

Isolation and Identification of Moderately Halophilic Bacteria from Soak Liquor Samples Collected of Leather Tanneries

by

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Abstract

Isolation and identification of protease and lipase producing moderately halophilic bacteria from soak liquor samples and studying their adverse effects to the sheepskin using scanning electron microscopy may provide critical data on decomposition of raw hide/skin materials during soaking process. Moreover, enzyme-production properties of the moderately halophilic isolates (such as catalase, oxidase, lipase, protease, urease, caseinase, amylase, cellulase, pullulanase, xylanase) were determined. The effects of different NaCl concentrations, pH and temperature values on the growth of moderately halophilic bacterial isolates were tested. In the present study, four moderately halophilic bacterial isolates were isolated and selected for further experiments. The isolated species designated as SLMHB5, SLMHB10, SLMHB12, SLMHB13 were similar to *Vibrio alginolyticus*, *Terribacillus halophilus*, *Vibrio alginolyticus*, and *Vibrio parahaemolyticus* species, respectively. Scanning electron micrographs of sheepskin samples demonstrated that enzymatic activities of moderately halophilic bacteria isolated from soak liquor samples which decomposed the skin structure. After 35-days storage period, the sheepskin sample showed bad odor, sticky appearance and hair slip. Hence, it is recommended to control these microorganisms during the soaking process with an effective antimicrobial agent.

Introduction

The leather industry is among the oldest traditional industries and it has a very important role in the world economy. The hides and skins, that are byproducts of the food industry, are processed and converted into valuable leather products in the leather industry. Fresh hides and skins are ideal growth environments for a wide variety of non-halophilic bacteria and yeasts originating from the animal itself and environmental sources.¹⁻³ If the fresh hides and skins are not preserved immediately, the enzymatic activities of these microorganisms may cause the deterioration of hides and skins. Traditionally, the hides and skins have been cured with salt or brine to remove water from the structure of hides and skins. However, the salt curing preservation process also causes contamination of hides and skins with moderately halophilic bacteria, halotolerant

bacteria, extremely halophilic archaea, and haloversatile bacteria in the leather industry.³⁻⁸ If the curing process is not accomplished adequately, metabolic activities of these microorganisms may cause bad odor, hair slip, and decomposition of hides and skins during storage and transportation periods which may decrease final leather quality.

The soaking process is the first stage in leather processing to remove preservation salt, non-collagenous proteins, and glycosaminoglycans. This process also cleans hides and skins from blood, manure, and dirt. In this process, salted hides and skins are rehydrated and collagen fibers return to the original hydrated structure by reabsorbing water.⁹ Various researchers have found large numbers of microorganisms in soaked hides and soak liquors in the leather industry.^{1,5,7,10-17} The microorganisms found on salted hide may grow in the soaking process.

Although moderately halophilic bacteria were reported on salted hides and skins, there are a limited number of studies related to moderately halophilic bacteria found in soak liquor.¹⁸ In one of those papers, motile and aerobic moderately halophilic bacteria were isolated from drained soak liquor.¹⁸ The researchers reported that total counts of moderately halophilic bacteria were detected as 2.3×10^5 CFU/mL- 7.9×10^5 CFU/mL in the soak liquor samples.¹⁸ In another preliminary study, total moderately halophilic bacterial counts, total proteolytic moderately halophilic bacteria, and total lipolytic moderately halophilic bacteria were investigated in twelve soak liquor samples collected from Tuzla Leather Organized Region (Tuzla, İstanbul/Türkiye).¹⁹ The researchers reported that total moderately halophilic bacterial counts and proteolytic moderately halophilic counts were found as 1.8×10^4 - 4×10^6 CFU/mL and 1.2×10^4 - 1.4×10^6 CFU/mL, respectively. Lipolytic moderately halophilic bacterial counts were detected as 1×10^4 - 2×10^6 CFU/mL on Tween80 Agar Medium and 1×10^3 - 5.1×10^5 CFU/mL on Rhodamine B-Olive Oil Agar Medium, respectively.¹⁹

Vibrio alginolyticus, *Terribacillus halophilus*, and *Vibrio parahaemolyticus*, which are the test isolates in the present study, have also been isolated from different samples by other researchers. For instance, *Vibrio alginolyticus* was reported as a halophilic bacteria causing various diseases in marine animals such as fish,

