ELSEVIER

Journal of Microbiological Methods 28 (1997) 169–178

Journal of Microbiological Methods

Fluorescent probes for wall porosity and membrane integrity in filamentous fungi

S. Brul*, J. Nussbaum, S.K. Dielbandhoesing

Microbiology and Preservation Section, Unilever Research, Vlaardingen Laboratory, 3133 AT Vlaardingen, The Netherlands

Received 18 August 1996; revised 12 December 1996; accepted 7 January 1997

Abstract

To assess the viability of moulds, quick detection methods using fluorescent probes were evaluated. A major hurdle in the use of intracellular probes can be the wall of filamentous fungi. We studied the permeability of the wall of germinating conidiospores with anionic ellipsoid FITC-dextran molecules and confocal scanning laser microscopy. In contrast to *S. cerevisiae*, filamentous fungi internalized FITC-dextran molecules up to 150 kDa (Stokes radius of 8–9 nm). We concluded that the wall of germinating conidia forms no size exclusion barrier for fluorescent viability probes.

To assess the viability of a suspension of germinating conidiospores, PI, CDFDA, and FUN1 were shown to be suitable. It was not possible to homogeneously stain fresh conidiospores with fluorescent viability probes. Nonetheless, fluorescence activated cell sorting was possible and allowed the sorting of different sub-populations. Whilst the presence of esterase activity (CDFDA staining) did not correlate with outgrowth potential, the uptake of PI did correlate with no outgrowth.

Keywords: Cell wall; CSLM; FACS; Fluorescent probes; Fungi; Viability

1. Introduction

Filamentous fungi (moulds) form an increasing spoilage problem in food manufacturing. Although conidiospores and vegetative hyphae of common genera, like *Penicillium* and *Aspergillus* are killed upon a 5 to 10 min heat treatment at 60°C, such a heating step is not always desirable. Moreover, the teleomorphs of fungal genera may form heat resistant ascospores [1]. Finally, yeast and moulds are also an increasing health hazard [2,3]. Clearly, effective antifungal treatments are required. The search for such treatments concentrates on agents that lyse the cell wall and plasma membrane or interfere with cell wall construction [4,5]. A reliable quick detection method will facilitate greatly the large scale testing of the emerging options. Fluorescent probes are a promising tool. They have shown their use in assessing membrane damage in bacteria [6].

In this paper we study the use of fluorescent probes for the assessment of viability of both freshly harvested conidiospores and germinating conidiospores from five common food spoilage moulds. As

*Corresponding author. Tel.: +31 10 4605173; fax: +31 10 4605188; e-mail: stanley.brul@unilever.com

0167-7012/97/$17.00
PII S0167-7012(97)00975-5

the viability probes have to permeate over the fungal envelope (wall+membrane), we also pay attention to the porosity of the cell wall of filamentous fungi. The wall of vegetative yeast cells is known to be permeable to FITC-dextrans up to 70 kDa [7]. Here we look at the FITC-dextran permeability of germinating conidiospores. It is known that the outer layer of mannoproteins plays a key role in determining the porosity in yeast. Mutants with a lower mannosylation on the wall proteins (*mnn9*) are characterized by a leaky cell wall [8]. To test whether also the wall of filamentous fungi contains mannoproteins we incubated germinating conidia with the lectin Concanavalin-A, which selectively binds mannose sugar residues [9].

Propidium iodide (PI) is used for a rapid viability assessment of various cell types [6]. The positively charged membrane-impermeable fluorochrome can pass only through the membrane of stressed, injured or dead cells. The dye binds with little or no base pair preference by intercalating into DNA and RNA where its red fluorescence is enhanced. Esterase activity is often used as an indicator for cell viability. A polar, membrane- impermeable fluorescent dye (generally a fluorescein or fluorescein derivative), is loaded into intact cells by incubation with the respective non-fluorescent membrane permeable acetyl or acetoxymethyl ester, like Carboxy-Dichloro-Fluorescein-Diacetate (CDFDA). Once inside the cells, the esters are hydrolysed into more polar fluorescent products and acetate by the non-specific intracellular esterases [10,11]. The fluorescent compound rapidly leaks from cells with compromised membranes. Noticeably, in *Saccharomyces cerevisiae* recently an active extrusion mechanism has been described for carboxyfluorescein [12].

Finally, FUN1, a membrane-permeant compound, stains cytoplasmic nucleic acids with a green fluorescence. In viable yeast cells, due to the activity of vacuolar enzymes, a distinct orange-red intravacuolar fluorescence is subsequently formed. For *S. cerevisiae* cells, metabolic activity (glucose utilisation) is linked to an increase of the red/green fluorescence ratio over time (product information from the manufacturer and own unpublished observations).

