Glaciochemistry and pigment producing ability of bacteria from roof of the world, the glaciers of Karakoram, Pakistan

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Research Article

Keywords: Bacterial diversity, carotenoid pigments, geochemistry, glaciers, Karakoram

Posted Date: March 4th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-271782/v1

Abstract

The Karakoram Mountain Range (KMR) is one of the largest mountain ranges in the world, with ~ 37% of its area glaciated. Here, we present the geochemistry of ice, sediment and meltwaters sampled from Ghulmet, Ghulkin and Hopar glaciers of the Karakoram Range, Pakistan, in addition to the first information on the diversity of pigmented bacteria evaluated using culture-dependent techniques. Geochemical analyses revealed Ca^{2+} and SO_4^{2-} to be the most abundant cation and anion species across all glacial samples, respectively. Total organic carbon (TOC), total nitrogen (TN) and total phosphorus (TP) were found in the sediments of all glaciers studied in current research. Bacterial species were capable of producing a variety of different pigments, including alloxanthin, astaxanthin, bacterioruberin, β-carotene, 19'-hexanoyloxyfucoxanthin, peridinin, violacein and zeaxanthin. Culturable bacterial diversity was studied using two molecular biomarkers, 16S rRNA and rpoB gene, with a total of 82 bacterial strains representing 25 genera identified across all glacial samples. This study provides the first characterization of glacier-associated, pigment-producing bacterial communities from the KMR. Findings are important for considerations of alternative sources of conventional pigment production in industrial fields.

Introduction

Around 15 million km^2 (10%) of the Earth's surface is *covered* by glaciers and ice sheets which are characterized by low temperatures (Anesio and Laybourn-Parry 2012; Stibal et al. 2015), which are characterized by low temperatures. In recent times, glaciers and ice sheets have been identified as a biome harboring microorganism that contribute to important biogeochemical processes globally (Hodson et al. 2008; Anesio and Laybourn-Parry 2012). Based on the study of Castello and Rogers (2005), glacier melt releases approximately 1×10^{17} to 1×10^{21} viable microbes at the global scale annually. Glaciers become habitable during summers as water availability leads to occurrence of microflora in various habitats including snow, ice, biofilms and cryoconite holes (Lutz et al. 2016). Among these habitats, cryoconite holes have been the most widely investigated habitat up to now (Edwards et al. 2014). They are formed on glacial surfaces because of melting of inorganic and organic debris into ice driven by solar radiation (Anesio et al. 2009).

Extremophiles existence has been observed in almost all ice phases described from glacial environments (Priscu and Christner 2004; Rafiq 2016, 2020, 2021), such as atmospheric particles (Sattler et al. 2001), snow (Takeuchi et al. 1998), glacier ice (Priscu et al. 2006), accretion ice (Priscu et al. 2006), glacial sediments (Hassan et al. 2017, 2018, 2020a) and basal ice/till mixtures (Skidmore et al. 2000; Foght et al. 2004). Pressure, solar radiation, rock-water contact, pH, ionic strength, reduction potential, moisture and nutrient content are some of the parameters that may differ immensely in supraglacial, englacial, and subglacial environments (Hodson et al. 2017). Such variety of abiotic conditions characteristic of glacial environments likely provides a wealth of niches inhabitable by a diversity of microorganisms. A combination of adaptation strategies for cellular metabolism has been observed in these organisms

(Cavicchioli et al. 2002), such as production of cold active enzymes, antifreeze proteins and exopolymeric substances to cope with cold tolerance (Siddiqui and Cavicchioli 2006; Hassan et al. 2016). Cell membrane fluidity and integrity is vital for membrane functions at low temperatures (Hassan et al. 2020b). It is known from previous studies that organisms cope with cold conditions by decreasing the average fatty acid chain length and increasing the proportion of unsaturated fatty acids, which leads toward the maintenance of cell membrane fluidity (Anesio et al. 2009: Hassan et al. 2016).

In addition, cold habitats with high elevation, are exposed to higher solar radiation especially between 280 and 400 nm (UV radiation) (Rothschild 1999). Increased UV radiation has extreme detrimental effects on microbial life inhabiting cold environments by directly damaging DNA, RNA, proteins, enzymes and lipids (Cockell and Knowland 1999; Rothschild 1999). Detrimental impacts of UV radiation can be evident following direct absorption by biomolecules or via indirect mechanisms, for example, by stimulating the production of reactive oxygen species (ROS) that ultimately cause oxidation of essential cell components (Moline et al. 2013). Accordingly, microbial adaptations have evolved in order to minimize such effects, including pigment production (Libkind et al. 2009). The higher occurrence of pigment production observed in bacteria isolated from ice cores, glaciers and marine surface waters (Foght et al. 2004; Agogue et al. 2005; Zhang et al. 2008) suggests a potential role for pigmentation in bacteria related to adaptation against UV radiation in cold habitats. Most importantly, carotenoid pigments have been found to modulate fluidity of cell membrane in bacteria residing cold environments by UV radiation (Cockell and Knowland 1999; Jagannadham et al. 2000). In addition, studies also reported carotenoid pigments as a protective tool for phytoplankton and non-photosynthetic bacteria in low temperature against UV radiation (Cockell and Knowland 1999).

