This document was prepared in conjunction with work accomplished under Contract No. DE-AC09-96SR18500 with the U.S. Department of Energy.

The Role of 4-Hydroxyphenylpyruvate Dioxygenase in Enhancement of Solid-Phase Electron Transfer by *Shewanella oneidensis* MR-1

Charles E. Turick[1+], Alex Beliaev[2], Daniel A. Lowy[3], Tara E. Poppy[4*], Andrea Malony[5#], and Amy A. Ekechukwu[1]

[1]Savannah River National Laboratory, Aiken, SC
[2]Pacific Northwest National Laboratory, Richland, WA
[3]Nova Research, Inc., Alexandria, VA
[4]University of South Carolina, Aiken, SC
[5]Winthrop University, Rock Hill, SC

[+] Corresponding author. Environmental Science and Biotechnology Department. Savannah River National Laboratory, Building 999W, Aiken, SC. 29808. Phone: 803-819-8407. Fax: 803-819-8432. Email: Charles.Turick@srnl.doe.gov

* present address- Department of Veterinary Medicine, University of Tennessee, Knoxville, TN.

present address – South Carolina Department of Health and Environmental Control, Columbia, SC

Key words: dissimilatory metal reducing bacteria, pyomelanin, electron shuttle, electron transfer, hydrous ferric oxide, iron minerals, anaerobic respiration, cyclic voltammetry.

Running title: 4-HPPD Enhances Solid-Phase Electron Transfer

Summary

While mechanistic details of dissimilatory metal reduction are far from being understood, it is postulated that the electron transfer to solid metal oxides is mediated by outer membrane-associated *c*-type cytochromes and redox active electron shuttling compounds. This study focuses on the production of homogensitate in *Shewanella oneidensis* MR-1, an intermediate of tyrosine degradation pathway, which is a precursor of a redox cycling metabolite, pyomelanin. In this study, we determined that two enzymes involved in this pathway, 4-hydroxyphenylpyruvate dioxygenase (4HPPD) and homogentisate 1,2-dioxygenase are responsible for homogentisate production and oxidation, respectively. Inhibition of 4-HPPD activity with the specific inhibitor sulcotrione ([2-(2- chloro- 4- methane sulfonylbenzoyl)-1,3-cyclohexanedione), and deletion of *melA*, a gene encoding 4-HPPD, resulted in no pyomelanin production by *S. oneidensis* MR-1. Conversely, deletion of *hmgA* which encodes the putative homogentisate 1,2-dioxygenase, resulted in pyomelanin overproduction. The efficiency and rates, with which MR-1 reduces hydrous ferric oxide, were directly linked to the ability of mutant strains to produce pyomelanin. Electrochemical studies with whole cells demonstrated that pyomelanin substantially increases the formal potential (E°′) of *S. oneidensis* MR-1. Based on this work, environmental production of pyomelanin likely contributes to an increased solid-phase metal reduction capacity in *Shewanella oneidensis*.

Introduction

Dissimilatory metal reducing bacteria (DMRB) contribute to biogeochemical cycling of both soluble and solid-phase metals, including Fe(III). DMRB gain energy for growth by catalyzing Fe(III) reduction with organic electron donors (Lovley et al., 1988), even though most of the Fe(III) in soils and sediments is found in the form of highly insoluble minerals (Schwertmann and Taylor, 1997). The specific strategies by which DMRB transfer electrons to insoluble minerals have not been completely characterized although substantial evidence points at outer membrane-associated *c*-type cytochromes as key mediators of the electron transfer to solid metal oxides (Lloyd, 2003; Gorby et al., 2006). Another strategy involves environmental humic compounds which serve as terminal electron acceptors and electron shuttles for iron reduction by DMRB (Lovley et al.,1996). Humic compounds are capable of transferring electrons to iron minerals due to the redox cycling capabilities of the quinone moieties that are associated with these heteropolymers (Scott et al., 1998). This scenario, however, is dependent on the quantity and redox cycling capability of humics present in the environment since redox activity varies for humic compounds from different locations (Scott et al., 1998). This raises the question of how solid-phase electron transfer can be accomplished efficiently when environmental humics are low in quantity or their redox cycling capacities are poor.

The ability for some DMRB to produce their own extracellular electron shuttling compounds for solid-phase mineral reduction offers another strategy for growth and survival of these microorganisms in the environment. It was shown that production of a specific redox cycling metabolite by *Shewanella algae* BrY, tentatively identified as pyomelanin, plays a role in dissimilatory metal reduction by serving as a soluble and cell-associated

electron shuttle (Turick et al, 2002; 2003). Quantities of pyomelanin as low as 3 fg/cell were calculated to be sufficient to increase iron oxide reduction rates by 10% (Turick et al., 2002). It should be noted that several types of melanin pigments exist in nature and are produced by various biochemical mechanisms. Pyomelanin originates from bacterial conversion of tyrosine and/or phenylalanine as part of the fumarate pathway (Lehninger, 1975) and is produced by numerous bacteria (Yabuuchi and Omyama, 1972; Kotob et al., 1995; Ruzafa et al.,1995; David et al., 1996; Sanchez-Amat et al., 1998) including members of the *Shewanella* genus (Fuqua et al., 1993; Coon,et al., 1994; Ruzafa et al., 1994). Complete breakdown of these precursors to acetylacetate and fumarate requires the enzymes 4-hydroxyphenylpyruvate dioxygenase (4-HPPD) and homogentisate oxidase (HGA-oxidase). In the absence of HGA-oxidase [or if homogentisate (HGA) production exceeds that of HGA-oxidase], HGA is over-produced and excreted from the cell (Coon et al., 1994). Auto-oxidation and self-polymerization of excreted HGA results in pyomelanin, a polyaromatic heteropolymer which consists of numerous quinone moieties (Ruzafa et al., 1995). This process occurs when oxygen concentrations decline, (Ruzafa et al., 1995) likely at the oxic/anoxic zones in the environment. Other types of melanins include eumelanin and allomelanin (Prota, 1992). Eumelanin results from the conversion of tyrosine to the eumelanin precursor 3,4-dihydroxyphenylalanine (DOPA), by the enzyme tyrosinase while allomelanins are produced by laccase conversion of phenolic compounds.

In this study, we elucidated the process of homogentisate production by *S. oneidensis* MR-1 and identified key steps involved in accumulation of pyomelanin. To better understand the environmental relevance of this redox shuttling metabolite, we employed genetic analysis and physiological characterization of recombinant *S. oneidensis* MR-1

strains deficient in or displaying substantially increased melanin production. The relative significance imparted by pyomelanin on solid-phase electron transfer was also addressed using an electrochemistry approach incorporating cyclic voltammetry to better understand the link between genetics and physiology to biogeochemical cycling, especially in relation to bioremediation of metal contaminated environments.

Results

Characterization of melanin production. Tyrosine supplemented lactate basal salts medium (LBSM) resulted in production of a reddish-brown pigment during late log and early stationary growth phases. At pH $\leq$2, the soluble cell-free pigment precipitated rapidly displaying melanin and humic-like properties (Ellis and Griffiths, 1974). Following precipitation, washing and dialysis, the dried (60°C) pigment resulted in a black powder with melanin-like characteristics based on the following properties (Ellis and Griffiths, 1974): insolublity in organic solvents (ethanol, chloroform and acetone); solubility in NaOH solutions at pH $\geq$10; decolorization in H_2O_2; precipitation by $FeCl_3$; and inability to filter through 8-kDa dialysis membrane.

High pressure capillary electrophoresis (HPCE) analyses of spent cell-free tyrosine-supplemented LBSM minimal medium demonstrated a peak that co-eluted with the homogentisic acid (HGA) standard indicating production of homogensitate, the precursor of pyomelanin (Fig. 1). In contrast, DOPA, the precursor of eumelanin was not detected. Laccase is inhibited at elevated glucose concentrations (Frases et al., 2007), but an equivalent degree of pigmentation was evident at both 5 and 25g/l glucose supplemented basal salts medium with tyrosine, indicating that laccase was not involved in pigment production (data

not shown). These data specifically implied the production of pyomelanin and hence the existence of 4-HPPD, which is responsible for HGA production in the tyrosine catabolic pathway.

To confirm the role of 4-HPPD in pyomelanin production, *S. oneidensis* MR-1 cultures were grown in tryptic soy broth (TSB) and tyrosine supplemented LBSM for 48 hr in the presence or absence of sulcotrione, a competitive inhibitor of 4-HPPD. Cultures grown in LBSM with both sulcotrione and tyrosine had no pigmentation relative to cultures without tyrosine (Table 1). Decreased pyomelanin production of spent cell free TSB was evident from sulcotrione-treated cultures. After 48 h of growth in LBSM with sulcotrione and no tyrosine, no pigmentation was detected (Table 1). Sulcotrione did not affect growth in these studies. Cell densities per ml from TSB ($1.6x10^8$ $\pm 3.5x10^7$) were similar to those from TSB + sulcotrione ($1.5x10^8$ $\pm 3.4x10^7$) and cell densities per ml from LBSM ($4.4x10^7$ $\pm 2.6x10^6$) were similar to those from LBSM + sulcotrione ($4.4x10^7$ $\pm 5.1x10^7$).

