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Abstract

The quality of leather manufactured from brine-cured cattle hides is known to deteriorate on prolonged storage of the untanned hides, particularly at elevated temperatures. This deterioration is probably due to the presence of proteolytic enzymes produced by microorganisms growing on the hide. Extensive research has been done on the identification of halophilic organisms in hides cured with solid salt, but much less information is available on the extremely halophilic organisms found on commercially brine-cured hides.

Brine-cured cattle hide samples were obtained from several commercial brine-curing operations over a period of one year. One hundred and thirty one samples were analyzed for halotolerant, slightly, moderately, and extremely halophilic organisms. Halophilic organisms of all three types as well as halotolerant organisms were found on almost every brine-cured cattle hide sample tested. More than half of the 332 extremely halophilic isolates tested positive for proteolytic activity based on a gelatin plate assay. The majority of the proteolytic positive isolates appeared to be archaeobacteria, based on antibiotic sensitivity and lipid analysis. Measurement of the growth of four randomly selected isolates over a wide range of temperatures indicates that these organisms grow rapidly at elevated temperatures (35°-42°C) and warrants further investigation to assess these organisms as a source of hide damage.

Introduction

A wide variety of bacteria are present on a cattle hide at the time of slaughter. Invasion of the skin and subsequent deterioration of the hide by these organisms begins immediately after the animal dies. Curing the hide with salt to arrest this degenerative process has been used for centuries. If the hide is properly salt cured, the activity of these bacteria are readily controlled. However, another group of bacteria, the halophiles, are frequently found on salt cured hides. These organisms are not present on the cattle hide but are found in the brine raceways and the salt used to cure the hides. The growth of these bacteria are not inhibited but rather are encouraged by the presence of salt¹.

* On leave from Marmara University, Istanbul, Turkey

Halophilic bacteria are capable of growing in environments containing a wide range of salt concentrations. True halophilic bacteria are classified as follows: (a) slightly halophilic, which require 0.2 to 0.5 M NaCl for growth; (b) moderately halophilic, which require 0.5 to 2.5 M NaCl for growth and (c) extremely halophilic which require 2.5 to 5.2 M NaCl. Another class of bacteria, halotolerant, do not require NaCl for growth, but can tolerate NaCl concentrations up to as much as 2.0 M and occasionally more^{2,3}. All other bacteria are non-halophiles and cannot grow in media containing more than 0.2 M NaCl. The emphasis of this study is on the extreme halophiles.

Extremely halophilic bacteria occur naturally in salt lakes, soda lakes and salterns. Solar salts may contain 10^5 to 10^6 viable halophilic bacteria g^{-1} , and may survive for many years under ambient storage conditions. Salt lakes may contain 10^7 to 10^8 cells ml^{-1} ⁴. Halophilic bacteria are generally capable of producing red to violet pigments and the presence of a red discoloration on the flesh surface of cured hides is an obvious indicator of the growth of halophilic bacteria^{1,5,6}. In addition to their presence on cattle hides, extremely halophilic bacteria are also found on other common proteinaceous commercial products, such as fish, that have been cured with solar salt.

The objectives of this research were to determine the extent to which salt cured hides in the United States are contaminated with extreme halophiles, to identify the predominant species present, and to determine their growth characteristics. The long range goal is to determine whether the presence of these organisms on cured hides reduces the value of the hide as a raw material for leather manufacture.

Materials and Methods

MOISTURE AND SALT SATURATION DETERMINATIONS

Ten grams of brine cured hide was removed with a quarter inch punch and the hair and any adhering flesh was removed with a razor blade. Hide samples were then weighed into tarred aluminum pans and placed in a Mettler LP16 Infrared Dryer** for sixty minutes. The loss in moisture and weight change of the sample were automatically recorded. The dry samples then were placed in ceramic crucibles and ashed in a muffle furnace. The furnace was programmed to increase the temperature over a period of 2 hours to 600° C and then to hold that temperature for 4 hours. After cooling, the samples were weighed to determine salt or ash content.

