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Screening of cytosolic enzymes for the identification of candidate biomarkers in the authentication of fresh and frozen-thawed buffalo meat

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Abstract
The goal of the current study was to screen candidate biomarker(s) to develop an enzymatic assay for rapid, easy, and reliable detection of fresh (chilled) and frozen-thawed buffalo meat in the supply chain. The method is based on analyzing and selecting a biomarker enzyme from a pool of cytosolic enzymes. The enzymes were chosen based on differences in the enzyme activity of different meat cuts (loin and shoulder) at different temperatures (-10, -20, and -40 °C) for different (45) days. Results showed that SOD and Catalase displayed significant visual as well as spectrometric differences (approx. four times) and proved tremendous potential as biomarkers since their activity was consistent in fresh as well as frozen-thawed meat cut stored samples as compared to other enzymes studied. The results showed that all antioxidant enzyme activities varied (p<0.05) until the end of the process. Loin meat cuts showed more consistent results than shoulder meat cuts due to muscle complexity causing muscle damage during post-mortem. The studies proved that the method developed targeting these two enzymes as biomarkers could be helpful for potential application in the differentiation of chilled or defrosted buffalo meat.

Keywords: Screening, cytosolic enzymes, meat, authentication, supply chain, enzyme method

1. Introduction
Quality assurance is critical for ensuring that food products satisfy the intended quality, consumer safety, regulatory compliance, supply chain efficiency, customer trust, dependability, and overall quality. In the present time, there is a need to encourage global commerce by assuring high-quality food items that fulfill regulatory requirements, customer expectations, and sustainability concerns. Globalization raises the danger of perishability, necessitating supply chain field monitoring (Biswas *et al*., 2023) [4].

India accounts for 20.45% of buffalo of the total livestock population (DADF, 2022) [7]. World meat production is 329 MMT whereas meat production in India is 9.29 MMT (BAHS, 2022) [6]. Buffalo meat is having higher demand in the global market because of its high quality and availability at competitive prices. Meat authentication is a broad range of efficient activities, that is rapidly growing due to increasing public awareness concerning meat quality and safety. Meat products can be verified based on their biochemical composition, and possible adulterations can be detected by modern analytical methods (Esteki *et al*., 2021) [9]. The Indian market focuses on fresh meat while frozen meat is mostly exported. Buffalo meat dominated the exports with a contribution of over 78.65% of total Animal products exported from India in 2022-23 (APEDA, 2023) [2]. Meat is exported in the form of Frozen and deboned-deglanded meat.

Chilled meat is regarded to be of excellent quality because it contains the most nutrients. However, as global trade expands and the distance between producer and consumer grows, so does the need for frozen beef in transit. Freezing plays an important function in this business in ensuring the safety of the meat products sent abroad. Even yet, the effects of freezing and thawing on meat quality remain a major concern. The fundamental purpose of freezing storage is to inhibit catabolic processes that have a negative influence on the quality and quantitative characteristics of microorganisms, as well as enzymatic processes of meat. Cold chain is highly desired commercially for handling, processing, and keeping fresh products. Stocking and excessive meat stacking are frequently done to produce strong demand with a significant price gain or to result in widespread meat freezing due to surplus supply. Furthermore, due to price instability, meat is frequently frozen in bulk and mislabeled as higher-value goods to

increase profit margins. Consumers rely on food labels to evaluate if the meat they are purchasing is safe and dependable. Even though cold chain solutions have been created to solve concerns of temperature abuse, the meat cold chain still has flaws, such as temperature variations during shipment, distribution, and unloading in small businesses (Kwon *et al*., 2022) [15]. Temperature changes cause tiny ice crystals to melt and form bigger, more irregular crystals, resulting in an accumulation of ice crystals with a mean size expansion (Zhang & Ertbjerg, 2019) [23]. After thawing, ice crystals strongly pressurize cellular components and impact microstructural healing, releasing some important enzymes, haem and non-haem iron, and pro-oxidative agents. Furthermore, it may boost microbial fecundity and enzyme activity, which may result in the production of carcinogenic chemicals in the meat (Huang *et al*., 2016) [12]. The meat industry's major goal is to offer exceptional goods with high commercial value that are both engaging and pleasant to the customer. As a result, it is critical to understand the effects of freezing and thawing on various meat varieties and cuts.

Frozen-thawed meat is nutritionally inferior because freezing disrupts the cell's microstructural array and integrity. Mechanical stress from ice crystals lowers myofibrils' water-holding capacity, contributing to drip loss and other quality degradation after thawing. (Zhang & Ertbjerg, 2019) [23]. The disruption of organelle membranes caused by ice crystals formed during freezing leads to the release of enzymes from cellular organelles (such as mitochondria and lysosomes) into meat exudates. Meat juice made from frozen-thawed meat has more enzymatic activity than fresh meat juice. These enzymes have the potential to be used as indicators for distinguishing fresh meat from previously frozen meat. The exudate (drip) that is lost during thawing comprises a high concentration of enzymes (mitochondrial and cytosolic) and proteins. Freezing-thawing also produces reactive oxygen species (ROS), which causes oxidative stress by creating an imbalance between increased ROS production and its clearance by antioxidant mechanisms. (Edge & Truscott, 2021) [8]. Superoxide Dismutase is the first line of defense against free radicals and reactive oxygen species (ROS). Furthermore, antioxidants suppress free radical formation and activate the antioxidant system. (Mu *et al*., 2018) [18].

