

Full Length Research Paper

Psychrotrophic bacteria isolated from -20°C freezer

Bashir Ahmad, Imran Javed, Aamer Ali Shah, Abdul Hameed and Fariha Hasan*

Microbiology Research Laboratory, Department of Microbiology, Quaid-i-Azam University, Islamabad 45320, Pakistan.

Accepted 28 December, 2009

Three psychrotrophic bacteria, morpho-physiologically, identified as *Bacillus subtilis* MRLBA7, *Bacillus licheniformis* MRLBA8 and *Bacillus megaterium* MRLBA9 were isolated from -20°C freezer of the Microbiology Research Laboratory (MRL), Quaid-i-Azam University, Islamabad, Pakistan. These strains were able to grow aerobically at 6°C but not at 40°C except MRLBA8 that could grow at 48°C. None of the isolates showed inhibition of growth in the presence of glycerol. Isolate MRLBA7, bearing central spore, grew in the presence of 30% glycerol at 0°C after 48 h of incubation and showed maximum growth without glycerol at 25°C after 24 h. Isolate MRLBA8 showed growth in the presence of 50% glycerol at 4°C after 72 h of incubation and maximum growth was observed at 20°C in the absence of glycerol. Isolate MRLBA9 showed growth at 6°C in the presence of 40% glycerol after 48 h of incubation and maximum growth was observed at 25°C in the absence of glycerol. Isolates were susceptible to antibiotics except *Bacillus subtilis* MRLBA7 that exhibited antibiotic resistance against penicillin and fosfomycin, *Bacillus licheniformis* MRLBA8 against aztreonam and fosfomycin, and *Bacillus megaterium* MRLBA9 against vancomycin and penicillin. The growth profile and biochemical characteristics of all the isolates were rather similar to that of mesophilic counterparts except adaptation to low temperature. These strains could be used as model microbial strains for characterization of food contaminants in freezers, to understand the mechanism of antibiotic resistance induced at low temperature and as a source of psychrotrophic enzymes.

Key words: Psychrotrophs, *Bacillus*, antibiotic resistance, growth characteristics.

INTRODUCTION

Ice whether in the form of tundra, glacier, snow, cloud or from a freezer, presents a special environment for microbial life (Staley et al., 2002). Cold active microorganisms may be isolated from cold storage, ice cabins, ice creams, deep freezers, frozen oceans and glaciers (Morgan et al., 1994; Hennessy et al., 1996; Christner et al., 2000).

Life on our planet has been adapted to different extreme environments like pH, pressure and temperature etc (Petegem et al., 2002). Psychrophiles ($\leq 20^{\circ}\text{C}$) and psychrotrophs ($\leq 37^{\circ}\text{C}$) are distinguished according to their range of temperature adaptation (Russell, 1990). They thrive in cold environments due to unique features like cold shock proteins, short and unsaturated fatty acids in membranes, enzymes with high specific activity, thermolability and genetic changes to thermal shifts (Margesin et al., 2007).

Psychrotrophs are more ubiquitous and successful

group of bacteria than psychrophiles, hence, notorious for food contamination in cold storage and freezing cabins like ice cream freezers (Kanbakan et al., 2004). Spores of various *Bacillus* species are metabolically dormant and extremely resistant to a variety of harsh treatments. Due to this extreme resistance, *Bacilli* are involved in spoilage of food in the fridges and refrigerators and emerging food-borne diseases (Ghosh et al., 2008). *Bacillus subtilis* secretes a membrane phospholipid desaturase during temperature triggered lipid adaptations which would be particularly important for growth in thermally unstable cold habitats (Aguilar et al., 1998).

Gram-positive bacteria are mostly treated with penicillins or with aminoglycosides combined with β -lactams. Antibiotic resistance is acquired through different mechanisms, such as reduced cell wall permeability, or multi-drug pumps (Hogan and Kolter, 2002), selective pressure and stress conditions that also help microbes in their survival. Also, the preservation of microorganisms and biomolecules at freezing temperatures is a technical area where viability, recovery and potent storage are major

*Corresponding author. E-mail: farihasan@yahoo.com.

concerns (Prescott, 2002).