GROWTH MEDIA — MODIFIED NORBERG MEDIA

The liquid growth media used in this study for isolation and growth of halophilic organisms was a modification of Norberg Medium⁷ containing higher levels of magnesium and calcium. The modified medium contained 250 g NaCl, 4.884 g $MgCl_2$, 5 g KCl, 0.175 g $CaCl_2 \cdot H_2O$, and 1 g of yeast extract (Difco, Detroit, MI) in 1 L of distilled water. We found that as a result of this change the growth rate was enhanced. Solid plate medium was prepared by the addition of 15 g of agar to 1 liter of the liquid medium.

ISOLATION OF HALOPHILIC BACTERIA

Ten grams of brine cured hide sample cut into half inch squares were placed in a flask containing 90 mL of 25% (w/v) sterile brine solution. The flask was shaken in a Lab Line shaker

incubator (Lab-Line Instrument, Inc., Melrose Park, IL) at 41°C for 5 days. One mL of the supernatant was diluted with sterile 25% brine to the desired bacterial concentration for inoculation. Plate inoculation was carried out with a Spiral Plater System, Model D (Spiral Systems, Inc., Silver Spring, MD) on the modified solid Norberg media containing 0%, 3%, 15% and 25% salt in order to isolate halotolerant, slightly, moderately and extremely halophilic bacteria. Inoculated plates were incubated at 41°C for one week and then bacterial counts were made. Representative colonies were picked, purified by streaking, and then further grown on modified Norberg media containing 25% NaCl.

GELATIN HYDROLYSIS ACTIVITY

Gelatin hydrolysis was determined on modified Norberg solid media containing 25% NaCl and 1% gelatin (Oxoid). Each isolate was inoculated in 9 mL of modified Norberg liquid medium containing 25% NaCl and incubated at 41°C for 4 days. Gelatin hydrolysis was determined by transferring 30 mL of culture medium into a 5 mm diameter well punched in modified solid Norberg Medium containing 25% NaCl and 1% gelatin. After incubation of the plates at 41°C for 2 additional days, a clear zone in the area around the wells was interpreted as positive gelatin hydrolysis.

SENSITIVITY TO ANTIBIOTICS

A single colony from the primary isolation medium was removed with a sterile loop and cultured in liquid Norberg medium containing 25% salt. After incubation at 41°C in an Orbital shaker at 140 rpm/min for 3 days, the resulting culture suspension was diluted to the recommended turbidity for the Bacto Antimicrobial Susceptibility Test System against a standard prepared from BaCl₂ and H₂SO₄. A sterile non-toxic cotton swab on a wooden applicator was used to inoculate the entire agar surface of a fresh plate by streaking in two directions. The inoculum was allowed to dry for 10 minutes and the antibiotic discs were then placed on the inoculated surface with a pair of sterile forceps. The antibiotics tested were Bacitracin (10 units), Chloramphenicol (30 mcg), Erythromycin (15 mcg), Novobiocin (30 mcg), Penicillin G (10 units), Streptomycin (10 mcg), and Tetracycline (30 mcg) (Difco Laboratories, Detroit, MI). The plates were incubated at 41°C for 3 days and the diameters of the inhibition zones was measured. The reaction of the isolates was reported as either resistant, intermediate or sensitive^{8,9}.

LIPID ANALYSIS

The extremely halophilic isolates were grown for two weeks in modified Norberg liquid growth medium at 41°C in an incubator shaker. Twenty mL of maximum growth culture was harvested by centrifugation at 4°C in a Sorvall SM 24 rotor at 10,000 rpm for 120 minutes in a Sorvall RC-5B refrigerated superspeed centrifuge (DuPont, Wilmington, DE). The supernatant was decanted carefully and the pellet was re-suspended in 1 mL of distilled water to lyse the bacteria and remove the salt. The bacterial cells were centrifuged again at top speed for 30 minutes in a clinical centrifuge, the supernatant was decanted and the pellet was re-suspended in 1 mL of absolute ethanol. After five minutes the sample was centrifuged again. The ethanol was decanted and 1 mL of methanolysis reagent [toluene/methanol/concentrated sulfuric acid 3:3:0.3 v/v/v] was added. After overnight incubation at 50°C, 1 mL of hexane was added to extract the methylated lipids. The extracts were chromatographed on a TLC Scanner II (Camag, Mütterz, Switzerland) using precoated HP TLC plates, 0.2 mm thick, 10 x 10 cm, silica gel (Merck, Darmstadt, Germany), without fluorescent indicator. Separated components were detected by fluorescence with a TLC Scanner II (Camag, Mütterz, Switzerland)^{10,11}.

