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Doctoral thesis

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Abstract

Rich mineral ores are depleting and the demand for minerals is increasing worldwide. Thus, there is need to liberate minerals from low-grade ores which requires processing of ores to fine and ultra-fine particles. But the flotation of fine mineral particles is challenging. Moreover, the technique uses reagents that have a large environmental imprint. Therefore, there is a need for environmentally friendly and an effective alternative. The use of microbes or microbe-derived metabolites have the potential to replace the conventional reagents as reviewed in the first paper of the thesis. Apart from many factors, two main governing factors for the separation of fine particles by flotation is the hydrophobicity of the particles and the size of the bubbles in the flotation cell to which particles can attach. One of the approaches suggested to improve this is to use flocculants or depressants that promote the selective flocculation of the fine particles and thus the aggregated particles being large can be floated.

Rhodococcus opacus is a Gram-positive bacterium which has a unique cell wall structure. It is prominently marked by the presence of mycolic acids (2-alkyl, 3-hydroxy fatty acids) in the cell envelope, which imparts hydrophobicity to it. Due to rich hydrophobic moiety in its cell envelope, it has been explored for the separation of various minerals. Here, we adapted *R. opacus* PD630 to the metal oxide nanoparticles, CuO and γ-Fe_2O_3 NPs (model system for the fine minerals) and studied the changes in surface properties of the cells and the interactions between the cells and NPs. Adapted cells showed increased affinity to the corresponding NPs compared to the wild type. They were more significantly aggregating NPs from the solution in just few minutes, thus acting as potential flocculating reagents for the corresponding fine and ultra-fine mineral particles. CuO adaptation resulted in a stable phenotype, and protein analysis of these cells indicated that some proteins were differentially expressed in response to the NPs stress. These proteins included copper transport proteins, catalase, signal transduction proteins and cell wall biogenesis proteins, mainly mycolic acid synthetic proteins. This showed that the *R. opacus* cell wall is important for its survival in the stressed conditions and acts a first line of defense. In a follow-up study, for testing the importance of mycolic acids in *R. opacus* and the mineral processing, we wanted to construct mycolic acid negative mutant. It is known that the species has low transformation and recombination frequencies. So, to improve this, we constructed conjugative conditional suicide plasmids that separated the process of plasmid transfer and recombination in time. We tried to knock out the last three genes of mycolic acid gene cluster, but it was not possible to knock out these genes without the presence of a complementation plasmid expressing the complete gene. The results indicated that mycolic acid is important for *R. opacus* viability like it is for *Mycobacterium* but not for *Corynebacterium*. Since *R. opacus* negative mutant was not possible, it could not be tested for mineral separation. The conditional suicide vector developed in this work can also be used to deliver recombinases, gRNA, and other gene products to *Rhodococcus*, *Mycobacterium, Corynebacterium,* and related genera.

Polysaccharides are also reported to work as good flocculating agents, therefore, further, we explored the flocculating potential of the fully characterized alginates from *Pseudomonas fluorescens* and *Laminaria hyperborea*, for metal oxide nanoparticles and found that it depends on the composition of alginates and the cations used for the formation of gel.

The above results suggested that *R. opacus* PD630 and the bacterial alginates could be used as good flocculating agents (depressants) for the ultra-fine mineral particles, thus settling them during the separation process and reducing the harmful impact of the toxic reagents used otherwise and also of the fine particles themselves. Moreover, aggregation increases the size of the fine particles and hence, it becomes easier for the hydrophobic particles to attach to the bubbles during separation in the flotation process. It might also eliminate the need to use special flotation techniques for the fine and ultra-fine particles like electroflotation.

List of Papers

Paper 1:

Garima Jain, Håkon Havskjold, Priyanka Dhar, Helga Ertesvåg, Irina Chernyshova, and Hanumantha Rao Kota* **Green Foam-Based Methods of Mineral and Ion Separation.** *Multidisciplinary Advances in Efficient Separation Processes.* American Chemical Society. (2020) **DOI:** 10.1021/bk-2020-1348.ch009

Paper 2:

Garima Jain, Vladislav Slabov, Irina Chernyshova, Hanumantha Rao Kota and Helga Ertesvåg* **Adaptation of *Rhodococcus opacus* to copper oxide and iron oxide nanoparticles.** *Manuscript prepared for submission.* (2022)

Paper 3:

Garima Jain, Helga Ertesvåg* **Improved site-specific mutagenesis in *Rhodococcus opacus* using a novel conditional suicide plasmid.** *Applied Microbiology and Biotechnology, Submitted.* (2022)

Paper 4:

Vladislav Slabov, Garima Jain, Irina Chernyshova, Hanumantha Rao Kota and Helga Ertesvåg*. **Alginates as green flocculants for metal oxide nanoparticles**. *Manuscript under construction.*

Paper not part of the thesis

Vladislav Slabov, Garima Jain, Erik Larsen, Irina Chernyshova* and Hanumantha Rao Kota*. **Eco-friendly collectors for flotation of fine hematite and malachite particles.** *Mining Metallurgy and Exploration, Submitted.* (2022)

Contribution to the papers

Paper 1: I have gathered all the information related to the use of microbes and microbially produced materials in sulfide mineral processing and drafted those sections of the paper. I was also actively involved in the writing during revisions of the manuscript.

Paper 2: I was the main person in designing and performing all the experiments in the present paper except the flotation experiment. I have analyzed most of the data, drafted the whole manuscript and responsible for all the subsequent revisions.

Paper 3: I was the main person in designing and performing experiments regarding the use of the suicide vector for homologous recombination and the deletion of mycolic acid gene cluster including the use of a complementing plasmid. I had drafted most of the manuscript and was a main writer during the revisions.

Paper 4: I have performed the experiments on the effect of different alginates and salts on flocculation using spectroscopy along with Vladislav. I was also involved in writing the manuscript along with the coauthors.

Conferences

Garima Jain and Helga Ertesvåg. **Construction and Use of a New Conditional Suicide Plasmid to Delete an Essential Gene for Mycolic Acid Biosynthesis in *Rhodococcus opacus.*** *i-poster presentation at WORLD MICROBE FORUM, 2021 (collaboration of ASM and FEMS).* (20/06/2021 – 24/06/2021)

Håkon Havskjold, Garima Jain, Priyanka Dhar, Helga Ertesvåg, Irina Chernyshova, and Hanumantha Rao Kota. **Toward sustainable recovery of Rare Earth Elements Separation of CeO_2, La_2O_3, and Fe_2O_3 nanoparticles using biosurfactants**. *Poster presentation at Norwegian Nanosymposium, Trondheim*. (16/10/2019 – 17/10/2019)

Abbreviations

EPSs	Extracellular polymeric substances
DLVO	Derjaguin–Landau–Verwey–Overbeek
XDLVO	Extended Derjaguin–Landau–Verwey–Overbeek
sp.	species
NPs	Nanoparticles
TAGs	Triacylglycerols
ALE	Adaptive laboratory evolution
IEP	Isoelectric point
REEs	Rare earth elements
4-HB	4-hydroxybenzaldehyde
AGs	Arabinogalactans
LAMs	Lipoarabinomannans
C atoms	Carbon atoms
CoA	Coenzyme A
FASs	Fatty acid synthase systems
KR	Ketoacyl reductase
ER	Enoyl reductase
DE	β-hydroxyacyl dehydrase
Kas	β-ketoacyl synthase
KasA/KasB	β-ketoacyl ACP synthases
ACPs	Acyl carrier proteins
Acyl-ACPs	Acyl-acyl carrier proteins
FabD	Malonyl-CoA: ACP transacylase
Fad32	Fatty acyl-AMP ligase
accD	Acyl-CoA carboxylase
FabH	β-ketoacyl-ACP synthase III
pks	Polyketide synthase
PPB	Phosphopantetheine binding
TE	Thioesterase
AT	Acyl transferase
KS	Ketoacyl synthase
MIC	Minimum inhibitory concentration
SDS	Sodium dodecyl sulfate
MS	Mass spectrometry
SLIC	Sequence and ligation independent cloning
aTc	Anhydrotetracycline
M	Mannuronic acid
G	Guluronic acid
SDBS	Sodium dodecyl benzenesulfonate
IMSA	Iron-oxide modified sericite alginate beads
TSC	Thiosemicarbazide
McAPT	Magnetic carboxyl-functionalized attapulgite
SEM	Scanning electron microscopy
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

CFUs	Colony forming units
Alg	Alginates
PfAc	*Pseudomonas fluorescens* acetylated alginates
PfdeAc	*Pseudomonas fluorescens* de-acetylated alginates

Table of Contents

1. Introduction

The present thesis highlights the potential of biotechnology as a promising tool to overcome some of the challenges faced by the mining industry. Hence, to give a background of the study, firstly, some of the major problems and challenges faced by the mineral processing industry and their potential remediation using microorganisms are being discussed. The main work has been carried out on an actinomycetes bacterium *Rhodococcus opacus* PD630 strain. In this context, its biotechnological significance, cell wall structure, and composition are being discussed in the following chapters. Then, its role in mineral processing and the change in surface properties of bacterial cells on interaction with minerals is summarized. Further, the structure and gelling properties of alginates and their use as a potential flocculating agent in mineral processing is discussed.

1.1 Mineral processing and flotation

Metals can occur as native elements, structural component of minerals or as substitutions in other minerals and ores. Generally, minerals of important metals do not occur in abundance, which makes their extraction non-economical (Whitworth et al., 2022).

Froth flotation is a physicochemical method used to extract valuable minerals from ores. The method is based on the difference in hydrophobicity of the valuable mineral and the gangue (non-valuable/waste). The surfactants are adsorbed on the surface of the valuable mineral making it more hydrophobic, and hence the particles attach to the gas bubbles in the flotation cell and are carried to the top of the cell, where they are collected (Figure 1) (Whitworth et al., 2022). The process also uses additional reagents, such as collectors and depressants to increase the contrast in hydrophobicity of the minerals. Most of these reagents are toxic to the environment. Hence, it is essential to replace these toxic reagents with environmentally friendly reagents that must also be as efficient as these conventional toxic agents (Jain et al., 2020).

Flocculation is also a physicochemical method of separation which occurs in the presence of flocculants that forms the aggregation of particles suspended in the liquid (Czemierska et al., 2016). Flocculants are high molecular weight, long-chain polymers that induce the formation of flocs by bridging the particles (Vajihinejad et al., 2018). The method can be used for the separation of fine particles or removal of fine particles waste produced in mineral processing (Somasundaran, 1980).

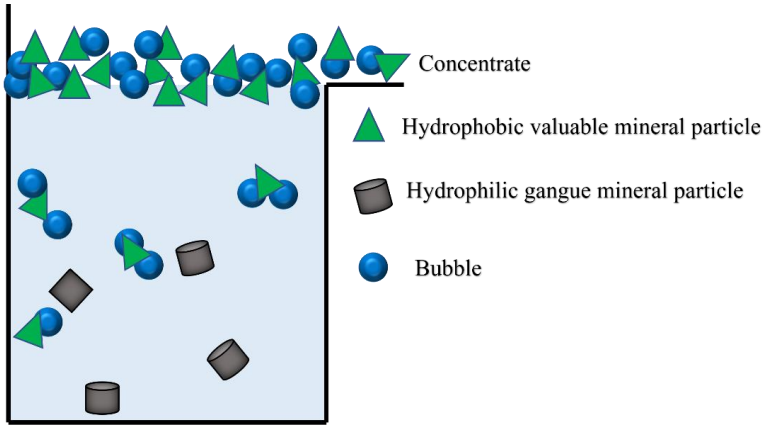


Figure 1 Process of froth flotation. Adapted from Paper 1 of the thesis Jain et al. (2020).

1.1.1 Microorganisms in mineral processing

The global demand for minerals is increasing and the high-grade ores, for example, uranium deposits (the Oklo deposits in the Gabon Republic in Africa), and copper and cobalt deposits (the Katanga Copperbelt) are being depleted (Stephen Herring, 2004, Putter et al., 2011). Therefore, lower-grade ores (for example, Aitik mine in Gällivare, Sweden having copper content less than 1%, and Talvivaara mine in Finland with Ni-Zn-Cu-Co complex ore deposit) must be used, which need to be processed to finer particles to liberate the minerals (Jain et al., 2020, Whitworth et al., 2022, Nkuna et al., 2022). With the conventional flotation method, it is difficult to recover fine particles as there would be a low probability of collision and adhesion between the bubbles and particles in the flotation cell (Farrokhpay et al., 2021). Multiple approaches have been suggested to overcome the problem of fine particles flotation, such as decreasing the bubble size (e.g. by using microbubbles via electroflotation), use of specific flotation cells, or by increasing the particle size via selective flocculation (Farrokhpay et al., 2021, Simões et al., 2021). After the aggregation of the fine or ultrafine particles selectively from a mixture (selective flocculation), the resulting flocs can be separated via flotation. The approach has been used in many studies for the flotation of fine particles (Song et al., 2001, Aruna and Shende, 2006).

The use of biotechnology (microorganisms) along with these approaches can help in the flotation of fine and ultrafine particles in an environmentally friendly and efficient way. Microbes and microbial products can aid in the mineral processing industry by selective bioflotation and bioflocculation of minerals of interest. Microbial exopolymers such as proteins and polysaccharides change the surface properties of the minerals, thus facilitating their separation. The use of microbes or their metabolites for the separation of various sulfide minerals has been summarized in the present thesis (Table 2, Paper 1).

Biosurfactants are amphiphilic surface-active molecules that originate from microbes (Jimoh and Lin, 2019). They are biodegradable, non-toxic, environmentally friendly and have extensive foaming capacity (Jimoh and Lin, 2019, Santos et al., 2016). Phospholipids, lipopeptides, and glycolipids are low molecular weight biosurfactants whereas polymeric

molecules such as polysaccharides and proteins constitute the high molecular weight biosurfactants (Cappelletti et al., 2020). Bioflocculants are naturally produced macromolecules, generally released by microorganisms that can flocculate solid particles suspended in the solution, e.g., sodium alginates (Abu Tawila et al., 2018). Biosurfactants and bioflocculants have industrial and environmental significance, such as wastewater treatment, and remediation of contaminants (Cappelletti et al., 2020). Moreover, biosurfactants and bioflocculants can replace the conventional toxic reagents used in the mining industry to separate and purify minerals.

Microorganisms such as *Bacillus subtilis*, *Rhodococcus opacus*, *Acidithiobacillus ferrooxidans*, *Paenibacillus polymyxa*, and *Mycobacterium phlei* have been studied for the separation of various mineral systems (Botero et al., 2008, Deo and Natarajan, 1999, Misra et al., 1996, Zheng et al., 2001). The adhesion or interaction between bacterial cells and the minerals can be evaluated by using Derjaguin–Landau–Verwey–Overbeek (DLVO) and extended Derjaguin–Landau–Verwey–Overbeek (XDLVO) theories, calculating the total energies between them (Botero et al., 2008). *A. ferrooxidans* has mostly been investigated for the flotation of sulfide minerals, galena (PbS), sphalerite (ZnS) and chalcopyrite ($CuFeS_2$) (Chandraprabha et al., 2005, Dwyer et al., 2012). These cells attach to the sulfide minerals to obtain the energy required for their growth. *M. phlei* has been tested for the flotation and flocculation of hematite particles (Dubel et al., 1992, Smith et al., 1993, Dwyer et al., 2012). Due to its hydrophobic surface, *M. phlei* can attach to the mineral and make it hydrophobic too, facilitating its separation via flotation. Moreover, it can attach to the fine hematite particles and form aggregate, thus acting as flocculating agent for the fine particles separation (Dwyer et al., 2012). Therefore, *M. phlei* acts as a flotation agent when larger particles are to be separated and flocculating agent when used for the separation of fine particles. Related to *Mycobacteria, R. opacus* has also been shown to separate hematite from hematite-quartz suspension (de Mesquita et al., 2003). The authors reported that the increased floatability of hematite over quartz was attributed to both physical and chemical adsorption of *R. opacus* on hematite, whereas only electrostatic interaction (physical adsorption) was involved between quartz and *R. opacus*. *R. opacus* has also been studied for the flotation of other mineral systems (Botero et al., 2008, Merma et al., 2017). This suggests that the bacterial cells with hydrophobic surfaces and a negative charge over a large range of pH values have great potential to be used in the mineral processing industry (Dwyer et al., 2012).

Gram-positive and Gram-negative bacteria have different cell surfaces. Gram-positive bacteria have a thicker peptidoglycan layer containing teichoic and lipoteichoic acids whereas Gram-negative bacteria have a thin peptidoglycan layer surrounded by outer phospholipid layer containing porins and lipopolysaccharides (Dwyer et al., 2012). Species belonging to the order *Mycobacteriales* have a different cell wall structure which is analogous to Gram-negative bacteria but not exactly like them (Chapter 1.3.2 of the thesis). The composition of the cell wall determines the charge and hydrophobicity of the surface of the cell. These properties of the cell surface are responsible for their interactions with the minerals (Dwyer et al., 2012). The presence of proteins or polysaccharides will govern the initial adhesion of the cell to the mineral particles, which is important for any bioflotation activity. The adhesion is the result of the interactions such as electrostatic, van der Waals interactions and acid-base interactions. After this initial binding, an irreversible bond is formed between mineral and bacterial cells via

binding through EPSs (extracellular polymeric substances) produced by the bacterial cells, possibly resulting in the formation of biofilm (Dwyer et al., 2012).
P. polymyxa has also been shown to interact with various minerals, hematite, corundum, calcite, kaolinite, and quartz. On interaction, surface changes are induced in bacterial cells and minerals. Quartz and kaolinite became hydrophobic due to the overproduction of proteins by bacterial cells on interaction, while the other minerals became hydrophilic due to polysaccharides excess (Deo and Natarajan, 1997, Deo and Natarajan, 1998, Deo and Natarajan, 1999). Differential surface properties of the minerals induced by the cells help in their separation. Adaptation of cells to specific minerals also influences their interaction with that mineral. Deo and Natarajan (1999) adapted *P. polymyxa* to corundum and found that corundum specific proteins were secreted on adaptation and the adapted strain was able to selectively flocculate corundum from the hematite and quartz mixture (Deo and Natarajan, 1999). This shows that mineral specific EPSs that are produced on interaction could also be used as flotation reagents. However, the overexpression of corundum specific proteins was temporary and ceased when the adapted strain was de-adapted by repeated culturing without corundum.
The use of bioreagents or biosurfactants offers multiple advantages. They have lower toxicity that promotes eco-friendly extraction from low-grade ores and are also biodegradable in nature and have rich molecular structures (Pereira et al., 2021, Jain et al., 2020). Biosurfactants such as rhamnolipids from *Pseudomonas aeruginosa* have been shown to be useful as a frother in the flotation of copper ore (Khoshdast et al., 2012). The surface activity of the rhamnolipids is comparable to that of the conventional frothers such as propylene glycol. Also, biosurfactants from *Bacillus* sp. and *Streptomyces* sp. have been shown to act as reagents in the flotation of serpentinite and quartz (Didyk and Sadowski, 2012). Biosurfactants extracted from *R. opacus* have been studied for the recovery of hematite from the iron ore tailings via flotation (Pereira et al., 2021).
The use of microorganisms in flotation and mineral processing has been reviewed in Paper 1.

1.2 Bacteria-nanoparticles interactions

Materials that have at least one of the dimensions in the nano-range (<100 nm) are known as nanomaterials. Metal and metal oxide nanoparticles (NPs) usually have antibacterial activity. The antibacterial activity is a sum of multiple mechanisms induced by NPs in the bacterial cells, such as oxidative stress, non-oxidative stress and release of metal ions (Wang et al., 2017). Due to this multi-mode of action by the NPs, it usually becomes difficult for the cells to develop resistance against them. Apart from antibacterial action, NPs have multiple industrial and biomedical applications, to name a few, drug delivery, cell imaging, and as fuel additives (Pelletier et al., 2010).
Bacteria-NPs interactions can be explored for bioremediation as well. Titanium dioxide NPs are a contaminant to the environment, which can be adsorbed by *R. opacus* cells (Sundararaghavan et al., 2020), but the binding between both is not electrostatic but rather coordinative as indicated by zeta-potential results. *R. opacus* being a resistant bacterium to most of the environmental stress was able to resist the toxicity and oxidative stress generated by the titanium dioxide NPs (Sundararaghavan et al., 2020).

When NPs are exposed to bacterial cells, attachment and internalization of NPs are two common phenomena (Sundararaghavan et al., 2020) that can lead to cell wall damage (Slavin et al., 2017). Transcriptomic and proteomic profiles of the bacterial cells also change when exposed to NPs, as a response to the toxicity of NPs (Slavin et al., 2017). In *Escherichia coli*, total of 188 genes were found to be regulated in common (161 genes upregulated and 27 genes downregulated) when exposed to silver nitrate and silver NPs. However, the response to Ag NPs was two times more in magnitude than silver nitrate, exclusively, 309 genes were regulated by Ag NPs and 70 genes by silver nitrate (McQuillan and Shaw, 2014). Another study by Pelletier et al. (2010) found that CeO_2 NPs exposure to *E. coli* resulted in 144 genes being differentially expressed. They found that many iron uptake genes were regulated on NPs treatment, indicating CeO_2 NPs disrupted the iron homeostasis (respiration) in *E. coli* (Pelletier et al., 2010). In another study, exposure of *Pseudomonas* sp. to Ag NPs resulted in the upregulation of proteins involved in translation and stress regulation (ribosomal proteins S2 and L9), proteins for oxidative stress (alkyl hydroperoxide reductase and thiol-specific antioxidant) and protein involved in sugar metabolism (ketohydroxyglutarate aldose) indicating metabolic changes resulting from Ag NPs (Soni et al., 2014). McQuillan et al. (2012) studied the effect of Ag NPs on *E. coli* cells and reported that the NPs attach to the cell walls, interact with the membrane and dissolve to release Ag^+ to the cells (McQuillan et al., 2012). Interestingly, they observed differential expression of genes related to the Cu^+ homeostasis and stress. Genes such as *copA*, *cusA* (copper sensitive) and *cueO* were upregulated. Therefore, the protein profile of Ag NPs exposed *E. coli* cells looked like as they were exposed to Cu-NPs, which could be due to both cations being isoelectronic, so cells are treating them the same (Slavin et al., 2017, McQuillan et al., 2012).
These studies show that when bacterial cells are exposed to NPs stress, they adapt by activating a network of genes, coping with that stress. Two important responses of bacterial cells to NPs are the upregulation of heat shock proteins and cell envelope proteins (Slavin et al., 2017) (Paper 2).

