# Prevention of halobacterial damage on hide caused by lipolytic halophilic archaea with halocins

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## SUMMARY

Kaldırım and Kayacık Salterns of Tuz Lake in Central Anatolia are the major salt sources of Turkey. Fifty five percent of salt need is supplied from these salterns. 492.000 and 620.000 tons of salt are produced from Kaldırım and Kayacık Salterns in 2004, respectively. Crude salt extracted from these salt sources is commonly used in hide preservation. Lipolytic activity of extremely halophilic microorganisms found in the salt can reduce hide quality. Many bactericides have been used in leather industry to prevent damage by halophilic microorganisms. Halobacterial growth on hides may be prevented with natural antimicrobial compound such as halocins. Extremely halophilic Archaea produce halocins to kill or inhibit the other halophilic Archaea in the same or different environmental niche. Hence, a microbial survey was conducted to examine whether halocins are used to control lipolytic strains in brine solutions or not. A total of 25 extremely halophilic strains were isolated from these salt sources. The percent of lipase producer strains in both salterns was almost similar. Thirty nine percent of Kaldırım Saltern's strains and fourty three percent of Kayacık Saltern's strains produced lipase. Eighty nine percent of Kaldırım Saltern's strains and twenty nine percent of Kayacık Saltern's strains produced effective halocin against each other. It is suggested that lipase negative halocin producers or their halocin extract may be used in preventing the halobacterial deterioration that can occur during brine curing of hide.

**Key Words:** halocin, lipase activity, extremely halophilic Archaea, Kaldırım and Kayacık Salterns.

*This work was supported by the Scientific Research Project Commission of Marmara University, Project No: Fen-125/081104, 2005.

## INTRODUCTION

Bacteriocins are proteins produced by 30 genera of the domain *Bacteria* that inhibit or kill closely related species or even different strains of the same species (Pelczar *et al.,* 1993; Shand *et al*., 1999). These agents are ribosomally synthesized peptides (Madigan and Martinko, 2006). Many of human bacterial flora synthesize and release bacteriocins (Prescott *et al*., 1993). There are many different bacteriocins including those produced by bacteria normally found in the intestine. Bacteriocins may give their producers an adaptive advantage against other bacteria. Sometimes, they may increase bacterial virulence by damaging host cells such as mononuclear phagocytes. Most of bacteriocins are produced by gram-negative bacteria. Bacteriocins are named according to the species of organisms that produces them. Thus, colicins are bacteriocins synthesized by *Escherichia coli*, klebicins are bacteriocins synthesized by *Klebsiella* spp (Shand *et.al.*,1999), subtilisin is bacteriocin synthesized by *Bacillus subtilis* (Madigan and Martinko, 2006). Bacteriocins often kill cells by forming channels in the plasma membrane that allowing potassium ions and protons to leak out, leading to a loss of the cell's energy forming ability. They may also degrade DNA and RNA, or attack peptidoglycan and weaken the cell wall (Prescott *et al.*, 1993). However, bacteriocin may be an endonuclease that can cleave cellular DNA or a nuclease that cuts at a specific site in 16S rRNA and inactivates ribosomes. In addition, bacteriocins may inhibit transcription, translation, DNA replication and peptidoglycan synthesis (Barefoot *et al.*, 1992; Shand *et al.*, 1999; Prescott *et al.*, 1993). The bacteriocins or bacteriocin-like agents of gram-positive bacteria are quite different from the colicin and some even have commercial value. For example, bacteriocin Nisin A produced by the lactic acid bacteria strongly inhibits the growth of a wide range of gram-positive bacteria and used as a preservative in food industry (Madigan and Martinko, 2006).

