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Reduction of anthranilic acid to 2-aminobenzaldehyde by the white-rot fungus *Bjerkandera adusta* DSMZ 4708

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ABSTRACT

The biocatalytic aerobic “in-water” reduction of anthranilic acid to 2-aminobenzaldehyde by growing cultures of the basidiomycetous white-rot fungus *Bjerkandera adusta* has been studied. The high specific activity of *Bjerkandera adusta* towards the carboxylic group of anthranilic acid that allows avoiding the formation of the corresponding alcohol has been demonstrated using different substrate concentrations. The presence of ethanol as co-solvent allows increasing the yield of target product. In contrast to chemical reducing agents that usually yield 2-aminobenzyl alcohol, an overreduction of anthranilic acid is completely suppressed by the fungus and gives the target flavor compound in satisfactory preparative yields. It was shown that the activity of *Bjerkandera adusta* towards anthranilic acid does not apply to its *m*- and *p*-isomers.

1. Introduction

2-Aminobenzaldehyde (**1**) is a pleasant fragrance component imparting a floral odor. It has been identified as an essential component of many flowers including broom (*Spartium junceum*), false acacia (*Robinia pseudoacacia*), European bird cherry (*Padus avium*), lily (*Lilium candidum*), seringat (*Philadelphus coronarius*), *Pittosporum tobira*, and *Hypecoum imberbe* (Joulain, 1987). In *Robinia pseudoacacia*, the biosynthetic pathway towards **1** includes the transformation of anthranilic acid (**2**) to indole (**3**) followed by oxidative ring opening and hydrolysis of the resulting *N*-formyl-2-aminobenzaldehyde (**4**) (Spiteller and Steglich, 2001). The chemical synthesis of **1** is based on the reduction of 2-nitrobenzaldehyde (**5**) with aqueous ferrous sulfate and ammonia (Scheme 1) (Foy et al., 1993). An alternative approach based on the reduction of the much less expensive precursor anthranilic acid (**2**) is not successful as it proceeds directly to 2-aminobenzyl alcohol due to overreduction (Wiklund and Bergman, 2006). This is due to the well-known fact that aldehydes are much more reactive towards reductants than carboxylic acids.

The preparation and handling of 2-aminobenzaldehyde (**1**), which is more reactive than many other aldehydes, is difficult. Additionally, it is complicated by the presence of both, amino and aldehyde groups, causing intermolecular condensation reactions. Formed under acidic conditions, the dimer **6** and the tetramer of **1** have been known for around 100 years, (Seidel, 1926) giving rise to McGeachin-type bisaminals chemistry (Chen et al., 2020). Several spectrophotometric assays are based on the ability of **1** to react spontaneously with δ-1-pyrrolines and δ-1-piperidines generating the triple aromatic ring structure **7** (Schöpf et al., 1948). For example, **1** reacts with pyrrolines forming yellow compounds that are used in spectrophotometric methods for the determination of diamine oxidase activity (Bolmstedt and Tham, 1959; Boehm et al., 2020a). The same characteristic of **1** makes it possible to simply and quickly determine the amount of δ-1-piperidine-6-carboxylate in the urine of patients with antiquitin deficiency, which causes the most common vitamin B_6-dependent epileptic encephalopathy (Boehm et al., 2020b). Moreover, **1** is routinely used as a highly specific tool for dihydrodipicolinate synthase purification due to the formation of the purple adduct (**8**) with 2,3-dihydrodipicolinate (Mitsakos et al., 2011).

Previous data suggest that utilization of living organisms for the preparation of 2-aminobenzaldehyde (**1**) should be pretty challenging. However, it is already known that **1** is produced by several basidiomycetous fungi, namely *Hebeloma Sacchariolens* (Wood et al., 1992) and *Wolfiporia cocos* (Sommer et al., 2021). Recently, we demonstrated the ability of white-rot fungi to reduce efficiently aromatic carboxylic acids to the corresponding aldehydes and alcohols, (Zhuk et al., 2021, 2022) and the aim of current study was to develop an effective and inexpensive

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method for the production of 2-aminobenzaldehyde with potential for direct use by humans. Herein, we propose the first biocatalytic approach to 2-aminobenzaldehyde (**1**) by selective reduction of anthranilic acid (**2**) with growing cultures of the white-rot fungus *B. adusta*.

