Reversal of pyrithiamine-induced growth inhibition of *Saccharomyces cerevisiae* by oxythiamine

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Summary. Oxythiamine reversed the growth inhibition of *Saccharomyces cerevisiae* caused by pyrithiamine, although oxythiamine alone inhibited yeast cell growth. This phenomenon was explained by thiamine production from these 2 thiamine antagonists which was demonstrated using cell suspensions and the crude extract of *S. cerevisiae*.

It has been generally accepted that microorganisms possess a specific mechanism for thiamine uptake[1-5]. In yeast, pyrithiamine, a potent antimetabolite of thiamine, was found to be taken up by the cells by a common transport system for thiamine; this uptake resulted in the inhibition of cell growth[6]. On the other hand, oxythiamine, another thiamine antagonist, has been reported to show thiamine activity in *Escherichia coli* after being converted to thiamine via thiaminosuccinic acid[7], whereas oxythiamine splits into pyrimidine and thiazole moieties in *Saccharomyces sake*[8].

In this communication we report that oxythiamine not only inhibits the growth of *Saccharomyces cerevisiae*, but also reverses pyrithiamine-induced growth inhibition of the yeast. Evidence indicating that thiamine is produced from these 2 thiamine antagonists in yeast cells is also presented.

As shown in table 1, oxythiamine in concentrations greater than 1 μM effectively inhibited the growth of *S. cerevisiae*. On the other hand, 10 μM oxythiamine reversed the growth inhibition of yeast induced by 1 μM pyrithiamine. This appeared to be paradoxical since each thiamine antagonist alone inhibits the growth of *S. cerevisiae*. However, it should be noticed that there is a different intact moiety of thiamine in each molecule; that is 2-methyl-4-amino-5-hydroxymethylpyrimidine in pyrithiamine and 4-methyl-5-hydroxyethylthiazole in oxythiamine, and the 2 moieties might be utilized to produce thiamine if they were released from the thiamine antagonists. Therefore, thiamine production from pyrithiamine and oxythiamine was investigated using yeast cell suspensions.

As shown in table 2 an appreciable amount of thiamine was produced form pyrithiamine and oxythiamine in the presence of 0.1 M glucose as an energy source by resting cells of *S. cerevisiae*. Pyrithiamine alone could not serve for thiamine production, whereas oxythiamine was found to convert into thiamine to some extent. Since thiamine production from oxythiamine and oxythiamine plus pyrithiamine was inhibited 87.9% and 87.1%, respectively, by 100 μM aminothiazole which is known to inhibit thiamine biosynthesis from its pyrimidine and thiazole moieties[10], the condensation of 2-methyl-4-amino-5-hydroxymethylpyrimidine derived from pyrithiamine and 4-methyl-5-hydroxyethylthiazole from oxythiamine was presumed to be the main pathway for thiamine production. In addition, a little oxythiamine appeared to be converted into thiamine by condensation of its thiazole moiety and 2-methyl-4-amino-5-hydroxymethylpyrimidine formed via 2-methyl-4-hydroxy-5-hydroxymethylpyrimidine derived from the antagonist.

Table 3 shows thiamine synthesis from pyrithiamine and oxythiamine in the presence of ATP and Mg^{2+} by the crude extract of *S. cerevisiae*. Enzyme activity was dependent on ATP and Mg^{2+} and it was strongly inhibited by aminothiazole, as also observed in the experiment using yeast cell suspensions. Thiamine synthesizing activity from oxythiamine alone by the yeast

Table 1. Effect of oxythiamine and pyrithiamine on the growth of *Saccharomyces cerevisiae*

Addition	Growth (optical density at 560 nm) − Pyrithiamine	+ Pyrithiamine
None	0.430	0.020
Oxythiamine (1 μM)	0.095	0.025
Oxythiamine (2 μM)	0.055	0.040
Oxythiamine (5 μM)	0.055	0.120
Oxythiamine (10 μM)	0.060	0.410

