DETERMINING THE IMPACT OF GROWTH CONDITIONS ON BACTERIAL GROWTH, MAGANESE(II) OXIDATION, AND ANIMAL HEMEPEROXIDASES EXPRESSION IN *ROSEOBACTER SPP. AZWK-3B*

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ABSTRACT

DETERMINING THE IMPACT OF GROWTH CONDITIONS ON BACTERIAL GROWTH, MANGANESE(II) OXIDATION, AND ANIMAL HEMEPEROXIDASES EXPRESSION IN *ROSEOBACTER SPP. AZWK-3B*

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Mn (III, IV) oxides are environmentally important minerals as they are capable of oxidizing complex organic compounds into simpler organic forms and also capable of influencing toxic metal mobility, such as chromium and lead. In this study, we examined a species of the *Roseobacter* clade, *Roseobacter* spp. AzwK-3b, which is capable of oxidizing Mn(II) to Mn (III, IV) oxides. *R.* AzwK-3b uses an extracellular enzyme, animal heme peroxidase (AHPs), to produce superoxide, which mediates the oxidation of Mn(II) to Mn(IV). However, very few studies have reported the involvement of bacterial heme peroxidases in reactive oxygen species (ROS) production. In contrast, ROS production by eukaryotic heme peroxidases is well documented. The physiological reason for expression of these energetically costly AHPs by *R.* Azwk-3b still remains elusive. This study hypothesized that AHPs expression in *R.* Azwk-3b depends upon the growth conditions. To examine this hypothesis, *R.* AzwK-3b was grown in various conditions and its growth, Mn (III, IV) oxides formation, and gene expression were monitored. The results show that changing the carbon and nitrogen sources can impact growth and Mn(II) oxidation Mn (III, IV) oxides formation), however the expression of AHPs is independent of these nutrients. In addition, AHPs expression is independent of presence of H_2O_2. Taken together, data suggests that the expression of this bacterial heme peroxidase is primarily for oxidative function instead of peroxidative function.

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CHAPTER I

INTRODUCTION

Manganese (Mn) is the fifth most abundant metal in Earth's crust and occurs in the environment as three oxidation states: Mn(II), Mn(III) and Mn(IV). Mn(II) is soluble and commonly present in the environment, whereas Mn(III) is unstable and found primarily with other organic ligands (Tebo, 2004). Mn(IV) is insoluble and forms oxides, which are strong oxidants, capable of oxidizing toxic metals like Cr(III) (Tebo, 2004). Mn(IV) oxides also play a role in breaking down many complex nutrients and organic compounds such as humic substances, polychlorinated biphenyls, phenol, chlorinated anilines and atrazine into simpler forms (Stone & Morgan 1984a, b; Stone 1987; Ulrich & Stone 1989). Although the oxidation of Mn(II) by oxygen is thermodynamically favorable, kinetics limit the reaction. It has been reported that Mn(II) does not get oxidized in the presence of oxygen, even after seven years (Diem and Stumm, 1984). In contrast, microbes are able to oxidize Mn(II) to Mn(IV) at a much faster rate (Nealson and Myers, 1992; Tebo, 2005).

The marine *Alphaproteobacteria*, *R.* AzwK-3b, produces an extracellular enzyme, animal heme peroxidase (AHPs), which can oxidize Mn(II) into Mn (III, IV) via the production of superoxide (Hansel et al., 2006; Learman et al., 2011a, Learman et al., 2013 Andeer et al., 2015). AHPs belong to the subfamily of bacterial peroxidases within the superfamily cyclooxygenase peroxidase, (Zámocký et al., 2008; Marchler-Bauer et al., 2009, Zámocký and Obinger, 2010; Santamaria-Hernando et al., 2012). *R.* AzwK-3b possesses four genes that code for AHPs within two different loci. The first locus contains only one gene, *ahp*L, and the second locus contains three different genes: *ahp*A, *ahp*B and *ahp*C (Andeer et al., 2015). There is 99.9% sequence

identity between *ahp*L and overlapping sequences of *ahp*A, *ahp*B and *ahp*C. However, the individual role and function of these AHPs related genes are yet to be elucidated.

Bacterial AHPs have been implicated to play a role in Mn(II) oxidation in other organisms, but they have been proposed to possess different mechanisms compared to *R.* AzwK-3b. Specifically, two *Alphaproteobacteria*, *Aurantimonas manganoxydans* strain SI85-9A1 and *Erythrobacter sp.* strain SD-21, have heme peroxidases which have been directly linked to catalyzing Mn(II) oxidation (Anderson et al., 2009). Contrary to *R.* AzwK-3b, which use superoxide for indirect Mn oxides formation, these bacteria use H_2O_2 as oxidant and directly oxidize Mn(II) (Nakama et al., 2014). Although *R*. AzwK-3b can oxidize Mn(II) via AHPs, the expression of AHPs by *R.* AzwK-3b is not dependent on the presence of Mn(II) (Learman and Hansel 2014).

Heme peroxidases are primarily linked to their peroxidative function, however, oscillation of heme peroxidase between oxidative and peroxidative function is not new in eukaryotic heme peroxidases (Scheeline et al., 1997). In fact, hemeperoxidases like horseradish peroxidase (HRP), lignin peroxidases, Mn peroxidases and myeloperoxidase are able to generate reactive oxygen species (ROS) (Rotilio et al., 1975; Wariishi and Gold, 1989; Scheeline et al., 1997; Watanabe et al., 2000; O'Brien et al., 2012). Recently, AHPs in *R.* Azwk-3b were reported to oscillate between their oxidative and peroxidative function (Andeer et al., 2015). Yet, the physiological role of AHPs in *R.* AzwK-3b remains poorly understood. It is still unclear whether AHPs are produced for H_2O_2 scavenging, superoxide generation, or other physiological functions.

In general, heme peroxidases mostly show different specificity for oxidizing different substrates (Battistuzzi et al., 2010). Extensively studied heme peroxidase, horseradish peroxide from different superfamily of bacteria, fungi and plant, has shown the substrate specificity even at

the level of structure of specific substrate (Gilabert et al., 2004). Furthermore, the fungal heme peroxidase, a DyP-type heme peroxidase from another superfamily, also shows the substrate specificity (Bloois et al., 2010). During heterologous expression in *Escherichia Coli,* extracellularly expressed DyP-type heme peroxidase is highly active against anthraquinone dyes but has low activity against standard heme peroxidase substrates like aromatic sulfides and azo dyes (Bloois et al., 2010; Kim and Shoda 1999; Sugano et al., 2000, 2004, 2007; Johjimajoh et al., 2003; Sturm et al., 2006; Zubieta et al., 2007 a, b). However, it is still unknown whether AHPs possess any substrate specificity and if the available substrates affect Mn(II) oxidation (Mn (III, IV) oxides formation). In addition, it is also unclear if the available substrate has an effect on the either oxidative or peroxidative function of AHPs.

To address the uncertainty regarding the role and function of AHPs, *R*. AzwK-3b was grown in minimal medium with various amendments and growth conditions (carbon, nitrogen, Fe (II) concentrations, H_2O_2, catalase, and high RPM). The impact of these conditions was evaluated by monitoring AHPs expression, growth, and Mn(II) oxidation (Mn (III, IV) oxides formation) rates. The findings suggest that the function of AHPs to oxidize Mn(II) to Mn (III, IV) is dependent upon the nature of available carbon sources. Furthermore, AHPs are expressed primarily for their oxidative but not for their peroxidative role. The data suggests these bacterial heme peroxidases have an oscillatory oxidative–peroxidative function that is similar to eukaryotic enzymes.

