Buffalo Bulletin (April-June 2025) Vol.44 No.2
https://doi.org/10.56825/bufbu.2025.4424224

DISTINCTION OF FRESH FROM FROZEN-THAWED BUFFALO MEAT USING B-HYDROXYLACYL-COA-DEHYDROGENASES (HADH) ASSAY

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Received: 23 March 2021
Accepted: 19 June 2025

ABSTRACT

The present study aimed to differentiate the fresh from frozen-thawed buffalo meat in the supply chain using a simple enzyme-based method. In the buffalo meat supply chain, due to faulty cold chain maintenance and irregular power supply, the meat gets repeatedly thawed which ultimately leads to quality deterioration. The buffalo meat samples were divided into three groups namely fresh (no freezing and thawing), 1st FT (meat was frozen at -18±2°C for 48 h followed by thawing at 4±1°C for 12 h), and 2nd FT (1st FT meat was again frozen at -18±2°C for 48 h followed by thawing at 4±1°C for 12 h). The meat press juice was collected from meat samples for HADH enzyme activity estimation. The meat quality parameters (pH, antioxidant activity, water holding capacity, cooking loss, thawing loss, extract release volume, microbiological count, and color) were also determined for correlation with HADH enzyme activity using Pearson's two-tailed correlation. The enzyme assay showed that HADH concentration significantly ($P<0.05$) increased from 2.82±0.06 U/mL (fresh) to 6.77±0.05 U/mL and 9.63±0.06 U/mL for 1st FT and 2nd FT, respectively signifying its dependency on freeze-thaw cycles. The findings of meat quality parameters obtained for buffalo meat at different freeze-thaw cycles correlate significantly ($r >0.90$) with the HADH activity thereby indicating the deterioration of meat quality with an increase in the number of freeze-thaw cycles. Therefore, the HADH enzyme assay in meat press juice and meat quality parameters could be used to differentiate fresh from frozen-thawed buffalo meat.

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Keywords: *Bubalus bubalis*, buffaloes, buffalo meat, frozen-thawed buffalo meat, enzyme-based

INTRODUCTION

Quality assurance and maintenance for meat and meat products is a crucial component from the point of production to the point of consumption in the supply chain. The application of quality assurance maintains desired standards, ensuring consumers' safety and government regulatory authority requirements. Fresh meat is a perishable product, and the various intrinsic and extrinsic factors affect the quality and safety of meat during storage mostly utilizing chilling and freezing during long-distance transit and supply chain (Zhou *et al*., 2010). It means that cold chain maintenance during long-distance transit and supply chain is very important for assuring the quality and safety of the meat and meat products. Due to a lack of proper cold chain maintenance and erratic power supply during transportation, distribution, loading, and unloading of meat products, the chances of thermal abuse are more which ultimately affecting the quality and safety of meat and meat products. Temperature fluctuations arose due to thermal abuses causes repeated freezing and thawing of meat result in small ice crystals to melt forming larger one affecting cellular structure and drip loss (Wei *et al*., 2017). Moreover, this process also causes the release of certain enzymes, pigments, and pro-oxidative factors causing a significant physiological and biochemical change in meat, such as a loss of water-holding capacity, color and texture deterioration, and lipid and protein oxidation (Zhang *et al*., 2018).

Frozen thawed meat is supposed to be nutritionally inferior because increased drip loss causes the release of various nutritive compounds in meat. This is a major concern for consumers and retailers from a nutritive and economic point of view, respectively. Therefore, creating probabilities of adulteration of fresh meat with frozen-thawed meat. As per Visciano and Schirone (2021) adulteration of fresh meat with frozen-thawed meat is a very common fraudulent practice existing in the wet market as the consumer generally prefers fresh meat to frozen meat, due to their specific eating habits and preference of taste. It occurs frequently in wet markets as frozen-thawed meat fetches very low prices and a price difference of about 10 to 30% between cheaper frozen-thawed meat and freshly slaughtered or chilled meat (Cheung *et al*., 2015). So, there is a need for a method that can differentiate fresh from frozen and thawed meat and that can work particularly in resource-constrained settings within in very short period.

It is not easy to differentiate objectively in any circumstances between fresh from frozen-thawed meat leading to serious fraudulent practices in the meat marketing trade. Various methods based on different scientific principles have been reported for the distinction of fresh from frozen-thawed or chilled meat *viz*., sensory evaluation, enzymatic assay, comet assay, nuclear magnetic resonance, reflectance microscopy, etc. (Cheung *et al*., 2015; Nicolalde *et al*., 2006; Boerrigter-Eenling *et al*., 2017; Sen and Sharma, 2005). As per the available literature globally, among enlisted reliable methods, the enzymatic assay has been used widely as a method of choice for the distinction of fresh from frozen-thawed or chilled meat (Ballin and Lametsch, 2008). Enzymatic assay distinction of meat is based on the measurement of various enzyme activities released during mitochondrial membrane rupture

