# Diversity of Cold-Adapted Bacteria from Batura Glacier Pakistan and use of Exopolysaccharide from *Pseudomonas* sp. BGI-2 for Cryopreservation.

By

**Pervaiz Ali**

**Department of Microbiology**
**Quaid-i-Azam University**
**Islamabad, Pakistan**
**2020**

# Diversity of Cold-Adapted Bacteria from Batura Glacier Pakistan and use of Exopolysaccharide from *Pseudomonas* sp. BGI-2 for Cryopreservation.

A thesis

Submitted in the Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

By

**Pervaiz Ali**

**Department of Microbiology**
**Quaid-i-Azam University**
**Islamabad, Pakistan**
**2020**

# DECLARATION

The material contained in this thesis is my original work and I have not presented any part of this thesis/work elsewhere for any other degree.

Pervaiz Ali

DEDICATED

TO

My beloved Mother

(Late Naseeb Sultana)

# CERTIFICATE

This thesis, submitted by **Mr. Pervaiz Ali** is accepted in its present form by the Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirement for the degree of Doctor of Philosophy (PhD) in Microbiology.

**Examiner:** ________________________________

**Examiner:** ______________________________

**Supervisor:** _______________________________
Dr. Aamer Ali Shah

**Chairperson:** ____________________________
Dr. Rani Faryal

**Dated:**

**CONTENTS**


| S. No. | Chapter No. | Title | Page No. |
|---|---|---|---|
| 1 | | List of Abbreviations | i |
| 2 | | List of Tables | iii |
| 3 | | List of Figures | iv |
| 4 | | Acknowledgements | vii |
| 5 | | Abstract | ix |
| 6 | Chapter 1 | Introduction | 2 |
| 7 | Chapter 2 | Review of Literature | 14 |
| 8 | Chapter 3 | A survey of culture – dependent and culture-independent diversity of bacteria from Batura Glacier in the Karakoram Range and their potential for industrial applications | 39 |
| 9 | Chapter 4 | Production, optimization and structural characterization of a cryoprotective EPS extracted from the cold-adapted bacterium *Pseudomonas* sp. BGI-2 | 79 |
| 10 | Chapter 5 | Cryopreservation of cyanobacteria and microalgae using exopolysaccharide extracted from the glacier isolate BGI-2 | 113 |
| 11 | Chapter 6 | Draft genome sequence of cold-adapted *Pseudomonas* sp. BGI-2 isolated from the ice of Batura glacier, Pakistan | 138 |
| 12 | Chapter 7 | Cold-adapted halotolerant *Rhodococcus* sp. BGI-11, a potential candidate for bioremediation of petroleum hydrocarbons in deep oceans | 157 |
| 13 | | References | 181 |
| 14 | | Appendices | 222 |

## List of Abbreviations

| | |
|---|---|
| ˚C | Degree Celsius |
| µL | Microliter |
| a.s.l | Above sea level |
| AFPs | Antifreeze proteins |
| ANI | Average nucleotide identity |
| BLAST | Basic local alignment search tool |
| CAPs | Cold acclimation proteins |
| CFU | Colony forming unit |
| COSY | Correlation spectroscopy |
| CPA | Cryoprotective agent |
| CPS | Capsular polysaccharide |
| CSPs | Cold-shock proteins |
| $D_2O$ | Heavy water |
| Da | Dalton |
| dDDH | Digital DNA-DNA hybridization |
| DEPT | Distortionless enhancement by polarization transfer |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic Acid |
| EPS | Exopolysaccharide |
| et al. | et alii/alia, and others |
| GOLD | Genomes online database |
| GTFs | Glycosyltransferases |
| HKH | Hindukush-Karakoram-Himalaya |
| HPAEC-PAD | High performance anion exchange chromatography with pulsed amperometric detection |
| HSQC | Heteronuclear single quantum coherence |
| Km | Kilometer square |
| LB | Luria Bertani |
| MEGA | Molecular evolutionary genetics analysis |
| MHz | Megahertz |
| mL | Milliliter |
| MSM | Mineral salt medium |
| NCBI | National center for biotechnology information |
| NGS | Next generation sequencing |
| NMR | Nuclear magnetic resonance |
| OD | Optical density |
| $OD_{600}$ | Optical density at 600nm |

| | |
|---|---|
| RGI | Randolph glacier inventory |
| rpm | Rotation per minute |
| rRNA | Ribosomal ribonucleic acid |
| TCA | Trichloroacetic acid |
| TOCSY | Total correlation spectroscopy |
| TSA | Tryptic soy agar |
| TSB | Tryptic soy broth |
| UDP | Uridine diphosphate |
| UV | Ultra violet |

## List of Tables


| Serial No. | Title | Table No. | Page No. |
|---|---|---|---|
| 1. | Summary of the Randolph glacier inventory (RGI) | 2.1 | 15 |
| 2. | Longest glaciers of the Karakoram range, Pakistan | 2.2 | 17 |
| 3. | Cold-adapted bacteria reported from various glaciers of the polar and non-polar regions | 2.3 | 21 |
| 4. | Overview of some of the most important bacterial EPS with the composition, physicochemical properties and applications | 2.4 | 34 |
| 5. | Physicochemical properties of glacier samples | 3.1 | 51 |
| 6. | Viable and microscopic bacterial counts of the glacier samples | 3.2 | 51 |
| 7. | Physiological characteristics of the glacial isolates | 3.3 | 53 |
| 8. | Phylogenetic affiliation of culturable isolates with closely related species in the GenBank database | 3.4 | 56 |
| 9. | Growth of glacial isolates on different culture media | 3.5 | 62 |
| 10. | Extracellular enzymes and antimicrobial activities demonstrated by the glacier isolates | 3.6 | 64 |
| 11. | Antibiotic susceptibility test of the glacier isolates using broad spectrum and narrow spectrum antibiotics. | 3.7 | 67 |
| 12. | Relative areas of seven major NMR resonances (800 MHz, $D_2O$) of anomeric positions ($O_2CH$-units) in EPS | 4.1 | 98 |
| 13. | Genomic features of the *Pseudomonas* sp. BGI-2 | 6.1 | 145 |
| 14. | dDDH and ANI values for *Pseudomonas* sp. BGI-2 compared with closely related mesophilic *Pseudomonas* species | 6.2 | 147 |
| 15. | dDDH and ANI values for *Pseudomonas* sp. BGI-2 compared with psychrophilic *Pseudomonas* species | 6.3 | 147 |
| 16. | Stress response genes comparison of cold-adapted *Pseudomonas* sp. BGI-2 with closely related mesophilic *Pseudomonas* species. | 6.4 | 149 |
| 17. | Osmotic stress response genes comparison of cold-adapted *Pseudomonas* sp. BGI-2 with closely related mesophilic *Pseudomonas* species. | 6.5 | 149 |
| 18. | Comparison of genes involved in the metabolism of various nutrients and aromatic compounds between *Pseudomonas* sp. BGI-2 and closely related mesophilic *Pseudomonas* species. | 6.6 | 150 |
| 19. | Physiological and biochemical characteristics of strain BGI-11 | 7.1 | 167 |
| 20. | Antimicrobial activities demonstrated by strain BGI-11 against pathogenic and non- pathogenic microorganisms | 7.2 | 171 |
| 21. | Extracellular enzyme activity demonstrated by strain BGI-11 using plate assay | 7.3 | 172 |
| 22. | Antibiotic susceptibility test of BGI-11 against narrow and broad spectrum antibiotics | 7.4 | 172 |

## List of Figures


| Serial No. | Title | Figure No. | Page No. |
|---|---|---|---|
| 1. | Panoramic view of the Batura glacier | 2.1 | 18 |
| 2. | Some of the important physiological changes observed in a psychrophilic bacteria at low temperatures | 2.2 | 25 |
| 3. | Microbial exopolysaccharide (EPS) biosynthesis | 2.3 | 30 |
| 4. | Picture of the sampling site in Batura glacier | 3.1 | 44 |
| 5. | Phylogenetic tree for isolates recovered from the glacier ice sample | 3.2 (a) | 58 |
| 6. | Phylogenetic tree for isolates recovered from the glacier sediment sample | 3.2 (b) | 59 |
| 7. | Phylogenetic tree for isolates recovered from the glacier meltwater sample | 3.2 (c) | 60 |
| 8. | Antibiotic susceptibility, cellulolytic activity and antimicrobial activities exhibited by the selected glacial isolates | 3.3 | 66 |
| 9. | Distribution of bacterial community from the glacier samples at the phylum level, using culture-independent method | 3.4 (a) | 69 |
| 10. | Distribution of bacterial community from the glacier samples at the genus level, using culture-independent method | 3.4 (b) | 69 |
| 11. | Mucoid phenotype of strain BGI-2, Exopolysaccharide (EPS) ethanol precipitation and lyophilization | 4.1 | 90 |
| 12. | Phylogenetic tree of *Pseudomonas* sp., BGI-2 using neighbor joining method | 4.2 | 91 |
| 13. | Growth of *Pseudomonas* sp. BG1-2 at different temperatures | 4.3 (a) | 93 |
| 14. | Growth of *Pseudomonas* sp. BG1-2 at different salinities | 4.3 (b) | 93 |
| 15. | Growth of *Pseudomonas* sp. BG1-2 at different pH | 4.3 (c) | 93 |
| 16. | Growth of *Pseudomonas* sp. BG1-2 at different concentration of molasses | 4.3 (d) | 93 |
| 17. | Growth of *Pseudomonas* sp. BG1-2 at different nitrogen sources | 4.3 (e) | 93 |
| 18. | Growth of *Pseudomonas* sp. BG1-2 at different glucose/yeast extract ratios | 4.3 (f) | 93 |
| 19. | EPS production by *Pseudomonas* sp. BG1-2 at different temperatures | 4.4 (a) | 95 |
| 20. | EPS production by *Pseudomonas* sp. BG1-2 at different salinities | 4.4 (b) | 95 |
| 21. | EPS production by *Pseudomonas* sp. BG1-2 at different pH | 4.4 (c) | 95 |
| 22. | EPS production by *Pseudomonas* sp. BG1-2 at different concentration of molasses | 4.4 (d) | 95 |
| 23. | EPS production by *Pseudomonas* sp. BG1-2 at different nitrogen sources | 4.4 (e) | 95 |
| 24. | EPS production by *Pseudomonas* sp. BG1-2 at different glucose/yeast extract ratios | 4.4 (f) | 95 |

| | | | |
|---|---|---|---|
| 25. | High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC- PAD) chromatogram of sugars used as standard | 4.5 (a) | 96 |
| 26. | Monosaccharide composition of EPS using HPAEC-PAD | 4.5 (b) | 96 |
| 27. | $^{1}$H NMR spectra of EPS | 4.6 (a) | 99 |
| 28. | $^{13}$C DEPT NMR spectra of EPS | 4.6 (b) | 99 |
| 29. | $^{1}$H, $^{1}$H TOCSY and $^{1}$H, $^{13}$C DEPT HSQC NMR spectra of EPS | 4.6 (c) | 100 |
| 30. | Overlay of $^{1}$H, $^{1}$H TOCSY and $^{1}$H, $^{1}$H NOESY NMR spectra of EPS-2 | 4.6 (d) | 101 |
| 31. | $^{1}$H, $^{1}$H NMR TOCSY NMR; $^{1}$H, $^{1}$H NMR TOCSY NMR and $^{1}$H, $^{13}$C DEPT HSQC NMR spectrum of EPS | 4.6 (e) | 102 |
| 32. | Selective excitation of 1D $^{1}$H TOCSY and 1D $^{1}$H NOESY NMR of BGI-2 with nominal $\delta_H$ of irradiation provided | 4.6 (f) | 103 |
| 33. | Freeze thaw survivability of *Pseudomonas* sp. BGI-2 | 4.7 | 104 |
| 34. | Picture of plates used for cryoprotective activity of EPS | 4.8 | 105 |
| 35. | Cryoprotective activity of different concentrations of EPS | 4.9 | 105 |
| 36. | Picture of a 24 well plate used for biomass recovery of the microalgal and cyanobacterial strains. | 5.1 | 120 |
| 37. | Cell morphology of the microalgal and cyanobacterial strains | 5.2 | 121 |
| 38. | Growth recovery for *Synechococcus* sp. CBW1003 after 05 days of cryopreservation | 5.3 (a) | 123 |
| 39. | Growth recovery for *Synechococcus* sp. CBW1003 after 15 days of cryopreservation | 5.3 (b) | 123 |
| 40. | Growth recovery for *Synechococcus* sp. CBW1003 after 09 months of cryopreservation | 5.3 (c) | 123 |
| 41. | Growth recovery for *Scenedesmus* sp. HTB1 after 05 days of cryopreservation | 5.4 (a) | 125 |
| 42. | Growth recovery for *Scenedesmus* sp. HTB1 after 15 days of cryopreservation | 5.4 (b) | 125 |
| 43. | Growth recovery for *Scenedesmus* sp. HTB1 after 09 months of cryopreservation | 5.4 (c) | 125 |
| 44. | Growth recovery for *Synechococcus* sp. CB0101 after 05 days of cryopreservation | 5.5 (a) | 127 |
| 45. | Growth recovery for *Synechococcus* sp. CB0101 after 15 days of cryopreservation | 5.5 (b) | 127 |
| 46. | Growth recovery for *Synechococcus* sp. CB0101 after 09 months of cryopreservation | 5.5 (c) | 127 |
| 47. | Growth recovery for *Microcystis aeruginosa* PCC 7806 after 05 days of cryopreservation | 5.6 (a) | 129 |
| 48. | Growth recovery for *Microcystis aeruginosa* PCC 7806 after 15 days of cryopreservation | 5.6 (b) | 129 |
| 49. | Growth recovery for *Microcystis aeruginosa* PCC 7806 after 09 months of cryopreservation | 5.6 (c) | 129 |

| | | | |
|---|---|---|---|
| 50. | Growth recovery for *Chlorella vulgaris* after 05 days of cryopreservation | 5.7 (a) | 131 |
| 51. | Growth recovery for *Chlorella vulgaris* after 15 days of cryopreservation | 5.7 (b) | 131 |
| 52. | Growth recovery for *Chlorella vulgaris* after 09 months of cryopreservation | 5.7 (c) | 131 |
| 53. | Growth recovery for *Synechococcus* sp. CB0101 after 09 months of cryopreservation in autoclaved and non-autoclaved EPS | 5.8 | 132 |
| 54. | Subsystem coverage and distribution of strain BGI-2 genome using Rapid annotation subsystem technology (RAST) | 6.1 | 146 |
| 55. | Colonies of strain BGI-11 on tryptic soy agar plate | 7.1 | 167 |
| 56. | Phylogenetic tree constructed for the glacial isolate BGI-11 using neighbor-joining method | 7.2 | 167 |
| 57. | Growth curve ($OD_{600}$) of BGI-11 at different temperatures | 7.3 (a) | 168 |
| 58. | Growth curve ($OD_{600}$) of BGI-11 at different pH | 7.3 (b) | 169 |
| 59. | Growth curve ($OD_{600}$) of BGI-11 at different salt concentrations | 7.4 | 169 |
| 60. | Growth curve ($OD_{600}$) of BGI-11 at different sugars used as carbon source | 7.5 (a) | 170 |
| 61. | Growth curve ($OD_{600}$) of BGI-11 at different carbon substrates | 7.5 (b) | 170 |
| 62. | Growth of BGI-11 in hexadecane supplemented medium | 7.6 (a) | 173 |
| 63. | Dry weight biomass of BGI-11 in hexadecane supplemented medium | 7.6 (b) | 173 |

## Acknowledgments

*From the depth of my heart I express my deep sincere gratitude to the* ***Almighty Allah****, for the Blessings He had bestowed upon me to do this work. I owe my deep respect to* ***Prophet Muhammad*** *(Peace Be Upon Him), the city of knowledge and mercy for the entire universe.*

*I am immensely pleased to place on record my profound gratitude and heartfelt thanks to my supervisor,* ***Dr. Aamer Ali Shah****, Associate Professor, Department of Microbiology, for his patience, motivation, enthusiasm, and guidance for the successful completion of my research work. The journey through my PhD degree would have been not possible without his support.*

*Sincere thanks also goes to my research supervisor in the States,* ***Dr. Feng Chen****, Professor, University of Maryland Center for Environmental Sciences (UMCES), for his inspiring guidance. Being a mentor he truly made a difference in my academic career and I deem it as my privilege to work under his able guidance.*

*I would like to express my sincere regards to* ***Dr. Fariha Hasan*** *for her valuable suggestions, constructive discussions and practical guidance during my entire research work. I would also like to express my appreciation to* ***Dr. Rani Faryal****, Chairperson, Department of Microbiology, for her appreciable cooperation and kind help.*

*Sincere thanks to all my lab fellows and friends (Both in the U.S and Pakistan), especially* ***Dr. Wasim, Ana Sosa, Taylor, Mengqi, Hualong, Daniela, Dr. Sahibzada,*** *and* ***Dr. Rafiq,*** *for the constructive discussions, good time, and all the fun we have had during this long journey.*

*Finally, my deepest gratitude goes to my beloved parents for their endless love, unceasing prayers, unequivocal support and encouragement to pursue the highest degree. The contribution of my late mother in my personal and academic growth is unmatchable and I call her as my best mentor. Sincere thanks goes to my wife for her unconditional support and consistent encouragement during the latter part of this degree. I also take this*

*opportunity to express the profound gratitude to all my brothers and sisters. I would like to extend my appreciation especially to my sister Nani for her guidance and support.*

*Finally, I express my gratitude and apology to all those who provided me the opportunity to achieve my goals but I missed to mention them personally.*

*Pervaiz Ali*

**Abstract**

Bacterial diversity of Batura glacier was investigated using culture-dependent and culture-independent methods. 27 cold-adapted bacteria (mostly psychrotrophic) were isolated using culture-dependent method. Majority of the isolates exhibited growth at a wide range of temperature (4-35˚C), pH (5.0–12.0) and salinity (1-6%). Culture-dependent diversity results revealed isolates from the three glacier samples belonged to 3 major phylogenetic groups: Actinobacteria (48%), Bacteroidetes (26%) and Proteobacteria (22%) while *Flavobacterium* (26%), *Arthrobacter* (22%) and *Pseudomonas* (19%) represented the dominant genera. Bacterial 16S rRNA gene sequences in the unculturable community were also dominated by the Proteobacteria, Actinobacteria and Bacteroidetes, while *Arthrobacter*, *Mycoplana*, *Ochrobactrum*, *Kaistobacter*, *Janthinobacterium* and *Flavobacterium* represented the dominant genera. The glacial isolates demonstrated antimicrobial activities against other microorganisms which also included the multidrug resistant strains. Majority of the isolates exhibited activities for more than one extracellular enzymes including lipases, proteases, cellulases and amylases. Almost, all the isolates were sensitive to the broad spectrum antibiotics (ofloxacin and imipenem), while resistance was found against the narrow spectrum antibiotics.

The isolate BGI-2 was selected for further studies based on its high abundance, relatively rapid growth at low temperatures and maximum EPS production among the 7 exopolysaccharide (EPS) producing isolates. Molecular identification using 16S rRNA gene sequencing placed BGI-2 in the genus *Pseudomonas* with the closest species *Pseudomonas mandelii* (99.59%) and *Pseudomonas frederiksbergensis* (99.59%). *Pseudomonas* sp. BGI-2 was able to grow in a wide range of temperature (4-35˚C), pH (5.0-11.0), and salinity (1-5%). The strain was able to use glucose, galactose, mannose, mannitol and glycerol as carbon sources. Maximum EPS production was observed at 15˚C, pH 6, NaCl (10 g $L^{-1}$), glucose as carbon source (100 g $L^{-1}$), yeast extract as nitrogen source (10 g $L^{-1}$) and glucose/yeast extract ratio (10/1). Under optimized conditions EPS production was 2.01 g/L, which is relatively high for a *Pseudomonas* species compared to previous studies using the same method for quantification. The EPS is a heteropolysaccharide with glucose, galactose and glucosamine as the main sugar monomers. The strain is also able to use molasses as a growth substrate, an alternative for

the relatively expensive sugars for large scale EPS production. Freeze-thaw survivability of BGI-2 was significantly higher than the non-EPS producing strains, including a psychrotroph BGI-11 isolated from the same environment and a mesophilic *Escherichia coli* strain K12. Also, the EPS produced by BGI-2 conferred significant cryoprotection for a mesophilic *E. coli* K12. Increase in EPS concentration from 1-5% significantly improved survivability of the *E. coli* K12, which was comparable to 20% glycerol.

We used the possibility of employing EPS from BGI-2 as a cryoprotective agent for the cryopreservation of two microalga and three cyanobacterial strains. The EPS extracted from the glacier bacterium *Pseudomonas* sp., BGI-2 worked well for the cryopreservation of cyanobacterial strains *Synechococcus* sp. CB0101, *Synechococcus* sp. CBW1003, and *Microcystis aeruginosa* PCC 7806 and one microalga strain, *Scenedesmus obliquus* HTB1. Overall, biomass recovery for the microalgae and cyanobacterial strains in EPS was better than 5% DMSO and 10% glycerol after 9 months of cryopreservation. Moreover, concentration of the EPS used as cryoprotective agent is critical and varies with different strains, depending on their sensitivity.

Successful colonization of the harsh cold environments is a result of the molecular evolution and adaptations. Draft genome sequence data revealed BGI-2 genome has 11 EPS-producing genes compared to none in the 6 closely related mesophilic *Pseudomonas* strains. We also found more stress response genes in the genome of BGI-2 than the closely related mesophilic counterparts. The stress response genes included osmotic stress, oxidative stress, cold shock, detoxification and carbon starvation. The genome also contains genes involved in cold adaptation, including desaturases for maintenance of membrane fluidity, production and uptake of compatible solutes, and production of exopolymers.

Finally, another isolate *Rhodococcus* sp. BGI-11 exhibited ability to utilize long chain hydrocarbon hexadecane as the carbon source. This strain also tolerated the maximum salt concentration (8%) among all the glacial isolates. The strain also demonstrated growth at a wide range of temperature (4-35°C) and pH (4-11). Thus, the cold-adapted halotolerant *Rhodococcus* sp. BGI-11 can be used as a potential candidate for bioremediation purpose in Deep Ocean, where accidental oil spills are threat to its biodiversity.

# Chapter 1

# 1. Introduction

A major part of the earth is cold; as high as 80% of the earth is constantly at temperature 5˚C or below. This cold environment ranges from the terrestrial and aquatic environments in the Arctic and Antarctic polar-regions, deep ocean, high mountains of alpine regions, upper atmosphere (stratosphere and mesosphere), cave systems and to some extent man made habitats such as refrigerated appliances. Deep sea represents the major fraction of this cold environment as oceans covers 71% of the Earth and 90% of its volume is at temperature <5˚C. Snow makes 35% of the land surface, followed by permafrost (24%), sea ice (13%) and glaciers (10%), (Casanueva et al., 2010; Margesin and Miteva, 2011; Rodrigues and Tiedje, 2008).

Geographically, glaciers and ice sheets cover around 15 million km$^2$, or approximately 10% of the land surface area of the Earth. According to the latest version of Randolph glacier inventory (RGI), there are approximately 198,000 glaciers in the world with an area cover around 726,800 km$^2$, which excludes the Greenland and Antarctic ice sheets (Pfeffer et al., 2014). According to RGI, the maximum number of glaciers lies in central Asia which numbers around 46,543 with an area 62,606 km$^2$. South Asian region, which also includes Pakistan has around 36,917 glaciers in its geographical boundary, with an area of 55,658 km$^2$. The north most territories of Pakistan consist of the greatest mountain ranges in the world, including the western Himalayas, Karakoram and Hindukush. The Himalaya-Karakoram-Hindukush (HKH) is the most glacierized area outside of the Polar Regions and is therefore considered the third pole of our planet (Zhang et al., 2015). These ranges host at least 5000 glaciers in the Pakistani geographical region and serve fresh water to a large portion of their population (Rasul et al., 2011).

The Karakoram mountain range is located between the borders of Pakistan, Afghanistan, India and China (Rankl et al., 2014). Some of the notable glaciers of the Karakoram includes, Siachen glacier (~ 75 km long), Biafo glacier (~ 68 km), Baltoro glacier (~ 62 km), and Batura glacier (~ 59 km) (Hewitt, 2014). Glaciers are used as an indicator for climate change, as there is a worldwide retreat of glaciers caused by the ever increasing global warming (Huss et al., 2017; Beniston et al., 2018). However, a significant number

of glaciers in the Karakoram are either stable or even growing contrary to the ice bodies worldwide that are receding due to global warming (Minora et al., 2016; Rankl et al., 2014). This unusual behavior of the Karakoram glaciers is known as the "Karakorum Anomaly" (Hewitt, 2005). Therefore, an understanding of the microbial communities and their ecological role in these habitats is of great interest for future studies.

Microbial biogeography is an emerging field which uses the combination of modern technologies and interdisciplinary approaches that allow the study of diverse environmental parameters and estimation of microbial diversity (Fierer, 2008; Green et al., 2008). The concept of glacial ecology has gained interest of researchers in recent years, mainly focusing on the two key glacier ecosystems; the supra-glacial and sub-glacial (Hodson et al., 2008). Cold-adapted microorganisms are involved in key ecological functions in the cryosphere, where photo-autotrophy is the basis of food webs in the pro-glacial and chemoautotrophy dominates the sub-glacier environments (Boetius et al., 2015). Microbial related studies of the glacier habitats have revealed their role in various metabolic processes, including methanogenesis, respiration, carbon fixation, and iron cycling (Hodson et al., 2008; Wadham et al., 2008; Anesio et al., 2009; Hood et al., 2009). Majority of the microbial studies have focused on the polar glaciers and less attention paid to mountain glaciers which covers a significant part of the cryosphere. The biodiversity (including the microbial life) in these glaciers faces a major threat due to the ever increasing global warming (Xu et al., 2009). The role of psychrophilic microorganisms in various processes linked with climate warming is still far from being clear.

Glaciers in the Pakistani geographical region are least explored in terms of their microbial diversity and functionality. The only few studies reported is limited to cultivation based approach for microbial isolation, hence not fully characterizing their diversity (Rafiq et al., 2017; Hassan et al., 2018). Also reports on the physiological characteristics of the microorganisms inhabiting these glaciers are rare. As discussed earlier, the Karakoram glaciers have exhibited quite resilience to the global warming compared to receding of the glaciers in other part of the world (Minora et al., 2016). It is therefore interesting to characterize the microbial diversity of these glaciers, which could have possible link of the microbial activity and climate change in future studies. The unexplored microbial life of

these glaciers also provides opportunity for bioprospecting of novel microbial metabolites such as antimicrobial compounds and cold-active enzymes, which can make their way into the biotechnological industry. Microbial enzymes represents one of the exciting products of biotechnology. Cold-active enzymes are especially appealing, as they maintain high catalytic activity at low temperatures. This negates heating requirement, resulting in a cost effective and sustainable industrial production (Siddiqui, 2015; Russell, 1998).

It is well known that only a small fraction (<1%) of microorganisms present in a sample can be grown in laboratory conditions (Amann et al., 1995). This limitation has been mitigated with the new tools introduced by next generation sequencing (NGS), which allow researchers to understand microbial communities and their lifestyles in different ecosystems. These tools have generated significant data from the cold biosphere over the last decade and helped in our understanding of microbial communities and adaptive strategies employed for their successful colonization. Therefore, culture-independent methods are indispensable for accurate characterization of microbial communities in any ecosystems (Cardenas and Tiedje, 2008). The various molecular methods have resulted in better understanding of the microbial diversity, their physiology and potential role in their respective ecosystem (Lam et al., 2015). Molecular analyses based on PCR is advantageous as it can detect DNA from dormant, damaged, live and dead cells, resulting in better characterization of the microbial diversity. Molecular analyses of extreme environments such as the glacier ice is challenging due to the low bacterial count which ranges from $10^2$ to $10^3$ $mL^{-1}$ (Bedrossian et al., 2018). Other limitations includes availability of relatively small volume samples and difficulties in cell lysis for DNA extraction. All these conditions significantly limits the quantity and quality of the extracted DNA for further cloning experiments. Whole genome sequencing is a viable solution to these challenges as it can reduce biases and generate a better representation of the microbial diversity present.

The advent of culture-independent methods have increased our understanding that only a small proportion of microorganisms in any environmental sample is culturable and the majority of these organisms go unnoticed. Isolation of microorganisms from frozen ice becomes more challenging due to the stressful conditions posed by this and related environments. The low culturability is majorly associated to the extreme conditions where

cells may be present in dormant or damaged state or incapable of growth on the media used for isolation. Therefore, new techniques of isolation and amendments in the composition of culture media should results in better recovery. One of the promising technique to improve cultivability is by ensuring culture conditions which mirrors those in their natural environments, including ambient temperature, pH, light, oxygen level, using water and nutrients from the same environment (Vester et al., 2015). Since extreme environments such as the cryosphere are oligotrophic in nature, extensive dilution of the culture media could be an important strategy for successful microbial recovery from these environments. Using low nutrient media such as R2A or extensively diluted nutrient rich media with long incubations (up to several months) has resulted in high bacterial recovery from the cold environments (Christner, 2002; Christner et al., 2003a; Miteva et al., 2004; Xiang et al., 2005). Another strategy employed for successful isolation of bacteria is enrichment of the sample in liquid media before plating. It was found that liquid media enrichment resulted in better recovery with an increased number as well as diversity of the isolates (Miteva et al., 2004). Although advent of DNA sequencing technologies has revolutionized our understanding of bacterial communities but cultivation based studies are still important in assessing microbial physiology (Prakash et al., 2013). The isolation of single species in a culture gives researchers the opportunity to do whole genome sequencing and sequentially test hypothesized functional capacities that results from metagenomic data (Giovannoni and Stingl, 2007). One major drawback of the metagenomic studies is in its inability to identify origin of the community DNA, whether from dead, living or dormant cells (Prosser, 2015).

Apart from their ecological role, psychrophilic microorganisms are rich source of enzymes and biological active compounds that finds their way into the biotechnological industry. Some of the applications of psychrophiles and their metabolic byproducts includes use of the cold-active enzymes in food, detergent and leather industry (Barroca et al., 2017). Production of the ice active substances such as antifreeze proteins and exopolymers for various industrial applications (Deming and Young, 2017; Munoz et al., 2017). Environmental applications of the psychrophiles and their enzymes includes biodegradation of petroleum oil and other wastes in cold environments (Greer and Juck, 2017), where their mesophilic counterparts may not even survive. Lastly, cold-adapted

bacteria are considered as novel source of antimicrobial compounds, as they live in nutrient limited environments, resulting in fierce competition among the microbes (Borchert et al., 2017). Enzymes from the psychrophiles are considered as next generation biocatalysts because of the huge potential they offer for various industrial processes, including applications in the textile, food, detergent, and animal feed industry (Barroca et al., 2017).

Microorganisms living in glaciers and other part of the cryosphere are subjected to various environmental stresses such as extreme low temperatures, desiccation, high UV radiations and often nutrient limitation (Margesin and Miteva, 2011). Psychrophiles have developed various physiological adaptations to combat the challenges inherent to low temperatures, which includes modification of the cell envelope, increased flexibility of their enzymes and production of cryoprotectants (Collins and Margesin, 2019). Maintenance of membrane fluidity at low temperature is achieved by increased unsaturation of the fatty acids, methyl branching and decrease in overall chain length (Siliakus et al., 2017; De Maayer et al., 2014). Enzymes from psychrophiles have more flexible structure which results in low substrate affinity and increased heat lability. The structural flexibility of cold-active enzymes is responsible for maintenance of high biochemical reaction rate at low temperatures (Feller, 2013). Other strategies employed includes, production of cold-acclimation and cold-shock proteins (Phadtare, 2004), antifreeze proteins (Lorv et al., 2014), compatible solutes (Hoffmann and Bremer, 2011) and exopolymers such as exopolysaccharides (Deming and Young, 2017). Antifreeze proteins (AFPs), also known as ice structuring proteins, binds to the ice surface in a non-colligative manner, resulting in the inhibition of ice growth and recrystallization (Kim et al., 2017). Compatible solutes are small organic compounds produced by a number of microorganisms including psychrophiles. The main functions ascribed to these organic compounds includes osmoprotection and cryoprotection (Govrin et al., 2019). Some of the well-studied compatible solutes includes trehalose, glycine, betaine, glycerol and sucrose. These compounds play a role in cryoprotection by reducing the freezing point of cytoplasmic water. Other functions assigned to the compatible solutes includes, scavenging free radicals, prevention of macromolecule aggregation and stabilization of cytoplasmic membrane in cold conditions (Collins and Deming, 2013). The role of exopolymeric substances in microbial cell protection against the harmful effects of cold temperature has

been well-studied (Krembs and Deming, 2008). Exopolysaccharide (EPS), a major component of the extracellular polymers are also considered an important strategy employed by psychrophilic microorganisms to cope with the extremely cold temperatures (Deming and Young, 2017; Casillo et al., 2017).

Production of exopolysaccharide (EPS) is common in all forms of microbial life and environments they inhabit. However, it is a hallmark of microorganisms living in extreme environments, where it is thought to play key role for their successful colonization. The purpose of EPS could be either provide individual protection or released in their surrounding environment for consortial arrangement through biofilm formation (Deming and Young, 2017). EPSs are extracellular polysaccharide polymers which are produced and secreted by microorganisms outside the cell (Sutherland, 1972). Microorganisms produce polysaccharide in two forms, exopolysaccharide (EPS) and capsular polysaccharide (CPS). The EPS or slime either remains loosely attached to the cells or is completely released into the surrounding environment. The CPS remains strongly associated to the cell envelope through covalent bond and plays role in pathogenesis. EPSs are comprised of repeating units of sugar moieties attached to a lipid carrier. The EPS can be associated to non-sugar components including organic and inorganic compounds. The most common monosaccharides reported in microbial EPS includes glucose, galactose, mannose, rhamnose, amino sugars (N-acetylglucosamine and N-acetylgalactosamine) and acidic sugars (glucuronic acid and mannuronic acid). Linkages between the monomeric units is also critical in the classification of these polymers which includes 1,4-β or 1,3-β and 1,2-α or 1,6-α linkages. The linkage 1,4-β or 1,3-β is characterized by a strong rigid structure while 1,2-α or 1,6-α provides a more flexible structure (McSwain et al., 2005; Nicolaus et al., 2010; Llamas et al., 2012; Staudt et al., 2012).

EPS and EPS-producing microorganisms are ubiquitous in both terrestrial and aquatic environments suggesting their importance in microbial ecology (Passow, 2000; Lasa, 2006). The functions assigned to EPS is majorly of protective nature, which helps microorganisms to evade predators (protozoans), surfactants and antimicrobial agents (Toska et al., 2018). EPS is also thought to provide protection by stabilizing the membrane structure against unfavorable environmental conditions which includes extremes of

temperature, pH, desiccations, and salinity (Knowles and Castenholz, 2008; Tamaru et al., 2005; Isfahani et al., 2018). The water holding capacity of EPS creates a hydrated environment enabling microorganism to successfully colonize dry and desiccated habitats. Other functions assigned to EPS includes attachment of the microorganisms to biotic and abiotic surfaces, uptake of nutrients and most significantly in biofilm formation (Janczarek et al., 2015; Rossi and De Philippis, 2015; Limoli et al., 2015; Bamford and Howell, 2016; Qin et al., 2007).

As discussed earlier the major functions designated to the EPS is of protective nature enabling the bacteria to proliferate in extremes of temperature, pH, and salinity. High concentration of exopolymeric substances from Arctic winter sea ice has been reported (Krembs et al., 2002). It was suggested that these exopolymers are produced by the active microorganisms in brine veins of the sea ice and could play cryoprotective role to microorganisms particularly the diatoms in the high salinity and low temperature environment (Krembs et al., 2002). It is now well established that the EPS has a critical role in survival of the cold-adapted microorganisms inhabiting the cryosphere (Deming and Young, 2017). EPSs from the psychrophiles have been reported for their cryoprotective role (Casillo et al., 2017; Marx et al., 2009; Arcarons et al., 2019; Carrion et al., 2015; Mancuso Nichols et al., 2004). The EPS in freezing environment protects microbial cells through ice recrystallization inhibition activity (Casillo et al., 2017). Also, the EPS matrix is thought to provide cryoprotection by lowering the freezing point of water and physically protects the microbial cells from the ice crystals by forming a protective shell around the cells (Caruso et al., 2018; Deming and Young 2017; Krembs et al., 2011).

In recent years, rapid depletion of natural resources and continued demand for polymers have led to an increased interest in the search of biopolymers produced by microorganisms (Schmid et al., 2016). Some of the benefits associated with bio-based polymers compared to the petroleum derived polymers is in their biocompatibility and biodegradability, making these polymers more eco-friendly. The increased demand of natural polymers has led to the exploration of EPS producing microorganisms from less explored extreme environments, which provide unique rheological functional properties for various industrial applications. EPSs are useful polymers with a vast range of applications which

extends from food to pharmaceuticals (Moscovici, 2015; Zannini et al., 2016), detoxification (Singh and Kumar, 2019), bioremediation (Sardar et al., 2019) and biotechnology (Barcelos et al., 2019). Several bacterial polysaccharides already have huge market values, while others are in process of development to make their way into the global market. The two well-known bacterial exopolysaccharides such as xanthan and gellan have found applications in food, pharmaceuticals, cosmetics, medicine and research (Becker et al., 1998; Ates and Oner, 2017). Microbial polysaccharide is expected to replace synthetic polymers due to the ease of production, wide range of physicochemical properties and less toxicity. A large portion of the microbial world is still unexplored due to the inability to culture these microorganisms in laboratory conditions (Amann et al., 1995). Therefore, intelligent screening of the microbial world will fuel discovery of novel exopolysaccharides with medical and industrial relevance.

Cryopreservation is an important technique for long term preservation of cells, tissues and organs. Polyhydroxyl compounds such as glycerol and sugars depresses the homogenous ice nucleation temperature more effectively then electrolytes. Freezing point of water can be further depressed by addition of large polymers in a system which has already polyhydroxyl compounds (Sutton, 1991). The cryoprotective agents could be penetrating or non-penetrating depending on their permeability to cross the membrane. Penetrating CPAs are a class of cryoprotectants that cross cell membranes such as ethylene glycol (EG), propylene glycol, dimethylsulfoxide (DMSO), glycerol and methanol. The large molecules or polymers that cannot enter cells are considered as non-penetrating CPAs and inhibit ice growth by the same mechanisms used by penetrating CPAs (Karlsson and Toner, 1996). Examples of the non-penetrating cryoprotectants include sucrose, trehalose, and polyethylene glycol (PEG) among others. Trehalose is a popular non-penetrating CPA, reported as less toxic and highly efficient in cryopreservation (Wen et al., 2016; Lee et al., 2013). The toxicity of penetrating cryoprotectants are higher compared to the non-penetrating cryoprotectants at the same concentration (Wowk, 2007). The toxicity of penetrating or permeating cryoprotectants have been summarized well in a review article (Best, 2015). EPSs produced in the sea ice acts as natural cryoprotectant by decreasing the freezing point of water. Global market for microbial exopolysaccharides depends on their unique biological activities and novel applications. Bacterial EPSs have already made their

impact on the global market with vast applications in food, health and environment, however its use as a cryoprotectant in cryopreservation will be a significant milestone.

Advent of genomic technologies has revolutionized our understanding of microbial diversity, evolution, adaptation and their functions in diverse habitats (MacLean et al., 2009). The data generated through genomic and metagenomic studies from cryosphere will aid to better understand cold-adaptation in psychrophiles (Mocali et al., 2017; Casanueva et al., 2010). Metagenomic studies from cold environments in the past decade has significantly increased resulting in more than thousand metagenome data accessible in the publicly available databases (Aliyu et al., 2017). Genomic data analysis from psychrophiles have revealed presence of a large number of genes involved in psychrophily. These included genes involved in maintenance of membrane fluidity, production and uptake of compatible solute, free radical scavenging system, production of cold-active enzymes and exopolysaccharides (Feng et al., 2014; Riley et al., 2008; Methe et al., 2005; Zhang et al., 2018a). The genomic data has contributed significantly in our understanding of the role of bacterial communities in cold environments. The reduced cost for next generation sequencing and availability of more genome sequence from psychrophiles will further help to better understand the molecular basis of cold-adaptation (Goordial et al., 2016).

Batura glacier with a length of ~ 59 km is one of the longest glacier outside the Polar Regions (Hewitt, 2014). The glacier is located in Passu village of the upper Hunza valley in the northern Pakistan. Hunza valley is located at the base of the Karakorum Range along the Karakorum Highway. Batura glacier (36°32′N, 74°40′E) has the highest elevation around 7795 m in the West and the lowest nearly 2570 m in the East (Hewitt, 2014). The glacier basin is ~ 48% glacierized and surrounded by several major peaks with altitude >7000 m, including Batura Muztagh ~ 7,795 m (Hodson et al., 2002). Sampling was done at an elevation ~ 3500 m towards the lower end of the glacier. Bacterial diversity from the three glacier samples including ice, melt-water and sediment were analyzed using a combination of culture-dependent and culture-independent methods. *Pseudomonas* sp. BGI-2 was selected for further studies among the culturable isolates based on its high abundance in the glacier ice sample, relatively rapid growth and maximum EPS production

at low temperatures. This isolate also exhibited various enzyme and antimicrobial activities against a number of bacteria including pathogenic strains, which is discussed in details in chapter 3.

Chapter 4 deals with extraction, purification and chemical characterization of the EPS from the psychrotrophic bacterium *Pseudomonas* sp. BGI-2. It further involves optimization of the physico-chemical conditions for growth and EPS production from the strain BGI-2. Since there is more temperature fluctuation in the mountain glaciers compared to the other cold environments (Polar Regions and deep seas), resulting in frequent freeze thaw cycles. We therefore tested the survivability of strain BGI-2 subjected to a number of freeze thaw cycles. EPS from BGI-2 was used as a cryoprotective agent for a mesophilic *Escherichia coli* K12 strain subjected to a number of freeze thaw events.

Cryopreservation of photosynthetic microorganisms such as cyanobacteria and microalgae is challenging, as these organisms are maintained in laboratories through serial sub-culturing. We used the possibility of EPS as a cryoprotective agent for successful cryopreservation of these photosynthetic organisms, which is discussed in chapter 5. Draft genome of BGI-2 was sequenced to explore some of the molecular aspects of cold-adaptation. Genome of the cold-adapted *Pseudomonas* sp. BGI-2 was compared with the closely related mesophilic *Pseudomonas* strains and the results are included in chapter 6. Finally, another isolate from the glacier ice *Rhodococcus* sp. BGI-11 demonstrated the ability to use long chain hydrocarbon hexadecane as carbon source. This strain also exhibited growth at low temperatures and high salinity. The potential use of this cold-adapted halotolerant strain in bioremediation of petroleum hydrocarbons in deep-ocean is discussed in chapter 7.

## Aim and Objectives

### Aim

The aim of the study was to characterize bacterial diversity of Batura glacier and explore some of their physiological characteristics for possible industrial applications.

### Objectives

The objectives of the study were

1. To evaluate bacterial diversity of Batura glacier using culture-dependent and culture-independent methods and their physiological characterization.
2. To perform initial screening of the glacial isolates for extracellular enzymes and antimicrobial activities.
3. To optimize exopolysaccharide (EPS) production by the cold-adapted *Pseudomonas* sp. BGI-2 and its structural characterization.
4. Cryopreservation of bacteria, cyanobacteria and microalgae using EPS from the glacial isolate *Pseudomonas* sp. BGI-2 as a cryoprotective agent (CPA).
5. Molecule insight into cold-adaptation of *Pseudomonas* sp. BGI-2 through draft genome sequencing.
6. Physiological characterization of a cold-adapted halotolerant isolate *Rhodococcus* sp. BGI-11, with ability to utilize hexadecane as carbon source.

# Chapter 2

# 2. Review of Literature

Cold environments represent a major fraction of the biosphere on the Erath. It is estimated that as high as 80% of the biosphere is constantly under 5˚C. It is obvious from the fact that ocean covers 71% of the Earth and 90% of its volume is below 5˚C. Therefore, deep sea represents major fraction of this cold environment followed by the snow, permafrost, sea ice and the glaciers. Glacier makes 10% of the land surface in the polar and non-polar regions. The cold habitats ranges from the terrestrial and aquatic environments in the Arctic and Antarctic polar regions, deep ocean, high mountains of alpine regions, upper atmosphere (stratosphere and mesosphere) and the cave systems. (Margesin and Miteva, 2011).

## 2.1. Glaciers of the world

Glacier represents the major fraction of the cryosphere, is a persistent body of dense ice that moves slowly over land. The term “glacier” comes from the French word *glace*, which means ice. Glaciers are often called “rivers of ice.” (National Geographic). Glaciers have long been known as the sensitive indicators of climate change.

The inventory for all glaciers of the world is provided by the Randolph Glacier Inventory (RGI) through a digital outlines, which excludes the two ice sheets of Greenland and Antarctica (Pfeffer et al., 2014). The total number of glaciers in RGI is around 200,000 (Table 2.1). These glaciers covers a total area of 726,800 km$^2$ and are organized into 19 regions of the world. The Antarctic and Subantarctic with a glacier area 132,900 km$^2$ constitutes the iciest region, followed by Arctic Canada North (104,900 km$^2$). Of the total extent, 44% is in the Arctic regions and 18% in the Antarctic and Subantarctic. The High Mountain Asia accounts for 16% which includes Central Asia, South Asia West and South Asia East whereas Alaska constitute for 12%. According to RGI the high Asia which also includes Pakistan has the maximum number of glaciers outside the polar-regions (Table 2.1). Alpine glaciers are called mountain glaciers or valley glaciers as they are formed on the high mountains with a downward movement through valleys. These glaciers creates valleys by pushing the soil and other materials out of their way during the downward movement. The three mightiest mountain ranges of the world including, the Karakoram,

Hindukush and Himalaya has the largest ice reserve in the non-polar region. These ranges extends border between China and the south Asian countries including India, Nepal, Pakistan, and Bhutan.

Table 2.1. Summary of the Randolph glacier inventory (RGI), version 3.2 (Pfeffer et al., 2014).

| No. | Region | Number of glaciers | All glaciers area (km$^2$) |
|---|---|---|---|
| 1 | Alaska | 26,944 | 86,715 |
| 2 | Western Canada and US | 15,215 | 14,559 |
| 3 | Arctic Canada North | 4538 | 104,873 |
| 4 | Arctic Canada South | 7347 | 40,894 |
| 5 | Greenland Periphery | 19,323 | 89,721 |
| 6 | Iceland | 568 | 11,060 |
| 7 | Svalbard and Jan Mayen | 1615 | 33,922 |
| 8 | Scandinavia | 2668 | 2851 |
| 9 | Russian Arctic | 1069 | 51,592 |
| 10 | North Asia | 4403 | 3430 |
| 11 | Central Europe | 3920 | 2063 |
| 12 | Caucasus and Middle East | 1386 | 1139 |
| 13 | Central Asia | 46,543 | 62,606 |
| 14 | South Asia West | 22,822 | 33,859 |
| 15 | South Asia East | 14,095 | 21,799 |
| 16 | Low Latitudes | 2863 | 2346 |
| 17 | Southern Andes | 16,046 | 29,333 |
| 18 | New Zealand | 3537 | 1162 |
| 19 | Antarctic and Subantarctic | 2752 | 132,867 |
| - | Total | 197, 654 | 726,792 |

## 2.2. Glaciers of Pakistan

Pakistan is located in Southern region of the Asian continent between 24°-37°N latitude and 66°-77°E. The Karakoram, Himalayas and Hindukush ranges with lofty mountains meet each other in the northern part of Pakistan. These ranges in the Pakistani geographical

boundary host more than 5000 glaciers and feed water to the Indus River System (Rasul et al., 2011). A significant number of the world's highest and most spectacular mountains lies in Pakistan. Among the 30 tallest peaks of the world, 13 lies in its geographical boundary. The notable peaks includes K-2 (8,611 m) in the Karakoram, the second highest peak in the world, Nanga Parbat (8,125 m) in the Himalayas, the ninth highest peak, and Tirich Mir (7,690 m) in the Hindukush. These ranges in the Northern Pakistan constitute an estimated glacier cover of 15,000 $km^2$ and among the three greatest mountain ranges, the Karakoram is the most glacierized region with 37% covered by glaciers (Rankl et al., 2014). The notable glaciers in this region includes Siachen glacier (~ 72 km), Biafo glacier (~ 68 km) and Baltoro glaciers (~ 62 km) (Hewitt, 2014).

### 2.3. Glaciers of the Karakoram Mountain range

The Karakoram together with the Hindukush and Himalaya makes the greatest mountain range of the world and known as Hindukush-Karakoram-Himalaya (HKH). These ranges meet each other at a junction point near Gilgit in the northern part of Pakistan, The Karakorum range is home to some of the longest glaciers in the non-polar region (Table 2.2). The Karakoram mountain range stretches over 500 km between borders of China and the south Asian countries including India, Pakistan, and Afghanistan (Rankl et al., 2014). The Karakoram Range hosts some of the highest mountains of the world with four peaks at heights more than 8000 m above sea level, including K-2 (8611 m). Major part of the Karakoram is covered by the glaciers with an estimated area of 17,946 $km^2$ and half of its surface lies above 5000 m (a.s.l) (Copland et al., 2011; Bolch et al., 2011). Some of the notable glaciers of the Karakoram includes, Siachen glacier (~ 72 km long), Biafo glacier (~ 68 km), Baltoro glacier (~ 62 km), and Batura glacier (~ 59 km) (Hewitt, 2014). Interestingly, glaciers of the Karakoram are stable or even growing contrary to the ice bodies worldwide that are receding due to global warming (Minora et al., 2016; Rankl et al., 2014). This unusual behavior of the Karakoram glaciers is known as the "Karakorum Anomaly" (Hewitt, 2005).

Table 2.2. List of the longest glaciers in the Karakoram region of Pakistan (Hewitt, 2014).

| Glacier | Length (km) | Basin area (km$^2$) |
|---|---|---|
| Siachen | 75 | 1400 |
| Biafo | 68 | 855 |
| Baltoro | 62 | 1270 |
| Batura | 59 | 710 |
| Hisper | 53 | 785 |
| Chiantar | 51 | 436 |
| Chogo L. | 47 | 690 |
| Rimo | 45 | 612 |
| Panmah | 44 | 680 |
| Skamri | 40 | 510 |
| Kondus-K. | 36 | 490 |
| Braldu | 36 | 430 |
| Virjerab | 36 | 389 |
| Sarpo Largo | 32 | 390 |

## 2.4. Batura Glacier

Batura glacier with a length of ~ 59 km is one of the longest glacier in the non-polar region. The glacier covers a basin area of 710 km$^2$, with the highest elevation around 7795 m in the West and terminates at an altitude of ~3000 m East in the Hunza valley of Gilgit Baltistan (Hewitt, 2014). As much as 48% of the glacier basin is glacierised and surrounded by several major peaks of the Karakoram that reach heights greater than 7000 m including, Batura Muztagh ~ 7,795 m (a.s.l). The glacier has ~ 700 m of clear white ice in the middle along much of its length and debris on either sides (Fig. 2.1) (Hodson et al ., 2002). Unlike other glaciers in the Karakoram, Batura glacier has been assessed and studies in detail, because of its accessibility from the Karakoram highway (KKH). A group of Chinese geologists and glaciologists investigated the glacier back in 1979 to investigate the possible advancement of the glacier, a threat to the Karakoram highway (BGIG, 1979). The glacier has 0˚C isotherm around 4,200 m and the snowline occurs at about 5,000 m. The upper reach of the glacier is therefore cold whereas the middle and lower reaches are temperate. The work of Chinese glaciologists gave evidence that the Batura glacier is not a surge-type

glacier (BGIG, 1979). However, they predicted that the glacier terminus would advance in the 1980s and then retreat in the 1990s. The predicted advance never happened rather the down-waste of the terminal ice cliff was recorded (Shroder, 1984).

Figure 2.1. Panoramic view of the Batura glacier in Passu village of the Hunza valley, showing a thin layer of white glacier in the middle and debris on both the sides.

### 2.5. Cold-adapted microorganisms

Microorganisms exhibiting growth near the freezing point of water have been known since the nineteenth century. The cold environments once seen as hostile habitats have been successfully colonized by a diverse group of organisms including the microorganisms. Various investigators have defined cold-adapted organisms in their own way. Schmidt-Nielson in 1902 proposed the term psychrophile for the first time for microorganisms able to grow near the freezing point of water. Ingraham (1958), defined psychrophiles as organisms that grow rapidly (in one or two weeks) at 0˚C. Later, Ingraham and Stokes (1959) used the term psychrophiles for bacteria that grow well near freezing point of water and produce visible colonies or turbidity within two weeks. They divided cold-adapted bacteria into obligate and facultative psychrophiles with the former having limited temperature range for growth while the later demonstrating growth at a wide range of temperatures. The current definition of cold-adapted microorganisms is proposed by Morita (1975) who divided cold-adapted organisms as psychrophiles or psychrotrophs. According to Morita's definition, psychrophiles are organisms growing optimally at 15˚C

or lower and a maximum growth temperature at about 20˚C. He proposed the term psychrotrophs for cold-tolerant organisms that can grow at low temperatures, but their optimal and maximal growth temperatures lies above 20˚C. Psychrophiles colonize permanently cold environments such as marine ecosystem (<5˚C) whereas psychrotrophs dominates habitats which experiences more thermal fluctuations such as the terrestrial environment (Russell, 1990).

**2.6. Cold-adapted bacteria in glaciers**

Psychrophilic bacteria have been routinely isolated from the glacier ice which is considered as one of the harsh environments of the biosphere. These bacteria have been studied to determine the limits of microbial life in the freezing temperatures (Bakermans, 2017). At present, a bacterial isolate from the permafrost held the lowest temperature limit for growth which is -15˚C (Mykytczuk et al., 2013). Protein and DNA synthesis have been reported at -20˚C (Junge et al., 2006; Tuorto et al., 2014), and respiration has been recorded at -33˚C (Bakermans and Skidmore, 2011). Since a major part of the earth is constantly under temperature 5˚C, therefore it is obvious that cold inhabiting bacteria play a significant role in global ecology. Within the past decade, a significant number of bacteria (including novel species) from permanently cold environments have been isolated and characterized including permafrost (Liang et al., 2019), glacier ice (Liu et al., 2019), Arctic sea ice (Junge et al., 2002), and from numerous cold ecosystems around the world (Shen et al., 2018; Pulschen et al., 2017). In the cold biosphere, glacier ice is considered as one of the harsh environments due to the extremely low temperature (which may go down as low as -56˚C), low nutrient and water availability, darkness and salinity (Margesin and Miteva, 2011). However, glaciers and other icy environments in the polar and non-polar regions harbors a huge number of microorganisms (~ $9.61\times10^{25}$ cells) which are naturally preserved in the ice for thousands of years (Priscu et al., 2007; Priscu and Christner, 2004). The abundance of microorganisms in glaciers have been reported at a range of $10^2$ to $10^7$ cells per mL, which varies between glaciers and also along the depth and altitude (Miteva et al., 2009; Xiang et al., 2005; Zhang et al., 2008a).

Cold-adapted bacteria have been frequently reported from the glaciers in the polar and non-polar regions (Table 2.3). These bacteria are represented by the dominant phylogenetic

groups including *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*. The physiological and cellular features shared by the bacteria from these icy environments include ability to grow at low temperatures in oligotrophic medium, pigmentation of their colonies, production of cryoprotectants, exopolymers, antifreeze proteins and cold-active enzymes (Cavicchioli, 2016; Pulschen et al., 2017; Kawahara, 2017; Deming and Young, 2017; Santiago et al., 2016: Munoz et al., 2017). Metagenomics studies of the cold environments have also revealed presence of a large number of genes related to psychrophily (Liljeqvist et al., 2015; Mackelprang et al., 2017; Varin et al., 2012).

Table 2.3. List of the dominant cold-adapted bacteria reported from various glaciers in the polar and non-polar regions

| **Glacier (Region)** | **Sample (s)** | **Dominant bacterial phylogenetic groups** | **References** |
|---|---|---|---|
| Yuzhufeng Glacier (Tibetan Plateau) | Ice core | *Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes* | Shen et al., 2018 |
| Tibetan Plateau | Ice core | *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Proteobacteria* | Liu et al., 2019 |
| Ecology (Antarctica) | Soil | *Arthrobacter* | Zdanowski et al., 2013 |
| Kuytun 51 (Sub-continent) | ice, fern, snow | *Proteobacteria* | Xiang et al., 2009 |
| East Rongbuk (Everest) | Ice core | *Firmicutes, Proteobacteria* | Hong Zhang et al., 2010 |
| Gulkana (Alaska) | snow, ice | *Betaproteobacteria, Gammaproteobacteria* | Segawa et al., 2011 |
| Austre Lovénbreen (Svalbard) | Ice core | *Bacteroidetes, Actinobacteria, Alphaproteobacteria* | Zeng et al., 2013 |
| John Evans (Canadian Arctic) | Water, sediment | *Betaproteobacteria (Comamonadaceae), Bacteroidetes (Flavobacterium)* | Cheng and Foght, 2007 |
| Midtre Lovenbreen (Arctic) | Meltwater | *Actinobacteria*, *Bacilli*, *Flavobacteria* and *Proteobacteria* | Reddy et al., 2009 |
| Puruogangri (Tibetan Plateau China) | Ice core | High-G + C gram-positives, Low-G + C gram-positives, *Proteobacteria* | Zhang et al., 2008a |
| East Rongbuk (Mount Everest) | Meltwater | α, β, and γ-*Proteobacteria*, *Actinobacteria*, *Firmicutes* | Liu et al., 2009a |
| Mount Everest | Moraine lakes, Meltwater | *Cytophaga-Flavobacterium-Bacteroides (CFB)* | Liu et al., 2006 |
| Greenland | Ice core | High-G + C gram-positives, Low-G + C gram-positives, *CFB, Proteobacteria* | Miteva et al., 2004 |
| Stubaier (Tyrolean Alps Austria) | Cryoconite | *Pseudomonas*, *Sphingomonas* | Margesin et al., 2002 |
| Mt. Qomolangma (Everest) | Ice | β, γ-*proteobacteria* and *Firmicutes* group | Zhang et al., 2008b |
| Pangi–Chamba Himalaya | Soil, compost, lake water | *Pseudomonas, Bacillus, Geobacillus, Arthrobacter, Paenibacillus, and Flavobacterium* | Thakur et al., 2018 |
| Antarctic | Multiple samples | *Arthrobacter*, *Psychrobacter*, *Pseudoalteromonas*, *Rhodococcus* | Silva et al., 2018 |
| Siachen (Karakoram) | Ice, meltwater, Sediment | γ-proteobacteria (*Pseudomonas*), *β-proteobacteria*, *Flavobacteria*, *Actinobacteria* (*Rhodococcus* and *Arthrobacter)* | Rafiq et al., 2017 |

## 2.7. Cold-adaptations in bacteria

Microorganisms including bacteria in nature are exposed to variations in temperature. Cold-adapted bacteria have evolved structural and physiological changes for successful colonization of the cold environments. One of the most well characterized changes is the alteration in physical property of cell membrane called membrane fluidity. Optimum fluidity is essential for membrane function, which is achieved at low temperature by changes in the structure of lipid constituents and fatty acids (Fig. 2.2). Maintenance of membrane fluidity at low temperature is achieved by following changes in phospholipids: (i) reduction in chain length, (ii) increased unsaturation of fatty acids and (iii) increase in methyl branching of fatty acids (Kralova, 2017; Siliakus et al., 2017; Shivaji and Prakash, 2010). At the enzymatic level, cold-adapted microorganisms have evolved a more flexible structure of their enzymes to cope with the reduced reaction rates at low temperatures. The high activity at low temperatures is maintained at the expense of a more flexible structure particularly around the active sites. This results in the high specific activity and increased thermolability of the cold-adapted enzymes. Enzymes from psychrophiles can be ten times more active at low temperature compared to their mesophilic homologues (Feller and Gerday 2003: Zheng et al., 2016: Siddiqui and Cavicchioli, 2006).

Change in protein profile is another strategy evolved by psychrophiles to cope with the cold shock. Two kinds of proteins are mainly synthesized called cold-shock proteins (CSPs) and cold-acclimation proteins (CAPs). Psychrophiles have a different cold shock response than their mesophilic counterparts. Psychrophiles produce cold acclimation proteins (CAPs) continuously when subjected to low temperatures and production of the housekeeping proteins continues after temperature downshift (Panoff et al., 1997). Therefore, production of cold shock proteins is decreased after continuous exposure to low temperature whereas cold acclimation proteins are synthesized continuously to maintain growth at low temperatures (Phadtare, 2004).

Bacteria adapted to grow at low temperatures have successfully evolved mechanisms to minimize the deleterious effects of the freezing conditions. One of the key strategy to counter the stressful subzero conditions is the production of antifreeze proteins (Olijve et

al., 2016). Antifreeze proteins (AFPs) also known as ice-binding proteins are a unique class of proteins synthesized by bacteria at low temperatures. AFPs have two major functions; it decreases the freezing point of cellular water through thermal hysteresis activity and also possesses ice recrystallization inhibition (RI) activity. AFPs acts as antifreeze agents and prevents growth of the ice crystal which otherwise is injurious to bacterial cells. AFPs have been characterized from Antarctic bacteria, including *Colwellia* sp. and *Marinomonas* sp. (Raymond et al., 2007; Gilbert et al., 2005).

Production of compatible solutes or organic osmolytes is another key adaptive strategy employed by bacteria for successful colonization of the hypersaline and freezing environments (Fig. 2.2) (Torstensson et al., 2019; Fedotova and Dmitrieva, 2016; Miladi et al., 2017). Compatible solutes are synthesized and accumulated by microorganisms to maintain cellular volume and osmotic balance in stressful conditions including freezing, high salinity and desiccation (Yancey et al., 1982). Some of the well-studied compatible solutes include quaternary amines (carnitine, betaine), tetrahydropyrimidines (ectoines), sugars (trehalose, sucrose), and amino acids (proline and glutamate) (Fig. 2.2). Role of compatible solutes in cryoprotection has been previously reported (Fedotova and Dmitrieva, 2016; Miladi et al., 2017). The osmolytes are also thought to protect macromolecules inside the cells and stabilize proteins in stressful conditions (Rabbani and Choi, 2018).

Productions of exopolymeric substances is a hallmark of the microorganisms in extreme environments including the cold. High concentrations of exopolymers have been reported from the winter ice cores. Apart from many other functions, these high molecular weight polymers in the brine veins of the sea ice are thought to play an important role in cryoprotection of the indigenous microorganisms against ice crystals (Krembs et al., 2002; Deming and Young, 2017; Krembs and Deming, 2008; Aslam et al., 2012). Recently, cryoprotective role of exopolysaccharides (EPSs) have been reported from bacteria isolated from various cold environments around the world. The EPS provided cryoprotection to the producer strains as well as to the other bacteria when added as a cryoprotective agent (Casillo et al., 2017; Carrion et al., 2015; Liu et al., 2013a; Marx et al., 2009).

The ability of psychrophiles to survive in the cold environments is a result of molecular evolution and adaptation in these microorganisms. The advancement of the ‘omic’ technology has significantly increased our knowledge of molecular based adaptation in psychrophiles. The different omic technology including genomics, transcriptomics and proteomics have produced results with better understanding of the cold-adaptation (Bowman, 2017; Nunn et al., 2015; Koh et al., 2017). The number of genomic and metagenomic data is ever increasing with the decrease in the sequencing costs. As many as hundred genomes from psychrophiles and thousand metagenome data from cold environments are now accessible in the publicly available databases (Bowman, 2017; Aliyu et al., 2017). Metagenomic data from a glacier ice revealed presence of a large number of genes involved in potential cold-adaptation (Simon et al., 2009). These included genes involved in the production of osmoprotectants and cryoprotectants such as glutamate, choline, betaine and glycine. The metagenomic data also revealed presence of genes responsible for increase in membrane fluidity by converting the saturated fatty acids to unsaturated such as the desaturases (Simon et al., 2009). These results have largely contributed to our understanding of the ecological role of psychrophiles in their respective environments and adaptive strategies employed to successfully colonize these stressful conditions (Boetius et al., 2015; Aliyu et al., 2017).

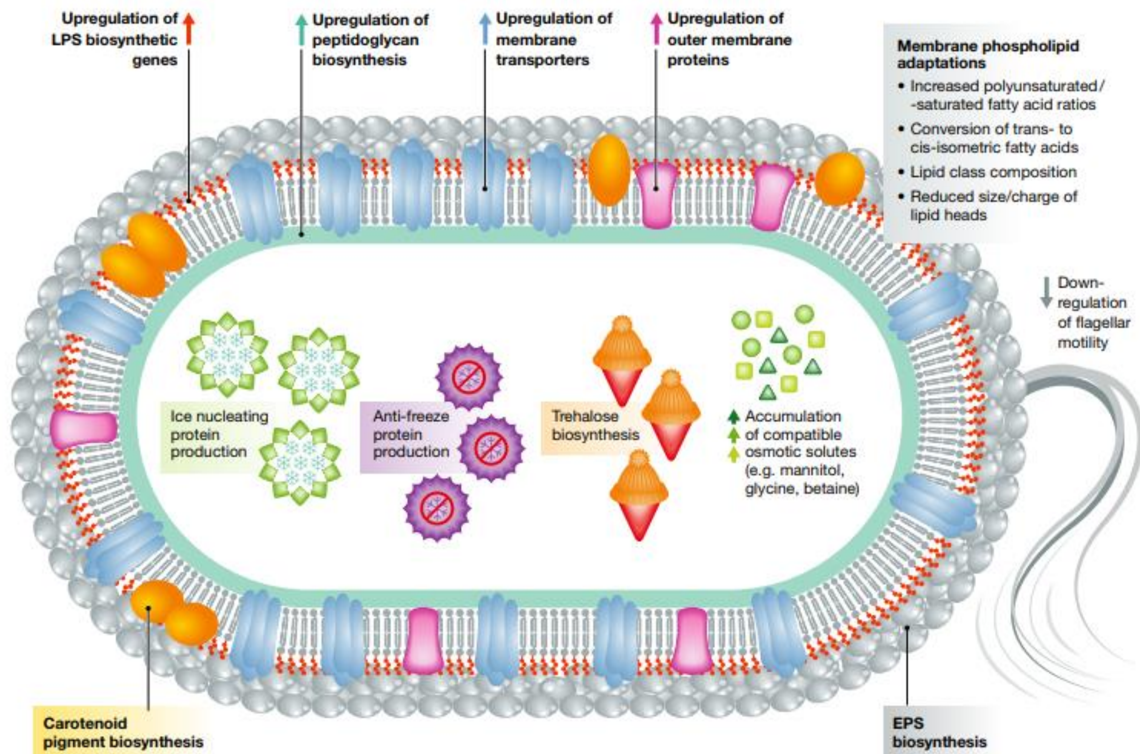



Figure 2.2. Some of the important physiological changes observed in a psychrophilic bacteria at low temperatures (De Maayer et al., 2014).

### 2.8. Applications of psychrophiles

Psychrophiles have great potential in biotechnological exploration and a strong interest is developing in cold-adapted microorganisms, resulting in isolation and screening for potential novel microbial species and their metabolites. The metabolites produced by psychrophiles with industrial relevance includes cold-active enzymes, antifreeze proteins (AFPs), compatible solutes, unsaturated fatty acids and extracellular polymeric substances and biosurfactants (Collins and Margesin, 2019).

Cold-adapted enzymes from the psychrophiles are considered as the next generation enzymes due to the huge potential it offers for the biotechnological industry. Enzymes from the cold-active microorganisms have plentiful applications in the textile, food, detergent, and environmental bioremediations, animal feed industry (Kavitha, 2016; Kuddus, 2018; Theerachat et al., 2019). This field is still at its infancy and needs more research as a small portion of organisms producing these enzymes have been identified and characterized.

More investigation is required regarding the diversity and abundance of cold loving microbes. Working with cold-adapted enzymes negates expensive heating requirements and saves energy. A very common example is addition of cold-active lipase in detergent formulation which efficiently removes stains from cloth fabrics during cold washing. In this way it saves energy as well textile fibers from wear and tear.

Antifreeze proteins (AFPs) are used in food industry for improved preservation during storage at subzero temperatures. They maintains smooth texture of the frozen foods and its use in the ice creams is well established (Munoz et al., 2017; Kaleda et al., 2018). AFPs helps in cryoprotection and this property is used for cryopreservation of various cells and tissues (Kim et al., 2017). The freeze tolerance of crops is improved and frost damage is prevented by the addition of AFPs, also applied in a similar way to transgenic aquaculture fish (Gupta and Deswal, 2014; Zbikowska, 2003). Another group of cryoprotectants called compatible solutes (which primarily acts as the osmoprotectants) have applications in cosmetics, skin care products, cryopreservation of food and biological materials (Hussain Wani et al., 2013).

Polyunsaturated fatty acids, a major component of the membrane lipids in psychrophiles have significant nutritional and pharmaceutical value (Yoshida et al., 2016). The nutritional value of PUFAs is remarkable and helps in the development and smooth functioning of the nervous system. For normal healthy diet, a balance intake of omega 3 such as ecosapentenoic acid (EPA) and docosahexaenoic acid (DHA) is recommended. Production of extracellular polymeric substances (EPSs) by cold-adapted microorganisms helps in colonization of the freezing environments (Deming and Young, 2017). These EPSs have numerous applications ranging from food, cosmetics, pharmaceuticals and environmental bioremediation. The EPSs are also used as bioflocculants and bioabsorbants in bioremediation of as well as in wastewater treatments (More et al., 2014). The reported cryoprotective role of the exopolysaccharides makes it a potential cryoprotectants for various types of cells and tissues (Caruso et al., 2018; Casillo et al., 2017).

Apart from industrial applications, extremophiles including the psychrophiles can serve as models for extraterrestrial life in other planets. The most promising planet to contain life is Mars, where water has been discovered recently. These planets have some features resembling the cold environments on Earth. Knowing the characteristics of extremophiles on the present day Earth, the habitability and physical environments of other planets can be investigated, using extremophiles as bioindicators (Merino et al., 2019; Cavicchioli, 2002).

### 2.9. Biopolymers

Biopolymers are polymers produced by living organisms or biological systems such as plants, animals and microorganisms. These polymers are also known as renewable polymers. This type of polymers are made up of the biological starting materials including monosaccharide sugars, amino acids and nucleotides. Hence, based on the starting materials biopolymers can be polysaccharide, polynucleotide and polypeptide (Tang et al., 2012). The rapid depletion of natural resources and continued demand of polymers have led to an increased interest in the search of biopolymers. Some of the benefits associated with bio-based polymers compared to the petroleum derived polymers include their biocompatibility and biodegradability, making these polymers more eco-friendly. The toxicity of petrochemical derived polymers to humans and environment is well established. Biopolymers from renewable resources are seen as the best alternatives for a more sustainable future (Zhu et al., 2016). Polysaccharides based biopolymers such as xanthan, gellan and alginate produced by bacteria have already a huge global market with applications ranging from food, pharmaceuticals, cosmetics, medicine and research (Ates and Oner, 2017; Rehm, 2009; Imeson, 2010).