1.3 *Rhodococcus opacus* PD630

1.3.1 Biotechnological significance

R. opacus is a Gram-positive bacterium with high G+C content and is resistant to various environmental stresses (Donini et al., 2021, Cappelletti et al., 2016, Cappelletti et al., 2020, LeBlanc et al., 2008). *Rhodococcus* spp. are biotechnologically important bacteria because of their biosynthetic and bioconversion abilities. They can help in waste bioremediation by the degradation of various organic and aromatic compounds. In addition to this, *Rhodococcus* spp. have the potential to produce various industrially valuable compounds namely, carotenoids, bioflocculants, biosurfactants, lipids, antibiotics, and metal nanostructures, mostly in response to stress conditions (Figure 2) (Cappelletti et al., 2020). Several strains of *Rhodococcus* produce biosurfactants when grown in the presence of water-insoluble compounds such as hydrocarbons (Whyte et al., 1999, Cappelletti et al., 2020).

Biosurfactants and bioflocculants produced by *Rhodococcus* strains are indeed eco-friendlier and less toxic than the synthetic surfactants, but sometimes they are even less toxic than the ones produced by the other bacterial species, for example, biosurfactants derived from *R. ruber* AC 235 are less toxic than rhamnolipids of *Pseudomonas aeruginosa* (Cappelletti et al., 2020).

Different *Rhodococcus* strains can produce different kinds of biosurfactants depending on the growth substrate in the medium. Biosurfactants from *Rhodococcus* spp. have complementary properties to that of the synthetic surfactants, like low critical micelle concentration, and ability to reduce interfacial tension (Lang and Philp, 1998, Cappelletti et al., 2020). *Rhodococcus* predominantly produces trehalose glycolipids with high structural diversity when grown in the presence of liquid hydrocarbons such as alkanes (Kuyukina and Ivshina, 2010, Cappelletti et al., 2020, Lang and Philp, 1998). These biosurfactants are bound to the cell surface and promote cell adhesion to the hydrophobic molecules. Trehalolipids are mainly produced by *Rhodococcus erythropolis*, and they are formed by esterification of trehalose moiety and fatty (mycolic) acid moiety, both of which are synthesized independently (Kuyukina and Ivshina, 2010). Some of the biosurfactants produced by *Rhodococcus* spp. are trehalose monomycolate, trehalose tetraesters, and succinoyl trehalose lipids. Some *Rhodococcus* strains have high flocculating activity and produce bioflocculants such as mycolate containing glycolipids. *R. erythropolis* S-1 is known to be one of the best flocculating species of *Rhodococcus*. (Cappelletti et al., 2020).

R. opacus is an oleaginous bacterium that mainly occurs in the soil. It is called oleaginous because it produces a large amount of triacylglycerols (TAGs), accounting for almost 87% of its dry weight. *Rhodococcus* spp. stores surplus carbon as lipid-based molecules, polyhydroxyalkanoates and TAGs (Donini et al., 2021). Kurosawa et al. (2010) showed increased TAGs production from *R. opacus* PD630 when grown on high glucose concentration and a certain C/N ratio, therefore the strain can be utilized as industrial biodiesel in the future (Kurosawa et al., 2010). *R. opacus* can be developed as a cell factory for lipid production (Firrincieli et al., 2022).

R. opacus PD 630 strains have also been engineered through heterologous gene expression or overexpression/deletion of native (autologous) genes, aiming to improve the utilization of cellulose, and its degradation products, resulting in increased lipid accumulation (Donini et al., 2021). *bglABC* operon was heterologously expressed in *R. opacus PD630* from *Thermobifida fusca* resulting in strain with increased growth on cellobiose and enhanced lipid accumulation (Hetzler and Steinbüchel, 2013). The Adaptive Laboratory Evolution (ALE) method has also been applied to obtain the *R. opacus* and *R. jostii* mutant populations that have increased TAGs production. The approach utilizes sub-culturing the strain in the new media after a fixed interval of time and in the presence of particular substrate and growth conditions until the selection of desirable mutants (Donini et al., 2021). Through ALE, Kurosawa et al. produced *R. opacus* strain with improved TAGs production and resistance to inhibitors such as phenols (derived from lignocellulose hydrolysis) by repeated culturing in the presence of increasing concentrations of lignin, syringaldehyde and 4-hyrdoxybenzaldehyde (4-HB) (Kurosawa et al., 2015). *R. opacus PD630* was adapted to phenol resulting in mutant strains with increased tolerance to phenol and two-fold higher lipid production compared to the wild type (Yoneda et al., 2016).

Metabolically, genetically, or adaptively engineered *R. opacus* strains can be used to convert the low-cost substrates to high value products, which highlights the great industrial and biotechnological significance of the genus *Rhodococcus*. Similarly, in the present work, we tried to engineer *R. opacus* PD 630 genetically and adaptively for its application in mineral processing (Paper 2 and 3).

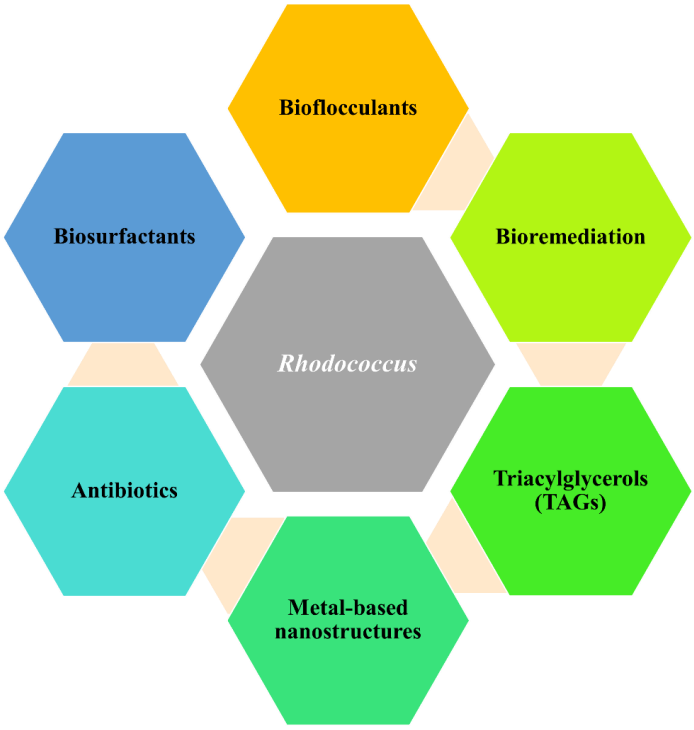


Figure 2 Biotechnology applications of *Rhodococcus* spp. Inspired from Cappelletti et al. (2020).

1.3.2 Cell wall structure, hydrophobic surface

Rhodococcus and its related species, *Mycobacterium*, *Corynebacterium, Gordona, Nocardia*, and *Tsukamurella* belonging to order *Mycobacteriales* (Gupta, 2019), are marked by the presence of typical cell envelope structure and composition, especially predominated by long chain 2-alkyl 3-hydroxy fatty acids called mycolic acids, due to which they are also collectively referred to as the 'mycolata' (Sutcliffe et al., 2010). The cell envelope of the mycolata is represented by the presence of mycolyl-arabinogalactan-peptidoglycan complexes that are covalently linked together (Sutcliffe, 1998, Sutcliffe et al., 2010).
The cell envelope organization in *Rhodococcus* is based on the Minnikin model and is a layered structure (Minnikin, 1991, Sutcliffe et al., 2010). The cell wall structure of *Mycobacteriales* is analogous to Gram-negative bacteria rather than Gram-positive bacteria. The plasma membrane is composed of simple polar lipids, mainly glycerophospholipids that form a lipid bilayer acting as a permeability barrier (Rahlwes et al., 2019). The plasma membrane is supported from the outside by peptidoglycans which are linked to arabinogalactans (AGs) that carries the mycolic acids perpendicular to the plasma membrane (Figure 3). The galactan part of the AGs is connected to peptidoglycans through linkers and the arabinan domain carries the mycolic acids via ester bond (Daffe et al., 1993, Sutcliffe, 1998, Sutcliffe et al., 2010). Mycolic acids are packed such that their main meromycolate chain and the saturated alkyl branch are parallel to each other, forming a monolayer of lipid permeability barrier in addition to the one

provided by the plasma membrane. The flexibility of the AGs contributes to such packing of the mycolic acids in the cell envelope (Sutcliffe, 1998). The peptidoglycan layer gives shape and strength to the cells and comprises of repeating units of N-acetylmuramic acid, β 1,4-linked N-acetylglucosamine and tetrapeptides (Rahlwes et al., 2019). However, in the mycolata except for *Corynebacterium*, instead of N-acetylmuramic acids, N-glycolylmuramic acids are present (Uchida et al., 1979, Vollmer, 2008). Teichoic acids, which are an important component in the peptidoglycan layer of Gram-positive bacteria are absent in the mycolata (Rahlwes et al., 2019).

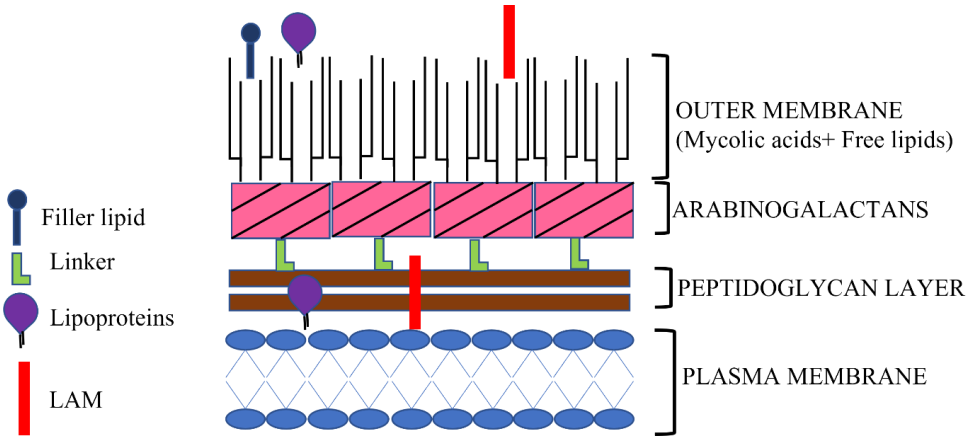


Figure 3 The cell envelope structure in *Rhodococcus* species. Mycolic acids form a hydrophobic domain 'mycolate or lipid permeability barrier'. Free lipids, lipoproteins and lipoarabinomannans (LAMs) are also present which can interact with the mycolic acids. Lipoproteins and LAMs either bind to the lipids in the plasma membrane or the mycolic acids in the outer region. The outermost layer may contain polysaccharides or capsules, not shown in the figure. Adapted from Sutcliffe et al. (2010), Minnikin (1991).

In addition to the mycolic acids, the outer membrane of the cell envelope contains diverse free lipids (Figure 3), also known as 'fillers' such as trehalose dimycolates, trehalose mycolates and glycerol monomycolates (Sutcliffe, 1998, Sutcliffe et al., 2010). The complex free lipids interact with the mycolic acid matrix. The localization of complex free lipids in the outer cell envelope structure is however not fully understood, and it is not clear whether they form the outer leaflet of the outer membrane (Minnikin, 1991) or whether they are intercalated in the mycolate layer (Zuber et al., 2008, Sutcliffe et al., 2010). There is also the presence of lipoproteins, lipoglycans and porins in the cell envelope of *Rhodococcus* but their localization is also not confirmed. Lipoglycans are lipopolysaccharides, some of which belong to the LAMs family.

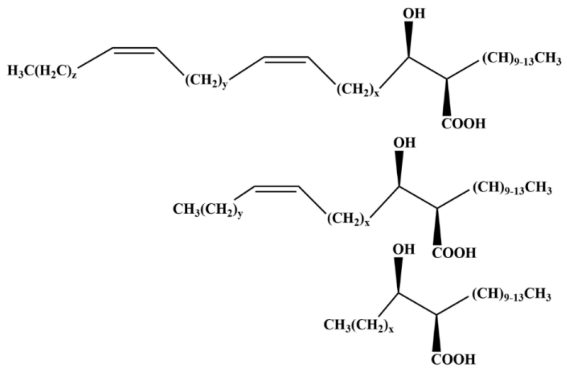
Lipoproteins are proteins with lipid molecules covalently attached to the cysteine residues (N-terminal) (Garton et al., 2002, Gibson et al., 2003, Sutcliffe et al., 2010). They can be found

either associated with the plasma membrane or with mycolic acids in the mycolate layer (Sutcliffe et al., 2010).

1.3.2.1 Mycolic acids

The size and the complexity of mycolic acids vary in different genera of mycolata (Goodfellow et al., 1982, Collins et al., 1982, Rahlwes et al., 2019). *Corynebacterium* spp. have the simplest mycolic acids with short carbon chain length, whereas *Mycobacterium* spp. have the most complex ones with long chain length, 60-90 carbon atoms (C atoms) long and different functional groups- keto, methoxy, and cyclopropane resulting in different types of mycolic acids found in *Mycobacterium tuberculosis*. (Portevin et al., 2004, Sutcliffe et al., 2010, Takayama et al., 2005). However, in *Rhodococcus,* mycolic acids have the intermediate chain length and complexity, they usually have 30-54 C atoms. The main meromycolate chain contains 20-42 C atoms and up to four C-C double bonds whereas the saturated alkyl branch is a shorter chain with 10-16 carbons (Figure 4) (Sutcliffe et al., 2010, Barry et al., 1998). In *R. opacus*, mycolic acids are usually longer compared to other *Rhodococcus* species (48-54 C atoms long), and the meromycolate chain in mycolic acid is at least 20 C atoms longer than the saturated alkyl chain whereas in the shorter rhodococcal mycolic acids the meromycolate main chain and the alkyl side chain have nearly the same number of C atoms (Klatte et al., 1994, Sutcliffe, 1998).

a)



b)

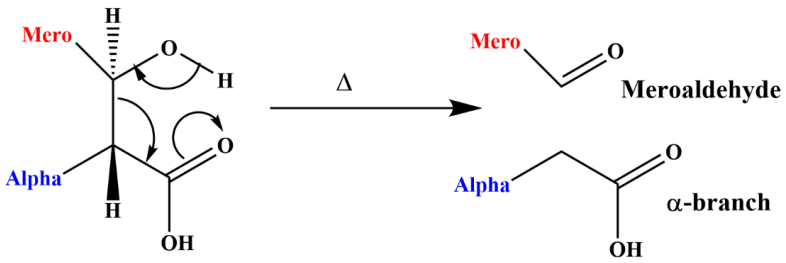


Figure 4 a) Structure of mycolic acids in *Rhodococcus* b) Cleavage of mycolic acid into meroaldehyde and α-branch. The branch containing meroaldehyde is called the meromycolate branch of mycolic acids. Adapted from a) Sutcliffe et al. (2010) b) Barry et al. (1998)

1.3.2.2 Mycolic acids synthesis

Mycolic acids synthesis in *M. tuberculosis* has been described in detail and based on the similarity of genes in *R. jostii*, a similar mechanism has been outlined for *Rhodococcus* as well (Sutcliffe et al., 2010, Takayama et al., 2005).

Mycolic acids synthesis occurs from two fatty acid synthase systems (FASs), a eukaryotic like FAS-I system where all the reactions are catalyzed by one multifunctional enzyme and the FAS-II system (bacteria-like) which follows the same reactions as FAS-I but using monofunctional enzymes forming one enzyme complex (Marrakchi et al., 2014). The four main enzymatic reactions that takes place in these two systems are keto reduction, enoyl reduction, dehydration, and condensation (Barry et al., 1998, Sutcliffe et al., 2010) through enzymes β-ketoacyl reductase (KR), enoyl reductase (ER), β-hydroxyacyl dehydrase (DE) and β-ketoacyl synthase (Kas), respectively (Barry et al., 1998). The reactions in both the systems results in chain elongation by the repeated addition of 2-carbon atoms from the malonyl-coenzyme A (CoA) to the growing acyl group (Figure 5). FAS-I produces long chain acyl-CoAs for the synthesis of alkyl branch of mycolic acids and also as the precursors for FAS-II. FAS-II further elongates acyl-CoAs from FAS-I and produces acyl-acyl carrier proteins (acyl-ACPs) for synthesizing the long meromycolic chain of the mycolic acids (Marrakchi et al., 2014, Daffé et al., 2019). The reaction occurs by repetition of several cycles, each of the four enzymatic

steps mentioned above. Malonlyl-CoA: ACP transacylase (FabD) transfers malonyl group to ACP and forms malonyl-acyl carrier protein (malonyl-ACP). Malonyl-ACP condenses with acyl-CoA via β-ketoacyl-ACP synthase III (FabH) and forms β-ketoacyl-ACP that enters FAS-II (Figure 5) (Marrakchi et al., 2014). This is the main enzyme linking FAS-I and FAS-II systems and is involved in the initial reaction entering from FAS-I to FAS-II system (Takayama et al., 2005). The β-ketoacyl-ACP synthases, KasA and KasB are responsible for acyl extension in FAS-II in *M. tuberculosis* (Sutcliffe et al., 2010). Intermediate length meromycolate precursors are extended by KasA, whereas the long chain meromycolate precursors are extended by KasB. The absence of KasB homologue in *Rhodococcus* attributes to its intermediate length mycolic acids rather than a long chain as in *Mycobacterium* (Sutcliffe et al., 2010).

Fatty acyl-AMP ligase, Fad32 converts meromycolyl-S-ACP (derived from FAS-II) to meromycolyl-AMP (Trivedi et al., 2004, Sutcliffe et al., 2010). Acyl-CoA carboxylase is responsible for the carboxylation of the precursor of the alkyl branch derived from FAS-I to carboxyl-acyl-CoA (Figure 5) (Gande et al., 2004, Sutcliffe et al., 2010, Takayama et al., 2005). The second last step involved in mycolic acid synthesis is the Claisen-type condensation carried by *pks13*, also known as 'Condensase' that is responsible for condensation of the two fatty acyl groups resulting in mycolic acids (Portevin et al., 2004). The enzyme has five domains, two non-equivalent phosphopantetheine binding (PPB), a thioesterase (TE), acyl transferase (AT), and ketoacyl synthase (KS) (Takayama et al. 2005). For the condensation reaction, the two substrates, meromycolyl-S-AMP and carboxyl-acyl-CoA are transferred to KS domain of *pks13* (Gande et al., 2004, Sutcliffe et al., 2010, Rahlwes et al., 2019). The product obtained from Pks13, β-keto-mycolate is reduced and then exported for integration into the cell envelope.

fad32, *pks*, and *accD3* forms the last three genes in mycolic acids gene cluster of *R. opacus*. We tried to knock out these genes to construct mycolic acids negative *R. opacus* mutant (Paper 3).

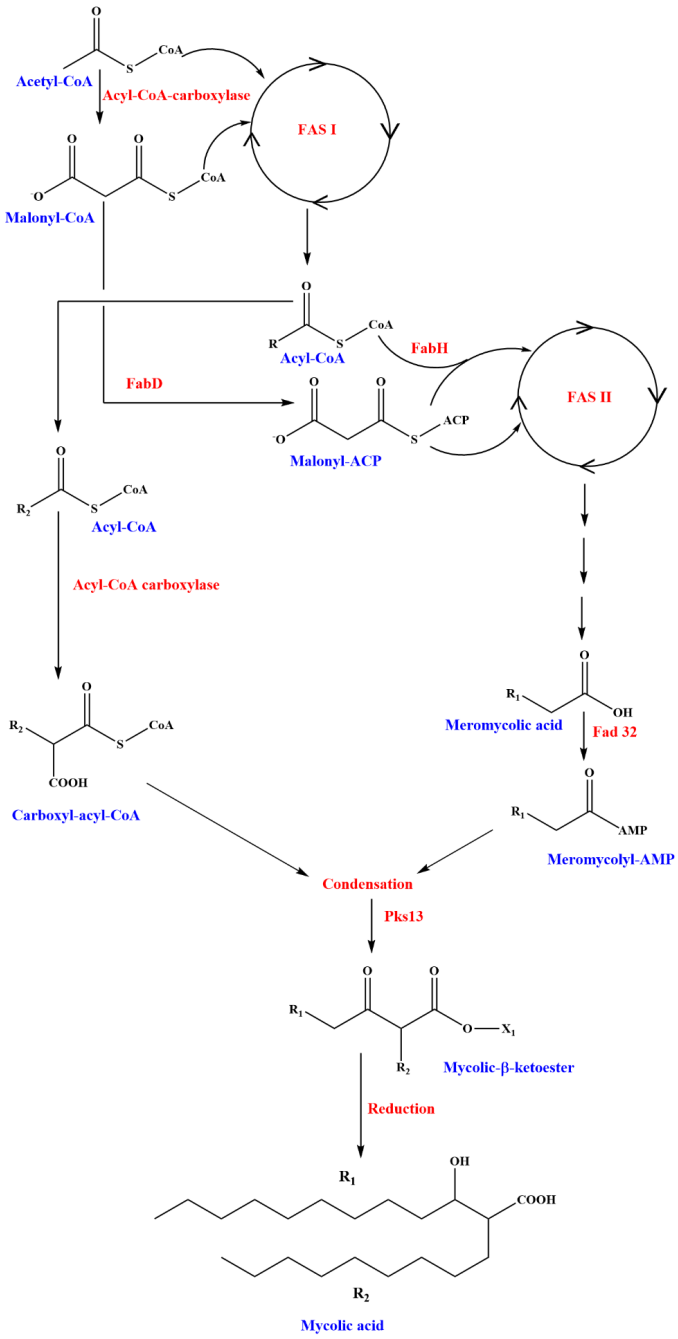


Figure 5 Scheme for the synthesis of mycolic acids. *fad32-pks13-accD3* forms the last three genes of the mycolic acid gene cluster in *R. opacus* that catalyzes the last steps of the synthesis. *pks 13* is responsible for the condensation of the substrates produced from FAS-I and FAS-II. Adapted from Marrakchi et al. (2014)

1.3.3 Genetic engineering of *R. opacus*

Rhodococcus spp. have large complex genomes with multiple extrachromosomal elements. *R. opacus* PD 630 was reported to contain one circular chromosome and nine plasmids (two circular and seven linear) (Chen et al., 2014).

