Isolation and Characterization of Two Novel local Psychrotolerant *Kocuria* spp. with High Affinity towards Metal Cations Biosorption

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Abstract: Two psychrotolerant bacteria were isolated from Nile river, Egypt during winter 2007. Based on 16S rRNA sequences, they were identified as members of the genus *Kocuria.*The yellow pigmented strain showed 99% similarity to *K.carniphila,* and was designated as *K. carniphila* MY, whereas the orange strain was similar to *Kocuria Polaris* by 99 % and was named *K.polaris* MO. Both strains grew best at pH 7.2. The optimum growth temperature was found to be 28°C and 20°C for *K.carniphila* MY and K.*polaris* MO, respectively. The two strains shared the same need to glycerol, fructose, glucose, xylose, mannose, cellulose, cyclohexane and benzene as carbon sources. Casein and peptone supportrd maximal growth, whilst inorganic nitrogen sources ($NaNO_3$, $(NH_4)_2SO_4$ and NH_4Cl) proved to be unsuitable for both strains.The two strains could tolerate high level of salinity reaching up to 7.5% and 10% NaCl for *K.carniphila* MY and *K.polaris* MO, respectively.They were sensitive to the tested antibiotics; Salbactam+ampicillin, Ceftazidim, Amikin, Ofloxacin, Kanamycin, Ceftriaxone, Chloramphenicol, Cefotaxim, Cefadroxil, Aztreonam, Cefalexin and Tetracycline and were resistant to nitrofurantoin, nalidixic acid and colistin.The metabolized cells of the isolates were used for metal biosorption, where it was noticed that *K.carniphila* MY and *K.polaris* MO highly biosorbed all of the five tested cations (Zn, Cu, Co, Cd, and Pb) with maximum affinity towards Zn and Cu, respectively.

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Introduction

More than three quarters of the earth's surface is occupied by cold ecosystems which have been successfully colonized by a class of extremophilic microorganisms that are known as psychrophiles.

There is a common misconception concerning the maximum, minimum, and optimum growth temperatures for psychrophilic bacteria. Psychrotolerant organisms, i.e. organisms that are capable of growth close to 0°C but with an optimum growth temperature of 20°C, have been isolated more often from permanently cold environments than psychrophilic organisms, which grow only at temperatures below 20°C.

Despite living at these low temperatures, psychrotolerant often have metabolic rates comparable with those displayed by microorganisms adapted to moderate temperatures. Psychrotolerant organisms are typically considered able to grow near freezing but with optimum growth 20°C (Feller and Gerday, 1997). The presence of psychrotolerant microorganisms in perennially cold waters, however, implies that these organisms are able to coexist with psychrophiles. Further, most studies concerning the relative abundance of the two groups have found that psychrotolerant bacteria are the dominant group (McMeekin, 1988; De Lille, 1992; Groudieva et al, 2004).

Psychrotolerant organisms and their products have potential applications in a broad range of industrial, agricultural and medical processes (Russell et al, 1998 and Asgarani et al, 2012). It has been reported that psychrotolerant bacteria can be used in the bioremediation of metal contaminated cold areas (Marion et al, 2000; Puyen et al, 2012).

The genus *Kocuria* was created from the genus *Micrococcus* on the basis of the phylogenetic and chemotaxonomic dissection of the genus *Micrococcus*. There are approximately 11 species in this genus: *K. aegyptia, K. carniphila, K. erythromyxa, K. himachalensis, K. kristinae, K. marina, K. palustris, K. polaris, K. rhizophila*, *K. rosea and K. varians* (Stackebrandt et al, 1995). All of the recognized species of *Kocuria* are coccoid, ranging from about 0.5 to 3 micrometers in diameter and typically appear in tetrads, Gram-positive, non-endospore forming, aerobic, that can be differentiated from other genera in the order Actinomycetales on the basis of their peptidoglycan type, the presence of galactosamine and glucosamine as their major cell-wall amino sugars (Mayilraj et al, 2006). Some species are pigmented when grown on solid media, such as *Kocuria carniphila* (yellow) and *Kocuria rosea* (red), produce yellow or red colonies (Yong et al, 2009).

This study aimed for the isolation and identification of some psychlotolerant bacteria from

aquatic environments, then evaluating their potentiality in removing metal ions from solutions.

2.Materials and Methods

Materials

Bacterial strains

The two *Kocuria* spp. used in this study were isolated from fresh water of Rasheed branch of The River Nile, at Fawa water treatment plant in Kafr El-Shakhe-Egypt, in the first of January (2007). Samples were collected near the inlet side of the plant.

Chemicals and reagents

Ingredients of media, reagents and solvents of analytical grade, were obtained from recognized chemical suppliers. Fine chemicals and reagents were of pure grade and were obtained from Sigma Chemicals Company, St. Louis, USA.

Metal stock solutions

Metal salts used in bioaccumulation experiments were copper and zinc sulfates, cobalt and cadmium chlorides and lead acetate. Metal solutions (0.1mM) were filter-sterilized through 0.22 µm pore size membrane filter and kept at 4°C **(**El-Sharouny, 2001).

Antibiotics

Susceptibility to antibiotics was determined with Difco disks of the following antibiotics: Fluoroquinolone, Intravenous β-lactam, β-lactam, Aminoglycoside, Cephalosporin, Tetracycline, Macrolide, β-lactam and cephalosporin, Synthetic Sulfamethoxazole, Synthetic quinolone, Lincosamide, and Polymyxin antibiotic. Antibiotics concentrations are mentioned in Table 3.

Methods

Isolation and purification of psychrotolerant bacteria

King's agar plates (King et al, 1954**)** composed of the following g/l:Peptone, 2.0, Glycerol, 1.0, K_2HPO_4, 0.15, $MgSO_4$, 0.15 and Agar, 1.5, were inoculated with fresh water samples collected from Nile River as previously mentioned. Plates were incubated at temperatures ranging from 10 °C to 30°C and checked every 24h for psychrotolerant bacterial growth. Isolated bacterial strains were purified by streak plate method and subcultured on slants of the same isolation media and preserved in refrigerator at 4°C with fresh transfers at bimonthly intervals. (Moss, 2002 and Minh, 2006).

16S r DNA sequence analysis

DNA was isolated from the purified isolates according to Sambrook et al (1989). A single colony was suspended in 100 ml of 33 mM Tris HCL buffer (pH 8.0). The bacterial suspension was incubated for 10 min at 95 °C and centrifuged to precipitate cell debris. The supernatant was transferred to a fresh tube and used as total genomic DNA sample. The bacterial 16 S rDNA was amplified from the total genomic DNA using universal eubacteria specific primers, designated to amplify 1500bp fragment of the 16 S rDNA regions. The forward primer was: 5'AGAGTTTGATCMTGGCTCAG3'and the reverse primer was 5'TACGGYTACCTTGTTACGACTT3'. The PCR mixture consists of 30 picomoles of each primer, 10ng of chromosomal DNA, 200 µM dNTPs and 2.5 Units of Taq polymerase in 50 µl of polymerase buffer. The PCR was carried out for 30 cycles in 94°C for 1 min, 55°C for 1 min and 72°C for 2 minutes. After completion, a fraction of the PCR mixture was examined using agarose gel electrophoresis (Ausubel et al, 1999) and the remnant was purified using QIAquick PCR purification reagents (Qiagen). DNA sequences were obtained using an ABI PRISM 377 DNA Sequencer and ABI PRISM BigDye Terminator Cycle Sequencing **(**Perkin Elmer**)**. The PCR product was sequenced using the same PCR primers. Blast program (www.nebi. nlm.nih.gov/BLAST) was used to assess the DNA similarities and multiple sequence alignment and molecular phylogeny were performed using BioEdit software (Hall, 1999). The phylogenetic tree was displayed using the TREEVIEW program.

