

GENETICALLY-ENGINEERED *CHROMOBACTERIUM VIOLACEUM* WITH
ENHANCED CYANIDE LIXIVANT PRODUCTION FOR BIOLEACHING OF
PRECIOUS METAL FROM ELECTRONIC WASTE

RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Application No. 61/844,550, filed on July 10, 2013. The entire teachings of the above application are incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] Conventional leaching (extraction) methods for gold recovery from electronic waste involve the use of large amounts of energy and/or strong acids and pose considerable threat to the environment. The alternative use of bioleaching microbes for gold recovery is non-pollutive and relies on the secretion of a lixiviant or (bio)chemical such as cyanide for extraction of gold from electronic waste. However, widespread industrial use of bioleaching microbes has been constrained by the limited cyanogenic capabilities of lixiviant-producing microorganisms. Accordingly, a need exists for improved methods of recovery of metals such as gold from, *e.g.*, electronic waste, preferably by bioleaching.

SUMMARY OF THE INVENTION

[0003] The invention provides, *inter alia*, improved methods of recovery of metals such as gold from sources such as electronic waste by bioleaching, as well as genetically engineered microbes for performing these methods. The invention is based, at least in part, on the construction of a metabolically-engineered strain of *Chromobacterium violaceum* that produces more (70%) cyanide lixiviant and recovers more than twice as much gold from electronic waste compared to wild-type bacteria. Our results demonstrated the utility and advantages of lixiviant metabolic engineering

in the construction of enhanced bioleaching microbes for the bioleaching of precious metals from electronic waste.

BRIEF DESCRIPTION OF THE DRAWINGS

[0004] The foregoing will be apparent from the following more particular description of example embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating embodiments of the present invention.

[0005] FIG. 1: Lixiviant metabolic engineering of *Chromobacterium violaceum* for enhanced cyanide production and gold recovery. Schematic of genomic region between locus tags CV_0677 and CV_0678 of wild-type and engineered *C. violaceum*. The duplicated *hcnABC* operon, with the respective *pBAD* or *pTAC* promoter, was inserted into the *glmS* gene using Tn7-based transposition.

[0006] FIG. 2: Cyanide production profiles of wild-type and engineered *Chromobacterium violaceum* strains. *pBAD* and *pTAC* strains were induced with 0.002% L-arabinose and 1 mM IPTG, respectively.

[0007] FIG. 3: Production of cyanide by *pBAD* strain was dependent on L-arabinose inducer concentrations.

[0008] FIG. 4: Production of cyanide by *pTAC* strain was dependent on IPTG inducer concentrations.

[0009] FIG. 5: Gold recovery profiles of wild-type and engineered *Chromobacterium violaceum* strains. Gold recovery from ESM was obtained using *pBAD* and *pTAC* strains that were induced with 0.002% L-arabinose and 1 mM IPTC, respectively.

[0010] FIG. 6: Decoupling cyanide production from quorum control. Cyanide production profiles of wild-type and engineered strains; *pBAD* and *pTAC* strains were induced with 0.002% L-arabinose and 1 mM IPTG, respectively.

[0011] FIG. 7: Comparative proteome analyses after increased cyanogenesis in *Chromobacterium violaceum*. The cyanogenic pathway is highlighted in blue. Up-regulations are represented in red, and down-regulations are represented in green. The identities of proteins and enzymes detected in the study are shown in Tables 1A-1C.

[0012] FIG. 8: Growth curves of wild-type and engineered *C. violaceum*. Wild-type *C. violaceum* was grown in the absence of gentamycin, while engineered *C. violaceum* strains were grown in the presence of gentamycin.

[0013] FIG. 9: Growth curves of wild-type and engineered *C. violaceum* in the absence of gentamycin. Due to the production of *C. violaceum*, growth progress was monitored as a function of colony forming units per mL of bacterial culture (CFU/mL).

[0014] FIG. 10: Cyanide production by wild-type and engineered *C. violaceum*.

DETAILED DESCRIPTION OF THE INVENTION

[0015] A description of example embodiments of the invention follows.

[0016] The invention provides, *inter alia*, synthetic microorganisms for enhanced bioleaching that include a heterologous *hcnABC* operon, which results in enhanced cyanide production by the microorganism, and methods of using these microorganisms for, *e.g.*, bioleaching of metals, such as precious metals, *e.g.*, gold, from sources such as electronic waste.

[0017] “Microorganism” refers to microscopic single-celled and multi-celled organisms and includes bacteria, algae, and fungi.

[0018] A “synthetic microorganism” is a microorganism that is engineered by the hand of man and does not exist in nature. A synthetic microorganism, in particular embodiments, comprises a heterologous nucleic acid sequence—either integrated in its genome or maintained as an extragenomic plasmid—where, in particular embodiments, the heterologous nucleic acid sequence confers a new or enhanced function on the microorganism, such as increased cyanide production.

[0019] An “*hcnABC* operon” is a nucleic acid comprising a regulatory sequence in operative association with a coding sequence that, together with the regulatory

sequence, provides the hydrogen-cyanide-producing function of the *hcnABC* operon in organisms, such as *Chromobacterium violaceum*. For example, in *Chromobacterium violaceum*, the *hcnABC* operon comprises three genes: 1) *hcnA* (NCBI GeneID 2548889, reference protein sequence NP_901354.1, comprising a conserved Fer2_4 superfamily domain, pfam13510; see also YP_610914.1 (*Pseudomonas entomophila*), NP_250883.1 (*Pseudomonas aeruginosa*), and WP_003180501.1 (*Pseudomonas fluorescens*)), 2) *hcnB* (NCBI GeneID 2549117, reference protein sequence NP_901353.1, comprising conserved NAD-binding8 domain (pfam13450), Fer2-BFD-like domain (pfam04324), and NAD(FAD)-dependent dehydrogenase (COG0446); see also WP_003223946.1 (*Pseudomonas fluorescens*) and YP_001348462.1 (*Pseudomonas aeruginosa*)), and 3) *hcnC* (NCBI GeneID 2548500, reference protein sequence NP_901352.1, comprising conserved hydroxyglutarate oxidase (PK11728) and DadA (COG0665) domains; see also WP_003180503.1 (*Pseudomonas fluorescens*) and WP_003132105.1 (*Pseudomonas aeruginosa*)).