CHAPTER II

MATERIALS AND METHODS

Growth in Carbon and Nitrogen Source Plates

Standard growth conditions for *Roseobacter sp.* AzwK-3b (from the -80 freezer stock) was in J-medium (75% artificial seawater (Waasbergen et al., 1993), 1.5 mM NH_4Cl, 10 mM $FeSO_4$, 2 mM HCO_3, 73 µM KH_2PO_4, and 10 ml of Wolfe's vitamin supplement, 10 mM acetate) and incubated at 30 °C. Initially, various carbon and nitrogen sources were evaluated to examine which would support growth (Table 1). For testing growth in different carbon sources, J-media plates (15g of agar per liter) were prepared with one form of carbon sources (listed in Table 1) and NH_4Cl was used as the sole nitrogen source. Similarly, when examining nitrogen sources, J-media plates only contained acetate as the sole carbon source with each type of nitrogen source in each plate.

Preparation of Culture Media and Bacterial Growth

For growth, oxidation, and RNA extraction, *R.* AzwK-3b was first grown in 25 ml of J-medium or K- medium (75% artificial seawater (Waasbergen et al., 1993), yeast extract, and peptone) at 30 °C and 150 rpm for 24 hours. Cultures grown overnight were inoculated in new J-media or K-media with initial $O.D._{600}$ of 0.02. J-medium with acetate and NH_4Cl (JAC) or K-medium was used as a positive control in all experiments. There were three replicates for each of the substrates and growth conditions.

Table 1. Carbon and Nitrogen Substrates used for *R.* Azwk-3b Growth in J-medium

Carbon Sources	No. of successful growth plates out of 4	Nitrogen Sources	No. of successful growth plates out of 4
Acetate	4 of 4	Glutamate	4 of 4
Glycerol	4 of 4	Alanine	4 of 4
Glucose	4 of 4	Glycine	4 of 4
Galactose	4 of 4	Serine	4 of 4
Sucrose	1 of 4	l-arabinose	4 of 4
Dextrose	2 of 4	Aspartic acid	0 of 4
Maltose	0 of 4	Dihydroxybenzoic acid	0 of 4
Citric acid	0 of 4	Arginine	4 of 4
Lactate	4 of 4		
Fructose	0 of 4		
Pyruvate	0 of 4		
Trehalose	0 of 4		
Succinate	0 of 4		
Putrescine	0 of 4		
Phenylalanine	0 of 4		
Oxalic acid	0 of 4		
Malic acid	0 of 4		

Monitoring Growth and Mn Oxidation

To monitor growth, absorbance readings were taken at 600 nm ($O.D._{600}$) using a spectrophotometer (Cary 60 UV–Vis spectrophotometer, Varian). To measure growth with Mn oxides in the growth medium, ascorbic acid (400 μM) was added to reduce the Mn oxides in

cuvette before absorbance readings were taken. Mn(II) oxidation was measured with the Leucoberbelin blue (LBB) assay (Krumbein and Altmann, 1973) and absorbance was measured at 620 nm using a spectrophotometer.

Growth in Carbon and Nitrogen Sources

R. Azwk-3b was grown in J-medium cultures with different forms of carbon sources (Table 1) on four replicate plates. The plates which exhibited significant growth (4 of 4) were further grown with NH_4Cl as) sole nitrogen source. Similarly, different forms of nitrogen sources were altered in J-medium cultures on four replicate plates to monitor growth and oxidation (Table 1). The plates which exhibited significant growth (4 or 4) were further grown with acetate as sole carbon source. $MnCl_2$ (100 μM) was added to each flask to monitor Mn oxidation. Growth was monitored at 24, 36, 48, 72 and 96 hours respectively.

Bacterial Growth and Mn Oxidation under different Growth Conditions

Bacteria growth and Mn oxidation was monitored in specific growth conditions. First, 2.5 mM of H_2O_2 was spiked at 48 hours in cultures grown in K-media. The growth OD was monitored at 24, 48, 49, 53 and 72 hours. Second, the growth and oxidation was monitored in catalase by adding 10, 20, 30 and 40 μg/ml of catalase every 24 hours to cultures grown in K-media. Third, *R.* Azwk -3b was grown in J-medium in four $FeSO_4$ concentrations: 0 mM, 10 mM, 20 mM and 30 mM to examine the effect of different concentrations of iron(II). The growth and oxidation was monitored every 24, 48, 72 and 94 hours for cultures grown in different concentrations of catalase and $FeSO_4$. Lastly, K-media cultures of *R.* Azwk -3b were incubated at 150, 250 and 300 rpm to monitor the effect of RPMs on growth and oxidation. Two treatments, without catalase and with

catalase (20 μg/L), were implemented to monitor whether presence of catalase altered the oxidation in different RPMs. Growth and oxidation was monitored every 24, 48 and 74 hours.

Expression of AHPs, RNA Extraction and RT-PCR

From the growth and oxidation experiment, the substrates and conditions that had the most effect on either growth or oxidation were further selected to monitor the expression of AHPs. For each substrate and growth condition, RNA was extracted during lag phase (before 24 hours/below 0.2 OD), log phase (24-60 hours/0.2-0.6) and stationary phase (after 60 hours/0.6 OD).

RNA was extracted from the suspension using UltraClean® Microbial RNA Isolation Kit. The extracted RNA concentration was measured and then normalized cDNA was generated by using reverse transcriptase, with the iScript™ cDNA Synthesis Kit. Previously designed primers for different *ahp* genes (*ahp*A, *ahp*B and *ahp*L) and reference genes (*rec*A, *rpo*D and *cat*) were obtained from Dr. Peter F. Andeer's unpublished work (Table 2). Different forward and reverse primers (Table 2); *ahp*A, *ahp*B and *ahp*L, were used to quantify the expression level of different fragments of AHPs related genes, where primers *ahp*A, *ahp*B and *ahp*L are associated with the unique fragment of *ahp*A and *ahp*B from one locus, and *ahp*L from the other locus respectively. *rec*A was used as the reference gene. AHPs gene expression was analyzed by qPCR using the StepOnePlus™ Real-Time qPCR system. Fast Plus EvaGreen® qPCR master mix was used as a fluorescent dye. The optimum annealing temperature for each sets of primers for each genes *ahp*A (51.8°C) , *ahp*B (56.1°C), *ahp*L (58.1°C), *rec*A (58.1°C), *rpo*D (60.1°C) and *cat* (60.2°C) and $MgCl_2$ concentration was determined for each primer set by amplifying genomic DNA fragments encoding AHPs with regular PCR. Fast Plus EvaGreen® qPCR master mix contained 2.5 mM $MgCl_2$ concentration was optimum for all the primers used except for primers of gene *ahp*B where

3 mM $MgCl_2$ concentration was used. The RT-PCR cycle was used as recommended in the protocol of Fast Plus EvaGreen® qPCR master mix with slight modifications. The initial holding time was set for 2 minutes at 95°C, with 40 cycles run with 5 seconds of denaturation 95°C, 10 seconds of annealing temperature and 25 seconds of extension at 72°C. The concentration of primers used was 2 μM and concentration of cDNA was 0.004 ng/μL. $2^{-\Delta CT}$ and $2^{-\Delta\Delta CT}$ were used to compare the relative gene expression of AHPs in different substrates and growth conditions.

Table 2. RT-PCR primer Sequences (Dr. Peter F. Andeer's unpublished work)

Name of Primers	Target gene Fragment	Sequence(5'-3')
QAHPSF	*Ahp*A	GCGCAAAACACTCTGACAGC
QAHPSR	*ahp*A	AGATCAACGGCGTTCTGC
QAHPMF	*ahp*B	CAATGGCAGTGCCGAGAT
QAHPMR	*ahp*B	GGGGTCCATCGTGTGAAC
QAHPLbF	*ahp*L	GGAAGAAGGGTTCGGGTTC
QAHPLbR	*ahp*L	GGATGCACCGACCATGTACT
QRECAF	*rec*A	GATCCGCATGAAGATTGGCG
QRECR	*rec*A	TATCCAGACGCACGGAACTG
QRPOD1F	*rpo*D	GACGATCAACAAGCTGGTGC
QRPOD1R	*rpo*D	CTTCATCACCTTGCGGACCT
QCATF	*Cat*	ATCCTTTGGCCGCTGAAGAA
QCATR	*Cat*	GCCCATCGCCTCATAAGACA

Flow Cytometry

R. Azwk-3b was grown in triplicates in J-medium with acetate, galactose, glycine and without $FeSO_4$. Bacterial cell count was monitored every 24, 36, 48 and 72 hours. SYTO® BC bacterial stain by Thermo Fisher was used to stain the live cells. SYTO® BC bacterial stain is responsible for green fluorescence which is excited at 480 nm wavelength and emitted at 500 nm wavelength. Microsphere standard of 6 μm and of 1×10^8 beads/ml concentration was used. Perpidium iodide from Thermo Fisher was used to stain dead cells which is excited at 533 nm

wavelength and emitted at 618 nm wavelength. Bacterial samples were incubated with 1 μl/ml SYTO® BC bacterial stain, 1×10^6 beads/mL and 3 μM of the propidium iodide for 15 minutes prior to measurement. The measurement was taken in Beckman Coulter CytoFLEX using CytExert. Green fluorescence was collected through the FITC laser channels and red fluorescence was monitored through the PE laser channels.

