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Scanning Electron Microscopy and Energy-Dispersive X-Ray Microanalysis of *Penicillium brevicompactum* Treated with Cobalt

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Abstract

Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX) were used to study the morphology and elemental composition of the conidia, phialids and hyphae of *Penicillium brevicompactum* grown in the presence of cobalt concentrations of 0, 50, 200, 500, 800 and 1000 ppm (mg/l). Cobalt uptake was through the hyphae, phialids and the conidia with maximum uptake being by the conidia at a concentration of 1000 ppm. EDX revealed the increase in the percentage of calcium and magnesium in the hyphae, conidia and phialids, compared to corresponding controls, accompanying the increase in cobalt uptake. Alternatively a decrease in sulfur percentage was observed. This study might reflect the possibility of using SEM-EDX as a new technique in understanding the mechanism of tolerance.

Key words: *Penicillium brevicompactum,* cobalt, elemental analysis, morphology, tolerance mechanism

Introduction

Cobalt belongs to Group VIII of the periodic classification of elements and shares properties with nickel and iron. Cobalt is a relatively rare element in the earth's crust (0.0023%) and is usually found in association with other metals such as copper, nickel, manganese, and arsenic. Release of cobalt to the environment occurs *via* soil and natural dust, seawater spray, volcanic eruptions, forest fires, and other continental and marine biogenic emissions

Anthropogenic sources include fossil fuel burning, processing of cobalt-containing alloys, copper and nickel smelting and refining, sewage sludge, and agricultural use of phosphate fertilizers (Eco-SSL, 2005).

Although cobalt is an essential nutrient, excessive oral doses result in a variety of adverse responses. The best characterized toxic responses are increases in red blood cell counts (polycythemia), cardiomyopathy, and effects on the male reproductive system (Paternain *et al*., 1988; Haga *et al*., 1996). In addition, reduced food and water intake and growth inhibition are commonly observed (Diaz *et al*., 1994a; 1994b).

Some heavy metals are essential for the fungal metabolism, whereas others have no known biological role. Both essential and nonessential heavy metals are toxic for fungi, when present in excess. Whereas fungi have metabolic requirements for trace metals, the same metals are often toxic at concentrations only a few times greater than those required (Hughes and Poole, 1991). The metals necessary for fungal growth include copper, iron, manganese, molybdenum, zinc, cobalt and nickel. Nonessential metals commonly encountered include chromium, cadmium, lead, mercury and silver (Gadd, 1993).

The contents of heavy metals in fungal mycelia reflect the metal concentrations in their environment and in several cases, metal-tolerant strains of fungi were isolated from contaminated sites (Gabriel *et al.*, 1997; Colpaert *et al.*, 2000). It should also be noted that it is possible to adapt fungi to higher heavy metal concentrations (Baldrian, 2000).

Fungal species and strains differ in their sensitivity towards metals and in the protection mechanisms involved (Baldrian, 2003). Baldrian and Gabriel (2002) reported the high variability of growth response in the case of Cd with the brown-rot fungus *Piptoporus betulinus*; Cd-tolerant as well as sensitive strains were found among 14 isolates from sites with different levels of air pollution by cadmium.

The interaction of fungi with heavy metals causes severe changes in the physiological processes and

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under certain circumstances it can even kill the mycelium. Therefore, fungi evolved active defense mechanisms that alleviate the toxicity of metals. The defense is usually based on immobilization of heavy metals using extracellular and intracellular chelating compounds. In many different taxonomic groups of fungi, heavy metals are intracellularly chelated by peptidic low molecular weight compounds-phytochelatins or metallothioneins (Tomsett, 1993; Baldrian, 2003). The binding properties of the cell wall could not be refrained together with its role as a mechanism of metal tolerance (Hall, 2002).

The wild type *Neurospora crassa* was found to remove cobalt from solutions having a cobalt content of 10 mg/l. The cobalt resistant mutant of *N. crassa* was shown to remove more than 90% of cobalt even from solution having Co concentrations as high as 500 mg/l (Karna *et al.,* 1996).

