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BIOACCUMULATION AND BIOSORPTION OF COPPER BY *PSEUDOMONAS* SPECIES

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Abstract

The toxic heavy metals resistance, their accumulation and biosorption by bacteria is a wide spread phenomenon that could be exploited for the betterment of global environment. This study describes investigation of metal resistant bacterial strains isolated from polluted industrial sites in Karachi. Biosorption and bioaccumulation were studied with reference to the cadmium, copper and chromium. The isolates were identified by 16S ribosomal gene analysis and by API kits. The *Pseudomonas* species were more prevalent, showing multiple metal resistances to copper, chromium, cadmium, nickel, zinc and cobalt salts. Maximum accumulation and biosorption of cadmium, copper and chromium was found in CMG64, CMG462, and CMG463, respectively. The biosorption and bioaccumulation were periodically monitored and the concentrations of metal were estimated by atomic absorption spectrophotometer (AAS) and by enzyme assays. The localizations or deposition of heavy metal inside/outside of the cell surface were further confirmed by electron microscopy and by energy dispersive x-ray analysis (EDX). These microbes are good candidates for bioremediation purposes.

Introduction

Prevalence of heavy metals in effluent is a major cause of environmental damages. The most prevalent ones include, cadmium, chromium, barium, copper, iron, manganese, lead, nickel and zinc. In bacteria toxic heavy metal resistance systems have been reviewed over the last decade (Brown, 1985; Foster, 1987; Misra *et al*., 1984; Silver, 1981; Mergaey, 1985). The resistance mechanisms against all these heavy metals are highly specific. There is no general mechanism for resistance to all heavy metals. Bacteria can physically remove heavy metals from solution through either bioaccumulation or biosorption (Gadd, 2000; Xaing, 2000; Badar *et al*., 1999). Bioaccumulation plays an important part in the detoxification of hazardous metals. The uptake of hazardous metal ions onto cell surfaces and their subsequent translocation into the cell are well known natural processes but are highly specific (Hughes & Poole, 1989). Microbial cells can intracellularly and extracellularly accumulate both essential and non-essential metals such as chromium, cadmium, copper, nickel, lead, iron, germanium, silver and zinc. Several species of bacteria have been reported for the accumulation/uptake of various metal e.g., *Citrobacter* species accumulated cadmium and uranium (Macaskie and Dean. 1984; Macaskie and Dean. 1987b; Macaskie, *et al*., 1988), *Pseudomonas syringae* accumulated copper (Cooksey & Azad, 1992), *Pseudomonas stutzeri* accumulated germanium (Dyke *et al*., 1990) etc.

Biosorption does not consume energy. Positively charged metal ions are sequestered primarily through the absorption of metal ions to the negative ionic groups on cell surfaces (Badar *et al*., 2000), such as coating of polysaccharide found on most forms of bacteria, or other extracellular structures such as slime layer or capsules. Metallic cations are attracted to the negatively charged sites at the surface of the cell (Beveridge & Murray, 1980). Anionic ligands such as phosphoryl, carboxyl, sulfbydryl, and hydroxyl group of membrane proteins also involve in metal binding to the cell surface (Volesky, 1990).

These processes are applied to clean the effluents, contaminated ground waters and soils. For the development of this technology microorganism especially bacteria are of great importance. They have the ability to reduce the toxicity of metals; this ability of bacteria can be harnessed in biotechnological applications for the removal/control of excess metal in various environments such as industrial and other wastes.

MATERIALS AND METHODS

Bacterial isolates and growth conditions: CMG64, CMG462, CMG463, local isolates, were used in this study. Nutrient broth (Oxoid) was used as a starter medium. Maximum tolerable concentrations of various heavy metals were estimated in tris-minimal medium (Mergaey, 1985).

