3.0 Materials and Methods...
3.1 Materials

3.1.1. Chemicals

Analytical reagents supplied by different companies as detailed below were used for the experiments.

Qualigens
Ammonium molybdate
Boric acid
Chloroform
Ethanol
Glacial acetic acid
Glycerol
Nitric acid
Petroleum ether
Potassium iodide
Sodium chloride
Sodium hydroxide
Sodium meta bisulphate
Sodium sulphate
Sulphuric acid
Trichloro acetic Acid

Merck
Acetonitrile
Coomassie Brilliant Blue R250
Hydrochloric acid
O –Phthalaldehyde
Parafilm M
Perchloric acid
Phenolphthalein
Potassium carbonate
Sodium dodecyl sulphate
Thio barbituric acid reagent
Sisco Research Laboratories (SRL)
2-mercaptethanol
Ammonium per sulphate
Ferric chloride
Glycine
L-Leucine
Methanol
Standards of Arsenic, Lead, Copper, Zinc, Cadmium and Chromium
Tryptophan
SD. Fine Chemicals
Bromophenol blue
Potassium bromide
Sodium hypochlorite
Tri sodium citrate
Sigma chemicals
Acrylamide /BIS Acrylamide
Amino acid standards
Gelatin from Bovine Skin Type B ~ 225 Bloom
Gelatin from Bovine Skin Type B ~ 75 Bloom
Gelatin from cold water fish skin
Gelatin from Porcine Skin Type A ~ 175 Bloom
Gelatin from Porcine Skin Type A ~ 300 Bloom
Gelatin standards
L -4- hydroxyproline
Wide Range Sigma Marker (Molecular weight 6500 – 200,000 Da)

3.1.2. Equipment and Glassware

Amino acid analyzer : HPLC- LC 10 AS, Schimadzu with FL6A fluorescence Detector and Shimadzu CR 6A Chrompac recorder

Atomic Absorption Spectrophotometer : Varian AA 420 ,USA

78
Bio-rad Tetra Mini Protean II unit with gel documentation system : Bio-Rad Laboratories, Hercules, CA

Bloom jars : Schott Duran, Germany

Bowl Chopper : Tecator 1094

Centrifuge : REMI Cooling centrifuge, Model CPR 24, Remi Instruments, India

Circulating water bath : (Haake D3, Germany)

Deionised water generation system : ELGA Purelab Ultra, UK.

Flake ice machine : F90 compact unit, Icematic, Italy

Freeze Drier : Martin Christ, Gamma 1-16 LSC, Germany

Gas Permeability Apparatus : Davenport, UK

Glass wares : Borosil Glass ware, India

Homogenizer : Ultra Turrax, T20 B IKA Labortechnik, Germany

Hot air oven : Beston hot air oven, India

Infrared Spectrophotometer : Nicolet Avatar 360 ESP

Micrometer Screw Gauge : Reston Equipment, India

Microwave Digester : Anton Paar, Germany

pH meter : Cyberscan 510 pH meter, Eutech Instruments, Singapore

Spectrocolorimeter : Hunter lab Miniscan® XE plus, UK

Spectrophotometer : Genesys 5, Spectronic Instruments, USA

Texture Analyser & Tensile Strength Tester : Lloyd Instruments, Model LRX Plus, U.K
Vacuum Chamber : Heraeus Vacutherm – Germany
Viscometer : Brookfield DV E Model, England
Water Bath : Julabo TW 20, Germany
Weighing Balance : Sartorius Electronic Balance, Germany

3.1.3 Fish Skin

The raw materials for the study were the skins of three cultured freshwater fishes viz., Rohu (*Labeo rohita* – Hamilton Buchanan), Common carp (*Cyprinus carpio*) and Grass carp (*Ctenopharyngodon idella*). Fish samples were procured from different freshwater farms located in Central Kerala. The size description of the species collected is given in Table 3.1.

**Table 3.1 Size description of the selected fish species**

<table>
<thead>
<tr>
<th>Species</th>
<th>Average size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rohu</td>
<td>Length: 55 ± 2.8 cm, Weight: 2500 ± 120g</td>
</tr>
<tr>
<td>Common carp</td>
<td>Length: 30 ± 3.5 cm, Weight: 1500 ± 65g</td>
</tr>
<tr>
<td>Grass carp</td>
<td>Length: 62 ± 2.2 cm, Weight: 2610 ± 140g</td>
</tr>
</tbody>
</table>

The fish was brought to the laboratory in iced condition. The samples were then filleted and the skinless boneless fillets were used for the preparation of value added products. The skin was collected, cleaned by removing scales, washed and blast frozen and stored at -18 °C with a maximum storage of less than two months before use.
3.2 Methods

3.2.1 Preparation of the Fish Skin for Gelatin Extraction (Zhou and Regenstein, 2004)

Frozen skins were thawed at 4°C for about 20 h, chopped into small pieces (about 2 to 3 cm), and washed with tap water (1:6 w/v) for 10 min. Washing was repeated 3 times. The cleaned fish skins were drained using cheesecloth for 5 min, and the cheesecloth containing the skins were squeezed by hand to remove liquid.

3.2.2. Process of Gelatin Extraction

3.2.2.1 Pretreatment of fish skins prior to main extraction

The gelatin extraction procedure followed was essentially as described by Grossman and Bergman (1992) with slight modifications. The cleaned and drained fish skins were given a pretreatment with an alkaline solution followed by an acid solution. The detailed steps were as follows: Cleaned skins (c.a 30.00 g) were taken in conical flask and treated with different concentrations of sodium hydroxide (1:6 w/v) for variable times. Then, the samples were rinsed with tap water and drained using cheesecloth. The above treatment was repeated for 2 times. Afterwards the samples were treated with different concentrations of sulphuric acid (1:6 w/v) for variable times. The samples were then rinsed with tap water and drained using cheesecloth. The acid treatment was also repeated two times. The treated samples were squeezed manually using cheesecloth to remove excess water prior to the extraction. The conditions followed for the pretreatment are given in Table 3.2.
Table 3.2 Process variables adopted for the pretreatment of fish skins.

<table>
<thead>
<tr>
<th>Process variables</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH concentration (mol/L)</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>H₂SO₄ concentration (mol/L)</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Pretreatment time (minutes)</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>60</td>
</tr>
</tbody>
</table>

3.2.2.2 Gelatin extraction

The pretreated fish skins were taken in flasks for gelatin extraction with varying volumes of deionized water. The flasks were covered with parafilm and the extraction was carried out in a water bath for variable times at variable extraction temperatures as given in Table 3.3. Finally, the gelatin solutions were filtered through 4 layers of cheesecloth, and freeze dried prior to further work.

Table 3.3 Process variables adopted for the extraction of gelatin.

<table>
<thead>
<tr>
<th>Process variables</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin/water ratio</td>
<td>1:4</td>
</tr>
<tr>
<td></td>
<td>1:5</td>
</tr>
<tr>
<td></td>
<td>1:6</td>
</tr>
<tr>
<td>Extraction time</td>
<td>6H</td>
</tr>
<tr>
<td></td>
<td>8H</td>
</tr>
<tr>
<td></td>
<td>10H</td>
</tr>
<tr>
<td>Extraction temperature</td>
<td>40 °C</td>
</tr>
<tr>
<td></td>
<td>50 °C</td>
</tr>
<tr>
<td></td>
<td>60 °C</td>
</tr>
</tbody>
</table>
Fig. 3.1 Flow chart for pretreatment and extraction of gelatin from fish skin

1. Cleaned Fish skin
2. Alkali pretreatment
   - Washing with water (Repeated two times)
   - Draining using cheesecloth
3. Acid pretreatment
   - Washing with water (Repeated two times)
   - Draining using cheesecloth
4. Extraction using deionized water
5. Filtration & Freezing
6. Freeze Drying
3.2.3 Experimental Design for Process Optimization

Optimization is the method of choice when seeking a best alternative from a specified set of alternatives. The experimental design for optimization is a two stage process. In the first stage called screening, the objective is to efficiently determine the critical control variables from a large number of potential variables. In the second stage of optimizing, the objective is to determine the optimum values for the critical control factors so that the desired quality objectives are met.

