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Biogenic production of cyanide and its application to gold recovery

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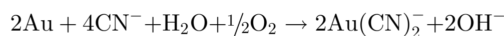
Chromobacterium violaceum **is a cyanogenic (cyanide-producing) microorganism. Cyanide is used on an industrial scale to complex and recover gold from ores or concentrates of ores bearing the precious metal. A potentially useful approach in gold mining operations could be to produce cyanide biologically in relatively small quantities at the ore surface. In this study, *C. violaceum* grown in nutrient broth formed a biofilm and could complex and solubilize 100% of the gold on glass test slides within 4–7 days. Approximately 50% of the cyanide-recoverable gold could be mobilized from a biooxidized sulfidic-ore concentrate. Complexation of cyanide in solution by gold appeared to have a beneficial effect on cell growth — viable cell counts were nearly two orders of magnitude greater in the presence of gold-coated slides or biooxidized ore substrates than in their absence. *C. violaceum* was cyanogenic when grown in alternative feedstocks. When grown in a mineral salt solution supplemented with 13.3% v/v swine fecal material (SFM), cells exhibited pigmentation and suspended cell concentrations comparable to cultures grown in nutrient broth. Glycine supplements stimulated production of cyanide in 13.3% v/v SFM. In contrast, glycine was inhibitory when added at the time of inoculation in the more concentrated SFM, decreasing cell numbers and reducing ultimate bulk-solution cyanide concentrations. However, aeration and addition of glycine to stationary phase cells grown on 13.3% v/v SFM anaerobically resulted in rapid production and high concentrations (up to 38 mg l^{-1}) of cyanide. This indicates that biogenesis of cyanide may be supported in remote areas using locally produced and inexpensive agricultural feedstocks in place of commercial media.** *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 134–139.

Keywords: bioleaching gold; *Chromobacterium violaceum*; cyanogenesis

Introduction

Cyanide is used in the mining industry as part of a leaching circuit for recovering gold from ores. Biooxidation utilizes iron and sulfur-oxidizing acidophilic bacteria to oxidize sulfides contained in certain refractory gold ores [21]. In this process, bacteria degrade the sulfide matrix surrounding gold, thus improving accessibility of gold to complexation by cyanide in alkaline solution [20]. Cyanide is one of the few reagents capable of forming water-soluble complexes with gold. The anodic and cathodic reactions characterizing the oxidative dissolution and complexation of gold by cyanide, as reviewed by Haque [9], are summarized in the following equation.



$$2Au + 4CN^- + H_2O + 1/2 O_2 \rightarrow 2Au(CN)_2^- + 2OH^-$$

A mathematical model proposed by Crundwell and Godorr [6] describes a two-stage electrochemical mechanism and indicates that the overall rate of reaction is one-half order with respect to the concentrations of oxygen and cyanide in solution, in contrast to older first-order, diffusion models [6].

HCN has a p*K*a of 9.4 [9] and the concentration of CN^- in solution is highly dependent on pH. High temperature, high solution concentration, and low pH promote the generation of gaseous HCN [20]. Commercial leaching operations are conducted at a pH greater than 10.3 to maximize the concentration of CN^- in solution and to prevent its loss by volatilization as HCN [20]. Alkaline pH (pH >9) also favors maintaining an E_h (400 mV) that will promote the cyanidation reaction [9].

C. violaceum is a mesophilic, motile, Gram-negative, facultative anaerobe. Strains of this microorganism are variably cyanogenic [10,18]. They can produce extracellular cyanide during mid-to-late logarithmic and briefly in early stationary phases [4,12,18]. Cyanide is generally considered a secondary metabolite because its production is dependent on the growth phase [4]. Phenotypic expression of cyanogenesis, as well as production of violaceum pigment [17,27], chitinolytic enzymes [5], and exoproteases [26] may be globally regulated in *C. violaceum* as part of a quorum sensing mechanism by *N*-acyl homoserine lactone signaling molecules [27]. However, like other secondary metabolite systems, cyanide production by *C. violaceum* is also influenced by factors such as pH, temperature, and the concentrations of oxygen and glycine [3,4,12,19,23]. The HCN synthase of *Pseudomonas fluorescens* has been studied to a greater extent than that of *C. violaceum*. The synthase is coded by the *hcn*ABC gene sequences. Glycine is metabolized to cyanide in reactions characterizing oxidative decarboxylation [13]. However, Laville *et al* [14] suggested that the synthase itself may act in part as dehydrogenase as glycine is converted to HCN and CO_2, because the nucleotide sequences exhibit FAD or NAD (P) binding motifs and extensive homology to other hydrogen-transfer enzymes. Overall, cyanogenesis occurs under microaerophilic conditions and requires the *anr* gene product [28]. Oxygen is, however, the ultimate electron acceptor. Glycine can partially stabilize the cyanide synthase in the presence of oxygen, and Castric [3] demonstrated that oxygen is only an indirect acceptor that

