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Complete List of Authors:	Sobhan, Abdus ; South Dakota State University Muthukumarappan, Kasiviswanathan; South Dakota State University, Agricultural and Biosystems Engineering Wei, Lin; South Dakota State Unversity, Zhou, Ruanbao; South Dakota State University, Biology and Microbiology Tummala, Hemachand; South Dakota State University - College of Pharmacy, Pharmaceutical Sciences

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Development of a polylactic acid-coated nanocellulose/chitosan-based film indicator for real-time monitoring beef spoilage

Abdus Sobhan[a], Kasiviswanathan Muthukumarappan[a], Lin Wei[a]*, Ruanbao Zhou[b], and Hemachand Tummala[c]

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[a]*Department of Agricultural & Biosystems Engineering, South Dakota State University,*

1400 North Campus Drive, Brookings, South Dakota 57007, United States

[b]*Department of Biology and Microbiology, South Dakota State University, 1400 North Campus Drive, Brookings, SD 57007, United State*

[c]*Department of Pharmaceutical Sciences, South Dakota State University, 1400 North Campus Drive, Brookings, SD 57007, United State*

* Correspondence: E-mail: Lin.Wei@sdstate.edu.; Tel: +1-605-688-4179; Fax: +1-605-688-6764.

Abstract. Food safety is one of the biggest challenges in global markets. There is a critical need to develop a simple, affordable, and environmentally friendly color indicator that can fast and conveniently monitor and indicate the quality of packaged food products at home, supermarkets, shops, etc. This study aimed to develop a nanocellulose/chitosan-based film coated with polylactic acid (PLA) to monitor beef spoilage in real-time. This film was fabricated by casting suspension of nanocellulose/chitosan mixture doped with methyl red, followed by a coating of PLA on the film surface, named PLA/NCM film. The film displayed a visible color change in response to different pH buffer solutions (2−10). The PLA/NCM film was applied to monitor the spoilage of beef at a refrigerant condition (4 °C) and showed an apparent color change after 5 days as a threshold for the beef spoilage. The color modulation of the PLA/NCM films was processed for each time via the colorimetric device and revealed substantial color difference values (ΔE) after 5 days of beef spoilage. The total viable microbial counts (TVC) and pH of the beef sample were determined, and the findings showed that the TVC and pH increased simultaneously during the beef spoilage. Although further research is necessary, the PLA/NCM film has the potential to be a color indicator for applications in both smart food packaging and real-time monitoring spoilage of beef and other meat products.

Keywords. Smart packaging; Indicator; Film; Nanocellulose; Food Spoilage

1. Introduction

Smart packaging is a packaging system which can monitor the condition of the packaged foods or food environments and improve the quality of food during transportation and storage.[1] The most common techniques used to monitor the condition of foods are time-temperature indicators, radio frequency identification, thermochromic ink, gas chromatography and electronic chemical nose.[2] These methods monitor the volatile and biogenic amines produced as metabolites related to food spoilage. The most common metabolites produced after meat and fish spoilage are ionized or deionized ammonia, histamine, tyramine, cadaverine and putrescine.[3–5] However, these methods have some drawbacks. For example, they require a higher level of expertise, are time-consuming and expensive, and have low precision.[6–10] In addition, these packaging

[a.] *Address here.*
[b.] *Address here.*
[c.] *Address here.*
† Footnotes relating to the title and/or authors should appear here.
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materials are not recyclable and are poorly biodegradable, led to unnecessary waste disposal and environmental contamination issues.[11] There is therefore a need for a fast, convenient, low-cost, and eye-catching method to assess the quality of packaged food products at home, supermarkets, shops, etc.

Chitosan is one of the copolymers that comprises of β-(1–4)-2-amino-d-glucose and β-(1–4)-2-acetamido-d-glucose units.[12] The polysaccharide backbones in chitosan have excellent physicochemical properties that make them ideal for use in food packaging, biomedical applications, and the chemical industry. Recently, chitosan has received an approval from the U.S. Food and Drug Administration (FDA) and regulatory agencies for their usage as the biomaterials in a variety of commercial food products.[13] In the previous studies, the use of chitosan has been reported with brown rice starch, Chinese root extract, alizarin, etc. for developing packaging film.[12,14,15] We have considered chitosan in our study because it owns excellent film formability, high transparency, biodegradability, and high mechanical property.[12]

The most popular organic polymer in nature is cellulose, which has attractive features such as biocompatibility, low cost, low density, and good mechanical properties.[16] Cellulose nanofibers (CNF) with a width of 50 nm have been identified as a reinforcing material for packaging film formation.[17] The ideal characteristics of CNF are transparency, inexpensive, low density, flexibility and good chemical stability.[18] In addition, CNF acts as an excellent additive modifier to improve the film's performance and has eco-friendly, and naturally degradable properties.[16] The physical combination of CNF and chitosan, along with methyl red (MR) as an acid-base indicator, can offer a potential pH-sensing nanocomposite for film preparation.

The first generation of biodegradable polyester is poly-lactic acid (PLA) and has been commonly used in the form of pure or blended polymer as food packaging film.[19,20] The reason for using PLA in this study is its biodegradable and biocompatibility properties which eliminates health challenges in food packaging, and its benefits due to its abundance, cleanliness, and reasonable cost. Furthermore, the coating of PLA as a thin layer enhances the mechanical strength of the film and considerably rises porosity upon the film, resulting in a higher surface area, which helps the sensing element to be entrapped onto the film surface.[19,21] Although different film indicators for food spoilage have been developed, such as pH indicator film,[22] anthocyanin film,[23] azo-anthraquinone film[24] and dye-based indicator film,[25] all of these films have lower mechanical strength, poor color stability, and difficulty to determine onset-detection associated with the spoilage threshold. To overcome these limitations, PLA/NCM film as an on-packaged indicator has been considered as a new alternative approach via eye-catching detection of meat spoilage. To the best of our knowledge, no studies have been reported so far on this functionalized dyestuff with nanocellulose/chitosan, so we decided to use this film to monitor meat spoilage. The main aim of this work is to develop PLA/NCM film as an on-packaged indicator for real-time monitoring of meat spoilage. The objectives of this work are to 1) develop PLA/NCM film and characterize the functional and microbial response properties, and 2) to employ the indicator film to monitor for meat spoilage.

2. Materials and methods

2.1. Materials

CNF's aqueous-based gel with 3 percent solid purity and 87 percent crystalline index was obtained from the University of Maine Process Development Center (Orono, ME, USA). Chitosan with a deacetylation degree of 75 % and Methyl red (MR) were acquired from Sigma-Aldrich (St. Louis, MO, USA). The stock solution of MR was made by dissolving MR into 70% (v/v) of ethanol solution to yield a concentration of 1 mg/mL. Polylactic acid (PLA) employed in this research was procured SOC3D Company (MI, USA) and the solution of PLA was prepared by dissolving PLA in chloroform (w/w). The density, melting point and glass transition temperature of PLA were 1.24 g/cm^3, 160°C and 57.8°C, respectively.

2.2. Preparation and fabrication of PLA/NCM film

The PLA/NCM film's solution was prepared as described below. Initially, 30 mL of MR suspension was prepared with 70% of ethanol, and concentrations of MR in ethanol were maintained at 1mg/mL. Second, 4.5 g of CNF gel was taken with MR solution and then slightly agitated for 20 min to produce an MR dopped CNF suspension. Then, 0.5 g of chitosan was added to the MR dopped CNF suspension and then homogenized with a handheld homogenizer (4000 rpm) for 15 min. The prepared homogenized mixture of MR, CNF and chitosan was then transferred into a petri-dish (diameter 13 cm) and dried two days at room temperature (21 °C) to get a dried MR dopped nanocellulose/chitosan film, which is renamed as NCM film. Afterward, the dried NCM film was dipped into 2% PLA (wt.%) solution and coated the film for 5 min to generate PLA/NCM film. Afterward, PLA/NCM film was dried for 8 h at room temperature (21 °C). Then, the PLA/NCM film was washed with deionized water (D.I) to eliminate unbound color residues within the film surface. Thereafter, PLA/NCM film was again dried for 8 h at room temperature and kept in a desiccator at 50–60% relative humidity (RH) for 2 days. A hand-held micrometer was used to measure film thickness (Esslinger, MN, USA). The synthesis process of PLA/NCM film was summarized and shown in Fig. 1.

