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Identification and Characterization of "Red Heat" Damage Causing Halophilic Microbes on Salted Sheepskin

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Abstract

A number of factors cause damage of skin and hide. Among these the major factor that did not get the attention of all stakeholders in Ethiopia is the "red heat" damage caused by halophilic microbes on salted skins and hides. Tanners and foreign buyers reject salted skins and hides affected by "red heat" damage. The objective of this study was to identify and characterize halophilic microbes causing "red heat" damage of salted skins and hides using culture-dependent and molecular methods. Halophilic and halotolerant bacteria and archaea were isolated and characterized from the "red heat" damaged salt cured sheepskins, with visible red patches. The enrichment microbial community was shifted from bacteria to archaea through time. A total of 85 isolates were obtained from 10%, 20% and 25% salt concentration enrichment microbial communities. Members of bacterial domain were the most abundant (89%), while members of domain Archaea were found to be 11% all belong to archaeal phylum Euryarchaeota. The enrichment bacterial isolates were clustered to three phyla, namely; Actinobacteria, Firmicutes and Proteobacteria. The bacterial enrichment isolates were affiliated to members of five genera: Halomonas, Bacillus, Salimicrobium, Nesterenkonia, Marinococcus, and Nesterenkonia. All the isolates belonged to Archaea were affiliated to members of genus Halococcus. Among the 17 representative isolates tested for their ability to degrade collagen, the archaeal isolate Halococcus sp. s2535 showed the maximum degree of hydrolysis followed by bacterial isolates Nesterenkonia sp.s211, Marinococcus sp. s2526 and archaeal isolate Halococcus sp. s2528. The phylogenetic analysis of isolates showed that most of the isolates were closely related to halophilic microbes obtained from various hypersaline environments, fermented foods and salted skins and hides. The result of this study indicates that microbes causing "red heat" damage of salted skin and hide are diverse and halotolerant. Although halophilic microbes growing at high salt concentration are the most efficient collagen degraders, those growing at low salt concentration also have certain degree of contribution. Therefore, an attempt to prevent the impact of halophilic microbes on salted skin and hide should address the diverse groups of microbes.

Keywords: skin defect, collagen, skin degradation, salt preservation, Skin and hide.

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List of Abbreviations

ARDRA	Amplified Ribosomal DNA Restriction Analysis
BSA	Bovine Serum Albumin
CSA	Central Statistical Agency
CTAB	Cetyl Trimethylammonium Bromide
DGGE	Denaturing Gradient Gel Electrophoresis
EU	European Commission
OD	Optical Density
OTU	Operational Taxonomic Unit
SDS	Sodium Dodecyl Sulfate
TDS	Total Dissolved Solids
TMPD	N,N,N',N'-Tertamethyl-p-phenylenediamine
UNIDO	United Nations Industrial Development Organization
USAID	United States Agency for International Development

1. Introduction

In Ethiopia, the leather tanning industry is an important industrial sector employing thousands of workers and serve as a major source of foreign currency earnings for the country (CSA, 2014). Recognizing the potential of the sector for growth and its contribution to national development, in recent years, the Government of Ethiopia put in place incentives to encourage further growth of the leather industry. As a result, in the last few years, new factories were established and existing factories significantly expanded their capacity to process daily 107,850 pieces of sheepskin, 51,550 pieces of goatskin and 9,800 hide (USAID, 2013). Leather tanning industries in Ethiopia have the capacity to process skin and hide to finished leather goods for the local and export markets (UNIDO, 2012).

Ethiopia is home to one of the highest number of animals in Africa (CSA, 2013). Despite the availability of huge resources and due attention given by the government, the contribution of leather and tanning industry to the national economic development is not as expected. As confirmed by tanneries, only 10 to 15% of harvested skins qualify for top grades, with the rest downgraded and rejected (Behailu Amde, 2017). The causes of damages are grouped into two categories. The first factors are grouped as those created or acquired during the life of the animal (Pre-mortem defects) and the second group being those that occur during and after slaughtering of animals (Post-mortem defects) (Tesfay Kahsay *et al*., 2015).

To avoid damage of skin and hide due to microbial putrefaction, different methods of preservation are employed. The most preferred method of skin and hide preservation practiced throughout the world is salting by adding ordinary salt (sodium chloride) shortly after flaying (UNIDO, 1995). Salt draws water out of the skin or hide thus reducing the water activity making it unfavorable for the growth of most microbial species. Moreover, salt acts as an inhibitor to most groups of microorganisms. Therefore, salt added to fresh skin or hide penetrates the skin and make the skin or hide unfavorable for microbial growth. The assumption is that in the absence of microbial growth, there will not be production of protein degrading enzymes and thus no damage to the skin or hide structure (Gudro, 2015).

However, certain groups of microorganisms, collectively known halophiles, optimally grow in the presence of high salt concentration. Naturally these microorganisms exist in hypersaline environments, such as salt lakes, marine environments, and salt concentration ponds and remain viable in the dry salt (Grant, 2004; Oren, 2014). When salt obtained from these sources is used for skin or hides curing, halophilic microorganisms present on the dry salt start to proliferate. The main nutrient available on animal skin and hide that can support the growth of halophilic microbes is protein that account for 33% of the fresh weight (or more than 90% of the dry weight) of the skin or hide (Gudro, 2015). Therefore, growth of halophilic microorganisms on salted skin or hide implies that they are capable of hydrolyzing the skin or hide protein. Indeed halophilic microorganisms that grow on salted skin and hide have been shown to produce protein-degrading enzymes, proteases. These protein degrading enzymes produced by halophilic microorganisms cause extensive damage to the collagen fiber, the part that is needed for leather production. If such skin or hide is processed, it leads to the production of very low or inferior quality leather (Welton and Woods, 1973).

Damage of salted skin and hide due to the growth of halophilic microorganisms has been reported from different parts of the world. At latter stages of growth (at or around the stationary growth phase) halophilic microorganisms give rise to the appearance of red patches on the cured skin or hide (Stuart *et al*., 1933). This happens due to the production of a red pigment by some halophilic microbial species at certain stage of their growth cycle. The appearance of red patches on salted skin or hide is a sign of damaged skin or hide by halophilic microorganisms and is commonly known as "red heat" damage. Because tanners are aware of damages caused by the action of such microbes, salted skin or hide having the so-called "red heat" damage (a sign of excessive growth of halophilic microorganisms) is often rejected (Akpole*et al*., 2015).

"Red heat" damage of salted skins and hides is a well recognized problem of the tanning industry in many countries of the world (Stuart *et al*., 1933). Similarly, in Ethiopia, tanners and local collectors have acknowledged the impact of "red heat" or "yekoda meklat" damage on the quality of salted skins and hides. Tanners reported that foreign buyers reject salted skins and hides affected by "red heat" damage. Tanners also reject salted skins and hides supplied by suppliers if the degree of "red heat" damage is high and visible by the naked eye as red patches. Therefore, in the absence of red pigment formation that gives the visible red patch ("red heat"), tanners are totally unable to distinguish whether or not salted skin or hide is damaged by the

growth of halophilic microbes. As a result, tanners consider all salted skins and hides showing no obvious red patches as normal, accepting them from suppliers, and processing them following the standard tanning procedure. After the end of the process, however, the tanner could discover that the resulting leather is of very low quality due to damage of the skin or hide by the growth of halophilic microorganisms (Addis Simachew and Amare Gessesse, 2016). Foreign buyers reject or provide low price for the exported finished leather after the skins and hides passed through the whole process of tanning and transported abroad, making the economic loss huge (Yakob Hailu, 2013).

Previously several studies have been conducted to identify the major factors that cause damage of skins and hides. These studies indicate that the defects observed pre-slaughter and post-slaughter include scratch, skin disease and flay cuts as the major causes of damage on skins and hides (e.g., Dawit Tesfaye *et al*., 2012; Behailu Amde, 2015; Tesfay Kahsay *et al*., 2015). Moreover, so far several attempts have been made to improve the quality of hides and skins by applying several intervention mechanisms on the problems associated to pre-slaughter and post-slaughter defects. However, the quality of hides and skins is decreasing from time to time suggesting that the major factors that are responsible for deterioration of the quality of hides and skins are not well known (Addis Simachew and Amare Gessesse, 2016). On the contrary, these earlier studies indicate that the impact of microbial putrefaction on the quality of skins and hides are low.

In a preliminary survey carried out recently by Addis Simachew and Amare Gessesse, (2016), the quality reports of finished leather products shows that all processed skins were of low quality with grades of V and below. None of the skins and hides processed reached grades of I-IV, grades for high quality leather tagged with the highest international prices. The results of the survey also show that although tanners and collectors acknowledge the huge impact of "red heat" damage on the quality of skins and hides, they did not have a clear understanding about the cause of "red heat" damage of skins and hides. Almost all stakeholders in the tanning industry believe that salted skins and hides are immune from microbial attack or "red heat" (Gudro, 2015). Tanners and other stakeholders involved in the sector wrongly believe that cause of development of "red heat" or what they call "yekoda mekelat" is the heat generated upon piling of skins and hides one over the other (Addis Simachew and Amare Gessesse, 2016).

This clearly shows the lack of basic but simple information that enables tanners and suppliers to carry out minimal precautions to prevent putrefaction of skins and hides by the growth of halophilic microorganisms. Moreover, the lack of this basic information shifted the focus of all stakeholders involved in the sector to deal with other problems, which have less impact on the quality of skins and hides. As a result the extent of damage of microbial putrefaction or "red heat" damage of skins and hides is not well known and ignored by all the stakeholders involved in the Tanning Industry. It is therefore, essential to identify and characterize "red heat" damage causing microorganisms as well as evaluating the impact of these microbes on the quality of hides and skins.

2. Objectives

2.1. General Objective

- The general objective of this study was to identify and determine the impact of halophilic microbes causing “red heat” damage of salted skins and hides using culture-based and molecular methods.

2.2. Specific objectives

The specific objectives of this study were to:

- Identify and assess the diversity of halophilic microbes causing “red heat” damage of salted sheepskins and hides from putrefied skins using culture-based and molecular methods.
- Evaluate the impact of the isolated halophilic strains on the damage of salted skins and hides.
- Determine the collagen degrading halophilic microbial community shift through time of incubation.
- Screen isolates that have proteolysis, gelatinolytic and lipolytic enzyme activity.

3. Literature Review

3.1. Skin and hide production

Archeological studies have shown that many activities of human being use skin and hide. Egyptian tombs, wall paintings and artifacts indicate that leather was used for sandals, clothes, gloves, buckets, bottles, shrouds for burying the dead and for military equipment (Lawal and Odums, 2015). The ancient Greeks and Romans also made extensive use of leather and it has remained an important industrial raw material since those times (Abraham Jemberu *et al*., 2017). Leather has been one of the most widely traded commodities in the world. The leather and leather products industry plays a prominent role in the world's economy, with an estimated global trade value of approximately US$100 billion per year (UNIDO, 2010; Coppeaux *et al*., 2016).

Currently leather is a highly versatile material used in various range of products including shoes, gloves, luxury bags, jackets, car and aircraft seats. The leather industry market in terms of overall turnover for all types of leather is dominated by China, Italy, India, and Brazil (Coppeaux *et al*., 2016). The biggest sources of skins and hides from domesticated animals are sheep, goat, and cattle, respectively (Abraham Jemberu *et al*., 2017). Over 95 % of hide and skin is obtained from the world's meat and dairy industry. Globally, 68.8 % leather is obtained from bovine and 59 % of the total global leather is used for footwear (Coppeaux *et al*., 2016).

3.2. Skin and hide production in Ethiopia

Ethiopia is believed to have the largest livestock population in Africa (CSA, 2013). According to the Central Statistical Authority 2017 report the county's livestock population is estimated to be 59.5 million cattle, 30.7 million sheep and 30.2 million of goats. For instance, according to the off-take rates of 7% for cattle, 33% for sheep and 37% for goat the countries potential was estimated to be 4.16 million cattle hides, 10.1 million sheep skins and 10.5 million goatskins in 2016/17 (CSA, 2017). Between 2006 and 2011, the total value of Ethiopia's exports of leather and leather products grew from US$66 million to US$112 million (UNIDO, 2012), almost doubled.

There are about 33 tanneries in Ethiopia which have the potential of 107,850 pieces of sheepskin, 51,550 pieces of goatskin and 9,800 hide for daily process (USAID, 2013). They are also largely export oriented by producing semi and fully processed leathers (UNIDO, 2012). However, they

are not working to their full capacity due to many reasons. The first reason is the skin and hides are available only when the meat is needed, especially in the Ethiopian holidays (Abraham Jemberu *et al*., 2017). Besides, the sector has been prevented from fully capitalizing on significant comparative advantages by the low level of competitiveness (UNIDO, 2012). Tanneries also cannot impose clear difference in price between different quality of the skins that they receive, because the quality can only be seen after shaving the hides and skins and treating with chemicals. The quality selection result end up by rejecting around 20% of skins and hides received from traders. In the 80% remaining skins and hides, grades 1-2-3 account for 10% all together, grade 4 accounts for 30%, grade 5 accounts for 30% and grade 6 accounts for 10% (Coppeaux *et al*., 2016) making the total proportion of skins and hides rejected or tagged with low price 90%. What makes worse is that the low quality of the skins and hides is detected after processing and a lot of chemical and labor cost is incurred.

