Quality determination of *Aureobasidium* cells in the fermentation liquid of a wood protective biofinish

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INTRODUCTION

Unprotected wood will usually be degraded during longer outdoor exposure. A new technology based on a protective and decorative microbial coating is in development to improve the service life of wood and reduce the maintenance costs. This biofinish protects wood against UV-radiation, wood-decay fungi and has self-repairing properties.[1,2] *Aureobasidium* occurs naturally on wood surfaces and coatings[3-6], and has been shown to be one of the most important fungi forming the biofinish.[1] *Aureobasidium* is also adaptable to various stressful conditions, it is considered to be polyextremotolerant.[7]

An important question in the development of the biofinish is the viability of *Aureobasidium* cells. To get an impression of the quality of the fungal suspension, cells of *Aureobasidium* were stained with a live/dead staining. For cell viability the stains acridine orange/ethidium bromide were used to stain the viable *Aureobasidium* cells.[8,9] This fluorescent staining is used to visualize changes in the nucleolus and allows therefore to distinguish between live and dead cells.[10,11]

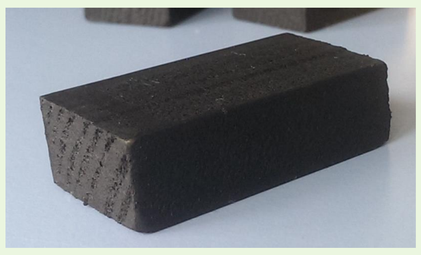


Figure 1: Wood sample (Pinus silvestris) treated with biofinish

Since at outdoor applications on dark surfaces Temperatures up to 70 °C can be created.[12] Different temperature treatments were used on *Aureobasidium* cells.

METHODS

Sample preparation

Aureobasidium cells were cultured from the microbial fermentation liquid. Cells were grown on malt extract agar (MEA) and obtained after a week of growth at 23 °C. Cells from the edge of the colony were placed on a microscope slide or dissolved in phosphate buffered saline (PBS) and treated at different temperatures (table 1).

Table 1: *Treatments of Aureobasidium cells*

Sample	Treatments
Aureobasidium cells on a microscope slide	Untreated
Aureobasidium cells on a microscope slide	Heat fixed
Aureobasidium cells on a microscope slide	3 hours at 70 °C
Aureobasidium cells on a microscope slide	3 hours at 120 °C
Aureobasidium cells on a microscope slide	Steam treated using a steam cooker (95 °C)
Aureobasidium cells solved in PBS	Untreated
Aureobasidium cells solved in PBS	3 hours at 70 °C
Aureobasidium cells solved in PBS	3 hours at 120 °C
Aureobasidium cells solved in PBS	Steam treated using a steam cooker (95 °C)

Acridine orange / ethidium bromide staining

The staining was prepared with 20 µl of 1% ethidium bromide in water and 20 µl of 5 mg/ml acridine orange was dissolved in 1 ml PBS.
Samples were diluted with 10 µl acridine orange/ethidium bromide staining.
Cells were visualized with a fluorescent microscope using a BP490 filter.

The amount of live and dead *Aureobasidium* cells dissolved in PBS was analysed by counting green and red cells with a Bürker-Türk counting chamber, 100 till 150 cells were evaluated on colour.

Colony Forming Units (CFU)

Cells from the edge of the colony were dissolved and vortexed in 1,5 ml 0,9% physiological saline solution (PSS). Prior to the CFU-test cells in 0,9% PSS were counted with a Bürker-Türk counting chamber. After counting the amount of cells, dilution series up to 10^{-6} were made in a 0,9% PSS. After 7 days of incubation the CFU were counted and after 14 days of incubation the CFU were controlled on more growth.

DISCUSSION AND CONCLUSION

In the context of this research the viability of *Aureobasidium* cells in the coating is a relevant factor. Therefore, the quality of *Aureobasidium* cells in the fermentation liquid of the biofinish is partially determined. A quantification method to stain live and dead *Aureobasidium* cells with acridine orange combined with ethidium bromide is developed. Living *Aureobasidium* cells seen with this staining were not in the definition viable. Viable cells are alive, but also capable of growth. Compared with de CFU-test results not all of the living cells were capable of growth.
Chlamydospores in the microbial fermentation liquid didn't colour with this staining, because their thicker dark coloured cell walls make it difficult for substances to penetrate.[13]

One of the selection factors for *Aureobasidium* growth on wood is probably the influence of temperature. Earlier studies show that *Aureobasidium* is able to survive at higher temperatures. Results of the live/dead cell counting reveal a high percentage of living cells at samples without any thermal treatment. A smaller percentage of living cells was found at samples treated at 70 °C. Earlier studies suggest that at outdoor applications on dark surfaces temperatures up to 70 °C or more can be created. Steam treatment of *Aureobasidium* cells on an microscope slide show more dead cells on top and living cells beneath. On a microscope slide cells are probably more protected during temperature exposure than in a suspension, which would explain the lower percentages of living cells in the suspension and the absence of culturable micro-organisms in the CFU test. This is emphasized by treatment of *Aureobasidium* cells at 120 °C with less living cells in the suspension compared to the microscope slide. In the CFU-test with the treated *Aureobasidium* cells no culturable micro-organisms were found. This suggests that after these treatments no viable *Aureobasidium* cells were present in the suspensions although the staining indicates that there were some living cells.

