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A Novel Psychrophilic *Janthinobacterium lividum* MMPP4 Isolated from Manimahesh Lake of Chamba District of Himachal Pradesh, India

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Abstract

We isolated psychrophilic bacterial strains from high altitude (elevation 13,390 ft) Manimahesh Lake of Chamba District of Himachal Pradesh. Based on phenotypic characteristics and growth appearance at 4ºC, four bacterial isolates (MMPP2, MMPP4, MMPP5, and MMPP7) were selected and showed optimum growth at 20ºC and no growth was detected at 25ºC. Qualitative assay showed that cell free medium of MMPP4 prevent inhibition of freezing (antifreeze activity) as determined by freezing of cell free medium at -20ºC for seven days. Proteinase K treatment and heat inactivation at 80ºC for 6 h resulted in the loss of antifreeze activity, suggesting a proteinaceous nature of antifreeze activity. MMPP4 produces protease, phytase and lipase enzymes, but did not produce amylase. Moreover, cell free supernatant of MMPP4 showed proteinaceous type of antimicrobial activity against *S. aureus* as measured by agar well diffusion assay. To identify the MMPP4 strain, 16s rDNA was amplified using 27F and 1492R standard primers and sequenced on both the strands. Nucleotide BLAST and phylogenetic analysis showed 99% identity to *Janthinobacterium lividum* strain and deposited in GenBank accession no. as KJ509870. The finding revealed a great potential of the *Janthinobacterium lividum* strain MMPP4 for biotechnological applications as a source of industrially important enzymes and antimicrobial compound.

Keywords: Antifreeze proteins, psychrophiles, 16S rDNA, Phylogenetic analysis, *Janthinobacterium lividum* MMPP4

Introduction

Life exists in a diverse range of habitats including extremes of temperature, pH, salt, pressure, etc, Organisms adapted to cold environment (e.g. Antarctic or Arctic cold) produce antifreeze proteins (AFP) that prevents the organisms from freezing and allows them to survive below 0ºC (Vries 1971). Antifreeze proteins were discovered in the early 1970s, in the blood of Antarctic fish. AFPs adsorb onto the ice surface and lower the freezing temperature which inhibits ice crystallization (Knight and Duman 1986). AFPs are produced by various organisms, such as *Solanum dulcamara* (Duman 1994), *Secale cereal* (Antikainen and Griffith 1997), *Daucus carota* (Worrall et al 1998), and *Lolium perenne* (Sidebottom et al 2000), fish (Tong et al 2000), and diatoms (Bayer-Girald et al 2010). Many bacteria, such as *Pseudomonas putida GR12-2*, isolated from soil from the Canadian High Arctic (Sun et al 1995), *Rhodococcus erythropolis* isolated from the midguts of beetlelarvae and *Micrococcus cryophilus* isolated from chilled sausages (Duman and Olsen 1993) and other bacteria (Raymond et al 2008; Raymond et al 2007) have been characterized for the production of AFPs. More recently, AFPs have recently been identified in yeast (Park et al 2011), fungi (Xiao et al 2010), and mushrooms (Raymond and Janech 2009). Structurally AFPs have evolved independently and has been classified into five groups (types I–IV and antifreeze glycoproteins) depending on their structural characteristics and source (Davies and Sykes 1997). AFPs from insects, including long horn beetle and spruce budworm have been isolated, cloned, expressed and studied extensively (Graether and Sykes 2004; Graether et al 2003). AFP finds varied applications in medicine (for improving cryosurgery; enhancing preservation of tissues for transplant or transfusion), food processing/production (lengthening the shelf life of frozen foods), and Agri/Aquaculture (increasing freeze tolerance of crop plants; improving farm fish production; extending the harvest season in cooler climates). Though, there is wealth of information about AFPs, but microbes from different climatic conditions largely remained unexplored for the production of AFPs. Moreover, microorganisms producing AFPs as well as antimicrobial compounds have not given much attention.

Here we report the isolation of *Janthinobacterium lividum*-MMPP4 strain, a psychrophilic bacterial strain from sub glacier Lake of Manimahesh of Chamba district of Himachal Pradesh, India. *Janthinobacterium lividum*-MMPP4 produces antifreeze protein(s) and antimicrobial compounds.

