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REVIEW

Mechanistic and Mathematical Inactivation Studies of Food Spoilage Fungi

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Brul, S. and Klis, F. M. 1999. Mechanistic and mathematical inactivation studies of food spoilage fungi. *Fungal Genetics and Biology* 27, 199–208. Fungal spoilage forms an increasing economic problem in the food industry. Chemical antifungals are becoming less attractive as food preservatives and hygiene agents due to the development of resistance and due to stricter legal regulations concerning the permitted concentrations. Finally, consumers tend to demand more "naturally preserved" or preservative-free products. Here we review our understanding of the mechanisms of action and resistance to classical antifungals. Next, we evaluate the scientific basis underlying the application of novel, natural antifungals. Finally, we discuss the mathematical modelling of fungal growth and the development of preliminary predictive lag-time models. The eventual aim of the reviewed work is to generate mathematical lag-time models in real foods that predict the microbiological stability of the food and are based on a mechanistic understanding of the chain of events that leads to cell death, or an extension of lag-time of the initiation of outgrowth.

Index Descriptors: fungi; spoilage; toxicity; hygiene; natural antifungals; synergy; resistance; inactivation; lag-time; modelling.

OVERVIEW OF FOOD SPOILAGE FUNGI

Fungi represent an increasing problem in our current society as spoilage organisms in food products, as in-house surface contaminants, and as important lethal human pathogens. In food manufacturing, fungal spoilage of products causes severe economic losses and also potential health hazards due to the possible production of mycotoxins (Samson *et al.*, 1994; Tournas, 1994; Samson *et al.*, 1995; Pitt and Hocking, 1997). An example of this is fungal spoilage of fresh cheese (Engel, 1986). Perhaps more significantly, in 1984 it was reported that several cases of food- and water-borne diseases in Canada were caused by yeasts (National Institute of Health U.S., 1984). In indoor environments fungal volatiles/mycotoxins have been shown to provoke adverse health effects (Samson *et al.*, 1994). Finally, the consumer trend toward reduced temperatures for laundry washing has led to increasing hygiene problems with fungi because mold spores are inactivated at 60°C but not at 40–45°C. These and other observations have led to increasing concern among health authorities (Sternberg, 1994). Additionally, fungi exhibit more and more resistance to established inhibitory treatments.

A list of typical spoilage fungi and associated products is shown in Table 1. Most are clearly related to the Ascomycete *Saccharomyces cerevisiae*, which is very well characterized both biochemically and genetically. Therefore, this organism is often used as a model system.

Current Antifungal Strategies and Their Disadvantages

Classical antifungal measures that extend both closed and open shelf-life of food products involve heating at high

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TABLE 1
Examples of Food Spoilage Fungi and Typically Affected Products

Organisms	Products typically affected
Aspergillus versicolor	Bread, dairy products
Aspergillus flavus	Cereals, nuts
Aspergillus niger	Spices
Byssochlamys fulva	Cereals in airtight packs
Fusarium oxysporum	Fruit
Neosartorya fischeri	Pasteurized foods
Penicillium roqueforti	Meat, eggs, and cheese
Penicillium expansum	Fruits and vegetables
Penicillium commune	Margarines
Penicillium discolor	Cheese
Saccharomyces spp.	Soft drinks
Trichoderma harzianum	Margarines
Zygosaccharomyces bailii	Dressings

Note. Data were taken from Samson *et al.* (1995) and Pitt and Hocking (1997).

temperatures to inactivate spores, weak acid preservatives (e.g., sorbic acid), or antibiotics such as natamycin to inhibit fungal outgrowth in fat spreads and cheese. Although mold spoilage can be controlled by these measures to some extent, three main issues ensure that there are several reasons why in the future novel strategies will be necessary (see, e.g., Gould, 1995). First, extensive heating negatively affects the organoleptic qualities of food products, and in some cases also their nutritional value. Second, current market trends favor fresh products containing less or no chemical preservatives. Third, some fungi have become resistant to levels close to the permitted levels of classical preservatives such as sorbic acid (see, e.g., Mihyar *et al.*, 1997). In addition, some molds can degrade sorbic acid to the malodorous pentadiene, while others are able to adapt and grow in the presence of high concentrations of sorbic acid (see below). Also, relatively natamycin-insensitive fungi have been identified. For example, despite the use of this antibiotic, *Penicillium discolor* is often found as a spoilage organism in the cheese industry (Frisvad *et al.*, 1997). In itself, sanitary improvements during and after the manufacturing process can lead to a significant extension of the closed shelf-life of products. However, cleaning procedures for packaging material often use harsh and environmentally unfriendly chemicals. The problems described have led to a search for novel, natural antifungal molecules to replace or complement current antifungal strategies. In addition, such antifungals may contribute to lowering the harmful environmental burden of cleaning chemicals.

