Growth and siderophores production in *Alcaligenes faecalis* is regulated by metal ions

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Abstract During stationary phase of growth under low stress of iron in succinic acid medium, *Alcaligenes faecalis* BCCM ID 2374 produced microbial iron chelators. Increase in iron concentration supported bacterial growth but suppressed siderophores production, 1 μM and 2 μM of iron was optimum for maximum siderophore yield, i.e. 354 and 360 $\mu\text{g/ml}$ in untreated and deferrated medium, respectively. Threshold level of iron, which suppressed siderophores production in *A. faecalis* BCCM ID 2374, was 20 μM . Ten micromoles and above concentration of CuCl_2 and CoCl_2 , and 20 μM of MgCl_2 , MgSO_4 , ZnCl_2 and ZnSO_4 severely affected siderophores production.

Keywords *Alcaligenes faecalis* · Growth · Siderophores · Iron · Heavy metals

Introduction

Rhizosphere is a dynamic environment having diverse groups of rhizobacteria capable of promoting plant growth this particular group of rhizobacteria have been referred as yield increasing bacteria (YIB) or plant growth promoting rhizobacteria (PGPR) [1]. Mechanisms involved in plant growth promotion by these PGPRs include increased uptake of nitrogen [2], iron through siderophores [1] and phosphorus [2] synthesis of phytohormones [3–4] and control of plant diseases [5–9]. Siderophores producing PGPR function as a biocontrol agent, by depriving the pathogen from iron nutrition thus resulting in increased yields of crop [1, 10]. Siderophores are iron-scavenging ligands synthesized under low iron stress for the solubilization and transport of iron inside the microbial cell [1]. Growth and siderophore production by PGPR is attributed to metal ions and several other components of root exudates [10–13] and metal ions accumulating due to the liberal use of chemical pesticides and fertilizers [8, 10] since siderophore synthesis occurs under low stress of iron, effect of metal ions on either the growth or siderophore production by PGPR would greatly influence the plant growth promoting and biocontrol potential of that PGPR [11, 14–16]. Present work focuses on the effect of various metal ions on growth and siderophore production in a rhizobacterium *Alcaligenes faecalis* BCCM ID 2374.

Materials and methods

Alcaligenes faecalis BCCM ID 2374 was obtained from School of Life Sciences, North Maharashtra University, Jalgaon. Screening for siderophores production was carried out by growing *A. faecalis* BCCM ID 2374 on

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CAS agar plate [17] and in iron free succinic acid medium [SM] [18] consisting of g/l, K_2HPO_4 ; 6.0, KH_2PO_4 ; 3.0, $MgSO_4 \cdot 7H_2O$; 0.2, NH_4SO_4 ; 1.0; succinic acid 4.0, at $28 \pm 2^\circ C$ with constant shaking at 120 rpm for 24–48 h.

A. faecalis BCCM ID 2374 was grown (6×10^7 cells/ml) in 100 ml of SM [18] at $28 \pm 2^\circ C$ at 120 rpm for 24–48 h. Samples withdrawn after 6 h interval were subjected for growth measurement at 620 nm by using double beam ultraviolet visible spectrophotometer. Cell free supernatant obtained by centrifugation ($7,000 g \times 15$ min) was assayed for the detection. Siderophores produced in culture broth were detected by CAS assay as per Schwyn and Neilands [19]. Quantitative estimation of siderophores was done by CAS-shuttle assay [20]. In which 0.5 ml of culture supernatant was mixed with 0.5 ml of CAS reagent, absorbance was measured at 630 nm against a reference consisting of 0.5 ml of uninoculated broth and 0.5 ml of CAS reagent. Siderophore content in the aliquot were calculated by using following formula:

$$\% \text{ siderophore units} = \frac{Ar - As}{Ar} \times 100$$

where

Ar = Absorbance of reference at 630 nm (CAS reagent)

As = Absorbance of sample at 630 nm

Threshold level of iron which suppressed siderophores biosynthesis in *A. faecalis* BCCM ID 2374 was determined by growing (6×10^7 cells/ml) it in untreated and deferrated SM at $28 \pm 2^\circ C$ at 120 rpm for 24 h. Both the media were externally supplemented with 1–100 μM of iron ($FeCl_3$). Deferration was carried out with 0.5% chloroform based 8-hydroxyquinoline [21].

For studying the influence of various heavy metal ions on growth and siderophores production, *A. faecalis* BCCM ID 2374 (6×10^7 cells/ml) was grown in SM, externally supplemented with 1–100 μM of various heavy metals like $CuCl_2$, $CoCl_2$, $MgCl_2$, $MgCl_2$, $MnSO_4$, MgO , $ZnCl_2$, $ZnSO_4$, $BaCl_2$, $CdNO_3$, $(CH_3COO)_2Pb$ and $Al_2(SO_4)_3$, at $28 \pm 2^\circ C$ at 120 rpm. Growth and siderophore were estimated as described earlier.

Results and discussion

Change in color of CAS agar and CAS reagent from blue to orange red indicated the ability of *A. faecalis* BCCM ID 2374 to produce and excrete siderophores [17].

