Instituto Tecnológico y de Estudios Superiores de Monterrey

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School of Engineering and Sciences

"Studies on the growth of *Magnetospirillum gryphiswaldense* under several conditions and its influence on the production of magnetosomes"

A thesis presented by

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Declaration of Authorship

I, Jesús Gilberto Rodríguez Ceja, declare that this thesis titled, “Studies on the growth of *Magnetospirillum gryphiswaldense* under several conditions and its influence on the production of magnetosomes” and the work presented in it are my own. I confirm that:

- This work was done wholly or mainly while in candidature for a Master of Science degree at this University.
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.
- Where I have consulted the published work of others, this is always clearly attributed.
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.
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Jesús Gilberto Rodríguez Ceja

Monterrey Nuevo León, May 27th, 2017

Dedication

To my family,

I dedicate this thesis, because with your faith, support, encouragement and for constantly teaching me to believe in myself to achieve my dreams. I will never finish to thank you for everything you have done for me.

To Aidee,

Who has accompanied me during this two years, being my girlfriend and my best friend.

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“Studies on the growth of *Magnetospirillum gryphiswaldense* under several conditions and its influence on the production of magnetosomes”

by

Jesús Gilberto Rodríguez Ceja

Abstract

*In recent years, Magnetospirillum gryphiswaldense has called attention because it is a bacteria capable of producing magnetic nanoparticles (magnetosomes) that can be used in a variety of applications, such as directed therapies for drug delivery. The production of magnetosomes has not reach threshole concentration to ensure feasible for industrial applications. Thus, it is of high importance to increase the magnetosome production. This research evaluated the effect of stirring rate (100, 200, 400 and 600 rpm) and hydrodinamic conditions based on Reynolds number for the grwoth and production of M. gryphiswaldense. Overall, the better results were obtained at 200 rpm, with correspond to higher biomass and oxygen transfer coeficient (K_{LA}). Optimal impeler stirring rate was selected to grow bacteria, followed by an anaerobic stage with feeding, to induce the production of magnetosomes. The best magnetosome yield obtained was 18.79 mg/mL*day in a fed-batch culture, 11.78 in continuous culture and 1.4 in batch culture. Their respective specific growth (μ) and generation time (min) were 0.083 and 8.31, 0.022 and 31.38, 0.02 and 34.65. Three different pellets were obtained and their magnetosome content was extracted to be analyzed in Z-sizer, resulting in an average size of 100 nm. Also aggregates of magnetosomes were found. The findings reported here could serve as a basis for the future scaling up of a biological factory for nanocarriers for multiple research and/or medical applications*.

Keywords: Magnetotactic bacterium, *Magnetospirillum gryphiswaldense*, magnetosomes, fed-batch, continuous, anaerobic bacteria, nanocarriers.

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Chapter I

1. Introduction

The magnetism is a force resulted of charged matter that creates an invisible field away from itself, and this includes the planet earth (Huang, 2007). Such magnetic field has diverse effects in animals and other organisms that are capable of detecting the direction of earth´s magnetic field. Among those organisms, we can find bacteria, chitons, sharks, honeybees, homing pigeons and dolphins. Such ability is because they have mechanisms that involve biologically precipitated ferromagnetic nanoparticles, mainly composed of magnetite (Fe_3O_4), that respond to magnetic fields. (Kirschvink & Gould, 1981).

Nanoparticles (NP) are those whose range is in scale of nanometers (10^{-9} m). Such particles are being used for biomedical and biotechnological applications (**Figure 1**) and, in the case of magnetic nanoparticles (MNPs), they have called attention because of their ability to carry molecules and to be controlled by magnetic fields to reach a specific point. Therefore, MNPs can bind and deliver molecules to sites, tissues or cells (Ito, Shinkai, Honda, & Kobayashi, 2005). A notable characteristic, is that the carried molecule can be protected from some degrading agents, for example, the DNA molecules may not be available for enzymatic degradation when bound to a nanoparticle (Bin, 2007).

Magnetic nanocarriers can be divided into synthetics and organics. The first ones are synthetized chemically, being the most used metals iron and gold (Laurent et al., 2008). Although the synthesis is easy, such molecules have shown toxicity if they are not properly coated with biocompatible materials (Neuberger, Scho, Hofmann, & Rechenberg, 2005). The organic nanocarriers are synthetized by bacteria, for example the genus *Magnetospirillum* (Nakamura, Hashimoto, & Matsunaga, 1991), *Desulfovibrio* (Sakaguchi, Arakaki, & Matsunaga, 2002), *Magnetovibrio*, *Magnetospira*

and *Magnetobacterium* (Lefèvre & Bazylinski, 2013). Previous works have demonstrated less toxicity of organic nanoparticles than synthetic ones (Bin, 2007; Sun, Duan, et al., 2008; Xiang et al., 2007). These bacteria are believed to direct their mobility using geomagnetic field and thus they are known as magnetotactic bacteria (MTB) (Lefèvre & Bazylinski, 2013).

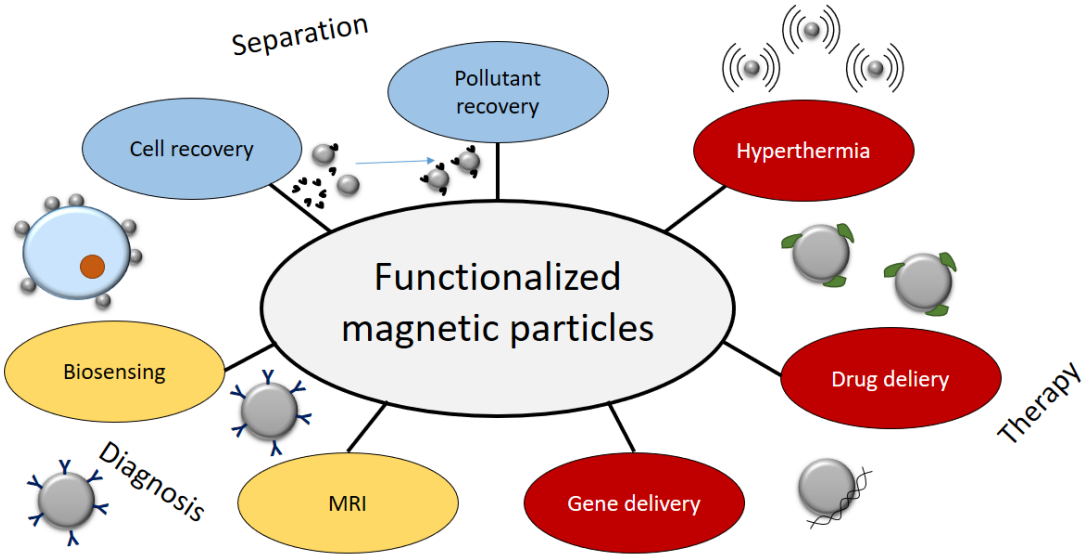

Figure 1. Applications of magnetic nanoparticles.

The MTB were discovered in 1975 in surface sediments collected from salt marshes from Cape Cod and surface layer of sedimentary cores in Buzzards Bay (both in Massachusetts, US) (Blakemore, 1975). The first MTB to be isolated in a defined medium was found in Cedar Swamp from Wood Hole (Massachusetts, US) and called *Aquaspirillum magnetotacticum*, which was later renamed as *Magnetospirillum magnetotacticum* (R. P. Blakemore, Maratea, & Wolfe, 1979; Schleifer et al., 1991). The first anaerobic facultative MTB was obtained from freshwater and sediment ponds in Koganei (Japan) and it was identified as *Magnetospirillum magneticum* AMB-1 (Tadashi Matsunaga & Kamiya, 1987; Tadashi Matsunaga, Sakaguchi, & Tadokoro, 1991). Later, a MTB was isolated from mud of the river Ryck (Greifswald, Germany), a strain that was described as an spirillum and named *Magnetospirillum gryphiswaldense* MSR-1 (Schleifer et al., 1991). A characteristic of the three previous bacteria (which are the commonly studied) is that they are gram negative bacteria, spirillum shape, in

the α-subidivision of proteobacteria, facultative anaerobic and nitrate reducing (Tadashi Matsunaga & Okamura, 2003).

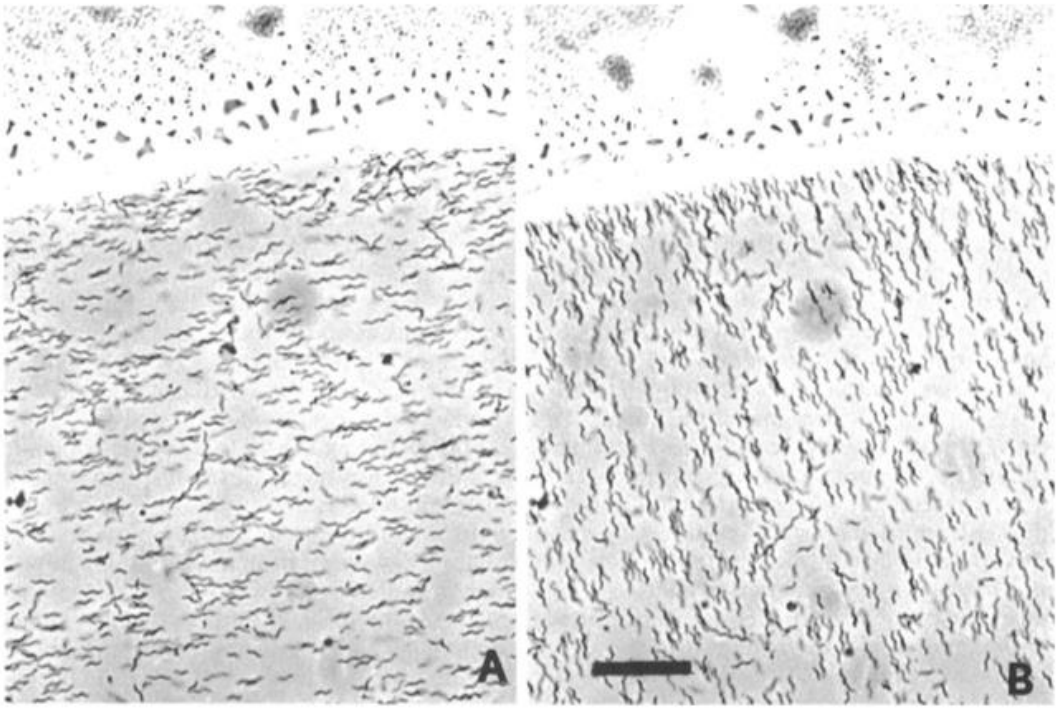

Figure 2. Response to magnetic field of *M. magnetotacticum*. Phase contrast of M. magneticum MS-1 obtained by using Zeiss standard research microscope. The grey bar represents 20 μm. A) Bacteria in steady state. B) Bacteria aligned under magnetic field effect. Figure taken from (R. P. Blakemore et al., 1979).

In MTB, the organelles responsible of magnetic response are called magnetosomes. The magnetosome is formed as a vesicle with lipid bilayer containing iron oxide (Fe_3O_4) nanocrystals inside. Such organelles-like are used to orientate in the Earth´s magnetic field, to help the cells to move to better oxygen and to growth in favoring zones. As the bacteria are in aquatic habitats, they can migrate using the pili, in a process called magnetotaxis (Jogler & Schüler, 2009). Their size range from 30 to 140 nm (Faivre & Schüler, 2008). The magnetosomes are aligned along actin-like cytoskeletal structures, perpendicular to cell axis (Schüler, 2008). The production of magnetosomes is related to aerotactic sensory mechanisms. When the oxygen and nitrogen source are below the optimal, the magnetosomes are produced and their biological usage is to help the

cell to migrate to better growing conditions (Jogler & Schüler, 2009; Tadashi Matsunaga, Tsujimura, & Kamiya, 1996).

Several strains have been studied recently, aiming to understand the molecular mechanisms for the formation of magnetosomes. Some of this magnetotactic bacteria includes: *Magnetospirillum magneticum* (Tadashi Matsunaga & Okamura, 2003) and *Magnetospirillum gryphiswaldense*. The second one have been used in several works that approached to increase the magnetosomes yield and it have shown to be better than *Magnetospirillum magneticum* (Liu et al., 2010; Sun, Zhao, et al., 2008; Zhang, Zhang, Jiang, Li, & Li, 2011). But to our knowledge, there is very limited information on growing models and kinetic parameters available in literature to control growth and magnetosome production in laboratory conditions. Therefore, a valuable information would be to have a better understanding of the operational conditions that eventually would lead to the use of *Magnetospirillum gryphiswaldense* as a commercial or research biofactory for magnetosome for diverse applications. Such information is the velocity of growth (μ and μmax), dissociation constant (Ks) and productivity ($Y_{x/y}$) for determined carbon and iron source, as well as Reynolds number (Re) and oxygen transfer constant (K_{LA}) for several stirring rates.