Genome modification in any species requires a library of promoter components that can control the expression of the targeted genes in a predictable way (Donini et al., 2021). Only limited genome editing of *Rhodococcus* spp. has been conducted due to its low transformation and recombination efficiency and high GC content (~70%) (DeLorenzo et al., 2018, Donini et al., 2021, Jiao et al., 2018, Liang et al., 2020). The lack of genetic engineering tools makes the industrial utilization of *R. opacus* difficult. *R. opacus* has been genetically engineered for the increased consumption of substrates such as xylose, cellobiose, etc. and increased production/accumulation of lipid-based molecules using the tools, and genetic parts borrowed from the related species belonging to Actinobacteria (DeLorenzo et al., 2018). However, there are some genetic parts that have been characterized in *R. opacus*, inducible promoters (pAcet, pTet, pBAD), constitutive promoter (pTac), plasmid backbones (pNG2, pGA1), selection markers (kanamycin, gentamycin, chloramphenicol) and reporters (lacZ, mCherry) (DeLorenzo et al., 2017, DeLorenzo et al., 2018). In addition, DeLorenzo et al. (2018) have significantly expanded this toolbox for genetic engineering of *R. opacus* by characterizing a predictable constitutive promoter library and optimizing two antibiotic selection markers (chloramphenicol resistance and hygromycin B).

Homologous recombination is a typical method employed for site-specific mutagenesis in bacterial species. The method uses an antibiotic selection marker for selecting the mutants. The DNA fragment to be inserted have arms homologous to the flanking region of the target site in the chromosomal DNA. *R. opacus* is found to have a high frequency of illegitimate recombination (integration of the fragment or DNA segment at the wrong site in the genome) (DeLorenzo et al., 2018). DeLorenzo et al. (2018) showed that the use of bacteriophage recombinases promoted site-specific recombination in the species, but the plasmid could not be cured out even after the removal of selection pressure (DeLorenzo et al., 2018). We tried to solve these challenges of low transformation and recombination frequencies in *R. opacus* by developing a conjugative conditional suicide vector for its genetic engineering via homologous recombination (Paper 3).

1.4 *R. opacus* in mineral processing

R. opacus might have an amphoteric character, with hydrophobic as well as hydrophilic properties, due to the presence of polysaccharides, lipids, peptidoglycans, and mycolic acids in the cell wall (de Mesquita et al., 2003, Merma et al., 2017, Stratton et al., 2002, Vásquez et al., 2007). This presents a variety of functional groups for interaction: amino (NH_2^+/NH_3^+), hydroxyl (OH^-), carboxyl (COO^-), sulfate (SO_4^{2-}) and phosphate (PO_4^{3-}) (Presentato et al., 2020). Based on this, *Rhodococcus* could potentially be used as flocculating as well as a flotation reagent as previously demonstrated for *M. phlei* (Dwyer et al., 2012, Smith et al., 1993). An acidic exopolymer with high flocculating activity was extracted and characterized from *R. opacus* (Czemierska et al., 2016). Kim et al. (2017) tested *R. opacus* as a collector for flotation in a binary system of malachite ($Cu_2CO_3(OH)_2$, valuable mineral) and silica (gangue). They achieved maximum recovery of malachite at pH 7 due to the maximum opposite charge between *R. opacus* and malachite at this pH value, favoring the electrostatic interactions (Kim et al., 2017).

Adaptation of bacterial cells to minerals is an approach used by many studies in mineral processing (Merma et al., 2017, Deo and Natarajan, 1999, Sharma et al., 2001, Sarvamangala and Natarajan, 2011, Sarvamangala et al., 2013, Vasanthakumar et al., 2013). Adaptation basically means the growing of cells in the presence of minerals by repeated culturing until the growth of bacteria reaches equal to that of the control strain. The above mentioned studies on the adaptation of cells to minerals reported that during growth with the minerals, bacterial cells produce certain metabolites, proteins, and polysaccharides specific to that mineral. Some authors also reported that there is change in the morphology of the cells on adaptation. Merma et al. adapted *R. opacus* cells to apatite and quartz (Merma et al., 2017). Adaptation of *R. opacus* to apatite and quartz induced change in surface charge and functional groups were modified. The surface tension of the stationary phase cells reduced, indicating production of metabolites. Apatite adapted cells promoted higher floatability of the apatite, whereas quartz adapted cells promoted higher floatability of quartz (Figure 6) but still, the flotation of apatite was more than that of quartz, hence allowing their separation. This shows that apatite adapted cells can be used as a collector at optimum pH 3 for the separation of apatite from quartz. It indicates that mineral specific metabolites produced can work as reagents improving the flotation (Merma et al., 2017).

These studies suggest that adaptation leads to the increased affinity. We wanted to float very fine mineral particles, therefore, in this work, we adapted *R. opacus* cells to metal-oxide nanoparticles as model systems for the fine mineral particles (Paper 2).

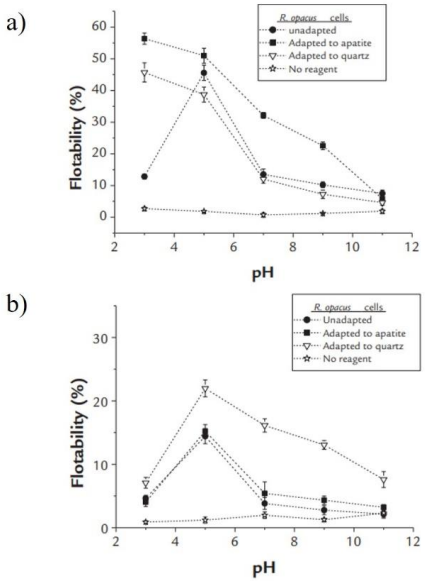


Figure 6 Flotation of the minerals, quartz and apatite using adapted and unadapted *R. opacus* cells. a) Apatite flotation, b) Quartz flotation. Bacteria concentration 0.15 g/L and flotation time 2 mins. Apatite floatability at pH 3, 55% with apatite-adapted cells. Copied from Merma et al. (2017). The figure is used under a Creative Commons License of the BY assignment type.

1.5 *R. opacus* for removal of heavy metals by biosorption

In addition to the methods such as precipitation, ion exchange, and flotation for the removal of contaminants (heavy metals) from waste water, there is another relatively simple and cost-effective method called 'biosorption' (Vásquez et al., 2007). The traditional methods usually face the challenges of incomplete removal and low selectivity. Using microorganisms for the adsorption or removal of heavy metal ions from wastewater can help to reduce these obstacles (Cayllahua et al., 2009). Electrostatic interactions between the metal ions and the functional groups (proteins, polysaccharides) of the bacterial surface are the main governing factors of biosorption (Presentato et al., 2020).

Heavy metal ions can be removed from the waste streams using *R. opacus* as adsorbent. It has been shown to have the potential for the removal of various ions of lead, copper, chromium, nickel, and aluminum (Bueno et al., 2008, Cayllahua and Torem, 2010, Cayllahua and Torem, 2011). The adsorption of metal ions on the bacterial surface depends on pH of the solution, concentration of metal ions and the cells, and the contact time (Bueno et al., 2008). To study the surface changes upon interaction of bacteria cells with the metal ions, zeta-potential studies are usually conducted. Bueno et al. observed the shift in isoelectric point (IEP) and zeta-potential of *R. opacus* treated with the metal ions. They found that the shift in IEP is due to the electrostatic adsorption of single metal ions and the change in zeta potential might occur due to the adsorption of metal hydroxy complexes that are formed from the adsorbed metal ions (Bueno et al., 2008).

pH plays an important role in the adsorption of metal ions on bacterial cells (Vásquez et al., 2007). Generally, IEP of the bacterial cells is below 4. From zeta-potential studies, it has been reported that IEP of *R. opacus* is approximately pH 3.2, above this value it is negatively charged (Cayllahua and Torem, 2010, Dwyer et al., 2012). Below IEP, the surface of the bacterial cells (bioadsorbent) will be positively charged, and it will repulse the cations, whereas above IEP, it will be negatively charged, and it will attract the cation metal species present in the solution. The use of *R. opacus* for bioremediation of heavy metals by biosorption phenomena suggests that *R. opacus* has the capacity to develop tolerance toward toxic metal ions (Paper 2). The main resistance mechanism of *Actinomycetes* to the metals are the production of EPSs, metal efflux, biosorption, bioaccumulation and extracellular sequestration by the production of siderophores (Figure 7) (Presentato et al., 2020).
The summary of the use of *R. opacus* cells in the separation of minerals and biosorption of metal ions is presented in Table 1.

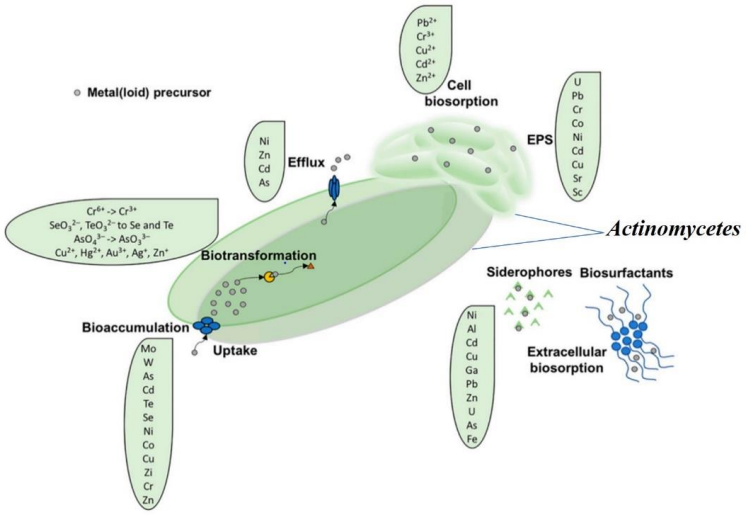


Figure 7 Biotic mechanisms upon *Actinomycetes* and metal interaction. Copied and modified from Presentato et al. (2020). The figure is used under the terms of the Creative Commons Attribution (CC BY) License (http://creativecommons.org/licenses/by/4.0/).

Table 1: Use of *Rhodococcus* cells/metabolites in the adsorption of metal ions or flotation of minerals.

***Rhodococcus* cells/EPSs**	**Minerals/Ions/ Nanoparticles**	**Comment**	**Reference**
R. opacus	Biosorption of Al (III) from water stream	Maximum sorption capacity: 41.59 mg/g at pH 5 in 20 mins, 25 °C.	(Cayllahua and Torem, 2010)
R. opacus	Biosorption of Pb (II), Cr (III) and Cu (II)	pH 5, Pb (II) 95% removal, and at pH 6, for Cu (II) 52% and Cr (III) 70% removal.	(Bueno et al., 2008)
R. opacus	Biosorption + flotation of Ni (II) and Al (III)	Flotation concentrations: 2 g/L of *R. opacus* and 5 mg/L Ni and 50 mg/L Al. 90% and 93% removal of Ni (II) and Al (III) after 15 minutes.	(Cayllahua and Torem, 2011)
R. opacus	Biosorption of Cd^{+2} and Zn^{+2} from liquid streams	At pH 7.0 and 26 °C, Cd^{2+} removal 60%, initial concentration: 15 ppm. Zn removal: 88% from an initial concentration of 5 ppm.	(Vásquez et al., 2007)
Biosurfactant from *R. opacus*	Hematite from iron ore tailings	pH 3, optimal condition for hematite recovery	(Pereira et al., 2021)
EPSs from *R. opacus* and *R. rhodochrous*	Adsorption of Cd (II), Pb (II), Ni (II), Co (II) and Cr (VI) ions.	Highest adsorption for Pb (II) and Cd (II) ions.	(Dobrowolski et al., 2017)
R. opacus	Ni (II) biosorption	Maximum sorption capacity- 7.63 mg/g at pH 5, spontaneous and endothermic sorption	(Cayllahua et al., 2009)
EPS from *R. opacus* immobilized on synthetic microspheres	Adsorption of Pb (II) and Cd (II)	Optimum adsorption pH for Pb (II): 5, and Cd (II): 6.5. Pb (II) adsorption capacity increased by 47% and 30% for Cd (II) after immobilization.	(Dobrowolski et al., 2019)
R. opacus	Biocollector in malachite–silica binary mixture and Cu oxide ore systems	pH 7: highest recovery and grade of malachite	(Kim et al., 2017)
R. opacus	Biosorption of Pb (II) ions	Maximum Pb (II) ion sorption capacity on *R. opacus* 89.7 mg/g.	(Bueno et al., 2011)

Different Rhodococcus species	Cesium ions accumulation/uptake from the medium	Optimal pH 7.8-8.6, amount of Cs uptake by the living cells increased first and then decreased but it remained constant for the dead cells. However, the uptake was more for live cells due to biosorption (non-specific sorption at cell surface) + bioaccumulation (active transport mechanism) whereas only biosorption in dead cells. Cesium concentration: 0.01 to 1.0 mM, above 5 mM inhibition of growth.	(Ivshina et al., 2002)
R. opacus	Removal of Cr (III) from liquid by biosorption and flotation	pH 5, biomass concentration 1.50 g dm^3, 96.30% of metal removal after 20 min of flotation. Sorption kinetics: 49.10% of metal removal in 200 min.	(Calfa and Torem, 2008)
Biosurfactant from *R. opacus*	Electroflotation of hematite fine particles	pH 3, biosurfactant concentration 300 mg/L, recovery of 78% and Fe grade of 59% achieved.	(Simões et al., 2021)
R. opacus PD630	TiO_2 NPs uptake	Exposure to UV increased the uptake of TiO_2 NPs, 57% in dark and 73% under CUV.	(Sundararaghavan et al., 2020)
R. opacus	Flotation collector for calcite and magnesite	Magnesite, floatability 93% for *R. opacus* concentration of 100 ppm, pH 5. Calcite: 55% for *R. opacus* conc. 220 ppm, pH 7. More affinity of *R. opacus* to magnesite than calcite.	(Botero et al., 2008)
R. opacus	Flotation reagent for hematite-quartz system	Increased floatability of hematite over quartz at neutral pH. 70% hematite was recovered.	(de Mesquita et al., 2003)
R. opacus	Electroflotation of fine hematite particles	Optimum pH 6, particle size : 20µm, *R. opacus* conc. 300 mg/L, 80% recovery with hydrogen and ~70% with oxygen bubbles.	(Hacha et al., 2018)
Adapted *R. opacus cells*	Flotation of quartz and apatite	pH 3, 55% floatability of apatite using apatite adapted cells as collector	(Merma et al., 2017)

1.6 Polysaccharides in the flocculation of minerals

As discussed in the previous chapter (1.1), flocculation is the aggregation of particles in the solution via large polymers making flocs that can be settled making the solution clear (Vajihinejad et al., 2018). But there is a slight difference between aggregation and flocculation, aggregation is a term used when the particles are just held together in any manner whereas the loose aggregation of the group of particles via polymers is called flocculation (Somasundaran, 1980). In mineral processing, flocculation is used for dewatering and the removal of fine particles that might be the toxic waste produced during mineral processing as well as for the separation of valuable fine mineral particles. The most employed flocculants in mineral processing are polyacrylamide based (Pearse, 2003, Hogg, 2000). Apart from them, alginates have also been explored as flocculants in mineral separation and removal of heavy metals for wastewater treatment. A summary of examples is presented in Table 2. However, from the table, it can be seen that most of the studies have used modified alginates.

Alginates are linear polysaccharides consisting of 1,4 linked mannuronic acid (M) and its C5-epimer guluronic acid (G) (Figure 8) (Ertesvåg, 2015). They are commercially produced from the brown algae though bacteria of genera *Pseudomonas* and *Azotobacter* also produce alginates. Different alginates have variable distribution of M and G residues and are composed of three kinds of blocks; G-blocks (continuous stretch of G residues), M-blocks (continuous stretch of M residues) and MG-blocks (alternating M and G residues). These blocks can be two to several hundred residues long (Maleki et al., 2017). It is known that the G-blocks of the alginates are very important as they can induce the formation of gels by crosslinking through divalent cations, e.g., Ca^{2+}. The cation can fit into the cavity formed by the G-blocks and allows the crosslinking of the polymer (Figure 9). The strength of the gel formed depends on the length of the G-blocks (Steigedal et al., 2008, Draget and Taylor, 2011). Alginates from brown algae and *Azotobacter* are rich in G-blocks, whereas they are absent in the alginates of *Pseudomonas* (Maleki et al., 2017). The G-residues in the alginates are formed via epimerization by mannuronan C-5 epimerases. AlgG is one of the epimerases that is present in both *Pseudomonas* (30%) and *Azotobacter vinelandii*. The enzyme AlgG can introduce only G-residues and not G-blocks in the alginates. In addition, *A. vinelandii* has a family of seven epimerases AlgE1-7, each of which have the capability to introduce different patterns of G-residues, some introduces G-blocks, and some MG-blocks (Steigedal et al., 2008).

In addition to the traditionally used Ca^{2+} ions, binding of alginates with other alkaline earth metals is also possible. The extent of binding of different ions varies with different blocks. Mørch et al. reported that Ca^{+2} binds to G and MG-blocks, Sr^{+2} to only G-blocks and Ba^{+2} to G and M-blocks (Mørch et al., 2006). The binding of trivalent cations to the alginates is less block structure selective than binding to the divalent cations (Hu et al., 2021). Trivalent cations can bind to the three carboxyl groups and induce cross-linking of the alginate chains (Menakbi et al., 2016). Therefore, the crosslinking and formation of the gel depends on the type of ions and composition of alginates used. We wanted to explore the effect of alginates composition on its flocculating properties for ultrafine and fine particles separation in mineral processing (Paper 4).

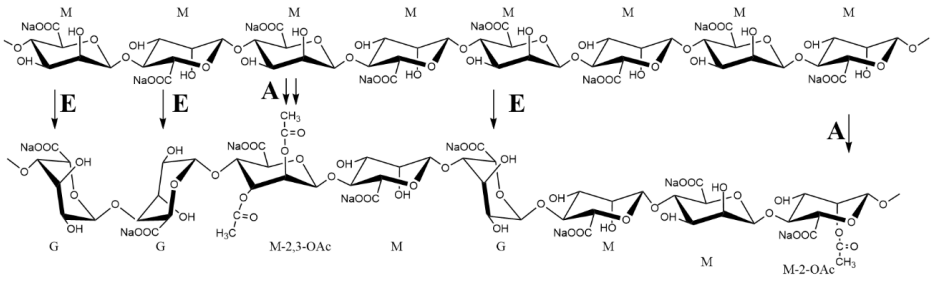


Figure 8 Structures of sodium alginates with their modifications. Copied from Paper 4 of the thesis.

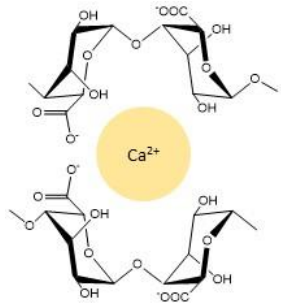


Figure 9 Cross-linking of two G-blocks via Ca^{2+}. Copied from Paper 4 of the thesis.

Table 2: Use of alginates for the selective removal of minerals/ions.

System	Main Results	Reference
Sodium alginate	Improves the removal rates of quartz and chlorite significantly. Increases the viscosity of the pulp and enhances the foam stability.	(Fu et al., 2018)
Flotation modifier: sodium alginate, Minerals: scheelite, calcite and fluorite, Collector: sodium oleate	Sodium alginate acts as selective depressant for calcite and fluorite, pH 7-12.	(Chen et al., 2017)
Anionic surfactant: sodium dodecyl benzenesulfonate (SDBS) and sodium alginate as stabilising agent.	A higher removal of lead and copper ions was obtained with addition of sodium alginate to SDBS.	(Corpuz et al., 2018)
Sodium alginate with thiosemicarbazide as flocculant	Removal rates of the flocculant for Pb^{2+}, Cd^{2+}, and Cu^{2+} reached up to 97.8%, 86.3%, and 80.0%, respectively.	(Tian et al., 2017)
Iron-oxide modified sericite alginate beads (IMSA).	Adsorption capacity of IMSA beads: 21.61 mg/g and 133.73 mg/g for As (V) and Pb (II), respectively.	(Lalhmunsiama et al., 2017)
Nanochitosan/sodium alginate beads using Ca^{2+} as a crosslinking agent.	Adsorption capacity of Pb (II) was 178.57 mg/g at 45°C.	(Ablouh et al., 2019)
Sericin-alginate	Bioadsorption kinetics is faster for Cr (III) than for Cr (VI) ions, occurrence of ion exchange mechanism with Ca^{+2}.	(de Andrade et al., 2018)
Sunflower waste carbon calcium–alginate beads	The maximum biosorption capacity for Cd (II) 23.6 mg/g.	(Jain et al., 2013)
Thiosemicarbazide (TSC) modified alginate.	TSC: crosslink agent. Removes lead and cadmium at high concentrations, 950 mg/g (at pH 3) and 300 mg/g (at pH 7) respectively.	(Córdova et al., 2018)
Composite absorbent (McAPT@Alg), magnetic carboxyl-functionalized attapulgite (McAPT) and sodium alginate (Alg).	Pb (II) removal efficiency of >70%	(Zou et al., 2018)

2. Aims of study

The depleting high-grade ores, use of toxic reagents for the separation of minerals, and the problem of fine particles flotation are some of the challenges faced by mineral processing industries. The aim of the thesis was to address these challenges with the help of biotechnology by developing potential microbe-based reagents. The detailed objectives are as follows:

1. The interactions between the bacterial cells and mineral particles results in the changed surface properties of both which facilitates the separation of minerals via flotation or flocculation. To better understand which microorganisms have been studied for which mineral systems and how they can be modified (adapted or genetically engineered) to facilitate the separation process, a detailed literature review is necessary, therefore, the present status of the use of microorganisms and their metabolites for the separation of minerals would be reviewed.
2. There are several microorganisms (*R. opacus, P. polymyxa, A. ferrooxidans,* and *P. fluorescens*) that have been studied for their potential as the bioreagents in mineral separation, but mostly for the micro-range minerals. Here, we aimed to test the separation of fine mineral particles using *R. opacus* as bioreagent. We wanted to test the potential of *R. opacus* cells for the flotation/flocculation of metal-oxide nanoparticles (used as model system for the fine mineral particles). From the previous studies by different research groups, it is known that the cells adapted to the specific minerals have increased affinity towards them compared to the unadapted cells, the aim was to adapt *R. opacus* to the metal-oxide nanoparticles and study the interactions between them and the change in their surface properties. Moreover, we wanted to study the response of the cells which makes them adaptable to the toxic NPs and test if the proteins regulated in the adaptation process are responsible for the changed surface properties. We wanted to identify if any specific protein was responsible for the increased affinity to the NPs and could be used directly for the separation of fine mineral particles. *R. opacus* have presence of mycolic acids in their cell wall which imparts hydrophobicity to them, the hypothesis was that it might make the mineral surface more hydrophobic that can help in their flotation. To test this hypothesis, we aimed to construct mycolic acid negative *R. opacus* mutant and wanted to see if mycolic acids could be useful as bioreagents for the separation of fine mineral particles.
3. For the separation of minerals, polysaccharides can also work as depressants or flocculants. Alginates are linear polysaccharides which are known to have gelling properties on the addition of divalent cations. Mostly, modified alginates have been used as flocculants for water purification. We aimed to use the bacterial alginates for the flocculation of fine mineral particles and test the effect of the addition of different cations on their flocculating potential towards the fine particles (nanoparticles).