3.3. Structure of skin and hide

Green hide and skins consist of three layers, the epidermis or thin outside layer, a second thicker layer known as the corium or true skin, and a third layer of adipose tissue or flesh (Fig. 1). The first and third layers are removed in the process of tanning. The epidermis is made up of cells, an under layer of living epithelial cells and an outer layer of dead cells. This outer layer consists mostly of an insoluble protein, keratin, and affords surface protection to the body (Abraham Jemberu *et al*., 2017). The grain layer provides the aesthetic value of leather. The strength and resiliency of leather comes from corium layer. It is rich in the protein collagen. Individual collagen molecules combine together in the corium to form very small fibrils that are in turn bound together to form collagen fibers. The strength of the skin and of leather is due to cross weaving of these fibers (Behailu Amde, 2015).

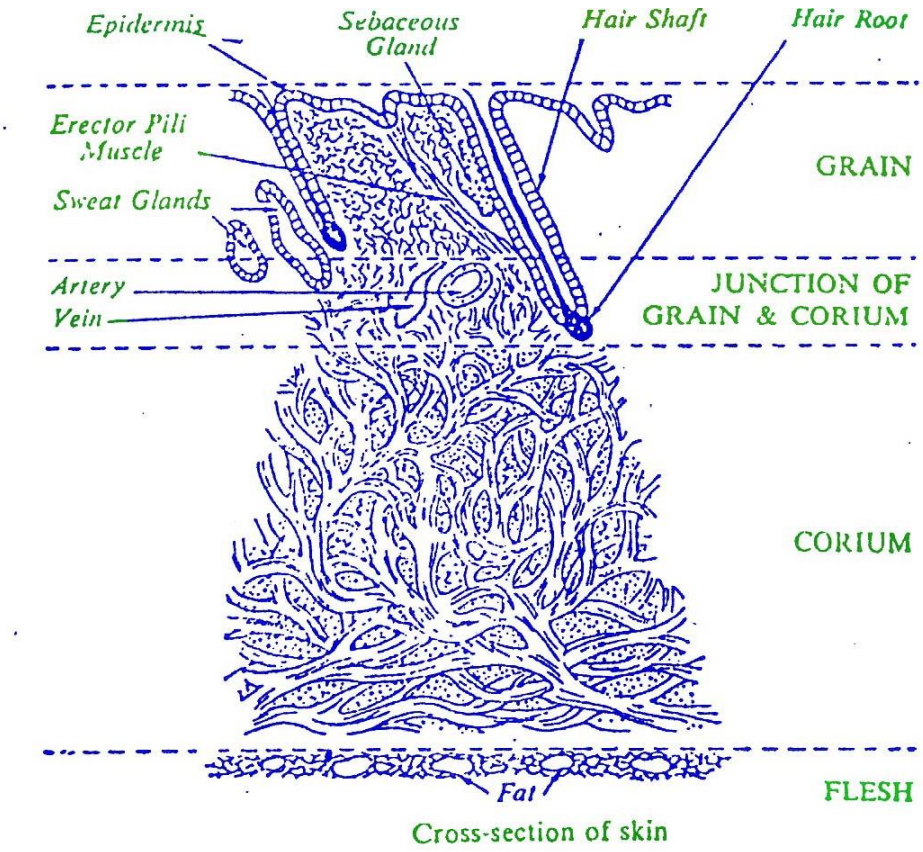

Figure 1. Cross section of the skin Source: http://trimax.yolasite.com/resources/PDF/Bag

The chemical composition of raw hides and skins is water 64 %, protein 33 %, fats 2 %, mineral salt 0.5 % and other substances 0.5 %. The most important constituent in skin is protein collagen which covers about 90 % of absolutely dry hide weight (Gudro, 2015). In this form after being removed from the carcass the hide is susceptible to bacterial activity within hours. The degradation of skins/hides is assumed to be by the combination of the bacteria which requires moisture to be available and tissue enzymes (Kudit *et al.*, 2013).

3.4. Preservation methods of skin and hide

Tanneries stock hide and skin to optimize the efficiency of their batch processes (EU, 2013). Preservation of raw skin and hide allow transport and storage by rendering the flayed pelt resistant to putrefaction. Therefore, preservation provides cured environment either by destroying active bacteria, by preventing bacterial activity or by preventing bacterial contamination (Gudro, 2015).

There are wide choices of preservation methods to prevent in transit of hide and skin that cannot process immediately. Usually the choice depends on the period of time envisaged for preservation (EU, 2013). The preservation techniques of hides and skins can be divided into long term preservation and short term preservation (Gudro, 2015). Widely used long term methods are drying methods (ground dried, sun dried, frame dried, shade dried); salting methods (dry salting, wet salting, re-use of salt and brining). The short term methods are cooling and deep freezing (UNIDO, 1995).

The popular method of preserving raw hides and skins is curing with sodium chloride due to ease, cost effectiveness, does not interfere with processes in the activated sludge treatment, non-toxic, hygienically acceptable when safety measures observed and widely available (UNIDO, 1995; Kudit *et al*., 2013). Sodium chloride used amounts 30-40 % of total flesh weight (Gudro, 2015). Salt acts by inhibiting the growth of bacteria by lowering the moisture content and changing the living condition in raw hide and skin.

In general, hides and skins are spreaded out, covered with salt and then stacked, sandwiched with more salt. The hide and skin may need to be re-salted if they are stored for a long time (EU, 2013). However, the reuse potential of salt is limited mainly due to risks associated with the increase of halophilic bacteria (Stuart *et al*., 1933). Disposal of the excess solid salt used and the pollution load of tannery effluent which becomes highly contaminated with increased total dissolved solids (TDS) and chlorides (Cl^-) is an important environmental problem as well (UNIDO, 1995).

Other simple method which does not require any chemicals is drying. Drying ways can be natural in sun or controlled drying (Gudro, 2015). The simplest and cheapest method is to dry hides/skins by evaporation under the sun. It is one of eco-friendly curing process (UNIDO, 1995). However, this method is practicable only in dry warm climate countries. The fresh skin can be dried in a controlled environment within 24 hour by using drying chamber at 26ºC and provides long term preservation. However, this method requires high installation and running cost. The high humidity may increase the drying time in the wet seasons which results in growth of bacteria, causing putrefaction; susceptibility to damage by insects, rats, etc; (Gudro, 2015).

Although salting and drying are the common curing methods several curing agents have been reported by researchers including potassium chloride, silica gel, herbal-based products, boric acid, borax, acetic acid, sodium sulphite, sulphur dioxide, sodium hypochlorite, dimethyldithiocarbamate and chlorinated phenols (UNIDO, 1995; Kudit *et al*., 2013).

3.5. Factors affecting quality of skin and hide in Ethiopia

Despite the huge livestock population and skin and hide production, Ethiopian leather industry is not developed as expected since all the resource is not valued as a raw material for the leather industry because of different factors that cause rejection or dawn grading of the skin and hide (Coppeaux *et al*., 2016). Skin and hide quality defects are responsible for the decline in quality grades which ultimately determines the benefit from the sector (Dawit Tesfaye *et al.*, 2012). The quality of skin and hide is affected in one of the two either pre-slaughter or post slaughter defects (Behilu Amde, 2017).

3.5.1. Pre-slaughter defect causes

Pre-slaughter period covers the greater part of the animal's life, from its birth to about the time it is collected for delivery to the butchery (Behilu Amde, 2017). In Ethiopia many hides and skins are discarded soon after slaughtering, but the major losses occur among materials which have been damaged before, during or after collection (Kassa *et al*., 1998; Tesfay Kahsay *et al*., 2015). The commonly observed pre-slaughter defects can be natural (poor nutrition, age, sex, breed and climate effects), mechanical damage (brand mark, scratches, horn rake, yoke mark etc.), or defects due to skin diseases and external parasitic infestation (Abraham Jemberu *et al*., 2017).

Biological factors like breed, gender and age have an effect on the weight and the strength of the skin and hide. The skins from male goats and sheep are heavy with a coarse grain. While female skins have better tensile strength. The skin structure of young animals tends to be fine, compact and have tight grain patterns. The grain surface becomes tougher and coarser grained as animals grow older. Besides aged animals accumulate more scars from brands, diseases, parasites, scratches and other injuries (Behilu Amde, 2017).

A considerable portion of the pre-slaughter defects that accounts for 65 % are directly associated with skin diseases initiated by the ectoparasites (mange, sheep ked, lice and ticks) or to the damage that occurs when the animal scratches itself to relief the itching (Behilu Amde, 2017). Cockle causes higher proportion of skins to fall into the lower grades. The defect results in huge

economic loss to tanneries and the country at large since the damage is detected after a lot of cost is incurred on the processing (Ahmed Husen *et al*., 2016).

3.5.2. Post-slaughter defects

Post-mortem defects are directly related to transportation, preservation and storage of the materials. It comprises of inadequate bleeding/veininess, poor pattern, gouge marks, flay cut/hole, heating/putrefaction, and beetle damages (Ahmed Husen *et al*., 2016). Most of the post-slaughter defects are due to improper curing or preservation and storage of hides and skins (Tesfay Kahsay *et al*., 2015).

Hide and skins should be preserved within short period of time, after removed from the carcass to avoid bacterial growth and decomposition of the skin that downgrade the quality of hide and skin (Ahmed Husen *et al*., 2016). If the carcass is not completely drained through proper bleeding at the time of slaughtering, blood remains in the vessel and capillaries of the hides and skins (Yakob Hailu, 2013). As a result bacteria then develop more rapidly (Behilu Amde, 2017). Also careless use of the knife or using unsuitable knives may cause the most serious mechanical defects on hides and skins (Ahmed Husen *et al*., 2016). If dried hides or skins are allowed to be flint dry before folding the fiber get ruptured and there will be cracks due to grain rupture (Behilu Amde, 2017).

3.6. Impacts of halophiles on the quality of skin and hides

Halophiles have mainly been isolated from saltern crystallizer ponds, the Dead Sea, solar lakes and hyper-saline lakes (Kumar *et al*., 2012). Halobacteria are extreme halophiles grow best at the highest salinities (3.4–5 M NaCl), forming dense blooms and resulting in the red color of many brines (DasSarma and Arora, 2001).

Stuart *et al*., 1933 reported that the origin, effect and prevention of reddening organisms for codfish and hide have been studied since 1980.These areas received considerable attention and reported on dry-salted skins and on domestic green-salted hides. The condition has been called in the trade as “frigorifico reddening”, “strawberry”, “reddening” “heating” and “yekoda meklat” (Stuart *et al*., 1933; Addis Simachew and Amare Gessesse, 2016). The condition appears a bright-red discoloration on the flesh side, in spots, streaks, and great blotches on commercially green-salted hides. The discoloration is frequently modified to a rather nondescript brown, when

the hide is partly dried out. It has been reported that bacteria causing red spots on the flesh side of salted hides or skins cause hair slip, epidermis and grain damage which diminish the sale value of the leather (Ventosa *et al*., 2015).

Researchers reported that the contamination of halophilic microorganisms found in salts, causes red colorations (Akpolat *et al*., 2015). It was found that 34 out of 35 samples of crude solar evaporated salts and 25 of 39 open-pan evaporated grainer salts contained red microorganisms (Stuart *et al*., 1933).

The development of hide and skin "red heat" causing organisms influenced by different factors. It develops in a wide range of temperature and high temperature is not essential for their development. Relatively high humidity is necessary and slightly alkaline conditions are preferred for the development of these organisms (Stuart *et al*., 1933).

Putrefaction (heat) damage causes 22% of sheepskin and 19% of the goatskin rejection of the total skin (Kidanu, 2001 cited in Yakob Hailu, 2013). Samples of hide showing "red heat" were found from a number of Australia, England, India, South America, and Spain tanneries (Anderson, 1954; Stuart *et al*., 1933). Anderson (1954) isolated 25 red pigmented and 8 colorless bacteria from hides with "red heat". These microorganisms required 15 % NaCl for growth. Some red pigmented and colorless microorganisms were able to digest protein. He also observed that these microorganisms grew very well in the medium containing sterile hide and 27.5 % NaCl; they produced red pigments in 2 weeks at 37 ^{o}C.

In other experiment conducted on salted sheepskin, according to the phenotypic characteristics and comparative partial 16S rRNA sequence analysis, 101 extremely halophilic archaeal and 78 moderately halophilic bacterial isolates were identified. These include genera of, *Pseudomonas*, *Acinetobacteria*, *Alkalibacillus*, *Salimicrobium*, *Marinococcus* and *Staphylococcus* belonging to moderately halophilic bacteria; and Genus of *Halorubrum*, *Natrinemapelli*, *Halococcus*, *Halostagnicol*, *Haloterrigena* and *Natrinema* belonging to extremely halophilic archaea were isolated from sheep skins (Akpolat *et al*., 2015).

3.7. Biotechnological applications of halophiles

Screening of new source of novel and industrially useful enzymes is a key research pursuit in enzyme biotechnology (Kumar *et al*., 2012). Halophiles have been considered over many years for their vast repository of novel enzymes. There diverse response mechanism for the high concentration of salt gives them to be the source of valuable molecules like stable enzymes (DNAases, lipases, amylases, gelatinases and proteases) under harsh physical and chemical conditions that lead to precipitation or denaturation of most proteins (DasSarma and Arora, 2001; DasSarma and DasSarma, 2015). Halophilic enzymes, therefore, offer important opportunities in many applications, such as environmental bioremediation, food industry, and waste-water treatment (Sirisattha *et al*., 2016).

