Food Packaging Formulation Using Chitosan and Bacteriocin as an Antimicrobial Agents

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Abstract: Food preservation has always been a concern for the secured quality of the food products. Of suggested many antimicrobial agents reported, the chitosan blend with bacteriocin based food packaging formulation for the preservation purpose solely depends upon the synergistic effect of both the molecules. The durability, resistance to the foodborne pathogens, external environmental factors such as pH, temperature, and changes in chemical properties makes chitosan more effective packaging material for the preservation of the food. The papaya preservation at room and refrigerated temperature showed that the preservation at refrigerated conditions showed more effectiveness. The chitosan and nisin based film showed no or less moisture content, water-solubility, no difference in antioxidant properties of the papaya fruit and was able to inhibit the microbial load due to its combine antimicrobial properties.

Keywords: Preservation, Chitosan, Nisin, Papaya

INTRODUCTION:

The sole purpose of food packaging is to ensure increased shelf-life, quality, and acceptability of the food product. The market has also increased by the fact of novel packaging of the food product **(Youssef, et al., 2019)**. The packaging ensures protection from microbial contamination, external environment, changes in chemical properties like pH, taste, smell and also prevent physical distortion of the food product **(Khaneghah et al., 2018) (Al-Tayyar et al., 2019)** and maintaining the integrity, nutrients, the texture of the food. The effective packaging also functions as protection from spillage during processing, storage, shipping, and distribution. The plastic is used in the food packaging purposed due to its low-cost of production and transportation, high-thermal stability, lightweight, better barrier properties, heat sealability and much more **(Muller, González-Martínez, & Chiralt, 2017)**. The use of such petroleum-based plastic for food packaging by the manufacturers of many industries over many years is now facing the concern of the health issues of the consumers and the disposing of such plastic-based packaging in the environment has led to accumulation problems. Due to such a problematic issue and the negative impact of public health and the environment has shifted the packaging from petroleum-based plastic towards the biological-based plastic also known as bio-plastic with antimicrobial properties.

There are many bio-polymers that can be used for packaging purposes having potential antimicrobial properties like chitosan, cellulose, agar and various proteins (casein) **(Fabra, López-Rubio, & Lagaron, 2014)**. The most widely used packaging polymer is the chitosan-based packaging along with additional bacteriocin as an antimicrobial agent. Chitosan, that is obtained from the deacetylation of the chitin which is normally found as the major cell wall component of all the fungal species. The US FDA has given chitosan a GRAS status and can be applied in the food processing sector **(No H.K. et al., 2007) (Nguyen et al., 2020)**. The application of chitosan in the food sector has been a success due to properties like biodegradability, biocompatibility with other chemicals, non-toxic to the consumers, and a broad range of antimicrobial activity against the foodborne pathogens **(Palmieri et al., 2018) (Pokhrel et al., 2015)**.

The chitosan blended with an antimicrobial agent like bacteriocin (nisin) that has been fabricated for the packaging purpose by techniques like solution casting, layer-by-layer and many more. The chitosan along with bacteriocin provide synergistic action against the food-borne pathogens and increases the shelf life of the food **(Hosseinnejad & Jafari, 2016)**. The present research aims for the novel packaging formulation using chitosan and bacteriocin (nisin) which is cast on LDPE (low-density polyethylene) as an effective packaging to increase the shelf life of post-harvest papaya fruit by monitoring the microbial load under the storage condition.

MATERIALS AND METHODS:

Sample procurement and its processing:

The isolated *Coprinopsis cinerea* strain BAF Ccult 4361-S1 (Coprinopsis-S1) was sub-cultured on wheat bran agar medium using the fungal isolation, purification technique **(Sharma et al., 2017)**.

Optimization, Production, and Extraction of chitosan:

The optimization, production, and the extraction process were done previously using the standard protocol **(Rohan et al., 2019)**

The formulation of chitosan with nisin:

Layering on LDPE:

The obtained chitosan-nisin solution was layered on the LDPE by the use of a brush. Single, double, triple and four layers were coated on each pre-measured LDPE respectively. The coated LDPE was dried at room temperature and was subjected to disinfectant by exposure to UV rays.

Characterization of the chitosan-nisin coated LDPE:

The following parameters were examined for the successful coating.