Genetic analysis of genes involved in melanin production. Obvious homologs of tyrosinase or laccase were not identified in the genome sequence of MR-1 although a putative *melA* (SO1962) gene encoding 4-HPPD was present. *melA* is located 120 base pairs downstream of an ORF encoding a conserved hypothetical protein (SO1963). To identify the potential function of SO1963, we conducted protein-protein BLAST search across the NCBI non-redundant database. The results of the search indicated that SO1963 is 45% identical and 61% similar to the putative homogentisate 1,2-dioxygenase from *Bacillus anthracis* strain Ames and 23% identical and 42% similar to the human homogentisate 1,2-dioxygenase. In many organisms, homogensitate 1,2-dioxygenase is a part of the tyrosine catabolic

pathway and is involved in the oxidation of homogentisic acid to maleyl-acetoacetate which is subsequently assimilated through the TCA cycle. The genome sequence suggests the presence of a complete tyrosine degradation pathway in *S. oneidensis* MR-1 and its components are identified in the KEGG database (http://www.kegg.com).

To elucidate the functions of SO1962 and SO1963 in melanin production, we generated in-frame deletion mutants lacking putative *melA* and *hmgA* genes. The resulting mutants, Δ*melA* and Δ*hmgA*, were tested for production of pyomelanin during growth on tyrosine supplemented LBSM and TSB media. Our results indicate that pyomelanin production was completely abolished in the Δ*melA* mutant, while Δ*hmgA* strain deficient in the putative HGA-oxidase displayed substantial overproduction of this electron shuttling compound relative to the wild-type *S. oneidensis* MR-1 (Table 1). Interestingly, the overproduction of pyomelanin was most notable in stationary phase when the Δ*hmgA* strain was grown on minimal medium without the addition of tyrosine exceeding the wild-type production levels by 5- to 8-fold (Table 1).

Pyomelanin production related to Fe(III)-oxide reduction. Anaerobic resting cell studies with *S. oneidensis* MR-1 and H_2 (as electron donor) were conducted to quantify the role of 4-HPPD on pyomelanin production relative and subsequent HFO reduction. For LBSM grown cultures, only those with tyrosine but without sulcotrione (melanized cells) demonstrated enhanced HFO reduction capacities compared to cultures grown with tyrosine + sulcotrione, no tyrosine, or sulcotrione only (non-melanized cells) (Fig. 2A). TSB grown cells with sulcotrione (non-melanized) also had a diminished capacity for HFO reduction compared to TSB grown cells without sulcotrione (Fig. 2B).

Resting cell studies of mutant cultures and *S. oneidensis* MR-1 grown in TSB and tyrosine supplemented LBSM demonstrated increased HFO reduction capacity as a function of pyomelanin production. Δ*melA* strain had a diminished capacity for HFO reduction relative to the control *S. oneidensis* MR-1 (Fig. 3A and 3B). In contrast, the pyomelanin overproducing strain (Δ*hmgA*) exceeded the HFO reduction capacity of *S. oneidensis* MR-1 (Fig.3A and 3B). Pyomelanin spiked cultures of Δ*melA* resulted in a substantial increase in HFO reduction compared to Δ*melA* without supplemental pyomelanin (Fig. 3B).

Electrochemical analysis of electron transfer by pyomelanin. Electrochemical studies were conducted in order to obtain a precise determination of electron transfer behavior at the cell surface relative to pyomelanin concentration. Cyclic voltammograms (CV) from concentrated whole cell suspensions demonstrated redox activity as a function of pyomelanin content. As shown in Figure 4, the wild-type and the two mutant strains demonstrated different CV as a function of pyomelanin production or exposure to pyomelanin during growth.

The wild-type strain, *S. oneidensis* MR-1, had a formal potential (E°′) of about -485 mV vs. Ag/AgCl, 3 M KCl reference, henceforth Ag(RE), as calculated from the oxidation peak (-423 mV vs. Ag(RE)) and reduction peak (-547 mV vs. Ag(RE)) (Fig. 4A). Another slight oxidation peak was produced by *S. oneidensis* MR-1 around 550 mV vs. Ag(RE) but a corresponding reduction peak was not detected (Fig. 4A). Only one redox couple was detected from Δ*melA* with a E°′ of about – 566 mV vs. Ag(RE) (Fig. 4B) and the current response was diminished compared to that of *S. oneidensis* MR-1(Fig.4A and 4B). Growth of Δ*melA* in the presence of 0.1 mg/ml of pyomelanin

resulted in an increase of the E°′ to approximately – 474 mV vs. Ag(RE), similar to *S. oneidensis* MR-1 and 92 mV greater than Δ*melA* grown without supplemental pyomelanin (Fig. 4C). In contrast, growth in the presence of pyomelanin resulted in increased current response of Δ*melA* (Fig 4C), indicating pyomelanin sorption to the cell surface. In addition a second, small oxidation peak was detected around 470 mV vs. Ag(RE) and may be a result of pyomelanin addition.

Two obvious redox couples were generated by CV of Δ*hmgA* (Fig. 4D). The formal potential of one redox couple near -474 mV vs. Ag(RE) was similar to that of *S. oneidensis* MR-1 and the pyomelanin exposed culture of Δ*melA* (Fig. 4A, C, and D). A second redox couple at E°′= 114 mV vs. Ag(RE) was likely due to excessive surface associated pyomelanin produced by this strain. To confirm this result, dried concentrated pyomelanin mixed with carbon paste was also analyzed. The pyomelanin/carbon paste electrode demonstrated redox activity with an oxidation peak similar to that of the second oxidation peak of Δ*hmgA*, but a more positive reduction peak relative to Δ*hmgA* (Fig. 4D and 4E) with the resulting E°′= 253 mV vs. Ag(RE) . The differences in the CV between the concentrated pyomelanin and Δ*hmgA* may be indicative of how pyomelanin is incorporated onto the cell surface.

Cell-free supernatant fluid from the suspensions analyzed above did not produce significant electrochemical activity, demonstrating that the CV of the cell suspensions were due to electrochemical activity at the cell surface and not electron shuttles in the bulk solution.

Prolonged growth in LBSM without tyrosine; pigment production and HFO reduction. In order to determine if pyomelanin production was possible without exogenous tyrosine, cultures were grown in LBSM without tyrosine for extended periods. Growth in LBSM did not result in any detectable pyomelanin production within the first 48 h by any of the strains (Table 1). The HFO reduction rates displayed by resting cells from these cultures were also similar (data not shown). However, when incubation was prolonged to 240 h, pyomelanin was detected in the spent medium and cell surfaces of Δ*hmgA* and *S. oneidensis* MR-1 (Table 1). In subsequent resting cell studies carried out as above, the Δ*hmgA* strain reduced HFO to the greatest degree, followed by *S. oneidensis* MR-1, with the least HFO reduction displayed by Δ*melA* (Fig. 5).

Discussion

Bacterial activity plays an increasingly appreciated role in biogeochemical cycling of metals in the environment. An understanding of specific mechanisms involved in this phenomenon is necessary to more fully address dissimilatory metal reduction and its role in biogeochemical cycling of metals and other key elements such as carbon, nitrogen, and sulfur. DMRB, especially in the genus *Shewanella*, possess a complex respiratory network and utilize a variety of strategies for metal reduction (Lloyd, 2003; Turick et al., 2003; Gorby et al., 2006). The delineation of these mechanisms is important in order to understand their complexity and possible synergy with the bacterial cell. Environmental nutrient availability and its effect on specific metal reduction mechanisms is important to understand in order to comprehend and predict biogeochemical changes, especially in relation to bioremediation.

Here we showed that *S. oneidensis* MR-1 utilizes tyrosine to produce HGA by way of the enzyme 4-HPPD. The resultant pigment, pyomelanin is also produced by *S. collwelliana* (Fuqua et al., 1993; Ruzafa et al., 1994) and was tentatively identified from *S. algae* BrY (Turick et al., 2002; 2003). The absence of HGA oxidase or its decreased activity relative to 4-HPPD results in the overproduction of HGA leading ultimately to extracellular pyomelanin formation. The absence of pyomelanin production from the Δ*melA* strain and the abundance of pyomelanin from the Δ*hmgA* strain confirmed the genetic pathway involved in pyomelanin production in *S. oneidensis* MR-1. The high concentration of pyomelanin produced by Δ*hmgA* compared to the wild type MR-1, indicated that the HGA oxidase is functional but its activity is lower than that of the upstream enzyme 4-HPPD in the wild type strain, resulting in excess HGA production.

When melanin is produced by DMRB, this redox active metabolite mediates electron transfer to solid-phase metal oxides by *S. algae* BrY (Turick et al., 2002; 2003). In this study we demonstrated the production of pyomelanin by *S. oneidensis* MR-1 and the relative significance of this metabolite as a mechanism for solid-phase electron transfer in relation to tyrosine concentrations. Tyrosine concentrations in both TSB and LBSM correlated to pyomelanin production and increased HFO reduction when pyomelanin production was inhibited and when the genetic mutants were incorporated into the studies. Addition of soluble pyomelanin to resting cell studies of Δ*melA* confirmed that pyomelanin was responsible for significant acceleration of HFO reduction by the pyomelanin over producer Δ*hmgA*.

Association of pyomelanin with bacterial surfaces also enhances electron transfer to solid-phase terminal electron acceptors (Turick et al., 2003). Due to the redox cycling

behavior of pyomelanin, its close association with the bacterial surface presents an efficient process that permits a low concentration of pyomelanin to be used repeatedly for electron transfer. Our electrochemical studies were developed to determine the degree of enhanced electron transfer conferred to the cell surface by pyomelanin. This technique has several advantages over conventional wet chemistry techniques for determining electron transfer kinetics. We were able to determine specific surface associated redox couples through CV and demonstrate the contribution pyomelanin made to enhanced electron transfer at the cell surface.

