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Effects of growth conditions on siderophore producing bacteria and siderophore production from Indian Ocean sector of Southern Ocean

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Iron is an important element for growth and metabolism of all marine organisms, including bacteria. Most (99.9%) of iron in oceans are bound to organic ligands like siderophores and siderophore-like compounds. Distribution of bioavailable iron mainly depends on pH and temperature of the ocean. Due to global warming and ocean acidification, bioavailability of iron may alter and in turn effect the response of marine bacteria. In this study, we investigated the effect of growth conditions like pH, temperature, and iron (III) concentrations on growth and siderophore production in selected heterotrophic bacteria isolated from waters around Kerguelen Islands (KW) and Prydz Bay (PB). Microcosm experiments were carried out on two KW-isolates (*Enterococcus casseliflavus* and *Psychrobacter piscatorii*) and five PB-isolates (*Pseudoalteromonas tetraodonis*, *Bacillus cereus*, *Psychrobacter pocilloporae*, *Micrococcus aloeverae*, and *Pseudomonas weihenstephanensis*) which produced either hydroxamate-type or catecholate-type siderophores. Increasing iron concentrations (10 nM to 50 μM) increased the growth rate of all isolates while siderophore production (% siderophore) generally reduced at higher iron concentration. Siderophore production peaked at early log phase, probably in response to higher iron-demand. Temperature and pH experiments showed that most isolates produced more siderophore at 15 and 25 °C temperature and pH 8.5. These results reveal that in future ocean conditions (warmer and acidified waters), bacterial growth and siderophore production may get affected and thereby influencing iron uptake and associated biogeochemical processes.

KEYWORDS
iron, microcosm, siderophore, Southern Ocean, warmer and acidified water

1 | INTRODUCTION

Iron is one of the most important trace elements required for growth of many terrestrial and marine microorganisms [1]. It is required in micro-molar concentrations for growth and essential metabolic activities of microorganisms [2]. Unlike terrestrial environment, concentrations of iron in open ocean is extremely low and its distribution is patchy [3]. Dissolved iron concentration is higher in marine waters closer to land and/or surrounded by land mass as compared to open ocean.

For example, dissolved iron concentration in Arabian Sea is greater and ranges from 1.25 to 60 nM [2] compared to open ocean waters of Indian Ocean sector of Southern Ocean (IOSO) (0.046–0.38 nM) [3].

In recent decades, studies based on high sensitive electrochemical methods suggests that 99.9% bio-available iron in ocean are attached to organic ligands [4]. These are differentiated into two classes viz., organic compounds strongly bound to iron (L1) and is present in photic layers of the water column, while second group of organic compounds (L2) which are weakly associated with iron and abundant in the entire water column [5,6]. Although the structure of these organic ligands is not clear, these compounds are considered to be siderophores or siderophore-like compounds due to their high affinity for iron [7]. Siderophores are low molecular weight organic ligands produced by bacteria (including some cyanobacteria), fungi, and some higher plants [8]. Bacteria produce siderophore for iron sequestration to support it's growth under iron limiting conditions (concentrations $<10^{-5}\,\mathrm{mol\,L^{-1}}$) in environment [8,9]. Some siderophore producing marine bacteria are also reported to stimulate the growth of other bacteria in iron deficient conditions by a process called siderophore signaling [10]. Apart from supporting the bacterial iron-demand, siderophore producing bacteria in iron deficient marine environment may synergistically support primary productivity by providing iron in exchange for organic carbon, thereby fueling the indirect nutrient requirement to support microbial loop [11]. In contrast, siderophore producing marine bacteria were found to compete with phytoplankton for available iron and vitamins, resulting in suppression of phytoplankton growth [12].

Bacterial siderophores are either hydroxamate or catecholate-type siderophores; some bacteria are also reported to produce carboxylate siderophore [13]. Two broad categories of siderophore viz. α-hydroxyaspartic acid or "citric acid" and amphiphilic siderophores have been abundantly reported from ocean regimes [11]. The α-hydroxyaspartic acid-type siderophores are photoreactive siderophores, which are prominent in surface ocean e.g., aerobactin, pertrobactins [14], while amphiphillic types contain peptide heads and fatty acid tails e.g., aquachelins [15].

IOSO waters encompass diverse oceanic regimes warm, nutrient limited frontal waters forming its northern limits to cold nutrient replete polar waters in the south. Nevertheless, these waters are considered to be high-nutrient low-chlorophyll (HNLC) waters wherein iron is considered to be a major growth limiting nutrient of phytoplankton [16]. The IOSO extends from 40°S to the coast of Antarctica and includes various frontal regimes with strong gradients in hydrography and nutrients [17]. Presence of islands like Kerguelen islands and Crozet islands are known to naturally fertilize these waters, resulting in sporadic blooms of phytoplankton [3]. Sequestration of iron by siderophores in these waters can play an influential role in regulating primary production and therefore affect the biological drawdown of atmospheric carbon [3,18]. Siderophore production is influenced by bioavailability of iron and growth conditions (temperature, nutrients, carbon source, etc.). Increasing atmospheric CO_2 levels are reported to cause an increase in the dissolution of CO_2 in surface oceans, especially in the cold waters of Southern Ocean (SO), altering the ocean pH [19]. Such decrease in pH may alter the concentrations of bio-available iron for bacteria in marine waters [20]. However, none of the previous studies in IOSO have highlighted the presence of siderophore producing bacteria or investigated the effect of growth conditions including pH, temperature, and iron concentrations on production of siderophores.

In the present study, we investigate the influence of different concentrations of iron (III), temperature, and pH on the growth and siderophore production of selected bacterial isolates obtained from marine waters near Kerguelen islands (KW) and Prydz Bay (PB).

2 | MATERIALS AND METHODS

2.1 | Sampling area

In Indian Ocean sector of Southern Ocean (IOSO), Kerguelen basin has a surface area of $7000\,\mathrm{km}^2$ swept by Antarctic Circumpolar Current (ACC), that is rich in nutrients [3] and naturally fertilized with iron from the islands, resulting in sporadic phytoplankton blooms while PB is the third most productive bay around Antarctica [21]. Sampling locations were selected based on the proximity to land, nutrient rich waters, possible higher iron inputs due to land mass effect (KW), and sea ice melting (PB). Moreover, both KW and PB stations reflect different regimes based on differences in ambient temperature, salinity, and nutrients.

