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Production and Optimization of Siderophore Production by*Bacillus tequilensis*

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Abstract

Bacillus tequilensis DB1 produced maximum siderophore production i.e. 65% siderophore unit (SU) in succinate medium.Optimization of cultural conditions were carried out to enhance the siderophore production. Maximum siderophore production 65.66% SU, 66.72% SU, 62.13% SU, 71% SU was observed at pH 6, temperature 30°C, incubation time (30 hrs), and inoculum size (2%) respectively. Succinic acid was found to stimulate the growth as well as siderophore production. Optimum siderophore production 72.77% SU was observed by adding 0.5% of succinic acid to the medium. Increase in iron concentration upto 100µM favored the growth of the isolate but drastically affects the siderophore production. Threshold level of iron concentration ($FeCl_3$) that affects the siderophore production was 20µM above which there will be decrease in yield. The present study reveals the classical method of optimization for increasing the yield of siderophore production by *Bacillus tequilensis* DB1.

Keywords:Siderophore; Optimization;*Bacillus tequilensis*; iron

Introduction

Siderophores are iron-chelating ligands synthesized under low iron stress for the solubilization and transport of iron (Fe III) inside the microbial cell (Sayyed et al. 2011).Siderophores are produced by various microorganisms and are classified into four main classes (carboxylate, hydroxamates, catecholates, and mixed type) on the basis of their structural features, functional groups, and types of ligands (Ali and Vidhale 2013; Kumar et al. 2017). Siderophore exhibit requisite; hydrophilic, lipophilic and hydro-lipo-phile properties for chelating the extracellular iron respectively from aqueous environment, through the lipoprotenaceous membrane receptors of the cell and from fatty environment (Sayyed et al. 2008).

Siderophores produced by PGPR help in fulfilment of the iron requirement of plants by causing its solubilisation and chelation from organic or inorganic complexes present in soil (Arora et al. 2013;

Singh et al. 2017).Siderophore producing rhizobacteria play vital role in stimulating plant growth and in controlling several plant diseases (Sahaet al.2016).They function as a biocontrol agent by depriving the pathogen from iron nutrition, this resulting in increased yield of crop.Siderophores have received much attention in recent years because of their potential roles and applications in various areas of environmental research. Their significance in these applications is because siderophores have the ability to bind a variety of metals in addition to iron, and they have a wide range of chemical structures and specific properties. Siderophore also used to reduce the level of metal contamination in environment specifically from soil and water (Mishra et al. 2017).

Present study focuses on the influence of cultural conditions like pH, temperature, incubation time, inoculum size, iron concentration and succinic acid concentration on growth and siderophore production by *Bacillus tequilensis* DB1.

Materials and Methods

Microorganisms

Rhizobacteria showing maximum siderophore production was isolated from the rhizosphere of cluster bean and was identified as *Bacillus tequilensis*DB1 on the basis of biochemical characteristics and 16sRNA sequencing and deposited to gene bank as *Bacillus tequilensis* DB1 under National Centre for biotechnology information (NCBI) accession number KY039155.

Detection and Quantification of Siderophore Production

Quantitative estimation of siderophore production by *Bacillus tequilensis*DB1 was carried out by CAS shuttle assay (Schwyn and Neilands, 1987). In this assay, the isolate was grown on succinate medium and incubated for 24-30 hrs at 28^0C at 120 rpm. After incubation the broth was centrifuged at 5000g x cm at 4^0C for 15 min. The assay was carried out by mixing 1ml cell free supernatant with 1ml CAS reagent. The intensity of the color produced was determined by measuring the absorbance at 630 nm using the spectrophotometer. 1 ml of uninoculated broth and 1 ml of CAS reagent was used as a reference. The percentage of siderophore units produced was estimated as the measure of proportion of CAS color shifted using the formula (Payne, 1994).

% SU= Ar-As/Ar X 100

Where, Ar = Absorbance of reference at 630 nm (CAS assay solution + uninoculated medium) and

As = Absorbance of the sample at 630 nm (CAS assay solution + cell supernatant)

Optimization of culture conditions

Cultural conditions of the medium like PH, time, temperature, Inoculum size, Succinic acid concentration &iron concentration optimized so study their effect on growth and siderophore production by *Bacillus tequilensis* DB1.