Bacterial diversity in polar and non-polar habitats across the Arctic (Bottos et al. 2008; Hell et al. 2012), Antarctica (Yergeau et al. 2007; Niederberger et al. 2008) and Himalayas (Pradhan et al. 2010) has been previously examined using culture-dependent and -independent techniques. In addition, glaciers located in non-polar habitats most famously of the Hindu Kush, Karakoram and Himalaya (HKKH) mountain range, have also been studied for the presence of bacterial populations (Branda et al. 2010; Gupta et al. 2015; Rafiq et al. 2017, 2019). Many researchers reported occurrence of bacteria, for example, *Exiguobacterium indicum* (Chaturvedi et al. 2006), *Dyadobacter hamtensis* (Chaturvedi et al. 2005), *Leifsonia pindariensis*, *Bacillus cecembensis* (Reddy et al. 2008), *Cryobacterium roopkundense* (Reddy et al. 2009), *Cryobacterium pindariense* (Reddy et al. 2010), *Paenibacillus glacialis* (Kishore et al. 2010) and *Serratia Marcescens* (Hassan et al. 2019) from various samples of glacial soil, snow, water and sediments. The main purpose of the current research work was to study geochemical properties of glacial samples which were not studied before and the culturable diversity of pyschrotolerant bacteria from glaciers of Karakoram Mountain range, Pakistan, for the first time. Moreover, these bacteria were studied for pigments production and analyzed for the effects of temperature on selected pigments production.

Material And Methods

Sampling sites and processing

Three different glaciers, named Ghulmet glacier (36°12.474 N, 74°29.035 E), Hopar (Bualtar) glacier (36.2108228 N, 74.7724664 E) and Ghulkin glacier (36.42791 N, 74.80659 E), located in the Karakorum Mountain Rang, Pakistan, were selected for this study (Fig. 1). Hopar glacier, also known as Baultar glacier, is positioned between two highest peaks, Diran peak (7257 m high) and Miyar peak (6824 m high), Hunza valley, Karakorum Mountains Range, Pakistan. Its total length is 18 km. In addition, Ghulkin glacier is located in Gojal, one of the biggest counties of the Gilgit-Baltistan, Pakistan. Its name is derived from two local Wakhi words, 'Ghulk', meaning 'well' and 'kin', meaning 'whose'. It starts from the Ultar Sar (north-eastern part).

Glacial ice, sediments and meltwater were sampled aseptically from each glacier. Approximately 300-400 g sediments were collected using sterilized gloves and scoops to avoid sample contamination. Individual samples were immediately placed into sterile Whirl-pack bags (Nasco, Fort Atkinson, WI) (for sediments) and sterilized polyethylene bottles (for ice samples). Temperature using Easy-Read thermometer (Sigma Aldrich) was recorded at the time of sampling (Table 1). All samples were transported on ice to the Department of Microbiology, Quaid-i-Azam University, Islamabad and stored at -20°C for subsequent analyses.

Determination of TOC, TN, TP and major ions content

Concentrations of major anions (Cl^- and SO_4^{2-}) and cations (Ca^{2+}, K^+, Mg^{2+}, Na^+ and NH_4^+) were conducted in all samples using a Dionex ICS-5000 (Thermo Scientific), while NH_3, NO_3^- and PO_4^{2-} were analyzed using a Gallery Plus automated photometric analyzer (Thermo Scientific). Water and ice after melting, were filtered prior all analyses processes. Specific retention times (40 min), an eluent gradient (KOH, MSA, K_2CO_3, and LiOH) as well as a limit of detection between 0.05-10 ppm were used for detection of ions (Rafiq et al. 2019). In addition, for sediment analysis, sediment was digested using aqua-regia (HCl: HNO3 = 3:1) method (Rafiq et al. 2019). Briefly, 15 mL aqua-regia reagent was mixed with 1 g of sediment and allowed to heating at 150°C. After appearance of brown fumes, 3 mL of 5% hypochloric acid were added and kept again at 150°C. Once the white fumes were appeared, mixture was finally filtered and diluted using double deionized water.

Total organic carbon (TOC) and total nitrogen (TN) were measured through FlastEA 1112 nitrogen and carbon elemental analyzer following procedure proposed by Hedges and Stern (1984), whereas, total phosphorus (TP) was determined according the protocol described by Koroleff (1976). Briefly, for TP analysis, samples were added into a solution of oxidizing reagent (g/L: 50 g potassium peroxodisulphate, 30 g boric acid and 15 g sodium hydroxide) and autoclaved at 121°C for 30 minutes and then analyzed by gallery™ plus automated photometric analyzer (Thermo Scientific). Prior to autoclaving, ratio of oxidizing reagent and samples was adjusted as 5:50, respectively. For TN and TOC analysis, 0.1g of sediment was mixed with 2ml of 1M HCL, agitated for 5 min using ultrasonic agitator and the dried at 50°C for 12 hrs. Lastly, dried samples were shifted to tin vials for analysis.

Determination of colony forming units

Colony forming units (CFU) per mL or g for each glacial sample was determined following protocol described by Rafiq et al. (2017). Approximately 100 µL of glacial ice, sediment and meltwater were taken and spread on 9 plates containing bacterial culture medium. A 1:10 ratio was adjusted by adding 1 mL (ice and meltwater) in sterile glass tubes containing 9 mL of normal saline, while 1 g of sediment was added in 10 ml of sterile normal saline. 5 and 25˚C were used for incubation of plates.

