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Siderophore production by *Alcaligenes faecalis* and its application for growth promotion in *Arachis hypogaea*

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A rhizobacterium isolated from groundnut rhizosphere and identified as *Alcaligenes faecalis* BCCM 2374 produced siderophore during 24 h submerged growth in modified succinic acid medium (SM) prepared in tap water without deferration. For the optimized production of siderophore in the presence of starch and sucrose, the optimum parameters found were as follows: slight acidic *p*H (5.5), 35°C temperature and 1% inoculum. While alkaline *p*H (8.5), 37°C temperature, 5% inoculum in the presence of fructose, xylose and glucose gave optimum growth, succinic and citric acids, NH_4SO_4, NH_4NO_3 and NH_4Cl supported growth as well as siderophoregenesis. Amino acids also showed varying effects on growth and siderophore production. Inoculation of *A. faecalis* enhanced seed germination (8.75%), root length (9.35%), shoot length (16%) and chlorophyll content (8.0%) in *Arachis hypogaea* over control treatment under pot culture conditions.

Keywords: *Alcaligenes faecalis*, *Arachis hypogaea*, siderophore, optimization, groundnut

Introduction

The term plant growth promoting rhizobacteria (PGPR) or yield increasing bacteria (YIB) refers to those rhizobacteria which directly or indirectly stimulate plant growth[1-2]. The mechanism of plant growth promotion by PGPR includes production of siderophores, increased uptake of Fe (through siderophores), production of phytohormones, solubilisation of insoluble nutrients like P and suppression of phytopathogens[1,3-7]. Siderophores are low molecular weight (<10,000 D), ferric specific ligands produced by microbes in order to combat the Fe insolubility[1]. Siderophore producing PGPR play a vital role in Fe nutrition of plants[8,9] and therefore in plant growth promotion leading to healthy plants, which are vital for increasing the crop/food yield[10].

Rhizosphere is a dynamic environment in which many factors affect structure and species composition of rhizobacteria. Since the rhizosphere is inhabited by PGPR, the selective effects of root exudates on rhizobacteria and therefore plant health are of prime importance. Growth and siderophore production by PGPR is attributed to organic acids, sugars, amino acids, minerals, enzymes and several other components of root exudates[11,12]. Any factor influencing either the growth or siderophore production by PGPR would greatly influence the efficacy of that PGPR in plant growth promotion and disease suppression[7,11]. Siderophore production of PGPR is influenced by C, N and minerals. Any factor influencing either the growth or siderophore production by a bacterial antagonist would greatly influence the efficacy of that antagonist in plant growth promotion and disease suppression[1].

Studies on the plant growth promoting potential of many PGPR have been done in recent times[1,3-7], however, the role of *Alcaligenes faecalis* as PGPR remained unexplored and, therefore, the present study was aimed towards the optimization of siderophore production and evaluation of its plant growth promoting potential.

Materials and Methods

Bacterial Culture and Screening for Siderophore

The isolate, *A. faecalis* BCCM 2374, obtained from rhizosphere of North Maharashtra University, was partially identified as per Bergey's manual[13]. It was confirmed on the basis of BIOLOG-GN microtitre plate analysis after growing the organism on biological universal growth medium (BUGM) (Biolog Inc., California, USA).

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Screening for siderophore production was carried out by growing the isolate on chrome azurol sulphonate (CAS) agar plates[14,15] and in Fe-free succinic acid medium (SM)[16] at 28±2°C at 120 rpm for 24 h. It was maintained on nutrient agar at 4°C.

Growth Measurement and Siderophore Quantification

A. feacalis BCCM 2374 was grown in 100 mL of sterile SM at 28±2°C at 120 rpm for 24 h followed by measuring the cell density at 620 nm. Cell-free supernatant was assayed for the production of siderophore both qualitatively and quantitatively by CAS-shuttle assay[17].

Estimation of Fe Content of Different Media

Fe content of different media like, nutrient broth (NB), MacConkey's broth (MB), SM, Barbhayya and Rao broth (BR) and Cas-amino acid broth (CAA) was determined on atomic absorption spectrometer (UNICAM, Model 969, UK).

