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Full paper

Isolation and Characterization of Metal and Antibiotic Resistant Psychrotrophic Bacteria from Refrigerated Spoiled Food

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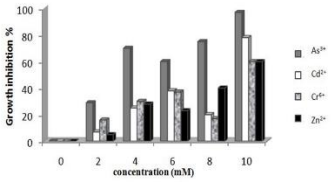
Graphical abstract



Abstract

The aim of this study was to isolate and characterize psychrotrophic bacteria resistant to antibiotic and metals from cold samples of refrigerated spoiled food and ice. Two isolates named H and F were successfully isolated from samples incubated at 10°C and 4°C, respectively. Both isolates were able to grow at 4°C, 10°C, 20°C and 30°C and exhibited multidrug resistance to antibiotics (chloramphenicol, ampicillin, tetracycline and kanamycin). Bacteria H and F were then further tested for their resistance to metal ions such as Cd^{2+}, Zn^{2+}, Cr^{6+} and As^{3+} at concentrations ranging from 0-10 mM. The results of the test revealed that the minimum inhibitory concentration (MIC) for isolate H was determined at 2 mM for all the metal ions. By comparison, isolate F was highly resistant to Cr^{6+}, Cd^{2+} and Zn^{2+} with MIC of 10 mM for these cations, while MIC for As^{3+} was 4 mM. 16S rRNA analysis revealed that isolate F shares a 98% identity match to *Bacillus* sp. This strain could be used as a model microbial strain to understand the mechanisms of metal resistance and as a source of psychrotrophic enzymes with potential application for bioremediation of heavy metals in cold environments.

Keywords: Psychrotroph; metal-resistant; bioremediation; heavy metals; *Bacillus*

Abstrak

Kajian ini bertujuan untuk memencilkan dan mencirikan bakteria psikrotrofik rintang antibiotik dan logam daripada sampel makanan rosak dalam peti sejuk dan ais. Dua pencilan dinamakan H dan F telah berjaya dipencilkan daripada sampel yang dieram pada suhu 10°C dan 4°C masing-masing. Kedua-dua pencilan dapat tumbuh pada 4°C, 10°C, 20°C dan 30°C dan mempamerkan rintangan pelbagai dadah terhadap antibiotik (kloramfenikol, ampisilin, tetrasiklin dan kanamisin). Bakteria H dan F kemudiannya terus diuji kerintangan mereka terhadap ion logam seperti Cd^{2+}, Zn^{2+}, Cr^{6+} dan As^{3+} dalam kepekatan antara 0-10 mM. Keputusan ujian menunjukkan bahawa kepekatan renjatan minimum (MIC) untuk bakteria H telah ditentukan pada 2 mM untuk semua ion logam. Sebagai perbandingan, bakteria F didapati lebih rintang terhadap Cr^{6+}, Cd^{2+} dan Zn^{2+} dengan MIC 10 mM untuk kation ini, manakala MIC bagi As^{3+} adalah 4 mM. Keputusan perbandingan gen 16S rRNA menunjukkan bahawa bakteria F mempunyai 98% kesamaan identiti dengan *Bacillus sp.* Spesis ini boleh digunakan sebagai model mikrob untuk memahami mekanisma kerintangan logam dan sebagai sumber enzim psikrotofik yang berpotensi untuk biopemulihan logam berat dalam persekitaran sejuk.

Kata kunci: Psikrotrofik; rintang logam; biopemulihan; logam berat; *Bacillus*

1.0 INTRODUCTION

Psychrotrophic bacteria are capable of living in cold environments. They can be found in soils, in surface and deep sea waters, in Antarctic ecosystems, and frozen foods. Psychrotrophs can grow at temperatures above refrigeration (25–30°C) optimally, but grow slowly at refrigeration temperatures (below 7°C) [1]. Psychrotrophs thrive in cold environments because of unique features like short and unsaturated fatty acids in membranes, cold shock proteins, enzymes, thermolability and genetic changes [2]. There is a huge untapped potential for exploitation of psychrotrophic microorganisms and their products and enzymes for use in biotechnological processes. Psychrotrophic bacteria with metal and antibiotic resistance have been isolated by Ahmad *et al.* [2], Souza *et al*. [3] and Beena *et al.*

[4]. Interestingly, metal and antibiotic resistance have also been linked to chromogenesis as previously reported [3]. Souza *et al.* also suggested that resistance to antibiotics and metals can be carried by plasmids [3].

The aim of this study was to isolate and characterize psychrotrophs and to subsequently screen their resistance to antibiotics and metals. Moreover, metal resistant psychroptrophs could be used in bioremediating heavy metal contaminated environments at low or elevated temperatures.

