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Magic Mushrooms: Screening for Novel Biocatalysts in the Phylum Basidiomycota

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Abstract

The ascending application of enzymes in organic synthesis creates a growing demand for novel biocatalysts. The applied methods for their identification range from microbial enrichment cultures over metagenome screenings to solely computational methods. In this communication, we demonstrate a straightforward screening approach for the detection of novel biocatalysts in fungi belonging to the phylum Basidiomycota. It basically relies on mincing of the whole fruit bodies of freshly collected mushrooms with subsequent direct screening. Suitability was demonstrated with eight different mushrooms which were investigated for carbonyl reductase activity on sterically demanding carbonyl compounds. The results indicate the presence of potentially useful carbonyl reductases (KREDs) in all tested fungi. Closer characterization of the preparation from pigskin poison puffball (*Scleroderma citrinum*) showed the presence of KRED exhibiting a broad substrate range. Thus, applicability of this low-tech screening approach could be verified in this study.

Keywords

Biocatalysis, Carbonyl Reduction, Screening, Biological Source

1. Introduction

Enzymes are an increasingly important tool for the synthesis of commodity as well as fine chemicals, applications ranging from lab to industrial scale [1] [2]. Thus the identification of novel enzymes with new characteristics and functionalities is required to meet the ever-changing/evolving requirements for the growing number of processes. Strategies for this task are numerous [3]. A classical approach is the utilization of enrichment cultures, where a biological sample is treated with a potential substrate. Microorganisms with an ability to metabolize the given substrate are enriched and may then be used as a source for enzymes modifying the given compound [4]. Another

prominent approach is the screening of organism or metagenome libraries for a desired enzyme activity [5]. All these strategies have in common that they mainly focus on microbial targets. In general, this is reasonable since bacteria, archaea and simple eukaryotic organisms provide a large part of the metabolic variety in the biosphere. Nevertheless, a part of this variety is neglected, since large eukaryotic organisms cannot easily be included in the screening procedures. For instance, fungi of the phylum of Basidiomycota are known to synthesize various complex compounds, some of pharmaceutical interest, within their secondary metabolism [6]. This requires an expanded set of enzymes to specifically catalyze the corresponding reactions. A prominent example is the fungal lignin degradation, performed by specific laccases and peroxidases [7] [8]. These metabolic abilities render Basidiomycota a promising source for novel biocatalysts.

An example for a successful Basiodiomycota derived biocatalyst is the laccase from *Trametes versicolor* [9]. Its presence could be demonstrated *in vivo* and *in vitro* by Evans [10]. Later the responsible enzyme was isolated [11] and has since been applied in various biotechnological and industrial applications such as dye decolorization, degradation of xenobiotics or Denim bleaching [12]. A screening for novel laccases in basidiomycetes was successfully conducted applying cell cultures as starting material [13].

Considering the difficulty in the cultivation of fungi and their high potential as source for biocatalysts for biotechnological applications a culture independent screening procedure for a specific enzyme activity in Basidiomycota would be useful.

In this study, we demonstrate a screening approach for enzyme activity based on the direct application of minced fruit bodies of freshly collected mushrooms from the phylum Basidiomycota. Simple as it is, this has to our knowledge never been described before. Sample candidates were screened for carbonyl reductase (KRED) activity on sterically demanding substrates. The chiral alcohols resulting from their reaction are relevant as chemical building blocks and precursors for pharmaceuticals [14] [15]. The extract from the most promising candidate, the pigskin poison puffball (*Scleroderma citrinum*, **Figure 1**), was characterized in detail.

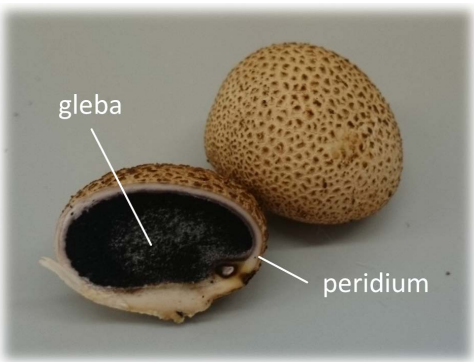


Figure 1. Pigskin poison puffball (*Scleroderma citrinum*) with gleba and peridium.

2. Materials and Methods

2.1. Provision of Biological Material

Mushrooms for the screening were collected at Dresdner Heide (51˚04'12.0"N, 13˚51'36.0"E) on the 12.10.2014. Fruit bodies from the species *Clitocybe nebularis*, *Entoloma undatum*, *Lactarius deterrimus*, *Laccaria amethystina*, *Amanatia muscarina*, *Boletus badius*, *Suillus bovinus* and *Scleroderma citrinum* were collected. Mushrooms were identified by phenotypical traits according to fungal determination literature [16] [17]. For detailed characterization of Scleroderma citrinum a second batch was collected on 24.10.2014 at the same location. In case of all candidates except for *Scleroderma citrinum*, complete fruit bodies without mycelium were cleaned by rinsing in water and minced for 30 sec with a Braun 160 Watt hand blender. The minced material was directly applied in the activity assays. For *Scleroderma citrinum* the tough outer wall (peridium) was removed leaving the soft gleba, which was minced and applied (**Figure 1**).