### 2.10. Exopolysaccharides (EPSs)

EPSs are extracellular polysaccharide polymers which are produced and secreted outside the cell by microorganisms (Sutherland, 1972). The EPS either remains loosely attached to the cells or is completely released into the surrounding environment (Tallon et al., 2003; Schmid et al., 2015). Microbial polysaccharides are divided into three categories based on the location with respect to the cell including, intracellular storage polysaccharide

(glycogen), capsular polysaccharides (covalently attached to the cell membrane) and exopolysaccharide (either remains loosely attached to the cell surface or released into the external environments) (Schmid et al., 2015). Another categorization of the EPS is based on the monosaccharide composition of these polymers called homopolysaccharides or heteropolysaccharides. Homopolysaccharides consists of only one type of sugar monomeric unit and examples in this group includes cellulose and levan. Cellulose consists of only glucose units whereas levan is composed of fructose monosaccharide. Heteroplysaccharide consists of more than one type of monosaccharide units and example of this type of polysaccharides include xanthan (glucose, mannose and glucuronic acid) and gellan (glucose, rhamnose and glucuronic acid). The most common monosaccharides reported in heteropolysaccharides includes glucose, galactose, mannose, rhamnose, amino sugars (N-acetylglucosamine and N-acetylgalactosamine) and acidic sugars (glucuronic acid and mannuronic acid). Some exopolysaccharides may also contains non-sugar substituent such as glycerol, acetyl and phosphate. Linkages between the monomeric units is also critical in classification of these polymers which includes 1,4-β or 1,3-β and 1,2-α or 1,6-α linkages. The linkage 1,4-β or 1,3-β is characterized by a strong rigid structure while 1,2-α or 1,6-α provides a more flexible structure.

Exopolysaccharides are produced by diverse group of microorganisms including bacteria (Sardari et al., 2017), cyanobacteria (Bemal and Anil, 2017), archaea (Squillaci et al., 2016), fungi (Sun et al., 2016), yeast (Han *et al*., 2018) and microalgae (García-Cubero et al., 2018). EPS producing microorganisms have been isolated from diverse extreme environments including marine hot springs and deep sea hydrothermal vents (Wang et al., 2017; Arena et al., 2009), polar and cold marine environments (Casillo et al., 2017; Liu et al., 2013a; Aslam et al., 2012) saline and hypersaline environments (Joulak et al., 2019: Biswas and Paul, 2017)**.**

### 2.11. Biosynthesis of exopolysaccharides

Exopolysaccharides are synthesized in the cell throughout log phase, or sometimes during late logarithmic or stationary phase (Sardari et al., 2017; Castellane et al., 2017).

Production of EPSs in bacteria is influenced by a variety of factors including, temperature, salinity, pH and nutrients particularly carbon and nitrogen sources (Kumar et al., 2007). Synthesis of bacterial polysaccharides takes place in four steps. The different steps are governed by specialized enzyme group located in the different regions of the cell (Fig. 2.3) (Kumar et al., 2007).

The first step of EPS synthesis involves uptake of a particular substrate (mainly a glucose sugar) into the cell. The sugar substrate enters the cell either through the process of diffusion, active transport or group translocation. The second step starts with the metabolism of the sugar moiety, resulting in its phosphorylation. This step is governed by group I enzymes called hexokinase, converting glucose to glucose-6-phosphate. The enzyme phosphoglucomutase converts glucose-6-phosphate to an intermediate glucose-1-phosphate. This intermediate may enter into the catabolic pathway for energy production or go into the anabolic pathways for exopolysaccharide synthesis. The entry of glucose-1-phosphate into the anabolic pathway is carried out by the group II enzymes. Here, UDP-glucose pyrophosphorylase enzyme acts to convert glucose-1-phosphate to uridine diphosphate glucose (UDP glucose). The UDP-glucose is either interconverted into other sugars and progress towards the catabolic pathways. For example, UDP-glucose is converted into UDP-galactose by UDP-Gal-4-epimerase. The UDP-glucose can also proceed into the anabolic pathways where polymerization of the sugar moieties takes place, resulting in polysaccharide synthesis (Fig. 2.3). Thus fate of the intermediate is decided by the physiological needs of the cells which is regulated by genes present in the genetic material. Thus, synthesis of the polysaccharide is governed by the group III enzymes known as glycosyltransferases (GTFs). In this stage, GTFs causes polymerization by adding sugar to a repeating unit attached to a glycosyl lipid carrier (isoprenoid alcohol). Thus in third step (polymerization), repeated units of sugar monomers are linked through glycosidic bond to each other, resulting in polysaccharide synthesis. In the fourth step of EPS synthesis, modification of the polysaccharide is carried out by enzymes which results in acetylation, sulphation and acylation of the newly synthesized polysaccharide. Lastly, the polysaccharide is carried to the cell surface and released in the form of slime or a capsule. This transfer of nascent polysaccharide is carried out by the group IV enzymes

such as permeases, flippases and ABC transporters (Mishra and Jha 2013; Kumar et al., 2007: Sutherland, 2001a).

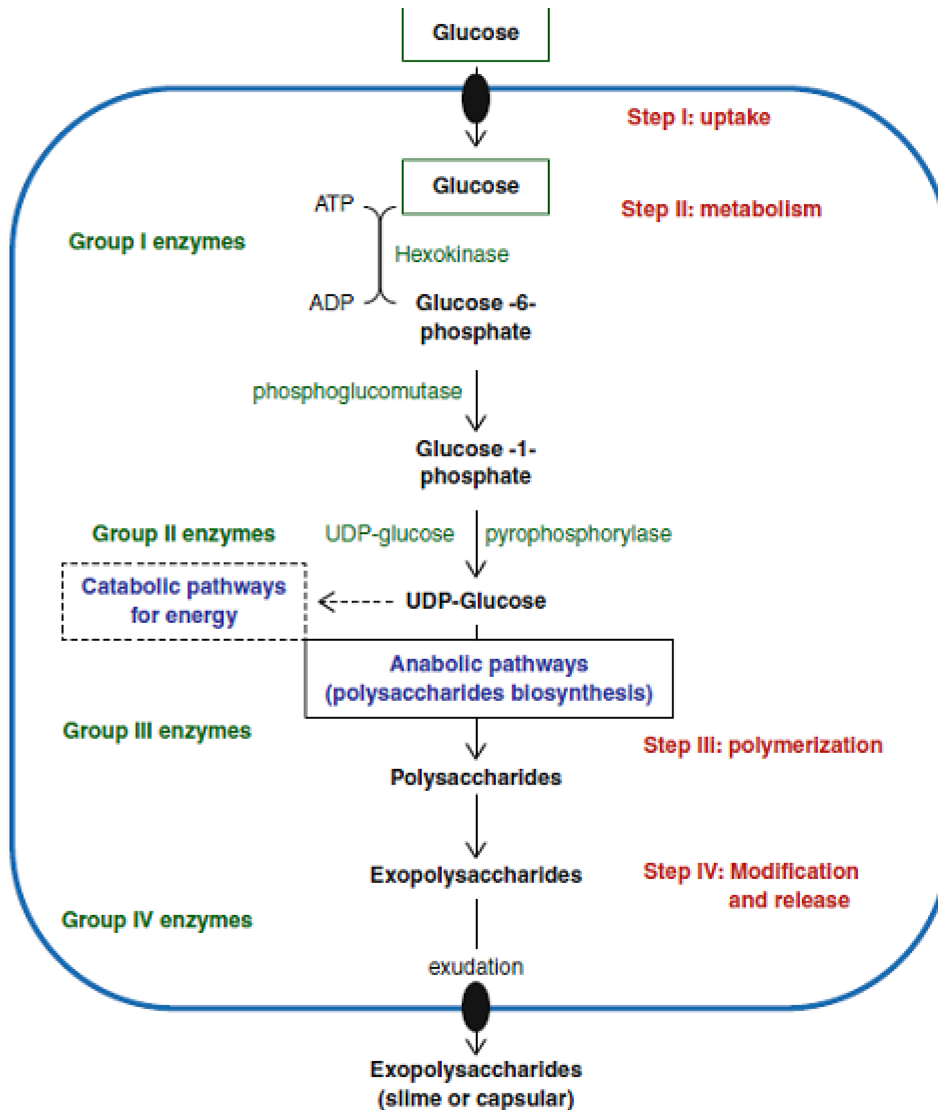



Figure 2.3. Microbial exopolysaccharide biosynthesis (Mishra and Jha, 2013)

### 2.12. Functions of exopolysaccharides

Production of exopolysaccharides by bacteria and other microorganism is thought to have a defensive function. The specific role of the EPS is majorly depended on the habitats or environments the microorganisms live in. For example, EPS from an Antarctic bacterium is thought to provide cryoprotection to the producer organism (Casillo et al., 2017: Carrion et al., 2015). Similarly EPS from a bacterium living in hypersaline environment protects it

from the damaging effects of high salt concentration (Isfahani et al., 2018). Therefore, the functions assigned to EPS is majorly of protective nature, which helps microorganisms to evade predators (protozoans), surfactants and antimicrobial agents (Toska et al., 2018). EPSs is also thought to provide protection by stabilizing the membrane structure against unfavorable environmental conditions which includes extremes of temperature, pH, desiccations, and salinity (Knowles and Castenholz, 2008; Tamaru et al., 2005; Isfahani et al., 2018). The water holding capacity of EPS creates a hydrated environments enabling microorganism to successfully colonize dry and desiccated habitats. Other functions assigned to EPS includes attachment of the microorganisms to biotic and abiotic surfaces, uptake of nutrients and most significantly in biofilm formation (Janczarek et al., 2015; Rossi and De Philippis, 2015; Limoli et al., 2015). In oligotrophic environments EPS therefore plays a critical role in the acquisition of the nutrients. EPS is the major component of microbial biofilms which forms a physical barrier between organism and its physical environment, enables them to survive and thrive in extreme niches.

Recently, EPS and EPS producing microorganisms have been used successfully in bioremediation of heavy metals from affected soils and water (Sardar et al., 2018; Mohite et al., 2017; Kalpana et al., 2018). The anionic nature of these EPS helps in capturing of essential nutrients and also enables removal of the positively charged heavy metals and ions from solutions. In pathogenic bacteria, capsular polysaccharide (CPS) helps in pathogenicity as the CPS mimics host cell surface and hence do not stimulate immune system.

### 2.13. Functions of exopolysaccharides in cold environments

A large number of EPS producing bacteria have been isolated from various extreme environments around the world and EPS is thought to play key role in their survival. As discussed earlier the major functions designated to the EPS is of protective nature enabling the bacteria to proliferate in extremes of temperature, pH, and salinity. It is now well established that the EPS has a critical role in survival of the cold-adapted microorganisms inhabiting the cryosphere. Mancuso Nichols et al., (2004) reported two EPS producing Antarctic isolates (CAM025, CAM036) from the genus *Pseudoalteromonas*. According to

their results, isolate CAM025 produced as many as 30 folds more EPS at -2 and 10˚C compared to 20˚C. The high yield of EPS production at extremely low temperature could be a cryoprotection strategy employed by the isolate CAM025. Krembs et al., (2002) reported high concentration of EPSs from Arctic winter sea ice. They suggested that these exopolymers are produced by the active microorganisms in brine veins of the sea ice. The high concentration of EPSs could play cryoprotective role to microorganisms particularly the diatoms in this high salinity and low temperature environment. They also suggested the high concentration of EPS with high polyhydroxyl content will decrease the freezing point of water in the freezing conditions of the sea ice and physically protects the microbial cells from the ice crystals.

In another study, EPS production by the cold-adapted *Colwellia psychrerythraea* 34H under different conditions which included temperature, pressure and salinity was checked (Marx et al., 2009). Their results revealed that extreme environmental conditions stimulated maximum EPS production. Also the strain was recovered well from deep freezer (-80˚C) when first supplemented with its own EPS than the common cryoprotectants used, such as glycerol. This study further demonstrated the role of EPS in survival of the stressful conditions including low temperature.

Recently, the cryoprotective role of EPS have been reported from psychrophiles isolated from cold environments in the polar and non-polar regions (Arcarons et al., 2019; Casillo et al., 2017: Deming and Young, 2017; Carrion et al., 2015). A psychrophilic *Colwellia psychrerythraea* 34H was reported for production of a cryoprotective exopolysaccharide (Casillo et al., 2017). This EPS also exhibited significant ice recrystallization inhibition activity, suggesting its role in cold-adaptation. An Antarctic bacterium *Pseudomonas* sp. ID1 isolated from marine sediment sample was reported for exopolysaccharide production (Carrion et al., 2015). The EPS was composed of glucose, galactose and fucose monosaccharides. The cryoprotective activity of this EPS was significantly higher than fetal bovine serum (FBS). In a recent study, cryoprotective role of exopolysaccharide from *Pseudomonas* sp. ID1in vitrification of cow oocytes was reported (Arcarons et al., 2019).

## 2.14. Application of exopolysaccharides

Microbial exopolysaccharides constitute an important group of industrial polymers with applications ranging from food to pharmaceuticals, and personal care products to environmental bioremediation (Barcelos et al., 2019; Sardar et al., 2018; Kielak et al., 2017). The use of these polymers in food industry is well established due to their gelling, emulsifying and stabilizing properties (Jindal and Khattar, 2018). These polymers are water soluble and increase viscosity of the solutions even at very low concentration, a potential replacement of polysaccharides from plant and animal origin. Some of the well-known microbial exopolysaccharides with already huge market values include xanthan, gellan, dextran, levan, bacterial cellulose and alginate (Table 2.4). Microbial polysaccharides is expected to replace synthetic polymers due to the ease of production, less toxicity and wide range of physicochemical properties.

Some of the microbial polysaccharide which also contains fucose and rhamnose as the monomeric units have been reported for biological activities including antioxidant, anticancer, antiviral, antimicrobial and antiulcer (Poli et al., 2010). These types of exopolysaccharide have wide commercial applications in cosmetics, pharmaceutical and functional food products to improve overall health. Fucose is a rare sugar used in the development of anti-inflammatory and antitumor drugs. It is also used in creams for speedy wound healing, as moisturizer and anti-aging substance (Peterszegi et al., 2003). Recently, EPS have been used successfully in bioremediation of heavy metals from affected soils and water (Sardar et al., 2018; Mohite et al., 2017; Kalpana et al., 2018). The anionic nature of these polymers helps in capturing of essential nutrients and also enables removal of the positively charged heavy metals and ions from solutions.

Table 2.4. Overview of some of the most important bacterial EPS with the composition, physicochemical properties and applications

| EPS | Monosaccharide Composition | Molecular weight (Dalton) | Charge | Properties | Applications | References |
|---|---|---|---|---|---|---|
| Xanthan | Glucose, Mannose, Glucuronic acid | $(2.0\text{-}50) \times 10^6$ | Anionic | High viscosity at low conc., Maximum stability at a wide pH and temp. range | Food, Pharmaceuticals, Cosmetics, Petroleum, Agriculture, and personal care products | (Rehm, 2009; Imeson, 2010; Petri, 2015; Ates and Oner, 2017) |
| Gellan | Glucose, Rhamnose, Glucuronic acid | $5.0 \times 10^5$ | Anionic | Gelling capacity, Stability over wide pH range | Foods, Pharmaceuticals, Research | (Rehm, 2009; Imeson, 2010; Fialho et al ., 2008; Bajaj et al ., 2007; Ates and Oner, 2017) |
| Alginate | Glucuronic acid, Mannuronic acid | $(0.3\text{-}1.3) \times 10^6$ | Anionic | Gelling capacity, Hydrocolloid, Film-forming | Food, Medicine (Surgical dressings, Wound management, Controlled drug delivery) | (Rehm, 2009; Imeson, 2010; Remminghorst and Rehm, 2006; Yang, 2011) |
| Cellulose | Glucose | $10^6$ | Neutral | High crystallinity, Insolubility in most solvents, High tensile strength | Foods, Biomedical (Wound healing, Tissue engineered blood vessels, Audio speaker diaphragms) | (Chawla et al ., 2009; Imeson, 2010; Yang, 2011; Cacicedo, 2016) |
| Dextran | Glucose | $10^6$ - $10^9$ | | Non-ionic, maximum stability, Newtonian fluid behavior | Foods, Pharmaceuticals (Blood volume expander), Chromatographic media | (Rehm, 2009; Yang, 2011; Huang and Huang, 2018) |
| Levan | Fructose | $3.0 \times 10^6$ | Neutral | Low viscosity, High water solubility, Biological activity (Anti-inflammatory, Anti-tumor activity), | Food (prebiotic), Feed, Medicines, Cosmetics | (Rehm, 2009; Srikanth et al., 2015; Oner et al ., 2016; Ates and Oner, 2017) |
| Curdlan | Glucose | $5\times 10^4$ - $2\times 10^6$ | Neutral | Gelling capacity, Water insolubility, Edible and non-toxic, Biological activity | Foods, Pharmaceutical industry, Heavy metal removal, | (Rehm, 2009; Yang, 2011; Ates and Oner 2017; Zhan and Zhang, 2017) |

### 2.15. Psychrophiles in bioremediation of hydrocarbons

Petroleum hydrocarbons are one of the major environmental pollutants which contaminate terrestrial and freshwater through accidental spills or shipping activities (Arulazhagan et al., 2010). Petroleum mainly consists of alkanes while n-hexadecane is a major component of alkanes. Alkanes are highly inflammable but one of the least reactive among organic compounds. The successful bioremediation of alkanes such as hexadecane is dependent on the accessibility of this compound to the microbes involved in degradation and optimum conditions for the degradation process (Kebria et al., 2009). Low molecular weight alkanes are degraded rapidly as compared to long chain and multiple branched alkanes (Stroud et al., 2007).

The indigenous psychrophilic microorganisms play key role in biodegradation of petroleum products and other pollutants in low temperature environments (Miri et al., 2019). This low temperature favors the growth of cold-adapted bacteria and hence the degradation activity. Low temperature biodegradation of petroleum components (n-alkanes, aromatic and polyclic aromatic hydrocarbons) has been reported in the Arctic (Garneau et al., 2016), Antarctic (Habib et al., 2018) and Alpine glacier (Margesin and Schinner, 2001a).

Biodegradation of diesel oil in an alpine glacier at an altitude of 3000 m (a.s.l) was investigated (Margesin and Schinner, 2001a). Results revealed that the pollutant can be efficiently removed even at extremely low temperature, which is an unfavorable condition for many microbes. In another study, investigated cold-adapted bacteria for their degradation potential from Alpine habitats were investigated (Margesin and Schinner, 1998). These bacteria were screened from 29 different locations from soil and glacier samples. They surprisingly recovered oil degrading bacteria not only from oil-contaminated samples but also from uncontaminated site. Degradation was efficient at low temperature as most inocula degraded 40-60% of the diesel oil within 8 days at 10°C. Degradation ability decreased at higher temperature (48˚C) to 20-40% after 8 days. It was also found that degradation of the hydrocarbon occurred mainly during the first 6 days of incubation, and no significant degradation was noticed after 6 days. Cold inhabiting

microorganisms have a tremendous potential for applications in bioremediation processes as they harbors enzymes which maintains high catalytic efficiency and unusual specificity at low temperatures. Introduction of specific cold adapted microorganisms in mixed cultures in contaminated environments is expected to improve biodegradation of organic pollutants.

### 2.16. Molecular basis of cold-adaptation in psychrophiles

Molecular evolution and adaptations have resulted in the ability of psychrophilic microorganisms to better survive and thrive in cold environments. Recently, few studies have revealed deep understanding regarding molecular conformity at subzero temperatures using genomic, transcriptomic and proteomic investigations (Bowman, 2017; Koh et al., 2017; Nunn et al., 2015). Over a hundred genomes have been obtained from psychrophiles with representation from major taxonomic groups (Bowman, 2017). Metagenomic studies from cold environments in the past decade has significantly increased resulting in more than thousand metagenome data accessible in the publicly available databases (Aliyu et al., 2017). These metagenomic data have contributed significantly in our understanding of the role of bacterial communities in cold environments. Also these data are very useful in our understanding of cold adaptation and ecological role played by the indigenous microorganisms (Aliyu et al., 2017; Boetius et al., 2015).

Metagenomics studies from various cold environments around the world has greatly increased our understanding of the cold-adaptation and the various ecological role played by the psychrophilic microbial communities. These includes studies from the Arctic region (Yergeau et al., 2010), Antarctic region (Koo et al., 2017) and the non-polar alpine region (Simon et al., 2009). Functional metagenomics of the cold environments revealed presence of various metabolites involved in cold-adaptation. Cold-active enzymes including lipases, cellulases, proteases and esterases have been routinely identified in the metagenomic data (Sindhu et al., 2017; Alma’abadi et al., 2015).

Genomics and metagenomics data analysis from various cold environments have revealed presence of a large number of genes involved in psychrophily. These included genes

involved in maintenance of membrane fluidity, production and uptake of compatible solute, free radical scavenging system, production of cold-active enzymes and exopolymers. Genome comparison of the psychrophilic *Psychroflexus torquis* with a closely related mesophilic counterpart revealed presence of genes responsible for polyunsaturated fatty acid (PUFA) and exopolysaccharide (EPS) biosynthesis (Feng et al., 2014). The genes involved in cold-adaptation were mostly absent in the non-psychrophilic species. In another study, genome of the sea ice psychrophilic *Psychromonas ingrahamii* was investigated and resulted in the identification of a large number of cyclic GDP regulators, suggesting production of extracellular polysaccharide. The EPS might play a role in cold-adaptation by depressing the freezing point of water around the cell. This cold-adapted bacterium also possessed genes involved in synthesis of the osmolyte betaine choline (Riley et al., 2008).

Genome analysis of the true psychrophilic bacterium *Colwellia psychrerythraea* 34H revealed presence of multiple genes involved in successful colonization of the permanently cold environments (Methe et al., 2005). The genome of this strain contains multiple copies of the catalase genes and two superoxide dismutase (SOD) genes. These enzymes are very essential for the cold-adapted microorganisms as decrease in temperature results in an increase in oxygen solubility which poses great risk from the reactive oxygen species (ROS). The genome of strain 34H also contained multiple genes involved in the synthesis and release of exopolymeric substances. The role of exopolymers in cold-adaptation has been discussed in detail earlier. Genes involved in the uptake of compatible solutes were also recovered which may have a role in osmoprotection and cryoprotection. Finally, genes involved in the maintenance of membrane fluidity (unsaturation of fatty acids) were also present (Methe et al., 2005).

In a recent study, cold-adaptation based on genome analysis in two psychrotolerant *Colwellia* species was investigated (Zhang et al., 2018a). The study found genes responsible for cold-adaptation including, uptake of compatible solutes and cold-shock proteins. Genes responsible for increasing membrane fluidity (desaturases) at low temperature were also detected.

# Chapter 3

# Chapter 3

## A survey of culture – dependent and culture-independent diversity of bacteria from Batura Glacier in the Karakoram Range and their potential for industrial applications

**Abstract**

High-altitude cold habitats of the Karakoram are rarely explored for their bacterial diversity and functionality. In the present study bacterial communities in ice, meltwater and sediments of Batura glacier were investigated using culture-dependent and culture-independent methods. 27 cold-adapted bacterial strains (mostly psychrotrophic) were isolated using R2A, tryptic soy agar (TSA) and Luria-Bertani (LB) media, at 4 and 15˚C through culture-dependent method. Most of the isolates exhibited growth at wide range of temperature (4-35˚C) with optimum at 20-30˚C, pH around 5-12 and tolerated salinity up to 6% (w/v). Among the culturable bacteria, 52% were identified as Gram-positive while 48% represented Gram-negatives. The results of phylogenetic analysis indicated that all the culturable bacteria belonged to 3 major phylogenetic groups: *Actinobacteria* (48%), *Bacteroidetes* (26%) and *Proteobacteria* (22%); while *Flavobacterium* (26%), *Arthrobacter* (22%) and *Pseudomonas* (19%) were represented as the dominant genera. Similarly, 16S rRNA gene sequences in the unculturable community were dominated by the same phylogenetic groups, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*, while *Arthrobacter*, *Mycoplana*, *Ochrobactrum*, *Kaistobacter*, *Janthinobacterium* and *Flavobacterium* were found the dominant genera. The culturable bacteria were further screened for production of extracellular enzymes and antimicrobial compounds as well as their resistance to commercial antibiotics. 67% of the isolates demonstrated activity for cellulases, 48% as lipases, 40% proteases, 41% DNases and only 7% for amylases. The glacial isolates demonstrated antimicrobial activity against other microorganisms such as *Staphylococcus aureus*, *Candida albicans*, *Bacillus* sp., *Pseudomonas aeruginosa*, *Acinetobacter* sp. and *Klebsiella pneumonia*. 67% of Gram-positive while 46% of Gram-negative glacial bacteria were resistant to trimethoprim/ sulfamethxazole. Resistance against methicillin and vancomycin among the Gram-positive isolates was 23% and 15%, respectively, while 11% of the Gram-negative isolates exhibited resistance against both colistin sulfate and nalidicixic acid.

**Keywords**: Karakoram, Glacier, Bacteria, Psychrophiles, Psychrotrophs, Metagenomics

## 3.1. Introduction

More than 80% of the biosphere is constantly at temperatures under 5˚C. The deep sea represents a major fraction of these cold environments as the oceans cover 71% of the planet and 90% of their volume is below 5˚C. Additionally, glaciers account for 10% of the land surface (Russell, 1990; Margesin and Miteva, 2011). The cold biosphere harbors a diverse group of cold-adapted microorganisms called psychrophiles or psychrotrophs, inhabiting terrestrial and aquatic environments in the Arctic and Antarctic polar regions as well as the deep ocean, high mountains of alpine regions, upper atmosphere and the cave systems. Successful colonization of the cold environments by psychrophilic microorganisms is the result of a vast array of adaptation strategies including: maintenance of membrane fluidity and structural flexibility of their enzymes, expression of the cold shock proteins, production of antifreeze proteins and compatible solutes (Collins and Margesin, 2019; Tribelli and López, 2018; Barria et al., 2013). Recently, the different omics technologies have given new insights into bacterial cold-adaptation (Goordial et al., 2016; Koh et al., 2017; Raymond-Bouchard et al., 2018).

Microbial biogeography is an emerging field, which uses the combination of modern technologies and interdisciplinary approaches that allow the study of diverse environmental parameters and the estimation of microbial diversity (Fierer, 2008; Green et al., 2008). The concept of glacial ecology has gained interest of researchers in recent years, mainly focusing on the two key glacier ecosystems; the supraglacial and subglacial (Hodson et al., 2008). The multidisciplinary study of proglacial and subglacial ecosystems has recently attracted scientific community and literature is rapidly expanding as a result of the potential role of microorganisms inhabiting these environments. Cold-adapted microorganisms are involved in key ecological functions in freezing environments where photoautotrophy is the basis of food webs in the proglacial and chemoautotrophy dominates the sub-glacier environments (Boetius et al., 2015). Glacier ice with an extremely low temperature is considered as one of the harshest environments on the biosphere. Apart from the extremely low temperatures, other limitations include low nutrient and water availability, frequent freeze-thaw events and increased darkness (Margesin and Miteva, 2011). However, despite all these limitations it is estimated that

glaciers and ice sheets around the world contain as many as $10^{29}$ microbial cells (Irvine-Fynn and Edwards, 2014). Glaciers are largely autotrophic systems where production exceeds respiration, acting as a carbon sink (Anesio et al. 2009). Studies on the structure of bacterial communities and their possible ecological role in cold environments have been reported from various glaciers of the polar and non-polar regions (Shivaji et al., 2011; Miteva et al., 2004; Segawa et al., 2011).

Extremophiles are organisms that can thrive in environments that are hostile or inhabitable to other forms of life (Horikoshi et al., 2010; Seckbach et al., 2013). Psychrophiles represent the most abundant group of extremophiles in terms of distribution, diversity and biomass (Struvay and Feller, 2012). Apart from their ecological role, cold-adapted microorganisms have shown tremendous potential for biotechnological applications ranging from enzymes and antimicrobial productions to environmental bioremediations (Feller and Gerday, 2003; Sánchez et al., 2009; Gratia et al., 2009; Cavicchioli et al., 2011; Margesin and Feller, 2010). Enzymes from the psychrophiles are considered as next generation biocatalysts because of the huge potential they offer for various industrial processes. The use of enzymes from cold-adapted bacteria could have some economic advantages over their mesophilic counterparts as they can maintain high catalytic activity at lower temperatures and save considerable energy by negating the heating process. (Russell, 1998).

Antibiotic resistance is part of natural evolution but its indiscriminate use has accelerated the process. The emergence of multi-drug resistant pathogenic strains is a global concern and this effect could be aggravated in the near future. Nature is considered the best reservoir for the discovery of industrially important metabolites. The stressful and inhospitable conditions of extreme environments have allowed these microorganisms the ability to produce unique compounds for their survival. Therefore, extreme environments are seen as new avenues for the bioprospecting of novel bioactive molecules including enzymes and antimicrobial compounds (Sánchez et al., 2009; Cavicchioli et al., 2002).

The north-most territories of Pakistan consist of the greatest mountain ranges in the world including the western Himalayas, Karakoram and Hindukush. The Himalaya-Karakoram-Hindukush (HKH) is the most glacierized area outside of the Polar Regions and is therefore considered the third pole of our planet. These ranges host at least 5000 glaciers in the Pakistani geographical region and serve fresh water to a large portion of their population (Rasul et al., 2011). The Karakoram mountain range is located between the borders of Pakistan, Afghanistan, India and China (Rankl et al., 2014). The Karakoram region is heavily glaciated with 37% of its area covered by the glaciers (Rankl et al., 2014). Some of the notable glaciers of the Karakoram includes, Siachen Glacier (~ 75 km long), Biafo Glacier (~ 68 km), Baltoro Glacier (~ 62 km), and Batura glacier (~ 59 km) (Hewitt, 2014). Interestingly, glaciers of the Karakoram are stable or even growing contrary to the ice bodies worldwide that are receding due to global warming (Minora et al., 2016; Rankl et al., 2014). This unusual behavior of the Karakoram glaciers is known as the "Karakorum Anomaly" (Hewitt, 2005). However, glaciers in the Karakoram are the least explored in terms of their bacterial diversity and functionality. It would be interesting to study the bacterial community of these glaciers that have exhibited resilience in climate change when other glaciers in the world are retreating.

Batura glacier in the Karakoram mountain range with a length of ~ 59 km is one of the longest glacier in the non-polar region (Hewitt, 2014). In this paper, we report diversity of the cold-adapted bacteria from Batura glacier in Pakistan using culture-dependent and culture-independent methods. It is well known that only a small fraction (<1%) of microorganisms in any environment is culturable in laboratory conditions and a large fraction remains unnoticed (Amann et al., 1995). Therefore, a combination of culture-dependent and culture-independent approaches should be employed to completely characterize the microbial diversity in any environment (Steven et al., 2007). Culturable bacteria were characterized based on their growth characteristics and exploited for extracellular enzymes and antimicrobial activities for potential future applications. Previously, we did genome sequencing of the glacial isolate *Pseudomonas* sp. BGI-2 and found 11 EPS-producing genes in its genome compared to none in the closely related

mesophilic *Pseudomonas* species (Ali et al., 2019). Also BGI-2 had more stress response genes compared to the closely related mesophilic counterparts it was compared with.

To the best of our knowledge, this is the first study in which culture-independent method was used along with culture-dependent method to characterize bacterial community in any glacier of the Karakoram in the Pakistani geographical region. The Karakoram glaciers are unique in their resilience to contemporary warming. Will this safeguard their microbial biodiversity? This study will serve as the basis for future diversity studies and comparisons of bacterial community change over time.

## 3.2. Materials and Methods

### 3.2.1. Description of the sampling site

Sampling was done in Batura glacier located in the Passu village of the upper Hunza valley in the northern Pakistan (Fig. 3.1). Hunza valley is located at the base of the Karakorum Range along the Karakorum Highway. Batura glacier (36° 32′ N, 74° 40′ E) is one of the longest glacier outside the Polar regions with the highest elevation around 7795 m in the West and the lowest nearly 2570 m in the East (Hewitt, 2014). Batura glacier basin is ~48% glacierized and surrounded by several major peaks with altitude >7000 m including, Batura Muztagh ~7,795 m (Hodson et al., 2002).

Figure 3.1. Picture of the sampling site in Batura glacier with Passu cathedral peak in the background, village Passu, district Hunza, Gilgit Baltistan.

### 3.2.2. Collection of samples

Sampling of ice, sediment and water was done in December of 2015. Mean temperature in winter (Oct. – Feb.) goes down as low as -20˚C at nights and up to 8˚C during the daytime. Ice, melt-water and sediment samples were collected aseptically in sterile 500 mL wide-mouth polypropylene sample bottles. Handling of samples was performed according to standard microbiological techniques. Sterilized tools were used for sampling of glacial ice and sediments. Surface ice and sediment up to 4-5 inches were removed and discarded. Underlying samples were collected into sterile bottles, temperature and pH were recorded at the time of sampling. The tubes were sealed, placed in an isothermal box and transported to Applied, Environmental and Geomicrobiology Laboratory, at the Department of Microbiology, Quaid-i-Azam University Islamabad and stored at -20˚C. Geographic coordinates were recorded at the time of sampling using a GPS device.

### 3.2.3. Physicochemical characteristics of the glacier samples

Analysis of samples for sulfates, phosphates, nitrate, nitrite, electrical conductivity, salinity, total dissolved solids (TDS) and pH were performed using Spectroquant (Pharo 100 Spectroquant® Merck, Germany) following the protocols from standard methods for the examination of water and wastewater by American Public Health Association (APHA).

### 3.2.4. Enumeration, isolation and viable bacterial count

Enumeration of culturable heterotrophic bacteria was carried out through the plate count method. Three general purpose culture media were used to isolate psychrophilic/ psychrotrophic bacteria from the samples including tryptic soy agar (Appendix Table A1), Luria Bertani agar (Appendix Table A2) and R2A agar (Appendix Table A3). 100 µL aliquots of the three glacier samples, or a 10 folds dilution of them, were spread onto agar plates using two different temperatures (4˚C and 15˚C) for incubations. Plates were incubated up to four weeks and observed daily for colony appearance. Plates were also incubated at 4˚C for an extended period up to several months for recovery of injured or compromised isolates. Morphologically different colonies from each plate were sub-cultured on separate fresh plates to obtain pure cultures. All the viable counts were performed in replicates and the results presented are the mean of duplicate assays.

### 3.2.5. Direct bacterial count using epifluorescence microscopy

Direct bacterial count from the three glacier samples was performed with the method used by Zhang et al., (2008a) and Chen et al., (2001) with minor modifications. 2 to 3 mL of glacier samples (melted ice and water) were filtered through 0.2 µm polycarbonate membrane filter (Millipore) under gentle vacuum pressure. CYBR Gold (Invitrogen) working solution (2X) was prepared from an original stock (10,000X) using TE buffer (10 mM Tris-Cl; 1 mM EDTA, pH 7.4-7.6). Bacterial cells on the filters were stained with 300 µL of the CYBR Gold solution (2X) for 15 minutes in the dark and the residual stain was removed by applying vacuum. 10 µL of mounting solution (50% glycerol and 50% TE buffer) was placed on the slides and stained filters were mounted on top of the drop with cover slips placed over it. The samples were examined under blue-green light excitation (480-495 nm) using epifluorescence microscopy (Axioplan, Zeiss). The number of bacterial cells per mL or g for each sample was calculated based on count of at least 20 randomly selected microscopic fields and the volume of each filtered sample.

### 3.2.6. Enrichment of ice sample

For ice samples, 100 µL of melted ice was directly plated on TSB, LB agar and R2A agar, and incubated at 4˚C for 9 months and 15˚C for 6 months. Enrichment was also carried out by adding 10 mL of melted ice sample to 90 mL of liquid culture media (R2A broth, TSB and LB broth). Enrichment samples were incubated in shaker incubators (150 rpm) at two different temperatures (4˚C and 15˚C). Samples were then visualized for bacterial growth (media turbidity) every day up to 2 to 3 weeks. 100 µL of diluted and undiluted samples from each enrichment culture were plated on their corresponding solid media for separate colonial growth. Morphologically different colonies observed were streaked on separate plates to obtain pure cultures. A similar procedure was used for enrichment of water and sediment samples for maximum recovery of bacterial isolates.

### 3.2.7. Identification of culturable bacterial strains

#### 3.2.7.1. Colony and cell morphologies

The bacterial isolates were differentiated and identified initially based on colony morphology (size, elevation, margin and pigmentation), cell morphology (cocci,

coccobacilli or rods), and biochemical characteristics (sugar fermentation, $H_2S$ production, catalase, oxidase, nitrate reduction and citrate utilization).

#### 3.2.7.2. Molecular identification of culturable isolates

Extraction of DNA from each isolate was carried out using a Genomic DNA isolation kit (Norgen Biotek Corporation, Canada). Sequencing of 16S rRNA gene was done by Macrogen Inc. Seoul, Korea. The ambiguous low quality aligned regions from sequences were removed using Chromas (version 2.6.6). 16S rRNA gene sequences for all the isolates were aligned in BLAST using EzBioCloud database (Yoon et al., 2017a). 16S rRNA gene sequences for all the isolates were submitted in the GenBank database of NCBI to obtain their accession numbers. Phylogenetic trees were constructed separately for isolates from the three glacier samples using neighbor-joining method (bootstrap value >50) in MEGA 7.0 software (Saitou and Nei, 1987).

### 3.2.8. Characterization of culturable bacteria

#### 3.2.8.1. Optimization of physicochemical culture conditions

Growth of the isolates was checked at different temperature (4-45˚C) and pH (3.0-13.0) on R2A agar plates. Isolates were checked for their ability to grow under halophilic conditions by streaking them on R2A agar plates supplemented with NaCl at concentration ranges from 2-10% (w/v). Growth medium was also optimized by inoculating on to different media named R2A (Difco), tryptic soy agar (Oxoid), nutrient agar (Oxoid), LB agar (Oxoid), sabouraud dextrose agar (Oxoid), mannitol salt agar (Oxoid), MacCkonkey agar (Oxoid) and Mueller Hinton agar (Oxoid). Specifications of the media are given in the Appendix (Table A4). For all the above tests, the result were analyzed after incubating plates at 15˚C for 5 days in duplicates. These tests were performed according to the methods described previously (Zhang et al., 2013: Shivaji et al., 2013; Shivaji et al., 2011).

#### 3.2.8.2. Antibiotic resistance

Antimicrobial susceptibility was determined on R2A and Mueller-Hinton agar plates using disc diffusion method (Bauer et al., 1966). All the isolates were grown in R2A broth at 15˚C for 48 hours. Inoculums were diluted to turbidity equivalent to 0.5 McFarland

standard and swabbed on the surface of agar plates using sterile cotton swabs. Seven antibiotics representing different classes were used including methicillin (10 µg), imipenem (10 µg), ofloxacin (5 µg), vancomycin (5 µg), colistin sulfate (10 µg), nalidixic acid (30 µg) and trimethoprim sulfamethaxazole (30 µg), specification of antibiotics is mentioned in Appendix (Table A5). All the plates were incubated at 15˚C and results were interpreted after 72-96 hours. The susceptibility of strains to each antibiotic was determined by measuring zone of inhibition.

#### 3.2.8.3. Extracellular enzyme activity

Isolates were screened for their ability to produce extracellular enzymes. Starch hydrolysis test was performed for amylase production (Priest, 1977). The bacterial isolates were streaked on agar surface supplemented with 1% starch and incubated at 15˚C for 5 days. The plates were later flooded with Lugol's iodine solution. The area of the medium containing un-hydrolyzed starch was stained dark purple while the hydrolyzed region around the growth was clear. Lipase activity was checked by streaking isolates on basal medium supplemented with 1% Tween-40 along with 0.001% rhodamine B and 0.01% $CaCl_2$, according to the method described previously (Booth, 1978). Formation of opaque hazy zone of calcium soap crystals around the growth indicated hydrolysis of Tween. Cellulase activity was demonstrated on carboxymethylcellulose (CMC) agar (Appendix Table A6) plates according to the method as previously described (Kasana et al., 2008). Isolates were spot inoculated on CMC agar and plates were incubated at 15˚C for 5 days. The plates were later flooded with Gram's iodine for 3 to 5 minutes. Zone of clearance around the isolates indicated a positive response for cellulase production (Fig. 3.3b). Protease activity was checked by the method as previously described by Priest (1977) with some modifications. Isolates were streaked on the basal medium supplemented with 1% casein. Plates were then incubated at 15˚C for 5 days and flooded with 1M acetic acid. A clear zone of hydrolysis around the growth demonstrated a positive response for protease production. For DNase test, isolates were spot inoculated on surface of DNase agar and plates were incubated at 15˚C for 5 days. Plates were then flooded with 1N hydrochloric acid. Hydrolysis of DNA was indicated by a clear zone around the growth of tested organism.

#### 3.2.8.4. Antimicrobial activity

Antimicrobial activity of all the glacial isolates was tested against a number of microorganisms including the multidrug resistance strains of *Candida albicans*, *Klebsiella pneumoniae*, *Acinetobacter* sp., and *Bacillus* sp. and two ATCC strains *Pseudomonas aeruginosa* (ATCC27853) and *Staphylococcus aureus* (ATCC6538). Antimicrobial activity was determined through spot inoculation as well as agar well diffusion methods (Balouiri et al., 2016). For spot inoculation, R2A agar and Mueller-Hinton agar plates were swabbed with overnight culture of indicator organisms and isolates were spot inoculated on plates. The plates were incubated at 15˚C for 3 to 5 days and observed for a clear zone around the isolates. For well diffusion method, both the indicator organisms and isolates were grown in their respective media. Cultures of indicator organisms were standardized to 0.5 McFarland standard ($10^8$ CFU/mL) and swabbed on agar plates. Wells of 10 mm diameter on the agar media were made using sterile steel borer. 100 µL of cell free supernatant for all the isolates was poured into their designated wells. Plates were incubated for 3 to 5 days at 15˚C and observed for zone of inhibition. The multiple drug resistance strains were provided by the Medical Microbiology Laboratory, in the Department of Microbiology, Quaid-i-Azam University, Islamabad.

### 3.2.9. Metagenomics Analysis of Glacier Samples

#### 3.2.9.1. Extraction of Community DNA and 16S rRNA Gene Sequencing

Whole DNA was extracted from each glacier sample using commercially available kits. DNA from ice and melt-water was extracted using DNeasy® PowerWater kit (Qiagen GmbH, Hilden, Germany). Community DNA extraction from sediment sample was done using DNeasy® PowerSoil® kit (Qiagen GmbH, Hilden, Germany). Quality and quantity of the extracted DNA were determined using NanoDrop 2000 spectrophotometer (Thermo Scientific). 16S rRNA genes for the three samples were amplified using the forward primer (5′GTGCCAGCMGCCGCGGTAA) and reverse primer (5′GGACTACHVGGGTWTCTAAT) designed for the V3 and V4 regions (Klindworth et al., 2013). Size of the PCR amplicons was checked through 1% agarose gel electrophoresis. Sequencing was done using MiSeq (Illumina, San Diego, CA) at the BioAnalytical Laboratory, Institute of Marine and Environmental Technology (IMET), University of

Maryland Center for Environmental Sciences (UMCES). The libraries were prepared using the Nextera XT kit (Illumina) and sequencing was done using 2×300 cycle run using the MiSeq reagent kit v.3. The 16S rRNA gene dataset consisted of 189,890 raw sequence reads for the ice sample, 451,614 for water and 225,910 reads for the sediment sample.

#### 3.2.9.2. Processing of 16S rRNA gene data

16S rRNA gene MiSeq sequencing reads for the three glacier samples were assembled and classified using QIIME software v 1.6.0 (Caporaso et al., 2010). Alignment of the unique sequences was performed with the 'align.seqs' command against a Greengenes database.

#### 3.2.9.3. Assignments of operational taxonomic unit (OTU)

The sequences after quality filter were grouped into OTUs based on sequence resemblances using a 97% similarity threshold against the respective reference databases (Marzorati et al., 2008). All the other sequences were clustered into denovo OTUs with UCLUST within QIIME. RDP classifier in QIIME was used for the assignment of taxonomy to these sequences.

#### 3.2.9.4. Nucleotide sequence accession numbers

The bacterial 16S rRNA gene raw data from MiSeq sequencing for the ice, water and sediment samples was deposited into the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the BioProject accession number PRJNA533649.

## 3.3. Results

### 3.3.1. Physicochemical properties of glacier samples

pH of the glacier samples was found to be slightly alkaline with low salinities. The concentration of sulfate was significantly higher compared to phosphate, nitrate and nitrite for the glacier samples (Table 3.1).

Table 3.1. Physicochemical properties of glacier samples

| Sample Type | Physical Properties | | Chemical Properties (mg/L) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | pH | Electrical Conductivity (µS/cm) | TDS | Phosphate | Sulfate | Nitrate | Nitrite | Salinity |
| Ice | 7.87 | 100 | 90 | 1.60 | 450 | 1 | 0.37 | 50 |
| Water | 7.50 | 170 | 121 | 2.62 | 375 | 1 | 0.06 | 80.9 |

### 3.3.2. Bacterial count from the glacier samples

Bacterial number in the glacier samples was estimated using viable as well as direct microscopic count. For viable count in the ice sample, no bacterial colony was observed on any of the three media used (R2A, TSA, LBA) even after 9 months of incubation at 4˚C and 6 months of incubation at 15˚C. Viable bacterial count from the melt-water and sediment samples varied depending on the incubation temperature and the type of medium used. Maximum viable count for the melt-water ($2.0 \times 10^3$ CFU $mL^{-1}$) and sediment ($1.8 \times 10^4$ CFU $g^{-1}$) samples was recorded when R2A medium was used at 15˚C (Table 3.2).

In case of direct bacterial count using epifluorescence microscopy revealed the presence of high number of bacterial cells in all three samples. The highest bacterial count was observed in sediment ($3.3 \times 10^6$ cells $g^{-1}$), followed by water ($2.8 \times 10^6$ cells $mL^{-1}$) and ice ($6.4 \times 10^5$ cells $mL^{-1}$) (Table 3.2).

Table 3.2. Bacterial counts from the three glacier samples (ice, water, sediment). Table also includes direct bacterial cell counts using CYBR Gold with epifluorescence microscopy.

| Sample Type | Viable count (CFU) at 4˚C (CFU/mL or g) | | | Viable count (CFU) at 15˚C (CFU/mL or g) | | | Epifluorescence microscopy (cells/mL or g) |
|---|---|---|---|---|---|---|---|
| | R2A | TSA | LBA | R2A | TSA | LBA | |
| Ice | - | - | - | - | - | - | $6.4 \times 10^5$ |
| Meltwater | $1.5\times10^3$ | $1.1\times10^3$ | $1.1\times10^3$ | $2.0\times10^3$ | $1.6\times10^3$ | $1.6\times10^3$ | $2.8 \times 10^6$ |
| Sediment | $1.1\times10^4$ | $1.0\times10^4$ | $8.9\times10^3$ | $1.8\times10^4$ | $1.4\times10^4$ | $1.1\times10^4$ | $3.3 \times 10^6$ |

### 3.3.3. Isolation of bacteria from glacier samples

A total of 27 bacterial isolates were recovered from three glacier samples, including 11 from melt-water and 8 from each ice and sediment samples. Most of the isolates were

recovered on the oligotrophic medium R2A, whereas the nutrient rich media (TSA and LBA) yielded less colonies. Most of the colonies appeared after 2 to 3 weeks of incubation (4˚C and 15˚C) and only few appeared in the first week. However, the same isolates formed visible colonies within 2 to 5 days on subsequent re-streaking. Initial identification was done based on the colony and cell morphologies, pigmentation and growth characteristics.

Direct plating of the melted ice sample on agar media (R2A, TSA and LBA) did not yield colonies on any of the plates even after 9 and 6 months of incubation at 4˚C and 15˚C, respectively. On the other hand, growth was observed in the second week (indicated by turbidity in the media) in the enrichment culture at 15˚C. When inoculated on the respective solid medium, colonies appeared within the first to 3$^{rd}$ week of incubation at 4˚C and 15˚C. A total of 8 different bacterial isolates were obtained after plating the liquid enrichment culture on agar media, six colonies from R2B and one each from TSB and LB enrichments were recovered.

Gram staining revealed Batura glacier is inhabited by representatives from both Gram-positive (14 isolates) and Gram-negative (13 isolates), with majority of the isolates exhibited coccobacillary cell morphologies (Table 3.3). Strains isolated from the glacier ice, water and sediment are named with the prefix BGI (Batura glacier ice), BGW (Batura glacier water) and BGS (Batura glacier sediment).

Table 3.3. Physiological characteristics of the glacial isolates (+ means sugar fermentation, catalase production or nitrate reduction, whereas – represents no activity).

| Isolates | Temperature Range (˚C) | pH Range | NaCl Range (%) | Biochemical Properties | | | | Cell Morphology (Gram Staining) |
|---|---|---|---|---|---|---|---|---|
| | | | | Citrate Utilization | Glucose/ Sucrose/ Lactose | Catalase Test | Nitrate Reduction | |
| BGI-1 | 4-30 | 5-11 | 0 – 4 | + | +/-/- | + | - | -ve (short rods) |
| BGI-2 | 4-35 | 5-11 | 0 – 5 | + | +/-/- | + | - | -ve (short rods) |
| BGI-4 | 4-40 | 4-12 | 0 – 2 | + | -/-/- | + | + | +ve (thin long rods) |
| BGI-5 | 4-40 | 5-12 | 0 – 4 | - | -/-/- | + | - | +ve (cocci) |
| BGI-7 | 4-25 | 4-12 | 0 – 4 | - | -/-/- | + | + | +ve (coccobacilli ) |
| BGI-10 | 4-35 | 3-13 | 0 – 8 | + | -/-/- | + | - | +ve (coccobacilli ) |
| BGI-11 | 4-35 | 3-13 | 0 – 8 | + | -/-/- | + | - | +ve (coccobacilli ) |
| BGI-14 | 4-25 | 3-12 | 0 – 2 | - | +/+/+ | + | + | +ve (cocci) |
| BGS-1 | 4-30 | 5-12 | 0 – 6 | + | +/-/- | + | - | +ve (coccobacilli) |
| BGS-2 | 4-30 | 5-12 | 0 – 4 | - | +/-/- | + | + | +ve (short rods) |
| BGS-3 | 4-35 | 6-11 | 0 – 2 | - | +/-/- | + | - | +ve (cocci in clusters) |
| BGS-5 | 4-30 | 5-12 | 0 – 4 | - | -/-/- | + | + | +ve (short rods) |
| BGS-6 | 4-35 | 4-13 | 0 – 8 | - | -/-/- | + | - | +ve (coccobacilli) |
| BGS-9 | 4-30 | 4-13 | 0 – 6 | - | -/-/- | + | + | +ve (cocci in clusters) |
| BGS-12 | 4-30 | 5-13 | 0 – 4 | + | -/-/- | + | - | +ve (coccobacilli) |
| BGS-13 | 4-35 | 4-12 | 0 – 6 | - | +/+/+ | + | - | +ve (bacilli) |

| BGW-1 | 4-25 | 5-11 | < 1 | - | +/-/- | + | + | -ve (coccobacilli) |
|---|---|---|---|---|---|---|---|---|
| BGW-2 | 4-25 | 5-11 | < 1 | - | -/-/- | + | - | -ve (short rods) |
| BGW-4 | 4-35 | 5-13 | 0 – 6 | + | -/-/- | + | - | -ve (coccobacilli) |
| BGW-5 | 4-35 | 5-13 | 0 – 6 | + | -/-/- | + | - | -ve (short rods) |
| BGW-7 | 4-20 | 5-12 | < 1 | - | -/-/- | + | - | -ve (coccobacilli) |
| BGW-8 | 4-35 | 4-13 | 0 – 4 | + | -/-/- | + | + | -ve (short rods) |
| BGW-10 | 4-35 | 4-13 | 0 – 4 | + | -/-/- | + | - | -ve (bacilli) |
| BGW-12 | 4-25 | 5-10 | < 1 | - | +/-/- | + | - | -ve (thick rods) |
| BGW-15 | 4-20 | 6-9 | < 1 | - | +/-/- | + | - | -ve (small cocci) |
| BGW-17 | 4-20 | 5-11 | 0 – 2 | - | +/+/+ | + | + | -ve (short rods) |
| BGW-18 | 4-20 | 5-10 | < 1 | - | +/+/+ | + | + | -ve (short rods) |

### 3.3.4. Phylogenetic classification of isolates based on 16S rDNA gene sequences

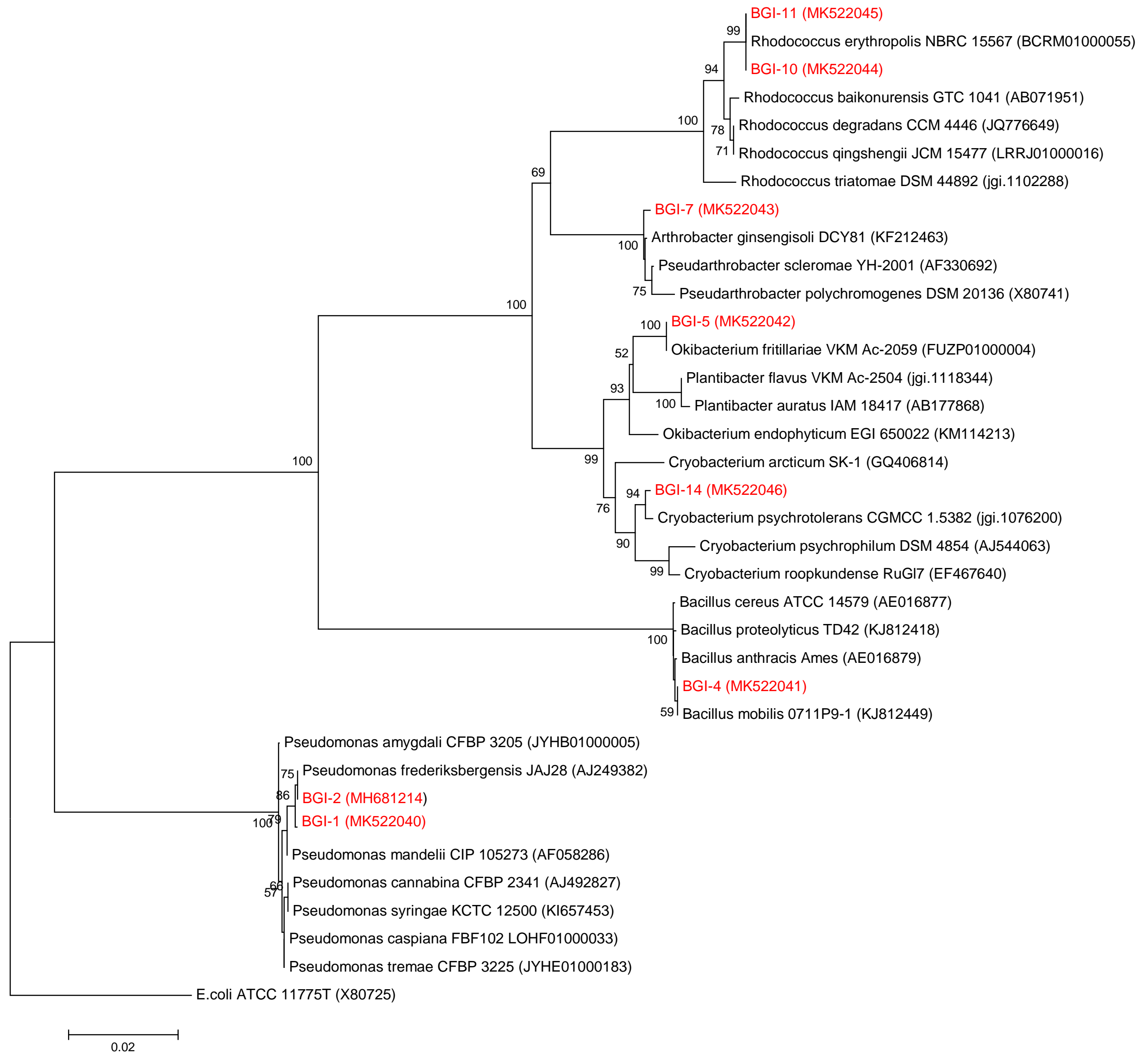
Molecular identification based on 16S rDNA gene sequences revealed Batura glacier is inhabited by a diverse group of bacteria. The isolates belong to 3 major phylogenetic groups; *Actinobacteria* (48%), *Bacteroidetes* (26%) and *Proteobacteria* (22%). The dominant genera are *Flavobacterium* (26%), *Arthrobacter* (22%) and *Pseudomonas* (19%). The accession numbers for isolates from different samples were designated as follows: bacteria from ice (MK522040 - MK522046); from water (MK558827 - MK558837) and from sediment (MK558838 - MK558845) (Table 3.4). A phylogenetic tree was constructed separately for isolates from each glacier sample to determine their affiliation with other bacteria in the NCBI GenBank database (Fig. 2). The majority of 16S rRNA sequences were similar to previously determined sequences with 98% or above identical values (Table 3.4). Some of the isolates had 98 to 99% 16S rRNA gene sequence similarity with psychrophilic bacteria isolated from various cold environments. BGI-14 was closely related to *Cryobacterium psychrotolerans* (>99%), BGW-1/BGW-17/BGW-18 to *Flavobacterium psychrolimnae* (>99%), BGW-2/BGW-7/BGW-12 to *Flavobacterium glaciei* (>98%) and BGW-5 to *Pseudomonas antarctica* (>99%).

Table 3.4. Phylogenetic affiliation of 16S rRNA gene sequences of culturable bacterial isolates from glacier samples with accession number and closely related species in the GenBank database with similarity index.

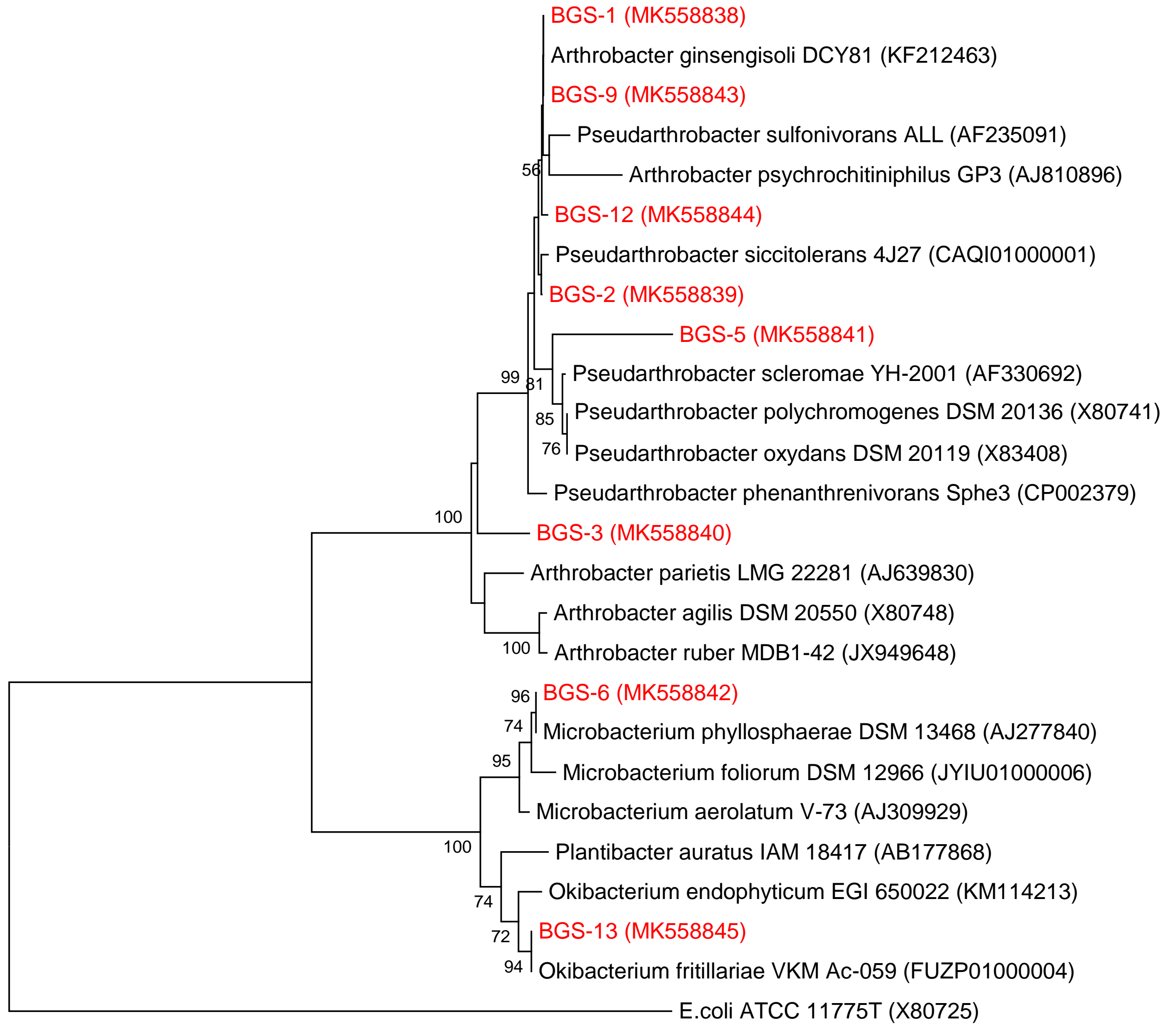
| **Isolate** | **Sample Type** | **Accession No.** | **Phylogenetic Group** | **Nearest phylogenetic neighbor with accession number** | **Similarity (%)** |
|---|---|---|---|---|---|
| BGI-1 | Ice | MK522040 | *Proteobacteria* | *Pseudomonas mandelii* (BDAF01000092) | 99.72 |
| BGI-2 | Ice | MH681214 | *Proteobacteria* | *Pseudomonas silesiensis* A3 (KX276592) | 99.66 |
| BGI-4 | Ice | MK522041 | *Firmicutes* | *Bacillus proteolyticus* (MACH01000033) | 99.81 |
| BGI-5 | Ice | MK522042 | *Actinobacteria* | *Okibacterium fritillariae* (FUZP01000004) | 99.81 |
| BGI-7 | Ice | MK522043 | *Actinobacteria* | *Arthrobacter ginsengisoli* (KF212463) | 98.84 |
| BGI-10 | Ice | MK522044 | *Actinobacteria* | *Rhodococcus erythropolis* (BCRM01000055) | 99.81 |
| BGI-11 | Ice | MK522045 | *Actinobacteria* | *Rhodococcus erythropolis* (BCRM01000055) | 99.76 |
| BGI-14 | Ice | MK522046 | *Actinobacteria* | *Cryobacterium psychrotolerans* (jgi.1076200) | 99.12 |
| BGS-1 | Sediment | MK558838 | *Actinobacteria* | *Arthrobacter ginsengisoli* (KF212463) | 99.44 |
| BGS-2 | Sediment | MK558839 | *Actinobacteria* | *Arthrobacter ginsengisoli* (KF212463) | 99.62 |
| BGS-3 | Sediment | MK558840 | *Actinobacteria* | *Arthrobacter agilis* (X80748) | 97.68 |
| BGS-5 | Sediment | MK558841 | *Actinobacteria* | *Pseudarthrobacter sulfonivorans* (AF235091) | 95.90 |
| BGS-6 | Sediment | MK558842 | *Actinobacteria* | *Microbacterium phyllosphaerae* (AJ277840) | 99.68 |
| BGS-9 | Sediment | MK558843 | *Actinobacteria* | *Arthrobacter ginsengisoli* (KF212463) | 99.38 |
| BGS-12 | Sediment | MK558844 | *Actinobacteria* | *Arthrobacter ginsengisoli* (KF212463) | 99.42 |
| BGS-13 | Sediment | MK558845 | *Actinobacteria* | *Okibacterium fritillariae* (FUZP01000004) | 99.91 |
| BGW-1 | Meltwater | MK558827 | *Bacteroidetes* | *Flavobacterium psychrolimnae* (AJ585428) | 99.03 |

| | | | | | |
|---|---|---|---|---|---|
| BGW-2 | Meltwater | MK558828 | *Bacteroidetes* | *Flavobacterium glaciei* (DQ515962) | 98.72 |
| BGW-4 | Meltwater | MK558829 | *Proteobacteria* | *Pseudomonas deceptionensis* (JYKX01000012) | 99.48 |
| BGW-5 | Meltwater | MK558830 | *Proteobacteria* | *Pseudomonas antarctica* (AJ537601) | 99.82 |
| BGW-7 | Meltwater | MK558831 | *Bacteroidetes* | *Flavobacterium glaciei* (DQ515962) | 98.74 |
| BGW-8 | Meltwater | MK558832 | *Proteobacteria* | *Janthinobacterium svalbardensis* (DQ355146) | 99.43 |
| BGW-10 | Meltwater | MK558833 | *Proteobacteria* | *Pseudomonas deceptionensis* (JYKX01000012) | 99.64 |
| BGW-12 | Meltwater | MK558834 | *Bacteroidetes* | *Flavobacterium glaciei* (DQ515962) | 98.94 |
| BGW-15 | Meltwater | MK558835 | *Bacteroidetes* | *Flavobacterium omnivorum* (AF433174) | 99.82 |
| BGW-17 | Meltwater | MK558836 | *Bacteroidetes* | *Flavobacterium psychrolimnae* (AJ585428) | 99.35 |
| BGW-18 | Meltwater | MK558837 | *Bacteroidetes* | *Flavobacterium psychrolimnae* (AJ585428) | 99.43 |

(a)



(b)



(c)

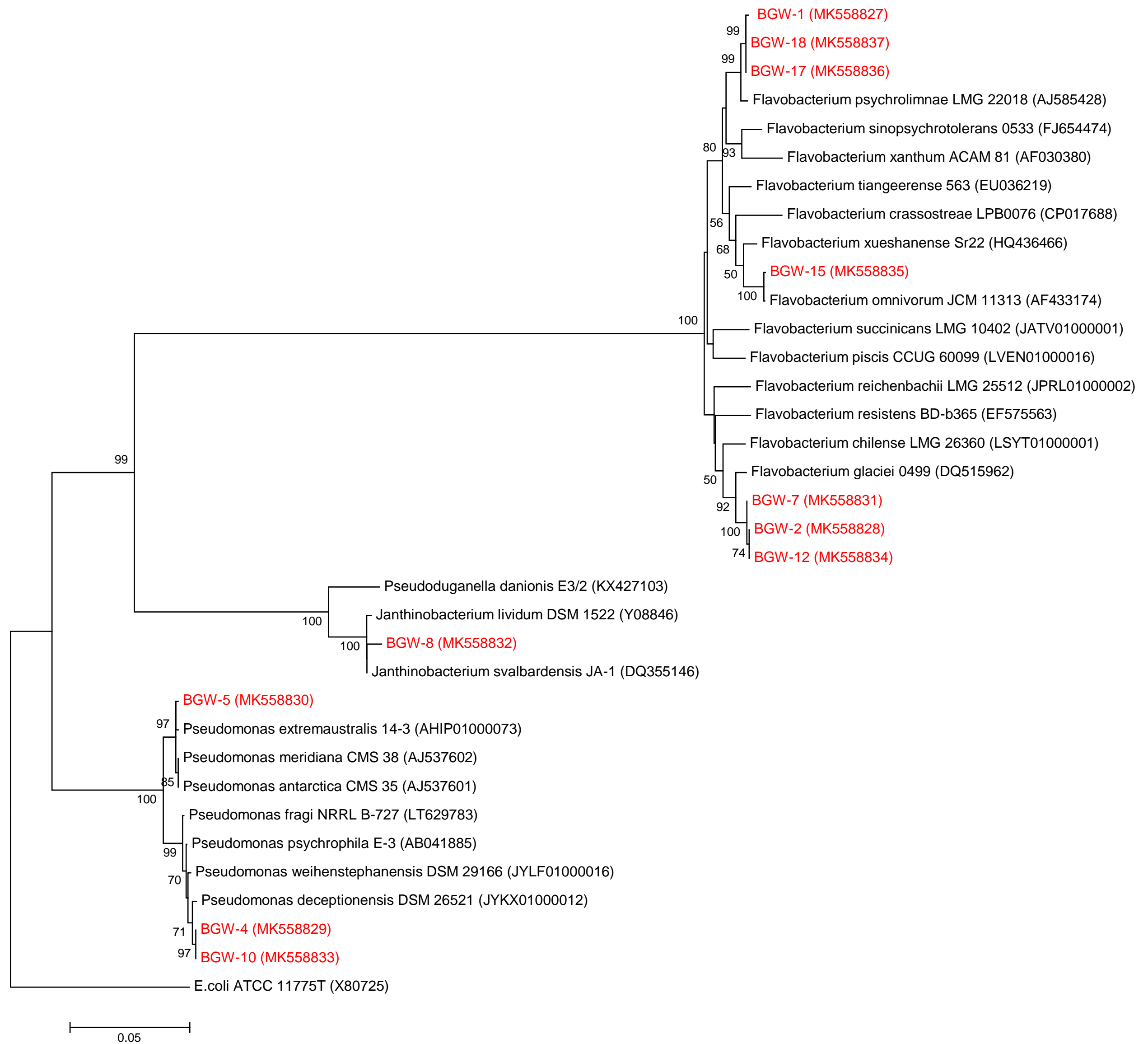



Figure 3.2. Phylogenetic tree of isolates recovered from the three glacier samples (a) ice, (b) sediment and (c) melt-water, using neighbor-joining method with a bootstrap value (%) greater than 50 from 1000 replicates. Numbers in the brackets are GenBank accession numbers for the 16S rRNA sequences and *E.coli* ATCC 11775T was used as an outgroup to root the tree.

### 3.3.5. Growth characteristics of the culturable bacteria

Phenotypic characteristics including temperature, pH, and salinity ranges for growth were determined. Results revealed that most of the isolates could grow at wide range of temperature (4–35˚C) and pH (5–12) (Table 3.3). A few strains, including BGI-10 and BGI-11, demonstrated the ability to grow at the extremes of pH range (3 and 13). The majority of the isolates were either slight-halophiles or halotolerant. BGI-10 and BGI-11 were able to grow at salt concentrations of up to 8% (w/v) but none of the isolates demonstrated growth at 10% NaCl. The majority of the isolates preferred the oligotrophic medium R2A but also demonstrated growth at nutrient rich media. BGI-10 and BGI-11 were able to grow in all the 8 media used (Table 3.5). The five *Pseudomonas* species BGI-1, BGI-2, BGW-4, BGW-5 and BGW-10 demonstrated growth on MacConkey's agar and could not ferment lactose. BGS-6, BGI-10 and BGI-11 demonstrated good growth on mannitol salt agar with high salt concentration (7.5% w/v) and deemed halotolerant (Table 3.5).

