0273-2289/02/102–103/0359/$12.00

Screening of Thermoacidophilic Autotrophic Bacteria for Covellite Solubilization

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Abstract

In an attempt to obtain suitable bacterial isolates for bioleaching of copper from chalcopyrite and covellite, soil samples taken from areas of the metal industry were screened using an enrichment procedure specially run at acidic pH and thermophilic temperature range, to overcome the limitations of mesophiles employed for the purpose besides having economic and environmental advantages. Of a total of 47 isolates, the most promising 3 having resistance to copper toxicity were evolved by subjecting them to gradually increasing concentrations of $CuSO_4$ by acclimatization runs conducted on an environmental shaker for 125 d at 65°C. The isolates, JVCu-8, JVCu-10, and JVCu-12, exhibited significantly enhanced bioleaching and copper tolerance ability at pH 3.5 and 60–70°C. The total solubilization of copper recorded was 87, 89.4, and 91.2% by JVCu-8, JVCu-10, and JVCu-12, respectively, and these isolates exhibited tolerance to $CuSO_4$ concentrations of 6.9, 7.2, and 7.2%, respectively. The isolates morphologically resembled *Thiobacillus* and *Sulfolobus*.

Index Entries: Bioleaching; acidothermophiles; covellite and chalcopyrite solubilization; copper tolerant bacteria.

Introduction

Chemolithoautotrophic bacteria play a key role in the geobiochemical cycling of elements by the oxidation of reduced compounds such as sulfide, methane, hydrogen, and ammonia *(1)*. There have been interests in the microbial community in the metallurgical and mining biosphere. The environment of this biosphere is the extreme world under high pressure, high temperature, and high acidity, under nourishment of organic content, ranging from aerobic to microaerobic to anaerobic condition, and so on. Varieties of microbes were isolated from such a biodiversified ecosystem *(2)*. The microorganisms are able to populate in various extreme environments. This also holds true for natural or man-made ecosystems *(3)*. The industrial zone of Rajkot City in Western India has man-made ecosystems comprising

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various metallurgical manufacturing. The soil environment of such an industrial area has considerably higher concentrations of various metals such as Fe > Al > Cu > Ni > Cr > Pb > Ag > Au in descending order in the form of sulfide, sulfate, nitrate, and chloride than those of natural mine samples. The prolonged exposure of microbial habitat to such a man-made environment might have resulted in the development of appreciable metal tolerance and metal biotransforming potency to the various metals *(4)*.

Increased research efforts in hydrometallurgy have produced techniques that are now used commonly in the extraction of copper and uranium *(5,6)*. The technique for the extraction of recalcitrant chalcopyrite has not been refined. The thermophilic acidophilic bacterial strain from thermal water from Yellowstone National Park enhanced the extraction of molybdenum, uranium, and copper from mine ores *(7)*. Flask studies indicated that the thermophilic *Sulfolobus* more readily extracted copper from minerals having chalcopyrite than did *Thiobacillus ferrooxidans (8)*. Strain *Cryptococcus albidus* IFO 0378 T can grow in the presence of 10 m*M* $CuSO_4$ concentration as copper-tolerant yeast isolated from the Japan Trench *(9)*. A strain of *T. ferrooxidans* was adapted to grow at a higher concentration of copper by single-step culturing in the presence of 20 g/L (0.314 mol/L) of cupric ions added to 9K medium *(10)*. The original culture of *Sulfolobus* had a low tolerance for copper (i.e., 3 g/L), and by progressive acclimatization procedure tolerance could increase to 27 g/L with about 50% Cu extraction *(11)*. Especially in the case of bioleaching of copper, along with the solubilization, the toxicity of solubilized copper plays an inhibitory role in bioactivities *(12)*.

This article reports on various attempts to develop covellite- and chalcopyrite-solubilizing extremophilic bacterial strains from an artificial metallurgically active site of Rajkot City.

Materials and Methods

Sampling

Soil and mud samples were collected from a metallurgically active industrial area of Rajkot where native copper and ferrous have been dealt with for many years. The physicochemical analysis of collected samples was carried out with special reference to the nature of their location, pH, temperature, and qualitative and quantitative estimation of certain metals *(13)*. The microbiologic analysis of collected samples was also carried out. Microscopic examination for total counts of bacteria per milliliter of sample was done with Neuber's improved double-ruled chamber and differential staining reaction for morphologic studies and categorization. The sample yielding 12–18 cells per microscopic field with ×100 objective were selected for primary enrichment.

