



MARMARA ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ



**INVESTIGATION OF ANTIBIOTIC RESISTANCE, BACTERIOCIN AND
ENZYME PRODUCTION OF HALOVERSATILE BACTERIA IN SALT USED
FOR SKIN PRESERVATION, CONTROLLING WITH AN ANTIMICROBIAL
SUBSTANCE**

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ÖZET

DERİLERİN KORUNMASINDA KULLANILAN TUZDA BULUNAN HALOVERSATİL BAKTERİLERİN ANTİBİYOTİK DİRENÇLERİNİN, BAKTERİYOSİN VE ENZİM ÜRETİMLERİNİN ARAŞTIRILMASI, BİR ANTİMİKROBİYAL MADDE İLE KONTROLÜ

Bu çalışmada 25 adet tuz örneği Tuzla/İstanbul'da bulunan üç tane deri fabrikasından toplanmıştır. Tuz örneklerinin pH'ı 7.60 ve 8.04 arasında saptanmıştır. Toplam haloversatil bakteri sayısı, toplam proteolitik haloversatil bakteri sayısı ve toplam lipolitik haloversatil bakteri sayısı sırasıyla 6×10^3 - 1.3×10^5 KOB/g, 1×10^3 - 7×10^3 KOB/g, 2×10^3 - 3.1×10^4 KOB/g olarak bulunmuştur. Toplam 35 adet haloversatil bakteri [*Staphylococcus epidermidis* (6 izolat), *Staphylococcus warneri* (2 izolat), *Corynebacterium aurimucosum* (2 izolat), *Staphylococcus caprae* (2 izolat), ve sadece birer izolat *Staphylococcus pasteurii*, *Peribacillus frigoritolerans*, *Staphylococcus haemolyticus*, *Lysinibacillus telephonicus*, *Kocuria rosea*, *Paenibacillus illinoisensis*, *Kocuria Polarıs*, *Bhargavaea ginsengi*, *Bacillus haynesii*, *Bacillus sonorensis*, *Bacillus licheniformis*, *Pseudomonas oryzihabitans*, *Bacillus subtilis*, *Bacillus pıscıs*, *Staphylococcus hominis*, *Micrococcus yunnanensis*, *Staphylococcus saccharolyticus*, *Pseudomonas iranica*, *Micrococcus luteus*, *Staphylococcus capitis* subsp. *urealyticus*, *Corynebacterium minutissimum*, *Bacillus paralicheniformis*, *Staphylococcus taiwanensis* türleri] tuz örneklerinden izole edilmiştir ve 16S rRNA gen dizilemesi ile moleküler olarak tanımlanmıştır. Tüm izolatlar *Bacteria* Domainine ait olarak bulunmuştur. Sadece iki tane tür [*Pseudomonas oryzihabitans* (PSS14a) ve *Pseudomonas iranica* (PSS21a)] Gram-negatif iken, kalan diğer 33 izolat Gram-pozitifdir. Haloversatil türler üç şubede sınıflandırılmıştır: *Firmicutes*, *Actinomycetota* ve *Proteobacteria*. *Staphylococcus* sp. (16 izolat), *Bacillus* sp. (6 izolat), *Peribacillus* sp. (1 izolat), *Lysinibacillus* sp. (1 izolat), *Paenibacillus* sp. (1 izolat), ve *Bhargavaea* sp. (1 izolat) cinslerine ait izolatlar *Firmicutes* şubesinin üyeleridir. *Pseudomonas* cinsine ait iki izolat *Proteobacteria* şubesine ait olarak saptanmıştır. *Actinomycetota* şubesinde *Corynebacterium* sp. (3 izolat), *Kocuria* sp. (2 izolat), and *Micrococcus* sp. (2 izolat) cinslerine ait toplam yedi adet izolat bulunmuştur. Tüm izolatlara yeni erişim numaraları alınarak NCBI ve GenBank Nükleotid Veritabanlarına kaydedilmiştir. Haloversatil izolatların hidrolitik enzim üretme yetenekleri araştırılmıştır. Enzimatik sonuçlar estera, ürea, protea, kazeina, lipa,

ksilanaz, selülaz, amilaz, DNaz ve oksidaz enzimlerinin sırasıyla izolatların %40, %66, %77, %6, %17, %6, %17, %20, %20 ve %40'ü tarafından üretilmiştir. İzolatların tamamı katalaz üretirken, hiçbir izolatın pullulanaz üretmediği görülmüştür. Ayrıca, lipaz üretimi için optimum koşulların Tween 80 Besiyerinde pH 7-8 değerinde, 1%-3% NaCl konsantrasyonda, 24-96 saatlik inkübasyon süresi boyunca, 37°C-50°C sıcaklıkta 30 dakikalık su banyosunda bekletme olduğu saptanmıştır. Lipaz ve proteaz enzimlerinin miktarları Bradford Metodu ile belirlenmiştir. *Staphylococcus warneri* (PSS1a), *Staphylococcus pasteurii* (PSS1b), *Staphylococcus warneri* (PSS2a), *Paenibacillus illinoisensis* (PSS7a) ve *Bacillus subtilis* (PSS16a) tarafından üretilen enzim miktarları sırasıyla 15.5 µg/ml, 13.25 µg/ml, 12.75 µg/ml, 12.5 µg/ml ve 12 µg/ml olarak saptanmıştır. Proteaz enzim miktarı *Staphylococcus warneri* (PSS1a) için 3.25 µg/ml, *Staphylococcus epidermidis* (PSS3a) için 2.5 µg/ml, *Staphylococcus epidermidis* (PSS3b) için 2.25 µg/ml, *Peribacillus frigoritolerans* (PSS4a) için 1.75 µg/ml, *Bacillus haynesii* (PSS11b) için 1.75 µg/ml, *Bacillus subtilis* (PSS16a) için 1.5 µg/ml, *Micrococcus yunnanensis* (PSS18b) için 2 µg/ml, *Staphylococcus saccharolyticus* (PSS20a) için 2 µg/ml, *Staphylococcus epidermidis* (PSS24a) için 2.5 µg/ml olarak bulunmuştur. Haloversatil izolatlar farklı şeker ve amino asit kaynaklarını kullanmıştır. Sırasıyla dekstroz, fruktoz, riboz, sellobiyoz ve sukroz izolatların 91%, 94%, 77%, 77% ve 91%'i tarafından kullanılmıştır. İzolatların tümü L-tirozin, L-prolin ve L-valini kullanmıştır. İzolatların %9 ve %14'ü sırasıyla L-lizin ve L-arjinin kullanılmıştır. *Staphylococcus*, *Corynebacterium*, *Micrococcus*, *Kocuria* ve *Bhargavaea* cinslerine ait türler hareketsiz iken diğer türler hareketliydi. Hücre şekilleri basil, çomak veya pleomorfikti. Optimum gelişim tüm izolatlar için pH 7, 1.17% NaCl, ve 35°C sıcaklıkta tespit edilmiştir. *Corynebacterium aurimucosum* (PSS2b), *Bacillus haynesii* (PSS11b), *Staphylococcus epidermidis* (PSS3a) ve *Peribacillus frigoritolerans* (PSS4a) türleri yüksek bakteriyosin üretirken, *Bacillus subtilis* (PSS16a), *Micrococcus luteus* (PSS21b) ve *Paenibacillus illinoisensis* (PSS7a) izolatları bakteriyosin üretmedi. *Corynebacterium aurimucosum* (PSS2b)'a ait bakteriyosinden etkilenen *Bacillus pascis* (PSS17a) izolatının etrafında 9 mm'lik inhibisyon zonu oluşmuştur. En düşük inhibisyon zonu (4 mm) sırasıyla *Corynebacterium aurimucosum* (PSS2b), *Bacillus haynesii* (PSS11b), *Pseudomonas iranica* (PSS21a), *Pseudomonas iranica* (PSS21a) izolatlarının bakteriyosinlerinden etkilenen *Pseudomonas iranica* (PSS21a), *Staphylococcus warneri* (PSS1a), *Bacillus haynesii* (PSS11b) etrafında ölçülmüştür. Koyun ve keçi derileri proteaz-lipaz üreten *Staphylococcus warneri* (PSS1a), *Staphylococcus pasteurii* (PSS1b),

Staphylococcus warneri (PSS2a), *Paenibacillus illinoisensis* (PSS7a), *Bacillus licheniformis* (PSS13a), *Bacillus subtilis* (PSS16a); proteaz üreten *Staphylococcus epidermidis* (PSS3b), *Bhargavaea ginsengi* (PSS11a), *Bacillus sonorensis* (PSS12a), *Bacillus haynesii* (PSS11b), *Corynebacterium aurimucosum* (PSS15a) izolatların karışık kültürleri ile muamele edilerek 40 gün 24°C'de depolanmıştır. Koyun ve keçi derilerindeki zararlı etkiler taramalı elektron mikroskopunda incelenmiştir. Bu zararlı etkiler, kıl dökülmesi, gözenekler, delikler, liflerin kopması, deri yapısındaki morfolojik değişiklikler olarak saptanmıştır. Tüm izolatlar Rifampisin (RD5, 5 µg), Amikasin (AK30, 30 µg) ve Neomisin (N30, 30 µg) antibiyotiklerinden etkilenmiştir. Sodyum dimetilditiyokarbamet içeren antimikrobiyal maddenin 22 farklı konsantrasyonu (0.00175, 0.0035, 0.007, 0.014, 0.028, 0.06, 0.12, 0.24, 0.48, 0.96, 1.96, 3.92, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000, 2000, 4000 µg/ml) *Staphylococcus warneri* (PSS1a), *Staphylococcus pasteurii* (PSS1b), *Paenibacillus illinoisensis* (PSS7a), *Bacillus subtilis* (PSS16a), *Micrococcus luteus* (PSS21b), *Staphylococcus epidermidis* (PSS15b), *Bacillus sonorensis* (PSS12a), *Corynebacterium aurimucosum* (PSS15a), ve *Staphylococcus capitis* subsp. *urealyticus* (PSS22a) izolatlarına karşı uygulanmıştır. Tüm test bakterilerinin gelişimleri (*Staphylococcus warneri* isolate PSS1a dışında) 125 µg/ml-4000 µg/ml konsantrasyonlarında inhibe edilmiştir. *Staphylococcus warneri* (PSS1a), *Staphylococcus pasteurii* (PSS1b), *Paenibacillus illinoisensis* (PSS7a), *Bacillus subtilis* (PSS16a), *Micrococcus luteus* (PSS21b), *Staphylococcus epidermidis* (PSS15b), *Bacillus sonorensis* (PSS12a) izolatlarının MİK değerleri sırasıyla 250 µg/ml, 62.5 µg/ml, 15.6 µg/ml, 7.8 µg/ml, 125 µg/ml, 15.6 µg/ml, 62.5 µg/ml olarak bulunmuştur. *Corynebacterium aurimucosum* (PSS15a) ve *Staphylococcus capitis* subsp. *urealyticus* (PSS22a) izolatlarının MİK değeri 3.92 µg/ml olarak bulunmuştur. Test bakterileri arasında bu iki izolat en düşük konsantrasyonda inhibe olduğu halde, *Staphylococcus warneri* (PSS1a) bu antimikrobiyal maddeye en dirençli tür olarak saptanmıştır.

Ekim, 2023

HAIDER SABAH ABDULHUSEIN

ABSTRACT

INVESTIGATION OF ANTIBIOTIC RESISTANCE, BACTERIOCIN AND ENZYME PRODUCTION OF HALOVERSATILE BACTERIA IN SALT USED FOR SKIN PRESERVATION, CONTROLLING WITH AN ANTIMICROBIAL SUBSTANCE

In the present study, 25 salt samples were collected from three leather industries in Tuzla/Istanbul. The salt samples' pH values were determined as between 7.60 and 8.04. The total numbers of haloversatile bacteria, the total numbers of proteolytic haloversatile bacteria and the total numbers of lipolytic haloversatile bacteria were respectively found as 6×10^3 - 1.3×10^5 CFU/g, 1×10^3 - 7×10^3 CFU/g, 2×10^3 - 3.1×10^4 CFU/g. Totally 35 haloversatile bacterial [*Staphylococcus epidermidis* (6 isolates), *Staphylococcus warneri* (2 isolates), *Corynebacterium aurimucosum* (2 isolates), *Staphylococcus caprae* (2 isolates), and only one isolate of the species *Staphylococcus pasteurii*, *Peribacillus frigoritolerans*, *Staphylococcus haemolyticus*, *Lysinibacillus telephonicus*, *Kocuria rosea*, *Paenibacillus illinoisensis*, *Kocuria polaris*, *Bhargavaea ginsengi*, *Bacillus haynesii*, *Bacillus sonorensis*, *Bacillus licheniformis*, *Pseudomonas oryzae*, *Bacillus subtilis*, *Bacillus pascalis*, *Staphylococcus hominis*, *Micrococcus yunnanensis*, *Staphylococcus saccharolyticus*, *Pseudomonas iranica*, *Micrococcus luteus*, *Staphylococcus capitis* subsp. *urealyticus*, *Corynebacterium minutissimum*, *Bacillus paralicheniformis*, *Staphylococcus taiwanensis*] were isolated from salt samples and molecularly identified by Sanger dideoxy sequence methods and 16S rRNA gene sequencing. All isolates were belonged to the Domain *Bacteria*. While only two species [*Pseudomonas oryzae* (PSS14a) and *Pseudomonas iranica* (PSS21a)] were Gram-negative, the remaining 33 isolates were Gram-positive. The haloversatile species have distributed in three phyla: *Firmicutes*, *Actinomycetota*, and *Proteobacteria*. *Staphylococcus* sp. (16 isolates), *Bacillus* sp. (6 isolates), *Peribacillus* sp. (1 isolate), *Lysinibacillus* sp. (1 isolate), *Paenibacillus* sp. (1 isolate), and *Bhargavaea* sp. (1 isolate) were determined as the members of the Phylum *Firmicutes*. Two isolates belonging to the Genus *Pseudomonas* were found as the members of the Phylum *Proteobacteria*. The Phylum *Actinomycetota* contained seven isolates such as *Corynebacterium* sp. (3 isolates), *Kocuria* sp. (2 isolates), and *Micrococcus* sp. (2 isolates). All isolates have been recorded in the NCBI and GenBank Nucleotide Databases with accession numbers. The ability

of haloversatile isolates to produce hydrolytic enzymes was evaluated. The enzymatic results showed that esterase, urease, protease, caseinase, lipase, xylanase, cellulase, amylase, DNase, and oxidase were respectively produced by 40%, 66%, 77%, 6%, 17%, 6%, 17%, 20%, 20%, and 40% of the isolates. While all isolates produced catalase, none of the isolates produced pullulanase. In addition, the optimal conditions for lipase production were found as pH 7-8, 1%-3% NaCl concentrations, an incubation period at 24 hours to 96 hours on Tween 80 Agar Media, and 30 minutes in a water bath, temperature between 37 °C and 50°C. The amounts of lipase and protease enzymes was determined by Bradford Method. The amounts of lipase enzyme produced by *Staphylococcus warneri* (PSS1a), *Staphylococcus pasteurii* (PSS1b), *Staphylococcus warneri* (PSS2a), *Paenibacillus illinoisensis* (PSS7a), and *Bacillus subtilis* (PSS16a) were detected as 15.5 µg/ml, 13.25 µg/ml, 12.75 µg/ml, 12.5 µg/ml, and 12 µg/ml. The amounts of protease enzyme were determined for *Staphylococcus warneri* (PSS1a) as 3.25 µg/ml, for *Staphylococcus epidermidis* (PSS3a) as 2.5 µg/ml, for *Staphylococcus epidermidis* (PSS3b) as 2.25 µg/ml, for *Peribacillus frigoritolerans* (PSS4a) as 1.75 µg/ml, for *Bacillus haynesii* (PSS11b) as 1.75 µg/ml, for *Bacillus subtilis* (PSS16a) as 1.5 µg/ml, for *Micrococcus yunnanensis* (PSS18b) as 2 µg/ml, for *Staphylococcus saccharolyticus* (PSS20a) as 2 µg/ml, and for *Staphylococcus epidermidis* (PSS24a) as 2.5 µg/ml. The haloversatile isolates were able to utilize different sugar and amino acid sources. Dextrose, fructose, ribose, cellobiose, and sucrose were respectively used by 91%, 94%, 77%, 77%, and 91% of the isolates. L-tyrosine, L-proline, L-valine were used by all isolates. L-lysine and L-arginine were used by 9% and 14% of the isolates, respectively. Although all species belonging to the Genus *Staphylococcus*, *Corynebacterium*, *Micrococcus*, *Kocuria*, and *Bhargavaea* were non-motile, the other isolates were motile. The cell shapes of isolates were bacil, rod, coccus, or pleomorphic. The optimum growth was observed at pH 7, 1.17% NaCl, and 35°C for all isolates. The results of bacteriocin experiments showed that while *Corynebacterium aurimucosum* (PSS2b), *Bacillus haynesii* (PSS11b), *Staphylococcus epidermidis* (PSS3a) and *Peribacillus frigoritolerans* (PSS4a) were able to produce high amount of bacteriocin, *Bacillus subtilis* (PSS16a), *Micrococcus luteus* (PSS21b) and *Paenibacillus illinoisensis* (PSS7a) could not produce bacteriocin. The highest inhibition zone was determined for *Bacillus piscis* (PSS17a) as 9 mm which was affected by *Corynebacterium aurimucosum* (PSS2b). The lowest inhibition zone was determined for *Pseudomonas iranica* (PSS21a), *Staphylococcus warneri* (PSS1a), *Bacillus haynesii* (PSS11b) as 4mm which were affected by

Corynebacterium aurimucosum (PSS2b), *Bacillus haynesii* (PSS11b), *Pseudomonas iranica* (PSS21a), *Pseudomonas iranica* (PSS21a), respectively. Sheepskin and goatskin samples, treated with the mix culture of enzyme producing haloversatile bacteria [protease and lipase producing *Staphylococcus warneri* (PSS1a), *Staphylococcus pasteurii* (PSS1b), *Staphylococcus warneri* (PSS2a), *Paenibacillus illinoisensis* (PSS7a), *Bacillus licheniformis* (PSS13a) and *Bacillus subtilis* (PSS16a); protease producing *Staphylococcus epidermidis* (PSS3b), *Bhargavaea ginsengi* (PSS11a), *Bacillus sonorensis* (PSS12a), *Bacillus haynesii* (PSS11b) and *Corynebacterium aurimucosum* (PSS15a)], were incubated 40 days at 24°C. The deleterious impacts in sheepskin and goatskin were clearly seen under scanning electron microscope. These deleterious effects were hair loose, pores in skin, cutting of skins' fibers, skin morphological changes. All isolates were affected by Rifampicin (RD5 µg), Amikacin (AK 30µg), and Neomycin (N 30µg). Twenty-two different concentrations of an antimicrobial agent containing sodium dimethyldithiocarbamate (0.00175, 0.0035, 0.007, 0.014, 0.028, 0.06, 0.12, 0.24, 0.48, 0.96, 1.96, 3.92, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000, 2000, 4000 µg/ml) were applied to *Staphylococcus warneri* (PSS1a), *Staphylococcus pasteurii* (PSS1b), *Paenibacillus illinoisensis* (PSS7a), *Bacillus subtilis* (PSS16a), *Micrococcus luteus* (PSS21b), *Staphylococcus epidermidis* (PSS15b), *Bacillus sonorensis* (PSS12a), *Corynebacterium aurimucosum* (PSS15a), and *Staphylococcus capitis* subsp. *urealyticus* (PSS22a). The growth of the test isolates were inhibited with the concentrations between 125 µg/ml and 4000 µg/ml except *Staphylococcus warneri* (isolates PSS1a). The MICs of *Staphylococcus warneri* (PSS1a), *Staphylococcus pasteurii* (PSS1b), *Paenibacillus illinoisensis* (PSS7a), *Bacillus subtilis* (PSS16a), *Micrococcus luteus* (PSS21b), *Staphylococcus epidermidis* (PSS15b), *Bacillus sonorensis* (PSS12a) were found as 250 µg/ml, 62.5 µg/ml, 15.6 µg/ml, 7.8 µg/ml, 125 µg/ml, 15.6 µg/ml, 62.5 µg/ml. The MICs of *Corynebacterium aurimucosum* (PSS15a) and *Staphylococcus capitis* subsp. *urealyticus* (PSS22a) were determined as 3.92 µg/ml. While these two species were affected by the lowest concentration of the antimicrobial agent, *Staphylococcus warneri* (PSS1a) was the most resistant species against this agent.

October, 2023

HAIDER SABAH ABDULHUSEIN

CLAIM FOR ORIGINALITY

INVESTIGATION OF ANTIBIOTIC RESISTANCE, BACTERIOCIN AND ENZYME PRODUCTION OF HALOVERSATILE BACTERIA IN SALT USED FOR SKIN PRESERVATION, CONTROLLING WITH AN ANTIMICROBIAL SUBSTANCE

This is the first study to isolate and identify haloversatile bacteria from salt samples collected from tanneries in Istanbul/Türkiye; to estimate the total number of haloversatile bacteria and the total number of lipolytic and proteolytic haloversatile bacteria in the salt samples; to perform conventional and molecular characterization of haloversatile bacteria using 16S rRNA gene sequence analysis; to examine the production properties of hydrolytic enzymes (lipase, proteinase, esterase, DNase, catalase, oxidase, xylanase, DNase, cellulase, urease, pullunase); to examine the utilization ability of haloversatile bacteria various amino acids and sugars; to examine the ability of haloversatile bacteria to produce bacteriocins; to investigate the activity of bacteriocins under various conditions (pH, temperature, NaCl); to investigated the antibiotic susceptibilities of haloversatile isolates against Cephalothin (30 µg, KF30), Cefotaxime (5µg, CTX5), Rifampicin (5 µg, RD5), Spectinomycin (100 µg, SH100), Sulbactam/Ampicillin (10 µg, SAM10), Amikacin (30 µg, AK30), Neomicin (30 µg, N30) and Tetracycline (30 µg, TE30) by Kirby-Bauer disc diffusion method; to investigate the MICs (Minimum Inhibitory Concentration) of antimicrobial agent containing sodium dimethyldithiocarbamate against haloversatile isolates by using the broth dilution method in a 96-well microtiter plate; to examine the harmful effect of enzyme-producing haloversatile isolates against sheepskin and goatskin samples under SEM (Scanning Electron Microscope).

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Assoc. Prof. Dr. Pınar ÇAĞLAYAN

Haider Sabah ABDULHUSEIN

SYMBOLS

°C	: Centigrade degree
cm²	: Centimeter square
cm	: Centimeter
h	: Hour
µl	: Microlitre
g	: Gram
µg	: Microgram
pH	: A unit of measure that express the degree of acidity or alkalinity of a solution
%	: Percentage
V	: Volt
ml	: Mililitre
µg	: Microgram
kg	: Kilogram
M	: Molar
N	: Normal
kb	: Kilo base
m²	: Square meter
µm	: Mikrometre

ABBREVIATIONS

ATP	: Adenosine tri-phosphate
ATCC	: American Type Culture collection
A	: Adenine
BaCl ₂	: Barium chloride
CFU	: Colony Formation Unite
rpm	: Revolutions per minutes
CO ₂	: Carbon dioxide
CaCO ₃	: Calcite
C	: Cytosine
CaCl ₂	: Calcium chloride
DNA	: Deoxyribonucleic acid
SDDC	: Sodium dimethyl dithiocarbomate
dNTP	: Deoxyribonucleic tri-phosphate
DNase	: Deoxyribonuclease
G	: Guanine
HCl	: Hydrochloric acid
HgCl ₂	: Silver chloride
H ₂ S	: Hydrogen sulfide
H ₂ O ₂	: Hydrogen peroxide
H ₂ SO ₄	: Sulfuric acid
EDTA	: Ethylene demine tetra acetic acid
K ₂ HPO ₄	: Potassium phosphate

ABBREVIATIONS

KNO ₃	:	Potassium nitrate
KCl	:	Potassium chloride
MgCl ₂	:	Magnesium chloride
MgSO ₄	:	Magnesium sulfide
MIC	:	Minimum Inhibition Concentration
NaBr	:	Sodium bromide
NaHCO ₃	:	Sodium bicarbonate
NaCl	:	Sodium chloride
NaClO ₂	:	Sodium chlorite
PCR	:	Polymerase chain reaction
TAE	:	Tris Acetate
rRNA	:	Ribosomal ribonucleic acid
T	:	Thiamine
NCBI	:	National Center Biotechnology Information
U	:	Uracil
SEM	:	Scanning Electron Microscope
TEM	:	Transmission Electron Microscope
kV	:	Kilovolt
SW	:	Salted water
PSS	:	Preservative Salt Sample
Na ₂ CO ₃	:	Sodium Carbonate

ABBREVIATIONS

NCBI	: National Center For Biochemical Information
mA	: miliAmper
BLAST	: Basic Local Alginment Search Tool
TAE	: Tris- Acetate and EDTA
ORCID	: Open Researcher and Contributor ID
HMDS	: Hexa-Methyl-Disilazane
EUCAST	: European Committee on Antimicrobial Susceptibility Testing
MgSO ₄ .7H ₂ O	: Magnesium Sulphate Heptahydrate (Epson salts)
GAM	: Gelatine Agar Medium
HAM	: Halophilic Agar Medium
HBM	: Halophilic Agar Medium
PE	: Primary Electrons
SE	: Secondary Electrons
PBS	: Phosphate Buffer Saline
Na ₂ HPO ₄	: Disodium Hydrogen Phosphate
CFS	: Cell Free Supernatant
TCA	: Trichloric Acitic Acid
<i>p</i> -NPP	: Para-NitroPhenyl-Palmitate
dH ₂ O	: Distillated Water
BOD	: Biochemical Oxygen Demand
COD	: Chemical Oxygen Demand
EPA	: American Environmental Protection Agency

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CHAPTER 1

1. INTRODUCTION

The leather sector has historically been recognized as a crucial economic backbone for a number of countries, as it has been considered to be an important source of income that remains stable against external factors. Moreover, current producers of leather include economically and industrially highly developed countries like China, the United States, India, and other countries. Türkiye is a prominent player in the global market for leather products, having a strong position as an exporter. Since 1970, the leather industry has played an essential role in Turkish economy and has emerged as a significant sector. In recent decades, there has been a notable increase in the economic importance of the leather sector, coinciding with the proliferation of rawhide resources in Türkiye. The leather industry has experienced significant export volumes, with a notable proportion of the necessary raw materials being sourced through imports (Leather and Leather Products Sector, 2022). Based on the data published by the Social Security Institution for the year 2021, it has been reported that there is an overall workforce of 71.685 individuals employed in 7,086 industries involved in the production of leather and leather-related goods in Türkiye. According to the Leather and Leather Products Sector (2022), Istanbul, Izmir, Bursa, Bolu, Hatay, Manisa, Isparta, Balıkesir, Uşak, Tekirdağ, and Ordu are the main cities in Türkiye that engage in the manufacturing of leather. The production of leather items and related goods has seen an evolution towards ecologically sustainable ways due to the adverse environmental effects associated with the leather industry throughout nations. This transformation has been supported by the creation of an environmental protocol, which was approved by the ministry of climate change. The year 2021 had a notable growth in the exports of leather and leather-related items, with a substantial increase of 38.6% amounting to a total value of \$1.9 billion. Shoes provide the largest proportion of exports in this industry, accounting for 56.9% of the total. Thus establishing shoes as the most significant export product (Leather and Leather Products Sector, 2022). The proportions of Turkish total exports related to leather goods, raw and processed leather goods, leather, and furs are 21%, 7%, 13%, and 8%, respectively (Leather and Leather Products Sector, 2022). The shoe industry in Türkiye has the largest share within the sub-sector of leather and leather goods exports, contributing a substantial amount of 1.1 million dollars, which represents around 56.9% of the overall exports in this category. The footwear industry in Türkiye is a significant contributor to the country's export sector and plays a crucial role in job

creation. Leather appears as the main product within the industry of shoe exports. The leather shoe exports had a notable increase of 31.1% in the year 2021 compared to the previous year, resulting in a total value of 466.8 million dollars. Russia represents the primary export market for the leather shoe company, constituting a significant proportion of the whole volume of exports, specifically 14.9%. Germany, Romania, Spain, England, and Italy are significant marketplaces (Leather and Leather Products Sector, 2022). Perform such types of research carries significant importance within the aforementioned conclusions, especially in the circumstances of Türkiye. The aims of the present study were to collect salt samples from leather tanneries; to determine pH of salt samples; the total numbers of haloversatile bacteria; to determine the total numbers of proteolytic and lipolytic haloversatile bacteria in the salt samples; to isolate haloversatile bacteria from salt samples; to characterize haloversatile bacteria molecularly; to determine their enzymatic activities; to determine the utilization of different amino acid and different sugar sources; to examine the effects of pH, salt and temperature on the growth of haloversatile bacteria; to investigate Gram Staining reactions, cell morphology and pigmentation; to investigate the effects of lipolytic and proteolytic haloversatile bacteria on sheep and goat skins; to examine the haloversatile bacteria isolated from salt samples under scanning electron microscope; to investigate the ability of bacteria to produce bacteriocin; to investigated the optimum conditions for bacteriocin production; to examine antibiotic tests; to detect the MIC of antimicrobial agents against haloversatile isolates. The present study covered important issues associated with the utilization of bacterial metabolic products, specifically bacteriocins, as an alternative for antibiotics in the control of bacterial activity.

1.1.THE SKIN

1.1.1. Definition and Function of Skin

Skin has various functions in live mammalian animals, such as providing resistance against cold and hot conditions, extra-mechanical impacts, and severe effects of microorganisms. Skin is considered the animal body's largest and most complex organ. Skin is also important in thermo-regulation and helps prevent dehydration in animals (Gustauson, 1956). It is an indispensable barrier that insulates the internal body and provides protection against harmful factors such as microorganisms, ultraviolet (UV) radiation and chemical materials (Menon, 2002; Krinsky *et al.*, 2004; Lademann *et al.*, 2011). Typically, as shown in Figure 1.1, skin has self-repair

mechanisms that respond depending on the depth and nature of the damage suffered. Moreover, it plays a valuable role in physical stress resistance (Horie, 1990).

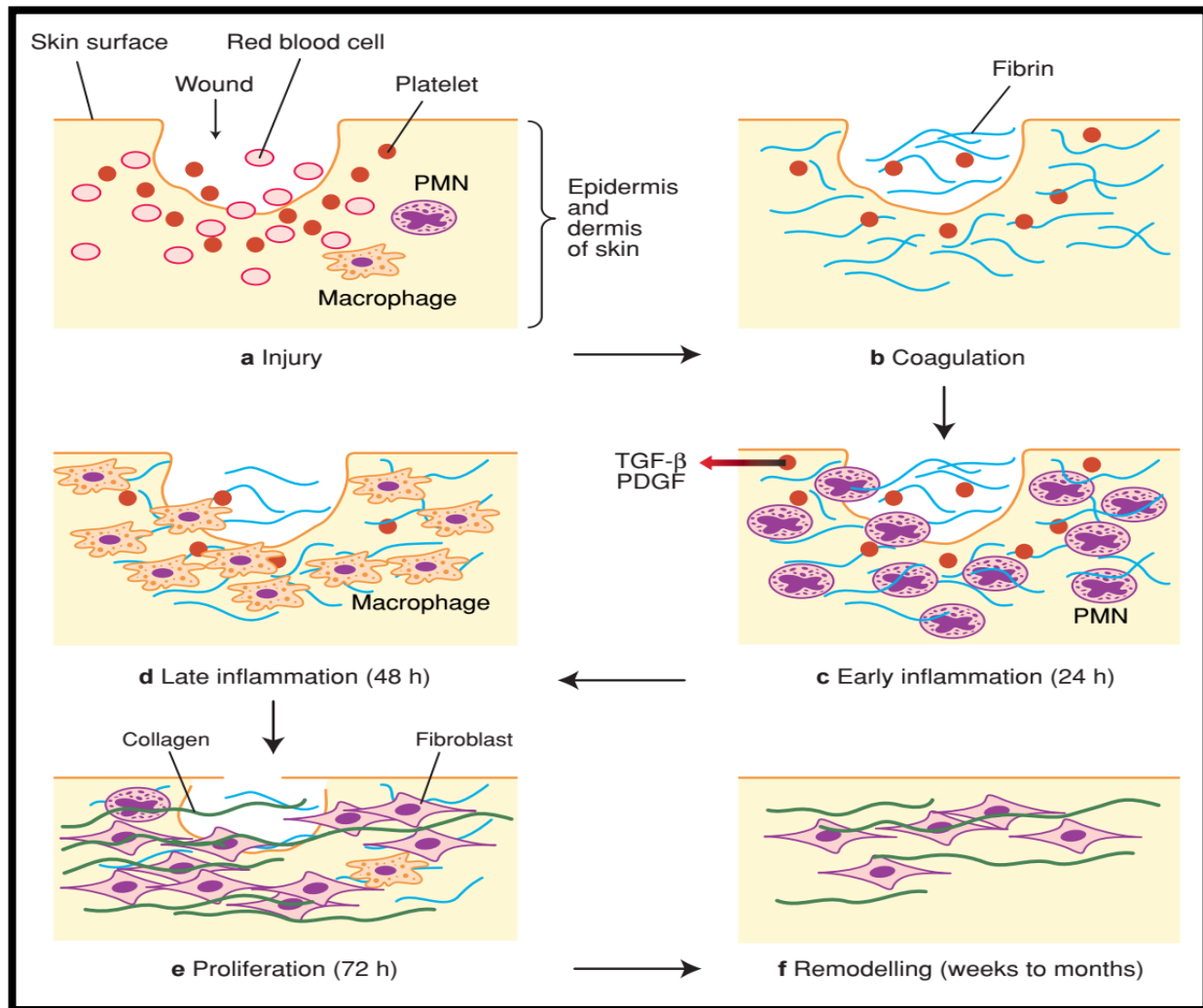


Figure 1.1. Healing of skin injury. Six phases illustrated as: (A) Extravagante of blood elements such as polymorphonuclear neutrophils (PMNs) and platelets; (B) Platelets and fibrin aggregate alongside to form coagulation; (C) During early inflammation, platelets liberate growth factors (PDGF) to attract PMNs to the wound location; (D) During late inflammation, macrophages eliminate debris, produce PDGFs (attracting fibroblasts) and begin reorganizing extracellular matrix; (E) After producing collagen by fibroblast cells, proliferation begins; (F) Finally, reorganization and collagen cross-linking lead to the remodeling phase (Beanes *et al.*, 2003).

Observations show that skin's size and thickness differ among animals, with further variations occurring depending on age, sex and species. Younger animals have thinner and smaller skin mass than aged ones, as characterized by a finer fiber network (Figure 1.2). Generally, The skin structure can be evaluated with three layers: the outer epidermal layer, the central dermal layer and the inner subcutaneous layer (Figure 1.3). Due to the complexity of these layers, animal hides require harsh treatments during processing in order to penetrate and affect the desired structural alterations in the hide. During the animal slaughter processes, especially in meat production industries, we get edible meats, offal and other non-edible remains including skins, blood, teeth, bones, horns. From a commercial point of view, edible and non-edible animal products are an estimated 44% and 33% of cattle and swine's live weight, respectively. Additionally, these materials are used in the cosmetics, pharmaceutical and leather sectors (Martie *et al.*, 2011; Mikkilineni, 2021). Skins and hides are common place byproducts of animal slaughter methods and thus are used in leather production (Masciana, 2015).

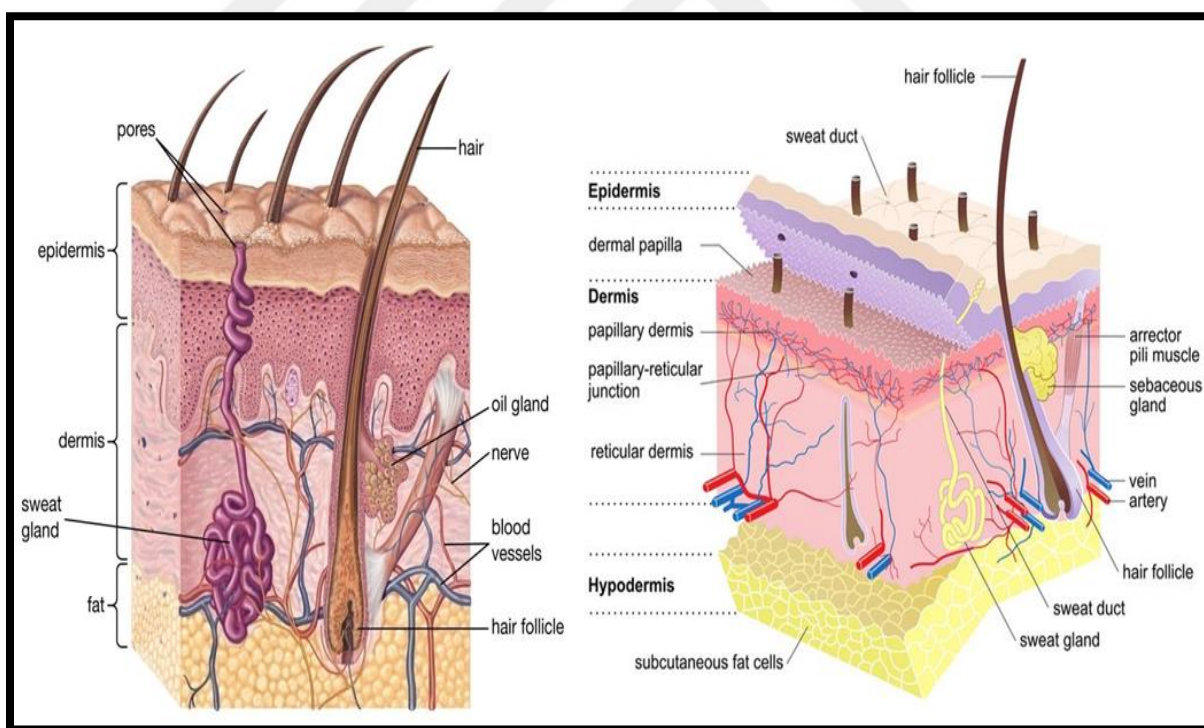


Figure 1.2. Cross section of sheepskin.
(Doherty *et al.*, 2021).

Figure 1.3. Main structure of skin
(<https://www.britannica.com/science/sebaceousgland>, Date of Access, May 2023).

1.1.2. Chemical and Biological Structure of Skin

Raw skin is composed of 64% water, 2% lipids and 33% proteins. A glycosaminoglycan composes the main structure of dermis layer that covers with connective tissue and is responsible for giving skin its substance. These carbohydrates are made of sulfate. There are other components including blood vessels, lymph vessels, elastic fibers, hair follicles, nerves, sebaceous glands and sweat glands (Polkade, 2007). Skin is composed of 43% water, 4% lipids, 13% hair and 40% other materials (Aquim, 2004). Skin compositions vary from one animal to another depending on many factors such as age, sex, animals' race and type (Table 1.1) (Gutterrens, 2003). The amount of water in skin is about 60-70% (Santos and Gutterres, 2007). Young animal skins contain much more water than old ones (Şenses, 1987). High water content of raw skins can negatively affect hides because it leads to the growth of destructive bacteria. As mentioned, the age and race of animal affect their hides; the amounts of lipids in sheep's skin (up to 30%) are fewer than in cattle. Types of fats found in raw hides are triglycerides, phospholipids and cholesterol. Fats and minerals are found in lesser proportions in hides than protein, so this characteristic makes it easy to convert rawhide into leather (Ockerman and Basu, 2014). There are nearly 2% fats in the structure of the skin (Kılıçoğlu, 1991). The main structure of raw hide consists of proteins, which make up nearly 80% of the dry weight of skin and are divided into two main types: fibrous and globular proteins (Şenses, 1987). Kılıçoğlu's results (1991) show that approximately 32.5% of a hide's weight comes from proteins.

Table 1.1. Chemical composition of hides (Moulton and Lewis, 1940; Aten *et al.*, 1955; Biedermann *et al.*, 1962; Henrickson *et al.*, 1984; Leather Industries of America and US Hide, 1993; Ockerman and Basu, 2014; Ockerman and Hansen, 2000).

Type and age of animal	Water (%)	Protein (%)	Fat (%)	Minerals (%)
Average slaughter cattle	62–70	36	8	1
Mature cattle hide, without hair	65	30	6	1
Very fat animal	55	19	10-12	0.1
Wet cattle hide	83	15.7	0.2	0.1
Air-dried cattle hide	9.1	89.9	0.2	0.8
Newborn calf	67.9	30.8	1	1
Three-month-old calf	66	31	1.6	1.4
Two-year-old steer	61.2	35	3.2	1.1
Four-year-old steer	55.6	38.2	6	1.1
Old cow	60.2	36	3.1	1.1
Sheepskin	54	39	30-50	0.2
Goatskin	60	42	3-10	0.1
Angora goatskin	43	41	6-17	1.3
Hog skin	37	14	30-50	0.5
Cured cattle hide	39-48	41	44-47	11-16

1.1.2.1. Globular Proteins

This section contains four types of proteins: globulin, mucin, mucous and albumin. These proteins are insoluble in water, yet they can be soluble in aqueous salt solution. These proteins dissolve when hide and skins are soaked. Mucoïds are easily removed by lime solution during calcination process because they dissolve in alkaline solutions (Şenses, 1987; Berber, 2009).

1.1.2.2. Fibrous Proteins

Fibrous proteins, the main structural components in leather production, are classified in four groups: collagen, keratin, reticulin and elastin (Şenses, 1987; Berber 2009; Çağlayan, 2014).

1.1.2.1. Collagen

The major building unit of hides and skins is collagen because it makes up 60-70% of skin's dry weight (Vankar and Dwivedi, 2009). Collagen contains 20 amino acids. In addition to hydroxylysine and hydroxyproline, collagen molecules synthesized from three α -helical polypeptide chains that have repeating glycine -X, Y sequencing, X and Y sequences represent proline and 4-hydroxyproline (Fratzl, 2008). Also, collagen consists of hydroxylated lysine that, when modified by monosaccharide, is necessary to produce collagen crosslinks (Fratzl, 2008). It is believed to be closely related to the hydrothermal stability of collagen, which mediates, via hydrogen bonding, the formation of a lattice of water molecules around and within the tropocollagen (Ramachandran *et al.*, 1973). The antecedent studies showed the main structure of collagen molecules aggregated and formatted suprastructures of straight fibrils during the production of collagen natural crosslinks (Fratzl, 2008). Collagen has a high molecular weight, which is part of the reason it is a major building block of gelatin. It represents the most significant proportion of protein within animal connective tissues, tendon and skin (Ramachandran *et al.*, 1968). Infact, collagen is soluble in water and low acidity organic solutions; however, it is insoluble in alkaloid and high acidic solutions. The structure of collagen is stable in pure water pH 7 up to 60°C, but it is convertible to gelatin structure and becomes soluble when heated in boiling water and the pH is lower than seven or more than seven. For this reason, pretanning processes are not carried out at temperatures above 30-35°C. Dried collagen is composed of 50.2% carbon, 17.8% nitrogen, 6.4% hydrogen, 25.4% oxygen and sulfur (Şenses, 1987). Due to the collagen is the most abundant protein in skin, it under pins healthy skin and is considered the key factor for maintaining skin firmness and elasticity (Fleischmajer, 1985; Sibilla, 2015). One study found that there are more than 29 types of collagen, which can be classified into two groups, fibril forming and non-fibril forming (Gelse *et al.*, 2003). Anatomically, the molecular structure and tissue distribution are [α(I)]₂α₂(I) for fibril forming collagen and [α(III)]₃ for non-fibril forming collagen. In fibril forming collagens, these are predominately distributed in bone, dermis, tendons, ligaments, cornea, and skin. In non-fibril forming collagens, they are distributed mostly in skin, vessel wall, reticular fibers of most tissues (lungs, liver, spleen, etc.) (Gelse *et al.*, 2003). Also, the data that obtain from (Nolte *et al.*, 2008) explains collagen type one pose 80-90% of skin structure which produce by fibroblasts, it is defined as a mesenchymal cells (Nolte *et al.*, 2008). In addition to collagen, fibroblasts produce elastin, a protein which is responsible for

skin flexibility, elasticity and allows tissue to return to original skin conformity after stretch and recoil (Sherratt, 2009; Weihermann *et al.*, 2017). This function of fibroblasts is an important for preserving the elasticity of skin (Figure 1. 4).

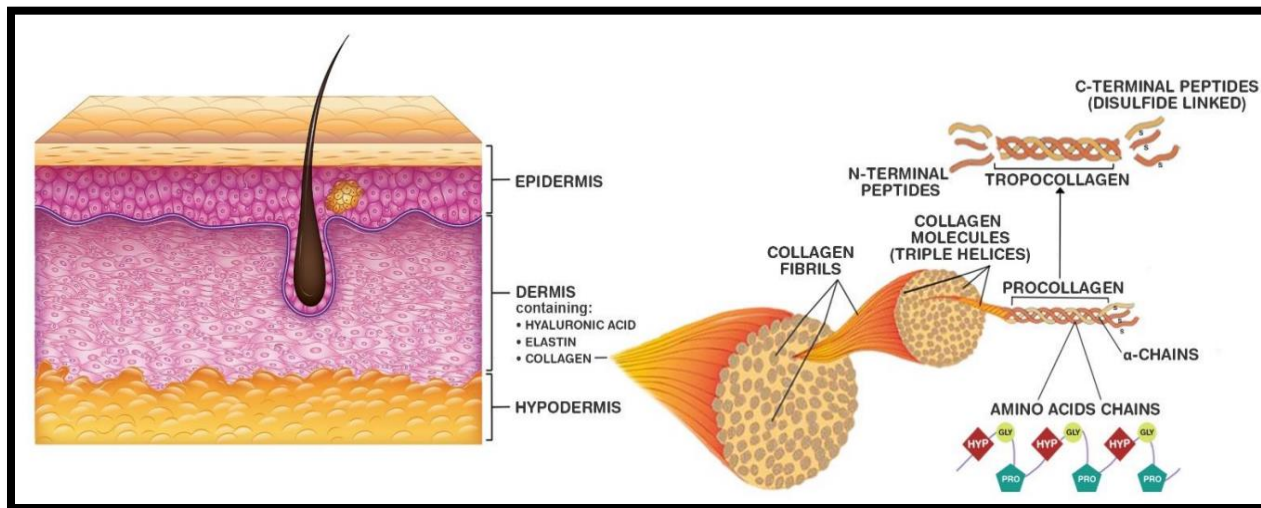


Figure 1.4. Molecular structure of skin-collagen (Tang *et al.*, 2017; Reilly and Lozano, 2021; Matinong *et al.*, 2022).

1.1.2.2. Keratin

The main protein in hair structure is keratin, which composes 6-10% of total hide protein. Keratin also represents 2% of dry skin weight and contributes to forming outer layer of hides. It is insoluble in water, alcohols and ether. However, keratin does dissolve in concentrated acid alkalis. Additionally, it is very sensitive to reducing agents such as Na_2S and CaS (Şenses, 1987). It is destroyed and dispersed by zinc. Keratin is characterized by tight binding of alpha helix and beta layer; it has cysteine bridges, hydrophobic interactions, high disulfide bonds and hydrogen bonding. Depending on these properties, keratin is considered highly resistant to microbiologic deterioration and mechanical damages. Keratin slightly swells in acids and alkalis (Cortezi *et al.*, 2008).

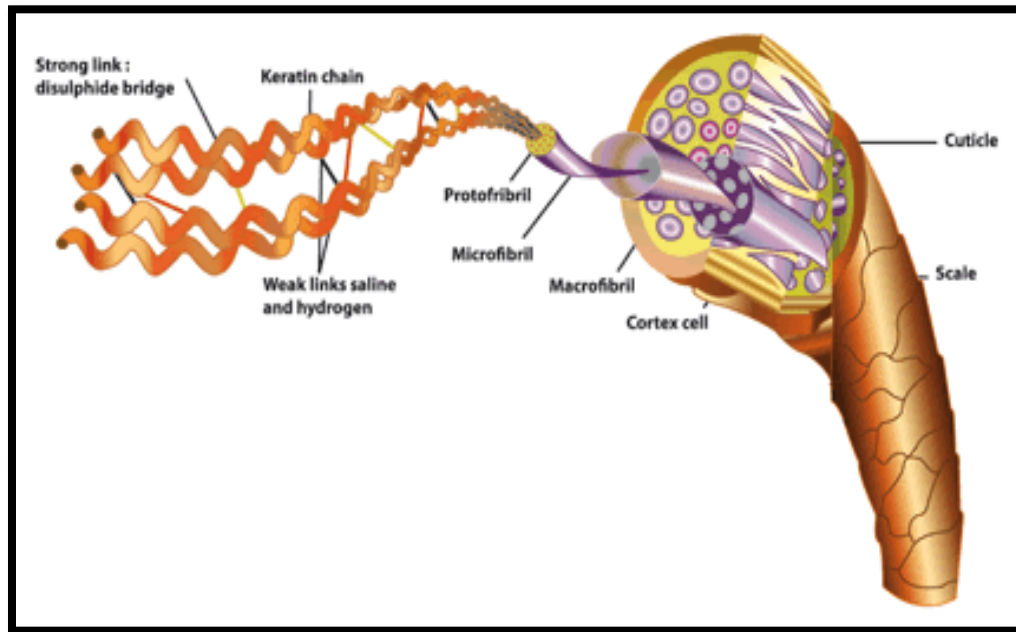


Figure 1.5. (A) Structure of hair and major structure of keratin (https://sites.google.com/site/chsanatomyphysiology27/_/rsrc/1468885277181/home/keratin/Keratin.png, Date of access, May 2023).

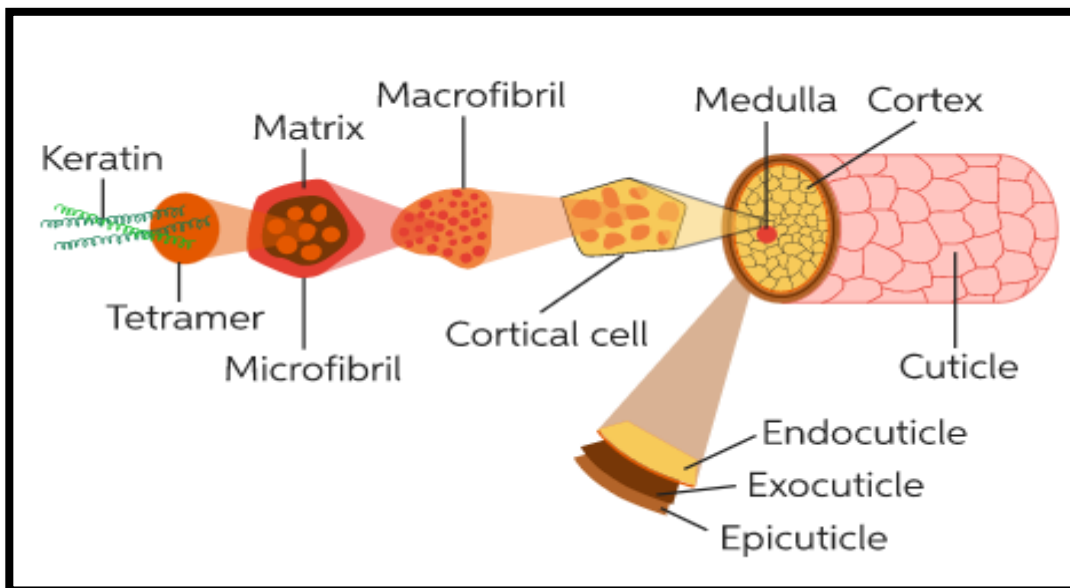


Figure 1.5. (B) Major details of how keratin participates in skin and the leather extensions (https://images.ctfassets.net/vrrt8fsfwf0e/hyB2WsW8uayfaaxVRXpdq/2202271ed68c3db6a14ce583e57e0daf/keratin_art_1.svg, Date of access, May 2023).

1.1.2.3. Reticulin

Reticulin is a fibrous protein that wraps around collagen fiber bundles to hold them together. Reticulin is more resistant to acid, alkalis and boiling water than collagen. It also swells slightly less than collagen. It is digestible by pepsin enzymes and extensively affected by bacterial activity (Şenses, 1987; Berber, 2009). Mammalian animal tissues, collagen and reticulin tissues have similar structure and amino acid types. Reticulin protein contains 7.7% alanine, 24.6% glycine, 2.13% valine, 2.85% leucine, 0.04% cystine, 7.7% proline, 1.27 % histidine, 0.24 % tyrosine, 6.1 % glutamic acid, 3.4 % serine 1.44 % phenylalanine, 1.43% isoleucine, 1.74% threonine, 4.21% aspartic acid, 8.6% hydroxyproline, 3.44% lysine, 14.4% arginine, 1.93% hydroxylysine and 0.68% methionine amino acids (Eastoe, 1955).

1.1.2.4. Elastin

Elastin makes up approximately 0.3 % of the total dry weight of skin. Its fibres are responsible for skin elasticity. They are insoluble in water and organic solvents. Indeed, dry elastin fibers are not elastic; unlike collagen proteins, they are more resistant to boiling water, acids, alkaline solvents and proteolytic enzyme (Şenses, 1987; Berber, 2009). Furthermore, the main structure of proteins in the mammalian raw hide's texture is made up of different types of amino acids including glycine (32.9%), proline (12.6%), alanine (10.9%), glutamic acid (7.4%), hydroxyproline (9.5%), arginine (4.9%), serine (3.6%), lysine (2.9%), aspartic acid (4.7%), leucine (2.4%), valine (2.2%), tyrosine (0.3%), histidine (0.5%), methionine (0.6%), cysteine (0.1%), phenylalanine (1.3%), isoleucine (1.1%), threonine (1.9%), isoleucine (9.5%) and hydroxylysine (9.5%) (Szpak, 2011). There are other substances in raw hides such as hydrogen, nitrogen, minerals (sulfate, iron, phosphorus, iodine, and chlorine) (Kılıçoğlu, 1991; Berber, 2009).

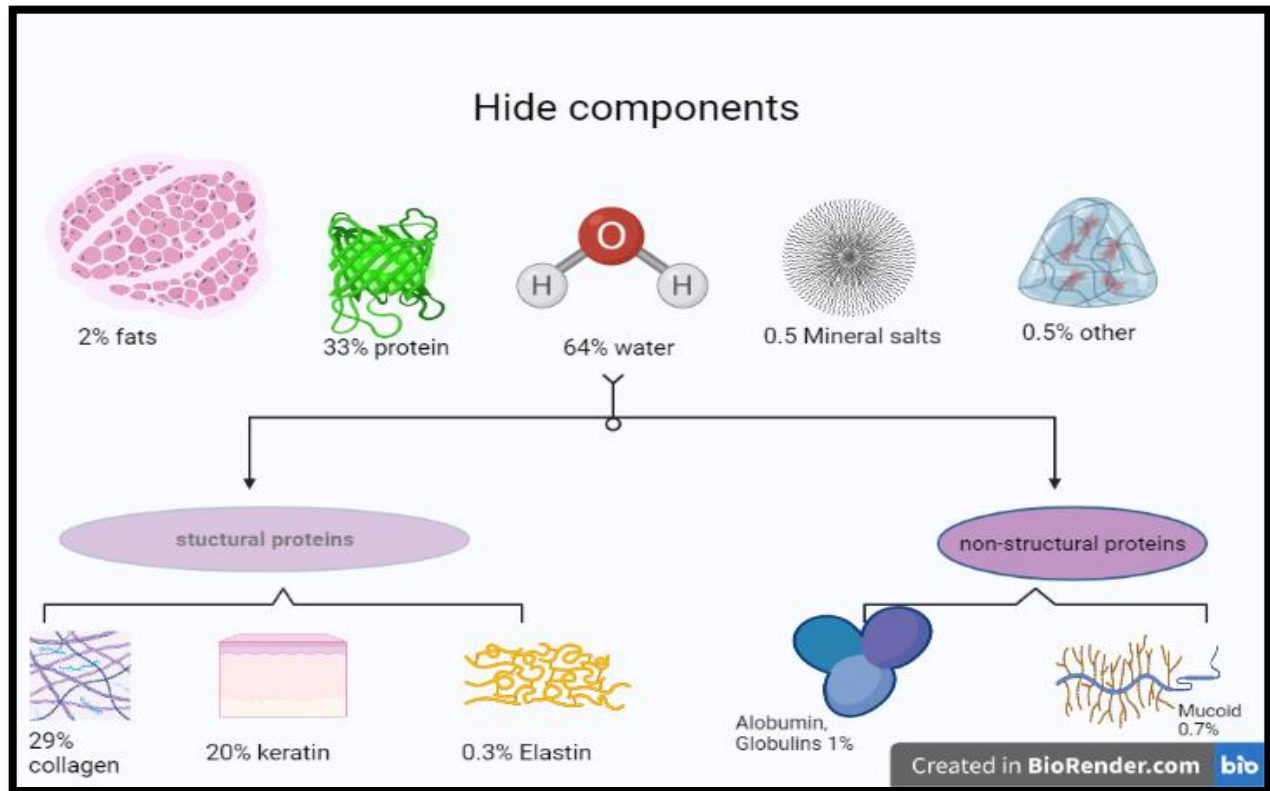


Figure 1.6. Estimated components of animal hides (Sharphouse, 1971).

1.1.3. Anatomical and Histological Structure of Skin

Mammal skins are composed of three major layers. The outer epidermis that represents the first protective layer and mainly consists of keratin-producing cells (keratinocytes) and fiber (the parts of hairshaft). The dermis or second layer occurs beneath the first layer and contains basement membrane (a specific structure synthesized especially from collagen type IV and VII), a main junction between first and second layer, and profusely glycosylated proteins named proteoglycans. Finally, there is the inner most hypodermis layer of skin (Breitkreutz *et al.*, 2013). The layers are shown in cross-section in Figure 1.7.

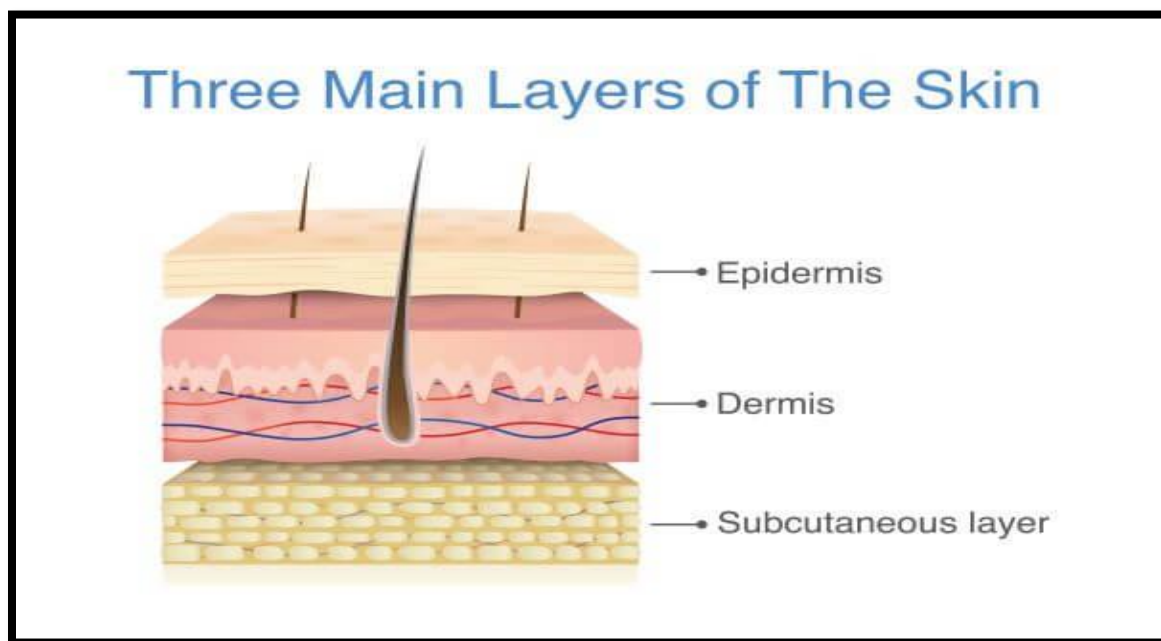


Figure 1.7. Three main layers of the skin. Adapted from (<https://biologydictionary.net/wp-content/uploads/2021/04/Skin-layers.jpg>, Date of access, May 2023).

1.1.3.1. Epidermis

This outer layer of skin is branched into five sub-layers with different thicknesses. The first surface layer, named *stratum corneum* (horny layer), is composed of dead cells without any organelles and nucleus. The thickness of this layer varies depending on age, and the major element of its composition is insoluble keratin (Hijazy, 1971; Horie, 1990). The horny layer has a crucial function to protect deeper skin components from infection, disease, mechanical stress, chemical substances and dehydration (Dale, 1978; Bediz-Ölçer and Gönül, 2002). The second layer is called the *stratum lucidum*, the third layer is the *stratum granulosum*, the next layer is *stratum spinosum* and eventually the last layer of epidermis is the *stratum basale (germinativum)* (Monteiro-Riviera *et al.*, 1997; Bediz-Ölçer and Gönül, 2002; Kennedy and Wess, 2003). Epidermis has sensory receptors to warn the nervous system of danger. The thickness of this layer is 1-2% of total skin thickness, thus making it a first line of defense for the body. From top to bottom, the epidermis is also sometimes categorized in a simplified 3-layer structure: horny, spiky and basal layer. The first layer is composed of keratin. The basal layer engages in prekeratin production and is also responsible for giving the skin specific color because its cells have pigments that are influenced by light. Finally, there are no blood vessels in the epidermis; it

is fed from dermis layer. Conventionally, the epidermis layer disappears during calcification processes (Bediz-Ölçer and Gönül, 2002).