Pure cytochromes of the DMRB *Goebacter sulferreducens* demonstrate electrochemical activity (Magnuson et al., 2001). In addition, cell suspensions of various strains of *S. oneidensis* exhibit different electrochemical responses with CV as a result of mutations in electron transfer capacity (Kim et al., 2002). Here, we demonstrated that pyomelanin production also affects solid-phase electron transfer behavior of *S. oneidensis*. The increase in E°′ of Δ*melA* grown in the presence of pyomelanin (estimated to be the same concentration of pyomelanin produced by *S. oneidensis* MR-1) essentially restored the E°′ of this strain to that of *S. oneidensis* MR-1 and the more negative redox couple of Δ*hmgA*. This provided strong evidence that existing electron transfer mechanisms are enhanced through surface complexation of pyomelanin.

A second redox couple at about +500 mV vs. Ag(RE), barely detectable in the CV of *S. oneidensis* MR-1 was obvious from the CV of the pyomelanin over producer Δ*hmgA* and can be attributed to pyomelanin. This is based on the CV of concentrated pyomelanin and the absence of such a redox couple from Δ*melA*. The difference in redox couples between the pyomelanin over producer and pyomelanin alone may be a result of

pyomelanin integration onto the bacterial membrane. Peak-to-peak separation of oxidation and reduction waves in Figs. 4D and 4E allowed for the calculation of apparent rate constants via Laviron's theory (Laviron, 1983; Finklea, 2001). Apparent rate constant (k^o) values obtained were 4.0×10^{-6} s^{-1} for Δ*hmgA* and 2.1×10^{-4} s^{-1} for pyomelanin in carbon paste. The 50 times greater k^o value obtained for concentrated pyomelanin may be indicative of the degree of hydration or how pyomelanin is incorporated into the cell surface, especially in relation to the potential insulating properties of exopolysaccharides (Finkenstadt, 2005).

A key nutritional requirement for pyomelanin production is tyrosine and/or phenylalanine, where phenylalanine is converted to tyrosine in this pathway (Lehninger, 1975). A portion of this present study focused on the complex medium TSB and the defined LBSM (with equivalent tyrosine concentrations to TSB) in order to delineate the role of tyrosine in both pyomelanin production and enhanced electron transfer. Enzyme inhibition of pyomelanin production as well as directed mutagenesis studies targeted 4-HPPD. Both approaches were successful in preventing pyomelanin production. The presence of tyrosine as a component of growth media from previous studies with *S. oneidensis* MR-1 likely contributed to pyomelanin production and hence influenced metal reduction rates. Increased pyomelanin production from TSB compared to LBSM with supplemental tyrosine may be a result of phenylalanine also present in TSB.

Environmental concentrations of tyrosine or phenylalanine are expected to promote pyomelanin production and thus contribute to increased biogeochemical cycling of metals. The presence of tyrosine and phenylalanine in soils varies and may play a useful role in predicting biogeochemical activity in the subsurface. For instance, tyrosine and

phenylalanine concentrations have been shown to range from 10 – 632 mg/kg in various soils (Martens and Loeffelmann, 2003: McLain and Martens, 2005). Since pyomelanin production is linearly related to tyrosine concentration and minute quantities of pyomelanin are needed per cell to increase electron transfer rates (Turick et al., 2002), tyrosine and phenylalanine concentrations reported from environmental studies would be sufficient to produce enough pyomelanin to accelerate metal reduction rates in the subsurface. Our evidence for pyomelanin production from prolonged incubation suggests that lysed cells in high concentration may provide enough amino acid precursors for pyomelanin production. The low concentration of pyomelanin associated with *S. oneidensis* MR-1 from prolonged growth in LBSM was enough to increase HFO reduction by about 10% and corroborates previous reports (Turick et al., 2002). This scenario is likely in the environment, especially in biofilms and could also contribute to accelerated electron transfer.

Experimental Procedures

Growth conditions. Cultures were maintained on tryptic soy agar throughout the study. Tryptic soy broth (TSB) and a lactate (70 mM) basal salts medium (LBSM) (Turick et al., 2002) with or without 300 mg/l of tyrosine were used for pyomelanin studies. Tyrosine concentrations of LBSM were based on that of TSB (McCuen, 1988). Growth conditions were at 25°C, 25 ml, and shaken (100 rpm) in an aerobic environment for 48- 72 h, unless specified otherwise. Specific growth media were inoculated with 24 h cultures grown in the same medium. To determine if pyomelanin production occurred without supplemental tyrosine, cultures were grown in LBSM for 48 and 240h under the same conditions above. For electrochemical studies cultures were grown in TSB (as above) for 72h. To determine the effect of surface associated pyomelanin on electron transfer, sterile pyomelanin (0.1 mg/ml) was added to some cultures of the pyomelanin deficient strain (Δ*melA*) after 24h of growth.

Pigment characterization. Cell-free spent growth medium (LBSM with 300 mg/l tyrosine) of *S. oneidensis* MR-1 were assayed for melanin precursors DOPA and/or HGA by high-pressure capillary electrophoresis (HPCE) with a Celect H150 C-8 bonded phase capillary column. Standards (DOPA and HGA) were dissolved in 4 mM ascorbate, (to prevent oxidation of precursors to melanin pigments) to a final concentration of 4 mM each. HGA and DOPA were also determined by colorimetric methods. DOPA analysis consisted of the DOPA nitration method (Waite and Benedict, 1984). HGA content was determined based on its reaction with cysteine to form 1, 4-thiazine, according to the methods of Fellman et al. (1972). To determine if laccase activity contributed to melanin formation cultures were

grown with 10 and 25 g/l of glucose in BSM with 300 mg/l tyrosine because laccase is inhibited above 20 g/l (Frases et al., 2007).

Characterization of spent cell-free media to determine humic and melanin type properties was performed as previously described (Ellis and Griffiths, 1974; Turick et al., 2002). Soluble pyomelanin was quantified spectrophotometrically (OD_{400}) as previously described (Turick et al, 2003). This technique incorporated known concentrations of concentrated bacterial pyomelanin and pure pyomelanin produced by the autooxidation of HGA that served as quantitative standards.

Cell associated pyomelanin was quantified initially as previously described (Turick et al., 2003) incorporating the nitro blue tetrazolium assay (Paz et al., 1991). Results from this assay compared well to a spectroscopic analysis (OD_{400}) of cell suspensions, which was used throughout the study. The methods included a known cell density of pyomelanin producing cells blanked against an equal cell density of the pyomelanin deficient strain (*ΔmelA*). Pyomelanin was quantified as described above and calculated per cell. Cell density was determined with acridine orange staining and an epifluorescence microscope (Hobbie et al., 1975)

Inhibition of pyomelanin production. Sulcotrione [2-(2- chloro- 4- methane sulfonylbenzoyl)-1, 3-cyclohexanedione)] (Zeneca Ag. Products, Richmond, CA) is a potent inhibitor of 4-HPPD (Schulz et al., 1993; Secor, 1988; Lee et al., 1997). *S. oneidensis* MR-1 was grown for 48 h in tyrosine-supplemented LBSM or TSB with 18 μM sulcotrione to test its effects on melanin production. Sulcotrione was added continuously with a syringe pump to maintain an 18 μM concentration. Melanin production was completely inhibited with 18

μM sulcotrione. Cell density and growth were not affected by the sulcotrione concentration tested (data not shown).

Construction of *melA* and *hmgA* mutants of *S. oneidensis* MR-1. In-frame deletion mutagenesis was performed using the method originally described by Link et al. (1997) with minor modifications. Upstream and downstream fragments flanking the target locus were PCR amplified using *S. oneidensis* MR-1 genomic DNA and fused via overlap extension PCR (Ho et al., 1989). The fusion PCR amplicon was ligated into *Xcm*I digested pDS3.1 (Wan et al., 2004). The resulting recombinant plasmids were used to transform *E. coli* ß-2155 (Dehio and Meyer, 1997) or WM3063 (Saltikov et al., 2003) and subsequently transferred to *S. oneidensis* MR-1 by conjugation. The primary integrants were selected by plating on LB medium containing 7.5 μg/ml gentamycin. Selection for a second homologous recombination to remove the plasmid sequence was accomplished by sucrose counter-selection (Blomfield et al., 1991). Sucrose-resistant colonies were screened for sensitivity to gentamycin and then screened for deletion of the gene of interest using PCR. The resulting PCR amplicon was then used as the template for DNA sequencing of the deleted and flanking regions involved in the recombination events (ACGT, Inc. Wheeling, IL).

Quantification of electron transfer. The Fe(III) oxide, hydrous ferric oxide (HFO) was used in resting cell studies in carbonate buffer as previously described (Turick et al., 2003) in order to quantify Fe(III) reduction by measuring Fe(II) evolution over time. An autoclaved pyomelanin solution (1mg/ml final concentration) was spiked into selected suspensions of Δ*melA* in order to determine its effect on HFO reduction. Cell densities in these studies were

normalized to10^8 cells/ml as determined with acridine orange staining and an epifluorescence microscope (Hobbie et al., 1975). Fe(II) was measured spectrophotometrically using the ferrozine assay as described elsewhere (Turick et al., 2003).

