Metals and Minerals as a Biotechnology Feedstock: Engineering Biomining Microbiology for Bioenergy Applications

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Abstract

Developing new feedstocks for the efficient production of biochemicals and biofuels will be a critical challenge as we diversify away from petrochemicals. One possible opportunity is the utilization of sulfide-based minerals in the Earth's crust. Non-photosynthetic chemolithoautotrophic bacteria are starting to be developed to produce biochemicals from CO₂ using energy obtained from the oxidation of inorganic feedstocks. Biomining of metals like gold and copper already exploit the native metabolism of these bacteria and these represent perhaps the largest-scale bioprocesses ever developed. The metabolic engineering of these bacteria could be a desirable alternative to classical heterotrophic bioproduction. In this review, we discuss biomining operations and the challenges and advances in the engineering of associated

chemolithoautotrophic bacteria for biofuel production. The co-generation of biofuels integrated with mining operations is a largely unexplored opportunity that will require advances in fundamental microbiology and the development of new genetic tools and techniques for these organisms. Although this approach is presently in its infancy, the production of biochemicals using energy from non-petroleum mineral resources is an exciting new biotechnology opportunity.

Introduction

The development of technologies for recycling CO₂ to create biofuels and biochemicals is a grand engineering challenge. Initial approaches have relied on photosynthesis to capture CO₂ and use photonic energy to generate chemical bonds which are then processed into biofuels directly or through heterotrophic bioprocessing. While plants and photosynthetic microorganisms are very good at capturing and using CO₂, the overall energy efficiency of photosynthesis is poor [1] and this approach competes with agriculture [2]. Ethanol is a first generation biofuel initially generated from food crops such as corn or sugarcane, and production of ethanol is moving towards exploiting cellulose as a feedstock. Non-photosynthetic autotrophic bacteria have also been explored for the fixation of CO₂ and the generation of useful chemicals and fuels [1,3,4]. Butanol is a second generation biofuel with a higher energy density and can be produced autotrophically by engineered bacteria [1,3-6].

Chemolithoautotrophs have evolved to oxidize inorganic materials and some of these bacteria, such as *Acidithiobacillus* spp. and *Leptospirillum* spp., are exploited in metal mining operations for increased leaching rates of metals from ores [7-9]. Given that the dimensions of a bioleaching heap can be as large as one trillion liters, it is thought that metal biomining with chemolithoautotrophic bacteria represents the largest-scale bioprocess ever developed [10]. These processes rely on native microbial consortia, but reports on the engineering of these cells are beginning to emerge [11-23]. Future work aimed at the metabolic engineering of these cells for co-generation of useful chemicals and fuels using atmospheric CO₂ and the energy available in metal sulfide feedstocks could represent a new paradigm in bioproduction. Currently, the globally

identified sulfur resources amount to approximately 5 billion tons [24] which can release up to 10^{14} MJ of energy during oxidation. These resources include elemental sulfur found in evaporite and volcanic deposits in addition to sulfur associated with natural gas, petroleum, tar sands, and metal sulfides [24]. The energy released during oxidation of sulfur [25] is comparable to other energy related materials such as petroleum and glucose [26,27] (Figure 1). This review describes the current state of biomining operations involving sulfide based feedstocks as well as recent advances in the genetic engineering of chemolithoautotrophic and related biomining bacteria with a focus on biochemical generation.

Figure 1. A comparison of energy densities from complete oxidation of petroleum, coal, ethanol, glucose and sulfur [25-27]. Although petroleum has a greater energy density than sulfur, metal sulfides are as abundant as petroleum and could represent an untapped feedstock for the production of biochemicals and biofuels.

Current Biomining Operations

Although the role of microorganisms in metal mining processes were undiscovered until approximately 70 years ago, it is likely these microbes have been contributing to the recovery of metals from ores for hundreds of years [7,28]. Biotechnological methods for recovery of metals are becoming increasingly important to current industrial applications. Copper and gold biomining practices are well-developed and accepted; in that globally, approximately 5% of gold, 15% of copper, and lesser percentages of the other base metals, are presently leached through the deliberate use of microorganisms [29]. Table 1 lists some examples of biomining operations that focus on bioleaching of metals or biooxidation of minerals for metal extractions.