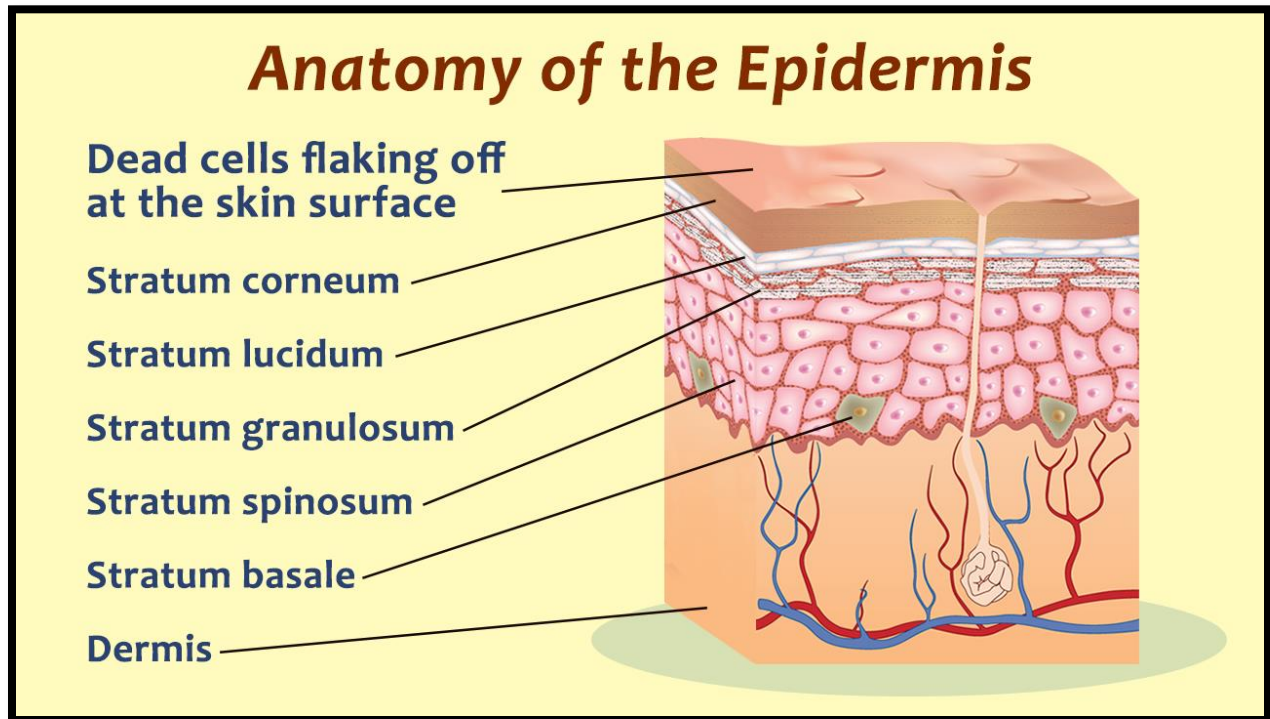


Figure 1.8. Structure of epidermis layer (<https://i.pinimg.com/originals/85/f7/d1/85f7d193416b9990f8627f287be36949.png>, Date of access, May 2023).

1.1.3.2. Dermal Layer

Anatomical studies of sheepskin have determined that the second layer of sheepskin has main contents including 53% water, 27.5% proteins, 18.3% fats and minerals. Furthermore, the main types of collagen found in dermis layer are types I and III, which compose nearly 60-70% of the skin's dry weight (Frantz *et al.*, 2010). This layer is the greatest remainder after skin processing and is considered the center and gist of skin structure in the leather and parchment manufacturing business. The upper part of dermis layer is called enamel because it contains a network of microfilament composed of collagen type VI (Edmonds *et al.*, 2008). The dermal layer forms about 98% of skin thickness. Hence, it provides the skin its structural and physical strength. It is composed of abundant collagen fibers, especially type I and type III collagen. Each one of these fibers is made up of three polypeptide chains, which are rich in proline and glycine (Maffia *et al.*, 2004; McEvoy *et al.*, 2003; Lunam and Weir, 2006). As noted by Horie (1990), the dermal layer

consists of subordinate layers. The superficial grain (papillary layer) is composed of fine fibers and a lot of polysaccharides. In contrast to the second layer, the main structure of the papillary layer is connective tissue. The deep layer is called the reticular dermis, which has structures as well as ruffinian corpuscles (Horie, 1990; Lunam and Weir, 2006). Controversially, other studies have elucidated that the dermal layer is composed of three layers and papillary layer located between the vitreous and reticular layers (Berber, 2009; T.C. Milli Eğitim Bakanlığı, Sama and Yağ Giderme, 2011). It is worth mentioning that the amount of water in dermal layer represents 80% of its consistency, and the remaining 20% is composed of other macromolecules such as lipids, elastin and collagen, which contains high amount of hyaluronic acid (Horie, 1990). Furthermore, the dermal layer contains other types of cell such as fibroblast cells, which produce collagen and elastin as extracellular proteins (Hijazy, 1971). Also, it has the hair follicle, which is covered by an epithelial tissue. The hair follicles participate in the synthesis and transfer of melanin pigments and keratin which are produced by melanocytes and keratinocytes respectively. As has been mentioned in a previous study, both melanocytes and keratinocytes are responsible for form, color and hair shaft (Hijazy, 1971). Finally, this layer's sebaceous gland synthesizes and secretes sebum with hair extensions (Hijazy, 1971). The dermis layer is located on the hypodermis layer (Figure 1.9). The thickness of animal skin depends on the type of animal (12-25% in bovine, 35-50% in goat, 60% in sheep). On the contrary, the papillary layer is an unstable layer. It becomes loose and changeable during calcification processes and removal of all non-collagenous molecules such as hair, sweat and sebaceous glands. Consequently, this layer is weaker than the reticular layer (Berber, 2009; T.C. Milli Eğitim Bakanlığı, Sama and Yağ Giderme, 2011).

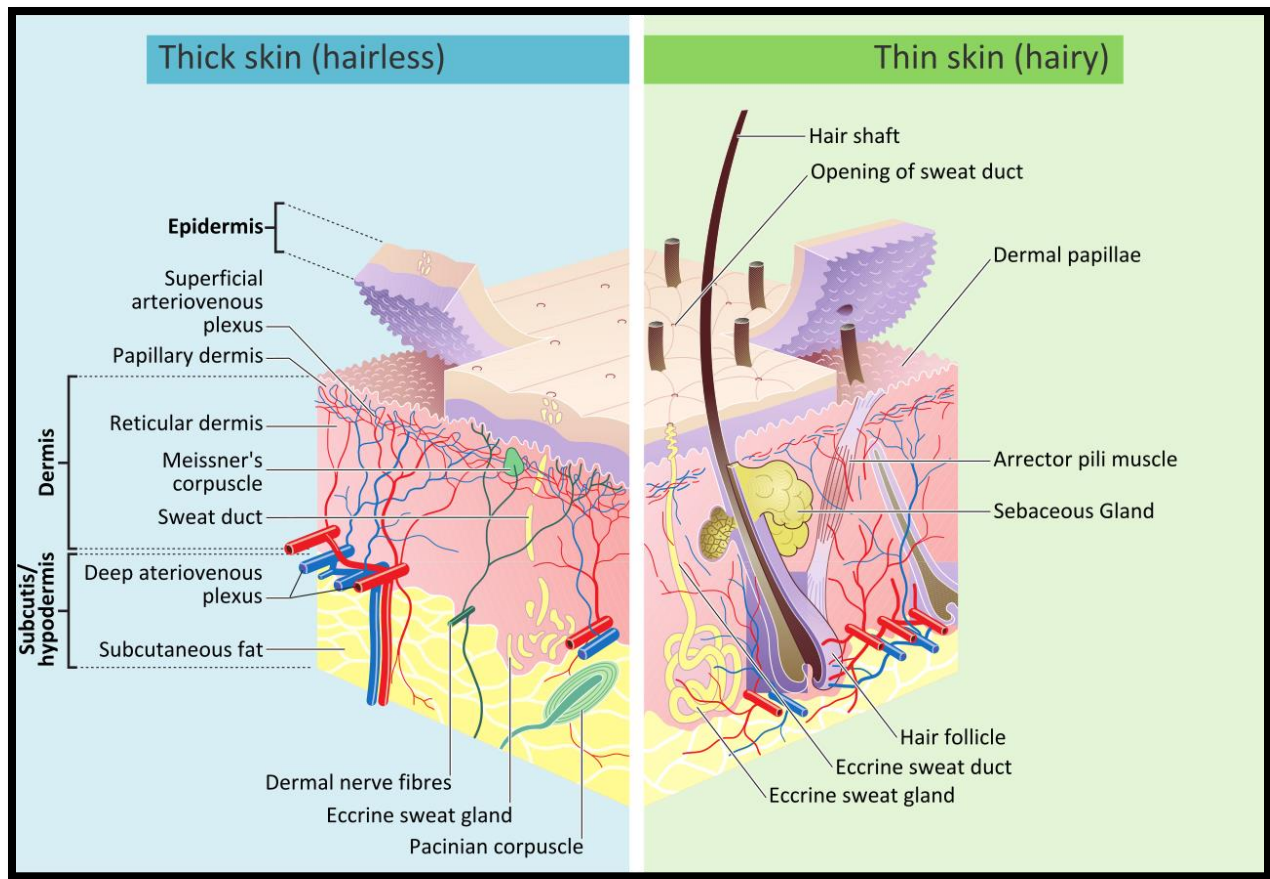


Figure 1.9. The differences between thick skin and thin skin (https://upload.wikimedia.org/wikipedia/commons/thumb/e/e1/Skin_layers.svg/1280px-Skin_layers.svg.png, Date of access, May 2023).

1.1.3.3. Hypodermis

It is mainly composed of fatty byproducts (lipid) and meat (Şenses, 1987). It consists of nerve cells, adipose cells and blood vessels. Depending on the health, age and species of animal, there is disparity of lipid amounts in subcutaneous layer. On the whole, the main function of this layer is to connect bones and muscle to skin. It is rich in fat and gives the shape and contour to skin. During skin fleshing operation, this layer protects the other main layers from knife cuts (Birbir, 1991). This layer is supported by collagen and elastic fibers (Figure 1.10) (Maxwell, 2007). Dermal layer and hypodermis layer are removed during leather processing. Merely the skin is taken off the animal after slaughtering processes; in warm weather it should quickly be made ready for transport to the tannery.

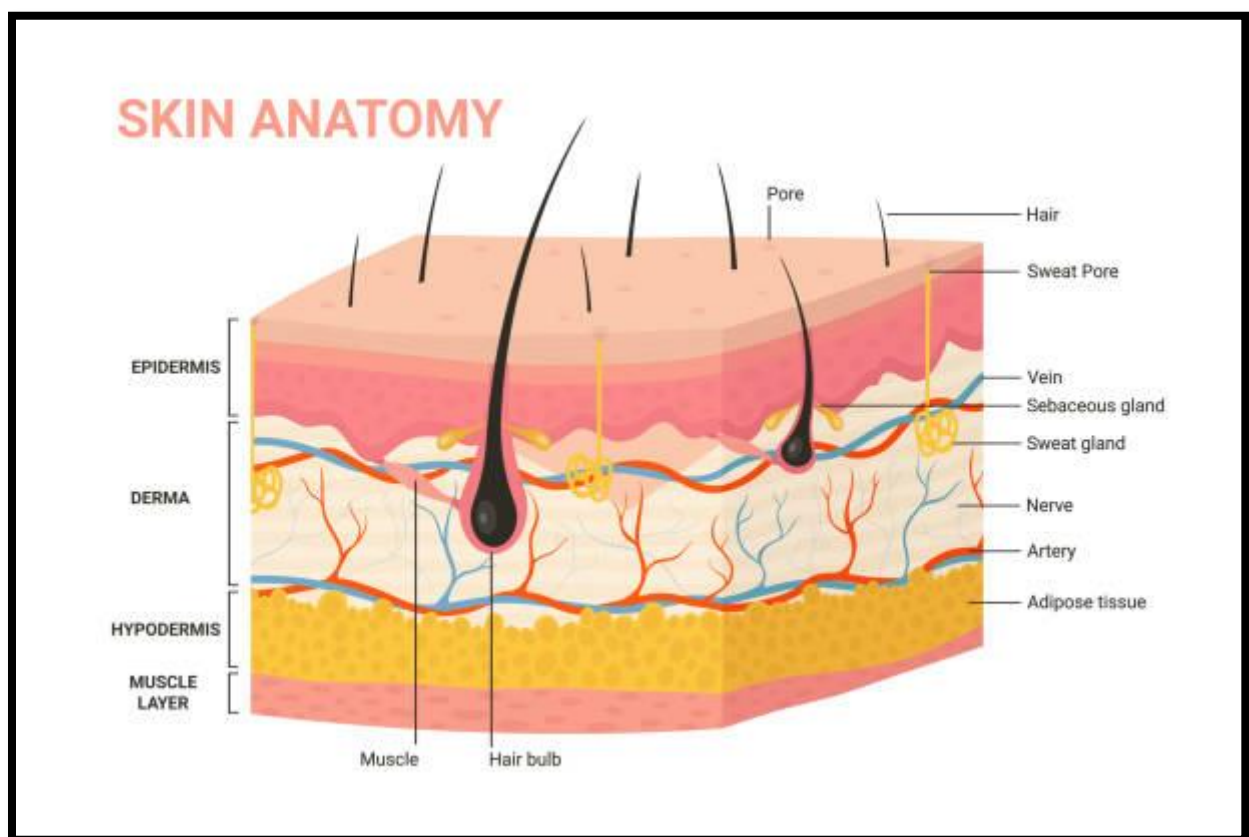


Figure 1.10. Subcutaneous layer (https://media.istockphoto.com/id/1325453968/vector/skin-layers-structure-anatomydiagram_human_skin_infographic_anatomical_background.jpg?s=612x612&w=0&k=20&c=A9ttgBSgehoxsEmA69XX_xVeKBsFqAkNbZQptM_gqxc=, Date of access, May 2023).

1.2. LEATHER

Skin from vertebrates such as sheep, cows, goat, and pig is used to produce different types of leather goods such as belts, shoes and bags through chemical and physical processes that affect fibrous protein collagen to allow the hides to reform into solid shape. India, U.S.A. and some West African nations have industries that extensively utilize skins of animals as raw material in leather manufacturing. Furthermore, animal skins are considered edible in different countries. For instance, in U.S.A. there is a taste for crispy pork rinds; in Jamaica and West Africa cow skin is used to makes up and stews (Haines and Barlow, 1975; Mikkilineni, 2021). Though many types of animal skin can be transformed into leather products, the most generally used skin is taken from sheep, cattle and goats. Pig skin is used to a lesser extent. All of them basically have

a hassling skin structure that consists of uncountable collagen bundles interweaving in a three-dimensional manner (Haines and Barlow, 1975). The major source of leather is skins acquired from pig, goat, deer and sheep; the second source is hides obtained from water buffalo hides and cattle; the last source is exotic animals such as crocodile, alligator, snake and ostrich (Scanes, 2018).

Leather is intensive, thick and powerful as well as mushy and malleable. It is characterized as a perfect thermal insulator. Also, it has many important features including resistance to tearing, scraping, fire, water and mold. It is valuable to humans and used it in synthesis of other goods such as clothes and shelter. Additionally, its appearance can be maintained through application of polish and dyes (Scanes, 2018). The dry weight proportions of hides from pig and sheep are 6% and 9%, respectively (Jayathilakan *et al.* 2012). However, in sheep it is 9%. According to data published by FAOStats in 2015, the total production of animal fiber-based outputs in 2013 were 9.19, 8.12, 2.13, 1.26, and 0.96 million metric tons of sheepskin, cattle hides, goatskins, buffalo hides and horse hair, respectively. The main countries that participate in the total leather production worldwide are China, U.S.A. and India with 1.63, 1.12 and 0.94 million metric tons respectively in 2013 (Scanes, 2018). The countries that mainly contributed to sheep skin and goatskin production in 2013 were New Zealand, that produces 7.33 million metric tons of sheep skin; and China, which produced 364.000 and 392.000 metric tons of sheep and goat skin respectively (FAQStats, 2015).

1.3. TANNING

For over a decade, Tanning process is applied to skin and hide employed to improve their mechanical, physical, biological and chemical features. The various phases use technologies to process pre-tanning, tanning and post-tanning methods (Maina *et al.*, 2019). It is believed that leather products have been used by humans for millennia (Carbonell *et al.*, 1999). Our ancestors used animal skins to create shelter, clothes, musical instruments and parchment, as well as more recently for sports products synthesis and to make gelatin (Scanes, 2018). Leather production has depended on the evolution of new types of skin scrapers (Carbonell *et al.*, 1999). The most basic definition of tanning is the process by which raw skin converts into leather (US EPA, 2015). To employ these processes, each is divided into sundry stages, such as deliming and bating, in order to obtain tanned leather (Covington and Wise, 2019). Bating and deliming are different, but they

are remarkably used in sequence. The purpose of deliming methods is to eliminate the lime found on the skin after applied liming process (Maina *et al.*, 2019). It has to be applied so that the skin does not become stiff and hard. The deliming process is achieved in two stages involving slightly alkaline and slightly acidic (Prayitno, 1998; Puvanakrishnan *et al.*, 2019). Once the first stage is finished, the bating stage has already begun, so there is interference between the two stages. Indeed, the former process relies on the latter to achieve the desired result. Basically, both of them are carried out under the same condition by using alkaline solutions.

Conversely, previous studies mentioned that the deliming stage can also be processed under acidic conditions. Nevertheless, some previous studies mentioned that bating process can also be carried out under acidic conditions. Bating has evolved to become an important process that converts the structure of skin and removes some skin components such as non-collagen materials (Covington and Wise, 2019). Also, by using specific enzymes within bating agents, the collagen fibers are perfectly opened (Kesarwani, 2015). These enzymes are extensively used in leather tanning industry; they can be isolated from microorganisms experimentally, including from yeast, fungi and bacteria (e.g. that obtained from *Rhizopus oligosporus*). Bating can furthermore synthesize lipase and proteinase under specific conditions such as pH 3-7 and 25-30°C, though it ideally works in optimum pH 5.5 (Rauf *et al.*, 2010; Irfan *et al.*, 2011; Waseem *et al.*, 2018). The aforementioned studies have shown how tanning achieves the required physical properties because it modifies the natural weave of skin. Therefore, the fine spaces between fibrils remain, so that the fibrils stay free to move over one another. This condition improves leather flexibility. If not produced so, the fibrils attached to each other within bundles would not be free to move, thus making the leather too stiff (Haines and Barlow, 1975). These spaces are easily noticed by using microscope in longitudinal sections. Industrially, tanning processes can be conducted by different approaches including chromium tanning, vegetable, aldehyde, smoke, alum, synthetic chemical and oil tanning processes. The most common method is chromium tanning process (FAQStats, 2015; Scanes, 2018). Recently, the world has witnessed an increase in the use of leather products, especially in pharmaceutical and cosmetic products such as capsule, conditioners, clean sponges and shampoos, as well as other hair outputs (Scanes, 2018). Leather manufacturing can be abbreviated by the following scheme:



Scheme 1.1. Simplified tanning processes (New England Tanners Club, 1994; Marmer, 1996; Ockerman and Hansen, 2000; FAQStats, 2015; Duraisamy *et al.*, 2016; Scanes, 2018).

1.4. SKIN PROTECTION PROCESSES

Skins and hides are produced from meat factories as secondary products. After slaughter process, the animal carcass must be completely bled out; otherwise, opportunistic bacteria transmitted from the environment and the carcass itself can damage the meat, skin and skin suffixes. Grain stains were seen on the final leather products, showing evidence of how the raw skins were broken down by proteolytic enzymes produced from microorganisms. This reduces there sistance of leather, and especially in summer this risk will be higher (Kılıçoğlu, 1991; Kanagaraj *et al.*, 2005). To solve these problems experts recommend using different methods such as keeping skin in an environment under 20°C, using antimicrobial materials to prevent growth of microorganisms, adding chemical materials to skins within half an hour after cutting, protecting by acids and salts, and cooling leather before processing. Also, skin can be kept at low temperatures during storage and transport methods (Adminisand Money, 2003).

Skin without preservation allows the growth of microorganisms immediately and causes skin deterioration within 5-6 h (Vankar and Dwivedi, 2009). According to results reported by Wolfgang and Hans-Ulrich (1990), gelatine liquefying bacteria, *Bacillus mycoides*, *Pseudomonas aeroginosa*, *Staphylococcus aureus*, *Escherichia coli* and *Enterobacter areogenes* are the preponderant bacteria found in soaking baths. The conventional dry salt sprinkling process has been used to keep skin through storage, transport and stacking of leather. The objective of this method is to reduce water content and prevent bacterial deterioration caused by normal flora found on the animal skin. (Vankar and Dwivedi, 2009). On the contrary, the massive amounts of salt used in leather industries cause increased levels of total dissolve salts (TDS) and chloride ions (Cl⁻) in effluents, thereby leading to harmful water pollution (Vankar and Dwivedi, 2009). Researchers have started to explore new techniques and materials to preserve skins (e.g. instant sodium sulphates salt). Researchers mentioned that sodium sulphates salt is not as dangerous a pollutant as NaCl and reduces the concentrations of TDS and TSS in water waste. It also efficaciously minimizes the level of chlorine in water waste (Vankar and Dwivedi, 2009). Some processes used in leather preservation and protection are explained below.

1.4.1. Salt Preservation Method

As mentioned above, salts have been used for skin and hides preservation by sprinkling salt on the surface of skin. Skin has high water content, so it is an environment highly favorable for growth and reproduction of harmful microorganisms (Wu *et al.*, 2017). To prevent microorganisms from damaging the skin's protein, brine should be applied in a short time after the animal has been bled. A sufficient amount of NaCl should be spread equally all over the raw hide. After the skin is salted, NaCl enters the layers and removes water from tissue. About 20% of the weight of leather brine should be used. There is no need to wash leather before using salt, but if the skin has more moisture the salt amount should be increased in order to remove the most amount of water (Money, 1994). Salt saturation should be $\geq 85\%$ and the content of water in salted leathers between 40 to 48%. Researchers found that when water content is less than 40%, it causes rehydration. When the moisture ratio is more than 48%, bacterial deterioration occurs even in salted skin (Bailey, 2003). This conventional method is not sufficient to save and protect the pigskin; instead of salt-cure, dehydrating solvents can be used (Ockerman and Basu, 2014). Considering the type of salt used in cure-salt and the weight of hides (kg), the duration will vary. For instance, the brine time is 16 h, but this is variable depending on skin thickness and salt concentration (Money, 1994). One per cent of NaF (sodium fluoride) or 1% of $C_{10}H_8$ (naphthalene) + 1% H_3BO_3 (boric acid) were used with NaCl in salt-pack curing method to preserve hides. Other chemicals, zinc oxide or sodium met bisulfate, for instance, have been used perfectly to preserve skin (Ockerman and Basu, 2014). In the procedure developed for fish skin preservation, the skin was pickled with 10% NaCl for 10 minutes, then repeat pickling step by using 1% HCl (hydrochloric acid) for one hour and 40 minutes, followed by an overnight brine bath (Songchotikunpan *et al.*, 2008; Karthikeyan *et al.*, 2011). The pH was adjusted at 2.0 by using HCl 1%, then the skin left another night to complete pickling. After that the skins were taken and saved for 10 days. Following the first pickling the skins had to undergo another pickling method in a bath with 8% NaCl and 80% water for 10 minutes; pH was adjusted by adding 1% of HCl solution and left soaking for 90 minutes. Previous studies discussed the side effects of NaCl on water pollution (Peng *et al.*, 2014) and alternate salt curing methods such as a salt-less curing approach by using different chemicals plus NaCl including NaCl+EDTA (Wu *et al.*, 2017). This method has been used by Russell *et al.* (1997) in South Africa to keep skin and hides 40% NaCl, 25% EDTA and 35% medium coarse. NaCl+Silica Gel a technique developed

by Kanagaraj *et al.* (2005), at the turn of the century by adding 5% silica gel to absorb the moisture. Five per cent of NaCl+ 0.5% sodium meta-bisulphite (SMBS) is an important method to reduce bacterial load and skin preservation without any effects on the quality of leather (Kanagaraj *et al.*, 2005a). Using a formula of 5% NaCl and 5% boric acid, the concentration of NaCl has been reduced from 40% to 5% and applied directly (Kanagaraj *et al.*, 2005b).

1.4.2. Drying

Drying has been demonstrated to be one of the oldest and most common preservation methods applied in open areas to reduce the relative humidity of hides. During this process the content of moisture should not be less than 10%. If the moisture level is too low it can cause damage in the leather, such as breaks in the leather fibers. This damage affects subsequent production stages (T.C. Milli Eğitim Bakanlığı, Küçükbaş Hayvan Derileri, 2011). Drying is not appropriate for cattle skin, in contrast to other animal skins such as goat, lambs, sheep, and rabbits. Generally, there are four drying techniques that can be applied to remove part of moisture from skin: 1) Hanging is a simple method using large drying oven, but it can also have negative effects on the quality of leather; 2) Toggling method uses special hooks to attach the leathers for drying; 3) Pasting is a popular method that use sa specially conditioned drying oven with 40% humidity, 60-66 degrees centigrade for 4-6 hours; 4) Vacuum technique preserves skin moisture content at 10-12% even after drying (Ockerman and Basu, 2014).

1.4.3. Refrigerating

This method depends on the climate and country (Woods *et al.*, 2002). Leathers can be stored for 21 days using refrigeration. They apply this technique in Australia by using cold water and ice to store kangaroo skins in cool weather. Refrigeration does not cause any type of pollution. In contrast to salt-curing method, it can be used for all kinds of skins and does not cause hair loss either. The skin should be in normal temperature before processed in tanneries. There are a few factors that may affect the refrigeration process, such as climate temperature and storage time. For instance, while 20°C is sufficient for one day in the refrigerator, a 3-week storage would require temperature under 0°C (Adminis and Money, 2003).

1.5. PROCESSING LEATHER STAGES

Various approaches, specific industrial and natural materials are used to preserve and soften skins while preventing spoiling by microorganisms (Koloka and Moreki, 2011). Regardless the type of tanning process and materials used in the first step, tanning has been considered an essential stage in leather production and heavily employed since old civilizations (Frederik *et al.*, 2011). Moreover, tanning is a process that induces fibrous proteins in skins to make durable structures against enzymatic biological deterioration, heat and mechanical stress (Musa and Gasmelseed, 2013). Commercially, chrome and vegetable tanning are the most used methods. Tanning stages and what happens at each stage have been extensively studied by many researchers as explained below:

1.5.1. Pre-tanning Stages (Beamhouse Processes)

Generally, these stages deal with preserved and salted skins as briefly explained below:

1.5.1.1. Wetting (Softening)

Water can be used in this step depending on the type and size of skin and amount of water limited. Indeed, this stage needs from 5 to 24 hours to complete the removal of non-collagenous proteins. During soaking step various types of wetting agents and surface active factors are used, such as sodium carbonate, sodium hydroxide and bactericides. The procedure that used in this step is 110% water at 22°C with 0.05%, 0.05% and 0.2% of Rokafenol, Biocid and Na₂CO₃ respectively. The main objective is to remove unwanted materials such as proteins, blood, additives, salts, dirt and manure. Also it eliminates oddly shaped and unworkable places of skin. Furthermore, it aims to remove urine and other extraneous material (T.C. Çevre ve Şehircilik Bakanlığı, Deri Sektörü, 2012; Daniels, 2003; Thanikaivelan *et al.*, 2005; Heidemann, 1993).

1.5.1.2. Unhairing Processes

Animal hair can be removed treating skin with lime [Ca(OH)₂] and sodium sulfide (Na₂S), or by using bacterial enzyme such as protease enzyme. The requirements of this stage are room temperature and duration between 14 to 24 hours. The ratio of water used is 80% at 28°C. Chemicals are 0.1% Rokafenol, 0.9% Erhait, 0.5% CaOH for one hour; 0.7% NaHS, 0.6% Na₂S and 2% calcium hydroxide for 20, 60 and 60 minutes, respectively. Lastly, repeat the floating step by water with different ratio of water 100% for 30 minutes, and calcium hydroxide with

1.5% for 18 hours. Actually, this step is applied to remove hair and wool from skin by breaking down the disulfide bridge (S-S) in keratin structure. Also in this way, non-structural proteins are eliminated (Frendrup, 2000; Heidemann, 1993; T.C. Milli Eğitim Bakanlığı, Kıl Giderme, 2008; Nugraha *et al.*, 2022).

1.5.1.3. Fleshing

By using piping machines on tough hypodermis, undesirable meat layers and hair are removed. The output of this process is called *tola* or sometimes “green fleshing”. It helps to remove excessive organic materials by washing with 200% water at 25°C for fifteen minutes, then repeating the last washing step at 30°C. An alternate method is called splitting process, by which the skin is divided into three layers. Successful execution of the fleshing process ensures that the chemicals to be applied in subsequent operating steps spread and penetrate the skin homogeneously (Ludvik and Buljan, 1998; Thanikaivelan *et al.*, 2005; T.C. Milli Eğitim Bakanlığı, Kıl Giderme, 2008; Mikkilineni, S., 2021).

1.5.1.4. Deliming (Descaling)

Descaling process is carried out by using ammonium salts (e.g. ammonium chloride and ammonium sulfate) as well as sodium bisulfide to achieve two goals: make pH level suitable to do subsequent processes and to clean hides of worthless substances. The process is achieved by using 100% water at 30°C, 0.5% Sodium metabisulphite $\text{Na}_2\text{S}_{20} 5$, at 30 min, and drain float. The major objectives of this step are to remove the lime used in the previous step and eliminate the preliminary descaling and alkalis from skin. This stage is pH neutral and reduces the level of pH raised during the previous step (Leafe, 1999; Lama, 2010; T.C. Milli Eğitim Bakanlığı, Kireç Giderme, Sama, Yağ Alma, 2009; Mikkilineni, 2021).

1.5.1.5. Bating

Bating uses special proteolytic enzymes such as trypsin or chymotrypsin instead of chemical materials. This stage has been considered one of the most important stages in tanners. These types of enzyme require a specific pH range to work (8-9 as optimum pH). Non-structural proteins in skin and epidermis residues are eliminated during this step. It has been explained that the obtained leather is soft, smooth and more elastic (Daniels, 2003; Leafe, 1999; Lama, 2010; Mikkilineni, 2021).

1.5.1.6. Degreasing

In some countries the fat layer in sheepskin is so thick that it is necessary to undergo a degreasing process in order to make skin ready for following steps. Degreasing also removes excessive natural fat (Covington and Wise, 2019).

1.5.1.7. Pickling

In this process the skin is prepared using sulfuric acid, formic acid solution and salt with pH 0.1-1.1. Through this process, 100% water, 10% salt, 1-2% sulfuric acid and 0.2-0.3% bactericide are added to the skin, which is kept in a boiler for 3-4 h. The procedure used in this step is 80% water, 20°C, 7% NaCl for 15 min. 1% HCOONa +1.2% H₂SO₄, (1:10) three times per 10 min. The aim is to permit the materials used in tanning process to penetrate the skin. To control acid swelling NaCl is used as a pH regulator (Kılıçoğlu, 1991; Saravanabhavan *et al.*, 2004; Leafe, 1999; Thanikaivelan *et al.*, 2005; Berber, 2009).

1.5.2. Tanning Processes

This is generally the main step in leather manufacture because it prevents leather from putrescence and converts the skin into stable form. Also it provides thermal stability of collagen matrix. The major objectives of this process are to make the skin resistant to temperature and microorganisms, to separate fibers from each other, and to connect the fiber bundles together. The resultant leather is durable and flexible (T.C Milli Eğitim Bakanlığı, Tabaklama, 2007).

1.5.2.1. Chrome Tanning

The chrome tanning method helps increase leather quality and produces desirable characteristics such as good flexibility, high thermal stability and perfect stability (Dasgupta, 2004; Elshahat *et al.*, 2012). It is demonstrably the most popular method used in approximately 85% of leather industries around the world (Haroun *et al.*, 2009). Sometimes it is called the “wet blue method” (Covington, 2001). Chrome tanning uses chemical materials as a heat stabilizer and non-leaching substance such as chrome Cr (III) and Cr (VI), which the American Environmental Protection Agency (EPA) rates as non-toxic. The mode of action of chrome tanning is a reaction between chromium salts and reducing sugar (such as maltose sugar) and sulfuric acid. The results are reduce chromium salts to chrome that can be directly added to hides with concentration 1.5-3%

(Ockerman and Basu, 2014). According to the method employed by Nugraha *et al.* (2022), a chromium was added for one hour with dose of about 8%, and sodium bicarbonate (baking soda) was added for 6 hours. This helps to increase the pH content to 3.4-3.6 and increases the interaction between collagen and chrome (Ockerman and Basu, 2014). On the other hand, tanning and pickling are the main source of salts in tannery wastes due to both of them using a lot of NaCl in order to reduce or prevent acid swelling. Chrome tanning and pickling are conducted in the same drum (IPPC, 2001; Lama, 2010).

1.5.2.2. Vegetable Tanning

One of the most important tanning processes is conducted by using bio-materials of vegetable origin such as root, leaves, wood and bark. Vegetable or bark tanning method can improve leather quality, durability and water resistance (Wemegah, 2014). It is preferred over other methods due because it produces high quality, unique, soft, heat resistant, flexible and non-putrefiable leather (Mikkilineni, 2021). Generally it is slower than chromium tanning, with a processing duration of several months (Ockerman and Basu, 2014). However, bark tanning finally results in firmer leather than chromium process. By using tannic acid extracted from plants or by mixed plant parts directly with skins and hides, the resultant leather is characterized by solidity and air permeability, another variation on this method is called “green tanning” (Kuria *et al.*, 2016). Tannin is an acid normally found in plants with molecular weight 500-20,000, and the chemical structure is polyphenolic compounds (Haroun, 2006). These compounds can be extracted and used in leather processes because their biodegradation is friendly to the environment. Also, the produced wastes are likewise easily degradable (Musa and Gasmelseed, 2013). Furthermore, there are other methods used in skin/hides tanning processes, including using cattle, deer and buffalo brains to give the leather greater wash ability and softness (Richter and Dettloff, 1995). Also, oils isolated from aquarium animals such as shark, cod, seal and whale can be used in leather tanning (Ockerman and Basu, 2014). The main objective of using animal brains is to provide oils that make the skin pliable. Microorganism enzymes such as protease, lipase, and amylases are furthermore applied in leather production, decreeing processes and skin softening respectively (Oijala, 2013). Moreover, mineral tanning substances such as zirconium (Zr) and aluminum are used in some cases. Particularly Zr is used with silica in pH 2 to tan skin speedily (Ockerman and Basu, 2014). Lastly, other methods are used such as formaldehyde, glutaraldehyde, silica, quinone, iron technique, and tungestun procedure.

1.5.3. Post-tanning Processes

According to Aravubdhan *et al.* (2007) post-tanning is a group of processes including wringing, neutralization, shaving, re-tanning, lubrication, coloring, as well as drying. The main objective of these processes is to grant additional features for leather. The leather made from these steps is called a “crust” (Saravanabhavab *et al.*, 2003a; Rao *et al.*, 2003).

1.5.3.1. Wringing

Wringing, is a process that removes the wrinkles from hides and expels the extra moisture. This method is achieved by a special wringing machine (Ockerman and Basu, 2014).

1.5.3.2. Neutralization

In this process the pH of leather is increased to 5-6 in order to allow chemicals to penetrate leather during retaining, lubrication, dyeing and finishing processes (T.C. Milli Eğitim Bakanlığı, Nötralizasyon, 2010).

1.5.3.3. Shaving and Splitting

In these processes a very sharp and flexible knife is used to detect the normal thickness of leather and obtain 8mm thickness. However, this may differ from one animal to another, even from one area to another on the same animal carcass (Marmer, 1996; Ockerman and Basu, 2014). The famous methods used in the U.S.A. are called hair side splitting and flesh side splitting (Tanners' Council of America, 1983; Leather Industries of America Research Laboratories, 1991; Ockerman and Hansen, 2000).

1.5.3.4. Re-tanning

Post-tanning or second tanning is a process by which leather is re-tanned by using the same tanning material from the previous step in order to change the leather's quality and increase its solidity. It takes only one or two hours of re-tanning to improve leather textures (Ockerman and Basu, 2014; Kleina *et al.*, 2021). Re-tanning plays an important role in preventing leaching of chrome and increasing the stability of chrome. At the end of this process, leather will have obtained a uniform structure.

1.5.3.5. Fat Liquoring (Lubrication)

The purpose of lubrication is to obtain suitable softness, increase flexibility, prevent the fibers in leather structure from sticking together and hardening, and to prepare leather for the dyeing stage. During fat liquoring, soluble oils are applied before the leather is dyed (T.C. Milli Eğitim Bakanlığı, Yağlama, 2009; Ockerman and Basu, 2014). The main materials used to achieve this process are mineral, animal oils and vegetable oils such as soaps, glycerin, waxes, egg yolk, neatsfoot oil and sulfated castor oil (New England Tanners Club, 1994; Marmer, 1996; Ockerman and Basu, 2014). This method depends on the type of animal skin. Cattle skins generally require less fat liquorthan does pigskin for instance.

1.5.3.6. Setting Out

Setting out is a process in which the leathers are contracted to attain acceptable moisture (60%) and increase their smoothness, softness and strength. This method also shrinks the leather and reduces grease (New England Tanners Club, 1994; Marmer, 1996; Ockerman and Basu, 2014).

1.5.3.7. Dyeing (Coloring)

By using dyes such as metalized, acid, basic and direct, the inner sides and outer sides of hides are stained before being converted into leather products. To attain an acceptable color, the leathers are usually dyed inside large blends (Ockerman and Basu, 2014).

1.5.3.8. Drying

For drying, hides are left outdoorsto undergo one of four techniques: hanging, toggling (by using special hooks), pasting (the popular method), and vacuum drying (Ockerman and Basu, 2014).

1.5.4. Finishing Process

In leather factories this method is the final step to confer the product's aesthetic value. Finishing also improves the quality and shape of resultant leathers through six steps including conditioning, staking and buffing (Aravubdhan *et al.*, 2007; Ockerman and Basu, 2014).

1.6. STRATEGIES TO PREVENT BACTERIAL GROWTH IN LEATHER PRODUCTION

As we generally know, the unique structure of animal skin foments bacteria growth and harmful effects that subsequently affect the leather industry. Researchers have paid close attention to this problem and used chemical and physical approaches in order to minimize the number of bacteria in the skin and in skin liquor process. The aim of using antibacterial agents is to prevent bacterial growth on skin, and a good biocide does not cause any problem during leather processing. Among the chemical materials that have been used and are quietly successful in salty water are hypochlorite, chlorine and quaternary amines.

Nevertheless, the high content of organic material renders these chemical substances ineffective in high salinity liquid. For this reason, ozone has been suggested as the most novel antibacterial factor. Despite the high antibacterial effectiveness of ozone, some factories cannot afford the high cost. In addition, other materials have been stated as antibacterial agents such as peroxide and hexavalent chromium compounds. These are excellent biocides, but their toxic side effects should be carefully considered (Kallenberger, 1984; Berber, 2009). Furthermore, many antibacterial agents have been used directly against proteolytic extreme halophilic bacteria isolated from leather brine. Some examples of these bactericides are naphthalene, 1-2 dichlorobenzene, trichloro-S-triazinetriene, sodium sulfate, sodium bisulfite, acetic acid, K-dimethyl dithrocarbonate, alkyl phenol ethoxylate, ortho-benzyl-para-chlorophenol, sodium-ortho-phenyl-phenate, thiocyanate and benzothiazole (Bailey and Birbir; 1993).

1.7. THE SIDE EFFECTS OF LEATHER INDUSTRIES ON TANNERY PERSONNEL AND THE ENVIRONMENT

The leather industry has escalated quickly and gained ground in the last decades. Hides are a byproduct of the meat industry and become raw material for leather production (Langmaier *et al.*, 1999). Due to the high amounts of water consumption during tanning processes, organic offal, odor and contaminated water are leather industrial wastes that cause pollution in rivers and water supplies. Leather industries have been demonstrated themselves to be high pollution sources (Haile *et al.*, 2018). Human beings have been using animal skin for daily requirements such as tools, shelter and clothing since 5000 BCE. Hides are useful because of their high stretch and tear strengths, insulating and water-resistant qualities. By 2027 it is predicted that global

leather industries will dramatically increase to a worth of USD 3061.1 billion in the world economy (GIA, 2021). However, there are big problems still facing the leather industry. Some of the biggest are environmental pollution caused by processing chemicals, solid and liquid waste discharges.

Turkish leather industry is considered an important sector in Turkey's economy. According to the export-based production model, Turkey's leather industry is mentioned among global markers. Nonetheless, due to the increasingly competitive market, with lower quality and prices coming from different countries, Turkish leather industry is facing challenges such as competition pressure (Turanl, 2018). The leather sector provides 2% of total employment in Turkey. The average wage of these workers is 50.93 TL (9.93 Euro) per hour (Republic of Türkiye Social Security Institution). Statistically, the numbers of students who attend leather industry-related vocational training have decreased in recent years; therefore, there may be negative consequences on qualified labor force (Turanl, 2018). Pollutants such as sodium sulfide and lime, which are extensively employed during the liming phase for unhairing hides, also indirectly affect the economy. The hair removed from skin by using sodium sulfide and lime has an unpleasant odor and makes highly alkaline byproduct that is dangerous for the environment and creates a waste disposal issue (Zambare *et al.*, 2007; Malathi and Dhar, 1987). Another factor that has to be considered is the amount of water used during leather manufacturing. Hence, leather industry workers and technologists have to use other environmentally friendly methods for hide maintenance and leather goods production (Tu *et al.*, 2022). Furthermore, the traditional process of leather manufacturing is to use a lot of salts (nearly 30-40%, w/w) and then large amounts of water to remove salts and rehydrate the hide. This causes excessive water usage and environmental pollution (Covington and Wise, 2019). Initially, to convert animal skins to leather, extensions such as hair and wool must be removed (depilation) by using natural substances such as enzymes. These are isolated from microorganisms or artificial chemical materials such as Na_2S , NaOH , NaHS , $\text{Ca}(\text{OH})_2$. These substances have pros and cons. Their strong alkaline consequences affect the follicles of hair and wool, causing keratin degradation. Lime solution and alkaline sulfide cause the concentrations of biological and chemical oxygen demand alike (Tu *et al.*, 2022). Furthermore, when the pH of wastewater falls below 9.5, H_2S is produced. This gas has been demonstrated to be very flammable and poisonous. In fact, the beam house process causes increases in the levels of BOD and COD in the liquid waste, even up to 70%. Even worse,

these substances interfere with the oxidation of wastes. Drainage wastewater from tanneries also causes air pollution because some leather factories burn animal hair to dispose of it. Similarly, chemical substances cause effluent pollution (Scanes, 2018). Keep in mind that not all chemicals used in leather processes are absorbed by the animal skin. On the contrary, a massive amount of these substances run off the leathers in effluent streams that cause water pollution. According to previous data observed by Ozgunay *et al.* (2007), nearly 30 to 35 m³ of water used to wet one ton of hide produce 600 kg of waste. There are different chemicals applied in tanneries (Figure 1.11) (Kanagaraj *et al.*, 2015).

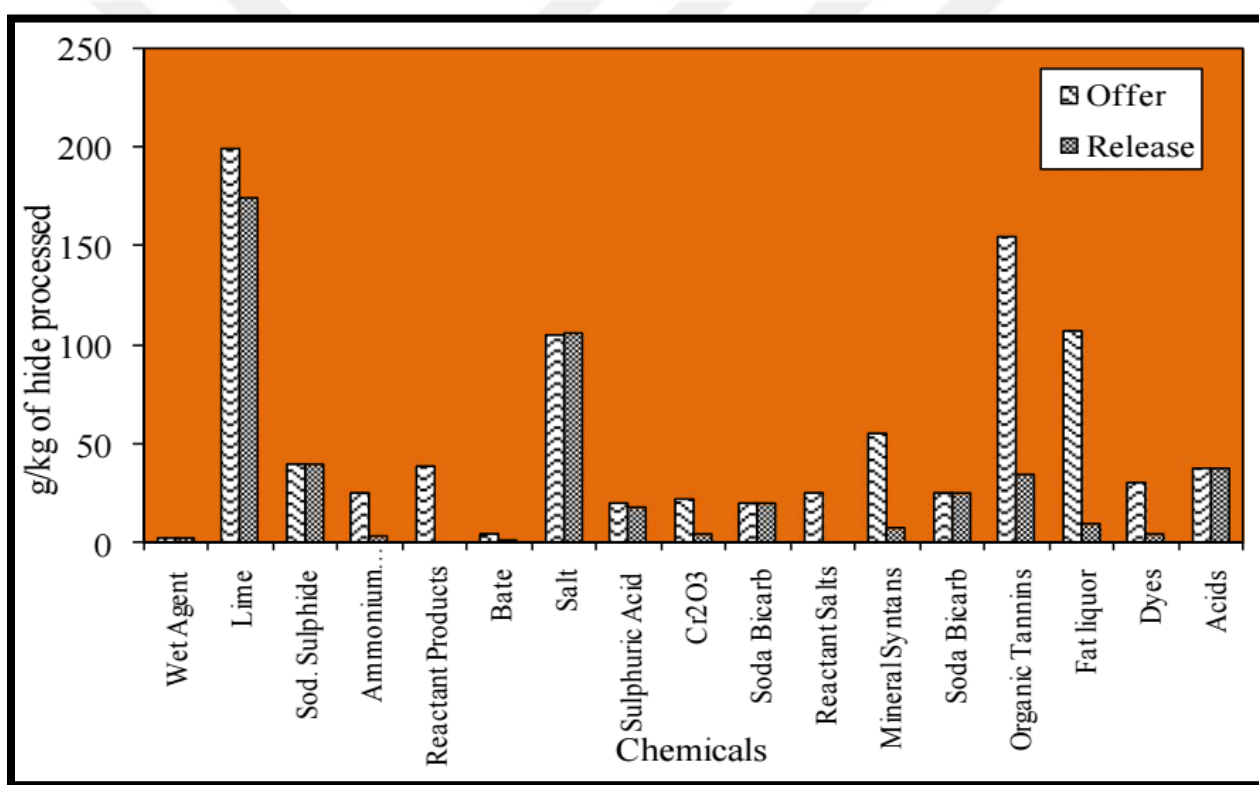


Figure 1.11. Chemical substances applied in leather industries (Kanagaraj *et al.*, 2015)

Normally, animal skins are inhabited by different kinds of microorganisms that are natural inhabiting or acquired from the external environment (Anderson, 1945; Berber and Birbir, 2010). The microorganisms found on animal skin have the ability to grow and rapidly reproduce because animal hides are rich in nutrients and the environment is characterized by adequate temperature, pH, and high moisture content (Kayalvizhi *et al.*, 2008; Oppong *et al.*, 2006).

Various types of bacteria have been isolated from hides and skins, most of them nonpathogenic types (Hanlin *et al.*, 1995; Wilson, 2005). But some of these isolated species are considered as pathogenic or opportunistic pathogenic. For example, *Escherichia coli* and *Bacillus cereus* were isolated from buffalo hides during ambient preserve (Shede *et al.*, 2008). *Pseudomonas aeruginosa* and *Staphylococcus aureus* were isolated by Bitlisli *et al.* in 2004. Furthermore, several experiments have been conducted on leather industry wastes and isolated pathogenic bacteria during discharge stages, especially those found in tannery effluents. Hence, these types of bacteria have caused not only water pollution but also infectious disease for tannery personnel (Lama *et al.*, 2013).

1.8. DESCRIPTION AND CHARACTERIZATION OF HALOVERSATILE BACTERIA

1.8.1. Halophilic Microorganisms

Halophiles are microorganisms able to grow in diverse concentrations of NaCl ranging from 0.2-0.5 % to more than 5%. According to their ability to thrive in salted environment and the concentrations of salts in which they grow, they can be classified as slightly halophilic bacteria, which can grow optimally in the 0.2-0.5 NaCl content; moderately halophilic bacteria that can thrive optimally in NaCl concentration 0.5-2.5; and extreme halophilic, which can grow and thrive in a wide range of NaCl (2.5-5.2 M) (Kushner and Kamekura, 1988). As seen in Figure 1.13, halophiles are distributed among three domains: *Archaea*, *Bacteria* and *Eukaryota* (Madigan *et al.*, 2009). Ecologically, halophiles are distributed in different ecosystems and isolated from a variety of extremely saline environments such as hypersaline soils, salted food, marine sediments (Dead Sea) (Wie *et al.*, 2015), salt lakes (e.g. Lake Urmia) (Mehrshad *et al.*, 2015a), Tuzkoy salt mine (Mutlu and Güven, 2015), solar salterns (Boujelben *et al.*, 2012) and Rambla Salada, Murcia (Luque *et al.* 2014). Naturally, no type of organism can survive for a long time in hypersaline environments unless it has an appropriate adaptation method that allows the bacterial cells to grow and thrive. The most substantial impact of hypersaline environments on bacterial cells is losing large amounts of water from the cell and causing bacterial shrive as shown in Figure (1.12A). Halophilic organisms can adapt in hypersaline environments such as thalassohaline and athalassohaline by using high salt and low salt strategies (Figure 1.12B). In high salt method the bacteria cell tries to accumulate as much salt as possible inside the cell in order to make a type of equilibrium between inside and outside the cell (Mongodin *et al.*, 2005).

Many kinds of archaeal species can survive in high salt concentration by making an osmotic balance between internal and external environments by pump potassium ions in the cell, as show in Figure 1.12C. Many archaeal cells accomplish this by using active pumping protons in the cell membranes, ATP synthase and N/H ions antiporter (Kixmuller and Greie, 2012). This occurs in archaeal species belonging to the *Halobacteriaceae* family and some bacterial species belonging to the *Halanaerbiales* family (Oren, 2008).

In the low salt method, sometimes called osmo-protect, bacteria and archaeal cells produce suitably protected organic materials such as betaine (*Actinopolyspora halophila* and *Halomonas elongate*) (Nyyssölä et al., 2000), ectoine (*Methyloarculamarina*, *Methyloarculaterricola*, *Methylophaga* sp.), hydroxyectoine (*Sporosarcina pasteurii*), glutamine, glutamate (*Halobacillus halophilus*), sucrose (*Synechocystis* sp.) and glycine betaine (*H. elongate*) (Mongodin et al., 2005). The high salt process is used by extreme halophilic microorganisms, while the second strategy is used by moderately halophilic bacteria such as *Halobacillus halophilus* (Saum and Müller, 2008), *Halorhodospira halochloris* (Galinski and Trüper, 1982), *Actinopolyspora halophila* and *Halomonas elongate* (Nyyssölä et al., 2000).

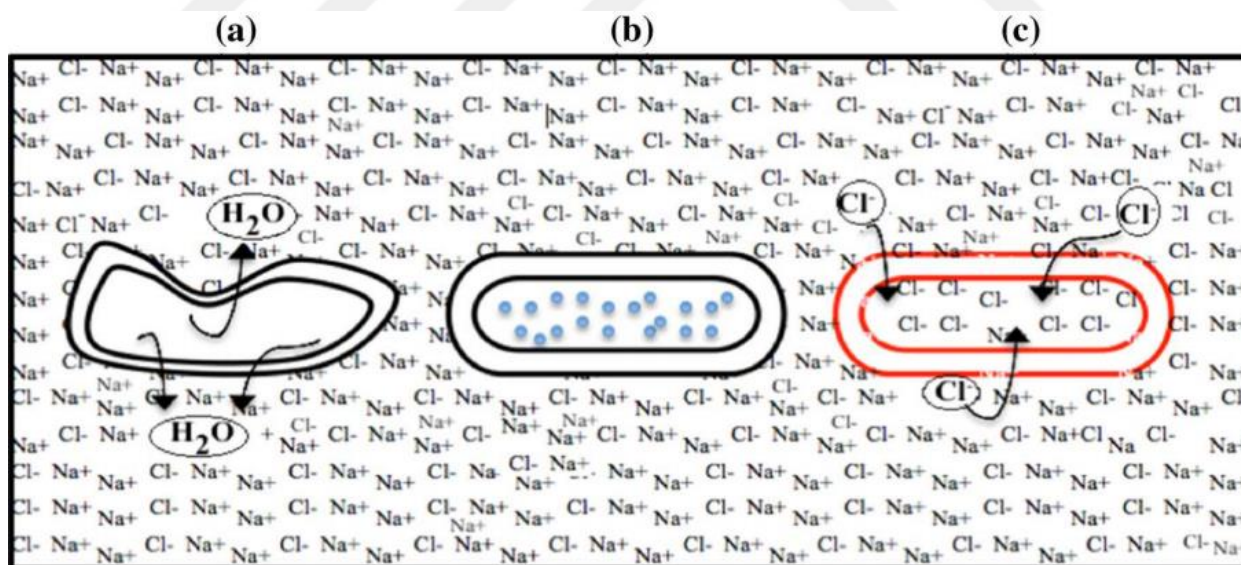


Figure 1.12. (A) The effects of high salt concentrations (losing cell water and cellular structures), (B) Protect cell by producing organic solvents, (C) Pumping ions into cell in order to make balance between inside and outside environment (Bell, 2012).

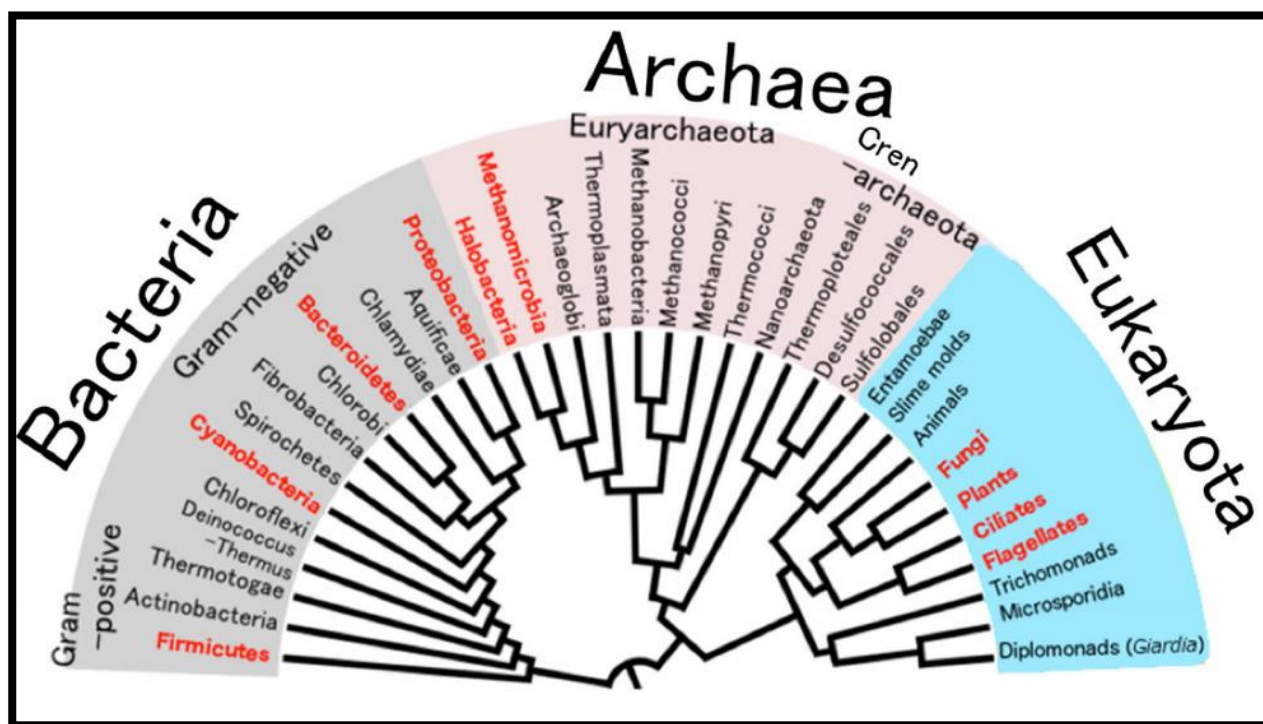


Figure 1.13. The distribution of halophilic microorganisms in three kingdoms (Ciccarelli *et al.*, 2006; Edbeib *et al.*, 2016).

1.8.2. Salinity and its Effect on Bacterial Activity

Salinity has been shown to be one of the important factors as well as decisive environmental determinant on the presence and biological activity of microorganisms. Salinity also affects microbial community composition and dispersal, thereby exerting a high evolutionary selection pressure among microorganisms (Lozupone and Knight, 2007). Relatively few microorganisms have been able to survive and adapt in extreme environments despite the high salinity. To avoid the aggregation of protein, many halophile and almost all halotolerant microorganisms tend to expend energy to exclude excessive salt content from cytoplasm. However, this type of acclimatization represents one of the most difficult environmental barriers for macro and microorganisms alike (Lee and Bell, 1999; Vermeij and Dudely, 2000; Logares *et al.*, 2009; Forster *et al.*, 2012). Of particular note is that the halophilic microorganism uses various sources of energy and covers aerobic and anaerobic species. It depends on different pathways to survive and can be classified into methanogenic, sulfate reducing, fermentative and phototrophic species (Ollivier *et al.*, 1994; Oren, 2002). Salinity has detrimental effects on some bacteria. These

species most likely perish if not counteracted. Similarly, the vast majority of halophiles are so fragile that when they are placed in distilled water they are unable to survive outside their salt-conditioned environments. This transfer may induce change in osmotic conditions and cause cell lysis. The high concentration of NaCl in the environment affects aerobic prokaryotes by limiting the availability of oxygen for respiration. It also causes many harmful effects on the macromolecules (protein, nucleic acid and others). Proteins aggregate and denature, as well lose their specific tertiary structure (Bolhuis, 2008; McGenity and Oren, 2008). The effects on enzyme activity were explained by Madern *et al.* (2000) and Lea *et al.* (2018). Nevertheless, there are many flourishing communities of microorganisms (bacteria, archaea and microbial eukaryotes) that have been identified in different salty environments worldwide (Boetius, 2009). Halophilic cells have different kinds of mechanisms to cope in high salinity and therefore resist losing water. Charged amino acids on their surfaces is one such coping mechanism. Microorganisms have developed two main ways to survive in high salinities and prevent desiccation through hyperosmotic. Both mechanisms occur by increasing the internal osmolarity of the cell.

One process is observed in some archaea, almost all halophilic bacteria and fungi as well. The organism accumulates organic compounds in the cytoplasm osmoprotectants which are known as compatible solutes. These can be either synthesized or accumulated from the environment (Santos and Da Costa, 2002). In the second process, many methanogenic microorganisms, including slightly and moderately halophilic organisms, have used mixed type of osmoregulation such as K⁺ ions accumulation inside the cell along with positively and negatively charged molecules as that employed by extreme halophiles (Martin *et al.*, 1999). This type of osmotic adjustment is specific to extremely halophilic archaeal and extremely halophilic bacteria such as *Halobacteriaceae*, *Salinibacter ruber*, respectively (Oren, 1999).

1.8.3. The Microbial Diversity in Saline Regions

A comparatively wide range of bacteria and archaea are halophilic, so the majority of the prokaryotic population is in hypersaline environments (Oren, 2002). The bacteria studied most extensively in order to understand the biochemical and physiological strategies used to survive in extreme and moderate salty lakes are *Halomonas elongate*, *Salinivibrio costicola* and *Halomonas israelensis* (Vreeland *et al.*, 1980). Liaw and Mah (1992) isolated *Haloanaerobacter*

chitinovorans from a solar saltern as well *Cyanobacteria* and members of the *Halobacteriaceae* from high salt concentration environments. According to statistical data in Türkiye, about 70-80% of leather production depends on processing the raw hides of sheep and goats. For that reason, the leather industry in Türkiye is considered an important sector that plays a role in economic development. Traditionally, salt is used in leather preservation to prevent fresh raw skins from damage immediately after peeling. Healthy animal skins are environment for numerous microorganisms named normal flora. These microorganisms do not harm the skin matrix. However, after skinning the normal flora starts to cause damage and lysis to the skin (Berber, 2009). Leather production has been classified as a highly polluting industry because of its negative influence on the environment (Kanagaraj *et al.*, 2002; Kanagaraj *et al.*, 2015; Dixit *et al.*, 2015).

The climate of some environments is represented by very cold and extreme drought. Due to these conditions, seawater is undergoes physical and chemical changes that change saline into hypersaline water bodies. Haloversatile bacteria inhabit extreme environments such as Antarctic saline lakes. Haloversatile bacteria are able to grow in high-salt environments (15% M NaCl to about 30% M NaCl). An example of this phenomenon is *Halomonas* species (James *et al.* 1990; Ventosa *et al.* 1998; Okamoto *et al.* 2004). Mixed microorganisms such as *Halobacterium lacusprofundi*, *Methanococcoides burtonii*, *Flavobacterium salegens*, *Halomonas subglaciescola*, *Halomonas meridiana*, *Flectobacillus glomeratus*, *Carnobacterium funditum*, *Carnobacterium alterfunditum*, *Flavobacterium gondwanense*, and *Vesiculatum antarcticum* were isolated by Ellis-Evans (1985), who discovered that these microorganisms have ability to cope under extreme conditions, including cold. Whereas many types of extremely halophilic archaea, extremely halophilic bacterium and anaerobic moderately halophilic bacteria are characterized by osmotic adjustments such as *Halobacteriaceae*, *Salinibacter ruber* and *Halanaerobiales*, respectively, the vast majority of microorganisms are not (Oren, 1999; Anton *et al.*, 2002; Oren and Mana, 2002). For that adaptation to live in high saline environment, it has necessarily undergone massive genetic alterations. As a prerequisite it also accumulates specific small molecules (Brown, 1976; Brown, 1990; Ventosa *et al.*, 1998).

