III. MATERIALS AND METHODS

3.1. Materials

Fresh ribbonfish (Trichiurus spp.) and bull’s eye fish (Priacanthus spp.) caught by trawl net along the coast off Mangalore, India, were used for the study. Immediately after harvest, fish were washed in fresh water and iced in the ratio of 1:1 (fish: ice) and transported to the laboratory. The whole fish was washed in chilled water prior to dressing. Head and entrails were removed manually and washed with chilled water. Separated meat from these fishes was used for the preparation of raw material for extruded products.

Rice flour, wheat semolina and ragi flour were procured from the local market for extrusion process.

3.1.1. Chemicals

All the chemicals and reagents used in the present study were either AR or GR grade. Sulfuric acid, hydrochloric acid, citric acid, acetic acid (glacial), acetone, petroleum ether, boric acid were procured from Qualigens fine chemicals, India. Sodium acetate trihydrate, (Mallinckrodt), Triethylamine (TEA – Waters Part Number 88121 or equivalent), Glacial acetic acid (J.T. Baker), Acetonitrile (Burdick and Jackson), Water (Milli-Q quality or equivalent), Disodium hydrogen phosphate (Na₂HPO₄, Mallinckrodt), Phosphoric acid (J.T. Baker), Methanol, Sodium Acetate (IM), derivatising agent (PTIC), Amberlite mixed resin were obtained from Fischer chemicals UK. Disodium hydrogen phosphate, sodium dihydrogen phosphate, copper sulphate, potassium sulphate, sodium chloride, ammonium per sulphate, ammonium sulphate, sodium hydroxide and selenium dioxide were procured from E.Merck (India) Limited, India. Bromophenol blue, methyl red, methylene blue, and glycerol were procured from Ranbaxy laboratories Limited, India. Sodium Dodecyl sulphate, TEMED, acrylamide, bis-acrylamide (N, N'-methylene-bis-acrylamide), 2-mercapto ethanol, Trizma base (tris [hydroxyl methyl] amino methane), Coomassie brilliant blue and standard markers were obtained from Sigma chemical Co., USA.

3.1.2. Equipments

The equipments used in the present investigation include a twin-screw extruder, Differential scanning calorimeter, Raman spectrometer, HPLC amino acid analyzer,
Varian gas chromatograph, Texture analyzer, Colorimeter, Controlled Stress Rheometer, Phase contrast microscope, Vacuum flash evaporator, Hot air oven, Soxhlet apparatus and other analytical equipments.

### 3.1.2.1. Extruder

The extruder used for the present study is a twin-screw extruder (Basic Technologies, Pvt., Ltd., Calcutta, India) (Fig. 2) with co-rotating three start screws (Fig. 3). The three-start screw of the extruder was 29.7 mm in diameter and 350 mm long. The pitch of the screw was 75 mm with a flight depth of 3.5 mm.

The drive system of the extruder is provided with ABB Frequency Converter to control the RPM precisely according to the need of process. Since this type of drive generates torque gradually, a bypass switch (installed at the side of control desk) is provided to directly apply the drive from motor, which gives the sudden application of torque necessary to clean the barrel from burnt-out products, by the “inching device”. The main drive is provided with a 5 HP Motor (400 V 3 Ph. 50 Hz). The out-put shaft of worm reduction gear is provided with a Torque limiter coupling. This device consists of a Torque limiter and a roller chain type coupling. The torque limiter is a protective device that limits torque transmitted by the out-put shaft of worm reduction gear. The torque limiter utilizes ‘spring loaded friction surface’, when there is overload, the friction surface slips and some smoke may come out if there is any oil contamination.

The out-put of the coupling rotates the input shaft of Duplex gear which has two out-put shafts to rotate two screws inside the barrel in co-rotating fashion. The barrel of the extruder receives the feed from a co-rotating feeder (variable speed). The knob of the controller controls the rated capacity of the feeder. The barrel is provided with two electric and band-heaters and two water cooling jackets. A temperature sensor is fitted on the front die plate, which is connected to temperature controller placed on the panel board. The second band heater is controlled by temperature controller (SUNVIC, UK).

