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Fungal Biodegradation of the Biocide 2-Methyl-4-Isothiazolin-3-One

Gomes E[1]*, Boscolo M[1], Da Silva R[1], Rodrigues A[2] and Orjuela GL[1]

[1]Laboratory of Biochemistry and Applied Microbiology, Universidade Estadual Paulista, Brazil

[2]Department of Biochemistry and Microbiology, Universidade Estadual Paulista, Brazil

***Corresponding author:** Eleni Gomes, Universidade Estadual Paulista-Unesp, Ibilce Rua Cristóvão Colombo, n. 2265, Jardim Nazareth, São José do Rio Preto, SP, CEP 15054-000, Brazil

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Abstract

Isothiazolinones are the broad spectrum of biocides, frequently used as a mixture of 2-methyl-4-isothiazolin-3-one (MIT) and 5-Chloro-2-Methyl-4-Isothiazolin-3-one (CMI), in personal hygiene products, air fresheners and wall paint. The aim of this work was to investigate the fungi present in wall paint and its components and evaluate the ability of them to grow in the presence of isothiazolinone and to degrade it. 30 strains were isolated and identified as being from 8 genera. *Trichoderma* was the most prevalent in the total range of isolated and *Aspergillus* was the most frequent in the deteriorated paint samples. *T. longibrachiatum* FB01, *F. solani* FB07 and *A. niger* FB14, that were able to grow on a solid medium containing 40 g L^{-1} of MIT, when cultivated in liquid media, were able to degrade this biocide. The growth of *T. longibrachiatum* FB01 was partially (25%) inhibited by the biocide. The growth of *F. solani* FB07 and *A. niger* FB14 were increased by the biocide by 32 and 50% in relation to the control. In the culture medium containing MIT after the cultivation of *A. niger* FB14, malonic, lactic, acetic, 2-oxi-butanoic and metoxi acetic acids were identified. In the culture samples of *F. solani* FB07, acetic, propionic, malonic, propanoic and 2-2-oxobutyric acids were identified and in the cultivation medium with *T. longibrachiatum* FB01, tartaric, acetic and 2-2-oxobutyric acids were found. It was proposed that the biodegradation of MIT by fungi generate similar organic acids determined in the photodegradation.

Keywords: Isothiazolinones; Biodegradation; Wall paint; Fungi

Introduction

Isothiazolinones are frequently used as a mixture of 2-methyl-4-isothiazolin-3-one (MIT) and 5-Chloro-2-Methyl-4-Isothiazolin-3-one (CMI) in a wide range industrialized products, including personal hygiene, air fresheners, perfume, deodorants, cosmetics and wall paint, due their broad spectrum of activity against bacteria and fungi [1,2,3]. The mechanism of action of these compounds is still not completely understood but has been described that they act in the inhibition of transport and metabolism of glucose and generation of energy in prokaryote and eukaryote cells [4] and in the citotoxicity, apoptosis and increasing of P-glycoprotein mRNA expression in eukaryotic cells [5,6].

Cases of Allergic Contact Dermatitis (ACD) in patients who used cosmetics with isothiazolinones have been reported [7,8]. The isothiazolinone derivatives such as Methylisothiazolinone (MIT), Methylchloroisothiazolinone (MCI), and Benzisothiazolinone (BIT) were detected as emissions from wall paint for several days after its application and from evaporation over a longer period, resulting in chronic exposure of people and being residual in the environment [9].

Although the dispersion of these compounds in the environment has been reported [10], fill information about the biogradation has been presented. In their production and storage, the wall paints can be contaminated by fungi and bacteria present in the environment which are involved in the biodegradation of the paint even with the presence of preservatives [11,12,13] and sometimes, the bacteria and fungi are able to degrade these biocides to use them as carbon and other nutrients [14].

The aim of this work was to investigate the mesophilic fungi present in water-based wall paint and its components and as well as to evaluate the ability of these fungi to grow in the presence of isothiazolinone biocides used in its formulation and to evaluate the biodegradation of these compounds.