1.8.4. Microorganisms Found in Hypersaline Environments

Hypersaline environments pose major challenges to their microbial residents. Based on experimental results, there were different isolates of haloversatile halophiles isolated from extreme environments such as the saline lake, Suribati Ike, near Syowa Station in Antarctic (Naganuma *et al.*, 2005). Saline lakes are distributed into seven groups according to depth related spreading. Among these, six represent new species of *Halomonas* and *Marinobacter*. These results explain the extreme conditions in Antarctic saline lakes responsible for selective unique haloversatile halophiles. Generally, hypersaline environments are inhabited by only a few types of organisms. The maximum salt concentration for living vertebrates such as *Tilapia* spp. is 10% above this value. Some types of invertebrates such as *Artemia salina* and *Dunaliella salina* can also survive in these environments. Similarly, these hypersaline ecosystems are inhabited by members of prokaryotes including *Haloanaerobiaceae*, *Halobacteriaceae*, *Methanogens*, *Cyanobacteria* etc. Despite the extreme conditions, the hypersaline ecosystem is indeed an adequate environment for Haloversatile bacteria growth because of the abundance of organic matter from dead cells and metabolites of other halophilic organisms such as algae and plants that precipitate after death. An example of this phenomenon occurs in hypersaline lakes of Africa during the rainy season (Reynaud and Roger, 1981). It is furthermore worth mentioning that bacteria, halophilic algae and some types of other invertebrates were reported as a source for oxidizable materials in haloversatile ecosystem. Organic compounds such as lipids, sugars and protein accumulate in significant quantities in these environments as a result of cell decomposition (Liaw and Mah, 1992). There are many studies applied on aerobic and heterophilic bacteria isolated from sea water. According to results, these bacteria can grow at 20-30% concentration of NaCl contents (Forsyth *et al.*, 1971). In addition, there are several types of bacteria such as *Halomonas elongate* (Vreeland *et al.*, 1980), *Halomonas israelensis* (Rafaeli-Eshkol and Avi-Dor, 1968), *Halovibrio denitrificans*, *Marinobacter* sp., *Pseudomonas halophila* and *Gammaproteobacteria* (Grant, 2004; Sorokin *et al.*, 2006) that were isolated from salty environments on Bonaire (Netherlands Antilles), in the Dead Sea, Mongolia, and Great Salt Lake, respectively. The researchers reported that halophilic bacteria found in salt can contaminate hides (Lloyd, 1929; Anderson, 1954; Kallenberger, 1985; Bailey and Birbir, 1993; Ventosa and Nieto, 1995; Birbir, 1997; Akpolat *et al.*, 2015; Caglayan *et al.*, 2015; Birbir *et al.*, 2015; Bilgi *et al.*, 2015). The same is true for various types of halophile microorganisms isolated

from salts and salted animals such as fish, sheep and goat. Published reports have shown the most common genera *Salinivibrio*, *Pseudomonas*, *Alteromonas*, *Alcaligenes*, *Acinetobacter*, and *Flavobacterium* were isolated from Alicante and Huelva Saltpans in Spain (Rodríguez-Valera *et al.*, 1985; Rodríguez-Valera, 1986; Márquez *et al.*, 1987; Rodríguez-Valera, 1988). Furthermore, *Alteromonas*, *Marinomonas*, *Salinivibrio* and *Actinomonas* types were isolated from the Atacama Desert in the north of Chile (Prado *et al.*, 1991). Birbir *et al.* (2004) isolated members of archaea such as *Natrinema*, *Haloarcula*, *Halobacterium* and *Halorubrum* from Tuzköy Salt Mine in Turkey. Within domain Archaea, a large number of halophilic genera that have high tolerance for different levels of salinity (e.g. *Halobacterium* and *Halococcus*) have distinct acidic proteins due to their ability to resist the denaturing by NaCl in hypersaline environments (Oren, 2014). Up to now, 15 genera of *Halobacteriaceae* are recognized as extreme halophilic archaea and isolated from saltern crystalliser ponds. Some of these are *Halogeometricum*, *Halococcus*, *Haloarcula*, *Halobacterium*, *Haloterrigena*, *Halorubrum* and *Haloferax* (Gutierrez *et al.*, 2002). Similarly, domain *Bacteria* have been reported as halophilic and halotolerant when isolated from different salt sources. These include a wide range of taxa such as *Bacteroidetes*, *Spirochaeta*, *Proteobacteria*, *Cyanobacteria*, *Actinobacteria* and *Firmicutes* (Grant, 2004). The genera of *Proteobacteria* are present in saline lakes. Aerobic organotrophic species *Halomonas*, *Chromohalobacter*, *Pseudomonas*, *Alteromonas*, and *Vibrio* members of the phylum *Gammaproteobacteria* are able to survive in saline environments (Pagaling, 2007). In Spain, halophilic bacterium such as *Salinibacter ruber* was isolated at 22.4-37% NaCl concentration from Bras del Port salt flats in Alicante (Antón *et al.*, 2002). *Salinibacter ruber*, a red bacterium, may constitute up to 25% of the prokaryotic population.

1.9. ENZYMES

Enzymes are proteins that achieve biochemical reactions within cells. They are extensively used to accomplish various reactions in industrial sectors. Enzymes are characterized by many crucial characteristics: very high catalytic activities, minimal undesirable byproducts, they are not depleted during reactions, high specificity, low cost, and can be obtained from different sources (microorganisms, animal cells and plants). Research shows that enzymes produced from microorganisms are preferred in contrast to enzymes obtained from plant and animal cells (Wiseman, 1987). The most substantial enzymes used in industry are protease, lipase, xylanase,

cellulase, and amylase (Kiran *et al.*, 2006). Enzymes have large-scale use in different sectors including wine, cheese, bread, and starch production. In view of an ideal role of enzymes in chemical reactions, they are used not only in biology but also in different fields such as biochemistry, engineering, and chemical engineering. In the second half of the twentieth century, enzyme research relevant to metabolic products and microorganisms drew a lot of clear attention (Beg *et al.*, 2001).

1.9.1. Halophilic Enzymes

It has been reported that metabolic materials isolated from microorganisms that have ability to grow in extreme environments can be used as enzymes and pigments. High salt concentration profoundly affects bacterial cells and causes dehydration in cells. Losing all water and subsequent loss of their cellular structure and function significantly affects cellular enzymes (Edbeib *et al.*, 2016). They have the ability to be stable in extreme halophilic environments and remain active for a long time. Cellular enzymes are not affected by high contents of salt ions. Halophilic enzymes have an important role in biotechnology activities in comparison to non-halophilic enzymes. Also, they are characterized by their ability to remain active in alkaliphilic and thermophilic environments (Amador *et al.*, 2009). All these features lead scientists to pay close attention to applied halophilic enzymes in textile and food production sectors, as well as detergents synthesis (Gomes and Steiner, 2004; Setati, 2010). Halophilic enzymes were isolated and purified from different environments with NaCl concentrations. Protease was isolated from *Halobacterium* sp. (Elbanna *et al.*, 2015) and *Alkalibacillus* sp. (Mesbah and Wiegel, 2014a) with NaCl concentrations 30% (w/v) and 6% (w/v) respectively. Amylase was produced by *Halobacillus* sp. (Amoozegar *et al.*, 2003) and *Amphibacillus* sp. (Mesbah and Wiegel, 2014b) in NaCl contents (5%, w/v) and (0-25%, w/v), respectively. Xylanase was isolated by Wejse *et al.* (2003), Prakash *et al.* (2009), and Poosarla and Chandra (2014) respectively from *Oceanospirillum linum*, *Chromohalobacter* sp. and *Gracilibacillus* sp. Esterase and cellulose were isolated from *Bacillus cereus* with 3-11% NaCl and *Gracilibacillus* sp. with 7.5-17.5% NaCl. Moreno *et al.* (2012) isolated DNase from *Halobacillus* sp. with high concentration of NaCl.

1.9.1.1. Lipase

Lipase is a hydrolysis enzyme with ability to catalyze the hydrolysis and transesterification of fatty acids esters. Lipase and esterase are categorized within the same hydrolyses family owing to the fact they share a lot of structural and functional features (Güçlü, 2019). Lipase is less kinetic than esterase and also differs in specificity of substrate (Chahinian *et al.*, 2009). Lipase isolation and purification from halophilic bacteria and archaea has been a saleable attendance lastly (Guzmán *et al.*, 2008). Esterase shows activity across a wide range of temperatures ranging from 40 to 100°C and in a wide range of pH ranging from 7 to 12, with optimum temperature and pH at 70°C and 11 respectively (Karan *et al.*, 2012). The results by Karan *et al.* (2012) exhibited esterase has ability to resist protease, and a variety of organic solvents also proved esterase can maintain stable activity in 58% NaCl. Esterase can provoke hydrolysis in short chain fatty acids that dissolve in water and consist of less than 10 carbon atoms; however, they do not hydrolyze insoluble triglycerides composed of more than 10 carbon atoms. Microbial esterase and lipase have been studied extensively and used in many sectors such as medicine, pharmaceutical material production, detergents production, and bioremediation (Wang *et al.*, 2016). Lipases were isolated from many sources such as plants, animals, fungi, bacteria *Salinivibrio* sp. (Amoozegar *et al.*, 2008) and *Natronococcus* sp. (Boutaiba *et al.*, 2006).

1.9.1.2. Protease

Proteases are the most useful enzymes in industry. Protease forms approximately 65% of the enzymes used in various sectors such as detergents, pharmaceuticals, nutrients production and decomposition of industrial wastes (Haki and Rakshit, 2003). Proteases are classified according to their mode of action and the location where they can break down the peptide bonds between amino acids. The first kind is exopeptidase, which has the ability to destroy peptide ponds between amino acids at the end of the protein chain. The second type is endopeptidase, which destroys peptide bonds between amino acids in the middle of protein chains (Rao *et al.*, 1998; Haki and Rakshit, 2003).

1.9.1.3. Cellulase

Enzymes have the ability to form colloidal solution in water, which can easily be separated by the same methods used for other proteins. These types of approaches have been usually considered long and complex, depending on the differences between solvents (Li and Yu, 2011). Due to the progressive demand on the enzyme in various aspects such as food, leather, paper, textile, medical sectors and etc., the researchers try to develop and discover new production and purification methods for enzymes (Xin and Hui-Ying, 2012). Generally, enzymes are produced from most organisms (viral, bacteria, fungi, plants, and animal). Cellulase is an enzyme produced from fungi and bacteria to decompose cellulose to its original form (glucose- β or oligosaccharides). Therefore, it is used in a variety of applications (Zou and Plank, 2015). Cellulase is classified into two groups: 1) Catalytic domain group (CD), which induces the hydrolysis of β -1,4 glucosidase among glucose molecules; and 2) CBMI (Carbohydrate Binding Model), which lacks stimulating activity (Tonozuka *et al.*, 2014). Cellulose is considered one of the most available organic molecules and is characterized by harsh structure in contrast to starch. This being the case, three enzymes must be provided in order to completely decompose cellulose. These are endoglucose, exoglucose and β -glycosidase.

1.9.1.4. Xylanase

Xylan is a complex polysaccharide consisting of xylose molecules connected by β -1,4-glycosidic bonds; xylan forms 30-35% of the dry weight of the plant by virtue of its inclusion in the structure of the cell wall as a hemicellulose polysaccharide (Beg *et al.*, 2001). It has wide industrial uses, such as paper production, and is also considered an additive substance for enhanced nutritional value in domestic animal feed. Moreover, it is used to increase the quality of grain products and is used alongside pectinase in fruit juice industry (Beg *et al.*, 2001). Undoubtedly, the first step in xylan hydrolysis is achieved by the activity of xylanase; therefore, Actinomycetes are the main source for xylanase. This enzyme shows activity in a wide pH value range from 7.0-11.0 (Fagerstrom *et al.*, 2008).

1.9.1.5. Amylase

It is an important enzyme extensively used throughout a variety of industrial methods such as food production, textile, paper production, pharmaceutical material and detergent production because of its ability to break down the α -1,4-glycosidic bonds in starch structure. This capacity to destroy complicated carbohydrates makes it favorable for extensive use in medicine and analysis chemistry (Vaidya *et al.*, 2015). The amylase group includes three kinds: α -amylase, β -amylase and gluco-amylase (Kadziola *et al.*, 1998). Amylase is isolated from various halophilic archaeal species such as *Halobacterium salinarum*, *Halomonas meridian*, *Nesterenkinia halobia*, *Haloferax mediterranei* and *Halobacillus* sp. (Karan *et al.*, 2012). It is also isolated from bacterial species such as *Thalassobacillus* sp. (Li and Yu, 2012b), *Nesterenkonio* sp. (Shafiei *et al.*, 2011), *Chromohalobacter* sp. (Prakash *et al.*, 2009a) and *Bacillus* sp. (Kiran and Chandra, 2008).

1.9.1.6. Chitinase

With an important role in chitin hydrolysis, chitinase is proven to be one of the important enzymes that recently applied in industrial applications. It is considered one of the enzymes that is thoroughly used by some microorganisms, especially by bacteria, to provide sources (nitrogen and carbon). The main structure of chitin is a polysaccharide containing N-acetyl-D-glucosamine linked by β -1,4 glycoside bonds. A coding gene, ChiN1 coding for chitinase enzyme has been found in a halophilic archaea *Halobacterium salinarum* strain NRC-1 and it is a structural homologue of its non-halophilic equivalent microorganism. This gene was also found in *Haloarcula japonica* strain TR-1, homogenously expressed with optimum activity at NaCl concentration 1M (Hatori *et al.*, 2006).

1.10. UTILITY OF HALOPHILIC BACTERIAL ENZYMES IN INDUSTRIAL AND CLINICAL ASPECTS

Halophilic microorganisms for a long time have been extremely used in biotechnological aspects such as industrial, clinical and food production sectors because they can be satiated in inordinate conditions such as extravagant salted environments, drought pond and very cold niches. Those qualities make them important in extreme technological processes such as high temperature and low-level water in high salt environments. Additionally, halophilic microorganisms are studied

not only for their crucial roles in ecology but also because they are considered an important source for many types of extracellular enzymes which can be applied in different sectors including leather, food, laundry detergent, biochemical and biomedical industries (Rothschild and Manicini, 2001; Moreno *et al.*, 2013).

1.10.1. Application in Leather Industries

Leather production is an ongoing process passing through stages of skin preservation, soaking, hair removal, calcification, descaling, sama, oil removal, pickling, tanning, dyeing and finishing. During some of these stages, microbial enzymes play an important role as alternative agents (Birbir *et al.*, 2016). Due to the adverse effects of chemical factors used in tanning, such as sodium sulfate, and subsequent rises in the environmental pollution, researchers found bacterial enzymes can attain these functions as they also increase the quality of leather (Rao, 1998). Various types of useful enzymes have been originated from microorganisms and used in leather processing, particularly in hair removing step (Khambhaty, 2020). Lipase has been used in leather tanning, especially during wetting stage in order to break down the lipids in hypodermis layer. Protease is applied to remove the hair from hides and make pores in skin in leather manufacturing (Güçlü, 2019). As far as known, the main structure of skin and hair is protein, so protease has influence in removing non-collagenous materials such as albumins and globulins (Rao, 1998). Protease is isolated from *Bacillus* sp. and used as alternative agent in order to decrease the percentages of BOD, COD, total nitrogen and sulfides in effluents resulting from leather processes (Dettmer *et al.*, 2013). But these enzymes produce spoilage on skin surface; hence, they are mixed with sulfide in order to raise the efficiency. Moreover, the application of enzyme should be under control and requires suitable pH and temperature. However, the prices of enzyme are high in contrast to those of conventional chemical items (Sujitha *et al.*, 2018).

1.11. BACTERIOCINS

Bacteriocin is a peptide biosurfactant derived from some types of bacteria. It deeply affects other types of bacteria that are close to the same species (Pato *et al.*, 2022). Basically, not all bacteria types have ability to produce bacteriocins, so the production of bacteriocins from bacteria has substantially taxonomic features. Researchers have paid close attention to bacteriocin production and identified sources in Gram-positive and Gram-negative bacteria, as

well as from archaea (Drider *et al.*, 2011). Bacteriocins have been ribosomally synthesized and may have bacteriostatic or bactericidal antimicrobial effects (Simons *et al.*, 2020). Generally, bacteriocins are used in food synthesis as a normal bio-preservation compound. The proteinaceous structure of bacteriocin is characterized by low molecular weight, so they cannot be applied in the therapeutic sector because they are sensitive to stomach acidity and also degradable peptides by proteases. Bacteriocins are used in cheese production in order to inhibit or kill pathogenic bacteria that causes spoilage in milk products (Barboza *et al.*, 2022; Aljohani *et al.*, 2023). Apparently bacteriocin is not used in therapeutic sectors with antibiotics. In order to prevent conflicts and confusion with antibiotic therapy, it could also be banned because of potential health risks for humans (Deraz *et al.*, 2005). Nevertheless, some researchers have classified bacteriocins as alternative antimicrobial agents for antibiotics and approved them for use in human therapy (Garneau *et al.*, 2003).

According to Klaenhammer's results (1993), 280 isolates were isolated, but only 5% of them have ability to produce bacteriocin. Eight different types of bacteriocin have been genetically detected (Skaugen *et al.*, 2003). By genetic and biochemical methods, various types of bacteriocins have been distinguished. Two naturally unique bacteriocins have been discovered: nisin A and nisin Z (Sahl *et al.*, 1987). Bacteriocins are categorized according to different criteria such as their mode of action, the type of bacteria produced, the sequence of amino acid in proteins, and their spectrum of effects. Generally, there are two types of bacteriocins. The first is produced from bacterial species and affects other genetically approximate bacteria. This type has an arrow spectrum effect. The second type of bacteriocin has wide spectrum effect against bacteria such as nisin that are produced from *Lactobacillus lactis* subsp. *lactis* and affects different types of Gram-negative bacteria (Moreno *et al.*, 2000).

The name "bacteriocin" is derived from the name of produced bacteria by adding suffix (in) or (cin) at the end of its name. For example, pediocin and colicin have been isolated from *Pediococcus* and *Escherichia coli* (Hanaa, 2008). Some types of bacteria have ability to produce more than one type of bacteriocin. *Lactobacillus acidophilus*, for example, can produce lactocin A, lacticin and acidophilin. In this case the nomenclature of bacteriocins depends on a specific character or particular trait that these bacteriocins possess (Gilliland, 1989). There are many types of bacteriocins produced in the laboratory, and their antibacterial activity was tested against other types of bacteria. The results were reasonable and acceptable. Torodov and Diks

(2004) isolated Plantaricin (35d) from *Lactobacillus plantarum*. It affects *Ayromonas hydrophilia*. Previous studies by Caridi (2002) explained the roles of bacteriocins isolated from *Lactobacillus paracasi* subsp. *paracase* against *Escherichia coli*. Thermophilin bacteriocin was isolated from *Streptococcus thermophilus* in order to inhibit *Escherichia coli*, *Yersinia*, *Staphylococcus typhimurium*, *Pseudotuberculosis*, *Listeria monocytogenus* and *Bacillus* (Ivanova *et al.*, 1998). Two types of bacteriocins, ST26MS and ST28MS, were produced from *Lactobacillus plantarum* and inhibit the growth of *Acinetobacter baumannii* and *Escherichia coli* (Lade *et al.*, 2006).

1.12. ELECTRON MICROSCOPE

Although the light or simple microscope enhances human eyesight considerably for discerning fine details, it is still unsuitable for magnifying small objectives. For this reason, we have seen a push to develop new systems in which electron beams are applied to provide us high quality pictures and magnification details (Bora, 2015). Scanning electron microscope (SEM) is used to magnify pictures by using electron beams as an illumination source accelerated in a vacuum instead of light sources. This advantage is the main difference between SEM and optical microscope (Kapakin, 2007). The principle of SEM depends on interactions between wavelengths that scatter from objects and matter to allow the image to be made in nanometer scales. Having a large depth SEM produces high magnification and creates high resolution images. Producing high quality image with high clarity and richness of details mainly depends on the resolution. The resolution of SEM is 2-20 Angstrom, while the resolution power of optical microscope is 0.5-1 (Kapakin, 2007). Generally, the Electron Microscope (EM) is divided into two types: Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) (Bora, 2015).

1.12.1. Scanning Electron Microscope (SEM)

The working principle of SEM depends on focusing the beam of accelerated electrons with a high voltage on the sample under scrutiny. The signals emit from the interaction between the atoms of material and the electron beam applied onto the material surface during material surface scanning. Figure 14 shows how these signals have an effect on appropriate sensors and transmission during a tube and aggregate on screen of cathode rays after passing through a

specific amplifier (Bora, 2015). SEM produces fictional three-dimensional images. Their depth of focusing is 300 more than an optical microscope and the visibility rate of SEM is 20-100,000 (Kapakin, 2007).

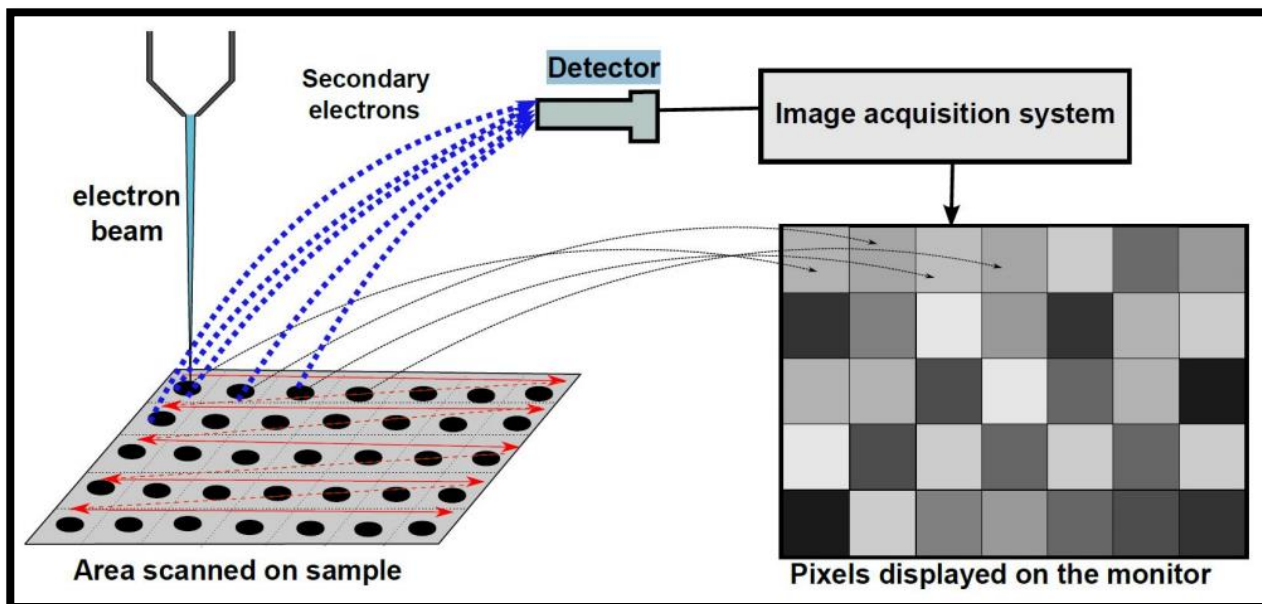


Figure 1.14. Formation of an image in scanning electron microscope (Maturi, 2013).

Four types of electrons and emissions have been produced as a result of the interaction between the fallen electron beam and the surface of specimen (Figure 1.15). This produced electron and emission is classified as follows:

- a- Light emissions (cathode rays) are emissions produced as a result of interaction between the outer surface of the specimen and the electron beam. These types of emissions give us information about the electronic structure of the specimen's outer layer.
- b- Primary electrons (Pe) belong to the electronic beam and give us information about the specimen's outer surface.
- c- Scattered electrons reflect from the surface of specimen.
- d- Secondary electrons (Se) come from the atoms of the specimen and give us information about the surface of specimen (Schatten, 2013).

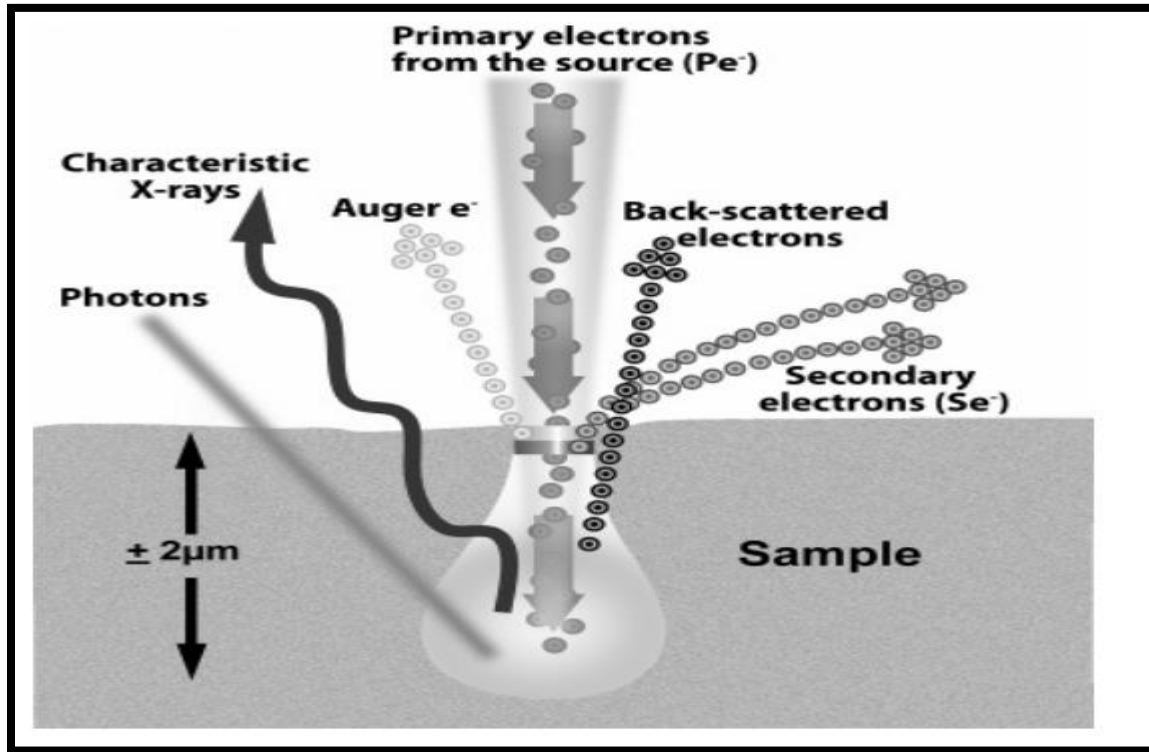


Figure 1.15. The production of electronic series from the inelastic collision between specimen and electronic beam (Schatten, 2013).

The energy of electrons in electronic beam ranges from approximately 1 to 40 kV. The material should be examined in a vacuum environment. The electronic beam produced from an electronic source will be focused after passing through a series of magnetic lenses, thereby producing an electronic series from the specimen as a result of inelastic collision between the electronic beam and the material. These produced electrons are called secondary electrons, which are finally installed by the secondary electronic sensor and transformed into images (Yalçınkaya, 2010).

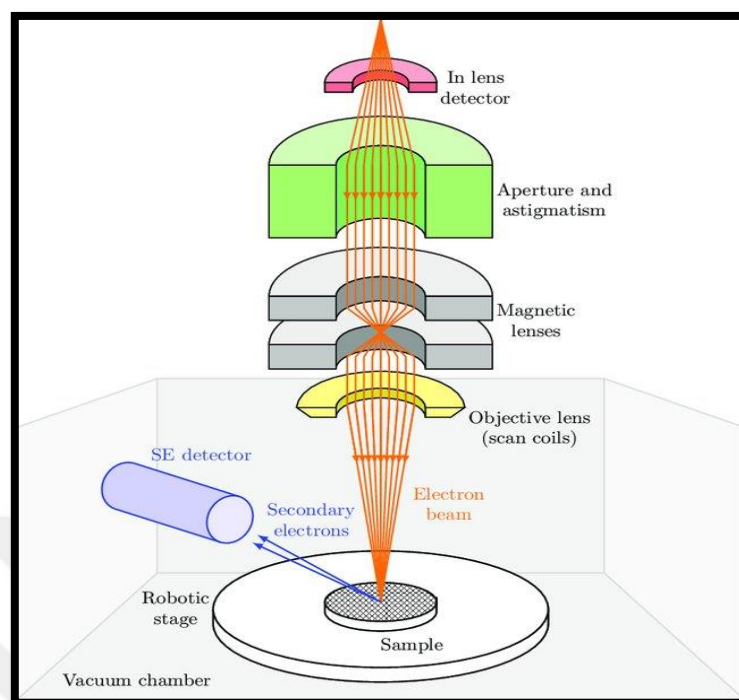


Figure 1.16. Major components of SEM (Kudryavtsev, 2017).

1.12.2. Transmission Electron Microscope (TEM)

TEM is used in a variety of sciences such as biology, botany, cytology, medicine (forensics, anatomy, microbiology, biochemistry, taxonomy, pathology), earth sciences and material sciences. It displays samples magnified more than 600,000 times. To be properly examined, samples should be thin, taken by ultra-microtome at 60 nm. TEM is used to examine samples at the ultra-structure level. Microorganisms, cell membrane, organelles, building blocks and nucleus are examined under TEM. This type of electronic microscope depends on passing a beam of electrons through the very thin section of specimen. Then, a two-dimensional image is created on phosphor-fluorescent screen. The clearness of the image is directly proportional to the quantity of electrons passed through the specimen (Kapakin, 2007). TEM is such a sensitive system that it can take pictures on an atomic level. As in SEM, electrons are used in TEM; however, the electrons pass through the specimen in this mechanism. The electrons passed through the specimen exhibit on a screen to thus produce an image of the specimen's structure. The energy of electrons in a TEM beam ranges approximately almost from 100 to 500 kV. The high-energy electron beam focused on a specimen after passing through a system of lenses is then further transmitted through another series of lenses before finally being projected onto a

specific screen. The examined specimen should be paper thin, with a thickness not exceeding some hundreds of nanometers, so that the electronic beam passes cleanly through the specimen (Yalçinkaya, 2010). The result of TEM examination depends on the method by which a specimen has been prepared (Karakulak, 2010).

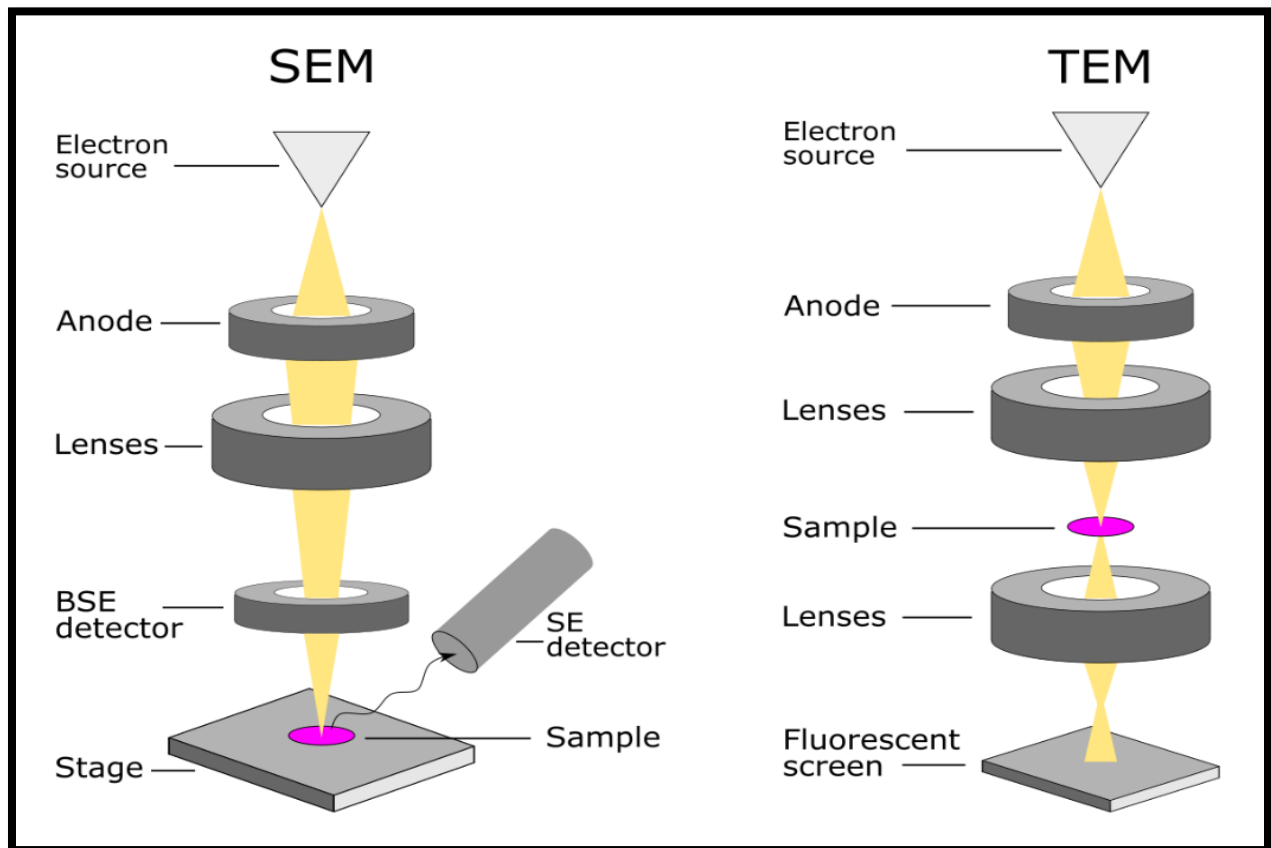


Figure 1.17. Comparison between the components and methods of SEM and TEM (<https://anapath.ch/electron-microscopy-2/>, Date of Access, May 2023).

1.13. GENERAL INFORMATION ABOUT *FIRMICUTES*, *ACTINOMYCETOTA* AND *PROTEOBACTERIA*

In our study, 25 salt samples were collected. From these samples, haloversatile isolates underwent DNA extraction and DNA sequencing depending on the sequence of 16S rRNA gene and the phylogenetically similar species were determined. In the salt samples the phyla *Firmicutes*, *Actinomycetota* and *Proteobacteria* were present (Figure 1.18).

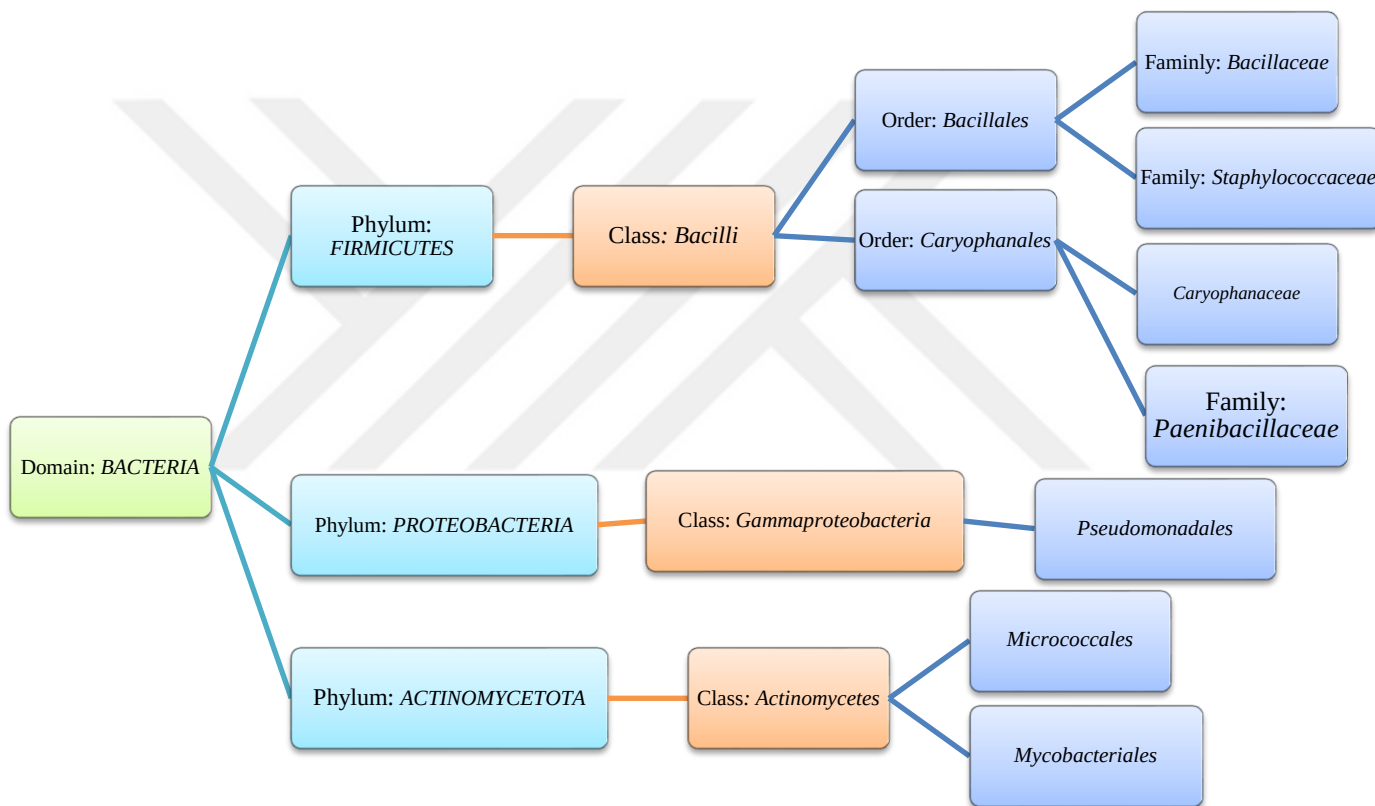


Figure 1.18. The phyla *Firmicutes*, *Actinomycetota* and *Proteobacteria*.

1.13.1. The Main Characteristics of Phylum *Firmicutes*

Madigan *et al.* (2015) described this phylum and divided it into six classes which including (*Clostridia*, *Mollicutes*, *Erysipelotrichia*, *Bacilli*, *Thermolithobacteria* and *Negativicutes*). All genera that belonging to this branch are Gram-positive, the percentage of GC in species inside firmicutes are low; also this branch involves lactic acid bacteria. Depending on previous studies and according to the major features of our isolates were classified after had compared the name of phylogenitically similar isolates in NCBI and GeneBank, all our isolates were classified depending on the results that exhibited and fixed in BacDive-The Bacterial Diversity Metadatabase (dsmz.de) (BacDive).

1.13.1.1. Genus *Bacillus*

The shape of *Bacillus* sp. cells is rod that is frequently observed in pairs or chains, a Gram-positive bacterium. These cells typically possess rounded or square ends and are commonly associated with the presence of a single endospore. Endospores typically have an oval shape, although they may occasionally appear round or cylindrical, and possess a remarkable ability to withstand harsh environmental circumstances. The process of sporulation remains undisturbed in the presence of atmospheric oxygen (Holt *et al.*, 1994).

1.13.1.1.1. Species: *Bacillus haynesii*

Bacillus haynesii has been isolated from different environments especially from soil. It has been identified as a species that exhibits facultative aerobic behavior, possesses Gram-positive characteristics, and is capable of generating spores. Furthermore, it is classified as a mesophilic bacterium, and its growth is observed within a pH range of 5.5 to 10. The colonies generated on the agar exhibit circular forms and possess a creamy white coloration (Parte *et al.*, 2020).

1.13.1.1.2. Species: *Bacillus sonorensis*

Bacillus sonorensis, a Gram-positive bacterium capable of generating spores and exhibiting facultative aerobic characteristics, has been obtained from soil samples collected in the Sonoran Desert, specifically from Tumamoc Hill. The mesophilic bacteria exhibit the ability to generate an irregular colony with a distinct yellow color (Palmisano *et al.*, 2001).

1.13.1.1.3. Species: *Bacillus paralicheniformis*

A bacterium with the characteristics of being Gram-positive, facultatively anaerobic, motile, rod-shaped, and capable of generating endospores was isolated from a fermented paste made from soybeans. The strain's closest phylogenetic relationship, as determined by analyzing the 16S rRNA gene, was shown to be with *Bacillus sonorensis* (Dunlap *et al.*, 2015).

1.13.1.1.4. Species: *Bacillus licheniformis*

It is a bacterium that has the ability to produce endospores and can survive in both aerobic and anaerobic conditions. It is widely distributed in various environments, such as plant material, and soil. It has been classified under the *Bacillus subtilis* group and has been used in the synthesis of enzymes or antibiotics, such as bacitracin. The bacterium *Bacillus licheniformis* has the potential to induce food-borne poisoning as a result of its ability to synthesize the surfactant known as lichenysin (Yeak *et al.*, 2022).

1.13.1.1.5. Species: *Bacillus piscis*

A bacterium that has been isolated from a type of fish (*Dissostichus mawsoni*) in Antarctica, it is characterized by the ability to grow in a wide range of temperature (Lee *et al.*, 2019).

1.13.1.1.6. Species: *Bacillus subtilis*

Bacillus subtilis is a Gram-positive, catalase-positive species that has been isolated from soil as well as the digestive tracts of ruminants, humans, and marine sponges. It is also known as the grass bacillus. It has a rod-like structure and the ability to generate a durable endospore, which serves as a protective barrier, enabling it to survive in harsh environments. Traditionally, it has been categorized as an obligatory aerobe; however, there is evidence suggesting that it is actually a facultative anaerobe. *Bacillus subtilis* is used as a model organism for research into the replication of bacterial chromosomes and the development of bacterial cells. It plays a significant role in the manufacturing of enzymes and is also utilized on an industrial basis by firms in the biotechnology industry (Errington *et al.*, 2020; Paul *et al.*, 2021).

1.13.1.2. Genus *Staphylococcus*

The bacterium was initially discovered by Rosenbach in 1884. Subsequently, it has been obtained from various sources including the urinary system, skin, respiratory tract, as well as milk and milk products. The microorganism was cultivated in a medium containing 7.5% NaCl, and it demonstrates the ability to survive in environments characterized by low water activity

(Madigan *et al.*, 2015). It is a Gram-positive bacterium that does not have the ability to produce spores and is non-motile. However, it possesses the capacity to manufacture various colors, as well as enzymes such as lipase, protease, gelatinase, coagulase, phospholipase, and esterase. It includes 53 species and 28 strains (Güçlü, 2019). *Staphylococcus capitis*, *Staphylococcus haemolyticus*, *Staphylococcus taiwanensis*, *Staphylococcus saccharolyticus*, *Staphylococcus caprae*, *Staphylococcus hominis*, *Staphylococcus warneri*, *Staphylococcus pasteurii*, and *Staphylococcus epidermidis* were isolated from salt samples during this study and identified depending on biochemical tests and molecular diagnosis. It was Gram-positive bacteria, non-spore forming and non-motile. All species were utilized L-tyrosine, L-proline, and L-valine as a nitrogen source. While were catalase positive, all these mentioned species were oxidase negative.

1.13.1.3. Genus *Peribacillus*

The only species *Peribacillus frigoritolerans* belonging to genus *Peribacillus* had been classified as Gram-positive, demonstrating a preference for aerobic conditions and mesophilic temperatures. It has been obtained by isolation procedures from soil samples (Li *et al.*, 2014).

1.13.1.4. Genus *Lysinibacillus*

Lysinibacillus is a genus that belongs to the *Bacillaceae* family. Traditionally, it has been classed as a bacillus. The primary distinguishing characteristic of *Lysinibacillus* bacteria, as compared to other members of the *Bacillus* genus, is the presence of peptidoglycan containing specific amino acids (including: lysine, aspartic acid, alanine, and glutamic acid) (Rahi *et al.*, 2017). *Lysinibacillus telephonicus* is a bacterium belonging to the genus *Lysinibacillus*. It is characterized as an aerobic bacterium that is gram-positive, aerobic, rod-shaped, motile, and endospore-forming as well. This particular species was isolated from the screen of a cellular phone (Ahmed *et al.*, 2007).

1.13.1.5. Genus *Bhargavaea*

The only species *Bhargavaea ginsengi* belonging to genus *Bhargavaea* had been identified during this study. It is characterized by its Gram-positive nature, moderately halophilic bacterium, and does not have ability to motile. It was initially identified from the roots of a plant (ginseng) in Beijing/China (Qiu *et al.*, 2009).

1.13.1.6. Genus *Paenibacillus*

It is a facultative anaerobic genus, which has been classified as a Gram-positive *Bacillus*, yet it was separated in 1993 main genus that belongs to the family of *Bacillaceae*, it is endospore-forming bacteria (Ash *et al.*, 1993a). *Paenibacillus* has been isolated from different sources such as clinical samples, vegetable matter, larvae of insect, forage, rhizosphere, water as well as (Ouyang *et al.*, 2009; Padda *et al.*, 2017). The nomenclature of paenibacilli is derived from the Latin term “paene”, which indicates “almost”, hence translating paenibacilli as “almost bacilli” in literal terms. The genus encompasses several important species, including *Paenibacillus larvae*, which is responsible for the development of American foulbrood disease in honeybees. Another species, *Paenibacillus polymyxa*, shows the ability to conduct nitrogen fixation, making it valuable in agricultural and horticultural practices (Ben-Jacob *et al.*, 1992; Ben-Jacob *et al.*, 1994; Ben-Jacob *et al.*, 1995).

1.13.2. The Main Characteristics of Phylum *Proteobacteria*

According to the results of 16S rRNA gene sequence Phylum *Proteobacteria* categorized into six classes which including *Gammaproteobacteria*, *Deltaproteobacteriaveya*, *Betaproteobacteria*, *Deltabacteria*, *Epsilonproteobacteria*, *Zetaproteobacteria* and *Alphaproteobacteria*. There are different species in all above mentioned classes except *Zetaproteobacteria*, which involves only one an iron oxide marine genus (*Mariprofundus ferroxydans*). Similar metabolic activities such as photorophy, nitrification and methyl trophy have been observed in different classes of *Proteobacteria* although the phylogenetic classification. This type of diversity in *Proteobacteria* branch because the horizontal genes transfer (Madigan *et al.*, 2015). The members of this branch had been defined as the most metabolically diverse; also this branch has members very important in the medicine, agriculture and industrial sector. All members of this branch classified as Gram-negative bacteria, it may be anaerobic, microaerophilic and facultative aerobe. The shapes of the members are straight rods, coiled rods, filaments, cocci and spiral. The type of nutrition also various it could be phototrophic, chemolithotrophic and chemoorganotrophic (Madigan *et al.*, 2015).

1.13.2.1. Genus *Pseudomonas*

The genus *Pseudomonas* contains Gram-negative bacteria that are classified into the family *Pseudomonadaceae* in the *Gammaproteobacteria* class. The genus, consisting of 313 species, (Parte *et al.*, 2020) has significant metabolic diversity, enabling them to successfully inhabit a broad spectrum of ecological niches (Madigan *et al.*, 2006). The genus *Pseudomonas* has become a prominent subject of scientific investigation due to its ability to easily grow in laboratory conditions and the growing availability of genome sequences for various *Pseudomonas* strains. *Pseudomonas* has been recognized early in the history of microbiology due to their extensive distribution in water and plant seeds, particularly dicots. The nomenclature “*Pseudomonas*” was initially established by Walter Migula in 1894 and 1900 to classify a group of bacteria. Migula’s definition of this genus was characterized by broad criteria, including Gram-negative staining, rod-shaped morphology, possession of polar flagella, and the presence of some species capable of sporulation (Migula, 1984; Migula, 1900). It is a rod-shaped bacterium characterized by its non-fermenting and produce yellow pigments. It has the potential to induce several medical conditions such as sepsis, peritonitis, endophthalmitis, and bacteremia (Freney *et al.*, 1988). It has been classified as an opportunistic pathogen, capable of infecting both people and warm-blooded animals. It can be found in various environmental reservoirs, ranging from soil to rice fields. *Pseudomonas oryzae* may be differentiated from other non-fermenting bacteria by its negative oxidase response and preference for aerobic conditions (Freney *et al.*, 1988). According to reports, it has the capability to cause infections in persons who suffer with significant medical conditions (Lejbkiewicz *et al.*, 2003). Based on the examination of 16S RNA, these bacteria have been classified under the taxonomic group known as *Pseudomonas putida*. *Pseudomonas oryzae* exhibits resistance to certain antibiotics including ampicillin, amoxicillin-clavulanic acid, and cefazolin (Marin *et al.*, 2000).

1.13.3. The Main Characteristics of Phylum *Actinomycetota*

The *Actinomycetota*, often known as *Actinobacteria*, represent a very varied phylum of Gram-positive bacteria characterized by a significant proportion of guanine and cytosine nucleotides (Gao and Gupta, 2012). It possesses the capability to inhabit in terrestrial or aquatic environments (Servin *et al.*, 2008). The species within this phylum play a crucial role in supporting agriculture and forests. Within the soil environment, microorganisms play a crucial

role in the decomposition process of organic matter derived from died creatures. This process facilitates the breakdown of complex compounds, allowing for their subsequent uptake and utilization by plants for growth and development. Although fungi also fulfill this duty, *Actinomycetota* are far smaller and are unlikely to occupy the same ecological niche. Their colonies frequently exhibit the growth of enormous mycelia, like that of a fungus.

1.13.3.1. Genus *Kocuria*

Two species (*Kocuria rosea* and *Kocuria polaris*) are belonged to the genus *Kocuria*. Those two species established by conducting a phylogenetic and chemotaxonomic analysis of the genus *Micrococcus*, as described by Stackebrandt *et al.* (1995). The genus *Kocuria* includes five distinct species, namely *Kocuria rosea*, *Kocuria varians*, *Kocuria kristinae*, *Kocuria palustris*, and *Kocuria rhizophila*. All recognized species of *Kocuria* exhibit a coccoid morphology and possess characteristics such as being Gram-positive, non-endospore-forming, aerobic bacterium. Furthermore, it should be noted that the species have been observed to exhibit mesophilic characteristics (Stackebrandt *et al.*, 1995).

1.13.3.2. Genus *Micrococcus*

The genus *Micrococcus* is classified within the family *Micrococcaceae*. *Micrococcus* is a genus of bacteria that can be found in different habitats, including water, dust, and soil. *Micrococci* are characterized by oxidase and catalase positive reaction; whereas, it is a citrate and indole negative reaction. In addition to their Gram-positive spherical cells, those have a diameter ranging from around 0.5 to 3 micrometers. These cells commonly occur in tetrads, which are groups of four cells. The bacterium *Micrococcus* possesses a significant cellular wall, which makes up as much as fifty percent of its whole cellular mass. The genomic composition of *Micrococcus* is characterized by a high proportion of guanine and cytosine (G-C), often ranging from 65% to 75% GC-content. Micrococci frequently contain plasmids, which exhibit a wide range of sizes, typically ranging from 1 to 100 MDa. These plasmids play a crucial role in providing advantageous characteristics to the microorganism. Certain species of *Micrococcus*, including *Micrococcus luteus* (yellow) and *Micrococcus roseus* (red), exhibit the ability to generate colonies with a yellow or pink color after cultivation on mannitol salt agar. It has been shown that strains of *Micrococcus luteus* have an enhanced production of riboflavin when cultivated in the presence of hazardous organic pollutants such as pyridine (Sims *et al.*, 1986).

1.13.3.3. Genus *Corynebacterium*

The genus *Corynebacterium* consists of Gram-positive bacteria, the majority of which exhibit aerobic metabolism. It exhibits a bacillary morphology, and during some stages of their life cycle, they assume a distinctive club-shaped appearance. This characteristic morphology served as the inspiration for the nomenclature of the genus, as the term “coryneform” denotes a shape like a club. *Corynebacterium* distributed in the natural environment, particularly among the microbiota of many species, including humans. They typically maintain a harmless presence and frequently establish commensal associations with their specific hosts. Certain species, such as *Corynebacterium glutamicum*, have significant economic and industrial value. Various pathogens have the potential to induce human illness, with one prominent example being diphtheria, a condition mostly attributed to the bacterium *Corynebacterium diphtheria*. Similar to numerous species of microbiota, including those found in the genera *Arcanobacterium* and *Trueperella*, these organisms are often non-pathogenic. However, they may occasionally invade tissues through wounds, or take advantage of damaged host defenses (Collins, 2004; Poetsch, 2011; Burkovski, 2008). Collins and Cummins (1986) provided a comprehensive description of the primary characteristics belonging to the genus *Corynebacterium*, it exhibits characteristics of being Gram-positive, catalase-positive, non-spore-forming, non-motile, and possess a rod-shaped morphology, appearing either straight or slightly curved (Collins and Cummins, 1986). Metachromatic granules are commonly observed as storage of phosphate deposits (Yassin, 2003).

CHAPTER 2

MATERIAL AND METHOD

2.1. MATERIAL

2.1.1. MEDIA

Different types of media and chemical substances were used in the present study to isolate and identify haloversatile bacteria:

Nutrient Agar Medium, NaCl-free Halophilic Agar Medium, Halophilic Agar Medium containing different NaCl Concentrations (0.5%, 1.17%, 3%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, and 25% NaCl), Gelatin Agar Medium, Tween 80 Agar Medium, DNase Agar Medium, Starch Agar Medium, Xylan Agar Medium, Casein Agar Medium, Pullulan Agar Medium, Urea Agar Medium, and Cellulose Agar Medium, amino acid decarboxylation and carbohydrate fermentation broths (Beşe, 1974; Harley and Prescott, 2002; Caglayan, 2019).

2.1.1.1. Nutrient Agar Medium

Nutrient agar	20 g
NaCl	8.5 g
Distilled water	1000 mL

The media powder and NaCl were dissolved in distilled water and sterilized in autoclave at 121°C for 20 minutes.

2.1.1.2. NaCl-free Halophilic Agar Medium

Yeast extract	10 g
Agar	20 g
Distilled water.....	1000 mL
Glucose.....	1 g
Proteinase peptone.....	5 g
NaHCO ₃	0.06 g
MgSO ₄ .7H ₂ O.....	1g
NaBr.....	0.23 g
FeCl.....	500 µL
KCl.....	2 g
CaCl ₂ .2H ₂ O.....	0.36 g

All powder ingredients were measured accurately before being added to a 2000 mL flask containing distilled water and stirred until the powder completely dissolved. The mixture was then sterilized in an autoclave at 121°C for 20 minutes (Ventosa *et al.*, 1982; Caglayan, 2019). The pH was adjusted to 7.0. The medium was then put into plates and stored in the cabin to harden before being used.

2.1.1.3. Halophilic Agar Medium Containing Different NaCl Concentrations (0.5%, 1.17%, 3%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, and 25% NaCl)

Yeast extract	5 g
Agar	10 g
Distilled water	500 mL
Glucose.....	0.5 g
Proteinase peptone.....	2.5 g
NaHCO ₃	0.03 g
MgSO ₄ .7H ₂ O.....	0.5 g
NaBr.....	0.12 g
FeCl.....	250 µL
KCl.....	1 g
CaCl ₂ .2H ₂ O.....	0.19 g
NaCl.....	(depending on the percentage of NaCl)

The quantities were dissolved in water and autoclaved at 121°C for 20 minutes. The pH was adjusted to 7.0. All ingredients were mixed together, and the required concentration of NaCl was added (Ventosa *et al.*, 1982; Caglayan, 2019).

Table 2.1. Media that were used to examine enzyme production properties of haloversatile bacteria.

2.1.1.4. Gelatine Agar Medium	
Yeast extract.....5 g	Gelatine agar medium was sterilized in an autoclave at 121°C for 20 minutes. The pH was adjusted to 7.0 (Beşe, 1974; Harley and Prescott, 2002; Caglayan, 2019).
Glucose.....1 g	
Agar.....20 g	
SW10.....1000 mL	
Gelatine.....20 g	
2.1.1.5. Tween 80 Agar Medium	
NaCl.....8.5 g	Tween 80 agar medium was sterilized in an autoclave at 121°C for 20 minutes. The pH was adjusted to 7.0 (Beşe, 1974; Harley and Prescott, 2002; Caglayan, 2019). Tween 80 added after sterilization.
Agar.....20 g	
Distil water.....1000 mL	
Yeast extract.....5 g	
Tween 80.....10 g	
2.1.1.6. DNase Agar Medium	
DNase agar.....42 g	DNase agar medium was sterilized in an autoclave at 121°C for 20 minutes. The pH was adjusted to 7.0 (Harley and Prescott, 2002).
Agar.....5 g	
SW10.....1000 mL	
2.1.1.7. Starch Agar Medium	
Starch agar.....25 g	Starch agar medium was sterilized in an autoclave at 121°C for 20 minutes. The pH was adjusted to 7.0 (Harley and Prescott, 2002).
Agar.....5 g	
SW10.....1000 mL	
2.1.1.8. Xylan Agar Medium	
Agar.....10 g	Xylan agar medium was sterilized in an autoclave at 121°C for 10 minutes. The pH was adjusted to 7.0 (Caglayan, 2019).
SW10.....1000 mL	
AZCL-Xilano.....1 g	
2.1.1.9. Casein Agar Medium	
Casein Medium A	Casein agar medium was sterilized in an autoclave at 121°C for 10 minutes. The pH was adjusted to 7.0 (Birbir <i>et al.</i> , 2003; Caglayan, 2019). A and B solutions mixed together after sterilization.
SW20.....500 mL	
Tryptone.....20 g	
Agar.....20 g	
Casein Medium B	
Skim milk.....20 g	
Distilled water.....500 mL	

Table 2.1. Continue.

2.1.1.10. Pullulan Agar Medium	
AZCL-pululano.....10 g	Pullulan agar medium agar was sterilized in an autoclave at 121°C for 10 minutes (Yılmaz, 2010). The pH was adjusted to 7.0.
SW10.....1000 mL	
Agar.....10 g	
2.1.1.11. Urea Agar Medium	
Urea Agar Medium A	Solution A was autoclaved for 15 minutes at 121°C. The pH was adjusted to 7.0, Solution B filtered through a 0.45 micron nitrocellulose membrane. Then, solutions A and B were mixed and placed in a test tube in an oblique position until the medium solidified (Christensen, 1946; Bilgehan, 2004).
Peptone.....1 g	
D-Glucose.....1 g	
KH ₂ PO ₄ 2 g	
Phenol red 0.2%.....6 mL	
SW10.....866 mL	
Agar.....20 g	
Urea Agar Medium B	
Urea.....20 g	
SW10.....134 mL	
2.1.1.12.Cellulose Agar Medium	
CaCl ₂ 0.3 g	All components were mixed together and put in sterile flask, then the mixture was sterilized in an autoclave for 15 minutes at 121°C. After incubation period the medium was dispensed in sterilized petri plates (Birbir <i>et al.</i> , 2007).
Carboxymethyl cellulose.....2 g	
KCl.....2 g	
MgSO ₄ .7H ₂ O.....20 g	
MgCl ₂ .6H ₂ O.....13.3 g	
NaBr.....0.2 g	
NaCl.....100 g	
Yeast extract.....1 g	
NaHCO ₃ 0.1 g	
Distilled water.....1000 mL	
Casamino acid.....1 g	
2.1.1.13. Carbohydrate Fermentation Medium	
Carbohydrate Fermentation broth	Carbohydrate fermentation medium was placed in tubes containing Durham tube, then autoclaved for 15 minutes at 121°C (Caglayan, 2019).
Phenol red.....0.001 g	
Yeast extract.....0.5 g	
SW10.....90 mL	

Table 2.1.Continue.

2.1.1.14.Amino acid Decarboxylase Medium		
Amino acid Decarboxylase	To prepare amino acid decarboxylase medium, 5 mL and 2.5 mL of Bromocresol purple solution and cresol red solution, were respectively added and thoroughly mixed. The mixture was separated into the tubes at a volume of 2.7 mL. After that, the tubes were autoclaved at 121°C for 15 minutes. an amount of 0.3 mL amino acid sterilized by the filtration through 45 µm membrane filter was added into the tubes, constituting 1% of the total volume.	
Glucose.....0.5 g		
Meat extract.....5 g		
Peptone.....5 g		
Pyridoxal.....0.005 g		
Distil water.....993 mL		
Bromocresol Purple solution		
Bromocresol Purple.....0.2 g		
Distil water.....100 mL		
Cresol Red Solution		
Cresol Red.....0.2 g		
Distil water.....100 mL		
2.1.1.15. Esterase Agar Medium		
0.8% (w/v) trypticase peptone, 0.4% (w/v) yeast extract, 3% (v/v) olive oil, 0.2% (w/v) Rhodamine B, 2% (w/v) agar , and 1.17% (w/v) NaCl (Gutierrez <i>et al.</i> , 2016).		

2.1.2. SOLUTIONS AND CHEMICAL SUBSTANCES

The following reagents and chemical solutions were used in this study: Sterile physiological salt water (containing 0.085%, 1.17%, 3%, 5%, 10%, and 25% NaCl), and dye solutions.

2.1.2.1. Sterile Physiological Saline Water

Six types of physiological water with different NaCl concentrations used in 0.85%,3%, 5%, 10%, 20% and 25% were prepare and sterilized by autoclave with 121°C for 20 minutes and adjusted at pH 7.0 (Ventosa *et al.*, 1989).

2.1.2.1.1. Sterile Physiological Saline Water Containing 0.85% NaCl

NaCl----- 0.85g
 Distilled water----- 100 mL
 pH ----- 7.0

2.1.2.1.2. Sterile Physiological Saline Water Containing 3% NaCl

NaCl----- 3g
 Distilled water----- 100 mL
 pH ----- 7.0

2.1.2.1.3. Sterile Physiological Saline Water Containing 5% NaCl

NaCl----- 5g
Distilled water----- 100 mL
pH ----- 7.0

2.1.2.1.4. Sterile Physiological Saline Water Containing 10% NaCl

NaCl----- 10 g
Distilled water----- 100 mL
pH ----- 7.0

2.1.2.1.5. Sterile Physiological Saline Water Containing 20% NaCl

NaCl----- 20g
Distilled water----- 100 mL
pH ----- 7.0

2.1.2.1.6. Sterile Physiological saline water containing 25% NaCl

NaCl----- 25 g
Distilled water----- 100 mL
pH ----- 7.0

2.1.2.2. Dye Solutions

Additionally, traditional chemical reactions and standard dye have been utilized for complete the diagnosis of haloversatile bacteria. Dyes include Gram staining and Safranin solution.

2.1.2.2.1. Gram Staining Solution

To distinguish between Gram-negative and Gram-positive bacteria we used gram stain solution, the main components of Gram stain are gram iodine, Crystal violet solution and Ethyl alcohol.

2.1.2.2.1.1. Gram's Iodine solution

This solution was prepared according to (Harley and Prescott, 2002), consist of crashed 1 g of Iodine crystals and 2 g of KI thoroughly in a mortar, then the powder dissolved in distil water and the volume completed to 300 mL distil water then the solution was stored in a brown bottle.

2.1.2.2.1.2. Crystal Violet Solution

By mix two solutions A and B (Harley and Prescott, 2002).

A- solution: Composed of 2 g of 85% crystal violet and 20 mL of 95% ethyl alcohol.

B- solution: Produced from dissolve 0.8 g of Ammonium oxalate in 80 mL of distil water.

2.1.2.2.1.3. 95% Ethyl Alcohol

Preparing by mix 95 mL of ethyl alcohol in distil water.

2.1.2.2.2. Safranin Solution: Preparing by dissolve 2.5 g of safranin in 100 mL of 95% ethyl alcohol and 100 mL distil water (Norrell and Messley, 1997).