The study conducted by Kumar *et al*. (2012) in saline habitats of India screened novel/ stable and maximum amylase and lipase from *Marinobacter* sp. and protease from *Bacillus* sp. Ramadas and Ramadoss (2012) also produced lipase enzyme from *Halobacterium* sp., which has the capacity for large scale culturing. Other research conducted in India also produced nobel amylase, protease, inulinase and gelatinase enzymes from extreme halophile *Salinicoccus sp*. JAS4 (Jayachandra *et al*., 2012). The enzyme α-amylases that are functional at high temperature and salt as well as stable towards organic solvents, have been produced from different halophilic sources such as *Natronococcus amylolyticus*, *Halomonas meridiana*, *Halothermo thrixorenii*, *Haloferax mediterranei*, *Nesterenkonia* sp. strain F and *Marinobacter* sp. EMB 8 (Kumar *et al*., 2016).

In addition, some halophilic microorganisms are capable of producing massive amounts of compatible solutes that are useful as stabilizers for biomolecules or stress-protective agents. They have been considered as the source of bioactive compounds like β-carotene and ectoin production, fermentation of soy and fish sauces (DasSarma and Arora, 2001; Oren, 2014).

Some halophilic microorganisms also have environmental applications in degradation of heavy oils. A halophilic bacterium, strain TM-1, was isolated from the reservoir of the Shengli oil field in East China. This strain can use a variety of organic substrates and can grow at up to 58ºC and in an 18% NaCl solution. It can degrade and change the properties of the oil and when grown on heavy oils it can lead to a loss of aromatic hydrocarbons (Hao and Lu, 2009).

4. Materials and Methods

4.1. Sample Collection

"Red heat" damaged salt cured sheepskins with visible red patches and bad odors were collected from the local collector's stores in Addis Ababa, Ethiopia. The samples were transported to the laboratory with icebox. These damaged sheepskins were used as source of inoculums for enrichment and isolation of halophilic microbes causing "red heat" damage on salted skins and hides.

4.2. Enrichment and enrichment medium preparation

To enrich halophilic microbes causing "red heat" damage of salt cured skins and hides, a medium containing (g/l) $MgSO_4.7H_2O$, 10 g; yeast extract, 1 g; K_2HPO_4, 0.2 g; $CaCl_2.2H_2O$, 0.2 g; and KCl, 2 g were prepared. Following this the medium was divided into three different flasks containing 10%, 20% and 25% NaCl and 2 g of fresh chopped sheepskin (de-haired and cleansed of fat and other dirt). The medium was autoclaved at 121 ^oC for 15 min. Then 10 ml /l filter-sterilized trace metals solution was added when it cooled to 50-60 ^oC. Afterwards a loopful of red material scraped from the 'red heat' damaged sheepskin was inoculated into each flask by leaving one flask as a control. Then it was incubated at 30 ^oC with 121 rpm in a shaker until the whole skin was hydrolyzed for 15 days. The growth of microorganism was monitored through visual observation and OD (optical density) reading (600 nm, UV-7804C Spectrophotometer).

4.3. Isolation of pure culture isolates

For isolation of pure cultures, to the same medium composition as the enrichment culture 2 % agar was added for solidification. The medium was heated until the agar and other salt components dissolved completely. Then the medium was autoclaved at 121 ^oC for 15 min. When the medium cooled down to 50-60 ^oC, 0.5% glucose, separately autoclaved, was added as carbon source. The medium was poured onto plates, approximately 25 ml per plate. The plates were left at room temperature overnight.

Enrichment cultures from the fifth day were serially diluted with sterile salted water (10 %, NaCl), (10^{-1} to 10^{-9}), and 100 µl spreaded onto agar plates. The plates were wrapped with a plastic bag and incubated at 30 ^oC until distinct colonies emerge. After five (10 % NaCl), ten (20 % NaCl) and twenty one (25 % NaCl) days of incubation 85 different shape and colors of single

colonies were picked randomly, and purified through repeated streaking. The purified pure colonies were preserved at 4 ºC for further study.

4.4. DNA Extraction from enrichment cultures for DGGE analysis

To extract enrichment microbial community genomic DNA cells were harvested from 1 ml enrichment cultures grown on the same medium composition and condition as indicated in section 4.2. To determine the microbial community shift 1.5 ml enrichment microbial community cultures were harvested in an Eppendorf tubes at different time intervals of incubation (on days; 1, 2, 3, 4, 5, 7, 10, 15 and 30) and pelleted by centrifugation at 13,000 rpm for 10 min (Wagtech, K2 series). On the cell pellets 250 µl of lysozyme/Rnase (1 mg lysozyme /ml TE, 0.5 mg Rnase A/ ml TE) solution was added and incubated at 37 ºC for 15 min. And then 10 µl of proteinase K (1 mg/ ml TE) was added and again incubated at 37 ºC for 15 min. The cell pellets were lysed with 0.25 ml preheated lyses buffer (10% SDS) incubated at 55 ºC for 15 min by gentle mixing occasionally. The cell lysate was incubated at 65 ºC for 10 min by adding 80 µl 5 M NaCl and 100 µl preheated CTAB (Cetyl Trimethylammonium Bromide) solution (10 % (w/v) CTAB and 0.7 % NaCl). Following this 500 µl of chloroform:isoamyl alcohol (24:1) was added and shaken for 10 min on a vortex. Phases were separated by centrifugation (5 min, 13,000 rpm) and the upper phase were removed to a new Eppendorf tube and 0.6 volume of isopropanol was added on it and incubated at room temperature for 10 min by mixing it carefully. Then it was centrifuged at 13,000 rpm for 10 min. The resulted pellet was washed in cold 70 % ethanol. Finally it was centrifuged again at 13,000 rpm for 10 min and the pellete was air dried. The dried pellet was resuspended by 50 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) buffer. The quantity and quality of extracted DNA was measured using a Nanodrop (NanoDrop™ 2000/c) and by running in 0.8% agarose gel electrophoresis. The extracted DNA was stored at -20 ºC for future experiment.

4.5. Genomic DNA extraction from pure isolates

Genomic DNA was extracted from pure isolates by following the freeze thaw method with a modification of a rapid boiling method designed by Moore *et al,* (1999). A loopful of colonies of the pure isolate were picked and suspended in 50 µl of TE buffer and then incubated in boiling water (92 ºC) for 5 min. After boiling the tubes were withdrawn and immediately incubated at -20 ºC for 15 min. Then, the boiling and freezing processes were repeated once again. The cell

lysate was centrifuged at 13000 rpm for 10 min and the supernatant was used as crude extract of genomic DNA. The supernatant was stored at -20 ^{o}C for future experiment.

4.6. PCR amplification for 16S rDNA

Table 1 shows the oligonucleotide primers that were used for PCR amplification and sequencing of 16S RNA genes in this study. Fragments of 16S rRNA gene were amplified from pure bacterial isolates using primer sets 338f-H1542r and A8f-H1542r (Giovannoni 1991; Øvreås *et al*. 1997) (Table 1). For archaeal isolates fragments of 16S rRNA gene were amplified using primer set Arch21F-Arch1542r (Giovannoni 1991; Delong 1992). Amplifications were carried out with 25 µl reaction volume (Nuclease free water; 19.625 µl PCR buffer; 2.5 µl DNTPs; 0.25 µl Forward primer; 0.125 µl Reverse primer; 0.125 µl Taq DNA polymerase; 0.125 µl BSA; 0.125 µl and Template DNA; 2 µl) in a thermal cycler (Techne TC-412, Barloworld Scientific) at 95 ^{o}C for 5 min initial denaturation, and 30 cycles of each 94 ^{o}C for 30 S, 54-55 ^{o}C for 30 S, 72 ^{o}C for 1 min for denaturation, annealing and elongation, respectively and final elongation at 72 ^{o}C for 7 min.

4.7. PCR amplification for DGGE analysis

Fragments of 16S rRNA gene (positions 1055-1392 and 931-1392, *Escherichia coli* numbering), were amplified from enrichment community DNA for bacterial and archaeal DGGE analysis using a primer sets B1055f-B1392r and Arch931f-Arch1392r, respectively. A40 bp long GC-clamp was attached to the 5′-end of the reverse primers (Table 1). Amplifications were carried out in thermal cycler (Techne TC-412, Barloworld Scientific) at 95 ^{o}C for 5 min initial denaturation, and 30 cycles of each 94 ^{o}C for 30 S, 52-53 ^{o}C for 30 S, 72 ^{o}C for 1 min for denaturation, annealing and elongation, respectively, and final elongation at 72 ^{o}C for 7 min.

Table 1. List of primers used for PCR and sequencing

Primer	Positions	Target	Annealingtmperature(°C)	Oligonucleotidesequences (5′-3′)	References
A8f	8-27	*Bacteria*	55	CTGAGCCAGGATCAACTCT	(Giovannoni 1991)
H1542r	1542-1522	*Bacteria*	55	TGCGGCTGGATCACCTCCTT	(Giovannoni 1991)
338f	338–357	*Bacteria*	55	ACTCCTACGGGAGGCAGCAG	(Øvreås *et al*. 1997)
B1055f	1055-1070	*Bacteria*	56	ATGGCTGTCGTCAGCT	(Ferris *et al*. 1996)
B1392r-GC	1392-1406	*Bacteria*	56	ACGGGCGGTGTGT AC	(Ferris *et al*. 1996)
Arch1392r-GCr	1392-1406	*Archaea*	53-59	ACGGGCGGTGTGTGC	(Ferris *et al*. 1996)
Arch21f	2-21	*Archaea*	52-56	TTCCGGTTGATCCYGCCGGA	(Delong 1992)
ARCH931f	915-931	*Archaea*	53-59	AGGAATTGGCGGGGGAGCA	(Nagy *et al*. 2005)
GC clamp				CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG	(Muyzer *et al*. 1993)

4.8. Agarose gel electrophoresis

All pure culture PCR products were applied onto 0.8% (w/v) agarose gels and run in 1x TBE buffer (10x TBE= Tris 108 g, EDTA 7.44 g, H_3BO_3 55 g, and H_2O 1000 ml) at 80 V for approximately 40 min. Gels were stained with ethidium bromide and bands were visualized and photographed by a gel documentation system (Gel DOC^{TM} XR, Bio-Rad, USA) using Quantity one software (Bio-Rad, USA) (Appendix 5). A 1Kb DNA ladder was used as molecular size marker.

4.9. Denaturing gradient gel electrophoresis (DGGE)

DGGE analysis was performed by loading 10 µl PCR products onto 10% polyacrylamide-bis (37.5:1) gels with denaturing gradients from 30 to 80% (where 100% is 7 M urea and 40% (vol/vol) deionized formamide (100 ml of 40% formamide with 3.8 g DOWEX 1x8)) in 0.5xTAE electrophoresis buffer (20 mM Tris, 10 mM acetate, 0.5 mM Na_2-EDTA (pH 7.4)). Electrophoresis was performed at 20 V first for 20 min and subsequently at 75 V and 60 °C for 13 h. After electrophoresis completed, the gels were soaked for 30 min in ethidium bromide (1:10 dilution), and photographed on a gel documentation system (Gel DOC^{TM} XR, Bio-Rad, USA) using Quantity one software (Bio-Rad, USA).

4.10. Restriction endonuclease digestion and electrophoresis

Enzymatic digestion of PCR products from pure isolates were carried out using restriction enzyme, TaqI (5′-T^CGA-3′) and (3′-AGC^T-5′). A 7.50 µl restriction reaction contained 0.5 µl 1U TaqI (Himedia, India), 1.50 µl 10x Hibuffer TaqI (Himedia, India) and 6.1 µl nuclease free water (Qiagen). Then 10 µl PCR products were added to each reaction mixture. The reactions were mixed very well and spinned with a microcentrifuge briefly (VWR, Galaxy Mini, Korea). The restriction digestions were carried out at 65 ^{o}C for 1 h on a thermocycler. Following the restriction digestion the enzyme were inactivated by incubating at 85 ^{o}C for 15 min. Then the entire restriction digest was loaded onto 3% agarose gel containing 3.5 µl ethidium bromide. Electrophoresis was run at 100 V for 2 h using 1kb DNA ladder as a size marker. ARDRA (Amplified Ribosomal DNA Restriction Analysis) patterns or OTUs (Operational Taxonomic Units) were determined for sequencing. One isolate from each OTU was selected for sequencing.

4.11. Purification of PCR products for sequencing

PCR products of pure isolates were cleaned using GenElute PCR Clean-Up Kit (Sigma) according to the manufacture's instruction.

4.12. Sequencing

Nearly full-length 16S rRNA gene was sequenced using primer pairs of A8f-H1542f for bacterial isolates and A21f-H1542r for archaeal isolates, from both directions at the sequence facility of the University of Calgary, Canada. For few bacterial isolates that did not give good quality PCR products with the primer set A8f-H1542R, primer 338f was used instead of A8f for PCR amplification and subsequent sequencing.

4.13. Sequence Analysis

The sequences obtained were edited manually by using Bioedit software. The sequences were then assembled by PRABI-Doua CAP 3 software and then aligned using ClustalW in Mega 6 alignment explore with the reference sequences retrieved from the NCBI GenBank database available at http://www.ncbi.nlm.nih.gov using BLAST Nucleotide (Basic Local Alignment Search Tool). The aligned sequences were then run to construct phylogenic tree using MEGA 6 software (Tamura *et al*., 2013). Phylogenetic relationship of sequences to closest matches in public database based on 16S rRNA gene sequences was constructed by using Maximum like hood method using distances calculated with Jukes-Cantor correction (Jukes and Cantor, 1969). The stability and reliability of the relationships of lineages on the inferred trees was tested by bootstrap analysis for 1000 replicates.

