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Review on *chromobacterium violaceum* for gold bioleaching from e-waste

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Abstract

Electronic waste, such as printed circuit boards, are an important secondary resource if processed with environment-friendly technologies for obtaining precious metal, such as gold. The gold bioleaching from electronic waste was recently getting paid attractive attention because its available deposit is limited. This review was focused on *Chromobacterium violaceum (C. Violaceum)*, which was a mesophilic, gram-negative, and facultative anaerobe. *C. violaceum* has the ability of producing CN^- which can dissolve gold from the metallic particles of crushed waste printed circuit boards. This article also provided an overview of cyanide-generation mechanism and the optimal conditions for *C. violaceum* to achieve maximum amount of cyanide generation. The past achievements and recently scenario of recovery studies carried out on the use of some other microorganisms were compared with *C. violaceum*. And recently some researchers proposed that combined *C. violaceum* with chemical methods or other mechanism such as iodide, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* which can reinforce the cyanide generation and improve gold-leaching efficiency. The factors affected the microorganisms on cyanide generation were summarized and the proper conditions were also discussed in this article. And present researches of *C. violaceum* in gold bioleaching had made good progress which the reported leaching efficiency of gold was over 70%.

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Keywords: Chromobacterium violaceum; e-waste; gold recovery; bioleaching; cyanide

1. Introduction

With the development of industrial processes, science and technology innovation, and modern business marketing strategy results in the development of electronic products that are cheaper, better and more readily available than older versions. As a consequence, the electronic devices become obsolete and redundant faster than ever and are treated as electrical waste (e-waste).

E-waste is considered to be one of the fastest growing trash[1], about 2.5 million tons of e-waste (both self-generated and imported from developed countries) appeared in Chinese mainland every year[2], and about 50 million tons of e-waste are

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generated annually for worldwide. This e-waste stream presents a major disposal challenge as e-waste contains toxic metallics such as lead, mercury arsenic, cadmium, selenium, and hexavalent chromium. In addition to its inherent toxicity, e-waste also contains significant amounts of precious metals such as gold (Au)[3]. However, according to the EPA report recently, only 18% e-waste was recycled and the remaining was incinerated or land filled[4]. Not only does the e-waste occupy large amount of land, but also the leachate can contaminate soil and groundwater and further pose a risk to human and environmental health. In fact, e-waste is a very significant secondary resources, precious metals content in e-waste is dozens of times the content of the metals in high-grade ore. Especially for gold, compared with natural gold ores which has about 0.5-13.5 grams gold per ton, e-waste has significantly higher gold content at around 10-10,000 grams gold per ton[5,6], making it an important source of economic can also be used as a substitute for gold mine. Through environmentally friendly manner to process e-waste can not only reduce environmental pollution, but also can achieve precious metals recovery.

There are a number of control technologies for gold recovery. Mechanical separation, pyrometallurgical, hydrometallurgical, and bio-hydrometallurgical technologies have been extensively used to recover gold from printed circuit boards (PCBs)[7]. Pyrometallurgical processes have been a traditional technology for recovery of precious metals from e-waste. However, such processes requiring high consumption of energy, inefficiently recover precious metals and contain halogenated flame retardants in the smelter feed that can lead to the formation of dioxins and furans[8]. Hydrometallurgical processes are mostly involved in cyanide leaching, halide leaching of precious metals. So the hydrometallurgical processes rise risks of environmental impact due to the toxicity of the reagents used and the large amount of by-products generated[5]. In the last decade, the biotechnology has been emerging as one of the most promising technologies for recovering metals from primary and secondary resources[9]. Biotechnology processes are often promoted as eco-friendly processes for the treatment of e-wastes. They are essentially hydrometallurgical processes using microorganisms such as bacteria, archae and fungi to enhance the dissolution of metals from ores, concentrates and wastes. In these processes, the exploitation of microorganisms is based on their inherent characteristics to oxidize or utilize inorganic and organic substrates so as to generate lixiviant for dissolution of metals[10].

1.1. Chromobacterium violaceum

C. violaceum is a Gram-negative bacterium (facultative anaerobe), that is considered to be nonpathogenic for humans and found in tropical and subtropical areas of several continents. However, in some cases it can act as an opportunist pathogen for animals and humans and cause fatal septicemia[11].This bacterium is capable of exploiting a wide range of energy resources by using appropriate oxidases and reductases. This allows *C. violaceum* to live in both aerobic and anaerobic conditions[12].