In order to more precisely characterize the role of pyomelanin as a surface associated electron shuttle, electrochemical studies were designed for whole bacterial cells. Cyclic voltammetry comprises electrochemical techniques that provide information of the electrochemical activities of materials by sweeping the potential back and forth at predetermined rates. Potential sweeps in the positive direction result in oxidation peaks and reduction peaks are evident when the potential is swept in the negative direction. Our studies incorporated concentrated suspensions (10 x) of whole bacterial cells under anaerobic conditions. Operational conditions included washed (3x) cell suspensions of cultures grown aerobically in TSB (above). Cultures were resuspended in phosphate buffered saline (PBS) for electrochemical studies. Cell suspensions were made anaerobic by a continuous nitrogen purge for 15 min. prior to electrochemical analyses. Electrochemistry studies included a Ag/AgCl reference electrode, a Pt counter electrode and carbon paste working electrode (Bioanalytical Systems), all immersed into the anaerobic cell suspension that was blanketed with nitrogen gas throughout the studies. Potential sweep originated in the positive direction at a rate of 850 mV/sec for all CV in this study, using a model 100B/A potentiostat (Bioanalytical Systems). A total of six sweeps were performed with each culture. Duplicate CV of each assay were averaged and the 5^{th} and 6^{th} averaged sweeps were reported here. For each set of experiments with each culture, working electrodes were rinsed in deionized water followed by a methanol rinse and then sonicated for 10 minutes in deionized water. The cleaned electrodes were then air dried prior to the next study. Each study included a CV of

the washed bare electrode in PBS that served as a no-cell control for the next series of voltammetry studies for each strain analyzed. In addition, cell free liquid from each cell suspension was analyzed (as above) for electrochemical activity in the bulk phase.

Apparent rate constant values (k^o values) were derived for a 1-electron transfer mechanism, by simulating the CV with the software provided for the Model 660a Electrochemical Workstation by CH Instruments (Austin, TX).

Acknowledgements

This research was supported in part through funding by the Department of Energy NABIR program, the Savannah River National Laboratory Independent Research and Development Program, and Soil and Groundwater Closure Projects of the Savannah River Site. The authors wish to thank Thomas H. Cromartie of Zenica Agrichemicals for furnishing the sulcotrione.

Table 1. Production of pyomelanin by wild-type and mutant strains of *Shewanella oneidensis* MR-1.

	Soluble pyomelanin (mg/ml)				Cell-associated pyomelanin (fg/cell)		
Strain	**TSB**	**LBSM + 300 mg/l Tyrosine (48h)**	**LBSM (48h)**	**LBSM (240h)**	**TSB**	**LBSM + 300 mg/l Tyrosine (48h)**	**LBSM no tyrosine (240h)**
MR-1	0.427 (0.046)	0.270 (0.025)	ND	0.032 (0.009)	66.32 (4.63)	36.50 (4.60)	4.14 (0.964)
MR-1 + sulcotrione	ND[1]	ND	ND	NI[2]	NI	NI	NI
ΔmelA	ND	ND	ND	ND	ND	ND	ND
ΔhmgA	0.987 (0.167)	0.680 (0.074)	ND	0.162 (0.003)	153.40 (16.87)	86.44 (16.70)	37.07 (3.214)

1. ND – not detected
2. NI – not included in assay

Standard deviation in parentheses

References

Blomfield, I. C., Vaughn, V., Rest, R.F., and Eisenstein, B. I. (1991) Allelic exchange in *Escherichia coli* using the *Bacillus subtilis sacB* gene and a temperature-sensitive pSC101 replicon. *Mol. Microbiol* **5**:1447-1457.

Coon, S.L., Kotob, S., Jarvis, B.B., Wang, S., Fuqua, W. C., and Weiner, R.M. (1994) Homogentisic acid is the product of MelA, which mediated melanogenesis in the marine bacterium *Shewanella colwelliana* D. *Appl Environ Microbiol* **60**:3006-3010.

Dehio, C., and Meyer, M. (1997) Maintenance of broad-host-range incompatibility group P and group Q plasmids and transposition of *Tn*5 in *Bartonella henselae* following conjugal plasmid transfer from *Escherichia coli*. *J Bacteriol* **179**:538-540.

David, C., Daro, A., Szalai, E., Atarhouch, T., and Mergeay, M. (1996) Formation of polymeric pigments in the presence of bacteria and comparison with chemical oxidative coupling-II. Catabolism of tyrosine and hydroxyphenylacetic acid by *Alcaligenes eutrophus* CH34 and mutants. *Eur Polym J* **32**:669-697.

Ellis, D.H., and Griffiths, D.A. (1974) The location and analysis of melanins in cell walls of some soil fungi. *Can J Microbiol* **20**:1379-1386.

Fellman, J.H., Fujita, T.S., and Roth, E.S. (1972) Assay, properties and tissue distribution of *p*-hydroxyphenylpyruvate hydroxylase. *Biochim Biophys Acta* **284**:90-100.

Finkenstadt, V.L. (2005) Natural polysaccharides as electroactive polymers. *Appl Microbiol Biotechnol* **67**:735-745.

Finklea, H. O. (2001) Theory of Coupled Electron-Proton Transfer with Potential-Dependent Transfer Coefficients for Redox Couples Attached to Electrodes. *J Phys Chem B* **105**: 8685.

Frases, S., Salazar, A., Dadachova, E., and Casadevall, A. (2007) *Cryptococcus neoformans* can utilize the bacterial melanin precursor homogentisic acid for fungal melanogenesis. *Appl Environ Microbiol* **73**:615-621.

Fuqua, W.C., and Weiner, R.M. (1993) The *melA* gene is essential for melanin production in the marine bacterium *Shewanella colwelliana*. *J Gen Microbiol* **139**:1105-1114.

Gorby, Y.A., Yanina, S., McLean, J. S., Rosso, K. M., Moyles, D., Dohnalkova, A., et al., (2006) Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. *Proc Nat Acad Sci* **103**:11358-11363.

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**:51-59.

Hobbie, J.E., Daley, R.J., and Jasper, S. (1977) Use of Nucleopore filters for counting bacteria by fluorescence microscopy. *Appl Environ Microbiol* **33**:1225 –1228.

Kim, H.J., Park, H.S., Hyun, M.S., Chang, I.S., Kim, M., and Kim, B.H. (2002) A mediator-less microbial fuel cell using a metal reducing bacterium, *Shewanella putrefaciens*. *Enzyme Microbial Technol* **30**:145-152.

Kotob, S.I., Coon, S.L., Quintero, E.J., and Weiner, R.M. (1995) Homogentisic acid is the primary precursor of melanin synthesis in *Vibrio cholera*, a *Hyphomonas* strain, and *Shewanella colwelliana*. *Appl Environ Microbiol* **61**:1620-1622.

Laviron, E. (1983) Electrochemical reactions with protonations at equilibrium: Part VIII. The 2 e, $2H^+$ reaction (nine-member square scheme) for a surface or for a heterogeneous reaction in the absence of disproportionation and dimerization reactions. *J Electroanal Chem* **146**: 15-36.

Lee, D.L., Prisbylla, M.P., Cromartie, T.H., Dargin, D.P., Howard, S.W., Provan, W.M. et al.,(1997) The discovery and structural requirements of inhibitors of *p*-hydroxyphenylpyruvate dioxygenase. *Weed Sci*. **45**:601-609.

Lehninger, A.L. (1975) Oxidative degradation of amino acids. In: *Biochemistry*. pp.568-570. Worth Publishers. N.Y. NY,

Link, A., Phillips, J., D., and Church, G. M. (1997) Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J Bacteriol* **179**:6228-6237.

Lloyd, J. R. (2003) Microbial reduction of metals and radionuclides. *FEMS Microbiol Rev* **27**:411-425.

Lovley, D.R., Coates, J.D., Blunt-Harris, E.L., Phillips, E.J.P, and Woodward, J.C. (1996) Humic substances as electron acceptors for microbial respiration. *Nature* **382**:445-448.

Lovley, D.R., and Phillips, E.J.P. (1988) Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. *Appl Environ Microbiol* **54**:1472-1480.

Martens, D.A., and Loeffelmann, K.L. (2003) Soil amino acid compostion quantified by acid hydrolysis and anion chromatography-pulsed amperometry. *J Agric Food Chem* **51**:6521-6529.

Magnuson, T.S., Isoyama, N., Hodges-Myerson, A.L., Davidson, G., Maroney, M.J. , Geesey, G.G., and Lovley, D.R. (2001) Isolation, characterization and gene sequence analysis of a membrane associated 89 kDa Fe(III) reducing cytochrome c from *Geobacter sulfurreducens. Biochem J* **359**:147-152.

McCuen, P.J. (1988) Culture media components In: *Manual of BBL Products and Laboratory Procedures.* Power, D.A. (ed). Beckton Dickinson Systems, Cockeysville, MD. pp. 293-294.

McLain, J.E.T., and Martens, D. A. (2005) Nitrous oxide flux from soil amino acid mineralization. *Soil Biol Biochem* **37**: 289-299

Paz, M.A., Fluckiger, R.A. , Boak, A., Kagan, H.M., and Gallop, P.M. (1991) Specific detection of quinoproteins by redox-cycling staining. *J Biol Chem* **266:**689-692.

Prota, G. (1992) Natural and synthetic melanins. In, *Melanins and Melanogenesis*. G. Prota (ed). San Diego, CA, USA. Academic Press, Inc. pp. 63-87.

Ruzafa, C., Solano, F., and Sanchez-Amat, A. (1994) The protein encoded by the *Shewanella colwelliana melA* gene is *p*-hydroxyphenylpyruvate dioxygenase. *FEMS Microbiol Lett* **124**:179-184.

Ruzafa, C., Sanchez-Amat, A., and Solano, F. (1995) Characterization of the melanogenic system in *Vibrio cholerae* ATCC 14035. *Pigment Cell Research* **8**:147-152.

Saltikov, C. W., Cifuentes, A., Venkateswaran, K., and Newman, D. K. (2003) The *ars* detoxification system is advantageous but not required for As(V) respiration by the

genetically tractable *Shewanella* species strain ANA-3. *Appl Environ Microbiol* **69**:2800-2809.