Table 3.5. Growth of isolates on different culture media with different nutritional components (+ Weak growth; ++ Moderate growth; +++ Good growth: NFL; Non-lactose fermenter).

| Isolates | R2A | Luria-Bertani Agar | Tryptic Soy Agar | Nutrient agar | Sabouraud dextrose agar | Mannitol salt agar | MacConkey agar | Mueller-Hinton agar |
|---|---|---|---|---|---|---|---|---|
| BGI-1 | +++ | +++ | +++ | +++ | - | - | (NLF) +++ | +++ |
| BGI-2 | +++ | +++ | +++ | +++ | - | - | (NLF) +++ | +++ |
| BGI-4 | +++ | +++ | +++ | +++ | - | + | (NLF) +++ | +++ |
| BGI-5 | +++ | +++ | +++ | +++ | - | + | - | +++ |
| BGI-7 | +++ | +++ | +++ | +++ | - | + | - | +++ |
| BGI-10 | +++ | +++ | +++ | +++ | +++ | +++ | (NLF) +++ | +++ |
| BGI-11 | +++ | +++ | +++ | +++ | +++ | +++ | (NLF) +++ | +++ |
| BGI-14 | +++ | + | + | ++ | - | ++ | - | ++ |
| BGS-1 | +++ | +++ | +++ | +++ | + | ++ | - | +++ |
| BGS-2 | +++ | +++ | +++ | +++ | + | + | - | +++ |
| BGS-3 | +++ | +++ | +++ | +++ | - | + | - | ++ |
| BGS-5 | +++ | +++ | +++ | +++ | - | + | - | +++ |
| BGS-6 | +++ | +++ | +++ | +++ | + | +++ | (NLF) +++ | +++ |
| BGS-9 | +++ | +++ | +++ | +++ | - | ++ | - | +++ |
| BGS-12 | +++ | +++ | +++ | +++ | + | + | + | +++ |
| BGS-13 | +++ | +++ | +++ | +++ | + | + | - | +++ |
| BGW-1 | + | - | - | +++ | - | - | - | ++ |
| BGW-2 | + | - | - | - | - | - | - | + |
| BGW-4 | +++ | +++ | +++ | +++ | + | - | (NLF) +++ | +++ |
| BGW-5 | +++ | +++ | +++ | +++ | + | - | (NLF) +++ | +++ |
| BGW-7 | +++ | + | - | - | - | - | - | + |
| BGW-8 | +++ | +++ | +++ | +++ | + | - | (NLF) +++ | +++ |
| BGW-10 | +++ | +++ | +++ | +++ | + | - | (NLF) +++ | +++ |
| BGW-12 | +++ | + | - | - | - | - | - | + |
| BGW-15 | +++ | + | - | - | - | - | - | + |
| BGW-17 | +++ | ++ | + | + | - | - | - | ++ |
| BGW-18 | +++ | ++ | +++ | +++ | - | - | - | + |

### 3.3.6. Extracellular enzymes and antimicrobials production

Isolates were screened for enzymatic and antimicrobial activities. Most of the isolates demonstrated the ability to produce one or more extracellular enzymes (Table 3.6). Enzymatic assays revealed that 67% of the strains produced cellulases, 48% lipases, 40% proteases, 41% DNases and only 7% amylases. Among the isolates, BGS-9 demonstrated the ability to produce 4 different enzymes including proteases, lipases, cellulases and DNases. BGI-1, BGI-2, BGS-2 and BGS-12 demonstrated activity for proteases, lipases and cellulases. BGW-4 demonstrated activity for lipases, proteases and DNases. BGW-5 produced proteases, lipases and cellulases whereas BGW-15 showed the ability to produce cellulases, amylases and DNases. *Flavobacterium* sp. BGW-15 and *Flavobacterium* sp. BGW-17 were the only strains able to produce amylases.

Antimicrobial activity of the glacial isolates were tested against a number of indicator organisms including representatives from Gram-positive and Gram-negative bacteria as well as a yeast. The majority of the glacier isolates demonstrated antimicrobial activity against one or more test microorganisms: 30% against *C. albicans*, 30% against *Bacillus* sp., 26% against *P. aeruginosa*, 26% against *S. aureus*, 22% against *Acinetobacter* sp. and only 4% against *K. pneumonia*. Isolates BGI-2, BGW-4 and BGW-5 demonstrated activity against 5 of the 6 test organisms used including *S. aureus*, *P. aeruginosa*, *C. albicans*, *Bacillus* sp. and *Acinetobacter* sp. (Table 3.6). BGI-1 demonstrated antimicrobial activity against 4 test organisms including, *S. aureus*, *P. aeruginosa*, *C. albicans*, and *Acinetobacter* sp. The isolates BGW-8 and BGW-10 demonstrated activities against 4 of the test organisms (Table 3.6).

Table 3.6. Extracellular enzymes and antimicrobial activities demonstrated by the glacier isolates (+ Enzyme activity; **-** No enzyme/antimicrobial activity).

| **Isolates** | **Enzyme Activities** | | | | | **Antimicrobial Production (Zone of Inhibition in mm)** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Lipases** | **Proteases** | **Amylases** | **Cellulases** | **DNases** | ***Staph. aureus*** | ***Pseudomonas aeruginosa*** | ***Bacillus* sp.** | ***Klebsiella Pneumoniae*** | ***Candida albicans*** | ***Acinetobacter* sp.** |
| BGI-1 | + | + | - | + | - | 12.94 | 15.22 | - | - | 28.35 | 12.14 |
| BGI-2 | + | + | - | + | - | 22.14 | 12.55 | 15.42 | - | 20.62 | 10.11 |
| BGI-4 | - | - | - | + | + | - | 11.76 | - | - | - | - |
| BGI-5 | - | - | - | + | + | - | - | - | - | 11.56 | - |
| BGI-7 | + | + | - | - | - | - | - | - | - | - | - |
| BGI-10 | + | - | - | - | + | - | - | 32.92 | - | 30.14 | - |
| BGI-11 | + | - | - | - | + | - | - | 17.32 | - | 25.87 | - |
| BGI-14 | + | - | - | + | - | - | - | - | - | - | - |
| BGS-1 | + | + | - | - | - | - | - | - | - | - | - |
| BGS-2 | + | + | - | + | - | - | - | - | - | - | - |
| BGS-3 | - | - | - | + | + | - | - | - | - | - | - |
| BGS-5 | - | + | - | + | - | 17.45 | - | 14.72 | - | - | 15.37 |
| BGS-6 | - | - | - | + | + | - | - | - | - | - | - |
| BGS-9 | + | + | - | + | + | - | - | - | - | - | - |
| BGS-12 | + | + | - | + | - | - | 11.86 | - | - | - | - |
| BGS-13 | - | - | - | + | + | - | - | - | - | - | - |
| BGW-1 | - | - | - | + | - | - | - | - | - | 20.03 | - |

| BGW-2 | - | - | - | + | - | - | - | - | - | - | - |
|---|---|---|---|---|---|---|---|---|---|---|---|
| BGW-4 | + | - | - | + | + | 18.71 | 12.41 | 25.38 | - | 24.55 | 14.11 |
| BGW-5 | + | + | - | + | - | 19.41 | 13.49 | 23.82 | - | 22.43 | 13.04 |
| BGW-7 | - | - | - | + | - | - | - | - | - | - | - |
| BGW-8 | + | + | - | - | - | 19.62 | 13.84 | 33.72 | - | - | 13.11 |
| BGW-10 | - | + | - | - | - | 21.42 | - | 21.65 | 11.64 | 22.47 | - |
| BGW-12 | - | - | - | + | + | - | - | - | - | - | - |
| BGW-15 | - | - | + | + | + | - | - | - | - | - | - |
| BGW-17 | - | - | + | - | - | - | - | - | - | - | - |
| BGW-18 | - | - | - | - | - | - | - | - | - | - | - |

### 3.3.7. Prevalence of antibiotic resistance in the glacier isolates

Antibiotic resistance to commercial antibiotics was found among the glacial isolates. As many as 86% of the Gram-positive and 46% of the Gram-negative bacteria demonstrated resistance to trimethoprim/sulfamethxazole (30 µg). From the Gram-negative bacteria, 14% were resistant to colistin sulfate (10 mcg) and nalidicixic acid (30 µg) each. Resistance against methicillin (10 mcg) and vancomycin (5 µg) among the Gram-positive isolates was 23% and 15%, respectively. All the Gram-negative bacteria were sensitive to the broad-spectrum antibiotics imipenem (10 µg) and ofloxacin (5 µg). Only one Gram-positive isolate showed resistance to imipenem (10 µg) and all the isolates were sensitive to ofloxacin (5 µg) (Table 3.7). *Bacillus* sp. BGI-4 and *Okibacterium* sp. BGI-5 demonstrated resistance against methicillin, vancomycin and trimethoprim/sulfamethxazole. *Okibacterium* sp. BGS-13 demonstrated resistance against methicillin, imipenem and trimethoprim/sulfamethxazole (Fig. 3.3a). *Rhodococcus* sp. BGI-10, *Rhodococcus* sp. BGI-11 and *Cryobacterium* sp. BG-14 demonstrated resistance against 2 antibiotics, methicillin and trimethoprim/sulfamethxazole. *Pseudomonas* sp. BGW-5 showed resistance against nalidicixic acid and trimethoprim/sulfamethxazole, while *Pseudomonas* sp. BGW-10 demonstrated resistance against colistin sulfate and trimethoprim/sulfamethxazole. Six isolates exhibited resistance to only 1 antibiotic and four isolates were sensitive to all antibiotics used.

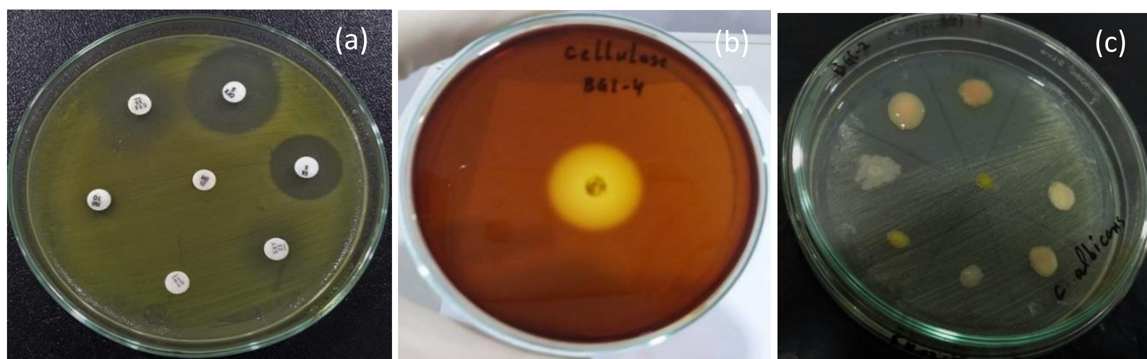



Figure 3.3 (a) Antibiotic susceptibility test for *Okibacterium* sp. BGS-13 shows resistance to methicillin, imipenem and trimethoprim sulfamethxazole (b) Cellulolytic enzyme activity by the *Bacillus* sp. BGI-4 on carboxymethylcellulose (CMC) plate (c) Antimicrobial activities by *Pseudomonas* sp. BGI-1 and *Pseudomonas* sp. BG-2 against *Candida albicans* by spot inoculation method using Mueller Hinton agar plate.

Table 3.7. Antibiotic susceptibility test of the glacier isolates using broad spectrum and narrow spectrum antibiotics. Table represents the number of isolates (in percentage) sensitive and resistance in each group (Gram positive and Gram negative bacteria).

| **Antibiotics** | **Gram Negative Bacteria** | | **Gram Positive Bacteria** | |
|---|---|---|---|---|
| | **Sensitive (%)** | **Resistance (%)** | **Sensitive (%)** | **Resistance (%)** |
| Imipenem | 100 | 0 | 92.31 | 7.69 |
| Ofloxacin | 100 | 0 | 100 | 0 |
| Trimethoprim/Sulfamethxazole | 33.33 | 66.67 | 53.85 | 46.15 |
| Vancomycin | NA | NA | 84.62 | 15.38 |
| Methicillin | NA | NA | 76.92 | 23.08 |
| Colistin Sulfate | 88.89 | 11.11 | NA | NA |
| Nalidicixic acid | 88.89 | 11.11 | NA | NA |

### 3.3.8. Industrially valuable strains

Some of the isolates demonstrated remarkable adaptability and metabolite production. *Pseudomonas* sp. BGI-2 was able to grow at a wide range of temperature (4-35˚C) and pH (5-11). This strain produced 3 out of the 5 extracellular enzymes tested: lipases, proteases and cellulases. The strain exhibited broad-spectrum antimicrobial activity against 5 of the 6 indicator organisms including Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus* sp.), Gram-negative bacteria (*Pseudomonas aeruginosa*, *Acinetobacter* sp.) and a yeast (*Candida albicans*) (Fig. 3.3c). BGI-2 displayed growth in 6 of the 8 media tested (Table 3.5). *Rhodococcus* sp. BGI-10 and BGI-11 can grow at a wide temperature range (4-35˚C). These strains can also grow at extreme pH conditions (3 and 13) and can tolerate salinity up to 8% (w/v). These strains produced 2 out of the 5 enzyme, lipases and DNases and demonstrated antimicrobial activities against *Bacillus* sp. and *Candida albicans.* BGI-10 and BGI-11 were the only strains that exhibited growth in all 8 of the tested media (Table 3.5) including oligotrophic medium (R2A), high salinity medium (mannitol salt agar, salinity 7.5%), acidic medium (sabouraud dextrose agar, pH 5.6) and medium that usually inhibits Gram-positives (MacConkey agar). The low pH of sabouraud dextrose agar usually favors fungi and yeast and inhibits bacteria but BGI-10 and BGI-11 were able grow in its acidic pH (Table 3.3). *Pseudomonas* sp. BGW-4 and *Pseudomonas* sp. BGW-5 demonstrated growth at wide ranges of temperature (4-35˚C) and pH (5-13) (Table 3.3).

Both these strains exhibited broad-spectrum antimicrobial activity. BGW-4 exhibited activity for lipases, cellulases and DNases and BGW-5 demonstrated lipases, proteases and cellulases activities (Table 3.6). BGW-4 and BGW-8 could tolerate salinity up to 6%.

### 3.3.9. Culture-independent diversity

The results from analysis of 16S rRNA clone libraries revealed *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* as major phyla from the three glacier samples. Proteobacteria dominated in all the three samples, representing 71% in ice, 62% in water and 50% in sediment. *Actinobacteria* represented the second major phyla with 26% in water, 20% in sediment and 12% in ice. *Bacteroidetes* was found to be the third major phyla with 13% representatives in ice, 10% in water and 7% in sediment samples. A small fraction of the bacterial communities was also represented by the *Saccharibacteria* and *Firmicutes* and a considerably large fraction of the sequences (20%) in the sediment sample were not unassigned to any group (Fig. 3.4a).

The sequences in glacier samples were assigned to 24 genera (> 0.5%) and the majority of them could not be refined to the genus level and were therefore grouped under the category "Bacteria (others)". The genera included: *Arthrobacter*, *Flavobacterium*, *Pseudomonas*, *Kaistobacter*, *Mycoplana*, *Ochrobactrum*, *Devosia*, *Rhodococcus*, *Rhodobacter*, *Janthinobacterium*, *Acinetobacter*, *Segetibacter*, *Flavisolibacter*, *Methylotenera*, *Polaromonas*, *Sphingomonas*, *Novosphingobium*, *Agrobacterium*, *Methylobacterium*, *Lysinibacillus*, *Chryseobacterium*, *Salinibacterium*, *Mycetocola* and *Microbacterium* (Fig.3.4b).

*Mycoplana* (16%) and *Arthrobacter* (12%) represented the major genera in the ice sample. *Kaistobacter* (20%) and *Arthrobacter* (16%) genera dominated in sediment sample; *Ochrobactrum* (30%), *Janthinobacterium* (24%) and *Flavobacterium* (11%) dominated in ice sample. *Arthrobacter* was found predominantly in all the three samples. Graphs (Fig. 3.4a and 3.4b) depict the phyla and genera with operational taxonomic units (OTUs) that have abundance greater than 0.5%.

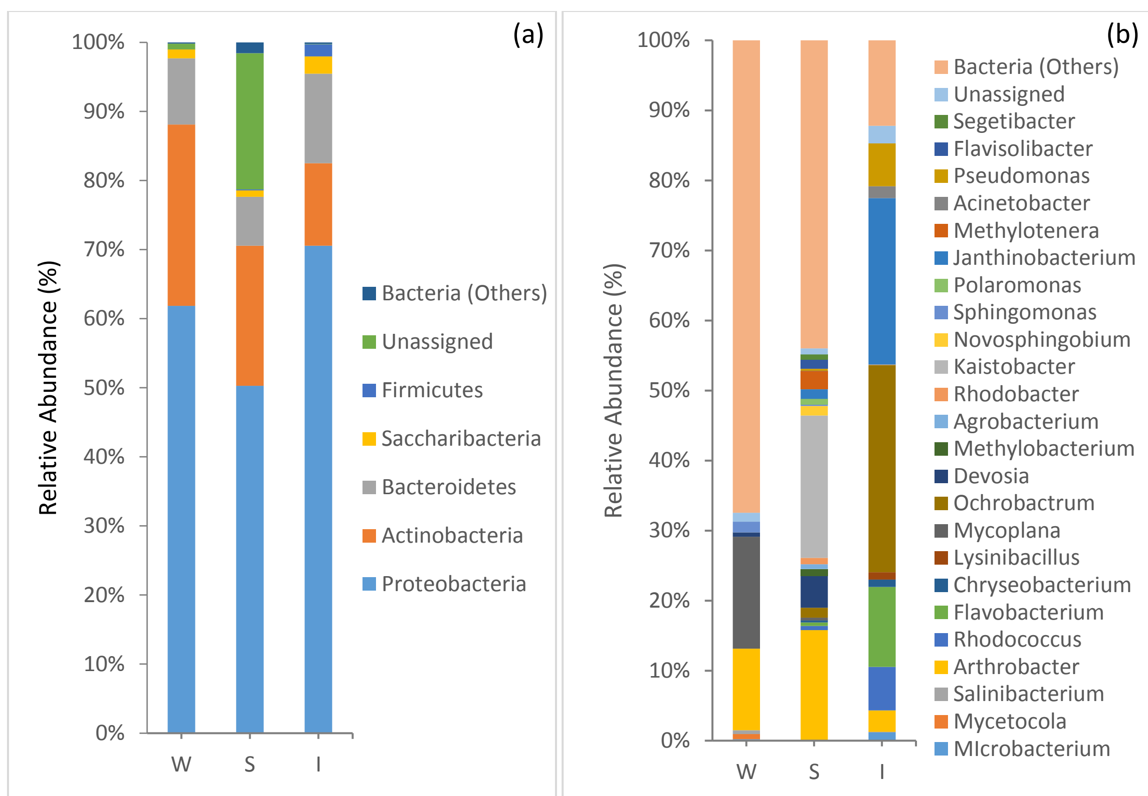



Figure 3.4. Distribution of bacterial community at the (a) phylum and (b) genus level in the three glacier samples (W=meltwater, S=sediment, I=ice)

### 3.4. Discussion

The glaciers of the Karakoram, particularly in the Pakistani geographical region are not commonly explored in terms of their bacterial diversity and functionality. To the best of our knowledge, this is the first study of bacterial diversity in Batura glacier involving culture-dependent and culture-independent methods. In the present study, we examined the possibility of using a glacier in the Karakoram Range of Pakistan as source for isolation of novel cold-adapted bacteria, identification and exploration of some of their metabolic potential to be used for biotechnological or industrial applications.

Three different media (R2A, TSA, and LBA) and two different temperatures (4 and 15˚C) were used for bacterial isolation. Viable count was highest on R2A among the three media and the majority of the isolates were recovered using this medium. R2A is a low nutrient medium designed for the recovery of bacteria from oligotrophic samples (Reasoner and Geldreich, 1985). R2A helps in the recovery of stressed or slow growers that would

otherwise be outgrown by fast-growing bacteria on nutrient rich media. Many studies have previously been reported where R2A is considered as the medium of choice for isolation of psychrophilic or psychrotrophic bacteria from cold environments (Christner et al., 2001; Segawa et al., 2011; Tam et al., 2015). Maximum viable counts were measured at $1.5 \times 10^3$ CFU $mL^{-1}$ (R2A, 15˚C) for meltwater and $1.1 \times 10^4$ CFU $g^{-1}$ (R2A, 15˚C) for sediment. A similar range of viable counts have been reported previously for samples from the cold habitats (Zhang et al., 2013; Segawa et al., 2011). Microscopic counts revealed the glacier harbors a high number of bacterial cells with maximum cell numbers found in the sediment ($3.3 \times 10^6$ cells $g^{-1}$) followed by meltwater ($2.8 \times 10^6$ cells $mL^{-1}$) and ice ($6.4 \times 10^5$ cells $mL^{-1}$). These numbers are close to microscopic counts previously reported from similar environments (Stibal et al., 2012; Reddy et al., 2009; Foght et al., 2004).

In the cold biosphere, glacier ice is considered as one of the harsh environments due to the extremely low temperature (which may go down as low as -56˚C), low nutrient and water availability, darkness and salinity (Margesin and Miteva, 2011). However, glaciers and other icy environments in the polar and non-polar regions harbors a huge number of microorganisms (~ $9.61\times10^{25}$ cells) which are naturally preserved in the ice for thousands of years (Priscu et al., 2007; Priscu and Christner, 2004). Viable microbes embedded in the ice have been reported and discussed previously in the scientific literature. Miteva et al., (2004) reported viable microorganisms deep Greenland glacier ice core (~ 3000 m), trapped in the ice at least 120, 000 years ago. They isolated nearly 800 aerobic organisms and studied some of their physiological properties which included growth temperature range, antibiotic resistance and enzyme activity. Similarly, Sheridan et al., (2003) reported high number of microorganisms ($6 \times 10^7$ cells $mL^{-1}$) from Greenland ice core that had remained at - 9˚C for over 100, 000 years. Successful colonization of the cryosphere by psychrophilic microorganisms is the result of a vast array of adaptation strategies employed by these organisms (discussed in detail in chapter 2). At present, a bacterial isolate from the permafrost held the lowest temperature limit for growth which is -15˚C (Mykytczuk et al., 2013). Protein and DNA synthesis have been reported at -20˚C (Junge et al., 2006; Tuorto et al., 2014), and respiration has been recorded at -33˚C (Bakermans and Skidmore, 2011). In our study, viable counts were slightly higher at 15˚C compared to those at 4˚C for meltwater and sediment samples. Margesin et al., (2002) also reported similar results

for alpine glacier cryoconite samples where higher viable counts were recorded at 20˚C compared to 2˚C.

Not a single bacterial colony was observed from the ice sample even after prolonged incubation on any of the 3 media and 2 different temperatures (4˚C and 15˚C) used. Even the direct and undiluted plating of the melted ice sample did not yield any colony on R2A, TSA and LBA agar media when incubated at 4˚C for 9 months and 15˚C for 6 months. Isolates from the ice were recovered through culture enrichment in liquid media which yielded 8 morphologically different isolates. Very similar results were reported by Antony et al., (2012). The authors were not able to recover any colonies when ice core samples were plated directly on agar plates even after prolonged incubations (6 months) and at 2 different temperatures (4˚C and 20˚C). They also used liquid media culture enrichment for the recovery of different isolates and diluted TSB worked the best for growth whereas in our study R2A was the medium of choice. Christner et al., (2001) used a wide range of growth media for the recovery of viable bacteria from accretion ice of Lake Vostok in the Antarctica. In this study, they were only able to recover 3 colonies on R2 agar medium and no colonies were observed on any of the other media after incubation for more than 3 months at 4 and 25˚C.  Only a small fraction of microorganisms in any environment is culturable and a large fraction remains uncultivable and extreme environments make it even harder for the isolation of bacteria; their nutritional requirements are limited and somewhat unknown. Extremely low viable bacteria counts have been reported previously from the samples. Hong Zhang et al., (2010) reported viable bacterial count in an ice core of East Rongbuk Glacier that ranged between 0 and 5.6 CFU $mL^{-1}$ on PYGV and R2A media. Zhang et al., (2008a) reported numbers of total microbial cells and viable bacterial cells (CFU) from glacier ice ranging from $10^4$ -$10^5$ cells $mL^{-1}$ and 0-760 CFU $mL^{-1}$, respectively. Shivaji et al., (2013) reported viable counts (CFU) in Antarctic ice cores varied from 0.02 to $5.8\times10^3$ $mL^{-1}$. In another study, the highest number of colony-forming units (CFU) from glacier sample was found around $10^4$ - $10^5$ CFU $mL^{-1}$ on diluted R2A agar plates (Segawa et al., 2011). The slightly lower viable count in meltwater and sediment samples in our study could be explained in two main ways. One, they used a diluted R2A medium which resulted in maximum recovery and two, they did sampling in the month of

August while we did ours in December. Time and space greatly influence bacterial communities and their overall abundance, as lower counts are observed in the winter.

Another feature which was predominant in the glacial isolates was the formation of pigmented colonies. More than half of the isolates formed pigmented colonies that appeared yellowish and orange. Some of the studies have indicated carotenoid pigmentation as a strategy to counter environmental stresses such as low temperatures (Chattopadhyay and Jagannadham, 2001; Dieser et al., 2010). Studies showing pigmented bacteria dominating bacterial communities have been reported from glaciers and other cold habitats (Shen et al., 2018; Bowman et al., 1997; Miteva et al., 2004; Christner et al., 2000; Antony et al., 2012). One of the earlier study established a correlation between temperature and carotenoid production (Fong et al., 2001). Low temperature cultivation resulted in increased carotenoid production, which is speculated to contribute in membrane stabilization at low temperatures.

In this study, 27 culturable bacteria were isolated from three glacier samples including ice, meltwater and sediment. All the culturable isolates belonged to 4 major phyla including *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes*. The predominance of these groups in glaciers and other cold environments have been previously reported (Silva et al. 2018; Shen et al., 2018; Liu et al., 2019; Anesio et al., 2017; Cheng and Foght, 2007). *Flavobacterium* (26%), *Arthrobacter* (22%) and *Pseudomonas* (19%) were the dominant genera in overall the three glacier samples representing 7, 6 and 5 isolates each. All the culturable isolates from sediment samples were members of *Actinobacteria* with 5 out of 8 (63%) representing the genus *Arthrobacter*. Bai et al., (2006) also found *Arthrobacter* as the dominant genus among the viable isolates in alpine permafrost from China. Results from another study in the Qinghai-Tibet plateau showed as high as 90% of the isolates were Gram positive bacteria with a high abundance of *Actinobacteria* (Zhang et al., 2007). Recently, study related to culturable bacterial diversity of soil in the Tianshan Mountains revealed 50% of the isolates belonged to the genus *Arthrobacter* (Zhao et al., 2018a). In our study, *Actinobacteria* dominated among isolates from the glacier ice. *Bacteroidetes* dominated isolates from the meltwater and all the isolates were members of the genus *Flavobacterium*. The predominance of some of the genera including, *Pseudomonas*,

*Arthrobacter* and *Flavobacterium* in samples of Batura glacier conforms previous reported results from cold habitats (Thakur et al., 2018; Bai et al., 2006; Reddy et al., 2009; Juck et al., 2000; Cheng and Foght, 2007).

Batura glacier is dominated by psychrotrophic bacteria as the majority of the isolates exhibited a wide growth temperature range (4- 30˚C or 4-35˚C). *Flavobacterium* isolates BGW-15, BGW-17 and BGW-18 could not grow above 20˚C and were determined to be representatives of psychrophilic bacteria. Schmidt-Nielsen, (1902) used the term psychrophiles for the first time to describe microorganisms that grow near the freezing point of water, but the upper limit of temperature was missing in his definition. The current definition of cold-adapted bacteria was proposed by Morita, (1975), dividing organisms into psychrophiles and psychrotrophs. According to his definition psychrophiles are microorganisms able to grow at 0˚C or below, with an optimum temperature around 15˚C and an upper growth temperature limit of 20˚C. Psychrotrophs were defined by him as microorganisms that can grow near the freezing point of water, but their optimum and maximum growth temperature lies above 20˚C. Therefore, based on Morita's definition, we consider the majority of the isolates recovered from Batura glacier as psychrotrophs. According to Russell (1990), psychrophiles are found in permanently cold environments and psychrotrophs dominate habitats which experience more thermal fluctuations. This is the case for non-polar glaciers like Batura glacier in the Karakorum, as it experiences wide seasonal temperature fluctuations. Therefore, psychrotrophic bacteria are expected to dominate over their psychrophilic counterparts. The dominance of psychrotrophic bacteria compared to true psychrophilic bacteria has been previously reported in cold environments (Liu et al., 2019; Thakur et al., 2018; Reddy et al., 2009; Steven et al., 2007).

One of the most intriguing findings about the glacier isolates were their ability to withstand multiple extreme conditions. Three isolates including, *Rhodococcus* sp. BGI-10, *Rhodococcus* sp. BGI-11 and *Microbacterium* sp. BGS-6 demonstrated growth at 3 different extreme conditions such as temperature, pH and salinity. These isolates exhibited growth at low temperature (4˚C), extremes of pH (3 and 13), and in presence of 8% salinity (Table 3). Similarly, *Microbacterium* sp. BGS-6 was able to grow well at 4˚C, pH 4 and 13, and 8% salinity. *Pseudomonas* sp. BGW-5 demonstrated growth at 4˚C, pH 13 and

salinity up to 6%. *Rhodococcus* sp. BGI-10, *Rhodococcus* sp. BGI-11 and *Microbacterium* sp. BGS-6 are representatives of Gram-positive bacteria. According to Cotter and Hill, (2003) Gram-positive bacteria are equipped with a number of acid resistance systems which help them survive in acidic environments. An extremophile is an organism that thrives in an extreme environment and if the organism endure multiple extremes then it is termed as polyextremophile (Rothschild and Mancinelli, 2001). Extremophiles represents untapped and innovative source of novel industrial enzymes. Extremozymes possess extraordinary properties with the ability to catalyze chemical reactions under harsh conditions, making them potential candidates for various industrial processes.

Culture-independent methods also revealed *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* as the dominant taxa in the glacier samples. *Proteobacteria* accounted for more than 50% of relative abundance in all the three glacier samples. *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* have been previously reported as the dominant phyla from glacier environments using culture-independent methods (Wilhelm et al., 2013; Xiang et al., 2009; Lopez et al., 2018; Zhang et al., 2016). *Mycoplana*, *Arthrobacter*, *Kaistobacter*, *Ochrobactrum*, *Janthinobacterium* and *Flavobacterium* represented the major genera in the glacier samples. The majority of the bacterial genera were different between the three glacial samples; however, there were some bacteria shared in all the samples. *Arthrobacter* represented a large fraction of bacterial abundance across all the three samples. *Arthrobacter* has been frequently reported as the dominant genus from cold environments (Thakur et al., 2018; Zhao et al., 2018a; Bai et al., 2006). Similarly, *Mycoplana* (Yang et al., 2016; Salwan et al., 2010), *Kaistobacter* (Yang et al., 2016), *Janthinobacterium* (Rassner et al., 2016), *Ochrobactrum* (Liu et al., 2009b; An et al., 2010) and *Flavobacterium* (Segawa et al., 2011) have also been reported previously from glacial environments. *Kaistobacter* was the dominant genera that accounted for 20% in the sediment sample. Yang et al., (2016) recently reported *Kaistobacter* as one of the dominant genera, among many others, from the glacial soil samples on the Chongce ice cap, West Kunlun Mountains. Interestingly, *Ochrobactrum* was the dominant genus among all in the ice samples and accounted for 30% of the relative abundance. Members of the genus *Ochrobactrum* have rarely been reported from glaciers and other cold environments. Some *Ochrobactrum* species are aerobic, Gram-negative bacteria inhabiting various

environments including water, soil, plants and animal tissue (Kämpfer et al., 2011; Bathe et al., 2006; Chain et al., 2011). Some members of the genus are human pathogens, examples of this are *Ochrobactrum anthropi* and *Ochrobactrum intermedium*. *Ochrobactrum* species are metabolically versatile and produce a variety of enzymes to degrade recalcitrant compounds including pesticides, phenols and petroleum hydrocarbons, making them potential candidates for bioremediation technologies (Ermakova et al., 2017; Zhang et al., 2006; Arulazhagan and Vasudevan, 2011).

Antibiotic resistance patterns in the glacier isolates revealed resistance against some commercial antibiotics. The highest resistance was documented against trimethoprim/sulfamethxazole with Gram-negative (67%) and Gram-positive (46%) bacteria. Resistance against the broad-spectrum antibiotics was the least observed, with all the isolates being sensitive to oflaxacin except for one Gram-positive bacterium (*Okibacterium* sp. BGS-13), all the isolates were sensitive to imipenem. Imipenem is a β-lactam antibiotic and resistant bacteria produce β-lactamases to inactivate the drug. In a recent study, Van Goethem et al., (2018) found a very low abundance of β-lactamase genes in the metagenome of a pristine cold habitat of Antarctica. 11% of the Gram-negative isolates demonstrated resistance against colistin sulfate and nalidixic acid. Resistance incidence against methicillin and vancomycin among the Gram-positive bacterial strains was 23.08% and 15.38% respectively. It is important to state that antibiotic resistance is not limited to clinical environments. Recently, antibiotic resistance genes and organisms have been reported from non-clinical and natural environments including the ocean and Polar Regions (Hatosy and Martiny, 2015; Van Goethem et al., 2018; Segawa et al., 2013). Microorganisms produce antibiotics as a defense strategy against other organisms to compete in limited nutrients. Thus, an antagonistic interaction among microbial populations can lead to the development of antibiotic resistance (Cordero et al., 2012). According to a recent metagenomic study of antibiotic resistance genes in a remote and pristine Antarctic glacier environment found 177 naturally occurring genes conferring resistance to natural antibiotics (Van Goethem et al., 2018). The resistance found in the remote pristine regions with no or less human impact suggests a natural process that is acquired by these microorganisms over time through the process of evolution and the passing off through generations. There are previous reports of high prevalence of antibiotic

resistance genes or bacteria from cold pristine environments in the polar and non-polar regions (Tam et al., 2015; Rafiq et al., 2018; Segawa et al., 2013). The resistance found in glacier isolates may be naturally occurring, as glacier environments are oligotrophic and there is more competition due to less resources, resulting in antibiotic production by some and resistance by others. Nevertheless, Batura glacier is located close to living populations and bacterial transfer through wind and birds to the glacier surface is a possibility.

Another feature of the glacier isolates was production of antimicrobial compounds with activity against representatives of yeast, Gram-positive and Gram-negative bacteria. Most of the isolates exhibited antimicrobial activities against one or more of the test organisms. All cultured *Pseudomonas* species demonstrated broad-spectrum antimicrobial activities. Isolates BGI-2, BGW-4, and BGW-5 belonging to the *Pseudomonas* species exhibited activity against 5 of the 6 test organisms. *Pseudomonas* sp. BGI-1 and *Pseudomonas* sp. BGW-10 exhibited activities against 4 out of the 6 test organisms (Table 3.6). Cold-adapted *Pseudomonas* species with wide-spectrum antimicrobial activities have been reported previously from Antarctica (Silva et al., 2018; Tedesco et al., 2016; O'Brien et al., 2004; Tomova et al., 2015). Emergence of multiple drug resistance in human pathogens is a serious health concern. Discovery of new antibiotics is therefore crucial to cope with increasing resistance in microorganisms with medical and industrial relevance.
Isolated strains from Batura glacier exhibited abilities to produce a variety of extracellular enzymes including cellulases, proteases, lipases, DNases and amylases. The majority of the isolates demonstrated cellulolytic activity (67%), similar to a recent study by Thakur et al., (2018); their result revealed 70.92% of cellulolytic bacteria in their strains using carboxymethyl cellulose (CMC) plates. *Arthrobacter* sp. BGS-9 isolated from the glacier sediments was the most useful strain in terms of enzymatic activity, producing 4 out of the 5 enzymes tested. Isolates from the *Pseudomonas* species (BGI-1, BGI-2, BGW-4, BGW-5), *Arthrobacter* species (BGS-2, BGS-12) and *Flavobacterium* sp. BGW-15 exhibited activities for 3 enzyme types each. Reddy et al., (2009) reported cold-adapted bacteria with lipase, protease and amylase activity from an Arctic glacier. Margesin et al., (2003) reported 71% activity of proteases, 38% of lipases and amylases and only 12% of cellulases among the cold-adapted bacteria. Overall, *Pseudomonas* species dominated among the glacier isolates in terms of enzymatic activities. *Pseudomonads* have been reported

previously as producers of lipases (Ganasen et al., 2016; Tanaka et al., 2012), proteases (Zeng et al., 2003) and cellulases (Menéndez et al., 2015).

### 3.5. Conclusions

The range of temperature for growth of the isolates indicated that Batura glacier is mainly inhabited by psychrotrophic bacteria. The physiological study of the cold-adapted bacteria suggested that they play a key role in nutrient cycling of organic and inorganic materials. Our results indicate that cold environments in the Karakoram Range are promising sources of cold-adapted bacteria with enormous potential for biotechnological exploitation. Most of the isolates produced one or more extracellular enzymes and exhibited antimicrobial activity against a number of microorganisms including multidrug resistant pathogenic strains. Some of the isolates demonstrated great versatility by withstanding and growing in more than one stressful condition and are therefore considered polyextremophiles. These organisms can be useful in industrial processes where normal organisms may not survive. More community studies will aid in linking bacterial diversity and functionality in cold environments across the world. This work will serve as a baseline for studies related to the diversity and potential functionality of bacteria inhabiting glaciers in the Karakoram region.

# Chapter 4

# Chapter 4

## Production, optimization and structural characterization of a cryoprotective EPS extracted from the cold-adapted bacterium *Pseudomonas* sp. BGI-2

**Abstract**

*Pseudomonas* sp. BGI-2 is a psychrotrophic bacterium isolated from the ice sample collected from Batura glacier, Pakistan. This strain produces highly viscous colonies on agar media supplemented with glucose. In this study, we have optimized growth and production of exopolysaccharide (EPS) by the cold-adapted *Pseudomonas* sp. BGI-2 using different nutritional and environmental conditions. *Pseudomonas* sp. BGI-2 is able to grow in a wide range of temperature (4-35˚C), pH (5-11), and salt concentrations (1-5%). Carbon utilization for growth and EPS production was extensively studied and we found that glucose, galactose, mannose, mannitol and glycerol are the preferable carbon sources. The strain is also able to use sugar waste molasses as a growth substrate, an alternative for the relatively expensive sugars for large scale EPS production. Maximum EPS production was observed at 15˚C, pH 6, NaCl (10 g $L^{-1}$), glucose as carbon source (100 g $L^{-1}$), yeast extract as nitrogen source (10 g $L^{-1}$) and glucose/yeast extract ratio (10/1). Under optimized conditions EPS production was 2.01 g $L^{-1}$, which is relatively high for a *Pseudomonas* species compared to previous studies using the same method for quantification. HPAEC-PAD analysis of EPS revealed glucose, galactose and glucosamine as the main sugar monomers. The EPS produced by BGI-2 conferred significant cryoprotection for a mesophilic *Escherichia coli* K12 which was comparable to glycerol, a common cryoprotective agent. Increased EPS production at low temperatures, freeze thaw tolerance of the EPS producing strain and increased survivability of *E.coli* in presence of EPS as cryoprotective agent supports the hypothesis that EPS production is a strategy for survival in extremely cold environments such as the glacier ice.

**Keywords:** Karakoram, Psychrotrophs, Exopolysaccharide (EPS), Glacier bacteria, Cryopreservation

## 4.1. Introduction

Low temperature is very common among extreme environments on the earth. About 85% of the earth's biosphere is permanently exposed to temperatures below 5˚C and glaciers accounts for 10% of it (Margesin and Miteva, 2011). The cold habitats have been successfully colonized by microorganisms which survive and even grow at temperatures near freezing point of water. The cold-adapted organisms are called psychrophiles or psychrotrophs with the former isolated from permanently cold environments and the latter tends to dominate environments that undergo thermal fluctuations. Cold-adapted microorganisms have evolved unique mechanisms to cope with the challenges. These adaptations include increased membrane fluidity through changes in the lipid profile of the membrane (Kralova, 2017; Siliakus et al., 2017; Chintalapati et al., 2004; Shivaji and Prakash, 2010), conformational flexibility and increased enzyme activity involved in key cellular processes such as transcription and translation (Russell 2000; Zheng et al., 2016; Lim et al., 2000), induction of cold-shock proteins (CSPs) (Phadtare, 2004; Tosco et al., 2003), production of antifreeze proteins (AFPs) (Olijve et al., 2016; Gilbert et al., 2004; Gilbert et al., 2005), and production of cryoprotectants such as exopolysaccharides (Deming and Young, 2017; Carrion et al., 2015; Aslam et al., 2012; Krembs et al., 2002).

EPSs are extracellular polysaccharide polymers which are produced and secreted outside the cell by microorganisms. The EPS either remains loosely attached to the cells or is completely released into the surrounding environment (Tallon et al., 2003; Schmid et al., 2015). Exopolysaccharides are produced by diverse group of microorganisms including bacteria (Sardari et al., 2017), cyanobacteria (Bemal and Anil, 2017), archaea (Squillaci et al., 2016), fungi (Sun et al., 2016), yeast (Han *et al*., 2018) and microalgae (García-Cubero et al., 2018). EPS provides protection against the predators, antimicrobial agents and assist microorganisms to endure extremes of temperature, salinity and desiccation. EPS is also essential for the attachment of microorganisms to other surfaces (biotic and abiotic), nutrient uptake and most importantly in biofilm formation (Casillo et al., 2017; Rossi and De Philippis 2015; Janczarek et al., 2015; Sutherland, 2001b; Tamaru et al., 2005; Knowles and Castenholz, 2008). EPS producing microorganisms have been isolated from diverse extreme environments including marine hot springs and deep sea hydrothermal vents (Wang et al., 2017; Arena et al., 2009; Arena et al., 2006), polar and cold marine

environments (Casillo et al., 2017; Mancuso Nichols et al., 2004; Liu et al., 2013a; Aslam et al., 2012) saline and hypersaline environments (Joulak et al., 2019: Biswas and Paul, 2017; Poli et al., 2007; Mata et al., 2008).

*Pseudomonas* species have been reported previously for the production of exopolysaccharides. These exopolysaccharides demonstrated a variety of functional activities ranging from metal removal (Sathiyanarayanan et al., 2016; Maalej et al., 2015), gelling and emulsification activity (Maalej et al., 2016; Carrion et al., 2015), biofilm formation (Jennings et al., 2016), antioxidant activity (Sirajunnisa et al., 2016) and antibacterial activity (Maalej et al., 2017). Analysis of monosaccharide units is critical for structural characterization of the exopolysaccharides. EPSs with only a single type of monomeric unit are called homopolysaccharides and more than one type of sugar monomeric unit is termed as heteropolysaccharides. Monosaccharide composition of the EPSs produced by the members the *Pseudomonas* species demonstrate diverse structural compositions. The EPS from *Pseudomonas stutzeri* AS22 was a heteropolysaccharide consisting of glucose, mannose and lactyl rhamnose (Maalej et al., 2014). Sathiyanarayanan et al., (2016) reported a heteropolysaccharide from the psychrotrophic *Pseudomonas* sp. PAMC 28620, with glucose, galactose, fucose, mannose, rhamnose and ribose as the major monomers. Antarctic bacterium, *Pseudomonas* sp. ID1 produced heteropolysaccharide with glucose, galactose and fucose sugar monomers (Carrion et al., 2015). *Psudomonas stutzeri* XP1 produced dextran, a homopolysaccharide consisting of glucose units (Zhao et al., 2018b).

The increased demand for natural polymers for various industrial applications has resulted in a remarkable interest in microbial polysaccharides. Bacteria produce diverse structural and functional EPSs that could play an important role in biotechnology and industry. Xanthan and gellan are two of the well-known commercially available bacterial EPSs with GRAS (generally regarded as safe) status. The application of xanthan ranges from foods to pharmaceuticals, cosmetics, personal care products, agriculture and petroleum industries (Ates and Oner, 2017; Rehm, 2009; Imeson, 2010; Yang, 2011; Petri, 2015). Gellan also has a wide range of industrial applications which includes, foods, pharmaceuticals, research and pet food (Ates and Oner, 2017; Rehm, 2009; Imeson, 2010; Fialho et al., 2008;

Prajapati et al., 2013). Dextran, alginate, curdlan and cellulose are other bacterial EPS that have significant commercial value. Some microbial EPSs have been reported for anticancer, antiviral, antibacterial, antiulcer, antioxidant and immunomodulation activities (Zhang et al., 2019; Zhu et al., 2018; Mahdhi et al., 2017; Arena et al., 2009; Arena et al., 2006; Novak and Vetvicka, 2009; Sun et al., 2016). This is why the beneficial effects of probiotics to human health are partly attributed to exopolysaccharides produced by bacteria.

Nature is the best reservoir and less explored extreme environments can be promising sources of microbial metabolites with potential industrial applications. Many extremophile bacteria produced EPS as a strategy to cope with the harsh environmental conditions. In contrast to other extreme environments, little has been reported on EPS producing bacteria from the cold environments either isolated from the deep sea or polar region. Hence there is a continuous interest for bioprospecting of the extreme environments as they are the least explored niche. Glaciers are considered harsh environments of the biosphere harboring a special biotic community of cold-adapted microorganisms. The multidisciplinary study of proglacial and subglacial ecosystems is only in its infancy and the literature is rapidly expanding as a result of the potential role of microorganisms inhabiting these environments. A great number of glaciers outside Polar Regions are located in the Karakoram Mountains, which also includes Batura glacier. There are very limited reports available on the diversity and biotechnological potential associated with bacteria inhabiting the Karakoram glaciers.

Karakoram, Himalaya and Hindukush are among the mightiest mountain ranges in the world and meet at a junction point in the northern part of Pakistan; they host more than 5,000 glaciers in its geographical range (Rasul et al., 2011). These glaciers are less explored compared to the polar glaciers in terms of its bacterial diversity and functionality. Batura glacier, with a latitude 36°32′N and longitude 74°40′E, is one of the longest non polar glacier in the world. We explored bacterial diversity of this glacier using culture dependent and culture independent methods. The glacier is dominated by a diverse group of psychrotrophic bacteria (In press). *Pseudomonas* sp., BGI-2 was used for detailed study among other bacteria due to its high abundance in the glacier ice sample and rapid growth

at low temperatures. *Pseudomonas* sp. BGI-2 produced highest EPS among the 7 EPS producing isolates from the glacier samples. Draft genome sequence data revealed BGI-2 genome has 11 EPS-producing genes compared to none in the 7 closely related mesophilic *Pseudomonas* strains (Ali et al., 2019). Previously, we also found more stress response genes in the genome of BGI-2 than the closely related mesophilic counterparts. BG1-2 is able to thrive in the glacier likely due to its resilience to cope with the harsh glacier conditions which includes freezing, frequent freeze-thaw cycle, high UV exposure, desiccation and low nutrient availability. Current study shows that *Pseudomonas* sp., BGI-2 exhibited high freeze thaw survivability and the EPS produced by BGI-2 conferred significant cryoprotection for a mesophilic *Escherichia coli* K12, suggesting its role in cryoprotection. This work will pave new avenues for the EPS as a cryoprotective gent and its application in the cryopreservation will be a significant milestone. Microbial EPS could thus be ecofriendly due to its biodegradability and nontoxicity, compared to the recalcitrant chemical cryoprotective compounds currently in use.

## 4.2. Materials and Methods

### 4.2.1. Sample collection and bacterial isolation

Samples were collected on December 24th 2015 in winter season. An ice axe wiped with 70% ethanol was used for drilling and sampling of the ice. Surface ice up to 5 inches was removed, discarded and the underlying ice was collected aseptically into sterile 500 mL polypropylene wide-mouth sample bottles. Handling of the samples was performed according to standard microbiological techniques to avoid any contamination. The tubes were sealed, placed in an isothermal box and transported to Microbiology Research Laboratory, Quaid-i-Azam University Islamabad, and stored at -20˚C. Geographic coordinates and height of the sampling location was measured using a GPS device.

For bacterial isolation, two different approaches were used. (a) Direct plating of the samples on R2A, TSA and LB agar (b) enrichment of the sample in R2A broth (Difco), tryptic soya broth (Oxoid) and Luria Bertani broth (Miller) before plating on agar plates. For direct plating, different dilutions of the sample were plated on agar plates using spread plate method. Plates were incubated at 4 and 15˚C and observed daily for colony appearance. For sample enrichment, 10 mL of melted ice sample was added in 100 mL of

R2A broth, tryptic soy broth and Luria-Bertani broth, and placed in shaker incubators (150 rpm) at 4 and 15˚C. Turbidity of the media after 2 weeks indicated bacterial growth. Different dilutions of the enrichment sample were plated on R2A, TSA and LB agar plates using spread plate method. Plates were incubated at 4 and 15˚C and observed daily for colony appearance. BGI-2 was among 8 bacteria isolated from ice sample through sample enrichment. The strain was purified according to streak plate method and cryopreserved at - 80˚C using 20% glycerol.

### 4.2.2. Identification of isolate

Preliminary identification of the isolate was done through colony morphology (size, elevation, margin and pigmentation), cell morphology (Gram staining) and using different biochemical tests (triple sugar iron test, catalase, oxidase, citrate utilization, nitrate reduction). Whole genome sequencing of BGI-2 was done in BAS Lab, Institute of Marine and Environmental Technology, University of Maryland System Centre for Environmental Sciences, using Illumina Miseq sequencing. Rapid Annotation using Subsystem Technology (Aziz et al., 2008) was used to get the complete 16S rRNA gene sequence and sequence was BLAST using EzBioCloud database (Yoon et al., 2017a). 16S rRNA gene sequence was submitted to NCBI GenBank with the accession number MH681214. Phylogenetic tree was constructed by neighbor-joining method using MEGA 7.0 software (Saitou and Nei, 1987).

### 4.2.3. Growth optimization of *Pseudomonas* sp. BGI-2

To determine the optimal growth condition, BGI-2 was cultivated at different temperatures (4-45˚C), pH (4-11), salinity (1-10%) and carbon sources (glucose, galactose, lactose, sucrose, mannitol, mannose, maltose, arabinose, xylose and starch), nitrogen sources (tryptone, peptone, yeast extract, urea, sodium nitrate and ammonium sulfate) and glucose/yeast extract ratios (1/1, 10/1, 20/1, 30/1,40/1 and 50/1). Effect of different concentration of molasses (1-5%) on the growth of BGI-2 was also investigated. Tryptic soya broth (17 g $L^{-1}$ tryptone, 3 g $L^{-1}$ soytone, 2.5 g $L^{-1}$ glucose, 5 g $L^{-1}$ sodium chloride, 2.5 g $L^{-1}$ dipotassium hydrogen phosphate) was used as a growth medium for temperature and pH optimization. Tryptic soya broth amended with different NaCl concentration was used for the salt requirement for optimal growth. For carbon utilization experiment, the

mineral salt medium was prepared according to Chayabutra and Ju (2000) (4 g $L^{-1}$ $NH_4Cl$, 2.5 g $L^{-1}$ $K_2HPO_4$, 0.5 g $L^{-1}$ NaCl, 0.3 g $L^{-1}$ $MgSO_4.7H_2O$, 0.03 g $L^{-1}$ $FeCl_3.6H_2O$, 0.01 g $L^{-1}$ $CaCl_2$, 0.01 g $L^{-1}$ $MnCl_2.4H_2O$) supplemented with different sugars. All the sugars were filter sterilized using 0.22 µm syringe filters and aseptically added to the mineral salt media. All optimization experiments were carried out in 250 mL flasks with 50 mL working volume in a refrigerated shaker incubator (Innova 4340) at 150 rpm and 15˚C. Optical density ($OD_{600}$) was used to monitor the growth every 24 hours up to 6 days. For optical density 48 well plates with 1 mL sample volume was used and readings were taken in a microplate reader (SpectraMax M5). All the tests were run in replicates. The growth rate was also determined by taking optical density at 600nm after every two hours during the exponential phase of the growth.

### 4.2.4. Optimization of EPS production

The effect of environmental and nutritional conditions on EPS production by *Pseudomonas* sp. BGI-2 was investigated at different temperatures (4-45˚C), pH (4-11), salinity (1-10 %), incubation periods (1-6 days) and carbon sources (glucose, galactose, lactose, sucrose, xylose, arabinose, mannitol, mannose, maltose, glycerol and starch), nitrogen sources (tryptone, peptone, yeast extract, urea, sodium nitrate and ammonium sulfate) and glucose/yeast extract ratios (1/1, 10/1, 20/1, 30/1,40/1 and 50/1). BGI-2 was also grown at different concentration (1-5%) of molasses to evaluate its effect on EPS production. All the experiments were conducted for 6 days at 15˚C in a refrigerated shaker incubator (Innova 4340) at 150 rpm. Estimation of EPS was conducted daily using a microplate based phenol sulfuric acid method.

### 4.2.5. Extraction, purification and estimation of EPS production

Extraction and purification were conducted following the method used previously with some modifications (Dabour and LaPointe, 2005). For EPS extraction, bacterial culture samples were centrifuged (10,000 rpm, 4˚C, 15 minutes) to pellet out the cells. Cells were discarded and EPS in the supernatant was precipitated using 2 volumes of chilled absolute ethanol and left overnight at 4˚C. The precipitated EPS was collected by centrifugation (10,000 rpm, 4˚C, 15 minutes) and dried at room temperature. The crude EPS was dissolved in deionized water, treated with 12% (v/v) chilled trichloroacetic acid (TCA) and

left at 4˚C for 1 hour. Precipitated proteins were removed by centrifugation (10,000 rpm, 4˚C, 15 minutes). The deproteinized EPS in the supernatant was precipitated by addition of 2 volumes of chilled ethanol after overnight incubation at 4˚C. The precipitated EPS was collected by centrifugation (10,000 rpm, 15 minutes, 4˚C) and dried at room temperature. The EPS was re-dissolved in deionized water and dialyzed against distilled water for 2 days at 4˚C using a dialysis membrane (Mw cut off: 12000 Da) to remove traces of TCA, salts and low molecular weight molecules and freeze-dried in a lyophilizer (FreeZone 2.5, LABCONO). The purified EPS was re-dissolved in deionized water and EPS concentration was determined by phenol sulfuric acid method (Dubois et al., 1956). For rapid quantification, all the measurements were done in 96 well microplates by the method described previously (Le Parc et al., 2014).

### 4.2.6. Structural Analysis of the EPS

#### 4.2.6.1. Analysis of monosaccharide composition of EPS using HPAEC-PAD

Monosaccharide composition of the EPS was analyzed using High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) adopted from the method used previously (Zhang et al., 2012). Hydrolysis of the EPS was carried out in trifluoroacetic acid (2 N) at 100˚C for 1 hour (Maalej et al., 2015). Glucose, galactose, arabinose, mannose, glucosamine, galactosamine, glucuronic acid, N-acetylneuraminic acid (Neu5AC) and N-glycolylneuraminic acid (Neu5GC) was run as standard.

#### 4.2.6.2. NMR spectroscopy of the EPS

800 MHz 1H NMR spectra with water suppression have been acquired from ~1 mg EPS-BGI-2 in ~400 mg CD3OD as described elsewhere (Hertkorn et al., 2013). 1-5 mg of solid EPS preparation were suspended in ~ 600 µL $D_2O$ (100% $^2H$; Merck, Darmstadt) with minuscule amounts of deuterated TMSP ($(H_3C)_3Si-CD_2-CD_2-COONa$, $\delta_H = 0$ ppm) added as reference and subjected to 5 minutes of ultrasonic bath. Not all EPS was dissolved under these conditions, but NMR spectra of EPS solution looked rather alike when prepared from different ratios of solid EPS and $D_2O$. The supernatant was collected after centrifugation and the solution was transferred to Bruker match tubes ranging from 2.5- 5 mm diameter, and sealed; typical solubility was estimated to ~30%. NMR spectra of aqueous EPS extracts

were acquired with a Bruker Avance III NMR spectrometer at 800.13 MHz ($B_0$ = 18.7 T) at 300 K with a 5 mm z-gradient $^{1}H$ / $^{13}C$ / $^{15}N$ / $^{31}P$ QCI cryogenic probe (90° excitation pulses: $^{13}C$ ~ $^{1}H$ ~ 10 μs. 1D $^{1}H$ NMR spectra were recorded with a spin-echo sequence (10 μs delay) to allow for high-Q probe ringdown, and classical pre-saturation to attenuate the residual water present "*noesypr1d*", typically 512 scans (5 s acquisition time, 5 s relaxation delay, 1 ms mixing time; 1 Hz exponential line broadening). Lorentzian lineshape fitting was performed with Bruker TopSpin software 2.3pl7. Selective excitation was performed with Bruker standard pulse sequences *"selnogpzs"* and *"seldigpzs"*, with aq = 3 s, d1= 0.1s, TOCSY mixing time d9 = 250 ms, NOESY mixing time d8 = 350 msec and 400 scans each. The one-bond coupling constant $^{1}J(CH)$ used in the 2D $^{1}H$,$^{13}C$ DEPT-HSQC NMR spectrum (*hsqcedetgpsisp2.2*) was set to 145 Hz; other conditions: $^{13}C$ 90 deg decoupling pulse, GARP (70 μs); 50 kHz WURST 180 degree $^{13}C$ inversion pulse (Wideband, Uniform, Rate, and Smooth Truncation; 1.2 ms); F2 ($^{1}H$): spectral width of 5981 Hz (11.96 ppm); 1.25 s relaxation delay; F1 ($^{13}C$): SW = 17607 Hz (140 ppm). HSQC-derived NMR spectra were computed to a 8192 × 1024 matrix. Phase sensitive echo-antiecho TOCSY spectra (*dipsi2etgpsi*) used a spectral width of 5498 Hz and were computed to a 16384 × 2048 matrix. The $^{13}C$ DEPT NMR spectrum was acquired with a Bruker Avance III NMR spectrometer at 125.77 MHz ($B_0$ = 11.7 T) at 300 K with a 5 mm z-gradient dual $^{13}C$ / $^{1}H$ / dual cryogenic probe (90° excitation pulses: $^{13}C$ ~ $^{1}H$ ~ 12 μs), with an acquisition time of 1 second and an interpulse delay of 2 seconds.

### 4.2.6. Freeze thaw survivability of EPS producing *Pseudomonas* sp. BGI-2

*Pseudomonas* sp. BGI-2 was subjected to freeze thaw cycles to check its survivability/tolerance against freezing and freeze thaw cycles without the addition of any external cryoprotective agent. This test was performed by colony count method as described previously with some modifications (Sleight et al., 2006**;** Shivaji et al., 2013)**.** Survivability of BGI-2 was compared with another psychrotrophic non-EPS producing *Rhodococcus* sp. BGI-11 isolated from the same environment and a mesophilic *Escherichia coli* K12. An overnight culture of these bacteria in late log phase was harvested through 5 minutes of centrifugation at 10,000 rpm. Cell pellets were re-suspended in 0.85% NaCl solution. 1mL of each culture was transferred to sterilized cryovials. CFU/mL using spread plate method was done to determine the original cell numbers. All the vials were

frozen at −80˚C in a deep freezer. After 24 hours, tubes were drawn out of the freezer and thawed for 25 minutes in a water bath at 25˚C. 100 µL of thawed culture was serially diluted in 0.9% saline. Survivability of these bacteria was determined by comparing the log CFU counts before and after the freeze thaw cycles. BGI-2 and BGI-11 were plated on TSA plates whereas *E.coli* diluted culture samples were plated on LB plates. Tubes were again frozen at −80˚C. This freeze thaw cycle was repeated after every 24 hours for one week and CFU/mL for all the three bacteria were determined using plate count method. The test was run in replicates for all the three strains.

#### 4.2.7. Cryoprotective activity of the EPS

The cryoprotective property of EPS using *Escherichia coli* as an indicator organism was determined by the method used previously, with little modifications (Dubey and Jeevaratnam, 2015). Precisely, an overnight culture of *E. coli* in late log phase was mixed with 1, 3, and 5% (w/v) EPS solution. The culture was first harvested through 5 minutes of centrifugation at 4˚C and 10,000 rpm. The cell pellets were re-suspended in 0.9% NaCl solution and used for the cryoprotection assay. An equal volume of *E. coli* cell suspension in 1.5 mL cryo-vials was mixed with EPS solution to make a final volume of 1 mL. Now 100 µL samples from all the tubes were taken, serially diluted and plated on LB plates using spread plate method to get the original bacterial count. All the vials were frozen at −80˚C in a deep freezer. After 24 hours, tubes were drawn out of the freezer and thawed for 25 minutes in a water bath at 25˚C. The freeze thaw cycle was repeated after every 24 hours for one week and CFU/mL of the indicator bacterium was determined using the plate count method. The survivability of *E.coli* K12 was determined by comparing the log CFU counts before and after the freeze thaw cycles. The cryoprotective effect of EPS was compared to 20% glycerol, which is a well-known cryoprotective agent for bacterial cryopreservation.

### 4.3. Results

#### 4.3.1. Screening and identification of *Pseudomonas* sp. BGI-2

BGI-2 was isolated from the ice sample of Batura glacier, Pakistan. Direct plating of the melted ice sample on TSA, R2A and LB agar plates did not yield any growth even after 9

months of incubation at 4˚C. Isolation was done using sample enrichment in R2A broth before plating on R2A agar plates. The enrichment culture media turned turbid (indicating bacterial growth) after two weeks of incubation at 15˚C. Plating of the enrichment sample on agar plates yielded 8 morphologically different bacterial colonies after one month of incubation at 4˚C and 15˚C. Colonies of BGI-2 appeared on Day 6 compared to other isolates which appeared only after 15-21 days. All the colonies were purified on separate plates and also cryopreserved at - 80˚C in 20% glycerol.

BGI-2 was the most abundant bacterial isolate, contributing more than 60% of all the colonies on each plate. BGI-2 produces large, circular, raised and slimy colonies on agar plates. The unique feature of this glacial isolate was production of extremely mucoid colonies when grown on R2A or tryptic soy agar medium supplemented with 1-5% glucose (Fig. 4.1a), indicating exopolysaccharide (EPS) production (Fig. 4.1b). Gram staining revealed BGI-2 as a Gram negative bacterium with rod shape. BGI-2 demonstrated positive test for citrate utilization and negative for nitrate reduction.  The triple sugar iron test revealed fermentation of glucose only and no fermnetation was observed for sucrose and lactose. BGI-2 also demonstrated positive results for catalase and oxidase enzymes.

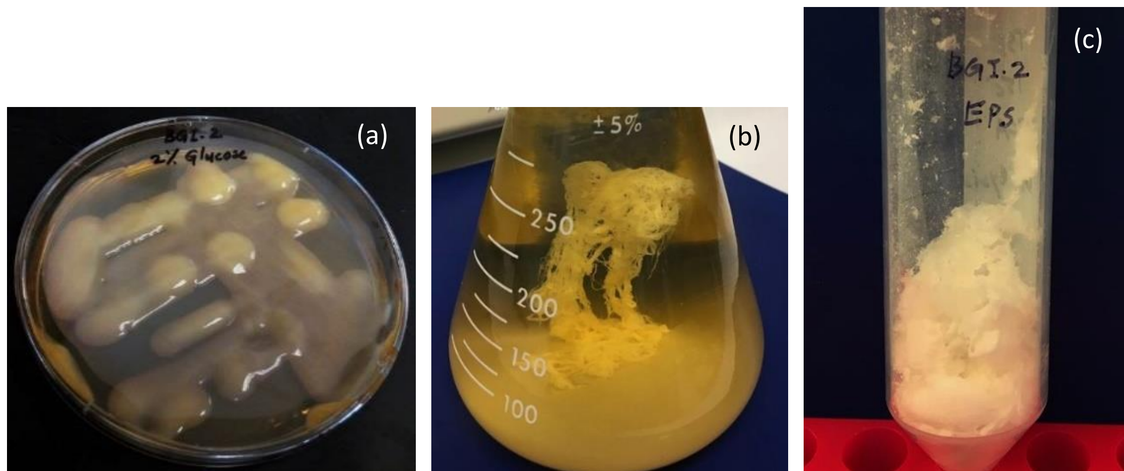



Figure 4.1. Exopolysaccharide production (a) Mucoid phenotype of BGI-2 on tryptic soya agar supplemented with 2% glucose (b) Ethanol precipitation of exopolysaccharide (c) Powedred EPS after lyophilization.

The sequence of 16S rRNA gene revealed strain BGI-2 clustered in to genus *Pseudomonas* (Fig. 4.2), with the nearest species: *Pseudomonas mandelii* (99.59%), *Pseudomonas*

*frederiksbergensis* (99.59%), *Pseudomonas caspiana* (99.45%), *Pseudomonas tremae* (99.38%) and *Pseudomonas amygdali* (99.32%).

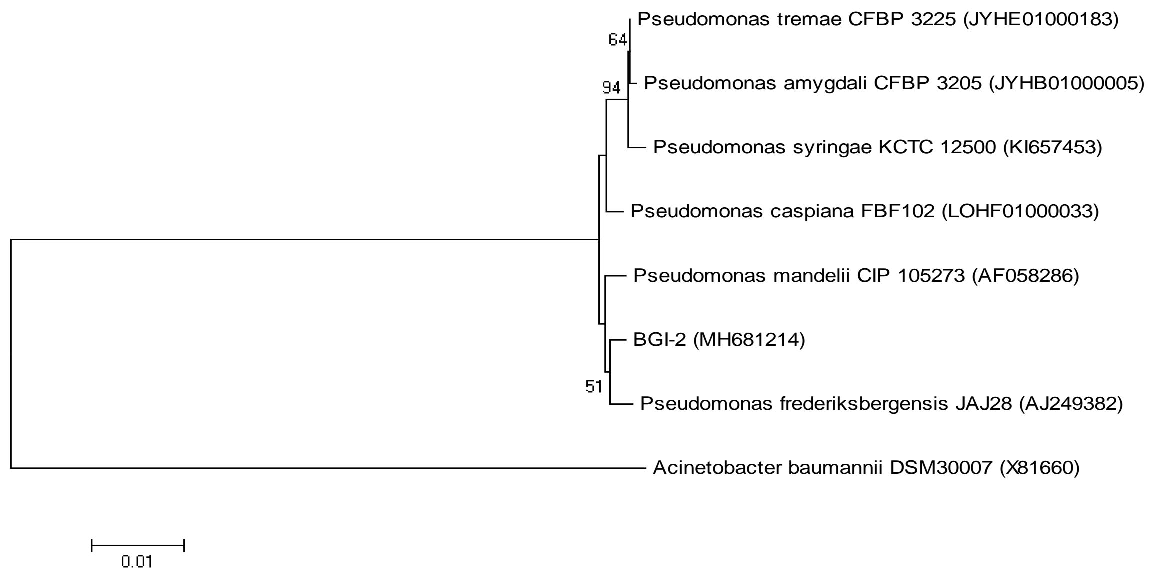



Figure 4.2. Phylogenetic tree of *Pseudomonas* sp., BGI-2 using neighbor joining method with bootstrap value (%) greater than 50 from 1000 replicates. Numbers in the brackets are GenBank accession numbers for the 16S rRNA gene sequences and *Acinetobacter baumannii* DSM 30007 is used as an outgroup to root the tree.

### 4.3.2. Growth optimization of *Pseudomonas* sp. BGI-2

The effect of temperature on the cell growth was tested at 5 different temperatures including 4, 15, 25, 35 and 45˚C. The optimal growth temperature for BGI-2 was found around 20 - 30˚C. Highest growth rate was observed at 25˚C with a mean exponential growth rate of 0.467 $h^{-1}$ and generation time 2.14 hours. Second highest growth was observed at 30˚C with a mean growth rate of 0.399 $h^{-1}$ (generation time 2.51 hours). Mean growth rate at 15˚C was 0.346 $h^{-1}$ (generation time 2.89 hours). The decrease in growth rate with increase in generation time was observed at 4˚C and 35 ˚C. No growth was observed at 45˚C (Fig. 4.3a). Effect of different NaCl concentration on the growth revealed that BGI-2 is able to grow at a salt range of 1-5% (w/v). The maximum growth was observed in control (no NaCl) with a mean exponential growth rate of 0.39 $h^{-1}$ (generation time 2.56 hours). The second highest growth was observed at 1% NaCl with a mean growth rate of 0.372 $h^{-1}$ (generation time 2.69 hours). The decrease in growth rate and increase in generation time was observed at higher concentration of NaCl used. No growth was

observed at 7% NaCl (Fig. 4.3b). BGI-2 demonstrated good growth at a wide range of pH 5 - 11. A similar growth pattern was observed for pH 6-10 during the first 2 days. The maximum growth was observed at pH 7 and pH 8. The mean growth rate at pH 7 was 0.397 $h^{-1}$ (generation time 2.52 hours) and pH 8 was 0.399 $h^{-1}$ (generation time 2.51 hours). A slight decrease in growth rate at low and high pH was observed. Growth rate decreased at pH 6 to 0.317 $h^{-1}$ (generation time 3.15 hours) and pH 9 to 0.221 $h^{-1}$ (generation time 4.5 hours). No growth was observed at pH 4 (Fig. 4.3c). Effects of different concentrations (1-5%) of molasses (as a carbon source) on the growth also was studied. The maximum growth was observed at 1% molasses with a mean growth rate of 0.318 $h^{-1}$ (generation time 3.14 hours) followed by 3% molasses 0.272 $h^{-1}$ (generation time 3.46 hours) and 5% molasses 0.252 $h^{-1}$ (generation time 3.97 hours). The least growth was observed in the control (no molasses) with the doubling time increased to 4.93 hours (Fig. 4.3d). For carbon utilization, BGI-2 strain was able to utilize glucose, galactose, mannose, mannitol, glycerol and molasses as carbon sources. The maximum growth was observed for molasses with a mean growth rate of 0.224 $h^{-1}$ (generation time 4.46 hours) followed by glucose 0.214 $h^{-1}$ (generation time 4.67 hours) and glycerol 0.163 $h^{-1}$ (generation time 6.13 hours). No growth was observed when lactose, sucrose, xylose, arabinose and maltose were used as carbon source. For nitrogen source, the maximum growth was observed for yeast extract followed by peptone and tryptone (Fig. 4.3e). Poor growth was recorded for the inorganic nitrogen sources such as sodium nitrate and ammonium sulfate. For glucose/yeast extract ratios, the maximum growth was observed at 10/1 followed by 20/1 and 1/1. Growth decreased at the higher glucose/yeast extract ratio (Fig. 4.3f).

