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Impact of growth medium on siderophore production by Vibrio campbellii: An experimental approach using universal CAS assay

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ABSTRACT

The growth medium is the sole source of nourishment for the bacteria cultured under laboratory conditions. This implies that the growth medium could largely influence the quality and quantity of secondary metabolites produced by the bacterium. Secondary metabolites are the weapons used by bacteria for survival in competitive ecosystems. In this regard, Siderophores are among the vital secondary metabolites of marine bacteria which help them in sourcing the minimal iron essential for their growth and virulence from the marine environment. Among the 11 different growth media evaluated for their impact on Siderophore production by the emerging marine pathogen Vibrio campbellii, MM9 native (minimal salt medium) was found to yield the maximum siderophore units (95.35%). The key factors influencing the growth of V. campbellii were found to be glucose and casamino acid. The determinant factors influencing Siderophore production were found to be glucose and iron-depleted composition of MM9 native medium.

Keywords— *MM9 medium, Secondary metabolite, Vibrio campbellii, Iron*

1. INTRODUCTION

Marine bacteria are among the highly diverse life forms in the ocean. They thrive on the nutrients available in the marine ecosystem and immensely contribute to the nutrient cycles, thus enabling to maintain the subtle balance in the rich and diverse aquatic systems. However, the marine bacteria have to face an important limiting condition in the marine system, i.e. iron limitation. Iron is an important bioactive metal absolutely necessary for the growth and metabolism of bacteria. Iron plays a key role in electron transport, oxidation-reduction reactions, and detoxification of oxygen radicals, synthesis of DNA precursors and in many other biochemical processes (Sandy and Butler, 2009). Marine microorganisms face unique challenges to obtain essential metal iron required to survive and grow in the ocean (Vraspir and Butler, 2009).To overcome this, microbes like bacteria and fungi produce a specific type of low molecular weight ligands, known as Siderophore. Siderophores, literally meaning iron carriers in Greek, are relatively low molecular weight, ferric ion specific chelating agents produced by bacteria and fungi growing under iron stress. The role of these compounds is to scavenge iron from the environment and to make it available for the microorganism (Neilands, 1984).

Vibrionaceae family are widely distributed in the marine environment comprising of several species which cause diseases to human beings and animals (Andrus, 1983). Subsequently, Vibrios have a profound ability to produce bioactive secondary metabolites (Mansson *et al.*, 2011). Like other pathogenic bacteria, Vibrios also require iron for their growth (Mey *et al*., 2005). In iron-deficient marine ecosystems, these bacteria produce a wide range of Siderophores (iron-chelators).

Growth media support for the growth of microorganisms under controlled conditions. This applies to the production of Siderophores also. Previous studies indicate large variations in Siderophore production by the same bacteria when grown on various media. Modified succinate medium plays the main role in the production of the iron chelating molecule and it has been proved (Sayyed et

al., 2005). MM9 medium is used for the optimization and standardization of siderophore production in *Pseudomonas putida* (Muruggapan *et al*., 2012). Iron-deficient (low nutrient sea water) based liquid medium IDSM is used under iron-limiting condition (Guan *et al.,* 2001). BOSS Medium is used specifically for luminescent bacteria (Klein *et al.,* 1998). R2A medium is used for enumeration of heterotropic bacteria from the portable water and was found to influence the production of siderophore (Reasoner *et al*., 1985). Since there are very few reports on siderophore production by *Vibrio campbellii*, the current study was undertaken to select an appropriate media for maximum Siderophore production by *V. campbellii*.

2. MATERIALS AND METHODS

2.1 Stock culture preparation of *Vibrio campbellii*

For the preparation of stock culture, *Vibrio campbellii* (NIOT/SID/43) were grown in the sterile Zobell Marine Broth (ZMB) under optimized conditions (8 days at 28°C and pH 5.5).

2.2 Preparation of various growth media

A total of 11 different media (250 ml each) were prepared separately in sterile conical flasks (500 ml, Erlenmeyer) and was autoclaved at 121ºC for 15 minutes. After sterilisation, the broth was cooled and inoculated with 500µl of *V. campbellii* strain. The flasks were incubated in shaking incubator at 220rpm at 28°C. The growth media selected for the experiment are listed in Table 1.