Identification of bacterial strains: The isolates (CMG462 and CMG463) were identified using partial l6S rRNA gene analysis. A small colony of each was suspended in 0.1 ml of deionised water (DW), mixed well and heated at 70°C for 10 mins for cell lysis. The cell lysate (0.2 µl) was added to sterilized deionized water (0.0 198 ml) and this lysate is used as a template in a polymerase chain reaction using the eubacterial 16S targeted PCR primers (pA and pH') as designed by Edwards (1989). These are known to amplify a 1,536 base pair (approx. 1.5 kb) length of 16S rDNA. The reaction mixture for amplification was as published by Bruce *et al*.,

(1992). For PCR MJ thermalcycler (Mi Research Inc., USA) was used under tube temperature control and using 30 cycles of the following program: 94°C for 40 sec, 55°C for I min, 72°C for 2 min and a final 10 min extension at 72°C. PCR products were cleaned using Sephacryl S400 columns (Pharmacia, Sweden), and partially sequenced using I6S sequencing primer 943 reverse (Lane *et al.*, 1985) by Alta Biosciences (University of Birmingham, UK). Sequences (up to 650 bp) were analysed using ADVANCED BLAST software to access the EMBL database, Heidelberg, Germany (Web ref: l-ITTP://www.ncbi .n lm.nih.gov/cgib in/Blast). Netscape browser interface was used. The isolate CMG64 was identified by API- kit.

Heavy Metal Resistance: To find out the maximum tolerable concentration (MTC) of metal salts such as $CuSO_4$, $NiCl_2$, $Pb(CH_3OO)_2$, $ZnSO_4$, Cr_2O_7, $CoCl_2$, and $CdCl_2$, bacterial culture were streaked on tris mineral medium plates with variable salt concentrations. The plates were then placed in an incubator at 37°C and growth was observed after 24-48 h.

Accumulation of copper: The accumulation of heavy metals was assessed by growing bacterial cultures in tris minimal broth. The 50ml tris minimal broth in 250ml flask supplemented with variable concentrations of metal salts such as cadmium and copper were inoculated with 1ml of pre grown culture and placed in shaking incubator (100rpm) at 37°C. Each day samples were collected for estimation of copper using copper assay and total cell protein content by protein assay using protein test kit (Sigma: TPRO 562).

Biosorption of copper: Biosorption of copper was estimated by suspending cells of CMG462 and CMG463 in saline water supplemented with 1mM $CuSO_4$. Loss of copper from solution was estimated after 5mins, 1h, 3h and 24h of time intervals by copper assay.

Copper assay: Copper was assayed by a method described by Macaskie (1995) with little modifications.

Transmission electron microscopy and energy dispersive x-ray analysis: Bacterial pellets were harvested after 48 h, washed with distilled water and fixed by immediate resuspension in glutaraldehyde (2.5% vol/vol) dissolved in sodium cacodylate (0.1 M) buffer having pH 7.2. The cells were treated for dehydration in an ethanol series (70, 90, and 100% ethanol: 15 min each), twice with propylene oxide (15min) and then in a mixture of propylene oxide/epoxy resin (1:1; 45min). Samples were then embedded in epoxy resin under vacuum in plastic moulds (20 min) and left to polymerize at atmospheric pressure (24 h, 60°C). Sections (70 nm) were cut, collected on copper grids/aluminium grids and examined by transmission electron microscopy. For energy dispersive X-ray analysis (EDX) thicker sections (200-300 nm) were cut and examined by scanning transmission electron microscopy (JEOL JEM-100CXII) using a LINK ISIS X-ray analyzer to determine elemental distribution in/on and around the cells.

RESULTS AND DISCUSSIONS

Bacterial strains: Three isolates were homologous to strains of *Pseudomonas* (Table 1) CMG462 and CMG463 were identified as *P. stutzeri* by 16S rRNA analysis. These *pseudomonads* were 99% similar to the matching sequences, while CMG64 was identified as *Pseudomonas aeruginosa* by API kits (Table 1).

Table 1. Source of bacterial isolates used in this study.

Strain code	Bacteria	Source
CMG462	*Pseudomonas stutzeri*	(Foundry soil, Karachi Shipyard and Engineering works)
CMG463	*Pseudomonas stutzeri*	(Foundry soil, Karachi Shipyard and Engineering works)
CMG64	*Pseudomonas aeruginosa*	Korangi Industrial Area, Sector 7-A, Karachi

Heavy metal resistance: The metal resistances were studied in tris-based medium because the complexation with heavy metals is minimum therefore the shown metal concentration is approximately the free metal concentration (Mergaey *et al*., 1985).

