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Effect of Cadmium and Copper on the Production of Citric Acid by *Aspergillus niger*

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ABSTRACT. Copper and cadmium inhibited the growth as well as citric acid production (depending on the heavy metal concentrations) by citric-acid-producing *Aspergillus niger*. Activity of citrate synthase was connected with citrate synthesis in the absence as well as in the presence of heavy metals. The activity of aconitase, and both NAD- and NADP-isocitrate dehydrogenases was strongly inhibited by copper. The contents of DNA and proteins in the cells decreased but the contents of lipids and polysaccharides increased considerably in the presence of both heavy metals.

Aspergillus niger has long been known for its ability to produce citric acid and the mechanism of this process is well documented (Kubicek and Röhr 1986; Röhr and Kubicek 1981; Legisa and Kidric 1989). As yet, only few attempts have been made to explore the control mechanism(s) by which this process is governed under the conditions of heavy metal toxicity. Some heavy metals (*e.g.* zinc or copper) are essential for fungi but most of them are toxic even at a low concentration. So far, the effect of heavy metals was mainly studied on growth (Rozicki 1992; Schmitz *et al.* 1993) and some changes in fungal morphology and physiology caused by these substances were reported (Baldrian *et al.* 1996; Baldrian and Gabriel 1997).

In this work, we studied the changes in fungal physiology and enzyme patterns with the age of mycelia of *Aspergillus niger* cultivated in the presence of copper and cadmium. The enzymes examined are those related to citrate cycle, which is known to play an important role in citric acid fermentation.

MATERIALS AND METHODS

Organism and cultivation. Aspergillus niger strain IM 13, from the *Culture Collection of the Institute of Microbiology, Bulgarian Academy of Sciences* (Sofia), was used as test organism. Spore suspensions for inoculation (spore concentration 1/nL) were obtained from cultures on agar medium of Moyer after 7-d cultivation at 30 °C. The growth medium for shake cultures contained (g/L): glucose 150, malt extract 1, NH_4NO_3 2, $MnSO_4 \cdot 4H_2O$ 0.25, $MgSO_4 \cdot 7H_2O$ 0.02, $FeSO_4 \cdot 7H_2O$ 0.02; pH 2.6. The metal ions (Cu^{2+} and Cd^{2+}) were added as $CuSO_4 \cdot 5H_2O$ and $CdSO_4 \cdot 8H_2O$ in two concentrations: 20 and 40 mmol/L for copper, and 50 and 100 mmol/L for cadmium, after 1 d of cultivation. The cultivation was carried out in Erlenmeyer flasks with 75 mL medium on a rotary shaker (frequency 3.7 Hz, 30 °C, 10 d).

Harvesting of mycelia and preparation of cell-free extracts. Every day (from the beginning of cultivation) mycelia were harvested at room temperature by vacuum filtration through Whatman no. 1 filter paper and washed thoroughly with distilled water. The resultant cell cake was weighed (wet mass) and used for preparation of cell-free extract. The samples were finely crushed while frozen and disintegrated thoroughly with quartz sand in a chilled mortar with a pestle. The homogenate was mixed with a small amount of appropriate buffer and centrifuged (5000 ***g***, 20 min). All operations were carried out at 1–3 °C. The supernatant was kept in an ice bucket and was used for enzyme assays as soon as possible.

Enzyme assay procedures. Spectrophotometric measurements were done with a Unicam Spectrophotometer (SP 500) holding two 10-mm cuvettes of 3 mL capacity. Tests were done at 25 °C, a period of 5 min being allowed before the addition of substrate to enable the temperature of the test system in the cuvettes to reach that of the water bath. The unit of activity was defined as an initial rate of increase in absorbance of 0.001 min^{-1} mL^{-1} of extract. Specific activity was expressed in katals

(nkat/mg protein). The activity of citrate synthase (CS; EC 4.1.3.7) was determined by the method of Srere and Kosicki (1961), absorbance being read at 412 nm. The activity of aconitase (AC; EC 4.1.2.3) was measured according to Anfinsen (1955), absorbance being read at 240 nm. The activity of both isocitrate dehydrogenases, (NAD-linked; ICDH), as well as NADP-linked (EC 1.1.1.41) were assayed according to La Nause (1966) by measuring the increase of absorbance at 340 nm.