The validity of the viability stains was tested on fresh and germinating conidiospores (swollen spores and unbranched hyphae).

2. Materials and methods

2.1. Culture media

Malt extract broth (MEB; 2%, Oxoid)

Malt extract agar (MEA): 2% Malt extract broth and 2% agar (Difco)

2.2. Strains

The following common food spoilage moulds were selected: *Penicillium roqueforti*, *Trichoderma harzianum*, *Paecilomyces variotti*, *Aspergillus niger*, *Aspergillus nidulans*. All strains are food product isolates and have been classified by the ‘‘Centraal Bureau voor de Schimmelcultures’’ in Baarn, The Netherlands.

2.3. Preparation of cellular samples

2.3.1. Fresh spores

Well sporulated cultures were obtained after five days at 25°C in MEA. Fresh spores were harvested by overlaying the fully sporulated plates with water and loosening the spores by gently scraping with a Drigalski spatula. The spore suspension was filtered over 2 layers of sterile paper tissue, microscopically examined and the titre was determined. The suspensions were routinely used immediately after harvesting.

2.3.2. Germinating spores

Spores were germinated in 25 ml of MEB. Their germination and growth were followed microscopically. Fresh germinating spores were defined as spores which had formed unbranched hyphae less than 2 or 3 times as large as the spore itself.

2.3.3. Protoplasts

Germinating fungal spores were incubated for 30 min at 37°C with 10 mg/ml lytic enzymes (Novozym 234, Novo Nordisk) in the presence of 0.6 M KCl. The suspension was subsequently washed twice with 0.6 M KCl.

2.4. Stainings

2.4.1. Con A-FITC

Germinating spores were harvested by centrifuging 1 min at 3,000 rpm. The pellet was resuspended in 10 mM potassium phosphate (KPi) buffer pH 7.8 and incubated with 20 μg/ml Con A-FITC (Sigma) for 30 min at 37°C. In a few experiments 20–50 μg/ml D(+)-mannose was added as competitor. The incubations were stopped by centrifuging the cells for 1 min at 3000 rpm, and washing four times with 10 mM KPi buffer, pH 7.8.

2.4.2. FITC-Dextran (Fd)

Germinating spores or protoplasts were harvested by centrifuging 1 minute at 3,000 rpm. The pellet was resuspended in 10 mM KPi buffer pH 7.8 (in experiments with protoplasts 0.6 M KCl was added as an osmoprotectant), and incubated with FITC-dextran (Sigma) of 10, 20, 40, 70, 150 and 500 kDa for 0, 5, 10, 15, 20, 40 or 60 min. The final concentrations Fd were 1.8, 3.57, 7.14, 12.5, 26.5 mg/ml and 25 mg/ml respectively. The incubations were stopped by centrifuging the cells for one minute at 3,000 rpm. The suspensions were washed at least four times before microscopical examination.

2.4.3. CDFDA and PI

1 ml suspensions of dormant or germinating spores were incubated in 50 mM KPi buffer, pH 6, with 5 μl CDFDA (1 mg/ml in dimethyl sulfoxyde (DMSO), Molecular Probes) and 5μl PI (1 mg/ml in 50 mM KPi buffer pH 6.0, Molecular Probes), at 30°C for 15 min. The staining solution was removed by centrifuging 1 minute at 5000 rpm and discarding the supernatant. Subsequently, the pellet was dissolved in 0.1 ml of KPi buffer and examined microscopically.

2.4.4. FUN1 and Calcofluor White

Suspensions of freshly harvested or germinating spores were incubated in *N*-[2-Hydroxyethyl]piperazine-*N′*-[2-ethanesulfonic acid] (HEPES) buffer (10 mM, pH 6) in the presence of 20 μM FUN1 (Molecular Probes, from a 10 mM stock in DMSO) and 25 μM Calcofluor White M2R (Molecular Probes, from a 5 mM stock in dH_2O) as counter stain, during 30 min at 30°C. Subsequently, these suspensions were examined microscopically.

Table 1
Excitation/emission wavelength of the fluorochromes

Fluorochrome	Excitation (nm)	Emission (nm)
CDFDA	485	530 (green)
PI	538	617 (red)
FUN1	485	530 (green)
	485	620 (red)
Calcofluor White	346	460 (blue)
FITC	485	530 (green)

The fluorescence characteristics of the probes are shown in Table 1. At least 200 individual freshly harvested or germinating spores of individual strains were examined with the fluorescence microscope (Zeiss).

2.5. ATP measurements

The ATP measurements were done with a bioluminescence technique using the luciferin/luciferase system according to Ref. [14]. The measurements used a LUMAC biocounter.

2.6. Flow cytometry

Flow cytometry and cell sorting were performed essentially as described in [6]. The flow cytometer used was an in-house modified Becton Dickinson FACS 2.

