JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Dec. 2014. Vol. **8**(6), p. 4851-4857

Estimation of Microbial Load in High Temperature Thawed Buffalo Meat using Fluorescein Diacetate (FDA) Hydrolysis Assay

M.R. Vishnuraj[1], G. Kandeepan[1]*, Vivek Shukla[1], S.K. Mendiratta[1], Arun T.R.[2] and R.K. Agarwal[2]

[1]Division of Livestock Products Technology, Indian Veterinary Research Institute, Izatnagar, Bareilly - 243122, India.
[2]Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar, Bareilly - 243122, India

(Received: 29 August 2014; accepted: 25 November 2014)

The aim of the study was to estimate the various microbial loads in different high temperature thawed frozen buffalo meat samples using fluorescein diacetate (FDA, 3', 6'-diacetyl-fluorescein) hydrolysis assay. Frozen buffalo meat (-18±1°C) samples was thawed at 25±1°C and 37±1°C for different durations in a temperature controlled incubator. All the samples were analyzed for various microbiological parameters using conventional plate count method and compared with FDA hydrolysis. Thawed samples were further stored at refrigeration temperature (4±1°C) and analyzed on alternate days. Significant ($P<0.05$) increase in FDA hydrolysis values were noticed after temperature abuse, where control sample showed an absorbance of 0.24±0.02 and most severely thawed sample showed a value of 0.48±0.02. Standard plate count (SPC), *pseudomonas* count, psychrophilic count and yeast-mold count increased significantly ($P<0.05$) after high temperature thawing. FDA hydrolysis showed significant ($P<0.05$) positive correlation with all the microbial parameters and highest degree of association was noticed between FDA and SPC (r = 0.97). Regression formula (Y = 0.08X-0.055, Y represents FDA, X represents SPC) was generated using FDA hydrolysis values and SPC value as dependent and independent variables respectively and found that predicted absorbance value of 0.5±0.05 was coinciding with upper limit of microbiological acceptance value (SPC) of $\log_{10}$ 7 cfu/g for frozen fresh meat.

Key words: FDA hydrolysis, buffalo meat, thawing, microbial load, correlation, refrigeration storage.

India is the largest exporter of buffalo meat in the world. India is exporting deboned and deglanded buffalo meat mainly by applying low temperature preservation methods. As per the present quality control and inspection rules for export of raw meat from India, temperature of meat should be - 8° C during storage and transportation[1]. Any fluctuations in the prescribed cold chain temperature at any point during transport or on subsequent storage will cause accelerated microbial proliferation and which may result in immature spoilage in meat[2]. Moreover temperature abuse can lead to food safety issues by enhancing pathogen growth [3]. Therefore to establish a microbiological acceptance criterion for fresh and frozen meat, ICMSF has put forward a total plate count value of $\log_{10}$ 7 cfu/g as the upper microbiological limit[4].

Determination of microbial load in meat samples using conventional plate count methods are time consuming and laborious. So meat industry and various inspection services are in

* To whom all correspondence should be addressed.
Mob.: 7599359005;
E-mail: drkandee@gmail.com

continuous search for rapid alternative methods to establish the microbial load in meat and meat products. Moreover such methods can also be used for monitoring HACCP units and sanitary standard operating procedures in meat plant.

Alternate methods to evaluate microbial load through estimation of physicochemical parameters like extract release volume (ERV) [5,6], total volatile basic nitrogen (TVBN) [7], D-glucose [7,8], pH [9], free amino acids (FAA) [8] and trimethylamine [10] were studied for protein rich foods but these assays need more time and intensive techniques. Some newer methods using high technology instruments were also utilized for spoilage detection in food. A rapid capillary electrophoresis (CE) assay was developed for quantification of *Lactobacillus acidophilus* and *Bifidobacterium infantis* for consumer products like powdered health supplements [11]. An electrical impedance based method was standardized for detection of microbial load in ice cream[12]. Similarly techniques like bioluminescence[13], biosensors[14], flow cytometric methods [15], electrochemical methods [16] and a hydrophobic grid membrane filtration (HGMF) [17] for raw beef were also developed for the quantification of microbial load.

