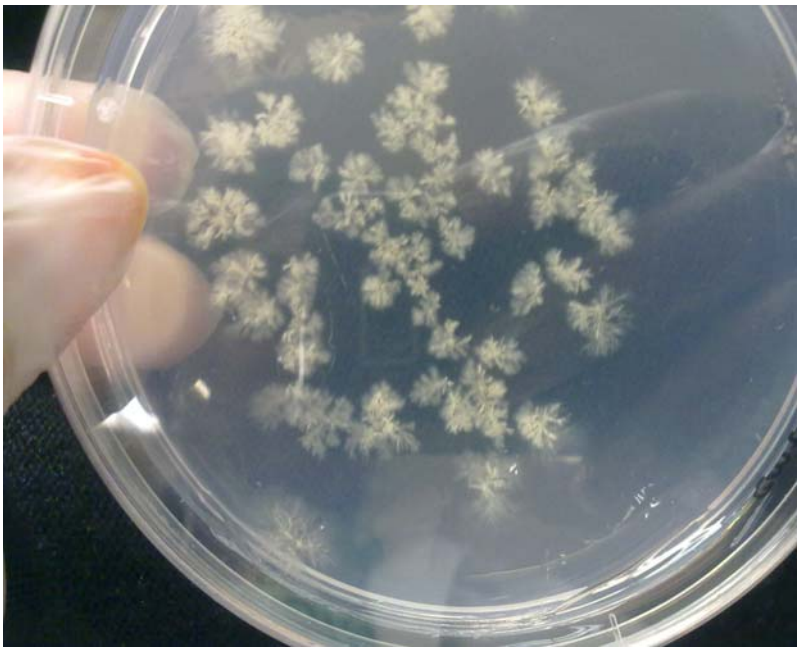

Stress tolerance and growth of trehalose and mannitol deficient mutants in *Aspergillus niger*

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Abstract:

There are several fungal species including *Aspergillus niger* that are responsible for food spoilage. The conidia of *Aspergillus niger* contain mannitol and trehalose and these compounds act as stress protectants and keep the cells viable. In my experiments, I observed the survival after stress in trehalose and mannitol deficient mutants of *Aspergillus niger* and compared with wild type strains. I applied different treatments like heat shock, freeze-drying and different energy sources on mutants and wild types spores at different ages. The results indicate that 1-month-old spores of mutant of Δ*tppB* and Δ*mpdA* are more vulnerable at high and low temperatures. These experiments indicate that the trehalose deficient mutant Δ*tppB* and the mannitol deficient mutant Δ*mpdA* are more sensitive compared to wild type and most probably due to their lower mannitol and trehalose levels.

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Introduction:

Food is expected naturally as nutritious and good energy source for microorganisms. The spoilage of food caused by fungi, yeasts and filamentous moulds, is very common and results in a huge economic loss in food industry all over the world. For this reason, consciousness is raised over safety food owing to the potential production of mycotoxins in foods. *Aspergillus niger* is a recognized spoilage mould causing post-harvest decay of fruit, and is also among the most common fungi isolated from nuts, especially peanuts, cereals, meat products and cheese (Pitt & Hocking, 1997). The spores are also responsible for several human diseases like severe lung disease (aspergillosis) and fungal ear infection (otomycosis).

The conidia of *A. niger* contain mannitol and trehalose, and these compounds are very important for stress tolerance. They support the spores against different stresses and keep them more viable. The gene *mpdA* is responsible for mannitol biosynthesis in *A. niger* (Ruijter et al 2003). The synthesis of mannitol occurs in two steps (Fig1). First step, fructose 6-phosphate is reduced to mannitol 1-phosphate by the enzyme of mannitol 1-phosphate dehydrogenase (MPD). Second step, formation of mannitol by the dephosphorylation of mannitol 1-phosphate, and this step is catalyzed by mannitol 1-phosphate phosphatase (MPP). Hult and Gatenbeck (1978) proposed that the phosphorylation of fructose by hexokinase enzyme helps to complete the mannitol cycle.

The D-mannitol is the major carbon-containing compound in the conidiospores of *A. niger* and it is transported through hyphae by redox balancing. Mannitol works as a good antioxidant for reducing power. Mannitol is also able to quench reactive oxygen species (ROS) and that is essential for fungal species to prevent oxidative damage.