2.2. Activity Assay

Activity was measured in 100 mmol·L^{-1} triethanolamin (TEA) buffer containing 3 mmol·L^{-1} substrate, 250 µmol·L^{-1} NADH and 5% (v/v) isopropyl alcohol in 500 µL scale as described previously [18]. Reactions were started by addition of 100 mg minced mushroom preparation and performed at 25˚C and 700 RPM for 22 h. Conversion and yield of each reaction was determined via chiral GC. For determination of the pH-profile, acetate buffer (pH 5.0), phosphate buffer (pH 5.0, 6.0) and TEA buffer (pH 7.0, 8.0, 9.0) were applied. The pH- and temperature profiles were measured with benzaldehyde as substrate. All assays except for the initial screening were performed in triplicates and the standard deviations are given as error bars in the figures.

2.3. Analytics

Determination of substrate and product concentrations was conducted by chiral GC analysis. The quantification of all compounds was performed on a Shimadzu GC2010 gas chromatography system with FID detection system and a Hydrodex γ DIMOM column. In all methods a sample volume of 2 µL was injected with a split ratio of 20. Nitrogen was applied as carrier gas for all measurements. For aromatic substrates the separation was conducted at 130˚C for 12 min. For [C5]-compounds a linear temperature gradient from 95˚C to 115˚C over 13 min was applied. For [C6]-compounds a linear temperature gradient from 98˚C to 122˚C over 12 min was applied.

3. Results and Discussion

3.1. Carbonyl Reductase Activity Screening

Eight different mushrooms from the phylum Basidiomycota were screened for carbonyl reductase activity. As substrates, the aromatic ketone acetophenone and the aliphatic diketone 3,4-hexanedione were tested. **Figure 2** shows the reaction yields achieved after 22 h reaction time. All tested preparations exhibited carbonyl reductase activity on

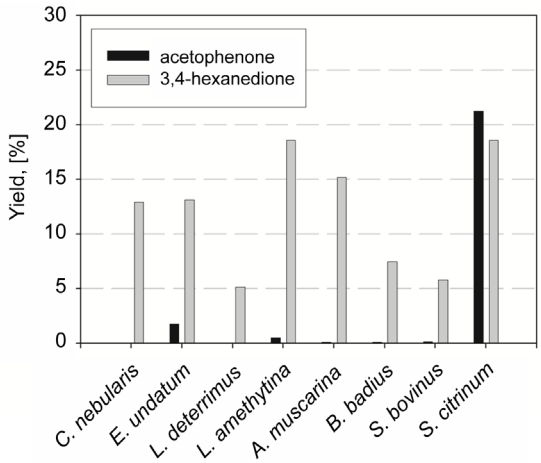
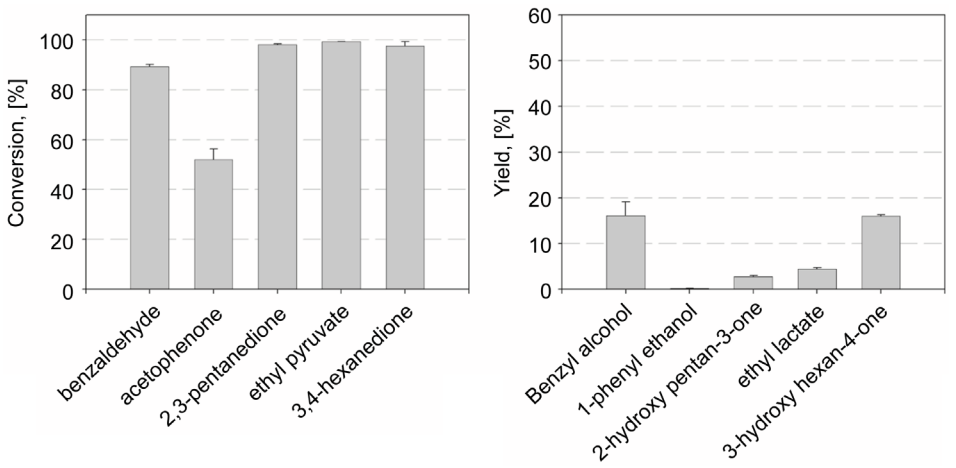


Figure 2. Conversions for the reduction of acetophenone and 3,4-hexanedione by various Basidiomycota preparations. Reactions were conducted for 22 h at 25˚C and a pH-value of 7.0.

3,4-hexanedione with yields ranging between 5 and 20%, which demonstrates the suitability of the screening procedure to render enzymes active. The preparations from *Entoloma undatum*, *Laccaria amethystine* and *Scleroderma citrinum* also exhibited significant activity on acetophenone as substrate, yielding 0.5 to 21% 1-phenyl ethanol. All three preparations showed (*S*)-selectivity with no detectable enantiomeric impurity. With conversions of 18.6% in the 3,4-hexanedione reduction and 21.2% in the acetophenone reduction, *Scleroderma citrinum* was the most promising candidate of this screening. Thus, a more detailed characterization of this preparation was conducted.