4.14. Hydrolysis of sheepskin

To identify the pure culture halophilic microbial strains responsible for the "red heat" damage of salt cured skins and hides, their capability to hydrolyze collagen was determined. Fresh de-haired sheepskin was chopped using sterile sizer into small pieces of approximately 2 cm^2 (Fig. 2) and added to a liquid mineral medium (prepared as described in section 4.2) as the sole carbon and nitrogen source. After autoclaving the flasks a loop full of refreshed pure culture strains were inoculated and incubated at 30 ^oC in 121 rpm shaker incubator for 15 days. The degrees of hydrolysis of pieces of sheepskins were monitored visually and the growth of microbes was observed by measuring the OD (600 nm, UV-7804C spectrophotometer).

Figure 2. Chopped sheepskin used as a sole carbon and nitrogen source for enrichment of "red heat" causing halophilic microbes

4.15. Biochemical tests for selected Operational Taxonomic Units (OTU)

Gram test and catalase test were carried out on the representatives of OTUs to check their biochemical characteristics. Gram test was carried out according to a method describe previously (Pacarynuk. *et al.*, 2005). A loopful of freshly grown colonies were picked from agar plates and added on a clean glass slide contained a drop of 3% potassium hydroxide (KOH) solution. The colonies were stirred for about a minute occasionally lifting the loop to check for the thickening and elasticity of the slurry. Catalase test was done using 3% H_2O_2 based on a method describe previously (Karen, 2010).

Lipase activity of the pure cultures of representative isolates were tested on twin 80 nutrient agar plates containing 0.5 % (v/v) of twin 80, supplemented with 10 %, 20 % and 25 % (w/v) NaCl (Akpolate *et al.,* 2015). Isolates that showed clear zones were read as lipase producer. Casein hydrolysis was also tested in the same way as lipase by changing the substrate to 0.5 % (w/v) casein. Gelatinase activity of the pure isolates were screened by the slight modification of Dela and Torres, (2012) nutrient gelatin stab method, by adding 1.5% gelatin in the same medium used in section 4.2 without glucose. After incubating for 12 days it was tested for liquefaction in ice bath. It was also tested if the gelatinase produced by the microorganisms is either extracellular or intracellular by testing liquefaction of 2 % gelatin by the filtrate of 10^{th} day culture after 2 h of incubation at 30 ^{o}C.

4.16. Data Analysis

The data collected from the domain level and the phylum level abundance were subjected to Chi-Square test by using SPSS software version 20.

5. Results

5.1. Growth of enrichment microbial community on chopped sheepskins

Figure 3 shows the growth of enrichment microbial community at three different salt concentrations using chopped sheepskin as the sole carbon and nitrogen source. In all the enrichments the chopped sheepskin was completely degraded faster by 10% salt concentration enrichment microbial communities followed by 20% and 25% salt concentration enrichment microbial communities. The chopped sheepskin in the control enrichment (without inoculums) was not degraded at all and remained intact at all salt concentrations (Fig. 3).

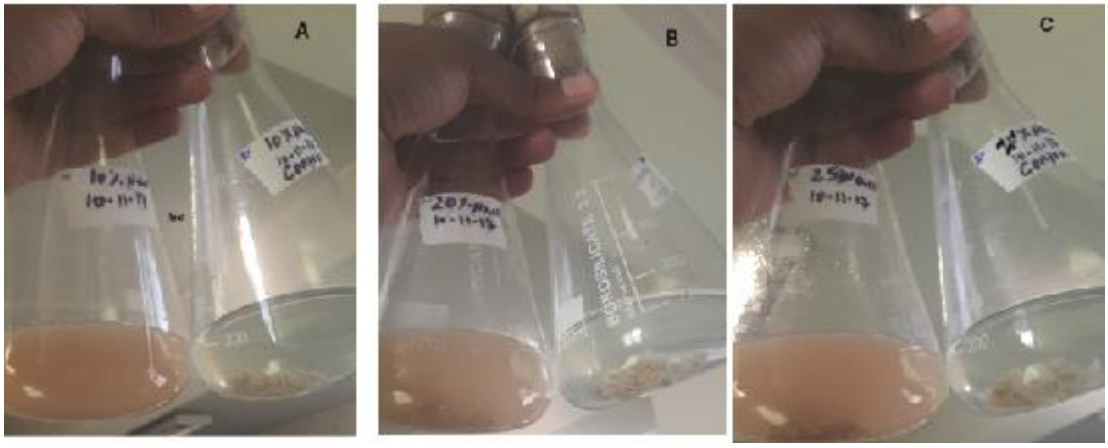

Figure 2. Halophilic microbial communities enriched at different salt concentrations using chopped sheepskins as sole carbon and nitrogen source. A. 10% salt; B: 20% salt; C: 25% salt (flask on right side control media and left inoculated)

The growth curve of the enrichment microbial community at different salt concentration shows that the microbial growth was fast at 10% salt concentration medium followed by 20% salt medium and it was slow at 25% salt concentration medium (Fig. 4). The 10% salt enrichment microbial community starts to grow at 24 hours of incubation (OD 0.6) and the growth reached maximum at the 5th day of incubation (OD 1.8) and then entered to its stationary phase. On the other hand appreciable growth of the enrichment microbial communities was observed after the 2nd and 3rd days of incubation at the 20% and 25% salt concentration medium, respectively. The enrichment microbial community growth continues exponentially until the 6th day of incubation and then slows to stationery. On the other hand the 25% salt concentration medium grows slowly until the 2nd day and continues to grow faster and did not reach to the stationary phase until the 8thday incubation (Fig. 4).

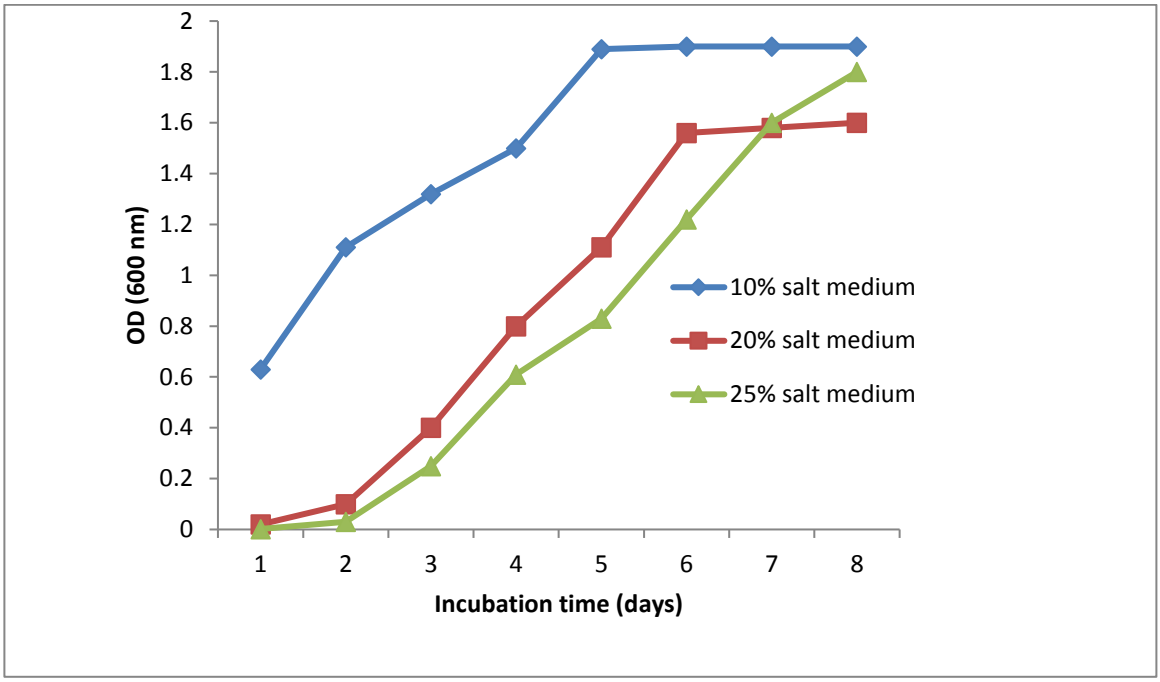

Figure 3. Growth curve of the enrichment microbial community at different salt concentration

5.2. Color change of enrichment microbial community over time

A clear and distinct color change was observed in enrichment microbial community along the salt concentration (Fig. 5). At 10 % salt concentration enrichment media, there was a slight color change while the medium completely changed to pink red at 20 % and 25 % salt concentration after seven days of incubation.

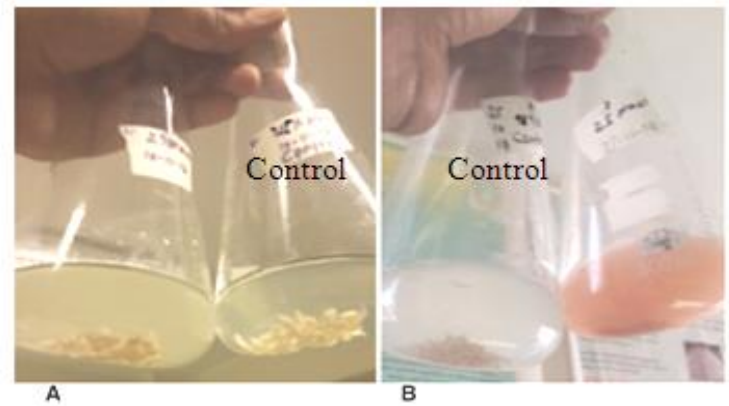

Figure 4. 25% salt enrichment culture and color change by red pigment producer microorganisms (A), 4th day culture and (B) 13th day culture.

5.3. Isolation of pure microbial strains from enrichment media

A total of 85 distinct colonies were picked from the three enrichment microbial communities (Table 2). From 10 % salt concentration medium 28 yellow and white isolates, distinct in shape were obtained. Twenty seven different isolates were obtained from 20% salt concentration medium. Thirty distinct red yellow and white isolates were also obtained from 25% salt of enrichments. Of the three salt concentration media, isolates from 25% salt media showed diversity in shape and color. Samples enriched on the 10%, 20% and 25% salt media grew in the agar media after five, ten and twenty one days of incubation, respectively at 30 ^{o}C.

Table 2. Pure culture halophilic isolates from "red heat" damaged sheepskins

EnrichmentSalinity (%)	Number of Isolates	Days of isolation
10	28	5
20	27	10
25	30	21
Total	**85**	

5.4. Microbial Community shift through time

As it can be seen from Figure 6A the enrichment bacterial microbial communities reached to a detectable level after 5th days of incubation and the enrichment bacterial communities reached maximal growth at the 7th days of incubation (Fig. 6A). The bacterial communities decreased over time and almost completely lost after the 10th days of incubation. However, one faint band (Band 7), which was not detected earlier, appears at the 10th days of incubation. Moreover, bands 1-5, which were relatively faint up to the 5th day of incubation, become more intense and dominant at the 7th day of incubation. On the other hand archaeal communities at both band positions exist from the 3rd to 10th days of incubation (Fig. 6B).The number of bands indicates there are at least 7 major bacterial populations and 2 archaeal populations in the enrichment microbial community.

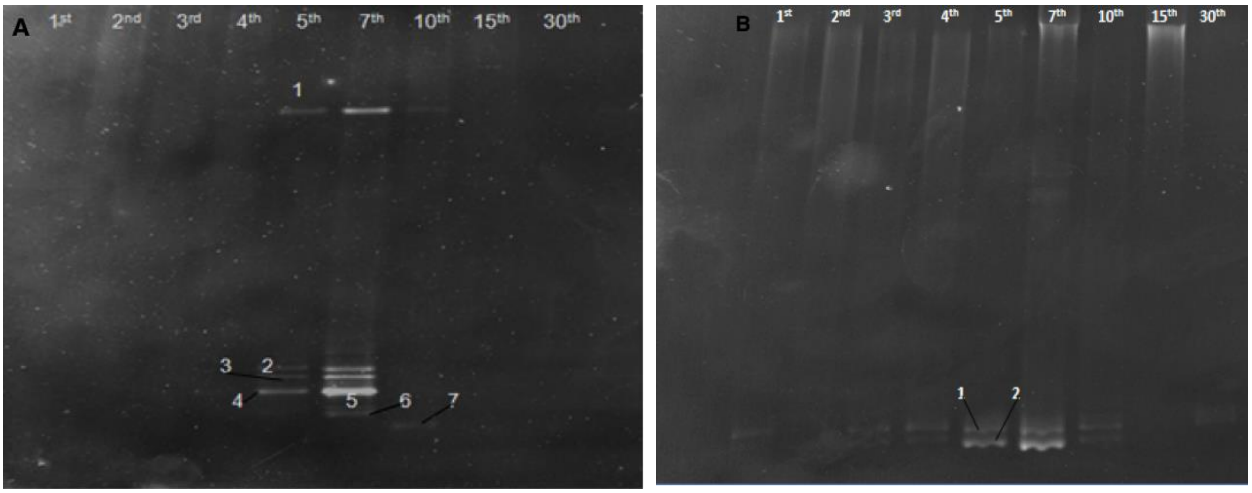

Figure 5. DGGE profile of the enrichment microbial communities showing the microbial community shift for 30 days. A:bacterial enrichment community; B: Archaeal enrichment communities.

5.5. Amplified Ribosomal DNA Restriction Analysis (ARDRA) grouping

To select representative isolates for sequencing restriction digestion of all PCR products was carried out using the restriction endonuclease enzyme, TaqI (Fig. 7). According to the 16S rDNA ARDRA profile (Appendix 6), the total possible operational taxonomic units (OTUs) obtained were 17 (7, 5 and 5 OTUs for 10%, 20 % and 25% salt concentration enrichments, respectively) (Appendix 7).

