1.1. Introduction: Background & aim of thesis

Wood under processing as well as in service especially when undergone chemical treatments can be a source of various risks for human, animal and environment health. Building and furnishing materials (wood) and their coatings (paints and varnishes applied, possibly wood preservatives) are major sources of VOCs (volatile organic compounds) emissions in the indoor environment (Franchi 2004). Solid wood, wood composites and their coatings for example emit different VOCs in the form of aldehydes, terpenes, alcohols, ketones and others (Wieslander et al. 1997, Fortmann et al. 1998, Risholm-Sundman et al. 1998, Baumann 1999, Hodgson et al. 2002, Manninen et al. 2002, Wiglusz et al. 2002, Makowski et al. 2005, Schneider & Dix 2006, Roffael 2006). α-pinene is an important constituent of monoterpenes released from fresh solid wood. Formaldehyde as the simplest and most common aldehyde is seen as the most important single indoor air pollutant (Franchi 2004). Fresh soft wood and wood composite products are among the sources of formaldehyde emissions (Hodgson et al. 2002, Wiglusz et al., 2002, Roffael 2006). Compared to natural wood, conventional wood composites release more formaldehyde due to the formaldehyde-containing bonding agents used in manufacturing of wood composites. Formaldehyde is known to be potential harmful compound released from wood. Treatments of wood with harmful chemical preservatives to repress microbial and insect attacks can also cause various environmental and health risks (Westlund & Nohrstedt 2000, Dahlgren et al. 2003, Dube et al. 2004, Stook et al. 2005). Wood in usage within the indoor environments contribute for the indoor air pollution with the emission of harmful VOCs, thereby causes illness in the dwelling people. Harmful VOCs from wood and related products cause sick building syndrome, sensory irritation and asthma related symptoms in the humans (Nishimoto et al. 2000). Thus, pollutants from wood and related sources need to be monitored.

Chemical detection methods such as gas chromatography (GC) and other conventional chromatography methods can identify a specific compound by comparison with suitable references (Van der Meer et al. 2004). Usually, conventional methods measure the quantity of pollutant accurately, but these chemical methods cannot reveal the potential harmful effects of the pollutants on living systems. Biomonitoring of hazardous compounds makes use of living organisms and their products. These respond to an individual harmful substance or a group of substances with similar harmful effects on the

biological system and also effects of compounds of unknown chemical nature. Most importantly, they detect only the bioavailable pollutants. Furthermore, biological test systems can access harmful effects of a sum of different hazardous compounds being individually present at very low concentrations (Rila & Eisentraeger 2003). The sensitivity and versatility of bioassays can thus exceed that of chemical methods. For example, a bioluminescent bacterium called *Vibrio fischeri* serves in a world wide accepted standard bioreporter test that is approved by product list for homeland security to evaluate the harmful effects of a large group of pollutants. The bacterial cells which produce detectable proteins can be used as bioreporters for detecting the harmful effects of pollutants (see chapter 2 of this thesis for details). A decrease in the bioluminescence gives the information on strength of the harmful effects of the analyte (Chang et al. 1981) (see chapter 2 of this thesis for further reading).

Free-living *V. fischeri* in the ocean waters inoculate the light organs of the juvenile squid and fish. Ciliated cells within the light organs selectively draw in the symbiotic bacteria *V. fischeri*. These ciliated cells promote the growth of the symbionts and actively reject any competitors. Through quorum sensing the bacteria cause these cells to die off once the light organ is sufficiently colonised. The light organ of certain squid contains reflective plates that intensify and direct the light produced, due to proteins known as reflectins. They regulate the light to keep the squid from casting a shadow on moonlit nights, (Visick et al. 2000). We can make use of the side effects of the natural bioluminescence system that it is not active in presence of pollutants. Whilst the *V. fischeri* system is a natural bioreporter without any genetic changes after being taken from the nature into laboratory,with modern gene technology genetic modifications by promoter changes or site-specific mutagenesis became possible in order to enhance the detection limits and specificities to types of pollutants and even to enable detection of new pollutants on which the system formerly might not have reacted to. The basic construction principle of the bioreporters is a regulatory promoter that is artificially fused with a promoter-less gene (reporter gene) coding for an easily measurable protein. Activation of the promoter by a transcription regulator via interaction with a chemical compound or analyte via a complex signaling chain leads to expression of the reporter gene yielding an output signal that can be detected (Leveau & Lindow 2002, Takahashi & Iwahashi 2004). Furthermore, gene technology allows development of completely new systems for biomonitoring which not existed before in nature. To this end, promoters and

