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20 L Scale Once-Fed-Batch Process Characterization of *Magnetospirillum Gryphiswaldense* Strain MSR-1 for Production of Magnetosomes

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Magnetite nanoparticles (mNPs; chains of magnetic crystals found in prokaryotes), especially the biomembrane-enclosed form of bacterial nanoparticles known as magnetosomes, have received keen commercial interest because of their narrow size range and good dispersibility. The biomineralization of magnetosomes is a highly controlled process regulated at the gene level that results in high-purity, single-magnetic-domain particles (i.e., magnetite [Fe_3O_4] or greigite [Fe_3S_4]). Because of the highly restrictive culture condition for magnetotactic bacteria in terms of slow growth, dissolved oxygen, and nutrient requirements, the yields of both magnetosomes and their host microorganisms under laboratory cultures tend to be low. In this effort, *Magnetospirillum gryphiswaldense* strain MSR-1 was used in a 20 L fermentor system to initially characterize the dissolved oxygen conditions for induction of magnetosome formation, establish a baseline magnetosome yield, and explore ways to increase the magnetosome yield over baseline culture conditions.

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PREFACE

The work described in this report was started in April 2018 and completed in October 2018. At the time this work was performed, the U.S. Army Combat Capabilities Development Command Chemical Biological Center (DEVCOM CBC; Aberdeen Proving Ground, MD) was known as the U.S. Army Edgewood Chemical Biological Center.

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20 L SCALE ONCE-FED-BATCH PROCESS CHARACTERIZATION OF *MAGNETOSPIRILLUM GRYPHISWALDENSE* STRAIN MSR-1 FOR PRODUCTION OF MAGNETOSOMES

1. INTRODUCTION

Magnetite nanoparticles (mNPs; chains of magnetic crystals found in prokaryotes), especially the biomembrane-enclosed form of bacterial nanoparticles known as magnetosomes, have received keen commercial interest for their narrow size range and good dispersibility.[1] Because of these desirable physical properties, previous studies have focused on using mNPs as catalysts and in a variety of applications, including wastewater treatment, magnetic resonance imaging, heavy metal removal, drug delivery, and even terabit magnetic storage devices. The size, quality, shape, and crystallization of these nanoparticles (NPs) greatly affect how they behave in different applications. In contrast, some chemical synthetic approaches to provide for optimal NP size control and crystallization are still greatly desired.[2] Industrial synthetic production of mNPs is generally labor-intensive, expensive, and hazardous to living organisms and the environment.

The biomineralization of magnetosomes is a highly controlled process regulated at the gene level that results in high-purity, single-magnetic-domain particles (i.e., magnetite [Fe_3O_4] or greigite [Fe_3S_4]). Because of the highly restrictive culture conditions for magnetotactic bacteria in terms of slow growth, dissolved oxygen, and nutrient requirements, the yields of both magnetosomes and their host microorganisms under laboratory cultures tend to be low.[3,4]

Thus far, biotechnological studies for magnetosomes have been focused on a limited number of strains of the genus *Magnetospirillum*, mostly *Magnetospirillum gryphiswaldense* strain MSR-1 and *Magnetospirillum magneticum* strain AMB-1,[3] which produces a chain of cubo-octahedral Fe_3O_4 magnetosome particles. For *M. gryphiwaldense*, a low dissolved oxygen concentration was determined to be necessary for the induction of magnetosome formation. For this study, *M. gryphiswaldense* strain MSR-1 was used in a 20 L fermentor system to initially characterize the Fe_3O_4 conditions for induction of magnetosome formation, establish a baseline magnetosome yield, and explore means to increase the magnetosome yield over baseline culture conditions.

2. MATERIALS AND METHOD

2.1 Bacterial Strain Source

M. gryphiswaldense strain MSR-1 was obtained from Deutsche Sammiung von Mikroorganismen und Zellkulturen (Brunswick, Germany).

2.2 Growth Medium Composition

The initial media used for 200 mL, 1.8 L flasks, and 20 L fermentation were the same and consisted of the following (per liter deionized [DI] water):

2.6 g sodium DL-lactate solution, 0.4 g NH_4Cl, 0.1 g yeast extract, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.5 g K_2HPO_4, 165 µmol as ferric quinate, and 0.5 mL mineral elixir.