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Materials and Methods

Sample collection site and isolation of bacterial strains

Water sample was collected aseptically from the Manimahesh Lake (15750 ft) situated in Chamba district of Himachal Pradesh (coordinates 32°23′42″N 76°38′14″E). 10 ml water sample was centrifuged at 8,000 rpm for 10 minutes and the microbial cell pellet thus obtained was resuspended in 1ml sterilized water and 200 µl of sample was spread on LB-agar plates and incubated at 4°C for one week. Four individual colonies were selected based on size, color, texture and morphology. The isolated colonies were purified by four successive streaking on nutrient agar plates and confirmed the growth at 4°C.

Biochemical characterization of microbial isolates

To determine the optimum temperature for growth, microbial strains were streaked on nutrient agar medium and incubated at 4, 20, 25, 30, and 37°C for 7 days and observed for growth. Gram's staining was performed as described (Madigan et al 2004) and shape and Gram's reaction was observed under the microscope. A drop of 3% hydrogen peroxide was placed on the smear of microbial strain and observed for the formation of bubbles of oxygen as an indicative of Catalase enzyme. Oxidase and motility tests were also performed as described previously (Pickett and Greenwood 1986).

Ice crystallization inhibition Assay

The Ice crystallization inhibition assay of all 4 isolates was tested qualitatively by the freezing assay (Hirano et al 1985) with modification. Isolates were grown in 20 ml nutrient broth and incubated at 4°C until the stationary phase is reached. Cells were removed by centrifugation at 8000 rpm for 5 min. and cell free supernatant was incubated at -20°C and observed for inhibition of ice crystallization for seven days. The sample showing inhibition of ice crystal growth (did not freeze considered as "active" for antifreeze proteins), whereas the sample showing no inhibition of ice crystal growth (freezes and considered as non-active for antifreeze proteins). The control was water sample or supernatant of DH5α laboratory strain, which was grown in LB medium at 37ºC. In order to determine the proteinaceous nature of the antifreeze activity, cell free supernatants were treated with proteinase K solution (1µg/ml) for the test sample and water was added for the control. Both samples were incubated at 37°C for 6 h, further incubated at -20°C and observed for inhibition of ice crystallization for seven days. Similarly, cell free supernatants were heat inactivated at 80^0C for 30 min, incubated at -20°C, and observed for inhibition of ice crystallization for seven days.

Enzymatic and Antimicrobial assays

The culture of MMPP4 strain was grown at 20ºC to an A_{600}nm = 1, whereas DH5α was used as control strain and grown to an equal cell density at 37ºC. The protease, amylase, lipase, and phytase activities were measured by supplementing LB agar medium with enzyme specific substrates such as casein (1% w/v), soluble starch (1% w/v), trybutyrin (1% v/v), and sodium phytate (0.25% w/v) respectively. Equal number of cells in 5µl of the culture was inoculated on the surface of the medium and plates were incubated at 20ºC for 12 h. The zone of clearance around the point of inoculation of culture indicated the presence of enzyme activity. Antimicrobial activity of the cell-free supernatant of the stationary phase grown culture of MMPP4 strain was evaluated by standard agar well diffusion assay (Perez et al 1990). The culture of MMPP4 strain was grown in LB broth medium at 20ºC to an A_{600}= 2.5, whereas DH5α was used as control strain and grown to an equal cell density at 37ºC. Wells of 6 mm in diameter were punched in LB agar medium in which top layer of soft agar (0.7%) was seeded with *S. aureus* as test organisms. 50 µl of cell-free supernatant `of stationery grown culture was added in the wells. As a control, filter disc of standard drug ampicillin (50 µg) was used. Cell-free supernatant was also heat inactivated at 80ºC for 30 min and tested alongside for antimicrobial activity. Plates were incubated at 20ºC for 18 h. Antimicrobial activity was measured as a zone of inhibition of microbial growth around the well. To assay the bactericidal or bacteriostatic activity, cells were carefully scraped from the zone of clearance around the well and streaked on nutrient agar plates, and observed for the growth after 24 h of incubation 37ºC.

