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Studies on Mercury Resistance in Yeasts Isolated from Natural Sources

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Mercury pollution is a worldwide problem. Although the sources of mercury pollution have been restricted in different parts of the world, still several reports indicate the existence of the mercury pollution in soil environment. Some studies from China and Poland also showed the accumulation of heavy metals in edible vegetables and fruits (Liu and Qing 2002; Nabrzyski and Gajewska 1982). Due to prolonged exposure to mercury compounds, microorganisms may develop mercury resistance. Plasmid-mediated mercury and organomercurial resistance in prokaryotes is well documented (Summers and Silver 1978; Pahan et al. 1994). Mercury and organomercurial resistant bacteria possess two inducible enzymes, namely, mercuric reductase (EC 1.16.1.1) and organomercurial lyase (EC 4.99.1.2) which volatilized Hg^{2+} to Hg^{o} state (Summers and silver 1978; Pahan et al. 1994). Mercury resistance has been reported so far in a limited number of eukaryotic fungi, namely, *Botrytis cinnerea* (Pary and Wood 1958), *Penicillium notatum, Sclerotinia fructicola* and *Stemphylium sarcinaeforme* (Patridge and Rich 1962), *Pyrenophora avenae* (Greenaway 1972), *Neurospora crassa* (Landler 1971) and *Cryptococcus* (Brunker and Bott 1974). All of these mercury resistant fungal strains were obtained by growing the organism in media supplemented with increasing concentrations of mercury compounds.

$HgCl_2$ is commonly used for the preservation of seeds in India and in many developing countries. Sometimes mercury compounds are used by the farmers for better preservation of fruits after harvesting and to inhibit the growth of microorganisms. We screened different rotten fruits, e.g. apples, guava and grapes collected from local markets. Mercury resistant yeast strains were isolated from rotten guava. Interestingly these rotten guava contained significant amount of mercury. In the present paper we report the isolation and characterization of some mercury-resistant yeast strains capable of tolerating very high doses of mercury compounds. These strains belonged to two genera, namely, *Rhodotorula* and *Saccharomyces*. We also determined their mercury and organomercurial resistance spectra, growth pattern in the presence and in the absence of mercury compounds. Our studies also revealed that mercury was not volatilized by these microorganisms indicating that pattern of mercury resistance in these microorganisms is distinctly different from that present in bacteria.

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MATERIALS AND METHODS

All chemicals and reagents used in the present study were of analytical grade (E. Merck, Germany and British Drug House, UK). All mercury compounds were purchased from Sigma Chemical Co., St. Louis, Missouri, USA.

Rotten fruit samples were collected from different markets of Calcutta and Barasat, a small township near Calcutta.

10 g of crushed rotten fruits were taken in an Erlenmeyer flask and 5 mL of concentrated HNO_3 was added and the contents were cooled in ice-water. To this, 5 mL of concentrated H_2SO_4 was added and the contents were mixed by gentle swirling. The flask was then placed in a waterbath at 60-70°C for 2 h with occasional swirling. The contents were then cooled in ice-water and 20 mL deionized water was added. The flask was cooled to room temperature and 5 mL of 5% (w/v) $KMnO_4$ solution was added to the mixture. The contents were left overnight. Next day 5% (w/v) hydroxylamine sulphate was added dropwise until the all brown MnO_2 and excess permanganate were reduced. The volume of solution was made upto 50 mL with deionized water. Mercury content was then determined by cold vapor atomic absorption spectrometric technique (Hatch and Welland 1968) using Mercury Analyzer [MA 5800D manufactured by Electronic Corporation of India (ECIL), Hyderabad] that can measure 20-200 ng of mercury present in the sample.

A buffer suspension of each rotten fruit sample was prepared by shaking 5 g of crushed fruits with 200 mL of sterile 0.1 M potassium phosphate buffer (pH 7.0) containing 50 μg/mL streptomycin sulphate and 20 units/mL benzyl penicillin for 24 h. 0.2 mL of each suspension and 0.2 mL of serially diluted suspension was plated on YPD agar medium (0.3% yeast extract, 1% peptone, 1% dextrose in distilled water, pH – 7.2, 2% agar) containing 50 μg/mL streptomycin sulphate and 20 units/mL benzyl penicillin.

Serially diluted suspensions were plated similarly for determining total viable counts of yeast cells per mL of undiluted suspension. In order to grow yeast colonies agar plates containing 0.2 mL of serially diluted suspension were incubated for 24 h at 30°C. For the growth of Hg-resistant yeast colonies, YPD agar plates containing 50 μg/mL of streptomycin sulphate, 20 units/mL of benzyl penicillin, 16.8 μg/mL of $HgCl_2$ and definite volumes of serially diluted suspension were incubated for 24 h at the same temperature. The total number of yeast colonies per gram of each rotten fruit sample was determined, with or without $HgCl_2$. In order to determine the total viable counts per gram of a rotten fruit sample, an average of six counts was used.