All the isolates of this study were originated from various metal contaminated sites of Karachi, Pakistan and showed multiple metal resistances (Table 2). The isolates CMG462, CMG463 and CMG64 showed resistance against cadmium chloride up to 2 mM whereas CMG462 and CMG463 exhibited highest resistance against copper i.e., 8 mM and 10 mM respectively with respect to other tested heavy metal salts in this study. The multiple metal resistance ability suggested the prior exposure of the isolates to these metals, which are present in the sampling sites; this phenomenon of multiple metal resistances has been reported by many workers.

Table 2. Maximum tolerable concentrations (MTC) of different metals.

Strain code	Metal concentration (mM)					
	$CdCI_2$	$CuSO_4$	$ZnCl_2$	Cr_2O_4	$CoCl_2$	$NiCl_2$
CMG462	2	8	0	1.5	0.5	0.5
CMG463	2	10	0.5	1.5	0.5	0.5
CMG64	2	1.5	2.5	-	2	2

Accumulations and biosorption of copper: One of the potential metal resistance mechanisms is accumulation or uptake of metal by bacterial cell. Metal accumulation of cadmium by CMG64 and copper by CMG462 and CMG463 was studied. Results (Table 3) show 40% of cadmium accumulation by CMG64, whereas CMG462 and CMG463 accumulated copper 90.7% and 97.4% respectively. Accumulation might be due to the presence of extracellular components such as proteins or polysaccharides. Falla & Block (1993) have reported that polysaccharide-producing strains are active metal accumulators. It is reported that the resistant strain have well developed mechanisms to prevent the shock, such as in *Pseudomonas* which accumulates metal in the periplasmic space that prevents the entrance of an excess amount of metal into the cytoplasm (Cooksey, 1990).

The observations of cell sections using TEM with energy dispersive X-ray analysis showed metal accumulation in these strains. Intracellular accumulation of cadmium in CMG64 was confirmed by TEM, which showed dark precipitates when bacterial cells grown in 0.1 mM cadmium while no precipitates were observed in absence of cadmium. CMG462 and CMG463 showed accumulation/removal of copper from media while growing at 1 mM $CuSO_4$. The concentration of copper removal was estimated by copper assay and calculated by copper standard curve. The intracellular/extracellular accumulation or the localization of copper was observed under electron microscope. Accumulation of copper from the medium was estimated through its removal form the medium which revealed the resistance mechanism. On the basis of highest percentage of copper removal, CMG463 would be selected for bioremediation studies i.e., for the development of biofilters.

Table 3. Accumulation of metals in tris-minimal medium.

Strain code	Metal salt	Cone. (mM)	Metal accumulation (%)
CMG64	$CdCI_2$	0.1	40.0
CMG462	$CuSO_4$	1.0	90.7
CMG463	$CuSO_4$	1.0	97.4

The localization of copper was determined in stained and unstained cells (Fig. 1). In CMG462 it was observed that the dark patches inside the cells i.e., in the cytoplasm and the darkly stained outer membrane was seen in the cells grown with copper (Fig. 1A, 1C). The EDX analysis also revealed the occurance of copper in the cell as well as at cell edges or the outer cell membrane (Fig. 2). In contrast with CMG463, the copper was bound with the cells extracellularly which was clearly observed in unstained cells (Fig. 1E). In EDX analysis the copper at the outer surface was extremely low or undetectable while it was detectable intracellularly (Fig. 2). Accumulation of copper has been observed in several species of *Pseudomonas* as well as copper resistant *Rhizobium loti* (Macaskie *et al*., 1988). Accumulation of copper from the medium was estimated through its removal form the medium that revealed the resistance mechanism. On the basis of highest percentage of copper removal CMG463 was selected for bioremediation studies i.e., for the development of cost effective bioreactors or filters.

Biosorption studies were conducted by using resting cell cell suspension of CMG462 and CMG463. The resting cell suspension of both the strains CMG462 and CMG463, showed immediate biosorption i.e., after 5min 66.7µM and 76.7 µM copper was lost respectively and most of the copper was removed after 24hr (Table 4).

The presence of multiple metal resistances suggested that these microbes are good candidates for exploitation of decontamination of cadmium, chromium, copper, cobalt and nickel contaminated sites.

Table 4. Biosorption of copper by resting cell from aqueous solution having initial concentration of $CuSO_4$ is 100 µM.