3.2.3.1 Screening Experimental Design

Screening was done to determine the critical variables for the extraction of gelatin from the skin of Rohu, Common carp and Grass carp with a fractional factorial design. Fractional factorial design consists of an appropriately chosen small fraction of the full factorial design which permits the study of a large number of variables in an economical number of trials. A fractional factorial design was used for screening. Six important factors (independent variables) that affect the extraction of gelatin from fish skin and their ranges between model levels described as −1 and +1 were selected for the screening experiments (Table 3.4). The design used in the study is a resolution three design (2^6−3/iii) in which the main effects are confounded with two factor interactions. The importance of these factors was evaluated based on the responses on two dependent variables selected. These selected dependent variables were gel strength (Bloom) and yield (%) which can be rated as the most commercially important physical properties of the extracted gelatin. A total of eight groups of extraction experiments were conducted using different combinations of these six factors (Table 3.4).

From the screening experiments, four factors were identified as critical variables that had a significant effect on the extraction of gelatin from the skin of Rohu, Common carp and Grass carp. These were Alkali pretreatment concentration (mol/L), Acid pretreatment concentration (mol/L), Pretreatment time (min), and Extraction temperature (°C).
Table 3.4 Independent Variables and their Levels in the 6 Factor, 2 Level Fractional Factorial (2^6-3 / II ) Screening Design

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Symbol</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-1</td>
</tr>
<tr>
<td>NaOH concentration (mol/L)</td>
<td>X1</td>
<td>0.1</td>
</tr>
<tr>
<td>H₂SO₄ concentration (mol/L)</td>
<td>X2</td>
<td>0.1</td>
</tr>
<tr>
<td>Pretreatment time (minutes)</td>
<td>X3</td>
<td>40</td>
</tr>
<tr>
<td>Skin/water ratio</td>
<td>X4</td>
<td>1:4</td>
</tr>
<tr>
<td>Extraction time (hours)</td>
<td>X5</td>
<td>6</td>
</tr>
<tr>
<td>Extraction temperature (°C)</td>
<td>X6</td>
<td>40</td>
</tr>
</tbody>
</table>

*No. of variables: 6, Levels: 2, Observations: 8, Resolution: 3: Wt. of sample: 30g for each run, Pretreatment ratio 1: 6.

Table 3.5 Fractional Factorial Screening Design in Coded Units

<table>
<thead>
<tr>
<th>Standard Order</th>
<th>X1</th>
<th>X2</th>
<th>X3</th>
<th>X4</th>
<th>X5</th>
<th>X6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>2</td>
<td>+1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
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<tr>
<td>3</td>
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<td>4</td>
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<td>5</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
<td>+1</td>
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<td>-1</td>
</tr>
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<td>6</td>
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<td>-1</td>
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<td>+1</td>
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<td>8</td>
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<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
</tr>
</tbody>
</table>
3.2.3.2 Optimization of Experimental Design

Once the important variables are determined by the screening, Response Surface Methodology was used for optimizing the process of extraction of gelatin. The independent variables and their levels used in the Design are given in table 3.6. A 4 factor, 5 levels Central Composite Rotatable Design was formulated which is given in Table 3.7. Here five levels were assigned for each factor instead of the two level design for screening experiments. To study the effect of the selected independent variables on the responses, a total of 31 runs which included seven centre point runs using the Central Composite Design were carried out. Experimental data were statistically analyzed using the software Design-Expert 6.0.11, (Stat-Ease, Inc., Minneapolis MN, USA).

Table 3.6 Independent variables and their levels in the 4 factor, 5 level Central Composite Rotatable Design for optimization of the extraction conditions of gelatin from fish skin

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Symbol</th>
<th>Code level</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH concentration(mol/L)</td>
<td>X1</td>
<td>coded</td>
</tr>
<tr>
<td></td>
<td></td>
<td>uncoded</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-2</td>
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<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>H2SO4 concentration(mol/L)</td>
<td>X2</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>Pretreatment time(minutes)</td>
<td>X3</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70</td>
</tr>
<tr>
<td>Extraction temperature(°C)</td>
<td>X4</td>
<td>30</td>
</tr>
<tr>
<td></td>
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<td>40</td>
</tr>
<tr>
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<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70</td>
</tr>
</tbody>
</table>
Table 3.7 Central Composite Design for Optimizing the Extraction Condition of Fish Skin Gelatin

<table>
<thead>
<tr>
<th>Standard Order</th>
<th>X1</th>
<th>X2</th>
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<td>07</td>
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<td>19</td>
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<td>-2</td>
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<tr>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
3.2.4 Gelatin Water Dessert Preparation (Zhou & Regenstein, 2007)

Gelatin water desserts were prepared by dissolving gelatins or their mixtures in a flavored orange drink (prepared from orange flavour instant drink mix, Kraft Foods Ltd., Thailand) heated 45 – 50 °C to compare the sensory and physical properties of gel desserts from various sources such as bovine, porcine and fish skin gelatin. The gelatin desserts prepared had the same gelatin concentration (3%w/w). Gelatin concentration lower than 3% resulted in desserts which are very soft and disintegrated immediately after formation, particularly in the case of fish skin gelatins. Gelatin concentration above 3% produced hard gels which is an undesirable feature for the desserts. Hence 3% concentration of gelatin was found to be the optimum concentration for the preparation of desserts. The final composition of desserts is shown in Table 3.8. The dessert solutions were then poured separately into 2 different containers: (1) standard bloom jars (112.5 g) for gel strength determination; (2) cylindrical plastic molds having diameter of 38 mm and a height of 22 mm for texture profile analyses. All samples were then matured at 2 - 4 °C for 20 - 24 hours before measurements were made.
Table 3.8 Composition and pH of Gelatin Desserts*

<table>
<thead>
<tr>
<th></th>
<th>BG</th>
<th>PG</th>
<th>RG</th>
<th>CG</th>
<th>GG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin (g)</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Water (g)*</td>
<td>87.0</td>
<td>87.0</td>
<td>87.0</td>
<td>87.0</td>
<td>87.0</td>
</tr>
<tr>
<td>Sugar (g)*</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Others (g)*</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Final pH</td>
<td>3.8</td>
<td>3.7</td>
<td>3.6</td>
<td>3.7</td>
<td>3.7</td>
</tr>
</tbody>
</table>

*BG = Bovine Skin Gelatin (225B); PG = Porcine Skin Gelatin (300B); RG = Rohu Skin Gelatin; CG = Common carp Skin Gelatin; GG = Grass carp Skin Gelatin

The amount of water and sugar are calculated based on the ingredient label of the flavored orange drink. The word "others" is based on the ingredient label of the flavored orange drink and refers to those compounds providing appropriate orange flavor and color, and are used to balance the pH, which include synthetic flavours (E102, E110, E171), acidity regulator (E330), minerals (E341)(0.57%), stabilizer (E415), vitamins (0.32%), Ferrous citrate (0.07%), edible salt and the total amount of these compounds is less than 1.0 g in 100 g of the final gelatin desserts.

3.2.5 Preparation of Gelatin Films (Sobral et al., 2001)

The gelatin films were prepared according to the casting technique described by Sobral et al., (2001) with slight modifications. This consists of dehydrating a filmogenic solution, conveniently applied on a support. The filmogenic solutions of gelatin were prepared under the following conditions: 7.5 g of gelatin / 105 ml distilled water (6.67% w/w) was mixed with 1.5 g glycerol as plasticizer at natural pH of the solution. The plasticizer used was 20% (w/w) of the gelatin. Initially the gelatin was hydrated at room temperature in water and solubilized later in a water bath with digital control (±0.5 °C) kept at 55 °C. After complete solubilisation, remaining water and glycerol were added, and the solution was kept in water bath under agitation for 30 minutes. The filmogenic solutions were degassed under vacuum and 25 ml of the solution was applied on High Impact Polypropylene trays of size 24 X 14 cm. The films were dried overnight at ambient temperature and manually peeled off from the surface.
3.2.6 Analytical Methods

3.2.6.1 Determination of yield (Muyonga et. al., 2004a).

The yield was calculated by taking 10 ml of gelatine solution in duplicate which was centrifuged, filtered and evaporated and used for solid concentration determination. The following equation was used for gelatine yield calculation:

\[ \text{Yield (\%)} = \frac{C \times V}{M} \times 100 \]

where \( C \) = light liquor concentration (g/ml), \( V \) = liquor volume, \( M \) = weight of sample (g) used for extraction.

