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Alkene cleavage by white-rot *Trametes hirsuta*: Inducing enzyme activity by a fungicide

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ABSTRACT

Alkene cleavage is a widely employed oxidation reaction in organic chemistry. An enzyme preparation of the wood degrading fungus *Trametes hirsuta* is known to cleave the C=C double bond adjacent to an aromatic ring to give the corresponding carbonyl compound at the expense of molecular oxygen as the sole oxidant. Lab-grown fungus cultures displayed varied activity and lost their alkene cleavage activity over generations of growth. *t*-Anethole, which is the best accepted substrate by the enzyme, is described as a major component of essential oils produced by certain plants with powerful fungicidal property. We could now show that the alkene cleaving activity was improved by the addition of the fungicide *t*-anethole during culture growth which represented to be an efficient method to produce cells possessing a consistent level of high alkene cleavage activity.

1. Introduction

Alkene cleavage to give the corresponding aldehydes or ketones is a very frequently used method in synthetic organic chemistry to (i) introduce oxygen functionalities into molecules, (ii) split large compounds selectively and (iii) remove protecting groups [1–5]. Employing biocatalysts [6–10], alkene cleavage has been reported as an undesired side-reaction catalyzed by some peroxidases [11–15] and certain mono- [16,17] or dioxygenases [18–23] with highly specific substrate scope.

Trametes hirsuta FCC 047 is a fungus belonging to the white-rot fungal family. A cell-free enzyme preparation of *Trametes hirsuta* was reported to cleave aryl alkenes possessing a C=C double bond adjacent to an aromatic ring [24,25] following a radical mechanism [26]. The substrate scope of the enzyme was found to be broad and the reactions were demonstrated on preparative scale [27,28]. *trans*-Anethole **1** was shown to be the best substrate for the biocatalytic alkene cleavage, affording *p*-anisaldehyde **2** as the sole detectable product (Scheme 1).

The genus *Trametes hirsuta* grows heterotrophically taking organic compounds as energy source. Several complex media providing different organic compounds were assayed to optimize the growth of *Trametes hirsuta* with respect to the alkene cleavage activity and cell mass (unpublished results). The finally employed complex medium consisted of yeast extract, L-aspargine monohydrate and glucose in addition to the basic assortment of salts. However, the alkene cleavage activity of the cultures varied from culture to culture. Additionally a decrease of activity was observed for the cells obtained after several rounds of re-cultivation. This affected the reproducibility of high conversions in biotransformations. In this study a method was sought to improve cultivation conditions to obtain and maintain a high level of alkene cleavage activity in the fungus.

2. Experimental

2.1. Materials

GC analysis for the biotransformation was carried out on Varian 3800 with a Varian chrompack 8200 autosampler and a DB1701 column. Sartorius MA 30 thermobalance was used for measuring the cell dry weight. Glucose concentration in the culture was measured employing a commercial blood sugar measuring device CONTOUR TS (Bayer). Unitron AJ200 shaker was used for the cultivation of shake flask cultures. Branson digital sonifier was used for cell breakage and Sorvall RC 5C PLUS cooling centrifuge for harvesting the culture.

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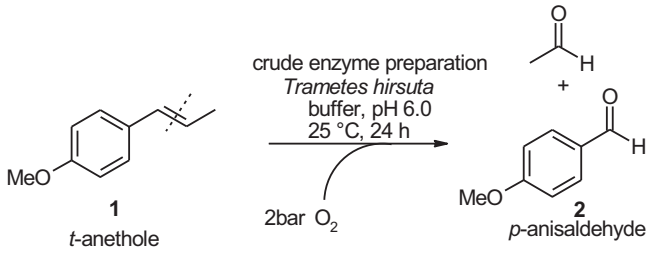


Scheme 1. Biocatalytic alkene cleavage of *t*-anethole to *p*-anisaldehyde by *T. hirsuta*.