- **Moisture content:**

  The moisture content was examined using a standard protocol by drying the layered LDPE and normal LDPE in the hot air oven at 50°C for 24 hours **(Singh et al 2015)**. The moisture content was calculated as:

  $$\textbf{Moisture content}\ (\%) = \frac{\mathbf{M1 - M2}}{\mathbf{M1}}\ \mathbf{x\ 100}$$

  Where M1 = weight of the subject before drying
  M2 = weight of the subject after drying

- **Water solubility:**

The water solubility was determined by solubilizing the pre-measured films in water. The solubility of chitosan-nisin coated LDPE and normal LDPE was tested. Both the films were immersed in 50 mL distilled water in 2 flasks each respectively. The flasks were kept in a shaking incubator for 24 hours. Both films were dried in a hot air oven for 24 hours to the constant weight **(Singh et al 2015)**. The final weight of both films was measured:

$$\textbf{Solubility}\ (\%) = \frac{\mathbf{W1 - W2}}{\mathbf{W1}}\,\mathbf{x\ 100}$$

where W1 = weight of the initial dry weight of the film
W2 = weight of the undissolved film

- **Fourier-transform infrared spectroscopy of films:**
  The infrared spectra of chitosan-nisin coated LDPE and LDPE was measure using FTIR SHIMADZU Japan Model Spectrum. The films were placed in the holder and exposed to a laser beam.
- **Scanning electron microscope of films:**
  The chitosan-nisin coated LDPE and LDPE were processed and observed in the SEM model JSM-IT300LV (JEOL).
- **Antimicrobial activity:**
  The antimicrobial activity was done in the previous study of **Rohan et al., 2019** against the foodborne pathogen such as *Escherichia coli*, *Bacillus subtilis*, *Listeria monocytogenes*, *Klebsiella pneumonia*.

Preservation of papaya:

Prior to packaging with the film, the papaya was first subjected to surface sanitization using chlorine of concentration 200 mg/L for 10 minutes. The papaya was then cut into 18 pieces each of 8 grams. Out of 18 pieces, 3 pieces were kept uncovered at room temperature and 3 pieces were kept in the refrigerator. The next 6 pieces were covered with LDPE and 3 of them were kept at room temperature and 3 were kept in the refrigerator. To the final 6 pieces of papaya were wrapped with chitosan-nisin coated LDPE and 3 of them were kept at room temperature and the last 3 were kept in the refrigerator. The preservation was observed for over 4 days **(Camatari et al., 2018)**.

Post Preservation parameters:

The following parameters were examined post preservation for the efficacy of the film for the packaging of the papaya.

- **Weight loss:**
  The weight loss was determined by taking the initial weight of papaya and then after wrapping, the weight of papaya was taken on each day till 4th day of preservation **(Camatari et al., 2018)**. Weight loss was expressed as %:

$$\textbf{Weight}\ (\%) = \textbf{Initial weight} - \textbf{Final weight}\ \mathbf{x\ 100}$$

- **Physical analysis:**

The physical characteristics like color, texture, and aroma of the preserved papaya of all conditions were examined and noted **(Camatari et al., 2018)**.

- **Antioxidant analysis**

  The total phenolic compounds was tested using the modified Folin-Ciolcalteu17 and the absorbance was taken at wavelength 765 nm. The total flavonoid content was analyzed using Chang et al18 method and the absorbance was taken at wavelength 415 nm. The DPPH activity was done using Blois's method with minor modification and the absorbance was measured at wavelength 515 nm by using UV-Vis spectrophotometer after the short incubation of 30 minutes **(Ruslan et al., 2016)**.

- **Microbial analysis:**

  The analysis was carried out for papaya wrapped with chitosan-nisin coated LDPE and with LDPE and for the unwrapped papaya. For the analysis, 3 tubes containing 9 mL of sterile peptone were prepared and the 1 gm of each referred papaya was cut and added to each tube of sterile peptone water respectively under aseptic condition. The papaya was mashed with sterile rod. 6 tubes of sterile peptone water was prepared for the dilution, 2 tubes for unwrapped papaya, 2 tubes for LDPE wrapped papaya and 2 tubes for chitosan-nisin coated LDPE wrapped papaya. 3 fold dilutions were prepared and the pour plate technique was done of 10^{-2} and of 10^{-3}. The plates incubated at 37˚C for 24 hours **(Camatari et al., 2018)** and CFU was calculated as:

$$\mathbf{CFU/mL} = \frac{\mathbf{No.\ of\ colonies\ counted\ x\ dilution\ factor}}{\mathbf{Volume\ plated}}\mathbf{x\ 100}$$

RESULT AND DISCUSSION:

Layering on LDPE:

Water solubility:

There was relatively less release of content from the coated LDPE in water after the 24 hours of shaking.

