ABSTRACT
Equilibrium and modelling study on copper biosorption by live cells of Penicillium cyclopium using fuzzy knowledge-based system was performed. Heavy metal uptake was found to be high, rapid and strongly dependent on both biomass and copper ion concentrations in the solutions. Most of total copper ions taken up by Penicillium cyclopium (up to 75%) were deposited in the cell surface during the first 5 min. The rest 25% were bound to the cells during the next 50 min. of the process. The largest amount of the heavy metal was removed from the solution with initial pH of the solution about 4.5. It is assumed that the copper biosorption by Penicillium cyclopium involves physico-chemical interaction between the metal ions and the functional groups present on the cell surface as well as the electrostatic attraction between groups plays an important role in this process.

Introduction
Many industrial facilities face wastewater treatment demand to prevent the anthropogenic release of heavy metals into local waterways. Regulatory agencies set effluents permit limits that can be difficult to meet for wastewater facilities receiving metal containing industrial wastes (1). Many studies have been performed on metal uptake by microorganisms, both living and nonliving (2, 3). Heavy metal’s biosorption is a potential and interesting alternative to conventional processes for removal of metals, such as ion exchange processes (4, 5).

Metal sorption performance depends on some external factors such as pH, concentration of biomass, concentration of the metal ions, temperature, other ions in solution, which may be in competition etc. (6). Different empirical or physical models in general represent experimental sorption data. Heavy metal adsorption is described by mathematical relationships between the concentration of the heavy metal in liquid and soiled phases, respectively in equilibrium conditions at constant temperature and pH (4, 7, 8). Although many experimental results have been published on metal ion biosorption much more effort should be addressed to the development of modelling procedures able to produce reliable experimental data in order to predict and explain metal ion biosorption in comparable conditions.

In this study a fuzzy knowledge-based system (KBS) was used to investigate copper biosorption by nonmetabolizing live biomass of Penicillium cyclopium and adsorption models of copper removal and uptake were determined. We have chosen this fungus due to its good adsorption properties expressed in term of heavy metal uptake. Moreover heavy metal biosorption by live cells of Penicillium cyclopium has not yet been reported. Copper was selected...
in the biosorption study because it is Environmental Protection Agency regulated heavy metal that is often used as anti-corrosion and decorative coating on metal alloys (9). Copper at excessive concentrations is toxic to a variety of living organisms from humans to bacteria, especially fish. Low exposures can impact respiration and affect cell walls of microorganisms.

Materials and Methods

Biomass cultivation

The fungus used in this study, *Penicillium cyclopium*, is deposited at the Collection of the Institute of Microbiology at the Bulgarian Academy of Sciences. Spores of 6-7 days old culture incubated on potato-glucose agar slants at 30 °C were used for inoculation (concentration of spore suspension 1x10⁶/ml). The growth medium contained (g/l): glucose - 20; NaNO₃ - 2; K₂HPO₄ - 1; MgSO₄ 7H₂O – 0.5; KCl – 0.5; FeSO₄ – 0.01; yeast extract – 1. The pH was adjusted to 5.0 before sterilization. The cultivation of *Penicillium cyclopium* was carried out in 500ml Erlenmeyer flasks with 100ml-growth medium on a rotary shaker at 30 °C. After 24h cultivation the mycelium was centrifuged (3000xg, 15 min.), washed with bidistilled water and used as a biosorbent.

Biosorption tests

Classically biosorption experiments were carried out in batches as follow: 1-4 g wet mass biomass was added to 100 ml metal ions solution, containing 50-250 mg/l copper (II) ions (as CuSO₄ 5H₂O) in bidistilled water in Erlenmeyer flasks. The pH was adjusted to the desired values 3.5-5.5 by adding the required amount of appropriately concentrated HCl or NaOH at the beginning of the experiment and then not further controlled. The variations in final pH values were less than 0.5 pH units, but this did not affect uptake values significantly. The content of the flasks then was agitated at 220 rpm on a rotary shaker until no more metal was removed from solution.

At the end of the process the content of the flasks was separated by filtration, using a Whatman №1 filter paper. The concentration of the metal ions in the filtrates was determined using an atomic absorption spectrophotometer with an air/acetylene flame (model 2380; Perkin Elmer, Überlinger, Germany). Blank samples were also considered to evaluate the eventual metal hydroxide precipitation.