2.2 | Sampling strategy

Seawater samples were collected from two stations each from PB (68°S 72°E and 68°S 76° E) and KW (47°S 64°E and 51°S 64°E) during the 9th Indian Expedition to SO (ISOE-9) onboard research vessel *SA Agulhas* in austral summer 2016–2017 (Fig. 1). Sea water samples were collected using clean 10 l Niskin bottles attached to CTD rosette equipped with Sea-Bird CTD system (SBE911 plus, Sea-Bird Electronics, USA) from three different depths (0 m, Deep Chlorophyll Maxima (DCM) and 200 m). Aliquots of water samples were collected for total cultivable bacteria (measured as colony forming units, CFU), isolation of siderophore producing bacteria, total bacterial abundance, chlorophyll *a*, and nutrients.

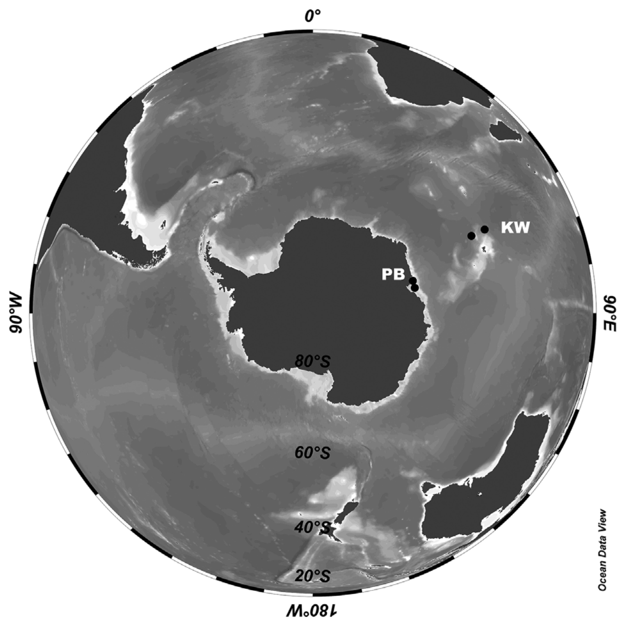

FIGURE 1 Sampling locations in Kerguelen waters (KW) and in Prydz Bay (PB) during 9th Indian Southern Ocean Expedition during austral summer 2017

2.3 | Oceanographic sample analysis

2.3.1 | Chlorophyll *a* (Chl *a*) in KW and PB waters

Three liters of water sample were collected, filtered through GF/F (Whatman, UK) filters paper, and transported in −20 °C to laboratory. For Chl *a* analyses, frozen samples were placed in 15 ml centrifuge tubes and extracted overnight at 4 °C using 10 ml of 90% acetone. The extracted samples were then transferred to 10 ml cuvette and measured fluorometrically using Turner's AU 10 Fluorometer (Turner Designs Inc., USA). Calibration was carried out using standard chlorophyll *a* pigment (Sigma–Aldrich, USA) as described [22].

2.3.2 | Total bacterial abundance in KW and PB waters

Aliquots of water samples (10 ml) were collected from different depths, fixed with 0.2 µm filtered formalin, and stored at 4 °C until analysis in laboratory. Samples were stained with DAPI (4′, 6-diamidino-2-phenylindole) and filtered through 0.22 µm pore size black polycarbonate membranes (BTBP, 2500, Millipore) placed on a support filter 5 µm polycarbonate membrane (GTTP, 2500, Millipore). After filtration black polycarbonate filter paper was mounted on glass slide with immersion oil for bacterial enumeration under epifluorescence microscope (Olympus BX 63, Japan) and counted at 100× magnification [23].

2.3.3 | Nutrient analyses of KW and PB waters

Aliquots (250 ml) of nutrient samples were collected in acid-cleaned bottle. Sample were stored at −20 °C and brought to the laboratory for analyses. The samples were thawed, brought to room temperature, and analyzed for dissolved nutrients (nitrate, nitrite, phosphate, silicate) using automated segmented nutrient analyzer (Seal UK; Model AA3), following standard colorimetric methods [24]. Standards were used to calibrate the auto-analyzer and frequent baseline checks were made. The standard deviation for all nutrients analyzed was ±1% and linearity of calibration curve yielded a correlation coefficient of $R^2 > 0.9990$.

2.4 | Culturable bacteria from KW and PB waters

Total heterotrophic culturable bacterial load in the sample was determined by serially diluting 100 µl sample into different dilution (10^3, 10^5, and 10^7) and spread on Zobell Marine Agar (ZMA, Hi-Media, India). The plates were incubated at room temperature (20 ± 2 °C) in the on-board laboratory. All well separated colonies were picked randomly on the basis of different morphology. All possible selected bacterial colonies were purified by re-streaking on ZMA plates. The cultures obtained were preserved at 4 °C (on-board) and used for screening siderophore producing bacteria in laboratory.

2.4.1 | Screening of siderophore producing bacteria from KW and PB waters

Siderophore producing bacterial isolates were separated from total culturable colonies by Chrome Azurol S (CAS) agar plate methods [25]. CAS agar plates incubated for 5 days at incubation temperature 22 ± 2°C. The CAS solution assay [9] was used to quantify siderophore activity in culture supernatant extracts by measuring the decrease in the absorbance of blue color at 630 nm (Basic Spectrophotometer, Eppendorf, Germany).

2.4.2 | Culture conditions and media for siderophore production

Iron deficient sea water-based media (IDSM) [26] was used for the growth experiments. The composition of IDSM included (in 1 L) NH_4NO_3 0.25 g; NaCl 7.5 g; $MgSO_4.7H_2O$ 0.125 g; KCl 0.075 g; K_2HPO_4 0.375 g; $CaCl_2$ 0.05 g and $C_8H_{18}N_2O_4S$ (HEPES) 2.38 g [8,27]. The medium was adjusted for pH and was de-ferrated by passing through a Chelex-100 (Sigma–Aldrich, USA) [28]. Filter sterilized (0.2 µm membrane filter) solutions of glucose (0.1% final concentration) and $FeCl_3$ were added to autoclaved de-ferrated IDSM. In order to remove iron contamination from the glass wares, all glass wares were soaked overnight in 6 N HCl followed by washing with deionized water.

