Scale-up and Optimization of Biogenic Magnetite Production

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Abstract

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Interest in biogenic magnetite nanoparticles is increasing as their potential applications are better understood. Magnetite can be produced by anaerobic Fe(III)-reducing and Fe(II)-oxidising bacteria. In this project the dissimilatory Fe(III)-reducing ability of *G. sulfurreducens* was harnessed for biogenic magnetite production. The main aim of this project was to scale up (from 5L to 50L, and potentially 750L volumes) and optimise the medium and growth conditions to achieve the highest biomass yield in the shortest time, while maintaining the Fe(III)-reduction ability of the grown cells at a high level. The results of this project suggest that, of the conditions tested, a defined “NBAF” medium containing 80 mM fumarate as the electron acceptor and 50 mM acetate as the electron donor, with 50 rpm stirring speed during incubation at 30°C produces the highest biomass yield. Using 20 mM bicarbonate buffer in the growth medium during the incubation period was sufficient to keep the pH between the ranges of 6.8-7.2. To optimise the process further, different concentrations of the electron shuttle (riboflavin) were used to find the optimal concentration to be used to accelerate the rate of Fe(III) reduction and subsequent biogenic magnetite production process. 10µM riboflavin concentration resulted in optimal magnetite production and therefore was considered as standard concentration for the purpose of this study. One of the most interesting observations of this project was the changes occurring in the growth pattern of the cells during the scale-up process. As the volume of the medium was increased, the lag phase of the cultures increased. This illustrates that the performance of microbial systems is not always predictable during scale-up. However, changes that were apparent during biomass preparation did not prevent the efficient production of bionanomagnetite at large-scale, which is an important step in the potential commercial use of these materials.
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Aims and objectives

The aims and objectives of the work described in this thesis were:

1. Optimization of growth medium to produce low cost, high biomass yields of the Fe(III)-reducing subsurface bacterium, *Geobacter sulfurreducens*.

2. Scale up of growth experiments from serum bottle (100 ml) to 5 L bioreactor and then to 50 L bioreactor, that latter with a commercial partner (Centre for Process Innovation).

3. Optimization of the conditions for subsequent magnetite production using washed cell suspensions of *G. sulfurreducens*.

4. Scale up of the biogenic magnetite production process from 10 ml bottles to 1 L and potentially 10 L vessels.
Literature review

Introduction

Interest in dissimilatory Fe(III) reduction has increased in recent decades as the importance of the Fe(III) reduction to the anaerobic degradation of organic matter and the geochemistry of soils and sediments has become apparent (Lovley et al., 2004, Lloyd, 2003).

Other factors that have promoted interest in this kind of respiratory process include potential practical applications such as the affects of dissimilatory Fe(III) reduction on the decomposition of organic matter in freshwater and marine sediments, aromatic hydrocarbon decomposition in contaminated aquifers, generation of high concentrations of dissolved iron Fe(II) in deep pristine aquifer, formation of other ferrous iron minerals such as siderite (FeCO₃) and vivianite (Fe₃(PO₄)₂•8(H₂O)), release of phosphate and trace metals into water supplies, soil gleying and the corrosion of steel (Lovley, 1993), the harvesting of electricity from aquatic sediments and waste organic matter (Bond and Lovley, 2003), coupling the oxidation of organic matter to the reduction of Fe(III) which results in generation and accumulation of magnetite in banded iron formations and also the production of commercially useful magnetic nanoparticles including magnetite Fe₃O₄ (Chaudhuri et al., 2001, Coker et al., 2007).

Since their discovery in the late 1980s (Lovley and Phillips, 1986), there are now many different species of Bacteria and Archaea known to have the ability to grow with Fe(III) as the sole terminal electron acceptor. The developments of new molecular tools and genomic data bases have accelerated the understanding of dissimilatory Fe(III) reduction, especially in species of Geobacter and Shewanella (Lovley et al., 2004).

In addition to significant advances in our understanding of the diversity and physiology of Fe(III)-reducing bacteria, there has been much interest in the products of Fe(III) reduction, especially in biogenic magnetite nanoparticles produced by these organisms. The potential applications of these novel biogenic magnetite nanoparticles (MNP) in different fields such as biomedical, biotechnology, engineering, material sciences, and environmental areas have attracted a lot of
attention recently. For example, they have the potential to overcome the limitations of conventional diagnostic techniques and offer a replacement to conventional therapeutic agents (Murbe et al., 2008, Nadejde et al., 2010). They also offer considerable potential for catalysing the reductive transformations of high valence toxic radionuclides, metals and organics, and could be applied during in situ and ex situ bioremediation programmes (Lloyd, 2003).

Dissimilatory Fe(III)-reducing microorganisms that have been studied extensively for their ability to produce crystals of magnetite as by-product of their metabolism, include Geobacter metallireducens, G. sulfurreducens and Shewanella oneidensis, which are members of the Proteobacteria. These bacteria combine the oxidation of fermentation products in anoxic environments to the reduction of Fe(III) as terminal electron acceptor and produce magnetite grains with sizes ranging from 10-50 nm (Majewskia and Thierrya, 2007, Coker et al., 2007).

The physical properties of the nanoparticles produced are of key importance, their small size and high surface to volume ratio provides unique properties such as superparamagnetism (Majewskia and Thierrya, 2007). Superparamagnetism is a property which allows a material to become magnetized under the influence of a magnetic field, with magnetization returning to zero once the field has been removed. It is a size dependent effect with superparamagnetism occurring in particles smaller than ~30nm. Studies have shown that 96% of magnetite nanoparticles produced by Geobacter metallireducens are superparamagnetic (Moskowitz et al., 1989).

Multiple factors affect the amount of magnetite produced by these microbial systems, for example the amount of Fe(III) present in the culture which is the primary factor in magnetite production (Frankel, 1987), and also the identity of the Fe(III) mineral (Cutting et al., 2009). Generally amorphous forms such as ferrihydrite are highly bioavailable for respiration, while crystalline phases such as hematite are not. Other factors such as the culture conditions (e.g. CO₂ concentrations) can also affect the size and crystallinity of the magnetite particles (Bazylinski et al., 2007). Although there are a lot of potential applications for magnetite nanoparticles, there is still question regarding the nano-materials biocompatibility and safety. One of the biggest challenges is engineering the surface of the nanoparticles to perform optimally in different in vitro and in vivo biological environments (Tsuji et al., 2006).
**Geobacter species**

The *Geobacteraceae* are a group of Fe(III)-reducing microorganisms which belongs to delta subclass of *Proteobacteria*. These microorganisms are classified as strict anaerobes, however, recent studies have showed that they readily tolerate oxygen exposure (Lin *et al*., 2004). *Geobacter metallireducens* was the first microorganism to be discovered that can completely oxidize organic compounds to carbon dioxide by reducing Fe(III) and other metal sources (Lovley and Phillips, 1988).

A key compound that acts as electron donor for these microorganisms is acetate, which is a central intermediate in the anaerobic metabolism of organic compounds in sedimentary environments (Lovley, 1995, Lovley, 2002). The *Geobacter* species have some unique properties such as completely oxidising organic compounds to carbon dioxide under anaerobic conditions coupled to the reduction of metals (Lovley and Phillips, 1988) that makes them an ideal candidate for bioremediation purposes.

*G. sulfurreducens* is another important member of the family *Geobacteraceae*, and is a mesophilic freshwater bacterium that can grow by coupling the oxidation of acetate to the reduction of Fe(III), sulphur, or fumarate (Caccavo *et al*., 1994, Galushko and Schink, 2000). This bacterium is used as a model to study *Geobacter* physiology as it is closely related to the *Geobacter* species that are predominant in the subsurface environment and also its complete genome is available (Methe *et al*., 2003).
Iron reduction

Iron is the most abundant metal on the planet and forms a wide range of mineral precipitates in different environments, such as iron oxides, hydroxides, or oxyhydroxide (Theil et al., 2010). Geological and microbiological evidence suggests that iron reduction may have been one of the earliest forms of respiration (Lovley, 2002). Geochemists suggest that high levels of ultraviolet radiation may have produced Fe(III) oxides and H$_2$ on the anoxic, prebiotic earth (Fig 1), which could have supported this form of microbial metabolism.

![Figure 1. Microbial metabolism on early biotic earth (Lovley, 2002)](Diagram)

There are two general mechanisms suggested for the iron reduction in the aquatic sediments:

1. Non-enzymatic reduction: where Fe(III) is reduced by either organic compounds or by development of low redox potential during microbial fermentation.
2. Enzymatic reduction: This can be carried out directly by Fe(III)-reducing microorganisms (Lovley et al., 1991b).

Until the last two decades, it was generally believed that most of Fe(III) reduction was the result of non-enzymatic reactions. This assumption was derived from three premises: 1. Reaction mechanisms demonstrated for Fe(III) oxides by some organic compounds under specific conditions (Zehnder, 1988). 2. The idea that, Fe(III)
reduction is a reversible redox reaction (Zehnder, 1988). Until the late 1980s, no organisms were known to be able to effectively couple the oxidation of organic compounds to the reduction of Fe(III).

However it has been indicated that the organic compounds capable of non-enzymatic reduction of iron are a small component of the organic matter in most sediments (Lovley, 1997). Studies have shown that very few organic compounds are capable of reducing iron non-enzymatically, however, in the presence of Fe(III)-reducing microorganisms, the oxidation of these organic compounds coupled to the reduction of Fe(III) may increase significantly (Lovley et al., 1991b).

Current information suggests that microorganisms that are not directly involved in Fe(III) reduction will break down the organic compounds to fermentation products, which will then be used by Fe(III)-reducers as primary electron donor (Lovley et al., 2004), which means they can work as a community.

Some bacteria indirectly reduce Fe(III) as a by-product of other modes of respiration, however, generally iron reduction serves two main purposes in bacterial metabolism. First, in a process termed assimilatory iron reduction, reduced iron is transported into the cell where it is utilized as cofactor in a range of cell processes including DNA synthesis, oxygen transport, and photosynthesis. Second, using dissimilatory Fe(III) reduction, ferric iron serves as a terminal electron acceptor, generating energy for cell growth (Theil et al., 2010). However, assimilatory iron reduction does not have a significant effect on the global ferric iron concentration, as the amount of iron needed by the bacteria is very small (Theil et al., 2010). Thus, dissimilatory iron reduction has a greater direct impact globally.

**Dissimilatory Fe(III) reduction**

Fe(III) reduction can have a significant impact on groundwater contaminated with waste materials such as petroleum and landfill leachates. In these kinds of environment, the increased microbial activity will deplete the oxygen, and Fe(III) is then generally the most abundant electron acceptor for organic compound degradation (Lovley et al., 2004). These activities convert the insoluble Fe(III) to the more soluble Fe(II), which can then move to the higher levels of the sediment where
oxygen is available and re-oxidises to form Fe(III)-bearing precipitates. This can clog wells and discolour the groundwater.

Most “conventional” terminal electron acceptors (TEAs) are usually soluble, readily taken up to microbial cells (by diffusion or transport processes) and then reduced at the cytoplasmic membrane, where the proton motive force-generating electron transport is located. However in case of insoluble Fe(III) oxides, the electrons located in the cytoplasmic membrane needs to pass through the periplasm to the outer membrane to reach the TEA. Here a wide range of c-type cytochromes are localised, and thought to play a role in “direct” electron transfer to the extracellular mineral as shown in Fig 2 (Lloyd, 2003, Pokkuluri et al., 2010).

In addition to the above direct mechanism of Fe(III) reduction, other recently identified electron transfer process can include;

Indirect mechanisms involving the use of electron shuttles such as humic acids and other quinone-containing compounds or secreted flavins such as FMN or riboflavin which can be reduced by the microbial cell and diffuse to the mineral substrate and transfer the electrons to mineral prior to returning to the microbial cell for further rounds of reduction and electron shuttling. The secretion of small organic chelate forming molecules that have high affinity for Fe(III) can also play a role, as these soluble forms of Fe(III) can then diffuse to the cell in a highly bioavailable form. Finally, the use of extracellular conductive pili (nanowires) to assist in electron transfer from the cell to the insoluble iron oxide (Reguera et al., 2005).
The last strategy mentioned has proved highly controversial. According to previous studies *G. sulfurreducens* does not have the ability to produce chelating agents or electron shuttles and can only reduce Fe(III) via direct contact. However, the bacterium can produce pili under some experimental conditions, in order to promote contact with Fe(III) minerals, when it is grown on insoluble iron oxides as electron acceptor but not soluble Fe(III) (Reguera *et al.*, 2005).

A more recent study (Malvankar *et al.*, 2011) has used *G. sulfurreducens* strain DL-1 and KN400 to confirm the role of pili in extracellular electron transfer. This study used microbial fuel cells with 10 mM acetate as the electron donor and an anode containing two probes with a distance of about 1.25 cm between them (Bridge gap) as electron acceptor. It was reported that when one of the probes was connected to the cathode, only that side of that probe was covered with a biofilm, and in the bridging part no biofilm was observed. However, when both probes were connected to the cathode, biofilms formed on both probes and the bridging gap and substantial DC conductance between the two electrodes was achieved. To test the role of the pili in electron transfer, the acetate was removed from the medium without a loss in conductivity, supporting a role of pili in electron transfer. To further test this hypothesis, a *G. sulfurreducens* strain was used that was genetically manipulated by

**Figure 2.** Different mechanisms for reducing insoluble iron oxides, a. direct contact between the cell and the surface of the particle, b. Using electron shuttling compounds (AQDS) to transfer the electrons, c. using chelating agent to solubilise the iron oxide (Lloyd, 2003).
deleting the genes coding four of the most abundant outer membrane $c$-type cytochromes (OmcB, OmcE, OmcS and OmcT). This strain had higher pilin content and also higher biofilm conductivity compared to the DL-1 strain.