But application of colorimetric dye reductions test which are normally fast and less laborious are more important in this area. A colorimetric alamar Blue assay for estimating the bacterial concentration and spoilage index in marine food was developed [18]. Resazurin reduction has been used to assess the bacteriological quality of fresh scallop[19], fresh beef [20] and milk [21]. A correlation coefficient value of 0.94 (r=0.94) was observed between resazurin reduction time and aerobic plate count (APC) in beef steaks under temperature abuse at 25° C [22]. Fluorescein diacetate (FDA) hydrolysis and its association with various microbial loads in biceps femoris steaks were also investigated [22]. The synthetic substrate fluorescein diacetate (FDA, 3', 6'-diacetyl-fluorescein) is hydrolyzed nonspecifically by microbial enzymes including proteases, lipases and esterases and the product fluorescein can be measured by spectrophotometry at 490 nm [23]. So the intensity of colour formation is an indirect indication of the microbial density in the meat sample. Therefore objective of this study was to evaluate whether FDA hydrolysis can be used as a rapid test to predict microbial load in different high temperature thawed buffalo meat.

MATERIALS AND METHODS

Buffalo meat sample

The meat samples were collected from buffalo slaughter house, Bareilly district, Uttar Pradesh State, India. The thigh muscles without excessive fat and connective tissue were collected from nine different buffaloes which were slaughtered according to traditional halal method and brought to the lab within 4 h of slaughter. Meat collected from nine different carcasses was packed separately in LDPE bags and kept in a chiller room maintained at 7-10°C for 24 h for the rigor mortis to complete so as to avoid cold shortening and excessive drip loss. After the initial chilling period, the total meat was divided into 3 replicates and each replicate represents meat from three randomly chosen carcasses. Each replicate of meat was divided into 7 experimental groups (C, T1 to T6) and packed in polystyrene foam tray covered with PVC cling film and stored in deep freezer at -18±1°C (Vest Frost, Denmark). These replicates of frozen meat were used for further experiments.

Experimental design

Temperature controlled incubators were used for the high temperature thawing of frozen buffalo meat, in which incubator 1 was maintained at 25±1°C and incubator 2 at 37±1°C respectively. Frozen buffalo meat undergone different thawing conditions as mentioned below:

C- Control; frozen meat kept at regular thawing temperature (4±1°C)

T_1, T_2, T_3 are frozen meat kept at higher thawing temperature of 25±1°C (incubator 1) for 6 h, 12h and 18h respectively

T_4, T_5, T_6 are frozen meat kept at higher thawing temperature of 37±1°C (incubator 2) for 4 h, 8h and 12h respectively

All the experimental groups (C, T1-T6) were analyzed for various microbiological parameters and FDA hydrolysis shortly after the experiment and reported as zero day results. All the experimental group (C, T1-T6) were further stored at refrigeration temperature (4±1°C) and the same parameters were analyzed further on alternate days (3d, 5d and 7d). Each experimental analyzes

were performed in duplicates in all three replicates (n=3).

Flourescein diacetate (FDA) hydrolysis

The FDA hydrolysis of meat samples were measured according to the procedure described by Venkitanarayanan *et al*.[22] with suitable modifications. One gram of meat was taken from each experimental group, observing necessary aseptic precautions. The samples were transferred into a tube containing 10 ml sterile peptone water (0.1%). The tubes were centrifuged at 100×*g* for 30 s to sediment meat particles. The supernatant was transferred into another tube and centrifuged at 3000×*g* for 30 min to pellet the bacterial cells. The supernatant was decanted and the bacterial pellet was washed and resuspended in 5 ml sterile sodium phosphate buffer (pH 7.6). The resultant 5 ml solution was sonicated in a bath sonicator (Soniprep 150 plus, MSE, U.K) in 4 episodes of 15 sec each. To 3 ml of the resulting clear solution, 100µL FDA reagent [500µg FDA/ml acetone] was added. The mixture was incubated at 25°C for 3 hr and the absorbance of the solution at 490 nm was recorded using a spectrophotometer (Model DU 640, Beckman, U.S. A). A tube containing 3 ml sterile phosphate buffer and 100 µl FDA reagent designated as ''blank'' was incubated simultaneously. The FDA hydrolysis was expressed as mean absorbance at 490 nm.