The die is fixed by a screwed nut tightened by a special wrench provided. The automatic cutting knife is fixed on a rotating shaft of knife drive assembly. The cutter is driven by a variable speed DC motor, which is controlled by a knob placed on the panel board. The Automatic cutter assembly is covered by a removable hinged guard.
Fig. 2: The full assembly of twin-screw extruder used for the study
Fig. 3 A: Barrel of the extruder with screw exposed
B: Dismantled screw of the extruder
3.2. Methods

3.2.1. Preparation of extruded products

3.2.1.1. Raw material preparation

Extruded products were prepared using a blend of rice flour/wheat semolina/ragi flour and fish mince (Ribbonfish/Bull’s eye fish). The initial moisture content of rice flour, wheat semolina and ragi flour was 5.5%, 7.5% and 11.37% respectively. The flours were mixed with fish meat at 10% and 20% (w/w) level. The final moisture content of the mixture with 10% (w/w) of fish mince was adjusted to 15% by addition of water. With the addition of 20% fish mince moisture will be slightly above 15% (16.5%). Appropriate controls were prepared wherein, no fish mince was added and the moisture content was kept at 15% by addition of water.

Ribbonfish and bull’s eye fish mince with moisture content of about 78.0% and 78.8% respectively were mixed with rice flour, wheat semolina and ragi flour separately and the final moisture content was adjusted to 15%. The blend was then mixed with sodium chloride at 2.5% (w/w) level. The mixture was thoroughly mixed and then sieved through a specific mesh to get uniform particle size. The sieved mixture was then kept for equilibration time of 30 minutes. After equilibration, once again the sample was sieved and kept ready for extrusion. The fish mince-flour mixture was used to prepare extruded products as a function of barrel temperature, screw speed and die diameter. Feeding rate of flour - fish mince mixture was adjusted to 120 g/minute (7.2 Kg/hr).

3.2.1.2. Extrusion process

The scheme used for the production of extruded is given in Fig. 4.

*Extruded products as a function of barrel temperature*

Extruded products from flour (rice flour, wheat semolina and ragi flour) with added fish (ribbonfish/bull’s eye fish) at various levels (10% and 20%) were prepared as a function of barrel temperature. Barrel temperature at the expansion section was maintained at 70°, 90° and 120°C respectively. Screw speed was maintained at a speed of 350 RPM.
DETAILS OF RAW MATERIALS USED FOR EXTRUSION

Flours used: Rice flour, Wheat semolina and Ragi flour (Finger millet)

Fish used: Ribbonfish (*Trichiurus* spp.) and Bull’s eye fish (*Priacanthus* spp.)

Percentage of fish mince added to flours/semolina: 10% and 20%
Control: Without fish mince

2.5% Sodium chloride added to all samples

Moisture content of the final mixture: 15% (for products with 10% fish mince and 16.5% (for products with 20% fish mince)

EXTRUSION VARIABLE DETAILS

Barrel temperatures used: 70°C, 90°C and 120°C

Screw speed used: 300 RPM, 350 RPM, 400 RPM and 500 RPM

Die diameter used: 3 mm, 4 mm and 5 mm

Fig. 4: Schematic representation of extrusion variables and raw materials used for extrusion using twin-screw extruder
and the die diameter used was 4 mm. At 120°C, only samples with 10% level fish mince has been carried out.

*Extruded products as a function of screw speed*

In order to find the effect of screw speed on the quality of extruded products, products were prepared from the three flour materials with 10% ribbonfish mince. Extrusion was carried out at 300, 400 and 500 RPM at a constant barrel temperature of 90°C and die diameter of 4 mm.

*Extruded products as a function of die diameter*

Extruded products were prepared from a mixture of flour (rice flour, wheat semolina and ragi flour) and ribbonfish mince (10% w/w) using different die diameters. The die diameters used were 3 mm, 4 mm and 5 mm. Extrusion was carried out at a constant barrel temperature of 90°C and screw speed was maintained at 350 RPM.