2.2. Methods

2.2.1. Growth medium

The growth medium for *T. hirsuta* FCC 047, the "sorbix" medium, contained glucose (15 g/L), L-asparagine monohydrate (3 g/L), KH_2PO_4 (1.5 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), yeast extract (6 g/L), 3,4-dimethoxybenzyl alcohol (0.1 mL) and 1 mL of trace element solution SL4 [EDTA (0.5 g/L), $FeSO_4{\cdot}7H_2O$ (0.2 g/L), $ZnSO_4{\cdot}7H_2O$ (0.1 g/L), $MnCl_2{\cdot}4H_2O$ (0.03 g/L), H_3BO_3 (0.3 g/L), $CoCl_2{\cdot}6H_2O$ (0.2 g/L), $CuCl_2{\cdot}2H_2O$ (0.01 g/L), $NiCl_2{\cdot}6H_2O$ (0.02 g/L), $Na_2MoO_4{\cdot}2H_2O$ (0.03 g/L)].

2.2.2. Strain maintenance

T. hirsuta FCC 047 was maintained on agar plates of sorbix medium (with 18 g/L of agar). The plates were stored at 4 °C. Additionally cells were stored as cryostocks of sorbix and a cryo solution [sodium glutamate (50 g/L), saccharose (50 g/L), dextrane (50 g/L), pH 6.4] in a ratio of 1:1.

2.2.3. Growth study

For the preculture sorbix medium (100 mL) in non-baffled flasks (300 mL) was used while for the main cultures 1 L non baffled flasks were employed (500 mL medium). The conditions for growth were 25 °C and 150 rpm on a rotary shaker. A piece of agar (1 cm^2) with the fungus was used to seed the preculture and grown for 5 days. A fraction of the preculture (25 mL) was employed for seeding the main cultures. In case of the culture grown in the presence of *t*-anethole, *t*-anethole (200 μL, dissolved in DMSO, 1 M stock) was added to set the concentration of *t*-anethole to 0.4 mM at 24 h. The same amount was also added after 48, 73 and 97 h of cultivation. The culture in the presence of *t*-anethole vapour was grown just beside the flask containing *t*-anethole. The flask in the absence of *t*-anethole was grown separately. Culture samples (50 mL) were withdrawn from all the three flasks after 24, 48, 73, 97 and 122 h. A fraction (5 mL) was used for cell dry weight determination and the remaining (45 mL) was prepared for the biotransformation (see Section 2.2.4).

2.2.4. Lyophilization of cells

The culture samples (45 mL each) were centrifuged (8000 rpm, 19,945 × *g*, 4 °C, 20 min). A fraction of the supernatant (1 mL) was taken for glucose determination. The remaining supernatant was discarded and the pellet was resuspended in Bis–Tris buffer (~50 mL, pH 6, 50 mM) for washing. After centrifugation, the pellet obtained was lyophilized and used for the biotransformation.

2.2.5. Glucose determination

CONTOUR TS (Bayer), a blood sugar measuring device, was used for determination of the concentration of glucose in the fungal growth medium. To adapt the protocol for glucose in the growth medium, a calibration curve ($y = 0.148x$, correlation coefficient $R^2 = 0.98$) showed linearity between 0 and 15 mg/mL. The supernatant after the first centrifugation step of the collected samples was diluted with water and tested for glucose concentration using the CONTOUR TS.

2.2.6. Cell dry weight determination

The CDW was determined using an automatic thermobalance (Sartorius MA 30 moisture analyzer). The filtered cells of 5 mL culture were heated until constant weight at 130 °C.

2.2.7. Optimized cultivation conditions

The preculture (100 mL sorbix medium in 250 mL flask), inoculated by a piece of agar with fungus (1 cm^2) was grown for 5 days at 25 °C and 150 rpm. The sample (16.5 mL) was used to seed the main culture (330 mL in 1000 mL non-baffled flask) which was incubated at 25 °C and 150 rpm. After 24, 48 and 72 h of growth, *t*-anethole (100 μL of 50 mM stock in ethylacetate) was added to the culture medium. After 96 h, the dosage was increased (300 μL of 50 mM stock in ethylacetate). The culture was finally harvested after 100 h of cultivation.