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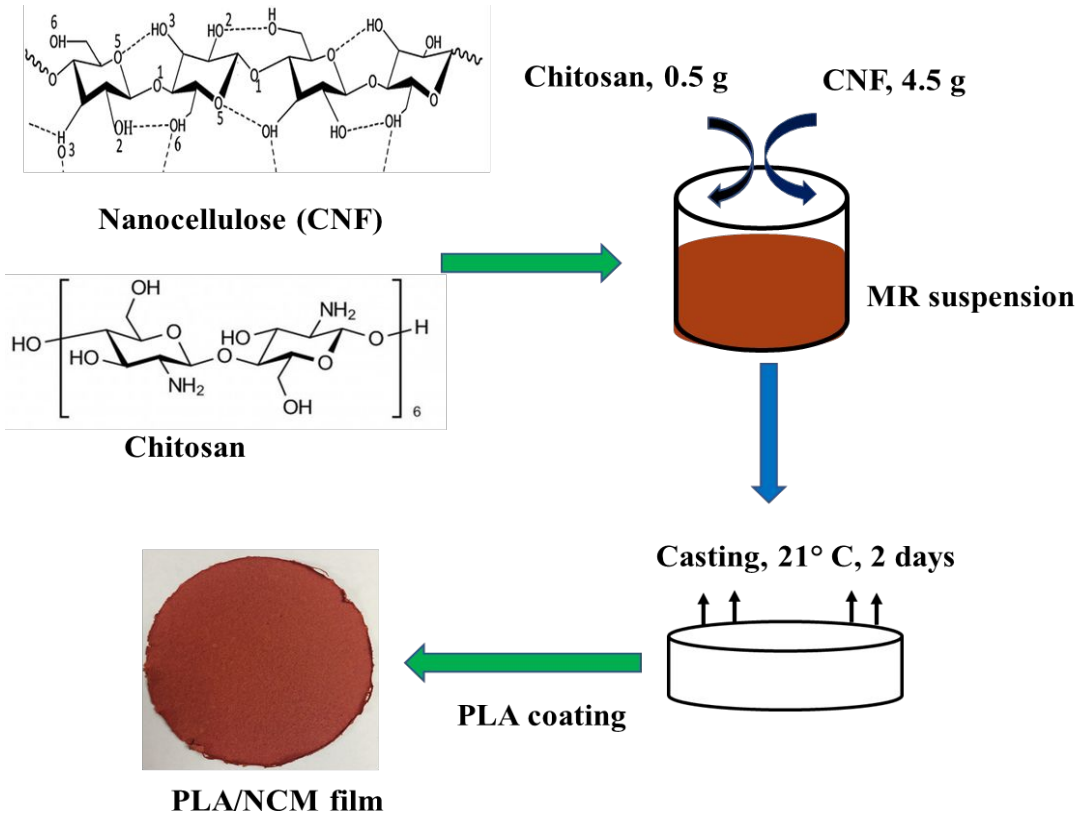

Fig. 1. Schematic diagram for the formation of PLA/NCM film using cellulose nanofiber (CNF), chitosan, methyl red and polylactic acid (PLA).

2.3. Characterization methods

2.3.1. Mechanical analysis

PLA/NCM film (50 mm long and 20 mm wide) was mechanically analyzed by following the method developed previously.[17] A texture analyzer fitted with Texture Exponent 32 software (Texture Technologies Corp., Scarsdale, NY, USA) was used to compute tensile force and deformation. The crosshead speed was set to 60 mm/min. Tensile strength (TS) and strain were determined based on the strength and deformation data recorded by the following equation.

$$TS = \frac{\text{Maximum applied force}}{\text{Film thickness} \times \text{Film width}} \quad \text{Eq. (1)}$$

$$\text{Strain} = \frac{\text{Elongation}}{\text{Original leanth of film}} \quad \text{Eq. (2)}$$

2.3.2. Photodegradation analysis

Photodegradation of the PLA/NCM film (2 cm x 2 cm) was evaluated by following the method described previously.[26,27] For this analysis, the film samples were placed at 38 cm distance from the aperture of visible light irradiation (40 W, halogen lamp, white color) at room temperature (21 °C) and atmospheric pressure. The color change of the film was measured using a hand-held color reader (Precise color reader, WR-10QC, China) on the daily basis.

2.3.3. Fourier transform infrared spectroscopy (FTIR)

FTIR analysis of the PLA/NCM film was conducted by following the Tensor 37 spectrophotometer to analyze the functional groups of the film components. The sample holder of the FTIR device was directed at 90° to the incident beam and parallel to the ground. The FTIR spectra were recorded at room temperature in the range of 500 to 4000 cm^{-1} and collected with 20 scans at 4.0 cm^{-1} resolution using the omnic spectroscopy software.

2.3.4. Color analysis of PLA/NCM film

Color analysis of PLA/NCM film was performed according to the previously described method.[4] First, a square piece of the PLA/NCM film (2 cm x 2 cm) was subjected to the various pH buffers (2-10). Afterward, the color change values (L*, a* and b*) of each PLA/NCM film were assessed via the hand-held color reader (Precise color reader, WR-10QC, China). A CIE-Lab color scale was applied to determine the degree of lightness (L*), redness (+a*) or greenness (−a*), and yellowness (+b*) or blueness (−b*) of the films. The color evaluation was carried out in three different points of the film. The total color difference (ΔE) value was determined by the following formula.

$$\Delta E = [(L^*_{film} - L^*_{standard})^2 + (a^*_{film} - a^*_{standard})^2 + (b^*_{film} - b^*_{standard})^2]^{0.5} \quad \text{Eq. (3)}$$

2.4. Application of PLA/NCM film for monitoring meat spoilage

2.4.1. Beef spoilage analysis using PLA/NCM film

Meat freshness refers to fresh meat that has not yet been spoiled or contaminated. Freshly sliced beef sample with a pH of 5.1 to 5.4 was obtained from Walmart Super Market, Brookings, SD and the sample was delivered to the lab within 30 min. The average postmortem time of this meat when bought was around 2 h. Then, 10 g of beef sample was taken into petri-dish and a thin strip of PLA/NCM film was placed inside of that petri-dishes in direct contact with beef in the petri-dish atmosphere. Afterward, the petri-dish was tightly packed with parafilm to eliminate air dispatch. Then, it was stored at refrigerant temperature (4 ± 0.2 °C) in order to assess the performance of the film for monitoring the beef spoilage. The temperature of the refrigerator was monitored using a thermometer during the storage period.

2.4.2. Microbial analysis of beef sample

The total viable count (TVC) of microorganisms in the beef samples was counted by following the previously described method with a minor modification.[28] To perform this analysis, approximately 10 g of beef samples were weighed and placed in sterilized Ziplock freezer bags.[22] Thereafter, 90 mL of peptone water solution was added and homogenized in a stomacher under the aseptic conditions for the 60 s at room temperature (Seward stomacher 400). The homogenized samples were then kept in the refrigerant condition (4 ± 0.2 °C) and allowed to deteriorate. During the beef spoilage trial, serial dilutions of the sample were prepared. Afterward, 0.1 mL of the appropriate dilutions were spread on the plate count agar (TSA; Merck, Darmstadt, Germany) and incubated the plates overnight at 25 °C. After overnight incubation, colonies on the agar plates were counted, and the results were correlated with the PLA/NCM film's response. Colonies were counted and reported as log colony forming units (CFU) per gram of beef.

2.4.3. pH and ammonium ions analysis in beef sample

The pH values of the beef sample were measured using a pH electrode (Mettler Toledo GmbH/Switzerland). To measure the pH values, nearly 10 g of beef samples was added in the 90 mL of DI water and then homogenized in a stomacher under the aseptic conditions for the 60 s at room temperature. The homogenized samples were then kept in the refrigerant condition (4 ± 0.2 °C). Afterward, the glass electrode of pH meter was dipped in the homogenate beef sample and then periodically recorded the pH values in relation to time and temperature. In a similar manner, to determine the ammonium concentrations in the beef sample, an ammonium electrode was used (Oakton, Cole-Parmer, Vernon Hills, IL, USA). The ammonium electrode response was measured by dipping the electrode in a homogenized beef sample. The electrode response values obtained were interpolated in the calibration curve that was constructed between 50 and 300 mg/L of standard ammonium solution (Standard ammonium chloride, Oakton, USA). The concentrations of the ammonium ion into the beef sample after their spoilage were calculated using the linear part of the calibration curve [R^2= 0.9628]. Each analysis was repeated three times.