### 3.2.2. Gelatin preparation

Fresh skins of bull’s eye fish were used for gelatin preparation following the method of Gudmundsson and Hafsteinsson (1997). Skin was separated from fish flesh and cleaned, washed several times with tap water and was dried in between two pieces of Whatman filter paper. Dried clean skins were cut into 2 x 2 cm pieces. Sodium hydroxide solution 1000 ml (1.5 g NaOH in 1000 ml distilled water) was added to 140 g of skins, shaken well and slowly stirred at 20°C for 40 minutes. The skin pieces were separated and rinsed with water. This procedure was repeated three times. Next the skins were treated, in the same way, but with 1000 ml sulphuric acid solution (1.5 ml H₂SO₄ in 1000 ml distilled water) and rinsed with water. This procedure was also repeated three times. Finally the skins were treated with 1000 ml citric acid solution (7 g citric acid in 1000 ml distilled water) with subsequent rinsing; this was also repeated three times. Fish skin was then extracted with distilled water in a 45°C water bath overnight (17 hrs) without stirring. The mixture was filtered using Buchner funnel with Whatman paper No. 4. The resultant filtrate was evaporated in a rotary evaporator at 45°C to reduce the volume to 1/10th the original volume.

To 25ml of # 5% gelatin solution 1g of amberlite mixed resin was added and stirred well. The mixture was heated at 65°C, for 30 minutes with constant stirring. The
conductivity of the solution was measured using a conductivity meter (Suntex Conductivity Meter SC-170, Taiwan). If the measured conductivity was found to be above 50µS/cm, further 1 g of resin was added and heated at 65°C for another 30 minutes, with constant stirring. The process was repeated till the measured conductivity value was below 50µS/cm. The solution was decanted and filtered through Whatman paper No. 4. The pH of the filtrate was measured at 30°C and adjusted to pH to 6.0 by using 0.1M sulphuric acid.

### 3.2.3. Proximate composition

Proximate composition of fresh fish (ribbonfish/Bull’s eye) was determined. Meat was separated and macerated well prior to proximate analysis. Proximate composition of the rice flour, wheat semolina and ragi flour were also determined (AOAC, 1995).

Moisture, protein, fat and ash content of extruded products and bull’s eye fish’s skin were estimated by the method as described in AOAC (1995). Moisture and protein content of gelatin was also estimated.

**Moisture**

Moisture content of the samples was determined according to the method of AOAC (1995). A known quantity (about 5g) of the sample was taken in moisture bottle, dried in hot air oven at 105°C for 10-12 hours and cooled in desiccator to a constant weight. The weight loss during this drying process is expressed as the moisture per 100g of sample.

**Total Nitrogen**

Total nitrogen was estimated by Kjeldahl method according to the method of AOAC (1995). About 1 g of sample was digested with 10ml concentrated sulphuric acid and a pinch of digestion mixture (Potassium sulphate: copper sulphate: selenium dioxide in the ratio of 100:10:2.5) until the digest becomes clear or colorless. After cooling the volume was made up to 100ml with distilled water. From this solution, 2 ml was taken for distillation along with 15ml of 40% sodium hydroxide in Kjeldahl distillation apparatus. The ammonia liberated during distillation process was absorbed in 2% boric acid containing mixed indicator [methyl red (0.6%): methylene blue (0.6%):: 2:1] and titrated against standard N/140 hydrochloric acid.
The nitrogen equivalence of standard hydrochloric acid was determined using ammonium sulphate solution containing 1 mg nitrogen/ml. The standard ammonium sulphate solution was distilled using Kjeldahl apparatus. The distillation was carried out and the amount of hydrochloric acid consumed to titrate was noted. From this, equivalence of hydrochloric acid for 1 mg nitrogen was calculated. One ml of N/140 HCl was equivalent to 0.1169 mg nitrogen. Crude protein content was determined by multiplying total nitrogen by a factor of 6.25. In case of crude protein content of gelatin the multiplying factor was 5.5.