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receives electrons *via* a cyanide-resistant branch of the electron transport system [3,13]. In studies of anaerobes using nitrate as an alternative terminal electron acceptor, little or no cyanide was produced [3,4]. However, anaerobically grown cells (using nitrate as a terminal electron acceptor) produced cyanide when shifted to anaerobic conditions, albeit at a lower level than anaerobically grown cells [4].

Cyanogenesis by *Pseudomonas aeruginosa* was greatest under pH and temperature conditions that were much more restrictive than those allowing for rapid growth of the organism. *P. aeruginosa* was also responsive to media trace metal concentrations, producing cyanide in proportion to the log of the Fe(III) concentration. The microorganism grew, but was not cyanogenic at phosphate concentrations between 90 and 300 mM [4].

Gold leaching by *C. violaceum* is highly variable depending on ore type and its gold content [15]. Lack of gold leaching was attributed to complexation of biogenic cyanide with iron. Still, there remains interest in the potential for using microorganisms such as *C. violaceum* in less alkaline environments to produce cyanide for use in leaching operations [11] because the most significant costs in many gold mining operations are for such chemical reagents. Cyanide would be produced within a biofilm at the ore surface. In this manner, relatively small amounts of cyanide would be generated and consumed. However, mining operations, especially in South America and Asia, may be located in remote areas, raising transportation costs to continuously import cyanide or the commercial feedstocks to support a cyanogenic bioprocess. Little information is available regarding secondary metabolite formation by *C. violaceum* under conditions likely to be encountered in an industrial application, i.e., exposure to material such as biooxidized ore and the use of alternative feedstocks. Here, we examined the use of the cyanogenic bacterium *C. violaceum* for gold solubilization under conditions and with substrates likely to be encountered in a field application. The goal was to determine the potential for an *in situ* cyanogenic process.

Material and methods

Organism and culture conditions

Chromobacterium violaceum ATCC 12472 was maintained on nutrient agar (Difco, Detroit, MI) at 37°C or grown in shake flasks containing nutrient broth (Difco). Incubation was at 25–27°C with shaking at 151 or 180 rpm. Long-term storage was at −70°C. Growth of the organism was monitored by absorbance at 660 nm (Klett colorimeter) or by plate counts on nutrient agar.

Enumeration of planktonic bacteria

Counts of viable suspended cells were done by the drop plate method of serial dilution on nutrient agar. The plates were incubated for 24 h at 32°C.

Enumeration of adherent cells

Counts of viable adherent cells were accomplished by scraping the biofilm off the glass slides with a sterile Chemware PTFE fluoropolymer resin policeman into a sterile beaker. Slides were then rinsed twice with 5 ml of sterile water, and the policeman was agitated in the collected volume. The solution was homogenized for 1 min (Janke and Kunkel IKA Labortechnik T25 basic homogenizer) with a 100 mm×8 mm O.D. tip. The homogenized sample was serially diluted and plated on nutrient agar using the drop plate method.

Gold coupon slide preparation

Glass microscope slides (7.6 cm×2.5 cm) were coated with gold under vacuum using an International Scientific Instruments PS-2 coating unit. The chamber was evacuated and the ambient atmosphere replaced by argon (0.2–0.1 Torr). An electrical current (24 mA) was applied and maintained for 10 or 30 min. Both faces of each slide were coated. The amount of gold deposited was monitored using inductively coupled plasma atomic emission spectroscopy (ICP-AES) by comparison of aqua regia digests of identically prepared slides.

