University of South Bohemia in České Budějovice
Faculty of science

Isolation and characterization of microorganisms producing extracellular polymeric substances (EPS) from permafrost

Bachelor's thesis

Simona Zelenková

Supervisor: doc. Ing Jiří Bárta Ph.D.
Supervisor specialist: MSc. Muhammad Waqas

České Budějovice 2022

Bibliographic references:

Zelenková, S., 2022: Isolation and characterization of microorganisms producing extracellular polymeric substances (EPS) from permafrost. Bc. Thesis, in English. – 27 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation:

Extracellular polymeric substances (EPS) are produced by microorganisms for several reasons. It has long been assumed that EPS are produced only for the purpose of protecting the cell (e.g., capsule, slime layer) and in the formation of biofilms. However, new studies show that EPS are also produced by various microorganisms in response to environmental stress caused by lack of water, nutrients, or extreme temperature. EPS contain mainly polysaccharides and proteins, but also DNA or RNA. They link different groups of microorganisms to form biofilms, facilitate nutrient transfer, allow efficient intercellular communication and defense against predators. However, EPS also link soil particles and organic matter, influencing the formation of soil aggregates and the stability of soil organic matter, which affects the amount of CO_2 that can be released into the atmosphere. In the permafrost of the Arctic, from which CO_2 is released due to warming from the decomposition of soil organic matter, understanding the influence of EPS-producing microorganisms is essential. The aim of this work was to isolate and identify EPS-producing microorganisms from different areas of degraded permafrost (i.e., dry, and wet scenarios) and evaluate their representation in the overall microbiome.

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

In České Budějovice,

Date: 8.12.2022 ……

Simona Zelenková

Acknowledgement

I would like to thank my supervisor doc. Ing Jiří Bárta Ph.D. and MSc. Muhamad Waqas for their guidance, imparting enthusiasm for research and all help with my thesis. With that I would appreciate help of RNDr. Eva Koutecká, Ph.D. and Ing. Monika Strejčková, Ph.D., who always led me in laboratory in times I was getting lost. My last but not least thanks belong to my loving family and friends which always kept cheering me and made me keep. I am immensely proud of all we did together for this thesis to be done and I pray for further successful results.

Table of Contents

List of abbreviation

Abbreviation	Meaning
A.dest	Distilled water
CFU	Colony-forming units
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
EPS	Extracellular polymeric substances
LB	Luria-Bertani medium
NC	Negative control
OUT	Operational taxonomic units
PC	Positive control
PCR	Polymerase chain reaction
NC	Negative control
OUT	Operational taxonomic units
PC	Positive control
PCR	Polymerase chain reaction
TGY	Tryptone glucan yeast
R2A	Reasoner`s 2A agar
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SOC	Soil organic carbon
SOM	Soil organic matter
Sp.	Species
Std I	Standard I
TAE	Tris-acetate EDTA

1. Introduction

1.1. Soil carbon content in permafrost

Due to permafrost characteristic features of low temperature, decomposition of soil organic matter is very slow which cause storing approx. 1500 Pg of organic carbon in northern cryosphere region. That includes surface (0-3m) to deeper layers to 25-50 meters in land and sea floor (McGuire et.al., 2010, Hugelius et.al., 2014). The depth of permafrost goes below to 700 meters containing ice wedges from repetitions of thawing and freezing. Variations in permafrost depth is affected by altitude and latitude, snow layer, soil-properties, temperature and also vegetation creating different zones of continuous and sporadic patches in higher and lower latitudes, respectively (Schaefer et.al.,2012). It was estimated that permafrost stores approx. 58% of soil organic carbon (SOC) pool (Mueller at.al., 2017). Permafrost also includes Yedoma, relict soils of steppe-tundra ecosystem from glacial periods, that has average of carbon up to 30 times higher than nonpermafrost soils (500 Gt difference). These depots were created by thawed soil layers becoming permanently frozen containing floral roots and faunal bones establishing enormous soil organic matter stocks (Zimov, Schuur and Stuart, 2006). Global warming is accelerating cryogenic disturbance and permafrost melting, leading to increased CO_2 and CH_4 emissions from active layers into the atmosphere (McGuire et.al.,2010).

1.2. Permafrost degradation

The warming of Artic is faster compared to other regions coming from solar radiation albedo of northern lands and surrounding oceans mostly because of sea-air interaction with atmosphere. In response, the soil temperature increases 0.03 to 0.06 °C every year (Kim et.al., 2019). According to Vasiliev at al. (2020), permafrost active layers in the western Russia deepened due to the warming from 1.2m to 1.8m during 2000 to 2016. The deepening of the active permafrost layer increases the activity of microorganisms that produce CO_2 as part of their metabolism. Therefore, thawing leads to the higher release of carbon (C) emissions from the permafrost C stocks. Microbial heterotrophic respiration increases not only in upper aerated/aerobic layers but also in anoxic lower parts increasing anaerobic production of CH_4 and CO_2. In the upcoming high warming scenario, permafrost is expected to lose 27.9-112.6 Pg CO_2 and CH_4 release from anoxic habitats may be up to 35% higher before 2100 (Koven at.al., 2015).

The active layer of permafrost includes the diverse community of microorganisms with distinct functions and metabolic abilities. It is the upper part of cryosphere affected by seasonally fluctuating temperatures. As a result of the warming of the Arctic and local hydrological and geomorphological conditions, moist water-saturated or drier drained soils are formed after permafrost thawing. These different conditions significantly affect the rate of decomposition of organic matter and the associated CO_2 and CH_4 emissions (Mauritz et al., 2019).

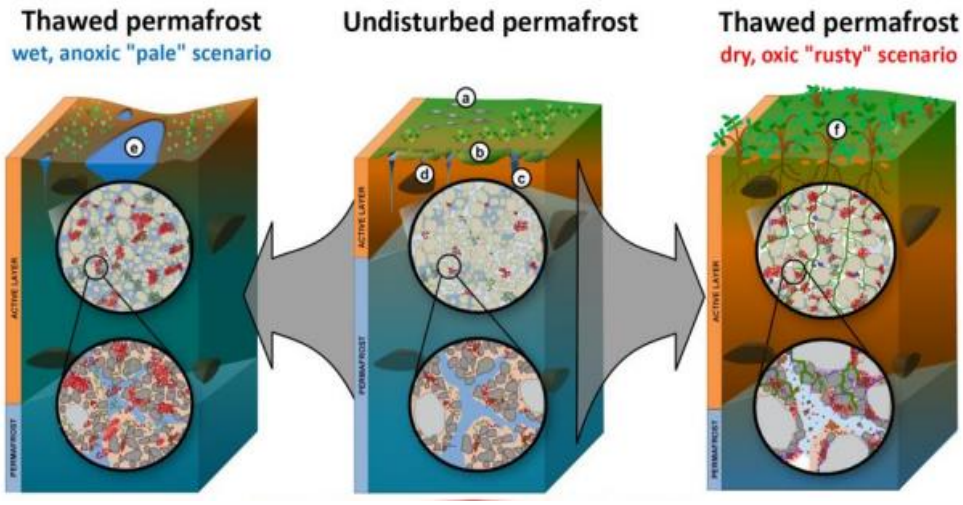

Figure 1: Two different hydrological scenarios of degraded permafrost soil.

