Investigations of hemolytic activity and iron utilization sources of *Vibrio alginolyticus* ATCC 17749

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Abstract
As iron plays an indispensable role in the various physiological process with the exception of Borrelia burgdorferi, it is a vital but constrained micronutrient. It is anticipated that the capability of this possible human pathogenic organism to use iron will be crucial for both the establishment of an infection in its hosts and for survival in the habitat. The current study aimed to determine the iron utilization spectrum and hemolytic activity of Vibrio alginolyticus ATCC 17749. The ability of V. alginolyticus ATCC 17749 to utilize iron-containing compounds was tested by agar plate growth promotion assay. It was also confirmed by liquid growth promotion assay where iron was restricted by 400 μM 2,2'-Bipyridyl. After that various iron sources of concentrations ranging from 0.1 μM to 10 μM were added for growth promotion of V. alginolyticus. Ferrous sulfate, ferrioxamine and hemin stimulated the growth of V. alginolyticus as a sole iron source while catechol and ferric dicitrate did not stimulate growth.

Vibrio alginolyticus supernatant was found to be hemolytic and on the blood agar plate, V. alginolyticus ATCC 17749 showed a hemolytic zone. According to our research, hemolysin synthesis is strongly influenced by the iron concentration of the growth medium. Hemin is used as the only source of iron and hemolysis might represent virulence traits of Vibrio alginolyticus. Iron utilization systems might be a potential target for antibiotics or a controlling point for hemolysin expression.

Keywords: Iron transport, Hemolysin, Hemolysis, Heme utilization.

Introduction

Iron is the most important micronutrient metal that catalyzes a broad spectrum of biochemical reactions, essential for all microorganisms except *Borrelia burgdorferi*[24]. It is essential for both the electron transport chain and the synthesis of DNA. With different biological donor ligands including nitrogen, sulphur and oxygen, iron readily forms complexes. The ability of iron to bind readily aids in its insertion into the active site of numerous metabolic proteins[11]. Ferrous iron (Fe^{2+}) and ferric iron (Fe^{3+}) which are both found in oxidising and reducing environments, are the two oxidation states in which iron may be found[12]. Iron used by pathogenic bacteria is necessary for both their survival inside the host and the spread of infection.

The bioavailability of iron in physiologically aerobic conditions is 10^{-18} M which is well below the 10^{-7} M minimum needed for bacterial growth[13]. Several bacteria have developed the ability to transport iron via absorbing heme, producing siderophores, low-molecular-weight iron chelators, or directly absorbing soluble ferrous iron[12,25]. In the development of bacterial pathogenicity, iron is an essential component[3,16,23].

The majority of the iron in the environment is present as ferric hydroxide, which is insoluble in water and biologically unavailable to bacteria. This low amount of free iron is insufficient for microbes to live. High-affinity iron-binding proteins such as transferrin and lactoferrin, bind to iron in the host including humans[10]. The concentration of bioavailable or free iron in human and other animals' body fluids at physiological pH is too low for the survival of bacterial growth[8,10]. In this level of iron deficiency, bacteria produce siderophores and high-affinity iron-binding proteins to scavenge iron from the growing environment. Complex of iron siderophores, or ferrisiderophores, is introduced into the bacteria using a particular iron transport system. The four elements of the iron or siderophore transport system are, successively, the outer membrane receptor, a periplasmic binding protein, the inner membrane permease and the ATPase protein[5,8].

Primarily species *V. alginolyticus* was proposed as the *Vibrio parahaemolyticus* biotype 2 members, but later these bacteria were classified under different species based on the fermentation of sucrose and other properties[19]. Many environmental surveys report the presence of *V. alginolyticus* as the predominant *Vibrio* in the various parts of the world[1,4,14]. It is a halophilic gram-negative bacterium that lives in marine settings including estuaries and coastal regions. It has been described as the most common pathogen isolated from diseased marine fish and shellfish with clinical symptoms of bacterial septicemia. *V. alginolyticus* is also responsible for ulcer disease in marine animals including fish[7].

In 1973, *V. alginolyticus* was first identified as a human pathogen after being isolated from patients with gastroenteritis[26] and then from the site after a 12-day calf cut[18]. Even after that, *V. alginolyticus* has now been isolated from the purulent drainage of leg stump wounds as well as from the purulent discharge of conjunctivae[21]. The rate of infection of *V. alginolyticus* highly increased in the summer

season[2]. Seawater usually contains *V. alginolyticus*, which causes otitis media and otitis externa, two types of ear infections[17].

