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Viability and physiological state transitions of *Rhizopus oligosporus* sporangiospores in tempe starter culture

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Abstract The viability and various physiological characteristics of individual sporangiospores of *Rhizopus oligosporus* in tempe starter cultures that had been stored for 8, 10, 16 and 30 months were examined by flow cytometry in combination with fluorescent dyes. Besides live, dead, and dormant spores we distinguished a category of sublethally damaged spores. Results indicated that the shelf-life of tempe starters was not limited by the death of spores, but by sublethal damage to spores as well as by dormancy which can be overcome by resuscitation, respiratory activation. During storage, the number of dormant and sublethally damaged spores increased: the longer the starter cultures were stored, the less dormant spores could still be activated. In contrast, the transition from sublethally damaged (spores that are not able to transform cFDA and emit green fluorescence except by activation treatment) to activated spores did not decrease with longer storage. However, after very long (30 months) storage, sublethally damaged spores could still be activated but could not germinate anymore. The shelf-life of spores in tempe starter is related to the physiological state of spores being sublethally damaged; a mechanism of physiological state transitions of *R. oligosporus* sporangiospores is proposed.

Keywords Activation · Dormancy · Fluorescent markers · Inoculum · Spores · Sublethal damage

Abbreviations

cFDA	carboxyfluoresceindiacetate
FCM	flow cytometry
PI	propidium iodide
TOTO-1	1-(4,4,7,7-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3dihydro (benzo-1,3-oxazole)-2-methylidene]-1-(3-trimethyl ammonium propyl)-pyridinium tetraiodide

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Introduction

Dormancy has been defined as a reversible state of metabolic shutdown or low metabolic activity (Kaprelyants et al. 1993; Kaprelyants and Kell 1993). This often corresponds to a state in which microbial cells ‘‘are not alive in the sense of being

able to form a colony when plated on suitable solid medium, but in which they are not dead in that when conditions are more favorable they can revert, by a process known as resuscitation, to a state of aliveness as so defined'' as cited from Kaprelyants et al. (1996). Dormant cells may require specific stimuli before they become active and culturable (Kell et al. 1998).

It is widely recognized that classic culture techniques may underestimate the numbers of viable microorganisms, especially when cells have been damaged by physical treatments. Fluorescent staining and detection by flow cytometry (FCM) are widely used (Ritz et al. 2001) as rapid, sensitive and quantitative techniques to detect microorganisms and assess their viability in the field of food microbiology, veterinary science and medical research (Chitarra and Van den Bulk 2003). This technique has demonstrated its potential as a means to assess the physiological state of damaged cells (Ueckert et al. 1997). FCM has been successfully used in several studies to assess the viability of microbial cells in probiotic products and dairy starters (Bunthof and Abee 2002), starved cultures (Kaprelyants and Kell 1993; Kaprelyants et al. 1996), cells having been exposed to antibiotics (Novo et al. 2000) or high hydrostatic pressure (Ritz et al. 2001).

A wide range of fluorescent dyes are available which aim at specific cellular targets, such as DNA, enzyme activities, internal pH, or the cytoplasmic membrane (Ueckert et al. 1995). Cell viability can be assessed using fluorescein derivatives, such as carboxyfluorescein diacetate (cFDA) or carboxyfluorescein succinimidyl ester (Breeuwer and Abee 2000; Davey and Kell 1996; Shapiro 1995). Membrane integrity and activity have also been described as good indicators of viability; these properties can be studied with fluorescent markers, including propidium iodide (PI) or TOTO-1{1-(4,4,7,7-tetramethyl-4,7-diaza-undecamethylene)-bis-4-[3-methyl-2,3dihydro (benzo-1,3-oxazole)-2-methylidene]-1-(3-trimethylammoniumpropyl)-pyridinium tetraiodide}. PI is a nucleic acid dye that generally does not cross intact cell plasma membranes and, hence, only enters into non-viable cells that have lost their membrane integrity. TOTO-1 is a nucleic acid dye that is excluded by intact cells and thus stains a cell only when the membrane is damaged. TOTO-1 is often preferred over PI because the excitation and emission spectra are suitable for FCM, it has a high fluorescence enhancement, and its molecular mass is approximately twice as high as that of PI (Bunthof et al. 2001; Chitarra and Van den Bulk 2003; Hirons et al. 1994).

