EFFECT OF THE IRON SOURCES ON THE SIDEROPHORE PRODUCED FROM *BACILLUS POLYMYXA*

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Abstract

To investigate the siderophore production from *Bacillus polymyxa*, an iron free combined carbon source medium has been used for cultivation. In the condition without addition of iron exhibit the long lag phase whereas $Na_2FeEDTA$, 0.1-2.0 μM $FeCl_3$. $6H_2O$ and DFOB can reduce the lag phase period. Siderophore produced from *B. polymyxa* was detected in the condition present only 10-20 ng/ml of DFOB during the stationary phase of growth.

Introduction

Iron, one of the essential elements for microbial growth and universally required by most living cells, exists in nature predominantly in the insoluble ferric(III) oxidation state which is not readily available for assimilation (7). The roles of iron in microbial metabolism are played mainly in the bioenergetic system such as being the important part of cytochromes in the respiratory chains. In addition, iron also involved in many enzymes regard to hydrogen peroxide and oxygen metabolism, tricarboxylic acid cycle, DNA biosynthesis and nitrogen fixation (8).

A great variety of microorganisms has been demonstrated to produce the iron (III) specific transport compounds to solubilize and sequester ferric iron, as defined in terms of siderophores. Siderophores are small molecules of low molecular weight (0.5-1.5 Kda) which act as ferric and ferrous iron-chelating agents. The major roles of siderophores are extracellular solubilization of iron from minerals or organic substrates under conditions of iron deprivation and specific transport of Fe^{3+} into cells (7).

Many siderophore producing microorganisms have been investigated and analysed from both bacterial and fungal genera. In this study, siderophore produced from the bacterium, *Bacillus polymyxa* will be focussed. Few diversifications of siderophores produced among this genus have been demonstrated. For example, *B. megaterium* has been shown to produce schizokinen (1) while *B. subtilis* produces 2,3 dihydroxybenzoylglycine (DHBG) (3). Moreover, *B. polymyxa* was reported to be a free living nitrogen fixing bacterium (2). This also provides preliminary study to further understanding the high complement of iron for nitrogenase enzyme involved in nitrogen fixation. In this study, isolation of siderophore from *B. polymyxa* will be elucidated.

Materials and Methods

1. Bacterial Strain and Growth Condition

B. polymyxa strain H30 (identification confirmed by the Deutche Sammlung fur Mikroorganismen, D-3300 Braunschweig, Germany) has been used throughout this study. Stock cultures were maintained on slant of nutrient agar (NA). The modified and deferrated combined carbon medium (solution I: manniol 5.0 g, sucrose 5.0 g, and sodium lactate 0.5 ml dissolved in deionized water 500 ml, deferrated with Chelex 100, solution II: K_2HPO_4 0.8g, KH_2PO_4 0.2 g, NaCl 0.1 g and NH_4Cl 0.2 g dissolved in deionized water 400 ml, solution III: $MgSO_4$. $7H_2O$ 0.2 g and $CaCl_2$ 0.06 g dissolved in deionized water 50 ml) were separately autoclaved. Solution IV (biotin 5.0 μg and PABA 10.0 μg dissolved in deionized water 50 ml) was sterilized by using

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membrane filtration and then all solutions were mixed together for further cultivation. (9).

To study the effect of iron and other siderophores on siderophore production from *B. polymyxa*, varied amounts of the $FeCl_3$. $6H_2O$ and (Des-) ferrioxamine B (DFOB) were added to the modified deferrated combined carbon medium. For cultivation, bacteria were cultured on a gyrotary shaker at 200 rpm at 30 Growth measurement was determined by optical density at 600 nm.