**Crude fat**

Crude fat was determined using Soxhlet apparatus according to the procedure of AOAC (1995). About 1 g of sample was taken in a thimble and placed in a Soxhlet extraction unit using petroleum ether (60-80°C) as solvent. The extraction was carried out for 16 hours. The solvent with dissolved fat in the receiver was evaporated and the residual solvent was removed by keeping in hot air oven at 60°C and cooled to a constant weight. The crude fat content was expressed as percentage.

**Ash**

Ash content of the sample was determined by method as described in AOAC (1995). About 1 g of moisture free sample was taken in a pre-weighed silica crucible. Preliminary ashing was done by slow heating on flame to allow smoking off fat without burning. Once the smoke stopped evolving from the sample, it was incinerated in a muffle furnace (SUNVIC, UK) at 500°C±10°C for 5 hours. The crucibles were removed and cooled in desiccator and weighed. Ash content was calculated from the weight difference of crucible and expressed as percentage on wet weight basis.

**3.2.4. Aminoacid composition**

Aminoacid composition of two selected fish species (0.2 g), and the gelatin extracted from bull’s eye fish skin was determined after derivatization with phenylisothiocyanate (PITC) according to the Waters Pico-Tag method as described by Bidlingmeyer *et al.* (1984) using the Waters Pico-Tag HPLC amino acid analyzer (Water Model 712 WISP, Waters, Watford, Herts., UK). Aminoacid composition of the extruded rice flour - ribbonfish / bull’s eye fish mince (added at a level of 10% and 20% w/w)
mixture processed at a barrel temperature of 90°C, was also determined. Weight of the sample taken for aminoacid analysis was 0.1 g of extruded product.

Sample (0.1/0.2 g) was weighed into screw cap pyrex vials, washed with 6N HCl, rinsed with deionised water and dried. 10 ml of 6 N HCl was added into the vials and flushed with nitrogen and quickly capped. Vials were then kept inside oven at 110°C for 24 hours. To 20µl of the sample/ Standard, 10µl drying solution (200µl Methanol, 200µl Sodium Acetate (IM), 100µl Triethylamine) were added and vortexed and dried. Derivatisation was carried out by adding 20µl derivatising agent (20µl PITC, 140µl Methanol, 20µl Triethylamine, 20µl Water) and dried for 10 minutes at ambient temperature. Further drying was continued by adding 10µl of methanol and vortexing. Reconstitution of dried material was carried out in 100µl eluent A (Sodium Acetate Trihydrate; pH = 6.4 (940 ml) + 60 ml Acetonitrile) and injected to the column. Column used was Nova-Pak C-18, 3.9 x 150 mm column, Waters. Flow rate was maintained at 1-1.5 ml/min and concentrations of eluted fractions were monitored at a wavelength of 254 nm.

3.2.5. Fatty acid composition

Fatty acid composition of fat extracted from ribbonfish and bull’s eye fish meat were determined. Transesterification was performed according to Schmarr et al. (1996). The prepared fatty acid methyl esters (1 µl) were then injected into a Varian gas chromatograph, Series 3600, with a hydrogen flame ionization detector using helium as a carrier gas. An initial temperature of 180°C for 4 minutes was raised to 250°C at a rate of 4°C/minute. Standard reference fatty acids were also chromatographed to identify the individual fatty acids.

3.2.6. Differential Scanning Calorimetry

The thermodynamic parameters of the samples were examined using a DSC VII calorimeter (Setaram, Lyon, France). The calorimeter include a detector micro DSC VII (-10° upto 120°C), a microprocessor for calorimeter operation and control of the gas and vacuum circuit, a gas circuit to protect, a Pentium 75 (TM) microcomputer, a multitasking and multimodulus software under Windows (TM) and a printer. To avoid steam condensation in the calorimeter wall especially at low temperature, a constant sweeping of inert gas (nitrogen) is used. The water circulation ensures the evacuation or supply of heat
to the two thermostatic walls. The calorimetric block of the Micro DSC VII is composed of metallic cylinder with a high thermal conductivity. Two blocks machined cavities take the measurement and reference experimental vessels. The vessels take 2 plates on the top as thermal buffers for a set of cylindrical covers. A peltier effect thermo element is placed between the block and the chamber to evacuate heat toward the intermediary chamber. The standard batch vessel is composed of a cylinder of 6.4 mm of internal diameter and useful height equivalent to 19.5 mm for the sample. The useful volume for the sample is equal to 1 cm$^3$.