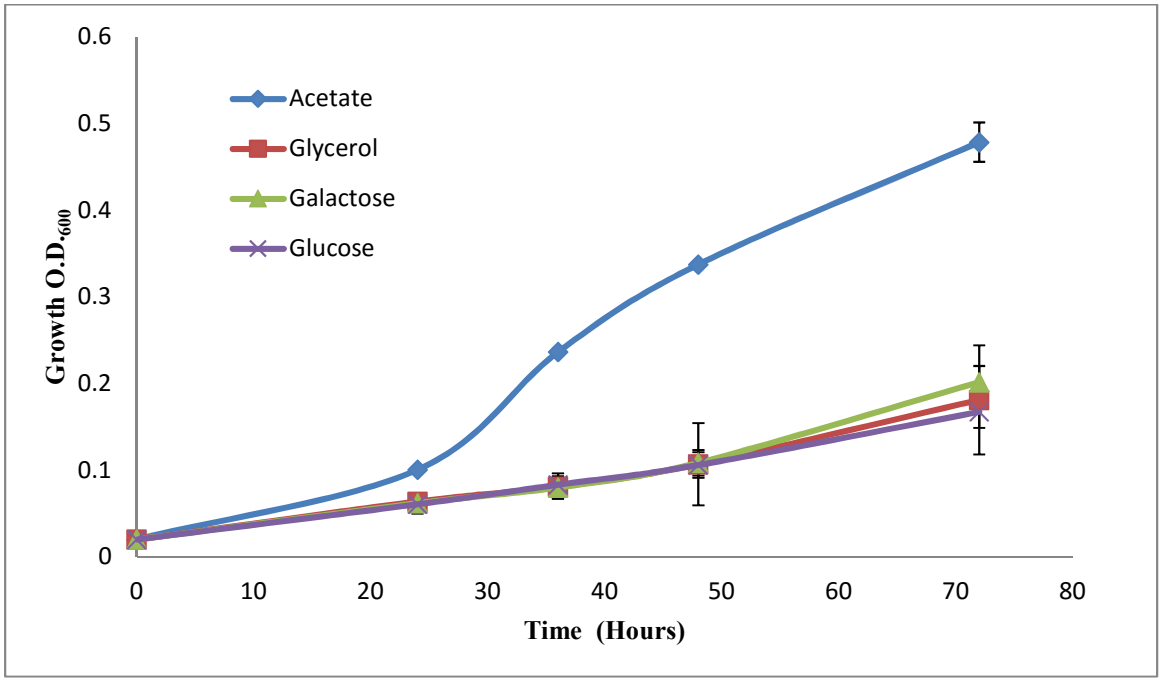
CHAPTER III

RESULTS AND DISCUSSION

Effect of Available Substrates on Bacterial Growth, Mn(II) Oxidation, and AHPs Expression

To assess the influence of available substrates in Mn(II) oxidation and AHPs expression, *R*. Azwk-3b was grown in different carbon and nitrogen sources and Fe concentrations. AHPs expression was not impacted by changing various carbon and nitrogen sources, however, growth and Mn(II) oxidation were affected by changing carbon sources. When grown in nutrient limited J medium, bacteria showed maximum growth in acetate among all the carbon sources (Fig. 1.a). When grown with glycerol, galactose, or glucose as the sole carbon sources, overall growth was low and lacked distinct log and stationary phases of growth. Even though growth was different, the oxidation was similar among carbon sources (Fig. 1.b). Although growth was higher in acetate, Mn(II) oxidation per cell per µl was distinctly higher in galactose than in acetate (Fig. 2). Conversely, growth in nitrogen sources showed distinct lag, log and stationary phases and growth and Mn(II) oxidation was relatively similar in all nitrogen sources (Fig. Glycine produced a higher growth and oxidation at 74 hours when compared to all other nitrogen sources (Fig. 3a and 3b). Mn(II) oxidation per cell per µl between nitrogen sources NH_4Cl and glycine was relatively similar (Fig. 2). Despite growth being significantly different among various carbon sources, there was no significant change in AHPs expression (Fig.4). Although growth was heavily affected by the alteration of carbon sources, there was minimal alteration in Mn(II) oxidation and AHPs expression. Mn(II) oxidation per cell per µl was relatively higher in galactose than in acetate, which indicates that Mn oxide formation is dependent upon the extracellular conditions.

a.



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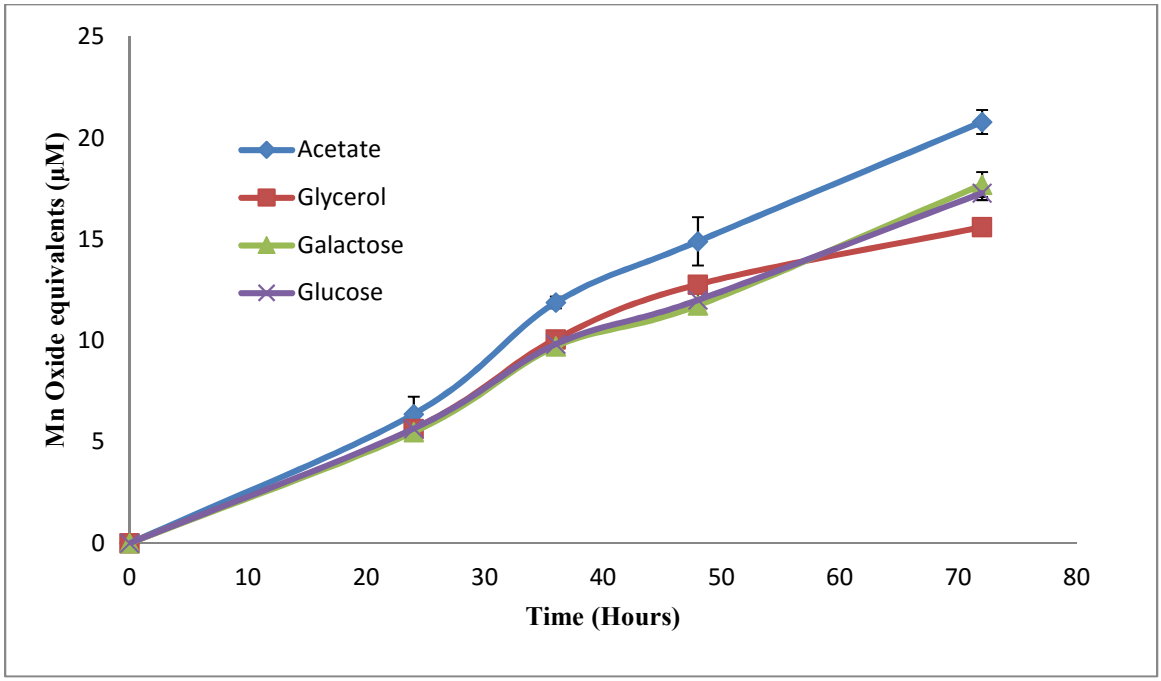


Figure 1. Carbon sources can influence the growth and oxidation in *R.* AzwK-3b: (a) Time series of growth of *R.* AzwK-3b in J-media with 10 mM carbon sources: acetate, glycerol, galactose and glucose. (b) Time series of Mn(II) oxidation (Mn (III, IV) oxides formation) assay of *R.* Azwk-3b cultures in J-media and different carbon sources: acetate, glycerol , galactose and glucose.