1.3. Microbial community in permafrost

Diversity of microorganisms in permafrost is comparable to temperate soils including bacteria, archaea, microalgae, filamentous fungi, and yeasts (Fierer et al.,2007). Degradation of permafrost affects microbiome structure, metabolism, and forces microbes to create distinct defense mechanisms to survive climate changes (Hu et al., 2015). Current widespread next-generation sequencing methods allow to study of complex microbial communities (Levy and Myers, 2016). In contrast, classical culture methods allow the isolation of specific groups of microorganisms and subsequent specific physiological and genomic studies (Schmit and Lodge, 2005).

Culturing from an extreme environment requires examination of many different types of favorable conditions for their growth, which is the most challenging and mostly still impossible task. Isolation of bacteria from permafrost is important to characterize the psychrophilic behavior of permafrost microorganisms and their ability to survive aging, low nutrient and metabolite supply, or biological stress (Vishnivetskaya et al., 2000). From active

layer of permafrost, up to 80% of total community can be represented by bacteria (Vishnivetskaya et al., 2014). In total amount it accounted 2.3 x 10^9 to 1.2 x 10^8 bacterial cells per gram of dry soil (Kobabe et al., 2004) which was comparable to temperate soils. The earliest studies focusing on pure bacteria isolation and culturing were made by Gilichinsky et al. (1989). Most of isolates were psychrotrophs but also mesophiles even few thermophiles were found. Depending on media used and metabolism, bacteria isolated from permafrost were aerobic heterotrophs further anaerobic, sulfur-reducing, sulfur-oxidizing, and nitrogen-fixing bacteria (Steven et al., 2006). Männistö and Häggblom (2006) found that most of the isolates belonged to gram-negative bacteria. Depending on different studies the most represented phylum was Proteobacteria among which Delta-Proteobacteria and Alfa-Proteobacteria dominated. These species belonged to main contributors of organic mass (carbon, nutrients) with high extracellular enzyme production (Männistö and Häggblom, 2006).

1.4. Extracellular polymeric substances (EPS) and their producers

Extracellular polymeric substances (EPS) are one of the defense system against biological stress for many microorganisms produced by bacteria, archaea, fungi, and few algae (Poli et al., 2010). It can be described as a hydrated matrix enveloping a cell forming microbial bond to other cells and an environment created by a mixture of polymers. There is still not known much about EPS due to its complex structure also due to distinct production pathways (Huang et al., 2022). The microbial aggregate constitutes of 10% of the dry matter with 90% of the matrix (EPS) formed by them containing mainly polysaccharides, DNA or RNA, and proteins (Flemming and Windenger, 2010). Most of the extracellular polymeric substances are synthesized intracellularly and are exported to the extracellular environment as macromolecules. There are few (e.g. levans and dextrans) which bacteria synthesised and polymerized outside of the cell by the action of secreted enzymes on substrates. The production of EPS requires complex pathway of sugar nucleotides synthesis which relies on different enzymes. EPS biosynthesis involves at least three steps: synthesis of monosaccharides, assembly of the monosaccharides into polysaccharides and transport of the polysaccharides to the cell surface. Type of polysaccharides divides EPS into two groups. Homopolysaccharides formed from monosaccharides connected by a single vase and heteropolysaccharides constructed from many individual oligosaccharides (Laws et al., 2001).

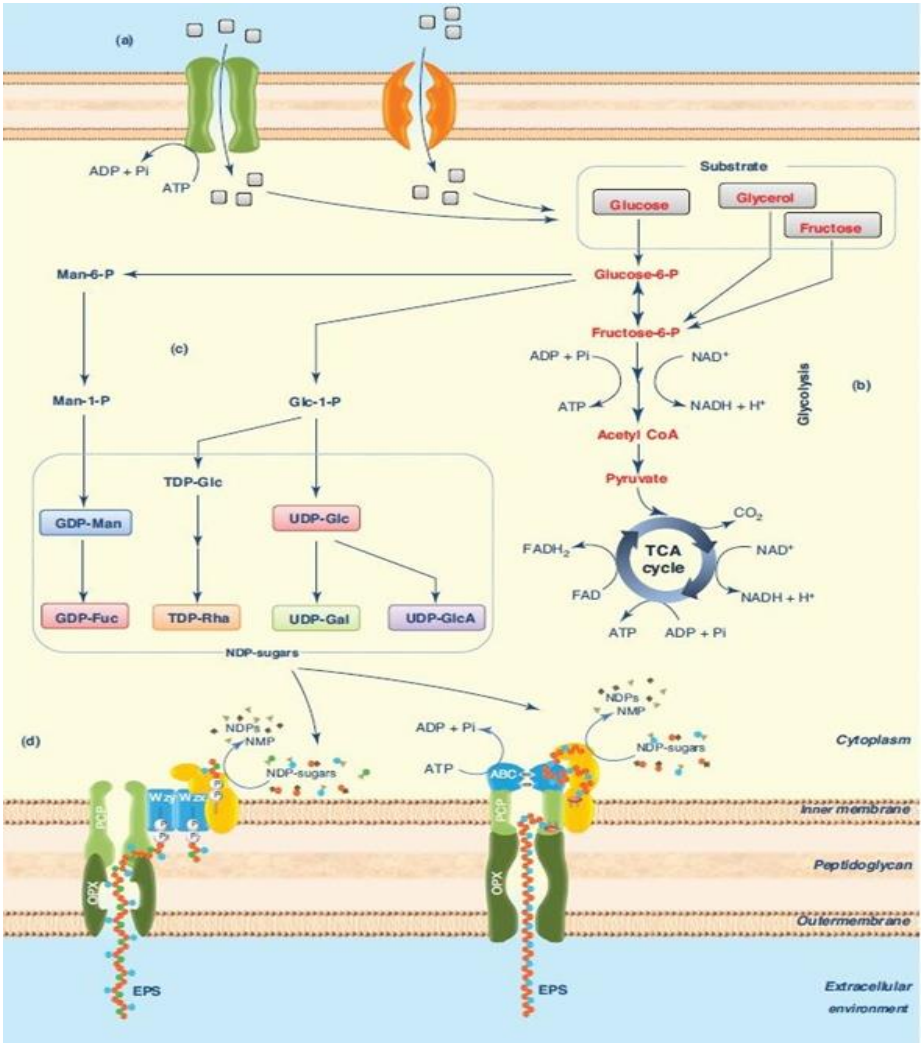

Figure 2: Bacterial biosynthetic pathways comprise substrate uptake, central metabolite pathway and polysaccharide and their secretion (Freitas et al., 2011).

