Antibiotic resistance profile of halophilic microorganisms isolated from tannery effluent

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Halophiles are defined as organisms showing considerable growth at salt concentrations higher than 100 g L\(^{-1}\). Based on the halophilicity, halophiles can be broadly classified as slightly, moderately or extremely halophilic depending on their requirement for NaCl. Halophilic microorganisms, metabolically diversified, comprising Archaea, Bacteria, and Eucarya, are found distributed all over the world in hypersaline environments including drained soak liquor and brine cured hides. Plasmids mediating resistance to antimicrobial agents have been found in many halophilic bacteria examined so far. For the purpose of protection of salt cured hides, adequate knowledge and exposure related to characteristics of halophilic bacteria is very important as halophilic microorganisms secrete bacterial collagenases responsible for collagen damage in the form of ‘Red heat’. All the halophilic bacteria isolated from the drained soak liquor used in these experiments were proved to be motile, aerobic and extremely pleomorphic Gram negative organisms. The growth curve of the halophilic bacteria showed slower growth profile at 37°C compared to \textit{E. coli}. Effective plasmid isolation further strengthened the antibiotic resistance of the halophiles. Analysis of drained soak liquor was followed to examine the related important features of the halophilic species. Optimum salinity of media and pleomorphic Gram-negative nature of halophiles were found as causative factors of insensitivity to antimicrobial agents (AMA). It was found that darkness and low temperature would resist ‘Red heat’ on hides.

**Keywords:** Antibiotic resistance, curing, Gram negative, halophiles, plasmid, red heat, tannery effluent

**Introduction**

Halophilic microorganisms, usually defined as salt-loving organisms that inhabit hypersaline environments all over the world, show considerable growth at salt concentrations within 0.2 to 5.1 mol L\(^{-1}\) NaCl. Halophilic microorganisms are found in all three domains of life: Archaea, Bacteria, and Eucarya\(^1\). According to Kushner, moderate halophiles are those organisms growing optimally between 0.5 and 2.5 M salt\(^2\). The metabolic diversity of halophiles includes oxygenic and anoxygenic phototrophs, aerobic heterotrophs, fermenters, denitrifiers, sulfate reducers, and methanogens. Halophilic organisms are frequently found in proteinaceous products (fish and hides) that are highly salted. Halophilic and halotolerant organisms are always found (in soak liquor) on brine cured hides, containing collagenases that could potentially digest the surface of a hide under long storage conditions at high temperatures\(^3\). Halobacterium glycoprotein requires high NaCl concentrations for structural stability. When suspended in low salt concentrations, the wall protein denatures and this leads to lysis and cell death\(^4\). Most proteins of the halobacteriales contain in excess the acidic amino acids, glutamate and aspartate and low amount of basic amino acids, lysine and arginine\(^5\). The repulsive interactions between the acidic residues at the protein surface were shown to be a major factor in the destabilization of halophilic proteins in low salt concentrations. The requirement of extremely high salt concentrations for structural stability of the protein be attributed to the low content of hydrophobic residues and accordingly weak hydrophobic interactions within the protein molecules. This feature of halophilic microorganisms has showed several biotechnological applications\(^6\).

To elucidate the organization of the genome of the halophilic bacteria, different workers found out the genome size of the said microorganism. Applying pulsed-field gel electrophoresis following restriction enzyme treatment the genome sizes of eleven \textit{Halomonas} strains were reported to be within the range of 1,450 to 2,830 kbp. Through a similar analysis, the genome size range of \textit{Chromohalobacter} strains was found between 1,770 and 2,295 kbp. The genome size of \textit{Salinivibrio costicola} strain E-367

