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THE INFLUENCE OF NICKEL(II) AND COPPER(II) COORDINATION COMPOUNDS BASED ON BENZOYLACETONE S-METHYLISOTHIOSEMICARBAZONE ON ENZYMATIC ACTIVITY OF MICROMYCETE *ASPERGILLUS NIGER* CNMN FD 10

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Mononuclear nickel(II) and copper(II) complexes with Schiff base ligands derived from benzoylacetone S-methylisothiosemicarbazone and 2,6-diformyl-4-methylphenol (CuL^1 and NiL^1) or 1-hydroxy-2-naphthaldehyde (CuL^2 and NiL^2) were previously synthesized and investigated.[1, 2] The aim of this work was to investigate the influence of previously obtained complexes on the enzymatic activity of cellulases and xylanases synthesized by fungal strain *Aspergillus niger* CNMN FD 10. It was found that the introduction of CuL^1 or NiL^1 complexes in the culture medium of micromycetes *Aspergillus niger* CNMN FD 10 causes a slight increase in the activity of β-glucosidases (2.81 U/mL compared with 1.59 U/mL in the control) and endoglucanases (6.36 U/mL compared with 5.71 U/mL in the control) at complex concentration of 1 mg/L. In the case of CuL^2 or NiL^2 complexes a positive effect was observed, *i. e.* the maximum activity of the enzymes was observed 24 hours earlier and it was significantly higher than in the controls. The maximum activity of β-glucosidases was 22.5% higher than in the control. The maximum activity of xylanases was 31.2% and 35% higher than in the control. Endogluconase's maximum activity was with 27.7% and 28.4% higher than in the control.

INTRODUCTION

For some time, semi- and thiosemicarbazones have been a subject of interest to researchers of different profiles. In view of the fact that these ligands form with many metals complexes of diverse chemical, physical and structural characteristics, they are of special interest to coordination chemists. Many of these ligands as well as their metal complexes, inclusively those with thiosemicarbazones, have shown a wide spectrum of biological activity, so that they have also become a subject of intense research for pharmacologists and microbiologist. Some authors noticed that the coordination compounds with thiosemicarbazones have an important role in the synthesis of bioactive substances, including of enzymes production by microorganisms. For example, it was shown that coordinative compounds of Cu and Ni with thiosemicarbazones increase the biosynthesis of pectolytic enzymes of fungal strains *Penicillium viride* CNMN FD 04 P with 58-74%.[3-5] Data of this kind are sporadic and don't show significant regularities of the interaction between complexes and biosynthetic activity of microorganisms.

Thiosemicarbazones possess a wide range of biological activity depending on the parent aldehyde or ketone. The biological properties of thiosemicarbazones are often related to metal ion coordination. Also, the metal complex can be more active than the free ligand, and some side effects may decrease upon complexation. In addition, the complex can exhibit bioactivities which are not shown by the free ligand.[6-8] The compounds mentioned above present an interest to

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microbiology both as free ligand or as Cu or Ni complexes having important role in mineral nutrition of microorganisms. Copper is a bioactive metal, being essential for copper-enzymes (polyphenoloxidases, tyrosinases, etc.), and for many proteins involved in oxidation-reduction reactions, linking or activating oxygen. It is known that in the presence of copper the iron absorption is facilitated as well as its incorporation into the cytochromes. Nickel is part of a series of enzymes (urease, NiFe hydrogenase, acetyl CoA decarbonylase/ synthase, dehydrogenase, metilCoM reductase, etc.). Nickel is essential in regulating transcription of genes which encode certain hydrogenases.[9, 10]

Although, Cu and Ni are essential for the vital activity of microorganisms, when present in high concentrations, they can be toxic. The toxicity of free metal ions is due to their ability to block functional groups, displacing an essential metal, modifying the active conformation of the molecule and, finally, denaturing proteins.[11]

