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Defined media for optimal pyoverdine production by *Pseudomonas fluorescens* 2-79

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Abstract. *Pseudomonas fluorescens* strain 2-79 (NRRL-15132) produces a fluorescent yellow-green pyoverdine when cultured on Fe(III)-poor medium. When cultured on Fe(III)-rich medium, strain 2-79 produces an antibiotic, phenazine 1-carboxylic acid, which is effective in suppressing plant fungal diseases such as take-all of wheat. A 2^3 factorial design was used to examine pyoverdine production as a function of the presence or absence of Bacto casamino acids, purines-pyrimidines and vitamins in an iron-deficient medium. Amino acids were found to be an important factor ($P=0.0002$). A Plackett-Burman design was used to identity eight amino acids, out of the 19 present in casamino acids, that were responsible for the increased pyoverdine production: methionine, valine, isoleucine, tyrosine, proline, phenylalanine, glutamic acid, and glycine. Biomass was enhanced only by glutamic acid.

Introduction

When grown under iron-poor conditions, many bacteria synthesize and secrete into the environment iron chelators that were termed siderophores by Lankford (1973). Siderophores are low-molecular-mass compounds that chelate the ferric ion with high specificity, and serve as a vehicle for the transport of (Fe(III) into microbial cells (Neilands 1981a, b). Pyoverdines are yellow-green pigments that fluoresce under ultraviolet light, representing one class of siderophores produced by fluorescent pseudomonads (Loper and Buyer 1991). Siderophore-mediated iron competition is believed to be a mechanism determining microbial interactions. This hypothesis is based on the observation that iron availability sometimes limits microbial growth and development on plant surface.

Supposedly, in response to iron-limiting conditions encountered either on aerial plant surfaces or in the rhizosphere, microbes produce siderophores in situ. Some investigators have generated evidence supporting the notion that siderophores play significant roles in the biocontrol of soil-borne diseases by adding purified siderophore or synthetic iron chelators to soil. For example, Kloepper et al. (1980) added purified pseudobactin to soil and successfully mimicked the effect of inoculating seed with *Pseudomonas* sp. strain B10 in growth promotion of potato. Although pyoverdines may not be universally implicated in the biocontrol by flourescent pseudomonads (Gutterson 1990; Weller et al. 1988), there is still a need to evaluate individual strains under different conditions since the role played by pyoverdine in the suppression of plant diseases may vary with soil environment, target pathogen, plant host, and the strain. However, as pointed out by Loper and Buyer (1991), no purified pyoverdine evaluation for plant growth promotion or biocontrol has been reported in the literature. This is in part due to the difficulty in obtaining the large quantities required.

The purpose of this study was to address the need to produce large quantities of pyoverdine. Our experimental approach involved developing an optimal medium formulation by adding or eliminating growth factors that influence pyoverdine production. At the first level, partial factorial experiments were conducted to determine whether amino acids, purines-pyrimidines, or vitamins were beneficial when added to the base growth medium. At the second level, a detailed screening for the individual beneficial components of the important growth factor(s) was accomplished.

Materials and methods

Microorganism and media. Pseudomonas fluorescens 2-79 (NRRL-15132, isolated by D. M. Weller and R. J. Cook, USDA, Pullman, Wash., USA) was obtained from the ARS Culture Collection at the National Center for Agricultural Utilization Research in Peoria, Ill., USA). Stock cultures were prepared as previously reported (Kisaalita et al. 1991). When this organism is cultured on Fe(III)-rich medium, it produces an antibiotic, phen-

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azine 1-carboxylic acid (PCA). PCA is effective in inhibiting the growth of plant pathogenic fungi such as *Gaeumannomyces graminis* var. *tritici, Rhizoctonia solani,* and *Pythium* spp. (Grusiddaiah et al. 1986). Strain 2-79 also produces a fluorescent yellow-green pyoverdine when cultured in Fe(III)-poor medium.