Growth and Siderophore Production in Various Media

Siderophore production occurs only under Fe-deficient conditions[16] hence different media like NB, MB, SM containing g L^{-1}: K_2HPO_4, 6.0; KH_2PO_4, 3.0; $MgSO4.7H_2O$, 0.2; NH_4SO_4, 1.0 and succinic acid, 4.0[16]; CAS: K_2HPO_4, 5.0 and $MgSO4.7H_2O$, 1.18; BR: K_2HPO_4, 0.25; KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 3.0; NH_4SO_4, 0.2 and succinic acid, 4.0[18], Cetrimide Broth: peptic digest of animal tissue, 10.0; NaCl, 5.0; beef extract, 10.0 and cetrimide, 0.3; Enrichment Medium (EM): Na_2HPO_4, 3.575; KH_2PO_4, 0.98; $MgSO_4.7H_2O$, 0.03; NH_4Cl, 0.5 and trace element 0.2 mL; carbon source (glucose/galactose); 4.0 mL[19], were separately inoculated and incubated to check their effect on growth and siderophore production by *A. faecalis* BCCM 2374.

Optimization of Physico-chemical Parameters

Influence of Water Source

Although the use of purest form of chemicals and water free from Fe in all media preparations has been reported[16], the use of deionized distilled water is a costly affair. Hence, SM prepared in raw water was evaluated for growth and siderophore production and compared with that prepared in deionized distilled water.

Influence of PO_4

To check the influence of PO_4, only one source of PO_4 was added at a time i.e. either K_2HPO_4 (1.0 to 6.0 g L^{-1}) or KH_2PO_4 (0.5 to 3.0 g L^{-1}). Following the incubation for 24 h at 28±2°C, growth measurement and siderophore quantification was done as described earlier.

Influence of N Source

N is important for soil fertility as well as for the growth of rhizobacteria, but siderophore production by PGPR is influenced by different N sources[1,11]. SM was prepared with 1.0 g L^{-1} each of NH_4SO_4, NH_4NO_3, NH_4Cl, NH_3, $NaSO_4$, urea, yeast extract, meat extract, beef extract, soy flour, groundnut oil, casein hydrolysate and corn steep liquor grown with *A. faecalis* BCCM 2374 for 24 h at 28±2°C. Urea and NH_4 were used with a view that the remaining or unutilized part of such nitrogenous compounds will function as a source of chemical N fertilizer when the fermented broth is used for field application.

Influence of pH and Temperature

Though Fe is the forth most abundant micronutrient, it is insoluble at neutral to alkaline *p*H in which the earth is bound. Solubility of Fe in media is also affected by *p*H. SM prepared with a *p*H range of 1-14 was checked for studying its effect on growth and siderophore production.

For studying the effect of incubation temperature on growth and siderophore production, *A. feacalis* BCCM 2374 was separately grown in SM at different temperatures (15-50°C) followed by growth and siderophore analysis.

Influence of Inoculum Level

The level of inoculum required for commercial fermentation is indirectly related to the cost of product. In order to determine the influence of inoculum levels on growth and siderophore excretion, SM was separately grown with *A. feacalis* BCCM 2374 at varying inoculum levels (1-5%) at 28±2°C followed by growth and siderophore measurement.

Influence of Sugars, Organic and Amino Acids

The easily assimilable sugars contribute to faster growth and thereby increased secondary metabolite (siderophore) production. For this purpose, 100 mL of SM was separately supplemented with 1 g L^{-1} each of D-glucose, D-sucrose, D-lactose, D-maltose, D-mannitol, starch, D-fructose, D-xylose, mesoinisetol and L arabinose. Each SM was separately grown with *A. faecalis* BCCM 2374 for 24 h at 28±2°C at 120 rpm, following the incubation growth and siderophore quantification was done as described earlier.

Nature of C compound determines the Fe requirement of cell and thus regulates siderophore production[11]. For this purpose, each 100 mL of SM (without succinic acid) was separately supplemented with 1 g L^{-1} each of succinic, citric, formic, propionic, lactic, ascorbic, D-malic, L-glutamic and L-aspartic acids and grown with *A. faecalis* BCCM 2374 at 28±2°C followed by growth and siderophore quantification.

