Title

White mutants of chloroperoxidase-secreting *Caldariomyces fumago* as superior production strains revealing an interaction between pigmentation and enzyme secretion

Authors

Markus Buchhaupt #, Sonja Hüttmann, Jens Schrader

DECHEMA Research Institute, Theodor-Heuss-Allee 25, 60486 Frankfurt am Main, Germany

Corresponding author e-mail address: buchhaupt@dechema.de

Journal section: biotechnology
Abstract

By mutant colony screening of *C. fumago* several white mutants were isolated that are superior strains for the production of the valuable enzyme chloroperoxidase (CPO). Their culture supernatant lacks the contaminating dark pigment, which simplifies downstream processing. Furthermore the CPO content increased significantly faster compared to the wild type, which uncovers possible interactions between pigmentation and enzyme secretion.
Results, discussion and methods

The filamentous fungus *C. fumago* is known for its ability to secrete the enzyme chloroperoxidase and is currently the only source for this highly versatile biocatalyst. The main reason, why CPO has not been used in large scale industrial processes yet is that it’s production costs are far too high. During optimization of the production medium, Pickard (6) found that addition of fructose instead of malt extract had the same CPO formation promoting effect and fortunately resulted in 95 % reduction of medium pigmentation simplifying enzyme purification. Alternative approaches for the removal of the contaminating black pigment are downstream processing steps like ethanol precipitation (5) or aqueous biphasic extraction (9).

A more straightforward approach to get completely rid of the dark pigment without producing additional costs would be the use of a nonpigmented strain. In order to isolate such a mutant of *C. fumago*, we plated spheroblasts on fructose minimal medium and afterwards mutagenized them by UV irradiation with a dose corresponding to a survival rate of 26%. From 26,000 single colonies that were visually inspected, six displayed an albino phenotype and were isolated. As all mutants behaved essentially identical in the following experiments, we show only the results obtained with strain white2. Although the mutant strain lacks the strong black color of the wild type and possessed a white to beige color on glucose minimal medium it had a reddish coloration after some days of growth on fructose minimal medium plates (Fig. 1A). This is most likely caused by the heme-containing CPO, whose formation is induced by fructose and repressed by glucose (1). All white mutant strains grew slightly slower in comparison to the wild type on fructose minimal medium (see also Fig. S1) as well as on other media (data not shown). After seven days of cultivation in preculture medium (glucose potato) the wildtype culture showed the usual black color whereas all mutant cultures looked white/beige (Fig. 1B). In fructose minimal medium inoculated with homogenized
glucose potato precultures the white mutants developed a red-brown color but did not produce the dark pigment present in the wild type culture.

The significant color change of the *C. fumago* mycelium after addition of 50 µg/ml of the DHN (dihydroxynaphthalene) melanin synthesis pathway inhibitor tricyclazole (Fig. 2) suggests that the black pigment of *C. fumago* is DHN melanin.

Analysis of CPO activities in culture supernatant samples not only demonstrated that the mutants produce maximal CPO levels similar to the wild type, but also revealed a different time course of enzyme production (Fig. 3A). Namely CPO activity in the white strain cultures increased more rapidly. Taking into consideration the lower growth rate (Fig. S2), this leads to a significant increase of the specific productivity of the white2 strain, being up to 2.3 fold higher compared to the wild-type value (at day 4).

Comparison of CPO protein levels in culture supernatants from day 3 by SDS-PAGE proved that the lower MCD assay activity of wild-type samples is not caused by inhibition of the enzyme by substances present only in the wild-type cultures, but comes from the lower amounts of enzyme secreted (Fig. 3B).

Such a positive effect of the loss of fungal cell pigmentation on protein production has, to our knowledge, not been described before. Although further research will be necessary to reveal the mechanism behind this phenomenon, we will discuss some explanatory hypotheses. One direct linkage between protein secretion and melanin is that secreted proteins have to pass the fungal cell wall, of which melanin is an integral component. Other reports discuss possible spatial overlaps between cell wall melanization and protein secretion activities in hyphal tips (4) and protein binding or inactivation activity of melanin (2, 3, 8). Another plausible explanation could be the lack of carbon or redox equivalent flux into the melanin polymer in the white mutants, which might limit CPO production in the early logarithmic growth phase.
of wild-type cells. Since fungal albino mutants often show pleiotropic phenotypes, the lack of melanin could also indirectly impact cellular processes associated with CPO synthesis or secretion. Our findings concerning the connection between fungal pigmentation and protein secretion brings up the question whether this is a relationship that exists also in other fungi.

Especially in case of A. niger, which is widely used as expression host for eukaryotic proteins, it is necessary to check nonmelanized mutants for a decreased lag phase with respect to protein secretion.

Less pigmented C. fumago mutants have been already isolated by Smith et al. (7), but, contrary to our findings, most of them were shown to exhibit a significantly decreased productivity and also other undesired properties. A direct comparison with our results is furthermore not possible as the authors did not specify the time point of sampling and CPO activity determination. As they measured enzyme activity only at one time point they were probably not able to recognize the decreased CPO production lag phase that we found with all our white mutants.

Besides the two features of the white C. fumago mutants that make them highly attractive as production organisms in order to produce CPO at lower costs – namely the lack of the contaminating pigment and the reduced production lag phase - they are ideal basic strains for further optimization approaches. In fact, the CPO content of a liquid culture or a colony grown on an agar plate can now be quantified by simple visual inspection due to the red-brownish color of CPO. This allows a fast screening for CPO overproduction or glucose repression mutants, which could be the next step towards a highly efficient CPO production strain.
Acknowledgments

Financial support from Arbeitsgemeinschaft Industrieller Fördervereinigungen e.V. (AiF) under the project 16152 N is gratefully acknowledged.

References


Legends

Fig. 1  Color comparison of *C. fumago* wild type (left) and white2 mutant (right) on solid and liquid media

A  Comparison of wild type strain and white2 mutant on solid medium. The strains were inoculated on minimal medium agar plates containing glucose or fructose with a piece of mycelium grown on a glucose potato agar plate and incubated at 24 °C for 20 days.

B  Comparison of wild type strain and white2 mutant in liquid medium. Flasks containing 100 ml glucose potato medium were inoculated with 1 cm² of mycelium grown on a glucose potato agar plate and incubated on a rotary shaker at 24 °C. Photographs were taken after seven days of incubation. After homogenization of the seven day-old glucose potato precultures 1 ml of the homogenates were used to inoculate flasks containing 100 ml fructose minimal medium. Incubation on a rotary shaker was performed at 24 °C. Photographs were taken after three days of incubation.

Fig. 2  Effect of tricyclazole addition on the pigmentation of *C. fumago*. 1 cm² of *C. fumago* wild type strain mycelium grown on a glucose potato plate was transferred to a glucose minimal plate (left picture) or to a glucose minimal plate containing 50 µg/ml tricyclazole (right picture). Photographs were taken after incubation of the plates for seven days at 24 °C.
Fig. 3  Comparison of CPO production characteristics of wild type and white2 strain.

A  Comparison of CPO activities in supernatant samples of wild type and white2 strain during cultivation in fructose minimal medium. Strain cultivation and determination of enzyme activity via the MCD assay was performed as described in the supplementary information. Data is expressed as mean of values from three shake flasks ± standard deviation.

B  Analysis of CPO protein levels in supernatant samples of wildtype and white2 strain by SDS-PAGE. 40 µl of supernatants from fructose minimal cultures grown for three days were subjected to an SDS-PAGE analysis using a 15 % gel followed by coomassie staining. The numbers indicate the sizes (in kDa) of the molecular weight marker proteins.