After 30 days of initial incubation, the average colony formation units (CFU/mL or g) was counted in order to determine number of culturable bacteria. Bacterial cultures displaying visually different morphologies were selected and further sub-cultured to obtain pure bacterial cultures.

Culturing condition and isolation of bacterial cultures

Isolation of bacterial cultures was carried out using Nutrient agar (NA) (g/L; D(+)-glucose 1, peptone 15, sodium chloride 6, yeast extract 3, Agar 15), Minimal salts medium (MSM) (g/L; yeast extract 3, Potassium phosphate monobasic 3, Magnesium sulphate, anhydrous 0.12, Calcium chloride dihydrate 0.05, Sodium phosphate dibasic 6, Agar 15) and Reasoner's 2A (R2A) (g/L; casein acid hydrolysate 0.5, dextrose 0.5, dipotassium phosphate 0.3, magnesium sulfate 0.024, protease peptone 0.5, sodium pyruvate 0.3, starch soluble 0.5, yeast extract 0.5, Agar 15). After initial incubation for 30 days, the distinct bacterial colonies were taken and sub-cultured using NA, R2A and MSM media. In order to examine the degree of psychrophily of isolates that were isolated at 5˚C, all bacterial isolates were grown at 25˚C for 10 days. Bacterial isolates were preserved in 30% glycerol using Nutrient Broth (NB) (g/L; D(+)-glucose 1, peptone 15, sodium chloride 6, yeast extract 3) for subsequent analyses.

Molecular identification of bacterial isolates

DNA extraction and PCR amplification of target gene

The genomic DNA of all the bacterial isolates was extracted using the Invitrogen PureLink Microbiome DNA Kit (Invitrogen) following the manufacturer’s instructions. T100 Thermal Cycler (Bio-Rad Laboratories, Inc) was used to amplify target genes of all the bacterial isolates. An already prepared PCR Master Mix (2X) (Thermo Fisher Scientific). PCR Master Mix contained all PCR reagents (0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, and 0.4 mM of each dNTP) except templet DNA and primers. In brief, 10 µM of each primer, 0.1 µL of templet DNA (20 ng), 50 µL of 2X PCR Master Mix was mixed with 50 µL nuclease-free water (Thermofisher scientific) to prepare a reaction mixture of 100 µL volume for amplification bacterial genes.

16S rRNAamplification

16S rRNA gene of all the bacterial isolates were amplified using primers 27F and 1492R (Table 2). PCR conditions used for 16S rRNA amplification was adjusted as preliminary denaturation at 94°C for 5 min, then 40 cycles of 94, 56 and 72°C each for 30s and a final step of extension at 72°C for 8 min. The amplified PCR products were confirmed via 1.2% (w/v) agarose gel electrophoresis.

rpoB gene *amplification*

Only those bacterial isolates who were not identified to the species level by 16S rRNA gene sequencing, were subjected to rpoB gene sequencing. Failure of 16S rRNA gene to identify bacterial isolates at species level could be possible of its presence as multiple intragenomic copies that could lead to differences in sequences, which ultimately result in multiple ribotypes identified for a single bacterium (Case et al. 2007). Therefore, many researchers have found the RNA polymerase β-subunit (rpoB) gene sequencing as a powerful tool to identify and classify taxonomically numerous bacterial species because rpoB gene existed as a single copy in genomes of bacteria (Adekambi et al. 2009; Alvarez-Buylla et al. 2012). Amplification of rpoB gene was done using primers rpoB1698F, rpoB2041R, rpoB1531F, rpoB2760R, rpoB2491F and rpoB3554R (Table 2). The rpoB gene was amplified using PCR program set as 94°C for 90s for initial denaturation, followed by 45 cycles of 94°C for 10s, 50°C for 20s and 72°C for 50s, as well as a final extension step at 72°C for 5 min.

Sequencing and phylogenetic tree analysis

The QIAquick PCR Purification Kit (QIAGEN) was used for purification of PCR products. Purified PCR products were then sent for sequencing to MRC PPU DNA sequencing and services, University of Dundee, Scotland, UK. The resultant sequences were trimmed and filtered using BioEdit software (Hall 1999). After quality filtering, remaining sequences were subjected to BLAST (Basic Local Alignment Search Tool) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for similarity index. The most similar sequences, related to the bacterial isolates of this study, were submitted to National Center for Biotechnology Information (NCBI) for getting accession numbers.

Quantitative pigment analysis

Pigment extraction

Pigment producing bacterial isolates were grown in NB for 7 days in 250 mL sterile flasks and incubated at 15°C. In addition, two isolates (GS_1 and GW_1) were incubated at four different temperature (5, 15, 25 and 35°C) in order to examine the effects of temperature on pigment production. These two isolates were able to grow well at all four temperature (5, 15, 25 and 35°C). In addition, their pigment were more intense in colors and found to cover whole petri plates as compare to others. Bacterial cultures grown on solid media, were transferred to 15 mL sterile falcon tubes and weighed. Before this, an empty similar falcon tube was weighed and the subtracted its weight from falcon tubes containing bacterial cultures and so on. Approximately 25 mg of cell biomass was mixed with 5 mL of ice-cold acetone and vortexed for 2 min. The falcon tubes were placed into chilled sonicator bath (<10°C) for 20 min. Following sonication, tubes were placed into fridge for 24 hours to allow extraction. After 24 hours, tubes were vortexed for 2 min and sonicated in chilled sonicator for 20 min. The extracts were filtered through 0.2 µm syringe filter into new 15 mL falcon tubes. Using a glass Pasteur pipette, ~ 1-2 mL of extracts were filtered into 2 mL amber glass screw vails (Thermo Scientific), and capped under nitrogen for storage at -20 until measurement. In parallel, n=3 blanks were prepared as above using extraction solvent.