2.4.3 | MM9 media

Bacterial strains were grown in modified M9 medium (MM9) without added iron [29] to test for siderophore production in isolates. Media comprised of KH_2PO_4 0.3 g, NaCl 0.5 g, NH_4Cl 1 g, $MgSO_4.7H_2O$ 493 mg, $CaCl_2$ 11 mg, $MnSO_4.H_2O$ 1.17 mg, H_3BO_3 1.4 mg, $CuSO_4.5H_2O$ 0.04 mg, $ZnSO_4.7H_2O$ 1.2 mg, $Na_2MoO_4.2H_2O$ 1 mg, cas-amino acids 3 g, PIPES 30.24 g, $FeCl_3.6H_2O$ 10 µM, EDTA 3.27 mg and glycerol 5 g. Siderophores were measured using chrome azurol S (CAS) assay [30].

2.4.4 | Assay for hydroxamate siderophore

Tetrazolium and Csáky tests were used for Hydroxamate siderophore assay. In tetrazolium assay, pinch of tetrazolium salt (~500 mg) was mixed with two drop of 2N NaOH and 0.1 ml of sample broth. Appearance of red color indicated Hydroxamate-type siderophore positive culture [31]. Csáky assay was used for confirmation of Hydroxamate siderophore production. Equal volumes of sample culture broth and 6 N sulfuric acid were mixed and autoclaved at 121 °C for 30 min. Subsequently, 1 ml of sulfanilic acid (1% w/v) prepared in acetic acid 30% (v/v) with 0.5 ml iodine (1.3% w/v) was mixed and this mixture was incubated for 5 min at room temperature. Further, excess iodine was removed by trisodium arsenite (Na_3AsO_2) (2% w/v) prepared in water. Finally, 1 ml solution of α-naphthylamine (0.3% w/v) was added to the solution resulting in change of color from orange to red which was measured at 256 nm absorbance after 30 min of incubation at room temperature [32].

2.4.5 | Assay for catecholate siderophore

Neilands' and Arnow's assays were used to confirm catecholate siderophore production. In Neilands' assay, reagent when added to the culture formed wine color complex in the presence of $FeCl_3$ which was then measured at 495 nm [33]. Arnow's assay was used as confirmatory test for catecholate-type siderophore production. In this assay 1 ml of sample filtrate was mixed with 1 ml of 0.5 M HCl. Further, 1 ml of nitrate molybdate is added to make the mixture solution turn yellow. Thereafter, 1 ml of 1 M of NaOH was added, mixed and incubated for 5 min at room temperature and the solution was measured spectrophotometrically at 510 nm [34].

2.5 | Growth conditions

2.5.1 | Effect of iron concentrations on growth and siderophore production

Selected bacterial isolates were grown in different iron concentration (10 nM, 100 nM, 1 µM, 10 µM, and 50 µM) in 250 ml acid cleaned flask containing IDSM. Twenty-four hour old bacterial cultures were used for inoculation of each flask and incubated for 360 h at 20 ± 2 °C and 200 rpm in an incubator. This experiment was used to ascertain the incubation time and iron concentration required for maximum siderophore production, which was then used for other experiments given below.

2.5.2 | Effect of temperature on siderophore production

The selected bacterial isolates were inoculated in MM9 media [25] and incubated at different temperature (5 ± 1, 15 ± 1, and 25 ± 1 °C) for 120 h at 200 rpm and siderophore production was analyzed.

2.5.3 | Effect of pH on siderophore production

Bacterial cultures were inoculated in MM9 media set at different pH (5.5, 6.5, 7.5, and 8.5) using 1N HCl and 1 N NaOH. The growth experiment was carried out for 120 h at 20 ± 2 °C in shaker incubator set at 200 rpm.

In all the above experiments, negative controls (plain media without any cultures) were also incubated for the said incubation time and under same conditions. All the experiments were carried out in duplicate, the samples for growth and siderophore production were analyzed spectrophotometrically (Basic Spectrophotometer, Eppendorf, Germany) at 540 nm and by CAS liquid assay at 630 nm [9], respectively.

2.6 | DNA isolation and 16S rDNA sequencing

Bacterial DNA from the selected cultures were isolated by GeneElute Bacterial genomic DNA kit (Sigma–Aldrich, USA) and 16S rRNA gene were amplified using Universal primers 27F (5′AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′TACGGTTAACCTTGTTACG ACTT-3′) [35]. The PCR reaction was carried out using Emerald Amp GT PCR Master Mix (Takara Bio Inc., Japan). DNA amplification was carried out by pre-denaturation at 95 °C for 5 min, followed by 25 reactions of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 1 min. Post-extensions were carried out for 10 min at 72 °C using CFX96 PCR (Bio-Rad Laboratories, US). Amplified PCR products were purified by PureLink PCR Purification Kit (Invitrogen, India) and sent for DNA sequencing (M/s

Agrigenome, India). The 16S rDNA gene sequences were then subjected to BLAST sequence similarity search to identify the nearest taxa, and then aligned with the sequences from the nearest taxonomic group using the CLUSTAL-X [36] before constructing phylo-genetic trees using MEGA-7.0.9 [37].

3 | RESULTS

3.1 | Oceanographic setting: hydrography, nutrients, Chl *a* and bacterial abundance

The average sea surface temperature (SST) of stations in the KW region was 4.7 °C. The temperature was almost the same (4.7 °C) in the DCM layer and then declined to 2.3 °C at around 200 m. However, in PB, the average SST (1.5 °C) at 0 m) was much lower than KW. At DCM, the temperature was −1.5, and −1.8 °C at 200 m. Average salinity (Table 1) did not vary much between KW and PB waters while nutrient concentrations (nitrate (NO_3), nitrite (NO_2), phosphate (PO_4), and silicate (SiO_4)) were not limiting in these stations at all depths (Table 1). The overall pH of seawater in these stations ranged from 8.08 to 8.28 (Table 1). Based on the Chl *a* concentration, PB water was more productive than KW waters (Table 1). Between the two regions, average Chl *a* concentration in surface, DCM and 200 m were 0.22 ± 0.001 & 0.314 ± 0.071, 0.348 ± 0.079 & 1.390 ± 0.454, and 0.029 ± 0.029 & 0.035 ± 0.005, respectively. The average bacterial abundance varied with depth with greater abundance ($149 \pm 26 \times 10^8$ cells/L in KW and $183 \pm 42 \times 10^8$ cells/L in PB) in DCM and minimal ($67 \pm 20 \times 10^8$ cells/L in KW and $86 \pm 6 \times 10^8$ cells/L in PB) at 200 m. Overall, abundance of bacteria was higher at PB than KW (Table 1).