Table 1. Bioleaching and Biooxidation Operations

Location	Company
Copper	
Morenci, Arizona	Freeport-McMoRan
Girilambone, Australia	Straits Resources & Nord Pacific
Lady Annie, Australia	NetMind Financial Holdings Ltd
Mount Gordon, Australia	Lighthouse Minerals and EMR Capital
Mt Leyshon, Australia	Formerly Normandy Poseidon
Birla Nifty, Australia	Aditya Birla
Whim Creek/Mons Cupri, Australia	Venturex Resources Ltd
Andacollo Cobre, Chile	Teck Resources
El Abra, Chile	Freeport-McMoRan
Escondida, Chile	BHP Billiton
Chuquicamata, Chile	Codelco
Cerro Colorado, Chile	BHP Billiton
Dos Amigos, Chile	CEMIN
El Abra, Chile	Freeport-McMoRan
Ivan-Zar, Chile	Compania Minera Milpro
Lince II, Chile	Antofagasta Plc
Lo Aguirre, Chile	Sociedad Minera Pudahuel
Lomas Bayas, Chile	Xstrata
Punta del Cobre, Chile	Sociedad Punta del Cobre
Quebrada Blanca, Chile	Teck Resources
Radomiro Tomic, Chile	Codelco
Spence, Chile	BHP Billiton
Tres Valles, Chile	Vecchiola Group
Zaldivar, Chile	Barrick Gold
Zijinshan Copper, China	Zijin Mining Group Co., Ltd
Skouriotissa Copper, Cyprus	Cyprus Mines Corporation
Monywa, Myanmar	Myanmar No.1 Mining Enterprise

Cerro Verde, Peru	Freeport McMoRan
Lisbon Valley, Utah	Lisbon Valley Mining Company
Jinchuan Copper, China	Zijin Mining Group
Skouriotissa Copper, Cyprus	Hellenic Copper
Cajamarca, Peru	Newmont Mining
Sotkamo, Finland	Talvivaara
Kasese town, Uganda	Kasese Cobalt Company Ltd
Gold	
Fairview, Barberton, South Africa	Pan African Resources
Sao Bento Brazil	AngloGold Ashanti
Wiluna, Australia	Apex Minerals
Ashanti, Obuasi, Ghana	AngloGold Ashanti
Youanmi, Australia	Apex Minerals
Ramboraque, San Mateo, Peru	Gold Hawk Resources
Beaconsfield, Tasmania, Australia	Beaconsfield Gold
Laizhou, Shandong Province, China	Eldorado Gold
Suzdal, Kazakhstan	Centroservice
Fosterville, Victoria, Australia	Northgate Minerals
Bogoso, Ghana	Golden Star Resources
Jinfeng, China	Eldorado Gold
Kokpatas, Uzbekistan	Navoi Mining and Metallurgy
Nickel/Zinc	
Sotkamo, Finland	Talvivaara Mining Plc
Adapted from [29] with modifications.	

Other metals, such as uranium, lead, manganese, and cobalt, have also been proven to be successfully biomined. Worldwide, there are over forty industrial plants targeting recovery of these various metals through bioleaching [30]. Additionally, although the process has not been proven on a large scale, there is promise for bioleaching of molybdenum [31]. Processing of waste streams is also becoming increasingly valuable through biomining. The metals in these materials were previously deemed uneconomical by non-biological methods. For example, today's copper tailings still contain about 0.1% weight copper; while older tailings, due to the inefficiency of prior processes, contain between 0.2 to 0.6% weight copper [32].

The large-scale engineering approaches to bioleaching or biooxidation in commercial applications include dump leaching, heap leaching, and continuous stirred tank

leaching. Another method, although not as commonly practiced in the present day, is *in situ* leaching. Dump bioleaching was first applied on an industrial scale around the mid-1900s [8]. This process is more predominant in the mining industry for bioleaching of copper. Dump leaching allows leaching of low-grade ores that cannot be economically processed by typical methods used to produce copper concentrate. These dumps are constructed by placing blasted below cut-off grade ore (anything below 0.6% wt. Cu [32]) on pads where it is treated with an acidic solution. This material is typically referred to as run-of-mine ore (ROM). Autotrophic microorganisms that are native to the environment catalyze the leaching process and become more prevalent as the conditions become more acidic and favorable for organism growth.

Although similar to dump bioleaching, heap bioleaching involves more planning and a greater depth of engineering in efforts to optimize the leaching conditions (Figure 2). Additional measures are taken in preparing the ore prior to stacking on a heap. Once the ore is blasted, it is crushed down to a smaller size. Crushing ore is dependent on ore grade, higher grade ore is crushed to a predetermined particle size. The ore is then conditioned by agglomeration, allowing finer particles to attach. Some operations condition the ore with indigenous microbes. Once the ore is conditioned, it is conveyed to specifically designed pads and stacked in lifts. Due to the oxidative conditions required for most metal dissolution reactions, these engineered heaps are aerated with external blowers. Forced air is a fairly common practice and is also important for the cultivation of microbes within the heap. Raffinate, a weak acidic solution, is applied and percolates throughout the heap. The leachate solution that is collected at the bottom of heaps, is typically processed through solvent extraction/electrowinning (SX/EW)