Characterization of bacterial strains

Morphology

Colony morphology, pigmentation, cell shape and arrangement were studied using light microscopy (model BH 2; Olympus) and Gram reaction was carried out by using the standard Gram stain and was confirmed by using the KOH lysis test.

Biochemical tests

Biochemical tests were performed according to Bergey's Manual of Systematic Bacteriology (2012).

Degradative enzymes

The ability to degrade starch by amylase was tested on MM agar containing 1% soluble starch (w/v). Clear zone was detected upon the addition of iodine solution. Urease detection was done by inoculation of bacteria on urea broth. Urease indicated by changing of phenol red color (Satta et al, 1979). Gelatinase detection was done in MM agar supplemented with gelatin. Positive results were indicated by liquefaction of gelatin around bacteria.

Lactase detection by addition of skimmed milk to MM agar, positive reaction noticed in litmus paper when it turned pink (Brown, 2007).

Inoculum preparation

Transfers 1mL of 65 CFU/mL (Abs550 = 0.5) from stock slants were taken into 50 mL of the King's broth in 250 mL conical flasks to intiate growth at 15°C. At late logarithmic phase (Abs550 = 0.5) standard inoculum of 1 % were used to cultivate growth media.

Growth condition

Growth of psychrotolerant bacteria was conducted by inoculation of 1 mL of 65 CFU/mL (OD

=0.5) in 50 mL of liquid King's medium preculture and incubated under standard conditions. Bacterial growth was estimated by measuring optical density of bacterial culture against the clear control medium at wavelength of 550 nm on Cecil spectrophotometer. Growth yield under different conditions were analyzed (Widdel, 2007). Minimal media (Difco, 1998) was used in the testing of growth on various carbon or nitrogen sources.

Physiological tests

Effect of pH

The effect of different starting pH values on the growth of isolated *Kocuria* sp. was investigated after 7 days incubation. Aliquots of the King's medium were initially adjusted to different pH values ranging from (4-11).*K. corniphila* MY was incubated at 28°C while *K. polaris* MO was incubated at 20°C. (Bhowmik & Elmer, 1989).

Carbon source

For carbon source requirement, minimal medium (Difco, 1998) was supplemented with different carbon sources (such as fructose, glucose, xylose, dextran, galactose, ribose, lactose, sucrose, cellulose, mannitol, arabinose, mannose, maltose, cellobiose, pectin, citrate, succinic acid,lactic acid, cyclohexan, benzene, kerosene and toluene). Growth was tested after 5 days incubation at optimum temperature. Carbon source (glycrol) constituent of the King's media was replaced by equivalent amount of previously mentioned carbon sources (Shirling and Gottlieb, 1966).

Nitrogen source

To determine growth in presence of different nitrogen sources, minimal medium (Difco, 1998) was supplemented with equivelant amounts of different nitrogen sources instead of the original one in King's medium. The organic nitrogen sources tested were peptone, tryptone, yeast extract, malt extract and beef extract, whereas, $NaNO_3$, $(NH_4)_2SO_4$, and NH_4Cl were used as inorganic nitrogen source.

Salinity tolerance

The effect of sodium chloride (NaCl) concentration on the growth of bacterial cells was studied. The two bacterial strains were grown in portions of King's broth with different salt concentrations (5, 7.5 and 10%,w/v) and incubated at 20°C for 5 days (Abdulkarim et al, 2009).

Antibiotic susceptibility

The standard disc-agar diffusion method described by kirby et al (1959), was employed for determining the susceptibility of the two *Kocoria* species towards tested antibiotics. The king's agar surface was then swabbed cross wise with a cell suspension prepared and antibiotic discs were set on the agar surface. Plates were then incubated at optimum temperature for 5 days (Hans-Jörg et al, 2000**).** The inhibition zones were carefully measured in mm. Results were recorded as susceptible for antibiotic (S) or resistant (R) according to manufacture instructions.

Bioaccumulation of Heavy Metals

Preparation of heavy metals solutions

Heavy metal solutions (0.1mM) were prepared by adding the equivalent weight for each metal cation in 50 mLof dionized water. Adjust pH to 6 by using 0.1N HCl and 0.1N NaOH. Solutions were sterilized by filteration (El-Sharouny, 2001).

Determination of metal bioaccumulation

In heavy metals uptake test, the amount of metals taken up by the cells was determined according to Nakajima and Sakaguchi (1986). Bacterial cultures (24-48hs) grown in King's broth were harvested, washed twice with distilled water, and 0.05g dry weight were re-suspended in 250 ml conical flasks each containing100 ml of different metal solution (0.1 mM copper, zinc, cobalt, cadmium and lead) adjusting pH to 6. The flasks were incubated on orbital shaker at 25ºC for 2 h. Thereafter, the metal solutions were centrifuged for 15 min at 5000 rpm. The supernatant was used to determine the amount of heavy metals using atomic absorption spectrophotometry. The biosorption percent was calculated using the following equation **(**El-Sharouny, 2001):

$$\% \text{ Biosorption} = \frac{\text{Initial metal concentration} - \text{Final metal concentration}}{\text{Initial metal concentration}} \times 100$$

The effect of pH on metal uptake was tested by being adjusted in the range from 1 to 10 before inoculation by the bacteria (Hemambika et al, 2011). The bioaccumulation of heavy metals was determined as previously described**.**

3.Results and Discussion

Isolation and selection of psychrotolerant bacteria

Water samples were characterized by water temperature of 17°C, pH 7.6 and dissolved oxygen of 7 ppm. Bacterial colonies developed on king agar plates. Two bacterial isolates were selected based on their good growth over a temperature range 5-20.They were coded on the basis of pigmentation as MO and MY. Several reports (Reddy et al, 2003; Satyanarayana et al,

2005; Asgarani et al, 2012) were published on the isolation of psychrotolerant bacteria from fresh water.

The growth pattern of isolates *Kocuria carniphila* MY.and *Kocuria Polaris* MO was determined over a range of temperature (10-30°C). Data in Fig.1a show that isolate MY grew best at 28°C followed by 20°C and 15°C. The lowest growth was observed at 10°C and 30°C. It is worthmentioning that maximum growth was recorded after 120 min, recording values of OD 1.15, 0.85 and 0.65 at 28°C, 20°C and 15°C, respectively.

On the other hand, Fig.1b shows that isolate MO grew best at 20°C followed by 25°C and15°C.The lowest growth was also observed at 10°C and 30°C and the maximum growth was recorded after 120 min, recording values of OD 1.15, 0.95 and 0.92 at 20°C, 25°C and 15°C, respectively.

These results were in close agreement with those reported by Nedwell and Rutter (1994) and Ruger et al (2000). The bacterial isolates named *K. corniphila* MY and *K. polaris* MO were found to be psychrotolerant rather than psychrophilic as they were normally growing at temperatures above 20°C or around, but also being able to grow at near 4°C or below.