The evaluation of the specific metal uptake \( q \) (mg/g dry weight of biomass) is obtained by using mass balances of metal and biomass in the system:

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q = \frac{(C_o - C_f)}{m},
\]

where \( C_o \) is initial Cu(II) concentration (mg/l), \( C_f \) – residual Cu(II) concentration (mg/l), \( m \) – biomass concentration as dry weight (g/l).

All experiments were carried out in duplicate. Values used in calculations were mostly the arithmetic average of the experimental data

Other methods

Dry weights were obtained by means of tarred aluminium foil cups dried at 85 °C for at least 48 h.

Modelling study

Introduction of new technologies in wide industrial area requires lot of experiments and training. Usually scientific research and preliminary investigations of complex, multivariable and nonlinear processes as well as biosorption processes are expensive because of great time, power and financial consumptions. Often the obtained experimental data are uncertain and incomplete, mutual relationships among the input/output variables are unstudied, the structure of any mathematical model is not known.

The main objective of our mathematical investigation was to create a modelling system which implementation reduces research expenses, gives simulation results with a high accuracy without a prior
knowledge of biosorption mechanism and shows the optimal technological conditions for the studied process.

A fuzzy knowledge-based system (KBS) was developed, which creation passed through five main stages: selection of the modelling approach, crisp data base development, fuzzification of crisp data (linguistic presentation by fuzzy membership functions), inference engine selection, rule base development, and adjustment of rules.

Results and Discussion

The search for new technologies involving the removal of toxic metals from wastewaters has directed attention to biosorption, based on metal binding capacities of various biological materials. The species of genus *Penicillium* are well known as producers of many biological active substances (10), but *Penicillium cyclopium* has not been investigated yet as a heavy metal biosorbent. That is why this microorganism has been chosen as a model in this study.

*Fig. 1* represents the purification of Cu(II) solution (100 mg/l) at two different biosorbent concentrations. The resulting curves share the same common characteristics of the process. Within the first 5 min. there was a steep decrease in Cu (II) content of the solution, due to a rapid uptake by the fresh biomass. Within the next 25 min. the rate of copper uptake gradually decreased. However, small amounts of the metal were sequestered even during the entire experimental observation period – 60 min. It could be assumed that true sorption equilibrium was not really established although the metal uptake rate was almost negligible, since there was no metabolizable substrate in the solution. The cells were starved and the metal uptake could not have been metabolically mediated. Residual enzymatic activity however, could not be entirely excluded.

The results obtained, showed that fungal biomass of *Penicillium cyclopium* is capable to remove copper ions from aqueous solutions and the process is represented by two phases: (*Fig. 1*) The first step of this mechanism was very fast, metabolism – independent and presumably represents cation binding to the cell surface. This was followed by slowly uptake of relatively small amounts of the cations. The second step can not be clearly labeled as metabolism – dependent or – independent. Most of the total copper ions taken up by *Penicil-
P. cyclopium (up to 75%) were deposited in the cell surface during the first phase and up to 25% were bound to the cells during the next 50 min of the process. Suh et al. (11) reported similar results on the penetration time of Pb(II) accumulation in Saccharomyces cerevisiae associated with the intracellular region (2h) that was quite different from the one in the extracellular region (3min). Probably, the metal uptake by P. cyclopium is a physico-chemical interaction between the metal and the functional groups present on the microbial cell surface. Microbial cell walls mainly composed of polysaccharide, proteins and lipids have abundant metal binding groups such as carboxyl, sulphate, phosphate and amino groups (12, 13, 14). The metal removal from solution may take place by ion-exchange mechanism and/or complex formation on the cell surface after the interaction between the metal and the active groups. This type of biosorption is relatively rapid and can be reversible.

The Cu (II)-uptake (q) at different values of m, pH and initial Cu (II)-concentrations are shown in Fig. 2. These experiments showed that copper uptake decreases when the biomass concentration rises and initial pH value of the solution decreases. Maximal values of the uptake (q_max) were obtained at pH about 4.5 when biosorbent concentration (m) was almost 1 g/l. (Fig. 2a). In Fig. 2b maximal values of the uptake were obtained at 150 mg/l Cu (II) - ions and biomass concentrations among 1-1.5 g/l. In opposite, at higher values of biomass and higher copper concentrations, the observed values of uptake decreased significantly. Copper uptake dependence on Co and pH is shown in Fig. 2c. Maximal uptake values are observed when pH is
about 4.5 at Cu (II) concentration about 150 mg/l. Better biosorption was achieved at pH 4.5 \(\approx 4 > 3.5 > 3\) (Fig. 2a, 2c) and hardly any was apparent at pH 2 (not shown). Minimal uptake values occur when the initial copper concentration increases higher than 160 mg/l, biomass concentrations rises more than 2.5 g/l and pH is between 3-3.5 (Fig. 2a, 2b).

