OPTIMIZING BIOGENIC MANGANESE OXIDE PRODUCTION

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Optimizing Biogenic Manganese Oxide Production. (May 2013)

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The presence of emerging organic contaminants in surface and ground water sources poses challenges for traditional drinking water treatments such as granular activated carbon and ozonation with sand filtration. Manganese (Mn) oxides can oxidize several organic compounds and may provide another avenue for removal of these pollutants. While the biological impetus is not completely understood, certain microorganisms are capable of oxidizing soluble manganese to produce Mn oxides (e.g. MnO_2). This study's model manganese-oxidizing bacteria (MOB) are *Bacillus* sp. strain SG-1 and *Pseudomonas putida* MnB1. *Bacillus* sp., a marine bacterium, forms spores upon which manganese is oxidized and precipitates. *P. putida*, a freshwater bacterium, deposits the oxidized mineral on the outer cell membrane.

The cultures were grown in a liquid medium, with temperature and pH as experimental factors. The optical density (OD) of each culture was regularly measured during incubation in order to develop growth curves and determine the maximum specific growth rates. Maximum specific growth rate was used to calculate the doubling time during the exponential growth phase.

The results showed that the growth of the MOB is favorable at ambient temperatures and near neutral pH. The optimal conditions for the growth of *Pseudomonas putida* in Luria-Bertani

broth were determined. The results also demonstrate that an alternative growth medium for *Bacillus* sp. SG-1 should be investigated. The results of this study provide insight for optimizing reactor conditions for the growth of MOB and production of Mn oxides, which is an important step toward their use in engineered systems.

DEDICATION

To my mother, Charlotte E. Parker, for her determination to change her family tree, which gave a brighter future to her children.

ACKNOWLEDGEMENTS

I would like to thank Dr. Bradley Tebo for generously donating the cultures of *Bacillus* sp. SG-1 and *Pseudomonas putida* MnB1.

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NOMENCLATURE

Abs	Absorbance
ASW	Artificial seawater
DI	De-ionized
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer)
M (mM, μM)	Molar (millimolar, micromolar)
Mn	Manganese
MOB	Manganese-oxidizing bacteria
OD	Optical density
t_d	Doubling time
WWTP	Wastewater treatment plants

CHAPTER I

INTRODUCTION

Challenges for traditional water treatment processes

The presence of emerging organic contaminants in surface and groundwater sources poses challenges for traditional water treatment. These contaminants are mostly anthropogenic in nature and commonly used in personal care products, pharmaceuticals, and pesticides. These emerging organic pollutants also may be carcinogenic, mutagenic, and endocrine disruptive in nature. Traditional treatments such as granular activated carbon (GAC), oxidation, and ozonation with sand filtration have not proven to effectively remove these pollutants. Furthermore, the periodic regeneration of GAC and ozone production can be costly, and ozone is unstable in aquatic systems.

Another concern for groundwater and surface water vulnerability is due to heavy metal toxicity, resulting from soil or water contamination. While precipitation and removal of the dissolved metals can be readily achieved in some aquatic systems, *in situ* remediation of polluted soil or groundwater sources is more difficult and not feasible at several locations. Reverse osmosis is not cost-effective on a large scale and the use of binding polymers or resins is equally costly; moreover, the spent polymers cannot usually be regenerated. Alternatively, the adsorption and oxidizing capacity of biogenic metal oxides can be harnessed to remove both organic and inorganic contaminants in natural and engineered aquatic systems.

Biogenic manganese oxides

The biological catalysis of reduction-oxidation reactions involving metal ions can be carried out by a variety of microorganisms. These biochemical reactions might result in metal oxides. When these bacteria alter the oxidation state of metals, they may yield metal oxides. Highly reactive metal oxides can oxidize both inorganic and organic compounds, including aromatic hydrocarbons, into lower molecular weight constituents (Bargar et al. 2006).