The purpose of this study was to characterize the viability and physiological states of *Rhizopus oligosporus* sporangiospores (spore subpopulations) in tempe starter cultures that were stored for 8, 10, 16 and 30 months. Flow cytometry in combination with cFDA, PI and TOTO-1 was used to assess the viability, membrane integrity and enzymatic activities of the spores before and after activation.

Materials and methods

Preparation of tempe starter

Tempe starter was prepared as follows (Thanh and Nout 2002): *R. oligosporus* LU 575 (NRRL 5905) was grown on malt extract agar (MEA, Oxoid CM59) for 1 week at 30°C, and spores were harvested by washing with sterile water. The spore suspension was diluted to approximately 10^5 spores per ml. This suspension ''A'' was used as inoculum for substrate used to prepare tempe starter. Polished broken rice was steam-sterilized and allowed to cool. To add nitrogen source and adjust the pH to 4, a volume of 1.5 ml of sterilized ammonium sulfate 1.5 M solution (0.0067 g $(NH_4)_2SO_4$ per g dry substrate) and 1 ml of sterilized H_2SO_4 0.5 M solution were added to the sterilized rice. pH 4 was reported as the optimum pH for spore germination of *R. oligosporus* (Breeuwer et al. 1997). Each batch of sterilized rice (50 g) was inoculated and thoroughly mixed with 0.4 ml of spore suspension ''A'' and incubated first at 40°C for 2 h, followed by 4 days at 30°C. The mouldy mass was broken up and dried at 42°C for 48 h in a forced-air drying cabinet. Crushing by sterile pestle and mortar, but no fine grinding was used in order to avoid misjudging between spores and rice particles with similar size and shape. The starter powder was stored in screw-cap glass tubes, protected from light, in a silicagel desiccator at 25°C.

Preparation of spore suspensions

Sporangiospores in crushed rice powder were suspended in sterile water to approximately 10^7spores/ml, by vigorous agitation and filtered through a coarse Millipore membrane filter [(fluorassure); Chem filter 15, REF: 100-C2003-01; Chemunex] to remove mycelium and other debris. Spore suspensions were washed twice by centrifugation at 13,000 × *g* for 3 min in an Eppendorf centrifuge (Greiner-labortechnik, 500 PP-Microcentrifuge tube 1.5 ml). After decanting, the spores in pellets were re-suspended to experimental suspensions according to the respective aims of studies: either in (a) phosphate buffer 50 mM of pH 7 for untreated and heat-killed samples or (b) in malt extract broth (MEB) for activation.

Heat-killed samples were made by heating spores (10^6/ml, in 50 mM potassium phosphate pH 7) at 100°C for 30 min in a water bath, in covered glass tubes. Activation samples were made by incubating spores (10^6/ml, in MEB) in a water bath at 37°C for 60 min in Eppendorf tubes.

Fluorescent labeling

cFDA [5-(and-6)carboxyfluorescein diacetate], PI and TOTO-1 were purchased from Molecular Probes Europe, Leiden, The Netherlands.

Single staining with cFDA, PI and TOTO-1 to be used as calibration controls

PI or TOTO-1 staining

PI was supplied by the manufacturer as a 1 mg/ml solution in distilled water. This was used as a working solution and stored in the refrigerator in the dark. TOTO-1 was supplied by the manufacturer as a 100 mM solution in dimethyl sulphoxide. The stock solution was further diluted in dimethyl sulphoxide to 100 μM to serve as the working solution. Heat-killed cell suspensions were exposed to 7.5 μM of PI or 1μM of TOTO-1. The mixtures were incubated for 30 min at 40°C in a water bath to stain the spores. Stained samples were kept on ice in the dark and used within 1 h for FCM analysis.

cFDA staining

A stock solution (10 mM) of cFDA was prepared by dissolving 4.6 mg of cFDA/ml in acetone and was stored at − 20°C in the dark. The stock solution was diluted further in acetone to 1 mM to serve as the working solution. Samples containing 10^7 cells/ml were incubated in potassium phosphate buffer (50 mM, pH 7) in the presence of 10 μM cFDA for 30 min at 40°C in a water bath. Stained samples were kept on ice in the dark and used within 1 h for FCM analysis.

