Biodegradation of the Fungicide Iprodione by *Zygosaccharomyces rouxii* Strain DBVPG 6399

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Iprodione is a contact fungicide used to control several pathogens such as *Botrytis cinerea*, *Monilia*, and *Sclerotinia*. This paper reports the ability of an iprodione-resistant strain of *Zygosaccharomyces rouxii* to degrade iprodione at a concentration of 1 mg L^{-1}. The yeast *Z. rouxii* was chosen also for its ability to grow at high osmolarity. Also of note is that in bioremediation situations and in the food industry such resistance could be important. The kinetic and metabolic behaviors of the fungicide in the media are described. The results show a new transformation pathway of iprodione by the yeast leading to the formation of *N*-(3,5-dichlorophenyl)-2,4-dioxoimidazoline, 3-isopropylhydantoin, and 3,5-dichloroaniline. These compounds were identified by ^{1}H NMR, ^{13}C NMR, and GC-MS analyses. This study provides a basis to employ yeast strains in biodegradation studies in relation to their ability in the disappearance and degradation of xenobiotics into simpler molecules.

KEYWORDS: Iprodione; yeast strain; metabolites; half-life

INTRODUCTION

Iprodione [3-[(3,5-dichlorophenyl)-*N*-(1-methylethyl)-2,4-di-oxo-1-imidazolidinecarboxamide], a fungicide that inhibits spore germination and fungal mycelial growth, is widely used in fruit growing, viniculture, and horticulture as a postharvest fungicide and to treat seeds.

Iprodione is considered as being mobile to highly mobile in some soils [log K_{oc} = 2.57–3.19, log K_f = −0.5−0.8 (*1*)] and will leach to groundwater. The presence of iprodione in the wastewaters from farms producing vegetables and flowers has been reported (*2*). Frequent applications or disregard for the recommended interval between treatments can result in residues of the fungicide being found in crops, wine, and compost (*3*, *4*).

Some studies have demonstrated that iprodione and its congeners undergo enhanced degradation in the field after successive treatments with the fungicide. This phenomenon seems to be due to its microbial metabolism. It has been reported that strains of *Pseudomonas*, *Bacillus*, and *Arthrobacter* degrade iprodione, and some of its metabolites have been isolated and identified (*5*−*8*). The metabolic fate of iprodione has also been studied in plants (*4*), compost (*2*), wine (*9*), and soil (*7*). In these cases the metabolites found were different from those isolated from bacterial degradation.

Most studies on the effect of iprodione on yeast cells have used *Saccharomyces cerevisiae* and the opportunistic pathogen *Candida albicans*. The results have shown that the former is insensitive even up to doses of 30 mg L^{-1}, whereas the latter is sensitive at the same dosage (*10*, *11*). Iprodione inhibited hyphal formation of *C. albicans* and stimulated the glycerol synthesis by interfering with the histidine-kinase signal transduction pathway, whereas it had no effect in *S. cerevisiae*, which has only one histidine-kinase gene (*10*). These findings raised the question of whether there could be other resistances in different yeast species that could have environmental biotechnological applications.

We directed our attention to *Zygosaccharomyces rouxii*, an osmotolerant species that is widely distributed in environments such as soils, vegetables, and prepared foods. This yeast was investigated for its resistance to iprodione and for its ability to degrade iprodione, aiming to a possible application in environmental bioremediation and in the food industry. Moreover, the enzymes involved in these degradation pathways could be isolated and used in those situations (such as the food industry) in which the yeast cells cannot be used directly.

Despite the fact that organic fungicides do not persist for a long time in the environment, transformation products are sometimes more recalcitrant than the parent compound. It is important to study the possibility of transforming the pollutants (agrochemicals, e.g.) into simpler intermediate products that do not have environmentally and toxicologically undesiderable characteristics.

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In this work the presence of iprodione and/or its biotransformation compounds was investigated in the distinct fractions of the culture medium (CM), washing liquid (WL), and raw extract (RE), and the degradative kinetics of the fungicide caused by the yeast cells was monitored.