2.1.2.3. Amino Acid Solution (1%)

Prepared five types of amino solutions separately by dissolve 10 gram of each type of amino acids (including L-arginine, L-lysine, L-valine, L-tyrosine and L-prolin) in 100 mL of sterile distil water.

2.1.2.4. 1 N HCl and 1 N NaOH Solutions

By dissolve 36.5 g of HCl in 1 liter sterile distil water and 39.99 g of NaOH in one liter sterile distil water.

2.1.2.5. Frazier Reagent

Prepared by mixed Mercuric chloride (HgCl_2) 12.4 g and hydrochloric acid (HCl) 16 mL and 80 mL distil water.

2.1.2.6. McFarland Turbidity Standard (0.5)

The McFarland solution was made by mixing 9.95 mL of 0.18 M Sulfuric acid (H_2SO_4) and 0.05 mL of 0.048 M Barium Chloride (BaCl_2) according to the process described by Etin (1980).

2.1.2.7. Antimicrobial Agents

200.000 mg of antimicrobial agent containing sodium dimethyldithiocarbamate (20%) was weighted and solved in 1000 mL of distilled water.

2.1.2.8. Solutions Used in the Enzyme Activity

All substrates and solutions that are used in this step were prepared according to standard methods and manufacturing instructions as explain below:

2.1.2.8.1. Preparation of Substrates

In order to detect the activity of lipase and protease produced by haloversatile halophilic bacteria, two kinds of substrates were used. Azocasein used in protease activity was prepared (2% Azocasein) by dissolving 0.5 g in 25 mL (500 mL of ethanol and 24.5 mL of Tris- HCl

buffer) at room temperature, mixed rapidly, and stored in the refrigerator until use (Caldas *et al.*, 2002). The p-NPP (paranitro phenyl palmitate) was used as a specific substrate for lipase and was prepared by dissolving 38 mg (14mM p-NPP) in 10 mL of phosphate buffer with a pH of 7.0 (final concentration 0.1 M).

2.1.2.8.2. Preparation of Trichloroacetic acid (TCA)

10% Trichloroacetic acid (TCA) was prepared by dissolving 2.5 g TCA in 25 mL ddH₂O and was used for to stop the reaction (Caldas *et al.*, 2002).

2.1.2.8.3. Tris-HCl Buffer (pH 8)

Tris-HCl buffer (pH 8.0) was prepared by dissolved 1.21 g in 100 mL. Then mixed thoroughly and stored in refrigerator until be used (Caldas *et al.*, 2002).

2.1.2.8.4. NaOH

1g of 1M NaOH was dissolved in 25 mL dH₂O, was mixed thoroughly and was kept until used.

2.1.2.9. Reagents and Buffers that were Used in the Molecular Diagnosis

Table 2.2. Reagents and Buffers that were used in molecular diagnosis.

Reagents	Components	Procedure
Primers	Primer.....12 µL Sterile deionizer distilled water....88 µL	About one µL of mixture was added to eppendorf tube and kept at -20 °C.
Ladder DNA	10µL of 1 kb DNA Ladder and 90 µL of Gel loading dye	One µL of mixture was put in eppendorf tube and kept at 4°C.
Agarose Gel	Agarose Sea Kem LE.....0.4 g 1 X TAE working buffer.....40 mL	The materials were placed in a flask and left in microwave for 1 minutes at 650°C. After 5 minutes of taking it out of microwave were poured it into the gel tray.
Nucleotides Mixture	125 µL of dNTP was mixed with 875 µL of sterile deionizer water	About one µL of mixture added to ependroff tube and kept at -20°C.
Gel loading dye	GelPilot 1 kb ladder (100) gel loading dye was mixed with the extracted DNA or PCR product prior to loading into the wells of the gel.	
1X Tris-acetate and EDTA (TAE) Working Buffer	100 mL of 10X TAE was mixed with 800 mL distil water	

2.1.3. THE TOOLS AND EQUIPMENTS

Our research was conducted by using equipment's from Marmara University, The Faculty of Science, The Department of Plant Disease, and The Microbiology. The instruments and apparatus used for this thesis are including Distilled Water Device (GFL), Balance (Sartorius Analytic/ China), Oven (Nüve EN500 Türkiye), Autoclave (Web MLW) (HIRAYAMA/ Japan), Pasteur Oven (Kermanlar), Vortex Tube Mixer (HIRAYAMA/Japan), Microscope (Olympus/Japan), Centrifuge (Sigma), Refrigerator (Arçelik/Türkiye), Shaking Oven (Edmund Buhler/Germany), Electrophoresis tank (BioRAD/USA), Power supply (BioRAD/USA), Microwave oven (Arçelik/Türkiye), pH Meter (WTW) (PT10, Sartorius Professional Meter PP-50 AG, Göttingen/Spain), Muffle furnace (Protherm), Biosafety Cabinet, PCR instrument (TECHNE TC-3000/ USA), Desiccator and Fume Hood, den-1 McFarland densitometer (Biosan), and Magnetic stirrer (HEIDOLPH/ Japan).

2.2. METHODS

Sixteen steps in the research process are listed below:

- 1) Collection of salt samples from leather tanneries.
- 2) Determination of pH of salt samples.
- 3) Determination of the total numbers of haloversatile bacteria in the salt samples.
- 4) Determination of the total numbers of proteolytic and lipolytic haloversatile bacteria in the salt samples.
- 5) Isolation of haloversatile bacteria from salt samples.
- 6) Molecular characterization of haloversatile bacteria isolated from salt samples.
- 7) Determination of enzymatic activities of haloversatile bacteria.
- 8) Utilization of different amino acid and different sugar sources by haloversatile bacteria.
- 9) Examining the effects of pH, salt and temperature on the growth of haloversatile bacteria.
- 10) Investigation of Gram staining, cell morphology and pigmentation of haloversatile bacteria.
- 11) Investigation the effects of lipolytic and proteolytic haloversatile bacteria on sheep and goat skins.
- 12) Examination the haloversatile bacteria isolated from salt samples under scanning electron microscope.
- 13) Investigation of the ability of bacteria to produce bacteriocin against each other.
- 14) Investigation of the optimum conditions for bacteriocin production of haloversatile isolates.
- 15) Antibiotic susceptibility tests.
- 16) MIC of antimicrobial agents against haloversatile isolates.

2.2.1. SAMPLING

Twenty-five salt samples, which were used in the preservation of skin and hides within the leather sector, were collected in aseptic conditions from various tanneries located in The Leather Organized Tannery Region (Tuzla-Istanbul, Türkiye). These samples were then carefully put in sterile bags. Subsequently, the salt samples were placed within a lightproof insulated container, accompanied by icepacks, and immediately delivered to the laboratory for further examination.

2.2.2. DETERMINATION OF THE SALT SAMPLES' pH

Each salt sample was weighed and deposited in a sterile flask containing 100 mL of sterile distilled water (dH₂O). In a shaking incubator; containers containing saline solution were shaken at 160 rpm for one hour. A pH meter (PT 10, Sartorius Professional Meter PP-50 AG, Gottingen, Germany) was used to measure the pH of each salt sample (Birbir and Ilgaz, 1996).

2.2.3. DETERMINATION OF TOTAL NUMBERS OF HALOVERSATILE BACTERIA IN THE SALT SAMPLES

By using spread plate technique, the total numbers of haloversatile bacteria that found in salt samples were determined on Halophilic Agar Medium (HAM) containing 11.7 g NaCl, 1 g MgSO₄·7H₂O, 0.36 g CaCl₂·2H₂O, 5 g protease peptone, 2 g KCl, 0.23 g NaBr, 1 g glucose, 10 g yeast extract, 0.06 g NaHCO₃, 20 g agar, FeCl trace element, 1000 mL distilled water (Norton and Grant, 1988; Caglayan, 2019). Twenty g of each salt sample were put into a flask containing 180 mL sterile 1.17% NaCl solution, and the flasks placed into a shaking incubator for 3 hours at 24°C and 160 rpm. Direct and serial dilutions of the bacterial suspension were spread on the surface of HAM. The petri plates were incubated at 35°C and 24h. After the incubation period, haloversatile bacterial colonies grown on the plates were counted (Norton and Grant, 1988; Caglayan, 2019).

2.2.4. DETERMINATION OF TOTAL NUMBERS OF PROTEOLYTIC AND LIPOLYTIC HALOVERSATILE BACTERIA IN THE SALT SAMPLES

The bacterial suspension was distributed onto the surface of Gelatine Agar Medium (GAM) to determine the total numbers of proteolytic haloversatile bacteria in the salt samples. GAM was prepared with 20 g gelatine, 11.7 g NaCl, 20 g agar, and 1000 mL of sterile distilled water. After 24 hours of incubation at 35°C, the plates were covered with Frazier solution. The transparent zones surrounding the colonies exhibited protease activity (Sánchez-Porroet *et al.*, 2003; Caglayan *et al.*, 2018; Caglayan, 2019). To detect the total numbers of lipolytic haloversatile bacteria in the salt samples, Tween 80 Agar Medium containing 5 g yeast extract, 11.7 g NaCl, Tween80 10 mL, 20 g agar, and 1000 mL distilled water was used (Sánchez-Porro *et al.*, 2003; Caglayan, 2019). Using the spread plate technique, direct and serial dilutions of bacterial suspensions were spread over the surface of the medium. The plates were incubated for 24 hours at 35°C. The opaque zones around the colonies on Tween 80 Agar Medium was interpreted as an evidence of lipase activity (Sánchez-Porro *et al.*, 2003; Caglayan *et al.*, 2018; Caglayan, 2019). The Colony-

Forming Unit (CFU) was estimated on the agar plates and was represented the number of viable cells in the salt samples. Each test was conducted in triplicate. In the current investigation, the pH of each test medium was adjusted to 7.0.

2.2.5. ISOLATION OF HALOVERSATILE BACTERIA FROM SALT SAMPLES

To isolate haloversatile bacteria, 10 grams of each salt sample were weighed and added into 90 mL of sterile physiological saline solution containing 1.17 % NaCl in an Erlenmeyer flask. The flasks were shaken for 3 hours in a shaking incubator at 24°C and 160 rpm. Using sterile physiological saline solution containing 1.17 % NaCl, direct and serial dilutions (from 10^{-1} to 10^{-6}) of the mixtures were prepared. To isolate haloversatile bacteria, 100 µL of bacterial dilutions were added to Halophilic Agar Medium using the spread plate technique. All Petri plates were incubated overnight at 35°C. Eventually, pure haloversatile bacterial colonies were obtained from Halophilic Agar Medium using the spread plate technique (Gonzalez *et al.*, 1978; Montalvo-Rodriguez *et al.*, 1998; Harley and Prescott, 2002).

2.2.6. MOLECULAR DIAGNOSIS OF HALOVERSATILE BACTERIA ISOLATED FROM SALT SAMPLES

In this step, molecular characterization of haloversatile bacteria isolated from salt samples was conducted.

2.2.6.1. Extraction and Purification of Chromosomal DNA from Haloversatile Bacteria

The extraction and purification methods were achieved by using specific kits such as QIAamp DNA Mini Kit (Qiagen, Germany) and QIAquick PCR Purification Kit (Qiagen, Germany) (Figure 2.1). The procedures were applied according to the instruction (QIAampDNA Mini and Blood Mini Handbook, 2010). Firstly, chromosomal DNA was extracted from pure cultured cells under sterilized conditions as shown below:

1. A 24-hour loop of haloversatile culture was mixed with 5 mL of Halophilic Broth Medium and subjected to incubation at 100 rpm for 24 hours in a shaker incubator. A volume of 1.5 mL of each liquid bacterial solution was carefully transferred into an eppendorf tube, followed by centrifugation at a speed of 7500 rpm for 10 minutes. In order to collect bacterial cells, the liquid supernatant was discarded, and the process of centrifugation was repeated three times.

2. After centrifugation, 180 μ L of enzyme solution containing 20 mg/mL lysozyme, 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 1.2% Triton was added to the remaining particle in the bottom of the eppendorf tube. The particles were homogenized with a micropipette.
3. Homogenized bacterial suspensions were incubated for 30 minutes at 37°C.
4. After incubation period, 20 μ L of proteinase K and 20 μ L of buffer were added on bacterial suspension and mixed with vortex. The mixture was incubated at 56°C for 30 minutes in an incubator and at 95°C for 15 minutes in a water bath.
5. Bacterial suspension was centrifuged at 7500 rpm for 2 minutes.
6. 200 μ L of the ethanol was added on the bacterial suspension and the mixture was vortexed for 15 seconds. The mixture was centrifuged at 7500 rpm for three minutes. Following these steps, 750 μ L of the bacterial suspension was placed in the QIAamp Mini spin column tube and centrifuged at 8000 rpm for one minute.
7. After the centrifugation process, the upper filtrate from the centrifuge tube was discarded and then transferred to sterilized collection tube. A volume of 500 μ L of Wash Buffer-1 was poured into the filtered mini spin column tube. Subsequently, the tube was subjected to centrifugation at a speed of 8000 rpm for one minute. The experiment was repeated by employing 500 μ L of Wash Buffer-2. The resulting mixture was subjected to centrifugation at a speed of 14000 rpm for duration of 3 minutes.
8. In order to extract DNA from the column, the QIAamp Mini spin column tube was placed on top of a sterile 1.5 mL eppendorf tube, 200 μ L of Elution Buffer was added, and the tube was centrifuged at 8000 rpm for 1 minute. Consequently, the DNA contained in the filter was collected in the microcentrifuge tube.
9. The collected DNA was stored at -20°C. The extracted pure DNA was run on an agarose gel to corroborate the presence of DNA bands, and DNA was then frozen at -20°C (Sambrook and Russell, 2001).

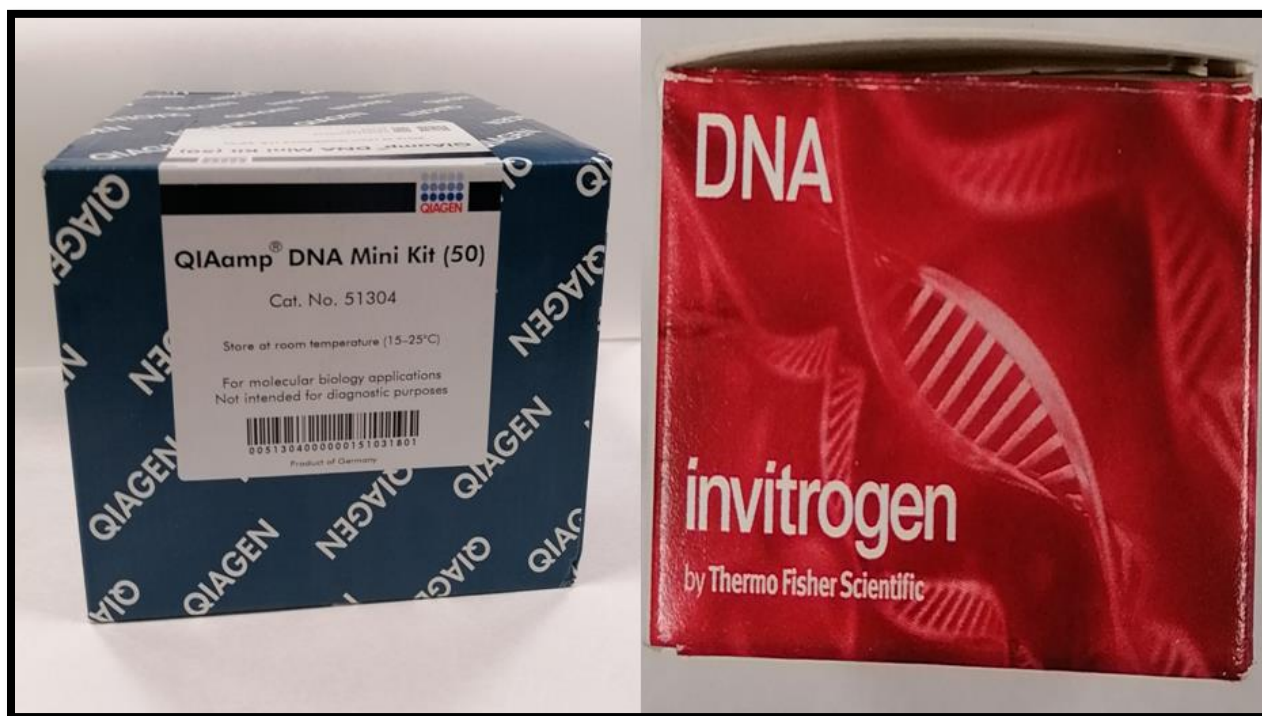


Figure 2.1.QIAamp DNA mini kit (Qiagen, Germany).

2.2.6.2.The Electrophoresis of DNA Extracted from Haloversatile Bacteria on an Agarose Gel

The gel was prepared by dissolving 0.4 g of agarose in 40 mL of 1X TAE working buffer and afterwards transferring the resulting solution into a flask. The agarose was dissolved by putting the mixture to microwave treatment for duration of 1 minute at a temperature of 560°C. A cooling interval of 1 to 2 minutes was selected for the Agarose solution, following which 0.5g/mL (2.5l) of ethidium bromide dye was added with the tip of a micropipette. The gel was then shaken by vigorously shaking the flask. Subsequently, the electrophoresis tank was filled with the mixture of agarose gel and ethidium bromide dye following the fixation of the comb. The electrophoresis tank was prepared by inserting a frozen agarose gel tray. Subsequently, specific combs were employed to construct wells within the gel tray. The wells were then positioned towards the negative pole (illustrated by the color black) due to the negative charge of the DNA molecules. Subsequently, the DNA molecules were observed to migrate from the cathode to the anode (shown by the red color) upon activation of the electric current within the gel electrophoresis apparatus. An adequate volume of recently made 1X TAE working buffer was added to the frozen agarose gel within the electrophoresis container, providing that it was submerged by about

1 cm. In order to facilitate a comparison between the 1500 bp band present in the isolated DNA and the DNA ladder, a volume of 10 µL of the DNA ladder was added into the first well of the agarose gel. A total volume of 5 µL of each extracted DNA sample was mixed with 5 µL of gel-loading dye using a micropipette on a parafilm surface. Subsequently, 5 µL of the resulting mixture was introduced into each of the wells of an agarose gel. The second step consisted of closing the lid of the electrophoresis tank, connecting the positive and negative cables, and then running the power source at 80 V and 100 mA for 30 minutes. The gel was then taken out from the electrophoresis container and examined after the power source was turned off (Caglayan, 2015).

2.2.6.3. PCR Amplification of 16S rRNA Isolated from Haloversatile Bacteria

Specific primer for gene 16S rRNA with forward 16F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer was 16R1488 (5'-CGGTTACCTTGTAGGACTTCACC-3') were used to amplify the DNA samples extracted from haloversatile bacteria (Sambrook and Russell, 2001; Fernández, 2012). The conditions of amplification and melting points were show in Table 2.3. The following components of PCR reaction 5 µL 10X PCR buffer (prepared by mixed 500 mM KCl and 100 mM Tris-HCl with pH 8.3), 8 µL dNTPs (10 mM), 0.5 µL Taq DNA polymerase, 1 µL genomic DNA, 28 µL ddH₂O, 2.5 µL MgCl₂ (25 mM), 2.5 µL forward primer and 2.5µL reverse primer are mixed together and the total amount in each tube was 50 µL (Mullis and Faloona, 1987).

Table 2.3. Thermo-cycle program (temperature and time) used in PCR technique.

No	Stage	Temperature °C	Time	Number of cycle
1	Initial Denaturation	95	5	1
2	Denaturation	94	1	25
3	Annealing	50	1	
4	Elongation	72	2	
5	Final Elongation	72	10	1

2.2.6.4. Execution of PCR Products of Haloversatile Halophilic Isolates on Agarose Gel

The electrophoresis procedure was carried out according to the method described by Sambrook and Russell (2001):

1. 1% agarose gel was prepared by dissolving 0.4 g of agarose in 40 mL of 1X TAE working buffer.
2. Place the mixture in a microwave oven at 560°C for one minute to dissolve the agarose powder.
3. The mixture was cooled down after being removed from the microwave oven.
4. After 2 minutes, 2.5 μ L (0.5 g/mL) of ethidium bromide dye was added.
5. After placing the comb in the electrophoresis tank, the mixture was transferred into the tank, and wells are created. After ten minutes, the comb was removed, and the substance completely hardened.
6. A sufficient quantity of 1X TAE working buffer (Figure 2.2) was added to the gel receptacle, covering it by approximately 1 cm.
7. Ten microliters of DNA ladder were added to the first well of the electrophoresis, and the remaining wells were filled with five microliters of DNA extraction and loading dye solutions.
8. The lid of the electrophoresis container was closed, and an 80 V, 100 mA electric current sources was turned on for 30 minutes.
9. The source of electric current was turned off, and the gel was investigated.



Figure 2.2. Buffers, microcentrifuge tubes and microcentrifuge device.

2.2.6.5. Purification of PCR Products

The QIAquick PCR Purification Kit (Qiagen) was used to purify the 16S rRNA PCR technique's products before sequencing. In this procedure, some unused dNTP, primer dimers, and other constituents such as Taq polymerase and NaCl were removed from the PCR product. Depending on the QIAquick PCR Purification Kit (Figure 2.3.), the procedure employed in this step is described in detail:

1. 45 μ L of PCR-derived product was added to 225 μ L of binding reagent (1/5). Micropipette was used to place the mixture in the column of the QIAquick tube. The QIAquick column tube is composed of two purple-colored parts: the inner part, which contains the filter, and the outer portion, which collects the waste liquid.
2. The QIAquick column tubes were centrifuged at 13000 rpm for one minute. Then, all the waste liquid that had collected in the outer tube was eliminated, and the two tubes were reconnected.
3. 750 μ L of washing buffer was added to the QIAquick column tube (Figure 2.3) and centrifuged at 13000 rpm for one minute in order to remove unwanted compounds from the PCR products.
4. The inner portion of the QIAquick column tube was transferred to a sterile 1.5 μ L eppendorf tube. To collect DNA into the bottom sterile microcentrifuge tube, 50 μ L of Elution buffer (10mM Tris-Cl, pH 8.5) was added and centrifuged for 1 minute at 13000 rpm (QIAamp DNA Mini and Blood Mini Handbook, 2010).
5. Electrophoresis was subsequently performed on the purified DNA.



Figure 2.3. QIAquick column tubes and buffers.

2.2.6.6. Determination of 16S rRNA Sequences of DNA from Haloversatile Isolates and Comparison with Sequences in the GenBank

- Once the DNA sequencing results were received in FASTA format, the sequences were examined on the NCBI site (<https://www.ncbi.nlm.nih.gov>, Date of access, May 2023) in order to detect phylogenetically similar species. This was accomplished by opening the NCBI site, selecting the Nucleotide BLAST option (Figure 2.4), copying the FASTA format, pasting it into the NCBI site, and clicking BLAST.

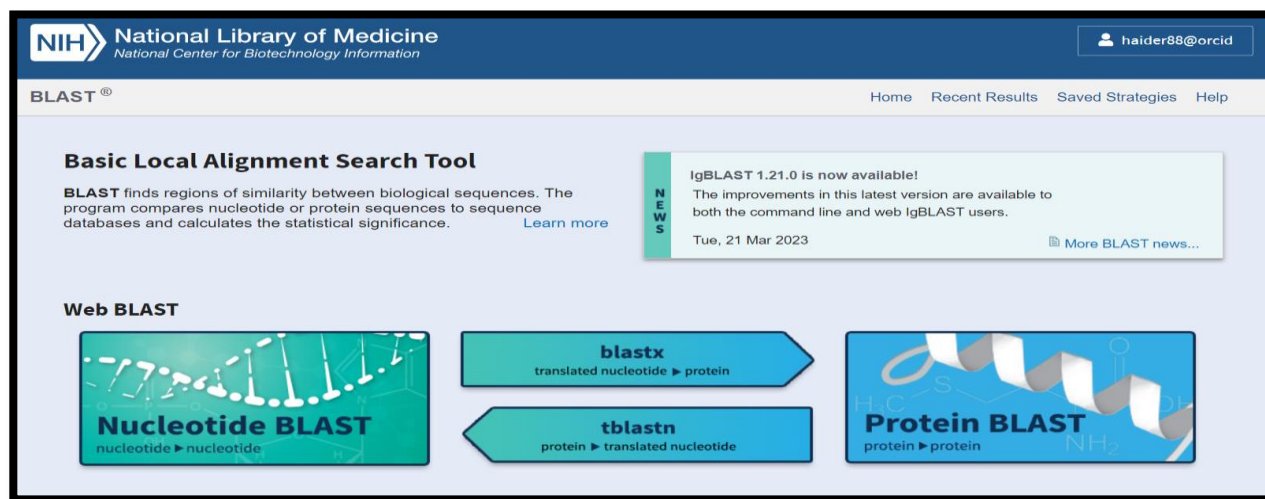


Figure 2.4. The main page of NCBI.

- Following entering BLAST (Figure 2.5), the results were explained within 5 minutes. According to the sequencing of our FASTA format, nearly 100 sequences were displayed. The sequence that was most similar to our own based on the value of E were chosen.

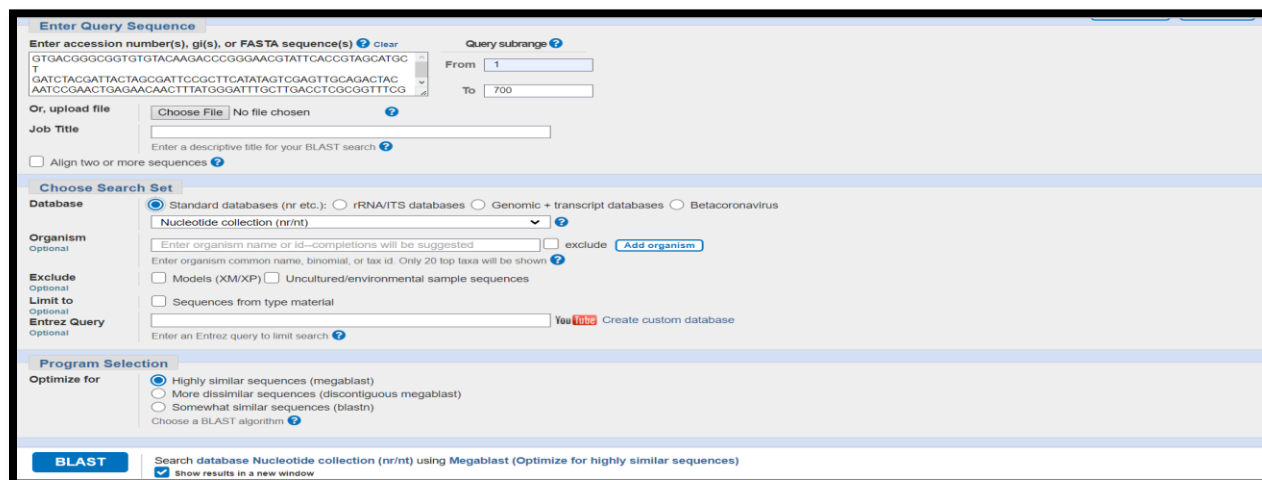


Figure 2.5. BLAST page in NCBI web site.

- From this drop-down menu, the species closest to our FASTA sequences were selected, and the strains were identified using the same ratio of identification (Figure 2.6).

The screenshot displays the NCBI BLAST search interface. The search parameters are as follows:

- Job Title:** Nucleotide Sequence
- RID:** 40Z0AVYA013 (Search expires on 04-21 07:40 am)
- Program:** BLASTN
- Database:** nt
- Query ID:** Ic|Query_40323
- Description:** None
- Molecule type:** dna
- Query Length:** 965

The **Filter Results** section includes options to filter by Organism (only top 20 will appear), Percent Identity, E value, and Query Coverage. The main results table, titled "Sequences producing significant alignments", shows 100 sequences selected. The table columns are: Description, Scientific Name, Max Score, Total Score, Query Cover, E value, Per Ident, Acc. Len, and Accession. The results list several *Staphylococcus epidermidis* strains with their respective accession numbers.

Figure 2.6. Selecting the more similar species from NCBI website.

2.2.6.7. Accession Number

The accession number of our isolates was obtained from the NCBI website (<https://www.ncbi.nlm.nih.gov>, Date of Access May 2023) (Figure 2.7) and GenBank by accessing the main page of NCBI and clicking on the submit option, then by using an ORCID account, entering our information and selecting a new submission option (Figure 2.8).

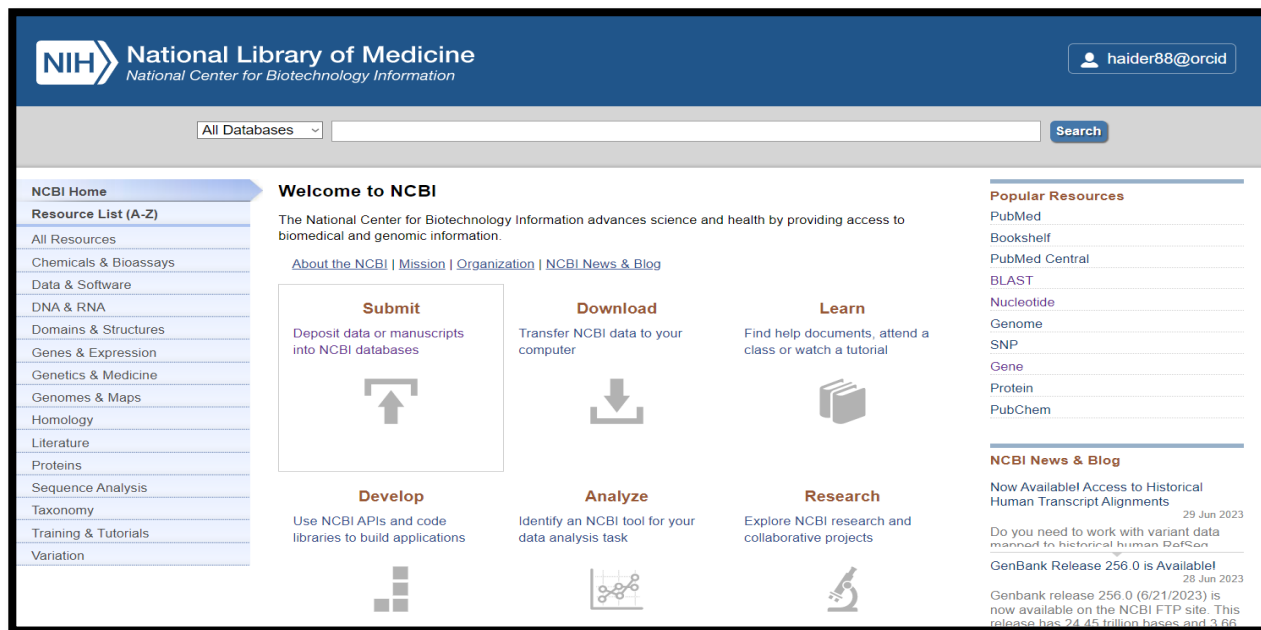


Figure 2.7. NCBI home page from which an accession number was obtained.

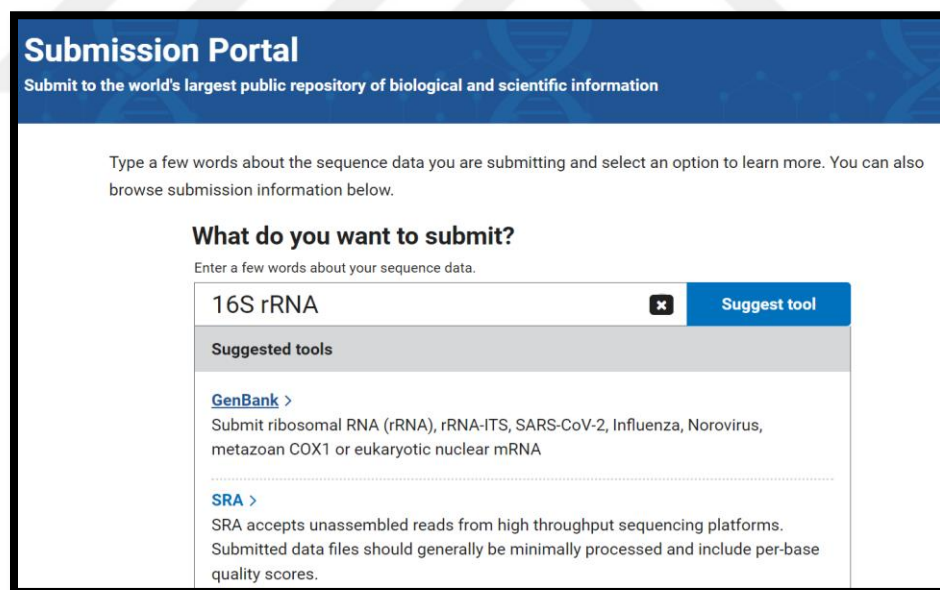


Figure 2.8. A new submission and register the isolates in GenBank.

Subsequently, a new page titled GenBank was opened, nine stages were displayed, and the procedure was initiated as described in detail below (Figure 2.9).

The screenshot displays the 'Submission Type' step of the GenBank submission process. At the top, a horizontal navigation bar shows nine steps: 1 SUBMISSION TYPE (active), 2 SUBMITTER, 3 SEQUENCING TECHNOLOGY, 4 SEQUENCES, 5 SEQUENCE PROCESSING, 6 SOURCE INFO, 7 SOURCE MODIFIERS, 8 REFERENCES, and 9 REVIEW & SUBMIT. Below the navigation bar, the title 'Submission Type' is followed by a note: 'Required fields are marked with * asterisk'. The main content area contains three sections of radio button options:

- * What do your sequences contain?**
 - ☒ ribosomal RNA (rRNA) or rRNA-ITS
 - ☐ COX1 from metazoan mitochondria
 - ☐ Eukaryotic nuclear mRNA (NEW)
 - ☐ SARS-CoV-2, Influenza, Norovirus, or Dengue virus
- * What type of ribosomal RNA (rRNA) or rRNA-ITS?**
 - ☒ Prokaryotic rRNA or rRNA-IGS
 - ☐ Eukaryotic nuclear rRNA or rRNA-ITS
 - ☐ Eukaryotic organelle rRNA (mitochondrial or chloroplast)
- * What do these prokaryotic rRNA or rRNA-IGS sequences contain?**
 - ☒ small subunit rRNA only (16S rRNA)
 - ☐ large subunit rRNA only (23S rRNA)
 - ☐ intergenic spacer (16S-23S rRNA IGS)

At the bottom of the form, there is a note: 'If none of the options above describe your sequences, use [BankIt](#) to submit.' Below this, a link reads 'Review requirements for prokaryotic rRNA or rRNA-IGS submissions' and a blue plus icon is visible in the bottom right corner.

Figure 2.9. Major steps in submission of FASTA format.

- ❖ After selecting the type of sequence and microorganisms and clicking the Continue button, the second phase was displayed.
- ❖ General information about the organization, including the organization's name, department, and address, as well as the author's email address and name, were included.
- ❖ Following the click on continue, the third step was accessed and the Sanger dideoxy sequence was chosen; the page was then transformed to the fourth step.
- ❖ Our isolates' FASTA format was uploaded, and the fifth phase was initiated.
- ❖ The name of the organism, the name of the strain, the host, the location, and the date of collections were written, as was the collector's name.
- ❖ The name of the author and the title of the investigation were chosen. After selecting the submit button, the principal report regarding our accession numbers was displayed.
- ❖ The accession numbers were finally received through email address.

2.2.6.8. Estimation the Molecular Weight of DNA Extracted from Haloversatile Bacteria

(https://www.bioinformatics.org/sms2/dna_mw.html, Date of Access May 2023) by clicking on this website the SMS (Sequencing Manipulation Suite) was opened. The molecular weight for each DNA sequence belonging to the haloversatile isolates was estimated depending on the given DNA FASTA format extracted from isolates and sequenced by Sanger dideoxy sequencing method (Figure 2.10).

The screenshot displays the SMS (Sequencing Manipulation Suite) website. The main heading is "Sequence Manipulation Suite: DNA Molecular Weight". Below this, a text box contains the instruction: "DNA Molecular Weight accepts one or more DNA sequences and calculates molecular weight. Sequences can be treated as double-stranded or single-stranded, and as linear or circular. Single-stranded sequences are assumed to have a 5' phosphate. Use DNA Molecular Weight when calculating molecule copy number." Below the text box, there is a form with two input fields for DNA sequences. The first field is labeled ">Sample sequence 1" and contains the text "garkbdctymvhu". The second field is labeled ">Sample sequence 2" and contains the text "ctymvhgarkbda". Below the input fields are three buttons: "Submit", "Clear", and "Reset". Below the buttons, there is a dropdown menu labeled "Treat sequences as" with options "single", "stranded", and "linear". Below the dropdown menu, there is a note: "*This page requires JavaScript. See browser compatibility." and a link: "*You can mirror this page or use it off-line." At the bottom of the page, there is a footer with the text "Sun 14 Jun 00:36:59 2020" and "Valid XHTML 1.0, Valid CSS".

Figure 2.10. Estimation of molecular weight of DNA extracted from haloversatile bacteria.

2.2.6.9. Alignment and Annotation

The alignment and annotation of sequences obtained from haloversatile bacteria were conducted using the freely available Jalview 2.11.2.7 programmer. Five reference sequences were obtained from the NCBI website and aligned with the sequence acquired from *Staphylococcus saccharolyticus* (isolate PSS20a). This was achieved by placing all sequences in the same FASTA folder. Subsequently, the Jalview software was used to perform the alignment. The alignment process involved selecting “input a new alignment from file list” and annotating the sequences to map the correct structures. Subsequently, the protein, wrap, and clustal options were selected from the View, Format, and Color menus, as illustrated in Figure 2.11.

2.2.7.1. Phenotypic Investigation of the Bioactive Producing Haloversatile Bacteria by Using Agar Plates and Test Tubes Methods

After preparing nine kinds of agar media, including starch, casein, Tween80, xylan, cellulose, gelatine, DNase, pullulan, and urea agar media, they were autoclaved at 121°C for 20 minutes. The haloversatile isolates were tested for the production of amylase, caseinase, lipase, xylanase, cellulase, protease, DNase, pullulanase, and urease on the aforementioned media. Each isolate was collected with a sterile loop and was spread on these media for the first eight media (starch, casein, Tween80, xylan, cellulose, gelatine, DNase, pullulan). A loop of each isolate was streaked on slant urea agar media in the test tube for urease activity. All Petri plates and test tubes were then incubated overnight at 35°C (Harley and Prescott, 2002). In addition, the ability of haloversatile bacteria to produce oxidase and catalase was examined using oxidase reagent (redox- tetramethyl-p-phenylene-diamine) and Hydrogen peroxide (H₂O₂), respectively. The Petri plates were individually tested and examined for each enzyme using standard procedures as described below.

2.2.7.1.1. Production of Esterase, Amylase, Caseinase, Lipase, Xylanase, Cellulase, Protease, DNase, Pullulanase, and Urease Enzymes by Haloversatile Bacteria

Gram's iodine solution was poured onto the starch agar medium to cover up the pure bacterial colonies that had grown there. Observing a transparent zone surrounding colonies on a starch agar medium indicates starch hydrolysis (Harley and Prescott, 2002). In a similar manner, the formation of a transparent zone around colonies grown on casein medium was regarded as evidence of positive caseinase activity (Harley and Prescott, 2002). The presence of opaque zones around grown colonies on Tween 80 Agar was interpreted as evidence of positive lipase activity (Sánchez-Porro *et al.*, 2003). The bacterial colonies which were grown on cellulose agar medium were stained with 0.1% of Congo red, and rinsed with a 1 M NaCl solution after 30 minutes. Clear zones surrounding bacterial colonies on agar plates were interpreted as evidence of positive cellulase activity (Limauro *et al.*, 2001). After the incubation period, Frazier reagent was added to the colonies that had been growing on Gelatin Agar Medium. Positive proteolytic activity was indicated by the presence of transparent zones around colonies (Harley and Prescott, 2002; Sánchez-Porro, 2005). After 24 hours of incubation, a specific volume of 1 N HCl was poured over colonies grown on DNase medium, and positive results were indicated by the formation of a transparent zone around the colonies (Bilgehan,

1992; Harley and Prescott, 2002). The presence of transparent zones around the colonies on pullulan and xylan media was accepted as positive test result (Sánchez-Porro, 2005). For urease production, inoculation was done in the agar slant tube and the agar slant tubes were incubated 24 hours. Observation of change color into pink color in urea agar slant tubes was indicated production of urease enzyme (Christensen *et al.*, 1946; Bilgehan, 1992). The isolates were inoculated onto the Olive Agar Medium, and the plates were incubated at 35°C for 24 hours. The orange fluorescence halos around the bacterial colonies under UV light was accepted as positive esterase activity (Gutierrez *et al.*, 2016).

2.2.7.1.2. Production of Oxidase and Catalase Enzymes by Haloversatile Bacteria

For oxidase test, tetra-dimethyl-para-phenylenediamine dihydrochloride was absorbed onto the filter paper. Then the bacterial colonies were smeared onto the filter paper. After that, the results were read by the formation of a blue-violet color within 20 seconds (Harley and Prescott, 2002). For catalase activity one or two drops of H₂O₂ (3%) were dripped onto the colonies on the medium and the positive isolates formed gas bubbles in the colony while the negative one did not produce any bubbles (Harley and Prescott, 2002).

2.2.7.2. Phenotypic Investigation of the Lipolytic and Proteolytic Activity of Haloversatile Bacteria by Using Spectrophotometer and Study the Effects of Different Factors

Lipase and protease activities were spectrophotometrically tested using specific substrates such as p-NFP (para nitro phenyl palmitate) and azocasein, respectively. Only *Staphylococcus warneri* (isolate PSS1a), *Staphylococcus pasteurii* (isolate PSS1b), *Staphylococcus warneri* (isolate PSS2a), *Paenibacillus illinoisensis* (isolate PSS7a), and *Bacillus subtilis* (isolate PSS16a) were selected as test bacteria in the lipase activity survey. The optimum conditions of enzymatic activity were investigated. The optimal conditions for detecting protease activity were examined for 24 isolates based on the diameter of the transparent zones around colonies on Gelatine Agar Medium.

2.2.7.2.1. Measuring the Activity of Lipase Enzyme

This method was done according to the procedure mentioned by Ozyilmaz (2009) with some modification, by dissolving 14.4 mM pNPP (*P*-Nitrophenyl palmitate) in 10 mL of phosphate buffer (0.1 M, pH 7.0). After the substrate was prepared, five lipase-positive isolates were centrifuged at 15000 rpm/5 minutes. Then, 50 µL of supernatant (crude enzyme) was added into

50 μL of pNPP. Also, 200 μL of phosphate buffer (100 mM) was added. Then, the mixture was incubated for 30 minutes at 37°C in water bath. In order to stop reaction, 500 μL of 0.5 M Na_2CO_3 was added and centrifuged at 5000 rpm/10 minutes (Table 2.4). Afterwards, 200 μL of the mixture was added into the well. The absorbance was read by spectrophotometer at 405nm. The standard was prepared under the same conditions.

Table 2.4. The amounts of solution prepared for measuring lipase activity using spectrophotometric method.

No	0.1 M pH 7.0 of phosphate buffer (μL)	Substrat pNPP (P- Nitrophenyl palmitate) (μL)	supernatant (crude enzyme) (μL)	Lipase from <i>Candida regusa</i> (μL)
Control	200	50	-	50
Sample1	200	50	50	-
Sample2	200	50	50	-
Sample3	200	50	50	-
Sample4	200	50	50	-
Sample5	200	50	50	-

2.2.7.2.2. Measuring the Activity of Protease Enzyme

This method was conducted with a little modification (Caldas *et al.*, 2002). The overnight culture of test bacteria which were grown in broth media was centrifuged at 15000 rpm/5 minutes. 50 μL of supernatant (crude enzyme) was added into the 50 μL substrate (2% Azocasein) and incubated for one hour at 37°C in the water bath. At the same time, the control samples were prepared by the same procedure, and unreacted substrate was precipitated by adding TCA (Trichloroacetic acid). After incubation 130 μL of TCA was added into the incubated samples and standard (control) and vortexed, then centrifuged for 10000 rpm/10 minutes. Then, 133 μL of NaOH was added into each well of plate and 67 μL of each sample and standard were added into wells. The plate was measured by spectrophotometer at 440nm. The standard in this procedure was made by mixing 50 μL of azocasein with 50 μL of enzyme and directly added 130 μL of Trichloroaceticacid (SIGMA-ALDRICH). The samples were centrifuged at the same conditions. The amounts and contents of the solutions were given in Table 2.5 and Figure 2.12.

Table 2.5. The amounts of solution prepared for measuring protease activity using spectrophotometric method.

Isolates	Crude enzyme (μL)	Substrates (azoxain) (μL)	Trichloroacetic (TCA) (μL)	acid NaOH (μL)	Mixture (μL)	Absorbance
Sample	50	50	130	133	67	440
Standard	50	50	130	133	67	440

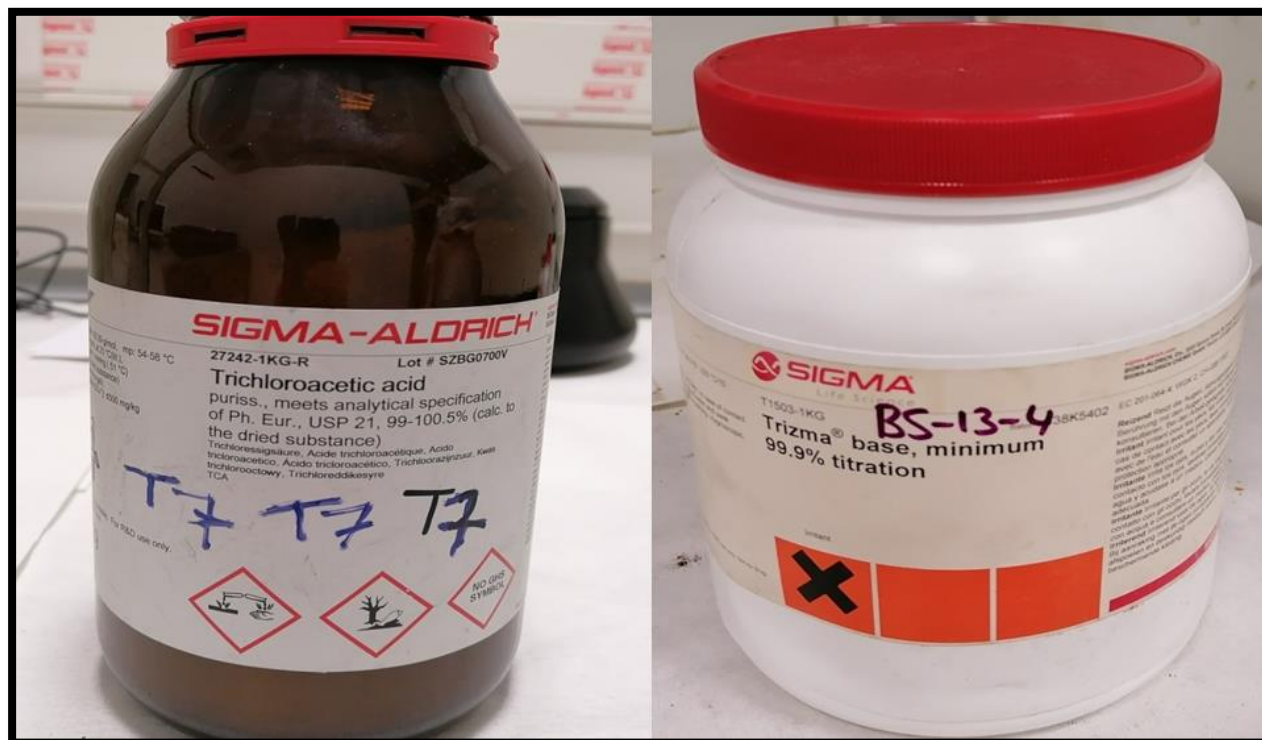


Figure 2.12. Chemicals used in the study of enzyme activity.

2.2.7.2.3. Determination of Lipase and Protease Contents Using the Standard Bradford Method

Given positive results for protease and lipase isolates were separately cultured in Halophilic Liquid Medium and incubated 37°C for 24 hours. Afterwards, 1 mL of each incubated isolates was taken and put in eppendorf tube. After centrifugation (15000 rpm/5 minutes), 2 μL of each bacterial supernatant fluid was taken and added onto 798 μL of distilled water, and 200 μL of Bradford reagent (Coomassie Brilliant Blue Dye, Bio-rad Quick StartTM) was added. The tubes were incubated from 10 minutes to one hour in the room temperature. The absorbance of each sample was read at 595 nm by spectrophotometer (Table 2.6) (Bradford, 1976).

Table 2.6. Preparing Bradford reaction components (Bradford, 1976).

Sample (μL)	Water (μL)	Bradford solution (μL)
2	798	200
Control (Bovine Serum Albumin, BSA)		
0	800	200
20	780	200
40	760	200
60	740	200
80	720	200

The lipase enzyme content in all samples was estimated based on the results of the Bradford reaction and the absorbance of Enzyme. Additionally, the graphic below was made using the absorbance values obtained from the Bradford reaction (Figure 2.13). By utilizing this equation ($y = 0.0345x + 0.0475$), and replacing the "y" value with the absorbance values of lipase, the protein content was calculated in each sample.

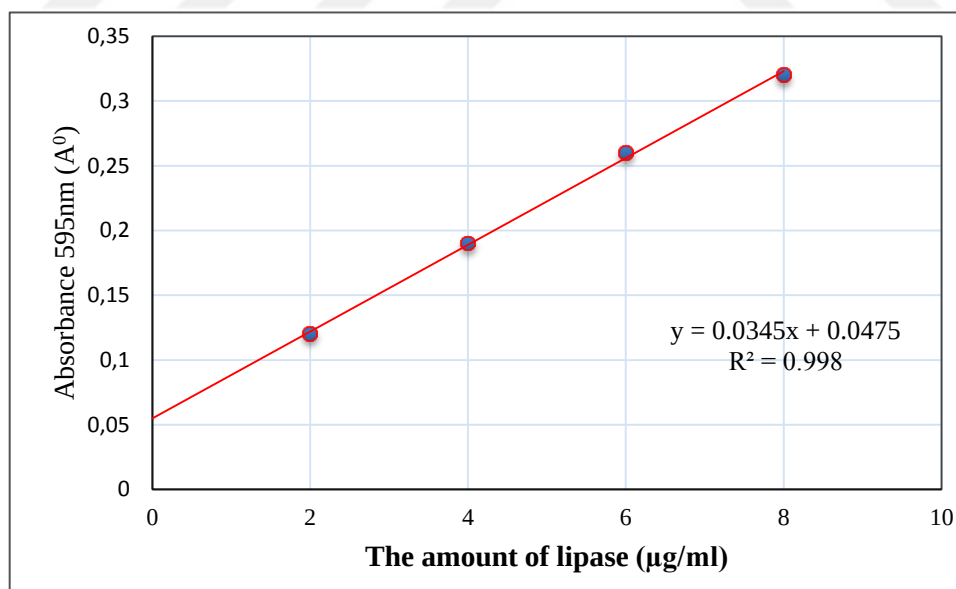


Figure 2.13. Standard chart prepared with ovalbumin (BSA), based on the absorbance of Enzyme and the amount of protein (Lipase).

The amount of protease enzyme in all samples was estimated based on the results of the Bradford reaction and the absorbance of enzyme. In addition to, the graphic below was made using the

absorbance values obtained from the Bradford reaction (Figure 2.14). By using this equation ($y = 0,0048x + 0,1283$), and replacing the "y" value with the absorbance values of protease, the protein content was calculated in each sample.

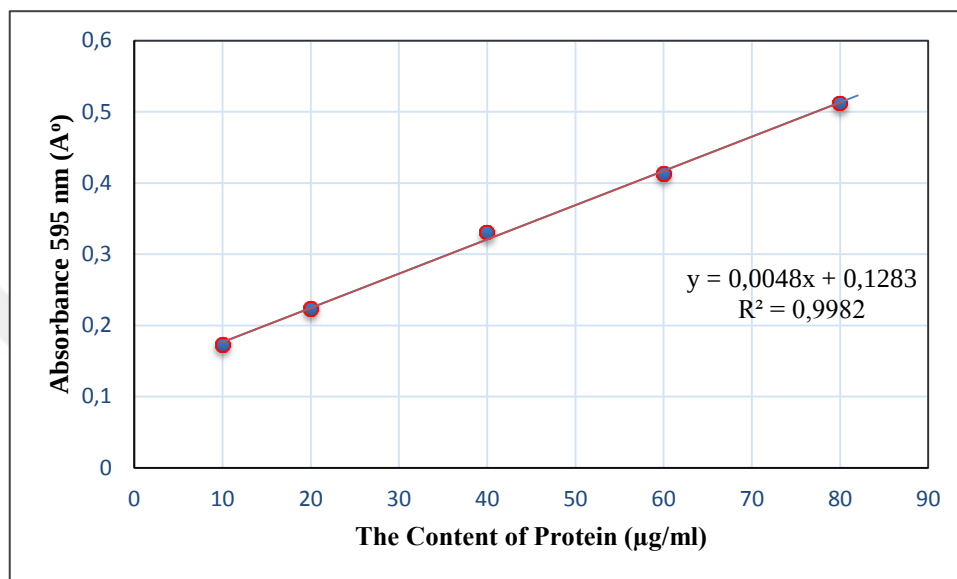


Figure 2.14. Standard chart prepared with ovalbumin (BSA), based on the absorbance of Enzyme and the amount of protein (Protease).

2.2.8.UTILIZATION OF DIFFERENT AMINO ACID AND DIFFERENT SUGAR SOURCES BY HALOVERSATILE BACTERIA

The metabolic activity of haloversatile bacterial isolates was evaluated using five amino acids and five carbohydrates. The amino acid sources (L-arginine, L-lysine, L-tyrosine, L-proline, and L-valine) had a concentration of 1% (w/v). The amino acid medium (1.17 % NaCl) contained 0.05% dextrose, 0.5% beef extract, 0.0005% pyridoxal, 0.001% bromocresol purple, 0.5% peptone, and 0.0005% cresol red. After adding bacteria to the test tubes, they were incubated at 35°C for 24 hours (Caglayan *et al.*, 2018; Harley and Prescott, 2002). The development of a purple color in the test tubes represented as a positive result. Using 1% (w/v) (dextrose, fructose, ribose, cellobiose, and saccharose), the utilization of five sugar sources by haloversatile isolates was examined independently. The sugar medium consists of phenol red at 0.001% (w/v) and yeast extract at 0.5% (w/v). The transformation of the medium from red to yellow was accepted as a positive result (Harley and Prescott, 2002; Caglayan *et al.*, 2018).

2.2.9. EXAMINATION OF THE EFFECTS OF pH, SALT AND TEMPERATURE ON THE GROWTH OF HALOVERSATILE BACTERIA

Halophilic agar media was prepared with 1.17% NaCl concentration and the pH values of the media were adjusted by pH meter into graduated values including pH 4.0, pH 5.0, pH 6.0, pH 7.0, pH 8.0, pH 9.0, pH 10.0, pH 11.0 and pH 12.0. The objective of this step was to detect the pH tolerance and pH requirement of haloversatile bacteria. After all media were prepared and autoclaved, the petri plates were inoculated with test isolates and were incubated at 35°C overnight. In order to investigate the ability of haloversatile bacteria to thrive in a wide range of NaCl concentration and to study their salt requirements, Halophilic Agar Media (HAM) were prepared with different content of salt including 0%, 0.17% NaCl, 1.17% NaCl, 2.5% NaCl, 5% NaCl, 7.5% NaCl, 10% NaCl, 12.5% NaCl, 15% NaCl, 17% NaCl, and 20% NaCl. All HAM's with different concentrations of NaCl were autoclaved and poured onto petri plates. After that, all test isolates were cultured and spread on the Petri plates. All petri plates were incubated at 35°C overnight. Furthermore, the growth ability of the isolates at different temperatures was tested for all haloversatile isolates at different temperatures such as 4°C, 10°C, 19°C, 22°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C (Caglayan, 2019).

2.2.10. INVESTIGATION OF CELL MORPHOLOGY AND PIGMENTATION OF HALOVERSATILE BACTERIA

The motility of haloversatile bacteria was tested by using slides. 100 µL of samples were taken by using pipette from liquid cultures in logarithmic growth phase and examined under the light microscope (Çetin, 1973). The morphology and pigmentation of bacterial colonies were investigated on petri plates after incubation period overnight at 35°C (Arahal *et al.*, 1996; Oren *et al.*, 1997). A loopful of overnight haloversatile bacterial cultures which were cultured under optimum conditions was taken and mixed with a physiological saline solution (1.17% of NaCl). One drop of bacterial solution was put on glass slide and slowly passed over the benzene flame. For Gram staining, first Crystal violet was poured directly on bacteria and waited one minute. Then, the slides were washed with sterile distilled water in order to remove unconnected crystal violet. Later, Gram' iodine was poured on the slides and left for one minute. Again, the slides were washed with sterile distilled water. 95% ethanol was applied (decolorization solution). Then, washed with sterile distilled water. Safranin dye as a counter stain was poured and waited one min. Then, the slides were washed and left air dry (Gram, 1884). In order to distinguish

Gram-negative and Gram-positive isolates and to study bacterial cell shapes light microscope (immersion lens) was used (Sánchez-Porro *et al.*, 2011).

2.2.11. INVESTIGATION THE EFFECTS OF LIPOLYTIC AND PROTEOLYTIC HALOVERSATILE BACTERIA ON SHEEPSKIN AND GOATSKIN

To examine negative impacts of lipolytic and proteolytic haloversatile bacteria on sheepskin and goatskin, the test isolates were grown in Halophilic Broth Medium. In this experiment, we selected four freshly slaughtered skins and studied them for organoleptic characteristics and a scanning electron microscope.

2.2.11.1. Sheepskin and Goatskin Curing Process and Storage

Initially, two pieces of freshly slaughtered sheepskin and two pieces of freshly slaughtered goatskin were obtained from a tannery. The samples were divided into two groups: the tested group and the control group. 10 g of sheepskin and 10 g of goatskin were weighed. Separately thirty milliliters of 15% NaCl brine solution was added to each vial. Each Petri plate contains a piece of sheepskin and the other flask contains a piece of goatskin as a control sample (Figure 2.15).



Figure 2.15. Pictures represent the control of goatskin and sheepskin treatment with physiological saline solution.

Eleven bacterial isolates [protease and lipase producing *Staphylococcus warneri* (PSS1a), *Staphylococcus pasteurii* (PSS1b), *Staphylococcus warneri* (PSS2a), *Paenibacillus illinoisensis*

(PSS7a), *Bacillus licheniformis* (PSS13a) and *Bacillus subtilis* (PSS16a); protease producing *Staphylococcus epidermidis* (PSS3b), *Bhargavaea ginsengi* (PSS11a), *Bacillus sonorensis* (PSS12a), *Bacillus haynesii* (PSS11b) and *Corynebacterium aurimucosum* (PSS15a)] were selected in this experiment, and the mixture was prepared with sterile physiological saline solution (15% NaCl). This bacterial suspension was adjusted as equivalent to 0.5 McFarland turbidity standards (10^8 CFU/mL). Ten milliliters of a mixed culture were added to flasks containing independently sheepskin and goatskin samples (Figure 2.16). All flasks containing inoculated skin samples and control skin samples were shaken for 18 hours at 50 rpm and 24°C. The inoculated sheepskin and goatskin samples were then removed from the flasks and stored at 24°C for 40 days. Following the period of storage, the sheepskin and goatskin samples were examined using a scanning electron microscope. In addition, organoleptic characteristics such as an unpleasant smell, a sticky appearance, and hair slip were tested in both test and control samples over a period of forty consecutive days (Bailey and Birbir, 1996).

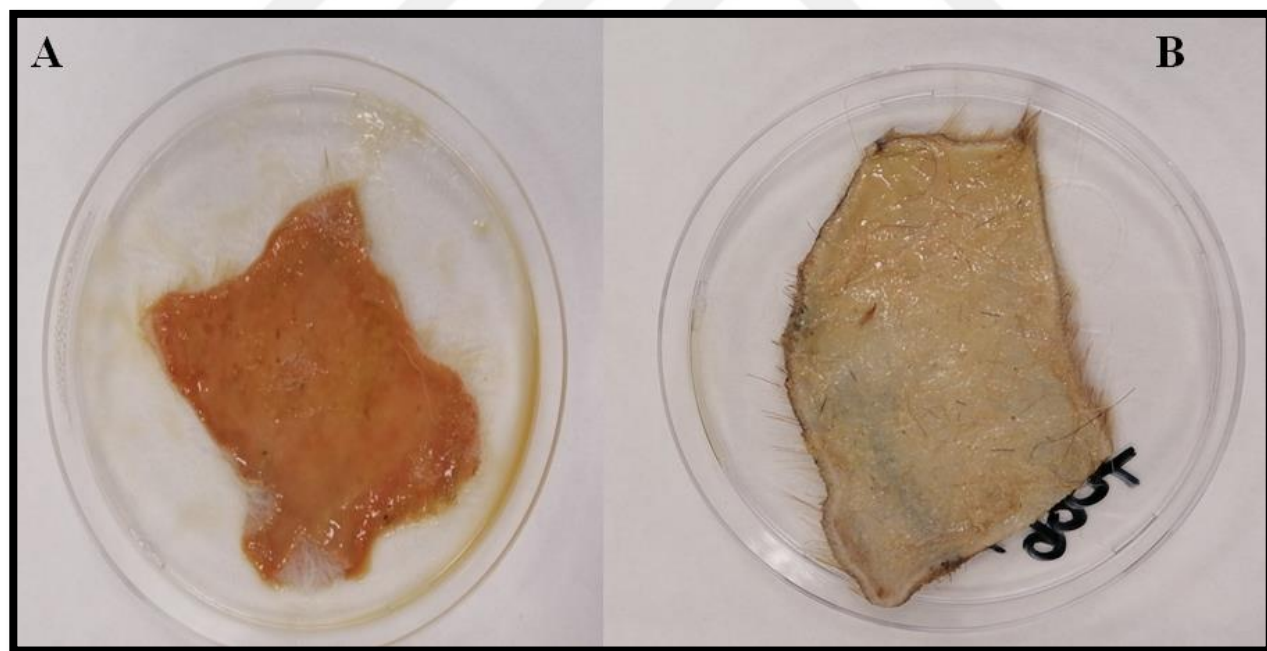


Figure 2.16. Pictures represent the test samples of sheepskin (A) and goatskin (B) treated with lipolytic and proteolytic bacteria.