3.2.6.2 Determination of pH (BS 757, 1975)

A 1% (w/v) solution of gelatin was prepared in distilled water at 60 °C, cooled to room temperature and the pH was measured using Cyberscan 510 pH meter.

3.2.6.3 Determination of Colour

Gelatin solutions (6.67% w/v) were used for the measurement of color. Colour analysis was performed with a Hunter lab Miniscan ® XE plus spectrophotometer (Hunter Associates Laboratory, Inc. Reston, Virginia, USA). Measurements were recorded using the \( L^* a^* b^* \) colour scale (CIE, 1986). Six repetitions of the different colour parameters were recorded.

3.2.6.4 Viscosity (Cho et. al., 2006)

Gelatin solutions at a concentration of 6.67%(w/v) were prepared by dissolving the dry powder in distilled water and heating at 60 °C. Viscosity (cP) of 10 ml of the solution was determined using Brookfield digital viscometer (Model DV E Brookfield Engineering, USA) equipped with a No.1 spindle at 30 ± 0.5 °C.
3.2.6.5 Clarity (ISO 7027:1999)

The clarity / turbidity of a 6.67 % gelatin solution was measured as the absorbance at 620 nm of a 6.67 % gelatin solution in 1 cm cuvettes against water using a spectrophotometer.

Results of spectrophotometric measurements can be expressed as absorbance (E) or transmission (T). Conversion of results is possible using the following formulas:

\[ E = \log \frac{1}{T} = \frac{1}{10E} \]

**Procedure**

7.50 g (± 0.01) gelatin was weighed into a 150 ml bottle and 105 ml (± 0.2) water was added. The solution was then stirred to moisten the gelatin completely, after which the bottle was covered with rubber stopper and allowed to stand at room temperature for 1 - 4h.

The bottle was then placed in a water bath at 65°C for about 20 min. for dissolving the sample. The bottle was shaken to dissolve the gelatine completely and to achieve a homogeneous solution. The completely dissolved sample was transferred to the cuvettes, and allowed to cool to room temperature. The absorbance at 620 nm was measured at room temperature against deionized water.

3.2.6.6 Foam Formation Capacity and Foam Stability (Cho et. al., 2004)

One gram of gelatin was placed in 50 ml distilled water and allowed to swell. The swollen sample was kept at 60 °C and the foam was prepared by homogenizing at 10,000 rpm for 5 min. in the Ultra Turrax homogeniser. The homogenized solution was poured into a 250 ml flask. The foam formation ability was calculated as the ratio of volume of foam to the initial volume of liquid. The foam stability was calculated as the ratio of the initial volume of foam to the final volume of foam after 30 min.

3.2.6.7 Water holding and Fat binding capacities (Cho et. al., 2004)

One gram of gelatin was taken in a centrifuge tube and weighed (tube with gelatin). For measuring water-holding capacity and fat-binding capacity, 50 ml distilled water or 10 ml sunflower oil was added respectively and held at room temperature for 1 h. The gelatin solutions were mixed in vortex mixer for 5 s every 15 min for one hour. The solutions were then centrifuged at 450g for
20 min. The upper phase was removed and the centrifuge tube was drained for 30 min on a filter paper after tilting to a 45° angle. Water holding and Fat binding capacities were calculated as the weight of the contents of the tube after draining divided by the weight of the dried gelatin, and expressed as the percentage of weight of dried gelatin.

3.2.6.8 Determination of Melting point (Wainewright, 1977)

Gelatin solutions 6.67% (w/w) were prepared and a 5-mL aliquot of each sample was transferred to a small culture test tube of 12 × 75 mm size. The samples were degassed in vacuum chamber (Heraeus vacutherm – Germany). The tubes were then covered with parafilm and heated in a water bath at 60 °C for 15 minutes. It was then cooled immediately in ice chilled water and matured at 10 °C for 16-18 hours. 5 drops of a mixture of 75% chloroform and 25% methyl red dye was placed on the surface of the gel. The gels were then put in a water bath (circulating bath – Haake D3) at 10 °C and the water heated at the rate of 0.2 °C per minute. The temperature at which the drops began to move freely down the gel was taken as the melting point.

3.2.6.9 Determination of Setting point and Setting time

Determination of setting point and setting time of gelatin was carried out as described by Muyonga et al., (2004a) but with slight modifications. Gelatin solutions of 10% (w/w) were prepared as described in Section 3.2.6.8 and transferred to thin wall (12 mm × 75 mm) test tubes. The dissolved samples were transferred to water bath held at 40 °C (circulating bath – Haake D3). The water bath was then cooled slowly at the rate of 0.2 °C per minute. A thermometer was inserted into the sample and lifted out at 30 seconds intervals. The temperature of the mixture at which the gelatin solution no longer dripped from the tip of the thermometer was recorded as the setting temperature.

Setting time was determined on samples prepared in the same way as those for the determination of the setting temperature. Samples were transferred to a water bath maintained at 10 °C (circulating bath – Haake D3). A rod was inserted in the gelatin solution and observed at intervals of 15 seconds. The time at which the rod could not detach from the gelatin sample was recorded as the setting time.
3.2.6.10 Gel strength determination (Jelly strength, Bloom) (BS 757: 1975)

**Definition**

The gel strength (Bloom) is the mass in grams necessary to depress a standard plunger 4 mm into the gel having a gelatin concentration of 6.67 % (w/v) and matured at 10.0°C for 17 h.

**Principle**

A 6.67 % solution of the gelatin sample is prepared in a wide-mouthed test bottle at 60°C, cooled to 10°C and kept for 17 h for maturation at this temperature. The resulting gel is tested using a Texture Analyzer.

**Equipment**

Texture Analyzer (Lloyd Instruments, Model LRX Plus, U.K).

Plunger: AOAC plunger, with 12.70 mm (0.500 inches) diameter, plane surface and sharp edge, no measurable radius.

Bloom jars (Schott Duran): The standard Bloom jar has a capacity of approximately 155 ml, internal diameter of 59 mm +/- 1 mm, overall height 85 mm and a flat bottom to ensure it does not rock on a flat surface.

Thermostatic water bath: held at 65 ± 2°C.

Balance: with a sensitivity of 0.01 g.

**Procedure**

7.50 ± 0.01 g gelatin was weighed into the Bloom bottle and 105 ± 0.2 ml deionized water was added and stirred. The bottle was covered with a rubber stopper and the sample was allowed to stand at room temperature for 4 hours. The bottles were then placed in water bath at 45°C for about 20 min with occasional shaking for complete dissolution. The bottles were allowed to cool for about 15 min. at room temperature, and then placed in chilled condition at 2-4°C for 17 h. For determining the gel strength, the plunger of the Texture Analyzer was set to move a distance 4 mm into the gel with a speed of 0.5 mm/sec. The sample bottle was removed from the chill condition and immediately placed at the centre of the platform of the Texture Analyzer so that the plunger contacts the sample as nearly at its midpoint as possible and the measurement was taken. The value given by the Texture Analyzer was the gel strength (Bloom).
3.2.6.11 Determination of Odour (Muyonga et. al., 2004a).