As in domain *Bacteria,* extremely halophilic Archaeal strains in domain *Archaea* produce antimicrobial substances called as halocins to kill or inhibit other haloarchaeons. Researchers have explained that halocin production was a near-universal feature of haloarchaeal rods and, based on antagonism studies, hundreds of different types have been found to exist (Price and Shand, 2000). Halocins always reduce competition among haloarchaeal strains. Morever, proteinaceous antimicrobials that can lyse competitors enrich the environment for the producer (Shand *et al*.,1999; Kis-Papo and Oren, 1999). While it is known a lot of halocin producer strains, a few of them were characterized at the protein level (halocin H4, H6 and R1). Only halocin H4 has been characterized at both the gene and mRNA transcript levels. Halocin H6 which is produced from *Haloferax gibbonsii* Ma 2.39 is a $Na^+/H^+$ antiporter inhibitor in other haloarchaeans (Meseguer *et al*., 1991; Cheung *et al*., 1997; Price and Shand, 2000). In mammal $Na^+/H^+$ antiporter inhibitors protect the myocardium against ischemia and reperfusion injury. Halocin H7 treatment protect the myocardium against deleterious effect of ischemia and reperfusion by decreasing infarct size and the number of ectopic beats. This finding has implications for reducing injury during organ transplantation (Meseguer *et al*., 1995; Alberola *et al*., 1998; O'Connor and Shand 2002).

It is known that source of proteolytic and lipolytic halophilic bacterial contamination on brine cured hides is the salt used in brine curing of hide (Lloyd *et al*.,1929; Kallenberger, 1984; Birbir *et al*., 2002; Birbir and Sesal, 2003; Birbir, 2004). Presence of proteolytic and lipolytic halophilic strains in salt affect hide quality adversely. Therefore, the number of extremely halophilic strains in Tuz Lake, Kaldırım and Kayacık Salterns of Tuz Lake and Tuzköy Salt Mine and protease and lipase activities of these isolated strains were examined in our previous study. The salt taken from the Tuz Lake, Kaldırım Saltern, Kayacık Saltern and Tuzköy Salt Mine contained $10^4$-$10^7$, $10^5$-$10^7$, $10^5$,$10^5$-$10^6$ colony- forming units (c.f.u.) of

extremely halophilic bacteria per gram, respectively. The brines taken from Tuz Lake and Kaldırım Saltern contained $10^3$-$10^5$ and $10^5$ c.f.u of extremely halophilic bacteria per ml, respectively. Fifty, twenty, nine and fifteen halophilic bacteria were isolated from Tuz Lake, Kaldırım Saltern, Kayacık Saltern and Tuzköy Salt Mine, respectively. 80% and 90% of Tuz Lake strains, 70% and 35% of Kaldırım Saltern strains, 78% and 33% of Kayacık Saltern strains, 67% and 100% of Tuzköy Salt Mine strains showed proteolytic and lipolytic activities. It was indicated that high numbers of proteolytic extremely halophilic strains were obtained in all salt samples collected from different locations. The numbers of lipolytic strains isolated from Tuz Lake and Tuzköy Salt Mine were higher than lipolytic strains of Kaldırım and Kayacık Salterns (Birbir, 2004).

Considerable attemps have been made to use bactericides during brine curing of hides (Vivian, 1969; Hendry *et al*., 1971; Birbir and Bailey, 2000). Effective bactericides have been recommended (Birbir and Bailey, 2000; Weiss and Thornton, 1984; Lollar and Kallenberger, 1986) but in recent years, the use of bactericides have been questioned due to different factors such as toxicity of bactericides and bacterial resistance to the bactericides on repeated use.

Halobacterial growth on hides may be prevented by using natural antimicrobial compounds such as halocins produced by extreme halophiles. In our previous research it was found that gelatinase negative halocin producers inhibited gelatinase positive strains. Therefore, it was suggested that these gelatinase negative halocin producers or their isolated halocins might be used in controlling the proteolytic halobacterial damage that can occur during brine curing of hides (Birbir *et al*., 2004) Although halocin production is widespread among *Halobactericaea* (O'Connor and Shand, 2002) there is no available information whether or not halocin producer strains may be used to control the growth of lipolytic halobacteria found in crude salt.