High-performance liquid chromatography (HPLC) analysis

A revised version of the HPLC method described by Van Heukelem and Thomas (2001) was used to analyze pigment extracts. An Agilent 1100 HPLC (HP, Waldbronn, Germany) equipped with photo-diode array detector (wavelength was set between 350 to 700 nm), C_8 column, chilled auto-sampler/injector slot with 900 µL syringe head, quaternary pump with in-line vacuum degasser and thermo-statted column slot, were used for pigment analysis. Identification and quantification of analyzed pigment was carried out by considering in-line photo-diode array spectra ranging from 350 to 700 nm and compared with analytical standards purchased from Sigma and using R_t (retention time). In the temperature experiment, the correlation between pigment and temperature were calculated using GraphPad Prism 5.0 software.

Results

Geochemistry of glacial samples

The measured concentrations of major ions (anions and cations) as well as TOC, TN and TP content of studied glaciers are given in (Tables 3 and 4). Sediments of all glaciers were found the most enriched for all major ions, TOC, TN and TP followed by meltwater and ice. Hopar glacier sediments showed the highest concentrations of Ca^{2+} (10016 µg kg^{-1}), K^+ (363 µg kg^{-1}), Mg^{2+} (1081 µg kg^{-1}) and PO_4^{2-} (0.7 µg kg^{-1}), whereas Ghulkin glacier sediments had the highest concentrations of NO_3^- (248 µg kg^{-1}), and sediments of Ghulmet glacier were enriched with Na^+ (1595 µg kg^{-1}). K^+ and PO_4^{2-} were not detected in all glacial samples collected from Ghulmet and Ghulkin glacier, respectively. Similarly, ice sampled from Ghulmet glacier did not contain measurable amounts of Mg^{2+} and SO_4^{2-}. PO_4^{2-} was not measurable in glacial ice sampled from from Hopar or Ghulmet glaciers. In addition, Ca^{2+} and SO_4^{2-} were dominant cation and anion in all glacial samples, respectively. NH_3 dominated glacial sediments, followed by water and ice. Overall, TOC was found in higher quantity in glacial samples as compared to TN and TP. The highest TOC, TN and TP were observed in sediments of all glaciers followed by meltwater and ice. TP content was below detection limits in ice from both Hopar and Ghulmet glaciers as well as in meltwater of Ghulkin glacier. TN content of glacial ice (28 µg g^{-1}) was very low as compared to glacial sediments and meltwater.

Molecular identification of bacterial isolates

Bacterial isolates with their homologous bacterial species and accession numbers (16S rRNA and rpoB genes) are given in (Supplementary Tables 1, 2 and 3). In the current study, a total of 82 bacterial isolates were isolated (30 isolates from Ghulmet glacier, 30 from Hopar glacier and 22 bacterial isolates from Ghulkin glacier). All isolated bacteria belonged to 5 different bacterial phyla. Proteobacteria accounted for 66% of total bacterial isolates followed by Actinobacteria (14% bacterial isolates), Firmicutes (10% isolates), Deinococcus-Thermus (6% bacterial isolates) and Bacteroidetes (4% isolates). Forty-eight of 82 bacterial isolates were isolated from glacial sediments, 23 from glacial meltwater, and 11 from glacial

ice. In addition, bacterial isolates GI_3, GI_4, GS_2, GS_3, GS_4, GS_{12}, GS_{14}, GW_2, GW_4, HI_2 and HS_{14} were not identifiable to the species level by 16S rRNA gene sequencing and accordingly, species level rpoB gene sequencing was undertaken (Supplementary Tables 1 and 3).

Quantitative pigment analysis

An overview of quantified and identified pigments produced by bacterial isolates is listed in (Table 5). Of 37 isolates, 26 were confirmed after HPLC to produce 7 different types of pigment including alloxanthin, astaxanthin, bacterioruberin, β,β-carotene, β,ε-Carotene, 19'-hexanoyloxyfucoxanthin, peridinin, violacein and zeaxanthin. Fifty percentage of bacterial isolates were able to produce astaxanthin, 69% β,β-carotene, 15% β,ε-carotene, 26% 19'-hexanoyloxyfucoxanthin, 38% peridinin and 30% zeaxanthin. Alloxanthin was produced only by bacterial isolate HS_8. Only bacterial isolate GS_1 and GW_1 were observed to produce bacterioruberin (138.42 µg g^{-1}) and violacein (352 µg g^{-1}), respectively. Moreover, it has been observed in quantitative analysis that xanthophylls were produced in higher quantities by bacterial isolates as compared to carotenes.