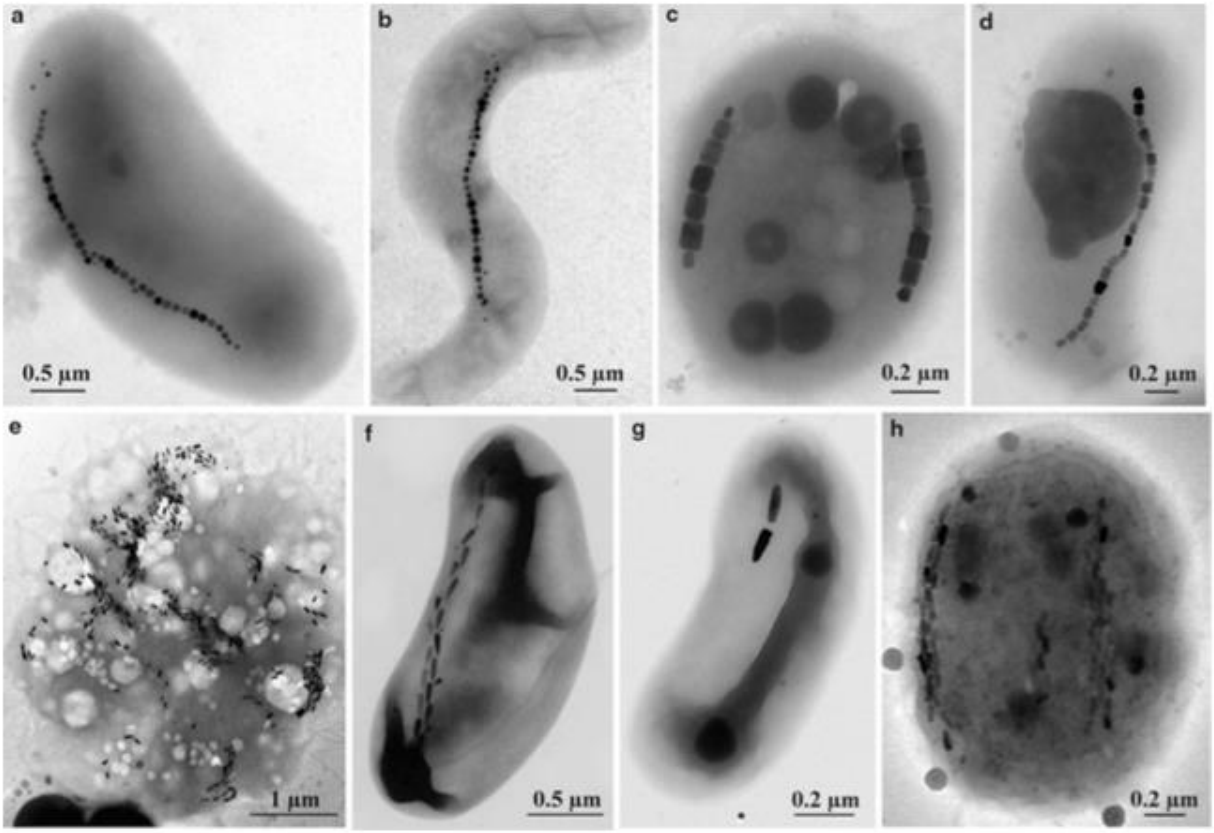

Figure 3. Several species of magnetotactic bacteria. Obtained using Brighfield TEM microscopy. a) gammaproteobacterium strain BW-2 isolated from Badwater Basin, California, USA. b) spirillum strain CB-1 isolated from Lake Mead, Nevada. c) unknown magnetotactic bacteria found in Mediterranian Sea at Marseille, France. d) gammaproteobacterium strain SS-5 isolated from Salton Sea, Califronia. e) uncultured multicellular magnetotactic prokaryote from Mediterranian Sea at Marseille, France. f) unknown magnetotactic bacterium from Mediterranian Sea at Marseille, France. g) thermophilic Magnetovibrio Candidatus strain HSMV-1. h) Magnetoovum mohavensis strain LO-1 found in Lake Mead, Nevada. Figure taken from (Bazylinski, Lefevre, & Schüler, 2013)

2. Background

2.1. Magnetosomes

Magnetosomes are nanoparticles that commonly have a range size of 30-140 nm depending on the producing organism and culture conditions. They can also be referred as bacterial magnetic particles (BMP) (Faivre & Schüler, 2008; Tadashi Matsunaga & Okamura, 2003).

The magnetosomes are vesicles which are made of a lipid bilayer composed of phospholipids to the same proportion as the *Magnetospirillum* sp. membrane (Grünberg et al., 2004; Nakamura et al., 1991). There have been identified specific proteins bound to magnetosome membrane and various from plasmatic membrane (Komeili, Li, Newman, & Jensen, 2006; Okamura, Takeyama, & Matsunaga, 2000).

In the lumen of magnetosomes there are ferric oxide (Fe_3O_4) crystals which have the magnetic quality (magnetite). The biological function of magnetosomes is to drive the cell to an optimal condition of dissolved oxygen (DO) to growth, which is approximately 1 ppm. When the oxygen concentration is below 1 ppm, the magnetosomes are produced to help the cell to navigate to better growing conditions. In laboratory conditions it has been reported that when the oxygen supply is reduced, the magnetosomes production increase (Ardelean et al., 2009; Yang et al., 2001).

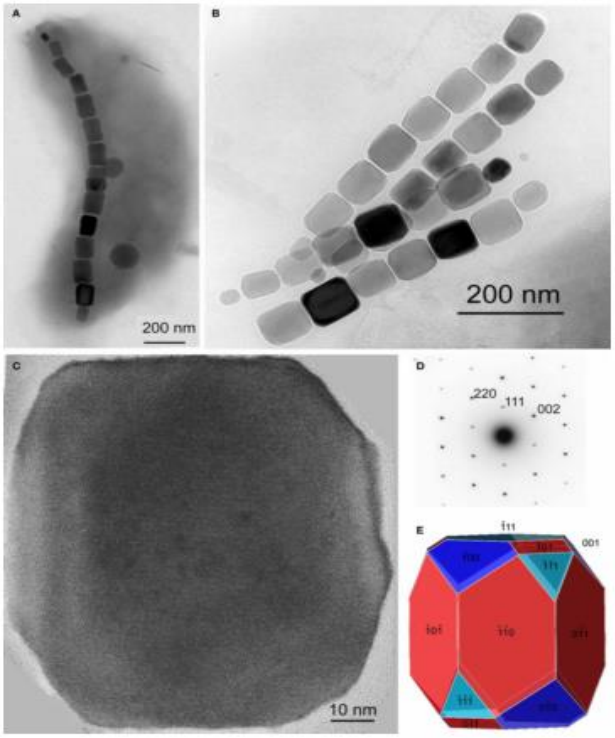

Figure 4. Isolated magnetosomes from alphaproteobacteria. Several TEM images showing magnetosomes. A) TEM image of magnetosomes of a vibroid MTB frzom Lake Mead, Nevada. B) TEM image of magnetosome chains of a freshwater coccus. C) TEM image of magnetosome from previous organism, D) and its selected area for electron diffraction pattern. E) morphological model of octahedron magnetosome. Figure taken from (Pósfai, Lefèvre, Trubitsyn, Bazylinski, & Frankel, 2013).

For *Magnetospirillum* genre, there are more than 20 proteins involved in formation, synthesis and function of the magnetosome. Such proteins have function in the: endocytosis and vesicle formation from cytoplasmic membrane (Mms24 and Mms16); iron accumulation inside the magnetosome (MagA, MamB and MamM); size controlling and crystallization (MamC, MamD, MamF and MamG); magnetosome aligning (MamF, MamJ, MamK); localization near the center of the cell and the septum of cell division (MamX, MamY and FtsZ). Also there are more proteins whose function that have not been clearly defined (Jogler & Schüler, 2009; Tadashi Matsunaga & Okamura, 2003; Murat, Quinlan, Vali, & Komeili, 2010). For example, MamE and MamO, that are HtrA-like serin proteases that possibly stabilize the magnetite crystals (Quinlan et al, 2011).

2.2. Magnetosome formation

It is well known that several magnetotactic bacteria start the magnetosome synthesis under absence of oxygen, which is the terminal electron acceptor during ATP production. Therefore, bacteria use nitrate as electron acceptor substitute. Although, when nitrogen has been consumed or there is not availability, MTB begin the iron uptake (Tadashi Matsunaga et al., 1996). The priority order of using those molecules for growing is due to the reduction potential. The MTB prefer molecules with high and positive reduction potential as electron acceptor. In the case of the couple ½ O2/H2O it is +0.82 volts, for NO_{3}^{-}/NO_{2}^{-} it is +0.42 volts and for the couple $Fe^{3+/}Fe^{2+}$ it is 0.2 volts (Liu et al., 2010).

It is remarkable that exist bacteria that produce magnetite under a controlled mechanism. This is also known as “biologically-controlled mineralization” (BCM). There are also bacteria capable of producing magnetite indirectly by a “biologically induced mineralization” (BIM) (Bazylinski, 1999). Such uncontrolled mechanism drives to poorly crystalized and not defined magnetite. In the case of *Magnetospirillum gryphiswaldense*, the mechanism is regulated by genes (Faivre & Schüler, 2008), so it is BCM.

Once the conditions and microorganisms are suitable for synthesis of magnetosomes, it starts the process, which can be divided in parallel 3 steps: iron take up, biomineralization and invagination for vesicle formation. Each step has involved proteins which are encoded by genes found in the magnetosome island (Li, Katzmann, Borg, & Schüler, 2012).

2.2.1. Iron uptake

The excess of intracellular iron can be harmful to the cell, so it has to be strictly controlled (Imlay, 2003). Moreover, iron concentration plays an important role in magnetosome synthesis because lack of iron reduce the magnetosome number but excessive iron prevents the bacteria growth (Heyen & Schüler, 2003; Schüler &

Baeuerlein, 1996). Two proteins have been found to be involved in iron uptake from extracellular media: FeoB and Fur-Like. Deletion in the gene *feo*B reduced the magnetosome production although not inhibition, so it is considered as an accessory protein (Rong et al., 2008). Deletion of *fur*-like gene resulted in abolition of magnetosome synthesis, so it has an important role in iron transporting (Voigt et al., 2010). For transporting iron from cytoplasm to magnetosome, there are two proteins, called MamB and MamM. It has been demonstrated that deletion of respective genes lead to dramatic magnetosome reduction (*mam*M) or inhibition (*mam*B) (Uebe et al., 2011). Also it has been found that those proteins have similarity to subfamily CDF3, which are proteins involved in "cation diffusion facilitator" activity (Faivre & Schüler, 2008). Therefore, the iron uptake process involves the transport of such metal inside the cell (in some moment it is reduced from Fe^{3+} to Fe^{2+}) and then the transport to the magnetosome.

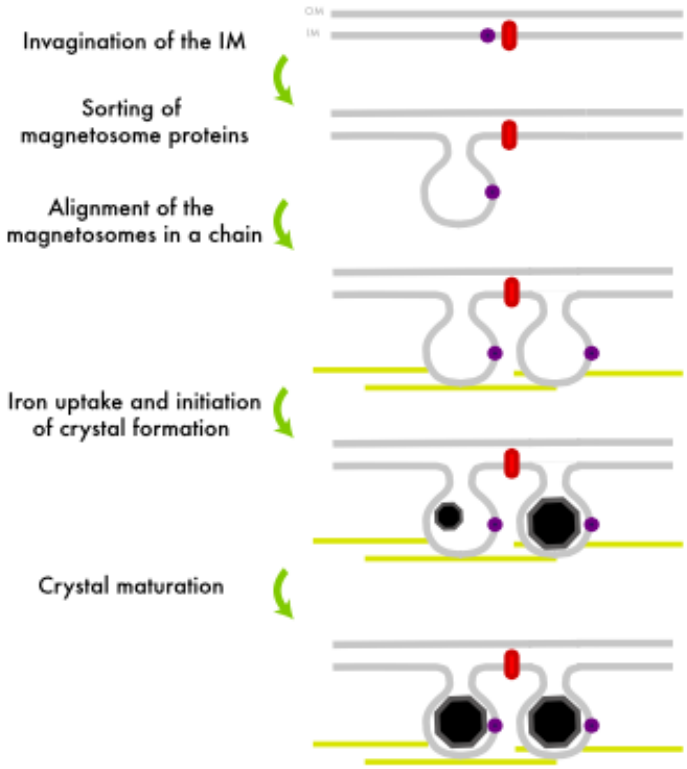

Figure 5. Steps involved in synthesis of magnetosomes. For every step, the key proteins involved in the process are shown. Figure taken and modified from (Murat et al., 2010).

2.2.2. Biomineralization

The ions are transported into an intracellular site located in the membrane, which is a compartment (the forming vesicle) and it has alkaline environment to enable the thermodynamic stability of magnetite. There are also ions coupled to membrane-associated ferritin. Then the magnetite precipitation starts by coprecipitation of Fe^{2+} and Fe^{3+}. The reaction is proposed in **Figure 6**.

$$Fe^{2+}A + 2Fe^{3+}B + (2x + y + 4)H_2O \rightarrow 2Fe(OH)_x^{3-x} + Fe(OH)_y^{2-y} + (2x + y)H^+ + A^{2-} + 2B^{3-} +$$

$$4H_2O \xrightarrow{-A^{2-} - B^{3-}} Fe_3O_4 + (2x + y)H_2O + 8H^+$$

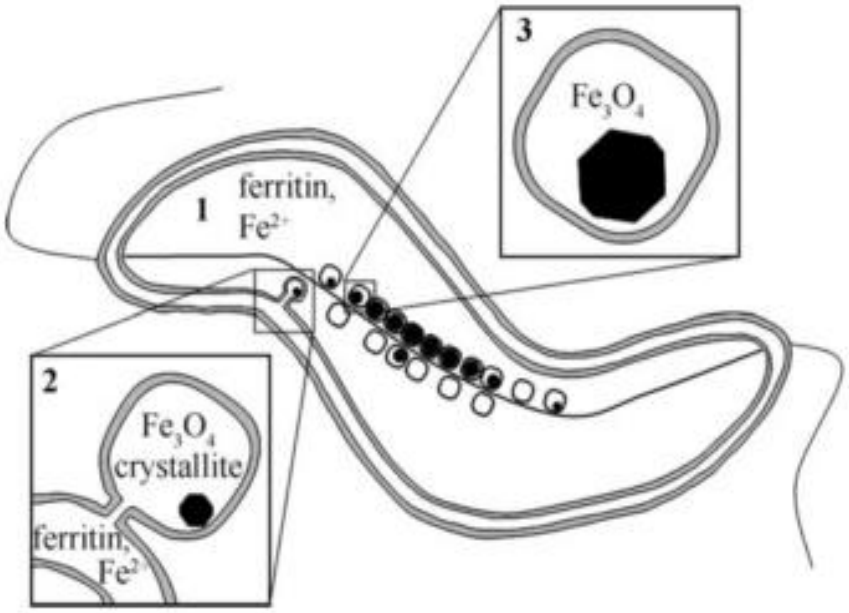

Figure 6. Model of iron precipitation and biomineralization. 1) Ferritin and Fe^{2+} ions are transported into the recently invagination. 2) Ions Fe^{2+} and Fe^{3+} start to coprecipitate forming the magnetite (Fe_3O_4). 3) The magnetite is fully mature, and the magnetosome is finished and aligned to cell membrane. Taken from: Faivre, Böttger, Matzanke, & Schüler (2007).