It has been observed that infection of *V. alginolyticus* infections results from exposure to cuts or bruising to seawater. The infections are easily treated using appropriate antibiotics but in rare cases or immunocompromised persons, it leads to bacteremia.

Material and Methods

Media, culture conditions and bacterial strains: *V. alginolyticus* strain ATCC 17749 was procured from American Type Culture Collection (ATCC). In Luria Bertani (LB) broth or LB agar plates with an additional 1.5 percent of sodium chloride, *V. alginolyticus* was cultivated at 37°C. Strain was maintained at - 80°C in their respective growing medium with 25% glycerol.

Preparation of reagents and iron sources compounds: 0.1 M stock solution of the iron chelator 2,2'-Bipyridyl; HiMedia Laboratories was prepared in 70% ethanol and kept at 4°C. The iron-limited condition was created by adding 400 µM 2,2'-Bipyridyl in Luria Bertani broth and 280 µM in Luria Bertani agar medium. The detection of hemolysin was carried out using blood agar plates with 5 percent cleaned sheep erythrocytes. All iron source compounds were obtained from HiMedia Laboratories except heme, which was obtained through the breaking of sheep erythrocytes with sterile deionized water. Ferrous sulfate, hemin and catechol were prepared freshly daily as mentioned by Dyer et al[6].

Iron utilization Assay: The ability of *V. alginolyticus* ATCC 17749 to utilize iron-containing compounds was tested by an agar plate growth promotion assay adapted and modified from Field et al[9]. Assay plates were filled with 30 ml of LB agar containing 1.5% extra sodium chloride and 280 µM 2,2'-Bipyridyl, seeded with 5×10^7 CFU of *V. alginolyticus* ATCC 17749 being tested.

The plates were kept at 37°C overnight for incubation and the growth in the spot area was monitored. Ferrous sulfate, ferric dicitrate, ferrioxamine, catechol and hemin were tested at a concentration ranging from 10 µM to 1 mM. Deionized water was used as the negative control. The utilization of iron-containing compounds was also confirmed by the liquid broth growth promotion assay.

Iron was restricted by 400 µM 2,2'-Bipyridyl in a liquid growth medium after that various iron sources, concentrations ranging from 0.1 nM to 1 mM were added for growth promotion of *V. alginolyticus*. The culture was incubated at 37°C and 200 rpm shaking for six hours and optical density was measured at 600 nm for cell density. The samples were tested in triplicate and the data represent the mean of three independent experiments combined with the standard error.

Growth curves in the presence of only one iron source: On LB agar plates, bacteria were cultivated. One colony was selected, added to LB broth and incubated at 37°C overnight. One iron source was used in the iron-restricted LB broth that contained one percent of the overnight-grown culture and optical density measurements at 600 nm were taken every hour for 24 hours. Growth induction of *V. alginolyticus* in the presence of ferrous sulfate, ferric dicitrate, ferrioxamine, catechol, hemin and heme was tested at a concentration ranging from 0.1 µM, 1.0 µM, 10 µM and 25 µM. Hemin and ferrioxamine were also tested at concentration 100 µM. Iron-restricted LB broth with 400 µM 2,2'-Bipyridyl used as a negative control, no iron restriction and no addition of additional iron sources were used as a positive control.

Hemolysis assay: Several pathogenic bacteria use hemin as an iron source and use it to produce hemolysin which is controlled by iron concentration[22]. *V. alginolyticus* is hemolytic on LB blood agar plates supplemented with 5% defibrinated sheep blood containing 1.5% extra sodium chloride. The culture of *V. alginolyticus* was grown overnight in 10 ml LB broth or LB broth with a low concentration of 2,2'-Bipyridyl. 200 µl of cleaned sheep erythrocytes were added to 800 µl of culture supernatant, the reaction mixture was centrifuged to remove any unlysed erythrocytes after being incubated at 37°C for three hours, adapted and modified from Mercurio and Manning[15].

The absorbance of the reaction mixture's supernatants was measured at 540 nm to determine how many erythrocytes were lysed. 100% lysis of erythrocytes was achieved by addition of 800 µl sterile deionized water into 200 µl washed sheep erythrocytes and taken as a positive control. As a negative control, LB broth was used.

Results and Discussion

Iron utilization Assay: In a plate experiment, the ability of the *V. alginolyticus* ATCC 17749 strain to acquire iron from several iron-containing compounds was evaluated. *V. alginolyticus* cells were seeded into an iron-limited agar plate where they were unable to proliferate without the addition of iron-containing substances as an iron source. Ferrioxamine, hemin, ferrous sulfate and heme induced growth whereas ferric dicitrate and catechol did not (Figure 1).