**Impact of nitrate on metal reduction**

There has been a lot of controversy regarding the inhibition of Fe(III) reduction by nitrate in the past decades. The first hypothesis was that a broad specificity nitrate reductase may transfer electrons to either $\mathrm{NO}_3^-$ or Fe(III) but prefer the nitrate over ferric iron (Ottow, 1970). Other studies suggested that the inhibition effect of nitrate is due to secondary chemical reoxidation of Fe(II) by $\mathrm{NO}_2^-$ that is produced by denitrifying bacteria (Obuekwe *et al.*, 1981). Other investigators have found that bacterially produced $\mathrm{NO}_2^-$ is responsible for inhibiting the Fe(III) reduction (DiChristina, 1992).

A different study proposed that reduction of $\mathrm{NO}_3^-$ in experimental or subsurface environments by microorganisms usually happens before ferric hydroxide reduction, mainly because $\mathrm{NO}_3^-$ is more available to the cells compared to ferric hydroxide which is insoluble in neutral pH (Achtnich *et al.*, 1995). On the other hand, more recent studies have reported that the presence of solid phase ferric hydroxide (goethite) inhibits the reduction of $\mathrm{NO}_3^-$ and $\mathrm{NO}_2^-$ by *S. oneidensis*, but enhances the production of $\mathrm{N}_2\mathrm{O}$ (Cooper *et al.*, 2003, Coby and Picardal, 2005). This behaviour can result from reactions between some of the biogenic Fe(II) produced with $\mathrm{NO}_2^-$, and formation of ferric hydroxide coating on the cell surface which inhibits the transportation of electron acceptors into the cell.

Any process that limits the $\mathrm{NO}_3^-$ reduction may have an important impact on the environment. For instance, the bioremediation of $\mathrm{NO}_3^-$ contaminated land may take longer while enhanced $\mathrm{N}_2\mathrm{O}$ production is undesirable as it is a potent greenhouse gas and believed to be involved in stratospheric ozone depletion (Crutzen, 1970).

**Bioremediation of uranium**

Uranium is a widespread contaminant of groundwaters at sites where uranium is mined or processed. Reduction and precipitation of uranium is an important process affecting the uranium levels in seawater and marine sediments. Current strategies for
treatment of uranium contaminated aquifers is based mainly on pump and treat technologies which are not efficient in keeping the groundwater uranium levels low (Abdelouas et al., 1999). However, an alternative remediation method is to reduce U(VI) to U(IV) which is insoluble and precipitates in subsurface (Lovley et al., 1991a, Lloyd, 2003). There are several mechanisms suggested for the reduction of U(VI) to U(IV) in anaerobic subsurface environments. Early studies suggested that sulphide may abiotically reduce U(VI) (Jensen, 1958), and more recent studies have suggested the reduction of U(VI) by Fe(II) and hydroquinones (Fredrickson et al., 2000). However, these proposals are debatable, as the results are from experiments that have been carried out with defined media and not with the actual sediments, and defined media may not truly replicate reactions that take place in situ (Lovley et al., 1991a).

Microbially-catalyzed reduction of U(VI) to U(IV) has been proposed as the most likely naturally occurring mechanism for the reduction of uranium (Lovley et al., 1991a). Many dissimilatory metal-reducing microorganisms can reduce uranium U(VI) which is soluble in water to the insoluble form U(IV). Most microorganisms that have the ability to conserve energy by oxidizing organic compounds or hydrogen coupled to reduction of Fe(III), can also use U(VI) as electron acceptor and reduce U(VI) to U(IV) and consequently precipitate and remove uranium from ground water (Lovley and Anderson, 2000). Furthermore, sulphate-reducing microorganisms have also shown the ability to reduce U(VI) (Lovley, 2002).

Recent studies have shown that microbial reduction of U(VI) can be stimulated in aquifer sediments with the addition of appropriate organic electron donors which will enhance the growth of organisms in the family Geobacteraceae and increase the rate of U(VI) reduction (Finneran et al., 2002). This may be an effective mechanism for removing U(VI) from contaminated groundwater. Finneran et.al, have also suggested, based on molecular characterization of the microbial community in the sediments, that Fe(III)-reducing bacteria were responsible for U(VI) reduction in the sediments.

Bioremediation of uranium offers several advantages over other technologies for uranium removal, including the following (Lovley and Phillips, 1992): precipitating uranium from U(VI)-carbonate complexes, highly concentrated and pure recovery of uranium, high uranium removal to biomass ratio, the ability to treat organic
contaminants and uranium at the same time by using the organic contaminant as electron donor to reduce uranium, and the potential for in situ ground water remediation.

In conclusion, it is now believed that microbial reduction of uranium can have an important role in the biogeochemical cycling of U, and it also can be used to remove and concentrate U from contaminated ground and surface waters or from contaminated soil wash through reductive precipitation.

**Bioremediation of chromium**

There are many different sources for chromium contamination including; manufacturing processes, tanning industry, domestic wastewater and the dumping of sewage sludge (Lovley, 1995). The chromium in natural waters is usually found in the form of Cr(VI) and/or Cr(III).

In the past decade, major concerns have arisen by environmental contamination of chromium, especially hexavalent chromium, Cr(VI). Cr(VI) is highly soluble, toxic and a potent carcinogen and mutagenic to humans and animals and may contribute to death if ingested in large doses. However, Cr(III) is much less toxic and forms insoluble hydroxides (Zayed and Terry, 2003). Therefore, reduction of Cr(VI) to Cr(III) can be a good mechanism for restoration of chromium-contaminated environments. Cr(VI) can be readily immobilized in soils by adsorption, reduction, and precipitation processes.

There are different factors affecting the microbial reduction of chromium in soil, including the concentration of organic compounds, and oxygen levels (Losi et al., 1994). Organic matter in the soil may act as an electron donor and enhance the microbial activity in the soil which will accordingly increase the reduction of Cr(VI). As the microbial activity increases, oxygen is depleted through microbial respiration creating reducing conditions. Many studies have demonstrated that there is a potential for the bioremediation of chromium-contaminated environments (Losi et al., 1994, Krishna and Philip, 2005).
Reduction of technetium 99

Technetium 99 is formed in large quantities during nuclear reactions and has been discharged into the environment during weapons testing and waste disposal. Due to these activities, $^{99}\text{Tc}$ has been found in ground water at locations where nuclear wastes are processed and stored (Macaskie, 1991). Factors that add to the importance of $^{99}\text{Tc}$-contaminated sites include, the long half-life of $^{99}\text{Tc}$ (2.13× $10^5$ years), formation of pertechnetate anion ($\text{TcO}_4^-$) which is highly mobile in the environment and also the potential uptake of pertechnetate by plants as an analog of sulfate and subsequent entry into the food chain (Cataldo et al., 1989).

Recent studies have shown that $^{99}\text{Tc}$ (as Tc(VII)) can be removed from contaminated solution by reduction of pertechnetate to insoluble TcO$_2$ (Lloyd et al., 2000). This can be attained under anoxic conditions by abiotic mechanisms mediated by zerovalent iron or Fe(II)-containing minerals. For example, Fe(II) minerals in igneous rocks can reduce pertechnetate and lead to sorption on mineral surfaces (Bondietti and Francis, 1979). Cui and Eriksen (Cui and Eriksen, 1996) have reported that magnetite also has the potential to reduce Tc(VII) with higher reduction rate than those for Fe(II)-containing minerals hornblende and chlorite. Soluble ferrous iron may also be able to reduce Tc(VII) but its reduction rate is too low to be used to control technetium mobility.

Studies have also shown an increase in Tc removal by adding sulphate-reducing bacteria to a mix culture of anaerobically grown soil bacteria (Henrot, 1989). The study assumed that removal of Tc was mediated by an indirect mechanism utilizing microbially generated H$_2$S. On the other hand, other studies have demonstrated a direct enzymatic reduction of Tc(VII) by sulfate-reducing bacteria (Lloyd and Macaskie, 1996, Lloyd et al., 1999). The results of the studies above suggest that anaerobic bacteria, including Fe(III)-reducing bacteria may play an important role in immobilizing technetium in sediments via direct and indirect mechanisms.

Magnetite nano particles

Magnetite is a form of iron-oxide mineral with the chemical formula Fe$_3$O$_4$, which shows strong magnetism. There are other minerals with magnetic properties such as ilmenite (FrTiO$_3$) and hematite (Fe$_2$O$_3$), yet, compared to magnetite they show a weak magnetism. Magnetite is usually found in nature as small particles. However,
they do appear as larger crystallized minerals in a form known as lodestone which has been used as compass by sailors in the past. Magnetite crystals are not only found in sedimentary environments but also in magnetotactic bacteria, and in the brains of bees, birds, termites, and also humans. It has been suggested that these magnetic crystals might be involved in magnetoreception, which has been lost by humans (Frankel, 1984).

Biogenic magnetic minerals are produced during microbial metabolism and can be found in different subsurface environments. These microbial activities may have a great influence on the environment by; 1) affecting the biogeochemistry and global cycle of iron (Lovley et al., 1987), 2) as they can use hydrocarbons as electron donors in their reduction activities it may also have an effect on the carbon cycle and 3) the Fe(II) present in the magnetite structure may also change the solubility of some trace metals through passive sorption and/or reductive mechanisms (Fredrickson et al., 2000).

Magnetite is a member of spinel group (MgAl₂O₄), with a cubic close packed oxygen array crystal structure (Matsuda et al., 1983) (Fig 3). The crystal contains sublattices in tetrahedral (Tₜ) and octahedral (Oₜ) coordinations. The unit cell of magnetite consists of eight Fe³⁺ₜ surrounded by four oxygen atoms, eight Fe³⁺ₒ and eight Fe²⁺ₒ each surrounded by eight oxygen atoms (Majewskia and Thierrya, 2007).

![Figure 3. Structure of magnetite, with Fe atoms in the octahedral and tetrahedral sites (PATTRICK et al., 2002).](image)

The magnetism of magnetite is dependent upon the location of the Fe²⁺ and Fe³⁺ within the crystal lattice. The magnetic orientations of octahedral and tetrahedral environments are anti-parallel, thus the magnetic moment from the Fe³⁺ₜ and
Fe\textsuperscript{3+}O\textsubscript{h} exactly cancel each other out due to their equal quantities per unit cell. The magnetism of magnetite can thus be considered to be entirely due to the magnetic moment from Fe\textsuperscript{2+} in the octahedral environment. This leads to the potentially significant effects on the particle magnetisation by the incorporation of transition metal dopants such as cobalt and zinc which substitute in place of either Fe\textsuperscript{2+}O\textsubscript{h} or Fe\textsuperscript{3+}T\textsubscript{d} within the crystal lattice (Coker et al., 2008).

**Synthesis of magnetite nano particles**

The high number of potential applications of magnetite nano particles (MNP) has resulted in several synthetic routes with different levels of control on the size, polydispersity, shape, and crystallinity.

**Co-precipitation method:** This method is based on precipitation of magnetite by adding a strong base to a solution of ferrous, ferric salts in water. By using this technique there is some control over the size and composition of particles through the ratio of Fe(II)/Fe(III) salts and also by changing the reaction conditions (e.g., pH, Temperature). The MNPs produced by this technique are highly aggregated and are generally between the size range of 15 to 50 nm, as a result they form polydisperse structure (Majewskia and Thierrya, 2007).

**Thermal decomposition method:** This method consists of thermal decomposition of iron-oleate complex derived from an iron precursor (e.g., iron acetylacetonate, iron cupferronates, and iron carbonyls) in hot organic solvents and presence of stabilizing surfactants such as oleylamine, oleic acid, and steric acid (Majewskia and Thierrya, 2007).

This method produces high quality monodisperse and monocrystalline iron oxide nanoparticles. The MNPs produced are in the 5 to 40nm size range and can be controlled by reaction time and temperature, and the ratio of iron / surfactant (Fig 4).

This method provides a much better control over size of the MNPs compared to the co-precipitation method. However, the potential issue of this technique is the use of solvents and surfactants that may reduce the biocompatibility of the product (Majewskia and Thierrya, 2007).
The reaction equation below shows that the iron precursor (iron oxo-hydrate) + surfactant (oleic acid), and solvent (1-octadecene) which produces an intermediate iron-oleate complex that is thermally decomposed upon heating the reaction mixture to the 320 °C temperature to form MNP (Fisher and Barron, 2009).

\[
\text{FeO(OH)} + \text{OA} \xrightarrow{\Delta} \text{Fe(Oleate)} + \text{H}_2\text{O} \xrightarrow{\Delta320^\circ\text{C}} \text{Fe}_3\text{O}_4 \text{ nanoparticles}
\]

**Figure 4.** The TEM pictures shows the effects of reaction time on the size of the MNPs (time decreases from left to right), the picture on the right shows surfactant coated MNP (Fisher and Barron, 2009).

**Biogenic magnetite:** Magnetite can be produced through biomineralization, which is a process by which organisms form minerals. There are two types of biomineralization; 1) biologically-induced mineralization (BIM) and 2) biologically-controlled mineralization (BCM). Magnetite is known to be produced via both mechanisms (Bazylinski *et al.*, 2007).

