RECONSTRUCCIÓN METABÓLICA Y ANÁLISIS DE FLUJOS METABÓLICOS
DE MICROORGANISMOS BOLIXIVIANTES EN CULTIVO PURO Y MIXTO

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METABOLIC RECONSTRUCTIONS AND METABOLIC FLUX ANALYSIS OF BIOLEACHING MICROORGANISMS IN PURE AND MIXED CULTURE

THESIS FOR THE DEGREE OF DOCTOR IN ENGINEERING SCIENCES MENTION CHEMISTRY

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Dedicado a mis papás y a mis negritos
This thesis project addresses a Systems Biology approach to metabolic modeling of biomining microorganisms. The work focuses on the study of three fundamental microorganisms of bioleaching processes, which coexist forming microbial communities: *Leptospirillum ferrooxidans*, *Leptospirillum ferriphilum* and *Ferroplasma acidiphilum*.

In order to provide a tool to understand how these biological systems work, and identify key interactions that occur in these microbial consortia, the main objective of this work was to obtain a representative metabolic map for these microorganisms in pure and mixed culture.

For the development of the stoichiometric models, a metabolic reconstruction was performed based on information available in literature and databases. In each case, the reconstruction considered the main routes of substrates consumption and product generation, central metabolism, synthesis of building blocks, macromolecules and finally biomass.

The first part of this work is about the metabolic reconstruction developed for *L. ferrooxidans*, which was evaluated by Metabolic Flux Analysis (MFA) with experimental data obtained from literature. Under the studied conditions, the model gave predictions consistent with data from literature for this bacterium. Furthermore, the model developed showed stable performance when subjected to a sensitivity analysis by imposing a random error in data inputs. Thus, it was concluded that the model of *L. ferrooxidans* is able to reproduce in silico the main metabolic characteristics of this bacterium.

In the second part of this work, the construction of a model for *L. ferrifilum* based on the metabolic reconstruction of *L. ferrooxidans* is shown, which considers the main metabolic differences between these species. Also, a metabolic model of *F. acidiphilum* was developed, and through a computational platform for metabolic networks, the stoichiometric models of *F. acidiphilum* and *L. ferrifilum* were implemented simultaneously, in order to represent a mixed culture.

Through Flux Balance Analysis (FBA) methodology, the models developed for *F. acidiphilum* and *L. ferrifilum* were evaluated, obtaining consistent data with experimental observations reported in the literature. Also, the model of *F. acidiphilum* was used to assess its growth in the presence of alternative substrates, thus it was possible to verify the chemomixotrophic behavior of this metabolic model, and acquire a notion of the appropriate composition of the culture medium to optimize the growth of this archaea. Finally, the robustness of both models was evaluated through simulations of knockouts on
different enzymes of central metabolism of both organisms, obtaining consistent results regarding the key enzymes for growth compared with information found in literature.

In the last part of this work, an experimental metabolic characterization of *Ferroplasma acidiphilum* BRL-115 was performed, confirming its chemomixotrophic behavior. Also, from the experiments of *L. ferriphilum* growing on *F. acidiphilum* supernatant and vice versa, the synergistic trend of both organisms growing in mixed culture was confirmed. Finally, tests were performed in batch cyclic operation with the aim of obtaining growth rates and specific consumption rates of ferrous iron of *F. acidiphilum* in pure culture and mixed with *L. ferriphilum*, with which reasonable results were achieved when used as input in the metabolic model of mixed culture.
RESUMEN PROYECTO DE TESIS

El presente proyecto de tesis aborda un enfoque de Biología de Sistemas para el modelamiento metabólico de microorganismos biomineros. El trabajo se centra en el estudio de tres microorganismos fundamentales en procesos de biolixiviación, los cuales coexisten formando comunidades microbianas: *Leptospirillum ferrooxidans*, *Leptospirillum ferriphilum* y *Ferroplasma acidiphilum*.

Con el propósito de entregar una herramienta para entender el funcionamiento de éstos sistemas biológicos y determinar las principales interacciones que se presentan en estos consorcios microbianos, el objetivo principal de este trabajo fue obtener un mapa metabólico representativo para estos microorganismos en cultivo puro y mixto.

Para el desarrollo de los modelos estequiométricos se realizó una reconstrucción metabólica basada en información disponible en literatura y bases de datos. En cada caso, dicha reconstrucción estuvo constituida por las principales vías de consumo de nutrientes y generación de productos, metabolismo central, síntesis de unidades estructurales, macromoléculas y finalmente biomasa.

La primera parte de este trabajo trata de la reconstrucción metabólica desarrollada para *Leptospirillum ferrooxidans*, la cual fue evaluada a través de Análisis de Flujos Metabólicos (MFA) con datos experimentales obtenidos de literatura. Bajo las condiciones estudiadas, el modelo entregó predicciones consistentes con información de literatura para esta bacteria. Asimismo, el modelo desarrollado mostró un comportamiento estable al ser sometido a un análisis de sensibilidad imponiendo un error aleatorio en los datos de entrada. De esta manera, se concluyó que el modelo de *L. ferrooxidans* es capaz de reproducir *in silico* las principales características metabólicas de esta bacteria.

En la segunda parte de este trabajo, se muestra la construcción de un modelo para *L. ferriphilum*, basado en la reconstrucción metabólica de *L. ferrooxidans*, para lo cual se consideraron las principales diferencias metabólicas entre ambas especies. Asimismo, se desarrolló el modelo metabólico de *F. acidiphilum*, y a través de una plataforma computacional para redes metabólicas, se implementaron los modelos estequiométricos de *F. acidiphilum* y *L. ferriphilum* de manera simultánea, con el fin de representar un cultivo mixto.

A través de la metodología de Balance de Flujos Metabólicos (FBA), se evaluaron los modelos desarrollados para *F. acidiphilum* y *L. ferriphilum*, obteniendo como resultado datos consistentes con observaciones experimentales publicadas en la literatura. Asimismo, se utilizó el modelo de *F. acidiphilum* para evaluar su crecimiento en presencia de sustratos alternativos, con lo cual fue posible corroborar el comportamiento...
quimiomixotrófico del modelo metabólico de esta arquea, y obtener una noción de la composición adecuada del medio de cultivo para optimizar su crecimiento. Finalmente, la robustez de ambos modelos fue evaluada a través de simulaciones de knockouts sobre distintas enzimas del metabolismo central de ambos microorganismos, obteniendo resultados coherentes respecto a las enzimas claves para el crecimiento encontradas en información de literatura.

En la última parte de este trabajo, se realizó una caracterización metabólica experimental de *Ferroplasma acidiphilum* BRL-115, ratificando su comportamiento quimiomixotrófico. Asimismo, a través de ensayos de crecimiento de cada microorganismo en presencia de sobrenadante del medio de cultivo del otro, se confirmó la tendencia sinergística de ambos microorganismos al desarrollarse en cultivo mixto. Finalmente, se realizaron ensayos de crecimiento en cultivo batch cíclico para la obtención de tasas de crecimiento y consumo de Fe$^{2+}$ de *F. acidiphilum* en cultivo puro y mixto con *L. ferriphilum*, con los cuales se lograron resultados razonables al utilizarslos como datos de entrada en el modelo metabólico del cultivo mixto.
I want to thank my supervisors Dr. Juan Asenjo and Dr. Barbara Andrews for their fundamental support for both my academic and personal development during these years. I warmly thank their willingness to converse and give me their always wise advices at every stage of my career.

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CHAPTER 1

Introduction and Objectives
1.1. INTRODUCTION

For many years, biology was studied under reductionist approaches, which successfully generated information about individual cellular components and their functions. Over the past decade, a new view of biology has emerged, the systems approach. Systems biology does not investigate individual genes or proteins individually. It rather investigates the behavior and relationships of all of the elements in a particular biological system while it is functioning. These data can then be integrated, graphically displayed, and ultimately computationally modeled (Ideker et al. 2001; Palsson 2000).

High throughput technologies have enabled the development of Systems Biology. Such technologies - genomic, transcriptomic, proteomic, and metabolomic - produce data in fundamentally different formats from previous approaches. To achieve a systematic understanding of a biological system requires the integration of different fields such as biology, computer sciences, mathematics and engineering. The ability to logically analyze high throughput data simultaneously remains a key challenge in the further development of systems biology.

In this report, a Systems Biology approach is developed to represent and analyze metabolic networks of bioleaching microorganisms, with the aim to advance the understanding of these biological systems, and thus provide new strategies to improve the bioleaching processes.
1.1.1. A Systems Biology Approach

There seems to be a consensus building around a definition for Systems Biology, that is comprised of four main factors: the enumeration of network components, the reconstruction of networks, the mathematical representation of networks, and their mathematical interrogation to assess their properties, and experiments to verify computational predictions (Palsson 2004).

1.1.1.1. Metabolic Reconstruction

It is becoming universally accepted that cells could be viewed as a system which represents complex networks of interacting gene products to produce physiological functions (Covert et al. 2001). The reconstruction of metabolic networks is motivated by the possibility of understanding these systems, and determining the relationship between the genome and the physiology of an organism.

The reconstruction of a metabolic network is the collection and visualization of all the cellular processes that are potentially relevant at a physiological level. It begins with a meticulous examination of the genome annotation of the microorganism. There are several high quality genomic databases and metabolic reconstruction web sites, which provide access to annotated genome sequences of many organisms, such as KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg), a bioinformatics database containing information on genes, proteins, reactions and pathways for general purposes; BioCyc Databases, which provides electronic reference sources on the pathways and genomes of different organisms. Particularly, MetaCyc describes enzymes and metabolic pathways from more than 1800 organisms that have been experimentally
elucidated. MetaCyc does not seek to model the complete metabolic network of each one organism, but it provides a comprehensive collection of experimental pathways (http://www.metacyc.org).

With these bioinformatic tools is possible to develop an automated reconstruction of a genome sequence, even if there is a partial annotation, obtaining a general backbone of an in silico organism. Automated reconstructions must be evaluated and curated according to experimental and literature data in order to build a more comprehensive metabolic network.

1.1.1.2. **Metabolic Flux Analysis (MFA) and Flux Balance Analysis (FBA)**

Mathematical models and their computer simulations are used to study the integrated function of the reconstruction of the metabolic network.

Metabolic fluxes are determinant for cell physiology because they show quantitatively the contributions of various pathways to overall cellular functions. Therefore, it is possible to correlate genotype to phenotype by analyzing the fluxes in the metabolic network (Covert et al. 2001).

**Metabolic flux analysis (MFA)** is a powerful methodology to determinate metabolic pathway fluxes. In this approach, the intracellular fluxes are calculated by using a stoichiometric model for the major intracellular reactions and applying mass balances around intracellular metabolites. A set of measured extracellular fluxes, typically uptake rates of substrates and secretion rates of metabolites, is used as input for calculations. The result of flux calculation is a metabolic flux map with the distribution of the biochemical
reactions included in the calculations, along with an estimation of the steady state rate (i.e., the flux) at which each reaction occurs.

With this methodology it is possible to obtain a metabolic flux map which contains the contribution of the pathways to the overall metabolic processes. However, the real value of such maps lies in the flux differences observed when flux maps are obtained with diverse strains under dissimilar conditions. With these comparisons, the impact of environmental or genetic perturbations can be fully evaluated, and the importance of specific pathways and reactions within such pathways can be accurately described (Stephanopoulos et al. 1998).

MFA starts with a description of the stoichiometry of the biological system, wherewith is described how metabolites are converted to biomass and products. The system of equations that describes mass balances for the metabolites is:

$$\frac{dX}{dt} = S \cdot v - b$$  \hspace{1cm} (1.1)

Where $X$ is the vector of metabolite amounts per cell, $S$ is the $n \times m$ stoichiometric matrix, $n$ is the number of metabolites and $v$ is the vector of $m$ reaction fluxes, and $b$ is the vector of known substrate consumption rates, waste production rates, and biosynthetic fluxes. The element $S_{ij}$ is the stoichiometric coefficient of the $i$th compound of the $j$th reaction. If the rate of volume expansion due to grow is slow compared to metabolic transients, it is assumed that the following equation can be used to calculate the steady state fluxes (Savinell and Palsson 1992a)

$$S \cdot v = b$$  \hspace{1cm} (1.2)
Equation (1.2) forms the basis of MFA for the determination of the unknown pathway fluxes in the intracellular rate vector $v$.

The number of reactions ($m$) is always greater than the number of pathway metabolites ($n$), so there is a certain degree of freedom in the set of algebraic equations given by:

$$f = m - n \quad (1.3)$$

Therefore, some elements of $v$ must be measured in order to allow the determination of the system. According to the number of measured rates, the system of equations can be determined, over determined or underdetermined (Stephanopoulos et al. 1998).

- If exactly $f$ fluxes or reaction rates in $v$ are measured, the system becomes determined and the solution is unique and rather simple to obtain.

- If more than $f$ fluxes are measured, the system becomes over determined, so extra equations exist that can be used for testing the consistency of the overall balances, the accuracy of the flux measurements, the validity of the pseudo steady state assumption, and the calculation of more accurate values for the unknown intracellular fluxes.

- If fewer than $f$ fluxes are measured, the system is undetermined and the unknown fluxes can be determined only if additional constraints are introduced or an overall optimization criterion is imposed in the metabolic balances.

For underdetermined systems, an infinite number of solutions exist for the network fluxes. In this case **Flux Balance Analysis (FBA)** could be used to determine the intracellular flux distribution.
FBA is a mathematical methodology for the estimation of metabolic pathway fluxes when there is not much detailed kinetic information. In this case, rather than attempting to calculate and predict exactly what a metabolic network does, it is possible to restrict the range of possible phenotypes that the metabolic system can display, based on the successive imposition of governing physicochemical constraints (Palsson 2000).

With this analysis, the solution of Equation (1.2) is formulated as a linear programming problem and uses the Simplex algorithm to find a solution (Savinell and Palsson 1992a):

\[
\text{Maximize } Z = \sum c_i \cdot v_i \tag{1.4}
\]

Where \( Z \) is the objective function which is represented as a linear combination, as defined by the weights in the vector \( c_i \), of the fluxes \( v_i \).

The optimization in Equation (1.4) is subject to the constraints:

\[
S \cdot v = b \tag{1.5}
\]

\[
\alpha_i < v_i < \beta_i, \quad i=1,2,\ldots,m \tag{1.6}
\]

The constraints from Equation (1.5) correspond to steady state flux balances. Moreover, capacity constraints from Equation (1.6) introduce the vector of parameters \( \alpha \) and \( \beta \), which represent the lower and upper bounds of fluxes through reactions \( v \), defining the allowable solution space. The value of these parameters is related to, for example, the maximum activity of the enzyme \( i \), which can be measured in vitro. The constraints represent the fact that fluxes through each enzymatic reaction are limited by the amount of that enzyme in the cell, as well as the rate with which the particular enzyme can react with substrate.
Flux based analysis methods have been used to generate quantitative hypotheses, giving to these techniques a wide range of applications in the field of Metabolic Engineering. For example, they have been used to study the synthesis of human superoxide dismutase (SOD) in *Saccharomices cerevisiae* in a glucose minimal medium, achieving to define the optimal conditions to favor the synthesis of SOD in this expression system (Gonzalez et al. 2003). They have also been used to describe and successfully simulate, for the very first time, the main aspects of the metabolism of two bioleaching bacteria, *Acidithiobacillus ferrooxidans* (Hold et al. 2009) and *Leptospirillum ferrooxidans* (Merino et al. 2010), to model penicillin production by *Penicillium chrysogenum* (Henriksen et al. 1996; Jorgensen et al. 1995), to improve the yield of aromatic amino acids in *Escherichia coli* (Liao et al. 1996) and lysine in *Corinebacterium glutamicum* (Stephanopoulos et al. 1998) through metabolic engineering of central metabolism, etc.

1.1.2. **Biomining**

Biomining is the generic term that describes the processing of metal containing ores and concentrates using (micro-) biological technology. Biomining has a particular application as an alternative to physical-chemical methods of mineral processing in a variety of niche areas (Rawlings and Johnson 2007). The use of microbes in ore processing has some distinct advantages over the traditional physicochemical methods. Microbial extraction procedures are more environmentally friendly. They do not require the high amounts of energy used during roasting or smelting and do not produce sulfur dioxide or other environmentally harmful gaseous emissions. Furthermore, mine tailings and wastes produced from physicochemical processes when are exposed to rain and air may be biologically leached, producing unwanted acid and metal pollution. Tailings from
biomining operations are less chemically active, and the biological activity they can support is reduced by at least the extent to which they have already been bioleached. Biomining also has a clear advantage in the extraction of metals from certain low grade ores, which are not economically recoverable by non biological methods (Rawlings 2002).

1.1.2.1. Biomining Microorganisms

Acidophilic prokaryotes involved in metal recovery from sulfide minerals include members of the Bacteria and Archaea domains. Some of these microorganisms catalyze the oxidation of ferrous iron and/or reduced sulfur, accelerating the oxidative dissolution of many sulfide minerals (Okibe and Johnson 2004). Other microorganisms utilize ferric iron or sulfur as electron acceptors in oxygen depleted environments (Bridge and Johnson 1998; Johnson et al. 2003; Segerer et al. 1988). Some of them are autotrophs, such as *Leptospirillum ferrooxidans*, others are heterotrophs, such as *Ferroplasma acidarmanus*, and a third group are mixotrophs. The differences in their physiologies facilitate the interactions between these microorganisms. Competing for substrates like ferrous iron, and synergistic and mutualistic interactions between these acidophiles, have been described (Johnson 1998).

The first bacterium discovered that was capable of oxidizing minerals was *Acidithiobacillus ferrooxidans* (Colmer and Hinkel 1947), a chemolithoautotrophic, γ-proteobacterium that obtains energy and electrons by the oxidation of reduced sulfur compounds to sulfate or Fe(II) to Fe(III). It is a mesophilic, facultative aerobe that fixes atmospheric CO$_2$ and N$_2$ to provide cellular carbon and nitrogen. It thrives in extremely acidic conditions (pH 1–2) and is often confronted with high concentrations of metals
including iron. This bacterium has the longest history of investigation; in fact, its complete genome sequence is accessible since a few years ago in the Comprehensive Microbial Resource (CMR) of the J. Craig Venter Institute web site (http://cmr.jcvi.org).

For many years, it was thought that *A. ferrooxidans* was the most significant microorganism in the leaching of sulfide minerals and the major contributor to acid mine drainage (AMD) (Rawlings 2002). Nowadays, it is increasingly clear that several other bacteria and archaea play crucial roles in mineral solubilization at different temperatures, and that metal solubilization is promoted by the concerted effort of a consortium of microorganisms (Johnson 1998).

Molecular phylogentic techniques have been applied to research the ecology of the mineral bioleaching environments. Thermophilic bacteria like *Leptospirillum* spp. and *Acidithiobacillus caldus* were found to be dominant at certain mineral leaching environments (Goebel and Stackebrandt 1994; Norris et al. 2000). Tyson et al. (2004) characterized a biofilm growing on the surface of AMD, revealing that all biofilms contained a mixture of microorganisms, mainly composed by *Leptospirillum* group I and II, *Sulfobacillus* spp. and archaea (*Ferroplasma* and other members of *Thermoplasmatales*). It was also observed that *Leptospirillum* is the predominant iron oxidizing in heap bioleaching operations (Pizarro et al. 1996). By PCR-DGGE analysis of a low-grade cooper sulfide run-of-mine test heap, it was found that *Ferroplasma* groups are quantitatively dominant at certain phases during bioleaching process (Demergasso et al. 2005).
**Leptospirillum spp.**

*Leptospirillum* type bacteria belongs to the genus *Nitrospiriceae*, of the order *Nitrospira*. Based in rRNA 16S gene phylogeny, they are classified into groups I, II and III. *L. ferrooxidans* is a representative of group I, *L. ferriphilum* is a representative of group II and a lately described *L. ferrodiazotrophum* belongs to group III (Tyson et al. 2005).