Fungi belonging to the genera *Rhizopus* and *Penicillium* have already been studied as a potential biomass for the removal of heavy metals from aqueous solution (Siegel *et al.*, 1990; Srivastava and Thakur, 2006a). Uptake of heavy metal ions by fungi may offer an alternative method for their removal from wastewater. The mechanisms of metal binding are not well understood due to the complex nature of the microbial biomass, which is not readily amenable to instrumental analysis. However, localization of metals has been carried out using electron microscopic and X-ray energy dispersive analysis studies (EDX) (Srivastava and Thakur, 2006b).

The current work aims at investigating the effect of different cobalt concentrations (0, 50, 200, 500, 800 and 1000 ppm) on the morphology and elemental composition of *P. brevicompactum* using scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX) as well as the possible use of these advanced techniques in elucidating fungal tolerance.

Experimental

Materials and Methods

Fungal isolate. *Penicillium brevicompactum* was obtained from the culture collection unit of the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University.

Media and growth conditions. Czapek's Dox medium was supplemented with different cobalt concentrations of 0, 50, 200, 500, 800 and 1000 ppm (mg Co/l). Except for the control, cobalt replaced ferrous sulfate. Cobalt chloride was the salt used to prepare the different cobalt concentrations. Media were autoclaved and poured into sterile petri dishes (two petri dishes for each cobalt concentration). The plates were inoculated with a 7-day old *P. brevicompactum* and incubated at 25°C for seven to fourteen days.

P. brevicompactum was grown on media supplemented with different cobalt concentrations (50, 200, 500, 800 and 1000 ppm). Morphological investigations were carried out using scanning electron microscopy. Energy-dispersive X-ray spectroscopy (EDX) was used to identify the elements associated with *P. brevicompactum* together with their percentage with respect to one another at the different investigated cobalt concentrations. The distribution of the elements in conidia, phialids and hyphae was also investigated.

Scanning electron microscopy (SEM). Blocks of the investigated fungal isolate were prepared for SEM at The Regional Center for Mycology and Biotechnology, Al-Azhar Univ. according to Zain, 1998). Fixation and dehydration procedures were performed using the programmable LEICA EM TP tissue processor model (A-1170), where six to eight millimeter squares of agar with fungal growth were cut from the cultures. The squares were then fixed by immersion in 2% (w/v) aqueous osmium tetroxide (OsO_4) at 4°C for 12 h. Fixed material was allowed to attain room temperature and then washed in distilled water (3 times, 10 min each) to remove excess of OsO_4. Fixed and washed materials were submerged and dehydrated through a graded, 10% steps, ethanol series from 10% to 90% and finally absolute ethanol. Dehydrated specimens were critical point-dried using the Critical Point Dryer EMS (Electron Microscopy Sciences) model EMS 850. The critical point-dried specimens were then attached to 0.9 mm diameter copper stubs using a carbon adhesive. Specimens were gold-coated (nearly 50 nm thickness) using an SPI Module^{TM} Sputter Coater and then examined using the high-vacuum mode of a JEOL JSM-5500LV Scanning Electron Microscope.

Energy-dispersive X-ray spectroscopy (EDX). Elemental analysis (the percentage of the detected elements with respect to one another) of the samples was carried out using the X-ray detector (INCAx-sight, Oxford Instruments) of the scanning electron microscope (Jeol JSM-5500LV). Window Integral was the mode of analysis. The given percentages represent the average of ten measurements for each of the conidia, hyphae and phialids.

Results

Scanning electron micrographs reveal the high tolerance of *P. brevicompactum* against cobalt (Fig. 1). The fungus was able to keep its penicillate form and conidial chain production even at the highest investigated cobalt concentration (1000 ppm) (Fig. 1i). A cobalt concentration of 50 ppm enhanced the growth of

