PERGAMON

Chemosphere 41 (2000) 1637–1642

CHEMOSPHERE

Yeasts as a model for assessing the toxicity of the fungicides Penconazol, Cymoxanil and Dichlofluanid

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Received 8 April 1999; accepted 18 January 2000

Abstract

In the present work the sensitivity of yeast strains of *Kluyveromyces marxianus*, *Pichia anomala*, *Candida utilis*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, to the fungicides cymoxanil, penconazol, and dichlofluanid, was evaluated. Dichlofluanid induced the most negative effects, whereas penconazol in general was not very toxic. Overall, our results show that the parameters IC_{50} for specific respiration rates of *C. utilis* and *S. cerevisiae* and C_D for cell viability of *S. cerevisiae* can be applied to quantify the toxicity level of the above compounds in yeast. Hence, could be explored as an alternative or at least as a complementary test in toxicity studies and, therefore, its potential for inclusion in a tier testing toxicity test battery merits further research.

1. Introduction

The ecotoxicity of chemical compounds or natural toxins can be assessed with vertebrate bioassays, in vitro bioassays or bioassays with invertebrates, bacteria or algae. Fungicides are widely used in agriculture, being released to the environment in large amounts. Studies on the evaluation of the toxicity of these compounds still depend extensively on the utilization of animals, but in the last years several publications have dealt with the development and appropriateness of several alternative methods for assessing toxicity, that either do not depend on animal utilization or explore the use of rapid and cost-effective alternatives (Guilhermino et al., 1994, 1996, 1999; da Silva et al., 1998). Yeast, as eucharyotics, are potentially good model systems particularly for the evaluation of citotoxicity. Furthermore, they are widely spread in nature playing important roles in many ecosystems. From a practical point of view they also present advantages, since they are easy to maintain and cultivate under controlled conditions, avoiding the problems of variability found with more complex organisms (for a review see Soares and Calow, 1993). Although Koch et al. (1993) has already proposed yeast as alternative organism for testing acute toxicity of drugs and environmental chemicals, the use of yeast as alternative toxicity test organisms or as tools for a preliminary toxicity screening or for inclusion in a tier testing battery has received little attention.

In this work, strains of five different yeast species were selected for testing toxicity of the fungicides penconazol, cymoxanil and dichlofluanid, evaluating their effects on the specific growth rate, the biomass production, the specific respiration rate and the yeast viability. The results will be compared with those reported for other organisms, and evidence is presented suggesting yeast as a model for assessing the toxicity of the fungicides tested.

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0045-6535/00/$ - see front matter
PII: S0045-6535(00)00039-4

2. Materials and methods

2.1. Microorganism and growth conditions

The yeast strains used were *Kluyveromyces marxianus* ATCC 10022, *Pichia anomala* IGC 3208, *Candida utilis* IGC 3092, *Schizosaccharomyces pombe* S2, *Saccharomyces cerevisiae* IGC 4072, IGC 3507 and IGC 3507-III, a respiratory deficient mutant of the strain IGC 3507. Yeast cultures were maintained in medium containing glucose (2%, w/v), peptone (1%, w/v), yeast extract (0.5%, w/v) and agar (2%, w/v). The IGC yeast strains used in this study were obtained from the Portuguese Yeast Culture Collection (PYCC), New University of Lisbon, Portugal. *Ss. pombe* S2 was isolated at the Institut Coopératif du Vin of Montpellier. *K. marxianus* ATCC 10022 was obtained from American Type Culture Collection.

2.2. Growth assays

For estimating the effects of the fungicides in the specific growth rates (μ_g) and the biomass production (X_f) of the yeast, cells were grown in a mineral medium with vitamins (van Uden, 1967) supplemented with glucose or acetic acid as the only carbon and energy source, in the absence and presence of fungicide in the following concentration range: cymoxanil, 5–100 mg/l; penconazol, 5–100 mg/l and dichlofluanid, 0.2–6 mg/l. For these assays an inoculum was prepared by growing cells at 26°C in 500 ml shake flasks (160 rpm) containing 200 ml of culture medium with glucose 2% (w/v). At exponential growth phase, cells were harvested, centrifuged, washed twice and suspended in ice-cold deionized water to a final optical density of 0.8–1.0. In the case of glucose-grown cells, the yeast suspension (0.5 ml) was mixed in tubes with 2.5 ml of growth medium. The volume was completed to 5 ml with water and/or fungicide solution according to the desired concentration of fungicide. For the non-fermentable carbon source, the assays were performed in Erlenmeyer flasks, and the volumes of all the solutions were increased by a factor of 5. The cultures were incubated at 26°C and at 160 rpm.