C. violaceum has been found to be the most effective for the bio-dissolution of gold from different materials because of its cyanide-associated metabolic activities[13]. There is an operon for HCN synthase (hcnA, hcnB and hcnC) in the *C. violaceum* genome, encoding a formate dehydrogenase and two amino acid oxidases, respectively, which are involved in cyanic acid synthesis[14]. In the cyanide synthesis process the four electrons produced by HCN synthase are transferred to oxygen, probably throughout the respiratory chain. These reactions occur at low levels of oxygen. So HCN is mainly generated in aerobic conditions[12].

These bacteria produce cyanide as a secondary metabolite. An important feature of cyanogenic bacteria is the inherent capacity to degrade cyanide. Previous studies have shown that *C. violaceum* and Bacillus megaterium are known to synthesize the enzyme b-cyanoalanine synthase which converts cyanide into b-cyanoalanine during the late stationary and death phase. Therefore, this strain can potentially be used in ecological Au recovery methods[11,14].

1.2. The Leaching Mechanism

Gold leaching by cyanogenic microorganisms generally involves an indirect process with microbial production of metabolites. These metabolites dissolve gold from minerals by the formation of soluble metallic complexes[15]. Au dissolution in cyanide solution consists of an anodic and a cathodic reaction and is summarized by Elsner's Eq. as follows[16]

$$4Au + 8CN^- \rightarrow 4Au(CN)_2^- + 4e^- \quad (1)$$

$$O_2 + 2H_2O + 4e^- \rightarrow 4OH^- \quad (2)$$

$$4Au + 8CN^- + O_2 + 2H_2O \rightarrow 4Au(CN)_2^- + 4OH^- \quad (3)$$

In contrast to existing processes, bioleaching, involving microorganisms such as *C. violaceum,* may allow metal recycling in a process analogous to natural biogeochemical cycles, and hence reduces the demand for resources such as ores, energy or space for landfills[15]. In a process similar to industrial Au cyanidation, cyanogenic microorganisms produce the cyanide lixiviant which then reacts with solid Au to complete the leaching process[5,17,18,14]. The cyanide lixiviant is derived from the secondary metabolite hydrogen cyanide (HCN) produced from glycine using the enzyme HCN synthase; however, the amount of lixiviant metabolite produced is limited (20 mg of cyanide per liter of bacteria culture, approximately 1×10^{16} colony forming units), and tight regulation of this operon under quorum control further restricts its widespread use in industrial Au recovery from electronic waste[19,20].

Cyanide is formed as secondary metabolite. It is assumed that its formation has an advantage for the organism by inhibiting competing microorganisms[21]. Cyanide occurs in solution as free cyanide which includes the cyanide anion (CN^-) and the non-dissociated hydrocyanic acid (HCN). At physiological pH, hydrogen cyanide has pKa of 9.3 at 26℃ and, therefore, cyanide is largely present as volatile HCN[14]. In the presence of salts this value decreases to approximately 8.3 and the volatility is reduced[22]. Generally, cyanide can interact with a series of metals. It is known that nearly all transitions metals (except lanthanides and actinides) form well-defined cyanides complexes which show often very good water solubility and a very high chemical stability[15,23]. However, until today a combination of this chemical knowledge with microbiological principles (biological HCN formation) regarding metal solubilization from metal-containing solids and the formation of water-soluble cyanide complexes has been considered very marginally. Only very few reports can be found in the literature describing the biological solublization of gold by *C. violaceum* and microbe-mediated formation of gold cyanide[24-26].

Since the leaching of metal ions in the solution and the formation of metal-cyanide complex may be toxic to the bacteria and adversely affect cyanide production, an approach to improve gold recovery without affecting bacterial growth is to decouple growth/cyanide production from bioleaching. Further, as base metals (mainly copper) present in the ESM (and at a higher concentration than gold) are capable of forming soluble metal-cyanide complexes, the bioleaching of gold may be enhanced through pretreatment of the ESM to reduce competition for cyanide ions from these metals[27].

2. Bioleaching Progress

The bioleaching process is now emerging as commercial exploitable technology applicable for metal extraction from electronic waste and low grade ores. Using bioleaching techniques, the efficiency of recovery of metals can be increased, as revealed in copper and gold mining where low grade ores are biologically treated to obtain metal values, which are not accessible by conventional treatments (mechanical and thermal)[28]. Although, this process has been successfully applied for the leaching of metals from ores[29], data pertaining to its application for the extraction of electronic waste is still scanty.

Currently study on gold bioleaching by *C. violaceum* Generally divided into one-step bioleaching, two-step bioleaching and spent medium leaching, according to whether ESM was added to the media as the second step when the maximum cell density and cyanide production was attained and cells were separated from the culture after it reached maximum cell density and cyanide production.