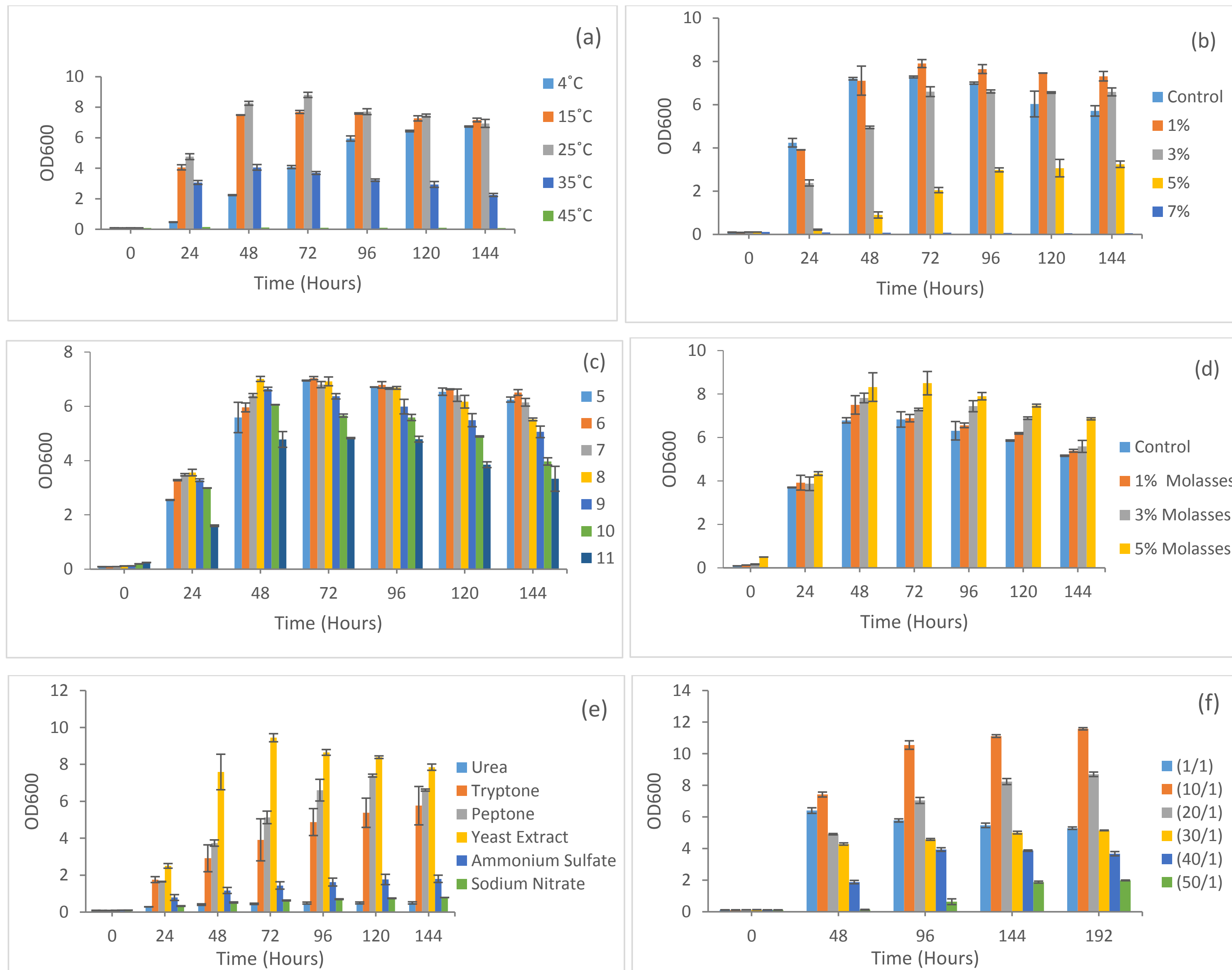



Figure 4.3. Growth comparison of BG1-2 in different conditions: (a) temperatures, (b) salinity, (c) pH, (d) molasses, (e) nitrogen sources, (f) glucose/yeast extract ratios.

### 4.3.3. EPS production by *Pseudomonas* sp. BGI-2

The maximum production of EPS was recorded at 15˚C (283 mg $L^{-1}$) during 96 hours (Fig. 4.4a). Likewise growth, EPS production was low at 4˚C during the first 72 hours and reached to the maximum during 144 hours (279 mg $L^{-1}$). The maximum production of EPS at 25˚C (209 mg $L^{-1}$) was observed during 72 hours. No EPS production was observed at 35˚C and above (Fig. 4.4a). For salinity the maximum EPS production was observed at 1% NaCl (287 mg $L^{-1}$) during 72 hours of incubation. The second highest yield of EPS was obtained at 3% NaCl (227 mg $L^{-1}$) followed by the control (198 mg $L^{-1}$) and 5% (161 mg $L^{-1}$). Likewise growth, no EPS production was observed for the treatment with 7% NaCl (Fig. 4.4b). The maximum EPS production was observed at pH 6 (273 mg $L^{-1}$), followed by pH 7 (263 mg $L^{-1}$) and pH 8 (256 mg $L^{-1}$) during the early stationary phase of growth. No EPS production was observed at the extremes of pH (pH 4 and pH 11) (Fig. 4.4c). The effects of different concentration of molasses on EPS production were also checked. Unlike the growth, EPS production increased with an increase in the concentration of molasses. The maximum EPS was observed at 5% molasses (675 mg $L^{-1}$) followed by 3% (451 mg $L^{-1}$) and 1% (296 mg $L^{-1}$). No EPS production was observed in the control (Fig. 4.4d). Although the strain BGI-2 demonstrated growth in MSM media supplemented with glucose, galactose, mannose, mannitol, glycerol and molasses; EPS production was negligible in the minimal medium.

For nitrogen source, the maximum EPS production was recorded for yeast extract (375 mg $L^{-1}$) followed by peptone (228 mg $L^{-1}$) and tryptone (217 mg $L^{-1}$) (Fig. 4.4e). Likewise the growth, no EPS production was recorded for the inorganic nitrogen sources (sodium nitrate and ammonium sulfate). For glucose/yeast extract ratio, the maximum EPS production was recorded at 10/1 (612 mg $L^{-1}$) followed by 20/1 (511 mg $L^{-1}$) and 30/1 (355 mg $L^{-1}$) (Fig. 4.4f). Putting all the optimized nutritional and environmental conditions in one experiment resulted in a high yield of EPS 2.01 g $L^{-1}$. The optimized conditions included temperature (15˚C), pH (6), NaCl (10 g $L^{-1}$), glucose as carbon source (100 g $L^{-1}$), yeast extract as nitrogen source (10 g $L^{-1}$) and glucose/yeast extract (10/1).

The effect of the incubation period on the growth as well as EPS production also was studied. For most of the optimization experiments, the maximum growth was observed from 24 to 48 hours and EPS production from 72 to 96 hours. Both the growth and EPS yield declined afterwards.

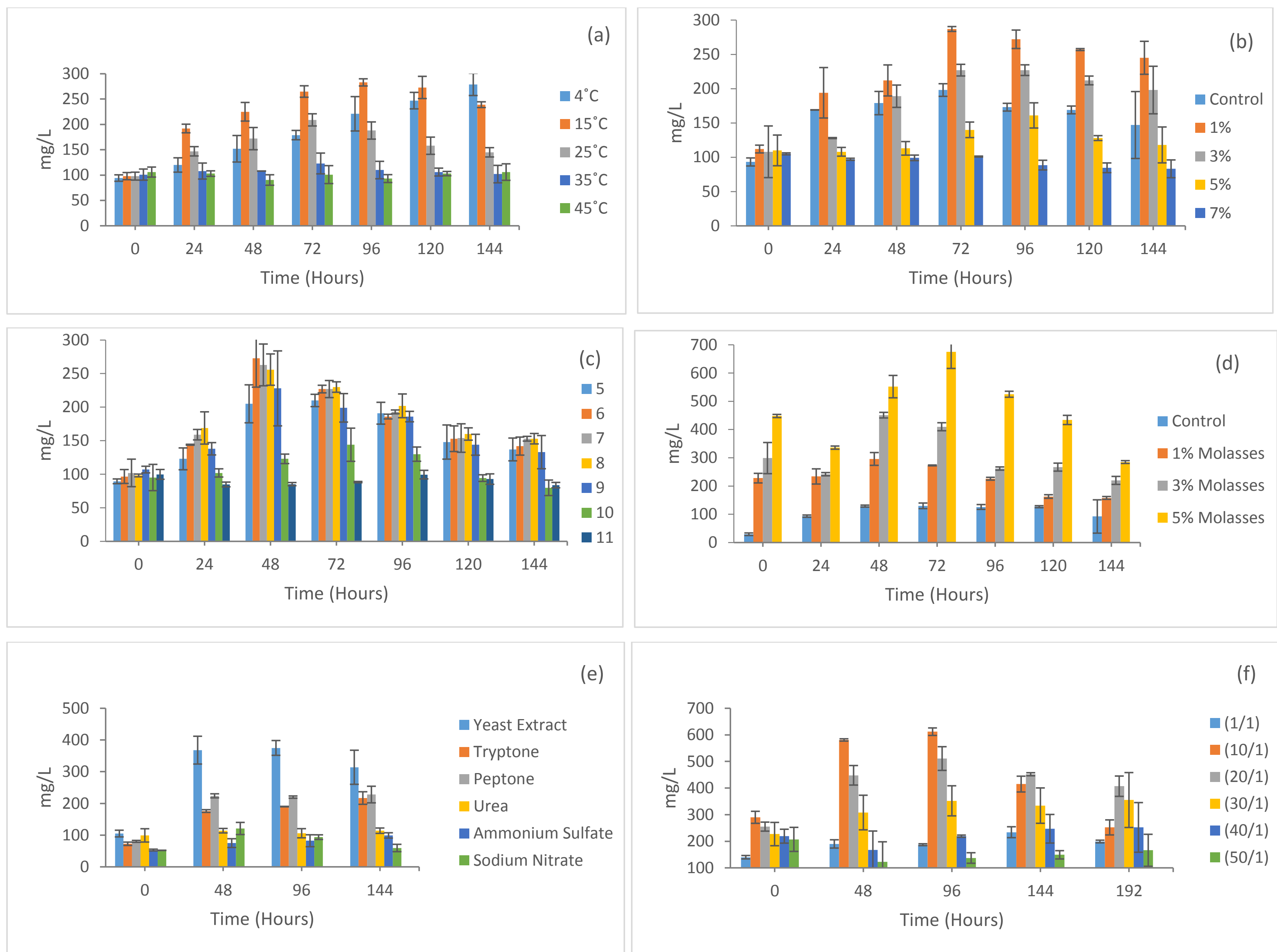



Figure 4.4. Production of EPS by strain BG1-2 under different growth conditions: (a) temperatures, (b) salinity, (c) pH, (d) molasses 1-5% with an increment of 2%, and (e) nitrogen sources, (f) glucose/yeast extract ratios

### 4.3.4. Monosaccharide composition of EPS using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC- PAD)

Analysis of the monosaccharide composition is very critical in characterizing the structure of complex carbohydrates. Results revealed the EPS is composed mainly of three sugar monomers including glucose, galactose and glucosamine (Fig. 4.5b). All the three sugars were characterized by appearance of peaks at different retention time when compared to the standards (Fig. 4.5a).

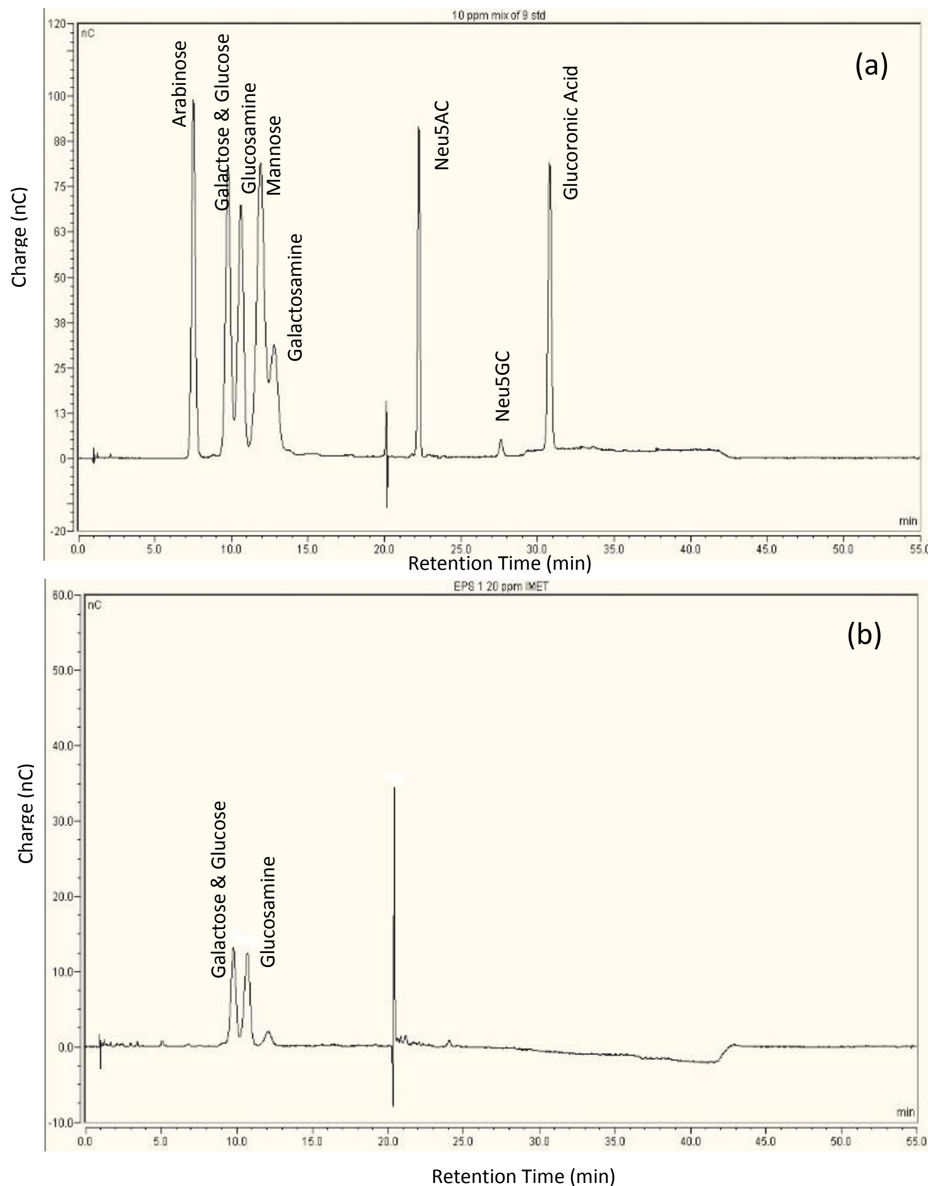



Figure 4.5. Monosaccharide composition of exopolysaccharide: (a) HPAEC- PAD chromatogram of 9 sugars used as standard, (b) HPAEC- PAD chromatogram of monosaccharides present in EPS.

### 4.3.5. NMR spectrometry of EPS

$^1H$ NMR spectra acquired from several EPS samples indicated the presence of very similar carbohydrates in all EPS preparations, with variable proportions of attendant aromatic and aliphatic compounds (data not shown). In all preparations of EPS, all carbohydrate-related NMR resonances showed only rather minor variations of relative NMR amplitude (<10%) at virtually identical $\delta_H$ (data not shown). This referred to the relative proportions of anomeric protons as well (Table 4.1) and implied production of an EPS molecule or a mixture of EPS molecules of rather uniform composition and structure, irrespective of details of culture conditions. The isolate BGI-2 showed seven major $^1H$ NMR resonances (denoted a-g in Fig. 4.6a) of anomeric $O_2CH$-units, the $^1H$ NMR integral ratio of $(OCH+OCH_2)/O_2CH$ units was ~6, suggesting presence of peptide-derived CONH-CαH NMR resonances as well (Fig. 4.6c).

$^1H$, $^1H$ TOCSY (Total correlation spectroscopy) and $^1H$, $^{13}C$ HSQC NMR spectra (heteronuclear single quantum coherence) showed that the major cross peaks of the EPS preparations divided into those derived from carbohydrates and those arising from peptides (Fig. 4.6c). The carbohydrates in EPS were itself complex, with >15 HSQC resolved cross peaks representing anomeric $O_2CH$-groups in $^1H$, $^{13}C$ HSQC NMR spectra (Fig. 4.6b). Among those, seven major cross peaks (peaks a-g; Fig. 4.6e) suggest either presence of a mixture of several carbohydrates or the presence of a single, uniform EPS molecule. If that would apply, a minimum of 19 anomeric positions is expected for this EPS (Table 4.1). It is however more likely that our current EPS isolates represent mixtures of different carbohydrate molecules at given ratios of abundance; an alternative option is the presence of some carbohydrate oligomers with repetitive connectivities but different overall size.

Table 4.1. Relative areas of seven major NMR resonances (800 MHz, $D_2O$) of anomeric positions ($O_2CH$-units) in EPS computed from lineshape-fitting of 1D $^{1}H$ NMR spectra; the nominal count refers to proposed minimum numbers of defined anomeric CH-units in a complex EPS, when this EPS would be a single compound. $\delta_C$ is derived from $^{1}H$, $^{13}C$ HSQC NMR spectra.

| **Number of anomeric HSQC cross peak** | **$\delta(^{1}H)$ [ppm]** | **Width [Hz]** | **% area** | **$\delta(^{13}C)$ [ppm]** | **% area** | **Nominal count of anomeric positions** |
|---|---|---|---|---|---|---|
| A | 5.301 | 9.4 | 19.0 | 103.45 | 19.0 | 4 |
| B | 5.151 | 7.7 | 8.3 | 105.14 | 8.3 | 1 |
| C | 5.123 | 9.2 | 14.8 | 101.14 | 14.8 | 3 |
| D | 5.098 | 10.0 | 15.3 | 101.12 | 15.3 | 3 |
| E | 5.062 | 5.9 | 13.1 | 105.08 | 13.1 | 2 |
| F | 5.052 | 7.9 | 22.7 | 105.08 | 22.7 | 5 |
| G | 4.907 | 5.5 | 6.6 | 102.32 | 6.6 | 1 |

$^{13}C$ DEPT-135 NMR spectra (Distortionless enhancement by polarization transfer) showed only protonated carbon atoms at a larger S/N ratio than available from single-pulse $^{13}C$ NMR spectra and allowed distinction of methylene (negative amplitude) and methine carbon atoms (positive amplitude) in carbohydrates. They indicated the presence of four major (groups of) carbohydrates as deduced from their distinct anomeric ($O_2CH$) $^{13}C$ NMR resonances, and several minor ones. The variable linewidth of the NMR resonances produced by anomeric carbon atoms most likely resulted from superposition of similar chemical environments in complex EPS (Fig. 4.6d) rather than from intrinsic effects of local mobility of defined chemical environments in pure polysaccharides (here, less mobile fragments would have produced NMR resonances with larger linewidths). The superposition of distinct anomeric positions in resonances of $^{13}C$ NMR spectra was also corroborated by $^{1}H$, $^{13}C$ HSQC NMR cross peaks (Fig. 4.6e); the seven major HSQC NMR cross peaks a-g projected on the four major $^{13}C$ NMR resonances (Fig. 4.6b).

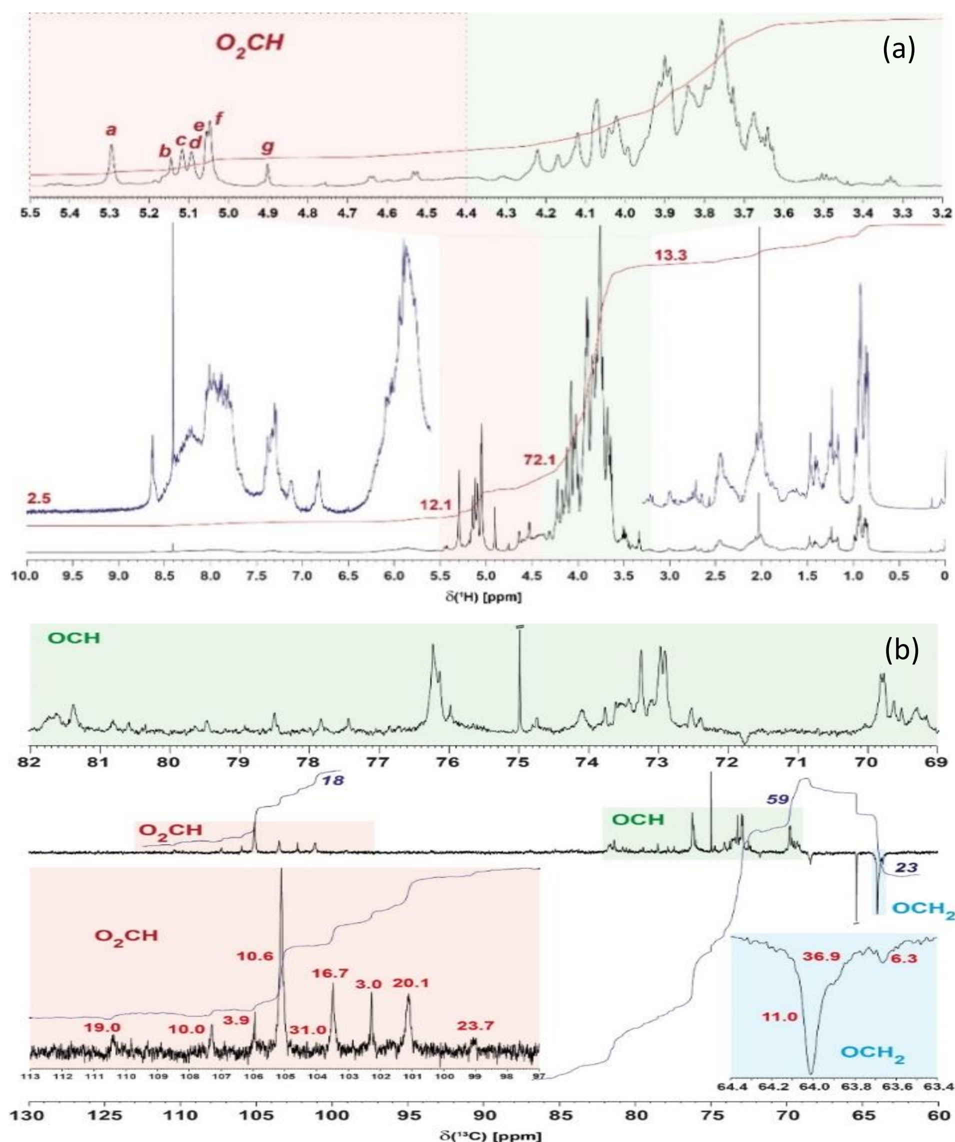



Figure 4.6. Chemical characterization of exopolysaccharide: (a) $^{1}H$ NMR spectra of EPS preparation BGI-2 (800 MHz, $D_2O$), with expansion of carbohydrate-derived NMR resonances shaded in orange (anomeric $O_2CH$-units) and green (OCH and $OCH_2$ units); major anomeric $^{1}H$ NMR resonances a-g are annotated (cf. text); numbers denote relative $^{1}H$ NMR section integrals (total: 100%), and (b) $^{13}C$ DEPT NMR spectra of EPS preparation BGI-2 (125 MHz, $D_2O$), with expansion of carbohydrate-derived $^{13}C$ NMR resonances shaded in orange (anomeric $O_2CH$-units) and green (OCH and $OCH_2$ units); red numbers indicate half-width [Hz] of anomeric and $OCH_2$ $^{13}C$ NMR resonances, most probably resulting from overlap of related but different chemical environments; blue numbers denote relative $^{13}C$ NMR section integrals (total: 100%, with respect to section shown).

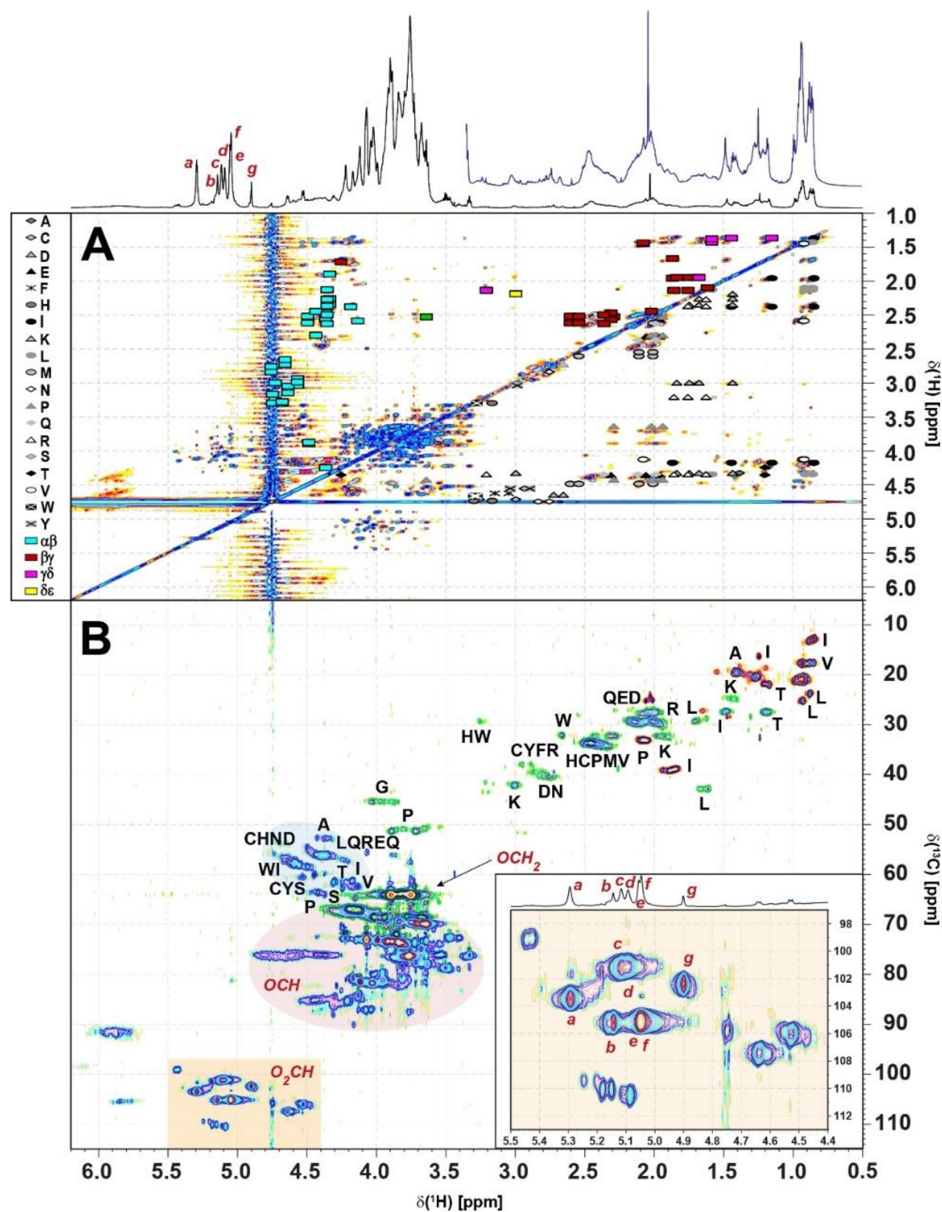


Figure 4.6 (c). (Panel A) $^{1}H$, $^{1}H$ TOCSY and (panel B) $^{1}H$, $^{13}C$ DEPT HSQC NMR spectra of EPS preparation, aliphatic section, with cross peaks of proteinaceous amino acids (see attendant single letter code) in proteins following alanine (A) annotated according to position and carbon multiplicity (Hertkorn et al., 2002). (Panel A): upper left half: amino acid-derived COSY cross peaks according to positioning in peptides; (panel A): lower right half: amino acid-derived TOCSY cross peaks according to individual amino acids (individual gray symbols; single letter code for amino acids). (Panel B): $^{1}H$, $^{13}C$ DEPT HSQC NMR spectrum; color code: blue: CH; green: $CH_2$, red: $CH_3$. Orange shaded insert denotes section of anomeric $O_2\underline{CH}$ cross peaks, with major cross peaks indicated by letters a-g; other carbohydrate-related substructures are oxyomethylene $O\underline{CH_2}$, largely from carbohydrates and oxymethine $OC_2\underline{CH}$ groups.

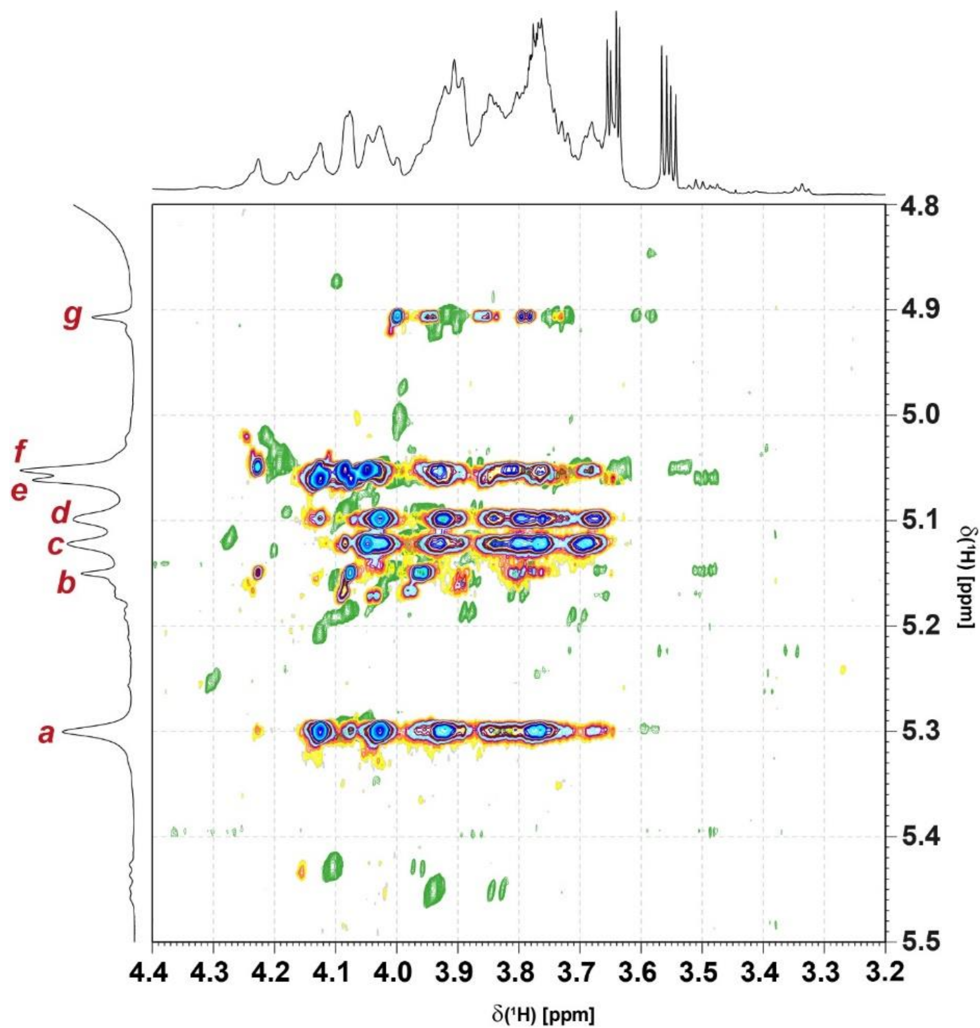



Figure 4.6 (d). Overlay of $^1H$, $^1H$ TOCSY and (green color) $^1H$, $^1H$ NOESY NMR spectra of EPS-2; section of anomeric $O_2CH$.units (F1: with annotated seven major NMR resonances) versus section of OCH and $OCH_2$-units (F2); expansive cross peaks indicate superposition of closely related chemical environments.

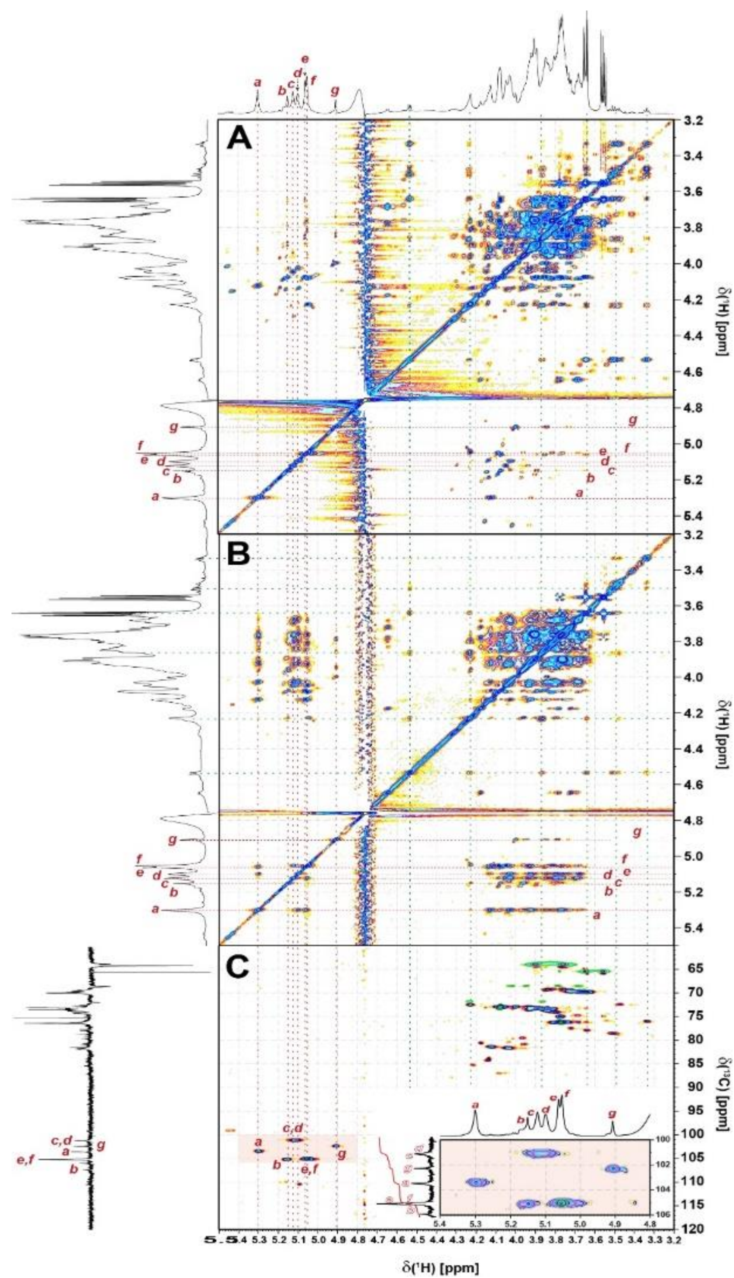


Figure 4.6 (e). (A) $^1H$, $^1H$ NMR TOCSY NMR (B) $^1H$, $^1H$ NMR TOCSY NMR and (C) $^1H$, $^{13}C$ DEPT HSQC NMR spectrum of EPS preparation BGI-2 (800 MHz, $D_2O$), with seven major anomeric NMR resonances of anomeric positions $O_2CH$, a-g, provided (cf. text).

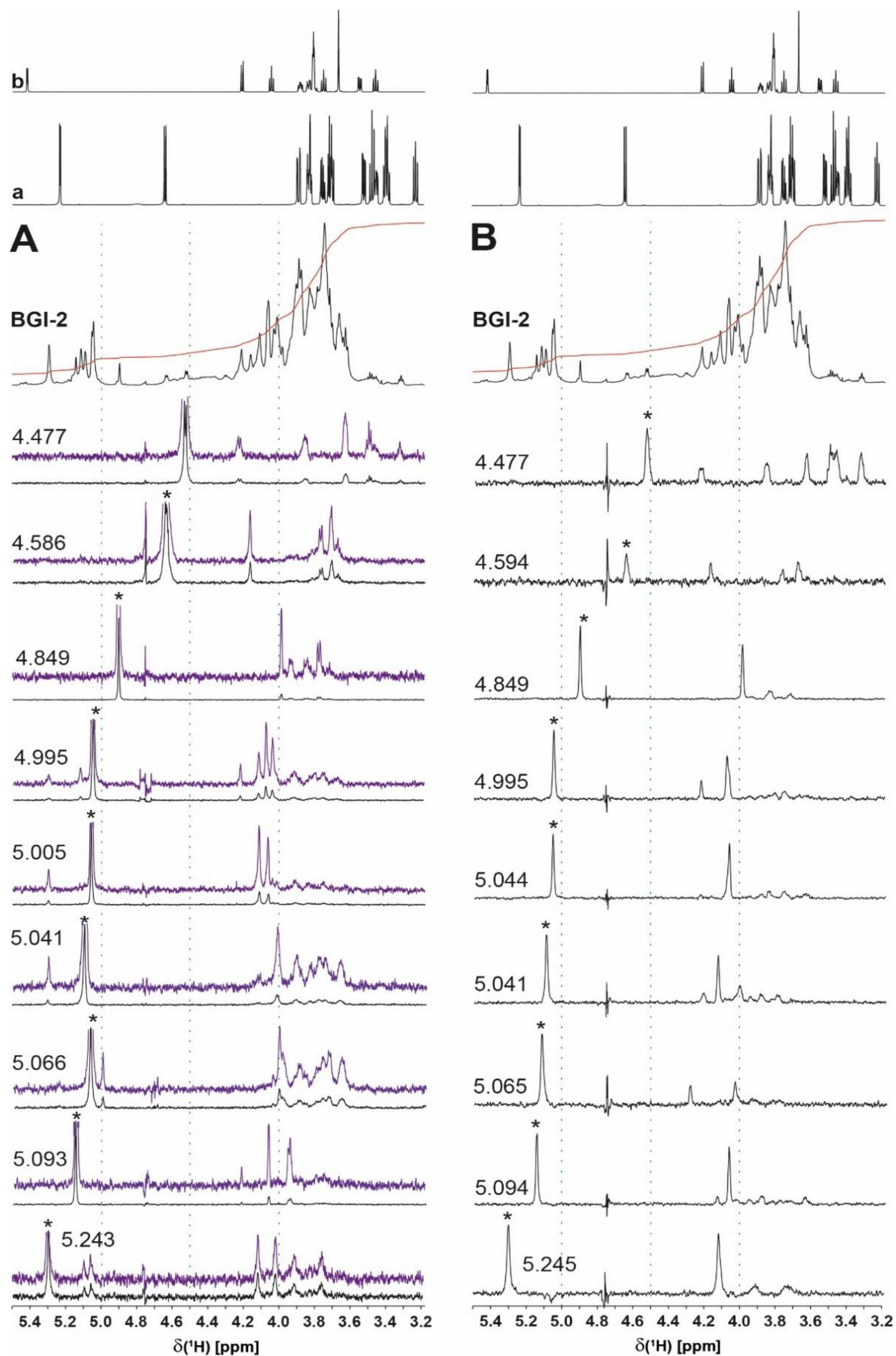



Figure 4.6 (f). Selective excitation of (panel A) 1D $^{1}$H TOCSY and (panel B) 1D $^{1}$H NOESY NMR experiments of BGI-2 with nominal $\delta_H$ of irradiation provided; top rows show standard $^{1}$H NMR spectra of (a) D-glucose, (b) sucrose, BGI-2.

### 4.3.6. Survivability of *Pseudomonas* sp. BGI-2 after freeze thawing without addition of an external cryoprotective agent (CPA)

The freeze thaw survivability of BGI-2 was compared to another non-EPS producing psychrotrophic strain *Rhodococcus* sp. BGI-11 isolated from the same environment and a mesophilic *E.coli* K12 strain. After 7 freeze thaw cycles, survivability of BGI-2 was 91.1% compared to 64.5% in BGI-11. Survivability of *E.coli* decreased significantly with the increase in freeze thaw cycle and no viability was observed after the 4$^{th}$ cycle. Overall survivability decreased with an increase in the freeze thaw events to variable degree depending on the strains (Fig. 4.7). BGI-2 demonstrate maximum tolerance compared to the other two bacteria when subjected to repeated freeze thaw cycles.

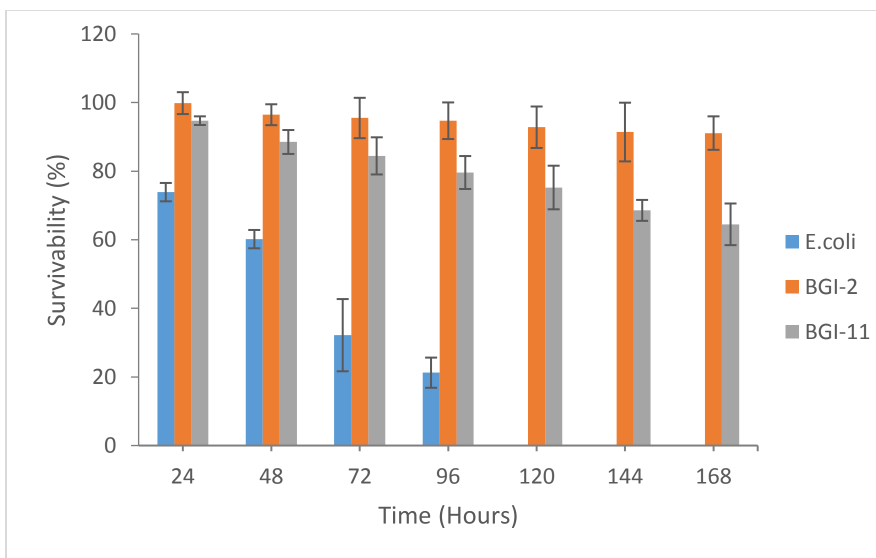



Figure 4.7. Freeze thaw survivability of *Pseudomonas* sp. BGI-2 compared to *Rhodococcus* sp. BGI-11 and *E.coli* K12 using plate count method.

### 4.3.7. Cryoprotective activity of EPS

Plate count was used to check the survivability of the indicator organism *E.coli* K12 for the cryoprotection assay (Fig. 4.8). We found that *E.coli* K12 strain recovered to varying degrees which depended on the concentration of EPS used and the number of freeze thaw cycles the strain was subjected. Survivability of *E.coli* K12 increased significantly with an increase in EPS concentration from 1 to 5% (w/v). The maximum survivability after 7 freeze thaw cycle was observed for 5% EPS (89.7%) followed by 3% EPS (61.4%) and 1% EPS (27.2%). Survivability was severely affected in the control (no cryoprotective agent)

with no viability observed after 4 freeze thaw cycles. Glycerol had the highest recovery where survivability was 93.9% (Fig. 4.9).

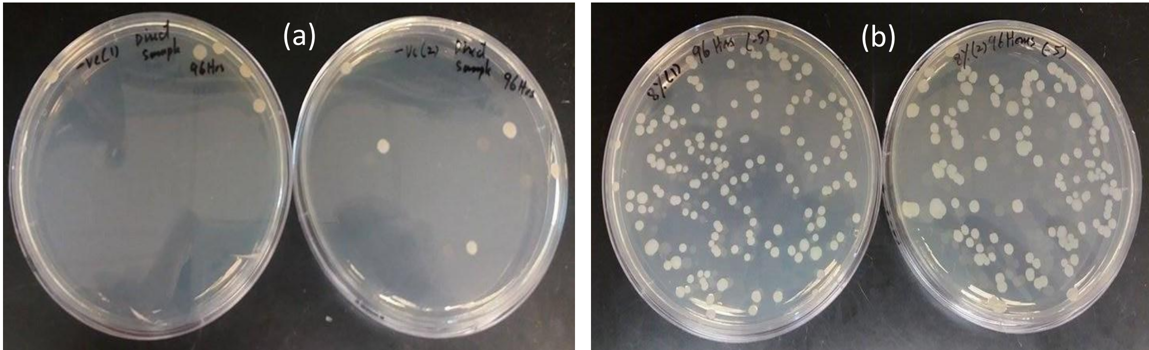



Figure 4.8. Cryoprotective activity of EPS using viable plate count: Recovery of *E.coli* K12 after 96 hours of cryopreservation (a) Control (no addition of EPS), (b) 5% EPS.

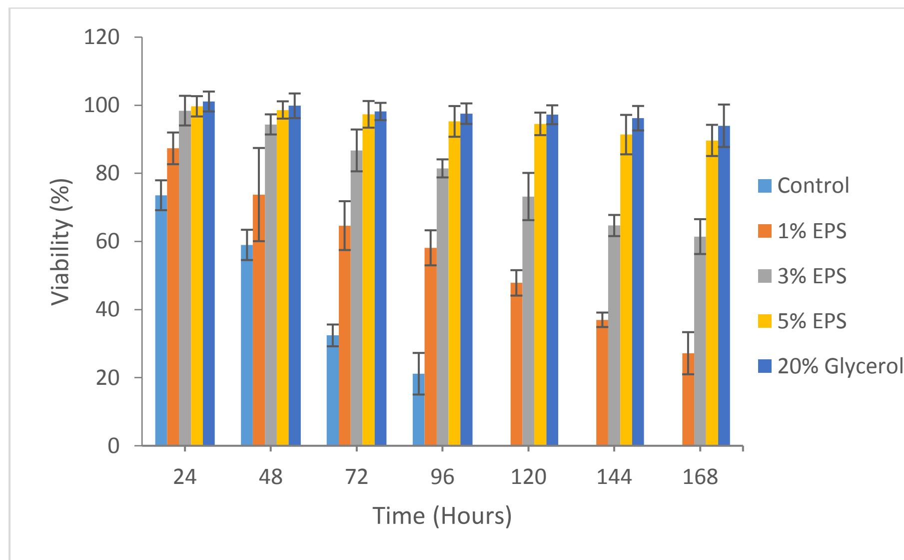



Figure 4.9. Cryoprotective effect of different concentrations of EPS (1 – 5%) on *E.coli* K12 subjected to 7 freeze thaw cycles using plate count method.

## 4.4. Discussion

The Karakoram is one of the most glaciated mountain ranges in the world outside the polar region, serving fresh water to a large population in the region. Batura glacier, one of the longest glacier outside the Polar Regions with a length of 59 km, also lies in the Karakoram (Hewitt, 2014). The glaciers in the Karakoram, particularly in the Pakistani geographical regions, are less explored in terms of its bacterial diversity and functionality. The very few studies of bacterial diversity are limited to sampling during the summer season. Here we

explored bacterial diversity within the ice of Batura glacier during the harsh winter season (In press). *Pseudomonas* sp. BGI-2 was selected for further studies based on its high abundance, relatively rapid growth at low temperatures and maximum EPS production compared to the other bacterial isolates. When glacier ice enrichment samples were streaked on agar plates, BGI-2 made up more than 60% of all bacterial colonies. Colonies of BGI-2 appeared on agar plates within the first week of plating enrichment samples whereas it took two to three weeks for the other glacial isolates to form visible colonies. The initial observations suggested how well this strain is adopted to the harsh environment characterized by frequent freeze thaw cycles, desiccation (low water activities), high UV radiation and low nutrient availability. Molecular characterization using complete 16S rRNA gene sequencing placed BGI-2 in the genus *Pseudomonas* with the closest species *Pseudomonas mandelii* and *Pseudomonas frederiksbergensis*. Recently, a psychrotrophic *Pseudomonas frederiksbergensis* ERDD5:01 was isolated from a glacial stream in the Himalaya and the strain exhibited high survivability/tolerance to stress conditions such as freezing, freeze thaw cycles and UV-C radiations (Kumar et al., 2019). *Pseudomonas* species are found in diverse environment due to their great metabolic flexibility**.** Cold adapted *Pseudomonas* species have been isolated from different cold environments around the world including the polar and non-polar regions (Vasquez-Ponce et al., 2017; Sathiyanarayanan et al., 2016; Jang et al., 2012). Männistö and Häggblom (2006) characterized the psychrotophic heterotrophic bacteria from Finish Lapland and found one third of the isolates as *Pseudomonads*, representing 60% of all isolates from alpine tundra soils. Meyer et al., (2004) found high prevalence of psychrotrophic *Pseudomonas* in alpine soil samples constituting key members of the bacterial community.

Temperature is an important factor determining the growth and metabolite productions in any organism. *Pseudomonas* sp. BGI-2 grew well at a temperature range of 4-35˚C. Since the growth rate was low at 15˚C compared to 25˚C and decreased above 30˚C, we assume therefore the optimum growth range lies within 20 - 30˚C. Morita (1975) defined psychrophiles as organisms having an optimal growth temperature around 15˚C or below and maximal growth temperature of 20˚C. Psychrotrophs were defined as organisms able to grow at low temperatures but have their optimal growth temperature above 20˚C.

According to these definitions BGI-2 falls in the category of psychrotrophs which is obvious from the fact that it was isolated from an environment that undergoes frequent temperature changes.

Although the optimum temperature for BGI-2 lies between 20 and 30˚C, however maximum EPS production was observed at suboptimal temperatures 15˚C and 4˚C. Nichols et al., (2005a) found a similar result while investigating the effect of incubation temperature on the growth and EPS production from a psychrotolerant sea ice bacterium. Their study revealed that EPS yields at - 2˚C and 10˚C were 30 times higher than at 20˚C, which is the optimum growth temperature for many psychrotrophic strains. The explanation for maximum EPS production at suboptimal conditions came from Sutherland (1982), suggested that there is a competition between the cell wall polymer biosynthesis (peptidoglycan and lipopolysaccharide) and EPS production as they use the same isoprenoid glycosyl lipid carriers. Therefore suboptimal growth temperature or any other physical factor that restrict the growth may enhance EPS production. Most of the optimization experiments revealed that the maximum production of EPS took place in the stationary phase which is in accordance with the previous studies (Nichols et al., 2005a; Samain et al., 1997; Moriello et al., 2003).

BGI-2 demonstrated the highest growth rate in the blank but maximum EPS production was observed at 1-3% NaCl, again supporting the explanation from Sutherland (1982) discussed above. Slight-halophiles grow optimally at 2-5% NaCl (w/v), moderate-halophiles 5-20% (w/v) and extreme-halophiles 20-30% (w/v) NaCl concentration. BGI-2 demonstrated growth at a salt range of 1- 5%, therefore we consider BGI-2 in the category of slight-halophile or halotolerant.

Carbohydrate sugars are the preferred carbon sources for EPS production. Among the many substrates tested, BGI-2 was able to use a broad range of carbon sources including glucose, galactose, mannose, mannitol, glycerol and molasses. This metabolic versatility of *Pseudomonas* facilitates its survival in different habitats and adaptation to varied environmental conditions. Although BGI-2 was able to grow in mineral salt medium

supplemented with glucose or galactose but growth and biomass production was not as good as in a nutrient rich medium (tryptic soy broth). The growth rate and biomass production of BGI-2 was two to three times higher in tryptic soy broth than in mineral salt medium. EPS production in MSM supplemented with either 1% glucose, galactose or molasses was negligible compared to the nutrient rich medium tryptic soy both where EPS yield was as high as 2.01 g $L^{-1}$. Tryptic soy broth has other organic nutrients to support the bacterial growth and biomass production. Tryptone and soytone in tryptic soy broth medium supply additional growth supplements for the bacterium, resulting in maximum cell biomass and EPS production. Production of metabolic products is very much dependent on the nutritional requirement of the bacteria. EPS production is highly influenced by the carbon and nitrogen sources in the fermentation medium. Maalej et al., (2015) found that organic nitrogen sources were more effective for growth and EPS production than inorganic source as no growth and EPS production was observed in presence of ammonium sulphate as a nitrogen source. According to Bajaj et al., (2006), organic nitrogen sources are complex containing amino acids and vitamins which improve the cell growth and EPS yield. Therefore production of EPS is influenced greatly by the carbon and nitrogen sources used. Carbohydrates is used as a carbon and energy source by most of the bacteria whereas, amino acids or inorganic ammonium salt serve as the nitrogen source (Sutherland, 1982).

Under optimized conditions, EPS production by *Pseudomonas* sp., BGI-2 was 2.01 g $L^{-1}$ which is significantly higher than some of the previous reports using the same method for EPS estimation (Phenol – sulfuric acid method). Celik et al., (2008) optimized EPS production for two *Pseudomonas* species and found xylose as the preferable carbon source. The maximum EPS yield for *Pseudomonas aeruginosa* G1 was recorded at 3% xylose (368 mg $L^{-1}$) and *Pseudomonas putida* G12 at 2% xylose (262 mg $L^{-1}$). Kılıç and Dönmez (2008) optimized EPS production for a chromium resistant *Pseudomonas aeruginosa* and maximum yield after 96 hours of incubation was recorded as 863.3 mg $L^{-1}$.

Nutritional as well as environmental conditions affect the overall yield of EPS production. The choice of a carbon source in culture medium is critical and can be sugar or non-sugar

depending on the strain. Sugar waste molasses can be used as a cheap carbon source for EPS production. The strain BGI-2 grew rapidly and produced high EPS yield in the medium supplemented with molasses as sugar source. BGI-2 had the highest growth rate in the MSM medium with molasses as the carbon source followed by glucose, glycerol and galactose. In our study, 5% molasses resulted in the maximum EPS production. The production cost is still a major obstacle for large scale production of EPS and industrial applications. The major nutrient for EPS production is the carbon sources used (mainly sugars) making the process costly. It is therefore important to optimize fermentation conditions and substitute nutrients with less expensive carbon substrates. Molasses is not only rich in sugars but also contains other essentials minerals which can be helpful in bacterial growth and EPS production. Likewise, BGI-2 also was able to use glycerol as a carbon source. Crude glycerol is generated as a waste in the biodiesel industry and can be employed as a cheap source of carbon for large scale EPS productions.

EPS is considered as stress molecule which assist microorganisms to cope with the extremes of temperatures, high salinity and desiccation. Therefore the survivability of BGI-2 against a series of freeze thaw cycles were compared to two other bacteria including a non EPS producing bacterium *Rhodococcus* sp., BGI-11 isolated from the same environment and a mesophilic *E.coli* K12. Survivability of EPS producing BGI-2 was significantly higher than BGI-11 and *E.coli*. After 7 freeze thaw cycles, survivability of BGI-2 was 91.1% compared to 64.5% for BGI-11. Whereas survivability decreased significantly for *E.coli* and not a single cell was recovered after 4 freeze thaw cycles. This suggests the role of EPS in protecting cells from damages caused by the freezing conditions and freeze thaw events. Casillo et al., (2017) demonstrated the cryoprotective role of an exopolysaccharide from cold-adapted bacterium *Colwellia psychrerythraea* 34H. Tamaru et al., (2005) studied the role of extracellular polysaccharides in desiccation and freeze tolerance in a terrestrial cyanobacterium *Nostoc commune.* They demonstrated that the EPS depleted cells were sensitive to desiccation and freeze thaw treatment compared to when the EPS was intact. Similarly, Aslam et al., (2012) proposed EPS production as a strategy by the polar diatoms for survival in the cold and saline environment of the sea ice.

Once it was established that EPS could have a role in cryoprotection, we used the possibility of EPS extracted from BGI-2 for the cryoprotection of a mesophilic bacterium *E.coli* K12. The effect of EPS on survivability of *E.coli* K12 after a series of freeze thaw cycles implied its role in cryoprotection. Survivability of *E.coli* depended on the concentration of EPS used and the number of freeze thaw cycles to which the strain was subjected. Survivability of *E.coli* K12 strain was improved as the concentration of EPS increased from 1% to 5% (w/v). After 7 freeze thaw cycles, the maximum survivability was observed for 5% EPS (89.7%). Survivability decreased significantly with the decrease in EPS concentration (1%) resulting in only 27.2% after 7 freeze thaw cycles. Similarly, survivability also decreased with the increase in freeze thaw cycles. For 3% EPS, survivability was 98.42% after the 1$^{st}$ freeze thaw cycle, decreased to 81.5% after 4 cycles and 61.4% after 7 cycles. Survivability was severely affected in the treatment with no addition of EPS where no viability was observed after 4 freeze thaw cycles. This is obvious as a freeze thaw cycle is injurious to cells, and bacteria inhabiting cold environments which undergo temperature fluctuation must adopt some strategies to cope with the change in temperature. Exopolysaccharides are thought to work as a cryoprotectant in such situations. The EPS demonstrated high cryoprotective activity based on the survivability of a mesophilic *E.coli* strain. Survivability in 5% EPS was comparable to 20% glycerol which is a common cryoprotectant used for bacterial cryopreservation. The addition of EPS significantly improved the tolerance of *E.coli* to the freeze-thaw cycle, indicating a universal cryoprotective role. These results are in accordance with previous studies which also demonstrated the cryoprotective role of EPS (Carrion et al., 2015; Marx et al., 2009; Dubey and Jeevaratnam, 2015; Liu et al., 2013a).

The current NMR data and auxiliary information are not sufficient for a definite analysis of EPS structural detail. This in part resulted from the paucity of meaningful cross peaks in $^{1}$H, $^{13}$C HMBC NMR spectra of BGI-2 (Heteronuclear multiple bond correlation; data not shown) which precluded definition of inter-residue linkages. Weak HMBC cross peaks might in part have resulted from fast transverse NMR relaxation which could indicate presence of rather larger size EPS molecules. $^{1}$H, $^{13}$C HSQC-TOCSY (data not shown), selective 1D $^{1}$H TOCSY and NOESY $^{1}$H NMR experiments (Nuclear overhauser effect

spectroscopy) were inconclusive as well (Fig. 4.6f). However, absence of major acetyl groups ($\delta_H$ ~ 2 ppm, appearing as singlet resonances) and other relevant lipid NMR resonances (Turska-Szewczuk et al., 2014) is proposed from NMR integral ratios of anomeric and the respective aliphatic NMR resonances; HSQC cross peaks are in agreement with galactose and glucose derivatives (Landersjö et al., 2002; Gerwig et al., 2013; Seviour et al., 2010), rhamnose will not be part of a major EPS molecule, because the conspicuous methyl NMR resonance ($\delta_H$ ~ 1.3 ppm; Casillo et al., 2015) is virtually absent in all EPS samples.

The $^1$H NMR resonance in region of δ 4.5-5.0 shows anomeric carbons of sugar molecules. The resonance or peaks in between δ5.37 and 4.97 show 1- linkages in glucose constituting the backbone of EPS polymers and other inter glycoside linkages. Fructose moiety reflected from the peaks fall in range of δ 3.0-3.5. Several other sugar moieties also reported from the HMBC spectrum with ß (2-6) glycosidic bonds. Peaks at 104 to 107 in $^{13}$C NMR reflects the presence of fructofurnosyl moieties thereby confirming ß configuration in long chain polymers. The downfield shift signals in $^{13}$C NMR 65-75 relating the presence of quaternary anomeric carbon presence. The intense signals of fructofuranosyl and other sugar residues overlapping the signals of α glucose moieties.

## 4.5. Conclusions

*Pseudomonas* sp. BGI-2 is a promising strain able to grow at a wide range of temperatures and pH. The strain produces high yields of a cryoprotective EPS at low temperatures and can use molasses or glycerol as cheap sources of carbon for large scale productions. Psychrotrophic *Pseudomonas* sp. BGI-2 can be used as a producer strain for the production of a cryoprotective EPS. Cryopreservation of cells and tissues is an essential tool in biotechnology and medicine, and further research can pave the way for EPS-BGI-2 as an effective cryoprotective agent. Cold-adapted microorganisms have many benefits over their mesophilic counterparts through energy saving such as production of essential metabolites at low temperature, negating the requirement for expensive heating steps, rapid and easy inactivation of their enzymes as they are heat-labile. Working at low temperature also minimizes undesirable chemical reactions and contaminations that usually occur at higher temperatures.

# Chapter 5

# Chapter 5

**Cryopreservation of cyanobacteria and microalgae using exopolysaccharide extracted from the glacier isolate *Pseudomonas* sp. BGI-2.**

**Abstract**

Long-term preservation of microbial cultures without changes in morphological, physiological and genetic traits is essential. Cryopreservation is the common method used for long term preservation, however this method does not work well for cyanobacteria and microalgae. Here we used exopolysaccharide (EPS) extracted from a glacier bacterium as a cryoprotective agent for the cryopreservation of three cyanobacteria; *Synechococcus* sp. CB0101, *Synechococcus* sp. CBW1003, and *Microcystis aeruginosa* PCC 7806; and two microalgae; *Scenedesmus obliquus* HTB1 and *Chlorella vulgaris* UTEX 2714. Previously, we used this EPS as a cryoprotectant for another bacterium *Escherichia coli* strain K12, where it helped in freeze thaw survivability of this bacterium. In the present study, growth recovery for the microalgal and cyanobacterial strains after 9 months of cryopreservation in EPS was better than 5% DMSO, a well-known cryoprotectant for various types of cells. Poor recovery was recorded for all the strains when 10% glycerol was used as a cryoprotective agent. The patterns of growth recovery for most of these strains were similar after 5 days, 15 days and 9 months of cryopreservation. Moreover, we found that concentration of EPS used as cryoprotective agent is critical and varies with different strains depending on their sensitivity. Except *Scenedesmus obliquus* HTB1, most of the strains recovered well at the higher concentration of EPS used. The EPS from psychrotrophic bacterium helped in long-term cryopreservation of cyanobacteria and microalgae, suggesting a potential natural cryoprotective agent for cryopreservation of cells.

**Keywords:** Psychrotrophs, Exopolysaccharide (EPS), Cryopreservation, Cryoprotective agent (CPA), Cyanobacteria, Microalgae

## 5.1. Introduction

Microalgae and cyanobacteria are photoautotrophs in the microbial world. They act as primary producers in the biospheres by converting $CO_2$ to organic compound using solar energy. The range of applications of the microalgae and cyanobacteria is vast ranging from food to health and energy to environment. These photosynthetic microorganisms have been potentially used for biofuel production (Chisti, 2007; Parmar et al., 2011), production of high value products (Mobin et al., 2019; Mobin and Alam, 2017), environmental bioremediation (Silkina et al., 2017; Xiong et al., 2018), and carbon sequestration (Kumar et al., 2011; Pires, 2019).

As microalgal and cyanobacterial biotechnology develops, there is a need to preserve the cultures for longer time ensuring viability, purity and genetic stability. Cryopreservation is maintenance of the biological samples in a state of 'suspended animation' at low temperatures (Karlsson and Toner, 1996). Unlike bacteria, cryopreservation is not always successful for the photosynthetic microorganisms, particularly microalgae with larger cells where less biomass recovery is recorded. These photoautotrophic organisms have been maintained in the laboratories through serial sub culturing (Lorenz et al., 2005). However, this method of culture maintenance has some drawbacks, which includes prone to contamination (either bacterial or cross contamination with other strains), time consuming, labor intensive and expensive when it involves large culture collections (Day and DeVille, 1995).

Cryopreservation is an important technique for long term preservation of cells, tissues and organs. However, the ultra-low temperature can cause formation of ice crystals in the cellular cytoplasm in absence of a suitable cryoprotective agent, which lyses the cell membrane (Bui et al., 2013). Cryoprotective agents (CPAs) are added to the culture media to protects the cells from cryo-injury by lowering the freezing point of water and inhibiting ice crystal formation in the suspension medium and cell interior (Chian, and Quinn, 2010). Dimethyl sulfoxide (DMSO), methanol, and glycerol are commonly used intracellular cryoprotectants while sucrose and large polymers provides extracellular protection as these polymers cannot permeate the cell membrane (Bui et al., 2013). CPAs at higher

concentration prevents ice formation more effectively in cells, tissues and organs during cryopreservation. However, the challenges lie in the fact that these CPAs become more toxic at higher concentration and negatively effects viabilities of the cryopreserved materials (Fahy, 1986). The successful cryopreservation therefore should involve strategies to eliminate the ice while minimizing the risk of CPA toxicity (Best, 2015). The various factors affecting successful cryopreservation depends on the types and concentrations of CPAs, storage time and temperatures. The method used for cryopreservation is also very critical, as many strains cannot withstand steps such as pre cooling and post thawing. Thus looking for alternative CPA with less toxicity and improving the protocols to avoid freezing damage can help in successful cryopreservation of more cells.

The use of glycerol and DMSO, which are regarded as universally useful CPAs for most samples, actually marked the beginning of modern cryopreservation technology. The cryoprotective agents could be penetrating or non-penetrating depending on their permeability to cross the membrane. Penetrating CPAs are a class of cryoprotectants that cross cell membranes such as ethylene glycol (EG), propylene glycol, dimethylsulfoxide (DMSO), glycerol and methanol. Non-penetrating CPAs are large molecules, usually polymers that inhibit ice growth by the same mechanisms as penetrating CPAs, but do not enter cells (Karlsson and Toner, 1996). Examples of the non-penetrating cryoprotectants include sucrose, trehalose, and polyethylene glycol (PEG) among others. Trehalose is a popular non-penetrating CPA, reported as less toxic and highly efficient in cryopreservation (Wen et al., 2016; Lee et al., 2013). Also cocktail of non-penetrating (trehalose) and penetrating (glycerol) CPAs resulted in efficient post cryopreservation recovery (Wen et al., 2016). Non-penetrating CPAs are usually less toxic than their penetrating counterparts at the same concentration (Wowk, 2007). The toxicity of penetrating or permeating cryoprotectants have been summarized well in a recent review article (Best, 2015).

Extreme environments are less explored niche and these untapped ecosystems offer novel microbial metabolites with promising industrial applications (Rampelotto, 2013). Cold temperature is widely distributed among the extreme environments on the earth. The cold

environments have been successfully colonized by the microorganisms, which not only survive but actively metabolize at these freezing conditions. They have developed strategies to successfully overcome the negative effects of the extreme low temperatures including intracellular freezing (Olijve et al., 2016). Production of exopolysaccharide (EPS) is one of the many other strategies microorganisms have evolved to cope with extremely low temperature (Deming and Young, 2017; Krembs and Deming, 2008; Carrion et al., 2015). Exopolysaccharides (EPSs) are glycopolymers secreted by microorganisms in their surrounding environment (Southerland, 1972). As discussed in the previous paper (Chapter 4), exopolysaccharides are produced by diverse group of microorganisms including bacteria, cyanobacteria, archaea, fungi, yeast and microalgae. Sea ice and ocean particles in the Antarctic marine environment have abundant microbial EPSs and they could play a role in survival and adaptation of microbial communities to cope with the extremes of temperatures and salinity (Krembs and Deming, 2008; Krembs et al., 2002; Nichols et al., 2005b).

Microorganisms are considered as hidden wealth due to the enormous biotechnological potential they offer. Despite the huge potential only few of the bacterial EPSs have made their way into the global market mainly because of their high production cost. Nevertheless bioprospecting for novel EPSs with unique functional properties and improving the production cost can pave the way for its commercialization. Biodiversity and functionality of the Karakoram glaciers have not been fully explored and hence provide an opportunity for the discovery of novel microorganisms or their metabolites. *Pseudomonas* sp. BGI-2 was isolated among 7 other psychrotrophic bacteria from the ice of Batura glacier, Pakistan. The strain BGI-2 was selected for further studies based on the maximum EPS production among the EPS producing isolates. Previously, we optimized production of EPS from the cold-adapted *Pseudomonas* sp. strain BGI-2 under different nutritional and environmental conditions. The strain was able to use molasses and glycerol as carbon source, which can be employed as a cheap alternate source of carbon substrate for large scale EPS production. Also, the EPS from BGI-2 provided significant cryoprotection to a mesophilic *E. coli* strain K12 subjected to a series of freeze-thaw cycles. In another study, we found 11 EPS-producing genes in the genome of BGI-2 compared to none in the 7 closely related

mesophilic *Pseudomonas* strains (Ali et al., 2019). All these findings helped in the hypothesis that production of EPS could be a strategy employed by microorganisms to avoid the deleterious effects of freezing conditions at low temperatures.

Although some bacterial EPSs (xanthan and gellan) have already huge global market but use of EPS as a cryoprotective agent will be a new addition to the cryopreservation industry. In this study, we used the possibility of employing a bacterial EPS for the cryopreservation of photosynthetic microorganisms, including cyanobacteria and microalgae. It seems the EPS from BGI-2 provided significant cryoprotection to the selected cyanobacterial and microalgal strains. Biomass recovery of the selected strains in presence of EPS as a cryoprotective agent was better than 5% DMSO and 10% glycerol. Cells and tissue cryopreservation is an important tool in research, medicine and biotechnology, hence further study can pave the way for EPS-BGI-2 in cryopreservation industry.

## 5.2. Material and Methods

### 5.2.1 Cyanobacterial and microalgal strains used for cryopreservation assay

Five strains including 3 cyanobacteria and 2 microalgae were used for this study. *Synechococcus* sp. CB0101, *Synechococcus* sp. CBW1003 and *Microcystis aeruginosa* PCC 7806 were cyanobacterial representatives whereas *Scenedesmus obliquus* HTB1 and *Chlorella vulgaris* UTEX 2714 represented microalgae. *Scenedesmus* sp. HTB1, *Chorella vulgaris* and *Mirocystis* sp. were grown in BG-11 medium (Allen, 1968) whereas SN15 (Waterbury and Willey, 1988) medium was used for the two *Synechococcus* strains. All the 5 strains are maintained in the laboratory at Institute of Marine and Environmental Technology (IMET), University of Maryland Centre for Environmental Sciences. *Microcystis aeruginosa* PCC 7806 is a fresh water cyanobacterium responsible for the major algal bloom around the world. *Synechococcus* sp. CBW1003 is a picocyanobacteria isolated from the Chesapeake Bay during the winter season (Xu et al., 2015). *Synechococcus* sp. CB0101 is another unicellular cyanobacterium isolated from the water of inner harbor Baltimore, Maryland (Chen et al., 2004). This strain is a common inhabitant of the Chesapeake Bay with a wide growth range for temperature, salinity and nutrients.

*Scenedesmus obliquus* HTB1 was isolated from the upper Chesapeake Bay (Back River) and has the ability to survive in high $CO_2$ concentration (Liu et al., 2013b). *Chlorella vulgaris* UTEX 2714 was bought from the UTEX culture collection of algae, at the University of Texas Austin, USA.

### 5.2.2. Cryopreservation of cyanobacteria and microalgae

Cryoprotective effect of EPS for cryopreservation of cyanobacteria and microalgae was determined by the method used previously with some modifications (Gaget et al., 2017). Three different concentrations of EPS (10%, 15% and 20%) from a stock solution (20 mg/mL) were used for cryopreservation. The cryoprotective effect of EPS was compared to 5% (volume/volume) dimethyl sulfoxide (DMSO) and 10% (volume/volume) glycerol, used as controls. Glycerol and EPS were sterilized using moist heat sterilization method (121˚C for 15 minutes) whereas DMSO was sterilized using 0.2µm filters. Each culture in their late log phase was transferred from flask into falcon tubes and centrifuged at 10,000 rpm for 5 minutes at room temperature. The supernatant was discarded and fresh medium was added on the ice. EPS was then added to a final concentration of 2 mg/ml, 3 mg/ml and 4 mg/ml. 5% DMSO and 10% glycerol was added in a similar way. Cells were re-suspended by gentle vortexing and immediately transferred to cryovials (1mL each). Cryovials were first incubated at 4˚C for 30 minutes and then stored in an ultra-low temperature freezer at - 80˚C.

### 5.2.3. Growth recovery

All the 5 strains were checked for growth recovery after 5 days, 15 days and 9 months of cryopreservation. For growth recovery, cryovials were taken out of the freezer and placed in a water bath at 35˚C for 5 minutes. Cultures were centrifuged at 8000 rpm for 3 minutes and supernatant discarded to remove any traces of the cryoprotective agents (CPAs). Cryopreserved cells were re-suspended in their respective media and transferred to 24 well plates. Cultures were kept in dark overnight in order to allow cells to recover under low light and under normal light at room temperature (21˚C) afterwards. Optical density was measured in a microplate reader (SpectraMax M5) to monitor the growth.

#### 5.2.4. Effect of heat sterilization on the cryoprotective activity of EPS

One set of experiment with three different concentration of EPS was also run without autoclaving the EPS stock to see the impact of heat sterilization on the cryoprotective activity of the EPS.

Well A1/B1 represents control (No EPS or cryoprotective agents), A3/B3 5% DMSO, A5/B5 10% glycerol, C1/D1 10% (2 mg/ml) autoclaved EPS, C2/D2 15% (3 mg/ml) autoclaved EPS, C3/D3 20% (4 mg/ml) autoclaved EPS, C4/D4 10% (2 mg/ml) non-autoclaved EPS, C5/D5 15% (3 mg/ml) non-autoclaved EPS, C6/D6 20% (4 mg/ml) non-autoclaved EPS (Fig. 5.1).

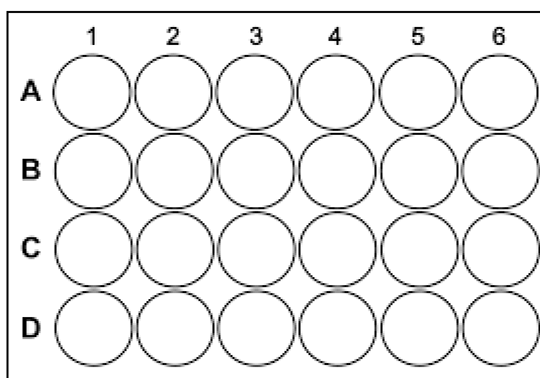



Figure 5.1. Picture of a 24 well plate used for biomass recovery test of the microalgal and cyanobacterial strains.