■2.0 MATERIALS & METHODS

2.1 Sample Collection

Two different samples were collected aseptically: ice from -20°C freezer of a research laboratory and refrigerated spoiled food from domestic sources.

2.2 Isolation

Ice from the -20°C was allowed to melt and then 100 ml aliquots were filtered under vacuum pump through a 0.20 micron membrane. The membrane was placed on Luria Bertani agar (LBA). A loopful of unfiltered melted ice water sample was also streaked onto LBA. One gram of solid refrigerated spoiled food and 0.1 ml of liquid spoiled sample (yoghurt) were separately mixed with 0.1 ml sterile distilled water before being cultured onto LBA. All samples were incubated at 5 different temperatures 4°C, 10°C, 20°C and 30°C till colonies were obtained. Single colonies were aseptically streaked onto LBA.

2.3 Antibiotic Resistance

Disc diffusion method (Kirby Bauer Method) was used to test the sensitivity of the isolates against antibiotics (ampicillin, kanamycin, chlorampenicol and tetracycline), by using the discs with 30 µg/mL concentration of all antibiotics [5]. Diameters of the zones of inhibition (mm) were measured after incubating plates for 24 h at 20°C.

2.4 Evaluation of Metal Resistance

The lowest concentration of an antimicrobial that will prohibit the growth of microorganisms after overnight incubation described as Minimum Inhibitory Concentration (MIC). The method of Courvalin *et al.* [6] was used in determining the MIC of isolated bacteria towards toxicants. LB medium supplemented with toxicants was inoculated with fresh overnight cultures to a final density of 10^7cfu/ml and then incubated at 20°C for 24 hours. The metal ions used for MIC determinations were Zn^{2+}, As^{3+}, Cr^{6+} and Cd^{2+}. MIC for all the metals was determined at 2 – 10 mM concentrations.

2.5 Morphological Characterization

Identification methods were based on Bergey's Manual of Determinative Bacteriology, 8th edition [7]. Morphological characteristics were specified according to colony morphology (colour, margins, elevation, shape and odour) and Gram staining and Spore staining.

2.6 Identification via 16s rRNA Molecular Analysis

Genomic DNA of the isolate was extracted with Promega Wizard DNA Purification Kit. The 16S rRNA gene of isolate was amplified using the universal primers 8F (5′ AGAGTTTGATCCTGGCTCAG) and 1541R (50-AAGGAGGTGATCCAGCCGCA-3′). The amplification was done by initial denaturation at 94°C for 4 min followed by 25 cycles of 94 °C for 4 min, 52 °C for 4 min, 72°C for 4 min and final extension at 72°C for 10 min. The purified PCR product was sequenced in First BASE Laboratories. Result of DNA sequencing was BLAST using NCBI BLAST program. The phylogenic relationship of the isolate was defined by comparing the sequencing data with sequences of some members of bacteria existing through the GenBank database of the National Center for Biotechnology Information (NCBI). The gene sequence of the isolated F obtained in this study was compared with known 16s rRNA gene sequences in the GenBank database.

■3.0 RESULTS & DISCUSSION

3.1 Isolation

After 48 hours, microbial growth was observed in the plates that had been incubated at 30°C and 20°C. Growth was observed at 4°C and 10°C after one week incubation. Table 1 summarizes the morphological characteristics of isolates observed at 4°C, 10°C, 20°C and 30°C.

The results showed that microbial growth was faster at 20°C and 30°C than 4°C and 10°C. Psychrotrophs have the ability to grow at low temperatures but have optimum growth temperature above 15°C [8]. Since the isolates grew faster at 20°C on LBA plates, growth and antibiotics and metal resistance tests of isolates were done at 20°C

Table 1 Morphological characteristics of isolates

Temperature	Growth after	Spoiled Refrigerated Liquid Food	Spoiled Refrigerated Solid Food	Membrane from Filtered Melted Ice from -20°C freezer	Non-filtered Melted Ice from -20°C freezer
4°C	7 days	F = White, round, smooth, gram +ve rods	J = white, irregular, undulate, gram +ve cocci	E = light yellow, round, smooth, gram –ve rods	-
10°C	7 days	H = pink, round, smooth, gram +ve cocci	G = lemon, round, entire, gram -ve rods	C = cream, round, smooth, gram +ve cocci	-
20°C	2 days	I = pink, round, smooth, gram +ve cocci	L = white, irregular, undulate,gram +ve cocci	A = buff, irregular, undulate, gram +ve rods	B = cream, round, smooth, gram +ve cocci
30°C	2 days	D = pink, round, smooth, gram +ve cocci	J = lemon, round, entire, gram -ve rods	K = buff, round, smooth, gram -ve rods	M = cream, round, smooth, gram +ve cocci