Identification of bacterial strain by 16S rDNA Gene Amplification by PCR and phylogenetic analysis

Total genomic DNA was isolated as described (Sambrook et al 1989) and electrophoresis was performed on 1% agarose gel, followed by staining with ethidium bromide (10 mg/ml) and visualized under gel documentation (Alpha Innotech). Genomic DNA was quantified using spectrophotometer by measuring ratio of absorbance at 260/280 nm. 16S rDNA sequence was amplified using universal bacterial specific forward primer 27F (5'-AGAGTTTGATCCTGGCTC-3') and reverse primer 1492R (5'- GGTTACCTTGTTACGACT-3'). The PCR reaction conditions were, initial denaturation for 2 minutes at 94°C followed by 35 cycles of 30 seconds at 94°C (denaturation), 30 seconds at 49°C (annealing), 2 minutes at 72°C (elongation) and 1 cycle of final extension at 72°C for 10 minutes. 16S rDNA amplified product was gel purified and sequenced on both the strands using 27 F and 1492 R primers at Eurofins, Bangalore, India (www.eurofins.com). Complete nucleotide sequence was generated by removing the overlapping nucleotides and was subjected to blastn (http://blast.ncbi.nlm.nih.gov) search at NCBI database. The first 20 hits showing greater than 95% similarity were used for the construction of dendrogram by using MEGA 4 software (http://www.megasoftware.net). The nucleotide sequence was submitted in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

Results

The current study was initiated to isolate the psychrophilic microorganisms from the water sample of Manimahesh Lake (sub Glacier Lake) and screen them for the presence of antifreeze proteins, which finds application in medicine, food processing, and agriculture. The lake is situated in Chamba District of Himachal Pradesh at an altitude of 13390 ft above the sea level. Mostly, the lake remains frozen from October through June and resulted in very scanty flora and fauna.

Microflora corresponding to 10 ml original water samples were plated on LB agar plates and incubated at 4°C for seven days. Distinct and visible bacterial colonies started appearing on fourth day. On seventh day of incubation (Fig. 1A), four colonies were selected based on the size and color and named MMPP2, MMPP4, MMPP5, and MMPP7 (Fig. 1A).

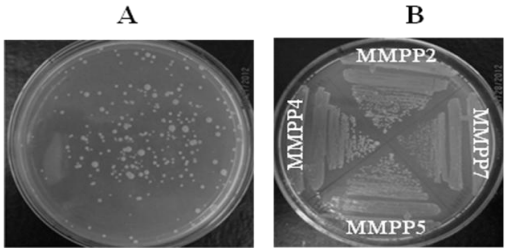

Figure 1: Isolation of bacteria from water sample of Manimahesh Lake. Water samples were spread on nutrient agar broth and incubated at 4°C for one week (A). Distinct and isolated colonies (named MMPP2, MMPP4, MMPP5 and MMPP7) were streaked on nutrient agar medium as indicated and incubated at 4°C for four days (B).

To test the limit of temperature for the growth, all the four isolates were streaked on LB agar plates and incubated at different temperature of 4, 20, 25, 30 and 37°C for four days. Growth was detected at 4 and 20°C, but no growth was detected at 25°C and higher temperature of incubation. Comparatively, the growth was more at 20°C than 4°C (Table 1). This suggests a psychrophilic nature of all the four isolates.

Table 1: Effect of Temperature on the growth of psychrophilic isolates

S. No.	Isolates	Growth (°C)				
		4	20	25	30	37
1	MMPP2	+++	++++	-	-	-
2	MMPP4	+++	++++	-	-	-
3	MMPP5	+++	++++	-	-	-
4	MMPP7	+++	++++	-	-	-

Plus sign (+) indicates the presence of visible growth and minus sign (-) indicates the absence of growth. Plus four signs (++++) indicates more visible growth as compare to plus three signs (+++).