Gold coupon slide reactors

Six gold-coated microscope slides were suspended in a jar reactor containing 300 ml of nutrient broth inoculated with *C. violaceum*. As controls, six uncoated slides were added to 300 ml of the same medium in a separate jar reactor that was inoculated with 1 ml of an actively growing culture of *C. violaceum*. The slides were situated so the buffed portions of the (coated) slides were just above the nutrient solution, and oriented parallel to the mixing motion of the fluid to reduce turbulence and splashing. Slides were removed from the reactors at timed intervals and analyzed for populations of attached bacteria (biofilm) by using adherent cell enumeration as described above. Gold in solution was also monitored with time. Aliquots of solution were removed, acidified with HCl–HNO_3 and analyzed for gold by ICP spectroscopy.

Analyses

Cyanide was analyzed colorimetrically at 578 nm using the pyridine barbituric acid colorimetric method [8]. Matrix corrections were done by diluting samples in nutrient broth. Gold content was analyzed by ICP-AES at Little Bear Laboratories (Golden, CO). ICP samples were preserved by the addition of 15% HCl+5% HNO_3 v/v and kept frozen before analysis. The pH was determined by EPA SW-846 method 9040 using meters equipped with a combination electrode.

Swine fecal material (SFM) slurry

Swine fecal material (SFM) was obtained from a commercial ranch. Of this sample, 1000 g were thoroughly mixed with 2000

Table 1 Bioreactor conditions for SFM

Reactor	% of pig fecal slurry	% of glycine	Milliliters of nutrient broth	Aeration
1	3.3	0	0	Yes
2	3.3	0.2	0	Yes
3	3.3	0.5	0	Yes
4	3.3	0.8	0	Yes
5	13.3	0	0	Yes
6	13.3	0.2	0	Yes
7	13.3	0.5	0	Yes
8	13.3	0.8	0	Yes
9	0	0.2	300	No
10	3.3	0.2	0	No
11	13.3	0.2	0	No

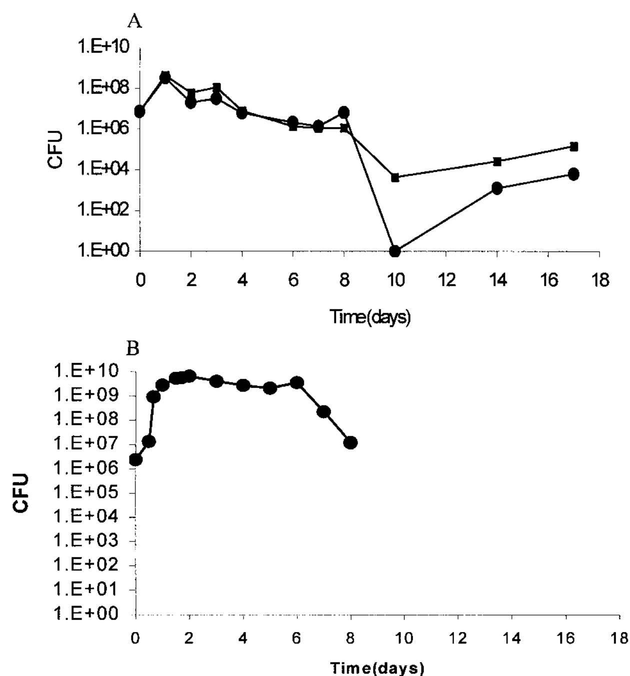

Figure 1 Growth of attached and planktonic cells of *C. violaceum*. (A) Average CFUs per slide for *C. violaceum*. (■) Reactor 1, containing six gold-plated slides and (●) reactor 4, all blank slides. (B) Average CFUs for planktonic cells of *C. violaceum*.

ml of modified M9 medium to create a 33% stock nutrient solution. This slurry was filtered through cheesecloth to eliminate particulate debris and then autoclaved for 40 min at 121°C and 15 psig. Modified M9 medium consists of, in grams per liter of solution: Na_2HPO_4, 6.0; KH_2PO_4, 3.0; NaCl, 0.5; NH_4Cl, 1.0; and, after autoclaving and cooling, 10 ml of a 0.01 M $CaCl_2$ stock solution [2].