The mineral elixir further consisted of the following (per liter DI water):

15.0 g of nitrilotriacetic acid, 3.0 g $MgSO_4 \cdot 7H_2O$, 5.0 g $MnSO_4 \cdot H_2O$, 10.0 g NaCl, 1.0 g $FeSO_4 \cdot 7H_2O$, 1.8 g $CoSO_4 \cdot 7H_2O$, 30.0 g $CaCl_2 \cdot 2H_2O$, 1.8 g $ZnSO_4 \cdot 7H_2O$, 0.1 g $CuSO_4 \cdot 5H_2O$, 0.2 g $KAl(SO_4)_2 \cdot 12H_2O$, 0.1 g H_3BO_3, 0.1 g $Na_2MoO_4 \cdot 2H_2O$, 0.25 g $NiCl_2 \cdot 6H_2O$, and 3.0 mg $Na_2SeO_3 \cdot 5H_2O$.[5]

In the 20 L once-fed-batch fermentation, 500 mL of concentrated ingredients were used, equivalent to the initial 20 L concentration, except the solution was supplemented with 10 g K_2HPO_4 when the carbon source was deemed to be depleted, as indicated by an increase in dissolved oxygen (DO) from the microaerophilic/anaerobic conditions.

2.3 20 L Fermentation

Twenty milliliters of frozen glycerol stock of *M. gryphiswaldense* MSR-1 was used to inoculate 200 mL of medium in a 500 mL flask. The 500 mL flask was incubated in an I2500 series incubator shaker (New Brunswick Scientific; Enfield, CT) at 30 ^oC and 225 rpm for at least 10 h. The optical density (OD) at 565 nm was allowed to reach a value greater than 1, then the 200 mL cultures were used to inoculate 1.8 L of medium in a 4 L flask. The 4 L flask was incubated at 30 ^oC and 175 rpm for at least 10 h. The OD value was again confirmed to be greater than 1, then a pre-sterilized 2 L transfer bottle was used to inoculate the Micros 30 L fermentor (New Brunswick Scientific), which had been prepared and steam-in-place sterilized with an 18 L medium, with the 2 L cultures. The operating parameters for the fermentor were 30 ^oC, 150 rpm, 2 standard liters per minute (SLPM), and 1 psi overhead pressure with a working volume of approximately 20 L.

The pH was monitored using a Mettler-Toledo pH 2100e transmitter (Columbus, OH) connected to a sterilizable, pre-pressurized, gel-filled electrolyte pH probe (Mettler-Toledo) and controlled at pH 7 with H_3PO_4 (3M; Saint Paul, MN) via a dedicated programmable logic controller (Allen Bradley; Milwaukee, WI). The DO was monitored using a Mettler-Toledo O2 4100e transmitter connected to an InPro 6800 polarographic oxygen sensor (Mettler-Toledo)

and displayed by a PanelView Plus 1000 operator interface terminal (Allen Bradley). The oxidation reduction potential (ORP) was measured using an Accumet 25 pH meter in mV mode (Fisher Scientific; Hampton, NH), connected to a sterilizable, pre-pressurized, gel-filled electrode ORP probe (Mettler-Toledo). All process data except the ORP, which was manually recorded periodically, were logged into a personal computer using the AVEVA Historian database (Cambridge, UK) for later retrieval and analysis.

Oxygen mass transfer coefficients (KLa') were determined for the Micros 30 fermentor with 20 L working volume of DI water at various combinations of agitation and air flow rates, according to chemical stripping of DO, reaeration, and monitoring DO method versus time.[6] A PowerFuge centrifuge (McMaster-Carr; Elmhurst, IL) was used to harvest the cells at the end of the 20 L fermentation cycle. The sample was continuously centrifuged at 2 L per min, then the cell paste was scraped off from the centrifuge cores and stored in a freezer at –80 ºC for later processing and purification of magnetosomes.