2.7. Confocal scanning laser microscopy

Confocal scanning laser microscopy (CSLM) was performed essentially as described in [13]. The CSLM used was a Bio-Rad MRC 1024 equipped with a Zeiss Axiovert microscope and a Krypton/Argon laser of 488 nm. The images were processed with the Lasersharp software package.

3. Results and discussion

In Con-A incubations with germinating conidiospores of the spoilage moulds, green fluorescence was visible on the germ tube and the original spore. Fig. 1 gives a typical example for *Aspergillus niger*. The

Fig. 1. Germinating conidia of *Aspergillus niger* stained with Concanavalin-A (magnification is identical to Fig. 2

fluorescence was competed away by D(+)-mannose confirming the specificity of the reaction (not shown). We concluded that a mannose rich protein layer is present on the walls of germinating conidia of these fungi. As, like *S. cerevisiae*, the selected moulds all belong to the Ascomycetes we infer that the mannose groups are attached to protein moieties and may act as a diffusion barrier [15]. Recent experiments performed by Frans Klis and co-workers and by our laboratory provide indeed immunobiochemical evidence for the presence of glucanase extractable cell wall mannoproteins in filamentous fungi [Brul et al., in preparation, [16]].

Subsequently we performed size exclusion experiments with fluorescently labelled dextrans (Fd) on germinating spores. In Fd uptake experiments, pH plays a major role. At a pH lower than ≈7, free FITC becomes uncharged which allows diffusion over the plasma membrane and thus an undisturbed entrance into the cell [17]. In our experiments we incubated cells with Fd at pH 7.8 to ensure that no free FITC entry could occur. Examination with the fluorescence microscope showed that up to an incubation time of 40 min, within the range of 10–150 kDa gradually more Fd is taken up. Fig. 2a shows fluorescence at $t=0$. Fig. 2b shows a typical example of the fluorescence after 40 min of incubation with Fd 150. While most of the Fd 500 is concentrated at the hyphal walls, standard fluorescence microscopy as shown in Fig. 2c suggests that also some uptake of label in cells takes place. In order to study the Fd labelling in more detail we performed CSLM on the samples. This three dimensional analysis clearly showed that Fd 500 always adhered to the hyphal walls and was not significantlly internalized (Fig. 3). Similar experiments with Fd150 did show uptake of the label (not shown). Within all colonies, independent of Fd size and incubation time, some of the germinating conidia showed a very bright fluorescence. Such germ tubes had a compromised plasma membrane indicated by a positive PI staining (see below). Fungal cells which had been treated with a cell wall lytic enzyme preparation took up similar amounts of Fd independent of size or incubation time. This was expected, since without the cell wall a major diffusion barrier disappears. Fig. 2d shows the uptake after 40 min of Fd 500 by such cells. In all cases uptake results after 60 min were essentially similar. In conclusion, in contrast to *S.cerevisiae* which took up Fd 70 [7], the spoilage moulds also took up Fd 150. A dextran molecule of 150 kDa represents a Stokes radius of 8.2 nm [18] so that the wall of (germinating) conidiospores does not form a size exclusion barrier for fluorescent viability probes.

Live/dead staining mixtures were subsequently examined using both fresh and germinating conidiospores. Fig. 4 shows the results for CDFDA/PI staining. The great differences observed between stained spore levels and colony forming units is one of the hurdles to the correlation of staining and outgrowth results. One explanation for this discrepancy is that spores of many strains are often clustered together in pellets (Santos da Silva M., personal communication) [19,20]. Secondly, the spores present in a single preparation have different maturation stages. *Aspergillus* strains for instance, have their spores arranged in long parallel chains on