In the present study, an attempt was made to isolate viable bacteria from ice collected from -20°C freezer used for preservation of bacterial isolates. The isolates were characterized for their optimal growth conditions and preservation requirements. Antibiotic resistance and sensitivity pattern was also studied in order to address any adaptation of microbes after long term preservation at low temperature.

MATERIALS AND METHODS

Collection of samples

Ice and ice melt water samples were collected aseptically from -20°C freezer in Microbiology Research Laboratory (MRL), Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan, during spring 2006. The sampling bottles were recapped inside the freezer to avoid any possible contamination and transferred to 4°C soon after the collection of the sample.

Media, culture conditions and isolation of bacteria

Small aliquots of 100 µL of ice melt water was spread on the surface of nutrient agar plates (Oxoid, Basingstoke, UK) in duplicate and incubated at 0, 4, 10, 20, 37 and 40°C. Only three bacterial isolates (MRLBA7, MRLBA8 and MRLBA9) were capable of growing at temperature <10°C and were selected for further study. Nutrient agar medium was prepared by addition of 1.8, 2 and 2.2% bacteriological agar (Oxoid, Basingstoke, UK). The cultures were maintained on nutrient agar slants at 4°C.

Morphological, biochemical and physiological characterization

Identification methods were adapted from Bergey's Manual of Determinative Bacteriology, 8th edition (Buchanan and Gibbons, 1974). Morphological characteristics were determined on the basis of colony morphology (color, shape, margins, elevation and odor) and Gram staining. Physiologically, the isolates were characterized by studying optimum pH, temperature and preservation at low temperature.

Viable cell count and enumeration of bacteria in ice samples

Viable cell count in ice samples was done by plate count method. Ten fold serial dilutions were made and plated on nutrient agar plates. The number of colonies (CFU/ml) was calculated using Beckman Colony Counter. The growth was also monitored by determining cell density of the broth culture spectrophotometrically (Agilent 8453, USA) at 600 nm.

Effect of glycerol on growth and preservation of bacteria

The effect of various concentrations of glycerol (10, 20, 30, 40, 50 and 60%) on growth of bacterial isolates was examined.

Optimization of temperature and pH

Isolates were cultivated in nutrient broth (Oxoid, Basingstoke, U.K.) and optimized for maximal growth at various temperatures (0, 4, 10, 25, 30, 37, 40 and 48°C) and pH (4, 5, 6, 7, 8, 9, 10 and 11). The

growth was monitored spectrophotometrically at 600 nm for 96 h.

Antibiotic sensitivity

Sensitivity of the isolates against different groups of antibiotics was tested by disc diffusion method (Kirby Bauer Method) (Drago et al., 1999), using antibiotic discs (Oxoid, U.K) like Aminoglycosides (neomycin, streptomycin), Glycopeptides (vancomycin, teicoplanin), β-Lactam (penicillin, aztreonam) and Phosphomycin (fosfomycin). Plates were incubated for 48 h at 25°C and diameters of zones of inhibition (mm) were measured according to National Committee for Clinical Laboratory Standards (NCCLS, 2002).

RESULTS

The morpho-physiological characteristics of isolates are summarized in Table 1. Colonies of all of the three isolates were white and the cells were gram positive rods with spores. The isolate MRLBA7 was non motile, chains present in late growth phase; grow at pH 4-11 and 2 - 20°C. However, isolate MRLBA8 was motile, grow at pH 4 - 10 and 4 - 30°C and isolate MRLBA9 was motile and grow at pH 5 - 10 and 4 - 35°C.

Viable cell count and enumeration of bacteria in ice samples

A total of 37, 49 and 33 colonies of the strains MRLBA7, MRLBA8 and MRLBA9, respectively, were observed when ice melt water was plated on nutrient agar medium and incubated at room temperature.

Biochemical characteristics

The three bacterial isolates showed activity of different enzymes in the cell free supernatant (Table 1). All the three isolates were positive for amylase and catalase tests, but, negative for urease and TSI tests. The Simons citrate test was positive only for MRLBA8. The gelatinase, methyl red and nitrate reductase tests were positive for isolate MRLBA7 only. However, Voges-Proskauer test was positive both, for isolate MRLBA7 and MRLBA8.