The main advantages of EPS for bacteria are protection against drought, salt osmotic stress, pH, extreme temperatures or even antimicrobials and predators. Other functions of this substances are possibilities of making symbionts with nitrogen-fixing plants and rhizobia and protect this symbiosis from pathogenetic factors (Morris et al., 1991). Microbial cells use the EPS to connect with each other and share their nutrient storages (cross-feeding). With this ability EPS can also act as carbon reserves in carbon-limited habitat (Costa et al.,2018).

EPS has also an important role in the improvement of soil structure, health, porosity, and fertility, therefore, the role of EPS matrix in soil aggregation has been in the focus of many scientists (Sandhya and Ali, 2015; Wingender et al., 1999). This is supported by experimental observations demonstrating that the amendment of soil with microbial EPS results in an increased soil aggregation. Microbial communities inside different soil aggregate structures

have been extensively studied, however, few studies evaluated which taxa in the microbial community could be involved in the soil aggregation (Kravchenko et al., 2014). Caesar et al., 2014, investigated soil aggregating bacteria in various soil aggregates using cultural-dependent techniques and they were able to isolate many bacterial species involved in EPS production. Among the bacterial EPS producers, the most studied bacterial species of *Pseudomonas*, *Bacillus*, and *Paenibacillus* genera which are easily grown in laboratory conditions, producing high amounts of EPS of different chemical compositions with for example different proportions polysaccharides and proteins (Saravanan et al., 2008). *Paenibacillus polymyxa* was found to be involved in the aggregation of root-adhering soil on wheat (Bezzate et al., 2000); *Pantoea agglomerans* on the other hand regulates rhizosphere's soil water content by improved soil macroaggregation (Kaci et al., 2005). Under the environmental stress conditions, bacteria produced more EPS, protecting it against environmental stress and contributing to soil structure. For example, *Pantoea putida* was capable of improving the soil stability by 150% in salinity, high temperatures, and drought conditions (Vardharajula and Ali 2015), *Bacillus amyloliquefaciens*, *B. licheniformis*, and *B. subtilis* also implicated in soil aggregate stability under drought stress (Vardharajula and Ali., 2014).

These findings suggest that microbial exudates such as EPS and their involvement in soil stability may have a role in the occlusion of SOM within the soil aggregate in degraded permafrost soil. Therefore, it is needed to identify key microbial taxa under wet and dry conditions of degraded permafrost which can influence stabilization processes of SOC.

The main goal of this study was to (i) isolate and identify EPS producing bacteria using different culture media and (ii) determinate the proportion of isolates in total microbial community from different degraded permafrost soils.

2. Materials and Methods

2.1. Soil sampling location

Soil samples were taken from permafrost soil located in Fairbanks, Alaska, USA in 2021. The samples were taken from two different scenarios dry and wet compared with control site (intact permafrost). One gram (g) of soil from each site were used in this study for isolation of bacteria.

2.2. Solid media

2.2.1. Media used for isolation of bacteria

Standard I (Std I), Reasoner´s 2A agar (R2A), Nutrient agar and Tryptone glucose yeast (TGY) agar were used for the isolation of bacteria from soil samples and Luria-Bertani (LB) broth only for liquid culturing of pure colonies. All components of media were completely dissolved in distilled water *(A. dest.)* and autoclaved for 20 minutes at 121 °C. For bacterial selection from soil samples antibiotics such as nystatin (final concentration 25 µg/ml) was added to each media after autoclavation. Nystatin is an antifungal that prevent the growth of fungi. Following solid media were utilized for isolation of bacteria and liquid media for bacterial enrichment. The nutrient composition of all used media is listened in following table 2.

Table 1: Nutrient composition of media used in the study

Media	Ingredients	Concentrations in 1L	Reference
Standard I	Glucose	1 g	(Carl Roth Gmbh and co. KG, Karlstruhe, Germany)
	Peptone	15 g	
	Yeast extract	3 g	
	NaCl	6 g	
	Agar	18 g	
Reasoner´s 2A agar (R2A)	Yeast extract	0.5 g	(Reasoner and Geldreich, 1985)
	Proteose peptone	0.5 g	
	Casamino acid	0.5 g	
	Glucose	0.5 g	
	Starch	0.5 g	
	K_2HPO_4	0.3 g	
	$MgSO_4$ x $7H_2O$	0.024 g	
	Sodium pyruvate	0.3 g	
	Agar	15 g	
Nutrient agar	Yeast extract	2g	(Oxid Ltd. Basingstoke, Hauts, UK)
	Peptone	5g	
	Lab-Lemco	1g	
	NaCl	5g	
	Agar	15g	
	(pH 7.4±0.2)		
Tryptone glucose yeast agar	Enzymatic digest of casein	5g	(Buchbinder et al., 1951)
	Yeast extract	2.5g	
	Glucose	1g	
	Agar	9g	
	(pH 7.0±0.2)		
Luria-Bertani broth (LB)	Casein enzymic hydrolysate	10 g	(Sezonov et al., 2007)
	NaCl	10 g	
	Yeast extract	5 g	
	(pH 7.5±0.2)		

2.2.2. EPS medium

Following table 3 contains ingredients of medium which was used for the screening of bacterial species for the EPS synthesis. Before putting all together, pH of solution 1-3 was adjusted to 7 with 4M NaOH and autoclaved separately. After the autoclave solution 1-5 were mixed together. EPS medium was prepared based on Rühmann et al. (2015).

Table 2: EPS medium ingredients

Solutions	**Ingredients**	**Concentrations in 1L**	
Solution 1	$MgSO_4$ x 7 H_2O	1.33g	900 ml autoclaved separately
	Peptone from Casein	1.5 g	
	Agar	10 g	
Solution 2	$CaCl_2$ x 2 H_2O	0.05g	80 ml autoclaved separately
	Glucose Monohydrate	11g	
Solution 3	KH_2PO_4	1.67 g	20 ml autoclaved separately
Solution 4	Vitamin solution	2 ml	
Solution 5	Trace elements	1ml	Sterile filtered
Trace elements	$MnCl_2$ x 2 H_2O	1.8 g	
	$FeSO_4$ x 7 H_2O	2.5 g	
	Boric acid	2.58 g	
	$CuSO_4$ x 5 H_2O	0.031 g	
	$ZnCl_2$	0.021 g	
	$CoCl_2$ x 6 H_2O	0.075 g	
	Na_2MoO_4 x 2 H_2O	0.023g	
	Sodium tartrate x 2 H_2O	2.1 g	

2.2.3. Preservation of bacterial cells

20 ml of glycerol were dissolved completely in 80 ml distilled water and autoclaved for 20 minutes at 121 °C. The solution was used for preservation of bacterial cells at -80 °C. 9 g of NaCl (0.9% saline solution) was prepared for soil samples dissolution and for serial dilution.