In the earth's crust, manganese (Mn) is the 12th most abundant element. In terrestrial environments, manganese exists in primarily as Mn(II), Mn(III), and Mn(IV); the higher oxidation states tend to manifest as precipitates, whereas Mn(II) is generally soluble. Manganese oxide minerals are also widely abundant, found in both soil and water environments, and are among the strongest oxidants (Tebo et al. 2004). The two-electron oxidation of Mn(II) to Mn(IV) (or MnO_2) is thermodynamically favored in the presence of oxygen, according to equation (1).

$$\mathbf{Mn(II) + 0.5O_2 + H_2O \rightarrow MnO_2 + 2H} \qquad \mathbf{(1)}$$

Biogenic (or bacteriogenic) oxidation occurs quite rapidly as opposed to abiotic oxidation in the presence of oxygen (Spiro et al. 2009). Biogenic oxidation may be the primary source of manganese (Mn) oxides found in natural settings; this hypothesis is based on the characteristics observed for biogenically formed minerals, such as poor crystallinity and high specific surface (Borch et al. 2009).

Biogenically produced MnO_2 tend to be amorphous and rife with defects. The amorphous nature and structural defects influence available surface area and adsorption capacity, which are much

higher than that of synthetic manganese oxides (Spiro et al. 2009). Biogenic manganese oxides in nature have the capacity to adsorb several metals such as selenium, lead, arsenic, and chromium ions (Borch et al. 2009; Dong et al. 2000).

In addition to adsorptive capacities, biogenic Mn oxides exhibit increased reactivity due to structural defects, such as cation vacancies (Spiro et al. 2009). Several organic and inorganic compounds, including dissolved heavy metals, can be chemically oxidized in the presence of MnO_2. During this process, insoluble MnO_2 is reduced into soluble Mn(II) (Hennebel et al. 2009). This process occurs quickly and at ambient temperatures and neutral pH.

Manganese-oxidizing bacteria

While some bacteria use the oxidation of metal ions as a means to gain energy or as protection mechanisms (Hennebel et al. 2009), the biological impetus for biogenic manganese oxide production is not universally understood. One explanation is that biologically induced mineralization may simply be an independent side-effect of metabolic processes, such as enzymatic activity (Frankel and Bazylinski 2003).

There are three commonly studied bacteria that oxidize Mn(II). A protein in the sheath of *Leptothrix discophora* catalyzes manganese oxidation and precipitation (Frankel and Bazylinski 2003). The outer cell membrane of *Pseudomonas putida*, a freshwater bacterium, is the site of oxidized manganese deposition (Villalobos et al. 2003). Lastly, *Bacillus* sp. strain SG-1 is a marine bacterium. On the surface of the dormant mature spores of this strain, manganese will bind and precipitate (Rosson and Nealson 1982).

Research objectives

In order to establish the preferred utility of using biogenic manganese oxides in an engineered water treatment system, the objectives of this research were to 1) determine optimum growth conditions for the MOB, 2) calculate the doubling time at optimum conditions and 3) characterize the biogenically formed manganese oxide minerals.

The effect of environmental factors and growth conditions were explored in order to optimize the yield of biogenic manganese oxides. Temperature and pH, as important biological parameters, were studied. Understanding the ideal physical and chemical conditions under which the growth of the MOB and the subsequent synthesis of manganese oxides occurs will drive the discussion for potential engineering applications. There is no assumption that both sets of conditions are comparable.

Another important consideration in using MOB is the characterization of the resultant minerals. The increased surface area and porosity, due to defects in the mineral's crystalline structure (Spiro et al. 2009), are a primary indication of the capacity for adsorption.

The insights from this study may provide inspiration for potential uses of biogenic metal oxides in water/wastewater treatment processes or *in situ* remediation of contaminated soil and groundwater sources. Water treatment with biogenic metal oxides may offer a novel way to remove contaminants using either adsorption or biochemical oxidation.

CHAPTER II

METHODS

Growth of model organisms

Bacillus sp. stain SG-1

Pure cultures of *Bacillus* sp. strain SG-1 were originally obtained from Dr. Bradley Tebo of the Oregon Health and Science University Institute of Environmental Health. The SG-1 strain was grown in a liquid K-medium, as modified from a published procedure (Bargar et al. 2005): 0.2 g peptone, 0.5 g yeast extract, 10 mM HEPES buffer, and 100 µM $MnCl_2$ in 75% strength artificial seawater (ASW). The pH was adjusted, as necessary, to 7.0.