3.2 | Total culturable bacteria and siderophore producing bacterial isolates

Total culturable bacteria were higher (1.8×10^4 CFU to 3.5×10^4 CFU) in KW compared to PB waters (0.75×10^4 CFU to 1.25×10^4 CFU) and decreased with depth in both locations (Fig. 2). A combined total of 323 morphologically different isolates were selected from KW and PB waters on the basis of colony characteristics and tested for siderophore production following CAS agar assay. In KW, 59 isolates out of 80 isolates (74%) were siderophore positive while in PB 114 out of 243 bacterial isolates (47%) were siderophore positive. All siderophore positive bacterial isolates were subjected to CAS liquid assay for quantification of siderophore production. The average siderophore production (expressed as %) of bacterial isolates in KW ranged from 18 to 40% (mean: 38%) compared to 22–30% (mean: 29%) in PB waters and decreased with depth. Although

TABLE 1 Average oceanographic setup of KW and PB at the sampling stations during ISOE-09 in austral summer, 2017

Location	Depth (m)	Temp. (°C)	pH	Salinity	Chl-a (μg L^{-1})	Bacteria (10^8 cells L^{-1})	NO_3 (μM)	NO_2 (μM)	SiO_4 (μM)	PO_4 (μM)
Kerguelen (KW)	0	4.7 ± 0.004	8.15 ± 0.121	33.8 ± 0.001	0.22 ± 0.001	96 ± 05	21.46 ± 3.94	0.26 ± 0.02	4.52 ± 4.43	1.44 ± 0.09
	DCM	4.7 ± 0.001	8.17 ± 0.083	33.8 ± 0.001	0.348 ± 0.079	149 ± 26	19.93 ± 7.08	0.22 ± 0.05	5.14 ± 4.47	1.39 ± 0.28
	200	2.3 ± 0.001	8.11 ± 0.037	33.9 ± 0.001	0.029 ± 0.029	67 ± 20	26.75 ± 1.90	0.03 ± 0.01	12.28 ± 3.50	1.71 ± 0.02
Prydz bay (PB)	0	1.5 ± 0.319	8.28 ± 0.035	32.8 ± 0.341	0.314 ± 0.071	129 ± 40	18.01 ± 3.10	0.16 ± 0.05	34.04 ± 0.75	1.22 ± 0.23
	DCM	−1.4 ± 0.394	8.21 ± 0.004	34.3 ± 0.053	1.390 ± 0.454	183 ± 42	24.61 ± 5.53	0.13 ± 0.06	42.28 ± 10.84	1.66 ± 0.32
	200	−1.8 ± 0.041	8.08 ± 0.003	34.5 ± 0.011	0.035 ± 0.005	86 ± 06	31.14 ± 1.50	0.09 ± 0.03	53.08 ± 3.47	2.03 ± 0.16

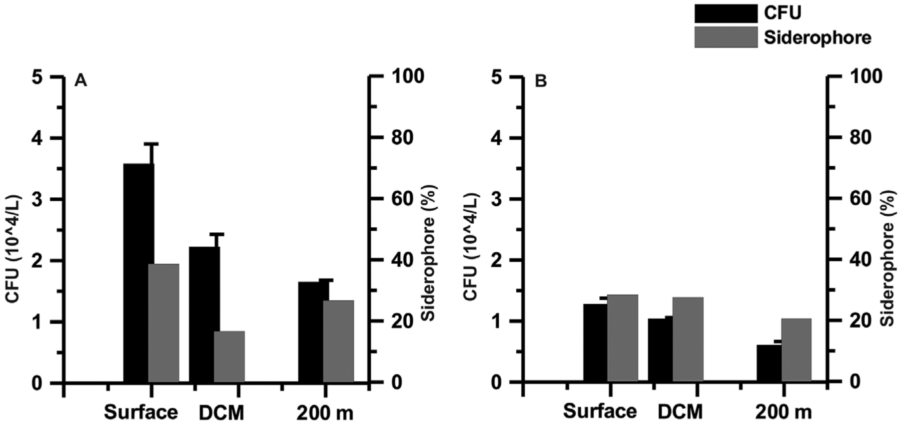

FIGURE 2 Vertical distribution of average colony forming unit (CFU) and average siderophore production by isolates obtained from surface, Deep Chlorophyll Maxima (DCM) and 200 m from (A) Kerguelen waters and (B) Prydz Bay

average siderophore production of KW was higher than PB, siderophore production in PB waters showed less variation with depth. Interestingly, maximum siderophore production was in surface waters compared to DCM. In PB, siderophore production in surface and DCM was similar and lowest at 200 m. Based on the siderophore production and growth, seven bacterial isolates (two from KW and five from PB) were selected for microcosm experiments.

3.3 | Identification of selected KW and PB isolates

Phylogenetic analysis based on 16S rRNA gene sequences indicated that seven selected siderophore producing bacteria is affiliated to three class namely γ-Proteobacteria, Bacilli, and Actinobacteria (Table 2). >98% Pairwise sequence similarity showed a coherent cluster with the closest strains related to *Enterococcus casseliflavus* (KW1) and *Psychrobacter piscatorii* (KW2). Bacteria isolated from KW belonged to Bacilli and γ-Proteobacteria, respectively. *Pseudoalteromonas tetraodonis* (PB1), *Bacillus cereus* (PB2), *Psychrobacter pocilloporae* (PB3), *Micrococcus aloeverae* (PB4), *Pseudomonas weihenstephanensis* (PB5) (Fig. 3) isolated from PB belonged to γ-Proteobacteria, Bacilli and Actinobacteria.