MATERIALS AND METHODS

Chemicals. Analytical grade iprodione (purity > 96%) and 3,5-dichloroaniline (3,5-DCA) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The analytical standard of 3-isopropylhydantoin was purchased from Acros-Organics (Geel, Belgium). Residue-analysis grade methanol, hexane, and acetone and HPLC-grade acetonitrile and water were purchased from Fluka Chemicals (Buchs, Switzerland). Chloroform-*d* for NMR spectroscopy was purchased from Sigma-Aldrich (St. Louis, MO). Orthophosphoric acid (extra pure), disodium hydrogen phosphate dihydrate, and sodium dihydrogen phosphate dihydrate were obtained from BDH (VWR International, U.K.).

Iprodione Degradation Products. An isomer of iprodione [*N*-(3,5-dichlorophenyl)-3-isopropyl-2,4-dioxo-1-imidazolidinecarboxamide] was obtained according to the procedure reported by Cooke et al. (*12*). The identity of the compound was confirmed by GC-MS and NMR analyses. ^{1}H NMR (CDCl$_3$) δ 1.4 (d, 6H, *J* = 7 Hz), 4.25 (s, 2H), 4.37 (m, 1H), 7.06 (bs, 1H, ArH$_4$), 7.42 (bs, 2H, ArH$_2$ ArH$_6$), 9.93 (s, 1H, −NH−); ^{13}C NMR (CDCl$_3$) δ 19.47 (Me), 44.1 (CH, Ipr), 47.45 (CH$_2$, C-5), 118.17 (CH, C-2, C-6), 124.43 (CH, C-4), 135.45 (C, C-3, C-5), 138.43 (C, C-1), 148.1 (C, C=O), 167.2 (C, C=O), 155.7 (C, C=O); GC-MS, [M$^+$] *m/z* 333/331/329 [Cl$_2$ pattern, (30)], 281 (3), 253 (2), 224 (2), 207 (5), 187 (100), 159 (30), 142 (15), 124 (65), 99 (22), 88 (13), 73 (20), 62 (25).

3-(Isopropylcarbamoyl)-5-(3,5-dichlorophenyl)hydantoic acid was obtained by chemical synthesis according to the method of Belafdal et al. (*13*). The identity of the compound was confirmed by NMR analysis. ^{1}H NMR (CDCl$_3$) δ 1.20 (d, 6H, *J* = 7.2 Hz), 3.25 (s, 2H), 4.07 (m, 1H), 7.16 (bs, 1H, ArH$_4$), 7.62 (bs, 2H, ArH$_2$ ArH$_6$), 8.03 (s, 2H, −NH−), 11.3 (bs, 1H, COO*H*); ^{13}C NMR (CDCl$_3$) δ 24.17 (Me), 28 (CH$_2$, C-2), 41.1 (CH, Ipr), 125.43 (CH, C-4); 128.17 (CH, C-2, C-6), 137.45 (C, C-3, C-5), 140.21 (C, C-1), 168.5 (C, C=O), 175.7 (C, C=O), 178.1 (C, C=O).

The identity of *N*-(3,5-dichlorophenyl)-2,4-dioxoimidazoline was confirmed by GC-MS and NMR analyses. ^{1}H NMR (CDCl$_3$) δ 4.25 (s, 2H), 7.06 (bs, 1H, ArH$_4$), 7.52 (bs, 2H, ArH$_2$ ArH$_6$), 10.03 (s, 1H, −NH−); ^{13}C NMR (CDCl$_3$) δ 53.1 (CH$_2$), 126.43 (CH, C-4); 127.17 (CH, C-2,C-6), 137.45 (C, C-3, C-5), 139.0 (C, C-1), 168.5 (C, C=O), 170.7 (C, C=O); GC-MS, [M$^+$] *m/z* 244/246/248 [Cl$_2$ pattern, (70)], 187 (100), 161 (38), 124 (55), 56 (15).

Strain. Twenty yeast strains (obtained from the Industrial Yeasts Collection DBVPG, http://www.agr.unipg.it/dbvba/sezf.htm) from 17 species were tested for their ability to grow on YEPD plates spiked with 1 and 10 mg L^{-1} iprodione.

Media and Growth Conditions. The yeast strain DBVPG 6399 was grown and maintained on YEPD (yeast extract 1% w/v, peptone 1% w/v, dextrose 2% w/v) solidified with 1.7% agar. Viability tests of cells growing on iprodione were carried out by methylene-blue staining followed by microscopical count. Viability was expressed as *V* = (100 × *W*)/(*B* + *W*), where *B* indicates the number of blue-stained dead cells and *W* the number of unstained live cells.