In the **Figure 6**, A and B are organic substrates at the cytoplasmic membrane and must be released in the magnetosome compartment. During this process, it is important the REDOX potential and pH because the magnetite is usually synthetized in the range -0.2 to -0.4 V. Also, protons are released during the biomineralization and a reductive molecule is needed (Faivre et al., 2007; Faivre & Schüler, 2008).

It has been discovered that the magnetotactic bacteria are capable of nitrate reduction during the process of magnetosome formation. Although the oxygen is necessary for optimal growth, it is not necessary for Fe_3O_4 biomineralization because oxygen used for Fe_3O_4 formation is derived from water (Mandernack, Bazylinski, Iii, & Bullen, 1999). Also, there are bacteria capable of biomineralization in anaerobic conditions. For example, *Magnetovibrio blakemorii* use N_2O as electron acceptor and *Desulfovibrio magneticus* RS-1 use sulfate and fumarate as electron acceptor (Sakaguchi et al., 2002). In the bacterium *Magnetospirillum gryphiswaldense* MSR-1, it has been found that the enzyme Nap (periplasmic nitrate reductase) is required for growth during anaerobic conditions and it is involved in redox control, which is also necessary for magnetosome synthesis (Li et al., 2012).

2.2.3. Invagination for vesicle formation

The magnetosome is considered an organelle, because the following biologic characteristics: A vesicle conformed by a lipid bilayer and specific soluble and transmembrane proteins (Komeili et al., 2006).

Basically the building of magnetosomes needs: Creation of a highly curved membrane controlled by the cell, selection and accumulation of the proper proteins in the vesicle lumen, separation of vesicle and chain organizing. For formation of membrane curvature and vesicle separation the proteins in charge are Mms24 16 (Murat et al., 2010).

MamK is an actin-like protein, which has been proposed for magnetosome location along the membrane. Such protein forms filaments that attach to the transmembrane protein MamJ of magnetosomes and, on the cellular membrane, MamK attaches to the protein MamI, so the magnetosomes can align tangentially to the inner curvature of the cell. MamI, MamJ and especially MamK, are not fundamental for magnetosome production, although it has been demonstrated that their absence leads to irregular distribution of vesicles and small magnetosome chains (Katzmann, Scheffel, Gruska, Plitzko, & Schüler, 2010; Komeili et al., 2006).

2.3. Growth medium

The understanding of proteins and mechanisms involved in magnetosome formation is important to know which are the best formulations and conditions for the growth medium. However, for optimization studies it is also important to consider the physical conditions as temperature and agitation, not only the chemical composition of medium. Therefore, this section will focus on carbon source, nitrogen source, iron source, REDOX potential and pH, dissolved oxygen, temperature and agitation.

2.3.1. Carbon source

The carbon source, as the main nutrient in a growth media, has been analyzed in several works to determine the best one for *M. gryphiswaldense*. In the beginning, it was observed that the bacterium was heterotrophic, because it broke down molecules to obtain energy. The first carbon sources used was sodium acetate (R. P. Blakemore et al., 1979) and sodium thioglycolate (Tadashi Matsunaga et al., 1996). More recently, lactate, pyruvate, acetate and succinate have been studied to achieve the best growth. It was found that lactate and pyruvate are better for growing the bacteria than acetate and succinate (Heyen & Schüler, 2003). The preference of bacteria to growth with lactate and pyruvate is because they have better potential redox (-190 mV) than acetate (-10 mV) and succinate (+33 mV), therefore they are better as electron donor for ATP synthesis (Thauer, Jungermann, & Decker, 1977). All those molecules are products of glucose and other carbohydrates during energy metabolism, so they have less potential as electron donors. Although carbohydrates need oxygen to be broken down and magnetotactic bacteria use to growth under microaerophilic conditions, they do not utilize sugar fermentatively neither oxidatively (Bazylinski, Williams, et al., 2013; Bazylinski & Williams, 2006).

It has been determined that 20 mM is the best concentration of sodium lactate for *Magnetospirillum* sp. growth in a batch culture. When the concentration increase, the bacteria yield decrease. But in a fed-batch, it is preferable to maintain low concentration

(2.3 mM), because this apparently allows faster growing (Liu et al., 2010; Zhang et al., 2011). Such behavior can be related to susceptibility of *M. gryphiswalde*nse to increased osmotic potential (Zhang et al., 2011).

2.3.2. Nitrogen source

The nitrogen source is very important for growth of magnetotactic bacteria, specially *Magnetospirillum* genre, because they are denitrifying bacteria (Bazylinski & Williams, 2006). As has been in 2.2 section, the couple NO_{3}^{-}/NO_{2}^{-} is better oxygen acceptor for bacteria growth than Fe^{3+}/Fe^{2+}. In fact, the magnetosome synthesis can be enhanced having the nitrogen source at low concentration (T Matsunaga, Kikuchi, & Tanaka, 2000). In 2010, it was discovered that NH_4Cl is better for growing *Magnetospirillum gryphiswaldense* than $NaNO_3$ (Liu et al., 2010). Although in more recent years it has been demonstrated a different result. There is a relationship of redox potential with nitrate reduction and magnetite production, which means that nitrate is better for magnetosome production.

For magnetite biomineralization it is required low redox potential. As it was explained in 2.2.2 section, if the cell is deficient in the nitrate reductase (Δnap) and cannot metabolize a nitrate source, the redox potential is not regulated and the result is low biomineralization (Li et al, 2012). In other situation, when *Magnetospirillum gryphiswaldense* cultures are deficient in nitrate source, the observed magnetosomes are reduced in number and they have flake shape. Additionally for those cultures, If nitrate concentration is increased, the magnetite crystal number and shape is restored, but if nitrite concentration is increased, the regular magnetosomes are not obtained (Raschdorf et al, 2013).

2.3.3. Iron source

As was explained in the iron uptake section, excess of iron in growth media can be harmful for the bacteria (Imlay, 2003). It has been determined that the iron saturation supply is between 10 and 20 μM. Increase in concentration results in iron precipitation (Schüler & Baeuerlein, 1996). In fact, slightly increase in iron concentration increase

the cell growth and magnetosome yield, but 100 μM reduce the magnetosome production and 200 μM or above damage the bacteria and reduce the growth (Heyen & Schüler, 2003; Schüler & Baeuerlein, 1996). However, some works report the iron concentrations above 20 μM and to up 200 μM using iron from different chemical sources, from $FeCl_3$ (C. Yang et al., 2001), ferric citrate (Heyen & Schüler, 2003; Liu et al., 2010; Sun, Zhao, et al., 2008) or increasing concentration of ferrous sulfate (Zhang et al., 2011), some of these as batch or fed-batch mode.

The iron source is important for magnetosomes production. It has been determined that ferrous sulfate is better than ferric gallate, ferric citrate, ferric malate and ferric quinate. Such behavior is because the reduced form (ferrous, iron II) is more soluble than the oxidized form (ferric, iron III). It also means that the reduced iron can be up taken more easily than the oxidized (C. Yang et al., 2001). However it has been described that for the production of magnetosomes, *Magnetospirillum gryphiswaldense* uses ferric (in ferritin form) and ferrous (soluble) ions at the same time (Faivre et al., 2007). At this point, the control of pH and REDOX potential plays an important role.

2.3.4. pH and REDOX potential

The organisms that produce magnetite, included *M. gryphiswaldense*, must be capable of coprecipitate Fe^{2+} and Fe^{3+}. For that, they need to control the intracellular REDOX potential through carbon metabolism and iron supply (Nishida & Silver, 2012), and both mechanism are involved in bacteria growth. The iron is used as an electron acceptor when there is no oxygen or nitrogen sources. When the iron accepts electrons, it converts from Fe^{3+} to Fe^{2+}. Finally, the Fe^{2+}/Fe^{3+} couple is equilibrated under a REDOX potential of 0.2 volts, near to pH 7.0. Such pH is usually used for magnetosomes production (Liu et al., 2010; Sun, Zhao, et al., 2008). Therefore, it is important to control the pH to facilitate the production of magnetosomes.

2.3.5. Dissolved oxygen

Dissolved oxygen has been determined as crucial factor for synthetizing magnetosomes and it is needed a strict control otherwise the iron uptake is repressed.

Some magnetotactic bacteria, for example in *M. gryphiswaldense*, growth preferably to aerobic conditions although they are also facultative anaerobic (Tadashi Matsunaga et al., 1991; Schleifer et al., 1991). In such cultures, when there is enough oxygen supply and the DO is above 0.4 ppm, the magnetosomes synthesis is reduced but the bacteria growth normally. On the other hand, low DO results in slow growing, meanwhile the magnetosome production tends to increase (C. Yang et al., 2001). Also, the color of cultures can vary depending Therefore, it is important to maintain controlled the DO to low ppm´s or to produce biomass aerobically and then to stress it changing to anaerobic condition (Liu et al., 2010; Sun, Zhao, et al., 2008).

2.3.6. Temperature

Temperature is not determinant for producing magnetosomes, but it is necessary to growth the bacteria properly. It has been used 28-30°C for *Magnetospirillum gryphiswaldense* as the optimal (Liu et al., 2010; Zhang et al., 2011). For *M. magneticum* has been used 25°C (C. Yang et al., 2001), 30°C for *M. magnetotacticum* and *Magnetovibrio blakemorei* at 28°C (Silva et al., 2013). Moreover, it was found that temperature above 28°C slightly improve growth but reduce the production of magnetosomes (Heyen & Schüler, 2003).

2.3.7. Stirring rate

Several reports use different agitation conditions, depending on the strain and the mode of culture, varying from 100 rpm in culture bottles to 200 rpm in agitated bioreactors (with Rushton impellers)(Rong et al., 2008). An increased growth of *M. gryphiswaldense* was observed when agitation was progressively raised from 200 to 400 rpm. (Sun, Zhao, et al., 2008). In another research with *M. gryphiswaldense*, it was discovered that stirring rate can affect directly the magnetic response. From 120 rpm to 200 the bacteria growth and it produced magnetosomes, but 300 rpm increased the bacteria yield but reduced magnetic response (J. Yang et al., 2013). To our knowledge, there is little or not research focusing on studying the effect of agitation speeds on the growth of MTB.

2.4. Applications

The magnetic nanoparticles have a good volume/mass relationship and this is one of the reasons why several potential applications have been proposed. Among them: hyperthermia treatment for tumor control, drug therapy using magnetic delivery and diagnosis using antibodies. Examples of recent reported applications include: gold magnetic nanoparticles in a immunoassay for antigen detection of bovine leukemia virus antigen *gp*51 (Baniukevic et al., 2013); hyperthermia generated by magnetic particles under a magnetic field to induce cell death in tumor tissue (Kobayashi, 2011). Such magnetic nanoparticles could be directed to specific sites or tissues using magnetic fields. However, magnetosomes have another reported advantage: its low toxicity compared with other chemically-synthesized nanoparticles. Such quality is because they are biologically made, covered by membrane and proteins, which are less antigenic than other free synthetic nanoparticles (Alphandéry, 2014; Bin, 2007; Xiang et al., 2007). Moreover, such magnetic nanoparticles can be directed to specific sites or tissues using magnetic fields. Although, magnetosomes have another interesting advantage: its lower toxicity when compared with other synthetic nanoparticles. Such quality is because they are biologically made, covered by membrane and proteins, which are less antigenic than free magnetic nanoparticles (Alphandéry, 2014; Bin, 2007; Xiang et al., 2007).

Magnetosomes have been used in a variety of applications, for example: Magneto-immuno polymerase chain reaction for detection of recombinant Hepatitis B surface antigen in human serum (Wacker et al., 2007); magnetosomes loaded with doxorubicin (compared with free doxorubicin) against H22 cells and cardiac cells increased antitumor activity and reduced toxicity to non-target cells (Sun, Duan, et al., 2008). Staphylococcal “protein A” has been expressed on surface of magnetosomes and attached G antibodies were used to magnetically trap, separate and detect *Vibrio parahaemolyticus* (Xu et al., 2014). Magnetosomes attached to hexahistidine were capable to bind Cd^{2+} (a pollutant heavy metal) and were magnetically recovered (Tanaka et al., 2008). Mice treated with magnetosome chains under alternating

magnetic fields showed disappearance of tumor cells after 30 days (Alphandéry, Faure, Seksek, Guyot, & Chebbi, 2011).

Although the magnetosomes have acquired importance in the nanocarriers area, the production is still not enough to cope with the quantities that are needed for large scale research. Several efforts have been done to produce large quantities of magnetosomes, there are just a few reports dealing with this issue, reaching about 170 mg/L/day (Alphandéry, Amor, Guyot, & Chebbi, 2012). Therefore, it is important to increase the production levels already achieved.