because of enlargement in ice crystals due to repeated freeze-thaw cycles. The β-hydroxyacyl-Coenzyme A-hydrogenase (HADH) activity can be measured spectrophotometrically and has been used to differentiate fresh and frozen beef (Nicolande *et al*., 2006), fish (Duflos *et al*., 2002), frog (Ramos *et al*., 2004), poultry (Boerrigter-Eenling *et al*., 2017), pork (Cheung *et al*., 2015) and shellfish meat (Fernandez *et al*., 1999). Moreover, Ottavian *et al.* (2014) used different analytical technologies, namely a portable visible/near-infrared spectrometer, a compact digital camera, and a texture analyzer for authentication of fresh /frozen-thawed west African goatfish (*Pseudupeneus prayensis*) fillets.

To the best of my knowledge, till date enzymatic assay method using the HADH enzyme has not been used for the distinction of fresh from frozen-thawed buffalo meat, hence representing the novelty of the present work. Therefore, this study was conducted to evaluate the applicability of the HADH enzyme assay as an analytical method for the distinction of fresh from frozen-thawed buffalo meat in the supply chain under limited resource conditions in a very short period.

MATERIALS AND METHODS

Animals and collection of muscle samples

The male buffalo reared under the same feeding and heeding conditions were randomly selected and *Halal* slaughtered at approximately 2.0 years of age with an average slaughter weight of 290.0±15.65 kilograms. After slaughter, the carcasses were dressed, and the *longissimus dorsi* muscle samples were collected immediately with hygienic precautions from the 10th to 11th rib region. The samples were packed in low-density polyethylene (LDPE) pouches and transported in an insulated icebox under controlled conditions to the laboratory.

Sample sets preparation

At 8 h of post-mortem, fat and connective tissues were removed from the muscle. Muscle was divided into 3.0 cm^3 chunk size by cutting perpendicular to muscle fibre orientation and divided into three groups namely fresh (no freezing and thawing of meat), 1st FT (meat was frozen at -18±2°C for 48 h followed by thawing at 4±1°C for 12 h) and 2nd FT (1st FT meat was again frozen at -18±2°C for 48 h followed by thawing at 4±1°C for 12 h). The time for freezing and thawing of buffalo meat was determined based on time to attain thermal centre during freezing, and time to thaw frozen meat at 4±1°C with consideration of thermal abuse conditions arose during the supply chain.

Collection and treatment of muscle press juice

Muscle chunks with approximate dimensions of 3.0 cm^3 were utilized for collection of press juice (2.0 mL) by compression method. The muscle cube was placed inside a stomacher which was positioned between two glass plates fitted with a T-clamp screw for exerting a pressure for three minutes in case of a fresh muscle sample and one minute in case of frozen-thawed sample. The press juice collected in the stomacher was transferred to a centrifuge tube with a sterile pipette and was centrifuged at 3000 rpm at 4°C for 5 minutes in centrifuge (Z 446, HERMLE Labortechnik, Wehingen, Germany) to precipitate debris and blood cells. 100 µL clear liquid aliquot of press juice was transferred into a 20 mL graduated tube and made up to the mark with 0.1 M of phosphate buffer (1:200 factor dilution). The treated muscle press juice solution was then utilized for the

spectrophotometric assay of the HADH enzyme.

Test indicators and methods

HADH enzyme Activity. HADH activity was determined as per the method described by Cheung *et al.* (2015) with slight modification. Measured 2.6 mL of 0.1 M phosphate buffer and 0.2 mL of 34.4 mM EDTA were added into a test tube, then it was mixed with an aliquot of 0.1 mL of diluted meat press exudate and 0.05 mL of 7.5 mM NADH. The mixture was shaken (Cyclo-mixer, HPCM-338, REMI equipment) for 10 seconds, transferred into a quartz cuvette, immediately followed by adding 0.05 mL of 5.9 mM Acetoacetyl-CoA. The obtained test solution was vortexed (Cyclo-mixer, HPCM- 338, REMI equipments) for 10 seconds and the cuvette was inserted into the cuvette holder in the UV-Vis spectrophotometer (Genesys 10 UV-Vis, Thermo Scientific, U.S.A). Measurements at 340 nm were recorded at the time, t = 1.0 and t = 4.0 minutes, with the temperature controlled at 20±5°C using the built-in thermostatted sample holders.

$$HADH\ activity = \frac{V}{\varepsilon\ X\ d\ X\ v}\ X\ k\ X \Delta E$$

The HADH activity was reported as mmole/minute/mL or unit/mL or U/mL where V = volume of the solution mixture in the cuvette (mL); ε = the extinction coefficient of NADH (6.22 $mmol^{-1}$ cm^{-1} at 340 nm); d = cuvette optical length (cm); v = diluted meat press volume (mL); ΔE = absorbance decreased rate per minute and k = dilution factor.

