Original Research

Estimation of Microbial Load in Anthocyanin-CMC Based Edible Film Coated Chicken Meat Slices in Refrigeration Storage Using Fluorescein Di-acetate (FDA) Hydrolysis Assay

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Abstract

The goal of the study was to estimate various microbial loads in anthocyanin-CMC based edible film coated chicken slices in refrigeration storage using fluorescein di-acetate (FDA, 3', 6'-diacetyl-fluorescein) hydrolysis assay. The samples were analyzed for various microbiological parameters using conventional plate count method and compared with FDA hydrolysis. Refrigerated (4±1°C) meat samples were analyzed up to 30 days for each 5 days interval. Significant (P<0.01) increase in FDA hydrolysis values were noticed in refrigeration storage condition both for control and treatments. Total plate count (TPC), pseudomonas count, psychrophilic count and Staphylococcus spp. count were increased significantly (P<0.01) in refrigerated storage condition for both control and treatments. FDA hydrolysis showed significant (P<0.01) positive correlation with all the microbial parameters and highest degree of association was noticed between FDA and TPC (r = 0.97).

Key words: Anthocyanin, Chicken Slices, Correlation, FDA Hydrolysis, Refrigerated Storage

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Introduction

Meat spoilage in supply chain creates great economic losses, by way of reduced product sale to producers at one end and also creates extra monitory burden for health care due to food poisoning among the

consumers. The microbiological safety and quality of poultry meat products are equally important to producers, retailers and consumers, and both involve microbial contaminants on the processed products (Mead GC, 2004). Therefore to establish a microbiological acceptance criterion for refrigerated chicken meat products, ICMSF has put forward a total plate count value of $\log_{10}5$ cfu/g as the upper microbiological limit (International Commission on Microbiological Specifications for Foods, 2002).

Edible films and coatings are an alternative to plastics extend the shelf life of animal originated foods and others by acting as barriers to water vapor, oxygen and carbon dioxide and as carriers of substances to inhibit pathogenic and spoilage microorganisms. Polysaccharides derived from cellulose, starch, alginate, and their mixtures have been used for edible films most frequently because of their excellent film-forming properties (Durango *et al.,* 2006). Anthocyanins, some natural pigments, have good potential to act as visual spoilage indicator in intelligent packaging system (Golasz *et al.,* 2013, Yoshida *et al.,* 2014; Shukla *et al.,* 2016). These types of packaging systems only can give us overview of quality status of products but laboratory based examinations are necessary for confirmation of spoilage.

Conventional microbiological analysis is extensively time-consuming where product demands are too high to supply. Meat industry and researchers are seeking for alternative rapid quantification methods to determine microbial loads in meat and meat products in supply chain. Some traditional physico-chemical parameters are there to evaluate microbial status in meat industry like pH, extract release volume (ERV), total volatile basic nitrogen (TVBN), tyrosine value, D-glucose, free amino acids (FAA) etc. especially for protein rich meat and meat products. Other than these, enumeration methods based on microscopy, ATP bioluminescence, and the measurement of electrical phenomena (Champiat *et al*., 2010), as well as detection methods based on either immunological or nucleic acid-based procedures (Scheu *et al.,* 1998), rapid capillary electrophoresis (CE) in powdered health supplements, Fourier transform infrared (FT-IR) spectroscopy, flow cytometric methods (Endo *et al.,* 2001), electrochemical methods (Ramsay *et al.,* 1988) and a hydrophobic grid membrane filtration (HGMF) (Greer *et al.,* 1997) for raw beef were also developed for the quantification of microbial load. These experiments need extensive instrumentations, skilled man power and availability of time.

FDA hydrolysis is an alternative informative colorimetric method for microbial spoilage detection in food system. Fluorescein is conjugated to two acetate radicals. This colour-less compound, fluorescein diacetate (FDA, 3', 6'-diacetyl-fluorescein) is hydrolysed by both microbial free exoenzymes and membrane bound enzymes releasing a coloured end product, fluorescein can be measured by spectrophotometry at 490 nm (Guilbault *et al.,* 1964; Stubberfield and Shaw, 1990). The intensity of the resulting yellow-green (fluorescein) color is indicative of the amount of enzymatic cleavage of the FDA molecule and the overall enzymatic activity in the sample (Adam and Duncan, 2001). Therefore, objective of this study was taken to

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evaluate microbial load of smart packaged chicken slices for one month refrigerated storage condition by FDA hydrolysis assay and correlate the result with conventional microbiological analysis method.