2.4 Analytical Methods

Cell concentration was monitored spectrophotometrically by absorbance at 565 nm using a Genesys 20 spectrophotometer (Thermo Fisher Scientific; Waltham, MA). The Fe^{2+}/Fe^{3+} concentration was measured using the 3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5″-disulfonic acid disodium salt (Ferene-S) assay method.[7] Two 1 mL samples, one untreated and the other spiked with 10 µL concentration HNO_3, were centrifuged (IEC Micro-MB centrifuge; International Equipment Company; Needham Heights, MA) at 10,000 rpm for 3 min. After centrifugation, supernatant from both samples was decanted for supernatant analysis. For the cell pellet, 0.1 mL concentration HNO_3 was added and digested using a Multi-Blok heater (Lab-Line Instruments; Melrose Park, IL) at 80 ºC for 2 h. The samples were allowed to cool, then 0.16 mL 1 N NaOH was added for neutralization, and the total volume of digested and neutralized sample was brought to 1 mL by adding 0.74 mL DI water. Either 1× or 2× working buffer was added to different dilutions of supernatant or 0.5 mL of digested samples and incubated in the dark for at least 20 h before absorbance readings were taken from the 96-well plate at 595 nm in triplicate at 0.3 mL per well using a SpectraMax 190 microplate reader (Molecular Devices; San Jose, CA). Iron standard (Sigma Aldrich; St. Louis, MO) in 0, 1, 2, 3, and 4 µg/mL concentrations was included for each set of 96-well microplate analyses.

The concentration of lactate in supernatant samples was measured using a lactate assay kit (Sigma Aldrich). Fifty microliters of a sample or dilution thereof was mixed with 50 µL of master reaction mix in each well and incubated for 30 min in the dark, then the SpectraMax 190 microplate reader was used to take a colorimetric reading at 570 nm. Lactate standard (Sigma Aldrich) in 0, 2, 4, 6, 8, and 10 nmol/µL concentrations was included for each set of microplate lactate analyses.

The concentration of ammonia in supernatant samples was measured using an ammonia assay kit (Sigma Aldrich) that was modified for use with the SpectraMax 190 microplate reader. Initially, each well to be analyzed was filled with 275 µL DI water and blanked at 340 nm. A new plate well pre-filled with 250 µL ammonia assay reagent was added with either 25 µL of a sample or a dilution thereof, 25 µL DI water for blank, or 25 µL ammonia standards and mixed well for 5 min before absorbance readings were taken at 340 nm. Immediately after, 2.5 µL of L-glutamate dehydrogenase solution was added to each well, mixed

well, and incubated for 5 min before absorbance readings were taken at 340 nm. Ammonia standards in 0, 2, 4, 6, 8, 10, and 15 µg/mL concentrations were included for each set of ammonia analyses.

2.5 Purification Method Protocol

Cell pastes were resuspended at a ratio of 1 g wet cell paste to 5 mL of phosphate-buffered saline (PBS, pH 7.2) and passed through a Microfluidizer fluid processor (model no. M110PII; Microfluidics; Westwood, MA) three times at 20,000 psi. Magnetosomes were separated from crude cell lysates using Dynabeads magnetic beads (Invitrogen; Waltham, MA). After separation, the supernatant was siphoned off, the magnetosome content was resuspended in PBS (pH 7.2), and the magnetosomes were separated out again. This resuspension and separation process was repeated ten times. The purified magnetosomes were dried at 70 ^{o}C overnight and weighed.

3. RESULTS AND DISCUSSION

Cell growth of *M. gryphiswaldense* strain MSR-1, monitored via OD at 565 nm and its DO profile in a 20 L fermentation system, is shown in Figure 1. An immediate decrease in DO in the 20 L fermentor (from 100 to approximately 75%) upon the inoculation of 1.8 L cultures was observed. This immediate DO drop could be due to a rapid DO uptake by oxygen-starved overnight cultures of the 1.8 L inoculum. The DO response immediately after the initial DO uptake within 10 min exhibited normal exponential decreases, as expected for exponentially growing batch cultures. During its exponential phase of growth, the maximum growth rate was estimated as 0.2/h. This value was comparable but slightly higher than the average growth rate of 0.13/h for the same strain using a 4 L oxystat fermentor.[8] The lower growth rate was most likely due to the oxygen-limiting factor under oxystat conditions. In fact, the growth rate of *M. gryphiswaldense* strain MSR-1 in this study also decreased to 0.076/h as oxygen became a limiting factor (i.e., 0% DO around elapsed fermentation time of 7.5 h and thereafter). Once the initial nutrients were depleted, as indicated by the DO increase (arrow in Figure 1), 500 mL of concentrated ingredients (equivalent to the initial 20 L concentration with the addition of 10 g K_2HPO_4) was aseptically added to maintain DO < 1% for the formation of magnetosomes.