Primary Enrichment

The selected 16 samples were used as 10% inoculum in flasks containing 100 mL of 9K *(14)* and MJ medium *(15,16)* individually enriched with

1%CuS. These flasks were incubated at pH 3.0 and 65°C for 25 d at 150 rpm on an environmental shaker. The uninoculated blank flasks were kept under the same conditions to determine autooxidation of metal sulfides owing to elevated temperature and acidity.

After incubation, all 32 primary-enriched flasks were subjected to quantitative and qualitative microbial analysis and tested for biooxidation of metal sulfides into soluble $CuSO_4$ content titrimetrically using the method of Vogel *(13)*.

Secondary Enrichment

The first 18 primary enrichment flasks showing higher biooxidation were used as inoculum for secondary enrichment with special emphasis on enhancement of copper tolerance and sulfide solubilization by the copper adaptation test described by Le Roux et al. *(11)* with certain modifications as described next.

Copper Adaptation Experiment

From among the primary enrichment flasks a 10-mL quantity was transferred to 90 mL of 9K or MJ basal medium enriched with 1% CuS at pH 3.5 and 65°C at 150 rpm on an environmental shaker until 70% solubilization of placed sulfide occurred. The solubilization of sulfide and cell count per milliliter were determined at 120-h intervals.

The second transfer was given by inoculating 1 mL from the previous flask into 99 mL of basal 9K or MJ medium. $CuSO_4$ was added to the flasks in proportion to the quantity oxidized/solubilized in the previous set of flasks. Oxidizable substrate CuS was uniformly added to all the flasks at the 1% (w/v) level. The flasks were incubated at the aforementioned experimental conditions until 70% solubilization of copper sulfide occurred.

The stated experimental conditions were subsequently repeated several times with increasing concentrations of $CuSO_4$ as necessary for a period of 54 mo.

The procedure yielding four consortium flasks having the highest biosolubilization and copper tolerance were used for pure-line culture and tested for bacterial colonization. The consortium was extremely diluted by serial dilution technique to yield about 3–5 bacterial cells/0.1-mL quantity. These extremely diluted 0.1-mL aliquots were aseptically inoculated into the following:

1. Ten milliliters of MJ medium supplemented with 0.1% agarose, 0.5% sodium sulfide, and bromophenol blue as pH indicator for column culture development at 65°C for 20 d.
2. Five milliliters of MJ medium, pH 3.5, supplemented with 25 mg of CuS in 50-mL flasks with the fractionation of 20 µL of 0.1 mL of inoculum through a fraction collector. The flasks were incubated at 65°C until the maximum solubilization of placed metal sulfide occurred. Uninoculated blank flasks were run to determine the autooxidation index.

3. A modified fabricated slide culture chamber as per von Holy et al. *(17)* to yield a well-isolated single cell per calibrated square of slide chamber. The selected slides were incubated at 65°C by placing them in a humidified slide rack chamber and were periodically examined to determine single-cell multiplication through phase contrast microscopy.

From these experiments, 12 bacterial cultures were selected qualifying single-cell line multiplicity with desired copper tolerance and metal sulfide biosolubilization. The most typical three of them are described.

Results and Discussion

The selected samples from metallurgically active industrial zone of Rajkot contained considerably high contents of Cu, Fe, Zn, Pb, SO_4, NO_3, Cl, and so on (Table 1) than did natural mine ore samples. The quantitative bacteriologic analysis of the samples showed 87×10^2 to 25×10^3 cells/g of soil. Qualitative bacteriologic microscopic analysis revealed the presence of Gram-positive spherical and rod-shaped as well as Gram-negative spherical, short and long-rod shaped bacteria ranging from 3 to 18 cells per microscopic field under ×100 objective. The exposure to high concentrations of metals for prolonged duration probably had induced survival potency among the various microorganisms.