Determination of odour by sensory evaluation was conducted using a seven member panel. Samples for sensory evaluation were prepared by dissolving 0.5 g of gelatin in 7 ml of distilled water, to obtain a solution containing approximately 6.67% gelatin. The samples were prepared in screw cap test tubes with and dissolved as described for the Bloom samples in Section 3.2.6.10. The samples were then held in a water bath at 50° C, with the screw caps lightly closed. Panelists were instructed to remove the screw caps, sniff the contents and identify the odour they perceived as well as indicate the odour intensity, using a five point scale (0 = no odour, 1 = very mild and only perceivable on careful assessment, 2 = mild but easily perceivable, 3 = strong but not offensive, 4 = strong and offensive, 5 = very strong and very offensive, )

3.2.6.12 Texture Profile Analysis (Muyonga et. al., 2004a)

TPA was measured using a Lloyds Texture Analyzer (Lloyd Instruments, Model LRX Plus, U.K.). The samples for Texture Profile Analysis were prepared in the same method as described in Section 3.2.6.10 for Bloom determination. The gel samples were then poured into cylindrical plastic containers with a diameter of 30 mm and a height of 40 mm and stored in a chilled room at 9–10 °C for 17 h. Before testing, the samples were equilibrated to room temperature for 30 min. The samples were removed from the plastic moulds and sections (20 mm length) cut off and tested by imparting a 50% strain, double compression, using 50 mm diameter cylindrical probe. Pre-test, test and post-test speed were set at 1 mm/s and trigger force at 5 g. The Hardness, Springiness index, Cohesiveness, Chewiness, Gumminess, Fracture Force, Adhesiveness and stiffness were determined as described by Pye (1996). From the TPA curve the mechanical textural parameters were calculated. Hardness is defined as the peak force (unit: g) in the first cycle; cohesiveness is defined as the ratio of the positive force area during the second cycle divided by the positive force area in the first cycle (A2/A1, a dimensionless quantity); gumminess is defined as hardness X cohesiveness (the unit for gumminess: g). Texture Profile Analysis result was tabulated using Nexygen Software.
3.2.6.13 Determination of Moisture (Method 934.01: AOAC, 2000)

5-10 g sample was weighed into pre-weighed clean petri dish. Dishes were placed in a hot air oven at 105±1°C for 6 hours. Dishes were cooled in desiccators and weighed to a constant weight. Moisture loss was calculated as:

\[
\% \text{ Moisture} = \frac{\text{Loss in weight} \times 100}{\text{Weight of the sample}}
\]

3.2.6.14 Determination of Crude Protein (Method 954.01: AOAC, 2000)

0.3 to 0.5 g of the moisture free gelatin sample was transferred into a digestion flask of 50 ml capacity. A few glass beads, a pinch of digestion mixture (8 parts K₂SO₄ & 1 part CuSO₄) and 10 ml concentrated sulphuric acid were added to the flask. It was digested over a burner until the solution turns colorless. The digest was transferred quantitatively into a 100 ml standard flask and made up to the mark. The 2 ml of well-mixed made-up solution was transferred to the reaction chamber of the Micro-Kjeldahl distillation apparatus, 2 drops of phenolphthalein indicator and 40% sodium hydroxide were added till the indicator changes to pink. Distillation was done for 4 minutes and ammonia liberated was absorbed into 2% boric acid containing a drop of Tashiro’s indicator. The amount of ammonia liberated was determined by titration with N/50 sulphuric acid. Percentage Crude protein was determined as:

\[
\% \text{ Crude protein} = \text{nitrogen content} \times 5.4 \quad \text{(Nitrogen conversion factor as per Eastoe & Eastoe, 1952)}
\]

3.2.6.15 Determination of Crude Fat (Method 991.36: AOAC, 2000)

About 1-2 g of accurately weighed moisture free sample was taken in a thimble plugged with cotton and was extracted with petroleum ether (40-60°C BP) in a Soxhlet apparatus for about 10 hrs, at a condensation rate of 5-6 drops per min. Excess solvent was evaporated and the fat was dried at 100°C to a constant weight. The crude fat was determined as:

\[
\% \text{ Crude fat} = \frac{\text{Weight of fat} \times 100}{\text{Weight of the sample}}
\]
3.2.6.16 Determination of Ash Content (Method 942.05:AOAC, 2000)

About 2-3 g of the moisture free sample was transferred into a previously heated, cooled and weighed silica crucible. The sample was charred at low red heat. Then the crucible was placed in a muffle furnace at 550°C for about 6 hours until a white ash was obtained. Crucible was cooled in a desiccator and weighed. Ash content was calculated as

\[
\text{\% Ash} = \frac{\text{Weight of residue} \times 100}{\text{Weight of the sample}}
\]

3.2.6.17 Estimation of Arsenic, Lead, Copper, Zinc, Cadmium and Chromium using Atomic Absorption Spectrophotometer (AOAC, 2000)

Reagents
1. Nitric acid
2. Perchloric acid
3. 1&2 mixed in 9:4 ratio
4. Stock solutions of Arsenic, Lead, Copper, Zinc, Cadmium and Chromium
   prepared by diluting concentrated solution of 1000 mg/L (SRL)

Procedure
1 g gelatin sample was used for the experiment. To the sample containing flask, 7 ml of nitric acid and perchloric acid (9:4) mixture was added, covered with a watch glass and left at room temperature overnight. The samples were then digested using a microwave digester (Anton Paar). The completely digested samples were allowed to cool at room temperature, filtered using glass and carefully transferred and made up into a clean 50 ml volumetric standard flask. The samples were analysed using Varian spectra AA 220, AAS equipped with deuterium background corrector, for the determination of Arsenic, Lead, Copper, Zinc, Cadmium and Chromium.

3.2.6.18 SDS-Polyacrylamide Gel Electrophoresis (SDS - PAGE)

Electrophoretic separation of gelatin proteins were separated by SDS-PAGE technique as described by Laemmli (1970). It is based on the principle that in the presence of 10% SDS and 2-mercaptoethanol, proteins dissociate into their sub units and bind large quantities of the detergent which mask the
charge of the proteins and giving a constant charge to mass ratio so that the proteins move according to their molecular weight in an electric field. In this discontinuous buffer system, the separating (resolving gels) and stacking gels are made up in the electrode buffer, Tris-glycine. During electrophoresis, the leading ion is chloride while the trailing ion is glycine. In this experiment, 7.5% gel concentration was used for the effective separation.

**Reagents**

1. Tris-HCl : 0.5M, pH 6.8
2. Tris-HCl : 1.5M, pH 8.8
3. SDS: 10%
4. Acrylamide /BIS: 30% T, 2.67% C
5. **Sample Buffer:**
   - **Distilled water:** 3.8 ml
   - **Tris-HCl :** 0.5M, pH 6.8, 1 ml
   - Glycerol: 0.8 ml
   - 10% SDS : 1.6 ml
   - 2-mercaptoethanol: 0.4 ml
   - 1% bromophenol blue: 0.4 ml
6. **Electrode Buffer:**
   - **Tris base:** 9g
   - **Glycine :** 43.2g
   - **SDS:** 3g

These reagents were dissolved in 600ml distilled water.

Working solution: Dilute 100ml from stock to 500ml with distilled water.

7. **Separating gel (7.5%)**:
   - 10% SDS : 100μl
   - Acrylamide :2.5ml
   - APS 10% : 50μl
   - **Distilled water:** 4.85ml
   - Tetramethylethylenediamine(TEMED) : 5 μl

Tris-HCl : 1.5M, 2.5ml

8. **Stacking Gel (4%)**
   - 10% SDS : 100μl
   - Acrylamide :1.33ml
APS 10% : 50µl
Distilled water:6.1ml
TEMED : 10 µl
Tris-HCl : 0.5M, 2.5ml

9. Ammonium per sulphate (APS): 10%

Procedure

The apparatus used was Bio-rad Tetra Mini Protean II unit (Bio-Rad Laboratories, Hercules, CA) with gel documentation system. Wide range sigma marker (mol. wt: 6500-200,00Da, S8445, Sigma Aldrich) was used in electrophoresis. Dry gelatin was dissolved in distilled water at 60°C to attain final concentration of 2 mg/ml of gelatine. 0.1 ml of the sample was taken in a micro centrifuge tube and added 0.1 ml of the sample buffer, heated in boiling water bath for 4 minutes, cooled and kept frozen pending analysis.

The separating gel was prepared without TEMED and APS. Degassed for 15 minutes to remove the air bubbles. Added TEMED and APS with intermittent shaking after each addition immediately transferred the solution to the apparatus. Added a little water on the top of the gel to level it and kept for 45 minutes. Prepared stacking gel after keeping the comb over the apparatus at 45°C, poured the gel slowly, and pressed the comb slowly and evenly. Marked the wells and kept for 45 minutes for setting. After removing the comb the whole apparatus was transferred to sandwich clamp assembly into the inner cooling core. Rinsed the apparatus and wells with electrode buffer and filled the inner chamber of the apparatus completely and the outer chamber to the optimum level. Injected 10 µl of the sample into the wells. The electrode lid was placed at proper position and connection was established. The power of 200V was supplied. Electrophoresis was carried out for 45 minutes approximately until the dye reaches the bottom. Subsequently, the gel was removed and was placed in a big petridish containing the stain Coomassie Brilliant Blue R250 to stain the protein bands. Kept for 30 minutes and transferred the gel to 7% acetic acid for destaining which was changed intermittently till complete destaining occurred.
3.2.6.19 Estimation of Amino Acids

Total amino acids in gelatin samples were determined as per the procedure of Ishida et. al., (1981).

