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Toxicities of Selected Phenols to Fermentative and Oxidative Yeasts

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Synthetic chemical contaminants, when released into the environment, affect the balance of natural biological communities (GOODMAN 1974). To reduce such environmental perturbations, numerous reports on toxicity bioassays using fish (KAISER et al. 1983), algae and protozoa (CAIRNS & LANZA 1972), mixed cultures of bacterial, mostly enteric (BROECKER & ZAHN 1976, KWASNIEWSKA et al. 1981) have been undertaken. Simplicity of the bacterial cell structure and ease to maintaining cultures makes them attractive organisms for screening chemicals for ecotoxicity.

Recently, yeasts have been used as model experimental eukaryotes to study the interaction with, and response of eukaryotic cells to toxicants (PEDZIWILK et al. 1980). They have a greater genetic complexity than bacteria and, therefore, offer certain biochemical advantages over bacteria. Furthermore, yeasts, or yeast-like fungi are ubiquitous in aquatic ecosystems and have been shown to possess a wide tolerance to various environmental conditions such as temperature, moisture, range of pH and high osmotic pressure (LODDER 1970, SPENCER et al. 1980). Their ability to attack complex nitrogen compounds, fats, polysaccharides and related substances may be important features of their role in biodegradation and detoxification processes in the natural environment. Despite their slower growth, compared to most bacteria, these and other properties, such as production of organic acids, alcohol and antibiotics, make yeasts very useful organisms in toxicity and biodegradation studies. In this report, two different groups of yeasts (fermentative and oxidative) have been used for measurement of toxicity of chlorophenols and para-substituted phenols, by the conventional turbidity test.

MATERIALS AND METHODS

Test chemicals: Ten chemicals were tested for toxicity towards yeasts: Phenol, p-chloro-phenol (MCP), 2,4-dichloro-phenol (DCP), 2,4,5-trichloro-phenol (TCP), pentachloro-phenol (PCP), p-methyl-phenol (p-cresol) (p-CH_3), p-cyano-phenol (p-CN), p-carboxy-phenol (p-COOH), p-nitro-phenol (p-NO_2) and p-amino-phenol (p-NH_2). All stock solutions, except for p-amino-phenol,

were prepared from their sodium salts. The solutions were sterilized by membrane filtration (0.45 μ) and stored at 4°C.

Microorganisms: Five strains of yeasts used in the toxicity test were selected from sewage at different stages of treatment Pichia denitrification stage (Brampton, Ontario); Torulopsis sp. recent chlorination, (Newmarket, Ontario); Rhodotorula sp. fresh activated sewage sludge of municipal sewage plant, (Burlington, Ontario). Others, Saccharomyces sp. from the top of plum jam, and Rhodotorula rubra from the shore of Lake Ontario near Grimsby (in August).

Medium: The culture medium was nutrient broth (NB) by Difco Co., fortified with 2% glucose. The medium was sterilized at 120°C for 15 minutes and the final pH of the medium was 7.0. The selective medium of Rose-Bengal with addition of 200 mg L^{-1} of chloramphenicol was used for isolating yeast strains. Purification and identification of the isolates were based on assimilation and fermentation of sugars and assimilation of nitrogen. Two of the isolates (Pichia and Saccharomyces sp.) were identified as fermentative strains and the other three (Rhodotorula sp., Rh. rubra and Torulopsis sp.) as oxidative. The strains described were inoculated and agitated in 125 mL Erlenmyer flasks containing 50 mL of NB fortified with 2% of glucose on a gyratory shaker (120 strokes min^{-1}) at 22 ± 2°C for 48 hours. To adapt the yeast strains to the medium, inoculum was transferred two to three times. The cultures from the stock were used for yeast seeding in all toxicity tests. Microscopic examinations were also made to determine if any significant change of yeast types took place during the experimental period.

Growth (turbidity) study: Since each strain has its own life cycle, it was essential to establish their growth curves prior to the toxicity tests. Based on their exponential growth between inoculation and approximately 24 hours, the time period of 10 to 12 hours was found to be most suitable with the yeasts, compared to four to five hours for bacteria (KWASNIEWSKA et al. 1981). Sequentially, the inoculum was added aseptically to each test solution in such amount as to give an initial reading of 10 Klett Summerson units (red filter). The aliquots were added aseptically to 250 mL flasks with side arms containing 20 mL of the liquid broth medium, with and without the addition of various amounts of the test substances. Ranges of the concentrations for chloro-phenols were 2 to 100 ppm and for phenol 10 to 1000 ppm. Those for para-substituted phenols (p-Cl, p-CN, p-NO_2, p-CH_3) were 5 to 150 ppm and 100 to 1000 ppm for p-COOH. The flasks were incubated on a gyratory shaker (120 strokes $min.^{-1}$) at 22 ± 2°C for 12 to 24 hours. At selected time intervals, the turbidity in each flask was measured in the side arm providing a direct measurement of the yeast growth. Despite their different physiological characters, each species was tested with the same experimental setup.