SFM as alternative carbon source

Eleven reactors were used in this experiment to evaluate the use of an agricultural waste as an alternative feedstock. Each bioreactor contained a total volume of 300 ml of SFM inoculated with 1 ml of an actively growing culture of *C. violaceum*. A range of concentrations of SFM and glycine was evaluated to achieve optimal cell density as well as cyanide production. The bioreactors were operated at 30°C and mixed on a rotary shaker at 151 rpm or were not aerated. The operating conditions are summarized in Table 1.

Leaching of gold concentrate ores

A biooxidized flotation concentrate of gold ore (5 g) containing 9.4 $\mu g\ g^{-1}$ of gold (0.3 troy oz per short ton, determined by fire assay) was added to 50 ml of nutrient broth. The pH was adjusted to 7.8 with NaOH and the flask was inoculated with 1 ml of an actively growing culture of the organism in nutrient broth. Aliquots of solution were removed with time, syringe filtered with a 0.2-μm pore-size bacterial filter, then acidified with HCl–HNO_3 and analyzed for gold by ICP spectroscopy. Controls were either not inoculated or inoculated in the absence of the ore concentrate. Experiments were conducted in duplicate for 8–10 days.

Results

Gold solubilization

C. violaceum readily colonized the gold-covered surfaces as well as the noncoated surfaces of glass slides. Within 24 h, a purple biofilm was visible on all slides and reactor walls at the air/fluid interface. Throughout the 17-day incubation, the biofilm persisted in the reactors containing gold-coated slides. The biofilm in the reactor containing uncoated slides diminished, and on day 10, viable bacteria were not detected. However, after day 10 a slight recovery of the bacteria occurred in the reactors containing uncoated slides achieving a final cell density less than that of the reactor containing gold-coated slides. Reactor 1, which contained only gold-coated slides, retained viable bacteria throughout the experiment. Growth curves from biofilm samples resembled growth curves of planktonic cells (Figure 1A and B). Comparable cyanide production was observed in reactors containing coated and uncoated slides. Peaks of 13.3 and 14.4 mg l^{-1}, respectively occurred on day 4 (Figure 2A). A large decrease of cyanide was observed between days 4 and 7 at which point 83% of the gold had been solubilized (Figure 2B). All of the gold was solubilized by day 17. No gold was detected in solution in a sterile control reactor containing gold-coated slides.

C. violaceum also solubilized gold from gold ore concentrate. Gold concentrations in solution increased steadily, reaching levels of 0.25 mg l^{-1} after 7 days in the first test and 0.34 mg l^{-1} after 10

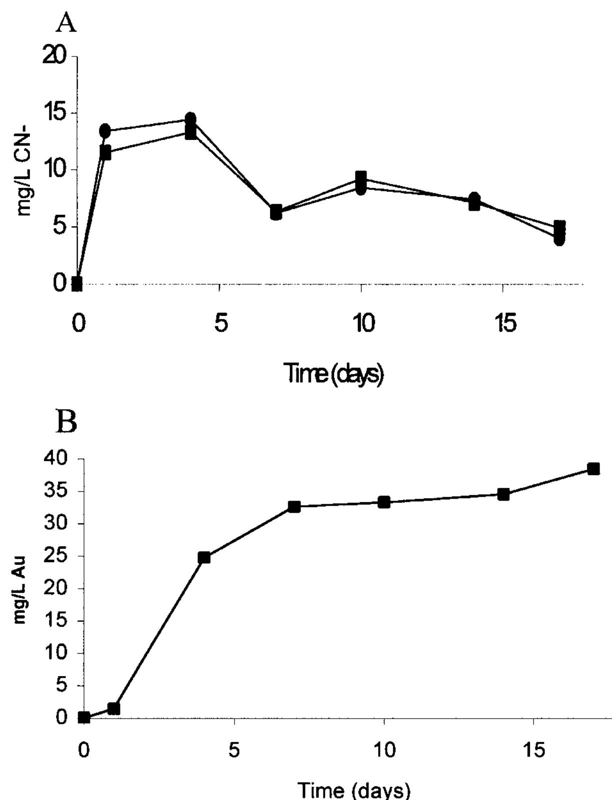

Figure 2 Cyanide production and gold solubilization in experiments involving glass slides. (A) Cyanide concentration in solution (average of duplicate experiments). (B) Gold concentrations in solution (average of duplicate experiments). (■) Reactor 1 containing six gold-coated slides and (●) reactor 4 containing no gold-coated glass slides.