Materials and Methods

All reagents were of analytical standard and HPLC grade solvents were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, U.S.). The wall-paint used was produced by a factory in São José do Rio Preto / SP/Brazil and it was composed of eight component groups: 1) solvent: water; 2) resin: styrene acrylate in aqueous dispersion (butyl acrylate); 3) additives: biocides (methylisothiazolinone, carbendazine and atrazine); 4) dispersant (sodium hexaphosphate), humectants (sodium dioctyl sulfosuccinate), 5) thickener (acrylic acid) 6) stabilizer (ammonia); 7) charges: mineral calcium carbonate and calcite; 8) pigment: titanium dioxide (white paint). All the individual components used in the experiments were supplied by the paint-manufacturer

Isolation of the fungi

The samples for isolation of the fungi were collected at a paint factory from deteriorated and freshly manufactured paint, water from the effluent treatment system and paint components (paint, acrylic resin, thickener and carbendazine, isothiazolinone and triazine

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Table 1: Tolerance of the fungi isolated from different sampling materials to 2-Methyl-1,2-Thiazol-3(2H)-one (MIT) concentrations.

		% growth in relation to the control)	
		MIT (g L^{-1})	
Isolated Fungi	**Sampling Material**	**30**	**40**
Acremonium sp. FB16	waste water		
Aspergillus flavipes FB25	deteriorated paint		
Aspergillus fresenii FB09	deteriorated paint	20	
Aspergillus niger FB14	isothiazolinone	95	90
Aspergillus sect. *flavipes* FB13	deteriorated paint		
Aspergillus niger. FB12	waste water		
Aspergillus terreus FB26	deteriorated paint		
Fusarium solani species FB07	deteriorated paint	90	65
Fusarium solani species FB19	waste water		
Fusarium solani species FB23	waste water		
Fusarium sp. FB06	equipments	50	
Fusarium sp. FB08	deteriorated paint	40	25
Mortierella alpina FB17	waste water		
Paecilomyces saturatus FB22	waste water	20	
Paecilomyces saturatus FB29	waste water	90	40
Paecilomyces saturatus FB30	equipment biofilms	85	50
Paecilomyces saturatus FB31	equipments	70	20
Paecilomyces sp. FB28	resin	70	
Syncephalastrum racemosum. FB24	fresh paint		
Syncephalastrum sp. FB27	resin		
Talaromyces sp. FB15	wastewater		
*Trichoderm harzianum*FB21	waste water	90	30
T. longibrachiatum FB01	carbendazine	90	70
T.longibrachiatum FB02	resin	90	10
T. longibrachiatum FB03	fresh paint	90	20
T. longibrachiatum FB04	deteriorated paint	95	15
T. longibrachiatum FB11	resin		
T. longibrachiatum FB18	deteriorated paint	40	
Trichoderma sp. FB05	deteriorated paint	90	10
Trichoderma sp. FB20	waste water		

biocides). One milliliter of each sample was transferred aseptically to tubes containing 9 mL of sterile distilled water and serial dilutions (10^{-2} to 10^{-5}) were carried out. Aliquots of 1 mL from each dilution were placed on Petri dishes containing a solid medium, as described by Kirk et al. [15] added of chloramphenicol and ampicillin at 0.2 g L^{-1}. The dishes were incubated at 28 ºC for 72 hours and the isolation of colonies was based on the appearance of the mycelium, anverse and reverse characteristics of the colonies and color of the spores. The cultures were re-inoculated by streaking until pure cultures were obtained. The overall purity was obtained from monosporic cultures and they were maintained in 2.0 mL of 15% glycerol at -80 ºC.

Fungi identification

All fungal strains were identified to genus level based on morphological characters, following molecular identification procedures. DNA extraction, quantification and sequencing followed protocols described in Arcuri et al. [16]. The strains were sequenced using the primer pair recognized as the best markers for defining species in each case: ITS4 and ITS5 for the internal transcribed spacer region of ribosomal DNA gene [17] TEF1R and EF1-986 for the elongation factor 1-α gene [18]) or βt2a and Bt2b for the β-tubulin gene [19].