Strain code	Biomass (mg/ml)	Loss of copper (µM)			
		5mins	1 hrs	3hrs	24hrs
CMG462	0.5	66.7	64	97.77	97.39
CMG463	0.5	76.67	52	95.53	99.63

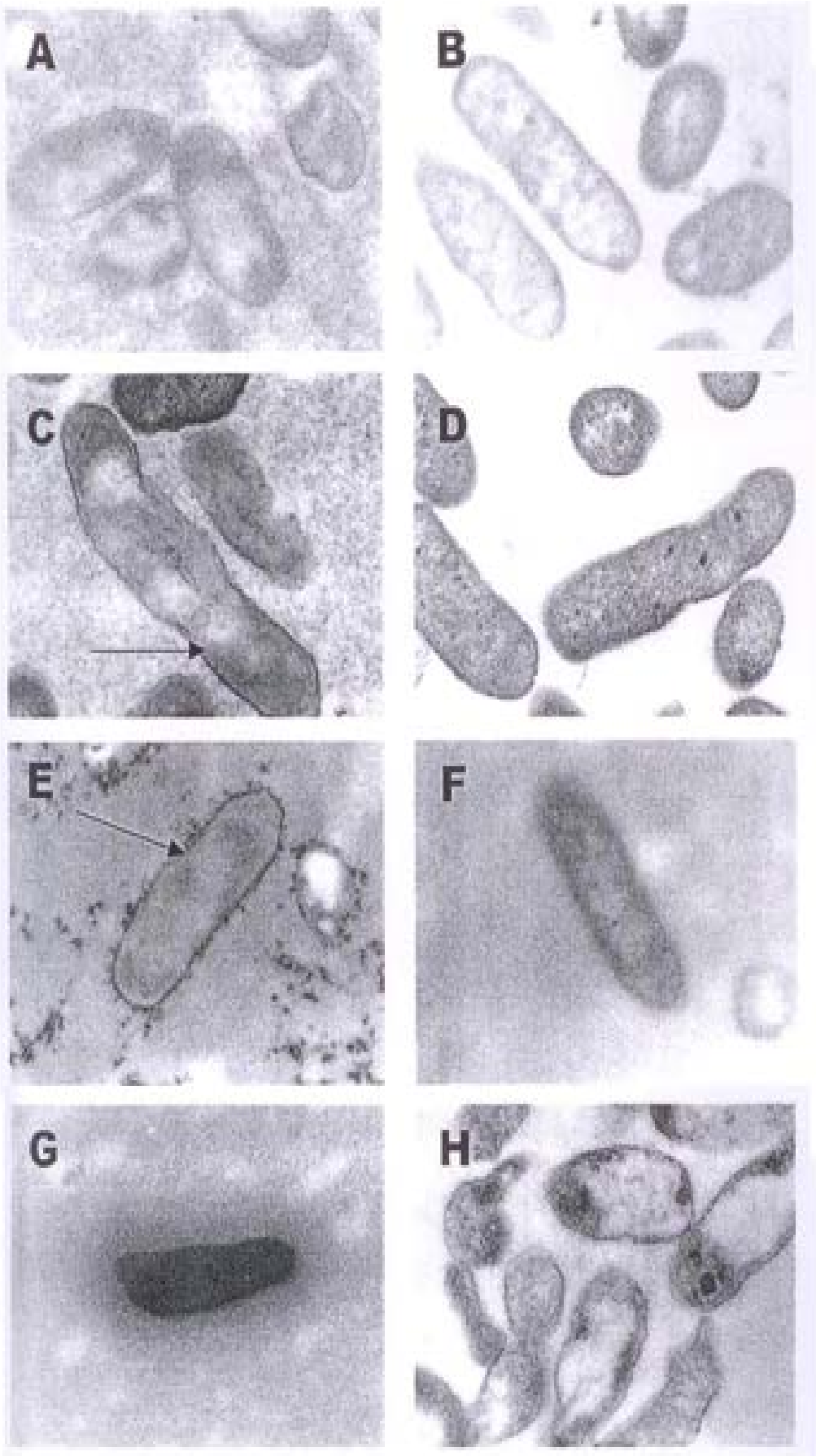

Fig. 1. Transmission Electron Micrographs (TEM). TEM of CMG462 (ABCD) and CMG463 (EFGH). ACEG showing bacterial cells grown in Copper supplemeted medium whereas BDFG, cells grown in copper un supplemented medium. ABEF are unstained cells, CDGH are stained cells. Arrow repreent electron opaque materials. Bar are 200nm.