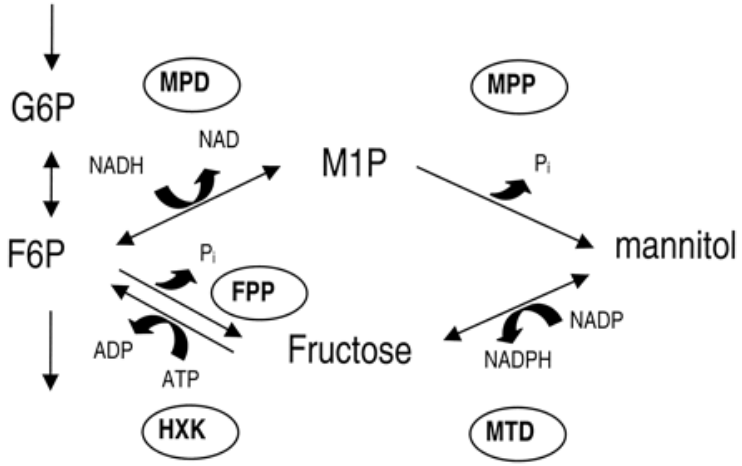

Fig 1: Mannitol metabolism in *A. niger* (From Ruijter et al., 2003).

Trehalose (a-D-glucopyranosyl-1, 1-a-D-glucopyranoside) is a non-reducing disaccharide, formed by α, α-1, 1-glucoside bond between two α-glucose units. In fungi, trehalose is accumulated in mycelia and conidia during the stationary phase and metabolized rapidly, once the growth is resumed or germination initiated (Wolscheck and Kubicek, 1996). It functions as a resistance against different physical stresses like heat, dehydration, and hyper-osmotic shock. For having a number of physico-chemical properties like high hydrophilicity, chemical stability, non-hygroscopic glass formation and the absence of internal hydrogen bond formation, trehalose is able to serve a unique role as stress metabolite (Arguelles, 2000; Thevelein, 1996). It also involved in metabolic signaling and regulation of carbohydrate metabolism (Hounsa et al., 1998; Arguelles et al., 2000). Trehalose is capable of protecting the integrity of the cell against various environmental damage and nutritional limitations.

The biosynthesis of trehalose in fungi occurs in two steps (Fig 2). First, glucose molecule is converted to glucose-6-phosphate by hexokinase enzyme. Then, trehalose-6-phosphate synthase (Tps) enzyme catalyzes the production of trehalose-6-phosphate (T6P) from glucose-6-phosphate and UDP-glucose. Finally, trehalose is formed by removing the

phosphate group from trehalose-6-phosphate and the enzyme of trehalose-6-phosphatephosphatase (Tpp) catalyzes this step (Nadia Al-Bader *et al*, 2010). The enzyme trehalase also recycles trehalose back to glucose when trehalose is required for energy. (Jorge *et al*, 1997)

The related enzymes of trehalose biosynthesis and trehalose hydrolysis have been well characterized in *Saccharomyces cerevisiae*. Biosynthetic genes include *GGS1/TPS1*, encoding trehalose-6-phosphate synthase, *TPS2*, encoding trehalose-6-phosphate phosphatase; *TSL1* and *TPS3*, redundant genes, probably encode a large regulatory subunit of the trehalose synthase complex (Nadia Al-Bader *et al*, 2010).These proteins are co-regulated both at the genetic and protein level and they form a protein complex for catalyzing trehalose biosynthesis (Bell *et al*.,1998; Winderickx *et al* .,1996).

In *Aspergillus nidulans,* the *orlA* (TppA) gene is important for normal development of the fungus. It is also necessary for their thermo-sensitive growth and response to sub-lethal exposure to oxidative stress.

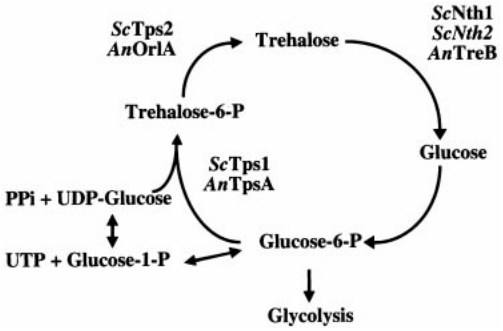

Fig 2: Trehalose metabolism in fungi. *Sc*Tps1 and *An*TpsA, trehalose-6-phosphate synthase; *Sc*Tps2 and *An*OrlA, trehalose- 6-phosphate phosphatase; *Sc*Nth1, *Sc*Nth2 and *An*TreB, neutral trehalase; *Sc*, *S. cerevisiae*; *An*, *A. nidulans* (Fillinger *et al*., 2001).