### 5.3. Results

#### 5.3.1. Cell morphology of the selected strains

We used the possibility of employing exopolysaccharide isolated from a psychrotrophic bacterium *Pseudomonas* sp. BGI-2 for the cryopreservation of cyanobacteria and microalgae. Cell morphology of cyanobacterial and microalgal strains is given below (Fig. 5.2).

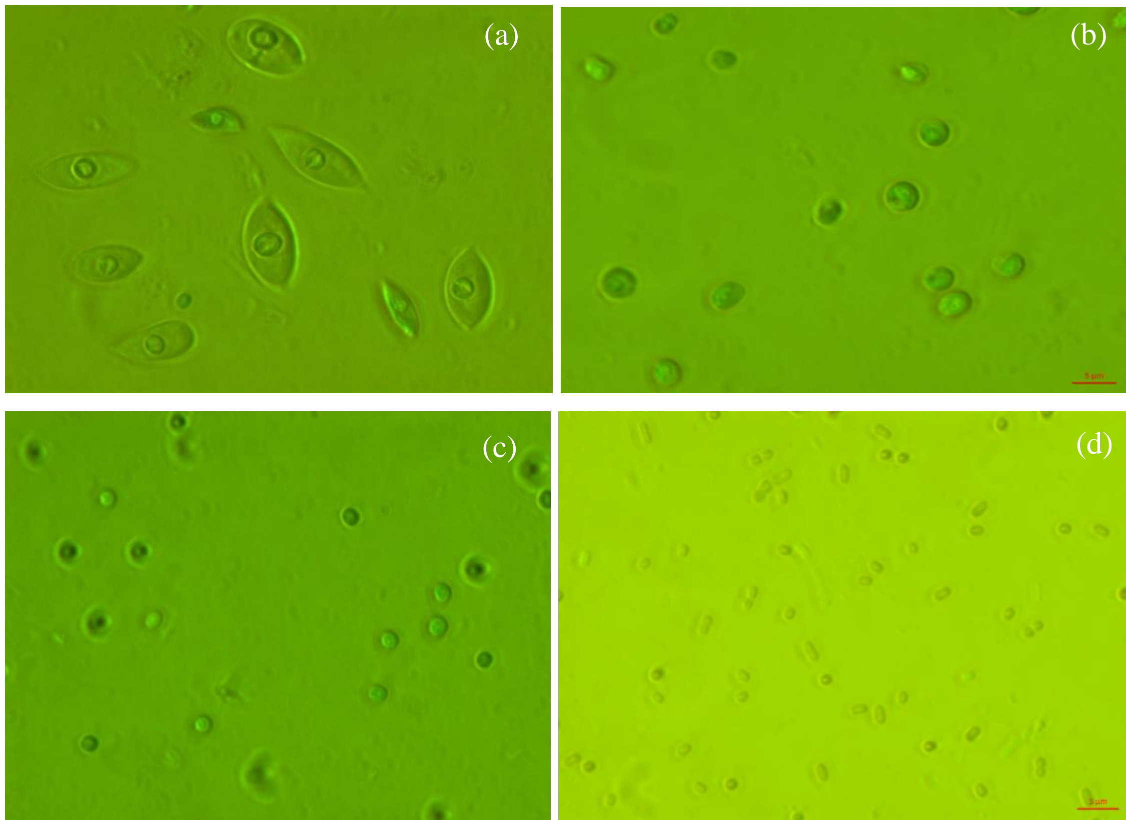



Figure 5.2. Cell morphology of the microalgal and cyanobacterial strains under 100X light microscopy (a) *Scenedesmus obliquus* HTB1 (b) *Chlorella vulgaris* UTEX 2714 (c) *Microcystis aeruginosa* PCC 7806 (d) *Synechococcus* sp. CBW1003

### 5.3.2. Growth recovery of cyanobacterium *Synechococcus* sp. BGW1003

The best result for cryopreservation was obtained with EPS for the cyanobacterial species *Synechococcus* CBW1003 after 5 days of cryopreservation at -80˚C. The maximum cell density was recorded at 4 mg/ml EPS ($OD_{570}$ 2.66), followed by 3 mg/ml EPS ($OD_{570}$ 2.13), 5% DMSO ($OD_{570}$ 2.1), 2 mg/ml EPS ($OD_{570}$ 1.98). Low biomass recovery was recorded in 10% glycerol ($OD_{570}$ 1.44) and in the control ($OD_{570}$ 0.95) after 18 days of incubation under normal shaking and light at room temperature (Fig. 5.3a).

The maximum biomass recovery after 15 days of cryopreservation was also recorded at 4 mg/ml EPS ($OD_{570}$ 3.32), followed by 3 mg/ml EPS ($OD_{570}$ 3.27), 5% DMSO ($OD_{570}$ 3.17) and 2 mg/ml EPS ($OD_{570}$ 3.01). Again low biomass recovery was recorded at 10% glycerol ($OD_{570}$ 1.24) and in control ($OD_{570}$ 1.52) as shown in Figure 5.3 (b).

The pattern of biomass recovery after 9 months of cryopreservation was similar to 5 and 15 days of cryopreservation. Growth after 9 months of cryopreservation showed the maximum recovery in 4 mg/ml EPS ($OD_{570}$ 2.32) and 5% DMSO ($OD_{570}$ 2.34). High cell density was also recorded at 3 mg/ml EPS ($OD_{570}$ 1.52) and 2 mg/ml EPS ($OD_{570}$ 1.52). Lowest biomass recovery was recorded at 10% glycerol ($OD_{570}$ 0.591) and in the control ($OD_{570}$ 0.761) as shown in Figure 5.3 (c).

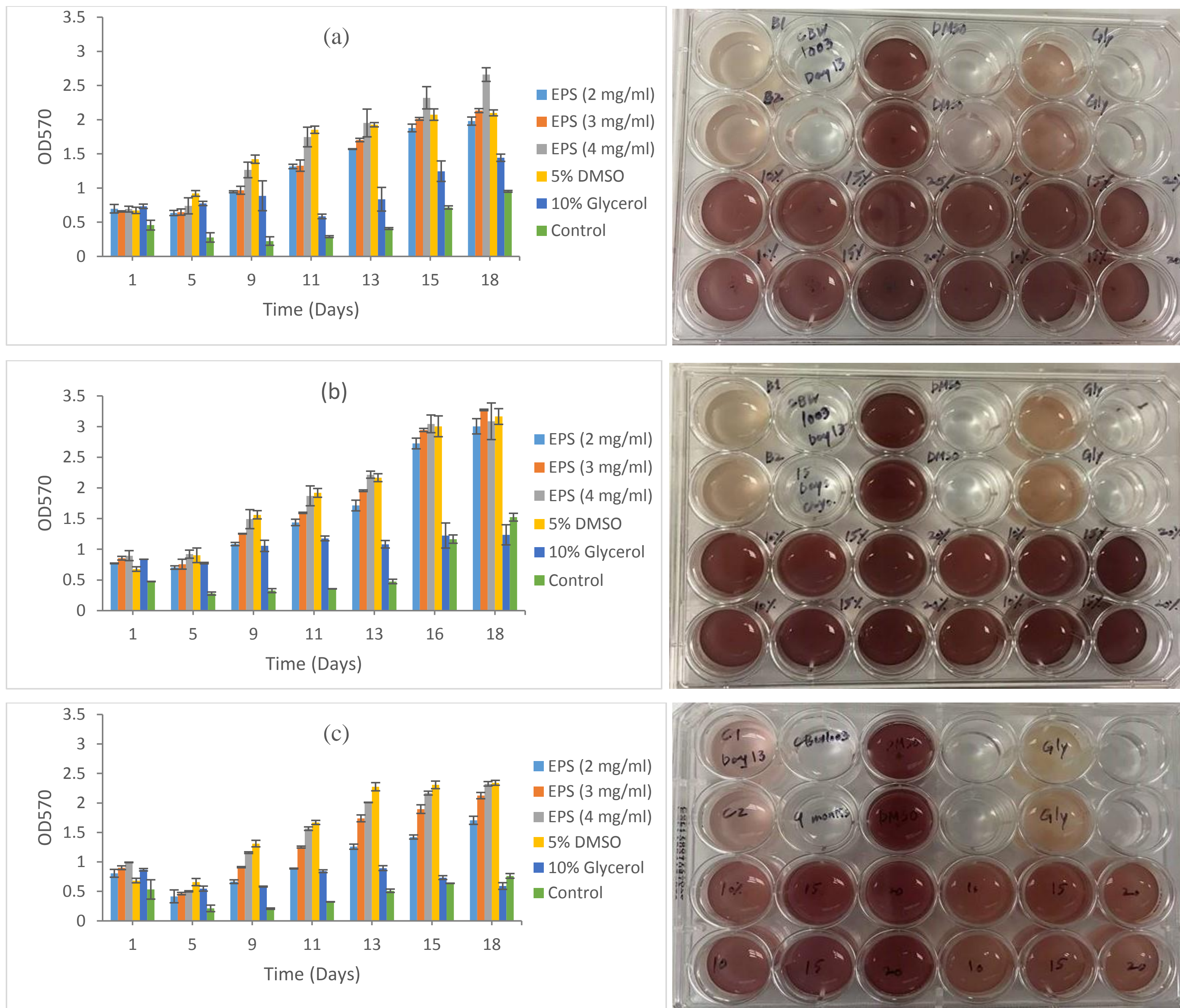



Figure 5.3. Growth recovery for *Synechococcus* sp. CBW1003 after cryopreservation (a) 5 days, (b) 15 days, and (c) 9 months.

### 5.3.3. Growth recovery of microalga *Scenedesmus* sp. HTB1

For the microalgal sp. *Scenedesmus* HTB1, the maximum growth recovery after 5 days of cryopreservation was observed at 5% DMSO ($OD_{680}$ 2.15) followed by 2 mg/ml EPS ($OD_{680}$ 2.04), 3 mg/ml EPS ($OD_{680}$ 1.96) and 4 mg/ml EPS ($OD_{680}$ 1.78). Poor biomass recovery in 10% glycerol ($OD_{680}$ 1.05) was observed. Viability and recovery was negligible in control with no addition of any cryoprotective agent (Fig. 5.4a). The optical density results presented here are over a period of 18 days of incubation under optimum conditions.

The maximum biomass recovery after 15 days of cryopreservation was again recorded at 5% DMSO ($OD_{680}$ 1.87) and 2 mg/ml EPS ($OD_{680}$ 1.86), followed by 3 mg/ml EPS ($OD_{680}$ 1.80) and 4 mg/ml EPS ($OD_{680}$ 1.78). Low biomass recovery was recorded at 10% ($OD_{680}$ 1.35) and control ($OD_{680}$ 1.07).

The pattern of biomass recovery after 9 months of cryopreservation was similar to 5 and 15 days of cryopreservation. Biomass recovery for HTB1 after 9 months of cryopreservation was the maximum at 2 mg/ml EPS ($OD_{680}$ 2.29) followed by 3 mg/ml EPS ($OD_{680}$ 2.19), 5% DMSO ($OD_{680}$ 1.99) and 4 mg/ml EPS ($OD_{680}$ 1.92). The lowest recovery was again recorded at 10% glycerol and control where optical density could not reach to 1 after 18 days of post cryopreservation incubation (Fig. 5.4c).

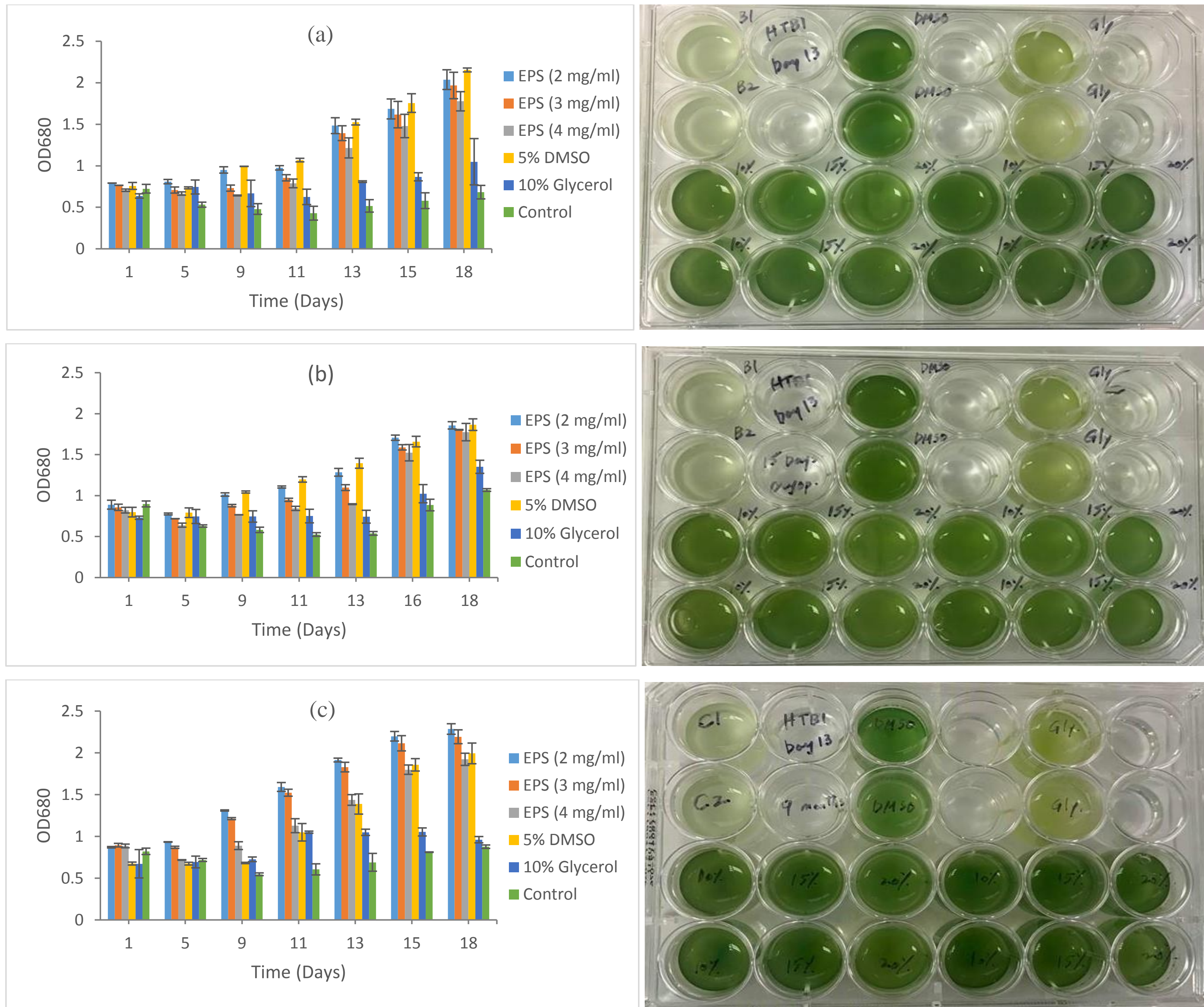



Figure 5.4. Growth recovery for *Scenedesmus* sp. HTB1 after cryopreservation (a) 5 days, (b) 15 days, and (c) 9 months.

### 5.3.4. Growth recovery of cyanobacterium *Synechococcus* sp. CB0101

For *Synechococcus* sp. CB0101, the maximum recovery was recorded in 4 mg/ml EPS ($OD_{435}$ 2.34), followed by 3 mg/ml EPS ($OD_{435}$ 1.98) and 2 mg/ml EPS ($OD_{435}$ 1.74) after 5 days of cryopreservation. Poor recovery was recorded for 10% glycerol and in the control. No biomass was recovered at 5% DMSO even after 20 days of post cryopreservation incubation under optimum conditions (Fig. 5.5a).

A similar recovery pattern was recorded after 15 days of cryopreservation with the maximum growth observed in cultures with EPS as the cryoprotective agent. The maximum biomass recovery was observed in the 4 mg/ml EPS ($OD_{435}$ 1.81) followed by 3 mg/ml EPS ($OD_{435}$ 1.45) and 2 mg/ml EPS ($OD_{435}$ 1.74). No biomass recovery was recorded at 5% DMSO, 10% glycerol and in the control (Fig. 5.5b).

Very similar results were obtained after prolong cryopreservation (9 months) of this strain. The maximum biomass recovery was recorded at 4 mg/ml EPS ($OD_{435}$ 2.26), followed by 3 mg/ml EPS ($OD_{435}$ 2.19) and 2 mg/ml EPS ($OD_{435}$ 1.30). Likewise no recovery was observed in 5% DMSO, 10% glycerol and control after 26 days of incubation under optimum conditions (Fig. 5.5c).

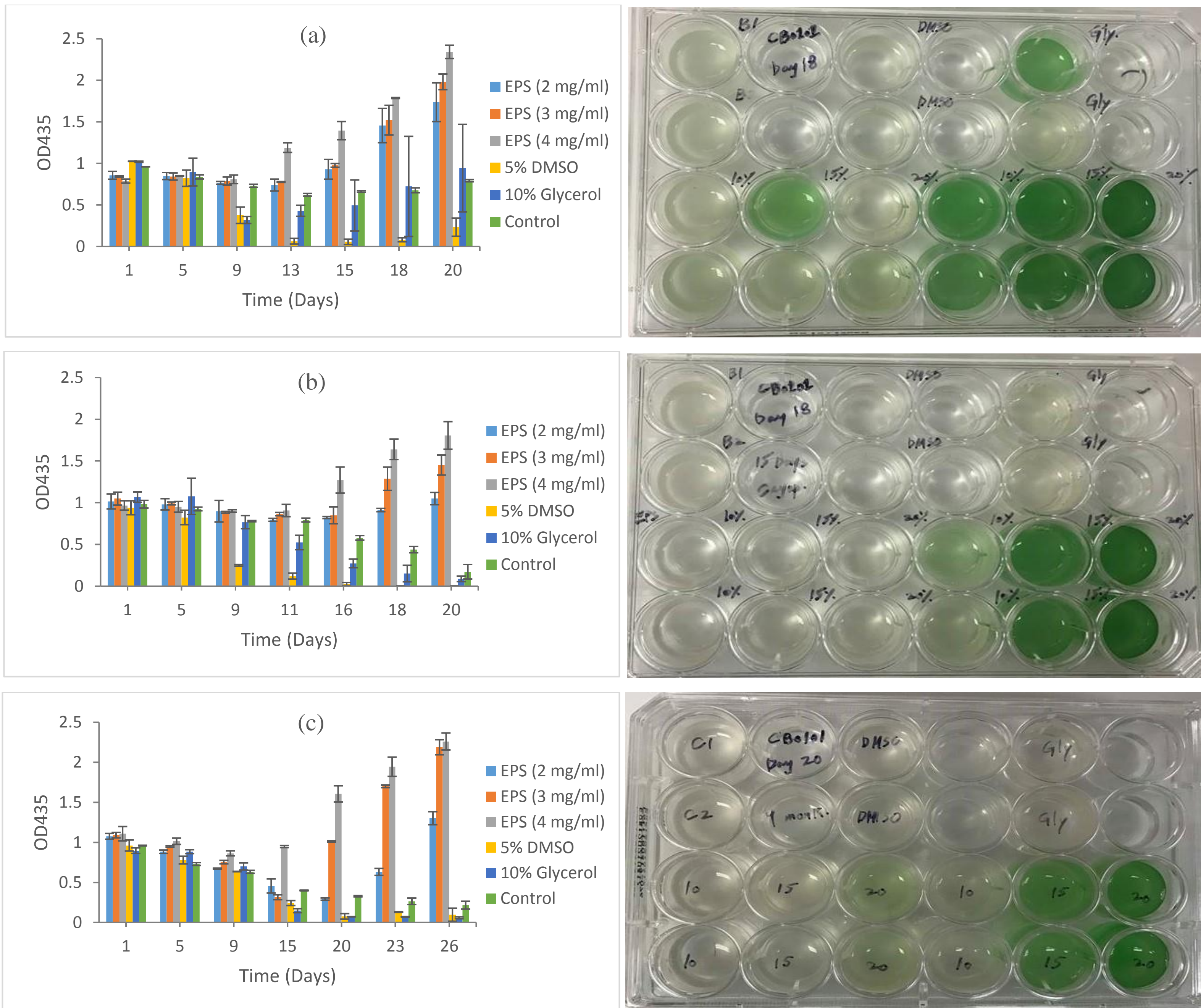


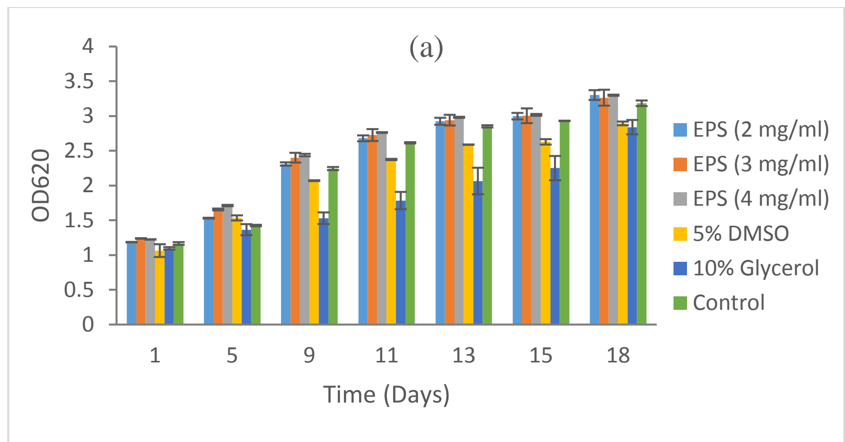

Figure 5.5. Growth recovery for *Synechococcus* sp. CB0101 after cryopreservation (a) 5 days, (b) 15 days, and (c) 9 months.

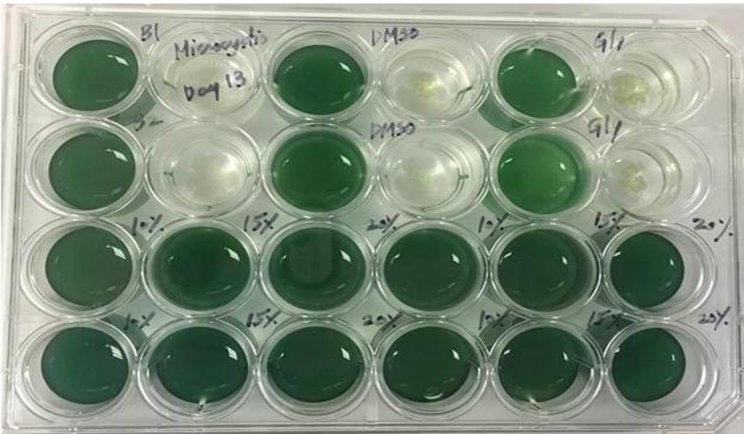
### 5.3.5. Growth recovery of cyanobacterium *Microcystis aeruginosa* PCC 7806

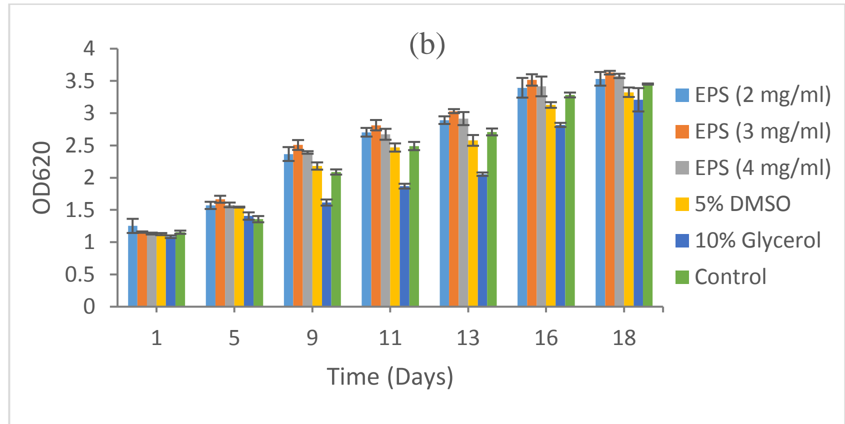
*Microcystis aeruginosa* PCC 7806 demonstrated good recovery in all treatments including the control. The maximum recovery after 5 days of cryopreservation was recorded at 4 mg/ml EPS ($OD_{620}$ 3.31), followed by 2 mg/ml EPS ($OD_{620}$ 3.30), 3 mg/ml EPS ($OD_{620}$ 3.26), control ($OD_{620}$ 3.18) and 5% DMSO ($OD_{620}$ 2.89). Recovery was low at 10% glycerol ($OD_{620}$ 2.84) compared to all other treatments (Fig. 5.6a).

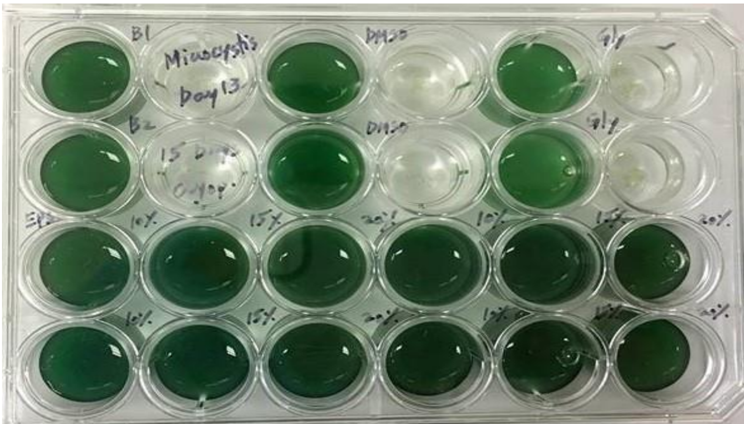
Recovery of this strain after 15 days of cryopreservation demonstrated a similar pattern with the maximum recovery recorded at 3 mg/ml EPS ($OD_{620}$ 3.63), followed by 4 mg/ml EPS ($OD_{620}$ 3.58) and 2 mg/ml EPS ($OD_{620}$ 3.53). Biomass recovery in the control ($OD_{620}$ 3.45) was better than 5% DMSO (OD620 3.33), and 10% glycerol ($OD_{620}$ 3.21) (Fig. 5.6b).

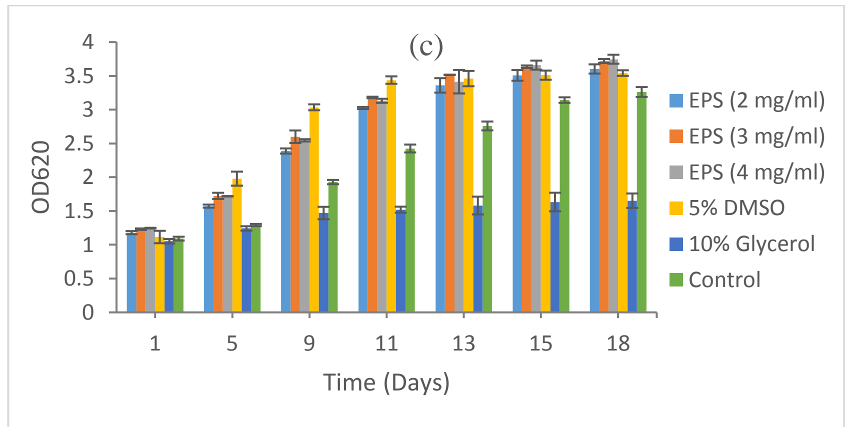
Biomass recovery after 9 months of cryopreservation again demonstrated the maximum growth in the treatment with 4 mg/ml EPS ($OD_{620}$ 3.75), 3 mg/ml ($OD_{620}$ 3.72) and 2 mg/ml EPS ($OD_{620}$ 3.60). The strain also recovered well in 5% DMSO ($OD_{620}$ 3.54) and even in the control ($OD_{620}$ 3.26). Poor recovery was observed in the 10% glycerol (Fig. 5.6c).











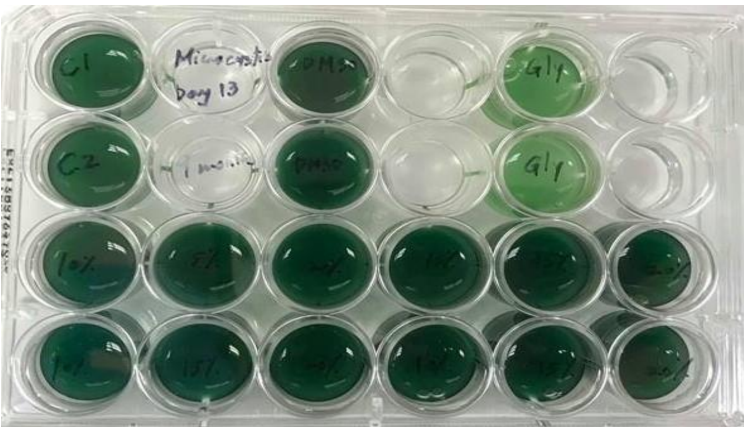



Figure 5.6. Growth recovery for *Microcystis aeruginosa* PCC 7806 after cryopreservation (a) 5 days, (b) 15 days, and (c) 9 months.

### 5.3.6. Growth recovery of microalga *Chlorella vulgaris*

*Chlorella vulgaris* clearly demonstrated the maximum recovery (after 5 days of cryopreservation) in presence of 5% DMSO ($OD_{680}$ 3.32) as a cryoprotective agent (CPA) followed by 10% glycerol ($OD_{680}$ 1.95) and control ($OD_{680}$ 1.67) after 18 days of incubation at optimum conditions. Poor recovery resulted for all concentrations of EPS used (Fig. 5.7a). The optical density results presented here are over a period of 18 days of incubation under optimum conditions.

15 days cryopreservation results again demonstrated 5% DMSO ($OD_{680}$ 3.26) as the choice of cryoprotective agent for this strain. Recovery was better in the control than the treatments containing EPS as cryoprotective agent (Fig. 5.7b). 5% DMSO worked well for the longer preservation of this strain.

After 9 months of cryopreservation, the maximum biomass was recovered from 5% DMSO ($OD_{680}$ 2.71), followed by 2 mg/ml EPS ($OD_{680}$ 2.13) and control ($OD_{680}$ 1.94). Recovery was low at all other concentrations of EPS and the glycerol (Fig. 5.7c). The optical density results presented here are over a period of 18 days of incubation at the optimum conditions.

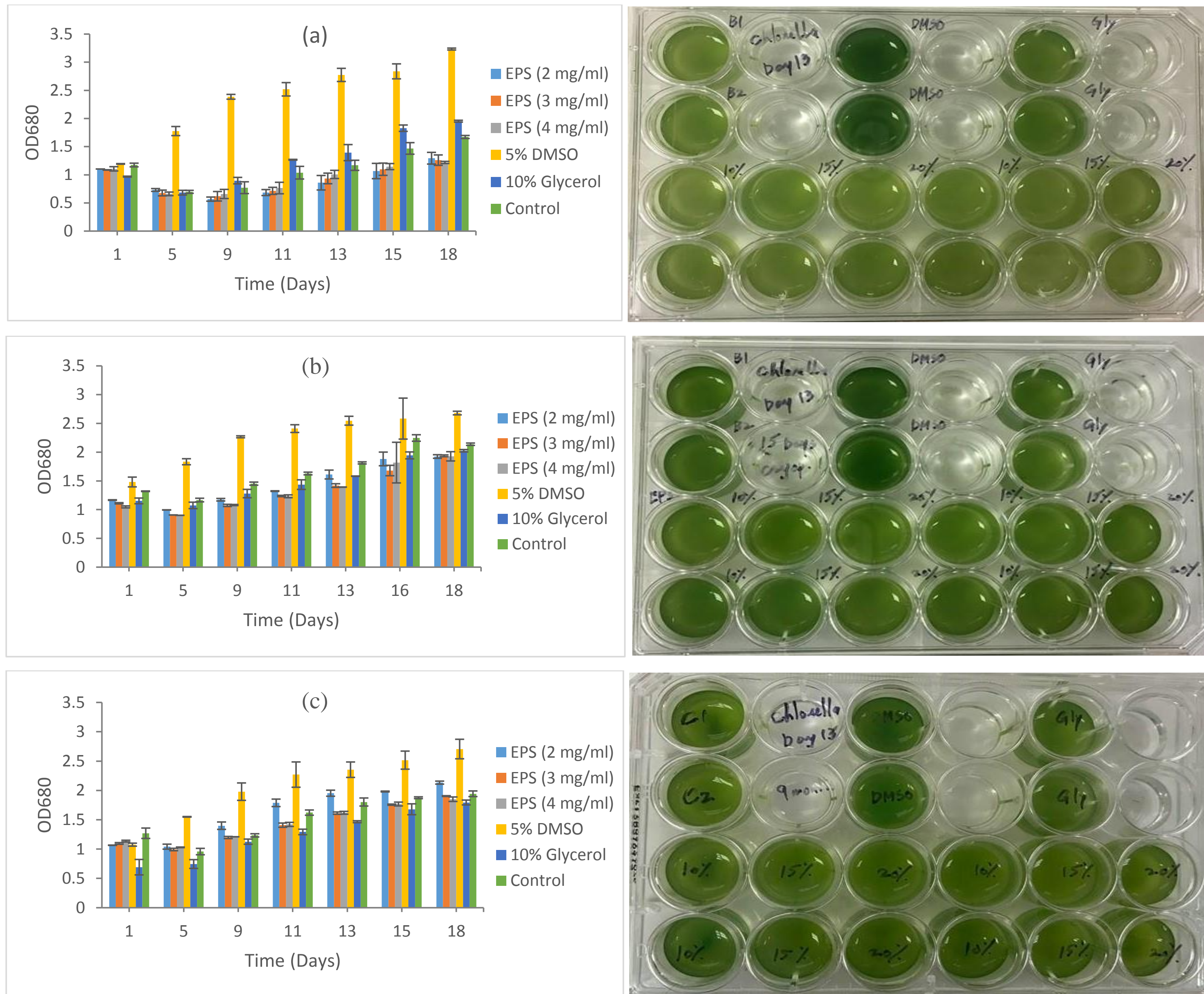



Figure 5.7. Growth recovery for *Chlorella vulgaris* after cryopreservation (a) 5 days, (b) 15 days, and (c) 9 months.

### 5.3.7. Effect of heat sterilization on the cryoprotective activity of the EPS

The autoclaving of EPS has no major effects on its cryoprotective activity for majority of the strains. However, in case of *Synechococcus* sp. CB0101, there was clear difference in biomass recovery in the treatments with autoclaved and non-autoclaved EPS. As discussed above, strain CB0101 demonstrated the maximum biomass recovery in the EPS while no recovery was recorded at 5% DMSO and 10% glycerol (Fig. 5.8). As clear, growth appears in the last three wells of last two rows represents non-autoclaved EPS (Fig. 5.5c). The maximum biomass recovery was recorded at 4 mg/ml non-autoclaved EPS ($OD_{435}$ 2.26). Comparatively biomass recovery was low in presence of autoclaved EPS as the cryoprotective agent (Fig. 5.8).

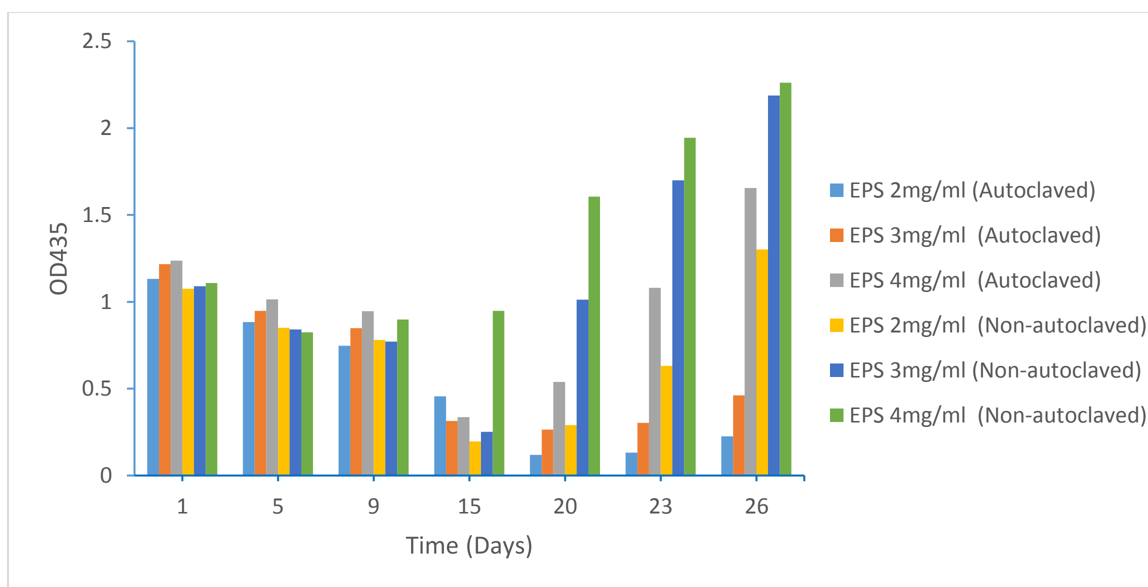



Figure 5.8. Growth recovery for *Synechococcus* sp. CB0101 after 9 months of cryopreservation in autoclaved and non-autoclaved EPS as the cryoprotective agent.

## 5.4. Discussion

Strain BGI-2 was isolated among many other bacteria from the ice of Batura glacier. The unique feature of this strain was production of high yield of a cryoprotective EPS at low temperatures. EPSs are considered as stress molecules, which assists microorganisms to cope with the extremes of temperatures, high salinity and desiccation (Deming and Young, 2017; Tamaru et al., 2005). Previously, survivability of the EPS producer BGI-2 against a series of freeze thaw cycles were compared to two other non-EPS producing bacteria including *Rhodococcus* sp., BGI-11 isolated from the same environment and a mesophilic

*E.coli*. Survivability of EPS producing strain BGI-2 was significantly higher than BGI-11 and *E. coli*. The EPS in our study also provided significant cryoprotection to another bacterium (*E.coli* K12), which was comparable to 20% glycerol. This demonstrated the role of EPS in protecting the cells from damages caused by the freezing conditions and freeze thaw events. Several studies have demonstrated the possible cryoprotective role of EPS in these cold and icy environments (Casillo et al., 2017: Deming and Young, 2017; Liu et al., 2013a). In another study, EPS from an Antarctic *Pseudomonas* species was reported for its cryoprotective role in the producer strain itself and to another bacterium (Carrion et al., 2015).

EPS from cyanobacteria and microalgae have been previously reported for its cryoprotective role in producer strains in extremely cold environments (Tamaru et al., 2005; Aslam et al., 2012). Keeping these in mind, we used the possibility of employing EPS-BGI-2 as a cryoprotective agent (CPA) for long term cryopreservation of the photosynthetic microorganisms where this type of preservation is not always successful. And to the best of our knowledge, there is not a single report of using a bacterial EPS for cryopreservation of the photosynthetic microorganisms including eukaryotic microalgae and prokaryotic cyanobacteria. Preservation of microalgae and cyanobacteria is a key step in basic research or industrially important strains. DMSO and methanol, the two most commonly used CPAs are toxic to cells at room temperature (Zhang et al., 2005). These chemicals are also hazardous to humans when ingested, inhaled or contacted through skin. We therefore used the possibility of employing a natural polymer (bacterial EPS) for cryopreservation of the photosynthetic organisms.

Overall, EPS worked well as the cryoprotective agent for strains including *Scenedesmus* sp. HTB1, *Synechococcus* sp. CBW1003, *Synechococcus* sp. CB0101 and *Microcystis* sp. 7806 EPS. For all these strains growth recovery was better than 5% DMSO, a common cryoprotective agent (CPA) used for the cryopreservation of various cells. Overall, poor recovery was recorded when 10% glycerol was used as the cryoprotective agent. There are conflicting reports regarding the performance and choice of CPAs for cryopreservation of the photosynthetic organisms. Gaget et al., (2017) used different concentrations of

methanol, DMSO and glycerol for the cryopreservation of 196 cyanobacterial isolates and found 5% DMSO as the preferable choice of CPA for most of the strains. In our study, 5% DMSO (except for strain CB0101) also worked well for most of the strains along with the EPS. In another study, Esteves-Ferreira et al., (2013) used five microalgae and cyanobacterial strains for cryopreservation and found 10% glycerol as the most efficient CPA. Some studies have demonstrated effectiveness of CPAs when used in combinations rather than alone. Nakanishi et al., (2012) demonstrated 50% survivability of the 4 microalgal strains when a combination of CPAs (5% DMSO, 5% Ethylene glycol, 5% proline) were used. According to their findings, little or no cryoprotection was observed when these CPAs were used alone. Aray-Andrade et al., (2018) found DMSO-sucrose and glycerol as the effective cryoprotective agents while working with cryopreservation of two *Chlorella* and one *Scenedesmus* species.

In *Synechococcus* sp. CB0101, EPS was the only cryoprotective agent where growth was recovered after 9 months of cryopreservation, whereas no recovery resulted in DMSO and glycerol. Interestingly, growth recovery was significantly higher in the non-autoclaved EPS compared to the autoclaved EPS. This pattern of growth recovery was recorded at all the three duration of cryopreservation (5 days, 15 days and 9 months). Also for strain CB0101, higher concentration of EPS (4 mg/ml) worked well whereas 5% DMSO had toxic effect. Although 5% DMSO is the preferable CPA but has been previously reported for its cytotoxic effect to the susceptible cyanobacterial strains (Esteves-Ferreira et al., 2013). Other studies have also reported toxicity of DMSO in their studies (Hanslick et al., 2009; Wang et al., 2007). For the successful post thaw survival rate of microalgae and cyanobacteria, the type and concentration of cryoprotectants are very critical. In our study, EPS overall worked well for all the strains except *Chlorella vulgaris*. Likewise, 5% DMSO performed well as a CPA for most of the strains except *Synechococcus* sp. CB0101. Similarly, the same cryoprotectant with different concentrations had different growth recovery. It is therefore critical to test the type and concentration of cryoprotectants for each strain before doing the long-term cryopreservation. Choice of cryoprotective agent (CPA), viability and biomass recovery is very much strain dependent (Saadaoui et al., 2016).

Microbial EPSs are biopolymers or natural polymers and have advantages over the synthetic cryoprotective agents in common use today. Natural polymers are considered less toxic and more biodegradable compared to the synthetic or man-made compounds. Also EPS are large polymers and acts as non-penetrating CPAs and these types of CPAs such as trehalose are considered less toxic than those, which penetrates through the membrane into the cells (Wen et al., 2016). Penetrating CPAs that cross cell membranes namely, ethylene glycol (EG), propylene glycol, dimethylsulfoxide (DMSO), glycerol and methanol have been reported for their cytotoxic activity (Best, 2005). The cytotoxic effects of various cryoprotectants to the flounder embryos found ethylene glycol with the most toxicity followed by glycerol and DMSO (Zhang et al., 2005).

The producer strain BGI-2 is a psychrotrophic bacterium capable of growing at low temperatures. This strain gives high yield of EPS at low temperatures, which negates the expensive heating steps required for working with mesophilic strains. Working at low temperatures conserve energy, minimizes contamination with other microorganisms and undesirable chemical reactions. In spite of the enormous biotechnological potential, only a handful of the bacterial EPS have been successfully commercialized. The major hindrance is the cost of production which can be overcome by successfully employing a number of measures including the use of cheaper substrate, optimizing fermentation conditions to improve product yield, improving the producer strain through mutagenesis, genetic and metabolic manipulations to enhance the productivity, improving downstream processing which involves extraction and purification.

### 5.5. Conclusions

Still many labs in the world uses sub-culturing as a method of choice for the maintenance of the cyanobacterial and microalga cultures, which has many pitfalls as discussed before. This study will contribute a step further in the cryopreservation of these photosynthetic microorganisms used in the field of basic research and applied biology. The concentration of EPS used as a CPA is critical depending on the sensitivity of the particular strain. Most of the strains demonstrated good recovery at the higher concentration of the EPS used. Therefore, more tests with increased EPS concentration will further improve biomass

recovery. Further research can pave the way for this natural polymer to replace the existing toxic chemicals in use today as the cryoprotective agents.

# Chapter 6

# Chapter 6

## Draft genome sequence of cold-adapted *Pseudomonas* sp. BGI-2 isolated from the ice of Batura glacier, Pakistan

## Abstract

*Pseudomonas* sp. BGI-2 is a psychrotrophic bacterium isolated from the ice of Batura glacier in the Karakoram mountain range. This strain produces high yield of exopolysaccharide (EPS) at low temperature and exhibits high freeze-thaw tolerance. Also increased survivability of *Escherichia coli* in presence of EPS-BGI-2 suggests its role in cryoprotection. Draft genome of BGI-2 consists of 6.3-Mb with a mean G+C content of 58.9%. The genome contains 11 EPS-producing genes, which are not found in the 6 closely related mesophilic *Pseudomonas* strains. Draft genome sequence data also revealed more stress response genes in BGI-2 than the closely related mesophilic counterparts. The stress response genes included cold shock, osmotic stress, oxidative stress, detoxification and carbon starvation. The genome also contains genes involved in cold adaptation, including desaturases for maintenance of membrane fluidity, production and uptake of compatible solutes, and production of exopolymers.

**Keywords:** Karakoram, Glacier, Cold-adapted bacteria, Exopolysaccharide (EPS), Psychrophiles

## 6.1. Introduction

Cold habitats have been successfully colonized by microorganisms known as psychrophiles or psychrotrophs, making it the most abundant extremophiles in terms of diversity, distribution and biomass (Struvay and Feller, 2012). Psychrophiles have developed a number of strategies to successfully colonize and thrive in the harsh cold environments (Tribelli and López, 2018; Rodrigues and Tiedje, 2008). These adaptive strategies are not limited to counter harmful effects of the cold rather a number of many other stresses associated with low temperature environments. These stressful conditions includes, high UV radiation, desiccation (low water availability), oligotrophic condition (low nutrient availability), increased salinity and osmotic pressure (D'amico et al., 2006; Margesin and Miteva, 2011; De Maayer et al., 2014). Their successful colonization of the harsh cold environments is a result of the molecular evolution and adaptations (Casanueva et al., 2010).

Molecular evolution and adaptations have resulted in the ability of psychrophilic microorganisms to better survive and thrive in cold environments. Recently, few studies have revealed deep understanding regarding molecular conformity at subzero temperatures using genomic, transcriptomic and proteomic investigations (Bowman, 2017; Koh et al., 2017; Nunn et al., 2015). Over a hundred genomes have been obtained from psychrophiles with representation from major taxonomic groups (Bowman, 2017). Metagenomic studies from cold environments in the past decade has greatly increased resulting in more than thousand metagenome data available in the publicly available databases (Aliyu et al., 2017). These metagenomic data have contributed significantly in our understanding of the role of bacterial communities in cold environments. Also these data are very useful in our understanding of cold adaptation and ecological role played by the indigenous microorganisms (Aliyu et al., 2017; Boetius et al., 2015).

Majority of the sequenced data from psychrophiles is originated from the marine environments in the polar-regions. The availability of a large number of genomic data from psychrophiles over the past decade has resulted in better understanding of the cold adaptation (Bowman, 2017). Comparative genome analysis of psychrophiles with their

mesophilic counterparts have resulted in identification of genes which are absent in the later temperature spectrum group. Genomic based cold-adaptation studies involves investigations of genes responsible for a variety of processes that are indispensable for growth at low temperature. These includes genes responsible for unsaturation of the membrane fatty acids (increased membrane fluidity), uptake and synthesis of small organic molecules (osmoprotection and cryoprotection) and production of exopolymers (cryoprotection) (Zhang et al., 2018b; Casanueva et al., 2010). Majority of the sequenced genome from psychrophiles are from the marine environments and less from the glaciers particularly in the non-polar region. A keyword search for "Bacteria" and "glaciers" against the GOLD database (Pagani et al., 2012) shows that only 4 complete or draft psychrophilic bacterial genomes from glaciers have been sequenced to date. These bacteria included *Chryseobacterium glaciei* IHBB 10212, *Sphingomonas glacialis* DSM 22294, *Paenisporosarcina* sp. TG20 and *Pseudomonas* sp. PAMC 25886. There is a need to sequence psychrophiles from the major cold environments all over the world and find what genes these organisms share for their successful colonization of these inhospitable environments.

Unicellular microorganisms such as bacteria are directly exposed to the external environments and therefore the cellular exterior is very critical in its defense against the harsh environmental conditions including the cold. EPS production in cold environments is considered as one key adaptation to withstand the damage caused by the freezing conditions (Deming and Young, 2017; Casillo et al., 2017; Liu et al., 2013a; Carrion et al., 2015; Aslam et al., 2015). The EPS also serves some other key functions including, nutrient uptake, addition to surfaces (biotic and abiotic), protection of extracellular enzymes and most importantly in biofilm formation (Qin et al., 2007; Janczarek et al., 2015; Limoli et al., 2015). The genetic basis of EPS production involves studying genes responsible for regulation, polymerization and export of EPS to the cellular exterior. The EPS biosynthesis is dependent on a number of factors such as presence of a suitable carbon source, which is majorly a sugar. It also depends on the stress conditions, as a number of studies have reported maximum EPS production at the sub optimal conditions (Nichols et al., 2005a; Marx et al., 2009a). Although a large number of microbial EPSs have been

recently reported but the genetic data is still limited (Donot et al., 2012). Production of EPS in microorganisms involves four group of enzymes which includes hexokinases, UDP-glucose pyrophosphorylase, glycosyltransferases (GFTs), and enzymes involved in the modifications of the EPS such as acetylation, sulphation and acylation. (Mishra and Jha, 2013; Kumar et al., 2007).

*Pseudomonas* is a genus of the *Gammaproteobacteria* known for its metabolic versatility and ability to inhabit diverse environments including the extremes (Peix et al., 2018). Cold-adapted *Pseudomonas* species have been isolated from different cold environments including polar and non-polar regions (Jang et al., 2012; Vasquez-Ponce et al., 2017). *Pseudomonas* sp. BGI-2 was isolated from the ice of Batura glacier using R2A medium (BD Difco™). Briefly, the ice sample was enriched in R2A broth as direct plating of the sample did not yield any colonies. The enriched culture was plated on R2A agar plates and incubated at 15˚C. Colonies of BGI-2 appeared in the first week and was re-streaked on separate plates to get pure culture. The strain is a halotolerant with a wide growth ranges for temperature (4 – 35˚C) and pH (5 – 11). The unique features of this strain are the production of a cryoprotective EPS at low temperatures and its resilience to freeze-thaw events. In this paper, comparative genomic data between the cold-adapted BGI-2 and closely related mesophilic *Pseudomonas* species is presented. The results revealed presence of more stress response genes in BGI-2 compared to their mesophilic counterparts. These included genes for cold shock, osmotic stress, oxidative stress, detoxification and carbon starvation. Interestingly, the genome of BGI-2 has 11 EPS-producing genes, which are not found in the closely mesophilic *Pseudomonas* strains. All these observations resulted in drawing the conclusion that EPS from BGI-2 likely contributes to its ability to survive in the harsh glacier environment.

### 6.2. Material and Methods

#### 6.2.1. Extraction of DNA

A pure culture of *Pseudomonas* sp. BGI-2 was grown in R2A broth at 15˚C and the genomic DNA was extracted from an overnight culture using UltraClean microbial DNA

isolation kit (MO BIO Laboratories). Concentration and purity of the extracted DNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

### 6.2.2. Sequencing of DNA

The DNA was sequenced by using Illumina MiSeq sequencing in BioAnalytical Services *Laboratory* (*BAS Lab.*), Institute of Marine and Environment Technology (IMET), University of Maryland Center for Environmental Sciences (UMCES). Library was prepared using Nextera XT DNA library prep kit (Illumina Inc., San Diego, CA) according to the manufacturer's protocol and sequencing was performed in a MiSeq 2 × 250-bp run.

### 6.2.3. Processing of the NGS data

Raw reads were processed for quality trimming and adapter removal using Trimmomatic-0.33 (Bolger et al., 2014). De novo assembly of the processed reads was performed using SPAdes v.3.10.0 (Nurk et al., 2013) with default settings.

### 6.2.4. Genome annotation

Rapid Annotations using Subsystems Technology (RAST v 2.0) server (Aziz et al., 2008) and the NCBI Prokaryotic Genome Annotation pipe line (PGAP v 4.7) (Tatusova et al., 2016) were used for the annotation of the assembled contigs to identify genes.

### 6.2.5. Overall genome relatedness indices (OGRI)

#### 6.2.5.1. Digital DNA-DNA hybridizations (dDDH)

Overall genome relatedness indices (OGRI) (Auch et al., 2010) between BGI-2 and the 7 most closely related mesophilic *Pseudomonas* species (Table 6.2) and between BGI-2 and 6 psychrophilic/psychrotrophic *Pseudomonas* species (Table 6.3) were calculated. Digital DNA-DNA hybridizations (dDDH) were determined online using the Genome-to-Genome Distance Calculator (GGDC) (Meier-Kolthoff et al., 2013). The estimated DDH values were calculated using formula two at the GGDC website (Auch et al., 2010; Meier-Kolthoff et al., 2013).

#### 6.2.5.2. Average nucleotide identity (ANI)

Average nucleotide identity (ANI) was calculated using the server-based software EzBioCloud (Yoon et al., 2017b). The proposed ANI and dDDH values for species boundary are 95-96% and 70%, respectively (Richter and Rosselló-Móra, 2009; Goris et al., 2007).

### 6.2.6. Genome Comparison of BGI-2 with mesophilic *Pseudomonas* species

Six closely related *Pseudomonas* species were chosen as mesophilic reference genomes for comparison with the cold-adapted *Pseudomonas* sp. BGI-2. These mesophilic strains included *Pseudomonas caspiana* FBF102, *Pseudomonas amygdali* CFBP 3205, *Pseudomonas cerasi* 58, *Pseudomonas congelans* DSM 14939, *Pseudomonas syringae* KCTC 12500, and *Pseudomonas fluorescens* DSM 50090. Complete genome sequence of these strains was downloaded from the National Center for Biotechnology Information (NCBI) database and included in the comparative genome analysis. These sequences were submitted to Rapid Annotation Subsystem Technology (RAST) for protein coding genes annotation.

### 6.2.7. Accession number(s)

The draft genome sequence has been deposited in the NCBI GenBank under the accession number SISB00000000, 16S rRNA gene sequence accession number MH681214, BioProject number PRJNA523205 and BioSample number SAMN10966221. The raw reads have been deposited at the NCBI Sequence Read Archive (SRA) with the accession number SRR8715451.

## 6.3. Results

### 6.3.1. General genome features

The draft genome sequence of *Pseudomonas* sp. BGI-2 consists of 6,267,352 bp with a G+C content of 58.9%. Assembly of the processed reads yielded 106 contigs. Annotation of the assembled contigs resulted in the identification of total 6015 genes; 5566 protein-coding genes, 73 RNA, 60 tRNA and 376 pseudogenes. Also some of the features of the

genome is given in (Table 6.1). Annotation results revealed almost 51% genes with unknown or predicted functions (Fig. 6.1).

### 6.3.2. Digital DNA-DNA Hybridization (dDDH) and Average Nucleotide Identity (ANI)

Overall the dDDH and ANI values were low for the closely related mesophilic *Pseudomonas* strains (Table 6.2) compared to the cold-adapted *Pseudomonas* strains (Table 6.3). *Pseudomonas* sp. BGI-2 was most closely related to the cold-adapted *Pseudomonas mandelii* JR-1 with the ANI and dDDH values of 91.89% and 46.2%, respectively (Table 6.3). These values are below the accepted threshold for species demarcation suggesting that *Pseudomonas* sp. BGI-2 could be a novel species in the genus *Pseudomonas*.

Table 6.1. Genomic features of the *Pseudomonas* sp. BGI-2.

| **Genomic Features** | **Numbers** |
|---|---|
| CDS (Coding sequence) | 6022 |
| Repeat region | 299 |
| tRNA | 60 |
| rRNA | 10 |
| Hypothetical proteins | 1485 |
| Proteins with functional assignments | 4537 |
| Proteins with EC number assignments | 1257 |
| Proteins with GO assignments | 1096 |
| Proteins with pathway assignments | 943 |

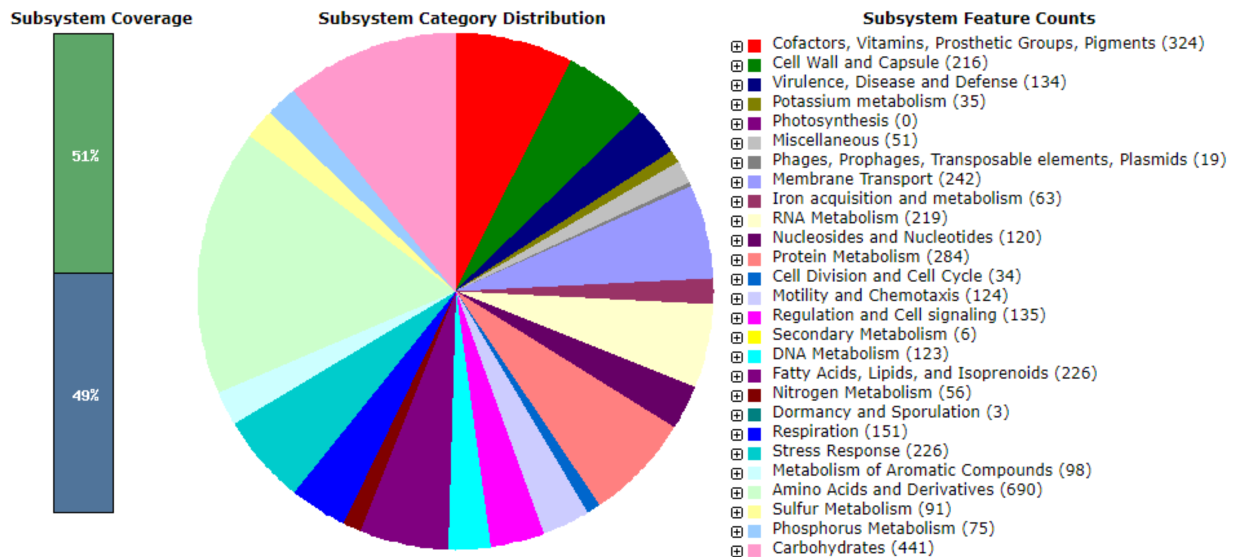



Figure 6.1. Subsystem coverage and distribution of BGI-2 genome using Rapid Annotation Subsystem Technology (RAST).

Table 6.2. dDDH and ANI values for *Pseudomonas* sp. BGI-2 compared with closely related mesophilic *Pseudomonas* species based on the 16S rRNA gene sequence similarity from EzBioCloud.

| **Bacteria** | **GenBank Accession Number** | **16S rRNA Gene Similarity (%)** | **dDDH (%)** | **ANI (%)** |
|---|---|---|---|---|
| *Pseudomonas caspiana* FBF102 | LOHF00000000 | 99.45 | 22.7 | 77.77 |
| *Pseudomonas amygdali* CFBP 3205 | JYHB00000000 | 99.32 | 22.6 | 77.99 |
| *Pseudomonas cerasi* 58 | LT222319 | 99.18 | 22.6 | 78.20 |
| *Pseudomonas congelans* DSM 14939 | FNJH00000000 | 99.18 | 22.5 | 78.19 |
| *Pseudomonas kilonensis* DSM 13647 | LHVH00000000 | 99.18 | 27.3 | 83.13 |
| *Pseudomonas syringae* KCTC 12500 | AYTM00000000 | 99.11 | 22.7 | 77.98 |
| *Pseudomonas fluorescens* DSM 50090 | LHVP00000000 | 98.56 | 24.8 | 81.10 |

Table 6.3. dDDH and ANI values for *Pseudomonas* sp. BGI-2 compared with other *Pseudomonas* species isolated from the cold environments.

| **Bacteria** | **GenBank Accession Number** | **dDDH (%)** | **ANI (%)** |
|---|---|---|---|
| *Pseudomonas antarctica* PAMC 27494 | CP015600 | 25.1 | 81.21 |
| *Pseudomonas frederiksbergensis* ERDD5:01 | CP017886 | 29.1 | 84.31 |
| *Pseudomonas mandelii* JR-1 | CP005960 | 46.2 | 91.89 |
| *Pseudomonas psychrophila* HA-4 | ALJC00000000 | 23.3 | 78.80 |
| *Pseudomonas putida* ATH-43 | LBME00000000 | 21.8 | 77.17 |
| *Pseudomonas* sp. PAMC 25886 | AHHC00000000 | 25.8 | 81.95 |

### 6.3.3. Stress response genes

In comparison to the 6 mesophilic *Pseudomonas* species, BGI-2 contains more stress response genes (Table 6.4). The genes for stress response included osmotic stress, oxidative stress, cold -shock, detoxification and carbon starvation. The number of osmotic stress and oxidative stress genes were significantly higher in BGI-2 compared to their mesophilic counterparts (Table 6.4).

The osmotic stress response genes included osmoregulation, synthesis of osmoregulated periplasmic glucans, coline and betaine uptake and betaine biosynthesis. BGI-2 has 5 genes for osmoregulation compared to 2 in the mesophilic *Pseudomonas* strains (Table 6.5). Similarly number of genes for uptake and biosynthesis of betaine were higher in the genome of BGI-2.

Overall *Pseudomonas* sp. BGI-2 has more genes for metabolism of various nutrients and aromatic compounds than the mesophilic *Pseudomonas* species (Table 6.6). These include genes for metabolism of potassium, phosphorus, nitrogen, DNA, and aromatic compounds. Also number of genes for polyhydroxybutyrate metabolism were significantly higher in BGI-2 compared to the mesophilic *Pseudomonas* strains (Table 6.6).

Table 6.4. Stress response genes comparison of cold-adapted *Pseudomonas* sp. BGI-2 with closely related mesophilic *Pseudomonas* species.

| **Bacteria** | **GenBank Accession Number** | **Stress Response (Total Genes)** | **Osmotic Stress** | **Oxidative Stress** | **Cold-Shock** | **Detoxifica-tion** | **Carbon Starvation** |
|---|---|---|---|---|---|---|---|
| *Pseudomonas* sp. BGI-2 | SISB00000000 | 226 | 38 | 104 | 6 | 27 | 7 |
| *Pseudomonas caspiana* FBF 102 | LOHF00000000 | 212 | 31 | 101 | 5 | 25 | 8 |
| *Pseudomonas amygdali* CFBP 3205 | JYHB00000000 | 188 | 33 | 82 | 5 | 25 | 6 |
| *Pseudomonas cerasi* 58 | LT222319 | 182 | 32 | 82 | 5 | 25 | 6 |
| *Pseudomonas congelans* DSM 14939 | FNJH00000000 | 183 | 30 | 84 | 5 | 25 | 6 |
| *Pseudomonas syringae* KCTC 12500 | AYTM00000000 | 179 | 32 | 79 | 5 | 25 | 6 |
| *Pseudomonas fluorescens* DSM 50090 | LHVP00000000 | 207 | 33 | 93 | 7 | 28 | 6 |

Table 6.5. Osmotic stress response genes comparison of cold-adapted *Pseudomonas* sp. BGI-2 with closely related mesophilic *Pseudomonas* species.

| **Bacteria** | **GenBank Accession Number** | **Osmotic Stress (Total Genes)** | **Osmo-regulation** | **Synthesis of Osmoregulated Periplasmic Glucans** | **Coline and Betaine Uptake and Betaine Biosynthesis** |
|---|---|---|---|---|---|
| *Pseudomonas* sp. BGI-2 | SISB00000000 | 38 | 5 | 5 | 28 |
| *Pseudomonas caspiana* FBF 102 | LOHF00000000 | 31 | 3 | 4 | 24 |
| *Pseudomonas amygdali* CFBP 3205 | JYHB00000000 | 33 | 2 | 5 | 26 |
| *Pseudomonas cerasi* 58 | LT222319 | 32 | 2 | 4 | 26 |
| *Pseudomonas congelans* DSM14939 | FNJH00000000 | 30 | 2 | 4 | 24 |
| *Pseudomonas syringae* KCTC 12500 | AYTM00000000 | 32 | 2 | 4 | 25 |
| *Pseudomonas fluorescens* DSM50090 | LHVP00000000 | 33 | 2 | 4 | 27 |

Table 6.6. Comparison of genes involved in metabolism of various nutrients and aromatic compounds between the cold-adapted *Pseudomonas* sp. BGI-2 and mesophilic *Pseudomonas* species.

| **Bacteria** | **Respiration** | **Potassium Metabolism** | **Phosphorus Metabolism** | **Nitrogen Metabolism** | **DNA Metabolism** | **PHB Metabolism** | **Metabolism of Aromatic carbon** |
|---|---|---|---|---|---|---|---|
| *Pseudomonas* sp. BGI-2 | 151 | 35 | 75 | 56 | 123 | 44 | 98 |
| *Pseudomonas caspiana* FBF 102 | 122 | 25 | 52 | 48 | 115 | 27 | 84 |
| *Pseudomonas amygdali* CFBP 3205 | 101 | 23 | 52 | 48 | 118 | 19 | 82 |
| *Pseudomonas cerasi* 58 | 106 | 25 | 52 | 51 | 139 | 18 | 70 |
| *Pseudomonas congelans* DSM 14939 | 105 | 24 | 53 | 49 | 110 | 15 | 70 |
| *Pseudomonas syringae* KCTC 12500 | 107 | 23 | 60 | 53 | 116 | 17 | 70 |
| *Pseudomonas fluorescens* DSM 50090 | 125 | 30 | 89 | 50 | 117 | 39 | 114 |

### 6.3.4. EPS producing genes

Interestingly, 11 EPS-producing genes were identified in the BG1-2 genome, while none of the 6 mesophilic *Pseudomonas* species contain these genes. The EPS genes included *eps*E (Undecaprenyl-phosphate galactosephosphotransferase), *cps*A (Capsular polysaccharide synthesis enzyme CpsA, sugar transferase), *cps*B (Capsular polysaccharide synthesis enzyme CpsB), *cps*C (Capsular polysaccharide synthesis enzyme CpsC, polysaccharide export), *cps*D (Capsular polysaccharide synthesis enzyme CpsD, exopolysaccharide synthesis), 3 genes of each *glt*1 (Glycosyl transferase, group 1 family protein) and *glt*2 (Glycosyl transferase, group 2 family protein).

## 6.4. Discussion

The draft genome of *Pseudomonas* sp. BGI-2 gives an insight into the psychrophilic lifestyle adapted by this strain to successfully colonize the glacier ice. Based on the whole genome comparison, *Pseudomonas* sp. BGI-2 was most closely related to the cold-adapted *Pseudomonas mandelii* JR-1 (Jang et al., 2012) with the ANI and dDDH values of 91.89% and 46.2%, respectively. BGI-2 shared genome relatedness more with the psychrophiles (Table 6.3) than the closely related mesophilic *Pseudomonas* strains (Table 2). Also, the proposed ANI and dDDH values for species boundary are 95-96% and 70%, respectively (Richter and Rosselló-Móra, 2009; Gorris et al., 2007). These values are below the accepted threshold for species demarcation suggesting that *Pseudomonas* sp. BGI-2 could be a novel species in the genus *Pseudomonas*. It is well established now that all the phenotypic characteristics of a bacterium used for its taxonomy is actually present in the nucleotide sequence of its genome. Therefore, relatedness between strains based on their genetic material is emerging as a powerful tool for classification of bacteria and other microorganisms. One of these tools is DNA–DNA hybridization (DDH) used by taxonomists for a long time as the gold standards for bacterial species delineation. Another method, which is relatively new is the average nucleotide identity (ANI) that measures genetic relatedness of the coding sequences in two or multiple genomes.

The genome analysis of BGI-2 revealed presence of a large number of genes involved in carbon metabolism and nutrient cycling. The BGI-2 genome has more genes for

metabolism of potassium, nitrogen and phosphorus than the mesophilic *Pseudomonas* stains (Table 6.6). Also, the number of genes for metabolism of aromatic carbons were significantly higher in BGI-2. Similarly, genome of strain BGI-2 contain more stress response genes than the mesophilic strains including genes for cold-shock, osmotic stress, oxidative stress and detoxification. Genes involved in cold-adaptation such as desaturases and synthesis and uptake of compatible solutes were also present in the BGI-2 genome. Genomics and metagenomics data analysis from various cold environments have revealed presence of a large number of genes involved in psychrophily. These included genes involved in maintenance of membrane fluidity, production and uptake of compatible solute, free radical scavenging system, production of cold-active enzymes and exopolymers. Genome comparison of the psychrophilic *Psychroflexus torquis* with a closely related mesophilic counterpart revealed presence of genes responsible for polyunsaturated fatty acid (PUFA) and exopolysaccharide (EPS) biosynthesis (Feng et al., 2014). The genes involved in cold-adaptation were mostly absent in the non-psychrophilic species. In another study, genome of the sea ice psychrophilic *Psychromonas ingrahamii* was investigated and resulted in the identification of a large number of cyclic GDP regulators, suggesting production of extracellular polysaccharide. The EPS might play a role in cold-adaptation by depressing the freezing point of water around the cell. This cold-adapted bacterium also possessed genes involved in synthesis of the osmolyte betaine choline (Riley et al., 2008).

Genome analysis of a true psychrophilic bacterium *Colwellia psychrerythraea* 34H revealed presence of multiple genes involved in successful colonization of the permanently cold environment (Methe et al., 2005). The genome of strain 34H contained multiple genes involved in the synthesis and release of exopolymeric substances. The role of exopolymers in cold-adaptation has been discussed in detail earlier. Genes involved in the uptake of compatible solutes were also recovered which play key role in osmoprotection and cryoprotection. Finally, genes involved in the maintenance of membrane fluidity (unsaturation of fatty acids) were also present (Methe et al., 2005). In a recent study, cold-adaptation based on genome analysis in two psychrotolerant *Colwellia* species was investigated (Zhang et al., 2018a). The study found genes responsible for cold-adaptation

including, uptake of compatible solutes and cold-shock proteins. Genes responsible for increasing membrane fluidity (Desaturases) at low temperature were also detected.

*Pseudomonas* sp. BGI-2 genome has 11 EPS-producing genes, which are not found in the closely related mesophilic counterparts. Previously we established high freeze thaw tolerance in BGI-2 compared to the non-EPS producing strains. Also the EPS from BGI-2 helped an *E. coli* strain from the freezing damage. Survivability of *E. coli* in 5% EPS was comparable to 20% glycerol after repeated freeze thaw cycles. Production of exopolysaccharide by microorganisms is considered as an adaptation to survive the freezing cold environments (Deming and Young, 2017; Carrion et al., 2015; Aslam et al., 2012). Since EPSs have high polyhydroxyl content, which is thought to depresses the freezing point and ice nucleation temperature of water. Recently, EPSs were reported for the improvement of the freeze thaw survivability in bacteria isolated from Antarctic sponge (Caruso et al., 2018). Similarly, EPS extracted from a cold-adapted bacterium *Colwellia psychrerythraea* exhibited cryoprotection by causing modifications in the desalination and microstructure of the growing ice resulting in an increase in the ice crystal disorder. Thus reduction of the ice permeability results in salt retention. Therefore, EPS might play a role by minimizing ice growth, which is essential for the successful colonization and survival of the microorganisms in sea ice and other parts of the cryosphere. (Ewert and Deming, 2011; Krembs et al., 2011). Bacterial EPS with cryoprotective activity have been reported previously from *Colwellia psychrerythraea* 34H (Marx et al., 2009), *Pseudomonas* sp. ID1 (Carrion et al., 2015) and *Pseudoalteromonas* sp. SM20310 (Liu et al., 2013a).