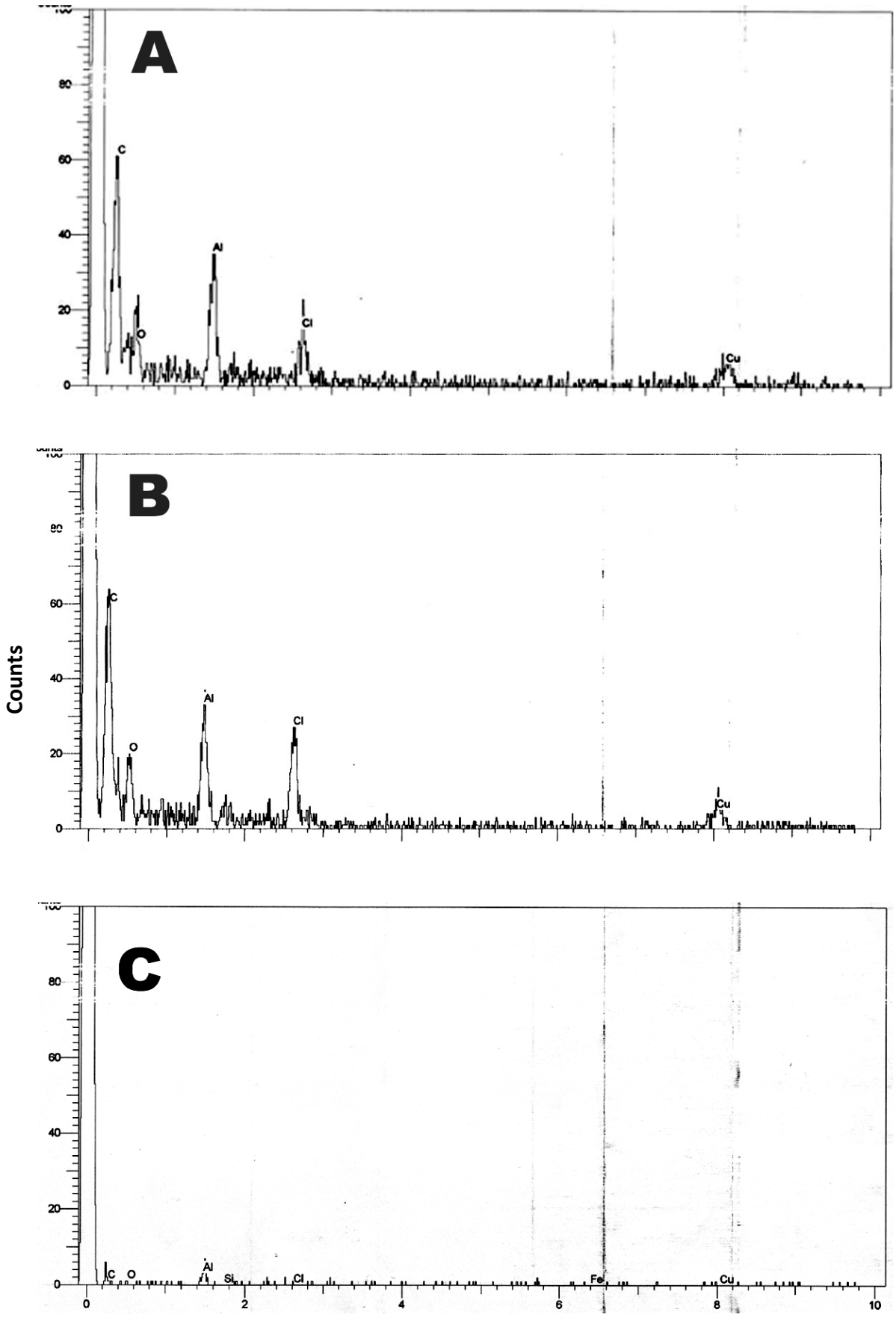

Fig. 2A. Energy Depressive x- ray Analysis of electron opaque materials CMG462 grown in presence of copper sulphate. A, analysis inside the cells, B, analysis at cell surface and C, control.

