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Effect of Iron on Some Parameters Recombinant *Pseudomonas aeruginosa* Carrying *Vitreoscilla* Hemoglobin Gene

Hüseyin KAHRAMAN *[1]

Abstract

In this study, the effects of iron presence on some bacterial parameters of *Pseudomonas aeruginosa* and its recombinant bacteria carrying *Vitreoscilla* hemoglobin on its chromosome were investigated for the first time. These parameters are; optical density, pH, glucose, trehalose production and biomass. Parameters; It was studied at 37 °C and 250 rpm ventilated conditions depending on time. Bacteria have developed mechanisms by which they can resist heavy metal stress with many other mechanisms, including making metals less toxic and excreting them out of the cell. The Efflux system is the most widely used mechanism. The bacterium that makes the best use of these mechanisms is *P. aeruginosa*, which has an environmental and versatile feature. In the presence of LB alone, an increase was observed in the first 48 hours and a decrease of 43% in the other time periods, especially in the 96th hour compared to the control. The highest increase was detected in the 48th time periods, up to 259% in the 3,32. When iron was added to the medium, significant increases were observed in all time periods compared to the controls and these increases reached 575% at 72 hours. In the same time periods, the maximum value of OD_{600} 4.55 was reached.

Keywords: *Pseudomonas aeruginosa*, *Vitreoscilla* Hemoglobin, Iron

1. INTRODUCTION

Iron is a cofactor for many enzymes involved in critical biological processes such as redox chemistry, tricarboxylic acid cycle, electron transport and DNA and RNA synthesis [1, 2]. The iron is not insoluble and bioavailability [3]. The iron is one of the elements necessary for the bacterial life and cannot be easily taken from the environment and may be below the concentration required for bacterial growth in many environments [4, 5]. Iron starvation can prevent bacterial growth. Recent studies have shown that these levels of metal function as a signal for biofilm development if there are enough irons to grow. It may be [6, 7]. It reacts with oxygen and water to form ferric (FeIII) oxides and hydroxides (e.g. rust) which limits the availability of living organisms [8]. For some microorganisms, it is also the key metal for the production of biological surfactants. Because of some bacteria may use

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iron as electron donor or acceptor in the energy metabolism, in the structure and activator of some enzymes [1, 9].

Working with a pathogen such as *Pseudomonas aeruginosa* in iron-related limiting conditions is due to the fact those it is a suitable bacterium to understand its behavior (Jimenez et al., 2010). Recent studies have shown that this metal serves as a signal for biofilm development in iron concentrations that do not limit growth. The production of rhamnolipid is stimulated under iron-limiting conditions and this causes an increase in twitching mobility [1, 2, 4, 8].

P. aeruginosa is an opportunistic pathogenic bacterium. Although it is a soil bacterium, it can be found in almost any habitat. This bacterium is a non-fermenting gram negative bacterium and, is able to survive in a variety of metabolic environments. *P. aeruginosa* has a natural resistance mechanism. Therefore, it is resistant to antimicrobial agents. It can transfer these resistance mechanisms in other bacteria. Iron regulation in *P. aeruginosa* occurs through a complex regulatory network [8], which may contain numerous environmental stimuli that allow virulence factors to be expressed on time. *P. aeruginosa* with biofilm is able to live on biotic and abiotic surfaces [1, 10].

Vitreoscilla hemoglobin provides oxygen to the respiratory chain and facilitates oxygenated growth and biological product synthesis. *Vitreoscilla* hemoglobin heterologous expression has an enhancing effect on cell growth and protein synthesis needed [11].

2. MATERIAL AND METHODS

2.1. Chemicals

L-(+)-Rhamnose monohydrate was purchased from MP Chemicals (USA). Phenol, H_2SO_4, and $NaHCO_3$ were purchased from Carlo Erba Chemicals. NaCl, L-(+)-glucose, $FeCl_3$ was purchased from Merck. Yeast and peptone were purchased from Mast Diagnostics. Antrone was purchased from Sigma-Aldrich. All other chemicals used were of analytical grade.

2.2. Bacterial Strains

P. aeruginosa (NRRL B-771) and its transposon mediated *vgb* transferred recombinant strain was used.

2.3. Culturing

Cells were maintained on agar plates at 4 ºC with transfers at monthly intervals. The liquid media used throughout the study was Luria- Bertani (LB) broth medium (g l^{-1}); peptone (10), NaCl (10), and yeast extract (5). The final pH values were adjusted to 7.0. 250 µl of overnight cultures was inoculated into 50 ml of the same medium in 150 ml volume flasks and incubated in at 37 °C in a 200 rpm (24, 48, 72 and 96 h). The concentration of iron compound based in preliminary experiments; in LB was 150 ppm iron. Stock iron solution was prepared in dH_2O and autoclaved. These solutions were kept at 4 ºC, and no longer than 1 month.

