Isolation and molecular characterization of some copper biosorped strains

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ABSTRACT: The biosorption of copper (II) from aqueous solution using different bacterial strains was studied. Copper-biosorbing bacteria were isolated from tannery effluent collected from Borg Al-Arab, Alexandria, Egypt. These isolates displayed different degrees of copper biosorption under aerobic conditions. Based on 16S rDNA gene sequence analysis, three of them (S2, S5 and S7) were identified as *Chryseobacterium sp.*, *Enterobacter sp.* and *Stenotrophomonas sp.*, respectively. Initial copper (II) ion concentrations from 25-250 mg/L at constant temperature 30 °C were studied. The residual copper (II) concentration and its toxicity effect in solution were determined using atomic absorption spectrophotometer and bioluminescent bioreporter. The bioluminescence inhibition of strain (S5) reached to 91.4 % as compared with the strain (S7) reached to 83.3 % at 225 mg/L of copper ion where the maximum biosorption efficiency for S5 and S7 were 71 % and 70.1 % correspondingly using atomic absorption. The bioluminescent bioreporter was proved to be fast and accurate technique for measurement the toxicity effect of residual copper (II) in solution.

Keywords: *Bacterial strains; Bioluminescent; Biosorption; Restriction fragment length polymorphism*

INTRODUCTION

Heavy metals are groups of pollutants, which are not biodegradable and tend to accumulate in living organisms (Kobya *et al.*, 2005). The concentrations of these metals need to be reduced to meet ever-changing legislative standards. According to the World Health Organization (WHO, 2010), the metals of most immediate concern include cadmium, chromium, cobalt, copper, lead, nickel, mercury and zinc. The presence of such metals in aquatic environments cause severe damage to aquatic life and killing microorganisms during biological water purification process (Vinodhini and Narayan, 2008). Moreover, these metals have exacting consequences on humans such as brain damage, reproductive failures, nervous system failures and tumor formation (Hamman, 2004; Mahvi, 2008). Copper, one of the most widely used heavy metals, is mainly employed in electrical and electroplating industries and in larger amounts is extremely toxic to living organisms. The presence of copper (II) ions, cause serious toxicological concerns, it is usually known to deposit in brain, skin, liver, pancreas and myocardium (Davis *et al.*, 2000).

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Conventional techniques for removing dissolved heavy metals include chemical precipitation, carbon adsorption, electrolytic recovery, ion-exchange, chelation and solvent extraction or liquid membrane separation (Vasudevan *et al.*, 2003; Lodeiro *et al.*, 2005; Liu *et al.*, 2006; Sharma *et al.*, 2006; Samarghandi *et al.,* 2007; Malakootian *et al.,* 2009) all exhibit several disadvantages, such as high cost, incomplete removal, low selectivity, high energy consumption (Panjeshahi and Ataei, 2008) and generation of toxic slurries that are difficult to be eliminated (Celaya *et al.*, 2000; Okafor and Opuene, 2007). Therefore, much attention has been paid to the removal of metal ions by microorganisms due to its potential applications in environmental protection and recovery of toxic or strategic heavy metals (Tsezos, 1985; Forest and Roux,1992; Chang and Hong, 1994; Chang *et al.*, 1997; Babel and Opiso, 2007; Zvinowanda *et al.,* 2009; Andreazza *et al.*, 2010). Certain types of microbial biomass are considered to retain relatively high quantities of metals by means of passive process known as biosorption. Biosorption utilizes various natural materials, including fungi, yeast and bacteria that have been studied to sequester metal ions from aqueous

solution and it represents a cheap alternative to conventional processes due to the use of a low cost sorbent material (Nameni *et al.,* 2008; Abdel-Ghani *et al.,* 2009; Silva *et al.,* 2009; Yahaya *et al.,* 2009). Biosorption is either metabolism independent, such as physical or chemical sorption onto the microbial cell walls, or metabolism associated, such as transport, internal compartmentalization and extracellular precipitation by metabolites. In addition, an important aspect of biosorption is that it can be carried out either with metabolically active or inactive cells (Kurek *et al.,* 1982; Brierley, 1990; Volesky, 1994; Chen *et al.,* 2000).