Growth induction by various iron sources: First, we determined the minimum concentration of 2,2'-Bipyridyl that restricts the growth of *V. alginolyticus* ATCC 17749. Growth of *V. alginolyticus* ATCC 17749 was observed in LB+1.5% NaCl alone and at various concentrations of 2,2'-Bipyridyl (Figure 2). Growth of *V. alginolyticus* ATCC 17749 was measured every hour by A_{600} nm. 400 µM of 2,2'-Bipyridyl restricted the growth. The ability of *V. alginolyticus* ATCC 17749 to be stimulated by various iron-containing compounds was further confirmed by growing in the liquid media. A single colony of *V. alginolyticus* ATCC 17749 from an LB agar plate was inoculated into LB broth,

then 1% of this culture was inoculated in the growth curve tube containing specific iron-containing compounds.

V. alginolyticus ATCC 17749 showed a significant level of growth induction by ferrous sulfate (Figure 6), ferrioxamine (Figure 5) and hemin (Figure 7) whereas catechol (Figure 3) and ferric dicitrate have shown little or no growth stimulation (Figure 4).

Hemolysin assay/activity: Hemolysis was also employed to test for hemolysin production and hemolytic activity was found. The zone of hemolysis appeared to be around colonies (Figure 8a). Production of hemolysins was significantly increased when cells were grown in different concentrations of 2,2'-Bipyridyl (Figure 8b).

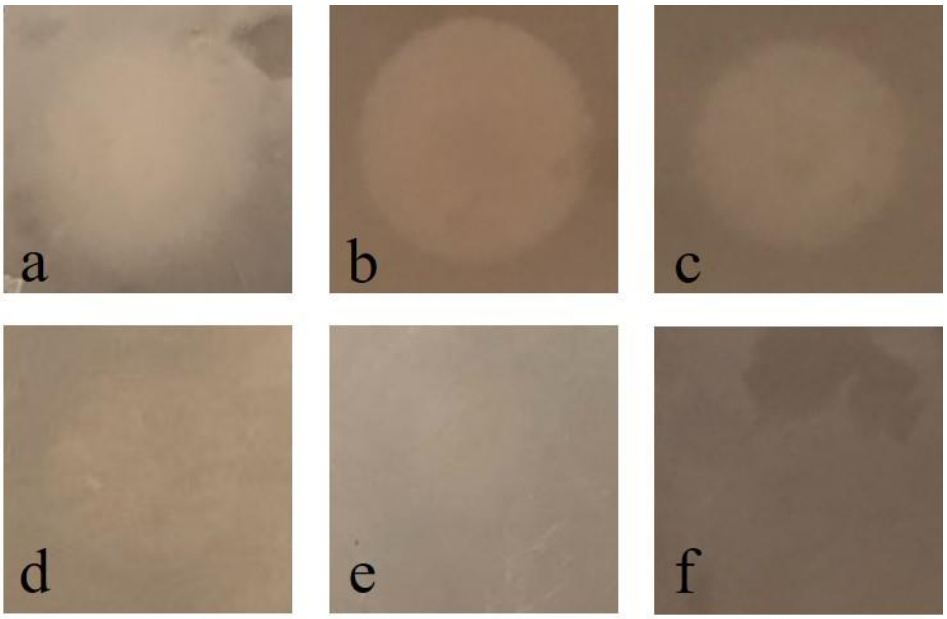

Figure 1: Iron utilization bioassay. *Vibrio alginolyticus* were seeded into LB medium supplemented with 1.5% NaCl and containing 400 µM iron chelator 2,2'-Bipyridyl. Iron sources were spotted on the agar plate a: Ferrioxamine; b: ferrous sulfate; c: hemin; d: catechol; e: ferric dicitrate; f: deionized water. Iron then diffuses onto the agar and gives bacterial growth as positive. The growth around each iron source was photographed after 24 hours of incubation at 37°C