technologies. Designed organic solvent extractants are used to release the metal of interest from the host solution under vigorous mixing. After a series of settling and mixing procedures, a highly concentrated solution of the desired metal is routed to the final step, electrowinning (EW). A metal anode sheet is placed in a cell with the high metal containing solution and a high voltage is applied to the cell. The metal ions adhere to the anode metal sheet, creating a cathode of the leached metal. This process is detrimental to the survival of microbes within the leachate. It is estimated that there is an approximate loss of 10^2 cells per milliliter throughout the solvent extraction process. However, once the recycled solution is reapplied to the heap, the microbial population increases over time to approximately 10^4 - 10^6 microbes per gram of ore [33].

Heap bioleaching has more recently been applied as a pre-treatment process in leaching gold ores. This technology is particularly used to oxidize sulfide minerals that are later treated with reagents in order to recover the gold [34]. Stirred tank reactors are another engineering application used in biomining. The tanks operate at moderate temperatures, typically constructed out of stainless steel, aerated, and agitated in order to keep the concentrate suspended. Driven by operational costs, this technology is typically used to biooxidize or bioleach mineral concentrates [34]. A final example of industrial biomining applications is *in situ* bioleaching. This method consists of leaching ore in its natural location, without excavation. A solution is pumped through passages that have been drilled within the ore, the leachate is collected and then processed for the recovery of metals.

There is also some headway in the bioprocessing of certain ores under reducing conditions, such as with lateritic materials [28]. Nickel and cobalt are examples of

metals that can be extracted under reducing conditions, unlike the oxidizing conditions used to bioleach copper and gold. Development of bioleaching processes is the key to recovering the metals in increasingly abundant low-grade ores. Bioleaching methods are less energy intensive, environmentally considerate, and have the potential to generate by-products that can be of value to other processes within the mine. For these reasons alone, new biotechnological advancements within biomining will likely have a tremendous impact in future metal recovery efforts.

Examples of mineral reactions <u>without</u> microbes	Examples of mineral reactions <u>with</u> microbes
Pyrite $FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow S_2O_3^{2-} + 7Fe^{2+} + 6H^+$ (1)	Pyrite $FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow SO_4^{2-} + 7Fe^{2+} + 6H^+$ (1) $2Fe^{2+} + \frac{1}{2}O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$ (4)
Sphalerite $ZnS + Fe^{3+} + H^+ \rightarrow Zn^{2+} + 0.5H_2S_n + Fe^{2+} (n \geq 2)$ (2) $H_2S_n + 2Fe^{3+} \rightarrow 0.25S_8 + 2Fe^{2+} + 2H^+$ (3)	Sphalerite $ZnS + Fe^{3+} + H^+ \rightarrow Zn^{2+} + 0.5H_2S_{n \geq 2} + Fe^{2+}$ (2) $H_2S_n + 2Fe^{3+} \rightarrow 0.25S_8 + 2Fe^{2+} + 2H^+$ (3) $0.5S_8 + 3O_2 + 2H_2O \rightarrow 2SO_4^{2-} + 4H^+$ (5)

Figure 2. Schematic of heap bioleaching, which is a common biomining practice, and the reactions involved for the recovery of copper along with other metals. **Top Panel 1:** Ore is drilled in an arranged pattern and then blasted (left). An open pit is created with benches around 50 ft. high (right). Ore is then transferred to either a heap or crusher. 2: A conveyor system stacks the ore. 3: Specially designed heaps are constructed for various ore grades, particle sizes, flow rates, air injections, and multiple other factors. A weak acid solution is irrigated from the top and percolates throughout the heap. 4: Leachate solution reports from the bottom of the heap and routed to collection ponds. 5: A solvent extraction (SX) circuit is used to release the metal of interest from the host solution (left). Solution is sent to the electrowinning (EW) area where a metal cathode is produced (right). 6: Cathode sheets are then sent to customers or rod mills. **Bottom Panel** Heap leaching reactions – Left: Two examples of chemical reactions that are found in heap leaching without microbe reactions. Pyrite, a non-metal mineral, once oxidized, is a source of ferric iron (Fe^{3+}) and sulfuric acid (H_2SO_4). Without microbes, the reaction stalls after the initial reaction (1). Sphalerite is a metal containing mineral and relies on the presence of ferric iron for metal liberation (2). The products of reaction (3) are left with elemental sulfur and ferrous iron (Fe^{2+}) which, are not further utilized without microbial interaction. Right: When microbes are present, the product of the

initial reaction can become the reactants. Ferrous iron, a product of the initial reaction, can be oxidized back to ferric iron through microbial metabolisms (4). Elemental sulfur can be further oxidized to sulfuric acid (5). Thus, the reactions by microbes act as catalysts to the initial (1), (2), and (3) reactions.

