

## Effect of microbial activities on stored raw buffalo hide

P.N. Shede<sup>1</sup>, P.P. Kanekar\*<sup>1</sup>, A.V. Polkade<sup>1</sup>, S.S. Sarnaik<sup>1</sup>, P.K. Dhakephalkar<sup>1</sup>,  
S.A. Chiplonkar<sup>2</sup> and S.S. Nilegaonkar<sup>1</sup>

<sup>1</sup>Microbial Sciences Division, <sup>2</sup>Biometry and Nutrition Group, Animal Sciences Division,  
Agharkar Research Institute, G. G. Agarkar Road, Pune - 411 004, India

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**Abstract:** 'Keeping qualities' of hides are dependent on the total microbial flora associated with the hides and the biochemical changes brought about by these microorganisms during short-term storage at ambient temperature ( $28 \pm 2^\circ\text{C}$ ). It was evident that within first 24 hr of hide's ambient storage, bacterial load was raised to  $8.8 \log \text{cfu g}^{-1}$  hide from  $6.1 \log \text{cfu g}^{-1}$  hide. Nonlinear parabolic increase in release of hydroxyproline and tyrosine from stored hide was observed starting from 0 hr and confirming proteolytic activities. Continuous release of  $\text{CO}_2$  from the stored hide suggested its mineralization. Exponential release of free fatty acids during storage indicated simultaneous lipolysis. Thus the process of biodegradation during the course of ambient storage of hide piece was found to progress steadily and seems to be interrelated as well as very complex. During the storage period, the liquefaction of hide piece was also observed visually within 96 hr. Present studies of assessment of bacterial activities on hide with respect to total bacterial load, release of amino acids, free fatty acids and evolved  $\text{CO}_2$ , provide data that can be used to formulate and evaluate hide curing agent(s) other than salt, thus rendering leather industry a platform to design bio-based technologies for efficient and ecofriendly preservation of raw materials.

**Key words:** Buffalo hide, Leather, Degradation, Hydroxyproline, Tyrosine, Ambient storage  
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### Introduction

Food and Agriculture Organization of the United Nations states that in the year 2005, total of 1.7 million pieces of raw hides were produced worldwide, out of which more than 1.6 million pieces were from Asian region. Asian water buffaloes and African wild buffaloes are considered to be the major source of hides. Out of the total buffaloes in the world, 97% are water buffalo primarily found in Asia and more than half of them are in India. Traditionally being a prominent exporter of leather to OECD market India has an influence on the leather trade world over (Rajaraman, 1993).

Earlier, the tannery effluents were proved to have acute toxic effects on human beings (Sahu *et al.*, 2007), animals (Muley *et al.*, 2007) and plants (Nath *et al.*, 2005, 2009). Therefore it was necessary for tanners to modify the conventional processes so as to minimize the polluting elements present in the effluents (Soyaslan and Karaguzel, 2008). Salting is the traditional method used for preservation of raw animal hide wherein ~35 to 40% (w/w) salt is used immediately after flaying of the animal. Reapplication of salt after 24 h is also a common practice. Presently the salt curing process is being challenged mainly because of presence of  $10^1 - 10^4 \text{cfu ml}^{-1}$  live bacteria in soak liquors (Birbir *et al.* 2008) and due to pollution caused by the effluent containing high amounts of chlorides and total dissolved solids (Kanagraj *et al.*, 2005). In order to have an

alternative method to preserve the animal hide by eliminating salting, it is quite necessary to know everything about the material to be preserved *i.e.* the changes occurring in the material when stored without any preservative and the components responsible for such type of changes etc. Ever since the science of leather manufacturing has been evolved, many researchers have extensively studied chemistry of tanning but the process of curing was neglected. The present information available on raw buffalo hide, as a material to be preserved is not adequate to develop and evaluate the alternative preservatives.