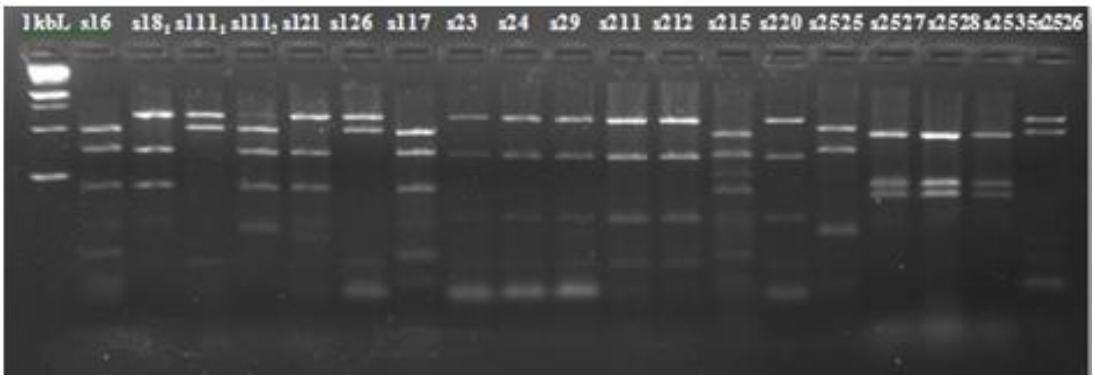

Figure 6. Image of restriction fragment patterns obtained using Taq I enzyme

5.6. Analysis of 16S rRNA gene sequence and phylogenic relationship

5.6.1. Domain and phylum level community abundance and composition

With 89 % (76 out of 85 isolates), members of bacterial domain were the most abundant (Fig. 8A). On the other hand, 11% (9 isolates) were found to be members of domain Archaea, affiliated to phylum *Euryarchaeota*. The P values were found to be 0.449 at 84 degree of freedom between the domains and 0.439 at 150 degree of freedom between the bacterial phylum abundance. All the 76 enrichment bacterial isolates characterized were affiliated to members of 3 bacterial phyla namely: *Actinobacteria*, *Firmicutes* and *Proteobacteria* (Fig. 8B). Members of *Firmicutes* and *Actinobacteria* were almost the same in abundance accounting for 45% (34 isolates) and 43% (33 isolates) of all bacterial isolates, respectively.

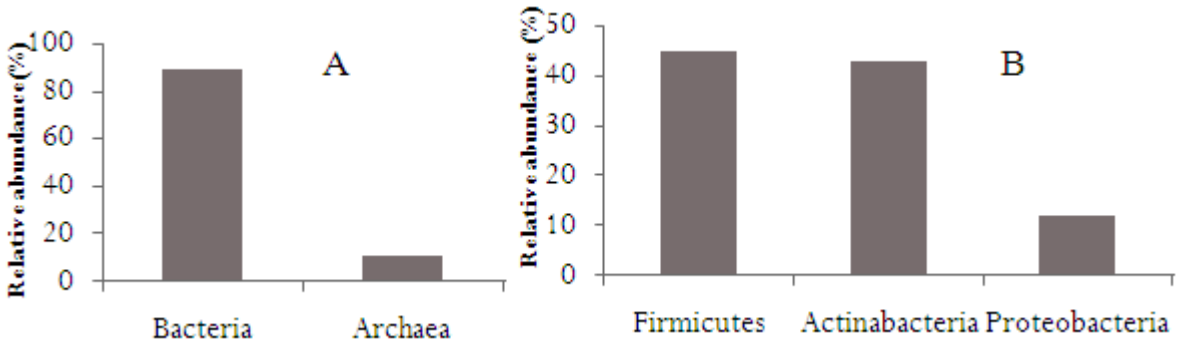

Figure 7. Relative abundance of microbial enrichment community; (A): Domain level, (B): Phylum level.

5.6.2. Genus level community abundance and composition

Based on the 16S rRNA gene sequences of representative isolates, all the isolates were grouped into six microbial genera, affiliated with known members of halophilic microbes (Table 3).The six microbial genera obtained were *Halomonas*, *Bacillus*, *Salimicrobium*, *Nesterenkonia*, *Marinococcus* and *Halococcus*. Member of the genus *Nesterenkonia* was the most abundant isolates 39% (33 isolates) followed by *Bacillus* 21% (18 isolates) and *Marinococcus* 15% (13 isolates). Members of the genera *Halomonas* and *Bacillus* were restricted to 10% medium while members of *Halococcus* and *Marinococcus* were restricted to medium with 25% salt concentration. On the other hand, members of the genera *Salimicrobium* and *Nesterenkonia* were found at two different salt concentrations.

Table 3. Genus level abundance of halophilic microbial isolates

S. No.	Genus	Salt conc. (%)			**Total abundance**	
		10	**20**	**25**	**No.**	**%**
1	*Halomonas*	9	-	-	**9**	11
2	*Bacillus*	18	-	-	**18**	21
3	*Salimicrobium*	1	-	2	**3**	3
4	*Nesterenkonia*	-	27	6	**33**	39
5	*Marinococcus*	-	-	13	**13**	15
6	*Halococcus*	-	-	9	**9**	11
Total		**29**	**27**	**29**	**85**	**100**

5.6.3. Phylogenetic analysis of bacterial isolates

The phylogenetic relationship of enriched bacterial isolates is presented in Figure 9A. Bacterial isolates affiliated with phylum *Firmicutes* were diverse grouped into three members of genera, namely *Bacillus, Salimicrobium* and *Marinococcus* (Appendix 1). The representative isolate, *Bacillus* sp. S181 representing 10 isolates of its ARDRA group, showed >99% 16S rRNA gene sequence similarity to its closest matches, *Bacillus* sp. M10 (2014) isolated from banana in Brazil (Souza *et al*., 2014), *Bacillus velezensis* strain AP183 isolated from cotton plant rhizophere in USA *(*Nasrin *et al*., 2015*)* and *Bacillus velezensis* strain WGB11 isolated from vegetable-soil of Baoding, Hebei (Lyu *et al*., 2018; GenBank).

Bacillus sp. s121 representing two isolates showed >99% 16S rRNA gene sequence similarity to *Bacillus* sp. strain PSUB1 isolated from Sewage sludge treatment plants (Poszytek *et al*., 2018 Gene Bank), *Bacillus amyloliquefaciens* strain ML274 isolated from multifloral honey (Sinacori, 2013 GenBank) and *Bacterium* strain ZY-7 isolated from *Dendrobium catenatum* as endophyte (Liu, 2017; GenBank).

Bacillus sp.s1112 representing one isolate showed >99 % 16S rRNA gene sequence identity to *Bacillus subtilis* strain CAB 1111 isolated from polluted river in South Africa (Benade *et al*., 2016), *Bacillus siamensis* strain JK-1 isolated from soil in China (Ouyang and Jiang, 2018

GenBank), and *Bacillus methylotrophicus* strain Psn214 isolated from *Panax ginseng* in China as endophyte (Lu, 2015; GenBank).

Salimicrobium sp. S126 represented one isolate showed >98%16S rRNA gene sequence identity with *Salimicrobium jeotgali* strain MJ3 isolated from salted, fermented Korean traditional seafood with the optimal growth on 10% salt concentration (Choi *et al*., 2014), *Salimicrobium salexigens* strain 29CMI isolated from salted hides in Spain (Rafael *et al*., 2011) and *Salimicrobium salexigens* strain YN7 isolated from the same habitat, salted sheepskins in Turkey (Caglayan *et al*., 2016).

Marinococcus sp. S2526 represented 13 isolates showed >99% identity of 16S rRNA gene sequence with *Marinococcus luteus* strain YIM 9109 isolted from soil from saline lake in China (Wang *et al*., 2007), *Nesterenkonia halobia* isolated from Salicornia, growing in Tunisian hypersaline soils and the isolate was found to be rhizobacteria, that have a potential for plant growth promotion (Mapelli *et al*., 2013) and *Marinococcus halophilus* strain HB68 isolated from 3500 years old wooden staircase in a salt mine in Austria (Pinar and Sterflinger, 2015 GenBank).

Bacterial isolates affiliated with phylum *Actinobacteria* were grouped into single genus, *Nesterenkonia* (Appendix 2). The representative isolate *Nesterenkonia* sp.s29 representing ten isolates of its ARDRA group showed identity >98% 16S rRNA gene sequence with *Nesterenkonia halophila* strain YIM 70179 isolated from saline soil in China (Li *et al.,* 2008), *Nesterenkonia halobia* strain DSM 20541 isolated from unrefined solar salt (Koch *et al*., 2015 GenBank) and *Nesterenkonia* sp. 118 JDE-2009 from the extreme environment presented by salt attacked monuments in Austria (Ettenauer, 2009 GenBank)

Nesterenkonia sp.s215 representing one isolate, showed >98% 16S rRNA gene sequence similarity to its closest matches *Nesterenkonia aethiopica* strain DSM 17733 isolated from the shore of Lake Abjata in Ethiopia (Delgado *et al*., 2006), *Nesterenkonia* sp. YIM 90725 *(*Zhi *et al*., 2006; GenBank) and *Nesterenkonia* sp. DY01 isolated from marin sedments of China *(*Huo, 2015; GenBank).

Nesterenkonia sp.s220 representing seven isolates of its ARDRA group showed >99% identity in 16SrRNA with its closest matches *Nesterenkonia sp.* Sl-1 isolated from Sambhar salt lake brine in India (Gehloth and Gaur, 2014 GenBank), *Nesterenkonia halobia* strain REG13 isolated from swamp and sandy soil in Ruoergai (Zhang and Chen, 2008. GenBank) and *Nesterenkonia flava* strain Nc5HA-1 isolated from surface seawater from the Drake Passage, Antarctica (Li *et al*., 2016 GenBank)

Nesterenkonia sp. s2525 representing six isolates is the only representative of genus *Nesterenkonia* isolated from 25% salt concentration. This isolate showed > 98% identity of 16S rRNA gene with closest matches *Nesterenkonia sp.* SK40 isolated from marine sponge (Tsubouchi *et al*., 2018; GenBank), *Nesterenkonia xinjiangensis* strain YIM70097 isolated from saline soils in the west of China (Li *et al*., 2004) and *Nesterenkonia suensis* strain Sua-BAC020 isolated from salt pan of South Africa (Govender *et al.*, 2002).

Bacterial isolates affiliated with phylum *Proteobacteria* were grouped into single genera, *Halomonas* (Appendix 3). *Halomonas* sp. S16 representing eight isolates of its ARDRA group showed >98% identity in 16S rRNA gene sequence with its closest matches *Halomonas halodenitrificans* strain L2 isolated from the same habitat of salted sheepskin in Turkey (Caglayan *et al*., 2016; GenBank), *Halomonas alimentaria* strain YKJ isolated from jeotgal, a traditional Korean fermented seafood (Yoon *et al*., 2002) and *Halomonas halodenitrificans* strain K2 isolated from salted goatskins (Caglayan *et al.,* 2016; GenBank).

Halomonas sp. S117 was a single isolate with >99% 16S rRNA gene sequence identity with *Halomonas halodenitrificans* isolated from salted sheepskins originating from different countries (Caglayan *et al.,* 2016; GenBank), *Halomonas halodenitrificans* strain DSM 735 (Miller *et al.,* 2017) and *Halomonas* sp. X45 isolated from Yuncheng Salt Lake of China (Li and Yu, 2015; GenBank).

5.6.4. Phylogenetic analysis of archaeal isolates

The phylogenetic relationship of enriched archaeal isolates is presented in figure 9B. Nine archaeal isolates were catagorized into two ARDRA groups and these two representative isolates were affiliated with extremely halophilic genera *Halococcus,* under phylum *Euryarchaeota* (Appendix 4). *Halococcus* sp. 2528 representing four isolates showed >99% identity of 16S rRNA gene sequence with its closest matches *Halococcus dombrowskii* strain H4 isolated from Permian alpine salt deposit in Austria (Stan-Lotter *et al*., 2002), *Halococcus dombrowskii* strain 519 isolated from salted hide in Turkey (Bilgi *et al.*, 2012; GenBank), *Halococcus morrhua* (Leffer and Garrett, 1984) and *Halococcus sediminicola* isolated from Marine sediment from the bay of Gangjin, Republic of Korea (Minegishi *et al*., 2014; GenBank).