Materials and Methods

Dressed chicken (WLH) were procured from Central Avian Research Institute (CARI), Izatnagar and these were deboned manually in the experimental abattoir of LPT Division , IVRI, Izatnagar by removing tendons, separable connective tissue and body fat and kept at -18°C until processed packed with LDPE films (200gauge). Carboxymethyl cellulose (CMC), plasticizers and other food grade chemicals (SD- Fine, MERCK, Sigma-Aldrich); Anthocyanin from red cabbage (*Brassica oleraceae),* (local market of Bareilly); condiments, spice mixes, refined wheat flour (local market of Bareilly), refined salts (Tata Chemicals Ltd., Mumbai) were used as raw materials for preparation of films and chicken slices. For microbiological studies media from Hi-Media Laboratories Pvt. Ltd., Mumbai was used.

Film Preparation

Films were prepared from a filmogenic suspension of CMC (2%), glycerol and poly ethylene glycol as plasticizers (0.75% each), and red cabbage extract containing anthocyanin (15%) using the casting technique. These films were prepared according to Ghanbarzadeh *et al.* (2010); Golasz *et al*. (2013) and Soni *et al*. (2016) with slight modifications.

Sample Preparation

Frozen chicken meat was partially thawed at refrigeration temperature (4±1ºC) for 16-18 h. Then cut into small cubes and double minced in a meat mincer (Hobart, US patentis, USA). Meat emulsion was prepared in a bowl chopper (Seydelmann K20, Ras, Germany). Pre-weighed quantity of minced chicken meat, salt, sodium tripolyphosphate (STPP) and sodium nitrite were added and chopping was done for about 2-3 minutes. It was chopped again for 2 minutes after the addition of ice flakes. Refined vegetable oil was slowly incorporated while chopping till it was completely dispersed in the batter. Condiment paste, dry spice mix, refined wheat flour and other ingredients were added. Chopping was continued till uniform dispersion of all the ingredients and desired consistency of the emulsion was achieved. The batter was stuffed in a stainless steel box smeared with oil inside and pressure free steam cooked for 45 min. Chicken meat blocks so obtained were cooled and sliced into each weighing 50 grams having 11 cm length, 4.8 cm width and 10 mm thickness with the help of meat slicer.

Experimental Design

Chicken meat slices were wrapped by anthocyanin-CMC based edible films as primary packaging materials and LDPE films (200 gauges) as secondary packaging material for maintenance of shelf life and they were

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assumed as treatments (T). Meat slices without edible films were treated as control (C) to compare the differences of microbial load. They were kept at refrigeration temperature (4±1°C) for one month of storage and were analyzed for FDA hydrolysis and microbiological parameters on every 5 days interval up to 30 days.

Flourescein diacetate (FDA) Hydrolysis

The FDA hydrolysis of meat product samples was measured according to the procedure described by Venkitanarayanan *et al.* (1997) with suitable modifications. The FDA hydrolysis was expressed as mean absorbance at 490 nm.

Microbiological Analysis

All the microbiological parameters (total plate count, psychrophilic count, pseudomonas count and staphylococcus count) of meat samples were determined as per methods described by APHA (2001).

Statistical Analysis

Three trials were conducted for each experiment with duplicate samples (No. of replicates=3). The data generated from various trials under each experiment were pooled and analysed by statistical method of one way-ANOVA, paired t-test and compare means as per the procedure of Snedecor and Cochran (1995) using IBM SPSS Statistics software (Version 20.0 for Windows; IBM SPSS Inc, Chicago, 111, USA). 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