2.3. Isolation of pure bacterial strains from soil samples

One gram of each soil sample was mixed in 9 ml saline solution. Serial dilutions (up to 10^{-6}) were prepared in a total volume of 1 ml. 100 µl of dilutions 10^{-2} to 10^{-5} were plated on Std I, R2A, Nutrient and TGY agar containing nystatin (final concentration 25 µg/ml). Each dilution was plated in triplicate and incubated for 7 days at 22 °C. The colony forming units (CFU) were counted and well-grown single colonies were streaked using sterile inoculum loop on fresh plates of Std I, and R2A, Nutrient and TGY agar and incubated for 3 days at 22 °C. After incubation, the single colonies were re-streaked on new fresh agar plates and this process was repeated three times to isolate pure colonies. Bacterial pure colony was inoculated into LB broth in 50ml sterile falcon tube and incubated on shaker at 160 rpm for 3 days. 1 ml of bacterial liquid cultured was centrifuged in 1.5ml sterile Eppendorf tube and the pellet was resuspended in 20 % glycerol and stored at -80 °C.

2.4. Molecular characterization of EPS-producing isolates

DNA extraction and PCR analysis will be conducted according to the standard protocol followed in the molecular lab, Faculty of science, USB. Bacterial DNA was extracted from species by following instructions from the DNeasy UltraClean microbial kit handbook (1/2020) of QIAGEN company. For the DNA quantification two solutions were prepared mentioned in table 3. Suppose that 1x TE buffer and Dye solutions were for 25 samples then the solution of 1x TE buffer was 250 µl 20x TE buffer and 4750 µl 0.1% DEPC treated water. For the Dye solution, 12.5 µl QuantiFluor Dye were added into 2487.5 µl 1x TE buffer. Afterwards 2 µl of STD or DNA sample were added to 98 µl 1x TE buffer with 100 µl Dye in one ml Eppendorf

tube. For a blank sample 100 µl 1x TE buffer and 100 µl Dye were used. The same procedure was followed for the PCR product quantification.

Table 3: Quantus fluorometer solution ingredients

Solutions	**Ingredients**	
1x TE Buffer	20x TE Buffer	10,0 µl
	H_2O	190,0 µl
QuantiFluor Dye	QuantiFluor Dye	0,5 µl
working solution	1x TE Buffer	99,5 µl

2.4.1. Polymerase chain reaction (PCR)

16s rRNA PCR amplification was carried out using master mix of 10.7 µl ddH_2O, 12,5 µl PCR buffer, 0.3 µl BSA, 0,25 µl of each primer: 9bfm (forward primer: 5'-GAGTTTGATYHTGGCTCAG3')and1512uR(reverseprimer:5'ACGHTACCTTGTTACGACTT-3') and 1,0 µl of DNA. For a positive control (PC) DNA of *Escherichia coli* (strain ATCC 9637) and for a negative control (NC) H_2O were used. The PCR cycler (PCR thermal cycler, Labcycle, SensoQuest GmbH, Germany) was performed as: initial denaturation for three minutes at 95°C, followed by 30 cycles of each denaturation for one minute at 95°C, annealing for one minute at 52°C and elongation for 90 seconds at 72°C. The final elongation was carried out for 10 minutes at 72°C.

2.4.2. Gel electrophoresis

For the gel electrophoresis 1 % agarose gel was prepared. Firstly 1 % agarose dissolved in 1xTAE buffer and after cooling mixed with 3 µl of LeliDNA fluorescent dye. The solution was poured to gel tray and left for 1 hour to completely solidify. 4 µl of PCR amplified product and 1 µl of Dye were loaded into the gel. 5 µl 1kb DNA ladder was used for the nucleotide length determination. Gel electrophoresis was run for 50 minutes at 120 V. The gel was visualized by transilluminator (Azure 200, Azure Biosystem, Inc, US).

2.4.3. Sequencing and sequence analysis of pure isolates

Purified PCR products were sent to SEQme sequencing company (Prague, CZ). The obtained sequence data were quality trimmed and joined the forward and reverse reads by Geneious Prime bioinformatics software. The nucleotide sequences were then searched in 16SrRNA gene NCBI database by standard nucleotide BLAST (Altschul et al., 1990).

2.5. Determination of proportion of EPS isolates in total community

To determine the relative abundance of the isolated bacteria in the total bacterial community, all 16SrRNA gene sequences from the pure isolates were blasted against a local database constructed from 16SrRNA sequences of operational taxonomic units (OTUs) from the total microbiome from 2021. The threshold from similarity between sequences was set at greater than 98%. For those OTUs that were more than 98% similar to the pure isolates, relative abundances at each site were calculated according to the OTU table. The proportion of isolated bacteria among the total bacterial community was statistically analysed by R Core Team 2020 (R v 4.0.2) using packages Phyloseq, Vegan and Tidyverse. The figure was generated via ggplot.

3. Results and Discussion

3.1. CFU determination

The CFU for the agar plates was counted to calculate the number of bacteria per gram of soil. The CFU/g of soil solution was calculated from the 10^3-fold diluted soil suspension which had the maximum number of colonies on agar plate as shown in the figure 3. The highest count of CFU/g was for the soil sample from wet site and then for the soil sample from intact site. While the least CFU/g was counted for the soil sample of dry site (Table 4). Different bacterial species were isolated from three different sites of permafrost using different cultivation media. The highest number of colonies was counted for the R2A agar such as $2.94*10^6$ CFU/g for wet site soil sample and lowest was counted for Nutrient agar $8*10^4$ CFU/g for dry site soil sample. Overall, for all the soil sample Std I agar plate had the minimum CFU/g count (Table 4 and Figure 3).

Reasoner`s 2A agar has rich density of nutrients which are good sources of nitrogen and carbon for heterotrophic bacterial growth. R2A agar is suitable for the bacteria from all environments and especially for slow growing bacteria (Reasoner and Geldreich, 1985). On the other hand, Std I and Nutrient agar have the low quantity of carbon and nitrogen compared to R2A. CFU count for the TGY agar was higher than the Std I and Nutrient agar media. It may be due to the enzymatic digest of casein which can enhance the growth of bacteria in a medium (Buchbinder et al., 1951).