Temperature effects on pigment production by GS_1 and GW_1 are shown in Fig. 2. A significant reduction in pigment quantity was observed in both bacteria isolates with increasing temperature. Pigment produced by GW_1 was 352 µg g^{-1} at 5°C and 81 µg g^{-1} at 35°C. Similarly, a 66% (81 µg g^{-1}) reduction in pigment production was observed when bacterial isolate GS_1 was grown at 35°C, compared to 66% (352 µg g^{-1}) increase production when it was grown at 5°C.

Discussion

The current study provides the first quantitative analysis of major anions and cations of glacial samples collected from Ghulkin, Ghulmit and Hopar glaciers situated in Karakoram Mountain Range (KMR), Pakistan. To the best of our knowledge, only Hodson et al. (2002) has examined major ions and minor elements content of meltwater originating from Batura glacier located in same KMR, Pakistan. Ca^{2+} was found as a major cation in all glacial samples, consistent with the findings of Hodson et al. (2002). Hasnain and Thayyen (1999) have also reported Ca^{2+} as a major cation from Dokriani glacier meltwaters, Himalaya. The excessiveness of Ca^{2+} in rocky glaciers could be the consequence of carbonate weathering coupled with one or all of following; gypsum dissolution, acid hydrolysis accompanied by sulphide oxidation or neutralization of acid aerosols containing NO_3^- and SO_4^{2-} (Hodson et al. 2002). Cations (K^+, Na^+ and Mg^{2+}) were observed in higher concentrations as compared to anions (Cl^-, PO_4^{2-} and SO_4^{2-}) during the present study, from our sample locations on glaciers situated in valleys. Similar trends have also been observed by previous authors, whereby valley glaciers were also shown to contain higher quantities of cations (most importantly Ca^{2+}) as compared to anions quantities (most importantly SO_4^{2-}) (Tranter et al. 1993, 1997). Interestingly, similar results with higher Ca^{2+} and SO_4^{2-} content were also reported from glaciers located in polar regions (Fortner et al. 2005; Yde et al. 2005).

Measurement of total organic carbon, total nitrogen and total phosphorus content of all glacial samples in the current study revealed that glacial sediments were enriched with C, N and P as compare to glacial ice and water. To the best of our knowledge, no previous study has assessed the TOC, TN and TP content of glaciers located in Karakoram Mountain Range. In comparison to values reported from Arctic glacial systems (Nash et al. 2018), TOC and TN concentrations recorded here were noticeably lower (Nash et al. 2018), potentially indicating a deficit in autochthonous and/or allochthonous nutrient accumulation within KMR glacial systems as compared to polar regions (Bradley et al. 2014). However, observed values matched generally with the C, N and P content reported for glacier forefields (Bradley et al. 2014). At present, no information is available on potential allochthonous delivery of nutrient resources to KMR glaciers, nor the potential for autochthonous production within these systems; areas that warrant future research in order to move toward a fully developed understanding of biogeochemical cycles within these changing systems.

During the present study, bacterial isolates were identified using 16S rRNA and rpoB genes sequencing. 16S rRNA gene based identification is commonly used to detect and measure the diversity of bacteria and archaea in variety of habitats (Peixoto et al. 2002; Vos et al. 2012). However, identification of many bacterial isolates at species level were not achieved with 16S rRNA sequencing in the current study, and thus rpoB gene sequencing was employed to expand our dataset. The bacterial species isolated and identified in the present research have been reported from various cold habitats in previous studies (Prasad et al. 2013; Lutz et al. 2015; Anesio et al. 2017; Rafiq et al. 2017). Bacterial isolates were recovered dominantly from glacial sediments, which corroborates with higher nutrient levels and potentially higher temperatures as compared to glacial ice and water (Parnell et al. 2016). It is well understood that Proteobacteria predominantly exist in glacial habitats throughout the world (Xu et al. 2011; Prasad et al. 2013; Lutz et al. 2015; Anesio et al. 2017; Rafiq et al. 2017) as also observed during the present study. Bacterial communities across disparate glacial systems show analogous composition (Prasad et al. 2013; Zhang et al. 2013; Leiva et al. 2015; Cuthbertson et al. 2017; Lee et al. 2017). To the best of our knowledge, we report for the first time bacterial isolates *Sphingobium xenophagum*, *Pseudarthrobacter scleromae* and *Stenotrophomonas maltophilia* from any glacier located in polar or non-polar regions.

In the current work, bacterial species isolated from glacial habitats were thoroughly screened for pigment production. Quantitative identification of pigments produced by bacterial isolates revealed that a large number of isolates were able to produce various pigments including astaxanthin and β-carotene, which act as a super antioxidant by preventing ROS formation resultant from UV radiation (Asker et al. 2018). Similar pigments have been reported from many bacteria inhabiting cold habitats (Lindquist et al. 2003; Zhu et al. 2003; Hong et al. 2008; Im et al. 2008; Asker et al. 2011; Mageswari et al. 2015; Masuelli et al. 2016; Asker 2017, 2018; Kanelli et al. 2018). However, to the best of our knowledge, none of *Paracoccus hibiscisoli* or *Paracoccus carotinifaciens* has been found to produce β-carotene and zeaxanthin. In addition, β-carotene and 19'hexanoyloxyfucoxanthin have not yet been identified from *Brevundimonas nasdae*, *Brevundimonas intermedia* and *Brevundimonas mediterranea* but astaxanthin producing *Brevundimonas vesicularis* has been reported from marine habitats (Asker 2017, 2018). It is well reported

that cold environments are exposed to higher UV radiation and detrimental effects of UVR especially with short wavelengths such as UV-B (280–320 nm) and UV-A (320–400 nm) on the microbial life inhabiting cold habitats are well documented (Jeffrey et al. 2000; Hader and Sinha 2005). To cope with the damaging effect of UVR, microbes have evolved various resistant mechanisms. Pigment production by microbial communities in cold environments is one of the strategy adapted by microbes to withstand increased UV radiation. Higher pigment production by microbial populations recovered from glaciers, ice cores and sea surfaces clearly indicates its role in adaptation to elevated UV radiation (Agogue et al. 2005; Zhang et al. 2008).