Table 1: Various growth media used for the optimisation of Siderophore production

Media No.	Growth Media particulars
M1	ZMB (full strength)
M2	ZMB (half strength)
M3	Modified Succinate deferrated medium (with and without chloroform extraction)
M4	Iron deficient low nutrient sea water based liquid medium(IDSM){1mMFe(III) fortified}
M5	MM9 medium (with low glucose 0.08%)
M6	MM9 medium (with high glucose strength 1%)
M7	BOSS medium
M8	MM9 native medium
M9	R2A medium without Iron
M10	R2A medium + 0.001%Fe
M11	MM9 (+HEPES)

2.3 Estimation of growth and biomass production by *V. campbellii*

V. campbellii strain was inoculated on the respective 11 different growth media plates (three replicates for each day of incubation) by spread plate technique and incubated for a maximum of 14 days at 28˚C. The plates were sampled every alternate day of incubation (i.e. 2, 4, 6, 8, 10, 12 and 14th day). After required incubation, the colony forming units (CFU) of each plate was counted.

Towards biomass estimation, *V. campbellii* strain was inoculated in 10 mL each of the 11 different growth media and incubated for a maximum of 14 days at 28˚C. The tubes were also sampled every alternate day of incubation (i.e. 2, 4, 6, 8, 10, 12 and 14th day). After required incubation, the Optical Density (OD) was measured using UV Spectrophotometer (Perkin Elmer; Sutton, 2011). Biomass was measured directly by dry weight process after washing with distilled water (Stone *et al*., 1992).

2.4 Estimation of *Siderophore* production by *V. campbellii*

The amount of siderophore production by *V. campbellii* grew on the 11 different growth media was estimated using universal chrome azurol sulfonate (CAS) assay (Schwyn and Neilands, 1987).

2.5 Preparation of CAS assay solution

To a 100 ml volumetric flask, 6ml of 10mM HDTMA solution was added. To this, 1.5 ml of iron (III) solution containing (1mM Fecl3. $6H_2O$, 10mM HCl) and 2mM aqueous CAS Solution 7.5 ml was added slowly under stirring. Further, anhydrous piperazine 4.307g was dissolved in water and 6.25 ml 12M hydrochloric acid was added carefully. The buffer solution maintained at pH 5.6 was rinsed into the volumetric flask. CAS Solution was made up to 100 ml using sterile distilled water. 5- sulfosalicylic acid was then added to the above solution as CAS shuttle solution at a concentration of 4 mM. The solution was stored in polyethene bottles and kept in the dark.

For estimation of siderophore concentration, 0.5ml of CAS solution was added to 0.5ml of culture supernatant of *V. campbellii* grown in the various media. The appearance of colour change from blue to orange indicates the presence of siderophore. The siderophore concentration was measured using UV-visible Spectrophotometer (Perkin Elmer) at 630 nm. The amount of siderophore produced was measured using the following formula:

$$\boldsymbol{Siderophore(SU)\% = \frac{Ar - As}{Ar \times 100}}$$

Where Ar = Absorbance of reference at 630 nm (CAS reagent), As = Absorbance of the sample at 630 nm.

Statistical analysis

The significant differences in siderophore production by various media and among the various periods of incubation were determined by one-way ANOVA followed by Tukey's post-hoc analysis (SPSS version 13).

3. RESULTS

V. campbellii exhibited significant growth on all the 11 media evaluated during the current study (Table 2). The maximum growth was observed on MM9 native medium, followed by MM9 medium with low glucose (0.08%) and MM9 medium with high glucose strength (1%), while ZMB (half strength) media recorded the minimum growth of *V. campbellii*. During the maximum incubation period of 14 days adopted for this study, significant differences were observed in the growth of *V. campbellii* on the different media. The three MM9 media exhibited a diauxic growth pattern (figure 1).