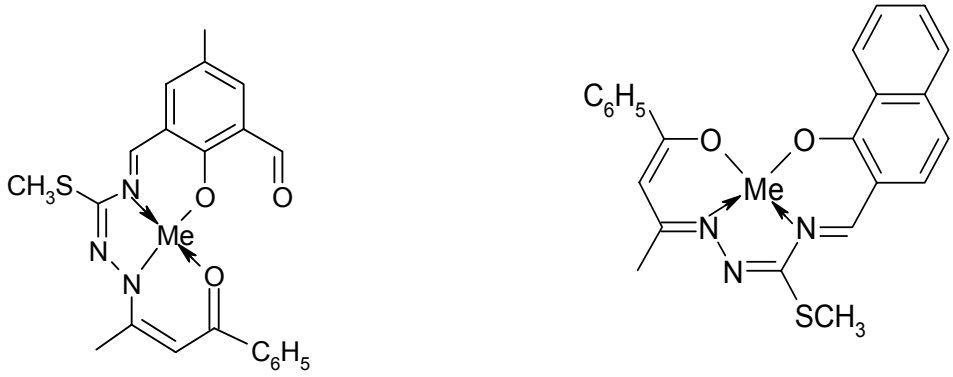
Chelation of free metal ions by polydentate ligands transforms them into stable, more coordination-rich particles which are not capable of destroying bicomplexes and, consequently, less toxic and more active.[12]

Preliminary toxicity studies of Ni(II) and Cu(II) complexes based on benzoylacetone S-methylisothiosemicarbazone, using biological test with ciliate *Paramecium caudatum*,[13] showed that they do not have any effect on the morphology, locomotor behavior and population density of paramecia even after 24-48 hours contact. Contrary, copper and nickel mineral salts, in similar concentrations, caused decrease in mobility of paramecia after 1 hour contact and cells lysis after 3 hours. The obtained results demonstrate that in the composition of coordination compounds the toxicity of metals is considerably reduced.

RESULTS

To identify the biological properties of the complexes CuL^1, NiL^1, CuL^2 and NiL^2 (Fig. 1), their influence on the activity of cellulosolytic enzymes synthesized by fungal strain *Aspergillus niger* CNMN FD 10 was investigated.

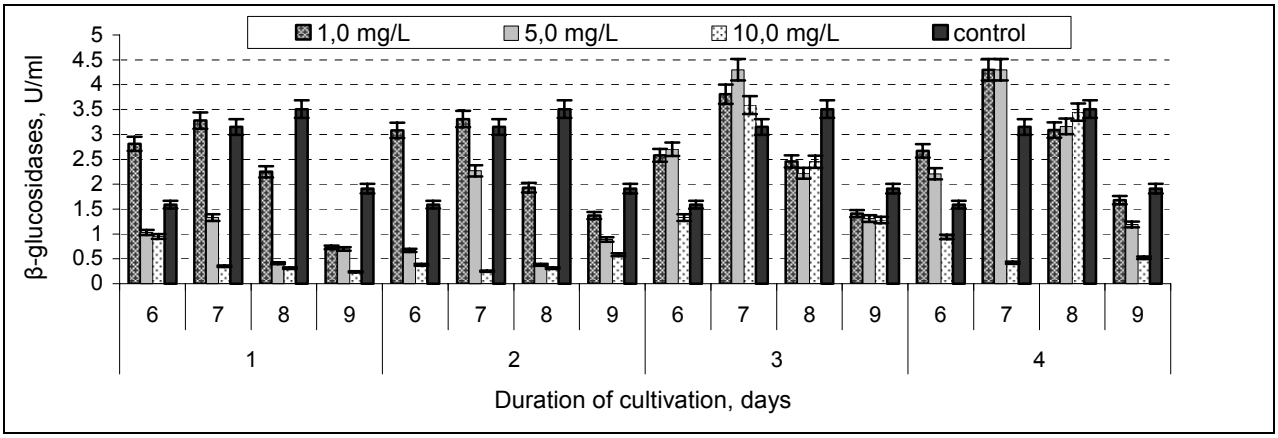
The strain is a producer of cellulases and xylanases, enzymes with wide application in the food industry for the production of fruit juices and preserves, and also in the winemaking where it is used to increase extraction and enhance the quality of the final product. Metals are characterized by a very narrow boundary between the concentrations necessary for vital activity of organisms and the toxic threshold. Therefore, when studying the biological properties of complex compounds (CC) of metals it is important to identify the optimal concentrations for their application. The results are presented in the Fig. 2 a-c.