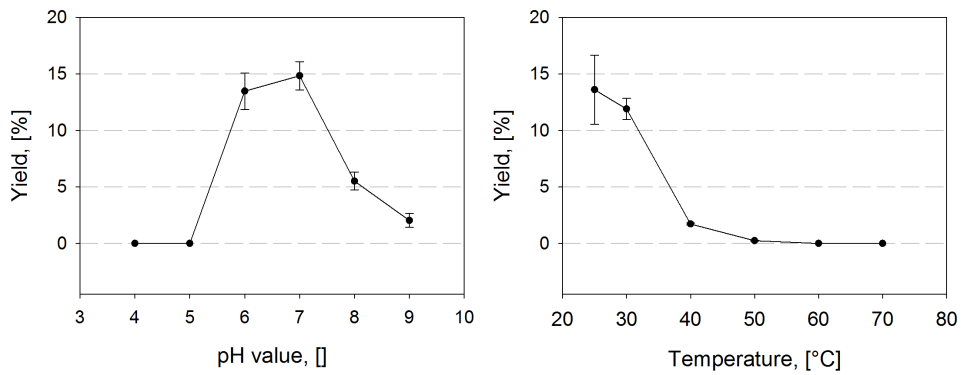
3.2. KRED-Activity of *Scleroderma citrinum*

The substrate specificity of the *Scleroderma citrinum* preparation was further characterized by determination of conversion and reaction yield for five carbonyl substrates, applying a preparation from a second batch of the mushroom. The reductions of ethyl pyruvate and 2,3-pentanedione yielded 3 to 5 % of the corresponding hydroxy ketones. Benzaldehyde and 3,4-hexanedione reduction exhibited the highest yields with 16% each (**Figure 3**). The reduction of acetophenone yielded 0.2% 1-phenylethanol. This is noteworthy, since the first preparation from Scleroderma citrinum showed equally high yields for the reduction of acetophenone and 3,4-hexanedione, whereas the second preparation showed significantly higher yields for the latter substrate. Since the mushroom preparations are biological samples, collected at a specific sampling time, the expression level of a certain protein may vary significantly. These variations may be caused by environmental influences such as humidity or temperature and by physiological influences such as the age of the specific mushroom and its stage of development [19]. In summary, the *Scleroderma citrinum* preparation showed carbonyl reductase activity on all tested substrates and the results from the screening could be confirmed by trend. Stereoselectivity was investigated for the reduction of acetophenone and 2,3-pentanedione, showing clear (*S*)-selectivity for both substrates.



(a) (b)

Figure 3. (a) Conversion and (b) yield of carbonyl compounds by *Scleroderma citrinum*. Reactions were conducted for 22 h at 25˚C and a value pH of 7.0.



(a) (b)

Figure 4. (a) pH- and (b) temperature profile of the Scleroderma citrinum catalyzed reduction of benzaldehyde. Reactions were conducted for 22 h at 25˚C (pH-profile) and a pH-value of 7.0 (temperature profile), respectively.

It is noteworthy, that the conversions were very high for all substrates ranging between 50 and 99% (**Figure 3**). Thus the largest part of the deployed substrate was converted without ending up as reaction product after 22 h. Since the preparations were minced gently it is likely that cells of the mushroom and here especially the spores were still intact to a certain degree. Thus, the consumption of the substrate may be interpreted as a metabolization of the compound by viable cells or a further degradation by released enzymes. The best example here is ethyl pyruvate, where 99% of the substrate was consumed, while only 4.4% of the reaction product ethyl lactate was yielded. Thus, 95% of the deployed substrate was converted otherwise. Depending on the target of the screening this effect has certainly to be taken into account.

The pH profile of the preparations shows maximum activity in a range between pH 6.0 and 7.0 (**Figure 4**). At higher pH values activity decreases to a residual yield of 14% compared to pH 7.0, while at lower pH values no product could be detected. The tem-

perature profile shows highest Yield at temperatures of up to 30˚C. At 40˚C the yield is reduced to 13% of the maximal value. Over 50˚C no product formation could be detected (**Figure 4**).

The characterization indicates that the preparation of Scleroderma citrinum contains at least one active carbonyl reductase. At this stage, of course, it is not clear if the described characteristics can be attributed to a single enzyme or if they are the merged characteristics of two or more enzymes. The likeability of the latter has for example been demonstrated for the carbonyl reductase activity of Candida parapsilosis described by Kula and coworkers in 1995 [20]. Only in 2011 it became clear, that the CPCR preparation actually contained two enzymes where one is mainly responsible for the characteristics that were initially described [21].

4. Conclusion

In this study, we demonstrate that crude mincing of freshly collected mushrooms is a viable screening approach to obtain valuable novel enzymes from Basidiomycota. Mining for NADH-dependent carbonyl reductases gave activity in all tested preparations with most promising results in the pigskin poison puffball (*Scleroderma citrinum*). In an appropriate season and location sufficient amounts for sampling can easily be collected. Despite natural sample related reproducibility issues, this screening approach is a useful low tech strategy for the detection of novel biocatalysts from higher fungi with potential for a high throughput method. Thus, it can help expanding the scope of available enzymes for biosynthetic use.

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