3. Hypothesis

The growing conditions of *Magnetospirillum gryphiswaldense* significantly influence the iron uptake and its capability of synthetizing magnetosomes. The bacterial growth is closely related to the stirring rate, playing an important factor in oxygen distribution and sheer stress, thus affecting iron uptake and magnetosome production.

4. Objectives

To improve the growth of *M. gryphiswaldense* by the modification of stirring rate in an aerobic culture and to design a fed-batch culture with adequate conditions to produce magnetosomes.

4.1. Specific objectives

- To determine the growth kinetic parameters of *M. gryphiswaldense* in shaking flask, considering the factors reported in literature in order to formulate growth medium.
- To evaluate the effect of agitation speed on the growth of *M. gryphiswaldense* growth in 1 L biorreactors from 100 to 600 rpm.
- To perform growth kinetics of batch and fed-batch cultures in 1 L volume, under aerobic conditions followed by anaerobic conditions to induce magnetosome production.
- To evaluate size distribution of isolated magnetosomes from different growing conditions using a z-sizer.
- To obtain kinetic values from cultures to propose models for growth and production of magnetosomes in 1 L bioreactor.

Chapter II.

5. Materials and Methods

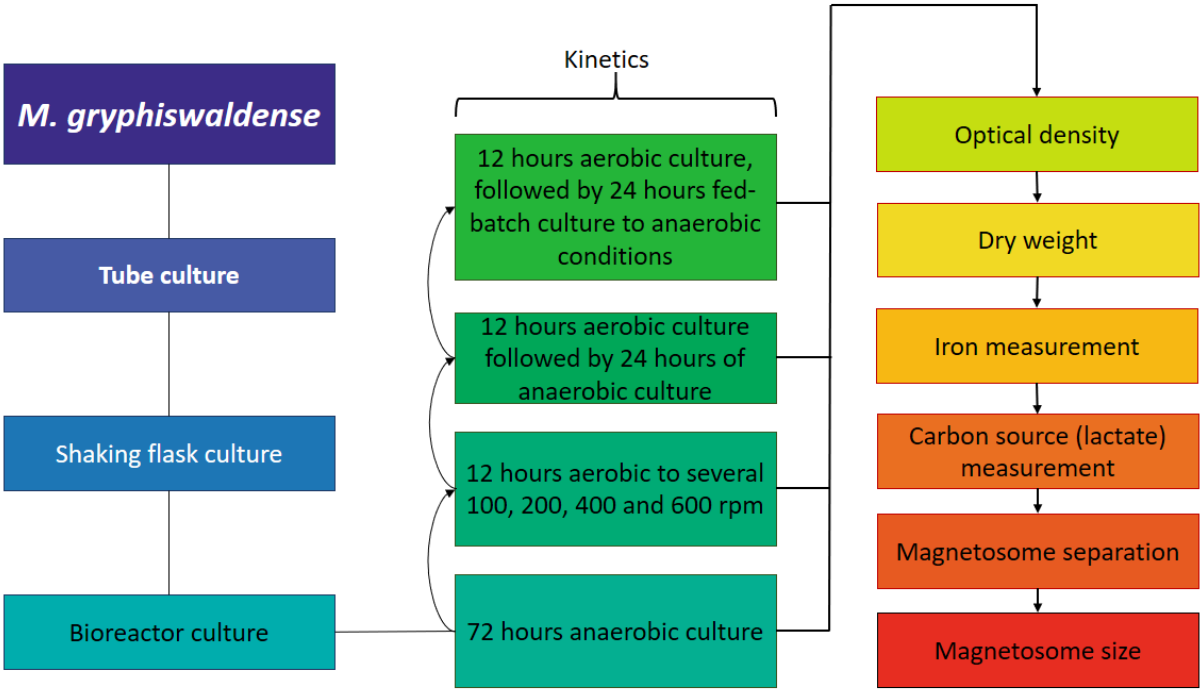

Figure 7. General overview of methodology.

5.1. Bacterial strain

Magnetospirillum gryphiswaldense strain MSR-1 was purchased from DSMZ ("Deutsche Sammlung von Mikroorganismen und Zellkulturen", Brunswick, Germany) a German collection of microorganisms. The catalog number is DSM6361.

5.2. Materials

The reagents for preparing the Magnetic Spirillum Growth Medium (MSGM) are: sodium lactate (L7022, Sigma Aldrich), NH_4Cl (A9434, Sigma-Aldrich), yeast extract (212750, Becton-Dickinson), $MgSO_4 \cdot 7H_2O$ (10034-99-8, DEQ), $K_2HPO_4 \cdot 3H_2O$ (7758-11-4, BDH), ferric citrate (F3388, Sigma-Aldrich), nitriloacetic acid (72560,

Sigma-Aldrich), $MnSO_4 \cdot H_2O$ (10034-96-5, DEQ), NaCl (7647-14-5, DEQ), $CoSO_4 \cdot 7H2O$ (C5421, DEQ), $CaCl_2 \cdot 2H_2O$ (10035-04-08, DEQ), $ZnSO_4 \cdot 7H2O$ (DEQ), $CuSO_4 \cdot 5H_2O$ (7758-99-8, DEQ), $KAl(SO_4)_2 \cdot 12H_2O$ (237086, Sigma-Aldrich), H_3BO_3 (A0140, DEQ), $NaMoO_4 \cdot 2H_2O$ (10102-40-6, DEQ), $NiCl_2 \cdot 6H_2O$ (N6136, Sigma-Aldrich) and $Na_2SeO_3 \cdot 5H_2O$ (214425, Sigma-Aldrich).

For iron uptake spectrophotometric method: ferrozine (160601, Sigma-Aldrich), (+)-ascorbic acid (A7631, Sigma-Aldrich), neocuprine (N1501-1G, Sigma-Aldrich), potassium permanganate (223468, Sigma-Aldrich), ammonium acetate (A1542, Sigma-Aldrich) and HCl (H1758, Sigma-Aldrich). For lactate consumption: lactate assay kit (MAK064, Sigma Aldrich).

5.3. MSGM preparation

To make the MSGM, the first step is to prepare a stock solution called mineral elixir. Such solution is prepared in 300 mL of distilled. The mineral elixir contains: 0.75 g nitriloacetic acid, 0.15 g $MnSO_4 \cdot H_2O$, 0.5 NaCl, 0.09 g $CoSO_4 \cdot 7H2O$, 1.5 g $CaCl_2 \cdot 2H_2O$, 0.09 g $ZnSO_4 \cdot 7H2O$, 5 mg $CuSO_4 \cdot 5H_2O$, 10 mg $KAl(SO_4)_2 \cdot 12H_2O$, 5 mg H_3BO_3, 5 mg $NaMoO_4 \cdot 2H_2O$, 12.5 mg $NiCl_2 \cdot 6H_2O$ and 0.15 mg $Na_2SeO_3 \cdot 5H_2O$. Then the solution was mixed vigorously to dissolve some crystals. Then it was autoclaved to 121°C during 15 minutes (Panasonic MLS-3781L) to dissolve completely.

One liter of MSGM is composed of: 2.6 g Sodium L-lactate, 0.4 g NH4Cl, 0.1 yeast extract, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.5 g $K_2HPO_4 \cdot 3H_2O$ and 3 mL of mineral elixir. The pH was adjusted to 6.7 using 1 M hydrochloric acid. Then in was autoclaved to 121°C during 15 minutes. (Zhang et al, 2011). For some assays solid medium was prepared containing 15 grams of agar per liter. The medium was stored at 4°C until its use.

5.4. Preparation of inoculum

5 mL of sterile MSGM were added to 6 corning tubes of 50 mL. Such tubes were placed in a shaking incubator (3621204 LABCONCO). The set conditions were 250 rpm, at 30°C, during 12 hours. 200 µL of the culture were taken and the optical density was measured to 620 nm (OD_{620}) (Epoch, BioTek). 200 µL of every tube was taken and properly mixed with sterile glycerol in a proportion 80:20 (culture:glycerol) and stored at -80°C in ultrafreezer (5656, WVR).

5.5. Culture Assays

To know the maximum growth and maximum optical density that can be reached under batch conditions, the following procedure was used: corning tubes of 50 mL were filled with 5 mL of MSGM and inoculated with bacteria previously stored in glycerol. They were placed in incubator to 250 rpm, at 30°C during 3 days. Every 12 hours 200 µL of the culture were taken and the optical density was measured to 620 nm. Finally, the corning tubes were centrifuged to 10,000 rpm during 10 minutes to 4°C (Allegra™ 64R Centrifuge, Beckman Coulter). The pellet characteristics were registered.

5.6. Shaking flask growth kinetic

For growth in shaking flasks, three test tubes of 50 mL were prepared with 5 mL of MSGM and inoculated. The tubes were incubated during 12 hours using the conditions previously described. These fresh cultures were used to inoculate 500 mL shaking flasks containing 120 mL of MSGM and they were set to 30°C, 250 rpm during 24 hours. Samples were taken every hour and the OD_{620} was measured immediately in spectrophotometer.

5.7. Bioreactor kinetics

5.7.1. Anaerobic kinetic in 1 L bioreactor with low agitation

Tubes with 5 mL of MSGM were inoculated and cultured for 12 hours. Then 500 mL shaking flask were prepared with 120 mL of MSGM and inoculated with 5 mL of the recently growth bacteria. The flask was cultured during 12 hours at the same conditions used in the 5.8 section. A 3 L bioreactor was prepared with 900 mL of sterile MSGM and connected to a fermentor (EZ Control, Applikon). The controllers were adjusted to keep the temperature to 28°C and the pH at 6.75 using HCl 1 M. The oxygen was removed injecting nitrogen (99%) until the sensor for dissolved oxygen showed 0%. The agitation was set to 50 rpm using Rushton turbine. Reactor was inoculated using 100 mL from shaking flask previously described (following the 10:90% relationship) with OD of 0.7. The culture time was of 72 hours and samples were taken every 12 hours. Bioreactors were run by triplicates.

At this point, the bioreactor was filled with a volume of 1 L. The Reynolds number can be calculated using the following data of bioreactor: 1 g/cm^3 water density, agitation of 47.5 revolutions per second (rps), the diameter of Rushton impeller was 4.75 cm and the viscosity at 28°C was 0.00833 g/(cm*s). Those data were used in the following equation:

$$Re = \frac{\rho v D}{\mu} \quad (1)$$

Where ρ is density of water, v the velocity of agitation, D is the diameter of Rushton impeller and μ is the dynamic viscosity of fluid. Also, the K_{LA} was calculated using the next equation:

$$K_{LA} = \frac{\ln(\frac{C_{AL} - C_{AL1}}{C_{AL} - C_{AL2}})}{t_2 - t_1} \quad (2)$$

Where C_{AL} is the DO at certain time point (t_n). The times used were the hour 1 and 2 of culture, and the hours were transformed into seconds.

5.7.2. Aerobic kinetics in 1 L bioreactor at several agitations speeds

To assess the effect of agitation on *M. gryphiswaldense* growth, 4 different agitations speeds were evaluated by triplicates: 100, 200, 400 and 600 rpm in 2.5 bioreactors adjusted as previously described. Also, the inoculum in corning tube and shaking flask followed the same procedure and OD. For these kinetics, pH was not controlled and the cultures were constantly sparged (2 L/min) with fishbowl air pumps (Elite 802, HAGEN). Bacterial growth was monitored for 12 hours taking samples every 2 hours, their optical density was measured and they were stored in freezer for further measurements. Dry weight was obtained of every sample taking 1.5 mL, centrifuged in microcentrifuge tubes to 10,000 x rpm during 10 minutes at 4°C. The supernatant was decanted, the pellet was dried in a drying stove (Shel Lab) overnight to 104°C, then the pellet was stabilized in a desiccator (Science Ware) for 20 minutes. The tubes were weighed in analytical balance (OHAUS-1, Pioneer) several times until constant weight was obtained, and the result was multiplied for the factor “1.5/1000” to obtain the biomass per liter (mg/L). The Reynolds number was calculated as described in 5.7.1 section, and the agitation velocities per second were: 95, 190, 380.15 and 570.22 rps (revolutions per second), that correspond to 100, 200, 400 and 600 rpm, respectively.

5.7.3. Aerobic culture followed by anaerobic stress

The three Applikon bioreactors were prepared and adjusted as described in 5.10.1 section. The agitation was set to 200 rpm, accordingly to results of 5.10.2 section and the inoculum was prepared in the same way as described before using the same OD (5.8 and 5.9 section). After inoculation of bioreactors, the culture was grown under aerobic (using the fishbowl pumps) for 12 hours. Then, air supply was eliminated, making the bacteria turn the bioreactor to anaerobic conditions. During the anaerobic phase, pH was controlled at 6.75 using 1 M HCl solution. Samples were taken before inoculation, and at hour 0, 12, 13, 14, 15, 16, 18, 20, 24 and 36 hour, when it was observed a lack of growth and any iron consumption. The optical density was measured, and samples were stored in the freezer.

5.7.4. Anaerobic Fed-batch culture

The three bioreactors were prepared with MSGM and inoculated with 100 mL (0.7 OD) of bacteria growth in shaking flasks. The first 12 hours were aerobic, then the air supply was eliminated and the bacteria lead the culture to anaerobic conditions (as indicated by the DO sensor). During the aerobic conditions, the pH was not controlled but in the anaerobic phase it was controlled to 6.75. One bioreactor was feed at hour 12, 24, 36 and 48 with 100 mL of concentrated 10X MSGM medium (having all the components with exception of mineral elixir). The second bioreactor was fed continuously (at 0.138 mL/min) with concentrated 10X MSGM medium (without mineral elixir), a peristaltic pump. The feed in both fed-batches were equivalent. The third bioreactor was prepared as the two previous and the samples were taken at the same time, but it was not fed. Samples were taken before inoculation, also to hour 0, 12, 13, 14, 15, 16, 18, 20, 24, 36, 42, 48, 62. The optical density was measured, then samples were stored in freezer (-20°C). The reason to have two ways of feeding was because some researches have used a continuous feeding, meanwhile several have used feeding in time lapses (fed-batch), so the results of both could be compared.