Microbiological analysis

All the microbiological parameters of meat samples were determined as per methods described by APHA [24]. Preparation of samples and serial dilutions were done near the flame of a horizontal laminar flow apparatus (model: YSI-188, Yarco Sales Pvt. Ltd., New Delhi) which were pre-sterilized by ultraviolet irradiation following all aseptic precautions. Duplicate plates were prepared and the counts were expressed as colony forming units (cfu) per gram. Plates for SPC were incubated at 37±1°C for 48 h and plates showing 30-300 colonies were counted. Psychrophiles were counted after incubation of plates at 4±1°C for 10-14 days. For yeast and mold count, the sterile cooled medium (potato dextrose agar) was acidified with sterilized 10% tartaric acid (1 ml/100 ml of media) to adjust the pH to 3.5. The plates were incubated at 25°C for 7 days and plates showing 10-150 colonies were counted. *Pseudomonas* count was carried out using *pseudomonas* agar base added with cetrimide supplement obtained from Sisco Research Laboratories Pvt Ltd., Mumbai (Code No. PM 028). The plates were incubated at 25±1°C for 48 h.

Statistical analysis

A randomized block design with three completely random replicates was used for experiments and the data generated for different meat quality parameters were compiled and analyzed using SPSS (version 20.0 for Windows; SPSS, Chicago. 111., U.S.A.). The data were subjected to analysis of variance, (two way ANOVA for storage data), and least significant difference for comparing the means to find the difference between groups and storage periods. The smallest difference ($D_{5\%}$) for two means was reported as significantly different ($P<0.05$). Pearson coefficient of correlation (r) between FDA hydrolysis and each of the microbial parameters was calculated. Regression equations were developed to predict various microbial parameters from the observed FDA hydrolysis values following linear regression model using FDA as independent variable and microbial parameters as dependent variable.

RESULTS AND DISCUSSION

FDA hydrolysis value (Mean ± S. E, Abs at 490 nm) was found to be significantly ($P<0.05$) higher in all higher temperature (25±1°C, 37±1°C)) thawed buffalo meat samples compared to a control sample, which was thawed at regular thawing temperature (4±1°C) (Table 1). Highest absorbance value of 0.48±0.02 was reported for samples subjected to T3 and T6 and lowest absorbance value of 0.24±0.02 for control sample just after the thawing period. And absorbance values of all the samples including control samples were found to be increased during refrigerated storage (4±1°C).

Various microbiological parameters evaluated using conventional methods for both treatment and control samples were presented in table 2 & 3. SPC value of all treatment samples were found to be increased after thawing period compared to control sample having a SPC count of 3.44±0.03 $\log_{10}$ cfu/g. A similar trend was observed with respect to yeast-mold count and *pseudomonas* count. In psychrophilic count there was no significant ($P<0.05$) difference was observed between C, T1 & T2 after temperature abuse, but

T3, T4, T5 & T6 has shown significantly (P<0.05) higher psychrophilic count compared to rest of the samples.

A highly significant (P<0.05) positive correlation (r = 0.97) was observed between SPC and FDA hydrolysis. Similarly FDA hydrolysis has shown positive correlation coefficient (r) of 0.91 with yeast-mold count, 0.86 with *Pseudomonas* count and 0.79 with psychrophilic count respectively (Fig 1&2). Since the SPC and FDA are

Table 1. FDA hydrolysis values of control and treatment samples after higher temperature thawing and on subsequent refrigeration storage at 4±1° C

Parameters/	Storage period (days)			
Groups	0	3	5	7
Flourescein diacetate (FDA) hydrolysis (Abs at 490nm)				
C	0.24±0.02^{d6}	0.30±0.02^{c6}	0.35±0.01^{b5}	0.49±0.01^{a2}
T1	0.27±0.02^{d4}	0.31±0.03^{c5}	0.41±0.02^{b4}	0.50±0.03^{a1}
T2	0.40±0.02^{c3}	0.48±0.02^{b3}	0.51±0.03^{a2}	ND
T3	0.48±0.02^{b1}	0.56±0.04^{a1}	ND	ND
T4	0.26±0.03^{d5}	0.36±0.03^{c4}	0.47±0.02^{b3}	0.51±0.03^{a1}
T5	0.42±0.01^{c2}	0.49±0.01^{b2}	0.53±0.02^{a1}	ND
T6	0.48±0.02^{b1}	0.56±0.02^{a1}	ND	ND

n=3. C=control; T1=Treatment 1 (thawed at 25±1°C for 6 h); T2=Treatment 2 (thawed at 25±1°C for 12 h); T3=Treatment 3 (thawed at 25±1°C for 18 h); T4=Treatment 4 (thawed at 37±1°C for 4 h); T5=Treatment 5 (thawed at 37±1°C for8 h); T6=Treatment 6 (thawed at 37±1°C for 12 h); ND=Not Detected due to visible spoilage development. Means with different superscripts (letters in the same row and numbers in the same column) indicate significance (P<0.05)