2.5. Statistical analysis

A fully randomized design was used to conduct all experiments. The statistical data analysis was carried out by one-way analysis of variance (ANOVA) using the sigma plot software (Version 14.0, Sigma plot., Chicago, IL, USA). The significant analysis of the data was performed by Tukey test with considering a defined significance level of $p < 0.05$.

3. Results and discussion

3.1. Functional properties of film

Mechanical properties of NCM and PLA/NCM film were determined to examine the tensile properties of the film with respect to applied force. As can be seen in **Fig. 2A**, PLA/NCM film showed higher tensile properties (i.e., tensile strength and strain) compared to the NCM film at the same time. This increase of tensile properties may allow the film to withstand the normal stress faced during the shipment, handling and transportation of foods.[29] The other possible reason for the increasing tensile properties of the film is related to interfacial interaction of PLA with NCM, because PLA is the plasticizer agent which may strictly bind film components during the film coating.[30] Therefore, it permits the higher tensile force during the film deformation; and resulted in an increase in tensile strength. Compared to the films, PLA/NCM film can be considered more suitable for food packaging applications.

The water solubility of the film was analyzed to define the comparative applications of NCM and PLA/NCM films for smart food packaging. The water solubility of the NCM film was higher than that of the PLA/NCM film (**Fig. 2B**). In comparison to PLA, chitosan and nanocellulose are highly susceptible to water molecules. Therefore, when the PLA was applied to the NCM film surface for coating, it reduced its water solubility performance**.** This is because the coating of PLA to the film can suppress the water diffusion rate. A similar phenomenon was observed in the previous study, where the water solubility of the film was decreased after PLA coating to the film,[21] which was consistent with the solubility results obtained from our test.

The photodegradation of the PLA/NCM film was carried out to characterize the color degradation properties of the film during light exposure at room temperatures. As can be seen in **Fig. 2C,** the color parameters (L*, a* and b*) for the PLA/NCM film did not potentially fluctuate during the exposition to light within 5 days. Whereas, after 3 days, the color parameters for NCM films fluctuated, and the color of NCM film degraded **(Fig. 2D)**. This phenomenon suggested that PLA coating upon the film surface has an effect to reduce the photodegradation.

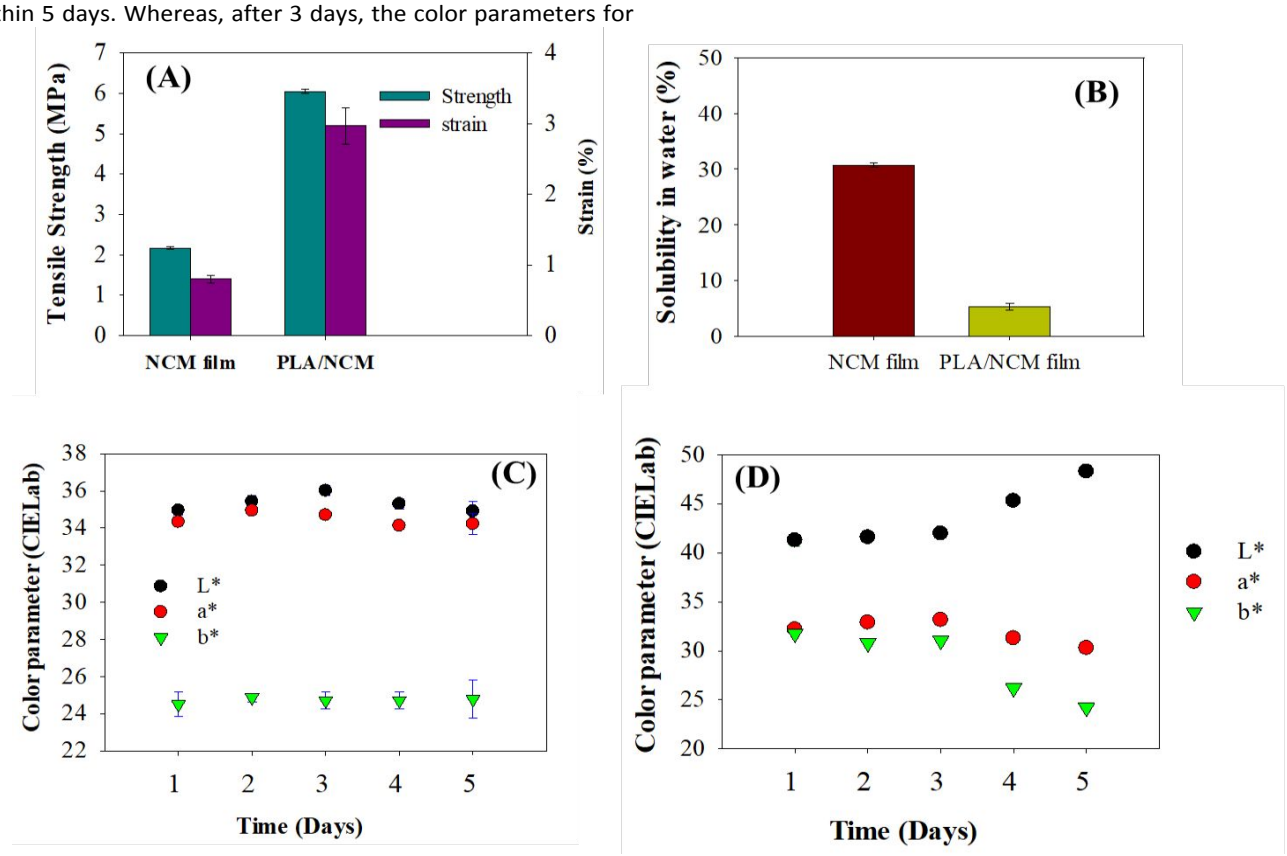

Fig. 2. (A) Mechanical properties of NCM and PLA/NCM films; (B) water solubility of NCM and PLA/NCM films; (C) Photodegradation of PLA/NCM film according to CIE-Lab coordinates; (D) Photodegradation of NCM film according to CIE-Lab coordinates exposed to visible light irradiation as a function of time at 21 °C.

3.2. FTIR spectra

Fig. 3 shows FTIR spectra of PLA/NCM film and film individual compositions such as PLA, nanocellulose and chitosan. As can be seen, PLA gives the peak at 1083 cm^{-1}, representing vibrational stretching of C-O (primary alcohol); 1731 cm^{-1}, representing stretching vibration of C=O (aldehyde); and 2956 cm^{-1}, representing C-H stretching vibration absorption (alkane). Nanocellulose gives a peak at 1011 cm^{-1}, representing C-F stretching (fluoro compounds); 2884 cm^{-1}, representing C-H stretching vibration absorption (alkane); and 3316 cm^{-1}, representing N-H stretching (aliphatic primary amine). Chitosan gives a peak at 1047 cm^{-1}, representing carbon-nitrogen (C-N, amine) stretching; 1623 cm^{-1}, representing C=C stretching vibration (conjugated alkene); 2920 cm^{-1}, representing carbon-hydrogen bonds (C-H, aliphatic). The characterized peaks of PLA/NCM film were displayed to 1047 cm^{-1}, 1731 cm^{-1}, and 2920 cm^{-1}, which is associated with the vibration absorption of carbon-nitrogen (C-N, amine), carbon oxygen double bonds (C=O, aromatic), and aliphatic carbon-hydrogen bonds (C-H), respectively. These observed peaks of PLA/NCM film indicated that strong interfacial adhesions were performed between C=O groups of PLA, N-H aliphatic functional groups of nanocellulose, and C-N functional groups of chitosan. A similar phenomenon has been previously observed in PLA coating polyphenol/chitosan film, in where the PLA copolymer contained carboxyl (C=O) groups in the polyphenol/chitosan films.[31]