Mode of Action and Resistance Development against Weak Organic Acids

Recent work carried out at the University College London in collaboration with Unilever Research represents an interesting example of how natural antifungals may complement current antifungal strategies. It has been shown that the plasma membrane H^+-ATPase, the principal enzyme in fungi responsible for maintenance of intracellular pH (pH_i) homeostasis, is required for optimal adaptation to sorbic acid (Holyoak *et al.*, 1996). The pH_i is believed to be a critical cellular parameter regulating both growth and metabolism. Past evidence has suggested that the inhibitory action of sorbic acid on yeast is due to reduction of pH_i per se. However, using a novel method to measure pH_i in growing cells little correlation was found between reduced growth rate on exposure to sorbic acid and reduction of pH_i (Bracey *et al.*, 1998a, b). In fact, growth inhibition correlated with an increase in the intracellular ADP/ATP ratio due to increased ATP consumption by the cells. This was partly attributed to the activation of protective mechanisms, such as increased proton pumping by the membrane H+-ATPase (Holyoak *et al.*, 1996), which ensured that pH_i did not decline when cells were exposed to sorbic acid. Therefore, the available evidence suggested that the inhibitory action of sorbic acid was due to the induction of an energetically expensive protective mechanism that compensated for any reduction in pH_i homeostasis but resulted in less available energy for normal growth. Interestingly, recent studies by Piper and co-workers have identified a membrane pump in yeast that is involved in the secretion of the anion of the weak organic acids sorbic acid, benzoic acid, and acetic acid into the surrounding cellular medium (Piper *et al.*, 1998). Figure 1 gives a schematic outline of the current view on how yeast cells respond to stress caused by weak organic acids. A similar dual mode of action can be envisaged for other weak organic acids. In short, at a medium pH equal to the pK of the weak acid, the undissociated form of the acid partitions into the membrane on the outside of the microorganism. The current view is that in itself this probably gives rise to some extent of membrane perturbation (see below). What this exactly means and how it is brought about is nonetheless still unclear. In the cell the molecule will partially dissolve in the cytoplasm where it meets a pH significantly higher than its pK. This leads to dissociation and consequent acidification of the cytoplasm. The cell is thought to counteract this phenomenon by both actively pumping out protons via the P-type plasma membrane ATP-ase and, as recently discovered, actively extrud-

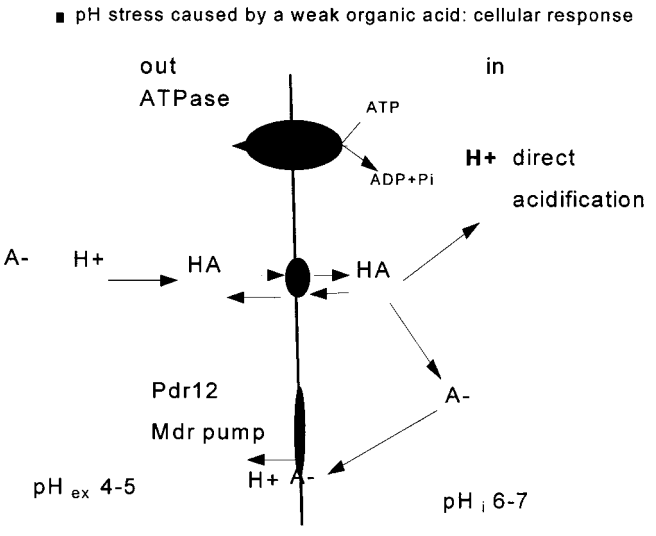

FIG. 1. A schematic impression of the stress response of a yeast cell challenged with weak organic acids (Piper *et al.*, 1998).