Earlier research has documented a range of analytical approaches for determining fresh vs. thawed meat authenticity, including DNA-based, spectroscopic, bioimaging, and sensory. Previous systems were complex and time-consuming, necessitating expensive apparatus and specialized staff for evaluation, rendering them unsuitable for field monitoring. They should be replaced by quick and dependable approaches that allow for quick decision-making. Over the last 10 years, research has proved the usefulness and dependability of peptide biomarker-based approaches for detecting relevant biomarkers and resolving the issue of frozen-thawed meat authenticity (Sentandreu and Sentandreu, (2011) [20].

As a result, the development of a system for certifying and distinguishing premium-quality meat from inferior meat is critical. Quick testing is necessary to prevent delaying both local and international shipping while meat samples are evaluated. As a result, enzymatic methods may be an excellent solution for distinguishing between fresh meat and previously frozen-thawed meat while avoiding the constraints of prior methodologies. When meat is defrosted, enzymes from the cytosol and mitochondrion are released into the sarcoplasm; enhanced enzyme activity in muscle press-juice implies tissue freezing and thawing. An increasing amount of enzyme and increased activity in the MEJ may be used to build a simple, visible, and spectrometric biochemical approach.

2. Materials and Methods

2.1 Materials

High purity (99%) chemicals like sodium phosphate buffer (99.5%), Tris buffer Hydrochloric acid (99.9%), Ethylenediaminetetraacetic acid (EDTA) (99.5%), 2-mercaptoethanol (99%), acetic acid glacial (99.7%), glycerol, glycine (99.5%), buffer components, β-Nicotinamide adenine dinucleotide disodium salt (reduced) NADH (98%), β-Nicotinamide adenine dinucleotide (oxidized) NAD^+, o-phenylenediamine (99%), NaOH (98%), phenazine methosulfate (90%), nitroblue tetrazolium were procured from Sisco Research Laboratories Pvt. Ltd, Mumbai. Sodium chloride (99.5%), hydrogen peroxide (30%), and enzyme standards were obtained from Sigma-Aldrich (Merck) Pvt. Ltd. USA. 1001W grade Whatman filter papers were procured from Axiflow, Axiva, Sonipat, India.

2.2 Sample collection

Fresh buffalo meat samples weighing 100 g each were procured from the regional Municipality slaughterhouse, Bareilly. The samples were collected in a cold room and brought to the lab in an ice box. The samples were cleaned of all visible fat, fascia, and connective components. A total of four different groups of samples were processed. In the first group, the control sample (fresh) was kept at room temperature (25 ^{C}) for 10–12 hours to allow for the onset of rigor mortis. After that, it was moved to a refrigerator for overnight PM ageing and then chilled (4±1 °C) for 1 day. The second group of samples, however, were moved to a deep freezer and kept at -20±2 °C for 7 days after overnight PM ageing. The third group of samples were kept at the same time and temperature as the second group, but they have undergone three cycles of repeated freezing and thawing. After being collected, the fourth group of samples was immediately stored in a deep freezer (without completing rigor) and preserved under the identical storage conditions as those described for the second group of samples. The enzyme activity was measured in terms of millimoles (mM) of product generated per minute per mL of MEJ for each sample, which was treated in triplicates.

For accurate detection and robustness, the screening of the cytosolic enzymes was done for the unknown meat samples which were taken randomly from the local market. For application in field samples a batch of a total of 33 meat samples for chilled and frozen-thawed each was procured to collect MEJ, from the open market (unknown samples), shifted into the laboratory under frozen conditions at -20$^{\pm}$2^{o}C for 7 days, and finally, analyzed for the authenticity of freezing.

2.3 Preparation of meat express juice (MEJ)

Meat express juice (MEJ) was prepared by immersing 10 g of meat samples in 10 mL of 50 mM Tris-HCl (pH 8.0) solution. The extracted MEJ was then centrifuged at 10,000 rpm in a chilled centrifuge (HERMLE Z32 HK, Germany) for 10 min after filtering with 1001W grade Axiflow Whatman filter paper (Axiva, Sonipat, India). For the preparation of extract from the frozen sample, it was first thawed in a refrigerator

and then used and both the fresh and frozen-thawed meat samples were handled separately throughout the extraction process, which was carried out at a temperature of 4 °C (Biswas *et al*., 2023) [4]. A total of five cytosolic enzymes were analyzed *viz*., superoxide dismutase (SOD), aldehyde dehydrogenase (ALDH), glutathione peroxidase (GPx), catalase (CAT) and alkaline phosphatase (ALP). For each enzyme, a different buffer solution was used.

2.4 Determination of enzyme activity

For identification of suitable biomarkers in the differentiation of fresh and frozen-thawed meat enzymes of two different sources were targeted- for cytosolic (antioxidant) origins. The cytosolic (antioxidant) enzymes were superoxide dismutase (SOD), aldehyde dehydrogenase (ALDH), glutathione peroxidase (GPx), catalase (CAT) and alkaline phosphatase (ALP). All these enzymes were analyzed, and among these enzymes, the two enzymes that showed greater and consistent activity were selected as candidate biomarkers in the differentiation of fresh and frozen-thawed buffalo meat

2.4.1 Superoxide dismutase (SOD)

The activity of the superoxide dismutase (SOD) enzyme was determined spectrophotometrically by employing the PMS–NADH–NBT system using the protocol (Khan *et al*., 2014) [14]. The reaction mixture consisted of 140 µL of 50 mM Tris-HCl buffer (pH 8.0), 20 µL of NADH (205 µM), 15 µL of NBT (184 µM) and 15 µL of PMS (1.9 µM). For assaying, the SOD enzyme was pipetted into a cuvette at room temperature (25 ^{C}) containing freshly prepared NBT and NADH. The reaction was initiated with the addition of freshly prepared PMS and the absorbance at 560 nm was continuously monitored as an index of NBT reduction using a UV-Vis spectrophotometer (Eppendorf Biospectrometer Basic, Germany). Reagent control as blank was taken which was lacking the enzyme. The amount of product formed was calculated by Beer-Lambert's equation using the molar extinction coefficient for the PMS-NADH-NBT system as 25000 (M^{-1} cm^{-1}).