Reagents

1. HCl : 6N
2. HCl : 0.05M
3. Buffer A: Dissolve tri sodium citrate (58.8g) in 2 L of double distilled water, add 210 ml ethanol of 99.5%, adjust the pH to 3.2 by adding 60% perchloric acid and make up to 3 L using double distilled water.
4. Buffer B: Dissolve tri sodium citrate, 58.8 g and boric acid, 12.4 g in double distilled water, adjust the pH to 10 by adding 4N NaOH, and make up the volume to 1L using double distilled water.
5. O-Phthalaldehyde (OPA) Buffer: Dissolve 122.1 g of Na₂CO₃, 40.7 g of H₃BO₃ and 56.4 g of K₂SO₄ in double distilled water and make up the volume to 3L.
6. O-Phthalaldehyde solution (OPA): Dissolve 400 mg OPA, 7 ml ethanol, 1 ml of 2-Mercaptoethanol and 2ml of 30% w/v Brij-35 in 500ml OPA buffer.
7. Sodium hypochlorite solution: 4% w/v Sodium hypochlorite in OPA buffer. i.e., 0.3ml Sodium hypochlorite in100ml OPA buffer.

Total amino acids

Principle

The amount of each amino acid present within a given protein does not vary from molecule and can provide useful information about the nature of the protein molecule. The sample was hydrolysed with 6N HCl at 1100 C (24h) so that the released amino acids can be assayed.

Sample preparation

100 mg gelatin sample was taken in a heat stable test tube; added 10ml 6N HCl and heat sealed the tube after filling with pure nitrogen gas. The
hydrolysis was carried out at 110°C for 24 hrs. After the hydrolysis, the contents were quantitatively transferred into a round bottom flask through Whatman filter paper. No 42 and washed the filter paper 2-3 times with distilled water. The contents were flash evaporated 2-3 times to remove traces of HCl. The residue was dissolved and made up to 10 ml with 0.05 M HCl.

**HPLC Analysis**

The sample was filtered through a membrane filter of 0.45 μm and inject 20 μl of this to an amino acid analyzer (HPLC- LC 10 AS) equipped with cation exchange column packed with a strongly acidic cation exchange resin i.e., styrene di vinyl benzene co polymer with sulphinic group. The column used was Na type i.e., ISC- 07/S 1504 Na having a length of 19 cm and diameter 5 mm.

The instrument was equipped with Shimadzu FL 6A fluorescence detector and Shimadzu CR 6A Chrompac recorder. The mobile phase of the system consists of two buffers, Buffer A and buffer B. A gradient system can be followed for the effective separation of amino acids. The oven temperature can be maintained at 60° C. The total run was programmed for 62 min. The amino acid analysis was carried out with non-switching flow method and fluorescence detection after post-column derivatization with o-phthalaldehyde. In the case of proline and hydroxyl proline, imino group is converted to amino group with hypochlorite. Amino acid standard (Sigma chemical Co., St. Louis, USA) was also run to calculate the concentration of amino acids in the sample.

**Quantification of amino acids**

The standard and the sample were analyzed under identical conditions. The elution time of the amino acids of the sample was compared and identified with those of the standard. Quantification of amino acid was done by comparing the respective peak areas in the chromatogram of the sample and the standard. The amino acid content was calculated as follows,

\[
\text{mg amino acid/gm tissue} = \frac{X \cdot \mu \text{mol} \cdot \text{mol wt} \cdot \text{volume made up} \cdot 1000 \cdot 100}{1000 \cdot 1000 \cdot 20 \cdot \text{wt. of sample}}
\]
The amount of each amino acid is expressed as mg amino acid/ gm tissue and mg amino acid/ ml serum.

3.2.6.20 Fourier Transform Infrared Spectroscopy (FTIR)

Spectral analysis of the fish skin gelatins were carried out as per the method described by Muyonga et.al., (2004b) using a Nicolet Avatar 360 ESP Infrared Spectrophotometer at a scanning range from 400 to 4000 cm\(^{-1}\) at data acquisition rate of 4cm cm\(^{-1}\) per point. FTIR spectra were obtained from discs containing 2 mg sample in approximately 100 mg potassium bromide (KBr). All spectra were obtained Background was subtracted using the Omnic software. Triplicate samples of gelatins were scanned for 32 times and the averaged spectrum was used for the analysis. The self deconvolution provided information on the number and location of components.

3.2.6.21 Methods for the Testing of Physical Properties of Gelatin Films.

Conditioning of the films

For characterization of functional properties the prepared films were conditioned at 22\(^{\circ}\) C and 58% of relative humidity in desiccators for 4 days.

3.2.6.21.1. Determination of Colour

Colour analysis of gelatin films was performed with a Hunter lab Miniscan \textsuperscript{®} XE plus spectrocolorimeter (Hunter Associates Laboratory, Inc. Reston, Virginia, USA). Measurements were recorded using the L* a* b* colour scale (CIE,1986). Six repetitions of the different colour parameters were recorded.

3.2.6.21.2. Determination of Thickness (IS: 1060-part l- 1966)

Thickness or Caliper is the perpendicular distance between the two principal surfaces of the gelatin film. Caliper of kraft liner for a particular grammage should be uniform across the sheet.

\textbf{Apparatus} : Micrometer Screw Gauge

\textbf{Report} : Corrected values of average, minimum and maximum obtained on each test specimen. The thickness of film is measured in mm or mils of points. (1 mil = 1 point = 1/1000 inch = 0.025mm).
3.2.6.21.3. Determination of Tensile Strength and Elongation at Break: (IS:2508-1984)

Tensile strength has been defined as the force parallel to the plane of the specimen required to produce failure in a specimen of specified width and length under specified condition of loading.

**Apparatus:**

Tensile Strength Machine

The machine used should be able to maintain a constant rate of traverse of one grip. The load scale should be accurate to within 1% or 0.1 N whichever is less. The load range should be such that the breaking load of the test pieces should fall between 15% and 85% of the full scale reading.

**Preparation of samples**

Samples were cut in lengthwise and crosswise direction, five numbers each with a minimum length 50 mm longer than the gauge length. The thickness was measured using a micrometer.

Gauge length of the Specimens: 50±1mm length x 15mm width

Traverse speed of machine: 500 mm/min.

The conditioned specimen was clamped between the grips of the machine. Machine was then switched on at the pre adjusted speed. The load and elongation at break were recorded.

**Calculation**

The tensile strength at break calculated in Kg/cm² from the original area of cross section. i.e., kgf/cross section area in cm². The mean of five results is expressed for the lengthwise and crosswise samples (MD and CD).

Cross Section area = width X thickness in cm.

Elongation at break is expressed as percentage of the original length between the reference lines. The mean value of the five results is expressed from MD & CD samples.

\[
\text{% ELB} = \frac{L_2-L_1 \times 100}{L_1}
\]

Where \( L_1 \) is the original length

and \( L_2 \) is the length at the time of break

**Breaking Length**

Breaking length (m) = \( \frac{\text{Tensile strength in kg} \times \text{Length of the strip in meters}}{\text{Wt. of strip in kg}} \)
3.2.6.21.4. Determination of Water Vapour Transmission Rate (ASTM 1989: E96-80)

This is an important property of the film under 3 mm thickness, to be considered in the selection of barrier materials for hygroscopic foods. It is measured as the quantity of water vapour in gms that will transmit from one side to the other of the film of an area of one sq. meter in 24 h. when the relative humidity difference between the two sides is maintained at 90±2% at 37°C.

**Apparatus:** Test Dishes

Shallow aluminium dishes of as large a diameter as a can was used. A wax seal between the test piece and the dish was given so as to prevent the transmission of water vapour at or through the edges of the sheet.