Forward and reverse sequences were assembled in contigs using BioEdit v.7.1.3 according to Hall [20]. These contigs were used to query for homologous sequences in the NCBI-GenBank database using the BLASTn tool (http://blast.ncbi.nlm.nih.gov/). Whenever possible, sequences of reference strains were retrieved (mostly from the CBS-KNAW Fungal Biodiversity Centre). The alignment was built using MAFFT v. 7158 9 [21]. The phylogenetic analysis was

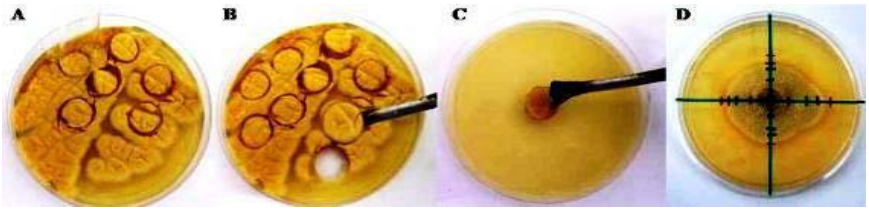

Figure 1: Petri dish preparation for the evaluation of fungi growth and inhibition in the presence of the biocide.

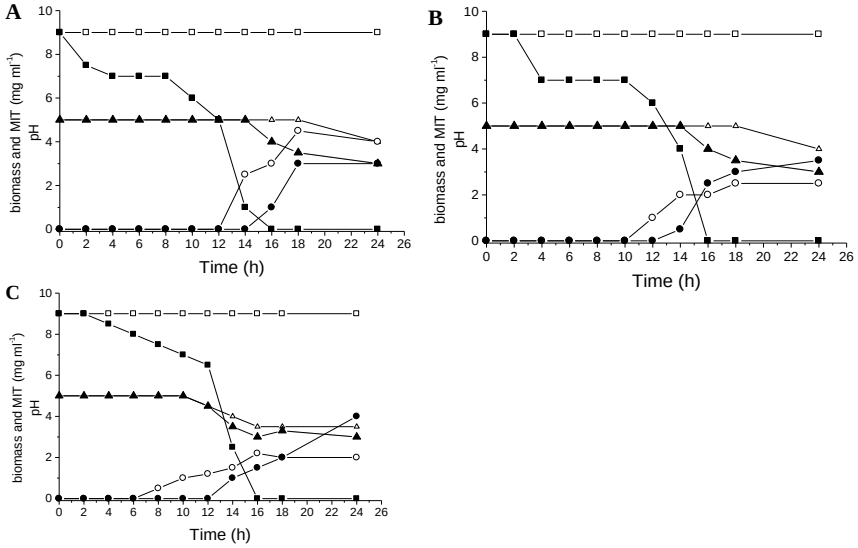

Figure 2: Cultivation of *T. longibrachiatum* FB01 (**A**), *F. solani* FB07 (**B**), *A. niger* FB014 (**C**) in the presence of 2-Methyl-1,2-thiazol-3(2H)-*one* -MIT. □= MIT in abiotic control; ■= MIT in the culture media; ∆= pH of culture media without MIT; ▲= pH of culture media with MIT; ●= fungal biomass in the culture with 10 ppm MIT; ○= fungal biomass in the culture with no MIT.

inferred in MEGA v.5.05 [22] using the neighbor-joining algorithm under the Kimura 2-parameters as the substitution model and gaps were excluded from the analysis. For branch support, 1000 bootstrap pseudo-replicates were calculated.

Tolerance of fungi to 2-Methyl-1,2-Thiazol-3(2H)-one (MIT) from the paint composition

The isolates from the paint and the biocides were first cultured in the Petri dish containing Potato Dextrose Agar (PDA) culture medium. After mycelia growth, disks of 10 mm diameter were cut and transferred to the center of a Petri dish with solid culture medium [15] containing MIT at 1.0; 3.0 and 4.0 $g.L^{-1}$. The dishes were incubated at 28 ºC, and the diameter of the colonies was measured at 12, 24, 48, 96 and 144 hours (Figure 1). The effect on the fungal growth was defined as the percentage of the growth in the presence of paint or biocides in relation of the control (100%) which consisted of fungal growth on a medium without the MIT.