Halococcus sp.2535 representing five archaeal isolates of its ARDRA group showed >99% identity in the 16S rRNA sequence with *Halococcus qingdaonensis* strain CM5 isolated from crude sea salt sample China (Wang *et al*., 2007), *Halococcus sp. Q65* isolated from Yipinglang Saline Mine, China (Chen *et at.*, 2017 GenBank), *Halococcus sp. CBA1201* isolated from plumage of captive flamingoes in Korea (Yim *et al.*, 2015) and *Halococcus thailandensis* strain JCM 13552 isolated from Fish sauce fermentation in Thailand (Minegishi *et al.*, 2015; GenBank)

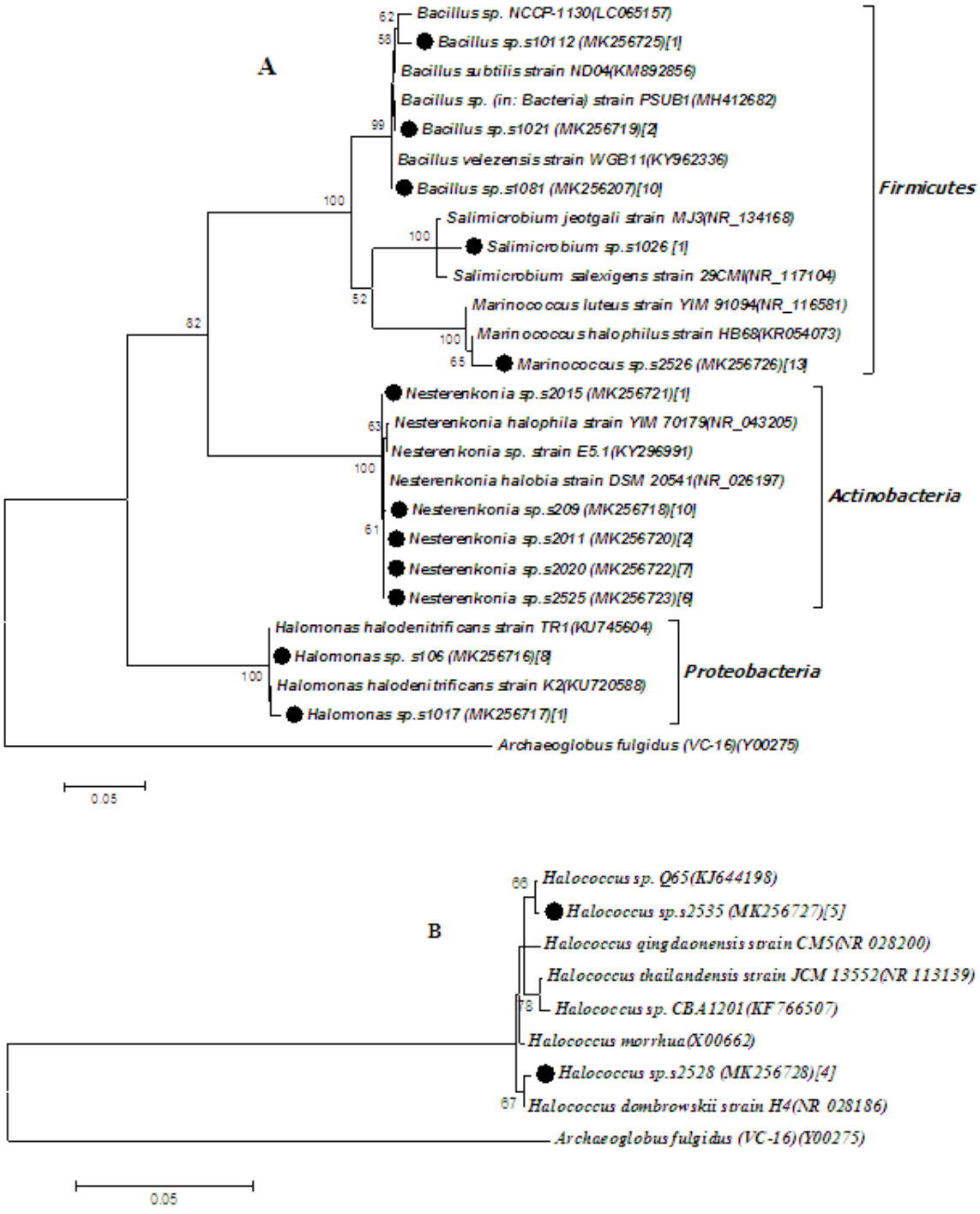

Figure 8. Phylogenic tree showing the evolutionary relation of enrichment microbial community. 16S rRNA gene sequence-based phylogenetic tree generated by using the Maximum like hood method based on the Jukes-Cantor model (Jukes and Cantor, 1969) showing the relationships between the strains studied and close matches. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees; only values greater than 50% are shown. Bar 0.05 number of substitutions per nucleotide position. Evolutionary analyses were conducted in MEGA6 (Tamura *et al* 2013). *Archeoglobus fulgidus* (vc-16) (y00275)was used as out-group. A: Enrichment bacterial community; B: Enrichment airchaeal community.

5.7. Degree of collagen hydrolysis by pure halophilic microbial isolates

The degree of collagen hydrolysis capability of all the 17 representative isolates sequenced were tested using chopped skin as the sole carbon and nitrogen source in the medium containing the salt concentration of their optimal growth. The degree of hydrolysis was monitored visually for 2 weeks (Table 4). Among the 17 isolates tested the archaeal isolate *Halococcus* sp.s2535 showed the maximum degree of hydrolysis of collagen (Fig. 10) followed by bacterial isolates *Nesterenkonia* sp.s211, *Marinococcus* sp. s2526 and archaeal isolate *Halococcus* sp. s2528. The rest isolates had lower degree of hydrolysis of collagen. Overall, isolates from 25% salt concentration microbial community were the most efficient collagen degraders and isolates from 10% salt concentration media were found to be the least degraders.

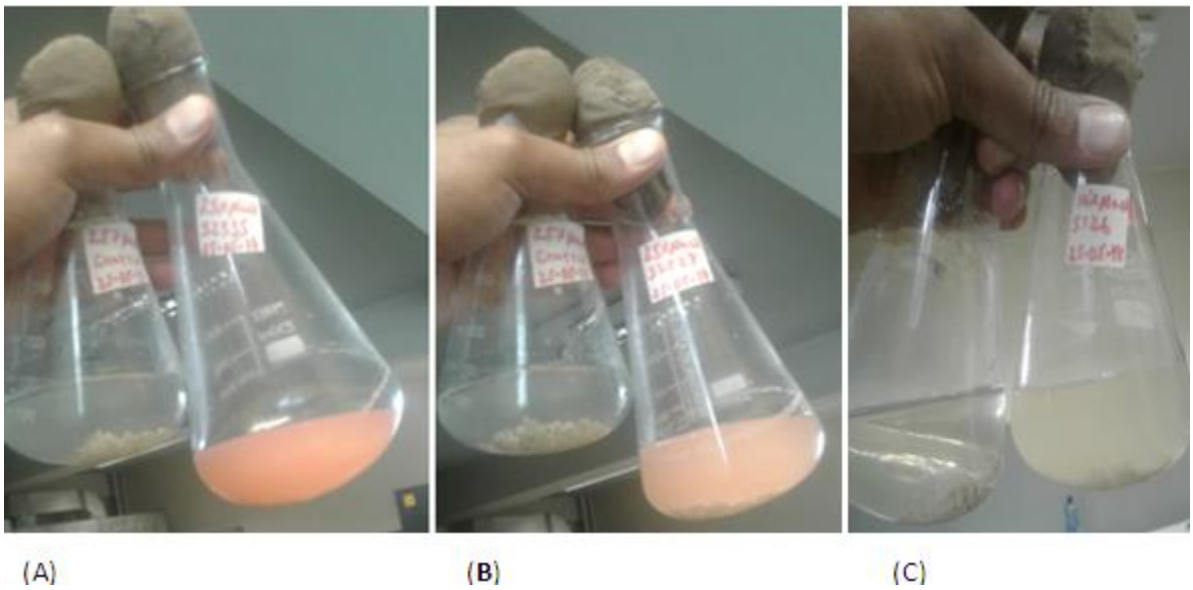

Figure 9. 15th day culture of the sheepskin hydrolysis test by individual isolates; A (*Halococcus* sp. s2535) best degrader and: B (*Halococcus* sp. s2528), and C (*Bacillus* sp. s126) examples of very good and good degraders respectively.(flasks on the left side; control and the right; inoculated)

Table 4. Degree of sheepskin degradation of the representative isolates

OUT group number	Name of isolate	Accession number	Salt concentration(%)	Degree of collagen degradation
1	*Halomonas* sp. s16	MK256716	10	**
2	*Bacillus* sp. s181	MK256207	10	*
3	*Bacillus* sp. s1111	---	10	**
4	*Bacillus* sp. s1112	MK256725	10	**
5	*Halomonas* sp. s117	MK256717	10	*
6	*Bacillus* sp. s121	MK256719	10	*
7	*Salimicrobium* sp. s126	---	10	**
8	*Nesterenkonia* sp. s23	---	20	**
9	*Nesterenkonia* sp. s29	MK256718	20	*
10	*Nesterenkonia* sp. s211	MK256720	20	***
11	*Nesterenkonia* sp. s215	MK256721	20	*
12	*Nesterenkonia* sp. s220	MK256722	20	**
13	*Nesterenkonia* sp. s2525	MK256723	20	*
14	*Marinococcus* sp. S2526	MK256726	25	***
15	*Salimicrobium* sp. s2527	MK256724	25	*
16	*Halococcus* sp. s2528	MK256727	25	***
17	*Halococcus* sp. s2535	MK256728	25	****

Key: *: Least degrader; **: moderate degrader; ***: very good degrader; ****: Maximum degrader

5.8. Biochemical and morphological characterization of pure isolates

The results obtained from biochemical tests and the morphological analysis are listed in Table 5. Most of the representative isolates tested were found to be gram positive. The catalase test revealed that all of the isolates were positive having the enzyme catalase. Of 17 representative isolates 9 of them showed gelatinase activity. Among the gelatinase positive isolates *Bacillus* sp.s1081, *Bacillus* sp.1112, *Nesternkonia* sp.s203 and *Halococcus* sp.s2535 were found to be extracellular gelatinase producer (Fig. 11). Any of the isolates did not show lipase activity. Casein was hydrolyzed by two strains namely *Bacillus* sp.s1111 and *Nesternkonia* sp.s220.

Figure 10. Gelatin liquefaction A; by *Halococcus* sp.s2535 cell and B; by the filtrate

Table 5. Biochemical and morphological characterization of representative isolates

Group number	Name of isolate	Pigmentation	Catalase Test	Gram Test	Lipase test	Gelatinase Test	Extracellular gelatinase Test	Casein hydrolysis
1	*Halomonas* sp. s16	Yellow	+	+	-	-	-	-
2	*Bacillus* sp. s181	Yellow	+	+	-	+	+	-
3	*Bacillus* sp. s1111	White	+	+	-	-	-	+
4	*Bacillus* sp. s1112	White	+	-	-	+	+	-
5	*Halomonas* sp. s117	Yellow	+	-	-	-	-	-
6	*Bacillus* sp. s121	Yellow	+	-	-	-	-	-
7	*Salimicrobium* sp. s126	Yellow	+	+	-	-	-	-
8	*Nesterenkonia* sp. s23	White	+	+	-	+	+	-
9	*Nesterenkonia* sp. s29	White	+	+	-	+	-	-
10	*Nesterenkonia* sp. s211	White	+	+	-	-	-	-
11	*Nesterenkonia* sp. s215	White	+	+	-	-	-	-
12	*Nesterenkonia* sp. s220	White	+	+	-	+	-	+
13	*Nesterenkonia* sp. s2525	White	+	+	-	+	-	-
14	*Marinococcus* sp. s2526	Yellow	+	+	-	+	-	-
15	*Salimicrobium* sp. s2527	White	+	+	-	-	-	-
16	*Halococcus* sp. s2528	Pink red	+	+	-	+	-	-
17	*Halococcus* sp. s2535	Pink red	+	-	-	+	+	-

Key: +: Positive for the test; –: Negative for the test

5.9. Growth of pure isolates at different salt concentration

The growth of each representative isolate on three different salt concentrations were tested (Table 6). Surprisingly, all of the isolates including those originally isolated at 20% and 25% salt concentration medium were able to grow at 10% salt concentration medium. However, except one isolate, *Salimicrobium* sp. s126, all the isolates originally isolated from 10% salt medium were unable to grow at

20% and 25% salt concentration medium. On the other hand isolates isolated at 25% salt concentration medium were able to grow at lower salt concentrations, 10% and 20%.

Table 6. Growth of isolates in different salt concentration within 21 days of incubation

OUT representatives	Salt concentration isolated (%)	Grows on concentration of NaCl		
		10 %	20 %	25 %
***Halomonas* sp. s16**	10	✓	×	×
***Bacillus* sp. s181**	10	✓	×	×
***Bacillus* sp. s1111**	10	✓	×	×
***Bacillus* sp. s1112**	10	✓	×	×
***Halomonas* sp. s117**	10	✓	×	×
***Bacillus* sp. s121**	10	✓	×	×
***Salimicrobium* sp. s126**	10	✓	✓	×
***Nesterenkonia* sp. s23**	20	✓	✓	×
***Nesterenkonia* sp. s29**	20	✓	✓	×
***Nesterenkonia* sp. s211**	20	✓	✓	×
***Nesterenkonia* sp. s215**	20	✓	✓	×
***Nesterenkonia* sp. s220**	20	✓	✓	×
***Nesterenkonia* sp. s2525**	25	✓	✓	✓
***Marinococcus* sp. s2526**	25	✓	✓	✓
***Salimicrobium* sp. s2527**	25	✓	✓	✓
***Halococcus* sp. s2528**	25	✓	✓	✓
***Halococcus* sp. s2535**	25	✓	✓	✓

Key: showed growth at the salt concentration tested; x: didn't grow at the salt concentration tested

6. Discussion

There are misunderstandings and lack of sound scientific information concerning the cause of putrefaction or "red heat" damage of skins and hides among all stakeholders in Ethiopia. Almost all stakeholders involved in the tanning industry believed that salted skins and hides are immune from microbial putrefaction. This study attempts to give clear and sound scientific evidence about the cause and impact of "red heat" damage causing halophilic microbes on salted skins and hides using culture dependent molecular methods.

