Aromatic amine biotransformation by *Trichoderma virens* and *Trichoderma reesei*

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Keywords: arylamine *N*-acetyltransferase; *Trichoderma*; aromatic amines; biotransformation; toxicity

Nowadays, pollution is a major concern. Human activities have resulted in xenobiotic accumulation in natural environments, particularly soil contamination by pesticides, industrial chemicals and their derivatives. Aromatics amines (AA) represent one of the most important classes of environmental pollutants. Many AA are toxic to most living organisms due to their genotoxic or cytotoxic properties. Living species use several xenobiotic metabolic pathways to protect themselves against the toxic effects of these pollutants. Arylamine *N*-acetyltransferase (NAT) proteins are xenobiotic-metabolizing enzymes (XME) which catalyze the transfer of an acetyl group from acetyl-coA (AcCoA) to AA and *N*-hydroxylated metabolites. The *N*-acetylation of these chemicals has been shown to detoxify them. The study of this mechanism underlying detoxification may pave the way for novel bioremediation applications. Although bacteria are the most common microorganisms used in bioremediation, fungi are described as promising tools (Silar *et al.*, 2011). NAT genes were indentified in fungal genome sequences and interestingly several *Trichoderma* species possess these genes (Martins *et al.*, 2010). We chose to study NAT enzyme from ascomycetes *Trichoderma virens* and *Trichoderma reesei*. These fungi have several characteristics interesting for bioremediation applications. *Trichoderma* sp. are abundant soil borne ascomycetes found all over the world. They are known as successful colonizers and efficiently competitors (Schuster & Schmoll, 2010). Moreover, *T. reesei* and *T. virens* are widely used in industry as source of cellulases and as biocontrol agent respectively. The aim of this project was (i) to characterize NAT enzymes of *T. virens* (TvirNAT) and *T. reesei* (TreeNAT), (ii) to study the fungal tolerance to AA, and (iii) to investigate their capacity to biotransform AA to acetyl AA.

To characterize NATs enzymes, recombinant proteins were produced and purified. NAT activity was measured in the 5,5'-dithiobis-(2-nitrobenzoic acid) assay with different aromatic substrates (drugs, pesticide residues and industrial chemical products). TvirNAT and TreeNAT were found to acetyl 10 substrates among the 18 substrates tested. These two recombinant NATs showed the same substrate specificity. Apparent Michaelis-Menten parameters V^{app}_m and K^{app}_m were determined for these 10 substrates by direct curve-fitting to Michaelis-Menten equation. Both NAT showed very similar catalytic efficiencies whereas TvirNAT efficiency was 0-7 fold higher for almost the 10 substrates tested here, compared to TreeNAT (Fig.1.1).However, the rate of 3,4-DCA acetylation by TvirNAT and TreeNAT was 225 and 509 times lower than *Podospora anserina* NAT (18 500 $nmol.min^{-1}.mg^{-1}$), which was the only fungal NAT described so far (Martins *et al.*, 2009).

AA tolerance of fungi was assessed in M2 agar medium in which increasing aromatic amine (3,4-DCA, Bz and 4-IPA) concentrations were added. Sensibility was screened by assessing radial growth for 3 days. When Bz and 4-IPA were added at 250 µM, the higher growth inhibition was 20% for the two strains, but the *T. reesei* and *T. virens* growth decreased 60% and 20% respectively with 200 µM of 3,4-DCA. In presence of acetyl AA, *T. virens* and *T. reesei* growth was globally increased by 7% and 11% respectively, compared to AA assays. These two *Trichoderma* appeared to be more sensitive to 3,4-DCA especially *T. reesei.* Moreover, acetyl-AA appeared to be less toxic for fungi than parent molecule. These results are consistent with previous data where aromatic compounds were described as more toxic than their corresponding acetyl metabolite (Tixier *et al.*, 2002).

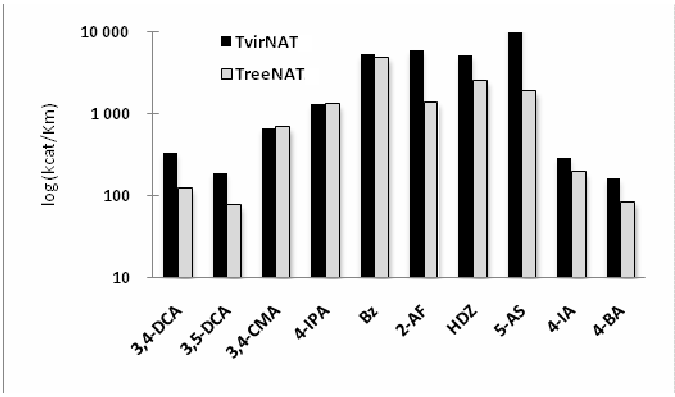

Figure 1.1. : Functional characterization of TvirNAT and TreeNAT. Comparison of catalytic efficiencies as estimated from log ratios of kinetic parameters (k_{cat}/K_m). Errors for triplicate values were at a maximum value of ±5%. 3,4-DCA, 3,4-dichloroaniline; 3,5-DCA, 3,5-dichloroaniline; 3,4-CMA, 3-chloro-4-methylaniline; 4-IPA, 4-isopropylaniline; Bz, benzidine; 2-AF, 2-aminofluorene; HDZ, hydralazine; 5-AS, 5-aminosalicylate; 4-IA, 4-iodoaniline; 4-BA, 4-bromoaniline.

To investigate the fungal biotransformation of AA, *T. reesei* and *T. virens* were grown in M2 liquid medium in which 250 µM of selected AA (3,4-DCA, Bz or 4-IPA) were added. The rate of acetylation of aromatic amines by fungi was measured by HPLC at different times of incubation. *T. virens* appears to be able to use these three AA. Bz, 4-IPA and 3,4-DCA levels decreased by 90%, 55% and 42% during assays. The dissipation of AA is partly explained by *N*-acetylation for Bz (42%) and 4-IPA (32%) but no acetyl DCA was detected in the medium. With *T. reesei*, concentrations of Bz, 4-IPA and 3,4-DCA decreased by 20%, 60% and 0% respectively. This strain could use Bz and 4-IPA although it does not significantly acetyl these compounds. Thus, *T. virens* seems to acetylate AA *in vivo* with higher efficiency compared to *T. reesei*. The mass balance between AA and acetyl AA could be explained by a rapid mineralization of acetyl aromatic compounds formed or by alternative AA metabolic pathways. Few studies reported the mineralization of aniline by fungi, only *Phanaerochaete chrysosporium* was described as mineralizing 3,4-DCA (Arjmand & Sandermann, 1985).

These results showed the capacity of *T. virens* and *T. reesei* NATs to acetylate several AA. Although *Trichoderma* NATs are less effective than *P. anserina* NAT, they show several characteristics potentially interesting for bioremediation. Indeed, *T. virens* and *T. reesei* have the capacity to degrade some AA by *N*-acetylation pathway or by alternative metabolic mechanisms. Moreover, *Trichoderma* sp. are successful colonizers, efficiently competitors and widely used in industry. Finally, to associate the properties of these fungi, a consortium with *Trichoderma* sp. and *P. anserina* could be considered for detoxification of contaminated soils.

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