**Method**

WVTR was determined by sealing the open end of the dish containing the desiccant (fused Calcium chloride) by the test specimen and exposing the dish to the desired RH and temperature conditions. For standard test this condition is 37°C and 92% RH, when the desiccant used exerts 2% RH. Increase in weight of the desiccant after a known period of time gives the amount of water vapour transmitted by the specimen.

\[
WVTR = \frac{Q \times 24 \times 90}{A \times t \times (H_1 - H_2)} \text{ g/m}^2/24 \text{ h. at 90±2% RH & 37°C.}
\]

Where;

Q - Quantity of water vapour pass through the test material of area A m² for t hours when the relative humidity on either side maintained at H₁ and H₂.

3.2.6.21.5 Determination of Gas Transmission Rate: (ASTM 1987: D1434)

The permeability of films by gases is described as the volumetric rate of transmission of the gas, under known pressure differential, through a known area of film and is usually expressed as the transmission rate in ml per square meter per 24 hrs per atmosphere (ml/m²/24 hrs. atmos). The permeability of plastic materials to different gases is of considerable significance in many
applications. It can often be desirable to achieve a certain degree of permeability to certain gases, rather than to produce an entirely impermeable pack.

The phenomenon of gas permeability is dependent on the physical nature of the film, its density, degree of crystallinity and thickness and on the other the size and mobility of the gas molecules. The degree of polarity of both plastic materials and gas molecules as well as their tendency to be either hydrophobic or hydrophilic do influence the permeability of films with respect to particular gases.

Apparatus
Gas Permeability Apparatus (Davenport-designed in general accordance with B.S.2782, method 514A, Procedure 2 and ASTM D 1434)

Procedure
Unscrew the bolts holding down the upper half of the permeability cell and remove it. As supplied, the apparatus will have the ‘X’ volume controlling insert correctly fitted in the lower half of the cell. A dried circular filter paper (Whatmann No.1) is placed on the top of the insert and the sample of film spread over the filter paper. The upper part of the film permeability cell is then replaced. The bolts are then reinserted and tightened up with the box spanner.

The test gas is now turned on and the cell ‘flushed out’ with a brisk stream of gas for a few seconds, after the flow may be reduced to a slow rate, to ensure that no air can diffuse back in to the cell (1 bubble/second through liquid paraffin). The lower part of the cell is then evacuated (using vacuum pump capable of giving a vacuum at least as low as 0.2 mm Hg. A vacuum gauge also be connected between the apparatus and the vacuum pump-Tipping Mc Leod gauge) as rapid as possible and as soon as the gauge indicates that the pressure is 0.2 mm Hg or lower. The apparatus is tilted to the left until the mercury runs out of the reservoir into the manometer, partially filling it. Return the apparatus to the normal position and immediately set the movable scale to a convenient starting point, start a stopwatch and begin to take readings, at suitable time intervals.
Calculation

\[ GTR = 273 \times p \times V \times 24 \times 10^4 \]

\[ A \times T \times P \]

where,

- **GTR** = Gas transmission rate in ml/m²/24 hrs at 1 atmosphere pressure difference.
- **p** = Rate of pressure change in capillary in cm Hg per hour.
- **V** = Total volume in ml of the space between the lower surface of the film and the top of the mercury column in the capillary.

This total volume expressed as,

- (a) The volume of cell cavity (i.e. 5, 10, 15 or 20)
- (b) The volume of capillary tube above the mercury level half way through test; as the area of cross section of the capillary is 0.018 cm², this volume will be 0.018 \( X \), when \( X \) is the length of the capillary above the mercury at the half way point in cm.
- (c) The 'free space' volume of filter paper - can be taken as 0.24 ml.

\[ A = \text{Area of the specimen} - 23.77 \text{cm}^2 \]

\[ T = \text{Temp. in °K (273+°C)} \]

\[ P = \text{Pressure difference = 1 atmosphere (76 cm Hg)} \]

\[ \text{i.e., } 273 \times p \times V \times 24 \times 10^4 \]

\[ 23.77 \times 76 \times (273+°C) \]

3.2.7. Microbiological Analyses

3.2.7.1 Total Aerobic Count at 30°C (**AFNOR, 1982: NFV 59-101**)

**Reagents and culture media**

Diluent: Dissolve in 1000 ml of distilled water Sodium monohydrogen phosphate (Na₂HPO₄, 12 H₂O) 9.0 g and Potassium dihydrogen phosphate (KH₂PO₄) 1.5 g. Adjust the pH so that the final value after sterilization will be 7.0 ± 0.1 at 25°C, dispense 180 ml in flasks, sterilize at 121°C for 20 min, store at 4°C.

Plate count agar: Dissolve in 1000 ml of distilled water: Tryptone 5.0 g, Yeast extract 2.5 g, Dextrose 1.0 g, Agar 15.0 g. Adjust the pH so that the final value after sterilization will be 7.0 ± 0.2, dispense 15 ml in tubes, sterilize at 121°C for 20 min, store for one month maximum at 4°C. Before use, regenerate for 20 min in boiling water bath, then cool to 45°C.
**Procedure**

Preparation of 1/10 test solution (S1): Under aseptic conditions, weigh out 20 g of gelatin, transfer to 180 ml of diluent, and shake to disperse. Leave the gelatin to absorb the diluent for 1 hour at room temperature, then place the flask in the 45°C water bath and shake gently to assure dissolution (maximum 1 hour) and obtain the test solution (S1).

Inoculation: Transfer 1 ml of (S1) in each of two sterile Petri dishes and add the content of a media tube. Homogenise and leave to cool on a flat surface and incubated at 30°C for 72 ± 3 h.

Result: After incubation, count the colonies in each plate. The arithmetic average of the counts were carried out and multiplied by 10 (inverse of the dilution factor of (S1) solution).

3.2.7.2 Coliforms (30°C), (AFNOR, 1982: NFV 59-102).

**Reagents and culture media**

Diluent: Dissolve in 1000 ml of distilled water Sodium monohydrogen phosphate (9 g) & Potassium dihydrogen phosphate (1.5 g). Adjust the pH to 7.0 ± 0.1 at 25°C, dispense 180 ml in flasks, sterilize at 121°C for 20 min, store at 4°C. Ingredients of Selective liquid culture medium is given in Table 3.9.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Lactose</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Sodium glutamate</td>
<td>6.35 g</td>
</tr>
<tr>
<td>Sodium formate</td>
<td>0.25 g</td>
</tr>
<tr>
<td>L-cystine</td>
<td>0.02 g</td>
</tr>
<tr>
<td>L (-) aspartic acid</td>
<td>0.024 g</td>
</tr>
<tr>
<td>L (+) arginine</td>
<td>0.02 g</td>
</tr>
<tr>
<td>Potassium monohydrogen phosphate</td>
<td>0.90 g</td>
</tr>
<tr>
<td>Bromocresol purple</td>
<td>0.01 g</td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.001 g</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.001 g</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>0.001 g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.10 g</td>
</tr>
<tr>
<td>Ammonium ferric citrate</td>
<td>0.01 g</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.01 g</td>
</tr>
<tr>
<td>Ammonium chloride</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Adjust the pH to 6.7 ± 0.1 at 25°C. Dispense 50 ml in flasks containing Durham tube. Sterilise at 116°C for 10 min, store for one month maximum at 4°C.

**Procedure**

Under aseptic conditions, weigh out 20 g of gelatin, transfer it in 180 ml of diluent, and shake to disperse. Leave the gelatin to absorb the diluent for 1
hour at room temperature, then place the flask in the 45°C water bath and shake gently to assure dissolution (maximum 1 hour) and obtain the test solution (S1).

**Inoculation**

Transfer 10 ml of (S1) in a flask containing 50 ml of the liquid culture medium. Mix the inoculum carefully into the culture medium, avoiding introduction of air into the Durham tube and incubated at 30°C and for 48 ± 2 hours.

**Result**

After the incubation period, the flasks were examined to detect the presence/absence of gas in the Durham tube. The presence of gas is always accompanied by an abundant culture of micro-organisms forming cloudiness and/or yellowing of the medium, which indicates the presence of at least one "coli" in the quantity of gelatin inoculated and the result is given as: Presence of 30°C developing coliforms in 1 g of gelatin.

3.2.7.3 Coliforms (44.5°C), (AFNOR, 1982: NFV 59-103).

**Reagents and culture media**

Diluent: Dissolve in 1000 ml of distilled water Sodium monohydrogen phosphate (9 g) and Potassium dihydrogen phosphate (1.5 g). Adjust the pH to 7.0 ± 0.1 at 25°C, dispense 180 ml in flasks, sterilize at 121°C for 20 min, store at 4°C. Ingredients for Selective liquid culture medium (double strength): lactose - sodium glutamate-ammonium chloride (LGA) is given in Table 3.10.