ing the remaining anion via the Pdr12 pump. To prevent futile cycling through active acid efflux followed by passive inward diffusion at an extracellular pH of 4–5, a final cellular adaptation reaction may exist, which results in alterations of its plasma membrane and/or cell wall structure such that inward diffusion of these antifungals is prevented or at least severely impaired. Indeed Loureiro-Dias recently discussed at the 19th ISSY in Braga, Portugal (September 1998), that preservative adapted cells are able to reduce the diffusion coefficient for benzoic acid such that presumably reaccess of effluxed preservative back into the cell is significantly impaired. This corroborates the notion that efflux of weak acids is a valid, nonfutile mechanism of adaptation. Bracey *et al.* (1998b) have shown that sorbic acid also acts as a membrane perturber (see additionally Stratford and Anslow, 1996). Hence, one approach to lower the concentration of sorbic acid in food products could be to combine it with other naturally occurring biomolecules that acts as membrane perturbers (peptides and some nonproteinaceous organic biomolecules). These can then be evaluated for synergy with the existing weak organic acids and derived aldehydes. A first example of such a combination may be the observed potentiation of the antifungal effect of sorbic acid by polygodial, a pungent principle from the spout of *Polygonum hydropiper* (Kubo and Lee, 1998). Clearly with this type of approach the required concentrations of the weak acids may be lowered and thus product quality can be improved and legal regulations with respect to the use of weak acids can be met.

Cell Wall-Degrading Enzymes

Natural antifungal agents from edible plants seem an attractive alternative for chemical food additives. Plants contain, e.g., many lytic enzymes that are able to effectively degrade the fungal cell wall and thereby impair growth. Studies on the fungal wall have benefited greatly from the insights on its structure and function gathered in various laboratories during the past 10 years (Klis, 1994; Cid *et al.*, 1995; Brul *et al.*, 1997a; Orlean, 1997; Van der Vaart and Verrips, 1998). Recently, the link between the wall polysaccharides and the major class of cell wall proteins has been identified (Kapteyn *et al.*, 1996; Kollar *et al.*, 1997). The latter are key in protecting the underlying cell wall and plasma membrane against environmental influences. The fungal wall of the filamentous Ascomycetes and the Ascomycete yeast *S. cerevisiae* consists of (galacto) mannoproteins, glucan polymers, and chitinous compounds. The glucan polymers are mainly α-(1,3)-, β-(1,3)-, and β-(1,6)-glucans, while the chitinous compounds are chitin (a polymer of *N*-acetylglucosamine) and chitosan (a polymer of glucosamine) (Klis, 1994). Figure 2 gives a schematic overview of the fungal cell wall of vegetative yeasts and molds. Wessels *et al.* (1997) have contributed greatly to our understanding of the fungal (conidio)spore outer wall, which contains as characteristic components many rather hydrophobic proteins called hydrophobins. However, nature-based mechanisms to interfere with the integrity of these hydrophobins still await discovery.

Enzymes capable of degrading glucan and chitinous compounds are available. Such glucanases and chitinases can be found in several microorganisms and plants. A microbial glucanase/chitinase cocktail significantly inhibits selected classes of spoilage fungi. Only the basic glucanases/chitinases from plant tissues are active as antifungals (see, e.g., Sela-Buurlage, 1996, and references therein). The purified enzymes inhibit fungal outgrowth at 1–10 ppm. The links between cell wall proteins and the β-(1,3)-glucan sugar backbone are through (1,6)-glucan (Kapteyn *et al.*, 1996). The nature of the linkage between β-(1,6)-glucan and wall proteins has recently been unravelled by Kollar *et al.* (1997), who showed that a remnant structure of the glycosylphosphatidylinositol anchor of cell wall proteins is involved: protein–ethanolamine–PO_4^-–Man_5–β-1, 6-glucan.

As the wall proteins form a crucial defence layer of the fungal cell, β-(1,6)-glucanases and proteases are expected to be key to facilitate the access of β-(1,3)-glucanases/chitinases and membrane active compounds to the underlying glucan/chitin sugar polymer layer and the plasma