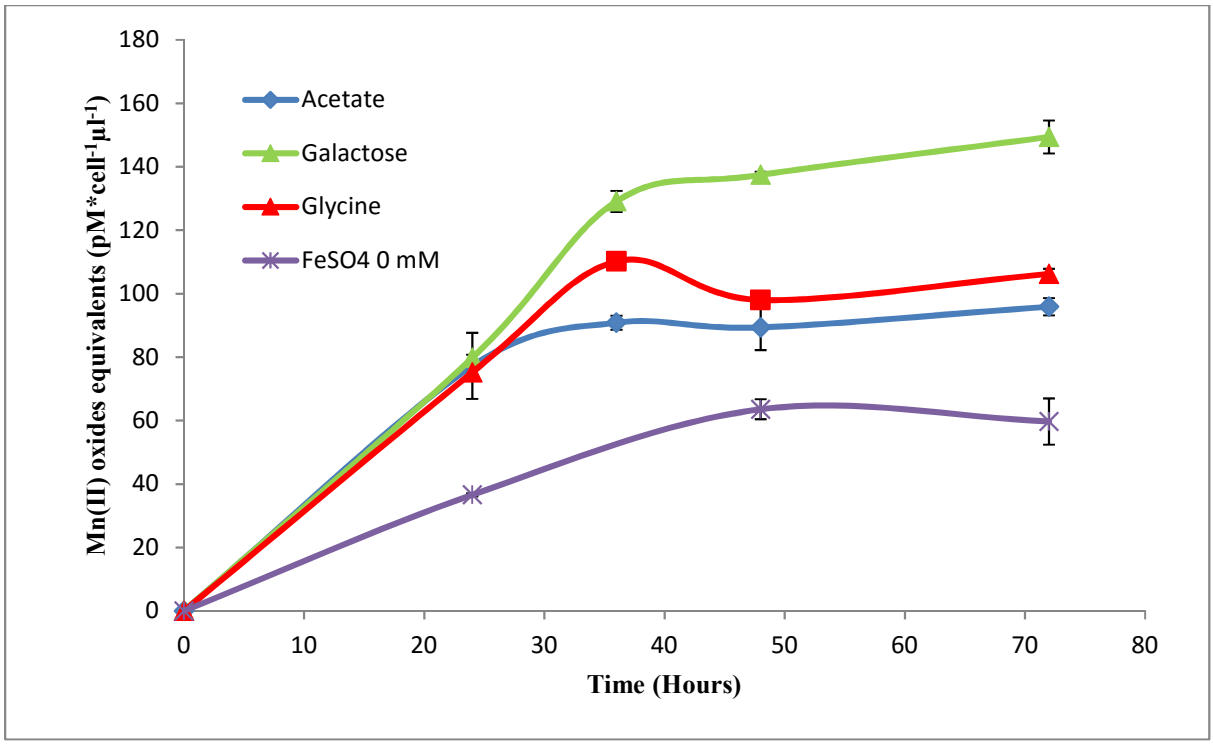
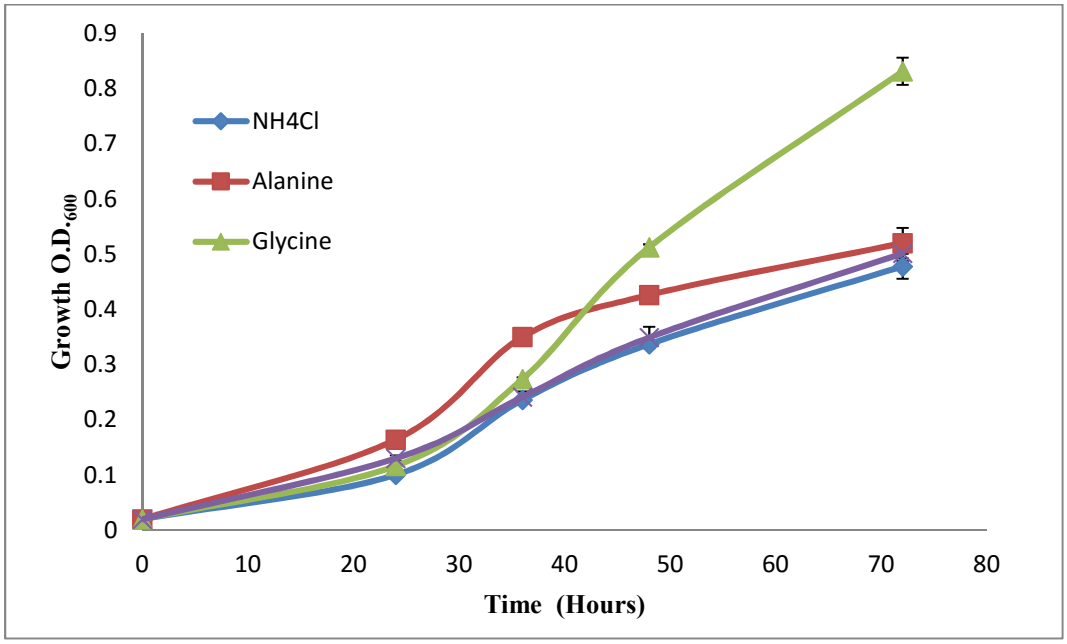


Figure 2. Mn(II) oxidation (Mn (III, IV) oxides formation) in *R.*AzwK-3b is affected by different growth conditions. a) Time series of Mn(II) oxidation (Mn (III, IV) oxides formation) per cell per µl of *R.* Azwk-3b in J-media with substrates: acetate, galactose and glycine and growth condition 0 mM $FeSO_4$.

a.



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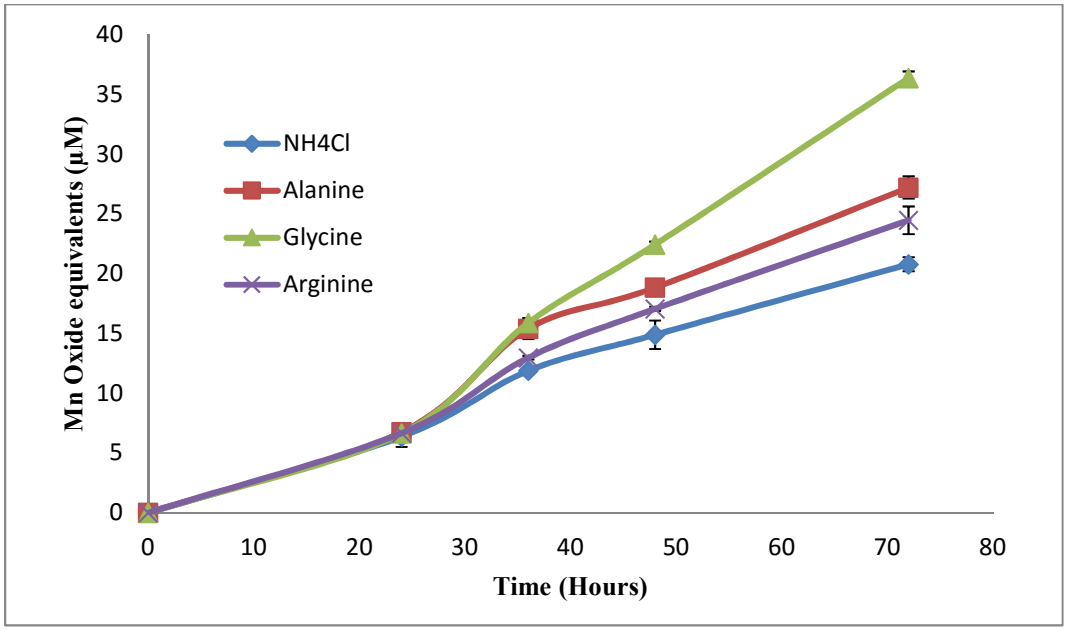


Figure 3. Mn(II) oxidation (Mn (III, IV) oxides formation) by *R.* AzwK-3b are independent of change in nitrogen source: a) Time series of growth of *R.* AzwK-3b in J-media with 1.5 mM nitrogen sources: NH_4Cl, alanine , glycine and arginine. b) Time series of Mn(II) oxidation assay of *R.* AzwK-3b cultures in J-media and different nitrogen sources: NH_4Cl, alanine, glycine and arginine.

