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Effect of Flutriafol on *Saccharomyces cerevisiae* During Continuous Cultivation at Different Dilution Rate

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Abstract

An increase in the concentration of Flutriafol in nutrient media decreased the content of protein, phosphorus, total ribonucleic acid, activity of pyruvate carboxylase and isocitrate lyase in cells of Saccharomyces cerevisiae parental strain and respiratory deficient (RD) mutant while the trehalose content increased at all investigated dilution rates. The respiration quotient value for the RD mutant was higher than for the parent strain. The RD mutant lacked cytochrome aa_3, while the contents of cytochromes c and b were lower than those of the parent strain at all investigated dilution rates. The values for specific oxygen uptake rate (Q_{O2}) and specific carbon dioxide production (Q_{CO2}) are dependent on the dilution rate.

Yeast, as eucharyotics, is a potentially good model system 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 [1]. Although Koch et al. [2] 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[3]).

Keywords: Saccharomyces cerevisiae, Flutriafol, Continuous Cultivation

Introduction

A large number of chemical compounds may be genotoxical to *Saccharomyces cerevisiae* [4,5]. 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. Fungicides have deserved a special interest in this context [6,7]. The degradation products of pesticides used for sugar-beet protection appear in molasses and may inhibit yeast growth. Zauner *et al.* (1979) investigated how the respiration intensity of *S. cerevisiae* cells depended on the concentration of different pesticides in molasses [8]. Fatichenti *et al.* (1983) investigated if the degradation of residual pesticides in *S.cerevisiae* fermentations was

influenced by the metabolism of yeast [9]. For instance, higher Benomyl concentration in nutrient media during continuous cultivation of *S. cerevisiae* could decrease the content of nucleic acid, protein and phosphorous in yeast cells [10]. Also, higher Flutriafol concentration in nutrient media during continuous cultivation of *S. cerevisiae* decreased the activity of pyruvate carboxylase and isocitrate lyase on cells of *S. cerevisiae* while the trehalose content increased [11].

Flutriafol is fungicide that inhibits ergosterol biosynthesis (steroid demethylation inhibitor), causing fungal cell wall collapse and inhibition of hyphal growth [12]. In the course of continuous cultivation of microorganisms, dilution rates represent an extremely important variable [13].

The aim of this investigation was to study the effect of Flutriafol on composition of the cells, respiration intensity, cytochromes content and the activities of metabolic enzymes in a parent and petit mutant strain of *S. cerevisiae* during continuous cultivation at different dilution rates.

Materials and Methods

Microorganism: Pure cultures of *S. cerevisiae* strain 163 (parent strain) and *S. cerevisiae* 14/I petit respiratory deficient (RD) mutant, a descendant of strain 163, were used [14]. Both microorganisms were from the collection of the *Faculty of Technology* in Novi Sad (Serbia). The pure cultures were maintained on a slants of van der Walt medium (1970) and, after incubation (30^{o}C, 2 d) stored at 4^{o}C. For inoculation of the fermenter one loopful of stock culture was tranferred to 50 mL of van der Walt medium in a 250-mL Erlenmeyer flask. The cultures were incubated with orbital shaking (3.3 Hz, 30^{o}C) for 1 d before inoculating of the fermenter.

Substrate preparation Flutriafol ((RS)-2.4'-difluoro-α-(1H-1,2,4-triazol-1-ylmethyl) benzhydryl alcohol; tradename Impact 25 SC; *Zeneca*) was added under sterile conditions.

Equipment and fermentation. Fermentation was carried out in a 10 L fermenter (*Chemap*, Switzerland), consisting of a top-driver stirrer, a water-cooled condenser on the air outlet, and antifoaming, temperature, pH and dissolved oxygen (DO) control systems. DO and pH were measured with a model 900 DO probe (*New Brunswick*, USA) and pH electrode (*Ingold*, UK), respectively. The working volume for all experiments was 5,0 L. Good mixing was assured by the rotation of two flat-blade agitators set at 10 Hz; the air flow rate being 240 L L^{-1} h^{-1}. Temperature was maintained at 30^{o}C. The fermenter was equipped with an additional unit that measured the mass (liquid) in the system, whereas the substrate mass in the fermenter was kept constant (chemostat). The in-and out-flowing air-streams were continuously monitored for oxygen and carbon dioxide concentrations. Gas analyzer *Hartmann and Braun* (Germany) was used as measuring apparatus. During the cultivation the flow was regulated to correspond to the selected dilution rate (D), *i.e.* the period spent in the fermenter unit. All liquids were pumped by means of peristaltic pumps.