Local collectors add large quantities of salt (30-40% of total flesh weight) (Gudro, 2015) to protect putrefaction of skins and hides, especially in order to store for a long period of time to search for a better price. However, this study shows that high salt concentration did not prevent putrefaction of skins and hides by halophilic microbes unless hides and skins processed within a short period of time. On the contrary as salt concentration increases, halophilic microbes that cause "red heat" damage of salted skins and hides get a safe haven to proliferate alone and cause "red heat" damage of salted skins and hides.

The sheepskin slices in the enrichment culture with 10% salt concentration were degraded completely at 13thday of incubation followed by 20% and 25% NaCl concentration cultures. This indicates that the rate of microbial growth as well as degree of collagen degradation decreases as salt concentration increases. However, the complete degradation of the chopped sheepskins lately at 20% and 25% salt medium indicates that although salt slows down the growth of collagen degrading microbes, it cannot immune skins and hides against putrefaction by halophilic microbes. On the other hand, halophilic microbes, particularly halophilic archaea are slow growers that prefer poor medium which contains low concentration of nitrogen and carbon sources (Addis Simachew *et al*., 2016). This implies that when local collectors store salted skins and hides for long period of time in search of good price, slow grower halophilic microbes get sufficient time to reach an exponential phase by producing high amount of enzymes including proteases to mobilize high amount of nutrients for their growth.

As stated, enrichment microbial communities grown at 10% salt concentration degraded chopped skins that were given as the sole carbon and nitrogen sources within 13 days. However, any individual isolate from 10% salt medium didn't degrade the chopped sheepskin even at a very

good scale. This could be due to the fact that microbes in the enrichment microbial community cooperates each other to degrade the chopped sheepskins (Calgayan *et al*., 2015).

On the other hand isolates from 25% salt medium were highly efficient to hydrolyze the sheepskin individually. All of the isolates (from 25% salt medium) were able to grow at wide range of salt concentration from 10 to 25% salt media, an indication that these isolates are probably the true collagen degrading microbes that were also responsible for the complete degradation of sheepskins at the 10 % salt concentration enrichment microbial communities.

However, moderately halophilic microbes isolated from 10 and 20 % salt concentration enrichment microbial communities were unable to grow on medium, with higher salt concentration (25 % salt concentration) than they were isolated. Since moderately halophilic isolates couldn't survive at extreme salt concentration and extremely halotolerant microbes can grow at lower salt concentrations, the microbial community diversity would be very high at lower salt concentration. This suggests that skin degradation would be very high when the skin is preserved with lower salt concentration.

This study shows that almost all halophilic microbial isolates obtained in this study have contributions in the collagen degradation and putrefactions of salted skins and hides and "red heat" damage of salted skins and hides. And there was no significant difference in the abundance of archaea and bacteria domains and also in the abundance of phylum within bacterial isolates. However, bacterial and archaeal isolates obtained at 20% and 25% salt concentration such as *Halococcus* sp. s2535, *Nesterenkonia* sp. s211, *Marinococcus* sp. s2526 and *Halococcus* sp. s2528 were the most important microbes responsible for putrefaction and "red heat" damage of salted skins and hides. Previous reports also indicate that members of microbes associated with the above genera are halophiles isolated from various saline environments including salted skins and hides (Stan-Lotter *et al*., 2002; Wang *et al*., 2009; Edouard *et al*., 2014; Yim *et al*., 2015). Interestingly, archaeal isolates, *Halococcus* sp.s2535 *and Halococcus* sp. s2528, show growth at all salt concentrations with efficient degradation of sheepskins individually. Moreover, *Halococcus* sp. s2535 liquefied gelatin and it was also found to be extracellular gelatinase producer which suggests that it is the best degrader as well as it leads the skin to be more prone

to bacterial attack by supplying the partially digested nutrient. This indicates that these two archaeal isolates are probably the real causes of "red heat" damage of salt cured skins and hides.

Moreover, archaeal isolates *Halococcus* sp.s2535 *and Halococcus* sp. s2528 were the only isolates which have a typical red color known for identification of "red heat" damaged salted skins and hides by tanners in the world (Stuart *et al*., 1933). Hence, although other bacterial isolates, especially those grown at high salt concentration, have some degree of contribution in the "red heat" damage of salted skins and hides. On the other hand, the enrichment microbial community efficiently degraded salted sheepskin compared to the pure isolates that indicates there is synergic effect in that each isolate in the microbial consortia helps each other.

Besides, the extracellular gelatinase enzyme producing isolates; *Bacillus* sp. s181, *Bacillus* sp.1112, *Nesternkonia* sp. s23 and *Halococcus* sp. s2535 may help to decompose skin and those microbes with gelatinase negative and fast grower bacteria may intake available digested nutrients which implies that the hydrolysis would be very easy and fast. This supports the suggestion that the isolates probably help each other to degrade the skin. Again the lower salted skin would be at high risk to be hydrolyzed by microbes with diverse community.

Halococcus sp. s2528 and *Halococcus* sp. s2535, isolated from 25% salt concentration enrichment culture, presented the typical red color pigment. This red-pigmented halophilic member of the archaea, found in saline environment and salt deposits, and have many physiological characteristics similar to the *Halococcus* sp. and are indeed the closest relative of the isolated strain (Stan-Lotter *et al*., 2002; Wang *et al*., 2007; Yim *et al*., 2015). Thus, it may be predicted that both groups share the same habitat which is a high salt concentration and they are the source of the red color pigment on the "red heat" damaged skin and hide. Moreover, it confirms that the source of halophilic microbes that cause "red heat" damage of salted skins and hides is the salt itself, which added to prevent the putrefaction of skins and hides (Stuart *et al*., 1933; Akpolat *et al*., 2015).

Unfortunately, red pigment production by extremely halophilic archaea *Halococcus* sp. that results in the appearance of the characteristic red patches on salted skins or hides starts late in the growth phase of the microorganisms. During the exponential growth phase, up to seven days of

incubation on the broth media these organisms do not produce any red color (Stuart *et al*., 1933). Moreover, all of the halophilic bacterial isolates in the present study do not produce red pigment even after reaching to the stationary phase. This insures that the tanners lose a huge amount of money by taking the defected skins and hides from suppliers and processing them because they are unable to distinguish damaged skin and hide unless they observe the red pigment (Addis Simachew and Amare Gessesse, 2016). After being processed the cured skins/hides end up having very low grade leather, making the loss of huge resources.

The majority of the isolates in the present study were closely related to bacterial strains isolated from salt cured skins and various saline environments widely distributed in the world such as Turkey, China and Africa. Most of them showed 98% to 100% 16S rRNA gene sequence similarity with halophilic bacterial and archaeal strains isolated from various saline environments, even in the salt deposits. This implicates that these bacterial and archaeal strains are extremely halotolerant that can stay a long period of time in the salt crystals and might contaminate hides and skins cured (Stuart *et al*., 1933; Akpolat *et al*., 2015).

Most of the isolates obtained in this study, affiliated to members of genus *Salimicrobium*, *Halomonas, Marinococcus,* and *Halococcus* isolated in the study conducted in Valencia, Spain (Akpolat *et al*., 2015) from deteriorated salted sheepskin. The deteriorated sheepskins in the experiment by Akpolat *et al*. (2015) were exhibited red and yellow discolorations, hair slips, slimy layers and bad odor. *Halomonas* sp., *Salimicrobium* sp. and *Marinococcus* sp. were also isolated from salted hides tanneries in Turkey (Caglayan *et al*., 2015).However, to the best of our knowledge, so far no members of genus *Bacillus* and *Nesterenkonia* reported from salted hides and skins suggesting that this study to be the first in reporting members of *Bacillus* and *Nesternkonia* from salted sheepskins.

All the isolates from 20% salt concentration enrichment culture were affiliated to members of the genus *Nesternkonia.* Moreover, 39% of the total isolate were affiliated to members of this genus which was isolated from Ethiopian soda lakes, *Nesterenkonia aethiopica* strain DSM 17733 with 98% identity (Delgado *et al*. 2015). However, it was reported that the optimal salt concentration for this isolate to be 2-3% salt which is less than the isolates obtained in this study (10-25% NaCl). On the other hand, *Nesterenkonia aethiopica* strain DSM 17733 was isolated from Soda

lakes which are alkaline with higher pH values compared to the isolate cultured in the present study. The other closest isolate with 99% identity is a gram positive moderately halophilic, *Actinobacterium, Nesterenkonia halophila* strain YIM 70179 isolated in saline soil of China (Li *et al.* 2008).

Among *Bacillus* strains isolated in the present study *Bacillus* sp. s181 and *Bacillus sp.* s1112 were obtained to be extracellular gelatinase producers, supporting the suggestion that these isolates supply partially digested nutrients for the fast growing microbes that facilitate the degradation of the skin by consuming the available nutrient.

On the other hand, the closest matches of isolates affiliated to genus *Bacillus* were isolated from plants as endophytes. Strain *Bacillus amyloliquefaciens* AP183 is a plant growth-promoting rhizobacterium (PGPR) isolated from a cotton plant rhizosphere and was found to be the closest match of *Bacillus* sp. s181 (99.4%). This strain was discovered to be resistance to methicillin, fosfomycin and fluoroquinolone and other antimicrobials (Nasrin *et al*., 2015). Besides *Bacillus* sp. M10 (2014) isolated from banana, which is a closest match (99.5%) with isolate *Bacillus* sp. s181 has exhibited antagonistic activities against *Fusarium oxysporum* and *Colletotrichum guaranicola*, with inhibitions ranging from 19 to 30% and 27 to 35%, respectively (Souza *et al*., 2014). This suggests that the isolated *Bacillus* species in this study may have applications as endophytes for adaptation of plants in saline environments, which is one of the major pressing problems that have been given much attention (Mapelli *et al*., 2013).

High number of visible dominant DGGE bands was observed in enrichment bacterial community compared with archaeal community. The number of bands increased through time and then declined. This shows that a certain group of microbial communities have been enriched and favored showing enrichment microbial community shift over time. The enrichment cultures were enriched using chopped sheepskin as sole nitrogen and carbon source suggesting that the common and dominant bands enriched over time might be the true collagen (the corium part of skin and hide) degrader microbial communities. Interestingly, the seventh day incubated microbial culture showed the dominant bands and then decreased drastically thereafter. This might be the fact that the enrichment microbial community shifted from bacteria to archaea. This is also supported by the presence of a relatively intense and more bands at the 10^{th} day of

incubation for the enrichment microbial communities amplified using archaeal DGGE primers compared to the same enrichment microbial communities amplified by using bacterial DGGA primers.

In this study, OTUs represented by isolates *Halococcus* sp. s2528 and *Halococcus* sp.s2535 were identical during ARDRA mapping, while in reality, these two groups show difference in their pigmentation as well as in their gram test. This supports the study conducted by Liu *et al*., (2008), who reported that the PCR-based ARDRA approach has low resolution display of microbial DNA fragments on gel. This indicates that it is difficult to state all OTUs in each ARDRA groups to be identical yet they are highly similar.

7. Conclusion

The “red heat” damage causing halophilic microbes isolated in this study belong to domains of bacteria and archaea to four different phyla, *Actinobacteria, Firmicutes, Proteobacteria* and *Euryarchaeota.* It can thus be concluded that halophilic microbes that cause “red heat” damage of salted hide and skin are highly diverse. Enrichment microbial community at all salt concentration fully hydrolyze the sliced sheepskin given as a sole carbon and nitrogen source so that it can be concluded that, the impact of halophilic microbes for the damage of salted skin and hide is very high. Archaeal strains obtained in this study are the major cause of “red heat” damage of salted skin and hide. Other bacterial strains including genus of *Bacillus, Salimicrobium, Marinococcus, Halomonas* and *Nesternkonia* are also responsible for the putrefaction process. The economical damage by “red heat” causing microbes is huge and faster when small amount of salt is added for preservation.

8. Recommendation

"Red heat" damage of salted skin and hide or "yekoda meklat" devalues or even destroy the leather industry's end product, which also lead the country to lose huge amount of money in the sector. Therefore, we suggest that inhibition of halophilic microorganisms in curing salt will help to avoid this problem. We also suggest that high salt concentration is better to use for preservation of hide and skin and the preserved material should not stay long for processing. High salt minimizes the degradation of the collagen by inhabiting the growth of fast grower microorganisms even it is heaven for slow grower true collagen degrader extreme halotolerants. Enzymes that can be active at high salt concentration have got high attention in their biotechnological applications. Hence, further work should be done to produce gelatinase enzyme from isolates; *Bacillus* sp. s181, *Bacillus* sp. s1112, *Nesternkonia* sp. s23, *Nesternkonia* sp. s29, *Nesternkonia* sp. s220, *Salimicrobium* sp. s2525, *Halococcus* sp. s2528, and *Halococcus* sp. s2535 and protease production from *Bacillus* sp. s1111 and *Nesternkonia* sp. s220 is needed.

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10. Appendix

Appendix 1. Table of Representative sequenced isolates in phylum *Firmicutes* with their closest matches.

