berepiki A, Barre A, et al. A novel family of dehydrin-like proteins is involved in stress response in the human fungal pathogen *Aspergillus fumigatus*. Mol Biol Cell. 2011;22:1896.

55. Suzuki S, Sarikaya Bayram Ö, Bayram Ö, Braus GH. *conF* and *conJ* contribute to conidia germination and stress response in the filamentous fungus *Aspergillus nidulans*. Fungal Genet Biol. Academic Press; 2013;56:42–53.

56. Navarro RE, Stringer MA, Hansberg W, Timberlake WE, Aguirre J. *catA*, a new *Aspergillus nidulans* gene encoding a developmentally regulated catalase. Curr Genet. 1996;29:352–9.

57. Sorger PK, Pelham HRB. Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. Cell. 1988;54:855–64.

58. Nicholls S, Leach MD, Priest CL, Brown AJP. Role of the heat shock transcription factor, Hsf1, in a major fungal pathogen that is obligately associated with warm-blooded animals. Mol Microbiol. 2009;74:844.

59. Fabri JHTM, Rocha MC, Fernandes CM, Persinoti GF, Ries LNA, Cunha AF da, et al. The heat shock transcription factor HsfA is essential for thermotolerance and regulates cell wall integrity in *Aspergillus fumigatus*. Front Microbiol. 2021;12:656548.

60. Hahn J-S, Hu Z, Thiele DJ, Iyer VR. Genome-wide analysis of the biology of stress responses through heat shock transcription factor. Mol Cell Biol. 2004;24:5249–56.

61. Calderwood SK, Xie Y, Wang X, Khaleque MA, Chou SD, Murshid A, et al. Signal transduction pathways leading to heat shock transcription. Sign Transduct Insights. 2010;2:13–24.

62. Chowdhary S, Kainth AS, Pincus D, Gross DS. Heat Shock Factor 1 drives intergenic association of its

target gene loci upon heat shock. Cell Rep. 2019;26:18.

63. Kren A, Mamnun YM, Bauer BE, Schüller C, Wolfger H, Hatzixanthis K, et al. War1p, a novel transcription factor controlling weak acid stress response in yeast. Mol Cell Biol. 2003;23:1775–85.

64. Kim MS, Cho KH, Park KH, Jang J, Hahn J-S. Activation of Haa1 and War1 transcription factors by differential binding of weak acid anions in *Saccharomyces cerevisiae*. Nucleic Acids Res. 2019;47:1211–24.

65. Gregori C, Schüller C, Frohner IE, Ammerer G, Kuchler K. Weak organic acids trigger conformational changes of the yeast transcription factor War1 in vivo to elicit stress adaptation. J Biol Chem. 2008;283:25752–64.

66. Piper P, Mahé Y, Thompson S, Pandjaitan R, Holyoak C, Egner R, et al. The Pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. EMBO J. 1998;17:4257–65.

67. van den Brule T, Punt M, Teertstra W, Houbraken J, Wösten H, Dijksterhuis J. The most heat-resistant conidia observed to date are formed by distinct strains of *Paecilomyces variotii*. Environ Microbiol. 2019;22:986–99.

68. Dijksterhuis J. The fungal spore and food spoilage. Curr Opin Food Sci. 2017;17:68–74.

69. Geoghegan IA, Stratford M, Bromley M, Archer DB, Avery S V. Weak acid resistance A (WarA), a novel transcription factor required for regulation of weak-acid resistance and spore-spore heterogeneity in *Aspergillus niger*. mSphere. 2020;5:e00685-19.

70. Fujikawa H, Itoh T. Tailing of thermal inactivation curve of *Aspergillus niger* spores. Appl Environ Microbiol. 1996;62:3745–9.

71. Hagiwara D, Sakai K, Suzuki S, Umemura M, Nogawa T, Kato N, et al. Temperature during conidiation affects stress tolerance, pigmentation, and trypacidin accumulation in the conidia of the airborne pathogen *Aspergillus fumigatus*. PLoS One. 2017;12:e0177050.

72. Teertstra WR, Tegelaar M, Dijksterhuis J, Golovina EA, Ohm RA, Wösten HAB. Maturation of conidia on conidiophores of *Aspergillus niger*. Fungal Genet Biol. 2017;98:61–70.