The purines-pyrimidines stock solution was prepared by adding 20 ml conc HCl, 20 mg each of adenine, thymine, cytosine and uracil to 200 ml distilled water. The solution was heated to dissolve all the components, adjusted to pH 7.0 with 3–4 M NaOH and the volume made up to 400 ml before sterilization by autoclaving. The amino acid stock solution was prepared by adding 50 g Bacto vitamin-free casamino acids (Difco, Detroit, Mich., USA), 500 mg tryptophan, 2 g cysteine to 200 ml distilled water and sterilized. The vitamin stock solution was freshly made by adding 25 mg each of thiamine, riboflavin, calcium pantothenate, niacin, pyridoxamine dihydrochloride, thioctic acid and 2.5 mg each of folic acid, vitamin B_{12} and biotin to 1 l distilled water and sterilized by filtration (0.22-μm disposable Nalgene filters). For pyoverdine production, the base-modified poor nutrient medium was made up of 6 g K_2HPO_4, 3 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.098 g $MgSO_4$, and 4 g succinic acid per liter and adjusted to pH 7.0 before sterilization (Meyer and Abdallah 1978). Various combinations of 40, 4, and 4 ml of purines-pyrimidines, amino acid and vitamin stock solutions, respectively, were added to the base media as called for by the experimental design described below.

Batch fermentation. Bacteria from slants served as inocula for 50-ml precultures in 125-ml flasks. Cells were harvested by centrifugation and washed three times with a 5% NaCl solution prior to use as inocula for 200 ml medium in 500-ml flasks. Both seed and experimental flasks were incubated at 25°C for 24 h at 200 rpm in a Model G27 New Brunswick Psychrotherm Shaker/incubator. Cell concentration, pH and pyoverdine formation were monitored daily. Samples for total iron analysis were taken at the beginning of each experiment.

Analyses. Total iron analyses were carried out by the inductively coupled plasma (ICP) atomic emission spectroscopic technique (EPA SW-846 Test Method no. 60-10) by Daily Analytical Laboratories (Peoria, Ill., USA). Total pyoverdine production was estimated by a fluorometer (Model LS-5B, Perkin Elmer, Norwalk, Conn., USA). LS-5B was set at the optimal excitation (403 nm) and emission (488 nm) wavelengths. Samples for pyoverdine measurements were centrifuged to remove all cells and buffered to control pH for fluorescence measurements by diluting 201 times with distilled water. Cell concentrations were monitored by optical density at 620 nm with a Beckman spectrophotometer, Model DU-70. Samples were diluted as appropriate to provide absorption readings in the region where absorbance and dry cell mass concentrations were linearly related.

Experimental designs. Pyoverdine production was examined as a function of the presence or absence of the three growth factors. Eight combinations of the three ingredients were tested in a 2^3 factorial design (Table 1), which addresses all of the main interactive effects (Webb 1971). In a follow-up experiment, the effect of the presence or absence of each of 19 amino acids present in Bacto casamino acids was examined using a Plackett and Burman (1946) multifactorial experimental design (Table 2). Two additional control flasks (experiment numbers 20B and 20C) were initially added to the design to enable the quantification of the effect of trace iron.

Statistics. With pyoverdine as the dependent variable, the analysis of variance (General Linear Model, GLM) procedure available in the SAS statistical package was used to determine the essential factors. For a factor to be essential, the significant probability (P) had to be ≤ 0.05. Since the media trace iron concentration influenced pyoverdine production and could not be controlled, it was measured at the beginning of each experiment and included in the analysis as a covariate (Tables 3 and 4). For statistical theory concerning GLM covariance analysis see the SAS/STAT User Guide for Personal Computers (SAS Institute, Cary, NC, USA).

Results

Tables 3 and 4 present the covariate media iron concentrations used in the first and second level statistical analyses. Table 5 shows analysis of variance results for the effect of the growth factors and their interactions on pyoverdine production. Amino acids were found to be beneficial ($P=0.0032$). Vitamins and the interaction between amino acids and purines-pyrimidines were borderline ($0.05<P<0.1$), whereas purines-pyrimidines and all other interactions were insignificant. Since the three-way interaction [amino acids – vitamins – purines-pyrimidines] was found to have a high P (0.24), it was not included in the final statistical analysis. GLM analysis with maximal recorded biomass as the dependent variable also revealed amino acids to be beneficial ($P=0.0004$). In order to identify the important amino acids among those found in Bacto casamino acids, a Plackett-Burman design (Table 2) was used. The absolute and relative amino acid concentrations were maintained at the same levels as in the first eigth experiments. The relative amounts were based on the