Growth studies were carried out using thiamine-omitted Wickersham's synthetic medium of the following composition: glucose, 10 g; $(NH_4)_2SO_4$, 1 g; KH_2PO_4, 875 mg; K_2HPO_4, 125 mg; $MgSO_4 \cdot 7H_2O$, 100 mg; $CaCl_2 \cdot 2H_2O$, 100 mg; NaCl, 100 mg; KI, 0.1 mg; $ZnSO_4 \cdot 7H_2O$, 0.07 mg; $FeCl_3 \cdot 6H_2O$, 0.05 mg; H_3BO_3, 0.01 mg; $CuSO_4 \cdot 5H_2O$, 0.01 mg; inositol, 2 mg; pyridoxine hydrochloride, 0.4 mg; D-pantothenic acid calcium salt, 0.4 mg; nicotinic acid, 0.4 mg; p-aminobenzoic acid, 0.2 mg; riboflavin, 0.2 mg; biotin, 0.002 mg; and distilled water to 1 l[6]. 5 ml of the medium with or without thiamine antagonist indicated was inoculated with a washed cell suspension of *S. cerevisiae* equivalent to 0.016 mg (dry wt), incubated at 30 °C without shaking. Growth after 18 h was measured turbidimetrically at 560 nm. Values are means from 2 experiments. Each experiment represents duplicate determinations.

Table 2. Thiamine production from pyrithiamine and oxythiamine by resting cells of *Saccharomyces cerevisiae*

Addition	Thiamine produced (nmole/mg dry wt/30 min)
None	0.10
Pyrithiamine (1 μM)	0.11
Oxythiamine (10 μM)	0.28
Pyrithiamine (1 μM) plus oxythiamine (10 μM)	0.56

Saccharomyces cerevisiae was grown in 100 ml of thiamine-omitted Wickerham's synthetic medium for 18 h at 30 °C with shaking, harvested and washed once in water. 5 ml of yeast cell suspension (3.5 mg dry weight) in 50 mM potassium phosphate buffer (pH 5.0) containing 0.1 M glucose and thiamine antagonist of the concentration indicated was incubated for 30 min at 37 °C with constant shaking. After the incubation thiamine in the cells was extracted by heating at 85 °C for 15 min and the amount of total thiamine was determined fluorometrically by the thiochrome method using cyanogen bromide after takadiastase hydrolysis[9]. Values are means from 2 experiments. Each experiment represents duplicate determinations.

Table 3. Thiamine biosynthesis from pyrithiamine and oxythiamine by the crude extract of *Saccharomyces cerevisiae*

Addition	Thiamine produced (nmole/mg protein/h)
Complete	0.30
minus oxythiamine	0
minus pyrithiamine	0.02
minus ATP	0.01
minus Mg^{2+}	0.04
Complete plus aminothiazole (100 μM)	0.02

The washed yeast cells grown in 500 ml culture as described in table 2 were suspended in 5 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM 2-mercaptoethanol and 1 mM EDTA, sonicated for 30 min at 10 kc, and then centrifuged at 15,000 × g for 20 min. The supernatant was used as the crude extract. The reaction mixture contained: 20 mM potassium phosphate buffer (pH 7.0), 10 μM oxythiamine, 10 μM pyrithiamine, 4 mM ATP, 4 mM $MgCl_2$ and the enzyme preparation (5.0 mg protein) in a total volume of 5 ml. After incubation for 1 h at 37 °C, the reaction was stopped by heating at 85 °C for 15 min after adjusting the pH of the mixture to 4.5. Thiamine in the reaction mixture was determined as described in table 2. The enzyme boiled for 2 min was used in blank experiment. Values are means from 2 experiments. Each experiment represents duplicate determinations.

extract was very much lower than that from oxythiamine and pyrithiamine, suggesting that the enzyme activity required for amination of 2-methyl-4-hydroxy-5-hydroxymethylpyrimidine to 2-methyl-4-amino-5-hydroxymethylpyrimidine is extremely weak in the cell-free extract of *S. cerevisiae*. From these results it was concluded that *S. cerevisiae* contains thiaminase II in addition to a thiamine synthesizing enzyme system to produce thiamine from pyrimidine and thiazole moieties of thiamine. Ozawa et al.[11] previously reported the presence of thiaminase activity in some yeast-like fungi. The precise enzymatic mechanism of thiamine synthesis from pyrithiamine and oxythiamine in *S. cerevisiae* remains to be established.