The primary enrichment cultivation qualified nine samples with 30–50% biosolubilization with a cell count of 10^8–10^{11} cells/mL after incubation for 25 d. The samples were recognized as positive only by yes/no type of assessment expressing desired copper sulfide biosolubilization in comparison to the autooxidation process, while the remaining sample would be conspicuous by the absence of interested microbial flora or their presence only in a very low number *(18)*.

During secondary enrichment, 18 flasks were exposed to copper adaptation test, which finally yielded four consortia flasks with a potency of 72–85% of CuS biosolubilization at pH 3.5, 65°C, and 150 rpm within 5 d.

All three techniques applied for colonization, cell isolation, and pure-line culture development proved equally satisfactory and 12 pure-line strains were selected (results are shown in Table 2).

The most outstanding strains, JVCu-8, JVCu-10, and JVCu-12, expressed enough biodiversity in terms of morphologic, cytologic, and physicochemical characteristics. The isolate JVCu-8 was Gram-negative, nonmotile granulated long rod-shaped bacteria occurring singly or in length of chain with two to three cells. They were capable of solubilizing 80% CuS and 80.12% $CuFeS_2$ with 6.9% $CuSO_4$ tolerance (Fig. 1). Strain JVCu-10 was Gram-negative, nonmotile, nongranulated, thin long-to-cylindrical rods occurring singly. They were capable of solubilizing 82.6% CuS and 82.8% $CuFeS_2$, with 7.2% soluble copper tolerance. Both these strains had no significant influence of Fe on its bioactivity and thus exhibited an Fe-independent direct CuS biooxidation mechanism. Strain JVCu-12 was

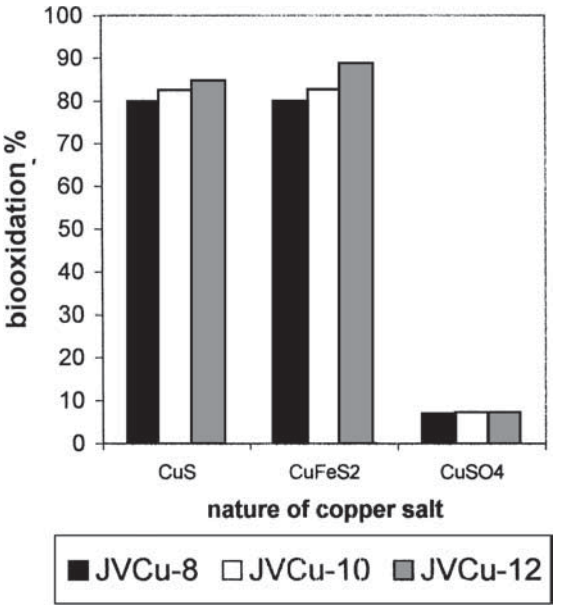
Table 1
Characterization of Sample

Site	No.	Nature	Temperature (°C)	pH	Metal elements present (ppm)
Gold refinery	1	Mud/soil	55	4.5	Cu: 1030; Fe: 740; Au: 57; Ag: 50; SO_4>NO_3>Cl
	2	Mud/soil	65	3.5	Cu: 1100; Fe: 580; Au: 78; Ag: 65; SO_4>NO_3>Cl
	3	Water	65	2.8	Cu: 960; Fe: 520; Au: 49; Ag: 48; SO_4>NO_3>Cl
Silver refinery	4	Mud/soil	60	5.8	Cu: 940; Fe: 520; Ag: 830; Zn: 640; NO_3>Cl
	5	Mud/soil	60	4.8	Cu: 920; Fe: 480; Ag: 960; Zn: 670; Ni: 375; Al: 468; NO_3>Cl
	6	Water	60	3.5	Cu: 840; Fe: 378; Ag: 857; Zn: 540; Ni: 225; Al: 350; Pb: 125; NO_3>Cl
	7	Water	62	2.8	Cu: 995; Fe: 430; Ag: 800; Zn: 450; Ni: 225; Al: 350; Pb: 120; SO_4>NO_3>Cl
Automobile spare parts foundries	8	Mud/soil	65	3.0	Cu: 1436; Fe: 1280; Al: 1840; Zn: 1520; Ni: 1450; Cr: 1050; Pb: 780; SO_4>Cl>NO_3
	9	Mud/soil	65	3.0	Cu: 1260; Fe: 1140; Al: 1650; Zn: 1310; Ni: 1240; Cr: 980; Pb: 620; SO_4>Cl>NO_3
Diesel engine alloys foundries	10	Mud/soil	85	3.5	Cu: 670; Fe: 6185; Al: 360; Zn: 310; Ni: 150; Cr: 150; Pb: 180; SO_4>Cl>NO_3
	11	Water	70	2.8	Cu: 540; Fe: 5800; Al: 360; Zn: 1310; Ni: 140; Cr: 210; Pb: 180; SO_4>Cl>NO_3
Diesel engine spare parts foundries	12	Mud/soil	65	3.8	Cu: 840; Fe: 6200; Al: 1250; Zn: 1060; Ni: 960; Cr: 980; Pb: 600; SO_4>Cl>NO_3