2.2.8. Biotransformations

For the determination of the conversion, a previous procedure [25–27] was adapted. Lyophilized cells (30 mg) were resuspended in Bis–Tris buffer (1 mL, 50 mM, pH 6.0). The cells were ultrasonicated (1 s pulse on, 4 s pulse off for a total time of 100 s, 50% amplitude) and centrifuged (4 °C, 8000 rpm, 20,000 × *g*, 20 min). The supernatant was used for the assay performed in 48 well RP plates. Each sample was tested in duplicates. The reaction mixture contained the cell free extract (900 μL) and *t*-anethole (5 μL, 4.94 mg, 33 μmol). In case of other tested substrates, an additional co-solvent (DMSO, 100 μL, 10%, v/v final concentration) was also added to improve solubility [28]. The reaction was run under oxygen pressure (2 bar) and shaken (170 rpm, GFL 3015 orbital shaker) at 21 °C. After 24 h, the samples were extracted with ethyl acetate (2× 500 μL) and analysed by GC.

The concentrations of the *t*-anethole and *p*-anisaldehyde were determined using a calibration curve. n-Decane was used as an internal standard in the solvent (ethyl acetate) which was used for the extraction of the samples.

3. Results

3.1. A fungicide as inducer

Literature suggested that *t*-anethole **1**, which was actually the best accepted substrate for alkene cleavage by *T. hirsuta*, is a very effective fungicide component present in essential oils produced by certain plants [29–31]. Therefore, we speculated that the fungus has developed the alkene cleavage activity in a response to this natural fungicide or related ones. To test the fungicide activity, *t*-anethole **1** was added to the liquid growth medium of the *T. hirsuta* (final concentration of 13.2 mM in 100 mL medium) which was inoculated with a sample of the fungus, which previously showed low alkene cleavage activity (17% conversion of *t*-anethole to *p*-anisaldehyde under standard reaction conditions). In parallel a control experiment was run in the absence of *t*-anethole. After 24 h, it was observed that the control flask was growing well, whereas the fungus in the presence of **1** did not grow. The minimal inhibitory concentration of *t*-anethole for the white-rot fungi *Trametes versicolor* was reported to be 1.25 mM [31]. Keeping this number in mind, the subsequent experiment was performed by a daily addition of a lower concentration of *t*-anethole (0.4 mM) to the growth medium right from the beginning. The daily addition was required due to the high volatility of *t*-anethole.

It was observed that after seven days of cultivation, the culture in the presence of **1** displayed lower final cell yield after lyophilization compared to the control flask. However, in the biotransformation

with the induced culture 95% *t*-anethole was consumed in contrast to just 67% by the non-induced culture after 26.5 h. Thus, higher concentrations of the product (*p*-anisaldehyde) could be obtained with the induced culture (7 mM) as compared to the non-induced culture (5.8 mM).

3.2. Growth study

3.2.1. Influence of t-anethole **1** on growth

The results obtained from the first tests, led to more detailed studies on the influence of **1** on the growth of the fungus and its ability to cleave alkenes. Consequently one culture was grown in the absence of **1** and one in the presence of **1**. Since **1** is very volatile and may already show an influence at rather low concentrations, a third culture was grown in the presence of *t*-anethole **1** vapour. To ensure comparability, the fungus from the same agar plate, which was used for the preliminary test was used for the growth study. A preculture (100 mL) was used to inoculate the experimental cultures (500 mL each). For the culture in the presence of **1**, **1** was added to the growth medium at 0.4 mM after 24 h. Furthermore the same amount was added at regular intervals (48, 73, and 97 h) to compensate evaporation. The culture in the presence of **1**-vapour was placed just beside the flask containing **1**. The glucose consumption and cell-dry weight were monitored until the glucose was completely depleted from the growth medium.

The graphs for the CDW of the cultures in the presence of **1** or **1**-vapour were found to be similar (Fig. 1). Addition of *t*-anethole to the growth medium starting after 24 h of growth did not seem to inhibit the rate of growth of the fungus, which was observed in the first test where *t*-anethole was added right in the beginning of cultivation. In the absence of **1**, a higher maximum CDW was reached. The glucose consumption was similar for all cultures, whereby glucose was completely consumed after 122 h.