Table 4: Calculation of CFU/g of soil solution

Soil sample	**Std I**	**R2A agar**	**Nutrient Agar**	**TYG agar**
Dry site	$2.1*10^5$	$3.5*10^5$	$8*10^4$	$2.1*10^5$
Wet site	$1.22*10^6$	$2.94*10^6$	$1.82*10^6$	$1.7*10^6$
Intact site	$3.1*10^5$	$1.27*10^6$	$3.6*10^5$	$4.5*10^5$

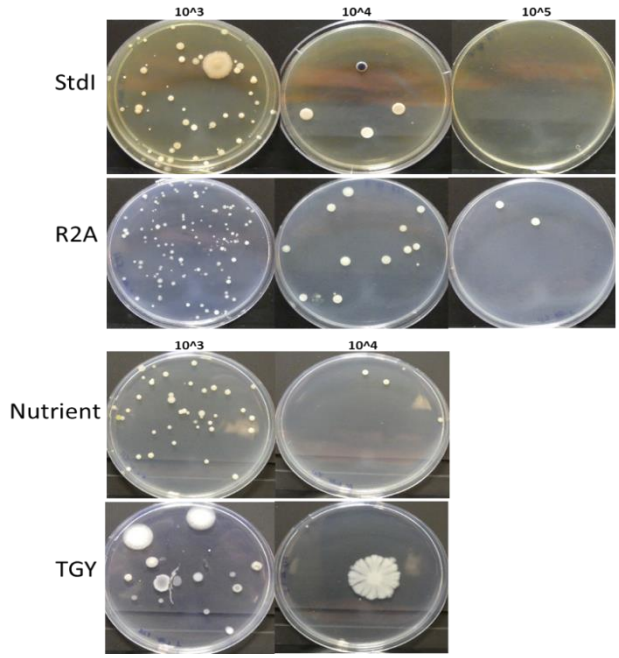

Figure 3: Different bacterial colonies number and morphology on serial diluted (103, 104,105) soil samples agar plates.

3.2. Isolation of bacteria from plates

Total of 57 bacterial colonies were picked from the serial diluted plates based on their distinct morphology and grown on fresh agar media. Representative agar plates are shown in the Figure 4.

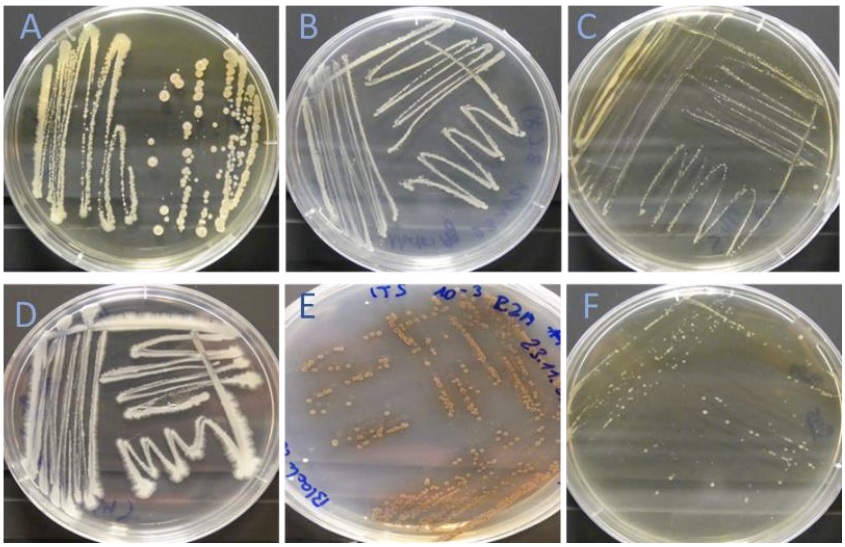

Figure 4: Pure bacterial colonies on agar plates: (A) *Curtobacterium sp*., (B) *Viridibacillus arenosi*, (C), *Bacillus subtilis*, (D) *Bacillus mycoides*, (E*) Bacillus sp*., (F) *Neobacillus bataviensis*.

3.3. Molecular characterization of soil isolates

Genomic DNA was isolated from all soil isolates and PCR was carried out using the universal primers. The PCR products were then visualized on 1 % gel. According to 16S rRNA PCR using universal bacterial primers 9bfm and 1512uR, the resulting PCR products have the expected size of about 1.5 kb DNA fragment of 16S rRNA gene comparing with 1kb DNA ladder (Figure 5).

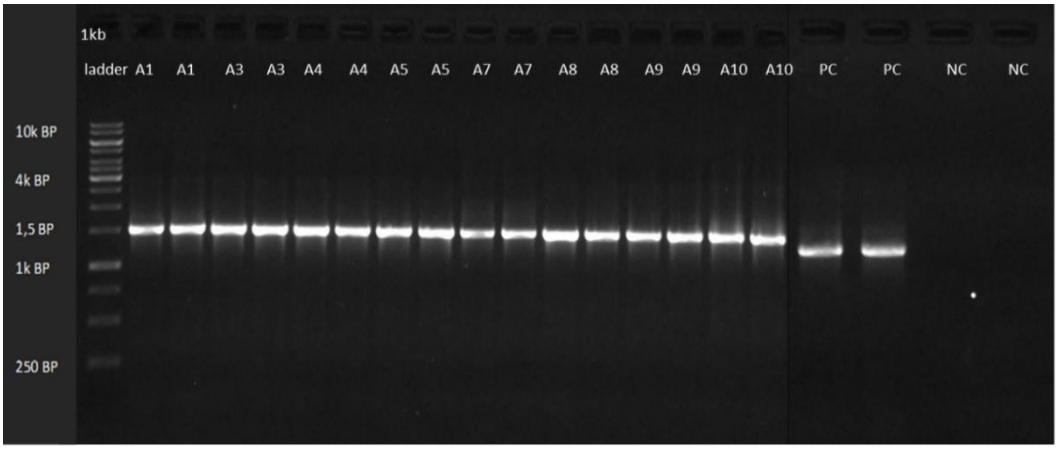

Figure 5: Representative agarose gel analysis of amplified 16S rRNA gene: (A1) *Paenibacillus lupini*, (A3) *Viridibacillus arvi*, (A4) *Viridibacillus arenosi*, (A5) *Bacillus subtilis*, (A7) *Staphylococcus edaphicus*, (A8) *Staphylococcus sp.*, (A9) *Bacillus mycoides*, (A10) *Neobacillus bataviensis*, (PC) positive control, (NC) negative control.