2.4.2 Aldehyde dehydrogenase (ALDH)

The MEJ was assessed for the activity of aldehyde dehydrogenase following the protocol of Laskar *et al*. (2017) [16] with suitable modifications. In the modified method, the reaction mixture contained 175 µL of 50 mM sodium phosphate buffer (pH 7.5), 10 µL of MEJ, 5 µL of 100 µM NAD^+ and 10 µL of 5 µM 6-methoxy-2-naphthaldehyde. The ALDH activity of MEJ was assayed spectrometrically (Eppendorf Biospectrometer Basic, Germany) using 6-methoxy-2-naphthaldehyde as substrate acting with NAD^+ as a coenzyme. The reaction was started by the addition of the enzyme at 25 ^{C} and monitored immediately after the addition of MEJ continuously for 5 min. Absorbance was measured before enzyme addition as control. Appropriate blanks were always taken and subtracted from the test (Arsalan *et al*., 2020) [3].

2.4.3 Glutathione peroxidase (GPx)

The MEJ samples were evaluated for the activity of Glutathione peroxidase following the protocol of (Ahmed *et al*., 2021) [1] with some slight changes. In the modified method, 600 µL of phosphate buffer solution (100 mM, pH 7.0) was added to the cuvette consisting of 1:1.5 volumes of A and B solutions, 40 µL of reduced glutathione (4 mM) and 20 µL of MEJ. The reaction was initiated by adding 20 µL of H_2O_2 (2 mM) and 300 µL of cupric reagent. The absorbance was taken at 450 nm and blank was taken without enzyme. Whenever the experiment was conducted, the fresh working cupric reagent was used.

2.4.4 Catalase (CAT)

The method was adopted after some modifications from the protocol (Hadwan, 2018) [10] depending on the conversion of the oxidation state of cobalt (II) to cobalt (III) by hydrogen peroxide in the presence of bicarbonate solution. This process ends with the formation of a carbonato-cobaltate (III) complex ([Co $(CO_3)_3$] Co). This end product has three absorption peaks at 407, 435 and 635 nm. The 435 nm wavelength had been used for the assessment of catalase activity. For analysis, 115 µL of phosphate buffer (pH 7.0, 50 mM) was taken in the cuvette to which 10 µL of MEJ was added. To initiate the reaction 125 µL of H_2O_2 (10 mM) and 750 µL of the working solution were added to the medium and the absorbance was taken at 0 min and after 5 min at 435 nm and blank was taken without enzyme. Catalase activity is always directly proportional to the rate of dissociation of hydrogen peroxide. The working solution consisted of 100 mL of cobalt (II) solution, 100 mL of Graham salt solution, and 1800 mL of sodium bicarbonate solution, which were mixed well after preparation.

2.4.5 Alkaline phosphatase (ALP)

The MEJ was analyzed for the activity of Alkaline phosphatase, following the method of Kanta *et al*. (2021) [13] with suitable modifications. ALP in the medium converts para nitrophenyl phosphate (PNPP) present in the AMP (2-amino 2-methyl 1-propanol) buffer to yellow-coloured para nitrophenol (PNP) at pH 10.5 which was read at 410 nm. At the end of incubation, just before stopping the enzyme activity with 0.104 mL 1.0 N of NaOH, 0.2 N HCl was added. For MEJ analysis, 400 µL of AMP buffer (pH 10.5) was taken in the cuvette, 50 µL of MEJ was added into the buffer and 50 µL of PNPP was added into the solution and measured the absorbance at 410 nm after the incubation of 30 min. of addition of substrate for 5 min. Blank was taken which lacked the enzyme same as the samples. Finally, the ALP activity was expressed as millimoles of PNP min^{-1} mL^{-1} of MEJ.

2.5 Statistical analysis

To have a total of six observations, data were extracted from each experiment, duplicate samples were taken for each parameter, and the whole set of experiments was independently performed three times. The average values and standard error were provided. The average of three replicates and the standard deviation were used to report the findings. The analysis of variance (ANOVA) was used to examine the data sets using the 'IBM-SPSS-Statistics-28.0 software packages per standard practices (Biswas *et al*., 2016) [5]. The means of enzyme activity were assessed using one-way ANOVA. Duncan's Multiple Range Test (DMRT) was used to analyze the statistical significance, which was calculated at the 5% level (p<0.05).