First, a stock supply of ASW was prepared according to Rosson and Nealon (1982). The ASW was formulated by adding 0.3 M NaCl, 0.01 M KCl, 0.05 M $MgSO_4$, and 0.01 M $CaCl_2$ in distilled de-ionized (DI) water. The yeast extract and peptone were added to a liter of 75% ASW and 25% DI water, then autoclaved in clear volumetric bottles at 121°C for 20 minutes. HEPES buffer and $MnCl_2$ were then added to the specified concentrations. The medium was inoculated with a loopful of SG-1 from an agar plate, using aseptic technique beneath a laminar flow hood. The bottles were "capped" with foam stoppers and placed on an agitating plate (150 rpm) at ambient conditions (approximately 24°C). Sporulation usually was visibly evident within four days.

Pseudomonas putida

Pure cultures of *Pseudomonas putida* MnB1 were also obtained from Dr. Bradley Tebo. The freshwater MOB culture was propagated by streaking nutrient agar plates. Incubation at room temperature was sufficient for growth. The plates were transferred to 4°C incubation after one or two days. During growth experiments, full-strength Luria-Bertani (LB) broth was used as the growth medium. LB broth consists of (by volume) approximately 40% tryptone, 20% yeast extract, and 40% sodium chloride. For full-strength medium, 20.0 g of powdered compound was added to 800 mL of purified water, mixed completely, then autoclaved.

Experimental setup

A 3^2 full-factorial experimental design was chosen. Both experimental factors, temperature and pH, were observed at three levels each. Due to space and time considerations, experiments were performed in temperature batches, with all respective replicates run concurrently.

Manipulation and control of experimental factors

The three levels of temperature were 25.0, 27.5, and 30.0°C. All experimental cultures were grown in a temperature-controlled incubator (0.1°C precision). Temperature was verified periodically with a separate thermometer. The three levels of pH were 6.0, 7.0, and 8.0. The pH of the medium was adjusted by the additional of NaOH and/or HCl. Any necessary pH adjustment occurred after the autoclaved (as appropriate) medium had cooled and prior to any inoculations. A recently calibrated pH meter (± 0.1 precision) was used to verify the desired initial pH. The medium was used within twelve hours of preparation.

Inoculation

For each temperature batch of experiments, two plated cultures were rinsed into approximately 30 mL of deionized water. The suspension was vortexed thoroughly to ensure reasonable homogeneity. Approximately 200 mL of prepared medium was inoculated with 3 mL of suspended culture. Inoculation was performed aseptically beneath a laminar flow hood immediately before the cultures were transferred to the incubation unit.

Development of growth curves

The optical density (OD or absorbance) of each replicate and control was measured at regular time intervals, based on the expected onset and duration of the exponential growth phase. Additionally, the initial absorbance was measured immediately after inoculation. Sterile, disposable pipettes were used to transfer 1.5 mL aliquots to single-use semi-micro polymethyl methacrylate cuvettes. The optical density was measured using a spectrophotometer (GENESYS 10S UV-Vis (Thermo Fisher Scientific Inc., Madison, WI) set at 600 nm using cuvettes with an optical length of 10 mm.

The absorbance at 600 nm (or OD600) was measured using DI water as the blanking sample. All growth experiments were carried out for one day, unless otherwise specified. The maximum specific growth rate (μ_{max}) was determined from the slope of the semi-logarithmic plot of OD600 versus time. The total absorbance did not include the contribution from the medium, found by measuring the control. Additionally, only measurements during the exponential growth phase were used to determine μ_{max} ($hour^{-1}$).

Calculation of doubling time

Assuming first-order growth kinetics, the doubling time ($t_{d,}$ hours) for each set of parameters was calculated from μ_{max} according to equation (2).

$$\mathbf{t_d = \frac{\ln(2)}{\mu_{max}}} \quad \mathbf{(2)}$$

The doubling time for each replicate, calculated independently, was used in the analysis performed in Design-Expert® 8.