Several toxicity bioassays have been described and different methods using microorganisms have been used to assess the acute toxicity of industrial wastewaters. Among these bioassays are bioluminescence tests (Kahru *et al.,* 2000). This test is based on the inhibition of the bioluminescence of luminescent bacteria (lights off). The lights off assays is an extension of the widely accepted microbial toxicity bioassays, which is based on the measurement of a decrease in light emission as a function of sample concentration by the wild type luminescent bacteria *Vibrio fischeri* following a short time exposure to the sample (Quershi *et al.,* 1998).

In the present study, the biosorption of Cu (II) from aqueous solution using different bacterial strains was studied. The RFLP using different enzymes was carried out, also the 16S rDNAs were amplified and partially sequenced, compared with sequences deposited in databases. The biosorption efficiency of different copper ion concentrations from loaded biosorbent was assessed using atomic absorption spectrophotometer, and bioluminescent biosensor technique.

This work was performed in Mubarak City for Scientific Research and Technology Applications, Alexandria, Egypt from 2008 to 2009

MATERIAL AND METHODS
Isolation of copper-resistant bacteria
Copper-resistant bacteria were isolated from tannery effluent obtained from a company at New Burg El-Arab City, Alexandria, Egypt. For the isolation and enumeration of bacteria, samples were serially diluted and plated on Luria–Bertani (LB) agar (tryptone: 10 g/L; yeast extract: 5 g/L; NaCl: 10 g/L; glucose: 0.1 g/L) adjusted at natural pH value of the medium from 6.5 to 7.0 without any additions. The molten medium was amended with Cu (II) as $CuSO_4$ to final concentration

50 mg/L using sterile filtered Cu (II) stock solutions. Plates were incubated at 30 ºC in the dark and were read after 2 days. Seven copper-resistant isolates representing different colony morphologies were purified on LB agar containing 50 mg/L of Cu (II).

Evaluation of Cu (II)-resistance
The minimum inhibitory concentration (MIC) of the seven Cu(II)-resistant isolates were determined by broth dilution method (Calomoris *et al.,* 1984) in LB medium with Cu (II) concentrations ranged from 50 to 250 mg/L. The minimum concentration of metal in the medium inhibiting complete growth was taken as the MIC. Out of these results three isolates (S2, S5 and S7) were selected for further analysis.

Copper biosorption by the isolates
Copper-resistant isolates (S2, S5 and S7) were inoculated into LB broth containing different concentrations of Cu (II) (from 25 to 250 mg/L) and incubated for 72 h at 30 ºC with orbital shaking (200 rpm). The inoculum was 2 % of total volume of media. Bacterial cell density (diluted 10-fold with water) of the liquid cultures was determined by measuring optical density at 600 nm by using UV/Vis spectrophotometer (DU.530 Beckman) (Andreazza *et al.,* 2010; Sultan and Hasnain, 2007). Residual of biosorped Cu (II) was determined from extracted solution by using a Perkin-Elmer Analyst 300 atomic absorption spectrophotometer (AAS). All the above experiments were performed in triplicates and the mean value was determined.

Residual of Cu (II) toxicity assay
The toxicity assay was performed using a bioluminescent bioreporter *Acinetobacter* strain DF4/PUTK2 that had been genetically modified by conjugation (Abd-El-Haleem *et al.,* 2006) to contain the PUTK2 plasmid (Burlage *et al.,* 1990), with the Tn4431 lux transposon downstream of a putative plasmid maintenance promoter to produce continual visible bioluminescence light. Bioluminescence was measured at 5 min regular time intervals from zero to 480 min by a luminometer (LumiStar, Galaxy, BMG, Germany). To perform the assay, a culture of the bioreporter strain DF4/PUTK2 was grown in LB medium on an orbital shaker at 200 rpm at 30 °C for 16 h. Subsequently, the cells were diluted 1:10 in LB medium and incubated on an orbital shaker at 30 °C to achieve