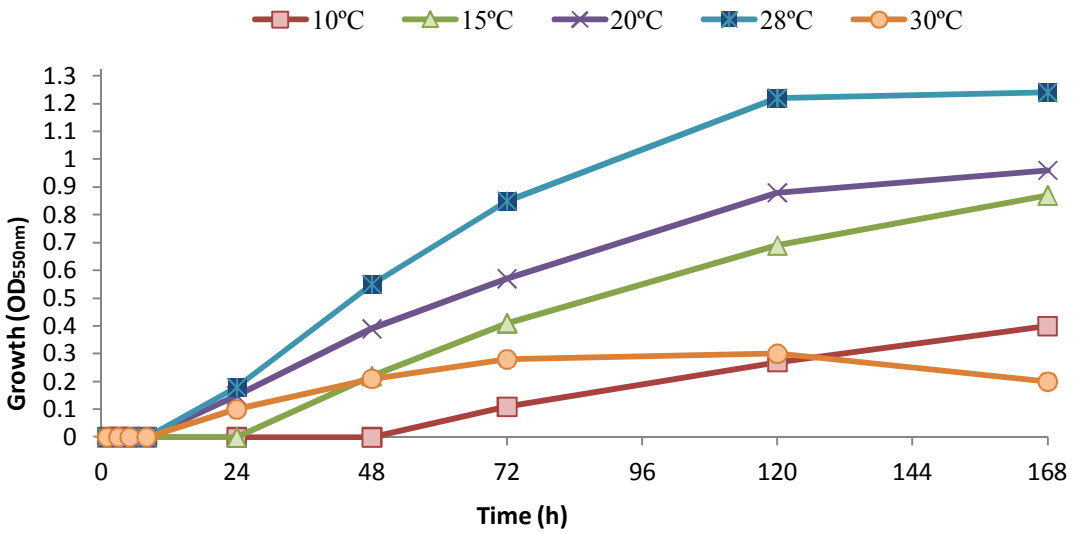

Figure. 1.a. Growth potential of *K.carniphila* MY at different temperatures at different time intervals

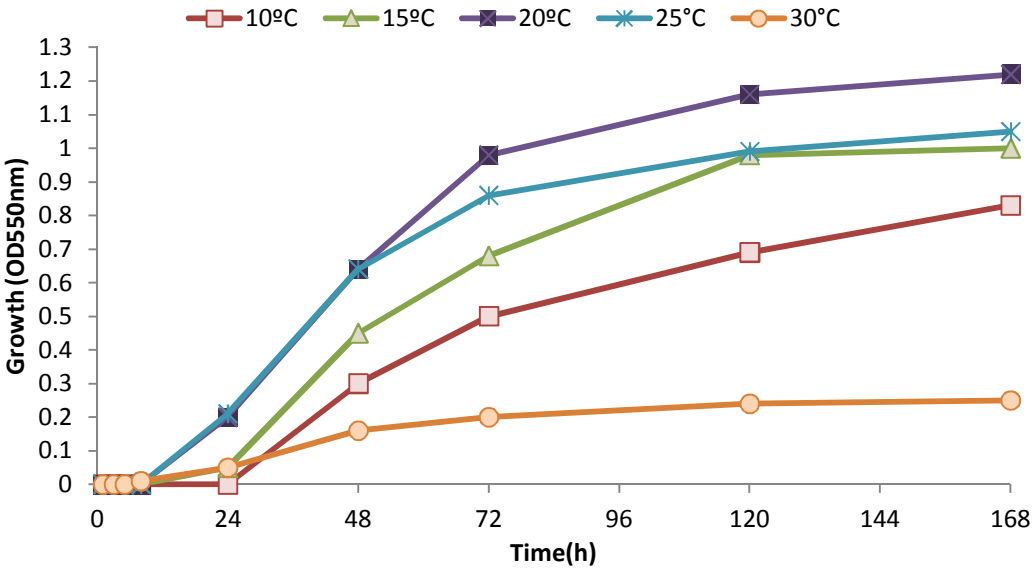

Figure.1.b. Growth potential of *K.polaris* MO at different temperatures at different time intervals

Molecular phylogeny of the selected bacteria

Genomic DNA of the bacterial strains was prepared, and a 1.5 Kb fragment coding for the 16S rRNA was specifically amplified using the universal primers F9 and R1525 shown in the Materials and Methods section. The PCR fragments amplified were purified and sequenced. The sequencing data were aligned using the BioEdit software.The phylogenetic relationships of the experimental isolates were analyzed by using the TREEVIEW program, and the results are presented in a phylogenetic cladogram (Figure 2.a and b). The 16S rRNA sequence for yellow colored isolate (MY) showed 99% identity to *Kocuria carniphila,* while in orange isolate (MO) showed 99% identity to *Kocuria polaris*. Genotypes recorded in the computer database. The experimental isolate differed by only 1 % from the 16S rRNA sequences of the closely related two *Kocuria* species (Table 1.a and b). Based on these phylogenetic characterization results, the experimental bacterial isolates were identified as a members of the genus *Kocuria*, so they were named as *Kocuria carniphila* MY and *Kocuria polaris* MO. They were submitted to the GenBank, where the sequence of the 16S rRNA gene is available under GenBank accession number JX485386 for *Kocuria Polaris* MO and JX485387 for *Kocuria carniphila* MY. 16S rRNA sequence comparison has been used as a powerful tool for establishing phylogenetic and evolutionary relationships among organisms (Lane et al, 1985; Woese, 1987; Govindaswami et al, 1993; Wu et al, 2008). An approach employed in many laboratories use the polymerase chain reaction (Saiki et al, 1998) to obtain 16S rRNA-specific genes for sequence analysis. The use of molecular genetic characteristics to classify an organism and place it in a map showing the relationship between this organism and other related ones, is called molecular phylogeny and a tree/map showing such a relationship is called phylogenetic tree (Li and Grauer, 1991**)**. Thus, molecular phylogeny is a combination between molecular biology and statistical techniques (Wang et al, 2001). The partial nucleotide sequence obtained revealed the affiliation of the two isolates to *Kocuria* with 99 % similarity level for each (*Kocuria carniphila* MY and *Kocuria polaris* MO).

Table 1.*a. Kocuria carniphila* strain showing 99 % identity to isolate MY

Accession	Description	Max ident
FJ607311.1	Kocuria sp. P30 16S ribosomal RNA gene, partial sequence	99%
EU196520.1	Kocuria sp. B3 16S ribosomal RNA gene, partial sequence	99%
EU196518.1	Kocuria sp. B1 16S ribosomal RNA gene, partial sequence	99%
AF385532.1	Kocuria sp. oral clone AW006 16S ribosomal RNA gene, partial sequence	99%
AM418389.1	Kocuria sp. Z21zhy 16S rRNA gene, strain Z21zhy	99%
AM237379.1	Kocuriacarniphila partial 16S rRNA gene, isolate OS-94	99%
AM237350.1	Kocuriacarniphila partial 16S rRNA gene, isolate OS-32.d1	99%
FM873332.1	Uncultured bacterium partial 16S rRNA gene, clone FD01H03	99%
NR_027193.1	Kocuriacarniphila strain CCM 132 16S ribosomal RNA, partial sequence >emb|AJ622907.1| Kocuriacarniphila partial 16S rRNA gene, strain CCM 132T	99%
AM237351.1	Kocuriacarniphila partial 16S rRNA gene, isolate OS-32.d2	99%
AM237391.1	Kocuriacarniphila partial 16S rRNA gene, isolate OS-155.b	99%
AM237385.1	Kocuriacarniphila partial 16S rRNA gene, isolate OS-130	99%