Abundance of Reduced Material in the Earth's Crust

There are no known global shortages of mineral resources, and the demand for these resources will continue to grow, requiring continued efforts in development and exploration [35,36]. Thus the processing of energy rich sulfide streams will continue to accelerate. According to the United States Geological Survey, upwards of 70 chemical elements and dozens of minerals are mined from over 100 different deposit types and geologic surroundings [35].

Metal sulfides, tar sands, petroleum, natural gas, and elemental sulfur resources in evaporite and volcanic deposits make up approximately 5 billion tons of Earth's sulfur [24]. Most sulfur production is a result of fossil-fuel related processes, where opportunities for improvement in sustainability and supply are abundant. Sulfur reserves in sulfide ores are large, with one of the largest physical reservoirs in the Earth's crust being found in pyrite (FeS_2). Metal sulfides make up the majority of the world supplies of non-ferrous metals and can be considered to be a key group of ore minerals [37]. The metals most commonly found in sulfide minerals includes copper, cobalt, iron, lead, nickel, silver, and zinc. Metal concentration contained in sulfide ores is relatively low, typically less than a few percent [37]. These sulfide minerals can undergo biological oxidation under both aerobic and anaerobic conditions. When oxygen is present, the

oxidation of sulfur produces sulfuric acid. Elemental sulfur generates 530 kJ of heat per mole of sulfur oxidized. This energy, which is typically released as heat, can be exploited [38].

Mining companies worldwide are faced with depleting oxide ore bodies and are tasked with finding ways to tap into the deeper, more complex, sulfide ores [34]. A primary copper sulfide, chalcopyrite, accounts for 70% of the world's copper reserves [39,40]. As of 2016 the world's total copper reserves have been estimated to contain about 5 billion metric tons of copper [24]. Considering 70% of this copper is from chalcopyrite, which contains about 35% by weight of sulfur, the amount of sulfur from chalcopyrite can be calculated to be around 3.5 billion metric tons which when oxidized (energy of oxidation of elemental sulfur being 530 KJ/mole) can release energy in the order of 10^{13} MJ. However, chalcopyrite is a refractory copper containing mineral that poses many challenges for effective leaching, such as a proposed passivation layer that renders the mineral insoluble [41]. These types of deposits, as well as below cut-off grade material for copper [32] are becoming targets for processing. Table 2 lists these types of sulfide minerals.

The most abundant minerals for biooxidation include pyrite, pyrrhotite, galena, sphalerite, chalcopyrite, and chalcocite [37]. Mechanisms for the microbial oxidation of sulfide materials are shown in Figure 3 [42]. Metal bearing minerals such as arsenopyrite and chalcocite generate heats of reaction of -9,415 kJ/kg and -8,201 kJ/kg, respectively. Whereas, pyrite and pyrrhotite, minerals that do not harbor metals desirable for mining, generate heats of reaction of -12,884 kJ/kg and -11,373 kJ/kg [33].

Table 2. List of common types of sulfide minerals.

Mineral	Formula
Ferrous Containing	
Arsenopyrite	$FeAsS$
Bornite	Cu_5FeS_4
Chalcopyrite	$CuFeS_2$
Pyrite	FeS_2
Pyrrhotite	Fe_7S_8
Non-Ferrous Containing	
Argentite	Ag_2S
Chalcocite	Cu_2S
Covellite	CuS
Digenite	Cu_9S_5
Galena	PbS
Millerite	NiS
Molybdenite	MoS_2
Orpiment	As_2S_3
Sphalerite	ZnS

Figure 3. Schematic of two different mechanisms of mesoacidophilic bioleaching showing the intermediate sulfur compounds formed. A.f, A.t and L.f stand for *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans*, *Leptospirillum ferriphilum* bacteria respectively [42].

These minerals are also prime pathways for the conversion of ferrous (Fe(II)) to ferric (Fe(III)) iron, which not only liberates the desired metal from its host mineral, but is exothermic, increasing the temperature and the leach rate of metal-containing minerals. Along with the aforementioned pyrite and pyrrhotite minerals, arsenopyrite, chalcocite, and chalcopyrite also generate energy during oxidation, with an average heat of reaction of -9,069 kJ/kg of mineral. Heap leaching systems are currently in place that target the oxidation of pyrite ores, specifically for heat generation [43]. However, no efforts have been made to date to capture this energy to drive chemical bioproduction.