an optical density of 0.6 measured with a spectrophotometer at 600 nm (Sultan and Hasnain, 2007; Andreazza *et al.*, 2010). After two washes in a sterile LB medium, the pellet was resuspended in 1 mL sterile LB. Subsequently, aliquots 100 µL of each sample mixed with 100 µL of a DF4/PUTK2 cell suspension were transferred to 96-well microtiter plates (Nunc) to produce 4 replications of each sample. Along with each treatment two controls were incorporated into each plate, the first contained 100 µL from the bioreporter DF4/PUTK2 mixed with 100 µl MilliQ water (no toxicity control), while the second control contained the bioreporter mixed with the original samples (non diluted toxic substances). Wells were then covered with transparent plate sealer and placed in the luminometer for luminescence detection at room temperature.

PCR-RFLP fingerprints of the 16S rDNA gene

The RFLP was carried out according to (Abd-El-Haleem *et al.*, 2003). EcoR1, BamH1, HaeIII, HindIII, HincII, and HinfI are the restriction enzymes used for RFLP of the 16S rDNA genes. A volume of 15 µL of amplified 16S rDNA was separately digested for 3 h at 37 °C with 2U of each restriction enzyme (GIBCO B.R.L). The fragments were separated by electrophoresis on 2 % agarose gels (FMC, Rockland, USA) containing Ethedium bromide at 0.1 µg/mL. Gels were run at 100V for 3 h in 1x TBE buffer and then visualized and photographed in the multiImage light cabinet (Alpha Imger ™ 1220). 100 bp and 1 kb ladder mix (Sigma, St. Louis, MO) were used as molecular weight marker

Molecular identification

Amplification of 16S rDNA with eubacterial universal primers 27F and 1492R was done (Lane, 1991). Genomic DNAs and/or PCRs were performed using EZ-10 Spin Column DNA purification kit according to the manufacturer's instructions (BIO BASIC INC.). Sequencing was performed using ABI PRISM dye terminator cycle sequencing kit with AmpliTaq DNA polymerase and an Applied Biosystems 373 DNA sequencer (Perkin-Elmer, Foster City, California).

The sequences were analyzed using the CHECK CHIMERA and the SIMILARITY RANK programs of the Ribosomal Database Project (Altschul *et al.*,1990) also analyzed using the BLAST program (National Centre for Biotechnology Information) to determine the closest available database sequences. Selected rDNA sequences were aligned using the Clustal W program (Hall, 1999). Published sequences were obtained from GenBank. A phylogenetic tree was constructed using Clustal W by distance matrix analysis and the neighbour-joining method (Saitou and Nei, 1987). Phylogenetic trees were displayed using TREEVIEW (Page, 1996).

RESULTS AND DISCUSSION

Isolation and evaluation

In search for copper-resistant microorganisms, a total of seven Cu-resistant bacteria were isolated from tannery effluent obtained from a company in Borg Al-Arab, Alex. Egypt. The effect of Cu (II) concentrations (MIC test) ranging from 50 to 250 mg/L on the growth of the isolates was evaluated according to Yahaya *et al.*, (2009) who used different Cu (II) concentration varying from 50 to 300 mg/L. From the results presented in Fig. 1, the most significant growth decreased after addition of 200 mg/L of Cu (II) was observed with isolates S1, S3, S4, S6 and S7. However, the growth of isolate S7 was more comparing to the other isolates at different copper concentrations. Isolates S2 and S5 were growing well when exposed to the highest copper concentration, till 250 mg/L. However, the MIC of strains S1, S3, S4, S6 and S7 was 200 mg/L, while it was 250 mg/L with strains S2 and S5, respectively more than 250 mg/L of Cu was toxic for the bacterial strains (data not shown). The detoxification efficiency of the seven respective isolates follows the sequence: S1 > S3 > S4 > S6 > S7 > S2 > S5. However, three isolates S2, S5 and S7 were the most resistant strains and selected for further analysis. Previously, Shakibaie *et al.* (2008) reported 9 strains able to resist Cu up to 0.5 mM according to their MIC.