3. Results and Discussion

3.1 Screening of cytosolic enzymes

Buffalo meat samples were screened to target and analyze selected cytosolic enzymes in the MEJ extracted from fresh and frozen-thawed buffalo meat samples

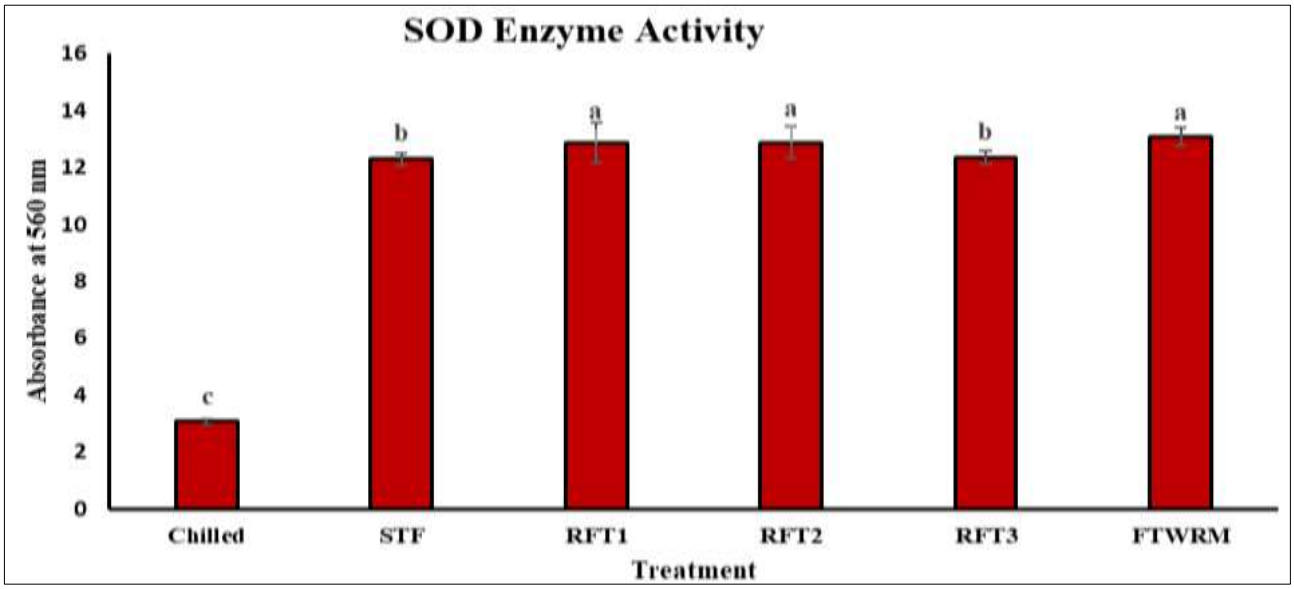

Fig 1: Screening of the SOD enzyme activity in MEJ extracted from fresh and frozen-thawed buffalo meat samples.

Results (Figure 1) show a significant increase in enzyme activity of frozen-thawed samples stored at (-20±2 °C for 7 days) concerning chilled samples. This must be because of higher oxidative stress levels in the cell caused by free radical generation during freezing and Reactive oxygen species (ROS) production, which on thawing leads to the release of enzymes in drip. No or negligible amount of meat exudate is seen in chilled samples signifying lesser damage. In the short-term freezing samples, significantly lesser enzyme activity was seen than in repeated freezing-thawing samples which might be due to the increased mean size of the ice crystals exerting much pressure to rupture the cell ultimately more enzyme release was seen (Ŝkorpilová *et al.*, 2014) [25]. More damage was seen in the meat samples which were frozen without completion of rigor mortis samples. Results displayed a significantly higher enzyme SOD activity approximately noted four times more in chilled than the frozen-thawed samples as oxidative stress triggers the activation of SOD i.e is the first line of defense in the cell (Halliwell, 2014) [11].

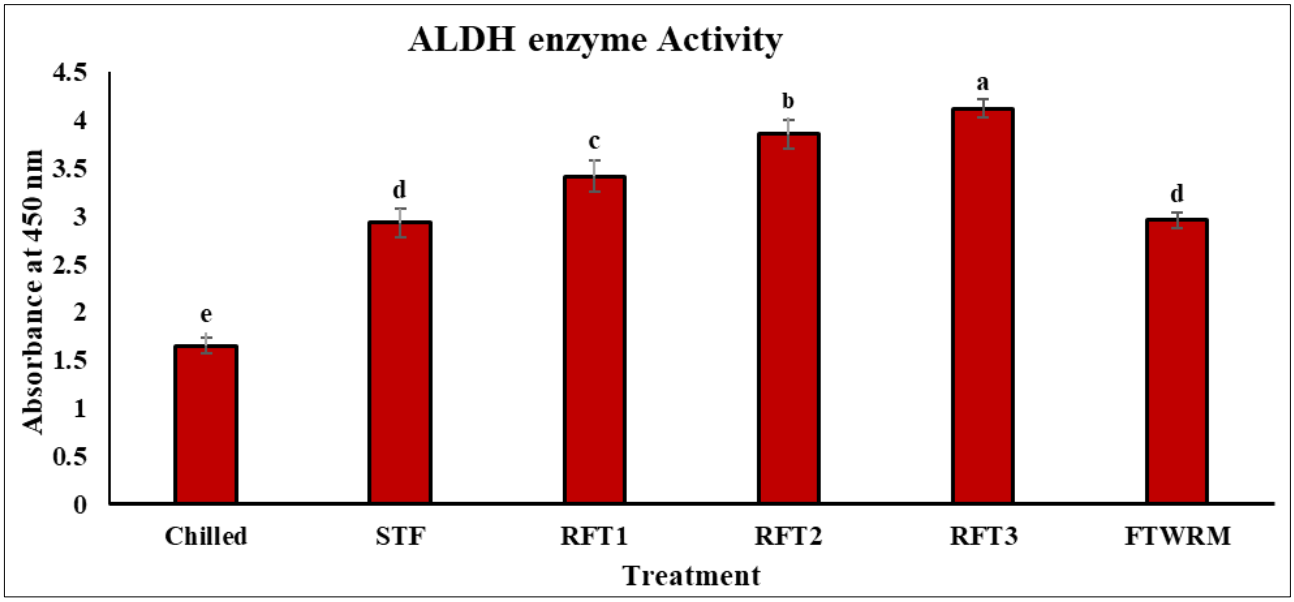

Fig 2: Screening of the ALDH enzyme activity in MEJ extracted from fresh and frozen-thawed buffalo meat samples.