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crustaceans and molluscs.^{20,21} Furthermore, *Vibrio alginolyticus* has been isolated from patients, brine shrimp, skin and sea water.²²⁻²⁴ The species can cause enteric diseases in humans and animals.²⁵ It is among the important opportunistic bacterial pathogens.²⁶ *Vibrio alginolyticus* was reported as a naturally occurring organism in seafood, estuarine water and coastal waters²⁵ and a reservoir of virulence genes in aquatic environments.²⁷ It was also isolated from seawater samples,²⁸ the primary organism from moribund clam larvae,²⁰ diseased aquatic animals,²⁷ and saltpan soil samples.²⁹ The researchers stated that *Vibrio alginolyticus* isolates were able to produce lipase, caseinase, amylase, lecithinase and protease enzymes.^{27,28} *Vibrio alginolyticus* isolates were able to grow at between pH 7.5-9, 10-40°C.²⁵

Essghaier and colleagues used moderately halophilic *Terribacillus halophilus* isolate to improve the growth of tomatoes.³⁰ The intracellular antifungal enzyme (chitinase) produced by *Terribacillus halophilus* caused the reduction of spore germination of *Botrytis cinerea* and the treated tomato with moderately halophilic *Terribacillus halophilus* isolate could grow.³⁰ The intracellular enzymes of moderately halophilic bacteria can be used in biotechnological applications.^{30,31} Pathogenic *Terribacillus halophilus* strains which are resistant to different disinfectants such as benzalkonium chloride, sodium hypochlorite and chloroxylenol were isolated from foods and surfaces of kitchen.³² Some of these chemicals have been used in the food industry as well as in the leather industry. Microorganisms may develop resistance against chemicals due to the misuse of these and other antimicrobial agents. Endospore-forming *Terribacillus halophilus* has been isolated from soil and saltpan samples.^{29,33} The investigators reported that *Terribacillus halophilus* showed growth at between pH 5-10, were motile and oxidase negative.³³ *Vibrio alginolyticus* and *Terribacillus halophilus* strains which were isolated from the saltpan soil samples collected from Vedaranyam were reported as protease and amylase producers.²⁹

Vibrio parahaemolyticus was reported as a human pathogen found in marine environments.³⁴ It is known as an important seafood-borne pathogen causing vomiting, abdominal cramps, headache and diarrhea.³⁵ Urease-positive *Vibrio parahaemolyticus* was isolated from frozen sea foods, patients and sea water samples.³⁶ *Vibrio parahaemolyticus* was isolated from raw seafoods³⁴ and oyster samples.³⁷ In another study, the *Vibrio parahaemolyticus* strains isolated from oyster samples were shown to produce protease, lipase, caseinase and amylase.³⁷

Due to the global economic importance of leather and related goods, determination of moderately halophilic bacteria in soak liquor is very important for leather industry to prevent their adverse effects. Consequently, in this study it was aimed to isolate and identify moderately halophilic bacteria from soak

liquor samples. The effects of different NaCl concentrations, pH and temperature values on the growth of moderately halophilic bacterial isolates were tested. Enzyme-production properties of the isolates were also investigated. Moreover, the adverse effect of protease and lipase producing moderately halophilic bacteria on the sheepskin samples were also assessed using scanning electron microscope.

Experimental

Soak Liquor Samples

Four soak liquor samples were obtained from Tuzla Leather Organized Region (Tuzla, Istanbul/Türkiye) and placed into sterile bottles. The soak liquor samples were immediately brought to the laboratory on ice.

Isolation of Moderately Halophilic Bacteria from Soak Liquors

Twenty mL of soak liquor samples were put into glass bottle containing 180 mL of Sterile Physiological Saline Solution (SPSS) (10% sodium chloride) and the glass bottles were placed in an orbital shaker at 150 rpm for two hours at 24°C. 100 µL of direct and serial dilutions (10^{-1} - 10^{-6}) of soak liquor solutions were spread onto the surface of the Complex Agar Medium (CAM) plates containing 5% yeast extract.^{38,39} Finally, the salt concentration of test media was adjusted to 10% (w/v) with the following ingredients (SW10, saline water): 0.7% (w/v) MgCl₂, 8.1% (w/v) NaCl, 0.96% (w/v) MgSO₄, 0.2% (w/v) KCl, 0.036% (w/v) CaCl₂, 0.006% (w/v) NaHCO₃, and 0.0026% (w/v) NaBr.³⁸ The CAM plates were incubated at 37°C for 24 hours. After the incubation, different bacterial colonies were selected according to their colony properties. The selected bacterial colonies were streaked again to obtain pure isolates. The pure cultures were phenotypically and genotypically analyzed.