[0020] An *hcnABC* operon for use in the present invention can, in some embodiments, comprise sequences at least 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, or 100% identical to the mature peptides described for any of the above organisms, or a functional fragment thereof that retains the ability to produce hydrogen cyanide in the context of the other genes in the operon. In particular embodiments, the functional fragment comprises about: 60, 65, 70, 75, 80, 85, 90, 95, or 100% of the length of the mature peptides described above. In certain embodiments, the functional fragments comprise the conserved domains described for the above proteins. An *hcnABC* operon for use in the invention can be from any organism, including a synthetic *hcnABC* operon engineered using any functional combination of the above-described sequences.

[0021] A “heterologous *hcnABC* operon” is an *hcnABC* operon contained in a microorganism—either by plasmid or genomic integration—that includes some inserted heterologous sequence. For example, in some embodiments, a heterologous promoter sequence, such as an inducible promoter sequence, can be integrated into a naturally-

occurring *hcnABC* operon locus to produce an inducible heterologous *hcnABC* operon. Alternatively, the coding sequence of an *hcnABC* operon can be inserted as a heterologous nucleic acid to be in operative association with an endogenous promoter in the microorganism. In other embodiments, a heterologous *hcnABC* operon can include the coding portion of an *hcnABC* operon in operative association with a regulatory sequence—inducible, repressible, derepressable, or constitutive—that is either heterologous or endogenous to the associated *hcnABC* operon coding sequence and is inserted together with it into the microorganism. For example, an inducible promoter can be appended to an organism's native *hcnABC* operon coding sequence and the resulting heterologous *hcnABC* operon can be inserted into the genomic DNA of the microorganism at an ectopic locus, or supported on an extragenomic plasmid in the microorganism.

[0022] A regulatory sequence is in “operative association” with a second nucleic acid when the regulatory sequence can drive the transcription of the second nucleic acid, such as a reporter molecule, under appropriate circumstances. In some embodiments, two sequences can be in operative association, and additional sequence elements such as enhancers or promoters may be present in the construct.

[0023] An organism which is “hypomorphic for cyanate hydratase” has reduced cyanate hydratase (*Chromobacterium violaceum* GeneID 2547440) activity by virtue of one or more of: reduced activity of the expressed protein (*e.g.*, a hypomorphic allele, including a fully null or knockout allele), reduced expression of the protein or nucleic acid encoding it, or a combination thereof.

[0024] “Pulp” is a slurry or suspension of a composition to be bioleached using the methods and synthetic microorganisms provided by the invention. In some embodiments, the slurry is of a solid, such as a powder, or otherwise dispersed material, such as crushed ore or electronic scrap material (ESM), mixed with growth medium for the synthetic microorganism. In other embodiments, whole ESM or large ore particles are contained in the suspension. The pulp density can be any density suitable for bioleaching of metals, such as gold. In some embodiments, the pulp density can be at

least about: 0.1, 0.125, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0% (W/V), or more, *e.g.*, in some embodiments, about .025 to about 1.0 %, *e.g.*, about 0.5%. Particles in the slurry can be any size. In particular embodiments, the pulp comprises particles with an average diameter of about: 5, 10, 12.5, 25, 50, 75, 100, 125, 150, 175, 200 μm , or less, *e.g.*, an average diameter of less than about 100 μm .

[0025] “Rare earth elements” refer to lanthanides plus scandium and yttrium.

EXEMPLIFICATION

INTRODUCTION

[0026] The ever increasing demand for electronics, fueled by a rising affluence in societies, coupled with the short lifetime of electronic devices, has resulted in the generation of a torrential electronic waste stream in our modern world. This electronic waste stream presents a major disposal challenge, as electronic waste contains toxic metallics, such as lead and mercury. In addition to its inherent toxicity, electronic waste also contains significant amounts of precious metals such as gold (Au). In fact, compared with natural Au ores, the Au content in electronic scrap material (ESM) is significantly higher (10 g to 10 kg Au per ton of ESM as compared to 0.5 g to 13.5 g Au per ton of natural Au ore), creating an economic driving force for the recycling of electronic waste.

[0027] In 2009, the United Nations Environment Program (UNEP) estimated the global electrical and electronic waste (e-waste) generated to be around 40 million tons annually. The same report projected that, by 2020, the waste stream would double in OECD countries and surge up to 500% from 2007 levels in developing nations. Electronic waste shows a higher growth rate than any other category of municipal waste. E-waste is growing at an alarming rate due to increased consumption of electronic devices and shorter life span of mobile phones, computers, televisions and other electronic devices. Manufacturing mobile phones and personal computers consumes 3% of gold and silver, 13% of palladium and 15% of cobalt mined worldwide each year.

[0028] Au is used in electronics for its excellent resistance to corrosion and high electrical conductivity. Existing processes for recovering Au from electronic waste are either pyrometallurgical (which are energy intensive) or hydrometallurgical (which use chemical lixiviants such as aqua regia, a mixture of concentrated nitric and hydrochloric acids), and are thus extremely pollutive and not environmentally sustainable.

[0029] In contrast to existing processes, bioleaching, involving microorganisms such as *Chromobacterium violaceum*, can 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. 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. The cyanide lixiviant is derived from the secondary metabolite hydrogen cyanide (HCN) produced from glycine using the enzyme HCN synthase (encoded by the *hcnABC* operon in cyanogenic *C. violaceum*); 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. Here we report the construction of two metabolically-engineered *C. violaceum* strains that produced more cyanide lixiviant, and recovered more Au from electronic waste, than wild-type bacteria. The metabolically-engineered microorganisms provided by the invention can be used in the recovery of commodity metals (such as Au, Fe and Cu) from electronic waste, the detoxification of toxic metals from waste and landfills, and the recovery and remediation of mine tailings.

RESULTS

Construction of metabolically-engineered *C. violaceum* strains

[0030] Two metabolically-engineered *C. violaceum* strains (L-arabinose and IPTG for *pBAD* and *pTAC*, respectively) were constructed by site-specific integration of the duplicated *hcnABC* operon under the transcriptional control of the exogenous promoters

pBAD (L-arabinose-inducible) and *pTAC* (Isopropyl-thiogalactoside/IPTG-inducible), respectively (FIG.1).