MEASUREMENT OF THE EFFECT OF TEMPERATURE ON GROWTH

Growth in liquid culture was determined over a temperature range from 19.3°C to 63.8°C in a 20 mL culture tube using a continuously rocking temperature gradient incubator Model TN-3F (ToyoRoshi International, Dublin, CA) for 15 days. The time of onset of turbidity at each temperature and the appearance of red color due to pigment formation in each tube was recorded.

Results

The moisture content of hide samples ranged from 25% to 50% with brine saturation varying from 55% to 100%. Forty-five percent of the hides samples had a salt saturation level of from 85% to 100%; 38% of the samples were from 70% to 85%; and the remainder between 55% to 70% brine saturation. These results show that the brine curing methods was generally good.

The presence or absence of slightly, moderately and extremely halophilic bacteria, as well as halotolerant bacteria, was determined on 131 individual hide samples representing 34 different hide curing facilities in the mid western portion of the United States. These samples were collected over a period of about ten months. The bacterial population found on these samples of brine cure hides was extensive. Fifty seven percent of the samples contained halotolerant bacteria, 80% contained slightly halophilic bacteria, 96% contained halophilic bacteria and 94% of the hide samples contained extremely halophilic bacteria.

A total of 332 colonies of extremely halophilic bacteria were isolated from the 131 brine cured hide samples. A maximum of four colonies was picked from each plate representing a single hide sample. Isolates were selected on the basis of distinct differences in either color or morphology.

The average brine cured hide sample contained between 10^3 to 10^4 viable halotolerant organisms gm^{-1} , 10^4 to 10^7 slightly, 10^5 to 10^8 moderately and 10^5 to 10^8 extremely halophilic bacteria gm^{-1} . As expected, there was a lower concentration of halophilic bacteria on cattle hides brined in the winter compared to hides brined in the summer.

Each isolate was numbered and tested for proteolytic activity and antibiotic sensitivity. Fifty-three percent of the extremely halophilic isolates demonstrated positive gelatinase activity (Figure 1). All of the extremely halophilic isolates that showed positive gelatinase activity were also tested for sensitivity to a variety of antibiotics. The results are shown in Table I. Complete resistance to Penicillium G is characteristic of archaeobacteria because of their cell wall structure. In addition, according to Barkows et al. (1981) archaeobacteria are completely resistant to Streptomycin and one hundred percent sensitive to Bacitracin, which is in line with our results. However, the response of these isolates to the remainder of the antibiotics was not consistent with those reported and may be due to species differences. Overall the results strongly suggest that all of the isolates are from the family Halobacteriaceae and are probably archaeobacteria.

Four randomly selected gelatinase positive isolates (8.1, 9.1, 11.1, 14.2) were examined in greater detail for lipid composition, pigment content and growth characteristics under different temperature conditions.

Lipid analysis of the four isolates was positive for the glycerol diether moieties (GDEM) described by Kates¹⁴ and found only in archaeobacteria. The fluorescence chromatographic pattern for these four isolates, three eubacteria (*Lysteria monocytogenes*, *Staphylococcus aureus* and *Halomonas elongata*) and one extremely halophilic organism obtained from the American Type Culture Collection (*Halobacterium saccharovorum*) is shown in Figure 2. All of the isolates and the known archaeobacteria contain a peak in the GDEM area and the other three organisms clearly contain FAME, fatty acid methyl esters.