Table: Water solubility

No. of sets	Initial weight of the chitosan-nisin coated LDPE (mg)	Final weight of the chitosan-nisin coated LDPE (mg)	Water solubility %
1	220	215	2.27 %
2	220	214	2.27 %
3	220	215	2.27 %

The less release of the content from the coated LDPE can be explained by the formation of strong hydrogen bonds between LDPE and chitosan-nisin solution and prevent the release from the LDPE. In the study of the incorporation of SCELE (13% to 28%) into chitosan films increased the water solubility of the chitosan film after the 24 hours of immersion in the water **(Nguyen et al., 2020)**. The study of chitosan-blend with chive root extract showed decreased water solubility due to the strong hydrogen bond between the two **(Riaz et al., 2020)**. The study of **Rahmani et al., 2017** noted an increased water solubility of chitosan-coated LDPE when incorporated with summer savory (*Satureja hortensis*) extract.

Moisture content:

There was no significant loss of moisture from the chitosan-nisin coated LDPE. The LDPE and chitosan-nisin were bound to each other. The possible explanation might be no bond formation of coated LDPE with the water molecules.

Table: Moisture content

No. of set	Initial weight (gms)	Final weight (gms)	Moisture content (%)
Set-1	0.8	0.8	0%
Set-2	1	1	0%
Set-3	0.8	0.8	0%

The study of **Singh et al., 2015** reported an increase in temperature increases the moisture content of the chitosan films. But in the case of chitosan-nisin LDPE, the film contains methylcellulose so there was no moisture content in the films. A similar study of increasing concentration of SCELE in the chitosan film decreased the moisture content of the film due to reduced availability of a hydrophilic functional group of chitosan to bind with water molecules **(Nguyen et al., 2020)**.

FTIR:

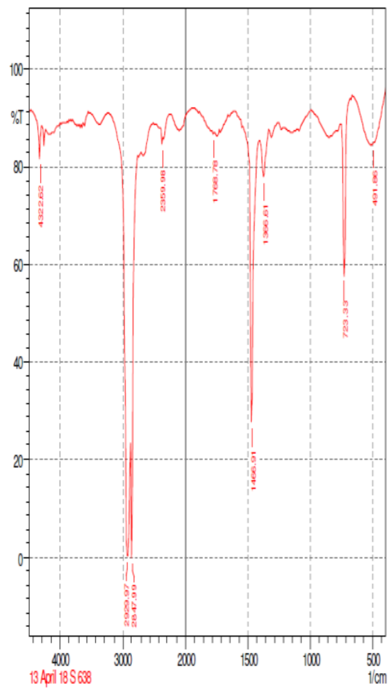

Fig: FTIR of LDPE

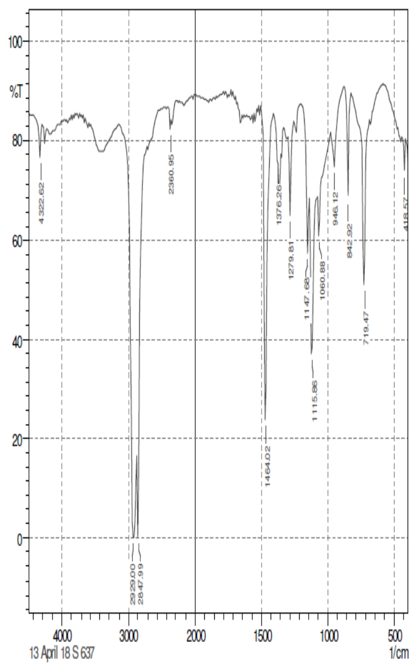

Fig: FTIR of single coated LDPE

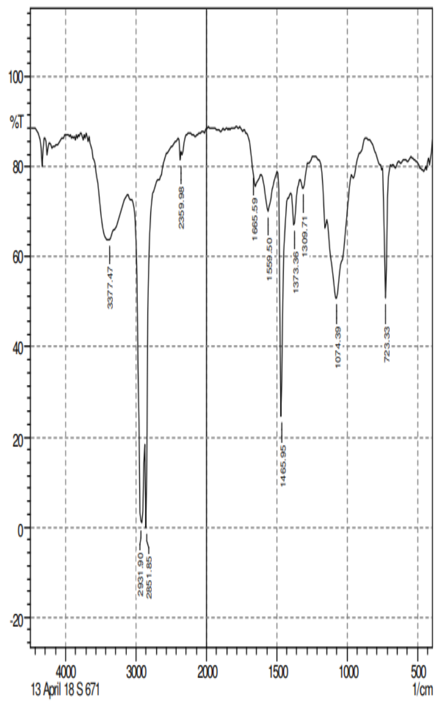

Fig: FTIR of triple coated LDPE

Scanning electron microscopy:

The layering of the chitosan-nisin on the LDPE was successfully incorporated which can be seen from the SEM reports.

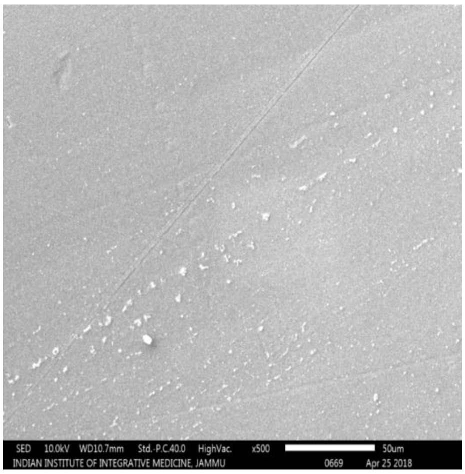

Figure: SEM of LDPE

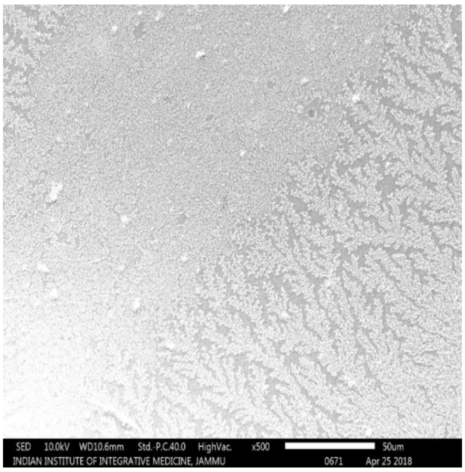

Figure: SEM of single-layered chitosan-nisin coated LDPE

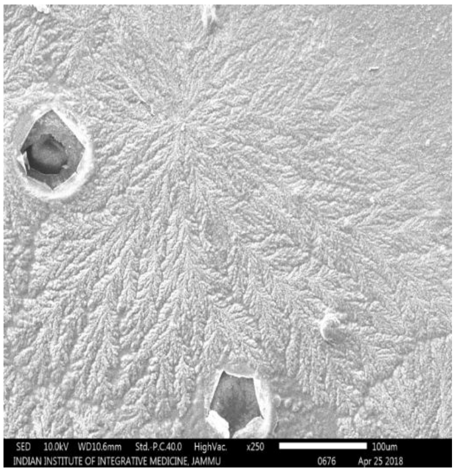

Figure: SEM of triple-layered chitosan-nisin coated LDPE

The SEM analysis of single-layered chitosan-coated LDPE shows discontinuous layering like some part smooth and some textured surface. The textured surface might be an aggregation of the coating solution on the LDPE. The SEM analysis of triple-layered coating on LDPE shows high textured formation which is a more like leafy structure most probably due to an aggregation of the coating solution.

Antimicrobial activity:

Preservation of Papaya:

- **Weight Loss:**
  `The table represents the weight loss measured for three consecutive days. The chitosan-nisin coated LDPE wrapped papaya showed significant ($P < 0.05$) reduced in the weight loss as compared to LDPE wrapped papaya and unwrapped papaya preserved at room temperature and no significant difference ($P > 0.05$) in the weight loss of chitosan-nisin coated LDPE wrapped papaya as compared to unwrapped papaya, LDPE wrapped papaya that was preserved at refrigerated temperature.