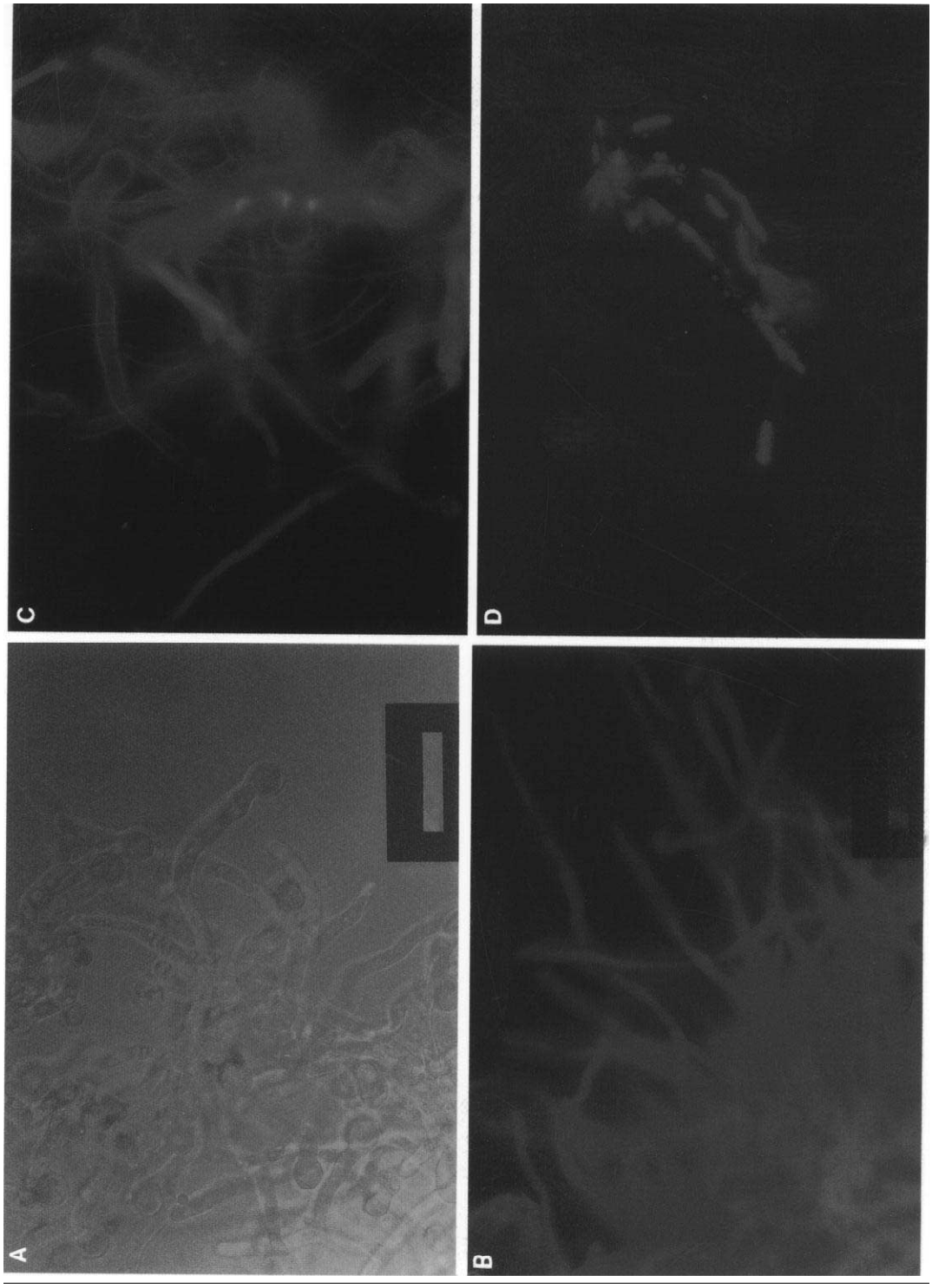

Fig. 2. Time course uptake experiments of Fd in *Aspergillus niger*. (a) $t=0$, (b) Fd 150 at 40 min, (c) Fd 500 at 40 min and (d) Fd 500 at 40 min for protoplasts, bar=25 μm.