Double staining

When dual labeling was performed, we used the same dye concentrations and incubation times as described above. Double staining was applied for untreated spore suspensions and activated spore suspensions of spores stored for 8, 10, 16 and 30 months. Each spore age sample was stained either with cFDA and PI or with cFDA and TOTO-1.

For multiparameter FCM analysis of double stained samples the following controls were used: nonstained, cFDA-stained, heat-killed-PI stained and heat-killed-TOTO-1- stained spore suspensions.

Flow cytometry

Flow cytometric analyses were performed as described recently (Bunthof et al. 2001; Chitarra et al. 2003) on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California) equipped with an air-cooled argon ion laser at 15 mW (excitation wavelength 488 nm). Sporangiospores of *R. oligosporus* at a concentration of 10^6 spores/ml were delivered at the low flow rate, corresponding to 150–500 spores/s. FSC (forward-angle light scatter), SSC (side-angle light scatter), and three fluorescence signals were measured. A band pass filter of 530 nm (515–545 nm) was used to collect the green fluorescence (FL1), a band pass filter of 585 nm (564–606 nm)

was used to collect the yellow-orange fluorescence (FL2), and a long pass filter of 670 nm was used to collect the red fluorescence (FL3). FSC was collected with a diode detector. SSC and the three fluorescence signals were collected with photomultiplier tubes. All signals were collected using the logarithmic mode. A combination of FSC and SSC was used to discriminate the spores from their background. Data were analyzed with the CELLQuest program (version 3.1f; Becton Dickinson) and the WinMDI program (version 2.8; Joseph Trotter, John Curtin School of Medical Research, Canberra, Australia).

Viable spores

Colony formation was measured after FCM analysis by spread-plating spore suspensions on malt extract agar (MEA, malt extract 30 g/l, mycological peptone 5 g/l, agar 15 g/l, Oxoid, CM 59). Colonies were counted after incubation at 37°C for 12 h.

Total spores

Spore suspensions were diluted as appropriate and counted using a Bürker-Türk counting chamber. Microscopic counts were also made similarly to determine fluorescent spores, as described previously (Thanh and Nout 2004).

Results

Differential staining of live and dead spores by cFDA, PI, and TOTO-1 was validated by testing the probes on the following treatments: spores (stored for 8, 10, 16, and 30 months) that were not treated and spores that were activated for 60 min in MEB at 37°C, as well as spores that were heat-killed at 100°C for 30 min (as a control for PI and/or TOTO-1 stained dead spores). FCM analysis of non-activated, unstained spores (stored for 8 months) showed minor (less than 1%) cFDA-, PI- or TOTO-1- fluorescent cells, which may be explained by natural autofluorescence as reported in fungi by Wu and Warren (1984). Single staining with cFDA of spores activated in MEB at 37°C for 60 min resulted only in 59.6% cFDA-stained, and 40.2% non-stained spores. After a thorough heat treatment (100°C, 30 min), single staining with PI or TOTO-1 resulted in 86.5% PI-stained or 84% TOTO-1-stained spores, respectively.

Double stainings with cFDA and PI, or with cFDA and TOTO-1 were used to differentiate viable (active), dead and dormant spores in non-activated and activated suspensions of spores that had been stored for 8, 10, 16, and 30 months. Retention of cFDA by cells indicates enzymatic activity and membrane integrity; retention of PI or TOTO-1 indicates loss of membrane integrity; unstained cells indicate dormancy. In Fig. 1, the dot plots show the unstained cells (indicated as R4) in the lower left corner. Spores stored for 8, 10, 16, and 30-months were exposed to phosphate buffer pH 7 (for non-activated) or MEB (for activation at 37°C for 1 h) and then stained simultaneously with cFDA and PI, or with cFDA and TOTO-1. FCM analysis showed that the cF- and the TOTO-1-labeled populations could be spatially resolved in dot plots shown in in Fig. 1a, b, e, f. R1 and R3 were separate subpopulations that had been calibrated using single stained controls. R2 (PI stained population) was very distinctly separated from others. It was shown earlier with lactic acid bacteria (Bunthof et al. 2001), that subpopulations can be resolved in dot plots using filters FL1 and FL2. PI-stained red fluorescent events could be detected by FL3 detector (Ben Amor et al. 2002). Our plots indicate that this is applicable to *Rhizopus* sporangiospores as well.