Identification of bacteria

Based upon Bergey's Manual of determinative bacteriology, isolate MRLBA7 was identified as *Bacillus subtilis*, isolate MRLBA8 as *Bacillus licheniformis* and isolate MRLBA9 as *Bacillus megaterium* (Table 1).

Optimization of temperature

B. subtilis MRLBA7 started to grow after 48 and 36 h when incubated at 0 and 4°C, respectively, but started to

Table 1. Morpho-physiological and biochemical properties of Isolates.

Characteristics	MRLBA7	MRLBA8	MRLBA9
Color	Off White	White	Creamy White
Margin and elevation	Irregular, flat elevation	Irregular form, lobate margin	Smooth entire margin, convex
Gram's staining	+	+	+
Shape	Cocco-bacilli	Rods	Long rods
Spore	+	+	+
Motility	-	+	+
pH range			
Lower limit	4	4	4
Upper limit	9	11	10
Temperature limits			
Lower limit (°C)	0	4	0
Upper limit (°C)	37	40	37
Biochemical tests			
Amylase	+	+	+
Catalase	+	+	+
Gelatinase	+	-	-
Simmon Citrate	-	+	-
Triple sugar Iron	-	-	-
Urease	-	-	-
Methyl Red	+	-	-
Voges-Proskauer	+	+	-
Nitrate Reductase	+	-	-
Identified microorganism	<i>B. subtilis</i>	<i>B. licheniformis</i>	<i>B. megaterium</i>

grow readily after 12 h prominently when incubated at 10, 25, 30, 37 and 40°C. The best growth (OD₆₀₀ 2.9) was observed at 25°C; where it started its growth after 6 h of incubation and showed exponential growth after 12 h up to 60 h (Figure 1).

B. licheniformis MRLBA8 started to grow after 96 and 36 h when incubated at 0 and 4°C, respectively. However, the strain exhibited its growth after 12 h when incubated at 10 and 25°C and 6 h when incubated at 30, 37, 40 and 48°C. Maximum growth (OD₆₀₀ 4.3) was observed at 37°C; where it started its growth after 6 h of incubation and predominantly showed its start of lag phase after 10 h that lasted till 72 h. The stationary phase continued till recorded time of 96 h (Figure 2).

B. megaterium MRLBA9 started to grow after 48, 24, 18, 12, 4, 6 h when incubated at 0, 4, 10, 25, 30, 37 and 40°C, respectively. The maximum growth (OD₆₀₀ 3.6) was observed at 30°C; where it started its growth after 4 h and predominantly showed its start of log phase after 12 h that lasted up to 72 h where stationary phase was reached that continued till 96 h (Figure 3).

Optimization of pH

B. subtilis MRLBA7 was able to grow at pH 4-9, with optimum growth (O.D. 2.5) at pH 7 at 25°C (Figure 1).

However, *B. licheniformis* MRLBA8 was able to grow at pH 4-11, with optimum growth (O.D. 2.9, at pH 9 at 37°C (Figure 2) and *B. megaterium* MRLBA9 was able to grow at pH 4 - 10, with optimum growth (O.D 2.4) at pH 8 at 30°C (Figure 3).

Antibiotic sensitivity

Seven β-lactam antibiotics (cell wall synthesis inhibitors and protein synthesis inhibitors) were tested against spore forming gram positive psychrotrophs (Table 2). *B. subtilis* MRLBA7 revealed sensitivity to neomycin (26 mm), streptomycin (23 mm); vancomycin (9 mm), aztreonam (11 mm) and tecoplanin (12 mm) but was resistant to penicillin and fosfomycin. *B. licheniformis* MRLBA8 was sensitive to neomycin (29 mm), streptomycin (25 mm), penicillin (21 mm), tecoplanin (11 mm) but resistant to aztreonam and fosfomycin. *B. megaterium* (MRLBA9) was sensitive to neomycin (27 mm), streptomycin (28 mm), tecoplanin (13 mm), aztreonam (9 mm) and fosfomycin (7 mm) but resistant to vancomycin and penicillin.