Representative sequenced isolate	Accession No.	No. of isolates	Closest matches	Identity (%)	Accession No.	Habitat	Reference
Bacillus sp. S181	MK256207	10	*Bacillus sp. M10(2014)*	99.53	KM925011	Banana Brazil	Souza, *et al*., 2014
			Bacillus velezensis strain AP183	99.65	CP029296	Cotton Plant Rhizosphere USA	Nasrin *et al*., 2015
			Bacillus velezensis strain WGB11	99.65	KY962336	vegetable-soil of Baoding, Hebei	Lyu *et al*., 2018 GenBank
Bacillus sp. S1111,		5	*Bacillus sp. NCCP-1130*	96.4	LC065157	Murtazabad Hot water spring soil Pakistan	Amin *et al*., 2015 GenBank
			Bacillus subtilis strain JPS1-2	96.2	JQ308564	geocarposphere soil of peanut growing area China	Wang *et al*., 2013
			Bacillus subtilis strain 30L2-1	96.2	JN366756	A cocoa powder production line in the Netherlands	Lima *et al*., 2012
Bacillus sp. s121	MK256719	2	*Bacillus sp. (in: Bacteria) strain PSUB1*	99.04	MH412682	Sewage sludge treatment plants	Poszytek *et al*., 2018 GenBank
			Bacillus amyloliquefaciens strain ML274	99.03	KC692159	multifloral honey	Sinacori 2013 GenBank
			Bacterium strain ZY-7	99.03	KY073345	*Dendrobium catenatum*	Liu 2017 GenBank
Bacillus sp.s1112	MK256725	1	*Bacillus subtilis strain CAB 1111*	98.8	KJ194590	polluted river South Africa	Benade *et al*., 2016

			Bacillus siamensis strain JK-1	98.78	KY807042	Soil	Ouyang and Jiang, 2018 GenBank
			Bacillus methylotrophicus strain Psn214	98.78	KT253247	Panax ginseng	Lu 2015 GenBank
Salimicrobium sp. S126		1	*Salimicrobium jeotgali strain MJ3*	97.3	NR_134168	salted, fermented seafoodKoria	Choi *et al*., 2014
			Salimicrobium salexigens strain 29CMI	97.1	NR_117104	salted hides Spain	Rafael *et al*., 2011
			Salimicrobium salexigens strain YN7	97.44	KU726512	salted sheep skins Turk	Caglayan et al., 2016
Salimicrobium sp. s2527		2	*Salimicrobium luteum strain BY-5*	96.6	NR_043659	marine solar saltern in Korea	Yoon *et al*., 2007
			Salimicrobium flavidum strain ISL-25	96.5	NR_104548	marine solar saltern South Korea	Yoon *et al*., 2007
Marinococcus sp. S2526	MK256726	13	*Marinococcus luteus strain YIM 9109*	99.08	NR_116581	soil from saline lake China	Wang *et al*., 2007
			Nesterenkonia halobia	98.89	HF678772	Salicornia growing in Tunisian hypersaline soils	Mapelli *et al*., 2013
			Marinococcus halophilus strain HB68	99.18	KR054073	wooden staircase in a salt mine	Pinar and Sterflinger, 2015 GenBank

Appendix 2. Table of Representative sequenced isolates in phylum *Actinobacteria* with their closest matches.

Representative sequenced isolate	Accession No.	No. of isolates	Closest matches	Identity (%)	Accession No.	Habitat	Reference
Nesterenkonia sp. S23		7	*Nesterenkonia aethiopica strain RMR30*	90.75	KT731541	oil polluted sea water India	Mohanram *et al.,* 2016
			Nesterenkonia sp. strain E5.1	91.2	KY296991	east coast of Hainan Island, China	Yuan 2017 GenBank
Nesterenkonia sp. s29	MK256718	10	*Nesterenkonia halophila strain YIM 70179*	98.98	NR_043205	saline soil China	Li *et al.,* 2008
			Nesterenkonia halobia strain DSM 20541	98.8	NR_026197	unrefined solar salt	Koch *et al*., 2015 GenBank
			Nesterenkonia sp. 118 JDE-2009	98.53	FN435921	salt attacked monuments Austria	Ettenauer, 2009 GenBank
Nesterenkonia sp. s215	MK256721	1	*Nesterenkonia aethiopica strain DSM 17733*	97.68	NR_042989	Ethiopian soda lake	Delgado *et al*., 2006
			Nesterenkonia sp. YIM 90725	98.43	EF151510	China	Zhi *et al*., 2006 GenBank
			Nesterenkonia sp. DY01	97.51	KF434120	Marine sediment	Huo 2015 GenBank
Nesterenkonia sp.	MK2	7	*Nesterenkonia sp.*	99.25	KJ9316	Sambhar salt lake	Gehloth and Gaur, 2014

s220	56722		*Sl-1*		68	brine India	GenBank
			Nesterenkonia halobia strain REG13	100	EU647699	swamp and sandy soil in Ruoergai	Zhang and Chen, 2008. GenBank
			Nesterenkonia flava strain Nc5HA-1	97.46	KP296217	surface seawater from the Drake Passage, Antarctica	Li *et al*., 2016 GenBank
Nesterenkonia sp. s211	MK256720	2	*Nesterenkonia sp. 118 JDE-2009*	99.2	FN435921	salt attacked monuments	Ettenauer *et al*., 2009. GenBank
			Nesterenkonia halobia strain REG13	99.8	EU647699	soil of Ruoergai wetland	Zhang and Chen, 2008. GenBank
			Nesterenkonia massiliensis strain NP1	97.7	NR_144709	Homo sapiens feces	Edouard *et al*., 2014
Nesterenkonia sp. s2525	MK256723	6	*Nesterenkonia halophila strain YIM 70179*	99.63	NR_043205	saline soil China	Li *et al.,* 2008
			Nesterenkonia xinjiangensis strain YIM70097	97.24	NR_029075	saline soils in the west of China	Li *et al*., 2004
			Nesterenkonia suensis strain Sua-BAC020	97.7	NR_116866	salt pan South Africa	Govender *et al.,* 2002

Appendix 3. Table of Representative sequenced isolates in phylum *Proteobacteria* with their closest matches.

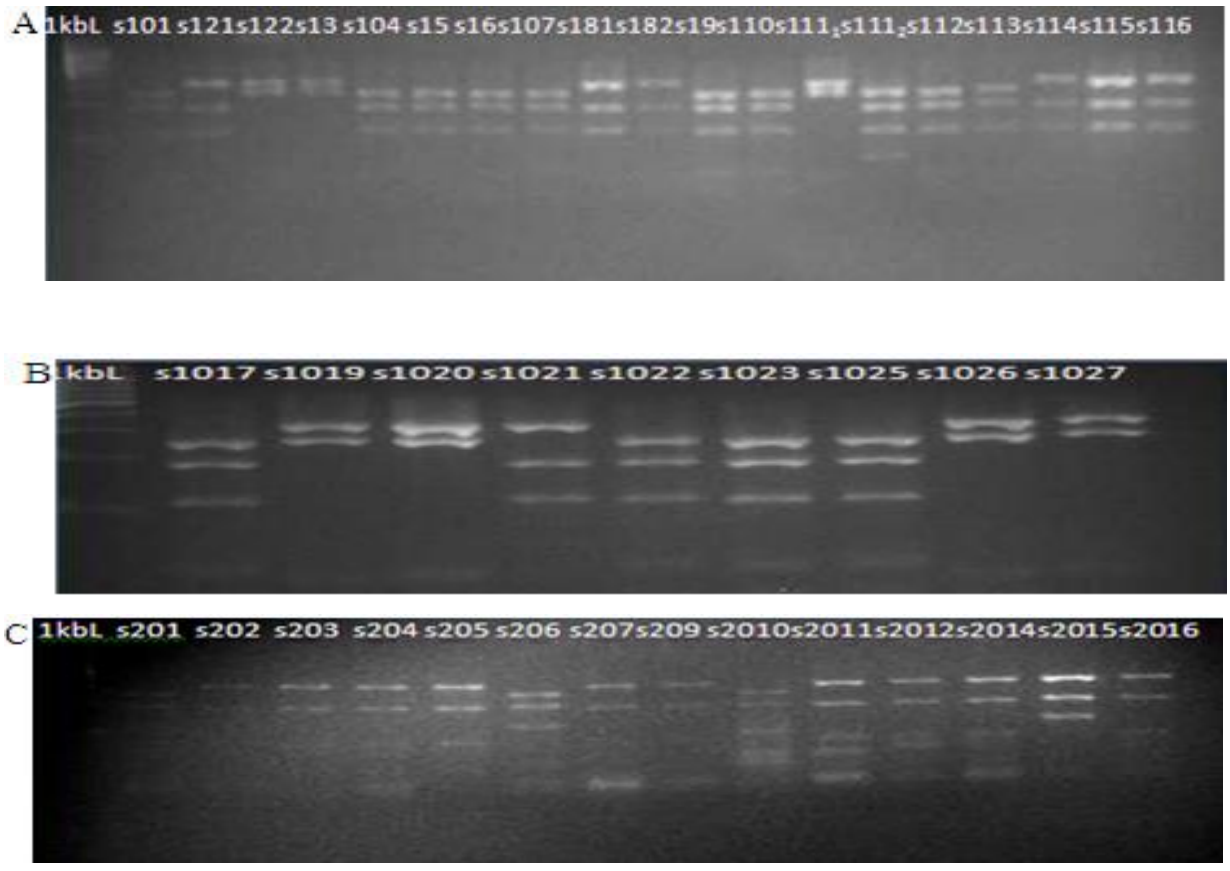
Representative sequenced isolate	Accession No.	No. of isolates	Closest matches	Identity (%)	Accession No.	Habitat	Reference
Halomonas sp. s16	MK256716	8	*Halomonas halodenitrificans strain L2*	99.8	KU745602	salted sheep skin Turkey	Caglayan *et al*., (2016) GenBank
			Halomonas alimentaria strain YKJ-	98	NR_025054	jeotgal, a traditional Korean fermented seafood.	Yoon *et al*., 2002
			Halomonas halodenitrificans strain K2	99.8	KU720588	salted goat skins	Caglayan *et al.*, 2016 GenBank
Halomonas sp. s117	MK256717	1	*Halomonas halodenitrificans*	99.63	KU745604	salted sheep skins originating in different countries	Caglayan *et al,*. 2016. GenBank
			Halomonas halodenitrificans strain DSM 735	98.9	NR_025890		Miller *et al.*, 2017
			Halomonas sp. X45	97.7	KM974797	Yuncheng Salt Lake China	Li and Yu, 2015 GenBank

Appendix 4. Table of Representative sequenced isolates in archaeal phylum *Euryarchaeota* with their closest matches.

Representative sequenced isolate	Accession No.	No. of isolate	Closest matches	Identity (%)	Accession No.	Habitat	Reference
Halococcus sp. 2528	MK256727	4	*Halococcus dombrowskii strain H4*	99.63	NR_028186	Permian alpine salt deposit Austria	Stan-Lotter *et al*., 2002
			Halococcus dombrowskii strain 519	99.84	JX481767	Salted Hide in Turkey	Bilgi *et al.*, 2012 GenBank
			Halococcus morrhua	99.28	X00662		Leffer and Garrett, 1984
			Halococcus sediminicola	98.26	AB935407	Marine sediment from the bay of Gangjin, Republic of Korea	Minegishi,*et al*., 2014 GenBank
Halococcus sp.2535	MK256728	5	*Halococcus qingdaonensis* strain CM5	98.99	NR_028200	crude sea salt sample China	Wang *et al*., 2007
			Halococcus sp. Q65	99.4	KJ644198	Yipinglang Saline Mine China	Chen *et at.*, 2017 GenBank
			Halococcus sp. CBA1201	99.3	KF766507	plumage of captive flamingoes Korea	Yim *et al.*, 2015
			Halococcus thailandensis strain JCM 13552	98.99	NR_113139	Fish sauce fermentation in Thailand	Minegishi *et al.*, 2015 GenBank

Appendix 5. Gel doc result of 338f and H1542r primers for ARDRA mapping and sequencing.

Appendix 6. Images of restriction fragment patterns obtained using Taq I enzyme (A&B; 10% NaCl isolates, C; 20% isolates, D; 25% isolates).



Appendix 7. ARDRA table: the table shows the isolates forming groups according to the cleavage pattern of Taq1 restriction enzyme and the salt concentration used for growth.

OUT	Salt concentration (%)	Isolate/s grouped in the same ARDRA	Representative isolate (sequenced)	Taxonomy
1	10	s11,s14,s15,s16,s17,s110, s122,s123	S16	*Halomonas sp. S16*
2	10	$s12_1$,$s18_1$, $s18_2$, s114, $s115_1$, $s115_2$, s119, s122, s123, s125	S181	*Bacillus sp. S181*
3	10	$S12_2$, s13, $s111_1$, s120, s127	S1111	*Bacillus sp. S1111,*
4	10	$s111_2$	S1112	*Bacillus sp.s1112*
5	10	s117,	S17	*Halomonas sp. S117*
6	10	S121, s19	S121	*Bacillus sp. s121*
7	10	S126	S126	*Salimicrobium sp. S126*
8	20	S22,s23, s218, s227, s230, s224, s228	S23	*Nesterenkonia sp.s23*
9	20	S25, s216, s217, s220, s24, s226,s229	S220	*Nesterenkonia sp.s220*
10	20	S27, s29, s222, s223, s210, s221, s225, S21, s26, s214,	S29	*Nesterenkonia sp.*
11	20	s211, s212	S211	*s29Nesterenkonia sp.s211*
12	20	S215	S215	*Nesterenkonia sp.s215*
13	25	S253, s2532, s2539, s2516, s2524, s2525,	S2525	*Nesterenkonia sp.s2525*
14	25	S257, s258, s259, s2511, s2512, s2513, s2514, s2515, s2518, s2520, s2526, s2530, s2544	S2526	*Marinococcus sp. S2526*
15	25	S2523, s2531, s2533, s2535,s2538	S2535	*Halococcus sp.s25*
16	25	s2527, s255	S2527	*Salimicrobium sp.s2527*
17	25	S2528, s2534,s2536, s254	S2528	*Halococcus sp. s252835*