Table 1. A set of eight experiments to determine whether amino acids (Bacto casamino acids), purines-pyrimidines, or vitamins are required in the growth medium for enhanced pyoverdine production by *Pseudomonas fluorescens* 2-79

Experiment no.	Growth factors			Pyoverdine fluorescence (replicates)			
	Amino acids	Purines-pyrimidines	Vitamins	1	2	3	4
1	+	+	+	110.6	120.2	182.1	129.5
2	−	+	+	88.4	76.4	91.6	86.7
3	+	−	+	94.7	118.0	222.0	150.4
4	+	+	−	108.9	99.7	186.7	132.7
5	−	−	+	184.8	109.9	150.8	62.6
6	−	+	−	81.8	109.5	65.9	66.4
7	+	−	−	124.5	125.7	200.6	150.7
8	−	−	−	145.5	188.4	137.3	63.7

+, presence; −, absence

Table 2. A 20-trial Plackett-Burman multifactorial design to screen for the essential amino acids among the 19 present in Bacto casamino acids for enhanced pyoverdine production by *P. fluorescens* 2-79

Experiment Number	Amino acids																			Pyoverdine fluorescence (replicates)		
	Alanine	Arginine	Aspartic acid	Cystine	Glutamic acid	Glycine	Cysteine	Histidine	Leucine	Isoleucine	Lysine	Methionine	Phenyl-alanine	Proline	Serine	Threonine	Tyrosine	Tryptophan	Valine	1	2	3
1	+	+	−	−	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	184.2	167.7	108.4
2	−	+	+	−	−	+	+	+	+	−	+	−	+	−	−	−	−	+	+	284.0	251.3	142.8
3	+	−	+	+	−	−	+	+	+	+	−	+	−	+	−	−	−	−	+	210.8	135.6	100.4
4	+	+	−	+	+	−	−	+	+	+	+	−	+	−	+	−	−	−	−	333.2	285.9	147.9
5	−	+	+	−	+	+	−	−	+	+	+	+	−	+	−	+	−	−	−	146.8	105.5	119.8
6	−	−	+	+	−	+	+	−	−	+	+	+	+	−	+	−	+	−	−	170.0	134.0	92.0
7	−	−	−	+	+	−	+	+	−	−	+	+	+	+	−	+	−	+	−	158.6	90.1	88.9
8	−	−	−	−	+	+	−	+	+	−	−	+	+	+	+	−	+	−	+	315.1	217.5	112.6
9	+	−	−	−	−	+	+	−	+	+	−	−	+	+	+	+	−	+	−	455.6	344.2	195.1
10	−	+	−	−	−	−	+	+	−	+	+	−	−	+	+	+	+	−	+	434.2	352.6	192.8
11	+	−	+	−	−	−	−	+	+	−	+	+	−	−	+	+	+	+	−	98.7	107.6	83.0
12	−	+	−	+	−	−	−	−	+	+	−	+	+	−	−	+	+	+	+	245.6	186.7	92.4
13	+	−	+	−	+	−	−	−	−	+	+	−	+	+	−	−	+	+	+	453.2	340.5	187.3
14	+	+	−	+	−	+	−	−	−	−	+	+	−	+	+	−	−	+	+	148.3	115.1	75.9
15	+	+	+	−	+	−	+	−	−	−	−	+	+	−	+	+	−	−	+	168.8	109.5	99.2
16	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	+	+	−	−	351.4	275.3	167.0
17	−	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	+	+	−	341.0	320.8	153.1
18	−	−	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	+	+	370.3	274.0	177.4
19	+	−	−	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	+	369.1	363.6	181.8
20A	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	112.1	103.6	80.0
20B	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	111.4	112.8	87.9
20C	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	123.2	111.7	86.0

+, presence; −, absence

Table 3. Media iron concentrations for experiments in Table 1

Experiment no.	Media trace iron in parts per billion (replicates)			
	1	2	3	4
1	270	350	730	230
2	200	360	690	340
3	190	280	380	330
4	230	360	640	480
5	240	740	50	480
6	260	670	220	1070
7	280	340	250	840
8	170	810	240	1100