Table: Weight loss

Room Temperature				**Refrigerated Temperature**			
Number of days	**Unwrapped papaya**	**LDPE-wrapped papaya**	**Chitosan-nisin coated LDPE-wrapped papaya**	**Number of days**	**Unwrapped papaya**	**LDPE-wrapped papaya**	**Chitosan-nisin coated LDPE-wrapped papaya**
D-1	8	8	8	**D-1**	8	8	8
D-2	7.3	7.5	8	**D-2**	7.6	8	8
D-3	5.5	6.9	7.1	**D-3**	7.1	7.7	7.9

Similar studies of **Narsaiah et al., 2015** reported that alginate-bacteriocin coating significantly ($P < 0.01$) reduced the weight loss of the papaya pieces as compared to the weight loss observed in the control (without coating) after the 21 days of the preservation.

Food preservation application:

The papaya possesses a high content of antioxidant properties that makes its packaging efficient to control and enhance the shelf-life. The chitosan-nisin application in the packaging of the papaya at room and the refrigerated temperature has been observed and noted in this study for four consecutive days. The physical analysis like color, smell, and texture of the papaya post preservation are been listed below.

DAY-1 of preservation:

Table: Preservation of fresh papaya at day-1

Fresh papaya	
Color	Amber to orange
Smell	Fruity
Texture	Soft

Figure: Preservation of fresh papaya

The physical appearance of the fresh papaya like color, smell, and texture was amber to orange, fruity smell, and smooth and soft texture.

DAY-4 of preservation:

Table: Preservation of coated LDPE wrapped papaya at room temperature

Chitosan-nisin coated LDPE wrapped papaya at room temperature	
Color	Red
Smell	Acidic
Texture	Very soft with watery surface

Figure: Preservation of coated LDPE wrapped papaya at room temperature

After the four days of the preservation of coated LDPE at room temperature, the color of the papaya became more prominent red with an acidic smell. The texture was distorted with a watery surface.

DAY-4 of preservation:

Table: Preservation of unwrapped papaya at room temperature

Unwrapped papaya	
Color	Red with black spots
Smell	Acidic
Texture	Hard shrink surface

Figure: Preservation of unwrapped papaya at room temperature

The preservation of the papaya without any coating at room temperature showed red coloration with black spots, the smell was acidic and the texture of the papaya was hard and had shrink surface.

DAY-4 of preservation:

Table: Preservation of LDPE wrapped papaya at room temperature

LDPE wrapped papaya at room temperature	
Color	Red
Smell	Acidic
Texture	Slimy

Figure: Preservation of LDPE wrapped papaya at room temperature

The LDPE wrapped papaya preserved at room temperature showed red color, acidic smell and slimy texture on the 4th day of the preservation.

DAY-4 of preservation:

Table: Preservation of coated LDPE wrapped papaya at refrigerator

Chitosan-nisin coated LDPE wrapped papaya at refrigerator	
Color	Orange
Smell	Fruity
Texture	Soft

Figure: Preservation of coated LDPE wrapped papaya at refrigerator

The coated LDPE preserved papaya under the refrigerated condition for 4 days showed the physical appearances that of the fresh papaya.

DAY-4 of preservation:

Table: Preservation of LDPE wrapped papaya at refrigerator

LDPE wrapped papaya at refrigerator	
Color	Reddish orange
Smell	Somewhat acidic
Texture	Hard

Figure: Preservation of **LDPE** wrapped papaya at refrigerator

The LDPE wrapped papaya at refrigerated condition showed reddish-orange color, somewhat acidic smell and hard texture.

DAY-4 of preservation:

Table: Preservation of unwrapped papaya at refrigerator

Unwrapped papaya at refrigerator	
Color	Red
Smell	Acidic
Texture	Hard and shrink surface

Figure: **Table:** Preservation of unwrapped papaya at refrigerator

The papaya preserved under the refrigerated condition showed shrinkage surface with an acidic smell and the color appeared red on the 4th day of the preservation.

The LDPE coated with chitosan and nisin for the preservation of the papaya showed the best preservation state under the refrigerated condition. The comparable result was obtained from the chitosan-nisin coated LDPE under room temperature. The duration of the preservation was for four consecutive days.

The shrink of the papaya pieces was due to the degradation of the cell wall during the ripening process **(Ali et al., 2013)**. The study of **Vilaplana et al., 2020** showed the papaya firmness was low (P < 0.05) treated with the hot water and chitosan. The results were compared with that of the chemical fertilizer which showed high firmness of the papaya when preserved at 10°C for 21 days.

