مشخصه‌یابی باکتری‌های قابل کشت سرماوست و مقاوم به سرما در خاک‌های آلپ ایران

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چکیده

مقدمه: در اکوسیستم‌هـای سـرد، باکتری‌هـا نقشـی حیـاتی در چرخـه‌هـای بیوشـیمیایی، تجزیـۀ زیسـتی آلاینـده‌هـا و زیست‌فناوری دارند؛ به‌همین‌علت در سال‌های اخیر، توجه زیادی به شناسایی و ارزیابی آنزیم‌هـای خارج‌سـلولی آنهـا شده است.

مواد و روش‌ها: در پژوهش حاضر، ۴۳ باکتری سرمادوست یا مقاوم به سرما از خاک‌های آلپی در محیط‌کشت‌های متفاوت جداسازی و مشخصه‌یابی شدند و توانایی باکتری‌های یادشده در تولید آنزیم‌های خارج‌سلولی مطالعه شد.

نتایج: باکتری‌هـای مطالعـه‌شـده بـه پـنج گـروه اصلـی شـامل *Actinobacteria، Firmicutes، αγ-proteobacteria* و *Bacteroidetes* تعلق داشتند و درمجموع، تعداد ۹ جنس شناسایی شـدند کـه جنس‌هـای *Arthrobacter، Bacillus* و *Pseudomonas* فراوانی بیشتری را نشان دادند.

بحث و نتیجه‌گیری: تمام سویه‌های به‌دست‌آمده توانایی تولید حداقل یک آنزیم خارج‌سلولی را داشتند و فعالیـت پروتئولیتیک و آمیلولیتیک در بین سویه‌ها بیشتر بود. فعالیت سلولاز و پکتیناز بـه‌ترتیب در ۴۴ و ۲۲ درصـد سـویه‌هـا مشاهده شد. مطالعۀ حاضر نشان داد هنگامی که علاقه‌مند به یافتن منابع زیستی برای تولید آنزیم‌های خارج‌سلولی فعال در سرما به‌منظور بازکردن پنجره‌های جدید برای کاربردهای زیسـت‌فنـاوری باشـیم، باکتری‌هـا در محیط‌هـای سـرد نامزدهای مناسبی برای جستجو هستند

واژه‌های کلیدی: آلپ ایران، آنزیم‌های فعال در سرما، باکتری‌های سرمادوست و مقاوم به سرما

Characterization of Psychrophilic and Psychrotolerant Cultivable Bacteria in Alpine Soil in Iran

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Abstract

Introduction: Bacteria in cold ecosystems play a vital role in biochemical cycles, the biodegradation of pollutants, and biotechnology. For this reason, the identification and evaluation of their extracellular enzymes have received a lot of attention in recent years.

Materials and methods: In this study, 43 psychrophilic or psychrotolerant bacteria were characterized from alpine soils on different culture media. The ability to produce extracellular enzymes by these bacteria was studied.

Results: These bacteria belonged to the four major phyla including *Actinobacteria, Firmicutes, Proteobacteria,* and *Bacteroidetes.* A total of nine genera were identified among which *Pseudomonas*, *Arthrobacter*, and *Bacillus* were the most abundant.

Discussion and conclusion: All of the obtained strains have the ability to produce at least one extracellular enzyme; proteolytic and amylolytic activities were the highest among these strains. Cellulase and pectinase activities were observed in 44 and 22 percentage of these strains. This study was the first report on psychrophilic and psychrotolerant bacteria in the Zagros Mountains (Oshtorankuh) and their extracellular enzyme production. The present study indicated that a wide range of bacteria in cold native ecosystems of Iran can be a suitable source for cold active enzymes.

Key words: Alpine Iran, Cold Active Enzyme, Psychrophile and Psychrotolerant Bacteria

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Introduction

Survival and growth in cold environments involve major challenges such as reduced enzyme reaction rate, limited nutrient sources, inappropriate pH, salinity, and reduced water activity. Psychrophilic and psychrotolerant bacteria have evolved for growth in low temperatures in terms of both structure and function. They are observed in a wide range of metabolic activities at sub-zero temperatures in cold ecosystems. Characteristics such as spore formation, pigmentation, enzymatic activity, membrane fluidity, and antifreeze proteins facilitate the survival of these bacterial communities in cold habitats (1) and the microbial activities play a significant role in the biogeochemical cycling of these ecosystems (2).