1. Biologically induced mineralization (BIM): *Geobacter* and *Shewanella* are two examples of well studied Fe(III)-reducing bacteria that can form magnetite as a byproduct of their metabolism. These bacteria have the ability to oxidise the fermentation products of other bacteria, coupled to the anaerobic reduction of Fe(III)-oxides and produce magnetite nanoparticles. MNPs produced by this route are epicellular, not aligned in chains, and are in the size range of 10-50 nm (Lovley *et al.*, 1987).

The amount and quality of the magnetite produced through this method is limited by the amount of Fe(III) present in the culture (environment) and also other growth conditions such as pH and temperature (Frankel, 1987). Moskowitz *et al.*, using magnetic analysis have shown that about 96% of MNPs produced by *Geobacter Metallireducens* in cultures are superparamagnetic (Moskowitz *et al.*, 1989).

2. Biologically controlled mineralization (BCM): what makes this type of mineralization different from the one mentioned above is that microbes show a
considerable active control over nucleation and different stages of mineral growth (Mann, 1988). A good examples for BCM is the deposition of intracellular magnetite by magnetotactic bacteria in organelles known as magnetosomes which are arranged in intracellular chains (Blakemore, 1982). These bacteria inhabit aerobic and anaerobic sediments and form single domain magnetic particles that show species specific morphology. Unlike BIM, magnetite crystals produced by BCM (magnetotactic bacteria) shows a high chemical purity. *Magnetospirillum magnetotacticum* and *Aquaspirillum magnetotacticum* are examples of magnetotactic bacteria (Blakemore, 1982).

Magnetite synthesis by magnetotactic bacteria involves a series of geochemical stages which starts with uptake of iron from the environment. It has been suggested that these bacteria excrete chelating agents (siderophores) that bind to the iron and increase its solubility, facilitating transportation into the cell. Once the siderophores-Fe(III) complex is formed, the next step is to transport the complex into the cell which is achieved by the specific receptor proteins located in the outer membrane which recognizes the Fe(III)-siderophore complex and with the help of other transporting proteins guides the complex into the cytoplasm (Neilands, 1989). The Fe(III) is then reduced to Fe(II) by Fe(III) reductase enzymes in the cell and transported into vesicle-like organelles known as magnetosomes. It is presumed that the Fe(II) in the magnetosome will re-oxidise to form Fe(III)-hydroxide using oxygen as terminal electron acceptor. The produced Fe(III)-hydroxide will then react with the excess Fe(II) and form magnetite (Bazylinski *et al.*, 2007).

Studies have also reported that some DIRB (e.g. *Shewanella oneidensis*) can produce a large amount of nanosize intracellular ferric hydroxide under anaerobic conditions, which are partially made of magnetite or maghemite and are surrounded by a membrane like structure (Glasauer *et al.*, 2002). This is an example of a type of biomineralization which is between BIM and BCM and the produced mineral shows qualities of minerals produce by both methods.

**Applications of magnetite nanoparticles**

There has been a lot of interest in the use of magnetic nanoparticles in recent years. These nanotechnology constructs have the potential to overcome the limitations of conventional diagnostic techniques and offer a replacement for conventional
therapeutic agents. Magnetite nanoparticles show a high potential to be used for in vivo applications such as contrasting agents for magnetic resonance imaging (MRI), magnetically targeted drug delivery, hyperthermia treatment processes, and thermoablation agents (Majewskia and Thierrya, 2007). They also have the potential for applications including bioremediation of contaminated ground waters and for magnetic data storage devices.

**Biomedical applications**

One of the main areas for MNP applications is in the development of bioassays, where magnetic properties of nanoparticles are used in vitro to manipulate the particles in a magnetic field. Two major factors for in vivo use of MNPs are their size and surface functionality (Faraji et al., 2010). Particles with the size range of 10-40 nm are optimal for prolonged blood circulation as they can cross capillary walls.

Reducing the none-specific adsorption events is another important requirement for nanoparticles to be used successfully in biomedical applications, for example adsorption of plasma proteins on to the MNPs surface which can reduce the efficiency of the bioassay. An essential requirement for nanoparticles to be used in vivo is their ability to resist opsonin adsorption which will cause rapid elimination of nano particles from blood by the mono nuclear phagocyte and results in accumulation of nanoparticles in reticuloendothelial system such as spleen and liver (Vonarbourg et al., 2006). The amount and types of plasma proteins adsorbing on the nano particles rely on the physicochemical characteristics of the particles and can be manipulated through surface engineering (Majewskia and Thierrya, 2007).

**Drug delivery**

There have been several attempts to functionalize the surface of MNPs with proteins, drugs, and genetic materials in order to attain localized delivery of therapeutic agents (Majewskia and Thierrya, 2007, Medarova et al., 2007). The benefit of this type of treatment is reducing the systemic toxicity of non-targeted administration of these agents resulting in reduced dosage required. Magnetite nanoparticles combined with an external magnetic field allow the delivery of the agent to the desired target while the medication is released locally.

Medarova et al., 2007, have shown the incredible potential of MNPs in medical applications by developing a dual purpose probe for in vivo transfer of siRNA and
the simultaneous imaging of its accumulation in tumours by high-resolution MRI and near-infrared in vivo optical imaging.

**Hyperthermia**

Hyperthermia is known to be an effective cancer therapy strategy. As cancer cells are sensitive to high temperature, when they are exposed to temperatures around 42-46 °C significant cell death can be achieved. The problem with heat based cancer therapy techniques is the ability to target only the cancer tissue and not damaging healthy cells.

A lot of interest has been focused on using MNPs to achieve intracellular hyperthermia for cancer therapy, for instance by targeting MNPs coated with monoclonal antibodies such as anti-HER2 (Rezaeipoor et al., 2009). When superparamagnetic iron oxide is placed in alternating current magnetic field the directions between parallel and antiparallel orientations randomly flips, which allows the transfer of magnetic energy to the particles in the form of heat that can result in hyperthermia (Faraji et al., 2010). This is a technique particularly suited for treatment of pancreatic and brain tumours where it is dangerous and difficult to perform invasive surgery.

**Bioseparation**

Magnetic separation has been used in many biotechnology applications such as cell sorting, purification, and immunoassays (Franzreb et al., 2006, Berensmeier, 2006). MNPs are ideal candidates for these purposes because they provide control over the transportation and separation of biomaterial using an external magnetic field. This procedure is based on labelling biological entities by superparamagnetic colloids and then separating them under an external magnetic field.

The small size and high surface area of nano particles provides a much better bioseparation compared to conventional micrometer-sized resins or beads. Some other advantages of using MNPs over conventional methods include, good dispersability, reversible and controllable flocculation, and faster and more effective binding of biomolecules (Faraji et al., 2010).

There are also commercially available products that can be used for applications such as DNA, RNA, and protein purification using MNPs. Using magnetite nanoparticles with the right buffer for separation of DNA will allow a quick and efficient
purification directly after extraction from cell extracts, which replaces the laborious and time consuming centrifugation steps. Moreover, the new approach has automated the entire process which makes the isolation of DNA from larger sample volumes a lot easier (Arakaki et al., 2008).

Environmental applications

Magnetic filtration has attracted a lot of interest as a tool for remediation of heavy metal contaminated water (Ambashta and Sillanpää, 2010). Yet, because these contaminants are not magnetic it is necessary to develop nanostructure filtration aids to adsorb them to help magnetic separation (Campos et al., 2010). Iron nano particles can be modified to improve their speed and efficiency of remediation (e.g. catalysed and supported nanoparticles). An example for environmental applications of MNPs is discussed below briefly.

Magnetite can be used to remove different types of substances from the water including, dissolved metal species, particulate matter, and organic and biological materials. Metal contaminants may have different sources such as 1) industrial wastes from the semiconductor, chrome plating, and mining industries, 2) the production of military equipment and weapons, 3) careless or intentional dumping of consumer and industrial products into municipal and residential water, 4) Agricultural runoff and leaches (Cotten et al., 1999).

In areas where it has a history of radioactive activities, for example production of materials for nuclear weapons or industrial wastes, different intensely radioactive fission products and byproducts can be found, among which $^{90}\text{Sr}$, $^{137}\text{Cs}$, and $^{60}\text{Co}$ are the most important (Ebner et al., 2001). Different strategies have been used to remove these species from aqueous solutions, such as precipitation, extraction, ion exchange, adsorption, bioaccumulation, and electric field assisted techniques.

Using magnetite as an adsorbent provides the advantage of being readily available and inexpensive (compared to other sources of adsorbent) and also its ferromagnetic properties improves its decontaminating activity. When magnetite is fixed with in a magnetic field, its magnetic characteristics will allow the retention of paramagnetic contaminant particles. Magnetite can be fixed in resins or inorganic sol-gels to be used in packed column which reduces the operational costs and improves its performance. Alternatively it can be used as metal ion adsorbent, where its
ferromagnetic properties will be used to recover it from the batch adsorption process by high gradient magnetic separation (Ebner et al., 2001).

Most metal ions in water supplies show substantial toxicity above critical concentrations. Therefore, there is a need to develop efficient procedures to remove metal ions from water and physiological fluids. For example, water polluted with arsenic adversely affects the health of millions of people (Berg et al., 2007, Samadder, 2010, Gunduz et al., 2010). Iron oxides can bind to arsenic in aqueous solutions. A recent study evaluated the effect of magnetite nanoparticles functionalized with oleic acid on the removal of arsenic from water (Mayo et al., 2007). This study reported, a complete removal of arsenic from the solution containing up to 45 μmol/L of arsenic by magnetite nanoparticles of about 12nm in diameter.

The high dispersion through a liquid with ease of recovery of MNPs makes it an ideal candidate to be used as support material for catalysts (Coker et al., 2010, Yoon et al., 2003, Lu et al., 2007). Coker et al, 2010, investigated a novel biotechnological route for the synthesis of a heterogeneous catalyst consisting of reactive palladium nanoparticles arrayed on a nanoscale biomagnetite support. This study tested the Pd0_biomagnetite for catalytic activity in the Heck reaction coupling iodobenzene to ethyl acrylate or styrene, and reported a near complete conversion to ethyl cinnamate or stilbene within 90 and 180 min, respectively. Thereby, using the above technique will not only facilitate the isolation and recovery of expensive catalysts, but also open a new avenue to regulate their activity and selectivity.

Biomagnetite may also be used for remediation of metal oxyanion-contaminated waters. For example it can be used for reduction and immobilization of Cr(VI). Chromium contamination of environment is a world-wide dilemma caused by the use of metal in a wide range of applications (more details is described in previous sections). Recent studies reported that biogenic MNPs produced from synthetic schwertmannite powder to be more efficient in reducing Cr(VI) than biogenic MNPs produced from a suspension of ferrihydrite or synthetic nano-scale Fe3O4 powder (Cutting et al., 2010).

The above studies suggest that with having tailored functionalities for the remediation of targeted pollutions, magnetite nanoparticles may have promising opportunities for applications of remediation of polluted environments.
Experimental design and methods

*Geobacter sulfurreducens* strain PCA was obtained from the Manchester University Geomicrobiology group culture collection and used for this study. Cells were grown in batch culture systems with medium containing a range of electron acceptor and donor ratios in order to optimise biomass yield.

All experiments were carried out with triplicate samples and the means are displayed in graphs with bars representing standard error at 95% (error bars not shown where smaller than marker).

Standard protocols

Media and growth conditions (standard protocol)

NBAF was used as the standard growth medium for *G. sulfurreducens* which contained, 4.64g/L (40mM) fumarate, 2.04g/L (25mM) sodium acetate, 10ml/L 100×NB Mix (appendix 2), 10ml/L NB mineral elixir (appendix 3), 15ml/L vitamin mix (appendix 4), 0.04g/L CaCl₂ 2H₂O, 0.1g/L MgSO₄ 7H₂O, 1.8g/L NaHCO₃, 0.5g/L Na₂CO₃, 1ml/L 1mM Na₂SeO₄. The medium was made according to the protocol included in appendix 1 and the pH was adjusted to 7.1 using 10N NaOH. Degassing was performed by purging with 80:20 N₂:CO₂ gas under sterile conditions for 45-60 min.

Microbial growth in 100 ml serum bottles (standard protocol)

Degassed NBAF was transferred into 120 ml serum bottles (90ml medium) under anaerobic conditions in an anaerobic cabinet, and finally bottles were autoclaved at 126°C for 15 min and kept at room temperature. Culturing was carried out by inoculating the prepared medium with a 10% vol/vol *G. sulfurreducens* using aseptic technique under a sterile stream of nitrogen, with incubation at 30°C under strictly anaerobic conditions.

Microbial growth in 6 L bioreactor (standard protocol)

For the purpose of this study a 6 L Applicon bioreactor was used (Figure 5) for degassing, pH control, temperature control and stirring during the incubation period.

4.5 L of NBAF was prepared as mentioned above and decanted into the Applicon bioreactor and autoclaved at 126°C for 15 min. Degassing the medium was performed using N₂ gas with 1bar pressure for 60 minutes. The pH of the medium was adjusted to 7.1 using sterile 1M MOPS and 3M NaOH solutions. Redox measurements were
taken using a Mettler Toledo M300 pH meter and measurements were saved on a computer using Bioexpert software.

Different media with various concentrations of electron donor and acceptor were used, in order to optimise biomass yields (described in more detail in the Optimization section).

**Figure 5.** Appicon bioreactor connected to the control centre, which was used for scaling up the growth of *G. sulfurreducens* to 5 L batch culture.