Biodegradation Studies of Iprodione. Biodegradation studies were carried out in 500 mL bottles containing 100 mL of YEPD with 1 mg L^{-1} iprodione (10% v/v acetone solution with 0.01% Tween) maintained under shaking at 150 rpm at 25 °C, in darkness. The cells for the inoculum were obtained from a 24 h YEPD preculture; cells were harvested by centrifugation (5 min at 3000*g*), washed with sterile water, counted with a Thoma-Zeiss counting chamber, and added to the YEPD−iprodione medium at a concentration of 10^7 cells mL^{-1}.

Samples (15 mL) were taken immediately (0) and then at 3, 6, and 9 days after inoculation and centrifuged (5 min at 3000*g*). The supernatant was collected for further analyses. This sample was referred to as culture medium (CM). The cells (≈2 × 10^9) were washed for 1 h with 5 mL of 0.5 M CaCl$_2$ and then centrifuged (5 min at 3000*g*); the supernatant washing liquid (WL) was collected. The pellet was subjected to cell-wall softening by a 2 h incubation at 37 °C in SPG buffer (sodium phosphate glycerol, 0.01 M NaH$_2$PO$_4$ in 50% glycerol) with 6 mg mL^{-1} lysing enzyme from *Trychoderma harzianum* (Sigma). Finally, the cells were broken open by beating the cells three times with 3 mL of glass beads (∅ 1−1.5 mm), at 1500 rpm for 2 min each time. The tubes were maintained at −20 °C for 5 min in the intervals between the beatings. The microscopic observation showed that >95% of the cells were broken open. After centrifugation (10 min at 3000*g*), the supernatant, referred to as raw extract (RE), was collected.

Iprodione Chemical Transformation Study. To monitor the chemical transformation of the fungicide, 0.1 mL of iprodione (1 mg mL^{-1}) was added to 100 mL of YEPD (pH 6.8) without cells [samples called uncultured medium (UM)], and 0.1 mL of iprodione (1 mg mL^{-1}) was added to 100 mL of aqueous buffered solution (0.01 M phosphate buffer at pH 6.8). The bottles containing the solutions were then incubated at 25 °C, in the dark. Aliquots (15 mL) of the solution were periodically sampled (0, 3, 6, and 9 days) and processed as the CM samples described above. The pH (6.8) and temperature (25 °C) were identical to those of the experiment with the yeast cells.

Isolation of Iprodione from Samples. All of the samples (CM, WL, RE, and UM) were filtered through a PTFE filter (pore size = 0.22 μm, Roth GmbH Karlsruhe, Germany) and mixed with acetonitrile, and then the two separated phases were analyzed by HPLC or by GC-MS, after evaporating the organic solvent to dryness and redissolving in hexane/acetone (1:1 v/v).

Recovery tests for YEPD CM samples were carried out for four fortification levels in the concentration range of 1−0.08 mg L^{-1} of iprodione. The mean percentage recovery for iprodione was found to be 96.8 ± 0.5% (*n* = 4).

HPLC Analysis. Analyses were performed on a Perkin-Elmer PE 200 system (autosampler, binary pump, and UV−vis detector) equipped with an Inertsil 5 ODS-3 (250 mm × 4.6 mm i.d. × 5 μm, Varian, Victoria, Australia) at a flow rate of 1 mL min^{-1}; the injection volume was 10 μL, and detection was made at 220 nm. The mobile phase of acetonitrile/water (0.05 M H$_3$PO$_4$) was previously filtered and degassed. The program was as follows: isocratic with 80% acetonitrile (18 min), linear gradient to 100% acetonitrile, 5 min isocratic with 100% acetonitrile. The compounds were identified by comparing the retention times with those of authentic reference compounds. The peaks were quantified by an external standard method, using the measurements of the peak areas and a calibration curve. Stock solutions of fungicide standards were prepared by diluting a solution (10 mg mL^{-1} in methanol) to obtain a range of concentrations from 0.01 to 1 mg mL^{-1}.

The limits of detection (LOD) were 0.05 mg L^{-1} for iprodione and 0.02 mg L^{-1} for 3,5-DCA and 3-isopropylhydantoin.