Svanström and Melin, (unpublished observation) mentioned that in *A. niger* six proteins are identified in the trehalose synthesis; TpsA - C and TppA – C. A mutant of tppB (that

we used in our experiment) contains a lower internal level of trehalose. According to Wolschek and Kubicek (1996), inactivation of the *tpsA* gene results in a loss of T6P activity. The exact roles of the other gene products in trehalose metabolism have not been examined yet.

The aim of this project is to observe the growth of trehalose and mannitol deficient mutants of *A. niger* in comparison with wild type strains. The characteristics of these mutants are not very well known. Therefore, the objective of my project work was to examine the characteristics of the reduced trehalose and mannitol mutants in *A. niger*. The strains Δ*tppB* (lower trehalose content), Δ*mpdA* (reduced mannitol content) and wildtypes *pyrG+* and *N402* were used in my experiments. Deletion of the *A. niger* kusA gene encoding the ortholog of the Ku70 protein in other eukaryotes (Meyer et al. 2007) stops heterologous recombination and enables more efficient gene targeting.

I have investigated: (1) if there is any difference between the different growth media used for regeneration of spores after stress treatment. Minimal agar media and malt extract agar media were used for this purpose. (2) Do the trehalose and mannitol contents affect the survival of spores after heat treatment or freeze-drying? Different temperatures with different exposure times were applied for investigating the survival capacity of these four strains. (3) What are the differences in survival of young, intermediate or old spores? Different aged spores were examined to identify the viability of young, intermediate and old spores.

Materials and Method:

Name of the strains and their genotypes:

Table 1: *A. niger* strains used in this study

Name	Genotype	Reference
N402	*cspA*	(Bos et al. 1988)
ΔmpdA	ΔmpdA:pyrG, ΔkusA:amdS, cspA, pyrG1.	(Meyer et al. 2007)
ΔtppB	ΔtppB:pyrG, ΔkusA:amdS, cspA, pyrG1	Svanström and Melin, (unpublished)
pyrG+	ΔkusA:amdS, cspA	Svanström and Melin, (unpublished)

Fungal maintenance and spore preparation:

The study was conducted with the four strains of *A. niger* Δ*tppB,* Δ*mpdA, pyrG+* and *N402.* The plates were prepared with minimal media; 6 g agar, 4g glucose and 400 ml H_2O. Minimal media was prepared by 16 ml 25× MN salts (120 g $NaNO_3$, 10.4 g KCL, 9.5 g KH_2PO_4 and total volume 800 ml was made up with M&M water). After that, autoclaved (121 ^{0}C) for 25 min. 8 ml 50×$MgSO_4$ (10.32 g $MgSO_4$.6 H_2O, total volume 400 ml was made up with de-ionized water and autoclaved (121 ^{0}C) for 25 minutes. 0.4 ml trace element (80 ml M&M, 2.2 g Zn SO_4.7 H_2O, 1.1 g H_3BO_3, 0.5 g $MnCl_2$.4H_2O, 0.5 g $FeSO_4$.7H_2O, 0.17 g $CoCl_2$.6H_2O, 0.16 g $CuSO_4$.5H_20, 0.15 g Na_2MoO4.2H_2O, 5.0 g EDTA.Na_2) and then boiled for 10 minutes, pH was adjusted with 1M and 10 M KOH and then total volume of 100 ml was prepared). The malt extract agar (MEA) media was prepared by 25 g MEA and the total volume was adjusted up to 500 ml M&M water. The MEA media was autoclaved (121 ^{0}C) for 20 minutes.

A. niger strains were grown on MEA by transferring spores at three distinct spots on the agar plate. The cultures were incubated at 25°C for up to four weeks before harvesting the conidia. Spores were taken out from plates and mixed with 500 µl tween buffer in a tube (1.5 ml) then vortex 1 minute. An aliquot of 50 µl spore suspension was transferred to another tube (1.5 ml). This 50 µl spore suspension was diluted with 450 µl NaCl and vortex again and was called the first dilution. 50 µl was loaded on a Bürker chamber from

the first dilution for counting the number of spores by Microscope. The spore suspension was adjusted by adding 0.15 M NaCl to get final a concentration 5×10^4 spores／ml, and this was designated as the control (untreated sample) in each experiment. Then the spore suspension was incubated at different temperatures for different times or subjected to freeze-drying.