Many studies performed in the past few decades have adopted the culture-dependent method to characterize the bacterial flora of snow-covered ecosystems (e.g. in the Arctic permafrost) (3), in high elevation grasslands of Central Asia (4, 5), South Shetland Islands, Antarctica (6), etc. These studies have reported that the most abundant bacterial community found in these extreme environments belongs to *Proteobacteria* (*Alpha*, *Beta,* and *Gamma*), gram-positive bacteria (*Actinobacteria*, *Firmicuts*), and *Bacteroidetes* (3-7). Alpine environments are sources of cold-tolerant bacteria that can potentially be important in fundamental and applied research. Although culture-dependent approaches have often been criticized for their selectivity, they remain essential in environmental microbiology (8). Isolation and cultivation methods are also essential in understanding the physiology and ecology of these bacterial communities.

The annual market for thermotolerant enzymes is $520 million. Considering the fact that there are many capabilities in cold active enzymes, it is possible that cold-adapted enzymes are of higher value (9). Cold-active enzymes have a high catalytic efficiency (K_{cat}/K_m) at low temperatures. Therefore, they result in the reduction of energy consumption since they do not need any heating, and the reaction efficiency increases which is more desirable in terms of cost (10). Using these enzymes would reduce the undesirable chemical reactions that occur at high temperatures and they can be inactivated easily by using high temperatures. Therefore, they are applicable in various industries such as food, textile, detergent, and pharmaceutical productions. The isolation and identification of psychrophilic bacteria that can produce different enzymes have been reported in many studies (11, 12). On the other hand, due to the challenges that exist in cold ecosystems, the role of these microorganisms has been studied in the bioremediation of pollutants and the effect of freeze-thaw cycles (12-14).

In this study, psychrophilic and psychrotolerant bacteria from Oshtorankuh (alpine soils) in Lorestan province were characterized and their ability to produce extracellular enzymes was evaluated.

Material and Methods

Sampling and Bacterial Isolation: Soil samples were collected under sterile conditions from the depth of 30 cm in the Oshtorankuh mountains (west of Iran, 48°58′ E, 33°11′ N). Serial dilutions (up to 10^{-6}) were plated on six different media and incubated aerobically at 4,20 and 37 °C for three weeks. Culture media containing Yeast Malt Extract agar 10% (per L: 4 g glucose, 4 g yeast extract, 10 g malt extract, 15 g agar), Tryptic Soy agar 10% (per L: 15 g Bacto treptone, 5 g bacto soytone, 5 g NaCl, 15 g agar), R_2A (per L: 0.5 g Yeast extract, 0.5 g proteose peptone, 0.5 g casamino acids, 0.5 g dexterose, 0.5 g soluble starch, 0.3 g sodium pyruvate, 0.3 K_2HPO_4, 0.05 g $Mg_3(PO_4)_2$, 15 agar), Soil Extract Agar (2 g glucose, 1 g yeast extract, 0.5 g K_2HPO_4, 100 ml soil extract, 15 agar) were used to isolate bacteria. Soil Extract (SE) was prepared as follows: 250 g of soil were re-suspended in 1000 ml of distilled water, homogenized by agitation with a stirring bar,

autoclaved at 121 °C for 20 min; the suspension was filtered through a 0.2-μm-pore-size filter, pH was adjusted to 7. Distinct colony types on the plates were purified and achieved for further studies by using diluted Tryptic soy agar plates.