Refrigerated chicken slices showed a significant (P<0.01) increase in microbial load in FDA hydrolysis (Table 1). The differences between control and treatment groups during FDA hydrolysis was also noticed significantly (P<0.01) during refrigerated storage period. The increased FDA hydrolysis values were observed in the present study was due to increased hydrolysis of FDA by enzymes like proteases, lipases and esterases released from bacterial cells. FDA hydrolysis by bacteria from meat samples showed increased absorbance at 490 nm as the total plate count of chicken slice sample increased during storage. Fluorescence colour development was clearly noticed in Fig. 1. In treatment, a slight decreased value of FDA hydrolysis was observed for first few days of storage that might be due to primary packaging. As bacterial load increased from 10^2cfu/cm^2 to 10^8cfu/cm^2, FDA activity (A_{490}) increased from 0.1 to 0.6 units (Venkitanarayanan *et al.,* 1997). Vishnuraj *et al.* (2014) reported that on subsequent refrigerated storage

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temperature abused buffalo meat sample at 4±1°C, the FDA hydrolysis increased significantly (p<0.05) with storage period. Sing *et al*. (2015) showed that exposure of chicken sausages to refrigerated temperature of 5±1°C showed a significant (P<0.05) increase in FDA hydrolysis.

Table 1: FDA hydrolysis values of control and treatment samples of chicken slices after refrigeration storage at 4±1° C

	FDA (Abs at 490nm)						
	0th day	**5th day**	**10th day**	**15th day**	**20th day**	**25th day**	**30th day**
C	0.02 ± 0.00^{fA}	0.11 ± 0.00^{eA}	0.13 ± 0.00^{dA}	0.14 ± 0.00^{dA}	0.19 ± 0.00^{cA}	0.23 ± 0.00^{bA}	0.31 ± 0.00^{aA}
T	0.06 ± 0.00^{fB}	0.05 ± 0.00^{fB}	0.09 ± 0.00^{eB}	0.12 ± 0.00^{dB}	0.17 ± 0.01^{cB}	0.24 ± 0.01^{bB}	0.33 ± 0.02^{aB}

**Mean±S.E. with different superscripts row wise (small alphabet) and column wise (capital alphabet) differ highly significantly (P<0.01); n =6; C=Control; T=Treatment*

Fig. 1: FDA hydrolysis in control and treatment groups on 30th day of refrigeration storage (1st bottle=blank, next two bottles=control, last two bottles= treatment)

Conventional microbiological analysis evaluated for both treatment and control samples were presented in Table 2 to compare with FDA hydrolysis. The total plate count (TPC) of chicken slices under refrigeration condition increased significantly (P<0.05) both in control and treatment groups. It reached to 5.61 ± 0.01 $\log_{10}$ cfu/g on 30th day of storage in treatment which was below permissible limit of $\log_{10}7$ cfu/g. Edible film of CMC was a base for growth of more microorganisms as CMC would act as simple nutrient. A significant (P<0.05) increase in total plate counts of chicken meat products stored under refrigeration was in agreement with Nath *et al*. (1995), Bhat *et al*. (2010), Bhat *et al.* (2013) who also reported similar results

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in chicken patties, chevon and chicken meat ball respectively. A similar result was seen for *pseudomonas* count, psychrophilic count and *Staphylococcus* count in this experiment (Table 2).

Table 2: Effect of refrigerated storage on microbiological characteristics of chicken slices packaged with Anthocyanin-CMC based edible film (Mean±S.E.)*