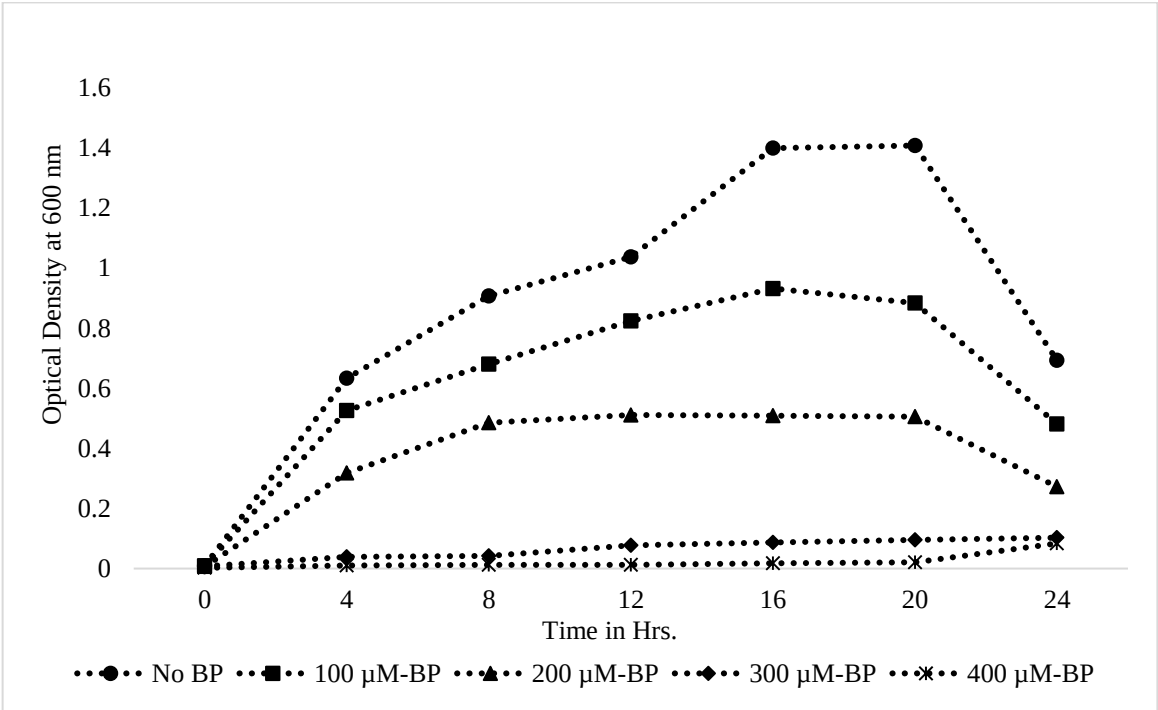

Figure 2: Growth restriction of *Vibrio alginolyticus* by 2,2'-Bipyridyl in LB medium supplemented with 1.5% NaCl