Each of the colonies showing opaque growth on the agar plate was streaked on YPD agar plate to isolate a pure culture. This process of single colony isolation was repeated thrice to have pure cultures. Organisms from ten such colonies were

Table 1. Physiological characteristics of *Saccharomyces* sp. and *Rhodotorula rubra*

Test	*Saccharomyces* sp.	*Rhodotorula rubra*
Fermentative utilization of carbon compounds	+	-
Utilization of inositol	-	+
Utilization of nitrite	-	-
Utilization of nitrate	-	-
Acid production	-	-
Production of starch	-	-
Hydrolysis of urea	-	+
Pigment formation	-	+

Symbol : +, all strains positive; -, all strains negative.

subsequently identified following the methods described by Lodder (1970) and by Campbell (1988).

Some cells of these isolates were spheroidal. Reproduction by multilateral budding was observed. They did not produce pseudomycelium. They produced asci with one to four oval shaped ascospores. Their characteristic properties were similar to those of *Saccharomyces* sp. and are shown in Table 1.

The other isolates were ovoidal cells. Reproduction by multilateral budding was observed. Formation of pseudomycelium was not observed and they did not produce ascospores. Their characteristic properties were similar to those of *Rhodotorula rubra* and are shown in Table 1.

Minimum inhibitory concentrations (MIC) of $HgCl_2$ and organomercurials such as merbromine (MB), p-hydroxy mercuribenzoate (pHMB), fluorescein mercuric acetate (FMA), phenyl mercuric acetate (PMA) and thimerosal (Tm) were determined for all yeast strains using the agar-cup method. Each cup contained 0.05 mL of test solution. Concentrations of mercuric chloride and organomercurials except PMA were as follows – 6.25, 12.5, 25, 50, 100, 150, 200, 300, 400, 500 and 600 nmoles per cup. For PMA the concentrations were 3, 5, 10, 15 and 20 nmoles per cup. Yeast strains were considered sensitive to $HgCl_2$ if the zone of inhibition of growth by the agar-cup method appeared at 12.5 nmoles per cup and sensitive to PMA if at 5 nmoles per cup of PMA (Schottel et al. 1974) the zone of inhibition of growth by the same method was prominent.

Typical growth patterns of yeast strains GVa_5 (*Rhodotorula rubra*) and App_2 (*Rhodotorula rubra*) were determined turbidimetrically in liquid YPD medium. Each of 20 mL liquid YPD medium containing 4.2 µg/mL, 8.4 µg/ml, 16.8 µg/ml of $HgCl_2$ was incubated separately with 20 µL of overnight culture. The control flask received only 20 µL of overnight culture. The cultures were incubated at 30°C on a rotary shaker (200 rpm) and growth was followed turbidimetrically.

To determine the binding of mercury by the mercury resistant yeast, six yeast strains were used. In the control flask, 3.33 mg of $HgCl_2$ were added to 200 mL of YPD

broth. In the experimental flasks an overnight culture of yeast cells was diluted 1 : 10 with sterile YPD broth to a final volume 200 mL and 3.33 mg of $HgCl_2$ were added. The organism was allowed to grow for 24 h on a rotary shaker (200 rpm) at 30°C and the control flask was also shaken similarly. Then the cells were harvested by centrifugation at 2500 × g for 5 min at 0-4°C and washed three times with deionized water. A weighed amount of wet cells, 2 ml of the supernatant after each cell harvesting and 2 ml of the control medium containing $HgCl_2$ were placed in 100 ml volumetric flasks. The mercury content of solutions was measured by cold vapor atomic absorption spectrometric technique as mentioned in the previous experiment (Hatch and Welland 1968) using Mercury Analyzer [MA 5800D manufactured by Electronic Corporation of India (ECIL), Hyderabad].

RESULTS AND DISCUSSION

Table 2 represents the mercury content as well as total viable counts of yeast per gram of rotten fruit sample. The juice of all rotten fruit samples was acidic in nature (pH 4.0-5.0). All the rotten fruit samples contained 28 ± 1 to 1067 ± 15 ng mercury per gram of rotten fruit sample. Two samples, S_2 and S_5 were found to contain significant amount of mercury. Apples and grapes were found to contain negligible amount of mercury (data not shown). It may be mentioned that the permissible limit of mercury in food is 0.5 ppm (WHO 1976). The ratio of total number of Hg-resistant yeasts to the total number of yeasts was rather low. In all the rotten fruit samples, the total viable counts of yeast were in range of 3.5×10^6 to 2.4×10^8 whereas Hg-resistant yeast ranged between 5.45×10^2 to 3.35×10^5.