Table 1.b. *Kocuria polaris* strain showing 99 % identity to isolate MO

Accession	Description	Max ident
GQ169064.1	Kocuria sp. CTDE1 16S ribosomal RNA gene, partial sequence	99%
EU045306.1	Kocuria sp. TK815 16S ribosomal RNA gene, partial sequence	99%
EU372960.1	Kocuria sp. A13 16S ribosomal RNA gene, partial sequence	99%
FJ745378.1	Kocuriarosea strain I-7R 16S ribosomal RNA gene, partial sequence	99%
EF540518.1	Kocuria sp. 4_O_59 16S ribosomal RNA gene, partial sequence	99%
NR_028924.1	Kocuriapolaris strain CMS 76or 16S ribosomal RNA, partial sequence >emb|AJ278868.1| Kocuriapolaris partial 16S rRNA gene, strain CMS76or	99%
EU372971.1	Kocuria sp. E7 16S ribosomal RNA gene, partial sequence	99%
FJ889675.1	Kocuria sp. ZS2-6 16S ribosomal RNA gene, partial sequence	99%

FJ267552.1	Kocuria sp. II_Gauze_W_10_8 16S ribosomal RNA gene, partial sequence	99%
AM418390.1	Kocuriasp. 29Y1zhy 16S rRNAgene, strain 29Y1zhy	99%
DQ060382.1	Kocuriarosea 16S ribosomal RNA gene, partial sequence	99%
DQ015980.1	Kocuriarosea isolate BCT-6 16S ribosomal RNA gene, partial sequence	99%
Y11330.1	K.erythromyxa 16S rRNA gene	99%
GU086386.1	Kocuria sp. enrichment culture clone NJ-4 16S ribosomal RNA gene, partial sequence	99%
EU977667.1	Kocuriarosea strain 2P03AA 16S ribosomal RNA gene, partial sequence	99%
FJ966940.1	Kocuria sp. Ld15 16S ribosomal RNA gene, partial sequence	99%

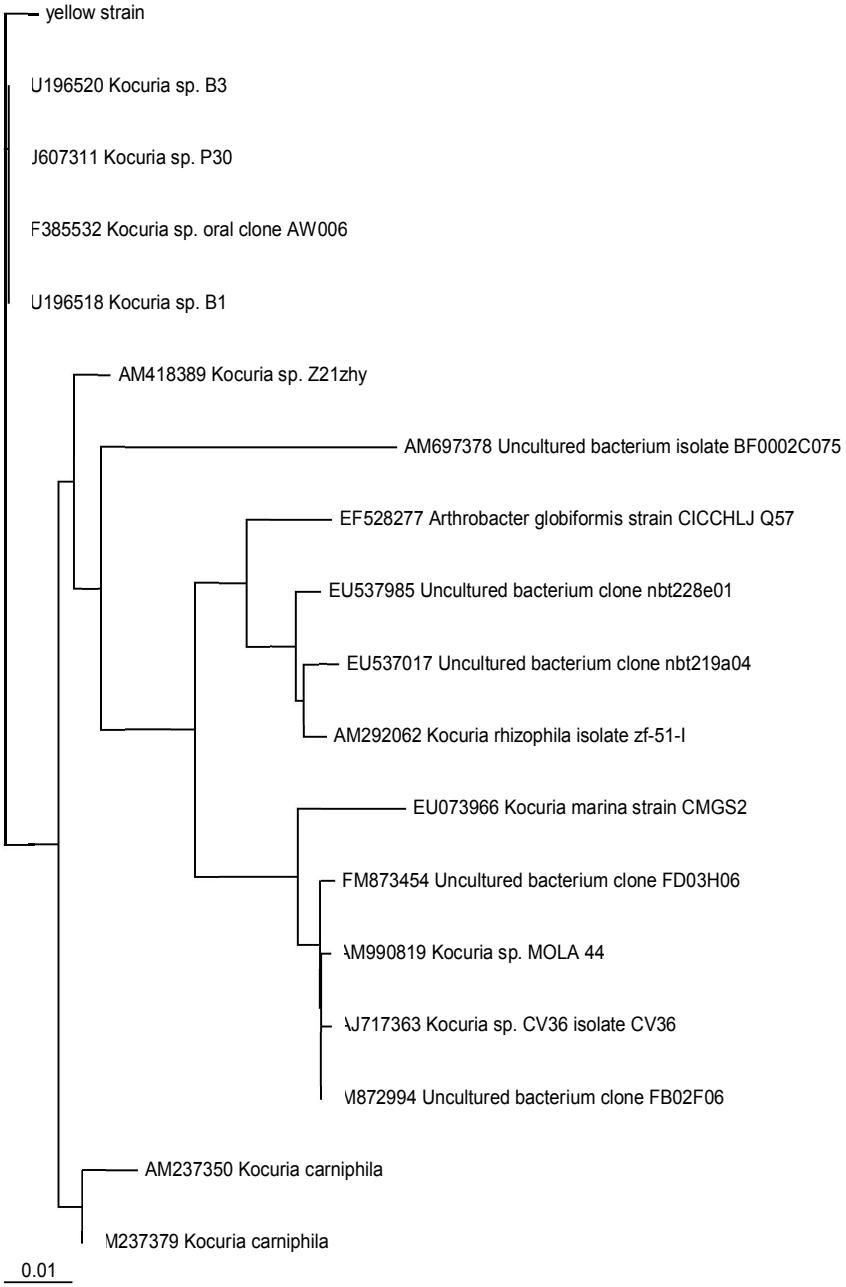

Figure 2.a. Phylogenetic relationships among representative experimental isolate MY and the most related bacteria based on 16S rRNA sequences.