Raw buffalo hide is a heterogeneous fibrous mass. It has been proved that skins and hides vary chemically not only from species to species but also with respect to location in the same animal (Muthian *et al.*, 1968). Raw buffalo hide is a biological matrix substantially loaded with protein (~33 percent) and the amount of fat or triglycerides (~2 percent) is also significant (Sharphouse, 1983). Considering this, the degradation of processed and/or unprocessed hide is to be attributed essentially to the proteolytic and lipolytic enzymes since some of the enzymes like esterase, phosphatase, oxidase, dehydrogenase etc. are known to be associated with animal skin/hide (Tancous and Jaysimhulu, 1973; Deasy *et al.*, 1968). Role of collagenase and other proteolytic enzymes in the dispersal of animal tissues was discussed by Kono (1969). Many researchers long back established this type of correlation in preserved hides (Tancous and Jaysimhulu, 1973) and processed leather (Birbir and Ilgaz,

\* Corresponding author: [kanekarp@rediffmail.com](mailto:kanekarp@rediffmail.com)

1996), which was portrayed mainly as an effect of microbial activities. Once the hide is removed from the animal, the inner surface of the hide becomes contaminated, bacteria penetrate the hide and their action rapidly overtakes that of autolysis (Haines, 1981). Chakraborty and Chandra (1990) have stated that many of non-specific proteinase can attack and solubilize hides and leather.

The hide protein undergoes various changes during its ambient storage. Quantitative determination of these biochemical changes could aid our understanding of the various ways that the protein of hide is affected during storage. Overall knowledge gained about this biodegradative process and timely progression of the said process can be used to formulate hide curing agent other than salt. More specifically progression studies of biodegradation process can also be used to define indicators for evaluation of any preservative formulation aimed to substitute salt. In the present study largely activities occurring on unpreserved raw buffalo hide stored at ambient temperature ( $28\pm 2^\circ\text{C}$ ) were monitored. The main objectives of the investigation were to monitor the physical, biochemical and biological changes occurring in unpreserved raw buffalo hide stored at ambient temperature ( $28\pm 2^\circ\text{C}$ ) taking in to account the microbial as well as the autolytic enzymes associated with the raw hide, to quantify the biochemical changes occurring as a result of proteolysis and lipolysis with respect to liberated amino acids and fatty acids, to determine total mineralization through quantitative detection of evolved  $\text{CO}_2$  and to examine overall effect on physical properties and timely changes in them to confirm progression of putrefaction.

### Materials and Methods

**Collection of sample:** Raw buffalo hide was collected in clean sampling bags, from a Pune Municipal Corporation slaughterhouse immediately after flaying the slaughtered animal and transported to the laboratory in ice bucket maintaining the temperature  $2-4^\circ\text{C}$ .

**Enumeration of bacteria:** The total viable count (TVS) of bacteria on raw buffalo hide stored at ambient temperature without any preservative was determined up to 144 hr at 24 hr intervals starting from zero hr. The method used for these enumerations was developed and validated by the authors (Shede *et al.*, 2008). The hide pieces of average weight 5 g and the size of 2.5 cm x 2.5 cm were placed in sterile petriplates without application of any preservative. The petriplates were kept at ambient temperature ( $28\pm 2^\circ\text{C}$ ) in a container wherein humidity was maintained. On each consecutive day, the hide pieces were transferred to conical flask containing 100 ml sterile saline (0.85 percent). The flasks were then kept on rotary shaker (100 rpm) at ambient temperature ( $28\pm 2^\circ\text{C}$ ) for 3 hr. The hide wash was then serially diluted. Selected dilutions were then plated on standard plate count (SPC) agar. The plates were incubated at

$37^\circ\text{C}$  and the number of colonies were counted after 48 hr. Total viable count of bacteria were determined in terms of colony forming units (CFU) per  $\text{cm}^2$  area of hide. The enumeration was made in duplicate and was repeated thrice as Hide I, II and III.

**Percentile count of bacteria:** To determine the percentile proportion of proteolytic and lipolytic bacteria, unit number of isolated colonies from the SPC plates were replicated on milk agar *i.e.* nutrient agar containing 1% (w/v) skimmed milk powder (for proteolytic) and tributyrine agar *i.e.* nutrient agar containing 1% (w/v) tributyrine (for lipolytic) using replica plate technique (Shede *et al.*, 2008).

**Biochemical changes:** The proteolysis and lipolysis occurring on the hide was quantitatively determined in terms of units of protease activity and units of lipase activity respectively. The hide wash prepared to enumerate the bacterial load was treated as an enzyme solution for these studies. Protease activity was assayed till 72 hr (Kanekar *et al.*, 2002) using casein as the substrate. One unit of protease activity (U) was defined as the amount of enzyme required to liberate  $1\ \mu\text{g}$  of tyrosine per min at  $37^\circ\text{C}$  and expressed as Units  $\text{ml}^{-1}\ \text{min}^{-1}$ . Tyrosine and hydroxyproline were also estimated as the products of proteolysis of total protein and collagen respectively. Before estimating the liberated amino acids from the suspension, it was treated with 5 percent TCA to precipitate proteins and polypeptides. The colorimetric method was used to estimate the liberated hydroxyproline (Woessner, 1961). Tyrosine liberated was estimated by colorimetric method (Lowery *et al.*, 1951).