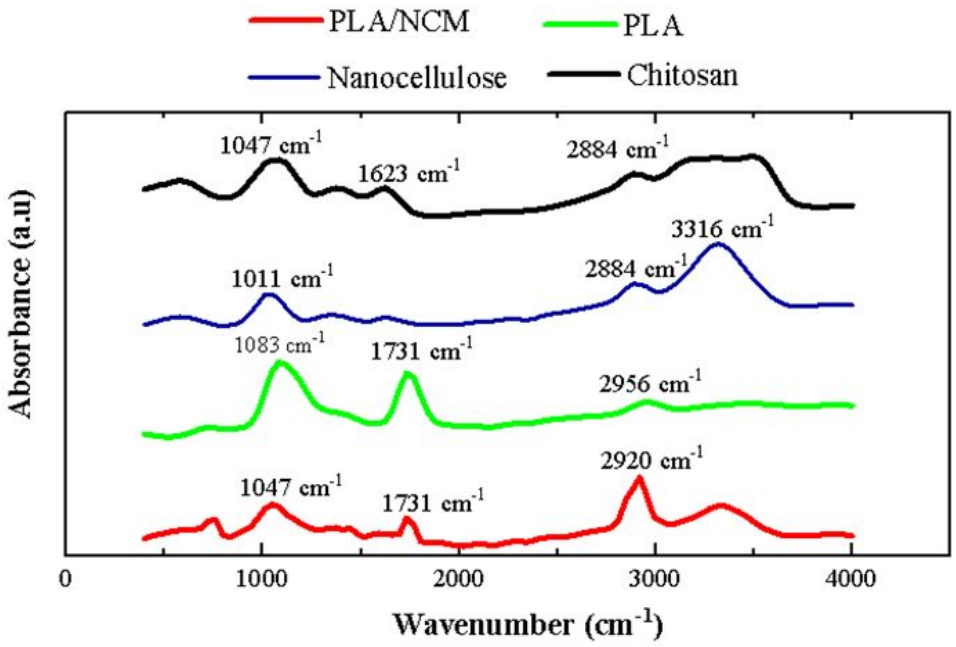

Fig. 3. Infrared spectroscopy (FTIR) spectra of PLA/NCM films. The films contained nanocellulose (CNF), chitosan and PLA contents.

3.3. pH sensing and optical properties of films

The color change properties of PLA/NCM film were evaluated depending upon the various pH buffer solutions (pH 2-10). As can be observed in **Fig. 4**, PLA/NCM film shows different color change properties, which are deep red color when in contact with pH 2-5.5, orange color when reacted to pH 6, and yellow color at pH 6.5-10. These color variations of PLA/NCM films are associated with the structural alteration of methyl red throughout the hydrazone-diazo tautomeric conversion when exposed to different pH levels.[32] When the pH is between 2-5.5, the PLA/NCM film appears dark red color because of the presence of quinone forms in the acidic condition (HMR+, dark red).[32] When the pH values increased between 6.5-10, the color of the film changed from dark red to yellow. This is because the structural alteration of methyl red (pK_a 5.1) occurred throughout the benzenoid formation in alkaline conditions (MR−, yellow).[32] The results of the colorimetric change were assessed using the CIELab scale (**Table 1**), which were consistent with those observed visually in **Fig. 4**. As can be seen that the lightness of the films (L*) was slightly affected with pH alterations. It shows that when the pH has varied from pH 5.5 to pH 10, PLA/NCM film tended to be the brightest (higher L* values). However, when the pH values varied from pH 2 to pH 5.5 or pH 6.5 to pH 10, no significant changes in the L* values were observed. The evaluation of a* values was not greatly affected with the pH variation. Nonetheless, b* values showed a trend color variation and tended to yellow at higher pH values (**Table 1**). Briefly, PLA/NCM film showed lower b* values at pH 2-5 (dark red color), and then showed increasing values of b* in pH 6-10, confirming that the visible and eye detected color has been formed. It is seen that the ΔE values increased as pH values in contact with film increased from pH 2 to 10. The higher ΔE values for PLA/NCM films after contact with pH buffer solutions (pH 6-10) indicated that the new color formation occurred that was identified by the human eye. According to these results, PLA/NCM film could be useful as a pH-indicator film with the future food packaging industry because it is very sensitive in a wide pH range. In addition, the current food industry is expanding worldwide, they need to use an eye-catching film with good mechanical properties to avoid false positive and negative results when determining the degree of freshness.[22] The produced PLA/NCM film could be potential for usage with food packaging materials for food industrial applications such meat industry due to its pH sensitive and colorful nature.

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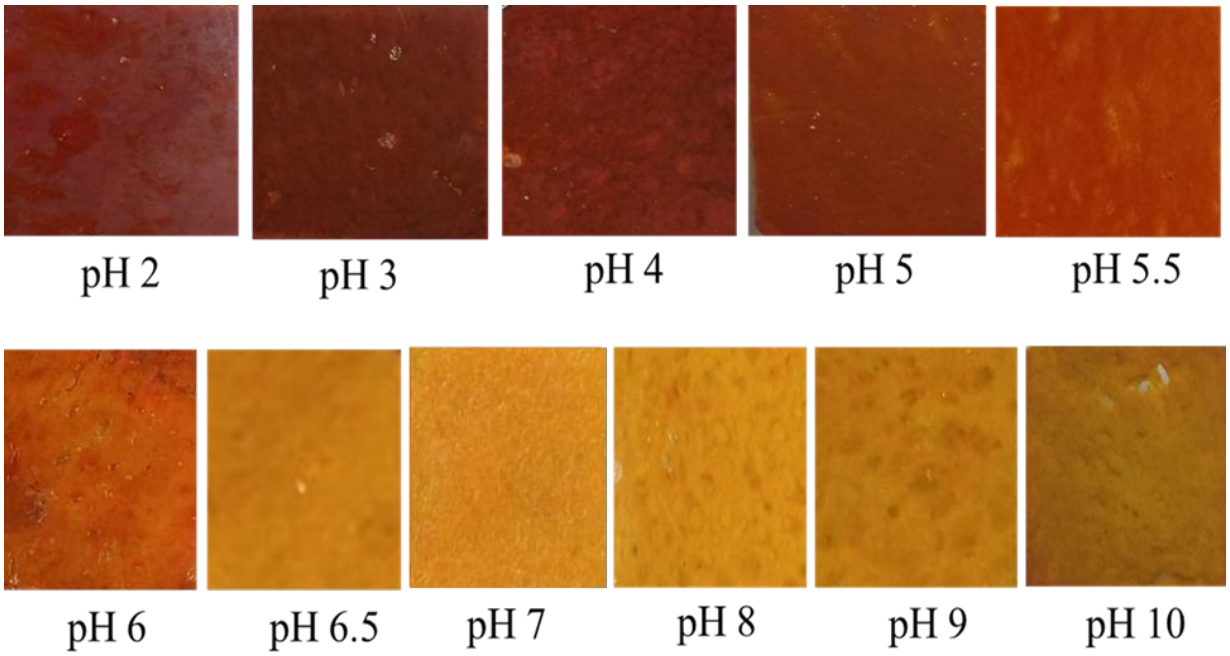

Fig. 4. The visible color response of the PLA/NCM film with respect to different pH buffer solutions (pH 2-10).

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Table 1. CIE-Lab color parameters (L*, a* and b*) of the PLA/NCM films after contact with different pH buffer solutions (pH 2-10).

	PLA/NCM Calorimetric indicator film			
	L*	a*	b*	ΔE
pH 2	33.56±0.54	16.88±0.14	5.79±0.34	
pH 3	32.43±0.35	13.64±0.13	6.48±0.51	3.87±0.76
pH 4	31.62±0.24	14.52±0.19	7.39±0.19	3.56±0.96
pH 5	31.03±0.03	13.22±0.02	7.65±0.13	4.93±0.53
pH 5.5	31.83±0.40	14.16±0.06	6.46±0.21	3.34±0.56
pH 6	37.98±0.15	22.56±0.41	19.14±0.49	15.17±0.70
pH 6.5	43.36±0.44	18.84±0.08	36.43±0.29	32.25±0.51
pH 7	43.33±0.24	18.27±0.27	33.07±0.31	29.02±0.64
pH 8	43.53±0.02	18.13±0.02	33.61±0.36	29.59±0.93
pH 9	43.04±0.49	16.29±0.06	33.80±0.29	29.62±0.67
pH 10	42.61±0.25	21.69±0.28	34.71±0.58	30.22 ±0.53

N.B: The ΔE values of films were calculated using Eq. (1) and considered the standard color parameters of films using pH 2 as a standard.