2. Materials and methods

2.1. Organism

The filamentous fungi *Bjerkandera adusta* (DSMZ, 4708), *Irpex consors* (DSMZ, 7382), *Trametes versicolor* (DSMZ, 11309), *Marasmius cohortalis* (DSMZ, 8257), *Stereum complicatum* (DSMZ, 5182), *Flammulina velutipes* (DSMZ, 1658), *Daedalea quercina* (DSMZ, 4953), *Pleurotus sapidus* (DSMZ, 8266), *Pleurotus erymgii* (DSMZ, 8264), *Pleurotus cornucopiae* (DSMZ, 5342), *Pleurotus ostreatus* (DSMZ, 1833), *Dichomitus squalens* (DSMZ, 9615), *Auricularia fuscosuccinea* (DSMZ, 9844), and *Auricularia polytricha* (DSMZ, 6918) were obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. The stock cultures were maintained on a solid medium containing 15 $g \cdot L^{-1}$ malt extract (Fluka, Neu-Ulm, Germany) and 15 $g \cdot L^{-1}$ agar-agar (Roth, Karlsruhe, Germany).

2.2. Chemicals

Anthranilic acid (99,5%), 2-aminobenzaldehyde (98%), 3-aminobenzoic acid (98%), and 4-aminobenzoic acid (99%) were purchased from Merck (Darmstadt, Germany) and used without further purification.

2.3. Screening of fungal reductive activity towards anthranilic acid using surface cultures

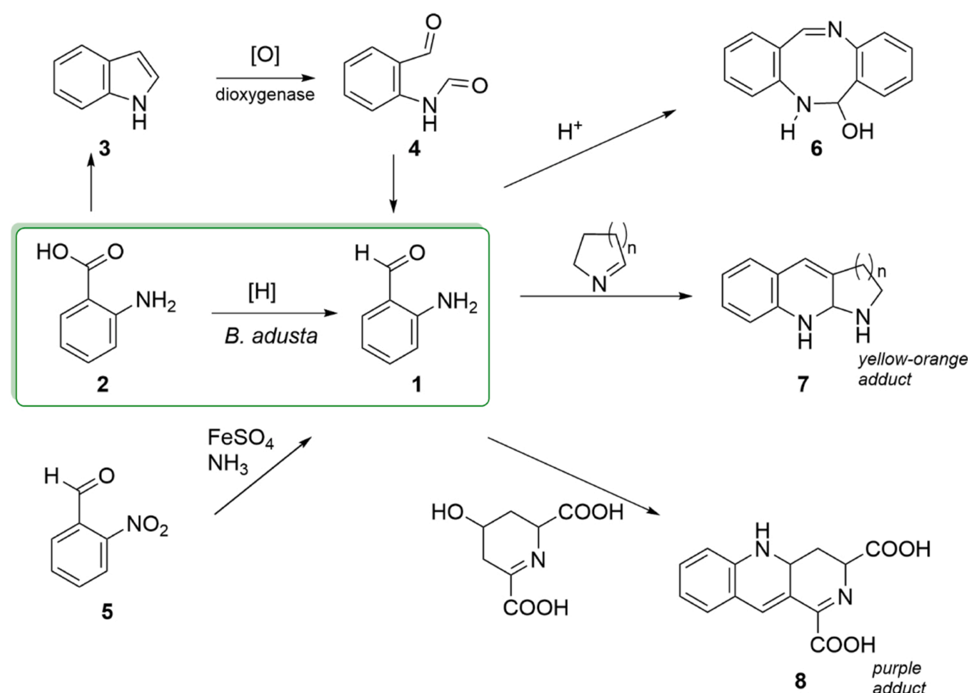
The fungi were maintained on malt extract agar (MEA; 20 $g \cdot L^{-1}$ malt extract, 15 $g \cdot L^{-1}$ agar-agar) or minimal medium (MMA; 15 $g \cdot L^{-1}$ agar-agar, 6.24 $g \cdot L^{-1}$ monosodium L-aspartate$\cdot H_2O$, 2.4 $g \cdot L^{-1}$ NH_4NO_3, 1.5 $g \cdot L^{-1}$ KH_2PO_4, 0.5 $g \cdot L^{-1}$ $MgSO_4 \cdot H_2O$, 400 $\mu g \cdot L^{-1}$ ethylenediaminetetraacetic acid (EDTA), 90 $\mu g \cdot L^{-1}$ $ZnSO_4 \cdot 7\ H_2O$, 80 $\mu g \cdot L^{-1}$ $FeCl_3 \cdot 6\ H_2O$, 30 $\mu g \cdot L^{-1}$ $MnSO_4 \cdot H_2O$, 5 $\mu g \cdot L^{-1}$ $CuSO_4 \cdot 5\ H_2O$, pH=6) without and with glucose (15 $g \cdot L^{-1}$). For inoculation, an agar plug (a 0.4 cm diameter) was transferred from a freshly overgrown plate to agar plates with corresponding media containing anthranilic acid (137 $\mu g \cdot L^{-1}$, approximately 3.5 mg per agar plate). As anthranilic acid is a toxic compound and poses danger in dust form, we kept it in agar layer. It is not volatile as it is characterized by a low vapor pressure of 0.001 hPa (52.6 °C). Cultivation was performed at 24 °C in the dark for up to 14 days. The formation of 2-aminobenzaldehyde was determined organoleptically, as the compound is characterized by specific strong floral odor.