The ALDH enzyme exhibited significantly increased approximately 3 times higher enzyme activity in frozen-thawed samples than the chilled ones. It was seen more in repeated- frozen-thawed samples (Figure 2). It might be due to higher stress levels due to cellular injury after repetitive freezing-thawing cycles, which come out from the cell cytoplasm in the MEJ. No visual changes were observed in the solution after incubation, only spectrometric change was there.

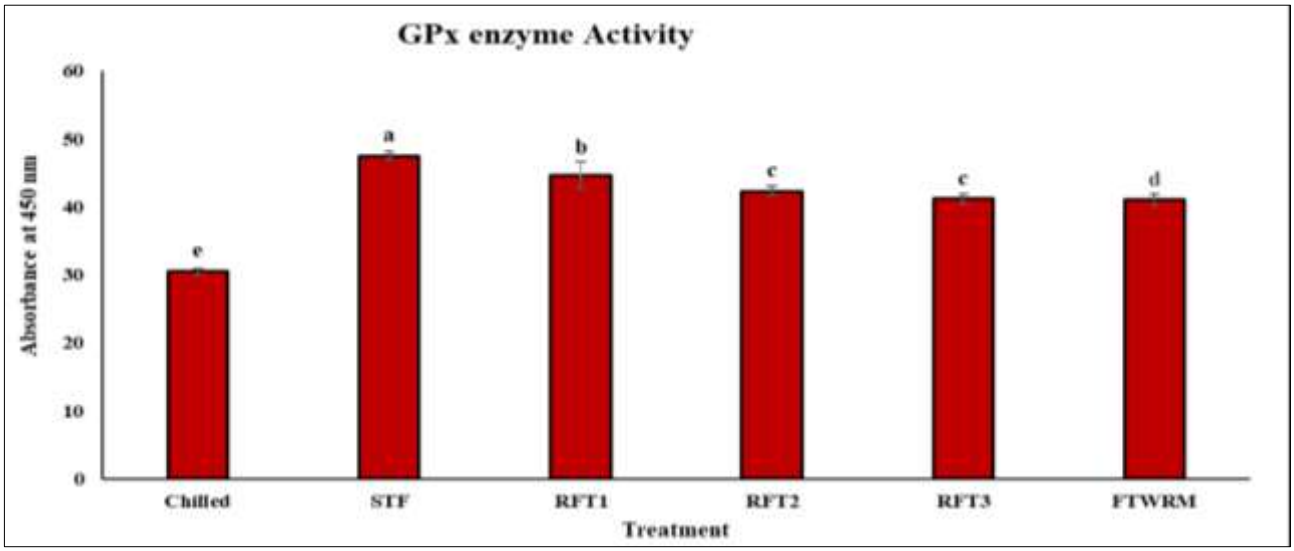

Fig 3: Screening of the GPx enzyme activity in MEJ extracted from fresh and frozen-thawed buffalo meat samples.

It was observed (Figure 3) that significantly higher (approx.1.5 times) enzyme activity was noted in frozen-thawed meat samples but an irregular pattern in absorbance was there in repeated frozen-thawed and frozen-thawed without rigor mortis meat samples. No colorimetric difference was seen among all the samples. Cytosolic enzyme glutathione peroxidase release was observed to be higher during combat with oxidative changes in cells during freezing-thawing.

