CHAPTER 3
MATERIALS AND METHODS

The study on various aspects and objectives related to the present research work was carried out during the year 2007 – 2011 at department of Food Processing Technology, A.D. Patel Institute of Technology, New Vallabh Vidyanagar, Anand, and Sophisticated Instrumentation Center for Applied Research and Testing, Vidyanagar, Anand.

The present investigation involved the application of edible coating treatment for fresh fruits and vegetables like tomato and papaya. The effectiveness of coating treatment and shelf life of sample was determined on the basis of various physicochemical, microbial and textural properties of samples. Another important aspect of current research project was to develop protein based edible film and to study various physical, mechanical and barrier properties of the film. This chapter deals with the description of materials, experimental set-ups, analytical techniques and processing and formulation techniques used in various experiments.

3.1 Materials

Soy Protein Isolate (90% protein) used in the experiment was purchased commercially and Table 3.1 indicates the detailed specification of SPI used in the experiment. Glycerol (LR grade) used as plasticizer, Carboxymethyl cellulose, oleic acid, sodium hydroxide (LR grade) used for pH adjustment, were purchased from local chemical supplier. The fruit and vegetable sample used in study were purchased from local market.
Table 3.1 Specifications of SPI Used in Experiment

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Test Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Colour</td>
<td>Straw-light yellow</td>
</tr>
<tr>
<td>2</td>
<td>Protein (d.b)</td>
<td>≥ 90%</td>
</tr>
<tr>
<td>3</td>
<td>Moisture</td>
<td>≤ 7.0%</td>
</tr>
<tr>
<td>4</td>
<td>pH</td>
<td>7± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>Ash</td>
<td>≤ 6.0%</td>
</tr>
<tr>
<td>6</td>
<td>Fat</td>
<td>≤ 0.5%</td>
</tr>
<tr>
<td>7</td>
<td>Particle size</td>
<td>≥ 96.0%(passing 100 mesh)</td>
</tr>
<tr>
<td>8</td>
<td>Total bacteria</td>
<td>≤ 1000 per g</td>
</tr>
<tr>
<td>9</td>
<td>Salmonella</td>
<td>0 per g</td>
</tr>
<tr>
<td>10</td>
<td>E-coli</td>
<td>≤ 2000 per 100 g</td>
</tr>
<tr>
<td>11</td>
<td>Yeast and Moulds</td>
<td>&lt; 100 per g</td>
</tr>
</tbody>
</table>

Courtesy: Certificate of analysis from supplier, Clarion Caseins Ltd.

3.2 Methodology for Application of Edible Coating and Shelf Life Study

3.2.1 Coating Solution Preparation and Application

Soy protein isolate, carboxymethyl cellulose and oleic acid in predetermined quantities were dispersed in 100 ml distilled water; glycerol was added as plasticizer followed by pH adjustment with 1N sodium hydroxide solution to 8. The other components like ascorbic acid and sodium benzoate were also added as per the formulations shown in Table 3.2 and coded as A, B and C. Samples dipped in distilled water were used as a control.

The solutions were then heated with constant stirring on heating mantle at 80 ± 5°C temperature for 15 ± 5 min. Samples were dipped in different coating solutions for 30s, the excess coating was drained and the coated samples were kept for surface drying under natural convection for 12 ± 2 hrs.
Materials and Methods

Table 3.2 Formulation of Edible Coating for Fresh Fruit and Vegetable

<table>
<thead>
<tr>
<th>Coating Treatment</th>
<th>SPI (%)</th>
<th>CMC (%)</th>
<th>Oleic Acid (%)</th>
<th>Sodium Benzoate (%)</th>
<th>Ascorbic Acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4</td>
<td>0.2</td>
<td>1</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>0.2</td>
<td>1</td>
<td>0.1</td>
<td>------</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>0.2</td>
<td>1</td>
<td>------</td>
<td>0.4</td>
</tr>
<tr>
<td>Control</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
</tbody>
</table>