Lipase activity in the hide wash was assayed titrimetrically till 72 hr on the basis of substrate hydrolysis (Dellamora-Ortiz *et al.*, 1997). The substrate used was tributyrine and the substrate emulsion was prepared in 2% polyvinyl alcohol (PVA) solution. PVA was dissolved in distilled water by boiling. One part of tributyrine and 4 parts of 2% PVA were mixed in glass beaker and sonicated for 5 min. Standardization of sodium hydroxide was carried out according to Standard methods (APHA, 2005). Five ml of substrate emulsion and 4 ml of phosphate buffer (0.1 M, pH 6.5) were added in test tubes and vortex mixed. The reaction mixture was incubated at  $37^\circ\text{C}$  for 10 min. One ml of enzyme solution was added to the reaction mixture and the incubation was continued further for next 20 min. To arrest the reaction, 20 ml of 1:1 acetone-alcohol was added after completion of 20 min. Release of fatty acids was estimated by titrating against standardized sodium hydroxide using phenolphthalein as an indicator. Blank was prepared using heat inactivated enzyme. One unit of the lipase activity was defined as the amount of enzyme required to liberate  $1\ \mu\text{mol}$  of free fatty acid per minute at  $37^\circ\text{C}$  and expressed as Units  $\text{ml}^{-1}\ \text{min}^{-1}$ . Liberated free fatty acids were estimated as a product of lipolysis by the above mentioned titrimetric method.

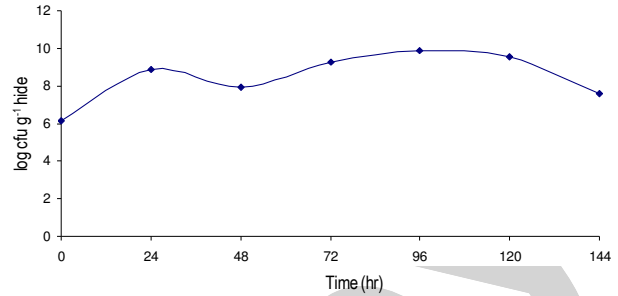
**Detection and determination of CO<sub>2</sub> evolved:** The CO<sub>2</sub> evolved was detected as a measure of mineralization. The hide pieces of average weight 1 g and the size of 0.5 cm x 0.5 cm were placed in sterile serum bottles (capacity 60 ml) along with appropriate controls. The serum bottles were sealed and kept at ambient temperature (28±2°C) in a container wherein humidity was maintained. On each consecutive day, the headspace of serum bottle was analyzed for the presence of CO<sub>2</sub> using gas chromatograph (Perkin Elmer, U.S.A.) equipped with thermal conductivity detector (TCD) using Porapak Q column and Hydrogen as carrier gas at the flow rate of 30 ml min<sup>-1</sup> (Bhadbhade *et al.*, 2002). The estimations were made in duplicate and the experiment was repeated thrice.

**Statistical analysis:** The data obtained for liberated hydroxyproline, tyrosine, free fatty acids and evolution of CO<sub>2</sub> from raw hide was statistically analyzed using SPSS Version 11.0. Average response for release of amino acids, FFA and evolved CO<sub>2</sub> was plotted with respect to time factor. The nature of response curve was determined by applying non-linear regression.

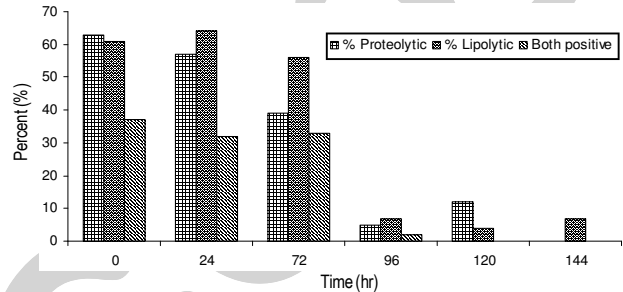
**Physical change(s) during preservation:** The hide pieces of average weight 5 g and the size of 2.5 cm x 2.5 cm were placed in sterile petriplates. The petriplates were kept at ambient temperature (28±2°C) in a container wherein humidity was maintained. On each consecutive day the hide pieces were observed for any physical change(s) with respect to three parameters as putritive odour, epidermis removal and hair loosening.