Microbiology in Mining Operations

The predominant microorganisms related to mining operations share some unique physiological characteristics: most are acidophilic (pH 1-3), have high metal ion resistance, are chemolithoautotrophic deriving carbon from CO₂, and derive energy from the oxidation of ferrous or reduced inorganic sulfur compounds (RISCs). The fixation of CO₂ in some of the chemolithoautotrophs including *Acidothiobacillus* is through the Calvin-Benson-Bassham pathway [3] which involves CO₂ capture using ribulose biphosphate carboxylase/oxygenase (RubisCo). Two structurally distinct forms of RubisCo (I and II), with different catalytic properties, are typically present in autotrophs and genes encoding both these forms has been found in *A. ferrooxidans* [10]. Other carbon fixation pathways identified in chemolithoautotrophs include the Wood-Ljungdahl pathway, the 3-hydroxy- propionate bicycle, the reductive tricarboxylic

acid cycle, 3-hydroxypropionate-4-hydroxy-butyrate cycle and 3-hydroxypropionate-4-hydroxy-butyrate cycle [3,44].

The optimal growth temperatures for these microorganisms vary considerably, such that mesophilic to extremely thermophilic microorganisms are involved. Various obligate acidophilic microbes, which naturally occur in mining environments, are listed in Table 3. Even though some of these microbes occur more frequently than the others, a naturally occurring microbial consortia is generally responsible for bioleaching and recovery of metals during mining [45].

The key mesoacidophilic microorganisms involved in biomining are *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans*, *Leptospirillum ferrooxidans* and *Leptospirillum ferriphilum*. Metal sulfide oxidation involved in heap bioleaching is exothermic, thus encouraging the growth of the thermoacidophiles. *Acidithiobacillus caldus* is common at moderately high temperatures (40-45 °C) while species from the genera *Metallosphaera*, *Sulfolobus* and *Acidianus* are considered to be the most important at temperatures higher than 60 °C [9,46].

Most of the biomining microorganisms derive energy from the oxidation of soluble ferrous or RISCs (summarized in Table 3) and generate ferric ions and sulfuric acid creating favorable conditions for bioleaching [47]. Most of these microorganisms are either iron oxidizers like *L. ferrooxidans* and *L. ferriphilum* or sulfur oxidizers like *A. thiooxidans* and *A. caldus* with the exception of *A. ferrooxidans*, which can oxidize both iron and sulfur.

Table 3. List of naturally occurring microbes within mining environments.

Species	Metabolism	Oxidize			Reduc e	Growth Range (°C)	Mode of Respiratio n	Genome
		Fe ²⁺ ₊	S ⁰	RIS C ⁱ	Fe ³⁺			Assembl y Level
<i>Acidiferrobacter thiooxydans</i>	Chemolithoautotroph ^a Diazotroph ^b	Yes	Yes	Yes	Yes	38-47	Facultativ e anaerobe	Contig
<i>Acidimicrobium ferrooxidans</i>	Mixotroph ^c	Yes	No	No	No	45-50	Aerobe	Complete
<i>Acidiphilium acidophilum</i>	Mixotroph ^c DIRB ^d	No	Yes	Yes	Yes	10-35	Aerobe	N/A
<i>Acidiphilium angustum</i>	Heterotroph ^e	No	No	No	No	20-42	Aerobe	Scaffold
<i>Acidiphilium cryptum</i>	Heterotroph ^e DIRB ^d	No	No	No	Yes	20-41	Aerobe	Complete
<i>Acidiphilium iwatense</i>	Chemoorganotroph ^f	No	No	No	No	22-35	Aerobe	N/A
<i>Acidiphilium multivorum</i>	Chemoorganotroph ^f	No	No	No	No	17-42	Aerobe	Complete
<i>Acidiphilium organovorum</i>	Heterotroph ^e DIRB ^d	No	No	No	Yes	30-42	Aerobe	N/A
<i>Acidiphilium rubrum</i>	Heterotroph ^e AAPB ^g (Zn-BChl <i>a</i>)	No	No	No	No	20-42	Facultativ e anaerobe	Contig
<i>Acidiphilium symbioticum</i>	Heterotroph ^e	No	No	No	No	30-37	Aerobe	N/A
<i>Acidithiobacillus albertensis</i>	Chemolithoautotroph ^a	No	Yes	Yes	No	25-30	Aerobe	N/A
<i>Acidithiobacillus caldus</i>	Mixotroph ^c	No	Yes	Yes	No	32-52	Aerobe	Complete
<i>Acidithiobacillus ferrovorans</i>	Chemolithoautotroph ^a Diazotroph ^b	Yes	Yes	Yes	Yes	4-37	Facultativ e anaerobe	Complete
<i>Acidithiobacillus ferridurans</i>	Chemolithoautotroph ^a	Yes	Yes	Yes	Yes	10-37	Facultativ e anaerobe	N/A
<i>Acidithiobacillus ferrooxidans</i>	Chemolithoautotroph ^a Diazotroph ^b	Yes	Yes	Yes	Yes	10-37	Facultativ e anaerobe	Complete
<i>Acidithiobacillus thiooxidans</i>	Chemolithoautotroph ^a	No	Yes	Yes	No	18-37	Aerobe	Contig
<i>Acidithrix ferrooxidans</i>	Heterotroph ^e DIRB ^b	Yes	No	No	Yes	10-30	Facultativ e anaerobe	Contig
<i>Acidobacterium capsulatum</i>	Chemoorganotroph ^f	No	No	No	No	25-37	Aerobe	Complete
<i>Acidocella facilis</i>	Chemoorganotroph ^f	No	No	No	No	25-37	Aerobe	Contig
<i>Alicyclobacillus disulfidooxidans</i>	Mixotroph ^c	Yes	Yes	Yes	No	4-40	Aerobe	N/A
<i>Alicyclobacillus tolerans</i>	Mixotroph ^c	Yes	Yes	No	Yes	20-55	Aerobe	Scaffold
<i>Ferrimicrobium acidiphilum</i>	Heterotroph ^e	Yes	No	No	Yes	30-37	Facultativ e anaerobe	Scaffold
<i>Ferrithrix thermotolerans</i>	Heterotroph ^e	Yes	No	No	Yes	37-50	Facultativ e	N/A