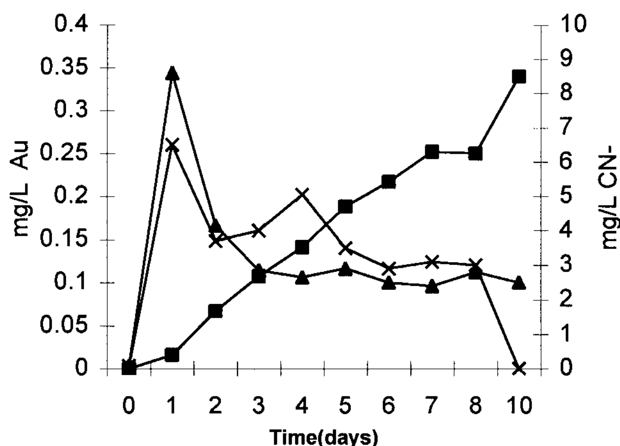

Figure 3 Cyanide production compared to gold leached from gold ore concentrates. (■) Average milligrams per liter of gold leached in two experiments, (▲) cyanide concentration averaged from duplicate experiments in the presence of gold. (×) Cyanide concentration averaged from duplicate experiments without the presence of gold.

days in the second test, corresponding to 28% and 38% gold extraction, respectively (Figure 3). The low amount of ore (5 g) employed in these tests precluded fire assay of the bioleached residue. Gold extraction from the concentrate with NaCN for 24 h (cyanide concentrations maintained at 1.0 g l^{-1} with NaCN) was 77%. Therefore, up to 50% of the extractable gold was removed at a relatively low concentration of microbially produced cyanide. In both cases, cyanide concentrations reached a maximum after 24 h and then declined. This decline was approximately at the point at which gold began to be leached, as shown for the second experiment using a separately prepared biooxidized ore concentrate (Figure 3). *C. violaceum* in the presence of gold ore concentrate experienced a prolonged stationary phase. Cell viability (colony-forming units, CFUs) of planktonic cells was up to four orders of magnitude higher when compared to a control lacking ore (Figure 4). A second control reactor containing ore but was not inoculated showed no microbial growth.

The use of SFM as a growth medium was also studied with respect to cyanogenesis. Use of a very dilute waste (1.3%) resulted in a loss of cell pigmentation and cyanogenic potential (data not shown). However, cyanide production was evident with a 13.3%

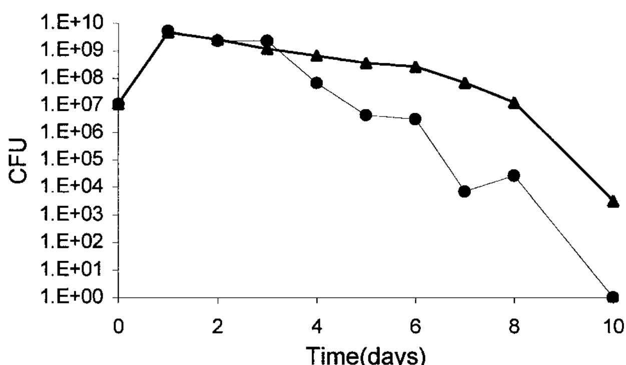

Figure 4 Average CFUs with or without the presence gold ore concentrate. (▲) CFUs averaged from duplicate experiments in the presence of gold ore concentrate. (●) CFUs averaged from duplicate experiments without the presence of gold ore.