3.4 | Identification of siderophore

All bacterial isolates grown on CAS agar medium produced an orange "halo" surrounding the bacterial colonies, which is indicative of the production of a Fe-binding compound. The selected siderophore producing isolates produced ≥60% siderophore when tested with CAS liquid assay. Both isolates from KW (KW1 and KW2) and two isolates from PB (PB1and PB3) tested positive with Csàky assay, confirming that these bacteria produced Hydroxamate-type siderophores. Rest three isolates from PB (PB2, 4 and 5) tested positive with Neilands and Arnow's assay for catecholate-type siderophores (Table 2).

3.5 | Effect of iron on growth and siderophore production

3.5.1 | Growth curve

The growth curve of KW isolates and PB isolates at five different iron concentrations is shown in Figs. 4 and 5 (A–E), respectively. The growth of all bacterial isolates at low iron concentration (10 nM, 100 nM, and 1 μM) was characterized by long lag phase of around 120–150 h

TABLE 2 Details of seven selected siderophore producing bacterial isolates used for the microcosm experiments

Strain	Accession number	Closest homolog	Pairwise similarity	Class	Siderophore production
KW-1	NR 114778.2	*Enterococcus casseliflavus*	97.85	Bacilli	Hydroxamate
KW-2	NR 112807.1	*Psychrobacter piscatorii*	99.26	γ Proteobacteria	Hydroxamate
PB-1	NR 041787.1	*Pseudoalteromonas tetraodonis*	98.33	γ Proteobacteria	Hydroxamate
PB-2	NR 113266.1	*Bacillus cereus*	100	Bacilli	Catecholate
PB-3	NR 153729.1	*Psychrobacter pocilloporae*	99.63	γ Proteobacteria	Hydroxamate
PB-4	NR 134088.1	*Micrococcus aloeverae*	99.56	Actinobacteria	Catecholate
PB-5	NR 148764.1	*Pseudomonas weihenstephanensis*	99.79	γ Proteobacteria	Catecholate

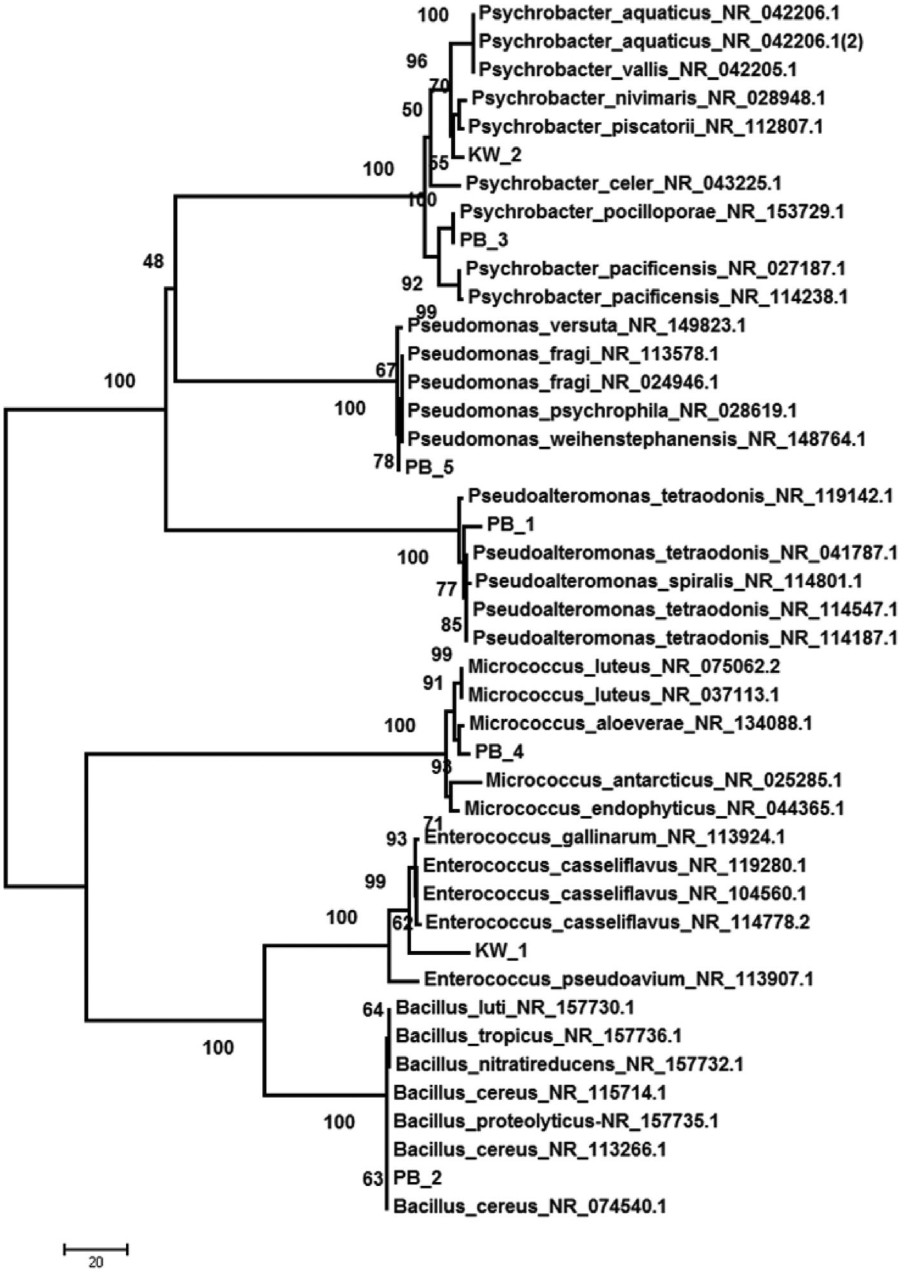

FIGURE 3 Neighbor-joining 16S-rRNA gene sequence tree showing the phylogenetic relationship of KW and PB strains and related taxa. Bootstrap replications were analyzed with uniform rate among sites MEGA-7.0.9

followed by log phase of up to 150–200 h. Stationary phase was attained within 250–300 h of incubation. At higher iron concentrations (10 and 50 μM), a short lag phase (50–100 h) and extended log phase (200–300 h) was observed.