Molecular Characterization of Moderately Halophilic Isolates

The genomic DNA extraction was performed according to the QIAamp DNA MiniKit (Qiagen, Hilden/Germany). 16S rRNA gene was amplified with the universal primers 16F27-16R1488. The reactions of Polymerase Chain Reaction (PCR) amplification (95°C for 5 min, 25 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, final 10 min extension at 72°C) contained forward primer (2.5 µL), reverse primer (2.5 µL), PCR buffer (5 µL), dNTPs (10 mM, 8 µL), MgCl₂ (25 mM, 2.5 µL), Taq DNA Polymerase (0.5 µL), template DNA (1 µL), distilled water (28 µL), in a final volume of 50 µL.⁴⁰ The PCR products were purified using QIAquick PCR Purification Kit. The 16S rRNA gene sequence analysis were performed by Macrogen Inc. (Seoul, Korea). The comparison of 16S rRNA gene sequencing among the isolates and closely related species were determined further using ChromasPro (South Brisbane, Australia) and EzTaxon-e tool (Seoul, Korea).⁴¹

GenBank Accession Number

16S rRNA sequence data of the isolates SLMHB5, SLMHB10, SLMHB12, SLMHB13 reported in this paper, have been deposited in NCBI and GenBank nucleotide sequence database under the respective accession numbers: OP435723; OP435724; OP435725 and OP435727.

Cell Morphology and Pigmentation of Moderately Halophilic Isolates

The cell morphology and pigmentation of overnight cultures of each test isolate were investigated under optimal conditions. Gram staining was applied to each test isolate according to standard procedures.⁴²

Effects of NaCl, pH and Temperature on Moderately Halophilic Bacterial Growth

Effect of different NaCl concentrations and optimum NaCl requirement of moderately halophilic bacteria were investigated on both CAM plates without salt and CAM plates containing different salt concentrations (0%, 1%, 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, 22.5%, 25%, 27.5%, 30%). Effect of different pH values and optimum pH requirement were tested on CAM plates containing 10% NaCl at different pH values (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, 13.0). Moreover, the effect of different temperature values such as (4°C, 10°C, 20°C, 25°C, 30°C, 37°C, 40°C, 45°C, 50°C, 55°C, 60°C).⁴²

Enzymatic Activities of Moderately Halophilic Isolates

Catalase activity

Catalase activity was tested by adding 3% H₂O₂ onto the bacterial colonies grown on CAM. The formation of bubbles on the bacterial colony was accepted as a positive catalase activity.⁴²

Oxidase activity

Oxidase activity was investigated by spreading the colony of test isolate with a sterile loop onto the filter paper dropped with oxidase reagent. Immediate color change from pink to dark purple was accepted as positive oxidase activity.⁴²

Lipase activity

Lipase activity was examined on the Tween80 Agar Medium (TW80M) containing Tween80 (1%, w/v). The inoculated agar plates were incubated for 48 hours at 37°C in an incubator. After incubation, the presence of opaque zones around the colonies on TW80M was taken as evidence of lipase activity.⁴³

Protease activity

Protease activity was determined using Gelatine Agar Medium (GAM) containing gelatine (2%, w/v). The inoculated agar plates were incubated for 48 hours at 37°C in an incubator. After incubation, the plates containing GAM were flooded with Frazier solution. Clear

zones around the colonies were accepted as an evidence of positive protease activity.⁴³

Urease activity

Urease activity was examined on Christensen Urea Agar. After growth was obtained, the test tubes were checked for red or pink color.⁴⁴

Caseinase activity

Caseinase production was tested on the Plate Count Agar medium containing 2% skim milk. After incubation, the clear zones around the bacterial colonies were accepted as positive activity of caseinase enzyme.⁴⁴

Amylase activity

Amylase activity was tested on CAM prepared with 0.5% (w/v) starch. The incubated plates were flooded with iodine. Clear halos around the colonies accepted as positive amylase activity.⁴³

Cellulase activity

Cellulase activity was tested on the cellulose agar medium prepared with 2 g carboxymethyl cellulose, 1 g casamino acid, 1 g yeast extract, 20 g agar and 1000 mL sterile saline water (10%).⁴⁴⁻⁴⁶ The inoculated plates were incubated at 37°C for 24 hours.⁴⁴⁻⁴⁶ After the incubation period, Congo Red (0.1%) solution was flooded on the bacterial colonies. Then, the plates were left for 30 minutes. After 30 minutes, the colonies on the plates were washed with 1 M NaCl solution. After the washing process, the clear zones around the bacterial colonies were accepted as positive cellulase activity.