3. Methods used in the study

This section briefly summarizes the different experimental approaches and methods used in Paper 2, Paper 3, and Paper 4.

In Paper 2, various instrumentation techniques were used to characterize nanoparticles, and adapted and unadapted bacterial cells. *R. opacus* cells were adapted to metal-oxide NPs, CuO and γ-Fe_2O_3 (maghemite) by repeated subculturing of the cells in the presence of these NPs at a selected fixed concentration of NPs until the cell count became closer to that of the wild type cells. After every passage, the cultures were plated to obtain CFU/mL. Since it is difficult to measure the optical density of cultures along with the NPs in the solution (because of the dark color produced by the NPs we used here), the protocols used in the paper always involved plating the cultures to get the viable cells count. The change in the affinity of the adapted cells to the NPs compared to the wild type cells was analyzed through adhesion tests and visualized via scanning electron microscopy (SEM). The protocol for the adhesion tests was established after many trials and from the literature available. Contrary to the literature, where they were able to separate the bacterial cells and the NPs under centrifugal force, we were unable to do that. So, we selected gravitational force as our separation force. The Fe-adapted cells and the wild type *R. opacus* cells were tested as collectors through the flotation tests with maghemite NPs and hematite fine (-20 μm) particles using XFG II flotation machine. The changed surface properties of the adapted cells were studied using zeta-potential measurements and protein analysis. The protein profiles of the wild type *R. opacus* and the adapted *R. opacus* cells were compared after protein extraction and their separation through sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The bands that appeared different from the wild type cells in the protein profile of the Cu-adapted cells were excised out and the proteins present in them were identified by mass spectrometry (MS). From the complete list of the proteins identified in the bands, they were further filtered, analyzed, and annotated based on their cut-off score and localization in the cell. Finally, from the sorted list, some proteins having a role in the cell wall biosynthesis were selected for overexpression in *R. opacus.* These genes were overexpressed by cloning the desired genes in the plasmid and transferring the plasmids to *R. opacus* via conjugation. The growth of the overexpressing strains was compared with the control strain by culturing them in the presence of NPs and plating after 24 h and 48 h to obtain the number of viable cells (CFU/mL). It was done to check if any single gene has a more significant effect in response to the NPs stress than others.

Paper 3 used molecular cloning techniques to construct various plasmids with desired genes of interest to make *R. opacus* mutant strain through homologous recombination. Various cloning methods used to construct the different plasmids were: restriction digestion and ligation cloning, sequence and ligation independent cloning (SLIC), and topo cloning. All the primers used in the study were designed using the Clone Manager 9 software from Sci Ed Software LLC, USA. For cloning, *E. coli* DH5α was used and for conjugation *E. coli* S17.1 strain was used, and both were cultured at 37 °C. The desired plasmids were transferred to *R. opacus* by conjugation from *E. coli* S17.1. The transconjugant colonies were selected using plasmid encoded antibiotic resistance and nalidixic acid (select against *E. coli*). Further, for homologous recombination, first crossover was initiated by removing the inducer, in our case, m-toluate and adding the repressor anhydrotetracycline, aTc to the culture medium so that plasmid does not replicate and gets integrated into the genome of *R. opacus*. After the second crossover, one would either get wild type or the mutant cells, so to select double recombinants the culture was

streaked on the plates with 6% sucrose, since we used *sacB* as a counterselection marker in all our study. *sacB* encodes levan sucrase that hydrolyses sucrose to levan, which is toxic for the bacteria. Sucrose resistant colonies were further selected for sensitivity to plasmid encoded antibiotic resistance and then tested by PCR to identify if the desired mutant strain was obtained.

For Paper 4, firstly, to test the influence of alginates on the nanoparticles, Fe_2O_3 and CeO_2 NPs, zeta-potential studies were carried out. The zeta-potential of NPs was measured in the presence and the absence of NPs. It was also measured after the addition of two different cations (Ce^{3+} and Ca^{2+}) we used in this study for the initiation of flocculating activity of alginates. Then, the flocculating activity of the alginates on NPs was tested just by a visual experiment, mixing of the NPs dispersions with the cations solution and then the addition of different alginates samples and afterwards keeping it for some time before observation. After the visual experiment, time-resolved flocculation experiment was done which involved measuring the optical density of the solution mix (alginates, cations, and NPs) via UV-vis spectroscopy over a fixed period of time and wavelength. Kinetic measurements were important to analyze the efficiency of the different alginates with different cations. To observe the difference in the appearance (shape and size) of the flocs, high speed camera images were recorded over ten seconds.

4. Summary of results and discussion

The present doctoral thesis includes one review paper and three research papers, the summary of the results and discussions of which are presented in this section. In the review paper (Paper 1), we summarized about the different methods and reagents used in mineral processing and the potential of microbe-based reagents to replace the conventionally used toxic reagents. This laid the foundation for the overall aim of the thesis which was to develop bioreagents for the separation of fine mineral particles. Paper 2 is the study of the potential of *R. opacus* cells as a bioreagent (biocollector or bioflocculant) for the separation of fine mineral particles. For studying the interfacial interactions between the *R. opacus* cells and metal oxide nanoparticles (model system for ultrafine mineral particles), the cells were adapted to the NPs, and we analyzed the change in surface properties of the cells and their affinity to the NPs. The results indicated that wild type *R. opacus* cells and even better NPs-adapted *R. opacus* cells could work as bioflocculants for the separation of fine mineral particles. Then to further explore the hydrophobic surface properties of the *R. opacus* cells, we tried to develop mycolic acid negative *R. opacus* mutant strain in Paper 3. However, it was not possible to construct such mutant strain. Then we explored the use of alginates with different G-distribution as bioflocculants for the separation of fine mineral particles. We found that the composition of alginates and the type of metal cations used for the gel formation in alginates are the important factors in deciding their flocculating activity towards the NPs (Paper 4).

4.1 Paper 1: Green foam-based methods of mineral and ion separation

This paper reviews the conventional and emerging trends and methods used in mineral separation. It discusses the conventional reagents used in the flotation methods for the separation of minerals, mainly sulfide minerals. Further, the paper highlights the knowledge on the use of green reagents in mineral processing, and it outlines the recent development and potential of using microbes and microbe-derived metabolites for the separation of sulfide minerals. It shows how the interaction between bacterial cells and the minerals is useful for making surface changes that help in separation. Further, in addition to the separation of minerals, we also discussed about the method used for the separation of metal ions, namely, foam flotation. Lastly, with the increasing demand for rare earth elements (REEs), this review also provides light on the knowledge available for the extraction of REEs.

Froth flotation is a conventional physicochemical method used in mineral processing for the extraction of minerals from ores (Figure 1(a), Paper 1). The method is based on the difference in hydrophobicity of the valuable minerals and the gangue. There is another method called foam flotation, which is also known as ion or precipitate flotation depending on what it separates from the aqueous solution: metal ions or their dispersed precipitate. (Figure 1(b), Paper 1). The method involves the passing of bubbles through an aqueous solution which contain metal ions of interest (colligend), a surfactant and some other background ions. The surfactant attaches to the bubbles with the polar headgroup pointing outwards into the solution to which the colligends are adsorbed and the foam is generated. The foam reaches the top of the flotation cell where the metal ions get concentrated. Unlike froth flotation, foam flotation method does not need any energy-intensive grinding operation. For the separation and purification of rare earth elements (REEs) hydrometallurgical methods are applied (solvent extraction, ion exchange, precipitation, electrolysis) after the froth flotation. The drawback of

the process is that it is not environmentally and economically sustainable, but foam flotation has the potential to make this process more sustainable. Some research results indicating the use of foam flotation in wastewater treatment and extraction of REEs (very few studies) are being summarized (Table 3, Paper 1).

The contrast in hydrophobicity/hydrophilicity of the mineral particles in froth flotation can be controlled by the selection of surfactants called collectors. However, the froth flotation depends on physical parameters as well, but it is easier to change the reagents than redesigning the flotation setup. Hence, design, selection, and development of new reagents remain very important for mineral processing (Fuerstenau and Pradip, 2019). Surfactant adsorption at the solid-water interface is governed majorly by electrostatic and chemical interactions. Other interactions include cation-dipole interactions, hydrogen bonding, solvation and desolvation, etc. (Somasundaran et al., 1998).

Sulfide ores are the important and major source of metals such as Cu, Ag, Au, Zn and Pb. To recover these metals, usually, froth flotation process is used and the major gangue minerals generally present with these valuable metal sulfides are silicate, iron, and clay minerals. The problem with the extraction of metal-sulfide minerals is that they undergo oxidation, which is not good for the flotation process. Traditional collectors employed for sulfide minerals flotation are sulfhydryl collectors, e.g., xanthates, xanthate ester, and dialkyl dithiocarbamates (Maree et al., 2017). Indeed, the sulfhydryl collectors offers advantages such as low cost and high selectivity but it has many hazards such as skin and eye irritation (National Industrial Chemicals and Assessment, 1995). Other type of collectors used for sulfide minerals separation are chelating collectors e.g., alkyl hydroxymates which have higher selectivity and are usually used for complex sulfide mineral separation (Table 1, Paper 1). Again, these are hazardous, toxic and they also bioaccumulates (Skipper et al., 1980). To replace these toxic reagents with environmentally friendly reagents, research is being conducted towards microbe-derived metabolites to work as 'Biosurfactants'.

Several microorganisms have been investigated to work as reagents for the flotation of sulfide minerals (chalcopyrite, sphalerite, etc.). These microorganisms include *Bacillus pumilus*, *A. ferrooxidans*, and *A. thiooxidans*. The cells either act as collector or depressants in the process of separation. The cells adhere to the mineral surface and produce EPSs that promote this interaction and change the surface properties of the cells and the mineral particles. The electrokinetic measurements and changes in isoelectric points of the cells and minerals after interaction indicate that surface properties tend to change due to this interaction. Interaction between the cells and minerals depends on many factors, like surface charge, and the presence of functional groups. (Figure 4, Paper 1). The formation of the insoluble sulfate on the surface of sulfide mineral particles by the cells turns their surface hydrophilic and thus, cells act as depressants (Figure 3, Paper 1). In acidic pH, the deposited sulfate will be insoluble hydrophilic metallosulfates, hence promoting depression. To act as a collector, cells make the surface of mineral hydrophobic by the formation of elemental sulfur on them. (Gardner and Woods, 1979, Otsuki, 2016, Pecina et al., 2009).

A summary of the use of biomolecules, EPSs, and whole cells for the flotation of sulfide minerals are presented in this paper (Table 2, Paper 1). Sulfide minerals are usually found in association with other minerals, and using bacterial cells makes the separation easier as they can preferentially adsorb on a particular mineral, thus creating a difference in their surface properties. For example, in a mixture of pyrrhotite, chalcopyrite and sphalerite, it was found that *A. ferrooxidans* adsorbed more on pyrrhotite, thus making it hydrophilic and depressing it

in the mixture, and chalcopyrite flotation was increased (Pecina-Treviño et al., 2012). More adsorption on pyrrhotite compared to chalcopyrite and sphalerite could be attributed to the higher iron content in the pyrrhotite. Thus, the interaction between bacterial cells and minerals also depends on the properties of the mineral particles.

Adaptation of the cells to the mineral particles is another approach that is being followed. It has been reported that bacteria adjust their EPSs production according to the mineral they are adapted to (Sarvamangala et al., 2013, Sharma et al., 2001). It might also be possible that some genomic changes take place during the adaptation that also promotes interactions (Sarvamangala and Girisha, 2015, Kondrat'eva et al., 2005). Bacterial EPSs have also been explored as reagents in sulfide minerals separation, e.g., single-stranded extracellular DNA from *B. megaterium* acted as biocollector for sphalerite (Vasanthakumar et al., 2014), and tricarboxylate sodium starch as a depressant for pyrite (Khoso et al., 2019). Biosurfactants such as fatty acids, rhamnolipids, sophorolipids, and lipoproteins are commonly used in flotation. They have an amphoteric character. In addition to biosurfactants being environmentally friendly, they have an added advantage, i.e., their diverse structure with bulky headgroups promotes multiple adhesions.

The process of foam flotation can also be improved using green reagents (Yuan et al., 2008, Salmani Abyaneh and Fazaelipoor, 2016). But the use of biosurfactants with foam flotation is not industrially feasible because of various reasons such as low efficiency, long flotation times and high surfactant dose (Peng et al., 2019). In most of the studies, microbes and microbe-derived metabolites are used along with the conventional reagents to increase the efficiency of flotation. But the use of biosurfactants definitely reduces the quantity of conventional reagents needed. Still, the use of microbes and biosurfactants in mineral processing is in the preliminary stages, but it has immense potential.

4.2 Paper 2: Adaptation of *Rhodococcus opacus* to copper oxide and iron oxide nanoparticles

Based on Paper 1, we wanted to explore the hydrophobic surface properties of *R. opacus* for the flotation of fine mineral particles. Therefore, in this paper, we first adapted *R. opacus* to the metal oxide nanoparticles (copper oxide nanoparticles and iron oxide nanoparticles), which are otherwise toxic to the species. For this, minimum inhibitory concentrations (MIC) were calculated to choose a concentration that inhibits the growth of the microbial cells without killing all the cells and we chose 6.25 mg/mL for CuO NPs and 25 mg/mL for γ-Fe_2O_3 NPs for adaptation (Table 1, Paper 2). Cells were adapted to γ-Fe_2O_3 NPs after the first passage (subculture), while it took more than 16 passages for adaptation to CuO NPs, owing to its greater toxicity and still the growth rate was not equal to the wild type cells (Figure 1a and Figure 2a, Paper 2). Three colonies from passage 17 plates were picked and tested, among them P17-3 was the best. Again, two colonies (P17i and P17ii) from P17-3 were picked and tested, P17ii showed better growth (Supplementary Fig S1b, Paper 2) so these cells were chosen, and stored for all the further experiments (Figure 10). When these cells were revived from -80 °C and tested again with the same concentration of the NPs after months, they still showed the same growth rate in the presence of CuO NPs as previously (Figure 2b and 2c, Paper 2). This indicated that cells might have retained their adaptability to the CuO NPs and is a stable phenotype which is useful when such cells are to be used for mineral processing. Similarly,

pure culture was stored for Fe-adapted cells also from Passage 2 (P2i) and used for the further experiments (Figure 1, Paper 2).

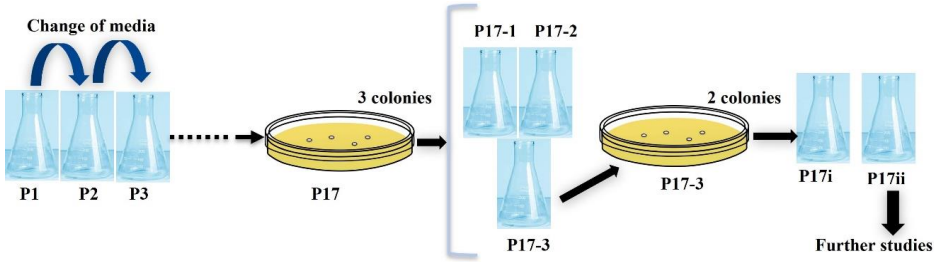


Figure 10 Adaptative evolution of *R. opacus* to toxic CuO NPs. All the cultures contained 6.25 mg/mL CuO NPs.

Then, to test surface changes in the adapted cells, zeta-potential studies were carried out, and adapted cells showed less negative zeta-potential than the wild type cells in the basic pH range. The isoelectric point of the Fe-adapted and the wild type cells were nearly the same while for Cu-adapted cells, there was a shift in the IEP (Figure 3, Paper 2).

To study and visualize the interactions between the NPs and the cells, adhesion and SEM studies were done, respectively. It was observed that the adhesion of NPs on the adapted cells was faster than on the wild type cells (Figure 4a and 4b, Paper 2). The adhesion of CuO NPs was more on the Cu-adapted cells than on the wild type cells. From the adhesion results and SEM images (Figure 4 and Figure 5, Paper 2), increased affinity between the adapted *R. opacus* and corresponding NPs was observed. These results suggested that adapted cells can work as a flocculating agent for the corresponding ultrafine mineral particles since the cells were able to precipitate NPs just in few minutes from the solution. This was also supported by the SEM image (Figure 5D, Paper 2) that indicated Cu-adapted cells had formed a network with the CuO NPs.

Adaptation or interaction of bacterial cells with NPs can result in changes in the gene expression of the cells to cope with stress. To see if this was the case, proteins were extracted from the adapted and the wild type cells and compared through SDS-PAGE. It was found that there were four different bands in the extracellular protein lane of the Cu-adapted cells (Figure 6, Paper 2). The proteins from these bands were identified through MS analysis. A total of 142 proteins were found, out of which 18 membrane proteins and 8 extracellular proteins were confirmed using various bioinformatics tools and the remaining were the result of autolysis (Supplementary Table S3, Paper 2). The differentially expressed proteins mainly consisted of cell wall/envelope synthesis proteins, catalase (act against reactive oxygen species generated by NPs), peptidyl cis-trans isomerase, histidine kinases and serine-threonine kinases (involved in signal transduction), and transporter proteins. We selected some genes and overexpressed them in *R. opacus* using plasmids to test if the overexpression of a single gene has more impact than the other in the process of adaptation to the NPs. The growth of these overexpressing strains was compared in the presence of adaptation concentration of CuO NPs and none of them had significantly higher growth than the control plasmids (Figure 8, Paper 2). This

indicated that single genes in one biosynthetic pathway could not have a measurable impact on the process of adaptation, however coping with stress is a combined action of several genes.

4.3 Paper 3: Improved site-specific mutagenesis in *Rhodococcus opacus* using a novel conditional suicide plasmid

Paper 2 showed that the cell envelope synthesis genes (mainly mycolic acids biosynthetic genes) were differentially expressed on CuO NPs adaptation. Moreover, mycolic acids impart hydrophobicity to the cell envelope of *R. opacus* cells, probably due to which it acted as a collector in the flotation of minerals in the studies conducted previously. Therefore, to test the significance of mycolic acids in *R. opacus* and their potential role in mineral processing for the separation of minerals, we wanted to construct mycolic acid negative mutant.

R. opacus is an industrially relevant species whose genetic engineering is limited due to low transformation and recombination frequency and illegitimate recombination. The other aim of this paper was also to improve the frequency of transformation and recombination in *R. opacus* for ease of its genetic manipulation. For this, we developed conjugative conditional suicide plasmids for *R. opacus* that improved the transformation and conjugation efficiency. Then, we tested these conditional plasmids for homologous recombination and the construction of *R. opacus* mycolic acid negative mutant.

First, we tested the strength of several promoters to find the best inducible system through luciferase assay. Plasmids with different promoters controlling the reporter luciferase gene were constructed: pHE511 (P_{lac} promoter), pHE518 (P_{myc1}/tetO), pRMG4 (P_m promoter, XylS controlled by P_{myc1}/tetO), and pHE513 ($P_{constit}$) (Figure 1a, Paper 3). It was found that $P_{constit}$ was strongest of all followed by P_{lac} and P_m (Figure 1b, Paper 3). Since we were looking for an inducible system and induction by m-toluate (activator of XylS) increased the expression level by five-fold while induction by IPTG or repression by aTc did not really have any significant effect on the expression levels, we selected the P_m promoter controlled by XylS for the construction of conditional suicide plasmid.

XylS/P_m is a *Pseudomonas putida* derived expression system that is positively regulated. The system is advantageous where one requires continuous low background expression. Benzoic acid derivatives such as m-toluic acid act as inducers of the XylS, which is an activator of P_m promoter which will control the expression of any gene of our interest. The use of XylS/P_m system has previously been demonstrated in Gram-negative bacteria (Gawin et al., 2017). But Dragset et al. (2015) have expanded the use of XylS/P*m* system for the conditional expression in *Mycobacterium* especially for low basal expression. The system is optimized for time and dose dependent induction in *Mycobacterium*. aTc acts as co-repressor for reverse TetR which controls P_{tet} (P_{myc1}/tetO), which in turn is controlling XylS. Therefore, the presence of m-toluic acid and the absence of aTc is required for the full activation of P_m by XylS and transcription of the gene of interest (Figure 11) (Dragset et al., 2015).

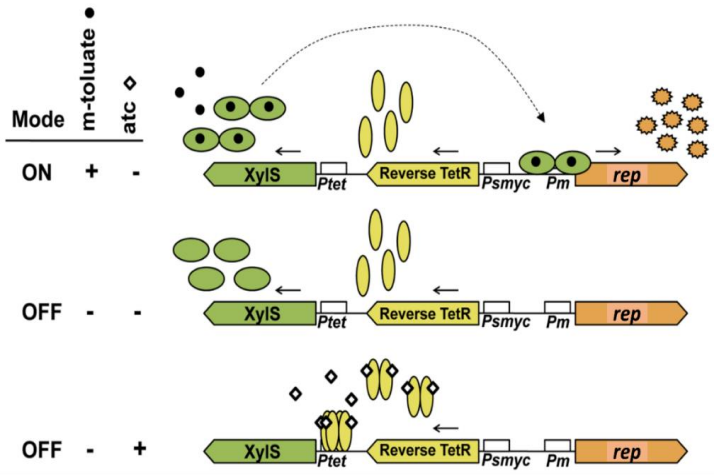


Figure 11 XylS/P_m, benzoic-acid inducible expression system optimized for *Mycobacterium*. XylS is the activator of P_m promoter which controls the expression of the gene of interest, in our case *rep*. XylS is controlled by P_{tet}. In the presence of anhydrotetracycline (aTc), corepressor of reverse TetR which control P_{tet}, *xylS* will not be transcribed. Moreover, XylS needs m-toluic acid for effective activation of P_m. Copied and modified from Dragset et al. (2015). The figure is used under the terms of the Creative Commons Attribution License.