Between KW and PB isolates, KW isolates had shorter lag phase at low iron concentration followed by longer log phase than PB isolates. In PB isolates, only *Pseudomonas weihenstephanensis* had relatively shorter lag phase at low iron concentration. At higher concentration of iron, growth pattern remained similar for all isolates.

3.5.2 | Siderophore production

Siderophore production expressed as % siderophore in five different iron concentrations is shown in Figs. 4 (KW) and 5 (PB) (F–J), respectively. The average siderophore production in KW and PB bacteria was higher at lag phase and early log phase (50–120 h) of bacterial growth, while the % siderophore concentration decreased with incubation time and increase in iron concentration. Maximum siderophore production was observed at low iron concentration (10 nM) and after 120 h of incubation.

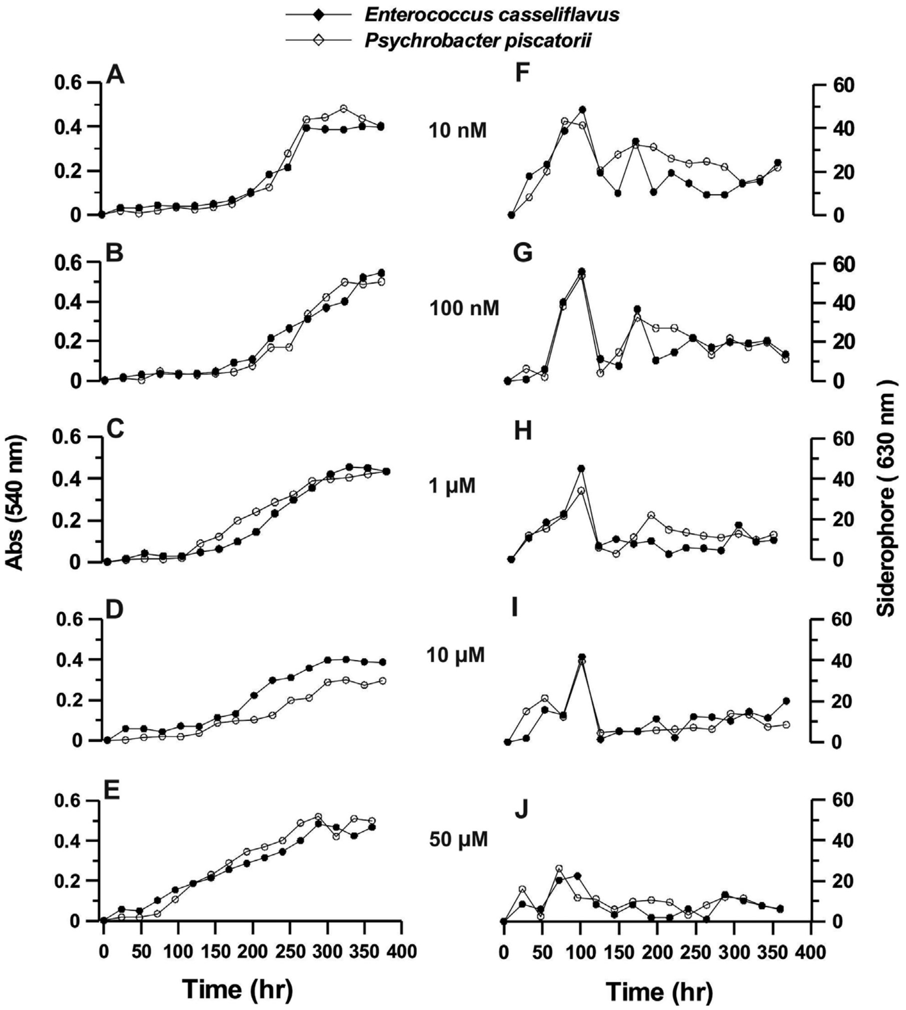

FIGURE 4 Growth (A–E) measured at 540 nm and siderophore production (F–J) measured at 630 nm of bacterial isolates obtained from Kerguelen waters (KW) grown at different iron concentrations (10 nM, 100 nM, 1 µM, 10 µM, and 50 µM)

3.6 | Effect of temperature

KW and PB isolates grown at three different temperatures (5, 15, and 25 °C) for 120 h showed varying growth responses (Fig. 6A–C). Bacterial growth increased from 0.1 to 0.3 OD at 5 °C to 0.2 to 0.6 OD at 15 °C, and 0.3 to 0.8 OD at 25 °C. Although cell density was least at 5 °C as compared at 15 and 25 °C, % siderophore was always >30% at all growth temperatures (Fig. 6D–F). The isolates from KW showed similar growth and siderophore production at different temperature compared to PB isolates.

3.7 | Effect of pH

At lower pH (5.5 and 6.5), bacterial growth (<0.2 O.D.) and siderophore production (<30%) was suppressed in all isolates (Fig. 7A, B, E, and F). At pH 7.5, all bacterial isolates except *P. tetradonis* (Fig. 7C) growth increased (>0.2 OD) while at pH 8.5 (Fig. 7D), all isolates flourished (>0.3 OD) after 120 h of incubation. Siderophore production showed an increase at pH 7.5 and ranged between 18% to 30% (Fig. 7G) while at pH of 8.5 siderophore production peaked, ranging from 30 to 60% (Fig. 7H).

The controls for the above experiments did not show any growth.