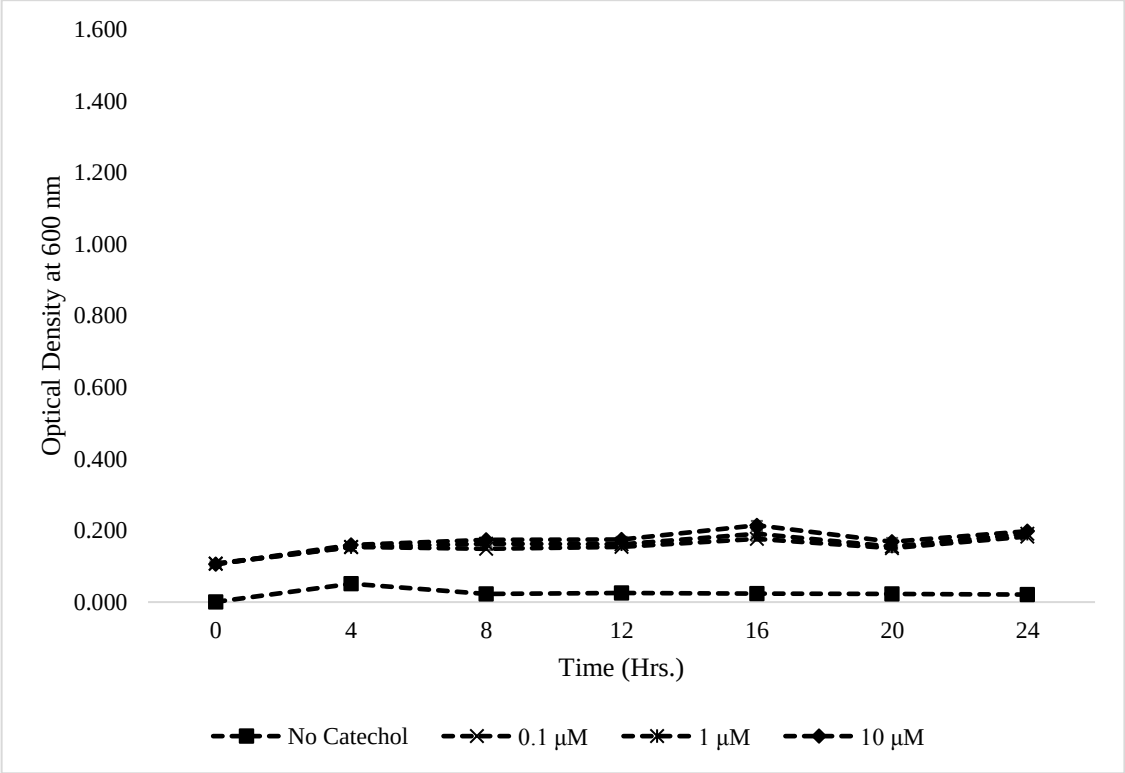

Figure 3: Growth induction of *Vibrio alginolyticus* by catechol in LB medium supplemented with 1.5% NaCl and 400 µM 2,2'-Bipyridyl. Filled square (■) symbol, 400 µM 2,2'-Bipyridyl but not supplemented with catechol; cross (×) symbol, supplemented with 400 µM 2,2'-Bipyridyl and 0.1 µM catechol; star (□) symbol, supplemented with 400 µM 2,2'-Bipyridyl and1 µM catechol, diamond (♦) symbol, supplemented with 400 µM 2,2'-Bipyridyl and10 µM catechol

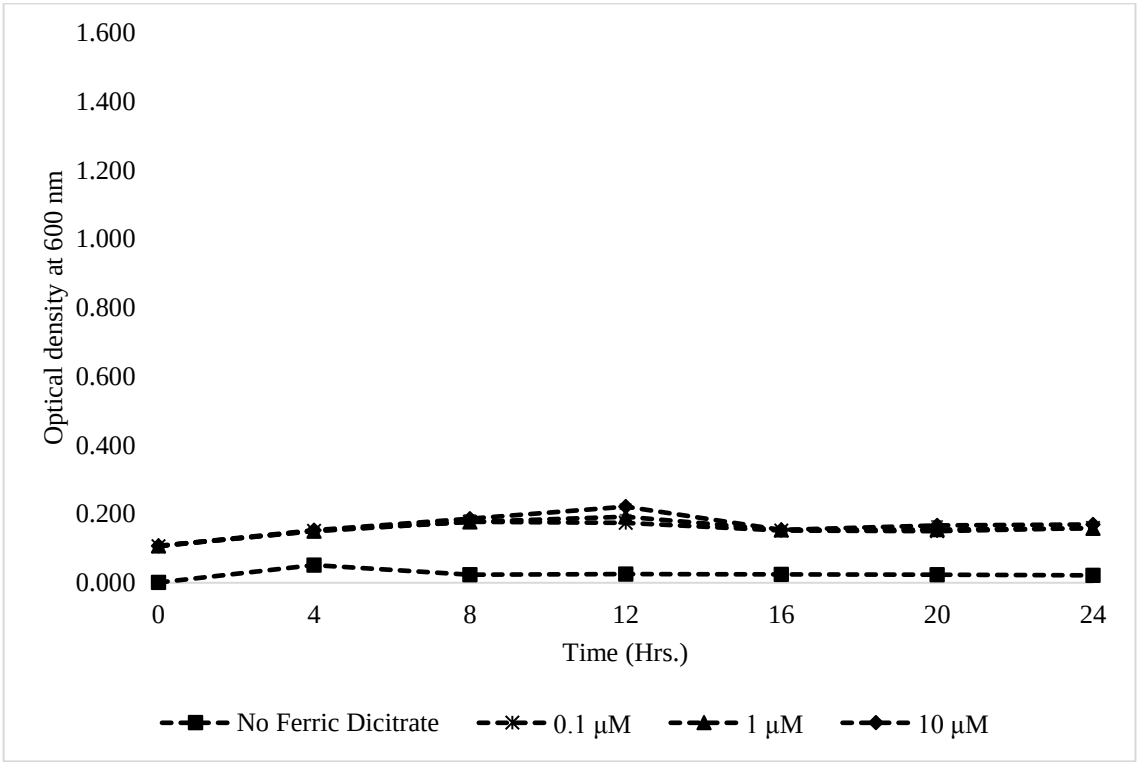

Figure 4: Growth induction of *Vibrio alginolyticus* by Ferric dicitrate in LB medium supplemented with 1.5% NaCl and 400 µM 2,2'-Bipyridyl. Filled square (■) symbol, 400 µM 2,2'-Bipyridyl but not supplemented with ferric dicitrate; star (□) symbol, supplemented with 400 µM 2,2'-Bipyridyl and 0.1 µM ferric dicitrate; triangle (▲) symbol, supplemented with 400 µM 2,2'-Bipyridyl and 1 µM ferric dicitrate; filled circle (●) symbols, supplemented with 400 µM 2,2'-Bipyridyl and 10 µM ferric dicitrate