2. Siderophore Assay

The CAS (chrome azurol S) universal chemical assay (10) for the detection of siderophores was prepared as follows; a 6 ml volume of 10 mM HDTMA solution was placed in a 100 ml volumetric flask and diluted with water. A mixture of 1.5 ml iron (III) solution (1 mM $FeCl_3$. $6H_2O$ in 10 mM HC1) and 7.5 ml 2 mM aqueous CAS solution was slowly added under sterring condition. A 4.307 g quantity of anhydrous piperazine was dissolved in water and 6.25 ml of 12 M HCl was carefully added. This buffer solution (pH 5.6) was rinsed into the volumetric flask which was then filled with water to afford 100 ml of CAS assay solution. The CAS shuttle solution was obtained by adding 5-sulfosalicylic acid to the above solution at a concentration of 4 mM. The solutions were stored in the dark. For the assays, 0.5 ml of CAS assay solution and 0.5 ml of CAS shuttle solution was mixed to 1.0 ml. Varied amounts of DFOB was added in modified deferrated combined carbon medium solution for standard curve determination. To examine the siderophore produced from *B. polymyxa*, 1.0 ml of the bacterial culture filtrate obtained after centrifugation at 12,000 rpm for 5 min was mixed with the assay solution as standard curve preparation, and the A_{630} was read after 2 h of incubation at room temperature.

3. Isolation of Siderophores

The culture filtrate obtained from 1 l nutrient medium was supplemented with $FeCl_3$. $6H_2O$ (330 mgl^{-1}) and the solution was stirred until a brown colour had developed. The siderophores were adsorbed onto XAD-2 (Sigma, Munich, Germany), washed with three volumes of deionized water and desorbed with one volume of methanol. Gel filtration on a sephadex LH20 column in methanol resulted in a further purification.

Results and Discussion

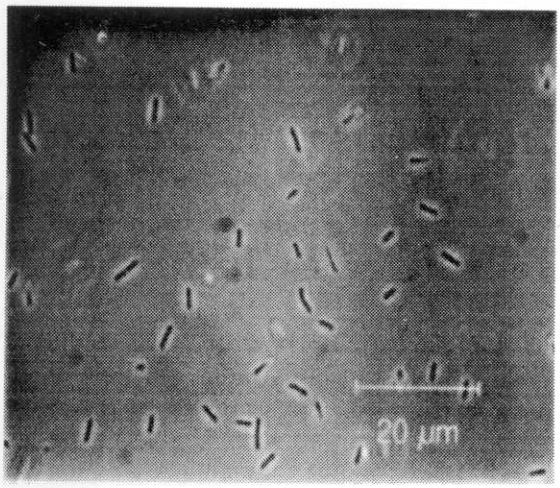
Growth of B. polymyxa in various kind of iron source

To investigate the effect of iron sources on the growth of *B. polymyxa* the cultivation in an iron free medium has been achieved. Stock culture of *B. polymyxa* in nutrient agar was transferred to fresh iron free combined carbon liquid medium. Prior to further inoculation the culture was washed three times with fresh liquid combined carbon medium. The culture was cultivated in an incubator shaker at 30 and 200 rpm for 2 days. 100 µl of culture was inoculated in 20 ml combined carbon medium throughout this study.

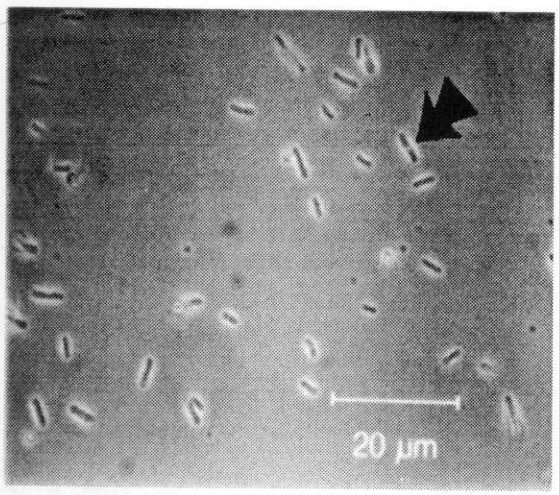
In the iron deprived condition the long lag phase was performed all 12 days of cultivation with an optical density value of 0.3 at 600 nm in average without spore formation (Fig.1). The addition of Na_2FeEDTA as chelated iron source showed every phase of growth (Fig.2). Therefore, it might be concluded that the chelated iron source plays a very important role in reducing the lag phase of *B. polymyxa*. This case is similar to the growth of *Anabaena flos-aquae* which exhibited a long lag phase and a reduced growth rate in an iron deprived condition (4,6). On the other hand, varied amounts of $FeCl_3$. $6H_2O$ were also used as the Fe(III) source. The result in figure 3 showed that a small amount of $FeCl_3$. $6H_2O$ such as 0.1 to 2.0 µM/ml can promote some growth of *B. polymyxa* whereas at concentration 3.0 µM did not show this effect. However, when the growth rates and growth yields between Na_2FeEDTA and $FeCl_3$. $6H_2O$ as different form of iron are compared, the results indicated that iron in chelated form able to enhance higher growth rate and cell number of *B. polymyxa* than non-chelated form. The same phenomena was also found when varied amounts of DFOB which contain some irons in chelated form were applied (Fig.4).