The conditional suicide plasmid pHE524 (Km^r) was constructed based on plasmid pGA1 from *C. glutamicum* which encodes a Rep protein needed for its replication (Nesvera et al., 1997). The copy number of the plasmid is controlled by ctRNA overlapping the start of the *rep*. To construct the conditional suicide plasmid, the forward promoter of the *rep* was replaced with P_m and the dual control inducible expression system XylS/P_m from *Mycobacterium* was used (Figure 2, Paper 1) (Dragset et al., 2015). Then pHE524 and a sister plasmid pHE523 not expressing ctRNA were transferred to *R. opacus* via conjugation. Several transconjugants were obtained which were cultured in medium with and without the presence of repressor aTc and plated on LA Km with and without aTc. It was seen that plasmids were lost at high frequency even in the absence of aTc in the medium (Table 1, Paper 3). pHE524 was lost 10 times more frequently than pHE523 that did not contain ctRNA, therefore pHE524 was considered as the best conditional suicide plasmid.
Then, we wanted to test such plasmid for homologous recombination in *R. opacus,* choosing a target protein known to be dispensable. Therefore, plasmid pGJ1 was constructed based on pHE524 but with the gene *PD630_RS00415* inactivated by insertion of Cm^r gene. *PD630_RS00415* putatively encodes fatty acyl CoA ligases in *R. opacus*. pGJ1 contains *sacB* as a counter selection marker for screening of second recombinants. Non-replicative plasmid pMV11 (Supplementary Fig. S2, Paper 3) containing the same copy of the inactivated gene *PD630_RS00415* was compared with conditional suicide plasmid pGJ1 to check for any improvements in conjugation frequency. Using the same batch of recipient cells, pMV11 showed no transconjugants, pGJ1 resulted in several hundred transconjugants and after growing under non-selective conditions, homologous recombinants were identified.

Now the tool was constructed that could be used for obtaining a mycolic acid negative mutant. Plasmid pGJ6 was constructed similarly to pGJ1 but for the complete deletion of *pks* gene (Supplementary Figure S5, Paper 3). Sucrose^r, Km^s colonies were tested by PCR to identify mutants. Only wild type strains resulted after the second crossover event. The experiment was repeated multiple times and several colonies were tested but no mutant was obtained. It is known that mycolic acid is essential in *Mycobacterium* but not for *Corynebacterium* (Portevin et al., 2004, Portevin et al., 2005, Bou Raad et al., 2010). These results indicated that mycolic acid might be essential for *R. opacus* as well. To confirm this, another plasmid pGJ7 was constructed where we deleted part of *accD3* gene (~533 nt) (Supplemental Fig. S5 and S7, Paper 3). Again, it was found that after the second crossover, only the first recombinants were obtained. However, when pGJ8 (Gm^r), a complementation plasmid expressing *accD3* from the constitutive promoter $P_{constit}$ was transferred to a first recombinant strain GJ7(7) and selected on Gm (Supplementary Fig. S8, Paper 3), mutants with an inactivated chromosomal *accD3* were obtained (Figure 4, Paper 3). The results indicated that deletion is possible only in the presence of a complementation plasmid that expresses *accD3* gene (Figure 3, Paper 3).
We found that mycolic acid is essential for *R. opcaus* viability like it is for *Mycobacterium.* The new conditional suicide plasmids increased the conjugation frequency by separating the process of plasmid transfer and recombination in time compared to the non-replicating standard suicide plasmids. We showed that inducible XylS/P_m system which was previously tested for *Mycobacterium* (Dragset et al., 2015) works well for *R. opacus* too. Also, such conditional suicide plasmids (pHE524) developed for *R. opacus* can be used to deliver transposons or genome-editing systems. Moreover, the system can be used for genome editing in other related species: *Corynebacterium*, and *Mycobacterium*. However, since we could not make the desired mutant, we could not test the importance of the mycolic acids on metal ion or metal NPs biosorption.

4.4 **Paper 4: Alginates as green flocculants for metal oxide nanoparticles**

Alginates are natural polymers that are reproducible and non-toxic in nature and can work as good bioflocculants to replace the conventional toxic reagents utilized in mineral processing. Alginates have excellent gelling property exhibited just by the addition of cations which makes them useful for multiple biomedical applications, like for encapsulation or as immobilization matrix. Alginates with abundant G-blocks are highly priced because of their high demand and easy binding to the divalent cations. Contrary to that, alginates with high M-blocks are cheaper and abundant. This makes them a preferable option for new large-scale applications, such as mineral processing and water purification. Moreover, most of the studies on the flocculating properties of alginates have utilized modified alginates, but here we wanted to use unmodified alginates as that would be cheaper and more environmentally friendly. In the present paper, we are exploring the potential of alginates as flocculants for the metal oxide NPs, CeO_2 and Fe_2O_3 NPs. Also, we wanted to see if the composition of alginates (having different G-content and G-distribution) has any effect on its flocculating property.
Three types of alginates were used in the study, LF10/60 from *Laminaria hyperborean* (having G- blocks, commercial), and other two derived from *Pseudomonas fluorescens* in the lab, acetylated (PfAc) and de-acetylated (PfdeAc) alginates, having no G-blocks (Methods section,

Paper 4). LF10/60 used in the study is at the higher end, *Azotobacter* would have been an alternative, but the current studies were early studies. LF 10/60 were readily available G-block alginates to compare with the bacterial alginates.

Zeta-potential measurements indicated that the NPs could be chemisorbed on all the alginate samples (Figure 2, Paper 4) but none of them showed flocculation without the addition of cations, indicating that cations are needed to induce the cross-linking of the alginate chains. Therefore, we studied the effect of cross-linking by addition of divalent (Ca^{2+}) and trivalent (Ce^{3+}) ions. Alginate LF 10/60 showed good flocculating properties when combined with Ca^{2+} for both iron oxide and cerium oxide NPs (Figure 4a and 4b, Paper 4). The alginates from *P. fluorescens* without any G-blocks do not show any flocculating properties, either in the presence or absence of Ca^{2+} metal cations (Figure 4 and Figure 5, Paper 4). Ca^{2+} acts as a cross-linking agent for the alginates having G-blocks but it does not bind M-blocks and weakly binds to MG-block (Mørch et al., 2006). The results obtained indicated the same that G-blocks of alginates are important for their gelation when Ca^{2+} ions are used.

Further, in the presence of Ce^{3+}, the formation of flocs was observed for all three alginates (Figure 4, Paper 4). Since the trivalent cation binding is not very block specific, this could be the reason for the formation of flocs in all the alginates, although differing in their composition (Hu et al., 2021). It was observed that flocculating property of LF 10/60 could be activated by either Ca^{2+} or Ce^{3+} but it was more with Ca^{2+} for iron oxide NPs and almost the same for cerium oxide NPs (Figure 5, Paper 4). De-acetylated bacterial alginates showed better flocculating properties than LF 10/60 for iron oxide NPs with Ce^{3+} but they showed weak precipitation with cerium oxide NPs and Ce^{3+} (Figure 5, Paper 4). Only LF10/60 alginate showed a good flocculating effect for cerium oxide NPs/ Ce^{3+}. Larger flocs were formed by PfdeAc with Fe_2O_3 NPs/Ce^{3+}compared to the other two alginates which explains their faster precipitating effect for Fe_2O_3 NPs than the other two alginates (Figure 6, Paper 4). Contrary to the Fe_2O_3 NPs, PfdeAc made fiber-like flocs and web-like structure in the dispersion with the cerium oxide NPs/Ce^{3+} which only slowly precipitated in the solution and thus exhibited a weak flocculating effect with cerium oxide NPs (Figure 6, Paper 4).

The results indicate that the mechanism of aggregation of alginates varies with different cations used, as observed by Chen et al. (2006) for the aggregation of alginate coated hematite NPs using different cations (Na^{+}, Ca^{2+}, Mg^{2+}). Alginates can be used for the flocculation of fine mineral particles and their flocculation efficiency depends on their composition and the cations used for gel formation. However, the potential of alginates as green flocculants for metal oxide NPs needs to be further explored. For future work, alginates with different G-contents can be obtained from *A. vinelandii*, which has previously been studied as a flocculating agent for the purification of water (Moral et al., 2016). The bacterial alginates used now are from *P. fluorescens* that have 30% G, so there would be quite a bit of MG-blocks. Hence, for further work, alginates composed purely of mannuronan (M) (for which we have mutants that make it) can be explored in order to probe the mechanisms for the Ce-interactions. No one knows if MG-blocks are necessary for gelling by Ce^{3+}, which needs to be answered.

5 Conclusion and recommendation for future research

In this thesis, we first summarized the current status of the use of microorganisms for mineral separation, and it was concluded that there is potential in microbes and microbe-based reagents to replace the conventional toxic reagents. Based on this, we studied *Rhodococcus opacus* PD630 and alginates from *Pseudomonas fluorescens* as bioreagents for the separation of fine mineral particles. The findings indicated that the adaptation of the *R. opacus* cells to the metal oxide nanoparticles (CuO and γ-Fe_2O_3, used as model systems for the ultrafine mineral particles) led to the changes in their surface properties and resulted in increased affinity between the cells and the respective NPs to which they were adapted to, as shown by the interaction studies (SEM and adhesion tests) between them. The protein profile of the Cu-adapted cells showed that eight extracellular and eighteen membrane proteins were regulated in response to the NPs stress. However, no single extracellular or membrane protein could be identified that was responsible for the increased affinity towards the toxic NPs and could be used alone to precipitate the fine mineral particles. Despite the increased affinity between the adapted cells and the NPs, Fe-adapted cells and the wild type cells did not show any collecting effect under the tested conditions for the flotation of maghemite NPs and the hematite fine mineral particles. However, from the adhesion studies, it was found that adapted cells and wild type cells both were able to aggregate the NPs and sediment them making flocs. The aggregation was relatively faster with the adapted cells than with the wild type cells. Therefore, the *R. opacus* strain showed its ability to be used as a flocculating agent for the separation of fine mineral particles. However, the previous studies using *R. opacus* have shown that they can act as a biocollector for flotation of various minerals, but the strain used in our study did not show any flotation effect for the fine mineral particles or NPs, despite its hydrophobic surface due to the presence of mycolic acids. This suggested that there is a great variation in the bioflotation activity of different strains of the same species. This can be further studied by taking different strains of *Rhodococcus* and testing the difference in their activity for the separation of minerals. Further, in order to verify the effect of the presence of mycolic acids in *R. opacus* on flotation, we tried to construct a mycolic acid negative *R. opacus* mutant using conditional suicide plasmids. We tried to knock out the last three genes of the mycolic acid gene cluster, but such a mutant was not possible, and we concluded that mycolic acids are important for the viability of *R. opacus* as it is for related *Mycobacterium*. Since the mutant could not be constructed, the importance of mycolic acids in flotation could not be tested.

Further, we studied *P. fluorescens* (acetylated and de-acetylated, no G-blocks) derived alginates and one commercial alginate, LF10/60 (having G-blocks). These alginates showed different flocculating effects with the addition of divalent (Ca^{2+}) and trivalent (Ce^{3+}) cations towards the metal-oxide NPs (CeO_2 and Fe_2O_3 NPs). In the presence of divalent ions, bacterial alginates did not show any flocculating effect. However, with the addition of trivalent cations, the de-acetylated bacterial alginates showed the best flocculating effect among all the alginates for Fe_2O_3 NPs. The results indicated that the G-blocks are important for the gel formation in alginates when divalent cations are used. The type of ions used and the composition of alginates will determine the binding nature of ions to the alginates and hence the mechanism of aggregation will vary according to that. Currently, in our study, we tested *P. fluorescens* alginates with 30% G content, but in the future, alginates with different G-contents from

Azotobacter vinelandii could also be tested. We could also test alginates composed purely of M-blocks and try to probe the mechanisms for Ce-interactions.

Altogether, the results revealed that *R. opacus* and bacterial alginates hold the potential to be used as promising bioflocculants in mineral processing for the separation of fine and ultrafine particles. However, they can be further tested for selective separation in mixed mineral systems. Moreover, Cu-adapted *R. opacus* cells showed more affinity than the wild type cells towards the CuO NPs and were able to precipitate the NPs from the solution very fast (in a few minutes). This new mutated strain could be explored as a bioreagent in a mixed mineral system of fine particles. The cells can be used for selective flocculation of fine particles and then aggregated particles can be collected via flotation (floc-flotation). Most of the previous studies using bio-based reagents in mineral processing have been done on micro-range minerals, but the bioflocculants recognized in our work have the potential for separating the mineral particles in the nano-range. This is a useful finding since the flotation of fines is a bottleneck in mineral processing industries. In addition to this, the flocculating activity of the relatively cheaper bacterial alginates (de-acetylated alginates with no G-bocks) was better than the costlier commercial alginates (with G-blocks) tested here for Fe_2O_3 NPs, which makes the former a promising candidate for large applications such as mineral processing. It is also possible to culture *R. opacus* on a large scale to produce sufficient biomass for industrial use. The work in the thesis is important, keeping in view its environmental significance. It lays down the foundation for future work towards the potential of bacteria and bacteria-derived metabolites to selectively flocculate or float the fine and ultrafine mineral particles, thus facilitating their purification from the mixtures in an eco-friendly way.

6 References

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7. Papers

Paper 1

Chapter 9

Green Foam-Based Methods of Mineral and Ion Separation

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We discuss an important gap in the literature on progress toward the development of green (microbial-derived) reagents in conventional (froth flotation) and emerging (foam flotation) separation methods. For comparison, we provide a brief overview of recent trends in development of conventional surfactants for froth flotation of sulfide minerals, as well as the current status of foam flotation in the removal of heavy metals from wastewater and selective extraction of rare earth elements.

Introduction

Global demand for industrial minerals has significantly increased in the past four decades and is projected to keep increasing. Froth flotation is one of the main physicochemical methods used in the mining industry to extract valuable minerals from ores (*1*). It is based on the selective adsorption of surfactants on dispersed particles of a valuable mineral, which makes them hydrophobic. In the dispersion the hydrophobic particles attach to gas bubbles that carry them to the top of the flotation cell, where they are skimmed off (Figure 1[a]). A surfactant is an amphiphilic molecule with a hydrophilic headgroup and a hydrophobic tail that adsorbs at interfaces and reduces interfacial tension (*2*). To increase the contrast in hydrophobicity of valuable and gangue minerals, the technology uses additional reagents (e.g., depressants) and regulators (e.g., hydrosulfides, cyanides, and dichromates) (*2*, *3*).

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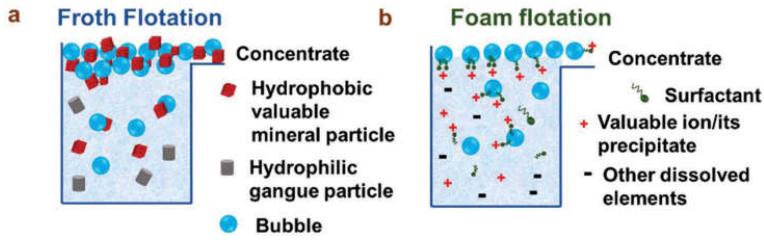

Figure 1. Principles of (a) froth and (b) foam flotation. Froth flotation relies on a difference in the hydrophobicity of different mineral particles dispersed in water that is controlled by selective adsorption of amphiphilic molecules (surfactants). Foam (ion or precipitate) flotation is applied to an aqueous solution containing dissolved ions or their precipitates and a surfactant.

Even though froth flotation has been used successfully for mineral separation for more than a century (*4*), the progressive reduction of ore grade forces miners to produce fine and ultrafine particles to liberate valuable minerals, which makes the optimal economic performance of the industry more and more challenging. Hence, the mainstream effort in this research area focuses on improving the cost-effectiveness of new froth flotation schemes and reagents, including surfactants (*5*, *6*).

In parallel, there is a growing effort toward the development of flotation reagents that are not only cost-efficient but also environmentally safe (nontoxic, nonhazardous, and environmentally benign). This effort is underpinned by the progressive tightening of environmental and health regulations, combined with low social acceptance of virgin mining (*7*). In fact, many of the conventional reagents are toxic, inflammable, and unstable. They can have adverse health effects in the workplace, preclude easy water reuse, and pollute the environment after disposal in tailing ponds. However, the low cost of conventional toxic reagents makes their replacement by green alternatives prohibitive unless the latter demonstrate superior separation properties and their cost measures up to the market demand. Evidently, this task is highly challenging. A very promising approach to address this challenge is to integrate the reagent development with biotechnology (*8*). Microorganisms have not only facilitated hydrometallurgical leaching operations but also have shown great promise in froth flotation. Several laboratory investigations have revealed that microorganisms and biomolecules derived from microorganisms could function like traditional reagents.

Apart from the separation of mineral particles, foams are capable of separating metal ions. The corresponding method was invented six decades ago and is called foam flotation (Figure 1[b]). This method can separate dissolved metal ions as such or their precipitates (called ion and precipitate flotation, respectively). Even though foam flotation has not yet been commercialized, it has been successfully applied to extract heavy metals and separate many metals, despite numerous issues that must be addressed. There is a rapidly increasing interest in improving foam flotation due to its potential to make hydrometallurgy economically and environmentally more sustainable. Hydrometallurgy extracts valuable elements from their matrices by chemical leaching that is traditionally followed by solvent extraction, cementation, adsorption, ion exchange, and electrowinning (*9*). Hydrometallurgy is currently a method of choice for post-flotation separation and purification of rare earth elements (REEs). It is also the main method of metal extraction from low-concentrated or low-volume streams that are difficult to process using pyrometallurgy. Such streams present many secondary sources including mining waste, wastewater, desalination brines, certain types of electronic waste, and sediments. Therefore, hydrometallurgy is likely to be the future of mining in a circular economy where the secondary streams are expected to become the main

sources of raw materials (*6*). However, thus far, hydrometallurgy does not demonstrate value when both economic and environmental factors are considered (energy, reagents, water, time, space, capital, and labor costs, combined with the generation of substantial waste streams, including toxic waste). To address these limitations, extensive research is underway toward innovating existing hydrometallurgical methods and introducing new ones. In this context, there has been an increasing interest in foam flotation.

This chapter presents an overview of recent progress in froth and foam flotation processes in the separation of minerals and ions, with a focus on the increase in the economic and environmental sustainability of both traditional petroleum-based and microbially-produced surfactants.

Conventional and Emerging Surfactants in Froth Flotation of Sulfide Minerals

The hydrophobic/hydrophilic properties of mineral particles selectively modified using surfactants ("collectors") are the main controlling factors for selectivity of mineral separation by froth flotation, even though froth flotation is strongly influenced by physical parameters (e.g., gas flow rate, particle size and shape, density, etc.). Because it is more convenient to alter the reagent scheme than to redesign the flotation flowsheet and/or install new equipment, the development of new collectors has been one of the major areas of research in adapting this century-old technology to the challenges of the unstable market, progressively stricter environmental regulations, and complex, difficult-to-process ores. Therefore, the design and development of collectors remains an important topic in current mineral processing research (*4*).

Past research has led to delineating the scientific basis for the development of flotation collectors in terms of the reactivity of the surfactant headgroup with the surface and colloid chemistry of the mineral surface, including its composition and crystallographic structure, solubility, and (electro)catalytic properties. The adsorption of a surfactant at the solid–water interface is driven by a decrease in the free energy of the system due to the following major interactions: electrostatic, chemical (including covalent, ionic, and coordination bonding), hydrogen (H) bonding, cation–dipole (e.g., C=O or OH) interactions, solvation/desolvation, and chain–chain or chain–surface hydrophobic interactions (*10*). Selectivity of the surfactant adsorption is controlled mostly by the electrostatic and chemical interactions. The electrostatic interaction is controlled by pH as it depends on the surface charge of the mineral (established by adsorption of H^+/OH^- or other potential determining ions) and the protonation state of the surfactant head group (controlled by its acid-base properties). Chemisorption is generally predicted by the hard and soft (Lewis) acid and bases concept pioneered by Pearson (*11*), or by the stability constants of the ligand–metal complexes (*12*). The qualitative difference between the adsorption of ligands and surfactants is underpinned by hydrophobic interactions. These interactions increase with increasing chain length and decreasing hydrophilic–lipophilic balance of a surfactant, driving the surfactant self-assembly (packing) at the mineral–solution interface (*13*). Self-assembly can be hampered by the incompatibility of the surfactant headgroup structure or size in the adsorbed state with the surface motif of the adsorption sites and the minimal required chain–chain distance (*14*). Additional complications can be imposed by (1) the metal-surfactant precipitation (*15*), (2) dissolution reprecipitation of metal ions as hydrolyzed species (autoactivation) (*1*), and (3) redox or catalytic interactions between the transition metal mineral and redox-active sulfhydryl collectors (see the following section for more detail) (*16–19*). The surfactant adsorption also depends on the composition of the "inert" electrolyte (*20*).

Sulfide ores are the major source of base and precious metals such as Cu, Pb, Zn, Au, and Ag. Froth flotation is an important process used by mineral processing industries and more than 95% of

metal sulfides are recovered by this process. The major sulfide mineralogy consists of valuable copper, lead, zinc, and nickel, while the gangue includes iron, silicate, and clay minerals. The problems of nonferrous metal sulfides selectivity against ferrous sulfides and fine particle flotation have been broadly studied (*3*, *21*–*23*). The usual procedure in the flotation of complex sulfide ore is floating Cu and Pb minerals in the basic pH region in the first stage, while a Zn mineral is floated in the second stage. It is well established that all metal sulfide minerals undergo oxidation, exhibiting oxide and hydroxide species on their surface after exposure to air or an aqueous solution, which is detrimental to flotation.

In the following, we briefly summarize recent progress in the development of traditional (petroleum-based) collectors in sulfide mineral flotation, while emerging biosurfactants are discussed in the next section (more-comprehensive recent reviews can be found in references (*7*) and (*24*)).

Sulfhydryl Collectors

Historically, the major class of surfactants used for floating sulfide minerals is sulfhydryl compounds containing a sulfur atom in their head group and a hydrophobic organic ligand. The majority of sulfhydryl surfactants are derivatives of carbonic acid, carbamic acid, phosphoric acid, urea, and alcohol (*21*). The most frequently used sulfhydryl surfactants for industry are alkyl dithiocarbonates (xanthates), xanthogen formats, xanthate ester, dialkyl and dicresyl dithiophosphates, dialkyl dithiophosphinates, and dialkyl dithiocarbamate. Xanthates are usually less selective than dithiophosphates and dithiocarbamates (*25*). In order to improve selectivity, mixtures of sulfhydryl collectors are used (*26*, *27*). Better results are observed when in the collector mixtures, xanthates are used as the primary collector while xanthic acid, xanthogen formats, dithiophosphates, and dithiophosphinates are used as the secondary collectors (*3*, *21*, *27*).