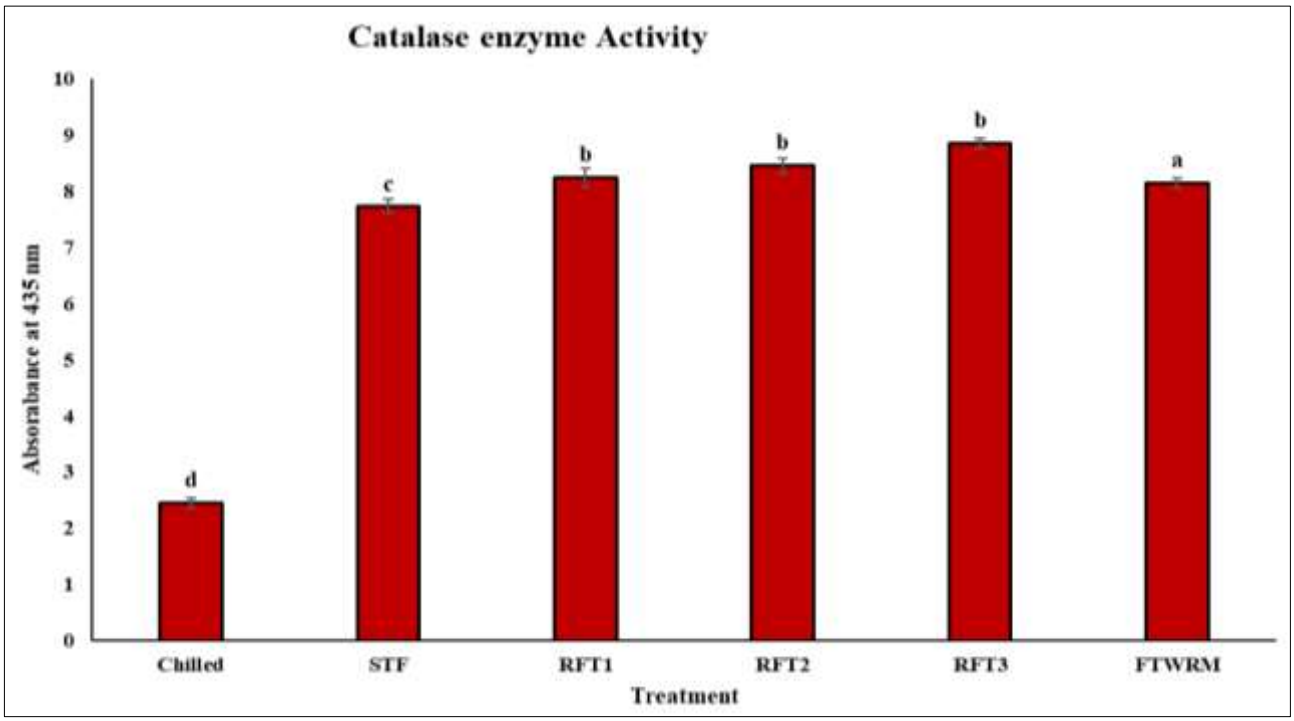

Fig 4: Screening of the Catalase enzyme activity in MEJ extracted from fresh and frozen-thawed buffalo meat samples.

Catalase is the second most abundant enzymatic antioxidant (after superoxide dismutase), which attenuates the levels of reactive oxygen. The activity of the enzyme is found (Figure 4) to be considerably higher approx. 3 times after freezing in response to increased levels of free radicals similar to SOD as both follow reverse assay. The clear visible difference as well as remarkable change in absorbance was observed in frozen-thawed than chilled samples.

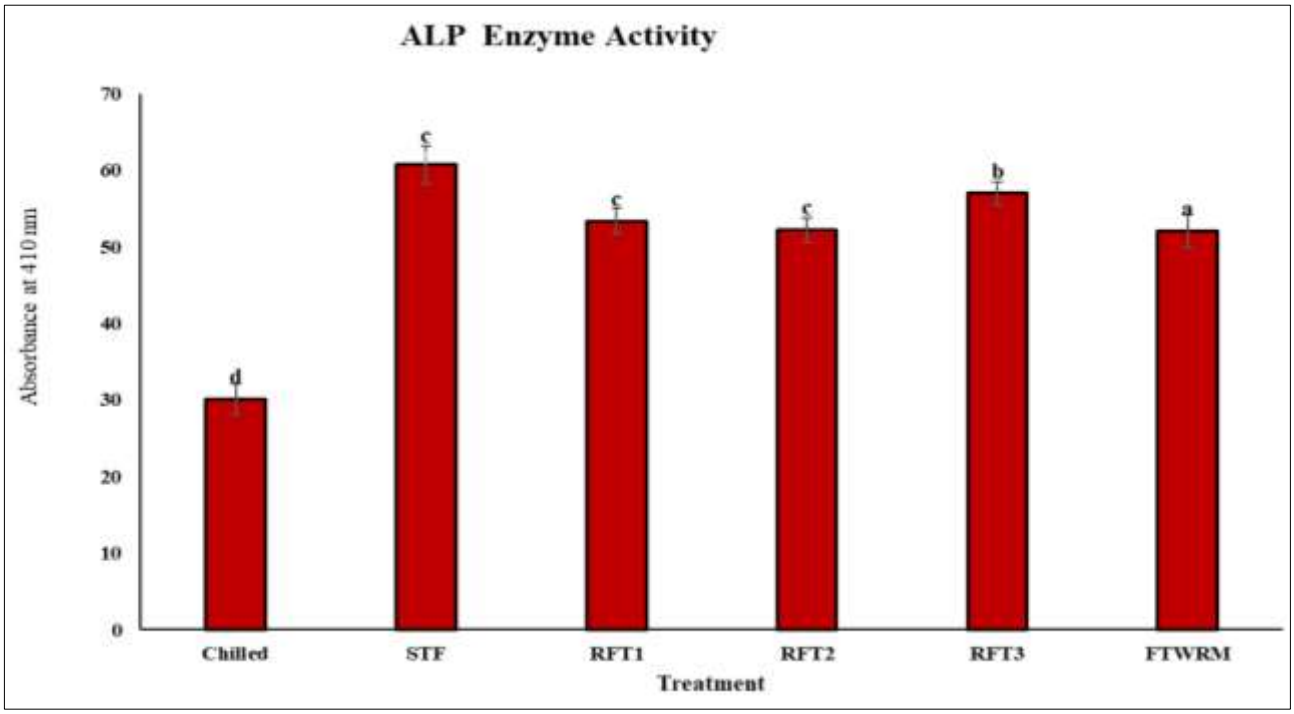

Fig 5: Screening of the ALP enzyme activity in MEJ extracted from fresh and frozen-thawed buffalo meat samples.

In ALP enzyme assay (Figure 5), it was observed that significantly increased enzyme activity (approx. 1.6 to 2 times) was found in RFT and FTWRM samples in comparison to chilled samples as needle-shaped ice crystals induced injury leads to more enzyme out of the cell after freezing period. The meat sample that was frozen without passing the rigor mortis showed the maximum damage with a higher release of the enzyme from the cytoplasm to the MEJ.