5.8. Dry weight

For dry weight determinations 2 mL Eppendorf tubes were labeled and weighted. Then, the tubes were placed inside an oven to 105°C for 8 hours. After that, they were placed in a desiccation chamber for 15 minutes. The tubes were weighted again to obtain the dry weight. They were placed inside the desiccation chamber for 15 minutes and weighted again. When the constant weight was reached, 1.5 mL of samples were taken and placed in the tubes. Then, they were centrifuged to 10,000 rpm during 10 minutes to 4°C and the supernatant was recovered for iron measurement (by ferrozine method), and the tubes were placed inside the oven during 8 hours to dry off the pellets. To measure the dry weight, the tubes were placed again inside the desiccation chamber for 15 minutes and then weighted. Such process was repeated two times to obtain the constant weight.

5.9. Ferrozine

A spectrophotometric method was used for measuring Fe^{2+} in solution. The recovered supernatants (50 µL) were placed on 2 mL tubes, then 50 µL of 50 mM NaOH were added and homogenized by inversion. Then 100 µL of 10 mM HCl and 100 µL of the iron releasing reagent (a freshly mixed solution of equal volumes of 1.4 M HCl and 4.5% (w/v) $KMnO_4$ in H_2O) were added. The tubes were placed in a thermoblock (Digital Heatblock, VWR) at 60°C for 2 hours. Since chlorine gas is produced during the reaction, the incubation was under a fume hood. After, the tubes were cooled to room temperature for 10 minutes. Then 30 µL of iron detecting solution (6.5 mM ferrozine, 6.5 mM neocuprine, 2.5 ammonium acetate, and 1 M of ascorbic acid and distilled water were added to each tube. The tubes were incubated at room temperature for 30 minutes and 200 µL were taken for absorbance reading at 550 nm (Epoch, Biotek). A calibration curve was made for ferrozine method by triplicates from 0 to 30 mg/L of ferric citrate.

5.10. Sodium lactate consumption

For this measurement, the Lactate Assay Kit was purchased from Sigma-Aldrich (MAK064). The vials were briefly centrifuged before opening. Then, reagents were prepared as follows: The Lactate Assay Buffer was melted to room temperature; Lactate Probe was warmed to room temperature, protected from light, aliquoted and diluted 1:10 with Lactate Assay Buffer; Lactate Enzyme Mix was reconstituted in 220 µL of Lactate Assay Buffer, aliquoted and stored to -20°C.

For standards and calibration curve, it was diluted 10 µL of the 100 nmol/µL Lactate standard with 990 µL of Lactate Assay Buffer to generate a 1 nmol/µL standard solution, then it was diluted again (10 µL in 990 µL of buffer) to obtain the concentration 1 pmol/µL. It was added 0, 2, 4, 6, 8 and 10 µL of the 1 pmol/µL Lactate standard into a 96 well plate. Lactate Assay Buffer was added to each well to bring the volume to 50 µL.

For sample preparation bacteria were homogenized in 4 volumes of the Lactate Assay Buffer. The samples were centrifuged to 13,000 x g for 10 minutes to remove insoluble material. 50 µL of soluble fraction were taken and added to the 96 wells plate.

Before any measure of standards or samples, it was necessary to prepare a Master Reaction Mix (MRM). For every reaction, the MRM needed 46 µL of Lactate Assay Buffer, 2 µL of Lactate Enzyme Mix and 2 µL of Lactate Probe. Then, 50 µL of MRM were added to every well with standard or sample. The plate was incubated to room temperature in horizontal agitation (VWR agitator) for 30 minutes, protecting it from the light. The fluorescence was measured to 535±25 (excitation) and 585±25 (emission) in plate reader.

5.11. Kinetic values

The natural logarithm of optical density and dry weight (y-axis) were graph versus the time (x-axis) to obtain the y = mx + b model, which is equivalent to Lineweaver-Burk model:

$$\frac{1}{v} = \frac{Ks}{\mu max} * \frac{1}{s} + \frac{1}{\mu max} \quad (3)$$

Such process was done for every kinetic to calculate the maximum velocity of growth per hour (µmax) and the saturation constant (Ks). The productivity of bacteria and magnetosomes yield also was obtained and expressed in mg per liter per day (mg/L*h). The consume rates were expressed as µM (or mM) per liter per hour. Finally, the growth dynamics were obtained using the following:

$$\mu = \frac{dx}{xdt} \quad (4)$$

$$T = \frac{ln_2}{\mu} \quad (5)$$

Where μ is the specific growth rate constant (h^{-1}), x is the mass in grams and T is the generation time.

5.12. Cell disrupting and magnetosome recovering

To recover the magnetosomes, it was necessary to disrupt the bacteria. Three different methods were considered to release magnetosomes from cells, and then used the disrupted extracts to measure the particle size of the recovered magnetic fraction.

Every culture was centrifuged in 50 mL corning tubes (already weighted) to 8,000 x g during 15 minutes at 4°C (Yang et al, 2001). The supernatant and pellet decanted and the supernatant was stored for further use if necessary. The pellet was weighted to obtain wet and dry weight.

5.12.1. French Press

The previously obtained pellet was washed with 20 mL of PBS buffer (pH 7.4, 5 mM/L) 3 times. Then 20 mL of PBS buffer were added to obtain a suspension. The disruption was made using a French press (EmulsiFlex-C3, Avestin) at $6.89X10^6$ Pa (Xiang et al., 2007). The lysate was stored to -8°C.

5.12.2. Alkaline lysis

The pellet was washed 3 times in PBS buffer. Then, the cleaned pellet was resuspended in 20 mL of NaOH 1N. The liquid is poured in a 250 mL baker and boiled in a hotplate (97042-602, VWR) during 20 minutes to lysate the cells (C. Yang et al., 2001). The lysate was stored in freezer to -8°C.

5.12.3. Ultrasound

As in the previous methods, the pellet was washed 3 times in 20 mL of PBS buffer. Then the newly obtained pellet was resuspended in 20 mL of PBS buffer and ultrasonicated (QSonica, Q125). Sonicator was set to 10 minutes, with On-Off cycles

of 30-30 seconds and amplitude of ultrasonication to 60%. To avoid overheating, the tubes were placed in an ice bath. The lysate was stored to -8°C.

5.12.4. Magnetosome separation and recovery

The newly obtained lysates are ready to be separated using a column filled with iron beads (MiniMACS, Miltenyibiotec). The column was attached in a magnetic adaptor and the iron beads become magnetized. A 15 mL corning tube was placed under the column to recover the liquid. Then 1-2 mL of lysate were added to the superior compartment and by gravity the liquid went through the column. When the superior compartment was almost empty, it was added more lysate. By the time all the liquid has crossed the column, 2 mL of PBS buffer were added to wash it. Then the column was removed from the magnetic adaptor and it is placed in a new corning tube. 2 mL of PBS buffer were added to remove the magnetosomes from the column. Finally, when there was no more leaking inside the tube, a piston was used to bring out the remaining PBS. Such process was repeated with every lysate using new columns (or optionally, using the same column washed with PBS 3 times).

Additionally, another separation method was used to isolate magnetosomes using a magnet. It consisted in using 2 mL tubes with an external magnet. The disrupted and homogenized pellets were placed in the tubes. The solution was decanted maintaining the magnets. Then the empty tube was filled with 2 mL of PBS buffer (pH 7.4), then it was mixed in vortex and the process of decanting with the magnet was used again. This process was repeated one more time, and finally the magnetosomes were resuspended in PBS buffer. All samples of three separation methods were stored in freezer to -8°C.

5.13. Magnetosome size

Samples of recovered fractions were taken and dissolved 1:10 and 1:100 in miliQ water at a final volume of 2 mL. Such solution was placed in plastic cells compatible with Z-sizer (ZSP NANO, Malvern). The measurement parameters for size were

maintained as recommended by the manufacturer. The refractive index for analysis was set to 2.42, which is for iron oxide (III).

Samples were additionally subjected to ultrasound to evaluate its effect on disaggregation of effect on magnetosomes. The ultrasound probe was set up to 60%, with two cycles of on:off pulses of 10:10 seconds. Finally, those samples were analyzed in z-sizer using the same parameters.

Chapter III.

6. Results and Discussion

6.1. Shaking flask growth kinetic

Under conditions described in sections 5.3, 5.4 and 5.5, *M. gryphiswaldense* growth to its maximum optical density (from 0.722 to 0.744) and dry weight (from 943 to 975 mg). Such methodology of growth has limitations because they were on batch culture with a determined concentration of carbon source (23 mM). It is qualified as low concentration, but it is preferably used under 20-27 mM because higher quantities inhibit the growth of *M. gryphiswaldense* (Heyen & Schüler, 2003; Liu et al., 2010; Zhang et al., 2011). Then, as it was found that the bacteria can growth in tube, such methodology was used to the inoculum for shaking flask.

The maximum growth observed in shaking flask was 1.0 optical unit, which is higher than the 0.7 previously obtained in 50 mL tube culture and this difference could be explained due to better oxygen transfer in shaking flask, besides the glycerol content in the starting inoculum not present in shaking flask when it was inoculated. For cultures in tube and shaking flasks it was measured the OD to monitor the growth and it was related to dry weight (Appendix C, **Figure 17**).

The growth of shaking flasks was in lag phase during the starting hours. At the 5th hour it reached the logarithmic phase and after 12 hours the stationary phase was evident (results shown in **Figure 8**). Apparently, the bacteria entered to death phase before hour 18, as shown by the optical density measurements, differing from pellet wet weight.

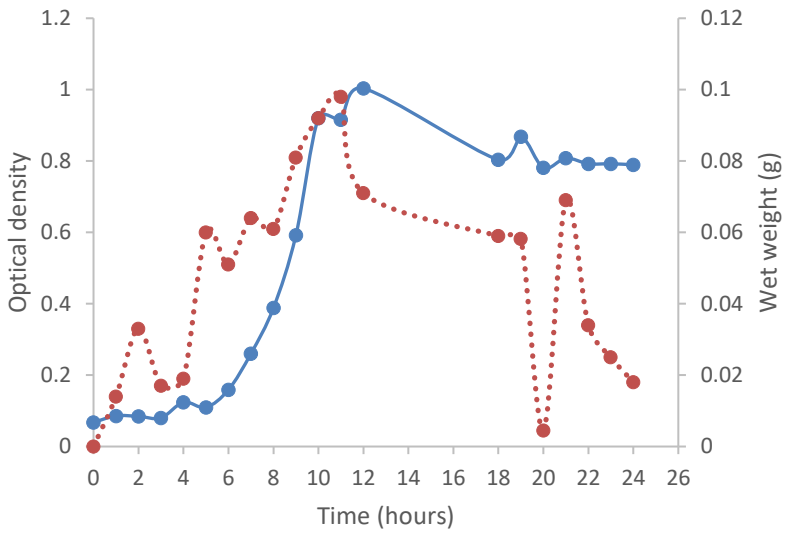

Figure 8. Kinetic curve of *M. gryphiswaldense* in shaking flask at 250 rpm. Solid line: optical density to 620 nm; square dots: wet weight.

During the first 0-12 hours, there is a relationship of optical density with wet weight. The following hours showed an optical density maintained at 0.6, meanwhile the wet weight was significantly reduced. The reason can be due to cell lysis in growth media avoiding the formation of pellets and increasing the resuspension after centrifugation, an effect not detected when optical measurements are taken.

6.2. Bioreactor kinetics

6.2.1. Anaerobic kinetic in 1 L bioreactor to low agitation

The results of growing *M. gryphiswaldense* during 72 hours to anaerobic conditions are observed in **Figure 9**. The growth was from 0.1 to 0.113, which is minimal when compared with results several from works. For example: 7 optical units after 40 hours (Sun, Zhao, et al., 2008), 6 after 55 hours (Liu et al., 2010) and 32 after 50 hours were reached (Zhang et al., 2011). Although the difference was that those cultures were not batch neither anaerobic. In the other hand, in this work, about 3-4 mg/L of iron were absorbed, that means the bacteria were consuming Fe to grow in the anaerobic conditions during 3 days. As the volume was 1 L, the theoretical magnetosome productivity was 0.065 mg/L*h.

For producing magnetosomes and improving the growth, important facts about the requirements of bacteria must be recognized. Mainly, the toxicity of sodium lactate to concentrations above 20 mM, but using low concentration results in low growing, therefore it is necessary to maintain it to optimal. For example, Liu et al. (2010) run batch cultures in shaking flasks and fed-batch in bioreactors using 2.3, 10, 20, 30, 40 and 50 mM/L sodium lactate, and the results were better growing in shaking flask at 20 mM/L than at the other concentrations. Additionally, authors reported 2.3 mM/L sodium lactate as better for rapid growth of *M. gryphiswaldense* when this concentration was constantly maintained. Also, other key factors as nitrogen source, oxygen and iron, need to be controlled as explained in sections 2.3.1, 2.3.2, 2.3.3 and 2.3.5, so batch cultures may not be appropriated the best for producing magnetosomes, because of the low concentrations of substrates resulting from rapid consumption from bacteria with minimal final growth.