2.2.11.2. Preparation of Goatskin and Sheepskin Samples for Scanning Electron Microscope

After 40 days of incubation, animal skin samples (sheepskin and goatskin) were treated for 30 minutes with a 4% solution of Glutaraldehyde prepared in a phosphate buffer (0.1M, pH 7.2) to stabilize the test and control samples. The samples were subsequently washed three times for ten minutes using a phosphate buffer (0.1M). Samples were treated with 1% osmium tetroxide in phosphate buffer (0.1M) at 24°C for one hour; osmium tetroxide increased the clarity of samples when examined by SEM (Scanning Electron Microscope) because it causes a yellow color in samples by binding to lipids and incorporating heavy metals in the cell membrane. The samples were subsequently washed twice by distilled water for 15 minutes, followed by washings with ethanol concentrations of 35%, 50%, 75%, 95%, and 100% in order to remove the water from the samples. During the drying procedure, samples were exposed to the air for three minutes. In order to increase drying of samples, they were washed with ethanol-HMDS (Hexa-Methyl-Disilazane) [1:2 (v/v)] for 30 minutes, ethanol-HMDS [1:1 (v/v)] for 30 minutes, and HMDS (2x30 min) (Figure 2.17).

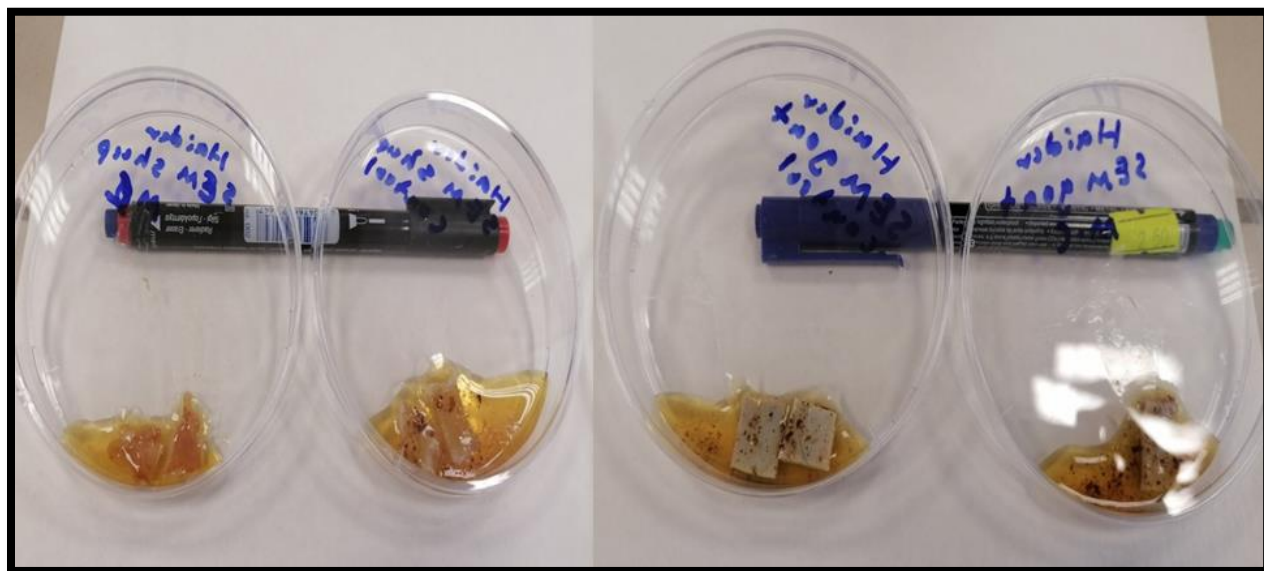


Figure 2.17. Animal skin samples after incubation period.

Subsequently, chemical solutions such as hexamethyldisilazane (HMDS) were eliminated through the process of subjecting sheepskin and goatskin samples to desiccation within a

desiccator for a duration of 14 hours. The parts which were covered with double-sided adhesive tape were employed for the purpose of affixing skin samples onto such components (Figure 2.18). The samples were examined using a Scanning Electron Microscope (Thermo-Fisher Scientific) (Das Murtey and Ramasamy, 2016).



Figure 2.18. Hexamethyldisilazane and SEM (Bogazici University, Istanbul, Türkiye).

2.2.12. EXAMINATION THE HALOVERSATILE BACTERIA ISOLATED FROM SALT SAMPLES UNDER SCANNING ELECTRON MICROSCOPE

To achieve a precise diagnosis, all of our isolates were prepared for scanning electron microscopy examination:

Bacteria cell preparation for scanning electron microscopy:

Bacterial cells were prepared for SEM examination through the combination of two procedures with a number of changes as described below (Ammar, 2017; Glauert, 1975a; Glauert, 1975b).

1. A fresh 24 h bacteria growth was prepared and centrifuged 10000 rpm for 5 minutes.
2. The supernatant was dispensed and the pellet was washed by using 0.2 M phosphate buffer saline of pH 7.2 (PBS) for 3 times preparing by dissolving 20.44g Na_2HPO_4 and 6.72 g NaH_2PO_4 in 500 mL of distilled water and the size was adjusted up to 1000 by using distilled water, then the concentration was diluted to access the demand concentration.
3. Depending on the size of diluted pellet adequate amount of 0.25% glutaraldehyde (which prepared by mixed 50 mL of 0.2 M Na-PBS with 10 mL of 25% glutaraldehyde then the total

volume was completed to 100 mL with distil water) was added and the solution was incubated overnight at 35°C.

3. Adding 0.25% glutaraldehyde (in Na-phosphate, pH 7.2) and incubated for 30 min at room temperature, then incubated 24h at 37°C.

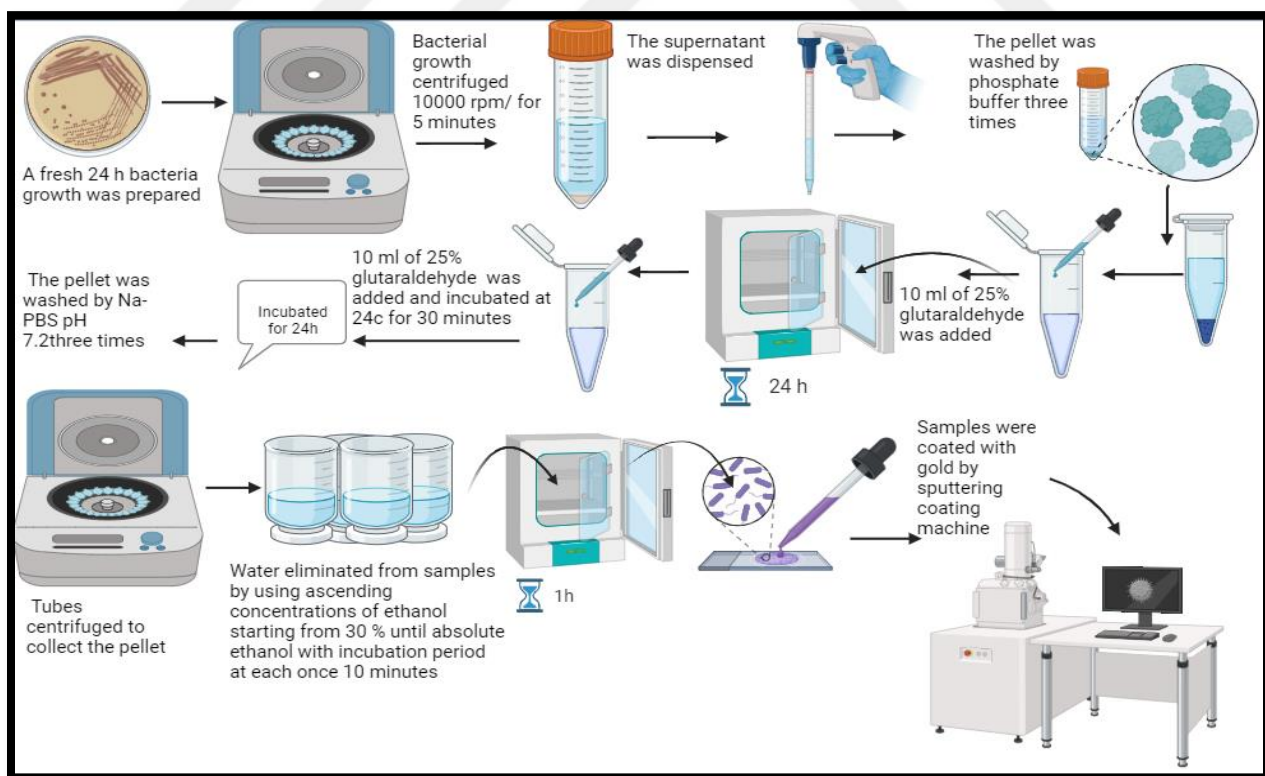
4. The solution washed 3 times by using Na-PBS pH 7.2 and centrifuged to collect the pellet.

5. Water eliminated from samples by using ascending concentrations of ethanol starting from 30% until absolute ethanol with incubation period at each once 10 minutes. The incubated at 100% for one hour.

6. One microliter of bacteria sample was spread on sterile and clean cover slide and left five minutes airdrying.

7. Bacterial samples were coated with gold by sputtering coating machine (this step was conducted in Faculty of Engineering/Bioengineering Department at Marmara University).

8. Samples were sent to Bogazici University (Istanbul, Türkiye) to be examined under SEM (Scheme 2.1.).



Scheme 2.1. Preparation of bacterial cells for SEM (Drawn by Haider Sabah Abdulhusein).

2.2.13. EXAMINATION OF THE ABILITY OF BACTERIA TO PRODUCE BACTERIOCIN AGAINST EACH OTHER

2.2.13.1. Preparation of Bacteriocin Solution

Each bacteriocin-producing haloversatile isolate was cultivated on Halophilic Broth Medium (HBM) (pH 7.0, 1.17% NaCl) and incubated overnight at 35°C to obtain a crude bacteriocin. The incubated solution was then centrifuged (8000 g for 15 minutes at 4°C) to remove all bacterial cells and yield cell-free supernatant (CFS) (Figure 2.19). To protect the development of bacteria from the inhibitory effects of acids, the pH was adjusted to 7.0 using 1N NaOH. Finally, supernatant was collected and filtered through a 0.22 µm Millipore membrane filter (Rajesh *et al.*, 2012).

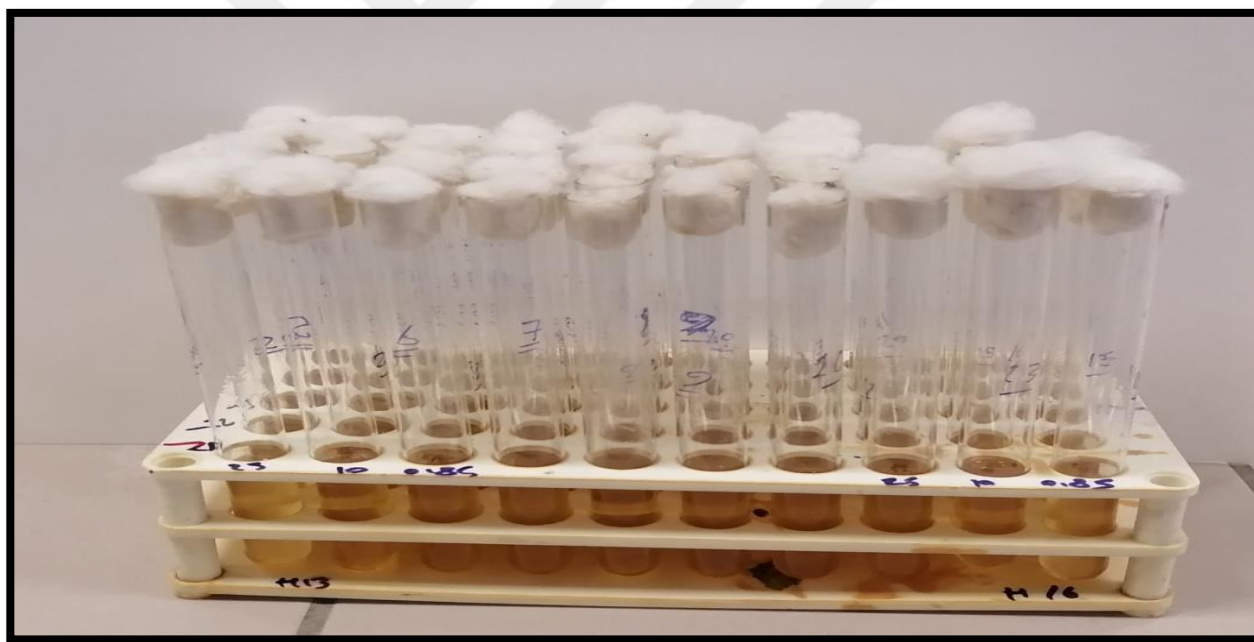


Figure 2.19. The haloversatile bacteria used in the bacteriocin production tests.

2.2.13.2. Screening of Antimicrobial Activity Among the Test Isolates

The antibacterial activity of bacteriocin was determined against every test isolate. The bacterial concentrations were individually adjusted to a concentration of 10^8 Colony-Forming Units per milliliter (CFU/mL) using a physiological saline solution. Petri plates containing Halophilic Agar Medium were inoculated with 100 µL aliquots of each adjusted bacterial suspension. In these inoculated petri plates, 3 µL of each bacteriocin were added. The diameters of the

inhibition zones (measured in millimeters) caused by the bacteriocin substance against each test isolate were measured following a 24-hour incubation period at a temperature of 35°C (Price and Shand, 2000). The bacteriocin-producing isolates, bacteriocin-susceptible isolates, and bacteriocin-resistant isolates were examined in this step. In the meantime, a particular group of bacteriocin-producing haloversatile isolates was chosen and subjected to further experiments. The inhibition effects of bacteriocins against lipase and protease producing isolates were investigated.

2.2.14. EXAMINATION OF THE OPTIMUM CONDITIONS FOR BACTERIOCIN PRODUCTION OF HALOVERSATILE ISOLATES

By using a sterile physiological solution, the bacterial concentration of each isolate was separately adjusted to 10^8 CFU/mL. Later, 3 mL of each bacterial solution was transferred into flasks which were containing 50 mL of HAM. Then, the flasks were incubated at six different temperature values; at the same time 3 mL of each adjusted bacterial solution was added to six flasks with different pH values and different concentrations of NaCl, and all flasks were incubated overnight at 35°C. The concentration of the tested isolates was independently adjusted to 10^8 CFU/mL by using a sterile physiological solution. Following that, 100 µL aliquots of each tested isolate was distributed on Halophilic Agar Medium Petri plates. On the inoculated petri plates, 3 mL of each bacteriocin was dropped. Bacteriocin inhibition zone diameters against each test isolate were evaluated after 24 hours at 35°C incubation (Price and Shand, 2000).

2.2.14.1. Investigation of the Effect of Heat on Bacteriocins Produced by Haloversatile Isolates

The bacteriocins that were produced by the haloversatile isolates were subjected to incubation at various temperatures ranging from 4°C to 100°C for duration of 15 minutes. Subsequently, the antibacterial activity of heat-treated bacteriocin samples was examined against test isolates using the agar spot diffusion method. The plates were incubated at 35°C for 24 hours. The diameters of the inhibition zones caused by bacteriocin against the tested isolates were measured following the incubation period (Caglayan and Birbir, 2018).

2.2.14.2. Investigation of the Effects of pH on the Bacteriocin Production Ability of Test Bacteria

The ability of bacteriocin production of bacteria was tested with different pH values ranging from 4 to 12. Bacteriocin producing isolates were kept at 25°C for 4 hours at different pH values. Then, the bacteriocin samples tested for antimicrobial activity against test isolates by agar spot diffusion assay. The inoculated Petri plates incubated at 35°C for 24 hours. After incubation period, the inhibition zone diameters of the bacteriocin against the test isolates were examined (Caglayan and Birbir, 2018).

2.2.14.3. Investigation of the Effects of NaCl Concentration on the Bacteriocin Production Ability of Test Bacteria

NaCl concentration of the bacteriocin of each isolate was adjusted to 1.17, 3, 5, 10, 15, 20, 25% NaCl. The bacteriocin samples tested for antibacterial activity against other test isolates by agar spot diffusion assay. All plates were incubated at 35°C for 24 hours. After incubation, the inhibition zone diameters were measured (Caglayan and Birbir, 2018).

2.2.14.4. Effect of Enzymes (Proteinase K and Lipase) on Bacteriocins Produced by Haloversatile Isolates

The inhibition impacts of two enzymes (Lipase and Proteinase K) against bacteriocin's effectiveness were investigated based on the method by Kindoli *et al.*, (2012). The source of both enzymes was *Candida rugosa* for lipase and *Tritirachium album* for proteinase K; the enzymes were purchased from Sigma Aldrich (St Louis, MO) (Rajesh *et al.*, 2012), prepared in 10 mM phosphate buffer (pH 7) and 0.05 M Tris HCl, 0.01 M CaCl₂, pH 8, and filtered through 0.22 µm pore size filter paper (HiMedia). One mL of sterile enzyme solution was added to the supernatant, and the mixture was incubated at 37°C for two hours. The activities of bacteriocins treated with enzymes were investigated using the agar spot diffusion method on test isolates that were isolated from salt samples. For 24 hours, the plates were incubated at 37°C. The inhibition zone diameters of the bacteriocin against the test isolates were evaluated following the incubation period. Negative controls for Proteinase K and Lipase were phosphate buffer (10 mM) and Tris HCl buffer (0.05 M), respectively (Kindoli *et al.*, 2012).

2.2.15. ANTIBIOTIC SUSCEPTIBILITY TESTS

This step carried out based on standard method by Bauer *et al.* (1996), the bacterial resistance and sensitive to antibiotics detected by Kirby-Bauer disc diffusion technique. All halophilic isolates grown in Mueller Hinton Broth (MHB) containing 1.17% NaCl were incubated overnight at 35°C the optical density was adjusted according to McFarland Standard No 0.5 (1×10^8 CFU/mL) with sterile saline solution (1.17% NaCl). Later, suspensions of isolates were spread on Mueller Hinton Agar. Different antibiotic discs such as Cephalothin (KF30, 30 µg), Cefotaxime (CTX5, 5µg), Rifampicin (RD5, 5 µg), Spectinomycin (SH100, 100 µg), Sulbactam/Ampicillin (SAM10, 10µg), Amikacin (AK30, 30 µg), Neomicin (N30, 30 µg) and Tetracycline (TE30, 30 µg) discs (Oxoid, UK) placed on surface of the inoculated Mueller Hinton Agar plates. The plates were incubated at 35°C for 24h. At the end of the incubation period, the diameters of the inhibition zones measured.

2.2.16. MIC OF ANTIMICROBIAL AGENTS AGAINST HALOVERSATILE ISOLATES

The MIC (Minimum Inhibitory Concentration) of an antimicrobial agent with sodium dimethyldithiocarbamate against haloversatile isolates were determined by using the broth dilution method in a 96-well microtiter plate. As explained in detail:

- At the first, protease-producing bacteria including *Staphylococcus warneri* (PSS1a), *Staphylococcus pasteurii* (PSS1b), *Paenibacillus illinoisensis* (PSS7a), *Bacillus subtilis* (PSS16a), *Micrococcus luteus* (PSS21b), *Staphylococcus epidermidis* (PSS15b), *Bacillus sonorensis* (PSS12a), *Corynebacterium aurimucosum* (PSS15a), and *Staphylococcus capitis* subsp. *urealyticus* (PSS22a) isolates were grown in Halophilic Broth Medium (HBM) containing 1.17% NaCl and incubated overnight at 35°C the optical density adjusted according to McFarland Standard No 0.5 (1×10^8 CFU/mL) with sterile saline solution (1.17% NaCl).
- Second, 100 µL of media broth added to each well in a 96- micro-titer (the first column left empty and the last column had 200 µL and), and then 100 µL of an antimicrobial agent with sodium dimethyldithiocarbamate was added to the first column A1 to H1, also 100 µL of an antimicrobial agent with sodium dimethyldithiocarbamate was added to the second column and mixed thoroughly by the tip of micropipette, and 100 µL was taken

and added to the next column, and then by the same token the process was continued until the tenth column and finally 100 μ L was dispensed out.

- Afterward, 100 μ L of an aliquot 24 h fresh bacterial suspension was added to all wells (based on the isolates' number), except the last column A12 to H12 which had just media broth; the column A11 to H11 it had only bacterial inoculants.
- Ultimately, the plates were incubated for 24h at 35°C, and (0.15%) 30 μ L of resazurin solution was added into each well. Thence, 96-well plates were left for 4 hours at room temperature, and then the result was checked for color changing; the pink color was accepted as a growth whereas the purple or blue color was interpreted as a non-growth and demonstrated a MIC of an antimicrobial agent with sodium dimethyldithiocarbamate (Elshikh *et al.*, 2016).
- All experiments were performed in triplicate.

CHAPTER 3

3. RESULTS AND DISCUSSION

3.1. DETERMINATION OF pH OF THE SALT SAMPLES

Twenty-five salt samples were collected from different tanneries from Tuzla/Istanbul, Türkiye. The pH values of all samples measured by using pH meter. The results express the pH values of all salt samples were close to each other and ranged from 7.60 to 8.04 (Table 3.1). The highest and the lowest pH were respectively measured for PSS7, PSS10 and PSS18 (Table 3.1).

Table 3.1. The pH values, total counts of haloversatile bacteria, proteolytic bacteria and lipolytic bacteria of the salt samples.

Salt Samples	pH values	Total counts of haloversatile bacteria (CFU/g)	Total counts of proteolytic bacteria (CFU/g)	Total counts of lipolytic bacteria (CFU/g)
PSS1	7.96	1×10^4	7×10^3	2×10^3
PSS2	7.84	6×10^3	1×10^3	1.4×10^4
PSS3	7.75	1.1×10^4	5×10^3	6×10^3
PSS4	7.94	1.2×10^4	3×10^3	7×10^3
PSS5	7.91	9×10^3	2×10^3	4×10^3
PSS6	7.89	8×10^3	5×10^3	4×10^3
PSS7	8.04	7×10^3	3×10^3	5×10^3
PSS8	7.93	6×10^4	1×10^3	4×10^3
PSS9	8.01	3×10^4	4×10^3	7×10^3
PSS10	7.60	1.1×10^4	4×10^3	8×10^3
PSS11	7.76	1.2×10^4	3×10^3	2×10^3
PSS12	7.83	1.1×10^4	2×10^3	3×10^3
PSS13	7.66	1×10^4	3×10^3	5×10^3
PSS14	7.84	2.8×10^4	4×10^3	8×10^3
PSS15	7.92	7×10^4	3×10^3	2×10^3
PSS16	7.67	1.3×10^5	5×10^3	3.1×10^4
PSS17	7.93	1.3×10^4	5×10^3	3×10^3
PSS18	7.60	7.2×10^4	6×10^3	5×10^3
PSS19	7.98	3×10^4	1×10^3	2×10^3
PSS20	7.91	6×10^4	1×10^3	4×10^3
PSS21	7.82	5×10^4	2×10^3	2×10^3
PSS22	7.85	1×10^4	1×10^3	2×10^3
PSS23	7.94	8×10^4	3×10^3	3×10^3
PSS24	7.95	1.2×10^4	2×10^3	4×10^3
PSS25	7.74	1.5×10^4	4×10^3	3×10^3

*PSS: Preservation Salt Sample

Berber and Birbir (2010) examined 40 salt, 36 salted hide, 34 soaked hide, and 19 soak liquor samples collected from Leather tanneries in terms of pH values and bacterial populations. The pH values of all samples (salt, salted hide, soaked hide, soak liquor) were reported between 6 and 9 (Berber and Birbir, 2010). The researchers reported that those pH values could support the bacterial growth (Berber and Birbir, 2010). While the samples were examined in detail, the bacterial numbers found supported that interpretation. The mesophilic bacterial counts were found as 10^2 - 10^4 CFU/g in salt samples, 10^2 - 10^6 CFU/g in salted hide samples (Berber and Birbir, 2010). The researchers reported that preservation method of skins and hides was not adequate due to the presence of protease and lipase producing mesophilic bacterial populations in soaked hide and soak liquor samples (Berber and Birbir, 2010). In an other previous study, 30 salt samples were examined for halotolerant bacteria which were collected from 14 leather factories in Tuzla and Corlu in Türkiye (Yilmaz and Birbir, 2019). In that study, total counts of halotolerant bacteria, the salt samples' pH values and moisture contents were reported as 10^4 CFU/g- 10^6 CFU/g, 6.23-7.22, 0.90-5.02, respectively (Yilmaz and Birbir, 2019).

3.2. DETERMINATION OF THE TOTAL NUMBERS OF HALOVERSATILE BACTERIA, THE TOTAL NUMBERS OF PROTEOLYTIC AND LIPOLYTIC HALOVERSATILE BACTERIA IN THE SALT SAMPLES

By using specific media such as Halophilic Agar Media with special contents, the total number of haloversatile bacteria were detected as 6×10^3 CFU/g to 1.3×10^5 CFU/g. While the total number of proteolytic haloversatile bacteria were ranged from 1×10^3 CFU/g to 7×10^3 CFU/g, the total number of lipolytic haloversatile bacteria were ranged from 2×10^3 CFU/g to 3.1×10^4 CFU/g by using Gelatin Agar Media and Tween 80 Agar Media, respectively (Table 3.1). Twenty and 4 salt samples were respectively contained 10^4 CFU/g and 10^3 CFU/g haloversatile bacteria. Only one salt sample contained 10^5 CFU/g haloversatile bacterial (Table 3.1). The total number of proteolytic haloversatile bacteria were detected as 10^3 CFU/g for all salt samples. The total number of lipolytic haloversatile bacteria were respectively found as 10^3 CFU/g and 10^4 CFU/g for 23 and 2 salt samples (Table 3.1).

3.3. ISOLATION OF HALOVERSATILE BACTERIA FROM SALT SAMPLES

Ten grams of each salt sample weighted and mixed with 90 mL of physiological saline solution (1.17%), then shacked for 3 hours at 35°C, 160 rpm. The serial dilutions were prepared and spread on the Halophilic Agar Media. The plates were incubated overnight at 35°C. After

incubation, the bacterial colonies on the Petri plates were counted and restreaked until pure colony for each isolate was obtained (Figure 3.1). In the present study, totally 35 isolates were obtained from 25 salt samples (Table 3.2).

Table 3.2. Haloversatile bacterial isolates.

Isolates numbers	Isolate codes	Phylogenetically similar to
1	PSS1a	<i>Staphylococcus warneri</i>
2	PSS1b	<i>Staphylococcus pasteurii</i>
3	PSS2a	<i>Staphylococcus warneri</i>
4	PSS2b	<i>Corynebacterium aurimucosum</i>
5	PSS3a	<i>Staphylococcus epidermidis</i>
6	PSS3b	<i>Staphylococcus epidermidis</i>
7	PSS4a	<i>Peribacillus frigoritolerans</i>
8	PSS4b	<i>Staphylococcus haemolyticus</i>
9	PSS5a	<i>Lysinibacillus telephonicus</i>
10	PSS6a	<i>Kocuria rosea</i>
11	PSS7a	<i>Paenibacillus illinoisensis</i>
12	PSS8a	<i>Kocuria polaris</i>
13	PSS9a	<i>Staphylococcus epidermidis</i>
14	PSS9b	<i>Staphylococcus caprae</i>
15	PSS10a	<i>Staphylococcus epidermidis</i>
16	PSS11a	<i>Bhargavaea ginsengi</i>
17	PSS11b	<i>Bacillus haynesii</i>
18	PSS12a	<i>Bacillus sonorensis</i>
19	PSS13a	<i>Bacillus licheniformis</i>
20	PSS14a	<i>Pseudomonas oryzae</i>
21	PSS15a	<i>Corynebacterium aurimucosum</i>
22	PSS15b	<i>Staphylococcus epidermidis</i>
23	PSS16a	<i>Bacillus subtilis</i>
24	PSS17a	<i>Bacillus pascalis</i>
25	PSS18a	<i>Staphylococcus hominis</i>
26	PSS18b	<i>Micrococcus yunnanensis</i>
27	PSS19a	<i>Staphylococcus caprae</i>
28	PSS20a	<i>Staphylococcus saccharolyticus</i>
29	PSS21a	<i>Pseudomonas iranica</i>
30	PSS21b	<i>Micrococcus luteus</i>
31	PSS22a	<i>Staphylococcus capitis</i> subsp. <i>urealyticus</i>
32	PSS23a	<i>Corynebacterium minutissimum</i>
33	PSS23b	<i>Bacillus paralicheniformis</i>
34	PSS24a	<i>Staphylococcus epidermidis</i>
35	PSS25a	<i>Staphylococcus taiwanensis</i>



Figure 3.1. Pure cultures of haloversatile isolates obtained from salt samples.

3.4. MOLECULAR STUDY

3.4.1. Determination of 16S rRNA Gene Sequences of DNA Belonging to the Haloversatile Isolates

A total of 35 isolates were obtained from salt samples and subjected to biochemical and molecular analysis. The findings of the molecular study, together with the identification of phylogenetically similar species to reference species available on NCBI sites, are presented in Table 3.3. All isolates studied were belong to the Domain *Bacteria*. According to the data analysis using 16S rRNA gene sequences, the haloversatile isolates grouped within three major phyla: *Firmicutes*, *Actinomycetota* and *Proteobacteria*. It was determined that 26 isolates (74%), 7 isolates (20%), and 2 isolates (6%) were respectively belonged to the Phylum *Firmicutes*, the Phylum *Actinomycetota*, and the Phylum *Proteobacteria* (Figure 3.2).

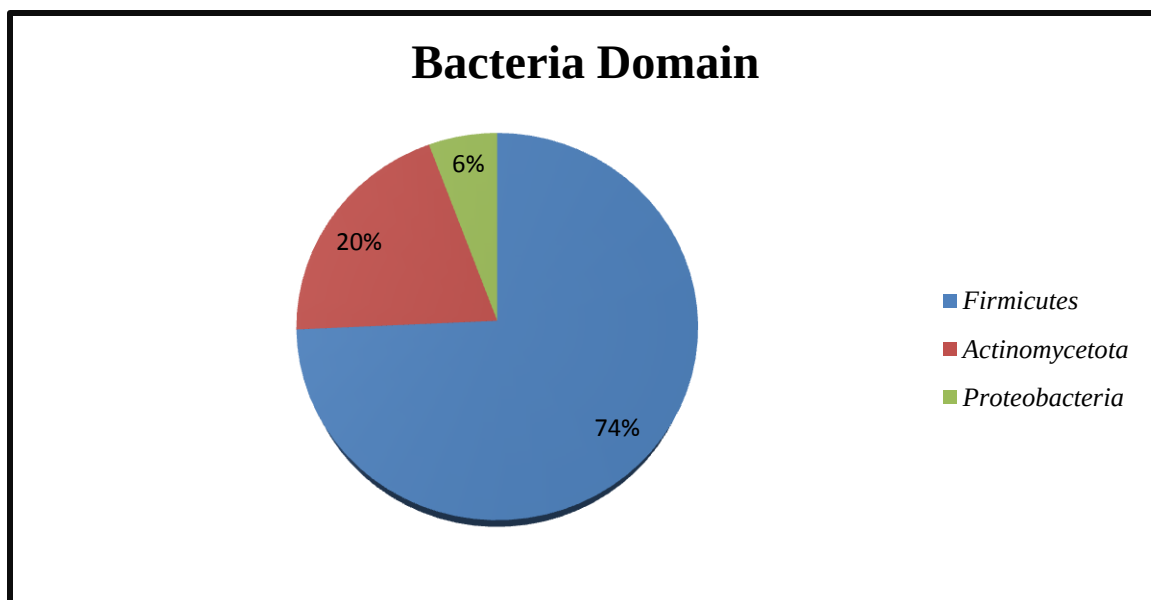


Figure 3.2. The classification of isolates according to phylogenetically similar species to phyla *Firmicutes*, *Actinomycetota* and *Proteobacteria*.

The isolates codes (PSSS1a-PSS25a), the base pair of 16S rRNA gene sequences (704-1119), the percent of haloversatile bacterial isolates' similarities (98-100%), the concentration of genomic DNA was between 30-100 ng/μl by using nanodrop (ND2000), the name of the species that haloversatile isolates phylogenetically similar to are shown in Table 3.3.

Table 3.3. Isolates codes, 16S rRNA sequence lengths (bp), similarity (%) and phylogenetically similar species of haloversatile bacteria isolated from salt samples.

Isolate codes	Base pair	Similarity	Similar species	Strain	Blast name of phylum
PSS1a	1040	99.81%	<i>Staphylococcus warneri</i>	RO8	Firmicutes
PSS1b	1041	99.33%	<i>Staphylococcus pasteurii</i>	ATCC 51129	Firmicutes
PSS2a	955	99.99%	<i>Staphylococcus warneri</i>	DK131	Firmicutes
PSS2b	704	99.57%	<i>Corynebacterium aurimucosum</i>	H2456	Actinomycetota
PSS3a	933	100%	<i>Staphylococcus epidermidis</i>	21DSE01	Firmicutes
PSS3b	1006	100%	<i>Staphylococcus epidermidis</i>	WHW31	Firmicutes
PSS4a	871	99.66%	<i>Peribacillus frigoritolerans</i>	DSM8801	Firmicutes
PSS4b	1082	99.34%	<i>Staphylococcus haemolyticus</i>	PL565	Firmicutes
PSS5a	1042	99.81%	<i>Lysinibacillus telephonicus</i>	S5H2222	Firmicutes
PSS6a	1054	99.62%	<i>Kocuria rosea</i>	DSM 20447	Actinomycetota
PSS7a	935	99.68%	<i>Paenibacillus illinoisensis</i>	NBRC 15959	Firmicutes
PSS8a	1054	99.72%	<i>Kocuria polaris</i>	CMS 76or	Actinomycetota
PSS9a	953	99.79%	<i>Staphylococcus epidermidis</i>	Fussel	Firmicutes
PSS9b	899	99.33%	<i>Staphylococcus caprae</i>	ATCC 35538	Firmicutes
PSS10	988	99.90%	<i>Staphylococcus epidermidis</i>	32FSE06	Firmicutes
PSS11	970	99.79%	<i>Bhargavaea ginsengi</i>	ge14	Firmicutes
PSS11	932	99.36%	<i>Bacillus haynesii</i>	NRRL B-41327	Firmicutes
PSS12	1057	99.34%	<i>Bacillus sonorensis</i>	NBRC 101234	Firmicutes
PSS13	1059	99.34%	<i>Bacillus licheniformis</i>	DSM 13	Firmicutes
PSS14	1065	99.06%	<i>Pseudomonas oryzihabitans</i>	IAM 1568	Proteobacteria
PSS15	1083	99.54%	<i>Corynebacterium aurimucosum</i>	IMMIB D-1488	Actinomycetota
PSS15	955	99.90%	<i>Staphylococcus epidermidis</i>	RG4	Firmicutes
PSS16	855	98.13%	<i>Bacillus subtilis</i>	P3	Firmicutes
PSS17	1098	99.64%	<i>Bacillus pascis</i>	16MFT21	Firmicutes
PSS18	942	99.48%	<i>Staphylococcus hominis</i>	FDAARGOS_74	Firmicutes
PSS18	1086	99.72%	<i>Micrococcus yunnanensis</i>	YIM 65004	Actinomycetota
PSS19	937	99.79%	<i>Staphylococcus caprae</i>	ATCC 35538	Firmicutes
PSS20	796	99.12%	<i>Staphylococcus saccharolyticus</i>	JCM 1768	Firmicutes
PSS21	1112	98.11%	<i>Pseudomonas iranica</i>	GH10	Proteobacteria
PSS21	1098	99.54%	<i>Micrococcus luteus</i>	Pso_Seed_1	Actinomycetota
PSS22	863	98.49%	<i>Staphylococcus capitis</i> subsp.	MAW 8436	Firmicutes
PSS23	1081	98.44%	<i>Corynebacterium minutissimum</i>	NCTC 10288	Actinomycetota
PSS23	966	99.69%	<i>Bacillus paralicheniformis</i>	KJ-16	Firmicutes
PSS24	965	99.79%	<i>Staphylococcus epidermidis</i>	8 Chromosome	Firmicutes
PSS25	1119	99.29%	<i>Staphylococcus taiwanensis</i>	NTUH-S172	Firmicutes

Figure 3.3. shows that 26 isolates (74%) were classified as members of the Phylum *Firmicutes* group. While 16 isolates were belong to Genus *Staphylococcus*, 6 isolates were belong to Genus *Bacillus*. Only one isolates was belong to Genus *Peribacillus*, Genus *Lysinibacillus*, Genus

Paenibacillus, and Genus *Bhargavaea* (Figure 3.3). Seven isolates belonging to the Phylum *Actinomycetota* (20%) were found, including Genus *Corynebacterium* sp. (3 isolates), Genus *Kocuria* sp. (2 isolates), and Genus *Micrococcus* sp. (2 isolates) as show in Figure 3.4. Genus *Pseudomonas* had two isolates belonging to the Phylum *Proteobacteria*.

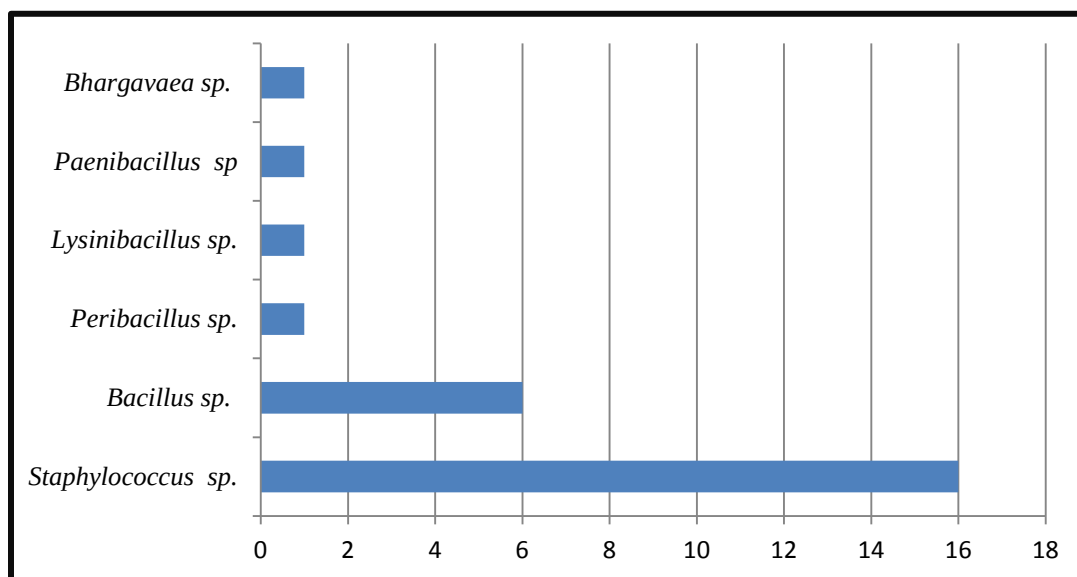


Figure 3.3. The number of bacterial genera which belong to the Phylum *Firmicutes* isolated from salt samples.

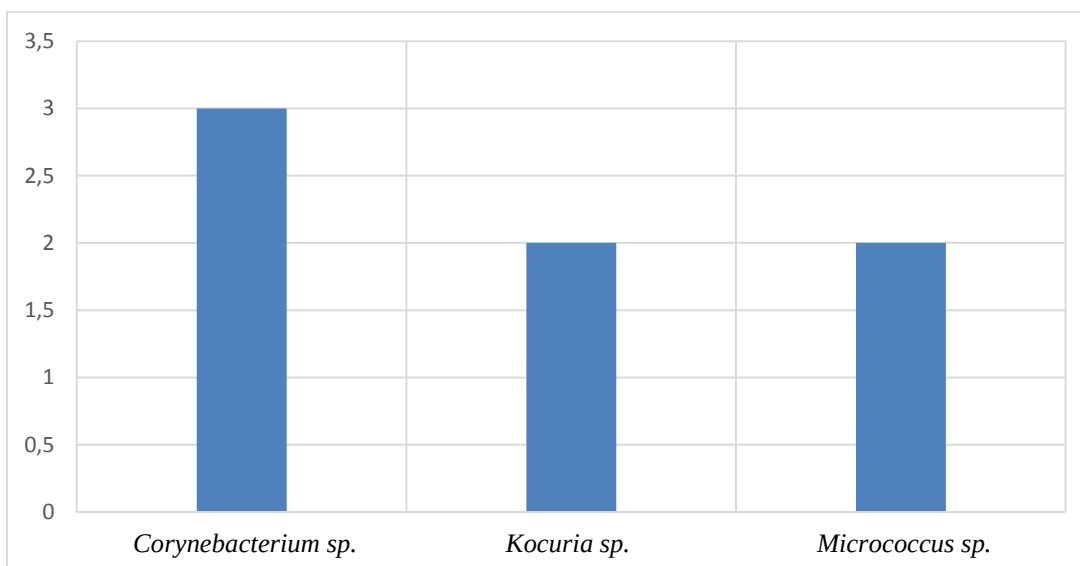


Figure 3.4. The number of bacterial genera which belong to the Phylum *Actinomycetota* isolated from salt samples.

Sixteen isolates were isolated from the salt samples belonging to the Genus *Staphylococcus* such as *Staphylococcus epidermidis* (6 isolates), *Staphylococcus caprae* (2 isolates), *Staphylococcus warneri* (2 isolates), *Staphylococcus capitis* subsp. *urealyticus* (1 isolate), *Staphylococcus haemolyticus* (1 isolate), *Staphylococcus taiwanensis* (1 isolate), *Staphylococcus saccharolyticus* (1 isolate), *Staphylococcus hominis* (1 isolate), *Staphylococcus pasteurii* (1 isolate) (Figure 3.5). Six species belonging to the Genus *Bacillus* such as *Bacillus pascis* (1 isolate), *Bacillus subtilis* (1 isolate), *Bacillus paralicheniformis* (1 isolate), *Bacillus haynesii* (1 isolate), *Bacillus sonorensis* (1 isolate) and *Bacillus licheniformis* (1 isolate) were isolated and identified (Figure 3.5). The other haloversatile species that isolated from salt samples belonging to the Phylum *Firmicutes* were *Bhargavaea ginsengi* (1 isolate), *Paenibacillus illinoisensis* (1 isolate), *Lysinibacillus telephonicus* (1 isolate) and *Peribacillus frigoritolerans* (1 isolate) (Table 3.3).

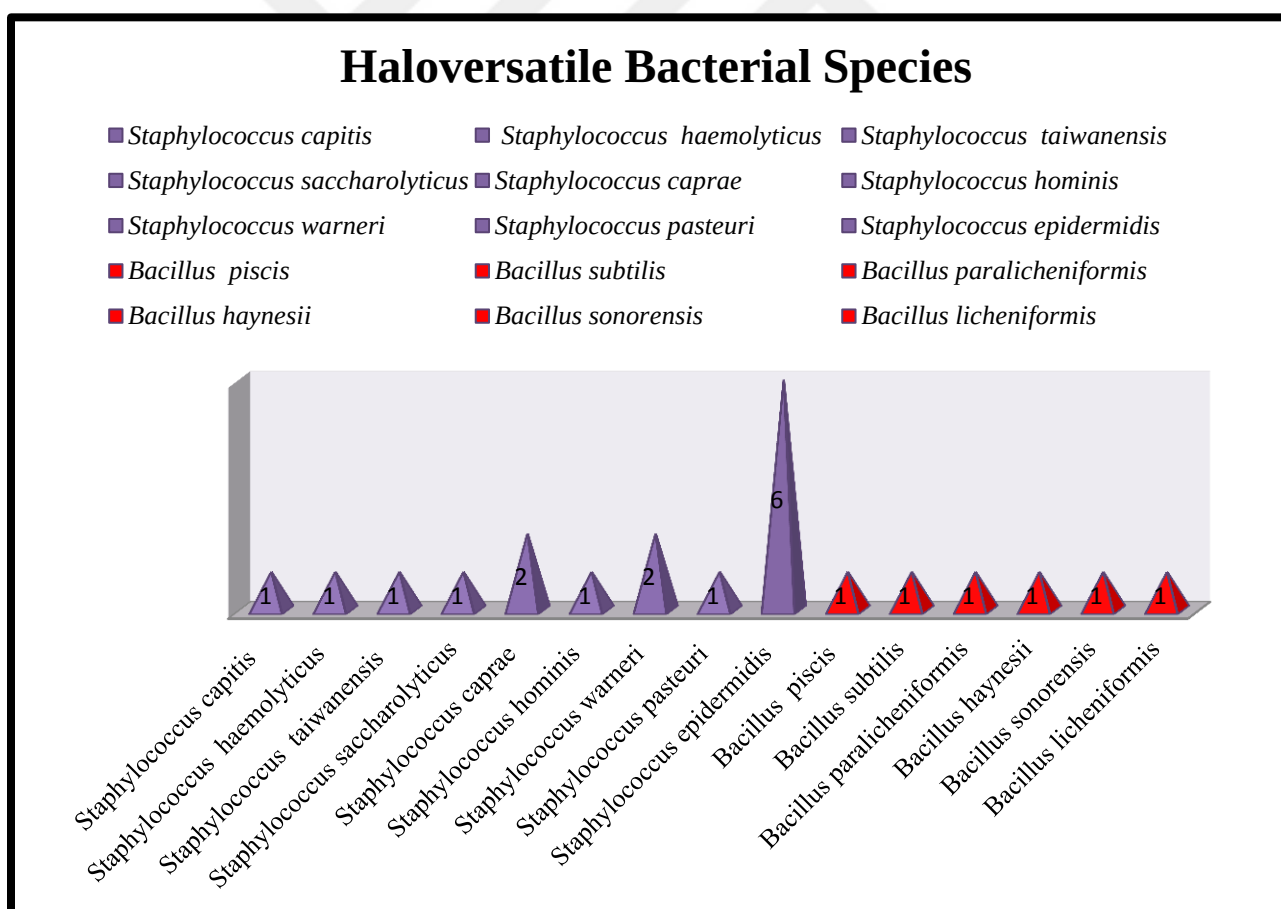


Figure 3.5. *Staphylococcus* and *Bacillus* species belonging to the Phylum *Firmicutes* isolated from salt samples.

Two isolates (*Pseudomonas oryzihabitans*, *Pseudomonas iranica*) were members of the Phylum *Proteobacteria*. Seven isolates with three genus and six species (*Corynebacterium aurimucosum* (2 isolates), *Corynebacterium minutissimum* (1 isolate), *Kocuria rosea* (1 isolate), *Kocuria Polar* (1 isolate), *Micrococcus yunnanensis* (1 isolate) and *Micrococcus luteus* (1 isolate) were belong to the Phylum *Actinomycetota* (Figure 3.6).

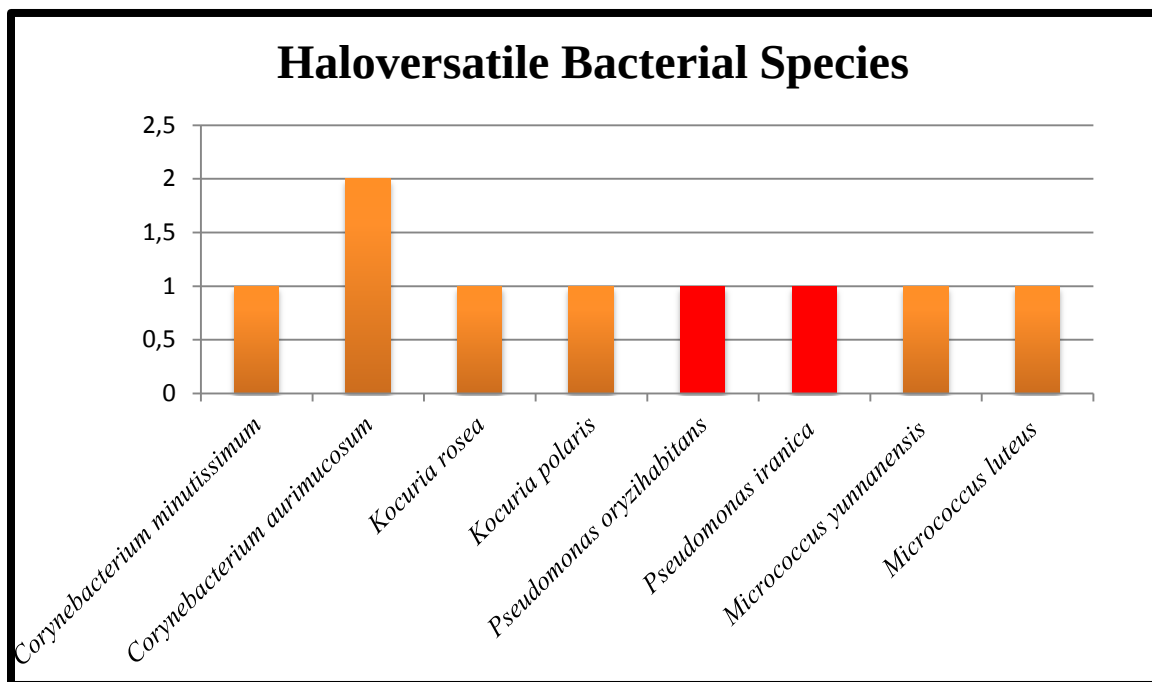


Figure 3.6. The species belonging to the Genera *Corynebacterium*, *Kocuria*, *Pseudomonas* and *Micrococcus* isolated from salt samples.

Caglayan (2019) reported that 69 haloversatile isolates were obtained from salt samples collected from Çamaltı Saltern in İzmir (Türkiye). This is the largest saltern originated from seawater. In that study, while 61 isolates were belong to the Phylum *Firmicutes*, 6 isolates were belong to the Phylum *Proteobacteria* (Caglayan, 2019). Likewise our study, the members of the Phylum *Firmicutes* was higher than other phyla.

3.4.2. Nucleotid Accession Number

All isolates sequenced depending on 16SrRNA gene which were reported under the specific codes (PSS1a, PSS1b, PSS2a, PSS2b, PSS3a, PSS3b, PSS4a, PSS4b, PSS95a, PSS6a, PSS7a, PSS8a, PSS9a, PSS9b, PSS10a, PSS11a, PSS11b, PSS12a, PSS13a, PSS14a, PSS15a, PSS15b, PSS16a, PSS17a, PSS18a, PSS18b, PSS19a, PSS20a, PSS21a, PSS21b, PSS22a, PSS23a, PSS23b, PSS24a, and PSS25a) were registered in NCBI and GenBank nucleotide data base under the respective accession numbers: OR016512, OR039442, OR036459, OR036460, OR036461, OR036876, OR036977, OR036978, OR036979, OR036980, OR036981, OR036982, OR036983, OR036984, OR036985, OR036986, OR036987, OR037108, OR037109, OR037110, OR037111, OR037112, OR037113, OR037114, OR037115, OR037116, OR037117, OR037118, OR037119, OR037120, OR037121, OR037122, OR037123, and OR037124, and OR016708 (Figure 3.7, Table 3.4).

Submission	Title	Group	Status	Updated
SUB13453463	Prokaryotic 16S rRNA		✓ GenBank: Processed OR039442 3 files: • AccessionReport.tsv • flatfile.txt • email.txt	May 24
SUB13447435	Prokaryotic 16S rRNA		✓ GenBank: Processed OR039438 3 files: • AccessionReport.tsv • flatfile.txt • email.txt	May 24
SUB13450664	Prokaryotic 16S rRNA		✓ GenBank: Processed OR037108-OR037124 3 files: • AccessionReport.tsv • flatfile.txt • email.txt	May 23
SUB13449683	Prokaryotic 16S rRNA		✓ GenBank: Processed OR036977-OR036987 3 files: • AccessionReport.tsv • flatfile.txt • email.txt	May 23
SUB13448388	Prokaryotic 16S rRNA		✓ GenBank: Processed OR036876	May 23

Figure 3.7. The accession numbers which were registered in NCBI and GenBank data bases.

The isolate codes of 35 haloversatile bacterial isolates, phylogenetically similar species, accession numbers and reference strains in GenBank and NCBI were shown in Table 3.4.

Table 3.4. Isolate codes, phylogenetically similar species, accession numbers and reference strains in GenBank and NCBI.

Isolate codes	Phylogenetically similar species	Accession numbers	Reference strains
PSS1a	<i>Staphylococcus warneri</i>	OR016512	ISIQ88
PSS1b	<i>Staphylococcus pasteurii</i>	OR039442	HAP2
PSS2a	<i>Staphylococcus warneri</i>	OR036459	3HAP
PSS2b	<i>Corynebacterium aurimucosum</i>	OR036460	4HAP
PSS3a	<i>Staphylococcus epidermidis</i>	OR036461	5HAP
PSS3b	<i>Staphylococcus epidermidis</i>	OR036876	HAP688
PSS4a	<i>Peribacillus frigoritolerans</i>	OR036977	7HAP
PSS4b	<i>Staphylococcus haemolyticus</i>	OR036978	8HAP
PSS5a	<i>Lysinibacillus telephonicus</i>	OR036979	9HAP
PSS6a	<i>Kocuria rosea</i>	OR036980	11HAP
PSS7a	<i>Paenibacillus illinoisensis</i>	OR036981	12HAP
PSS8a	<i>Kocuria polaris</i>	OR036982	13HAP
PSS9a	<i>Staphylococcus epidermidis</i>	OR036983	14HAP
PSS9b	<i>Staphylococcus caprae</i>	OR036984	15HAP
PSS10a	<i>Staphylococcus epidermidis</i>	OR036985	16HAP
PSS11a	<i>Bhargavaea ginsengi</i>	OR036986	17HAP
PSS11b	<i>Bacillus haynesii</i>	OR036987	19HAP
PSS12a	<i>Bacillus sonorensis</i>	OR037108	21HAP
PSS13a	<i>Bacillus licheniformis</i>	OR037109	22HAP
PSS14a	<i>Pseudomonas oryzae</i>	OR037110	23HAP
PSS15a	<i>Corynebacterium aurimucosum</i>	OR037111	24HAP
PSS15b	<i>Staphylococcus epidermidis</i>	OR037112	26HAP
PSS16a	<i>Bacillus subtilis</i>	OR037113	27HAP
PSS17a	<i>Bacillus pascalis</i>	OR037114	28HAP
PSS18a	<i>Staphylococcus hominis</i>	OR037115	28HAP
PSS18b	<i>Micrococcus yunnanensis</i>	OR037116	30HAP
PSS19a	<i>Staphylococcus caprae</i>	OR037117	31HAP
PSS20a	<i>Staphylococcus saccharolyticus</i>	OR037118	32HAP
PSS21a	<i>Pseudomonas iranica</i>	OR037119	33HAP
PSS21b	<i>Micrococcus luteus</i>	OR037120	34HAP
PSS22a	<i>Staphylococcus capitis</i> subsp. <i>urealyticus</i>	OR037121	35HAP
PSS23a	<i>Corynebacterium minutissimum</i>	OR037122	38HAP
PSS23b	<i>Bacillus paralicheniformis</i>	OR037123	40HAP
PSS24a	<i>Staphylococcus epidermidis</i>	OR037124	41HAP
PSS25a	<i>Staphylococcus taiwanensis</i>	OR016708	HAP4488

Table 3.5 shows the length, starting sequences and molecular weight of DNA extracted from haloversatile isolates obtained from the salt samples in the present study.

Table 3.5. The length, starting sequences and molecular weight of DNA extracted from isolates obtained from salt samples.

No	Isolates	Length (bp)	Starting sequences	Molecular weight
PSS1a	<i>Staphylococcus warneri</i>	1040	"GTGACGGGCG"	319776.68 Da
PSS1b	<i>Staphylococcus pasteurii</i>	1041	"GTGACGGGCG"	320129.92 Da
PSS2a	<i>Staphylococcus warneri</i>	955	"CACCGTAGCA"	293534.78 Da
PSS2b	<i>Corynebacterium aurimucosum</i>	704	"ACTTCATGGG"	215986.30 Da
PSS3a	<i>Staphylococcus epidermidis</i>	933	"ACCGTAGCAT"	286714.34 Da
PSS3b	<i>Staphylococcus epidermidis</i>	1006	"ACCGTAGCAT"	309158.85 Da
PSS4a	<i>Peribacillus frigoritolerans</i>	871	"CCCTTTGTAC"	267316.49 Da
PSS4b	<i>Staphylococcus haemolyticus</i>	1082	"CCACCGGCTT"	332742.04 Da
PSS5a	<i>Lysinibacillus telephonicus</i>	1042	"GACGGGCGGT"	320307.70 Da
PSS6a	<i>Kocuria rosea</i>	1054	"GACTTGACGG"	323606.44 Da
PSS7a	<i>Paenibacillus illinoisensis</i>	935	"ACCGGCTTTT"	286909.06 Da
PSS8a	<i>Kocuria polaris</i>	1054	"GACTTGACGG"	323576.40 Da
PSS9a	<i>Staphylococcus epidermidis</i>	953	"ACCGTAGCAT"	292940.42 Da
PSS9b	<i>Staphylococcus caprae</i>	899	"ATTCCAGCTT"	276115.45 Da
PSS10a	<i>Staphylococcus epidermidis</i>	988	"ACCGTAGCAT"	303582.24 Da
PSS11a	<i>Bhargavaea ginsengi</i>	970	"ACCGCGGCAT"	297968.03 Da
PSS11b	<i>Bacillus haynesii</i>	932	"GTGACGGGCG"	286526.78 Da
PSS12a	<i>Bacillus sonorensis</i>	1057	"ACCTCACCGA"	324846.47 Da
PSS13a	<i>Bacillus licheniformis</i>	1059	"ACCTCACCGA"	325438.87 Da
PSS14a	<i>Pseudomonas oryzihabitans</i>	1065	"GACTAGCTAC"	327312.25 Da
PSS15a	<i>Corynebacterium aurimucosum</i>	1083	"GTGACGGGCG"	332493.31 Da
PSS15b	<i>Staphylococcus epidermidis</i>	995	"GTGACGGGCG"	305967.81 Da
PSS16a	<i>Bacillus subtilis</i>	855	"ACCGCGGCAT"	262519.30 Da
PSS17a	<i>Bacillus piscis</i>	1098	"AGAACCTCAC"	337529.70 Da
PSS18a	<i>Staphylococcus hominis</i>	962	"GAAGGTTTTT"	298855.73 Da
PSS18b	<i>Micrococcus yunnanensis</i>	1086	"AATAGGCCAC"	333475.86 Da
PSS19a	<i>Staphylococcus caprae</i>	937	"ACCGTAGCAT"	287934.14 Da
PSS20a	<i>Staphylococcus saccharolyticus</i>	796	"ACCGTAGCAT"	244598.11 Da
PSS21a	<i>Pseudomonas iranica</i>	1112	"AAAGACAGCT"	341303.28 Da
PSS21b	<i>Micrococcus luteus</i>	1098	"GGCCACCGGC"	337239.29 Da
PSS22a	<i>Staphylococcus capitis</i> subsp. <i>urealyticus</i>	863	"CACCGTAGCA"	265007.28 Da
PSS23a	<i>Corynebacterium minutissimum</i>	1081	"ACGGGCGGTG"	331834.89 Da
PSS23b	<i>Bacillus paralicheniformis</i>	966	"ACGGGCGGTG"	296902.47 Da
PSS24a	<i>Staphylococcus epidermidis</i>	965	"GTGACGGGCG"	296722.79 Da
PSS25a	<i>Staphylococcus taiwanensis</i>	1119	"TCCACCGGCT"	344161.39 Da

3.4.3. Alignment and Annotation

The alignment and annotation of 16S rRNA gene sequences derived from haloversatile bacteria were performed using the freely available software Jalview 2.11.2.7 (Waterhouse *et al.*, 2009). Additionally, the codes involved for protein synthesis were identified and depicted in Figures 3.8 and 3.9. The alignment exhibited distinct gaps, mismatches, and matches, hence facilitating the researcher's comprehension of the similarity among the analyzed species. It is well understood that each of the three nucleotides plays a crucial role in the synthesis of a particular amino acids. Consequently, the depiction of nucleotides that encode for a certain amino acid is presented in the attached illustrations. The process involves vertically alignment the sequences and performing a comparison of their nucleotide compositions. The process of genome assembly and annotation involves the systematic arrangement of small sequences and the utilization of computational methods to comprehensively characterize the functional sections of genes and locate previously unidentified genomic fragments. Alignment is commonly regarded as one of the main goals within the field of bioinformatics. Alignment performs multiple purposes and demands a detailed understanding of the element contained by life information. Merely identifying the sequence in isolation does not provide any knowledge. The process referred to as pairwise sequence alignment, wherein two sequences are aligned, is widely recognized as a fundamental bioinformatics analysis. This analysis has significant importance as it serves as the initial stage in exploring the characteristics and functions of unknown sequences. The method of line-up in multiple sequence alignment is well recognized as the basis for inferring the relationship between two sequences being compared. If the two sequences exhibit significant degrees of similarity or symmetry, it is reasonable to infer that they share a common evolutionary ancestry. The proper alignment of fixed components, such as domains, which are often functionally significant, is considered crucial for creating relationships and evolutionary trees through the analysis of domain sequence rows. The multiline approach enables the identification of crucial activity sites, including as binding sites, enzyme active sites, and other active sites involved in certain functional processes. There are a couple of different techniques for aligning a pair of sequences, namely global alignment, which is the more frequently used approach, and local alignment. Moreover, the primary aim of a pair of sequences is to identify the optimal pair of sequences that exhibit the highest feasible count of amino acid similarities (Al-Kafaji and Ibrahim, 2012).