Evaluation of 2-methyl-1,2-thiazol-3(2H)-one degradation

The ability of fungi to degrade the MIT was evaluated in submerged fermentation using medium composed of: ($g.L^{-1}$): 0.5 of glucose, 2.0 $(NH_4)_2SO_4$, 2.0 KH_2PO_4, 0.4 $CaCl_2$, 1.0 $MgSO_4.7H_2O$, 0.1 peptone, 3.0 $(NH_4)H_2PO_3$, 0.5 yeasts extract, 5.0 mL of micronutrients ($g.L^{-1}$): (22 $ZnSO_4.7H_2O$; 11 H_3BO_3; 5 $MnCl_2.4H_2O$, 5 $FeSO_4.7H_2O$; 1.6 $CoCl_2.5H_2O$; 1.6 $CuSO_4.5H_2O$; 1.1 $(NH_4)Mo_7O_{24}.4H_2O$; 50 EDTA) and 10 $mg.L^{-1}$ of MIT The medium was inoculated with a conidial suspension in 0.05% Tween 80 in a proportion of 107 spores mL^{-1} and incubated at 28 ºC under shaking at 120 rpm. The fermented material was filtered using a Whatman nº 1 filter paper. The biomass was dried at 105 ºC and used for fungal growth quantification and the liquid was used for quantification of the biocides and their derivatives.

A culture without the biocides was used as biotic control and the media containing the biocides but not inoculated was considered to be the abiotic control.

Analytical methods

The HPLC analyses were realized with a 1220 Infinity LC system (Agilent Technologies, Santa Clara, U.S.) equipped with a column oven, autosampler and UV-Vis diode array detector. The samples were previously filtered through a 0.22 µm polyethylene membrane. Aliquots of 30 µL were injected into the system. 0.05% trifluoroacetic acid (A) solution/methanol:acetonitrile (B, 60:40 v/v) were used as the gradient of elution. The gradient used was: 1 mL min^{-1} of 15% B to 30% B (9.5 min), 30% B to 50% B (12 min) and 50% B to 75% B (13 min). 2-Metil-3-Isotiazolinone (MIT) analyses were done using a ZORBAX Eclipse Plus C18 analytical column (25 cm × 4.6 mm i.d. × 5 µm) (Agilent Technologies) at 25 ºC and recorded at λ 275 nm.

Metabolite identification was accomplished by injection of 1 µL aliquots (splitless mode) into a Clarus 680 gas chromatography system (PerkinElmer, Waltham, U.S.) equipped with a Clarus 600T mass-

selective detector (PerkinElmer) and 70 eV was used for ionization. Separation was performed in an Elite 5-MS column (30 m × 0.25 mm i.d. × 1 μm, PerkinElmer). The oven temperature was programmed as follows: 50 °C (3.5 min); 20 °C min^{-1} to 80 °C (6 min); 15 °C min^{-1} to 250 °C (10 min). The inlet and detector interface temperatures were 250 and 280 °C, respectively. Helium was used as the carrier gas.

The GC-MS analyzes were performed on a Perkin Elmer 680 gas chromatograph equipped with a Perkin Elmer - Clarus 600T mass selective detector. The analysis were carried out using an Elite 5-MS capillary column (Perkin Elmer - 30 × 0.25 mm, 1 μm) in splitless mode using helium as carrier at a flow rate of 1.0 $mL.min^{-1}$. The injected sample volume was 1 μL. The column temperature profile was programmed as follows: hold at 40 °C for 4 min; increase of 15 °C min^{-1} to 250 °C hold for 5 min; increase of 25 °C min^{-1} to 330 °C hold for 10 min. The injector temperature was set at 270 °C. The interface temperature and ion source temperature were both set to 280 °C. Mass spectra were obtained using scan mode (mass range m/z 50 to 300) and SIR mode for monitoring the molecular ion and its fragments, with electron energy 70 eV, and using Turbo Mass software.