The effect of pH at 5.0, 6.0, 7.0 and 8.0 on siderophore production was studied on Succinate medium by adjusting the pH before inoculating the strain with 1 N HCl or 1N NaOH and keeping all other conditions constant.

Bacillus tequilensis DB1was separately grown in SM (Succinate Medium) with constant shaking for 48hrs. Samples were withdrawn after every 6 hrs interval and were estimated for siderophore production.

The effect of temperature on siderophore production was studied on SM by inoculating the isolates and incubating at different temperature $20^{o}C$, $25^{o}C$, $30^{o}C$, $35^{o}C$, $40^{o}C$.

Siderophore production was studied by adding inoculum size in the range 1 to 5% in succinate medium followed by incubation. Samples were withdrawn and siderophore units were measured by CAS shuttle assay.

Siderophore production was studied by varying concentration of succinic acid by 0.2, 0.3, 0.4, 0.5, 0.6 gm/100ml in the medium. Samples were withdrawn and estimated for siderophore production.

Bacillus tequilensis DB1 was grown is succinate medium with externally supplemented iron ($FeCl_3.6H_2O$) in the range of 1-100µM concentration. Samples were withdrawn and estimated for siderophore production.

Results and Discussion

Siderophore detection and quantification

Siderophore production by the isolate *Bacillus tequilensis* DB1 was studied in succinate medium containing chrome azurol dye. Yellow colour around the colonies indicates siderophore production by the isolate.

The quantitative estimation of siderophore production was determined by CAS shuttle assay. Colour changes from blue to pink indicatessiderophore production. Isolate *Bacillus tequilensis* DB1 showed 65% of siderophore production.

Optimization of culturalconditions

Influence of pH on siderophore production

Siderophore production 65.66% SU was found to be maximum at pH 6.0 (Fig.1). With increasing pH towards alkalinity siderophore production found to be decreasing.This results showed that slightly acidic condition favors the siderophore production than neutral and alkaline conditions.pH plays a vital role in solubilizing iron and make it available to organisms and plants. Iron is present in insoluble at slightly acidic and neutral pH and absence of iron enhances the siderophore production. Increasing pH towards alkalinity helps in solubilizing the iron which increases iron content in the medium and thus siderophore production decreases (Olsen et al. 1981).

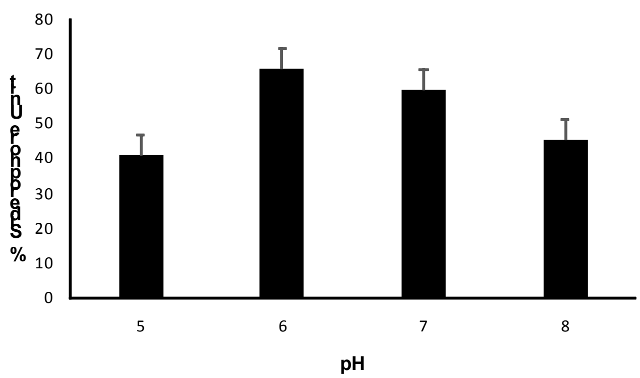

Figure 1: Influence of pH on siderophore production by *Bacillus tequilensis* DB1

Influence of time on siderophore production

During the intial phase of the growth siderophore production was not observed by the isolate. *Bacillus tequilensis* DB1 showed siderophore production at 18 hrs which increased upto 24-30hrs after that production decreases (Fig.2). Optimum siderophore unit (62.13% SU) was observed at 30hrs of incubation.