Enhanced cyanide lixiviant production in *pBAD* and *pTAC* strains

[0031] Under optimal inducer concentrations, the metabolically-engineered *pBAD* strain produced 68% more cyanide lixiviant compared to wild-type bacteria, whilst the *pTAC* strain produced 51% more cyanide lixiviant than the non-engineered wild-type cyanogenic bacteria (FIG. 2). The differing times for production of cyanide between wild-type and engineered strains of *C. violaceum* is a result of the differing growth rates and induction times between the strains (FIG. 8). Wild-type *C. violaceum* was grown in media without antibiotic supplementation, while engineered strains of *C. violaceum* were grown in media with gentamycin supplementation; the absence or presence of antibiotics in the growth media resulted in the differing growth rates. Both *pBAD* (FIG. 3) and *pTAC* (FIG. 4) strains exhibited typical dose-responses (cyanide production) to varying concentrations of the inducer (L-arabinose for *pBAD*, and IPTC for *pTAC*).

Enhanced Au recovery from electronic waste by metabolically-engineered strains

[0032] The engineered *pBAD* and *pTAC* *C. violaceum* strains were used to recover precious Au from ESM. Bioleaching studies revealed that there were significant increases in the recovery of Au from the ESM electronic waste for both strains of engineered *C. violaceum*, as compared to wild-type bacteria. After 8 days of bioleaching treatment, an Au recovery of 30% and 25% of the total amount of Au present in the electronic waste was achieved with the engineered *pBAD* and *pTAC* strains, respectively (FIG. 5); using wild-type bacteria under identical bioleaching conditions, an Au recovery of 11% of the total amount of Au present in the ESM was obtained. This represented increases of 170% and 120% in Au recovery from ESM by the *pBAD* and *pTAC* strains, respectively.

Was cyanide production decoupled from quorum control?

[0033] In order to decouple lixiviant production from quorum control, exogenous promoters *pBAD* and *pTAC* were used to drive the expression of the *hcnABC* operon. When early induction (prior to mid-logarithmic phase) was performed, we observed the

early production of cyanide lixiviant by the metabolically-engineered bacteria (FIG. 6). These observations, coupled with the observations of tunable, dose-responsive (under varying inducer concentrations) cyanide production, demonstrated that cyanide production in the metabolically-engineered *pBAD* and *pTAC* strains was effectively decoupled from quorum control.

Comparative proteomics study of cyanogenically-enhanced *C. violaceum*

[0034] Using iTRAQ (isobaric Tags for Relative and Absolute Quantitation) (Treumann, A., and Thiede, B, “Isobaric protein and peptide quantification: perspectives and issues,” *Expert Review of Proteomics*, 7, 647-653 (2010)) labeling of peptide digests of the wild-type and *pBAD* proteomes, coupled with mass spectrometric analyses, quantitative proteomics maps of the engineered and wild-type strains were obtained (Tables 1A-1C).

DISCUSSION

[0035] Here we report the construction of two metabolically-engineered *C. violaceum* strains (*pBAD* and *pTAC*) that produced significantly more cyanide lixiviant compared to wild-type bacteria. Lixiviant production was effectively decoupled from quorum control through the use of exogenous promoters. In corroboration with the observed increase in cyanide production by the engineered strains, Au recovery from ESM was significantly increased in the engineered strains. Our efforts in modulating the lixiviant metabolism of *C. violaceum* demonstrate that enhanced bioleaching microbes can be constructed as sustainable means of recovering precious metals such as Au from electronic waste.

[0036] Wild-type *C. violaceum* produces cyanide from glycine for short periods at the early stationary phase in its growth. The reliance of lixiviant production on cell population density is quorum-controlled. In an effort to obtain suitable strains of *C. violaceum* for bioleaching of Au from electronic waste, we examined if lixiviant production can be decoupled from quorum control and, if so, whether cyanide production can be increased by modulating the lixiviant metabolic pathways of the organism. Normal cyanide metabolism is maintained by combined actions of the

cyanogenic *hcnABC* operon and the cyanolytic *cynTSX* operon. We sought to engineer a tightly-regulated and tunable (responsive to varying concentrations of inducer) bioleaching strain by integrating a single copy of the *hcnABC* operon, under the transcriptional control of the exogenous promoters, into the bacterial genome. This site-specific genomic integration of an inducible cyanogenic operon was performed using Tn7-mediated transposition. We inserted a duplicate copy of the *hcnABC* operon (under the respective transcriptional control of exogenous promoters *pBAD* or *pTAC*) to decouple cyanogenesis from quorum control.

[0037] By design, genomic insertions can be made at specific Tn7 attachment (*attTn7*) sites located downstream of the highly conserved *glmS* gene (encoding for glucosamine 6-phosphate synthetase) in bacteria. The *glmS* gene of wild-type *C. violaceum* (GI: 34496132) is located upstream of a functionally unassigned gene (GI: 34496133) with a 45 bp intergenic region that is predicted to contain the *attTn7* site. However, in contrast to studies with other bacterial species genomic integration in *C. violaceum* did not occur at the intergenic region downstream of the *glmS* gene; instead, the transposable segment was inserted within the 3' region of the *glmS* gene (after 1607 bp of the 1830 bp intact *glmS* gene). Despite disruption of the *glmS* gene, bacterial fitness in the engineered *C. violaceum* strains was not compromised (growth curves of wild-type and engineered strains are virtually identical (FIG. 9)).

[0038] In attempts to increase the production of the cyanide lixiviant, we used a range of inducer concentrations to obtain dose-responsive profiles of cyanide production. Using L-arabinose as the exogenous inducer, cyanide production was increased above wild-type levels (FIG. 3), with maximal cyanide production (relative to uninduced levels) observed with 0.002% L-arabinose. An analogous dose-responsive profile was obtained with IPTG as the exogenous inducer (FIG. 4), where the addition of 1 mM IPTG resulted in maximal cyanide production, compared to uninduced or wild-type levels. Our observations indicated that the engineered *pBAD* and *pTAC* strains exhibited tunable, enhanced cyanide lixiviant production. The observed prototypical dose-responsive profiles also suggested that lixiviant production in these strains

had been decoupled from quorum-control. A comparison of the lixiviant profiles of wild-type *versus* engineered strains revealed that the *pBAD* and *pTAC* strains produced peak concentrations (at 30 hrs after inoculation) of 34.5 mg/L and 31 mg/L of cyanide, respectively (representing significant increases over the wild-type peak concentration of 20 mg/L of cyanide (FIG. 2)).