**Preparation of bacterial cells for inoculation into iron oxide containing medium**

The preparation of *G. sulfurreducens* for inoculation into iron oxide containing medium was carried out as follows throughout the study:

After incubation under anaerobic conditions at 30ºC for 24 hours, the bacteria were prepared for inoculation into bottles (10 ml) containing insoluble iron oxide (ferrihydrite) by pelleting the cells by centrifugation at 5000 RCF at 4ºC using a Sigma centrifuge 6K15 for 20 mins. After centrifugation, the supernatant was removed and the cell pellets were washed with degassed and sterile NaHCO₃ buffer (30 mM), resuspending the biomass in 100 ml of NaHCO₃ under anaerobic conditions (under N₂ gas) and centrifuging again as before.

The washing step was repeated twice and between each wash the supernatant was removed and replaced with new NaHCO₃ buffer. After the final spin the supernatant was removed and the remaining cell pellet was suspended in 2 ml buffer and transferred to a previously prepared sterile, sealed and degassed (N₂ gas) 10 ml
bottle. The OD of the cell suspension was recorded at 600nm wavelength using a Camspec M501 spectrophotometer. Based on the OD of the solution, the correct amount of buffer was added to the cell suspension to optimise the OD to $OD_{600nm} = 0.4$ for use in Fe(III) reduction experiments.

**Standard preparation of ferrrihydrite (“Iron Gel”)**

FeCl$_3$ was used to synthesise insoluble Fe(III) in this experiment. Ferrrihydrite was prepared according to the protocol described in appendix 6, by the controlled addition of base, and the ferrozine-based Fe(II) assay was used to quantify total iron levels in order to make the right concentration of iron gel. 100 µl of “iron gel” suspension was added to 200 µl of newly made 0.07 M hydroxylamine and 4.7ml of 0.5M HCl. The solution was left overnight and the next day, it was diluted to 1/50 using 0.5M HCl (100 µl of the solution + 4.9 ml HCl). The ferrozine assay was then carried out by adding 50 µl of the solution to 2.45 ml of Ferrozine and the absorption was read using a Camspec M501 spectrophotometer at wavelength of 562nm.

Ferrrihydrite suspensions for microbial reduction were made by dissolving 1.64g/L sodium acetate, 2.52g/L NaHCO$_3$ buffer, and appropriate amount of mineral suspension (from an approx 1 mole litre$^{-1}$ stock solution) to reach a final concentration of 50 mM litre$^{-1}$ ferrrihydrite in DI water (1 litre). Degassing the FeGel was carried out by bubbling 80:20 N$_2$:CO$_2$ through the suspension for 1h. The ferrrihydrite suspension was decanted into 10 ml vials under anaerobic conditions (anaerobic cabinet) and sealed to be autoclaved for 15min at 126ºC. After sterilization, each bottle was injected with filter sterilised riboflavin (10uM).

**Media and other optimization experiments**

The individual experiments carried out during this project are described in discrete sections:

1. **Optimization of ferrrihydrite bioreduction**

1.1 **Number of washes needed for preparation of ferrrihydrite solution**

The lab-scale production of amorphous iron oxyhydroxide (ferrrihydrite) by published methods (Schwertmann and Cornell, 2007) is slow and manually intensive, requiring centrifugation six times, with the iron mineral pellets resuspended after each spin.
The centrifugation step is performed to remove Cl− ions associated with the Fe mineral. In order to find the optimal number of centrifugation and washing steps and also simplify the published protocol, seven different sets of ferrihydrite was prepared with differences in the number of centrifugation and washing steps that were done during the preparation, with 0-6 washing cycles tested.

The ferrihydrite suspensions were then inoculated with the correct volume (to give the final OD_{600nm} = 0.4) of an overnight (late log phase) culture of *G. sulfurreducens* and incubated at 30ºC under anaerobic conditions with 20 mM acetate as an electron donor in 30 mM bicarbonate buffer (pH = 7.1). Samples were taken under anaerobic conditions (under a stream of N₂) at time intervals and analysed for Fe(II) using the ferrozine assay to quantify the rate of Fe(III) reduction.

1.2 Mediator optimization

Serum bottles containing ferrihydrite suspensions in carbonate buffer supplemented with acetate as described previously were supplemented with the following to determine the optimal electron mediator to accelerate magnetite synthesis from Fe(III) bioreduction; 10µM AQDS, 10µM riboflavin or 50µM riboflavin as mediator (with a negative control containing no mediator). All incubations were carried out in triplicate. The bottles were inoculated with an overnight culture of *G. sulfurreducens* and incubated at 30ºC.
2. *G. sulfurreducens* (100 ml Batch culture optimization)

The table below (Table.1) shows a summary of different 100 ml-scale experiments carried out for optimization of the medium, more detailed descriptions can be found in the following sections.

### Table 1 Summary of different types and combinations of electron donor/acceptor and other amendments in 100 ml-scale experiments.

<table>
<thead>
<tr>
<th>Experiment section</th>
<th>Electron Acceptor</th>
<th>Electron Donor</th>
<th>NB Mix</th>
<th>Mineral Elixir</th>
<th>Vitamin Mix</th>
<th>Yeast Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Section 2.1</td>
<td>40, 100, 150 mM fumarate, 40 mM potassium nitrate</td>
<td>25 mM acetate</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Section 2.2</td>
<td>40, 100, 150 mM fumarate</td>
<td>25, 50 mM acetate</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Section 2.4</td>
<td>40 mM fumarate</td>
<td>25 mM acetate</td>
<td>Yes</td>
<td>Yes</td>
<td>varied</td>
<td>varied 0.01%, 1%</td>
</tr>
<tr>
<td>Section 2.5</td>
<td>40, 80 mM fumarate</td>
<td>25, 50 mM acetate</td>
<td>varied</td>
<td>varied</td>
<td>varied</td>
<td>0.1%, 0.5%, 1%</td>
</tr>
</tbody>
</table>
2.1 Optimization of electron acceptor
An experiment was carried out by setting up 4 sets of 120 ml bottles (in triplicate) containing 90 ml of NBAF medium containing 25 mM acetate as the electron donor, and different concentrations of fumarate (40 mM, 100 mM or 150 mM) or nitrate (40 mM) as the electron acceptor.

The bottles were inoculated with a 10% vol/vol inoculum of an overnight culture of G. sulfurreducens and incubated at 30°C. Samples (1ml) were taken in different time intervals and the OD$_{600nm}$ recorded using a Camspec M501 to monitor growth.

2.2 Optimization of fumarate/acetate ratios
This experiment was carried out by setting up 5 sets of 120 ml bottles (in triplicate) of NBAF containing different concentrations of fumaric acid and sodium acetate to identify the ratio of electron acceptor/donor that provides the highest biomass yield. The combinations tested were 40 mM fumarate / 25 mM acetate, 100 mM fumarate / 25 mM acetate, 150 mM fumarate / 25 mM acetate, 100 mM fumarate / 50 mM acetate and 150 mM fumarate / 50 mM acetate.

The bottles were inoculated with a 10% vol/vol inoculum of an overnight culture of G. sulfurreducens and incubated at 30°C. Samples (1 ml) were taken in different time intervals and the OD$_{600nm}$ recorded using a Camspec M501 to monitor growth.

2.3 Investigating Fe(III) reduction capability of cells grown with various fumarate/acetate ratios
Five sets of bottles were prepared containing NBAF with the combination of electron acceptor/donor ratio as described for the previous experiment (2.2). The bottles were inoculated with 10% vol/vol inoculum of an overnight culture of G. sulfurreducens and incubated at 30°C for 23 hours under anaerobic conditions. After the incubation period the cells were centrifuged and washed 6 times.

Ferricyride suspensions were then inoculated with an overnight culture of G. sulfurreducens (to a final OD$_{600nm}$ of X) and incubated at 30°C under anaerobic conditions with acetate as an electron donor in 30 mM bicarbonate buffer (pH = 7.1). 10 µM riboflavin was used as mediator in all the bottles (the experiment was carried out in triplicate). Samples were taken under anaerobic conditions (under a stream of N$_2$) at time intervals and analysed for Fe(II) using the ferrozine assay to quantify the rate of Fe(III) reduction.
2.4 Replacing the vitamin mix in the NBAF with yeast extract

Four sets of 120 ml bottles of NBAF medium with nutrient additions were prepared (in triplicate) with no vitamin mix, with vitamin mix (standard), with 0.01% yeast extract (YE) replacing the vitamin mix and 1% YE replacing the vitamin mix. The bottles were inoculated with a 10% vol/vol inoculum of an overnight culture of *G. sulfurreducens* and incubated at 30°C. Samples (1ml) were taken in different time intervals and the OD_{600nm} recorded using a Camspec M501 to monitor growth.

2.5 Further optimisation of fumarate/acetate ratios and YE supplementation

This experiment was carried out by setting up 5 sets of 120 ml bottles (in triplicate) of NBAF containing different concentrations YE replacing the vitamin and mineral elixir to identify the best medium that provides the highest biomass yield. The combinations tested were 40 mM fumarate / 25 mM acetate, 80 mM fumarate / 50 mM acetate, 80 mM fumarate / 50 mM acetate with 0.1% YE replacement, 80 mM fumarate / 50 mM acetate with 0.5% YE replacement and 80 mM fumarate / 50 mM acetate with 1% YE replacement. The bottles were inoculated with a 10% vol/vol inoculum of an overnight culture of *G. sulfurreducens* and incubated at 30°C. Samples (1 ml) were taken in different time intervals and the OD_{600nm} recorded using a Camspec M501 to monitor growth.

2.6 Investigating Fe(III) reduction capability of cells grown with YE supplementation

5 sets of bottles were prepared containing NBAF with the exact combination of electron acceptor/donor ratio as in the previous experiment (2.5). The bottles were inoculated with 10% *G. sulfurreducens* and incubated at 30°C for 23 hours under anaerobic conditions. After the incubation period the cells were centrifuged and washed (Standard washing protocol). Ferrihydrite suspensions were then inoculated with *G. sulfurreducens* and measurements were taken as mentioned (2.3).
3. *Geobacter sulfurreducens (bioreactor batch culture optimization)*

Table 2 shows a summary of different experiments carried out on the 6 litre bioreactor for optimization of the medium for biomass production for biomagnetite synthesis. More detailed descriptions can be found in the following sections.

Table 2. Summary of different types and combinations of electron donor/acceptor, medium component, and different growth conditions for *G. sulfurreducens* examined in the bioreactor.

<table>
<thead>
<tr>
<th>section</th>
<th>Electron acceptor</th>
<th>Electron donor</th>
<th>Yeast extract</th>
<th>NB mix</th>
<th>Vitamin mix</th>
<th>Mineral elixir</th>
<th>Stirrer</th>
<th>pH control (6.8 - 7.1)</th>
<th>Acid for pH control</th>
</tr>
</thead>
<tbody>
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<td>100 rpm</td>
<td>Yes</td>
<td>1 M MOPS</td>
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<td>50 mM acetate</td>
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<td>No</td>
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3.1 Growth on NBAF 80F 50A with HCl as acid
The bioreactor was set up according to the standard bioreactor setup procedure. The NBAF medium used for this experiment consisted of 80 mM fumarate as the electron acceptor and 50 mM acetate as electron donor and the rest of the components were set up according to the standard setup protocol (page 31). The pH control was left ON during the incubation period, using 2M NaOH as base and 2M HCl as acid to maintain the pH as). The stirrer was also kept ON during the incubation period at 100rpm. Samples were taken from the bioreactor periodically from the sample collection point. The first two 20 ml samples were discarded and the third used to determine the OD$_{600\text{nm}}$ of the culture.

3.2 Growth on NBAF 80F 50A with MOPS as acid
The bioreactor was set up according to the standard bioreactor setup procedure. 1 M MOPS was used for pH adjustment during the incubation period. The NBAF used for this experiment consisted of 80 mM fumarate as the electron acceptor and 50 mM acetate as the electron donor and the rest of the components were set up according to the standard protocol (page 31). The stirrer was kept ON during the incubation period at 100 rpm. Sample collection and OD$_{600\text{nm}}$ measurements were as described above.

3.3 Growth on NBAF 80F 50A with no pH control
The bioreactor was set up according to the standard bioreactor setup procedure (page 31). The NBAF used for this experiment consisted of 80 mM fumarate as electron acceptor and 50 mM acetate as electron donor and the rest of the components were set up according to the standard setup protocol. The pH was adjusted to 7.1 using 2 M NaOH as base and 1 M MOPS as acid (before the inoculation step). The pH control on the bioreactor was turned OFF during the incubation period and the stirrer was kept ON at 100 rpm. Sample collection and OD$_{600\text{nm}}$ measurements were as described above.

3.4 Growth on NBAF 80F 50A with 1% YE supplement
The bioreactor was set up according to the standard bioreactor setup procedure with 1% YE replacing the vitamin and mineral elixir mix. The NBAF used for this
experiment consisted of 80 mM fumarate as electron acceptor and 50 mM acetate as electron donor and the rest of the components were set up according to the standard setup protocol (page 31). The pH was adjusted to 7.1 using 2 M NaOH as base and 1 M MOPS as acid (before the inoculation step). The pH control was turned OFF during the incubation period and the stirrer was kept ON with 100 rpm speed. Sample collection and OD_{600nm} was taken as mentioned (3.1).

3.5 Growth on NBAF 80F 50A with 0.5% YE supplement
The bioreactor was set up according to the standard bioreactor setup procedure with 0.5% YE supplement. The NBAF used for this experiment consisted of 80 mM fumarate as electron acceptor and 50 mM acetate as electron donor and the rest of the components were set up according to the standard setup protocol. The pH was adjusted to 7.1 using 2 M NaOH as base and 1 M MOPS as acid (before the inoculation step). The pH control and the stirrer with 100 rpm were kept ON during the incubation period. Sample collection and OD_{600nm} was taken as described above.