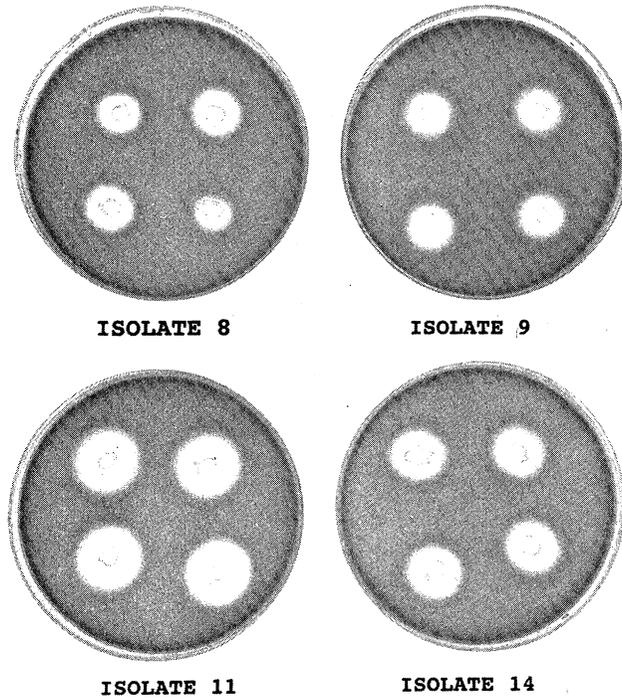


FIG. 1. — Gelatinase activity plate assay of isolates 8.1, 9.1, 11.1 and 14.2. Plate contains solid modified Norberg medium containing 25% sodium chloride and 1% gelatin. Thirty mL of halophile isolate culture was placed in a 5 mm diameter well and stored for 48 hours at ambient temperature. Clear area is the result of gelatin digestion.

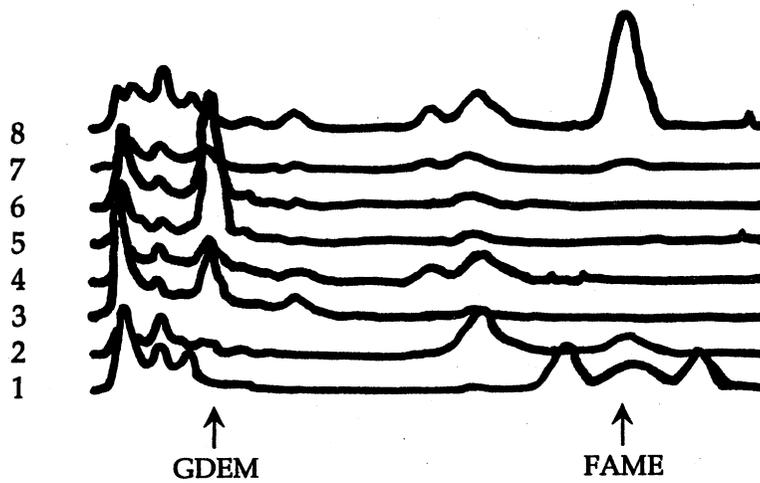


FIG. 2. — Fluorescence pattern from planar chromatography separation of methanolysis products on silica gel developed with hexane:diethyl ether (90/10). GDEM-glycerol diether moieties. FAME - fatty acid methyl ester. Samples: 1. *Listeria monocytogenes*, 2. *Staphylococcus aureus*, 3. Isolate 8.1, 4. Isolate 9.1, 5. Isolate 11.1, 6. Isolate 14.2, 7. *Halobacterium saccharovorum*, 8. *Halomonas elongata*.

TABLE I

Comparison of antibiotic sensitivity of extremely halophilic isolates and reported literature values for a variety of halophilic archaeobacteria. Isolates refers to the organisms isolated in this work and Archaea refers to values obtained in the literature.

Antibiotic Tested	Penicillin G Isolates/Archaea	Bacitracin Isolates/Archaea	Streptomycin Isolates/Archaea	Chloroamphenicol Isolates/Archaea	Erythromycin Isolates/Archaea	Novobiocin Isolates/Archaea	Tetracycline Isolates/Archaea
Sensitivity							
Resistant	100% / 100%		100% / 100%	62%	98.9% / 40%	14% / 0%	91% / 60%
Intermediate				18%		32%	6%
Sensitive		100% / 100%		19% / 100%	1.1% / 60%	54% / 100%	3% / 40%