Analytical procedures. Citric acid was measured by the enzymic method of Dagley (1974), total polysaccharides according to Trevelyan and Harrison (1952), and proteins by the method of Bradford (1976). Cell growth was measured by drying of washed mycelia at 105 °C to constant mass. Total lipids were estimated as follows: 1 g wet biomass was suspended in 30 mL chilled 10 % trichloroacetic acid. After 30 min it was filtered and washed by the series 15 mL ethanol, ethanol–chloroform (3 : 1) and ethanol–diethyl ether (3 : 1). The extracts obtained were collected and the organic solvents were evaporated. The residue was estimated by weighing as lipids, but mycelium was used for determination of nucleic acids. It was treated with 0.25 mol/L $HClO_4$ according to Kuboye *et al.* (1976). The amounts of DNA and RNA were determined by the method of Barton (1965).

RESULTS

The growth and citric acid production are shown in Table I. Both ions inhibited the synthesis of biomass, depending on the concentration of heavy metals. The biomass quantity in a medium with

Table I. Effect of copper and cadmium on growth, citric acid production and specific activity of citrate synthase, aconitase, NAD-ICDH and NADP-ICDH in *Aspergillus niger* after 2, 4, 7, and 9 d[a]

Treatment		2	4	7	9	2	4	7	9
		Growth, g/L				Citric acid, g/L			
None[b]		5.2	17.7	20.8	22.4	0	0	20.0	33.0
Cu^{2+}	2 mmol/L	2.3	12.7	14.8	15.6	0	0	10.1	15.1
	4 mmol/L	2.1	12.0	13.5	14.3	0	0	6.2	10.1
Cd^{2+}	50 µmol/L	4.7	15.9	20.3	20.8	0	0	15.6	25.7
	100 µmol/L	3.6	12.7	17.7	17.7	0	0	12.5	19.5
		Citrate synthase[c]				Aconitase[c]			
None[b]		1.7	8.1	12.9	9.0	2.0	2.9	1.5	1.3
Cu^{2+}	2 mmol/L	1.3	6.4	8.5	4.5	3.9	1.6	1.4	0.1
	4 mmol/L	0.9	5.7	6.1	2.9	1.6	1.3	0.8	0.9
Cd^{2+}	50 µmol/L	0.8	5.5	9.2	6.5	5.1	3.7	3.2	2.2
	100 µmol/L	1.1	3.8	6.2	4.4	2.7	2.7	2.4	1.8
		NAD-ICDH[d]				NADP-ICDH[d]			
None[b]		660	730	105	105	1040	1200	710	530
Cu^{2+}	2 mmol/L	0	340	52	0	220	550	350	400
	4 mmol/L	0	165	52	0	290	250	230	175
Cd^{2+}	50 µmol/L	760	490	240	47	880	960	710	270
	100 µmol/L	680	850	190	15.6	1120	1090	780	280

[a]The concentration of biomass and citric acid in the cultural liquid was determined during the growth phase (2 and 4 d) and during the citrate formation phase (7 and 9 d); means (n = 5), SD < 10 %.
[b]Control. [c]nkat/mg protein. [d]pkat/mg protein.

50 μmol/L Cd^{2+} is little different from the control sample, whereas the higher concentration as well as both concentrations of Cu^{2+} (2 and 4 mmol/L) provoked a visible decrease of the biomass. The dynamics of citrate production showed that the excretion of the product in the media started at the end of exponential growth (4 d). The product accumulation at both kinds of ions was inhibited to a higher degree than was the biomass. In the case of Cu^{2+} ions, the end concentration of citric acid was about 2–3 times lower than in the control.

The activity of citrate synthase increased and reached maximum values in the phase of active acid production (7 d). The level of aconitase activity was higher during the growth phase, when citric acid did not accumulate in the medium. After that, the enzyme activity decreased and at the end of the acid formation, their values were about twice lower than at the end of exponential growth.