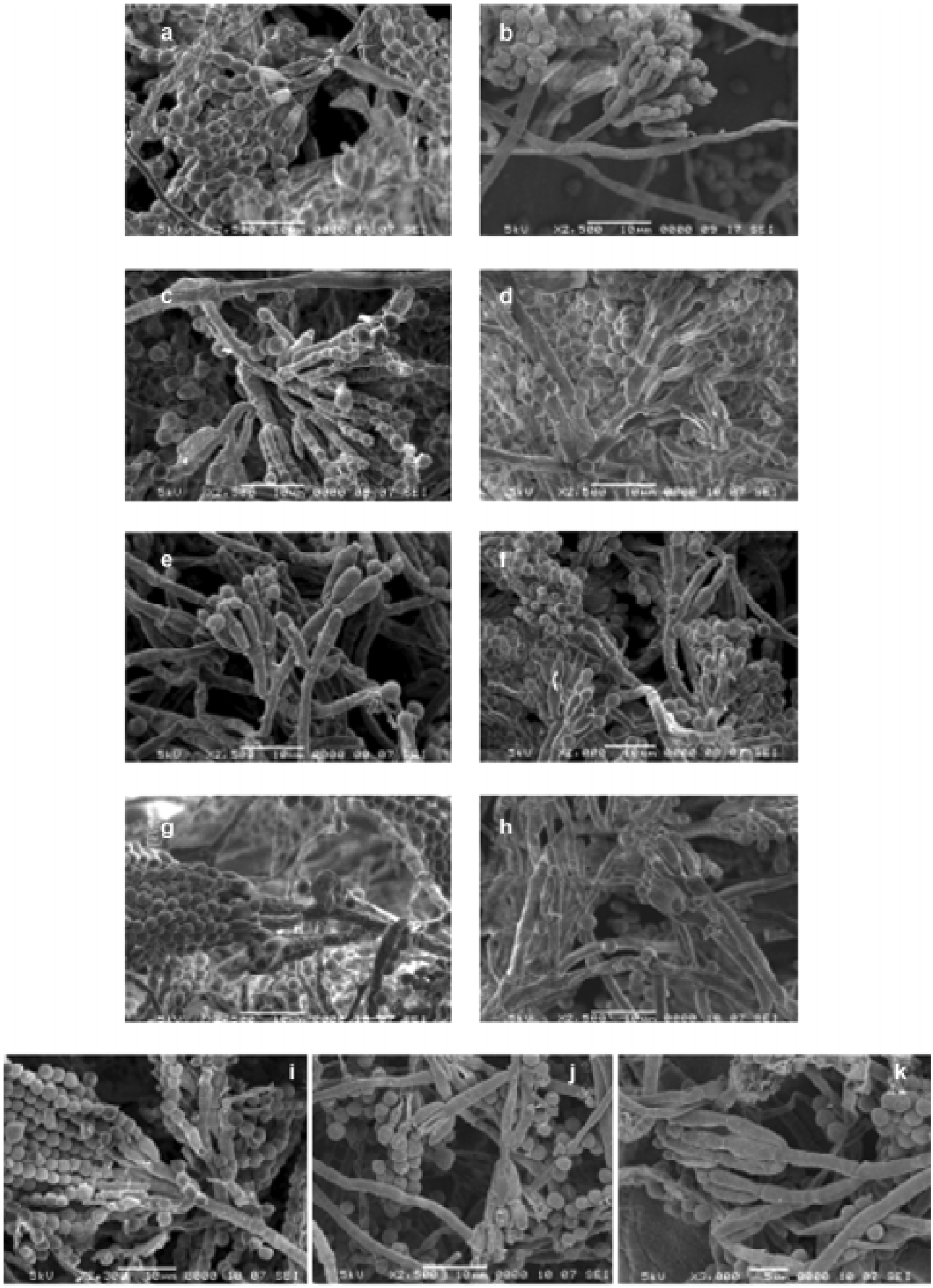

Fig. 1. Scanning electron micrographs of *P. brevicompactum* grown on Dox medium amended with cobalt concentrations of 0 (a), 50 (b), 200 (c, d), 500 (e, f), 800 (g, h) and 1000 ppm (i, j, k).

Table I
The percentage of elements detected by SEM-EDX in the conidia, phialids and hyphae of *Penicillium brevicompactum* grown under cobalt concentrations of 0, 50, 200, 500, 800 and 1000 ppm (mg/l)

Cobalt conc. (ppm) / element	0 (control)			50			200			500			800			1000		
	C	P	H	C	P	H	C	P	H	C	P	H	C	P	H	C	P	H
Na	1.8	4	1.6	0.7	0.2	4.9	6.2	12.7	7.9	2	1.9	2.7	1	2.5	1	0.5	1.1	1
Mg	3.8	2	4.5	4.5	3.7	5.7	5.5	5.9	9.8	6.3	13	10.7	6.5	6.8	5.7	15.5	13.4	12.6
S	67	65.7	68.6	57	64.9	18.7	71.9	12.4	34.7	32.9	31.3	42.2	42.9	38.9	40	0.2	23	30
K	4.8	10.8	5.1	5.5	3.8	5	2.7	7.2	1.3	2	5	3.4	1.7	5.2	1.8	5.2	7.3	4.3
Ca	22	17.5	20.2	23	19.9	59.5	6.5	49.1	37	39.8	29.6	27	23.6	22.3	26.5	40.6	32.4	27.3
Co	0	0	0	9.1	7.5	6.3	7.2	12.7	9.2	17	19.2	13.8	24.3	24.3	25	38	22.8	24.8

C, conidia; P, phialids; H, hyphae

the fungus and the fungus grew without obvious morphological changes (Fig. 1b).