Pullulanase and xylanase activities

Pullulanase and xylanase activities of the test isolates were examined on the plates containing the substrates azurine-cross-linked (AZCL)-pullulan and AZCL-xylan, respectively. Transparent zones observed around the colonies on xylan and pullulan media were accepted as positive xylanase and pullulanase activities.⁴³

Curing Skins with Enzyme Producing Moderately Halophilic Isolates and Storage Period

The damage caused by the mixed culture of enzyme producing moderately halophilic bacterial isolates (*Vibrio alginolyticus*, *Terribacillus halophilus*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*) isolated from four soak liquor samples was investigated. Two pieces of freshly butchered sheep skin sample were obtained from the tannery. Thirty mL of brine solution (20% NaCl) and 10 g sheep skin pieces were mixed in two flasks. The sheep skin sample treated with only sterile brine solution was prepared as control. In sterile physiological saline solution (20% NaCl), each enzyme-producing isolates was suspended and this bacterial suspension adjusted to 0.5 McFarland turbidity standard (10⁸ CFU/mL). Ten mL of each bacterial suspension was added

into the flask including brine solution and sheep skin sample. The flasks were shaken at 70 rpm at 24°C for 18 hours. Then, the cured sheep skin samples were taken from the flasks and stored at room temperature for 35 days. After the storage period, the samples were examined under the scanning electron microscope.¹⁵ In addition, the organoleptic features (bad odor, sticky appearance, hair slip) were observed on the cured sheep skin samples during 35 days.

Preparation of Stored Skin Samples for Scanning Electron Microscope

Glutaraldehyde solution (4%) prepared with phosphate buffer (0.1 M, pH 7.2) was applied to sheep skin samples for 30 minutes. The sheep skin samples were washed three times for 10 min with the phosphate buffer (0.1 M). Osmium tetroxide (1%) prepared in phosphate buffer (0.1M) was applied to the sheep skins for one hour at 24°C. The samples were washed two times in sterile distilled water for 15 min and 95%, 75%, 50%, 35%, and absolute ethanol.

The mixture of the ethanol-HMDS [1:2 (v/v)] (1×30 min), ethanol-hexamethyldisilazane (HMDS) [1:1 (v/v)] (1×30 min) and HMDS (2×30 min) were used during the air drying process. The sheepskin samples were put in a desiccator for 14 h. Then, the sheepskin samples were observed by Scanning Electron Microscope (Fei Quanta 450 FEG ESEM SEM, Model FEG 450).⁴⁷

Results and Discussion

In the present study, three different moderately halophilic bacterial species -*Vibrio alginolyticus* (two isolates), *Vibrio parahaemolyticus* (one isolate), and *Terribacillus halophilus* (one isolate)- were isolated and identified from four soak liquor samples obtained from Tuzla Leather Organized Region (Tuzla, Istanbul/Türkiye) (Table I). All test isolates showed yellow pigmentation on the Complex Agar Medium. *Vibrio alginolyticus* and *Vibrio parahaemolyticus* were Gram-negative, *Terribacillus halophilus* was Gram-positive (Table

Table I
Phenotypic characteristics of the moderately halophilic bacterial isolates

Characteristics	<i>Vibrio alginolyticus</i>	<i>Terribacillus halophilus</i>	<i>Vibrio alginolyticus</i>	<i>Vibrio parahaemolyticus</i>
Isolate code	SLMHB5	SLMHB10	SLMHB12	SLMHB13
Pigmentation	yellow	yellow	yellow	yellow
Gram staining	-	+	-	-
Cell morphology	curved-rod	rod	curved-rod	curved-rod
NaCl range (%)	3-15	3-15	3-15	3-20
Optimum NaCl (%)	10	10	10	10
Temperature range (°C)	20-40	20-40	20-40	20-45
Optimum temperature (°C)	37	37	37	37
pH range	6-9	6-9	6-9	5-9
Optimum pH	7.0	7.0	7.0	7.0
Endospore formation	-	+	-	-
Motility	+	-	+	+
Production of indole	+	-	+	+
Citrate utilization	+	-	+	+
Hydrolytic Activities	Catalase	+	+	+
	Oxidase	+	-	+
	Lipase	+	+	+
	Protease	+	+	+
	Urease	+	+	+
	Caseinase	+	+	+
	Amylase	+	+	+
	Cellulase	-	-	-
	Pullulanase	-	-	-
	Xylanase	-	-	-