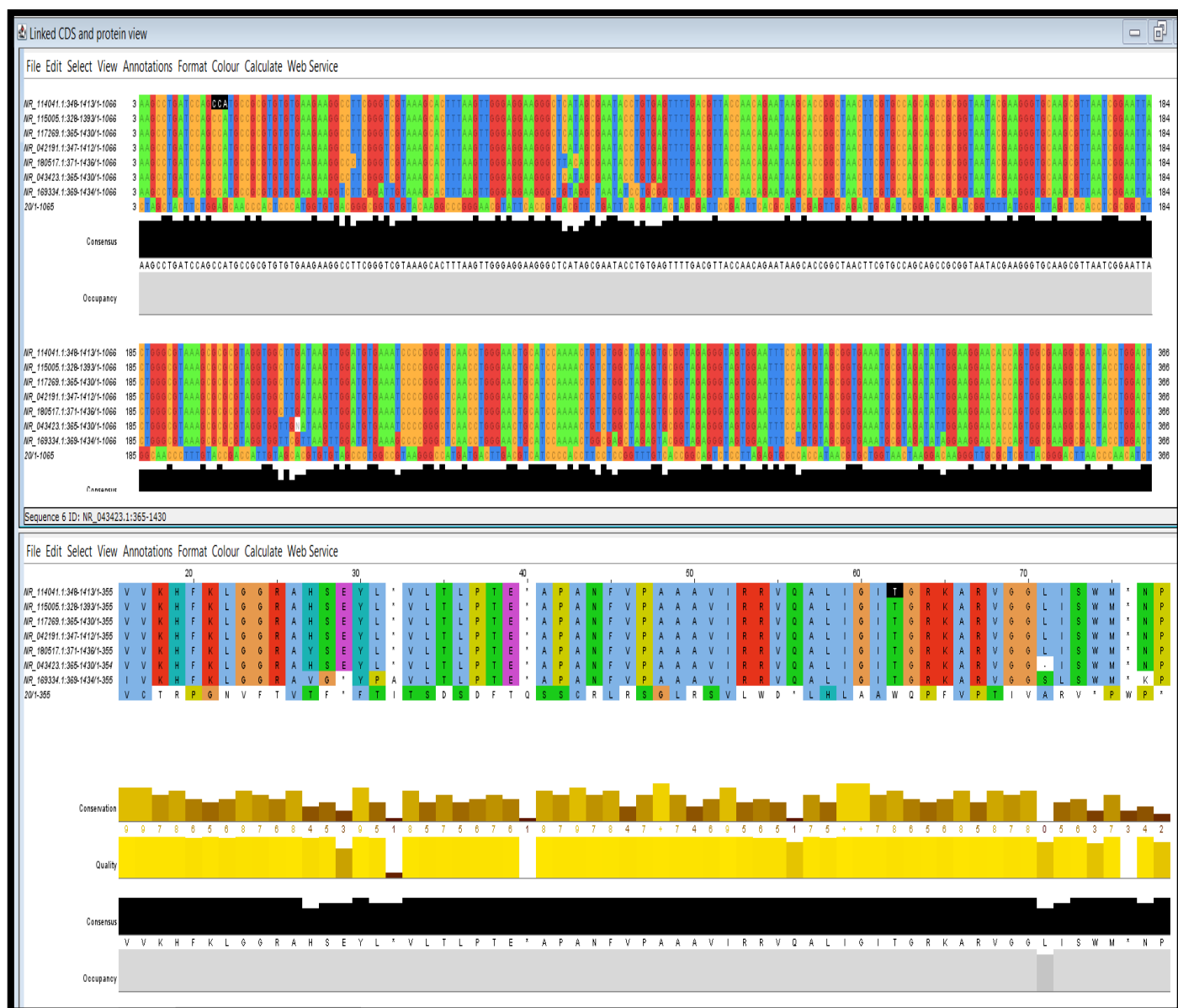


Figure 3.8. The alignment of the first segment of DNA sequence obtained from haloversatile *Staphylococcus saccharolyticus* (isolate PSS20a) by using the overview window.

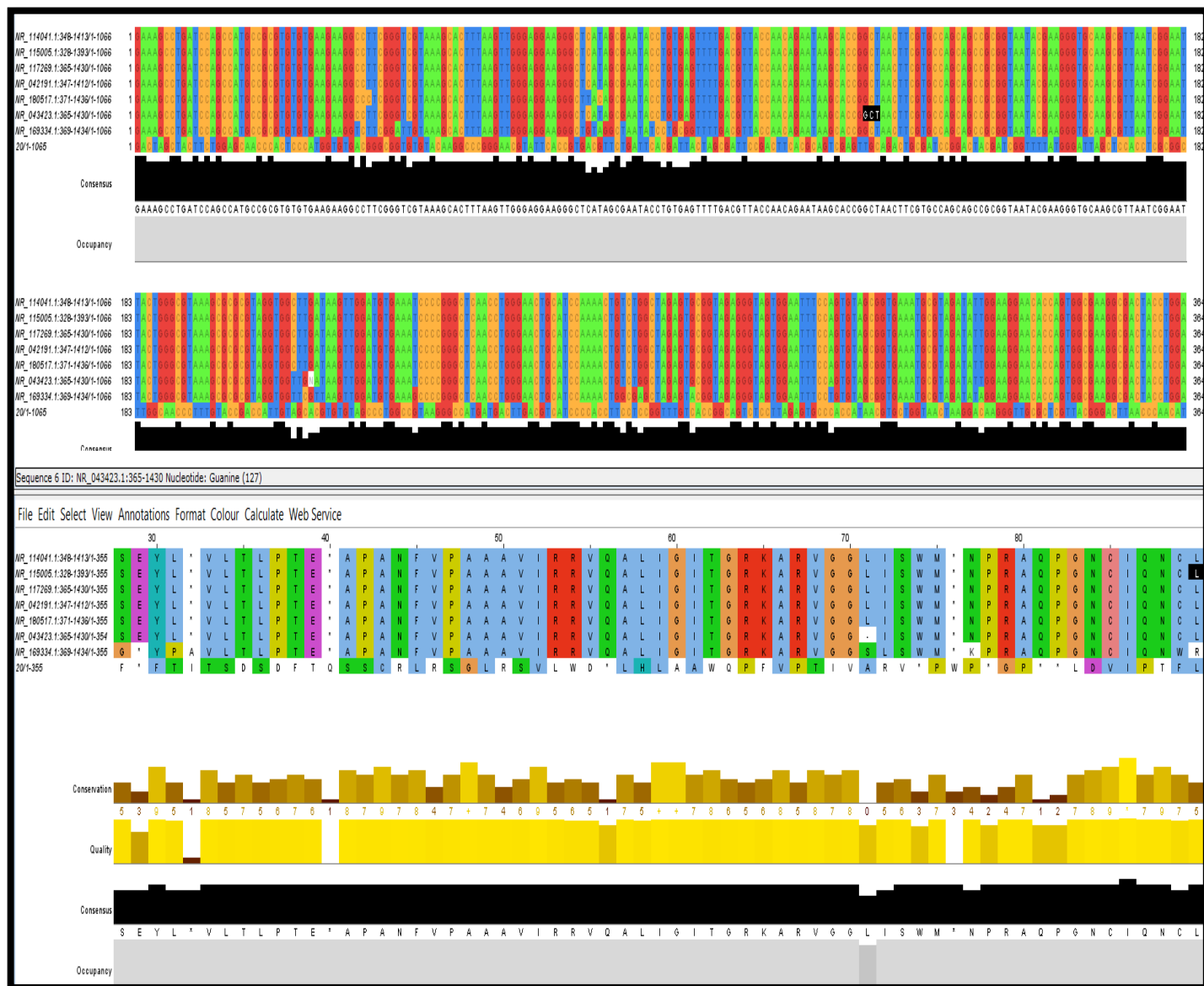


Figure 3.9. Alignment of the last segment of DNA sequence obtained from haloversatile *Staphylococcus saccharolyticus* (isolate PSS20a) by using the overview window.

3.5. CHARACTERIZATION OF HALOVERSATILE BACTERIA

3.5.1. Investigation of Cell Morphology, Pigmentation And Motility Of Haloversatile Bacteria

According to Gram-staining results, the isolates were separated into two groups: Gram-positive and Gram-negative. Only *Pseudomonas oryzihabitans* (isolate PSS14a) and *Pseudomonas iranica* (isolate PSS21a) were Gram-negative (6% of all isolates), while the other 33 isolates (94%) were Gram-positive. The cell shapes of isolates were bacil, rod, coccus, or pleomorphic (Figure 3.10, Table 3.6). Moreover, the shape of colonies detected with naked eyes on the agar plates and the motility of our isolates were detected (Figure 3.10, Table 3.6). Also all *Staphylococcus* sp., *Corynebacterium* sp., *Micrococcus* sp., *Kocuria* sp. and *Bhargavaea* sp. that were isolated in this study were non-motile, and other isolates were motile.

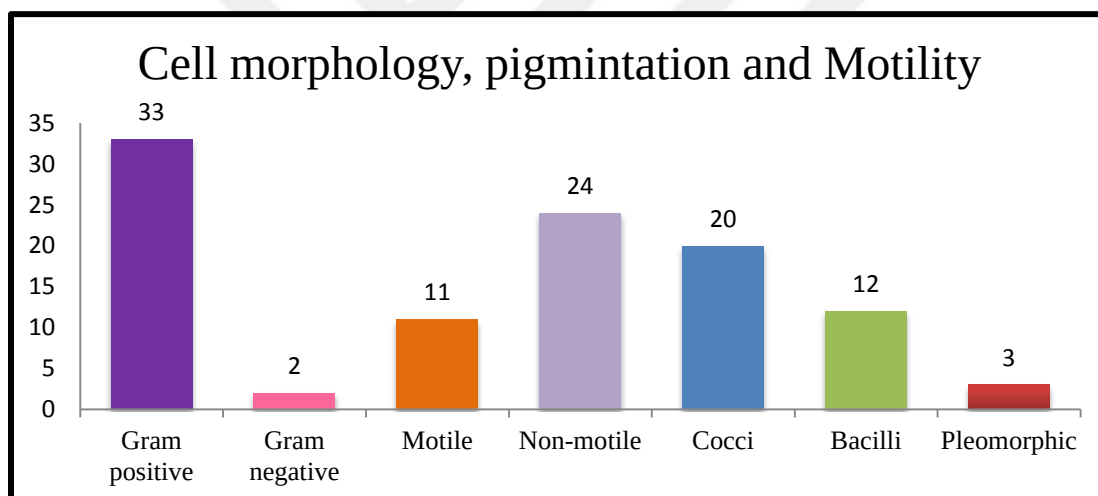


Figure 3.10. Gram reactions, cell morphologies, and motilities of haloversatile isolates.

Table 3.6. Gram staining, cell morphology, motility of haloversatile bacterial isolates.

No	Codes	Species	Gram staining	Cell	Motility
1	PSS1a	<i>Staphylococcus warneri</i>	+	Coccus	None-motile
2	PSS1b	<i>Staphylococcus pasteurii</i>	+	Coccus	None-motile
3	PSS2a	<i>Staphylococcus warneri</i>	+	Coccus	None-motile
4	PSS2b	<i>Corynebacterium aurimucosum</i>	+	Pleomorphic	None-motile
5	PSS3a	<i>Staphylococcus epidermidis</i>	+	Coccus	None-motile
6	PSS3b	<i>Staphylococcus epidermidis</i>	+	Coccus	None-motile
7	PSS4a	<i>Peribacillus frigoritolerans</i>	+	Bacil	Motile
8	PSS4b	<i>Staphylococcus haemolyticus</i>	+	Coccus	None-motile
9	PSS5a	<i>Lysinibacillus telephonicus</i>	+	Bacil	Motile
10	PSS6a	<i>Kocuria rosea</i>	+	Coccus	None-motile
11	PSS7a	<i>Paenibacillus illinoisensis</i>	+	Bacil	Motile
12	PSS8a	<i>Kocuria polaris</i>	+	Coccus	None-motile
13	PSS9a	<i>Staphylococcus epidermidis</i>	+	Coccus	None-motile
14	PSS9b	<i>Staphylococcus caprae</i>	+	Coccus	None-motile
15	PSS10a	<i>Staphylococcus epidermidis</i>	+	Coccus	None-motile
16	PSS11a	<i>Bhargavaea ginsengi</i>	+	Rod-shaped	None-motile
17	PSS11b	<i>Bacillus haynesii</i>	+	Rod-shaped	Motile
18	PSS12a	<i>Bacillus sonorensis</i>	+	Rod-shaped	Motile
19	PSS13a	<i>Bacillus licheniformis</i>	+	Bacil	Motile
20	PSS14a	<i>Pseudomonas oryzae</i>	-	Rod-shaped	Motile
21	PSS15a	<i>Corynebacterium aurimucosum</i>	+	Pleomorphic	None-motile
22	PSS15b	<i>Staphylococcus epidermidis</i>	+	Coccus	None-motile
23	PSS16a	<i>Bacillus subtilis</i>	+	Rod-shaped	Motile
24	PSS17a	<i>Bacillus pascis</i>	+	Rod-shaped	Motile
25	PSS18a	<i>Staphylococcus hominis</i>	+	Coccus	None-motile
26	PSS18b	<i>Micrococcus yunnanensis</i>	+	Coccus	None-motile
27	PSS19a	<i>Staphylococcus caprae</i>	+	Coccus	None-motile
28	PSS20a	<i>Staphylococcus saccharolyticus</i>	+	Coccus	None-motile
29	PSS21a	<i>Pseudomonas iranica</i>	-	Rode-shaped	Motile
30	PSS21b	<i>Micrococcus luteus</i>	+	Coccus	None-motile
31	PSS22a	<i>Staphylococcus capitis</i> subsp. <i>urealyticus</i>	+	Coccus	None-motile
32	PSS23a	<i>Corynebacterium minutissimum</i>	+	Pleomorphic	None-motile
33	PSS23b	<i>Bacillus paralicheniformis</i>	+	Rod-shaped	Motile
34	PSS24a	<i>Staphylococcus epidermidis</i>	+	Coccus	None-motile
35	PSS25a	<i>Staphylococcus taiwanensis</i>	+	Coccus	None-motile

3.6. THE EFFECTS OF pH, SALT AND TEMPERATURE ON HALOVERSATILE BACTERIAL GROWTH

In the present study, three environmental factors that profoundly affect all microorganisms have been studied under different pH (4-12), salt (0-20%) and temperature values (4°C-65°C) on Halophilic Agar Media. The optimum growth was observed at pH 7, 1.17% NaCl, and 35°C for all isolates. The growth was not observed at pH4, pH12, 20% NaCl, 25% NaCl, 65°C (Table 3.7)

Table 3.7. The effects of pH, NaCl contents and temperature values on bacterial growth.

No	Isolate code	Isolate name	Optimum pH	Range of pH	Optimum NaCl%	Range of NaCl %	Optimum temperature°C	Range of temperature°C
1	PSS1a	<i>Staphylococcus warneri</i>	7	5-11	1.17	0-17	35	19-55
2	PSS1b	<i>Staphylococcus pasteurii</i>	7	5-11	1.17	0-17	35	19-60
3	PSS2a	<i>Staphylococcus warneri</i>	7	5-11	1.17	0-17	35	19-55
4	PSS2b	<i>Corynebacterium aurimucosum</i>	7	5-11	1.17	0-17	35	19-60
5	PSS3a	<i>Staphylococcus epidermidis</i>	7	5-11	1.17	0-17	35	19-55
6	PSS3b	<i>Staphylococcus epidermidis</i>	7	5-11	1.17	0-17	35	19-60
7	PSS4a	<i>Peribacillus frigoritolerans</i>	7	5-11	1.17	0-17	35	19-60
8	PSS4b	<i>Staphylococcus haemolyticus</i>	7	5-11	1.17	0-17	35	19-60
9	PSS5a	<i>Lysinibacillus telephonicus</i>	7	5-11	1.17	0-17	35	19-60
10	PSS6a	<i>Kocuria rosea</i>	7	5-11	1.17	0-17	35	19-55
11	PSS7a	<i>Paenibacillus illinoisensis</i>	7	5-11	1.17	0-17	35	19-60
12	PSS8a	<i>Kocuria polaris</i>	7	5-11	1.17	0-17	35	19-55
13	PSS9a	<i>Staphylococcus epidermidis</i>	7	5-11	1.17	0-17	35	19-60
14	PSS9b	<i>Staphylococcus caprae</i>	7	5-11	1.17	0-17	35	19-60
15	PSS10a	<i>Staphylococcus epidermidis</i>	7	5-11	1.17	0-17	35	19-60
16	PSS11a	<i>Bhargavaeaginsengi</i>	7	5-11	1.17	0-17	35	19-60
17	PSS11b	<i>Bacillus haynesii</i>	7	5-11	1.17	0-17	35	19-55
18	PSS12a	<i>Bacillus sonorensis</i>	7	5-11	1.17	0-17	35	19-55
19	PSS13a	<i>Bacillus licheniformis</i>	7	5-11	1.17	0-17	35	19-60
20	PSS14a	<i>Pseudomonas oryzae</i>	7	5-11	1.17	0-17	35	19-55
21	PSS15a	<i>Corynebacterium aurimucosum</i>	7	5-11	1.17	0-17	35	19-55
22	PSS15b	<i>Staphylococcus epidermidis</i>	7	5-11	1.17	0-17	35	19-60
23	PSS16a	<i>Bacillus subtilis</i>	7	5-11	1.17	0-17	35	19-60
24	PSS17a	<i>Bacillus pascalis</i>	7	5-11	1.17	0-17	35	19-60
25	PSS18a	<i>Staphylococcus hominis</i>	7	5-11	1.17	0-17	35	19-60
26	PSS18b	<i>Micrococcus yunnanensis</i>	7	5-11	1.17	0-17	35	19-60
27	PSS19a	<i>Staphylococcus caprae</i>	7	5-11	1.17	0-17	35	19-60
28	PSS20a	<i>Staphylococcus saccharolyticus</i>	7	5-11	1.17	0-17	35	19-60
29	PSS21a	<i>Pseudomonas iranica</i>	7	5-11	1.17	0-17	35	19-60
30	PSS21b	<i>Micrococcus luteus</i>	7	5-11	1.17	0-17	35	19-60
31	PSS22a	<i>Staphylococcus capitis</i> subsp. urealyticus	7	5-11	1.17	0-17	35	19-60
32	PSS23a	<i>Corynebacterium minutissimum</i>	7	5-11	1.17	0-17	35	19-60
33	PSS23b	<i>Bacillus paralicheniformis</i>	7	5-11	1.17	0-17	35	19-60
34	PSS24a	<i>Staphylococcus epidermidis</i>	7	5-11	1.17	0-17	35	19-55
35	PSS25a	<i>Staphylococcus taiwanensis</i>	7	5-11	1.17	0-17	35	19-60

3.7. Determination of Enzymatic Activities of Haloversatile Bacteria

The results of enzymatic activities indicated that the isolates produced various enzymes (Figure 3.11). Esterase, urease, protease, caseinase, lipase, xylanase, cellulase, amylase, DNase and oxidase were respectively produced by 40%, 66%, 77%, 6%, 17%, 6%, 17%, 20%, 20% and 40% of the isolates (Table 3.8). Although all isolates exhibited catalase activity, none of the isolates produced pullulanase enzyme. Some of the positive activities of protease, lipase, amylase and caseinase of haloversatile isolates on the Petri plates were shown in Figure 3.12A, Figure 3.12B, Figure 3.12C, Figure 3.12D, respectively.

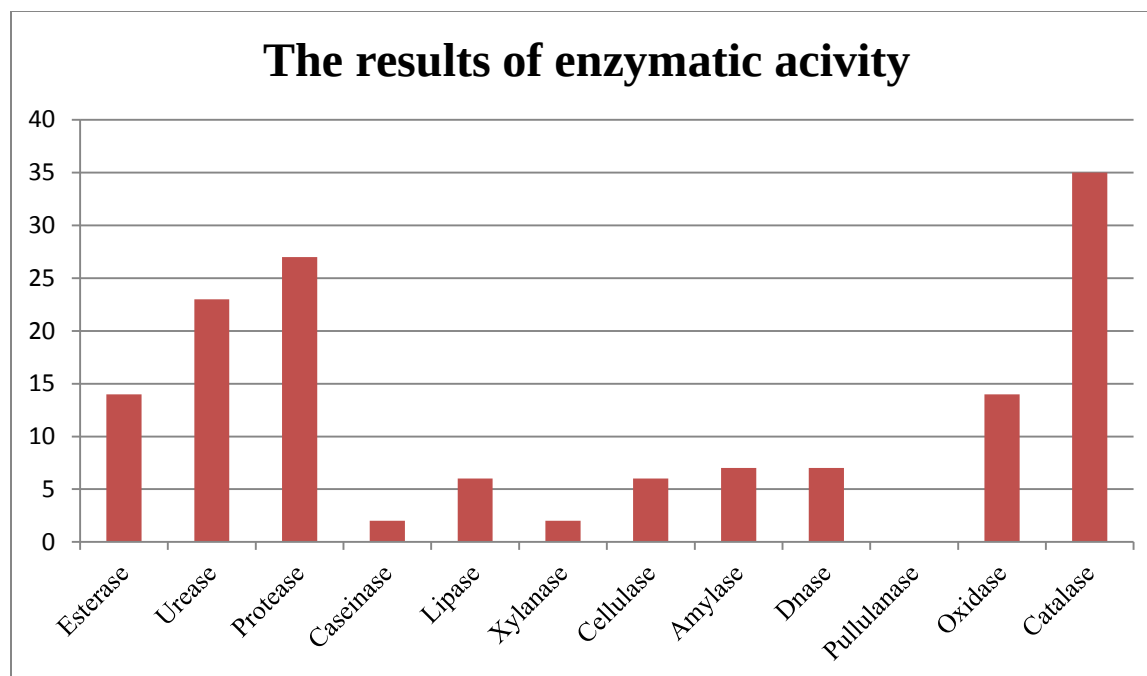


Figure 3.11. The positive numbers of haloversatile isolates for different types of enzymes.

Table 3.8. Enzymatic properties of haloversatile bacterial isolates.

Isolate code	Phylogenetically similar to	Esterase	Urease	Protease	Caseinase	Lipase	Xylanase	Cellulase	Amylase	DNase	Pullulanase	Oxidase	Catalase
PSS1a	<i>Staphylococcus warneri</i>	+	+	+	-	+	-	-	-	-	-	-	+
PSS1b	<i>Staphylococcus pasteurii</i>	+	+	+	-	+	-	-	-	-	-	-	+
PSS2a	<i>Staphylococcus warneri</i>	+	+	+	-	+	-	-	-	-	-	-	+
PSS2b	<i>Corynebacterium aurimucosum</i>	-	-	-	-	-	-	-	-	-	-	-	+
PSS3a	<i>Staphylococcus epidermidis</i>	+	+	+	-	-	-	-	-	-	-	-	+
PSS3b	<i>Staphylococcus epidermidis</i>	+	+	+	-	-	-	-	-	-	-	-	+
PSS4a	<i>Peribacillus frigoritolerans</i>	-	+	+	-	-	-	-	-	-	-	-	+
PSS4b	<i>Staphylococcus haemolyticus</i>	+	-	-	+	-	-	-	-	-	-	-	+
PSS5a	<i>Lysinibacillus telephonicus</i>	-	-	+	+	-	-	+	-	+	-	+	+
PSS6a	<i>Kocuria rosea</i>	-	-	-	-	-	-	-	-	-	-	+	+
PSS7a	<i>Paenibacillus illinoisensis</i>	+	+	+	-	+	+	-	+	-	-	+	+
PSS8a	<i>Kocuria polaris</i>	-	-	+	-	-	-	-	-	-	-	-	+
PSS9a	<i>Staphylococcus epidermidis</i>	+	+	+	-	-	-	-	-	-	-	-	+
PSS9b	<i>Staphylococcus caprae</i>	-	+	+	-	-	-	-	-	-	-	+	+
PSS10a	<i>Staphylococcus epidermidis</i>	+	+	+	-	-	-	-	-	-	-	-	+
PSS11a	<i>Bhargavaea ginsengi</i>	-	-	+	-	-	-	-	-	+	-	+	+
PSS11b	<i>Bacillus haynesii</i>	-	+	+	-	-	-	-	+	-	-	-	+
PSS12a	<i>Bacillus sonorensis</i>	-	-	+	-	-	-	+	+	+	-	+	+
PSS13a	<i>Bacillus licheniformis</i>	-	+	+	-	+	+	+	+	+	-	+	+
PSS14a	<i>Pseudomonas oryzae</i>	-	+	-	-	-	-	-	-	-	-	-	+
PSS15a	<i>Corynebacterium aurimucosum</i>	+	-	+	-	-	-	-	-	-	-	+	+
PSS15b	<i>Staphylococcus epidermidis</i>	+	+	+	-	-	-	-	-	-	-	-	+
PSS16a	<i>Bacillus subtilis</i>	-	+	+	-	+	-	+	+	+	-	+	+
PSS17a	<i>Bacillus pascalis</i>	-	+	+	-	-	-	+	+	+	-	-	+
PSS18a	<i>Staphylococcus hominis</i>	-	+	-	-	-	-	-	-	-	-	-	+
PSS18b	<i>Micrococcus yunnanensis</i>	-	+	+	-	-	-	-	-	-	-	+	+
PSS19a	<i>Staphylococcus caprae</i>	-	-	+	-	-	-	-	-	-	-	-	+
PSS20a	<i>Staphylococcus saccharolyticus</i>	-	+	+	-	-	-	-	-	-	-	-	+
PSS21a	<i>Pseudomonas iranica</i>	-	+	-	-	-	-	-	-	-	-	+	+
PSS21b	<i>Micrococcus luteus</i>	+	+	+	-	-	-	-	-	-	-	+	+
PSS22a	<i>Staphylococcus capitis</i> subsp. <i>urealyticus</i>	-	+	+	-	-	-	-	-	-	-	-	+
PSS23a	<i>Corynebacterium minutissimum</i>	-	-	-	-	-	-	-	-	-	-	+	+
PSS23b	<i>Bacillus paralicheniformis</i>	-	-	+	-	-	-	+	+	+	-	+	+
PSS24a	<i>Staphylococcus epidermidis</i>	+	+	+	-	-	-	-	-	-	-	-	+
PSS25a	<i>Staphylococcus taiwanensis</i>	+	-	-	-	-	-	-	-	-	-	-	+
Number of positive isolates		14	23	27	2	6	2	6	7	7	0	15	35
Percent of positive isolates (%)		40	66	77	6	17	6	17	20	20	0	43	100

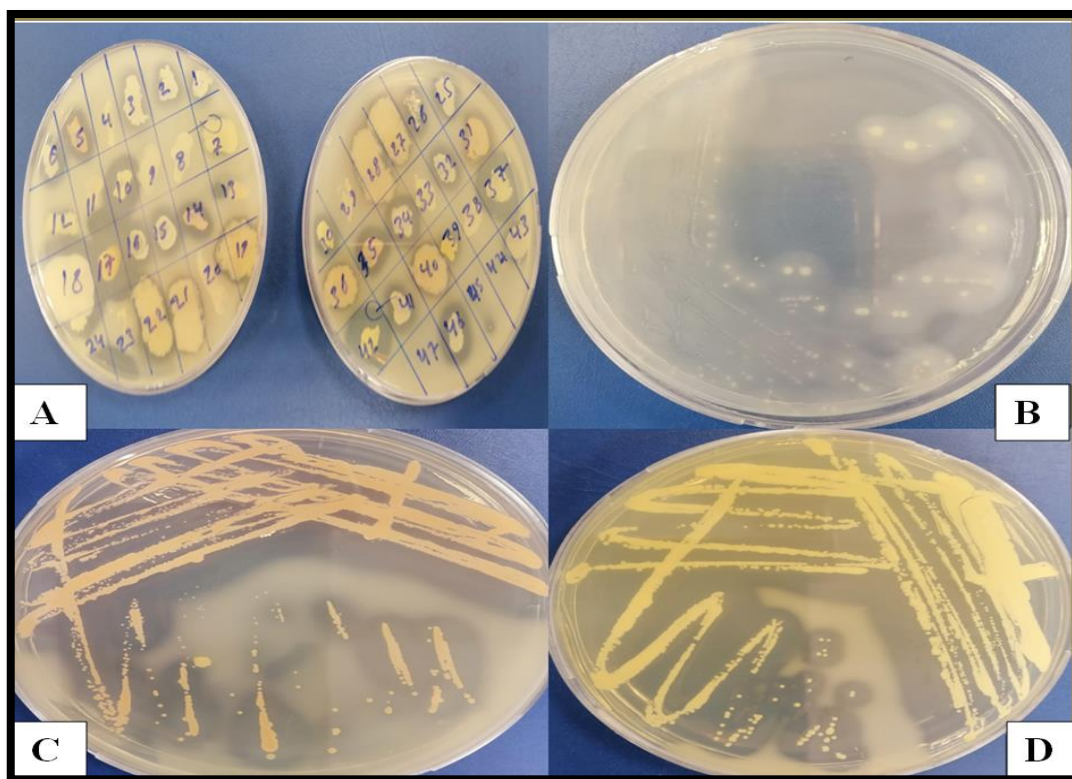


Figure 3.12. (A) Protease positive and negative isolates, (B) Positive lipase activity, (C) Positive protease activity, (D) Positive DNase activity of the haloversatile isolates.

While Figure 3.13A shows positive urease activity, Figure 3.13B shows positive esterase activity.

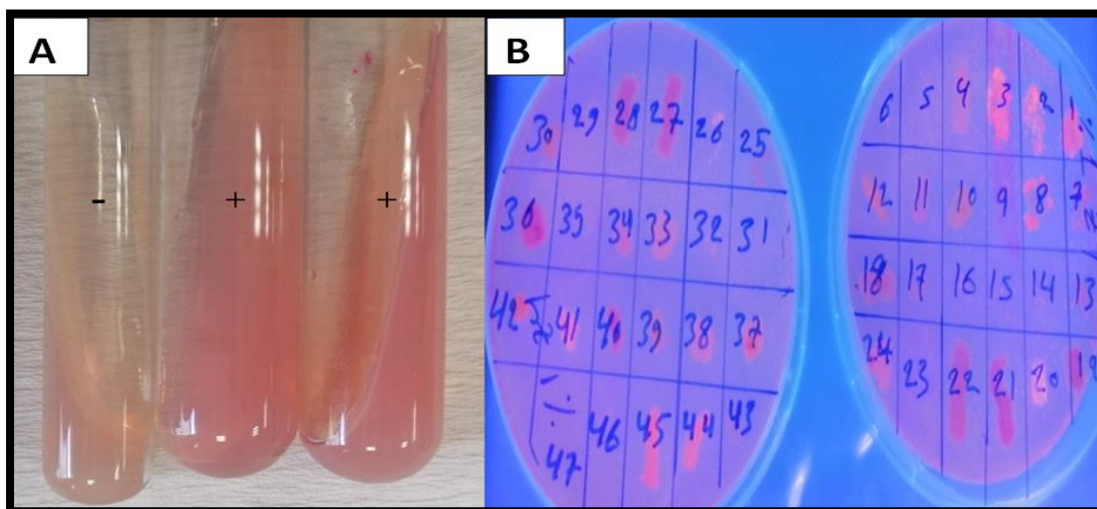


Figure 3.13. (A) Positive urease activity, (B) Positive esterase activity of haloversatile bacteria.

Due to hydrogen peroxide (H_2O_2) is harmful to bacterial cells, all isolates had the ability to produce catalase enzyme (to protect itself from H_2O_2), transforming it into water and oxygen (Figure 3.14).

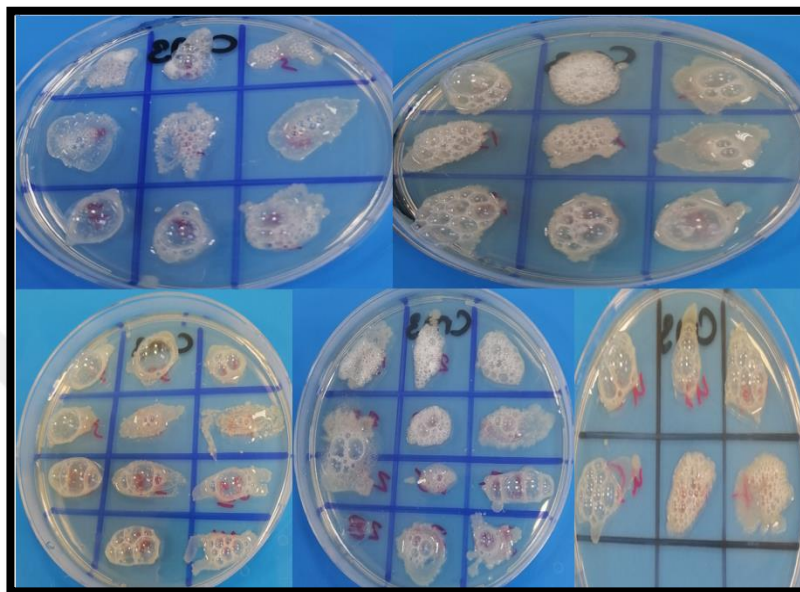


Figure 3.14. Catalase activity of haloversatile bacteria.

The ability to synthesize this enzyme is widespread among bacteria, including both aerobic and facultative anaerobic species. Only 14 isolates were able to produce cytochrome-oxidase enzyme, as evidenced by the colonies becoming a deep blue-purple color when treated with oxidase test reagent (redox-tetramethyl-p-phenylene-diamine). Caglayan (2019) isolated 40 isolates from 20 salt samples collected from Çamaltı Saltern in Izmir/Türkiye. The researcher reported that 14 genus of haloversatile bacteria such as *Bacillus* (23 isolates), *Kocuria* (9 isolates), *Paracoccus* (6 isolates), *Micrococcus* (4 isolates), *Microbacterium* (4 isolates), *Brevibacterium* (2 isolates), *Staphylococcus* (12 isolates), *Acinetobacter* (1 isolate), *Exiguobacterium* (3 isolate), *Gordonia* (1 isolate), *Pseudomonas* (1 isolate), *Agrococcus* (1 isolate), *Sanguibacter* (1 isolate), and *Virgibacillus* (1 isolate) were isolated from the salt samples taken from Çamaltı Saltern in Izmir/Türkiye (Caglayan, 2019). The experimental results showed that 100%, 80%, 52%, 51%, 33%, 23%, 17%, 10%, 7%, 7%, 4%, and 4% of the isolates were respectively produced catalase, oxidase, protease, lecithinase, cellulase, lipids, deoxyribonuclease, and pullulanase, amylase, caseinase, and urease (Caglayan, 2019). In the

study conducted by Ozbay and Caglayan (2022), six haloversatile isolates [*Terribacillus halophilus* (1 isolate), *Brevibacterium luteolum* (2 isolates), *Bacillus australimaris* (1 isolate), *Bacillus siamensis* (1 isolate), *Bacillus mojavensis* (1 isolate)] were obtained from soak liquor samples taken from the tanneries in The Leather Organized Region in Türkiye. In that study, 17% of the isolates produced cellulase, 83% of the isolates produced protease and lipase, 67% of the isolates produced caseinase, and 50% of the isolates produced amylase (Ozbay and Caglayan, 2022). The researchers reported that the haloversatile isolates could not produce urease, DNase, xylanase, and pullulanase (Ozbay and Caglayan, 2022).

3.7.1. Investigation of the Activity of Lipase Enzyme Produced from Haloversatile Isolates

Based on the lipase test, only five isolates exhibited lipase activity on Tween 80 Agar Media (Table 3.8). Due to the interest for microbial lipases, five best lipase-producing haloversatile isolates (*Staphylococcus warneri* isolate PSS1a, *Staphylococcus pasteurii* isolate PSS1b, *Staphylococcus warneri* isolate PSS2a, *Paenibacillus illinoisensis* isolate PSS7a, *Bacillus subtilis* isolate PSS16a) were selected and tested by spectrophotometer. The results found that the activity of lipase was high within *Staphylococcus warneri* isolate PSS1a, *Staphylococcus pasteurii* isolate PSS1b, *Staphylococcus warneri* isolate PSS2a, which represent 23 U/ml, 25 U/ml, and 24.8 U/ml, respectively in comparison with the control (Lipase from *Candida regusa*). Whereas, the activity of lipase was low by *Paenibacillus illinoisensis* isolate PSS7a, *Bacillus subtilis* isolate PSS16a, which represent 15 U/ml, and 7.9 U/ml, respectively (Table 3.9).

Table 3.9. The absorbance, control, absorbance-control and activity of lipase.

No	Isolate code	Species	Absorbance U/ml	Control (Lipase from <i>Candida regusa</i>) U/ml	The activity of lipase U/ml
1	PSS1a	<i>Staphylococcus warneri</i>	0.426	0.195	23
2	PSS1b	<i>Staphylococcus pasteurii</i>	0.447	0.195	25
3	PSS2a	<i>Staphylococcus warneri</i>	0.444	0.195	24.8
4	PSS7a	<i>Paenibacillus illinoisensis</i>	0.345	0.195	15
5	PSS16a	<i>Bacillus subtilis</i>	0.275	0.195	7.9

Earlier study by Güçlü, (2019) found that the activity of lipase produced by moderately halophilic bacteria isolated from salted animal skin was 21.00 U/ml of *Staphylococcus nepalensis* (isolate ISR2) and 24.18 U/ml of *Idiomarina loihiensis* (isolate GA3) at pH 7, 10% NaCl, and 37°C for 24 hours. Other study found that the activity of lipase by *Pseudomonas* sp. (isolate LAB34) was 104.66 ± 22.91 U/ml at pH value was 7 and by using p-nitrophenyl palmitate (Vargas *et al.*, 2004).

3.7.2. Investigation of the Activity of Protease Enzyme Produced from Haloversatile Isolates

The spectrophotometer was applied to determine the activity of protease enzyme produced by 24 highest protease-positive haloversatile isolates (depending on the primary results on GAM). The results of this experiment indicated that all tested isolates produced high amount of protease enzyme in Gelatin Broth Media also was characterised with protease enzyme activity by using spectrophotometer. The specific isolates that exhibited the high protease activity were *Staphylococcus warneri* (PSS1a) 35.4 U/ml, *Staphylococcus epidermidis* (PSS3a) 24 U/ml, *Staphylococcus epidermidis* (PSS3b) 32.3 U/ml, *Peribacillus frigoritolerans* (PSS4a) 19.7 U/ml, *Bacillus haynesii* (PSS11b) 22.5 U/ml, *Bacillus subtilis* (PSS16a) 21.3 U/ml, *Micrococcus yunnanensis* (PSS18b) 17.2 U/ml, *Staphylococcus saccharolyticus* (PSS20a) 21.7 U/ml, and *Staphylococcus epidermidis* (PSS24a) 15.9 U/ml (Table 3.10).

Table 3.10. The absorbance, control, absorbance-control and activity of protease.

Isolate codes	Isolates	Absorbance U/ml	Control U/ml	Absorbance-control	Activity of protease U/ml
PSS1a	<i>Staphylococcus warneri</i>	0.475	0.121	0.354	35.4
PSS1b	<i>Staphylococcus pasteurii</i>	0.190	0.08	0.11	11
PSS2a	<i>Staphylococcus warneri</i>	0.200	0.096	0.104	10.4
PSS3a	<i>Staphylococcus epidermidis</i>	0.337	0.097	0.24	24
PSS3b	<i>Staphylococcus epidermidis</i>	0.420	0.097	0.323	32.3
PSS4a	<i>Peribacillus frigoritolerans</i>	0.288	0.091	0.197	19.7
PSS9a	<i>Staphylococcus epidermidis</i>	0.203	0.086	0.117	11.7
PSS9b	<i>Staphylococcus caprae</i>	0.181	0.089	0.092	9.2
PSS10	<i>Staphylococcus epidermidis</i>	0.174	0.082	0.092	9.2
PSS11	<i>Bhargavaea ginsengi</i>	0.179	0.085	0.094	9.4
PSS11	<i>Bacillus haynesii</i>	0.301	0.076	0.225	22.5
PSS12	<i>Bacillus sonorensis</i>	0.160	0.094	0.066	6.6
PSS13	<i>Bacillus licheniformis</i>	0.153	0.080	0.073	7.3
PSS15	<i>Corynebacterium aurimucosum</i>	0.141	0.079	0.062	6.2
PSS15	<i>Staphylococcus epidermidis</i>	0.175	0.075	0.1	10
PSS16	<i>Bacillus subtilis</i>	0.313	0.1	0.213	21.3
PSS17	<i>Bacillus piscis</i>	0.160	0.08	0.08	8
PSS18	<i>Micrococcus yunnanensis</i>	0.247	0.075	0.172	17.2
PSS19	<i>Staphylococcus caprae</i>	0.166	0.073	0.093	9.3
PSS20	<i>Staphylococcus saccharolyticus</i>	0.304	0.087	0.217	21.7
PSS21	<i>Micrococcus luteus</i>	0.137	0.086	0.051	5.1
PSS22	<i>Staphylococcus capitis</i> subsp. <i>urealyticus</i>	0.194	0.081	0.113	11.3
PSS23	<i>Bacillus paralicheniformis</i>	0.225	0.103	0.122	12.2
PSS24	<i>Staphylococcus epidermidis</i>	0.232	0.073	0.159	15.9

The activity of protease produced by moderately halophilic bacteria isolated from salted animal skin was 32.86 U/ml by *Marinacoccus tarijensis* (isolate GAM3), 28.50 U/ml by *Staphylococcus equorum* (isolate GA7), 19.25 U/ml by *Halomonas eurihalina* (isolate BL5), and 25.90 U/ml by *Chromohalobacter canadensis* (isolate TR6) at pH value 7, 37°C for 24 hours, and 5% NaCl. Whereas, the the activity at pH value 8, 37°C for 12 hours, and 5% NaCl was 17.53 U/ml by *Marinacoccus tarijensis* (isolate GAM3), 19.0 U/ml by *Staphylococcus equorum* (isolate GA7), 14.35 U/ml by *Halomonas eurihalina* (isolate BL5), and 19.95 U/ml by *Chromohalobacter canadensis* (isolate TR6) (Öztürk, 2019).

3.7.3. Estimation the Amount of Lipase and Protease Produced by Haloversatile Isolates

The amount of lipase in each sample was measured depending on the standard graphic, and the results showed that all test isolates produced high amount of lipase enzyme, which represent 15.5 µg/ml, 13.25 µg/ml, 12.75 µg/ml, 12.5 µg/ml, and 12 µg/ml by *Staphylococcus warneri* (PSS1a), *Staphylococcus pasteurii* (PSS1b), *Staphylococcus warneri* (PSS2a), *Paenibacillus illinoisensis* (PSS7a), and *Bacillus subtilis* (PSS16a) (Table 3.11). Furthermore, the results indicated that the optimum conditions for lipase production were observed at pH 7-8, 1%-3% NaCl within 24 and 96 hours on the Tween 80 agar media. The water bath incubation period for 30 minutes at the temperature values ranging from 37°C to 50°C were also found to be optimal for lipase production.

Table 3.11. The amount of lipase enzyme produced by haloversatile isolates.

No	Isolate code	Species	Absorbance	The content of protein (µg/ml)
1	PSS1a	<i>Staphylococcus warneri</i>	0.120	15.5
2	PSS1b	<i>Staphylococcus pasteurii</i>	0.111	13.25
3	PSS2a	<i>Staphylococcus warneri</i>	0.109	12.75
4	PSS7a	<i>Paenibacillus illinoisensis</i>	0.108	12.5
5	PSS16a	<i>Bacillus subtilis</i>	0.106	12

Similarly, the amount of protease was calculated using the formula of the calibration of concentration and absorbance of Bradford reaction; that mean the amount of protease enzyme was estimated in all isolates by µg/ml, and the result showed that all isolates have capability to produce protease enzyme in optimum condition pH 7, temperature 37°C for 60 minutes in the water bath (Table 3.12). The isolates that exhibited the high protease content were *Staphylococcus warneri* (PSS1a) 3.25 µg/ml, *Staphylococcus epidermidis* (PSS3a) 2.5 µg/ml, *Staphylococcus epidermidis* (PSS3b) 2.25 µg/ml, *Peribacillus frigoritolerans* (PSS4a) 1.75 µg/ml, *Bacillus haynesii* (PSS11b) 1.75 µg/ml, *Bacillus subtilis* (PSS16a) 1.5 µg/ml, *Micrococcus yunnanensis* (PSS18b) 2 µg/ml, *Staphylococcus saccharolyticus* (PSS20a) 2 µg/ml, and *Staphylococcus epidermidis* (PSS24a) 2.5 µg/ml (Table 3.12).

Table 3.12. The amount of protease enzyme produced by haloversatile isolates.

codes	Isolates	Absorbance	The content of protein (µg/ml)
PSS1a	<i>Staphylococcus warneri</i>	0.141	3.25
PSS1b	<i>Staphylococcus pasteurii</i>	0.133	1.25
PSS2a	<i>Staphylococcus warneri</i>	0.136	2
PSS3a	<i>Staphylococcus epidermidis</i>	0.138	2.5
PSS3b	<i>Staphylococcus epidermidis</i>	0.137	2.25
PSS4a	<i>Peribacillus frigoritolerans</i>	0.135	1.75
PSS9a	<i>Staphylococcus epidermidis</i>	0.134	1.5
PSS9b	<i>Staphylococcus caprae</i>	0.132	1
PSS10a	<i>Staphylococcus epidermidis</i>	0.13	0.5
PSS11a	<i>Bhargavaea ginsengi</i>	0.132	1
PSS11b	<i>Bacillus haynesii</i>	0.135	1.75
PSS12a	<i>Bacillus sonorensis</i>	0.129	0.25
PSS13a	<i>Bacillus licheniformis</i>	0.13	0.5
PSS15a	<i>Corynebacterium aurimucosum</i>	0.129	0.25
PSS15b	<i>Staphylococcus epidermidis</i>	0.132	1
PSS16a	<i>Bacillus subtilis</i>	0.134	1.5
PSS17a	<i>Bacillus pascis</i>	0.131	0.75
PSS18b	<i>Micrococcus yunnanensis</i>	0.136	2
PSS19a	<i>Staphylococcus caprae</i>	0.134	1.5
PSS20a	<i>Staphylococcus saccharolyticus</i>	0.136	2
PSS21b	<i>Micrococcus luteus</i>	0.129	0.25
PSS22a	<i>Staphylococcus capitis</i> subsp. <i>urealyticus</i>	0.132	1
PSS23b	<i>Bacillus paralicheniformis</i>	0.136	2
PSS24a	<i>Staphylococcus epidermidis</i>	0.138	2.5

Previous study found that the amounts of protease produced by *Marinacoccus tarijensis* (GAM3), *Staphylococcus equorum* (GA7), *Halomonas eurihalina* (BL5), and *Chromohalobacter canadensis* (TR6) at pH 7, 37°C for 24 hours, and 5% NaCl were 1.38 µg/ml, 2.04 µg/ml, 4.25 µg/ml, and 0.77 µg/ml, respectively (Öztürk, 2019). Whereas, the protease contents were respectively, 1.52 µg/ml, 0.68 µg/ml, 3.55 µg/ml, and 0.74 µg/ml produced by the above mentioned isolates at pH 8, 37°C for 24 hours, and 5% NaCl.

3.8. UTILIZATION OF DIFFERENT AMINO ACID AND SUGAR SOURCES OF HALOVERSATILE BACTERIA

The results showed that some of our isolates were able to ferment carbohydrates under aerobic conditions and to produce acids in the media. So that, the color of the media changed from red to yellow (Figure 3.15A). However, some of these isolates could not use the tested sugars as a carbon source. Furthermore, some of the isolates could utilize amino acids as a single energy

source. In the positive result, the isolate using amino acid changed the color of medium into purple, while the negative one was yellow (Figure 3.15B). The results of sugar and amino acid utilization were presented in Tables 3.13 and 3.14.

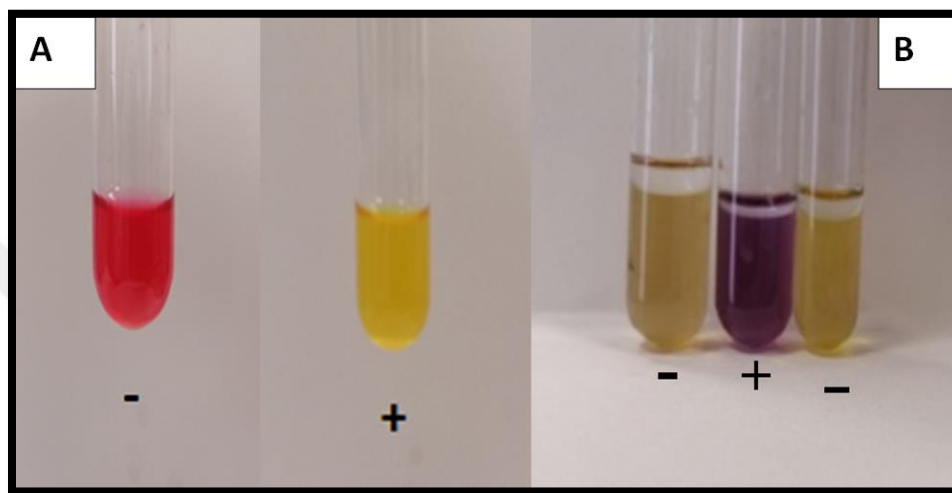


Figure 3.15. (A) Utilization of different sugar sources, (B) Utilization of different amino acid sources by haloversatile bacteria.

Dextrose, fructose, ribose, cellobiose, and sucrose were respectively used by 91%, 94%, 77%, 77%, and 91% of the isolates in this investigation (Tables 3.13 and 3.14). *Bacillus piscis* (isolate PSS17a), *Staphylococcus capitis* subsp. *urealyticus* (PSS22a), *Corynebacterium minutissimum* (isolates PSS23a); *Staphylococcus haemolyticus* (isolate PSS4b), *Kocuria rosea* (isolate PSS6a) respectively did not utilize dextrose, fructose (Table 3.13).

Table 3.13. Utilization of different sugars by haloversatile isolates.

Isolate code	Species	Dextrose	Fructose	Ribose	Cellobiose	Sucrose
PSS1a	<i>Staphylococcus warneri</i>	+	+	+	-	+
PSS1b	<i>Staphylococcus pasteurii</i>	+	+	-	-	+
PSS2a	<i>Staphylococcus warneri</i>	+	+	+	-	+
PSS2b	<i>Corynebacterium aurimucosum</i>	+	+	+	+	+
PSS3a	<i>Staphylococcus epidermidis</i>	+	+	+	+	+
PSS3b	<i>Staphylococcus epidermidis</i>	+	+	+	+	+
PSS4a	<i>Peribacillus frigoritolerans</i>	+	+	-	+	-
PSS4b	<i>Staphylococcus haemolyticus</i>	+	-	-	-	+
PSS5a	<i>Lysinibacillus telephonicus</i>	+	+	+	+	+
PSS6a	<i>Kocuria rosea</i>	+	-	-	-	-
PSS7a	<i>Paenibacillus illinoisensis</i>	+	+	+	+	+
PSS8a	<i>Kocuria polaris</i>	+	+	+	+	+
PSS9a	<i>Staphylococcus epidermidis</i>	+	+	+	+	+
PSS9b	<i>Staphylococcus caprae</i>	+	+	+	+	+
PSS10a	<i>Staphylococcus epidermidis</i>	+	+	+	+	+
PSS11a	<i>Bhargavaea ginsengi</i>	+	+	+	+	+
PSS11b	<i>Bacillus haynesii</i>	+	+	+	+	+
PSS12a	<i>Bacillus sonorensis</i>	+	+	+	+	+
PSS13a	<i>Bacillus licheniformis</i>	+	+	+	+	+
PSS14a	<i>Pseudomonas oryzae</i>	+	+	+	+	+
PSS15a	<i>Corynebacterium aurimucosum</i>	+	+	+	+	+
PSS15b	<i>Staphylococcus epidermidis</i>	+	+	+	+	+
PSS16a	<i>Bacillus subtilis</i>	+	+	+	+	+
PSS17a	<i>Bacillus pascalis</i>	-	+	+	+	+
PSS18a	<i>Staphylococcus hominis</i>	+	+	+	-	+
PSS18b	<i>Micrococcus yunnanensis</i>	+	+	-	+	+
PSS19a	<i>Staphylococcus caprae</i>	+	+	-	-	+
PSS20a	<i>Staphylococcus saccharolyticus</i>	+	+	+	+	+
PSS21a	<i>Pseudomonas iranica</i>	+	+	+	+	+
PSS21b	<i>Micrococcus luteus</i>	+	+	+	+	+
PSS22a	<i>Staphylococcus capitis</i> subsp. <i>urealyticus</i>	-	+	+	+	+
PSS23a	<i>Corynebacterium minutissimum</i>	-	+	-	-	+
PSS23b	<i>Bacillus paralicheniformis</i>	+	+	+	+	+
PSS24a	<i>Staphylococcus epidermidis</i>	+	+	+	+	+
PSS25a	<i>Staphylococcus taiwanensis</i>	+	+	-	+	-
Number of positive isolates		32	33	27	27	32
Percent of positive isolates		91%	94%	77%	77%	91%

Table 3.14. Utilization of different amino acids by haloversatile isolates.

No	Isolate code	Species	L-lysine	L-tyrosine	L-arginine	L-proline	L-valine
1	PSS1a	<i>Staphylococcus warneri</i>	-	+	-	+	+
2	PSS1b	<i>Staphylococcus pasteurii</i>	-	+	-	+	+
3	PSS2a	<i>Staphylococcus warneri</i>	-	+	-	+	+
4	PSS2b	<i>Corynebacterium aurimucosum</i>	-	+	-	+	+
5	PSS3a	<i>Staphylococcus epidermidis</i>	-	+	-	+	+
6	PSS3b	<i>Staphylococcus epidermidis</i>	-	+	-	+	+
7	PSS4a	<i>Peribacillus frigiditolerans</i>	-	+	-	+	+
8	PSS4b	<i>Staphylococcus haemolyticus</i>	-	+	-	+	+
9	PSS5a	<i>Lysinibacillus telephonicus</i>	-	+	-	+	+
10	PSS6a	<i>Kocuria rosea</i>	-	+	+	+	+
11	PSS7a	<i>Paenibacillus illinoisensis</i>	-	+	-	+	+
12	PSS8a	<i>Kocuria polaris</i>	-	+	-	+	+
13	PSS9a	<i>Staphylococcus epidermidis</i>	-	+	-	+	+
14	PSS9b	<i>Staphylococcus caprae</i>	+	+	+	+	+
15	PSS10a	<i>Staphylococcus epidermidis</i>	-	+	-	+	+
16	PSS11a	<i>Bhargavaea ginsengi</i>	-	+	-	+	+
17	PSS11b	<i>Bacillus haynesii</i>	-	+	-	+	+
18	PSS12a	<i>Bacillus sonorensis</i>	-	+	-	+	+
19	PSS13a	<i>Bacillus licheniformis</i>	+	+	+	+	+
20	PSS14a	<i>Pseudomonas oryzae</i>	-	+	-	+	+
21	PSS15a	<i>Corynebacterium aurimucosum</i>	-	+	-	+	+
22	PSS15b	<i>Staphylococcus epidermidis</i>	-	+	-	+	+
23	PSS16a	<i>Bacillus subtilis</i>	+	+	+	+	+
24	PSS17a	<i>Bacillus pascis</i>	-	+	-	+	+
25	PSS18a	<i>Staphylococcus hominis</i>	-	+	-	+	+
26	PSS18b	<i>Micrococcus yunnanensis</i>	-	+	-	+	+
27	PSS19a	<i>Staphylococcus caprae</i>	-	+	-	+	+
28	PSS20a	<i>Staphylococcus saccharolyticus</i>	-	+	+	+	+
29	PSS21a	<i>Pseudomonas iranica</i>	-	+	-	+	+
30	PSS21b	<i>Micrococcus luteus</i>	-	+	-	+	+
31	PSS22a	<i>Staphylococcus capitis</i> subsp. <i>urealyticus</i>	-	+	-	+	+
32	PSS23a	<i>Corynebacterium minutissimum</i>	-	+	-	+	+
33	PSS23b	<i>Bacillus paralicheniformis</i>	-	+	-	+	+
34	PSS24a	<i>Staphylococcus epidermidis</i>	-	+	-	+	+
35	PSS25a	<i>Staphylococcus taiwanensis</i>	-	+	-	+	+
Number of positive isolates			3	35	5	35	35
Percent of positive isolates			9%	100%	14%	100%	100%

L-tyrosine, L-proline, L-valine were utilized by all isolates (Table 3.14). L-lysine and L-arginine were respectively used by 9% and 14% of the isolates (Table 3.13). While L-lysine was used by *Staphylococcus caprae* (isolate PSS9b), *Bacillus licheniformis* (isolate PSS13a), and *Bacillus subtilis* (isolate PSS16a), L-arginine was utilized by *Kocuria rosea* (isolate PSS6a), *Staphylococcus caprae* (isolate PSS9b), *Bacillus licheniformis* (isolate PSS13a), *Bacillus subtilis* (isolate PSS16a), *Staphylococcus saccharolyticus* (isolate PSS20a) (Table 3.14). *Staphylococcus caprae* (isolates PSS9b), *Bacillus licheniformis* (isolates PSS13a), and *Bacillus subtilis* (isolate PSS16a) were used all tested amino acid sources (Table 3.14).

3.9. INVESTIGATION OF THE ABILITY OF HALOVERSATILE TO PRODUCE BACTERIOCINS AGAINST EACH OTHER

Thirteen isolates [*Staphylococcus warneri* (PSS1a), *Corynebacterium aurimucosum* (PSS2b), *Staphylococcus epidermidis* (PSS3a), *Kocuria polaris* (PSS8a), *Bacillus haynesii* (PSS11b), *Pseudomonas oryzihabitatus* (PSS14a), *Bacillus subtilis* (PSS16a), *Bacillus pascis* (PSS17a), *Pseudomonas iranica* (PSS21a), *Micrococcus luteus* (PSS21b), *Bacillus paralicheniformis* (PSS23b), *Peribacillus frigoritolerans* (PSS4a), *Paenibacillus illinoisensis* (PSS7a)] were chosen as the experimental group (Table 3.15). All of the isolates were tested against one isolate to one isolate. The optimal conditions for bacteriocin production were also examined. The bacteriocins of *Corynebacterium aurimucosum* (PSS2b), *Bacillus haynesii* (PSS11b), *Staphylococcus epidermidis* (PSS3a), *Peribacillus frigoritolerans* (PSS4a), *Staphylococcus warneri* (PSS1a), *Pseudomonas oryzihabitatus* (PSS14a), *Pseudomonas ironica* (PSS21a), *Kocuria polaris* PSS8a, *Bacillus pascis* PSS17a, *Bacillus paralicheniformis* PSS23b were respectively affected six, five, four, four, three, three, three, two, two, two isolates (Table 3.15). In a previous study it was mentioned that bacteriocins could affects the bacteria from the closed genus and the closed species (Pato *et al.*, 2022). Bacteriocin-producing bacteria have a unique feature by which can protect itself against the harmful effects of bacteriocin and this feature has considered a distinguish characteristic from antibiotics (Abee, 1995). Bacteriocin-producing bacteria have a setrategy by which can protect itself from bacteriocin, by producing two types of proteins which act synergistically to protect the cell from bacteriocin, one of them integrate with cytoplasmic-membrane to prevent bacteriocin making pores while bacteriocin attach the cytoplasmic membrane whereas the another protein transports the bacteriocin outside by using ATP-binding cassette (ABC) system (Siegers and Entian, 1995). The results found that the *Bacillus subtilis* PSS16a, *Micrococcus luteus* PSS21b, and *Paenibacillus illinoisensis* PSS7a could not produce bacteriocin. We concluded that, not all isolates can produce bacteriocin and not all produced bacteriocin can affects all type of bacteria. In addition, there are different factors may affect bacteriocin production such as temperature, pH, and NaCl content that we investigated during this study. Immune mechanisms (Cotter *et al.*, 2005) are the primary barrier that protects bacteriocin-producing bacteria from the effects of their own metabolites. Certain mechanisms of bacteriocin production have been identified and can be mediated by specific proteins, such as that of the enterocin CRL35, in which its immunity protein interacts with the forming pore,

blocking it (Barraza *et al.*, 2017) or that of nisin, in which an extracellular membrane-anchored NisI protein can block nisin molecules. The action mechanism of the majority of bacteriocins is inadequately understood (Hacker *et al.*, 2015). The (ATP-binding cassette) transporter is another mechanism, which pushes bacteriocin through the cell membrane.

Table 3.15. The antimicrobial activity of bacteriocins produced from haloversatile isolates against other isolates.

Species affected by bacteriocins	Bacteriocin producers isolates												
	<i>Staphylococcus warneri</i> PSS1a	<i>Corynebacterium aurimucosum</i> PSS2b	<i>Staphylococcus epidermidis</i> PSS3a	<i>Kocuria polaris</i> PSS8a	<i>Bacillus haynesii</i> PSS11b	<i>Pseudomonas oryzihabitus</i> PSS14a	<i>Bacillus subtilis</i> PSS16a	<i>Bacillus piscis</i> PSS17a	<i>Pseudomonas ironica</i> PSS21a	<i>Microccus luteus</i> PSS21b	<i>Bacillus paralicheniformis</i> PSS23b	<i>Peribacillus frigoritolerans</i> PSS4a	<i>Paenibacillus illinoisensis</i> PSS7a
<i>Staphylococcus warneri</i> (PSS1a)	+	-	-	-	+	-	-	-	+	-	+	+	-
<i>Corynebacterium aurimucosum</i>	+	+	+	-	-	+	-	-	+	-	+	+	-
<i>Staphylococcus epidermidis</i> (PSS3a)	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Kocuria polaris</i> (PSS8a)	-	+	+	-	-	-	-	-	-	-	-	+	-
<i>Bacillus haynesii</i> (PSS11b)	+	+	+	-	-	+	-	-	+	-	-	+	-
<i>Pseudomonas oryzihabitus</i> (PSS14a)	-	-	+	-	-	-	-	-	-	-	-	-	-
<i>Bacillus subtilis</i> (PSS16a)	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Bacillus piscis</i> (PSS17a)	-	+	-	+	+	-	-	+	-	-	-	-	-
<i>Pseudomonas iranica</i> (PSS21a)	-	+	-	+	+	-	-	-	-	-	-	-	-
<i>Microccus luteus</i> (PSS21b)	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Bacillus paralicheiniformis</i> (PSS23b)	-	-	-	-	+	-	-	-	-	-	-	-	-
<i>Peribacillus frigoritolerans</i> (PSS4a)	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Paenibacillus illinoisensis</i> (PSS7a)	-	+	-	-	+	+	-	+	-	-	-	-	-

The highest inhibition zone was determined for *Bacillus piscis* (PSS17a) as 9 mm which was affected by *Corynebacterium aurimucosum* (PSS2b) (Table 3.16). The lowest inhibition zone was determined for *Pseudomonas iranica* (PSS21a), *Staphylococcus warneri* (PSS1a), *Bacillus haynesii* (PSS11b) as 4mm which were affected by *Corynebacterium aurimucosum* (PSS2b), *Bacillus haynesii* (PSS11b), *Pseudomonas iranica* (PSS21a), *Pseudomonas iranica* (PSS21a), respectively (Table 3.16). *Bacillus subtilis* (PSS16a), *Micrococcus luteus* (PSS21b) and *Paenibacillus illinoisensis* (PSS7a) did not produced bacteriocin (Tables 3.15 and 3.16).