However, more divergent ramulli, metullae and phialids were observed at cobalt concentrations of 200 ppm (Fig. 1c and d), 500 ppm (Fig.1e and f), 800 (Fig. 1e and f) and 1000 ppm (Fig. 1j and k). Distortions in branching were also observed.

At 200, 500, 800 and 1000 ppm, different distorted phialid shapes were observed; enlarged, slender as well as diminished.

Table I shows the results of the elemental analysis (SEM-EDX) of conidia, phialids and hyphae of *P. brevicompactum* grown in the presence of different cobalt concentrations (0, 50, 200, 500, 800 and 1000 ppm).

At a cobalt concentration of 50 ppm, the percentage (%) of sulfur (S) remained elevated in both the conidia and phialids while the hyphae (possessing the highest S% at the control) experienced a great decrease in the percentage of S compensated for by an elevation in the percentage of calcium (Ca) which was also elevated in the conidia and phialids when compared to the control. The percentage of cobalt (Co) was highest in the conidia followed by phialids and then the hyphae. Regarding magnesium (Mg), there was an increase in its percentage with a value higher than the corresponding control.

At a cobalt concentration of 200 ppm, the percentage of S was greatly reduced in the phialids (12.4%) followed by the hyphae (34.7%) while was elevated in the conidia (53.9%) when compared to the corresponding controls (65.7%, 68.6% and 67.6% respectively). Ca % was the highest in the phialids followed by the hyphae and then the conidia in which the Ca % reached 6.5 compared to a control % of 22. Maximum cobalt uptake at such concentration was by the phialids followed by the hyphae and then the conidia.

At a cobalt concentration of 500 ppm, the percentage of S in the three investigated parameters was decreased with values less than their corresponding controls. Alternatively, the Ca and the Mg percentages were increased with values greater than their corresponding controls. The same pattern was followed at a concentration of 800 ppm regarding S, Ca and Mg. However for Co uptake, no marked differences were observed; 24.3, 24.3 and 25 represented the percentage of Co in conidia, phialids and hyphae respectively.

Again at a concentration of 1000 ppm, the percentages of S were reduced when compared to their corresponding controls with the conidia being the structures possessing the least sulfur content as detected by SEM-EDX (0.2% compared to a control value of 67.6%). Also, the % of Ca and Mg were increased over their corresponding controls. Maximum Co uptake was by the conidia followed by hyphae and then the phialids.

It could be concluded that maximum Co percentage (38%) which was detected in the conidia of *P. brevicompactum* was accompanied by elevated Ca as well as Mg levels. Alternatively, extremely reduced percent of S was detected.

It was observed that increasing the concentration of cobalt in the growth medium resulted in increasing its uptake by the fungus. No clear cut could be concluded regarding the best structure uptaking cobalt; conidia was the best at 50 ppm, phialids at 200 and 500 ppm, hyphae at 800 ppm and again conidia at 1000 ppm. However, it is clearly observed that the best uptake was by conidia at 1000 ppm followed by hyphae, phialids and conidia at 800 ppm.

Discussion

Fungi frequently display a higher affinity for metal ions compared to other microbial groups and can accumulate metals from their external environment by means of physicochemical and biological mechanisms (Khoo and Ting, 2000; Cabuk *et al*., 2004; Preetha and Viruthagiri, 2005).

Razak *et al.* (1990a, b, c and d; 1993) directed the attention towards heavy metal uptake and metabolism in microorganisms and the microbial role mobilizing and immobilizing them as: Se, Te, Cd, Ni, Pb,

Co, Cu and Hg. Recently, the ability of microorganisms to take up metals has been demonstrated (Filipovic-Kovacevic *et al.*, 2000; Costa and Duta, 2001; Yalcinkaya *et al*., 2002; Hussein *et al.*, 2004; Preetha and Viruthagiri, 2005).

