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EXPERIMENTAL ARTICLES

# Thiomorpholine Transformation by the Fungus *Bjerkandera adusta*

**I. T. Ermakova[a,1], N. G. Vinokurova[a], N. F. Zelenkova[a], B. P. Baskunov[a], and A. A. Leont'evskii[a,b]**

[a] *Skryabin Institute of Biochemistry and Physiology of Microorganisms, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia*
[b] *Pushchino State University*

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**Abstract**—A screening of lignin-degrading basidial fungi that can grow in the presence of thiomorpholine derivatives (the mixture of 1,4-perhydrothiazines) has been performed. Strain *Bjerkandera adusta* VKM F-3477 was shown to have the maximal rate of growth in the presence of these compounds, and its capacity for thiomorpholine degradation was studied. The methods of quantitative analysis of thiomorpholine and its degradation products on the basis of thin layer chromatography and high-performance liquid chromatography were developed. It was shown that the *B. adusta* strain did not utilize thiomorpholine as a carbon source but transformed it into thiomorpholine sulfoxide that accumulated in the medium. Mn peroxidase produced by *B. adusta* in the course of thiomorpholine transformation is not directly involved in its oxidation.

*Key words: Bjerkandera adusta*, thiomorpholine, 1,4-perhydrothiazines, thiomorpholine sulfoxide, transformation.

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Heterocyclic compounds of the class of morpholines, including thiomorpholine (TM), are widely used in production of pesticides, drugs, rubber, dyes, and as anticorrosion agents in boiler systems [1]. Furthermore, TM and its derivatives, 1,4-perhydrothiazines (PHT), are among the products of mustard gas neutralization, which are formed in the course of the Russian procedure for toxic agent elimination as a result of yperite interaction with monoethanolamine, a component of the decontaminating mixture [2].

Wide occurrence of morpholines arouses the risk of environmental contamination (of soils and water reservoirs). The most efficient approach to solving such ecological problems is the development of biotechnologies based on microbial destructive activity resulting in mineralization of toxic agents or their transformation into less dangerous compounds.

Efforts to find microbial degraders of morpholines have been unsuccessful for a long time [3], until bacterial strains growing on morpholine as a carbon, nitrogen, and energy source were isolated from the water treatment facilities of Monsanto (United States), a producer of various biocides. They were classified within the genus *Mycobacterium* [4]. By now, other bacteria of the genera *Mycobacterium*, *Arthrobacter*, and *Pseudomonas* have been found which degrade heterocyclic compounds of the class of morpholines [5, 6].

The pathway of morpholine degradation by mycobacteria was studied in detail. The initial reaction is the rupture of the C–N bond resulting in formation of an amino acid, which is then deaminated and transformed into glycolic acid. In the case of TM, a sulfur atom is at first oxidized with the formation of TM sulfoxide and then, after the rupture of the C–N bond and deamination, thiodiglycolic acid (TDGA) is formed and later involved in cell metabolism. Cytochrome P450 is supposed to participate in morpholine transformation in these organisms [7].

The pathway of rupture of the C–S bond in a polycyclic aromatic compound, dibenzothiophene, has been described. The initial stage of microbial attack on dibenzothiophene molecule is oxidation of the sulfur atom by dibenzothiophene oxygenase with successive formation of sulfoxide, sulfone, and 2-hydroxybiphenyl-2-sulfinate. The C–S bond is ruptured by 2-hydroxybiphenyl-2-sulfinate lyase with elimination of the sulfur atom as a sulfate ion and formation of 2-hydroxybiphenyl in *Rhodococcus rhodochrous* and *Gordona aichiensis* [8], or of benzoate in *Brevibacterium* sp. [9].

Ligninolytic fungi (basidiomycetes) are well known as effective destructors of stable compounds. As a rule, degradation of various xenobiotics by these fungi occurs as a result of expression of extracellular ligninolytic enzymes, which combine high redox potential and broad substrate specificity. However, stable compounds of some classes can be degraded by ligninolytic

[1] Corresponding author; e-mail: ermakova@ibpm.pushchino.ru

fungi without involvement of the ligninolytic enzyme system [10].

Mustard gas and its derivatives, including thiodiglycol as one of the products of yperite neutralization, are rather easily degraded by ligninolytic fungi [11, 12]. Ligninolytic fungus *Coriolus versicolor* was used in the study of degradation of heterocyclic sulfur-containing thiophene derivatives as well as transformation of benzothiophene and its derivatives with formation of water-soluble sulfoxides [13]. There are no reports on participation of ligninolytic fungi in TM or PHT transformation.