3.4. Assessment of color specificity of PLA/NCM film

The key components of foods are known as polysaccharides, lipids, amino acids and vitamins, and they act as precursors for assessing the quality of foods.[24] During the food spoiling, these components are broken down and thereby they produce various organic and inorganic volatile compounds. For example, during the beef, fish and seafood spoilage, various types of biogenic amines are released such as ionized or unionized ammonia (NH_3), histamine, tyramine, and ethylamine compounds.[4] During the fruit spoilage, various types of small molecular volatile compounds are evolved such as different kinds of aldehydes, ketone, carbon dioxide, acetic acid, and a small amount of HCl.[24]

To evaluate the color specificity of the PLA/NCM film, the PLA/NCM film was applied with different commercial basic biogenic amines and organic or inorganic compounds such as ammonia (NH_3), histamine, tyramine, acetic acid and hydrochloric acid (HCl) to observe the film's color change. The apparent color change of the PLA/NCM film in contact with different commercial basic biogenic amines and organic or inorganic compounds has been shown in **Fig. 5A**. The PLA/NCM film was brown in color under HCl and acetic acid conditions. However, the PLA/NCM film showed yellow color when the PLA/NCM film was applied in the biogenic amine compounds such as ammonia, tyramine, and histamine. All these color changes of the film were detected by the naked eye. There is no dramatic difference in color change among histamine, tyramine and ammonia. This indicates that the PLA/NCM film prepared in this study has a selective color change property from red to yellow to the biogenic amine compounds which are normally produced in the alkaline condition. This result also demonstrated that the PLA/NCM film could be a suitable candidate as the indicator label for detecting beef, fish and seafood spoilage, because these protein-rich food products produce biogenic amine compounds during their spoilage.

The color index of the film was quantitatively analyzed by color parameters (CIEb*). It showed that PLA/NCM film showed significant yellow color (+*b) to histamine, tyramine and ammonium condition (**Fig. 5B**). The higher *b means more obvious yellow color in the films were produced. On the other hand, PLA/NCM film showed lower *b values in response to HCl and acetic acid and thus it can have a great potential for detection of food spoilage.

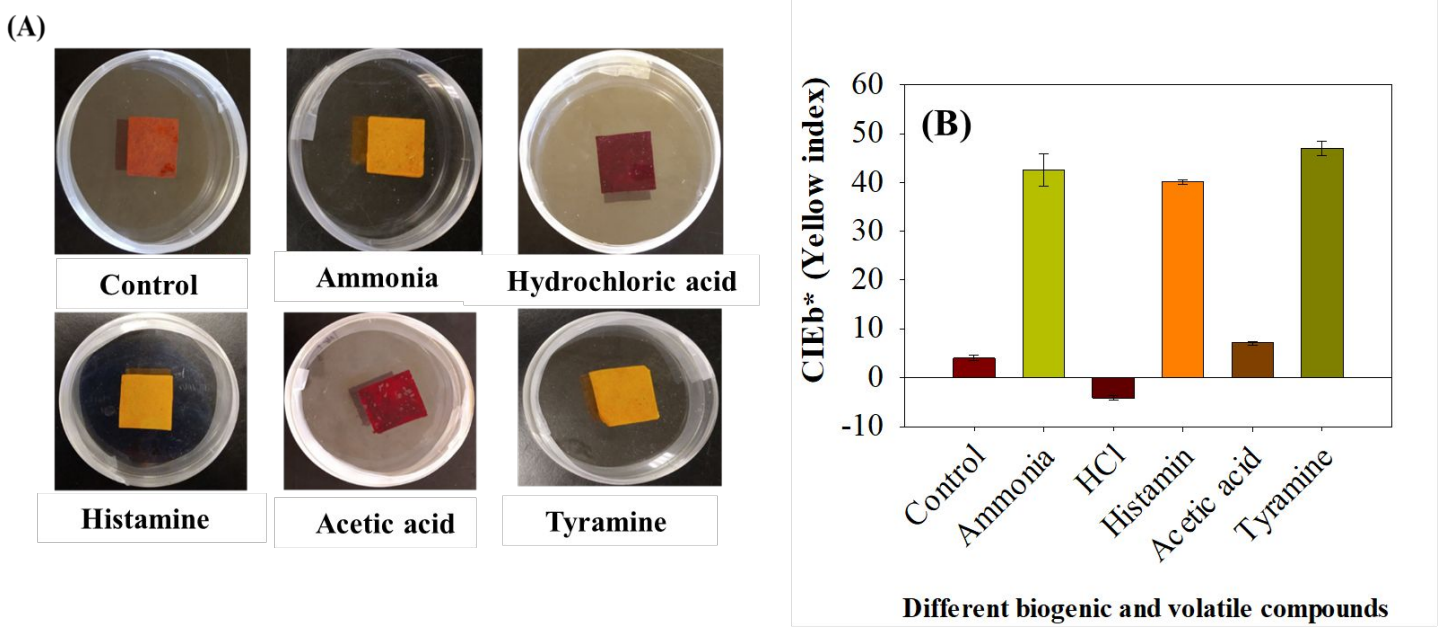


Fig. 5. (A) The color change of PLA/NCM film at different simulated conditions; (B) CIEb* color parameters of the PLA/NCM films at different simulated conditions. Control film denotes PLA/NCM film without any sample treatment.

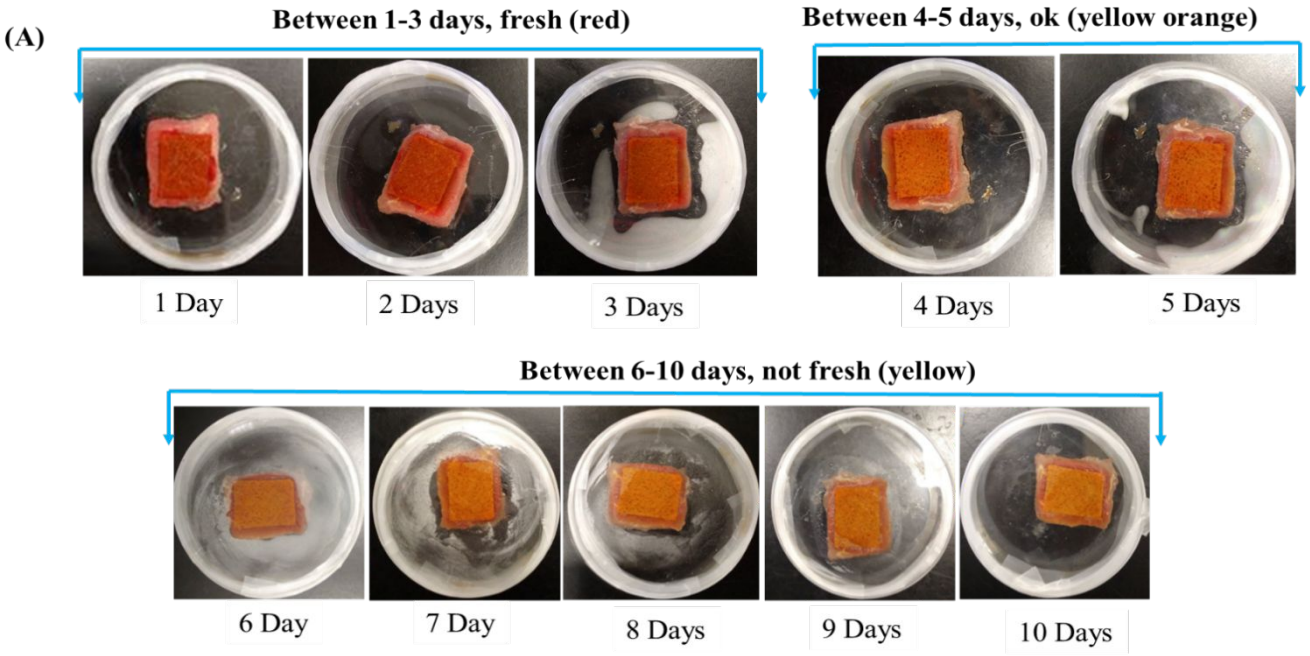
3.5. Application of PLA/NCM film for beef spoilage trial

3.5.1. Assessment of beef spoilage using PLA/NCM film

The PLA/NCM film was placed in contact with beef samples, allowing the film to respond to the spoiled beef with a very distinct color change from red to yellow under the refrigerant condition (4 ± 0.2 °C). These color changes of the film were monitored periodically until no further color change observed during the beef spoilage (**Fig. 6A**). In the fresh state of the beef, PLA/NCM film showed red color between 1-3 days and then turned slightly to orange-yellow between 3-5 days. Thereafter,

the color of the PLA/NCM film changed significantly to yellow between 6-10 days. Furthermore, no color change was inspected between 6-10 days. The color changes of the PLA/NCM film were distinguished by the naked eye in every state of beef spoilage. Based on the color change properties, it can be assumed that within 1–3 days, the quality of beef might be fresh, and its quality gradually decreased after 3 days at refrigerant condition. At room temperature (22 ± 2 °C), in the fresh state of the beef, PLA/NCM film showed red color between 0-8 h and then slightly turned into orange-yellow from 8 h to 12 h (Fig. S1). Thereafter, the color of the PLA/NCM film turned completely to yellow color between 16-24 h.