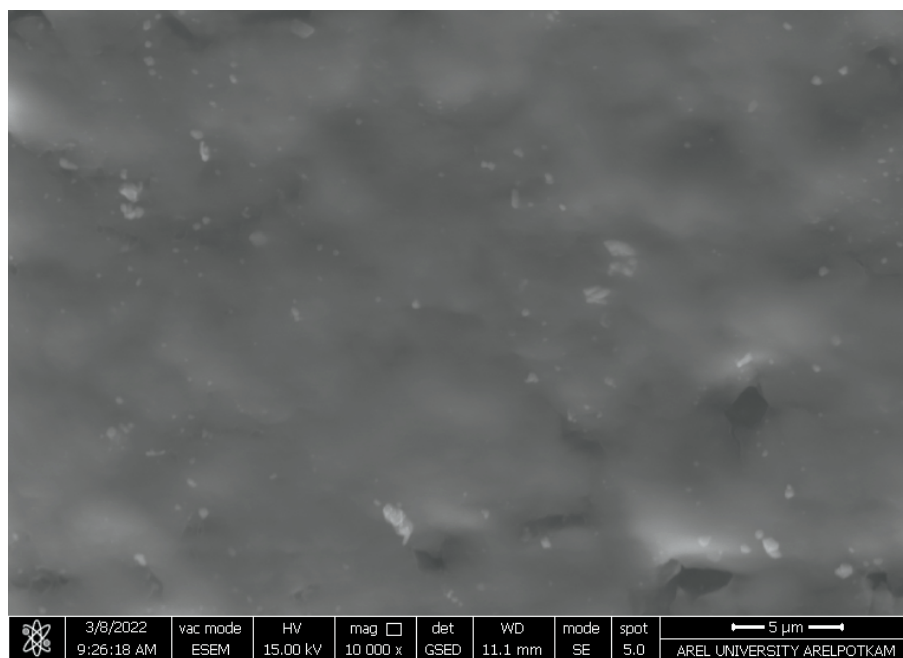


Figure 1. SEM micrographs of sheepskin sample treated with the sterile saline solution (control). The bar = 5 µm

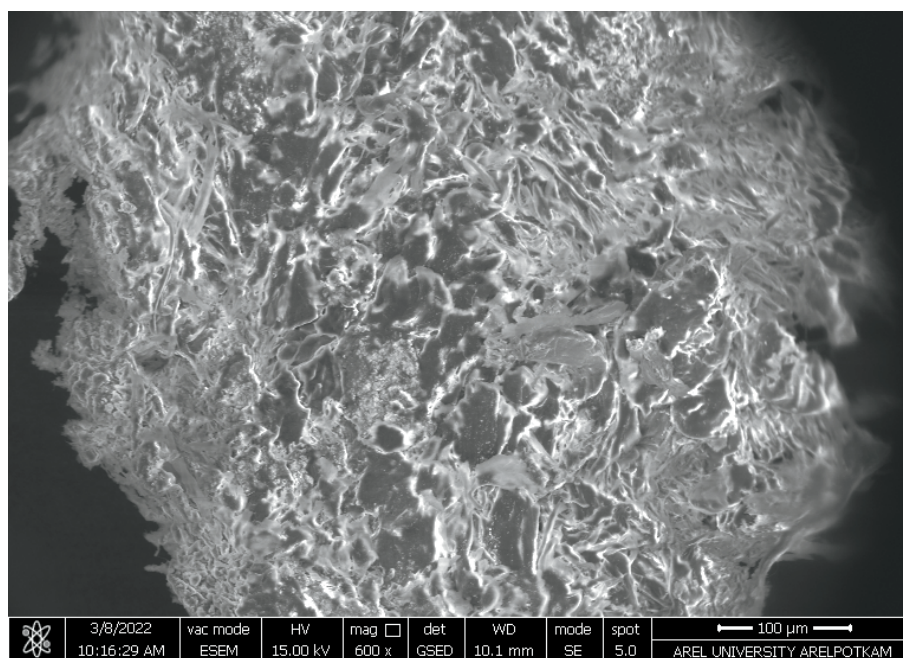


Figure 2. SEM micrographs of sheepskin sample treated with a mixed culture of moderately halophilic *Vibrio alginolyticus*, *Terribacillus halophilus*, and *Vibrio parahaemolyticus*. The bar = 100 µm