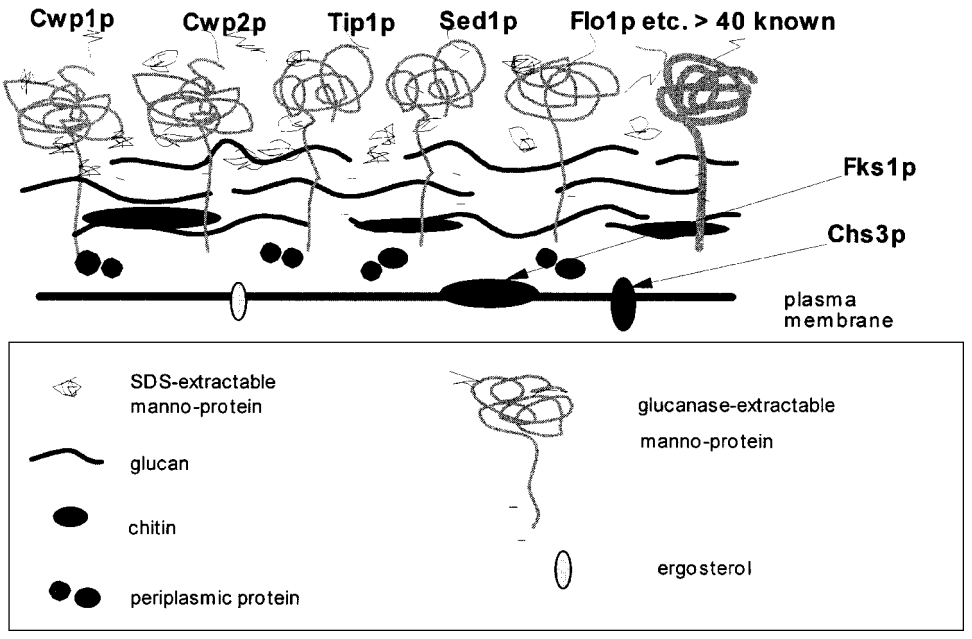

FIG. 2. Schematic overview of the cell wall of *Saccharomyces cerevisiae.* For more details, see Kapteyn *et al.* (1998). The filamentous fungi from the Ascomycetes are proposed to build their cell wall a long similar lines as *Saccharomyces cerevisiae.* Cwp1, cell wall protein 1; Cwp2, cell wall protein 2; Tip1, temperature-induced protein 1; Sed 1, multicopy suppressor of *erd2*; Flo1, flocculin 1, Fks1, glucan synthase 1; Chs3: chitin synthase 3.

membrane. This is indeed true as synergy is observed in lysing fungal cells upon addition of, e.g., β-(1,3)-glucanases/chitinases and protease or β-(1,6)-glucanases (see, e.g., Van der Vaart and Verrips, 1998, and references therein; Brul *et al.*, 1997a, Bom *et al.*, 1998). Note, that recently a further class of cell wall proteins has been described which is induced upon wall stress. These PIR (proteins with internal repeats) proteins are most likely not β-(1,6)-glucan linked to the wall (Mrsa *et al.*, 1997; Kapteyn *et al.*, 1998).

Wall lytic enzymes are not the only compounds that cause wall weakening in fungal cells. It is, for instance, known that the antifungal effect of 1,10-*o*-phenanthroline and EDTA is mediated through zinc chelation, which impairs the construction of a normal cell wall (Brul *et al.*, 1997d).

Resistance Mechanisms Against Wall Weakening

Fungi are able to adapt themselves to the presence of cell wall-degrading enzymes. This was first observed in *Fusarium* strains by Sela-Buurlage (1996) and later also in other *Ascomycete* fungi (S. J. M. C. Oomes, S. K. Dielbandhoesing, and S. Brul, unpublished observations). When macroconidia of *Fusarium solani* were exposed to sublethal concentrations of cell wall-degrading enzymes, and later when challenged again, they became resistant to much higher concentrations that are lethal to noninduced fungi. Recent molecular evidence for the presence of a repair system in fungal walls came from studies by Ram *et al.* (1998). These authors observed that yeast mutants with lower levels of wall β-(1,3)-glucan also displayed secondary wall related phenotypes which are an indirect effect of cell wall weakening. First, the yeast walls of low glucan mutants were enriched in chitin. This phenotype is consistent with the notion that the cell deposits more chitin in its walls to compensate for the observed loss of strength of the wall. Second, the walls contained considerably more Cwp1 mannoprotein than wild-type cells. In this respect, it is worth noting that recently Dielbandhoesing *et al.* (1998) obtained results indicating that both Cwp1p and Cwp2p play a major role in limiting the yeast wall permeability for the naturally occurring membrane-active peptide nisin. Moreover, Yun *et al.* (1997) showed that yeast wall PIR proteins are involved in the protection of the yeast cell against plant antifungal osmotin, and Shimoi *et al.* (1998) showed that a major cell wall protein Sed1p protects yeast in the stationary phase against the action of lytic enzymes. The overall contribution of cell wall proteins to the

organization of the yeast cell wall has been recently reviewed by Kapteyn *et al.* (1998).