3.2 Antibiotic Resistance

The antibiotic resistance of the isolates (based on the diameter of the zone of inhibition) is shown in Table 2. Generally, resistance to some or all four antibiotics was observed. Most interestingly, no zones of inhibition were observed for isolates H and F indicating high antibiotic resistance. Previous studies have reported that antibiotic resistance is associated with metal tolerance [9, 10]. Correlation exist between metal tolerence and antibiotic resistance in bacteria because of the likelihood that resistance genes to both may be located on the same plasmid. Under stressful conditions, microorganisms possessing both metal and antibiotic resistance are most likely able to grow faster by mutation and natural selection [11]. Hence, bacteria H and F were further screened for metal resistance.

3.3 Metal Resistance

The Minimum Inhibitory Concentration (MIC) of isolates H and F for the metal ions As^{3+}, Cd^{2+}, Cr^{6+} and Zn^{2+} was determined in this study. Zn^{2+}, As^{3+}, Cr^{6+} and Cd^{2+} were selected as they are typically associated with heavy metal pollution of soil and wastewater. Generally, both isolates H and F were able to tolerate relatively high concentrations of metal ions. Bacterial growths were inhibited by different concentrations of metals. Growth of isolating H was inhibited at 2 mm concentration of As^{3+}, Cd^{2+}, Cr^{6+} and Zn^{2+} with percentage growth inhibitions of 66%, 75%, 88% and 61%, respectively. In comparison, MIC test results for isolate F showed that bacterial growth was inhibited at 4 mM concentration of As^{3+} and 10 mM concentrations of Cd^{2+}, Cr^{6+} and Zn^{2+}. The most important observation that can be made is that isolate F is capable of resisting higher concentrations of metal ions than isolate H. Percentages of inhibition for different concentrations of the ions (As^{3+}, Cd^{2+}, Cr^{6+} and Zn^{2+}) for isolating F and H are shown in Figures 1 and 2, respectively.

Table 2 Antibiotic resistance of isolates

Antibiotic	Ampicillin (Am) (30 µg)		Chloramphenical (Cl)(30 µg)		Kanamycin (Ka) (30 µg)		Tetracycline (Te) (30 µg)	
Bacteria	Zone Size (mm)	R, S or I?	Zone Size (mm)	R, S or I?	Zone Size (mm)	R, S or I?	Zone Size (mm)	R, S or I?
G	0	R	0	R	9	S	19	R
K	0	R	10	R	7	I	17	R
F	0	R	0	R	0	R	0	R
L	0	R	15	I	0	R	0	R
C	0	R	7	R	0	R	0	R
E	0	R	7	R	9	I	17	R
B	9	R	16	I	12	R	28	S
J	0	R	7	R	7	R	24	S
A	0	R	20	S	15	I	30	S
H	0	R	0	R	0	R	0	R
I	0	R	13	R	0	R	7	R
D	7	R	7	R	0	R	7	R

S: Susceptibility R: Resistance I: Immediate

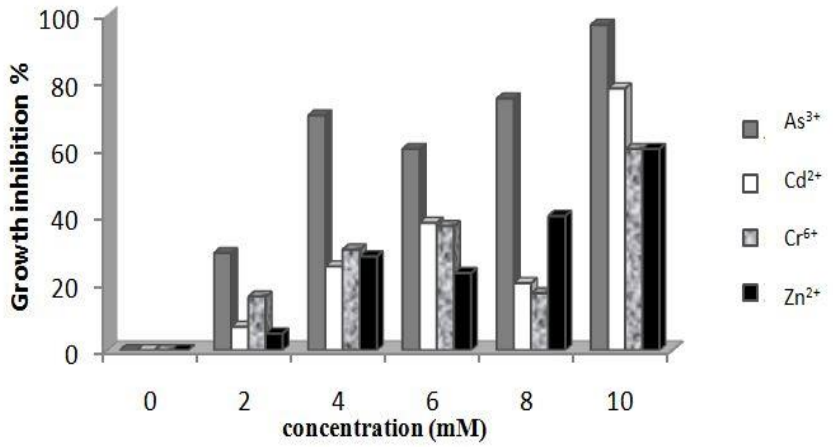


Figure 1 Percentages of inhibition by different concentrations of the ions (As^{3+}, Cd^{2+}, Cr^{6+} and Zn^{2+}) on isolate F