Treatment	Refrigerated Storage Period (Days)						
	0	**5**	**10**	**15**	**20**	**25**	**30**
Total Plate Count (log_{10} cfu/g)							
C	2.20 ± 0.02^{gA}	3.01 ± 0.02^{fA}	3.36 ± 0.02^{eA}	3.52 ± 0.02^{dA}	3.81 ± 0.02^{cA}	4.47 ± 0.01^{bA}	5.45 ± 0.01^{aA}
T	2.60 ± 0.04^{fB}	3.14 ± 0.02^{eB}	3.15 ± 0.04^{eB}	3.43 ± 0.03^{dB}	4.23 ± 0.01^{cB}	4.58 ± 0.01^{bB}	5.61 ± 0.01^{aB}
Psychrophilic Count (log_{10} cfu/g)							
C	ND	ND	1.35 ± 0.43^{dA}	2.67 ± 0.04^{cA}	3.24 ± 0.06^{bA}	3.55 ± 0.03^{abA}	3.95 ± 0.03^{aA}
T	ND	ND	0.98 ± 0.43^{dA}	2.16 ± 0.06^{cB}	3.24 ± 0.02^{bA}	3.50 ± 0.02^{bA}	4.04 ± 0.03^{aA}
***Staphylococcus spp.* Count (log_{10} cfu/g)**							
C	1.35 ± 0.43^{aA}	2.66 ± 0.07^{cA}	2.84 ± 0.06^{bcA}	2.67 ± 0.06^{cA}	3.13 ± 0.07^{abcA}	3.31 ± 0.02^{abA}	3.46 ± 0.03^{aA}
T	0.33 ± 0.32^{cA}	1.73 ± 0.35^{bB}	2.26 ± 0.07^{abB}	2.50 ± 0.06^{abA}	2.55 ± 0.07^{abB}	2.28 ± 0.46^{abA}	3.06 ± 0.03^{aB}
***Pseudosomans spp.* Count (log_{10} cfu/g)**							
C	2.30 ± 0.07^{fA}	2.57 ± 0.06^{eA}	2.85 ± 0.04^{dB}	3.05 ± 0.05^{dA}	3.26 ± 0.07^{cB}	4.51 ± 0.12^{bA}	5.15 ± 0.01^{aA}
T	2.38 ± 0.08^{fA}	2.90 ± 0.10^{eA}	3.14 ± 0.02^{dA}	3.35 ± 0.05^{dB}	3.92 ± 0.09^{cA}	4.57 ± 0.07^{bA}	5.17 ± 0.06^{aA}

**Mean±S.E. with different superscripts row wise (small alphabet) and column wise (capital alphabet) differ significantly (P<0.05); n =6; ND= not detected; C=control; T=treatment (edible film coated)*

The psychrophilic count was not observed on 0 and 5th day of storage in control and treatment although thereafter, psychrophilic count increased significantly (P<0.05) at each refrigerated storage interval. In the present study, psychrophilic counts always remained below the threshold level of acceptability of cooked meat products that have been reported as log_{10} 4.6 cfu/g (Cremer and Chipley, 1977). This result was expected since psychrotrophic microorganisms dominate the microbiota of protein food stored under refrigeration, and they are the main microorganisms responsible for the deterioration of chilled meat (Nychas *et al*., 2008; Jay, 2000). In case of *Staphylococcus* count, treatment showed slight lower values in refrigerated storage than control, lower counts might be due to antibacterial effects of anthocyanin. El-Nashi *et al*. (2015) found a similar result in beef sausage as incorporated with pomegranate rind extract showed less *Staphylococcus aureus count.* Saravana kumar (2015) stated that a potential bioactive anthocyanin compounds from the flower petals of *Sesbania sesban* was antimicrobial compound against Gram positive bacteria like *Staphylococcus aureus* and *Staphylococcus saprophyticus*.

A highly significant (*P*<0.01) positive correlation (r = 0.97) was observed between TPC and FDA hydrolysis. Similarly, FDA hydrolysis had shown positive correlation coefficient (r) of 0.92 with *Pseudomonas* count, 0.89 with *Psychrophilic* count and 0.57 with *Staphylococcus* count respectively in treatments (Table 3).

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Table 3: Pearson correlation coefficients between FDA hydrolysis and microbiological characteristics of chicken slices at refrigeration storage periods (4±1^{0}C)

Correlations					
		TPC	**PSY**	**STAPH**	**PSEUDO**
C	**FDA**	0.986**	0.868**	0.790**	0.966**
T	**FDA**	0.970**	0.894**	0.572**	0.921**

***Correlation is significant at the 0.01 level (2-tailed).*

Prediction of TPC and *Pseudomonas* count from FDA hydrolysis by using linear regression coefficient equation and correlation coefficient was showed in Table 4 for both control and treatment. Prediction of TPC and *Pseudomonas* count with FDA from linear regression equation showed positive linear relation of 97% and 92% prediction respectively and the actual values were close to the predicted values as observed in Fig. 2 and Fig. 3.

