

TOLERANCE AND IMMOBILIZATION OF COBALT BY SOME BACTERIA FROM FERROMANGANESE CRUSTS OF THE AFANASIY NIKITIN SEAMOUNTS

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Bacteria isolated from cobalt enriched ferromanganese crusts on the Afanasiy Nikitin Seamounts in the Equatorial Indian Ocean were examined for their ability to tolerate, and immobilize cobalt in unamended seawater and seawater amended with 0.01% glucose. Retrievable bacterial counts in the form of CFU (colony forming units) on media supplemented with 1 mmol Co l⁻¹ (58 mg Co l⁻¹) and 1 mmol Mn l⁻¹ (54 mg Mn l⁻¹) were in the range of 1.71x10⁴ to 1.05x10⁵ gm⁻¹ (wet wt) of crust, respectively. Most of the isolates (14/24) were pigmented and showed taxonomic affinities to *Flavobacterium sp.* Two representative isolates were tested for their tolerance of cobalt. We observed that in amended medium, the isolates tolerated upto 1 mmol Co l⁻¹, whereas in unamended medium they tolerated upto 10 mmol Co l⁻¹. Microscopic observations of cultures incubated with 10 mmol Co l⁻¹ showed the occurrence of an extracellular slime layer, which may be responsible for immobilizing the cobalt from the liquid phase. In the unamended medium, the tolerance and stimulation in total cell counts was similar to that in amended medium or sometimes greater. Total cell counts peaked at 100 μmol Co l⁻¹ for incubations in unamended medium (1.1-2.5x10¹¹ cells l⁻¹) and at 0.1-1 μmol Co l⁻¹ for incubations in amended medium (1.5-2.6x10¹¹ cells l⁻¹). Counts of formazan-stained respiring cells of both the isolates in the unamended medium reached up to a maximum of 2.9–7.8x10¹⁰ l⁻¹ after incubation for 10 days at 23(± 1)^oC. In the amended medium cell counts of respiring cells attained a maximum in the range of 4.6-15.8 x10¹⁰ l⁻¹ at 100 μmol Co l⁻¹. The Co immobilization rate was on average 82 (± 87.9, n = 24) μmol of Co d⁻¹. Since the isolates were naturally occurring bacteria from crusts, they could be more environmentally acceptable and safe if used for metal recovery and bio-leaching.

Keywords Cobalt, tolerance, immobilization, ferromanganese crust

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Introduction:

Ferromanganese crusts containing on an average > 1% of cobalt can be designated as cobalt crust. They appear as slabs capping the seamounts at shallow water depths (< 2500m). The Afanasiy Nikitin Seamount in the Equatorial Indian Ocean have been studied by Banakar et al. (1997) to elucidate the palaeoceanographic conditions during the genesis of the cobalt enriched ferromanganese crusts. Recently, Rajani et al. (2005) reported the compositional variation in relation to the hydrography of the region. At abyssal depths, cobalt may occur in ferromanganese nodules.

All microbes, whether prokaryotic or eukaryotic, employ some metal species for structural functions and/or catalytic functions (Ehrlich 1997). Some microbes may form metabolic products, such as acids or ligands, which have the capacity to dissolve base metals such as Fe, Cu, Zn, Ni, Co and others contained in minerals (Ehrlich 1996). In metal-microbe redox interactions, the metal ion may serve as the electron donor or acceptor depending on its oxidation state. Iron and manganese ions have well-established roles in chemosynthesis and anaerobic respiration. (For e.g. *Sulfolobus acidocaldarius* and *Acidianus brierleyi* are able to obtain all their energy for growth from the oxidation of Fe (II) to Fe (III)). The use of metals or metalloids as electron donors or acceptors in energy metabolism of eukaryotes is unknown (Ehrlich 1996). Although the role of cobalt as an electron source or acceptor in bacteria is not so well understood, it is essential as a trace nutrient that is required by all organisms. The levels at which it occurs rarely exceed the limits of tolerance. Lee and Tebo (1994) suggest that some microbes may directly oxidize Co (II). Moffett and Ho (1996) have reported that cobalt uptake onto suspended particles in Waquoit Bay was dominated by microbial oxidation. Some important mechanistic similarities between Mn and Co oxidation by fungi and bacteria were described by Souren (1998) and Moffet (1998).