Another feature of the BGI-2 genome was presence of genes involved in unsaturation of the membrane fatty acids such as fatty acid desaturase (EC 1.14.19.1) and sterol desaturase. One of the challenges encountered by singled cell microorganism at low temperature is decrease in cytoplasmic membrane fluidity, which severely affects transportation of nutrients across the membrane. Therefore, psychrophiles have successfully overcome this challenge by incorporating short chain unsaturated fatty acids (De Maayer et al., 2014). The presence of multiple genes for fatty acid desaturases have been reported from the cold-adapted bacteria (Rong et al., 2019; De Freitas et al., 2017). The desaturases are

essential for growth at low temperatures as they also provide protection against increased concentrations of reactive oxygen species. Genes involved in isoprenoid biosynthesis were also found in the genome of strain BGI-2. These genes included, Undecaprenyl diphosphate synthase (EC 2.5.1.31) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (EC 1.1.1.267). The role of isoprenoid in maintenance of membrane fluidity at low temperature has been reported previously (Seel et al., 2018).

Accumulation of compatible solutes is a strategy employed by microorganisms particularly in hypersaline and cold environments where they are majorly involved in osmoprotection and cryoprotection. Some of the well-studied compatible solutes includes trehalose, mannitol, ectoine, betaine, glycine, choline, sucrose and sorbitol. At extremely low temperature, these highly poly-hydroxylated compounds lowers the freezing point of cytoplasmic water and also thought to play role in the stability of the cellular macromolecules such as the enzymes (Torstensson et al., 2019; Miladi et al., 2017; Methe et al., 2005; Collins and Deming, 2013). Comparative genomics revealed more osmotic stress response genes in BGI-2 compared to the closely related mesophilic counterparts. Furthermore, genome mining of BGI-2 revealed presence of genes responsible for trehalose biosynthesis such as trehalose synthase (EC 5.4.99.16), trehalose-6-phosphate hydrolase (EC 3.2.1.93), and Malto-oligosyltrehalose synthase (EC 5.4.99.15). Genes for choline and betaine uptake and betaine biosynthesis including choline dehydrogenase (EC 1.1.99.1), phosphatidylcholine synthase (EC 2.7.8.24), betaine aldehyde dehydrogenase (EC 1.2.1.8), and L-Proline/Glycine betaine transporter ProP, L-proline glycine betaine ABC transport system permease protein ProW (TC 3.A.1.12.1) were also present.

Finally, genome study of the BGI-2 demonstrated its potential role in bioremediation as number of genes for catabolism of aromatic compounds were significantly higher than the mesophilic *Pseudomonas* strains. *Pseudomonas* species from cold environments have been reported for their ability to efficiently degrade petroleum hydrocarbons (Wang et al., 2016; Ma et al., 2006).

## 6.5. Conclusion

We found evidence for the presence of many cold-adaptation genes in the genome of the cold-adapted *Pseudomonas* sp. BGI-2. Genomic comparison revealed more stress response genes in the genome of BGI-2 compared to the mesophilic *Pseudomonas* species. Some of these stress response genes such as genes for osmotic and oxidative stress are essential for successful colonization of the cold environments. Interestingly, BGI-2 has 11 EPS producing genes compared to none in their mesophilic counterparts. Thus EPS production by the BGI-2 strain likely contributes in the successful colonization of the glacier ice. As previously reported, production of EPS seems to be an adaptive strategy employed by the microorganisms to colonize the extreme environments, including the cryosphere. In-depth study of the genomic data further will help better understand the molecular basis of cold adaptation.

# Chapter 7

# Chapter 7

## Cold-adapted halotolerant *Rhodococcus* sp. BGI-11, a potential candidate for bioremediation of petroleum hydrocarbons in deep oceans

**Abstract**

*Rhodococcus* sp. BGI-11 was isolated among many other psychrotrophic bacteria from the ice of Batura glacier in the mighty Karakoram Range. Phylogenetic analysis using 16S rRNA gene revealed the strain is most closely related to *Rhodococcus* erythropolis with a similarity index of 99.76%. BGI-11 was able to grow at a temperature range of 4-35°C, pH 4-11 and high salinity up to 8% (w/v). Results for carbon utilization revealed the strain is able to use glucose, sucrose, maltose, galactose and lactose sugars. This strain also demonstrated the ability to use glycerol and molasses as growth substrates. The strain demonstrated growth in all the 8 culture media tested including, nutrient rich and the selective media. Antimicrobial activity was checked against multidrug resistant Gram-positive, Gram –negative bacteria, and a yeast. BGI-11 exhibited antimicrobial activity against *Bacillus* sp. and multidrug resistant *Candida albicans*. This strain also demonstrated positive activity for lipase and DNase enzymes. Antibiotic susceptibility pattern revealed the strain is resistant to methicillin and trimethoprim/sulfamethxazole and sensitive to imipenem, vancomycin, and ofloxacin. The most exciting physiological characteristic exhibited by this strain was its ability to use long chain hydrocarbon hexadecane as a carbon substrate. Thus, the cold-adapted halotolerant *Rhodococcus* sp. BGI-11 can be used as a potential candidate for bioremediation purpose in Deep Ocean where accidental oil spills are menace to the biodiversity.

**Keywords:** Batura glacier, Cold-adapted bacteria, Halotolerant, Enzymes, Antibiotic resistance

## 7.1. Introduction

Among the extreme environments on the earth, low temperature contributes the majority. Around 85% of the earth is permanently or periodically exposed to temperatures below 5˚C. Deep oceans makes a large part of this cold environments as 71% of the earth is covered by ocean and 90% of its volume is permanently below 5˚C, followed by Arctic and Antarctic polar regions, the high mountains and cave system (Margesin and Miteva, 2011). These extreme environments have been successfully inhibited by bacteria and other microorganisms, which not only survive but actively metabolize at such low temperatures.

The ability of bacteria to grow at low temperatures or freezing conditions have been known long ago. Since significant part of the Earth's biosphere is permanently exposed to low temperatures (<5˚C), therefore it is obvious that psychrophiles play critical role in global ecology. Current definitions of the cold-adapted microorganisms is provided by Morita (1975). He defined true psychrophiles as bacteria that grow near the freezing point of water, has an optimal temperature for growth near 15˚C or below and cannot grow above 20˚C. Psychrotrophs, although able to grow near the freezing point of water but have wide growth temperature range, with the optimum and maximum temperature lies above 20˚C (Morita, 1975). The psychrotrophs are phylogenetically diverse as compared to true psychrophiles, and often found in environments with seasonal temperature fluctuation (Margesin et al., 2007).

Glacier accounts for 10% of the land surface and is considered as one of the harsh environments on biosphere. Microorganisms inhabiting cold environments encounter some challenges such as extreme low temperatures which may ranges from -56°C to -10°C. Other limitations include low nutrient and water availability, reduced enzymatic reaction rates, darkness and salinity (Margesin and Miteva, 2011). These extreme environments are inhabited by microorganisms either in dormant and vegetative forms and adapted to this extremely cold environment by evolving different mechanisms for survival. One of such challenges at low temperatures is decrease in membrane fluidity and permeability. The fluidity of the membrane at low temperature is maintained by increase in unsaturated fatty acids (Siliakus et al., 2017). Other adaptations include increased structural flexibility of

cold active enzymes to increase activity, production of cold shock and cold acclimation proteins which functions with high activity even at low temperatures (Phadtare, 2004). Production of antifreeze proteins (AFPs) constitute another class of proteins which lowers the freezing point of water through the mechanism of thermal hysteresis (Lorv et al., 2014). Production of compatible solutes such as trehalose and betaine to counter the osmotic pressure exerted by the outside freezing conditions (Hoffmann and Bremer, 2011). Production of exopolysaccharides, which helps in cryoprotection of bacterial or other microbial cells from the damaging effects of the freezing conditions (Deming and Young, 2017).

Psychrophiles have great potential in biotechnological exploration. Thus a strong interest is developing in cold adapted microorganisms, resulting in isolation and screening for potential novel microbial species and its metabolites. Within the past decade, a significant number of bacteria including novel species from permanently cold environments have been isolated and characterized including, permafrost (Bakermans et al., 2003), glacier ice (Miteva et al., 2004), Arctic sea ice (Junge et al., 2002), and from numerous ecosystems in Antarctica (Christner et al., 2003b). Enzymes from the cold-active microorganisms have plentiful applications in the textile, food, detergent, and animal feed industry (Gerday et al., 2000), nevertheless this field is still at its infancy as a small portion of organisms producing these enzymes have been identified and characterized.

Petroleum hydrocarbons are one of the major environmental pollutants, which contaminate terrestrial and freshwater through accidental spills or shipping activities (Arulazhagan et al., 2010). Petroleum mainly consists of alkanes while n-hexadecane is a major component of alkanes. Alkanes are highly inflammable but one of the least reactive among organic compounds. The success of bioremediation of alkanes such as hexadecane is dependent on the accessibility of the compound to the degrading microorganisms, inherent biodegradability of the pollutant and on the optimum condition for the degradation process (Kebria et al., 2009). Low molecular weight alkanes are degraded rapidly as compared to long chain and multiple branched alkanes (Stroud et al., 2007).

The indigenous cold adapted microorganisms play a key role in biodegradation of petroleum products and other pollutants in low temperature environments. This low temperature favors the growth of psychrophiles and psychrotrophs and hence the degradation activity. The aerobic biodegradation of a large number of petroleum components at low temperatures has been reported in Arctic, Antarctic and alpine regions. These component includes n-alkanes, aromatic and polycyclic aromatic hydrocarbons (Margesin and Schinner, 2001b). Cold inhabiting microorganisms have a tremendous potential for applications in bioremediation processes as they harbors enzymes which maintains high catalytic activity and unusual specificity at low temperatures. Introduction of specific cold adapted microorganisms in mixed cultures in contaminated environments is expected to improve biodegradation of organic pollutants.

The northmost territories of Pakistan consists of tangled mountains including the western Himalayas, Karakoram and Hindukush ranges. The three mightiest mountain ranges of the world meet at a junction point near Gilgit, Pakistan and considered as world's most heavily glaciated area outside of the Polar Regions. These ranges in Pakistani geographical boundary host more than 5000 glaciers and feed water to the Indus River System (Rasul et al., 2011). Batura glacier with a length of ~ 59 km (Hewitt, 2014) is one of the longest glaciers outside the polar region. This glacier is located in Passu village of Hunza valley, in the Karakorum Rang of Pakistan.

In this paper, we reported the isolation, identification, phylogenetic and physiological characterization of a cold-adapted bacterium isolated from ice of Batura glacier. BGI-11 is a versatile glacier isolate with diverse metabolic activities which also includes antimicrobial and enzyme activities. The strain is able to grow in MSM with hexadecane as the chief source of carbon substrate. Among the 8 glacial isolates BGI-11 could grow well at 8% salinity. The tolerance of BGI-11 to salinity and cold, ability to use long chain hydrocarbon (hexadecane) makes it a potential candidate for biodegradation of petroleum products in cold environments particularly the deep oceans.

## 7.2. Materials and Methods

### 7.2.1. Enrichment of ice sample and isolation of BGI-11

BGI-11 was isolated among 7 other bacteria from the ice of Batura glacier through sample enrichment method (in press). Plating of direct or diluted sample did not yield any colonies even after prolong incubations for months at 4˚C and 15˚C. For enrichment of the sample, 10 mL melted ice was added to 90 mL of R2A, tryptic soy broth (TSB) and Luria-Bertani broth (LB) and incubated at 4˚C and 15˚C in a shaker incubator. Once the broth media turned turbid, 100 µL samples from each inoculating media were plated on their respective agar medium (in press). Morphologically different colonies observed were streaked on separate agar plates containing same medium to obtain their pure culture including the isolate BGI-11.

### 7.2.2. Identification of strain BGI-11

Primary identification of strain BGI-11 was done on the basis of colony morphology, cell morphology, pigmentation, growth characteristics and biochemical characteristics. Isolate BGI-11 was later identified through 16S rRNA gene sequencing.

### 7.2.3. DNA extraction, 16S rRNA sequencing and phylogenetic analysis

DNA was extracted using UltraClean microbial DNA isolation kit (MO BIO Laboratories), according to the manufacturer instructions. Amplification of 16S rRNA gene was performed through PCR using universal primers 27F and 1492R. The alignment was thoroughly analyzed and the ambiguous aligned regions were removed from the sequence using Chromas (version 2.6.6). 16S rRNA gene sequence of BGI-11 and the most similar sequences were identified through BLAST in the EzBiocloud database (Yoon et al., 2017a). 16S rRNA gene sequence of BGI-11 was deposited in the NCBI GenBank database with the accession number MK522045. Finally, phylogenetic tree was constructed for BGI-11 using neighbor joining method with bootstrap values in the MEGA 7 software (Saitou and Nei, 1987).

### 7.2.4. Physiological characterization of *Rhodococcus* sp. BGI-11

#### 7.2.4.1. Growth at different Temperatures

Temperature growth range of the BGI-11 was checked at 5-45˚C with an increment of 5 on R2A agar plate in duplicates. Result interpretation was done after incubating plates at their respective temperatures for one week (Miteva et al., 2004: Zhang et al, 2013). Growth was also checked in liquid culture at 5, 15, 25, 35 and 45˚C. Overnight culture of BGI-11 in exponential phase of growth was used for inoculation and flasks were incubated in shaker incubators at 150 rpm. Optical density ($OD_{600}$) was used daily to monitor the growth for 6 days. For this purpose, 1mL of culture from each flask was taken in 48 well plate and readings were taken on a microplate reader (SpectraMax M5).

#### 7.2.4.2. Growth at different pH

Growth of the BGI-11 was checked at different pH ranging from 3 to 13 with an increment of 1 on R2A agar plates in duplicates. Result interpretation was done after incubating plates at 15˚C for one week (Zhang et al., 2013: Shivaji et al., 2013). pH optimization was also performed in the broth culture in replicates. Overnight culture of BGI-11 was inoculated in R2A broth with pH ranging from 4 – 11. All the flasks were incubated at 15˚C in a refrigerated shaker incubator at 150 rpm. Optical density ($OD_{600}$) was used every day for 6 days to monitor the growth and reading were taken in a microplate reader.

#### 7.2.4.3. Salt Tolerance

BGI-11 was checked for its ability to grow under halophilic conditions. Both broth culture and plate techniques were used. For plate streaking method, the BGI-11 was streaked on R2A agar plates supplemented with 2, 4, 6, 8, and 10% NaCl (w/v) and incubated at 15°C. Growth was checked after an incubation time of one week (Zhang et al, 2013: Shivaji et al., 2013). Isolate was also grown in broth culture supplemented with different concentration of salt up to 10% (w/). Flasks were kept in shaker incubator at 15˚C and optical density was measured in a microplate reader daily up to 7 days.

#### 7.2.4.4. Growth on different media

Growth on different media were checked using nutrient rich, oligotrophic and selective media. The media included R2A (Difco), tryptic soya agar (Oxoid), nutrient agar (Oxoid), Lauria bertani agar (Oxoid), saboraud dextrose agar (Oxoid), mannitol salt agar (Oxoid), MacCkonkey agar (Oxoid) and Mueller Hinton agar (Oxoid). Growth on all media were assessed by streaking BGI-11 on the respective agar plates and were incubated at 15˚C for one week (Zhang et al, 2013).

#### 7.2.4.5. Utilization of carbon sources

The ability of BGI-11 to use different sugars as carbon source was assessed in R2A broth. The sugars used were glucose, galactose, sucrose, maltose and lactose. R2A broth was prepared without adding dextrose and instead all these sugars at a concentration 2% (w/v) were added. BGI-11 culture in log phase was used as inoculum and flasks were incubated at 15˚C in shaker incubator (150 rpm) for one week. Optical density ($OD_{600}$) was used every day to monitor growth in microplate reader. Ability of BGI-11 to use molasses and glycerol as carbon substrates was also checked in a similar method discussed for sugars utilization.

#### 7.2.4.6. Antibiotic resistance/Antibiotic susceptibility testing

Disc diffusion method (Bauer et al., 1966) was used for the susceptibility test of BGI-11 to antibiotics using Mueller-Hinton agar plate. Strain BGI-11 inoculum was standardized to $10^8$ CFU McFarland standard. Two narrow spectrum antibiotics including methicillin (10 µg) and vancomycin (5 µg), 3 broad-spectrum antibiotics including, imipenem (10 µg), ofloxacin (5 µg), and trimethoprim sulfamethaxazole (30 µg) were used. Plates were incubated at 15˚C and results were interpreted after 24 to 48 hours. The susceptibility of BGI-11 to each antibiotic was determined from measurement of the zone of inhibition.

### 7.2.5. Activities of *Rhodococcus* sp. BGI-11

#### 7.2.5.1. Enzyme activity

BGI-11 was screened for its ability to produce extracellular enzymes. Lipase activity was checked by streaking BGI-11 on R2A supplemented with 1% Tween-40 along with 0.001%

rhodamine B and 0.01% $CaCl_2$ (Booth, 1971). Formation of opaque hazy zone of calcium soap crystals around the growth indicated hydrolysis of the Tweens. For amylase production, starch hydrolysis test was performed by the method used preciously (Priest, 1977). Briefly, BGI-11 was streaked on R2A agar surface supplemented with 1% starch. Plates were incubated at 15˚C for 05 days. Lugol's iodine solution was flooded on the surface of the plates and observed for change in color. The areas of the medium containing unhydrolysed starch was stained dark purple while hydrolyzed zones around growth were clear. Carboxymethylcellulose (CMC) agar plates were used to test cellulase activity (Kasana et al., 2008). *Rhodococcus* sp. BGI-11 was spot inoculated on CMC agar and plates were incubated at 15˚C for 05 days. Plates were later flooded with Gram's iodine for 3 to 5 minutes. Zone of clearance around the growth indicated positive activity for cellulase production. Protease activity was checked by the method described previously with some modifications (Priest, 1977). Colonies of BGI-11 were streaked on the R2A agar supplemented with 1% casein. Plates were then incubated at 15˚C for 05 days and flooded with 1M acetic acid. Clear zone of hydrolysis around the growth demonstrated positive for protease production. For DNase test, isolates were spot inoculated on the surface of DNase agar and plates were incubated at 15˚C for 05 days. Plates were then flooded with 1N hydrochloric acid. Hydrolysis of DNA is indicated by clear zone around the area of growth.

#### 7.2.5.2. Antimicrobial activity

Antimicrobial activity of BGI-11 was determined through spot inoculation method. The indicator microorganisms included the multidrug resistance strains of *Candida albicans*, *Klebsiella pneumoniae*, *Acinetobacter* sp., and *Bacillus* sp. and two ATCC strains, *Pseudomonas aeruginosa* (ATCC27853) and *Staphylococcus aureus* (ATCC6538). Antimicrobial activity was determined through spot inoculation method. Mueller-Hinton agar plates were swabbed with overnight culture of indicator organisms and BGI-11 was spot inoculated on the plates. The plates were incubated at 15˚C for 3 to 5 days and observed for a clear zone around the BGI-11 growth.

### 7.2.6. Utilization of Hexadecane as carbon substrate

BGI-11 was tested for its ability to degrade hexadecane. Mineral salt medium (MSM) was used as growth medium with hexadecane as the only carbon source. MSM was prepared as described previously (4 g $L^{-1}$ $NH_4Cl$, 2.5 g $L^{-1}$ $K_2HPO_4$, 0.5 g $L^{-1}$ NaCl, 0.3 g $L^{-1}$ $MgSO_4.7H_2O$, 0.03 g $L^{-1}$ $FeCl_3.6H_2O$, 0.01 g $L^{-1}$ $CaCl_2$, 0.01 g $L^{-1}$ $MnCl_2.4H_2O$) (Chayabutra and Ju, 2000). Two different concentrations of hexadecane were used, i.e. 0.5% and 1%. MSM with hexadecane was inoculated with a suspension of BGI-11 cells, which were pre-grown in R2A broth. The cells were centrifuged and washed 3 times in normal saline to remove any traces of R2A broth before inoculation. All the flaks were incubated at 15˚C in a shaker incubator at 150 rpm. Growth ($O.D_{600}$) was checked after every 48 hours for 10 days in a microplate reader. Dry weight was also measured at each time point after drying the sample in a lyophilizer (FreeZone 2.5, LABCONO). Two controls were used in the experiment including, MSM with no addition of hexadecane and un-inoculated MSM with hexadecane addition. The second control was to make sure that degradation is caused by the inoculated strain BGI-11. The experiment was carried out at 15˚C with initial pH 7.3 and salinity 0.03 %.

## 7.3. Results

### 7.3.1. Colony, cell morphologies and biochemical tests

BGI-11 was isolated from the ice of Batura glacier through sample enrichment technique. Colonies with morphologies similar to BGI-11 were present in abundant on the agar plates. The colonies of BGI-11 appeared milky, raised and circular (Fig. 7.1). Gram staining revealed BGI-11 as Gram-positive, coccobacilli cell morphology. Isolate BGI-11 demonstrated positive results for catalase and citrate utilization and negative for nitrate reduction (Table 7.1).

### 7.3.2. Molecular identification through 16S rRNA gene based phylogenetic analysis

The 16S rRNA gene sequence of BGI-11 was submitted to EzBiocloud data base to get the most closely hit strains. BLAST results revealed BGI-11 as closely related to *Rhodococcus erythropolis* species with similarity index >99%. Phylogenetic tree was also constructed and the bootstrap value for BGI-11 and *Rhodococcus erythropolis* NBRC 15567 was 100 (Fig. 7.2).

Figure 7.1. Pure culture of BGI-11 colonies on tryptic soy agar plate.

Table 7.1. Physiological and biochemical characteristics of strain BGI-11.

| Temperature Range (˚C) | pH Range | NaCl Range (%) | Biochemical Properties | | | Cell Morphology (Gram Staining) |
|---|---|---|---|---|---|---|
| | | | Citrate Utilization | Catalase Test | Nitrate Reduction | |
| 4-35 | 4-11 | 0 – 8 | + | + | - | +ve (coccobacilli ) |

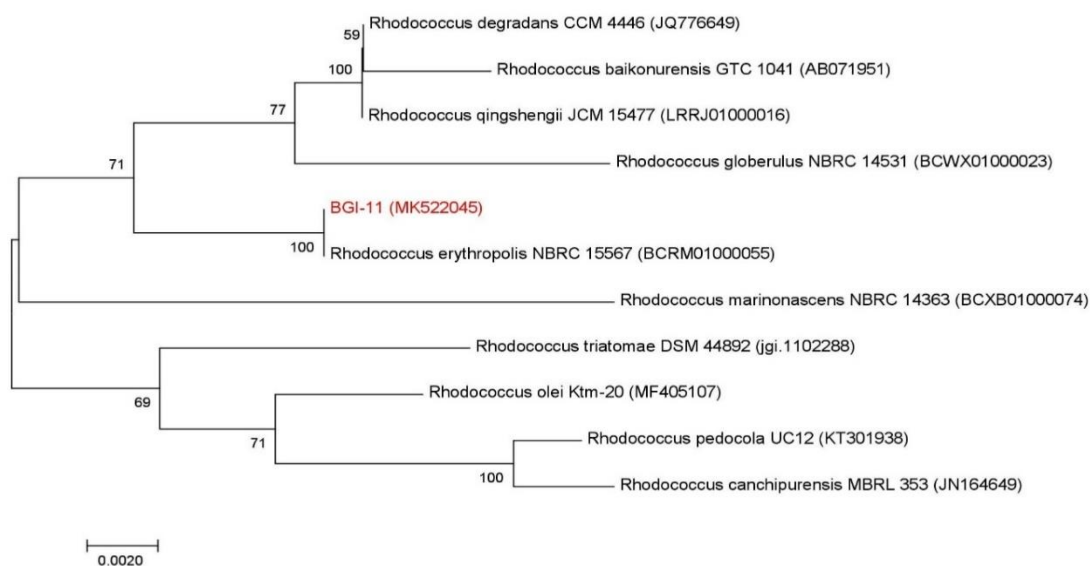


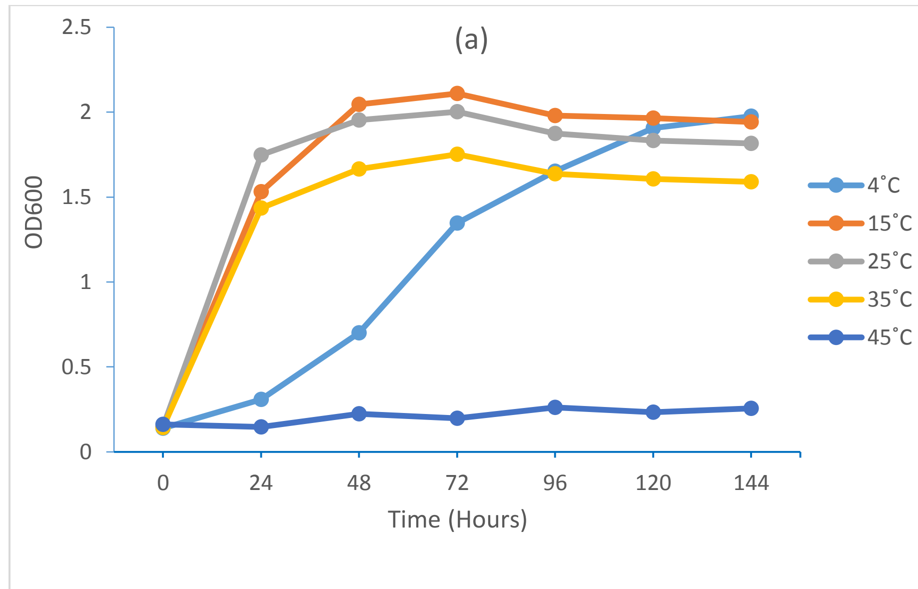

Figure 7.2. Phylogenetic tree constructed for the glacial isolate BGI-11 (red color) and closely hit strains, using neighbor-joining method with a bootstrap value (%) greater than 50 from 1000 replicates. Numbers in the brackets are GenBank accession numbers for the 16S rRNA sequences.

### 7.3.3. Growth characteristics of BGI-11

#### 7.3.3.1. Effect of temperature and pH

**S**train BGI-11 grew well at a temperature range of 4-35˚C. Growth pattern was similar at 15, 25 and 35˚C during the first 24 hours, demonstrating the exponential phase of the growth. Growth was slow at 4˚C during the first 24 hours and increased significantly after 48 hours and afterwards. No growth was observed at 45˚C (Fig. 7.3a). Stationary phase was reached during 72 hours for temperatures 15, 25, and 35˚C. Optimum temperature was found above 20˚C. Temperature optimization on agar plates also indicated a wide temperature range for growth, with no growth visible on plates at 45˚C.

BGI-11 demonstrated good growth at a pH range of 4-11. Growth curve pattern was similar at pH 6-11. However, slow growth in the first 24 hours was observed at pH 4 and 5 (Fig. 7.3b). Stationary phase was reached during 72 hours for pH 6-11. Increase in growth was observed at low pH after 24 hours of incubation. Plate streaking results also demonstrated a wide pH range for BGI-11. On the plates BGI-11 produced visible colonies at the extremes of pH including 3 and 13.



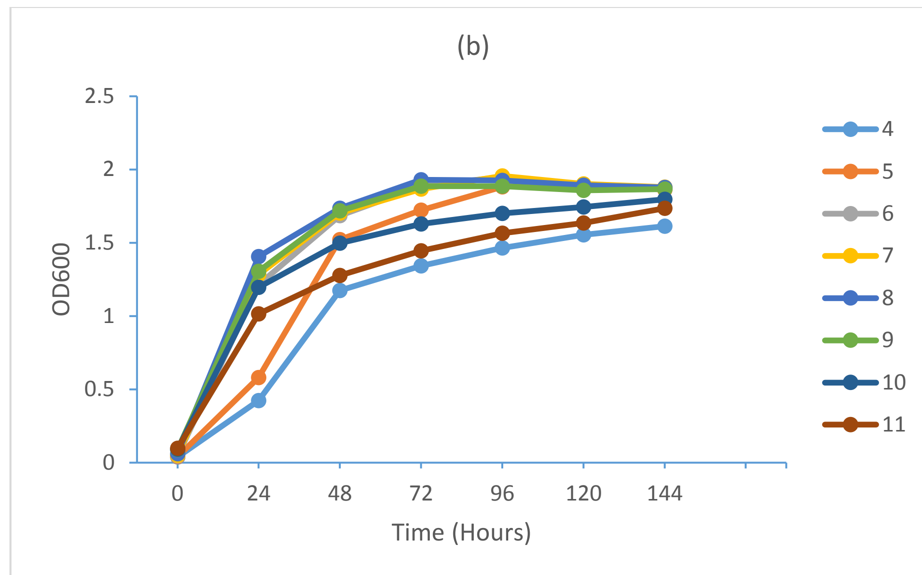



Figure 7.3. Growth curve ($OD_{600}$) of BGI-11 at different (a) temperatures (4-45˚C) and (b) pH (4-11)

### 7.3.3.2. Effect of salt concentration

*Rhodococcus* sp. BGI-11 demonstrated good growth at a salinity range up to 7% in the broth media. Maximum optical density was achieved at 1-5% (w/v) NaCl during 72 hours. Growth was also observed at 10% NaCl but could not reach high optical density even after one week (Fig. 7.4). BGI-11 also produced visible growth on agar plates supplemented with 8% NaCl, indicating a moderate salinity range. The strain was therefore considered as moderate halophile or a halotolerant.

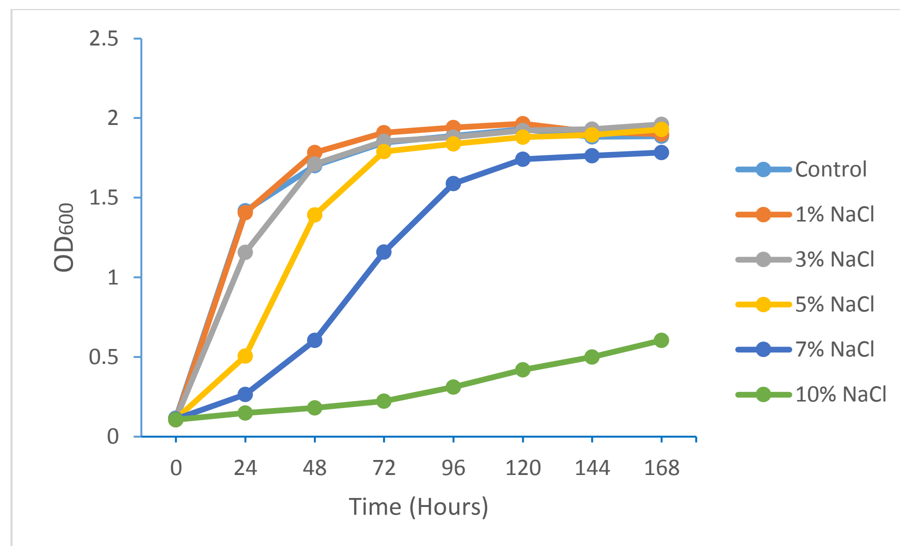



Figure 7.4. Growth curve using optical density ($OD_{600}$) of BGI-11 at different salt concentrations

### 7.3.3.3. Effect of carbon sources

BGI-11 demonstrated great versatility in utilization of sugars as growth substrate. The strain was able to use glucose, galactose, lactose, maltose and sucrose as carbon source. The growth pattern was similar in all the sugars supplemented media. Growth with high cell densities were observed for all the sugars (Fig. 7.5a). Glycerol and sugar waste molasses were used as substrate for the growth of BGI-11. The strain demonstrated good growth in glycerol and molasses (Fig. 7.5b). Maximum growth was observed in presence of 2% molasses after 144 hours, sustaining growth for a longer time. Also tryptic soy broth, a nutrient rich medium was used as a control. Maximum growth was observed in MSM with glycerol and molasses than tryptic soy broth.

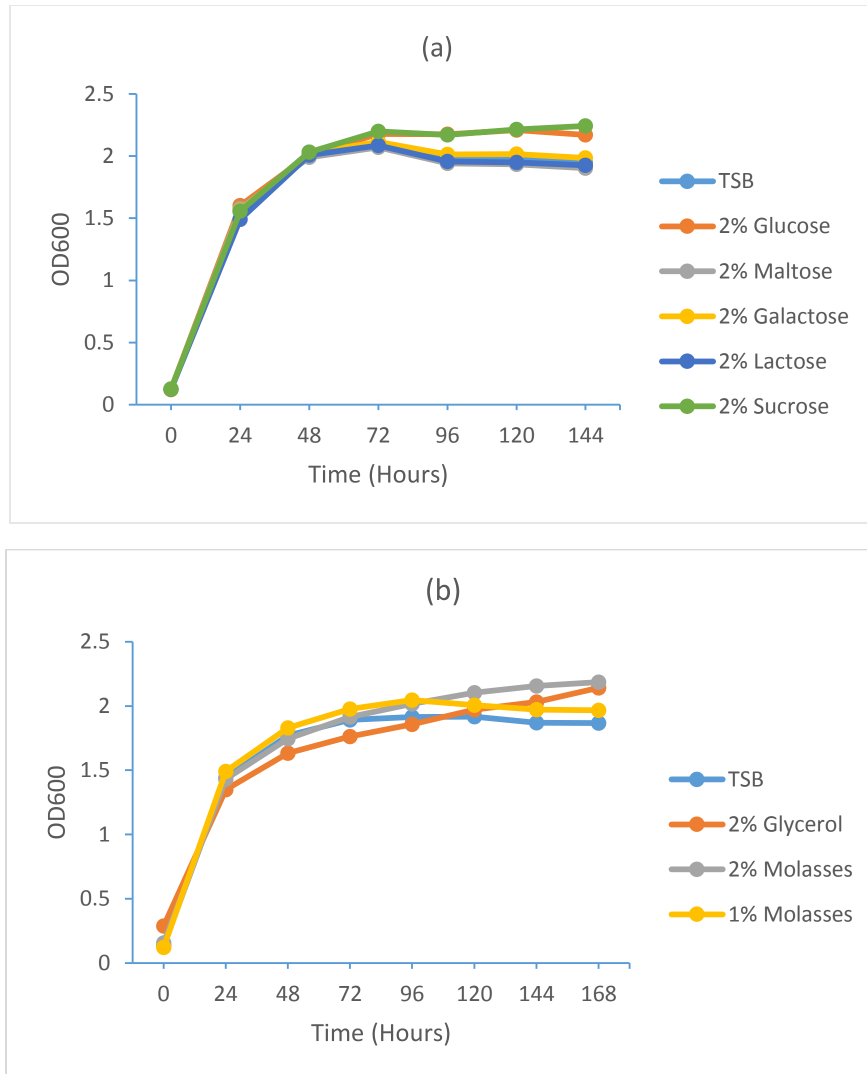



Figure 7.5. Growth curve using optical density ($OD_{600}$) of BGI-11 at different (a) carbon sources and (b) different concentration of molasses and glycerol.

#### 7.3.3.4. Growth at different culture media

BGI-11 was able to grow at all the culture media including R2A, Luria bertani agar, tryptic soy agar, nutrient agar, Sabouraud dextrose agar, mannitol salt agar, MacConkey agar and Mueller Hinton agar. Surprisingly, the strain was also able to grow in sabouraud dextrose agar (SDA) which is designed for growth of yeast and fungi. The low pH of this medium favors growth of fungi and inhibits bacteria. *Rhodococcus* sp. BGI-11 was able to grow well in SDA as this strain can tolerate very low pH. The strain also demonstrated growth on MacConkey agar, a selective medium which inhibits Gram-positives and favors Gram-negative bacteria. Growth on R2A was observed within 2 days of streaking and R2A is a medium of choice for isolation of bacteria from oligotrophic environments such as the glacier.

### 7.3.4. Activities of *Rhodococcus* sp. BGI-11

#### 7.3.4.1. Antimicrobial activity

BGI-11 demonstrated antimicrobial activity against 2 of the 5 indicator microorganisms including *Bacillus* sp. and *Candida albicans*. No activity was observed for the other three bacteria (Table 7.2). Activity against *Candida albicans* was surprising as this strain was multidrug resistant acquired from a tertiary care hospital.

Table 7.2. Antimicrobial activities demonstrated by BGI-11 against pathogenic and non-pathogenic strains of bacteria and a yeast using spot inoculation and well diffusion methods at 15˚C.

| **Antimicrobial activity (mm)** | | | | | |
|---|---|---|---|---|---|
| ***Staphylococcus aureus*** | ***Pseudomonas aeruginosa*** | ***Bacillus* sp.** | ***Klebsiella pneumoniae*** | ***Candida albicans*** | ***Acinetobacter* sp.** |
| - | - | 17.32 | - | 25.87 | - |

#### 7.3.4.2. Extracellular enzyme activity

BGI-11 demonstrated activity for 2 enzymes including, lipases and DNases. No activity was observed for proteases, cellulases and amylases (Table 7.3). Cold active enzymes maintain high catalytic activity at low temperatures and have relevance to many industrial processes. The high activity at low temperature negates the heating steps, saving energy. BGI-11 demonstrated lipase activity suggests its use in oil (fats) removal.

Table 7.3. Extracellular enzyme activity demonstrated by BGI-11 using plate assay at 15˚C.

| **Enzyme activities** | | | | |
|---|---|---|---|---|
| **Lipases** | **Proteases** | **Cellulases** | **Amylases** | **DNases** |
| + | - | - | - | + |

### 7.3.5. Antibiotic susceptibility test

Antibiotic susceptibility test revealed BGI-11 resistant to 2 antibiotics including, methicillin and trimethoprim sulfamethxazole. The strain was found sensitive to imipenem, ofloxacin and vancomycin (Table 7.4). The broad-spectrum oflaxacin and narrow spectrum vancomycin had the maximum zone of inhibition against BGI-11. Antibiotic resistance is common in oligotrophic environments as the scarcity of nutrients creates a competition among the microbes, resulting in production of antimicrobial compounds by some and development of resistance by the others.

Table 7.4. Antibiotic susceptibility test of BGI-11 against narrow and broad spectrum antibiotics using disc diffusion method at 15˚C.

| **Antibiotics (Zone of inhibition in mm)** | | | | |
|---|---|---|---|---|
| **Methicillin (10 µg)** | **Imipenem (10 µg)** | **Ofloxacin (5 µg)** | **Vancomycin (5 µg)** | **Trimethoprim Sulfamethxazole (30 µg)** |
| R | 19.80 | 31.27 | 27.66 | R |

### 7.3.6. Hexadecane utilization

Hexadecane was used as representative petroleum product to check the ability of BGI-11 for the utilization of long chain hydrocarbons. The strain demonstrated maximum growth in the test flask with 1% hexadecane, reaching to an optical density of 3 in 144 hours. The strain also grew well in the MSM with 0.5% hexadecane (Fig. 7.6a). No growth was observed in the two controls throughout the growth period. These controls included MSM with no hexadecane and MSM with 1% hexadecane lacking the BGI-11 inoculum (Fig. 7.6a). The second control was added to make sure the turbidity is solely cause by the cell growth.

Likewise, maximum biomass dry weight was observed in MSM with 1% hexadecane, reaching to 2.13 g/L after 144 hours. Maximum dry weight in 0.5% hexadecane supplemented MSM was 1.75 g/L after 192 hours. No biomass production was observed in the controls (Fig. 7.6b).

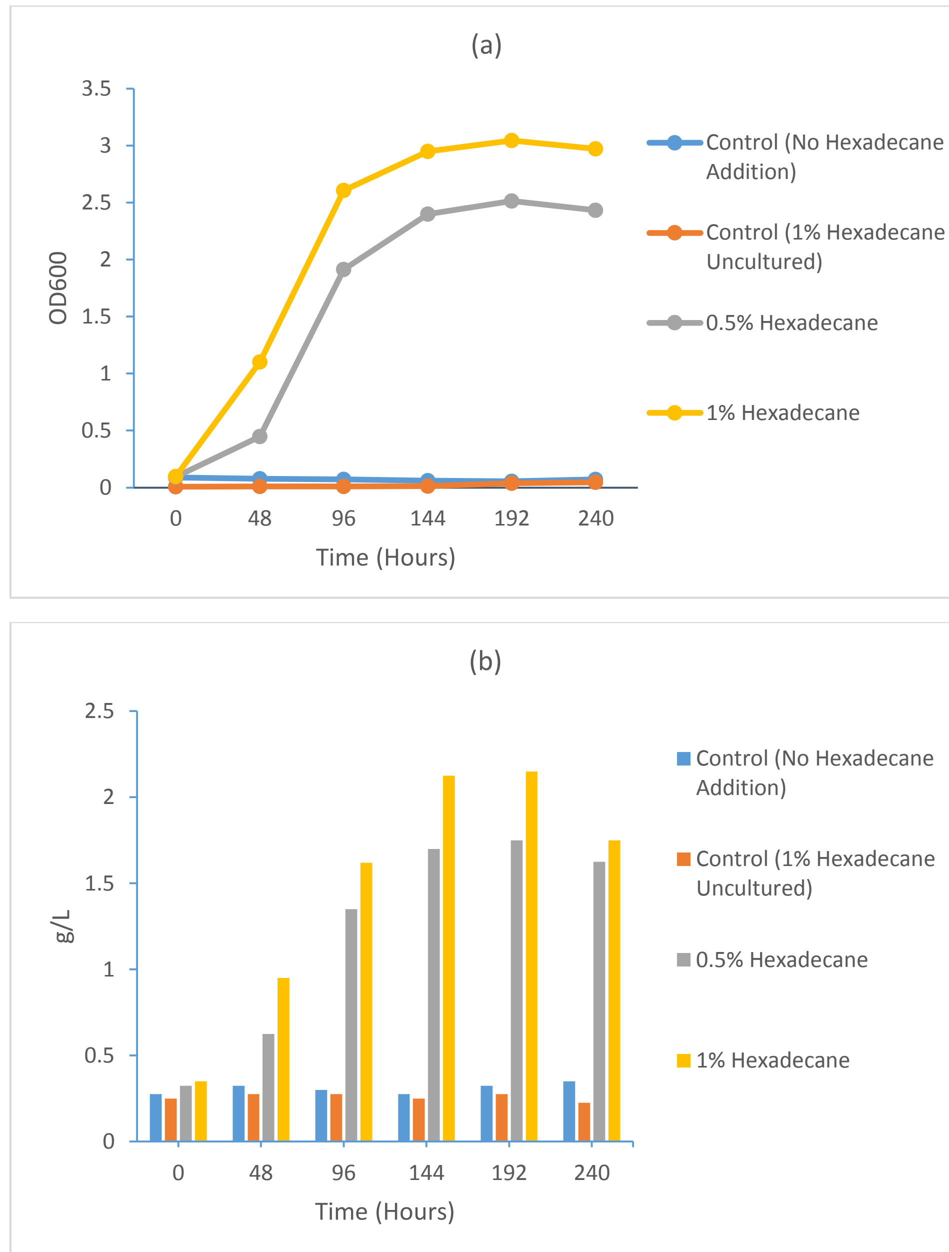



Figure 7.6. Growth of BGI-11 in 0.5% and 1% hexadecane, controls include no addition of hexadecane and 1% uncultured hexadecane supplemented medium (a) Growth curve ($OD_{600}$) (b) Dry weight biomass (g/L). The experiment was carried out at 15˚C with initial pH 7.3 and salinity 0.03 %.

## 7.4. Discussion

Present study involved isolation of a cold-adapted bacterium from glacier in the Karakoram to explore some of its metabolic potential for industrial and environmental applications. Compared to the other cold environments, glaciers in the high Karakorum are less explored in terms of their microbial diversity and functionality. Strain BGI-11 was most closely related to *Rhodococcus* erythropolis based on the 16S rRNA gene sequence analysis. *Rhodococcus* species are widely distributed and the bacteria have been routinely reported from cold environments (Maharana and Singh, 2018; Ruberto et al., 2005; Rapp et al., 2003).

These cold-adapted organisms are called psychrophiles or psychrotrophs, and various investigators have defined them in their own way, Morita's definition is widely used and accepted. According to Morita (1975) psychrophiles grow optimally at 15˚C or lower and a maximum growth temperature at about 20˚C. Psychrotrophs also grow near the freezing point of water but their optimal and maximum temperature for growth lies above 20˚C. According to Morita's definition, BGI-11 falls in the category of psychrotrophs as it demonstrated a wide growth range 4-35˚C. This is also true as unlike deep sea, terrestrial cold environments have more temperature fluctuation, which results in high abundance of the psychrotrophic organisms (Russell, 1990).

BGI-11 exhibited great versatility to withstand and grow in multiple stressful conditions. These physicochemical conditions included low temperature, extremes of pH and high salt concentration. BGI-11 is therefore considered as a polyextremophile. Organisms which grow in more than one extreme conditions are called polyextremophile (Pikuta et al., 2007). Extreme environments of the planet earth are considered as the best analogs to the extraterrestrial environments and extremophiles are used as biomarkers for the search of life in the extraterrestrial environments such as Mars (Cavicchioli, 2002). The bioactive compounds produced by polyxtremophilic microorganisms have plentiful applications in the biotechnological industry (Rekadwad and Khobragade, 2017). Many industrial processes require extreme conditions, where normal microbes may not even survive. Extremophiles or their products become potential candidate to carry on the reaction in such

inhospitable conditions. *Rhodococcus* species have been reported previously from various extreme habitats, including cold (Maharana and Singh, 2018), saline (Zolfaghar et al., 2019), and hydrocarbon contaminated (Quatrini et al., 2008) environments.

Bioremediation is an efficient and ecofriendly process for removal of the toxic compounds. This process is carried out by the indigenous psychrophilic microbes inhabiting the cold environments. Margesin and Schinner, (1998) investigated cold-adapted bacteria for their degradation potential from Alpine habitats. These bacteria were screened from 29 different locations from soil and glacier samples. They surprisingly recovered oil degrading bacteria not only from oil-contaminated samples but also from uncontaminated site. Degradation was efficient at low temperature as most inocula degraded 40-60% of the diesel oil within 8 days at 10˚C. Degradation ability decreased at higher temperature (48˚C) to 20-40% after 8 days. It was also found that degradation of the hydrocarbon occurred mainly during the first 6 days of incubation, and no significant degradation was noticed after 6 days.

Environmental pollution with organic compound is a global threat particularly, hydrocarbon contamination in the marine environments is increasing. Extremophilic bacteria with the ability to cope the harsh environmental conditions becomes crucial for the cleanup process. In our study, *Rhodococcus* sp. BGI-11 demonstrated good growth and biomass production in hexadecane supplemented medium. *Rhodococcus* species are one of the well-studied bacteria for biodegradation due to their ability to use a variety of hydrocarbons. Recently, a book has been published on bioremediation of contaminated environments using *Rhodococcus* (Kuyukina and Ivshina, 2010). Cold-adapted *Rhodococcus* species have been reported for hydrocarbon degradation in cold environments (Bej et al., 2000; Whyte et al., 1999; Ruberto et al., 2005). A psychrotrophic *Rhodococcus* species isolated from the Arctic soil utilized a variety of hydrocarbons including, hexadecane and cyclohexane at 5˚C (Whyte et al., 1998). In another study, two strains identified as *Rhodococcus* fascians utilized a number of aliphatic and aromatic hydrocarbons (including hexadecane) as principal carbon source at a range of 4-35˚C (Yakimov et al., 1999).

Petroleum hydrocarbons are one of the most common contaminant of soil and water. Many environments contaminated with hydrocarbon may have other stressful conditions including low temperature, low or high pH, increased pressure and salinity (Margesin and Schinner, 2001b). Bioremediation of the hydrocarbons in such environment needs microorganisms which have the capacity to deal with more than one stressful conditions. BGI-11 demonstrated this ability as it grew well at low temperature, extremes of pH and moderate salinity. Temperature is key to the metabolism of hydrocarbons and psychrophilic or psychrotrophic organisms produce cold-active enzymes which can perform degradation efficiently at low temperature. Margesin and Schinner, (2001b) for the first time worked on biodegradation of diesel oil in an alpine glacier which was at an altitude about 3000 m above the sea level. They concluded that the pollutant can be efficiently removed even at extremely low temperature which is an unfavorable condition for many microbes.

For successful bioremediation, it is necessary to select bacteria with high capacity and versatility to degrade large components of petroleum products. Bacteria able to grow and utilize petroleum products as its energy source becomes acclimatize to this process and acquire the ability to degrade a large number of other hydrocarbons. The metabolic versatility and adaptations to extreme conditions makes BGI-11 a potential candidate for bioremediation of hydrocarbons in cold environments particularly the deep ocean which is also characterized by low temperature and moderate salinity. Another exciting feature of BGI-11 was its ability to produce lipases. Lipases are an important class of hydrolytic enzymes which catalyzes hydrolysis of fats (lipid). Lipases have been reported for reduction of hydrocarbons from the oil contaminated soils indicating their potential role in bioremediations (Riffaldi et al., 2006: Margesin et al., 1999).

## 7.5. Conclusions

*Rhodococcus* sp. BGI-11 is a psychrotrophic bacterium with wide temperature and pH range for growth. The most exciting characteristic of this strain is its ability to utilize hexadecane as carbon substrate. This strain is able to grow at low temperature and can tolerate salt concentration upto 8% (w/v). It can utilize sugar waste molasses and also

glycerol as cheap growth substrates. All these characteristics makes it a potential candidate for bioremediation in Deep Ocean where accidental oil spills is an ever increasing concern. The deep oceans are also characterized by low temperature and moderate salinity, which makes it a perfect playground for BGI-11 to perform biodegradation of the hydrocarbons. Also molasses can be added as a cheap growth substrate to speed up the cleaning process through biostimulation.

## Overall conclusions

- Batura glacier in the Karakoram Range was the first time explored for their bacterial diversity using culture-independent method.
- Culture-dependent studies resulted in isolation of 27 cold-adapted bacterial strains.
- All the isolates were psychrotrophic in nature, with wide growth temperature range.
- Majority of the isolates demonstrated antimicrobial activity against a number of indicator organisms which also included multidrug resistant strains. Also, some isolates demonstrated activities for more than one extracellular enzymes.
- Some of the isolates exhibited ability to withstand more than one extreme condition and therefore considered as polyextremophile.
- Culture-independent study revealed high relative abundance of some genera rarely reported from the glacier environments including *Ochrobactrum*, *Kaistobacter*, and *Mycoplana*.
- The glacial isolate BGI-2 was found with high yield of EPS under optimized conditions and considered as relatively higher than the previous reports from *Pseudomonas* species using the same method for quantification.
- The EPS from BGI-2 provided significant cryoprotection to a mesophilic *Escherichia coli* K12 strain which was comparable to 20% glycerol.
- The EPS-producing isolate BGI-2 exhibited maximum freeze thaw survivability.
- The isolate BGI-2 used molasses and glycerol as cheap sources of carbon.
- The EPS from the cold-adapted bacterium BGI-2 helped in the long term cryopreservation of the selected cyanobacterial and microalgal strains.
- Genome analysis of BGI-2 revealed presence of genes essential for cold-adaptation, including genes involved in the unsaturation of membrane fatty acids, uptake and synthesis of compatible solutes and production of exopolysaccharide.
- Comparative genomics revealed presence of more stress tolerance genes in the genome of cold-adapted BGI-2 than the 6 closely related mesophilic *Pseudomonas* strains.
- Genome of BGI-2 has 11-EPS producing genes compared to none in their mesophilic counterparts.

- The presence of EPS genes in BGI-2 and its cryoprotective activity likely contributes in its successful colonization of the glacier ice.
- Another glacier isolate *Rhodococcus* sp. BGI-11 exhibited ability to utilize hexadecane as carbon source as well as growth at low temperatures and moderate salinity 8% (w/v). All these characteristics makes it a potential candidate for bioremediation of petroleum hydrocarbons in Deep Ocean which is also characterized by similar conditions.

## Future prospects

- The Karakoram Range of Pakistan hosts some of the longest glaciers of the world. These glaciers needs to be further explored for their microbiological diversity and functionality.
- Glaciers in the Karakoram are either stable or growing (the Karakoram anomaly) unlike glaciers receding in other part of the world due to global warming. It would be interesting to explore the microbial diversity and activity in these glaciers linking it to the climate change.
- Most of the bacterial and cyanobacterial strains demonstrated good post cryopreservation recovery at the higher concentration of EPS used. Therefore, more tests with increased EPS concentration will further improve biomass recovery.
- Perform cytotoxicity test of the EPS for its uses as a cryoprotective agent for human cells and tissues. Further research can pave way for the replacement of existing toxic chemical cryoprotective agents with this natural polymer.
- In-depth study of the genomic data from strain BGI-2 will help to better understand the molecular basis of cold adaptation.

## References

**Ali, P., Shah, A.A., Hasan, F., Cai, H., Sosa, A. and Chen, F.,** 2019. Draft genome sequence of a cold-adapted *Pseudomonas* sp. strain, BGI-2, isolated from the ice of Batura glacier, Pakistan. *Microbiology Resource Announcements*, *8*(19), pp.e00320-19.

**Aliyu, H., De Maayer, P., Sjöling, S. and Cowan, D.A.,** 2017. Metagenomic analysis of low-temperature environments. In: Margesin, R. (ed.) *Psychrophiles: from biodiversity to biotechnology*, 2nd edn. Springer, Berlin, pp.389-421.

**Allen, M.M.,** 1968. Simple conditions for growth of unicellular blue-green algae on plates. *Journal of Phycology*, *4*(1), pp.1-4.

**Alma'abadi, A.D., Gojobori, T. and Mineta, K.,** 2015. Marine metagenome as a resource for novel enzymes. *Genomics, Proteomics & Bioinformatics*, *13*(5), pp.290-295.

**Amann, R.I., Ludwig, W. and Schleifer, K.H.,** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiology and Molecular Biology Reviews*, *59*(1), pp.143-169.

**An, L.Z., Chen, Y., Xiang, S.R., Shang, T.C. and Tian, L.D.,** 2010. Differences in community composition of bacteria in four glaciers in western China. *Biogeosciences*, *7*(6), pp.1937-1952.

**Anesio, A.M., Hodson, A.J., Fritz, A., Psenner, R. and Sattler, B.,** 2009. High microbial activity on glaciers: importance to the global carbon cycle. *Global Change Biology*, *15*(4), pp.955-960.

**Anesio, A.M., Lutz, S., Chrismas, N.A. and Benning, L.G.,** 2017. The microbiome of glaciers and ice sheets. *NPJ Biofilms and Microbiomes*, *3*(1), p.10.

**Antony, R., Krishnan, K.P., Laluraj, C.M., Thamban, M., Dhakephalkar, P.K., Engineer, A.S. and Shivaji, S.,** 2012. Diversity and physiology of culturable bacteria associated with a coastal Antarctic ice core. *Microbiological Research*, *167*(6), pp.372-380.

**Aray-Andrade, M.M., Uyaguari-Diaz, M.I. and Bermudez, J.R.,** 2018. Short-term deleterious effects of standard isolation and cultivation methods on new tropical freshwater microalgae strains. *PeerJ*, *6*, p.e5143.

**Arcarons, N., Vendrell-Flotats, M., Yeste, M., Mercade, E., López-Béjar, M. and Mogas, T.,** 2019. Cryoprotectant role of exopolysaccharide of *Pseudomonas* sp. ID1 in the

vitrification of IVM cow oocytes. *Reproduction, Fertility and Development*, *31*(9), pp.1507-1519.

**Arena, A., Gugliandolo, C., Stassi, G., Pavone, B., Iannello, D., Bisignano, G. and Maugeri, T.L.,** 2009. An exopolysaccharide produced by *Geobacillus thermodenitrificans* strain B3-72: Antiviral activity on immunocompetent cells. *Immunology Letters*, *123*(2), pp.132-137.

**Arena, A., Maugeri, T.L., Pavone, B., Iannello, D., Gugliandolo, C. and Bisignano, G.,** 2006. Antiviral and immunoregulatory effect of a novel exopolysaccharide from a marine thermotolerant *Bacillus licheniformis*. *International Immunopharmacology*, *6*(1), pp.8-13.

**Arulazhagan, P. and Vasudevan, N.,** 2011. Biodegradation of polycyclic aromatic hydrocarbons by a halotolerant bacterial strain *Ochrobactrum* sp. VA1. *Marine Pollution Bulletin*, *62*(2), pp.388-394.

**Arulazhagan, P., Vasudevan, N. and Yeom, I.T.,** 2010. Biodegradation of polycyclic aromatic hydrocarbon by a halotolerant bacterial consortium isolated from marine environment. *International Journal of Environmental Science & Technology*, *7*(4), pp.639-652.

**Aslam, S.N., Cresswell-Maynard, T., Thomas, D.N. and Underwood, G.J.,** 2012. Production and characterization of the intra-and extracellular carbohydrates and polymeric substances (EPS) of three sea-ice diatom species, and evidence for a cryoprotective role for EPS. *Journal of Phycology*, *48*(6), pp.1494-1509.

**Ates, O. and Oner, E.T.,** 2017. Microbial xanthan, levan, gellan, and curdlan as food additives. In: Gupta, V.K., Treichel, H., Shapaval, V.O., de Oliveira, L.A. and Tuohy, M.G. (eds.) *Microbial functional foods and nutraceuticals.* Chichester, UK: Wiley, pp. 149–173.

**Auch, A.F., von Jan, M., Klenk, H.P. and Göker, M.,** 2010. Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. *Standards in Genomic Sciences*, *2*(1), p.117-134.

**Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M. and Meyer, F.,** 2008. The RAST Server: rapid annotations using subsystems technology. *BMC Genomics*, *9*(1), p.75.

**Bai, Y., Yang, D., Wang, J., Xu, S., Wang, X. and An, L.,** 2006. Phylogenetic diversity of culturable bacteria from alpine permafrost in the Tianshan Mountains, northwestern China. *Research in Microbiology*, *157*(8), pp.741-751.

**Bajaj, I.B., Saudagar, P.S., Singhal, R.S. and Pandey, A.,** 2006. Statistical approach to optimization of fermentative production of gellan gum from *Sphingomonas paucimobilis* ATCC 31461. *Journal of Bioscience and Bioengineering*, *102*(3), pp.150-156.

**Bajaj, I.B., Survase, S.A., Saudagar, P.S. and Singhal, R.S.,** 2007. Gellan gum: fermentative production, downstream processing and applications. *Food Technology and Biotechnology*, *45*(4), pp.341-354.

**Bakermans, C. and Skidmore, M.,** 2011. Microbial respiration in ice at subzero temperatures (−4 ˚C to −33˚C). *Environmental Microbiology Reports*, *3*(6), pp.774-782.

**Bakermans, C.,** 2017. Determining the limits of microbial life at subzero temperatures. In: Margesin, R. (ed.) *Psychrophiles: from biodiversity to biotechnology*. Springer, Berlin, pp.21-38.

**Bakermans, C., Tsapin, A.I., Souza-Egipsy, V., Gilichinsky, D.A. and Nealson, K.H.,** 2003. Reproduction and metabolism at -10˚C of bacteria isolated from Siberian permafrost. *Environmental Microbiology, 5*(4), pp.321-326.

**Balouiri, M., Sadiki, M. and Ibnsouda, S.K.,** 2016. Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, *6*(2), pp.71-79.

**Bamford, N.C. and Howell, P.L.,** 2016. Adhesive bacterial exopolysaccharides. In: Smith, A.M. (eds.). *Biological adhesives*. Springer, Cham, pp.1-24.

**Barcelos, M.C., Vespermann, K.A., Pelissari, F.M. and Molina, G.,** 2019. Current status of biotechnological production and applications of microbial exopolysaccharides. *Critical Reviews in Food Science and Nutrition*, pp.1-21.

**Barria, C., Malecki, M. and Arraiano, C.M.,** 2013. Bacterial adaptation to cold. *Microbiology*, *159*(12), pp.2437-2443.

**Barroca, M., Santos, G., Gerday, C. and Collins, T.,** 2017. Biotechnological aspects of cold-active enzymes. In: Margesin, R. (ed.) *Psychrophiles: from biodiversity to biotechnology*, 2$^{nd}$ edn. Springer, Cham, pp.461-475.

**Bathe, S., Achouak, W., Hartmann, A., Heulin, T., Schloter, M. and Lebuhn, M.,** 2006. Genetic and phenotypic microdiversity of *Ochrobactrum* spp. *FEMS Microbiology Ecology*, *56*(2), pp.272-280.

**Batura Investigations Group,** 1979. The Batura glacier in the Karakoram Mountains and its variations. *Scientia Sinica*, *22*(8), pp.958-974.

**Bauer, A.W., Kirby, W.M.M., Sherris, J.C. and Turck, M.,** 1966. Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*, *45*(4_ts), pp.493-496.

**Becker, A., Katzen, F., Pühler, A. and Ielpi, L.,** 1998. Xanthan gum biosynthesis and application: a biochemical/genetic perspective. *Applied Microbiology and Biotechnology*, *50*(2), pp.145-152.

**Bedrossian, M., Deming, J.W. and Nadeau, J.,** 2018. Methods for collection and characterization of samples from icy environments. In: Trevors, J.T. and Gurtler, V. (eds.) *Microbiology of Atypical Environments*. Academic Press, pp. 293-321.

**Bej, A.K., Saul, D. and Aislabie, J.,** 2000. Cold-tolerant alkane-degrading *Rhodococcus* species from Antarctica. *Polar Biology, 23*(2), pp.100-105.

**Bemal, S. and Anil, A.C.,** 2017. Effects of salinity on cellular growth and exopolysaccharide production of freshwater *Synechococcus* strain CCAP1405. *Journal of Plankton Research*, *40*(1), pp.46-58.

**Beniston, M., Farinotti, D., Stoffel, M., Andreassen, L.M., Coppola, E., Eckert, N., Fantini, A., Giacona, F., Hauck, C., Huss, M. and Huwald, H.,** 2018. The European mountain cryosphere: a review of its current state, trends, and future challenges. *Cryosphere*, *12*(2), pp.759-794.

**Best, B.P.,** 2015. Cryoprotectant toxicity: facts, issues, and questions. *Rejuvenation Research*, *18*(5), pp.422-436.

**Biswas, J. and Paul, A.K.,** 2017. Diversity and production of extracellular polysaccharide by halophilic microorganisms. *Biodiversity International Journal*, *1*(2), pp.32-39.

**Boetius, A., Anesio, A.M., Deming, J.W., Mikucki, J.A. and Rapp, J.Z.,** 2015. Microbial ecology of the cryosphere: sea ice and glacial habitats. *Nature Reviews Microbiology*, *13*(11), p.677-690.

**Bolch, T., Pieczonka, T. and Benn, D.I.,** 2011. Multi-decadal mass loss of glaciers in the Everest area (Nepal Himalaya) derived from stereo imagery. *The Cryosphere*, *5*(2), pp.349-358.

**Bolger, A.M., Lohse, M. and Usadel, B.,** 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), pp.2114-2120.

**Booth, C.,** 1971. *Methods in microbiology* (Vol. 4). Academic Press, London.

**Borchert, E., Jackson, S.A., O'Gara, F. and Dobson, A.D.,** 2017. Psychrophiles as a source of novel antimicrobials. In: Margesin, R. (ed.) *Psychrophiles: from biodiversity to biotechnology*, 2nd edn. Springer, Cham, pp.527-540.

**Bowman, J.P.,** 2017. Genomics of psychrophilic bacteria and archaea. In: Margesin, R. (ed.) *Psychrophiles: from biodiversity to biotechnology*, 2nd edn. Springer, Cham, pp.345-387.

**Bowman, J.P., McCammon, S.A., Brown, M.V., Nichols, D.S. and McMeekin, T.A.,** 1997. Diversity and association of psychrophilic bacteria in Antarctic sea ice. *Applied and Environmental Microbiology*, *63*(8), pp.3068-3078.

**Bui, T.V., Ross, I.L., Jakob, G. and Hankamer, B.,** 2013. Impact of procedural steps and cryopreservation agents in the cryopreservation of chlorophyte microalgae. *PloS one*, *8*(11), p.e78668.

**Cacicedo, M.L., Castro, M.C., Servetas, I., Bosnea, L., Boura, K., Tsafrakidou, P., Dima, A., Terpou, A., Koutinas, A. and Castro, G.R.,** 2016. Progress in bacterial cellulose matrices for biotechnological applications. *Bioresource Technology*, *213*, pp.172-180.

**Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I. and Huttley, G.A.,** 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), pp.335-336.

**Cardenas, E. and Tiedje, J.M.,** 2008. New tools for discovering and characterizing microbial diversity. *Current Opinion in Biotechnology*, *19*(6), pp.544-549.

**Carrion, O., Delgado, L. and Mercade, E.,** 2015. New emulsifying and cryoprotective exopolysaccharide from Antarctic *Pseudomonas* sp. ID1. *Carbohydrate Polymers*, *117*, pp.1028-1034.

**Caruso, C., Rizzo, C., Mangano, S., Poli, A., Di Donato, P., Finore, I., Nicolaus, B., Di Marco, G., Michaud, L. and Giudice, A.L.,** 2018. Production and biotechnological potential of extracellular polymeric substances from sponge-associated Antarctic bacteria. *Applied and Environmental Microbiology*, *84*(4), pp.e01624-17.

**Casanueva, A., Tuffin, M., Cary, C. and Cowan, D.A.,** 2010. Molecular adaptations to psychrophily: the impact of 'omic' technologies. *Trends in Microbiology*, *18*(8), pp.374-381.

**Casillo, A., Parrilli, E., Filomena, S., Lindner, B., Lanzetta, R., Parrilli, M., Tutino, M. and Corsaro, M.,** 2015. Structural investigation of the oligosaccharide portion isolated from the lipooligosaccharide of the permafrost psychrophile *Psychrobacter arcticus* 273-4. *Marine Drugs*, *13*(7), pp.4539-4555.

**Casillo, A., Parrilli, E., Sannino, F., Mitchell, D.E., Gibson, M.I., Marino, G., Lanzetta, R., Parrilli, M., Cosconati, S., Novellino, E. and Randazzo, A.,** 2017. Structure-activity relationship of the exopolysaccharide from a psychrophilic bacterium: a strategy for cryoprotection. *Carbohydrate Polymers*, *156*, pp.364-371.

**Castellane, T.C.L., Campanharo, J.C., Colnago, L.A., Coutinho, I.D., Lopes, É.M., Lemos, M.V.F. and de Macedo Lemos, E.G.,** 2017. Characterization of new exopolysaccharide production by *Rhizobium tropici* during growth on hydrocarbon substrate. *International Journal of Biological Macromolecules*, *96*, pp.361-369.

**Cavicchioli, R.,** 2002. Extremophiles and the search for extraterrestrial life. *Astrobiology*, *2*(3), pp. 281-292.

**Cavicchioli, R.,** 2016. On the concept of a psychrophile. *The ISME Journal*, *10*(4), pp.793-795.

**Cavicchioli, R., Charlton, T., Ertan, H., Omar, S.M., Siddiqui, K.S. and Williams, T.J.,** 2011. Biotechnological uses of enzymes from psychrophiles. *Microbial Biotechnology*, *4*(4), pp.449-460.

**Cavicchioli, R., Siddiqui, K.S., Andrews, D. and Sowers, K.R.,** 2002. Low-temperature extremophiles and their applications. *Current Opinion in Biotechnology*, *13*(3), pp.253-261.

**Celik, G.Y., Aslim, B. and Beyatli, Y.,** 2008. Characterization and production of the exopolysaccharide (EPS) from *Pseudomonas aeruginosa* G1 and *Pseudomonas putida* G12 strains. *Carbohydrate Polymers*, *73*(1), pp.178-182.

**Chain, P.S., Lang, D.M., Comerci, D.J., Malfatti, S.A., Vergez, L.M., Shin, M., Ugalde, R.A., Garcia, E. and Tolmasky, M.E.,** 2011. Genome of *Ochrobactrum anthropi* ATCC 49188T, a versatile opportunistic pathogen and symbiont of several eukaryotic hosts. *Journal of Bacteriology, 193*(16), pp.4274-4275.

**Chattopadhyay, M. and Jagannadham, M.,** 2001. Maintenance of membrane fluidity in Antarctic bacteria. *Polar Biology*, *24*(5), pp.386-388.

**Chawla, P.R., Bajaj, I.B., Survase, S.A. and Singhal, R.S.,** 2009. Microbial cellulose: fermentative production and applications. *Food Technology & Biotechnology*, *47*(2), 107-124.

**Chayabutra, C. and Ju, L.K.,** 2000. Degradation of n-hexadecane and its metabolites by *Pseudomonas aeruginosa* under microaerobic and anaerobic denitrifying conditions. *Applied and Environmental Microbiology*, *66*(2), pp.493-498.

**Chen, F., Lu, J.R., Binder, B. and Hodson, R.E.,** 2001. Enumeration of viruses in aquatic environments using SYBR gold stain: application of digital image analysis and flow cytometer. *Applied and Environmental Microbiology*, *67*(2), pp.539-545.

**Chen, F., Wang, K., Kan, J., Bachoon, D.S., Lu, J., Lau, S. and Campbell, L.,** 2004. Phylogenetic diversity of *Synechococcus* in the Chesapeake Bay revealed by Ribulose-1, 5-bisphosphate carboxylase-oxygenase (RuBisCO) large subunit gene (rbcL) sequences. *Aquatic Microbial Ecology*, *36*(2), pp.153-164.

**Cheng, S.M. and Foght, J.M.,** 2007. Cultivation-independent and-dependent characterization of bacteria resident beneath John Evans Glacier. *FEMS Microbiology Ecology*, *59*(2), pp.318-330.

**Chian, R.C. and Quinn, P.,** 2010. Cryobiology: an overview. In: Chian, R.C. and Quinn, P. (eds.), *Fertility cryopreservation*, Cambridge: Cambridge University Press, pp. 1-9.

**Chintalapati, S., Kiran, M.D. and Shivaji, S.,** 2004. Role of membrane lipid fatty acids in cold adaptation. *Cellular and Molecular Biology (Noisy-le-Grand, France)*, *50*(5), pp.631-642.

**Chisti, Y.,** 2007. Biodiesel from microalgae. *Biotechnology Advances*, *25*(3), pp.294-306.

**Christner, B.C., Mosley-Thompson, E., Thompson, L.G., Zagorodnov, V., Sandman, K. and Reeve, J.N.,** 2000. Recovery and identification of viable bacteria immured in glacial ice. *Icarus*, *144*(2), pp.479-485.

**Christner, B.C., Mosley-Thompson, E., Thompson, L.G. and Reeve, J.N.,** 2001. Isolation of bacteria and 16S rDNAs from Lake Vostok accretion ice. *Environmental Microbiology*, *3*(9), pp.570-577.

**Christner, B.C., Mosley-Thompson, E., Thompson, L.G., Zagorodnov, V., Sandman, K. and Reeve, J.N.,** 2002. Isolation and identification of bacteria from ancient and modern ice core archives. In: Casassa, G., Sepúlveda, F.V. and Sinclair, R.M. (eds.). *The Patagonian ice fields: a unique natural laboratory for environmental and climate change studies.* Kluwer Academic/Plenum Publishers, New York. pp.9-16.

**Christner, B.C., Mosley-Thompson, E., Thompson, L.G. and Reeve, J.N.,** 2003a. Bacterial recovery from ancient glacial ice. *Environmental Microbiology*, *5*(5), pp.433-436.

**Christner, B.C., Kvitko, B.H. and Reeve, J.N.,** 2003b. Molecular identification of bacteria and eukarya inhabiting an Antarctic cryoconite hole. *Extremophiles*, *7*(3), pp.177-183.

**Collins, R.E. and Deming, J.W.,** 2013. An inter-order horizontal gene transfer event enables the catabolism of compatible solutes by *Colwellia psychrerythraea* 34H. *Extremophiles*, *17*(4), pp.601-610.