3.4. Sequencing data analysis

The nucleotide sequence data from NCBI blast reviled that the majority of soil isolates belonged to phyla Bacillota, Actinomycetota, Firmicutes and Pseudomonata in total number of 57 species. Species of phylum Bacillota were high in member in all three sites and then followed by species from phylum Actinomycetota (Table 4).

All species of Bacillota are mesophilic that can grow easily at moderate temperature between 20 to 45 °C and create highest abundant in soil generally (Fan et al., 2022). They are Gram-positive bacteria with spores-producing ability that helps them to survive in harsh conditions (Douglas and Waldrop, 2019).The phylum Actinomycota belonging to Gram-positive bacteria are cold-adaptive and producing natural organic products protecting them from environment stress

(Benaud et al.,2022). Three species of phylum Pseudomonadota (mostly Gram-negative bacteria) were isolated from wet site, and none was isolated from control (intact site). This could be, the Pseudomonata has higher abundance in roots microbiome than the ones with intact permafrost soil characteristic, where it may be due to the low availability of nutrients (Dennert et al., 2018). Firmicutes phylum are Gram-positive species represented with one specie in each permafrost soil site (Table 5). Firmicutes has lower potential for utilizing soil nutrients than other phylum and correspond differently on carbon-rich aggregates (Li et al., 2020).

Table 5: Number of different soil isolates isolated from two different scenarios of degraded permafrost (dry, wet) and control site (intact soil).

	Number of bacteria according to permafrost soil sites		
Phylum level	Dry site	Wet site	Intact site
Actinomycetota	4	4	6
Bacillota	11	12	14
Firmicutes	1	1	1
Pseudomonata	1	3	0

At genus level most of the isolates were belonged to genera *Bacillus, Streptomyces, Viridibacillus, Staphylococcus, Neobacillus, Peribacillus, Phyllobacterium, Pseudarthrobacter* and *Frigoribacterium* (Table 6), which are represented as common species in permafrost soil with optimum growth temperature of 22°C. The reason behind their abundance in permafrost soil are their spore formations that can prevent survive of the cells as well as their adapted metabolic systems (Bakermans et al., 2003). Single isolated species were belonged to genera *Micromonospora, Micrococcus, Microbacterium, Mesobacillus, Luteimonas, Leclercia* and *Domibacillus* (Table 6).

Bacillus mycoides was the most isolated specie from the wet and dry as well as from the control (Intact site). The most represented genus *Bacillu*s is able to adapt to extreme environment and had high success rate of growth on different utilized media. Same result came out from Douglas and Waldrop (2019), who focused on microbial community structure across permafrost soils. The phylogeny of *Bacillus* is difficult since new close-related species were

clarified into this genus even though depending on branching in the 16S rRNA gene trees they only belong in family Bacillaceae. Therefore, our second most contained genera *Peribacillus* and *Viridibacillus* are not often named in publications, but still concluded in *Bacillus* genus (Patel and Gupta, 2020).

The last dominant species were from the genus *Streptomyces* (Table 5). Most of the species from this genus do not divide by binary fusion but have complex life cycle and known as spore forming bacteria. They have very high biological activity with abilities to survive in unfavorable conditions. Pan et al., 2021 found out the predominant genus for cold region highland barley in Qamdo mountains was *Streptomyces* with 16% higher abundance than *Bacillu*s. *Streptomyces* were found more in isolates incubated at low temperature, but the rate of growth was lower than other this is due to their complex life cycle (Wolanski et al., 2011).

One of the least represented ones was the specie from genus *Luteimonas* (Table 6). The reason why such as species were not present much in our finding is because their optimal growth temperature is too high. For example, optimal temperature for *Luteimonas* species is 30°C, and we incubated our species at 22°C (Mu et al., 2016).

Table 6: Results of bacterial species identification based on 16SrRNA gene sequencing

Strain	16s RNA sequencing	Identity %	Strain	16s RNA sequencing	Identity %
1	*Paenibacillus lupini*	98,3%	30	*Micrococcus yunnanensis*	99,3%
2	*Microbacterium flavescens*	98,3%	31	*Viridibacillus arvi*	99,5%
3	*Viridibacillus arvi*	100%	32	*Streptomyces laculatispora*	99,5%
4	*Viridibacillus arenosi*	99,5%	33	*Staphylococcus epidermidis*	99,8%
5	*Bacillus subtilis*	99,1%	34	*Streptomyces luozhongensis*	99,5%
6	*Bacillus proteolyticus*	98%	35	*Pseudarthrobacter sulfonivorans*	98,6%
7	*Staphylococcus edaphicus*	94,2%	36	*Paenisporosarcina indica*	98,8%
8	*Staphylococcus saprophyticus*	100%	37	*Frigoribacterium faeni*	99,4%
9	*Bacillus mycoides*	100%	38	*Frigoribacterium faeni*	99,5%
10	*Neobacillus bataviensis*	99,2%	39	*Neobacillus pocheonensis*	99,1%
11	*Streptomyces brevispora*	99,5%	40	*Streptomyces luozhongensis*	99,2%
12	*Luteimonas arsenica*	99,8%	41	*Peribacillus simplex*	99,3%
13	*Bacillus mycoides*	100%	42	*Pseudarthrobacter sulfonivorans*	98,7%
14	*Bacillus mycoides*	100%	43	*Viridibacillus arvi*	100%
15	*Curtobacterium oceanosedimentum*	99,3%	44	*Bacillus subtilis*	99,9%
16	*Micromonospora fulva*	98,6%	45	*Peribacillus simplex*	99,6%
17	*Bacillus subtilis*	99,8%	46	*Bacillus mycoides*	100%
18	*Tumebacillus permanentifrigoris*	99,3%	47	*Bacillus mycoides*	99,9%
19	*Peribacillus simplex*	99,9%	48	*Viridibacillus arenosi*	99,4%
20	*Phyllobacterium loti*	99%	49	*Bacillus mycoides*	100,%
21	*Phyllobacterium endophyticum*	99,5%	50	*Peribacillus simplex*	99,7%
22	*Bacillus mycoides*	100%	51	*Bacillus mycoides*	99,9%
23	*Neobacillus bataviensis*	99%	52	*Paenibacillus odorifer*	98,8%
24	*Peribacillus simplex*	96,2%	53	*Domibacillus mangrovi*	98,7%
25	*Peribacillus simplex*	95,6%	54	*Bacillus mycoides*	97,6%
26	*Leclercia adecarboxylata*	91,3%	55	*Bacillus subtilis*	99,2%
27	*Mesobacillus subterraneus*	98,2%	56	*Bacillus subtilis*	98%
28	*Curtobacterium oceanosedimentum*	98,8%	57	*Bacillus subtilis*	99,6%
29	*Streptomyces luozhongensis*	99,5%			

3.5. Growth of bacteria on EPS medium

For the screening of EPS production by bacterial species, we cultured all isolates on specific EPS medium. We have found that most of our bacterial species about 48 out of 57 were able to grow on EPS medium which considered as potential EPS producers. Most of the species from genus *Bacillus*, *Peribacillus*, *Pseudoarthrobacter*, *Frigoribacterium*, *Streptomyces* and *Viridibacillus* were able to grow on EPS medium that could be the potential EPS producers. These species are able to grow and produce high number of EPS at laboratory condition under certain parameters (Rühmann et al., 2015). In Deka et al.(2019), bacterial species were tested for EPS production which are the same finding to ours, the highest abundance had *Bacillus* species in screening for EPS production.