*L. ferrooxidans* and *L. ferriphilum* show similar properties. Both are small spiral-shaped cells of 0.3-0.6 µm wide and 0.9-3.0 µm long, Gram-negative, and motile by means of a single polar flagellum. They grow in chemolitoautotrophic conditions, fixing CO₂, using ferrous iron as electron donor, and oxygen as electron acceptor. Both grow at a similar pH ranges and for temperatures between 30°C-37°C, and some isolates are capable to grow at 45°C (Coram and Rawlings 2002).

**Ferroplasma acidiphilum**

*Ferroplasma acidiphilum* is an archeobacterium that belongs to the genus of *Ferroplasmaceae*, of the order *Thermoplasmatales*. They do not have a cell wall and are pleomorphic of 0.3-1.0 µm wide and 1.0-3.0 µm long. Different strains of *F. acidiphilum* have been isolated and characterized (Dopson et al. 2004; Golyshina et al. 2000; Golyshina and Timmis 2005; Pivovarova et al. 2002). These strains were classified by Dopson et al. (2004) as chemooorganotrophic, because they are able to grow in yeast extract or another source of organic carbon as the only energy source, and also chemomixotrophic, because they are capable to grow with ferrous iron as electron donor, but it requires a small amount (0.02% wt/vol) of yeast extract. Unlike other members of *Thermoplasmatales*, *Ferroplasma* is a mesophile, growing in a range of temperatures between 20°C-40°C, with an optimum of 35°C approximately. The optimal pH of growth varies in the range of 1.3-2.2, growing optimally at about 1.7 (Golyshina et al. 2000).
In this context, this project is focused in the study of the metabolism of three of the dominant species founded in extremely acid bioleaching environments, which have a great potential on commercial applications (Okibe and Johnson 2004; Rawlings et al. 1999) : 

*Leptospirillum ferrooxidans, Leptospirillum ferrphilum* and *Ferroplasma acidiphilum*. 
1.2. OBJECTIVES

The main goal of this research work is to obtain a representative metabolic map of three different bioleaching microorganisms, *Leptospirillum ferrooxidans*, *Leptospirillum ferrphiilum* and *Ferroplasma acidophilum*, which complemented with experimental data and flux-based analysis simulate the metabolic behavior of these strains growing in pure and mixed culture.

The specific aims of this project are the following:

- Develop a detailed metabolic model for each microorganism
- Develop mathematical analysis with the metabolic models and compare their predictions with experimental data to evaluate their robustness and consistency
- Use the models developed to propose improvements to the bioleaching processes.
CHAPTER 2

Stoichiometric Model and Metabolic Flux Analysis for
*Leptospirillum ferrooxidans*

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2.1. ABSTRACT

A metabolic model for *Leptospirillum ferrooxidans* was developed based on the genomic information of an analogous iron oxidizing bacteria and on the pathways of ferrous iron oxidation, nitrogen and CO$_2$ assimilation based on experimental evidence for *L. ferrooxidans* found in the literature. From this metabolic reconstruction, a stoichiometric model was built, which includes 86 reactions describing the main catabolic and anabolic aspects of its metabolism. The model obtained has 2 degrees of freedom, so two external fluxes were estimated to achieve a determined and observable system. By using the external oxygen consumption rate and the generation flux biomass as input data, a metabolic flux map with a distribution of internal fluxes was obtained. The results were verified with experimental data from the literature, achieving a very good prediction of the metabolic behavior of this bacterium at steady state.
2.2. INTRODUCTION

Bioleaching is the oxidation process of metallic sulfide to soluble metallic ions and sulfuric acid, catalyzed by microorganisms (Schippers and Sand 1999). The most commonly encountered bacteria in this environment are Acidithiobacillus ferrooxidans, Leptospirillum sp., Acidithiobacillus thiooxidans and Acidithiobacillus caldus (Hallberg and Lindstrom 1994; Kelly and Harrison 1989; Olson et al. 2003; Rohwerder et al. 2003; Schippers and Sand 1999). The genus Leptospirillum is composed of three groups, according to their 16S rRNA phylogeny (Bond and Banfield 2001): group I represented by L. ferrooxidans, group II represented by L. ferriphilum, and group III represented by L. ferrodiazotrophum (Tyson et al. 2005). Leptospirillum ferrooxidans is a Gram-negative chemolithoautotrophic bacterium; it utilizes the energy and reducing power derived from iron oxidation for several metabolic processes, including CO$_2$ fixation and acquisition of some nitrogen sources (Holmes and Bonnefoy 2007) (Levican et al. 2008; Rawlings 2005). Assuming a biomass composition represented by CH$_{1.8}$O$_{0.5}$N$_{0.2}$ (Jones and Kelly 1983; Roels 1983), the stoichiometric equation for biomass formation, obtained for elemental and charge balance can be written in terms of the following reactions (depending on the nitrogen source):

\[
\begin{align*}
\text{CO}_2 + \left(1 + \frac{4.2}{Y_{\text{SX}}} \right) \text{Fe}^{2+} + 0.2 \text{NH}_4^+ + \left(1 - \frac{4.2}{Y_{\text{SX}}} \right) \text{O}_2 + \left(\frac{1}{Y_{\text{SX}}} - 0.2\right) \text{H}^+ & \rightarrow \text{CH}_{1.8}\text{O}_{0.5}\text{N}_{0.2} + \frac{1}{Y_{\text{SX}}} \text{Fe}^{3+} + \left(\frac{1}{2Y_{\text{SX}}} - 0.6\right) \text{H}_2\text{O} \quad (2.1) \\
\text{CO}_2 + \left(1 - \frac{4.8}{Y_{\text{SX}}} \right) \text{Fe}^{2+} + 0.1 \text{N}_2 + \left(\frac{1}{Y_{\text{SX}}} - \frac{4.8}{Y_{\text{SX}}} \right) \text{O}_2 + \frac{1}{Y_{\text{SX}}} \text{H}^+ & \rightarrow \text{CH}_{1.8}\text{O}_{0.5}\text{N}_{0.2} + \frac{1}{Y_{\text{SX}}} \text{Fe}^{3+} + \left(\frac{1}{2Y_{\text{SX}}} - 0.9\right) \text{H}_2\text{O} \quad (2.2)
\end{align*}
\]
In Equations (2.1) and (2.2) \( Y_{sx} \) represents the biomass yield on ferrous iron (C-mol/mol Fe\(^{2+}\)).

*L. ferrooxidans* has been identified as one of the dominant ferrous iron oxidizing microorganisms present in biomining consortia (Rawlings 1995; Rawlings et al. 1999), and it plays an important role in bioleaching processes given its ability to attach to sulfide mineral, its high affinity for ferrous iron, and its low sensitivity to inhibition by ferric iron (Norris et al. 1998; Rawlings et al. 1999). Both kinetic studies (Boon et al. 1999b; Boon et al. 1999c; Breed et al. 1999; Breed and Hansford 1999; van Scherpenzeel et al. 1998) and *in situ* detection methods have demonstrated the importance of *L. ferrooxidans* in these acidic environments rich in iron (Okibe et al. 2003; Schrenk et al. 1998; Tyson et al. 2004). In this context, *L. ferrooxidans* has become an important objective of biological research, since a better understanding of its metabolic behavior will provide new strategies to improve the productivity of bioleaching process.

A powerful tool for this purpose is Metabolic Flux Analysis (MFA), whereby internal fluxes of a metabolic network are estimated using stoichiometric reaction models for the major intracellular reactions, mass balances for metabolites, and thermodynamics (biochemical reaction directionality) (Stephanopoulos et al., 1998, chapter 8). The set of constraints imposed by the stoichiometry on the distribution of resources through the metabolic network is one aspect of the overall mechanism for cellular control regulation. Therefore, an improved understanding of the stoichiometry is essential for greater understanding of the mechanisms that regulate the cell behavior, and to predict the effect of addition or removal of nutrients or related substances on cell metabolism (Savinell and Palsson 1992b).
In this work, we investigated the main metabolic pathways of *L. ferrooxidans* with the purpose of developing a stochiometric model of its catabolism and anabolism. In addition, we performed a MFA to obtain a flux map of the biochemical reactions involved. Such a model will have important applications in seeking conditions to improve practical bioleaching operations. For example, with linear programming (Edwards et al. 2002) it is possible to determine the flux distribution of the cells in a bioleaching tank by optimizing an objective function, such as maximizing growth rate, or minimizing consumption rates of nutrients, like CO$_2$ (Edwards et al. 2001; Knorr et al. 2007; Oliveira et al. 2005; Schuetz et al. 2007).
2.3. MATERIALS AND METHODS

2.3.1. Metabolic Reconstruction

The whole genome sequence and annotation of *L. ferrooxidans* is not publicly accessible, so a first metabolic reconstruction for this bacterium was done using as reference microorganism *Acidithiobacillus ferrooxidans* strain ATCC 23270, whose genome was sequenced by The Institute for Genomic Research (TIGR, www.tigr.org) and Integrated Genomics, Inc. (IG, www.integratedgenomics.com). A metabolic reconstruction of *A. ferrooxidans* is available in MetaCyc (Multiorganism Metabolic Pathways and Enzyme Database, www.metacyc.org), and a stoichiometric model of its central metabolism was recently developed by Hold et al. (2009).

In the present metabolic reconstruction, a manual revision of the enzymes of most conserved pathways was made based on the *A. ferrooxidans* genome annotation. To accept a pathway, the criterion was that the majority of the enzymes (more than 50%) must be present. Likewise, for alternative pathways all the enzymes of each one were sought in the genome annotation for *A. ferrooxidans*, and the one with more identified enzymes was accepted. The information of ferrous iron oxidation, nitrogen fixation and carbon dioxide assimilation pathways were included, based on experimental evidence for *L. ferrooxidans* found in the literature (Holmes and Bonnefoy 2007; Parro and Moreno-Paz 2004; Parro et al. 2007).
2.3.2. In Silico Model Construction and MFA

The stoichiometric model was implemented in the software INSILICO Discovery 1.1. (Stuttgart, Germany, www.insilico-biotechnology.com), which is a computational tool for graphically oriented reconstruction, management and engineering of large-scale cellular networks. With this platform, a graphical representation of the network was obtained, mass and charge balance of the system were checked, and topological and mathematical analysis were performed to determine the internal fluxes of the metabolic network. Finally, a sensitivity analysis of the model was performed according to Nielsen et al. 2003. A random percentage of error in the range of 9% to 10% over the calculated rates was assumed in different simulations. In order to analyze the sensitivity of the fluxes associated to the experimental error, the resulting flux distributions were compared with the distribution without error.

2.3.3. Theory

By quantifying intracellular fluxes it is possible to analyze nutrient requirements for both anabolic and catabolic process, and so redesign the culture medium, identify metabolic pathways that limit growth or production, and understand the biochemistry of the cell at a quantitative level. Mathematical modeling and analysis tools like MFA to estimate internal fluxes are of great value for these purposes.

The starting point of MFA is the reaction network describing how substrates are converted into products and biomass. A set of measured extracellular rates are used as input calculations (Stephanopoulos et al., 1998, Chapter 8). The basis of flux determination is a mass balance specified by the stoichiometry of the biochemical network, and the
assumption of pseudo-steady state of intracellular metabolites. The general equation that 
describes the cell metabolism is given by

\[ S \cdot v(x) = b \]  

(2.3)

Where \( S \) is the stoichiometric matrix, \( v(x) \) holds reactions fluxes in steady state, and \( b \) 
contains experimentally measured exchange rates between the medium and the cell 
(Savinell and Palsson 1992c).

The degree of freedom \( f \) of this linear system of equations is given by the difference 
between pathway fluxes and pathway metabolites. So, if exactly \( f \) fluxes of \( v(x) \) are 
measured, the system becomes determined, and the solution is unique and simple to obtain.

If more than \( f \) fluxes are measured, the system becomes over determined, so extra 
equations exists that can be used for testing the consistency of the system. If fewer than \( f \) 
fluxes are measured, the system is under determined and additional inputs are needed to 
calculate the unknown fluxes (Stephanopoulos et al., 1998, Chapter 8).

Also, an observability test must be applied to the system to establish if the solution could 
be calculated from the experimentally determined fluxes. It is important to note that a 
determined or over-determined system is not necessarily observable. If experimental data 
are redundant, the system as a whole will be not observable. To perform this test, Equation 
(2.3) is rearranged by collecting all metabolic substrates, products and intermediates in the 
matrix \( S \) and rewritten in order to differentiate measured fluxes with calculated fluxes 
(Nielsen et al. 2003):

\[ S_m \cdot v_m + S_c \cdot v_c = 0 \]  

(2.4)

Where the subscript \( m \) indicates measured rates (in vector \( v_m \)) and measurable compounds 
(in \( S_m \)), and \( c \) indicates the rates to be calculated in \( v_c \) and nonmeasurable compounds in 
\( S_c \). Equation (2.4) can be solved with:
\( v_c = -(S_c^T \cdot S_c)^{-1} \cdot S_c^T \cdot S_m \cdot v_m \) \hspace{1cm} (2.5)

The system will be observable if the matrix \( S_c \) is invertible, so the system can be determined by the relationship:

\[ v_c = -S_c^{-1} \cdot S_m \cdot v_m \] \hspace{1cm} (2.6)
2.4. Stoichiometric Model Development

A metabolic reconstruction for *L. ferrooxidans* was performed, including principal pathways of its metabolism. Conserved pathways like central metabolism (Embden-Meyerhof-Parnas, TCA cycle, pentose phosphate pathway and anaplerotic reactions), and building blocks biosynthesis pathways (amino acid, nucleotide and phospholipids) were reconstructed on the basis of the *A. ferrooxidans* genome annotation. Figure 2.1 shows the pathways included in the model.

**Figure 2.1.** Schematic representation of the main pathways included in the metabolic model of *L. ferrooxidans*. External specific rates are named $q_i$ and internal flux rates as $v_i$. 
2.4.1. First Assumptions

In order to obtain a simple but meaningful model, some assumptions were made to decrease its extension without loss of important information:

i. Most of the sequential reactions have been lumped into a single reaction step by eliminating intermediate metabolites that do not participate in other reactions of the network. This process reduces the number of reaction steps without affecting the flux results obtained. It is assumed that lumped reactions proceed at the same rate, and intermediate metabolites are in a steady state.

ii. To avoid linear dependences in the stoichiometric matrix, only one cofactor was included in the model. Coenzymes NAD$^+$/NADH and NADP$^+$/NADPH are interconvertible by the action of the enzyme nicotidamide nucleotide transhydrogenase, so only NAD$^+$ and NADH were used as substrates for fueling and biosynthetic reactions respectively (Stephanopoulos et al. 1998).

iii. ATP and GTP were pooled together, so we considered ATP/ADP as the only energy transporters. Also, pyrophosphate was interpreted as two single phosphates.

iv. Nucleotides, as anabolic building blocks, are represented as monophosphates to avoid phosphorylation/dephosphorylation steps. Therefore, the energy exchange is considered in the polymerization reactions of DNA and RNA.

v. Reversible and irreversible reactions were differentiated in the stoichiometric model, thus, irreversibility constraints were included to solve the system. All the reactions involved in production or consumption of ATP were stated as irreversible in a thermodynamically feasible direction. Moreover, all reactions of assimilation were restricted by the direction of fixation.
Table 2.1 lists the stoichiometric equations included in the metabolic model, and Table A.1 (Appendix A) shows the nomenclature of its components. A deeper analysis of the pathways is presented in the following section.

2.4.2. Fueling Reactions

Ferrous Oxidation and ATP/NAD(P)H Production:

A unifying characteristic of the leptospirilli bacteria is that they are capable of oxidizing ferrous iron as an electron donor to obtain energy for growth and maintenance, using O$_2$ as electron acceptor (Rawlings 2002).

The electron transport inside the cell occurs through an enzymatic system composed of a series of cytochromes and quinone pools, being a red cytochrome the initial Fe$^{2+}$ oxidizer (Parro et al. 2007; Tyson et al. 2004). This process is described by the following equation:

$$2\text{Fe}^{2+} + \frac{1}{2}\text{O}_2 + 2\text{H}^+ \rightarrow 2\text{Fe}^{3+} + \text{H}_2\text{O}$$

(2.7)

The majority of the protons consumed in the reaction shown in Equation (2.7) have entered the cell via the ATP synthetase complex embedded in the inner membrane (Holmes and Bonnefoy 2007). The synthesis of ATP is coupled to the extrusion of 3H$^+$ in iron oxidizing bacteria, as shown in the following equation (White 1995):

$$3\text{H}^+_{\text{EXT}} + \text{ADP} + \text{Pi} \rightarrow \text{ATP} + 3\text{H}^+_{\text{INT}}$$

(2.8)

Additionally, in Equation (2.9) the formation of reductive power by the electron transfer from ferrous iron to a NADH ubiquinone, to reduce NAD(P) is included (Ferguson and Ingledew 2008; Holmes and Bonnefoy 2007).
Nitrogen assimilation

*L. ferrooxidans* is capable of assimilation of nitrogen from different sources. It can either reduce atmospheric N\(_2\) to NH\(_4^+\) using the nitrogenase enzyme complex, or assimilate NH\(_4^+\) from the culture media, which is taken into the cell by ammonia permeases (Norris et al. 1995; Parro and Moreno-Paz 2004; Parro et al. 2007; Tyson et al. 2004). However, for this analysis it was considered that the nitrogenase complex is inactivated because of its high sensitivity to oxygen, and the elevated energy requirements that implies. Therefore, ammonia is assimilated by the GDH pathway (reaction 2.10), assuming no ammonia limitation in the culture media (Kanamori et al. 1987)

\[
\text{NH}_4^+ + \text{AKG} + \text{NAD}(P)H + H^+ \rightarrow \text{GLUT} + \text{NADP}^+ + H_2O \tag{2.10}
\]

**CO\(_2\)** Assimilation

According to Parro et al., 2007, *L. ferrooxidans* assimilates CO\(_2\) through the reductive acetyl-CoA pathway, where one CO\(_2\) is captured by a special tetrahydrofolate cofactor and reduced to a methyl group (reactions 4 to 7 in Table 2.1). The other CO\(_2\) is reduced to a carbonyl group by the enzyme CO dehydrogenase, which is then combined with the methyl group to form acetyl-CoA by a collection of enzymes called the acetyl-CoA synthetase complex (reaction 8, Table 2.1). This pathway seems to require hydrogen gas as the electron donor and it is very efficient, requiring only 4 H\(_2\) per acetate formed (Hugler et al. 2003; Menon and Ragsdale 1999). ATP and NAD(P)H required for this process are obtained from ferrous iron oxidation.
2.4.3. Synthesis of Precursor Metabolites

Pentose Phosphate Pathway (PPP)

Oxidative and non oxidative branches of this pathway were included in the stoichiometric model.

According to the simplification criterion described previously, the oxidative branch was reduced to a single reaction (reaction 9), and all reactions of the non oxidative branch were incorporated into the model (reactions 10 to 14).

Embden-Meyerhof-Parnas Pathway (EMP)

Several chemolithoautotrophic microorganisms are capable of operating their central carbohydrates metabolism in anabolic and catabolic directions in order to achieve long and short term adaptation. An important regulation point of this pathway is the PEP/pyruvate interconversion reaction (Tjaden et al. 2006).

In chemolithoautotrophic iron/sulphur oxidizing bacteria, like *L. ferriphilum*, *A. ferrooxidans* and *A. thiooxidans*, it has been demonstrated that genes of phosphoenolpyruvate synthetase, which catalyzes the conversion of pyruvate to PEP, are present. However, the genes of the reverse reaction, catalyzed by the enzyme pyruvate kinase, have been identified only in *A. ferrooxidans* and *A. thiooxidans*. Furthermore, genes of phosphoenolpyruvate diquinase, which catalyzes this reaction in a bidirectional way, were also missing in *L. ferriphilum* (Levican et al. 2008). Therefore, the EMP pathway in *Leptospirillum* probably works preferentially in an anabolic direction, so this pathway was included with these directional restrictions (reactions 15 to 19).
TCA cycle

Although the TCA cycle in *L. ferriphilum* runs in a reductive manner to fix CO₂ (Levican et al. 2008), it was incorporated into the model in an oxidative direction (reactions 20 to 26) because it is thought that *L. ferrooxidans* fixes CO₂ through an acetyl-CoA reductive pathway.