Preparation for measuring radial growth:

To measure the radial growth, 3 day old spores in suspension were inoculated at the center of MEA plates and incubated at 25°C. The colony diameters were then measured after 5, 10, 15 and 20 days of incubation.

Preparation for Heat treatments and freeze-drying:

To find out the effect of stress tolerance of the spores, different temperatures like 45°C, 50°C and 55°C were used. For all treatments, 1 ml spore suspension (10^4 spores／ml) was held in a 2 ml plastic tube. After incubation in 45°C for 72 h, the spores were collected for inoculation. For 50°C treatments, the spores were collected after 4 h incubation. For the treatment 55°C, the total incubation time was 2 h and within this period, the spores were collected at 30 min intervals for inoculation.

To analyze the effect of freeze-drying on the spores, 1ml of samples (10^4 spores／ml) was transferred to glass vials and refrigerated at 4°C before freeze-drying. The samples were prepared as the same way as previously described. After 24 h, vials were placed in the freeze dryer. The samples were freeze-dried using the following protocol: initial freezing -50°C, 40 mTorr 60 min; primary drying -20°C 40 mTorr 120 min, +5°C min^{-1} to 0°C and maintained for 1,000 min (5.3 Pa); secondary drying +0.1°C min^{-1} to 10°C and kept for 240 min, then +0.1°C min^{-1} to 15°C and maintained for 240 min (5.3 Pa). After completion of the freeze-drying, the temperature was set to 10°C 40 mTorr and the vials were sealed before releasing the vacuum. The freeze-dried samples were put again in fridge for further analysis. Before plating out the freeze-dried spores samples, 1ml

sterilized water was added to each freeze-drying vial. After 1 minute at room temperature the suspensions were diluted and plated on MEA media.

Calculation of percentage spore survival

To calculate the ratio of survived spores after temperature or freeze-drying stress, 100 µl of spore suspensions from control and treated spores were transferred to 900 µl 0.15 M NaCl and then serially diluted in NaCl. For each treatment, 100 µl was spread on three replicate MEA plates. After 72 hours of incubation at 25°C the numbers of growing colonies were counted. The percentage (%) of survival was calculated as the proportional number of colonies from treated spores relative to the number of colonies from the control.

Results:

Radial growth of different strains:

The radial growth of different strains of *A. niger* were evaluated on malt extract agar to identify any differences in morphology and growth rate. The inoculated strains were observed during the 20 days and all colony diameters were between 30–35 mm (Fig 3). Also, I could not detect any differences in morphology between the four strains.

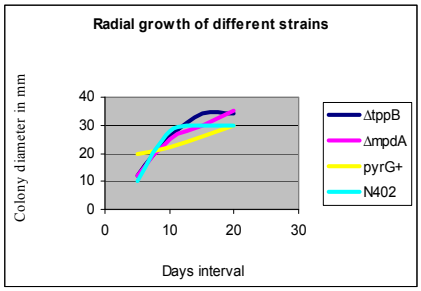

Fig 3: Colony diameter of the four different strains of *A. niger* on MEA

Survivability in different growth media:

Which growth media was more favorable at 50 ^{0}C for the survival of different strains after heat-treatment at 50 ^{0}C, was the objective of this experiment. The spores used in this trial were 10 days old. Results showed that the survival rate of wild type strain *N402* was high in both nutrient sources compare to other strains, and Δ*tppB* had a poorer survival rate. However, strain *N402* grew slightly better in glucose media compared to malt media but other strains did not show much different survival rate (%) in glucose and malt media (Fig 2).

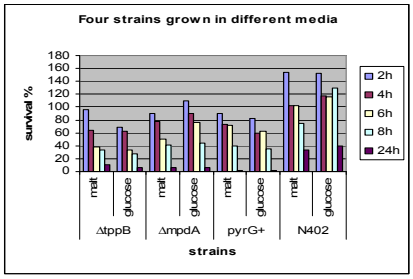

Fig 4: Survival of spores from the four tested strains treated at 50°C and re-plated on either glucose containing minimal media or malt containing MEA.