The goal of this research was to reveal the ability of thiomorpholine degradation in ligninolytic fungi and to elaborate analytical methods for determination of substrates and identification of intermediate degradation products.

## MATERIALS AND METHODS

Strains of basidiomycetous fungi *Cerena unicolor* F-3196, *Agaricus arvensis* F-1589, *Flammulina velutipes* F-1996, *Coprinus comatus* F-2940, *Fomes fomentarius* F-3203, *Kuehneromyces mutabilis* F-3215, *Pleurotus ostreatus* F-3468, *Daedalea quercina* F-1655, *Ganoderma lucidum* F-1720, *Bjerkandera adusta* F-3477, *Acremonium arxii* F-2717, and *Armillaria mellea* F-1163 were provided by the All-Russian Collection of Microorganisms (VKM, Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences).

Strains resistant to morpholines were preselected according to their growth rate on plates of glucose–potato agar (GPA) containing a model mixture that simulated the composition of reaction masses of yperite neutralization (PHT, 0.15%; monoethanolamine, 0.25%; and ethylene glycol, 0.025%) [2], with dilution 1 : 100. PHT contained 20% N-(2-hydroxyethyl)thiomorpholine, 57% N-(2-hydroxyethyl)-2-methylthiomorpholine, 4% N-(2-hydroxyethyl)-2,6-dimethylthiomorpholine, 12% N-(2-hydroxyethyl)-3-methylthiomorpholine, and 7% N-(2-hydroxyethyl)-2,5-dimethyl thiomorpholine [14].

The dynamics of activity of ligninolytic enzymes in the fungi *D. quercina, G. lucidum*, and *B. adusta* was studied in the course of their cultivation on a modified Kirk medium [15] containing 1% glucose as a carbon source, 0.05% Tween-80, pH 5.0, at high (9.0 g/l of asparagine and 4.8 g/l of $NH_4NO_3$) or low (0.9 g/l of asparagine and 0.48 g/l of $NH_4NO_3$) concentrations of nitrogen sources. Other media used included GD medium (mineral components and trace elements of the Kirk medium, 1% glucose, 0.02% yeast extract, 0.045 g/l $MnSO_4$ in 20 mM phthalate buffer, pH 4.5); and GDP medium (GD medium with 0.5% peptone).

Biomass growth, TM utilization and accumulation of its degradation products were studied during cultivation of *B. adusta* on the modified Kirk medium with "high" nitrogen. As a Mn peroxidase inducer, 50 mg/l of Mn (0.169 g/l $MnSO_4 \cdot H_2O$) was added. TM (0.1% vol/vol) was introduced into the Kirk medium immediately before inoculation.

Cultivation was carried out in a shaker (200 rpm) at 29°C in 750-ml flasks with 100 ml of the medium. The medium was inoculated with homogenized mycelium grown for 7 days under analogous conditions on a soya–glycerol medium containing the following (g/l): $NH_4NO_3$, 0.2; $KH_2PO_4$, 0.2; $K_2HPO_4$, 0.02; $MgSO_4$, 0.1; peptone, 0.5; soya flour, 0.5; glycerol, 0.4 ml.

Commercial preparations of thioglycolic acid (TGA) and TDGA (Sigma and Aldrich, United States) were used as a carbon sources during cultivation of the fungi (TM) and as standards for chromatographic analysis.

TM sulfoxide was obtained by TM oxidation with *m*-chloroperbenzoic acid in methylene chloride [7]. Its structure was confirmed by the results of mass-spectrometric analysis. Mass spectra were registered in a tandem mass spectrometer LCQ Advantage MAX (ThermoFinnigan, Germany) using a single-channel syringe pump for direct sample infusion.

Fungal biomass was assayed by weighing.

Mn peroxidase activity was measured by the method of $H_2O_2$-dependent oxidation of $Mn^{2+}$ with the formation of $Mn^{3+}$/malonate complex [16]; laccase activity was measured by the rate of 2',2'-azino-*bis*(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) oxidation at 436 nm [17]; and lignin peroxidase activity was measured by the rate of veratric alcohol oxidation [18].

TM transformation products were identified by thin-layer chromatography (TLC).