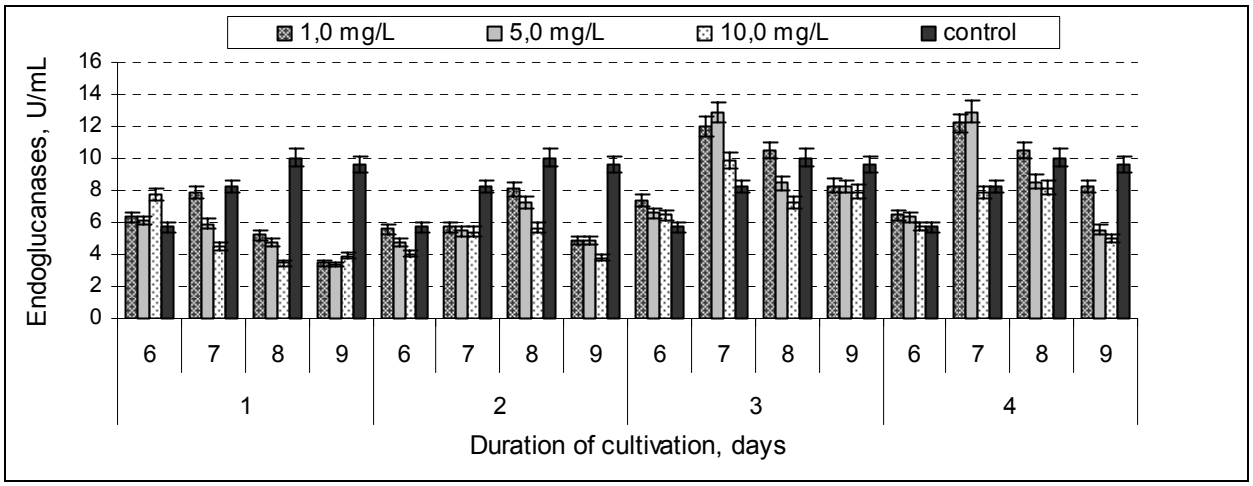
Me= Ni (NiL¹)
Me= Cu (CuL¹)

Me= Ni (NiL²)
Me= Cu (CuL²)

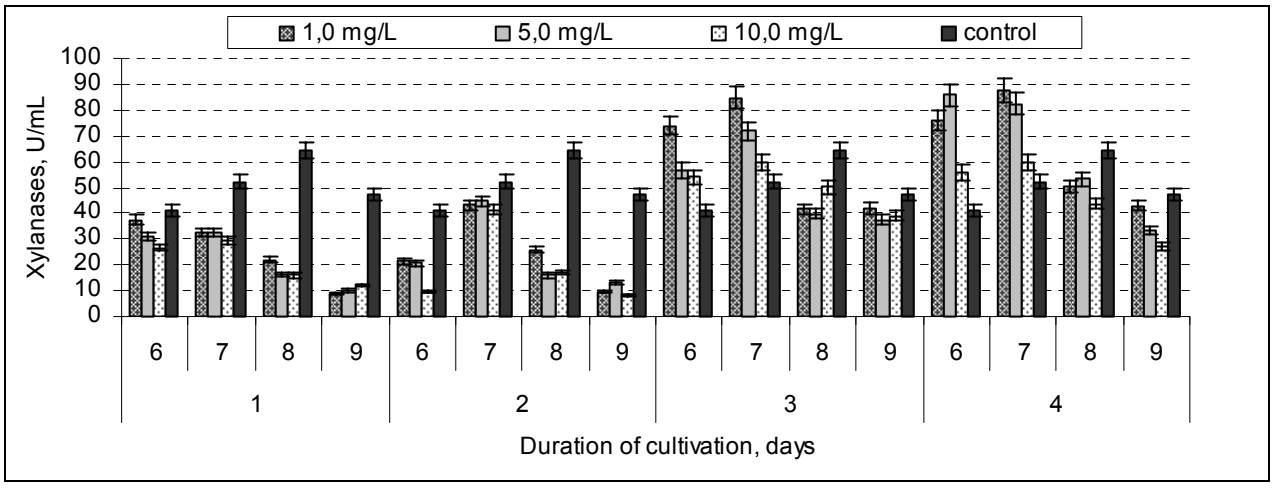
Fig. 1 – Structure of complexes CuL^1, NiL^1, CuL^2 and NiL^2. [1, 2]