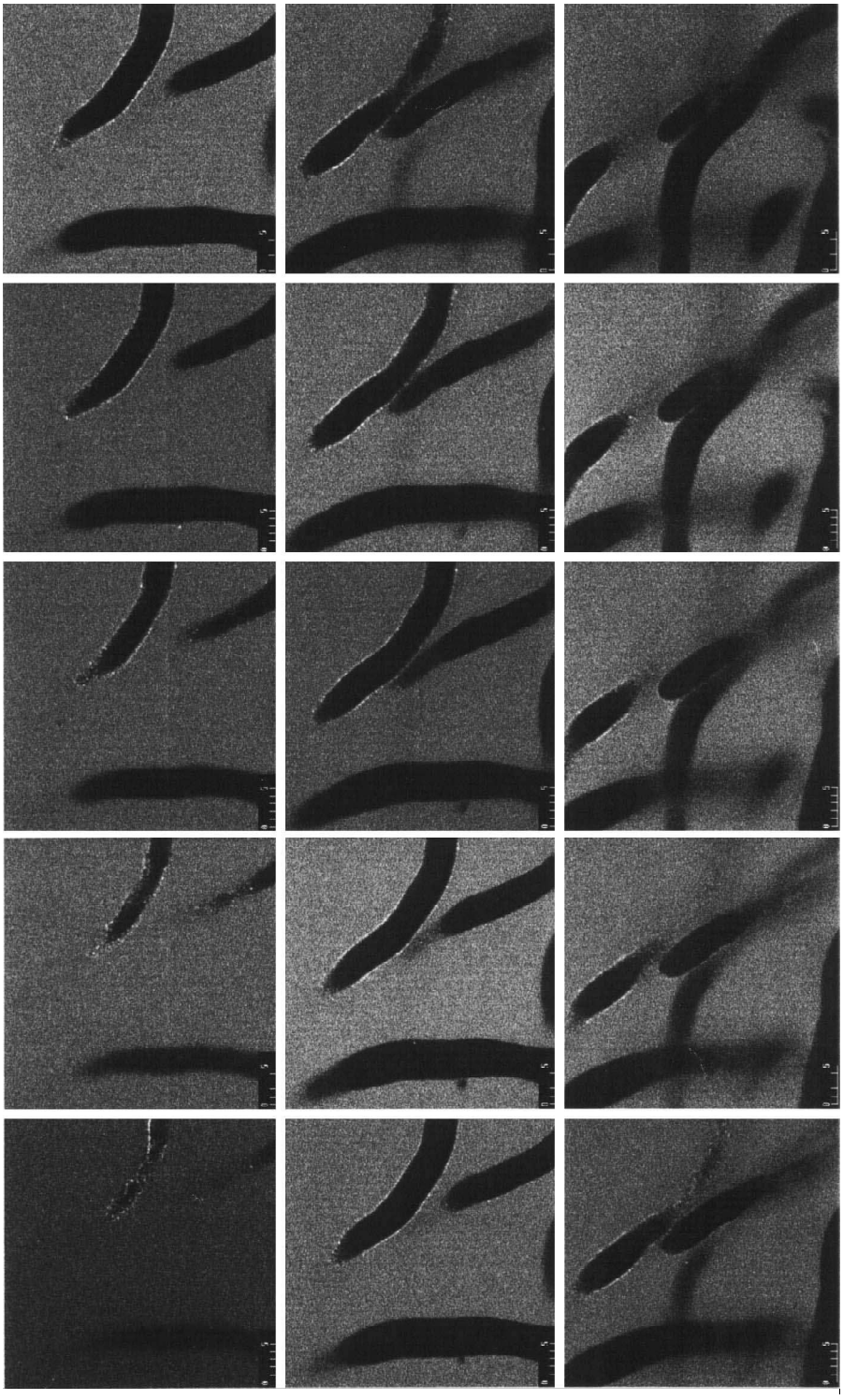

Fig. 3. Confocal scanning laser microscopy showing the distribution of Fd 500 upon a 40 min incubation on *Aspergillus niger*. Optical coupes were taken every 0.2 μm, bar=5 μm.