Proposed signal transduction mechanisms activating wall repair all include the PKC1 pathway. Kamada *et al.* (1995) have postulated that cell wall weakening induced by a mild heat treatment results in swelling of cells and that the corresponding membrane stretch activates the PKC1 pathway. Cells with a nonfunctioning PKC1 pathway can survive only in osmotically stabilized media and are sensitive to centrifugational forces corroborating the importance of the PKC1 pathway for cellular integrity. The walls of pkc1Δ cells are weak and disorganized, which leaves the plasma membrane under continuous stretch. Fungal plasma membranes contain stretch-activated channels which, most likely, can activate the PKC1 pathway. It is speculated that the Wsc protein family is involved in the activation of the PKC1 pathway upon membrane perturbation (Verna *et al.*, 1997). Recently, this concept has been further elaborated in a review by Banuett (1998) on signalling in the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* and filamentous fungi. Indeed experiments by Zhao *et al.* (1998) have now unequivocally shown that the expression of a gene involved in cell wall synthesis under stress conditions, FKS2, is at least partially regulated by the PKC1 pathway. Preliminary experiments by de Nobel and Oomes (H. de Nobel and S. J. C. M. Oomes, unpublished observations) have furthermore shown that mpk1Δ and pkc1Δ are much more sensitive to wall lytic enzymes than wild-type cells or fks1Δ cells which are deficient in β-1,3-glucan. In the latter cells, FKS2 is induced among others through the action of the PKC1 pathway (Zhao *et al.*, 1998; see also Mazur *et al.*, 1995). Gas1Δ cells lack a proper cross-linking of wall proteins to the β-glucan wall sugars (Popolo *et al.*, 1997; Ram *et al.*, 1998), are hypersensitive to further wall weakening, e.g., through the addition of wall lytic enzymes (H. de Nobel, personal communication) or the inhibition of chitin synthesis (Popolo *et al.*, 1997), and are hypersensitive to a perturbation of the PKC1 pathway, indicating that this pathway is involved in the basal regulation of the Gas1p protein expression (see also Igual *et al.*, 1996). Further data by de Nobel *et al.* (H. de Nobel, personal communication) show that treatment of yeast cells with wall lytic enzymes leads to the induction of a reporter fusion protein FKS2–LacZ. Figure 3 summarizes the proposed chain of events in case of cell wall damage.

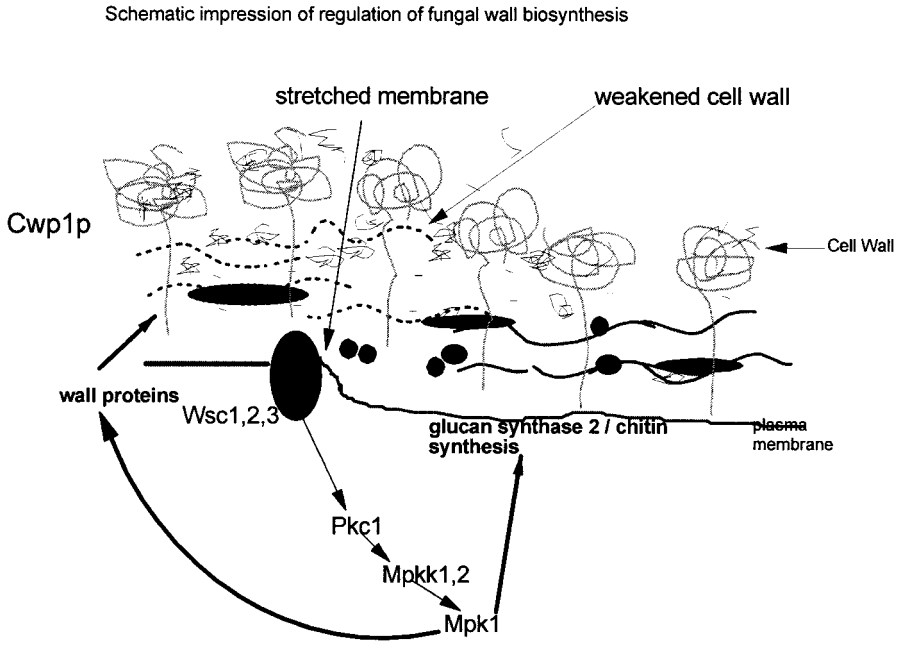

FIG. 3. Cell wall compensation mechanism induced by wall weakening treatments. Speculative scheme of events (see also Banuett, 1998). Local perturbation of the cell wall is proposed to result in membrane stretch, which activates members of the Wsc protein family. This would then activate the Pkc1 pathway, resulting in activation of among others glucan synthase 2 expression and increased chitin synthesis next to positively influencing at least Cwp1 expression.