a.



b.



c.

Fig. 2 – The activity of extracellular cellulases (a, b) and xylanases (c) synthesized by micromycete *Aspergillus niger* CNMN FD 10 cultivated in the presence of coordination compounds (**1 –** CuL^1; **2 –** NiL^1; **3 –** CuL^2; **4 –** NiL^2) in different concentration – 1.0 – 10.0 mg/L and control (without CC).

DISCUSSION

Our results show that the studied complex compounds of copper and nickel have different effects on the activity of extracellular cellulases, synthesized by micromycetes *Aspergillus niger* CNMN FD 10 depending on the applied concentration and the presence in them of different ligands of thiosemicarbazide nature (L_1, L_2).

The results mark the inhibitory action of complexes of both metals – Cu and Ni with L^1 ligand – on the activity of all components of the cellulases complex (β-glucosidases, endoglucanases) and xylanases synthesized by micromycetes *Aspergillus niger* CNMN FD 10. It was found that the addition of CuL^1 or NiL^1 complexes to the culture medium of micromycete *Aspergillus niger* CNMN FD 10 COP provides only a slight increase in the activities of β-glucosidases (2.81 U/ml compared with 1.59 U/mL in the control) and endoglucanases (6.36 U/mL compared with 5.71 U/mL in the control) in the early stage – 6^{th}, 7^{th} day of producer cultivation at a concentration of COP of 1 mg/L (Fig. 1a, b). During the next days the activity of synthesized enzymes becomes equal to the control, with a subsequent reduction, the activity level remaining during the entire cultivation under the level of control at the day of maximal biosynthesis (8^{th} day).

In the case of utilization of CuL^2 or NiL^2 complexes a positive effect was observed, consisting in the fact that the maximum activity of the enzymes was observed 24 hours earlier and it was significantly higher than in the controls.

Thus, the maximum activity of β-glucosidases in the control is 3.51 U/mL (8 days), and in optimized media – 4.30 U / mL (day 7), which is 22.5% higher. The maximum activity of xylanases in the control is 64.46 U/mL (8 days), but in the optimized media – 84.56 U/mL and 87.59 U/mL (day 7), which is with 31.2% and 35.9 % higher (Fig. 1c). Endogluconase's maximum activity in the control is 10.06 U/mL, but in optimized media – 12.85 U/mL and 12.92 U/mL – which is with 27.7% and 28.4% higher.

The effect exerted by coordination compounds varies depending on their concentration. The optimal concentrations of coordinative compounds that provide higher level of enzymatic activity are 1-5 mg/L. Increasing of metal complexes concentration to 10 mg/L causes a significantly diminution (with 20-90%) of cellulosolytic activity.