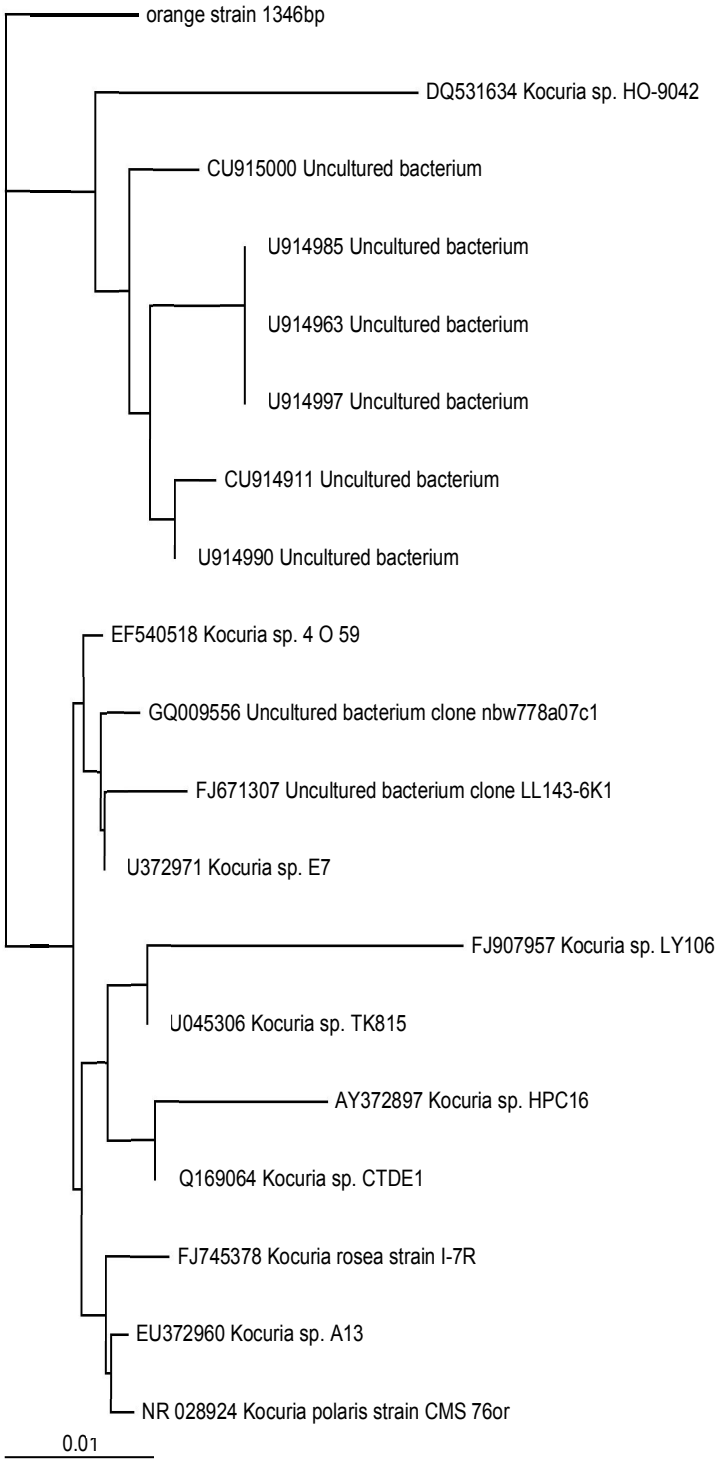

Figure 2.b. Phylogenetic relationships among representative experimental isolate MO and the most related bacteria based on 16S rRNA sequences.

Phenotypic Characterization

Isolate MY formed yellow color, rounded smooth, convex and opaque, uniformly edged colonies when grown on King's agar. Microscopic examination revealed that cells were Gram-positive cocci, occurring in tetrads. On the other hand, colonies of *K.polaris* MO were orange, spherical, and appeared smooth, round, uniformly edged, translucent and mucoid. Cells were

also coccoid, occurring in pairs, tetrads or clusters, Gram-positive, produced water insoluble pigment.

Tvrzova et al (2005) described Kocuria carniphila colonies as yellow, circular, convex and opaque and obligately aerobic, cells were coccoid, (1.5 µm in diameter), occurring in pairs or tetrads and Gram-positive, non-motile, non-acid-fast and non-spore-forming, having a GC content of 65-75 mol%. Cells contain water-insoluble pigments that are soluble in methanol and acetone. Colonies are yellow, circular, convex and opaque. Obligately aerobic, good growth at 28–37 °C, Growth over pH range 7.0–9.1. A very weak growth is in the presence of 10% (w/v) NaCl.

On the other hand, Reddy et al (2003) described *Kocuria polaris* colonies on peptone/yeast extract medium as 0.1–2.0 mm in diameter and appear orange in color, smooth, round, uniformly edged, translucent and mucoid, cells are coccoid (1.0–1.5 µm in diameter), occurring in pairs, tetrads or clusters and Gram-positive, produce water insoluble pigment.Grow between 5 and 30°C, with optimum growth at 20°C. Grows in media adjusted to pH 7–12 and tolerates up to 10% NaCl.

Sensitivity of psychrotolerant *Kocuria* species towards different antibiotics

Both *Kocuria* isolates (*Kocuria carniphila* MY and *Kocuria polaris* MO) were sensitive to the same antibiotics (Table 3):

Licomycin, Gatifloxacin, Pefloxacin, Ciprofloxacin, Imipenem, Tobranycin, Norfloxacin, Garamycin, Azithromycin, Salbactam+ampicillin, Ceftazidim, Amikin, Ofloxacin, Kanamycin, Ceftriaxone, Chloramphenicol, Cefotaxim, Cefadroxil, Aztreonam, Cefalexin, Tetracycline, Cefuroxime, Doxycycline, Cefadroxil and Sulbactam + Cefoperazone. However, they found to be resisting to nitrofurantoin, nalidixic acid and colistin.

According to Hans-Jörg et al (2000) and Tvrzova et al,(2005) who used disks loaded with the various amounts of antibiotics, and found that *Kocuria carniphila* was sensitive to Ampicillin, Cefoperazone, Co-trimoxazole, Amikin, Cefazolin, Ciprofloxacin, Penicillin, Vancomycin, Tetracycline, Lomefloxacin, Erythromycin, Roxithromycin, Tobramycin, Cefotaxim, Licomyci, Chloramphenicol, Streptomycin, Kanamycin, Norfloxacin, Amoxycillin and Cefuroxime, but it was resistant to Nitrofurantoin, Nalidixic acid and Colistin. Reddy et al, (2003) stated the same results for *Kocuria polaris*.

Physiological Characterization

Effect of pH

From the results obtained, it is evidence that the growth of *Kocuria* spp. responded differently to the reaction of the fermentation medium. They failed to grow at pH 4.0 and growth started at pH 5. It was shown that at low pH values, growth of bacteria was slow until the optimum is reached at pH 7.2 (neutral) for both *Kocuria* spp., therefore, pH 7.2 was used for further experiments. At pH values higher than 8, growth of both *Kocuria* species declined. Growth of *K.corniphila* MY was completely vanished at pH 10 while growth of *K. polaris* MO failed at pH>11.

Tvrzova et al (2005**)** found that *Kocuria carniphila* MY exerted relatively good growth between pH ranges 7.0-9.1. Reddy et al, (2003) stated that a pH range of (7-12) was the most convenient for the growth of *Kocuria polaris* MO.

Carbon source utilization

The effect of utilization of different carbon sources on the growth of the experimental psychrotolerant bacterial isolates was investigated and shown in Table 2. The carbon source (glycerol) constituent of King's agar medium was replaced by equivalent amount of one at a time of fructose, glucose, xylose, dextran,galactose, ribose, lactose, sucrose, cellulose, mannitol, arabinose, mannose, gelatin, maltose, cellobiose, pectin, casein, citrate, succinic acid, lactic acid, cyclohexan, benzene, kerosene and toluene, and amount of peptone was reduced to modified the medium.

Kocuria carniphila MY can not be able to grow on medium supplemented with mannitol, sucrose, gelatin, pectin, kerosene or toluene as a carbon sources, whereas a good biomass yield was obtained when culture medium was supplemented with glycerol, fructose, glucose, xylose, dextran, lactose, mannose, cellulose, casein, peptone, cyclohexane and benzene. These results were approved by Tvrzova et al, (2005) who found that *Kocuria carniphila* gave no growth when used pectin, gelatin, toluene, kerosene, mannitol or sucrose as acarbon source, while it was able to utilize mannose, casein peptone, xylose, cellulose, glucose, fructose, lactose, dextran, cyclohexan and benzene.