Table 2. Total viable counts of yeasts and total mercury content per gram of the rotten guava

Sample No.	pH of the rotten guava	Mercury content per gram of rotten guava† (ng/g of rotten guava samples)	Total number of yeasts per gram of rotten guava*	Total number of Hg-resistant yeasts per gram of rotten guava*	Ratio of total number of Hg-resistant yeast : Total number of yeasts
S_1	4.5	37 ± 1	8.2×10^7	4.25×10^3	$1 : 1.93 \times 10^4$
S_2	4.0	1067 ± 15	1.8×10^7	3.35×10^5	$1 : 5.37 \times 10^1$
S_3	4.5	28 ± 1	2.4×10^8	2.5×10^3	$1 : 9.6 \times 10^4$
S_4	5.0	46 ± 1	3.5×10^6	5.45×10^2	$1 : 6.4 \times 10^3$
S_5	4.5	142 ± 2	1.73×10^7	1.4×10^5	$1 : 1.23 \times 10^2$

* Average of six separate determinations is presented.

† Mean ± SD of six determinations.

Mercury and organomercurial resistance spectra of ten yeast strains are shown in Table 3. All the strains were resistant to $HgCl_2$, pHMB, merbromine and FMA. Among these five strains App_1, Ap_3 and App_2 displayed low resistance to $HgCl_2$, pHMB, merbromine and FMA. The strains Ap_3 and App_2 were found to tolerate

Table 3. Mercury resistance spectra of Hg-resistant yeasts isolated from guava.

Strain No.	Identified as	Mercury resistance (MIC)*				
		$HgCl_2$	PMA	PHMB	MB	FMA
Ap_3	*Saccharomyces* sp.	100	3	12.5	25	25
Ap_6	*Saccharomyces* sp.	300	5	100	400	100
Ap_7	*Saccharomyces* sp.	200	5	100	300	300
App_1	*Saccharomyces* sp.	50	0	25	50	50
App_2	*Rhodotorula rubra*	50	3	25	50	50
App_3	*Saccharomyces* sp.	300	3	100	200	200
App_4	*Saccharomyces* sp.	200	3	100	300	200
App_8	*Rhodotorula rubra*	300	5	150	400	300
GV_5	*Rhodotorula rubra*	300	3	100	300	200
GVa_5	*Rhodoturula rubra*	400	5	150	400	300

* in nmoles per cup containing 0.05 mL.

3 nmoles of PMA per cup containing 0.05 mL and 6.25 nmoles of thimerosal per cup containing 0.05 mL. The strains App_8 and GVa_5 which showed high resistance to $HgCl_2$, were also highly resistant to merbromine and FMA. However, they were moderately resistant to pHMB and weakly resistant to PMA. These strains were capable of tolerating 6.25 nmoles of thimerosal per cup containing 0.05 mL. Earlier

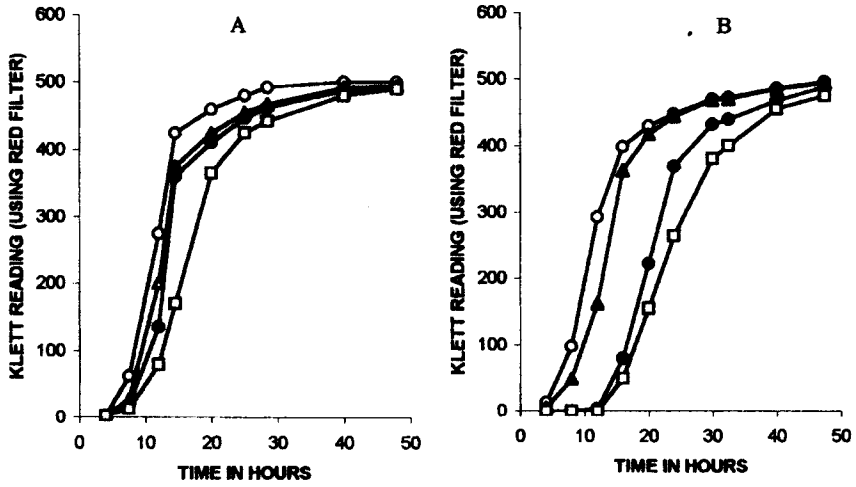

Figure. 1 Growth pattern of (A) *Rhodotorula rubra* GVa_5 and (B) *Rhodotorula rubra* App_2 in the presence of different concentrations of $HgCl_2$ at 30°C in liquid YPD medium. ○—○ Control in absence of $HgCl_2$; ▲—▲ in presence of 4.2 μg/mL $HgCl_2$; ●—● in presence of 8.4 μg/mL $HgCl_2$;□—□ in presence of 16.8 μg/mL $HgCl_2$.