Table 4: Prediction of Total plate count (TPC) and *Pseudomonas spp*. count from FDA hydrolysis by using linear regression coefficient and correlation coefficient

	Linear Regression Coefficient	Correlation Coefficient
C	Y(TPC)=10.996X+1.851$_{(X=FDA)}$+C.F(.292)	0.986
	Y(PSEUDO)=7.492X+3.031$_{(X=FDA)}$+C.F(.316)	0.966
T	Y(TPC)=9.033X+2.381$_{(X=FDA)}$+C.F(.356)	0.97
	Y(PSEUDO)=6.214X+3.363$_{(X=FDA)}$+C.F(.417)	0.921

C=Control; T=Treatment; C.F=Correction factor (i.e. S.E)

Laboratory for Environmental Pathogens Research, Department of Environmental Sciences, University of Toledo has given the regression equation, Y= -0.02+9.3×O.D490 for quantification of fluorescein produced by microbial action in various samples. A correlation coefficient of 0.92 was reported between FDA value and aerobic plate count (Venkitanarayanan *et al.,* 1997).

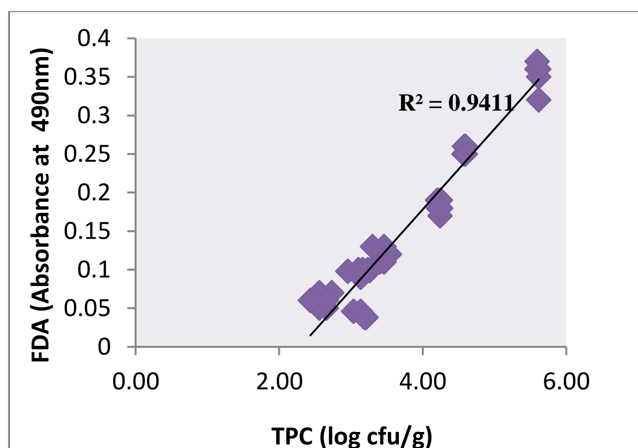

Fig. 2: Prediction of TPC from Fluroscent diacetate hydrolysis (FDA) values in treatment sample during refrigerated storage by using linear regression coefficient and correlation coefficient

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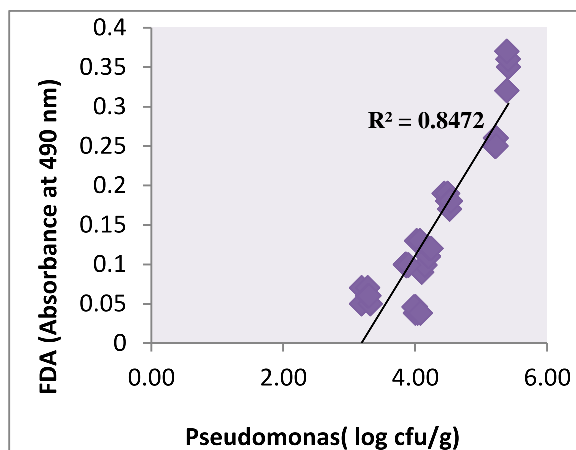

Fig. 3: Prediction of Pseudomonas from Fluroscent diacetate hydrolysis (FDA) values in treatment sample during refrigerated storage by using linear regression coefficient and correlation coefficient

A linear increase in FDA activity was observed in soil and litter with increase in *Fusarium culmorum* and *Pseudomonas denitrificans* biomass (Schnurer *et al*., 1982). Positive correlation coefficient of 0.97, 0.86, and 0.79 was reported for FDA with TPC, Pseudomonas and Psychrophilic counts respectively by Vishnuraj *et al*. (2014).

Conclusion

Demand for chicken meat products has been increasing day by day in market. Therefore, the quality and safety issues are also challenging for meat researchers now a days for consumers health benefit. Among the several rapid microbial quantification techniques, FDA hydrolysis is one of the rapid, cost effective colorimetric microbial quantification method can be used easily for food borne microorganisms' detection. In this current study, it was evaluated that edible film coated chicken slices showed more microbial counts at refrigerated storage except *Staphylococcus* count as Red cabbage extract was used here as natural indicator of meat spoilage rather than it's antimicrobial activity. In this study, a highly positive correlation was found between TPC and FDA. Various microbial loads in chicken slices were predicted from regression analysis with FDA hydrolysis. Therefore, this experiment will help to explore the microbial quality of the meat products to the producers, processors and consumers of whole meat industry.

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