CHAPTER III

RESULTS AND DISCUSSION

Early screening experiments with *Bacillus* sp. SG-1

Initial experiments with *Bacillus* sp. in the modified K-medium were promising: the presence of dark brown precipitates (presumably Mn oxides) after approximately five days indicated sporulation. However, it was noted that the control medium did not remain stable, often appearing "cloudy" or discolored within 24 hrs. The irregularities did not appear to happen at a specific time, and the manifestation often differed: thick, white flocculation or brown translucent coloring, for example. In light of the attention given to aseptic techniques, contamination was not initially considered as the cause.

The first formal growth experiment with *Bacillus* was conducted at 25°C and 7.0 pH, with three replicates and two identical controls. After ten hours, thick clumping white precipitates were evident in the first control; a similar condition occurred in the second control approximately eight hours later. Moreover, after ten hours the optical density measurements of the replicates started to vary greatly due to the presence of flocculants within the medium. Consideration was given to using cell dry weight instead of spectrophotometry to quantify growth. However, the flocculants would contribute to the total suspended solids, creating significant error in final oven-dried weights.

Alteration of the Bacillus medium

The initial preparation of the medium, as published, included the additional of HEPES and MnCl after autoclaving. Several changes to the preparation were attempted such as autoclaving or not autoclaving each/all components, adjusting the molar concentration of HEPES, and use of autoclaved foam stoppers. While the time of onset varied, ultimately, the medium did not remain translucent and the white flocculants formed.

Suspecting that the concentration of peptone and/or yeast extract could not remain soluble in a highly saline solution, such as the modified K-medium, those components were added to both autoclaved ASW and autoclaved DI water. These "control" samples were placed on an agitating plate (150 rpm) at ambient temperatures. Within three days, each exhibited the white cloudiness as previously witnessed. Suspecting bacterial contamination, each of these "control" samples was grown on solid medium. Two plates each, one nutrient agar and the other a simplified K-medium, were inoculated with one milliliter from each sample. Within three days, contamination was confirmed as bacterial growth was visibly significant on all four plates, more so for the nutrient agar than the simplified K-medium.

Growth experiments with *Pseudomonas putida*

A benefit of using LB broth as the medium was the absence of significant amounts of Mn(II). The rich color of any precipitated Mn(IV) would have been a source of absorbance that would otherwise require quantification and removal from the measured OD.

The growth experiments for *P. putida* were performed for 24 hrs, since the primary focus was growth during the exponential phase. Viewing the plots of OD versus time, the deceleration phase onset occurred within 24 hrs. Since the onset and duration of the exponential phase may have differed, the range from 4 to 8 hr was chosen as exhibiting exponential growth for all trials. A typical growth curve for this experiment is displayed in Figure 1. The doubling times shown in Table 1 were calculated based on OD measurements during this range.