The absorbance decreased rate per minute, ΔE (min^{-1}) is calculated by:

$$\Delta E = (A0 - A3)/t$$

Where, A0 = Absorbance at 1 minute, A3 = Absorbance at 4 minutes and t = Reaction time (3 minutes).

pH value

The pH of the buffalo meat sample was determined by Troutt *et al.* (1992). Five grams of sample was blended with 25 ml of distilled water using Ultra Turrax Tissue Homogenizer (Model T-25, Janke and Kenkel, IKA Labor Technik, Germany) for 1 minute and the pH was determined by using a digital pH meter with a penetration probe and automatic temperature compensation probe (Hanna Instruments, HI200201, Woonsocket, RI-USA) standardized at pH 4, 7 and 10.

TBARS (2-thio barbituric acid reactive substances) value determination

The distillation method of Tarlagdis *et al.* (1960) was followed to estimate the TBARS value. Ten grams of meat sample was taken in a 100 mL beaker and blended with 50 mL distilled water in an Ultra Turrax tissue homogenizer (Model T25, Janke and Kenkel, 1 KA Labor Technik, Germany) for 2 minutes and slurry was quantitatively transferred to a 500 mL Kjeldahl flask to which was added with 45 mL of distilled water, 5 mL 6 N HCl, liquid paraffin to prevent frothing. A few glass beads were also added to prevent bumping during heating at 60°C and 50 mL distillate was collected in a graduated beaker. 5 mL of distillate and 5 mL of TBA reagent (0.02 M 2-thiobarbituric acid in 90% glacial acetic acid) were added to each test tube, blank was prepared using 5 mL of distilled water and 5 mL TBA reagent, tubes were kept in a boiling water bath for 35 minutes. The test tubes were cooled for 10 minutes under running tap water and the optical density (OD) was recorded at 538 nm using a spectrophotometer (GENESYS

10S UV-VIS, Thermo Scientific). The OD was multiplied by a factor of 7.8 and the TBARS value was expressed as mg malonaldehyde/kg of sample.

Water Holding Capacity (WHC)

WHC was calculated as weight loss percentage based on measurements before and after compression of meat and expressed as:

$$WHC\ (\%) = \frac{T1 - T2}{T1 \times 100}$$

Where, T1 = Initial weight of meat before application of pressure; T2 = Final weight of meat after application of pressure.

Thawing loss

The thawing loss was calculated by weighing different treatments of meat samples before freezing and after thawing.

$$Thawing\ loss\ (\%) = \frac{T1 - T2}{T1 \times 100}$$

Where, T1 = Meat sample initial weight before freezing; T2 = Meat sample final weight after thawing.

Cooking loss

5.0±1.0 g of meat sample was weighed, packed in a heat-stable LDPE pouch, and kept at 80°C for 30 minutes in a water bath. The surface of the meat sample was dried, weighed and calculated as follows:

$$Cooking\ loss\ (\%) = [(W2 - W3) / W2] \times 100$$

Where, W2 = meat weight before cooking (g) and W3 = meat weight after cooking (g).

Extract Release Volume (ERV)

ERV was determined for buffalo meat samples by following the method of Strange *et al.* (1977). 25.0 grams of buffalo meat was blended with 100 mL distilled water for 2 minutes in an Ultra Turrax tissue homogenizer (Model T25, Janke and Kenkel, 1 KA Labor Technik, Germany). The homogenate was poured directly into a funnel equipped with folded Whatman filter paper No.1. The filtrate collected in a 100 mL graduated cylinder for 15 minutes was taken as ERV and expressed in mL.

Aerobic Plate Count (APC)

APC of buffalo meat samples was determined using the APHA (2001) method.

Preparation of samples and serial dilutions

The meat sample was opened in an inoculation chamber of laminar flow (Model: RH-58-03 Science tech, India) pre-sterilized by ultra-violet (UV) radiation. Ten grams of sample was aseptically weighed and transferred to a pre-sterilized mortar containing 90 mL of sterile 0.9% saline solution. The sample was triturated using a sterile pestle for 2 minutes to uniform dispersion to obtain 10^{-1} dilution. To prepare 10^{-2} dilutions, 1 mL of this diluted solution was quantitatively transferred and then mixed uniformly in a test tube containing 9 mL of sterile 0.9% saline solution. Again 1 mL of 10^{-2} dilution was added to 9 mL 0.9% sterile saline solution and mixed to obtain 10^{-3} dilution and so on.

Procedure of aerobic plate count

Accurately weighed 23.5 g of Plate Count Agar was dissolved in 1000 mL of distilled water in a flask and plugged with gauze cotton and autoclaved at 15 psi for 30 minutes and cooled

to 45°C in a bacteriological water bath (NSW Model-133, India) till it was used. One mL of each serial dilution was transferred aseptically to sterile Petri plates in duplicate. The plates were then poured with 10 to 15 mL molten agar medium at 45°C. To ensure proper sample mixing, the Petri plates were rotated 10 to 12 times each in clockwise-anticlockwise directions and allowed to be set. After solidification, the Petri plates were incubated at 37°C for 48 h. After incubation, the Petri plates containing 30 to 300 colonies were selected. The average numbers of colonies were multiplied with the dilution factor to obtain the total count as a colony-forming unit (CFU) per g of the sample. This count was then expressed as $\log_{10}$ CFU/g of the meat sample.