All the four isolates were whitish in color. MMPP4, MMPP5 and MMPP7 are Gram's negative, whereas MMPP2 showed Gram's positive reaction. MMPP4 is a rod shaped bacterium, whereas MMP2, MMP5, and MMP7 are Cocci. MMPP2 and MMPP5 showed the presence of catalase enzyme, but oxidase enzyme test was positive only for MMPP2. MMPP2 and MMPP5 are non-motile, whereas MMPP4 and MMPP7 are motile bacteria (Table 2).

Table 2: Biochemical characteristics of psychrophilic isolates

Isolates	Shape	Gram's reaction	Catalase/ Oxidase test	Motility test	Color
MMPP2	Cocci	+	+/+	-	Off white
MMPP4	Rods	-	-/-	+	Off white
MMPP5	Cocci	-	+/-	-	Transparent
MMPP7	Cocci	-	-/-	+	White

Plus sign (+) indicates the presence and minus sign (-) indicates the absence of reaction/test

Cell-free media of MMPP2, MMPP4, MMPP5, and MMPP7 were recovered from the stationary phase of growth and examined for antifreeze activity by incubating at -20°C and compared to cell free supernatant of DH5α as control strain over a period of one week. The assay is based on the principle of ice crystal formation and the presence of antifreeze proteins results the inhibition of freezing. Cell free media with antifreeze activity appeared clear, whereas media without antifreeze activity appeared turbid when photographed. Interestingly, cell free media of MMPP4 did not freeze over a period of one week, whereas MMPP2 forms ice crystals by seventh day of incubation. In contrast, the cell free media of MMPP5 and MMPP7 turn into ice crystals by third and fourth day respectively and appeared turbid. Moreover, the control sample freezes within 5-6 h of incubation (Table 3).

Table 3: Antifreeze activity of Psychrophilic isolates

S. No.	Isolates	Freezing (-20°C) days					
		1st	2nd	3rd	4th	5th	7th
1	MMPP2	-	-	-	-	-	+
2	MMPP4	-	-	-	-	-	-
3	MMPP5	-	-	+	+	+	+
4	MMPP7	-	-	-	+	+	+
5	DH5α	+	+	+	+	+	+

Plus sign (+) indicated freezing and minus sign (-) indicates the absence of freezing.

We further analyzed the cell-free supernatant of MMPP2 and MMPP4 after treatment of cell free medium with proteinase K (1mg ml^{-1} for 30 min) and heat inactivation (80ºC for 30 min) to destroy proteins. This resulted in loss of antifreeze activity and led to freeze the sample within 5-6 h of incubation at -20°C (Table 4). This indicated that the antifreeze activity is proteinaceous in nature.

Table 4: Effect of proteinase K and heat treatment on antifreeze activity of psychrophilic bacterial isolates

Isolates	Treatment		Freezing (-20°C) days						
			1	2	3	4	5	6	7
MMPP2	Protease K	+	+	+	+	+	+	+	+
		-	-	-	-	-	-	-	+
	80°C for 30 min	+	+	+	+	+	+	+	+
		-	-	-	-	-	-	-	-
MMPP4	Proteinase K	+	+	+	+	+	+	+	+
		-	-	-	-	-	-	-	-
	80°C for 30 min	+	+	+	+	+	+	+	+
		-	-	-	-	-	-	-	-
DH5α	Proteinase K	+	+	+	+	+	+	+	+
		-	+	+	+	+	+	+	+
	80°C for 30 min	+	+	+	+	+	+	+	+
		-	+	+	+	+	+	+	+

Plus sign (+) indicates freezing/treatment and minus sign (-) indicates no freezing/no treatment as indicated.