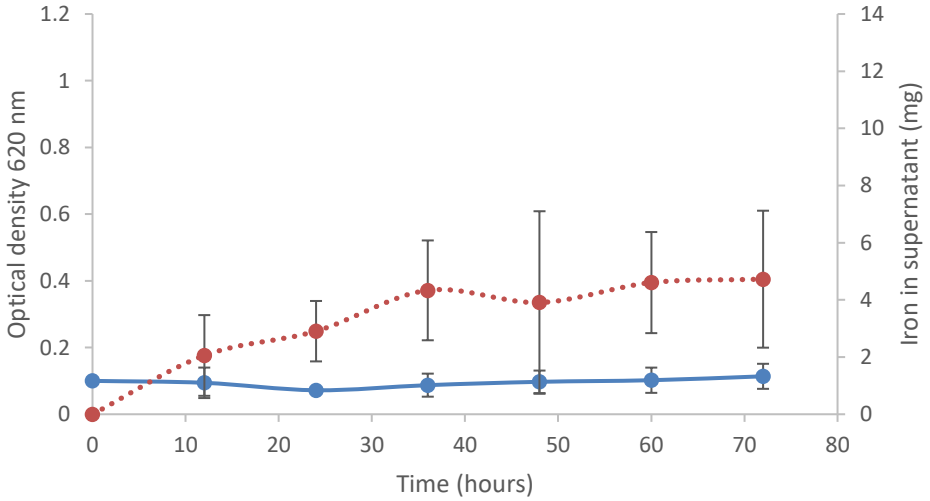

Figure 9. Bioreactor kinetic in anaerobic conditions at low stirring rate (50 rpm). Solid line: Bacterial growth; dotted line: iron in supernatant.

Considering the previous results and the literature, it was proceeded to perform kinetics studies to increase *M. gryphiswaldense* growth at different bioreactor conditions.

6.2.2. Aerobic kinetics in 1 L bioreactor at several agitations speeds

Several nutrients have been determined to be adequate to increase bacterial growth. For example, sodium lactate as carbon source (Liu et al., 2010; Zhang et al., 2011); NH_4Cl as nitrogen source (Liu et al., 2010); ferric citrate, $FeCl_3$ and $FeSO_4$ for synthesis of magnetosomes (C. Yang et al., 2001). But there is not enough information about agitation speed, which is necessary for homogeneous mixture of nutrients and it has been found to be determinant for oxygen transfer in bioreactor conditions. Therefore, it was decided to perform triplicates of aerobic batch cultures at 4 agitation speeds: 100, 200, 400 and 600 rpm. The results are shown in **Figure 10**. The soluble iron was not consumed during these experiments because they were in aerobic conditions, similar to what happens before (C. Yang et al., 2001).

As can be observed in figure **Figure 10A**, a stirring speed of 200 rpm had the best effect on growth (1 optical unit). The use of 100 or 400 rpm resulted in decrease of optical density in a range from 0.6 to 0.8 and the worst growth was obtained at 600 rpm. Such behavior is distinct of most bacteria which tend to growth better when stirring is increased because they have strong cell walls and the mixing is improved. Also, when working with pellets of cultures from 600 rpm, it was observed that they dissolved rapidly, that is also a characteristic of shear sensitive organisms (Winkler, 1990). The Re number of those stirring speeds was calculated and it is showed in **Table 1**, and it can be observed that all those of 100 and 200 rpm are in the transition state (between Re = 10^3 - 10^4), so it is considered that they were properly mixed. On the other hand, the stirring rates 400 and 600 rpm had Reynold numbers above 10^3 and it means they were turbulent and mixed better than 100 and 200 rpm (Narasimha, 1985).

The increase in stirring rate may have better effect on *M*. *gryphiswaldense* growth than decreasing, but it was observed that the bacteria growth was reduced from 200 to 400 rpm, and almost completely reduced from 400 to 600 rpm, because the biomass increased slightly since hour 0 to hour 12 (**Figure 10**). Such behavior has been observed in sensitive bacteria to hydrodynamic stress (Reyes et al., 2017),

also known as sheer bacteria, for example: *E. coli* DH5A-1 (Mohd-Rusli, Shamzi-Mohamed, Mohamad, Tri-Puspaningsih, & Arif, 2009), *Lactococcus lactis* NHD1 (Ibrahim, Abdul Rahman, Mohamad, & Abdul Rahim, 2010), *Pseudomonas* sp. (Munna et al., 2014).

Table 1. Kinetic parameters of agitations speeds**.**

	Stirring rate (rpm)			
	100	**200**	**400**	**600**
μ_{max} (h^{-1})	0.2064	0.2089	0.2248	0.2063
K_s	0.0452	0.0432	0.0409	0.0042
Re	4514	9028	18057	27085
K_{LA}	$1.1x10^{-5}$	$1.62x10^{-4}$	$3.57x10^{-5}$	$3.88x10^{-5}$
Growth model	y= 0.2191x + 4.843	y= 0.207x + 4.786	y= 0.1822x + 4.447	y= 0.0205x + 4.846

Based in the previous information, the behavior of *M. gryphiswaldense* to growth at 200 rpm is a combination of susceptibility to hydrodynamic stress and proper mixing. The optical density reached at 200 rpm was better than previously obtained in shaking flask and anaerobic bioreactor, which means an improvement in methodology because it helped to increase the oxygen transfer or K_{LA} (Micheletti et al., 2006). For 100 and 400 rpm, the growth was similar to those in shaking flask, probably because at 100 rpm the K_{LA} is similar to shaking flask and at 400 rpm the stress was reducing the growth. The calculated K_{LA} of stirring rates can be found in the **Table 1**, and the higher was obtained at 200 rpm and this value explain the better performance of this experiment compared with the other stirring rates.

The μmax (representing the maximum velocity of growth) was similar for all the experiments, that means they can reach the same behavior at any stirring rate. Although they can be at the same maximum velocity, they are not in it during the

same time (Sibbald & Albright, 1988), being the culture at 200 rpm the only one that maintained its growth rate during more time (**Figure 10**).

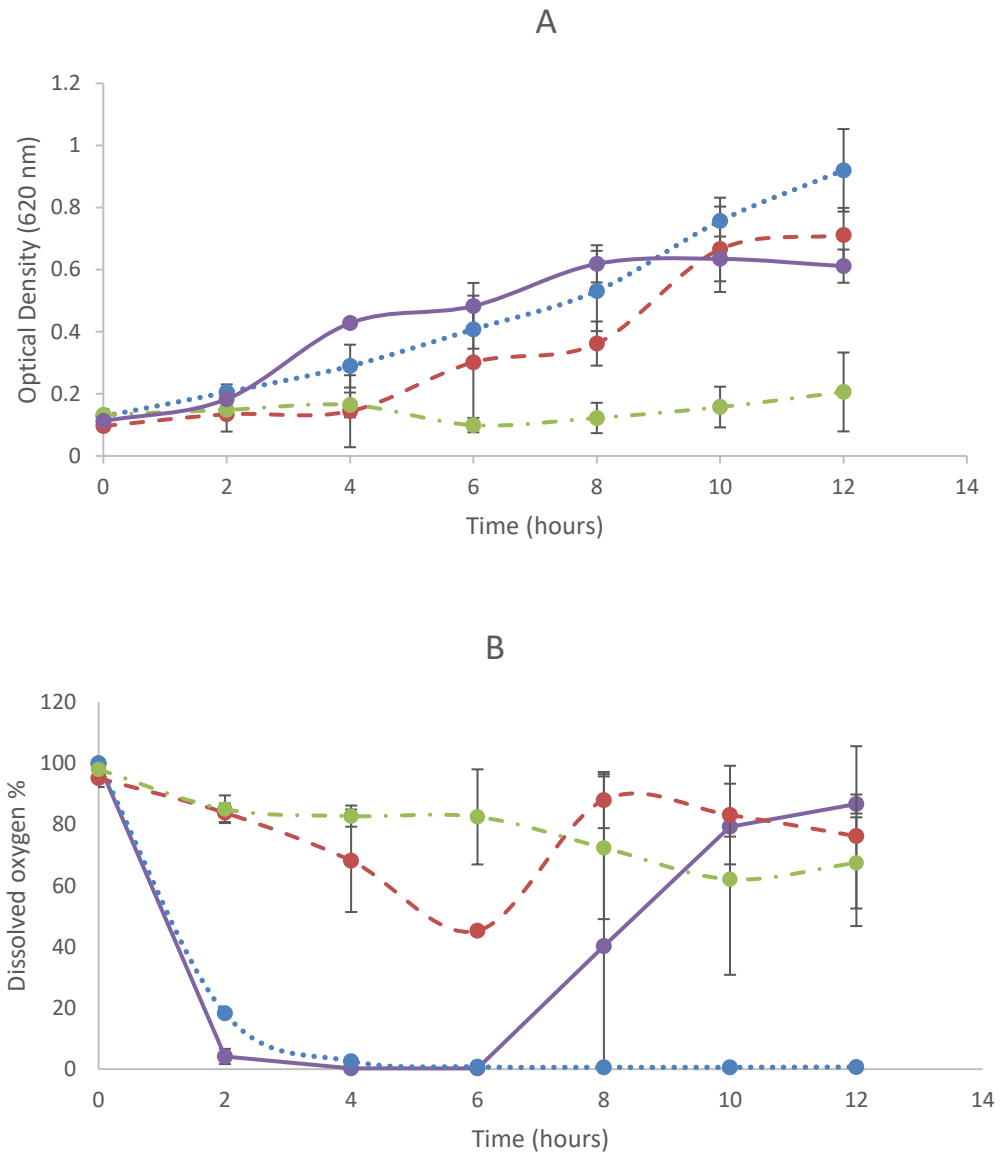

Figure 10. The effect of agitation speed and oxygen consumption on *M. gryphiswaldense* growth. Solid line: 100 rpm; dotted line: 200 rpm; dashed line: 400 rpm; dashed-squared line: 600 rpm. A) The effect of agitation speeds over *M. gryphiswaldense* growth during 12 hours. B) Oxygen consumption during 12 hours.

6.2.3. Aerobic culture followed by anaerobic stress

As it was determined in previous experiments, the best stirring rate was determined to be 200 rpm and the other culture conditions were decided to be the same. Therefore, the experiments were performed to 12 hours of aerobic culture to reach the maximum optical density (about 1.0), followed by anaerobic stress disconnecting the air supply. Nitrogen was not used because the bacteria have demonstrated to consume the oxygen leading to anaerobic conditions, as observed in previous experiments and references (Zhang et al., 2011). The dry weight was similar to that obtained at 200 rpm to aerobic conditions, that is over 1000 mg per liter of culture. The results are shown in **Figure 11.**

The iron consumption was measured, but it was found that iron was not consumed (data ton shown) by bacteria and the dry weight did not increase during the culture time after 12 hours. Both results are corresponding, because the bacteria use the iron as electron acceptor during growth while there is not oxygen neither nitrogen. But if the bacteria are not growing, the iron is not consumed. For this case, the carbon source must have been consumed and the carrying stationary phase reached. Those results lead to the decision of another phase of experiments including fed-batch cultures, because the conditions used at this point were not enough to induce production of magnetosomes.

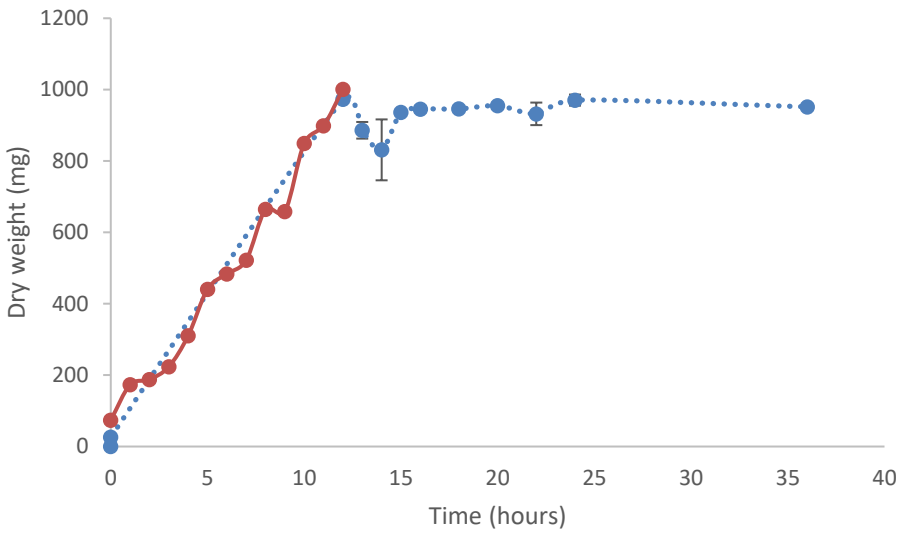

Figure 11. Kinetic of *M. gryphiswaldense* in aerobic conditions during 12 hours and anaerobic during 24 hours. Solid line: 12 hours of aerobic conditions; dotted line: Aerobic culture followed by anaerobic stress

6.2.4. Anaerobic fed-batch culture

The bioreactors were run simultaneously in fed-batch during 62 hours in two modes: feeding every 12 hours (in duplicates) and a constant fed-batch of 0.138 mL/min flux. Also, those cultures were run together with a control bioreactor without feeding. The culture medium used for feeding was the same, with the exception it was 10X times more concentrated to avoid a significant increase of volume.

Contrasting with previous results, iron consumption was observed during the culture time. Although the optical density and dry weight was not observed to increase (**Figure 12**). In similar works, when culture medium was continuously supplied the bacteria growth and iron consumption were linked to the production magnetosomes (Liu et al., 2010; Zhang et al., 2011).