2.4. Submerged cultures

The culture medium was prepared by dissolving malt extract (20 g) in 1 L of deionized water. For the preparation of the precultures, a 1 cm^2 agar plug from the leading mycelial edge was transferred into 100 mL medium (in a 250 mL Erlenmeyer flask) and then homogenized with a T 25 digital Ultra-Turrax homogenizer (IKA, Staufen, Germany; 30 s, 10.000 $r \cdot min^{-1}$). The precultures were grown on an incubation shaker (Orbitron, Infors HAT, Bottmingen, Switzerland; 150 $r \cdot min^{-1}$, deflection 25 mm) under the exclusion of light at 24 °C for 7 days. Subsequently, the precultures were homogenized, and 10% (v/v) of the homogenate was inoculated for submerged cultivation on 100 mL (in 250 mL Erlenmeyer flasks) scale.

2.5. Biotransformation of anthranilic acid with growing cultures

Anthranilic acid (22 mg (1.5 mM), 30 mg (2 mM), 37.7 mg (2.5 mM), 45 mg (3 mM), 52.7 (3.5 mM), 60 mg (4 mM)) was added to submerged cultures of *B. adusta* (110 mL) grown in malt extract (2%) medium in 250 mL Erlenmeyer flasks on the 3rd culture day. The reaction mixture was placed on an incubation shaker at 150 $r \cdot min^{-1}$ (deflection 25 mm) under exclusion of light at 24 ºC for 2 days. After supplementation of the growing culture with the substrate, samples (3 mL) were taken every 4 h. 0.5 g of NaCl was added to the sample, and the mixture was stirred for 5 min at 800 rpm (magnetic stirrer). For extraction, 3 mL Et_2O that contained 2.5 mM internal standard was added, and the resulting mixture was stirred for 10 min at 800 rpm



Scheme 1. Strategies for the production of 2-aminobenzaldehyde (**1**) and some chemical properties.

(magnetic stirrer) and centrifuged for 5 min at 3000 × g to separate the organic layer. The extraction was repeated three times. The combined organic layers were washed with brine (1 × 20 mL) and water (1 × 20 mL), dried over Na_2SO_4, concentrated to 2 mL, and analysed by GC-MS (Agilent Technologies 7890 A GC, column Agilent VF-WAXms (30 m × 0.25 mm, 0.25 μm) and Agilent 5975 C MSD Triple-Axis mass spectrometer). Every experiment was repeated three times to verify the reproducibility of the results.

2.5.1. Biotransformation of anthranilic acid with growing cultures: blank experiments

A. The experiment described in Section 2.5 has been performed with autoclaved media without fungus and repeated three times. No traces of product have been identified.

B. The experiment described in Section 2.5 has been performed with growing culture autoclaved prior to the addition of substrate and repeated three times. No traces of product have been identified.