Cell-free supernatant of MMPP4 was also analyzed for antimicrobial activity using agar well diffusion assay against *Staphylococcus aureus* strain. It was observed that cell free supernatant of MMPP4 showed remarkable inhibition of *Staphylococcus aureus* growth similar to ampicillin around the well indicating the production of antimicrobials (Fig. 2A, compare well number 1 with well no 2 and 3). The size of the zone of inhibition was 10 mm in the presence of ampicillin (15 µg). On the other hand, 7 mm and 12 mm zone of inhibition was observed, when 5 µl (5 µg) or 10 µl (10 µg) of cell free supernatant of MMPP4 isolate was respectively used. Cell-free supernatant of MMPP4 strain was further analyzed for antimicrobial activity after heat treatment at 80ºC for 30 min. More interestingly, the antimicrobial activity was completely inhibited, when sample was heat inactivated as compared to untreated sample (5 mm) as shown in Figure 2B (compare well no. 1 with 2). This further reinstates the protein nature of antimicrobial activity. To study the mechanism of growth inhibition, cells were carefully scraped from the zone of growth inhibition and further streaked on fresh LB agar medium. Interestingly, no growth was observed for the untreated sample in contrast to the heat inactivated sample (Fig. 2C, compare 1 and 2). This demonstrates the bacteriocidal nature of the antimicrobial compound produced by MMPP4 strain.

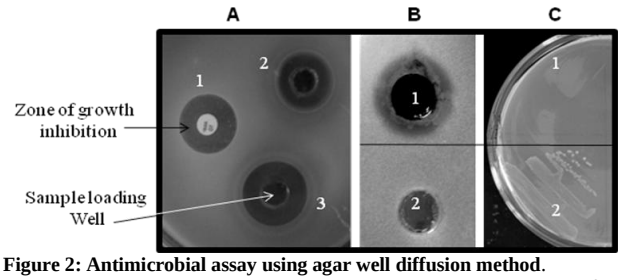

Figure 2: Antimicrobial assay using agar well diffusion method.
(A) MMPP4 strain was grown in LB broth at 20ºC to an optical density of 1.0 at A_{600} nm. Cell-free medium, 5 µl in well number 2 and 10 µl in well number 3 was loaded. A filter disc of ampicillin (15 µg) was used as control (well no. 1). (B) Heat inactivated cell free medium (well no. 2) was assayed as compared to untreated sample shown in well no. 1. (C) Cells were scraped around the zone of growth inhibition (B, well no. 1) and further streaked on LB agar medium in comparison to treated sample.

Furthermore, cell free media of MMPP4 strain was tested for different enzymatic activities using agar well diffusion assay in the presence of specific substrates. The results showed the production of hydrolytic enzymes such as protease (3 mm zone of clearance), lipase (3 mm zone of clearance), and phytase (5 mm zone of clearance) in LB agar media supplemented with casein (1% w/v), Tributyrin (1%), and sodium phytate (0.25% w/v) respectively (Fig. 3 A, B and D). Surprisingly, amylase activity was not detected in the MMPP4 strain when assayed in the LB medium supplemented with 1% starch (w/v) and iodine staining (Fig. 3C).

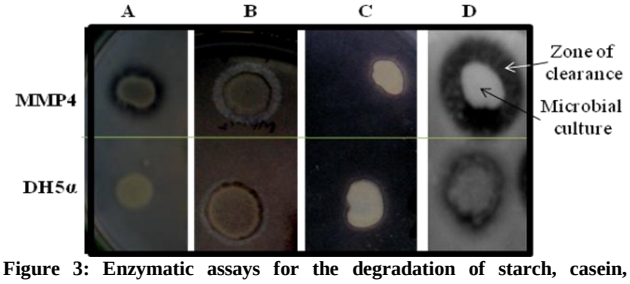

Figure 3: Enzymatic assays for the degradation of starch, casein, tributryin and phytate. MMPP4 strain was grown in LB broth at 20ºC to an optical density of 1.0 at A_{600} nm. 5 µl of cell suspension was spotted on LB agar medium supplemented with 1% casein (A), 1% tributryin (B), 1% soluble starch (C), and 0.25% sodium phytate (D). The equal density cell suspension of DH5α strain grown at 37°C was used as control.