Enzyme Activity: The presence of extracellular enzymes was examined on Mineral Salt Medium (MSM) agar supplemented with starch (1 %w/v), skim milk (2 %w/v), and Tween 80 (0.5 %v/v) for amylase, protease, and lipase, respectively. DNase test agar medium (42 g/L) was used for DNase activity. The ability to degrade urea was detected using urea broth medium (g/L) (yeast extract, 0.1: K_2HPO_4 9.5: KH_2PO_4, 9.1: Urea, 20: and phenol red, 0.01). Cellulase analysis was done using culture media containing (g/L): carboxymethyl cellulose (CMC), 5: $NaNO_3$, 1: K_2HPO_4, 2: glucose, 2: and agar, 15. Pectinolytic activity was carried using culture media containing pectin 5 g/L: $(NH_4)_2SO_4$ 1.4 g/L, K_2HPO_4 2 g/L, $FeSO_4.7H_2O$ 4 mg/L, $MnSO_4.H_2O$ 1.6 mg/L, $ZnSO_4.7H_2O$ 1.4 mg/L, $CaCl_2$ 2 mg/L and agar, 15 g/L.

These strains were kept at 4 or 20 °C for 7–10 days. Observation of a clear halo around the colonies or precipitation or coloration was indicative of the positive activity with the decomposition of substrates.

PCR Amplification of Strains: Genomic DNA was extracted from fresh bacterial cultures using boiling protocol. The 16S rRNA gene was amplified using universal primers 27F and 1492R. Gene amplification of 16S rRNA was performed using 1.5 mM $MgCl_2$, 30 mM KCl, 10 mM Tris-HCl, 2.5 mM of each dNTP, 10 pmol of each primer, 0.2U of ampliqon Taq DNA polymerase and template DNA (~50 ng). PCR amplification was performed under the following conditions: initial denaturation at 95 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 30s, primer annealing at 54 °C for 30s and extension at 72 °C for 50s. The final cycle included an extension at 72 °C for 7 min. The sequencing was carried out at Macrogen, South Korea.

Phylogenetic Analysis and Sequence Comparison: The partial 16S rRNA sequences of the isolates were compared with Gene Bank database using the National Centre for Biotechnology Information (NCBI) and EZ-Taxon. The method of Kimura 2-parameter was used to calculate evolutionary distances. Phylogenetic trees were constructed by the Neighbour Joining method and were evaluated by performing bootstrap analysis of 1000 data sets using MEGA 7.0 software (15).

Results

Counts and Phenotypic Characterization of the Isolates: A total of 411 bacterial isolates were isolated using four different media cultures. The bacteria were divided into psychrotolerant, psychrophile, and mesophile groups based on their growth under different temperature conditions. Psychrotrophs were identified by the growth of isolates at 4 °C and 20 °C but not at 37 °C. Strains growing only at 4 °C were considered as psychrophiles. Mesophiles were identified by the growth of isolates at 20 °C and 37 °C. Psychrotrophs had a higher abundance than psychrophiles in all the tested media, and mesophile bacteria were removed from the experiment. The bacterial counts and diverse phenotypes were higher in soil extract agar, and lower in TSA, and more abundant at 20 °C than at 4 °C (Fig. 1). Colony-forming unit (CFU) and pigmented colonies counts displayed a different repartition according to culture media at the two tested temperatures. The effect of isolation media is significant particularity at 20 °C, especially between soil extract agar and tryptic soy agar. The number of CFU was higher at 20 °C than at 4 °C.
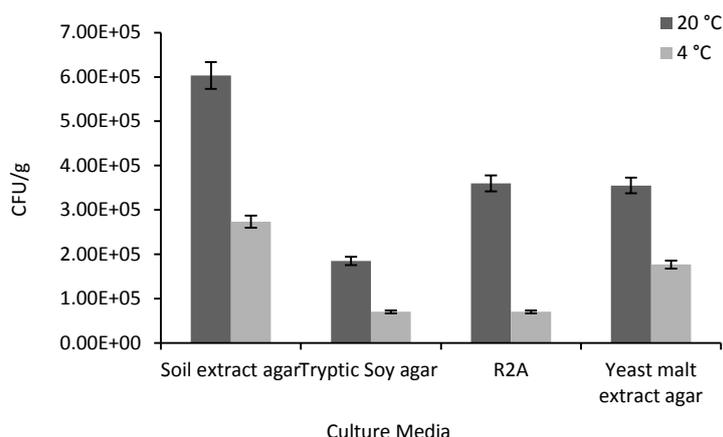
Fig. 1- Number of CFU in Different Culture Medium and at Two Different Temperatures