<i>Ferrithrix thermotolerans</i>	Heterotroph ^e	Yes	No	No	Yes	37-50	Facultative anaerobe	N/A
<i>Ferroplasma acidarmanus</i>	Mixotroph ^c	Yes	No	No	Yes	23-46	Facultative anaerobe	Complete
<i>Ferroplasma acidiphilum</i>	Chemolithoautotroph ^a	Yes	No	No	No	15-45	Aerobe	N/A
<i>Ferrovum myxofaciens</i>	Chemolithoautotroph ^a	Yes	No	No	No	5-30	Aerobe	Contig
<i>Leptospirillum ferriphilum</i>	Diazotroph ^b							
	Chemolithoautotroph ^a	Yes	No	No	No	25-45	Aerobe	Complete
<i>Leptospirillum ferrodiazotrophum</i>	Chemolithoautotroph ^a	Yes	No	No	No	37-42	Aerobe	N/A
	Diazotroph ^b							
<i>Leptospirillum ferrooxidans</i>	Chemolithoautotroph ^a	Yes	No	No	No	22-35	Aerobe	Complete
	Diazotroph ^b							
<i>Sulfobacillus acidophilus</i>	Mixotroph ^c	Yes	Yes	No	Yes	28-62	Facultative anaerobe	Complete
<i>Sulfobacillus benefaciens</i>	Facultative Autotroph ^h	Yes	Yes	Yes	Yes	30-47	Facultative anaerobe	N/A
	Mixotroph ^c							
<i>Sulfobacillus sibiricus</i>	Mixotroph ^c	Yes	No	No	No	30-55	Aerobe	N/A
<i>Sulfobacillus thermotolerans</i>	Mixotroph ^c	Yes	Yes	Yes	No	20-60	Aerobe	N/A

Adapted from Panda et al. [9] with modifications.

^a Chemolithoautotroph = Obtains energy by breaking down chemicals and carbon dioxide (CO₂) as carbon source

^b Diazotroph = Able to fix nitrogen (N₂) from the atmosphere

^c Mixotroph = Obtains energy and carbon source by heterotrophic and autotrophic metabolisms

^d DIRB = Dissimilatory iron reducing bacteria

^e Heterotroph = Unable to fix carbon dioxide (CO₂) and must use organic carbon as carbon source

^f Chemoorganotroph = Obtain energy and carbon source from organic carbon

^g AAPB = Aerobic Anoxygenic Photosynthetic Bacteria

^h Autotroph = Uses CO₂ as carbon source and produces organic substances

ⁱ RISC = Reduced inorganic sulfur compounds

* AAPB is stated to be present within all *Acidiphilium* species [60]

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While indigenous mesophilic (20-45 °C) and moderate thermophilic (45-60 °C) mining microbes are efficient in leaching metals, increasing temperature can escalate overall reaction kinetics. Extremely thermophilic archaea, which thrive at temperatures higher than 60 °C, have been reported to enhance leaching even further [46]. The rate of

metal sulfide oxidation is higher in case of the thermoacidophiles as compared to mesoacidophiles. The use of thermophilic archaea cultures is limited due to the fact that these microorganisms are not naturally occurring in mine sites and optimum temperatures can be hard to achieve in the field. Thermophilic heap bioleaching can be a faster alternative for chalcopyrite processing only if the required temperatures for growth and activity of these organisms can be achieved. Some of the other limiting factor of these organisms include lower metal tolerance and greater cell wall sensitivity to higher pulp densities [9]. However, BHP Billiton has succeeded in the commissioning of an aerated stirred-tank reactor using archaea of the genera *Acidianus*, *Metallosphaera*, and *Sulfolobus*. The tank operated at 78 °C with a pulp density of 12% (w/w) and recovered 98% copper from a chalcopyrite concentrate [48].