Table 6: Growth of soil isolates on EPS medium

Strain	16s RNA sequencing	Strain	16s RNA sequencing
2	*Microbacterium flavescens*	29	*Streptomyces luozhongensis*
3	*Viridibacillus arvi*	30	*Micrococcus yunnanensis*
4	*Viridibacillus arenosi*	31	*Viridibacillus arvi*
5	*Bacillus subtilis*	32	*Streptomyces laculatispora*
6	*Bacillus proteolyticus*	34	*Streptomyces luozhongensis*
7	*Staphylococcus edaphicus*	35	*Pseudarthrobacter sulfonivorans*
8	*Staphylococcus saprophyticus*	37	*Frigoribacterium faeni*
10	*Streptomyces brevispora*	38	*Frigoribacterium faeni*
12	*Luteimonas arsenica*	40	*Streptomyces luozhongensis*
13	*Bacillus mycoides*	41	*Peribacillus simplex*
14	*Bacillus mycoides*	42	*Pseudarthrobacter sulfonivorans*
15	*Curtobacterium oceanosedimentum*	43	*Viridibacillus arvi*
16	*Micromonospora fulva*	44	*Neobacillus bataviensis*
18	*Tumebacillus permanentifrigoris*	46	*Peribacillus simplex*
19	*Peribacillus simplex*	47	*Bacillus mycoides*
20	*Phyllobacterium loti*	48	*Bacillus mycoides*
21	*Phyllobacterium endophyticum*	49	*Viridibacillus arenosi*
22	*Bacillus mycoides*	50	*Bacillus mycoides*
23	*Neobacillus bataviensis*	51	*Peribacillus simplex*
24	*Peribacillus simplex*	52	*Bacillus mycoides*
25	*Peribacillus simplex*	53	*Paenibacillus odorifer*
26	*Leclercia adecarboxylata*	54	*Domibacillus mangrovi*
27	*Mesobacillus subterraneus*	45	*Bacillus mycoides*
28	*Curtobacterium oceanosedimentum*		

3.6.Comparison of classic media cultures with EPS medium

The bacterial species were grown on EPS medium and Std I medium for comparison of their colony's morphology (Figure 6). Most of the species were able to grow as a slimy colony on EPS medium which were different from ones on standard medium (Figure 7). The species of *Bacillus, Pseudarthrobacter Frigoribacterium* and *Paenibacillus* showed slimy colonies on EPS medium, but the slimy texture was not that much dense as Rühmann et al., (2015) have found. Slimy layer on bacteria colony is manly composed of polysaccharide and indication of EPS production. However, the high/low production does not depend on the amount of slimy colony on agar plates. Most of the bacteria are produce more EPS but have less dense in slimy layer when grow on EPS specific agar (Rühmann et al., (2015). Similarly, *Staphylococcus, Arthrobacter* and *Paenibacillus* species were found in permafrost thaw lake sediments and grew as slimy colony on simple carbon sources agar plates that detected EPS production, same way as our respective bacterial species (Finore at al., 2020).

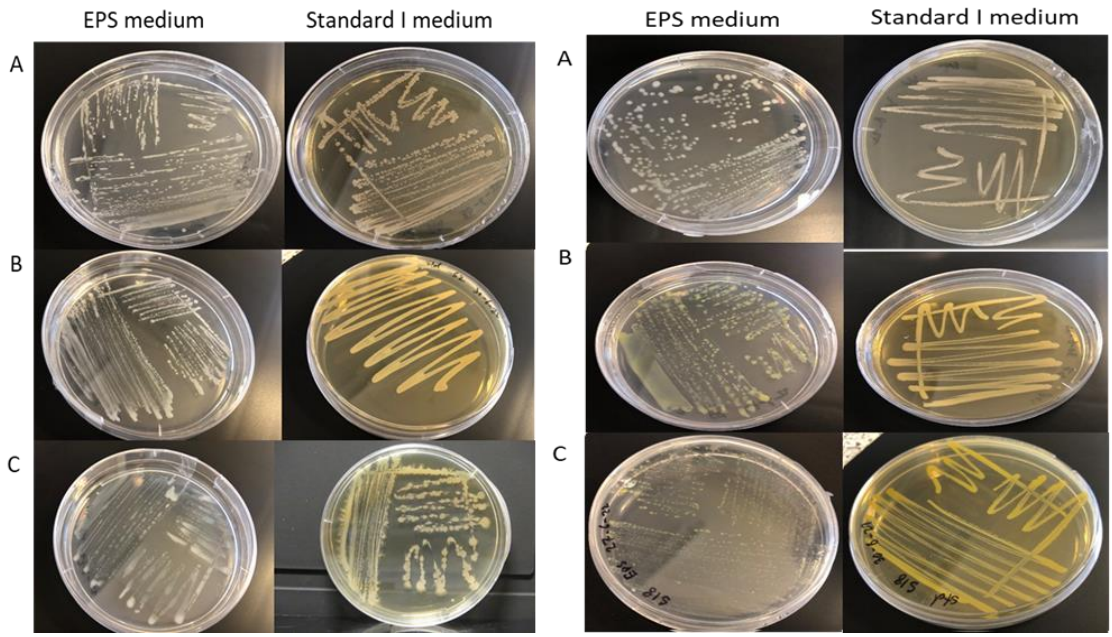

Figure 6: Comparison of bacterial colonies on EPS medium with Standard I medium: on the left side bacterial species are A – 4: *Viridibacillus arenosi*, B – 22*: Bacillus mycoides*, C – 5: *Bacillus Subtilis* and on the right side are A- 7: *Staphylococcus edaphicus*, B – 35: *Pseudarthrobacter sulfonivorans*, C – 38: *Frigoribacterium faeni*.