PCR-RFLP fingerprints of the 16S rDNA gene

In order to evaluate the applicability of restriction enzyme analysis of the examined isolates and to select the restriction enzyme that gives the highest level of discrimination, PCR-RFLP analysis was performed using each of the following restriction endonucleases: BamH1, EcoR1, HaeIII, HindIII, HincII, Hinf1. No and/or poor digestion was observed with EcoRI, HindIII, BamHI and HincII. However, the most differentiating banding pattern was showed with enzymes HaeIII and HinfI (Fig. 2). Previously, Abd-El-Haleem *et al.* (2003), reported that *Hinf*I is the most suitable enzyme for RFLP analysis for the 16S rDNA amplicons.

Identification and phylogenetic analysis

The 16S rDNA genes of the isolates were partially sequenced following PCR amplification and compared with sequences deposited in databases. Totally ~ 700
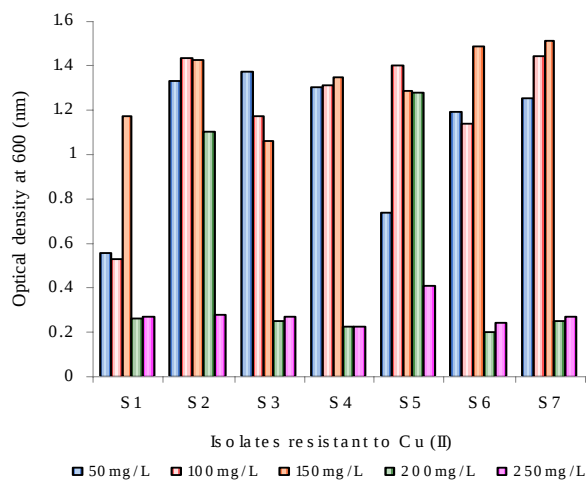
Fig. 1: Effect of different Cu (II) concentrations on the growth of seven selected isolates

bp of the 16S rDNA of isolates S2, S5 and S7 was determined. The phylogenetic tree (Fig. 3) showed that isolate S2 was closely related to *Chryseobacterium sp.* exhibiting similarity values greater than 94 %. Isolate S5 was belonging to *Enterobacter* sp. with similarity value > 98 %. However, isolate S7 was related to *Stenotrophomonas sp.* with similarity value > 87 %. The nucleotide sequence data reported in this study have been deposited in the NCBI nucleotide sequence database (Gen Bank) under the accession number of FJ827754, FJ827755 and FJ827756, respectively. It was previously emphasized that some strains related to the genus *Enterobacter* and *Stenotrophomonas* have been described for their ability to biosorp copper ion (Alluri *et al.*, 2007; Parungao *et al.*, 2007). Historically, the 16S rDNA gene has been chosen by many workers for phylogenetic studies because of its relatively small size which facilitates sequence analysis. As a result, several thousands of 16S rDNA sequences are available (Abd-El-Haleem *et al.*, 2002a and b; 2003).

Determination of accumulation efficiency of the isolates

The ability of cupper-resistant bacterial strains (S2, S5 and S7) for reduction of different concentrations of Cu (II) ranged from 25 - 250 mg/L were determined spectrophotometrically readings after 72hrs at 30ÚC in LB media. At different concentrations of Cu (II), Fig. 4 and Fig. 5 show cellular growth and biosorption efficiency (%) of *Chryseobacterium sp.* strain S2, *Enterobacter sp.* strain S5, and *Stenotrophomonas sp.* strain S7, respectively. A progressive decrease was

showed in growth by increasing Cu(II) concentrations (Fig. 4). The result obtained pointed out that the exact MIC for strains S2, S5 and S7 was 225, 250 and 200 mg/L, respectively. These selected strains according to their MIC similarly to Vullo *et al.* (2008) who chosen five of eleven strains due to their highest MIC for heavy metal and highest biomass yield in constant incubation time.