Growth course of *A. faecalis* BCCM ID 2374 showed a log phase of about 5 h. Siderophore excretion began from 6 h, continued up to 24 h and started declining after 24 h. Eighteen to 24 h incubation proved to be optimum for

maximum siderophores excretion and 30–36 h resulted in optimum growth. pH profile at different time intervals revealed a gradual increase in pH from acidic range towards alkalinity, optimum siderophore 92.25% units, and siderophore yield (dry weight) 347 $\mu g/ml$ was recorded at neutral pH during 24 h of incubation. Sayyed et al. [11] have also reported optimum siderophores yield during 24 h growth of *P. fluorescens* NCIM 5096 in SM.

Increase in growth with increasing concentration of iron showed the iron requirement of organism for cellular processes (Fig. 1). However, higher concentrations of iron repressed siderophores production. One μM and 2 μM of iron ($FeCl_3$) resulted in optimal siderophore yield, i.e. 354 and 360 $\mu g/ml$ in untreated and deferrated medium, respectively. Thirty μM iron concentration appeared to be threshold level, which repressed siderophore synthesis in both untreated and deferrated media suggesting that the organism has achieved iron sufficiency. Decreased siderophore production with increasing concentrations of iron has been reported by Budde and Leong [22].

Fig. 1 Influence of iron on growth and siderophore production in *A. faecalis* BCCM ID 2374.

Repression of siderophore synthesis with high concentration of iron (above 27 μM) has also been reported in fluorescent *Pseudomonas* [23]. Repression of siderophore synthesis at high concentration of iron is due to the fact that the bacterial gene expression in the rhizosphere is regulated both by endogenous and exogenous signals. Exogenous regulatory signal(s) activate LemA, a membrane-bound sensor kinase, which in turn regulates the production of bacterial autoinducers that control the biosynthesis of antifungal metabolites like siderophore/antibiotics [24] and the transcription of iron-regulated gene is under the negative control of *fur* protein [repressor] with Fe^{2+} as an essential co-repressor. Increase in the concentration of iron in cell cytoplasm cause iron toxicity to the cell [22, 25].

Table 1 Influence of metal ions on growth and siderophore production in *A. feacalis* BCCM ID 2374

Metal ions (μM)	1		10		20		50		100	
Metal ions	Abs	Sd ($\mu\text{g/ml}$)	Abs	Sd ($\mu\text{g/ml}$)	Abs	Sd ($\mu\text{g/ml}$)	Abs	Sd ($\mu\text{g/ml}$)	Abs	Sd ($\mu\text{g/ml}$)
Control	0.92	347								
CuCl_2	1.10	352 (0.71)	0.78	246 (0.68)	0.54	–	0.00	–	0.00	–
CoCl_2	0.53	209 (0.60)	0.54	253 (0.68)	0.55	–	0.28	–	0.06	–
MgCl_2	0.79	248 (0.68)	0.74	293 (0.70)	0.45	303 (0.65)	0.43	–	0.41	–
MnSO_4	0.77	325 (0.65)	1.13	323 (0.69)	0.61	276 (0.70)	0.76	–	0.53	–
MgO	0.91	256 (0.72)	1.20	294 (0.70)	1.04	270 (0.70)	0.78	283 (0.70)	0.99	295 (0.70)
ZnCl_2	0.79	248 (0.68)	0.81	293 (0.70)	0.99	303 (0.65)	0.13	–	0.99	–
ZnSO_4	0.74	332 (0.69)	0.61	325 (0.65)	0.46	292 (0.70)	0.63	–	0.65	–
BaCl_2	1.07	321(0.66)	0.96	317 (0.66)	1.06	315 (0.66)	0.80	310 (0.67)	0.65	225 (0.68)
CdNO_3	1.08	337 (0.67)	1.00	305 (0.65)	0.83	304 (0.65)	0.37	–	0.09	–
$(\text{CH}_3\text{COO})_2\text{Pb}$	0.68	99 (0.25)	0.82	305 (0.65)	1.03	199 (0.35)	1.12	336 (0.69)	1.32	339 (0.46)
$\text{Al}_2(\text{SO}_4)_3$	0.87	271 (0.70)	1.01	269 (0.69)	0.85	259 (0.72)	0.98	299 (0.70)	0.85	288 (0.68)

Abs = Absorbance, Sd = Siderophore, figures are the average of three replicates, figures in parentheses indicate standard deviations.

One μM concentration of CuCl_2 , MgCl_2 and ZnSO_4 resulted in optimal siderophores yield. A concentration of 10 μM of CuCl_2 and CoCl_2 , and 20 μM of MgCl_2 , MgSO_4 , ZnCl_2 and ZnSO_4 , drastically affected siderophore synthesis. It has been proposed that Zn^{2+} and Mn^{2+} may substitute for Fe^{2+} in the intracellular control of siderophores synthesis and are responsible for repressing siderophore synthesis [26]. Huyer and Page [27–28], and Page [29] have also reported the inhibition of azotobactin, azotochelin and aminochelin synthesis in *Azotobacter vinelandii* at elevated levels of Mn^{2+} and Zn^{2+} . Maximum growth with good siderophoregenesis was obtained even at high concentration (50 μM) of $(\text{CH}_3\text{COO})_2\text{Pb}$ (Table 1).

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