Table 2. SPC and Psychrophilic count of control and treatment samples after higher temperature thawing and on subsequent refrigeration storage at 4±1° C

Parameters/	Storage period (days)			
Groups	0	3	5	7
Standard plate count (log cfu/g)				
C	3.44±0.03^{d5}	3.95±0.11^{c6}	5.00±0.07^{b5}	6.96±0.06^{a2}
TI	4.21±0.02^{d4}	4.63±0.05^{c5}	5.62±0.02^{b4}	7.02±0.03^{a2}
T2	5.61±0.03^{c3}	6.32±0.03^{b3}	7.40±0.02^{a2*}	ND
T3	6.20±0.01^{b2}	8.57±0.06^{a2}	ND	ND
T4	4.29±0.04^{d4}	5.24±0.02^{c4}	6.31±0.02^{b3}	7.12±0.04^{a1}
T5	6.24±0.02^{c2}	6.46±0.02^{b3}	7.59±0.04^{a1*}	ND
T6	6.86±0.02^{b1}	8.81±0.04^{a1}	ND	ND
Psychropholic count (log cfu/g)				
C	3.56±0.04^{d5}	3.67±0.05^{c6}	4.64±0.01^{b4}	6.01±0.01^{a3}
T1	3.60±0.02^{d5}	4.54±0.08^{c5}	6.26±0.12^{b3}	6.61±0.05^{a2}
T2	3.51±0.03^{c5}	6.49±0.03^{b3}	7.68±0.06^{a1*}	ND
T3	5.21±0.04^{b3}	8.50±0.12^{a1}	ND	ND
T4	4.03±0.06^{d4}	4.43±0.09^{c5}	6.15±0.03^{b3}	7.24±0.03^{a1}
T5	5.47±0.06^{c2}	5.67±0.03^{b4}	7.91±0.04^{a2*}	ND
T6	5.71±0.03^{b1}	7.97±0.03^{a2}	ND	ND

n=3. C=control; T1=Treatment 1 (thawed at 25±1°C for 6 h); T2=Treatment 2 (thawed at 25±1°C for 12 h); T3=Treatment 3 (thawed at 25±1°C for 18 h); T4=Treatment 4 (thawed at 37±1°C for 4 h); T5=Treatment 5 (thawed at 37±1°C for8 h); T6=Treatment 6 (thawed at 37±1°C for 12 h); ND=Not Detected due to visible spoilage development. Means with different superscripts (letters in the same row and numbers in the same column) indicate significance (P<0.05)

showing the highest degree of correlation, a linear regression equation was developed by keeping FDA as dependent and SPC as independent variable ($Y = 0.08X-0.055$, Y represents FDA and X represents SPC). From this equation an FDA hydrolysis value of 0.5±0.05 was found to be the upper limit of acceptability for fresh meat. This FDA hydrolysis value was found to be coinciding with an SPC of $\log_{10}$ 7 cfu/g, which is the upper limit of microbiological acceptance of fresh frozen meat [4].Therefore the present study established a marginal FDA hydrolysis of 0.5±0.05 (mean ± s. e, Abs at 490 nm) which coincided with the microbiological rejection of fresh meat. And it was observed that the FDA hydrolysis value reached beyond the acceptable value (0.5±0.05) for samples T3 and T6 on 3rd day, T2 and T5 on 5th day and T1 and T4 on 7th day of refrigeration storage. To predict various microbial loads in higher temperature thawed buffalo meat, regression equations were developed between FDA hydrolysis as independent variable and different microbial

Table 3. Yeast-mold count and *Pseudomonas count* of control and treatment samples after higher temperature thawing and on subsequent refrigeration storage at 4±1° C