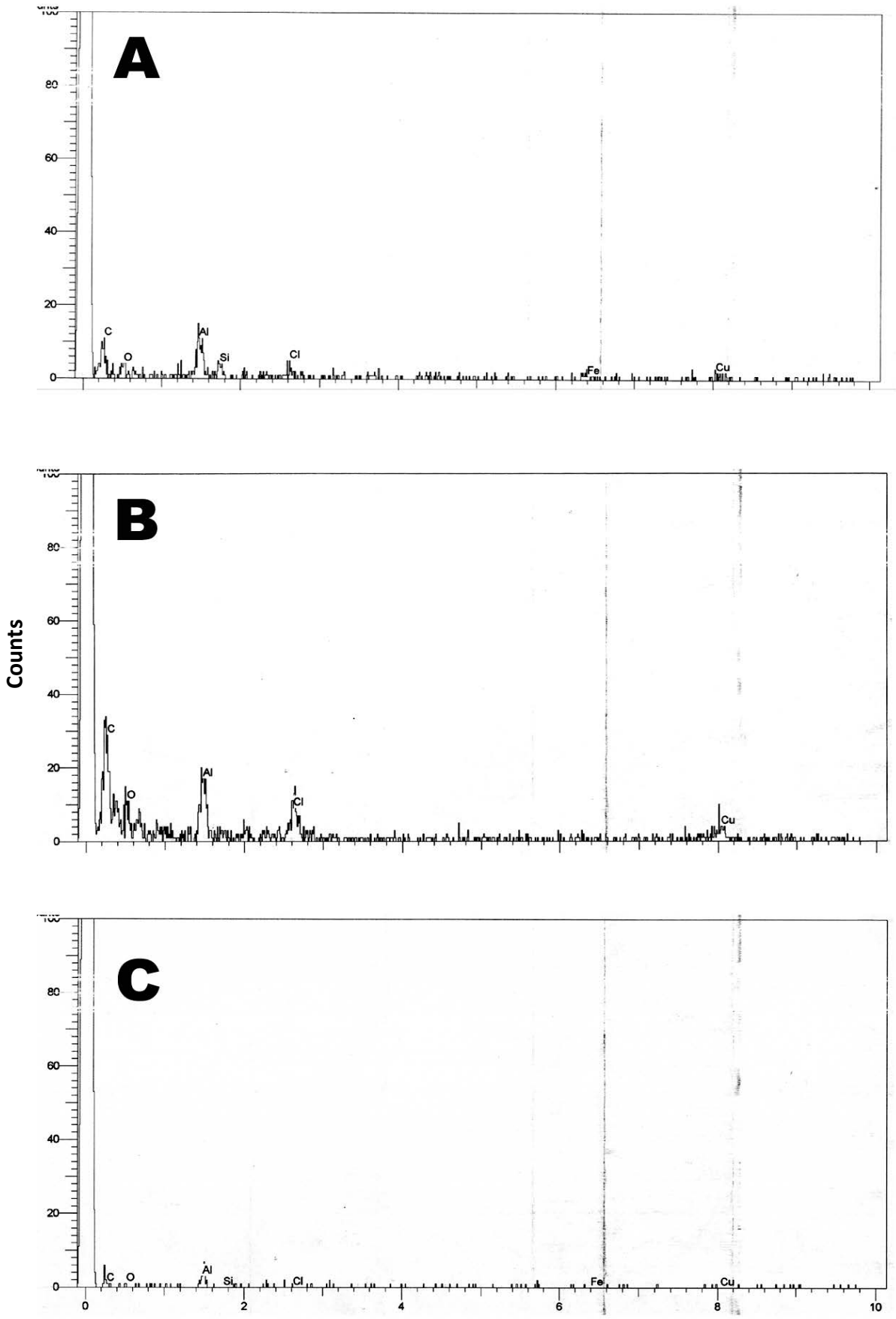

Fig. 2B. Energy Depressive x- ray Analysis of electron opaque materials CMG463 grown in presence of copper sulphate. A, analysis inside the cells, B, analysis at cell surface and C, control.

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