Hence, a microbiological study was conducted to find the most effective lipase negative halocin producers in Kayacık and Kaldırım Salterns in Turkey and to determine the lipase negative halocin producers which can be used in inhibiting lipolytic strains in brine solution.

## MATERIAL AND METHODS

### Extremely Halophilic Strains, Media, and Growth Conditions

The extremely halophilic strains used in this research were isolated from Kaldırım and Kayacık Salterns of Tuz Lake, Turkey in our previous study (Birbir *et al*., 2001). These strains were grown aerobically at 40°C in liquid Brown medium containing (per liter): yeast extract, 5 g; tri-Na-citrate, 3 g; $MgSO_4.7H_2O$, 20 g; KCl, 2 g; NaCl, 250 g in a orbital shaking incubater (Edmund Bühler, Germany) at 100 rpm until the cells reach stationary phase (Birbir *et al.*, 2004).

### Lipase Activity

Lipase activity of each test strain was determined on modified Brown agar medium containing (per liter): yeast extract, 5 g; tri-Na-citrate, 3 g; $MgSO_4.7H_2O$, 20 g; KCl, 2 g; NaCl, 250 g; $CaCl_2.2H_20$, 1 g; Tween 80 (v/v), 1 ml. Each strain was streaked onto surface of the Brown agar medium and incubated at $40^oC$ for 30 days. After incubation period,

presence of halos around the colonies were interpreted as positive lipase activity (Norris and Ribbons, 1971; Gonzales *et al.*, 1978).

**Halocin Activity**

The strains were screened for halocin production against each other. 10µl aliquots of liquid culture from the 25 extremely halophilic strains were spotted onto top-agar lawns of the other halophilic strains. The plates were incubated and inspected for the presence of zones of inhibition in the indicator lawn. Uninoculated media (10 µl) used to grow each strain were also spotted onto plates as a control. The plates were incubated at 40$^{o}$C for 7 days. The presence of zone of inhibition on double layer agar plates was used as indicator for halocin production (Shand *et al*., 1999; Price and Shand, 2000; O'Connor and Shand, 2002).

**RESULTS AND DISCUSSION**

Salt lakes and crude solar salt produced from them contain large numbers extremely halophilic Archaea of the family *Halobacteriaceae* and unprocessed solar salt often contains $10^5$-$10^6$ c.f.u. per gram. Members of the *Halobacteriaceae* are the dominant microorganisms in hypersaline environments worldwide, including salt lakes, crystallizer ponds of solar salterns, salt mines, as well as hypersaline soda lakes (Grant *et al*., 1998; Oren, 2002). Salt extracted from these hypersaline environments are used for brine curing of hides in all over the world. Halophilic bacterial population and especially the presence of high number lipolytic extreme halophiles in these environments directly effect hide quality.

When crude salt obtained from natural salt sources is directly used in brine curing of hides, lipolytic extreme halophiles in the salt will give damage of hide thus lowering the value of leather and representing a huge annual loss to world tanning industry. Conservation defects on hide and skins affect the fixing properties of dyestuff to leather substract and accordingly, even dyeing and homogeneity of the parties in a negative way. Microorganisms attacks, that occur in hides and skins especially during conservation, storing and transportation, cause undyed areas or light stains on the leather grain. Depending on the damage caused by microorganisms with proteolytic and lipolytic effect on the flesh side of skins, less dyestuffs may be fixed during dyeing process and this state may be observed as light stains on the suede surface (Eitel, 1987; Bitlisli *et al*., 2004). Therefore, before using the salt in hide preservation, extremely halophilic microbial content of the salt and lipolytic activity of the strains in the salt should be controlled to prevent hide damage. It is possible to reduce extreme halophile numbers in salt by kilning or treating the salt with effective bactericides (Birbir and Bailey, 2000). The control of bacterial population by natural antimicrobial substances such as chitin or chitosan in textile products is today an important alternative to toxic chemicals. Halocins are natural proteinaceous antimicrobials which were first discovered by Francisco Rodriguez-Valera and *et al*., in 1982 (Rodriguez -Valera *et al.,* 1982). The main reason for the existence of halocins has always been that they reduce competition by lysing competitors and enrich the environment for the producer strains (Rodriguez -Valera *et al.,* 1982). In that research, 40 extreme halophiles were screened against each other for production of halocins; seven of 40 were found to produce effective halocin against other 39 halobacterial strains. Five of the 7 producers inhibited a large number (19 to 35) of the 40 strains, while the remaining two inhibited only a few (1 to 3) (Rodriguez -Valera *et al.,* 1982).