In addition, it has been observed that bacterial isolates *Arthrobacter agilis* GS_1 and *Janthinobacterium lividum* GW_1) have reduced quantity of produced pigment with raising temperature but enhanced pigments production at lower temperature. The bacterial isolate GW_1 has produced high pigment at 5°C (352 μg g^{-1}) as compare to 35°C (81 μg g^{-1}). Similarly, GS_1 isolates also shown same pattern. These results highlight the possible role of pigment to stabilize the cytomembrane at lower temperature as cell membrane faces fluidity maintenance at reduced temperature. It has been previously linked to a relationship between increased pigment production and decreased temperature, probably related to the maintenance and increased firmness of the cytomembrane (Foght et al. 2004). UV radiation could contribute to oxidize bio-molecules within cells which associate with increased level of reactive oxygen species (ROS) that damage cell components harshly (Moline et al. 2013). Therefore, pigments have been found to protect microbial cells by neutralizing ROS. Few researchers have also suggested psychrophilic microbes that produce pigments in order to protect organelles from oxidation resultant from extreme UV radiation such as reported from the glaciers of the Tibetan Plateau (Remias et al. 2010: Boric et al. 2011). Gorton and Vogelmann (2003) reported carotenoid pigment protecting cytoplasm of *Chlamydomonas nivalis* from UV-B but also suggested role of integral parts of the cell in protection against UV radiation.

Conclusion

It is concluded from the current study that 82 different types of bacterial strains were isolated from Ghulmet, Hopar and Ghulkin glaciers, located in Karakorum mountain range, Pakistan. Glaciochemistry of samples showed Ca^{2+} and SO_4^{2-} to be present in higher concentrations in all glacial samples. 16S rRNA and rpoB gene sequencing revealed most of the bacterial isolates belonged to genus *Pseudomonas*. In addition, bacterial isolates were found to produce both types of pigments including carotenes and xanthophylls but most of bacterial isolates produced β-carotene followed by zeaxanthin. Glaciochemistry would help in future to monitor any detrimental effect of climate change on the health of glaciers located in Karakoram mountain range, Pakistan, as they are at high risk to global warming. Likewise, this research work reported bacterial diversity in glaciers with enhanced ability to produce variety of carotenoid pigments that would be an alternative to the pigments extracted from synthetic and other (e.g. plants).

Declarations

Acknowledgments

Funds for the research work was provided by Commonwealth Scholarship Commission, United Kingdom. In addition, we would like to acknowledge Dr. Simmon Cobb, Senior Teaching Laboratory Technician and Mr. James Williams, the Analytical Research Technician in LOWTEX, Bristol Glaciology Centre, School of Geographical Sciences, Faculty of Science, University of Bristol, United Kingdom, for providing technical help during research work.

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Tables

Table 1 Colony forming unit (CFU)/mL or gm and coordinates of samples

Glaciers	Samples	pH	Temperatures (˚C)	CFU/gm or mL at different temperatures		Elevation in meters (m)	GPS coordinates
				5˚C	25˚C		
Ghulkin	Sediment	7	1	3.73×10^5	2.36×10^7	3083	36.42791 N
	Meltwater	7	1	3.95×10^3	3.87×10^5		74.80659 E
Ghulmit	Ice	7	–13	1.34×10^2	6.50×10^4	3072	36°12.474 N
	Sediment	7	3	2.73×10^6	1.36×10^9		74°29.035 E
	Meltwater	7	1	4.73×10^4	2.85×10^6		
Hopar	Ice	7	–12	3.50×10^2	7.60×10^4	2963	36.2108228 N
	Sediment	7	2	3.55×10^7	1.65×10^9		74.7724664 E
	Meltwater	7	1	4.50×10^3	2.65×10^6		

Table 2 The primers details used for the PCR amplification of 16S rRNA and rpoB genes of bacterial isolates

Primers	Target position[1]	Target gene	Sequence (5′–3′)	References
27F[2]	7–27	16S rRNA	AGAGTTTGATCMTGGCTCAG	Paju et al. (2003)
1492R[3]	1492–1510	16S rRNA	GGTTACCTTGTTACGACTT	Paju et al. (2003)
rpoB1698F	1698–1715	rpoB	AACATCGGTTTGATCAAC	Dahllof et al. (2000)
rpoB2041R	2041–2060	rpoB	CGTTGCATGTTGGTACCCAT	Dahllof et al. (2000)
rpoB1531F[2]	1531–1552	rpoB	TGGCCGAGAACCAGTTCCGCGT	Tayeb et al. (2005)
rpoB2760R[3]	2760–2781	rpoB	CGGCTTCGTCCAGCTTGTTCAG	Tayeb et al. (2005)
rpoB2491F[2]	2491–2511	rpoB	AACCAATTCCGTATIGGTTT	Michel and Raoult (2002)
rpoB3554R[3]	3554–3573	rpoB	CCGTCCCA AGTCATGAAAC	Michel and Raoult (2002)

[1] rpoB and 16S nucleotides numbered used *E.*coli, *Pseudomonas aeruginosa* and *Pseudomonas putida* as reference.