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was estimated at 2,505 kbp (using SfiI restriction enzyme) and 2,259 kbp (using MboI restriction enzyme)\(^7\). Analysis of the restriction profiles led to the conclusion that strain E-367 harboured three different plasmids (pVC1, pVC2 & pVC3) as well as a megaplasmid. Plasmids have been detected in many halophilic bacteria examined so far. Other plasmids that have been isolated and characterized are pH11 from Chromobacter israelensis (48 kb), pHS1 from Halomonas subglaciescola (about 70 kb)\(^8\). The first plasmid characterized from a halophilic bacterium was pMH1, a 11.5 kbp plasmid, isolated from a strain of Halomonas elongata. It mediates resistance to kanamycin, tetracycline and neomycin\(^9\). Aminoglycoside antibiotics including kanamycin and neomycin are active primarily against aerobic Gram negative bacilli. It has been reported that members of Halobacteriaceae are typically resistant to such bacteria specific antibiotics viz., penicillin, ampicillin, cycloserine, kanamycin, neomycin, polymixin and streptomycin\(^10\)\(^,\)\(^11\). Most are sensitive to novobiocin and bacitracin. The transcriptional induction of purple membrane (expression of hop gene) and gas vesicle synthesis (expression of gvpA) in Halobacterium salinarum, an important halophilic strain as inhabitant of hide surfaces, is blocked by novobiocin\(^12\). Exposure to bacitracin, which interferes with the glycosylation of the protein, caused the formation of spherical cells, thus supplying further documentation for the structural shape-maintaining role of the H. salinarum glycoprotein. H. salinarum is further sensitive to haloquinone, an antibiotic produced by Streptomyces venezuelae subsp. xanthophaeus that affects DNA synthesis also in some bacteria. Cerulenin, the fatty acid biosynthesis inhibitor, inhibits growth of H. salinarum, and this inhibition can be relieved by the addition of stearate or oleate (but not by palmitate) to the medium\(^14\). The existence of another species, Natrinema pellirubrum, was demonstrated in the salted hides. The strain was originally deposited as H. salinarum NCIMB 786. Vincristine, an antitumour drug that targets tubulin, affects the structure of the fibrocrystalline body in the halophiles. The drug causes fragmentation of the fibrocrystalline body, changes cell shape and leads to growth inhibition\(^15\) of halophilic organisms including H. salinarum.

The necessity behind our study of halophilic microorganisms related to antibiotic resistance is that ‘red heat’, an important post-mortem defect of hides and skins, which arises as a result of degrading action of halophilic microorganisms on the hide surface. The red colour has been attributed to the presence of pigments inside growing halophilic bacteria\(^16\)\(^,\)=. Most representatives of the Halobacteriaceae are brightly red-orange coloured due to a high content of carotenoid pigments in their cell membrane. The carotenoid pigments of H. salinarum were also claimed to protect the cells against UV radiation and aid in photoreactivation\(^17\). A protective role of bacteriorubin by providing resistance to DNA damaging agent like mitomycin-C was also shown in H. salinarum. Prior to actual leather processing, during the brine curing process, the presence of salty environment proved to be ideal for the growth of halophiles on the hides. Therefore, halophilic microorganisms and their characterization, based on pigmentation, growth and the antibiotic resistance, was relevant for this study. Above all, various factors in effluent may help mutation in halophilic strains with surprising sensitivity to common antimicrobial agents.

Materials and Methods

Materials
Horizontal Gel Electrophoresis apparatus and Trans-illuminator (Biotech R&D Laboratories), Optical microscope (Leica DM LB2) with attached Leica DFC 320 camera have been used in this study. Different inorganic and organic chemicals and antibiotics used in these experiments were collected from Glaxo, Rankrm, Loba, Sigma, Merk and Sisco Research. Collection of Soak Liquor as Tannery Effluent containing Halophilic Microorganisms
Initially, soak liquor samples were collected at 29.5°C in sterilized test tubes from a paddle located at Nudrat Tanning Syndicate, Plot No. 98, Zone-2, Kolkata Leather Complex, Bantala and were thereafter despatched to laboratory in ice containers.

Preparation of Selective Culture Media & Plates for Halophilic Bacteria
An aqueous salt solution (1 L) was prepared according to the composition depicted in Table 1. 1.5 g yeast extract was added to the salt solution and pH of the mixture was adjusted to 7.2 by careful addition of 5(N) NaOH solution. 100 mL of the mixture was distributed in 20 test tubes each containing 5 mL of the mixture. The rest 900 mL mixture was divided into two conical flasks each
containing 600 mL & 300 mL, and 12 g & 6 g agar powder was added to them, respectively. Tests tubes and conical flasks, containing media were autoclaved at 15 lb/inch pressure, 120°C for 15 min. Ampiciline, neomycin and kanamycin solutions with concentrations of 30, 50 and 30 mg/mL, respectively were added to conical flasks containing 300 mL of sterilized culture media at 40°C and then the same media were distributed in 10 Petriplates in laminar air flow chamber (S A Industries), and stored at 4°C. Meanwhile, the 600 mL autoclaved solution was distributed in 20 Petriplates and stored at 4°C.