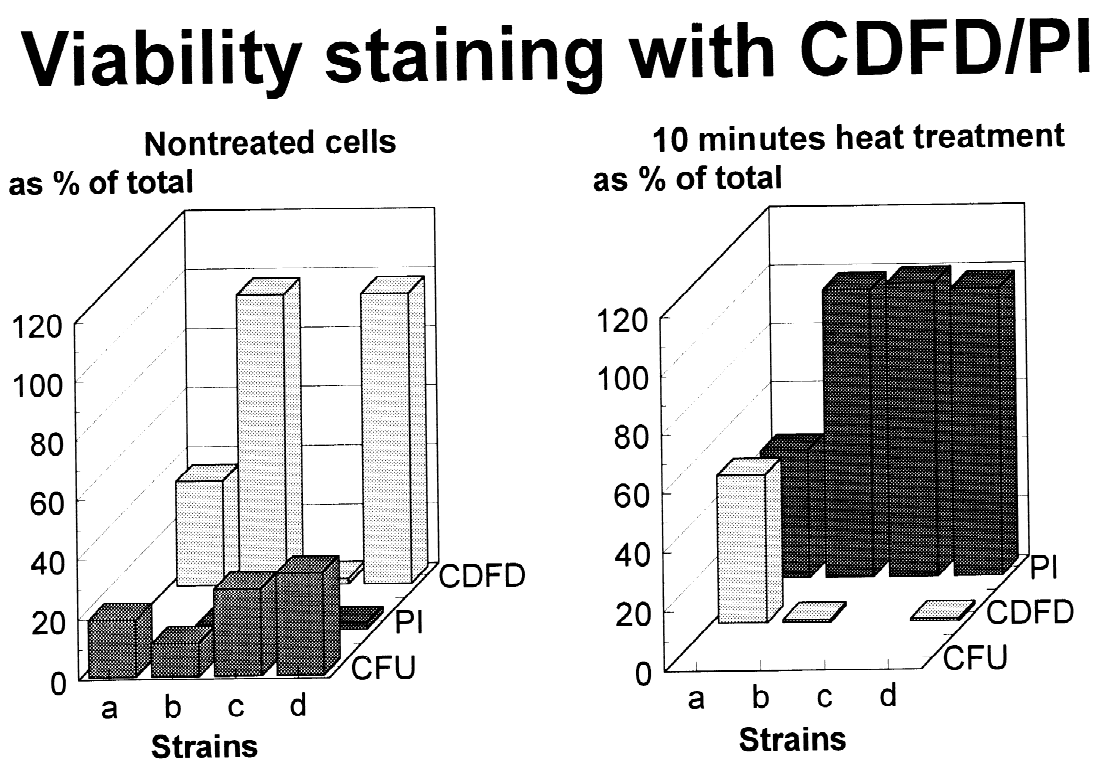

Fig. 4. Viability staining using CDFDA/PI on freshly harvested (a,c) and germinating (b,d) conidiospores of *Paecilomyces variotti* (a,b) and *Aspergillus niger* (c,d). The left panel shows untreated samples while the right panel shows the same samples after a 10 min treatment at 65°C. Values are given as percentage of total freshly harvested/germinating spores present.

the spore head. All the spores in a chain are derived from division of a single nucleus in the phialide at the base of that chain. The spores which loosen from the end of the chain probably do not have the same maturation stage (metabolic activity and physical properties) as the ones just formed on the phialide [19].

Fig. 4 (a,c) shows a typical result of the staining of a suspension of *P. variotti* and *A. niger* conidiospores. As expected, both untreated samples showed no PI staining. Similar results were obtained with *A. nidulans* and *P. roqueforti*. Remarkably, 30% of freshly harvested *T. harzianum* spores already stain with PI indicating an altered membrane permeability (not shown). *P. variotti* showed 35% CDFDA positive spores while only 2% of the untreated *A. niger* spores were stained with CDFDA. *A. nidulans* behaved essentially as *P. variotii* while the other two strains where analogous to *A. niger*.

Upon a 10 minute treatment at 65°C *A. niger* spores were all PI positive. However, *P. variotti* spores were 45% PI positive and remarkably 55% CDFDA positive. Indeed, heat treatment can activate the metabolism of fungal spores [20], possibly enhancing endogenous esterase enzyme activity. *P. roqueforti* and *A. nidulans* behaved as *A. niger* while *T. harzianum* gave similar results a *P. variotii* (not shown). None of these fungal condia gave rise to colonies. In conclusion, (1) untreated conidiospores are not all stained with similar efficiency and (2) upon a severe heat treatment the viability indicator CDFDA still stains some spores.

Fig. 4 (b,d) shows similar experiments performed with a suspension of germinating conidiospores. Here, all untreated *P. variotti* and *A. niger* germinating spores are stained with CDFDA while PI staining remains below 1%. Upon a 10 min heat treatment the situation is reversed, i.e. all germinating spores are PI positive while CDFDA staining remains below 1%. Similar results were obtained with the other

three strains. In conclusion, (1) all germinating spores stain and (2) their staining characteristics are as expected. Experiments with the live/dead stain FUN-1 gave similar results although generally more, but not all, freshly harvested conidia were stained (not shown).

The low frequency of CDFDA staining observed for freshly harvested conidiospores of most strains may be caused by a dormancy state of some of the spores within a population. These may not express their esterase activity fully leading to inhomogeneous CDFDA staining. In order to examine the CDFDA/PI staining of freshly harvested conidia in more detail we performed fluorescence activated cell sorting experiments. Fig. 5 shows that CDFDA stained almost all *P. variotti* spores. Window F contains 98% of all cellular events. However, clearly the staining is heterogeneous. Similar results were obtained with the *A. nidulans* strain. Presumably, the cells stained with a low intensity are scored as unstained upon examination with the fluorescence microscope due to its lower sensitivity. Upon sorting the non-uniformly CDFDA stained population, 20% of the sorted conidiospores gave rise to colonies. This is comparable to the outgrowth frequency of an unstained *P.variotti* spore suspension (see above for discussion). After 1 minute heat treatment, the fluorescence distribution and outgrowth potential were essentially the same. After 5 min at 65°C, 25% of the population was stained in red. Upon sorting this cluster 0.01% of these spores formed colonies. 70% of the spores were stained intense green and weak red. 0.3% of