The quantitative analysis of TM and TM sulfoxide was performed by HPLC reversed-phase liquid chromatography (LC) in a high-pressure chromatograph equipped with a UV-detector with variable wavelength and LKB-Pribori A integrator (Sweden). A Symmetry 300™ C4 column, 150 ± 3.9 mm (Waters, United States) was used in the work. The working wavelength was 206 nm; the analysis was performed at room temperature; the consumption of eluent (methanol : water : 25% ammonium, 2 : 98 : 0.036) was 0.5 ml/min. Calculations were made by the external standard method. The holding time of TM sulfoxide and TM was 6.21 and 22.69 min, respectively. This technique makes it possible to determine these metabolites in the range of concentrations from 30 to 1000 μg/ml with the relative error of determination (%) 15.2 (TM sulfoxide) and 13.5 (TM) at $n = 5$, $P = 0.95$.

## RESULTS AND DISCUSSION

**Screening of the fungi growing in the presence of PHT.** With the purpose of revealing fungi that could

**Table 1.** Fungal growth on agarized medium in the presence of PHT (cm)*

| Strain | GPA (control) | GPA + PHT mixture | Strain | GPA (control) | GPA + PHT mixture |
|---|---|---|---|---|---|
| *A. arxii* | 3.5 | 0.5 | *D. quercina* | 10.0 | 10.0 |
| *A. arvensis* | 10.0 | NG** | *F. velutipes* | 10.0 | 0.1 |
| *A. mellea* | 0.5 | 0.1 | *F. fomentarius* | 10.0 | NG** |
| *B. adusta* | 10.0 | 10.0 | *G. lucidum* | 10.0 | 10.0 |
| *C. unicolor* | 10.0 | 3.0 | *K. mutabilis* | 0.1 | NG** |
| *C. comatus* | 10.0 | NG** | *P. ostreatus* | 10.0 | 2.5 |

* Colony diameter after 20 days of cultivation.
** NG, no growth.

grow in the media with heterocyclic compounds, the strains obtained from VKM were screened by cultivation on a rich GPA medium in the presence of a chemically synthesized PHT mixture.

Fungal growth was assessed by colony diameter (cm) after 20 days of cultivation at 29°C. GPA medium without PHT was used as a control. The data on the screening of the fungal strains are presented in Table 1. The screening revealed three strains (*Daedalea quercina* F-1655, *Ganoderma lucidum* F-1720, and *Bjerkandera adusta* F-3477) forming colonies of the same size on the medium with PHT as in the control.

**Selection of cultivation conditions that yielded maximal activity of the fungal ligninolytic enzymes.** In selected fungal strains, the activities of ligninolytic enzymes (lignin peroxidase, Mn peroxidase, and laccase) were determined at a low and high nitrogen concentration, i.e., under conditions favorable for the activation and suppression of the total fungal ligninolytic activity, respectively. In addition, manganese was introduced as a Mn peroxidase inducer; yeast extract (GD medium) and peptone (GDP medium) were assessed as potential "inducers" of the enzymes. Under the above conditions, none of the three selected fungal strains showed the activities of lignin peroxidase and laccase.

Mn peroxidase activity was maximal in the fungus *B. adusta* at a high nitrogen concentration in the medium (Table 2). This culture was chosen as a subject of further research.

**Methods of monitoring of TM degradation and accumulation of its metabolic products.** Supposed products of TM degradation may belong to different classes of chemical compounds: secondary amines, organic acids, sulfides, and sulfoxides. The optimal conditions of TLC analysis were developed for identification of each group of compounds.

After analysis of the above compounds using thin layer plates of different manufacture, PTCH-P-V Sorbfil plates (Sorbpolimer, Russia) were chosen for further work.

The most satisfactory results of amines separation were obtained with the system of chloroform : methanol : 25% ammonia (80 : 20 : 2). In this system, the $R_f \times 100$ values were 53, 32, and 26 for TM, its sulfoxide, and sulfone, respectively. The reagent for detection of N-methylamino acids, containing equal volumes of 0.33% ninhydrin solution in tret-butanol and a mixture of glacial acetic acid : water : pyridine (1 : 5 : 5), was used for detection of these substances. The plate was sprayed and heated for several minutes at 70–80°C. The limit of TM detection by this reagent was below 1 μg.