[0039] The engineered *pBAD* and *pTAC* *C. violaceum* strains were used to recover precious Au from ESM. Bioleaching studies revealed that there were significant increases in the recovery of Au from the ESM electronic waste for both strains of engineered *C. violaceum*, as compared to wild-type bacteria (FIG. 5). There have been studies on the use of cyanogenic bacteria in the recovery of Au from solid waste; the reported modest recovery (up to 14.9% of total amount of Au present) corroborates with our observation (11% of total amount of Au present) on the limited utility of wild-type cyanogenic bacteria in bioleaching. In comparison, we were able to achieve Au recovery in excess of 30% of total amount of Au present using engineered *C. violaceum*. These results illustrate using bioleaching as a sustainable means of recovering precious metals from electronic waste.

[0040] We sought insights into the modulations of the lixiviant metabolic network of the *pBAD* strain through a comparative proteomics study of the cyanogenically-enhanced variant against the wild-type *C. violaceum* strain (FIG. 7). We examined if the observed levels of cyanide produced in the engineered strain could be further increased with future metabolic engineering.

[0041] Cyanide production through the actions of HCN synthase can be decreased by decreasing the availability of the substrate glycine; consequently, glycine flux away from cyanogenesis can be independently achieved through the actions of enzymes such as serine hydroxymethyltransferase (glyA, in the biosynthesis of serine from glycine) (Motokawa, Y., and Kikuchi, G., "Glycine metabolism in rat liver mitochondria: V. Intramitochondrial localization of the reversible glycine cleavage system and serine hydroxymethyltransferase," *Archives of Biochemistry and Biophysics*, 146: 461-466 (1971)), glycine decarboxylase (gcvP, in the glycine cleavage system) (Hiraga, K., and

Kikuchi, G., “The mitochondrial glycine cleavage system: Purification and properties of glycine decarboxylase from chicken liver mitochondria,” *J. Biol. Chem.*, 255: 11664-11670 (1980)), serine dehydratase (sdaA1, in serine catabolism) (Yoshida, T., and Kikuchi, G., “Comparative Study on Major Pathways of Glycine and Serine Catabolism in Vertebrate Livers,” *Journal of Biochemistry*, 72: 1503-1516 (1972)), threonine aldolase (CV_4309, in the biosynthesis of threonine) and glycine C-acetyltransferase (kbl, in the biosynthesis of threonine) (Miller, D. A., and Simmonds, S., “The Metabolism of L-threonine and Glycine by *Escherichia coli*,” *Proceedings of the National Academy of Sciences*, 43: 195-199 (1957)). Our comparative proteomics study revealed that upon an increase in cyanide production in the engineered strain, there were no significant changes in the protein levels of these enzymes (directly and indirectly) associated with the cyanolytic pathways (FIG. 7). Cyanolysis can also be directly achieved through the actions of cyanase (Carepo, M. S., *et al.*, “Identification of *Chromobacterium violaceum* genes with potential biotechnological application in environmental detoxification,” *Genetics and Molecular Research*, 3: 181-194 (2004)); our study did not detect significant changes in the levels of cyanase in the engineered strain, compared to wild-type *C. violaceum*. In addition, we did not observe any significant changes in the protein levels of enzymes associated with glycine biosynthesis, and, hence, cyanogenesis.

[0042] Cyanogenesis in bacteria and plants is associated with virulence and defense; in the context of *C. violaceum*, our comparative proteomics study revealed significant modulations in metabolic pathways associated with metabolic dormancy and energy conservation. Taken together, an increase in nucleotide salvage (represented by a decrease in the enzyme purine nucleoside phosphorylase (Agarwal, K. C., *et al.*, “Purine nucleoside phosphorylase: VI. Microheterogeneity and comparison of kinetic behavior of the enzyme from several tissues and species,” *Biochemistry*, 14: 79-84 (1975))/pnp), a decrease in deoxyribonucleotide biosynthesis (represented by a decrease in thioredoxin/trxA levels, the essential cofactor for ribonucleotide reductase (Ashley, G. W., and Stubbe, J., “Current ideas on the chemical mechanism of ribonucleotide

reductases,” *Pharmacology & Therapeutics*, 30: 301-329 (1985))), a decrease in the capacity for oxidative stress response (represented by decreases in superoxide dismutase/sodB1 and thioredoxin/trxA levels) (Camhi, S. L., *et al.*, “The oxidative stress response,” *New Horizons*, 3: 170-182 (1995)), and an increase in fatty acid biosynthesis (represented by an increase in enoyl-ACP reductase/CV_3743 levels, an essential enzyme in the biosynthesis of saturated straight-chain fatty acids; and a decrease in 3-hydroxyacyl-CoA dehydrogenase/fadB levels, an enzyme associated with β -oxidation of fatty acids) (Mead, J. F., “Lipid Metabolism,” *Annual Review of Biochemistry*, 32: 241-268 (1963)) suggested that the increased levels of cyanide production were within the cyanolytic capacity of the engineered strain. Our hypothesis was further corroborated by the observation that there was no significant change in protein levels of cioA (Zlosnik, J. E. A., *et al.*, “Investigation of the physiological relationship between the cyanide-insensitive oxidase and cyanide production in *Pseudomonas aeruginosa*,” *Microbiology*, 152: 1407-1415 (2006)), a cyanide-insensitive terminal cytochrome oxidase in the respiratory electron transport chain of *C. violaceum*; if cyanide levels were to exceed the inherent cyanolytic capacity of the bacterium, the expectation would be a significant up-regulation of cioA, so that aerobic respiration (in particular, cytochrome c oxidase) would not be inhibited.

[0043] We have demonstrated that lixiviant metabolism in *C. violaceum* can be engineered for enhanced cyanide production; a decoupling of cyanogenesis from quorum control resulted in a significant increase in cyanide production, and, correspondingly, an increase in Au recovery from electronic waste. Comparative proteomics analyses suggested that further increase in cyanogenesis is possible, and our results highlight the utility of lixiviant metabolic engineering in the construction of next-generation bioleaching microbes for the recovery of precious metals such as Au from electronic waste.