Different amino acids were checked for their utilization as C and N source. SM separately fortified with 1 g L^{-1} each of L-glycine, L-cystine, L-cysteine, L-alanine, L-methionine, L-luecine, L-isoleucine, L-tyrosine, L-histidine, L-proline, L-phenylalanine, L-valine, L-serine, L-arganine and L-threonine was grown with *A. faecalis* BCCM 2374 at 28±2°C. Following the incubation of inoculated SM at 28±2°C, growth and siderophore content were measured.

Influence of *A. feacalis* Inoculation on Germination and Growth of Groundnut

Surface sterilized groundnut seeds (*Arachis hypogaea*) were mixed/immersed for 10 min in siderophore rich broth of *A. feacalis* (8×10^7 cells mL^{-1}) grown in SM for 30 h. Control was prepared by adding 20 µ*M* of Fe in siderophore containing SM to remove siderophores. Bacterized seeds were sown (5 seeds/pot) in pots containing sterile soil and were intermittently moistened. Observations like root length, shoot length, % rate of germination and chlorophyll content[19] were recorded after 30 d of sowing.

Results and Discussion

Screening and Production of Siderophore

Change in the colour of CAS agar from blue to orange red and golden yellowing of SM in shake flasks confirmed the ability of *A. feacalis* to produce and excrete the siderophore[14].

Estimation of Fe Content of Different Media

Amongst the different media used, MB was found to contain maximum (0.2049 ppm) amount of Fe whereas SM was found to be Fe deficient as it contained only 0.0903 ppm of Fe. The Fe content of rest of the media used for siderophore production was NB= 0.1312, CAA= 0.0911 and BR= 0.0738 ppm.

Growth and Siderophore Production on Various Media

NB and MB supported luxurious growth, however, there was no detectable siderophore production in these media; this may be due to their high Fe content. SM was found to give maximum (92.25%) siderophore production in comparison to 88.00% in BR medium, 79.00% in CAA broth and 48% in EM (Table 1).

Influence of Water Source

Although, it has been already reported that the use of purest Fe free media ingredients can result in maximum siderophore production, our isolate gave better siderophore yields (92.25%) in raw water based SM *vis-a-vis* 86.00% siderophore production in distilled water based SM.

Optimization of Physico-chemical Parameters

Influence of PO_4 Levels

Amongst the various levels of PO_4 used, 6.0 g L^{-1} of K_2HPO_4 and 2.0 g L^{-1} KH_2PO_4 appeared to be optimum. SM without PO_4 content gave 85.30% siderophore (Table 1) and was found to be more cost effective medium. Siderophore production in low concentration of PO_4 has been reported by Barbhayya and Rao[18].

Influence of N Source

NH_4SO_4 yielded maximum siderophore units (88.21%) and good growth (Table 1). However, complex N sources could not boost growth and siderophore excretion as well, which may be due to their Fe richness. Utilization of urea by isolate suggested its possible exploitation for bioremediation of alkaline soil by reducing the excess amount of urea present in the soil. Sayyed *et al* have suggested this approach as cost effective and eco-friendly[1].

Influence of pH and Temperature

Alkaline *p*H (8.5) favoured growth while slight acidic *p*H (6.5) supported siderophoregenesis (81.69 %) with minimum growth (Fig. 1). It is known that alkaline *p*H helps in more solubilization of Fe, thus increasing the Fe content of media[21]. Growth and siderophoregenesis was affected at lower (15-20°C) as well as higher temperature (40-50°C). A temperature of 37°C and 35°C was optimum for growth and optimal siderophore (93.30%) yield (Fig. 2). Temperature of incubation close to the maximum temperature tolerated for growth is reported to be inhibitory for siderophore production[21].