GC-MS Analysis. GC-MS analyses were performed on a Saturn II GC-MS system (Varian, Walnut Creek, CA). Saturn WS software was used to check the instrument and collect the data. The GC was equipped with a ZB-5 column (30 m × 0.25 mm i.d. × 0.25 μm film thickness, Phenomenex, Torrance, CA). The carrier gas was ultrapure helium at a flow rate of 1 mL min^{-1}.

The temperature program was from 80 °C (1 min) to 180 °C (10 min) at a rate of 25 °C min^{-1} and increased to 270 °C (1 min) at a rate of 5 °C min^{-1}. The MSD was operated in electron impact (EI) mode at 70 eV. The MSD transfer line and injector temperatures were 280 and 250 °C, respectively.

NMR Analysis. The ^{1}H and ^{13}C NMR spectra were obtained with a Bruker DRX-Advance 400 MHz spectrometer.

Statistical Analysis. Data sets were submitted to one-way analysis of variance. Standard errors of a mean were calculated and reported in graphs as a measure of data variability.

RESULTS AND DISCUSSION

Biotransformation of Iprodione in Culture Medium. Table 1 shows the results of the microbial resistance screening. All strains were resistant to the fungicide with the exception of a *Pichia kluyveri* representative, suggesting that this fungicide is more active with fungi other than yeast. Among all resistant strains, *Z. rouxii* DBVPG 6399 was chosen as representative
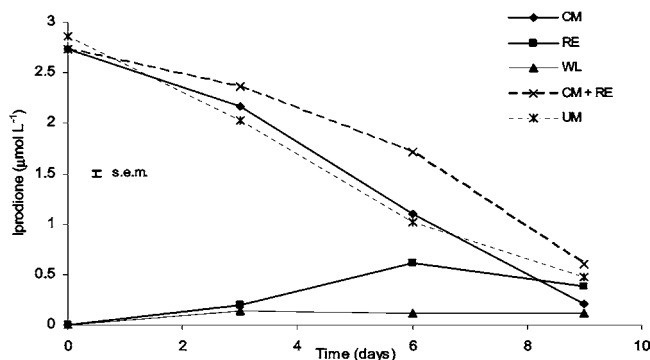
Figure 1. Concentrations of iprodione in the fractions culture medium (CM), row extract (RE), and washing liquid (WL) and kinetic behavior of iprodione in the fractions CM + RE, CM, and UM. Each point represents three replicate experiments.

of a generally regarded as safe (GRAS) yeast that could adapt to several extreme conditions in possible environmental biodegradations due to its high resistance to osmotic pressure.

During the biotic degradation cell viability ranged ≈50% without significant differences between the cells growing in the medium with and without iprodione, showing that this fungicide is indeed ineffective against this strain.

A procedure was developed to monitor the presence of iprodione in the three culture fractions (CM, WL, and RE). **Figure 1** shows that the concentration of iprodione decreased from 2.8 to 0.21 μmol L^{-1} in CM, whereas a gradual increase from 0.20 to 0.38 μmol L^{-1} was observed in RE over the time of the experiment. The quantity of iprodione detected in WL was very low and almost constant. This value can represent the quantity of iprodione potentially adsorbed onto the cell surface.