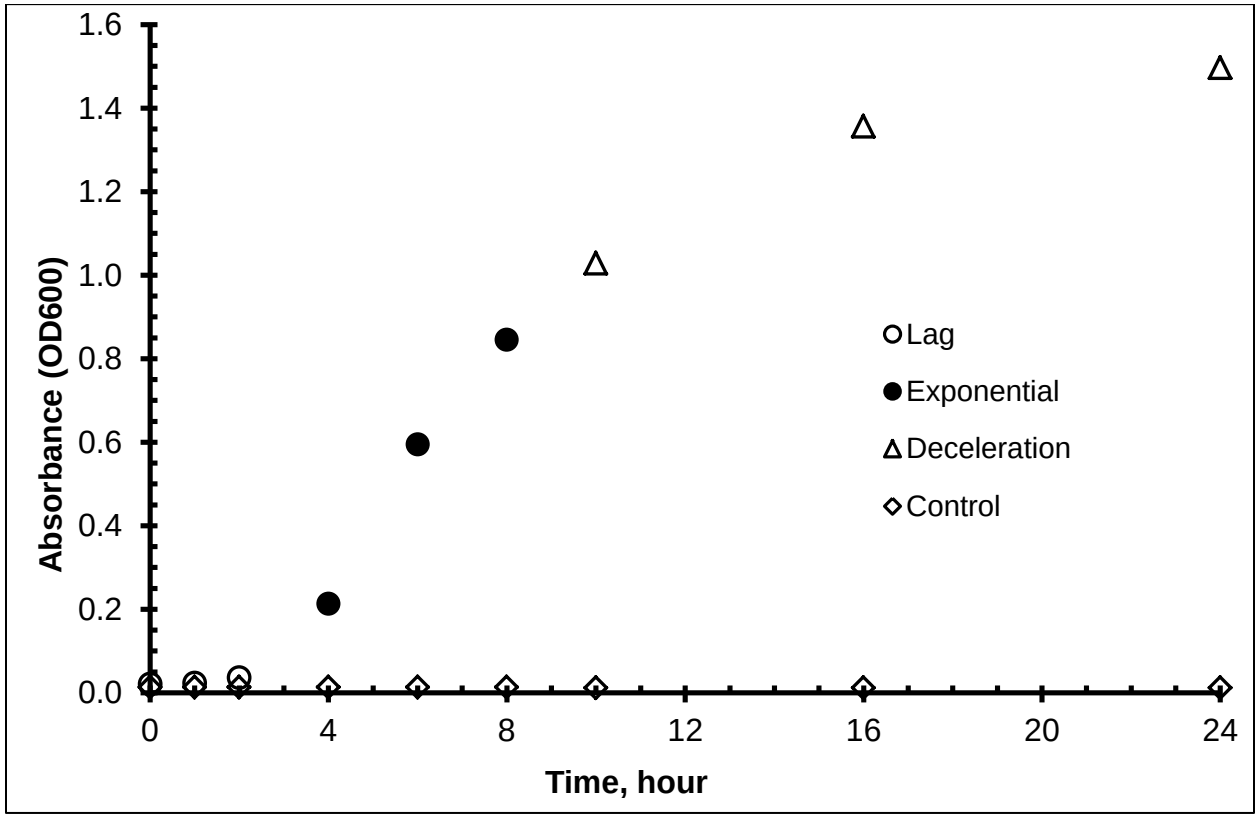

Figure 1. Growth curve for *P. putida* at 25°C and pH 6.0 using average absorbance values.

Table 1. Mean doubling times for *P. putida* in LB broth.

Doubling time (hours)		Temperature (°C)		
		25.0	27.5	30.0
pH	6.0	1.87	2.24	2.81
	7.0	2.38	2.50	3.33
	8.0	2.15	2.31	3.32

Data analysis

Analysis of variance was performed in Design-Expert® 8. As seen in Figure 2, both temperature and ph as well as the second-order terms were significant factors effecting the doubling time, while their interaction was not. The doubling times for all replicates were included in this analysis.

Transform: Inverse Sqrt Constant: 0

ANOVA for Response Surface Reduced Quadratic Model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	0.085	4	0.021	56.54	< 0.0001
A-pH	*5.515E-003*	*1*	*5.515E-003*	*14.62*	*0.0009*
B-Temperature	*0.067*	*1*	*0.067*	*178.16*	*< 0.0001*
A2	*8.451E-003*	*1*	*8.451E-003*	*22.41*	*0.0001*
B2	*4.135E-003*	*1*	*4.135E-003*	*10.96*	*0.0032*
Residual	8.298E-003	22	3.772E-004		
Lack of Fit	*2.712E-003*	*4*	*6.779E-004*	*2.18*	*0.1119*
Pure Error	*5.586E-003*	*18*	*3.103E-004*		
Cor Total	0.094	26			

Figure 2. Analysis of variance for the effect of temperature and pH on doubling time.

Additionally, both the contour plot and the response curve (Figures 3 and 4, respectively) show that the shortest doubling time occurred at the low levels for each experimental factor: 25.0 °C and pH 6.0.