Differences in the activity of both enzymes (CS and AC) in the presence of Cu^{2+} and Cd^{2+} were observed. Lower levels of citrate synthase in the presence of heavy metals were observed, whereas Cd^{2+} ions provoked increased aconitase activity during the two phases of the process. The activity of both ICDH, *viz.* NADP-ICDH as well as NAD-ICDH, showed that the activity of the first one was significantly higher than the NAD-linked enzyme and was influenced by the presence of Cu^{2+} ions considerably. No similar effect with Cd^{2+} ions was observed.

The quantitative analysis of contents of nucleic acids, proteins, polysaccharides and lipids in cells of *A. niger* from 7-d-old mycelium showed that the contents of DNA as well as the proteins decreased considerably with increasing concentration of Cu^{2+} and Cd^{2+} (Table II). In contrast, the contents of total lipids as well as polysaccharides were higher than in the controls.

Table II. Effect of copper and cadmium on the contents of total lipids (TL), polysaccharides (PS), protein (PT), DNA, and RNA in 7-d-old mycelium of *A. niger*[a]

Treatment		TL[c]	PS[d]	PT[d]	DNA[e]	RNA[e]
None[b]		21.1	0.10	0.44	100	100
Cu^{2+}	2 mmol/L	48.4	0.11	0.39	60	94
	4 mmol/L	61.0	0.15	0.23	34	76
Cd^{2+}	50 μmol/L	47.3	0.13	0.28	39	50
	100 μmol/L	55.3	0.14	0.26	31	47

[a]Means (n = 5), SD < 10 %. [b]Control. [c]mg/g dry cells. [d]g/g dry cells. [e]Relative %.

DISCUSSION

We found that cadmium is more toxic than copper for *A. niger*. The same effect was described in selected species of basidiomycetes (Sanglimsuwan *et al.* 1993; Baldrian and Gabriel 1997). Our results demonstrate that the reduction of growth caused by copper and cadmium is accompanied by a decreased production of DNA and protein, as well as increased production of lipids and polysaccharides. Hockertz *et al.* (1987) postulate that lower synthesis of DNA encourages citric acid accumulation by *A. niger*. They found reduced ribonucleotide reductase activity when the fungus was cultivated under conditions of Mn^{2+} deficiency, which is a prerequisite for enhancing citric acid production (Kubicek and Röhr 1977). This phenomenon could be exploited by using growth media with high concentrations of Cu^{2+} or Cd^{2+}.

We think that an increase of cell lipids and polysaccharides is also a reason for the changed acidogenesis of *A. niger* in the presence of heavy metals. Copper and cadmium probably influence the lipid composition of the membranes. Since the involvement of lipids in solute transport is well documented in yeasts (Prasad and Rose 1986), different lipid composition in cells might influence the activity of transport carriers and thus cause changes of the excretion of citric acid by the mycelium.

This influenced balance of cell biopolymers probably reflects the metabolic activity of the mycelia in the presence of Cu^{2+} or Cd^{2+}. The activity of citrate synthase is highest among the other citrate-cycle enzymes investigated. It could be assumed the citrate, which passes freely through the

mitochondrial membrane, has a significant role in the transfer of acetyl-SCoA from the mitochondria to the cytoplasm and it would be a reason for the highest activity of citrate synthase in comparison with the other citrate-cycle enzymes. It should be noted that the only factor controlling the activity of this enzyme is the concentration of its substrates. It is well known that the K_m for acetyl-SCoA depends on the concentration of oxaloacetate. Therefore, it is assumed that the critical factor controlling the enzyme activity is the presence of oxaloacetate (Kubicek and Röhr 1982). A higher amount of it reflects pyruvate carboxylase, the activity of which is in direct ratio to the concentration of the citric acid formed (Wongchai and Jeferson 1974). The lower level of citrate synthase in the presence of Cu^{2+} or Cd^{2+} would be explained by some inhibition effect of these ions on the activity of pyruvate carboxylase or by the increased cell needs from its substrates as biosynthetic intermediates. Thus, more enzymes are synthesized to scavenge the remaining citrate to produce isocitrate and 2-oxoglutarate for biosynthesis. As a response the activity of aconitase as well as isocitrate dehydrogenases is higher during the growth phase in comparison with the productive phase. Probably, during the period of intense citrate formation the activity of these enzymes (particularly NAD-ICDH) is inhibited by citrate.

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