Antimicrobial Peptides and Small Organic Biomolecules

The plasma membrane forms a lipid bilayer of approximately 7.5 nm width that acts as a barrier to separate the cytosol from the external medium. Fungal membranes contain as major phospholipid species phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), and phosphatidylglycerol (PG). In contrast to higher eukaryotes, the membrane contains predominantly ergosterol and not cholesterol (Rest *et al.*, 1995). Similar to the situation in all other living cells, the plasma membrane contains a variety of integral membrane proteins that perform roles in energy and signal transduction, and uptake and excretion of solute and metabolites, etc. To perform these functions, the membrane maintains an electrochemical gradient of protons (proton-motive force) that is positive outside versus negative inside, and acidic outside versus neutral inside. The proton-motive force drives the uptake of solutes into the cell against a concentration gradient. Moreover, by regulating the flow of protons across the membrane, the proton-motive force ensures that the intracellular pH is maintained at values near neutrality irrespective of the medium pH. Efficient energy transduction requires that the membrane is endowed with a low permeability to protons and ions. Any perturbation affecting the barrier function rapidly leads to a collapse of the proton-motive force. Subsequently, intracellular metabolites are lost ultimately causing a cessation of growth and cell death.

Many antimicrobial biomolecules kill because they interfere with the barrier function of the membrane by perturbation of its structure or permeability. Normally, free access to the membrane is impaired by the previously discussed cell wall. We will now discuss two classes of membrane-lytic biomolecules, antimicrobial peptides (AMP) and nonproteinaceous organic biomolecules.

Antimicrobial Peptides (AMP)

Experiments in the past at many research laboratories both academic and industrial have focused on the antibacterial effects of nisin. This cationic polycyclic lantibiotic is secreted by a number of lactic acid bacteria and has already been used in food products for some time as a preservation biomolecule (Hurst, 1981; Delves-Broughton, 1990; Hansen, 1993). Nisin has found application in the prevention of late-blowing of cheese by inhibiting the outgrowth of *Clostridium* spores. In Europe, the use of nisin in processed cheese has been approved by eight EC countries (Belgium, France, Ireland, Portugal, and Spain) for fresh cheese, quark (The Netherlands), processed vegetables (Italy), and canned foods (United Kingdom) as part of "botulinum-safe" thermal processing ($F° > 3.0$). The molecule permeabilizes bacterial membranes (see Driessen *et al.*, 1995, and references therein), and synergy has been reported with lysozyme, an enzyme degrading the cell wall of bacteria. Recent work by Dielbandhoesing *et al.* (1998) has shown, as mentioned above, that in yeast cells specific cell wall proteins confer resistance to nisin. Interestingly, also in this case synergy between specific cell wall protein removal and the action of nisin has been observed (S. K. Dielbandhoesing and S. Brul, unpublished observations). Finally, the established antimicrobial peptide dermaseptin from frog skin shows in combination with mild heat clear synergy in the inactivation of *S. cerevisiae* cells (Coote *et al.*, 1998b).

Disulfide-containing antimicrobial peptides have been isolated from several plants (see Table 2 and, e.g., Cammue *et al.*, 1995; Nielsen *et al.*, 1996). Well known are the thionins, a family of peptides ranging from 37 to 45 residues occurring in the tissues of many cereals such as barley and wheat. Antimicrobial peptides have also been purified from seeds of many different plant species. On the basis of similarities in amino acid sequence, these peptides can be divided into six distinct classes. They have several properties in common such as a relatively small size (<10 kDa), a high isoelectric point, and an abundance of cysteine residues. Structural studies on a protein isolated

TABLE 2
Antimicrobial Peptides (AMP's) from Plants

Source species	AMP	Class	Activity µg/ml (=ppm)[a]
Mirabilis jalapa	Mj-AMP2	Mirabilis type	1.5
Amaranthus caudatus	Ac-AMP2	Hevein type	0.75
Raphanus sativus	Rs-AFP1	Plant defensin	6
Raphanus sativus	Rs-AFP2	Plant defensin	1.5
Dahlia merckii	Dm-AMP1	Plant defensin	<0.75
Allium cepa	Ace-AMP1	Nonspecific lipid transfer protein type	<0.75
Raphanus sativus	RS-2S4	2S albumin	>100
Impatiens balsamina	Ib-AMP2	Impatiens type	<0.75

Note. The concentration required to give for 50% growth inhibition is given (IC_{50} values in µg/ml).