Anaplerotic Reactions

In order to keep a constant level of intermediary metabolites of the TCA cycle, to maintain metabolic balance in the cell, two anaplerotic reactions were included in the stoichiometric model, catalyzed by the enzymes pyruvate carboxylase and phosphoenolpyruvate carboxylase (reactions 27 and 28). Other anaplerotic reactions have not been included to avoid the introduction of reaction cycles, which could lead to observability problems in the mathematical analysis.

2.4.4. Biosynthesis of Building Blocks

Amino Acid Biosynthesis

Amino acid biosynthesis reactions were classified into five families, according to the specific precursor metabolite or amino acid that serves as the starting point for their synthesis (Stephanopoulos et al., 1998, Chapter 2, p.60). The reactions included are considered as standard reactions, so no further analysis will be incorporated (Reactions 29 to 51, Table 2.1).
Nucleotide Biosynthesis

Standard pathways of pyrimidine and purine nucleotides biosynthesis were incorporated into the stoichiometric model (reactions 52 to 61). All nucleotides were expressed as monophosphates, assuming that their activation to triphosphates is carried out on polymerization reactions to RNA and DNA.

Fatty Acid Biosynthesis

It was assumed that *L. ferrooxidans* only synthesized fatty acids as building blocks for its cell membrane phospholipids (Hold et al. 2009). As the composition of fatty acid and their length is unknown for *L. ferrooxidans*, for simplicity, a synthetic-theoretical fatty acid, called C17-CoA was considered, composed of equal proportions of the major fatty acids in bacteria, C16:0 and C18:0 (Hold et al. 2009; Stephanopoulos et al. 1998) represented in reactions 62 to 64.

2.4.5. Synthesis of Macromolecules

Protein Biosynthesis

Protein synthesis reactions were obtained assuming an analogous composition of amino acids to *E. coli*. Therefore, the protein was considered as a polypeptide of standard composition, which requires 4.3 ATP for the correct addition of one amino acid to the existing protein (reaction 65) (Stephanopoulos et al., 1998, Chapter 2, p. 68; Hold et al., 2009).
DNA Biosynthesis

As reported by Sand et al. (1992), the G+C content of \textit{L. ferrooxidans} fluctuates in the range of 53.9\% and 56.4 \%. Thus, for the stoichiometric model we assumed an average composition of 55.2\% of dGMP and dCMP and 44.8\% of dAMP and dTMP per base pair. Also, it was considered that 2 ATPs were consumed per nucleotide incorporated as the total energetic cost of unwinding the double helix before DNA replication, 4 ATP to activate monophosphates to triphosphates, and 0.8 ATPs for proofreading (Stephanopoulos et al., 1998, Chapter 2, pp.69-70). Hence, the energy requirements for one base pair formation are 6.8 ATPs. Consequently reaction 66 represents the elongation of the DNA chain by one base pair.

RNA Biosynthesis

Ribonucleic acid (RNA) is a polymer based on nucleotides, such as DNA, but has only one strand. Considering the number of genes being transcribed in parallel, homogeneity in the nucleotides can be assumed and the composition can be approximated by values from DNA (Hold et al. 2009), so GMP and CMP constitutions were set at 27.6\%, and 22.4\% for AMP and UMP. As stated by Ingraham et al. (1983), the energetic cost for the incorporation of one ribonucleotide as monophosphate is 0.4 ATP. Also, as mentioned before, 2 ATPs are required to activate monophosphate to triphosphates, hence 2.4 ATPs are required to synthesize one nucleotide of RNA (reaction 67).

Lipid Biosynthesis

As mentioned above, it was assumed that \textit{L. ferrooxidans} only synthesizes membrane-forming phospholipids, because the production of energy storage molecules on this
bacterium is unknown (Hold et al. 2009). Thus, a general phospholipid was created, composed of a 76.2% phosphatidylethanolamine, 14.3% phosphatidylglycerol and 9.5% cardiolipin (Stephanopoulos et al., 1998, Chapter 2, p. 64) (reactions 68 to 73).

*Carbohydrate Biosynthesis*

Carbohydrates are defined as molecules with an approximate stochiometric formula $C_n(H_2O)_m$ and they mainly play a role in three aspects: as part of the cell wall (murein), as energy storing molecules and finally as metabolites. A general carbohydrate was built as is shown in reaction 74.

**TABLE 2.1.** Reactions used in the stoichiometric model of *Leptospirillum ferrooxidans*. Irreversible reactions are indicated with an arrow (=>), and reversible reactions with an equal (=).

**Ferrous Oxidation and ATP/NADH Production:**

1. $4*F$ERROUS$_{EXT} + 0.5*O_2 + NAD^+ + 3*H$_{IN}$ => $4*F$ERRIC$_{EXT} + NADH + H_2O$
2. $3*H$_{EXT} + ADP + P + H => $3*H$_{CYT} + ATP + H_2O$

**NH$_4$ assimilation**

3. $NH_4 + AKG + NADH + H^+ => GLUT + NAD^+ + H_2O$

**CO$_2$ assimilation**

4. $CO_2 + H_2 = FOR + H^+$
5. $FOR + THF + H^+ = FTHF + H_2O$
6. $SER + THF = GLY + MTHF + H_2O$
7. $FOR + THF + ATP + H^+ + NADH => MTHF + NAD^+ + H_2O + ADP + P$
8. MTHF + 2*FERR\textsubscript{H2} + CO\textsubscript{2} + COA + H\textsubscript{2} => ACCOA + THF + 2*FERROX + H\textsubscript{2}O + 2*H\textsuperscript{+}

**Pentose Phosphate Pathway**

9. G6P + 2*NAD + H\textsubscript{2}O => RIBU5P + 2*NADH + CO\textsubscript{2} + 2*H\textsuperscript{+}
10. RIBU5P = XYL5P
11. RIBU5P = RIB5P
12. ERY4P + XYL5P = GAP + F6P
13. XYL5P + RIB5P = GAP + SED7P
14. GAP + SED7P = ERY4P + F6P

**Embden-Meyerhof-Parnas Pathway**

15. PYR + ATP + H\textsubscript{2}O => PEP + AMP + P + 2*H\textsuperscript{+}
16. PEP + H\textsubscript{2}O = G3P
17. G3P + ATP + NADH + H\textsuperscript{+} => GAP + NAD\textsuperscript{+} + P + ADP
18. 2*GAP + H\textsubscript{2}O = F6P + P
19. F6P = G6P

**TCA cycle**

20. ACCOA + CO\textsubscript{2} + 2*FERR\textsubscript{H2} = PYR + 2*FERROX + COA + 3*H\textsuperscript{+}
21. FUM + H\textsubscript{2}O + NAD => OAC + NADH + H\textsuperscript{+}
22. SUC + NAD\textsuperscript{+} = FUM + NADH + H\textsuperscript{+}
23. SUCCOA + P + ADP => SUC + COA + ATP
24. AKG + NAD\textsuperscript{+} + COA => SUCCOA + NADH + CO\textsubscript{2}
25. CIT + NAD\textsuperscript{+} => AKG + NADH + CO\textsubscript{2}
26. \( \text{H}_2\text{O} + \text{OAC} + \text{ACCOA} \Rightarrow \text{CIT} + \text{H}^+ + \text{COA} \)

**Anaplerotic Reactions**

27. \( \text{PYR} + \text{CO}_2 + \text{ATP} + \text{H}_2\text{O} \Rightarrow \text{OAC} + \text{ADP} + \text{P} + 2*\text{H}^+ \)

28. \( \text{PEP} + \text{CO}_2 + \text{H}_2\text{O} = \text{OAC} + \text{P} + \text{H}^+ \)

**Amino Acid Biosynthesis**

29. \( \text{GLUT} + \text{ATP} + 2*\text{NADH} + \text{H}^+ \Rightarrow \text{PRO} + \text{ADP} + 2*\text{NAD}^+ + \text{P} + \text{H}_2\text{O} \)

30. \( 2*\text{ATP} + \text{GLUM} + \text{CO}_2 + 2*\text{H}_2\text{O} \Rightarrow \text{GLUT} + 2*\text{ADP} + \text{P} + \text{CARP} + 3*\text{H}^+ \)

31. \( 2*\text{GLUT} + \text{ACCOA} + \text{ASPT} + 2*\text{ATP} + 2*\text{H}_2\text{O} + \text{NADH} + \text{CARP} \Rightarrow \text{ARGI} + \text{FUM} + \text{AMP} + \text{ADP} + \text{COA} + \text{NAD}^+ + \text{ACET} + \text{AKG} + 4*\text{P} + 4*\text{H}^+ \)

32. \( \text{OAC} + \text{GLUT} = \text{ASPT} + \text{AKG} \)

33. \( \text{ASPT} + \text{ATP} + \text{NH}_4^+ + \text{H}_2\text{O} \Rightarrow \text{ASN} + 2*\text{P} + \text{AMP} + \text{H}^+ \)

34. \( \text{ASPT} + \text{ATP} + 2*\text{NADH} + 2*\text{H}^+ + \text{PYR} + \text{SUCCOA} + \text{GLUT} \Rightarrow \text{LYS} + \text{ADP} + 2*\text{NAD}^+ + \text{P} + \text{COA} + \text{AKG} + \text{SUC} + \text{CO}_2 \)

35. \( \text{ASPT} + \text{ATP} + 2*\text{NADH} + 2*\text{H}^+ \Rightarrow \text{HSER} + \text{P} + 2*\text{NAD}^+ + \text{ADP} \)

36. \( \text{HSER} + \text{ATP} + \text{H}_2\text{O} \Rightarrow \text{THR} + \text{ADP} + \text{P} + \text{H}^+ \)

37. \( \text{THR} + \text{PYR} + \text{NADH} + 2*\text{H}^+ + \text{GLUT} \Rightarrow \text{ILE} + \text{NH}_4^+ + \text{CO}_2 + \text{NAD}^+ + \text{H}_2\text{O} + \text{AKG} \)

38. \( \text{NH}_4 + \text{GLUT} + \text{ATP} \Rightarrow \text{GLUM} + \text{ADP} + \text{P} \)

39. \( \text{HSER} + \text{ACCOA} + \text{NADH} + \text{MTHF} + \text{HS} + \text{H}^+ \Rightarrow \text{MET} + \text{THF} + \text{ACET} + \text{COA} + \text{NAD}^+ \)

40. \( 2*\text{PEP} + \text{ERY4P} + \text{NADH} + \text{ATP} \Rightarrow \text{CHOR} + \text{ADP} + \text{NAD}^+ + 4*\text{P} \)
41. CHOR + GLUM + PRPP + SER ⇒ TRYP + PYR + GLUT + 2*P + H₂O + CO₂ + GAP + 2*H⁺
42. CHOR + NAD⁺ + GLUT ⇒ TYR + AKG + NADH + CO₂
43. TYR + NAD⁺ + H₂O ⇒ PHEN + NADH + O₂ + H⁺
44. 2*PYR + NADH + 2*H⁺ ⇒ KIV + NAD⁺ + CO₂ + H₂O
45. KIV + GLUT = VAL + AKG
46. KIV + H₂O + ACCOA + NAD⁺ + GLUT ⇒ LEU + COA + NADH + H⁺ + CO₂ + AKG
47. PYR + GLUT = ALA + AKG
48. G3P + GLUT + NAD⁺ + H₂O ⇒ SER + AKG + NADH + P + H⁺
49. SER + ACCOA + HS ⇒ CYS + COA + ACET
50. RIB5P + ATP ⇒ PRPP + AMP + H⁺
51. PRPP + GLUM + ATP + 2*NAD⁺ + 5*H₂O ⇒ HIS + AICAR + AKG + 2*NADH + 7*H⁺ + 5*P

**Nucleotide Biosynthesis**

52. CARP + ASPT + 1/2*O₂ + PRPP ⇒ UMP + H₂O + CO₂ + 3*P + H⁺
53. UMP + NH₄⁺ + ATP = CMP + ADP + P + H⁺
54. UMP + 2*ATP + 2*NADH + H₂O + MTHF ⇒ DTMP + 2*ADP + 2*NAD + 2*P + THF
55. CMP + NADH + H⁺ ⇒ DCMP + NAD + H₂O
56. PRPP + 2*GLUM + GLY + 5*ATP + ASPT + FTHF + 4*H₂O + CO₂ ⇒ AICAR + 5*ADP + 7*P + 2*GLUT + THF + FUM + 9*H⁺
57. AICAR + FTHF = THF + IMP + H₂O + 2*H⁺
58. IMP + ASPT + ATP => AMP + ADP + P + FUM + H⁺
59. IMP + NAD + 2*H₂O + ATP + NH₄⁺ => GMP + AMP + NADH + 2*P + 3*H⁺
60. AMP + NADH + H⁺ => DAMP + NAD⁺ + H₂O
61. GMP + NADH + H⁺ => DGMP + NAD⁺ + H₂O

Fatty Acid Biosynthesis

62. 9*ACCOA + 16*NADH + 8*ATP + H₂O + 8*H⁺ => C18COA + 8*COA + 16*NAD⁺ + 8*ADP + 8*P
63. 8*ACCOA + 14*NADH + 7*ATP + H₂O + 7*H⁺ => C16COA + 7*COA + 14*NAD⁺ + 7*ADP + 7*P
64. 0.5*C16COA + 0.5*C18COA => C17COA

Protein Biosynthesis

65. 0.096*ALA + 0.055*ARGI + 0.045*ASN + 0.045*ASPT + 0.017*CYS + 0.049*GLUT + 0.049*GLUM + 0.115*GLY + 0.018*HIS + 0.054*ILE + 0.084*LEU + 0.064*LYS + 0.029*MET + 0.035*PHEN + 0.041*PRO + 0.04*SER + 0.047*THR + 0.011*TRYP + 0.079*VAL + 0.026*TYR + 4.3*ATP + 4.3*H₂O => PROT + 4.3*ADP + 4.3*H⁺ + 4.3*P

DNA Biosynthesis

66. 0.4485*DAMP + 0.5515*DCMP + 0.5515*DGMP + 0.4485*DTMP + 6.8*ATP + 6.8*H₂O => DNA + 6.8*ADP + 6.8*P + 6.8*H⁺

RNA Biosynthesis
67. \[ 0.27575 \times \text{CMP} + 0.27575 \times \text{GMP} + 0.22425 \times \text{AMP} + 0.22425 \times \text{UMP} + 2 \times \text{ATP} + 2 \times \text{H}_2\text{O} \rightarrow \text{RNA} + 2 \times \text{ADP} + 2 \times \text{P} + 2 \times \text{H}^+ \]

**Lipid Biosynthesis**

68. \[ \text{GAP} + \text{NADH} + \text{H}^+ \rightarrow \text{GLYC3P} + \text{NAD}^+ \]
69. \[ \text{GLYC3P} + 2 \times \text{C17COA} + \text{ATP} + \text{H}_2\text{O} \rightarrow \text{DIAGLYC} + 2 \times \text{COA} + 2 \times \text{P} \]
70. \[ \text{DIAGLYC} + \text{GLYC3P} + \text{H}_2\text{O} \rightarrow \text{PGLYC} + \text{AMP} + \text{P} \]
71. \[ \text{PGLYC} \rightarrow 0.5 \times \text{GLYC} + 0.5 \times \text{CLIPIN} \]
72. \[ \text{DIAGLYC} + \text{SER} \rightarrow \text{PETH} + \text{CO}_2 + \text{AMP} \]
73. \[ 0.762 \times \text{PETH} + 0.143 \times \text{PGLYC} + 0.095 \times \text{CLIPIN} \rightarrow \text{LIP} \]

**Carbohydrate Biosynthesis**

74. \[ \text{G6P} + \text{ATP} + \text{H}_2\text{O} \rightarrow \text{CARBOH} + \text{ADP} + \text{H}^+ \]

**Acetyl-CoA Synthetase**

75. \[ \text{ACET} + \text{COA} + \text{ATP} + \text{H}_2\text{O} = \text{ACCOA} + \text{AMP} + 2 \times \text{P} + \text{H}^+ \]

**Glycerol Synthesis**

76. \[ \text{GAP} + \text{NADH} + \text{H} + \text{H}_2\text{O} = \text{GLYC} + \text{NAD}^+ + \text{P} \]

**ATP Maintenance**

77. \[ \text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{P} + \text{H}^+ \]

**Assimilative Reduction of SO\(_4\)\(^{2-}\)**

78. \[ \text{SO}_4^{2-} \times \text{ATP} + 4 \times \text{NADH} + 2 \times \text{H}^+ \rightarrow \text{HS} + 4 \times \text{NAD}^+ + \text{ADP} + \text{AMP} + 3 \times \text{P} + \text{H}_2\text{O} \]

**Biomass Formation**

79. \[ 0.03 \times \text{DNA} + 0.16 \times \text{RNA} + 0.52 \times \text{PROT} + 0.17 \times \text{CARBOH} + 0.09 \times \text{LIP} \rightarrow \text{BIO} \]
Extracellular Transport

80. $\text{NH}_4^+_{\text{EXT}} + 4\text{ATP} + 4\text{H}_2\text{O} \rightarrow \text{NH}_4^+_{\text{CYT}} + 4\text{ADP} + 4\text{P} + 4\text{H}^+$

81. $\text{CO}_2_{\text{EXT}} = \text{CO}_2_{\text{CYT}}$

82. $\text{H}_2\text{O}_{\text{EXT}} = \text{H}_2\text{O}_{\text{CYT}}$

83. $\text{O}_2_{\text{EXT}} = \text{O}_2_{\text{CYT}}$

84. $\text{G6P}_{\text{EXT}} = \text{G6P}_{\text{CYT}}$

85. $\text{SO}_4^{2-}_{\text{EXT}} + \text{ATP} + \text{H}_2\text{O} \rightarrow \text{SO}_4^{2-}_{\text{CYT}} + \text{ADP} + \text{P} + \text{H}^+$

86. $\text{PE}_{\text{EXT}} + \text{ATP} + \text{H}_2\text{O} \rightarrow \text{P}_{\text{CYT}} + \text{ADP} + \text{P} + \text{H}^+$

87. $\text{H}_2_{\text{EXT}} = \text{H}_2_{\text{CYT}}$
2.5. RESULTS AND DISCUSSION

2.5.1. Stochiometric Model

The *in silico* metabolic network of *L. ferrooxidans* was constructed from database and literature information from an analogous iron oxidizing bacteria (see materials and methods for detail). When an incomplete pathway arises, the principal criterion to approve it was that more than 50% of the enzymes involved must be present. Thus, standard pathways, for example biosynthesis of amino acid phenylalanine, lysine, methionine and alanine were incomplete; however iron oxidizing bacteria like *A. ferrooxidans* and *L. ferrooxidans* must contain these pathways because they are capable of growth in basal medium 9K (Silverman and Lundgren 1959). Nevertheless, the opposite case is also possible, but further biochemical confirmation, through enzymatic activity assays for example, is necessary to complete the pathways validation.

A simplification process was performed in order to reduce the complexity of the stoichiometric model. With the purpose to reduce the number of metabolites and reactions, sequential reactions were grouped in one net reaction in conserved pathways, like the EMP, the pentose phosphate and the amino acid biosynthesis pathways. Also linearly dependent reactions were eliminated to avoid observability problems of the model. That is the case of the anaplerotic reactions of pyruvate carboxylase and the glyoxylate shunt, which when included together, make the system non observable since the latter is a linear combination of the pyruvate carboxylase reaction and the TCA cycle. Hence, the glyoxylate shunt was considered to be inactive in our simulations, assuming that the corresponding enzymes operate under different environmental conditions.
The metabolic model developed includes the main sources for catabolic and anabolic processes of *L. ferrooxidans*. The main catabolic substrates considered are ferrous iron, as energy source in aerobic conditions and CO$_2$ from the air as sole carbon source. Also, nitrogen assimilation was assumed to occur from NH$_4^+$ as the unique source. In addition, the main anabolic final products considered are the macromolecules DNA, RNA, protein, carbohydrates and lipids constituting one C-mol of biomass according to reaction 79 in Table 2.1. A schematic representation of the reconstructed metabolic network is shown in Figure 2.1.