Colour estimation

CIE (Commission Internationale de l'Eclairage) color parameters L* (lightness), a* (red-green), and b* (yellow-blue) were estimated using a portable colorimeter (MiniScan EZ, 4500L, USA). The colorimeter used for this study had a D65 illuminant with a 10 observer angle and an orifice diameter of 8 mm (Mishra *et al*., 2023). The equipment was calibrated against a white and black tile before measurement.

Statistical analysis

The data generated for different parameters were analyzed and subjected to Pearson's two-tailed correlation using SPSS (version 26.0 for Windows, SPSS, Chicago. 111. (U.S.A.). The data was subjected to analysis of variance and Tukey's HSD tests for comparing the means to find the difference among groups. The smallest difference between the two means was reported as significantly different ($P<0.05$).

RESULTS AND DISCUSSIONS

HADH enzyme assay

Freeze-thaw cycles affected significantly ($P<0.05$) the HADH enzyme activity in frozen-thawed buffalo meat. The HADH activity measured in meat press juice obtained from fresh, 1st FT, and 2nd FT cycles of buffalo meat was 2.82±0.06 U/mL, 6.77±0.05 U/mL, and 9.63±0.06 U/mL respectively (Figure 1). The HADH activity was higher in the 2nd FT meat samples than fresh meat and 1st FT samples. The increased enzyme activity with the progression of the freeze-thaw cycle could be due to the progressive growth of ice crystals causing considerable cell and muscle fibre disruption (Leygonie *et al*., 2012). The disruption occurs mainly in intact mitochondrial membranes causing the release of mitochondrial enzymes in sarcoplasm. The increased mitochondrial enzyme activity in meat press juice indicated that the meat was frozen and thawed repeatedly (Hamm, 1979). A similar increase in the activity of HADH enzymes during freeze-thaw cycles to differentiate between fresh from frozen-thawed meat was reported by Boerrigter-Eenling *et al.* (2017) for poultry, Chen *et al.* (1988) for beef, and Toldra *et al*. (1991) for pork. Chen *et al.* (1988) reported the HADH values for fresh beef in the range of 3.21 to 3.99 units/cm^3 which significantly increased to the value between 16.95 to 18.44 units/cm^3. Similarly, the increase in HADH enzyme activity in unfrozen pork from 3.09 U/mL to >6.0 U/mL for frozen thawed pork (Toldra *et al*., 1991). Here, the significant variation in HADH values of frozen and thawed meat of different food animal species might be due to differences in their muscle cellular integrity and response to large ice crystals at repeated freezing and thawing. Thus, the findings of HADH enzymes in buffalo meat and reported

previous evidence in poultry, pork, and beef, it can be said that freezing followed by thawing was the reason that affected muscle cell membrane and mitochondrial integrity that caused the release of HADH enzymes in meat press juice that acts as a potential marker for differentiation of fresh from frozen-thawed buffalo meat. To correlate the HADH enzyme-based assay with meat quality, various physicochemical, microbiological, and colour parameters were estimated at different points of the supply chain to determine the effects of repeated freezing and thawing on the quality of buffalo meat.

Effect of freeze-thaw cycles on ph of buffalo meat

The effects of freeze-thaw cycles on the pH of buffalo meat are presented in Table 1. The initial pH of the fresh buffalo meat sample was 6.19 indicating the normal pH of buffalo muscle within 2 h of slaughter. Repeated freezing-thawing affects the pH of buffalo meat significantly ($P<0.05$) and it drops to 5.58 (2^{nd} FT) from 5.90 (1^{st} FT). Frozen-thawed meat has a lesser pH than fresh meat and it results from the formation of lactic acid due to anaerobic glycolytic activity in muscle (Wei *et al*., 2017). The decrease in pH value was due to an increase in the concentration of solutes caused by the water loss and the release of hydrogen ions caused by protein denaturation during freeze-thaw cycles. The pH reported for buffalo meat in the present research agrees with Rahman *et al.* (2015) which showed a pH decrease from an initial 6.15 of fresh beef to <5.4 after three cycles of freezing and thawing. The pH of meat is a very important parameter determining the other sensory attributes like water holding capacity, cooking loss, and colour.