The decrease in the levels of pesticide residue in CM can be due to a breakdown of the molecule by the yeast cells. The degradation rate kinetics of iprodione by the yeast culture was investigated to determine its half-life. Iprodione disappeared at a rate similar in CM and in biotic system CM + RE, the half-lives were very similar (6 and 7 days, respectively), and up to 92% of the added fungicide was metabolized at the end of the experiment (9 days). These results suggest that the yeast cells are not only able to degrade iprodione but can also take it up from the medium. Supposing that the decrease of iprodione in CM can be due to a partial chemical transformation and to an uptake of the fungicide molecule by the *Z. rouxii* cells, which could be used by the molecule as nutrient, the presence of biotransformation products is therefore expected. The proof of microbial degradation was obtained when the presence of degradation products was ascertained. Iprodione metabolites were quantitatively and qualitatively estimated in the cell medium and were identified by using HPLC, GC-MS, and NMR techniques and by comparing the results with the authentic standards. It was found that over time, the *Z. rouxii* metabolized iprodione into different degradation products. Two metabolites appeared after 3 days of incubation. The HPLC profile of the samples gave two peaks at t_R = 4.5 and 8 min, whereas the uprodione peak was at t_R = 6 min. The compound with a t_R longer than that of iprodione was immediately identified as its structural isomer, whereas the compound eluting at t_R = 4.5 min was referred to as metabolite A. The amount of this compound increased during the experiment; it was observed that the RE had more metabolite A than the CM. Metabolite A was identified as *N*-(3,5-dichlorophenyl)-2,4-dioxoimidazoline through its fragmentation pattern, and the chemical structure was confirmed by ^{1}H NMR and ^{13}C NMR spectra. It was assumed
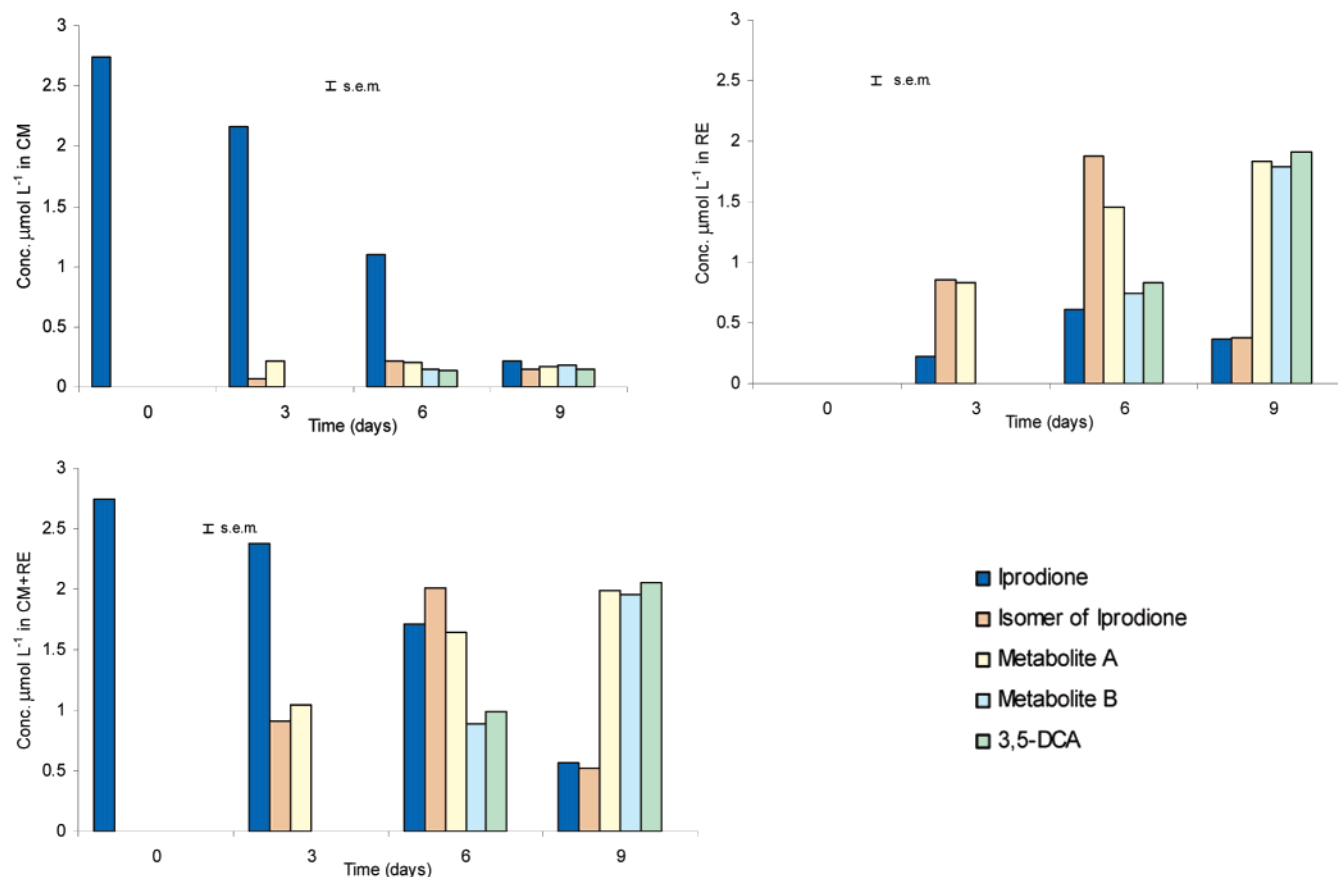