Effect of glycerol on growth and preservation of Isolates

There was no inhibitory effect of glycerol on viability of

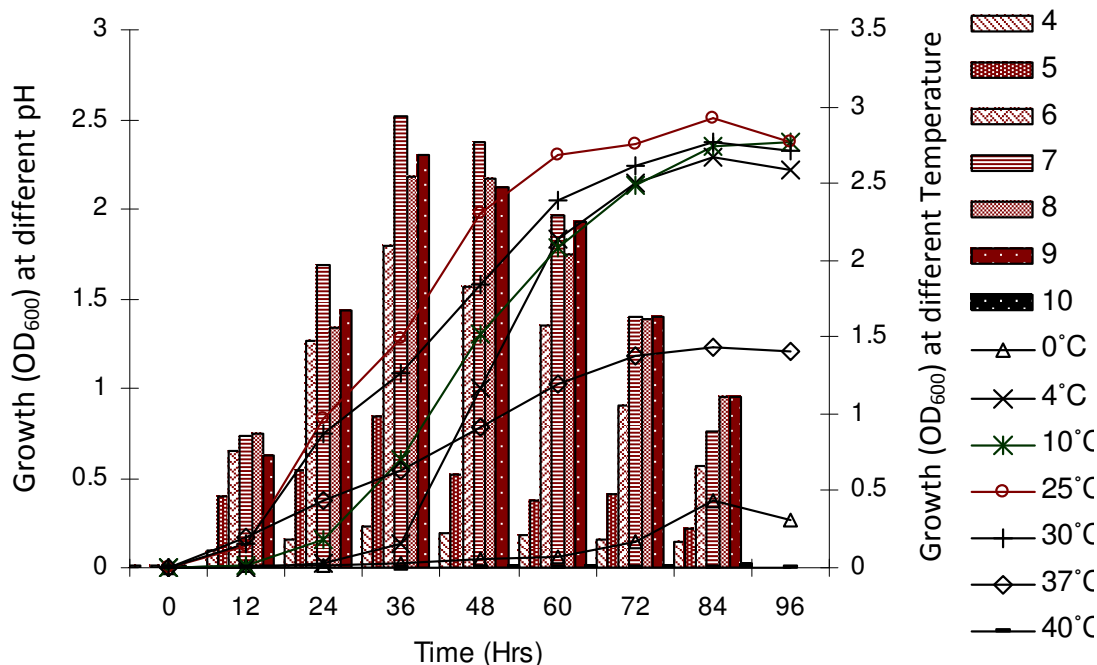


Figure 1. Optimization of pH and temperature: *Bacillus subtilis* MRLBA7.

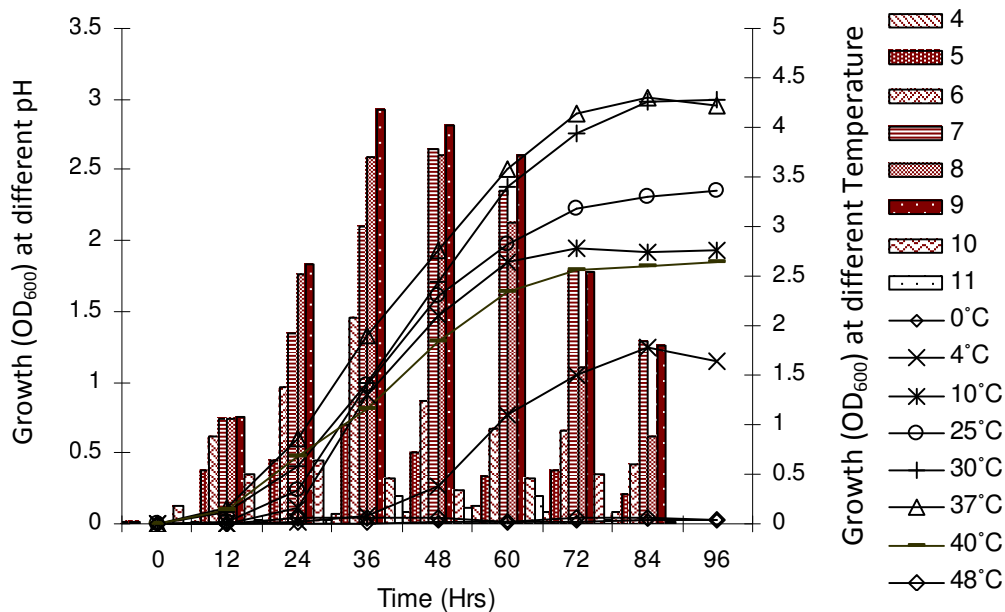


Figure 2. Optimization of pH and temperature: *Bacillus licheniformis* MRLBA8.