Siderophore production from B. polymyxa

The siderophore production by *B. polymyxa* was found only in the condition treated with 10 and 20 ng/ml DFOB. For addition of 10 ng of DFOB siderophore was detected at day 9 of cultivation and gave the maximum yield approximately 3.3 µg/ml in day 10 of cultivation and decreased about 1.0 µg/ml the day after. This probably due to metabolic re-utilization of the siderophore. Whereas at the concentration of 20 ng/ml DFOB, the produced siderophore was detected at 2 days later than 10 ng/ml DFOB amendment. In this condition performed lower yield 2.25 µ/ml and unable to detect in the day after (Fig. 5). On the other hand when observed at the lag phase period in this condition, we found



(A)



(B)

Fig. 1 **(A) Cells of *B. polymyxa* cultured in iron deprived condition for 12 days.**
(B) Spore of *B. polymyxa* (indicated by arrow) cultured on nutrient agar for 12 days.

that this was shorter than other conditions carried out in this study. This case showed the same as some reports which demonstrated that the growth and production of siderophores of some bacteria may be stimulated by other siderochromes or by other chelating agents, even when these organisms are known to produce such substances or, at least, have not been demonstrated to be iron-chelate auxotrophs. For several such non-auxotroph that have been studied, a variety of chelating agents other than siderophores may be effective, although the latter generally are far more potent. For example *B.*

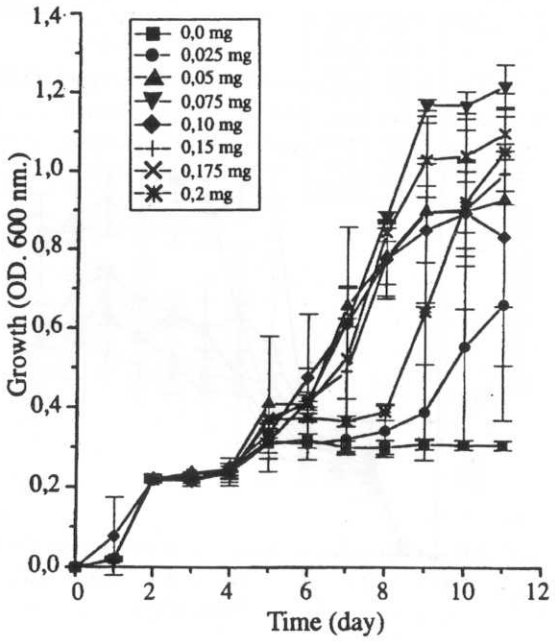


Fig. 2 **Growth curve of *B. polymyxa* in combined carbon medium with varied amount of $Na_2FeEDTA$ (0-2.0 mg/ml) incubated at 30 and 200 rpm. Values represent means and standard deviation of three replications.**