A toxic metal species may be converted to a less toxic or non-toxic entity by enzymatic oxidation or reduction (Wang and Shen 1995). This process may be linked to respiration or de-linked, as in the case of mercury resistance (Robinson and Tuovinen 1984). Microorganisms play important roles in the fate of metals in the environment by employing different physio-chemical and biological mechanisms to bring about transformations between soluble and insoluble phases.

In nature, noticeable microbial interaction with metals frequently manifests itself through metal immobilization and mobilization (Ferris et al.1989; Ghiorse and Ehrlich 1992; Ehrlich 1996). Metal immobilization may be through cellular sequestration and accumulation, or through extra-cellular precipitation. Metal mobilization results from dissolution of insoluble metal-containing phases. These processes are central in controlling biological availability of metals in sediment and water (Ehrlich and Brierley 1990). Sedimentary metal oxides and sulfides include extracellular metal accumulations that result from respiration or biosorption (Schultze-Lam et al.1996).

The geochemical cycling of cobalt has been considered to be controlled by the scavenging and oxidation of Co(II) on the surface of manganese oxides (Moffett and Ho 1996; Lee and Tebo 1994). The aim of the present study is to quantify the tolerance limits of the native bacterial flora to cobalt in the aqueous phase and delineate the ability of the bacterial flora to scavenge the ions.

Material and Methods:

Sample collection and processing:

A chain bag dredge was used to collect cobalt crust samples from Afanasiy Nikitin Seamounts in the Equatorial Indian Ocean during the cruises CC1 and CC2 on February and July 2003, respectively, on board *A. A. Sidorenko*. The crust samples were transported to the lab in sterile polyethylene bags under refrigeration. For experimentation, about 1-2 gm of crust was rinsed with sterile seawater (100ml) and pulverized by mechanically crushing using a clean sterile mortar and pestle. The crushed material was aseptically transferred to dilution blanks. It was then suspended by vigorous shaking for two minutes and allowed to settle for one minute before withdrawing aliquots for inoculation.

Inoculation, enumeration, isolation and identification:

Culturable bacteria associated with crust were enumerated in the form of colony forming units (CFU). Inocula of 100 µl were surface plated onto diluted nutrient agar (diNA i.e. 25% nutrient broth + 2% agar) and full strength nutrient agar (fNA i.e. 100% nutrient broth + 2% agar). A concentration of 100% corresponds to 8g nutrient broth (HiMedia

Laboratories Pvt Ltd. Bombay, India) per 1000ml seawater. Cobalt ($1 \text{ mmol Co l}^{-1} = 58 \text{ mg Co l}^{-1}$) and manganese ($1 \text{ mmol Mn l}^{-1} = 54 \text{ mg Mn l}^{-1}$) supplemented diNA (pH 7.2) was used to enumerate culturable cobalt and manganese tolerant bacteria separately. Counts of colonies formed on the medium by bacteria associated with particles of crushed ferromanganese crust were recorded after a period of 15 days of incubation at $23(\pm 1)^{\circ}\text{C}$. Representative cultures representing different colony morphologies on the plates were isolated. Each isolate represented a definite fraction of the plate count having similar colony and cell morphology. Isolates were identified by standard biochemical tests (Oliver 1982).

Cobalt tolerance:

Experiments were conducted to estimate the limits of cobalt tolerance. Two dominant representative isolates (Table 1) CC10a (Cobalt Crust isolate no. 10a) and CC23b were tested for their tolerance of cobalt (cobaltous chloride hexahydrate salt) added to medium at concentrations of 1,10 and 100 nmol of Co l^{-1} ; 1,10 and 100 $\mu\text{mol of Co l}^{-1}$; and 1,10 and 100 mmol of Co l^{-1} . Henceforth incubations with cobalt will be referred to as '+Co' and those without cobalt addition shall be referred as '-Co'. The cultures were microscopically examined to check their purity and measured inocula spread on plates of diNA medium and incubated for 48 hours at $23(\pm 1)^{\circ}\text{C}$. The cells were then scraped and suspended in sterile physiological saline (100ml). The suspension was centrifuged at 5000 r.p.m for 15 minutes. Cell pellets were washed twice with sterile saline and resuspended. Direct cell counts in a cell counting chamber were used to measure the inoculum concentration. A volume of 100 μl of inoculum contained $2.7\text{-}3.8 \times 10^{10}$ cells l^{-1} . Experiments were conducted with 10ml of media prepared with seawater containing the concentrations of cobalt mentioned above. The tests were run in triplicate at pH 7.2 in the presence (+ G) and absence (-G) of glucose (0.01%). Triphenyl tetrazolium chloride (TTC) (0.025%) was used to enumerate respiring cells, which convert colourless TTC to red Triphenyl formazan (Lenhard, 1956). The sizes of formazan crystals were measured using a calibrated ocular micrometer. The Cell counts (respiring and total) were done using a cell counting chamber after an incubation of 10 days. The culture was spun at 5000 r.p.m for 15 minutes and a representative portion of the cell pellet was examined microscopically under a bright field

(Olympus BHF –342) microscope. Another portion of the cell pellet was dried overnight at 37°C and observed under Scanning Electron Microscope (JEOL- JMS-5800LV)

Cobalt immobilization:

We chose the Co concentration of 10 mmol l⁻¹ from our previous experiments to examine the capacity of our test cultures to immobilize cobalt. All of the 24 isolates were examined. Inocula of the isolates were prepared as described above. After 37 days, the cultures were spun at 5000 r.p.m for 15 minutes. The supernatant was analyzed spectrophotometrically at 510 nm to estimate the residual cobalt (Stewart et al.1974). Immobilization rates were calculated based on the measurements of colour imparted by residual cobalt. The detection limit was 1 mmol Co l⁻¹.

Results

Bacterial enumeration, isolation and identification:

The retrievable counts in the form of CFU on both cobalt and manganese supplemented media were 1.71 (± 1.2) x10⁴ and 1.05(± 0.5) x10⁵ gm⁻¹ (wet weight) of crusts, respectively. On diNA and fNA media the retrievable counts were 1.01 (± 0.65) x10⁵ and 5.27(± 3.62) x10⁴ gm⁻¹ wet weight of crust respectively.

Characteristics of the isolates are presented in Table 1. All of the isolates were short gram-negative rods (generally 1.3µ x 0.3µ). Most of the isolates (14/24) were pigmented and showed taxonomic affinities to *Flavobacterium sp.* They represented 33% to 84% of the total population on fNA medium and Co-supplemented diNA medium respectively. The rest showed affinities to *Pseudomonas sp.* and *Acinetobacter sp.* Both CC10a and CC23b resembled *Flavobacterium sp.* except that CC23b was motile.

Cobalt tolerance:

In the presence of cobalt, the total cell counts in ‘-G’ medium peaked at a cobalt concentration of 100 µmol Co l⁻¹, whereas in ‘+G’ medium the peak counts were observed at lower concentrations of cobalt (0.1-1µmol Co l⁻¹). Table 2A and Figures 1 A-D list the total and respiring counts over a wide range of cobalt amendments. In the presence of cobalt, the respiring cell counts peaked at a concentration of 100 µmol Co l⁻¹ (Table 2B).

No trend was noted in the proportion of respiring cells to total cells of either strain in response to different amounts of added cobalt in ‘-G’ and ‘+G’ medium. Microscopic observations of cultures on 10 mmol Co l⁻¹ showed very large formazan crystals approximately 10-20 times the size of the cells (Figure 2) and an extracellular slime layer (Figure 3)

Cobalt immobilization:

Immobilization tests (Table 3) showed that 17 of the 24 cultures were positive and exhibited an average immobilization rate of 82 (\pm 87.9, n = 24) $\mu\text{mol Co d}^{-1}$. The specific activity of the cultures varied from 0.02-0.76 $\times 10^{-12}$ $\mu\text{mol Co cell}^{-1} \text{ day}^{-1}$. CC23b exhibited the greatest capacity to immobilize cobalt.