RESULTS AND DISCUSSION

The five strains of yeasts used in the toxicity test were identified as Pichia, Rhodotorula sp. and Torulopsis sp. The strains Pichia and Saccharomyces sp. were classified as spore-forming fermentative, and the red pigmented Rh. rubra, Rhodotorula sp. and Torulopsis sp. as oxidative. Higher aerobic conditions were favourable for growth in biomass to both oxidative and fermentative, whereas, alcohol production of fermentative strains was prevented by aeration at 120 strokes/min. Thus, the direct effects of selected chemicals on the growth rates of single strains of yeast are shown in Table 1. The inhibition concentrations (IC50, mg L^{-1}) were determined at a 50% reduction of growth relative to uninhibited controls at 12-hour incubation (log phase). Of the phenols studied, PCP and TCP were found to be the most toxic and p-carboxy-phenol the least toxic. The data shown in Table 1 indicate that the sensitivities of the strains to pentachloro-phenol (PCP) increase in the following sequence:

$$\text{Saccharomyces sp.} < \text{Pichia sp.} < \text{Torulopsis sp.} < \text{Rhodotorula sp.} < \text{Rhodotorula rubra}$$

It is evident that the oxidative strains such as Rhodotorula rubra, Rhodotorula sp. and Torulopsis sp. are more sensitive than the fermentative Saccaromyces sp. and Pichia sp.

TABLE 1. Concentrations of phenols producing a 50 percent growth reduction of different strains of yeast after 12 hr incubation (log phase); concentrations in $mg \cdot L^{-1}$.

Chemicals*	Pichia ferm.	Sacch.sp. ferm.	Rh.rubra oxid.	Rh.sp. oxid.	Torul.sp oxid.
Phenol	900.0	900.0	1000.0	500.0	460.0
MCP	145.0		62.5		
DCP	42.5		16.5		
TCP	4.3		2.0		
PCP	10.5	15.0	2.2	6.0	8.5
p-methyl-phenol	400.0		200.0		
p-cyano-phenol	270.0		165.0		
p-carboxy-phenol	>1000.0	>1000.0	>1000.0	>1000.0	>1000.0
p-nitro-phenol	60.0	1000.0	150.0	500.0	150.0
p-amino-phenol	ND**	ND	ND	ND	ND

* MCP: p-chloro-phenol; DCP: 2,4-dichloro-phenol; TCP: 2,4,5-trichloro-phenol; PCP: pentachloro-phenol.
** ND: not determined due to rapid oxidation.

In general, a toxicant becomes effective when it diffuses into the cell and changes a microorganism's growth, or reproduction.

The rates of diffusion greatly depend on the chemical's solubility and lipophilicity which is correlated with the degree of chlorination of the phenol molecule (LIU et al. 1982) as well as the selective permeability of the cell membranes of the tested microorganisms. It appears that the probability of permeation of toxicants into spore-forming fermentative yeasts is therefore markedly less than in oxidative yeasts because of the former having a secondary highly polymerized spores membrane. Further, fermentative yeasts have a different complex of enzymes which makes them more resistant and able to withstand much higher concentrations of toxicants. It can be seen from Table 1 that PCP and TCP are most toxic to the oxidative strain Rhodotorula rubra. The inhibition levels were determined as 2 mg/L, while the other oxidative strains required PCP levels of 8.5 and 6 mg/L to produce the same inhibition.

Different strains of yeasts such as fermentative Pichia and oxidative Rh. rubra were found to have similar relative sensitivities to a series of phenols. Table 2 illustrates the relative sensitivities of the various species to the same series of chloro-phenols. The following sequence of tolerance by these organisms was found in increasing order of toxicity:

$$\text{Phenol} < \text{4-MCP} < \text{2,4-DCP} \leq \text{PCP} < \text{2,4,5-TCP}$$