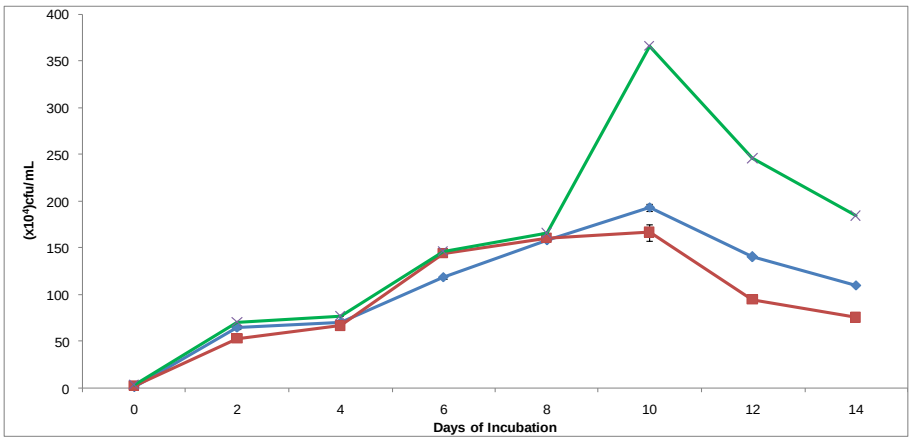

Fig. 1: Diauxic growth pattern of *V. campbellii* grown on three different MM9 media *(blue line, M5 (MM9 medium with low glucose 0.08%); red line, M6 (MM9 medium with high glucose strength 1%); green line, M8 (MM9 Native medium)*

Table 2: The growth pattern of *V. campbelli* on various media after incubation of 0 to 14 days

Growth Media	(x10⁴) cfu/mL							
	0 Day	2 Days	4 Days	6 Days	8 Days	10	12	14
M1	2	52	80	84	84	86	73	52
M2	1	43	55	58	56	50	39	38
M3	2	24	50	55	62	59	41	33
M4	1	34	45	52	58	64	48	35
M5	1	65	70	119	158	193	140	110
M6	2	53	66	144	160	166	95	75
M7	2	24	50	54	63	65	54	31
M8	3	70	77	146	165	366	245	184
M9	2	35	51	53	62	63	53	31
M10	1	35	42	52	59	64	52	33
M11	1	44	51	56	74	76	58	48

M1, ZMB (full strength); M2, ZMB (half strength); M3, Modified Succinate deferrated medium; M4, IDSM{1mMFe(III) fortified}; M5, MM9 medium (with low glucose 0.08%); M6, MM9 medium (with high glucose strength 1%); M7, BOSS medium; M8, MM9 Native; M9, R2A medium without Iron; M10, R2A medium with 0.001% Iron; M11, MM9 (+HEPES)

Among the different growth media, maximum biomass of *V. campbellii* was recorded on MM9 native media after 14 days of incubation (98.685 mg/mL), while ZMB (half strength) media exhibited minimum biomass (6.42 mg/mL) even after 14 days of incubation (Table 3).

Table 3: Increase in biomass concentration of *V. campbelli* on various media after incubation of 0 to 14 days

Growth Media	mg/mL							
	0 Day	2 Days	4 Days	6 Days	8 Days	10	12	14
M1	0	2.28	4.63	4.61	5.45	6.24	6.74	7.38
M2	0	2.10	4.73	4.65	5.68	6.42	7.32	7.49
M3	0	1.05	5.50	8.70	9.44	10.45	10.76	9.96
M4	0	0.77	5.57	6.59	7.77	8.39	9.00	8.33
M5	0	3.06	12.27	13.31	57.19	56.98	61.05	62.03
M6	0	3.03	9.60	12.07	35.25	47.87	49.04	48.72
M7	0	2.45	4.67	5.43	9.15	12.52	13.22	12.53
M8	0	3.38	12.55	17.98	67.85	98.69	101.38	101.94
M9	0	1.06	3.74	4.97	10.97	22.50	22.72	22.55
M10	0	1.28	2.73	3.63	4.71	7.22	7.84	8.52
M11	0	2.39	8.66	11.52	12.34	15.24	16.06	16.47

M1, ZMB (full strength); M2, ZMB (half strength); M3, Modified Succinate deferrated medium; M4, IDSM{1mMFe(III) fortified}; M5, MM9 medium (with low glucose 0.08%); M6, MM9 medium (with high glucose strength 1%); M7, BOSS medium; M8, MM9 Native; M9, R2A medium without Iron; M10, R2A medium with 0.001% Iron; M11, MM9 (+HEPES)

Among the 11 growth media evaluated for their role on siderophore production by *V. campbellii*, maximum siderophore production was recorded on MM9 native media after 8 days of incubation, while MM9 (+HEPES) media exhibited minimum siderophore production even after 14 days of incubation. The maximum siderophore production by *V. campbellii* on MM9 native medium was observed during late log phase. The analysis of siderophore production using CAS assay is depicted in Figure 2 & Figure 3. On day 0, there was no significant difference in siderophore production among any of the 11 media ($p>0.05$). However, during all the other incubation periods, there was a significant difference in siderophore production among the 11 media ($p<0.05$).