**Results and Discussion**

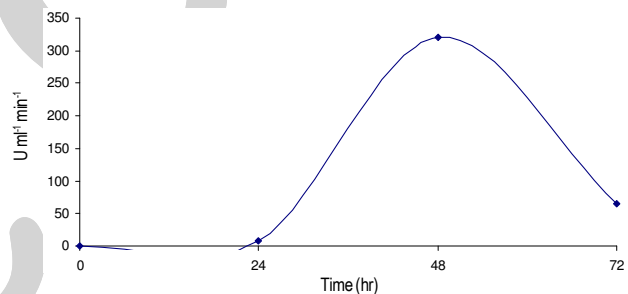
During ambient storage of raw hide, its putrefaction is essentially attributed to degradation of heterogeneous fibrous matrix. Many a times, aerobic and facultative anaerobic organisms are found to be associated with the raw buffalo hide, which can cause only partial hydrolysis of hide proteins. Bacterial growth varies from hide to hide depending upon hide environment, sampling site and pre- and post mortem history (Reid *et al.*, 2002a). Since the raw buffalo hide is rich in protein and contains high amount of water with some amount of fats, mineral salts and have pH near neutrality, the hide becomes a congenial medium for bacteria to grow. Birbir and Ilgaz (1996) also have carried out studies on bacterial flora of the hide samples and the chemical and histopathological changes occurring in the hide samples. The authors found that various bacterial species were associated with the hide samples and among them the bacterial community belonging to the genus *Bacillus* caused deterioration of hide samples which in turn adversely affected the quality of leather. Predominant bacteria present in the cattle hide were characterized by Hanlin and co-workers (1995) and the authors have also demonstrated the use of bacteriocin based preservative for



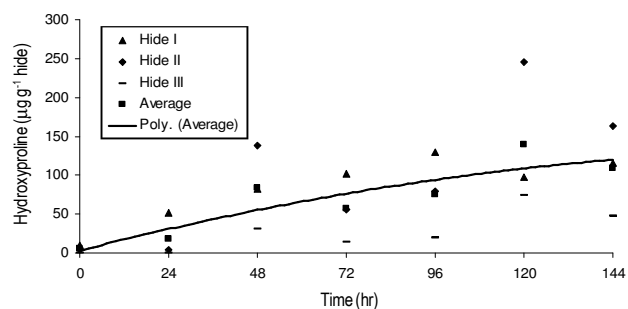
**Fig. 1:** TVC of bacteria on raw buffalo hide at different time intervals during storage



**Fig. 2:** Percentage of proteolytic and lipolytic bacterial isolates in the successive microbial communities



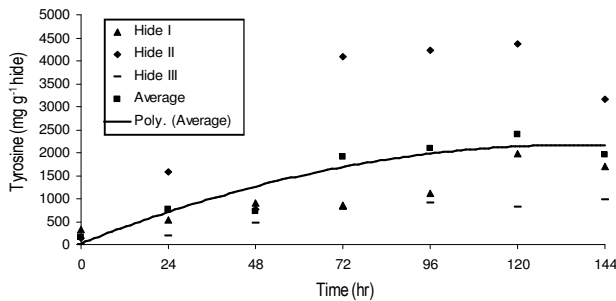
**Fig. 3:** Protease activity during storage of raw buffalo hide



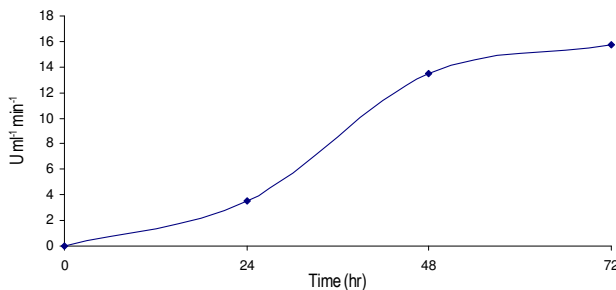
**Fig. 4:** Nonlinear (Parabolic) increase of liberated hydroxyproline with time \*Poly (Average) = polynomial (second order) fitted to average response

inhibition of these bacteria. Presence of food born pathogens was described by Reid and others (2002b).