A similar study was done by **Xiong et al., 2019** for the preservation of the pork loin. The edible coating of the pork was a blending of chitosan-gelatine, nisin and grape seed, and the preservation was done for 20 days. The color of the coated pork was slightly yellow due to the color of the chitosan kept for preservation. They compared the result with the control which showed brownish coloration due to breakdown of the oxyhemoglobin and deoxyhemoglobin to the methemoglobin which gives a brown color.

The work of **Cao et al., 2019** reported the preservation of pork loin under high oxygen modified atmosphere packaging over a period of 20 days. The pork loin was coated with the different solutions of chitosan, chitosan/gallic acid, chitosan/nisin, and chitosan/gallic acid/nisin. The lightness of the pork loin coated with chitosan, chitosan/gallic acid, chitosan/nisin showed increased results as compared to control which suggested the increase in the meat color. The chitosan/gallic/nisin reported a decrease in the lightness (P < 0.05) lightness value. The redness of the pork coated with chitosan/gallic acid and chitosan/nisin remained red on the 20 days of the preservation as compared to control which showed decreased redness after 10 days. The chitosan and chitosan/gallic acid/nisin showed normal red coloration between 5 and 10 days but reduced coloration on 15 and 20 days. The blends of nisin and gallic acid with chitosan inhibit discoloration under the high oxygen modified atmosphere packaging.

The preservation of the pork with chitosan and gallic acid blends under high oxygen modified atmosphere packaging was reported by **Fang et al., 2018**. The lightness of the pork of control and coated chitosan showed an increase in the values (P < 0.05) and chitosan/gallic acid-coated pork did not increase significantly (P > 0.05) due to the potential slow down process of the chitosan/gallic acid in the increment of the lightness values during the high oxygen modified atmosphere packaging on 20th day of the storage. The redness of the control pork, chitosan-coated pork, and chitosan/gallic acid-coated pork remained till the 5th day of the storage however it was decreased after. The decrease in the red color was slower in the chitosan/gallic acid-coated pork than the control and chitosan-coated pork. The shear force value for the analysis of texture showed low value for the chitosan-coated and chitosan/gallic acid-coated pork on the 20th day as compared to the high shear force value of the control pork.

Estimation of antioxidants:

Table: Antioxidant test on day-1

Fresh papaya

Total phenolics	DPPH	Flavonoids
0.825	0.591	0.802
0.804	0.584	0.798
0.815	0.589	0.788

The fresh papaya was initially tested for antioxidants such as total phenolics, DPPH, and flavonoids having mean values of 0.814, 0.588, 0.796 respectively.

Table: Antioxidant test on day-4

Total phenolics

Refrigerated temperature			Room temperature		
Unwrapped papaya	**LDPE wrapped papaya**	**Coated LDPE wrapped papaya**	**Unwrapped papaya**	**LDPE wrapped papaya**	**Coated LDPE wrapped papaya**
0.623	0.680	0.829	0.523	0.647	0.669
0.685	0.744	0.801	0.500	0.662	0.680
0.675	0.698	0.819	0.528	0.649	0.670

The antioxidant, total phenolics post preservation of coated LDPE wrapped papaya for four days showed no significant difference in the values when stored at refrigerated conditions having mean values of 0.816. However, there was a decreased mean value of 0.673 of total phenolics of coated LDPE wrapped papaya for four days when stored at room temperature condition.

Table: Antioxidant test on day-4

DPPH

Refrigerated temperature			Room temperature		
Unwrapped papaya	**LDPE wrapped papaya**	**Coated LDPE wrapped papaya**	**Unwrapped papaya**	**LDPE wrapped papaya**	**Coated LDPE wrapped papaya**
0.208	0.243	0.589	0.789	0.870	0.618
0.243	0.230	0.581	0.779	0.868	0.600
0.230	0.220	0.586	0.790	0.861	0.605

The antioxidant, DPPH post preservation of coated LDPE wrapped papaya for four days showed no significant difference in the values when stored at refrigerated conditions having mean values of 0.585. However, there was an increased mean value of 0.607 of DPPH of coated LDPE wrapped papaya for four days when stored at room temperature condition.

Table: Antioxidant test on day-4

Flavonoids

Refrigerated temperature			Room temperature		
Unwrapped papaya	**LDPE wrapped papaya**	**Coated LDPE wrapped papaya**	**Unwrapped papaya**	**LDPE wrapped papaya**	**Coated LDPE wrapped papaya**
0.189	0.204	0.806	0.645	0.651	0.874
0.178	0.230	0.796	0.623	0.808	0.749
0.184	0.233	0.781	0.648	0.683	0.811

The antioxidant, flavonoids post preservation of coated LDPE wrapped papaya for four days showed no significant difference in the values when stored at refrigerated conditions having mean values of 0.794. However, there was an increased mean value 0.811 of total phenolics of coated LDPE wrapped papaya for four days when stored at room temperature condition.