The usage of carbon (lactate) and nitrogen (ammonia) substrates by the growing cultures along with OD data are shown in Figure 2. Based on the lactate utilization data with OD increases, the cell yield was estimated to be 0.34 OD cell per gram of sodium lactate in the exponential phase of growth and 0.45 OD cell per gram of sodium lactate under the oxygen-limiting conditions. These yield values are in very good agreement with the 0.42 ± 0.10 yield previously reported for *M. gryphiswaldense* strain MSR-1.[3] The reason for the difference in cell yield values between the exponentially-growing and oxygen-limiting cultures is not clear. The remaining concentration of ammonia (around 20 µmol/mL) indicated that ammonia was not limiting growth throughout the batch and subsequent once-fed-batch period.

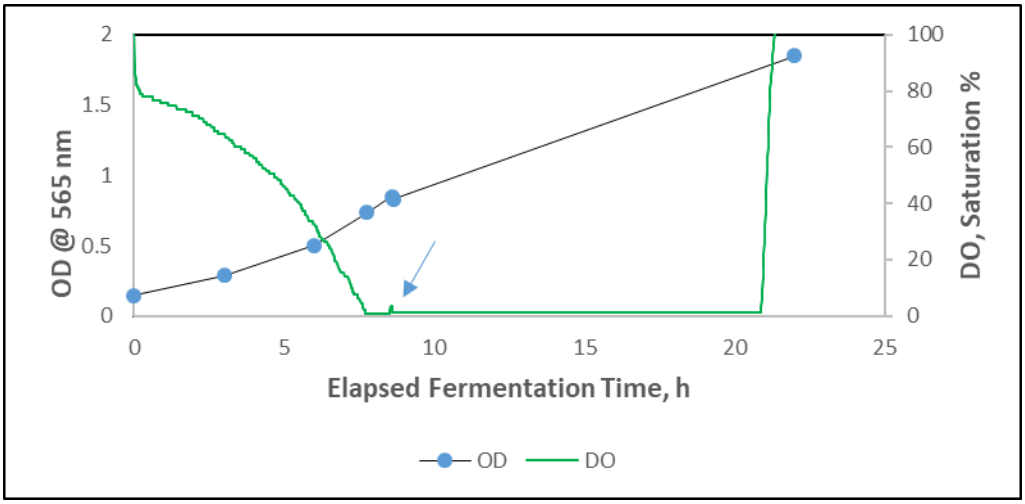

Figure 1. Cell growth as measured by OD at 565 nm and corresponding DO response. Arrow indicates the point at which initial nutrients were depleted.

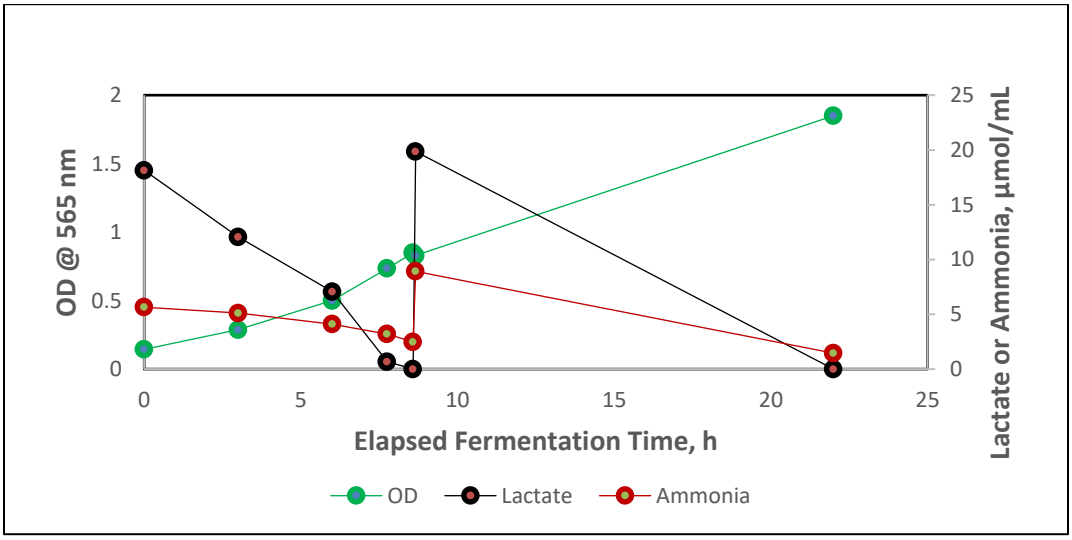

Figure 2. Lactate and ammonia consumption during cell growth of *M. gryphiswaldense* strain MSR-1.