I). The cells of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* were curved-rod, the cells of *Terribacillus halophilus* was rod-shaped. All isolates showed growth at 3-15% NaCl, 20-40°C and pH 6-9, *Vibrio parahaemolyticus* also showed growth at 20% NaCl, 45°C and pH 5. All test bacteria were able to grow at 10% NaCl, pH 7, 37°C (Table I). Hence, these isolates were considered as moderately halophilic. *Terribacillus halophilus* exhibited endospore formation. All isolates were motile, produced indole, and utilized citrate except *Terribacillus halophilus*. While all isolates produced catalase, lipase, protease, urease, caseinase, amylase, they did not produce cellulase,

pullulanase and xylanase. Only *Terribacillus halophilus* was oxidase negative (Table I).

To examine adverse effect of lipase and protease producing moderately halophilic soak liquor isolates to the structure of sheepskin, sterile and freshly slaughtered sheep skin samples were cured with a mixed culture of test bacteria (*Vibrio alginolyticus*, *Terribacillus halophilus*, *Vibrio parahaemolyticus*) for 35 days at room temperature. As shown in Figure 1, the curing process of the sheepskins sample with the sterile saline solution protected the sheepskin structure against the

microbial damage during 35 days of storage period.

Figure 2 clearly demonstrates that the destructive effects of moderately halophilic bacterial cells of the mixed culture on the sheepskin structure. The mixed culture of the test isolate caused weakening fibers in the corium and splitting (Figure 2).

Changes in the organoleptic characteristics such as sticky appearance, hair slip, bad odor, and compactness of the sheepskin structure were also observed on the sheepskin sample treated with a mixed culture of moderately halophilic *Vibrio alginolyticus*, *Terribacillus halophilus*, and *Vibrio parahaemolyticus* (Figure 2). After the storage period, only the control sample (cured with sterile brine solution) did not show hair slip, bad odor and sticky appearance (Figure 1). In Figure 2, the compactness of collagen fibers of sheepskin sample was adversely affected by the enzymes produced by the test bacteria.

In a previous study, SEM micrographs showed that hides cured with protease producing extremely halophilic archaeal isolates caused red discoloration and grain damage after 49 days of storage time at 41°C.¹⁵ In the study conducted by Birbir and her colleagues (2020), proteolytic and lipolytic extremely halophilic archaea (*Haloarcula salaria* AT1, *Halobacterium salinarum* 22T6, *Haloarcula tradensis* 7T3) were isolated from deteriorated salted sheepskin samples having red discolorations.⁴⁸ In that study, the freshly slaughtered sheepskin samples were cured with each test strain (*Haloarcula salaria* AT1, *Halobacterium salinarum* 22T6, *Haloarcula tradensis* 7T3), and with their mixed culture for 47 days at 33°C.⁴⁸ The researchers reported that organoleptic changes were closely related to enzymatic activities of the microorganisms.⁴⁸ They also reported that electron micrographs of each test isolate and their mixed culture destroyed the skins' collagen fibers.⁴⁸ In the present study, the structural damage of the sheepskin was due to the proteolytic and lipolytic activities of moderately halophilic bacteria,

Conclusion

This is the first study that investigates moderately halophilic bacterial species (*Vibrio alginolyticus*, *Terribacillus halophilus*, and *Vibrio parahaemolyticus*) found in the soak liquor samples obtained from Tuzla Leather Organized Region. All isolates produced catalase, lipase, protease, urease, caseinase and amylase enzymes. SEM micrographs of sheepskin sample treated with the mixed culture of moderately halophilic bacterial isolates also showed that these enzyme-producing isolates damaged the structure of the raw sheepskin sample. The experimental data obtained from this study clearly showed that inadequate preservation during soaking process may cause serious quality defects on crude sheepskins. Hence, effective antimicrobial applications should be applied in the

soaking process to obtain high quality leather product in the leather industry.

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