The model has 86 reactions, where 10 of them are transport reactions of nutrients and products (reactions 1, 2 and 80 to 87). It is composed of 100 metabolites, of which 88 are intermediate metabolites, and must be balanced. Consequently, the resulting system has 2 degrees of freedom, thus 2 external fluxes must be estimated to achieve a determined system and so calculate the internal fluxes of Figure 2.1.

### 2.5.2. Proof of Concept

In order to prove the functionality and feasibility of the metabolic model developed, a proof of concept was made. For this purpose, 2 external fluxes were estimated from published data. The calculation of the internal fluxes was made assuming a specific growth rate of *L. ferrooxidans* on ferrous iron $\mu=0.05$ (h$^{-1}$) (Boon 1996; Kleerebezem and van Loosdrecht 2008). A specific oxygen consumption rate was estimated through a relationship between this and the dilution rate in continuous culture (van Scherpenzeel et al. 1998):

$$ q_{O2} = \frac{D}{0.047} + 0.057 $$  \hspace{1cm} (2.11)
A value of $q_{O_2} = 1.12$ (mol O$_2$/C-mol·h) was obtained for $D = 0.05$ (h$^{-1}$) (equal to the specific growth rate $\mu$ for a steady state continuous culture).

The biomass generation at $D=0.05$ (h$^{-1}$) was obtained using the biomass composition of Table 2.2.

Table 2.2. Assumed percentage of each macromolecule ($x_i$) in cell composition of *L. ferrooxidans* (taken from Stephanopulos (1998), chapter 2, p.75 for *E. Coli*).

<table>
<thead>
<tr>
<th>Macromolecule</th>
<th>$x_i$ (g/g$_{cdw}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>0.52</td>
</tr>
<tr>
<td>DNA</td>
<td>0.03</td>
</tr>
<tr>
<td>RNA</td>
<td>0.16</td>
</tr>
<tr>
<td>Lipids</td>
<td>0.09</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>0.17</td>
</tr>
</tbody>
</table>

In order to obtain a determined and observable system, the estimated specific oxygen consumption rate and the generation rate of biomass were used as input data for the MFA, considering a standard deviation of 10%. With this analysis, a flux distribution was obtained, which is shown in Figure 2.2.

In Figure 2.2 it is possible to observe a scheme of the contribution of the different pathways to the global metabolic process. It can be seen that the TCA cycle is running in a reductive manner, which is very reasonable, because it would be inefficient for *L. ferrooxidans* to use the regular TCA cycle releasing the CO$_2$ needed for growth. This behavior is consistent with that observed for *Leptospirillum ferriphilum* in Levican et al. (2008).
Main uptake fluxes predicted with MFA are shown in Table 2.3.

Table 2.3. Calculated uptake specific rates with MFA for *L. ferrooxidans* using NH$_4^+$

<table>
<thead>
<tr>
<th>Calculated flux value</th>
<th>mol/C-mol·h</th>
</tr>
</thead>
<tbody>
<tr>
<td>qCO$_2$</td>
<td>0.04</td>
</tr>
<tr>
<td>qNH$_4^+$</td>
<td>0.01</td>
</tr>
<tr>
<td>qFe$^{2+}$</td>
<td>6.72</td>
</tr>
<tr>
<td>qATP</td>
<td>1.68</td>
</tr>
</tbody>
</table>

The growth yield on ferrous iron was calculated with the equation

$$Y_{SX} = \frac{\mu}{q_S} \quad (2.12)$$

Consequently, a yield of *L. ferrooxidans* on ferrous iron of $Y_{SX} = 0.007$ (C-mol/mol Fe$^{2+}$) was obtained, which compared with $Y_{SX} = 0.006$ (C-mol/mol Fe$^{2+}$) for *L. ferrooxidans* in Breed et al. (1999), $Y_{SX} = 0.010$ (C-mol/mol Fe$^{2+}$) for *Leptospirillum* sp. (van Scherpenzeel et al. 1998), and $Y_{SX} = 0.006$ (for iron oxidizing bacteria) (Mignone and Donati 2004), indicating a very good prediction of the model for this iron oxidizing bacteria growing on ferrous iron.

A sensitivity analysis was done using a random percentage of error in the range of 9% to 10% of the input data. Three simulations were carried out considering different measurement errors, and the flux distribution obtained in each case was very stable since the direction of the fluxes remains equal to the original distribution, and the calculated rates reveal only slight variations.
The elimination of the anaplerotic reactions catalyzed by the enzymes pyruvate carboxylase and phosphoenolpyruvate carboxylase was explored, determining that the elimination of any of these enzymes makes impossible the biomass generation by the metabolic network. The activity of both enzymes is necessary because they are the only way to generate oxaloacetate, which is required in biomass biosynthesis.

A simulation was made activating the nitrogenase enzyme to compare the flux distribution in the presence and absence of this pathway. When the enzyme is activated one degree of freedom is added to the metabolic system, so 3 external fluxes are needed to solve it. Considering a specific growth rate of $\mu = 0.05 \ (h^{-1})$, and the stoichiometric coefficients of Equation (2.2), a specific nitrogen consumption rate of $q_{N_2} = 0.005 \ (mol \ N_2/C-mol*h)$ was calculated. Thus, the input data is the same as in the other condition, but including a nitrogen reduction rate. With this test we observed that the model predicts that the nitrogenase reaction runs in the opposite direction, which indicates that *L. ferrooxidans* cannot use this nitrogen source for growth under the stated conditions, because of the high energetic requirements.
Figure 2.2: Metabolic flux distribution of \textit{L. ferrooxidans} in (mol/C·mol·h of biomass).
2.6. CONCLUSIONS

A description of the main metabolic pathways of *L. ferrooxidans* was made. The principal uptake pathways considered in the metabolic reconstruction were ferrous iron oxidation to gain energy for all anabolic and catabolic processes, CO$_2$ assimilation through the acetyl-CoA reductive pathway, and ammonia assimilation by the GDH pathway. A stoichiometric model composed of 86 reactions and 2 degrees of freedom was obtained. The model was used to calculate the internal flux distribution by MFA. With these data, an estimation of growth yields of *L. ferrooxidans* on ferrous iron was made, obtaining $Y_{sx} = 0.006$ (C-mol/mol Fe$^{2+}$), showing a very good behavior when compared with experimental data from the literature. A sensitivity analysis was performed, and the model shows a very stable behavior when a random error on the input data is considered. In conclusion, the metabolic model developed is capable of reproducing the main aspects of the metabolic behavior of *L. ferrooxidans*, thus, it could be used as a first approach to create new strategies to improve the productivity of the bioleaching processes.
CHAPTER 3

Stoichiometric Model and Flux Balance Analysis for a Mixed Culture of *Leptospirillum ferriphilum* and *Ferroplasma acidiphilum*
3.1. ABSTRACT

The oxidation process of sulfide minerals in natural environments is achieved by microbial communities comprised by Archaea and Bacteria domains. A metabolic reconstruction of two dominant species, which are always found together as a mixed culture in this natural environments, *Leptospirillum ferriphilum* and *Ferroplasma acidiphilum*, was made. The metabolic model, composed of 152 internal reactions and 29 transport reactions, describes the main interactions between these species, assuming that both use ferrous iron as energy source, and *F. acidiphilum* takes advantage of the organic compounds secreted by *L. ferriphilum* for chemomixotrophic growth.

A first metabolic model for a mixed culture used in bacterial leaching is proposed in this paper, and it was evaluated with literature data through Flux Balance Analysis (FBA) using as objective function the maximization of biomass production. The growth yields on ferrous iron obtained for each microorganism are consistent with literature data, and the flux distribution obtained allowed understanding of the metabolic capabilities of both microorganisms growing together in a bioleaching process.

The metabolic model was used to simulate the growth of *F. acidiphilum* on different substrates, with the aim of determining in silico which compounds maximize cell growth rate, and which are essential for growth. With this methodology it was possible to suggest a suitable composition of culture media to optimize cell growth.

Knockout simulations were carried out for *L. ferriphilum* and *F. acidiphilum* metabolic models, predicting key enzymes of central metabolism. The results of this analysis are
consistent with experimental data from literature, showing a robust behavior of the metabolic model.
3.2. INTRODUCTION

Bioleaching is the oxidation process of metallic sulfide to soluble metallic ions and sulfuric acid, catalyzed by microorganism consortia (Schippers and Sand 1999). Possibly, the main role of these microorganisms in bioleaching operations is to oxidize iron and sulfur containing minerals (Holmes and Bonnefoy 2007).

Oxidation of ore by consortia of microorganisms generally takes place at a higher rate than in pure cultures, however, the specific role of each microorganism in these natural environments remains unclear, as well as the factors that enable their growth in communities. Therefore, it is essential to identify the microorganisms that compose these consortia, study their interactions in bioleaching operations, and then predict and explore the metabolic details that control these communities.

Microorganisms involved in metal recovery from sulfide minerals include members of the Bacteria and Archaea domains. Species of *Leptospirillum* and *Ferroplasma* genus are part of the dominant members of microbial communities in acid mine drainage and bioleaching environments (Baker and Banfield 2003; Ram et al. 2005; Tyson et al. 2004).

*Leptospirillum ferrphilum* is an autotrophic, mesophilic, acidophilic, iron oxidizing bacteria. It plays an important role in bioleaching processes given its ability to attach to sulfide mineral, its high affinity for ferrous iron, and its low sensitivity to inhibition by ferric iron (Rawlings et al. 1999)

*Ferroplasma acidiphilum* is a recently described mesophilic, acidophilic, ferrous iron oxidizing, and cell wall-lacking archaea. Different strains of *F. acidiphilum* have been
isolated and characterized (Dopson et al. 2004; Golyshina et al. 2000; Golyshina and Timmis 2005; Pivovarova et al. 2002). These strains were classified by Dopson et al. (2004) as chemoorganotrophic, because they are able to grow on yeast extract or another source of organic carbon as the only energy source, and also chemomixotrophic, because they are capable of grow on ferrous iron as electron donor, but require a small amount (0.02% wt/vol) of yeast extract which, in this case, is essential for growth.

The main objective of the reconstruction of metabolic pathways is determining the relationship between genome and physiology of a microorganism. Through metabolic reconstruction it is possible to break down the metabolic pathways of an organism into its respective reactions and enzymes, allowing their analysis into a complete network context.

Information from fully sequenced genomes allows the strain specific global reconstruction of metabolic networks, permitting further structural and functional studies for a particular microorganism. These models have been proven to be an effective strategy to enhance physiological studies of environmentally relevant microorganisms and predict physiological responses of these microorganisms to environmental or genetic stimuli (Mahadevan et al. 2006).

The whole metabolism of two bioleaching bacteria, *A. ferrooxidans* (Hold et al. 2009) and *L. ferrooxidans* (Merino et al. 2010), were recently developed and evaluated through Metabolic Flux Analysis (MFA) to predict their metabolic behavior. In this work, a metabolic model of a mixed culture of bioleaching microorganisms composed of *L. ferrphilum* and *F. acidiphilum* was developed for the first time, in order to define the metabolic characteristics and molecular mechanisms underlying of these biomining species, and predict their behavior when they are growing in a mixed culture.
3.3. MATERIALS AND METHODS

3.3.1. Metabolic Reconstruction

A metabolic reconstruction of *L. ferriphilum* was developed based on a previous metabolic model of *L. ferrooxidans* (Merino et al. 2010).

The metabolic model of *F. acidiphilum* was built considering the main metabolic pathways of this archaea described in literature. Also, the reconstruction was based on the analogous archaea *Ferroplasma acidarmanus* Fer1 genome annotation, which is available in the Doe Joint Genome Institute website (JGI, http://www.jgi.doe.gov/genome-projects/).

3.3.2. Flux Balance Analysis

The intracellular flux distribution for an underdetermined system of equations can be estimated by linear programming. This methodology uses flux balances to define limits on metabolic capabilities, therefore is possible to obtain a unique solution for intracellular fluxes by optimizing an objective function, subject to the constraints of mass balances.

The general steady state equation that describes cell metabolism is written as:

\[ S \cdot v(x) = b \]  

(3.1)

where *S* is the stoichiometric matrix of the metabolic network, \( v(x) \) is the vector of reaction fluxes, and *b* is the net output from cellular metabolism (Varma and Palsson 1993).

The difference between pathway fluxes and pathway metabolites correspond to the degree of freedom, *f*, of the linear system. If the number of fluxes exceeds the number of metabolites, then the system is underdetermined. In these cases the system is solved by
linear optimization by stating an objective function, maximization of biomass in this case, and searching this maximal value within the stoichiometrically defined domain.

3.3.3. Modeling and Simulation Software

The metabolic model was implemented in the software INSILICO Discovery 3.2 (Stuttgart, Germany, www.insilico-biotechnology.com). Mathematical modeling, system analysis and flux balance analysis were made using this software.
3.4. RESULTS AND DISCUSSION

3.4.1. Metabolic Reconstruction of *L. ferrophilum*

The model of *L. ferrophilum* was modified in a few aspects with respect to the *L. ferrooxidans* model of Merino et al. (2010), recently developed by our group. The TCA cycle was included in a reductive manner (Levican et al. 2008) for CO$_2$ assimilation, therefore it was not included the reductive acetyl-CoA pathway. Ammonia fixation was considered to occur through the GDH pathway. In addition, EPS formation by the Leloir pathway was incorporated into the metabolic model (Barreto et al. 2005). Canonical pathways (other fueling reactions, synthesis of precursor metabolites, biosynthesis of building blocks, and macromolecules biosynthesis) were considered to be analogous in both types of *Leptospirillum*.

3.4.2. Metabolic Reconstruction of *F. acidiphilum*

3.4.2.1. Fueling Reactions

**Glycolysis:** According to Dopson et al. (2005) *F. acidiphilum* growing in chemomixotrophic conditions up-regulates the enzymes fructose 1.6-bisphosphatase and phosphoglycerate mutase, so it is assumed that the conversion of glucose to pyruvate occurs via the Embden-Meyerhof-Parnas (EMP) pathway (reactions 1 to 6 in Table 3.1)

**Pentose Phosphate Pathway (PPP):** All autotrophic and heterotrophic archaea must be able to generate NAD(P)H and pentoses as building blocks for the biosynthesis of nucleotides and certain amino acids, so oxidative and non oxidative branches of the PPP were included in the stoichiometric model (reactions 7 to 12).
**TCA Cycle:** All the enzymes of the TCA cycle were identified in the genome annotation of *Ferroplasma acidarmanus* Fer1, and then included in the stoichiometric model (reactions 13 to 19).

**Anaplerotic Reactions:** In order to regenerate oxaloacetate, anaplerotic reactions catalyzed by pyruvate carboxylase and phosphoenolpyruvate carboxylase were included in the stoichiometric model. Other anaplerotic reactions, such as the glyoxylate shunt, have not been included to avoid the introduction of reaction cycles, which could lead to observability problems in the mathematical analysis (reactions 20 and 21).

**Oxidative Phosphorylation:** The electron transport chain involved in iron oxidation was approximated to the one of *Ferroplasma acidarmanus* Fer1 for chemomixotrophic growth, which contains a putative haeme-copper terminal oxidase, cytochrome b and associated Rieske iron-sulphur proteins, and a blue copper protein (Dopson et al. 2005). The net reaction of electron transport from Fe\(^{2+}\) is the following:

\[
2 \text{Fe}^{2+} + \frac{1}{2} \text{O}_2 + 2\text{H}^+ \rightarrow 2 \text{Fe}^{3+} + \text{H}_2\text{O}
\]  

(3.2)

The electrons derived from organic compound oxidation fed both an NADH ubiquinone oxidoreductase complex to generate reducing power, and a succinate dehydrogenase to oxidize succinate to fumarate, and transfer the electrons to a coenzyme Q which is reduced to a coenzyme QH\(_2\). Then, the electrons are carried to the rest of the previously mentioned transport chain. Also, the NADH ubiquinone oxidoreductase pumps protons to the external space as it transfers the electrons to the transport chain. The amount of pumped protons to the periplasmic space was taken from the proposed transport chain for *Ferroplasma*
*Ferroplasma acidarmanus* Fer1 available on http://www.microbesonline.org/. These reactions are summarized as follow:

\[
\text{NAD}^+ + 4 \text{H}^+_{\text{INT}} + \text{coQ} \rightarrow \text{NADH} + 4\text{H}^+_{\text{EXT}} + \text{coQH}_2 \quad (3.3)
\]

\[
\text{SUC} + \text{coQ} \rightarrow \text{FUM} + \text{coQH}_2 \quad (3.4)
\]

On the other hand, it was assumed that *Ferroplasma acidiphilum* produces ATP by an ATP synthetase coupled to the extrusion of 3 H⁺

\[
\text{H}^+_{\text{EXT}} + \text{ADP} + \text{Pi} \rightarrow \text{ATP} + 3 \text{H}^+_{\text{INT}} \quad (3.5)
\]

### 3.4.2.2. Biosynthetic Reactions

**Amino Acids Biosynthesis:**

The pathways for the synthesis of 20 standard amino acids were included in the stoichiometric model. The enzymes involved in each pathway were verified in the genomic annotation of *Ferroplasma acidarmanus* Fer1. Amino acids and their main precursors are shown in reactions 25 to 50.

**Nucleotide Biosynthesis:**

Nucleotides in the form of ribonucleotides and deoxyribonucleotides were included as building blocks for RNA and DNA. All nucleotides were expressed as monophosphates, assuming that their activation to triphosphates is carried out by polymerization reactions to RNA and DNA (reactions 51 to 59).
Archeal Phospholipid Biosynthesis

Membrane lipids in Archaea are composed of ether lipids containing saturated isoprenyl chains, archaeol, and caldarchaeol (Nemoto et al. 2003). The lipid composition of *Ferroplasma acidiphilum* is constituted by two main glycopospholipids, which comprised 90% of the total lipids. These main components are β-D-glucopyranosylcaldarchaetidylglycerol and a triglycoside of caldarchaetidylglycerol (Batrakov et al. 2002; Pivovarova et al. 2002); both components were included in the model as the only lipids in *F. acidiphilum*. The common precursor of these lipids is archaeatidylglycerol, so it was included as the only phospholipid in the model.

The biosynthetic pathway of archaeatidylglycerol begins with the synthesis of the G-1-P backbone, which is formed from dihydroxyacetonephosphate (DHAP) by the enzyme G-1-P dehydrogenase (Nishihara et al. 1999). In the model, DHAP was replaced by its isomer GAP (glyceraldehyde 3-phosphate), getting the formation reaction 60 in Table 3.1.

On the other hand, the isoprenoid biosynthetic pathway starts from acetyl-CoA to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) through the classical mevalonate (MVA) pathway, which has been identified in archaea (Koga and Morii 2007; Nemoto et al. 2003). DMAPP is condensed consecutively with several IPP molecules resulting in the synthesis of polyisoprenyl diphosphate compounds. For the model the formation of the 20 carbon geranylgeranyl diphosphate (GGPP) molecule was considered as the precursor of archeatidylglycerol. Furthermore, GGPP molecules were expressed as a function of IPP molecules as is shown in reaction 61. GGPP is transformed to CDP-archaeol through the consecutive action of GGGP, DGGGP (archaetidic acid) and
CDP-archaeol synthases, which is finally converted to archaetidylglycerol by archaetidylglycerophosphate synthase and phosphatase (Koga and Morii 2007).