2.3. Measurement of specific respiration rates

For estimating the specific respiration rates of glucose (μ_{O_2}) a Clark electrode connected to a YSI 5300 monitor and to a recorder (Kipp & Zonen), was used. The electrode was immersed in a water-jacketed chamber provided with magnetic stirring. To the chamber 4.7 ml of deionized water and 0.3 ml of yeast suspension were added, and a baseline was obtained. Subsequently 20 μl of glucose 2 M was added, and the oxygen consumption was followed in the recorder. The values of μ_{O_2} were calculated from the difference in the slopes of the trace before and after the addition of glucose. To measure the effects of fungicides, in a first set of experiments the yeast cells were grown in the absence of fungicide. At exponential growth phase, cells were harvested, centrifuged, washed twice with ice-cold deionised water and then incubated in the presence of the fungicide for different time periods, before the respiration rates were measured. In a second set of experiments, the yeast suspensions were obtained by growing cells in mineral medium with vitamins and glucose 2% (p/v), and the desired concentration of fungicide. At exponential growth phase, cells were harvested, centrifuged, washed twice with ice-cold deionized water and suspended in deionized water to a final concentration of about 40 mg dry weight ml^{-1}.

2.4. Viability experiments and calculation of parameters

Loss of viability of glucose-grown cells of *S. cerevisiae* strains induced by dichlofluanid and cymoxanil was measured by the methods described earlier for alkanol- and short-chain weak acids and ethanol induced death (Leão and van Uden, 1986; Cardoso and Leão, 1992). The specific death rates (k_d) were calculated by least-square fitting of the linear parts of the semilog survival plots according to

$$\ln N_t = \ln N_0 - k_d t,$$

where N_0 and N_t represent average colony counts on the linear parts at time zero and time t, respectively.

2.5. Calculation of IC_{50} values

The concentrations of fungicide that induced an inhibition of 50% (IC_{50} values) on μ_g, X_f or μ_{O_2} were determined from the semilog plots of the relative values of each parameter as a function of the fungicide concentrations.

2.6. Chemicals

Fungicides were obtained from Riedel-deHaën. All other chemicals were reagent grade and were obtained from commercial sources.

2.7. Reproducibility of the results

All the experiments were repeated at least three times, and the data reported are mean values.

3. Results and discussion

Effects of the fungicides cymoxanil, penconazol, and dichlofluanid on growth parameters. The effects of the

Table 1
Effects of cymoxanil (5–100 mg/l), penconazol (5–100 mg/l) and dichlofluanid (0.2–6 mg/l) on the specific growth rates (μ_g) and on the biomass production (X_f) expressed as the concentration of fungicide that induced an inhibition of 50% (IC_{50}), in glucose-grown cells, at 25°C[a]

	IC_{50} (mg/l)					
	Cymoxanil		Penconazol		Dichlofluanid	
	μ_g	X_f	μ_g	X_f	μ_g	X_f
S. pombe S2	o.r.	o.r.	50	o.r.	2.3	2.9
S. cerevisiae IGC 4072	8	50	o.r.	o.r.	5.0	6.0
S. cerevisiae IGC 3507	4	8	o.r.	o.r.	2.6	o.r.
S. cerevisiae IGC 3507-III	25	5	o.r.	5	2.7	o.r.
K. marxianus ATCC 10022	a.i.	a.i.	a.i.	o.r.	2.6	a.i.
P. anomala IGC 4380	a.i.	a.i.	o.r.	23	o.r.	a.i.
C. utilis IGC 3092	a.i.	53	18	8.9	3.2	o.r.