METHODS

Construction of *Chromobacterium violaceum* strains

[0044] Wild-type *C. violaceum* (ATCC 12472) was obtained from ATCC. The *hcnABC* operon was PCR amplified from the genomic DNA isolated from *C. violaceum* (ATCC 12472) using Platinum Pfx DNA polymerase (Invitrogen) and the primer sets *hcnABC*-XbaI-F/*hcnABC*-XhoI-R (Table 2). Exogenous inducible promoters *pBAD* and *pTac* were cloned upstream of the *hcnABC* operon. The *pBAD* and *pTac* promoters were PCR amplified from pBAD33 and pET15b vectors, respectively. After PCR amplification, the respective promoters were cloned upstream of the *hcnABC* operon into pUC18-mini-Tn7T-Gm vectors. The Tn7-based genomic transposition method as described by Choi and Schweizer (Choi, K. H., *et al.*, “A Tn7-based broad-range bacterial cloning and expression system,” *Nat. Methods*, 2: 443-448 (2005)) was used for genomic integration of the duplicated *hcnABC* operon into the genome of *C. violaceum*. Genomic integration was confirmed by DNA sequencing of the insertion sites using the primer sets P-Tn7L and P-0678 (Table 2).

Electronic Scrap Material (ESM)

[0045] The electronic waste (ESM) used in this study was supplied by Cimelia Resource Recovery (Singapore). The grey dust-like ESM was generated by shredding and other mechanical separation processes during mechanical recycling of ESM parts such as printed circuit boards. ESM used in this study were of particle size less than 100 μm . We used the same batch of ESM for all our bioleaching experiments. To check on the uniformity in the composition of gold, we conducted acid digestion experiments after every few batches of bioleaching runs. The initial amount of gold present in the ESM was 0.24 ± 0.04 mg/g of ESM. Gold content in this ESM from printed circuit board scrap was higher than that reported by Ilyas, *et al.*, Liang, *et al.*, and Xiang, *et al.* (Ilyas, S., *et al.*, “Bioleaching of metals from electronic scrap by moderately thermophilic acidophilic bacteria,” *Hydrometallurgy*, 88: 180-188 (2007); Liang, G., *et al.*, “Novel strategies of bioleaching metals from printed circuit boards (PCBs) in mixed cultivation of two acidophiles,” *Enzyme and Microbial Technology*,

47: 322-326 (2010); Xiang, Y., *et al.*, “Bioleaching of copper from waste printed circuit boards by bacterial consortium enriched from acid mine drainage,” *Journal of Hazardous Materials*, 184: 812-818 (2010)), and was comparable with ESM reported by Hagelüken (Hagelüken, C., “Recycling of electronic scrap at Umicore’s integrated metals smelter and refinery,” *World of Metallurgy – ERZMETALL*, 59: 152-161 (2006)).

Metal analyses

[0046] ESM samples with weight 1.000 ± 0.005 g were added in 250 mL Erlenmeyer flasks. Each sample was subjected to aqua regia [mixture of concentrated nitric acid (69% m/v) and hydrochloric acid (37% m/v) at 1:3 ratio] leaching, and the resulting residue was sent for Inductively-Coupled Plasma Mass Spectrometry (ICP-MS) metal-analysis using an Agilent 7500a ICP-MS spectrometer. The residue was dried and weighed to determine the mass of the solubilized metal.

Growth and induction of bacteria cultures

[0047] Wild-type and engineered *C. violaceum* were inoculated and grown in LB media from single colonies. The engineered *pBAD* and *pTAC* strains were grown in LB supplemented with gentamycin at 15 μ g/mL. From an overnight confluent culture (1×10^7 cfu/mL), a 1% (v/v) inoculum was used to inoculate 100 mL of LB medium in a 250 mL Erlenmeyer flask; the culture was incubated at 30°C, with shaking at 170 rpm; and, for the engineered strains, respective inducers (0.0002%, 0.002%, 0.02% and 0.2% L-arabinose for *pBAD*; 0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM and 1 mM IPTG for *pTAC* strains) were added to the cultures during mid-log phase (12 hrs). To demonstrate decoupling of cyanide production from quorum control for the engineered strains, respective inducers were added to the cultures pre mid-log phase (6 hrs).

Cyanide analyses

[0048] Free cyanide present in samples was measured using a cyanide electrode and an Ion Selective Electrode (ISE) meter (Thermoscientific Orion). Six-point calibrations (0, 0.5, 1, 5, 10, 25 ppm) were performed prior to cyanide measurements. Dilutions

were done to fit the measurements within the dynamic range of the calibrated electrode when cyanide concentrations were above 25 ppm.

Bioleaching studies

[0049] Bioleaching of ESM was carried out with wild-type and engineered *C. violaceum* in 250 mL Erlenmeyer flasks with 100 mL of Luria-Bertani (LB) at a pulp density of 0.5% (w/v). A two-step bioleaching process was performed during Au bioleaching of ESM: bacteria were initially cultured in LB in the absence of ESM, and sterilized ESM was then added to the bacterial cultures upon cyanide production. The cultures were incubated at 30°C, with shaking at 170 rpm, over eight days after ESM addition. Samples were taken daily, with debris removed by centrifugation (11,000 g for 15 mins), and cyanide and metal analyses were performed as previously described. The cyanide produced over eight days after ESM addition is shown in FIG. 10.

Comparative proteome analyses

[0050] Proteome analyses were performed using three biological replicates each of wild-type and engineered *C. violaceum*; a technical replicate for each biological replicate was also performed. The growth of wild-type and *pBAD* strains were performed as previously described. For the engineered *pBAD* strain, 0.002% L-arabinose was used to induce the bacteria cultures at mid-log phase. After 16 hours post-induction (corresponding to maximal cyanide production), the cells were harvested by centrifugation, washed with M9 minimal media, and resuspended in lysis buffer (8 M urea, 25 mM TEAB, 2% Triton X-100, 0.1% SDS, 1X protease inhibitor cocktail and 50 µg/mL of DNase and RNase) (Sadowski, P. G., *et al.*, “Quantitative proteomic approach to study subcellular localization of membrane proteins,” *Nat. Protoc.*, 1: 1778-1789 (2006)). The cells were lysed by sonication, lysates were clarified by centrifugation, and the supernatants were collected and subjected to iTRAQ labeling.

[0051] For iTRAQ labeling, 50 µg of proteins were reduced and alkylated prior to trypsin digestion; the resulting peptides were then lyophilized and reconstituted before labeling, according to the manufacturer’s instructions (AB SCIEX). Protein identification and relative iTRAQ quantitation were performed using an AB SCIEX

TripleTOF 5600 mass spectrometer, with PROTEINPILOT™ software version 4.5 using PARAGON™ Algorithm 4.5.0.0; a search was made on the *C. violaceum* Uniprot complete proteome database entry version 19-Oct-2011, and on a reversed database to deduce the global false discovery rate. Sample type was iTRAQ 8plex (peptide labeled) with MMTS cysteine alkylation and an ID Focus on biological modifications. The cut-off value used for protein regulation was 1.3 for up-regulation and 0.7 for down-regulation (Tan, H. T., *et al.*, “Quantitative and temporal proteome analysis of butyrate-treated colorectal cancer cells,” *Molecular & Cellular Proteomics*, 7: 1174-1185 (2008)).