Sanchez-Amat, A., Ruzafa, C., and Solano, F. (1998) Comparative tyrosine degradation in *Vibrio cholerae* strains. The strain ATCC 14035 as a prokaryotic melanogenic model of homogentisate-releasing cell. *Comp Biochem Physiol Part B* **119**:557-562.

Secor, J. (1994) Inhibition of barnygrass 4-hydroxyphenylpyruvate dioxygenase by sulcotrione. *Plant Physiol* **106**:1429-1433.

Schulz, A., Ort, O. Beyer, P., and Kleninig, H. (1993) SC-0051, a 2-benzoyl-cyclohexane-1,3-dione bleaching herbicide, is a potent inhibitor of the enzyme *p*-hydroxyphenylpyruvate dioxygenase. *FEBS Lett* **318**:162-166.

Schwertmann, U., and Taylor, R.M. (1977) Iron oxides. In: *Minerals in Soil Environments*. Dixon, J.B and Weed, S.B. (eds). Madison WI: Soil Science Society of America, pp 145-180.

Scott, D.T., McKnight, D.M., Blunt-Harris, E. L., Kolesar, S.E., and Lovley, D.R. (1998) Quinone moieties act as electron acceptors in the reduction of humic substances by humics-reducing microorganisms. *Envion Sci Technol* **32:**2984-2989.

Turick, C.E., Tisa, L.S., and Caccavo Jr, F. (2002) Melanin production and use as a soluble electron shuttle for Fe(III) oxide reduction and as a terminal electron acceptor by *Shewanella algae* BrY. *Appl Environ Microbiol* **68**:2436-2444.

Turick, C.E., Cacavo, F. Jr., and Tisa, L.S. (2003) Electron transfer from *Shewanella algae* BrY to hydrous ferric oxide is mediated by cell-associated melanin. *FEMS Microbiol Lett* **220**:99-104.

Waite, J.H. and Benedict, C.V. (1984) Assay of dihydroxyphenylalanine (Dopa) in invertebrate structural proteins. *Meth Enzymol* **107**:397-413.

Wan, X. F., Verberkmoes, N. C. McCue, L. A. Stanek, D. Connelly, and H. Hauser, et al. (2004) Transcriptomic and proteomic characterization of the Fur modulon in the metal-reducing bacterium *Shewanella oneidensis*. *J Bacteriol* **186**:8385-8400.

Yabuuchi, E., and Omyama, A. 1972. Characterization of "pyomelanin"-producing strains of *Pseudomonas aeruginosa. Internat J Syst Bacteriol* **22**:53:64.

Figure Legends

Fig. 1. HPCE analysis for melanin precursors. After 18 h incubation of *S. oneidensis* MR-1, spent culture supernatant liquids were analyzed as described in the experimental procedures section. Panels: (A) 18 h *S. oneidensis* MR-1 (B) HGA and DOPA standards.

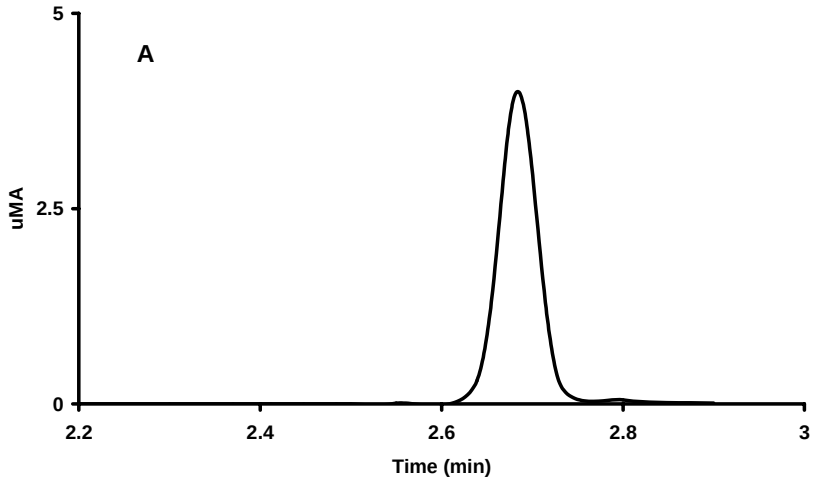
Fig. 2. Resting cell studies demonstrated inhibitory effects of sulcotrione on pyomelanin production and HFO reduction by *S. oneidensis* MR-1. (A) Cells pregrown in LBSM (●), LBSM and sulcotrione (Δ), LBSM with tyrosine (■), and LBSM with tyrosine and sulcotrione (□); (B) Cells pregrown in tryptic soy broth with (□) and without (■) sulcotrione.

Fig. 3. HFO reduction by 3 strains of *S. oneidensis*. Resting cell studies of cells pregrown in (A) TSB and (B)LBSM + 300 mg/l tyrosine, demonstrated increased HFO reduction efficiency by the pyomelanin over-producing strain (Δ*hmgA*) (♦) relative to MR-1(■) and the pyomelanin deficient mutant (Δ*melA*) (▲), which demonstrated the least amount of HFO reduction. Addition of pyomelanin (10μg/ml) to resting cell cultures of Δ*melA* (Δ) pregrown in LBSM demonstrated drastically increased levels of HFO reduction.

Fig. 4. Cyclic voltammetry of whole cell suspensions. Cultures grown in TSB for 72 hr, washed and centrifuged 3 times in PBS then concentrated 10 fold. Voltamograms were produced with a carbon paste working electrode, Pt counter electrode andAg/AgCl ref. electrode. Sweep rate was 850 mV/sec. Redox activity of whole cell suspensions was a

function of pyomelanin content.(A) *S. oneidensis* MR-1; (B) Pyomelanin deficient strain (Δ*melA*); (C) Pyomelanin deficient strain (Δ*melA*) incubated with 10 μg/ml pyomelanin; (D) Pyomelanin overproducing strain (Δ*hmga*). Pyomelanin increased formal potential of redox couples and increased current response from cells. (E) Electrochemical activity of dried pyomelanin incorporated into a carbon paste electrode.

Fig. 5. HFO reduction by 3 strains of *S. oneidensis* after prolonged incubation (10 days) in LBSM without exogenous tyrosine. Resting cell studies of cells pregrown in LBSM but without tyrosine, demonstrated increased HFO reduction efficiency by the pyomelanin over-producing strain (Δ*hmga*) (♦) relative to MR-1(■) and the pyomelanin deficient mutant (Δ*melA*) (▲), which demonstrated the least amount of HFO reduction.


A
5
2.5
0
uMA
2.2
2.4
2.6
2.8
3
Time (min)

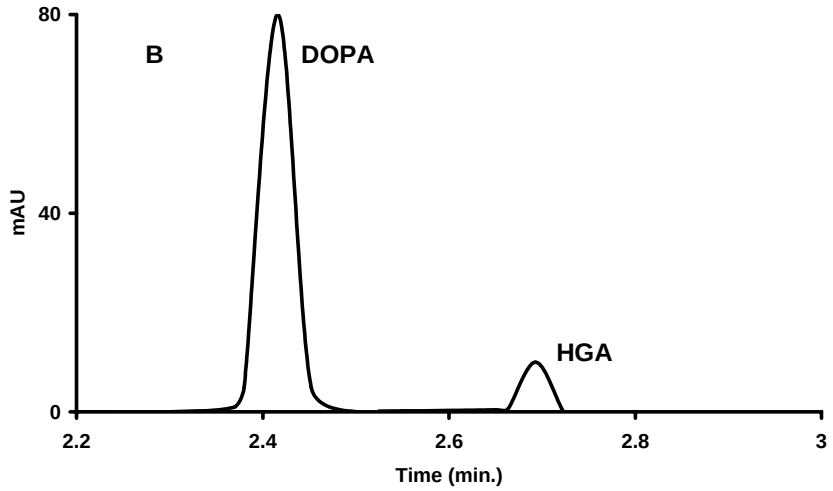
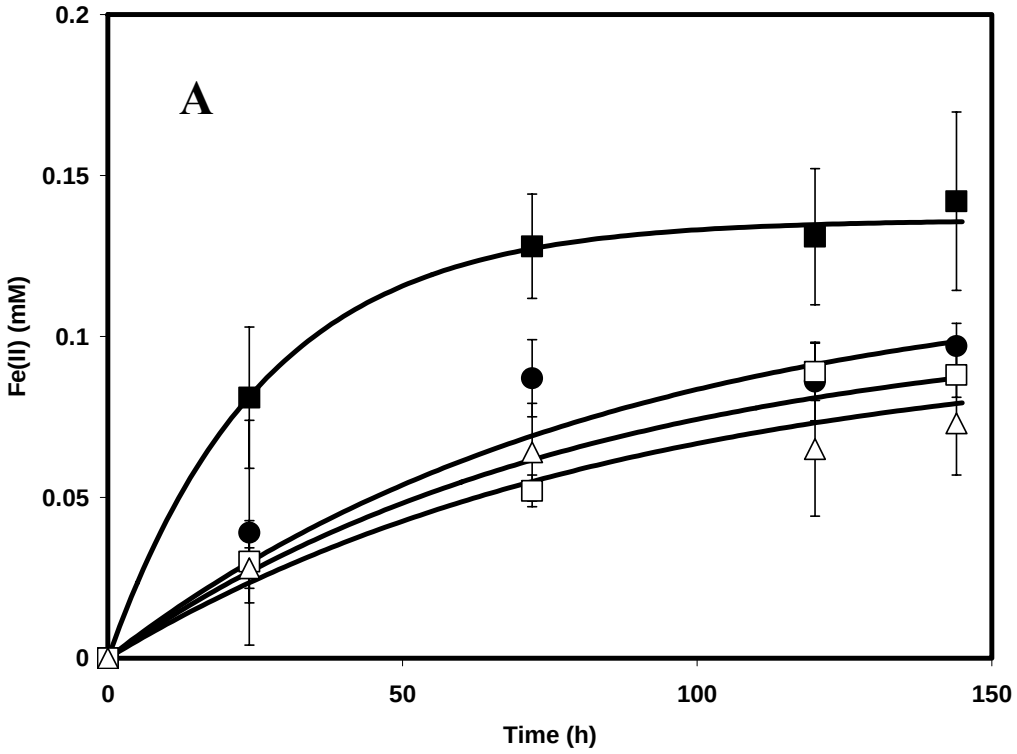
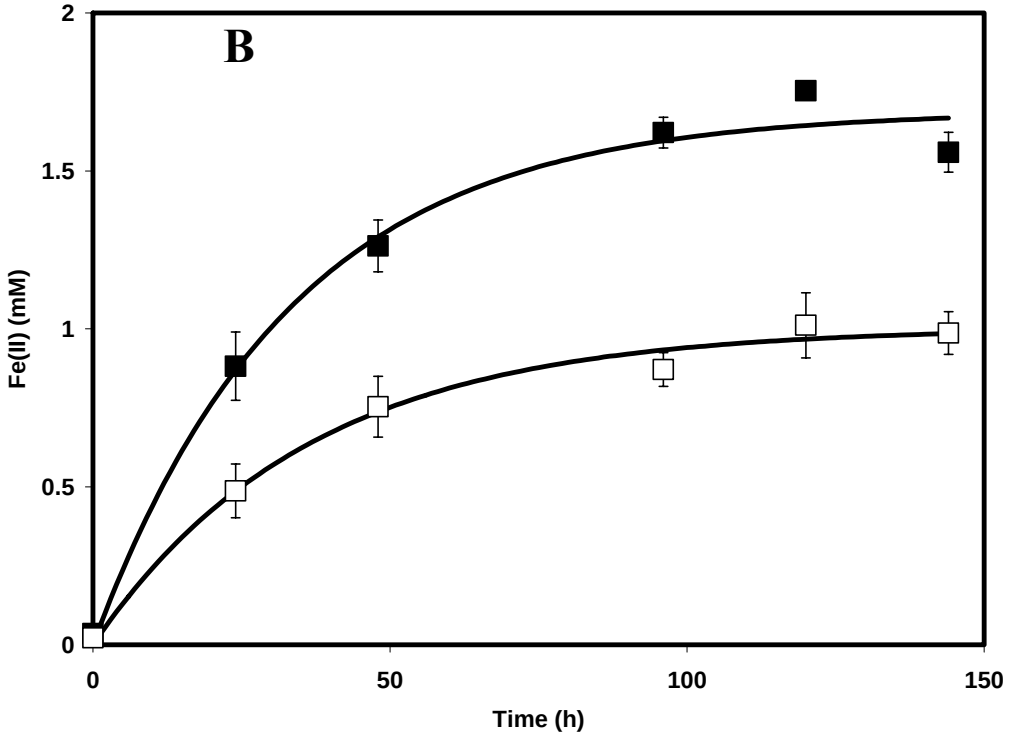