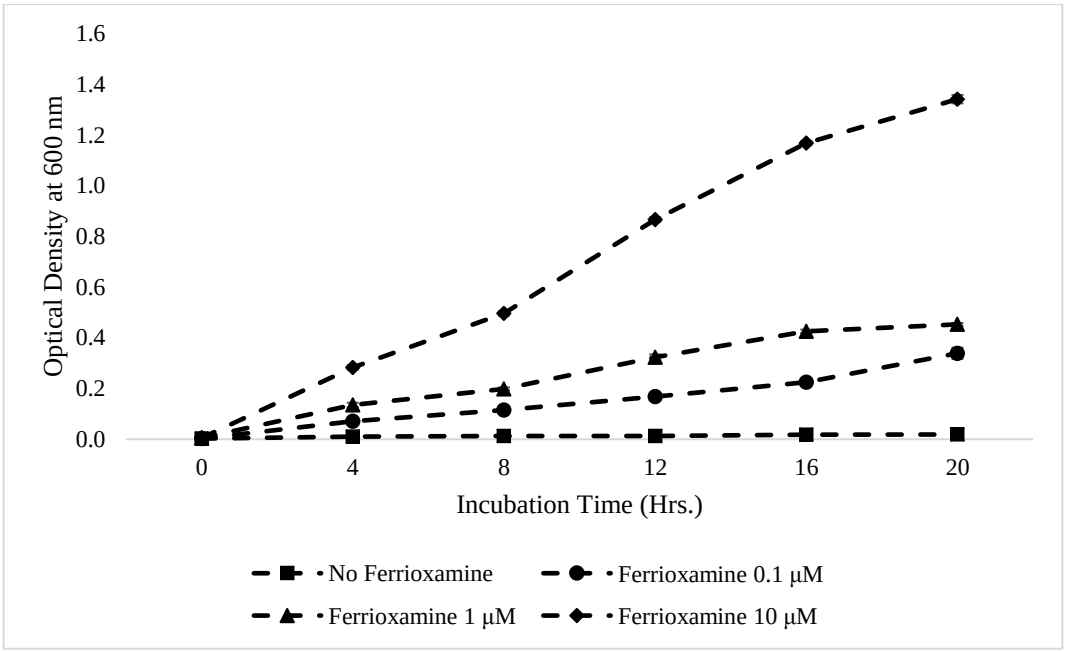

Figure 5: Growth induction of *Vibrio alginolyticus* by ferrioxamine in LB medium supplemented with 1.5% NaCl and 400 µM 2,2'-Bipyridyl. Filled square (■) symbol, 400 µM 2,2'-Bipyridyl but not supplemented with ferrioxamine; filled circle (●) symbol, supplemented with 400 µM 2,2'-Bipyridyl and 0.1 µM ferrioxamine; triangle (▲) symbol, supplemented with 400 µM 2,2'-Bipyridyl and 1 µM ferrioxamine; diamond symbol (♦) symbols, supplemented with 400 µM 2,2'-Bipyridyl and 100 µM ferrioxamine