3.4.2.3. **Synthesis of Macromolecules**

**Protein Biosynthesis**

Protein amino acid composition of *F. acidiphilum* was approximated with an average composition obtained from the proteomic analysis of different species of archaea (Pe'er et al. 2004). Protein biosynthesis requires 4.3 ATPs for the correct addition of one amino acid to the existing polypeptide (Stephanopoulos et al. 1998) (reaction 64, Table 3.1).

**DNA Biosynthesis**

The G+C chromosomal composition of *F. acidiphilum* is approximately of 35% (Pivovarova et al. 2002), so a composition of dGMP + dCMP of 35% and dAMP + dTMP of 65% per base pair were assumed. A consumption of 6.8 ATPs was calculated as the energy requirement for 1 bp formation of DNA (reaction 65). This value takes into account the ATP necessary for nucleotide incorporation, the separation of the double helix, the activation of monophosphates to triphosphates, and the energetic cost of proofreading (Stephanopoulos et al. 1998).

**RNA Biosynthesis**

From DNA composition, GMP + CMP nucleotide composition was approximated to 0.175% and 0.325% for AMP + UMP. An energetic cost of 2.4 ATPs was assumed for the synthesis of one nucleotide of RNA, 2 from the activation of monophosphates to
triphosphates and 0.4 for the incorporation of one ribonucleotide (Ingraham et al. 1983) (reaction 66).
Lipid Biosynthesis

Two glycophospholipids are formed from archaetidylglicerol (β-D-glucopyranosylcaldarchaetidylglycerol and a triglycoside of caldarchaetidylglycerol, named LIPI and LIPII in reactions 67 to 69). The composition of each one is 55% of LIPI and 35% of LIPII (Pivovarova et al. 2002), comprising 90% of the total lipids in *F. acidiphilum*.

Carbohydrate Biosynthesis

Carbohydrates for cell wall biosynthesis, energy storing molecules and metabolites were defined as molecules C\(_n\)(H\(_2\)O)\(_m\), and arise from the polymerization of glucose-6-phosphate (reaction 70).

Biomass Formation

Biomass relative composition of Protein, RNA, DNA, lipids and carbohydrates were estimated for an average prokaryotic cell, obtaining 63% protein, 24% RNA, 4% DNA, 5% lipids, carbohydrates less than 1% and 4% of soluble pools (Feist et al. 2006). This composition was used in the *F. acidiphilum* metabolic model.

**TABLE 3.1.** Reactions used in the stoichiometric model of *Ferroplasma acidiphilum*. Irreversible reactions are indicated with an arrow (=>), and reversible reactions with an equal (=).

Glycolysis

1. GLC + ATP => G6P + ADP + H\(^+\)
2. F6P = G6P
3. 2*GAP + H\(_2\)O = F6P + P
4. GAP + NAD\(^+\) + P + ADP => G3P + ATP + NADH + H\(^+\)
5. \( \text{PEP} + \text{H}_2\text{O} = \text{G3P} \)
6. \( \text{PEP} + \text{AMP} + \text{P} + 2*\text{H}^+ \Rightarrow \text{PYR} + \text{ATP} + \text{H}_2\text{O} \)

**Pentose Phosphate**

7. \( \text{G6P} + 2*\text{NAD}^+ + \text{H}_2\text{O} \Rightarrow \text{RIBU5P} + 2*\text{NADH} + \text{CO}_2 + 2*\text{H}^+ \)
8. \( \text{RIBU5P} = \text{XYL5P} \)
9. \( \text{RIBU5P} = \text{RIB5P} \)
10. \( \text{XYL5P} + \text{RIB5P} = \text{GAP} + \text{SED7P} \)
11. \( \text{ERY4P} + \text{XYL5P} = \text{GAP} + \text{F6P} \)
12. \( \text{GAP} + \text{SED7P} = \text{ERY4P} + \text{F6P} \)

**TCA cycle**

13. \( \text{PYR} + \text{COA} + \text{NAD}^+ \Rightarrow \text{ACCOA} + \text{CO}_2 + \text{NADH} \)
14. \( \text{H}_2\text{O} + \text{OAC} + \text{ACCOA} \Rightarrow \text{CIT} + \text{H}^+ + \text{COA} \)
15. \( \text{CIT} + \text{NAD}^+ \Rightarrow \text{AKG} + \text{NADH} + \text{CO}_2 \)
16. \( \text{AKG} + \text{NAD}^+ + \text{COA} = \text{SUCCOA} + \text{NADH} + \text{CO}_2 \)
17. \( \text{SUCCOA} + \text{P} + \text{ADP} \Rightarrow \text{SUC} + \text{COA} + \text{ATP} \)
18. \( \text{SUC} + \text{COQ} = \text{FUM} + \text{COQ}_2 \)
19. \( \text{FUM} + \text{H}_2\text{O} + \text{NAD}^+ \Rightarrow \text{OAC} + \text{NADH} + \text{H}^+ \)

**Anaplerotic Reaction**

20. \( \text{PYR} + \text{CO}_2 + \text{ATP} + \text{H}_2\text{O} \Rightarrow \text{OAC} + \text{ADP} + \text{P} + 2*\text{H}^+ \)
21. \( \text{PEP} + \text{CO}_2 + \text{H}_2\text{O} \Rightarrow \text{OAC} + \text{P} + \text{H}^+ \)

**Oxidative Phosphorylation**
22. \[2\text{FERROUS} + 0.5\text{O}_2 + 2\text{H}_\text{IN} \rightarrow 2\text{FERRIC} + \text{H}_2\text{O}\]

23. \[\text{NADH} + \text{COQ} + 4\text{H}_\text{IN} + \text{H}^+ \rightarrow \text{COQ}_2\text{H}_2 + \text{NAD}^+ + 4\text{H}_\text{OUT}\]

24. \[3.3\text{H}_\text{OUT} + \text{ADP} + \text{P} + \text{H}^+ \rightarrow 3.3\text{H}_\text{IN} + \text{ATP} + \text{H}_2\text{O}\]

**Amino Acid Biosynthesis**

25. \[\text{NH}_4^+ + \text{AKG} + \text{NADH} + \text{H}^+ \rightarrow \text{GLUT} + \text{NAD}^+ + \text{H}_2\text{O}\]

26. \[\text{NH}_4^+ + \text{GLUT} + \text{ATP} \rightarrow \text{GLUM} + \text{ADP} + \text{P}\]

27. \[\text{GLUT} + \text{ATP} + 2\text{NADH} + \text{H}^+ \rightarrow \text{PRO} + \text{ADP} + 2\text{NAD}^+ + \text{P} + \text{H}_2\text{O}\]

28. \[2\text{ATP} + \text{GLUM} + \text{CO}_2 + 2\text{H}_2\text{O} \rightarrow \text{GLUT} + 2\text{ADP} + \text{P} + \text{CARP} + 3\text{H}^+\]

29. \[2\text{GLUT} + \text{ACCOA} + \text{ASPT} + 2\text{ATP} + 2\text{H}_2\text{O} + \text{NADH} + \text{CARP} \rightarrow \text{ARGI} + \text{FUM} + \text{AMP} + \text{ADP} + \text{COA} + \text{NAD}^+ + \text{ACET} + \text{AKG} + 4\text{P} + 4\text{H}^+\]

30. \[\text{ASPT} + \text{ATP} + 2\text{NADH} + 2\text{H}^+ + \text{PYR} + \text{SUCCOA} + \text{GLUT} \rightarrow \text{LYS} + \text{ADP} + 2\text{NAD}^+ + \text{P} + \text{COA} + \text{AKG} + \text{SUC} + \text{CO}_2\]

31. \[\text{OAC} + \text{GLUT} = \text{ASPT} + \text{AKG}\]

32. \[\text{ASPT} + \text{ATP} + 2\text{NADH} + 2\text{H}^+ \rightarrow \text{HSER} + \text{P} + 2\text{NAD}^+ + \text{ADP}\]

33. \[\text{HSER} + \text{SUCCOA} + \text{CYS} + \text{MYTHF} + \text{H}_2\text{O} \rightarrow \text{MET} + \text{COA} + \text{SUC} + \text{PYR} + \text{NH}_4^+ + \text{H}^+ + \text{THF}\]

34. \[\text{HSER} + \text{ATP} + \text{H}_2\text{O} \rightarrow \text{THR} + \text{ADP} + \text{P} + \text{H}^+\]

35. \[\text{THR} + \text{PYR} + \text{NADH} + 2\text{H}^+ + \text{GLUT} \rightarrow \text{ILE} + \text{NH}_4^+ + \text{CO}_2 + \text{NAD}^+ + \text{H}_2\text{O} + \text{AKG}\]

36. \[\text{ASPT} + \text{ATP} + \text{NH}_4^+ + \text{H}_2\text{O} \rightarrow \text{ASN} + 2\text{P} + \text{AMP} + \text{H}^+\]

37. \[2\text{PEP} + \text{ERY4P} + \text{NADH} + \text{ATP} \rightarrow \text{CHOR} + \text{ADP} + \text{NAD}^+ + 4\text{P}\]

38. \[\text{CHOR} + \text{H}^+ + \text{GLUT} \rightarrow \text{PHEN} + \text{CO}_2 + \text{H}_2\text{O} + \text{AKG}\]

39. \[\text{CHOR} + \text{NAD}^+ + \text{GLUT} \rightarrow \text{TYR} + \text{AKG} + \text{NADH} + \text{CO}_2\]
40. CHOR + GLUM + PRPP + SER $\Rightarrow$ TRYP + PYR + GLUT + 2*P + H$_2$O + CO$_2$ + GAP + 2*H$^+$

41. 2*PYR + NADH + 2*H$^+$ $\Rightarrow$ KIV + NAD$^+$ + CO$_2$ + H$_2$O

42. KIV + GLUT = VAL + AKG

43. KIV + H$_2$O + ACCOA + NAD$^+$ + GLUT $\Rightarrow$ LEU + COA + NADH + H$^+$ + CO$_2$ + AKG

44. PYR + GLUT = ALA + AKG

45. G3P + GLUT + NAD$^+$ + H$_2$O $\Rightarrow$ SER + AKG + NADH + P + H$^+$

46. SER + THF = GLY + MTHF + H$_2$O

47. SER + ACCOA + HS $\Rightarrow$ CYS + COA + ACET

48. RIB5P + ATP $\Rightarrow$ PRPP + AMP + H$^+$

49. PRPP + 2*GLUM + GLY + 5*ATP + ASPT + FTHF + 4*H$_2$O + CO$_2$ $\Rightarrow$ AICAR + 5*ADP + 7*P + 2*GLUT + THF + FUM + 9*H$^+$

50. PRPP + GLUM + ATP + 2*NAD$^+$ + 5*H$_2$O $\Rightarrow$ HIS + AICAR + AKG + 2*NADH + 7*H$^+$ + 5*P

Nucleotide Biosynthesis

51. AICAR + FTHF = THF + IMP + H$_2$O + 2*H$^+$

52. IMP + ASPT + ATP $\Rightarrow$ AMP + ADP + P + FUM + H$^+$

53. AMP + NADH + H$^+$ = DAMP + NAD$^+$ + H$_2$O

54. IMP + NAD$^+$ + 2*H$_2$O + ATP + NH$_4^+$ $\Rightarrow$ GMP + AMP + NADH + 2*P + 3*H$^+$

55. GMP + NADH + H$^+$ $\Rightarrow$ DGMP + NAD$^+$ + H$_2$O

56. CARP + ASPT + 1/2*O$_2$ + PRPP $\Rightarrow$ UMP + H$_2$O + CO$_2$ + 3*P + H$^+$
57. UMP + 2*ATP + 2*NADH + H₂O + MTHF => DTMP + 2*ADP + 2*NAD⁺ + 2*P + THF
58. UMP + NH₄⁺ + ATP = CMP + ADP + P + H⁺
59. CMP + NADH + H⁺ => DCMP + NAD⁺ + H₂O

Phospholipid Biosynthesis

60. GAP + NADH + 2*H⁺ = GLYC1P + NAD⁺
61. 3*ACCOA + 2*NADH + 3*ATP + H₂O + H⁺ => 3*COA + 2*NAD⁺ + 3*ADP + CO₂ + P + IPP
62. GLYC1P + 8*IPP + CMP + 2*ATP + 9*H₂O = CDPARCH + 2*ADP + 17*H⁺ + 18*P
63. CDPARCH + GLYC1P + H₂O = ARCHGLYC + CMP + P + H⁺

Protein Biosynthesis

64. 0.078*ALA + 0.06*ARGI + 0.034*ASN + 0.056*ASPT + 0.008*CYS + 0.074*GLUT + 0.02*GLUM + 0.076*GLY + 0.016*HIS + 0.076*ILE + 0.096*LEU + 0.058*LYS + 0.025*MET + 0.038*PHEN + 0.045*PRO + 0.06*SER + 0.05*THR + 0.01*TRYP + 0.082*VAL + 0.038*TYR + 4.3*ATP + 4.3*H₂O => PROT + 4.3*ADP + 4.3*P + 4.3*H⁺

DNA Biosynthesis

65. 0.65*DAMP + 0.65*DTMP + 0.35*DCMP + 0.35*DGMP + 6.8*ATP + 6.8*H₂O
=> DNA + 6.8*ADP + 6.8*P + 6.8*H⁺
RNA Biosynthesis

66. \[0.175 \cdot \text{CMP} + 0.175 \cdot \text{GMP} + 0.325 \cdot \text{AMP} + 0.325 \cdot \text{UMP} + 2 \cdot \text{ATP} + 2 \cdot \text{H}_2\text{O} \rightarrow \text{RNA} + 2 \cdot \text{ADP} + 2 \cdot \text{P} + 2 \cdot \text{H}^+\]

Lipid Biosynthesis

67. \[2 \cdot \text{ARCHGLYC} + \text{GLC} \rightarrow \text{LIPI} + \text{GLYC1P}\]
68. \[2 \cdot \text{ARCHGLYC} + 3 \cdot \text{GLC} \rightarrow \text{LIPII} + \text{GLYC1P}\]
69. \[0.55 \cdot \text{LIPI} + 0.35 \cdot \text{LIPII} \rightarrow \text{LIP} \]

Carbohydrate Biosynthesis

70. \[\text{G6P} + \text{ATP} + \text{H}_2\text{O} \rightarrow \text{POLYS} + \text{ADP} + \text{H}^+\]

Biomass Formation

71. \[0.040 \cdot \text{DNA} + 0.24 \cdot \text{RNA} + 0.62 \cdot \text{PROT} + 0.01 \cdot \text{POLYS} + 0.09 \cdot \text{LIP} \rightarrow \text{BIO}\]

3.4.3. **Simplification criterion**

The stoichiometric models were subjected to a general reduction process of matrix \(S\); the simplification criterion consisted basically in:

i. Sequential reactions were lumped into a single reaction and therefore reduce the number of reaction steps without affecting the resulting fluxes. These reactions are assumed to be at steady state, as well as the metabolites participating in them. That is the case of the intermediate metabolites that have only one route of generation and one route of consumption, for example intermediate metabolites for amino acids biosynthesis.
ii. ATP and ADP were considered the only energy transporters. Pyrophosphate was interpreted as two single phosphates.

iii. Only NADH/NAD$^+$ were considered as cofactors for fueling and biosynthetic reactions, because it was supposed the action of nicotidamide nucleotide transhydrogenase, so NADH/NAD$^+$ are assumed to be equivalent to NADPH/NADP$^+$.

iv. Irreversibility was included as a constraint in both stoichiometric models. Reactions involved in consumption or production of ATP (stated according to the thermodynamically feasible direction), were considered as irreversible, substrate consumption reaction were restricted to the assimilation direction, and production of biomass components were stated in the synthesis direction.

v. Metabolites that are freely interconvertible were represented as a single metabolite. For example DHAP and G3P were both represented as G3P in the models.

vi. The EPS formation flux by \textit{L. ferriphilum} is equal to the EPS degradation flux by \textit{F. acidiphilum} because it is assumed that the operation is in steady state.

vii. EPS were expressed as glucose-6-phosphate in both models.

\textbf{3.4.4. Flux Balance Analysis for a Mixed Culture}

The underdetermined system of stoichiometric equations was solved using linear programming through Flux Balance Analysis (FBA) (Varma and Palsson 1993).

A FBA was performed on the simple model of \textit{L. ferriphilum}. The system of equations was solved in order to obtain the unknown fluxes by using as objective function the
maximization of biomass. The constraints of the system were given by the steady state flux balances, from Equation (3.1).

From the stoichiometric model, a standard composition of biomass of *L. ferriphilum* was determined as \( \text{CH}_{1.9}\text{O}_{0.7}\text{N}_{0.2}\text{P}_{0.06}\text{S}_{0.002} \), therefore a molecular weight of 29 GDW per mole of carbon was calculated. This value is the biomass Yield coefficient per carbon, \( Y_{cx} = 29 \left( \frac{\text{GDW}}{\text{mole-C}} \right) \).

Considering a specific growth rate of 0.081 (1/h) (Gahan et al. 2010), the specific carbon uptake rate was calculated with the equation:

\[
q_{\text{CO}_2} = \frac{\mu}{Y_{cx}} \left( \frac{\text{mole}}{\text{GDW-h}} \right)
\]  

(3.7)

Therefore, a specific consumption rate of \( \text{CO}_2 \), \( q_{\text{CO}_2} = 2.75 \) (mmol/GDW/h) was used as input data for a flux balance analysis of *L. ferriphilum*. This analysis showed that the maximization of biomass is achieved for a null flux of glucose through the Leloir pathway for EPS formation, which is very reasonable since this pathway results in a leak of carbon resources. Hence, the system was analyzed including the constraint of EPS formation flux, and also the sensitivity of the objective function with respect to this constraint was studied. This analysis showed that for fluxes greater than 0.5 (mmol glucose/GDW/h) in the Leloir pathway, the system becomes unfeasible.

With the results of the sensitivity analysis, a FBA was executed for the mixed model of *L. ferriphilum* and *F.acidiphilum*, using as input data the consumption rate of \( q_{\text{CO}_2} = 2.75 \) (mmol \( \text{CO}_2 \)/GDW/h) of *L. ferriphilum* and the different fluxes of EPS formation from the sensitivity analysis. In this case, the objective function was the maximization of biomass of
both populations, and the constraints were specified by the steady state mass balances of each stoichiometric model. Under the specified conditions mentioned above, the variation of the specific growth rates of *L. ferriphilum* and *F. acidiphilum* related to the EPS flux are shown in Figure 3.1.

![Figure 3.1](image.png)

**Figure 3.1.** Sensitivity analysis of EPS flux formation in specific growth rates of *L. ferriphilum* and *F. acidiphilum*.

As it was expected, increasing the production flux of EPS in *L. ferriphilum* results in a decrease of its specific growth rate, because more carbon is directed to this purpose and not to biomass production. Likewise, the specific growth rate of *F. acidiphilum* increases the higher the EPS flux, because it has more carbon source available.
A growth rate of 0.016 (h\(^{-1}\)) was assumed for *Ferroplasma acidiphilum* for chemomixotrophic growth (Pivovarova et al. 2002). With this data and Figure 1, an EPS flux distribution of \(q_{\text{EPS}} = 0.127\) (mmol EPS/GDW/h) is obtained. Under this scenario, the metabolic fluxes for both networks were determined through FBA and the resulting fluxes are shown in Figure 3.2.

With this flux distribution a biomass yield on ferrous iron was calculated for both populations, resulting in \(Y_{\text{FeX}} = 0.0016\) (mol-C/mol Fe\(^{2+}\)) for *L. ferrirphilum* and \(Y_{\text{FeX}} = 0.037\) (mol-C/mol Fe\(^{2+}\)) for *F. acidiphilum*, which are very realistic values compared with yields obtained for *L. ferrirphilum* (\(Y_{\text{FeX}} = 0.002\) (mol-C/mol Fe\(^{2+}\)) by Gahan et al. (2010) and for *F. acidiphilum* (\(Y_{\text{FeX}} = 0.043\) (mol-C/mol Fe\(^{2+}\)), considering a composition of 62% of proteins) by Golyshina et al. (2000).