**Table 3.10 Ingredients for Selective liquid culture medium-LGA**

<table>
<thead>
<tr>
<th>Lactose : 20.0 g</th>
<th>Sodium glutamate : 12.70 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium formate : 0.50 g</td>
<td>L-cystine : 0.04 g</td>
</tr>
<tr>
<td>L (-) aspartic acid : 0.048 g</td>
<td>L (+) arginine : 0.04 g</td>
</tr>
<tr>
<td>Thiamine : 0.002 g</td>
<td>Nicotinic acid : 0.002 g</td>
</tr>
<tr>
<td>Pantothentic acid : 0.002 g</td>
<td>Magnesium sulphate : 0.20 g</td>
</tr>
<tr>
<td>Ammonium ferric citrate : 0.02 g</td>
<td>Calcium chloride : 0.02 g</td>
</tr>
<tr>
<td>Potassium monohydrogen phosphate : 1.80 g</td>
<td>Bromocresol purple : 0.02 g</td>
</tr>
<tr>
<td>Ammonium chloride : 5.0 g</td>
<td>Distilled water : 1000 ml</td>
</tr>
</tbody>
</table>

Adjust the pH to 6.7 ± 0.1 at 25°C, dispense 100 ml in flasks containing Durham tube, sterilise at 116°C for 10 min, store at 4°C.
**Procedure**

Preparation of the 1/10 test solution (S1): Under aseptic conditions, weigh out 20 g of gelatin, transfer it in 180 ml of diluent, and shake to disperse. Leave the gelatin to absorb the diluent for 1 hour at room temperature, then place the flask in the 45°C water bath and shake gently to assure dissolution (maximum 1 hour) and obtain the test solution (S1).

*Inoculation*: Transfer 100 ml of (S1) in a flask containing 100 ml of the double strength liquid culture medium. Mix the inoculum carefully into the culture medium, avoiding introduction of air into the Durham tube and incubated at 44.5°C for 48 ± 2 hours.

**Result**

After the incubation period, examine the flask to detect the presence/absence of gas in the Durham tube. The presence of gas is always accompanied by an abundant culture of micro-organisms forming cloudiness and/or yellowing of the medium, which indicates the presence of at least one "coliform" in the quantity of gelatin inoculated and the result is given as: Presence of 44.5°C developing coliforms in 10 g of gelatin.

**3.2.7.4 Sulphite-Reducing Anaerobic Spores (37°C), (AFNOR, 1982: NFV 59-106).**

**Reagents and culture media**

Diluent: Dissolve in 1000 ml of distilled water Sodium monohydrogen phosphate (9 g) and Potassium dihydrogen phosphate (1.5g). Adjust the pH so that the final value after sterilisation will be 7.0 ± 0.1 at 25°C, dispense 180 ml in flasks, sterilize at 121°C for 20 min, store at 4°C. Ingredients for Beef extract-yeast extract-sulphite-iron agar is given in Table 3.11.

**Table 3.11 Ingredients for Beef extract-yeast extract-sulphite-iron agar**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>6.0 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Ammonium ferric citrate</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Cysteine hydrochloride</td>
<td>0.3 g</td>
</tr>
<tr>
<td>Soluble starch</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Sodium metabisulphite</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>12.0 g</td>
</tr>
</tbody>
</table>
Adjust the pH to 7.6 ± 0.2 at 25°C, dispense 20 ml in tubes, sterilise at 115°C for 30 min, store for 1 week only at 4°C. Before use, regenerate for 20 min in boiling water bath, then cool to 60°C.

**Procedure**

Preparation of the 1/10 test solution (S1): Under aseptic conditions, weigh out 20 g of gelatin, transfer it in 180 ml of diluent, and shake to disperse. Leave the gelatin to absorb the diluent for 1 hour at room temperature, then place the flask in the 45°C water bath and shake gently to assure dissolution (maximum 1 hour) and obtain the test solution (S1).

Treatment of the test solution: Pour 25 ml of (S1) in a tube and place in water bath to pasteurise at 80°C for 10 min and cool rapidly in 45°C water bath.

**Inoculation**

Transfer 5 ml of the pasteurised solution (6.2) into each of 2 tubes of culture medium at 60°C prepared and regenerated as previously described. Mix carefully the inoculum with the medium using circular movements without letting any air into the culture medium and incubated the tubes for 72 ± 3 hours at 37°C.

**Result**

Verify the absence of gas production and proteolytic action in the culture medium, then, count the black halo surrounded colonies which are present in the 2 tubes, and report.

**3.2.7.5 Clostridium perfringens Spores (AFNOR, 1982: NFV 59-107).**

**Reagents and culture media**

Diluent: Dissolve in 1000 ml of distilled water Sodium monohydrogen phosphate (9 g) and Potassium dihydrogen phosphate (1.5 g). Adjust the pH to 7.0 ± 0.1 at 25°C, dispense 180 ml in flasks, sterilise at 121°C for 20 min, store for one month maximum at 4°C. The ingredients of Enrichment broth (Thioglycolate resazurine double strength medium) is given in Table 3.12.
Table 3.12 Ingredients for Enrichment broth

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>30.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>11.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Sodium thioglycolate</td>
<td>1.0 g</td>
</tr>
<tr>
<td>L-cystine</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Resazurine</td>
<td>0.002 g</td>
</tr>
<tr>
<td>Agar</td>
<td>1.0 g</td>
</tr>
</tbody>
</table>

Adjust the pH to 7.1±0.2 at 25°C. Dispense 10 ml in tubes, sterilise at 121°C for 15 min, store at 4°C in the dark. Before use regenerate for 5 min in a boiling water bath (10 min if pink colour on more than 1/3 high), then cool to 45°C. Ingredients for lactose-sulphite specific medium (LS medium) is given in Table 3.13.

Table 3.13 ingredients for LS medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsic casein peptone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Lactose</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Cystein hydrochloride</td>
<td>0.3 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Adjust the pH to 7.1±0.1 at 25°C, dispense 8 ml in tubes containing Durham tubes, sterilise at 121°C for 15 min, store at 4°C. Before use, regenerate 5 min in a boiling water bath, then cool to 45°C; prepare separately the two following aqueous solutions:

Sodium bisulphite 12.0 g/l
Ferric ammonium citrate 10.0 g/l

The complete LS medium, prepared immediately before use, contains: Basic medium regenerated 8.0 ml, sulphite solution 0.5 ml and ferric solution 0.5 ml.

Procedure

Preparation of the 1/10 test solution (S1): Under aseptic conditions, weigh out 20 g of gelatin, transfer it in 180 ml of diluent, and shake to disperse. Leave the gelatin to absorb the diluent for 1 hour at room temperature, then place the flask in the 45°C water bath and shake gently to assure dissolution (maximum 1 hour) and obtain the test solution (S1).
**Enrichment inoculation**: Transfer 10 ml of (S1) in a tube containing 10 ml of the double strength enrichment broth described previously. Mix slowly and place in water bath to pasteurize at 80°C for 10 min, then cool quickly to 45°C.

**Enrichment incubation**: Cover the broth with a melted paraffin layer, and after solidifying, place the tube in the 46°C regulated water bath. Incubate for 24 hours (and eventually 48 hours).

**LS broth inoculation**: Subculture the enrichment tube that exhibits production of gas under the paraffin after 24 hours (or eventually 48 hours). Perforate the paraffin and transfer 1 ml into a tube of complete LS medium. Incubate in the 46°C water bath for 24 hours.

**Result**

After the specified LS incubation period, the simultaneous presence of gas in the Durham tube and of a black iron sulphide precipitate indicate the presence of at least one Clostridium perfringens spore in the amount of seeded gelatin. The result is given as: Presence of Clostridium perfringens spores in 1 g of gelatin.


**Reagents and culture media**

Diluent: Dissolve in 1000 ml of distilled water Sodium monohydrogen phosphate (9 g) and Potassium dihydrogen phosphate (1.5 g). Adjust the pH so that the final value after sterilisation will be 7.0 ± 0.1 at 25°C, dispense 180 ml in flasks, sterilize at 121°C for 20 min, store at 4°C. The ingredients for Selective liquid enrichment broth - highly salted lactose broth is given in Table 3.14.