Results and Discussion

Fungi isolation and identification

30 filamentous fungi and two yeasts were isolated and identified as 8 genera (Table 1). Fungi of the same genera, but isolated from different locations and samples, were considered to be distinct strains.

Genera *Trichoderma*, *Aspergillus*, *Paecilomyces* and *Fusarium* were the most common isolated (28, 19, 16 and 15%, respectively). Deteriorated paint samples allowed the isolation of the biggest number of specimens (35%), followed by effluent water samples (31%). Genus *Trichoderma* was the most prevalent in the total of isolated fungi but *Aspergillus* was the most frequent in the deteriorated paint samples. *Aspergillus* and *Penicillium* were reported as involved in the water-based paint biodegradation process by Saad et al. [23], Adeleye and Adeleye [24] and Grant et al. [25]. The genera *Trichoderma*, *Aspergillus*, *Fusarium*, and *Penicillium* were also detected by Aina et al. [13] in wall paint.

The biocides used in the formulations, time of exposure to these compounds, concentrations and type of aseptic procedure used in the factory may determine the differences in the fungi species isolated. Paint contamination during the production process or in storage, have been responsible for a lot of damage in the paint factories.

Growth/inhibition evaluation of fungi by 2-methyl-1,2-thiazol-3(2H)-one

Among all the 30 fungi analyzed, 13 strains were completely inhibited by the 2-methyl-1,2-thiazol-3(*2H*)-one at concentrations of 10 g L^{-1}. The *Aspergillus* strains were the most affected although they had been the most frequently isolated strains except for *A. niger* FB14 that grew at a concentration of 40 g L^{-1} of the biocide. This fungus was isolated from the MIT used as an additive of the paint in the factory (Table 1).

The strains *Paecilomysces* sp. FB28, *P. saturatus* FB29, FB30 and FB31, *Fusarium* sp. FB06 and FB08; *F. solani* FB07, *Trichoderma longibrachiatum* FB01, FB02, FB03 and FB04, *Trichoderma* sp. FB05 and FB20 *T. harzianun* FB21all showed high growth rates at concentrations of 30 to 40 g L^{-1}. The majority of them were isolated from deteriorated paint indicating that the tolerance to the biocide allows the fungal growth in the product resulting in its deterioration.

The maximum concentration of 2-methyl-1,2-thiazol-3(*2H*)-one allowed in paints in several countries, especially Europe and Brazil is 15 g L^{-1} [26,27] and, according to the data obtained in this study, this concentration is not sufficient to inhibit fungal growth in paint. Due to the prohibition of higher concentrations of methyl isothiazolinone in paint formulations, the factory has chosen to add carbendazine, a fungicide with a broad spectrum of action, which enhances the effect of methyl isothiazolinone.

Biodegradation of 2-Methyl-1,2-thiazol-3(2H)-one (MIT) in submerged fermentation

In the MIT biodegradation assay, the strains that were best able to grow on a solid medium containing 40 g L^{-1} of this biocide, i.e. *T. longibrachiatum* FB01, *F. solani* FB07 and *A. niger* FB14, were cultivated in liquid media with an initial concentration of 10 g L^{-1} for seven days. All the fungi were able to degrade the MIT in 16 h (Figures 1A, B,C,D).

The growth of *T. longibrachiatum* FB01 (Figure 2A) was partially inhibited by the biocide. The final biomass was 25% lower than the control and the acceleration of the growth was observed after 14 h of cultivation in the presence of the biocide compared with 12 h in the control. The growth of *F. solani* FB07 (Figure 2B) and *A. niger* FB14 (Figure 2C) were increased by the biocide by 32 and 50% in relation to the control, although the acceleration of the growth for both fungi started after 12h of cultivation.

For the three fungi, the accelerated growth after 12h was coincident with a decrease in the MIT concentration in the media. An increase in biomass production could only be observed after the MIT had disappeared from the media. The use of MIT as carbon or nitrogen sources could be considered. There is no information about the route for the microbial biodegradation of MIT in the literature but, in the photolytic degradation of this compound, studied by Krzeminski et al. [28]; Fewings and Menné [29] and Park et al [30] organic acids were detected as derivatives. This oxidation process is similar to the pathway proposed for the ring cleavage of isothiazolinones in the photocatalytic degradation put forward by Kandavelu et al. [31]. They postulated that ring cleavage of isothiazolinones led to the formation of amine products, which were slowly oxidized to CO_2.