Since MMPP4 strain showed highest antifreeze protein activity, this strain was selected for the PCR amplifications of the 16S rDNA gene using total genomic DNA (Figure 4A, Lane 2) and standard primers 27F and 1492R as described previously (24). A PCR product of approximately 1400 base pairs was amplified (Figure 4B, Lane 3). It was further gel purified and DNA sequencing of both the strands was performed. We obtained 1395 bps nucleotide sequence. BLAST analysis revealed that the DNA sequence of MMPP4 was most similar (99 % identity) to 16S rDNA sequences of *Janthinobacterium lividum* strain DSM 1522 (GenBank acc no. NR_026365). The nucleotide sequence has been submitted to the GenBank with accession no. as KJ509870. Phylogenetic tree of first 20 bacterial spp showing >95% similarity indicates that *J. lividum* MMPP4 form a separate clad with *J. lividum* DSM 1522 (Figure 4C).

Discussion

Temperature is one of the environmental factors that set the distribution of flora and fauna. Low temperature affects the organism by dehydration and crystallization of cellular

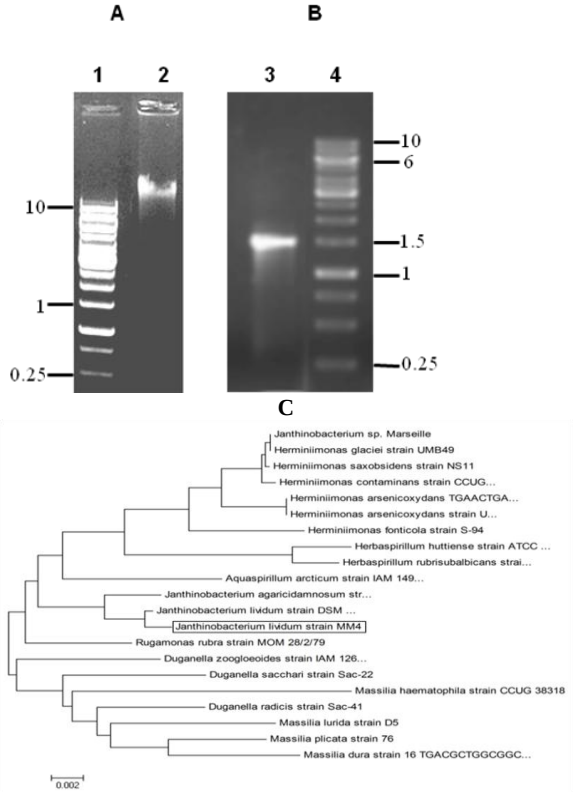

Figure 4: 16s rDNA amplification and phylogenetic analysis of MMPP4 isolates. 16s rDNA was amplified using total genomic DNA of MMPP4 (A). Lane 1 is size DNA marker (kbs) as indicated and lane 2 represents genomic DNA. 16s rDNA amplified PCR product is shown in lane 3 and molecular size marker in lane 4 (B). The DNA sequence was subjected to BLAST in NCBI database and first 20 hits showing greater than 95% similarity were subjected to phylogenetic analysis using UPGMA (C). *J. lividum* strain MMPP4 is shown in the rectangular box.

components (Beck et al 2004). In contrast, life of the organisms has been reported in Antarctic or Arctic cold environments, due to their ability to produce proteins associated with freezing resistance. Cold-adapted microorganisms increase their survival at sub-zero temperatures by producing proteins that bind to and inhibit the growth of ice crystals. Cold biosphere which is highly rich in microbial diversity would be most appropriate to screen microbes for the production of AFPs. Moreover, research on psychrophilic microorganisms is far behind that of thermophiles. Keeping in mind the potential applications of psychrophilic microbes and their biomolecules, we isolated four bacterial strains (MMPP2, MMPP4, MMPP5, and MMPP7) from water sample of Sub-glacier Lake of Manimahesh situated in Chamba District of Himachal Pradesh. This region is a part of Himalayas, which remained unexplored for microbial diversity. All the four bacterial showed growth at 4ºC and no growth was detected above 20ºC suggesting the psychrophilic nature. Microorganisms have been documented in ancient glacier ice (Christner et al 2003; Miteva and Brenchley 2005; Bidle et al 2007), sub glacier water (Mikucki et al 2009), basal ice (Sheridan et al 2003; Miteva et al 2004), sub glacier lakes and accreted ice (Karl et al 1999; Priscu et al 1999; Gaidos et al 2004), and sea ice (Junge et al 2004; Brown and Bowman 2006). Cell free medium of MMPP4 did not freeze at -20ºC for seven days, whereas MMPP2, MMPP5, and