Figure 1 indicates the existence of four main subpopulations: R1: cFDA-stained (active), R2: PI-stained (presumed dead), R3: TOTO-1-stained (dead) and R4: non-stained (dormant). R1 (Fig. 1a, c, e, g) increased enormously by activation (Fig.1b, d, f, h) and R2 (Fig. 1c, g) decreased after activation (Fig. 1d, h), while R3 (Fig. 1a, b) remained similar in size. Similar FCM results were found in 10 and 16-months stored spores (not shown).

In Table 1, double staining data with cFDA and PI on non-activated and activated spore suspensions are shown. Spores stored 8 months revealed considerable transitions: cFDA-stained spores increased from 8.9% to 73.2%, while

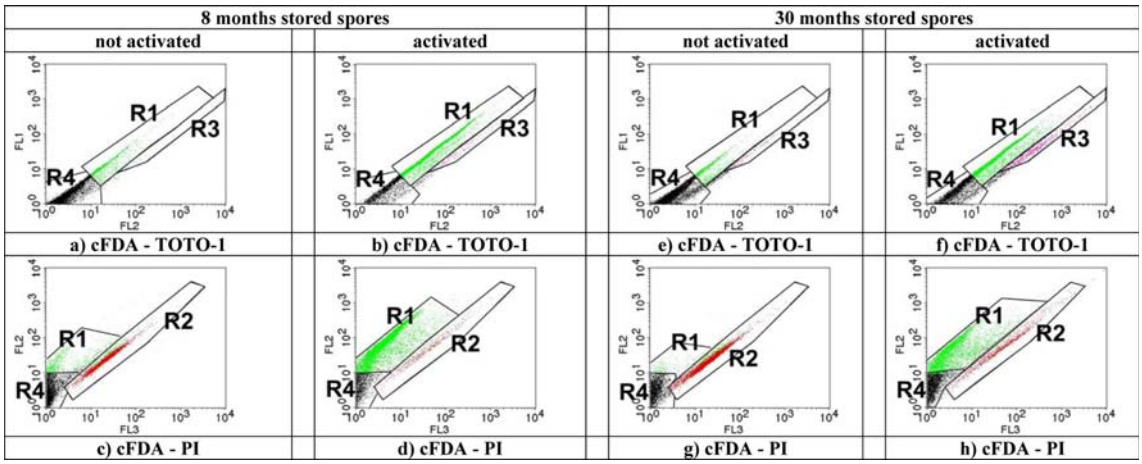

Fig. 1 FCM multiparameter analysis of staining with cFDA, PI, TOTO-1 of 8-months stored spores (**a**–**d**) and 30-months stored spores (**e**–**h**) before and after activation in MEB at 37°C, 60 min. R1: cF stained (metabolically active) population; R2: PI stained (dead + sublethally damaged) population; R3: TOTO-1 stained (dead) population; R4: non-stained (dormant) population. FL1 = fluorescence intensity at 515–545 nm; FL2 = intensity at 564–606 nm; FL3 =intensity at 670 nm

unstained spores decreased from 63.1% to 21.6% and PI-stained spores decreased from 28% to 5.2%. The increase of metabolically active (cFDA-stained) spores approximately represented the sum of the decreases of non-stained and PI-stained cells. Using the same samples and same treatment, we observed that the number of PI stained spores strongly decreased after activation, while the TOTO-1 stained spores remained similar before or after activation (0.7% and 2.3 %, respectively).

In 30 months old spores, cFDA-stained (metabolically active) spores increased considerably from 4.3% to 44.8% by activation, while the number of unstained (dormant) spores increased only a little from 38.8% to 42.8%; PI-stained spores however, decreased strongly from 56.9% to 12.5%, whereas TOTO-1-stained (dead) spores did not change significantly. Similar trends were observed for spores stored during 10 and 16 months (with the exception that unstained spores decreased still considerably after activation).