(1) One-step bioleaching

Bioleaching was carried out in disinfection erlenmeyer flasks with YP media and 1 g/L $MgSO_4 \cdot 7H_2O$ at 15 g/L pulp density while adding 1 mL *C. violaceum* culture under log phase. The flasks were incubated at 30℃ and the desired pH in an orbital motion shaker. With the pH increased from 8.0 to 11.0 in 8 days and the gold leaching efficiency increased from 7.78% to 10.9%[30]. The Eh-pH diagramme[31] showed that $Cu(CN)_2^-$ is more stable at pH less than 9.0, while $Cu(CN)_3^-$ and $Cu(CN)_4^{3-}$ will be formed in larger amounts at pH above 9.0 in presence of high cyanide; formation of higher cyanide complexes will be unlikely with low cyanide. Thus, high stability of metal cyanide complexes[32] and increased stability of HCN at pH > 10 improved the metal leaching at higher pH. As regards gold, $Au(CN)_2^-$ is formed at higher pH and higher DO is reported to favour gold dissolution[33].

Addition of 0.004% H_2O_2 increased gold recovery increased marginally from 8.1% to 11.32% with the rise in pH from 8.5 to 11.0. The DO played a definite role during the leaching which decreased significantly within 24 h because of respiration of .bacteria. Preferred copper leaching than gold may be the result of high copper present in the sample consuming cyanide produced at higher DO level. To improve gold bioleaching copper content must be decreased by a suitable method prior to bacterial leaching[30].

While the gold bioleaching process using a microorganism may be considered one of the most ecofriendly options, the dissolution rate may not be as fast as that in the case of direct addition of cyanide salts. Earlier researches[25,33] have shown that the presence of some metal ions can improve the bacterial cyanidation process. An enhanced rate of gold dissolution was observed with cyanide solutions in presence of small amounts of $Pb(NO_3)_2$, which activated the gold surface, leading to higher reactivity and faster leaching[34,35].

As investigated by Tran et al[36], cyanide generation by cyanogenic bacteria—*C. violaceum* in YP medium can be enhanced by adding a low amount of metal ions to the culture medium. This is due to a catalytic effect enhancing the enzymatic process[25]. The addition of $MgSO_4$ and $FeSO_4$ to the medium was found to be equally effective for cyanide generation by the bacteria, and the presence of Na_2HPO_4 and $Pb(NO_3)_2$ enhanced cyanide generation further. The dissolved oxygen concentration did not affect cyanide generation by *C. violaceum*. The results of bioleaching of valuable metals from waste mobile phone PCBs showed that the maximum amount of gold could be leached out (11% in 8 d) at pH 11.0 in the presence of $4.0\times10_{-3}$ mol/L $MgSO_4$ in the YP medium. The presence of phosphate and $Pb(NO_3)_2$ in the medium favored copper bio-dissolution, but it was not effective for gold leaching. The low concentration of cyanide generated by the metabolic activity of *C. violaceum*, and dissolved oxygen, favored copper bioleaching at the expense of gold from the PCBs[36].

(2) Two-step bioleaching

In two-step bioleaching, *C. violaceum* was initially cultured in Luria Bertani, Miller (LB) media in the absence of ESM. Sterilized ESM was added to the media as the second step when the maximum cell density and cyanide production was attained (in the early stationary phase). Two-step bioleaching was adopted in order to minimize inhibition of cyanide production owing to toxicity of ESM[37]. A two-step bioleaching process is believed to be appropriate to increase the metal leaching efficiency of micro-organisms from electronic waste[38]. For more efficient metal mobilization, direct growth of micro-organisms in the presence of electronic waste is not advisable due to its toxic effects[39]. Microorganisms were grown in the absence of electronic waste to produce biomass followed by the addition of different concentrations of electronic waste for metal mobilization for an additional time period of 7 days.

The cyanide concentration profiles of all the pure cultures generally showed a similar trend with its peak cyanide production occurring at the onset of the stationary growth phase. It was also observed that there was a subsequent decline of free cyanide concentration which could be attributed to the volatility of hydrogen cyanide and/or cyanide degradation by the bacteria. Studies have shown that *C. violaceum* converts cyanide to β-cyanoalanine[14] during its stationary and death phases. The highest cyanide concentration of 20 mg/l was observed in the pure culture of *C. violaceum* during early stationary phase (20 h after inoculation). At a pulp density of 0.5% w/v, *C. violaceum* showed highest gold bioleaching (11.3%)[37].