[2,3]F (forward) and R (Reverse) representing primers direction relating to the *rRNA*.

Table 3 Major ions content of glacial samples

Glaciers	Samples	Concentrations (μg l^{-1}/kg^{-1})									
		Ca^{2+}	K^{+}	Mg^{2+}	Na^{+}	NH_4^{+}	Cl^{-}	NO_3^{-}	PO_4^{2-}	SO_4^{2-}	NH_3
Hopar	Ice	2598	79	180	390	197	143	36	ND	112	280
	Sediment	10016	363	1081	1015	378	28	145	0.7	760	625
	Meltwater	7076	260	809	734	284	224	103	0.2	996	95
Ghulkin	Sediment	9009	225	856	1324	464	76	248	ND	645	741
	Meltwater	5008	204	707	946	308	100	81	ND	884	310
Ghulmet	Ice	2257	ND[1]	ND	441	221	121	58	ND	ND	51
	Sediment	8348	ND	469	1595	398	163	207	0.5	558	569
	Meltwater	6229	ND	929	990	271	ND	94	0.4	852	276

[1]Not detected

Table 4 Total organic carbon, total nitrogen and total phosphorus content of glacial samples

Glaciers	Samples	Concentrations (µg ml^{-1}/g^{-1})		
		TOC	TN	TP
Hopar	Ice	486	57	ND[1]
	Sediment	7543	993	5.2
	Meltwater	2771	125	3.4
Ghulkin	Sediment	6897	1139	1.5
	Meltwater	1799	101	ND
Ghulmet	Ice	557	28	ND
	Sediment	9174	1040	4.1
	Meltwater	2005	141	3.8

[1]Not detected

Table 5 An overview of quantified and identified pigments produced by bacterial isolates

Isolates	Pigments (µg g^{-1})						
	Alloxanthin	Astaxanthin	β-Carotene	β,ε-Carotene	19'-Hexanoyloxy fucoxanthin	Peridinin	Zeaxanthin
GI_1	–	–	4.04	–	8.51	–	–
GI_4	–	–	7.84	–	11.23	–	2.99
GS_5	–	–	4.14	–	110.5	–	9.70
GS_7	–	–	12.04	–	350.40	7.45	10.69
GS_9	–	8.13	191.82	–	–	–	30.49
GS_{14}	–	–	–	21.32	–	–	–
GS_{18}	–	38.43	4.38	–	61.68	124.03	–
GS_{19}	–	16.87	29.66	–	–	23.91	24.08
GS_{21}	–	39.59	2.95	–	–	49.05	–
GW_4	–	54.45	5.39	–	–	178	–
GW_7	–	31.03	–	–	–	–	–
GW_8	–	140.68	–	–	–	–	–
GW_9	–	–	–	101.32	–	–	–
GhS_1	–	56.28	–	–	–	–	–
GhS_4	–	–	2.65	–	34.74	151.54	–
GhS_8	–	36.10	4.12	–	–	103.64	–
GhW_6	–	21.15	5.64	–	–	312.96	–
GhW_7	–	8.88	3.01	–	–	128.06	–
HI_2	–	–	5.55	–	–	–	–
HI_3	–	–	–	31.42	–	–	

HS_3	–	–	3.17	–	–	–	351.67
HS_8	48.46	–	50.77	–	–	–	72.01
HS_{14}	–	–	–	10.37	–	–	–
HS_{16}	–	14.54	45.03	–	–	–	14.12
HW_5	–	–	–	–	15.49	–	–
HW_6	–	6.59	4.14	–	–	108.26	–