Recent Advances in Synthetic Biology

There has been a good deal of recent effort aimed at engineering biomining associated microbes [11-23] and understanding their unique physiology relevant for biomining operations, and more recently for developing new bioenergy applications [11,49,50]. Developing genetic tools and synthetic biology techniques for biomining microbes has been challenging because only a limited number of selection markers, plasmids and gene transfer techniques are suitable for these extremophilic organisms [8]. A major reason is their unique growth conditions, involving low pH and high concentrations of metal ions. These bacteria in general have high restriction barriers to foreign DNA entry and thus a limited number of gene transfer techniques have been developed [3,50]. In the case of *A. ferrooxidans*, *A. thiooxidans* and *A. caldus*, gene transfer based on

conjugation with *E. coli* has been successfully demonstrated [14,19,21-23]. More recently, electroporation has been developed in *A. caldus* [17] with high transformation efficiencies (3.6×10^4 transformants per μg of plasmid DNA) and this has been used to introduce genetic modifications [18]. Genetic engineering techniques have not been reported for *Leptospirillum* although some of their genes related to arsenic resistance [51] and nickel resistance [52,53] have been cloned and expressed in *E. coli*.

Plasmids of IncQ, IncP and IncW groups have been shown to function in some of the *Acidithiobacillus* species [14,21], the IncQ plasmids being the most stable and having the highest conjugative transfer efficiencies [12]. Gene knockout techniques based on homologous recombination have also been shown to generate new strains of *A. ferrooxidans* [13,15,16], *A. thiooxidans* [23] and *A. caldus* [20]. A recent model-driven study of genes in *A. ferrooxidans* suggested that an increase in extracellular polymer substances (EPS) could be achieved by introducing several genetic knock downs [54]. EPS is important in bioleaching applications as it facilitates bacterial adhesion to the sulfide minerals [54]. Other gene disruption studies and subsequent responses to various metal ions have been reported for *Metallosphaera sedula* and development of a more characterized genetic system is underway [55].

Another interesting example of genetic engineering in *A. thiooxidans* was the heterologous expression of *E. coli* phosphofructokinase-I or *pfkA* gene in these organisms [22]. Phosphofructokinase is a key enzyme in glucose metabolism and introduction of this gene significantly stimulated cell growth in the presence of glucose which is otherwise lacking in this chemolithoautotrophic bacteria. In a related approach, the presence of a potential phosphofructokinase-2 gene (*pfkB*) gene was predicted in

the *A. ferrooxidans* strain ATCC 23270 genome. In order to elucidate the role of the *pfkb* gene, a mutant strain of *A. ferrooxidans* was constructed by knocking out this gene using a markerless gene replacement technique exploiting the homing endonuclease I-Sce-1 [15]. Interestingly, the mutant strains showed reduced growth in sulfur media compared to wild type cells. Further studies showed that the growth of both wild type and mutant strains could be stimulated by adding glucose in the sulfur media but the extent of stimulation was much lower for the mutant. Overall, only limited glucose utilization was observed in both the cases.

More recently, the expression of green fluorescent protein (GFP) as well as two heterologous biosynthetic metabolic pathways have been reported in *A. ferrooxidans*. These pathways enable the production of either isobutyric acid or heptadecane [11,56]. These pathways were expressed by inserting codon-optimized genes into the pJRD215 (IncQ group) plasmid backbone which was delivered into *A. ferrooxidans* by conjugation with transformed *E. coli* cells [11]. For production of isobutyric acid, a 2-keto decarboxylase from *L. lactis* was used. For heptadecane production, genes from *S. elongatus* encoding acyl-ACP reductase and aldehyde deformylating oxygenase were used. This is the first report of the metabolic engineering of *A. ferrooxidans* to produce exogenous biochemicals from CO₂ using energy obtained from the oxidation of iron. Now efforts are underway to expand the genetic toolbox available for the engineering of *A. ferrooxidans*. New sulfur-responsive promoters to drive inducible gene expression in *A. ferrooxidans* have been characterized [57]. And recently, Yu et al. [16] constructed a new strain of *A. ferrooxidans* by knocking out the gene tetrathionate hydrolase (*tetH*). The *tetH* knockout mutants lost the ability to grow on tetrathionate (S₄O₆²⁻) as an energy

source. This approach could be used as a new selection strategy for these acidophilic bacteria given the limitations of antibiotic selections due to their unique growth conditions.