The study of **Abdipour et al., 2020** reported high retention of total phenols in chitosan-coated cherry fruit that was treated with UV-C radiation. However, no significant difference was seen in the same chitosan-coated cherry fruit when treated with UV-B radiations. The aloe-vera and chitosan-coated tomatoes showed no significant difference in total phenols content when stored for 42 days **(Khatri et al.,**

2020). The increased total phenols are due to the activity of ethylene in fruits **(Chrysargyris et al., 2016)**.

Microbial analysis:

Table: Microbial load of fresh papaya

Fresh papaya			
Dilutions	**No. of colonies**		**CFU**
10^{-2}	15	16	$1.5x10^4$
10^{-3}	6	8	$7x10^3$

The microbial analysis of the freshly cut papaya was examined using a spread plate technique on the first day before preservation and the microbial load was found to be around 1.5 logs CFU/g.

Table: Microbial load on 4th day under refrigerated condition

Unwrapped papaya at refrigerator			
Dilutions	**No. of colonies**		**CFU**
10^{-2}	90	88	$8.9x10^4$
10^{-3}	30	35	$3.25x10^5$

LDPE wrapped papaya at refrigerator			
Dilutions	**No. of colonies**		**CFU**
10^{-2}	55	57	$5.6x10^4$
10^{-3}	20	25	$2.25x10^5$

Coated LDPE wrapped papaya at refrigerator			
Dilutions	**No. of colonies**		**CFU**
10^{-2}	10	11	$1.05x10^4$
10^{-3}	5	6	$0.55x10^5$

The microbial load of coated and uncoated papaya was examined over the period of storage under refrigerated conditions. The microbial load of the uncoated papaya was high around 8.9 logs CFU/g followed by the LDPE wrapped papaya was 5.56 logs CFU/g, and the coated LDPE wrapped papaya showed 1.05 logs CFU/g.

Table: Microbial load on 4th day under the room temperature condition

Unwrapped papaya at room temperature			
Dilutions	**No. of colonies**		**CFU**
10^{-2}	TNTC	TNTC	TNTC
10^{-3}	TNTC	TNTC	TNTC

LDPE wrapped papaya at room temperature			
Dilutions	**No. of colonies**		**CFU**
10^{-2}	TNTC	TNTC	TNTC
10^{-3}	TNTC	TNTC	TNTC

Coated papaya at room temperature			
Dilutions	**No. of colonies**		**CFU**
10^{-2}	TNTC	TNTC	TNTC
10^{-3}	250	255	2.52×10^{-2}

The microbial load of coated and uncoated papaya was examined over the period of storage under refrigerated conditions. The microbial load of the uncoated papaya was more than 300 colonies so it was recommended as TNTC (Too Numerous To Count) followed by the LDPE wrapped papaya was also recommended as TNTC, and the coated LDPE wrapped papaya showed 2.52 logs CFU/g.

The coated LDPE wrapped papaya stored under refrigerated conditions for four consecutive days showed an overall and effective method in increasing the shelf life of the papaya fruit. Similar studies were done by **Tabassum and Khan, 2019** showing the effective way to increase the shelf life and preservation period of the papaya fruit through the coating of the edible film composed of alginate and essential oils of thyme and oregano. The effectiveness of the preservation of the apple was seen in the increasing order of CHS (chitosan-stevia coated) > CH (chitosan-coated) > control which was found to be significant ($P \leq 0.05$) **(Karagöza and Demirdöven, 2019)**.

CONCLUSION

The chitosan-nisin based antimicrobial agent layered on LDPE for the purpose of the packaging of post-harvest papaya has been successfully effective in increasing the shelf life of the papaya upto four consecutive days that was preserved under refrigerated conditions. The papaya warapped with chitosn-nisin based LDPE packaging preserved under refrigerated coditions showed effective results in terms of papaya nutrient values, stability of the packaging films against the external paramters like contaminating microorganisms. However, research concerning the effectiveness of chitosan-nisin combination should be evaluated on other fruits which are at risk of contamination.

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