2.4. Cell Densities and Dry Weight

The cell densities were measured at OD_{600} every 24 h using spectrophotometer. For determination of the cell dry weight, bacterial suspensions were centrifuged at 10.000 rpm for 20 min at 20 ºC, then cell pellets produced after centrifugation were dried at 105 ºC for 5 hours and then measuring the cell dry weight.

2.5. Glucose Detection

Soluble glucose was quantified according to the anthrone method. Colorimetric response was compared to a standard curve based on glucose, and total carbohydrate content was expressed as mg/ml of glucose [12, 13].

2.6. Rhamnose

Rhamnose concentration was using the phenol-sulphuric method [14, 15]. Rhamnose concentration was quantified by a colorimetric method as rhamnose content using a rhamnose standard [16-19].

2.7. Statistics

Student's *t*-test was used for comparing and significant difference was claimed when $P < 0.05$.

In the presence of LB alone, an increase was observed in the first 48 hours and a decrease of 43% in the other time periods, especially in the 96th hour compared to the control. The highest increase was detected in the 48th time periods, up to 259% in the 3,32. When iron was added to the medium, significant increases were observed in all time periods compared to the controls and these increases reached 575% at 72 hours. In the same time periods, the maximum value of OD_{600} 4.55 was reached.

3. RESULTS

3.1. OD_{600}

While wild bacteria caught the highest value at the 48th hour with 0.935 [in iron], recombinant bacteria (PaJC) reached the 72nd hour with a value of 4,55. Especially recombinant bacteria reach the value of 4,55 with an increase of 5 times after 48 hours. Likewise, a five-fold increase was observed in the wild bacteria at the 48th hour.

When we look at the graphics, recombinant bacteria give an advantageous OD_{600} value in the first 48 hours, while wild bacteria give this increase after 72nd hours. In the iron medium, an increase of OD_{600} up to 500% is observed from the 48th hour. In LB environment, recombinant bacteria reach the highest OD_{600} value with max 3,32, while wild bacteria reach 2,86 at 96th hour.

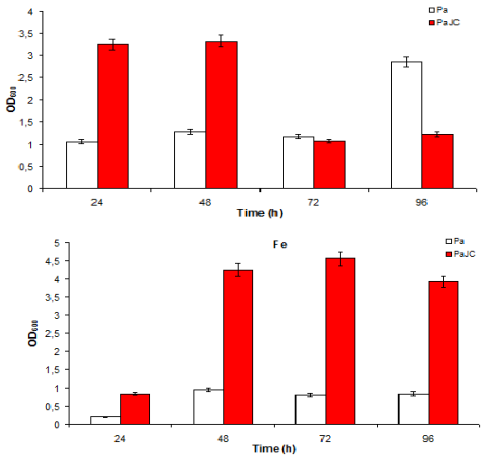

Figure 1 Growth rate of *P. aeruginosa* and its recombinant strain cells LB (top) and under LB+Fe-replete conditions (bottom).

In the presence of iron, the recombinant bacteria reach the OD_{600} value of 4.55 by the 72nd hour. Wild bacteria reach the 48th hour with 0.955 (Figure 1).

3.2. pH

Although there is an increase in the presence of PaJC in pH changes in LB environment, these increases are not significant. The highest pH increase was measured at PaJC with 8,9 at 96 hours. In the presence of iron, significant differences and pH increases occurred in the presence of PaJC. A pH increase of 133% was observed in the presence of PaJC at all times periods.

There was no significant difference in both wild bacteria and PAJC in all time frames. When wild bacteria and PaJC are compared, 1,33 times difference is seen as constant in all time frames. Wild tip bacteria reached the highest pH value again at the 96th hour with 6,71. Especially for recombinant bacteria, the pH value remained high in the presence of iron and did not show a significant change depending on the time (Figure 2).