Discussion

The retrievable counts on both manganese supplemented media and diNA were in the order of 10⁵ gm⁻¹ wet wt. of crust. However, the counts on cobalt supplemented media and fNA medium were in the order of 10⁴ gm⁻¹ wet wt. of crust. This difference seems to correlate with the oligo- and lithotrophic nutrient conditions prevailing on the seamounts. It is possible that manganese tolerant forms were more widespread than cobalt tolerant ones. The growth of both CC10a and CC23b was stimulated in terms of respective counts of respiring and total cells. The counts of respiring cells were enhanced by 200-250% at a concentration of 100 $\mu\text{mol Co l}^{-1}$ in both ‘+ G’ and ‘-G’ media at the end of 10 days. For both CC10a and CC23b the stimulation in total cell counts was greater (200-250%) in ‘-G’ medium than in ‘+ G’ medium (150%).

The tolerance limit for Co of cultures belonging to the *Flavobacterium* sp. incubated in ‘+G’ medium was 1mmol Co l⁻¹ whereas in ‘-G’ medium it was 10 mmol Co l⁻¹. This enhanced Co tolerance exhibited by pigmented bacteria agrees with earlier reports (Nair et al 1992). The peak in total cell count in ‘+ G’ medium was 35% (CC10a) and 85% (CC23b) lower than in ‘-G’ medium. The tolerance limits reported here are comparable to earlier reports (17mM of Co) by Bruhn and Wichlacz (1986) on the extreme acidophiles *Thiobacillus ferrooxidans* and the genus *Acidiphilium*. Lower concentrations of metal in

'-G' media seem to have a negative influence on cell counts of respiring and total cells. But in general the abundance of both respiring and total counts are proportional. The respiring cell count peaks appear to be less defined as compared to the total cell count peaks probably suggesting that percentage of respiring cells among the total counts did not show any trend with respect to the concentration of the metal used nor the culture. The percentage of respiring cell counts comprised about 26-49% of the total cell counts in amended incubations whereas it was 13-43% under unamended conditions. Cultures incubated with 10 mmol Co l⁻¹ showed an occurrence of extracellular slime, which may have been responsible for immobilizing the cobalt from the liquid phase for a period of 10 days. It was observed that the average size of red formazan crystals increased in size with an increase in cobalt concentration in the medium (Fig.4). The size of the crystals increased from 4 μ at nM/ μM concentrations to nearly 20 μ at mM concentration.

Tolerance of cobalt by gram-negative bacteria has been explained on the basis of transenvelope efflux. Resistance seems always to be linked to another heavy metal resistance, either to nickel (Liesegang 1993; Schmidt and Schlegel 1994) or to zinc (Nies et al. 1987). Hence it is probable that the cultures may respond in a similar way to enhanced levels of iron and manganese, which are dominant in cobalt crusts. Moreover, the occurrence of a slime layer indicates their potential for scavenging metal ions from the aqueous phase thereby immobilizing them. As these cultures represent a large fraction of our retrievable counts, it is possible that they play a significant role in the dynamics of cobalt in the seamount environment.

At present, the most important biotechnological applications or potential applications of metal/microbe interactions are in bioleaching or biobeneficiation of ores and in bioremediation of metal polluted sites and mineralization of polluting organic matter (Ehrlich 1997). This takes on increasing significance for metals like cobalt. Cobalt is a 'strategic' metal, vital for the manufacture of super-alloys in jet engines and other high performance applications. Recent discoveries of cobalt-rich nodules around the Cook Islands of the south Pacific have aroused particular interest among some mining companies (Clark et al. 1995).

The native bacterial flora of crusts tolerated cobalt up to 10 mmol Co l⁻¹ and immobilized it at a rate of 82 μmol Co d⁻¹. It may be possible to harness this feature in an environmentally acceptable and safe way for efficient metal recovery and bio-leaching.