3.2.2. Alkene cleavage with induced and non-induced cultures

Various samples taken during growth in the presence as well as in the absence of *t*-anethole **1** were tested for alkene cleavage. Due to low cell density, no samples were collected after 24 h of growth; thus biotransformations were performed for samples collected after 48, 73, 97 and 122 h of growth. From this study it was observed that for the same amount of cells the conversion increased with increasing CDW of the culture and reached its maximum when

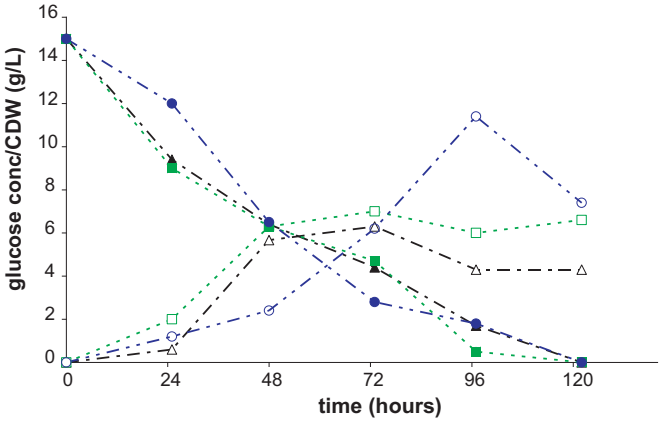


Fig. 1. Effect of *t*-anethole **1** on cell dry weight (CDW) and glucose consumption. Open symbols: CDW, closed symbols: glucose. Squares: presence of **1**; triangles: vapour of **1**; circles: absence of **1**.

the CDW was at its peak (Fig. 2). The highest values of CDW and conversion were reached after 72 h in case of the cultures grown in the presence of **1** and vapour of **1**. Whereas for the culture grown in the absence of **1**, the maximum was reached after 96 h. After this maximum, the conversion decreased for all samples. Interestingly, this decrease was moderate for the culture grown in the presence of **1**, more pronounced for the culture in the presence of vapour of **1** and very clear in the case of the culture grown in the absence of **1**. The conversions obtained for the 122 h culture samples, when glucose was completely consumed, was highest for the culture grown in the presence of **1** (86% conversion) followed by the culture in the presence of vapour of **1** (40%). The lowest conversion was obtained for the culture grown in the absence of **1** (24%).

3.3. Induction protocol and application

The final protocol was slightly modified. After a preculture (100 mL) was grown for 5 days, a fraction (16.5 mL) was used to seed the main culture (330 mL in 1000 mL flask). After 24, 48 and 72 h of growth, **1** (15 μM) was added to the culture medium. At 96 h, the dosage was increased to raise the concentration by 45 μM. The cells were harvested after 100 h of cultivation. Improved conversions could also be demonstrated for other previously published substrates [27,28] as shown in Table 1; thus the improved conversion

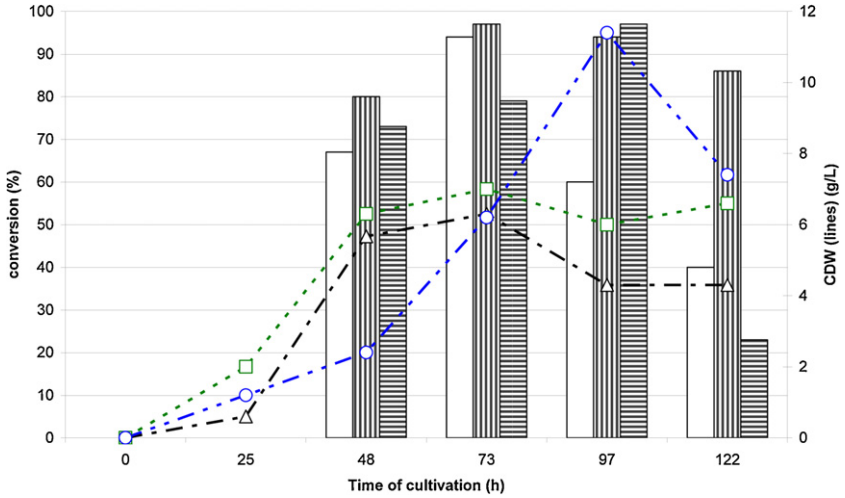
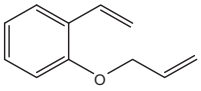
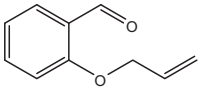
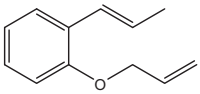
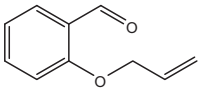
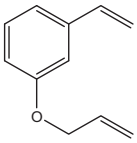
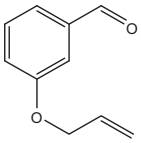