Our activation and FCM experiments resulted in a pattern of transition of physiological states, related with spore age (8, 10, 16, and 30 months). The longer the storage period, numbers of active spores decreased from 73.2% to 44.8%, and dormant and sublethally damaged spores increased from 21.6% to 42.8% and from 22.8% to 44.5%, respectively.

Discussion and conclusions

In a previous study (Thanh and Nout 2004) we showed that malt extract broth is a good activation medium. During activation with this medium, some interesting transitions took place: the proportion of dormant spores decreased very rapidly, with a concomitant increase of metabolically active spores as measured by fluorescent microscopy using fluorescent markers (Thanh and Nout 2004).

We now examined the usefulness of FCM for viability assessment of *R. oligosporus* sporangiospores. Flow cytometry was chosen for its capacity of multiparameter analysis and the possibility of using several fluorescent viability markers, to determine cell numbers, to measure various physiological characteristics of individual cells, and to distinguish between viable and non-viable cells.

cFDA was tested as a viable cell stain for *R. oligosporus* sporangiospores. cFDA is an esterase substrate that needs both enzyme activity to yield

Table 1 FCM analysis of non-activated and activated spores in tempe starter preparations

PI + cFDA staining

Spore age (months)	Not activated				Activated			
	cF-stained: (active/viable) (%) (Avg. ± SD)*	PI-stained (sublethally damaged) (%) (Avg. ± SD)	Autofluores cent (%) (Avg. ± SD)	Not stained (dormant) (%) (Avg. ± SD)	cF-stained: (active/viable) (%) (Avg. ± SD)	PI-stained (sublethally damaged + dead) (%) (Avg. ± SD)	Autofluorescent (%) (Avg. ± SD)	Not stained (dormant) (%) (Avg. ± SD)
8	8.9^{ab} ± 1.07	28.0^{c} ± 1.87	0.9	63.1^{a} ± 1.85	73.2^{a} ± 1.21	5.2^{c} ± 1.13	0.6	21.6^{c} ± 1.57
10	6.0^{bc} ± 1.79	29.6^{c} ± 1.98	1.2	64.5^{a} ± 1.77	67.8^{b} ± 2.18	6.6^{bc} ± 1.51	0.9	25.6^{c} ± 2.12
16	11.0^{a} ± 2.08	40.7^{b} ± 1.83	1.1	48.3^{b} ± 2.09	54.8^{c} ± 2.25	9.3^{ab} ± 1.58	1.1	35.9^{b} ± 2.06
30	4.3^{c} ± 0.80	56.9^{a} ± 1.37	1.0	38.8^{c} ± 1.51	44.8^{d} ± 1.64	12.5^{a} ± 1.18	1.6	42.8^{a} ± 1.65

TOTO-1 + cFDA staining

Spore age (months)	Not activated				Activated			
	cF-stained: (active/viable) (%) (Avg. ± SD)*	Autofluorescent (%) (Avg. ± SD)	TOTO-stained (dead) (%) (Avg. ± SD)	Not stained (dormant) (%) (Avg. ± SD)	cF-stained: (active/viable) (%) (Avg. ± SD)	Autofluorescent (%) (Avg. ± SD)	TOTO-stained (dead) (%) (Avg. ± SD)	Not stained (dormant) (%) (Avg. ± SD)
8	8.6^{a} ± 1.28	0.1	0.7^{b} ± 0.08	91.4^{a} ± 1.10	70.5^{a} ± 1.46	0.0	2.3^{b} ± 0.54	29.5^{c} ± 1.69
10	8.8^{a} ± 1.46	0.0	0.3^{c} ± 0.02	91.2^{a} ± 1.21	67.2^{b} ± 1.18	0.1	1.8^{b} ± 0.23	32.7^{c} ± 1.52
16	9.4^{a} ± 1.22	0	0.3^{c} ± 0.04	90.7^{a} ± 1.64	58.2^{c} ± 0.73	0.5	1.7^{b} ± 0.12	41.3^{b} ± 1.63
30	8.5^{a} ± 1.28	0.1	1.7^{a} ± 0.13	91.5^{a} ± 1.13	45.3^{d} ± 1.75	0.1	6.5^{a} ± 1.48	54.7^{a} ± 1.41

* Data reported as means of triplicates with standard deviation. In each column, data having the same indicators are not significantly different ($p \leq 5\%$, one-tail unrelated *t* test)

the fluorescent compound and membrane integrity to retain the compound in the cell. Esterase activity is an indicator of metabolic activity, and comparing this activity before and after activation can help to assess the physiological state of spore cells.