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Table No.1 Characteristics of gram-negative short rods (~1.3μ x 0.3μ) isolated from cobalt crust

Culture No.	Media used for Isolation	CFU Morph*	Motility	Catalase	Oxidase	Oxidation (O)/Fermentation/No Reaction (NR)	Biochemical identity similar to:	Abundance (%) of the isolate among the total CFU #
CC1b	Co1	Small/pink/round	-	+	-	O	Flavobacterium	48.83 (1.3)
CC3b	diNA	Small/brown/round	-	+	+	O	?	-
CC4a	Co1	Medium/brown/round	-	+	-	O	?	-
CC5a	diNA	Medium/yellow/round	+	+	+	O	Flavobacterium	43.07 (3.7)
CC5b	Co1	Medium/yellow/round	+	+	+	O	Flavobacterium	43.07 (3.7)
CC6	Co1	Small/pink/round	+	+	+	O	Flavobacterium	50.00 (2.4)
CC8a	diNA	Medium/yellow/round	-	+	-	O	Flavobacterium	65.62 (6.6)
CC8b	diNA	Medium/yellow/round	-	+	-	O	Flavobacterium	65.62 (6.6)
CC10a	Co1	Large/pink/irregular	-	+	-	O	Flavobacterium	82.89 (2.4)
CC10b	Co1	Large/pink/irregular	-	+	-	O	Flavobacterium	82.89 (2.4)
CC11	fNA	Large/white/irregular	-	+	-	O	Acinetobacter	22.22 (0.6)
CC14b	Co1	Small/red/round	+	+	+	O	Pseudomonas	70.58 (0.2)
CC18b	fNA	Small/white/irregular	+	+	+	O	Pseudomonas	11.11 (0.6)
CC23b	Co1	Large/pink/round	+	+	-	O	Flavobacterium	83.82 (11.3)
CC26b	Mn1	Large/orange/irregular	-	+	+	O	Flavobacterium	77.5 (3.5)
CC28	fNA	Large/light brown/irregular	+	+	+	O	Pseudomonas	35.71 (1.1)
CC30	fNA	Medium/white/irregular	-	+	-	NR	Acinetobacter	25.71 (2.8)
CC31	diNA	Small/orange/round	-	+	-	NR	Flavobacterium	64.28 (3.7)
CC32	Mn1	Small/dark brown/round	-	+	-	O	?	-
CC33	Mn1	Medium/dark brown/round	-	+	+	O	Pseudomonas	22.36 (1.8)
CC34	diNA	Small/pink/round	+	+	-	NR	Pseudomonas	34.88 (1.8)
CC35	fNA	Large/white/irregular	+	+	+	O	Flavobacterium	68.42 (0.6)
CC36	fNA	Large/light brown/irregular	-	+	+	O	Flavobacterium	33.33 (1.1)
CC37	diNA	Small/pink/round	-	+	-	O	Flavobacterium	36.84 (3.7)

fNA – 8g/l nutrient broth and 2% agar , diNA – 2g/l nutrient broth and 2% agar, Co1 – diNA with a final concentration of 1 mM Co, Mn1 – diNA with a final concentration of 1 mM Mn, *CFU diameter: Small: < 1mm, Medium > 1mm and < 3 mm, Large > 3mm, ? – Not identified, # Values in parentheses corresponds to CFU x 10⁴/gm wet wt. of crust corresponding to 100%

Table No.2A Highest cell counts at the end of 10 days on different media

Isolates	Total counts ($\times 10^{11}$ cells Γ^{-1})				
	+ G + Co		+G -Co	- G + Co	-G -Co
	100nM Co*	1 μ M Co*		100 μ M Co*	
CC10a		2.6	1.5	2.3	1.2
CC23b	2.2		1.5	2.5	1.1

+ Amended, - Unamended, G -Glucose (0.01%), * Concentration of cobalt in the test medium

Table No.2B Highest respiring cell counts at the end of 10 days on different media

Isolates	Respring counts ($\times 10^{10}$ cells Γ^{-1})			
	+ G + Co	+G-Co	- G + Co	-G-Co
	100 μ M Co*		100 μ M Co*	
CC10a	15.8	5.3	6.5	3.3
CC23b	9.8	4.6	7.8	2.9