[a] o.r. – out of range (inhibition less than 50% for the concentrations of fungicide tested); a.i. – absence of inhibition.

fungicides on growth of the strains *K. marxianus* ATCC 10022, *P. anomala* IGC 3208, *C. utilis* IGC 3092, *Ss. pombe* S2, *S. cerevisiae* IGC 4072, IGC 3507 and IGC 3507-III, were analysed with respect to the specific growth rate (μ_g) and the final biomass concentration (X_f). In glucose-grown cells, cymoxanil induced significant inhibition only for *S. cerevisiae* (Table 1), the most severe effects being observed for the strain IGC 3507 (Fig. 1). *C. utilis* exhibited some sensitivity especially in what concerns the biomass production, while a slight inhibition by this fungicide was observed for *Ss. pombe*. In turn, growth of *K. marxianus* and *P. anomala*, were not inhibited by cymoxanil. However, the sensitivity of these yeast to the fungicide increased when they were grown in acetic acid-medium (Table 2). For the other yeast strains tested, the change from a fermentable substrate (Table 1) to a respiratory one (Table 2) did not produce a significant change on the response to the fungicide.

In glucose-grown cells, penconazol strongly decreased μ_g and X_f of *C. utilis*, and X_f of *S. cerevisiae* IGC 3507-III and of *P. anomala* (Table 1). The strains IGC 4072 and IGC 3507 of *S. cerevisiae* were not very sensitive to penconazol, the effects increasing for the strain IGC 3507 in acetic acid-grown cells (Table 2). For *K. marxianus* at the fungicide concentrations tested, an inhibition of 50% was never reached. These results are in agreement with the described activity of penconazol as an inhibitor of ergosterol biosynthesis in fungi (Köller, 1992). In fact, cells may accumulate ergosterol reserves in the membranes, and the azole-induced depletion of these reserves may take some hours (Scheven and Schwegler, 1995). This would allow growth to proceed normally for some time, after which it would arrest. To test the possible limiting role of ergosterol in cell growth, two yeast strains (*Ss. pombe* and *C. utilis*) were grown in the presence of penconazol in glucose-media supplemented with ergosterol. For *C. utilis* ergosterol could avoid completely the toxic effects induced by penconazol (results not shown). In the case of *Ss. pombe* although a lower toxicity was observed in the presence of ergosterol, some inhibition still occurred (results not shown).

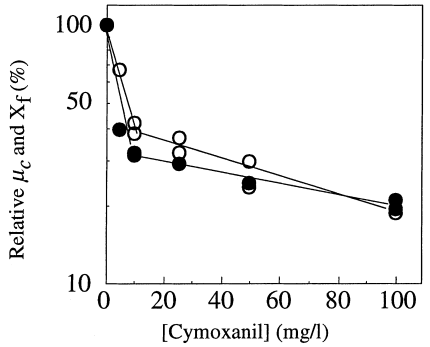

Fig. 1. Effects of cymoxanil on growth parameters of the yeast *S. cerevisiae* IGC 3507 in a medium with glucose (2%, w/v), 25°C. Relative specific growth rates (●) and biomass production (○) as a function of cymoxanil concentration.

Growth of the yeast *K. marxianus* in the presence of dichlofluanid only affected μ_g, no effects on the final biomass production being observed, over the concentration range of fungicide tested (Tables 1 and 2). In what concerns the other yeast strains, both μ_g and X_f were negatively affected by dichlofluanid, although the inhibition of X_f was lower. The only exception was observed with *P. anomala* for which the inhibition on X_f was higher in acetic acid-medium. For the yeast *C. utilis*, *S. cerevisiae* IGC 4072 and IGC 3507 the strongest effects were found in acetic acid-grown cells, the former strain being the most sensitive species.

Effects of the fungicides on the specific respiration rates. The yeast *C. utilis* and *S. cerevisiae* IGC 3507 were further tested for the fungicide effects on the specific respiration rates (μ_{O_2}) of glucose. In glucose-grown cells, none of the fungicides, when added after growth, had a significant effect on μ_{O_2}. However, when the cells were

Table 2
Effects of cymoxanil (5–100 mg/l), penconazol (5–100 mg/l) and dichlofluanid (0.2–6 mg/l) on the specific growth rates (μ_g) and on the biomass production (X_f) expressed as the concentration of fungicide that induced an inhibition of 50% (IC_{50}), in acetic acid-grown cells, at 25°C[a]

	IC_{50} (mg/l)					
	Cymoxanil		Penconazol		Dichlofluanid	
	μ_g	X_f	μ_g	X_f	μ_g	X_f
S. cerevisiae IGC 4072	10	25	o.r.	o.r.	3.2	3.4
S. cerevisiae IGC 3507	3	16	16	72	4.0	4.0
K. marxianus ATCC 10022	o.r.	a.i.	o.r.	o.r.	2.8	a.i.
P. anomala IGC 4380	o.r.	45	26	17	a.i.	3.9
C. utilis IGC 3092	o.r.	o.r.	<10	9.8	1.0	1.1

[a] o.r. – out of range (inhibition less than 50% for the concentrations of fungicide tested); a.i. – absence of inhibition.