2.6. Biotransformation of anthranilic acid with growing culture in the presence of ethanol

Anthranilic acid (30 mg (2 mM), 37.7 mg (2.5 mM)) was dissolved in ethanol (2 mL) and added to submerged culture of *B. adusta* (110 mL) on the 3rd culture day, and biotransformation proceeded for 2 days. The reaction mixture was processed and analysed as described in Section 2.5. Every experiment was repeated three times to verify the reproducibility of the experiments.

2.7. Up-scaled biotransformation of anthranilic acid

Anthranilic acid (120.7 mg, 0.88 mmol, final concentration of 2 mM) dissolved in 2 mL of ethanol was added to 440 mL submerged cultures of *B. adusta* grown in malt extract medium in 1 L Erlenmeyer flasks on the 3rd culture day. The reaction mixture was placed on an incubation shaker at 150 $r \cdot min^{-1}$ (deflection 25 mm) under exclusion of light at 24 °C for 2 days. 20 g of NaCl was added to the medium, and the mixture was stirred for 15 min at 800 rpm (magnetic stirrer). For extraction, 150 mL Et_2O was added, and the resulting mixture was stirred for 20 min at 800 rpm (magnetic stirrer), and layers have been separated. The extraction was repeated 5 times. The combined organic phases were dried over Na_2SO_4 and evaporated to dryness. The product was isolated by column chromatography on silica gel (hexane/ether = 1/1) to give 2-aminobenzaldehyde (41 mg, 39%) as a yellow solid. The identity of the solid has been confirmed by comparison of its ^1H and ^{13}C spectra with those of an authentic reference compound.

2.8. Biotransformations of isomers of anthranilic acid with growing culture

3-Aminobenzoic acid (30 mg, (2 mM)) was added to submerged culture of *B. adusta* (110 mL) on the 3rd culture day, and biotransformation proceeded for 2 days. The reaction mixture was processed and analysed as described in Section 2.5.

4-Aminobenzoic acid (30 mg, (2 mM)) was added to submerged culture of *B. adusta* (110 mL) as described above. The reaction mixture was processed and analysed as described in Section 2.5.

Anthranilic acid (15 mg, (1 mM)) and 4-aminobenzoic acid (15 mg, (1 mM)) were added to submerged culture of *B. adusta* (110 mL) as described above. After 24 h the reaction mixture was processed and analysed as described in Section 2.5.

Every experiment was repeated three times to verify the reproducibility of the experiments.

2.9. Biotransformation of anthranilic acid with resting cells

The mycelium was collected from the submerged cultures of *B. adusta* after three days of growing and washed three times with sterilized distilled water. Then the corresponding sterilized buffer was added (the volume was twice less then volume of supernatant). The next buffers have been tested: HEPES (pH = 7, 0.05 mM and 0.1 mM), phosphate (pH = 7, 0.05 mM and 0.1 mM), TRIS-HCl (pH = 7, 0.05 mM and 0.1 mM). Then anthranilic acid (2 mM) was added. The reaction mixtures were placed on an incubation shaker at 150 $r \cdot min^{-1}$ (deflection 25 mm) under exclusion of light at 24 °C for 20 h. After 24 h the reaction mixture was processed and analysed as described in Section 2.5. Every experiment was repeated three times to verify the reproducibility of the results.

3. Results and discussion

3.1. Screening of basidiomycetous fungal reductive activity towards anthranilic acid (2)

In a broad screening of various basidiomycetes, 14 different fungi from the orders Agaricales, Polyporales, Russulales and Auriculariales were tested for the reduction of **2**. Either malt extract (ME) or minimal medium with or without glucose were tested for fungal growth. The first screening round has been performed using surface cultures, and *B. adusta* has been identified as the most promising fungus for the reduction of anthranilic acid (**2**). The results of the screening have been presented in Table S1. Then the process has been transferred into submerged cultures in which 2-aminobenzaldehyde (**1**) has been identified without traces of the corresponding alcohol as a product of further reduction of the aldehyde group. The most efficient reduction of anthranilic acid (**2**) was obtained in malt extract (2%) medium and addition of **2** on the third culture day.