The product of TM biodegradation in mycobacteria is TDGA, which, in turn, is metabolized to TGA and acetic acid [7]. Chromatographic analysis of thioacids was performed in a system of isopropanol : 25% ammonia (70 : 30). The 0.8% alcohol solution of bromocresol

**Table 2.** The maximal activity of Mn peroxidase at cultivation of the fungus on different media

| Medium | Mn-peroxidase activity, U/ml | | |
|---|---|---|---|
| | *G. lucidum* | *B. adusta* | *D. quercina* |
| GDP | ″ | 0.1 | ″ |
| GDP | ″ | 0.8 | ″ |
| Kirk medium with a high nitrogen concentration | 2.0 | 7.0 | ″ |
| Kirk medium with a low nitrogen concentration | ″ | 3.5 | ″ |

″ stands for not detected.

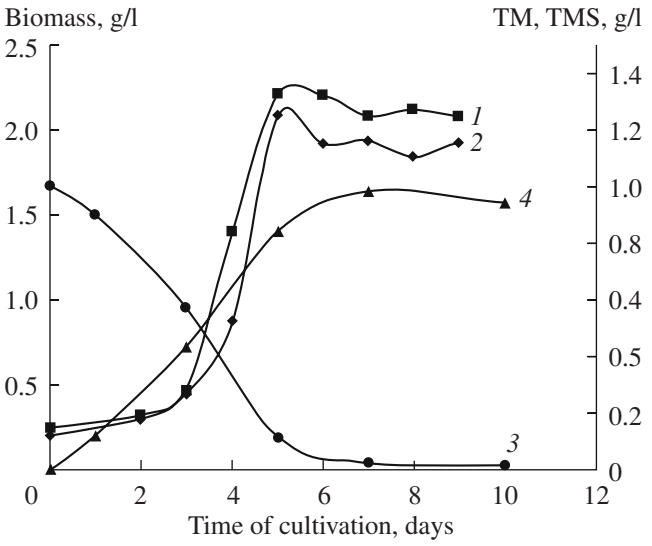


**Fig. 1.** TM transformation in the growth dynamics of the fungus *B. adusta*: biomass in the medium without TM (*1*); biomass in the medium with TM (*2*); TM (*3*); and TM sulfoxide (*4*).

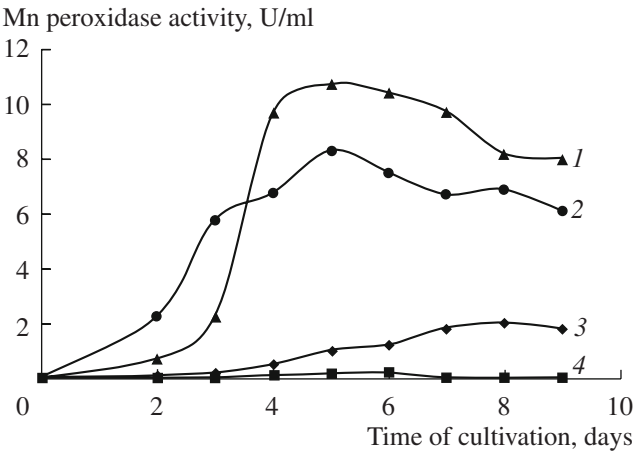


**Fig. 2.** Mn peroxidase activity at cultivation of the fungus *B. adusta* in the medium with Mn: with TM (*1*) and without TM (*2*); in the medium without Mn: with TM (*3*) and without TM (*4*).

green was used as an indicator. The values of $R_f \times 100$ were 32 and 41 for TDGA and TGA, respectively.

The following special reagent was additionally used for detection of compounds containing bivalent sulfur (TM, TDGA, and TGA): 0.002 M iodine solution in 50% ethanol with 1.5% sodium azide.

Besides, the method of ion exchange LC was used in model systems to select the conditions of separation of possible TM biodegradation products of acidic nature. The column was Repro-Gel H, 250 × 8 mm (Elsiko, Russia), the UV detector working wavelength was 206 nm, the column thermostat temperature was 65°C, and the mobile phase (0.01 N sulfuric acid) feed rate was 1 ml/min. The time of elution of the analyzed substances under these conditions was (min) 6.92 for TFGA, 8.90 for TGA, 8.4 for acetic acid, 30.79 for TM, and 30.95 for TM sulfoxide.

**Identification of the products of TM degradation by the fungus *B. adusta*.** The culture liquid of *B. adusta* grown in the Kirk medium with glucose in the presence of TM was analyzed by the TLC and LC techniques developed for this purpose. The TLC method showed the presence of a compound with the chromatographic mobility coinciding with the chromatographic mobility of the synthesized TM sulfoxide.