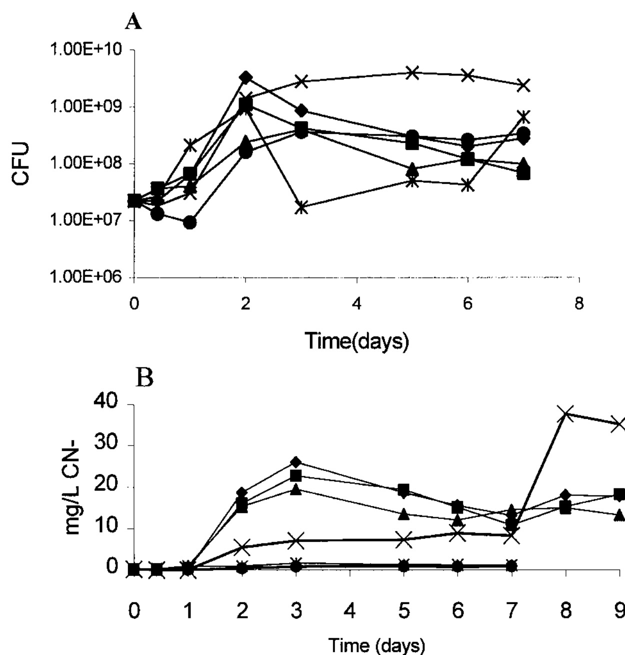

Figure 5 (A) CFUs of *C. violaceum* in 13.3% SFM. (B) Milligrams per liter of CN^- produced in the same sample. Percent of glycine and growing conditions of reactors: (×) 0.0% glycine, (◇) 0.2% glycine, (■) 0.5% glycine, (▲) 0.8% glycine (all reactors run aerobically). (*) anaerobic control reactor with nutrient broth and 0.2% glycine, and (●) anaerobic control reactor with 0.2% glycine. A spike of 0.2% glycine (final concentration) was added to all reactors at day 7.

SFM, and with the addition of glycine, pigmentation was restored with a maximal cell density reached approximately 48 h following inoculation (Figure 5A). The reactors with 13.3% SFM demonstrated an extended lag phase in comparison to the reactor operating with nutrient broth.

In a final set of reactors, maximum cyanide concentrations for the aerated reactors containing a 3.3% SFM occurred 48 h following inoculation, but only after 3 days for the 13.3% SFM. In reactors containing 3.3% SFM, concentrations of glycine up to 0.5% were stimulatory to cyanogenesis (data not shown). With a 13.3% SFM, increased levels of glycine generally resulted in less cyanide being produced and lower cell densities (Figure 5A and B). On day 7 of the 9-day experiment, all reactors were spiked with a glycine solution resulting in an addition of 0.2% glycine. Overall, there was no effect on cyanide concentration except in the reactor containing a 13.3% SFM, but no initial glycine addition at the start of incubation. This reactor showed an immediate increase of solution cyanide from 8.3 to 37.7 mg l^{-1} within a 24-h period. This was the highest level of cyanide observed in any of the experiments involving SFM. In addition, the 37.7 mg l^{-1} of cyanide measured is the highest level measured in any of the previous experiments involving nutrient broth. In those experiments involving nutrient broth, cyanide concentrations generally ranged from 20 to 25 mg l^{-1} at neutral pH with a highest measured level of 28 mg l^{-1} (data not shown). Preliminary experiments were done to evaluate growth and cyanide production in nutrient broth at increased pH levels. At an inoculation pH of 8.5, cyanide concentration was measured at 35 mg l^{-1} after 24 h. Although our preliminary experiments were not buffered against pH change and an NaOH spike was needed to maintain initial pH levels; our initial

results concerning cyanide production at pH 8.5 are in agreement with those of Lawson *et al* [15].

Discussion

Our findings are in general agreement with earlier literature reports concerning cyanogenesis by this organism. Cyanide production in commercial nutrient broth was highest within the first 3 days of incubation and corresponded to the logarithmic to stationary growth phase transition period [4,12]. Typical cyanide concentrations in this medium were somewhat higher at a near neutral pH than at pH 9. Unexpectedly, glycine addition to the medium appeared to have had little stimulatory effect on cyanide production. However, nutrient broth contains 5 g/l of peptone and 15.59% of peptone is glycine [7]. Thus a cyanide production response may be negligible due to the high level of glycine that already exists before the addition of 0.2–0.8% glycine. After several days, the cyanide concentration decreased rapidly in the presence or absence of glycine. Based on published reports, such a decrease was probably a result of its incorporation into compounds such as β-cyanoalanine [22] or, because the p*K*a of hydrocyanic acid is 9.4 [9], in part to a loss as HCN gas. Most industrial leaching operations are run at a pH of 10.3 or higher to minimize cyanide loss through volatilization of HCN [20].

However, these data indicate that a suitable process could be operated over a range from pH 7 to 9, although below the p*K*a of HCN. Because the E_h at pH 9 would favor oxidative dissolution of gold [9], a biological approach would require that a compromise be made between cell physiological requirements and complexing efficiency.