The batch culture in bioreactor showed less growing (400 mg/L) and iron consumption (<5 mg/L) than the fed-batch cultures of every 12 hours. Although the bioreactor with continuous feeding had similar maximum growth than batch bioreactor. On the other hand, the cultures with feeding every 12 hours had maximum growth of 800 and 1050 mg/L (**Figure 12**, C & D). For iron consumption, the accumulation of continuous culture was 37 mg and 59 mg for bioreactor with feeding every 12 hours. Their theoretical yield (mg/L*day) for production of magnetosomes are 1.4 (batch), 11.78 (continuous feeding), 18.79 (feeding every 12 hours).

The observed tendency of growth (**Figure 12**) demonstrate that cultures reached the stationary phase after 12 hours, although adding culture medium did not helped them to keep growing, because they went to death phase. Therefore, to probe if the bacteria was growing or maintaining the stationary phase, it was measured the consumption of lactate as the sole carbon source. The results are shown in **Figure 13**.

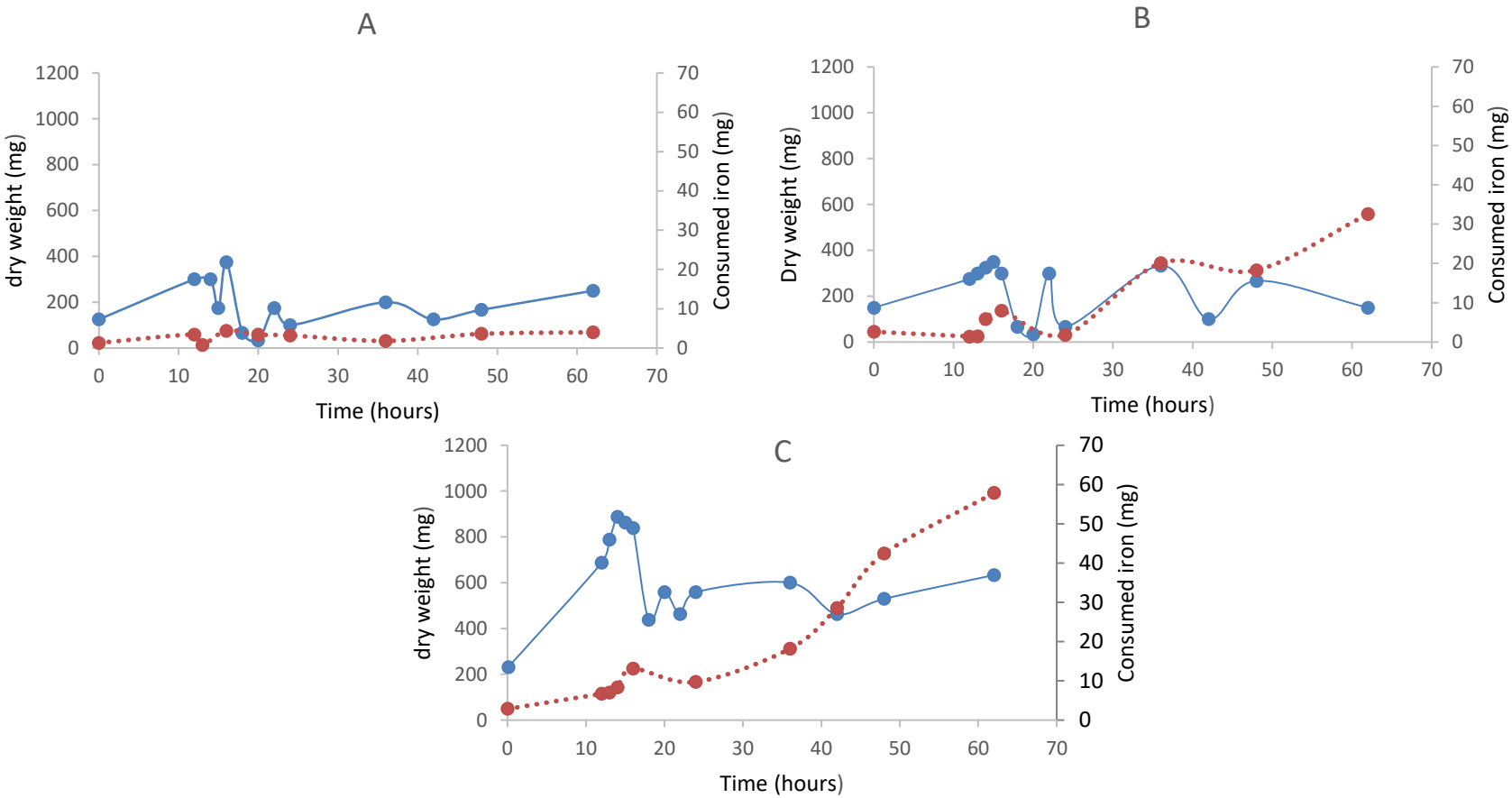

Figure 12. Growth and iron consumption of several cultures of 62 hours in 1 L volumes. A) Batch culture. B) Continuous feeding of culture medium. C) Fed batch with addition of culture medium every 12 hours. Solid line: dry weight; dotted line: consumed iron.

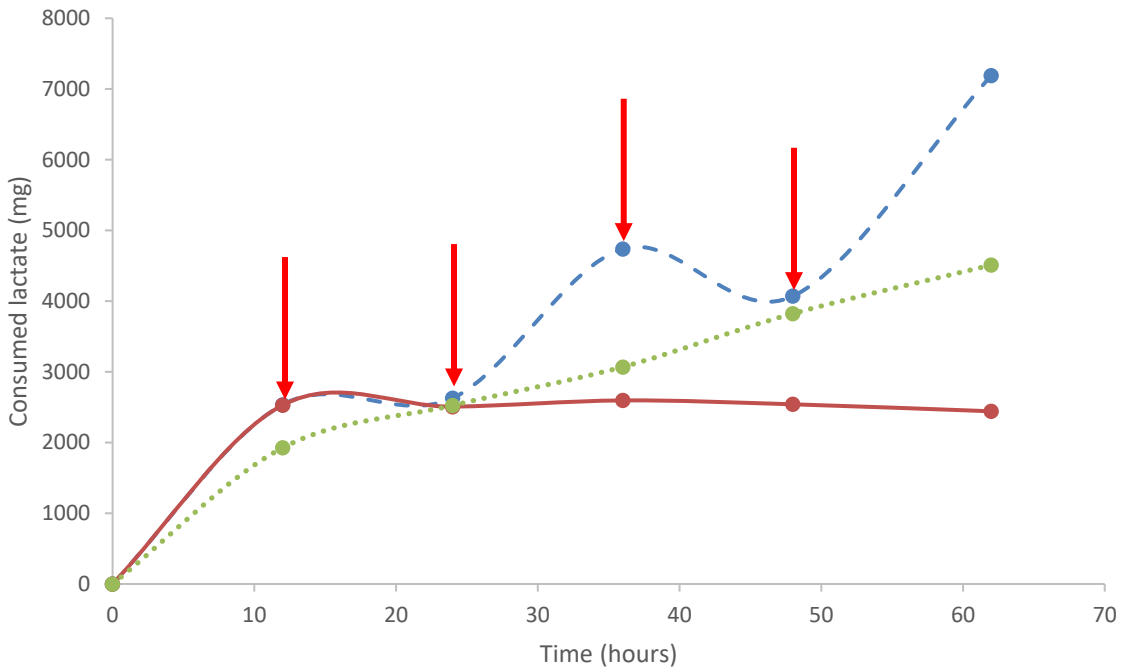

Figure 13. Lactate consumption during 62 hours. The points are for sample measurements at 0, 12, 24, 36, 48 and 62 hours. Solid line (red): batch culture; dotted line (green): continuous feeding; dashed line (blue): fed-batch culture, where the addition of MSGM medium to cultures was after every sample was taken, and such points are indicated by red arrows

The tendency showed that when the air supply was stopped the bacteria was dying, and they started to recover during the following hours. This could mean that feeding was probably initiated in a late point during the cycle of the bacterial growth. Comparing the results of **Figure 12** and **Figure 13,** it was observed that bacteria was consuming the carbon and iron source during all the culture time, therefore, they were maintaining a stationary phase. From lactate and iron consumption data, the kinetic parameters were obtained as shown in **table 2**.

The overall results of growth, iron consumption and lactate consumption could mean that bacteria with fed every 12 hours were growing and producing magnetosomes better than continuous feeding culture. The explanation of such behavior may be a methodology issue, because for both strategies the feeding started at 12 hours, but for the fed-batch it was a complete addition of culture medium, but for continuous

feeding the nutrient addition was equivalent until 12 hours passed by, that is another feeding point for both. So, the continuous feeding was delayed compared against fed-batch culture.

Table 2. Kinetic values of growth cultures.

Lactate consumption			
	Fed every 12 hours	Continuous feeding	Batch culture
μ_{max} (h^{-1})	0.0027	0.135	0.114
Ks	0.132	0.0022	-5.7E-5
Model	Y = 0.0204x + 7.5294	Y = 0.017x + 7.398	Y = -0.0005x +8.751
Lactate uptake rate (µM/h*cell)	$9.47x10^{-10}$	$1.94x10^{-9}$	$1.16x10^{-9}$
Iron consumption			
μ_{max} (h^{-1})	3.31	0.7903	3.861
Ks	0.2091	0.03	0.281
Model	Y = 0.063x + 0.302	Y = 0.038x + 1.2653	Y = 0.0728x + 0.259
Iron uptake rate (µM/h*cell)	$3.27x10^{-12}$	$6.02x10^{-12}$	$8.17x10^{-13}$
Growth dynamics			
Specific growth rate ($\mu = h^{-1}$)	0.083	0.022	0.02
Generation time (T = min)	8.31	31.38	34.65

Schüler & Baeuerlein (1996) described a high velocity and low affinity for the iron uptake of *M. gryphiswaldense* and the data they obtained were K_m = 3 µM Fe and V_{max} = 0.86 (nmol/min). Converting the results of table 2, the best K_m (K_s) was 0.122 µM Fe (continuous feeding) and its respective V_{max} was 3.225 µmol/min and on the

other hand, the worst K_m was 1.146 µM Fe and respectively V_{max} was 15.75 µmol/min (batch culture). Both results were better than reported and comparing such results, the K_s of fed batch culture is better than K_s of batch, but this one has better V_{max}.
In a recent work, using the same concentration of iron (60 µM) they found similar dynamic data: 0.108 Specific growth rate constant, 6.4 generation time and $6.7X10^{-12}$ of iron uptake rate (Taylor et al., 2011). The results of fed-batch cultures with addition every 12 hours (**Table 2**) are similar to those of Taylor et al (2011).

6.3. Cell disruption, magnetosome recovering and magnetosome size

As it was proved that fed-batch cultures in bioreactor consumed iron, they were selected to extract magnetosomes. For cell disruption tests, first it was necessary to obtain pellets of cultures. The selected cultures and their pellets are shown in **Figure 14** and the color in each one were: Brownish, pink and pink-grey. According to literature, the color is a qualitative result that indicates the presence of magnetosomes. It has been reported that cells growth at 1 µM $FeSO_4$ were colored from orange to red-rusty, but increase of concentration to 100 µM resulted in brown to blackish pellets with high magnetic content (Schüler & Baeuerlein, 1996). A similar result was observed in another research, where *M. gryphiswaldense* was growth to 500 µM of ferric citrate and the bacteria was described as dark-brown when produced magnetosomes and white-creamy when not produced (Schultheiss & Schüler, 2003). Such behavior of changing color has been also observed in other species, as *M. magneticum* AMB-1 that is black-brown when it produces magnetosomes and white if not (Tadashi Matsunaga et al., 1991).

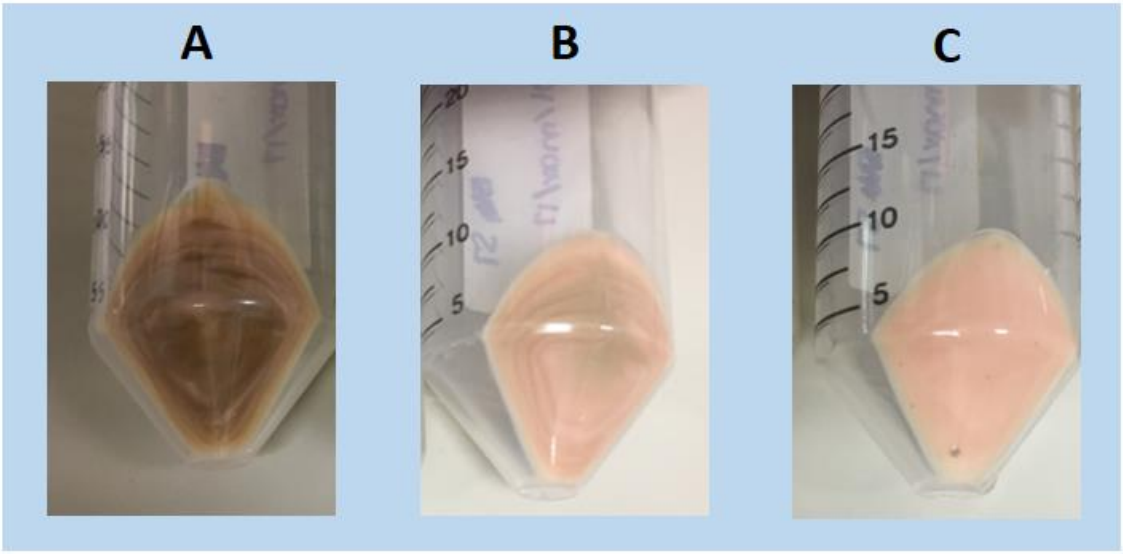

Figure 14. Pellets of *M. gryphsiwaldense* bacteria. A) Fed-batch with addition of culture medium every 12 hours. B) Continuous culture. C) Batch culture.