B
DOPA
HGA
80
40
0
mAU
2.2
2.4
2.6
2.8
3
Time (min.)

Figure 1

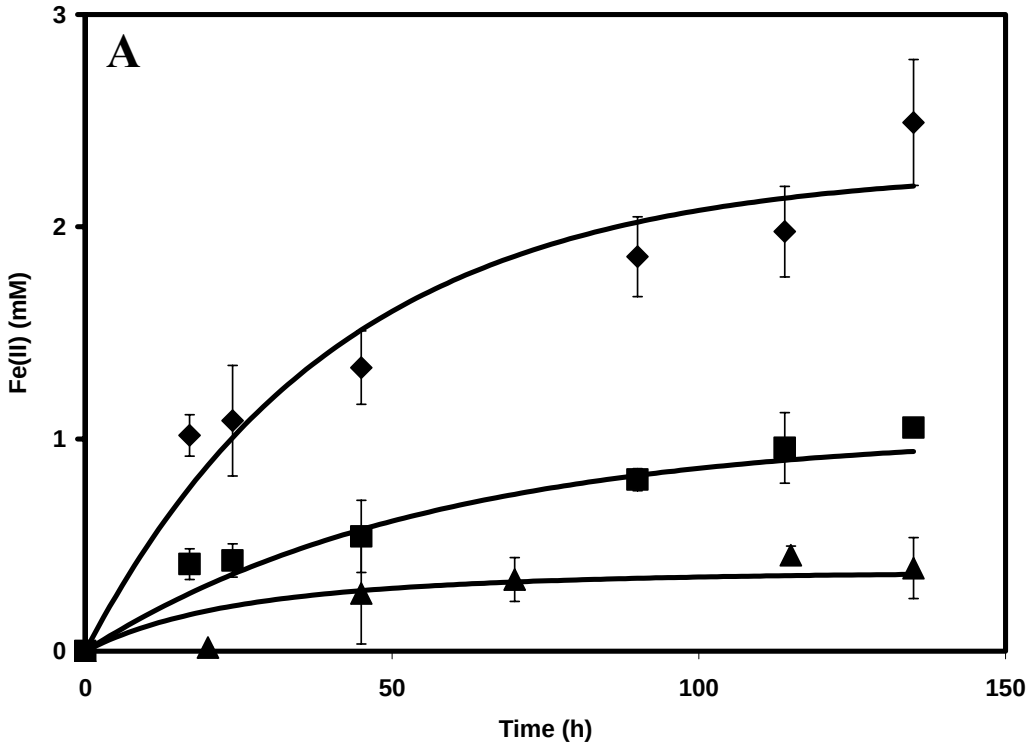

A
Fe(II) (mM)
Time (h)
0
0.05
0.1
0.15
0.2
0
50
100
150

1

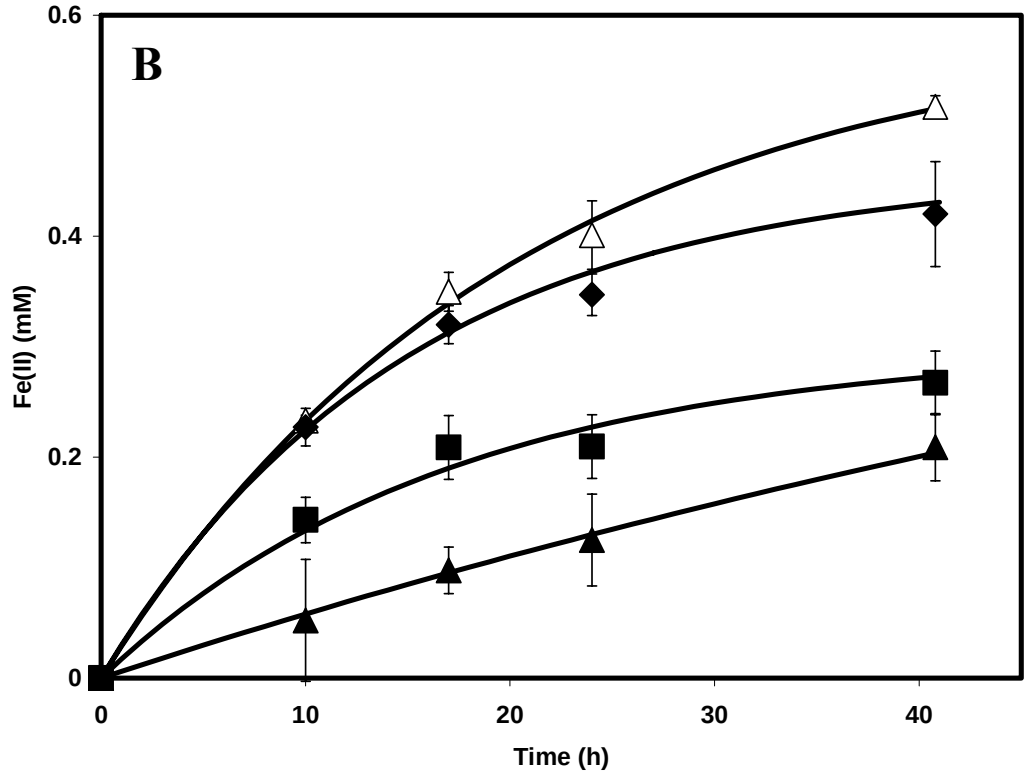

B
Fe(II) (mM)
Time (h)
0
0.5
1
1.5
2
0
50
100
150

2
3

1 Figure 2.
2



3



1
2Figure 3.