4 | DISCUSSION

Several marine heterotrophic bacteria have been reported to produce siderophores in marine water; especially in HNLC regions [2,38–40]. Heterotrophic bacteria from KW and PB were chosen as these bacteria belong to polar environment, which are productive and influenced by land mass and sea ice effects (Fig. 2). The observed anomaly in CFU between the two regions could be due to differences in sampling temperature and incubation temperature. KW sampling location is close to northern boundary of polar front in

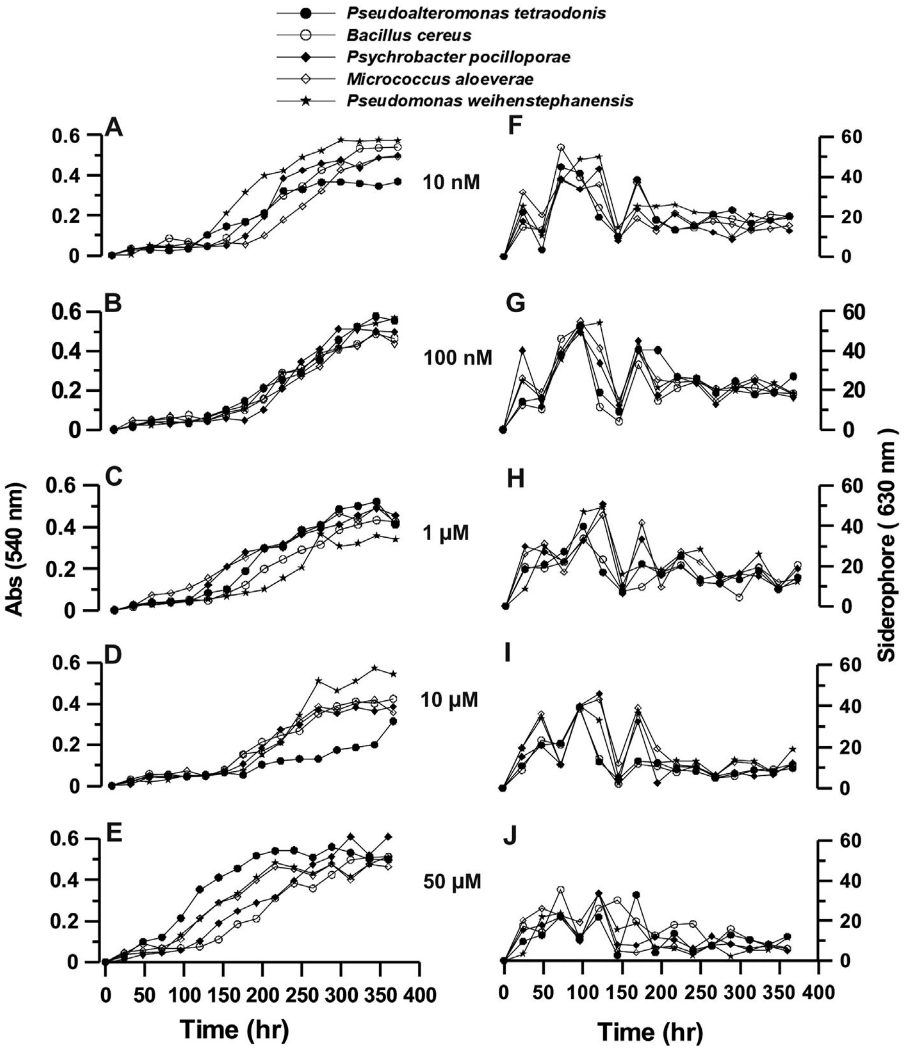

FIGURE 5 Growth (A–E) measured at 540 nm and siderophore production (F–J) measured at 630 nm of bacterial isolates obtained from Prydz bay grown at different iron - concentrations (10 nM, 100 nM, 1 µM, 10 µM, and 50 µM)

IOSO, wherein average SST was 4.7 °C in austral summer while average summer temperature of PB water was 1.5 °C. However, incubation temperature to grow the isolates in laboratory onboard *SA Agulhas* was 20 ± 2 °C, which might be favorable for thermotolerant isolates and may have selectively prevented growth of temperature sensitive bacteria in both locations since both sites had similar nutrient concentrations. Nutrient availability did not appear to influence siderophore production in bacteria. Similarly, maximum siderophore producing isolates were formed in less productive (lower Chl *a*) KW waters. The average siderophore production was greater in KW isolates (38%) compared to PB isolates (29%). Such variability in percentage of siderophore producing bacteria could probably be due to combination of ambient environmental conditions and species specific physiological traits.

Iron concentration strongly influenced the growth of bacterial isolates from KW and PB of IOSO. The growth curve differed for each isolate under different iron concentration, which could be species specific trait. Nevertheless, bacterial growth was significantly delayed in low iron concentrations while siderophore production was greater at lower iron concentrations. The lower iron concentrations (10 nM) is close to ambient iron concentrations and was probably low enough for bacteria to produce siderophore in the early log phase to meet their iron requirements. Iron acts as growth regulating factor throughout the life-cycle of bacteria [41–43]. Most of the bacterial isolates produced siderophore at early stage of log phase, probably to meet their iron demand for metabolic processes including nucleic acid synthesis and respiration. At higher iron concentrations, the siderophores production was suppressed without reducing the growth rate, which may be due to various reasons. Siderophores like aerobactin (hydroxamate-type) are regulated by specific repressor proteins (Fur protein) which uses Fe (II) as a natural co-repressor of aerobactin operon expression [33].

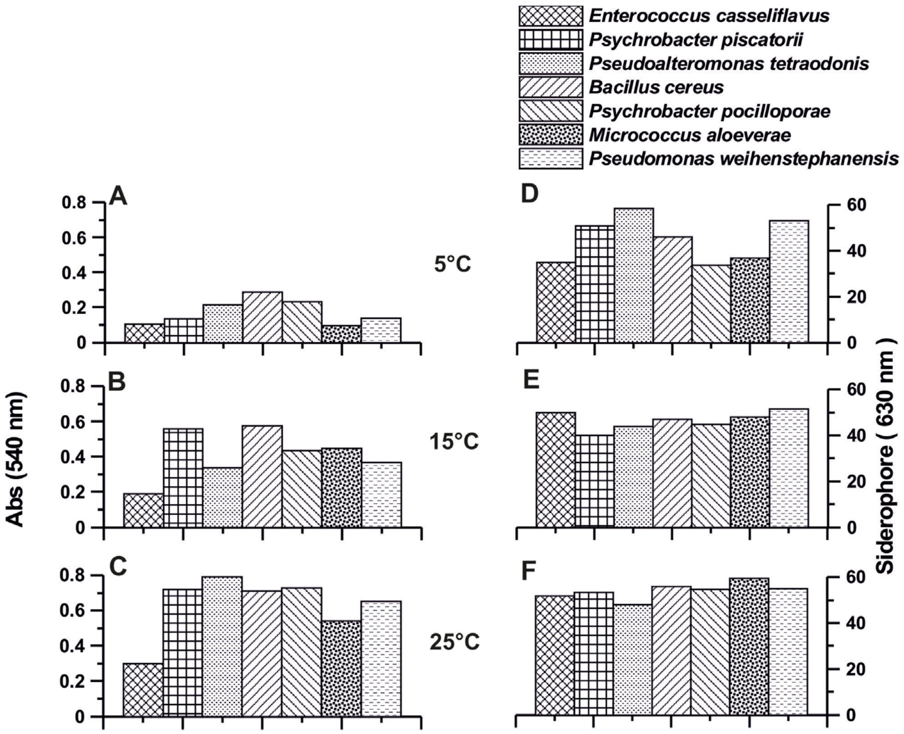

FIGURE 6 Gowth measured as absorption at 540 nm (A–C) and siderophore production as absorption at 630 nm (D–F) of select isolates obtained from KW and PB grown at different temperature (5, 15, and 25 °C)