Table 3.16. The inhibition zone diameters (mm) of bacteriocins produced from haloversatile isolates against other isolates.

Species affected from bacteriocins	Bacteriocin producers isolates												
	<i>Staphylococcus warneri</i> PSS1a	<i>Corynebacterium aurimucosum</i> PSSb2	<i>Staphylococcus epidermidis</i> PSS3a	<i>Kocuria polaris</i> PSS8a	<i>Bacillus haynesii</i> PSS11b	<i>Pseudomonas oryzihabitatus</i> PSS14a	<i>Bacillus subtilis</i> PSS16a	<i>Bacillus piscis</i> PSS17a	<i>Pseudomonas ironica</i> PSS21a	<i>Micrococcus luteus</i> PSS21b	<i>Bacillus paralicheniformis</i> PSS23b	<i>Peribacillus frigoritolerans</i> PSS4a	<i>Paenibacillus illinoisensis</i> PSS7a
<i>Staphylococcus warneri</i> (PSS1)	5	-	-	-	4	-	-	-	4	-	8	8	-
<i>Corynebacterium aurimucosum</i>	6	8	6	-	-	5	-	-	5	-	5	5	-
<i>Staphylococcus epidermidis</i> (PSS3a)	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Kocuria polaris</i> (PSS8a)	-	6	8	-	-	-	-	-	-	-	-	8	-
<i>Bacillus haynesii</i> (PSS11b)	8	8	8	-	-	5	-	-	4	-	-	8	-
<i>Pseudomonas oryzihabitatus</i> (PSS14a)	-	-	8	-	-	-	-	-	-	-	-	-	-
<i>Bacillus subtilis</i> (PSS16a)	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Bacillus piscis</i> (PSS17a)	-	9	-	8	8	-	-	5	-	-	-	-	-
<i>Pseudomonas ironica</i> (PSS21a)	-	4	-	8	8	-	-	-	-	-	-	-	-
<i>Micrococcus luteus</i> (PSS21b)	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Bacillus paralicheniformis</i> (PSS23b)	-	-	-	-	5	-	-	-	-	-	-	-	-
<i>Peribacillus frigoritolerans</i> (PSS4a)	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Paenibacillus illinoisensis</i> (PSS7a)	-	6	-	-	8	5	-	5	-	-	-	-	-

3.10. THE IMPACTS OF NaCl, pH AND TEMPERATURE VALUES ON BACTERIOGIN PRODUCTION

Based on the results mentioned above in Tables 3.15 and 3.16 the bacterial species which were produced high bacteriocin were selected [*Corynebacterium aurimucosum* (PSSb2), *Staphylococcus epidermidis* (PSS3a), *Bacillus haynesii* (PSS11b), and *Peribacillus frigoritolerans* (PSS4a)]. In addition, the bacteriocins' inhibition was investigated under different values of temperature, pH and NaCl against *Kocuria polaris* (PSS8a), *Staphylococcus warneri* (PSS1a), *Bacillus haynesii* (PSS11b), and *Peribacillus frigoritolerans* (PSS4a) which were sensitive to bacteriocins. The results indicated that the bacteriocin produced by these isolates could affect the tested isolates in a wide range of NaCl content and basically depends on the bacterial species and it was different from one genus to another one, (Table 3.17).

Table 3.17. The effect of NaCl concentrations on bacteriocins produced by haloversatile isolates.

		The isolates affected from bacteriocin				
NaCl contents		<i>Kocuria polaris</i> (PSS8a)	<i>Staphylococcus warneri</i> (PSS1a)	<i>Bacillus haynesii</i> (PSS11b)	<i>Peribacillus frigoritolerans</i> (PSS4a)	
Bacteriocin producing isolates	<i>Corynebacterium aurimucosum</i> (PSSb2)	0.85%	-	-	-	-
		3%	+	+	+	+
		5%	+	+	+	+
		10%	+	+	+	+
		20%	+	+	+	+
	<i>Staphylococcus epidermidis</i> (PSS3a)	0.85%	-	-	-	-
		3%	+	+	+	+
		5%	+	+	+	+
		10%	+	+	+	+
		20%	+	+	+	+
	<i>Bacillus haynesii</i> (PSS11b)	0.85%	-	-	-	-
		3%	+	+	+	+
		5%	+	+	+	+
		10%	+	+	+	+
		20%	+	+	+	+
	<i>Peribacillus frigoritolerans</i> (PSS4a)	0.85%	-	-	-	-
		3%	+	+	+	+
		5%	+	+	+	+
		10%	+	+	+	+
		20%	+	+	+	+

The bacteriocins produced by the four isolates, including *Corynebacterium aurimucosum* (PSSb2), *Staphylococcus epidermidis* (PSS3a), *Bacillus haynesii* (PSS11b), and *Peribacillus frigoritolerans* (PSS4a), exhibited a significant impact on the other four tested isolates within a wide range of NaCl concentrations, ranging from 0.85% to 20%. This shows that the protein structure of the bacteriocin is not affected by high NaCl concentrations. In fact, the bacteriocin demonstrated enhanced efficacy in higher NaCl concentrations. The study investigated the influence of pH values on the efficacy of bacteriocins produced by *Corynebacterium aurimucosum* (PSSb2), *Staphylococcus epidermidis* (PSS3a), *Bacillus haynesii* (PSS11b), and *Peribacillus frigoritolerans* (PSS4a). The results indicated that these proteins exhibited varying effects on the tested species at pH levels of 4, 5, 6, 7, 8, and 9. In contrast, the bacteriocin produced by all the isolates provided had no efficacy when tested at pH values of 10, 11, and 12 (Table 3.18).

Table 3.18. The effect of pH on bacteriocin produced by haloversatile isolates.

		The isolates that were affected from bacteriocin				
pH values		<i>Kocuria polaris</i> (PSS8a)	<i>Staphylococcus warneri</i> (PSS1a)	<i>Bacillus haynesii</i> (PSS11b)	<i>Peribacillus frigoritolerans</i> (PSS4a)	
Bacteriocin producing isolates	<i>Corynebacterium aurimucosum</i> (PSSb2)	4	-	-	+	+
		5	-	+	+	+
		6	+	+	+	+
		8	+	+	+	+
		9	+	+	+	+
		10	-	-	-	-
		11	-	-	-	-
		12	-	-	-	-
	<i>Staphylococcus epidermidis</i> (PSS3a)	4	+	-	+	+
		5	+	+	+	+
		6	+	-	+	+
		8	+	+	+	+
		9	-	+	+	-
		10	-	-	-	-
		11	-	-	-	-
		12	-	-	-	-
	<i>Bacillus haynesii</i> (PSS11b)	4	-	+	+	+
		5	+	+	+	+
		6	+	+	+	+
		8	+	+	+	+
		9	+	+	+	+
		10	-	-	-	-
		11	-	-	-	-
		12	-	-	-	-
	<i>Peribacillus frigoritolerans</i> (PSS4a)	4	-	-	-	-
		5	+	+	+	+
		6	+	+	+	+
		8	+	+	+	+
		9	-	-	-	-
		10	-	-	-	-
		11	-	-	-	-
		12	-	-	-	-

The initial experiments revealed that there are two categories of bacteriocins, namely lantibiotics and non-lanthionine low molecular weight bacteriocin-like molecules. These bacteriocins are characterized by their cationic nature and exhibit significant activity at low pH levels, specifically below pH 5. Additionally, it was observed that the adsorption of these bacteriocins to bacterial cells was enhanced at pH 6 compared to pH 2 (Jack *et al.*, 1995). Additionally, the results of the present study indicate that the bacteriocins continue to exhibit their effects even after undergoing incubation at various temperature levels, specifically 18°C, 20°C, 37°C, 40°C, 45°C, 50°C, and 55°C. In contrast, the bacteriocin's antibacterial activity was found to be diminished at a temperature of 4°C (Table 3.19).

Table 3.19. The effects of temperature on bacteriocin produced by haloversatile isolates.

The Isolates affected by bacteriocin					
Temperature values		<i>Kocuria polaris</i> (PSS8a)	<i>Staphylococcus warneri</i> (PSS1a)	<i>Bacillus haynesii</i> (PSS11b)	<i>Peribacillus frigoritolerans</i> (PSS4a)
Bacteriocin producing isolates	<i>Corynebacterium aurimucosum</i> (PSS2b)	4°C	-	-	-
		18°C	+	+	+
		20°C	+	+	+
		37°C	+	+	+
		40°C	+	+	+
		45°C	+	+	+
		50°C	+	+	+
		55°C	+	+	+
	<i>Staphylococcus epidermidis</i> (PSS3a)	4°C	-	-	-
		18°C	+	+	+
		20°C	+	+	+
		37°C	+	+	+
		40°C	+	+	+
		45°C	+	+	+
		50°C	+	+	+
		55°C	+	+	+
	<i>Bacillus haynesii</i> (PSS11b)	4°C	-	-	-
		18°C	+	+	+
		20°C	+	+	+
		37°C	+	+	+
		40°C	+	+	+
		45°C	+	+	+
		50°C	+	+	+
		55°C	+	+	+
	<i>Peribacillus frigoritolerans</i> (PSS4a)	4°C	-	-	-
		18°C	+	+	+
		20°C	+	+	+
		37°C	+	+	+
		40°C	+	+	+
		45°C	+	+	+
		50°C	+	+	+
		55°C	+	+	+

Azemin (2015) mentioned that the bacteriocins produced from certain archaeal and bacterial species such as *Halomonas* sp., *Vibrio* sp., *Staphylococcus* sp., *Micrococcus* sp., *Streptococcus* sp., *Bacillus cereus*, *Escherichia coli*, *Serratia*, *Proteus*, *Aeromonas*, *Enterobacter*, and *Plesiomonas* had effectiveness at a pH 7-8.5, temperature started from the ambient temperature to 55°C and 2.9 M of NaCl content. Other previous study reported that the bacteriocins are constat at 100°C for 10 minutes, hence it is used in food preservation methods (Moradi *et al.*, 2021). Furthermore, in previous study it was reported that the bacteriocins have antimicrobial activity at 10°C-60°C, pH 6.0-8.0, and 3%-20% salt concentrations against all test isolates (Caglayan and Birbir, 2018).

3.8.1. The Effects of Lipase and Proteinase-K Enzymes on Bacteriocin

Generally, the main structure of bacteriocin is protein, thus it is easily inactivated by proteinase K enzyme while it does not affected by lipase enzyme. Bacteriocins obtained from *Corynebacterium aurimucosum* (PSSb2), *Staphylococcus epidermidis* (PSS3a), *Bacillus haynesii* (PSS11b) and *Peribacillus frigoritolerans* (PSS4a) were inactivated after being treated with proteinase K enzyme; while lipase had no apparent impact on their bacteriocin structure or activity. These results were matched the results that obtained by Caglayan and Birbir (2018) in which the bacteriocin lost their effectiveness after being treated with proteinase K.

3.11. THE EFFECTS OF LIPOLYTIC AND PROTEOLYTIC BACTERIAL ACTIVITY ON SHEEPSKIN AND GOATSKIN SAMPLES AND EXAMINATION THE SKIN SAMPLES UNDER SCANNING ELECTRON MICROSCOPE

Two pieces of sheepskin and two pieces of goatskin were treated with the mix culture of enzyme producing haloversatile bacteria [protease and lipase producing *Staphylococcus warneri* (PSS1a), *Staphylococcus pasteurii* (PSS1b), *Staphylococcus warneri* (PSS2a), *Paenibacillus illinoisensis* (PSS7a), *Bacillus licheniformis* (PSS13a) and *Bacillus subtilis* (PSS16a); protease producing *Staphylococcus epidermidis* (PSS3b), *Bhargavaea ginsengi* (PSS11a), *Bacillus sonorensis* (PSS12a), *Bacillus haynesii* (PSS11b) and *Corynebacterium aurimucosum* (PSS15a)], while two pieces of them treated with physiological saline solution and incubated 40 days at 24°C. After that the skin samples were prepared according to the standard methods in order to examine under scanning electron microscope. The results indicated that the sheepskins and goatskins (control groups) which were treated with only 15% NaCl brine solution were not affected during the incubation periods as shown in the Figures (3.16, 3.17, 3.18, 3.19, 3.20 and 3.21). This demonstrated that the brine curing 15% NaCl was adequate for forty-days skin preservation.

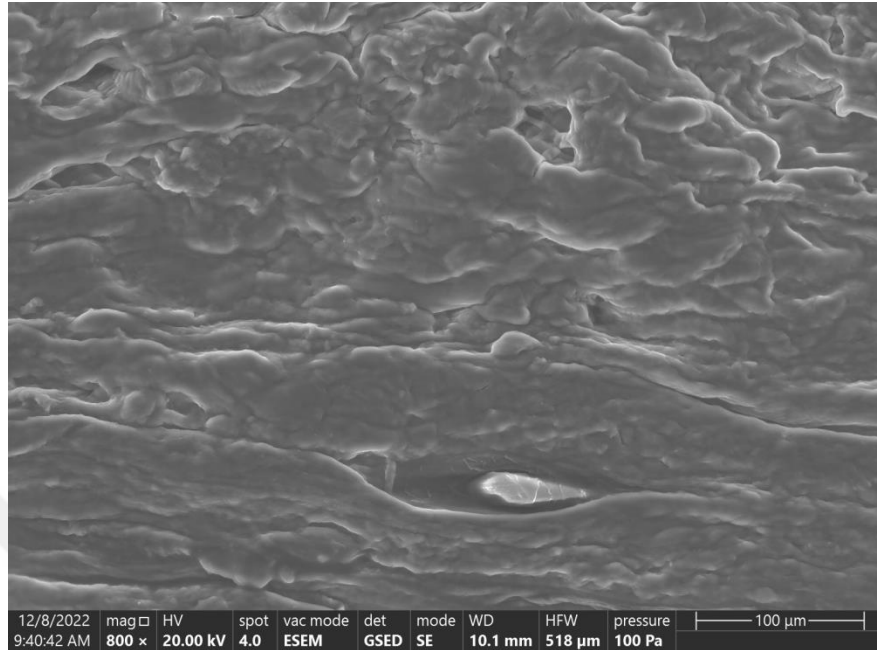


Figure 3.16. The control sample (sheepskin) treated with physiological saline solution (15% NaCl) and incubated for 40 days (The bar=100μm).

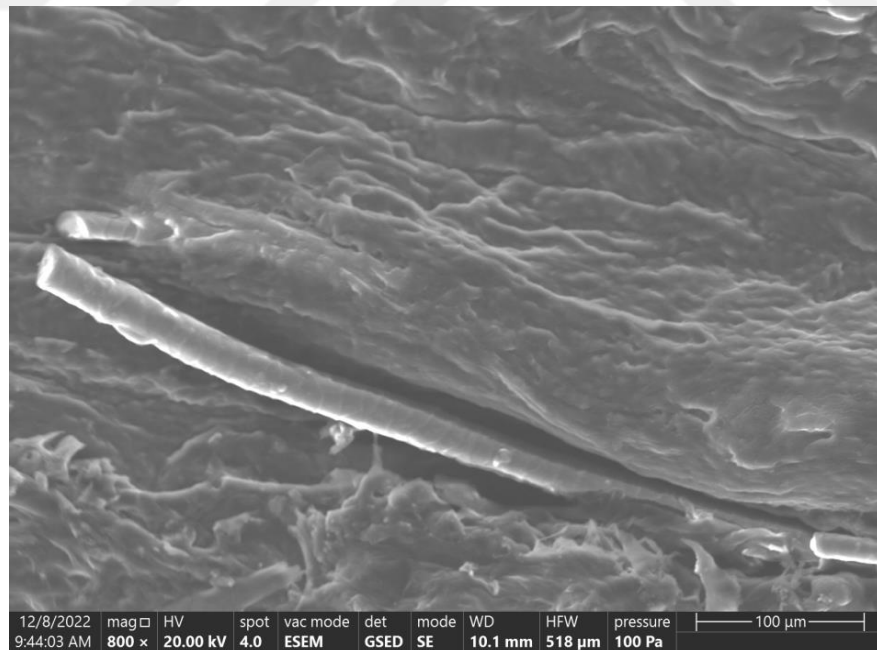


Figure 3.17. The control sample (sheepskin) treated with physiological saline solution (15% NaCl) and incubated for 40 days (The bar=100μm).

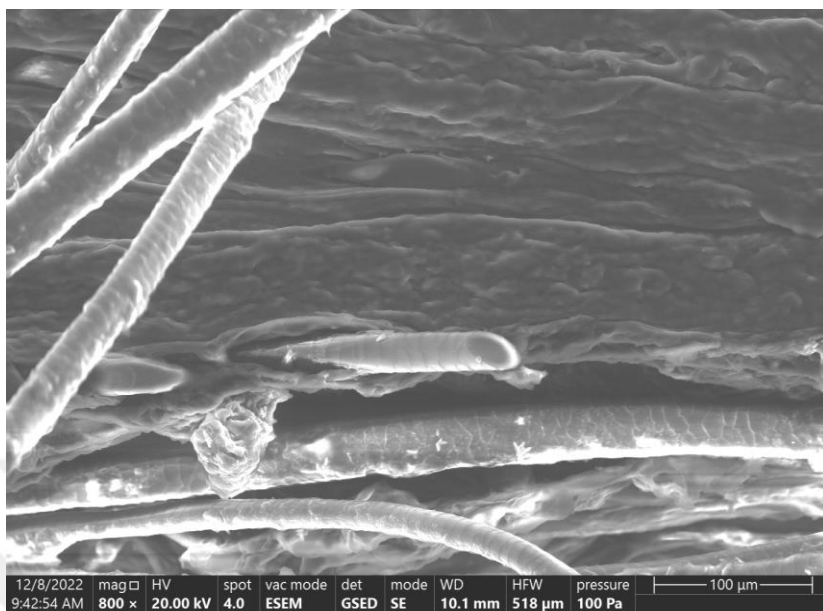


Figure 3.18. The sheepskin control sample treated with physiological saline solution and incubated for 40 days (The bar=100μm).

Figures 3.16, 3.17 and 3.18 demonstrate that the structural composition of the skin, including its fibers and hairs, remained constant during the storage period. This observation suggests that the preservation of the sheepskin can be attributed to the presence of a normal saline solution containing 15% NaCl.

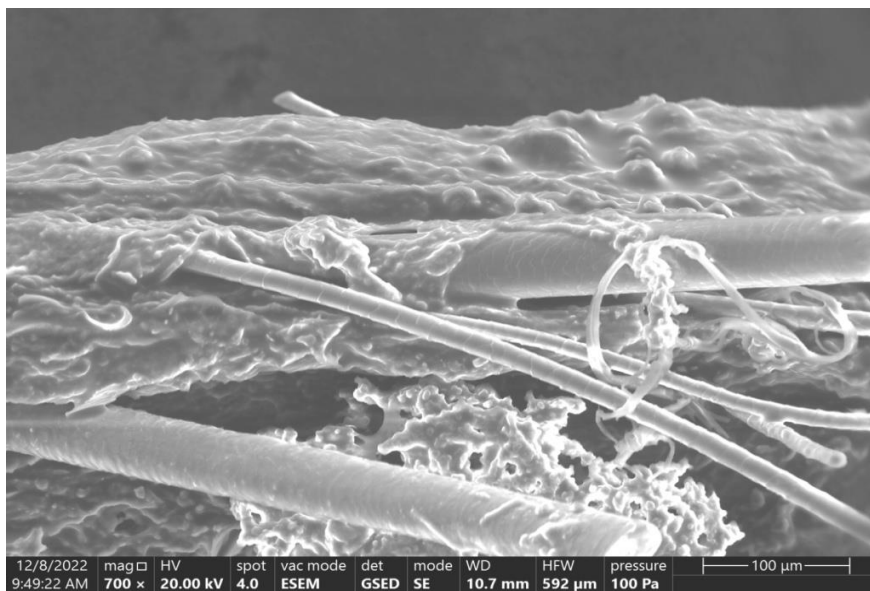


Figure 3.19. The goatskin (control sample) treated with physiological saline solution and incubated for 40 days (The bar= 100 μm).

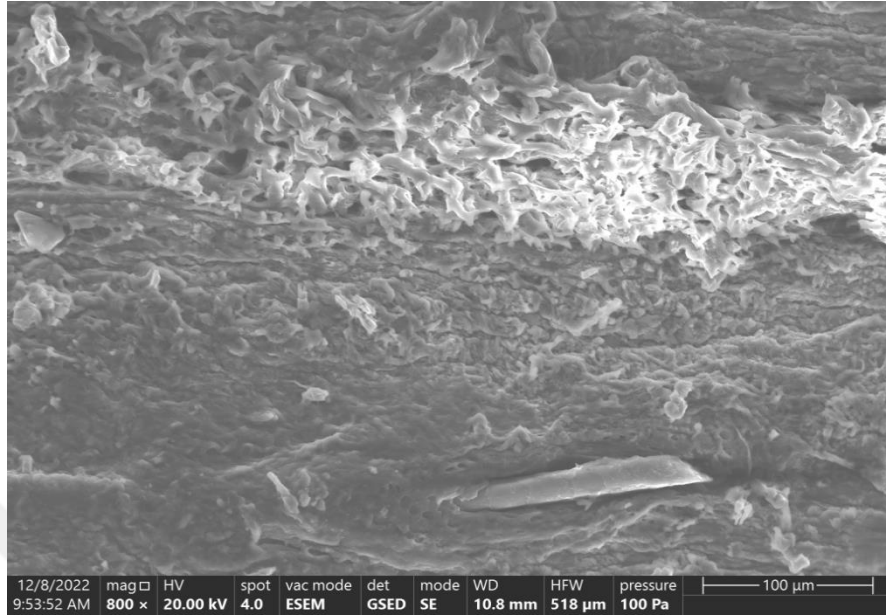


Figure 3.20. Illustrations image depicts goatskin (control) exhibiting its cut natural hair (The bar=100 μm).

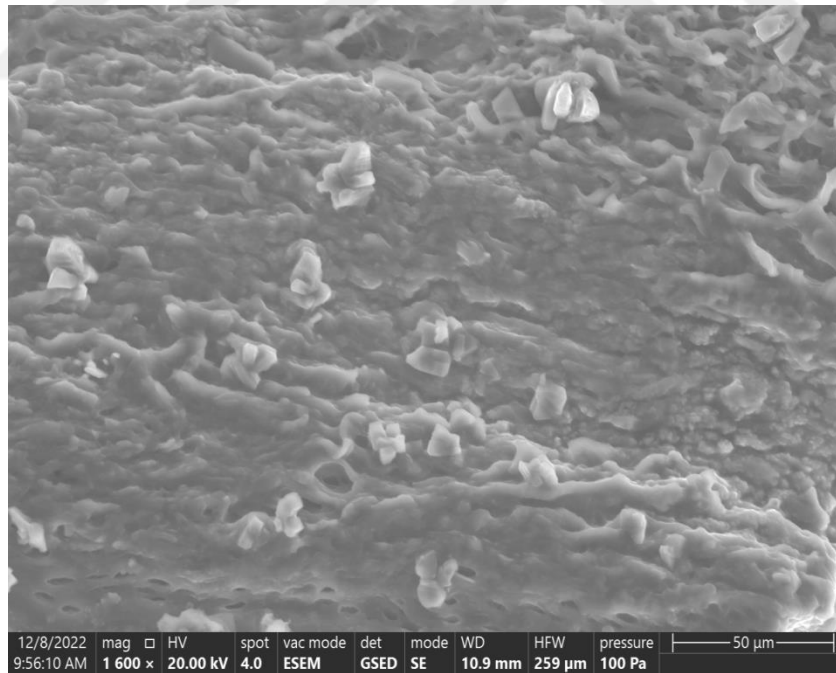


Figure 3.21. The control sample (goatskin) that was treated with physiological saline containing a particle of NaCl (The bar=50 μm).

Figures 3.19, 3.20, and 3.21, represent the normal structure of goatskin treated with 15% NaCl (control) and incubated for 40 days. The images demonstrated that there were no changes on the structure of the animal's epidermis, particularly the structure of the hair. On the contrary, the presence of a combination of lipolytic and proteolytic haloversatile bacteria had negative impact on the composition as well as the structure of the skin. This was visible by microscopic analysis, which demonstrated a significant decrease in skin hairs and fibers. Furthermore, alongside the observable alterations at a macroscopic level during the incubation period, there was also the discernible presence of a viscous texture on the outer layer of the skin and an unpleasant scent. The Figures (3.22, 3.23, 3.24, 3.25, 3.26, and 3.27) illustrate the harmful impacts of protease and lipase enzymes produced by haloversatile bacteria on animal epidermis samples treated with lipolytic and proteolytic bacteria during the incubation period. The observed effects included hair loss, a viscid aspect, and an increase in dermal pores.

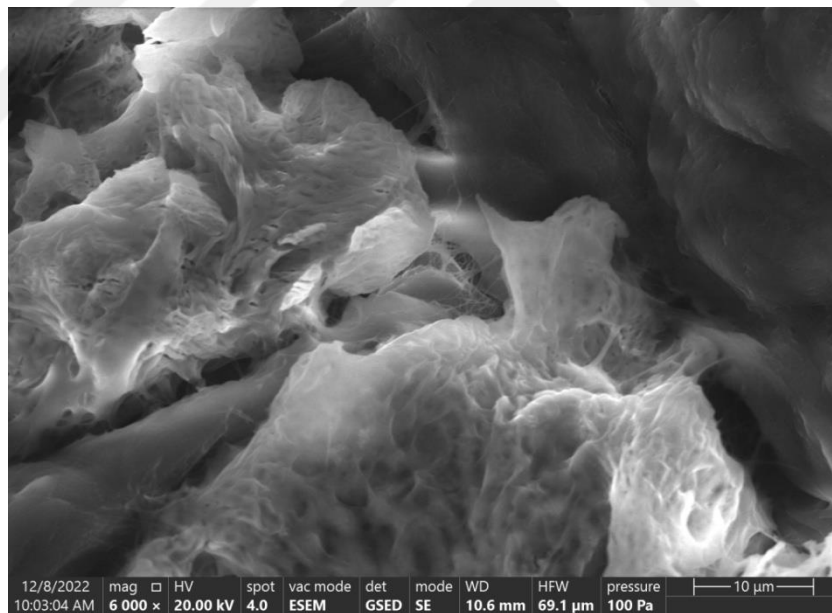


Figure 3.22. The inner layer of goatskin treated with the mixed culture of enzyme-producing haloversatile isolates after 40 days storage (The bar=10μm).

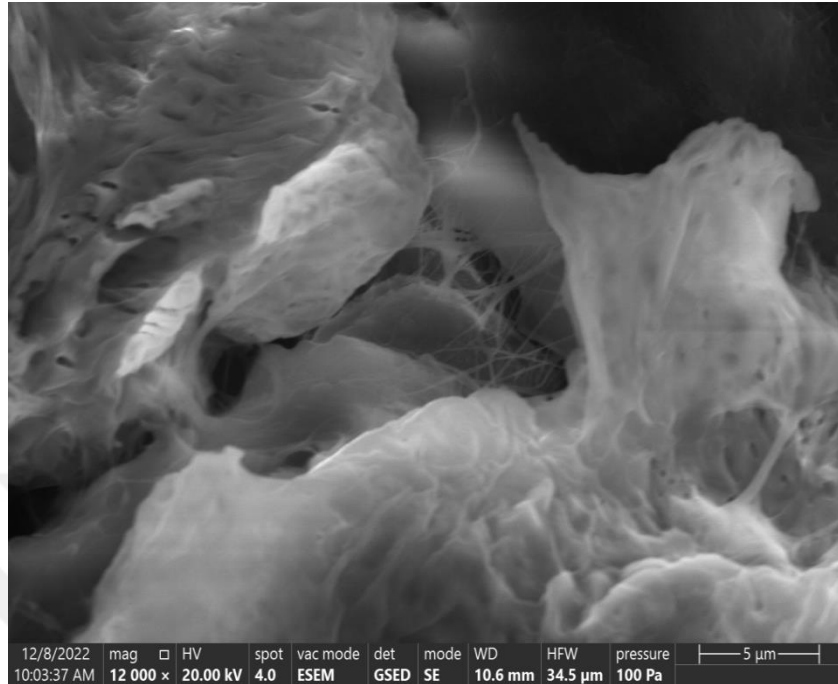


Figure 3.23. The goatskin sample treated with the mixed culture of enzyme-producing haloversatile isolates after 40 days storage (The bar=5μm).

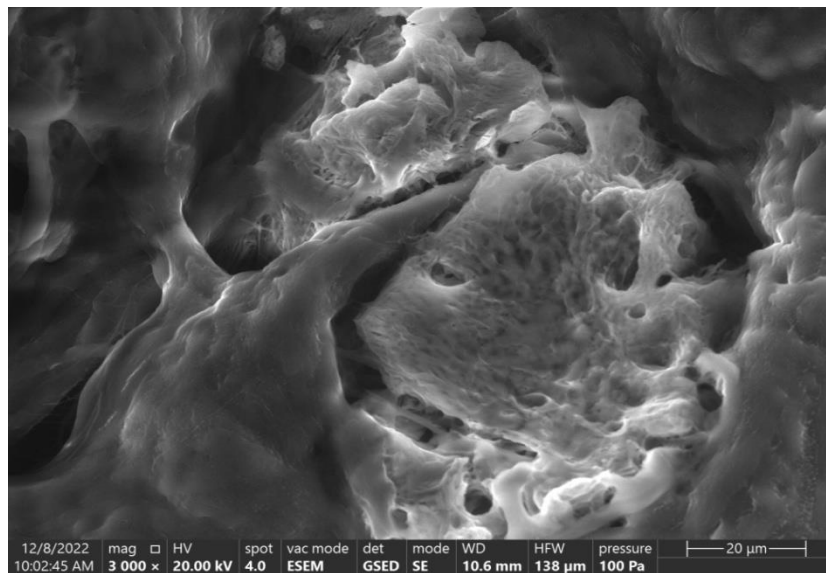


Figure 3.24. The holes in the structure of goatskin treated with the mixed culture of enzyme-producing haloversatile isolates after 40 days storage (The bar=20μm).

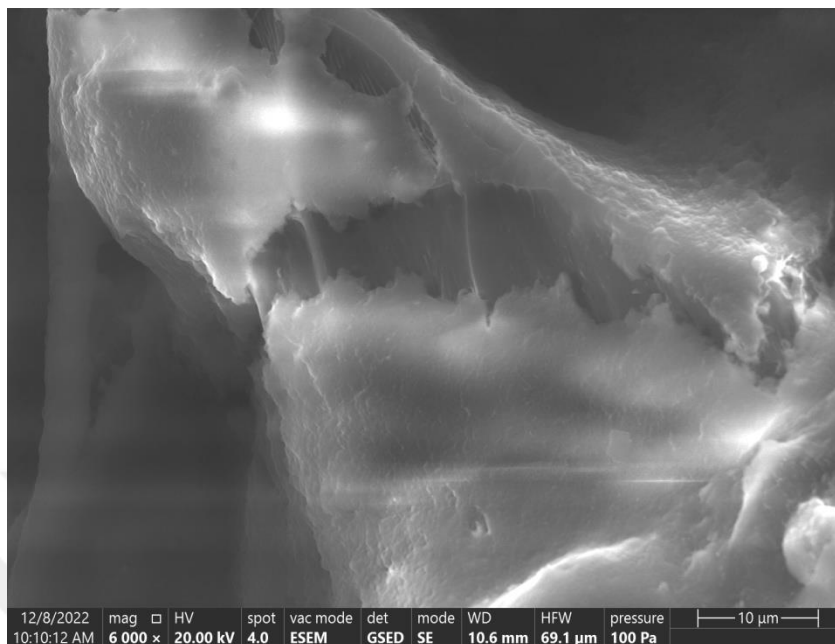


Figure 3.25. Sheepskin's components affected by mixed culture of enzyme-producing haloversatile isolates after 40 days storage (The bar=10μm).

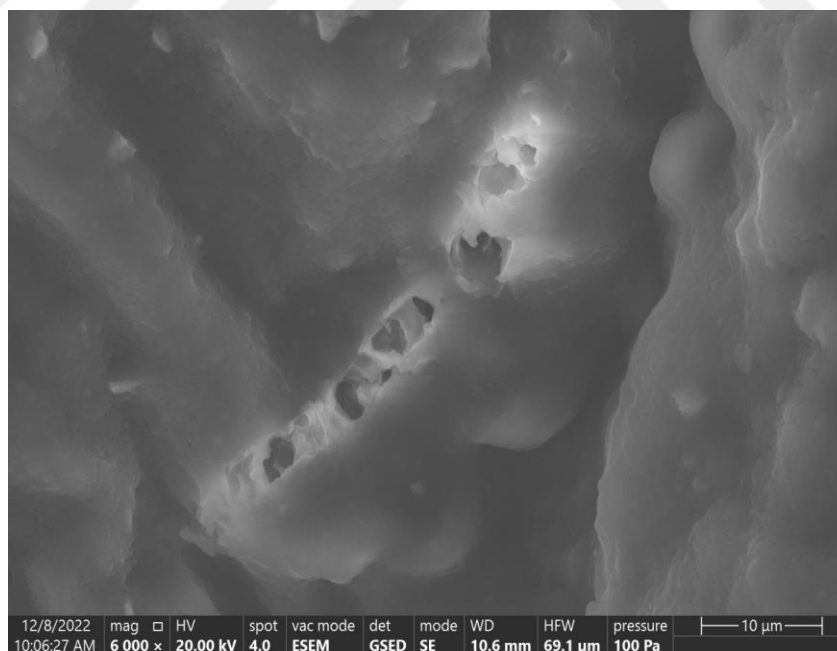


Figure 3.26. The harmful impacts of sheepskin's hair treated with mixed culture of enzyme-producing haloversatile isolates after 40 days storage (The bar=10μm).

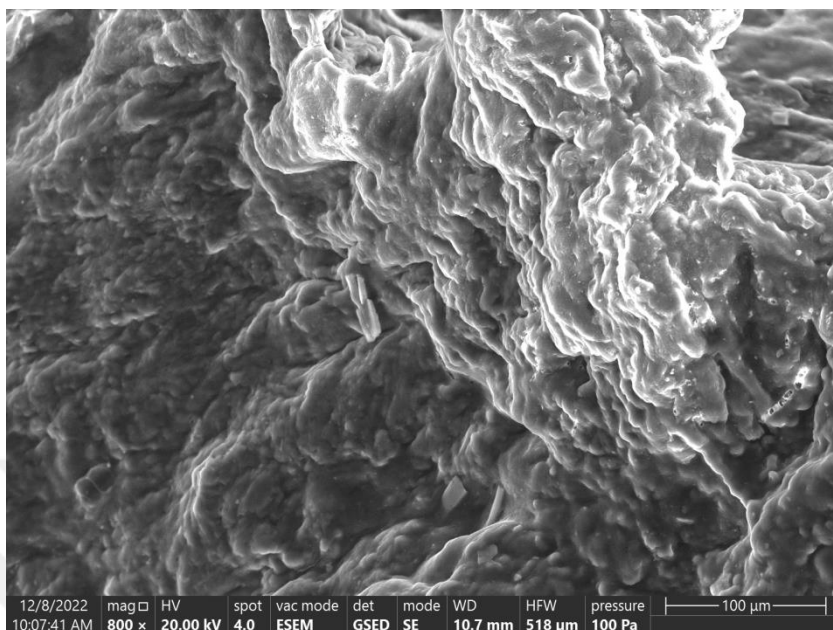


Figure 3.27. The sheepskin sample treated with mixed culture of enzyme-producing haloversatile isolates after 40 days storage (The bar=100μm).

Previous researches have demonstrated that hydrolytic enzymes produced by haloversatile bacteria have adverse impacts on animal epidermis. Salt, salted skins, salted hides, and soak liquors have all been found to contain a variety of salt-loving bacteria and archaea despite the severe effects of salts on microorganisms (Birbir *et al.*, 2007; Caglayan *et al.*, 2015; Gutiérrez-Arnillas *et al.*, 2016; Yilmaz and Birbir, 2019). Due to the proteinaceous structure and high lipid content of animal skin, numerous lipase and protease-producing halophilic bacteria can proliferate and decompose the hide and epidermis structure (Ozbay and Caglayan, 2022).

3.12. ANTIBIOTIC SENSITIVITY TEST

In the present study, Cephalothin (30 µg, KF30), Cefotaxime (5µg, CTX5), Rifampicin (5 µg, RD5), Spectinomycin (100 µg, SH100), Sulbactam/Ampicillin (10 µg, SAM10), Amikacin (30 µg, AK30), Neomicin (30 µg, N30) and Tetracycline (30 µg, TE30) were applied to 35 haloversatile isolates by Kirby-Bauer disc diffusion method. The results were presented as mm (Table 3.20). Inhibition zones were produced by all isolates against Rifampicin (RD5, 5µg), Amikacin (AK30, 30µg), and Neomycin (N30, 30µg) (Table 3.20). The inhibition zones of *Staphylococcus warneri* PSS1a, *Staphylococcus warneri* PSS2a, *Staphylococcus pasteurii* PSS1b, *Corynebacterium aurimucosum* PSS2b, *Corynebacterium aurimucosum* PSS15a, *Kocuria rosea* PSS6a, *Paenibacillus illinoisensis* PSS7a, *Bacillus sonorensis* PSS12a, *Bacillus pascalis* PSS17a, *Micrococcus yunnanensis* PSS18b, *Staphylococcus caprae* PSS19a against test antibiotics were 9-30mm, 12-26mm, 8-22 mm, 8-31mm, 8-32mm, 15-35mm, 15-33mm, 8-30mm, 11-30mm, 7-30mm, 11-43, respectively (Table 3.20). The isolates PSS3a, PSS3b, PSS9a, PSS10a, PSS15b, PSS24a which belong to *Staphylococcus epidermidis* species showed inhibition zones as 15-31mm, 14-45mm, 15-31mm, 12-48mm, 13-33mm, 17-39mm against test antibiotics (Table 3.20). Isolates *Peribacillus frigoritolerans* PSS4a, *Staphylococcus haemolyticus* PSS4b, *Kocuria polaris* PSS8a, *Bacillus licheniformis* (PSS13a), *Staphylococcus hominis* (PSS18a), *Staphylococcus saccharolyticus* PSS20a showed inhibition zones such as 7-25mm, 7-25mm, 10-24mm, 12-25mm, 14-31mm, 10-35mm against Cephalothin (30 µg, KF30), Rifampicin (5 µg, RD5), Spectinomycin (100 µg, SH100), Sulbactam/Ampicillin (10 µg, SAM10), Amikacin (30 µg, AK30), Neomicin (30 µg, N30) and Tetracycline (30 µg, TE30) (Table 3.20). *Lysinibacillus telephonicus* PSS5a and *Bhargavaea ginsengi* PSS11a showed inhibition zones as 14-30mm and 6-24mm against Cephalothin (30 µg, KF30), Cefotaxime (5µg, CTX5), Rifampicin (5 µg, RD5), Spectinomycin (100 µg, SH100), Amikacin (30 µg, AK30), Neomicin (30 µg, N30) and Tetracycline (30 µg, TE30), respectively (Table 3.20). The inhibition zone diameters of *Bacillus haynesii* PSS11b, *Pseudomonas oryzae* PSS14a, *Bacillus subtilis* PSS16a, *Pseudomonas iranica* PSS21a, *Micrococcus luteus* PSS21b, *Staphylococcus capitis* subsp. *urealyticus* PSS22a, *Corynebacterium minutissimum* PSS23a, *Bacillus paralicheniformis* (PSS23b), *Staphylococcus taiwanensis* (PSS25a) were also presented in Table 3.20.

Table 3.20. Diameters of Inhibition Zones (mm) of the test isolates against antibiotics.

Code	Isolates	Antibiotics								
		Cephalothin	Cefotaxime	Rifampicin	Spectinomycin	Sulbactam / Ampicilin	Amikacin	Neomycin	Tetracycline	Antibiotic number that affect each isolate
PSS1a	<i>Staphylococcus warneri</i>	20	9	30	9	15	18	19	24	8
PSS2a	<i>Staphylococcus warneri</i>	26	12	29	12	19	16	20	20	8
PSS1b	<i>Staphylococcus pasteurii</i>	18	8	22	12	11	12	16	15	8
PSS2b	<i>Corynebacterium aurimucosum</i>	21	8	31	17	12	24	25	25	8
PSS15a	<i>Corynebacterium aurimucosum</i>	24	8	32	15	10	20	26	21	8
PSS3a	<i>Staphylococcus epidermidis</i>	23	19	31	16	27	15	23	30	8
PSS3b	<i>Staphylococcus epidermidis</i>	25	17	45	14	25	20	26	25	8
PSS9a	<i>Staphylococcus epidermidis</i>	24	20	31	15	23	20	25	29	8
PSS10a	<i>Staphylococcus epidermidis</i>	25	23	48	12	25	20	29	27	8
PSS15b	<i>Staphylococcus epidermidis</i>	26	21	33	13	29	19	28	32	8
PSS24a	<i>Staphylococcus epidermidis</i>	26	25	39	17	25	20	21	24	8
PSS4a	<i>Peribacillus frigoritolerans</i>	8	-	18	18	7	25	20	20	7
PSS4b	<i>Staphylococcus haemolyticus</i>	15	-	25	7	7	16	20	17	7
PSS5a	<i>Lysinibacillus telephonicus</i>	20	14	30	15	-	25	28	19	7
PSS6a	<i>Kocuria rosea</i>	35	30	25	15	15	15	25	16	8
PSS7a	<i>Paenibacillus illinoisensis</i>	15	18	18	27	20	19	33	20	8
PSS8a	<i>Kocuria polaris</i>	10	-	21	15	10	18	20	24	7
PSS9b	<i>Staphylococcus caprae</i>	13	14	20	-	20	15	10	-	6
PSS11a	<i>Bhargavaea ginsengi</i>	24	7	15	6	-	15	22	7	7
PSS11b	<i>Bacillus haynesii</i>	-	12	16	15	-	20	20	18	6
PSS12a	<i>Bacillus sonorensis</i>	20	10	30	10	8	20	21	18	8
PSS13a	<i>Bacillus licheniformis</i>	15	-	21	14	12	18	19	25	7
PSS14a	<i>Pseudomonas oryzae</i>	-	-	19	28	-	30	30	24	5
PSS16a	<i>Bacillus subtilis</i>	16	-	19	17	-	20	21	30	6
PSS17a	<i>Bacillus pascis</i>	15	16	29	15	11	20	30	21	8
PSS18a	<i>Staphylococcus hominis</i>	17	-	31	20	14	17	18	29	7
PSS18b	<i>Micrococcus yunnanensis</i>	20	13	30	10	7	16	20	10	8
PSS19a	<i>Staphylococcus caprae</i>	25	13	43	11	15	18	25	16	8
PSS20a	<i>Staphylococcus saccharolyticus</i>	21	-	35	10	12	19	22	25	7
PSS21a	<i>Pseudomonas iranica</i>	18	15	40	-	19	20	13	29	7
PSS21b	<i>Micrococcus luteus</i>	20	10	28	12	-	15	18	-	6
PSS22a	<i>Staphylococcus capitis</i> subsp. <i>urealyticus</i>	13	-	35	12	-	16	14	27	6
PSS23a	<i>Corynebacterium minutissimum</i>	17	12	40	-	18	19	21	21	7
PSS23b	<i>Bacillus paralicheniformis</i>	18	-	40	-	8	19	20	-	5
PSS25a	<i>Staphylococcus taiwanensis</i>	15	-	25	10	-	15	20	23	6

3.13. THE MIC (MINIMUM INHIBITORY CONCENTRATION) OF ANTIMICROBIAL AGENT WITH SODIUM DIMETHYLDITHIOCARBAMATE AGAINST HALOVERSATILE BACTERIA

Twenty-two different concentrations of an antimicrobial agent containing sodium dimethyldithiocarbamate (0.00175, 0.0035, 0.007, 0.014, 0.028, 0.06, 0.12, 0.24, 0.48, 0.96, 1.96, 3.92, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000, 2000, 4000 $\mu\text{g/ml}$) were applied to *Staphylococcus warneri* (PSS1a), *Staphylococcus pasteurii* (PSS1b), *Paenibacillus illinoisensis* (PSS7a), *Bacillus subtilis* (PSS16a), *Micrococcus luteus* (PSS21b), *Staphylococcus epidermidis* (PSS15b), *Bacillus sonorensis* (PSS12a), *Corynebacterium aurimucosum* (PSS15a), and *Staphylococcus capitis* subsp. *urealyticus* (PSS22a). The main objective of this experiment was to determine the MIC and to reduce consumption of antimicrobial agent in the leather industry. The results showed that the growth of all tested isolates were inhibited upon exposure to the concentrations started from 125 $\mu\text{g/ml}$ until 4000 $\mu\text{g/ml}$ except *Staphylococcus warneri* (isolates PSS1a). The MICs of *Staphylococcus warneri* (PSS1a), *Staphylococcus pasteurii* (PSS1b), *Paenibacillus illinoisensis* (PSS7a), *Bacillus subtilis* (PSS16a), *Micrococcus luteus* (PSS21b), *Staphylococcus epidermidis* (PSS15b), *Bacillus sonorensis* (PSS12a) were found as 250 $\mu\text{g/ml}$, 62.5 $\mu\text{g/ml}$, 15.6 $\mu\text{g/ml}$, 7.8 $\mu\text{g/ml}$, 125 $\mu\text{g/ml}$, 15.6 $\mu\text{g/ml}$, 62.5 $\mu\text{g/ml}$ (Table 3.21). The MICs of *Corynebacterium aurimucosum* (PSS15a) and *Staphylococcus capitis* subsp. *urealyticus* (PSS22a) were determined as 3.92 $\mu\text{g/ml}$ (Table 3.21). While these two species were affected by the lowest concentration of the antimicrobial agent, *Staphylococcus warneri* (PSS1a) was the most resistant species against this agent (Table 3.21).

Table 3.21. The minimum inhibitory concentrations of the antimicrobial agent containing sodium dimethyldithiocarbamate against haloversatile test strains.

Concentration of sodium dimethyldithiocarbamate (µg/ml)	<i>Staphylococcus warneri</i> (PSS1a)	<i>Staphylococcus pasteurii</i> (PSS1b)	<i>Paenibacillus illinoisensis</i> (PSS7a)	<i>Bacillus subtilis</i> (PSS16a)	<i>Micrococcus luteus</i> (PSS21b)	<i>Staphylococcus epidermidis</i> (PSS15b)	<i>Bacillus sonorensis</i> (PSS12a)	<i>Corynebacterium aurimucosum</i> (PSS15a)	<i>Staphylococcus capitis</i> subsp. <i>urealyticus</i> (PSS22a)
4000	-	-	-	-	-	-	-	-	-
2000	-	-	-	-	-	-	-	-	-
1000	-	-	-	-	-	-	-	-	-
500	-	-	-	-	-	-	-	-	-
250	-	-	-	-	-	-	-	-	-
125	+	-	-	-	+	-	+	-	-
62.5	+	+	-	-	+	-	+	-	-
31.2	+	+	-	-	+	-	+	-	-
15.6	+	+	+	-	+	+	+	-	-
7.8	+	+	+	+	+	+	+	-	-
3.92	+	+	+	+	+	+	+	-	-
1.96	+	+	+	+	+	+	+	+	+
0.96	+	+	+	+	+	+	+	+	+
0.48	+	+	+	+	+	+	+	+	+
0.24	+	+	+	+	+	+	+	+	+
0.12	+	+	+	+	+	+	+	+	+
0.06	+	+	+	+	+	+	+	+	+
0.028	+	+	+	+	+	+	+	+	+
0.014	+	+	+	+	+	+	+	+	+
0.007	+	+	+	+	+	+	+	+	+
0.0035	+	+	+	+	+	+	+	+	+
0.00175	+	+	+	+	+	+	+	+	+
Positive control*	+	+	+	+	+	+	+	+	+
Negative control**	-	-	-	-	-	-	-	-	-

*Positive control: Media + Bacteria, **Negative control: Only media

3.14. EXAMINATION THE HALOVERSATILE BACTERIA ISOLATED FROM SALT SAMPLES UNDER SCANNING ELCECTRON MICROSCOPE

The isolates were examined under SEM (Scanning Electron Microscope) and the results supported the diagnosis of haloversatile bacteria by clarifying the cell molphology. As shown in Figures 3.28, 3.29, 3.30, 3.31, 3.32, 3.33, and 3.34 bellow:

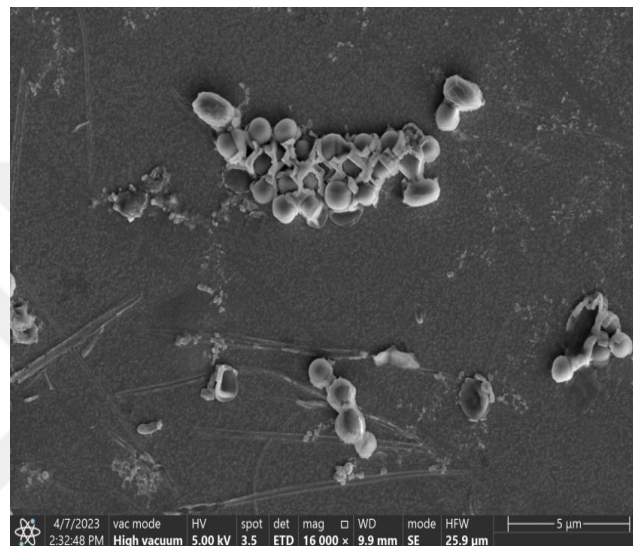


Figure 3.28. The cells of *Staphylococcus warneri* (isolate PSS2a) under SEM (The bar=5µm).

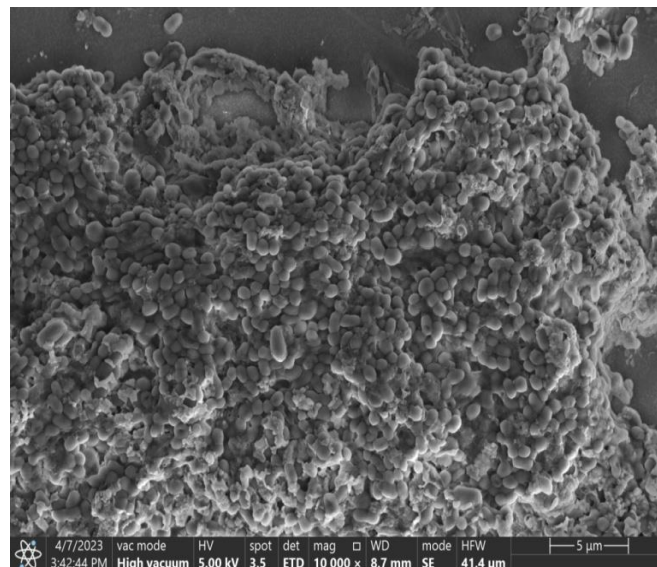


Figure 3.29. The cells of *Kocuria rosea* (isolate PSS6a) under SEM (The bar=5µm).

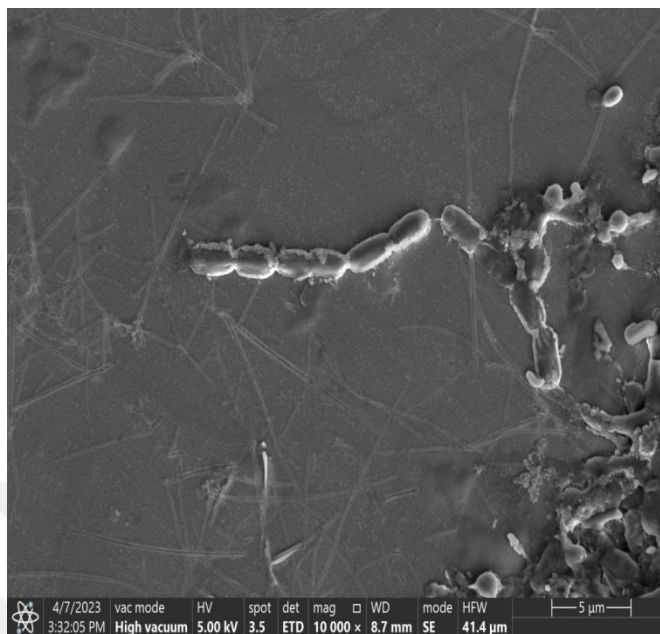


Figure 3.30. The cells of *Bhargavaea ginsengi* (isolate PSS11a) under SEM (The bar=5μm).

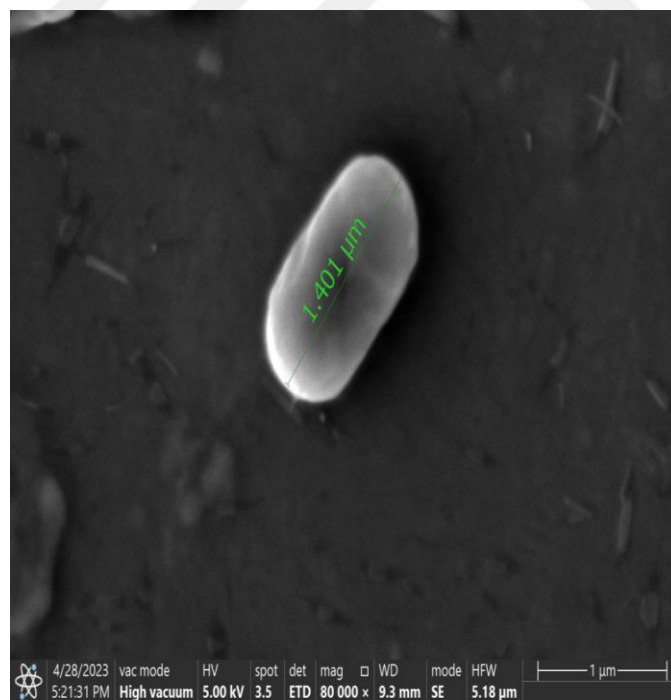


Figure 3.31. The cells of *Bacillus licheniformis* (isolate PSS13a) under SEM (The bar=1μm).

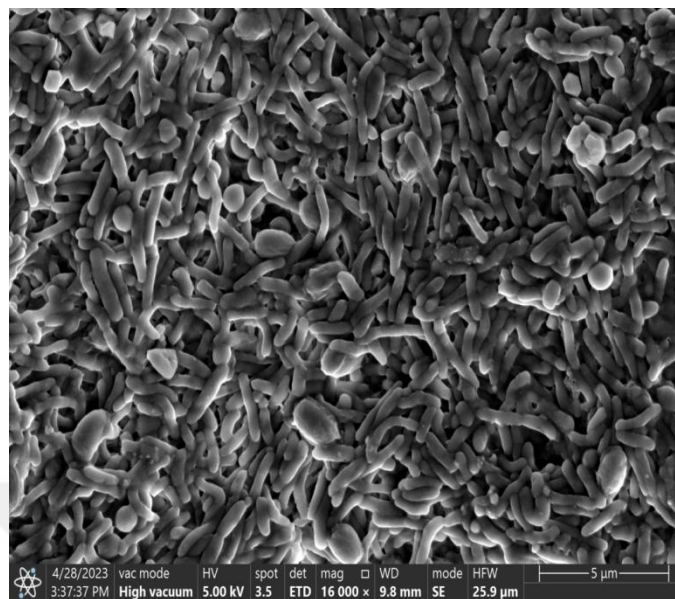


Figure 3.32. The cells of *Corynebacterium aurimucosum* (isolate PSS15a) under SEM (The bar=5μm).

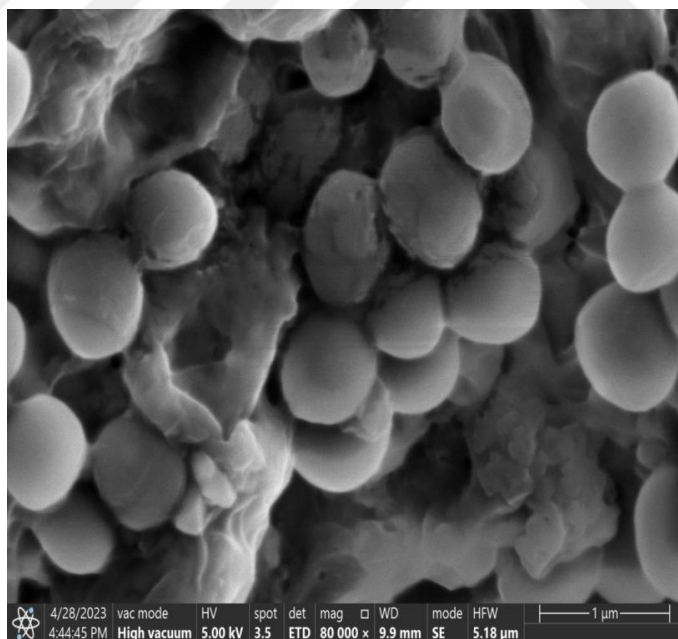


Figure 3.33. The cells of *Staphylococcus epidermidis* (isolate PSS9a) under SEM (The bar= 1 μm).

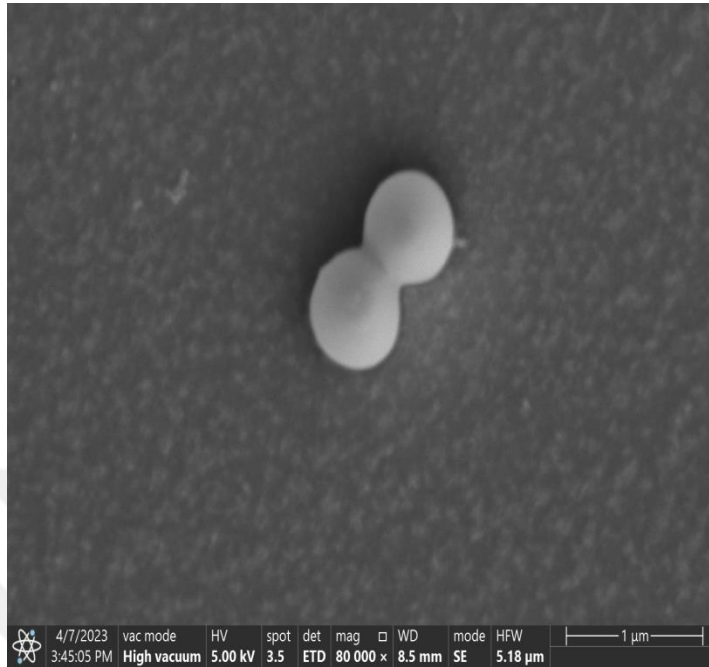


Figure 3.34. The diplococcus of *Staphylococcus epidermidis* (isolate PSS9a) under SEM (The bar= 1 μm).

CHAPTER 4

Conclusions

The total counts of haloversatile bacteria was found as between 6×10^3 and 8×10^4 CFU/g, while the lipolytic and proteolytic haloversatile bacterial counts were found as 2×10^3 - 3.1×10^4 CFU/g and 1×10^3 - 7×10^3 CFU/g, respectively. The microscopical investigation indicated a considerable loss of skin hairs and fibers, indicating that the enzyme-producing isolates had negative effects on the skin's structure and components. In addition to the macroscopic changes the incubation time indicated that the animal skin had become sticky and had an unpleasant odor. Proteases and lipases produced from bacterial can degrade skin quality by breaking down the skin's structure. Our research found that employing the dry salt curing approach to preserve animal skin direct after slaughter in a short time while reducing the load of microorganisms to an acceptable level was difficult. The pH of salt samples was found to range between 7.60 and 8.04, and the sheep skin structure was rich in organic compounds such as proteins and lipids. The pH of salt, the moisture content of animal skins, and the proteinaceous structure were all ideal for bacterial development. Despite the fact that the salt had been applied to fresh animal skin, the skins' moisture level was not particularly low. This promotes the development of salt-loving bacteria. As a result, the harm effects caused by microorganisms' activity can be prevented by using antimicrobial agents besides salt curing on the raw hide during storage and transportation until it is processed. 35 isolates were isolated from salt samples and which including [*Staphylococcus epidermidis* (6 isolates) was the most encountered species. *Staphylococcus warneri*, *Corynebacterium aurimucosum*, *Staphylococcus caprae* species were identified as two isolates. Only one isolate of the species *Staphylococcus pasteurii*, *Peribacillus frigoritolerans*, *Staphylococcus haemolyticus*, *Lysinibacillus telephonicus*, *Kocuria rosea*, *Paenibacillus illinoisensis*, *Kocuria Polar*, *Bhargavaea ginsengi*, *Bacillus haynesii*, *Bacillus sonorensis*, *Bacillus licheniformis*, *Pseudomonas oryzihabitans*, *Bacillus subtilis*, *Bacillus piscis*, *Staphylococcus hominis*, *Micrococcus yunnanensis*, *Staphylococcus saccharolyticus*, *Pseudomonas iranica*, *Micrococcus luteus*, *Staphylococcus capitis* subsp. *urealyticus*, *Corynebacterium minutissimum*, *Bacillus paralicheniformis*, *Staphylococcus taiwanensis* had been identified. All our isolated genus could grow at 19-55°C 0-17% NaCl, and pH 5-11. All isolates optimally showed growth at 1.17% NaCl. Hence, these isolates were accepted as haloversatile. In the present study, the enzymatic results showed that esterase, urease, protease,

caseinase, lipase, xylanase, cellulase, amylase, DNase, and oxidase were respectively produced by 40%, 66%, 77%, 6%, 17%, 6%, 17%, 20%, 20%, and 40% of the isolates. While all isolates produced catalase, none of the isolates produced pullulanase. The optimal conditions for lipase and protease production were at pH at 7-8, NaCl concentration at 1% to 3%, an incubation period at 24 to 96 hours on Tween 80 Agar Media and Gelatine Agar Media, respectively. Whereas, 30 minutes in a water bath, and a temperature between 37°C to 50°C for lipase and one hour, at 37°C to 50°C in water bath for protease. The concentrations and activities of lipase enzyme and protease were determined by using of the Bradford reaction and the absorbance using a Spectrophotometer. It is worth noting that the results of lipase and protease are regarded significant and suggest further exploration in future studies. Furthermore, it can be used as an alternative to chemicals in the leather industry. The result exhibited that the isolates were capable of fermenting carbohydrates under aerobic conditions. Furthermore, a significant portion of the isolates might utilise tested amino acids such as proline, tyrosin, lysin, and argenin, which are the essential amino acids in the proteins reticulin, elastin, keratin, and collagen. Considering these bacterial species that we isolated in our study while applying the preservation procedures and selecting the antimicrobial agent will also increase the effectiveness of the preservation method. Bacteriocin investigations showed that some of our isolates are capable of producing proteins with antibacterial activity, which can be employed as an alternative to antibiotics and may be applicable in the food preservation and leather sectors. The most suitable circumstances for bacteriocin production were also studied, and the results demonstrated that it was worthy of publication and beneficial to future research. The optimal circumstances for the development and flourish of haloversatile bacteria in salt have been identified, which could be helpful in the prevention of their proliferation in salts and animal skins. Furthermore, broth dilution method was used to examine antibiotic sensitivity and detect MIC of antimicrobial agent with SDDC active ingredients; the results revealed that the growth of haloversatile bacteria was inhibited at a specific antimicrobial agent concentrations and by using a specific antibiotic disc. These findings will be extremely useful with the leather industries in Istanbul/Türkiye in order to lower the load of lipolytic and proteolytic haloversatile bacteria in salt and skin samples during transformation and storage. We assume that the information collected in our study will be useful in future studies involving the leather industry.