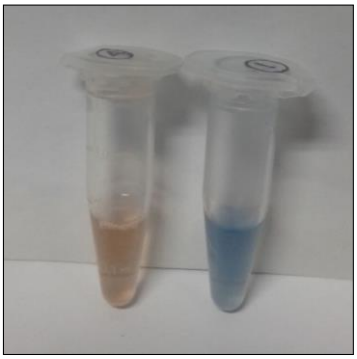

Fig. 2: Colour change (blue to orange) exhibited during CAS assay by *V. campbellii* grown on MM9 media. The blue coloured vial is the control CAS solution.

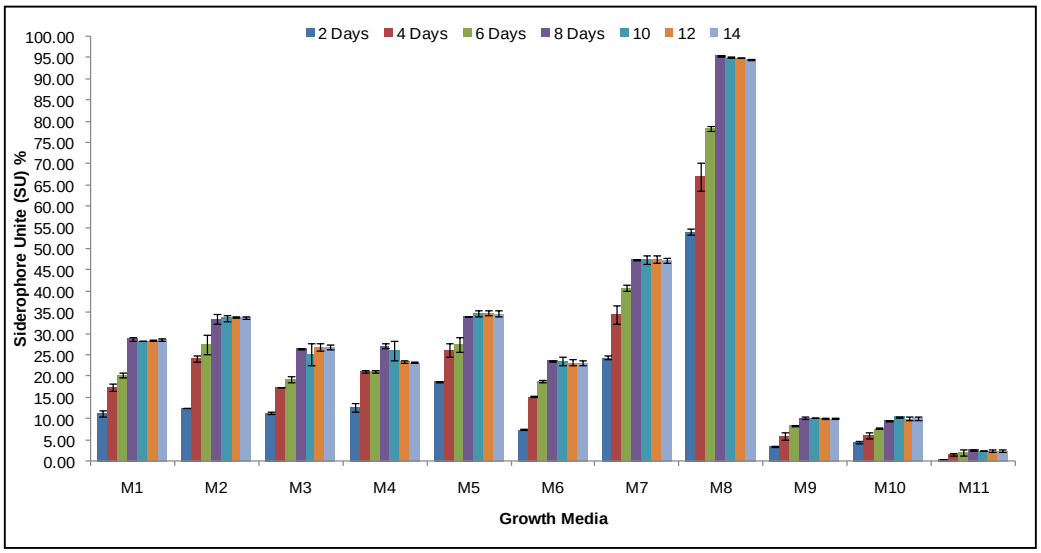

Fig. 3: Differences in siderophore production by *V. campbellii* grew on 11 different growth media

M1, ZMB (full strength); M2, ZMB (half strength); M3, Modified Succinate deferrated medium; M4, IDSM{1mMFe(III) fortified}; M5, MM9 medium (with low glucose 0.08%); M6, MM9 medium (with high glucose strength 1%); M7, BOSS medium; M8, MM9 Native; M9, R2A medium without Iron; M10, R2A medium with 0.001% Iron; M11, MM9 (+HEPES)

4. DISCUSSION

The siderophore production in bacteria is mostly mediated by the nutrient source and growth conditions, along with the genetic make-up. The growth media provides the bacteria with most of its nutrient requirements and hence play a major role in siderophore production, as well. Various growth media have been found to be suitable for maximum siderophore production by different types of bacteria. For example, *Pseudomonas aeruginosa* FP6 exhibited maximum siderophore production in succinate medium (Sasirekha and Srividya, 2016). Many bacilli have also exhibited high siderophore production using succinate media (Santos et. al., 2014). *Pseudomonas flurosces* and *P. putida* produced hydroxamate siderophore under the iron-limiting condition in the modified succinate medium (Sayyed et al., 2005). The common aspect of all these media is the low iron content in their composition.

In the current study, the growth and siderophore production potential of *V. campbellii* was evaluated using 11 different media. *V. campbellii* is closely related to *V. harveyii* and has been found to be pathogenic to aquatic organisms like shrimp (Wang et al., 2015). Thiosulfate-citrate-bile salts-sucrose (TCBS) agar has been widely used as a medium for isolation and enumeration of Vibrio species from marine and estuarine ecosystems. However, it has also been found that TCBS agar is inhibitory to many of the Vibrio species occurring in the marine environment. Subsequently, a non-inhibitory medium was developed for the selective enumeration and isolation of Vibrionaceae in seawater (Simidu and Tsukamoto, 1980, Harris et al., 1996). Later, with an increase in pathogenic outbreaks caused by *V. harveyi* and *V. campbellii*, various efficient growth media were developed to facilitate early detection and employment of preventive measures.