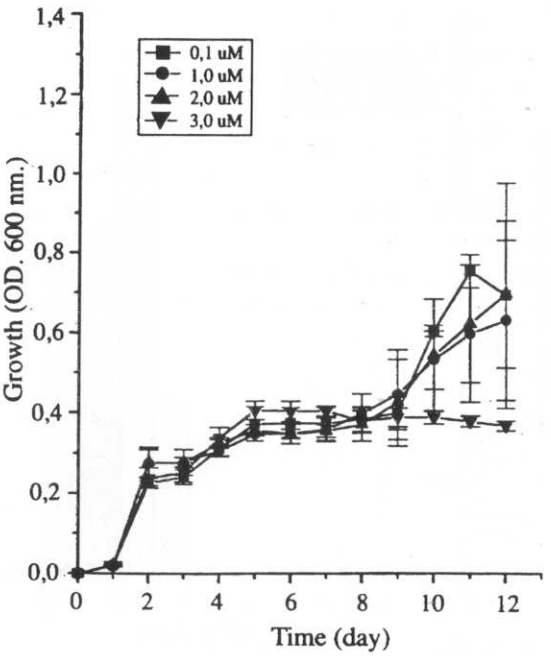


Fig. 3 **Growth curve of *B. polymyxa* in combined carbon medium with varied amount of $FeCl_3$. 6 H_2O (0.1-3.0 µM) incubated at 30 and 200 rpm. Values represent means and standard deviation of three replications.**

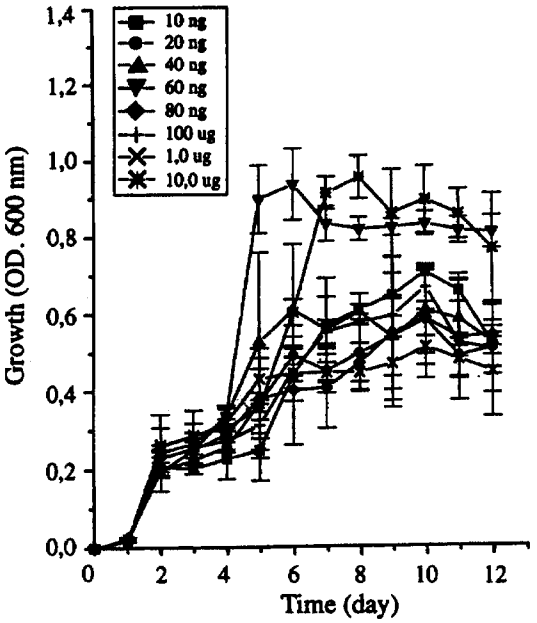


Fig. 4 Growth curve of *B. polymyxa* in combined carbon medium with varied amount of DFOB(10 ng-10 μg) incubated at 30 and 200 rpm. Values represent means and standard deviation of three replications.

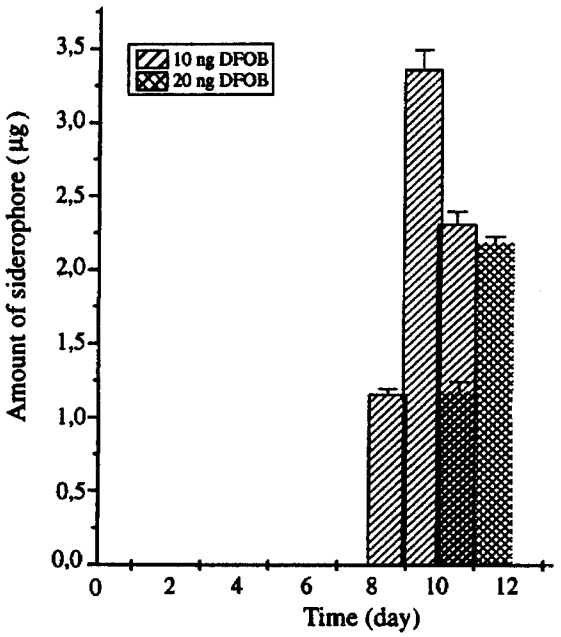


Fig. 5 Siderophore production by *B. polymyxa* in combined carbon medium with varied amount of 10 ng and 20 ng of DFOB incubated at 30 and 200 rpm. Values represent means and standard deviation of three replications.

megaterium ATCC19213 can be stimulated by ferrioxamine B, Ferrichrome etc. and the lag phase was eliminated (5).

The siderophore in these conditions was produced during the stationary phase of growth which shown the different from other siderophores. Hence iron is a very important requirement for growth and many enzymes in metabolic pathways thus many siderophores were found to be produced along with the growth of microorganisms (8). The siderophores produced from *B. polymyxa* in this study were extracted and partially purified along with the method number 3. Further investigation will be focussed on chemical structure analysis for further understanding of the relationship between the complement of iron and nitrogen fixation reaction.

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