Figures

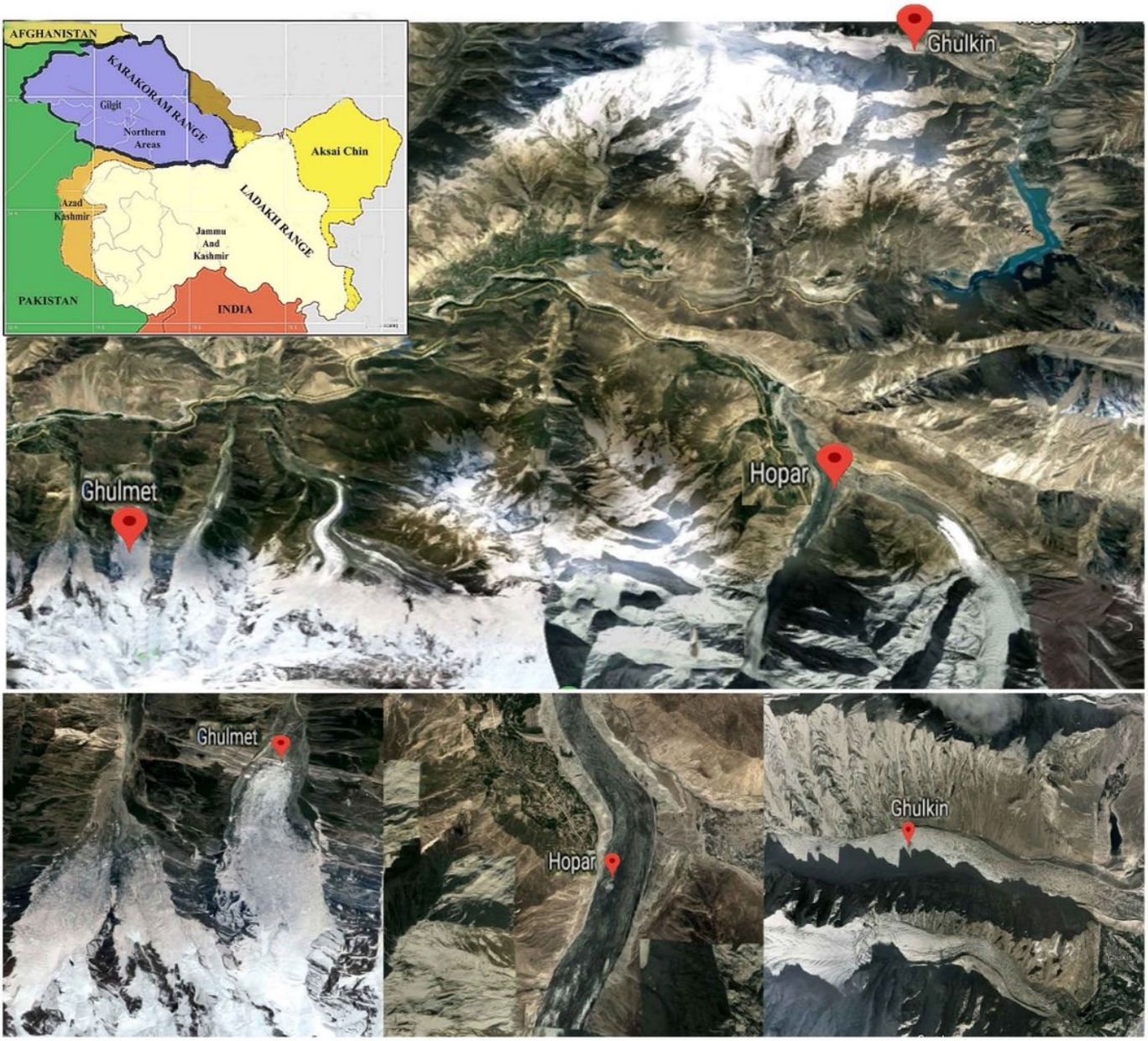

Figure 1

Topographic representation of sampling sites located in Karakoram Mountain Range, Pakistan. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

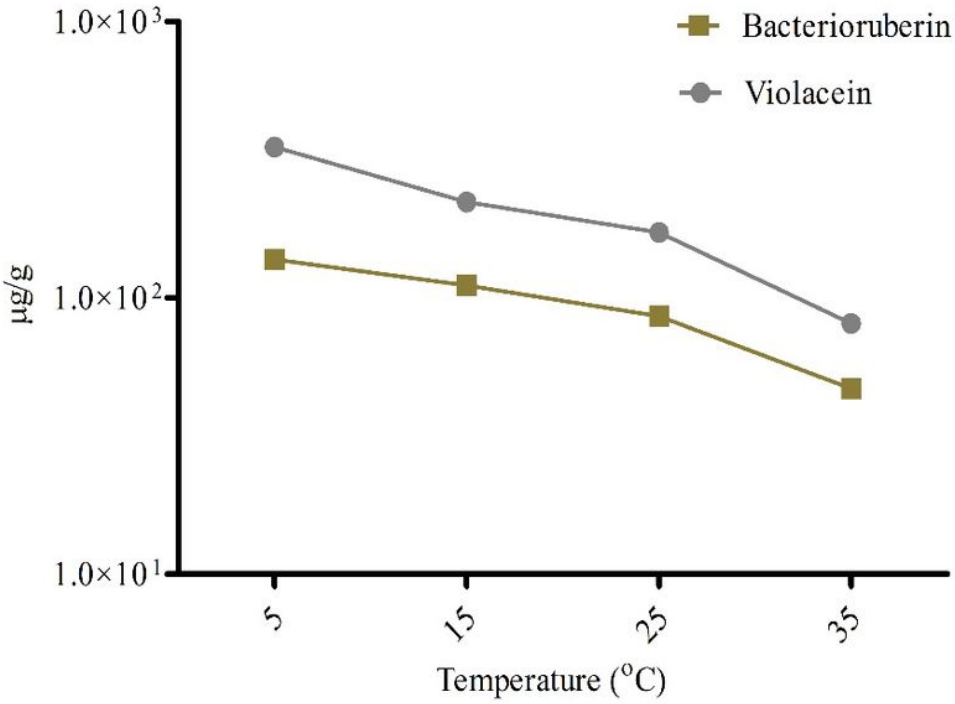

Figure 2

Pearson correlation of violacein (r = 0.980, P = 0.021) and bacterioruberin (r = 0.979, P = 0.021) produced by Arthrobacter agilis GS1 and Janthinobacterium lividum GW1, respectively, at different temperature

Supplementary Files

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