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ATTACHMENTS

Base pair sequences of 16SrRNA genes of halophilic bacteria isolated from salt samples:

Appendix: 1

Number of Bases: 1040

Phylogenetically similar species: *Staphylococcus warneri* strain RO8 16S rRNA

Reference: (Gungor, 2019).

>PSS1a

```
GTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGTAGCATGCT
GATCTACGATTACTAGCGATTCCAGCTTCATGTAGTCGAGTTGCAGACTA
CAATCCGAAGTGAACAACCTTTATGGGATTTGCTTGACCTCGCGGTTTA
GCTGCCCTTTGTATTGTCCATTGTAGCACGTGTGTAGCCCAAATCATAAG
GGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTACCGGC
AGTCAACTTAGAGTGCCCAACTTAATGATGGCAACTAAGCTTAAGGGTTG
CGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAA
CCATGCACCACCTGTCACCTTTGTCCCCCGAAGGGGAAGACTCTATCTCTA
GAGCGGTCAAAGGATGTCAAGATTTGGTAAGGTTCTTCGCGTTGCTTCGA
ATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCGTCAATTCCTTTGA
GTTTCAACCTTGCGGTCGTAATCCCCAGGCGGAGTGCTTAATGCGTTAGC
TGCAGCACTAAGGGGCGGAAACCCCTAACACTTAGCACTCATCGTTTAC
GGCGTGGACTACCAGGGTATCTAATCCTGTTTGATCCCCACGCTTTCGCA
CATCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCT
CCATATCTCTGCGCATTTACCGCTACACATGGAATTCACCTTTCCTCTT
CTGCACTCAAGTTTTCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGG
GCTTTCACATCAGACTTAAAAAACCGCCTACGCGCGCTTTACGCCCAATA
ATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTA
GTTAGCCGTGGCTTTCTGATCAGGTACCGTCAAGATGTGCACAGTTACTT
ACACATTTGTTCTTCCCTGATAACAGAGTTTTACGATCCGAAAACCTTCAT
CACTCACGCGGCGTTGCTCCGTACAGGCTTTCGCCATTGCG
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Appendix: 2

Number of Bases: 1041

Phylogenetically similar species: *Staphylococcus pasteurii* strain ATCC 51129 16SrRNA

Reference: (Gory *et al.*, 1998)

>PSS1b

```
GTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGTAGCATGCT
GATCTACGATTACTAGCGATTCCAGCTTCATGTAGTCGAGTTGCAGACTA
CAATCCGAAGTGAACAACCTTTATGGGATTTGCTTGACCTCGCGGTTTA
GCTGCCCTTTGTATTGTCCATTGTAGCACGTGTGTAGCCCAAATCATAAG
GGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTACCGGC
AGTCAACTTAGAGTGCCCAACTTAATGATGGCAACTAAGCTTAAGGGTTG
CGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAA
CCATGCACCACCTGTCACCTTTGTCCCCCGAAGGGGAAGACTCTATCTCTA
GAGCGGTCAAAGGATGTCAAGATTTGGTAAGGTTCTTCGCGTTGCTTCGA
ATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCGTCAATTCCTTTGA
GTTTCAACCTTGCGGTCGTAATCCCCAGGCGGAGTGCTTAATGCGTTAGC
TGCAGCACTAAGGGGCGGAAACCCCTAACACTTAGCACTCATCGTTTAC
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GGCGTGGACTACCAGGGTATCTAATCCTGTTTGATCCCCACGCTTTCGCA
CATCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCCT
CCATATCTCTGCGCATTTACCGCTACACATGGAATTCCACTTTCCTCTT
CTGCACTCAAGTTTTCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGG
GCTTTCACATCAGACTTAAAAAACCGCCTACGCGCGCTTTACGCCCAATA
ATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTA
GTTAGCCGTGGCTTTCTGATCAGGTACCGTCAAGATGTGCACAGTTACTT
ACACATTTGTTCTTCCTGATAACAGAGTTTTACGATCCGAAGACTTCATC
ACTCACGCGGCGTTGCTCCGTCAGCTTTCGCCATTGCGGAA

Appendix: 3

Number of Bases: 955

Phylogenetically similar species: *Staphylococcus warneri* strain DK131 16S rRNA

Reference: (Dehimi, 2020).

>PSS2a

CACCGTAGCATGCTGATCTACGATTACTAGCGATTCCAGCTTCATGTAGT
CGAGTTGCAGACTACAATCCGAACCTGAGAACAACCTTTATGGGATTTGCTT
GACCTCGCGGTTTAGCTGCCCTTTGTATTGTCCATTGTAGCACGTGTGTA
GCCCAAATCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCC
GGTTTGTACCCGGCAGTCAACTTAGAGTGCCCAACTTAATGATGGCAACT
AAGCTTAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACA
CGAGCTGACGACAACCATGCACCACCTGTCACTTTGTCCCCCGAAGGGGA
AGACTCTATCTCTAGAGCGGTCAAAGGATGTCAAGATTTGGTAAGGTTCT
TCGCGTTGCTTCGAATTAACACCATGCTCCACCGCTTGTGCGGGTCCCC
GTCAATTCTTTGAGTTTCAACCTTGCGGTCTGACTCCCCAGGCGGAGTG
CTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAG
CACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGATC
CCCACGCTTTCGCACATCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCG
CCACTGGTGTTCTCCATATCTCTGCGCATTTACCGCTACACATGGAAT
TCCACTTTCCTCTTCTGCACTCAAGTTTTCCAGTTTCCAATGACCCTCCA
CGGTTGAGCCGTGGGCTTTCACATCAGACTTAAAAAACCGCCTACGCGCG
CTTTACGCCCAATAATTCGGGATAACGCTTGCCACCTACGTATTACCGCG
GCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGATCAGGTACCGTCAAGAT
GTGCACAGTTACTTACACATTTGTTCTTCCTGATAACAGAGTTTTACGAT
CCGAA

Appendix: 4

Number of Bases: 704

Phylogenetically similar species: *Corynebacterium aurimucosum* strain H2456 16S rRNA

Reference: (Nouioui *et al.*, 2018).

>PSS2b

ACTTCATGGGGTCGAGTTGCAGACCCCAATCCGAACCTAAGGCCGGCTTTC
AGCGATTGCTCCACCTCACAGTGTGCTGCGCGTTGTACCGACCATTGT
AGCATGTGTGAAGCCCTGGACATAAGGGGCATGATGATTTGACGTCATCC
CCACCTTCCTCCGAGTTAACCCCGGCAGTCTCTCATGAGTCCCCAACCAA
ATGCTGGCAACATAAGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAA

CATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTACACCAACC
ACAAGGGACACTACATCTCTGCAGCAATCTGGTGTATGTCAAGCCCAGGT
AAGTTCTTCGCGTTGCATCGAATTAATCCACATGCTCCGCCGCTTGTGC
GGGCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTAATCCCCAG
GCGGGGCGCTTAATGCGTTAGCTACGGCACAGAAGACGTGGAAGCCCCCT
ACACCTAGCGCCACCGTTTACGGCATGGACTACCAGGGTATCTAATCCT
GTTTGCTACCCATGCTTTCGCTCCTCAACGTCAGTAACTGCCCAAAGACC
TGCCTTCGCCATCGGTGTTCTCCTGATATCTGCGCATTTACCGCTACA
CCAGGAATTCCAGTCTCCCCTACAGCACTCAAGTTATGCCCGTATCACCT
GCAC

Appendix: 5

Number of Bases: 933

Phylogenetically similar species: *Staphylococcus epidermidis* strain 21DSE01 16S rRNA

Reference: (Cole, *et al.*, 2019).

>PSS3a

ACCGTAGCATGCTGATCTACGATTACTAGCGATTCCAGCTTCATATAGTC
GAGTTGCAGACTACAATCCGAAGTGAACAACCTTTATGGGATTTGCTTG
ACCTCGCGGTTTCGCTGCCCTTTGTATTGTCCATTGTAGCACGTGTGTAG
CCCAAATCATAAGGGGCGATGATGATTTGACGTCATCCCCACCTTCCTCCG
GTTTGTCACCGGCAGTCAACTTAGAGTGCCCAACTTAATGATGGCAACTA
AGCTTAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACAC
GAGCTGACGACAACCATGCACCACCTGTCACCTCTGTCCCCCGAAGGGGAA
AACTCTATCTCTAGAGGGGTCAGAGGATGTCAAGATTTGGTAAGGTTCTT
CGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCG
TCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGC
TTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCTAACACTTAGC
ACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGATCC
CCACGCTTTCGCACATCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGC
CACTGGTGTTCTCCATATCTCTGCGCATTTACCGCTACACATGGAATT
CCACTTTCCTCTTCTGCACTCAAGTTTTCCAGTTTCCAATGACCCTCCAC
GGTTGAGCCGTGGGCTTTCACATCAGACTTAAAAAACCGCCTACGCGCGC
TTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGG
CTGCTGGCACGTAGTTAGCCGTGGCTTTCTGATTAGGTACCGTCAAGACG
TGCATAGTTACTTACACATTTGTTCTTCCCTAA

Appendix: 6

Number of Bases: 1006

Phylogenetically similar species: *Staphylococcus epidermidis* strain WHW31 16S rRNA

Reference: (Abdelall *et al.*, 2021).

>PSS3b

ACCGTAGCATGCTGATCTACGATTACTAGCGATTCCAGCTTCATATAGTC
GAGTTGCAGACTACAATCCGAAGTGAACAACCTTTATGGGATTTGCTTG
ACCTCGCGGTTTCGCTACCCCTTTGTATTGTCCATTGTAGCACGTGTGTAG
CCCAAATCATAAGGGGCGATGATGATTTGACGTCATCCCCACCTTCCTCCG
GTTTGTCACCGGCAGTCAACTTAGAGTGCCCAACTTAATGATGGCAACTA
AGCTTAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACAC

GAGCTGACGACAACCATGCACCACCTGTCACTCTGTCCCCGAAGGGGAA
AACTCTATCTCTAGAGGGGTCAGAGGATGTCAAGATTTGGTAAGGTTCTT
CGCGTTGCTTCGAATTAACCACATGCTCCACCGCTTGTGCGGGTCCCCG
TCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGC
TTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCTAACACTTAGC
ACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGATCC
CCACGCTTTTCGCACATCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGC
CACTGGTGTTCCTCCATATCTCTGCGCATTTACCGCTACACATGGAATT
CCACTTTCTCTTCTGCACTCAAGTTTTCCAGTTTCCAATGACCCTCCAC
GGTTGAGCCGTGGGCTTTCACATCAGACTTAAAAAACCGCCTACGCGCGC
TTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGG
CTGCTGGCACGTAGTTAGCCGTGGCTTTCTGATTAGGTACCGTCAAGACG
TGCATAGTTACTTACACATTTGTTCTTCCCTAATAACAGAGTTTTACGAT
CCGAAGACTTCATCACTCACGCGGCGTTGCTCCGTCAGGCTTCGCCATT
GCGGAA

Appendix: 7

Number of Bases: 871

Phylogenetically similar species: *Peribacillus frigoritolerans* strain DSM 8801 16S rRNA

Reference: (Beesley *et al.*, 2010)

>PSS4a

CCCTTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGC
ATGATGATTTGACCTCATCCCCACCTTCCTCCGGTTTGTACCGGCAGTC
ACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCT
CGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCAT
GCACCACCTGTCACTCTGTCCCCGAAGGGGAAAGCCCTATCTCTAGGGT
TGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTA
AACCACATGCTCCACCGCTTGTGCGGGCCCCCGTTAATTCCTTTGAGTTT
CAGCCTTGCGGCCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCA
GACTAAAGGGCGGAAACCCCTCTAACACTTAGCACTCATCGTTTACGGCG
TGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTC
AGTGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCTCCAA
ATCTCTACGCATTTACCGCTACACTTGGAATTCACCTTTCCTCTTCTGC
ACTCAAGTTCCCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTT
TCACATCAGACTTAAGGAACCACTGCGCGCGCTTACGCCCAATAATTC
CGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTA
GCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTACCAGCAGTTACTCTGGT
ACTTGTCTTCCCTAACAACAGAACTTTACAACCCGAAGGCCTTCTTCGT
TCACGCGGCGTTGCTCCGTC

Appendix: 8

Number of Bases: 1082

Phylogenetically similar species: *Staphylococcus haemolyticus* strain PL565 16S rRNA

Reference: (Jasim, 2020).

>PSS4b

CCACCGGCTTCGGGTGTTACAACTCTCGTGGTGTGACGGGCGGTGTGTA
CAAGACCCGGGAACGTATTCACCGTAGCATGCTGATCTACGATTACTAGC

GATTCCAGCTTCATGTAGTCGAGTTGCAGACTACAATCCGAACTGAGAAC
AACTTTATGGGATTTGCTTGACCTCGCGGTTTCGCTGCCCTTTGTATTGT
CCATTGTAGCACGTGTGTAGCCCAAATCATAAGGGGCATGATGATTTGAC
GTCATCCCCACCTTCCTCCGGTTTGTACCCGGCAGTCAACTTAGAGTGCC
CAACTTAATGATGGCAACTAAGCTTAAGGGTTGCGCTCGTTGCGGGACTT
AACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCA
CTTTGTCCCCCGAAGGGGAAGGCTCTATCTCTAGAGTTGTCAAAGGATGT
CAAGATTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCC
ACCGCTTGTCGGGTCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTC
GTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCG
GAAACCCCTAACACTTAGCACTCATCGTTTACGGCGTGGAATACCAGGG
TATCTAATCCTGTTTGATCCCCACGCTTTCGCACATCAGCGTCAGTTACA
GACCAGAAAGTCGCCTTCGCCACTGGTGTTCTCCATATCTCTGCGCATT
TCACCGCTACACATGGAATTCACCTTTCCTCTTCTGCACTCAAGTTTTC
AGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTT
AAAAAACCGCCTACGCGCGCTTACGCCCAATAATTCCGGATAACGCTTG
CCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTCT
GATTAGGTACCGTCAAGACGTGCATAGTTACTTACACGTATGTTCTTCCC
TAATAACAGAGTTTACGATCCGAAGACTTCATCACTCACGCGGCGTTGC
TCCGTCAGGCTTTCGCCATTGCGGAAGATTCC

Appendix: 9

Query length: 1042

Phylogenetically similar species: *Lysinibacillus telephonicus* strain S5H2222 16S rRNA

Reference: (Rahi *et al.*, 2017).

>PSS5a

GACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGA
TCCGCGATTACTAGCGATTCCGCTTCATGTAGGCGAGTTGCAGCCTACAA
TCCGAACTGAGAACGGTTTTATCGGATTGGCTCCCCCTCGCGGGTTGGCA
ACCGTTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGG
CATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGT
CAGATTAGAGTGCCCAACTGAATGATGGCAACTAATCTCAAGGGTTGCGC
TCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCA
TGCACCACCTGTACCGCTGTCCCCGAAGGGAAGGCTATGTCTCCATAGC
GGTCAGCGGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATT
AAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTT
TCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGC
AGCACTAAGGGGCGGAAACCCCTAACACTTAGCACTCATCGTTTACGGC
GTGGACTACCAGGGTATCTAATCCTGTTTGTCTCCACGCTTTCGCGCCT
CAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCTCCA
AATCTCTACGCATTTACCGCTACACTTGGAATTCACCTTTCCTCTTCTG
CACTCAAGTCCTCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCT
TTCACATCAGACTTAAAGGACCGCCTGCGCGCGCTTACGCCCAATAATT
CCGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTT
AGCCGTGGCTTTCTAATAAGGTACCGTCAAGGTACAGCCAGTTACTACTG
TACTTGTTCTTCCCTTACAACAGAGTTTTACGATCCGAAAACCTTCTTCA
CTCACGCGGCGTTGCTCCATCAGGCTTTCGCCCATTGTGGAA

Appendix: 10

Query length:

Phylogenetically similar species: *Kocuria rosea* strain DSM 20447 16S rRNA

Reference: (Stackebrandt *et al.*, 1995).

>PSS6a

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GACTTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTACCGCAGCGT
TGCTGATCTGCGATTACTAGCGACTCCGACTTCATGAGGTTCGAGTTGCAG
ACCTCAATCCGAACCTGAGACCGGCTTTTTGGGATTAGCTCCACCTCACAG
TATCGCAACCCTTTGTACCGGCCATTGTAGCATGCGTGAAGCCCAAGACA
TAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGAGTTGACCC
CGGCAGTCTCCTATGAGTCCCCACCATCACGTGCTGGCAACATAGAACGA
GGGTTCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGA
CGACAACCATGCACCACCTGTCCACCAGCCCCGAAGGGAAACCCCATCTC
TGGGGCGGTCCGGTGAATGTCAAGCCTTGTAAGGTTCTTCGCGTTGCAT
CGAATTAATCCGCATGCTCCGCCGCTTGTGCGGGCCCCCGTCAATTCCTT
TGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGCACTTAATGCGTT
AGCTACGGCGCGGAAAACGTGGAATGTCCCCCACACCTAGTGCCCAACGT
TTACGGCATGGACTACCAGGGTATCTAATCCTGTTTCGCTCCCCATGCTTT
CGCTCCTCAGCGTCAGTAACAGCCCAGAGACCTGCCTTCGCCATCGGTGT
TCCTCCTGATATCTGCGCATTTACCGCTACACCAGGAATTCCAGTCTCC
CCTACTGCACTCTAGTCTGCCCGTACCCACTGCAGACCCGGGGTTGAGCT
CCGGGCTTTACAGCAGACGCGACAAACCGCCTACGAGCTCTTTACGCCC
AATAATTCCGGACAACGCTTGCGCCCTACGTATTACCGCGGCTGCTGGCA
CGTAGTTAGCCGGCGCTTCTTCTGCAGGTACCGTCACTCTCGCTTCTTCC
CTACTGAAAGAGGTTTACAACCCGAAGGCCGTCATCCCTCACGCGGCGTC
GCTGCATCAGGCTTCCGCCCATTTGTGCATATTCCCACTGCTGCCTCCCGT
AGGA
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Appendix: 11

Query length: 935

Phylogenetically similar species: *Paenibacillus illinoisensis* strain NBRC 15959 16S rRNA

Reference: (Nakagawa *et al.*, 2011).

>PSS7a

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ACCGGCTTTTTAGGATTCGTTCCACCTCGCGGCTTCACAGCCCGTTGTAC
CGGCCATTGTAGTACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTT
GACGTCATCCCCACCTTCCTCCGGTTTGTACCGGCAGTCACCTTAGAGT
GCCCCACCGGAAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGG
ACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCT
GTCTCCTCTGTCCCGAAGGAAAGCCCTATCTCTAGGACGGTCAGAGGGAT
GTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATACT
CCACTGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGA
CCGTACTCCCCAGGCGGAATGCTTAATGTGTAACTTCGGCACCAAGGGT
ATCGAAACCCCTAACACCTAGCATTCATCGTTTACGGCGTGGACTACCAG
GGTATCTAATCCTGTTTGTCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTA
CAGCCCAGAGAGTCGCTTCGCCACTGGTGTTCCTCCACATCTCTACGCA
TTTACCGCTACACGTGGAATTCACCTCTCCTCTTCTGCACTCAAGTCAT
CCAGTTTCCAGTGCGATCCGGGGTTGAGCCCCGGGATTAAACACCAGACT
TAAATGACCGCCTGCGCGCGCTTTACGCCCAATAATCCGGACAACGCTT
GCCCCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTTC
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TTCTCAGGTACCGTCACTCCTTGAGCAGTTACTCTCAAGGACGTTCTTCC
CTGGCAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGCATT
GCTCCGTCAGGCTTTGCGCCATTGCGGAAAATTCC

Appendix: 12

Query length: 1054

Phylogenetically similar species: *Kocuria polaris* strain CMS 76or 16S rRNA

Reference: (Reddy *et al.*, 2003).

>PSS8a

ACCGTAGCATGCTGATCTACGATTACTAGCGATTCCAGCTTCATATAGTC
GAGTTGCAGACTACAATCCGAAGTGAACAACCTTTATGGGATTGCTTG
ACCTCGCGGTTTCGCTGCCCTTTGTATTGTCCATTGTAGCACGTGTGTAG
CCCAAATCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCG
GTTTGTACCCGGCAGTCAACTTAGAGTGCCCAACTTAATGATGGCAACTA
AGCTTAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACAC
GAGCTGACGACAACCATGCACCACCTGTCACTCTGTCCCCCGAAGGGGAA
AACTCTATCTCTAGAGGGGTCAGAGGATGTCAAGATTTGGTAAGGTTCTT
CGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCG
TCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGC
TTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCTAACACTTAGC
ACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGATCC
CCACGCTTTGCGACATCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGC
CACTGGTGTTCCTCCATATCTCTGCGCATTTACACGCTACACATGGAATT
CCACTTTCCTCTTCTGCACTCAAGTTTCCAGTTTCCAATGACCCTCCAC
GGTTGAGCCGTGGGCTTTACATCAGACTTAAAAAACCGCCTACGCGCGC
TTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACGCGG
CTGCTGGCACGTAGTTAGCCGTGGCTTTCTGATTAGGTACCGTCAAGACG
TGCATAGTTACTTACACATTTGTTCTTCCTAATAACAGAGTTTTACGATC
GAA

Appendix: 13

Query length: 953

Phylogenetically similar species: *Staphylococcus epidermidis* strain Fussel 16S rRNA

Reference: (Takahashi *et al.*, 2010).

>PSS9a

ATTCCAGCTTCATATAGTCGAGTTGCAGACTACAATCCGAAGTGAACA
ACTTTATGGGATTTGCTTGACCTCGCGGTTTCGCTGCCCTTTGTATTGTC
CATTGTAGCACGTGTGTAGCCCAAATCATAAGGGGCATGATGATTTGACG
TCATCCCCACCTTCCTCCGTTTGTACCCGGCAGTCAACTTAGAGTGCCC
AACTTAATGATGGCAACTAAGCTTAAGGGTTGCGCTCGTTGCGGGACTTA
ACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCAC
TCTGTCCCCCGAAGGGGAAAACCTCTATCTCTAGAGGGGTCAGAGGATGTC
AAGATTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCA
CCGCTTGTGCGGGTCCCCGTCAATTCTTTGAGTTTCAACCTTGCGGTCG
TACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGG
AAACCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGT

ATCTAATCCTGTTTGATCCCCACGCTTTCGCACATCAGCGTCAGTTACAG
ACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTGCGCATTT
CACCGCTACACATGGAATTCCACTTTCCTCTTCTGCACTCAAGTTTCCA
GTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTA
AAAAACCGCCTACGCGCGCTTTACGCCCAATAATTCCGGAAAACGCTTGC
CACCTACGTATTACCGCGGCTGCTGGCAGCTAATTAGCCCTGGCTTTCTG
ATTAGGTACCGTCAAGACCTGCATAGTTACTTACCCATTTGTTCTTCCC

Appendix: 14

Query length: 899

Phylogenetically similar species: *Staphylococcus caprae* strain ATCC 35538

Reference: (Takahashi *et al.*, 1999).

>PSS9b
ATTCCAGCTTCATATAGTCGAGTTGCAGACTACAATCCGAAGTGAACA
ACTTTATGGGATTTGCTTGACCTCGCGTTTCGCTGCCCTTTGTATTGTC
CATTGTAGCACGTGTGTAGCCCAAATCATAAGGGGCATGATGATTTGACG
TCATCCCCACCTTCCTCCGGTTTGTACCGGCAGTCAACTTAGAGTGCCC
AACTTAATGATGGCAACTAAGCTTAAGGGTTGCGCTCGTTGCGGGACTTA
ACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCAC
TCTGTCCCCCGAAGGGGAAAACCTCTATCTCTAGAGGGGTCAGAGGATGTC
AAGATTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAACCACATGCTCCA
CCGCTTGTCGGGTCCCCGTCAATTCTTTGAGTTTCAACCTTGCGGTCG
TACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGG
AAACCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGT
ATCTAATCCTGTTTGATCCCCACGCTTTCGCACATCAGCGTCAGTTACAG
ACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTGCGCATTT
CACCGCTACACATGGAATTCCACTTTCCTCTTCTGCACTCAAGTTTCCA
GTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTA
AAAAACCGCCTACGCGCGCTTTACGCCCAATAATTCCGGAAAACGCTTGC
CACCTACGTATTACCGCGGCTGCTGGCAGCTAATTAGCCCTGGCTTTCTG
ATTAGGTACCGTCAAGACCTGCATAGTTACTTACCCATTTGTTCTTCCC

Appendix: 15

Query length: 988

Phylogenetically similar species: *Staphylococcus epidermidis* strain 32FSE06.

Reference: (Pitton, 2023).

>PSS10a
ACCGTAGCATGCTGATCTACGATTACTAGCGATTCCAGCTTCATATAGTC
GAGTTGCAGACTACAATCCGAAGTGAACAACCTTTATGGGATTTGCTTG
ACCTCGCGGTTTCGCTGCCCTTTGTATTGTCCATTGTAGCACGTGTGTAG
CCCAAATCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCG
GTTTGTACCGGCAGTCAACTTAGAGTGCCCAACTTAATGATGGCAACTA
AGCTTAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACAC
GAGCTGACGACAACCATGCACCACCTGTCACTCTGTCCCCCGAAGGGGAA
AACTCTATCTCTAGAGGGGTCAGAGGATGTCAAGATTTGGTAAGGTTCTT
CGCGTTGCTTCGAATTAACCACATGCTCCACCGCTTGTGCGGGTCCCCG
TCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGC
TTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCTAACACTTAGC

ACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGATCC
CCACGCTTTTCGCACATCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGC
CACTGGTGTTCCTCCATATCTCTGCGCATTTCACCGCTACACATGGAATT
CCACTTTCTCTTCTGCACTCAAGTTTTCCAGTTTCCAATGACCCTCCAC
GGTTGAGCCGTGGGCTTTACATCAGACTTAAAAAACCGCCTACGCGCGC
TTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGG
CTGCTGGCACGTAGTTAGCCGTGGCTTTCTGATTAGGTACCGTCAAGACG
TGCATAGTTACTTACACATTTGTTCTTCCCTAATAACAGAGTTTTACGAT
CCAAGACCTTCATCACTCACGCGGCGTTGCTCCGTCAG

Appendix: 16

Query length: 970

Phylogenetically similar species: *Bhargavaea ginsengi* strain *ge14* 16S rRNA

Reference: (Verma *et al.*, 2009).

>PSS11a

ACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGC
GAGTTGCAGCCTGCAATCCGAACGGTTTTCTGGGATTGGCTCC
CCCTCGCGGGTTCGCAGCCCTCTGTACCGTCCATTGTAGCACGTGTGTAG
CCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCG
GTTTGTACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTA
AGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACAC
GAGCTGACGACAACCATGCACCACCTGTCACTCTGTCCCCCGAAGGGGAA
GGCCCTGTCTCCAGGGTGGTCAGAGGATGTCAAGACCTGGTAAGGTTCTT
CGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCG
TCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAGTGC
TTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCTAACACTTAGC
ACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCC
CCACGCTTTTCGCGCCTCAGCGTCAGTTACAGACCAGAAAGCCGCCTTCGC
CACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATT
CCGCTTTCTCTTCTGCACTCAAGTCCCCCAGTTTCCAATGACCCTCCAC
GGTTGAGCCGTGGGCTTTACATCAGACTTAAAGGACCGCCTGCGCGCGC
TTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGG
CTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTAAGGTACCGTCAGGGCA
CCGGCAGTTAACCGATGCTTGTCTTCCCTTACAACAAAGCTTTACGACC
CGAAGGCCTTCTTCCCTCAC

Appendix: 17

Query length: 932

Phylogenetically similar species: *Bacillus haynesii* strain *NRRL B-41327* 16S rRNA

Reference: (Dunlap *et al.*, 2017).

>PSS11b

GTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCT
GATCCGCGATTACAGCGATTCCGCTTCACGCAGTCGAGTTGCAGACTGCG
ATCCGAACCTGAGAACAGATTTGTGGGATTGGCTTAGCCTCGCGGCTTCGC
TGCCCTTTGTTCTGCCATTGTAGCACGTGTGTAGCCAGGTCATAAGGG
GCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTACCGGCAG
TCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCG

CTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACC
ATGCACCACCTGTCACTCTGCCCCGAAGGGGAAGCCCTATCTCTAGGGT
TGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTA
AACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTT
CAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTTGCTGCA
GACTAAAGGGCGGAAACCTCTAACACTTAGCACTCATCGTTTACGGCG
TGGACTACCAAGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCGCCTC
AGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCTCCAC
ATCTCTACGCATTTACCGCTACACGTGGAATTCCACTCTCCTCTTCTGC
ACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTT
TCACATCAAACCTTAATAAACCGCCTGCGCGCGCTTTACGCCCAATAATTC
CGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTATTTA
GCCGTGGCTTCTGGTTAGGTACCGTCAAGGT

Appendix: 18

Query length: 1057

Phylogenetically similar species: *Bacillus sonorensis* NBRC101234 16S rRNA

Reference: (Nakagawa *et al.*, 2014).

>PSS12a

ACCTCACCGACTCGGGTGTACAAACTCTCGTGGTGTGACGGGCGGTGTG
TACAAGGCCCGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACCA
GCGATTCCCGCTTACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGA
ACAGATTTGTGGGATTGGCTTAGCCTCGCGGCTTCGCTGCCCTTTGTTCT
GCCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTG
ACGTCATCCCCACCTTCCTCCGTTTTGTCACCGGCAGTCACCTTAGAGTG
CCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGAC
TTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGT
CACTCTGCCCCCGAAGGGGAAGCCCTATCTCTAGGGTTGTCAGAGGATGT
CAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCC
ACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACC
GTACTCCCCAGGCGGAGTGCTTAATGCGTTTGCTGCAGCACTAAAGGGCG
GAAACCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGG
TATCTAATCCTGTTCGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTACA
GACCAGAGAGTCGCCTTCGCCACTGGTGTTCTCCACATCTCTACGCATT
TCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCC
AGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTT
AAGAAACCGCCGCGCGCGCTTACGCCCAATAATTCCGGACAACGCTTG
CCACCTACGTATTACCGCGGCTGCTGGCACGTATTTAGCCGTGGCTTTCT
GGTTAGGTACCGTCAAGGTACCGCCCTATTCGAACGGTACTTGTTCTTCC
CTAACAACAGAGTTTACGATCCGAAAACCTTCATCACTCAGGCGGCGTTG
CTCCGTC

Appendix: 19

Query length: 1059

Phylogenetically similar species: *Bacillus licheniformis* strain DSM 13 16S rRNA

Reference: (Ludwig *et al.*, 1992).

>PSS13a

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ACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGT
GTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACT
AGCGATTCCGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAAGTGA
ACAGATTTGTGGGATTGGCTTAGCCTCGCGGCTTCGCTGCCCTTTGTTCT
GCCCATTGTAGCACGTGTGTAGCCAGGTCATAAGGGGCATGATGATTTG
ACGTCATCCCCACCTTCCTCCGTTTGTACCGGCAGTCACCTTAGAGTG
CCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGAC
TTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGT
CACTCTGCCCCCGAAGGGGAAGCCCTATCTCTAGGGTTGTCAGAGGATGT
CAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCC
ACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACC
GTACTCCCCAGGCGGAGTGCTTAATGCGTTTGTGTCAGCACTAAAGGGCG
GAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGG
TATCTAATCCTGTTTCGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTACA
GACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATT
TCACCGCTACACGTGGAATTCACCTCTCCTCTTCTGCACTCAAGTCCCC
AGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCACACTT
AAGAAACCGCCGGCGCGCGCTTACGCCCAATAATTCCGGACAACGCTGG
CCACCTACGTATTACCGCGGCTGCTGGCACGTATTTAGCCCTGGCTTTCT
GGTTAGGTACCGTCAAGGTACCGCCCTATTCGAACGGTACTTGTCTTCC
CTAACAACAGAGTTTTACGATCCGAAAACCTTCATCACTCAGGCGGCGTT
GCTCCGTCA
```

Appendix: 20

Query length: 1065

Phylogenetically similar species: *Pseudomonas oryzae* strain IAM 1568 16S rRNA

Reference: (Tvrtkova *et al.*, 2006).

>PSS14a

```
GACTAGCTACTTCTGGAGCAACCCACTCCCATGGTGTGACGGGCGGTGTG
TACAAGGCCCGGGAACGTATTCACCGTGACGTTCTGATTCACGATTACTA
GCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCGGACTACGA
TCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTACC
GACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTG
ACGTCATCCCCACCTTCCTCCGTTTGTACCGGCAGTCTCCTTAGAGTG
CCCACCATAACGTGCTGGTAACCTAAGGACAAGGGTTGCGCTCGTTACGGG
ACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCT
GTGTCTGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCAGCAT
GTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCT
CCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTAAACCTTGCGG
CCGTAATCCCCAGGCGGTCAACTTAATGCGTTAGCTGCGCCACTAAGATC
TCAAGGATCCCAACGGCTAGTTGACATCGTTTACGGCGTGGACTACCAGG
GTATCTAATCCTGTTTGTCTCCCCACGCTTTCGACCTCAGTGTCAGTGTC
```

AGTCCAGGTAGTCGCCTTCGCCACTGGTGTTCTTCCAATATCTACGCAT
TTCACCGCTACACTGGAAATTCCTACTACCCTCTACCGCATTCTAGCCAGA
CAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGATTTACATCCAACCT
TATCAAGCCACCTACGCGCGCTTTACGCCCAGTAATCCAATAAACGCTT
GCACCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTAT
TCTGTGCGTAACGTCAAACTCACAGGTATTCGCTGTAAGCCCTTCCTCC
AACTTAAAGTGCTTTACAACCCGAGGGCCTTCTTCACACACGCGGCAGGG
CTGGATCAGGCTTTC

Appendix: 21

Query length: 1083

Phylogenetically similar species: *Corynebacterium aurimucosum* strain IMMIB D-1488 16S

rRNA

Reference: (Yassin *et al.*, 2002).

>PSS15a

GTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCATTGC
TGATCTGCGATTACTAGCGACTCCCACTTCATGGGGTCGAGTTGCAGACC
CCAATCCGAACCTAAGGCCGGCTTTCAGCGATTGCTCCACCTCACAGTGT
CGCTGCGCGTTGTACCGACCAATTGTAGCATGTGTGAAGCCCTGGACATAA
GGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGAGTTAACCCCGG
CAGTCTCTCATGAGTCCCCAACCAATGCTGGCAACATAAGACAAGGGTT
GCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACA
ACCATGCACCACCTGTACACCAACCACAAGGGACACTACATCTCTGCAGC
AATCTGGTGTATGTCAAGCCCAGGTAAGGTTCTTCGCGTTGCATCGAATT
AATCCACATGCTCCGCCGCTTGTGCGGGCCCCCGTCAATTCCCTTTGAGTT
TTAGCCTTGCGGCCGTACTCCCCAGGCGGGGCGCTTAATGCGTTAGCTAC
GGCACAGAAGACGTGGAAGCCCCCTACACCTAGCGCCCACCGTTTACGGG
ATGGACTACCAGGGTATCTAATCCTGTTTGCTACCCATGCTTTCGCTCCT
CAGCGTCAGTAAGTGGCCAGAGACCTGCCTTCGCCATCGGTGTTCTCCT
GATATCTGCGCATTTACCGCTACACCAGGAATTCCAGTCTCCCCTACAG
CACTCAAGTTATGCCCCGTATCGCCTGCACGCCCGAAGTTAAGCCCCGGAA
TTTCACAGACGACGCGACAAACCACCTACGAGCTCTTTACGCCCAGTAAT
TCCGGACAACGCTCGCACCCCTACGTATTACCGCGGCTGCTGGCACGTAGT
TAGCCGGTGCTTCTTATACAGGTACCGTCACTTGCGCTTCGTCCCTGTCG
AAAGGAGTTTACAACCCGAAGGCCGTATCCCCACGCGGCGTCGCTGCA
TCAGGCTTCCGCCCATTTGTGCAATATCCCACTGCTGCCTCCCGTAGGAA
TCTGGGCCGTATCTCAGTCCCAATGTGGCCGTA

Appendix: 22

Query length: 995

Phylogenetically similar species: *Staphylococcus epidermidis* strain RG4 16SrRNA

Reference: (Dogruoz, 2019).

>PSS15b

GTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGTAGCATGCT
GATCTACGATTACTAGCGATTCCAGCTTCATATAGTCGAGTTGCAGACTA
CAATCCGAACCTGAGAACAACTTTATGGGATTTGCTTGACCTCGCGGTTTC
GCTGCCCTTTGTATTGTCCATTGTAGCACGTGTGTAGCCCAAATCATAAG

GGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTACCCGGC
AGTCAACTTAGAGTGCCCAACTTAATGATGGCAACTAAGCTTAAGGGTTG
CGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAA
CCATGCACCACCTGTCACCTCTGTCCCCCGAAGGGGAAAACTCTATCTCTA
GAGGGGTCAGAGGATGTCAAGATTTGGTAAGGTTCTTCGCGTTGCTTCGA
ATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGA
GTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGC
TGCAGCACTAAGGGGCGGAAACCCCTAACACTTAGCACTCATCGTTTAC
GGCGTGGACTACCAGGGTATCTAATCCTGTTTGATCCCCACGCTTTCGCA
CATCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCT
CCATATCTCTGCGCATTTACCGCTACACATGGAATTCCACTTTCCTCTT
CTGCACTCAAGTTTTCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGG
GCTTTCACATCAGACTTAAAAAACCGCCTACGCGCGCTTTACGCCCAATA
ATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTA
GTTAGCCGTGGCTTTCTGATTAGGTACCGTCAAGACGTGCATAGTTACTT
ACCCATTTGTTCTTCCCTAATAACAGAGTTTTACGATCCGAAAAC

Appendix: 23

Query length: 855

Phylogenetically similar species: *Bacillus subtilis* strain P3 16SrRNA

Reference: (Ash *et al.*, 1991).

>PSS16a

ACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTC
GAGTTGCAGACTGCGATCCGAAGTGAACAGATTTGTGGGATTGGCTTA
GCCTCGCGGCTTCGCTGCCCTTTGTTCTGCCCATTGTAGCACGTGTGTAG
CCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCG
GTTTGTACCCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTA
AGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACAC
GAGCTGACGACAACCATGCACCACCTGTCACCTCTGCCCCCGAAGGGGAAG
CCCTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCG
CGTTGCTTCGAATTAACACATGCTCCACCGCTTGTGCGGGCCCCCGTC
AATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTT
AATGCGTTTGCTGCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCAC
TCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTGCTCCCC
ACGCTTTCGCGCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCA
CTGGTGTTTCTCCACATCTCTACGCATTTACCGCTACACGTGGAATTCC
ACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGG
TTGAGCCGGGGGCTTTCACATCAAAATTAAAAAACCGCCTGCGCCCCCT
TTACGCCCAATAATTCGGACAACGCTTGCCACCTACGTATTACCCCGGCT
GCTGG

Appendix: 24

Query length: 1098

Phylogenetically similar species: *Bacillus piscis* strain 16MFT21 16S rRNA

Reference: (Lee *et al.*, 2016).

>PSS17a

AGAACCTCACCGACTTCGGGTGTTACAACTCTCGTGGTGTGACGGGCGG

TGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATT
ACTAGCGATTCCGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTG
AGAACAGATTTGTGGGATTGGCTTAGCCTCGCGGCTTCGCTGCCCTTTGT
TCTGCCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGAT
TTGACGTCATCCCCACCTTCCTCCGGTTTGTACCGGCAGTCACCTTAGA
GTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGG
GACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACC
TGTCACCTCTGCCCCCGAAGGGGAAGCCCTATCTCTAGGGTTGTCAGAGGA
TGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGC
TCCACCGCTTGTGCGGGCCCCCGTCAATTCTTTGAGTTTCAGTCTTGCG
ACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTTGCTGCAGCACTAAAGG
GCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTIONACCA
GGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTT
ACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCTCCACATCTCTACGC
ATTTACCGCTACACGTGGAATTCACCTCTCTCTTCTGCACTCAAGTTC
CCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGA
CTTAAGAAACCGCCGGCGCGCGCTTACGCCCAATAATTCCGGACAACGC
TTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTT
TCTGGTTAGGTACCGTCAAGGTACCGCCCTATTGCAACGGTACTTGTCT
TCCCTAACAAACAGAGTTTTACGATCCGAAAACCTTCATCACTCAGGCGGC
GTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCTACTGCTGCT

Appendix: 25

Query: 942

Phylogenetically similar species: *Staphylococcus hominis* strain FDAARGOS_745

Reference: (Patel *et al.*, 2019).

>PSS18a

GAAGGTTTTCGGATCGTAAAATTCTGTTATTAGGGAAGAACAACGTGTAAGTAACTGTG
CACGTTCTGACGGTACCTAATCAAAAAGCCACGGCTAATTACGTGCCAGCAGCCGCGGTA
ATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTT
TTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAAGGGTCATTGGAAACTGGAAAAC
TGAGTGCAGAAGAGGAAAGTGGAATTCATGTGTAGCGGTGAAATGCGCAGAGATATGGA
GGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAAGTACGCTGATGTGCGAAAGCGT
GGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTG
TTAGGGGGTTTTCCGCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGT
ACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATG
TGGTTTAATTGCAAGCAACGCGAAGAACCCTTACCAAATCTTGACATCCTTTGACCCTTCT
AGAGATAGAAGTTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGC
TCGTGTGCTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCC
ATCATTAAGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATG
ACGTCAAATCATCATGCCCTTATGATTTGGGCTACACACGTGCTACAATGGACAATACA
AAGGGCAGCGAAACCGCGAGGTCAAGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGT
AGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACG
GT

Appendix: 26

Query: 1086

Phylogenetically similar species: *Micrococcus yunnanensis* strain YIM 65004 16Sr RNA

Reference: (Qin *et al.*, 2009).

>PSS18b

```
AATAGGCCACCGGCTTCGGGTGTTACCAACTTTCGTGACTTGACGGGCGG
TGTGTACAAGGCCCCGGAACGTATTCACCGCAGCGTTGCTGATCTGCGAT
TACTAGCGACTCCGACTTCATGGGGTCGAGTTGCAGACCCCAATCCGAAC
TGAGACCGGCTTTTTTGGGATTAGCTCCACCTCACAGTATCGCAACCCATT
GTACCGGCCATTGTAGCATGCGTGAAGCCCAAGACATAAGGGGCATGATG
ATTTGACGTCGTCCTCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCCAT
GAGTCCCCACCACTACGTGCTGGCAACATGGAACGAGGGTTGCGCTCGTT
GCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCAC
CACCTGTGAACCCGCCCAAAAGGGGAAACCGTATCTCTACGGCGATCGAG
AACATGTCAAGCCTTGGTAAGGTTCTTCGCGTTGCATCGAATTAATCCGC
ATGCTCCGCCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAGCCT
TGCGGCCGTACTCCCCAGGCGGGGCACTTAATGCGTTAGCTGCGGCGCGG
AAACCGTGGAATGGTCCCCACACCTAGTGCCCAACGTTTACGGCATGGAC
TACCAGGGTATCTAATCCTGTTGCTCCCCATGCTTTCGCTCCTCAGCGT
CAGTTACAGCCCAGAGACCTGCCTTCGCCATCGGTGTTCTCCTGATATC
TGCGCATTCCACCGCTACACCAGGAATTCCAGTCTCCCCTACTGCACTCT
AGTCTGCCCCGTACCCACCGCAGATCCGGGGTTAAGCCCCGGACTTTCACG
ACAGACGCGACAAACCGCCTACGAGCTCTTTACGCCCAATAATTCCGGAT
AACGCTCGCACCCCTACGTATTACCGCGGGCTGCTGGCACGTAGTTAGCCGG
TGCTTCTTCTGCAGGTACCGTCACTTTTCGCTTCTTCCCTACTGAAAGAGG
TTTACAACCCGAAGGCCGTCATCCCTCAAGCGGCGTCGCTGCATCAGGCT
TTCGCCCATTTGTGCAATATTCCCCTGCTGCCTCCC
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Appendix: 27

Query length:

Phylogenetically similar species: *Staphylococcus caprae* strain ATCC 35538 16S rRNA

Reference: (Takahashi *et al.*, 1999).

>PSS19a

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ACCGTAGCATGCTGATCTACGATTACTAGCGATTCCAGCTTCATATAGTC
GAGTTGCAGACTACAATCCGAAGTGAACAACCTTTATGGGATTTGCTTG
ACCTCGCGGTTTCGCTGCCCTTTGTATTGTCCATTGTAGCACGTGTGTAG
CCCAAATCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCG
GTTTGTACCCGGCAGTCAACTTAGAGTGCCCAACTTAATGATGGCAACTA
AGCTTAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACAC
GAGCTGACGACAACCATGCACCACCTGTCCTCTGTCCCCCGAAGGGGAA
AACTCTATCTCTAGAGGGGTCAGAGGATGTCAAGATTTGGTAAGGTTCTT
CGCGTTGCTTCGAATTAACACCATGCTCCACCGCTTGTGCGGGTCCCCG
TCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGC
TTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCTAACACTTAGC
ACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGATCC
CCACGCTTTCGCACATCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGC
CACTGGTGTTCCTCCATATCTCTGCGCATTTACCGCTACACATGGAATT
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CCACTTTCCTCTTCTGCACTCAAGTTTTCCAGTTTCCAATGACCCTCCAC
GGTTGAGCCGTGGGCTTTCACATCAGACTTAAAAAACCGCCTACGCGCGC
TTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGG
CTGCTGGCACGTAGTTAGCCGTGGCTTTCTGATTAGGTACCGTCAGACGT
GCATAGTTACTTACACATTTGTTCTTCCCTAATAACA

Appendix: 28

Query: 796

Phylogenetically similar species: *Staphylococcus saccharolyticus* strain JCM 1768 16S rRNA

Reference: (Sakamoto and Ohkuma, 2011).

>PSS20a

ACCGTAGCATGCTGATCTACGATTACTAGCGATTCCAGCTTCATATAGTC
GAGTTGCAGACTACAATCCGAAGTGAACAACCTTATGGGATTGCTTG
ACCTCGCGGTTTCGCTGCCCTTTGTATTGTCCATTGTAGCACGTGTGTAG
CCCAAATCATAAGGGGCGATGATGATTTGACGTCATCCCCACCTTCCTCCG
GTTTGTACACCGGCAGTCAACTTAGAGTGCCCAACTTAATGATGGCAACTA
AGCTTAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACAC
GAGCTGACGACAACCATGCACCACCTGTCACTCTGTCCCCCGAAGGGGAA
AACTCTATCTCTAGAGGGGTCAGAGGATGTCAAGATTTGGTAAGGTTCTT
CGCGTTGCTTCGAATTAACCACATGCTCCACCGCTTGTGCGGGTCCCCG
TCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGC
TTAATGCGTTAGCTGCAGCACTAAGGGGGCGGAAACCCCTAACACTTAG
CACTCATCGTTTACGGGGTGGACTACCAGGGTATCTAATCCTGTTTGATC
CCCACGCTTTCGCACATCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCG
CCACTGGTGTTCCTCCATATCTCTGCGCATTTACCGCTACACATGGAAT
TTCCACTTTCCTCTTCTGCACTCAAGTTTTCCAGTTTCCAATGACCCTCC
ACGGTTGAGCCCTGGGCTTTCATCAAACCTAAAAAACCGCCTAC

Appendix: 29

Query: 1112

Phylogenetically similar species: *Pseudomonas iranica* strain GH10 16S rRNA

Reference: (Parsia *et al.*, 2013).

>PSS21a

AAAGACAGCTACTTCTGGAGCAACCCACTCCCATGGTGTGACGGGCGGTG
TGTAACAAGGCCCGGAACGTATTCACCGTGACATTCTGATTCACGATTAC
AGCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCGGACTACG
ATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTAC
CGACCATTGTAGCACGTGTGTAGCCCAGGCCGTAAGGGCCATGATGACTT
GACGTCATCCCCACCTTCCTCCGTTTGTACACCGGCAGTCTCCTTAGAGT
GCCCACCATTACGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGG
GACTTAACCCAACATCTCACGACACGAGCTGACGACCGCCATGCAGCACC
TGTGTCAGAGCTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCTGCA
TGTC AAGGCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAACCACATGC
TCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCG
GCCGTACTCCCCAGGCGGTGCACTTAATGCGTTAGCTGCGCCACTAATAT
CTCAAGGATCCCAACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAG
GGTATCTAATCCTGTTTGTCTCCCCACGCTTTCGCACCTCACTGTCAGTAT
TAGCCCAGGTGGTCGCCTTCGCCACTGGTGTTCCTTCTATATCTACGCA
TTTACCGCTACACAGGAAATTCACCACCCTCTGCCATACTCTAGCTTG

CCAGTTTTGAAAGCAGTTCCCAGGTTGAGCCCGGGGCTTTCAC TTCCAAC
TTAACAAACCACCTACGCGCGCTTTACGCCAGTAATTCCAATAAACGCT
TGCACCCTTCGTATTACCGCGGCTGCTGGCACGAAATTAGCCGGTGCTTA
TTCTGTCGGTAACGTCAAAACAGCAGGGTATTAAC TTACTGCCCTTCCCC
CCAAC TTAAAGTGCTTTACAATCCGAAA ACTTCTTCACCCACCCGGCATG
GCTGGATCAGGCTTTCCCCATTGTCCAAAATTCCCCACTGCTGCCTCCCC
GAAGAAATCTGG

Appendix: 30

Query length: 1098

Phylogenetically similar species: *Micrococcus luteus* strain *Pso_Seed_1* 16S rRNA

Reference: (Chiellini and Pistelli, 2022).

>PSS21b

GGCCACCGGCTTCGGGTGTTACCAACTTTTCGTGACTTGACGGGCGGTGTG
TACAAGGCCCGGGAACGTATTCACCGCAGCGTTGCTGATCTGCGATTACT
AGCGACTCCGACTTCATGGGGTCGAGTTGCAGACCCCAATCCGAACTGAG
ACCGGCTTTTTGGGATTAGCTCCACCTCACAGTATCGCAACCCATTGTAC
CGGCCATTGTAGCATGCGTGAAGCCCAAGACATAAGGGGGCATGATGATTT
GACGTCGTCCTCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCCATGAGT
CCCCACCATTACGTGCTGGCAACATGGAACGAGGGTTGCGCTCGTTGCGG
GACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACC
TGTGAACCCGCCCAAAGGGGAAACCGTATCTCTACGGCGATCGAGAACA
TGTCAAGCCTTGTAAGGTTCTTCGCGTTGCATCGAATTAATCCGCATGC
TCCGCCGCTTGTGCGGGCCCCCGTCAATTCTTTGAGTTTTAGCCTTGCG
GCCGTACTCCCCAGGCGGGGCAC TTAAATGCGTTAGCTGCGGCGCGGAAAC
CGTGGAATGGTCCCCACACCTAGTGCCCAACGTTTACGGCATGGACTACC
AGGGTATCTAATCCTGTTTCGCTCCCCATGCTTTCGCTCCTCAGCGTCAGT
TACAGCCCAGAGACCTGCCTTCGCCATCGGTGTTCTCCTGATATCTGCG
CATTCCACCGCTACACCAGGAATTCCAGTCTCCCCTACTGCACTCTAGTC
TGCCCGTACCCACCGCAAATCCGGGGTTAAGCCCCGGACTTTACGACAG
ACGCGACAAACCGCCAACGAGCTCTTTACGCCCAATAATCCGGATAACG
CTCGCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCT
TCTTCTGCAGGTACCGTCACTTTCGCTTCTTCCCTACTGAAAGAGGTTTA
CAACCCGAAGGCCGTATCCCTCAAGCGGCGTCGCTTCATCAGGCTTTTCG
CCCATTTGTGCAATATTCCCCTGCTGCCTCCCGTAGGAATCTGGGCCG

Appendix: 31

Query length: 863

Phylogenetically similar species: *Staphylococcus capitis* subsp. *urealyticus* strain *MAW 843616S*
rRNA

Reference: (Takahashi and Gakuen, 1997).

>PSS22a

CACCGTAGCATGCTGATCTACGATTACTAGCGATTCCAGCTTCATATAGT
CGAGTTGCAGACTACAATCCGAACTGAGAACA ACTTTATGGGATTTGCTT
GACCTCGCGGTTTCGCTGCCCTTTGTATTGTCCATTGTAGCACGTGTGTA
GCCCAAATCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCC
GGTTTGTACCGGCAGTCAACTTAGAGTGCCCAACTTAATGATGGCAACT

AAGCTTAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACA
CGAGCTGACGACAACCATGCACCACCTGTCACTCTGTCCCCGAAGGGGA
AAACTCTATCTCTAGAGGGGTCAGAGGATGTCAAGATTTGGTAAGGTTCT
TCGCGTTGCTTCGAATTAACACATGCTCCACCGCTTGTGCGGGTCCCC
GTCAATTCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTG
CTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAG
CACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGATC
CCCACGCTTTCGCACATCAACGTCAGTTACAGACCAAAAAGTCGCCTTCG
CCACTGGTGTTCCTCCATATCTCTGCGCATTTACCGCTACACATGGAAT
TCCACTTTCCTCTTCTGCACTCAAGTTTTCCAGTTTTCCAATGACCCTCC
CACGGTTGAACCCTTGGGCTTTCACATCAGAATTAATAAACCGCCTACGC
GCGCTTTACGCCCAATAATTCCGGAAACGCTTGCCACCTACGTATTACC
GCCGCCTGCTGGC

Appendix: 32

Query length: 1081

Phylogenetically similar species: *Corynebacterium minutissimum* strain NCTC 10288 16SrRNA

Reference: (Ruimy *et al.*, 1995).

>PSS23a

ACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCATTGCTGA
TCTGCGATTACTAGCGACTCCGACTTCATGGGGTCGAGTTGCAGACCCCA
ATCCGAATAAGGCCGGCTTTCAGCGATTGCTCCACCTCACAGTGTCGC
TGCGCGTTGTACCGACCATTGTAGCATGTGTGAAGCCCTGGACATAAGGG
GCATGATGATTTGACGTCATCCCCACCTTCCTCCGAGTTAACCCCGGCAG
TCTCTCATGAGTCCCCAACCAAAATGCTGGCAACATAAGACAAGGGTTGCG
CTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACC
ATGCACCACCTGTACACCAACCACAAGGGACACTACATCTCTGCAGCAAT
CTGGTGTATGTCAAGCCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAT
CCACATGCTCCGCCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTT
GCCTTGCGGGCGTACTCCCCAGGCGGGGCGCTTAATGCGTTAGCTACGGC
ACAGAAGACGTGGAAGCCCCCTACACCTAGCGCCCACCGTTTACGGCATG
GACTACCAGGGTATCTAATCCTGTTTGCTACCCATGCTTTCGCTCCTCAG
CGTCAGTAACTGCCCAGAGACCTGCCTTCGCCATCGGTGTTCCCTCCTGAT
ATCTGCGCATTTACCGCTACACCAGGAATTCAGTCTCCCCTACAGCAC
TCAAGTTATGCCCCGATCGCCTGCACGCCCCGAAGTTAAGCCCCGGAATTT
CACAGACGACGCGACAAACCACCTACGAGCTCTTTACGCCCAGTAATTCC
GGACAACGCTCGCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAG
CCGGTGCTTCTTATACAGGTACCGTCACTTGCGCTTCGTCCCTGTCGAAA
GGAGTTTACAACCCGAAGGCCGTCATCCCCACGCGGCGTCGCTGCATCAG
GCTTCCGCCCATTTGTGCAATATTCCCACTGCTGCCTCCCGTAGAATCTGG
GCCGTATCTCAGTCCCATGTGGCGTAACCCT

Appendix: 33

Query length: 966

Phylogenetically similar species: *Bacillus paralicheniformis* strain KJ-16

Reference: (Dunlap *et al.*, 2015).

>PSS23b

ACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGAT

CCGCGATTACTAGCGATTCCGCTTCACGCAGTCGAGTTGCAGACTGCGAT
CCGAACTGAGAACAGATTTGTGGGATTGGCTTAGCCTCGCGGCTTCGCTG
CCCTTTGTTCTGCCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGC
ATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTACCCGGCAGTC
ACCTTAGAGTGCCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCT
CGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCAT
GCACCACCTGTCACTCTGCCCCGAAGGGGAAGCCCTATCTCTAGGGTTG
TCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAA
CCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCA
GTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTTGCTGCAGC
ACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTG
GACTACCAGGGTATCTAATCCTGTTGCTCCCCACGCTTTCGCGCCTCAG
CGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACAT
CTCTACGCATTTACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCAC
TCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTC
ACATCAGACTTAAGAAACCGCCTGCGCGCGCTTACGCCCAATAATTCCG
GACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAATTAGC
CGTGGCTTTCTGTTAGGTACCGTCAAGGTACCGCCCTATTCGAACGGTA
CTTGTTCTTCCTAACA

Appendix: 34

Query length: 965

Phylogenetically similar species: *Staphylococcus epidermidis* strain 8 chromosome

Reference: (Abdalabbas and Abdulwahid, 2021).

>PSS24a

GTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGTAGCATGCT
GATCTACGATTACTAGCGATTCCGCTTCATATAGTCGAGTTGCAGACTAC
AATCCGAAGTGAACAACCTTTATGGGATTTGCTTGACCTCGCGGTTTCG
CTGCCCTTTGTATTGTCCATTGTAGCACGTGTGTAGCCCAAATCATAAGG
GGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTACCCGGCA
GTCAACTTAGAGTGCCCCAACTTAATGATGGCAACTAAGCTTAAGGGTTGC
GCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAAC
CATGCACCACCTGTCACTCTGTCCCCGAAGGGGAAACTCTATCTCTAG
AGGGGTCAGAGGATGTCAAGATTTGGTAAGGTTCTTCGCGTTGCTTCGAA
TTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAG
TTTCAACCTTGCGGTGCTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCT
GCAGCACTAAGGGGCGGAAACCCCTAACACTTAGCACTCATCGTTTACG
GCGTGGAATACCAGGGTATCTAATCCTGTTTGATCCCCACGCTTTCGCAC
ATCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTC
CATATCTCTGCGCATTTACCGCTACACATGGAATTCCACTTTCCTCTTC
TGCACTCAAGTTTTCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGG
CTTTCACATCAGACTTAAAAAACCGCCTACGCGCGCTTACGCCCAATAA
TTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAA
TTAGCCGTGGCTTTCTGATTAGGTACCGTCAAGACGTGCATAGTTACTTA
CACATTTGTTCTTCC

Appendix: 35

Query length: 1119

Phylogenetically similar species: *Staphylococcus taiwanensis* strain NTUH-S172

Reference: (Ruimy *et al.*, 1995).

>PSS25a

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TCCACCGGCTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGT
ACAAGACCCGGAACGTATTCACCGTAGCATGCTGATCTACGATTACTAG
CGATTCCAGCTTCATGTAGTCGAGTTGCAGACTACAATCCGAACTGAGAA
CAACTTTATGGGATTTGCTTGACCTCGCGGTTTCGCTGCCCTTTGTATTG
TCCATTGTAGCACGTGTGTAGCCCAAATCATAAGGGGCATGATGATTTGA
CGTCATCCCCACCTTCCTCCGGTTTGTACCGGCAGTCAACTTAGAGTGC
CCAACCTTAATGATGGCAACTAAGCTTAAGGGTTGCGCTCGTTGCGGGACT
TAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTC
ACTTTGTCCCCCGAAGGGGAAGGCTCTATCTCTAGAGTTGTCAAAGGATG
TCAAGATTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAACACCATGCTC
CACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGT
CGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGC
GGAAACCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGG
GTATCTAATCCTGTTTGATCCCCACGCTTTCGCACATCAGCGTCAGTTAC
AGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCCTCCATATCTCTGCGCAT
TTCACCGCTACACATGGAATTCCACTTTCCTCTTCTGCACTCAAGTTTTC
CAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACT
TAAAAAACCGCCTACGCGCGCTTACGCCCAATAATTCCGGATAACGCTT
GCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTC
TGATTAGGTACCGTCAAGACGTGCATAGTTACTTACACGTATGTTCTTCC
CTAATAACAGAGTTTACGATCCGAAGACTTCATCACTCAGGCGGCGTTGC
TCCGTCAGGCTTTCGCCATTGCGGAAGATTCCTACTGCTGCCTCCCGTAG
GAATCTGGACCGGGTCTCA
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RESUME

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High School	General	Ibrahim Al-Khalil School	2006-2007
Bachelor degree	Biology	University of Thi-Qar	2010-2011
Master degree	Immunology and Parasitology	University of Thi-Qar	2016-2017

Articles:

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