Temperature effects on survival:

To find a suitable temperature for the heat stress assays I tested 3 day, 10 day and 1 month old spores from the four different strains at 45 ^{0}C, 50 ^{0}C and 55 ^{0}C. After incubation, the survivability was analyzed on MEA media. After 4 h incubation at 45^{0}C, most of the spores were viable (results not given). But, after 72 h incubation the survivability of mutant type strains were lower than the wild types strains. The survivability of mutant type strains Δ*tppB* and Δ*mpdA* were 20% and 14% respectively (Fig 5). The survivability of all types of strains were very good at 50 ^{0}C for 4 h incubation (results are not given). Finally I found that 55°C was a suitable temperature to perform the experiments at because here a decline of viable spores could be observed after a shorter period compared to treatment at 45°C or 50°C.

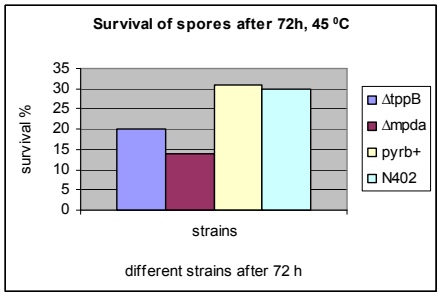

Fig 5: Survival of spores at 45 ^{0}C for 72h

Stress tolerance of different days old spores:

The stress tolerances of *A. niger* were observed among different aged spores at 55 ^{0}C. For the case of 3 day old spores, viability decreased gradually with time interval after incubation 55 ^{0}C at 30, 60, 90, 120 min. Results indicated that after 120 min of incubation at 55 ^{0}C, all spores of all strains were going to die. The number of viable spores of Δ*mpdA* strain was 125 cfu/mL before heating and after heating that decreased to 40, 39, 28 and 18 cfu/mL with time, respectively. However, this strain showed the highest survivability rate compared to other strains. So we can conclude that 3 day old Δ*mpdA* strains was less sensitive and *pyrG*+ was highly sensitive at 55^{0}C (Fig 6). For the case of 10 day old spores, Δ*mpdA* was more sensitive than *pyrG*+ strain (Fig 7). However, the 1-month-old strain *N402* was less sensitive and Δ*tppB* was more sensitive. The number of 1 month-old spores of Δ*tppB* was 1933 cfu/mL before heating and after heating, only 41 cfu/mL were viable (Fig 8).

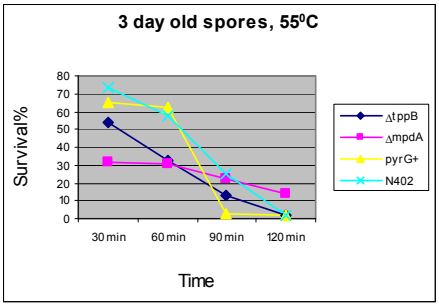

Fig 6: Growth of 3 day old spores at 55 ^{0}C.

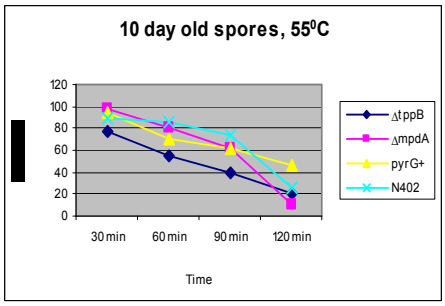

Fig 7: Growth of 10 day old spores at 55 ^{0}C.

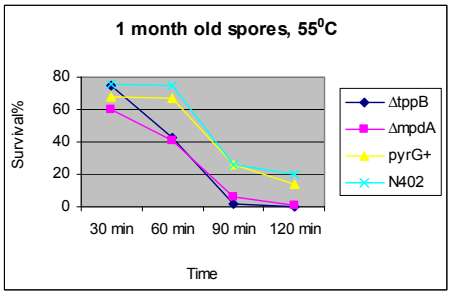

Fig 8: Growth of 1month old spores at 55 ^{0}C.

Freeze-drying effect:

Survival of young, intermediate and old spores:

By stressing the spores in the freeze-dryer, I wanted to check if there were any differences in tolerance between the stains and if the maturation of the spores had any effect. Freeze dried 1 month old spores are more sensitive than 3 day old spores and 10 day old spores are less sensitive. Generally, Δ*tppB* and Δ*mpdA* mutants of all ages are more sensitive than others are (Fig 9). I counted the all freeze-drying spores at two times. At first, I inoculated the freeze-drying samples on plates immediately after freeze-drying. Then put the replicates of the same freeze-drying samples in fridge and inoculated them again on plates after 10 days. So, one replicate was plated directly after freeze drying and other replicates put in fridge for 10 days and then inoculated on plates. Interestingly I got some variation of results although I worked with same freeze drying samples. The number of 3 day and 10 day old spores decreased which were not plated directly. But the survivability of 1-month-old spores increased after putting them in fridge (Fig 10).