The prerequisite for onset of magnetosome formation in *M. gryphiswaldense* strain MSR-1 was previously demonstrated to be prolonged exposure of the cultures (e.g., several hours) under microaerophilic or anaerobic conditions.[3,8] A DO cascade control using multi-gas approaches with an amplified DO signal was previously implemented to maintain the prerequisite microaerophilic conditions.[5] However, conventional DO cascade control using either agitation or airflow was not feasible due to insufficiently accurate DO probes[8] or too-short dynamic response times involved under microaerophilic conditions (this study, data not shown). Hence, the best practical approach for achieving microaerophilic/anaerobic culture conditions appeared to be allowing the actively growing cultures to keep DO tension under oxygen-limiting conditions below 1% DO with a continuous supply of nutrients. In fact, several studies have

applied a pH-stat using the carbon source (i.e., lactate) fed-batch strategies to maintain the DO tension below 1% and increase density of cultures, positing that higher cell density would lead to higher magnetosome formation.[3,5]

To balance high DO for cell growth and <1% DO for magnetosome formation, the (KLa') value for a given operating condition of fermentation cultures becomes critical. Different (KLa') values at different combinations of agitation speed and airflow rate with the Micros 30 fermentor are shown in Figure 3. At the 150 rpm agitation speed used in this study, the (KLa') values (0.0673 to 0.0845) did not significantly increase as air supply was increased from 2 to 8 SLPM; changes were noted at higher agitation speed over the same 2–8 SLPM air supply range. An agitation speed of 150 rpm and air supply of 2 SLPM were chosen for this study because these parameters allowed a reasonable amount of time to deplete DO before the onset of magnetosome formation. The (KLa') value of 0.0673/h at 150 rpm and 2 SLPM of air supply was hence used in conjunction with the DO profile for the estimation of DO uptake and usage by the batch cultivation of *M. gryphiswaldense* strain MSR-1.

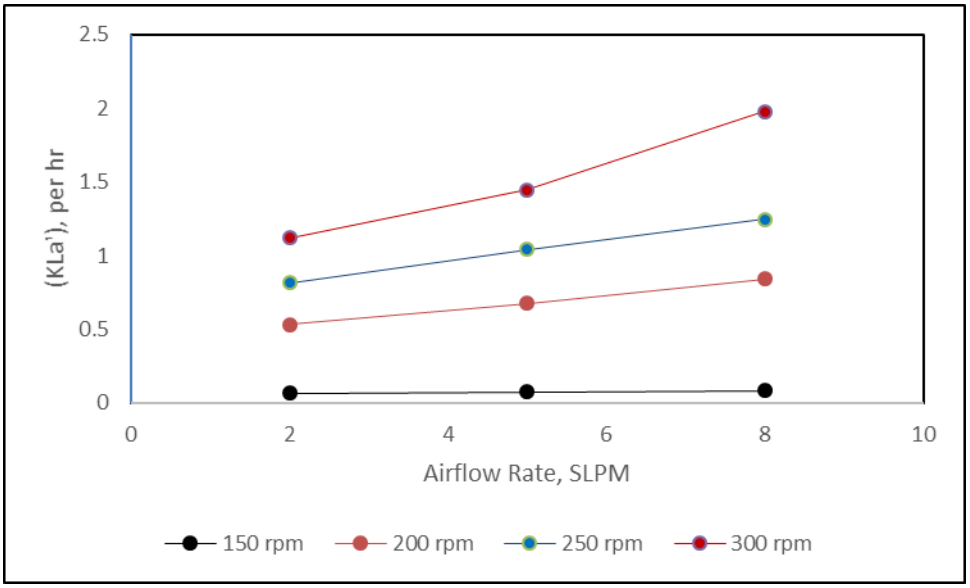

Figure 3. Experimentally determined oxygen mass transfer coefficient values at various combinations of mixing speeds and airflow rates.