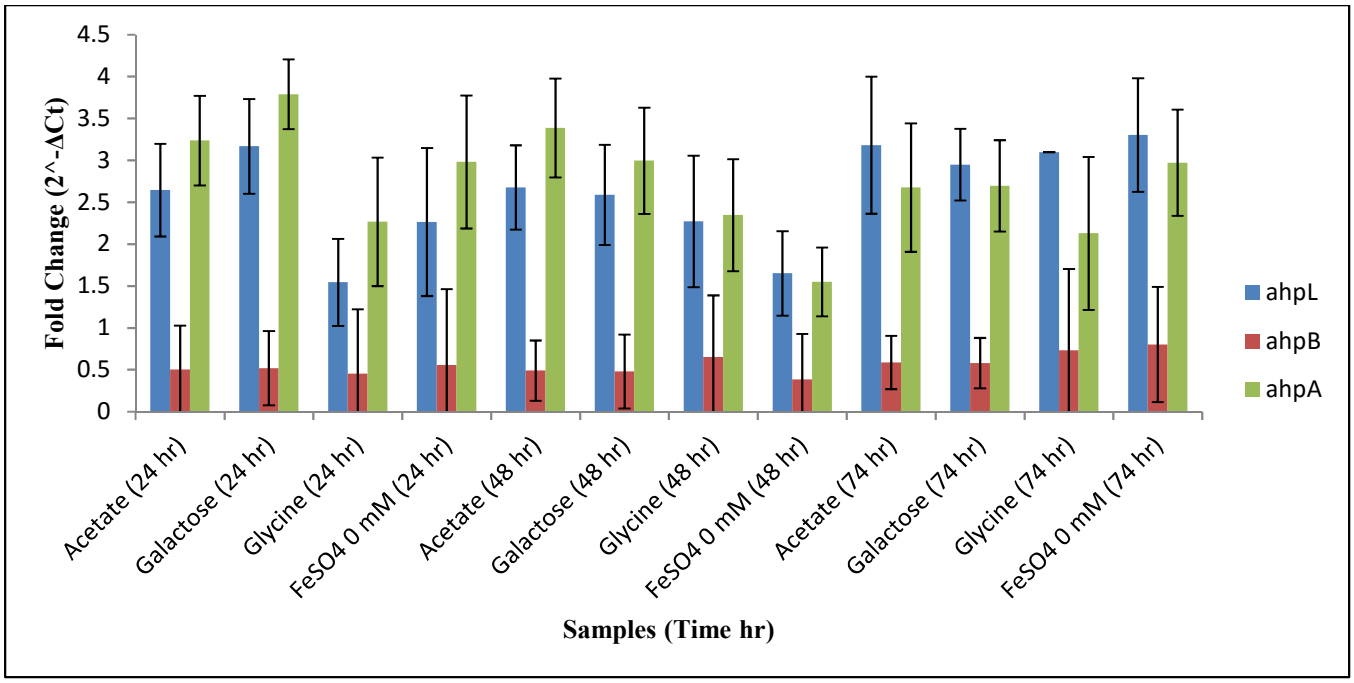


Figure 4. Altering carbon and nitrogen sources do not alter the expression of AHPs in *R.* AzwK-3b. Change in expression of *ahp*A, *ahp*B, and *ahpL*, and catalase in different carbon sources: acetate and galactose during the different growth phases of *R.* Azwk-3b: Lag phase (24 hr), Log phase (48 Hr) and Stationary phase (74 hr).

The growth, oxidation, and expression data suggests that Mn oxide formation by *R.* AzwK-3b is not controlled via AHPs expression but indirectly through solution chemistry (growth media and excreted metabolites). Previous studies report that extracellular parameters like oxygen, CO_2, pH, surface nutrients, have an effect on Mn(II) oxidation rates (Emerson et al., 1984; Marshall, 1979). Other studies have shown that mineral catalyzed abiotic Mn(II) oxidation rates can be dependent upon the size of nanoparticles of available minerals (Madden and Hochella, 2005). In addition, solution chemistry has always been an important factor for the Mn(II) oxidation process by *R.* azwk-3b (Learman et al., 2011b). Superoxide generated by *R.* Azwk-3b initially reacts with Mn(II) and forms colloidal hexagonal birnessite. These birnessite can induce further oxidation of Mn(II) (Learman et al., 2011b). The variation in rates of Mn(II) oxidation by birnessite was

observed upon different conditions such as change in media or availability of organic compounds (Learman et al., 2011b). In addition, the presence of organic compounds along with birnessite can increase the rate of Mn(II) oxidation (Learman et al., 2011b). The organic radicals formed from available organic compounds are speculated to aid birnessite in Mn(II) oxidation. It has been reported that the Mn(II) oxidation rates can be accelerated in complexation with organic compounds (Duckworth and Sposito, 2005). Also, there was a documented association between biotically produced Mn oxides and organic compounds (Estes, et al., 2016), which further supports the importance of organics to Mn mineralization. Our data suggests Mn(II) oxidation per cell per ul was relatively higher in galactose than in acetate. Thus, different carbon sources could alter solution radical chemistry, secondary mineralization, or Mn(III) oxidation to Mn oxides, altogether changing overall Mn(II) oxidation.

Effect of Oxidative Stress on AHPs Expression and Function

Peroxidases are classically known to aid oxidation stress via the scavenging of hydrogen peroxide, however, the role of AHPs in oxidative stress is unknown. To test this, *R.* Azwk-3b cultures were spiked with H_2O_2 at various times of growth. There was no significant change in AHPs expression when spiked with H_2O_2 (Fig. 5). However, there was a significant effect of H_2O_2 on growth (data not shown). While it was speculated that AHPs primary function will be to degrade H_2O_2, the unaltered AHPs expression with H_2O_2 supplemented cultures indicate that *R.* Azwk-3b does not express AHPs solely for its peroxidative role. Although growth and oxidation has always been impaired by H_2O_2, previous studies have also not connected H_2O_2 to the function of AHPs (Learman et al., 2011a; Andeer et al., 2015). Cell free extracts are not able to degrade H_2O_2, whereas the cultures can degrade H_2O_2 very efficiently (Learman et al., 2011a; Andeer et al.,

2015). Further, H_2O_2 concentration only increased when cell free extract was supplemented with Mn(II) (Learman et al., 2011a). Efficient removal of H_2O_2 in cell cultures but not in cell free extracts might be due to increase in expression of anti-oxidant genes, like catalase, when spiked with H_2O_2 as seen in our preliminary data. Similar results were observed when manganese oxidizing protein MopA and its AHPs domain from *Erythrobacter* sp. strain SD21 were heterologously expressed in *E. coli* (Nakama et al., 2014). There was no stimulus effect with H_2O_2, instead Mn(II) oxidation decreased upon addition of H_2O_2 (Nakama et al., 2014). H_2O_2 has no role in AHPs expression, which clearly indicates that AHPs are not expressed for a peroxidative role in *R.* Azwk-3b.