3.6 Growth on NBAF 80F 50A with pH and stirrer OFF
The bioreactor was set up according to the standard bioreactor setup procedure (page 31). The NBAF used for this experiment consisted of 80 mM fumarate as electron acceptor and 50 mM acetate as electron donor and the rest of the components were set up according to the standard setup protocol. The pH was adjusted to 7.1 using 2 M NaOH as base and 1 M MOPS as acid (before the inoculation step). The pH control and the stirrer were turned OFF during the incubation period. The stirrer was turned ON for 1 min before the sampling in order to homogenise the media and avoids false reading and turned back OFF. Sample collection and OD_{600nm} was taken as described above.

3.7 Growth on NBAF 80F 50A with 50rpm stirrer
The bioreactor was set up according to the standard bioreactor setup procedure (page 31). The NBAF used for this experiment consisted of 80 mM fumarate as electron acceptor and 50 mM acetate as electron donor and the rest of the components were set up according to the standard setup protocol. The pH was adjusted to 7.1 using 2
M NaOH as base and 1 M MOPS as acid before inoculation (before the inoculation step). The pH control was turned OFF and the stirrer was kept ON during the incubation period with 50 rpm. Sample collection and OD was taken as mentioned (3.1).

3.8 Growth on NBAF 80F 50A with 0.5% YE supplement and 50rpm Stirrer

The bioreactor was set up according to the standard bioreactor setup procedure with 0.5% YE supplement. The NBAF used for this experiment consisted of 80 mM fumarate as electron acceptor and 50 mM acetate as electron. The pH was adjusted to 7.1 using 2 M NaOH as base and 1 M MOPS as acid (before the inoculation step). The pH control was turned OFF and the stirrer was kept ON at 50 rpm during the incubation period. Samples were collected and OD600nm was taken as described above.

3.9 Growth on NBAF 80F 50A in a 10L bottle with pH and stirrer OFF

5L NBAF 80 mM fumarate/ 50 mM acetate was made according to standard protocol (page 31). 4.5 L was decanted in a sterile 10 L bottle and autoclaved. The medium was purged with 80:20 N2:CO2 gas for 1 hour and the pH was adjusted to 7.1 using CO2 and N2 gas. 500 ml of the remaining NBAF was decanted in a 500 ml duran bottle and autoclaved separately. The 500 ml autoclaved medium was degassed using the same procedures and inoculated with 10% vol/vol overnight grown G. sulfurreducens and incubated at 30°C to be used as inoculum. Inoculation of the 10 L bottle was carried out the next day using the prepared inoculum. The medium was incubated at 30°C with no pH control or Stirring. Vigorous shaking to the bottle was applied before every sampling point to homogenise the medium and avoid false reading. Samples were taken periodically and OD600nm was and the growth curve was plotted respectively.

4. Scale-up to 50 L fermenter

This experiment was set up to scale up the growth of G. sulfurreducens to a 50 L fermenter with commercial partners (The Centre for Process Innovation, Wilton) and also scale up the biogenic magnetite production from the biomass produced, moving
from 10 ml to 10 L vessels to test the iron reduction ability of the grown cells at larger scale.

NBAF (4.5 litres) with 80 mM fumarate as the electron acceptor and 50 mM acetate as the electron donor was made up according to standard protocol, and inoculated with 10% (500 ml) of a late log G. sulfurreducens culture and incubated at 30°C for 48 hours under anaerobic conditions. This 5L starter culture was incubated in a 10L Biostat Cplus fermenter (Appendix 9, Figure H) at 30°C with a 50 rpm stirring speed for 48 hours to be used as an inoculum for the 50 L fermenter. The headspace of the 10 L bioreactor was purged with N₂ gas during the incubation period to keep the culture anaerobic.

45 L of NBAF with 80 mM fumarate as the electron acceptor and 50 mM acetate as the electron donor was then prepared and transferred into a 50 L Sartorius fermenter (Appendix 9, Figure I) and the pH adjustment and degassing steps was carried out according to standard protocol. The medium was inoculated with 10% vol/vol inoculum of G. sulfurreducens, using the 5 L, 48 hours grown inoculum that was prepared in the 10 L fermenter). The incubation was carried out at 30°C and the stirring speed was set to 100 rpm to match the 50 rpm in the 5 L fermenter. Due to volume increase the speed of the stirrer was converted based on the following equation. \( N_1 = N_2 \times \left( \frac{V_1}{V_2} \right)^{0.29} \)

The culture was incubated for 48 hours and then the cells harvested and centrifuged at 5000 rpm for 20 min and washed according to standard protocol (page 31) to be inoculated into ferrihydrite supplemented buffer supplemented with acetate (as described previously). Different sized bottles, with different volumes of ferrihydrite in buffer were tested, including 2× 10 L, 3× 1 L, 3× 100 ml, and 3× 10 ml bottles to quantify the rate of Fe(III) reduction, and generate samples for mineralogical analysis produced at various scales. After inoculation (to a final biomass OD₆₀₀nm of approximately 0.4), the bottles were incubated at 30°C and samples were taken aseptically in time periods to quantify Fe(III) reduction (using the ferrozine assay).
Results and Discussion

Optimization of synthesis of insoluble amorphous Fe(III) oxyhydroxide for bioreduction

This experiment described in section 1.1 was carried out to investigate the effect of number of washes (during ferrihydrite preparation) on the overall rate of Fe(III) bioreduction and the production of magnetite.

The results (Figure 6) suggest that the maximum rate of Fe(III) bioreduction and subsequently magnetite production are achieved after 6 washes to remove residual chloride ions. Based on these results, 6 washes were used as the standard procedure for the production of insoluble Fe(III) oxyhydroxide throughout the experimental programme.

![Figure 6. Optimization of Fe(III) mineral preparation procedure (number of washes needed) for Fe(III) reduction by *G. sulfurreducens*.](image)
Mediator optimization
The Fe(III) reduction ability of *G. sulfurreducens* was tested (as described in section 1.2) with different types and concentrations of electron mediator to find the optimal conditions to be used as standard. Comparison of the data in Figure 7 shows clearly the advantage of the addition of electron mediators in enhancing the Fe(III) reduction rate.

Addition of 10 µM of the commonly used electron mediator and humic analogue AQDS resulted in a much higher reduction rate over 28 hours of anaerobic incubation at 30°C, when compared to the no mediator controls. However, the rates of reduction achieved by the addition of 10 and 50 µM riboflavin were quite similar to those of the humic analogue, confirming that both riboflavin and AQDS have similar redox mediating properties. This is most likely related to the more or less equal redox potentials ($E'_0$) of both compounds (−208 and −225 mV, respectively) (Sober, 1970). As the difference between reduction rate of 10 and 50 µM riboflavin is not significant, and as riboflavin is much more inexpensive than AQDS, 10 µM of the riboflavin was considered as standard for subsequent experiments.

![Figure 7](image_url)

*Figure 7.* Examining the effect of different redox mediators on the Fe(III) reduction rate of *G. sulfurreducens.*
Optimization of growth medium components (120ml bottle experiments)

*G. sulfurreducens* was grown in NBAF medium (in 120ml serum bottles) with different concentrations of fumarate (as the electron acceptor) and acetate (as the electron donor) under standardised incubation conditions, to find the ratio providing the highest growth rate and biomass yield.

The first experiment was carried out by keeping the concentration of acetate constant at 25 mM and only changing the fumarate concentration (Figure 8). The result of this experiment shows that the 100 mM fumarate provides the highest biomass compared to the standard concentration of 40 mM. On the other hand, cells grown on potassium nitrate as electron acceptor showed no growth, which suggests that potassium nitrate is not an efficient electron acceptor to support anaerobic cell growth.

Using 150 mM fumarate as the electron acceptor showed a negative effect on cell growth and also a slower growth rate. The latter set of experiments (150 mM fumarate) was repeated again due to high variability (SD= 0.001 - 0.2) of the results.

![Figure 8. Impact of different ratios of acetate: fumarate and also potassium nitrate as an electron donor on the growth of *G. sulfurreducens*.](image-url)
The second experiment (Figure 9) was carried out using different fumarate: acetate ratios to help identify more clearly the optimal ratio of electron acceptor to electron donor for biomass generation. In this experiment, 40 mM fumarate resulted in the lowest biomass yield and cells grown on 150 mM fumarate showed the highest biomass yield. As the acetate concentration was increased from 25 mM to 50 mM for these samples, a decrease in growth rate was observed but the final biomass yield stayed the same which suggests that the increase of electron donor to 50 mM with 150 mM fumarate as electron acceptor has a negative effect on the growth rate of the cells.

The 100 mM fumarate results also illustrate the same growth pattern; with the increase in acetate concentrations from 25 mM to 50 mM the growth rate was decreased but the final biomass yield was the same.

![Figure 9](image-url). Comparison of growth of *G. sulfurreducens* on different concentrations and ratios of fumarate and acetate.
Samples from the last experiment were tested for their Fe(III) reduction capability by spinning down and washing the pre-grown cells using degassed sterile carbonate buffer (Figure 10). Cells grown on 100 mM fumarate/50 mM acetate showed the highest Fe(III) reduction and subsequently higher magnetite production. However, the cells that were grown on 150 mM fumarate/50 mM acetate showed the slowest Fe(III) reduction rate.

Both batches of cells grown on 150 mM fumarate show a decrease in their iron reduction ability despite providing the highest biomass yield (Figure 9). The results suggest that increase of electron donor and acceptor to very high levels during growth periods have a significant negative effect on the iron reduction ability of the grown cells. However, cells grown on 40 mM fumarate/25 mM acetate showed the fastest Fe(III) reduction rate but due to their low biomass yield (Figure 9) it was not considered as the optimal combination.

Based on the results of these experiments, as the 100 mM fumarate/50 mM acetate provides a high biomass yield and also shows the highest iron reduction ability, it was considered as the optimal concentrations for the purpose of this study.

![Figure 10. The impact of electron donor/acceptor ratios on the rate of Fe(III) reduction by G. sulfurreducens.](image)
Yeast extract supplement

This experiment investigated the effects of replacing the vitamin mix in standard NBAF with different concentrations of yeast extract on the growth of *G. sulfurreducens*. After 48 hours of incubation under anaerobic conditions there was a significant variation in the growth pattern of the cells on the different media tested (Figure 11). The medium without the vitamin mix and yeast extract provided the lowest biomass yield and the original standard NBAF medium (40 mM fumarate / 25 mM acetate) containing the vitamin mix provided the highest biomass yield and growth rate.

The results show that the presence of the vitamin mix has a significant positive effect on the growth rate of the organism and the subsequent biomass yield (final OD$_{600\text{nm}}$ value of X). However, the medium containing 0.01%, and 0.1% of yeast extract also enhanced cell growth (when compared to the control with no vitamin additions) with final OD$_{600\text{nm}}$ values of 0.72 and 0.92 respectively.

Figure 11. Investigating the effects of vitamin mix replacement with yeast extract on the growth of *G. sulfurreducens*.
A second experiment was setup to compare the growth pattern of cells grown on NBAF with varied fumarate/acetate ratio to ones that were grown on NBAF with different concentrations of yeast extract replacing vitamin mix and mineral elixir.

As it is illustrated on the graph (Figure 12), the 80 mM fumarate/50 mM acetate provided the highest biomass yield while the NBAF medium with 1% yeast extract replacement resulted in the lowest biomass yield over the incubation period.

Cells grown on 40 mM fumarate/25 mM acetate have a very short lag phase of about 3 hours while the cells grown on 80 mM fumarate/50 mM acetate show a lag phase of about 6 hours. These results suggest that although the increase of electron donor to electron acceptor ratio may provide higher biomass yield but it will also show a negative effect on the growth pattern of the cells by increasing the lag phase and subsequently the incubation period.

Cells grown on NBAF with yeast extract replacement had a different growth pattern, as the concentration of yeast extract increased from 0.1% to 1%, the lag phase increased and the biomass yield decreased. This suggests that high concentrations of yeast extract in the medium may have a negative effect on the growth of the organism.

![Graph comparing growth pattern](image.png)

**Figure 12.** Comparing growth pattern of *G. sulfurreducens* on NBAF with varied fumarate/acetate ratio to NBAF with yeast extract replacing vitamin mix and mineral elixir.
To test the iron reduction ability of cells grown on different media, an experiment was carried out by spinning down the cells from different media after 27 hours anaerobic incubation at 30°C to be used as inoculum. The cells were prepared for inoculation into FeGel bottles according to standard protocol with 10 µM riboflavin as mediator.

The results (Figure 13) show that cells grown on NBAF 40 mM fumarate/ 25 mM acetate provide the highest and fastest Fe(III) reduction while the next best result is achieved by the cells grown on 80 mM fumarate/ 50 mM acetate. The lowest reduction was achieved by the cells grown on NBAF with 1% YE replacement which suggests that too much yeast extract during growth period will significantly reduce the iron reduction ability of the cells.

Although the cells grown on 0.1% and 0.5% YE replacement show a high Fe(III) reduction rate after 18h of incubation, but compared to results of other cells it takes too long to reach the ideal level. Moreover, there is a significant variation between every batch of cells grown on yeast extract, which results in varied growth pattern and will not provide reproducible results. Due to this problem the yeast extract was not considered as the ideal growth medium.