Kocuria polaris MO *could grow on* glycerol, fructose, glucose, *xylose,* galactose, mannose, *cellulose, pectin, casein peptone, toluene and cyclohexane,* as sole carbon source, but could not utilize dextran, ribose, *mannitol,* sucrose, citrate, succinic acid, lactic acid, *benzene or kerosene*. The same results were stated by Reddy et al, (2003). Also, it was noticed that Kocuria polaris was able to degrade *cyclohexan and toluene.*

Nitrogen source utilization

To examinat the effect of the nature of the nitrogen source on the growth of *Kocuria* spp.,the nitrogen source constituent of King's medium (peptone) was replaced by nitrogen equivalent amounts

of one at a time of $NaNO_3$, $(NH_4)_2SO_4$ and NH_4Cl. Also, complex substances were used as nitrogen source and added one at atime to the growth medium. Thus, the formulation of the medium was modified by separate addition of peptone, beef extract, yeast extract and malt extract at 20 g/l. The pH value of the medium was adjusted to 7.2 and cultures were incubated for 7 days at 28°C for *Kocuria carniphila* MY cultivation and at 20°C for the cultivation of *Kocuria polaris* MO. The results given in Figure 3 reveal that peptone supported the maximal growth (OD_{550nm}= 1.2). Lower biomass yield was recorded with other nitrogen sources, whilst inorganic nitrogen sources ($NaNO_3$, $(NH_4)_2SO_4$ and NH_4Cl) proved to be unsuitable for both *Kocuria carniphila* MY *and Kocuria polaris* MO.

King et al (1954) found that King's medium highly enhanced the growth of *Kocuria* sp. They advised to use glycerol,10, peptone,20, K_2HPO_4, 1.5 and of $MgSO_4$, 1.5 for better growth. Medicharla et al (1991) used medium containing peptone (0.5%), yeast extract (0.2%), and soil extract (5%) for the isolation of the psychrophilic pigmented *Micrococcus roseus* strain (*Kocuria polaris*).

Enzymes production

For amylase production, *Kocuria carniphila* MY gave a negative reaction, while a positive results was recorded for *Kocuria polaris* MO. Catalase was positive and oxidase was negative for both *Kocuria carniphila* MY *and Kocuria polaris* MO.This was formerly confirmed by Reddy et al, (2003) and Tvrzova ***et*** al, (2005). Production of lactase was also noticed by *Kocuria carniphila* MY *and Kocuria polaris* MO, but both gave no results for urease, gelatinase and phosphatase testes.

Biochemical testes

Sugar fermentation was carried out in test tubes containing sugar broth and incubated at 28°C for *Kocuria carniphila* MY and 20 °C for *Kocuria polaris* MO.

Strong acidification of glucose and lactose was done by *Kocuria carniphila*, while weak acid production was noticed on fermenting sucrose. On the contrary, negative results were found on fermenting mannitol. Gas accompanied acid production in case of lactose and sucrose.Tvrzova et al, (2005) found that acidification of glucose and lactose is positive for *Kocuria carniphila*, with weak acid production from sucrose. Acid production was negative for mannitol.

Kocuria polaris MO fermented mannitol, lactose, sucrose and glucose, but did not produce gas from glucose, mannitol, lactose or sucrose. Similary, Reddy et al (2003) found that *Kocuria polaris* could ferment mannitol, lactose, sucrose and glucose without gas production.

In the present study, Kocuria carniphila MY gave negative resultes for Voges–Proskauer test, indole test and the methyl red test, nitrate is reduced to nitrite. Nitrite is not any more reduced. On the other hand, Kocuria Polaris MO gave negative results in the indole test, methyl red test and the Voges–Proskauer test, nitrate is reduced to nitrite. Nitrite is reduced to ammonia.

Toleranc to salinity

The two isolates were grown on King's medium at pH 7.2 supplemented with NaCl (5 -10 %), *Kocuria carniphila* MY incubated at 28°C while *Kocuria polaris* MO was incubated at 20°C for 7 days.

Kocuria carniphila MY grew optimally at 5 and 7.5% NaCl, and fail to grow above this salt concentration. The same findings were recorded by Tvrzova et al, (2005) who found that *Kocuria carniphila* gave a very weak growth in the presence of 10% NaCl in medium.Whereas *Kocuria polaris* MO was successfuly grown, tolerating up to 10% NaCl. The same results obtained by Reddy et al, (2003**)** who found that *Kocuria polaris* tolerates up to 10% NaCl. Also, these data coincided with those of Kloos et al, (1974) who stated that *Kocuria* can grow well in environments with little water or high salt concentrations.

Li et al (2006), isolated A coccoid, non-motile actinobacterium, designated strain YIM 70003T, was isolated from a saline, alkaline, desert-soil sample from Egypt. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the organism formed a distinct phyletic line within the genus *Kocuria* and was most closely related to *Kocuria polaris* DSM 14382T (98.6% sequence similarity) and *Kocuria rosea* DSM 20447T (98.2 %).They named it *Kocuria aegyptia* sp. nov. where its cells are Gram-positive, coccoid, occur in pairs, tetrads or clusters, are non-motile and do not form endospores. Colonies are pink, circular, opaque and approximately 2 mm in diameter. Maltose, D-glucose, D-cellobiose, D-trehalose, D-sorbitol,

D-fructose, D-mannose and dextrin can each be utilized as a sole carbon source; acid is produced only from D-fructose. The G+Ccontent of the DNA is 73.0 mol%.The type strain is YIM 70003T (=CCTCC AA203006T=CIP107966T=KCTC 19010T=DSM 17006T) and was isolated from a saline, alkaline, desert-soil sample collected from Egypt.

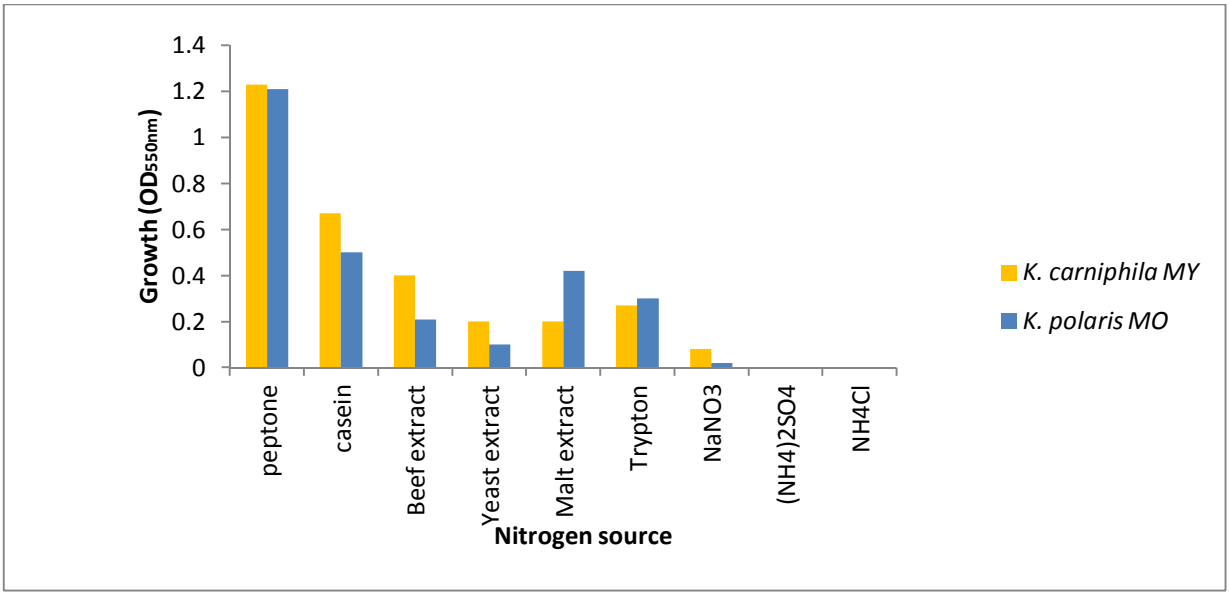

Figure.3. Growth potential of*Kocuria carniphila* MY and *Kocuria Polaris* MO in MM to which different nitrogen sources were added.