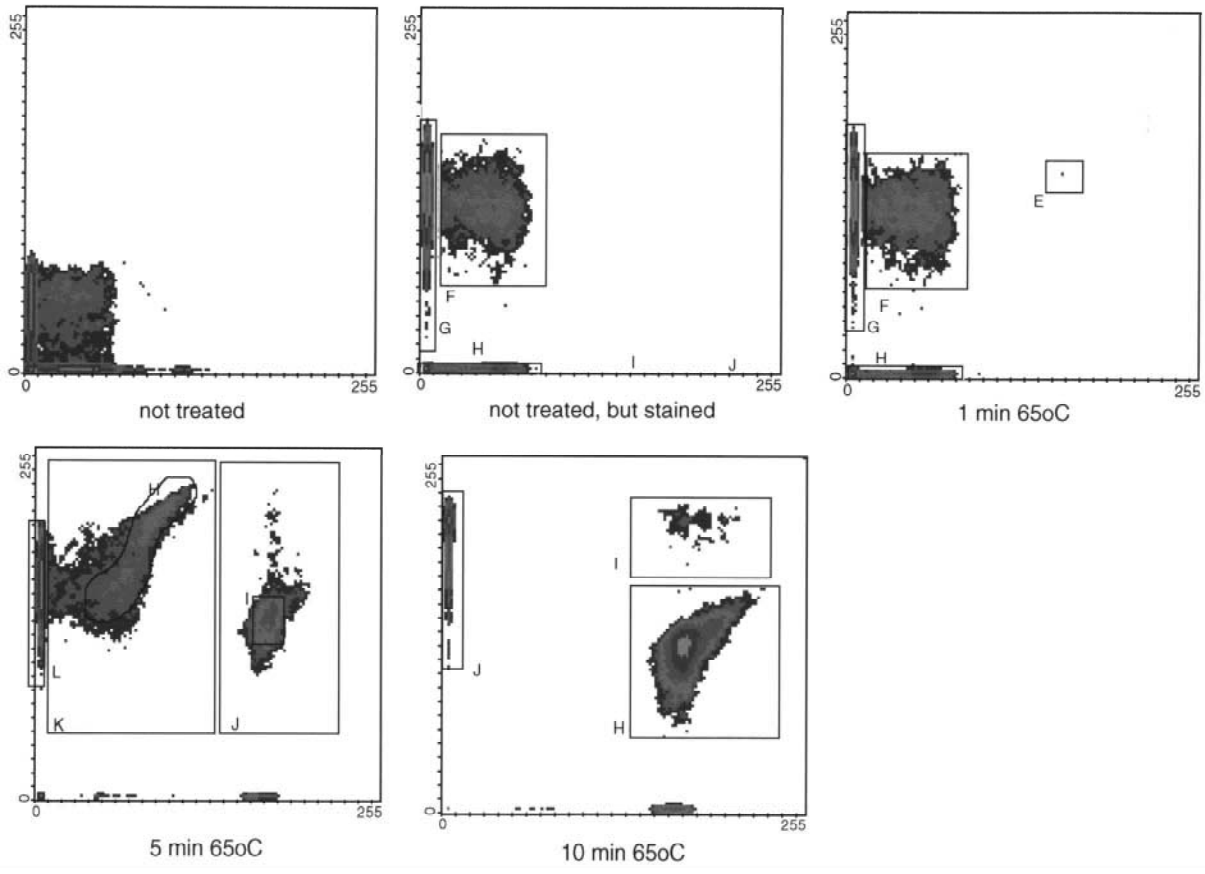

Fig. 5. Fluorescence activated cell sorting (FACS). *Paecilomyces variotti* spores (untreated and treated for various min at 65°C) were stained with CDFDA/PI and analyzed with the FACS. The windows indicate populations which were sorted (see text for further details). The vertical axis gives the intensity of green (CDFDA) staining, and the horizontal axis gives the intensity of red (PI) staining.

these cells grew out. Surprisingly, after 10 min 65°C, the spore population was divided in 12% of high green intensity (window J in Fig. 5), and 80% yellow/red fluorescence (windows H and I in Fig. 5). Neither the former not the latter of these populations showed outgrowth, in accordance with the intracellular ATP content which decreased in both cases to 3% of the initial ATP level.

In conclusion, for freshly harvested conidia CDFDA staining is no indication for outgrowth potential while PI staining indicates absence of outgrowth potential. Further work on the wall composition of fresh fungal spores is in progress in order to identify the cellular structures which cause the heterogeneity of their staining. Possibly, hydrophobins, characteristic proteins present in the outer layer of the conidial wall, are involved [21].

4. Notation

Fluoresceinisothiocyanate (FITC)
Propidium iodide (PI)
Carboxydichlorofluoresceindiacetate (CDFDA)
*Fungo*light (FUN-1)

Acknowledgments

Thanks to Jürg Ueckert and Ad Bos for their support with the FACS experiments and Han Blonk for his help with the CSLM. Drs Theo Verrips, Martin Cole and Frans Klis are thanked for critically reading the manuscript.