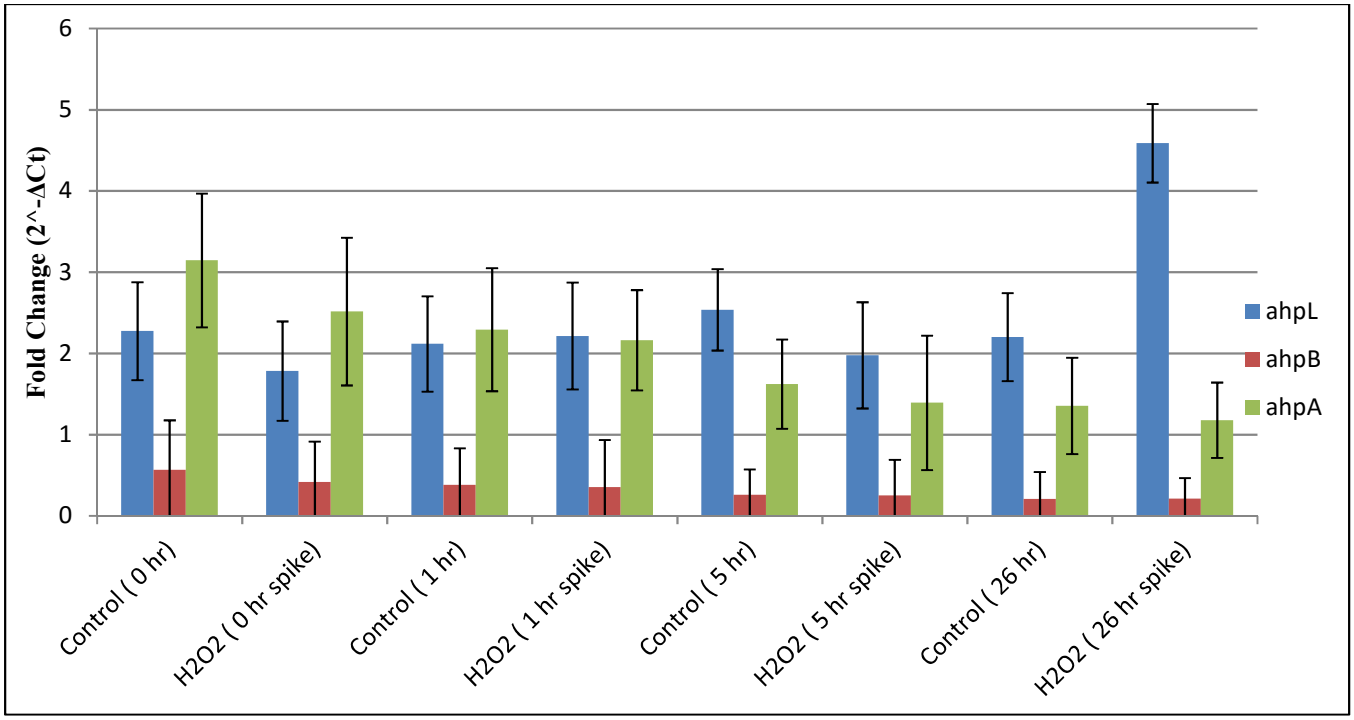
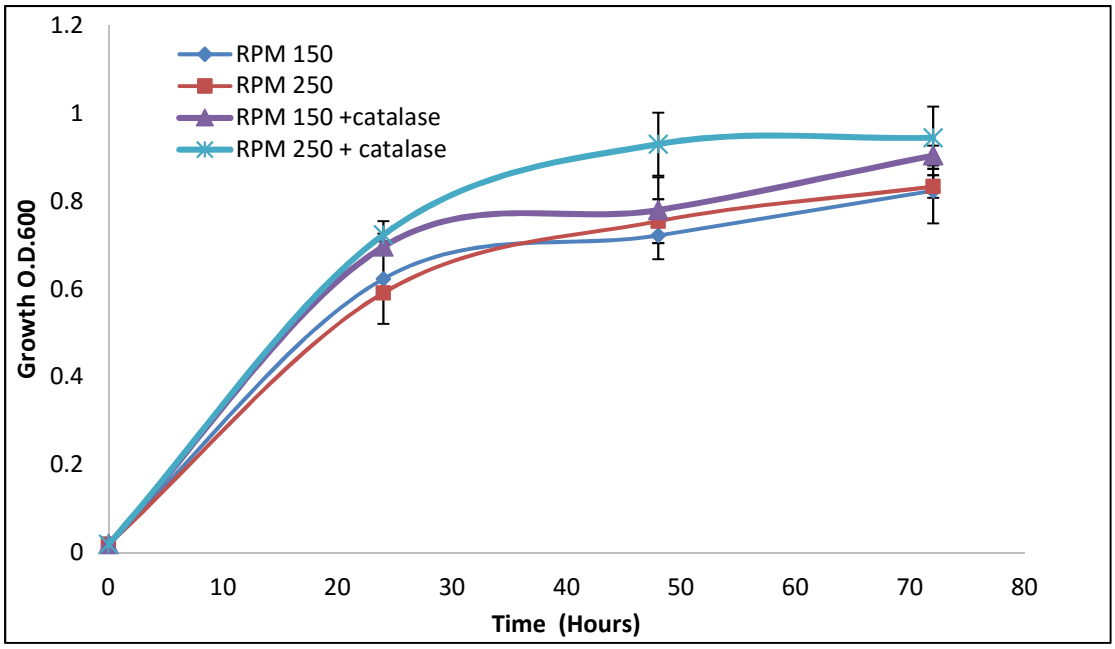


Figure 5. Addition of H_2O_2 does not alter AHP expression. Change in expression of *ahp*A, *ahp*B, and *ahpL*, before addition of H_2O_2 at time 0 hr, after one hour of addition of H_2O_2 at time 1 hr, after five hours of addition of H_2O_2 at time 5 hr and after 26 hours of addition of H_2O_2 at time 26 hr.

Oscillation of AHPs between Oxidative and Peroxidative Function

Previous studies have shown that microbial Mn(II) oxidation can be affected by the presence of oxygen and H_2O_2. The presence of oxygen can act as a stimulus and increase the Mn(II) oxidation rate (Clement et al., 2009; Nealson et al., 1978). In addition, AHPs in *R.* AzwK-3b have been shown to generate superoxide (Andeer et al., 2015). Thus, experiments were conducted to evaluate how increased dissolved oxygen (via shaking), may influence growth and oxidation. When cultures were grown with different shaker RPMs, there was no significant difference in growth (Fig. 6.a), however, Mn(II) oxidation was significantly decreased during log phase when *R*. Azwk-3b was grown in higher RPM (>150 RPM) (Fig. 6.b). If catalase was added to the cultures, growth and oxidation were the same (Fig. 6. a and b). The presence of catalase restored Mn(II) oxidation at higher RPMs to our standard conditions (150 RPMs). This result further suggests that Mn(II) oxidation is affected by the solution chemistry (O_2/H_2O_2). Growth was not impacted in higher RPMs, whereas Mn(II) oxidation was, indicating that the alteration in extracellular condition due to the increased dissolved oxygen or H_2O_2 could have altered Mn(II) oxidation. Since catalase significantly increased the Mn(II) oxidation in higher RPM 250, it is speculated that the accumulation of H_2O_2 might have resulted in decreased in Mn(II) oxidation (or the reduction of Mn oxides) in cultures grown in higher RPMs without catalase. In support of this, a previous study has shown that accumulation of H_2O_2 can cause the reverse reaction from Mn(III) to Mn(II) (Hansard et al 2011).

a.