Fig. 2. Effect of addition of *t*-anethol **1** to the cultivation medium on alkene cleavage conversion depending on the time and cell dry weight (CDW). Lines: CDW (Squares: presence of **1**; triangles: vapour of **1**; circles: absence of **1**.); bars: conversion (white bar: vapour of **1**; vertical lines: presence of **1**; horizontal lines: absence of **1**).

Table 1
Conversion for various substrates employing induced and non-induced lyophilized cultures of *T. hirsuta*.

Substrate	Product	Conversion (%)	
		Induced culture	Non-induced culture
		98[a]	<1
		>99[a]	2
		98[a]	1.5

[a] Reaction described in Ref. [28].

was observed for a broad scope of substrates and not only for *t*-anethole **1**, thus by induction the previously reported broad alkene cleaving activity was induced and not a novel *t*-anethole **1** specific activity. The compounds involved are volatile and have poor solubility in aqueous medium. Further investigation on the engineering aspects of the biotransformation reactor setup to minimize this issue is underway.

4. Discussion

Essential oils and their constituents have a long history of applications as antimicrobial agents. *t*-Anethole **1**, occuring widely in nature in essential oils, contributes to the distinctive flavours of various plants [29–31]. In a study [31] it was shown that among several essential oil components tested for anti-fungal properties against a white-rot *Trametes versicolor* and a brown-rot *Coniophora puteana*, *t*-anethole was one of the best antifungal agents with a very low minimal inhibitory concentration (MIC), e.g. for *T. versicolor* the MIC value was 1.25 mM. From our previous study it was known that the cleavage of the C=C double bond of *t*-anethole was catalyzed by an intracellular enzyme in *T. hirsuta* [24]. A possible reason why this fungus has the ability to cleave alkenes is probably a stress response to degrade a powerful fungicide, giving the fungus a significant advantage in the fight of the survival of the fittest. From the preliminary study it was observed that the addition of *t*-anethole in the growth medium resulted in higher alkene cleaving activity. For better insight, it was necessary to perform growth studies, which showed that the alkene cleavage activity in the culture increased with increasing CDW and reached its maximum activity when the CDW was highest. In the presence of *t*-anethole and in the presence of *t*-anethole vapour, the maximum CDW and enzyme activity were obtained after 73 h of cultivation, whereas in the absence of *t*-anethole, the maximum enzyme activity and CDW were obtained after 97 h of growth.

Irrespective of the time required to reach highest CDW, the alkene cleavage activity of the culture which was under constant stress by regular addition of *t*-anethole retained its ability for alkene cleavage even after 5 days (when glucose was completely consumed). In contrast, in the absence of the inducer the conversion (24 h) started to drop significantly as soon as it reached highest CDW. Furthermore, in the presence of *t*-anethole vapour the decrease of conversion was less pronounced than in the absence of any *t*-anethole. The investigation of the substrate scope showed that indeed the previously described alkene cleavage activity was induced and not a specific activity for *t*-anethole.

5. Conclusions

t-Anethole **1** is a strong fungicide component present in essential oils produced by certain plants. *t*-Anethole **1** is also an excellent substrate for the biocatalytic alkene cleavage catalyzed by an enzyme preparation from *Trametes hirsuta*. The enzyme is able to cleave C=C double bonds adjacent to an aromatic moiety with molecular oxygen as the sole oxidant. By the addition of *t*-anethole **1** to the growth medium at low concentration (15 μM) the alkene cleavage activity can be maintained during a long time window. The thereby produced biocatalyst still displayed a broad substrate scope. This study demonstrates that the fungal stress response to the fungicide induced a very useful biocatalyst.

Acknowledgements

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