Effect of freeze-thaw cycles on tbars of buffalo meat

The effect of freeze-thaw cycles on the TBARS value of buffalo meat is mentioned in Table 1. The TBARS value seems to increase significantly ($P<0.05$) with the progression of repeated freeze-thaw cycles. The TBARS values measured for fresh, 1^{st} FT, and 2^{nd} FT cycles buffalo meat were 0.11, 0.27, and 0.54 mg malonaldehyde/kg of meat, respectively. The increase in the value of TBARS might be due to accelerated oxidative activity during repeated freeze-thaw cycles as it causes some loss of muscular integrity (Jeong *et al*., 2011). The enlarged ice crystals (because of recrystallization) damaged the cellular membranes and caused the release of various pro-oxidants *viz.* oxidative enzymes, haem iron, and free radicals required for accelerating oxidation (Leygonie *et al*., 2012). The values of TBARS for buffalo meat obtained in this research showed increasing trends, as reported for TBARS value of porcine *longissmus dorsi,* ovine *longissmus dorsi,* and beef *semimebranosus* muscle during freezing and thawing (Cheng *et al*., 2019; Xia *et al*., 2012; Qi *et al*., 2012). But the values of TBARS reported for buffalo meat are not at par with porcine *longissmus dorsi* (Fresh: 0.18 mg/kg; Five freeze-thaw cycles: 5 mg/kg), ovine *longissmus dorsi* (Fresh: 0.08 mg/kg muscle; Seven freeze-thaw cycles: 0.16 mg/kg) and beef *semimebranosus* muscle (Fresh: 1.82 mg/kg; Fifteen freeze-thaw cycles: 13.78 mg malonaldehyde/kg) during multiple freeze-thaw cycles. The difference reported in TBARS value of different food animal species meat could be due to variations in fat content of muscle, sensitivity of fat, and location of muscle chosen for study along with differences in number of freezing and thawing cycles considered for study.

Effect of freeze-thaw cycles on aerobic plate count of buffalo meat

The microbiological quality of any food is very important for determining the safety and quality aspects during the supply chain. The most common parameter for estimating the microbiological quality is aerobic plate count (APC). The findings of the effect of freezing-thawing on the microbiological count of buffalo meat *i.e.*, APC is mentioned in Table 1. The APC measured for fresh, 1st FT and 2nd FT cycles of buffalo meat were 4.50, 5.16, and 6.41 $\log_{10}$CFU/g respectively. The APC of buffalo meat samples determined at different stages of freezing-thawing increased significantly ($P<0.05$). The increased APC at the 2nd FT cycle (>6 $\log_{10}$CFU/g) indicates incipient spoilage and deterioration in meat quality. The increase in APC (>6 $\log_{10}$CFU/g) at 2nd FT cycles of buffalo meat indicated that the microbial quality of the meat was not up to the mark after the second freeze-thaw cycle (FSSAI, 2016). This might be due to handling contamination during repeated freezing and thawing processes with drip loss that provides a favourable medium for the growth of the microbes. The thawing process caused thermal abuse and renders the increase in temperature above the freezing point of meat which leads to the growth of spoilage organisms. The cold chain temperature maintenance remains limitations and frozen meat may undergo temperature fluctuations during handling at transportation, storage, and display at the purchase shop, resulting in increased APC before being processed by consumers (Du *et al*., 2020; Li *et al*., 2019). Bae *et al.* (2014) reported an increase in APC from 2.52 log cfu/g for deboned fresh chicken thigh meat to 3.61, 5.11, and 7.36 log cfu/g for frozen thawed on the 1st, 3rd, and 7th day of storage which varies significantly from the present findings of buffalo meat APC. It means that the microbiological count for meat could vary as per species, number of freezing and thawing cycles, and storage period.

Effect of freeze-thaw cycles on extract release volume (erv) of buffalo meat

Extract release volume is the volume of extract released by a homogenate of meat when allowed to pass through the filter paper for a given period. It is inversely proportional to the extent of spoilage. The ERV is an important parameter determining the quality of meat during repeated freezing and thawing. The effect of freeze-thaw cycles on the ERV values of buffalo meat is represented in Figure 2. ERV measured for fresh, 1st FT, and 2nd FT cycles buffalo meat were 35.05 mL, 30.65 mL, and 19.87 mL respectively, which decreased significantly ($P<0.05$) with an increase in freezing and thawing cycles. The reported ERV values for fresh and 1st FT buffalo meat were within the prescribed meat safety limit (>20 mL) as suggested by the Food Safety and Standards Authority of India (2016) thereby indicating the absence of incipient spoilage, whereas 2nd FT showed ERV value <20 mL indicating presence of spoilage. The decline in ERV values might be due to hydrolysis of meat protein due to endogenous enzymes and microbiological action during repeated freezing and thawing of meat (Jay, 1964). Furthermore, repeated freezing and thawing cycles induced the release of proteinase to extracellular spaces, leading to the hydrolysis of muscle proteins. A similar decreasing trend in ERV of frozen chicken meat during different thermal abuse conditions for the development of lipase-based enzymatic time temperature indicator was reported by Jaiswal *et al.* (2020). The ERV decreased from 30.10 mL for frozen chicken meat at -18±2°C to 23.86 mL, 21.10 mL, 19.58 and

20.93 mL at thermal abuse temperature of 5±1°C, 15±1°C, 25±1°C and 35±1°C, respectively. There is a difference in the ERV values of both the meat types but the point to be noted here is that if ERV values are <20 mL, there are chances of incipient spoilage affecting the quality of meat.