After the pellets were homogenized and disrupted, the extracts were passed through MiniMacs® columns to separate the magnetosomes. Then, the extracts were analyzed in Z-sizer and it was observed that those proceeding from ultrasound treatment were showing signal in a range around 100 nm (**Figure 15**), but those from French press and alkaline lysis did not showed such result (data not shown). In literature, can be found the reported size of magnetosomes obtained by TEM, which is about 30-140 nm (Bazylinski, 1999; Faivre & Schüler, 2008). The observed peak in the range of 200-600 nm was obtained in every analysis, including the negative control of solely PBS buffer. Therefore, the peak corresponded to components of used buffer.

Another method for separating magnetosomes from extracts was to use a magnet and decanting the supernatant. Such method was preferred instead MiniMacs, because the analyzes showed that columns can trap magnetosomes inside the iron packing by magnetic adsorption.

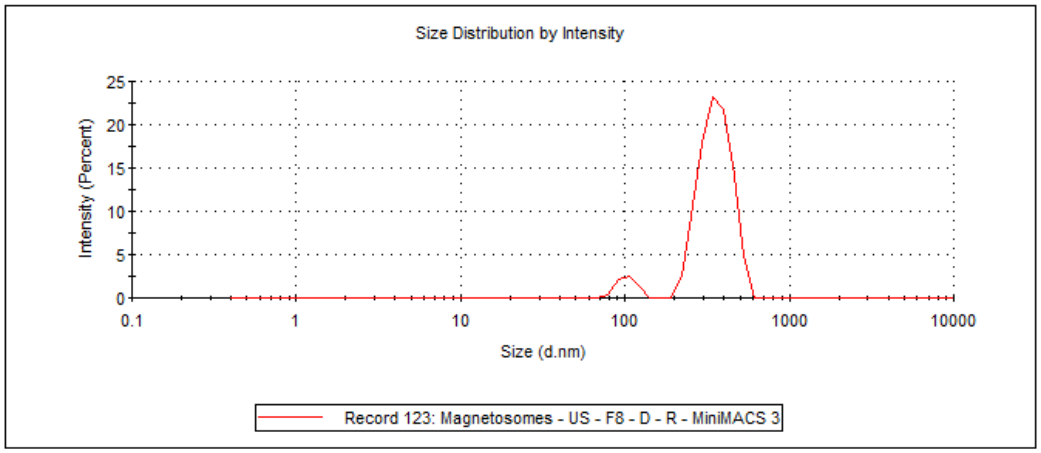

Figure 15. Particle size magnetic fraction obtained from ultrasound treated sample using magnets.

Finally, the extracts were analyzed using Z- sizer and the results obtained are those shown in **Figure 16**. The intensities in the range of 100 nm show the detection of magnetosomes and as all those cultures consumed iron, they are expected to produce magnetosomes.

Comparing the results of both ways of fed-batch cultures (**Figure 12**, **Figure 13**, **Figure 14** and **Figure 16**) it can be observed a similar behavior of results. Moreover, the fed-batch had better performance of growth than the continuous feeding, and nevertheless, the first one was the best in iron consuming. Both methodologies for fed-batch were tested under the same conditions of temperature, pH, time supplementing oxygen, culture medium, stirring and volume, but when observing the difference of color in pellets and the results of Z-sizer, it is assumed that both produced magnetosomes although the performance was different. In the **Figure 16** it can be observed a peak in an average size of 600-1,000 nm, which can mean an aggregation of magnetosomes after disruption. As the Z-sizer uses the refraction index to measures the size and percentages of particles, it is probable that the peak is represented by magnetosomes. Also, as the percentage intensity of the 600-1,000 nm peak is higher than the 100 nm peak, most of the magnetosomes must be aggregated. Additionally, it was observed that size variability of aggregated fraction increased in batch and continuous feeding cultures.

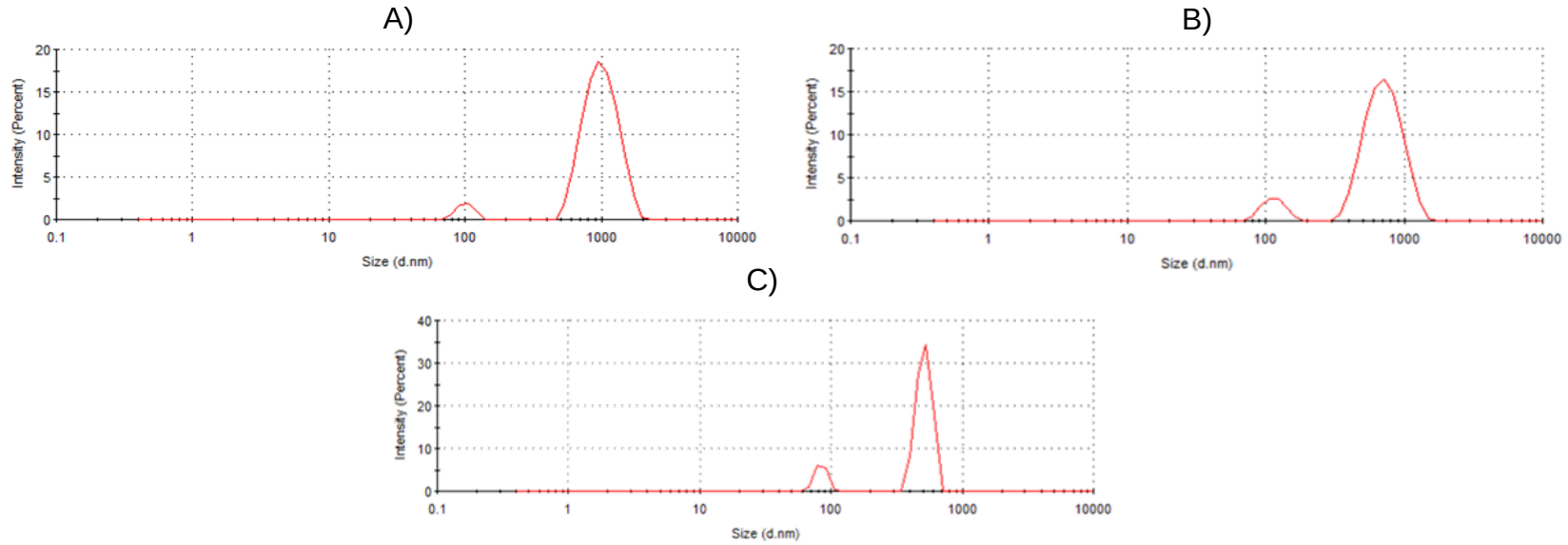

Figure 16. Distribution size of magnetosomes from batch and fed/batch cultures. Fractions recovered from decanted pellets using external magnet A) Batch culture; B) Continuous feeding; C) Fed-batch by addition of culture medium every 12 hours.

7. Conclusions

Magnetospirillum gryphiswaldense was tested under 100, 200, 400 and 600 stirring rates to improve the growth. It was found that *Magnetospirillum gryphiswaldense* grown at 200 rpm (using Rushton impellers) has better oxygen transfer ($1.62x10^{-4}$) than using lower or higher stirring rates. Also, from 400 to 600 rpm the Reynolds number increase (18,057 and 27,085) but also increasing the shear stress for the bacteria growth. This is the first time that such information is reported and can be valuable for future scale-ups in other bioreactors for bacterial growth and magnetosomes production models.

Magnetosomes were produced using a combination of aerobic growth, anaerobic stress after 12 hours and feeding of culture with growth medium. Indirect methods of detection were used in order to extract and isolate magnetic fraction from *M. gryphiswaldense* cultures. Iron consumption from fed-batch cultures followed by cell disruption and magnetic separation resulted in an isolated fraction corresponding to a clean mixture of particles of two main sizes: 100 nm and 600 nm. These correspond to the reported size of magnetosomes produced by *Magnetospirillum gryphiswaldense*. The average peak of 600 nm was probably an aggregate of magnetosomes.

The obtained experimental data becomes relevant to calculate the kinetic values that can be used for scaling-up and optimization to produce magnetosomes from laboratory to industry. This latter could be useful to have large quantities of magnetosomes to be used as nanocarriers for delivering molecules in therapies, attaching antibodies for diagnosis and specific cell isolation and for environmental applications such as pollutant recovery. More alternatives that are promising include as hyperthermia coadjutants, magnetic resonance and magnetic imaging.

8. Recommendations & future work

In this work, it was showed that *M. gryphiswaldense* can reduce its population dramatically when stressed by a transition from aerobic to anaerobic conditions. In yeast, when it is used the transition from aerobic to anaerobic conditions the temperature is decreased to reduce the death velocity and allow the cells to adapt new conditions. It is recommended for future works with this bacterium to reduce the temperature after the oxygen supply is stopped.

Another recommendation is to make fed-batch and continuous feeding by adding MSGM medium before hour 12, for example, at hour 8 and 10, when the bacteria are in logarithmic phase. Then the implementation of continuous culture will be useful to increase the production of magnetosomes. This methodology must include a technique of particle dispersion and avoid the aggregation of magnetosomes.

For researching the kinetic values for growing the magnetotactic bacteria it can be used microfluidics systems, because it allows better control of physical and chemical parameters than using big bioreactors. Additionally, because of the size, many mini bioreactors can be set up and the cost of culture medium per experiment is reduced.

The production of magnetosomes is important, but also it is necessary to separate them properly. Most methods used have used magnets for recovering the magnetosomes and some have used magnetic columns. An alternative solution could be to have a continuous system, that recover cells and lysate them, to separate the debris from magnetosomes and isolate them magnetically. For example, the use of CD-microfluidics, which can be adapted for different volumes and fluxes.

9. Appendix

9.1. Appendix A

List of Abbreviations

BCM	Biologically-controlled mineralization
BIM	Biologically-induced mineralization
BMP	Bacterial magnetic particles
MNPs	Magnetic nanoparticles
MTB	Magnetotactic bacteria
NP	Nanoparticles
M	Specific growth rate / dynamic viscosity
DO	Dissolved oxygen
MSGM	Magnetic Spirillum Growth Medium
rpm	Revolutions per minute
rps	Revolutions per second
OD	Optical density
ppm	Parts-per million
pH	Potential of hydrogen
REDOX	Oxidation-reduction
MRM	Master Reaction Mix
TEM	Transmission Electron Microscopy

9.2. Appendix B

List of symbols

°C	Celsius degrees
µL	Microliter
µM	Micromolarity
µmax	Velocity of growth per hour
ρ	Density
CAL	Dissolved oxygen at certain time point
cm	Centimeters
D	Diameter of Rushton impeller
g	Grams
h	Hour
K_{LA}	Oxygen transfer constant
Ks	Dissociation constant
L	Liters
m	Meters
M	Molarity
mg	Milligrams
min	Minute
mL	Milliliters
mM	Millimolar
mV	Millivolts
N	Normality
nm	Nanometers
pmol	Picomole
Re	Reynolds number
T	Generation time
tn	Time point n
V	Velocity of agitation
w/v	Weight/volume percentage
Xg	G-force
Yx/y	Productivity

9.3. Appendix C

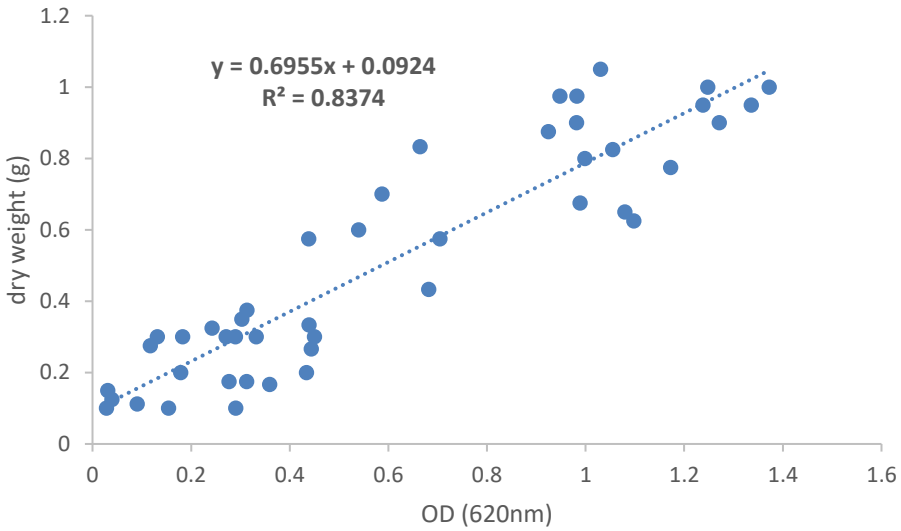

Figure 17-Appendix. Calibration curve with the relationship of dry weight and optical density. *The equation is shown inside the graph.*

9.4. Appendix D

Table 3-appendix.

	Stirring rate			
	100	200	400	600
Sample				
1	0.613	0.78	0.632	0.35
2	0.556	1.014	0.805	0.109
3	0.663	0.826	0.698	0.159
ANOVA details				
Source	*SS*	*df*	*MS*	
Between-treatments	0.7276	3	0.2425	*F*= 23.07883
Within-treatments	0.0841	8	0.0105	
Total	0.8117	11		
	p value = 0.000271			
	There is significant difference			

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11. Curriculum vitae

Jesús Gilberto Rodríguez Ceja was born in September 16, 1992, in Monterrey, Nuevo León. He graduated from the *Universidad Autonóma de Nuevo León* and earned the Genomic Biotechnologist degree in June, 2015. After starting his M.Sc. of Biotechnology, he focused his research on the project of this thesis. During his project and studies, he participated as speaker at a conference in Research and Development Conference in the Instituto *Tecnológico de Estudios Superiores de Monterrey*, Campus Monterrey, exposing the project “Optimizing the culture conditions of *Magnetospirillum gryphiswaldense* to produce magnetosomes”.