Table.2. Growth potential of the two bacterial isolates in MM with various organic carbon sources added as a sole source of carbon and energy

Carbon sources	MY isolate	MO isolate
Monosaccharides		
Glucose	+	+
Fructose	+	+
Xylose	+	+
Arabinose	-	-
Ribose	-	-
Mannose	+	+
Galactose	-	+
Glycerol	+	+
Disaccharides		
Lactose	+	-
Sucrose	-	-
Polysaccharides		
Cellulose	+	+
Dextran	+	-
Casein	+	+
Carboxylic acids		
lactic acid	-	-
Succinic Acid	-	-
Citrate	-	-
Polymers		
Kerosene	-	-
Benzene	+	-
Toluene	-	+
Cyclohexane	+	+
Gelatin	-	-
Pectin	-	+
Alcohols		
Mannitol	-	-

Table.3. Susceptibilty of bacterial isolates to different antibiotics

Antibiotic name	Mode of action		Conc. (ppm)	MY isolate		MO isolate	
				Sensitivity	I.Z. (cm)	Sensitivity	I.Z. (cm)
Aminoglycoside antibiotic							
Tobranycin		TOB	10	S	2.50	S	2.65
Tobramycin	protein synthesis	TOB	30	S	3.65	S	3.65
Kanamycin	inhibitors	K	30	S	4.15	S	4.25
Garamycin		CN	10	S	3.35	S	3.65
Streptomycin		S	30	S	3.64	S	3.60
Amikin		AK	30	S	5.35	S	5.55
Tetracycline antibiotics	protein synthesis						
Tetracycline	inhibitors	TE	30	S	3.35	S	3.15
Doxycycline		DO	30	S	4.35	S	4.55
β-lactam antibiotic	Inhibiting cell wall						
Salbactam+ampicillin	biosynthesis	SAM	20	S	4.35	S	4.50
Aztreonam		ATM	30	S	2.85	S	3.20
β-lactam &cephalosporin antibiotic	Inhibiting cell wall biosynthesis						
Sulbactam+Cefoperazone		CES	105	S	4.95	S	5.10
Cephalosporin antibiotic							
Ceftazidim		CAZ	30	S	3.35	S	3.50
Cefazolin	Inhibiting cell wall	CFP	30	S	4.05	S	4.25
Ceftriaxone	biosynthesis	CRO	30	S	2.75	S	2.95
Chloramphenicol		C	30	S	5.35	S	5.20
Cefotaxim		CTX	30	S	6.10	S	6.00
Cefadroxil		CFR	30	S	4.95	S	5.00
Cefadroxil		CFR	75	S	5.80	S	6.10
Cefalexin		CL	30	S	2.85	S	3.00
Cefuroxime		CXM	30	S	4.35	S	4.50
Fluoroquinolone antibiotics							
Gatifloxacin		GAT	5	S	4.35	S	4.20
Ofloxacin	nucleic acid	OFX	20	S	3.35	S	3.65
Pefloxacin	inhibitors	PEF	5	S	4.00	S	4.15
Lomefloxacin		LOM	30	S	2.80	S	3.05
Ciprofloxacin		CIP	5	S	4.95	S	5.10
Norfloxacin		NOR	10	S	3.35	S	3.85
Glycopeptide antibiotic vancomycin	Inhibiting cell wall biosynthesis	VER	30	S	3.50	S	3.90
Intravenous β-lactam antibiotic	Inhibiting cell wall						
Imipenem	biosynthesis	IPM	10	S	5.85	S	6.00
Lincosamide antibiotic	protein synthesis						
Licomyci	inhibitors	L	2	S	4.35	S	4.50
polymyxin antibiotic	Inhibiting cell wall						
Colistin	biosynthesis	COL	30	R	0.00	R	0.00
macrolide antibiotic	protein synthesis						
Roxithromycin	inhibitors	RXM	30	S	3.50	S	3.65
Erythromycin		E	15	S	2.90	S	3.35
Azithromycin		AZM	15	S	4.35	S	4.55
Sulfamethoxazole	nucleic acid						
Co-trimoxazole	inhibitors	SXT	30	S	3.30	S	3.55
Synthetic antibiotic	nucleic acid						
Nitrofurantoin	inhibitors	FD	30	R	0.00	R	0.00
Synthetic quinolone antibiotics	nucleic acid						
Nalidixic acid	inhibitors	NA	30	R	0.00	R	0.00

I.Z. width = Inhibition zone width R = Risistant S = Sensitive

Table 4. Comparison of main characteristics of different local *Kocuria* species.

Characteristic	*Kocuria aegyptia*	*Kocuria Polaris* MO	*Kocuria carniphila* MY
Shape of cells	Coccoid	Coccoid	Coccoid
Site of isolation	Saline, alkaline, desert-soil sample -Egypt	Fresh water, Nile river -Egypt	Fresh water, Nile river - Egypt
Colour of colonies	Pink	Orange red	Yellow
Optimum temperature for growth	28°C	20°C	28°C
Range of temperature	20-40°C	5-30°C	10-33°C
Range of pH	5-12	5-11	5-10
Optimum pH	10-10.5	7.2	7.2
Oxidase test	-ve	-ve	-ve
Salinity tolernace	1-5% NaCl	5-10% NaCl	5-7.5% NaCl
Urease	-ve	-ve	-ve
Voges-Proskauer	-ve	-ve	-ve
Catalase	+ve	+ve	+ve
Starch hydrolysis	-ve	+ve	-ve
Nitrate reduction	-ve	+ve	+ve
Methyl Red	-ve	-ve	-ve
Gelatin liquifaction	-ve	-ve	-ve
Casein hydrolysis	-ve	+ve	+ve

Bioaccumulation of heavy metals

Heavy metal solutions of 0.1mM were prepared by adding the equivalent weight for each metal in definite volume of dionized water (El-Sharouny, 2001). It was noticed that both isolates moderately biosorbed all of the five tested cations (Zn, Cu, Co, Cd, and Pb) with maximum affinity towards Zn in case of *K.carniphila*, MY. While the other strain *K.polaris* MO had the highest affinity towards Cu and the lowest towards Pb (Table 5).

Puyen et al, (2012**)** stated that *Micrococcus luteus* DE2008 has the ability to absorb lead and copper. They also investigated the effect of these metals on biomass and viability of this microorganism and the removal of the metals from culture media was determined.

In general, bacteria have genes for most elements known to mankind. These genes determine transport systems for uptake of needed nutrients, including for example potassium, phosphorous and iron, for maintaining equilibrium intracellular concentrations balancing needs, and for detoxification or elimination of purely toxic elements such as Hg, Pb, As, Cr, Cd, and Ag (Umrania, 2006**).** Since life arose in an inorganic environment, these genes and the proteins they determine are found in all types of microbes, including prokaryotes, archaea, and eukaryotes. Some

of these genes and proteins show possibilities for their use in environmental measurements, and for clean up or detoxification of waste from human impact (Umrania et al., 1998; Umrania and Joshi, 2002; Umrania and Agate, 2003).