Figure 2. Concentrations of biotransformation compounds of iprodione in the CM, RE, and CM + RE fractions during the experiment.
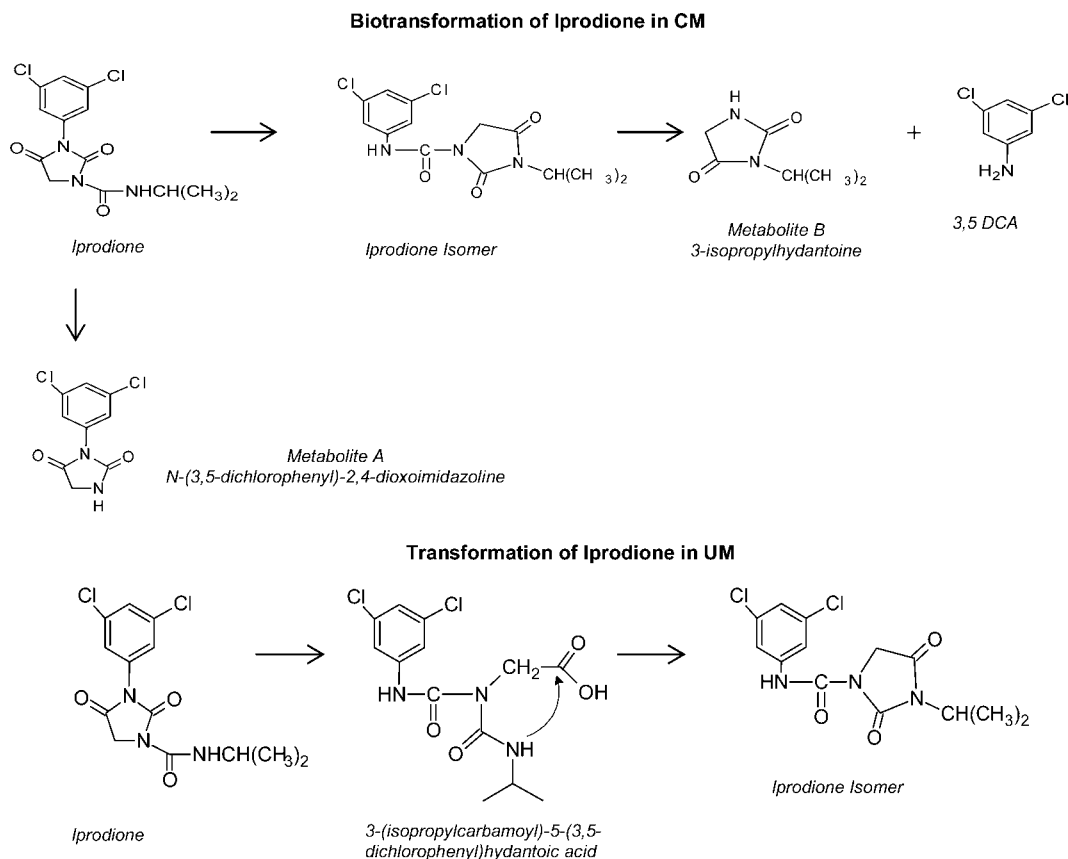
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Biotransformation of Iprodione in CM

Transformation of Iprodione in UM

Figure 3. Transformation pathway of iprodione in culture medium (CM) and in uncultured medium (UM).

Table 1. Screening on the Resistance of Different Yeast Species to Iprodione at 10 mg L^{-1}

species	DBVPG no.[a]	growth
Candida blankii	7130	1
Candida galacta	6802	1
Candida glabrata	3178	1
Candida haemulonii	6958	1
Candida humilis	7219	1
Candida magnoliae	3187	1
Candida melibiosica	6144	1
Candida membranaefaciens	3915	1
Candida parapsilosis	4330	1
Candida parapsilosis	4331	1
Candida parapsilosis	6150	1
Candida salmaticensis	6026	1
Candida terebra	6028	1
Candida tropicalis	3224	1
Candida versatilis	3731	1
Debaryomyces hansenii	6050	1
Pichia kluyveri	3634	0
Pichia membranaefaciens	3619	1
Zygosaccharomyces rouxii	6399	1
Zygosaccharomyces rouxii	6440	1

[a] Accession numbers of the strains, taken from the ollection DBVPG (see Materials and Methods).

that this metabolite was derived directly from the parent compound after the hydrolysis of the amidic side chain. *N*-(3,5-Dichlorophenyl)-2,4-dioxoimidazoline has also been found as a metabolite of iprodione in plants (*4*) and in bacterial strains isolated from soil (*6, 7*).