(continued)

Table 1 *(continued)*

Site	No.	Nature	Temperature (°C)	pH	Metal elements present (ppm)
	13	Water	65	3.5	Cu: 780; Fe: 5800; Al: 1120; Zn: 900; Ni: 900; Cr: 910; Pb: 580; SO_4>Cl> NO_3
Diesel engine brass works	14	Mud/soil	60	3.8	Cu: 3800; Fe: 560; Al: 360; Zn: 2010; Ni: 1700; Cr: 950; Pb: 570; SO_4>Cl>NO_3
	15	Water	60	3.2	Cu: 3650; Fe: 560; Al: 300; Zn: 1950; Ni: 1540; Cr: 1060; Pb: 890; SO_4>Cl>NO_3
	16	Water	60	2.5	Cu: 3650; Fe: 580; Al: 360; Zn: 1250; Ni: 1500; Cr: 1280; Pb: 640; SO_4>Cl>NO_3



	CuS	$CuFeS_2$	$CuSO_4$
JVCu-8	80	80.12	6.9
JVCu-10	82.6	82.8	7.2
JVCu-12	85	89	7.2

Fig. 1. Biosolubilization of covellite and chalcopyrite by acidothermophilic isolates at pH 3.5, 65°C, 150 rpm, and 120 h.

Table 2
Biosolubilization of CuS by Isolated Acidothermophiles at pH 3.5, 65°C, and 150 rpm Within 120 h[a]

	Biosolubilization		Autooxidation		Total solubilization	
Strain	mg of Cu/100 mL	%	mg of Cu/100 mL	%	mg of Cu/100 mL	%
JVCu-1	477	71.5	47	7.05	524	78.6
JVCu-2	480	72	46	6.9	526	78.9
JVCu-3	493	74	47	7.05	540	81.0
JVCu-4	498	74.7	45	6.75	543	81.4
JVCu-5	513	77	46	6.9	559	83.8
JVCu-6	510	76.5	46	6.9	556	83.4
JVCu-7	485	72.8	44	6.6	529	79.3
JVCu-8**	533	80	47	5.05	580	87
JVCu-9	520	78	46	6.9	566	84.9
JVCu-10**	550	82.6	46	6.9	596	89.4
JVCu-11	527	79.1	45	6.75	572	85.8
JVCu-12**	566	85	46	6.9	612	91.8

[a]1000 mg of CuS ≅ 663 insoluble Cu content/100 mL. ** = selected isolates.

Gram-negative nonmotile, nongranulated spherical-to-oval-shaped bacteria occurring singly, in pairs, or irregular cell clusters. They were capable of biosolubilizing 85% CuS and 89% $CuFeS_2$ in the presence of 7.2% soluble copper. This strain had a significant effect of Fe on its bioactivity and thus exhibited an Fe-dependent indirect biooxidation mechanism *(19)*. These selected strains need to be further characterized for their physicochemical optimization in order to justify their use as highly potential bioleaching starters for commercial exploitation.

Acknowledgments

The authors are thankful to Dr. A. D. Agate, Former Director, Agharkar Research Institute, Pune; Dr. S. R. Dave, Head and Reader, Dept. of Microbiology, Gujarat University Ahmedabad for their encouragement and valuable guidance. They also thank Principal and Staff members of MVM Sc. & HSc College, Rajkot.

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