Sulfhydryl collectors can be adsorbed on metal sulfides through chemical mechanisms (*28*), including chemisorption (*29*, *30*), dissolution–precipitation (e.g., oxidative dissolution of the sulfide mineral followed by precipitation of the metal collector complex) (*15*), ion exchange reaction (*31*), and redox reactions with Cu^{2+} cations (*16*). Complexes of sulfhydryl compounds with metals can have a broad diversity of coordination modes including monodentate, chelating, bidentate, and bridging (bimetallic biconnective) (*32*, *33*). The principal adsorption mechanism for redox-active sulfhydryl collectors, such as xanthates, dithiophosphates, and dithiophosphinates, is the electrochemical (mixed potential) mechanism (*18*, *19*, *28*, *34*, *35*). According to the widely accepted words of Woods (*17*–*19*), the mineral–sulfhydryl interaction can be treated as the sum of two coupled heterogeneous electrocatalytic reactions (Figure 2). The first is the oxidation of deprotonated sulfhydryl anion X^-:

$$X^- = X_{ads} + e^- \quad \text{and} \tag{1a}$$

$$2X^- = X_2 + 2e^-. \tag{1b}$$

The other is the oxygen reduction reaction (*18*, *19*):

$$O_2 + 4e^- + 4H^+ = 2H_2O \tag{2}$$

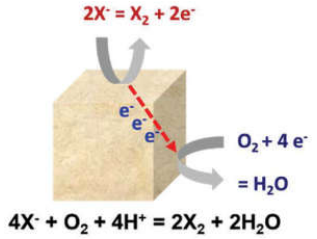

Figure 2. Electrochemical (mixed potential) mechanism of the interaction of a sulfhydryl collector X^- with a semiconducting metal sulfide particle.

Reactions (1a) and (1b) take place on the anodic (electron-poor) spot of the sulfide particle, making this spot hydrophobic. Reaction (1a) presents the underpotential oxidation of the collector, leading to its chemisorption. Reaction (1b) presents its bulk oxidation to a disulfide dimer with an S–S bond X_2 (e.g., dixanthogen), which is physiosorbed on the mineral surface. Reaction (2) takes place on a cathodic (electron-rich) spot of the mineral particle. The anodic and cathodic spots are coupled electronically, meaning that there is electron transfer from the anodic to the cathodic spot due to the electronic conductivity of the sulfide.

It should be noted that since the electrochemical mechanism of reactivity of semiconducting metal sulfides was established in mineral processing, it has been adopted by the geochemistry and catalysis communities (*36*–*38*). In return, research on froth flotation has begun to adopt principles of electrocatalysis. In particular, it has been demonstrated that catalytic activity of metal sulfides in their electrochemical interaction with xanthates can be described by their activity in an oxygen reduction reaction (*39*).

The main advantages of sulfhydryl collectors include their low cost and high selectivity to the metal sulfides, in spite of being short hydrocarbon chain collectors. Their selectivity can be further improved by attaching additional active sites to the sulfhydryl group (*40*). However, many of these collectors, such as xanthates, pose occupational hazards to the workers as their decomposition products (CS_2 and OCS) are strong eye and skin irritants (*41*). At the same time, dialkyl dithiophosphinates are considered as less problematic, which has led to a growing interest in them as substitutes for more toxic or hazardous sulfhydryl collectors (*30*).

Chelating Collectors

Chelating collectors have been used industrially as secondary collectors to increase the selectivity of sulfide mineral separation (*26*, *42*). These collectors present polydentate ligands capable of forming strong coordination complexes with multivalent metal ions. They coordinate with the metal ions via two or more functional groups, forming a chelate-ring that renders them a strong specific affinity for certain mineral surfaces (*43*). Most chelating agents form a stable four- or six-membered complex (*44*, *45*). The formation of several bonds with the mineral surface makes chelating collectors generally more selective in the flotation of complex sulfide mineral systems when compared to sulfhydryl collectors (*45*). In the flotation of metal sulfides, three major classes of chelating collectors include: (1) alkyl hydroxymates, (2) oximes, and (3) mercapto compounds (*46*). Table 1 summarizes recently used chelating collectors in sulfide flotation, along with their main separation results.

Table 1. Summary of Tailor-Made Chelating Reagents Used in Metal Sulfide Flotation

Collector	*Ores*	*pH*	*Flotation outcomes*	*Ref.*
Alkoxycarbonyl Alkyl Thiourea	Cu–Au ores Cu–Fe ores	8–9	Float chalcopyrite more efficiently than chalcocite, bornite, and covellite	(*71*)
N-propyl-N-ethoxycarbonyl thiourea	Cu ores	8.5	Cu-mineral flotation against Fe-sulfides	(*72*)
3,3-diethyl-1,1-oxydiethylenedicarbonyl bis(thiourea)	Complex sulfide ores	<9.0	Selective Cu-mineral flotation against pyrite, sphalerite, and galena	(*73*)
3-hexyl-4-amino-1,2,4-triazole-5-thione	Complex sulfide ores	4–8	Selective chalcopyrite flotation from iron and other gangue minerals	(*74*)
Taurin, $NH_2(CH_2)_2SO_3H$	Oxidized Pb ore		Celestite flotation	(*7*)
L-dioxime	Ni ore	11–12	Effective nickel and cobalt flotation from iron impurity minerals	(*3*)
Hydroxy oximes	Cu ore with Si impurities	9–11	Effective flotation of chalcopyrite, chalcocite, and crysocolla over quartz	(*3*)
8-Hydroxyquinoline	Cu–Zn ores, Mixed CuS ores	4–6	More specific to chalcopyrite than other copper minerals	(*3*, *7*)
2,3-Alkyldione dioxime	Ni ore	4–9	As effective as xanthate for pentlandite flotation	(*75*)
1-Nitroso-2-napthol	Co–Ar ore; Co–Fe ore	8–9	Selective flotation of Co containing minerals from pyrite, pyrrhotite, and chalcopyrite	(*75*, *76*)
Amino thiophenol	Pb and Zn minerals	6–8	Selective flotation of marmatite over jamesonite	(*77*)
Mercaptobenzoxazole	Cu ore	5.5–6	Selective flotation of copper minerals from quartz, other silicates	(*2*)

Table 1. (Continued). Summary of Tailor-Made Chelating Reagents Used in Metal Sulfide Flotation

Collector	*Ores*	*pH*	*Flotation outcomes*	*Ref.*
Mercaptobenzothiozoles	Pb ore	7–10	Selective flotation of lead from chalcopyrite and pyrite	(*2*)
Diisothiocyanate diester derivative	Cu ore, Cu–Au ore, Cu–Fe ores, Pb–Fe ore, Zn–Fe ore	5–11	Float chalcopyrite, lead sulfide minerals, or zinc sulfide minerals activated by copper ions, nickel sulfide minerals as well as noble metal minerals (e.g., gold, silver, etc.) and rejects gangue sulfide minerals (e.g., iron pyrites, pyrrhotite, etc.)	(*78*)
Diacyl dithiourea sulfide	Cu ore, Pb–Fe, Zn–Fe ores	<11	Float copper, lead, zinc minerals selectively from iron sulfides	(*79*)
1,3,4-thiadiazole-2-thione	Cu, Ag, Au minerals	6–11	Enrichment and recovery of copper, silver, or gold	(*80*)
1,3,4- thiadiazole	Pb–Fe, Zn–Fe ores	4–10	Selective flotation of lead zinc minerals from pyrite	(*81*)
dialkyloxycarbonyl bisthiourea	Cu, Pb, Zn sulfide ores	0–11.5	Selective flotation of nonferrous ores from ferrous impurity minerals	(*82*)
n-octyl hydroxamate	Cu–Fe ores	7–10	Selective separation of chalcopyrite and bornite from magnetite	(*83*)

Chalcopyrite: $CuFeS_2$; Pyrite: FeS_2′ Sphalerite: (Zn–Fe)S; Galena: PbS; Chalcocite: Cu_2S; Pyrrhotite: $Fe_{(1-x)}S$; Marmatite: (Zn–Fe)S; Jamesonite: $Pb_4FeSb_6S_{14}$; Celestite: $SrSO_4$; Pentlandite: $(Fe,Ni)_9S_8$; Chrysocolla: $Cu_{2-x}Al_x(OH)_4{\cdot}nH_2O$ or $(Cu, Al)_2H_2Si_2O_5(OH)_4{\cdot}nH_2O$; Quartz: SiO_4.; Metal sulfide: Me-S.

The major advantage of using chelating collectors is the remarkably high stability of their complexes with selected metal cations. These collectors produce better results at acidic or neutral pH as compared to the traditional sulfhydryl collectors, which are only used at basic pH. Additionally, it is relatively more straightforward to assess the complexation of these collectors using thermodynamic and density functional theory (DFT) modeling as compared to the redox-active sulfhydryl collectors which can undergo catalytic oxidation (Figure 2). The major disadvantage is that chelating collectors are also toxic, hazardous, and most are not biodegradable so, as a result, they can bioaccumulate. For example, occupational and environmental hazards are linked to one of the most effective chelating surfactants: alkyl hydroxymates (*47*, *48*).

Despite some progress, the development of more effective flotation collectors has been relatively slow (*49*). Modifying the flotation strategy based on comparison of metallurgical data (e.g., bulk flotation followed by separation vs sequential flotation) might lead to better economic and environmentally friendly results. In addition, collector development can be accelerated by employing the mineralogical and automated mineralogical analyses of the flotation to detect and assess problems arising due to the dynamic nature of the process (*50*, *51*). To better understand the effect of reagents and other variables during flotation, it can also be useful to compare the flotation feed, flotation concentrates, and tailings at the end of each stage (*21*, *52*). Another approach to the development of more effective collectors could be based on experimentally validated theoretical (DFT and molecular dynamics) modeling approaches capable of ranking the interfacial activity of different surfactants and their mixtures. However, computational ranking thus far has been limited to case studies of a subset of conventional surfactants and mineral systems, while most of these studies instead explore mechanistic aspects of the selectivity of already established surfactants (*45*, *53*–*66*). Unfortunately, the predictive power of this straightforward approach has been very limited due to the variety of mineral systems and the condition-dependent multiplicity of interfacial variables. These drawbacks are anticipated to be addressed in the future with growing computational power and adaptation of tools developed for computational materials discovery in catalysis (*67*–*69*) and drug design (*70*). In particular, it has recently been demonstrated that the design and synthesis of new flotation collectors can be guided by computer-aided drug design tools such as quantitative structure-activity relationship (QSAR) (*70*). Hence, the new era in the development of novel, more effective conventional surfactants and biosurfactants could be associated with developmental progress of computational tools.

Microorganisms in Sulfide Mineral Flotation

The use of microbes and microbial metabolites for effective and selective separation of minerals from ores has gained interest due to the imperiling effect of conventional flotation reagents such as hydrosulfides, cyanides, dichromates, and more, on the environment (*84*). Several species of microorganisms—*Leptospirillum ferrooxidans, Bacillus pumilus, Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans, Paenibacillus polymyxa,* and others—have been reported in the selective separation of various sulfide minerals such as chalcopyrite, pyrrhotite, pyrite, and sphalerite (*85*–*87*). The bacteria interact with the mineral particles to act as either biocollectors or depressants in the bioflotation process. This interaction can be due to the production of extracellular polymeric substances (EPSs) or biosurfactants from the bacteria, which bind to and change the surface properties of the minerals (*87*).

The beneficiation of sulfide minerals from ore using microorganisms as collectors, depressants, or modifiers for more selective, efficient, and environmental-friendly separation is termed bioflotation. It involves the adhesion of bacteria on the mineral surface and the formation of biofilm, which changes the surface chemistry of the mineral particles. The interaction between bacteria and mineral surface depends on many factors based on the surface properties of the bacteria and the mineral particles (e.g., surface charge, presence of hydrophobic or hydrophilic groups, polymers on the surface of bacteria, acid-base components of surface energy, etc.) (*85*).

The progress of microbial flotation of sulfide minerals is reviewed in this chapter along with an overview of biomolecules, biosurfactants, whole cells, and EPSs studied so far in the flotation of sulfide minerals.

Mechanisms of Sulfide Mineral Flotation and Depression by Deposition of Sulfates

The depression of sulfide minerals by microorganisms primarily takes place by means of the formation of insoluble sulfate on the surface of sulfide mineral particles. The reaction can take place in two different ways via the formation of two types of intermediates; polysulfide or thiosulfate (Figure 3). Acid insoluble metal sulfides (FeS_2, MoS_2, WS_2) usually follow the thiosulfate pathway, whereas the polysulfide mechanism mainly occurs for acid-soluble metal sulfides (*88*). In both cases, Fe^{3+} ions are necessary to initiate and propagate the reactions and, at acidic pH and in the absence of bacteria, elemental sulfur is produced upon the dissolution of sulfide minerals (*88*). Elemental sulfur is quite stable in nature; therefore, it can only be efficiently oxidized in the presence of some sulfur-oxidizing agents such as sulfur-oxidizing bacteria (e.g., *Acidithiobacillus spp, Sulfolobus spp*) (*89*). These bacteria may theoretically interact with the sulfide minerals via three mechanisms: direct contact, indirect contact, and noncontact (*90*). Both in direct and indirect contact mechanisms, bacteria attach to the surface of mineral particles, form a biofilm, and secrete EPSs (e.g., carbohydrates, nucleic acids, fatty acids, and proteins). The only difference between the mechanisms is that in direct contact there is no need of ferric or ferrous ions, while in the latter two bacteria oxidize ferrous to ferric ions, which then leach the mineral.

When the pH is sufficiently low, the resulting sulfate will be deposited on the mineral surface as insoluble hydrophilic metallosulfates. As described above, increased hydrophilicity causes the mineral to be more likely to be depressed during flotation (*91*, *92*). In contrast to the depression mechanism, one possible mechanisms proposed for the raising or flotation of sulfide minerals by microorganisms is through the formation of elemental sulfur that makes the surface of the mineral particles hydrophobic and, hence, they can be floated during separation (*91*, *93*, *94*).

Flotation Selectivity

Sulfide minerals are often found as mixtures (e.g., chalcopyrite is usually associated with sphalerite and pyrrhotite in the deposits). Given that the separation of minerals from complex mixtures is a key to efficient mineral processing, many studies have been performed to provide evidence that microorganisms may be used to increase the variation in floatability between different minerals. Often the cells' function is to depress one mineral and thus collect the more valuable by flotation (see Table 2 for examples). The most common trend that is followed in sulfide mineral bioflotation is the depression of pyrite minerals from other associated sulfide mineral particles.

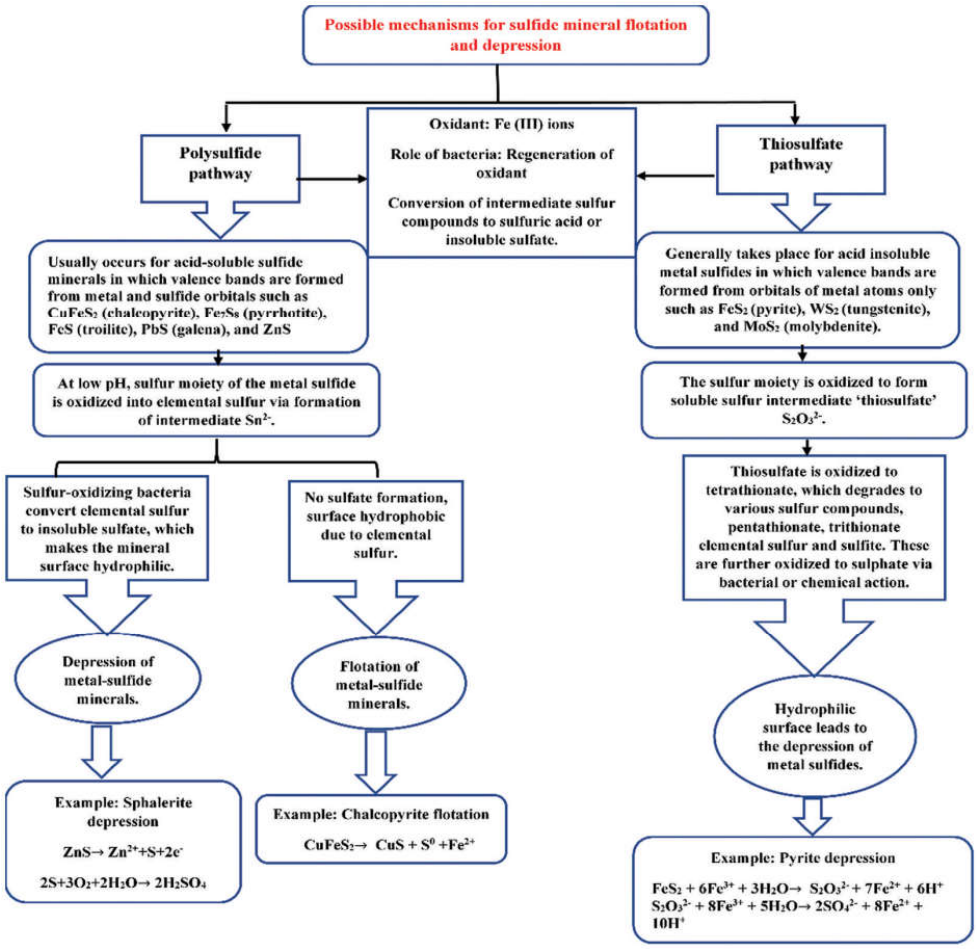

Figure 3. Possible mechanisms for sulfide minerals flotation and depression.

A. ferrooxidans is an acidophilic bacterium that derives its energy from the oxidation of ferrous, sulfur, and reduced sulfur compounds (*95*). Chandraprabha et al. (*96*) studied the role of *A. ferrooxidans* in the separation of pyrite associated with other sulfide minerals, chalcopyrite, and arsenopyrite. Flotation in the presence of both collectors and bacterial cells made the process quite efficient, overcoming the need to use environment unfriendly depressants. The change in electrophoretic mobility and the isoelectric points of the minerals and the cells upon interaction indicates the changes in their surface properties. The electronegative character of the minerals was reduced after interaction. Among the three minerals, the reduction in the electrokinetic behavior of the pyrite and pyrite-interacted bacterial cells was at the maximum, whereas it was at the minimum for the arsenopyrite. This contrast suggests only minimal interaction of arsenopyrite with the bacterial cells. These results clearly indicate the specific adsorption and the selectivity of the bacterial cells toward a particular mineral.

Table 2. Microbes and Metabolites Studied for their Effect on the Flotation of Different Sulfide Minerals

Bacterium (ref)	*Biological system*	*Mineral systems*
Alicyclobacillus ferrooxydans (*87*)	Cells	Pyrite
Effect/results: Bacterial cells produced a significant amount of biosurfactants that changed the properties of the surface of mineral (pyrite) and thus acted as a biocollector or depressant with respect to incubation time.		
A. ferrooxidans (*96*)	Cells	Pyrite, chalcopyrite, arsenopyrite
Effect/results: The conventional collector xanthate was used along with bacterial cells. Cells made the surface of pyrite hydrophilic, which remained the same even in the presence of the collector, hence depressed pyrite from other minerals.		
A. ferrooxidans (*86*)	Cells	Pyrrhotite, chalcopyrite,sphalerite
Effect/results: The order of adhesion of the cells to the mineral surface is: pyrrhotite > chalcopyrite > sphalerite. Due to acidic pH, sphalerite oxidizes to soluble species of zinc, therefore cells are weakly bonded to the mineral as the dissolution process continues. Toxicity of Cu ions in chalcopyrite results in lower adsorption of cells on the surface of the mineral. Therefore, maximum adsorption of cells occurs on the surface of pyrrhotite, resulting in its depression.		
A. thiooxidans (*107*)	Cells	Pyrite, chalcopyrite
Effect/results: Flotation of minerals with bacterial cells and conventional collector potassium isopropyl xanthate. Chalcopyrite was floated, whereas pyrite was depressed.		
A. thiooxidans (*84*, *92*)	Cells	Galena, sphalerite
Effect/results: At both alkaline and acidic pH, sphalerite oxidizes to soluble species and galena to insoluble ones, resulting in greater adsorption of cells on the surface of galena than on sphalerite. After the interaction with cells, the isoelectric point of the minerals shifts to higher values pH values. Interaction between cells and minerals occurs via hydrogen bonding. Decrease in the absolute magnitude of zeta potential values of the cells due to interaction. Obtained nearly identical flotation recoveries of the minerals pretreated with the cells in the presence as well as the absence of collector (PIPX) and activator ($CuSO_4$).		
Bacillus megaterium (*113*)	Extracellular ssDNA	Galena, sphalerite
Effect/results: Recovery of about 95% sphalerite from the mixture in the presence of ssDNA and other bacterial components with a selectivity index of 19.1. ssDNA acted as the biocollector and other components like polysaccharides and teichoic acids acted as depressants. DNA can act as a potential biocollector in bioflotation process owing to its amphipathic nature due to the presence of polyphosphate groups and hydrophobic bases.		
Bacillus pumilus (*87*)	Cells	Pyrite
Effect/results: Acts as biocollector/depressant according to the duration of incubation with the mineral. SEM analysis revealed that the pyrite particles were attached to the cell walls of the bacterial cells. Filaments were observed, which can be attributed to the EPSs produced by the cells.		
Bacillus subtilis (*24*)	Cells	Quartz, calcite, pyrite, galena
Effect/results: Pyrite was depressed and separated from the oxide mineral particles (quartz and calcite) and the sulfide mineral (galena). Mineral-specific proteins and polysaccharides were secreted by the cells, making		

Table 2. (Continued). Microbes and Metabolites Studied for their Effect on the Flotation of Different Sulfide Minerals

Bacterium (ref)	*Biological system*	*Mineral systems*
their surfaces either hydrophilic or hydrophobic. Larger amounts of polysaccharides were secreted in the presence of pyrite, which made its surface hydrophilic and hence depressed it during flotation.		
L. ferrooxidans (*94*)	Cells	Pyrrhotite, chalcopyrite, sphalerite
Effect/results: Studied the effect of cells on the floatability of the minerals using xanthate as collector. Least adsorption of the cells on the surface of sphalerite due to its lowest iron content (0.62 wt %). Hydrophobicity of the chalcopyrite was found to increase after contact with the bacteria and reached its maximum at 20 mins interaction. Bacteria acted as a weak depressing agent for pyrrhotite when conditioned with the mineral for more than 60 mins. Sphalerite floatability was not much affected by the cells. The authors stated that bacterial cells bring only the apparent changes on the surface of minerals due to their oxidation.		
L. ferrooxidans (*85*)	Cells	Pyrrhotite, chalcopyrite
Effect/results: The cells have a greater affinity for pyrrhotite than chalcopyrite and chalcopyrite recovery increased from 80 to 95% in the presence of bacterial cells. However, it acted as a weak depressant for the pyrrhotite.		
P. polymyxa (*108*)	Cells and cell metabolites	Pyrite, chalcopyrite
Effect/results: Pyrite-adapted cells have the highest amount of cell surface secretions (proteins and polysaccharides) in comparison to chalcopyrite-adapted and unadapted cells. Flotation recovery was found to be higher in the presence of unadapted cells compared to adapted cells and was at its lowest in the case of chalcopyrite-adapted cells.		
P. polymyxa (*109*)	Bacteria and proteins secreted by it	Pyrite, quartz, calcite
Effect/results: Affinity of bacterial cells to minerals: Pyrite >calcite >quartz. Flotation recovery of quartz was 96% and for pyrite 7.8% with the use of a small amount of collector (hexamine) after the interaction of the minerals with bacteria. With extracellular bacterial protein, the recovery of quartz was 82.2%.		
P. polymyxa (*109*, *135*, *136*)	Protein	Pyrite, chalcopyrite, galena, quartz, calcite
Effect/results: Increased hydrophobicity of galena, quartz, calcite. A depressant for pyrite and chalcopyrite. As a function of pH, the adsorption density of cells on the surface of chalcopyrite was more than that of galena. The adsorption density of extracellular proteins (bioproteins) was higher on chalcopyrite than that of galena in acidic and alkaline pH. Adsorption densities of bacterial cells and metabolites were found to be greater on the pyrite surface than that of galena for all pH levels studied.		