In view of the fact that the siderophore production potential of *V. campbellii*, along with biomass production is envisaged, 11 different media with nil to varying proportions of iron were selected for the present study. As anticipated, only 3 [MM9 medium (with low glucose 0.08%), MM9 medium (with high glucose strength 1%) and MM9 native medium] out of 11media selected exhibited considerable increase in growth and biomass production during an incubation period of 14 days, with MM9 native medium recording the highest growth and biomass production. While all the selected media have been previously used for enumeration of marine bacteria (Zobell et al., 1935; Reasoner et al., 1985; Klein et al., 1998; Guan et al., 2001; Sayyed et al., 2005; Muruggapan et al., 2012), the relatively higher growth and biomass production exhibited by MM9 media could be attributed to their specific glucose concentrations and diauxic growth. Glucose has been reported to cause diauxic growth in *Pseudomonas fluorescens* grown in LB medium (Jonathan and Richard, 2011). Another important constituent of MM9 media facilitating higher growth could be casamino acid, which provides a completely hydrolyzed protein nitrogen source. Growth rates of cultured bacteria have been reported to be highest in complex media and in media supplemented by amino acids (Marr 1991).

Subsequently, the maximum siderophore production was also exhibited by MM9 native medium. There are only a few earlier reports that acknowledge the siderophore-production potential of MM9 medium. Soto-Rodriguez et al. (2012) used iron-deficient MM9 broth to elucidate the siderophore-production in *V. harveyi*. The siderophore-producing bacteria, *Xylella fastidiosa* was also grown in the iron-limiting condition in MM9 medium (Stenico et al., 2005) and *Pseudomonas putida* was standardized for siderophore production using MM9 medium with the different condition of carbon, nitrogen, and amino acid sources (Murugappan et al., 2012).

The higher siderophore production by MM9 native over the other two media exhibiting relatively higher growth of *V. campbellii* (MM9 medium with low glucose 0.08% and MM9 medium with high glucose strength 1%) could also be attributed to the concentration of glucose in MM9 native medium (0.4%) along with iron depletion. The mode of action needs to be further explored. Also, the relatively higher growth and biomass production by *V. campbellii* grown in MM9 native medium could have possibly resulted in higher siderophore yield. In general, the minimal media, MM9 resemble the marine environment in its composition and this could have enhanced the biological potential and siderophore production efficiency of *V. campbellii* (Gram, 1996; Murugappan et al., 2012). Further, the maximum siderophore production during late log phase by *V. campbellii* indicates the critical demand for iron during this particular phase of the growth cycle of the bacterium. Similar observations have earlier been reported for *Pseudomonas putida* (Murugappan et al., 2012).

Another interesting observation during the present study was the considerable yield of siderophore (~ 48% SU) by *V. campbellii* grown on a BOSS medium, in spite of relatively low growth and biomass production. Klein *et al.,* 1998 has reported the use of BOSS Medium specifically for luminescent bacteria. In this case, we have not observed considerable growth or biomass production, but only siderophore production was relatively high. One possible cause could be that *V. campbellii* exhibited synchronous growth in BOSS medium, such that at any given point of time, all the bacterial cells would be in the same stage of the cell cycle (Harvey, 1972). In such a scenario, the siderophore production of the total cell population would be depicted during a given time. Normally, the cells in culture would be in various stages of the growth phase and therefore, the siderophore produced may be the effort of only a certain percentage of the population.

The growth and siderophore production potential of *V. campbellii* on 11 different growth media were evaluated and significant differences were observed. Among the selected media, only three media, namely, MM9 medium with low glucose (0.08%), MM9 medium with high glucose strength (1%) and MM9 native medium, exhibited a considerable increase in growth and biomass production during an incubation period of 0 to 14 days. This observation is attributed to the specific glucose concentrations of the MM9 media, resulting in diauxic growth and also the presence of casamino acid, a rich source of nitrogen for the bacteria. MM9 native medium exhibited the highest siderophore production potential, followed by the BOSS medium. The siderophore production efficiency of MM9 native medium is attributed to its specific glucose concentration (0.4%) and iron depletion. In the case of a BOSS medium, the considerable yield of siderophore in spite of relatively low growth and biomass yield is hypothesized to be the result of synchronous growth.

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