Han et al [32] (2011) proposed a pathway for electrochemical oxidation of isothiazolin-ones. The initial step would be the release of chlorine atoms from 5-chlorine-2-methyl-4-isothiazolin-3-ones with the formation of 2-methyl-4-isothiazolin-3-ones. The cleavage of the ring and loss of the sulphur element leads to the formation of N-methyl-crylic-acetone. The formation of 1-nitro-2-crylic-acetone, acetic acid and formic acid was observed in this sequential oxidation route.

Based on these data, the structure of the compounds and the acidification of the media during the degradation, one could expect the metabolites to be short chain organic acids. The organic acids were analyzed by HPLC and by GC-MS.

In the culture medium containing MIT, after cultivation of *A.*

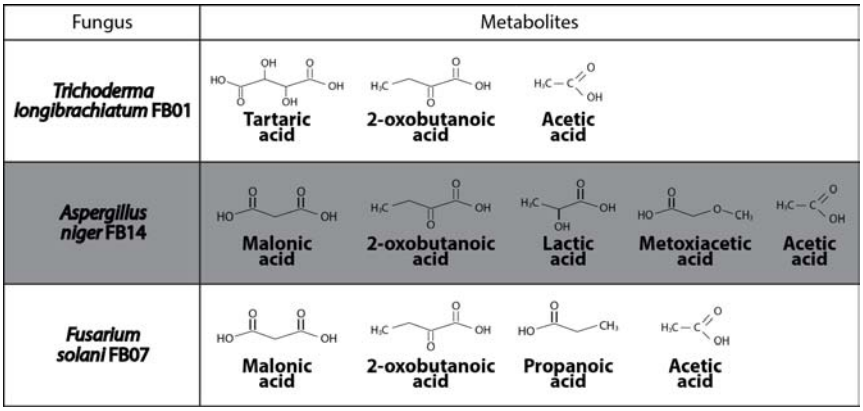

Figure 3: Organic acid produced by degradation of 2-Methyl-1,2-Thiazol-3(2H)-one (MIT) by *T. longibrachiatum* FB01, *F. solani* FB07 and *A. niger* FB014. Only acetic acid was detected in the cultivation with no MIT.

niger FB14 malonic, lactic, acetic 2-oxobutyric and metoxi-acetic acids were identified. Only acetic acid was detected in the control medium. In the culture samples of *F. solani* FB07 acetic, propionic, malonic, propanoic and 2-oxobutyric were identified. Isopropyl alcohol and acetic acid were also detected in the biotic control. The metabolites identified in the cultivation medium of *T. longibrachiatum* FB01were tartaric, acetic, 2-oxobutyric and only acetic acid was detected in the control (Figure 3).

MIT has been considered a broad-spectrum biocide that acts in prokaryotic and eukaryotic cells. It enters the microbial cell through a typical diffusion process and causes a rapid disruption of the central metabolic pathways by inhibition of specific dehydrogenase of the tricarboxylic acid cycle (α-ketoglutarate, pyruvate dehydrogenase, succinate dehydrogenase, and lactate dehydrogenase) and the concomitant generation of energy (NADH dehydrogenase) which react with isothiazolones. The ATP synthesis is rapidly inhibited in microbial aerobic and anaerobic cells. In high concentrations of the biocide, the cell death results from the progressive loss of protein thiols due free radicals produced in the cell as answer of the cell disrupted metabolism. Isothiazolones also inhibit ATP utilization, which affects the potential of the cells to maintain the energy balance for growth and cell repair [33]. Due this complex effect of MIT on microbial cells, the resistance to this compound is not considered to be common. However, the results here showed the ability of filamentous fungi both to tolerate and also to degrade it.

Although MIT retards the growth acceleration, after its degradation there seems to be an improvement in the biomass production perhaps by supplying assailable carbon sources as the organic acids detected in the culture medium.

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