Analytical methods. During continuous cultivation, samples were taken for the determination of dry mass [15] of trehalose [16], nitrogen and phosphorus [17], cytochromes [18], and content of total ribonucleic acids [19]. The activity of pyruvate carboxylase (EC 6.4.1.1) and isocitrate lyase (EC 4.1.3.1) were determined according to [20]. All chemicals were of analytical grade if possible, otherwise of the highest purity available. Carbon dioxide production and oxygen uptake rate were measured in Warburg respirometer [21].

Results and Discussion

Increasing Flutriafol concentrations in the nutrient medium resulted in decreasing protein, phosphorus and ribonucleic acids contents in the cells of both the parent and the RD mutant strain at all investigated dilution rates (Table I). A possible explanation is that Flutriafol is an inhibitor biosynthesis of essential amino acids (*e.g.* isoleucine and methionine) and of the biosynthesis of the RNA purine. In fact, cells may accumulate ergosterol reserves in the membranes, and the azole-induced depletion of these reserves may take some hours [22]. The trehalose content increased with increasing of the Flutriafol concentrations at all investigated dilution rate.

Table I Effect of Flutriafol concentration (mg/L) on the cell composition of parental strain *S. cerevisiae* 163 and an RD mutant 14/1 at different dilution rate[a].

Flutriafol	Dry mass, %		Proteins[b]		Phosphorus[b]		Trehalose[b]		Total ribonucleic acids[b]	
	163	RD	163	RD	163	RD	163	RD	163	RD
Dilution rate D = 0.1 /h										
0	27.0	26.5	53.6	44.8	2.9	2.5	0.6	1.5	7.4	5.1
3	26.5	26.1	51.8	43.7	2.6	2.1	0.9	1.8	6.1	4.5
6	26.0	25.4	50.4	42.2	2.4	2.0	1.5	2.3	5.0	3.4
9	25.7	24.2	49.5	40.6	2.2	1.8	1.7	2.5	4.2	2.9
Dilution rate D = 0.2 /h										
0	26.5	26.1	52.4	43.8	2.8	2.3	0.5	1.2	7.0	4.9
3	26.2	25.7	50.2	42.2	2.4	1.9	0.6	1.6	5.2	3.8
6	25.7	25.0	49.1	40.5	2.1	1.7	1.2	2.1	4.8	2.7
9	25.1	24.6	48.8	39.0	1.9	1.6	1.5	2.3	3.7	2.4
Dilution rate D = 0.3 /h										
0	25.2	24.9	50.6	41.5	2.5	2.2	0.2	0.9	5.9	4.2
3	24.9	24.2	48.2	40.1	2.0	1.8	0.4	1.2	4.8	3.0
6	24.5	23.8	46.6	38.2	1.7	1.6	0.6	1.5	4.4	2.5
9	24.2	23.5	45.1	37.7	1.6	1.5	1.1	1.8	3.2	2.1

[a] At the new steady-state in fermenter; mean values of five exchange of fermenter content with the same Flutriafol concentration
[b] % in dry mass

Trehalose is a storage disaccharide, which is potential factor in stress tolerance of yeast. Previous studies demonstrated that this sugar accumulated in *S. cerevisiae* [23] and S*chizosaccharomyces pombe* [24] during exposure to noxious agents, indicating that it might play a role in the inducible stress response of yeasts. Stress tolerance of *S. cerevisiae* varies

considerably with the physiological state and the cultural conditions [25]. For example, in the cells with exponential growth phase are more sensitive to stress caused by physical and chemical mutagens [26] than cells that are growing more slowly or are resting.

The specific oxygen uptake and specific carbon dioxide release were adversely related to the Flutriafol concentrations in the parent strain (Fig 1. *A*). The RQ values <1 t all investigated Flutriafol concentrations and dilution rates, indicated that oxidative glucose degradation was intensified. This quotient appears to be a function of the citrate cycle and respiratory chain activity [27]. The Q_{O2} of the RD mutant cells was approximately six times lower compared to the parent strain. This could be attributed to the loss or severe damage of the respiratory chain in the RD mutant. Increasing Flutriafol concentration increased both the Q_{CO2} and RQ values (RQ>1) while the Q_{O2} further decrease with increasing dilution rate (Fig. 1. *B*). This means that glucose degradation developed further towards anaerobic metabolism.