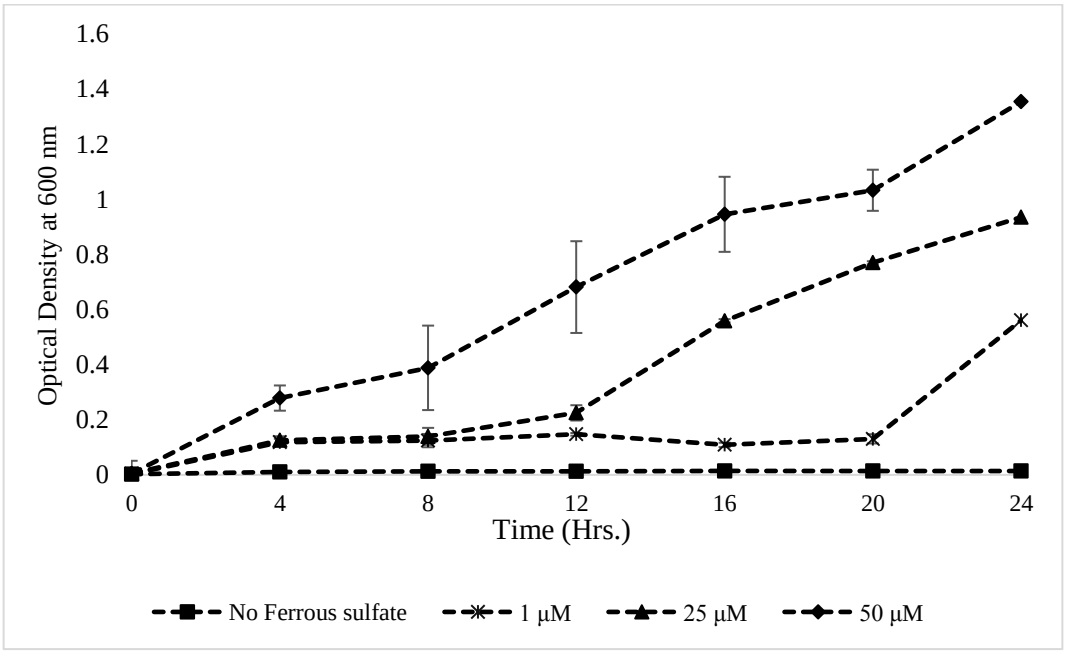

Figure 6: Growth induction of *Vibrio alginolyticus* by ferrous sulfate in LB medium supplemented with 1.5% NaCl and 400 µM 2,2'-Bipyridyl. Filled square (■) symbol, 400 µM 2,2'-Bipyridyl but not supplemented with ferrous sulfate; star (□) symbol, supplemented with 400 µM 2,2'-Bipyridyl and1 µM ferrous sulfate; triangle (▲) symbol, supplemented with 400 µM 2,2'-Bipyridyl and 25 µM ferrous sulfate; diamond (♦) symbol, supplemented with 400 µM 2,2'-Bipyridyl and 50 µM ferrous sulfate

Hemolysin production by *V. alginolyticus* increased two-fold and four-fold when it was grown in iron restricted medium created by adding 150 µM and 200 µM 2,2'-Bipyridyl respectively. These results strongly recommended regulation of hemolysin production as related to iron availability.

The results from the present study showed that *V. alginolyticus* easily obtains iron from hemin, heme, ferrioxamine and ferrous sulfate. In the case of catechol, faint growth stimulation was observed only in the spot assay. Catechol and ferric dicitrate were not utilized by the *V. alginolyticus* in the liquid broth assay. Growth stimulation

by various iron sources utilized by the *V. alginolyticus* shows a different pattern. Ferrioxamine and ferrous sulfate stimulate the growth of *V. alginolyticus* approximately as equal as control. Hemin stimulates the growth of *V. alginolyticus* slightly less than the control in 24 hours of incubation.

The data represented here indicate that *V. alginolyticus* produces a hemolysin. In a similar approach, hemolytic activity was found in bacteria that do make hemolysin used to identify hemolysins in such bacteria[15]. The level of hemolysis by *V. alginolyticus* was detected here as that mentioned for *Shigella flexneri*[15,20]. Production or expression of hemolysin by the *V. alginolyticus* is regulated by the iron because the low iron concentration in the medium induces the high production of hemolysins whereas high iron concentration shows less production of hemolysins (Figure 8b). These results suggested that the hemolysin gene does not constitutively express but it might contribute to pathogenesis in ways other than iron acquisition.

Conclusion

Ferrous sulfate, hemin, ferrioxamine and heme are utilized by the *V. alginolyticus* as a sole source of iron whereas catechol and ferric dicitrate are not utilized. Growth stimulation of *V. alginolyticus* by the ferrous sulfate and ferrioxamine was similar to control experiment.