In another halocin study conducted by Torreblance (Torreblance *et al*., 1994), 147 extremely halophilic strains were screened against each other for production of halocins; 144 of the 147 were found to produce halocins and 20 of the 144 were sensitive to their own halocin and none of the isolates was completely insensitive to all halocin. The results of Torreblance's study showed that some of the strains inhibited nearly all the strains and the others inhibited only a few. None of the strains was completely insensitive to all halocins. Both studies indicated that there were numerous classes or groups of halocins and the halocin production was a pratically universal feature of haloarchaea (Rodriguez -Valera *et al.*, 1982; Torreblance *et al*., 1994).

In our previous study, the number of halocin producers were lowest in Tuz Lake (37%) and highest in Kaldırım Saltern (89%). Raising the temperature increases the bacterial population and, as a consequence, the level of nutrients in the restricted area decreases. Thus, survivors will compete with each other by releasing halocins. This may explain the dominance of halocin producers as competitors in Kaldırım Saltern (Birbir *et al*., 2004).

Most of halocin producers were pleomorphic cells. All the strains grew at 10, 15 and 25% NaCl but optimum growth occurred at 25% NaCl at 40$^{o}$C and a pH of 7.5. In accordance with cell morphologies and salt requirements reported in previous studies, the isolated strains were certainly defined as extremely halophilic microorganisms (Grant *et al.,*1998).

As susceptibility toward antibiotics may serve as a guideline for grouping microorganisms as *Archaea* or *Bacteria*, tests with various antibiotics were used to distinguish Archael and Bacterial strains. Results revealed that most of halocin producers were resistant to antibiotics that inhibit the growth of halophilic bacteria. In other words, they all belong to *Archaea* domain. Since only one extremely halophilic *Archaea* family was defined in Bergey's Manual, all Archaeal strains were placed in family *Halobactericeae* (Grant *et al.,*1998).

In our previous research, although gelatinase negative and gelatinase positive halocin producers were found at the same number in Kayacık Saltern, gelatinase positive halocin producers were common in Tuz Lake, Kaldırım Saltern and Tuzkoy Salt Mine (Birbir *et al.*, 2004). 1KYS1 strain was found to be the most effective strain in inhibiting all gelatinase positive strains of Kayacık Saltern and some of the other gelatinase positive strains which could not be inhibited by Tuz Lake and Tuzköy Salt Mine strains. It was suggested that gelatinase negative halocin producers or their halocin extracts might be used in preventing the proteolytic halobacterial damage that can occur during burine curing of hides (Birbir *et al.*, 2004).