**Table 3.14 Ingredients for Selective liquid enrichment broth**

<table>
<thead>
<tr>
<th>Beef extract</th>
<th>3.0 g</th>
<th>Lactose</th>
<th>7.5 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10.0 g</td>
<td>Sodium chloride</td>
<td>75.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>0.5 g</td>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Adjust the pH to 7.4 ± 0.1 at 25°C, dispense 190 ml in flasks, sterilise at 121°C for 20 min, store for 1 month maximum at 4°C.

Isolation medium - Baird-Parker medium
1) Base medium: In 1000 ml of distilled water dissolve, Tryptone (10 g), Yeast extract (1 g), Beef extract (5 g), Glycine (12 g), Lithium chloride (5 g), Sulphamezathine solution (27.5 ml), Agar (15 g) and adjust the pH to 7.2 ± 0.2 at 25°C, dispense 90 ml in flasks, sterilise at 121°C for 2 min, store at 4°C.
2) Sulphamezathine solution: In 100 ml of distilled water dissolve Sulphamezathine (0.2 g) & 10.0 ml (0.1 M) Sodium hydroxide solution. Store for 1 month maximum at 4°C.
3) Potassium tellurite solution: In 100 ml of distilled water dissolve 1.0 g Potassium tellurite and sterilise by filtration, store for 1 month maximum at 25°C.
4) Sodium pyruvate solution: in 100 ml of distilled water dissolve 20 g Sodium pyruvate and sterilise by filtration, store for 1 month maximum at 25°C.
5) Egg yolk emulsion: commercial preparation - 20 %.
6) Complete Baird-Parker medium
   Base medium (1) 90 ml
   Potassium tellurite solution (3) 1 ml
   Sodium pyruvate solution (4) 5 ml
   Egg yolk emulsion (5) 5 ml
Melt the base medium, then cool to about 50°C in a water bath, add successively the solutions, with effective mixing after each addition, and cool at 45°C in a water bath, pour 15-20 ml of the complete medium into sterile Petri dishes and allow to solidify. Dry the surface of the medium, cover removed an dishes turned downwards, in a 50°C regulated oven for 30 min, store the dishes for 24 hours maximum at 4°C.
Coagulase test
1) Brain-heart broth: In 1000 ml distilled water dissolve, Peptone (10 g), Calf brain extract (12.5 g), Beef heart extract (5 g), Dextrose (2 g), Sodium chloride (5 g), Sodium monohydrogen phosphate (2.5 g). Adjust the pH to 7.4 ± 0.2 at 25°C, dispense 10 ml in tubes, sterilize at 121°C for 20 min, store for 1 month maximum at 4°C.
2) Rabbit plasma: rehydrated commercially available rabbit plasma.

Procedure

Preparation of the 1/10 test solution (S1)
Under aseptic conditions, weigh out 20 g of gelatin, transfer it in 180 ml of diluent, and shake to disperse. Leave the gelatin to absorb the diluent for 1 hour at room temperature, then place the flask in the 45°C water bath and shake gently to assure dissolution (maximum 1 hour) and obtain the test solution (S1). Inoculation
Transfer 10 ml of (S1) in a flask containing 190 ml of the selective liquid enrichment broth. Mix the inoculum carefully into the culture medium and incubated at 37°C and for 48 ± 2 hours.

Isolation
After incubation transfer a loopful of the flask (6.3.) and streak on the surface of a dish of isolation medium. Return the dish and incubate at 37°C for 24-48 hours. From plate examine for the presence of characteristic colonies black, shining and convex, 1-1.5 mm diameter after 24h and 1.5-2.5 mm diameter after 48 hours incubation, surrounded by a clear partially opaque zone. Select at random five suspect-colonies, pick each with a sterile inoculating needle and inoculate tubes of brain-heart broth. After incubation for 24 hours at 37°C add 0.1 ml of the culture in each tube to 0.3 ml of rabbit plasma. Incubate for 4-6 hours at 37°C and examine the tubes for plasma coagulation. Coagulase reaction is positive if a large quantity of coagulum is formed (about 3/4 of the volume). Check negative reaction with 0.1 ml of sterile brain-heart broth.

Result
The result is given as: Presence/absence of S. aureus in 1 g of gelatin

3.2.7.7 Salmonella (AFNOR, 1982: NFV 59-104).
Reagents and culture media

Pre-enrichment medium - Buffered peptone water Dissolve in 1000 ml of distilled water: Peptone 10.0 g Sodium chloride 5.0 g Sodium monohydrogen phosphate 9.0 g Potassium dihydrogen phosphate 1.5 g Adjust the pH so that the final value after sterilisation will be 7.0 ± 0.1 at 25°C, dispense 225 ml in flasks, sterilise at 121°C for 20 min, store at 4°C.

Enrichment media-
(i) Rapaport-Vassiliadis broth - green malachite-magnesium chloride broth
Solution A: In 1000 ml of distilled water dissolve: Tryptone (5 g) Sodium chloride (8 g) Potassium dihydrogen phosphate (1.6 g)
Solution B: 400.0 g Magnesium chloride dissolved in 1000 ml of distilled water.
Solution C: 0.4 g Green malachite oxalate dissolved in 100 ml of distilled water. Complete medium (RV 10 formula): Take Solution A 1000 ml, Solution B 100 ml and Solution C 10 ml and adjust the pH to 5.2 ± 0.1 at 25°C, dispense 10 ml in tubes, sterilise at 115°C for 15 min, store at 4°C.

(ii) Selenite-cystine broth
Base medium: In 1000 ml of distilled water dissolve Tryptone (5g), Lactose (4 g), Sodium monohydrogen phosphate (10 g), Sodium monohydrogen selenite (4 g).
Dissolve the first three ingredients in water and boil for 5 min, cool and add the selenite salt.
Cystine solution: In 100 ml sterile distilled water in a sterile flask dissolve L-cystine 0.1 g 1 mol/l sodium hydroxide solution 15.0 ml. Do not sterilise.
Complete medium: Under sterile condition, add cystine solution (10 ml), to the cooled base medium (1000 ml) and adjust the pH to 7.0 ± 0.2 at 25°C, dispense 20 ml in sterile tubes.

Isolation media:
Phenol red-brilliant green-agar (PRBG agar): In 1000 ml of distilled water dissolve, Tryptone (10 g) Beef extract (5 g) Yeast extract (3 g) Lactose (10 g) Dextrose (10 g) Sodium hydrogen phosphate (1.0 g) Sodium dihydrogen phosphate (0.6 g) Phenol red (0.09 g) Brilliant green (0.005 g) Agar 14.0g. Adjust the pH to 6.9 ± 0.2 at 25°C, boil gently to dissolve the components, do not sterilise, cool to 45°C. Dispense 15 ml in Petri dishes, cool to room temperature and dry the plates in the oven before use, store 24 hours maximum at 4°C.

Procedure
Pre-enrichment
Weigh 25 g of gelatin and transfer into 225 ml of pre-enrichment medium, and shake to disperse. Allow to stand for 1 hour at room temperature. Then place the flask in the 45°C water bath and shake gently to assure the dissolution is
complete and incubate at 37°C for at least 16 hours but not more than 20 hours.

*Enrichment*

Seed 10 ml of Rapaport-Vassiliadis broth with 0.1 ml of the culture (6.1), and incubate at 42°C for 24 to 48 hours. Seed 20 ml of selenite broth with 2 ml of the culture and incubate at 37°C for 24 to 48 hours. 6.3. Isolation After 24 h, and after 48 h, transfer a loopful of the two flasks and streak on the surface of the PRBG agar and of the optional agar. Return the plates and incubate at 37°C for 24 to 48 hours.

*Confirmation*

Characteristic colony was submitted to confirmatory analysis, by means of purification sub-culture, biochemical identification, and serological confirmation.

*Result*

The result is given as Presence/absence of salmonella in 25 g of gelatin.

3.3. Statistical Analysis

Results are expressed as mean or mean log ± SD for biochemical & microbiological parameters. Statistical analysis between the means using ANOVA and Dunken’s’ multiple test were carried out to test the significance of variance. Statistical package used in the study is SPSS, 10.