Fig. 5 represents the percentage of copper biosorption efficiency using atomic absorption spectrophotometer. The % of biosorption efficiency was increased as the initial copper ion concentration increased. The result implies that the gradual increase in the efficiency of the strain shows nearness to saturation of the available binding sites. These results agree with the result of Adesola Babarinde *et al.* (2007). The maximum biosorption efficiency of 225 mg/L Cu by isolates S2, S5 and S7 was 48 %, 71 % and 70.1 %, respectively. It is established that biosorption of metal ions has been reported to be biphasic (Liu *et al.*, 2006). The initial fast phase occurs due to surface adsorption on the biomass. The subsequent slow phase occurs due to diffusion of the metal ions into the inner part of the biomass. In the present study (Fig. 5), it was observed that the metal biosorption rate was high at the beginning of the process but plateau values were obtained after constant concentration, similar to what was reported by Liu and coworkers (Liu *et al.*, 2006). Also Andreazza *et al.* (2010) reported that the rate of bacterial growth, Cu (II) removal and Cu (II) bioreduction were directly related to Cu (II) concentration. Increasing the concentration of Cu (II) lowered growth due to its toxic effects. However, increasing concentration increased Cu (II) reduction and removal. A similar relationship between concentration and bioremoval was reported for a heavy metal by Okeke *et al.* (2008) and Okeke (2008). At the highest copper concentration, isolate NA strongly reduced and removed copper indicating high capacity for Cu (II) biotransformation.

Bioluminescence assay

The environmental protection against the harmful industrial wastes requires fast and reliable techniques to assess the wastes in order to apply the necessary bioremediation measures in the appropriate time (Abd-El-Haleem *et al.*, 2006; Zaki *et al.*, 2008). The residual Cu (II) concentrations was evaluated using bioluminescent biosensor, this techniques are gaining wide acceptance in environmental studies. The bioluminescence assay
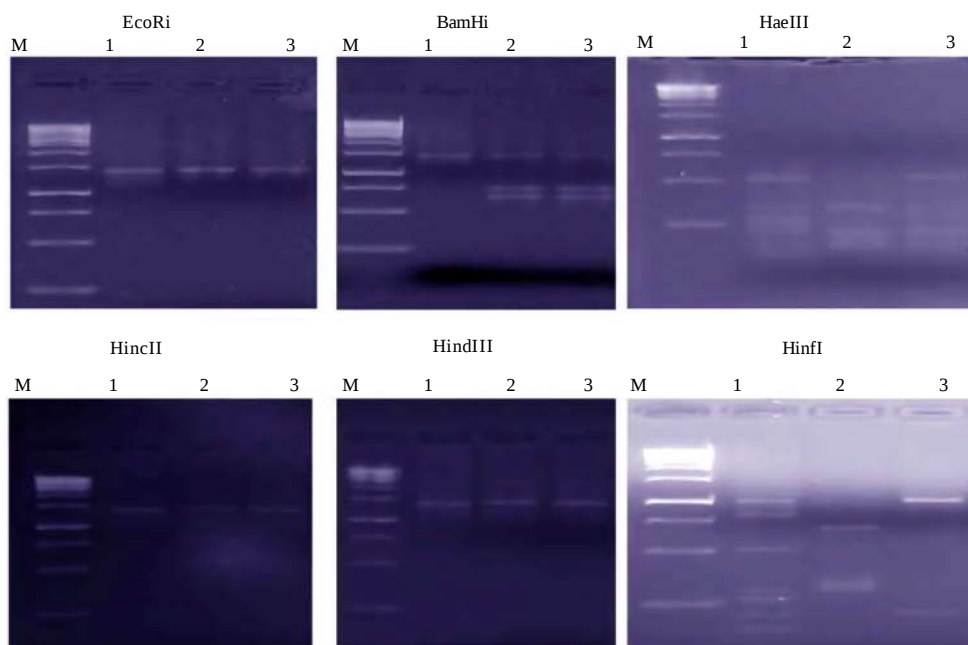
Fig. 2: Gel electrophoresis RFLP of 16S rDNA
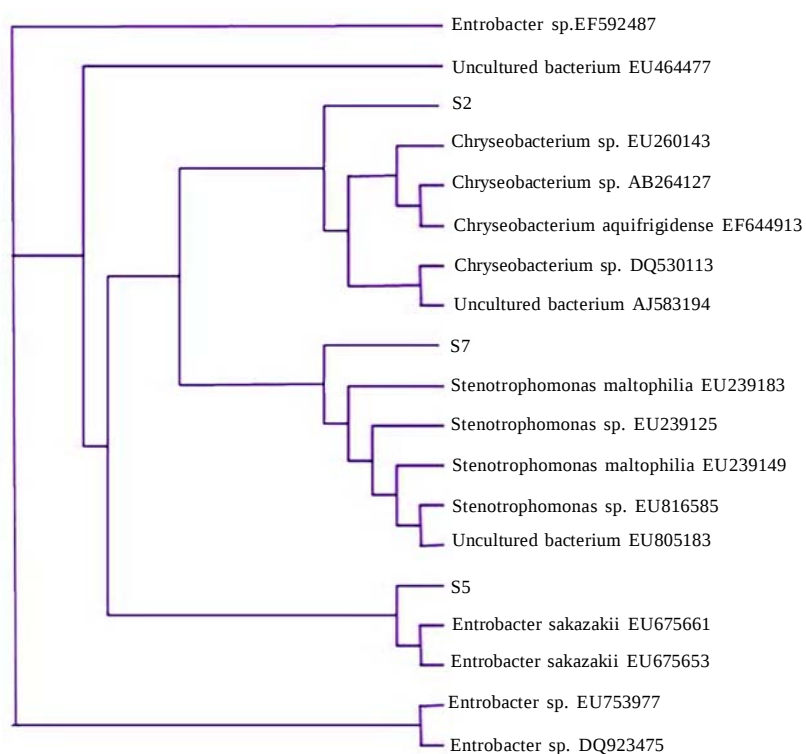
Fig. 3: Phylogenetic tree showing the relationships among selected isolates