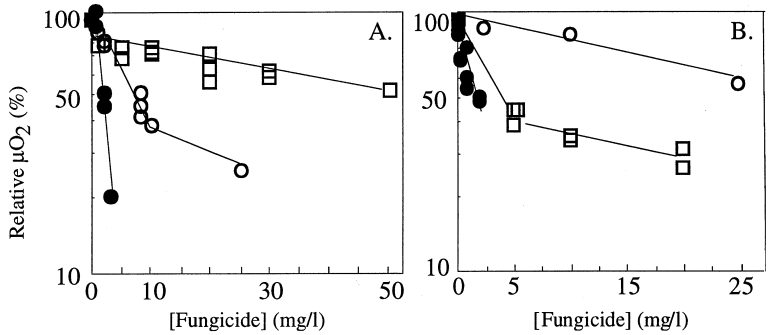

Fig. 2. Specific respiration rates of the yeasts *S. cerevisiae* IGC 3507 (A) and *C. utilis* IGC 3092 (B) grown in a medium with glucose (2%, w/v), 25°C, in the presence of cymoxanil (○), penconazol (□) and dichlofluanid (●).

Table 3
Toxicity indexes of the fungicides cymoxanil, penconazol and dichlofluanid obtained for the yeast strains tested in comparison with those published for fishes and daphnids

	Toxicity index		
	Dichlofluanid (mg/l)	Cymoxanil (mg/l)	Penconazol (mg/l)
S. cerevisiae IGC 3507	C_D – 1.0	C_D – 105	n.d.
S. cerevisiae IGC 3507 (μ_{O_2})	IC_{50} – 1.9	IC_{50} – 7.5	$IC_{50} \geqslant 50$
C. utilis IGC 3092 (μ_{O_2})	IC_{50} – 1.8	$IC_{50} \geqslant 25$	IC_{50} – 3.7
Fish[a]	LC_{50} – 0.05	LC_{50} – 13.5	LC_{50} – 2.1–2.8
	LC_{50} – 0.25	LC_{50} – 18.7	LC_{50} – 1.7–1.3
	LC_{50} – 0.3		LC_{50} – 3.8–4.6
Daphnids[a]	$LC_{50} > 1.8$	$LC_{50} > 30$	LC_{50} – 7–11

[a] From literature information of commercial fungicides.

grown in glucose and in the presence of the fungicides a significant decrease of the μ_{O_2} was observed (Fig. 2; Table 3). For both species, the effects on μ_{O_2} were stronger than those observed for μ_g and X_f, but the relative toxicity of the fungicides was maintained as follows: for *S. cerevisiae*, dichlofluanid > cymoxanil > pencanozol; for *C. utilis*, dichlofluanid > pencanozol > cymoxanil.

Effects of dichlofluanid on cell death. Death experiments in the presence of fungicide and under isothermic conditions, were performed in glucose-grown cells of the three strains IGC 4072, IGC 3507 and IGC 3507-III of *S. cerevisiae*. Semilogarithmic survival curves typically produced an initial shoulder (indicative of deathless period) followed by a straight line with negative slope that indicates an exponential death (not shown); the slope of this line corresponds to k_d, the specific death rate. Under these conditions, k_d values were exponential functions of the fungicide concentration. Fig. 3 shows the results of typical experiments obtained at 25°C. For