Physico-chemical Parameter Analysis of Soak Liquor Sample

The pH, conductivity, total dissolved solids (TDS), and dissolved O₂ (DO) were measured potentiometrically during sample collections using Multiline P4. For determining the Cl⁻ content, Hg²⁺ ion was reacted with Cl⁻ to form practically undissociated HgCl₂. The excess Hg²⁺ together with 1-5, diphenylcarbazide as indicator was allowed to form a blue-violet complex in nitric acid.

Determination of Metals by Atomic Absorption Spectrophotometer

Some metals like Cr, Pb, Zn, Fe, Mn and Cu were detected by atomic absorption spectrophotometer (Perkin-Elmer Analyst-100 with interfacing AA Winlab Software), using element specific hollow cathode lamps in default condition, by flame absorption mode. Each time the concentration was determined using nonlinear calibration with 3 replicates each and 3.0 s integration time.

Estimation of Microbial Population

The samples of soak liquor in various dilutions ranging from raw to 1:1000 were inoculated at designated Petriplates and tubes using needle and spreader and incubated at 37°C for several days. Every day after inoculation, the growth of the colonies was closely observed and as the sufficient number of colonies was formed, colony counting was conducted for each Petriplates. Replica plating was followed in antibiotic containing plates.

Determination of Growth Curve of Halophilic Bacteria

Two colonies collected separately from each of the Petriplates, inoculated in 10 mL culture media, kept at 37°C in a shaking incubator (MARS Technocommercial) overnight. Next day, 50 mL sterilized culture media was inoculated with 2 mL overnight culture and allowed to grow at 37°C with a control in the same media without any bacteria. 2 mL of bacterial culture taken out from each of the conical flasks was measured spectroscopically at 600 nm and those conical flasks containing bacterial cultures were placed in a shaking incubator at 45 min intervals until sufficient number of readings were obtained for evaluating the growth curve.

Plasmid DNA Isolation & Purification

Using the plasmid isolation kit and the associated instruction manual supplied by Sigma the isolation and purification of plasmid was carried out.

MIC (Broth Tube Dilution Method)

Minimum inhibitory concentration (MIC) of halophile isolates carrying plasmids was determined according to the method of J M Andrews using different concentrations of kanamycin and neomycin.

Gram Staining

Gram staining was done following the protocol of Bergey et al.

Results and Discussion

The present paper deals with the study of different physico-chemical parameters as well as metal concentrations (Tables 1 & 2) and some microbiological characteristics in the soak liquor of a leather processing industry. The colony forming unit

<table>
<thead>
<tr>
<th>No.</th>
<th>Parameters</th>
<th>NTS (R1)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Total dissolved solids</td>
<td>1.04 g/L</td>
</tr>
<tr>
<td>2</td>
<td>Conductivity</td>
<td>2.08 ms</td>
</tr>
<tr>
<td>3</td>
<td>Chloride (Cl⁻)</td>
<td>610 mg/L</td>
</tr>
<tr>
<td>4</td>
<td>Total hardness</td>
<td>640.8 mg/L</td>
</tr>
<tr>
<td>5</td>
<td>Carbon hardness</td>
<td>291.92 mg/L</td>
</tr>
<tr>
<td>6</td>
<td>Phosphate (PO₄³⁻)</td>
<td>13.3 mg/L</td>
</tr>
<tr>
<td>7</td>
<td>Nitrate (NO₃⁻)</td>
<td>50 mg/L</td>
</tr>
<tr>
<td>8</td>
<td>Carbonate alkalinity</td>
<td>0.3 mg/L</td>
</tr>
<tr>
<td>9</td>
<td>Total alkalinity</td>
<td>6.3 mg/L</td>
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<tr>
<td>10</td>
<td>Acidity</td>
<td>0.00 mg/L</td>
</tr>
<tr>
<td>11</td>
<td>Temperature at the time of sample collection</td>
<td>29.5°C</td>
</tr>
<tr>
<td>12</td>
<td>pH</td>
<td>8.25</td>
</tr>
<tr>
<td>13</td>
<td>BOD₅</td>
<td>114.12 mg/L</td>
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</table>