[0052] The bars in FIGs. 2 to 6 represent the standard deviation for the various points in the plots for the various experiments.

Construction of *Chromobacterium violaceum* strains

[0053] Wild-type *C. violaceum* (ATCC 12472) was obtained from ATCC. The *hcnABC* operon was PCR amplified from the genomic DNA isolated from *C. violaceum* (ATCC 12472) using Platinum Pfx DNA polymerase (Invitrogen) and the primer sets *hcnABC*-XbaI-F/*hcnABC*-XhoI-R (Table 2). The PCR reaction (100 µL) contained 1 ng of genomic DNA, 10 µL of 10X *Pfx* Amplification Buffer, 1 mM MgSO₄, 0.4 mM of each dNTP, 40 pmol of each primer, and 5 U of Platinum *Pfx* DNA polymerase. The gene was amplified using a PTC-0200G Thermal Cycler (Bio-Rad Laboratories), with the following parameters: 94°C for 1 min followed by 40 cycles of: 94°C for 1 min, 55°C for 1 min and 15 sec, 68°C for 5 min, and a final extension of 68°C for 10 min. Exogenous inducible promoters *pBAD* and *pTac* were cloned upstream of the *hcnABC* operon. The *pBAD* and *pTac* promoters were PCR amplified from pBAD33 and pET15b vectors, respectively. The PCR reaction (100 µL) contained 1 ng of plasmid DNA, 10 µL of 10X *Pfx* Amplification Buffer, 1 mM MgSO₄, 0.4 mM of each dNTP, 40 pmol of each primer, and 5 U of Platinum *Pfx* DNA polymerase. The gene was amplified using a PTC-0200G Thermal Cycler (Bio-Rad Laboratories), with the following parameters: 94°C for 1 min followed by 40 cycles of: 94°C for 1 min, 55°C for 1 min and 15 sec, 68°C for 3 min, and a final extension of 68°C for 10 min.

Table 1A

Uniprot Accession #	Name	Relative expression to wild-type	
Proteins Up-regulated			S.D.
Q7M7F1	Elongation factor Tu (tuf1)	1.477	0.186
Q7NQ65	Glutathione synthetase (gshB)	1.366	0.245
Q7NQG9	30S ribosomal protein S5 (rpsE)	1.456	0.270
Q7NXP1	Transcription termination factor Rho (rho)	1.537	0.254
Q7NXX7	Nsuccinylglutamate 5-semialdehyde dehydrogenase (astD)	1.562	0.401
Q7NY10	Purine nucleoside phosphorylase (pnp)	2.094	0.548
Q7NYY5	Acetaldehyde dehydrogenase (adhE)	1.838	1.141
Q7P1M2	Transketolase 1 (tktA)	2.457	1.334
Q7P255	Probable site-specific DNA-methyltransferase, cytosine-specific (CV_0006)	3.064	1.889
Q7NRN7	Enoyl-[acyl-carrier-protein] reductase [NADH] (CV_3743)	1.333	0.118

Table 1B

Uniprot Accession #	Name	Relative expression to wild-type	
Proteins with no detected change			
Q7NRJ9	Arginine deiminase (<i>arcA</i>)	1.115	0.385
Q7NUY9	ATP-dependent Clp protease proteolytic subunit (<i>clpP</i>)	1.043	0.190
Q7NWN7	Chaperone protein ClpB (<i>clpB</i>)	0.899	0.142
Q7NXU5	Acetate kinase (<i>ackA</i>)	1.153	0.272
Q7NYC6	Threonyl-tRNA synthetase (<i>thrS</i>)	0.926	0.073
Q7NYI8	Serine hydroxymethyltransferase (<i>glyA</i>)	1.012	0.189
Q7NYR7	Methionyl-tRNA synthetase (<i>metG</i>)	0.888	0.189
Q7NZF7	Adenosylhomocysteinase (<i>ahcY</i>)	1.118	0.186
Q7P0W5	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase (<i>dapD</i>)	1.207	0.123
Q7P1M4	Phosphoglycerate kinase (<i>pgk</i>)	0.977	0.203
Q7NQP9	Probable transcriptional regulator (<i>CV_4088</i>)	0.944	0.165
Q7NR07	Putative uncharacterized protein (<i>CV_3978</i>)	0.941	0.320
Q7NR66	Probable glutaryl-CoA dehydrogenase (<i>CV_3918</i>)	1.037	0.172
Q7NRJ7	NAD(P)H-flavin reductase (<i>ubiB</i>)	1.108	0.172
Q7NRS1	Phosphoenolpyruvate synthase (<i>ppsA</i>)	0.938	0.221
Q7NS39	Putative uncharacterized protein (<i>CV_3588</i>)	0.950	0.215
Q7NS77	Catalase (<i>katE</i>)	0.921	0.265
Q7NSI0	Chemotaxis protein CheV (<i>cheV2</i>)	1.092	0.226
Q7NTH1	Glutamate dehydrogenase (<i>gdhA</i>)	0.935	0.160
Q7NU29	L-serine dehydratase (<i>sdaA1</i>)	1.065	0.170
Q7NUM7	Putative uncharacterized protein (<i>CV_2670</i>)	0.963	0.129
Q7NV74	Aconitate hydratase 2 (<i>acnB</i>)	1.042	0.212
Q7NWD9	Probable zinc-containing alcohol dehydrogenase (<i>CV_2051</i>)	1.058	0.274
Q7NWT8	Probable outer membrane protein (<i>CV_1891</i>)	0.933	0.097
Q7NXH6	Glycine C-acetyltransferase (<i>kbl</i>)	0.881	0.102
Q7NZ48	Dihydrolipoyl dehydrogenase (<i>lpdA2</i>)	0.955	0.207
Q7NZ51	2-oxoglutarate dehydrogenase E1 component (<i>sucA</i>)	1.084	0.281
Q7NZ52	Citrate synthase (<i>gltA</i>)	0.984	0.127
Q7P022	Probable transformylase (<i>CV_0747</i>)	0.978	0.219
Q7P0M6	Putative uncharacterized protein (<i>CV_0540</i>)	1.153	0.131
Q7P182	Aromatic-amino-acid transaminase (<i>tyrB2</i>)	0.882	0.215
Q7P1T7	Ribonuclease G (<i>cafA</i>)	0.920	0.168
Q7P210	Uroporphyrin-III C-methyltransferase (<i>hemX</i>)	1.275	0.362
Q7NQE4	50S ribosomal protein L10 (<i>rplJ</i>)	0.979	0.220
Q7NQF7	50S ribosomal protein L22 (<i>rplV</i>)	0.841	0.145
Q7NRX0	Cyanide insensitive terminal oxidase (<i>cioA</i>)	1.002	0.066
Q7NQ31	L-allo-Threonine aldolase (<i>CV_4309</i>)	1.135	0.214