Refer footnote of Table No.2A for legends

Table No.3 Immobilization rates of the isolates

Culture ID	Cobalt removal ($\mu\text{mol Co day}^{-1}$)	Specific activity: Cobalt removal ($\times 10^{-12} \mu\text{mol Co cell}^{-1} \text{day}^{-1}$)	Culture ID	Cobalt removal ($\mu\text{mol Co day}^{-1}$)	Specific activity: Cobalt removal ($\times 10^{-12} \mu\text{mol Co cell}^{-1} \text{day}^{-1}$)
CC1b	ND	-	CC18b	11.76	0.03
CC3b	96.56	0.19	CC23b	266.14	0.76
CC4a	223.74	0.39	CC26b	54.16	0.26
CC5a	96.56	0.36	CC28	107.15	0.45
CC5b	101.85	0.22	CC30	11.76	0.04
CC6	6.47	0.02	CC31	ND	-
CC8a	ND	-	CC32	170.75	0.40
CC8b	ND	-	CC33	101.85	0.41
CC10a	170.75	0.63	CC34	ND	-
CC10b	11.76	0.03	CC35	213.14	0.69
CC11	107.15	0.26	CC36	ND	-
CC14b	223.74	0.93	CC37	ND	-

ND Not Detected.

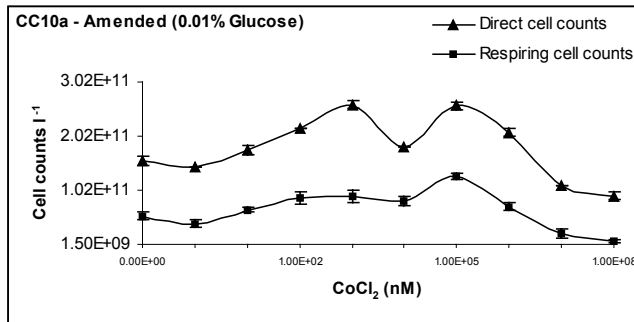


Fig.1A

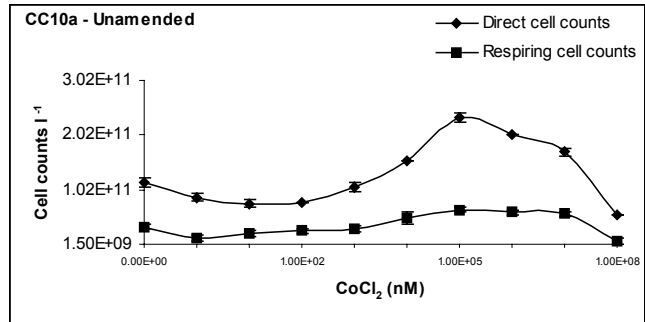


Fig.1B

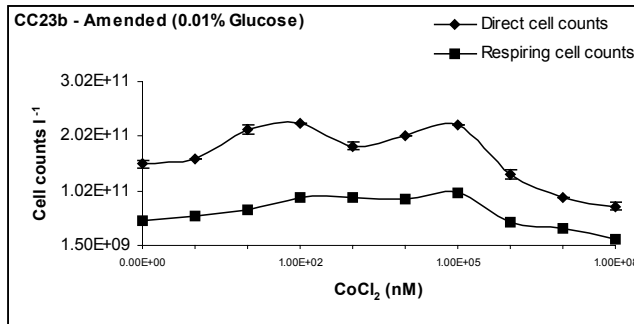


Fig.1C

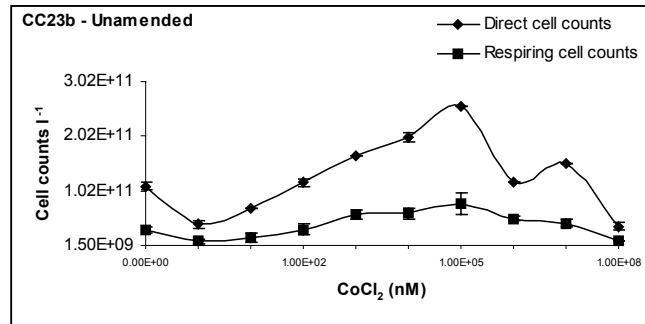


Fig.1D

Figure1: Effect of various concentrations of CoCl₂.6H₂O on the growth and respiration of the isolates CC10a and CC23b. (A,C) amended medium, (B,D) unamended medium. Error bars (±) represent standard error of the mean (n = 3).