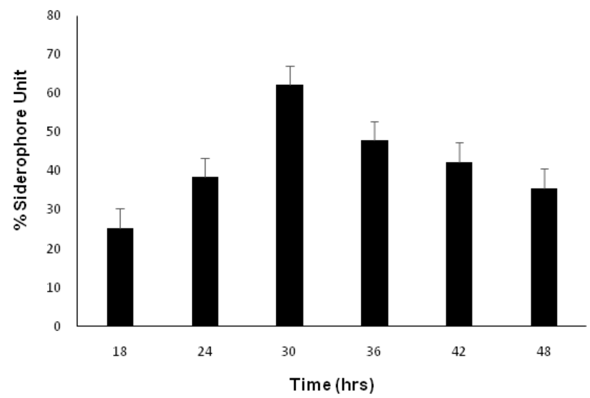

Figure 2: Influence of time on siderophore production by *Bacillus tequilensis* DB1

Influence of Temperature on Siderophore production

To determine the effect of different temperatures on siderophore production, the production medium was incubated at different temperature in the range of 20^{0}C, 25^{0}C, 30^{0}C, 35^{0}C, 40^{0}C, After incubation for 30 hrs with pH 6 and constant shaking at 120 rpm on rotary shaker, it was found that maximum production of siderophore was observed at 30^{0}C (66.72% SU). This result indicates that optimum incubation temperature required to produce maximum siderophore is 30^{0}C increase in temperature showed decrease in yield (Fig.3).

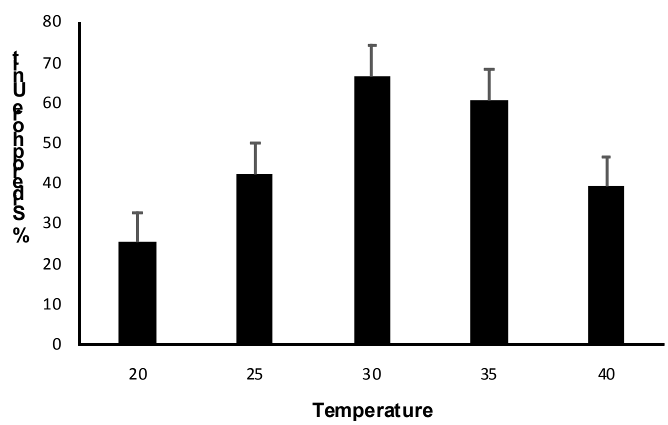

Figure 3: Influence of temperature on siderophore production by *Bacillus tequilensis* DB1

Influence of inoculum size

Amount of cell mass present in the inoculum determies the growth and the production of secondary metabolites i.e. siderophore. For optimum siderophore production (71% SU), 2% of inoculum was found to be satisfactory. Increasing inoculum size beyond this leads to decrease in siderophore production (Fig.4)

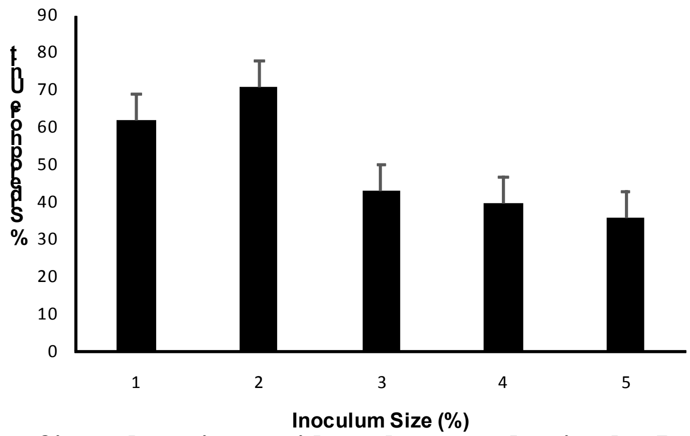

Figure 4: Influence of inoculum size on siderophore production by *Bacillus tequilensis* DB1

Siderophore production at different succinic acid concentration

Succinic acid is an important component that supports the growth of the isolate and also the siderophore production. Optimum siderophore prodcution (72.77% SU) was observed at 0.5% of succinic acid in the growth medium. Incorporation of increased concentration above 0.5% of succinic acid in the medium decreases the siderophore prodcution (Fig.5).