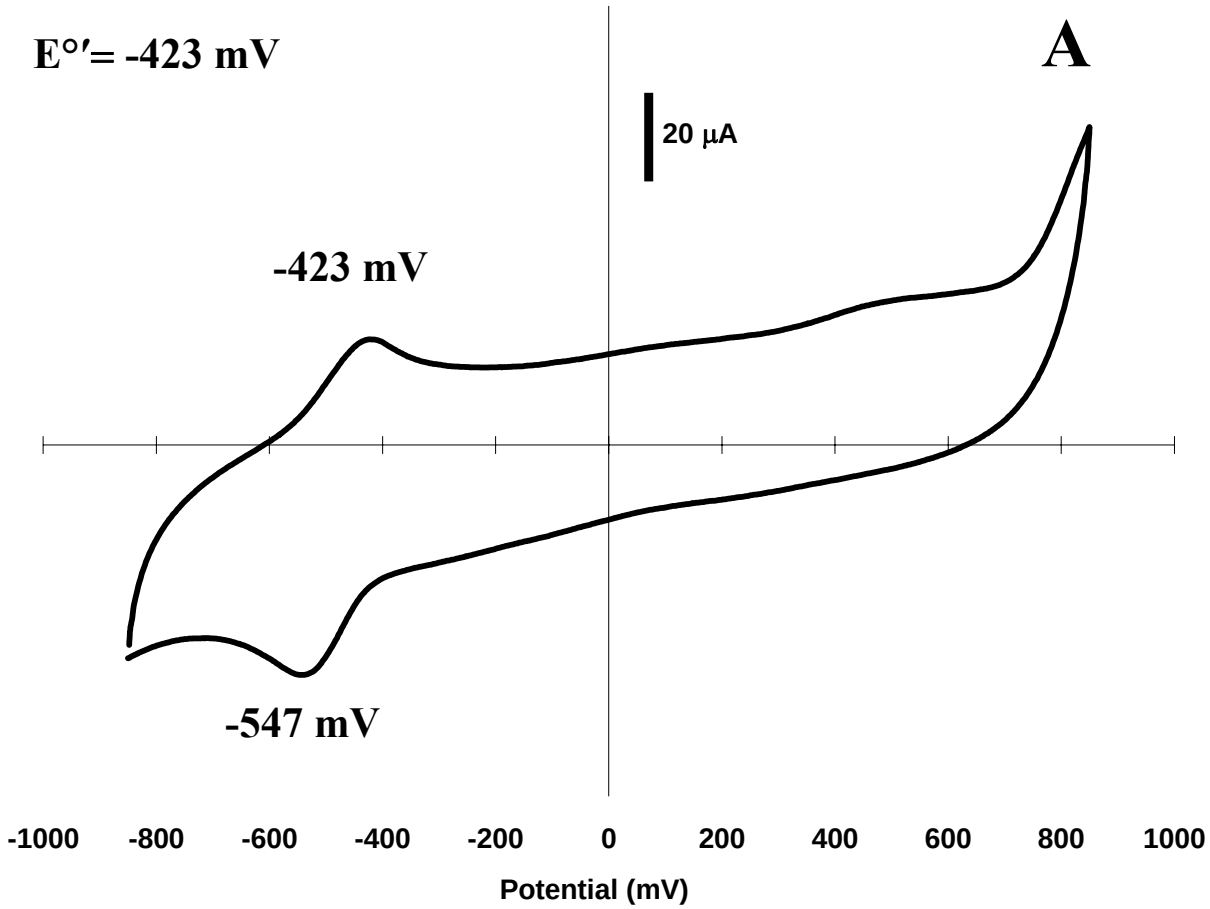
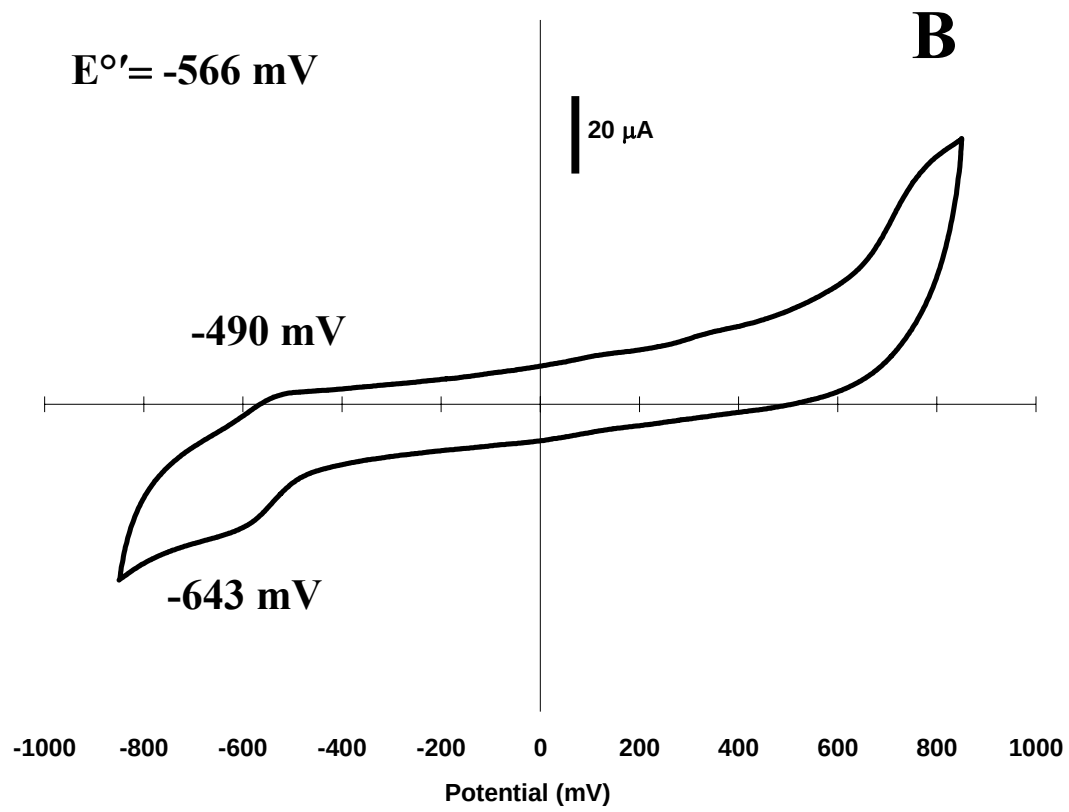

E°′= -423 mV
A
20 μA
-423 mV
-547 mV
-1000
-800
-600
-400
-200
0
200
400
600
800
1000
Potential (mV)

Figure 4.

1


B
E°′= -566 mV
20 µA
-490 mV
-643 mV
-1000
-800
-600
-400
-200
0
200
400
600
800
1000
Potential (mV)

2

3 Figure 4.

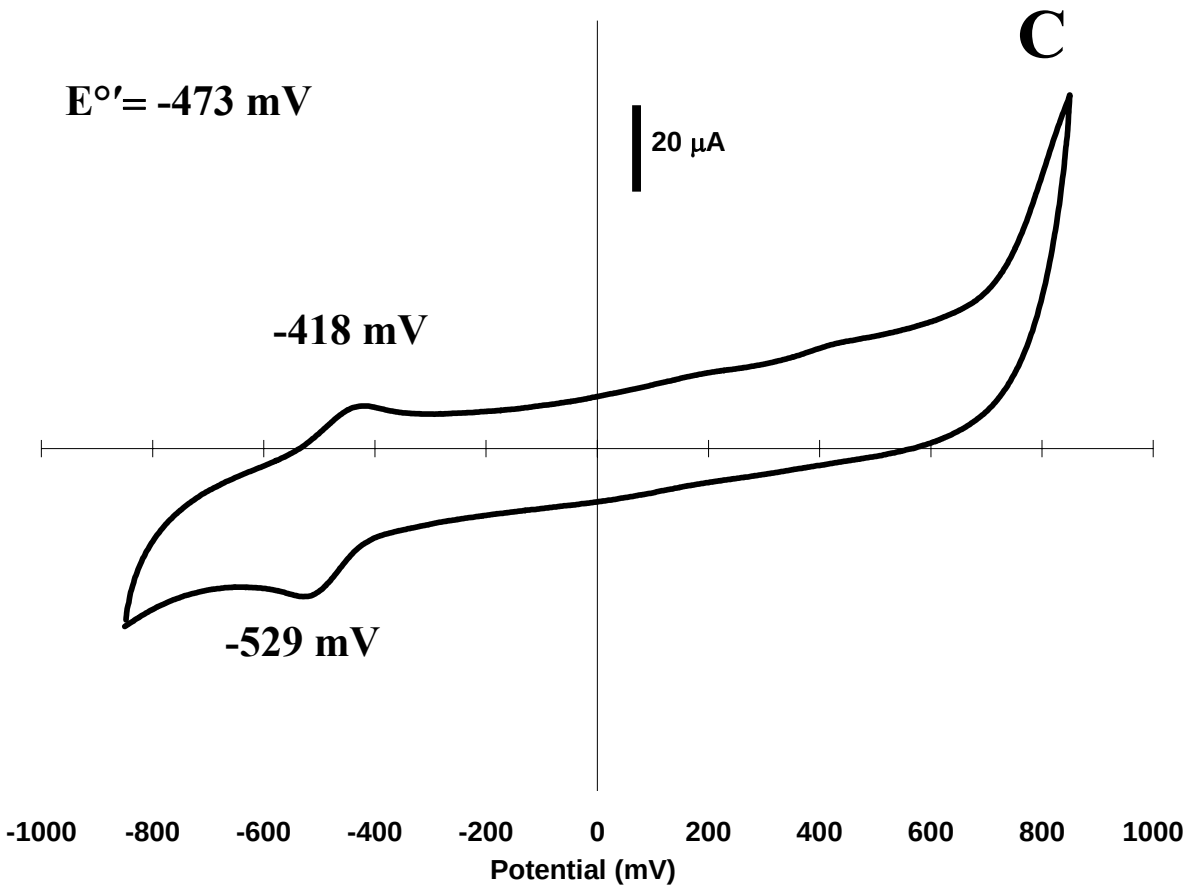

C
E°′= -473 mV
20 μA
-418 mV
-529 mV
-1000
-800
-600
-400
-200
0
200
400
600
800
1000
Potential (mV)

Figure 4.