The reductive activity of *B. adusta* towards **2** may be associated with the presence of specific carboxylic acid reductases (CARs), that are common enzymes for white-rot fungi and other organisms. CARs are large multi-domain proteins that catalyze the ATP- and NADPH-dependent reduction of a wide range of acids to the corresponding aldehydes. Carbonyl reductase activity towards the corresponding aldehyde **1** was not observed, as no traces of the alcohol have been identified in the reaction mixtures.

3.2. Time course for anthranilic acid (2) biocatalytic reduction

In the next step, *B. adusta* was grown submerged in malt extract medium in 250 mL Erlenmeyer flasks (110 mL medium). The formation of 2-aminobenzaldehyde (**1**) was monitored quantitatively using o-xylene as an internal standard by GC-MS analysis. The range of initial concentration of anthranilic acid (C_0) was 1.5 mM - 4 mM (Fig. 1). Samples was taken every four hours during 2 days. The maximum concentrations of **1** in A (C_0 = 1.5 mM) and B (C_0 = 2 mM) were achieved after 8 h and 24 h, and amounted to 0.85 mM and 1.1 mM respectively, corresponding to roughly 55% yield. In C (C_0 = 2.5 mM) and D (C_0 = 3 mM), the highest concentrations of **1** were observed after 28 h (0.78 mM and 0.54 mM). In E (C_0 = 3.5 mM) the maximum concentration of **1** (1.3 mM) was found after 32 h of transformation. With C_0 = 4 mM (F) the concentration of **1** was very low (0.2 mM).

The above experiments clearly showed that the time to reach the maximum concentration increased with increasing substrate concentration. This could be a result of toxic effects of anthranilic acid on the culture that slow down the expression of the corresponding reductases, or the gradual dissolution of the anthranilic acid (**2**) at higher concentrations. Also, the yield of the aldehyde **1** decreased with increasing substrate concentrations. The optimal concentration was C_0 = 2 mM, when the yield of **1** was roughly 55%. It was clearly shown that after reaching the maximum, the concentration of **1** decreased till its complete disappearance in all experiments. This may be caused by further transformation of the aldehyde **1**, e.g., by oxidative degradation or condensation. The further reduction of **1** can be excluded, as no traces of