Effect of freeze-thaw cycles on water holding capacity, thawing and cooking loss of buffalo meat

Water holding capacity, thawing, and cooking loss are important parameters which inform about the sensory quality of meat. During, the freezing and thawing of meat, a large amount of water is lost with nutrients as drip loss which affects the juiciness and yield of meat. Therefore, estimation of these parameters can correlate with HADH enzyme activity during freezing and thawing cycles, indirectly predicting the sensory quality of meat. The effect of freeze-thaw cycles on the WHC, thawing loss, and cooking loss of buffalo meat is presented in Figure 2. The WHC measured for fresh, 1st FT, and 2nd FT buffalo meat was 65.26, 52.12 and 42.38%, respectively which decreased significantly ($P<0.05$) with the progression of each freezing and thawing cycle. Higher amounts of thawing and cooking losses were also observed with the increase in number of freeze-thaw cycles. The thawing and cooking loss after one freeze-thaw cycle were 29.46 and 46.95% respectively, which increased to 46.11 and 60.68%, ($P<0.05$) respectively after the second freeze-thaw cycle. From the above finding, it was observed that, with an increase in freezing and thawing cycles, WHC decreases with an increase in thawing and cooking losses. Repeated freezing and thawing cause melting and recrystallization phenomena which lead to large-size ice-crystal formation resulting in mechanical disruption of cell membranes and consequently loss of WHC (Srinivasan *et al*., 1997).

The change in the intracellular structure of the muscle cells during repeated freezing-thawing affects the ability of muscle cells to hold water and has major implications on meat product yield, appearance, and juiciness (Huff-Lonergan *et al*., 2005). Thawing loss and cooking loss can be called variations in the water-holding capacity of meat and have a direct impact on meat weight and loss of proteins that influence economic loss to the meat industry, including decreased meat quality. The loss of water during repeated freezing and thawing of meat causes the loss of specific tasteful components *viz*., several amino acids, nucleotide fragments, etc which are responsible for the flavour and acceptability of meat (Xia *et al*., 2009). Therefore, higher cooking loss of frozen-thawed meat may be due to the effect of increased myosin denaturation and increased weakening of the myofibril lattices due to protein degradation. Similar trends of freeze-thaw effects on WHC, thawing, and cooking loss have been reported in shrimp muscle and porcine *longissimus dorsi* muscle (Srinivasan *et al*., 1997; Xia *et al*., 2009). The thawing losses and cooking loss reported in porcine *longissimus dorsi* muscle were 3.51% and 30.69%, respectively after one freeze-thaw cycle which increased ($P<0.05$) to 18.27% and 46.22%, respectively after five freeze-thaw cycles. The thawing and cooking loss found for buffalo meat differed from porcine muscle due to differences in species, fat content, and cellular membrane integrity but their values seem to increase with freezing and thawing cycles. Therefore, as the number of freeze-thaw cycles increases, the water-holding capacity, thawing loss, and cooking loss increases significantly, thereby worsening the water retention properties of meat and meat

products (Pan *et al*., 2021).

Effect of freeze-thaw cycles on meat colour parameters of buffalo meat

The colour of meat and meat products plays a significant role in the appearance, presentation, and acceptability and freeze-thaw cycles have a significant effect on the meat colour leading to differences in the acceptability of meat by consumers (Cheng *et al*., 2019). The changes in colour parameters of buffalo meat during different freeze-thaw cycles were measured in terms of L*, a*, and b* values (Figure 3). Lightness (*L**) values measured for fresh, 1st FT, and 2nd FT cycles buffalo meat were 52.76, 36.47, and 24.90, respectively which decreased significantly with the progression of each freeze-thaw cycle suggesting repeated freeze-thaw cycles make the meat appear darker than meat without freezing and thawing treatment. The decrease in *L** value could be due to ultra-structural change and loss of drip during repeated thawing resulting in the decline in light reflectance. The decreased L* value might have occurred due to the degradation of meat proteins resulting in increased diffusion of incident light (Vatavali *et al*., 2013). A similar trend of decrease in *L** value was observed in the ovine *longissimus dorsi* muscle in response to the first five initial freeze and thaw cycles (Xia *et al*., 2009). *L** value reported for ovine *longissimus dorsi* muscle decreased significantly from 48.26 to 35.02 which is different from the findings of present research. Redness (*a**) related to redness-greenness is used as an indicator of colour stability in meat and meat products (Mishra *et al*., 2023). a* measured for fresh, 1st FT, and 2nd FT cycles buffalo meat were 19.63, 12.08, and 7.69, respectively which decreased significantly ($P<0.05$) with an increase in the number of freeze-thaw cycles. The reduction in *a** value might be due to the loss of myoglobin with the drip during thawing and the formation of metmyoglobin. *a** value decreased with increasing cycles, which is in accordance with Xia *et al*. (2009); Qi *et al.* (2012). The b* value indicative of yellowness of the buffalo meat increased significantly ($P<0.05$) with the progress of freeze-thaw cycles. However, yellowness (*b**) measured for fresh, 1st FT, and 2nd FT cycles buffalo meat were 10.17, 18.80, and 26.60, respectively which might be due to lipid oxidation and browning reaction (Karakosta *et al*., 2022).