To further assess the color change properties of the PLA/NCM film, the ΔE value, which is the color difference value of the PLA/NCM film, was counted (**Fig. 6B**). As can be seen, between 1-5 days, ΔE values slightly increased and then rapidly increased after 5 days and continued to 10 days. No drastic color change was observed between 6-10 days. The color change of the film was significant when the ΔE value was greater than 7.36± 0.23 after 5 days, belonging to a different color space. In addition, at the room temperature (22 ± 2 °C), ΔE values were nearly identical between 0 and 8 h. After 8 h, ΔE slightly rose until 12 h, and the rapidly rose from 12 h to 24 h (Fig. S2).



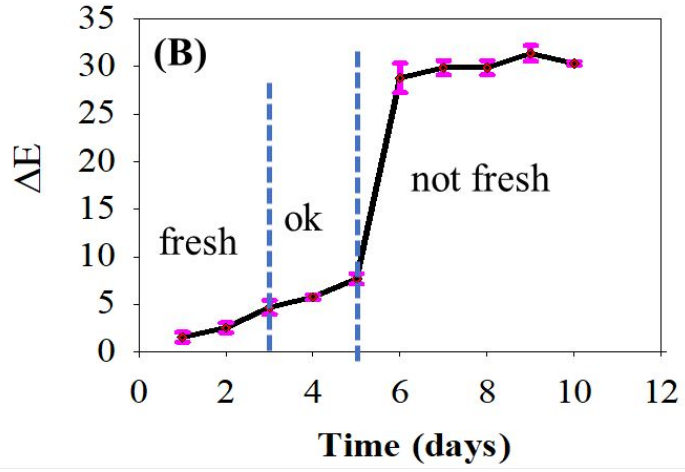


Fig. 6. (A) Color variation of PLA/NCM film with respect to beef spoilage at refrigerant condition (4±0.2°C); **(B)** The color change (ΔE) of PLA/NCM film towards the spoiling beef at refrigerant condition (4 ± 0.2°C).

3.5.2. Assessment of microbial degradation of beef using PLA/NCM film

As can be seen in **Fig. 7A,** the initial TVC count in beef was 1.95 log (CFU/g) and gradually reached at 8.67 log (CFU/g) by 10 days. According to international trade, when the TVC count exceeds the normal level of TVC [6 logs (CFU/g)], meat is considered to be a poor quality product and unfit for consumption.[33] It has been noted that the TVC count exceeded 6 log (CFU/g) after 5 days at 4 °C. After 5 days, the TVC count in beef was 6.9 log CFU/g, which was unsafe for consumption and exceeded the minimum level of beef acceptability.

The ΔE of PLA/NCM film was correlated with the TVC count and it was found that TVC values were consistent with ΔE of the PLA/NCM film **(Fig. 7B and C).** When ΔE value is 9.2 ± 0.96 at 5 days, the cut-off value for TVC [Log (CFU/g)] was 4.8± 0.96. After this point, ΔE values suddenly rose and exceeded the threshold level of the TVC [6 log CFU/g)]. Which is why this point can be used as an onset of detection for PLA/NCM film as the threshold of spoilage at refrigerant condition, and this value was termed as the onset of detection or cut-off value for PLA/NCM film. This result was in good agreement with the findings of Taherkhani et al., who reported a beef at chiller condition can last for 5 days for consumption.[23] From this perspective, 5 days at refrigerant condition has been considered as the threshold for beef consumption. Thus, it can be proven that the PLA/NCM film can be used to indicate the presence of high microbial populations in packaged beef by the color change that can be seen using the naked eye for visual detection.

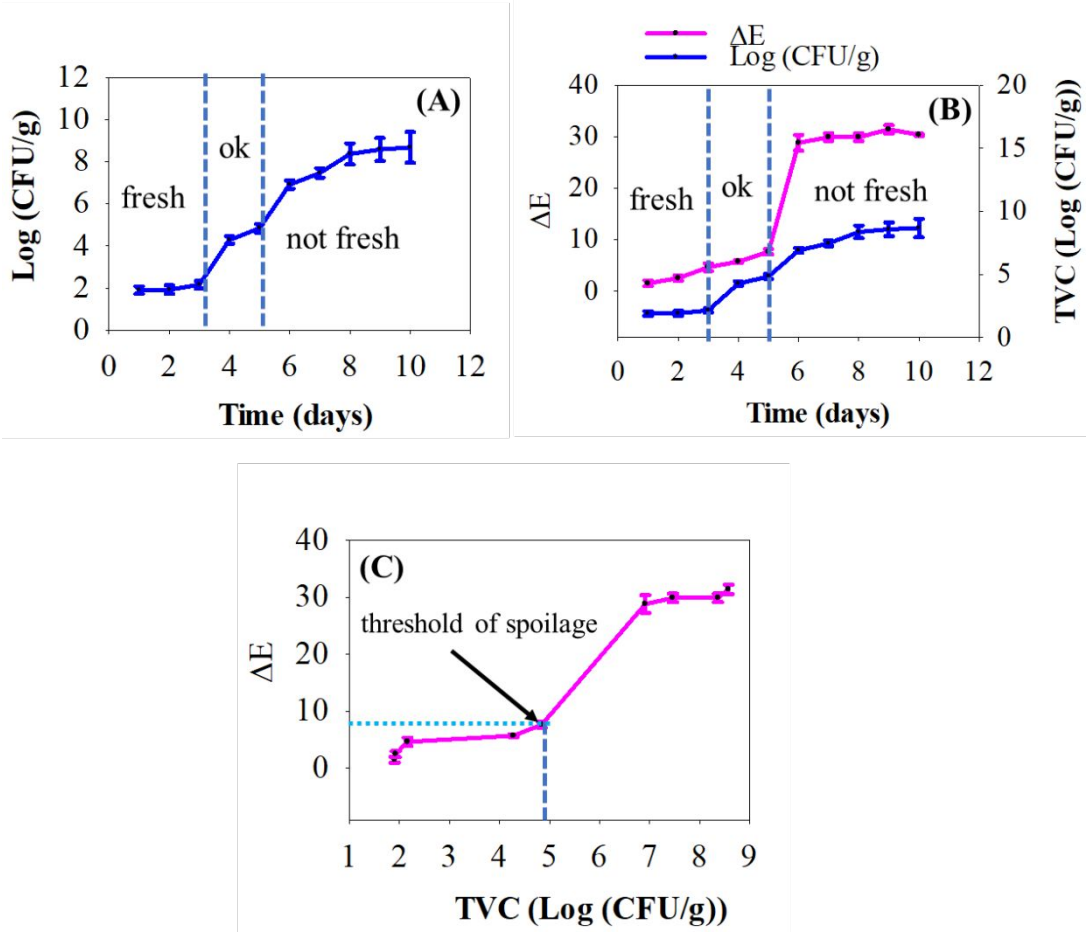


Fig. 7. (A) Total viable count (TVC) of microorganism in the beef sample in the refrigerant condition; (B) TVC correlated with color change value (ΔE) of PLA/NCM film between 1-10 days; (C) ΔE correlated with TVC in the refrigerant condition.

3.5.3. Assessment of pH in spoiling beef with color response of PLA/NCM film

The pH value of beef sample was evaluated with regard to ΔE of the PLA/NCM film, and the results were shown in **Fig. 8**. As can be observed in **Fig. 8A**, the pH values increased depending on the condition of spoiled beef. At a fresh stage, the

pH of the beef was 5.58 and increased to pH 5.93 until 5 days, while ΔE of the PLA/NCM film increased and reached at 9.2 as the threshold of consumption at 5 days. It is noted that the increase of pH was consistent with the increase of ΔE of the film (**Fig. 8B and C**). Therefore, the pH of beef was considered to be one of the parameters to indicate the beef spoiled or deteriorated. In general, the cell densities of fresh beef are higher at normal pH levels (< 6). However, when pH increases and exceeds normal pH levels (> 6), the cell densities of the meat decreases and gets spoiled or deteriorated.[5] Previous studies have shown a similar phenomenon, while the pH of beef increased with the increase of beef spoilage.[22] While the meat products decompose, the basic alkaline amine compounds are produced, thereby they raise the pH of the spoiling beef sample.