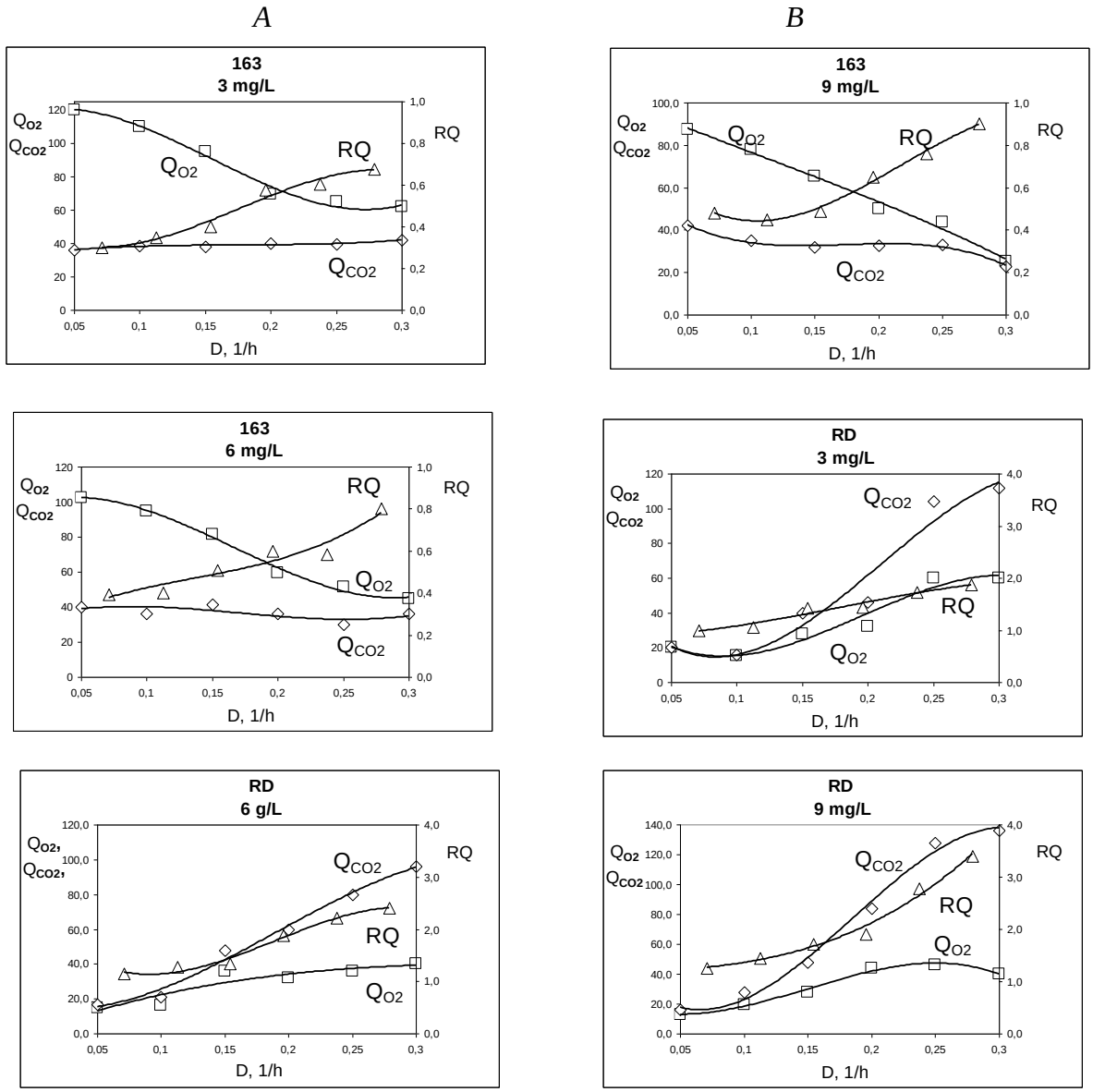

Fig. 1. Specific oxygen uptake rate (Q_{O2}) specific CO_2 production rate (Q_{CO2}, both mg/g per h) and respiratory quotient (RQ) at different dilution rates (D) in Flutriafol (mg/L) supplemented continuous cultivations of strains *S. cerevisiae; A*: parent strain 163, *B*: respiratory deficient mutant 14/1

Cytochromes aa_3, *b* and *c* were found in *S. cerevisiae* parent strain (Table II). The RD mutant lacked cytochrome aa_3, and the content cytochromes *b* and *c* were lower than those of the parent strain at all investigation dilution rates. Increasing Flutrifol concentrations resulted in decreasing cytochrome contents during continuous cultivation of both strains at all investigated dilution rates while the cytochrome contents decreased at higher dilution rates. These results are in accordance with the data of Oura (1974) who suggested that cytochrome content depends on cultivation conditions [28]. The presence or absence of cytochromes may also be used for the estimation of the possible genotoxic effect of different chemicals.