studies in this laboratory showed that bacterial strains exhibiting high resistance to $HgCl_2$ were often highly resistant to thimerosal, PMA and other organomercurials

Table 4. Binding of mercury from $HgCl_2$ containing YPD broth of mercury-resistant yeast

Experimental sets	MIC (in nmole per cup containing 0.05 mL)	Wet weight of the cells (g)	Total mercury bound by cells (mg)	Total mercury bound per gram cell mass (mg)	Total mercury retained in 200 mL YPD broth after volatilization (mg)	% of mercury volatilization
Control (without organism)	-	-	-	-	2.21 ± 0.03	10.9
Saccharomyces sp. App_1	50	3.36 ± 0.05	1.04 ± 0.07	0.31 ± 0.02	1.23 ± 0.03	8.5
Rhodotorula rubra App_2	50	3.42 ± 0.07	1.01 ± 0.05	0.30 ± 0.01	1.28 ± 0.02	7.7
Saccharomyces sp. Ap_3	100	3.49 ± 0.05	0.98 ± 0.06	0.28 ± 0.01	1.32 ± 0.05	7.3
Rhodotorula rubra App_8	300	3.76 ± 0.07	0.69 ± 0.05	0.18 ± 0.01	1.50 ± 0.02	11.7
Rhodotorula rubra GV_5	300	3.67 ± 0.08	0.63 ± 0.06	0.17 ± 0.01	1.62 ± 0.03	9.3
Rhodotorula rubra GVa_5	400	3.53 ± 0.08	0.53 ± 0.04	0.15 ± 0.01	1.73 ± 0.03	8.9

In this experiment mercury-resistant yeast strains were grown for 24 hours. The amount of total mercury initially present in 200 ml YPD broth was 2.48 ± 0.02 mg.

Values represent the mean ± SD for six determinations.

(Pahan et al. 1994). Growth curves of the strain GVa_5 which was highly resistant to $HgCl_2$ and that of strain App_2 which showed mild Hg-resistance are shown in Fig. 1. Their growth patterns were similar. In the presence of 4.2 μg/mL $HgCl_2$ there was no growth inhibition observed in case of GVa_5. However at higher concentration e.g. 8.4 μg/mL and 16.8 μg/mL $HgCl_2$, their growth was affected at early log phase. But in the late log phase very little inhibition was observed as indicated by the Klett Reading. There is no report regarding the existence of any mercury volatilizing enzymes like bacteria found in yeast. So it is possible to speculate the fact that mercury tolerant elements which are synthesized during log phase of growth enable the cells to get protected against the toxic effect of mercury.

Table 4 shows the pattern of mercury binding by the whole cell in different mercury-resistant yeasts. In the control set of experiments without organisms, 10.9% of the total mercury was lost from the medium during 24 h incubation at 30°C under shaking condition. In the experimental set containing yeast cells *Saccharomyces* sp. App_1, *Rhodotorula rubra* App_2 and *Saccharmyces* sp. Ap_3, significant amount of mercury were bound to the cell mass and only 8.5%, 7.7% and 7.3% mercury were lost from the medium like the control set. Interestingly these three species were low mercury resistant organisms (MIC value 50-100 nmole/cup containing 0.05 mL). However, high mercury-resistant organism *Rhodotorula rubra* App_8, *Rhodotorula rubra* GVa_5 and *Rhodotorula rubra* GV_5 also showed similar mercury utilization profile, only 11.7%, 8.9% and 9.3% of mercury were lost from the medium under the same experimental conditions. It is presently unclear why total mercury bound per gram cell-mass was lower in case of high mercury-resistant organism than the low one.

It is further interesting to note that mercury reductase activity could not be detected in mercury induced yeast cells exhibiting high resistance to mercury compounds. So the high resistance towards mercury may be associated with the binding of Hg^{2+} by cell wall or by the cytoplasmic membrane of yeast (Brunker and Bott 1974, Murray and Kidby 1975). In yeast cells the existence of Cu^{2+} and Zn^{2+} metallothionein have been reported by several groups (Ecker et al. 1986; Winge et al. 1985). But there is no report regarding mercury binding protein or mercury metallothionein in yeast cells. In heavy metal toxicity cellular redox thiol pool plays important role in protecting the microorganism. This was also reported earlier in bacterial systems (Boyland and Chasseaud 1969; Gachhui et al. 1991). In higher eukaryotic cells also there is an important role of cellular thiol towards metal toxicity (Addya et al. 1984). Yeast cell wall may also play a significant role in protection against Hg^{2+} toxicity by acting as an efficient adsorption filter (Ono et al. 1988).

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