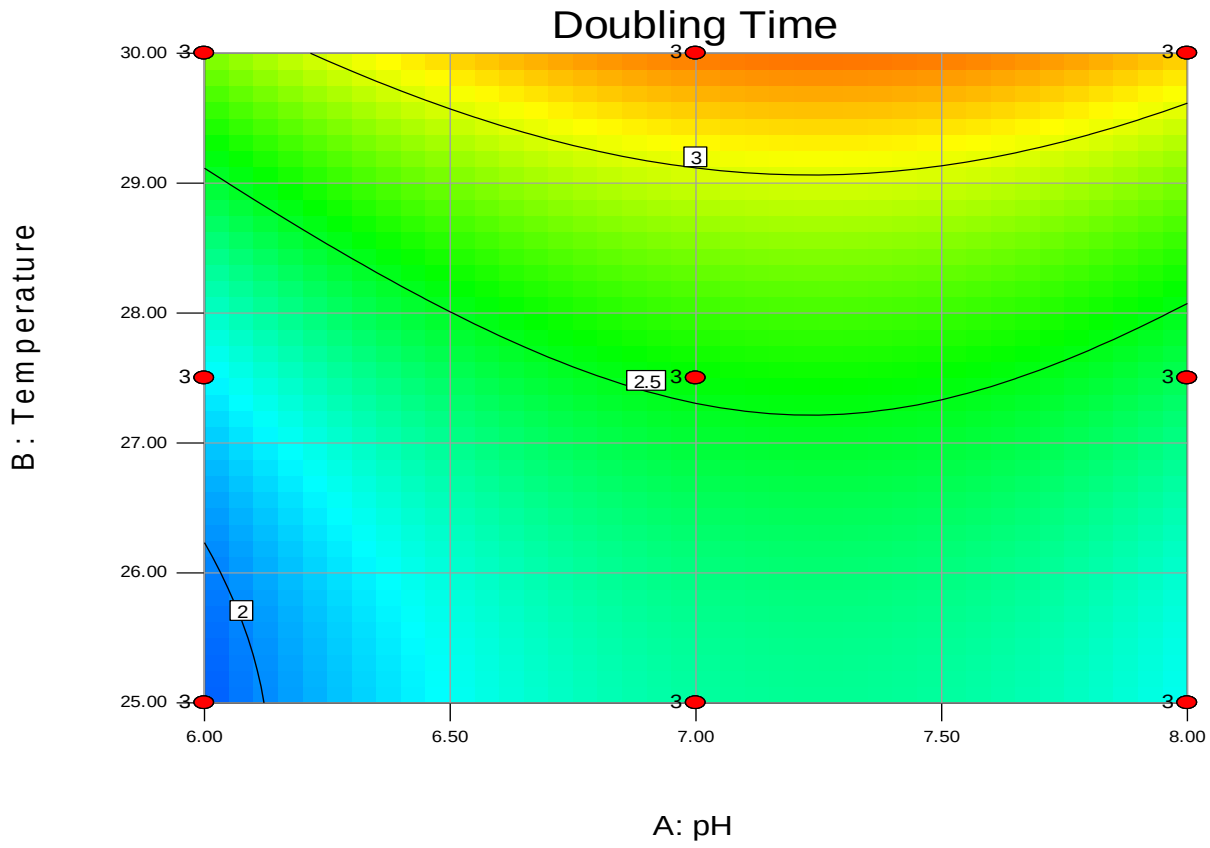

Figure 3. Contour plot of the effect of pH and temperature on doubling time of *P. putida*.