It is known that microorganisms require metals in trace quantities for metabolism and growth, but higher concentrations can be toxic. Copper toxicity is based on the production of hydroperoxide radicals and on interaction with the cell membrane.[14] Ni ions in high concentrations interact with cellular components like organic acids, nucleotides, amino acids etc., resulting in disturbance of physiological and biochemical processes.[15]

Microelements in chelated form act in the body as a factor which, as it appears, alleviates the high sensitivity of cells to trace elements by creating concentration gradient. Regulation of the concentration gradient outside and inside the cells is a promising direction in biotechnology of obtaining important substances from producers' cells.[16]

It is difficult to explain the mechanism of metalocomplexes influence on the activity of enzymes produced by microorganisms. The trace elements may be included (direct and/ or indirectly) in various biosynthetic processes enhancing metabolic processes of various bioactive principles (in this case of enzymes), on the other hand they may act on already synthesized enzymes, having the role of activators, stabilizers or inhibitors.

In micromycetes the synthesis of extracellular enzyme proteins is carried out in the form of inactive precursors in ribosomes; subsequently, they are attached to the cytoplasmic membrane, afterwards the enzyme adopts its characteristic configuration of an enzymatically active globular protein inside the membrane and is transported in the environment.[17] Since the studied complex compounds were added to the culture medium simultaneously with the inoculation material (suspension of spores), there are two possible factors stimulating their impact on ferments production:

– Penetration of complex compounds through the cell membrane and their impact on the regulatory mechanisms responsible for the synthesis of cellulolytic enzymes, the activation and the formation of their spatial configuration;

– The impact of complex compounds on the properties of the cell membrane and cytoplasmic membrane (chemical composition, charge, pH), which contributes to an increase in their permeability to maintain the dynamic equilibrium of life processes, start-up of intracellular processes such as metabolism, growth, development, division, and secretion (release from cells of synthesized secondary metabolites, including enzymes and proteins).

Furthermore, the presence of complex compounds in the environment can have a stabilizing effect on secondary and tertiary structures of the cellulolytic enzymes. Cellulases, as well as other enzyme proteins have different structural elements (catalytic, cellulose-binding and connecting them) with different functional active groups. In the stabilization processes, mainly those groups of proteins that are not essential for the catalytic functions may be involved.[18,19] Thus, a strict geometric

complementarity is required between the structure of complex compounds and the structure of sites of reacting biological molecules. Obviously, complex compounds of copper and nickel containing in their structures ligand L_2 better meet all these requirements than the complex compounds of copper and nickel with ligand L_1, providing a significant increase in the activity of cellulases.

EXPERIMENTAL

The submerged cultivation of the strain was carried out in 0.5 L Erlenmeyer flasks at temperature of 28-30°C on shakers (180-200 rot/min.) during 6, 7, 8 and 9 days.

The nutrient medium of the following chosen composition (g/L) was used as the control: beet pulp – 20.0; wheat bran – 10.0; apple or grape marc – 10.0; KH_2PO_4 – 1.0; $CaCl_2 \cdot 2H_2O$ – 0.1; KCl – 0.1; $MgSO_4 \cdot 7H_2O$ – 0.3; $NaNO_3$ – 2.5; $FeCl_3$ – 0.01; pH – 5.5-6.0.

The tested compounds were introduced into the nutritive medium in concentrations of 5, 10 and 15 mg/L. Coordination compounds were added to the sterile cultivation medium (after medium autoclaving) in the form of solutions prepared as follows: 100 mg of the compound was dissolved in 10 mL methyl sulfoxide and was completed to 100 mL with sterile distilled water.

Activity of cellulosolytic enzymes in culture filtrates was assayed by measuring the amount of reducing sugars released from the corresponding substrates: β-D-phenyl glucopyranoside – for β-glucosidases; Na-carboxymethyl cellulose – for endoglucanases; oat spelt xylan – for xylanases. The determination of glucose liberated from the substrate was measured calorimetrically using the Somogy-Nelson copper method. [20, 21]

CONCLUSIONS

Since there is no positive effect observed when using CuL^1 and NiL^1 complexes, but it occurs when introducing into the nutritive media the complexes CuL^2 and NiL^2, we can conclude that the presence of the positive effect is determined by the structure and characteristics of metalocomplexes of the ligand L^2.

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