Influence of Inoculum Level

Inoculum (1%) was optimum and its further increase did not support growth and siderophoregenesis (Table 1). Sayyed *et al*[1] have

Table 1—Influence of various physico-chemical factors on growth and siderophoregenesis in *A. feacalis*

Factors and growth and siderophore yield

Factor	Growth	% SU	Factor	Growth	% SU	Factor	Growth	% SU	Factor	Growth	% SU
K_2HPO4			KH_2PO_4			N_2 Source			Sugars		
1.0	1.5	Nd	0.0	1.0	88 (0.15)	AS	1.3	88 (0.33)	Starch	1.7	88 (0.39)
2.0	2.1	Nd	1.0	0.9	84 (0.11)	AN	1.3	86 (0.61)	Sucrose	1.1	84 (0.71)
3.0	0.7	88 (0.21)	2.0	0.9	75 (1.21)	AC	--	Nd	Lactose	1.5	83 (2.1)
4.0	0.6	92 (0.23)	3.0	0.8	85 (0.42)	AM	--	Nd	Glucose	1.4	79 (1.5)
5.0	0.7	79 (0.11)	4.0	0.7	86 (0.31)	SS	1.3	85 (0.39)	Fructose	1.7	78 (1.2)
6.0	0.4	48 (0.34)	5.0	0.6	85 (0.27)	U	--	Nd	Maltose	1.3	78 (1.1)
			6.0	0.6	84 (0.24)	YE	0.3	Nd	Mannitol	1.3	
Inoculum (%)			KH_2PO_4								
1	1.0	93	0.0	1.0	85 (0.08)	ME	0.3	Nd	Arabinose	1.0	48 (0.34)
2	1.3	91	0.5	0.8	77 (0.51)	BE	--	Nd	Xylose	1.4	34 (0.31)
3	1.4	90	1.0	0.8	86 (0.17)	SF	0.1	Nd	Meso-inosetol	0.4	34 (0.31)
4	1.4	90	1.5	0.8	87 (0.29)	GM	0.1	Nd			
5	1.5	90	2.0	0.6	88 (0.41)	CH	0.2	Nd			
			2.5	0.6	86 (0.64)	CSL	0.3	Nd			
			3.0	0.7	85 (0.33)						
Organic acid			Amino acid		*p*H				Temp (^oC)		
SA	1.0	88 (0.73)	Gly	1.2	80 (0.64)	1.0	Nd	Nd	15	Nd	Nd
CA	1.2	61 (0.51)	Cys	1.7	57 (0.58)	2.0	Nd	Nd	20	Nd	Nd
LA	1.9	56 (0.44)	Ala	1.3	87 (0.72)	3.0	Nd	Nd	25	1.3	84 (071)
PA	0.1	52 (0.32)	Met	1.5	87 (0.70)	3.5	Nd	Nd	30	1.1	71 (0.38)
MA	--	47 (0.29)	Ile	1.3	82 (0.66)	4.0	Nd	Nd	37	1.3	93 (0.75)
FA	--	44 (0.27)	Tyr	1.4	86 (0.71)	4.5	Nd	Nd	40	0.2	Nd
AA	--	--	His	1.9	74 (0.44)	5.0	0.3	25 (0.22)	45	1.3	Nd
			Lys	1.6	86 (0.71)	5.5	0.6	67 (0.27)	50		
			Pro	1.4	74 (0.44)	6.0	1.0	81 (0.41)			
			Pal	1.3	79 (0.49)	6.5	1.3	79 (0.44)			
			Val	1.3	79 (0.49)	7.0	1.1	74 (0.47)			
			Ser	1.3	84 (0.73)	7.5	1.6	72 (0.48)			
			Leu	1.4	74 (0.44)	8.0	1.5	71 (0.38)			
			Cys	1.5	81 (0.71)	8.5	1.8	70 (0.36)			
			Arg	1.4	84 (0.47)	9.0	1.6	71 (0.36)			
			Thr	1.5	79 (0.49)	9.5	1.5	79 (0.44)			
			Asp	1.6	59 (0.27)	10	1.3	Nd			
						11	Nd	Nd			
						12	Nd	Nd			
						13	Nd	Nd			
						14	Nd	Nd			

Values in the parenthesis indicate standard deviation (SD).
Values are the mean of three replicates
SU = Siderophore units, Nd= Not detected,
AS= Ammonium sulphate, AN= Ammonium nitrate, AC= Ammonium Chloride, AM= ammonia, SS = Sodium sulphate, U= Urea, YE= Yeast extract, ME= Meat extract, BE=Beef extract, SF= Soy flour, GM= Groundnut meal, CH= Casein hydrolysate, CSL= Corn steep liquor.
SA= Succinate, CA= Citrate, LA= Lactate, PA= Propionate, MA= Malate, FA= Fumarate, AA=Ascorbate
Gly=L-glycine, Cys= L-cysteine. Ala= L-alanine, Met=L-methionine, Ile= L-isoleucine, Tyr= L-tyrosine, His= L-histidine, Lys= L-lysine, Prol= L-proline, Pal, L-phenylalanine, Valine = L-valine, ser= L-serine, Leu= L-leucine, Cys= L-cystine, Arg= L-arganine, Thr= L- threonine, Asp= L-aspartic