Pearson two-tailed correlation analysis

The correlation between HADH enzyme assay in meat press juice data and physicochemical, microbiological, and color properties of buffalo meat at various points of supply chains were analyzed by Pearson two-tailed correlation. The coefficient of correlation (r) obtained for HADH enzyme assay and TBARS, thawing loss, cooking loss, b* value, and APC for buffalo meat after two freezing and thawing cycles were found positive ($r >0.90$). Other parameters like pH, extract release volume, water holding capacity, L* and a* values showed negative correlation with HADH enzyme assay ($r >0.95$ except for pH, $r = 0.54$). From the above correlation analysis, it can be said that the HADH enzyme assay in meat press juice and meat quality parameters could be used to monitor the quality of buffalo meat affected due to repeated freezing and thawing as a simple and rapid analytical tool in resource-constrained establishments.

CONCLUSION

The exposure of buffalo meat to different freezing and thawing cycles during the supply chain had a significant influence on its quality.

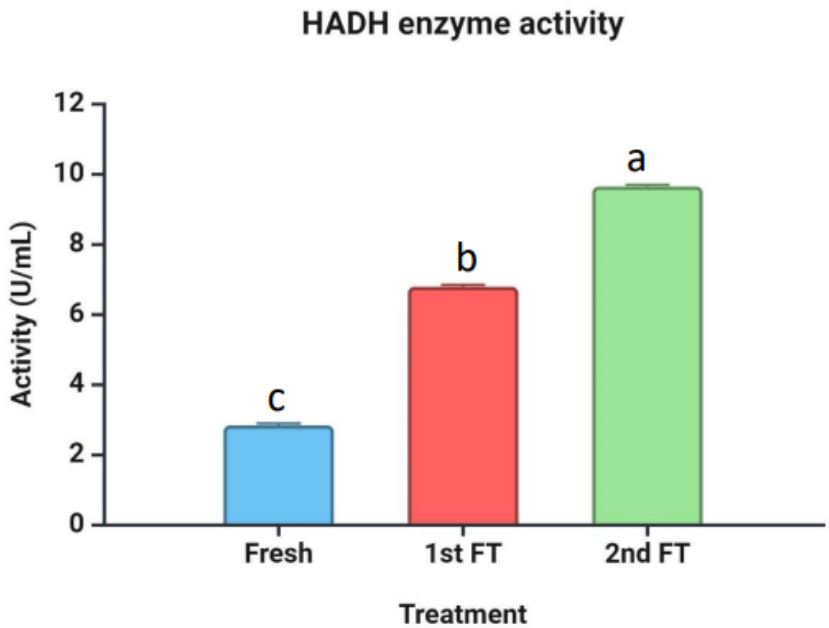

Figure 1. Activity of HADH enzyme in buffalo meat press juice at different stages of freeze-thaw cycles during supply chain. Different letters in the same row indicate the significant differences (a, b, c: $P<0.05$).

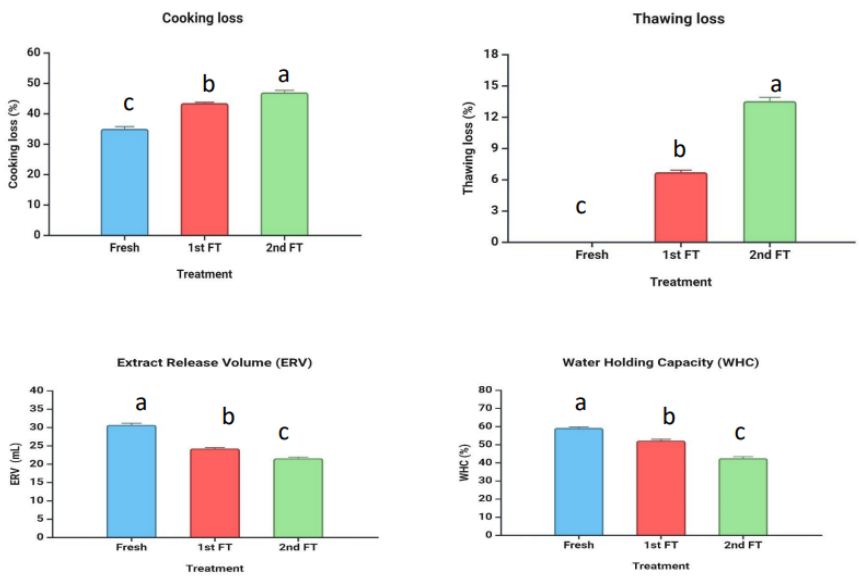

Figure 2. Freeze-thaw cycles correlation with cooking loss, thawing loss, extract release volume (ERV) and water holding capacity (WHC) of buffalo meat at different stages of freeze-thaw cycles during supply chain. Different letters in the same row indicate the significant differences (a, b, c: $P<0.05$).