**Enumeration of bacteria :** Conventionally, wet/dry swabbing or hair clipping is the sampling method used to study the skin microflora. In order to have a true picture of the bacterial load associated with raw buffalo hide, entire hide pieces were used in the present studies to account for not only the bacteria present on the hide but also the



**Fig. 5:** Nonlinear (Parabolic) increase in liberated tyrosine with time  
\*Poly (Average) = polynomial (second order) fitted to average response



**Fig. 6:** Lipase activity during storage of raw buffalo hide

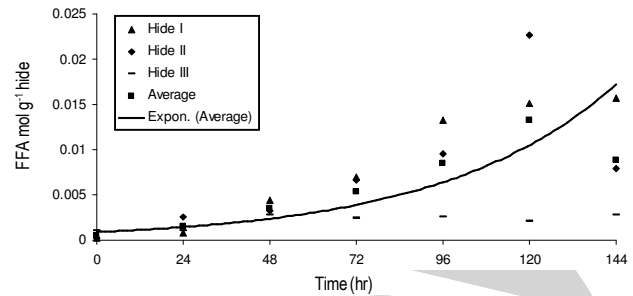
bacteria contributed by intestinal contents of the animal, flaying operations, handling of flayed hide etc. (Reid *et al.*, 2002a,b; Hanlin *et al.*, 1995).

The bacterial load was found to increase from 6.1 log cfu g<sup>-1</sup> hide to 8.8 log cfu g<sup>-1</sup> hide within first 24 hr of ambient storage. Subsequently, it increased 10 fold to 9.8 log cfu g<sup>-1</sup> hide at 96 hr and was 9.5 log cfu g<sup>-1</sup> hide at 120 hr. Later on, it declined to 7.5 log cfu g<sup>-1</sup> hide at 144 hr (Fig. 1).

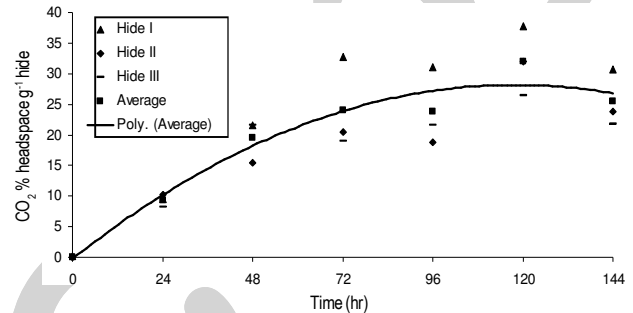
Birbir *et al.* (2008) have described the presence of mesophilic Gram -ve bacterial population to the extent of 10<sup>1</sup> – 10<sup>4</sup> cfu ml<sup>-1</sup> and also the halotolerant bacterial load of the magnitude 10<sup>3</sup> - 10<sup>4</sup> cfu ml<sup>-1</sup> in both main and first hide-soak liquors exhibiting proteolytic and lipolytic activities. The authors have attempted application of direct electric current to inactivate this bacterial population and minimize the metabolic activity of proteolytic and lipolytic bacteria so as to improve the quality of leather.

#### Percent population of proteolytic and lipolytic bacteria:

The proteolytic and lipolytic bacterial isolates constituted close to 60% of the microbial population associated with raw buffalo hide at zero hr while 37% of the bacterial isolates produced both, protease and lipase. However, this population decreased with the time. At 72 hr, 39% of the isolates were the protease producers while 56% were the lipase producers. Both proteolytic as well as lipolytic bacterial counts considerably decreased after 72 hr incubation of the hide, indicating that the proteolytic and lipolytic bacteria played an important role in the initial phase of hide decay (Fig. 2). These results are in accordance with the findings



**Fig. 7:** Exponential increase in liberated free fatty acids (FFA) with time  
\* Expon. (Average) = exponential (second order) fitted to average response



**Fig. 8:** Nonlinear (Parabolic) increase of CO<sub>2</sub> evolved with time  
\*Poly (Average) = polynomial (second order) fitted to average response

of Birbir *et al.* (2008) who have described the prevalence of proteolytic and lipolytic mesophilic and halotolerant bacteria in main hide-soak liquor as compared to first hide-soak liquor. However, continued increase in total bacterial load even after 72 hr indicated that the non-proteolytic and non-lipolytic bacteria thrived on raw buffalo hide after initial prominence of proteolytic bacteria on the raw buffalo hide.