3.2 Long-term Storage Studies

The results of the different cytosolic enzyme activity of long-term stored buffalo meat cuts are displayed in (Table 1). Since loin muscles are less compact and more susceptible to ice crystal-induced injury than shoulder muscles, the loin muscle demonstrated slightly higher enzyme activity than the shoulder muscle. There is a more than four-fold increase in the activity of SOD in meat express juice obtained from frozen-thawed meat samples. Samples stored at different freezing temperatures showed enzyme activity as the freezing process causes ice crystals with sharp edges causing more damage. The activity of enzymes varied with the time, a small increase in the activity was observed when the samples were stored for different time intervals. The long-term storage study suggests that the SOD and Catalase enzymes can be considered as potential biomarkers for the differentiation of fresh and frozen-thawed meat. However, the process cannot tell much difference between storage days and freezing temperature (Zhang *et al*., 2018) [24].

Table 1: Determination of the activity of cytosolic enzymes in the meat express juice extracted from buffalo meat. Samples were stored for different times under different temperature conditions.

Enzyme Activity in Buffalo Meat Express juice											
Temperature (°C)	Time (Days)	SOD		ALDH		GPx		Catalase		ALP	
		Loin	Shoulder	Loin	Shoulder	Loin	Shoulder	Loin	Shoulder	Loin	Shoulder
4	1	3.16±0.09^{F}	2.97±0.07^{D}	1.62±0.06^{E}	1.60±0.08^{D}	31.72±0.98^{E}	29.65±1.15^{E}	2.54±0.14^{G}	2.26±0.16^{G}	31.28±1.44^{D}	29.54±1.12^{D}
-10	15	13.23±0.31^{B}	12.74±0.51^{A}	2.97±0.09^{B}	2.89±0.10^{A}	50.27±4.13^{A}	50.09±5.17^{A}	7.97±.53^{B}	7.89±0.18^{B}	62.27±4.14^{A}	60.09±3.32^{A}
-10	30	13.37±0.31^{B}	12.87±0.26^{A}	3.04±0.11^{B}	2.91±0.19^{A}	50.74±4.86^{A}	50.43±4.66^{A}	8.05±0.27^{B}	7.93±0.09^{A}	64.43±3.87^{A}	62.02±2.27^{A}
-10	45	13.71±0.33^{A}	12.89±0.28^{A}	3.19±0.37^{A}	2.97±0.16^{A}	52.09±3.51^{A}	51.85±4.83^{A}	8.51±0.49^{A}	7.97±0.04^{A}	66.23±4.09^{A}	63.76±3.04^{A}
-20	15	12.30±0.42^{C}	12.11±0.47^{B}	2.83±0.14^{C}	2.79±0.17^{B}	48.25±3.11^{B}	41.12±1.92^{C}	7.81±0.27^{C}	7.25±0.34^{D}	58.87±2.99^{B}	57.32±3.12^{B}
-20	30	12.35±0.43^{C}	12.16±0.32^{B}	2.89±0.24^{B}	2.82±0.18^{B}	48.38±3.47^{B}	43.82±3.89^{B}	7.86±0.23^{C}	7.46±0.12^{C}	59.12±3.02^{B}	58.31±4.05^{B}
-20	45	12.85±0.24^{D}	12.27±0.19^{B}	2.92±0.18^{B}	2.83±0.19^{B}	49.04±3.13^{B}	44.81±2.74^{B}	7.87±0.14^{C}	7.52±0.18^{C}	59.66±4.07^{B}	59.02±3.78^{B}
-40	15	11.42±0.32^{E}	11.39±0.23^{C}	2.70±0.17^{D}	2.69±0.13^{C}	44.84±2.25^{C}	40.21±1.01^{D}	6.85±.09^{F}	6.68±0.24^{F}	55.22±2.25^{C}	55.99±2.67^{C}
-40	30	11.67±0.19^{E}	11.52±0.18^{C}	2.71±0.16^{D}	2.70±0.11^{C}	42.45±2.31^{D}	40.37±1.99^{D}	7.10±0.08^{E}	6.88±0.17^{E}	55.98±3.03^{C}	56.08±2.45^{C}
-40	45	11.88±0.23^{E}	11.66±0.22^{C}	2.72±0.23^{D}	2.72±0.20^{C}	41.78±1.98^{D}	40.39±1.60^{D}	7.29±0.11^{D}	6.89±0.23^{E}	56.30±3.13^{C}	56.47±2.13^{C}

n = 6; Mean±S.E. with different superscript column-wise differ significantly (p<0.05).
*Enzyme activity was expressed in terms of µmoles of product formed per min per ml of meat express juice.

Results (Table 1) indicate significantly increased enzyme activity of frozen-thawed samples stored at (-10, -20 and -40 °C for 45 days) as compared to chilled samples. The shoulder meat showed slightly lower activity than the loin meat. Defrosted meat showed significantly higher activity of SOD at different storage temperatures for different time periods as compared to chilled meat. This must be due to damage caused by the formation of ice crystals and ROS production, which leads to the formation of free radicals and increased oxidative stress in the cell during freezing, exerting a great deal of pressure on cell organelles, resulting in the release of enzymes and cytoplasmic content of the cell in the form of drip on defrosting. Chilled samples show no or a minor quantity of flesh exudate, indicating less damage. Results displayed a significantly higher enzyme activity approximately noted three times more in chilled than the frozen-thawed samples as

oxidative stress triggers the activation of SOD (Mondola *et al*. 2016) [17].