Isolation and Identification of Bacteria: A total of 117 pure bacterial strains were isolated from Oshtorankuh Mountains from four different media. Based on strains morphology, 26 psychrotolerants and 17 psychrophiles were selected for the molecular identification. The identification of the strains was carried out using the 16S rRNA gene. The results revealed that the cultivated strains belonged to four major lineages of the bacterial phyla, namely *α-γ-Proteobacteria,* *Bacteroidetes,* *Actinobacteria,* and *Firmicutes* and corresponded to 9 different genera. *γ-Proteobacteria* with 39.5 %, *Actinobacteria* with 32.5%, and *Firmicutes* with 11.5% were the most frequently-observed ones (Table 1).

Table 1- Type and Number of Psychrophilic and Psychrotolerant Cultivable Bacteria isolated from Oshtorankuh Mountains (Alpine Soils)

Phylum	Psychrotolerant	Number	Psychrophiles	Number
α-Proteobacteria	*Sphingomonas*	1	*Sphingomonas*	2
γ-Proteobacteria	*Pseudomonas*	7	*Pseudomonas*	10
Bacteroidetes	*Flavobacterium*	1	*Pedobacter* *Flavobacterium*	2 1
Actinobacteria	*Arthrobacter* *Streptomyces* *Pseudarthrobacter* *Kocuria*	8 2 1 1	*Arthrobacter* *Pseudarthrobacter*	1 1
Firmicutes	*Bacillus*	5	-	-
Total number of the strains		26		17

Fourteen isolates were affiliated with the *Actinobacteria* phylum. Molecular characterization based on 16S rRNA gene sequencing analysis revealed that the strains of OSIP1 and OSNP14 belonged to *Actinobacteria* phylum. The strain OSIP1 shared the highest similarity (100%) with *Streptomyces subrutilus* (X80825). The strain OSNP14 showed the highest similarity (99.2%) with *Streptomyces* *cirratus* (AY999794). Strains of EL1 and EL24 showed 99.3% similarity with *Arthrobacter sulfonivorans* (AF235091). EL30 shared the highest similarity (97.7%) with *Arthrobacter ginsengisoli* (KF212463). EL20, EL43, EL51, EL58, EL90, and BS1 showed 100% similarity with *Arthrobacter oryzae* (AB279889). Strains of EL22 and BS2 revealed 100% similarity with *Pseudarthrobacter oxydans*

(X83408). The strain of OSRP3B revealed 98.9% similarity with *Kocuria salina* (LT674162) (Fig. 2).

Five isolates were affiliated with the *Firmicutes* phylum. EL85 and EL17 showed the highest sequence similarity of 100 and 99.7 percent with *Bacillus*