PI is a red fluorescent phenanthridinium dye intercalating nucleic acid, that generally does not traverse intact cell membranes and, hence, only enters into non-viable cells with damaged membranes, or into dead cells (Chitarra and Van den Bulk 2003; Lopez-Amoros et al. 1995; Mortimer et al. 2000). Also, TOTO-1, a yellow fluorescent dimeric cyanine dye was used because the excitation and emission spectra are suitable for FCM, it has a high fluorescence enhancement, and its molecular mass is approximately twice as high as that of PI (Bunthof et al. 2001; Hirons et al. 1994).

The seemingly low levels of cFDA-stained spores after activation (Table 1) should be considered in the light of their storage history (8 months or longer); values obtained are in good agreement with our previous results of direct microscopic counting: about 60% fluorescent spores of 10 months old sample in comparison with 93.3% in freshly harvested spores (Thanh and Nout 2004).

As we considered PI-stained spores as representing dead spores, their number would be expected to remain independent of activation treatment. Thus, the decrease of the number of PI-stained spores after activation required further investigation. Therefore, instead of PI, TOTO-1 was used together with cFDA for double staining of the same (8-month stored spores) sample (Table 1). Remarkably, the number of TOTO-1 stained cells was of the same order of that of PI stained cells after activation (2.3% and 5.2%, respectively). We deduce that TOTO-1 is superior to PI in discriminating intact and dead *Rhizopus* spores, as was reported earlier (Bunthof et al. 2001) for lactic acid bacteria.

This may be caused by TOTO-1 being larger than PI; the molecular masses are 1,303 and 668 Da, respectively. Furthermore, the very high fluorescence enhancement of TOTO-1 enables good distinction of nonlabeled and labeled cells in the FCM. The labeling with TOTO-1 gave clear discrimination between live and dead cells, whereas PI gave less contrast. The decrease of the number of PI-stained spores probably was caused by a transition from sublethally damaged cells with reversible membrane permeability into active cells, disabling entry of PI. Seriously damaged spores (with bigger and irreversible membrane damage) were stained with TOTO-1 (as well as with PI) and could be considered as truly dead spores. These could not be resuscitated anymore: the number of TOTO-1 stained spores remained almost similar before or after activation.

It is probable that the simultaneous decrease of PI-stained spores and increase of cF-stained spores is caused by dried cells being able to recover from certain membrane damage during incubation in malt extract broth (MEB). It also clearly shows that PI is not an accurate cell death indicator in fungal cells (spores). This would be in agreement with the resuscitation behavior of *Micrococcus luteus* in activation medium (Kaprelyants and Kell 1993) that was associated with a restoration of their membrane barriers (Kaprelyants et al. 1996). It was concluded (Kaprelyants et al. 1994; Votyakova et al. 1994) that viable cells might produce factors that stimulate the resuscitation of dormant cells and promote the transition of cells from a state in which they are incapable of growth and division to one in which they are capable of colony formation. However, it is unknown whether this happens as well in fungal spores.

In the present study, we considered the resuscitated spores to have been sublethally damaged. Thus, besides viable, dead, and dormant spores in tempe starter cultures we were able to distinguish one more subpopulation, namely sublethally damaged spores. The number of sublethally damaged spores could be quantified by FCM by comparing responses to PI and TOTO-1. Whereas metabolically active (living) spores would be cFDA-positive and negative for TOTO-1 and PI, dormant (inactive but still living) spores are negative for all these stains, sublethally damaged spores (gradually dying but can still be resuscitated, i.e. reversible situation) are cFDA (–), TOTO-1 (–) but PI (+), and dead (irreversible) spores are cFDA (–), TOTO-1 (+) and PI (+).