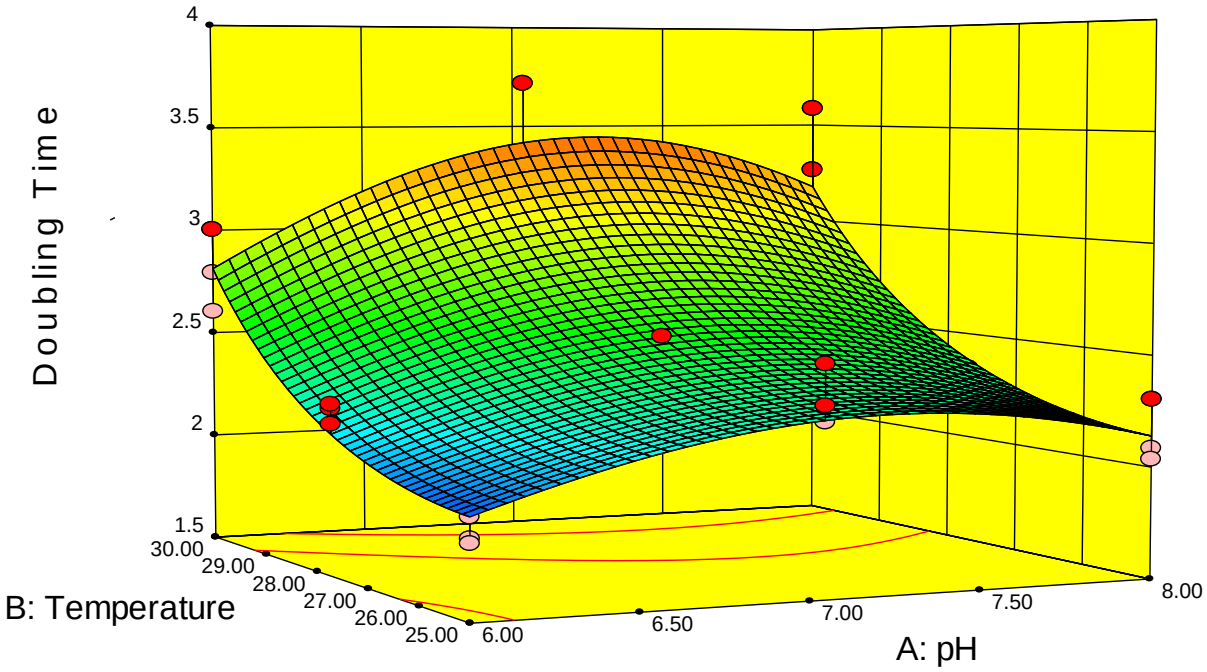

Figure 4. Second-order response surface for the effects on the doubling time of *P. putida*.

Future modifications to experimental method

For each experiment, only the initial pH was measured. Future experiments at a minimum should include a final pH measurement to determine whether carbon dioxide from cellular respiration has an effect on the pH of the medium.

Another modification would be to measure OD more frequently, until the onset of deceleration. The additional information would permit a better delineation of the onset and duration of the exponential growth phase, thus a more precise calculation of the maximum specific growth rate. The specific growth rate is an important design parameter as it determines reactor retention time. Lastly, a standard curve relating optical density to cell density (g/mL) needs to be developed.

CHAPTER IV

CONCLUSIONS

. The growth experiments for *Bacillus* were not successful, but that may have been serendipitous. As noted in the discussion regarding the growth of *P. putida*, the presence of darkly colored oxidized manganese would have been a source of interference in measured absorbance.

Although it is fair to conclude contamination of at least one compound (either peptone or yeast extract) contributed to the difficulty in the *Bacillus* growth experiments, for future study an alternative medium should still be developed. As in the case of *P. putida*, a medium that is not amended with a source of Mn(II) could permit a more direct quantification of bacterial growth. As first posited in the introduction, quantifying the growth characteristics is an important step in determining the feasibility of a scaled operation.

Another important consideration is realizing that optimum conditions for MOB growth may not necessarily coincide with the optimum conditions under which the MOB produces the desired Mn oxides. Under this assumption, the production of biogenic manganese oxide might best be accomplished in two separate reactors: 1) maintain the MOB in the exponential growth phase; and 2) Mn oxidation by the MOB in the presence of a suitable form and concentration of Mn(II).

The shortest doubling time (and hence, fastest growth) for *P. putida* in the LB broth occurred at an initial pH of 6.0 and 25°C. Far from being quantitative absolutes, these results are

nevertheless important. These findings demonstrate that the MOB grows well at circumneutral pH and ambient temperatures. Moreover, a relatively short doubling time (from 1.5 to 3.5 hrs) may favor a larger-scale operation.

Future study is needed to characterize the biogenic Mn oxides and quantify the yield in terms of both MOB and Mn(II) substrate concentrations. The yield coefficient may perhaps be less than that for abiotic manganese oxidation. However, it is anticipated that the superior mineralogy of biogenic manganese oxides, including greater specific surface area and oxidative capacity, and faster production at more favorable conditions will outweigh a potentially smaller yield coefficient.

The biogenic production of manganese oxide warrants further study to assess feasible use in the removal of emerging contaminants in a drinking water treatment process.

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