Table 4. Media trace iron concentrates for experiments in Table 2

Experiment no.	Media trace iron in parts per billion (replicates)		
	1	2	3
1	120	310	730
2	84	180	840
3	90	230	830
4	150	270	1180
5	140	230	1100
6	300	210	810
7	230	170	1060
8	210	170	1010
9	220	380	590
10	240	300	610
11	—	270	530
12	220	310	680
13	260	250	810
14	280	200	620
15	31	160	610
16	54	200	430
17	31	180	1520
18	128	150	270
19	111	220	1160
20A	200	260	730
20B	170	260	600
20C	130	180	790

Table 5. Analysis of variance results for the effect of three growth factors (amino acids, purines-pyrimidines, and vitamins) and their interactions on pyoverdine production by *P. fluorescens* 2-79

Growth factor and/or Interactions	Significance probability (P)
Amino acids	0.0032
Purines-pyrimidines	0.2348
Vitamins	0.0651
Amino acids and purines-pyrimidines[a]	0.0772
Amino acids and vitamins	0.6792
Purine-pyrimidines and vitamins	0.9309

[a] Interaction between amino acids and purines-pyrimidines

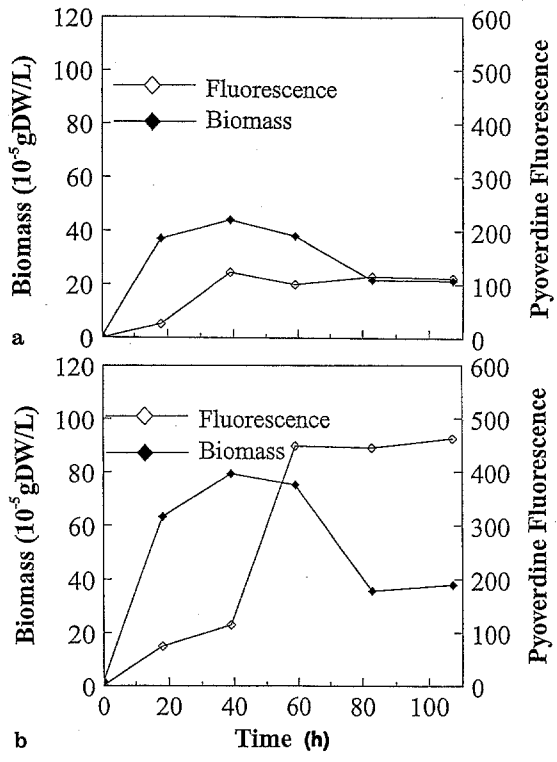

Fig. 1a, b. Comparison of pyoverdine and biomass production on amino-acid-supplemented and basal med: DW, dry weight

composition of hydrolyzed casein (Zabriskie et al. 1980). As shown in Table 2 for the three replicates, experiment number 9 yielded the maximum pyoverdine fluorescence. Table 4 presents the covariate iron concentrations. Growth and pyoverdine production curves for experiment numbers 20 (control) and 9 are compared in Figs. 1a and b, respectively. A four- to fivefold increase in pyoverdine fluorescence is evident. Eight amino acids [methionine ($P=0.0001$), valine ($P=0.0003$), isoleucine ($P=0.0003$), tyrosine ($P=0.0005$), proline ($P=0.0008$), phenylalanine ($P=0.0158$), glutamic acid ($P=0.0291$), and glycine (0.0377)] were found to be beneficial (Table 6). Glutamic acid was found to be the only essential amino acid ($P=0.0001$) for enhanced biomass production (biomass data not presented).

Discussion

Out of the three growth factors tested, only amino acids were found to enhance pyoverdine production, while purines-pyrimidines, vitamins and all the interactions had either negligible or no effect. Structural elucidation of pyoverdines isolated from several strains of fluorescent pseudomonads belonging to different species has revealed that these pyoverdines are polypeptides, consisting of six to ten hydrophilic amino acids bound to a chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline (Demange et al. 1989). This is consistent with the importance of amino acids with respect to pyoverdine production found in this study. The identification of eight essential amino acids out of

Table 6. Analysis of variance results for the effect of individual amino acids on pyoverdine production by *P. fluorescens* 2-79