**Biochemical changes:** Structural proteins viz. collagen (29%), elastin (0.3%) and keratin (2%), being the major components of raw buffalo hide are likely to be affected by proteolytic enzymes. Collagen, a key protein from hide is characterized by the presence of hydroxyproline, an amino acid whose occurrence is confined exclusively to collagen (Siddiqi and Alhomida, 2003). The total protease activity was manifested within 24 hr and was present till 72 hr (Fig. 3).

The activity was found to be highest during 48 hr. In the initial phase of storage of hide, there was less release of hydroxyproline, while the release suddenly increased at 48 hr. Increase in the release was then observed at 120 hr with a subsequent decrease in the released hydroxyproline level at 144 hr (Fig. 4). Muthian and others (1968) have demonstrated the biochemical changes occurring in the skin samples collected from different sites from different animals.

The average response of liberated hydroxyproline with respect to time was found to be parabolic in nature and followed an equation  $y = -0.0028t^2 + 1.2218t + 3.049$  with  $R^2 = 0.798$  ( $p = 0.041$ ). Quantification of tyrosine, an amino acid could be the measure

of both structural and non-structural protein degradation. The release of tyrosine was considerably increased within 24 hr followed by slight decrease at 48 hr. Further, a marked increase in the tyrosine level was observed which continued till 72 hr and after that it remained constant (Fig. 5). The results in the present paper are similar to those reported by other workers. Kono (1969) has described the role of collagenases and other proteolytic enzymes in the dispersal of animal tissues.

The average response of liberated tyrosine with respect to time was found to be parabolic in nature and followed an equation  $y = -0.1151t^2 + 31.452t + 20.312$  with  $R^2 = 0.89$  ( $p = 0.012$ ).

Lipolysis of fat from hide will result in release of the free fatty acids in the microenvironment and their quantification is the measure of lipolysis occurring on hide. The total lipase activity was found to be continuously increasing right from 0 hr till 72 hr (Fig. 6).

The presence of bacteria could be the one reason for profuse activity of degradative enzymes viz. protease and lipase in initial phase of ambient storage *i.e.* during 0 hr to 48 hr. A continuous increase in the level of released free fatty acids was observed from 0 hr to 120 hr (Fig. 7). The average response of liberated FFA with respect to time was found to be exponential in nature and followed the equation  $y = \exp(-7.0328 + 0.0206t)$  with  $R^2 = 0.857$  ( $p = 0.003$ ). The release of free fatty acids (FFA) can be considered as the activity of lipases or esterases.

However in above studies, the direct quantitative estimation of total protease and total lipase enzyme takes into account the microbial as well as lysosomal enzymes.

**Detection and determination of CO<sub>2</sub> evolved:** In case of evolution of CO<sub>2</sub>, a continuous increase was observed from 0 hr to 120 hr with a slight decrease after that (Fig. 8).

The average response of CO<sub>2</sub> evolved with respect to time was found to be parabolic in nature and showed equation  $y = -0.002t^2 + 0.4818t - 0.2165$  with  $R^2 = 0.9562$  ( $p = 0.002$ ). The parabolic increase in the evolved CO<sub>2</sub> itself is an indication that the mineralization of biological matrix has progressed during storage period.

**Physical change(s) of hide:** An undemanding way to solicit putrefaction of raw buffalo hide is a visual observation. Any sort of physical damage is believed to be due to the degradative enzymes unless the hide has been tampered mechanically. The sequential changes in physical properties as existence of putrid odour, loosening of hairs and epidermis layer affirmed the onset of putrefaction immediately after 24 hr.

The continual decline in overall hide texture was evident by increased fluffiness in the due course of storage. This finally resulted in complete liquefaction of hide immediately after 144 hr.

Preservation of raw animal hide primarily depends upon the bacterial load associated with the animal hide, operational conditions during flaying and handling of the flayed hide. The biochemical changes can be attributed to the metabolic activities of the bacterial load. It can be concluded from the present studies that when the buffalo hide is stored at ambient temperature ( $28 \pm 2^\circ\text{C}$ ) without any preservative, the bacterial count increased; about 60% bacterial population was proteolytic and lipolytic in nature and the appreciable release of hydroxyproline, tyrosine and free fatty acids was the cumulative effect of bacterial metabolic activity. The data thus generated will be useful for developing an effective alternative to salt curing used for preservation of raw buffalo hide.

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