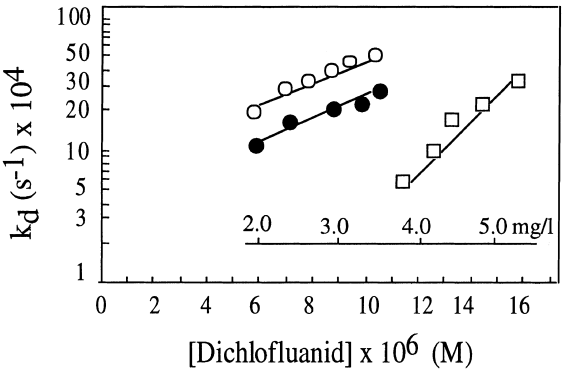

Fig. 3. Dependence on the extracellular concentration of dichlofluanid of the specific death rates (k_d) of three strains of *S. cerevisiae* at constant temperature, 25°C. Symbols: ○ – IGC 3507; ● – IGC 4072; □ – IGC 3507-III.

each yeast strain tested an estimate of the exponential enhancement constant was calculated from the slope of the respective linear isothermic death plot according to

$$\ln k_d^x = \ln k_d^{x_m} + k_1(x - x_m), \quad (1)$$

where $k_d^{x_m}$ and k_d^x are the specific death rates in the presence of x_m and x fungicide concentration, being x_m the minimum concentration above which the enhancement of death was measurable and k_1 is the exponential enhancement constant of cellular death induced by the fungicide.

For the three strains of *S. cerevisiae* tested, the values of k_1 (l mol^{-1}) for dichlofluanid calculated from Eq. (1) were: IGC 4072, 197×10^3, IGC 3507, 213×10^3 and IGC 3507-III, 367×10^3. According to these results, the parameter k_1 allowed to differentiate the relative sensitivity of the different strains to the fungicide, the strain IGC 3507-III being the less resistant. Besides this parameter, k_1, from the kinetics underlying the exponential cellular death stimulation by dichlofluanid, under isothermic conditions, it was possible to introduce other parameters that could be more directly correlated with the toxicity indexes (LC_{50} and IC_{50}) normally presented in the literature. One of these parameters that can be defined, is the fungicide concentration necessary to stimulate N times the value of k_d (C_N) which can be calculated according to the equation:

$$C_N = \frac{\ln N}{k_1} \quad (2)$$

and, if $N = 2$

$$C_D = \frac{\ln 2}{k_1} \quad (3)$$

where C_D is the fungicide concentration necessary to duplicate the value of k_d under well defined experimental conditions.

Table 3 shows the values obtained for C_D by applying Eq. (3) to the experimental isothermic death plots for dichlofluanid and cymoxanil in *S. cerevisiae*. At least in the case of dichlofluanid a good correlation between C_D in yeast and LC_{50} in fishes was found.

4. Concluding remarks

On the initial screening for the assessment of the sensitivity of different yeast strains to fungicides, we used cell growth and final biomass production as indicators of the overall effects of the fungicide on the metabolic activity of the cells. All the yeast tested was sensitive at least to one of the fungicides, the effects depending on the strain and culture conditions. However, the yeast strains that exhibited the highest sensitivity were *C. utilis* and *S. cerevisiae* IGC 3507. In both species, the specific respiration rates were also affected in the presence of the fungicides tested, the IC_{50} values estimated for this process being well correlated with LC_{50} values present in the literature for other organisms. Specially, in the case of *C. utilis* the IC_{50} values for μ_{O_2} were very similar to the described for LC_{50} in daphnids (Table 3). The three strains of *S. cerevisiae* were further investigated for their sensitivity to fungicides in terms of cell viability. From these experiments, it was possible to define the parameter C_D that in addition to express the relative toxicity of the fungicides for the yeast strains, allowed the comparison with the parameters IC_{50} and LC_{50}. Table 3 shows these results. The values of C_D estimated for cymoxanil and for dichlofluanid in *S. cerevisiae* are in the range of those of LC_{50} published for fishes. Overall, our results show that the parameters IC_{50} for specific respiration rates of *C. utilis* IGC 3092 and *S. cerevisiae* IGC 3507 and C_D for cell viability of *S. cerevisiae* IGC 3507, can be applied to quantify the toxicity level of the above compounds in yeast and, therefore could be explored as an alternative or at least as a complementary test in toxicity studies. The potential of these parameters for inclusion in a tier testing toxicity test battery merits further research, which is under development.

Acknowledgements

This study was supported by a Portuguese Research Grant (PAMAF contract n° 6088).

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