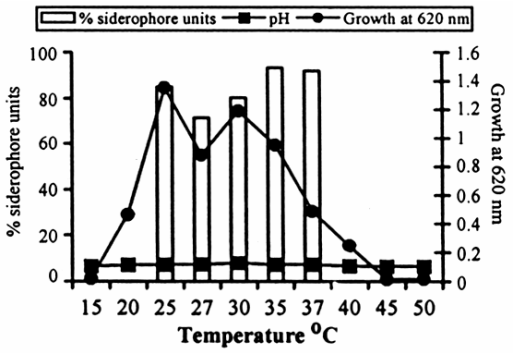

Fig. 1—Influence of *p*H on growth and siderophore production.

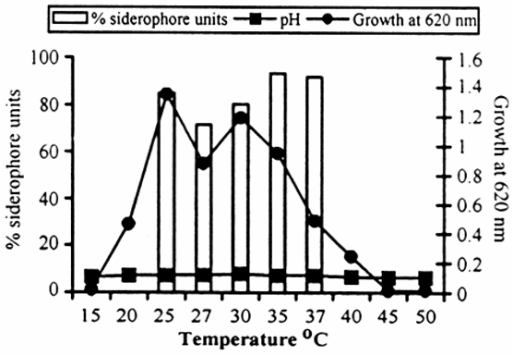

Fig. 2—Influence of temperature on growth and siderophore production.

Table 2—Influence of *A. feacalis* inoculation on groundnut germination and growth

Treatment	Germination (%)	Root length (mm)	Shoot length (mm)	Chlorophyll content (mg g^{-1})
Control	75 (0.67, 22.33)	2.6 (0.14, 4.66)	6.6 (0.38, 1.17)	3.2 (0.28, 7.14)
Test	100 (0.67, 30.66)	5.6 (0.28, 17.00)	21.2 (0.25, 5.75)	4.3 (0.32, 8.75)

Values in the parenthesis indicate standard deviation (SD).
Values are the mean of three replicates

reported optimum siderophore production with 1% inoculum level in *P. fluorescens* NCIM 5096.

Influence of Sugars, Organic and Amino Acids

Disaccharide (fructose, xylose, maltose and lactose), monosaccharides (glucose) supported growth while polysaccharides (starch) supported both growth and siderophore yield (88.02%) (Table 1).

Succinic, citric and lactic acids, which are the major organic compounds in root exudates, are also supportive for growth and siderophore production. Lactic acid supported growth while succinic acid favoured siderophore production (Table 1).

Cysteine and histidine boosted the growth while methionine, isoleucine, tyrosine and proline enhanced siderophore production (Table 1). These results are in accordance with the earlier results[1,11] that alanine, tyrosine, arginine, lysine and succinic acid were supportive for growth, fluorescence and siderophore production in *Pseudomonas* sp. Root exudates containing sugars, organic acids and amino acids are essential constituents in rhizosphere which influence the growth, metabolite production and root colonization of PGPR[23].

Influence of *A. feacalis* Inoculation on Growth of Groundnut

A 8.75% increase in the rate of germination, 9.35% increase in the root length and 16% increase in the shoot length and 8% increase in chlorophyll content was evident in *A. feacalis* inoculated seeds of groundnut over the control (Table 2). This is in accordance with the results obtained by Manwar *et al*[24] and Sayyed *et al*[1,3, 5-6].

Conclusion

A. faecalis BCCM 2374 may prove vital for, plant growth promotion, increased crop yield and as a biofertilizer to provide Fe nutrition to plants. Though the decomposition of cystine, did not favour much siderophore excretion but it resulted in H_2S evolution, which is one of the important steps in sulfur nutrition and indicative of possible exploitation of isolate as sulfur biofertilizer besides its applicability as a bioinoculant for Fe nutrition.

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