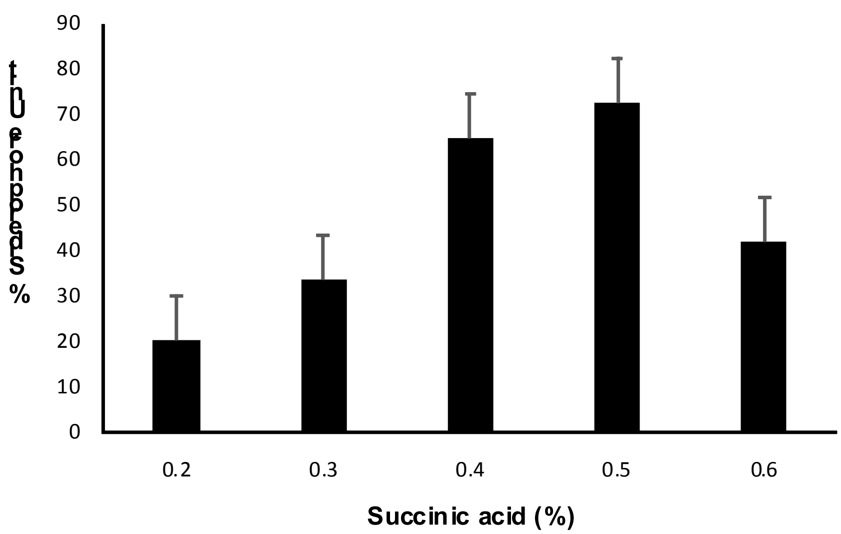

Figure 5: Influence of succinic acid concentration on siderophore production by *Bacillus tequilensis* DB1

Influence of iron on siderophore production

As shown in Fig. 6 as the iron concentration increases in the succinate medium the growth of *Bacillus tequilensis* DB1 increases. However siderophore production decreases with increase in iron concentration. *Bacillus tequilensis* DB1 showed maximum siderophore production 53% SU at iron concentration of 1µM. Increase in growth with increase in iron concentration reflects the requirement of iron for cellular processes in organisms (Sayyed et al. 2005). No siderophore production was observed at and above 20µM of iron concentration. Dave and Dube have reported the repression of siderophore production with increasing concentration of iron and reported 27µM of iron as threshold level at which flourescent *Pseudomonas* stopped siderophore production.

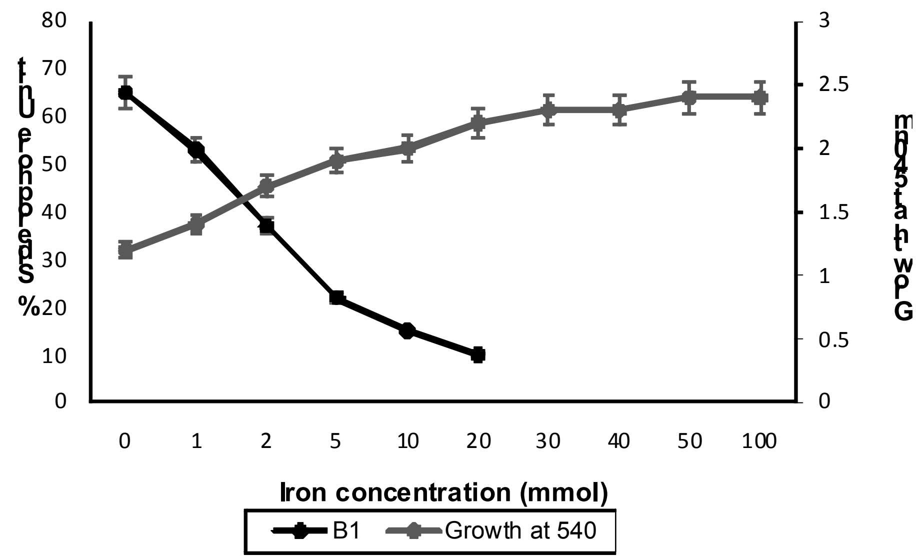

Figure 6: Influence of iron concentration on siderophore production by *Bacillus tequilensis* DB1

Conclusion

By using classical approach method we can get the important parameter affecting the siderophore production. Siderophore production by the isolate *Bacillus tequilensis* DB1 was found to be maximum after 18hours of incubation with the medium at pH 6, temperature of 30°C, after incubation time of 30 hrs by adding 2% inoculum size and succinic acid concentration of 0.5 gm%. Thus optimization of cultural conditions results into increased production.

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