For the leaching process to occur, cyanide must be in close association with the gold surface for the gold to be oxidized and solubilized [9]. A bacterial biofilm, an attached community comprised of the organisms within layers of polysaccharides, would create a close association. Our data show that *C. violaceum* not only can colonize a gold surface, but can also solubilize the metal from the artificial substrates. This is consistent with an earlier report by Smith and Hunt [23] using sponge gold. Most cyanide formation occurred in mid-to-late log phase, again corresponding to earlier reports on extracellular cyanide production [4,12,18,23]. Most of the gold was solubilized from the slides within a 7-day period, which correlated directly with a drop in the concentration of cyanide in solution.

Initial studies on this bench-scale system using a biooxidized gold ore concentrate were encouraging. Cyanide concentrations were somewhat less in the gold ore reactors in comparison to the study of gold-plated slides. However, almost 50% of the cyanide-extractable gold was solubilized from one biooxidized ore concentrate. Although the ultimate percentage of available gold solubilized from ore was less than that from the gold-plated slides, this could be due to the decreased surface area of the exposed gold particle in relation to the gold slides. Leaching of an ore or gold concentrate is dependent on the reaction rate, being influenced partly by the amount of CN^- present, but also the size of the exposed gold surface [6]. Lawson *et al* [15] obtained highly variable results in leaching gold form ores with *C. violaceum*. The lack of gold bioleaching from certain iron-containing gold ores after 10 to 20 days was attributed to complexation of biogenic cyanide by iron. The gold ore concentrate used in the present study contained over 10 wt.% Fe, yet good gold extraction was achieved.

Experiments with the gold coupon and with sulfide ore indicated that the presence of gold appeared to have a beneficial physiological effect on *C. violaceum*, as measured by recoveries of viable adherent cells. This benefit may be attributed to gold complexing with cyanide, reducing the physiological effect of cyanide on the cell. Our findings are somewhat at odds with previous findings by Michaels and Corpe [18], who found no effect on the viability and total cell yield of planktonic *C. violaceum* cells at cyanide concentrations comparable to those in this study. Furthermore, Castric [4] found that Fe(III) had a stimulatory effect on cyanide production by *P. aeruginosa*. An argument could be made that the complexation of cyanide by gold reduced the toxicity of cyanide, providing a beneficial physiological effect to *C. violaceum* that was not noted in the absence of gold.

Cell pigmentation and cyanogenic potential were lost if the cells were grown in very dilute (1.3%) SFM, suggesting either an overall deficiency of nitrogen in the system or, specifically, a tryptophan deficiency, because this compound is an immediate precursor of violacein [24]. Increasing the concentration of SFM from 1.3% and 3.3% to 13.3% restored cell pigmentation. This indicated that the SFM itself and not the M9 diluent supplied one or more nutrients. Solution cyanide levels were comparable to those achieved in nutrient broth. However, in 13.3% SFM, increased levels of glycine generally decreased viable cell concentrations and cyanide production. Following a lag in the onset of cyanogenesis, solution cyanide concentrations maximized then decreased over a period of days, as in the case of nutrient broth-grown cells, and then remained fairly constant for the remainder of the experiment. However, the most dramatic improvement in cyanide production was noted when the addition of the glycine supplement was delayed until stationary phase in media supplemented with 13.3% SFM. This may be a result of simply having a larger population of viable cells available to effect the transformation. Cyanide is produced throughout mid-log phase and early stationary phase; thus cyanide levels would be subject to volatilization by low pH and by cell metabolism [16], resulting in a lower maximum level of cyanide. By delaying the addition of glycine, the precursor for cyanide formation, until the onset of stationary phase, a more efficient transformation to cyanide may result.

There are several possible applications of this organism to gold extraction. First, the organism might be used in heaps to produce cyanide [11], especially if slower gold-extraction rates could be tolerated. The organism might also be used to generate cyanide in continuous feed bioreactors at remote mine sites where it could be stripped from solution and concentrated. This approach may be most applicable where transportation costs are high. The very low solution concentrations of cyanide, which were effective in mobilizing approximately 50% of the gold in a biooxidized sulfidic ore could potentially reduce environmental hazards and treatment costs associated with conventional cyanide leaching operations.

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