Amino acid	Significance probability (*P*)
1. Methionine	0.0001
2. Valine	0.0003
3. Isoleucine	0.0003
4. Tyrosine	0.0005
5. Proline	0.0008
6. Phenylalanine	0.0158
7. Glutamic acid	0.0291
8. Glycine	0.0377
9. Cysteine	0.0735
10. Serine	0.1042
11. Threonine	0.1048
12. Leucine	0.1540
13. Alanine	0.1830
14. Aspartic acid	0.3800
15. Lysine	0.4558
16. Tryptophan	0.5716
17. Histidine	0.5923
18. Cystine	0.6546
19. Arginine	0.8299

19 tested may suggest that these amino acids used by the organism as building blocks for pyoverdine, since the number eight is consistent with six to ten hydrophilic amino acids required for pyoverdine. Structural elucidation of *P. fluorescens* 2-79 pyoverdine is underway to confirm this hypothesis. However, in a separate study (Slininger and Jackson 1992) of *P. fluorescens* 2-79 nutritional factors regulating biomass and the production of phenazine 1-carboxylic acid (PCA), amino acids supported biomass and PCA production as the sole nitrogen source. In this study, only glutamic acid was found to be essential for enhanced biomass production suggesting that the bulk of the amino acids were not required as a nitrogen source. The processes of pyoverdine and PCA production are expected to be fundamentally different in terms of the dominant metabolic pathway, one being triggered by black of iron and the other by the presence of iron. Thus differences in amino acid requirements for PCA and pyoverdine production are to be expected, just as nutritional differences for biomass and metabolite production are typical, as seen here and elsewhere (Slininger and Jackson 1992).

As to the pyoverdine quantification, it's fluorescence has been assumed to represent pyoverdine concentration for purposes of determining the essential growth factors. Caution should be exercised in interpreting these data. For example, doubling of pyoverdine fluorescence does not necessarily imply the doubling of pyoverdine production. This is because it is likely that at certain concentrations the relationship between fluorescence intensity and pyoverdine is non-linear. It is also possible that strain 2-79 produces more that one pyoverdine, in which case the observed fluorescence intensity is a sum of the individual pyoverdine intensities. Evidence supporting the notion that the observed pyoverdine fluorescence from fermentation broths is a sum of all contributions from a number of fluorescent compounds produced by the organism was first reported by Meyer and Abdallah (1978). In their final chromatographic purification step for pyoverdine produced by *P. fluorescens* CCM 2799, two fractions were observed. They postulated that the front-running was a degradation product of the main pyoverdine. Also, Philson and Llinás (1982) isolated four distinct fluorescent compounds produced by *P. fluorescens* ATCC 13525. In addition, Menhart et al. (1991) have most recently shown that *Azotobacter vinelandii* ATCC 12837 produces more than one pyoverdine and that these compounds differ in their peptide components. However, they postulated that only one of these compounds is a true pyoverdine while the others are metabolic by products that are produced possibly due to intrinsic limitations of the synthetase complex involved in their biosynthesis. However, the use of total fermentation fluorescence is considered sufficient in the absence of pure components. Besides, we are not aware of any evidence in the literature to suggest that fluorescent pseudomonads produce other fluorescent compounds distinct from pyoverdine. In fact in a recent study, the structure of pyoverdines occurring in iron-deficient cultures of *P. fluorescens* ATCC 13525 were elucidated and also found to contain a common partly cyclic peptide (Linget et al. 1992). This peptide contained a thirteen-member ring bound to different substituted chromophores derived from 2,3-diamino-6,7-dihydroxyquinoline.

Although the approach to optimizing media adopted in this study has been tested with *P. fluorescens* 2-79, it should yield valid results for other fluorescent pseudomonads. Should pyoverdine be conclusively implicated in biocontrol by fluorescent pseudmonads, a need for including pyoverdine in biocontrol agent formulations will justify pyoverdine commercial process development. In addition, pyoverdines may have other uses such as the development of kits for rapid quantitation of iron (Kisaalita et al., unpublished data) or drag transport across cell membranes (Miller et al. 1991). Therefore, our findings are not only useful to researchers needing large quantities of pyoverdine, but may be a starting point for commercial pyoverdine processes development.

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