The flux distribution is shown in Figure 3.2. Under the described condition, the only active anaplerotic enzyme in *L. ferrirphilum* and *F. acidiphilum* is phosphoenolpyruvate carboxylase, whereas pyruvate carboxylase turns out to be inactive for optimization purposes.

Little is known about archaeal enzymes involved in pentose conversion. Genes encoding canonical enzymes of the oxidative branch of the pentose phosphate pathway have not been identified in archaea (Makarova et al. 1999; Selkov et al. 1997; She et al. 2001). In this simulation, the operation of the oxidative branch of the pentose phosphate pathway in *F. acidiphilum* is inactive. Therefore, it is proposed that the non-oxidative branch for pentose formation is fed from fructose 6-phosphate of the Embden-Meyerhof pathway by transketolase activity (Verhees et al. 2003).
Figure 3.2. Flux distribution for a mixed culture of *L. ferriphilum* and *F. acidiphilum* in (mmol/GDW/h)
3.4.5. **Flux Balance Analysis of *F. acidiphilum* in pure and mixed culture**

Using as input data the specific consumption rates obtained in the previous simulation, the metabolic model of *F. acidiphilum* was used to simulate growth of this archaea on different substrates.

First of all, the effect of chemoautotrophic growth of *F. acidiphilum* on biomass generation by suppressing the EPS consumption flux on the model was explored, considering CO₂ assimilation through the reductive Acetyl-CoA pathway (Equation 3.8) and ferrous iron oxidation as energy source. In this scenario it was found that biomass formation is not possible, so input of an organic compound such as glucose as carbon source for *F. acidiphilum* is necessary, reaffirming a chemomixotrophic growth as described by Dopson et al (2004).

\[
\text{Methyl-THF} + 2 \text{NADH} + \text{CO}_2 + \text{CoA} + 2 \text{H}^+ \rightarrow \text{Ac-CoA} + 2 \text{NAD}^+ + \text{THF} + \text{H}_2\text{O} \quad (3.8)
\]

It has been shown that *Ferroplasma acidiphilum* requires a small amount of yeast extract for growth. However, it is not clear which component of the yeast extract is being used as growth factor, remaining uncertain if it is a specific amino acid, vitamins or the organic compounds from the yeast extract.

Then, simulations of growth on different substrates were tested in order to get a notion about the substrates from yeast extract that could be used for growth. With this aim, exchange reactions for specific amino acids and sugars were added to the metabolic model. The simulations were performed with FBA by maximization of *F. acidiphilum* biomass production rate as the objective function, and the maximum substrate uptake rate was set
on an arbitrary value of 10 (mmol/GDW/h) for every substrate. The specific growth rates obtained in each simulation are shown in the table below.

Table 3.2. Specific growth rate of *F. acidiphilum* on different substrates estimated by FBA

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Growth Rate (h&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base</td>
<td>0.015</td>
</tr>
<tr>
<td>Fructose-6-phosphate</td>
<td>4.66</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.018</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.018</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.015</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.015</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>0.017</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.015</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.015</td>
</tr>
<tr>
<td>Serine</td>
<td>0.537</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.015</td>
</tr>
<tr>
<td>Valine</td>
<td>0.015</td>
</tr>
</tbody>
</table>

All simulations were carried out considering an input flux of EPS, expressed as glucose, of 0.127 (mmol EPS/GDW/h), because it was observed that in the absence of this input, biomass generation is not possible.

It was assumed that the addition of cysteine or methionine as organic sulphur sources could increase the specific growth rate of *F. acidiphilum*; however, according to the estimations shown in Table 3.2, the addition of these amino acids, as well as asparagine, phenylalanine, valine and tyrosine do not cause any increase in cell growth.
Other substrates such as glutamic acid, glutamine and aspartic acid cause a slight rise in specific growth rate, because these amino acids are connected with the biosynthesis of important precursors of building blocks like oxaloacetate and α-ketoglutarate, and also aspartic acid is related to the biosynthetic pathways of nucleotides and other amino acids.

An important increase in specific growth rate is observed with serine, which is related to biosynthesis of purines and pyrimidines and with the synthesis of tryptophane, cysteine and glycine. It is also involved in the metabolism of tetrahydrofolate, which acts as a coenzyme transporting carbon groups.

The most significant rise in growth rate is obtained with fructose-6-phosphate as an additional carbon organic source to glucose, which is logical since these are the only carbon sources for biomass formation. However, it is important to note that the metabolic model does not consider substrate inhibition effects; therefore, in the simulations, the specific growth rate of biomass will be higher the larger the specific sugar consumption rate.

The exchange of metabolites between both species was explored. For this purpose, transport reactions of several amino acids (cysteine, glutamate, glutamine, asparagine, aspartate, methionine, phenylalanine, serine, tyrosine and valine) were included in both stoichiometric models, and the exchange fluxes were unconstrained, so free fluxes of these amino acids between the microorganisms was tested by FBA. It was observed that the calculated exchange fluxes are very small and do not affect considerably the growth rate of \textit{L. ferriphilum} and \textit{F. acidiphilum}, obtaining a specific growth rate of 0.018 (h$^{-1}$) and 0.014 (h$^{-1}$) for \textit{L. ferriphilum} and \textit{F. acidiphilum} correspondingly.
Assuming that *F. acidiphilum* grows using the biological matter generated by *L. ferriphilum*, amino acids exchange fluxes were tested only in one direction, secreted from *L. ferriphilum* and consumed by *F. acidiphilum*. In this case, the FBA predicts that the only amino acids that could be exchanged are glutamate, glutamine and serine, causing a slight increase in the growth rate of *F. acidiphilum* to 0.015 (h\(^{-1}\)) in the case of glutamate and glutamine, and 0.019 (h\(^{-1}\)) for serine, and a decrease in the specific growth rate of *L. ferriphilum* to 0.015 (h\(^{-1}\)) and 0.012 (h\(^{-1}\)) respectively.

### 3.4.6. Knockout Simulations

To simulate enzyme knockouts, the associated reaction to the enzyme was constrained by setting the upper and lower bounds of the reaction to 0 (mmol/GDW/h). The enzyme knockouts tested corresponded to main enzymes involved in central metabolic pathways such as glycolysis/gluconeogenesis, pentose phosphate pathway and TCA (or reductive TCA cycle for *L. ferriphilum*), anaplerotic reactions and respiration.

Knockout simulations were carried out for each model with FBA methodology, using as input data the consumption rates of carbon and energy sources (\(q_{CO2}\) and \(q_{Fe2+}\) for *L. ferriphilum*, \(q_{EPS}\) and \(q_{Fe2+}\) for *F. acidiphilum*) to a maximum value of 10 (mmol/GDW/h). The optimal value of the objective function for each deletion or “mutant” was evaluated and compared with the “wild type” value to determine the effect of the enzyme deletion on the optimal growth rate (Edwards and Palsson 2000). The ratio between the objective function with the enzyme deletion (\(Z_{mut}\)) and the wild type (\(Z_{wt}\)) were calculated and plotted as shown in Figures 3.3 and 3.4 for *L. ferriphilum* and *F. acidiphilum* respectively.
According to Figure 3.3, the action of enzymes PEPS, ENO and PGM, which participate in the Embden-Meyerhof-Parnas pathway, are essential for cell growth. PEPS catalyzes the anabolic conversion of pyruvate to phosphoenolpyruvate, and therefore defines the anabolic characteristic of the pathway, allowing the generation of glucose from CO\textsubscript{2}. ENO and PGM catalyze the formation from phosphoenolpyruvate of glyceraldehyde 3-phosphate, which is an important intermediate that participates in the pentose phosphate pathway and lipid generation.
Under the studied conditions, all the enzymes of the reductive TCA cycle (FUM, MDH, FDR, SUC, OGOR, IDH, ACO, CCS, CCL, POR) are found to be necessary for growth, because this pathway is the only way to fix CO₂ for *L. ferrophilum*. Experimentally, four enzymes of this cycle are described to be essential and make possible the reversal behavior of this cycle: FDR, OGOR, CCS, CCL and POR (Campbell and Cary 2004; Hugler et al. 2003; Levican et al. 2008).

From the pentose phosphate pathway, only the deletion of RPI was found to be lethal for cell growth. This enzyme is the only source of D-Ribose 5-phosphate formation, which is precursor of 5-phosphoribosyl-1-pyrophosphate (prpp) from which nucleotides and several amino acids are generated.

Respiration enzymes were symbolized by REDCYT as the ferrous oxidizer, CYTcbb3 as the electron transporter from ferrous iron to O₂, and NDH for the formation of reductive power by the reverse electron transfer from ferrous iron to NDH ubiquinone. These three enzymes, as well as ATP synthetase, result to be indispensable for growth, because ferrous iron is the only source of energy and reducing power on this bacterium, and the exchange of protons derived from this process, executed by ATP synthetase, is the main source of ATP for the cell.

The other enzymes studied correspond to anaplerotic reactions PC and PEPC whose deletion does not affect cell growth because they compensate the knockout of each other. The rest are enzymes that catalyze reactions in the pentose phosphate pathway, whose removal results in a smaller growth rate, so other reactions could compensate their knockout.
Several differences between both microorganisms appear when the results of the previous analysis are compared. For *F. acidiphilum* more enzymes of glycolysis are necessary for growth, like GAP, PGK, ENO, PGM, PYK and PFK because this pathway is its only way to obtain energy from organic carbon sources. Of these enzymes, PFK and PYK have been described as key enzymes of this pathway (Verhees et al. 2003). Moreover, respiration
enzymes for reductive power and ATP generation (NDH and ATP) appear to be essential, however the knockout of enzymes involved in ferrous iron oxidation (symbolized by BLUECYT for iron oxidation and UBIQ for electron transport) results in a slight decrease in cell growth of about 6%, due to the chemoorganotrophic capacities of this microorganism, so it could obtain energy from organic carbon metabolism by glycolysis.

It is also observed that almost all TCA cycle enzymes are essential except SUC, because there is an alternative reaction to generate succinate from lysine synthesis from aspartate, which compensates the knockout of SUC, thus supporting cell growth. If both reactions are deleted, growth is not possible for this archaea.

From this analysis, it is possible to observe that the growth predictions restricted to enzyme deletions are consistent with literature information about the key enzymes for growth in central metabolism of both microorganisms, showing a good performance of the metabolic models.
3.5. CONCLUSIONS

A metabolic model for a mixed culture composed by *L. ferriphilum* and *F. acidiphilum* was reconstructed from literature and data base information. With this model and a Flux Balance Analysis methodology, the biomass yields for both populations growing on ferrous iron were estimated, obtaining very reasonable values.

A metabolic model of *L. ferriphilum* was developed based on a stoichiometric model of similar specie, *Leptospirillum ferrooxidans*, previously elaborated. For this end, the model was modified considering the specific metabolic differences between these species described in literature data.

Specific features of *Ferroplasma acidiphilum* metabolism were included in the metabolic model taken from literature data, for instance the chemomixotrophic capacity of this archaea to grow on ferrous iron and also organic carbon as energy sources. Specifically, it was simulated that *F. acidiphilum* can use the EPS secreted by *L. ferriphilum*, and ferrous iron from culture media for growth.

Cell growth of *F. acidiphilum* on different substrates was also tested, obtaining specific growth rates for each one. With this methodology, it is possible to obtain a notion about useful substrates that could maximize cell growth, and then design and suggest an appropriate composition of the culture media.

From knockout enzyme simulations it was possible to determine the essential enzymes for growth, and therefore, predict which enzymes could be removed without affecting (or only slightly affecting) cell growth. From this analysis, most of the key enzymes predicted were
consistent with experimental literature data information, indicating a good performance of both metabolic models.

This work describes the first metabolic model available for a mixed culture of *L. ferriphilum* and *F. acidiphilum*. This first approach allowed obtaining a deeper understanding of the metabolism of these microorganisms growing together, such as their main interactions and metabolites exchange.

Further experimental work will be necessary to fully address the predictive capacity of the model. More extensive studies of these microorganisms growing alone and together are required to evaluate the predicted values and determine the composition of the culture media which enables the maximum growth rate of both species, and therefore provide new strategies to enhance and optimize productivity on bioleaching processes.
CHAPTER 4

Characterization of *Ferroplasma acidiphilum* growing in pure and mixed culture with *Leptospirillum ferriphilum*
4.1. ABSTRACT

Bioleaching metal recovery from sulfide minerals is promoted by the concerted effort of a consortium of acidophile prokaryotes, comprised by members of the Bacteria and Archaea domains. *Ferroplasma acidiphilum* and *Leptospirillum ferriphilum* are two of the dominant species in extremely acid environments and have great potential in bioleaching applications (Okibe and Johnson 2004; Rawlings et al. 1999).

*Ferroplasma acidiphilum* (BRL-115) is able to grow chemoorganotrophically with yeast extract or another organic carbon source, and chemomixotrophically using a carbon source or yeast extract as growth factor, and ferrous iron as electron donor. The effect of different substrates on growth was tested and compared with a condition supplemented only with yeast extract. It was observed that *F. acidiphilum* is able to grow with sucrose, cysteine and casamino acids chemoorganotrophically and on the same substrates for chemomixotrophic growth with ferrous iron, but cell growth is lower compared to the condition supplemented with only yeast extract at a concentration of 0.04%.

From the experiments of *L. ferriphilum* growing on *F. acidiphilum* supernatant and vice versa, it was observed that in both cases cell growth is favorably affected by the presence of the filtered medium of the other microorganism, assuming a synergistic interaction between these species.

The experimental results obtained in this chapter are consistent with the simulations performed with the metabolic models of *L. ferriphilum* and *F. acidiphilum* previously developed by our group, obtaining a reasonable behavior of the single and mixed models.
4.2. INTRODUCTION

The ability of certain microbes to solubilize metals from ores has given rise to the bioleaching technology, which is the use for commercial purposes of microbes to extract metals from ores, by harnessing their natural oxidative dissolution capacity (Rawlings 2002). The use of microbes in ore processing holds environmental advantages over the traditional physicochemical methods, because bioleaching processes do not require the high amounts of energy used for roasting and smelting, and do not produce dangerous gaseous emissions. Thus, microbial technology could be used for metal recovery from low-grade ores and dumps which are not economically profitable to treat by traditional chemical methods.

Bioleaching processes occur in extremely acidic environments, and at elevated concentrations of soluble metals and metalloids, therefore microorganisms metabolically active in such processes are extremophiles, highly acidophilic, tolerant to dissolved metals, and generally thermophilic (Okibe and Johnson 2004). Prokaryotic acidophiles from eubacteria and archaea domains, have been the focus of much of the research activity in this area, primarily because of the importance of these microorganisms in commercial biological processing of metal ores, and in environmental generation of acid mine drainage (AMD) (Johnson 1998).

Acidophilic microorganisms exist as mixed populations in bioleaching environments, where community-essential roles are distributed between the different species involved. Extremely acidic environments could be classified as oligotrophic because of the low concentration of organic carbon, therefore autotrophic, heterotrophic and mixotrophic
species can be found in these locations. Autotrophic organisms are able to assimilate CO$_2$ from the air-water interface, heterotrophic organisms use the organic matter originating from lysis products or secreted products from the chemolithotrophic acidophiles. Chemomixotrophic organisms are described as capable of growth with organic carbon and an inorganic electron donor, such as ferrous iron or inorganic sulfur compounds (Dopson et al. 2004).

This work is focused on the metabolic characteristics of two dominant species of microbial communities in AMD (Tyson et al. 2004), *Leptospirillum ferriphilum* and *Ferroplasma acidiphilum*.

*L. ferriphilum* is described as a chemolithoautotrophic organism. It uses ferrous iron as electron donor and CO$_2$ and NH$_4^+$ as carbon and nitrogen sources (Levican et al. 2008). On the other hand, *Ferroplasma acidiphilum* has been described as capable of growth either with organic carbon as electron donor and carbon source (chemoorganotrophic) and ferrous iron as energy source plus yeast extract as organic carbon source respectively (chemomixotrophic) (Dopson et al. 2004).

In this chapter, an experimental metabolic characterization of *F. acidiphilum* strain BRL-115 is developed to evaluate the stoichiometric mixed model detailed in Chapter 3. Also, a Flux Balance Analysis with experimental data is performed in order to predict the metabolic behavior of the mixed culture.
4.3. MATERIALS AND METHODS

4.3.1. Isolated strains

BRL-115 and BRL-111 strains were used in this study, provided by BioSigma S.A. A DGGE analysis revealed that these strains belong to *Ferroplasma acidiphilum* and *Leptospirillum ferriphilum* species respectively.

4.3.2. Growth conditions

Strains were grown in KMD medium, a variation of medium 9KF described in the literature (Silverman and Lundgren 1959). The composition of medium KMD, in grams per liter is as follows: \((\text{NH}_4)_2\text{SO}_4\), 0.99; \(\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}\), 0.1275; \(\text{MgSO}_4\cdot7\text{H}_2\text{O}\), 0.10; \(\text{KH}_2\text{PO}_4\), 0.0525 and \(\text{CaCl}_2\cdot2\text{H}_2\text{O}\), 0.021. For all the experiments, KMD medium was adjusted to pH 1.4 with \(\text{H}_2\text{SO}_4\), and autoclaved. Different substrates were added for the characterization. Each substrate were previously filtered (0.22 µm Millipore filter) to maintain sterile conditions. Experiments were inoculated with 10% (vol/vol) of viable cell mass. For mixed cultures, biomass concentration of the species in fresh inoculums was estimated by cell counting under a microscope, in order to add similar initial number of cells per ml of each strain. Experiments of growth of *F. acidiphilum* under different substrates and concentrations of yeast extract were done in 250 (ml) flasks, incubated at 30°C with agitation of 250 rpm. For the studies of growth of *L. ferriphilum* on *F. acidiphilum* supernatant and vice versa, an equivalent method was used in 500 (ml) flasks. The determination of growth parameters in pure cultures of *F. acidiphilum* and mixed cultures with *L. ferriphilum* were done in a 1 (L) bubble column reactor operated in batch cyclic mode at 30°C.
4.3.3. **Protein concentration**

2 (ml) of culture sample were centrifuged at 4°C and 8000 rpm for 15 minutes to separate the cells. The cell pellet was washed and resuspended in 1 (ml) of KMD medium at pH 1.4 twice. After the second wash step, cells were suspended in 200 µl of Phosphate Buffered Saline (PBS) (Composition in grams per liter: NaCl, 9.91; KCl, 0.291; K₂HPO₄, 1.591; KH₂PO₄, 0.249). The proteins from the resuspended pellet were extracted by ultrasound application, with a rod sonicator applying 4 pulses of 1 minute at 50% of power, with 1 minute breaks between each pulse. Then, the solution was centrifuged at 4°C and 14.000 rpm for 20 minutes, and the supernatant was stored at -80°C for protein quantification with the enhanced protocol of Pierce® BCA Protein Assay Kit (Smith et al. 1985).

4.3.4. **Ferrous iron concentration**

Fe²⁺ in solution was determined by the o-phenantroline method (Kolthoff and Sandell 1963) and total iron concentration was measured by atomic absorption spectroscopy (Perkin-Elmer Instruments, AAnalyst 400).

4.3.5. **Growth of *F. acidiphilum* with different concentrations of yeast extract and sucrose**

KMD medium was supplemented with different concentrations of yeast extract, and with yeast extract and sucrose, to determine the optimal concentration of yeast extract, and to evaluate the effect of an alternative organic carbon source on growth. The explored conditions were as follow: 0%; 0.005%; 0.01%; 0.02%; 0.03%; 0.04%; 0.06%; 0.08% and 0.1% (wt/vol) of yeast extract, and one condition with 0.04% (wt/vol) of yeast extract supplemented with 0.02% (wt/vol) of sucrose. The cultures were incubated for 400 h and
biomass concentration was determined by total protein concentration (method described above).