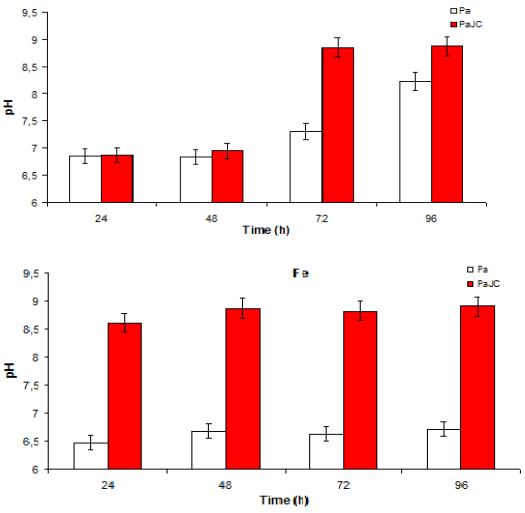

Figure 2 pH rate of *P. aeruginosa* and its recombinant strain cells LB (top) and under LB+Fe-replete conditions (bottom).

3.3. Glucose

While there were no significant differences in LB control environment, decreases in glucose production up to 712% compared to controls were recorded especially in the 96th period. In the presence of iron, the highest difference was obtained in PaJC with 204% and 387,01 mg / ml. These values decreased up to 3-fold according to LB environment. Iron reduces glucose production. Glucose production causes a decrease in both bacteria [in iron]. It is seen that it reaches the highest value with 387 mg / ml only in the presence of recombinant bacteria iron at 24 hours (Figure 3).

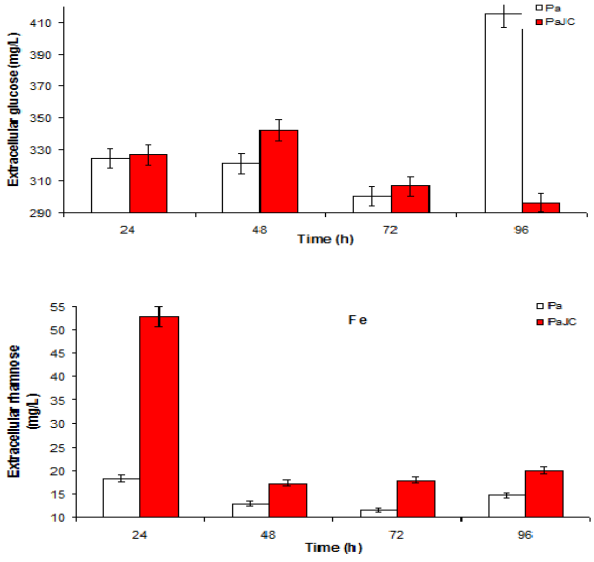

Figure 3 Extracellular glucose rate of *P. aeruginosa* and its recombinant strain cells LB LB (top) and under LB+Fe-replete conditions (bottom).

3.4. Rhamnose

Iron ramnose production decreased by 2-fold compared to the control environment. However, it appears to be advantageous in the presence of both LB and LB + iron compared to PaJC wild strain. The highest difference was 289% and 52,77 mg / ml and 24 hours in the presence of LB + iron. Rhamnose production decreases in the presence of both bacteria iron (Figure 4).

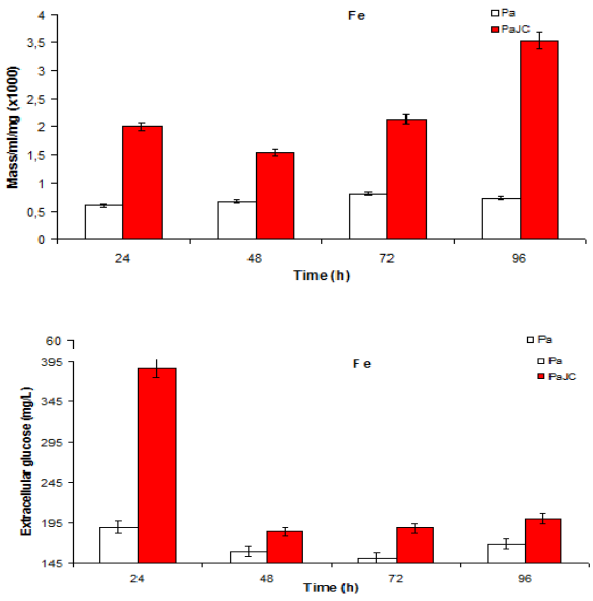

Figure 4 Extracellular rhamnose rate of *P. aeruginosa* and its recombinant strain cells LB (top) and under LB+Fe-replete conditions (bottom).

3.5. Mass

The addition of iron in the biomass measurement, one of the most important parameters, resulted in a difference between PaJC and wild strain. The difference was the highest with 204% and highest 500%. These ratios have reached only 288% in LB environment. PaJC showed differences in biomass growth up to 3-fold in the presence of LB and LB + iron. The highest biomass formation is reached by the 96th hour, by far, by recombinant bacteria. However, in the presence of iron, it causes an up to 3-fold decrease in wild-type bacteria (Figure 5).