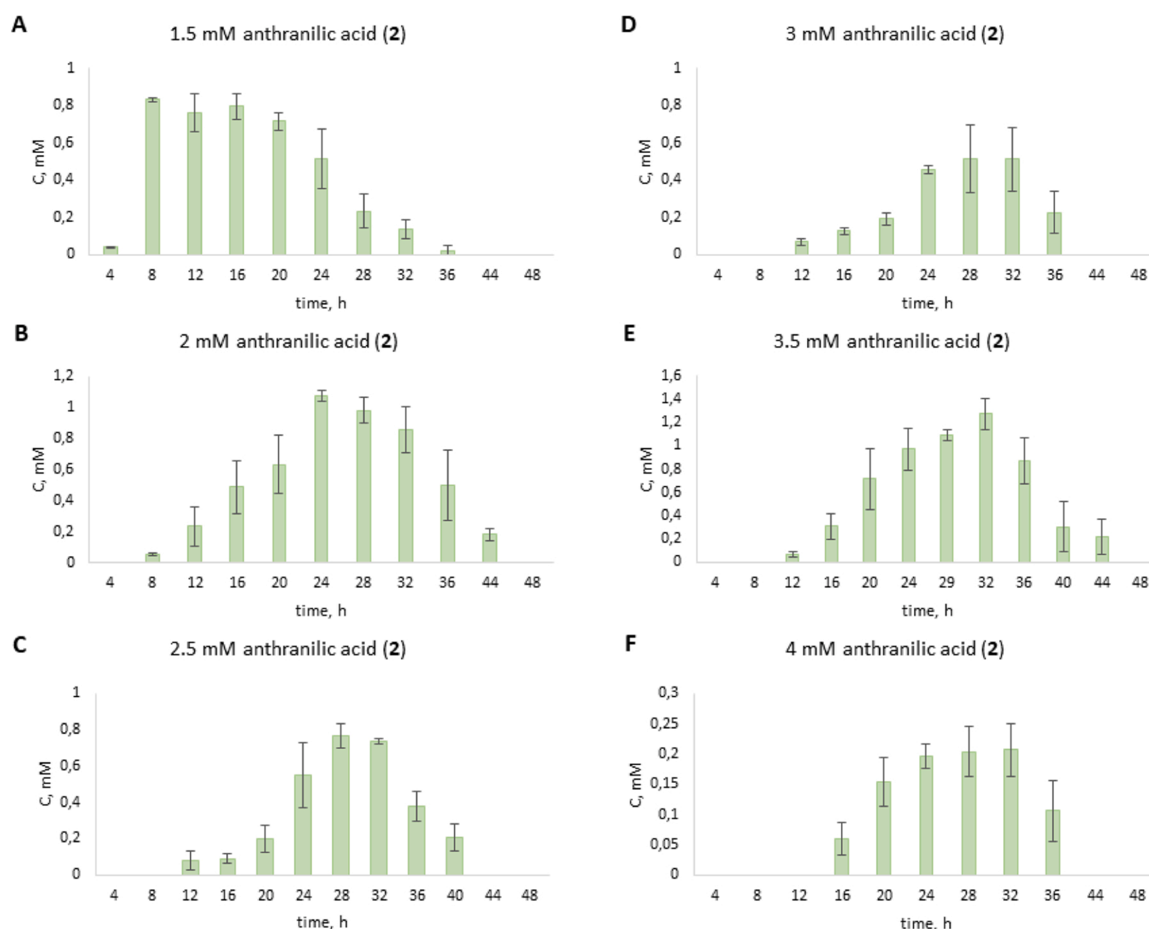


Fig. 1. Formation of 2-aminobenzaldehyde (**1**) in submerged cultures of *B. adusta* containing varying initial concentrations of anthranilic acid (**2**) as precursor: A) 1.5 mM, B) 2 mM, C) 2.5 mM, D) 3 mM, E) 3.5 mM, F) 4 mM. Error bars represent standard deviations based on triplicate experiments.

the corresponding alcohol have been detected in the reaction mixtures. The further transformation of 2-aminobenzaldehyde in cultures of BAD are subject of our future research.

3.3. Reduction of anthranilic acid (2) in the presence of ethanol as co-solvent

Previously, it was shown that white-rot fungi can survive and efficiently transform the compounds of interest in the presence of alcohols as co-solvents (Zhuk et al., 2015). Moreover, the presence of co-solvents may increase the yields of the biotransformation reactions. Therefore, the reduction of **2** was studied in the presence of ethanol. The yields of 2-aminobenzaldehyde (**1**) were significantly increased, and the presence of the co-solvent substantially decreased the reaction times. The observed effect of the presence of ethanol as co-solvent could be a result of better and faster dissolution of the substrate in the culture media (Fig. 2).

3.4. Preparative transformations

To evaluate the potential of the developed biotransformation for preparative applications, the reduction of anthranilic acid (**2**) by *B. adusta* in the presence of ethanol as a co-solvent was scaled-up (Scheme 2). Using 2 mM as the initial concentration, acid **2** was added

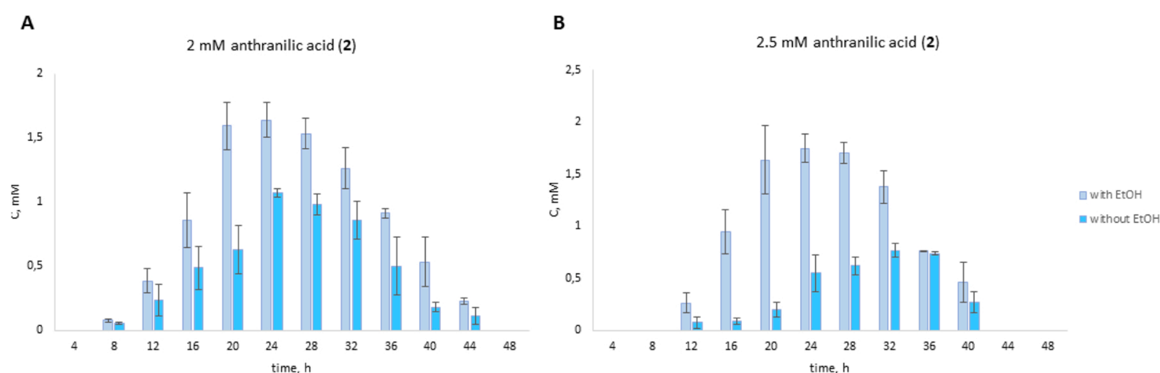
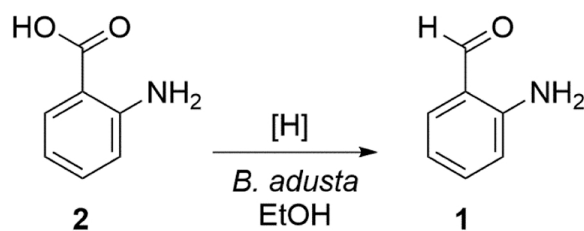