![Figure 13](image.png)
Bioreactor optimization experiments

Optimization of acid for pH control

Based on the results from the 120 ml serum bottle experiments, 80 mM fumarate and 50mM acetate was considered as the optimal electron acceptor and electron donor ratio for the bioreactor optimization and scale-up experiments respectively. The first two experiments using the 5 L bioreactor were carried out to examine which acids to be used for the pH control of the bioreactor before and during the incubation period.

In both experiments the bioreactor was setup according to a standard protocol (page 27). The results from the experiment with 2 M HCl as acid for pH control showed an inhibition effect on the growth of the cells and after 27 hours of incubation the cells reach the stationary phase. On the other hand, when 1 M MOPS was used for pH control the cell yield was increased from $OD_{600nm}$ 0.38 to 0.82 after 38 hours incubation.

The results of this experiment suggest that 1 M MOPS to be ideal for controlling the pH and keeping it at a neutral value. The reasons for its better performance are not clear, but it is interesting to note its effectiveness and in other studies (Moon et al., 2010).

![Figure 14](image_url). Comparison of 2M HCl and 1M MOPS for controlling the pH of NBAF 80 mM fumarate/50 mM acetate in a 5L bioreactor experiment.
**Effect of pH control on cell growth**

In order to examine the effects of controlling the pH of the medium on the biomass yield during incubation period, the bioreactor was set up according to the standard protocol with 100rpm stirring and the pH control was kept off during incubation period. The results of this experiment (Figure 15) illustrated a high biomass yield (OD$_{600nm} = 1.038$) after 45 hours of incubation at 30°C with pH varying between the range of 7.1 - 6.94, which suggests that the bicarbonate buffer present in the NBAF medium is sufficient to keep the pH at around neutral conditions during the incubation period and there is no need to control the pH using external reagents.

![Graph](Figure 15.png)

**Figure 15.** Investigating the effect of controlling pH cell growth and also testing the efficiency of the buffer in the NBAF to keep the pH at neutral range.

A second experiment was carried out by setting up the bioreactor with NBAF 80 mM fumarate/ 50 mM acetate according to standard protocol with 1% yeast extract replacing the vitamin mix and mineral elixir. The pH was adjusted to 7.0 before inoculation and during incubation the pH control was kept off to examine the cell growth pattern.
During the incubation period the pH changed significantly between the ranges 7.0 – 6.29, and affected the growth of *G. sulfurreducens* and resulted in an OD$_{600nm}$ = 0.7 after 46 hours of incubation at 30°C (Figure 16).

Comparing the biomass yield of the standard NBAF medium (OD$_{600nm}$ = 1.0 at 45 h) with the results from the same medium but with 1% YE replacing the vitamin mix (OD$_{600nm}$ = 7.0 at 46 hours), it was concluded that the 1%YE replacement has a negative effect on cell growth which confirms the results presented from the 100 ml microcosm experiments (Figure 12). This may be due to the yeast extract in the medium causing pH variation during incubation period, as the pH varied between the ranges of 6.26 – 7.0 in this experiment.

![Graph](image)

**Figure 16.** Growth of *G. sulfurreducens* grown on NBAF with 1% YE replacing vitamin mix and mineral elixir, while keeping the pH control OFF during the incubation period.

**Effects of Stirring speed on cell growth**

This experiment was carried out to check the effect of stirring on cell growth. The bioreactor was set up with NBAF 80 mM fumarate/ 50 mM acetate according to the standard protocol. The pH was adjusted to 7.2 using 1M MOPS and after inoculation with a log phase starter culture (10% vol/vol) and during the incubation period, the pH control was kept off. The stirrer was also kept off during the incubation period and only turned on for 1 minute at 100 rpm before sampling points to mix the culture and avoid any sampling errors.
During the incubation period, the pH was between the ranges of 7.19 – 6.87 which is similar to the other experiment with pH control off (Figure 15). However comparing the results of the two experiments carried out without pH control, there was a slight decrease in biomass yield when the stirrer was turned off (Figure 17).

Another interesting observation includes the variation of temperature by 3 - 4°C during sampling points when the stirrer was turned on for 1 minute (homogenization step). These temperature changes raised the hypothesis that as the temperature of the bioreactor is controlled via a heat jacket, turning the stirrer off may change the distribution of heat throughout the medium which subsequently resulted in the decrease of biomass yield.

![Figure 17. Growth of G. sulfurreducens while the pH control was kept off during incubation.](image)

To test the above hypothesis, an experiment was setup by preparing the NBAF 80 mM fumarate/ 50 mM acetate medium according to standard protocol and decanting it in a 10 L sterile bottle to be used instead of the bioreactor vessel. The pH of the medium was adjusted to 7.1 and inoculated with 10% vol/vol G. sulfurreducens late log culture and incubated at 30°C.
The results of this experiment shows a pH variation between 7.04 – 7.28 with highest $OD_{600\,nm} = 0.885$ after 48 hours of incubation (Figure 18).

From comparisons of the results of this experiment (Figure 18) to the ones in the bioreactor (Figure 17), it was concluded that temperature distribution in the bioreactor did not inhibit cell growth, and the heat jacket used in the bioreactor is sufficient to control the medium temperature.

This suggestion leaves only one potential factor that may affect the cell growth and that’s the stirring speed which was set to 100 rpm during the previous bioreactor experiments.

![Graph showing the growth of G. sulfurreducens](image)

**Figure 18.** Examining the effect of equal temperature distribution on growth of *G. sulfurreducens* while the pH control and stirring is off during incubation period.

To further test the effects of stirring speed on the cell growth the next experiment was carried out to examine the cell growth at 50 rpm speed (Figure 19).
During the incubation period the pH was between the ranges of 6.87 – 7.1 which is consistent with the results from the previous experiment (Figure 15). Comparison of the biomass yield of the two experiments show similar results for (OD$_{600nm}$=1.038 after 45 hours of incubation) with 100 rpm stirring and (OD$_{600nm}$ = 1.120 after 46 hours of incubation) 50 rpm of stirring.

Therefore as running the stirrer at 50rpm provides the same biomass yield as the 100rpm speed and also it is more cost effective (less electricity used during incubation), it was used as the standard stirring speed during scale up and bioreactor experiments.

The final experiment was carried out to examine the growth pattern of \textit{G. sulfurreducens} on NBAF 80 mM fumarate/ 50 mM acetate with yeast extract replacing the vitamin mix and mineral elixir. The experiment was carried out twice with equal conditions and only the stirrer speed was varied.

On the first experiment the stirrer was set to 100rpm and on the second repeat it was set to 50rpm to examine the effect of stirring speed on cell growth.

The experiment with 100 rpm stirrer resulted in a slightly higher biomass yield with the final OD$_{600nm}$=0.635 after 48 hours of incubation (Figure 20), compared to the final OD$_{600nm}$=0.501 after 48 hours of incubation for cells grown with 50 rpm stirring (Figure 21).
Figure 20. Examining the effect of stirrer speed on *G. sulfurreducens* grown on NBAF 80 mM fumarate/50 mM acetate 5 L bioreactor with stirring at 100 rpm and no pH control during incubation period at 30°C.

Figure 21. Examining the effect of stirrer speed on *G. sulfurreducens* grown on NBAF 80 mM fumarate/50 mM acetate 5 L bioreactor with stirring at 50 rpm and no pH control during the incubation period at 30°C.
The results of these two experiment confirms the previous findings, that the stirring speeds of 50 rpm and 100 rpm provide almost the same biomass yield, and therefore 50 rpm was used as the standard stirring speed for scale-up experiments.

**Overall comparison of the bioreactor results**

The graph below (Figure 22) illustrates the results from the different bioreactor experiments carried out during this project, for comparison purposes. The experiment carried out with pH control off and 100 rpm stirring during the incubation period shows a longer lag phase which can be due to the high stirring speed, as the stirring speed is reduced to 50 rpm the lag phase time is reduced effectively.

The biomass yields of the bioreactor experiments carried out with different concentrations of yeast extract is not as high as the ones with the standard NBAF medium with vitamin additions. Therefore it was concluded that although the yeast extract supports cell growth to some levels and shows the lowest lag phase during growth but it can’t replace the standard medium as the final biomass yield is not high enough, thus it is not sufficient for the purpose of this study.

As the experiment with pH control off and 50 rpm stirring speed during incubation provides a high biomass yield, it was considered to as the best conditions for cell growth and thus it was selected to be used as standard protocol for the scale-up purposes.
**50 L Scale-up experiment**

The results of this experiment shows that, the cells had a long lag phase of around 17 hours with the maximum biomass yield \((\text{OD}_{600nm}= 0.466)\) achieved after 48 hours of incubation. The pH of the medium was varied between 7.09 - 7.34 during the incubation period. These results again highlight the increase of the lag phase with scale up, which means as the medium volume increases the lag phase of the cells increases respectively.

During this experiment an error occurred with the fermenter. The aeration valves were set on automatic and due to a configuration error, for a small period of time oxygen was pumped into the medium which changed the redox potential in the medium and may have also affected the cell growth. To solve the problem \(\text{N}_2\) was pumped in the system to maintain the anoxic conditions. However this action did increase the pH to above neutral level which again may have influenced the cell growth.
After 48 hours of incubation, the grown cells were collected by centrifugation, washed and inoculated into 10 ml, 100 ml, 1 L and 10 L bottles of bicarbonate buffer supplemented with ferrihydrite as discussed previously according to standard protocol.
Figure 24. Ferrihydrite-containing bottles with different volumes ranging from 10 ml - 10 L were prepared for inoculation, in order to examine the Fe(III) reduction capability of *G. sulfurreducens* grown in the 50 L fermenter.

Figure 25. Ferrihydrite-containing bottles after inoculation with *G. sulfurreducens* and anaerobic incubation for 72 hours at 30°C, show biogenic magnetite production (black precipitate).
Figure 26. Fe(III) reduction of *G. sulfurreducens* cells grown at 50 L scale, washed and inoculated into bottles with different volumes ranging from 10 ml to 10 L.

The iron reduction experiment results (Figure 26) shows that after 24 hours of anaerobic incubation at 30°C, biogenic magnetite production can be achieved in the 10 L and 120 ml bottles while it took 72 hours for the 1 L bottle to reach the same level of magnetite production. However, the 10 ml bottle showed the lowest activity which contradicts the previous findings of this project and may be due to experimental error (for example, lower biomass inoculation or insufficient degassing of the bottle during the preparation process).

X-ray diffraction (XRD) was carried out to determine the mineral phases present in the material produced (Figure 27). Powder X-ray diffraction (PXRD) measurements were carried out using a Bruker D8Advance with Cu *Kα1* source. Data was acquired over a 2θ range of 5°-70° with a step size of 0.02°.
Figure 27. XRD patterns show characteristic spectra of magnetite (Fe$_3$O$_4$). Results indicate that there is little variation between the spectra, however the appearance of an additional mineral phase siderite becomes more prominent as volume increases (dashed line).

The data shows that magnetite is produced in all of the samples, however there is also the presence of a small amount of siderite (FeCO$_3$) which is an Fe(II) mineral.

Although the 10 ml bottle did not generate the expected results, the main purpose of this experiment was to successfully scale-up the biogenic magnetite production to 10 L bottles which was successfully achieved.
Conclusion

Based on the results of this project several conclusions were made including:

- NBAF medium with 80 mM fumarate as electron acceptor and 50 mM acetate as electron donor was considered as standard medium to support the growth of *G. sulfurreducens*, as it provides the highest biomass yield when incubated in large volumes (5 L and above) at 30°C with 50 rpm stirrer speed, and has sufficient activity against Fe(III) to generate biomagnetite.

- Cultures that were grown under conditions of electron acceptor (fumarate) limitation, had higher activities against Fe(III), but lower biomass yields.

- There is a direct relation between the volume of the medium used for the cell growth and the lag phase period. As the reactor volume increases, a longer lag phase is observed.

- 10µM riboflavin is a sufficient replacement for AQDS, to be used as electron shuttling compound during biogenic magnetite production.

- Although using low concentrations of yeast extract did enhance cell growth to some levels, results were not as good as with a vitamin mix added. It was thought that there was a significant variation between batches of yeast extract used, and the results were not reproducible, Yeast extract was not considered as an ideal replacement of vitamin and mineral elixir mix.

- The optimization, production and scale-up of bionanomagnetite from 10 ml serum bottles to 1 L vessels was carried out efficiently, which is an important step in the potential commercial use of these materials. And finally it was concluded that when providing optimal growth conditions, *G. sulfurreducens* is an excellent candidate for biogenic magnetite production at industrial-scale.
Future Work

- As the growth pattern of *G. sulfurreducens* on Yeast extract replacement was variable between every batch the results was not reproducible, as a result yeast extract replacement was not considered to provide the best medium for cell growth. However the Fe(III) reduction ability of the cells grown on 0.01% YE replacement was promising. Therefore more experiments are suggested to further examine the potential of yeast extract.

- Scale-up of the process from 50 L fermenter to 750 L fermenter and subsequently further scale up of magnetite production from 10 L to 100 L volume.

- Investigation into the use of *Shewanella oneidensis*, a facultative anaerobe for biogenic magnetite production purposes.