Results obtained for dependence of q on pH of the solutions (Fig. 2a, 2c,) indicated that the mechanism of adsorption includes participation of functional groups of the biomass which could be weakly acidic protonated at pH 2.0. However, at higher pH values the coordinating ligand may be deprotonated and available to bind positively charged ions such as Cu (II). Since carboxylic groups generally have pKa values about 4, they are most likely responsible for the Cu(II) adsorption by *Penicillium cyclopium*, as previously found for Cu(II) binding by the other kind of biomass (15).

The effects of varying of pH solution have indicated that the heavy metals accumulation by the biomass is occurring through surface functional groups via ion-exchange type process and most likely involves carboxyl groups. The experimental data related to heavy metal biosorption by *S. natans* showed that the cell wall seems to have two or more main functional groups responsible for heavy metal uptake (16). Esposito *et al.* (13) have shown that the biomass cell wall is characterized by two main kinds of weakly acidic groups with pKa=4.9 and pKa=7.8. According to the chemical composition of a bacterial cell wall, these two sites could be a carboxylic and a phosphate group respectively (14, 17). The pH optimum value obtained in our study was found to be about 4.5 for Cu (II) accumulation by *Pen. cyclopium* at all experimental conditions (Fig. 2a, 2c). This indicated that the binding sites in *Pen. cyclopium* could involve carboxylic groups. Fourest *et al.* (12) also reported that carboxylic groups present on the fungal cell wall of different species of *Rhizopus, Mucor, Penicillium* and *Trichoderma* contributed from 30 to 70% to the total heavy metal binding at the fungal wall, depending on the species.

Biomass concentration in solution seems to influence the specific uptake: for lower values of biomass concentrations, there is an increase in the copper uptake by *Pen. cyclopium* (Fig. 2a). The reduction of the uptake with raising the biomass concentration more than 2 g/l is attributed to electrostatic interaction between functional groups of the cell surface. This suggestion about interference between the binding sites may be a significant factor in the biomass dependence of metal adsorption at copper concentration higher than 140-150 mg/l also (Fig. 2b). Reduction in biomass concentration in the suspension at a given metal concentration enhances the metal/biosorbent ratio, and thus increases metal uptake per gram of biosorbent as long as the latter is not saturated. Illustration of this behaviour is given in the investigation of Fourest and Roux (18) showing Zn(II) sorption by *R. arrhizus*. Hence, this factor needs to be taken into consideration in any application of microbial biomass as biosorbent. Therefore it is not useful to increased the biomass concentration beyond 2.25 – 2.5 g/l to purify 100 – 110 mg/l copper solutions at pH near to 4.5 (Fig. 3a, 3b)

Fig. 3 represents the purification of Cu (II) solutions at both different biosorbent concentrations and pH of the solutions. These experiments show that residual copper concentrations (Cf) in solutions generally decrease, when pH is about 4.5 and m is near to 2.5 g/l or more, but the copper concentrations must be about 100 mg/l (Fig. 3a, 3b). At these conditions, copper removal from the solutions seems to be successful.

The influence of the initial copper concentration on copper uptake (Fig. 2b, 2c) at different values of m and pH suggests that
adsorption of Cu (II) ions by no metabolizing live cells of *Penicillium cyclopium* is a process that follows a chemical, equilibrated and saturable mechanism on the cell surface. The copper uptake values increase with increasing the copper concentrations until reaching saturation of the biomass. The reduction in Cu (II) uptake by higher free cell concentrations was attributable to electrostatic interaction between free cells as previously reported (3, 19). This causes the three dimensional structure of the cell wall, thus reducing Cu (II) diffusion through the structure and the accessibility of the binding sites for adsorption (20).

**Conclusions**

Hence, it is possible to remove copper ions from the solutions using living cells of *Penicillium cyclopium* at optimized parameters of the process. Therefore, it is critical to determine the copper concentration, pH and biomass concentration in the system to be achieved a successful removing of the metal in an environmentally appropriate manner. The modeling technique described allowed to optimize this process at low number of experiments and for a short experimental time. Introduction of artificial intelligence methods and knowledge-based systems is efficient for scientific research of unstudied dependence among different by nature variables (physical, chemical, biological, etc.), for solving tasks of inferential measurement and process optimization without requiring a big amount of precise experimental data, without knowing the structure of the mathematical model, which looks up for the detailed recognition of process mechanisms.

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