After the coating process, one lot of sample was stored at ambient conditions at a temperature of 35 ± 3 deg Celsius and RH of 70 ± 5 per cent for 9 days. Another lot of sample was stored at 10 ± 3 deg Celsius and RH of 85 ± 5 per cent for 21 days. For each treatment 30 individual samples were coated and three replications per treatment were analyzed after predetermined frequency. The sampling frequency for tomatoes was 1, 3, 5, 7 and 9th and 1, 5, 9, 13, 17 and 21st day of storage for samples stored at ambient and refrigerated conditions respectively. The sampling frequency used for papayas stored at refrigerated conditions was 1, 3, 5 and 7th day of storage. During storage period the quality parameters tested were as follows.

Physico-chemical properties

1. Titratable Acidity
2. Vitamin C content
3. Total Sugars
4. Reducing Sugars
5. Total soluble solids
6. pH
7. Weight loss
8. Sugar to acid ratio

Textural properties

1. Stiffness
2. Chewiness
3. Cohesiveness
Microbial analysis
1. Standard plate count
2. Yeast and mold count

3.2.2 Titratable Acidity
The titratable acidity (expressed as citric acid %) was determined by extracting known weight of sample into distilled water and titrating with 0.1 N sodium hydroxide, using phenolphthalein as an indicator to an end-point of pH 8.1 (AOAC, 2000).

3.2.3 Vitamin C
Vitamin C (ascorbic acid) content was determined by using titrimetric method with the titration of filtrate against 2, 6-dichlorophenol indophenol and the results of vitamin C content were expressed as mg/100 g (AOAC, 2000)

\[
\text{Ascorbic acid content (mg/100 gm of fruit pulp)} = \frac{(T \times D \times V_1 \times 100)}{(V_2 \times W)}
\]

Where,
- \(T\) = Titre,
- \(D\) = Dye factor,
- \(V_1\) = Volume made up,
- \(V_2\) = Volume of extract taken for estimation
- \(W\) = Weight of sample taken for estimation.

3.2.4 Total and Reducing Sugars
The reducing and non-reducing sugar contents were determined by following the Shaffer-Somogyi method as described by Ranganna 2000.

3.2.5 pH and TSS
The pH of the sample was determined by the method described by Ranganna 2000. The pH of tomato juice was recorded by using digital pH meter. The pH meter was standardized with the help of buffer solution.

The Total Soluble Solid (TSS) content of tomato fruit pulp was determined by using Hand refractometer by placing a drop of pulp
solution on its prism. The percentage of TSS was obtained from direct reading of the refractometer. Temperature correction was made by using methods described by Ranganna 2000.

3.2.6 Weight Loss

To determine the effectiveness of edible coatings as moisture-barriers, the weight of five samples in each treatment was monitored during storage. It was assumed that weight loss corresponded entirely with water loss. For analysis of weight loss separate lot of about 100 gm from each treatment was used during study. The weight loss percent relative to initial weight was calculated by weighing the samples every 2 days.

3.2.7 Sugar Acid Ratio

The sugar acid ratio also termed as Brix : Acid ratio was calculated by taking the ratio of total soluble solid and acidity of respective samples.

3.2.8 Measurement of Textural Properties

The textural properties of fruit and vegetable sample were determined with 5 mm cylindrical probe by using texture analyzer TA plus (Lloyds, England). The crosshead speed used was 5mm/min and deformation per cent was kept at 30. The stiffness, chewiness and cohesiveness were determined directly from the stress - strain curves using the software Nexgen V 4.5.