[a] Assays performed in microplate cultures using an isolate of *Fusarium oxysporum* from banana. Details on the assays are given in Terras *et al.* (1992) and in Cammue *et al.* (1995), and on the outcome in Novel Antifungal Proteins: applications in crop protection, final technical report EU-project PL-00075.

from radish (Rs-AFP1) show that internal cross-linking between cysteine residues gives a characteristic, stable 3-D structure. Other antimicrobial peptides are from seeds from *Amaranthus caudatus* (see, e.g., Broekaert *et al.*, 1992). In addition, a novel pathogen- and wound inducible chitin-binding tobacco protein with antifungal activity has been identified (Ponstein *et al.*, 1994). Structural and immunological data suggest that this *Nicotiana tabacum* protein belongs to the Pathogenesis Related-4 group of proteins. The protein inhibits growth of several fungi and acts synergistically with both a tobacco class I chitinase and a class I β-1,3-glucanase. All AFPs are heat-stable and easily purified from plant seed extracts. Resistance development against these plant antifungal peptides has been discussed above. Interestingly, it has recently been reported by Yun *et al.* (1997) that specific fungal phosphomannoproteins are crucial in the protection of yeasts against the plant antifungal molecule osmotin.

Nonproteinaceous Antimicrobial Biomolecules

Finally, several nonproteinaceous plant biomolecules have strong antifungal activity (Nychas, 1995). For instance Unilever published a patent in 1997 on the synergistic combination of wall lytic enzymes and herbs (Brul *et al.*, 1997b). In herbs, the essential oils and related compounds are believed to be the active components. The membrane-permeabilizing effect of these biomolecules was demonstrated in a variety of ways. For example, upon culture of fungal cells in the presence of carvacrol, the cells take up the membrane-impermeable fluorescent compound propidium iodide (S. J. C. M. Oomes and S. Brul, unpublished observations). Apart from carvacrol, there are many structurally analogous molecules that also have clear antimicrobial effects (see, e.g., Helander *et al.*, 1998; Kubo and Lee, 1998).

Modeling

Modelling of the interactions between various physiological parameters requires a detailed knowledge of the interactions discussed on the previous pages. Although it is true that much is already known, many questions remain. We may have a generally agreed hypothesis on how hypotonic conditions lead to activation of the Pkc1 signal transduction route, which eventually leads to changes in cell growth and cell wall biosynthesis, but the molecular details of how many other cellular signal transduction systems become activated are still largely enigmatic (Banuett, 1998). We should also bear in mind that such systems look at processes involved in growth regulation and not at germination of fungal spores or the release of G1 arrest in vegetative yeast cells. A proper description of the initial physiological processes that relieve this "dormancy" is now emerging especially through the advent of fluorescent probes that allow physiological measurements at the single cell level. However, translation to the molecular level is a major task often still to be done. Work by Breeuwer and co-workers shows that the germination of sporangiospores of *Rhizopus* correlates with a rise in intracellular pH (Breeuwer *et al.*, 1997). Indeed also actively growing yeast cells have a higher pH_i than lag-phase or stationary-phase cells (see, e.g., Imai and Ohno, 1995). Whether the rise in pH_i is a cause or a consequence of the onset of outgrowth is not yet clear.

Given the fact that mechanistic interaction models are still ambitious and under development, what can we do now to come to a quantitative predictive description of fungal growth and lag time? Often a prediction based on mathematic analysis of available data can be very helpful. In fact, such an approach also allows for a proper analysis of the most crucial factors in a hurdle approach toward food preservation (Gould, 1995). The basic principle of the hurdle approach in food presentation is that individual treatments may not give adequate preservation, but upon proper combination will give a sufficient preservation while leaving as much as possible the organoleptic characteristics of a food product intact. Hurdles can include lowering of the temperature (chilled distribution of food products), water activity (for example by the addition of NaCl or sugar), or pH; minimal heating profiles (mild pasteurization conditions); or the addition of (natural) preservatives.