The accumulated DO uptake in milligrams per liter by the batch cultures of *M. gryphiswaldense* strain MSR-1 and computed specific DO uptake/usage per OD at 565 nm are shown in Figure 4. During the exponential phase of growth, the specific oxygen uptake/usage by the cultures averaged 220 mg/L·OD at 565 nm. However, as the cultures entered into oxygen-limiting conditions, the specific DO uptake/usage per OD went up to an average of 380 mg/L·OD. The reason for the higher specific DO uptake/usage per OD during oxygen-limiting conditions is not clear. Perhaps the oxygen demand for the oxidation of ferrous ions to ferric ion for magnetosome formation may have exerted higher specific DO per OD.

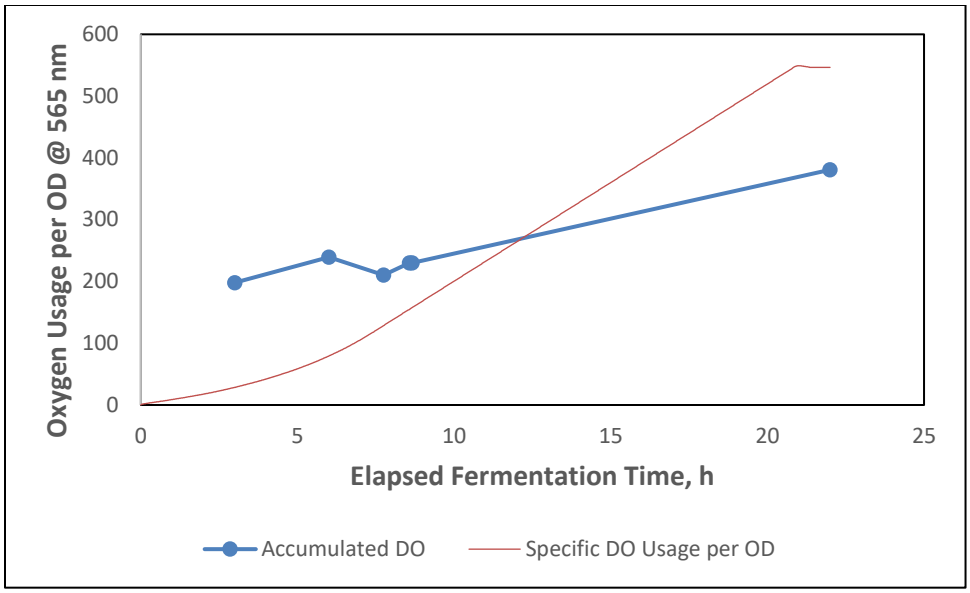

Figure 4. Accumulated DO and specific DO usage per OD during batch cultures of *M. gryphiswaldense* strain MSR-1.

Time-dependent concentration of Fe^{+2}/Fe^{+3}in solution and intracellular iron accumulated by the cell cultures are shown in Figure 5. As expected, a sharp increase in solution iron concentration was noted upon the addition of 500 mL of concentrated one-batch-equivalent nutrients. Over time, the iron concentration of the solution decreased, and the intracellular iron concentration increased overnight.