Furthermore, the activity of ALDH was determined in the buffalo meat of similar meat cuts and it was observed that shoulder and loin meat's MEJ was showing similar activities in almost every set and a considerable increase (approximately 1.8 times) in the activity was observed in the ALDH activity in the frozen-thawed meat as compared to the chilled meat in the shoulder meat cuts stored at -10±1ºC for 15 days (Table 1). It might be related to increased oxidative stress following storage, which causes greater enzyme release from the cell cytoplasm in the MEJ. Its role is to catalyze the reversible conversion of lactate to pyruvate with the reduction of NAD+ to NADH, as well as to consider substrate oxidation and combat reactive oxygen species (Zanoni *et al*. 2022) [22]. The pattern of ALDH activity throughout the study remained uniform but no further differentiation was observed within the frozen-thawed samples stored under different conditions. No colour change was noticeable in after incubation, only a change in absorbance was observed.

While analyzing GPx activity (Table 1), the frozen-thawed meat showed significantly higher activity (approx. 1.6 times) as compared to the chilled one. However, no remarkable changes were observed in the GPx activity while examining loin and shoulder meat cut samples. The change in optical density was seen without any change in visual appearance with chilled samples. GPx activity was inversely correlated with the decrease in absorbance of the coloured Cu(I)–neocuproine complex (Orange coloured) (Ahmed *et al*. 2021) [1]. The production of cytosolic enzyme glutathione peroxidase becomes higher with an increase in oxidative stress during freezing-thawing. The enzyme is responsible for the reduction of hydroperoxides. As a result, it preserves membrane lipids and hemoglobin against peroxide oxidation (Orian *et al*. 2015) [19].

The activity of the CAT enzyme was in a similar pattern like SOD (Table 1). The activity of the enzyme is found to be considerably higher approx. 3-4 times after freezing due to more oxidative stress in defrosted meat than in chilled meat. Although both cuts have equal enzyme activity, the loin cut had greater enzyme activity than the shoulder meat samples. The chilled samples (intense olive-green) and frozen-thawed samples (colorless) showed a clear difference. The reduction in colour intensity can be used to signify an increase in catalase activity. It is a cytoplasmic enzyme that dissociates hydrogen peroxide (H_2O_2) into molecular oxygen (O_2) and water (H_2O) in a chemical process controlled by catalase. The absorbance of the carbonato-cobaltate (III) complex as a function of time was measured. Catalase activity is usually exactly proportional to the rate of hydrogen peroxide dissociation and grows as oxidative stress on meat storage (freezing) increases (Hadwan 2018) [10].

ALP enzyme assay measures the conversion of para-nitrophenyl phosphate to a yellow-coloured compound, i.e. para-nitrophenol (PNP) (Kanta *et al*. 2021) [13]. It was observed (Table 1) that significantly increased (approx. 2 times) enzyme activity was found in the frozen-thawed samples in comparison to chilled samples, this might be related to increased ALP enzyme release from the cytoplasm following cell structural integrity loss. The changes in enzyme activity in meat express juice isolated from fresh and frozen-thawed meat were entirely dependent on the kind of meat cut and muscle tissue complexity (Biswas, 2023) [4].

This could be due to differences in the size of ice crystals formed. It is well known that the size of ice crystal formation inside cells is significantly dependent on the kind and rate of freezing. Slow freezing (− 10±1 ºC) imposed more pressure to rupture muscle cells and released more enzymes than fast freezing (− 20±2 ºC and − 40±2 ºC) because larger ice crystals formed (Ŝkorpilová *et al*., 2014) [25].

3.3 Application in field samples

The same methodology for calculating enzyme activity was applied to unknown meat samples. The enzyme activity was investigated (Table 2) for cytosolic enzymes in MEJ, found to be significantly higher in defrosted than chilled samples but much difference was noticed in SOD and catalase enzymes between the two samples. This is possibly due to more enzyme release because of more damage after repeated freezing of meat samples than the fresh meat making it inferior to use than the chilled one. Therefore, it was concluded that these two enzymes can be reported to be potential biomarkers for further studies.

Table 2: Determination of the activity of cytosolic enzymes in the MEJ extracted from unknown buffalo meat samples. Samples were stored for 7 days at (-20 ºC) temperature conditions.

Sample/Enzymes	SOD	ALDH	GPx	Catalase	ALP
Fresh	3.06±0.08^{b}	1.65±0.08^{b}	30.47±0.41^{b}	2.43±0.10^{b}	30.08±1.97^{b}
Frozen-thawed	12.16±0.72^{a}	2.94±0.14^{a}	47.52±0.63^{a}	7.73±0.12^{a}	52.06±2.16^{a}

Data (n=33) represents cytosolic enzyme activity units (mean±SE) in terms of millimoles of product formed per min per ml of MEJ for different samples superscripts column-wise differ significantly. (p<0.05).

4. Conclusion

The MEJ from fresh/chilled and frozen-thawed buffalo meat samples was evaluated for cytosolic enzymes from various meat cuts, with SOD and Catalase exhibiting increased enzyme activity and being shown to be efficient and fast to reveal the findings. These enzymes may be a sign of muscle injury. This is a low-cost, straightforward, and dependable method for distinguishing between fresh and frozen-thawed meat. Visually and spectrophotometrically, the SOD and catalase exhibited encouraging findings for future kit development preparation. These enzymes may be utilized to create tools for meat authentication and regulatory purposes, making it a simple procedure that could find a place in field monitoring.

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6. Declaration of Competing Interest

The authors declare no conflict of interest, financial or otherwise.

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8. Highlights

- Screening of cytosolic enzymes was done to investigate potential biomarkers.
- Frozen-thawed meat displayed higher enzyme activity as compared to chilled samples.
- SOD and Catalase were found to be potential biomarkers among the cytosolic enzymes in meat samples, as they showed huge differences between chilled and frozen-thawed meat samples.
- The methods can be used for kit development

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