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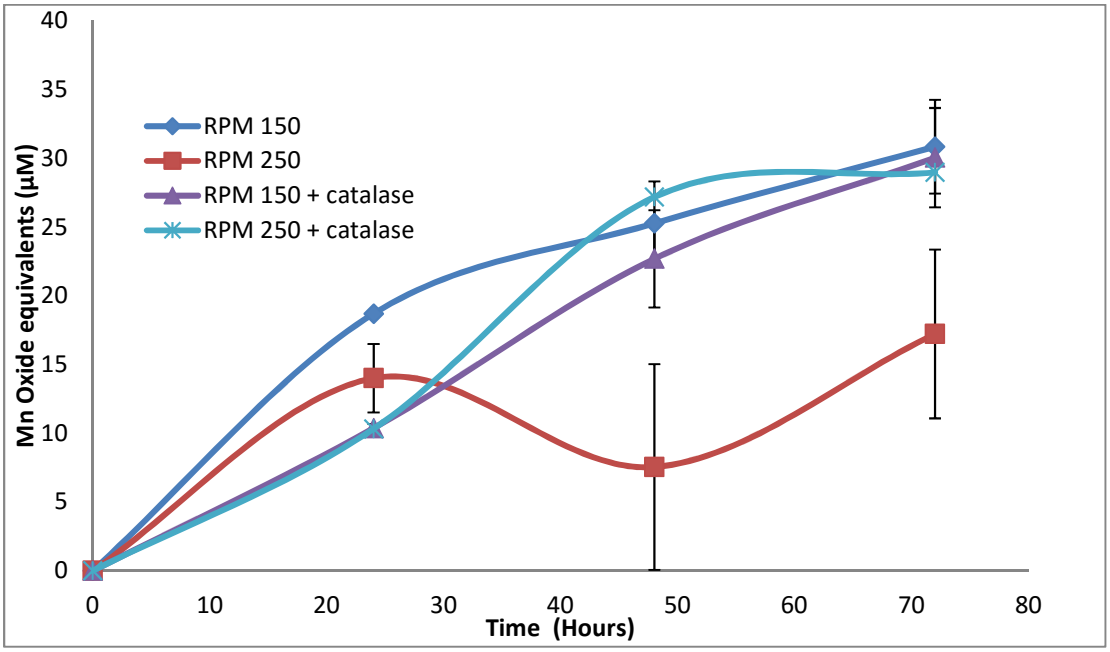


Figure 6. Addition of catalase increase Mn(II) oxidation (Mn (III, IV) oxides formation) in higher RPM. a) Time series of growth of *R.* AzwK-3b in K-media with and without catalase in 150 RPM and 250 RPM. b) Time series of Mn(II) oxidation (Mn (III, IV) oxides formation) assay of *R.* Azwk-3b in K-media with and without catalase in 150 RPM and 250 RPM.

To further investigate the impact of removing H_2O_2 (via the addition of catalase) *R*. Azwk-3b, additional experiments were conducted with catalase supplemented. Interestingly, when catalase was added every 24 hours starting from the beginning of the experiment, AHPs expression was significantly increased during the log phase at 48 hours (Fig. 7). *ahp*A expression increased nearly five folds higher, *ahp*B expression increased around 2.5 fold higher, and *ahp*L expression increased three times higher than the control (Fig. 7). There was no significant change in expression of AHPs during lag phase and stationary phase (Fig. 7). There was significant increase in catalase expression upon H_2O_2 addition (Fig. 5).

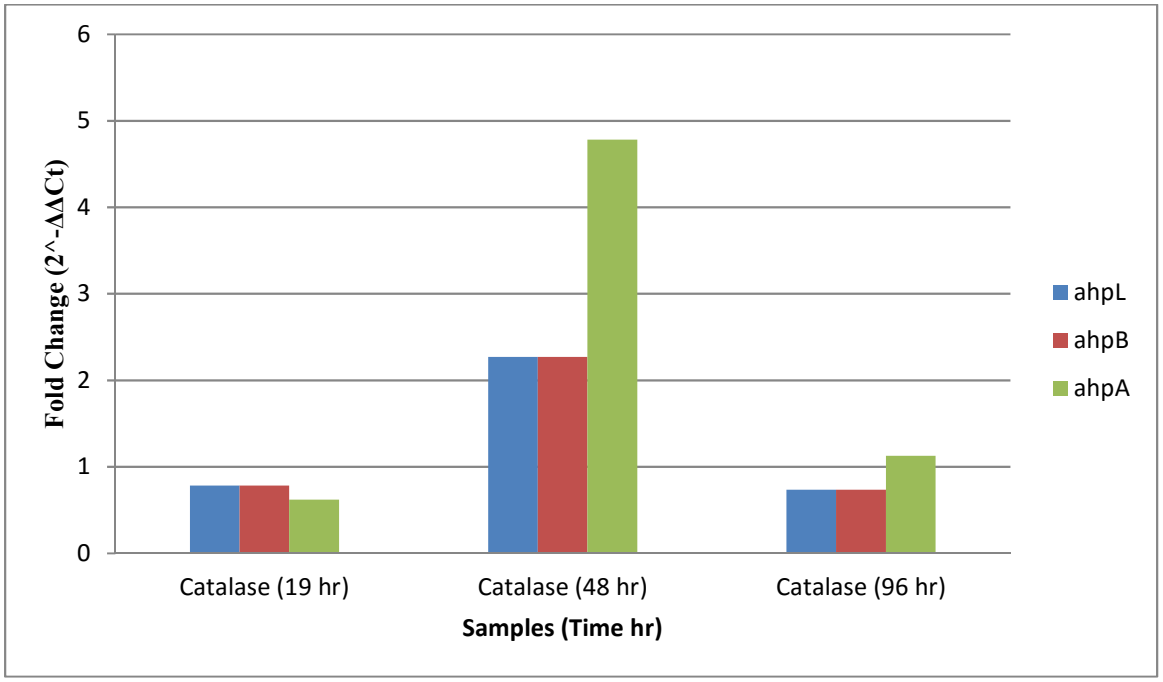


Figure 7. Addition of catalase increase the expression of AHPs. Change in expression of *ahp*A, *ahp*B, and *ahpL*,in addition of catalase at time 0 hr, 24 hr, 48 hr and 72 hr and RNA extracted at 19 hr, 48 hr and 92 hr

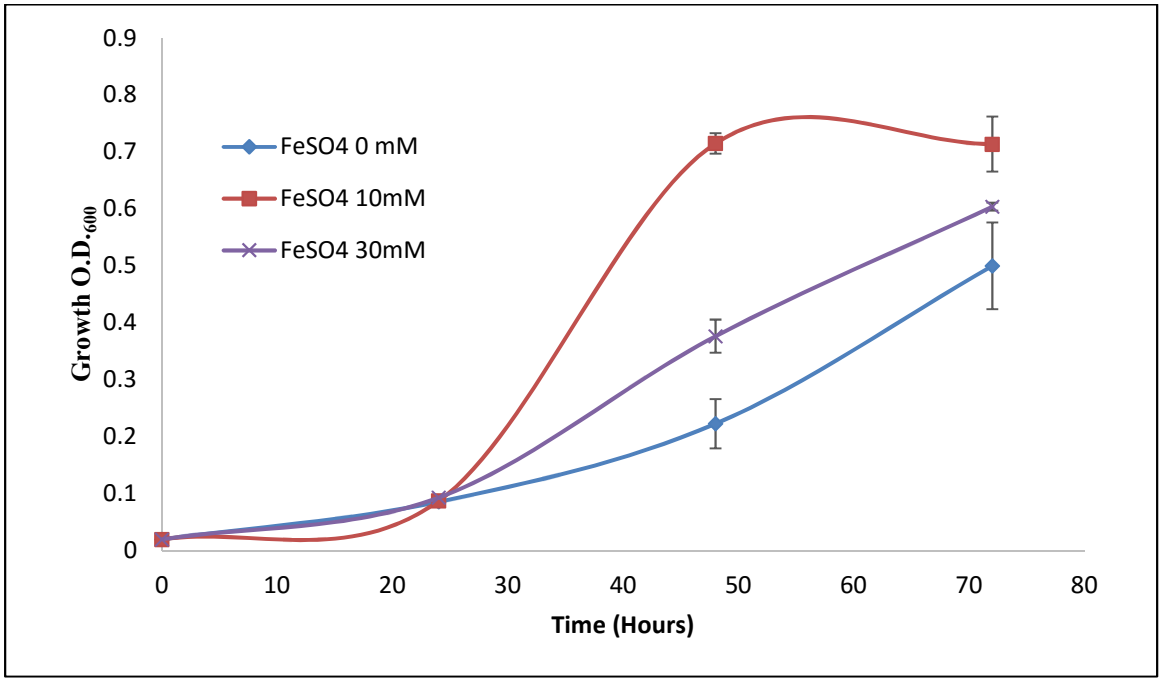
Taken together, this data suggests that AHPs are most likely expressed for the oxidative function in *R.* Azwk-3b. The increase in AHPs expression when supplemented with catalase, does

suggest a role of H_2O_2 degradation in AHP expression and/or function. Since AHPs expression increased when the ROS scavenging enzyme catalase is introduced, it indicates that AHPs has least peroxidative role and might have primary role of ROS generation. Furthermore, molecular oxygen could be responsible for AHPs expression, since catalase hydrolyses H_2O_2 into water and oxygen. In addition, previous studies have shown that molecular oxygen is a key ingredient for AHPs function and superoxide production (Li et al., 2014). *R.* Azwk-3b not only oxidizes Mn(II), but also indirectly oxidizes iodine via extracellular superoxide (Li et al., 2014). Complete inhibition of iodine oxidation was observed when *R.* Azwk-3b was grown anaerobically (Li et al., 2014). Also, isolated AHPs were able to degrade H_2O_2 enough to be able to oxidize Mn(II) in gel assay (Andeer et al., 2015). Thus, this work further supports the conclusion made in Andeer et al., (2015) that AHPs in *R*. Azwk-3b oscillate between both oxidative and peroxidative function to oxidize Mn(II).