It was observed in Pradhan's[24] investigation that significantly high concentration of Cu (77.12%w/w) and Au (63.14% w/w) were leached out by the mixed cultures of *P. fluorescens* and *C. violaceum*. The Cu exhibited maximum leachability followed by Au, Zn, Fe and Ag. The precious metal Au leached out around 73.17% w/w by mixed cultures of *P. aeruginosa* and *C. violaceum* and 69.3% w/w by *C. Violaceum* alone, which were the most noteworthy results. It was observed that mixed cultures of *P. aeruginosa* and *C. violaceum* exhibited more leaching capability for all metals than the other combinations of mixed cultures as well as the single cultures. This might be due to a higher tolerance to metal toxicity, formulation of stable metal complexes in the presence of electronic waste releasing additional secondary metabolites for metal leaching and enhanced growth in comparison with other biological treatments[40,41]. Higher metal bioleaching capabilities were achieved by using combinations of mixed cultures of cyanogenic bacterial strains. *Pseudomonas aeruginosa* was used for the bioleaching study of electronic waste for the first time and in combination with *C. violaceum* exhibited higher metal leaching capabilities than other combinations[24].

In Li's[42] investigation, in order to study the experimental factors on the leaching rate of gold, the experiment selected pretreatment, particle size, nutritive salts, pH, DO, the amount of bacteria, addition amount of powder and reaction temperature to achieve maximum gold leaching efficiency. In the growth phase, *C. violaceum* consumed dissolved oxygen for bacterial respiration, which made a rapid decrease of DO. This decrease restrained the gold leaching. After oxygen supplement, gold leaching efficiency of every set had a dramatic increase in 7 days. Without pretreatment, the gold leaching efficiency is only 19.8 %. There are many toxic substances, such as mercury, cadmium, lead, arsenic and fire retardant, which are toxic to microorganisms. Pretreated at optimum conditions by *At. ferrooxidans*, gold leaching efficiency is 40.1 %, and the total cyanide amount after 7 days can reach 2.155 mg. Gold bioleaching by *C. violaceum* and the cyanide amount can be enhanced by adding a low amount of nutritive salts into the culture medium. After the addition of NaCl and $MgSO_4 \cdot 7H_2O$, there was an obvious increase in gold leaching efficiency. The maximum gold leaching efficiency can reach 70.6 % and 52.4 %, respectively. The optimum amount of $MgSO_4 \cdot 7H_2O$ and NaCl is 4×10^{-3} mol/L, 1.7×10^{-1} mol/L, respectively.

(3) spent medium leaching

In spent medium leaching, cells were separated from the culture after it reached maximum cell density and cyanide production (16–20 h), and only cell-free metabolites were used for (spent medium) leaching experiments.

As investigated by Natarajan et al[37], spent medium leaching for both gold and copper showed higher metal recovery than in two-step bioleaching, at all pulp densities. In all instances, the recovery remained relatively constant beyond Day 1. Unlike in two-step bioleaching where oxygen is consumed by the bacteria, oxygen is utilized in gold complex formation (in the absence of bacteria) in spent medium leaching. The role of oxygen in gold cyanide complex formation has been shown earlier in Elsner equation (Eq. (3)).

Maximum cyanide production occurs during the early stationary phase. In two-step bioleaching, cyanide is also consumed, via conversion to β cyano-alanine during the mid and late stationary phase, and via complexation with metals present in ESM during bioleaching. As there is no consumption of cyanide by the bacteria in spent media leaching, the biogenic cyanide may be fully utilized in the leaching of gold compared to two-step bioleaching where growth/cyanide production is not decoupled from bioleaching process[37].

Significant amount of metals from ESM were found to be biosorped and bioaccumulated in two-step bioleaching. As metals were solubilized via metal-cyanide complex formation, the bacteria continue to bioaccumulate gold (and possibly other metals). Biosorption of gold on the inactivated bacteria after bioleaching reduces the concentration of gold in the bioleached solution. These reasons might explain the enhanced performance of spent medium leaching over two-step leaching for gold recovery [37].

The present study has some important implications. Spent medium leaching yields comparable or higher gold recovery than two-step bioleaching, confirming that only the metabolites (i.e. cyanide) produced by *C. violaceum* are involved in bioleaching. Spent medium bioleaching has significant advantages. As the bacteria are not in direct contact with ESM, a continuous or fed-batch culture may be developed where the spent medium is harvested under growth condition resulting in maximum cyanide production (in the absence of ESM). Leaching takes place in a second stage. Spent medium leaching removes the limitation on pulp densities loading and may be operated at higher pH and pulp densities, an approach not possible with two-step and one-step bioleaching system owing to toxicity of the ESM.