Similar results were reported by Pecina-Treviño et al. (*86*), who studied the role of *A. ferrooxidans* in the bioflotation of sphalerite, pyrrhotite, and chalcopyrite separately and also in the mixture. They found that chalcopyrite flotation was increased and pyrrhotite was depressed, resulting in their separation. The mechanism appeared to be that, because the number of *A. ferrooxidans* cells adhering to pyrrhotite was higher compared to those adhering to sphalerite and chalcopyrite, pyrrhotite was depressed due to the hydrophilic nature of the *A. ferrooxidans* cells.

Interaction of Sulfide Minerals and Bacterial Cells

The interaction between the sulfide mineral particles and the bacterial cells is governed by several factors, summarized in Figure 4. In addition to the functional groups present on the cell's surface, the interaction of the bacteria with the mineral particles depends on the characteristics of the mineral particles, such as their chemical composition and dissolution, the nature of the products formed on the surface of particles as a function of pH, and toxicity of the mineral substrate (*84*, *86*). If the oxidation product formed on the surface of a sulfide mineral is soluble, then the number of bacteria adsorbed on its surface would be lower because of the dissolution. This is the case for sphalerite, which is oxidized to soluble zinc sulfate and zincate ions at acidic and alkaline pH, respectively. However, there will be greater adsorption of cells if the oxidation product formed on the surface is insoluble, as in the case of galena, where there is a formation of insoluble lead sulfate and lead hydroxide at acidic and alkaline pH, respectively. Therefore, Santhiya et al. (*84*) reported that there is higher adsorption of *A. thiooxidans* cells on the surface of galena than onto sphalerite during their separation. Minerals that release toxic ions, such as Cu^{2+}, might also attract fewer cells. On the other hand, such minerals could be an ecological niche for bacteria resistant to that particular ion. Moreover, for iron- and sulfur-oxidizing bacteria, access to these energy sources will promote growth and thus enhance the cell number. This might explain why the number of *L. ferrooxidans* cells on the surface of pyrrhotite that has 58.99 wt% iron was found to be higher than the number found on chalcopyrite and sphalerite, which contain 32.58 wt% and 0.62 wt% iron, respectively (*94*).

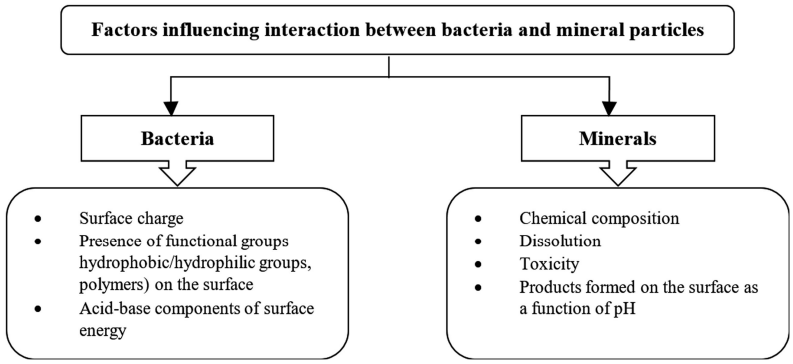

Figure 4. Factors influencing the interaction between bacteria and mineral particles.

It is reported that the EPS released by the bacteria mediate the interaction between the cells and mineral particles via formation of a biofilm (*97*). Within this biofilm, a microenvironment is created that can promote attachment and a high concentration of cells, retain ions (e.g., Fe^{2+} and Fe^{3+}), absorb harmful substances such as heavy metal ions, and more. *A. ferrooxidans* can oxidize a variety of sulfide minerals (e.g., chalcopyrite, sphalerite, galena, pyrite, etc.) and it is used for the extraction of sulfide minerals (*98*). Devasia et al. (*99*) studied the difference in the surface properties of *A. ferrooxidans* cells grown in the presence of sulfide minerals to those grown in the presence of ferrous iron. The cells grown in the presence of sulfur and sulfide minerals (pyrite and chalcopyrite) were more hydrophobic than ferrous ion-grown cells, which accounts for their better adhesion. Devasia et al. suggested the formation of a proteinaceous component on the surface of cells grown in the presence of the minerals that acts as a support for the adhesion of cells with the mineral particles. The change in hydrophobicity and the electrokinetic potential of the cells may be due to this new

component. It has also been reported that the initial cell adhesion occurs due to the hydrophobic and electrostatic interactions, followed by absolute adhesion due to the proteinaceous component's role as a support (*99*, *100*). FTIR studies revealed the presence of functional groups (NH_2, CONH, CO, CH, CH_2, CH_3, NH_3, and COOH) corresponding to proteins on the surface of *A. ferrooxidans* cells grown in the presence of sulfur and sulfide minerals (*99*). Other bacterial species have also been found to adjust their EPS production to the mineral to which they are adapted (*101*, *102*). Genomic changes have been found in such adapted bacteria (*103*, *104*), indicating that it might be possible to obtain strains with improved properties by following this approach.

A. thiooxidans are also exploited for the separation of sulfide minerals. This bacterium uses elemental sulfur or reduced sulfur compounds as substrates for its growth and oxidizes elemental sulfur to sulfate (*105*). The bacterium must first adsorb on the surface of the sulfur particles to oxidize the elemental sulfur. The adhesion process is pH-dependent and occurs via thiol groups present in the bacterium's cell envelope. The attachment of these cells to the sulfur particles is an energy-dependent process and requires an optimum pH of 2–5 (*84*, *106*). Chandraprabha and Natarajan (*107*) studied the interaction of *A. thiooxidans* with pyrite and chalcopyrite mineral particles and the surface changes taking place on these mineral particles due to the interaction. They found that the surface of both the bacterial cells and the mineral particles changed upon interaction, with an increase in the isoelectric points of both and a reduction in electrophoretic mobility. They suspected that the reason for this reduction was the secretion of proteinaceous compounds from the cells after interaction with mineral particles. They also noticed a higher cell density on the surface of pyrite than chalcopyrite because of the toxic copper ions present in the chalcopyrite (*107*). *A. thiooxidans* have also been studied for the separation of other minerals (Table 2).

Sarvamangala and Girisha studied the role of *Bacillus subtilis* and its metabolic products in the separation of pyrite from calcite and quartz (oxides) and also galena (sulfide mineral) (*103*). The minerals were adapted to the bacterial cells prior to flotation. Mineral-specific proteins and polysaccharides were secreted by the bacterial cells, making the surface of the mineral particles either hydrophobic or hydrophilic. On interaction with calcite, quartz, and galena, *B. subtilis* produced more proteins while, during interaction with pyrite, the bacterium produced higher quantities of exopolysaccharides. This rendered the surfaces of calcite, quartz, and galena hydrophobic, and that of pyrite hydrophilic. Therefore, the pyrite was depressed after interaction with bacteria during flotation.

Paenibacillus polymyxa or *Bacillus polymyxa* (its superseded nomenclature) is a gram-positive, periflagellated heterotrophic bacteria that is associated with several mineral deposits. It secretes metabolic products such as polysaccharides, proteins, formic acids, and others (*108*) and has been explored for bioflotation of various sulfide minerals (Table 2). Patra and Natarajan studied the role of *P. polymyxa* and its metabolites in the separation of pyrite from quartz and calcite (*109*). They observed a higher adsorption of cells on the surface of pyrite than on quartz and calcite for all pH values tested. However, with an increase in pH, the number of cells adhering to the surface of pyrite and quartz decreased due to electrostatic repulsion. The zeta potential of the cells shifted to a positive value after interaction with pyrite due to the proteinaceous compound secreted by the cells upon interaction. During flotation, the pyrite was selectively depressed whereas the quartz was floated because of its more hydrophobic surface after interaction with bacteria and bacterial metabolites, thus resulting in their separation (*109*). The same authors have also reported the role of this bacterium and its metabolites in the flocculation and flotation of sulfide minerals, chalcopyrite, and galena (*110*).

Another study carried out by Sharma and Rao reported the role of *P. polymyxa* in the flotation of pyrite and chalcopyrite (*108*). The cells were adapted to the mineral particles prior to the flotation experiment. The surface properties of the bacterial cell depend on its growth conditions. They conducted FTIR studies to deduce the nature of functional groups present on the surfaces of mineral-adapted cells. They found that pyrite-adapted cells have the highest quantity of cell surface secretions (proteins and polysaccharides) when compared to chalcopyrite-adapted and -unadapted cells. All the peaks were more intense in adapted cells compared to unadapted ones. The quantity of proteins and polysaccharides present on the surface of bacterial cells determines their hydrophobicity. Therefore, the chalcopyrite-adapted cells were more hydrophobic than the pyrite-adapted cells because the amount of polysaccharide on the surface of chalcopyrite-adapted cells was lower. The authors also observed a change in the isoelectric points of the minerals, which became nearly the same as the isoelectric point of the bacterial cells, indicating a specific adsorption of cells on these mineral particles. There was a difference in the isoelectric points of the unadapted and mineral-adapted bacterial cells, which indicates the difference in their surface properties and, hence, their interaction with the minerals, during flotation. The flotation recovery was found to be higher in the presence of unadapted cells compared to adapted cells and it was lowest in the case of chalcopyrite-adapted cells. In the presence of cell metabolites, the flotation recovery was lower than when whole cells were used. Metabolites from adapted cells were still more efficient in depressing pyrite than those from unadapted cells (*108*). Therefore, cells of *P. polymyxa* can potentially be used to depress pyrite during the extraction of important sulfide minerals.

Additional reasons for the preferential adsorption of cells on the pyrite surface are the presence of toxic copper ions on the surface of chalcopyrite, and poisonous arsenic species on the surface of arsenopyrite, hindering adhesion. Chandraprabha et al. (*96*) reported that the adsorption density of cells on the surface of pyrite was twice as high as that on chalcopyrite and four times higher than on arsenopyrite. It was also suggested that this specific binding on pyrite is due to the protein aporusticyanin (*96*, *111*). Pyrite was depressed even in the presence of a collector after conditioning with bacterial cells. Arsenopyrite needed conditioning with activator prior to treatment with the collector to activate the copper ions that increase its floatability. Activated copper ions adsorb into the arsenic sites and form arsenide (Cu_3As and Cu_3As_2) that holds the collector xanthate firmly and floats the arsenopyrite (*96*). We now know that the preferential adsorption of bacterial cells on a particular mineral particle is influenced by several factors: nutrient availability, toxicity, and the properties of the macromolecules secreted by the cell.

Bacterial EPSs in the Flotation of Sulfide Minerals

Several studies began with the observation that bacteria help in separating minerals and then proceeded with extracting the EPSs (carbohydrates, uronic acids, proteins, etc.). Such components may later be overproduced and used in sulfide mineral processing. Govender and Gericke (*112*) isolated EPS from a mixture of bioleaching cultures containing mesophiles, moderate thermophiles and extreme thermophiles growing in the presence of various sulfide minerals (sphalerite, chalcopyrite, and pyrite). They also studied the effect of this free EPS and cell-bound EPS in the bioflotation of chalcopyrite for the separation of pyrite and chalcopyrite. They found that the EPS extracted from bacteria growing in the presence of chalcopyrite had a higher affinity toward chalcopyrite than the EPS obtained from other systems. Free EPS was more efficient than cell-bound EPS in the flotation of chalcopyrite. The ratio of carbohydrates to protein is also an important role in the flotation process. The proteins tend to impart hydrophobicity, whereas polysaccharides make

the surface hydrophilic, but acidic polysaccharides may also mask positive charges on the mineral. Accordingly, Govender and Gericke observed higher flotation recovery of chalcopyrite with EPSs containing high amounts of proteins and acidic polysaccharides (*112*).

Patra and Natarajan studied the role of extracellular proteins and polysaccharides from cells of *P. polymyxa* in the separation of several sulfide minerals (Table 2). When studying chalcopyrite and galena (*110*), they reported that the bacterial cells adhered strongly to both the minerals. The adsorption of extracellular polysaccharides was almost the same on the surface of both galena and chalcopyrite in the acidic as well as alkaline pH range. The adsorption density of extracellular proteins (bioproteins), however, was always higher on chalcopyrite than on galena. During flotation, prior interaction of minerals with bioproteins made the surface of galena hydrophobic, therefore it was preferentially floated over chalcopyrite up to pH 6. But the flotation recovery of galena was found to decrease upon interaction with extracellular polysaccharides and bacterial cells (*110*).

Vasanthakumar et al. (*113*) studied the role of cell wall components, bacterial metabolites, and extracellular secretions of bacteria *Bacillus megaterium* for the flotation of sphalerite ([Zn,Fe]S) and galena (PbS). To study the effect of cell wall components (proteins, polysaccharides, teichoic acids, and nucleic acids) on the flotation recovery, these surface biopolymers were digested with the corresponding degradation enzymes. It was found that out of all biofilm components, extracellular DNA played a major role in the selective flotation of sphalerite. Moreover, due to the amphipathic nature of single-stranded DNA (ssDNA), it was more efficient than double-stranded DNA in the flotation process (*113*, *114*). The preferential flotation of sphalerite from the sphalerite–galena mixture resulted in a recovery of about 95% sphalerite from the mixture in the presence of ssDNA and other bacterial components with a selectivity index of 19.1. In this case, ssDNA acted as a biocollector and other components like polysaccharides and teichoic acids acted as depressants, resulting in selective flotation. With the increase in the concentration of extracellular proteins the recovery of sphalerite increased and become constant after 300 ppm, whereas the extracellular polysaccharides did not notably affect the flotation recovery of any mineral. Their study indicated that ssDNA was an effective collector in the flotation of such sulfide minerals (*113*, *114*). Still, the stability of ssDNA under actual mineral processing conditions might be too low.

Once it was shown that isolated polysaccharides and proteins can be used in mineral separation, another possibility would be to screen known polysaccharides for their ability to bind to mineral particles and act as collectors or depressants. Hayat et al.[5] reported that the positively charged polysaccharide chitosan was more effective in depressing pyrite than the conventionally used depressant sodium cyanide (*5*). They studied the depressing role of chitosan in the flotation of sulfide minerals—galena, chalcopyrite, pyrite, and sphalerite—and the depression effect was seen at a pH at which chitosan is positively charged. Khoso et al. (*115*) synthesized biopolymer tricarboxylate sodium starch for the separation of the sulfide minerals pyrite and chalcopyrite in flotation. They observed that the biopolymer depressed pyrite more than chalcopyrite because the biopolymer adsorbed more strongly on pyrite than on the chalcopyrite (*115*).

Biosurfactants in Sulfide Mineral Processing

The harmful environmental impacts of chemically synthesized surfactants have made it desirable to use nature-friendly biosurfactants with very little toxicity and high selectivity toward the target ions. Biosurfactants are biologically derived surfactant molecules produced by microorganisms, animals, plants, and more. They are primarily neutral or anionic in nature and only a few of them are cationic (those containing amine groups) (*116*, *117*). The hydrophilic head of biosurfactants

can be composed of amino acids, peptides, phosphate, carboxylic acid, sugars, sugar derivatives, or alcohols, whereas the hydrophobic part is mainly long-chain fatty acids (*114*, *116*–*118*). In ion flotation, the polar head of the biosurfactant molecule attaches to the oppositely charged target ion, exposing the nonpolar hydrophobic tail. This exposed hydrophobic tail helps the surfactant-target ion molecule attach to the air bubbles produced in the flotation cell. Therefore, the biosurfactant molecules demonstrate the dual nature of the collector, as also serving as a frother in the flotation process (*116*, *119*). The most commonly used biosurfactants are rhamnolipids, sophorolipids, fatty acids, phospholipids, lipopeptides, and lipoproteins (*116*, *118*).

Biosurfactants are biocompatible, biodegradable, and have extensive application in the food, mining, metallurgical, and pharmaceutical industries (*120*). Various biosurfactants have been produced by microorganisms for various applications (*40*, *121*). Biosurfactants are used as additives for flocculation in mineral beneficiation and also as adsorbents for environmental remediation (*116*, *117*, *122*). Didyk and Sadowski (*123*) studied the role of biosurfactants in the flotation of quartz and serpentinite using nickel ions as the activator. The change in zeta potential values and the isoelectric points of the minerals indicate the change in their surface properties after interaction with the biosurfactants produced by *Bacillus circulans* and *Streptomyces sp.* Thus, the floatability of the minerals increased and resulted in their separation (*123*). Menezes et al. (*124*) reported the treatment of aqueous effluent containing heavy metals from acid mine drainage using biosurfactants produced by the yeast cells *Candida lipolytica* and *Candida sphaerica.* (*124*). Dhar et al. (*125*) have found that, unlike the traditional sulfhydryl surfactants, glycolipid biosurfactants (sophorolipid, glucolipid, and glucoside) make the mineral surface hydrophobic at alkaline pH, due to adsorption via both surface precipitation and hydrogen bonding (*125*).

Although adsorption and self-assembly of biosurfactants have been studied at the liquid–liquid and air–liquid interfaces in the past two decades, very little is known about their interaction with minerals (*126*). Some reports argue the importance of the ion–dipole interactions and chemisorption (*127*–*130*), while others advocate for hydrogen bonding interactions (*131*, *132*). Given the rich molecular structures and large headgroups of biosurfactants, as well as the complexity of their self-assembly processes in solution (*133*), one can expect that their self-assembly at the mineral–water and air–water interfaces to be quite different from that of conventional surfactants. Conventional surfactants containing one head group and a tail comprising the hydrocarbon chain adsorb at the air–water interface with their polar head group interacting with water and the hydrocarbon chain protruding toward the air (*134*). In contrast, biosurfactants with polar groups at both ends of a long hydrocarbon chain can self-assemble at the air–water interface in such that both the polar groups point toward water (*125*). However, this interesting phenomenon is poorly understood. Hence, more spectroscopic (especially in situ) and computational modeling studies are required to shed light on the conformations of adsorbed biosurfactants.

Foam Flotation

Foam flotation is used here as an umbrella term for adsorptive bubble separation processes that are also referred to as ion, foam, and precipitate flotation, as well as foam fractionation. Foam flotation is an important and versatile separation process with multiple uses, as it is relatively cost-effective and can be optimized for many different applications (*9*, *137*, *138*). In general, the process can be applied to water purification and concentration of ions (*9*). The latter process is called *beneficiation* by the mining community, where the benefit is increased concentration of the target

ion. Purification and concentration both aim to remove ions from the solution. In purification, the final water phase is the primary focus, with selectivity and grade of extracted ions being less important. In concentration, the extracted ions are the focus where selectivity and grade are the decisive parameters.

Foam flotation can selectively extract dissolved ions or their dispersed precipitates from a complex solution. The corresponding techniques are called ion and precipitate flotation, respectively. In foam flotation, bubbles are passed through an aqueous solution containing a surfactant, the ion of interest (*colligend*), and background ions (Figure 1[b]). Driven by the tendency to reduce surface tension, the surfactants are automatically adsorbed at the bubble–solution interface, with the surfactant polar headgroup pointing toward the solution. This process can be accompanied by two useful phenomena: (1) it can generate a foam (gas dispersion) and (2) the colligends can be preferentially adsorbed, either physically or chemically, by the surfactant headgroups at the bubble surfaces. The foam reaching the top of the flotation cell is manifold enriched in the colligend when compared to the initial solution.

Figure 5 shows the interaction between a positive-charged colligend and an anionic collector in the wall of a gas bubble. An effective collector interacts preferentially with the colligend(s) compared to the competing ions, including the collector's counterion (*139*). As a rule, the colligend is a metal cation (Table 3). The surfactants used in foam flotation are typically ionic surfactants with the opposite charge of the colligend. An increase in concentrations of the metal cation and surfactant may lead to precipitation of the metal complexes. In general, the precipitate can be formed by adding any compound, including a hydroxyl ion and a chelating ligand, that forms an insoluble complex with the metal. The foam flotation carried out under these conditions is called precipitate flotation (*140*). In many aspects, precipitate flotation resembles froth flotation where the hydrophobic phase is a solid particle.

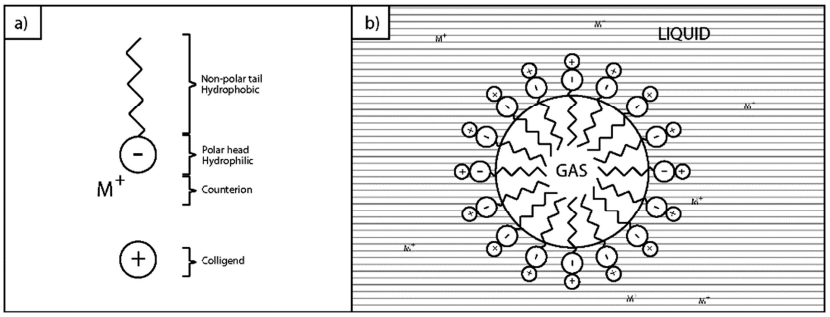

Figure 5. Illustration of interactions between surfactant and colligend. (a) Nomenclature of simplified anionic surfactant (e.g., a collector, with opposite charge colligend) and (b) Surfactant–colligend interaction on gas bubble in liquid phase.