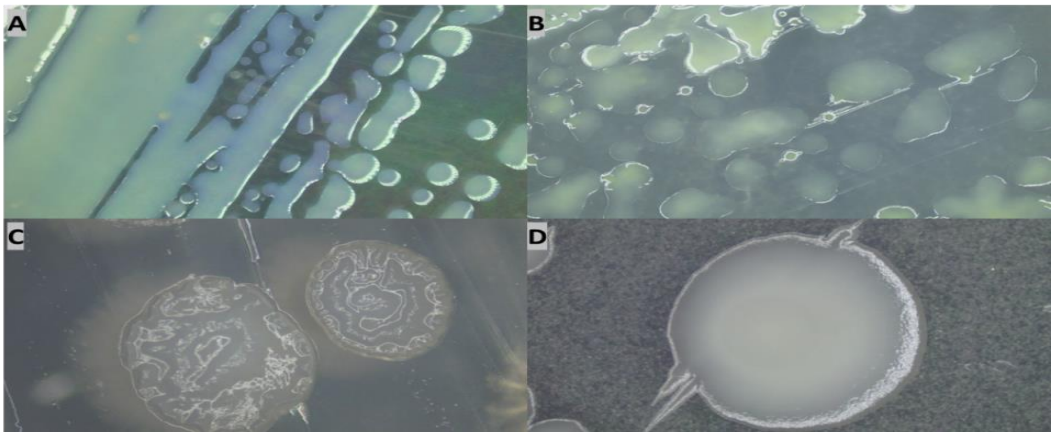

Figure 7: Closer photo of bacterial slimy colonies on EPS medium by microscope with camera and 0.5x zoom lens (Hotair,CZ) : Bacterial species are A – 22: *Bacillus mycoides*, B – 38: *Frigoribacterium faeni*, C – 35: *Pseudarthrobacter sulfonivorans*, D – 4: *Viridibacillus arenosi*.

3.7. Proportion of potential EPS-producing bacteria in total bacterial community

We compared the soil isolates (potential EPS-producers) 16S rRNA gene with the database of total bacterial community in soil sample at genus level from 2021 data (Figure 8). About 20% of total bacterial community in soil sample covered by the potential EPS-producing bacteria for the dry and about 5% for wet site compared to the control (Intact site) which covered about 4%. Dry scenario of the permafrost soil has more vegetation compared to the control (Intact site) (Mauritz et al., 2019). In this condition bacteria have high metabolic activity, can utilize available nutrients, and might produce EPS. That could be the reason why the proportion of potential EPS-producers was higher in dry site.

While the proportion of EPS-producing bacteria were different among the three sites but the most interesting is that structure of genera were almost similar. There could be several reasons for the presences of bacteria from same genera among all sites, such as a diversity of vegetation, different water content and temperature (Mauritz et al., 2019).

There were 17 genera after blasting 16SrRNA gene sequences from the pure isolates against the database. The species of *Bacillus* were more prominent followed by species of genus *Klebsiella* and *Cohnella* in the dry site compared to the control (Intact site). Species of *Bacillus, Domibacillus, Phyllobacterium* and *Planococcus* had almost the same proportion in the wet site but lower than dry and higher than control site. The abundance of *Planococcus, Domibacillus,*

and *Bacillus* was lower in control (Intact soil), while *Phyllobacterium* was not even found. In control (Intact site) the highest proportion had *Leifsonia*, which was not found in dry and wet site. (Figure 8).

From this analysis we have found that the isolated potential EPS-producers were present in all three permafrost sites in certain proportion. These bacteria may have role in occlusion of organic matter within soil aggregates by production of EPS.

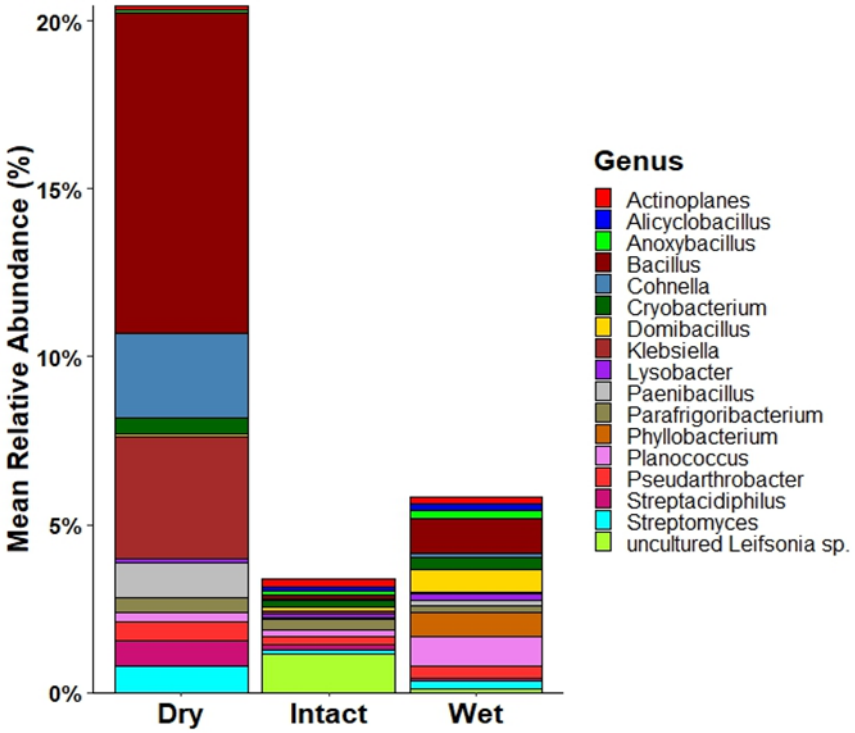

Figure 8: Relative proportion of potential EPS producers at genus level in total bacterial community of three different sites of permafrost soil samples of 2021.

4. Conclusion

The aim of this study was to isolate EPS producing bacteria from the different site of permafrost soil using different media. From the utilized media, R2A had highest for bacterial growth from permafrost soil due to high nutrients rich media and high in amount of carbon and nitrogen.

In our finding, 48 out of the 57 bacterial species were able to grow on EPS specific medium that were considered as potential EPS-producers. Comparing the soil isolates (potential EPS-producers) 16S rRNA gene with the database of total bacterial community in soil sample 2021 at genus level. There was difference in the proportion as well as the structure of EPS-producing genera among the three sites of permafrost soil. The potential EPS producing bacteria cover at genus level over 20% of total bacterial community in dry site, 5% for wet site, and below 4 % for the control (Intact site).

The result of our project contributes to find and understand the microbial EPS production in permafrost effected soil and their relations with the stabilization of SOM. The problem is also that the active layer of permafrost is constantly changing and everything inside with it, that is why we have differences in bacterial community structure among the three sites of permafrost soil.

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