reporter genes (possibly from different organismal origin) are combined and transformed into a host either where the promoter came from or, into another more sensitive species in which the promoter is known to be active (for further reading see chapters 2 and 5 of this thesis). Usually in a genetic engineering approach, new bacterial strains have been designed to act as bioreporters for sensing the harmful effects caused by pollutants or environmental stressors (Leveau & Lindow 2002). The harmful effects of a substance can be same or may vary on each and every organism. Effects may vary in particular from prokaryotes to eukaryotes. In forests, many examples of eukaryotic organisms can be found being potential bioindicators such as trees or parts of trees, herbal plants, tissues of animals, and algae in lakes and in other stagnant water. In most instances, these bioindicators are already natural habitants in the biotops. However, there are also possibilities to actively bring a new organism into the biotope for biomonitoring (see chapter 2 of this thesis). In many instances, eukaryotic organisms (e.g. trees and other plants) are large and react upon contaminants in a large area (e.g. in the air space, in soil regions) but usually in a comparatively slow manner. Quick tests however should also be possible, preferentially with small amounts of contaminated samples. Fungi, particularly unicellular yeasts, are organisms that might be taken into the laboratory for developing biological tests. Many cellular functions are conserved among eukaryotic organisms from simple yeast over filamentous fungi to mammals and, genes from higher eukaryotes can often complement those of lower eukaryotes (Botstein et al. 1997). Fungi can therefore serve as model to detect effects of harmful effects of pollutants on eukaryotic organisms including humans. In fungi, there are no obvious naturally available easy to follow signaling systems for environmental biomonitoring such as the bioluminescence system in *V. fischeri*. To design best reacting detection systems, promoter-reporter constructs need to be constructed by genetic engineering and transformed into a fungal host. The best studied fungus with the most sophisticated transformation system is the yeast *Saccharomyces cerevisiae*. Like bacteria, *S. cerevisiae* is unicellular, easily handled in culture and can readily be genetically modified. The yeast grows very fast overnight in liquid culture and we know the complete genome of this species and therefore have an easy access to genes which react on presence of pollutants (Suter et al. 2005). Currently, a few yeast reporter systems for detection of pollutants are available. There is a genetically modified system for detection of genotoxicity of chemicals like methyl methane sulfonate (MMS), ethyl methane sulfonate (EMS), 1,2-dimethyl hydrazine (SDMH), hydrogen peroxide (H_2O_2) etc. (see chapter 7 of this thesis for references and details).