The concentration of the isomer increased up to 6 days (0.73 μmol L^{-1}) and then decreased (0.2 μmol L^{-1}), which indicated a further transformation into other metabolites. In fact, two compounds were detected in both the CM and RE. Evidence that the respective concentrations were similar suggested that

the compounds were derived from the iprodione isomer by cleavage of the amide bond. The compound with a $t_R = 3.3$ min was identified as 3,5-DCA, and the compound (referred to as metabolite B) was identified as 3-isopropylhydantoin by comparing it with authentic standards.

The quantities of biodegradation compounds recovered from the CM treated with 1 mg L^{-1} of iprodione for different time intervals are presented in **Figure 2**, and on the basis of these findings, a degradation pathway of iprodione by *Z. rouxii* was postulated (**Figure 3**).

Transformation of Iprodione in Uncultured Medium. To confirm the postulated degradation pathway of iprodione by *Z. rouxii*, further experiments were also carried out with yeast cells in YEPD solution without iprodione, in YEPD solution with iprodione but without yeast cells, or in a buffered solution (pH 6.8) with iprodione. The fungicide was added at the same concentration as that used in the culture medium. The kinetic behavior of the fungicide was evaluated, and the chemical transformation of iprodione could be described by the most common first-order kinetic model.

As shown in **Figure 1**, the half-life of iprodione in the UM was ≈5 days, and the concentration decreased from 0.43 to 0.05 μmol L^{-1} during the experiment. After 9 days, 83% of the added fungicide had disappeared. In fact, the concentration of iprodione decreased rapidly. A compound with a retention time (by HPLC analysis) lower than that of iprodione appeared on the third day, and its concentration increased as the iprodione concentration decreased; at the end of the experiment, its concentration was negligible. A new compound with a t_R corresponding to the iprodione isomer started to form on the sixth day. The concentration of the isomer increased steadily from 0.15 to 0.33 μmol L^{-1} at the end of the experiment (**Figure 4**); small amounts of the other compounds were also present.
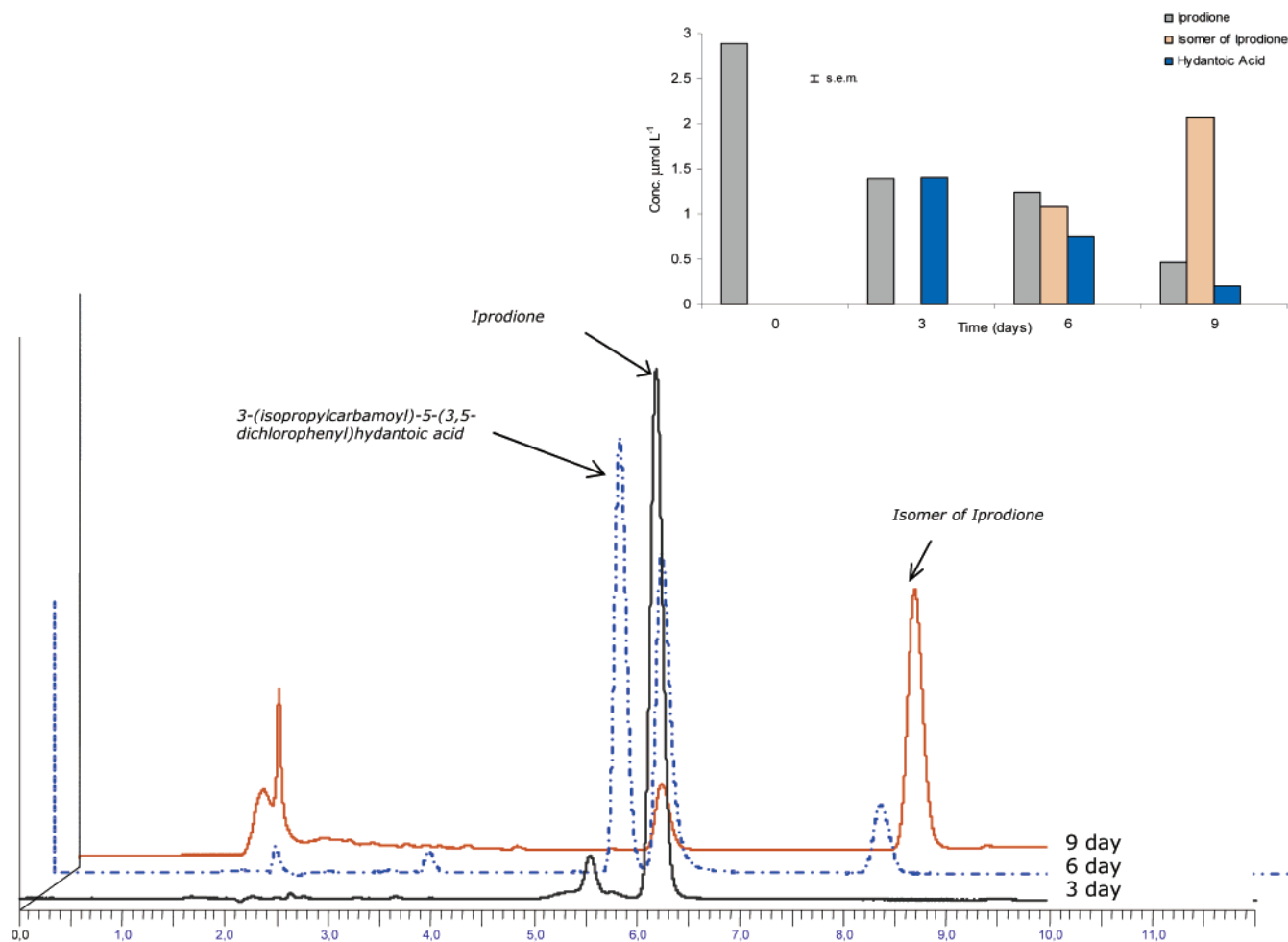
Figure 4. HPLC chromatograms overlaid to show the behavior of iprodione and concentrations of iprodione transformation compounds in uncultured medium (UM).