4.3.6. **Growth of *F. acidiphilum* on different substrates**

With the purpose of verifying chemoorganotrophic and chemomixotrophic growth of *F. acidiphilum*, experiments with different organic and inorganic substrates were carried out. The evaluated conditions were: 0.1% Casamino Acids, 0.01% L-cysteine and 0.02% sucrose. Every condition was evaluated with and without the addition of ferrous iron at a concentration of 45 (g/L) of FeSO$_4$·7 H$_2$O. Biomass concentration was determined by protein concentration, ferrous iron oxidation was estimated by Eh measurement and ferrous iron concentration was quantified through the *o*-phenanthroline method described previously.

4.3.7. **Growth of *L. ferriphilum* on *F. acidiphilum* supernatant and growth of *F. acidiphilum* on *L. ferriphilum* supernatant**

The hypothesis of these experiments is that *F. acidiphilum* uses the organic matter secreted by *L. ferriphilum* (EPS) for growth, maintaining low levels of organic compounds in the culture medium, preventing their toxic effects on *L. ferriphilum*. To test this hypothesis, two assays were done with *L. ferriphilum* on *F. acidiphilum* supernatant and vice versa. To this end, a fresh inoculum of *L. ferriphilum* was grown in 9KMD medium (basic salts KMD with 9 (g/L) of ferrous iron) until the Eh of the culture reached 650 mV, therefore, ferrous iron was completely oxidized. Subsequently the culture medium was filtered using a 0.22 µm Millipore filter in sterile conditions, and was supplemented with ferrous iron (9 g/L), fresh KMD medium, the pH was adjusted to 1.4, and was inoculated with a fresh culture of *F. acidiphilum*. For *F. acidiphilum* growing on
*L. ferriphilum* supernatant, an equivalent method was used. Control conditions were tested in both cases.

### 4.3.8. Determination of growth parameters in a bioreactor

Three different conditions were explored. In the first phase of this experiment, a culture of *F. acidiphilum* growing on 0.04% of yeast extract and 9 (g/L) of ferrous iron in batch cyclic mode was performed. Batch cyclic operation means that 500 (ml) of culture were replaced by the same volume of fresh medium every time the Eh exceeded 600 mV. This process was continued until the culture was synchronized. At this point, it is assumed that the culture is at steady state, and thus growth parameters are measured. In the second phase of this experiment, the replacement medium was KMD supplemented with 0.04% of yeast extract, 9 (g/L) of ferrous iron and 0.02% of sucrose. The same procedure as mentioned above was used, until the culture entered steady state and growth parameters were measured.

Finally, growth parameters were calculated for a *F. acidiphilum* and *L. ferriphilum* mixed culture with KMD, supplemented only with 9 (g/L) of ferrous iron.

### 4.3.9. Modeling and Simulation Software

The metabolic model was implemented in the software INSILICO Discovery 3.2 (Stuttgart, Germany, [www.insilico-biotechnology.com](http://www.insilico-biotechnology.com)). With this computational platform, a graphical representation of the network was obtained, mass and charge balance of the system were checked, and topological and mathematical analysis were performed to determine the internal fluxes of the metabolic network.
4.4. RESULTS

4.4.1. Optimal concentration of yeast extract for *F. acidiphilum* strain BRL-115.

In Figure 4.1 the growth curves for different concentrations of yeast extract are shown. For biomass estimation it was considered that the protein composition of BRL-115 is 63% of biomass, and the weight of one cell is $10^{-13}$ (g/cell). Experimental data was fitted through a logistic model of growth (Zanoni et al. 1993).

It is possible to observe that the condition which maximizes growth is the one supplemented with 0.04% (wt/vol) of yeast extract and 0.02% (wt/vol) of sucrose, which indicates that BRL-115 is able to grow chemoorganotrophically using sucrose as carbon source, and yeast extract as growth factor. For conditions supplemented only with yeast extract, growth is maximized at a concentration of 0.04% (wt/vol) of this substrate.
Figure 4.1. Growth curve of strain BRL-115 under different concentrations of Yeast Extract on KMD medium, pH 1.4 and 30°C of temperature. Experimental data and fitted curves are shown. ★, 0% YE; ●, 0.02% YE; ◇, 0.03% YE; ▽, 0.04% YE; ■, 0.04% YE+0.02% Suc; ▲, 0.05% YE; ○, 0.06% YE; ✱, 0.1% YE.
In Figure 4.2 is shown the effect of yeast extract on growth.

![Graph showing the effect of yeast extract on biomass growth of BRL-115](image)

**Figure 4.2.** Effect of yeast extract and sucrose addition on biomass growth of BRL-115, on KMD medium, pH 1.4 and 30°C of temperature. Protein concentration values are the difference between the final protein concentration, after 250 hours of growth, and the initial concentration.

As mentioned above, maximum growth is achieved with 0.04% of yeast extract and 0.02% of sucrose. In the absence of an organic carbon source, BRL-115 uses only yeast extract as energy source. For concentrations higher than 0.04% of yeast extract, a decrease in biomass production is observed, possibly because of the presence of extracellular metabolites in inhibitory concentrations in the growth medium. Particularly, the presence of organic acids from yeast extract, which at this low pH (1.4) are not dissociated and thus they could diffuse through the plasmatic membrane, causing cellular lysis.
4.4.2. *Growth of F. acidiphilum on different substrates*

With the purpose of determining which component of yeast extract is essential for growth, the effect of different substrates on growth of BRL-115 was tested, and the results are shown in Figure 4.3. From these results it is concluded that neither organic sulfur from cysteine, nor organic carbon from sucrose, or organic nitrogen from casamino acids are key substrates. These three conditions were analyzed with the addition of ferrous iron as energy source, but growth was not significantly higher. For all the conditions studied, growth was considerably lower than the culture supplemented only with yeast extract. Vitamins as supplements for growth were not studied, however according to Golyshina (2000) with these substrates, a lower yield of biomass is achieved compared with yeast extract.

![Figure 4.3. Effect of different substrates on *F. acidiphilum* growth](image)

**Figure 4.3.** Effect of different substrates on *F. acidiphilum* growth

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4.4.3. **Growth of L. ferriphilum on F. acidiphilum supernatant**

Figure 4.4 shows that *L. ferriphilum* growing on supernatant of *F. acidiphilum* achieves the same final biomass concentration as in the control condition. However, the specific growth rate for the non control condition is higher (0.065 (h$^{-1}$) compared with 0.037(h$^{-1}$) for the control). It is possible that this result is due to the extracellular proteins secreted by *F. acidiphilum* that are present in its supernatant. These proteins could enhance the interaction with ferrous iron or facilitate substrate transport or electron transport chain in this microorganism. This phenomenon was observed in other biomining microorganisms such as *A. thiooxidans* (Babadilla et al. 2010) and *A. ferrooxidans* (Chi et al. 2007).

![Figure 4.4. Growth curves of L. ferriphilum. □, L. ferriphilum growing on filtered supernatant of F.acidiphilum at pH 1.4, supplemented with KMD and 9 (g/L) of Fe(II); ●, control (KMD medium supplemented with 9(g/L) of Fe(II))](image-url)
4.4.4. Growth of *F. acidiphilum* on *L. ferriphilum* supernatant

In this case it is important to note that the filtered supernatant of *L. ferriphilum* was supplemented only with KMD salts and ferrous iron, no yeast extract was added to the non control condition in order to analyze the effect of organic components of the supernatant on *F. acidiphilum* growth.

Figure 4.5 shows the growth curves for the studied conditions. With the control condition, a higher final biomass concentration is reached (near to $1 \cdot 10^9$ (cells/ml)), with a specific growth rate of 0.133 (h$^{-1}$). For the non control condition, a final biomass concentration of about $1 \cdot 10^8$ (cells/ml) is achieved with a specific growth rate of 0.039 (h$^{-1}$). Despite the lower biomass growth in the non control condition, it is possible to conclude that this archaea uses the organic compounds present in *L. ferriphilum* supernatant, because it was observed previosuly that *F. acidiphilum* is not capable of growth only with ferrous iron as energy source (data not shown).

![Figure 4.5. Growth curves of *F. acidiphilum*. □, *F. acidiphilum* growing on filtered supernatant of *L. ferriphilum* at pH 1.4, supplemented with KMD and 9 (g/L) of Fe(II); ●, control (KMD medium supplemented with 9(g/L) of Fe(II) and 0.04% of yeast extract )](image-url)
4.4.5. **Determination of growth parameters in a bioreactor operated in batch cyclic mode**

For pure and mixed cultures, a batch cyclic methodology was used (detailed in materials and methods).

For *F. acidiphilum* growing with 0.04% of yeast extract, a synchronization of the culture was attained at 800 hour of operation. In the synchronized culture, all ferrous iron was oxidized within 48 hours. In Figure 4.6 the exponential growth and ferrous iron consumption curves obtained after synchronization are shown.

![Figure 4.6](image)

**Figure 4.6.** ●, exponential growth curve of *F. acidiphilum* on KMD medium supplemented with 9 (g/L) of ferrous iron and 0.04% of yeast extract, on batch cyclic operation; ▽, ferrous iron consumption curve.
After the first synchronization of the culture growing on Fe\(^{2+}\) and yeast extract, the reactor was fed with a fresh medium also supplemented with sucrose. Under these conditions, after 300 hours, the culture was synchronized again and thus remained at steady state, so new parameters were measured. The results of biomass growth and Fe\(^{2+}\) consumption are shown in Figure 4.7.

![Graph showing biomass concentration and Fe\(^{2+}\) consumption over time.](image)

**Figure 4.7.** ●, exponential growth curve of *F. acidiphilum* on KMD medium supplemented with 9 (g/L) of ferrous iron and 0.04% of yeast extract and 0.02% of sucrose on batch cyclic operation; ▽, ferrous iron consumption curve.

In addition, *F. acidiphilum* and *L. ferriphilum* were grown in mixed culture in batch cyclic operation. The synchronization of the culture was achieved after 150 hours of operation. The growth curve at steady state and ferrous iron consumption were determined, and are shown in Figure 4.8.
Specific reaction rates

With bioreactor growth data it is possible to calculate the specific rates, which are the measured fluxes that will be used as input data for the estimation of the flux distribution of the metabolic network. The specific rate of substrate consumption or product formation is given by the equations:

\[
    r_S = -\frac{1}{X} \frac{dC_S}{dt} \quad \text{and} \quad r_P = \frac{1}{X} \frac{dC_P}{dt}
\]  \hspace{1cm} (4.1)

Where \( r_S \) and \( r_P \) are the specific rates of substrate consumption and product synthesis respectively, \( X \) is cellular concentration and \( C_S \) and \( C_P \) are substrate and product concentration respectively. If sufficient measurements of cellular concentration, substrates and products are available, their time curves could be fitted to a polynomial expression.

Figure 4.8. ●, exponential growth curve of \( F. \ acidophilum \) and \( L. \ ferrophilum \) on KMD medium supplemented with 9 (g/L) of ferrous iron on batch cyclic operation; ▼, ferrous iron consumption curve
Therefore, Equation (4.1) could be used directly for the specific rates calculation (Papoutsakis and Meyer 1985). If there are not sufficient experimental measurements to fit the curves, the rates are calculated considering two consecutive points in time, doing an approximation to linear behavior. Then, Equation (4.1) is rearranged as:

\[ r_S = -\frac{1}{X} \frac{\Delta C_S}{\Delta t} \quad \text{and} \quad r_P = \frac{1}{X} \frac{\Delta C_P}{\Delta t} \]  

(4.2)

With these equations, the consumption of ferrous iron was calculated and the results are shown in Table 4.1.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>( \mu_{\text{max}} ) (h(^{-1}))</th>
<th>Yield (GDW/mol Fe(^{2+}))</th>
<th>qFe(^{2+}) (gFe(^{2+})/GDW*h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( F.\ acidiphilum + YE + Fe^{2+} )</td>
<td>0.027</td>
<td>16.60</td>
<td>0.78</td>
</tr>
<tr>
<td>( F.\ acidiphilum + YE + Sucrose + Fe^{2+} )</td>
<td>0.043</td>
<td>12.28</td>
<td>0.78</td>
</tr>
<tr>
<td>( F.\ acidiphilum + L.\ ferrifilum + Fe^{2+} )</td>
<td>0.073</td>
<td>0.72</td>
<td>4.52</td>
</tr>
</tbody>
</table>

Assuming that the EPS formation depends only on cell growth, the specific formation rate of EPS on \( L.\ ferrifilum \) could be defined as:

\[ q_{\text{EPS}} = f_{\text{EPS}} \cdot \mu \left( \frac{\text{\text{gEPS}}}{\text{GDW-h}} \right) \]  

(4.3)

For the iron oxidizing bacteria \( A.\ ferrooxidans \) growing on ferrous iron a value of 250 ± 30 (µg EPS/10\(^{10}\) cells) was experimentally obtained (Gehrke et al. 1998). Considering a cell weight of 10\(^{-13}\) (g/cell) and a composition of 75% water in the cell, a value of \( f_{\text{EPS}} = 0.86 \) (gEPS/GDW) is obtained. Assuming a similar production of EPS on \( L.\ ferrifilum \)
growing on ferrous iron, and a specific growth rate of 0.037 (h\(^{-1}\)) for this bacteria (from experimental data of section 4.4.3) an EPS specific production rate of 

\[ q_{EPS} = 0.03 \left( \frac{g_{EPS}}{GDW \cdot h} \right) \]

is calculated.

A simulation with the metabolic model of the mixed culture was made using as input data the calculated EPS specific production rate of \( L. \) _ferriphilum_, and the global specific consumption rate of ferrous iron of \( q_{Fe^{2+}} \) obtained experimentally for the mixed culture (Table 4.1). The simulation was developed with FBA methodology, using as objective function the growth maximization of both microorganisms. The constraints of the linear equations system are represented by the mass balances of the metabolites at steady state.

With this simulation, a specific consumption rate of ferrous iron for \( L. \) _ferriphilum_ of

\[ q_{Fe^{2+}Lf} = -4.32 \left( \frac{g_{Fe^{2+}}}{GDW \cdot h} \right) \]

was obtained. As expected, this value is much higher than the obtained for \( F. \) _acidiphilum_ of \( q_{Fe^{2+}Fa} = -0.18 \left( \frac{g_{Fe^{2+}}}{GDW \cdot h} \right) \), because \( L. \) _ferriphilum_ requires ferrous iron for growth, unlike \( F. \) _acidiphilum_, which can use the EPS as energy and carbon source, and only oxidize ferrous iron as an extra source of energy.

It is observed in the flux distribution of the simultaneous system that the specific consumption rate of \( L. \) _ferriphilum_ is \( q_{CO_{2}Lf} = -0.34 \left( \frac{g_{CO_{2}}}{GDW \cdot h} \right) \), and the majority of CO\(_2\) for growth is obtained from the external medium, and only a small amount is exchanged from \( F. \) _acidiphilum_ production (\( q_{CO_{2}Fa} = 0.03 \left( \frac{g_{CO_{2}}}{GDW \cdot h} \right) \)).

Growth under anaerobic conditions was also explored, and showed that the mixed culture could not grow in the absence of oxygen. The same results were obtained in the absence of
ferrous iron for the whole system, because ferrous iron is indispensable for growth of *L. ferriphilum*, which at the same time is essential for growth of *F. acidiphilum* in this scenario, because of the exchange of EPS.

With the previous analysis, it has been possible to evaluate the metabolic model for the mixed culture composed of *L. ferriphilum* and *F. acidiphilum*. The usefulness of the developed model is that it allows exploring the metabolic capabilities of these microorganisms in specific conditions for a determined goal, and thereby defining methodologies to achieve the optimization purposes, like a genetic manipulation or a suitable composition of the culture media.
Figure 4.9. Flux distribution for a mixed culture of *L. ferriphilum* and *F. acidiphilum* in (g/GDW/h)
4.5. CONCLUSIONS

With the results obtained for the metabolic characterization of *Ferroplasma acidiphilum* BRL-115 it was observed that this strain requires yeast extract for growth, and it is able to exploit it as a carbon source or as a growth factor if it is in the presence of another energy source such as ferrous iron or sugars.

If only organic sulfur, nitrogen or carbon is added to the culture medium, there is not a significant increase in growth. The effect of vitamins or other single amino acids like serine on growth remains unclear. From the experimental results, it is concluded that this strain takes advantage of the different components of the yeast extract for growth. Therefore, *Ferroplasma acidiphilum* is a chemomixotrophic organism, because it can use inorganic and organic energy sources, and it always requires the presence of yeast extract in small concentrations (0.04% wt/vol).

From the experiments of *L. ferrirphilum* growing in *F. acidiphilum* supernatant it was observed that this condition yields a higher growth than the control condition, which can be attributed to the presence of extracellular proteins of the "secretome" of *F. acidiphilum* that favor the growth of *L. ferrirphilum*. Moreover, in the case of *F. acidiphilum* growing on supernatant of *L. ferrirphilum*, a lower growth was observed in comparison to the control condition, indicating that although growth is not maximized, *F. acidiphilum* would be using the EPS produced by *L. ferrirphilum* as a source of organic carbon.

Growth parameters for a cyclic batch reactor in pure culture of *F. acidiphilum* with yeast extract and Fe$^{2+}$; yeast extract, sucrose and Fe$^{2+}$; and in mixed culture with *Leptospirillum* sp. in medium supplemented only with Fe$^{2+}$ were determined (Table 4.1). It is observed
that *F. acidiphilum* reaches a higher specific growth rate with yeast extract, sucrose and Fe$^{2+}$ in comparison to the sucrose condition, as it has an available organic energy source.

The results obtained from the experiments in batch cyclic mode of the mixed culture were used as input for a simulation on the simultaneous metabolic model, obtaining a reasonable behavior of the metabolic fluxes and the system as a whole.

The metabolic model developed for the mixed culture was assessed by FBA using as objective function the biomass maximization. It is important to note that such models can be used for different purposes through an appropriate definition of the objective function and the constraints of the system, and using a suitable set of experimental data. For example, it is possible to determine the flux distribution for an optimal use of resources such as nutrients, ATP, redox potential, or more specific, to increase the leaching of minerals through the maximization of bacterial oxidation ferrous ion. Therefore, with this tool it is possible to postulate different strategies to optimize the yield on ferrous iron, like genetic manipulation, different composition of culture medium or by the addition of chemicals which influence the enzymatic activity by blocking a specific reaction or enhancing its velocity.
CHAPTER 5

Use of *Leptospirillum ferrooxidans* model: a practical example
In this section, a practical application of the *L. ferrooxidans* model is shown. For this purpose, the model developed in Chapter 2 was slightly modified according to the results obtained. Two changes were made:

- The nitrogenase complex remains inactive because of the high energy cost that its action involves, so nitrogen is assimilated as ammonia by the GDH pathway.
- The TCA cycle was included in a reductive direction to fix CO$_2$.

Accordingly, the model has 2 degrees of freedom, so the specific consumption rate of $q_{CO_2} = 0.04$ (mol/C-mol·h) and $q_{O_2} = 1.12$ (mol/C-mol·h) (values taken from Chapter 2) were used as input data for MFA, obtaining a determined and observable system. The resulting metabolic distribution of this analysis is considered as the base case.

Furthermore, the metabolic model was analyzed by linear programming using the same specific consumption rate of oxygen, $q_{O_2}$ from Chapter 2, and stating as objective function the maximization of ferrous iron oxidation. The restrictions of the system were given by the mass balance of each metabolite at steady state. Therefore, a flux distribution under optimal conditions was obtained.

Figure 5.1 shows the distribution of metabolic fluxes obtained by solving the system with MFA (upper value) and FBA (inferior value in bold and italics), summarizing the difference in the external and the main internal fluxes of *L. ferrooxidans* obtained with the two analyses.
Figure 5.1. Distribution of metabolic fluxes obtained by MFA (upper values) and FBA (inferior values in bold and italic). Fluxes are expressed in (mol/C-mol·h)
The fluxes obtained with MFA represent what the cell really does under the stated conditions, and the fluxes obtained with FBA correspond to an optimal scenario of maximization of ferrous iron oxidation.