MMPP7 cell free medium freezes on 7th, 3rd and 4th day respectively. This indicates MMPP4 either secrete AFPs extracellularly in large amounts or different AFPs from MMPP2, MMPP5, and MMPP7 bacterial isolates. It is also possible that MMPP2, MMPP5, and MMPP7 produce AFPs at temperature below 20ºC. *P. putrid GR12-2* synthesizes and secretes its AFP into the culture medium only when grown at cold temperatures (Sun et al 1995). This suggests that antifreeze proteins secreted extracellularly may alleviate cell damage by preventing recrystallization of extracellular ice. The antifreeze activity present in the cell free medium of MMPP4 isolate was inactivated after treatment with proteinase K or heat inactivation at 80ºC, suggesting a proteinaceous nature of AFP activity. Antifreeze protein has been isolated from *Marinomonas primoryensis*, isolated from a brackish, ice-covered lake in Antarctica. The protein is exceptionally large (ca. 1.5 MDa) with Ca^{2+}-dependent antifreeze activity (Garnham et al 2008). Out of fourteen of the bacterial and yeast isolates, one bacterial strain recovered from glacial ice at a depth of 3,519 m, just above the accreted ice from Sub glacial Lake Vostok, was found to produce a 54 kDa ice-binding protein (GenBank EU694412) that is similar to ice-binding proteins previously found in sea ice diatoms, a snow mold, and a sea ice bacterium. AFPs have been recently demonstrated in Antarctic lake bacteria (Gilbert et al 2004), one of which, from *Marinomonas primoryensis*, is Ca^{2+}-dependent and hyperactive (Gilbert et al 2005). The AFP from the Arctic plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2 shows both antifreeze and ice-nucleating activities (Muryoi et al 2004). It has been postulated that microorganisms produces hydrolytic enzymes to effectively respond to the changing environmental conditions (Dang et al 2009; Dias et al 2009; Srinivas et al 2009). Likewise, we showed that all the four microbial isolates secrete extracellular protease, lipase, and phytase enzymes, but fail to secrete amylase (Fig. 3). More interestingly, MMPP44 strain showed bacteriocidal type of antimicrobial activity against *S. aureus*. There is no repot in the literature about a psychrophilic strain producing AFPs as well as antimicrobial agents. 16SrDNA analysis identified MMPP4 as *Janthinobacterium lividum.* As the name indicates, *Janthinobacterium* are violet colored rod, not all known species exhibit a violet phenotype. *J. lividum MMPP4* is a Gram negative rod and off white color. It was shown that the genus *Janthinobacterium* produces different pigments that exhibit antibiotic activity, such as violacein (Becker et al 2009; Pantanella et al 2007), purple violet pigment (Mojib et al 2010), bluish-purple pigments (Shirata et al 2000). In addition, certain antibiotics or antifungal metabolites such as prodigiosin (Schloss et al 2010) and the peptide lactone antibiotics janthinocin A, B and C (Johnson et al 1990). Though, *J. lividum* MMPP4 did not show violet pigmentation, but showed proteinaceous type of antimicrobial activity against *S. aureus*. Further investigations are required for the purification and characterization of antifreeze and antimicrobial activity of *J. lividum* MMPP4.

Conclusions

In conclusion, four psychrophilic bacterial strains (MMPP2, MMPP4, MMPP5, and MMPP7) were successfully isolated from the water sample of Manimahesh Lake of Chamba district of Himachal Pradesh. MMPP4 was identified as *Janthinobacterium lividum* and showed proteinaceous type of antifreeze and antimicrobial activity, which have potential applications in medicine, food processing/production, and Agri/Aquaculture. Moreover, presence of phytase activity may be useful to enhance the bioavailability of phosphorous to agriculture crops of the colder regions. In order to harness the full potential of *Janthinobacterium lividum MMPP4* isolate, pure and sufficient quantities of these compounds are required for research to further investigate their applications.

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