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Volatile metabolites of aspergilli in relation to spore germination of some keratinophilic fungi[1]

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Summary. The effect of volatile metabolites produced by 8 Aspergilli (i.e., *Aspergillus candidus, A. chevalieri, A. flavus, A. fumigatus, A. nidulans, A. niger, A. ochraceous* and *A. tamarii*) on spore germination were tested against *Auxarthron conjugatum, Chrysosporium pannicola, Keratinomyces ajelloi* and *Microsporum gypseum*. The volatile metabolites inhibited the spore germination of all the test fungi.

During the microbial degradation of organic waste a number of volatile compounds are produced. These volatiles are either produced by microorganisms or by decaying organic matter. Soil-inhabiting keratinophilic fungi in all habitats live in a atmosphere of volatile organic compounds, which are known to play an important role in soil fungistasis[2–6]. To add to the knowledge available on this topic, the production of volatiles by Aspergilli was investigated and their inhibitory/stimulatory effect against keratinophilic fungi was evaluated.

The volatile metabolites produced by 8 Aspergilli (table) were tested against *Auxarthron conjugatum, Chrysosporium pannicola, Keratinomyces ajelloi* and *Microsporum gypseum* isolated during a survey of soil-inhabiting keratinophilic fungi. The effect of volatiles on spore germination was evaluated by 2 assay techniques. *Sterile cellophane agar diffusion (SCAD) method*[4]. In this method agar discs (6 mm diameter and 2–3 mm thick) made from 2% Difco purified agar were placed on sterile cellophane (2 × 2 inches) in contact with 35 g of sterilized soil in the petri dishes. The petri dishes were inoculated with individual *Aspergillus* species. Inoculations were made 10 days before adding the cellophane paper and discs. The agar discs were preactivated in this manner for 24 h at 28 °C. They were transferred to a sterile glass slide in a glass tube kept in a moistened petri dish and then inoculated with spore suspension of a 10-day-old test organism grown on Sabouraud's, dextrose agar medium. After 24 h incubation at 28 °C, the agar discs were examined for spore germination of the test fungi. Approximately 200 conidia were observed in each count.

Soil emanation agar (SEA) method[4]. In this method a sterile glass slide was attached in the center of the inside of a petri dish cover with masking tape, and the agar discs were placed on its free surface. The cover of the petri dish with the slide and agar discs was replaced on the bottom plate containing a pure culture of 10-day-old Aspergilli. The agar discs were thus exposed to the volatile substances emanating from the culture. Preactivation and the remaining procedures were the same as described in the previous method.

The result presented in the table showed that the volatile metabolites inhibit the spore germination of all the test fungi. The volatiles produced by *A. chevalieri* and *A. ochraceous* could cause 100% inhibition in spore germination of *K. ajelloi*, while in the case of *M. gypseum* the volatiles produced by *A. cheva-*

Sporostatic effect of volatiles produced by different species of *Aspergillus*

S. No.	*Aspergillus* spp.	% inhibition in spore germination of keratinophilic fungi							
		A. conjugatum		*C. pannicola*		*K. ajelloi*		*M. gypseum*	
		A	B	A	B	A	B	A	B
1	*Aspergillus candidus*	44	53	52	59	68	73	48	40
2	*A. chevalieri*	59	41	64	61	100	100	100	100
3	*A. flavus*	52	49	63	57	49	53	74	83
4	*A. fumigatus*	33	19	76	71	25	07	70	68
5	*A. nidulans*	70	67	90	93	78	75	54	58
6	*A. niger*	70	66	72	76	59	63	77	72
7	*A. ochraceous*	74	88	62	55	100	100	95	93
8	*A. tamarii*	58	54	45	43	68	64	60	56

A, Sterile cellophane agar diffusion method; B, soil emanation agar method. Each figure is an average of 3 determinations.