pacificus (KJ812450). EL5 and EL21 had 98.5 and 99.7% sequence similarity to *Bacillus australimaris* (JX680098), respectively. OSRP78C was closely related to *Bacillus safensis* (KY990920) with a 98.8% sequence similarity (Fig. 2).
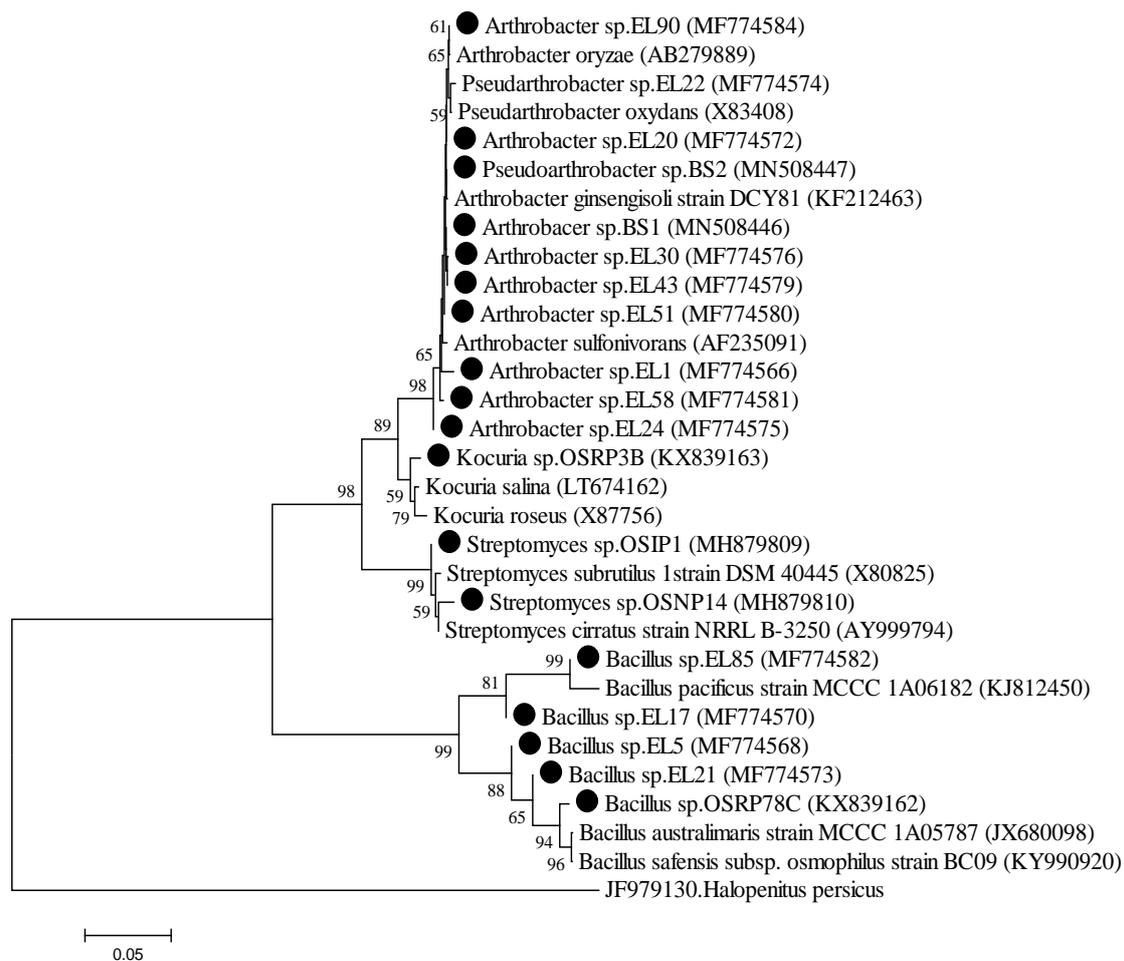

Fig. 2- Neighbour joining phylogenetic tree of the 16s rRNA of gram-positive bacteria

The sequences of the *Halopenitus persicus* (JF979130) was used as the/an out-group. Bootstrap values (%) are based on 1000 replicates. Seventeen isolates were affiliated with the gamma-subdivision of *Proteobacteria*. EL4 was most closely related to *Pseudomonas migulae* (AF074383) (99.5% sequence similarity). EL7, BS5, BS6, BS7, BS8, BS9, BS10, BS11, BS12, BS13, and BS14 were most similar (100%) to a psychrotolerant

bacterium isolated from a subantarctic environment *Pseudomonas yamanorum* (EU557337), reported by Arnau et al. (2014) (16). Two strains of EL19 and EL34 were related to *Pseudomonas helmanticensis* (HG940537) isolated from forest soil Ramírez-Bahena et al. (2014) (17) with 99.4 and 100% similarity, respectively. EL86 can be considered as belonging to *Pseudomonas bohemia* (MG190030), with 99.7% of sequence

similarity. The remaining one, EL86, showed 98.7% of sequence similarity to *Pseudomonas silesiensis* (KX276592) (Fig. 3). Three strains belonged to *Bacteroidetes* phylum. The strain of OSRP78A was most similar (98.33%) to a psychrophilic bacterium isolated from alpine soil *Sphingomonas alpine* (GQ161989) reported by Margesin et al. (2012) (18) (Fig. 3). Strains of BS3 and BS4 were most similar to *Sphingomonas aurantiaca* (AJ429236) one orange-pigmented and psychrotolerant bacterium isolated from Antarctic with 99.35 % similarity (Fig. 3) (19).