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<tr>
<th>No.</th>
<th>Metal name</th>
<th>Concentration (mg/L)</th>
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<tbody>
<tr>
<td>1</td>
<td>Chromium</td>
<td>0.675</td>
</tr>
<tr>
<td>2</td>
<td>Manganese</td>
<td>0.450</td>
</tr>
<tr>
<td>3</td>
<td>Iron</td>
<td>7.150</td>
</tr>
<tr>
<td>4</td>
<td>Copper</td>
<td>0.000</td>
</tr>
<tr>
<td>5</td>
<td>Lead</td>
<td>0.650</td>
</tr>
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</table>
(CFU) in different salt agar plates varied from $2.3 \times 10^5$ to $7.9 \times 10^5$ CFU after overnight incubation in 37°C. Replica plating from salt agar plates in kanamycin and neomycin salt media showed few colonies from which plasmids were isolated and compared with Amp plasmid in *E. coli*. The size of the kanamycin plasmid appeared smaller than the neomycin as shown in gel picture. The nature of the growth curve obtained spectrophotometrically has been shown in Fig. 1. The confirmed existence of plasmids in halophilic bacterial cell has been represented in Fig. 2. Pleomorphic, Gram-negative nature of the mixed strain of halophilic bacteria via Gram staining and subsequent morphological study has been elucidated in Fig. 3. The MIC of plasmid containing isolates in kanamycin (1.2 mg/L) and neomycin (31 mg/L) and *E. coli* ATCC strain has been used as reference.

The halophilic species present in both brine cured hide and soak liquor are altogether aerobic in nature as can be exemplified from the nature of environment they live in and the conditions maintained during their incubation. In all the culture plates incubated in dark, red coloration like 'red heat' was absent suggesting that the pigmentation could not occur unless proper conditions are maintained. The decrease in colour, with pigmented halophilic bacteria, was earlier reported to occur with the removal of optimal conditions. Under proper conditions, *H. salinarum*, was also reported to be present on the flesh side of the salted hides, producing large quantities of purple pigmented bacteriorhodopsin. The synthesis of bacteriorhodopsin in *H. salinarum* is directed by the *bop* gene cluster. This cluster contains at least three genes: *bop*- the gene encoding the bacterio-opsin (the protein backbone of bacteriorhodopsin), *brp*- a bacterio-opsin related protein, and *bat*- the bacterio-opsin activator. Expression of the *bop* gene cluster is induced by low oxygen tension and by light. When grown under high oxygen tensions in the dark, the transcript levels of *bop* and *bat* were low during the exponential growth phase, and they increased about 29 and 45 fold respectively, upon entering the stationary phase. The *brp* gene transcription level remained low during all the stages of growth. Exposure to high light intensities stimulated expression of all the three genes, even in the presence of high oxygen levels. In our experiment, the incubation was done in dark and, therefore, the expression of gene clusters to produce

![Fig. 1—Growth-curve of halophiles.](image1)

![Fig. 2—Existence of antibiotic resistant plasmids in the cells of halophiles: Lane 1-no sample; lane 2-kanamycin +ve halophilic sample (plasmid band clearly visible); lane 3-neomycin +ve halophilic sample (plasmid band present); lane 4-marker DNA band (5 Kilo base pairs); lane 5-ampicilline +ve *E. coli* sample; lane 6-no sample; lane 7-no sample; & lane 8-no sample.](image2)

![Fig. 3—Gram negative, pleomorphic characteristics of halophiles counterstained by safranine.](image3)
bacteriorhodopsin was inhibited. Hence, all the colonies were found as colourless. The other reason might be the red pigmented halophiles frequently require special media wherein enrichment techniques necessitate 7 to 10 d for good growth\(^{20}\).

The importance of temperature together with hydration on the proton pump activity of bacteriorhodopsin is well known\(^ {25}\). As reported by Zaccai, the inhibition of bacteriorhodopsin activity at low temperatures or in the dry state could be due to a reduction of motions from the close packing of lipids around the protein in purple membrane\(^ {26}\). Here, the influence of both moisture content and temperature behind pigmentation were minimum since 37°C temperature was maintained throughout the experiment and relative humidity was recorded as high as 95%.