3. The Comparison of Gold Bioleaching Efficiency in Different Conditions

Gold bioleaching processes from ores have been used successfully on a commercial scale, but it is just getting started from e-waste. These processes are based on the natural ability of chemoautotrophic bacteria, which are able to use either organics or inorganics as their energy source and which can transform solid nutritive salts to its soluble and extractable form [43]. So the process is limited by many factors, such as microbial properties, nutriment, DO, pH, pulp density and particle size etc. If the optimum combination of all these factors is explored, biotechnology will be one of the most promising technologies in metallurgical processing. Following is a comprehensive comparison of gold bioleaching efficiency in different conditions (Table 1).

4. Conclusion

Microorganisms play many roles in the biogeochemical cycling of gold and can be utilised in a number of ways for gold processing and recovery and *C. violaceum* is one of the most effective microorganisms for the bio-dissolution of gold. Biohydromellurgy can be regarded as a rapidly evolving technology. It has revolutionized metal extraction as an alternative method, which can overcome problems associated with conventional pyrometallurgy and chemical hydrometallurgy in the recovery of metal values. Before the recovery of gold by *C. Violaceum*, a pretreatment step is required. Based on the differences of the physical properties (gravity, magnetism, electrical conductivity, etc.) different materials can be separated by multi-crushing, grinding, electrostatic separation, gravity separation, fluid-bed separation, density-based separation, and magnetic separation.

A direct comparison of gold recoveries from bioleaching studies is difficult since the metal composition of ESM is heterogeneous and varies with age, origin, and manufacturer, and the acid digestion protocols used by different researchers are dissimilar. Moreover other factors such as growth medium, bioleaching period and composition of toxic metals/non-metallic elements in ESM affect cyanide production and gold recovery. Therefore, we can not simply compare the leaching rate level to determine which method is better.

Additional research would be needed in searching and modifying a biomass to have a high uptake capacity and good biosorption characteristics to recover gold from secondary sources.

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Table 1 Comparison of the highest gold bioleaching efficiency in different conditions

Bacteria	Particle size	Pretreatment	Progress	Pulp density	Nutrient salts	Oxygen supplement	The highest efficiency	Reference
C.violaceum	1×1mm	-	One step	1.5% w/v	1 g/L $MgSO_4 \cdot 7H_2O$	-	10.90%	[30]
C.violaceum	1×1mm	-	One step	1.5% w/v	1 g/L $MgSO_4 \cdot 7H_2O$	0.004%(v/v) H_2O_2	11.30%	[30]
C.violaceum	1×1mm	-	One step	1.5% w/v	4.0×10^{-3} mol/L $MgSO_4$	-	11.80%	[36]
C.violaceum	1×1mm	-	One step	1.5% w/v	1.0×10^{-2} mol/L Na_2HPO_4	-	9.20%	[36]
C.violaceum	1×1mm	-	One step	1.5% w/v	3.0×10^{-6} mol/L $Pb(NO_3)_2$	-	10.10%	[36]
C.violaceum *C.violaceum*	1×1mm	-	One step	1.5% w/v	1.0×10^{-2}mol/L Na_2HPO_4 3.0×10^{-6} mol/L $Pb(NO_3)_2$	-	11.00%	[36] [36]
C.violaceum	1×1mm	-	Repeated batch culture		10 g/L NaCl	-	68.50%	[36]
C.violaceum	1.1µm	-	Repeated batch cultures	1.0mmol/L	1 g/L $MgSO_4 \cdot 7H_2O$	Aerated with sterilized air	60.00%	[23]
C.violaceum	<75µm	Biooxidzed by *At. ferrooxidans*	Two step	0.5%w/v	-	-	11.50%	[6]
C.violaceum	37 to 149 µm	-	Two step	1% w/v	-	-	69.30%	[24]
P. aeruginosa, C.violaceum	37 to 149 µm	-	Two step	1% w/v	-	-	73.20%	[24]
P. fluorescens, C.violaceum	37 to 149 µm	-	Two step	1% w/v	-	-	63.10%	[24]
C.violaceum	74 to 400 µm	Biooxidzed by *At. ferrooxidans*	Two step	0.5% w/v	0.984 g/L $MgSO_4 \cdot 7H_2O$	homemade sterile oxygenator	70.60%	[42]
C.violaceum	<100µm	nitric acid	Two step	0.5% w/v	-	-	11.30%	[37]
C.violaceum	<100µm	nitric acid	spent medium leaching	0.5% w/v	-	-	30%	[37]

Note that “-” denote “absence” of the items or “the literature did not mention”

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