3.2.9 Microbiological Analysis

Samples were analyzed for microbial quality at a predetermined time interval during storage. Sample of 1 cm X 1 cm size was first mixed with 99 ml sterile water, which was further serial diluted up to desired dilution factor (10⁻³). For determination of Standard Plate Count (SPC) Nutrient agar (Hi-Media Laboratories Pvt. Ltd., Mumbai), and for yeast and mold count (YMC) freshly prepared acidified (pH adjusted to 3.5 by sterile 10 per cent tartaric acid solution) potato dextrose agar (Hi-Media Laboratories Pvt. Ltd., Mumbai) was used as medium for growth.
Plate 3.1 Texture Analyzer used for Textural Studies

Plate 3.2 Texture Probe Used for Textural Studies
3.2.9.1 Standard Plate Count

A 15 ml of molten nutrient agar was poured aseptically to the 1 ml of the dilution kept in sterile petri plates. The contents were mixed and plates were cooled. The plates then were inverted and incubated in an incubator maintained at 37 ± 0.5 deg Celsius for 24 hrs and number of colony forming units was calculated.

3.2.9.2 Yeast and Mold Count

The 5 ml of sample prepared as described in previous section was used for plating in duplicate and thereafter 15 ml of molten PDA was poured aseptically to plates. The contents were mixed and plates were cooled and were incubated at 25 ± 0.5 deg Celsius for 72 hrs and number of colony forming unit was calculated.

3.2.10 Statistical Analysis

Analysis of variance (ANOVA) was used to detect treatment effect. Mean separation was performed by using least significance difference (LSD) at the p< 0.05 level.

3.3 Development of Protein Based Edible Film by Using RSM

3.3.1 Experimental Design and Statistical Analysis

RSM was used to generate the experimental designs, statistical analysis and regression model with the help of Design Expert Software Version 8 (Statease Inc.).

The Central Composite Rotatable Design (CCRD) with a quadratic model (Box & Draper, 1987) was employed. Three independent variables namely Soy Protein Isolate (SPI) concentration ($x_1$), plasticizer concentration ($x_2$) and pH ($x_3$) were chosen. Each independent variable had 3 levels which were -1, 0 and +1. A total of 20 different combinations (including six replicates of the centre point each signed the coded value (0) were chosen in random order according to a CCRD configuration for three factors divided in three blocks (Cochran & Cox, 1957). The α-values in the design outside the ranges were selected for rotatability of the design (Thompson et al, 1982).
Table 3.3 The Central Composite Experimental Design Employed for Preparation of Edible Film.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>SPI Concentration (%)</th>
<th>Plasticizer Concentration (% of SPI)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$X_1(x_1)$</td>
<td>$X_2(x_2)$</td>
<td>$X_3(x_3)$</td>
</tr>
<tr>
<td>1</td>
<td>8(0)</td>
<td>50(0)</td>
<td>9(0)</td>
</tr>
<tr>
<td>2</td>
<td>8(0)</td>
<td>50(0)</td>
<td>9(0)</td>
</tr>
<tr>
<td>3</td>
<td>8(0)</td>
<td>50(0)</td>
<td>9(0)</td>
</tr>
<tr>
<td>4</td>
<td>8(0)</td>
<td>50(0)</td>
<td>9(0)</td>
</tr>
<tr>
<td>5</td>
<td>8(0)</td>
<td>50(0)</td>
<td>9(0)</td>
</tr>
<tr>
<td>6</td>
<td>8(0)</td>
<td>50(0)</td>
<td>9(0)</td>
</tr>
<tr>
<td>7</td>
<td>8(0)</td>
<td>33(-α)</td>
<td>9(0)</td>
</tr>
<tr>
<td>8</td>
<td>10(+α)</td>
<td>50(0)</td>
<td>9(0)</td>
</tr>
<tr>
<td>9</td>
<td>8(0)</td>
<td>67(+α)</td>
<td>9(0)</td>
</tr>
<tr>
<td>10</td>
<td>8(0)</td>
<td>50(0)</td>
<td>11(+α)</td>
</tr>
<tr>
<td>11</td>
<td>6(-α)</td>
<td>50(0)</td>
<td>9(0)</td>
</tr>
<tr>
<td>12</td>
<td>8(0)</td>
<td>50(0)</td>
<td>7(-α)</td>
</tr>
<tr>
<td>13</td>
<td>9(+1)</td>
<td>60(+1)</td>
<td>8(-1)</td>
</tr>
<tr>
<td>14</td>
<td>9(+1)</td>
<td>40(-1)</td>
<td>10(+1)</td>
</tr>
<tr>
<td>15</td>
<td>7(-1)</td>
<td>60(+1)</td>
<td>10(+1)</td>
</tr>
<tr>
<td>16</td>
<td>7(-1)</td>
<td>40(-1)</td>
<td>8(-1)</td>
</tr>
<tr>
<td>17</td>
<td>7(-1)</td>
<td>60(+1)</td>
<td>8(-1)</td>
</tr>
<tr>
<td>18</td>
<td>9(+1)</td>
<td>40(-1)</td>
<td>8(-1)</td>
</tr>
<tr>
<td>19</td>
<td>7(-1)</td>
<td>40(-1)</td>
<td>10(+1)</td>
</tr>
<tr>
<td>20</td>
<td>9(+1)</td>
<td>60(+1)</td>
<td>10(+1)</td>
</tr>
</tbody>
</table>