Parameters/	Storage period (days)			
Groups	0	3	5	7
Yeast and mold count (log cfu/g)				
C	3.07 ± 0.03^{d6}	3.44 ± 0.05^{c7}	4.32 ± 0.08^{b4}	4.68 ± 0.09^{a3}
TI	3.57 ± 0.05^{d4}	4.15 ± 0.05^{c6}	4.80 ± 0.09^{b3}	$5.33\pm0.10^{a2*}$
T2	3.60 ± 0.01^{c4}	4.69 ± 0.04^{b4}	$5.54\pm0.05^{a2*}$	ND
T3	5.05 ± 0.02^{b2}	$5.53\pm0.05^{a2*}$	ND	ND
T4	3.37 ± 0.06^{d5}	4.41 ± 0.04^{c5}	4.70 ± 0.06^{b3}	$6.19\pm0.04^{a1*}$
T5	4.58 ± 0.05^{c3}	5.30 ± 0.03^{b3}	$6.19\pm0.02^{a1*}$	ND
T6	5.57 ± 0.02^{b1}	$6.23\pm0.02^{a1*}$	ND	ND
Pseudomonas count (log cfu/g)				
C	3.33 ± 0.03^{d4}	3.80 ± 0.11^{c6}	5.17 ± 0.03^{b5}	6.31 ± 0.03^{a3}
T1	3.42 ± 0.05^{d4}	4.19 ± 0.04^{c5}	5.81 ± 0.10^{b4}	$6.61\pm0.10^{a2*}$
T2	4.53 ± 0.02^{c3}	6.10 ± 0.03^{b3}	$7.13\pm0.06^{a2*}$	ND
T3	5.26 ± 0.06^{b2}	$8.23\pm0.05^{a1*}$	ND	ND
T4	4.58 ± 0.05^{d3}	5.18 ± 0.05^{c4}	6.45 ± 0.04^{b3}	$7.11\pm0.02^{a1*}$
T5	5.30 ± 0.07^{c2}	6.31 ± 0.10^{b2}	$7.58\pm0.09^{a1*}$	ND
T6	5.81 ± 0.04^{b1}	$8.32\pm0.07^{a1*}$	ND	ND

n=3. C=control; T1=Treatment 1 (thawed at 25±1°C for 6 h); T2=Treatment 2 (thawed at 25±1°C for 12 h); T3=Treatment 3 (thawed at 25±1°C for 18 h); T4=Treatment 4 (thawed at 37±1°C for 4 h); T5=Treatment 5 (thawed at 37±1°C for8 h); T6=Treatment 6 (thawed at 37±1°C for 12 h); ND=Not Detected due to visible spoilage development. Means with different superscripts (letters in the same row and numbers in the same column) indicate significance ($P<0.05$)

Table 4. Linear regression equations of various microbiological counts from observed FDA values from higher temperature thawed buffalo meat

Microbiological parameter	Regression equation
Standard plate count (SPC)	$Y_1 = 11.863X+0.943$ (Y1=SPC, X= FDA)
Yeast-mold count (YMC)	$Y_2 = 8.182X+1.135$ (Y2=YMC, X= FDA)
Pseudomonas count (PSD)	$Y_3 = 7.896X+1.728$ (Y3=PSD, X= FDA)
Psychrophilic count (PSY)	$Y_4 = 7.406X+1.743$ (Y4=PSY, X= FDA)

Standard error of estimate and level of significance at $P<0.05$ for the regression constant is 0.329 and 0.103 for SPC, 0.441 and 0.139 for YMC, 0.502 and 0.066 for PSD, 0.654 and 0.128 for PSY respectively.

parameters as dependent variable (Table. 4).

Laboratory for Environmental Pathogens Research, Department of Environmental Sciences, University of Toledo has given the regression equation, $Y= -0.02+9.3\times O.D_{490}$ for quantification of flourescien produced by microbial action in various samples. A similar finding was observed for beef steaks during thawing and exposure to 25°C, where FDA activity increased from 0.1 to 0.6 units as bacterial load increased from 102 cfu to 108 cfu/ cm2 [22]. In one such study a higher degree of FDA hydrolytic activity was observed in non irradiated meat samples compared to irradiated meat samples [25], due to higher microbial load in non irradiated meat. A correlation coefficient of 0.92 was reported between FDA value and aerobic plate count [22]. Association between fungal biomass and FDA activity in soil were also reported [26]. Moreover the same researchers established the association between biomass and FDA activity using microbial respiration as µL O_2/h/g of dry weight. A linear increase in FDA activity was observed in soil and litter with increase in *Fusarium culmorum* and *Pseudomonas denitrificans* biomass [26].