Similarly, bacteria are also known to acquire iron using alternate pathways like transferrin/lactoferrin, heme uptake system, etc. [44,45]. Nevertheless, the actual reason for the low siderophores expression could not be ascertained in this study.

Changes in incubation temperature affected the growth of all the isolates but did not alter their siderophore production (>30%). All isolates except *E. casseliflavus* showed three- to four fold increase in growth at 25 °C, which could be attributed to species specific physiological traits (Fig. 5). Since all the cultures were isolated at higher incubation temperatures (20 ± 2 °C), it appears that these isolates were mesophiles that could adapt to higher temperatures. Interestingly, siderophore production in these isolates was independent of cell growth as siderophore production did not vary with temperature. The expression of operon controlling Fur repressor protein is reported to be independent of growth temperature in case of pathogenic bacteria [46]. Such a genetic regulation may be one of the reasons for the observed siderophore production even in low growth conditions. Our results also suggest that siderophore production by these isolates (especially PB isolates) might not be very different in the natural environment.

Changes in pH of ambient environment affects both growth of microbes and the bioavailability of iron [47]. Experiments evaluating pH effect clearly showed that pH close to ambient conditions (8–8.2) showed maximum growth and siderophore production (Fig. 7). Siderophores are produced to bind Fe^{3+} which are sparingly available in oxygenated alkaline seawater [48]. In acidic conditions, solubility of Fe^{3+} increases but reduces chelation by organic ligands like siderophores due to increased competition for binding iron from free hydroxide ions [49]. In our experiment, bacterial growth and siderophore production too got affected at lower pH (5.5, 6.5, and 7.5). The decreased siderophore production at lower pH could be a combination of altered bioavailability of iron, effect on iron binding by siderophores and poor growth of bacterial isolates.

In conclusion, iron chelation in polar waters (especially HNLC waters) play a crucial role in sustaining phytoplankton blooms in these waters and may influence the carbon sequestered by the “biological pump” in SO. Our microcosm experiments clearly show that among the three key parameters tested for influencing the bacterial siderophore production, impact of pH was clearly evident amongst all isolates. Increasing atmospheric CO_2 concentrations is expected to lower the ocean pH to 7.8 by year 2100 [50]. Our data suggest that bacterial isolates and siderophore production could adjust to warming conditions but acidification of ocean may drastically affect their growth and ability to chelate iron; and in turn affect the carbon biogeochemistry in polar waters.

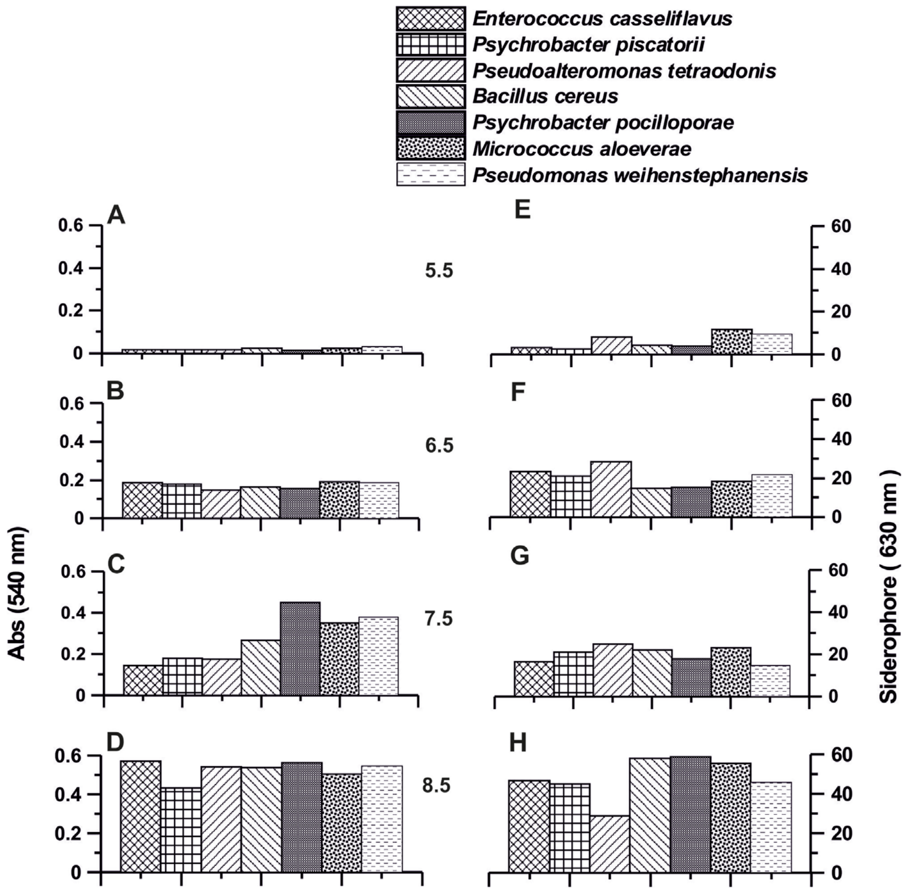

FIGURE 7 Gowth measured as absorption at 540 nm (A–D) and siderophore production as absorption at 630 nm (E–H) of select isolates obtained from KW and PB grown at different pH (5.5, 6.5, 7.5, and 8.5)

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CONFLICTS OF INTEREST

Authors have no conflict of interest, financial or otherwise.

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