The specific metabolic rates of the optimal solution are markedly higher than the base case. According to these results, it is possible to increase the ferrous iron oxidation about 14 times by ensuring the availability of the key nutrients in the culture media, like \( \text{O}_2 \) and \( \text{CO}_2 \), whose consumption rates rise in the same proportion. Also, the metabolic fluxes of the central metabolism show an increase of 10 orders of magnitude because these pathways must be capable of processing a larger amount of substrate under the optimal conditions. Likewise, the specific growth rate of *L. ferrooxidans* increases from 0.05 (h\(^{-1}\)) to 0.67 (h\(^{-1}\)).

The maximization of biomass was checked to compare the results with the previous analysis, and a similar flux distribution was obtained as in the case of the optimization of ferrous iron oxidation. The reason for this result is that cell growth depends directly on ferrous iron oxidation, since a major ATP and redox potential are produced.

In this particular case, an improved yield of the cell on ferrous iron only depends on the increase of the specific rates of ferrous and key nutrients, so the availability of these substrates in the culture media and the activity of the specific enzymes involved in their processing (respiration enzymes or reverse TCA cycle for \( \text{CO}_2 \) fixation) must be ensured.

It is important to note that this optimization analysis does not consider capacity restrictions of the enzymes; this is limitations of the amount of the enzymes in the cell or activity restrictions, which are important in proposing a genetic modification strategy. However, with this practical example it is possible to observe *in silico* that a simple modification of
the culture medium can guarantee an optimal yield of the cell with a specific substrate, also giving a notion about the magnitude of the fluxes that must be processed for this purpose.

Also, with MFA it is possible to estimate the required increase in the flux of nutrients to reach a specific increase in the flow of iron oxidation. For example, if it is desired to increase the flux of ferrous iron oxidation in 30%, with MFA methodology it was obtained that the fluxes of nutrients must be increased as follows: \( \text{O}_2 \) in 17% and \( \text{CO}_2, \text{NH}_4^+ \) and \( \text{SO}_4^{2-} \) in 16%. With this, it is also obtained an increase of 18% in biomass production.

Therefore, the FBA and MFA analysis are useful tools to propose specific strategies to improve the productivity of the cells. In this case, the methodology to be applied after these mathematical analyses would be:

1. Increase the concentration of nutrients to maximize ferrous iron oxidation according to the mathematical analyses.
2. Measure the improved rates to analyze the effect of the new rates on metabolism.
3. Evaluate the possibility of cloning one or more enzymes in specific pathways to achieve an increase in productivity.

With further experimental information this analysis could be extended to the mixed model developed in Chapter 4, where it can be used also to maximize the oxidation rate of ferrous iron to ferric iron by \textit{L. ferriphilum}, and estimate its implications on the growth of \textit{F. acidiphilum}, allowing prediction of which metabolic pathways can be intervened for this purpose. Therefore, it will be possible to postulate promising strategies to inactivate dispensable routes increase the activity of key metabolic pathways or define an optimal composition of the culture medium.
GENERAL CONCLUSIONS
In this work, a metabolic reconstruction of three bioleaching microorganisms, *Leptospirillum ferrooxidans*, *Leptospirillum ferriphilum* and *Ferroplasma acidiphilum*, was developed. For each one, a metabolic model was built comprising the most important features of their metabolism. Main pathways of central metabolism, biosynthesis of building blocks, macromolecules and biomass, and the transport reactions of nutrients and products, were included in the metabolic models.

For *Leptospirillum ferrooxidans*, the main uptake pathways considered in the metabolic reconstruction were ferrous iron oxidation to gain energy for all anabolic and catabolic processes, CO$_2$ assimilation through the acetyl-CoA reductive pathway, and ammonia assimilation by the GDH pathway. With the metabolic information collected, a stoichiometric model composed of 86 reactions and 2 degrees of freedom was obtained.

To estimate the internal flux distribution, this model was analyzed by Metabolic Flux Analysis (MFA) with experimental data from literature, obtaining an estimation of a growth yield of *L. ferrooxidans* on ferrous iron of $Y_{FeX} = 0.006$ (C-mol/ mol Fe$^{2+}$), showing a very good behavior of the metabolic model compared with literature data. Also, a sensitivity test was executed, obtaining a very stable behavior when a random error in the input data was considered. In conclusion, the metabolic model developed was able to reproduce the main aspects of the metabolic behavior of *L. ferrooxidans*.

Additionally, a stoichiometric model for *Leptospirillum ferriphilum* and *Ferroplasma acidiphilum* was constructed. The three individual models included the main routes of central metabolism of each organism and the particular pathways that differentiate them. The metabolic model of *L. ferriphilum* incorporated certain features that differentiate it
from *L. ferrooxidans*, since they are organisms of the same genus but from different groups. Both are chemolithotrophic iron oxidizers; however *L. ferrooxidans* can fix environmental N\(_2\) and also assimilate NH\(_4^+\), unlike *L. ferriphilum* which has no nitrogenase enzyme complex, and therefore only assimilates NH\(_4^+\) from the culture medium. Also, the CO\(_2\) fixation mechanism differs between the two species, since *L. ferrooxidans* uses the reductive acetyl-CoA pathway and *L. ferroxidans* the reductive TCA cycle. On the other hand, canonical pathways of central metabolism were considered similar in both species due to their phylogenetic proximity.

On the other hand, the metabolic model of *Ferroplasma acidiphilum* differs from the previously mentioned models in several aspects. Since it is a mixotrophic organism, the EMP pathway was included in the catabolic direction; therefore this model considers the use of an organic carbon source and CO\(_2\) generation, unlike *Leptospirillum*, where the EMP pathway runs in an anabolic manner. The TCA cycle was included in the forward direction (oxidative) and not in a reductive way, as in the case of *Leptospirillum*. Likewise, the oxidation of iron was added as an alternative source of energy and not as the sole energy source as in the models of *Leptospirillum*.

Formation reactions of DNA, RNA, proteins and lipids were differentiated according to the specific composition of each species. The formation of lipid pathways is particularly different between the *Leptospirillum* and *F. acidiphilum* models, because is an archaea, so their membrane lipid compositions are quite different compared to bacteria.

With the aforementioned considerations, it was possible to obtain a simple and representative metabolic model of these three fundamental microorganisms.
Subsequently, a simultaneous metabolic model for *Leptospirillum ferriphilum* and *Ferroplasma acidiphilum* was developed. It is comprised of 152 internal reactions and 53 transport reactions, with the aim of simulating their growth and main interactions as a mixed culture.

These two metabolic models were analyzed alone and together in a mixed culture with Flux Balance Analysis (FBA) methodology to estimate internal fluxes and growth yields, and also its robustness was probed with knockouts simulations. With these results it was possible to demonstrate a very good behavior of the models for pure and mixed cultures.

For *F. acidiphilum* growing in pure culture, a series of simulations were carried out in order to determine useful substrates to maximize growth. Some of the results obtained were experimentally probed in the last part of this work, reaffirming the predictions of the metabolic model.

To integrate the metabolic models of *L. ferriphilum* and *F. acidiphilum*, a compartmentalized system was created including two inner compartments corresponding to each microorganism, and a top compartment corresponding to the culture media, which separates the internal from the external space. Transport reactions of nutrients and products were defined in the boundaries of each compartment. The species that are transported from the exterior to the top compartment can be considered as the reactor feed. Similarly, the culture media was separated from each microorganism by the inner compartments or cytoplasmic membranes, through which nutrients and products are transported.

This simultaneous model incorporates the interactions between both microorganisms. Every nutrient entering the system is shared between the species in stoichiometric
proportion. Likewise, the exchange of several compounds between both microorganisms was considered, such as organic carbon from *L. ferriphilum* to *F. acidiphilum*, CO$_2$ from *F. acidiphilum* to *L. ferriphilum* and some amino acids.

Therefore, a metabolic model was obtained for a simultaneous culture capable of simulating the growth in a mixed system where both populations are differentiated according to their macromolecular composition. With this model and a FBA analysis, it was possible to assess the main interactions between the participating species, predict key enzymes for growth and suggest an optimal composition of the culture media for *F. acidiphilum*.

An experimental characterization of *F. acidiphilum* was developed, corroborating that it is a chemomixotrophic archaea which is capable to grow either with organic carbon sources or inorganic energy sources, such as ferrous iron, but requiring a small amount (0.04% wt/vol) of yeast extract.

It was shown that the addition of only organic sulfur, nitrogen or carbon has no significant effect on the growth of *Ferroplasma acidiphilum* BRL-115, remaining unclear the effect of vitamins or other single amino acids like serine. From these results, it was concluded that this strain takes advantage of the different components of the yeast extract for growth.

With the experimental information it was also shown that *F. acidiphilum* and *L. ferriphilum* grow interacting synergistically, where *L. ferriphilum* provides organic matter in form of EPS to *F. acidiphilum*, and the latter helps to maintain low levels of organic matter in the culture media. It was also suggested that they could exchange extracellular proteins which can favor their interaction with substrate.
Growth parameters for a batch cyclic reactor in pure culture of *F. acidiphilum* with yeast extract and Fe$^{2+}$, yeast extract, sucrose and Fe$^{2+}$, and in mixed culture with *Leptospirillum* sp. in medium supplemented only with Fe$^{2+}$, were determined. The results showed that *F. acidiphilum* reaches a higher specific growth rate with yeast extract, sucrose and Fe$^{2+}$, in comparison to the condition without sucrose, since having available an alternative organic energy source, it promotes the consumption of it.

The results obtained from the experiments in batch cyclic mode of the mixed culture were used as input for a simulation on the simultaneous metabolic model, obtaining a reasonable behavior of the metabolic fluxes and the system as a whole.

From simulations made with the mixed model in chapter 3, it was observed that the additional input of amino acids, except for serine, does not significantly increase the specific growth rate of *F. acidiphilum*, which was experimentally corroborated with cysteine and casamino acids in Chapter 4. Therefore, with the developed model it was possible to get a preliminary idea about a suitable composition of the culture medium of *F. acidiphilum*.

In this work, metabolic models of these biomining microorganisms were developed for the very first time, acquiring a deeper understanding of their metabolism whilst growing in pure and mixed culture. With this kind of methodology complemented with experimental information, it is possible to suggest new strategies to improve productivity in bioleaching processes, such as an optimal culture media to maximize cell growth.


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APPENDIX
APPENDIX A

Table A.1. Abbreviation of components included in the metabolic models of *Leptospirillum ferrooxidans* and *Ferroplasma acidiphilum*.

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>ACCOA</td>
<td>Acetyl-CoA</td>
</tr>
<tr>
<td>ACET</td>
<td>Acetate</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AICAR</td>
<td>5’-phosphoribosyl-5-amino-4-imidazolecarboxamide</td>
</tr>
<tr>
<td>AKG</td>
<td>Alpha-ketoglutarate</td>
</tr>
<tr>
<td>ALA</td>
<td>L-alanine</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
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<td>ARCHGLYC</td>
<td>Archaetidylglycerol</td>
</tr>
<tr>
<td>ARGI</td>
<td>Arginine</td>
</tr>
<tr>
<td>ASN</td>
<td>L-asparagine</td>
</tr>
<tr>
<td>ASPT</td>
<td>Aspartate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BIO</td>
<td>Biomass</td>
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<tr>
<td>C16COA</td>
<td>Palmitic acid Coa</td>
</tr>
<tr>
<td>C17COA</td>
<td>Synthetic fatty acid</td>
</tr>
<tr>
<td>C18COA</td>
<td>Steric acid Coa</td>
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<td>CARBOH</td>
<td>Carbohydrates</td>
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<td>Carbamoyl phosphate</td>
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<td>Cdp-archaeol</td>
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<td>CHOR</td>
<td>Chorismate</td>
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<td>CIT</td>
<td>Citrate</td>
</tr>
<tr>
<td>CLIPIN</td>
<td>Cardiolipin</td>
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<tr>
<td>CMP</td>
<td>Cytidine-3’-monophosphate</td>
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<td>CO₂</td>
<td>Carbon dioxide</td>
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<tr>
<td>COA</td>
<td>Coenzyme A</td>
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<tr>
<td>COQ</td>
<td>coenzyme Q (oxidized)</td>
</tr>
<tr>
<td>COQH₂</td>
<td>coenzyme Q (reduced)</td>
</tr>
<tr>
<td>CYS</td>
<td>L-cysteine</td>
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<td>DAMP</td>
<td>2’-deoxyadenosine 5’-monophosphate</td>
</tr>
<tr>
<td>DCMP</td>
<td>2’-deoxycytidine 5’-monophosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>DGMP</td>
<td>2'-deoxyguanosine 5'-monophosphate</td>
</tr>
<tr>
<td>DIAGLYC</td>
<td>CDP-diacylglycerol</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid base pair</td>
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<tr>
<td>DTMP</td>
<td>Deoxymethylidine 5'-monophosphate</td>
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<tr>
<td>ERY4P</td>
<td>D-erythrose 4-phosphate</td>
</tr>
<tr>
<td>F6P</td>
<td>Fructose-6-phosphate</td>
</tr>
<tr>
<td>FERR$_{H2}$</td>
<td>Reduced ferredoxin</td>
</tr>
<tr>
<td>FERRIC</td>
<td>Ferric iron</td>
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<tr>
<td>FERROUS</td>
<td>Ferrous iron</td>
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<td>FERROX</td>
<td>Oxidized ferredoxin</td>
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<td>FOR</td>
<td>Formate</td>
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<td>Glucose-6-phosphate</td>
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<td>Hydrogen</td>
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<td>Proton</td>
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<td>H$_{OUT}$</td>
<td>External proton</td>
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<td>Sulfide</td>
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<td>ILE</td>
<td>Isoleucine</td>
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<tr>
<td>IMP</td>
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<td>KIV</td>
<td>2-keto-isovalerate</td>
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<td>LEU</td>
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<td>LIP</td>
<td>Synthetic lipid molecule</td>
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<tr>
<td>LIPI</td>
<td>β-D-glucopyranosylcaldarchaetidylglycerol</td>
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<tr>
<td>LIPII</td>
<td>Triglycoside of caldarchaetidylglycerol</td>
</tr>
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<td>L-lysine</td>
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<td>L-methionine</td>
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<td>MTHF</td>
<td>5,10-methylene tetrahydrofolate</td>
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<tr>
<td>N₂</td>
<td>Nitrogen</td>
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<tr>
<td>NAD⁺</td>
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<td>Nicotinamide adenine dinucleotide (reduced)</td>
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<td>Succinate</td>
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<td>L-tryptophan</td>
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<tr>
<td>VAL</td>
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<tr>
<td>XYL5P</td>
<td>D-xylulose 5-phosphate</td>
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## APPENDIX B

### Table B.2. Nomenclature

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<tr>
<td>S</td>
<td>Stoichiometric matrix</td>
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<tr>
<td>$v(x)$</td>
<td>Vector of intracellular reaction rates</td>
</tr>
<tr>
<td>$b$</td>
<td>Vector of extracellular reaction rates (substrate consumption and products formation)</td>
</tr>
<tr>
<td>$X$</td>
<td>Cellular concentration</td>
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<tr>
<td>$Z$</td>
<td>Objective function</td>
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<tr>
<td>$\alpha_i, \beta_i$</td>
<td>Capacity constraints of reaction i</td>
</tr>
<tr>
<td>$f$</td>
<td>Degree of freedom</td>
</tr>
<tr>
<td>$m$</td>
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<td>$v(x)$</td>
<td>Vector of intracellular reaction rates</td>
</tr>
<tr>
<td>$b$</td>
<td>Vector of extracellular reaction rates (substrate consumption and products formation) (mol/C-mol·h)</td>
</tr>
<tr>
<td>$q_{O_2}$</td>
<td>Specific oxygen consumption rate (mol $O_2$/C-mol·h)</td>
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<tr>
<td>$D$</td>
<td>Dilution rate (1/h)</td>
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<tr>
<td>$r_i$</td>
<td>Generation rate of macromolecule i (mol i/C-mol·h)</td>
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<tr>
<td>$x_i$</td>
<td>Proportion of macromolecule i (mol/C-mol)</td>
</tr>
<tr>
<td>$Y_{SX}$</td>
<td>Growth yield in substrate (ferrous iron) (C-mol/mol Fe$^{2+}$)</td>
</tr>
<tr>
<td>$q_s$</td>
<td>Specific substrate consumption rate (eg. ferrous iron) (mol Fe$^{2+}$/C-mol·h)</td>
</tr>
<tr>
<td>$\mu$</td>
<td>Specific growth rate (1/h)</td>
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</table>

<table>
<thead>
<tr>
<th>Nomenclature Chapter 3</th>
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<tbody>
<tr>
<td>S</td>
<td>Stoichiometric matrix</td>
</tr>
<tr>
<td>$v(x)$</td>
<td>Vector of intracellular reaction rates</td>
</tr>
<tr>
<td>$b$</td>
<td>Vector of extracellular reaction rates (substrate consumption and products formation) (mol/GDW·h)</td>
</tr>
<tr>
<td>$Y_{CX}$</td>
<td>Growth yield in carbon (GDW/C-mol)</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>$Y_{FeX}$</td>
<td>Growth yield in ferrous iron (C-mol/mol Fe$^{2+}$)</td>
</tr>
<tr>
<td>$q_{CO2}$</td>
<td>Specific consumption rate of CO$_2$ (C-mol/GDW·h)</td>
</tr>
<tr>
<td>$q_{EPS}$</td>
<td>Specific production rate of exopolysaccharides by L. ferrophilum (mol EPS/GDW·h)</td>
</tr>
<tr>
<td>$q_{Fe2+}$</td>
<td>Specific consumption rate of ferrous iron (mol Fe$^{2+}$/GDW·h)</td>
</tr>
<tr>
<td>$\mu$</td>
<td>Specific growth rate (1/h)</td>
</tr>
<tr>
<td>$Z_{mut}$</td>
<td>Objective function with enzyme deletion (1/h)</td>
</tr>
<tr>
<td>$Z_{wt}$</td>
<td>Objective function for wild type strain (1/h)</td>
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**Nomenclature Chapter 4**

<table>
<thead>
<tr>
<th>Symbol</th>
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<tbody>
<tr>
<td>$r_S$</td>
<td>Specific rate of substrate consumption (mol/GDW·h)</td>
</tr>
<tr>
<td>$r_P$</td>
<td>Specific rate of product synthesis (mol/GDW·h)</td>
</tr>
<tr>
<td>$X$</td>
<td>Cellular concentration (GDW/L)</td>
</tr>
<tr>
<td>$C_S$</td>
<td>Substrate concentration (mol/L)</td>
</tr>
<tr>
<td>$C_P$</td>
<td>Product concentration (mol/L)</td>
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<tr>
<td>$\mu_{max}$</td>
<td>Maximum specific growth rate (1/h)</td>
</tr>
<tr>
<td>$f_{EPS}$</td>
<td>EPS production amount (g EPS/GDW)</td>
</tr>
<tr>
<td>$q_{EPS}$</td>
<td>Specific production rate of exopolysaccharides by L. ferrophilum (g EPS/GDW·h)</td>
</tr>
<tr>
<td>$q_{Fe2+M}$</td>
<td>Specific consumption rate of ferrous iron of the mixed culture (g Fe$^{2+}$/GDW·h)</td>
</tr>
<tr>
<td>$q_{Fe2+Lf}$</td>
<td>Specific consumption rate of ferrous iron of L. ferrophilum (g Fe$^{2+}$/GDW·h)</td>
</tr>
<tr>
<td>$q_{Fe2+Fa}$</td>
<td>Specific consumption rate of ferrous iron of F. acidiphilum (g Fe$^{2+}$/GDW·h)</td>
</tr>
</tbody>
</table>
APPENDIX C

Publication *in extenso* originated by this thesis