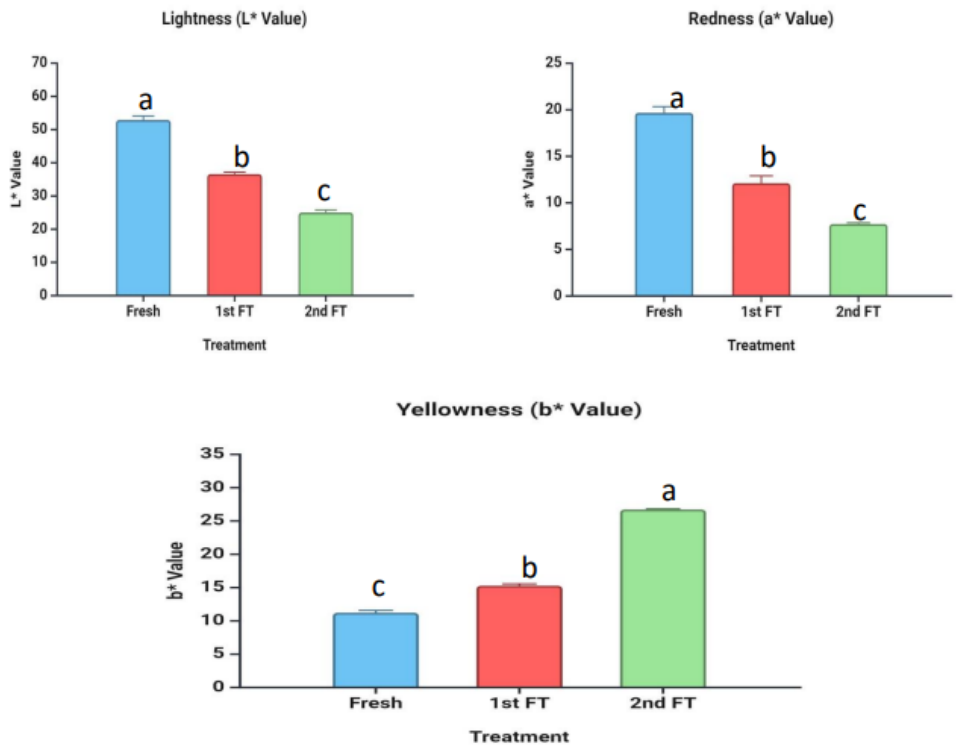

Figure 3. Freeze-thaw cycles correlation with colour parameters of buffalo meat at different stages of freeze-thaw cycles during supply chain. Different letters in the same row indicate the significant differences (a, b, c: P<0.05).

Table 1. Physiochemical and microbiological characteristics of buffalo meat at different stages of freeze-thaw cycles during supply chain.

Parameters	Fresh meat	First freeze-Thaw cycle	Second freeze-Thaw cycle
pH	6.19±0.03^{a}	5.90±0.02^{b}	5.58±0.01^{c}
TBARS (mg/Kg)	0.11±0.01^{c}	0.27±0.02^{b}	0.54±0.03^{a}
APC (log_{10} CFU/g)	4.50±0.38^{c}	5.16±0.25^{b}	6.41±0.16^{a}

Values are expressed as the Mean ± Standard Error (N=6). Different letters on the shoulder mark indicate significant differences (P<0.05).

The meat press juice collected from the one-time freeze-thaw cycle (1st FT) and two times freeze-thaw cycles (2nd FT) treated buffalo meat showed higher HADH activity than fresh buffalo meat press juice. The HADH enzyme assay activity showed a positive correlation (r>0.90) with TBARS, thawing loss, cooking loss, b* value, and APC whereas a negative correlation (r >0.95) with extract release volume, water holding capacity, L* and a* values of buffalo meat. The research findings suggested that the measurement of HADH activity in meat press juice can differentiate fresh from frozen-thawed buffalo meat. Therefore, the HADH activity assay and meat quality parameters could be utilized for discriminating fresh from frozen-thawed buffalo meat as a simple analytical method. In the future, studies on differentiating fresh from frozen-thawed buffalo meat shall be done with ELISA-based immunological methods.

ACKNOWLEDGEMENTS

The authors are thankful to Director, ICAR-Indian Veterinary Research Institute, Bareilly-243122, Uttar Pradesh, India for financial support and Head, Department of Livestock Products Technology for providing Fresh Meat Technology Laboratory for conducting experiments.

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