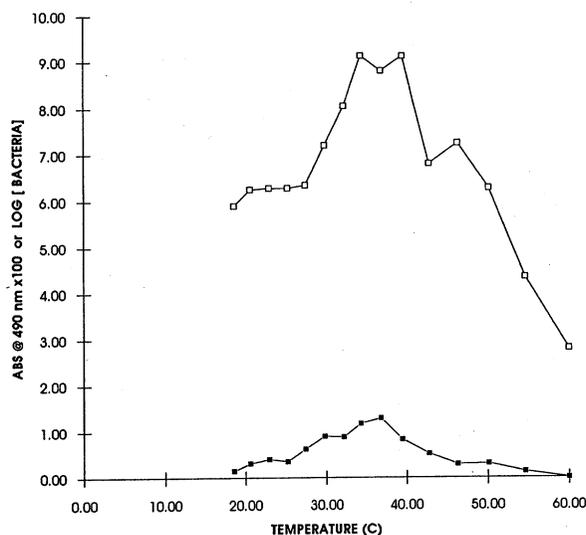


FIG. 3. — Relationship between growth (solid markers) and pigment formation (open markers) of isolate 8.1 vs temperature. Relative pigment concentration was measured by absorption (ABS) at 490 nm. Growth was measured by plate count and plotted by log of count.

Cultures of each of the isolates were centrifuged and the pellet extracted by the method of Oren¹⁴ and the absorption pattern was measured spectrophotometrically. One of the isolates (8.1) contained sufficient pigment to obtain a typical absorption pattern as described by Oren (1983) with peaks at 468, 493 and 528 nm. Two of the other extracts had a smaller amount of pigment and the third did not appear to have any. The spectra further identified two of these isolates as archaeobacteria.

The growth of the four isolates was compared at temperatures ranging from 25 to 55°C. Growth and pigment production at 15 days are shown in Figure 3. The temperature range examined was between 25°C to 55°C. Growth between 25°C and 30°C was slight compared to the maximum that occurred at 40°C. Results were similar for all four isolates. The appearance of pigment generally followed the growth curve and appeared several days to one week after the initial turbidity.

Conclusions

The most interesting and significant conclusion of these experiments is that ninety-four percent of the brine cured cattle hide samples tested positive for extremely halophilic bacteria. The twelve hides that did not, came from six different brine curing facilities. Other brined hide samples from all six of these operations did contain halophiles. This indicates that every brine curing raceway that produced hide samples for these tests contained significant numbers of halophiles and contaminated almost every hide. It also means that the usual brine raceway treatments used to control hide bacteria during curing do not control halophilic bacteria.

Halophilic archaeobacteria are biochemically unique from eubacteria in several ways. They lack murein in their cell walls and thus are insensitive to penicillin^{4,12}. Their cell walls contain ether-linked phosphoglyceride lipids, which are readily separated by thin layer chromatography from the normal ester lipids of the eubacteria¹³. The results of the lipid analysis and antibiotic

sensitivity tests suggest that most, if not all, of the extremely halophilic isolates in this study were archaeobacteria in the family Halobacteriaceae.

The growth temperature profile of the four isolates from brine cured hides permits a crude estimation of the potential growth of these bacteria under commercial conditions of transportation and storage. At room temperature the growth of these organisms is very slow and it would be unlikely that they would affect hide quality. Nevertheless, if the brine cured cattle hides were shipped in hot climates over a period of several weeks, this would increase the growth of halophiles, thereby increasing the potential for deterioration of the hides. It remains to be seen if this increased growth actually has an impact on the quality of the leather produced from this raw material.

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Interpretive Summary

The predominant raw material used for leather manufacture in the United States, brine (salt) cured cattle hides, is known to deteriorate on prolonged storage, particularly at elevated temperatures. One possible cause of this damage is the microbial population present on the stored hides. Bacteria that can grow in the presence of high concentrations of salt are called halophilic bacteria. Over a period of one year, 131 samples of brine cured hides representing 34 different hide curing facilities were tested for halophilic bacteria and these bacteria were isolated from every hide sample. More than half of these isolated bacteria tested positive for enzymes that can break down proteins. Those enzyme-producers appeared to be archaeobacteria, based on antibiotic sensitivity and an analysis of the unique fat content of the specialized organisms. It was found that under conditions of elevated temperatures (35°-42°C) these organisms can grow very quickly. These findings warrant further investigation to assess these organisms as the possible source of the observed deterioration of salt cured hides.

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