**Collins, T. and Margesin, R.,** 2019. Psychrophilic lifestyles: mechanisms of adaptation and biotechnological tools. *Applied Microbiology and Biotechnology*, *103*(7), pp.2857-2871.

**Copland, L., Sylvestre, T., Bishop, M.P., Shroder, J.F., Seong, Y.B., Owen, L.A., Bush, A. and Kamp, U.,** 2011. Expanded and recently increased glacier surging in the Karakoram. *Arctic, Antarctic, and Alpine Research*, *43*(4), pp.503-516.

**Cordero, O.X., Wildschutte, H., Kirkup, B., Proehl, S., Ngo, L., Hussain, F., Le Roux, F., Mincer, T. and Polz, M.F.,** 2012. Ecological populations of bacteria act as socially cohesive units of antibiotic production and resistance. *Science*, *337*(6099), pp.1228-1231.

**Cotter, P.D. and Hill, C.,** 2003. Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiology and Molecular Biology Reviews*, *67*(3), pp.429-453.

**Dabour, N. and LaPointe, G.,** 2005. Identification and molecular characterization of the chromosomal exopolysaccharide biosynthesis gene cluster from *Lactococcus lactis subsp. cremoris* SMQ-461. *Applied and Environmental Microbiology*, *71*(11), pp.7414-7425.

**D'Amico, S., Collins, T., Marx, J.C., Feller, G. and Gerday, C.,** 2006. Psychrophilic microorganisms: challenges for life. *EMBO Reports*, *7*(4), pp.385-389.

**Day, J.G. and DeVille, M.M.,** 1995. Cryopreservation of algae. In: Day, J.G. and Stacey G. (eds), *Cryopreservation and freeze-drying protocols*. Humana Press Inc., Totowa, NJ, pp.81-89.

**De Freitas, R.C., Odisi, E.J., Kato, C., da Silva, M.A.C. and de Souza Lima, A.O.,** 2017. Draft genome sof the deep-sea bacterium *Moritella* sp. JT01 and identification of biotechnologically relevant genes. *Marine Biotechnology*, *19*(5), pp.480-487.

**De Maayer, P., Anderson, D., Cary, C. and Cowan, D.A.,** 2014. Some like it cold: understanding the survival strategies of psychrophiles. *EMBO Reports*, *15*(5), pp.508-517.

**Deming, J.W. and Young, J.N.,** 2017. The role of exopolysaccharides in microbial adaptation to cold habitats. In: Margesin, R. (ed.), *Psychrophiles: from biodiversity to biotechnology*, 2$^{nd}$ edn. Springer, Cham, pp.259-284.

**Dieser, M., Greenwood, M. and Foreman, C.M.,** 2010. Carotenoid pigmentation in Antarctic heterotrophic bacteria as a strategy to withstand environmental stresses. *Arctic, Antarctic, and Alpine Research*, *42*(4), pp.396-405.

**Donot, F., Fontana, A., Baccou, J.C. and Schorr-Galindo, S.,** 2012. Microbial exopolysaccharides: main examples of synthesis, excretion, genetics and extraction. *Carbohydrate Polymers*, *87*(2), pp.951-962.

**Dubey, A.K. and Jeevaratnam, K.,** 2015. Structural characterization and functional evaluation of an exopolysaccharide produced by *Weissella confusa* AJ53, an isolate from fermented Uttapam batter supplemented with Piper betle L. leaves. *Food Science and Biotechnology*, *24*(6), pp.2117-2124.

**Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.T. and Smith, F.,** 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), pp.350-356.

**Ermakova, I.T., Shushkova, T.V., Sviridov, A.V., Zelenkova, N.F., Vinokurova, N.G., Baskunov, B.P. and Leontievsky, A.A.,** 2017. Organophosphonates utilization by soil

strains of *Ochrobactrum anthropi* and *Achromobacter* sp. *Archives of Microbiology*, *199*(5), pp.665-675.

**Esteves-Ferreira, A.A., Corrêa, D.M., Carneiro, A.P., Rosa, R.M., Loterio, R. and Araújo, W.L.,** 2013. Comparative evaluation of different preservation methods for cyanobacterial strains. *Journal of Applied Phycology*, *25*(4), pp.919-929.

**Ewert, M. and Deming, J.W.,** 2011. Selective retention in saline ice of extracellular polysaccharides produced by the cold-adapted marine bacterium *Colwellia psychrerythraea* strain 34H. *Annals of Glaciology*, *52*(57), pp.111-117.

**Fahy, G.M.,** 1986. The relevance of cryoprotectant "toxicity" to cryobiology. *Cryobiology*, *23*(1), pp.1-13.

**Fedotova, M.V. and Dmitrieva, O.A.,** 2016. Proline hydration at low temperatures: its role in the protection of cell from freeze-induced stress. *Amino acids*, *48*(7), pp.1685-1694.

**Feller, G. and Gerday, C.,** 2003. Psychrophilic enzymes: hot topics in cold adaptation. *Nature Reviews Microbiology*, *1*(3), p.200-208.

**Feller, G.,** 2013. Psychrophilic enzymes: from folding to function and biotechnology. *Scientifica*, *2013*.

**Feng, S., Powell, S.M., Wilson, R. and Bowman, J.P.,** 2014. Extensive gene acquisition in the extremely psychrophilic bacterial species *Psychroflexus torquis* and the link to sea-ice ecosystem specialism. *Genome Biology and Evolution*, *6*(1), pp.133-148.

**Fialho, A.M., Moreira, L.M., Granja, A.T., Popescu, A.O., Hoffmann, K. and Sá-Correia, I.,** 2008. Occurrence, production, and applications of gellan: current state and perspectives. *Applied Microbiology and Biotechnology*, *79*(6), pp.889-900.

**Fierer, N.,** 2008. Microbial biogeography: patterns in microbial diversity across space and time. In: Zengler, K. (ed.), *Accessing uncultivated microorganisms: from the environment to organisms and genomes and back*. ASM press, Washington DC, pp. 95-115.

**Foght, J., Aislabie, J., Turner, S., Brown, C.E., Ryburn, J., Saul, D.J. and Lawson, W.,** 2004. Culturable bacteria in subglacial sediments and ice from two southern hemisphere glaciers. *Microbial Ecology*, *47*(4), pp.329-340.

**Fong, N., Burgess, M., Barrow, K. and Glenn, D.,** 2001. Carotenoid accumulation in the psychrotrophic bacterium *Arthrobacter agilis* in response to thermal and salt stress. *Applied Microbiology and Biotechnology*, *56*(5-6), pp.750-756.

**Gaget, V., Chiu, Y.T., Lau, M. and Humpage, A.R.,** 2017. From an environmental sample to a long-lasting culture: the steps to better isolate and preserve cyanobacterial strains. *Journal of Applied Phycology*, *29*(1), pp.309-321.

**Ganasen, M., Yaacob, N., Rahman, R.N.Z.R.A., Leow, A.T.C., Basri, M., Salleh, A.B. and Ali, M.S.M.,** 2016. Cold-adapted organic solvent tolerant alkalophilic family I. 3 lipase from an Antarctic *Pseudomonas*. *International Journal of Biological Macromolecules*, *92*, pp.1266-1276.

**García-Cubero, R., Cabanelas, I.T.D., Sijtsma, L., Kleinegris, D.M. and Barbosa, M.J.,** 2018. Production of exopolysaccharide by *Botryococcus braunii* CCALA 778 under laboratory simulated Mediterranean climate conditions. *Algal Research*, *29*, pp.330-336.

**Garneau, M.È., Michel, C., Meisterhans, G., Fortin, N., King, T.L., Greer, C.W. and Lee, K.,** 2016. Hydrocarbon biodegradation by Arctic sea-ice and sub-ice microbial communities during microcosm experiments, Northwest Passage (Nunavut, Canada). *FEMS Microbiology Ecology*, *92*(10), p.fiw130.

**Gerday, C., Aittaleb, M., Bentahir, M., Chessa, J.P., Claverie, P., Collins, T., D'Amico, S., Dumont, J., Garsoux, G., Georlette, D. and Hoyoux, A.,** 2000. Cold-adapted enzymes: from fundamentals to biotechnology. *Trends in Biotechnology*, *18*(3), pp.103-107.

**Gerwig, G.J., Dobruchowska, J.M., Shi, T., Urashima, T., Fukuda, K. and Kamerling, J.P.,** 2013. Structure determination of the exopolysaccharide of *Lactobacillus fermentum* TDS030603—A revision. *Carbohydrate Research*, *378*, pp.84-90.

**Gilbert, J.A., Davies, P.L. and Laybourn-Parry, J.,** 2005. A hyperactive, Ca2+-dependent antifreeze protein in an Antarctic bacterium. *FEMS Microbiology Letters*, *245*(1), pp.67-72.

**Gilbert, J.A., Hill, P.J., Dodd, C.E. and Laybourn-Parry, J.,** 2004. Demonstration of antifreeze protein activity in Antarctic lake bacteria. *Microbiology*, *150*(1), pp.171-180.

**Giovannoni, S. and Stingl, U.,** 2007. The importance of culturing bacterioplankton in the'omics' age. *Nature Reviews Microbiology*, *5*(10), p.820-826.

**Goordial, J., Raymond-Bouchard, I., Zolotarov, Y., de Bethencourt, L., Ronholm, J., Shapiro, N., Woyke, T., Stromvik, M., Greer, C.W., Bakermans, C. and Whyte, L.,** 2016. Cold adaptive traits revealed by comparative genomic analysis of the

eurypsychrophile *Rhodococcus* sp. JG3 isolated from high elevation McMurdo Dry Valley permafrost, Antarctica. *FEMS Microbiology Ecology*, *92*(2), fiv154.

**Goris, J., Konstantinidis, K.T., Klappenbach, J.A., Coenye, T., Vandamme, P. and Tiedje, J.M.,** 2007. DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. *International Journal of Systematic and Evolutionary Microbiology*, *57*(1), pp.81-91.

**Govrin, R., Obstbaum, T. and Sivan, U.,** 2019. Common Source of Cryoprotection and Osmoprotection by Osmolytes. *Journal of the American Chemical Society*, *141*(34), 13311-13314.

**Gratia, E., Weekers, F., Margesin, R., D'Amico, S., Thonart, P. and Feller, G.,** 2009. Selection of a cold-adapted bacterium for bioremediation of wastewater at low temperatures. *Extremophiles*, *13*(5), pp.763-768.

**Green, J.L., Bohannan, B.J. and Whitaker, R.J.,** 2008. Microbial biogeography: from taxonomy to traits. *Science*, *320*(5879), pp.1039-1043.

**Greer, C.W. and Juck, D.F.,** 2017. Bioremediation of petroleum hydrocarbon spills in cold terrestrial environments. In: Margesin, R. (ed.), *Psychrophiles: from biodiversity to biotechnology*. Springer-Verlag, Berlin, pp.645–660.

**Gupta, R. and Deswal, R.,** 2014. Antifreeze proteins enable plants to survive in freezing conditions. *Journal of Biosciences*, *39*(5), pp.931-944.

**Habib, S., Ahmad, S.A., Johari, W., Lutfi, W., Shukor, M.Y.A. and Yasid, N.A.,** 2018. Bioremediation of petroleum hydrocarbon in Antarctica by microbial species: an overview. *Pertanika Journal of Science & Technology*, *26*(1), pp.1-20.

**Han, M., Xu, J.Z., Liu, Z.M., Qian, H. and Zhang, W.G.,** 2018. Co-production of microbial oil and exopolysaccharide by the oleaginous yeast *Sporidiobolus pararoseus* grown in fed-batch culture. *RSC Advances*, *8*(6), pp.3348-3356.

**Hanslick, J.L., Lau, K., Noguchi, K.K., Olney, J.W., Zorumski, C.F., Mennerick, S. and Farber, N.B.,** 2009. Dimethyl sulfoxide (DMSO) produces widespread apoptosis in the developing central nervous system. *Neurobiology of Disease*, *34*(1), pp.1-10.

**Hassan, N., Hasan, F., Nadeem, S., Hayat, M., Ali, P., Khan, M., Sajjad, W., Zada, S. and Rafiq, M.,** 2018. Community analysis and characterization of fungi from Batura

glacier, Karakorum mountain range, Pakistan. *Applied Ecology and Environmental Research*, *16*(5), pp.5323-5341.

**Hatosy, S.M. and Martiny, A.C.,** 2015. The ocean as a global reservoir of antibiotic resistance genes. *Applied and Environmental Microbiology*, *81*(21), pp.7593-7599.

**Hertkorn, N., Guenzl, A., Freitag, D. and Kettrup, A.,** 2002. Nuclear magnetic resonance spectroscopy investigations of silylated refractory organic substances. In: Frimmel, F.H., Abbt-Braun, G., Heumann, K.G., Hock, B., Luedemann, H.D. and Spiteller, M. (eds.) *Refractory organic substances in the environment*. Wiley-VCH, Weinheim, pp.129–145.

**Hertkorn, N., Harir, M., Koch, B., Michalke, B. and Schmitt-Kopplin, P.,** 2013. High-field NMR spectroscopy and FTICR mass spectrometry: powerful discovery tools for the molecular level characterization of marine dissolved organic matter. *Biogeosciences*, *10*(3), pp.1583-1624.

**Hewitt, K.,** 2005. The Karakoram anomaly? Glacier expansion and the 'elevation effect' Karakoram Himalaya. *Mountain Research and Development*, *25*(4), pp.332-341.

Hewitt, K. 2014. *Glaciers of the Karakoram Himalaya: Glacial environments, processes, hazards and resources*. Springer, Netherlands.

**Hoffmann, T. and Bremer, E.,** 2011. Protection of *Bacillus subtilis* against cold stress via compatible-solute acquisition. *Journal of Bacteriology*, *193*(7), pp.1552-1562.

**Hodson, A., Anesio, A.M., Tranter, M., Fountain, A., Osborn, M., Priscu, J., Laybourn-Parry, J. and Sattler, B.,** 2008. Glacial ecosystems. *Ecological Monographs*, *78*(1), pp.41-67.

**Hodson, A., Porter, P., Lowe, A. and Mumford, P.,** 2002. Chemical denudation and silicate weathering in Himalayan glacier basins: Batura Glacier, Pakistan. *Journal of Hydrology*, *262*(1-4), pp.193-208.

**Hoffmann, T. and Bremer, E.,** 2011. Protection of *Bacillus subtilis* against cold stress via compatible-solute acquisition. *Journal of Bacteriology*, *193*(7), pp.1552-1562.

**Hong Zhang, S., Gui Hou, S., Li Yang, G. and Hui Wang, J.,** 2010. Bacterial community in the East Rongbuk Glacier, Mt. Qomolangma (Everest) by culture and culture-independent methods. *Microbiological Research*, *165*(4), pp.336-345.

**Hood, E., Fellman, J., Spencer, R.G., Hernes, P.J., Edwards, R., D'Amore, D. and Scott, D.,** 2009. Glaciers as a source of ancient and labile organic matter to the marine environment. *Nature*, *462*(7276), pp.1044-1047.

**Horikoshi, K., Antranikian, G., Bull, A.T., Robb, F.T. and Stetter, K.O.** 2010. *Extremophiles handbook*. Springer, Berlin, Germany.

**Huang, G. and Huang, H.,** 2018. Application of dextran as nanoscale drug carriers. *Nanomedicine*, *13*(24), pp.3149-3158.

**Huss, M., Bookhagen, B., Huggel, C., Jacobsen, D., Bradley, R.S., Clague, J.J., Vuille, M., Buytaert, W., Cayan, D.R., Greenwood, G. and Mark, B.G.,** 2017. Toward mountains without permanent snow and ice. *Earth's Future*, *5*(5), pp.418-435.

**Hussain Wani, S., Brajendra Singh, N., Haribhushan, A. and Iqbal Mir, J.,** 2013. Compatible solute engineering in plants for abiotic stress tolerance-role of glycine betaine. *Current Genomics*, *14*(3), pp.157-165.

**Imeson, A.** 2010. *Food stabilisers, thickeners and gelling agents*. Chichester, United Kingdom: Wiley-Blackwell, pp.275-292.

**Ingraham, J.L. and Stokes, J.L.,** 1959. Psychrophilic bacteria. *Bacteriological Reviews*, *23*(3), pp.97-108.

**Ingraham, J.L.,** 1958. Growth of psychrophilic bacteria. *Journal of Bacteriology*, *76*(1), pp.75-80.

**Irvine-Fynn, T.D. and Edwards, A.,** 2014. A frozen asset: the potential of flow cytometry in constraining the glacial biome. *Cytometry Part A*, *85*(1), pp.3-7.

**Isfahani, F.M., Tahmourespour, A., Hoodaji, M., Ataabadi, M. and Mohammadi, A.,** 2018. Characterizing the new bacterial isolates of high yielding exopolysaccharides under hypersaline conditions. *Journal of Cleaner Production*, *185*, pp.922-928.

**Janczarek, M., Rachwał, K., Cieśla, J., Ginalska, G. and Bieganowski, A.,** 2015. Production of exopolysaccharide by *Rhizobium leguminosarum bv. trifolii* and its role in bacterial attachment and surface properties. *Plant and Soil*, *388*(1-2), pp.211-227.

**Jang, S.H., Kim, J., Kim, J., Hong, S. and Lee, C.,** 2012. Genome sequence of cold-adapted *Pseudomonas mandelii* strain JR-1. Journal of Bacteriology, *194*(12), p.3263.

**Jennings, L.K., Storek, K.M., Ledvina, H.E., Coulon, C., Marmont, L.S., Sadovskaya, I., Secor, P.R., Tseng, B.S., Scian, M., Filloux, A. and Wozniak, D.J.,** 2015. Pel is a

cationic exopolysaccharide that cross-links extracellular DNA in the *Pseudomonas aeruginosa* biofilm matrix. *Proceedings of the National Academy of Sciences*, *112*(36), pp.11353-11358.

**Jindal, N. and Khattar, J.S.,** 2018. Microbial polysaccharides in food industry. In: Grumezescu, A.M. and Holban A.M. (eds.) *Biopolymers for Food Design*, 1st edn. Elsevier (Academic Press), Cambridge, USA, pp. 95-123.

**Joulak, I., Finore, I., Nicolaus, B., Leone, L., Moriello, A.S., Attia, H., Poli, A. and Azabou, S.,** 2019. Evaluation of the production of exopolysaccharides by newly isolated *Halomonas* strains from Tunisian hypersaline environments. *International Journal of Biological Macromolecules, 138*, pp.658-666.

**Juck, D., Charles, T., Whyte, L.G. and Greer, C.W.,** 2000. Polyphasic microbial community analysis of petroleum hydrocarbon-contaminated soils from two northern Canadian communities. *FEMS Microbiology Ecology*, *33*(3), pp.241-249.

**Junge, K., Eicken, H. and Deming, J.W.,** 2004. Bacterial activity at −2 to −20˚C in Arctic wintertime sea ice. *Applied and Environmental Microbiology*, *70*(1), pp.550-557.

**Junge, K., Eicken, H., Swanson, B.D. and Deming, J.W.,** 2006. Bacterial incorporation of leucine into protein down to − 20˚C with evidence for potential activity in sub-eutectic saline ice formations. *Cryobiology*, *52*(3), pp.417-429.

**Junge, K., Imhoff, F., Staley, T. and Deming, W.,** 2002. Phylogenetic diversity of numerically important Arctic sea-ice bacteria cultured at subzero temperature. *Microbial Ecology*, *43*(3), pp.315-328.

**Kaleda, A., Tsanev, R., Klesment, T., Vilu, R. and Laos, K.,** 2018. Ice cream structure modification by ice-binding proteins. *Food Chemistry*, *246*, pp.164-171.

**Kalpana, R., Angelaalincy, M.J., Kamatchirajan, B.V., Vasantha, V.S., Ashokkumar, B., Ganesh, V. and Varalakshmi, P.,** 2018. Exopolysaccharide from *Bacillus cereus* VK1: Enhancement, characterization and its potential application in heavy metal removal. *Colloids and Surfaces B: Biointerfaces*, *171*, pp.327-334.

**Kämpfer, P., Huber, B., Busse, H.J., Scholz, H.C., Tomaso, H., Hotzel, H. and Melzer, F.,** 2011. *Ochrobactrum pecoris* sp. nov., isolated from farm animals. *International Journal of Systematic and Evolutionary Microbiology*, *61*(9), pp.2278-2283.

**Karlsson, J.O. and Toner, M.,** 1996. Long-term storage of tissues by cryopreservation: critical issues. *Biomaterials*, *17*(3), pp.243-256.

**Kasana, R.C., Salwan, R., Dhar, H., Dutt, S. and Gulati, A.,** 2008. A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. *Current Microbiology*, *57*(5), pp.503-507.

**Kavitha, M.,** 2016. Cold active lipases–an update. *Frontiers in Life Science*, *9*(3), pp.226-238.

**Kawahara, H.,** 2017. Cryoprotectants and ice-binding proteins. In: Margesin, R. (ed.) *Psychrophiles: from biodiversity to biotechnology*, Springer, Cham, pp.237-257.

**Kebria, D.Y., Khodadadi, A., Ganjidoust, H., Badkoubi, A. and Amoozegar, M.A.,** 2009. Isolation and characterization of a novel native *Bacillus* strain capable of degrading diesel fuel. *International Journal of Environmental Science & Technology*, *6*(3), pp.435-442.

**Kielak, A.M., Castellane, T.C., Campanharo, J.C., Colnago, L.A., Costa, O.Y., Da Silva, M.L.C., Van Veen, J.A., Lemos, E.G. and Kuramae, E.E.,** 2017. Characterization of novel Acidobacteria exopolysaccharides with potential industrial and ecological applications. *Scientific Reports*, *7*, p.41193.

**Kılıç, N.K. and Dönmez, G.,** 2008. Environmental conditions affecting exopolysaccharide production by *Pseudomonas aeruginosa*, *Micrococcus* sp., and *Ochrobactrum* sp. *Journal of Hazardous Materials*, *154*(1-3), pp.1019-1024.

**Kim, H., Lee, J., Hur, Y., Lee, C., Park, S.H. and Koo, B.W.,** 2017. Marine antifreeze proteins: structure, function, and application to cryopreservation as a potential cryoprotectant. *Marine Drugs*, *15*(2), p.27.

**Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glöckner, F.O.,** 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, *41*(1), pp.e1-e1.

**Knowles, E.J. and Castenholz, R.W.,** 2008. Effect of exogenous extracellular polysaccharides on the desiccation and freezing tolerance of rock-inhabiting phototrophic microorganisms. *FEMS Microbiology Ecology*, *66*(2), pp.261-270.

**Koh, H.Y., Park, H., Lee, J.H., Han, S.J., Sohn, Y.C. and Lee, S.G.,** 2017. Proteomic and transcriptomic investigations on cold-responsive properties of the psychrophilic Antarctic bacterium *Psychrobacter* sp. PAMC 21119 at subzero temperatures. *Environmental Microbiology*, *19*(2), pp.628-644.

**Koo, H., Hakim, J.A., Morrow, C.D., Eipers, P.G., Davila, A., Andersen, D.T. and Bej, A.K.,** 2017. Comparison of two bioinformatics tools used to characterize the microbial diversity and predictive functional attributes of microbial mats from Lake Obersee, Antarctica. *Journal of Microbiological Methods*, *140*, pp.15-22.

**Kralova, S.,** 2017. Role of fatty acids in cold adaptation of Antarctic psychrophilic *Flavobacterium* spp. *Systematic and Applied Microbiology*, *40*(6), pp.329-333.

**Krembs, C. and Deming, J.W.,** 2008. The role of exopolymers in microbial adaptation to sea ice. In: Margesin, R., Schinner, F., Marx J.C. and Gerday, C. (eds.) *Psychrophiles: from biodiversity to biotechnology*. Springer, Berlin, Heidelberg, pp.247-264.

**Krembs, C., Eicken, H. and Deming, J.W.,** 2011. Exopolymer alteration of physical properties of sea ice and implications for ice habitability and biogeochemistry in a warmer Arctic. *Proceedings of the National Academy of Sciences*, *108*(9), pp.3653-3658.

**Krembs, C., Eicken, H., Junge, K. and Deming, J.W.,** 2002. High concentrations of exopolymeric substances in Arctic winter sea ice: implications for the polar ocean carbon cycle and cryoprotection of diatoms. *Deep Sea Research Part I: Oceanographic Research Papers*, *49*(12), pp.2163-2181.

**Kuddus, M.,** 2018. Cold-active enzymes in food biotechnology: An updated mini review. *Journal of Applied Biology & Biotechnology*, *6*(3), pp.58-63.

**Kumar, A.S., Mody, K. and Jha, B.,** 2007. Bacterial exopolysaccharides–a perception. *Journal of Basic Microbiology*, *47*(2), pp.103-117.

**Kumar, K., Dasgupta, C.N., Nayak, B., Lindblad, P. and Das, D.,** 2011. Development of suitable photobioreactors for $CO_2$ sequestration addressing global warming using green algae and cyanobacteria. *Bioresource Technology*, *102*(8), pp.4945-4953.

**Kumar, R., Acharya, V., Mukhia, S., Singh, D. and Kumar, S.,** 2019. Complete genome sequence of *Pseudomonas frederiksbergensis* ERDD5: 01 revealed genetic bases for survivability at high altitude ecosystem and bioprospection potential. *Genomics*, *111*(3), pp.492-499.

**Kuyukina, M.S. and Ivshina, I.B.,** 2010. Application of *Rhodococcus* in bioremediation of contaminated environments. In: Alvarez, H. (eds.) *Biology of Rhodococcus*. Springer, Berlin, Heidelberg, pp.231-262.

**Lam, K.N., Cheng, J., Engel, K., Neufeld, J.D. and Charles, T.C.,** 2015. Current and future resources for functional metagenomics. *Frontiers in Microbiology*, *6*, p.1196.

**Landersjö, C., Yang, Z., Huttunen, E. and Widmalm, G.,** 2002. Structural studies of the Exopolysaccharide produced by *Lactobacillus rhamnosus* strain GG (ATCC 53103). *Biomacromolecules*, *3*(4), pp.880-884.

**Lasa, I.,** 2006. Towards the identification of the common features of bacterial biofilm development. *International Microbiology*, *9*(1), pp.21-28.

**Le Parc, A., Lee, H., Chen, K. and Barile, D.,** 2014. Rapid quantification of functional carbohydrates in food products. *Food and Nutrition Sciences*, *5*(1), p.71-78.

**Lee, Y.A., Kim, Y.H., Kim, B.J., Kim, B.G., Kim, K.J., Auh, J.H., Schmidt, J.A. and Ryu, B.Y.,** 2013. Cryopreservation in trehalose preserves functional capacity of murine spermatogonial stem cells. *PloS one*, *8*(1), p.e54889.

**Liang, R., Lau, M., Vishnivetskaya, T., Lloyd, K.G., Wang, W., Wiggins, J., Miller, J., Pfiffner, S., Rivkina, E.M. and Onstott, T.,** 2019. Predominance of anaerobic, spore-forming bacteria in metabolically active microbial communities from ancient Siberian permafrost. *Applied and Environmental Microbiology*, pp.AEM-00560.

**Liljeqvist, M., Ossandon, F.J., González, C., Rajan, S., Stell, A., Valdes, J., Holmes, D.S. and Dopson, M.,** 2015. Metagenomic analysis reveals adaptations to a cold-adapted lifestyle in a low-temperature acid mine drainage stream. *FEMS Microbiology Ecology*, *91*(4), p.fiv011.

**Lim, J., Thomas, T. and Cavicchioli, R.,** 2000. Low temperature regulated DEAD-box RNA helicase from the Antarctic archaeon, *Methanococcoides burtonii*. *Journal of Molecular Biology*, *297*(3), pp.553-567.

**Limoli, D.H., Jones, C.J. and Wozniak, D.J.,** 2015. Bacterial extracellular polysaccharides in biofilm formation and function. *Microbiology Spectrum*, *3*(3).

**Liu, Y., Yao, T., Jiao, N., Kang, S., Zeng, Y. and Huang, S.,** 2006. Microbial community structure in moraine lakes and glacial meltwaters, Mount Everest. *FEMS Microbiology Letters*, *265*(1), pp.98-105.

**Liu, Y., Yao, T., Jiao, N., Kang, S., Huang, S., Li, Q., Wang, K. and Liu, X.,** 2009a. Culturable bacteria in glacial meltwater at 6,350 m on the East Rongbuk Glacier, Mount Everest. *Extremophiles*, *13*(1), pp.89-99.

**Liu, Y., Yao, T., Jiao, N., Kang, S., Xu, B., Zeng, Y., Huang, S. and Liu, X**., 2009b. Bacterial diversity in the snow over Tibetan Plateau Glaciers. *Extremophiles*, *13*(3), pp.411-423.

**Liu, S.B., Chen, X.L., He, H.L., Zhang, X.Y., Xie, B.B., Yu, Y., Chen, B., Zhou, B.C. and Zhang, Y.Z.,** 2013a. Structure and ecological roles of a novel exopolysaccharide from the Arctic sea ice bacterium *Pseudoalteromonas* sp. strain SM20310. *Applied and Environmental Microbiology*, *79*(1), pp.224-230.

**Liu, Z., Zhang, F. and Chen, F.,** 2013b. High throughput screening of $CO_2$-tolerating microalgae using GasPak bags. *Aquatic Biosystems, 9*(1), p.23.

**Liu, Y., Priscu, J.C., Yao, T., Vick-Majors, T.J., Michaud, A.B. and Sheng, L.,** 2019. Culturable bacteria isolated from seven high-altitude ice cores on the Tibetan Plateau. *Journal of Glaciology*, *65*(249), pp.29-38.

**Llamas, I., Amjres, H., Mata, J.A., Quesada, E. and Béjar, V.,** 2012. The potential biotechnological applications of the exopolysaccharide produced by the halophilic bacterium *Halomonas almeriensis. Molecules*, *17*(6), pp.7103-7120.

**Lopez, E.G., Rodriguez-Lorente, I., Alcazar, P. and Cid, C.,** 2018. Microbial communities in coastal glaciers and tidewater tongues of Svalbard Archipelago, Norway. *Frontiers in Marine Science*, *5*, p.512.

**Lorenz, M., Friedl, T. and Day, J.G.,** 2005. Maintenance of actively metabolizing microalgal cultures. In: Andersen, R. A. (ed.) *Algal Culturing Techniques*, Elsevier, pp.145-156.

**Lorv, J.S., Rose, D.R. and Glick, B.R.,** 2014. Bacterial ice crystal controlling proteins. *Scientifica*, *2014*, 976895.

**Ma, Y., Wang, L. and Shao, Z.,** 2006. *Pseudomonas*, the dominant polycyclic aromatic hydrocarbon-degrading bacteria isolated from Antarctic soils and the role of large plasmids in horizontal gene transfer. *Environmental Microbiology*, *8*(3), pp.455-465.

**Maalej, H., Boisset, C., Hmidet, N., Buon, L., Heyraud, A. and Nasri, M.,** 2014. Purification and structural data of a highly substituted exopolysaccharide from *Pseudomonas stutzeri* AS22. *Carbohydrate Polymers*, *112*, pp.404-411.

**Maalej, H., Hmidet, N., Boisset, C., Buon, L., Heyraud, A. and Nasri, M.,** 2015. Optimization of exopolysaccharide production from *Pseudomonas stutzeri* AS 22 and examination of its metal-binding abilities. *Journal of Applied Microbiology*, *118*(2), pp.356-367.

**Maalej, H., Hmidet, N., Boisset, C., Bayma, E., Heyraud, A. and Nasri, M.,** 2016. Rheological and emulsifying properties of a gel-like exopolysaccharide produced by *Pseudomonas stutzeri* AS22. *Food Hydrocolloids*, *52*, pp.634-647.

**Maalej, H., Boisset, C., Hmidet, N., Colin-Morel, P., Buon, L. and Nasri, M.,** 2017. Depolymerization of *Pseudomonas stutzeri* exopolysaccharide upon fermentation as a promising production process of antibacterial compounds. *Food Chemistry*, *227*, pp.22-32.

**Mackelprang, R., Burkert, A., Haw, M., Mahendrarajah, T., Conaway, C.H., Douglas, T.A. and Waldrop, M.P.,** 2017. Microbial survival strategies in ancient permafrost: insights from metagenomics. *The ISME Journal*, *11*(10), p.2305-2318.

**MacLean, D., Jones, J.D. and Studholme, D.J.,** 2009. Application of 'next-generation' sequencing technologies to microbial genetics. *Nature Reviews Microbiology*, *7*(4), p.287-296.

**Maharana, A.K. and Singh, S.M.,** 2018. Cold active lipases produced by *Cryptococcus* Sp. Y-32 and *Rhodococcus erythropolis* N149 isolated from Nella Lake, Antarctica. *International Journal of Current Microbiology and Applied Sciences*, *7*, pp.1910-1926.

**Mahdhi, A., Leban, N., Chakroun, I., Chaouch, M.A., Hafsa, J., Fdhila, K., Mahdouani, K. and Majdoub, H.,** 2017. Extracellular polysaccharide derived from potential probiotic strain with antioxidant and antibacterial activities as a prebiotic agent to control pathogenic bacterial biofilm formation. *Microbial Pathogenesis*, *109*, pp.214-220.

**Mancuso Nichols, C.A., Garon, S., Bowman, J.P., Raguenes, G. and Guezennec, J.,** 2004. Production of exopolysaccharides by Antarctic marine bacterial isolates. *Journal of Applied Microbiology*, *96*(5), pp.1057-1066.

**Männistö, M.K. and Häggblom, M.M.,** 2006. Characterization of psychrotolerant heterotrophic bacteria from Finnish Lapland. *Systematic and Applied Microbiology*, *29*(3), pp.229-243.

**Margesin, R. and Schinner, F.,** 1998. Oil biodegradation potential in alpine habitats. *Arctic and Alpine Research*, *30*(3), pp.262-265.

**Margesin, R., Zimmerbauer, A. and Schinner, F.,** 1999. Soil lipase activity–a useful indicator of oil biodegradation. *Biotechnology Techniques*, *13*(12), pp.859-863.

**Margesin, R. and Schinner, F.,** 2001a. Bioremediation (natural attenuation and biostimulation) of diesel-oil-contaminated soil in an alpine glacier skiing area. *Applied and Environmental Microbiology*, *67*(7), pp.3127-3133.

**Margesin, R. and Schinner, F.,** 2001b. Biodegradation and bioremediation of hydrocarbons in extreme environments. *Applied Microbiology and Biotechnology*, *56*(5-6), pp.650-663.

**Margesin, R., Zacke, G. and Schinner, F.,** 2002. Characterization of heterotrophic microorganisms in alpine glacier cryoconite. *Arctic, Antarctic, and Alpine Research*, *34*(1), pp.88-93.

**Margesin, R., Gander, S., Zacke, G., Gounot, A.M. and Schinner, F.,** 2003. Hydrocarbon degradation and enzyme activities of cold-adapted bacteria and yeasts. *Extremophiles*, *7*(6), pp.451-458.

**Margesin, R., Neuner, G. and Storey, K.B.,** 2007. Cold-loving microbes, plants, and animals—fundamental and applied aspects. *Naturwissenschaften*, *94*(2), pp.77-99.

**Margesin, R. and Feller, G.,** 2010. Biotechnological applications of psychrophiles. *Environmental Technology*, *31*(8-9), pp.835-844.

**Margesin, R. and Miteva, V.,** 2011. Diversity and ecology of psychrophilic microorganisms. *Research in Microbiology*, *162*(3), pp.346-361.

**Marx, J.G., Carpenter, S.D. and Deming, J.W.,** 2009. Production of cryoprotectant extracellular polysaccharide substances (EPS) by the marine psychrophilic bacterium *Colwellia psychrerythraea* strain 34H under extreme conditions. *Canadian journal of microbiology*, *55*(1), pp.63-72.

**Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D. and Verstraete, W.,** 2008. How to get more out of molecular fingerprints: practical tools for microbial ecology. *Environmental Microbiology*, *10*(6), pp.1571-1581.

**Mata, J.A., Béjar, V., Bressollier, P., Tallon, R., Urdaci, M.C., Quesada, E. and Llamas, I.,** 2008. Characterization of exopolysaccharides produced by three moderately halophilic bacteria belonging to the family Alteromonadaceae. *Journal of Applied Microbiology*, *105*(2), pp.521-528.

**McSwain, B.S., Irvine, R.L., Hausner, M. and Wilderer, P.A.,** 2005. Composition and distribution of extracellular polymeric substances in aerobic flocs and granular sludge. *Applied and Environmental Microbiology*, *71*(2), pp.1051-1057.

**Meier-Kolthoff, J.P., Auch, A.F., Klenk, H.P. and Göker, M.,** 2013. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics*, *14*(1), p.60.

**Menéndez, E., Ramírez-Bahena, M.H., Fabryová, A., Igual, J.M., Benada, O., Mateos, P.F., Peix, A., Kolařík, M. and García-Fraile, P.,** 2015. *Pseudomonas coleopterorum* sp. nov., a cellulase-producing bacterium isolated from the bark beetle *Hylesinus fraxini*. *International Journal of Systematic and Evolutionary Microbiology*, *65*(9), pp.2852-2858.

**Merino, N., Aronson, H.S., Bojanova, D.P., Feyhl-Buska, J., Wong, M.L., Zhang, S. and Giovannelli, D.,** 2019. Living at the extremes: extremophiles and the limits of life in a planetary context. *Frontiers in Microbiology*, *10*, p.780.

**Methe, B.A., Nelson, K.E., Deming, J.W., Momen, B., Melamud, E., Zhang, X., Moult, J., Madupu, R., Nelson, W.C., Dodson, R.J. and Brinkac, L.M.,** 2005. The psychrophilic lifestyle as revealed by the genome sequence of *Colwellia psychrerythraea* 34H through genomic and proteomic analyses. *Proceedings of the National Academy of Sciences*, *102*(31), pp.10913-10918.

**Meyer, A.F., Lipson, D.A., Martin, A.P., Schadt, C.W. and Schmidt, S.K.,** 2004. Molecular and metabolic characterization of cold-tolerant alpine soil *Pseudomonas sensu stricto*. *Applied and Environmental Microbiology, 70*(1), pp.483-489.

**Miladi, H., Elabed, H., Slama, R.B., Rhim, A. and Bakhrouf, A.,** 2017. Molecular analysis of the role of osmolyte transporters opuCA and betL in *Listeria monocytogenes* after cold and freezing stress. *Archives of Microbiology*, *199*(2), pp.259-265.

**Minora, U., Bocchiola, D., D'Agata, C., Maragno, D., Mayer, C., Lambrecht, A., Vuillermoz, E., Senese, A., Compostella, C., Smiraglia, C. and Diolaiuti, G.A.,** 2016. Glacier area stability in the Central Karakoram National Park (Pakistan) in 2001–2010: the "Karakoram Anomaly" in the spotlight. *Progress in Physical Geography*, *40*(5), pp.629-660.

**Miri, S., Naghdi, M., Rouissi, T., Kaur Brar, S. and Martel, R.,** 2019. Recent biotechnological advances in petroleum hydrocarbons degradation under cold climate conditions: A review. *Critical Reviews in Environmental Science and Technology*, *49*(7), pp.553-586.

**Mishra, A. and Jha, B.,** 2013. Microbial exopolysaccharides. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E. and Thompson, F. (eds.) *The Prokaryotes*. Springer, Berlin, Heidelberg, pp.179-192.

**Miteva, V., Teacher, C., Sowers, T. and Brenchley, J.,** 2009. Comparison of the microbial diversity at different depths of the GISP2 Greenland ice core in relationship to deposition climates. *Environmental Microbiology*, *11*(3), pp.640-656.

**Miteva, V.I., Sheridan, P.P. and Brenchley, J.E.,** 2004. Phylogenetic and physiological diversity of microorganisms isolated from a deep Greenland glacier ice core. *Applied and Environmental Microbiology*, *70*(1), pp.202-213.

**Mobin, S. and Alam, F.,** 2017. Some promising microalgal species for commercial applications: A review. *Energy Procedia*, *110*, pp.510-517.

**Mobin, S.M., Chowdhury, H. and Alam, F.,** 2019. Commercially important bioproducts from microalgae and their current applications–A review. *Energy Procedia*, *160*, pp.752-760.

**Mocali, S., Chiellini, C., Fabiani, A., Decuzzi, S., de Pascale, D., Parrilli, E., Tutino, M.L., Perrin, E., Bosi, E., Fondi, M. and Giudice, A.L.,** 2017. Ecology of cold environments: new insights of bacterial metabolic adaptation through an integrated genomic-phenomic approach. *Scientific Reports*, *7*(1), p.839.

**Mohite, B.V., Koli, S.H., Narkhede, C.P., Patil, S.N. and Patil, S.V.,** 2017. Prospective of microbial exopolysaccharide for heavy metal exclusion. *Applied Biochemistry and Biotechnology*, *183*(2), pp.582-600.

**More, T.T., Yadav, J.S.S., Yan, S., Tyagi, R.D. and Surampalli, R.Y.,** 2014. Extracellular polymeric substances of bacteria and their potential environmental applications. *Journal of Environmental Management*, *144*, pp.1-25.

**Moriello, V.S., Lama, L., Poli, A., Gugliandolo, C., Maugeri, T.L., Gambacorta, A. and Nicolaus, B.,** 2003. Production of exopolysaccharides from a thermophilic microorganism isolated from a marine hot spring in flegrean areas. *Journal of Industrial Microbiology and Biotechnology*, *30*(2), pp.95-101.

**Morita, R.Y.,** 1975. Psychrophilic bacteria. *Bacteriological Reviews*, *39*(2), p.144-167.

**Moscovici, M.,** 2015. Present and future medical applications of microbial exopolysaccharides. *Frontiers in Microbiology*, *6*, p.1012.

**Munoz, P.A., Márquez, S.L., González-Nilo, F.D., Márquez-Miranda, V. and Blamey, J.M.,** 2017. Structure and application of antifreeze proteins from Antarctic bacteria. *Microbial Cell Factories*, *16*(1), p.138.

**Mykytczuk, N.C., Foote, S.J., Omelon, C.R., Southam, G., Greer, C.W. and Whyte, L.G**., 2013. Bacterial growth at − 15˚C; molecular insights from the permafrost bacterium *Planococcus halocryophilus* Or1. *The ISME Journal*, *7*(6), p.1211-1226.

**Nakanishi, K., Deuchi, K. and Kuwano, K.,** 2012. Cryopreservation of four valuable strains of microalgae, including viability and characteristics during 15 years of cryostorage. *Journal of Applied Phycology*, *24*(6), pp.1381-1385.

**Nichols, C.M., Bowman, J.P. and Guezennec, J.,** 2005a. Effects of incubation temperature on growth and production of exopolysaccharides by an Antarctic sea ice bacterium grown in batch culture. *Applied and Environmental Microbiology*, *71*(7), pp.3519-3523.

**Nichols, C.M., Guezennec, J. and Bowman, J.P.,** 2005b. Bacterial exopolysaccharides from extreme marine environments with special consideration of the southern ocean, sea ice, and deep-sea hydrothermal vents: a review. *Marine Biotechnology*, *7*(4), pp.253-271.

**Nicolaus, B., Kambourova, M. and Oner, E.T.,** 2010. Exopolysaccharides from extremophiles: from fundamentals to biotechnology. *Environmental Technology*, *31*(10), pp.1145-1158.

**Novak, M. and Vetvicka, V.,** 2009. Glucans as biological response modifiers. *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders)*, *9*(1), pp.67-75.

**Nunn, B.L., Slattery, K.V., Cameron, K.A., Timmins-Schiffman, E. and Junge, K**., 2015. Proteomics of *Colwellia psychrerythraea* at subzero temperatures–a life with limited movement, flexible membranes and vital DNA repair. *Environmental Microbiology*, *17*(7), pp.2319-2335.

**Nurk, S., Bankevich, A., Antipov, D., Gurevich, A.A., Korobeynikov, A., Lapidus, A., Prjibelski, A.D., Pyshkin, A., Sirotkin, A., Sirotkin, Y. and Stepanauskas, R.,** 2013. Assembling single-cell genomes and mini-metagenomes from chimeric MDA products. *Journal of Computational Biology*, *20*(10), pp.714-737.

**O'Brien, A., Sharp, R., Russell, N.J. and Roller, S.,** 2004. Antarctic bacteria inhibit growth of food-borne microorganisms at low temperatures. *FEMS Microbiology Ecology*, *48*(2), pp.157-167.

**Olijve, L.L., Meister, K., DeVries, A.L., Duman, J.G., Guo, S., Bakker, H.J. and Voets, I.K.,** 2016. Blocking rapid ice crystal growth through non-basal plane adsorption of antifreeze proteins. *Proceedings of the National Academy of Sciences*, *113*(14), pp.3740-3745.

**Oner, E.T., Hernández, L. and Combie, J.,** 2016. Review of levan polysaccharide: from a century of past experiences to future prospects. *Biotechnology Advances*, *34*(5), pp.827-844.

**Pagani, I., Liolios, K., Jansson, J., Chen, I.M.A., Smirnova, T., Nosrat, B., Markowitz, V.M. and Kyrpides, N.C.,** 2011. The Genomes OnLine Database (GOLD) v. 4: status of genomic and metagenomic projects and their associated metadata. *Nucleic Acids Research*, *40*(D1), pp.D571-D579.

**Panoff, J.M., Corroler, D., Thammavongs, B. and Boutibonnes, P.,** 1997. Differentiation between cold shock proteins and cold acclimation proteins in a mesophilic

gram-positive bacterium, *Enterococcus faecalis* JH2-2. *Journal of Bacteriology*, *179*(13), pp.4451-4454.

**Parmar, A., Singh, N.K., Pandey, A., Gnansounou, E. and Madamwar, D.,** 2011. Cyanobacteria and microalgae: a positive prospect for biofuels. *Bioresource Technology*, *102*(22), pp.10163-10172.

**Passow, U.,** 2000. Formation of transparent exopolymer particles, TEP, from dissolved precursor material. *Marine Ecology Progress Series*, *192*, pp.1-11.

**Peix, A., Ramirez-Bahena, M.H. and Velazquez, E.,** 2018. The current status on the taxonomy of *Pseudomonas* revisited: an update. *Infection, Genetics and Evolution*, *57*, pp.106-116.

**Peterszegi, G., Isnard, N., Robert, A.M. and Robert, L.,** 2003. Studies on skin aging. Preparation and properties of fucose-rich oligo-and polysaccharides. Effect on fibroblast proliferation and survival. *Biomedicine & pharmacotherapy*, *57*(5-6), pp.187-194.

**Petri, D.F.,** 2015. Xanthan gum: A versatile biopolymer for biomedical and technological applications. *Journal of Applied Polymer Science*, *132*(23).

**Pfeffer, W.T., Arendt, A.A., Bliss, A., Bolch, T., Cogley, J.G., Gardner, A.S., Hagen, J.O., Hock, R., Kaser, G., Kienholz, C. and Miles, E.S.,** 2014. The Randolph Glacier Inventory: a globally complete inventory of glaciers. *Journal of Glaciology*, *60*(221), pp.537-552.

**Phadtare, S.,** 2004. Recent developments in bacterial cold-shock response. *Current Issues in Molecular Biology*, *6*(2), pp.125-136.

**Pikuta, E.V., Hoover, R.B. and Tang, J.,** 2007. Microbial extremophiles at the limits of life. *Critical Reviews in Microbiology*, *33*(3), pp.183-209.

**Pires, J.C.,** 2019. Biosequestration of carbon dioxide from flue gases by algae. In: Hosseini, M. (ed.) *Advances in feedstock conversion technologies for alternative fuels and bioproducts*. Woodhead Publishing, pp.105-118.

**Poli, A., Anzelmo, G. and Nicolaus, B.,** 2010. Bacterial exopolysaccharides from extreme marine habitats: production, characterization and biological activities. *Marine Drugs*, *8*(6), pp.1779-1802.

**Poli, A., Esposito, E., Orlando, P., Lama, L., Giordano, A., de Appolonia, F., Nicolaus, B. and Gambacorta, A.,** 2007. *Halomonas alkaliantarctica* sp. nov., isolated from saline

lake Cape Russell in Antarctica, an alkalophilic moderately halophilic, exopolysaccharide-producing bacterium. *Systematic and Applied Microbiology*, *30*(1), pp.31-38.

**Prajapati, V.D., Jani, G.K., Zala, B.S. and Khutliwala, T.A.,** 2013. An insight into the emerging exopolysaccharide gellan gum as a novel polymer. *Carbohydrate Polymers*, *93*(2), pp.670-678.

**Prakash, O., Shouche, Y., Jangid, K. and Kostka, J.E.,** 2013. Microbial cultivation and the role of microbial resource centers in the omics era. *Applied Microbiology and Biotechnology*, *97*(1), pp.51-62.

**Priest, F.G.,** 1977. Extracellular enzyme synthesis in the genus *Bacillus*. *Bacteriological Reviews*, *41*(3), pp.711-753.

**Priscu, J.C. and Christner, B.C.,** 2004. Earth's icy biosphere. In: Bull, A.T. (ed.) *Microbial diversity and bioprospecting*. Washington, DC: ASM Press, pp.130-145.

**Priscu, J.C., Christner, B.C., Foreman, C.M. and Royston-Bishop, G.,** 2007. Biological material in ice cores. *Encyclopedia of Quaternary Sciences*, *2*, pp.1156-1166.

**Prosser, J.I.,** 2015. Dispersing misconceptions and identifying opportunities for the use of 'omics' in soil microbial ecology. *Nature Reviews Microbiology*, *13*(7), p.439-446.

**Pulschen, A.A., Bendia, A.G., Fricker, A.D., Pellizari, V.H., Galante, D. and Rodrigues, F.,** 2017. Isolation of uncultured bacteria from Antarctica using long incubation periods and low nutritional media. *Frontiers in Microbiology*, *8*, p.1346.

**Qin, G., Zhu, L., Chen, X., Wang, P.G. and Zhang, Y.,** 2007. Structural characterization and ecological roles of a novel exopolysaccharide from the deep-sea psychrotolerant bacterium *Pseudoalteromonas* sp. SM9913. *Microbiology*, *153*(5), pp.1566-1572.

**Quatrini, P., Scaglione, G., De Pasquale, C., Riela, S. and Puglia, A.M.,** 2008. Isolation of Gram-positive n-alkane degraders from a hydrocarbon-contaminated Mediterranean shoreline. *Journal of Applied Microbiology*, *104*(1), pp.251-259.

**Rabbani, G. and Choi, I.,** 2018. Roles of osmolytes in protein folding and aggregation in cells and their biotechnological applications. *International Journal of Biological Macromolecules*, *109*, pp.483-491.

**Rafiq, M., Hayat, M., Anesio, A.M., Jamil, S.U.U., Hassan, N., Shah, A.A. and Hasan, F.,** 2017. Recovery of metallo-tolerant and antibiotic resistant psychrophilic bacteria from Siachen glacier, Pakistan. *PloS one*, *12*(7), p.e0178180.

**Rampelotto, P.,** 2013. Extremophiles and extreme environments. *Life (Basel), 3*(3), 482-485.

**Rankl, M., Kienholz, C. and Braun, M.,** 2014. Glacier changes in the Karakoram region mapped by multimission satellite imagery. *The Cryosphere*, *8*(3), pp.977-989.

**Rapp, P. and Gabriel-Jürgens, L.H.,** 2003. Degradation of alkanes and highly chlorinated benzenes, and production of biosurfactants, by a psychrophilic *Rhodococcus* sp. and genetic characterization of its chlorobenzene dioxygenase. *Microbiology*, *149*(10), pp.2879-2890.

**Rassner, S.M., Anesio, A.M., Girdwood, S.E., Hell, K., Gokul, J.K., Whitworth, D.E. and Edwards, A.,** 2016. Can the bacterial community of a high Arctic glacier surface escape viral control?. *Frontiers in Microbiology*, *7*, p.956.

**Rasul, G., Chaudhry, Q.Z., Mahmood, A., Hyder, K.W. and Dahe, Q.,** 2011. Glaciers and glacial lakes under changing climate in Pakistan. *Pakistan Journal of Meteorology*, *8*(15), pp.1-8.

**Raymond-Bouchard, I., Goordial, J., Zolotarov, Y., Ronholm, J., Stromvik, M., Bakermans, C. and Whyte, L.G.,** 2018. Conserved genomic and amino acid traits of cold adaptation in subzero-growing Arctic permafrost bacteria. *FEMS Microbiology Ecology*, *94*(4), p.fiy023.

**Raymond, J.A., Fritsen, C. and Shen, K.,** 2007. An ice-binding protein from an Antarctic sea ice bacterium. *FEMS Microbiology Ecology*, *61*(2), pp.214-221.

**Reasoner, D.J. and Geldreich, E.E.,** 1985. A new medium for the enumeration and subculture of bacteria from potable water. *Applied and Environmental Microbiology*, *49*(1), pp.1-7.

**Reddy, P.V.V., Rao, S.S.S.N., Pratibha, M.S., Sailaja, B., Kavya, B., Manorama, R.R., Singh, S.M., Srinivas, T.N.R. and Shivaji, S.,** 2009. Bacterial diversity and bioprospecting for cold-active enzymes from culturable bacteria associated with sediment from a melt water stream of Midtre Lovenbreen glacier, an Arctic glacier. *Research in Microbiology*, *160*(8), pp.538-546.

**Rehm, B.H.** 2009. *Microbial production of biopolymers and polymer precursors: applications and perspectives*. Caister Academic Press.

**Rekadwad, B. and Khobragade, C.,** 2017. Marine polyextremophiles and their biotechnological applications. In: Kalia, V.C. and Kumar, P. (eds.) *Microbial applications vol. 1: bioremediation and bioenergy*. Springer, Cham, pp 319–331.

**Remminghorst, U. and Rehm, B.H**., 2006. Bacterial alginates: from biosynthesis to applications. *Biotechnology Letters*, *28*(21), pp.1701-1712.

**Richter, M. and Rosselló-Móra, R.,** 2009. Shifting the genomic gold standard for the prokaryotic species definition. *Proceedings of the National Academy of Sciences*, *106*(45), pp.19126-19131.

**Riffaldi, R., Levi-Minzi, R., Cardelli, R., Palumbo, S. and Saviozzi, A.,** 2006. Soil biological activities in monitoring the bioremediation of diesel oil-contaminated soil. *Water, Air, and Soil Pollution*, *170*(1-4), pp.3-15.

**Riley, M., Staley, J.T., Danchin, A., Wang, T.Z., Brettin, T.S., Hauser, L.J., Land, M.L. and Thompson, L.S.,** 2008. Genomics of an extreme psychrophile, *Psychromonas ingrahamii*. *BMC Genomics*, *9*(1), p.210.

**Rodrigues, D.F. and Tiedje, J.M.,** 2008. Coping with our cold planet. *Applied and Environmental Microbiology*, *74*(6), pp.1677-1686.

**Rong, J.C., Liu, Y., Yu, S., Xi, L., Chi, N.Y. and Zhang, Q.F.,** 2019. Complete genome sequence of *Paenisporosarcina antarctica* CGMCC 1.6503 T, a marine psychrophilic bacterium isolated from Antarctica. *Marine Genomics, 49,* (In press).

**Rossi, F. and De Philippis, R.,** 2015. Role of cyanobacterial exopolysaccharides in phototrophic biofilms and in complex microbial mats. *Life*, *5*(2), pp.1218-1238.

**Rothschild, L.J. and Mancinelli, R.L., 2001.** Life in extreme environments. *Nature*, *409*(6823), pp.1092-1101.

**Ruberto, L.A., Vazquez, S., Lobalbo, A. and Mac Cormack, W.P.,** 2005. Psychrotolerant hydrocarbon-degrading *Rhodococcus* strains isolated from polluted Antarctic soils. *Antarctic Science*, *17*(1), pp.47-56.

**Russell, N.J.,** 1990. Cold adaptation of microorganisms. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, *326*(1237), pp.595-611.

**Russell, N.J.,** 1998. Molecular adaptations in psychrophilic bacteria: potential for biotechnological applications. In: Antranikian, G. (ed.) *Biotechnology of extremophiles:*

*Advances in biochemical engineering/biotechnology*. Springer, Berlin, Heidelberg, pp.1-21.

**Russell, N.J.,** 2000. Toward a molecular understanding of cold activity of enzymes from psychrophiles. *Extremophiles*, *4*(2), pp.83-90.

**Saadaoui, I., Al Emadi, M., Bounnit, T., Schipper, K. and Al Jabri, H.,** 2016. Cryopreservation of microalgae from desert environments of Qatar. *Journal of Applied Phycology*, *28*(4), pp.2233-2240.

**Saitou, N. and Nei, M.,** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), pp.406-425.

**Salwan, R., Gulati, A. and Kasana, R.C.,** 2010. Phylogenetic diversity of alkaline protease-producing psychrotrophic bacteria from glacier and cold environments of Lahaul and Spiti, India. *Journal of Basic Microbiology*, *50*(2), pp.150-159.

**Samain, E., Miles, M., Bozzi, L., Dubreucq, G. and Rinaudo, M.,** 1997. Simultaneous production of two different gel-forming exopolysaccharides by an *Alteromonas* strain originating from deep sea hydrothermal vents. *Carbohydrate Polymers*, *34*(4), pp.235-241.

**Sanchez, L.A., Gómez, F.F. and Delgado, O.D.,** 2009. Cold-adapted microorganisms as a source of new antimicrobials. *Extremophiles*, *13*(1), pp.111-120.

**Santiago, M., Ramírez-Sarmiento, C.A., Zamora, R.A. and Parra, L.P.,** 2016. Discovery, molecular mechanisms, and industrial applications of cold-active enzymes. *Frontiers in Microbiology*, *7*, p.1408.

**Sardar, U.R., Bhargavi, E., Devi, I., Bhunia, B. and Tiwari, O.N.,** 2018. Advances in exopolysaccharides based bioremediation of heavy metals in soil and water: a critical review. *Carbohydrate Polymers*, *199*, pp.353-364.

**Sardari, R.R., Kulcinskaja, E., Ron, E.Y., Björnsdóttir, S., Friðjónsson, Ó.H., Hreggviðsson, G.Ó. and Karlsson, E.N.,** 2017. Evaluation of the production of exopolysaccharides by two strains of the thermophilic bacterium *Rhodothermus marinus*. *Carbohydrate Polymers*, *156*, pp.1-8.

**Sathiyanarayanan, G., Bhatia, S.K., Kim, H.J., Kim, J.H., Jeon, J.M., Kim, Y.G., Park, S.H., Lee, S.H., Lee, Y.K. and Yang, Y.H.,** 2016. Metal removal and reduction potential of an exopolysaccharide produced by Arctic psychrotrophic bacterium *Pseudomonas* sp. PAMC 28620. *RSC Advances*, *6*(99), pp.96870-96881.

**Schmid, J., Sieber, V. and Rehm, B.,** 2015. Bacterial exopolysaccharides: biosynthesis pathways and engineering strategies. *Frontiers in Microbiology*, *6*, p.496.

**Schmid, J., Fariña, J., Rehm, B. and Sieber, V.,** 2016. Microbial exopolysaccharides: From genes to applications. *Frontiers in Microbiology*, *7*, p.308.

**Schmidt-Nielsen, S.,** 1902. Ueber einige psychrophile Mikroorganismen und ihr Vorkommen. *Zentr. Bakteriol. Parasitenkd. Infektionsk. Hyg. Abt.*, *9*, pp.145-147.

**Seckbach, J., Oren, A. and Stan-Lotter, H.** 2013. *Polyextremophiles: life under multiple forms of stress*. Springer Science & Business Media.

**Seel, W., Flegler, A., Zunabovic-Pichler, M. and Lipski, A.,** 2018. Increased isoprenoid quinone concentration modulates membrane fluidity in *Listeria monocytogenes* at low growth temperatures. *Journal of Bacteriology*, *200*(13), pp.e00148-18.

**Segawa, T., Yoshimura, Y., Watanabe, K., Kanda, H. and Kohshima, S.,** 2011. Community structure of culturable bacteria on surface of Gulkana Glacier, Alaska. *Polar Science*, *5*(1), pp.41-51.

**Segawa, T., Takeuchi, N., Rivera, A., Yamada, A., Yoshimura, Y., Barcaza, G., Shinbori, K., Motoyama, H., Kohshima, S. and Ushida, K.,** 2013. Distribution of antibiotic resistance genes in glacier environments. *Environmental Microbiology Reports*, *5*(1), pp.127-134.

**Seviour, T., Lambert, L.K., Pijuan, M. and Yuan, Z.,** 2010. Structural determination of a key exopolysaccharide in mixed culture aerobic sludge granules using NMR spectroscopy. *Environmental Science & Technology*, *44*(23), pp.8964-8970.

**Shen, L., Liu, Y., Wang, N., Jiao, N., Xu, B. and Liu, X.,** 2018. Variation with depth of the abundance, diversity and pigmentation of culturable bacteria in a deep ice core from the Yuzhufeng Glacier, Tibetan Plateau. *Extremophiles*, *22*(1), pp.29-38.

**Sheridan, P.P., Miteva, V.I. and Brenchley, J.E.,** 2003. Phylogenetic analysis of anaerobic psychrophilic enrichment cultures obtained from a Greenland glacier ice core. *Applied and Environmental Microbiology*, *69*(4), pp.2153-2160.

**Shivaji, S. and Prakash, J.S.,** 2010. How do bacteria sense and respond to low temperature? *Archives of Microbiology*, *192*(2), pp.85-95.

**Shivaji, S., Pratibha, M.S., Sailaja, B., Kishore, K.H., Singh, A.K., Begum, Z., Anarasi, U., Prabagaran, S.R., Reddy, G.S.N. and Srinivas, T.N.R.,** 2011. Bacterial

diversity of soil in the vicinity of Pindari glacier, Himalayan mountain ranges, India, using culturable bacteria and soil 16S rRNA gene clones. *Extremophiles*, *15*(1), pp.1-22.

**Shivaji, S., Begum, Z., Rao, S.S.S.N., Reddy, P.V.V.V., Manasa, P., Sailaja, B., Prathiba, M.S., Thamban, M., Krishnan, K.P., Singh, S.M. and Srinivas, T.N.,** 2013. Antarctic ice core samples: culturable bacterial diversity. *Research in Microbiology*, *164*(1), pp.70-82.

**Shroder, J.F.,** 1984. Batura glacier terminus, 1984, Karakoram Himalaya. *Geological Bulletin University of Peshawar*, *17*, pp.119-126.

**Siddiqui, K.S. and Cavicchioli, R.,** 2006. Cold-adapted enzymes. *Annual Review of Biochemistry*, *75*, pp.403-433.

**Siddiqui, K.S.,** 2015. Some like it hot, some like it cold: Temperature dependent biotechnological applications and improvements in extremophilic enzymes. *Biotechnology Advances*, *33*(8), pp.1912-1922.

**Siliakus, M.F., van der Oost, J. and Kengen, S.W.,** 2017. Adaptations of archaeal and bacterial membranes to variations in temperature, pH and pressure. *Extremophiles*, *21*(4), pp.651-670.

**Silkina, A., Nelson, G.D., Bayliss, C.E., Pooley, C.L. and Day, J.G.,** 2017. Bioremediation efficacy—comparison of nutrient removal from an anaerobic digest waste-based medium by an algal consortium before and after cryopreservation. *Journal of Applied Phycology*, *29*(3), pp.1331-1341.

**Silva, T.R., Duarte, A.W., Passarini, M.R., Ruiz, A.L.T., Franco, C.H., Moraes, C.B., de Melo, I.S., Rodrigues, R.A., Fantinatti-Garboggini, F. and Oliveira, V.M.,** 2018. Bacteria from Antarctic environments: diversity and detection of antimicrobial, antiproliferative, and antiparasitic activities. *Polar Biology*, *41*(7), pp.1505-1519.

**Simon, C., Wiezer, A., Strittmatter, A.W. and Daniel, R.,** 2009. Phylogenetic diversity and metabolic potential revealed in a glacier ice metagenome. *Applied and Environmental Microbiology*, *75*(23), pp.7519-7526.

**Madhavan, A., Sindhu, R., Parameswaran, B., Sukumaran, R.K. and Pandey, A.,** 2017. Metagenome analysis: a powerful tool for enzyme bioprospecting. *Applied Biochemistry and Biotechnology*, *183*(2), pp.636-651.

**Singh, S. and Kumar, V.,** 2019. Mercury detoxification by absorption, mercuric ion reductase, and exopolysaccharides: a comprehensive study. *Environmental Science and Pollution Research*, pp.1-21.

**Sirajunnisa, A.R., Vijayagopal, V., Sivaprakash, B., Viruthagiri, T. and Surendhiran, D.,** 2016. Optimization, kinetics and antioxidant activity of exopolysaccharide produced from rhizosphere isolate, *Pseudomonas fluorescens* CrN6. *Carbohydrate Polymers*, *135*, pp.35-43.

**Sleight, S.C., Wigginton, N.S. and Lenski, R.E.,** 2006. Increased susceptibility to repeated freeze-thaw cycles in *Escherichia coli* following long-term evolution in a benign environment. *BMC Evolutionary Biology*, *6*(1), p.104.

**Squillaci, G., Finamore, R., Diana, P., Restaino, O.F., Schiraldi, C., Arbucci, S., Ionata, E., La Cara, F. and Morana, A.,** 2016. Production and properties of an exopolysaccharide synthesized by the extreme halophilic archaeon *Haloterrigena turkmenica*. *Applied Microbiology and Biotechnology*, *100*(2), pp.613-623.

**Srikanth, R., Reddy, C.H.S., Siddartha, G., Ramaiah, M.J. and Uppuluri, K.B.,** 2015. Review on production, characterization and applications of microbial levan. *Carbohydrate Polymers*, *120*, pp.102-114.

**Staudt, A.K., Wolfe, L.G. and Shrout, J.D.,** 2012. Variations in exopolysaccharide production by *Rhizobium tropici*. *Archives of Microbiology*, *194*(3), pp.197-206.

**Steven, B., Briggs, G., McKay, C.P., Pollard, W.H., Greer, C.W. and Whyte, L.G.,** 2007. Characterization of the microbial diversity in a permafrost sample from the Canadian high Arctic using culture-dependent and culture-independent methods. *FEMS Microbiology Ecology*, *59*(2), pp.513-523.

**Stibal, M., Telling, J., Cook, J., Mak, K.M., Hodson, A. and Anesio, A.M.,** 2012. Environmental controls on microbial abundance and activity on the Greenland ice sheet: a multivariate analysis approach. *Microbial Ecology*, *63*(1), pp.74-84.

**Stroud, J.L., Paton, G.I. and Semple, K.T.,** 2007. Microbe-aliphatic hydrocarbon interactions in soil: implications for biodegradation and bioremediation. *Journal of Applied Microbiology*, *102*(5), pp.1239-1253.

**Struvay, C. and Feller, G.,** 2012. Optimization to low temperature activity in psychrophilic enzymes. *International Journal of Molecular Sciences*, *13*(9), pp.11643-11665.

**Sun, K., Chen, Y., Niu, Q., Zhu, W., Wang, B., Li, P. and Ge, X.,** 2016. An exopolysaccharide isolated from a coral-associated fungus and its sulfated derivative activates macrophages. *International Journal of Biological Macromolecules*, *82*, pp.387-394.

**Sutherland, I.W.,** 1972. Bacterial exopolysaccharides. In: Rose, A.H. and Tempest, D.W. (Eds.) *Advances in microbial physiology*. Academic Press, London – New York, pp.143–213.

**Sutherland, I.W.,** 1982. Biosynthesis of microbial exopolysaccharides. In: Rose, A.H. and Morris, J.G. (eds.) *Advances in microbial physiology*. Academic Press, London, pp.79–150.

**Sutherland, I.W.,** 2001a. Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology*, *147*(1), pp.3-9.

**Sutherland, I.W.,** 2001b. Microbial polysaccharides from Gram-negative bacteria. *International Dairy Journal*, *11*(9), pp.663-674.

**Sutton, R.L.,** 1991. Critical cooling rates to avoid ice crystallization in solutions of cryoprotective agents. *Journal of the Chemical Society, Faraday Transactions*, *87*(1), pp.101-105.

**Tallon, R., Bressollier, P. and Urdaci, M.C.,** 2003. Isolation and characterization of two exopolysaccharides produced by *Lactobacillus plantarum* EP56. *Research in Microbiology*, *154*(10), pp.705-712.

**Tam, H.K., Wong, C.M.V.L., Yong, S.T., Blamey, J. and González, M.,** 2015. Multiple-antibiotic-resistant bacteria from the maritime Antarctic. *Polar Biology*, *38*(8), pp.1129-1141.

**Tamaru, Y., Takani, Y., Yoshida, T. and Sakamoto, T.,** 2005. Crucial role of extracellular polysaccharides in desiccation and freezing tolerance in the terrestrial cyanobacterium *Nostoc commune*. *Applied and Environmental Microbiology*, *71*(11), pp.7327-7333.

**Tanaka, D., Yoneda, S., Yamashiro, Y., Sakatoku, A., Kayashima, T., Yamakawa, K. and Nakamura, S.,** 2012. Characterization of a new cold-adapted lipase from *Pseudomonas* sp. TK-3. *Applied Biochemistry and Biotechnology*, *168*(2), pp.327-338.

**Tang, X.Z., Kumar, P., Alavi, S. and Sandeep, K.P.,** 2012. Recent advances in biopolymers and biopolymer-based nanocomposites for food packaging materials. *Critical Reviews in Food Science and Nutrition*, *52*(5), pp.426-442.

**Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E.P., Zaslavsky, L., Lomsadze, A., Pruitt, K.D., Borodovsky, M. and Ostell, J.,** 2016. NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Research*, *44*(14), pp.6614-6624.

**Tedesco, P., Maida, I., Palma Esposito, F., Tortorella, E., Subko, K., Ezeofor, C., Zhang, Y., Tabudravu, J., Jaspars, M., Fani, R. and de Pascale, D.,** 2016. Antimicrobial activity of monoramnholipids produced by bacterial strains isolated from the Ross Sea (Antarctica). *Marine Drugs*, *14*(5), p.83.

**Thakur, V., Kumar, V., Kumar, S. and Singh, D.,** 2018. Diverse culturable bacterial communities with cellulolytic potential revealed from pristine habitat in Indian trans-Himalaya. *Canadian Journal of Microbiology*, *64*(11), pp.798-808.

**Theerachat, M., Guieysse, D., Morel, S., Remaud-Siméon, M. and Chulalaksananukul, W.,** 2019. Laccases from marine organisms and their applications in the biodegradation of toxic and environmental pollutants: a review. *Applied Biochemistry and Biotechnology*, *187*(2), pp.583-611.

**Tomova, I., Stoilova-Disheva, M., Lazarkevich, I. and Vasileva-Tonkova, E.,** 2015. Antimicrobial activity and resistance to heavy metals and antibiotics of heterotrophic bacteria isolated from sediment and soil samples collected from two Antarctic islands. *Frontiers in Life Science*, *8*(4), pp.348-357.

**Torstensson, A., Young, J.N., Carlson, L.T., Ingalls, A.E. and Deming, J.W.,** 2019. Use of exogenous glycine betaine and its precursor choline as osmoprotectants in Antarctic sea-ice diatoms. *Journal of Phycology*, *55*(3), pp.663-675.

**Tosco, A., Birolo, L., Madonna, S., Lolli, G., Sannia, G. and Marino, G.,** 2003. GroEL from the psychrophilic bacterium *Pseudoalteromonas haloplanktis* TAC 125: molecular characterization and gene cloning. *Extremophiles*, *7*(1), pp.17-28.