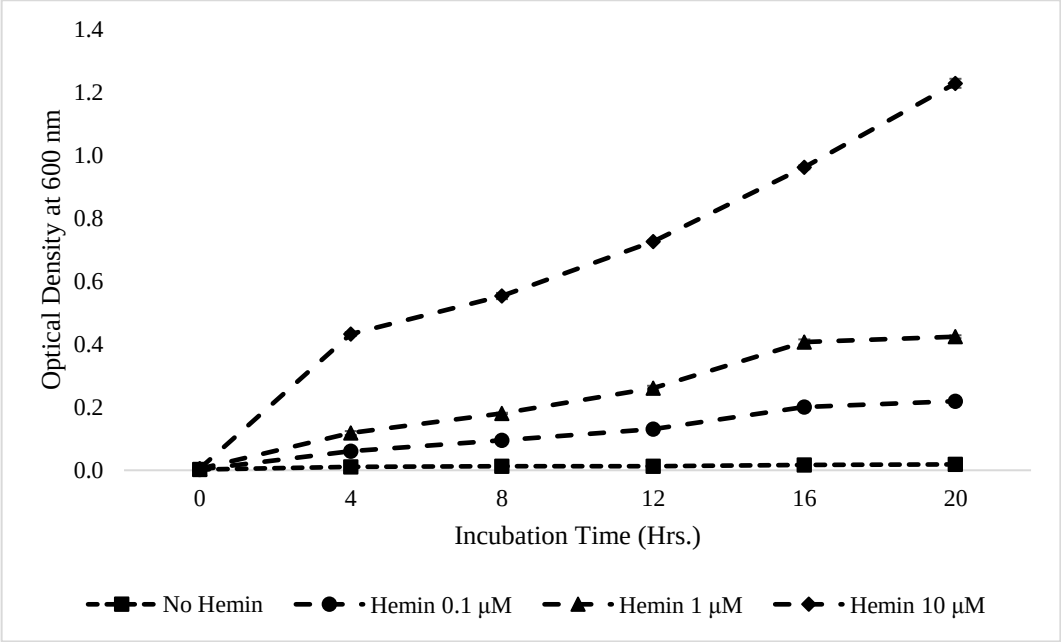

Figure 7: Growth induction of *Vibrio alginolyticus* by hemin in LB medium supplemented with 1.5% NaCl and 400 µM 2,2'-Bipyridyl. Filled square (■) symbol, 400 µM 2,2'-Bipyridyl but not supplemented with hemin; filled circle (●) symbol, supplemented with 400 µM 2,2'-Bipyridyl and 0.1 µM hemin; triangle (▲) symbol, supplemented with 400 µM 2,2'-Bipyridyl and 1 µM hemin; diamond (♦) symbols, supplemented with 400 µM 2,2'-Bipyridyl and 100 µM hemin

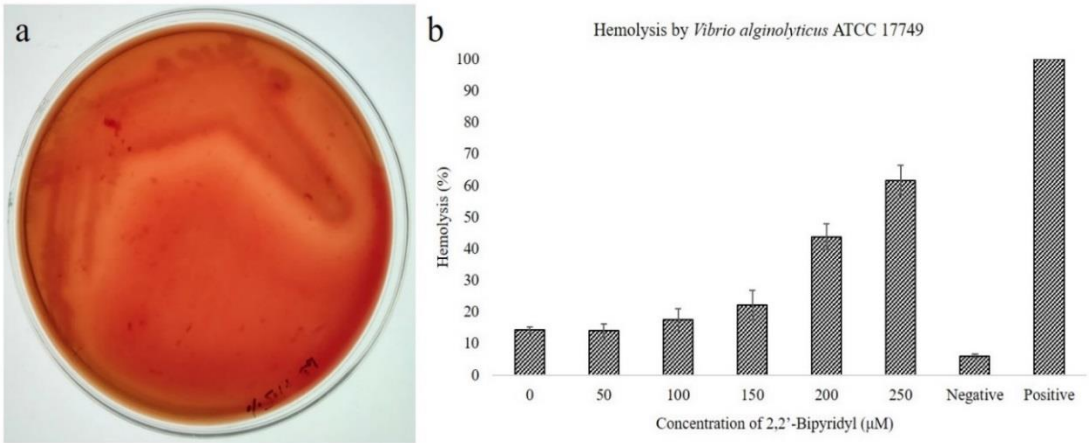

Figure 8: *Vibrio alginolyticus* ATC17749 showing hemolysis on sheep blood agar plate. (a) Production of hemolysin from *V. alginolyticus*, grown in different concentrations of 2,2'-Bipyridyl. (b) Medium without bacterial culture was used as negative control and 100 % hemolysis was achieved by deionized water used as a positive control. The percentage hemolysis was calculated for each condition and shown as means of triplicates (+/- SE)

Growth stimulation by the hemin is slightly less than the control whereas heme stimulates the growth more than the negative control after 24 hours of incubation. The results recommended the hypothesis that the concentration of iron in the growth medium regulates the expression of hemolysin.

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