In the current study, the growth of *P. brevicompactum* was not greatly affected by the investigated cobalt concentrations. Cobalt was distributed among the hyphae, phialids and conidia with little morphological distortions where the penicillate form together with long conidial chains of the fungus were maintained even at the highest investigated concentration (1000 ppm). A number of *Penicillium* spp. were also reported to show high tolerance against different heavy metals, *e.g.*, Razak *et al*. (1993) reported that *P. chrysogenum* was able to keep its penicillate form even at the highest investigated tellurium concentration (0.5%).

In this study, long chains of conidia which were still produced even at 1000 ppm might reflect their role in metal uptake which was confirmed by SEM-EDX where maximum cobalt uptake (38%) was by conidia at a concentration of 1000 ppm.

SEM-EDX has been used to identify elements associated with microorganisms and wetland plants (Srivastava and Thakur, 2006b).

In the current work, SEM-EDX was used to study the distribution of cobalt in *P. brevicompactum* and its effect on the percentage of the other detected elements (Ca, Mg, S, Na and K). The most obvious result was the increase in the percentage of Ca and Mg accompanying the increase in cobalt uptake, where Ca and Mg were always higher than their values in the corresponding controls. This agrees with the results of Latha *et al*. (2005) who reported that cobalt taken up by *Neurospora crassa* was largely surface bound (>90%), resulting in a release of calcium and magnesium. Also, formerly (Karamushka and Gadd, 1994*)*, Ca and Mg were reported to have a protective effect on proton efflux from *Saccharomyces cerevisiae* influenced by the heavy metal copper. It was concluded that the protective effect of Ca and Mg is mediated by competitive and stabilizing interactions at the cell surface as well as physiological functions of Ca and Mg.

Another noticeable result was the decrease in the percentage of sulfur with increasing the cobalt concentration in the culture medium. This might be attributed to the synthesis of sulfhydryl-rich peptides. Glutathione (γ-L-glutamyl-L-cysteinylglycine) is responsible for the synthesis of these sulfhydryl-rich peptides in plants and fungi. These peptides are produced in response to metal stress where metals are chelated through coordination with the sulfhydryl groups in cysteine. An intracellular complex formed by these thiol peptides is thought to detoxify the metal by sequestration in the vacuole (Prasad, 2004). The sulfur in such intracellular complexes formed inside the vacuole could not be detected by SEM-EDX as the penetration power of the electron beam cannot reach that depth into the cytoplasm and vacuoles (*Oxford Operation Manual,* 2000), and thus cannot detect the sulfur in such compartments resulting in a decreased sulfur percentage. It should be noted that the penetration power of the electron beam cannot also allow the detection of the cobalt sequestered in the vacuole by the thiol peptides, however the percentage of cobalt was still high accompanying the treatments with low S%. This could lead to the conclusion that cell wall might play the major role in cobalt uptake by *P. brevicompactum*, this agrees with the results of Latha *et al*. (2005)**.**

The increase in the percentage of Ca and Mg, with values greater than the corresponding controls, by increasing the percentage of cobalt in the medium in the current study might also reflect the ability of the highly tolerant *P. brevicompactum* to sequester cobalt through the formation of thiol peptides where Mariano-da-Silva *et al*. (2007) reported that accumulation of heavy metals in the vacuole may cause calcium displacement from the vacuole, increasing free Ca^{+2} ions in the cytosol. Mg^{+2} ions are also displaced from the vacuole, passing to the cytoplasm.

Also, Tsekova *et al.* (2006) reported that biosorption of copper and cobalt ions displaced K^{+}, Mg^{2+} and Ca^{2+} present on *Penicillium cyclopium* indicating that biosorption took place as a result of an ion-exchange process.

The presence of two techniques for heavy metal sequestering has been reported; *e.g*., detoxification of Cd in *Paxillus involutus* involved binding of Cd to the cell wall and accumulation of Cd in the vacuole (Blaudez *et al.*, 2000).

Conclusively, SEM-EDX might reflect the possible presence of two mechanisms conferring tolerance to *P. brevicompactum* against cobalt; cell wall and thiol peptides. Further confirming studies will be conducted using transmission electron microscope and biochemical studies.

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