Q7NSJ5	Glycine dehydrogenase [decarboxylating] (<i>gcvP</i>)	1.223	0.625
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Table 1C

Uniprot Accession #	Name	Relative expression to wild-type	
Proteins Down-regulated			
Q7P190	Urocanate hydratase (<i>hutU</i>)	0.651	0.114
Q9ZHI1	Acetyl-CoA acetyltransferase (<i>phaA</i>)	0.445	0.099
Q7NPX8	Putative uncharacterized protein (<i>CV_4364</i>)	0.426	0.208
Q7NR08	Putative uncharacterized protein (<i>CV_3977</i>)	0.387	0.042
Q7NS55	Outer membrane protein A (<i>rmpM</i>)	0.250	0.160
Q7NSF9	Transmembrane protein (<i>elaB</i>)	0.434	0.059
Q7NTK8	30S ribosomal protein S1 (<i>rpsA</i>)	0.723	0.123
Q7NUH8	3-hydroxyacyl-CoA dehydrogenase (<i>fadB</i>)	0.637	0.096
Q7NV31	Amidophosphoribosyltransferase (<i>purF</i>)	0.704	0.136
Q7NV42	Superoxide dismutase (<i>sodB1</i>)	0.551	0.068
Q7NVG0	Putative uncharacterized protein (<i>CV_2383</i>)	0.637	0.403
Q7NWA2	Acetyl-CoA C-acetyltransferase (<i>atoB</i>)	0.523	0.163
Q7NWA4	3-hydroxybutyryl-CoA dehydrogenase (<i>paaH</i>)	0.637	0.156
Q7NWH0	Probable aldehyde dehydrogenase (<i>CV_2019</i>)	0.676	0.085
Q7NX40	Protein kinase (<i>prkA</i>)	0.745	0.113
Q7NXP2	Thioredoxin (<i>trxA</i>)	0.435	0.194
Q7NYA8	Probable phasin (<i>CV_1366</i>)	0.523	0.150
Q7NYB1	Probable trans-acting regulatory HvrA protein (<i>CV_1363</i>)	0.412	0.076
Q7NYU5	Probable small heat shock protein (<i>CV_1177</i>)	0.338	0.099
Q7NZK6	NADP-dependent malate dehydrogenase (<i>maeB</i>)	0.535	0.106
Q7NZQ3	Putative uncharacterized protein (<i>CV_0868</i>)	0.288	0.086
Q7P1C4	Glutathione S-transferase family protein (<i>gst2</i>)	0.386	0.263
Q7NQX1	60 kDa chaperonin 2 (<i>groL2</i>)	0.561	0.185

Table 2

Name of Primer	Sequence	Remarks
henABC-XbaI-F henABC-XhoI-R	5' GACACCTGTATCGCTCTAGAGAACCGGGCAATGGCGGAACCC 3' (SEQ ID NO: 1) 5' GGCTCAGTGAGAGGACTCGAGTCAGGCGTGCAAGCGCGAAATCC 3' (SEQ ID NO: 2)	Forward and reverse primers for cloning <i>henABC</i> into pUC18-mini-Tn7T-Gm
pBAD BamHI pBAD XbaI	5' ATTGTCTGATTTCGTGGATCCTTATGACAACTTGACGGGTAC 3' (SEQ ID NO: 3) 5' CAAAATTATTGAGCTCTCTAGAGCTAGCCCAAAAAACGGG 3' (SEQ ID NO: 4)	Forward and reverse primers for cloning PBAD promoter into pUC18-mini-Tn7T-Gm
pTAC BamHI pTAC XbaI	5' CGTTGGGATCCCTGCCCGCTTCCAGTCGGGAAACCTGTCGTGCCAGCTGC 3' (SEQ ID NO: 5) 5' CTAGTCTAGAACATTATACGAGCCGATGATTAATTGTCAGTGAAACCAACGTTATACG 3' (SEQ ID NO: 6)	Forward and reverse primers for cloning Ptac promoter into pUC18-mini-Tn7T-Gm
P-Tn7L P-0678	5' ATTAGCTTACGACGCTACACCC 3' (SEQ ID NO: 7) 5' TCGAGCCAAGGCCGACGAAC 3' (SEQ ID NO: 8)	Forward and reverse sequencing primers specific for site of Tn7L integration

[0054] It should be understood that for all numerical bounds describing some parameter in this application, such as “about,” “at least,” “less than,” and “more than,” the description also necessarily encompasses any range bounded by the recited values. Accordingly, for example, the description “at least 1, 2, 3, 4, or 5” also describes, *inter alia*, the ranges 1-2, 1-3, 1-4, 1-5, 2-3, 2-4, 2-5, 3-4, 3-5, and 4-5, *et cetera*.

[0055] For all patents, applications, and other references cited herein, such as non-patent literature and reference sequence or chemical information, it should be understood that it is incorporated by reference in its entirety for all purposes as well as for the proposition that is recited. Where any conflict exists between a document incorporated by reference and the present application, this application will control.

[0056] Headings used in this application are for convenience only and do not affect the interpretation of this application.