can in short time (45 min only) show the response of the bioreporter strain DF4/PUTK2 to the toxic effect of certain pollutants. Similar bioassay methods were also based on microbial bioassays and biosensors were presented elsewhere (Abd-El-Haleem *et al.*, 2006; Zaki *et al.*, 2008). In present study, from the results obtained in Fig. 5 represent that strains S5 and S7 were the most potent for Cu biosorption so we selected them for bioluminescence assay. Fig. 6 shows that the bioluminescence of the bioreporter strain DF4/PUTK2

was decreased with increasing the concentrations of Cu from 25-250 mg/L. The results show that the bioluminescence inhibition of strain (S5) reached to 91.4 % as compared with the strain (S7) reached to 83.3 % at 225 mg/L of Cu ion. Finally, the data presented in this research indicated that with the use of metal resistant bacteria, the bioremediation of heavy metals from effluents of the factories can considerably be enhanced and the disposal problems of the waste with little expense is improved.
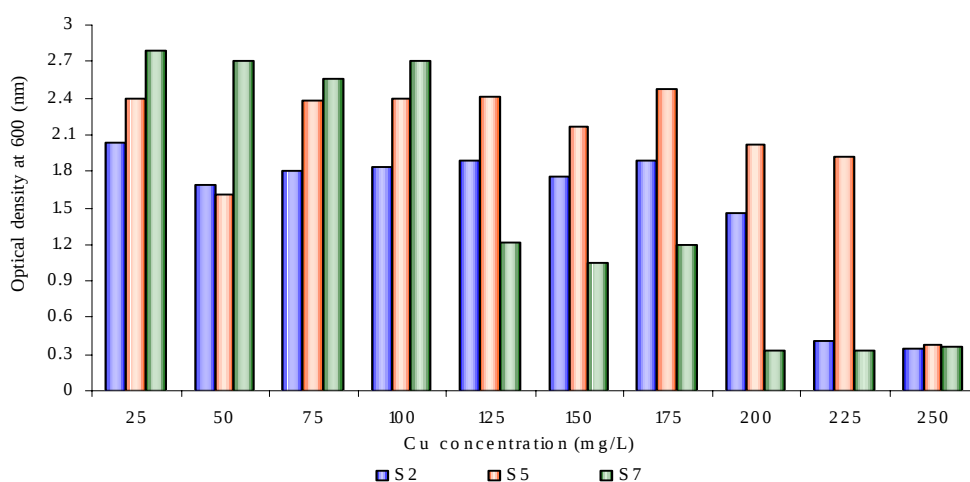
Fig. 4: Effect of various concentrations of Cu (II) (mg/L) on the growth of strains S2, S5 and S7
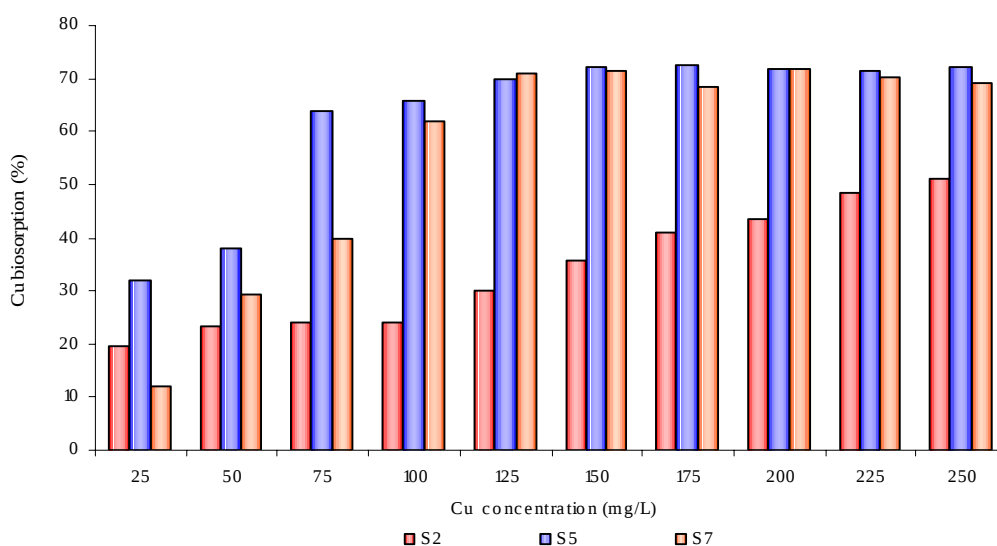
Fig. 5: Influence of initial Cu (II) concentrations on the rate of copper biosorption by S2, S5 and S7
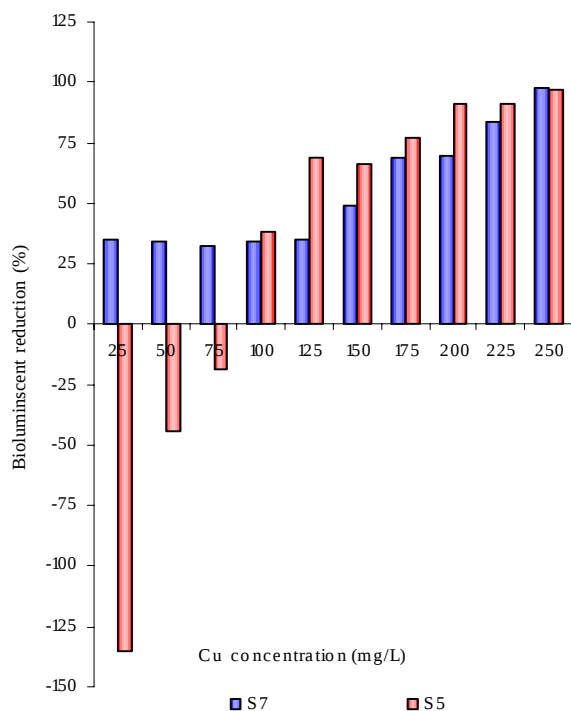
Fig. 6: Bioluminescent reduction efficiency

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