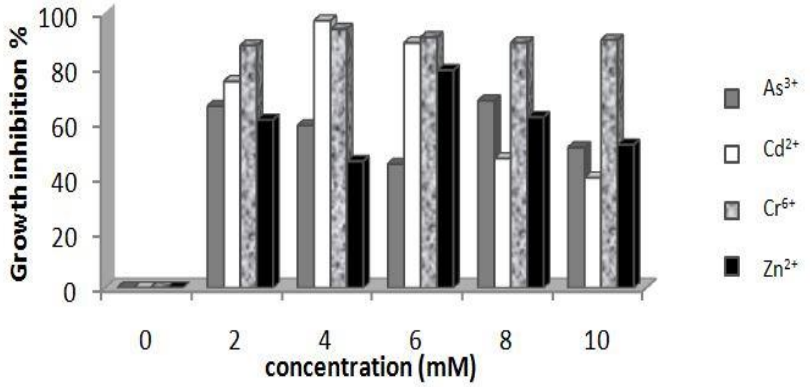


Figure 2 Percentages of inhibition by different concentrations of the ions ($As^{3+,}$ Cd^{2+}, Cr^{6+} and Zn^{2+}) on isolate H

3.4 Morphological Characterization

Identification of isolate F was done since this isolate showed higher tolerance to metal ions than isolate H. *Bacillus* species are gram-positive spore-forming bacilli. Moreover, most members of this genus are saprophytic organisms prevalent in soil, water, and air, like *Bacillus cereus* and *Bacillus subtilis*. Different *Bacillus* species' spores are very resistant to adverse environmental conditions and are metabolically inactive, so they are compromised to be a type of bacteria involved in food spoilage and food poisoning [12]. It is also worth noting that previous work by Ahmad *et al.* (2010) [2] reportedly isolated several psychrotrophic *Bacillus* species from cold environments. Isolate F is an aerobic microorganism and can grow at low temperature (4ºC) with the optimum growth at 20ºC. According to the staining results (spore and gram positive) it is most plausible that isolate F is a *Bacillus* sp. Gram staining and spore staining results are shown in Table 3.

3.5 Bacterial Identification via 16s rRNA

PCR amplification and sequencing of the 16S rRNA gene were done after DNA extraction and gel electrophoresis Figure 3 shows the amplified 16S rRNA gene for isolate F. The results of comparing 16S rRNA genes revealed that isolate F has 98% identity match with *Bacillus sp*. MML1(Figure 4).

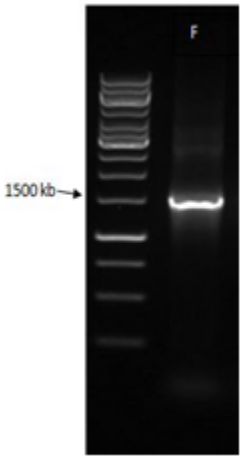


Figure 3 Gel electrophoresis of amplified 16S rRNA

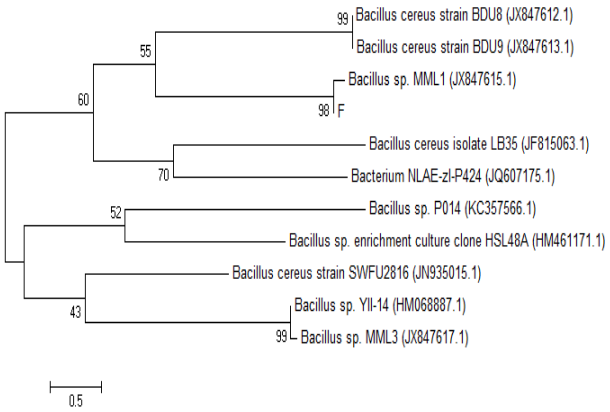


Figure 4 Phylogenetic trees for the taxonomic location of isolate F

Table 3 Staining characteristics of isolate F

Test	Result	Description
Gram staining	+	The shape was round-ended cylinder (bacillus), color of cells were purple.
Spore staining	+	Endospores were observed within rods.

4.0 CONCLUSION

In this study, psychrotrophic bacteria, F and H, were successfully isolated from spoiled yoghurt on LBA Agar using spread plate and streaking method at 4°C. Antibiotic and metal resistance screening showed that high degree of heavy metals resistance is associated with multiple antibiotic resistances for both isolates H and F. Isolate F was generally found to be more resistant than isolate H to higher concentrations of metal ions tested. According to the 16s rRNA and staining test results it is most plausible that isolate F is a *Bacillus species.* The results of this preliminary study suggest that F is a psychrotrophic bacterium since it was able to grow at low temperatures (4°C, 10°C and 20°C) and thus making it possible to be applied for bioremediation of heavy metals in cold environments. This strain could also be used as a model microbial strain to understand the mechanisms of metal resistance induced at low temperatures.

Acknowledgements

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