any of the isolates during preservation at freezing temperature for storage. Maximum growth was observed in the presence of 30, 50 and 40% of glycerol for *B. subtilis* MRLBA7, *B. licheniformis* MRLBA8 and *B. megaterium* MRLBA9, respectively, when incubated at their optimum temperatures required for growth (Figure 4). The isolates were inoculated in optimized glycerol containing media

and preserved at -20°C (Figure 4).

DISCUSSION

The normal flora entombed in ice of freezers may have adapted the severe physiological conditions and scarce

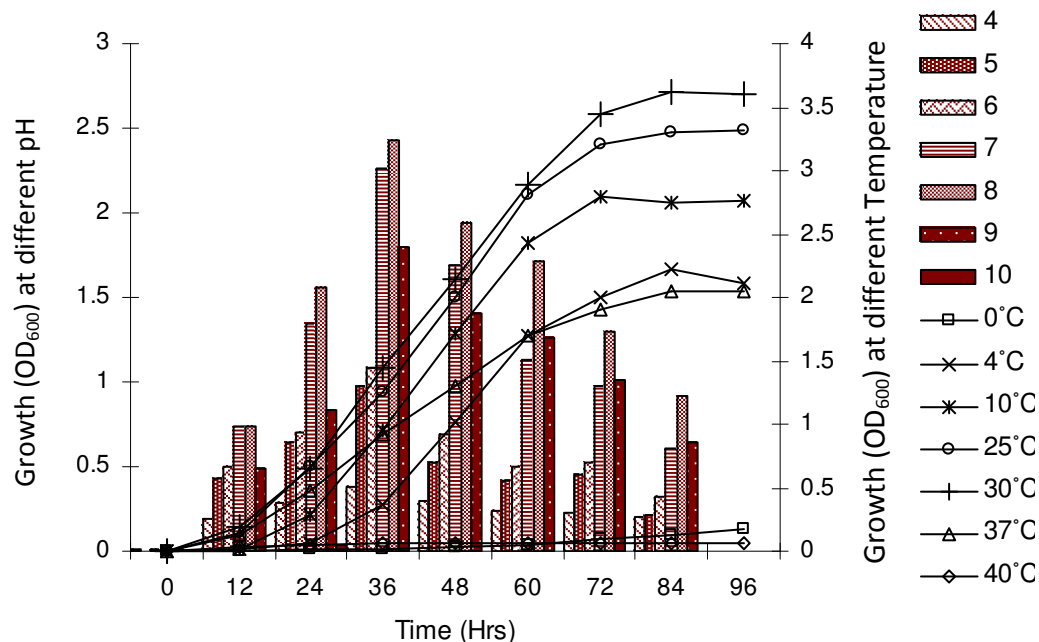


Figure 3. Optimization of pH and temperature: *Bacillus megaterium* MRLBA9.

Table 2. Antibiotic sensitivity of isolates.

Class	Antibiotics (groups of antibiotics)	Zone of Inhibition (mm)		
		MRLBA7	MRLBA8	MRLBA9
Protein synthesis inhibitors	Streptomycin (aminoglycoside)	S (23)	S (25)	S (28)
	Neomycin (aminoglycoside)	S (26)	S (29)	S (27)
Cell wall synthesis inhibitors	Vancomycin (glycopeptide)	S (9)	S (20)	R
	Tecoplanin (glycopeptide)	S (12)	S (11)	S (13)
	Penicillin (β -lactam)	R	S (21)	R
	Aztreonam (synthetic monocyclic β -lactam)	S (11)	R	S (9)
	Fosphomycin (phosphonomycin)	R	R	S (7)

R = Resistant, S = Susceptible.

source of macronutrients for their survival. Secretion of secondary metabolites as an adaptation mechanism for survival, secreted by some of these survivors may have been habituated by competitors due to their continuous exposure. Spore forming bacteria should have better chances to survive and reproduce because they have sufficient time to opt the physiological changes while they are safe in spores or transition of vegetative phase. Except for time span and atmospheric pressure, freezers may be considered as mini glaciers. The studies on survival and physiological adaptation of isolates from freezers should have similar results as exhibited by those isolated from glaciers.