The values in bracket i.e. $x_1$, $x_2$ and $x_3$ represents the coded values for respective factors.
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The centre points for these designs were selected with ingredients at levels expected to yield satisfactory experimental results. The experimental design in the coded (x) and actual (X) levels of variables is shown in Table 3.3. The responses function (y) measured were Thickness, Tensile strength, Young’s Modulus and Elongation at break of the edible film. These values were related to the coded variables (xi, i = 1, 2 and 3) by a second degree polynomial using the equation below.

\[ y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \]

The coefficients of the polynomial were represented by b0 (constant term), b1, b2 and b3 (linear effects), b11, b22 and b33 (quadratic effects), and b12, b13 and b23 (interaction effects). The analysis of variance (ANOVA) tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms in the polynomial were judged statistically by computing the F-value and compared with standard significance level of 0.1%, 1% and 5%. The regression coefficients were then used to make statistical calculation to generate contour maps from the regression models.

3.3.2 Film Formation and Formulation

Soy protein isolate with desired concentration was dispersed in 100 ml distilled water, then glycerol was added as plasticizer followed by pH adjustment with 1N sodium hydroxide solution to the desired value as per formulations given in Table 3.3. The solutions were heated on heating mantle with continuous stirring at 85±5 degree Celsius temperature for 15±5 min and kept at room conditions for 5 min to allow bubbling to dissipate prior to pouring. All of the solutions in the beakers were poured onto glass plate of 24” x 24” to control film thickness, the quantity of each film forming solutions poured onto these plate were always and dried overnight at room temperature. The typical laboratory scale protocol for preparation of protein based edible film is given in Fig 3.1
SPI

Addition of glycerol

pH adjustment using 1N sodium hydroxide

Make up volume to 100ml with distilled water

Heating with constant stirring at 85±5°C temperature for 15±5 min

Casting on glass plate

Drying at ambient conditions

Peeling off the film

Self supporting film

Fig. 3.1 Lab Scale Process for Formation of Soy Protein Based Edible Film

The various parameters studied for the edible film during optimization are as follows

1. Thickness
2. Tensile strength
3. Elongation at break
4. Young’s Modulus

3.3.3 Methodology for Measurement of Edible Film Properties

3.3.3.1 Measurement of Film Thickness

Film thickness was measured with a micrometer (No. 7327, Mitutoyo Manufacturing Co. Ltd., Tokyo, Japan) to the nearest 0.001 mm around the film testing area at 5 random positions.
3.3.3.2 Measurement of Mechanical Properties

Tensile strength, elongation at break and Young’s modulus are the most commonly reported responses to describe mechanical properties of edible films and coatings. These parameters were determined according to the standard method D882-95 (ASTM, 1995), taking an average of three determinations in each case. The films were cut into 25 mm wide and 125 mm long strips using a scalpel, and mounted between the grips of the texture analyzer TA plus (Lloyds, England). The initial grip separation was set at 100 mm and the crosshead speed at 50 mm/min. The tensile strength and elongation at break were determined directly from the stress - strain curves using the software Nexgen V 4.5.