- Characterization, including particle size, surface reactivity (of surface Fe(II)) against pollutants and magnetic properties of the magnetite during scale-up.
Appendix

Appendix 1

Preparation Protocol for NBAF 40F, 25A

1. Add the following into a bottle.

<table>
<thead>
<tr>
<th>Component</th>
<th>Per 1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>900ml</td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>4.64g</td>
</tr>
<tr>
<td>100× NB Mix (appendix 2)</td>
<td>10ml</td>
</tr>
<tr>
<td>NB Mineral Elixir (appendix 3)</td>
<td>10ml</td>
</tr>
<tr>
<td>Vitamin Mix (appendix 4)</td>
<td>15ml</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.04g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.1g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>1.8g</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>0.5g</td>
</tr>
<tr>
<td>1mM Na₂SeO₄</td>
<td>1ml</td>
</tr>
<tr>
<td>Sodium Acetate</td>
<td>2.04g</td>
</tr>
</tbody>
</table>

2. Adjust the pH to 7.1 using 10N NaOH

3. Bring out the final volume

4. Degas the media using 80:20 N₂:CO₂ and readjust the pH to 7.1 using the gas

5. Place the medium into 100ml bottles or the Bioreactor and autoclave
# Appendix 2

## 100X NB Mix (for 10L PCA Media)

<table>
<thead>
<tr>
<th>Component</th>
<th>Per 1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH$_2$PO$_4$(monobasic)</td>
<td>42.0 g</td>
</tr>
<tr>
<td>K$_2$HPO$_4$(dibasic)</td>
<td>22.0 g</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>20.0 g</td>
</tr>
<tr>
<td>KCl</td>
<td>38.0 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>36.0 g</td>
</tr>
</tbody>
</table>
Appendix 3

**NB Mineral Elixir**

<table>
<thead>
<tr>
<th>Components</th>
<th>Per 1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTA</td>
<td>2.14 g **</td>
</tr>
<tr>
<td>MnCl$_2$ • 4H$_2$O</td>
<td>0.1 g</td>
</tr>
<tr>
<td>FeSO$_4$ • 7H$_2$O</td>
<td>0.3 g</td>
</tr>
<tr>
<td>CoCl$_2$ • 6H$_2$O</td>
<td>0.17 g</td>
</tr>
<tr>
<td>ZnSO$_4$ • 7H$_2$O</td>
<td>0.20 g</td>
</tr>
<tr>
<td>CuCl$_2$ • 2H$_2$O</td>
<td>0.03 g</td>
</tr>
<tr>
<td>AlK(SO$_4$)$_2$ • 12H$_2$O</td>
<td>0.005 g</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>0.005 g</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$</td>
<td>0.09 g</td>
</tr>
<tr>
<td>NiSO$_4$ • 6H$_2$O</td>
<td>0.11 g</td>
</tr>
<tr>
<td>Na$_2$WO$_4$ • 2H$_2$O</td>
<td>0.02 g</td>
</tr>
</tbody>
</table>

**After adding NTA, pH to 8-8.5 with NaOH to dissolve NTA.**

- Remember to use free acid NTA and not the trisodium salt.

Allow each ingredient to dissolve completely before adding the next.
Appendix 4

**VITAMIN MIX (DL)**

<table>
<thead>
<tr>
<th>Components</th>
<th>Mg per 1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin</td>
<td>2.0</td>
</tr>
<tr>
<td>Folic acid</td>
<td>2.0</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>10.0</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>5.0</td>
</tr>
<tr>
<td>Thiamine</td>
<td>5.0</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>5.0</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>5.0</td>
</tr>
<tr>
<td>B-12</td>
<td>0.1</td>
</tr>
<tr>
<td>p-aminobenzoic acid</td>
<td>5.0</td>
</tr>
<tr>
<td>Thiocytic acid</td>
<td>5.0</td>
</tr>
</tbody>
</table>
## Appendix 5

Preparation Protocol for Minimal Media

<table>
<thead>
<tr>
<th>Solution 1</th>
<th>g/L</th>
<th>Mr</th>
<th>mM</th>
<th>50x stock g/l</th>
<th>In 1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄</td>
<td>1.189</td>
<td>132.1</td>
<td>9.0</td>
<td>59.463</td>
<td>20ml</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.993</td>
<td>174.2</td>
<td>5.7</td>
<td>49.641</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.449</td>
<td>136.1</td>
<td>3.3</td>
<td>22.455</td>
<td></td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.168</td>
<td>84.0</td>
<td>2.0</td>
<td>8.401</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution 2</th>
<th>g/L</th>
<th>Mr</th>
<th>mM</th>
<th>100x stock g/l</th>
<th>In 1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄. 7 H₂O</td>
<td>0.246</td>
<td>246.5</td>
<td>1.0</td>
<td>24.647</td>
<td>10ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution 3</th>
<th>g/L</th>
<th>Mr</th>
<th>mM</th>
<th>100x stock g/l</th>
<th>In 1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂. 2 H₂O</td>
<td>0.072</td>
<td>147.0</td>
<td>0.5</td>
<td>7.203</td>
<td>10ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Trace element solution (20x):</th>
<th>g/L</th>
<th>Mr</th>
<th>uM</th>
<th>200x stock g/l</th>
<th>In 1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂EDTA</td>
<td>0.023</td>
<td>336.2</td>
<td>67.2</td>
<td>4.519</td>
<td>10ml</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.003</td>
<td>61.8</td>
<td>56.6</td>
<td>0.700</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>0.001</td>
<td>58.4</td>
<td>10.0</td>
<td>0.117</td>
<td></td>
</tr>
<tr>
<td>FeSO₄. 7 H₂O</td>
<td>0.002</td>
<td>278.0</td>
<td>5.4</td>
<td>0.300</td>
<td></td>
</tr>
<tr>
<td>CoCl₂. 6 H₂O</td>
<td>0.001</td>
<td>237.9</td>
<td>5.0</td>
<td>0.238</td>
<td></td>
</tr>
<tr>
<td>NiCl₂. 6 H₂O</td>
<td>0.001</td>
<td>237.7</td>
<td>5.0</td>
<td>0.238</td>
<td></td>
</tr>
<tr>
<td>Na₂MoO₄. 2H₂O</td>
<td>0.001</td>
<td>205.9</td>
<td>3.9</td>
<td>0.161</td>
<td></td>
</tr>
<tr>
<td>Na₂SeO₄</td>
<td>0.000</td>
<td>143.1</td>
<td>1.5</td>
<td>0.043</td>
<td></td>
</tr>
<tr>
<td>MnCO₃</td>
<td>0.000</td>
<td>223.1</td>
<td>1.3</td>
<td>0.058</td>
<td></td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0.000</td>
<td>287.6</td>
<td>1.0</td>
<td>0.058</td>
<td></td>
</tr>
<tr>
<td>CuSO₄. 5 H₂O</td>
<td>0.000</td>
<td>249.7</td>
<td>0.2</td>
<td>0.010</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amino acid solution (1000x):</th>
<th>g/L</th>
<th>Mr</th>
<th>uM</th>
<th>1000x stock g/l</th>
<th>In 1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-arginine hydrochloride</td>
<td>0.020</td>
<td>20.000</td>
<td>20.000</td>
<td>1ml</td>
<td></td>
</tr>
<tr>
<td>L-glutamate</td>
<td>0.020</td>
<td>20.000</td>
<td>20.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-serine</td>
<td>0.020</td>
<td>20.000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Electron donor</th>
<th>ml/L</th>
<th>Mr</th>
<th>mM</th>
<th>25x stock ml/l</th>
<th>In 1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-lactate</td>
<td>15.435</td>
<td>100</td>
<td>385.875</td>
<td>40ml</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Electron acceptor</th>
<th>g/L</th>
<th>Mr</th>
<th>mM</th>
<th>25x stock g/l</th>
<th>In 1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>fumarate</td>
<td>2.320</td>
<td>116.1</td>
<td>20</td>
<td>58.035</td>
<td>40ml</td>
</tr>
</tbody>
</table>
Appendix 6

Amorphous FeOOH suspension (iron gel) preparation

- Separate batch in two and only add NaOH to one so that if the pH goes too high, you can bring it down with the reserve.
- Rinse the pH electrode frequently while preparing FeGel.

- Pour 1000 ml Q H$_2$O in Fe contaminated beakers
- Dissolve $\approx$162 g FeCl$_3$
- Separate into two beakers
- Adjust the pH to 7 with 10N NaOH (approx. 75 ml)
- Allow suspension to sit for ½ hour then re-pH
- It will become very thick, ensure continual stirring
- Pour into Fe-contaminated centrifuge bottles filling to 2/3 volume
- Balance centrifuge bottles in pairs
- Spin @ 1700 RCF for 20 minutes
- Pour off supernatant
- Wash 6 times* (see below)

*WASH:

Add Q H$_2$O to 2/3 level,

Resuspend Fe gel

Rebalance pairs and centrifuge

Pour off the final supernatant and scrape out all the sludge into one bottle.

- Measure the total Fe$^{3+}$ concentration using hyroxylamine:
  Add 100$\mu$l Fe gel to 4.7 ml 0.5N HCl and 200$\mu$l $\approx$6N Hydroxylamine.
  Leave overnight in closed scintillation vial. Dilute 1:50 in 0.5 N HCl. Test 50$\mu$l of mix in 2.45 ml Ferrozine
- Add Q H$_2$O so that the final concentration is approximately 1 M.
Appendix 7

*Shewanella* Species

One of the most studied microorganisms capable of dissimilatory Fe(III) reduction are *Shewanella* species which are from the gamma subclass of *Proteobacteria*. A good example is *Shewanella oneidensis*, one of the most fascinating characteristics of this genus is its ability to use oxygen as electron acceptor and grow under oxic conditions and in the absence of oxygen it can reduce Fe(III) and continue growing.

Another factor that makes this organism a very important candidate for studies is the availability of its complete genomic sequence (Heidelberg *et al.*, 2002). On the other hand, molecular studies have found that this organism is not a significant component of communities in a wide range of environment where Fe(III) reduction is important. One of the reasons supporting this suggestion is that *Shewanella* would prefer lactate as electron donor, which is not an important intermediate for anaerobic metabolism in sedimentary environments (Lovley, 2002).

Due to the limited time for this project, the project was mainly focused on optimisation of media and incubation conditions for *G. sulfurreducens* to be used as the candidate organism. However, some experiments were carried out on *S. oneidensis* to test its iron reduction ability and growth rate to be used for the magnetite production purposes in the future.

**Media and Growth Conditions**

Different media were tested on *S. oneidensis* to find the optimal media providing the highest biomass yield. Iron reduction experiments were also carried out on grown cells to test their iron reduction ability.

**Minimal Medium**

Standard growth medium used for *S. oneidensis* was Minimal Media which contained, 2.32 g/L (20 mM) fumarate, 15.44 g/L (100 mM) lactate, 20 ml/L solution 1, 10 ml/L solution 2, 10 ml/L solution 3, 10 ml/L solution 4, 1 ml/L solution 5 (appendix 5). Minimal media was prepared according to the protocol and the pH was adjusted between 7.2-7.4 using 10 N NaOH solution. The prepared medium was separated into 50 ml batches and were decanted in 250 ml conical flasks in order to
leave some head space for aeration purposes. The flasks were then autoclaved at 126ºC for 15 mins.

The standard procedure for setting up the inoculum for different experiments includes; inoculating the prepared medium with 4% S. oneidensis and incubating in Stuart Orbital Incubator SI500 at 30ºC with 150 rpm shaking for 24 hours. However for other experiments, 1% inoculum was used as standard to start the growth.

**S2 Medium**

This medium was tested for *S. oneidensis* to compare its biomass yield against minimal medium. The medium contained the following solutions:

<table>
<thead>
<tr>
<th>solution</th>
<th>Per litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>K2HPO4</td>
<td>95g</td>
</tr>
<tr>
<td>NaH2PO4</td>
<td>78g</td>
</tr>
<tr>
<td>(NH4)2SO4</td>
<td>360g</td>
</tr>
<tr>
<td>MgSO4 7H2O</td>
<td>400g</td>
</tr>
<tr>
<td>FeCl3</td>
<td>9.7g</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Trace element solution (per litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuSO4 5H2O</td>
</tr>
<tr>
<td>MnSO4 4H2O</td>
</tr>
<tr>
<td>ZnSO4 7H2O</td>
</tr>
<tr>
<td>CaCl2</td>
</tr>
<tr>
<td>Conc. H2SO4</td>
</tr>
<tr>
<td>S2 Medium (per litre)</td>
</tr>
<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td>K2HPO4</td>
</tr>
<tr>
<td>NaH2PO4</td>
</tr>
<tr>
<td>(NH4)2SO4</td>
</tr>
<tr>
<td>MgSO4 7H2O</td>
</tr>
<tr>
<td>FeCl3</td>
</tr>
<tr>
<td>Trace element solution</td>
</tr>
</tbody>
</table>

The S2 medium was made based on above table and then 2 g/litre yeast extract was added to the medium and the pH was adjusted to 7.2 using 10 N NaOH. The prepared medium was autoclaved at 126°C for 15 minutes.

**Growth experiment**

Minimal media was made according to standard protocol. 3 bottles of minimal media were inoculated with 1% overnight grown inoculum and incubated in Stuart Orbital Incubator SI500 at 30°C with 150 rpm shaking. Samples were taken in sterile conditions in different periods and OD_{600nm} was taken using Camspec M501. Growth curve was plotted based on the readings of the sample (Figure A).
Effect of trace metal solution on growth

This experiment was set up to investigate the effects of trace metal solution on growth of *S. oneidensis*. 2 bottles of minimal was prepared, one containing trace metal solution and the other without the trace metal solution. The media was autoclaved and inoculated with 1% over night grown inoculum. OD$_{600nm}$ was checked periodically. The results suggest that the presence of trace metal solution in the medium is essential and it has a significant positive effect on the growth rate and biomass yield (Figure B).