However, absolute sensitivities of these strains differ by a factor of approximately five with Rhodotorula rubra being more sensitive than Pichia. Toxic compounds with phenolic groups, such as phenol and cresol, generally can alter surface activities and therefore have a capacity to affect protein denaturation, enzyme deactivation and disruption of cell membranes (HAWKER & LINTON 1971). It may explain why they are more toxic to oxidative than spores-forming fermentative yeasts. Some oxidative yeasts, such as Rh. rubra, have carotenoid lipophilic pigments which may facilitate the diffusion of lipophilic contaminants through the cell walls. The growth inhibition of both fermentative and oxidative yeasts was also corroborated by the microscopic examination which showed morphological changes after exposure to TCP and PCP at concentrations above 5 ppm and with 20 ppm DCP. However, the degree of chlorine substitution of the phenol does not entirely go parallel to their toxicities as TCP is slightly more toxic than PCP. This is evident from the data in Table 2 where 2 ppm of TCP inhibit Pichia 28.5% and Rh. rubra 85%, while PCP was found to be not toxic to either yeast at this concentration.

Table 3 presents data on the growth inhibition of fermentative and oxidative yeasts by the para-substituted phenols. The results indicate that the toxicities increase in the following substituent order:

$$\text{COOH} < \text{H} < \text{CH}_3 < \text{CN} \leq \text{Cl} \leq \text{NO}_2$$

TABLE 2. Percent growth inhibition of two strains of yeast at different concentrations of chloro-phenols.

	Pichia, ferm.					*Rhodotorula rubra*, oxid.				
Conc. $mg \cdot L^{-1}$	Phenol %	MCP %	DCP %	TCP %	PCP %	Phenol %	MCP %	DCP %	TCP %	PCP %
2				28.5	0				85.0	0
5	0			87.0	32.5		4.0	18.5	95.0	66.5
10	0		1.5	91.0	47.5	6.0	8.0	37.0		81.0
20	0		10.0	93.0	75.0	21.0	15.5	92.5		85.5
50	-7.0	1.5	64.0			3.0	39.0			100.0
100	4.5	15.0				0	78.0			
1000	54.5	95.0				64.0				

TABLE 3. Percent growth inhibition of two strains of yeast at different concentrations of para-substituted phenols.

	Pichia, ferm.					*Rhodotorula rubra*, oxid.				
Conc. $mg \cdot L^{-1}$	p-Cl %	p-CN %	p-NO_2 %	p-CH_3 %	p-COOH %	p-Cl %	p-CN %	p-NO_2 %	p-CH_3 %	p-COOH %
5			3.0			4.0		4.0		
10			7.5			8.0		8.0		
20			13.5			15.5	-2.0	10.0		
50	1.5		25.0			39.0	36.0	12.0	7.0	0
100	15.0	11.5		14.0	-7.6	78.0	59.0	44.5	18.5	0
150									33.0	
200		31.5		26.0						
300		54.0		37.0						
1000	95.0			91.0	-7.6					0

As apparent from the data, the addition of 50 to 1000 ppm of p-carboxy-phenol to either *Pichia* or *Rh. rubra* culture did not result in any growth inhibition. In fact, a certain stimulation was observed for the fermentative yeast.

As for the chloro-phenols, the oxidative *Rh. rubra* is also more sensitive than fermentative *Pichia* to para-substituted phenols. For example, at a concentration of 100 ppm, the p-Cl, p-CN, p-NO_2, and p-CH_3 substituted phenols produced inhibitions of 19 to 70% on *Rh. rubra* and only 12 to 15% on *Pichia*.

Recently, LIU et al. (1982) gave quantitative structure-toxicity equations of chloro- and bromo-phenols to a *Bacillus* sp. from activated sludge. The octanol/water partition coefficient (P) and Hammett's constant for ortho substitution were found to be

physical parameters correlating with the observed toxicites. A similar examination can be performed on the yeast data presented here. After conversion of the IC50 data given in Table 1 ($mg \cdot L^{-1}$) to molar concentrations C ($mmole \cdot L^{-1}$), the following structure-toxicity equation was found for the effects of both para- and chloro-substituted phenols to Rhodotorula rubra:

$$\log \frac{1}{C} = -1.82 + 0.72 \log P + 1.48 F \qquad (1)$$

$$(n = 8,\ r^2 = 0.93,\ s.d. = 0.30)$$

where F is the field constant as given by HANSCH & LEO (1979). A similar equation can be calculated for the IC50 data for Pichia sp., however, the level of significance is much lower than that of equation 1.

The results of this study demonstrate that the yeasts offer a promising potential for the study of both toxicity and biodegradation rates of environmental contaminants. Furthermore, their relatively short growth cycles make yeasts useful for the study of structure-toxicity correlations. Finally, their highly adaptive O/R enzyme system fills the existing gap in the study of contaminant ecology.

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