Effect of pH on bacterial uptake of heavy metals

The effect of pH on the bacterial uptake of heavy metals was investigated. It could be noticed that the maximum average rate of metal cation biosorption was achieved at pH 6 for *K.carniphila* MY and pH 4 - 6 for *K.polaris* MO. At low pH values both bacterial strains were able to biosorb low amounts of all tested metal cations Cu, Zn, Co, Cd and Pd and by increasing pH value Co, Cu, Cd and Pd uptake was increased till reaching its maximum at pH 6 where after, they all started to decrease again (Figure 4.a and 4.b).

Leung et al (2000) studied *Kocuria* species efficiency for metal biosorption to achieve low cost biosorption. He found that biosorption capacity of these strains was more effective in use for five different heavy metals he used (copper, cadmium, zinc, lead and cobalt) which were determined at pH 5 and initial metal concentration 100 mg/L. His study of pH effect on metal removal for these strains indicated that the metal biosorption increased with increasing pH from 2 to 6.

Conclusively, the main aim of the present study was to explore our natural environments searching for psychrotolerant species with special efficiency in environmental bioremediation in which it can be used to achieve low cost and safe ways.

Table 5. Amount of metal uptaken by bacterial isolates

Metal cation	*K. carniphila* MY (metal biosorped %)	*K. polaris* MO(metal biosorped%)
Copper	70.3%	74.0%
Zinc	79.6%	72.6%
Cobalt	78.5%	70.3%
Cadmium	74.6%	74.14%
Lead	46.6%	34.2%

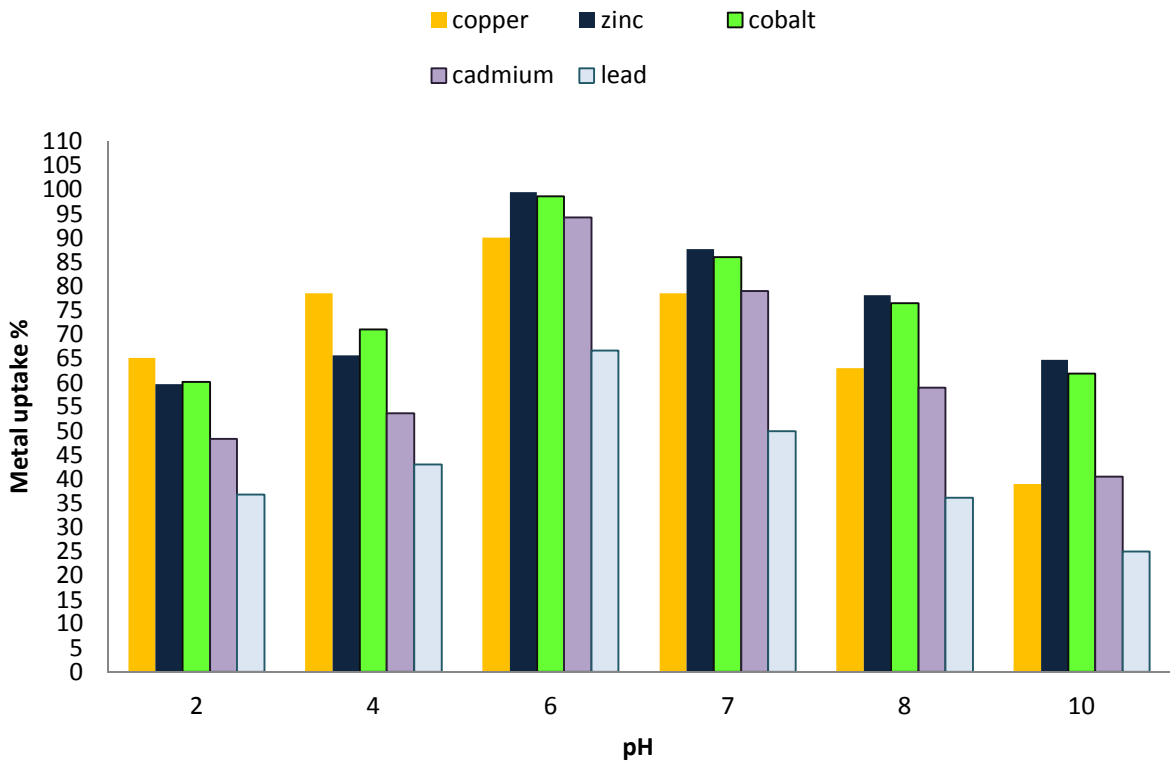

Figure.4.a.Effect of different pH values on metal cation uptake by *K. carniphila* MY

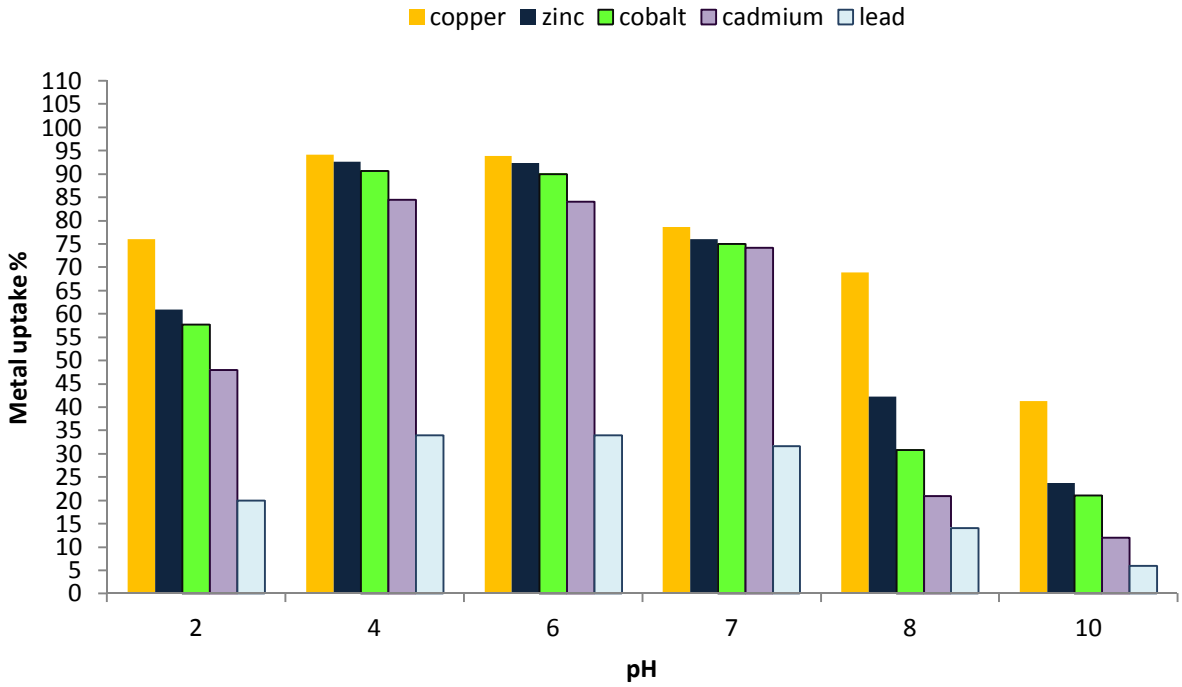

Figure.4.b.Effect of different pH values on metal cation uptake by *K.polaris* MO

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