In comparison to conventional growth curve of E. coli, the log phase of our growth curve was found to be of variable slope and this slope irregularity stressed upon the fact that the exponential phase might have constituted more than one sub phases. This observation is in accordance with an earlier work which showed that when H. salinarum was grown in a defined medium containing inorganic salts, five nucleosides, 21 amino acids, glycerol and the vitamins (folic acid, thiamine and biotin), a complex growth curve was obtained. In this curve a number of phases could be discerned within the “exponential” growth phase, each with a different growth rate\(^ {27}\). The slower growth profile enunciated in consequence to the comparatively lower average slope of the log phase gives an idea of duration of cell cycle. Eventually, the cell cycle is the fundamental process of how cellular life generates offspring. Organisation of the cell cycle is highly complex and correct performance and timing of each stage is vital for duplicating the genetic material and producing daughter cells. The process is in essence, the same for all life on Earth, though the variations on the theme are extensive\(^ {28}\). The cell cycle is easily affected by the composition and pH of growth medium, temperature, oxygen access, etc., which should be taken into consideration while interpreting data. Multiple genome copies have also been detected in the Haloarchaea (H. salinarum and Haloferax volcanii)\(^ {29}\). Interestingly, reduced growth rate does not affect the number of genome copies in M. jannaschii, H. volcanii or H. salinarum\(^ {29,30}\), indicating that they do not have overlapping rounds of replication like fast-growing E. coli\(^ {31}\). Incidentally, the replisome DNA synthesis rate, an important part of cell growth, has only been determined for P. abyssi, an archaea, which synthesizes ~330 bp/s\(^ {32}\), similar to C. crescentus but significantly higher than the 30–50 bp/s for eukaryotes and lower than the 1000 bp/s for E. coli\(^ {33}\). Similar events might have occurred in the cell cycles of our experimental halophilic strains comprising both archaea and bacteria.

Looking into the effect of salt concentration on growth, it has been reported that the salt requirement and tolerance of many species vary according to growth conditions such as temperature and medium composition\(^ {34}\). Not only that, salt requirement and tolerance are highly variable among the different species but also these parameters are by no means constant since they may vary according to the growth temperature and the nature of the nutrients available\(^ {35}\). It has been observed that at low salt concentrations, the moderately halophilic bacteria won the competition, while at the highest salinities, the pigmented archaea outcompete the bacteria. Within the intermediate salt concentration range (20 to 30%), temperature was the decisive factor determining the outcome, as the bacteria are favoured by low temperatures\(^ {34}\). In our experiment, the salt concentration was maintained between 20% and 30%. From this angle, it can be presumed that our mixed culture constituted both types of halophilic microorganism e.g. haloarchaea and halobacteria and in the given salt concentration, the population of non-pigmented, moderately halophilic bacteria might have outweighed the population of pigmented archaea like H. salinarum since the given salt concentration was ideal for superior growth of moderately halophilic bacteria compared to haloarchaea. Moreover, in some species, chloride salts proved especially inhibitory (with NH\(_4\)Cl, KCl and NaCl being increasingly toxic), and hardly any activity was observed in the presence of 0.6 M Cl\(^ -1 \). Chloride was found to prevent the attachment of the 50S ribosomal subunit to the 30S subunit mRNA complex and also displaced already bound ribosomes. The accuracy of the translation process was not affected. However, the inhibitory effect of Cl\(^ -1 \) could be partially reversed by glycine betaine or glutamate. When Cl\(^ -1 \) was replaced by other anions, such as glutamate, sulfate or acetate, excellent in vitro protein synthesis activity was found at cation
concentrations as high as 0.6 M, both in \textit{S. costicola} and in \textit{H. canadensis}^{36-40}. Actually, Cl$^1$ content played an important role in the translation, thereby cell cycle and growth of halophiles in the culture medium.

Since the species in our study showed resistance towards ampicillin, kanamycin and neomycin, existence of intracellular plasmid was presumed (Fig. 2). Therefore, the plasmid of the halophilic species might contain the antibiotic resistant genes since the plasmid mediated resistance is the main mechanism behind aminoglycoside resistance$^{12}$. Both Gram negative characteristics of the cell wall and the plasmid contribute substantially in the ampicillin resistance of the halophilic organism, though aminoglycosides like kanamycin, neomycin are effective primarily against Gram-negative organisms. It is also known that antimicrobial resistance shown by halophiles depends considerably on the salinity of the culture media. It has been already reported that at optimal salinity, moderate halophiles generally tolerate high concentrations of most antimicrobial agents$^{41-43}$. For nalidixic acid, spectinomycin, and tetracycline, the effect of salinity was less pronounced and strain dependent. All moderate halophiles tested showed a high sensitivity to rifampicin and trimethoprim, regardless of the salt concentration$^{44}$. In the present study, optimal salinity of culture media, plasmid mediated antibiotic resistance coupled with Gram-negative cell wall barrier might have contributed to the observed insensitivity to most antimicrobial agents.

We may conclude that (1) the pleomorphic, Gram-negative nature of the halophilic bacteria can be reaffirmed on the basis of microscopical observation; (2) the antibiotic resistance profile and MIC with respect to kanamycin and neomycin gives the findings that these resistant strains may contain multiple plasmids as seen in case of other antibiotics; and (3) curing should be practised in dark and at low temperature to avoid ‘Red heat’ specially on bovine hides.

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\section*{References}