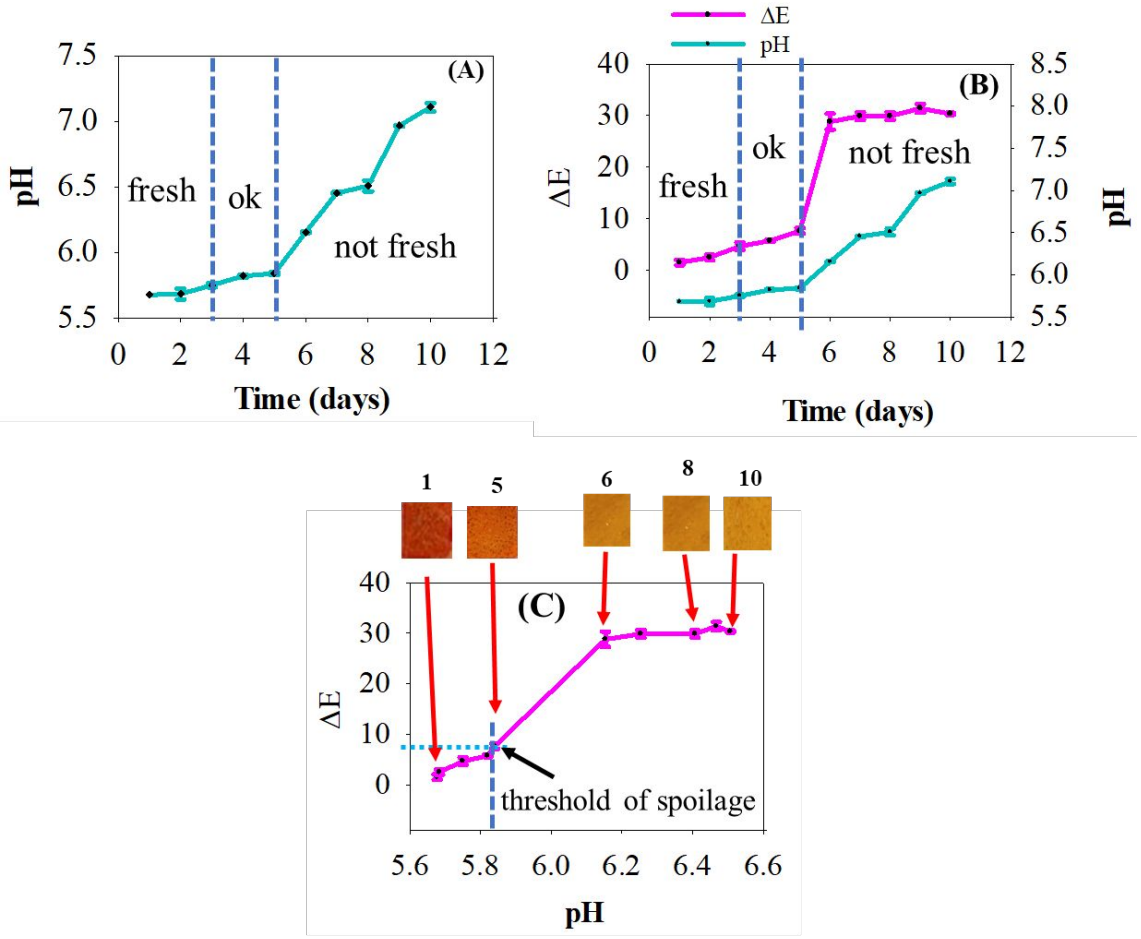


Fig. 8. (A) Determination of the pH of beef sample depending on their spoilage between 1 and 10 days; **(B)** pH of spoiling beef correlated with the color change value (ΔE) between 1-10 days; **(C)** pH of spoiling beef sample correlated with the color change value (ΔE) and images of PLA/NCM film after beef spoilage at 1, 5, 6, 8 and 10 days.

3.5.4. Assessment of ammonium concentrations in spoiling beef

Meat and meat products are a good source of protein with high biological value (26-30% of protein in beef meat, w/w).[34] After protein deamination, the peptide nucleotide catabolites and amino acids of beef tissue are degraded because of microbial deterioration, and thereby ammonia along with other different volatile basic amines are evolved. [22] The most common volatile basic amines after beef deamination are termed as ionized or deionized ammonia, methylamine, histamine, putrescine, tyramine, and cadaverine.[35,36]

The ammonia electrode was used to reliably monitor the increased levels of evolved volatile basic ammonia ions released and correlated it with the film color change (ΔE). Within 1-5 days, no significant ammonium ion concentration was detected in the beef sample (**Fig. 9A and B**). After 5 days, the ammonium ion concentrations greatly increased and varied from 41 to 111 mg/L between 6 and 10 days. According to European Food Safety Authority, a level of ammonium concentration between 0.5 mg/L and 5 mg/L in water can pose a risk to human health.[37] Which is why beef was considered as an unsafe product for consumption after 5 days and exceeded the minimum level of beef acceptability. In general, in these refrigerant conditions, deamination of protein in beef tissue slowly occurs, during

which volatile basic amines are slowly formed.[38] Therefore, the ammonium concentrations in beef slowly rose during the period of beef monitoring at refrigerant conditions.

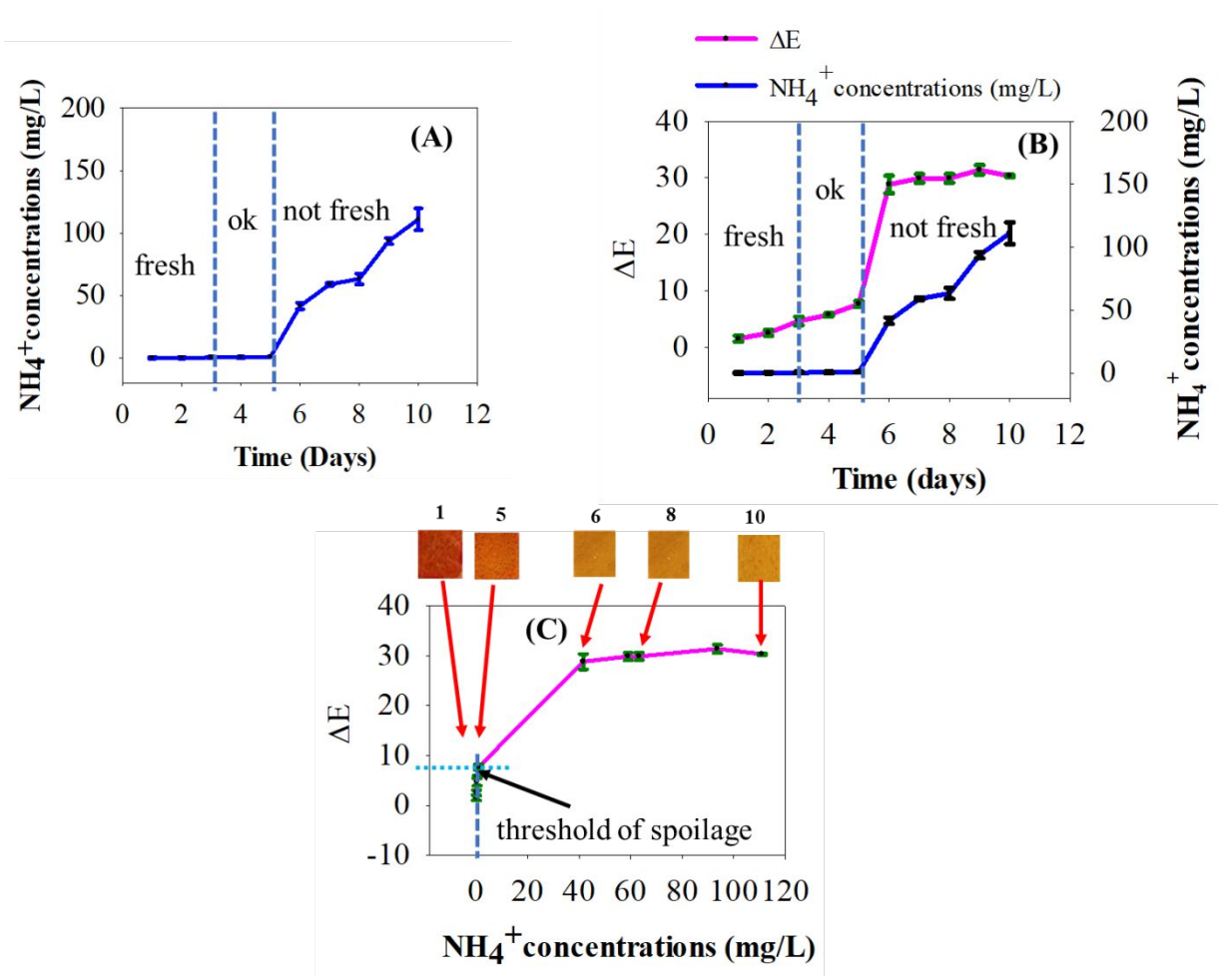


Fig. 9. (A) Determination of evolved ammonium concentration in spoiled beef samples; **(B)** evolved ammonium concentration correlated with color change value (ΔE) between 1-10 days; **(C)** evolved ammonium concentration of beef sample correlated with color change value (ΔE) and images of PLA/NCM film after beef spoilage at 1, 5, 6, 8 and 10 days.