It is important to note (Table 1) that the increase of activated 8-months-old spores (73.2–8.8 = 64.3%) is due to disappeared dormant (41.6%) and sub-lethally damaged spores (22.8%) and likewise for the spores of other ages. This suggests that not only dormant, but also sublethally damaged spores may transit into viable spores.

It was observed that starved *M. luteus* cells may contain a mixture of dormant and injured cells; moreover, dormant cells could have injuries as well. The resuscitation may actually represent their recovery from injury, or the repair of the membrane barrier (Kaprelyants et al. 1996; Kell et al. 1998). Our data indicate a similar situation for *R. oligosporus*.

The decrease of active spores during storage appears to be related with the decreased transition of dormant spores. In contrast, transitions of sublethally damaged spores did slightly increase. In particular, of the 30 months old starter, 0% of dormant spores made the transition but many sublethally damaged spores (40.5%) were still able to transit to active spores; we presume that the slight increase of dormant spores observed (from 38% to 42.8%, Table 1) could be caused by a low number of sublethally damaged spores that repaired their membrane function but lack metabolic activity thus remaining unstained with cFDA.

This indicates that the transition from dormant to activated spores depends on spore age, being gradually disabled in very old (30 months) spores. On the contrary, the transition of sublethally damaged spores still occurred after long-term storage.

In the proposed model (Fig. 2) transitions may simultaneously take place during activation, from dormant spores to active spores, as well as from sublethally damaged spores (a) to dormant spores that have slightly recovered but that do not stain detectably with dyes and further to active spores, or from sublethally damaged spores (b) directly to active spores. Our data show that depending on age, either all sublethally damaged spores may transit to active spores (8–16 months), or most of them transit to active and a minority to dormant spores (30 months).

The effect of storage on the viability of spores was reported by other investigators. At room temperature, a significant decrease in viability of *R. oligosporus* spores was noted already after 2 months (Wang et al. 1975); germination percentages decreased rapidly at first, followed by a leveling off of the germination percentage, and subsequently declined to very low levels (Rusmin and Ko 1974).

It is interesting that while the numbers of dead spores was quite low (6.5%) after 30 months of storage, and activation is still effective (44.8% of spores activated), only 4.4% of spores were still able to form colonies, a level that is similar to 4.3% of non-activated spores (data not shown).

It can be concluded that after 30 months, spores in starter culture could still be activated but that these active spores have lost the ability to germinate, which may be due to the fact that the activated spores originated from sublethally damaged ones, unlike other younger spores that were activated from dormant spores.

In conclusion, sublethally damaged spores were found in tempe starter cultures whereas the number of dead spores was not high, even after 30 months of storage. The shelf-life of spores in tempe starter is related to the physiological state of sublethally damaged and dormant spores. Both dormant and sublethally damaged spores can be resuscitated and become active spores. Our previous results (Thanh and Nout 2004) and data

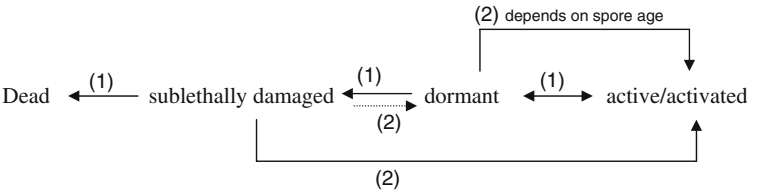

Fig. 2 Flow-sheet of transitions of spore populations

(1) Natural transition during storage
(2) Transitions by activation

in this paper showed that long-stored dormant spores can be activated and can give rise to high numbers of colonies.

This indicates that traditional starters for tempe manufacture which are stored as dry spores, will gradually become sublethally damaged. Up to a rather long storage period (at least 1 year) a majority of these sublethally damaged spores can be resuscitated into metabolically active spores that can germinate. Our earlier results (Thanh and Nout 2002) indicated that storage at ambient temperatures (25–30°C) is less damaging than refrigerated storage. We found that certain amino acids, e.g. alanine, are functional as resuscitating agents (Thanh et al. 2005). It will be of interest to the industry to optimize cheap media for the resuscitation of stored spore starters.

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