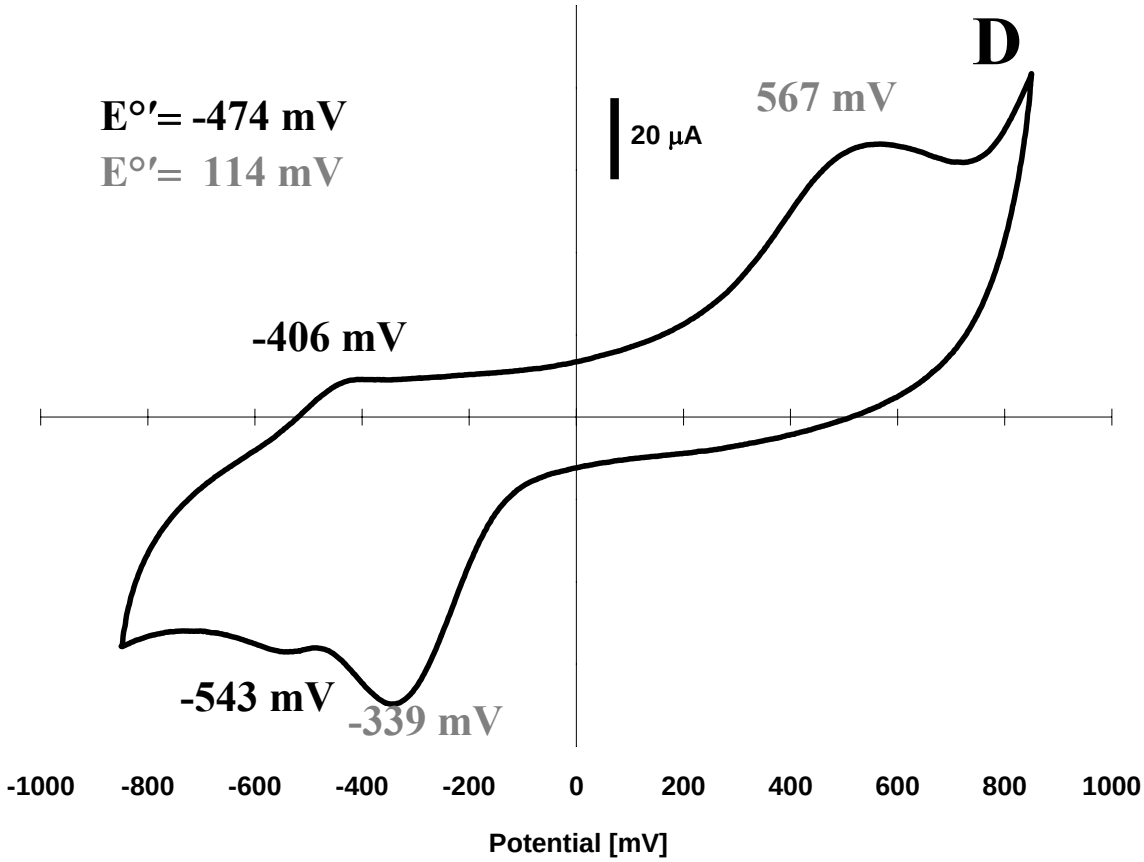

D
E°′= -474 mV
E°′= 114 mV
20 µA
567 mV
-406 mV
-543 mV
-339 mV
-1000
-800
-600
-400
-200
0
200
400
600
800
1000
Potential [mV]

Figure 4.

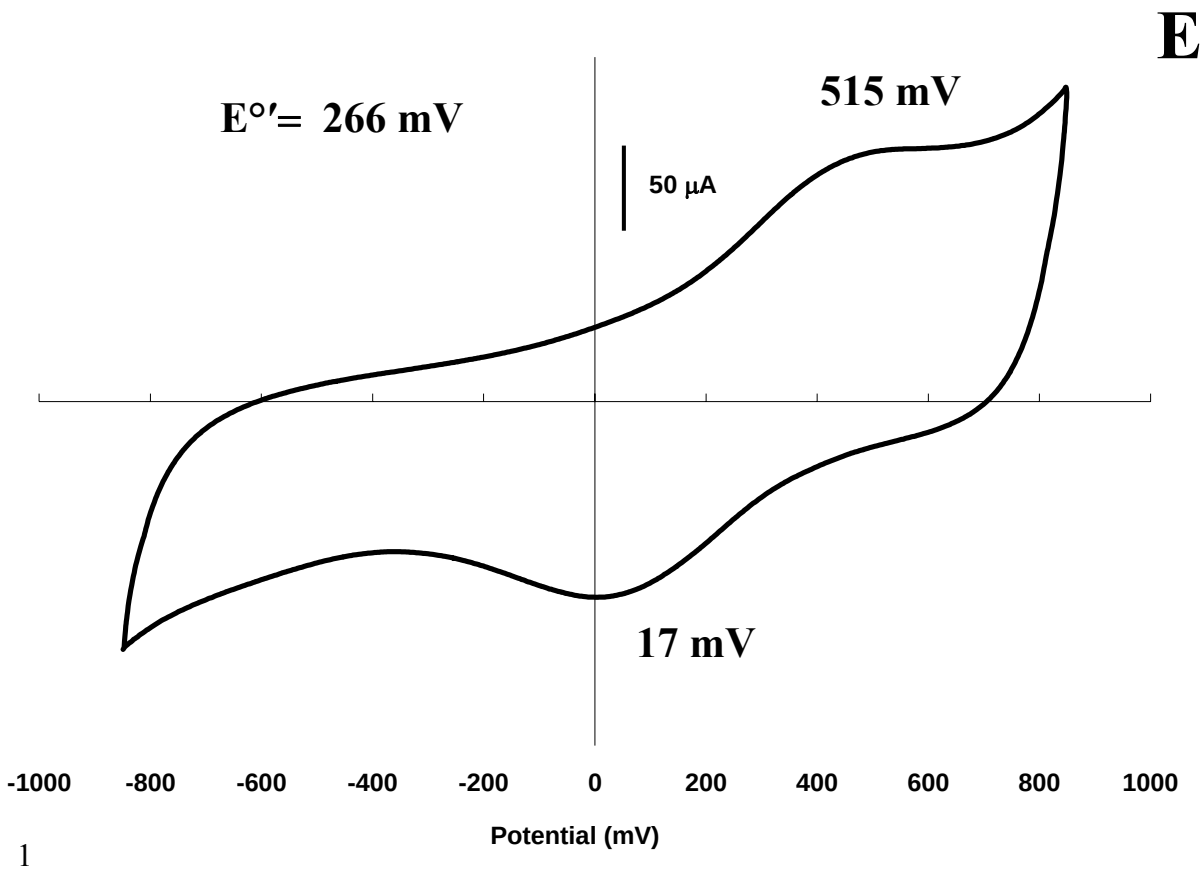

E
E°′= 266 mV
515 mV
50 μA
17 mV
-1000
-800
-600
-400
-200
0
200
400
600
800
1000
Potential (mV)

Figure 4.

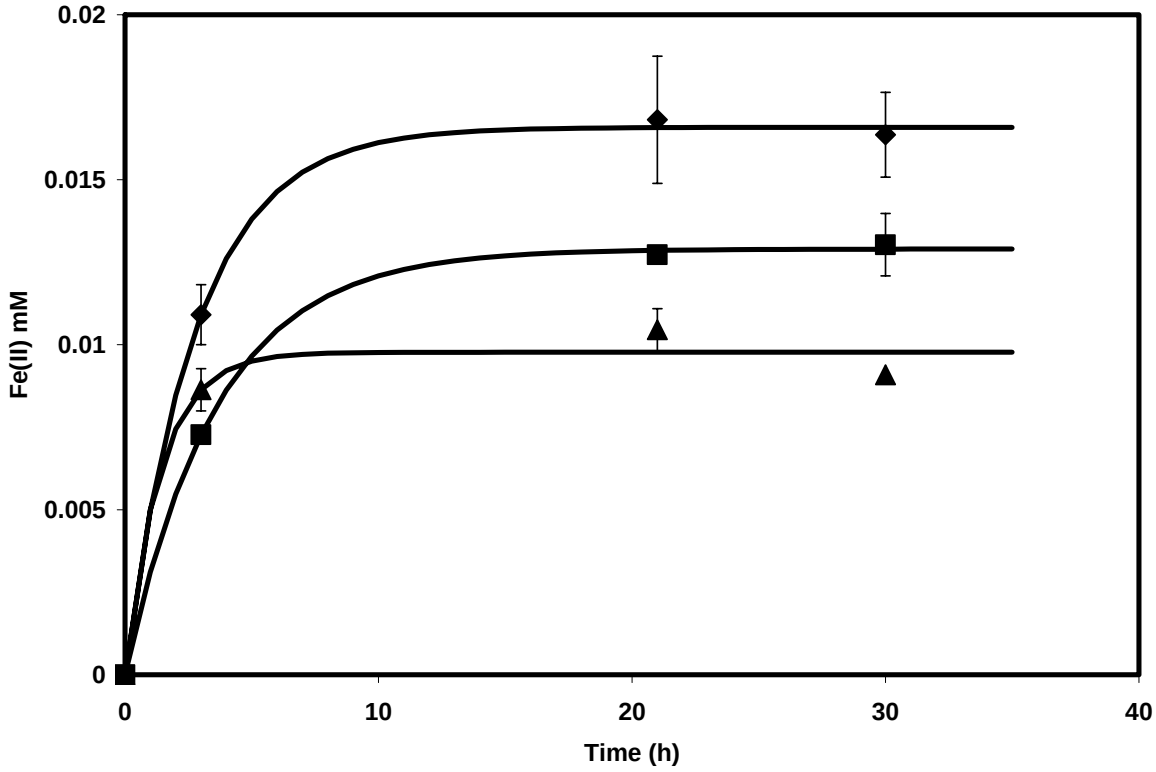


Figure 5.