Samples of ice were obtained aseptically from a -20°C freezer. Bacteria have been isolated from glacier ice, sub glacial ice cores and ice cream freezers previously (Abyzov, 1993; Hamilton and Lenton, 1998). Christner et al.

(2000) documented the recovery of viable, *B. subtilis* and *B. licheniformis* and phage (tomato mosaic genomic segments) from *B. subtilis* (Castello et al., 2005) from 100,000 years old ice core.

Plate count method as used in this study, was fast, inexpensive and can directly provide information on the active, heterotrophic component of the population (Bing-Ru et al., 2006).

Survival in low temperature environment requires physiological and metabolic adaptations (Thomas and Diekmann, 2002). Low temperature dwellers possess high specific activity (K_{cat}) at low temperature, weak thermostability and incomplete adaptation to temperature optima (D'Amico et al., 2002).

The range of temperature required for the growth of the three strains were observed as; 0 - 37°C for *B. subtilis* MRLBA7, 4 - 40°C for *B. licheniformis* MRLBA8 and

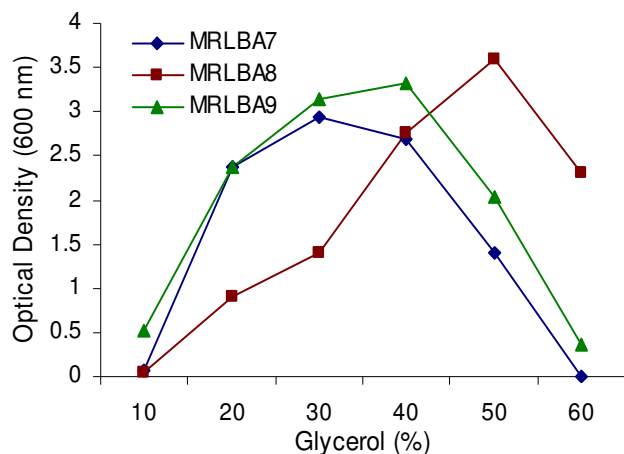


Figure 4. Growth pattern of *B. subtilis* MRLBA7, *B. licheniformis* MRLBA8 and *B. megaterium* MRLBA9 in different concentrations of glycerol.

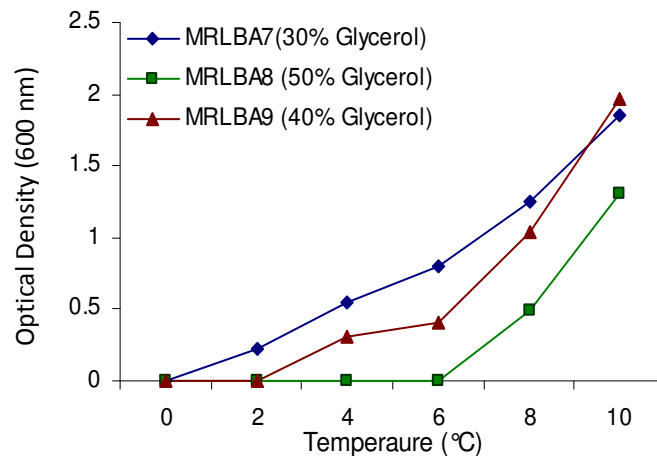


Figure 5. Viability of *B. subtilis* MRLBA7, *B. licheniformis* MRLBA8 and *B. megaterium* MRLBA9 at low temperature (0 - 10°C) in presence of glycerol.