3.4 Study of properties of optimized Protein Based Edible Film

The formulation for protein based edible film was optimized by using RSM tool and three best formulations were identified as shown in Table 3.4 to obtain edible film with maximum tensile strength and elongation at break and minimum thickness and Young’s modulus, respectively. These optimized formulations were used during subsequent investigation to study barrier properties and moisture content of edible film.

Table 3.4 Optimized Formulation of Protein Based Edible Film by Using RSM

<table>
<thead>
<tr>
<th>Formula</th>
<th>SPI Conc. (%)</th>
<th>Plasticizer Conc. (% of SPI)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8.4</td>
<td>60</td>
<td>8.8</td>
</tr>
<tr>
<td>B</td>
<td>8.6</td>
<td>60</td>
<td>9.0</td>
</tr>
<tr>
<td>C</td>
<td>8.7</td>
<td>60</td>
<td>9.1</td>
</tr>
</tbody>
</table>

3.4.1 Methodology for Barrier Properties and Moisture Content

3.4.1.1 Measurement of Water Vapor Transmission Rate

Water vapor transmission rate was measured by according to ASTM D1653/E96 using Labthink instrument model No. TSY-T1. The preconditioned sample was used for measurement. The specimen size was 100 mm and actual test area was 63.58 cm². The test parameters
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used were temperature of 38 deg Celsius and relative humidity of 90 per cent.

WVP was calculated from equation

\[ WVP = WVTR \times \frac{L}{\Delta P} \]

Where
- WVP is Water Vapor Permeability (g.mm m\(^{-2}\)h\(^{-2}\)kPa\(^{-1}\))
- WVTR is Water Vapor Transmission Rate (g.m\(^{-2}\)h\(^{-2}\))
- L is the film thickness (mm),
- \(\Delta P\) is the partial water vapour pressure difference (kPa) between the two sides of the film.

**Principle**

Saturated water vapor transmits through specimen in a unit time under specified conditions of temperature and humidity. Determine the transmitted mass by testing the decreasing weight of distilled water with time going.

3.4.1.2 Measurement of Oxygen Permeability

The oxygen transmission rate was measured by using Labthink instrument model VAC-VBS according to standard method ASTM D 1434. The preconditioned sample was used for measurement. The specimen size was 80 mm and actual test area was 28.3 cm\(^2\). The test was conducted under proportional mode. The gas pressure used was between 4-6 kg/hr and vacuum used was less than 26 Pa. The oxygen permeability was calculated from the oxygen transmission rate and the measured thickness of the films, by using equation

\[ OP = OTR \times \frac{L}{\Delta P} \]

Where
- OP is Oxygen Permeability (cm\(^3\) µm/(m\(^2\) d kPa)
- OTR is Oxygen Transfer Rate (cm\(^3\)/(m\(^2\) d kPa)
- L is thickness in µm
- \(\Delta P\) is partial pressure (kPa) difference between both sides of film
Principle

Put the sample pre-conditioned between the upper and lower chambers, clamp tightly, open the valve of the lower chamber to vacuum the lower chamber, and then vacuum the whole system. When the vacuum degree reaches the certain data, close the valve of the lower chamber. Feed certain pressure of testing gas into the upper chamber to generate a constant pressure difference between the two chambers (adjustable). The gas will penetrate the film from the higher pressure side to the lower pressure side under the function of the pressure gradation. Get the gas transmission rate through inspecting and processing the pressure of the lower chamber.

3.4.1.3 Measurement of Moisture Content

Film samples were weighed into aluminum dishes and dried in an air-circulating oven at 105°C for 24 h. Moisture content (MC) was determined as percentage of initial film weight lost during drying and reported on a wet basis. Triplicate measurements of MC were obtained for each type of film with individually prepared films as replicated experimental units and three specimens tested from each film.

3.4.2 Statistical Analysis

Analysis of variance (ANOVA) was used to determine effect of formulations. Mean obtained were compared by using Tukey’s multiple comparison test at p< 0.05 level by using Daniel’s XL Toolbox version 4 software.