The revealed substance was isolated by the method of preparative chromatography on a Sorbfil plate, eluted from the carrier by methanol, and concentrated on a rotor evaporator to solid residue. The mass spectrum of the isolated metabolite was identical to the mass spectrum of the synthesized TM sulfoxide and characterized by the presence of a molecular ion $(M + H)^+ = 120$ and fragmentary ions with *m/z*: 102, 76, and 56. The multiplicity of the molecular ion signal (*m/z* 122) unambiguously points to the presence of one sulfur atom in the molecule.

The products formed after the rupture of thiomorpholine ring (TGA, TDGA) have not been found in the culture liquid of the fungus *B. adusta* by any of the methods applied.

The dynamics of fungal growth in the media with and without TM showed almost equal amounts of biomass in both variants. Hence, TM was not a carbon source for growth. However, quantitative analysis of the culture liquid by the method of reversed-phase LC showed a gradual decrease of TM content with concurrent increase of TM sulfoxide (Fig. 1). In the control variants, for incubation of the media without microorganisms or with inactivated mycelium, TM sulfoxide was not found.

These results suggest that the basic reaction of TM transformation by *B. adusta* is oxidation of the sulfur atom with the formation of TM sulfoxide. Similar results were obtained for transformation of benzothiazines by the fungus *C. versicolor*, when the respective sulfoxides were found as products in the culture liquid [13].

**TM biotransformation and ligninolytic enzymes.** Enhanced expression of *B. adusta* F-3477 Mn peroxidase in "non-ligninolytic" conditions (at a high nitrogen concentration in the medium, Table 2) is not an abnormal case for fungi of this genus. The expression of lignin peroxidase under cultivation on rich media has been noticed previously in *Bjerkandera* sp. BOS55 [19] and *B. adusta* IFO 4983 [20]. It seems that the fungi of this genus are a comparatively rare exception from the number of typical ligninolytic basidiomycetes, where the production of ligninolytic peroxidases is triggered by nitrogen or carbon starvation [9].

The studies were performed to examine the interrelation between the ability of *B. adusta* to transform TM into TM sulfoxide and the activity of Mn peroxidase. The enzyme activity was determined in the dynamics of culture growth in the presence and absence of TM in the media with and without Mn, an inducer of the enzyme. The findings (Fig. 2) show that the enzyme yield steadily increased by 20–30% for growth of the fungus in the medium with 1 g/l TM and Mn. At the same time, at the beginning of cultivation (1–3 days), when TM was already being actively transformed into sulfoxide, the enzyme activity in the medium with TM was 2.5 times lower than in the medium without TM, even though the amounts of mycelium biomass were almost the same (Fig. 1). The enzyme yield drastically increased in the following period, after TM concentration had become nearly 2 times lower.

Expression of Mn peroxidase in the presence of TM took place in the medium without its inducer, Mn (Fig. 2); noticeable enzyme activity was detected, like in the medium with Mn, only after the decrease of TM concentration (data not shown).

These data demonstrate that TM present in the medium in concentrations of 1 to 0.5 g/l inhibits the expression of Mn peroxidase but, at the same time, is actively transformed into sulfoxide. Hence, the enzyme does not directly catalyze the reaction of transformation.

This is confirmed by the fact that no TM transformation products were revealed upon introduction of 0.1% TM into the culture liquid of *B. adusta* (Mn-peroxidase activity, 7 U/ml) concentrated 20-fold by ultrafiltration and containing the necessary components of the reaction mixture [14] after incubation for 24 h at 30°C.

However, the enhanced yield of the enzyme in the medium with TM and Mn and its induction in the medium without Mn allow us to believe that there is a mediated interrelation between the enzyme expression and the reactions of toxicant transformation.

The study of thiophene transformation by a *C. versicolor* fungal culture has shown no direct participation of ligninolytic enzymes in the oxidation of such substrates, in spite of accelerated transformation of some thiophene derivatives under conditions providing the expression of the ligninolytic enzyme system [13].

Associations of ligninolytic fungi transforming substances of the TM class with bacterial cultures that can break heterocycles followed by assimilation of the degradation products will possibly contribute to solution of the problem of utilizing these toxicants in development of biotechnological methods for the cleaning of polluted environmental objects.

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