Fig. 2. Formation of 2-aminobenzaldehyde (**1**) in submerged cultures of *B. adusta* with ethanol as co-solvent. Initial concentrations of anthranilic acid (**2**): A) 2 mM, B) 2.5 mM. Error bars represent standard deviations based on triplicate experiments.



Scheme 2. The reaction of preparative biotransformation of anthranilic acid (**2**).

to 440 mL culture on the 3rd culture day. The concentration of aldehyde **1** increased during 2 days, and started to decrease after 48 h. The reaction was stopped by the addition of NaCl to the reaction medium. The extraction of the reaction mixture (including culture supernatant and mycelium) with diethyl ether resulted in 50 mg of crude extract. After combining all organic extracts and purification by column chromatography, 41 mg of pure 2-aminobenzaldehyde (**1**) with GC-MS and NMR spectra identical to those of the reference compound were obtained. To the best of our knowledge, this is the first example of the preparative biocatalytic reduction of anthranilic acid that allows the production of 2-aminobenzaldehyde (**1**) without formation of even traces of 2-aminobenzyl alcohol.

3.5. Biocatalytic reduction of isomers of anthranilic acid (2)

In order to clarify if the developed approach could be also applied for the synthesis of isomers of **1**, the following experiments were performed. Under the conditions developed for the reduction of **2**, isomers of anthranilic acid, namely 3-amino- and 4-aminobenzoic acid were subjected to the biotransformation process. After supplementation of 3-aminobenzoic acid, only trace amounts of the corresponding aldehyde were detected, and 4-aminobenzoic acid was not reduced to the corresponding aldehyde at all. 3- and 4-aminobenzyl alcohol were also not detected in the reaction mixtures. Experiments with simultaneous addition of anthranilic and 4-aminobenzoic acid to the fungal culture were also performed. **1** was identified as a single aldehyde in reaction mixture.

3.6. Biotransformation of anthranilic acid with resting cells

We decided to analyse the ability of resting cells to reduce the anthranilic acid (**2**). We tested three buffers keeping neutral pH in every case. Neutral pH should help to avoid the further transformations of the target product 2-aminobenzaldehyde (**2**). In the reaction mixtures with HEPES and phosphate buffers only the traces of **2** have been detected. With TRIS-HCl buffer the yield of **2** was 15–20% according to GC-MS. These results clearly show that responsible for the biotransformation enzymes are produced by *B. adusta* not only in presence of anthranilic acid (**1**), but they are its regular enzymes.

4. Conclusions

Growing cultures of the basidiomycetous fungus *B. adusta* demonstrate the ability to selectively reduce anthranilic acid to 2-aminobenzadehyde with satisfactory chemical yields and without overreduction to the corresponding benzyl alcohol. This biocatalytic approach is potentially useful for the production of this highly demanded aroma component with a floral scent by the aerobic "in-water" approach. In addition, our experimental results offer clear paths for the further development this green technology for the production of 2-aminobenzadehyde, *e.g.,* through the use of co-solvents and/or identification and further application of the enzyme responsible for the disclosed biotransformation.

CRediT authorship contribution statement

Yuliya Kurtash: Methodology. **Holger Zorn:** Writing – review & editing, Resources, Conceptualization. **Valeriia Babkina:** Writing – original draft, Visualization, Methodology. **Yana Hajduk:** Methodology. **Tatyana Zhuk:** Writing – review & editing, Validation, Supervision, Project administration, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiotec.2024.03.015.

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