Table II. Effect of Flutriafol concentration (mg/L) on the cytochrome (cyt aa_3, *b* and *c*) content of *Saccharomyces cerevisiae* 163 (parent) and RD mutant 14/I at different dilution rate[a]

Flutriafol	cyt aa_3				cyt *b*				cyt *c*				Total	
	605[b]		444[b]		550[b]		520[b]		560[b]		532[b]			
	163	RD	163	RD	163	RD	163	RD	163	RD	163	RD	163	RD
Dilution rate D=0.1 /h														
0	0.5	0	1.7	0	3.1	2.6	4.6	3.5	2.8	2.3	6.1	4.9	18.8	13.3
3	0	0	1.0	0	1.6	1.1	3.4	1.8	2.2	1.2	4.5	2.4	12.7	6.5
6	0	0	0.5	0	0.9	0.5	1.6	1.2	1.1	0.7	2.2	1.3	6.3	3.7
9	0	0	0.2	0	0.3	0	1.1	0.2	0.4	0.2	1.1	0.6	3.1	1.0
Dilution rate D=0.2 /h														
0	0.4	0	1.4	0	2.6	2.2	4.0	2.7	2.4	1.6	5.6	4.0	16.0	10.5
3	0	0	0.7	0	1.4	0.9	2.1	1.5	1.6	1.2	3.4	2.0	9.2	4.6
6	0	0	0.3	0	0.7	0.4	1.3	0.9	0.9	0.5	2.1	1.1	5.4	2.9
9	0	0	0.1	0	0.3	0	0.8	0.4	0.2	0.1	0.9	0.3	2.3	0.8
Dilution rate D=0.3 /h														
0	0.3	0	0.9	0	2.0	1.7	3.6	2.0	2.2	1.4	5.4	3.4	14.4	8.5
3	0	0	0.7	0	1.0	0.7	1.5	1.1	1.3	0.8	2.8	1.8	7.3	4.4
6	0	0	0.2	0	0.5	0.2	0.9	0.6	0.8	0.4	1.5	0.7	3.9	1.9
9	0	0	0	0	0.1	0	0.2	0.1	0.1	0	0.5	0.3	0.9	0.4

[a] See footnote a to Table I [b] Wavelength (nm)

The amount of produced ethanol increased with increasing Flutriafol concentration for RD mutant cells at all investigated dilution rates (Table III); this could be a consequence of a higher intensity of anaerobic metabolism (*see* Fig. 1 *B*). The activity of pyruvate carboxylase and isocitrate lyase is dependent on Flutriafol concentration in nutrient media in both strains at all investigated dilution rates. With increasing Flutriafol concentration and dilution rate, the activities of these enzymes decrease, especially in RD mutant cells. Flutriafol probably influences the activity of pyruvate carboxylase in continuous cultivation conditions. A decrease of the activity of isocitrate lyase point to the influence of Flutriafol on the enzymes of glyoxylate cycles in continuous cultivation. These results are in agreement with data given [29].

Table III. Effect of Flutriafol concentration (mg/L) on the ethanol production (g per L) and pyruvate carboxylase (μkat/g protein) activity and isocitrate lyase (nkat/g protein) activity in *S. cerevisiae* strain 163 (parent) and RD mutant 14/1 at different dilution rate[a]

Flutriafol	Ethanol		Pyruvate carboxylase		Isocitrate lyase	
	163	RD	163	RD	163	RD
Dilution rate D=0.1 /h						
0	0.1	1.9	267	200	150	83
3	0.3	2.1	243	146	138	48
6	0.5	2.6	205	121	110	29
9	0.7	2.9	192	90	68	17
Dilution rate D=0.2 /h						
0	0.5	2.6	258	189	142	75
3	0.8	3.2	237	139	128	36
6	1.1	3.8	198	110	99	18
9	1.3	4.3	177	82	59	11
Dilution rate D=0.3 /h						
0	1.0	3.2	225	129	130	69
3	1.2	4.3	186	115	118	27
6	1.6	4.8	175	99	89	15
9	1.9	5.3	162	67	48	8

[a] See footnote a to Table I

Conclusions

Increasing Flutriafol concentrations in the nutrient medium resulted in decreasing protein, phosphorus and ribonucleic acids contents in the cells of both the parent and the RD mutant strain at all investigated dilution rates.

The trehalose content increased with increasing of the Flutriafol concentrations at all investigated dilution rate.

The values for specific oxygen uptake rate (Q_{O2}) and specific carbon dioxide production (Q_{CO2}) are dependent on the dilution rate.

Increasing Flutrifol concentrations resulted in decreasing cytochrome contents during continuous cultivation of both strains at all investigated dilution rates while the cytochrome contents decreased at higher dilution rates.

The activity of pyruvate carboxylase and isocitrate lyase is dependent on Flutriafol concentration in nutrient media in both strains at all investigated dilution rates. With increasing Flutriafol concentration and dilution rate, the activities of pyruvate carboxylase and isocitrate lyase decrease, especially in RD mutant cells.

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