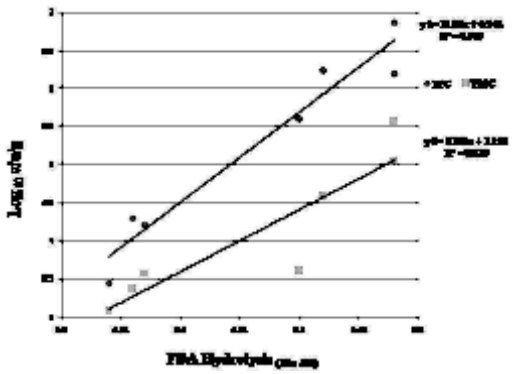

Fig. 1. Correlation of FDA hydrolysis with Total Plate Count (TPC) and Yeast and Mold Count (YMC), Y1 represents TPC and Y2 represents YMC

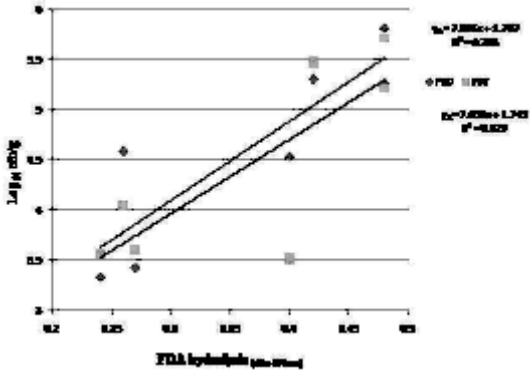

Fig. 2. Correlation of FDA hydrolysis with *Pseudomonas* count (PSD) and Psychrophilic Count (PSY), Y3 represents PSD and Y4 represents PSY

CONCLUSION

The demand for rapid, cost effective and less labour intensive methods for quantification of food borne microorganisms is increasing. Since temperature abuse in the form of indiscriminate thawing is a common cause of buffalo meat spoilage, rapid methods are required to assess the microbial safety of buffalo meat. The present study establishes usefulness of FDA hydrolysis as a rapid test since it shows a higher degree of correlation with SPC. Regression analysis showed that FDA activity can be used to predict the various microbial loads in temperature abused buffalo meat samples. Therefore the present findings will help the Indian buffalo meat industry by providing a rapid and accurate decision making tool regarding microbial safety of meat.

ACKNOWLEDGEMENTS

The authors are thankful to the Director, Indian Veterinary Research Institute, Izatnagar for the facilities provided. The work was carried out using the institute fund provided for the project approved by The Director and The Joint Director (Research), Indian veterinary Research Institute.

REFERENCES

1. APEDA. Export of agro and processed food products including meat and meat products (2012). Agricultural and Processed Food Products Export Development Authority. Ministry of Commerce, Government of India.
2. Nychas, G.J. E., Skandamis, P. N., Tassou, C. C., & Koutsoumanis, K. P. Meat spoilage during distribution. *Meat Sci.*, 2008; **78(1-2)**: 77-89.
3. Ingham, S. C., Fanslau, M. A., Burnham, G.

M., Ingham, B. H., Norback, J. P., Shafner, D. W. Predicting pathogen growth during short term temperature abuse of raw pork, beef, and poultry products: Use of an isothermal based predictive model. *J. Food. Protect.*, 2007; **70(6)**: 1445-1454.