Much work has been done to develop models for bacterial growth and death as a function of temperature, pH and a_w (Cuppers and Smelt, 1993; McMeekin *et al.*, 1987; Rosso *et al.*, 1995; Wijtzes *et al.*, 1995; Zwietering *et al.*, 1994). Recently, also fungal growth has been studied along similar lines (Gibson *et al.*, 1994; Brul *et al.*, 1997c; Cuppers *et al.*, 1997).

Gibson *et al.* (1994) were the first to assess and model both growth rate and the time needed for visible colony formation (lag time) as a tool to monitor spoilage. Visible colonies were thereby defined as colonies of 3-mm diameter. This criterium was also used by Cuppers *et al.* (1997) and Brul *et al.* (1997c) in their experiments. The model by Gibson *et al.* uses linear regression models for growth rate at different water activity values. Cuppers *et al.* compared in their experiments different NaCl concentrations, transformed into water activity values, and different culture

temperatures for five spoilage molds (Cuppers *et al.*, 1997). The models they used were both the generally used Ratkowsky square root model (Ratkowsky *et al.*, 1983) and the more recently proposed model by Rosso *et al.* (1993). The latter model uses the three cardinal temperatures (T_{max}, T_{min}, and T_{opt}) and the growth rate at the optimum temperature (r_{opt}) as parameters. A great advantage of the Rosso model is that it contains parameters that have a physiological meaning which clearly facilitates initial parameter estimations and may also aid in future incorporation into the model of underlying cell biological mechanisms. Although a statistical comparison of the results of the Rosso and Ratkowsky models showed no significant differences, the Rosso model is to be preferred based on both the fact that it contains biologically interpretable parameters and mathematically analytical solutions rather than numerical ones.

Brul *et al.* (1997c) have made a first attempt to develop lag-time models based on the results of Cuppers and coworkers to predict the timespan to visible fungal colony surface growth. They used a variation of the basic model by Zwietering *et al.* (1994) for bacterial growth in a liquid culture which assesses the time span to turbidity. The variation consists in the addition of a power function to the inverse proportionality between growth rate and lag time. The derived equation is thus log (lag) = k1-k2 log (growth rate). This relation describes the timelag to visible colony growth of the same five spoilage moulds that were studied by Cuppers *et al.* (1997) satisfactory.

Cuppers *et al.* (1997) used in their modeling studies the notion developed by Gibson *et al.* (1994) that the logarithm of the growth rate of fungal cells on a surface, i.e. the increase in colony diameter, shows a parabolic relationship with the square root of $1\text{-}a_w$. This characteristic makes extrapolations to very high salt concentrations possible without getting odd values for growth rates, However, it is dangerous to extrapolate these kinetic models outside their measurement areas as mechanistic understanding of the models is still lacking.

CONCLUDING REMARKS

What is needed to develop mechanistic growth models is a proper understanding of the primary cellular inactivation targets of the particular treatment chosen. As discussed extensively in this review, such knowledge often is available where it concerns biological inactivation treatments. Also heat inactivation, especially where it concerns mild-heat inactivation, is well covered (see, e.g., Banuett, 1998, and references therein). The newest microbiological insights in inactivation by ultra-high pressure were recently reviewed by Smelt (1998). Work in both bacteria (Wouters *et al.*, 1998) and yeast (A. Rommens, personal communication) has shown that membrane proteins are the most likely primary targets of ultra-high pressure. The plasma membrane-bound bacterial F_1F_0-ATPase in bacteria is presumably inactivated and/or dislocated by pressure. Regarding the most recent physical alternatives for heat treatments such as pulsed electric fields, not much detail is known yet about how these may lead to microbial inactivation (see a recent review by Wouters and Smelt, 1997). It will only be through a thorough microbiological analysis of cellular physiology under different culture conditions, coupled with mathematical model development, that we will be able generate robust predictive models that can be used in the future for the prediction of the fungal vulnerability of food products.

The now available computer power combined with modern single-cell fluorescent analysis techniques (Brul *et al.*, 1997e) and high throughput screening (Oomes and Brul, 1998) will allow us to include in these models a significant number of "hurdles" such that hurdle technology for food preservation with its inherent benefits of enhanced product quality will become more widely applicable.

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