Table 3. Selection of Foam Flotation Research Results

Colligend	*C. Conc. [μM]*	*R/R (%)*	*Appl.*	*Flot.*	*Foreign ion(s)*	*Collector*	*Ratio**	*Precipitant*	*Ratio**	*Gas [ml/min]*	*pH*	*Duration [min]*	*Year*	*Ref.*
Cd^{2+}	750	100	WWT	IF	-	AEC	1: 5–10			N_2: 200	7.5	10	2015	(*166*)
	500	73–99	WWT	PF	Ni^{2+} and Co^{2+}, increase with very low conc. of Al^{2+} and Na^{+}, otherwise decrease	SDS	1: >2	KEtX	1: >2	50	6.25	30	2015	(*153*)
	500	>99	WWT	PF	Ni^{2+} and Co^{2+}, decrease with Al^{2+} and Na^{+}	HDTMA	1: >3	KEtX	1: >2	50	3.2–7.4	30	2015	(*153*)
	60	>99	WWT	IF	Cd^{2+}, Cr^{3+}, Pb^{2+}, Ni^{2+}, Zn^{2+}	EHDABr	1:4			60	9	60	1979	(*167*)
Cu^{2+}	5000	>99	WWT	IF	$Mn^{2+} + Fe^{3+} + Pb^{2+} + SO_4 + Ca^{2+} + Mg^{2+}$	Xanthates	1: ~5			100	2.5–5.5	5	2004	(*168*)
	300	>95	WWT	IF	-	SDS	1:2			N_2: 10.8	6.8	20–40	1966	(*145*)
	790	96.4	WWT	IF	-	DEDTK	1:1			1800	3	10	2010	(*169*)
Ge^{4+}	700	100	WWT	PF	Many (fly ash)	DA	1:3	Catechol	1:3	-	4–7	30	2006	(*170*)
	100	>90	WWT	PF	-	DA	1:2	Pyrogallol	1:3	N_2: 200	7	5	1988	(*171*)
Ni^{2+}	170	88	WWT	IF	-	SDS	1:3			1.8	3	60	2015	(*172*)

Table 3. (Continued). Selection of Foam Flotation Research Results

Colligend	*C. Conc. [μM]*	*R/R (%)*	*Appl.*	*Flot.*	*Foreign ion(s)*	*Collector*	*Ratio**	*Precipitant*	*Ratio**	*Gas [ml/min]*	*pH*	*Duration [min]*	*Year*	*Ref.*
Pb^{2+}	240	85	WWT	IF	-	SDS	2:1			1000	8	30	2013	(*173*)
	100	>95	WWT	IF	-	SDS	1:2			N_2: 24.8	8.2	2040	1969	(*174*)
U^{4+}	100	>99	WWT	IF	-	HDTMA	1:5			N_2: 52	-	20	1973	(*175*)
Zn^{2+}	150	95	WWT	IF	-	SDS	1:3			1.8	3	60	2015	(*172*)
Y^{3+}	1000	>99	Conc.	IF	-	SDS	1:3			-	>5	10	2008	(*176*)
Ce^{3+}	1000	>99	Conc.	IF	-	SDS	1:3			-	>6	10	2008	(*176*)
La^{3+}	1000	UC: 513x	Conc.	IF	-	SDS	1:3			-	8.6	5	2012	(*177*)
Ho^{3+}	1000	UC: 27x	Conc.	IF	-	SDS	1:3			-	7	5	2012	(*177*)
Ag^{+}	460	>95	Conc.	PF	-	DA	4:3	Th	3:1	100	4–12	5	1995	(*178*)
Au^{+}	2.4	>80	Conc.	IF	Selective over $Ag(CN)_2^-$	HDTMA	1:57			6.48 mm/s	8	Continuous	1991	(*179*)
REEs	400	87100	Conc.	IF	See Figure 7	OIMBP	1: 2.5			N_2: 15	-	20	1992	(*163*)

* Molar or wt. ratio, colligend (C.): surfactant. **Abbreviations used:** R/R: Removal/Recovery; WWT: Wastewater treatment; Conc.: Concentration (flotation); UC: Up-concentration; Appl.: Application, Flot.: Flotation type; IF: Ion Flotation; PF: Precipitate Flotation; N_2: Nitrogen; AEC: Sodium trideceth-4 carboxylate; SDS, (NaLS): Sodium dodecyl sulfate (Sodium lauryl sulfate); HDTMA: cetyltrimethylammonium bromide, Hexadecyltrimethylammonium bromide (Cetyl trimethyl ammonium bromide); EHDABr: Ethylhexadecyldimethylammonium bromide; DEDTK: Sodium diethyldithiocarbamate; DA: Dodecylamine; OIMBP: KEtX Potassium Ethyl Xanthate; Th: Thiosulphate.

Even though adsorption of alkali metal cations by headgroups of surfactant monolayers self-assembled at the air–water interface dates back to 1900 (*141*), adsorption of heavy metals was described by Langmuir and Schaefer in 1937 (*142*). In 1959, Felix Sebba suggested using ion flotation to separate metal ions from water, with seawater being the primary example (*143*). In the same paper, a purification process is proposed using ions to remove surfactants from sewage water. In 1962, Sebba expanded this subject and published his results in book form (*144*).

As a separation method, foam flotation offers many advantages, including low energy requirements, low cost, simplicity, relatively fast operation, small space requirements, applicability to a variety of colligends in different concentration ranges, a small volume of sludge, low residual concentration, and recyclability of water and collector (*9*, *140*). In contrast to froth flotation, foam flotation does not require the energy-heavy grinding operation. In contrast to solvent extraction, it does not use organic solvents and can be applied to a wide range of ion concentrations. Finally, it is expected that foam flotation has taken from froth flotation its flexibility and adaptivity to different sources of metal ions, which can be achieved through tuning the solution conditions, reagent regime, and physical parameters of the foam (bubble size and flow rate).

Selectivity and grade are the main challenges for efficient concentration of metal ions using foam flotation. These characteristics are controlled not only by the selectivity of the colligend–collector interaction, but also the foaming properties of the collector. If the foam is wet it can trap a substantial amount of solution and hence nontargeted ions, which reduces selectivity (*145*, *146*). Hence, foam drainage is even more important as compared to froth flotation because there is an intrinsic trade-off between yield and selectivity. Drainage of foams produced by surfactants in an electrolyte is a complex phenomenon that depends on the surfactant and the ions, their concentration, and the method by which bubbles are generated (*147*). In particular, it has been shown that salts and pH affect film thickness and drainage in the case of foam flotation using a pH-sensitive surfactant, nonaoxyethylene oleylether carboxylic acid as a collector (*148*). Na^+ and Ca^{2+} ions increase drainage, while Nd^{3+} and low pH reduce it. An increase in the ionic strength of the solution generally has an adverse effect on ion flotation, although it can be compensated for (at least partly) by increased surfactant concentration (*149*). The selectivity of the foam flotation methods can be promoted by adding chelating ligands (*150*).

Another detrimental process is coalescence of the bubbles within the rising foam, because it releases adsorbed material causing internal reflux (*141*). The presence of surfactant micelles in the reflux has an additional negative impact. To overcome this problem, several techniques have been proposed such as enriching, stripping, and multi-stage modes (Figure 6) (*141*, *146*). These techniques can be useful if the bubble surface in the rising foam is not saturated with the colligend. In the enriching technique, the drainage from a collapsed foam is refluxed into the rising foam. In the stripping technique, the feed enters the flotation column closer to its top and hence drains down through the rising foam. As a result, the depleted solution is continuously replaced by the feed, which can lead to better purification of the bottom solution as compared to the simple mode. Finally, in the combined technique, an enriching section is placed on top of a stripping one.

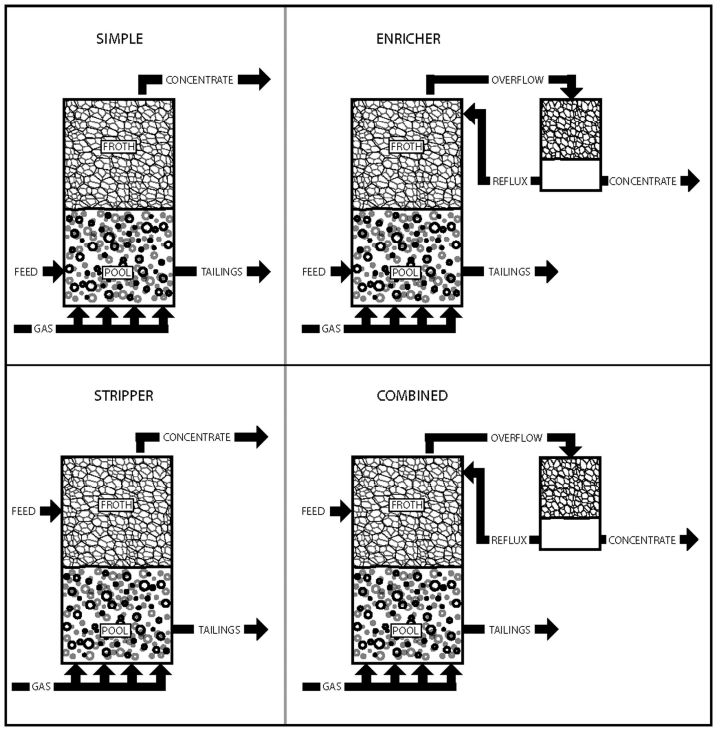

Figure 6. Four modes for continuous foam fractionation, transferrable to foam flotation. Illustration based on figures by Lemlich (141). The simple mode is the most common, introducing the feed in the liquid pool. In the enriching mode, the concentrate is returned to the foam phase for further concentration. Another configuration that can improve recovery is the stripper mode, where the feed is directed to the foam phase. Finally, there is a mode that combines stripper and enricher modes (141). Reproduced with permission from ref (141). Copyright 1968 American Chemical Society.

Surfactants used in foam flotation have recently been reviewed by Chang et al. (*9*) and Peng et al. (*116*) The most commonly used surfactant for metal cation flotation is sodium dodecyl sulfate (SDS) (Table 3).

The current research on foam flotation focuses predominantly on purification, specifically wastewater treatment with heavy metal cations as the colligends (*9*). In concentration, REEs are the most studied colligends. Below, we briefly review applications of foam flotation to wastewater treatment and recovery of REEs, as well as emerging demand for more environmentally friendly reagents: biosurfactants.

Removal of Heavy Metals from Wastewater

Heavy metals such as Pb, Cd, and As are toxic for plants and animals and are bioaccumulative (*151*). Other heavy metals such as Cu, Zn, Cr, and Ni (among others) are also problematic at high concentrations (*151*). Hence, these metals must be removed before disposing of wastewater.

Heavy metals can be selectively removed from water using adsorption, ion exchange, electrodeposition, chemical precipitation, carbon adsorption, evaporations, membrane methods, and more (*152*). However, these methods may be relatively resource- and energy-intensive for high throughput of low concentration solution. Foam flotation, on the other hand, can be designed for

specific species present in the wastewater and can potentially reduce reagent and energy use. With research steering toward biosurfactants, the waste product may also be more environmentally friendly. A selection of water treatment results achieved using foam flotation is shown in Table 3.

Precipitate flotation using xanthate as precipitant has shown promising results for Cd, Ni, and Co (see Table 3). For synthetic wastewater (tap water with added heavy metal ions), hexadecyltrimethylammonium bromide (HDTMA) was shown to be better or equally good compared to SDS as collectors, achieving >91% of each of the contaminants (*153*). For these systems, foreign ions were problematic and high concentrations of Na^+ and Al^{3+} reduced the recovery from around 95% to around 70% at 0.015 M foreign ions. It may be of interest to test the performance of other sulfhydryl collectors and their mixtures with chelating surfactants that have been employed in froth flotation of copper sulfides (Table 1).

Given the risks associated with exposure and nonrenewable origin of xanthates a more sustainable direction is shown in the employment of green surfactants (surfactants produced from renewable resources or by plants or microbes) as collectors. Apart from the evident environmental benefits such a surfactant can collect several metal ions due to its bulkier headgroups with their multiple adhesion or coordination sites. In particular, high separation efficiencies ($R > 98\%$) have been reported for the separation of Cr(III) by ion and precipitate flotation with caffeic acid (3-(3,4-dihydroxyphenyl)-2-propenoic acid) as the collector (*154*). This molecule contains three electron donor groups capable of complexing metal ions. Similar compounds are present in plants and are by-products resulting from pharmaceutical plant processing.

It has also been shown that small admixtures of conventional surfactants can boost performance of green surfactants and hence reduce their consumption in foam flotation of heavy metals. Specifically, the synergistic effect of binary surfactant mixtures of sapindus saponin (a plant-derived green surfactant) and cetyltrimethylammonium bromide has been demonstrated in the removal of Cr(VI) from its aqueous solution (*155*). Interestingly, the experimental results indicate that the attachment of Cr(VI) on the gas–liquid interface contributed to improving foam drainage, resulting in a low liquid holdup (*155*).

Concentration of Rare Earth Elements (REEs)

Economic recovery of REEs is recognized as one of the greatest challenges of industrial inorganic separation. This challenge originates from the unique mineralogy, occurrence, and physicochemical properties of REEs. As the name REE implies, the number of explorable REE deposits (with a predictably distributed rare earth oxide grade higher than 1.5–2.0%) is rare (*156*). As a result, REE separation requires the processing of large quantities of ores. Moreover, REEs occurs naturally in more than 250 different minerals (carbonates, phosphates, silicates, halides, and oxides), and several different REE minerals are always found in the same deposit. These minerals and many associated minerals have similar surface chemistry, magnetic susceptibility, specific gravity, and electrical conductivity. Moreover, two REEs adjacent to each other in the periodic table differ subtly in terms of their ionic radii and atomic weights. This translates into a subtle difference in their electronegativity (the power of an atom to attract electrons to itself), solubility, crystallization, complexation, and diffusion properties, which substantially complicates their separation from one another.

REE-bearing minerals are industrially concentrated using froth flotation aided by physical separation methods (e.g., multi-gravity, magnetic and electrostatic techniques) (*156*, *157*). Afterward, the concentrate is treated with a mineral acid to leach REEs out, followed by their further purification using a hydrometallurgical method such as precipitation, solvent extraction, ion

exchange, or electrolysis (*158*). However, both froth flotation and hydrometallurgy face the challenges of high cost and a large environmental footprint. Foam flotation may be a more environmentally friendly alternative. Firstly, in contrast to froth flotation, which requires liberation of <100 μm particles of REE minerals, foam flotation avoids the energy cost, occupational hazard, and environmental footprint associated with grinding, processing, and disposing of the fine particles. Secondly, ion flotation is able to concentrate REEs at low concentrations that cannot easily be accessed by other separation methods (*140*). This advantage has been employed in analytical chemistry to preconcentrate ions. Ion flotation can be applied to REE coproduction and extraction from mining tailings and other secondary sources where the traditional technologies are not economical. On the other hand, precipitate flotation can be applied to molar concentrations (*159*), which makes foam flotation a universal with respect to REE concentrations.

Despite the great promise of foam flotation in the separation of REEs, only a few studies have been carried out to test it; Table 3 provides some examples. In particular, it has been shown to be possible to separate Sm^{3+}, Ce^{3+}, and Eu^{3+} from ca. 150 ppm solutions of their salts using SDS as a collector (*159*, *160*). Furthermore, lauryl phosphoric acid can extract 97–98% Eu^{3+} at 150 ppm concentrations, while the REE concentration can be increased up to 76% using diphosphine dioxide as a chelating agent (*161*). Micheau et al. (*148*, *162*) have demonstrated that nonaoxyethylene oleylether carboxylic acid (trade name AKYPO® RO 90 VG) separates 0.2–0.3 mM individual REEs from alkali cations. The cation specificity order follows the surfactant–ion affinity at the air–water and micelle–water interfaces as $Na^+ < Li^+ < Sr^{2+} < Ca^{2+} < Cu^{2+} < Nd^{3+} < Eu^{3+}$ (*162*). This trend correlates with the zeta potential of the surfactant micelles in the presence of the corresponding metal salts, which reflects the actual charge of the micelles. The latter depends on the condensation of metal cations in the electric double layer of the micelles. It also has been found that, if experiments were conducted at the initial pH below the surfactant pK_a, the final pH always decreases as the cation charge increases. This result has been explained by the concomitant increase in the cation–surfactant binding interaction, which leads to greater deprotonation of the carboxylic group of the surfactant. Interestingly, the observed selectivity trend differs from that predicted by metal ion complexation constants of acetate, which is not surface active. Also of interest is the fact that the observed selectivity is not influenced by the micelle surface curvature. Collectively, these results suggest that increasing the cation charge enhances its electrostatic interaction with the anionic headgroup of the surfactant, thereby modifying its critical micelle concentration, zeta potential of the micelles, and the foam film thickness. The most pronounced change in pH and zeta potential has been observed for Nd^{3+}, the weakest one for Na^+. Hence, surfactant self-assembly in the solution and at the interface plays a critical role in the selectivity of ion flotation.

As follows from the works of Micheau et al. (*148*, *162*), pH is a decisive factor for colligend–surfactant interaction. Sawaji et al. (*163*) have shown that it is possible to manipulate pH to alter hydrophobicity (i.e., recovery) such that selectivity may be achieved for most REEs using a single collector. According to the paper, this is due to the dissociation of the surfactant, octadecyliminobismethylene-bisphosphonic acid (OIMBP), at different pH levels. OIMBP acid (H_4A) dissociates into H_3A^-, H_2A^{2-}, HA^{3-}, and A^{4-} at increasing pH (*163*). The proposed successive flotation process is illustrated in Figure 7, where the pH of the flotation stages with the corresponding separation of elements is shown. Also, an important achievement, this work shows that foam flotation can separate two REEs with close mass numbers.

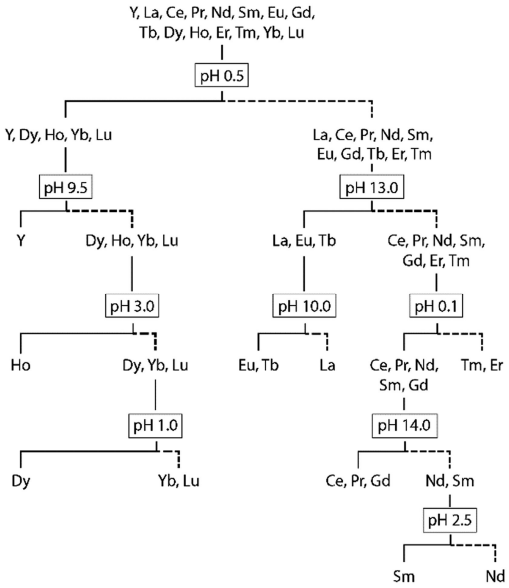

Figure 7. A proposed procedure to separate REEs using OIMBP as an anionic collector (*163*). *The ions on the left side, drawn with a full line, represent the liquid phase. The ions on the right side, drawn with a dashed line, represent the froth phase. This work shows that foam flotation can separate two REEs with close mass numbers. Reprinted with permission from ref.* (*163*). *Copyright 1992 Japan Society for Analytical Chemistry.*

To date, several works have demonstrated that foam flotation can be improved using green surfactants as collectors. In particular, tea saponin (a plant-derived surfactant) has been shown to be successful for the flotation of Cd, Pb, and Cu (*149*). More than 80% of the three metals were removed at 3:1 collector to metal ratio at pH 4, with FTIR results indicating complexation between the carboxylate group and the divalent metal ions (*149*). Abyaneh et al. (*164*) found that rhamnolipids were suitable as collector of Cr when using $FeSO_4$ as precipitant. The highest removal in the study was 96.1%, at low airflow rate (50 mL/min), very low collector to metal ratio (rhamnolipids: Cr ratio of 1:100) at pH 8. Only two pH levels were tested, pH 5 and 8, leaving room for improvement.

The need for improvement to the process is also evident from another foam flotation study that attempted to recover Cr using a series of glycolipid surfactants derived from renewable resources (e.g., vegetable oils, carbohydrates, and amino acids) (*165*). It found that the Cr removal efficiency depends on the foamability, foam stability, and number of chromium complexing sites per glycolipid molecule; however, the best Cr recovery, observed at pH 3, was only ca. 40% and took 4 h of flotation.

Even though foam flotation was invented more than half a century ago, it has attracted increased interest in the past several years with the growing need to find a more environmentally friendly alternative to solvent extraction of REEs and other critical elements. This method has already demonstrated impressive results with conventional surfactants. However, as pointed out by Peng et al. (*116*), industrial applications of foam flotation with biosurfactants are limited because of problems such as large surfactant dosage, low efficiency, and long flotation time. At the same time, the number of studies on foam flotation is still limited and many fundamental aspects as well as the efficiency, of mixed surfactants and separation of critical elements from base metals remain largely unknown.

Therefore, further systematic, experimental, and theoretical research is required to understand whether biosurfactants are beneficial for foam flotation or not.

Conclusions and Research Needs

To summarize, the design and development of new reagents in froth flotation mainly focuses on improving the performance of conventional (petroleum-based) surfactants, while progress is relatively slow. The main trend is to use surfactant mixtures (e.g., sulfhydryl collectors or sulfhydryl and chelating collectors), while reducing the use of xanthate (e.g., by less toxic dithiophosphinates). To improve recovery, new chelating surfactants selective to nonferrous metal sulfides have been designed. There has also been an effort to accelerate surfactant development using computational screening, along with automated mineralogical analyses of the flotation results.

Foam flotation is of interest in the separation of ions from aqueous solutions because it can extract elements from wastewater and leachates of secondary sources of valuable elements without using toxic and hazardous organic solvents and is technically simple. However, the capacity and limitations of this method have so far been studied in a limited number of works focused primarily on the removal of heavy metals. Moreover, there is very little data on the collector mixtures. Hence, more experimentally derived correlations and a better understanding of the purification and concentration processes in the ion and precipitation regime are required to realize whether foam flotation can be commercialized for a particular separation system or not.

Recent studies have also demonstrated that conventional collectors in both froth and foam flotation can be replaced in the future by their environmentally more sustainable alternatives including microbes and microbial metabolites (e.g., proteins, polysaccharides, nucleic acids, EPSs, etc.). Microbes can produce a wide range of surface-active compounds with diverse applications. They can be effectively used for the removal of heavy metal ions from the effluents of the mining industry and their eco-friendly separation. However, in most of the bioflotation tests, some amount of conventional flotation reagents has also been used along with the bacterial cells and metabolites for increasing the efficiency of the flotation recovery. This means that these bacteria and bacterial products only partially change the surface of mineral (hydrophilic or hydrophobic). So, further studies must be conducted on whether the effect of bacteria on mineral surfaces is partial or complete. Moreover, it is not known if mineral-adapted cells are mutated or if it is just a temporary metabolic adjustment to the environment. These studies are essential to realize efficient and greener bioflotation processes.

In this context, it currently seems more feasible to replace conventional surfactants with biosurfactants produced by microorganisms. These biosurfactants can be retrieved and concentrated for the application in mineral processing, but still, they are not produced in large quantities and at a market-attractive price. Another hurdle is the poor understanding of the interaction of biosurfactants with mineral particles, complexation with metal ions, and their foaming properties. In particular, based on the rich variety of unique self-assembled packing structures observed for biosurfactants in solution, we expect that self-assembly of biosurfactants at the mineral– and air–water interfaces will also be versatile, unique, and stimuli-responsible, which can translate in the unique selectivity mechanisms which are yet to be discovered.

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Paper 2

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Paper 3

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