4. ICMSF. 2002. Microorganisms in foods-2. Sampling for microbiological analysis: Principles and specific applications. 2nd (edn), Blackwell scientific publications.
5. Jay, J.M., Loessner, M. J., & Golden, D. A. (2005). *Modern Food Microbiology*. 7th edition. New York : Springer.
6. Kandeepan, G., Biswas, S. Effect of domestic refrigeration on keeping quality of buffalo meat. *J Food Technol*, 2007; **5(1)**, 29-35.
7. Byun, J.S., Min, J. S., Kim, I. S., Kim, J.W., Chung, M.S., Lee, M. Comparison of Indicators of microbial quality of meat during aerobic cold storage. *J Food Protect*, 2003; **66**, 1733-1737.
8. Nychas, G.J. E., Skandamis, P. N., Tassou, C. C., Koutsoumanis, K. P. Meat spoilage during distribution. *Meat Sci*, 2008; **78(1-2**), 77-89.
9. Nychas, G-J.E., Marshall, D., & Sofos, J. (2007). Meat poultry and seafood. In M. P. Doyle, L. R. Beuchat. and T. J. Montville, (eds.). *Food Microbiology: Fundamentals and Frontiers*, Washington DC: ASM press.
10. Gram, L., Huss, H. Microbiological spoilage of fish and fish products. *Int J Food Microbiol*, 1996; **33**: 121-137.
11. Armstrong, D. W., Schneiderheinze, J. M., Kullman, J. P., He, L. Rapid CE microbial assays for consumer products that contain active bacteria. *FEMS Microbiology Letters*, 2001; **194**: 33-37.
12. Grossi, M., Lanzoni, M., Pompei, A., Lazzarini, R., Matteuzzi, D., Ricc'o, B. Detection of microbial concentration in ice-cream using the impedance technique, *Biosensors and Bioelectronics*, 2008; **23,** 1616–1623
13. Karl, D. M. Cellular nucleotide measurements and applications in microbial ecology. *Microbiol Rev*, 1980; **44**, 739-785.
14. Hoshi, M., Nishi, H., Hayashi, T., Okuzumi, M., Watanabe, E. Development of a biosensor for the determination of total viable count. *Nippon Suisan Gakkaishi*, 1991; **57**: 281-285.
15. Endo, H., Nagano, Y., Ren, H., Hayashi, T. Rapid enumeration of bacteria growth on surimi based products by flowcytometry. *Fisheries Science* , 2001; **67**, 959-974.
16. Ramsay, G., Turner, A. P. E. Development of an electrochemical method for the rapid determination of microbial concentration and evidence for the reaction mechanism. *Analytica Chimica Acta*, 1988; **215**, 61-69.
17. Greer, G., Gordon, D., Bryan, D. Enumeration of meat borne spoilage bacteria with hydrophobic grid membrane filtration. *J Food Protect*, 1997; **3**, 1388-1390
18. Takashi, K., Toshihiro. Colorimetric alamarBlue assay as a bacterial concentration and spoilage index of marine foods, *Yano Food Control*, 2003; **14**, 455–461
19. Webbs, N.B., Thomas, F.B., Busta, F.F., Kerr, L.S. Evaluation of scallop meat quality by there sazurin reduction technique. *J Milk Food Technol*, 1972; **35**, 664–668.
20. Dodsworth, P.J., Kempton, A.G. Rapid measurement of meat quality by resazurin reduction II. Industrial Application. *J Inst Can Sci Technol Aliment*, 1977; **10**, 158–160.
21. Kroll, R.G. Dye reduction and other colorimetric methods for the assessment of microbial contamination. In *Rapid Methods in Food Microbiology*, M.R. Adams, and C.F.A. Hope, (Ed.), 1989; 191–237. Elsevier Publ. Amsterdam.
22. Venkitanarayanan, K. S., Faustman, C., Hoagland, T., Berry, B. W. Estimation of spoilage bacterial load on meat by fluorescein diacetate hydrolysis or resazurin reduction. *J Food Sci*, 1997; **62**, 601-604.
23. Guilbault, G.G., Kramer, D.N. Fluorometric determination of lipase, acylase, alpha and gamma chymotrypsin and inhibitors of these enzymes, *Anal Chem*, 1964; **36**, 409–412.
24. APHA. Compendium of methods for the microbiological examination of foods, 2001; 4th edition. American Public Health Association, Washington DC, USA.
25. Venkitanarayanan, K.S., Faustman, C., Berry, B.W. 1993. Rapid methods to assess spoilage and predict shelf–life of beef. Proceed Food Preservation 2000 Conf., Natick, MA. II: 671–677.
26. Schnurer, J., Roswall, T. Fluorescein diacetate hydrolysis as a measure of total microbial activity in soil and litter. *Appl Environ Microbiol*, 1982; **43**, 1256–1261.