The compounds were identified by NMR analysis and by comparison with synthetic reference compounds. In the uncultured medium and in the buffered solution, iprodione was transformed into an acyclic intermediate compound, 3-(isopropylcarbamoyl)-5-(3,5-dichlorophenyl)hydantoic acid, that was then converted into a stable compound identified as a structural isomer of iprodione [*N*-(3,5-dichlorophenyl)-3-isopropyl-2,4-dioxo-1-imidazolidinecarboxamide].

The kinetic data for the UM are in agreement with those reported in the literature (abiotic hydrolysis: $t_{1/2} = 3$ days at pH 7), and the formation of the iprodione isomer as the final transformation product has also been found in other biotic and abiotic systems such as water and soil (*14*).

For this purpose it is important to consider that the degradation of iprodione in soil and water environments is mainly referred to as the disappearance of the chemical ($t_{1/2} = 20-160$ days). In these cases, the parent molecule does not transform into breakdown products but undergoes only a structural rearrangement.

Conclusions. In this study we have observed not only the resistance of the yeast *Z. rouxii* DBVPG 6399 to iprodione but even its ability to degrade the chemical. The question about a possible energetic catabolic cometabolism cannot be answered by our experiments and is beyond the scope of this paper. Unfortunately, the extremely low water solubility of this chemical prevents it from reaching a concentration adequate to support yeast growth as the only carbon source. Thus, no conclusion can be drawn regarding the possibility that this degradation has an energetic function.

The transformation products isolated in these experiment, *N*-(3,5-dichlorophenyl)-2,4-dioxoimidazoline, 3-isopropylhydantoin, and 3,5-dichloroaniline, are the result of an extensive degradation of the chemical by the yeast. The degradation chemicals have also been found by other authors in plants and in soil bacteria. These results shed light on the evolution and adaptive significance of these metabolisms and indicate the potential biotechnological application that yeast may have as bioremediation agents. From an environmental point of view the most complete metabolism of an organic xenobiotic is desired if one is interested in avoiding the generation of potentially hazardous pesticide intermediates.

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