[0057] Preferred features of each of the aspects provided by the invention are applicable to all of the other aspects of the invention *mutatis mutandis* and, without limitation, are exemplified by the dependent claims and also encompass combinations and permutations of individual features (*e.g.*, elements, including numerical ranges and exemplary embodiments) of particular embodiments and aspects of the invention, including the working examples. For example, particular experimental parameters exemplified in the working examples can be adapted for use in the claimed invention piecemeal without departing from the invention. For example, for materials that are disclosed, while specific reference of each of the various individual and collective combinations and permutations of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein, as are methods of making and using such compounds. Thus, if a class of elements A, B, and C is disclosed as well as a class of elements D, E, and F, and an example of a combination of elements, A-D, is disclosed, then, even if each is not individually recited, each is individually and collectively contemplated. Thus, in this example, each of the combinations A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are specifically contemplated and should be considered disclosed from disclosure of A, B, and C; D, E, and F; and the example

combination A-D. Likewise, any subset or combination of these is also specifically contemplated and disclosed. Thus, for example, the sub-groups of A-E, B-F, and C-E are specifically contemplated and should be considered disclosed from the disclosure of A, B, and C; D, E, and F; and the example combination A-D. This concept applies to all aspects of this application, including elements of a composition of matter and steps of method of making or using the compositions.

[0058] The forgoing aspects of the invention, as recognized by the person having ordinary skill in the art following the teachings of the specification, can be claimed in any combination or permutation to the extent that they are novel and non-obvious over the prior art. Thus, to the extent an element is described in one or more references known to the person having ordinary skill in the art, they may be excluded from the claimed invention by, *inter alia*, a negative proviso or disclaimer of the feature or combination of features.

[0059] While this invention has been particularly shown and described with references to example embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

What is claimed is:

1. A synthetic microorganism engineered at genome level for enhanced bioleaching comprising a heterologous hcnABC operon integrated in the synthetic microorganism's genome that results in enhanced cyanide production by the microorganism, wherein

the heterologous hcnABC operon is not subject to quorum control;

the cyanide production is decoupled from quorum control through use of exogenous promoters, *pBAD* and *pTAC*; and

the microorganism is *Chromobacterium violaceum*.

2. The synthetic microorganism of Claim 1, wherein the *Chromobacterium violaceum* is ATCC accession number ATCC 12472 transformed with the heterologous hcnABC operon.

3. A synthetic microorganism engineered for enhanced bioleaching comprising a heterologous hcnABC operon contained on an extragenomic plasmid that results in enhanced cyanide production by the microorganism, wherein

the heterologous hcnABC operon is not subject to quorum control;

the cyanide production is decoupled from quorum control through use of exogenous promoters, *pBAD* and *pTAC*; and

the microorganism is *Chromobacterium violaceum*.

4. The synthetic microorganism of Claim 1, wherein the hcnABC operon is integrated in the synthetic microorganism's genome by site-specific genomic integration.

5. The synthetic microorganism of Claim 4, wherein the site-specific genomic integration is by Tn7-mediated transposition.

6. The synthetic microorganism of Claim 5, wherein the site-specific integration is at the glmS locus.
7. The synthetic microorganism of any one of the preceding claims, wherein the heterologous hcnABC operon comprises a heterologous regulatory sequence in operative association with an hcnABC operon.
8. The synthetic microorganism of Claim 7, wherein the heterologous regulatory sequence is an inducible regulatory sequence.
9. The synthetic microorganism of Claim 8, wherein the inducible regulatory sequence is selected from an IPTG (isopropylthiogalactoside)-inducible, L-arabinose-inducible, tetracycline-inducible, lactose-inducible, N-acyl homoserine lactone-inducible, propionate-inducible, rhamnose-inducible, hypoxia-inducible, or galactose-inducible regulatory sequence.
10. The synthetic microorganism of Claim 9, wherein the inducible regulatory sequence is selected from an IPTG-inducible or L-arabinose-inducible regulatory sequence.
11. The synthetic microorganism of any one of the preceding claims, wherein the heterologous hcnABC operon is an endogenous hcnABC operon in operative association with a heterologous inducible regulatory sequence.
12. The synthetic microorganism of any one of Claims 1-10, wherein the heterologous hcnABC operon is a heterologous hcnABC operon in operative association with a heterologous inducible regulatory sequence.
13. The synthetic microorganism of any one of the preceding claims, wherein the microorganism comprises 1, 2, 3, 4, 5, 6, 7, 8, or more heterologous hcnABC operons.

14. The synthetic microorganism of any one of the preceding claims, wherein the microorganism is hypomorphic for cyanate hydratase.
15. The synthetic microorganism of any one of the preceding claims, wherein the microorganism is placed into contact with a pulp comprising one or more metals under conditions for enhanced cyanide production by the heterologous hcnABC operon.
16. The synthetic microorganism of Claim 15, wherein the pulp has been pretreated by oxidation.
17. The synthetic microorganism of Claim 16, wherein the oxidation is by biooxidation.
18. The synthetic microorganism of Claim 17, wherein the biooxidation is with a microorganism capable of redox-based biooxidation, such as *Acidithobacillus ferrooxidans*.
19. The synthetic microorganism of any one of Claims 15-18, wherein the pulp density is between about 0.1 to about 5.0 % (W/V).
20. The synthetic microorganism of any one of Claims 15-19, wherein the pulp comprises particles with an average diameter of about: 5, 10, 12.5, 25, 50, 75, 100, 125, 150, 175, 200 μm , or less.
21. The synthetic microorganism of Claim 18, wherein the pulp comprises particles with an average diameter of less than about 100 μm .
22. The synthetic microorganism of any one of Claims 15-21, wherein the pulp comprises a mineral ore.

23. The synthetic microorganism of any one of Claims 15-22, wherein the pulp comprises electronic scrap material (ESM).
24. The synthetic microorganism of any one of Claims 15-23, wherein the one or more metals include one or more of gold, silver, platinum, copper, aluminum, nickel, zinc, iron, rare earths elements, other heavy metals, or a combination thereof.
25. A method of recovering one or more metals from a pulp comprising contacting the pulp with the synthetic microorganism of any one of Claims 1-22 under conditions for enhanced cyanide production by the heterologous hcnABC operon to solubilize the one or more metals and isolating the one or more solubilized metals.
26. A method of recovering gold from a pulp comprising electronic scrap material (ESM) comprising contacting the pulp with the synthetic microorganism of any one of Claims 1-22 under conditions for enhanced cyanide production by the heterologous hcnABC operon to solubilize the gold and isolating the solubilized gold.

4459 1081-001 FINAL SEQUENCE LISTING

SEQUENCE LISTING

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Wen Shan Yew
Yen Peng Ting
Song Buck Terence Tay
Gayathri Natarajan

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violaceum with Enhanced Cyanide Lixiviant Production for
Bioleaching of Precious Metal from Electronic Waste

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