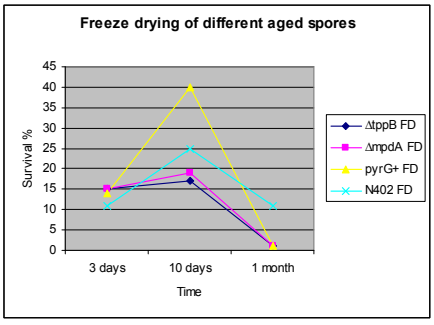

Fig 9: Growth of spores immediately after freeze-drying.

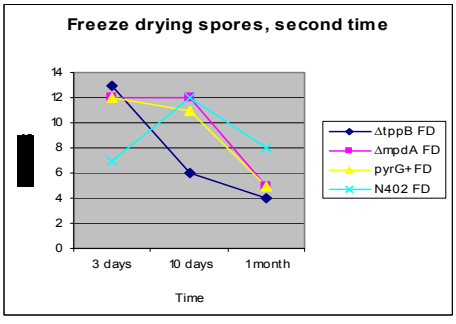

Fig 10: Growth of spores after of freeze-drying, and storage for 10 days at 4°C.

Discussion:

In my experiments, there are no differences in survival dependent on which media the spores were grown. For the sensitivity test of the different days old spores at 55 ^{0}C, 3 day old Δ*mpdA* strain showed less sensitivity than wild type *pyrG+*. The reason could be the trehalose in spores increases to very high levels after 3 days of growth (Svanström and Melin, unpublished data). This trehalose level is high when the spores are younger. For this reason, 3 day old Δ*mpdA* was less sensitive to heat stress. For some reason the *pyrG+* strain is often extra sensitive and in young conidia the trehalose level is very low. With increasing age, the trehalose level in *pyrG+* moves towards the wild type. For this reason, 10 day old *pyrG+* strain had the greatest survival rate than other three strains after heat stress and freeze-drying. In my experiment, I found that 1-month-old *N402* are less sensitive and Δ*tppB* are much more sensitive. These spores are the oldest spores of my experiment. The reason could be the mannitol and trehalose levels in mutants increase with age up to 2 weeks then it starts to decline. For this reason, Δ*tppB* and Δ*mpdA* are much more sensitive compared to wild types.

Spores from plates that were incubated 1 month, show less viability after freeze-drying and heat shock compared to 3 day and 10 day old spores. In the plate at first, there were enough places for growing spores and sufficient nutrient for survive. When number of spores increase, then their availability of getting nutrient decreased. It is not possible to grow many spores in a fixed growing place. Therefore, the number of viable spores going to decrease day by day and it occurs naturally. This idea suggested that 3 day and 10 day old spores have a higher viability than 1-month-old spores. There is no obvious reason why freeze-dried spores in fridge, were less viable compared to spores that were plated directly. One explanation could be that the damaged spores could not recover in the fridge and eventually the ability to germinate was lost. In contrast, the spores directly transformed to the rich medium had better chance to repair and germinate. I found 10 day

old *N402* freeze-drying spores had the best survivability both with direct plating and after storage.

The *A.niger mpdA* gene has been investigated by Ruijter *et al*. (2003), who cloned and characterized the *mpdA* gene encoding mannitol 1-phosphate dehydrogenase (MPD) from *A*. *niger*. These authors incubated the spores for 1 h at 50 ^{0}C and found that only 5% of Δ*mpdA* conidia were viable and the viability for wild type conidia was 100%. They did not mention the ages of spores. I have used the same temperature 50 ^{0}C for 3 day old spores and found most of them alive. Storage of conidia in suspension for up to 24 days at 25°C, they did not find any loss of viability. But I found the loss of viability of 1-month-old spores when they were heated with 55 ^{0}C. The differences between my results and their study could be for the differences for strains and genotypes. However, in their study the *mpdA* mutant was made in a target strain with a different genetic background to the mutant strain that I used. The age of spores is also an important matter for different results. In my experiment, I worked with single mutants. Double mutants can give good results. Another thing is that the analysis were not done by statistics, if it possible then it can provide the more identical and significant results. The weakness in my experiments is that the experiments were not repeated or replicated. It is necessary when the differences in viabilities among the strains are quite low.

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