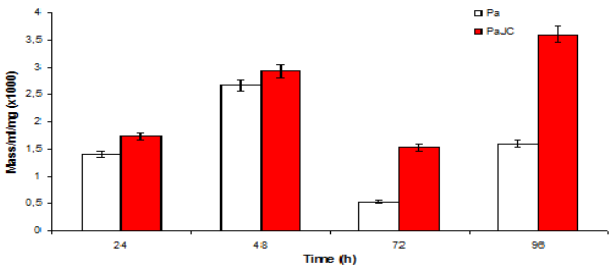

Figure 5 Biomass of *P. aeruginosa* and its recombinant strain cells LB (top) and under LB+Fe-replete conditions (bottom).

P. aeruginosa was chosen because it is widespread in nature and it has been often used in bioremediation studies [20].

4. DISCUSSION

Bacteria have developed mechanisms by which they can resist heavy metal stress with many other mechanisms, including making metals less toxic and excreting them out of the cell. The Efflux system is the most widely used mechanism. The bacterium that makes the best use of these mechanisms is *P. aeruginosa*, which has an environmental and versatile feature [21].

VHb contributes to aerobic growth and biological product synthesis by providing O_2 to the respiratory chain. Heterologous expression of VHb increases cell growth and total protein synthesis.

Iron plays a very important role in cell growth, especially in the presence of hydrobobic substrates. Some studies show that iron deficiency in the environment inhibits growth. In another study, it was stated that the growth of *P. syrigae* increased in the presence of iron. However, it has been reported that its excess causes toxicity. For example, ATCC15442 strain has been shown to affect gene expression in the presence of $FeCl_2$ and FCL_3 (0.025-0.075 and 0.1 g / L [16].

Iron is an essential growth factor for most microorganisms. *P. aeruginosa* has many iron absorbing systems to reduce under iron limited conditions. These systems; (1) siderophore system (reduces ferric iron via endogenous siderophores and xenosiderophores); (2) the heme system that obtains iron from the heme group, and (3) the iron-bearing iron-absorbing system [22, 23].

Iron restriction limits the growth rate of *P. aeruginosa*. This may be due to the iron-containing respiratory system. As a result, there is a decrease in the production of TCA cycle enzymes. Iron is already known to be a helpful factor in many biological processes. Iron is important for all organisms as it also increases resistance to DNA replication, electron transfer system and oxidative stress. Iron is essential to all organisms due to its role in DNA replication, electron transfer, and oxidative stress resistance etc [22, 24].

Due to its role in both electrons transfer and resistance to oxidative stress, it is vital that iron is present in a certain amount in microorganisms. It has been stated that the reasons for the decrease in the growth rate of *Pseudomas* produced in iron-limited conditions may be related to the decreased expression of iron-carrying TCA cycle enzymes (such as aconitase and succinate dehydrogenase). My work also supports this view (Fig. 1). Again, in this study, it is seen that the recombinant bacteria are not affected by this problem (Figure 1-5).

Apart from this, it was stated that there was a decrease in the biomass of *P. putida* grown in a limited iron environment and this was related to the ED pathway. No such determination was made in this study. On the contrary, I detected a decrease in biomass in the presence of iron (Fig. 5).

Rhamnose production decreases in the presence of both bacteria Fe. This causes a decrease in rhamnolipid formation. Although iron is a key nutrient for most organisms.

It causes an up to two-fold decrease in Fe-glucose production. We think this may be due to Cyt enzymes. It also causes a decrease in Fe-OD_{600} and mass; A decrease of up to three times is observed in the production of rhamnose.

5. CONCLUSION

While there is a stable pH in both bacteria in FE-pH, pH overlap occurs from the 72^{nd} hour in the LB environment. However, in the LB environment, the recombinant bacteria reach pH values in the presence of iron at 72 hours. This can be explained by the production of VHb to provide the oxygen needed.

In the presence of Fe, the recombinant bacterium seems to be more advantageous than the wild strain. This may be because the VHb molecule has reached a high level of iron binding capacity. As a result, has more oxygen uptake ability. Normally the presence of a plasmid or episome-shaped genetic load can be a burden for

metabolism in bacteria. However, we don't see that here.

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The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the author.

Authors' Contribution

Author have contributed in experimental study and writing of the manuscript.

The Declaration of Ethics Committee Approval

Author declare that this study doesn't require ethics committee approval and any special permission.

The Declaration of Research and Publication Ethics

The authors of the paper declare that they comply with the scientific, ethical and quotation rules of SAUJS in all processes of the paper and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

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