The specific aims of this thesis were to evaluate effects from VOCs from wood and wood-related sources on a simple eukaryotic organism acting as a model for other eukaryotes including humans and to use this simple organism to construct a bioreporter system for the tested VOCs. By the reasons listed above, we have chosen *S. cerevisiae* as a host strain for bioreporter construction. In this study, first the harmful effects of specific VOCs from wood and wood-related sources on the yeast is analysed in a complete genome DNA microarray analysis. To construct an efficient and specific bioreporter system and to evaluate the harmful effects of VOCs from wood and related sources, we need to select a promoter from the yeast genome whose gene product is strongly and specifically reacting on the substances. Such strongly reacting promoters were selected from the microarray study, in addition to a number of promoters known from literature to react on various types of harmful compounds (see chapter 5 of this thesis for specific list of promoters). As a reporter gene, the *Escherichia coli lacZ* gene present in the yeast shuttle vector YEp356R was selected, upstream of which selected promoters were cloned (see chapter 5 of this thesis for details). The gene *lacZ* encodes the enzyme β-galactosidase which can be assayed easily by measuring spectro-photometrically the absorbance of the formed colored product upon the degradation of the chromogenic substrate, X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) by the β-galactosidase enzyme. Cloned behind a fungal promoter the *lacZ* gene gives a functional protein in *S. cerevisiae*. With X-gal as a substrate, β-galactosidase activity can be followed on plates by coloring yeast colonies blue. β-galactosidase is a cytoplasmic enzyme. Upon breaking yeast cells from liquid cultures, the cellular extract can be used in photometric tests with ONPG (*O*-nitro phenyl β-D galactopyranoside) as a substrate to quantify the amount of enzyme produced and thereby indirectly the activity of the promoter cloned upstream of *lacZ* in plasmid YEp356R (Myers et al. 1986) (see chapter 5 of this thesis for further details).

After transforming the recombinant promoter-*lacZ* constructs into *S. cerevisiae*, β-galactosidase assays on solid plates were performed, exposing cells to different concentrations of formaldehyde, a potential harmful VOC from wood and wood composites and α-pinene as a natural VOC released from solid wood in order to evaluate the principle suitability of the various constructs obtained in this study together with yeast as a bioreporter system for detection of formaldehyde and α-pinene and other VOCs from wood, respectively.

In this study, a total of 11 constructs visibly reacted to different concentrations of formaldehyde. On X-gal plates, those of the constructs tested with the strongest reaction were further tested for sensitivity of the reaction. Sensitivity of α-pinene could not be further studied by the lack of a visible blue reaction on the colonies.

Attempts to further increase the sensitivity of the system targeted at the host itself. The sensitivity of the host strain to detect effects of harmful chemicals at lowest possible concentrations might be increased by deleting non-essential genes involved in drug resistance and/or stress responses. We chose to knockout the gene *GTT1* that encodes glutathione S-transferase involved in the drug detoxification (Sharma et al. 2003). In addition from the microarray analysis, the involvement of ergosterol in blocking the negative effects of the VOCs became clear (see chapter 7 of this thesis). Therefore, as a second gene for improving the sensitivity of the bioassay, gene *ERG6* for a ΔC-24 sterol methyltransferase was knocked out. Preliminary sensitivity tests for these knock-out mutants were performed (see chapter 6 of this thesis for further details).

Amongst other projects, our laboratory also works on fruiting body development of the *Coprinopsis cinerea* (Kües 2000). Recently, an essential gene *cfs1* for initiating fruiting body development was cloned and found to encode a cyclopropane fatty acid synthase. *ERG6* of *S. cerevisiae* is closely related to *cfs1* of *C. cinerea* (Liu et al. 2006). Cyclopropane fatty acid synthases are further known in various bacteria where the enzymes modify lipids in the cell membrane in order to protect the cells against external stresses including harmful chemicals (Dufourc et al. 1984, Perly et al. 1985). In stress protection, the membrane properties alter by decreasing the membrane fluidity (Couto et al. 1996, Sajbidor 1997, Chang & Cronan 1999). A similar reaction is observed in yeast, when ergosterol is lacking (Gaber et al. 1989). By this similarity in membrane alterations, as an extra aim of this thesis, effects of expressing the *C. cinerea cfs1* gene in the *S. cerevisiae erg6* knock-out mutant was tested, and in parallel in the wild type (see chapter 7 of this thesis for details).

In conclusion from the experimental work of this thesis, the main objective of the thesis, construction of a eukaryotic bioreporter system for VOCs from wood and wood-related sources was achieved in the case of formaldehyde. In future, this system will need further modification to enhance the sensitivity. The approaches taken in this direction for further

studies are partially promising but also tell that the task of increasing the sensitivities is not an easy, quickly to resolve problem.

1.2. References

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