References

[1] Scholte, R.P.M. (1995) Spoilage fungi in the industrial processing of food. In: Introduction to Food-borne Fungi (Eds. Samson, R.A., Hoekstra, E.S., Frisvad, J.C. and Filtenborg, O.,). Centraalbureau voor Schimmelcultures, Baarn, Delft, pp. 275–288.
[2] Van den Bossche, H., Marichal, P. and Odds, F.C. (1994) Molecular mechanisms of drug resistance in fungi. Trends Microbiol. 2, 393–400.
[3] Sternberg, S. (1994) The emerging fungal threat. Science 266, 1632–1634.
[4] Harman, G.E., Lorito, M., Di Pietro, A. and Hayes, C.K. (1994) Nagase and glucosidase isolated from *Trichoderma harzianum* and antifungal synergistic combinations of fungal cell wall degrading enzymes. Patent WO 94/24271.
[5] Georgopapadakou, N.H. and Tkacz, J.S. (1995) The fungal cell wall as a drug target. Trends Microbiol. 3, 98–104.
[6] Ueckert, J., Breeuwer, P., Abee, T., Stephens, P., Nebe von Caron, G. and ter Steeg, P. (1995) Flow cytometry applications in physiological study and detection of foodborne microorganisms. Int. J. Food Microbiol. 28, 317–326.
[7] De Nobel, J.G., Dijkers, C., Hooijberg, E. and Klis, F.M. (1989) Increased cell wall porosity in *Saccharomyces cerevisiae* after treatment with Dithiothreitol or EDTA. J. Gen. Microbiol. 135, 2077–2084.
[8] van der Rest, M.E., Kamminga, A.H., Nakano, A., Anraku, Y., Poolman, B. and Konings, W.N. (1995) The plasma membrane of *Saccharomyces cerevisiae*: structure, function, and biogenesis. Microbiol. Rev. 59, 304–322.
[9] Tkacz, J., Cybulska, E. and Lampen, J.O. (1971) Specific staining of wall mannan in yeast cells with fluorescein-conjugated concanavalin A. Journal of Bacteriology 105, 1–5.
[10] Breeuwer, P. Drocourt, J.L., Bunschoten, N., Zwietering, M.H., Rombouts, F.M. and Abee, T. (1995) Characterisation of uptake and hydrolysis of fluorescein and carboxyfluorescein diacetate by intracellular esterases in *Saccharomyces cerevisiae* which result in accumulation of fluorescent product. Appl. Environ. Microbiol. 61, 1614–1619.
[11] Yang, H.C., Nemoto, Y., Homma, T., Matsuoka, H., Yamada, S., Sumita, O., Takatori, K. and Kurata, H. (1995) Rapid viability assessment of spores of several fungi by an ionic intensified fluorescein diacetate method. Curr. Microbiol. 30, 173–176.
[12] Breeuwer, P., Drocourt, J.L., Rombouts, F.M. and Abee, T. (1994) Energy-dependent, carrier-mediated extrusion of carboxyfluoresceindiacetate by intracellular esterases in *Saccharomyces cerevisiae* allows rapid assessment of cell viability by flow cytometry. Appl. Environ. Microbiol. 60, 1467–1472.
[13] Coote, P.J., Billon, C. M-P., Pennell, S., McClure, P.J., Ferdinando, D.P. and Cole, M.B. (1995) The use of confocal scanning laser microscopy (CSLM) to study the germination of individual spores of *Bacillus cereus*. J. Microbiol. Methods 21, 193–208.
[14] Imai, T. and Ohno, T. (1995) The relationship between viability and intracellular pH in the yeast *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 61, 3604–3608.i
[15] Klis, F.M. (1994) Cell wall assembly in yeast. Yeast 10, 851–869.
[16] Schoffelmeer, E., Kapteyn, J.C., Montijn, R.C., Cornelissen, B.C. and Klis, F.M. (1996) Glucosylation of fungal cell wall proteins as a potential target for novel anti-fungal agents. In: Fungicides and Antifungal Compounds (Eds. Lyr, H., Russel, P.E. and Sisler, H.D.) Intercept, Hampshire UK (in press).
[17] Preston, R.A., Murphy, R.F. and Jones, E.W. (1987) Apparent endocytosis of fluoresecein isothiocyanate-conjugated dextran by *Saccharomyces cerevisiae* reflects uptake of low molecular weight impurities, not dextran. J. Cell Biol. 105, 1981–1987.

[18] Granath, K.A. (1958) Solution properties of branched dextrans. J. Coll. Sci.e 13, 308–328.
[19] Ward, M. (1991) *Aspergillus nidulans* and other filamentous fungi as genetic systems. In: Modern Microbial Genetics (Eds. Streips, U.N. and Yasbin, R.E.), Wiley-Liss, New York pp. 455–496.
[20] Griffin, D.H. (1994) Fungal Physiology. 2nd ed., Wiley-Liss, New York .
[21] Stringer, M.A., Dean, R.A., Sewall, T.C. and Timberlake, W.E. (1991) Rodletless, a new *Aspergillus* developmental mutant induced by site directed gene inactivation. Genes Dev. 5, 1161–1171.