The challenges and recent advances made in engineering other chemolithoautotrophic hosts (not involved in biomining applications) as production platforms for useful compounds have also been reviewed in detail [1,3]. A *Ralstonia*, *Rhodobacter* and *Clostridium*, are some of the major non-metal oxidizing chemolithoautotrophic bacteria that have been engineered to produce fuels and other useful chemicals from CO₂ [3,4,50,55,58]. Microbial electrosynthesis has also been developed in which electroactive bacteria capable of accepting electrons directly and fixing CO₂ have been used to produce useful compounds. Detailed discussions on this are beyond the scope of this review and can be found elsewhere [49,50]. A list of some of the useful compounds generated by some genetically modified chemolithoautotrophs are provided in Table 4.

Table 4. List of compounds generated by genetically modified chemolithoautotrophs.

Organism	Compound Produced	Energy Source	Titers (g/L)	Batch/Continuous	Reference
<i>R. eutropha</i>	Methyl Ketones	Hydrogen	0.18	4 days batch	[61]
	Cyanophycin	Hydrogen	0.13 ± 0.03*	6 days* batch	[62]
	Isobutanol	Formate from electricity	0.846 ± 0.05*	6 days batch	[6]
	3-methyl-1-butanol	Formate from electricity	0.570 ± 0.05*	6 days batch	[6]
<i>R. capsulatus</i>	Botryococcene	Hydrogen	0.06	Continuous	[63]
			0.11		
<i>C. ljungdahlii</i>	Butyrate	Syngas (CO ₂ & H ₂)	1.24*	15 days* batch	[64]
	Butanol		0.15	6 days* batch	[5]
<i>C. acetium</i>	Acetone	Syngas (CO ₂ & H ₂)	0.008	6 days* batch	[4]

<i>A. woodii</i>	Acetate	Syngas (CO ₂ & H ₂)	50	4 days batch	[65]
<i>A. ferrooxidans</i>	Isobutyric acid	Fe(II)	0.00057 ± 0.00035	continuous	[11]
			0.0069 ± 0.0005	3 days batch	
	Heptadecane	Fe(II)	(4.2 ± 0.9) × 10 ⁻⁶	3 days batch	[11]

* Estimated from graphical data

Conclusions and Future Prospects

The search for new routes for the generation of chemicals and fuels is critical as we transition away from petroleum resources. Energy-rich sulfide materials are as abundant as petroleum in the Earth's crust. These materials could eventually be developed for dual use: as resources for metal recovery and as feedstocks for CO₂ fixation into biochemicals and biofuels. Thus, genetically engineered chemolithoautotrophic bacteria could open up a new paradigm for industrial bioproduction (Figure 4). These advances could also be leveraged to improve metal solubilization and recovery.

Figure 4. Co-generation of biofuels and other chemicals by genetically modified chemolithoautotrophs during biomining.

Chemolithoautotrophic microorganisms release energy during the oxidation of iron and reduced sulfur compounds during the course of metal mining, most of which is essentially lost into the environment. As shown in Figure 1, the free energy released during oxidation of sulfur is comparable to that released during the combustion of petroleum. Co-generation of fuels from these microorganisms during mining operations can serve as a means of harnessing this virtually untapped terrestrial energy resource. The efficiency of conversion of this energy by the microorganisms and the titers of biochemicals produced are important factors which will impact the commercial feasibility of these co-generation activities. To date, the production of isobutyric acid by genetically engineered *A. ferrooxidans* has only been characterized using Fe^{2+} as an energy source, and the Gibbs free energy efficiencies for incorporation of this energy into the isobutyric acid product are on the order of 1% [11,56,59]. In order to develop a commercially viable processes, these efficiencies will need to be increased when using mining process streams as feedstocks.

Model autotrophs like *Ralstonia* and *Clostridium*, which have emerged as promising candidates for biofuel production, have standard and established genetic modification protocols. On the other hand, metabolic engineering of the biomining organisms has been more difficult due to a lack of established genetic tools and because of their unique growth conditions. However, as advances in this space continue to accelerate,

more genetic tools will become available for the biomining associated chemolithoautotrophs.

As chemolithoautotrophic bioprocesses are developed, new opportunities for their utilization will also arise. For example, there is an increasing interest in the exploration of Mars, and chemolithoautotrophic conversion of CO₂ using non-terrestrial minerals may become a critical future technology for these endeavors.

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