0 - 37°C for *B. megaterium* MRLBA9, with maximum growth at 25, 37 and 30°C and pH 7, 9 and 7, respectively. Psychrotrophs can grow near zero but have optimum temperature for growth above 20°C (Russell, 1990; Hebraud and Potier, 1999) and continue to adapt further (Gerday, 2000). Survival at temperature like -20°C and scarce inorganic nutrients, stresses the marine protozoans to develop a robust resistant cyst (Stoecker et al., 1998). Alteration of physiology like solubility, reaction kinetics, membrane fluidity and protein conformation are adaptations to cope with concurrent changes in physical and biochemical parameters (Hebraud and Potier, 1999). The long term viability of only spore former, gram positive bacteria seem to be adaptive of the environment and acquired the physiological attributes like psychrotrophy.

Presence of an appreciable number of viable cells of spore forming bacteria (dormant in ice or in transition of growth) was due to the combination of mechanisms for adaptation in addition to the role of spores. The membrane fluidity and cold shock response are key factors for adaptation to such environments. Membrane fluidity at low temperature can be achieved by increasing the ratio of unsaturated fatty-acyl residues and/or cis double bonds, chain shortening and sometimes by methyl branching (Russell, 1990; Hebraud and Potier, 1999). Other adaptive mechanisms of psychrotrophs include cold shock proteins (CSPs) (Schroder et al., 1993; Berger et al., 1996; Michel et al., 1997) and cold acclimation proteins (CAPs) (Roberts and Inniss, 1992; Hebraud and Potier, 1999; Berger et al., 1996). The cold shock response is a complex process, connected with heat shock and general stress response associated with cold induced proteins (CIPs) in *B. subtilis* and bears a broad spectrum of functions. A sudden decrease of temperature (10 - 15°C) in *B. subtilis* creates a stress situation to which cells respond by specific adaptive mechanisms that allow their subsequent growth at the lower temperature.

To examine the levels of antibiotic sensitivity and resistance of microbes entombed away from natural exposure of communities and in the absence of current use of antibiotics, two major groups of antibiotics, that is, protein and cell wall synthesis inhibitors was studied. All the three isolates were found susceptible to neomycin and streptomycin. The tecoplanin and fosfomycin, bearing a different β -lactam structural property among cell wall inhibitor group, showed weak potency against these isolates. Three antibiotics, that is, vancomycin, penicillin and aztreonam also showed some resistance and susceptibility but less than that of protein synthesis inhibitors. Cell wall synthesis inhibitors were not effective class of antibiotics against low temperature dwelling *Bacillus* spp. studied here. Levy (2002) described that antibiotics were pivotal in the selection of bacterial resistance and spread of the resistant genes. Many psychrotrophs exhibiting susceptibility/resistance also showed multiresistance to β -lactams (Munsch-Alatossava and Alatossava, 2007; Miller et al., 2009).

To study the proliferation and preservation of strains near freezing temperature, growth of isolates in the presence of glycerol in media was optimized. The *B. subtilis* MRLBA7, *B. licheniformis* MRLBA8 and *B. megaterium* MRLBA9 grew to maximum number in 30, 50 and 40% of glycerol at 25, 37 and 30°C, respectively. Also, they were able to grow at 2, 4 and 8°C, respectively, when incubated in media containing optimized concentration of glycerol. Previously, (Rashid et al., 1999) characterized KB700A showed severe inhibition of growth in the presence of 10% glycerol but Howard (1956) preserved different bacteria in 15% glycerol at -70°C for 2 months and -10°C without losing viability. Nanninga (1971) preserved *B. subtilis* in 20% glycerol concentration during physical and chemical fixation protocols (Figure 5).

In summary, three bacteria from ice obtained from -20°C freezer has been isolated. The morpho-physiological

characterization of the isolates, render the MRLBA7, MRLBA8 and MRLBA9 as *B. subtilis*, *B. licheniformis* and *B. megaterium*, respectively. Candidates were characterized for their antibiotic resistance profile if only exposed strains become resistant to antibiotics used commonly. The isolates were found psychrotrophic and able to grow in presence of glycerol around 0°C. These isolates should be explored further for novel enzymes, antibiotics and biomass applications.

ACKNOWLEDGMENT

The authors wish to express their profound gratitude to the Higher Education Commission of Pakistan for their contribution to the success of this work.

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