Figure A. Growth curve of *S. oneidensis* grown aerobically for 27h on Minimal media at 30°C with 150rpm shaking.

Figure B. Growth curve of *S. oneidensis* grown on minimal media, with and without trace metal solution.
S2 Medium Experiment

S2 medium was made according to standard protocol mentioned above. After autoclaving the medium, sterile glucose and glycerol solution was added to different bottles to give the final concentration of 20 g/litre, in order to test for their biomass yield. Minimal media was also made according to standard protocol with different concentrations of glucose and glycerol as carbon source to be tested for their biomass yield and compared to S2 media. A bottle of minimal media without fumarate was also made separately to investigate the effect of fumarate on cell growth.

The result of this experiment (Figure C) shows that the highest biomass yield was achieved by minimal media without fumarate and the minimal media with 50 mM glucose, with the lowest biomass yield being the minimal media with 100, 200, and 300 mM glucose.

It seems that too much glucose not only won’t enhance the growth but will also inhibit the growth of *S. oneidensis* significantly. However, comparing the results of standard minimal media with the minimal media containing the 50mM glucose, it seems that low concentrations of glucose may increase the growth rate and consequently increase the biomass yield over time.

The cells growth pattern on salt media shows a very low lag phase and a high growth rate during the first 6h of incubation following by a gradual decrease in biomass yield and finally reaching death phase, which suggests that salt medium is not the best possible medium for growing *S. oneidensis*. Another interesting finding of this experiment is the growth results of minimal media without fumarate which is higher than the biomass yield of standard minimal media, and suggests that presence of fumarate in the minimal medium not only will not support the growth of *S. oneidensis* but has a negative effect on cell growth and biomass yield.
Figure C. Comparing the biomass yield of S. oneidensis grown on minimal media and S2 media with different concentrations of glucose and glycerol as carbon source.

Future work:
Scale up and optimization of minimal medium from 50 ml to 5 L and potentially 50 L fermenter to test for biomass yield and iron reduction ability of the cells.
Scale up of magnetite production from 10 ml bottles to 100 ml, 1 L, and potentially 10 L bottles.
Examining different incubation conditions (for example, incubation under aerobic for a period and switching to anaerobic for a further duration) to enhance the growth and iron reduction ability of the cells.
Appendix 8

Purification of *G. sulfurreducens* stock

Identification of the contaminant:

Samples were taken from the contaminated *G. sulfurreducens* batch and tested as below:

1. Gram staining was carried out on the samples.
2. Samples were streak plated onto agar plates and incubated aerobically along with a control plate at 30°C under sterile conditions.
3. Samples were taken from the single colonies on the aerobically grown plates for identification purposes.

Identification was performed through the following steps:

- **DNA Extraction:** A single colony was taken from the agar plates that were incubated aerobically, and added 20 µl of DI water and mixed in a PCR tube. The mix was loaded on to a PCR machine programmed with 5 mins boiling at 99°C and kept at 4°C, to extract the DNA.

- **Amplification of the DNA (PCR):** The PCR was carried out by making up the reaction mix which includes 12.5 µl of Sigma PCR Ready mix, 0.5 µl of 16S forward primer (8F), 0.5 µl of 16S reverse primer (1492R), 10 µl of DI water, and 2 µl of the extracted DNA to be used as template. The tube was kept on an ice tray during the preparation procedure. Sample was loaded on a PCR machine programmed as, Initial 96°C for 2 minutes, then 30 cycles of (96°C for 40 seconds, 50°C for 15 seconds, 60°C for 3 minutes) and finally cools to 4°C.

- **Agarose Gel Electrophoresis:** The agarose gel was prepared by adding 1g of agarose to 100 ml of 1xTAE buffer in a 250 ml conical flask and heating it up in a microwave on full power until the mixture starts to bubble (approx 50 seconds). The flask was set on a side for a few minutes to cool down. 4 µl of Ethidium Bromide solution was added into the gel and mixed well by swirling. The agar gel was then gently poured into the pre-prepared gel casting tray and left to set for 30 minutes. 600 ml of 1X TAE Buffer was poured into a submarine gel electrophoresis tank. Once the gel was casted, it was transferred to the electrophoresis tank and the comb was removed. Samples were prepared by
adding 2 µl of 5X Gel Loading Solution to 8µl of the PCR products and mixing it by pipetting it up and down a few times. The mix was then loaded in the wells and the system was set to run at 80 Volts for approximately 40 minutes. Hyper LadderI was used as ladder. Photographs were taken under UV light to confirm the bands.

- **Product Precipitation:** The PCR product was transferred to a 1.5 ml eppendorf and then the following reagents were added: PCR product (20 µl), 2 µl of 125 mM EDTA, 2 µl of 3 M Na Acetate (pH 4.5), 0.5 µl GlycoBlue, and 50 µl of 95% Ethanol. The tube was mixed and left for 10 minutes then spun at 13,000rpm for 30 minutes. Supernatant (excess reaction mix / salt / ethanol) was removed with pipette and the remaining pellet was washed with 250 µl 70% ethanol and spun again at 13,000 rpm for 15 minutes. Supernatant was removed from the tube and then left opened on a heated block at 90°C for 1 minute to drive off excess ethanol. Sample was then kept in the dark at 4°C until sent for sequencing.

**Purification procedures:**

NBAF medium containing 40 mM fumaric acid and 25 mM acetate and other standard components was prepared as mentioned above (page 31). The medium was degassed and autoclaved according to standard protocol. 2% agar was added to the NBAF medium and the final solution was decanted into separate Petri dishes under anaerobic conditions. Samples from the contaminated *G. sulfurreducens* NBAF medium were taken and streak plated on 2 NBAF agar plates, and a third plate was left blank to be used as control. The plates were placed in an anaerobic jar and incubated at 30°C for 5 days. Subcultures from the grown single colonies was made onto NBAF agar and incubated in an anaerobic jar at 30°C for 5 days.

Samples from the single colonies on the last NBAF agar plate was taken and tested as below:

1. 3 bottles (100 ml) of sterile and degassed NBAF 40 mM fumaric acid and 25 mM acetate was inoculated using the single colonies and incubated at 30°C under anaerobic conditions for 5 days.

2. Samples were taken from each of the bottles for gram staining purposes.
3. Sample was taken from the bottles and streak plated on to an agar plate and incubated at 30°C aerobically along with a control plate under sterile conditions.

4. Cells from each of the bottles were spun down using standard protocol and inoculated into ferrihydrite suspensions (10 ml) with 10 µM riboflavin as mediator to test the Fe(III) reduction capability of the cells.

**Results and Discussion**

The gram staining of the *G. sulfurreducens* stock streak plated and incubated aerobically at 30°C showed a rod shaped, Gram positive, spore like forming bacteria which was suspected to be a Bacillus species (Figure D).

![Figure D](image_url)

Figure D. A sample from the *G. sulfurreducens* stock was Gram stained and observed under light microscope with special filters.

Samples were taken from single colonies on the aerobically grown agar plates and amplified using protocols mentioned above and sent for identification. The results suggested that the contaminant was *Bacillus bataviensis* strain BAC3053 (Heyrman *et al.*, 2004), with the partial 16s ribosomal sequence as below:

```
CAGTCGAGCGGACTTTAAAAGCTTGCTGTAAAAGTTAGGCGGCGGACGG
GTGAGTAACACGTGGGCAACCTGCCTGTAAAGACTGGGATAACTTCGGGA
AACCAGGACCTAACCCGATAATCTTTCCACCACATGTTGGAAAAAGCTG
AAAGACGTCTTCTGGCTGTCCCTTACAAATGGGCCCCGGCGGCGCATTAACCT
AGTGGGGAGGTAAACGGTTCAACCAAAGGCAACAATGCGTACCCAACCTGA
AAGGGGATCGGCCCACACTGGGACTGAAACACGCGCCAAACTCTACGG
GAGGCAGCAGTGAGGAAATCTTCCGCAATGGGACAAAAAGTCGACGGGACCA
CCGCCCCGGGAGTGATGAAAGGTGTTTCGGATCGTAAAGTTCTGTGTTTAGG
GAAAAAAGTGACAAAAAGTACTGCTTGTACTTGAGGTACTTAACCA
```
Samples were taken from the single colonies of the aerobically grown cells and subcultured onto NBAF agar plates to be incubated anaerobically according to protocol mentioned above. After the incubation period, samples were taken from single colonies on the plate and Gram stained to check for purity. The cells appeared to be Gram negative rods (Figure E) which match with *G. sulfurreducens* specification.

To make sure the cells were *G. sulfurreducens*, samples were taken from the single colonies grown anaerobically on NBAF agar and subcultured into NBAF 40 mM fumaric acid and 25 mM acetate (100 ml bottle) and incubated anaerobically at 30°C. After 7 days of incubation the NBAF changed colour to pink (Figure F) which indicated the presence of *G. sulfurreducens*. 

Figure E. Samples from the purified stock was Gram stained to check for purity, the cells appeared as Gram negative rods.

GAAAGCCACGGTTAACTACGTGCCAGCAGCGCGGTATTACGTAGGTGG
CAGGGTAGGGTCCGGAATTATGG

GAAAGCCACGGTTAACTACGTGCCAGCAGCGCGGTATTACGTAGGTGG
CAGGGTAGGGTCCGGAATTATGG
Figure F. Samples from the single colonies on NBAF agar and inoculated into NBAF 40 mM fumaric acid and 25 mM acetate and incubated anaerobically at 30°C for 7 days to check for any changes in colour.

Samples were taken and inoculated into ferrihydrite suspensions according to standard protocol to test the iron reduction ability of the cells. Transformation and biogenic magnetite production achieved (Figure G) after 24 h of anaerobic incubation at 30°C which confirmed the presence of G. sulfurreducens.

Figure G. Ferrihydrite suspensions were inoculated with the cell samples to check for their Fe(III) reduction ability (original iron gel bottle on the left, biogenic magnetite produced by the cells on the right side of the picture).
Appendix 9

Scale up to 50L Fermenter

Figure H. 10L BIOSTAT C plus fermenter used for incubating *G. sulfurreducens* to be used as inoculum for the 50L scale up experiment.

Figure I. 50L Sartorius fermenter used to incubate *G. sulfurreducens* for the scale up experiment.
Appendix 10

Metabolomic Experiment

Comparison of the metabolomic fingerprints of *G. sulfurreducens* grown in 100 ml microcosms to 5 L bioreactor over time.

This experiment was set up to compare the metabolomic changes of *G. sulfurreducens* over time during the scale up process from 100 ml bottles to 5 L bioreactor.

7 L of NBAF (80 mM fumaric acid/ 50 mM acetate) was made according to protocol (appendix1). 4.5 L of the prepared medium was decanted into the bioreactor and the setup procedure was carried out according to standard bioreactor setup protocol.

630 ml of the medium was decanted into a 1 L bottle, the bottle was autoclaved and the medium degassed using 80:20 N₂:CO₂ for 1h in sterile conditions. The degassed medium was then inoculated with 70 ml of overnight grown *G. sulfurreducens* and incubated at 30°C for 40 hours to be used as the main inoculum in this experiment.

1.7 L of the NBAF was decanted into a 2 L duran bottle and degassed using 80:20 N₂:CO₂ for 1 hour under sterile conditions. The degassed medium was then decanted into 100ml bottles under anaerobic conditions (anaerobic cabinet). The bottles were then autoclaved for 15 min at 126°C.

The bioreactor and the bottles were inoculated with 10% *G.sulfurreducens* from the main inoculum. Bioreactor was set to incubate at 30°C with 50rpm stirring and the pH control OFF, and the bottles were incubated in a Stuart Orbital SI500 incubator at 30°C and no shaking. The bottles were setup in triplicate.

Samples were taken from the bottles and the bioreactor in different time intervals (Table 3) to be prepared for FTIR analysis. Samples were centrifuged at 4°C at 12000 g for 20 using Sigma 6K15 centrifuge. The supernatant was removed and cell pellets were resuspended in 5ml of sterile 0.9% NaCl solution and centrifuged again at 4°C and 5240 g for 10 min, supernatant was removed and the last spinning step was repeated again. After the final spin, supernatant was removed and the cell pellets were flash frozen using liquid nitrogen for 2 mins and kept at -80°C freezer for the FTIR analysis to be carried out.
A 96 well sample plate was washed with 2-propanol and deionised water and air dried at room temperature for FTIR purposes. 20 µl (0.016g biomass) of each bacterial sample was evenly applied onto the plate. The plate was dried in an oven at 60°C consequently for 10 min and loaded into the FT-IR spectroscopy motorized module. Spectra were collected over the wavelength range of 4000 to 600 cm\(^{-1}\) under the control of a computer programmed with Opus 4. Spectra were acquired at a resolution of 4 cm\(^{-1}\) and 64 spectra were co-added and averaged to improve the signal-to-noise ratio.

**Table 3.** Samples were taken in different time intervals from the 100ml bottles and bioreactor for metabolomic analysis.

<table>
<thead>
<tr>
<th>Samples</th>
<th>T(_{0h})</th>
<th>T(_{4h})</th>
<th>T(_{24h})</th>
<th>T(_{3d})</th>
<th>T(_{7d})</th>
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</thead>
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<td>100ml Bottles</td>
<td>×3</td>
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<td>×3</td>
<td>×3</td>
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<tr>
<td>Bioreactor</td>
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<td>×3</td>
<td>×3</td>
<td>×3</td>
<td>×3</td>
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