RESULTS

Impression of *Aureobasidium* cells and live/dead staining

Figure 2 gives an impression of *Aureobasidium* cells taken directly from MEA plate and treated on a microscope slide at different temperatures. Acridine orange/ethidium bromide stained live and dead *Aureobasidium* cells. Black chlamydospores didn't colour with this staining, so these cells weren't quantified.

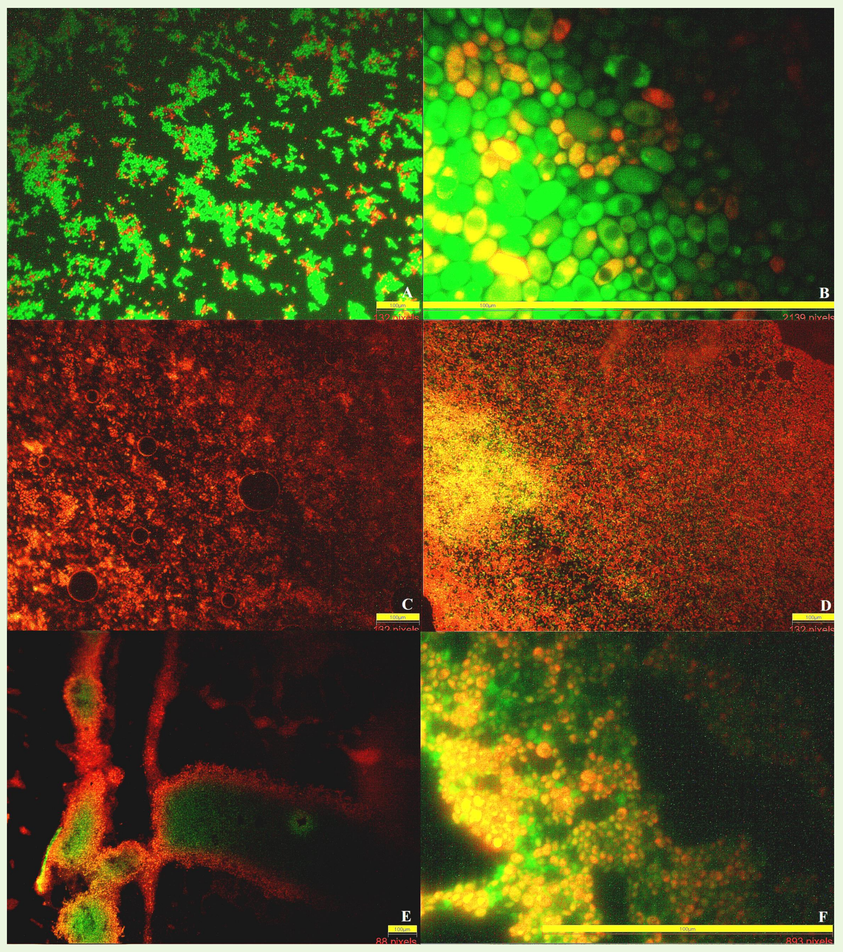


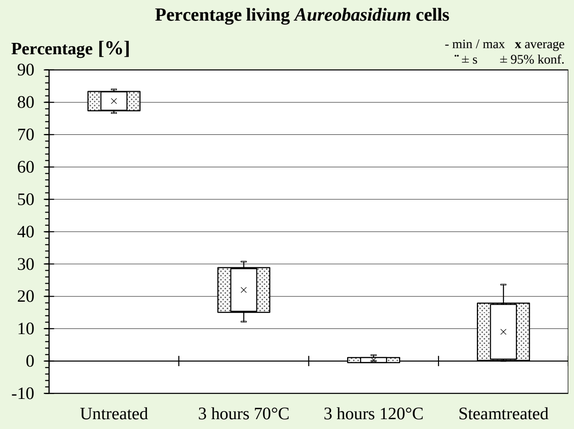
Figure 2: Global impression of Aureobasidium cells treated with acridine orange/ethidiumbromide. A/B) untreated ([a] 100x magnification, [b] 1000x magnification); C) heat fixed (100x magnification; D) incubated for 3 hours at 70 °C (100x magnification); E) incubated for 3 hours at 120 °C (40x magnification); F) steam treated (400x magnification)

Percentage living *Aureobasidium* cells

Figure 3 shows the percentages of living *Aureobasidium* cells dissolved in PBS and treated at different temperatures.
Results of staining and counting live and dead cells showed:

- Around 80% living *Aureobasidium* cells (untreated)
- Around 25% living *Aureobasidium* cells at a temperature of 70 °C
- A few living cells at a temperature of 120 °C
- Around 10% living *Aureobasidium* cells with a steam treatment

Figure 3: Percentage living Aueobasidium cells solved in PBS, stained with acridine orange/ethidium bromide and counted with a Bürker-Türk counting chamber



Colony Forming Units (CFU)

- Counted cells before CFU-test: $1{,}2*10^6$ cells/ml.
- CFU/ml for the untreated samples: $5{,}5*10^4$ CFU/ml

This means an average percentage of 5% growing *Aureobasidium* cells.

At conditions of 70 °C, 120 °C and steam treatment no culturable micro-organisms were found using the CFU method.

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