In this research, sixteen of 18 strains produced halocin against each other (Table I). Three of the sixteen producers inhibited a large number (9 to 13) of the 18 strains, while the remaining thirteen inhibited only a few (1 to 7). Seven strains (6KS1, 1KS3, 2KS3, 3KS4, 5KS4, 6KS4, 7KS4) were lipase positive (Table I). Strain 3KS4 produced halosin against 13 of the 18 strains but this strain was lipase positive. Lipase negative 1KS1 strain produced halosin against 11 of the 18 strains, and 5 (6KS1, 2KS3, 5KS4, 6KS4 and 7KS4) of these 11 strains were lipase positive. Lipase negative 2KS1, 2KS4 and 3KS1 strains produced halosin against 7, 6 and 5 strains, respectively. Lipase negative six strains (4KS1, 6KS1, 1KS2, 3KS2, 1KS4 and 4KS4) were found effective against a few number of (1-4 strains) the 18 strains. Halocin produced from 3KS4 which was lipase positive was found to be effective against lipase positive 1KS3 strain. Strain 2 KS1 was found to be effective against lipase positive 3KS4 strain. Our research results showed that lipase positive 7 extremely halophilic strains were inhibited by halocin produced from lipase negative strains in Kaldırım saltern.

Three (1KYS1, 2KYS1, 4KYS1) of 7 Kayacık Saltern strains were found as lipase positive. Two of 7 produced halocin against each other (Table II). Lipase negative 3KYS1

strain produced halocin against five strains. Strain 3KYS1 could be used to inhibit all of lipase positive strains in Kayacık Saltern.

**Table I**

Lipase activity and sensitivities of Kaldırım Saltern strains against Kaldırım Saltern halocin producers

**Halocin producers**

| Kaldırım Saltern Strains | Lipase Activity | 1KS1 | 2KS1 | 3KS1 | 4KS1 | 6KS1 | 7KS1 | 1KS2 | 3KS2 | 1KS3 | 2KS3 | 1KS4 | 2KS4 | 3KS4 | 4KS4 | 5KS4 | 7KS4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1KS1 | - | - | + | + | + | - | - | - | - | + | + | - | - | + | - | + | + |
| 2KS1 | - | + | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - |
| 3KS1 | - | + | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - |
| 4KS1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 6KS1 | + | + | - | + | - | - | + | - | + | + | - | - | - | - | + | - | - |
| 7KS1 | - | - | + | + | - | - | - | - | + | + | + | - | + | + | + | - | - |
| 1KS2 | - | + | + | - | + | - | - | - | - | + | + | - | + | + | - | - | - |
| 3KS2 | - | - | + | - | - | + | - | - | - | + | + | + | + | + | + | - | - |
| 1KS3 | + | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - |
| 2KS3 | + | + | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - |
| 3KS3 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 1KS4 | - | + | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - |
| 2KS4 | - | + | - | - | - | - | - | + | - | - | - | - | - | + | - | - | - |
| 3KS4 | + | - | + | - | - | - | - | - | - | + | + | - | + | + | - | - | - |
| 4KS4 | - | + | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - |
| 5KS4 | + | + | + | + | - | + | - | - | + | + | - | + | + | - | + | - | - |
| 6KS4 | + | + | - | - | - | - | - | - | - | + | - | - | + | - | - | - | - |
| 7KS4 | + | + | + | + | - | - | + | - | + | + | + | - | - | + | - | + | - |

**Table II**

Lipase activity and sensitivities of Kayacık Saltern strains against Kayacık Saltern halocin producers

**Halocin producers**

| Kayacık Saltern strains | Lipase Activity | 1KYS1 | 3KYS1 |
|---|---|---|---|
| 1KYS1 | + | - | + |
| 2KYS1 | + | + | + |
| 3KYS1 | - | + | - |
| 4KYS1 | + | + | + |
| 2KYS2 | - | + | + |
| 3KYS2 | - | - | - |
| 4KYS2 | - | + | + |

As conclusion, it was found that lipase negative halocin producers may inhibit lipase positive strains. Especially these strains or their concentrated halocins may be used in controlling the extremely halophilic bacterial damage that can occur during brine curing of hides.

## ACKNOWLEDGEMENTS

We thank the Scientific Research Project Commision for supporting this research. We also thank Professor Richard F. Shand, Miss Lejla Pasic, Dr. Manuel Sanchez, Professor Rashid Anwar and Miss Hüsniye Hacıoğlu for their helpful guidance.

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