4. Conclusions

The PLA/NCM film was created by film casting methodology by adding methyl red (MR) to the nanocellulose/chitosan blend for the real-time monitoring of beef spoilage. This indicator film exhibited distinct color change properties depending on the different pH buffer solutions in the range of 2-10. The FTIR spectra of the PLA/NCM film confirmed the successful coating of PLA on the film. The PLA/NCM film revealed the distinct color change in contact with biogenic amines and the significant color change properties were achieved. The ΔE of PLA/NCM film showed a good relationship with the TVC and confirmed the safe level of TVC in the beef was achieved at 5 days. The pH and ammonium concentration increased depending on the beef spoiling level and a relationship between film color change (ΔE) and pH in spoiled beef was established. This study provided a promising indicator for monitoring the spoilage of high protein foods such as meat and seafood to inform consumers in real-time of product quality and safety.

Author Contributions

Abdus Sobhan designed this works, conducted the experiments and wrote the original draft. Dr. Lin Wei and Dr. Kasiviswanathan Muthukumarappan supervised and provided advice and guidance during competition of this study. Dr. Ruanbao Zhou and Dr. Hemachand Tummala provided advice during competition of this study.

Conflicts of interest

There are no conflicts to declare.

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References

1 Z. Fang, Y. Zhao, R. D. Warner and S. K. Johnson, *Trends Food Sci. Technol.*, 2017, **61**, 60–71.
2 B. Kuswandi, Y. Wicaksono, Jayus, A. Abdullah, L. Y. Heng and M. Ahmad, *Sens. Instrum. Food Qual. Saf.*, 2011, **5**, 137–146.
3 Y. Zhang, Y. Mao, K. Li, P. Dong, R. Liang and X. Luo, *Asian-Australasian J. Anim. Sci.*, 2011, **24**, 713–722.
4 M. Alizadeh-Sani, M. Tavassoli, E. Mohammadian, A. Ehsani, G. J. Khaniki, R. Priyadarshi and J. W. Rhim, *Int. J. Biol. Macromol.*, 2021, **166**, 741-750, DOI:10.1016/j.ijbiomac.2020.10.231.
5 B. Kuswandi and A. Nurfawaidi, *Food Control*, 2017, **82**, 91–100.
6 B. Schumann and M. Schmid, *Innov. Food Sci. Emerg. Technol.*, 2018, **47**, 88–100.
7 A. Sobhan, J. Lee, M.-K. Park and J.-H. Oh, Rapid detection of Yersinia enterocolitica using a single–walled carbon nanotube-based biosensor for Kimchi product, LWT, 2019, **108**, 48-54.
8 A. Sobhan, J. H. Oh, M. K. Park and J. Lee, *Microelectron. Eng.*, 2020, **225**, 111281.
9 A. Sobhan, J.-H. Oh, M.-K. Park, S. W. Kim, C. Park and J. Lee, *Biosci. Biotechnol. Biochem.*, 2018, **8451**, 1–9.
10 A. Sobhan, J.-H. Oh, M.-K. Park and J. Lee, *J. AOAC Int.*, 2018, **101**, 1558–1565.
11 C. Vasile, *Materials (Basel).*, 2018, **11**, 1834. DOI:10.3390/ma11101834.
12 P. Ezati and J. W. Rhim, *Food Hydrocoll.*, 2020, **102**, 105629.
13 L. J. R. Foster, S. Ho, J. Hook, M. Basuki and H. Marçal, *PLoS One*, 2015, **10**, 1–22.
14 M. Hasan, D. A. Gopakumar, N. G. Olaiya, F. Zarlaida, Alfian, C. Aprinasari, T. Alfatah, S. Rizal and H. P. S. A. Khalil, *Int. J. Biol. Macromol.*, 2020, **156**, 896–905.
15 A. Riaz, C. Lagnika, H. Luo, Z. Dai, M. Nie, M. M. Hashim, C. Liu, J. Song and D. Li, *Int. J. Biol. Macromol.*, 2020, **150**, 595–604.
16 Y. Qian, L. Bian, K. Wang, W. Y. Chia, K. S. Khoo, C. Zhang and K. W. Chew, *Chemosphere*, 2020, **266**, 128948.
17 A. Sobhan, K. Muthukumarappan, Z. Cen and L. Wei, *Carbohydr. Polym.*, 2019, **225**, 115189.
18 A. Sobhan, K. Muthukumarappan, L. Wei, T. Van Den Top and R. Zhou, *Mater. Today Commun.*, 2020, 101124.
19 A. Khosravi, A. Fereidoon, M. M. Khorasani, G. Naderi, M. R. Ganjali, P. Zarrintaj, M. R. Saeb and T. J. Gutiérrez, *Food Packag. Shelf Life*, 2020, **23**, 100429.
20 V. Sessini, I. Navarro-Baena, M. P. Arrieta, F. Dominici, D. López, L. Torre, J. M. Kenny, P. Dubois, J. M. Raquez and L. Peponi, *Polym. Degrad. Stab.*, 2018, **152**, 126–138.
21 G. Chen, Y. Chen, N. Jin, J. Li, S. Dong, S. Li, Z. Zhang and Y. Chen, *Ind. Crops Prod.*, 2020, **150**, 112382.
22 B. Kuswandi and A. Nurfawaidi, *Food Control*, 2017, **82**, 91–100.
23 E. Taherkhani, M. Moradi, H. Tajik, R. Molaei and P. Ezati, *Int. J. Biol. Macromol.*, 2020, **164**, 2632–2640.
24 H. Zhang, A. Hou, K. Xie and A. Gao, *Sensors Actuators, B Chem.*, 2019, **286**, 362–369.
25 D. Kim, S. Lee, K. Lee, S. Baek and J. Seo, *Food Sci. Biotechnol.*, 2017, **26**, 37–42.
26 Y. A. R. Gaviria, N. S. N. Palencia, C. Capello, T. C. Trevisol, A. R. Monteiro and G. A. Valencia, *Starch - Stärke*, 2021, **2000208**, 2000208.
27 B. Merz, C. Capello, G. C. Leandro, D. E. Moritz, A. R. Monteiro and G. A. Valencia, *Int. J. Biol. Macromol.*, 2020, **153**, 625–632.
28 H. Chen, M. Zhang, B. Bhandari and C. Yang, *LWT - Food Sci. Technol.*, 2019, **99**, 43–49.
29 M. Chaichi, M. Hashemi, F. Badii and A. Mohammadi, *Carbohydr. Polym.*, 2017, **157**, 167–175.
30 C. Yang, H. Tang, Y. Wang, Y. Liu, J. Wang, W. Shi and L. Li, *Food Packag. Shelf Life*, , DOI:10.1016/j.fpsl.2019.100393.
31 J. Ye, S. Wang, W. Lan, W. Qin and Y. Liu, *Int. J. Biol. Macromol.*, 2018, **117**, 632–639.
32 M. R. Plutino, E. Guido, C. Colleoni and G. Rosace, *Sensors Actuators, B Chem.*, 2017, **238**, 281–291.
33 P. Ezati, H. Tajik and M. Moradi, *Sensors Actuators, B Chem.*, 2019, **285**, 519–528.
34 Y. Zhang, B. W. B. Holman, Y. Mao, X. Chen, X. Luo, D. L. Hopkins and Y. Zhang, *Meat Sci.*, 2021, **172**, 108347.
35 V. R. Heerthana and R. Preetha, *Rev. Aquac.*, 2019, **11**, 220–233.
36 M. Papageorgiou, D. Lambropoulou, C. Morrison, E. Kłodzińska, J. Namieśnik and J. Płotka-Wasylka, *TrAC - Trends Anal. Chem.*, 2018, **98**, 128–142.
37 E. Food and S. Authority, *EFSA J.*, 2012, **10**, 1–16.
38 S. Talegaonkar, H. Sharma, S. Pandey, P. K. Mishra and R. Wimmer, *Food packaging*. Academic Press, 2017, 79-110.