Four strains belonged to *Bacteroidetes* phylum, EL97 can be considered belonging to *Flavobacterium sinopsychrotolerans* (FJ654474) with 97.35% similarity. This bacterium was isolated from a glacier reported by Xu et al. (2011) (20) (Fig. 3). Strains of BS15 and BS16 were most similar to *Pedobacter cryoconitis* (AJ438170). The strain of BS17 showed 99.18% similarity with *Flavobacterium branchiicola* (HE612102). The GenBank accessions of the sequences are shown in the phylogenetic tree.

Fig. 3- Neighbour joining phylogenetic tree of the 16s rRNA of gram-negative bacteria. The sequences of the *Halopenitus persicus* (JF979130) was used as the/an out-group. Bootstrap values (%) are based on 1000 replicates.

Extracellular Enzyme Production: In this study, the ability of extracellular hydrolytic enzymes (amylase, protease, pectinase, lipase, gelatinase, CMCase, urease, and DNase) of psychrophilic and psychrotolerant bacteria isolated from Oshtorankuh Mountains was investigated.

Screening of the bacteria showed that all strains produced at least one extracellular hydrolytic enzyme. Protease was the most dominant extracellular hydrolytic enzyme produced by psychrotolerant, while amylase was the abundant enzyme produced by psychrophilic strains.
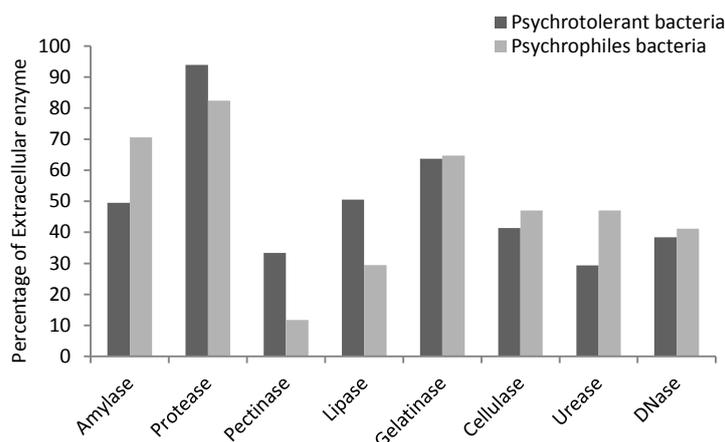

Fig. 4- The Percentage of Production of Extracellular Enzymes in Psychrophilic and Psychrotolerant Cultivable Bacteria from Oshtorankuh Mountains

Discussion

Bacterial species cultivated using classical methods represented less than 1% of the total bacteria. Still, the study of cultivable bacterial diversity is important to obtain new strains for the study of adaptation as well as models for metagenomic studies or even for biotechnology purposes. In recent decades, microorganisms in cold ecosystems have been studied using a variety of methods (4, 5). Reported values ranged from 10^1 - 10^3 to 10^2 - 10^8 CFU/gr of dry soil. Our estimates (10^5 - 10^7) are comparable to those of the permafrost in Tianshan (10^5 - 10^6 (4)) and Spitsbergen in Norway (10^5 - 10^6 (3)). It seems that the number of bacteria depends on the substrate of the environment from which they are obtained. Temperature incubation seems to have a stronger impact on bacteria isolation. The number of CFU was higher at 20 °C than at 4 °C. This phenomenon has also been observed in other cold habitats. These

isolates were psychrotrophic (cold-tolerant) rather than psychrophilic (cold-adapted). It seems that even in cold habitats, the bacterial isolates are more psychrotrophs than psychrophiles.

The authors of the present study have also observed many pigment-containing bacteria at our study sites were identical to other alpine locations. It has been suggested that the presence of pigments plays a role in protection against oxidative stress caused by the high levels of UVB radiation (21). These results supported the importance of pigmentation as a mechanism for bacterial survival in alpine tundra.