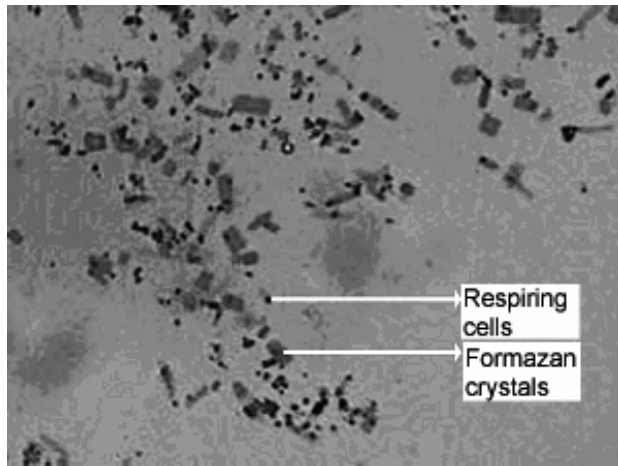


Figure 2 (40X)

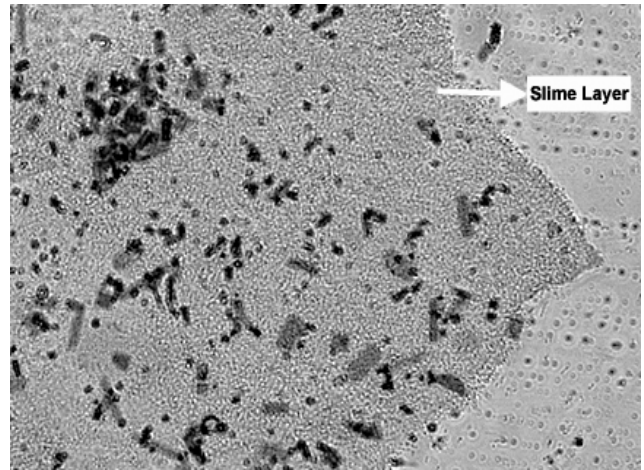


Figure 3 (40X)

Figure 2: Gray scale image of red formazone crystals, an index for respiratory activity along with respiring (red) cells of CC10a

Figure 3: Photograph of CC10a showing the occurrence of cells bound by an extracellular slime layer

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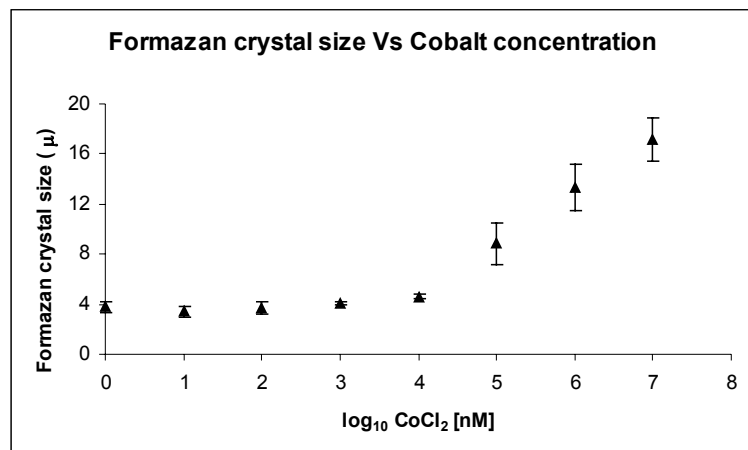


Figure 4: Effect of various concentrations of CoCl₂.6H₂O on the formazan crystal size. Error bars (±) represent standard error of the mean (n=40).