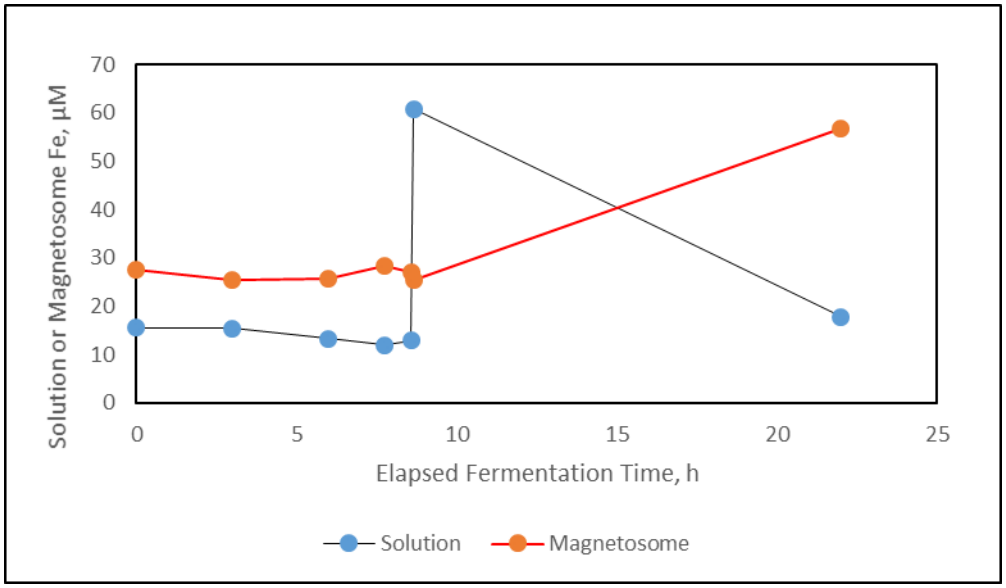

Figure 5. Solution iron uptake and intracellular iron accumulation during batch cultures of *M. gryphiswaldense* strain MSR-1.

The reason for the presence of intracellular iron from the start of 20 L fermentation, when DO was not limited, is not clear at this time. It could possibly be attributed to the 2 L inoculation cultures, which presumably had been exposed to microaerophilic/anaerobic conditions. As previously shown in Figure 1, the immediate DO uptake by 2 L cultures in the start of 20 L fermentation indicated that the overnight 2 L cultures were oxygen deprived, which had to be conducive to magnetosome formation.

Overall, about 60 µM of intracellular iron, as shown in Figure 5 (3.35 mg iron/L, 1.81 mg iron/OD, or 67 mg total iron), was obtained at the end of 20 L fermentation based on the iron analyses. A total of 76.5 g wet cell paste was obtained from the 20 L fermentation; therefore, the intracellular iron yield per gram of wet cell paste was determined to be 0.86. Assuming that >99% of the total cellular iron is magnetosome-bound,[7] 20 µM of Fe_3O_4 would be expected since three Fe are required to form Fe_3O_4. Hence, 4.63 mg Fe_3O_4/L, 1.2 mg Fe_3O_4/g wet cell paste, 1.85 mg Fe_3O_4/OD, or 92.6 mg total Fe_3O_4 are estimated based on the solution iron analyses. The estimated yield of 1.2 mg Fe_3O_4/g wet cell paste can be compared with the actual purification yield of magnetosomes from three different portions of cell paste (Table 1).

Table 1. Data on Magnetosome Purification Yield

Wet cell weight (g)	**Dry magnetosome weight (mg)**	**Yield (mg magnetosome/g wet cell weight)**
14.8	59.4	3.9
13.76	16.1	1.17
11.0	50	4.54

Two of the three yield values were much higher than the estimated Fe_3O_4 yield based on the intracellular iron concentration. It would be expected that purified membrane-enclosed magnetosome would result in a higher yield value since the weight of the membrane was included as well. Other residual matter from the purification process may also have contributed to the magnetosome yield being higher than estimated based on intracellular Fe_3O_4. Nevertheless, the yield values are in the same order of magnitude, and the average yield was 3.2 mg magnetosome/g wet cell paste. This magnitude could not be directly compared to other studies,[8] which were performed on a dry cell basis. However, it is expected that 3.2 mg magnetosome/g wet cell paste is comparable to 5.3–14.2 mg magnetosome/g dry cells under varying DO tensions, considering that more than 70% of wet cell paste consists of water. Assuming 70% water in wet cell paste in this study, 10.6 mg dry weight cells are estimated from 3.2 mg wet cell paste.

The change in ORP during the batch cultures of *M. gryphiswaldense* strain MSR-1 are shown in Figure 6. As expected, the ORP exponentially decreased along with DO. However, as soon as the second-batch equivalent of medium containing ferrous ion was added, the ORP immediately dropped to –118 mV, most likely due to the reduced form of ferrous iron added. Unfortunately, the ORP under DO-limiting conditions could not be monitored. The next day, when the DO had risen back to 100% due to nutrient depletion, the ORP also increased to

200 mV, indicative of the oxidative state of the spent culture medium. As a result, the effect of lowered ORP during the microaerophilic/anaerobic conditions as opposed to the addition of ferric ions (i.e., oxidized form) could not be ascertained because of the lack of data points in between. Nevertheless, this study has shown that the addition of ferrous ion, which is more soluble than ferric ion, could also serve as an iron source for magnetosome formation in *M. gryphiswaldense* strain MSR-1.