As in other surveys conducted in cold ecosystems (3, 4, 7), the authors have found that members of the *Actinobacteria* and *γ-Proteobacteria* were dominant among cultivable bacteria. The abundance of members of the *Actinobacteria* phylum has been observed in studies of cold ecosystems especially in soils and sediments (4, 22). The high G+C content,

the specific composition of the cell wall, and the ability of this group to form dormant cells provided the capability to survive under harsh conditions (23). Along the same line, the high frequency of the isolation of *Arthrobacter* species from cold environments has already been reported (3, 4, 7, 23).

Moreover, the study of bacterial diversity in these ecosystems using culture-dependent approaches has a different perspective. Although *Actinobacteria* can be one of the dominant groups at the phylum level, the genera *Streptomyces* and *Arthrobacter* using metagenomics and cloning methods have little abundance (24). Classical cultivation captures a fraction of the total diversity of the sample represented by heterotrophic bacteria, because conventional culture methods are designed to isolate this kind of bacteria. It was also proposed that bacterial cells can be present in active and quiescent forms. Anyway, isolation by cultivation provides the analysis of the complete gene and allows the identification of physiological and biochemical capabilities of microorganisms.

Among Gram-negative bacteria, *Pseudomonas* was the most-represented genus in the culturable bacteria isolated from alpine soil habitats 40% (17 of 43). The increased abundance of members of *Pseudomonas* compared to other members of *γ- Proteobacteria* was consistent with previous results from Tian Shan Mountains, north-western China (4), Arctic permafrost soil from Spitsbergen (3), alpine soil (25), Qinghai-Tibet Plateau permafrost region (5), King George Island, Antarctica (26) and Collins glacier in Antarctica (27).

Production of Enzymes: Since low temperatures can have destructive effects on the cellular and enzymatic structure of microorganisms, psychrophilic and psychotolerant have mechanisms to overcome them (28). Increasing flexibility,

thermolability, and electrostatic interactions in psychrophilic enzymes allow them to have a higher level of activity at low temperatures. On the other hand, the use of cold-adapted enzymes in heat-sensitive reaction allowed the reaction to carry out at lower temperatures and prevent undesirable chemical changes that can occur at higher temperatures. For this reason, a lot of attention has been paid to the isolation and identification of cold active extracellular enzymes producing bacteria (10, 29, 30). The strains obtained from both locations had the ability to produce at least one hydrolase enzyme. Protease and amylase enzymes were the most abundant of them.

The reason for less cellulolytic activity observed compared with other enzymes could be due to humification and litter decomposition processes. A predominance of amylase in soil samples was evaluated by Alves et al. (2014) (31).

The cellulase activity of the microbial population in the winter has a significant increase compared with the summer. This is due to their ability to grow on phenolic, cellulose, and vanillic acid compounds (32).

The high abundance of protease, gelatinase, and amylase producing bacteria probably indicates the existence of large amounts of protein and starch in the locations of soils sampling and the dynamics of the bacteria even under snow. Among the strains, the ability to produce several enzymes was observed in various types of *Arthrobacter, Bacillus,* and *Pseudomonas* that is in agreement with the findings of Lamilla et al. (2017) (6) and is probably due to the high physiologic and genetic diversity of these strains.

Our data indicated that cold ecosystems were appropriate locations for the screening and identification of bacteria that have a high capability of biodegradation of bio-macromolecules. The microorganisms isolated from cold ecosystems can be a

good source to produce cold active enzymes that are of high value in reducing costs and energy consumption.

Conclusion

The objective in the present study was to obtain an overview of psychrophilic and psychrotolerant bacterial diversity in alpine tundra of Oshtorankuh Mountain, using traditional culture-dependent methods. The authors of the present study obtained a wide variety of cultivable bacteria belonging to the four major phyla including *Actinobacteria*, *Firmicutes*, *Proteobacteria,* and *Bacteroidetes*. Analysis of their extracellular enzymes showed that proteolytic and amylolytic activities were higher among the strains. However, cellulase and pectinase activities were significant in some strains. This study showed that these strains can be considered in biotechnology and bioremediation applications.

Acknowledgments

Conflict of Interest

The authors declared no conflicts of interest.

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