**Toska, J., Ho, B.T. and Mekalanos, J.J.,** 2018. Exopolysaccharide protects Vibrio cholerae from exogenous attacks by the type 6 secretion system. *Proceedings of the National Academy of Sciences*, *115*(31), pp.7997-8002.

**Tribelli, P. and López, N.,** 2018. Reporting key features in cold-adapted bacteria. *Life*, *8*(1), p.8.

**Tuorto, S.J., Darias, P., McGuinness, L.R., Panikov, N., Zhang, T., Häggblom, M.M. and Kerkhof, L.J.,** 2014. Bacterial genome replication at subzero temperatures in permafrost. *The ISME Journal*, *8*(1), pp.139-149.

**Turska-Szewczuk, A., Duda, K., Schwudke, D., Pekala, A., Kozinska, A. and Holst, O.,** 2014. Structural studies of the lipopolysaccharide from the fish pathogen *Aeromonas veronii* strain Bs19, serotype O16. *Marine Drugs*, *12*(3), pp.1298-1316.

**Van Goethem, M.W., Pierneef, R., Bezuidt, O.K., Van De Peer, Y., Cowan, D.A. and Makhalanyane, T.P.,** 2018. A reservoir of 'historical' antibiotic resistance genes in remote pristine Antarctic soils. *Microbiome*, *6*(1), p.40.

**Varin, T., Lovejoy, C., Jungblut, A.D., Vincent, W.F. and Corbeil, J.,** 2012. Metagenomic analysis of stress genes in microbial mat communities from Antarctica and the High Arctic. *Applied and Environmental Microbiology*, *78*(2), pp.549-559.

**Vasquez-Ponce, F., Higuera-Llantén, S., Pavlov, M.S., Ramírez-Orellana, R., Marshall, S.H. and Olivares-Pacheco, J.,** 2017. Alginate overproduction and biofilm formation by psychrotolerant *Pseudomonas mandelii* depend on temperature in Antarctic marine sediments. *Electronic Journal of Biotechnology*, *28*, pp.27-34.

**Vester, J.K., Glaring, M.A. and Stougaard, P.,** 2015. Improved cultivation and metagenomics as new tools for bioprospecting in cold environments. *Extremophiles*, *19*(1), pp.17-29.

**Wadham, J.L., Tranter, M., Tulaczyk, S. and Sharp, M.,** 2008. Subglacial methanogenesis: a potential climatic amplifier? *Global Biogeochemical Cycles*, *22*(2).

**Wang, X., Hua, T.C., Sun, D.W., Liu, B., Yang, G. and Cao, Y.,** 2007. Cryopreservation of tissue-engineered dermal replacement in $Me_2SO$: Toxicity study and effects of concentration and cooling rates on cell viability. *Cryobiology*, *55*(1), pp.60-65.

**Wang, J., Wang, J., Zhang, Z., Li, Y., Zhang, B., Zhang, Z. and Zhang, G.,** 2016. Cold-adapted bacteria for bioremediation of crude oil-contaminated soil. *Journal of Chemical Technology & Biotechnology*, *91*(8), pp.2286-2297.

**Wang, L., Zhang, H., Yang, L., Liang, X., Zhang, F. and Linhardt, R.J.,** 2017. Structural characterization and bioactivity of exopolysaccharide synthesized by *Geobacillus* sp. TS3-9 isolated from radioactive radon hot spring. *Advances in Biotechnology & Microbiology*, *4*, pp.1-8.

**Waterbury, J.B. and Willey, J.M.,** 1988. Isolation and growth of marine planktonic cyanobacteria. *Methods in enzymology, 167*, pp.100-105.

**Wen, Y.Z., Su, B.X., Lyu, S.S., Hide, G., Lun, Z.R. and Lai, D.H.,** 2016. Trehalose, an easy, safe and efficient cryoprotectant for the parasitic protozoan *Trypanosoma brucei*. *Acta Tropica*, *164*, pp.297-302.

**Whyte, L.G., Hawari, J., Zhou, E., Bourbonnière, L., Inniss, W.E. and Greer, C.W.,** 1998. Biodegradation of variable-chain-length alkanes at low temperatures by a psychrotrophic *Rhodococcus* sp. *Applied and Environmental Microbiology*, *64*(7), pp.2578-2584.

**Whyte, L.G., Slagman, S.J., Pietrantonio, F., Bourbonniere, L., Koval, S.F., Lawrence, J.R., Inniss, W.E. and Greer, C.W.,** 1999. Physiological adaptations involved in alkane assimilation at a low temperature by *Rhodococcus* sp. strain Q15. Applied and Environmental Microbiology, *65*(7), pp.2961-2968.

**Wilhelm, L., Singer, G.A., Fasching, C., Battin, T.J. and Besemer, K.,** 2013. Microbial biodiversity in glacier-fed streams. *The ISME Journal*, *7*(8), p.1651-1660.

**Wowk, B.,** 2007. How cryoprotectants work. *Cryonic*, *28*, pp.3-7.

**Xiang, S., Yao, T., An, L., Xu, B. and Wang, J.,** 2005. 16S rRNA sequences and differences in bacteria isolated from the Muztag Ata glacier at increasing depths. *Applied and Environmental Microbiology*, *71*(8), pp.4619-4627.

**Xiang, S.R., Shang, T.C., Chen, Y., Jing, Z.F. and Yao, T.,** 2009. Dominant bacteria and biomass in the Kuytun 51 Glacier. *Applied and Environmental Microbiology, 75*(22), pp.7287-7290.

**Xiong, J.Q., Kurade, M.B. and Jeon, B.H.,** 2018. Can microalgae remove pharmaceutical contaminants from water? *Trends in Biotechnology*, *36*(1), pp.30-44.

**Xu, J., Grumbine, R.E., Shrestha, A., Eriksson, M., Yang, X., Wang, Y.U.N. and Wilkes, A.,** 2009. The melting Himalayas: cascading effects of climate change on water, biodiversity, and livelihoods. *Conservation Biology*, *23*(3), pp.520-530.

**Xu, Y., Jiao, N. and Chen, F.,** 2015. Novel psychrotolerant picocyanobacteria isolated from Chesapeake Bay in the winter. *Journal of Phycology*, *51*(4), pp.782-790.

**Yakimov, M.M., Giuliano, L., Bruni, V., Scarfi, S. and Golyshin, P.N.,** 1999. Characterization of Antarctic hydrocarbon-degrading bacteria capable of producing bioemulsifiers. *The New Microbiologica*, *22*(3), pp.249-256.

**Yancey, P.H., Clark, M.E., Hand, S.C., Bowlus, R.D. and Somero, G.N.,** 1982. Living with water stress: evolution of osmolyte systems. *Science*, *217*(4566), pp.1214-1222.

**Yang, G.L., Hou, S.G., Le Baoge, R., Li, Z.G., Xu, H., Liu, Y.P., Du, W.T. and Liu, Y.Q.,** 2016. Differences in bacterial diversity and communities between glacial snow and glacial soil on the chongce ice cap, west Kunlun Mountains. *Scientific Reports*, *6*, p.36548.

**Yang, S.T.** 2011. *Bioprocessing for value-added products from renewable resources: new technologies and applications*. Elsevier, Amsterdam.

**Yergeau, E., Hogues, H., Whyte, L.G. and Greer, C.W.,** 2010. The functional potential of high Arctic permafrost revealed by metagenomic sequencing, qPCR and microarray analyses. *The ISME Journal*, *4*(9), pp.1206-1214.

**Yoon, S.H., Ha, S.M., Kwon, S., Lim, J., Kim, Y., Seo, H. and Chun, J.,** 2017a. Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *International Journal of Systematic and Evolutionary Microbiology*, *67*(5), p.1613-1617.

**Yoon, S.H., Ha, S.M., Lim, J., Kwon, S. and Chun, J.,** 2017b. A large-scale evaluation of algorithms to calculate average nucleotide identity. *Antonie Van Leeuwenhoek*, *110*(10), pp.1281-1286.

**Yoshida, K., Hashimoto, M., Hori, R., Adachi, T., Okuyama, H., Orikasa, Y., Nagamine, T., Shimizu, S., Ueno, A. and Morita, N.,** 2016. Bacterial long-chain polyunsaturated fatty acids: their biosynthetic genes, functions, and practical use. *Marine Drugs*, *14*(5), p.94.

**Zannini, E., Waters, D.M., Coffey, A. and Arendt, E.K.,** 2016. Production, properties, and industrial food application of lactic acid bacteria-derived exopolysaccharides. *Applied Microbiology and Biotechnology*, *100*(3), pp.1121-1135.

**Zbikowska, H.M.,** 2003. Fish can be first–advances in fish transgenesis for commercial applications. *Transgenic Research*, *12*(4), pp.379-389.

**Zdanowski, M.K., Żmuda-Baranowska, M.J., Borsuk, P., Świątecki, A., Górniak, D., Wolicka, D., Jankowska, K.M. and Grzesiak, J.,** 2013. Culturable bacteria community development in postglacial soils of Ecology Glacier, King George Island, Antarctica. *Polar Biology*, *36*(4), pp.511-527.

**Zeng, R., Zhang, R., Zhao, J. and Lin, N.,** 2003. Cold-active serine alkaline protease from the psychrophilic bacterium *Pseudomonas* strain DY-A: enzyme purification and characterization. *Extremophiles*, *7*(4), pp.335-337.

**Zeng, Y.X., Yan, M., Yu, Y., Li, H.R., He, J.F., Sun, K. and Zhang, F.,** 2013. Diversity of bacteria in surface ice of Austre Lovénbreen glacier, Svalbard. *Archives of Microbiology*, *195*(5), pp.313-322.

**Zhan, X. and Zhang, H.,** 2017. Curdlan Oligosaccharides: Production and application. In: Chen, J., Zhu, Y. and Liu, S. (eds.) *Functional carbohydrates: Development, characterization and biomanufacture*. CRC Press, pp.43-92.

**Zhang, Y.Z., Zhang, S.C., Liu, X.Z., Xu, Y.J., Hu, J.H., Xu, Y.Y., Li, J. and Chen, S.L.,** 2005. Toxicity and protective efficiency of cryoprotectants to flounder (*Paralichthys olivaceus*) embryos. *Theriogenology*, *63*(3), pp.763-773.

**Zhang, X.H., Zhang, G.S., Zhang, Z.H., Jian-Hong, X.U. and Shun-Peng, L.I.,** 2006. Isolation and characterization of a dichlorvos-degrading strain DDV-1 of *Ochrobactrum* sp. *Pedosphere*, *16*(1), pp.64-71.

**Zhang, G., Ma, X., Niu, F., Dong, M., Feng, H., An, L. and Cheng, G.,** 2007. Diversity and distribution of alkaliphilic psychrotolerant bacteria in the Qinghai–Tibet Plateau permafrost region. *Extremophiles*, *11*(3), pp.415-424.

**Zhang, X.F., Yao, T.D., Tian, L.D., Xu, S.J. and An, L.Z.,** 2008a. Phylogenetic and physiological diversity of bacteria isolated from Puruogangri ice core. *Microbial Ecology*, *55*(3), pp.476-488.

**Zhang, S., Hou, S., Wu, Y. and Qin, D.,** 2008b. Bacteria in Himalayan glacial ice and its relationship to dust. *Biogeosciences*, *5*(6), pp.1741-1750.

**Zhang, Z., Khan, N.M., Nunez, K.M., Chess, E.K. and Szabo, C.M.,** 2012. Complete monosaccharide analysis by high-performance anion-exchange chromatography with pulsed amperometric detection. *Analytical Chemistry*, *84*(9), pp.4104-4110.

**Zhang, D.C., Brouchkov, A., Griva, G., Schinner, F. and Margesin, R.,** 2013. Isolation and characterization of bacteria from ancient Siberian permafrost sediment. *Biology*, *2*(1), pp.85-106.

**Zhang, G., Yao, T., Xie, H., Wang, W. and Yang, W.,** 2015. An inventory of glacial lakes in the Third Pole region and their changes in response to global warming. *Global and Planetary Change*, *131*, pp.148-157.

**Zhang, B., Wu, X., Zhang, W., Chen, X., Zhang, G., Ai, X., Sun, L., Zhang, B., Liu, G., Chen, T. and Dyson, P.,** 2016. Diversity and succession of Actinobacteria in the forelands of the Tianshan Glacier, China. *Geomicrobiology Journal*, *33*(8), pp.716-723.

**Zhang, C., Guo, W., Wang, Y. and Chen, X.,** 2018a. Draft Genome sequences of two psychrotolerant strains, *Colwellia polaris* MCCC 1C00015T and *Colwellia chukchiensis* CGMCC 1.9127 T. *Genome Announcement*, *6*(4), pp.e01575-17.

**Zhang, C., Guo, W., Wang, Y. and Chen, X.,** 2018b. The complete genome sequence of *Colwellia* sp. NB097-1 reveals evidence for the potential genetic basis for its adaptation to cold environment. *Marine Genomics*, *37*, pp.54-57.

**Zhang, J., Liu, L., Ren, Y. and Chen, F.,** 2019. Characterization of exopolysaccharides produced by microalgae with antitumor activity on human colon cancer cells. *International Journal of Biological Macromolecules*, *128*, pp.761-767.

**Zhao, Y., Song, C., Dong, H., Luo, Y., Wei, Y., Gao, J., Wu, Q., Huang, Y., An, L. and Sheng, H.,** 2018a. Community structure and distribution of culturable bacteria in soil along an altitudinal gradient of Tianshan Mountains, China. *Biotechnology & Biotechnological Equipment*, *32*(2), pp.397-407.

**Zhao, F., Guo, C., Cui, Q., Hao, Q., Xiu, J., Han, S. and Zhang, Y.,** 2018b. Exopolysaccharide production by an indigenous isolate *Pseudomonas stutzeri* XP1 and its application potential in enhanced oil recovery. *Carbohydrate Polymers*, *199*, pp.375-381.

**Zheng, Y., Li, Y., Liu, W., Chen, C.C., Ko, T.P., He, M., Xu, Z., Liu, M., Luo, H., Guo, R.T. and Yao, B.,** 2016. Structural insight into potential cold adaptation mechanism through a psychrophilic glycoside hydrolase family 10 endo-β-1, 4-xylanase. *Journal of Structural Biology*, *193*(3), pp.206-211.

**Zhu, Y., Romain, C. and Williams, C.K.,** 2016. Sustainable polymers from renewable resources. *Nature*, *540*(7633), pp.354-362.

**Zhu, Y., Wang, C., Jia, S., Wang, B., Zhou, K., Chen, S., Yang, Y. and Liu, S.,** 2018. Purification, characterization and antioxidant activity of the exopolysaccharide from *Weissella cibaria* SJ14 isolated from Sichuan paocai. *International Journal of Biological Macromolecules*, *115*, pp.820-828.

**Zolfaghar, M., Amoozegar, M.A., Khajeh, K., Babavalian, H. and Tebyanian, H.,** 2019. Isolation and screening of extracellular anticancer enzymes from halophilic and halotolerant bacteria from different saline environments in Iran. *Molecular Biology Reports*, *46*(3), pp.3275-3286.

## Appendices

Table A1. Composition of tryptic soy agar (Oxoid).

| **Constituent** | **Quantity (g/L)** |
|---|---|
| Pancreatic digest of casein | 17.0 |
| Enzymatic digest of soya bean | 3.0 |
| Sodium chloride | 5.0 |
| Dipotassium hydrogen phosphate | 2.5 |
| Glucose | 2.5 |
| Agar | 15.0 |

Table A2. Composition of Luria Bertani agar (Miller).

| **Constituent** | **Quantity (g/L)** |
|---|---|
| Tryptone | 10.0 |
| Yeast extract | 5.0 |
| Sodium chloride | 10.0 |
| Agar | 15.0 |

Table A3. Composition of R2A (Difco).

| **Constituent** | **Quantity (g/L)** |
|---|---|
| Yeast extract | 0.5 |
| Proteose peptone | 0.5 |
| Casamino acids | 0.5 |
| Dextrose | 0.5 |
| Soluble starch | 0.5 |
| Sodium pyruvate | 0.3 |
| Dipotassium phosphate | 0.3 |
| Magnesium sulfate | 0.05 |
| Agar | 15.0 |

Table A4. Specifications of the culture media used for nutritional requirement test.

| Media | Manufacturer | Lot# | Expiry Date |
|---|---|---|---|
| R2A | Difco | - | - |
| Tryptic Soya Agar | Oxoid | 1435041 | 12/2018 |
| Nutrient Agar | Oxoid | 1315904 | 03/2018 |
| Luria Bertani Agar | Oxoid | - | - |
| Sabouraud Dextrose Agar | Oxoid | 1163557 | 03/2017 |
| MacConkey Agar | Oxoid | 1123606 | 11/2016 |
| Mueller Hinton Agar | Oxoid | 1626184 | 02/2020 |
| Mannitol Salt Agar | Oxoid | 1217383 | 07/2017 |

Table A5. Specifications of the commercial antibiotics used for antibiotic susceptibility test.

| Antibiotics | Manufacturer | Lot# | Expiry Date |
|---|---|---|---|
| Methicillin (10 µg) | Bioanalyse | 141211F | 02/2016 |
| Trimethoprim/Sulfamethoxazole (25 µg) | Liofelchem | 061614066 | 06/2016 |
| Colistin (10 µg) | Bioanalyse | 140102 | 06/2016 |
| Nalidixic Acid (30 µg) | Liofelchem | 112911028 | 11/2013 |
| Ofloxacin (5 µg) | Liofelchem | 040414071 | 04/2016 |
| Vancomycin (5 µg) | Liofelchem | 032714074 | 03/2016 |
| Imipenem (10 µg) | Liofelchem | 102214049 | 10/2016 |

Table A6. Composition of carboxymethylcellulose (CMC) agar.

| Constituent | Quantity (%) |
|---|---|
| Sodium nitrate | 0.2 |
| Dipotassium hydrogen phosphate | 0.1 |
| Magnesium sulfate | 0.05 |
| Potassium chloride | 0.05 |
| CMC sodium salt | 0.2 |
| Peptone | 0.02 |
| Agar | 1.7 |

Table A7. Growth ($OD_{600}$) of *Pseudomonas* sp. BGI-2 at different temperatures.

| **Time (Hours)** | **4˚C** | | | **15˚C** | | | **25˚C** | | | **35˚C** | | | **45˚C** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 0 | 0.085 | 0.087 | 0.086 | 0.084 | 0.082 | 0.083 | 0.087 | 0.082 | 0.085 | 0.09 | 0.09 | 0.09 | 0.085 | 0.083 | 0.084 |
| 24 | 0.481 | 0.455 | 0.468 | 3.94 | 4.18 | 4.06 | 4.89 | 4.62 | 4.76 | 3.15 | 2.97 | 3.06 | 0.171 | 0.134 | 0.152 |
| 48 | 2.22 | 2.26 | 2.24 | 7.48 | 7.50 | 7.49 | 8.35 | 8.17 | 8.26 | 4.21 | 3.95 | 4.08 | 0.151 | 0.08 | 0.12 |
| 72 | 4.01 | 4.15 | 4.08 | 7.76 | 7.62 | 7.69 | 8.93 | 8.69 | 8.81 | 3.79 | 3.66 | 3.73 | 0.101 | 0.098 | 0.099 |
| 96 | 5.83 | 6.07 | 5.95 | 7.56 | 7.62 | 7.59 | 7.57 | 7.84 | 7.71 | 3.28 | 3.17 | 3.23 | 0.101 | 0.10 | 0.10 |
| 120 | 6.47 | 6.40 | 6.44 | 7.15 | 7.39 | 7.27 | 7.39 | 7.52 | 7.46 | 3.11 | 2.84 | 2.98 | 0.097 | 0.095 | 0.096 |
| 144 | 6.76 | 6.71 | 6.74 | 7.08 | 7.25 | 7.17 | 6.75 | 7.12 | 6.94 | 2.33 | 2.19 | 2.26 | 0.087 | 0.095 | 0.091 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A8. Growth ($OD_{600}$) of *Pseudomonas* sp. BGI-2 at different pH.

| **Time (Hours)** | **5** | | | **6** | | | **7** | | | **8** | | | **9** | | | **10** | | | **11** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 0 | 0.09 | 0.08 | 0.09 | 0.093 | 0.091 | 0.092 | 0.095 | 0.094 | 0.095 | 0.114 | 0.119 | 0.117 | 0.122 | 0.124 | 0.123 | 0.199 | 0.191 | 0.195 | 0.245 | 0.244 | 0.245 |
| 24 | 2.56 | 2.54 | 2.55 | 3.29 | 3.26 | 3.28 | 3.51 | 3.45 | 3.48 | 3.64 | 3.47 | 3.55 | 3.24 | 3.31 | 3.28 | 2.98 | 2.99 | 2.99 | 1.62 | 1.58 | 1.6 |
| 48 | 5.19 | 5.98 | 5.59 | 5.84 | 6.07 | 5.96 | 6.35 | 6.45 | 6.40 | 7.07 | 6.94 | 7.01 | 6.68 | 6.59 | 6.64 | 6.06 | 6.05 | 6.06 | 4.98 | 4.57 | 4.78 |
| 72 | 6.96 | 6.94 | 6.95 | 7.00 | 7.08 | 7.04 | 6.88 | 6.72 | 6.80 | 7.15 | 6.92 | 7.035 | 6.30 | 6.44 | 6.37 | 5.62 | 5.70 | 5.66 | 4.84 | 4.81 | 4.83 |
| 96 | 6.70 | 6.71 | 6.71 | 6.70 | 6.87 | 6.79 | 6.68 | 6.64 | 6.66 | 6.64 | 6.71 | 6.68 | 6.18 | 5.80 | 5.99 | 5.51 | 5.67 | 5.59 | 4.86 | 4.71 | 4.79 |
| 120 | 6.44 | 6.63 | 6.54 | 6.62 | 6.64 | 6.63 | 6.57 | 6.25 | 6.41 | 6.00 | 6.33 | 6.17 | 5.66 | 5.32 | 5.49 | 4.87 | 4.90 | 4.89 | 3.94 | 3.79 | 3.85 |
| 144 | 6.18 | 6.31 | 6.25 | 6.58 | 6.43 | 6.51 | 6.25 | 6.05 | 6.15 | 5.48 | 5.55 | 5.52 | 5.21 | 4.91 | 5.06 | 3.87 | 4.06 | 3.97 | 3.65 | 3.00 | 3.33 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A9. Growth ($OD_{600}$) of *Pseudomonas* sp. BGI-2 at different salinities.

| Time (Hours) | Control | | | 1% NaCl | | | 3% NaCl | | | 5% NaCl | | | 7% NaCl | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R1 | R2 | M | R1 | R2 | M | R1 | R2 | M | R1 | R2 | M | R1 | R2 | M |
| 0 | 0.088 | 0.106 | 0.097 | 0.082 | 0.089 | 0.086 | 0.106 | 0.112 | 0.109 | 0.109 | 0.111 | 0.11 | 0.131 | 0.09 | 0.112 |
| 24 | 4.10 | 4.38 | 4.24 | 3.90 | 3.91 | 3.91 | 2.28 | 2.48 | 2.38 | 0.204 | 0.24 | 0.22 | 0.105 | 0.088 | 0.097 |
| 48 | 7.16 | 7.24 | 7.20 | 7.58 | 6.63 | 7.11 | 4.99 | 4.91 | 4.95 | 0.798 | 1.00 | 0.89 | 0.093 | 0.077 | 0.085 |
| 72 | 7.31 | 7.25 | 7.28 | 7.77 | 8.03 | 7.90 | 6.76 | 6.44 | 6.60 | 1.96 | 2.13 | 2.05 | 0.080 | 0.075 | 0.077 |
| 96 | 6.96 | 7.03 | 6.99 | 7.79 | 7.50 | 7.645 | 6.66 | 6.56 | 6.61 | 3.05 | 2.92 | 2.99 | 0.070 | 0.058 | 0.064 |
| 120 | 6.45 | 5.61 | 6.03 | 7.46 | 7.46 | 7.46 | 6.53 | 6.58 | 6.56 | 3.35 | 2.78 | 3.07 | 0.059 | 0.057 | 0.058 |
| 144 | 5.88 | 5.54 | 5.71 | 7.47 | 7.16 | 7.315 | 6.46 | 6.72 | 6.59 | 3.14 | 3.35 | 3.25 | 0.055 | 0.051 | 0.053 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A10. Growth ($OD_{600}$) of *Pseudomonas* sp. BGI-2 at different concentrations of molasses.

| Time (Hours) | Control | | | 1% Molasses | | | 3% Molasses | | | 5% Molasses | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R1 | R2 | M | R1 | R2 | M | R1 | R2 | M | R1 | R2 | M |
| 0 | 0.093 | 0.093 | 0.093 | 0.124 | 0.129 | 0.127 | 0.153 | 0.174 | 0.164 | 0.497 | 0.499 | 0.498 |
| 24 | 3.71 | 3.69 | 3.70 | 4.16 | 3.68 | 3.92 | 3.65 | 4.09 | 3.87 | 4.28 | 4.40 | 4.34 |
| 48 | 6.71 | 6.88 | 6.79 | 7.80 | 7.20 | 7.50 | 7.66 | 7.97 | 7.82 | 8.78 | 7.85 | 8.32 |
| 72 | 6.58 | 7.08 | 6.83 | 6.77 | 7.01 | 6.89 | 7.33 | 7.25 | 7.29 | 8.88 | 8.12 | 8.50 |
| 96 | 6.01 | 6.61 | 6.31 | 6.63 | 6.48 | 6.56 | 7.62 | 7.26 | 7.44 | 8.02 | 7.78 | 7.90 |
| 120 | 5.84 | 5.88 | 5.86 | 6.16 | 6.21 | 6.19 | 6.85 | 6.93 | 6.89 | 7.42 | 7.51 | 7.47 |
| 144 | 5.14 | 5.18 | 5.16 | 5.35 | 5.44 | 5.39 | 5.78 | 5.39 | 5.59 | 6.89 | 6.82 | 6.86 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A11. Growth ($OD_{600}$) of *Pseudomonas* sp. BGI-2 at different carbon substrates.

| Time (Hours) | Control | | | 1% Glucose | | | 1% Galactose | | | 1% Mannose | | | 1% Mannitol | | | 1% Glycerol | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 0 | 0.076 | 0.074 | 0.075 | 0.082 | 0.079 | 0.08 | 0.078 | 0.075 | 0.077 | 0.077 | 0.076 | 0.077 | 0.078 | 0.077 | 0.079 | 0.084 | 0.085 | 0.085 |
| 24 | 0.074 | 0.071 | 0.0725 | 1.31 | 1.27 | 1.29 | 0.74 | 0.77 | 0.755 | 0.95 | 0.94 | 0.945 | 0.85 | 0.82 | 0.835 | 1.17 | 1.13 | 1.15 |
| 48 | 0.065 | 0.068 | 0.0665 | 2.56 | 2.49 | 2.525 | 2.37 | 2.41 | 2.39 | 2.22 | 2.18 | 2.2 | 2.32 | 2.27 | 2.295 | 2.46 | 2.44 | 2.45 |
| 72 | 0.074 | 0.073 | 0.0735 | 3.56 | 3.49 | 3.525 | 3.51 | 3.4 | 3.455 | 3.19 | 3.11 | 3.15 | 3.34 | 3.21 | 3.275 | 3.47 | 3.35 | 3.41 |
| 96 | 0.08 | 0.077 | 0.0785 | 3.69 | 3.75 | 3.72 | 3.54 | 3.49 | 3.515 | 3.37 | 3.29 | 3.33 | 3.45 | 3.39 | 3.42 | 3.42 | 3.5 | 3.46 |
| 120 | 0.075 | 0.077 | 0.076 | 3.61 | 3.52 | 3.565 | 3.52 | 3.44 | 3.48 | 3.32 | 3.17 | 3.245 | 3.35 | 3.24 | 3.295 | 3.43 | 3.27 | 3.35 |
| 144 | 0.068 | 0.071 | 0.0695 | 3.45 | 3.36 | 3.405 | 3.3 | 3.19 | 3.245 | 3.22 | 3.19 | 3.205 | 3.26 | 3.18 | 3.22 | 3.34 | 3.25 | 3.295 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A12. Production of EPS by *Pseudomonas* sp. BGI-2 at different temperatures, using phenol sulfuric acid method.

| Time (Hours) | 4˚C | | | 15˚C | | | 25˚C | | | 35˚C | | | 45˚C | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 0 | 98.67 | 89.67 | 94.17 | 93 | 103 | 98 | 92 | 103 | 97.5 | 93.47 | 109 | 101 | 113 | 99 | 106 |
| 24 | 130 | 110 | 120 | 198 | 186 | 192 | 153 | 140 | 147 | 119 | 96.67 | 108 | 107 | 99.33 | 103 |
| 48 | 170 | 133 | 152 | 238 | 212 | 225 | 156 | 187 | 172 | 108 | 108 | 108 | 97.67 | 83 | 90.34 |
| 72 | 172 | 185 | 179 | 273 | 257 | 265 | 217 | 200 | 209 | 137 | 108 | 123 | 113 | 88 | 101 |
| 96 | 245 | 197 | 221 | 288 | 278 | 283 | 176 | 200 | 188 | 122 | 97.67 | 110 | 87.67 | 98.67 | 93.17 |
| 120 | 235 | 258 | 247 | 288 | 257 | 273 | 146 | 170 | 158 | 111 | 100 | 106 | 106 | 100 | 103 |
| 144 | 294 | 263 | 279 | 243 | 235 | 239 | 151 | 138 | 145 | 114 | 89.67 | 102 | 117 | 94 | 106 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A13. Production of EPS by *Pseudomonas* sp. BGI-2 at different pH, using phenol sulfuric acid method.

| Time (Hours) | 5 | | | 6 | | | 7 | | | 8 | | | 9 | | | 10 | | | 11 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 0 | 87 | 92 | 89.5 | 104 | 89.38 | 96.69 | 88 | 117 | 102.5 | 96.8 | 99.67 | 98.24 | 110 | 103 | 106.5 | 81.33 | 109 | 95.17 | 94.67 | 105 | 99.835 |
| 24 | 134 | 111 | 122.5 | 144 | 143 | 143.5 | 164 | 153 | 158.5 | 152 | 186 | 169 | 131 | 144 | 137.5 | 97.33 | 106 | 101.67 | 87.33 | 82.33 | 84.83 |
| 48 | 225 | 185 | 205 | 242 | 303 | 272.5 | 285 | 241 | 263 | 239 | 272 | 255.5 | 188 | 267 | 227.5 | 118 | 128 | 123 | 87 | 83.67 | 85.34 |
| 72 | 203 | 216 | 209.5 | 223 | 231 | 227 | 236 | 218 | 227 | 224 | 235 | 229.5 | 184 | 214 | 199 | 126 | 161 | 143.5 | 89 | 87.67 | 88.34 |
| 96 | 179 | 202 | 190.5 | 188 | 183 | 185.5 | 191 | 195 | 193 | 214 | 189 | 201.5 | 191 | 180 | 185.5 | 137 | 122 | 129.5 | 95 | 104 | 99.5 |
| 120 | 130 | 166 | 148 | 139 | 166 | 152.5 | 169 | 139 | 154 | 166 | 153 | 159.5 | 155 | 133 | 144 | 97 | 90 | 93.5 | 87.33 | 98.33 | 92.83 |
| 144 | 125 | 149 | 137 | 132 | 151 | 141.5 | 150 | 155 | 152.5 | 147 | 158 | 152.5 | 150 | 115 | 132.5 | 71.67 | 88 | 79.84 | 86.67 | 81 | 83.84 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A14. Production of EPS by *Pseudomonas* sp. BGI-2 at different salinities, using phenol sulfuric acid method.

| Time (Hours) | Control | | | 1% | | | 3% | | | 5% | | | 7% | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 0 | 97.3 | 89.03 | 93.17 | 116 | 108 | 112 | 80.73 | 134 | 107.37 | 93.37 | 125 | 109.19 | 106 | 104 | 105 |
| 24 | 169 | 169 | 169 | 168 | 220 | 194 | 127 | 128 | 127.5 | 112 | 103 | 107.5 | 98.17 | 96 | 97.09 |
| 48 | 191 | 167 | 179 | 196 | 228 | 212 | 200 | 177 | 188.5 | 106 | 120 | 113 | 96.33 | 102 | 99.165 |
| 72 | 204 | 191 | 197.5 | 289 | 284 | 286.5 | 221 | 233 | 227 | 148 | 132 | 140 | 100 | 101 | 100.5 |
| 96 | 177 | 169 | 173 | 281 | 262 | 271.5 | 232 | 221 | 226.5 | 174 | 148 | 161 | 93.57 | 83.6 | 88.58 |
| 120 | 165 | 173 | 169 | 258 | 256 | 257 | 207 | 216 | 211.5 | 125 | 130 | 127.5 | 89.67 | 79.67 | 84.67 |
| 144 | 181 | 112 | 146.5 | 262 | 228 | 245 | 222 | 173 | 197.5 | 136 | 99 | 117.5 | 92.33 | 74 | 83.165 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A15. Production of EPS by *Pseudomonas* sp. BGI-2 at different concentrations of molasses, using phenol sulfuric acid method.

| Time (Hours) | Control | | | 1% | | | 3% | | | 5% | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 0 | 32.66 | 26.26 | 29.46 | 216 | 240 | 228 | 338 | 260 | 299 | 452 | 444 | 448 |
| 24 | 96 | 90.4 | 93.2 | 252 | 214 | 233 | 246 | 238 | 242 | 332 | 340 | 336 |
| 48 | 126 | 131 | 128.5 | 312 | 280 | 296 | 444 | 458 | 451 | 580 | 524 | 552 |
| 72 | 137 | 123 | 130 | 274 | 272 | 273 | 400 | 420 | 410 | 716 | 633 | 674.5 |
| 96 | 132 | 120 | 126 | 222 | 229 | 225.5 | 258 | 266 | 262 | 532 | 517 | 524.5 |
| 120 | 124 | 129 | 126.5 | 167 | 158 | 162.5 | 277 | 257 | 267 | 445 | 422 | 433.5 |
| 144 | 50.6 | 134 | 92.3 | 161 | 154 | 157.5 | 230 | 210 | 220 | 288 | 281 | 284.5 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A16. Production of EPS by *Pseudomonas* sp. BGI-2 under optimized conditions, using phenol sulfuric acid method.

| Time (Hours) | Replicate 1 (R1) | Replicate 2 (R2) | Mean (M) |
|---|---|---|---|
| 24 | 1015 | 948 | 982 |
| 72 | 1345 | 1389 | 1367 |
| 120 | 1421 | 1505 | 1463 |
| 168 | 1308 | 1223 | 1265 |
| 216 | 1088 | 1013 | 1050 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A17. Freeze thaw survivability of *Pseudomonas* sp. BGI-2 compared to *Rhodococcus* sp. BGI-11 and *Escherichia coli* K12, using plate count method.

| Time (Hours) | ***Pseudomonas* sp. BGI-2** | | | ***Rhodococcus* sp. BGI-11** | | | ***Escherichia coli* K12** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 24 | 102.4 | 97.89 | 100.145 | 93.66 | 95.44 | 94.55 | 75.45 | 71.66 | 73.55 |
| 48 | 99.88 | 95.57 | 97.725 | 90.17 | 85.24 | 87.705 | 62.45 | 58.67 | 60.56 |
| 72 | 99.89 | 91.57 | 95.73 | 88.55 | 80.89 | 84.72 | 45.45 | 30.56 | 38.01 |
| 96 | 98.21 | 90.66 | 94.435 | 82.56 | 75.77 | 79.165 | 25.78 | 19.56 | 22.67 |
| 120 | 96.32 | 87.77 | 92.045 | 80.44 | 71.45 | 75.945 | - | - | - |
| 144 | 97.34 | 85.24 | 91.29 | 70.88 | 66.56 | 68.72 | - | - | - |
| 168 | 95.46 | 88.56 | 92.01 | 68.47 | 59.88 | 64.175 | - | - | - |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A18. Cryoprotective effect of different concentrations of EPS on *E.coli* K12, subjected to 7 freeze thaw cycles using plate count method.

| Time (Hours) | **Control** | | | **20% Glycerol** | | | **1% EPS** | | | **3% EPS** | | | **5% EPS** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 24 | 76.67 | 70.46 | 73.565 | 103.2 | 99.06 | 101.13 | 90.63 | 84.05 | 87.34 | 101.5 | 95.34 | 98.42 | 97.58 | 101.8 | 99.69 |
| 48 | 62.13 | 55.84 | 58.985 | 102.4 | 97.28 | 99.84 | 83.44 | 64.09 | 73.765 | 96.46 | 92.28 | 94.37 | 96.81 | 100.4 | 98.61 |
| 72 | 34.67 | 30.15 | 32.41 | 99.96 | 96.37 | 98.165 | 69.72 | 59.58 | 64.65 | 91.08 | 82.39 | 86.74 | 100.1 | 94.56 | 97.33 |
| 96 | 25.47 | 16.83 | 21.15 | 99.66 | 95.41 | 97.535 | 54.49 | 61.77 | 58.13 | 83.33 | 79.58 | 81.46 | 98.44 | 92.06 | 95.25 |
| 120 | - | - | - | 99.17 | 95.25 | 97.21 | 50.47 | 45.19 | 47.83 | 78.09 | 68.28 | 73.185 | 96.87 | 92.19 | 94.53 |
| 144 | - | - | - | 98.74 | 93.65 | 96.195 | 38.49 | 35.48 | 36.99 | 66.87 | 62.48 | 64.675 | 95.47 | 87.28 | 91.38 |
| 168 | - | - | - | 98.38 | 89.57 | 93.97 | 31.55 | 22.80 | 27.18 | 65.05 | 57.81 | 61.43 | 92.91 | 86.43 | 89.67 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A19. Growth recovery ($OD_{570}$) for *Synechococcus* sp. CBW1003 after 5 days of cryopreservation.

| Time (Days) | Control | | | 5% DMSO | | | 10% Glycerol | | | 2 mg/ml EPS | | | 3 mg/ml EPS | | | 4 mg/ml EPS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 1 | 0.508 | 0.407 | 0.458 | 0.641 | 0.706 | 0.674 | 0..765 | 0.711 | 0.734 | 0.654 | 0.742 | 0.698 | 0.664 | 0.652 | 0.658 | 0.662 | 0.722 | 0.692 |
| 5 | 0.326 | 0.229 | 0.278 | 0.951 | 0.892 | 0.922 | 0.753 | 0.797 | 0.775 | 0.609 | 0.662 | 0.636 | 0.681 | 0.617 | 0.649 | 0.657 | 0.823 | 0.740 |
| 9 | 0.269 | 0.181 | 0.225 | 1.465 | 1.380 | 1.423 | 0.733 | 1.042 | 0.888 | 0.956 | 0.936 | 0.946 | 0.927 | 1.009 | 0.968 | 1.185 | 1.346 | 1.266 |
| 11 | 0.299 | 0.280 | 0.290 | 1.891 | 1.816 | 1.854 | 0.562 | 0.609 | 0.586 | 1.339 | 1.293 | 1.316 | 1.270 | 1.387 | 1.329 | 1.646 | 1.849 | 1.748 |
| 13 | 0.398 | 0.417 | 0.408 | 1.905 | 1.949 | 1.927 | 0.714 | 0.959 | 0.837 | 1.567 | 1.574 | 1.571 | 1.686 | 1.721 | 1.704 | 1.809 | 2.096 | 1.953 |
| 15 | 0.7 | 0.733 | 0.717 | 2.016 | 2.134 | 2.075 | 1.414 | 1.353 | 1.247 | 1.840 | 1.919 | 1.880 | 1.999 | 2.027 | 2.013 | 2.206 | 2.436 | 2.321 |
| 18 | 0.961 | 0.943 | 0.952 | 2.070 | 2.131 | 2.101 | 1.406 | 1.481 | 1.444 | 1.938 | 2.022 | 1.980 | 2.113 | 2.154 | 2.134 | 2.590 | 2.731 | 2.661 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A20. Growth recovery ($OD_{570}$) for *Synechococcus* sp. CBW1003 after 15 days of cryopreservation.

| Time (Days) | Control | | | 5% DMSO | | | 10% Glycerol | | | 2 mg/ml EPS | | | 3 mg/ml EPS | | | 4 mg/ml EPS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 1 | 0.475 | 0.474 | 0.475 | 0.652 | 0.704 | 0.678 | 0.837 | 0.838 | 0.838 | 0.766 | 0.774 | 0.770 | 0.834 | 0.876 | 0.855 | 0.954 | 0.839 | 0.897 |
| 5 | 0.296 | 0.261 | 0.279 | 0.818 | 0.986 | 0.902 | 0.767 | 0.783 | 0.775 | 0.722 | 0.683 | 0.703 | 0.700 | 0.814 | 0.757 | 0.967 | 0.874 | 0.921 |
| 9 | 0.349 | 0.303 | 0.326 | 1.515 | 1.610 | 1.563 | 0.991 | 1.120 | 1.056 | 1.104 | 1.067 | 1.086 | 1.250 | 1.257 | 1.254 | 1.601 | 1.382 | 1.492 |
| 11 | 0.354 | 0.357 | 0.356 | 1.869 | 1.970 | 1.920 | 1.150 | 1.205 | 1.178 | 1.475 | 1.401 | 1.438 | 1.587 | 1.601 | 1.594 | 1.986 | 1.757 | 1.872 |
| 13 | 0.501 | 0.450 | 0.476 | 2.213 | 2.122 | 2.168 | 1.039 | 1.125 | 1.082 | 1.775 | 1.652 | 1.714 | 1.945 | 1.964 | 1.955 | 2.304 | 2.129 | 2.217 |
| 16 | 1.118 | 1.214 | 1.166 | 3.125 | 2.885 | 3.005 | 1.08 | 1.369 | 1.225 | 2.786 | 2.663 | 2.725 | 2.927 | 2.963 | 2.945 | 3.148 | 2.987 | 3.068 |
| 18 | 1.478 | 1.568 | 1.523 | 3.074 | 3.255 | 3.165 | 1.120 | 1.352 | 1.236 | 3.093 | 2.917 | 3.005 | 3.28 | 3.263 | 3.272 | 3.395 | 3.243 | 3.319 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A21. Growth recovery ($OD_{570}$) for *Synechococcus* sp. CBW1003 after 09 months of cryopreservation.

| Time (Days) | Control | | | 5% DMSO | | | 10% Glycerol | | | 2 mg/ml EPS | | | 3 mg/ml EPS | | | 4 mg/ml EPS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R1 | R2 | M | R1 | R2 | M | R1 | R2 | M | R1 | R2 | M | R1 | R2 | M | R1 | R2 | M |
| 1 | 0.417 | 0.649 | 0.533 | 0.657 | 0.711 | 0.684 | 0.853 | 0.882 | 0.868 | 0.854 | 0.76 | 0.807 | 0.879 | 0.924 | 0.902 | 0.989 | 0.995 | 0.992 |
| 5 | 0.173 | 0.251 | 0.212 | 0.701 | 0.615 | 0.658 | 0.517 | 0.577 | 0.547 | 0.493 | 0.339 | 0.416 | 0.449 | 0.477 | 0.463 | 0.508 | 0.494 | 0.501 |
| 9 | 0.215 | 0.199 | 0.207 | 1.272 | 1.35 | 1.311 | 0.576 | 0.587 | 0.582 | 0.687 | 0.643 | 0.665 | 0.918 | 0.904 | 0.911 | 1.147 | 1.167 | 1.157 |
| 11 | 0.321 | 0.325 | 0.323 | 1.645 | 1.694 | 1.670 | 0.826 | 0.859 | 0.843 | 0.883 | 0.892 | 0.888 | 1.263 | 1.24 | 1.252 | 1.543 | 1.585 | 1.564 |
| 13 | 0.491 | 0.531 | 0.511 | 2.226 | 2.324 | 2.275 | 0.866 | 0.924 | 0.895 | 1.228 | 1.289 | 1.259 | 1.78 | 1.698 | 1.739 | 2.011 | 2.006 | 2.009 |
| 15 | 0.639 | 0.635 | 0.637 | 2.261 | 2.352 | 2.307 | 0.757 | 0.711 | 0.734 | 1.398 | 1.447 | 1.423 | 1.947 | 1.844 | 1.896 | 2.144 | 2.19 | 2.167 |
| 18 | 0.791 | 0.735 | 0.763 | 2.312 | 2.371 | 2.342 | 0.631 | 0.551 | 0.591 | 1.657 | 1.754 | 1.706 | 2.162 | 2.087 | 2.125 | 2.298 | 2.351 | 2.325 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A22. Growth recovery ($OD_{680}$) for *Scenedesmus* sp. HTB1 after 05 days of cryopreservation.

| Time (Days) | Control | | | 5% DMSO | | | 10% Glycerol | | | 2 mg/ml EPS | | | 3 mg/ml EPS | | | 4 mg/ml EPS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R1 | R2 | M | R1 | R2 | M | R1 | R2 | M | R1 | R2 | M | R1 | R2 | M | R1 | R2 | M |
| 1 | 0.761 | 0.687 | 0.724 | 0.728 | 0.787 | 0.758 | 0.655 | 0.618 | 0.637 | 0.791 | 0.792 | 0.792 | 0.765 | 0.764 | 0.765 | 0.716 | 0.696 | 0.706 |
| 5 | 0.553 | 0.510 | 0.532 | 0.743 | 0.726 | 0.735 | 0.685 | 0.804 | 0.745 | 0.791 | 0.828 | 0.810 | 0.734 | 0.682 | 0.708 | 0.652 | 0.680 | 0.666 |
| 9 | 0.525 | 0.434 | 0.480 | 0.994 | 0.992 | 0.993 | 0.555 | 0.780 | 0.668 | 0.977 | 0.923 | 0.950 | 0.757 | 0.705 | 0.731 | 0.646 | 0.639 | 0.643 |
| 11 | 0.488 | 0.370 | 0.429 | 1.084 | 1.054 | 1.069 | 0.560 | 0.691 | 0.626 | 0.994 | 0.957 | 0.9755 | 0.882 | 0.829 | 0.856 | 0.825 | 0.751 | 0.788 |
| 13 | 0.570 | 0.464 | 0.517 | 1.502 | 1.551 | 1.527 | 0.802 | 0.816 | 0.809 | 1.551 | 1.416 | 1.484 | 1.146 | 1.330 | 1.393 | 1.301 | 1.13 | 1.216 |
| 15 | 0.647 | 0.513 | 0.580 | 1.676 | 1.835 | 1.756 | 0.902 | 0.833 | 0.868 | 1.770 | 1.60 | 1.685 | 1.729 | 1.504 | 1.617 | 1.579 | 1.380 | 1.480 |
| 18 | 0.740 | 0.625 | 0.683 | 2.138 | 2.171 | 2.155 | 1.246 | 0.852 | 1.049 | 2.122 | 1.954 | 2.038 | 2.08 | 1.855 | 1.968 | 1.859 | 1.695 | 1.777 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A23. Growth recovery ($OD_{680}$) for *Scenedesmus* sp. HTB1 after 15 days of cryopreservation.

| Time (Days) | Control | | | 5% DMSO | | | 10% Glycerol | | | 2 mg/ml EPS | | | 3 mg/ml EPS | | | 4 mg/ml EPS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 1 | 0.924 | 0.873 | 0.899 | 0.757 | 0.840 | 0.799 | 0.699 | 0.761 | 0.730 | 0.928 | 0.851 | 0.890 | 0.830 | 0.884 | 0.857 | 0.847 | 0.798 | 0.823 |
| 5 | 0.639 | 0.619 | 0.629 | 0.833 | 0.750 | 0.792 | 0.685 | 0.806 | 0.746 | 0.768 | 0.784 | 0.776 | 0.717 | 0.719 | 0.718 | 0.657 | 0.622 | 0.640 |
| 9 | 0.605 | 0.559 | 0.582 | 1.035 | 1.053 | 1.044 | 0.696 | 0.794 | 0.745 | 0.999 | 1.028 | 1.014 | 0.888 | 0.867 | 0.878 | 0.765 | 0.767 | 0.766 |
| 11 | 0.541 | 0.510 | 0.526 | 1.174 | 1.221 | 1.198 | 0.692 | 0.809 | 0.751 | 1.096 | 1.114 | 1.105 | 0.962 | 0.935 | 0.949 | 0.826 | 0.862 | 0.844 |
| 13 | 0.555 | 0.523 | 0.539 | 1.438 | 1.352 | 1.395 | 0.687 | 0.798 | 0.743 | 1.251 | 1.318 | 1.285 | 1.073 | 1.123 | 1.098 | 0.899 | 0.892 | 0.896 |
| 16 | 0.935 | 0.835 | 0.885 | 1.614 | 1.705 | 1.660 | 0.945 | 1.102 | 1.024 | 1.729 | 1.684 | 1.707 | 1.612 | 1.566 | 1.589 | 1.594 | 1.453 | 1.524 |
| 18 | 1.057 | 1.080 | 1.069 | 1.916 | 1.816 | 1.866 | 1.295 | 1.408 | 1.352 | 1.889 | 1.826 | 1.8575 | 1.806 | 1.800 | 1.803 | 1.850 | 1.700 | 1.775 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A24. Growth recovery ($OD_{680}$) for *Scenedesmus* sp. HTB1 after 09 months of cryopreservation.

| Time (Days) | Control | | | 5% DMSO | | | 10% Glycerol | | | 2 mg/ml EPS | | | 3 mg/ml EPS | | | 4 mg/ml EPS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 1 | 0.848 | 0.794 | 0.821 | 0.662 | 0.687 | 0.675 | 0.552 | 0.792 | 0.672 | 0.876 | 0.864 | 0.870 | 0.88 | 0.91 | 0.895 | 0.87 | 0.901 | 0.886 |
| 5 | 0.730 | 0.705 | 0.718 | 0.685 | 0.662 | 0.674 | 0.644 | 0.743 | 0.694 | 0.935 | 0.931 | 0.933 | 0.878 | 0.86 | 0.869 | 0.72 | 0.715 | 0.718 |
| 9 | 0.558 | 0.535 | 0.547 | 0.687 | 0.676 | 0.682 | 0.745 | 0.706 | 0.726 | 1.315 | 1.305 | 1.31 | 1.204 | 1.221 | 1.213 | 0.922 | 0.856 | 0.889 |
| 11 | 0.652 | 0.558 | 0.605 | 1.123 | 0.975 | 1.049 | 1.041 | 1.059 | 1.050 | 1.629 | 1.554 | 1.592 | 1.552 | 1.493 | 1.523 | 1.187 | 1.069 | 1.128 |
| 13 | 0.765 | 0.614 | 0.690 | 1.473 | 1.302 | 1.388 | 1.022 | 1.075 | 1.049 | 1.93 | 1.898 | 1.914 | 1.87 | 1.789 | 1.830 | 1.481 | 1.391 | 1.436 |
| 15 | 0.813 | 0.809 | 0.811 | 1.909 | 1.804 | 1.857 | 1.019 | 1.087 | 1.053 | 2.239 | 2.158 | 2.199 | 2.179 | 2.05 | 2.115 | 1.838 | 1.759 | 1.799 |
| 18 | 0.891 | 0.862 | 0.877 | 2.081 | 1.906 | 1.994 | 0.934 | 0.987 | 0.961 | 2.330 | 2.241 | 2.286 | 2.251 | 2.134 | 2.193 | 1.974 | 1.871 | 1.923 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A25. Growth recovery ($OD_{435}$) for *Synechococcus* sp. CB0101 after 05 days of cryopreservation.

| Time (Days) | Control | | | 5% DMSO | | | 10% Glycerol | | | 2 mg/ml EPS | | | 3 mg/ml EPS | | | 4 mg/ml EPS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 1 | 0.958 | 0.959 | 0.959 | 1.026 | 1.023 | 1.025 | 1.014 | 1.025 | 1.019 | 0.890 | 0.823 | 0.857 | 0.835 | 0.848 | 0.842 | 0.804 | 0.770 | 0.787 |
| 5 | 0.820 | 0.855 | 0.838 | 0.752 | 0.891 | 0.822 | 0.778 | 1.014 | 0.896 | 0.978 | 0.818 | 0.848 | 0.873 | 0.812 | 0.843 | 0.848 | 0.853 | 0.851 |
| 9 | 0.744 | 0.718 | 0.731 | 0.306 | 0.446 | 0.376 | 0.289 | 0.349 | 0.319 | 0.778 | 0.754 | 0.766 | 0.822 | 0.753 | 0.788 | 0.845 | 0.772 | 0.809 |
| 13 | 0.611 | 0.634 | 0.623 | 0.040 | 0.088 | 0.064 | 0.383 | 0.477 | 0.430 | 0.790 | 0.689 | 0.740 | 0.780 | 0.772 | 0.776 | 1.231 | 1.149 | 1.190 |
| 15 | 0.657 | 0.671 | 0.664 | 0.033 | 0.079 | 0.056 | 0.711 | 0.277 | 0.494 | 1.013 | 0.845 | 0.929 | 0.992 | 0.960 | 0.976 | 1.472 | 1.317 | 1.395 |
| 18 | 0.697 | 0.656 | 0.677 | 0.064 | 0.098 | 0.081 | 1.149 | 0.297 | 0.723 | 1.602 | 1.310 | 1.456 | 1.647 | 1.394 | 1.521 | 1.791 | 1.782 | 1.787 |
| 20 | 0.783 | 0.802 | 0.793 | 0.155 | 0.310 | 0.233 | 1.316 | 0.571 | 0.944 | 1.902 | 1.572 | 1.737 | 2.048 | 1.915 | 1.982 | 2.399 | 2.286 | 2.343 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A26. Growth recovery ($OD_{435}$) for *Synechococcus* sp. CB0101 after 15 days of cryopreservation.

| Time (Days) | Control | | | 5% DMSO | | | 10% Glycerol | | | 2 mg/ml EPS | | | 3 mg/ml EPS | | | 4 mg/ml EPS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 1 | 1.015 | 0.950 | 0.983 | 0.999 | 0.881 | 0.940 | 1.111 | 1.023 | 1.067 | 1.079 | 0.952 | 1.016 | 1.104 | 0.995 | 1.050 | 1.007 | 0.928 | 0.968 |
| 5 | 0.941 | 0.910 | 0.926 | 0.885 | 0.763 | 0.824 | 1.230 | 0.923 | 1.077 | 0.934 | 1.029 | 0.982 | 1.004 | 0.980 | 0.992 | 0.906 | 0.997 | 0.952 |
| 9 | 0.774 | 0.785 | 0.780 | 0.244 | 0.258 | 0.251 | 0.823 | 0.712 | 0.768 | 0.807 | 0.990 | 0.899 | 0.879 | 0.894 | 0.887 | 0.912 | 0.891 | 0.902 |
| 11 | 0.809 | 0.776 | 0.793 | 0.096 | 0.147 | 0.122 | 0.463 | 0.585 | 0.524 | 0.783 | 0.808 | 0.796 | 0.850 | 0.878 | 0.864 | 0.959 | 0.855 | 0.907 |
| 16 | 0.599 | 0.560 | 0.580 | 0.040 | 0.015 | 0.028 | 0.237 | 0.310 | 0.274 | 0.814 | 0.832 | 0.823 | 0.921 | 0.778 | 0.850 | 1.382 | 1.161 | 1.272 |
| 18 | 0.465 | 0.412 | 0.439 | 0.007 | 0.008 | 0.007 | 0.222 | 0.082 | 0.152 | 0.927 | 0.899 | 0.913 | 1.386 | 1.189 | 1.288 | 1.727 | 1.553 | 1.640 |
| 20 | 0.235 | 0.112 | 0.174 | 0.003 | 0.004 | 0.003 | 0.112 | 0.065 | 0.089 | 1.102 | 0.998 | 1.050 | 1.537 | 1.367 | 1.452 | 1.923 | 1.689 | 1.806 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A27. Growth recovery ($OD_{435}$) for *Synechococcus* sp. CB0101 after 09 months of cryopreservation.

| Time (Days) | Control | | | 5% DMSO | | | 10% Glycerol | | | 2 mg/ml EPS | | | 3 mg/ml EPS | | | 4 mg/ml EPS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 1 | 0.957 | 0.963 | 0.960 | 1.010 | 0.912 | 0.961 | 0.872 | 0.921 | 0.897 | 1.05 | 1.101 | 1.076 | 1.115 | 1.066 | 1.091 | 1.045 | 1.172 | 1.109 |
| 5 | 0.717 | 0.742 | 0.729 | 0.813 | 0.745 | 0.779 | 0.867 | 0.901 | 0.884 | 0.868 | 0.898 | 0.883 | 0.941 | 0.955 | 0.948 | 1.042 | 0.986 | 1.014 |
| 9 | 0.619 | 0.645 | 0.632 | 0.634 | 0.638 | 0.636 | 0.668 | 0.731 | 0.699 | 0.668 | 0.677 | 0.673 | 0.738 | 0.769 | 0.754 | 0.885 | 0.841 | 0.863 |
| 15 | 0.402 | 0.396 | 0.399 | 0.220 | 0.264 | 0.242 | 0.129 | 0.164 | 0.147 | 0.393 | 0.518 | 0.456 | 0.336 | 0.293 | 0.315 | 0.939 | 0.958 | 0.949 |
| 20 | 0.322 | 0.334 | 0.328 | 0.100 | 0.051 | 0.076 | 0.073 | 0.070 | 0.072 | 0.281 | 0.301 | 0.291 | 1.018 | 1.006 | 1.012 | 1.535 | 1.678 | 1.607 |
| 23 | 0.235 | 0.292 | 0.264 | 0.125 | 0.133 | 0.129 | 0.067 | 0.073 | 0.070 | 0.662 | 0.600 | 0.631 | 1.711 | 1.689 | 1.700 | 1.860 | 2.030 | 1.945 |
| 26 | 0.181 | 0.250 | 0.216 | 0.154 | 0.039 | 0.097 | 0.066 | 0.049 | 0.058 | 1.360 | 1.245 | 1.303 | 2.122 | 2.255 | 2.189 | 2.186 | 2.336 | 2.261 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A28. Growth recovery ($OD_{620}$) for *Microcystis aeruginosa* PCC 7806 after 05 days of cryopreservation.

| Time (Days) | Control | | | 5% DMSO | | | 10% Glycerol | | | 2 mg/ml EPS | | | 3 mg/ml EPS | | | 4 mg/ml EPS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 1 | 1.180 | 1.152 | 1.166 | 0.999 | 1.129 | 1.064 | 1.083 | 1.109 | 1.096 | 1.188 | 1.183 | 1.186 | 1.236 | 1.242 | 1.239 | 1.226 | 1.222 | 1.224 |
| 5 | 1.417 | 1.432 | 1.425 | 1.508 | 1.560 | 1.534 | 1.309 | 1.421 | 1.365 | 1.526 | 1.535 | 1.531 | 1.665 | 1.646 | 1.656 | 1.719 | 1.705 | 1.712 |
| 9 | 2.260 | 2.228 | 2.244 | 2.074 | 2.066 | 2.070 | 1.589 | 1.471 | 1.530 | 2.293 | 2.327 | 2.310 | 2.450 | 2.351 | 2.401 | 2.451 | 2.426 | 2.439 |
| 11 | 2.621 | 2.608 | 2.615 | 2.368 | 2.381 | 2.375 | 1.872 | 1.696 | 1.784 | 2.649 | 2.709 | 2.679 | 2.787 | 2.667 | 2.727 | 2.759 | 2.766 | 2.763 |
| 13 | 2.863 | 2.840 | 2.852 | 2.590 | 2.586 | 2.588 | 2.200 | 1.930 | 2.065 | 2.889 | 2.960 | 2.925 | 2.995 | 2.886 | 2.941 | 2.976 | 2.987 | 2.982 |
| 15 | 2.928 | 2.932 | 2.930 | 2.601 | 2.656 | 2.629 | 2.374 | 2.127 | 2.251 | 2.965 | 3.031 | 2.998 | 3.079 | 2.929 | 3.004 | 3.026 | 3.008 | 3.017 |
| 18 | 3.155 | 3.212 | 3.184 | 2.875 | 2.913 | 2.894 | 2.767 | 2.913 | 2.840 | 3.252 | 3.351 | 3.302 | 3.345 | 3.183 | 3.264 | 3.305 | 3.293 | 3.299 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A29. Growth recovery ($OD_{620}$) for *Microcystis aeruginosa* PCC 7806 after 15 days of cryopreservation.

| Time (Days) | Control | | | 5% DMSO | | | 10% Glycerol | | | 2 mg/ml EPS | | | 3 mg/ml EPS | | | 4 mg/ml EPS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 1 | 1.172 | 1.137 | 1.155 | 1.137 | 1.114 | 1.126 | 1.07 | 1.099 | 1.085 | 1.175 | 1.33 | 1.253 | 1.164 | 1.149 | 1.157 | 1.144 | 1.124 | 1.134 |
| 5 | 1.391 | 1.323 | 1.357 | 1.539 | 1.549 | 1.544 | 1.365 | 1.446 | 1.406 | 1.61 | 1.532 | 1.571 | 1.704 | 1.627 | 1.666 | 1.604 | 1.553 | 1.579 |
| 9 | 2.117 | 2.060 | 2.089 | 2.141 | 2.221 | 2.181 | 1.648 | 1.581 | 1.615 | 2.443 | 2.292 | 2.367 | 2.561 | 2.453 | 2.507 | 2.405 | 2.377 | 2.391 |
| 11 | 2.536 | 2.446 | 2.491 | 2.423 | 2.513 | 2.468 | 1.895 | 1.842 | 1.869 | 2.752 | 2.657 | 2.705 | 2.871 | 2.757 | 2.814 | 2.733 | 2.613 | 2.673 |
| 13 | 2.747 | 2.672 | 2.710 | 2.519 | 2.637 | 2.578 | 2.073 | 2.036 | 2.055 | 2.934 | 2.850 | 2.892 | 3.054 | 3.010 | 3.032 | 2.988 | 2.845 | 2.917 |
| 16 | 3.256 | 3.309 | 3.283 | 3.096 | 3.158 | 3.127 | 2.841 | 2.796 | 2.819 | 3.502 | 3.286 | 3.394 | 3.578 | 3.453 | 3.516 | 3.523 | 3.307 | 3.415 |
| 18 | 3.458 | 3.445 | 3.452 | 3.273 | 3.377 | 3.325 | 3.336 | 3.08 | 3.208 | 3.608 | 3.458 | 3.533 | 3.648 | 3.608 | 3.628 | 3.601 | 3.554 | 3.578 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A30. Growth recovery ($OD_{620}$) for *Microcystis aeruginosa* PCC 7806 after 09 months of cryopreservation.

| Time (Days) | Control | | | 5% DMSO | | | 10% Glycerol | | | 2 mg/ml EPS | | | 3 mg/ml EPS | | | 4 mg/ml EPS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 1 | 1.071 | 1.111 | 1.091 | 1.180 | 1.05 | 1.115 | 1.029 | 1.076 | 1.053 | 1.163 | 1.196 | 1.179 | 1.223 | 1.239 | 1.231 | 1.243 | 1.250 | 1.247 |
| 5 | 1.304 | 1.281 | 1.293 | 2.052 | 1.905 | 1.979 | 1.219 | 1.265 | 1.242 | 1.588 | 1.556 | 1.572 | 1.756 | 1.691 | 1.724 | 1.719 | 1.717 | 1.718 |
| 9 | 1.951 | 1.906 | 1.929 | 3.066 | 3.004 | 3.035 | 1.405 | 1.535 | 1.470 | 2.415 | 2.361 | 2.388 | 2.665 | 2.534 | 2.599 | 2.558 | 2.534 | 2.546 |
| 11 | 2.466 | 2.384 | 2.425 | 3.399 | 3.477 | 3.438 | 1.551 | 1.488 | 1.519 | 3.036 | 3.014 | 3.025 | 3.188 | 3.173 | 3.181 | 3.110 | 3.152 | 3.131 |
| 13 | 2.713 | 2.805 | 2.759 | 3.380 | 3.540 | 3.460 | 1.674 | 1.488 | 1.581 | 3.435 | 3.283 | 3.359 | 3.516 | 3.515 | 3.516 | 3.537 | 3.291 | 3.414 |
| 15 | 3.113 | 3.171 | 3.142 | 3.462 | 3.558 | 3.510 | 1.730 | 1.536 | 1.633 | 3.563 | 3.451 | 3.507 | 3.623 | 3.649 | 3.636 | 3.706 | 3.611 | 3.659 |
| 18 | 3.210 | 3.313 | 3.262 | 3.512 | 3.571 | 3.542 | 1.728 | 1.577 | 1.653 | 3.651 | 3.554 | 3.603 | 3.741 | 3.699 | 3.720 | 3.793 | 3.699 | 3.746 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A31. Growth recovery ($OD_{680}$) for *Chlorella vulgaris* after 05 days of cryopreservation.

| **Time (Days)** | **Control** | | | **5% DMSO** | | | **10% Glycerol** | | | **2 mg/ml EPS** | | | **3 mg/ml EPS** | | | **4 mg/ml EPS** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 1 | 1,197 | 1.151 | 1.174 | 1.197 | 1.190 | 1.194 | 0.973 | 0.962 | 0.968 | 1.103 | 1.103 | 1.103 | 1.088 | 1.084 | 1.086 | 1.079 | 1.133 | 1.106 |
| 5 | 0.679 | 0.716 | 0.698 | 1.720 | 1.834 | 1.777 | 0.653 | 0.712 | 0.683 | 0.748 | 0.713 | 0.731 | 0.713 | 0.641 | 0.677 | 0.639 | 0.684 | 0.662 |
| 9 | 0.697 | 0.845 | 0.771 | 2.356 | 2.416 | 2.386 | 0.856 | 0.937 | 0.897 | 0.542 | 0.594 | 0.568 | 0.678 | 0.559 | 0.619 | 0.602 | 0.717 | 0.660 |
| 11 | 0.958 | 1.117 | 1.038 | 2.435 | 2.603 | 2.519 | 1.262 | 1.272 | 1.267 | 0.647 | 0.722 | 0.685 | 0.759 | 0.670 | 0.715 | 0.693 | 0.717 | 0.660 |
| 13 | 1.105 | 1.232 | 1.169 | 2.692 | 2.857 | 2.775 | 1.293 | 1.495 | 1.394 | 0.768 | 0.950 | 0.859 | 1.002 | 0.869 | 0.936 | 0.955 | 1.058 | 1.007 |
| 15 | 1.396 | 1.541 | 1.469 | 2.744 | 2.932 | 2.838 | 1.788 | 1.868 | 1.828 | 0.971 | 1.163 | 1.067 | 1.176 | 1.022 | 1.099 | 1.108 | 1.184 | 1.146 |
| 18 | 1.653 | 1.691 | 1.672 | 3.246 | 3.222 | 3.234 | 1.941 | 1.964 | 1.953 | 1.221 | 1.368 | 1.295 | 1.326 | 1.195 | 1.261 | 1.206 | 1.233 | 1.220 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A32. Growth recovery ($OD_{680}$) for *Chlorella vulgaris* after 15 days of cryopreservation.

| **Time (Days)** | **Control** | | | **5% DMSO** | | | **10% Glycerol** | | | **2 mg/ml EPS** | | | **3 mg/ml EPS** | | | **4 mg/ml EPS** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 1 | 1.316 | 1.323 | 1.319 | 1.421 | 1.541 | 1.481 | 1.117 | 1.187 | 1.152 | 1.17 | 1.163 | 1.167 | 1.103 | 1.118 | 1.111 | 1.060 | 1.036 | 1.048 |
| 5 | 1.145 | 1.189 | 1.167 | 1.796 | 1.870 | 1.833 | 1.113 | 1.034 | 1.074 | 0.996 | 0.994 | 0.995 | 0.908 | 0.906 | 0.907 | 0.9 | 0.902 | 0.901 |
| 9 | 1.433 | 1.471 | 1.452 | 2.255 | 2.279 | 2.267 | 1.331 | 1.228 | 1.280 | 1.157 | 1.188 | 1.173 | 1.06 | 1.087 | 1.074 | 1.073 | 1.087 | 1.08 |
| 11 | 1.612 | 1.645 | 1.629 | 2.362 | 2.457 | 2.410 | 1.494 | 1.376 | 1.435 | 1.326 | 1.318 | 1.322 | 1.229 | 1.245 | 1.237 | 1.25 | 1.215 | 1.233 |
| 13 | 1.80 | 1.826 | 1.813 | 2.492 | 2.601 | 2.547 | 1.582 | 1.582 | 1.582 | 1.665 | 1.556 | 1.611 | 1.441 | 1.396 | 1.419 | 1.392 | 1.392 | 1.392 |
| 16 | 2.206 | 2.286 | 2.246 | 2.331 | 2.835 | 2.583 | 1.986 | 1.901 | 1.944 | 1.966 | 1.798 | 1.882 | 1.744 | 1.616 | 1.680 | 2.068 | 1.568 | 1.818 |
| 18 | 2.152 | 2.123 | 2.138 | 2.658 | 2.702 | 2.680 | 2.01 | 2.039 | 2.025 | 1.946 | 1.902 | 1.924 | 1.947 | 1.925 | 1.936 | 1.948 | 1.87 | 1.927 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*

Table A33. Growth recovery ($OD_{680}$) for *Chlorella vulgaris* after 09 months of cryopreservation.

| Time (Days) | Control | | | 5% DMSO | | | 10% Glycerol | | | 2 mg/ml EPS | | | 3 mg/ml EPS | | | 4 mg/ml EPS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** | **R1** | **R2** | **M** |
| 1 | 1.207 | 1.332 | 1.269 | 1.057 | 1.093 | 1.075 | 0.598 | 0.784 | 0.691 | 1.067 | 1.063 | 1.065 | 1.106 | 1.086 | 1.096 | 1.146 | 1.125 | 1.136 |
| 5 | 0.996 | 0.919 | 0.958 | 1.553 | 1.546 | 1.549 | 0.692 | 0.798 | 0.745 | 1.071 | 1.011 | 1.041 | 0.978 | 1.007 | 0.993 | 1.033 | 1.026 | 1.029 |
| 9 | 1.253 | 1.214 | 1.234 | 2.084 | 1.874 | 1.979 | 1.095 | 1.157 | 1.126 | 1.353 | 1.443 | 1.399 | 1.184 | 1.210 | 1.197 | 1.209 | 1.210 | 1.209 |
| 11 | 1.653 | 1.590 | 1.622 | 2.422 | 2.117 | 2.269 | 1.325 | 1.259 | 1.292 | 1.743 | 1.833 | 1.788 | 1.382 | 1.433 | 1.408 | 1.396 | 1.445 | 1.421 |
| 13 | 1.852 | 1.754 | 1.803 | 2.446 | 2.260 | 2.353 | 1.455 | 1.478 | 1.467 | 1.916 | 1.989 | 1.953 | 1.599 | 1.627 | 1.613 | 1.602 | 1.636 | 1.619 |
| 15 | 1.864 | 1.886 | 1.875 | 2.625 | 2.407 | 2.516 | 1.744 | 1.613 | 1.679 | 1.976 | 1.986 | 1.981 | 1.764 | 1.750 | 1.757 | 1.745 | 1.791 | 1.768 |
| 18 | 1.977 | 1.909 | 1.943 | 2.823 | 2.588 | 2.706 | 1.823 | 1.765 | 1.794 | 2.115 | 2.151 | 2.133 | 1.899 | 1.908 | 1.904 | 1.823 | 1.87 | 1.850 |

*Note: R1 = Replicate 1, R2 = Replicate 2, M = Mean*