Effect of Fe in Bacterial Growth, Oxidation and Expression

As a heme containing protein, Fe is an integral to the biosynthesis of AHPs (Banci, 1997). To investigate how Fe would impact AHP expression in *R.* Azwk-3b, cultures were grown under various Fe ($FeSO_4$) concentrations. Growth and oxidation in cultures without $FeSO_4$ and in 30 mM $FeSO_4$ were slow and distinctly lower than in 10 mM $FeSO_4$ (Fig. 8. a and b). The optimum growth and oxidation was found in 10 mM $FeSO_4$. The Mn(II) oxidation per cell per µl in cultures without $FeSO_4$ was drastically lower than cultures with 10 mM $FeSO_4$ (Fig. 2). Additionally, AHPs expression was impacted when the cells were grown in limited Fe conditions Fig. 4). Relative to the optimal condition of 10 mM $FeSO_4$, cells grown in 0 mM $FeSO_4$ had nearly half fold less AHP expression during the log phase (Fig. 4).

a.



b.

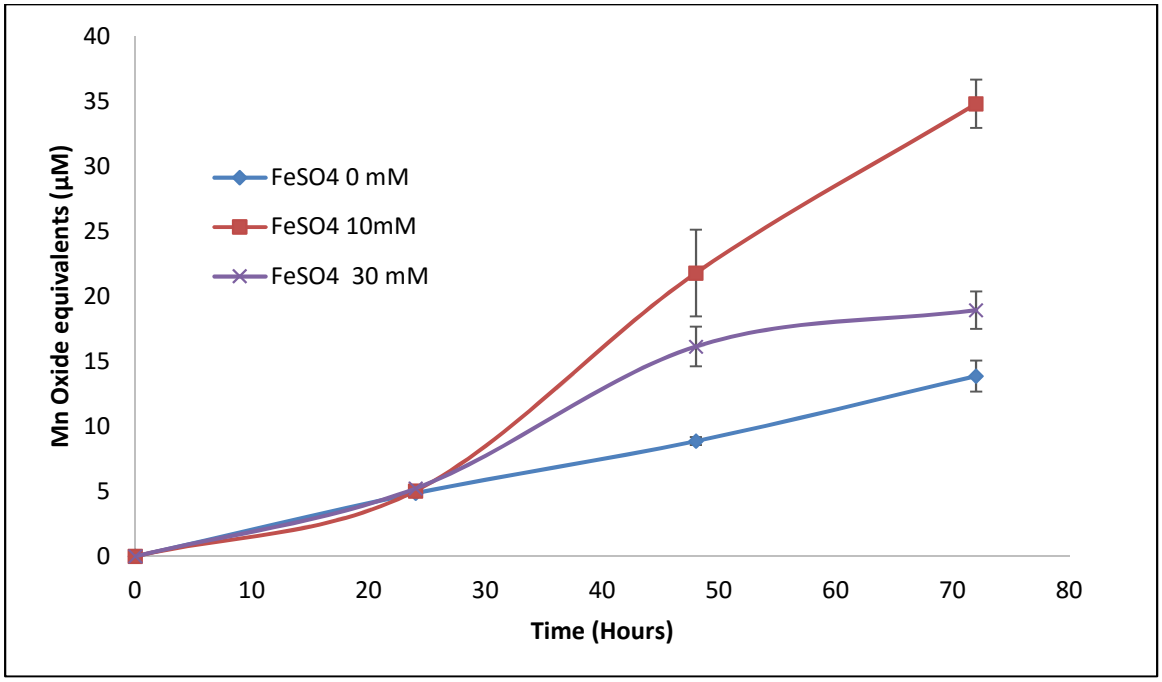


Figure 8. $FeSO_4$ concentration influence the growth and Mn(II) oxidation (Mn (III, IV) oxides formation) in *R.* AzwK-3b. a) Time series of growth of *R.* AzwK-3b in J-media with 0mM $FeSO_4$, 10 mM $FeSO_4$ and 30 mM $FeSO_4$. b) Time series of Mn(II) oxidation assay of *R.* Azwk-3b cultures in J-media with 0mM $FeSO_4$, 10 mM $FeSO_4$ and 30 mM $FeSO_4$.

Growth, oxidation and expression were affected in different Fe concentrations. Growth declined drastically in lower $FeSO_4$ concentration indicating that Fe is required for normal growth of *R*. Azwk-3b. The expression declined during the log phase, which showed that Fe is required in actively replicating bacteria to make AHPs. Mn(II) oxidation per cell per µl was drastically lower in samples without $FeSO_4$, which might be due to decrease in expression of AHPs during the log phase. Furthermore, a deficiency in Fe can result in impaired growth and metabolism as previously seen in *Escherichia coli* (Hubbard et al., 1986; Johnova et. al., 2003). The decline in growth in higher $FeSO_4$ concentration might be due to the increased toxicity by Fe. Increase in toxicity is expected as Fe catalyzes the Fenton reaction, which results in highly reactive hydroxyl radical (Byers et al., 1998). Dissolved iron concentration in oceans is below 0.2 nm (De Baar and Dejong, 2001; Boyd and Ellwood, 2010). As Fe is found in scarce amounts, the investment of Fe to make AHPs might point towards the important function of AHPs in *R*. Azwk-3b besides its role in iron metabolism.

Updated Mn(II) Oxidation Mechanism for R. AzwK-3b

Growth in different carbon sources clearly indicates that the Mn(II) oxidation is dependent upon medium constituents or excreted metabolites. There are two possible mechanisms where the nature of the medium could affect the function of heme peroxidases to oxidize Mn(II): substrate specificity of AHPs and/or stability of Mn(III) by ligand binding. AHPs are speculated to oxidize organic compounds into organic radicals, which further react with molecular oxygen generating superoxide. AHPs may have substrate specificity towards certain compounds, yielding higher amount of organic radicals and superoxide eventually. Substrate preference by other heme peroxidases has been commonly observed, as other heme peroxidases have previously shown a

different specificity for oxidizing different substrates like indole, phenols, aromatic amines, lignin, and other proteins (Battistuzzi et al., 2010). In addition, Mn(III) is a highly unstable compound and short living, so the oxidation of Mn(II) to Mn oxides also depends upon the stabilization of Mn (III), which could be influenced by organic compounds (Learman et al., 2013; Tebo, 2007). Citrate has previously been shown to form complexes with Mn(III) and increase the stability of Mn(III) (Klewicki and Morgan, 1998). Citrate has also been shown to increase abiotic Mn oxide formation that was driven by superoxide **(**Learman, 2013). So, it is possible that both, substrate specificity and organic ligand complex formation with Mn(III), is able to increase the Mn(IV) oxides formation. This result demonstrates that carbon compounds glucose, glycerol and galactose either are preferred substrate by AHPs and/or they might have an oxidized form that can stabilize Mn(III) better than acetate, therefore yielding higher oxidation per growth OD

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