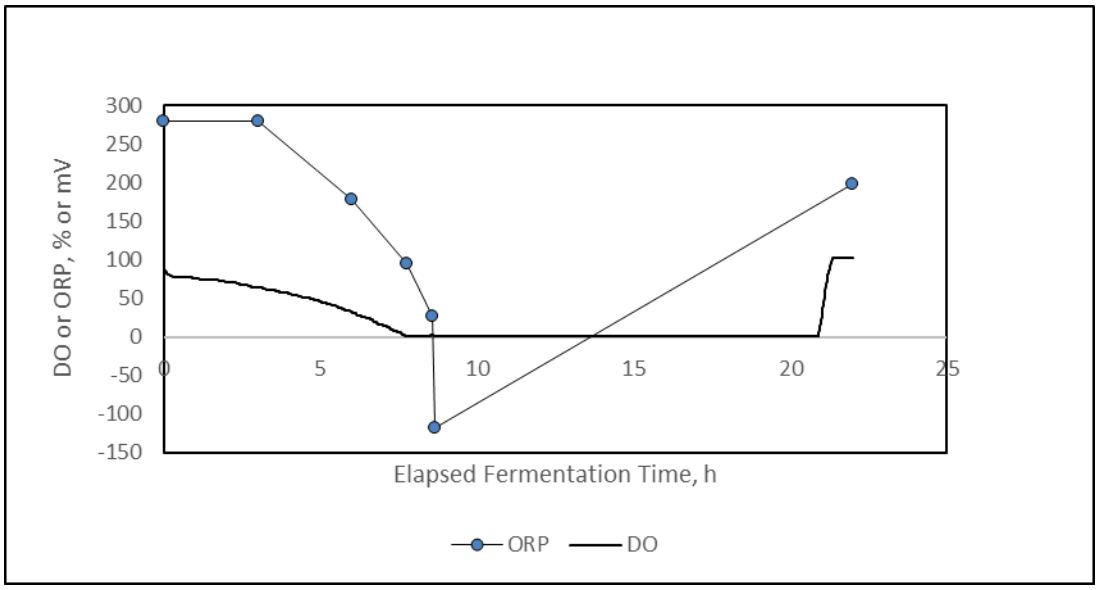

Figure 6. DO and ORP profiles during cell growth of *M. gryphiswaldense* strain MSR-1.

4. CONCLUSION

Batch cultures of *M. gryphiswaldense* strain MSR-1 in a 20 L working volume were performed to characterize the nutritional requirement and onset of magnetosome formation. Since the initial DO present in the 20 L fermentation vessel along with nutrients will be used for growth-related metabolism until the DO is depleted below 1%, a second dose of 500 mL concentrated 20 L batch-equivalent nutrients was aseptically added when the DO began to rise, indicating nutrient depletion. The addition of a second batch of nutrient (i.e., once-fed-batch) allowed DO to be maintained at <1% for onset of magnetosome formation.

Batch growth characteristics of *M. gryphiswaldense* strain MSR-1 in terms of growth rate and cell yield value were 0.2/h and 0.34 OD cell/g lactate consumed, respectively, during the exponential growth phase without DO limitation. Under DO limiting conditions (i.e., DO < 1%), the growth rate decreased to 0.075 per, as expected, but the cell yield increased to 0.45 OD cell/g lactate; the reason for this increase is not known at this time.

Overall, an average of 3.2 mg magnetosome/g wet cell paste was purified in this study. This was estimated to be equivalent to 10.6 mg magnetosome/g dry weight cells, which is comparable to results from previous studies performed on a dry cell basis.

The most practical strategy for maintaining DO < 1% for the formation of magnetosomes appeared to be feeding fresh nutrients continuously, as in a fed-batch mode. However, due to less reliable DO readings at levels below 1% and its response time, a manual intervention based on DO reading may be necessary to achieve and maintain fermentation conditions for the formation of magnetosomes. Furthermore, the linear growth rate data and yield information during the DO-limiting conditions could be used to implement a fed-batch mode using a linear growth model for future scale-up efforts in production of magnetosomes.

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ACRONYMS AND ABBREVIATIONS

DI	deionized
DO	dissolved oxygen
Fe_3O_4	magnetite
Fe_3S_4	greigite
Ferene-S	3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5″-disulfonic acid disodium salt
(KLa')	oxygen mass transfer coefficients
mNP	magnetite nanoparticles
NP	nanoparticle
OD	optical density
ORP	oxidation reduction potential
PBS	phosphate-buffered saline
SLPM	standard liters per minute

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