The thermodynamic parameters of the fishes (Ribbonfish, Bull’s eye fish) and the flours (rice, wheat semolina, ragi) were examined using a DSC VII calorimeter (Setaram, Lyon, France). Flour materials were mixed with water to get a 10% (w/v) solution and this is loaded to the sample container. Water was kept as reference for all the samples. Heating rate was 0.5$^\circ$C/min from 10$^\circ$C to 90$^\circ$C. Heat absorbed or released by the sample results in either endothermic or exothermic peaks as function of temperature. For fish meat, the temperature reached when half of the sample is denatured is referred to as the transition temperature and was measured at the tip of the peak ($T_m$). The energy required to denature the sample, the enthalpy change ($\Delta H$), was measured by integrating the area under the peak using the Setaram DSC software. The onset temperature ($T_o$) and peak gelatinization temperature ($T_m$) of the flour samples were measured from the endothermic curve in the thermogram. The energy required for gelatinization of flours was measured by integrating the peak area using the software.

### 3.2.7. FT-Raman spectroscopy

The Perkin Elmer Fourier-Transform (FT) near infrared (NIR) Raman spectrometer (System 2000) is a system that can collect both Raman and NIR spectra, using different sample chambers. The front-center chamber is used for the NIR path. The rear left chamber is attached to a continuous wave Nd.YAG (Neodymium depedyttrium aluminium garnet) laser operating at 1.064 $\mu$m (9398.5 cm$^{-1}$). Within the chamber, the laser beam emerges through an aperture and is steered by a mini prism attached to the collecting lens onto the sample. The beam comes to a gentle focus (c.a.100 $\mu$m) about 50 mm from the prism. The scattered light is collected (180$^\circ$ back scattering) by the collecting lens and Raman components passes through set of low-pass filters into the spectrometer that
produces an interferogram, detected on an InGaAs detector. The spectrometer is controlled by PC using a proprietary PE software package (Spectrum-V3).

Frozen fish (Ribbonfish and Bull’s eye fish) samples were thawed and the meat was taken in 7 ml glass containers (FBG-Anchor, Cricklewood, London) on a Perkin-Elmer system 2000 FT-Raman spectrophotometer with excitation from a Nd:YAG laser at 1064nm. Starchy materials (rice, wheat and ragi) were also analysed both in native and gelatinized state using Raman spectrophotometer. Frequency calibration of the instrument was carried out using the sulfur line at 217 cm$^{-1}$. Triplicate analysis was carried out. The relative intensity has been calculated as the mean of the three trials. Laser power was set at 1500 mW for the fish sample and 800 mW for the starchy samples. The spectra were an average of 64 scans which was baseline corrected and smoothed. Gelatinization of the starchy materials has been carried out by heating the sample at 80°C for 30 minutes. For gelatinized samples the spectra were an average of 128 scans. The spectra of fish were normalized to the intensity of the phenylalanine band at 1004 cm$^{-1}$ (Howell and Li-Chan, 1996) and the spectra of the starchy materials were normalized to the intensity of the band at 480 cm$^{-1}$ (Dupuy and Laureyns, 2002). The recorded spectra were analysed using Grams 32 (Galactic Industries Corp., Salem, NH) software. Assignments of the bands were done based on the literature (Schuster et al., 2000; Howell and Li-Chan, 1996; Li-Chan et al., 1994; Careche et al., 1999) (Table 1).

3.2.8. Phase contrast microscopy

Changes in the physical structure of flour samples before and after gelatinization has been studied using Transmitted light microscope – Leitze (Leitz-laborlux D) with eyepiece power of 10x and objective (160/0.17, PLAN, 40/0.65, Phaco 2) power of 40x. Gelatinization of the starchy materials has been carried out by heating the sample at 80°C for 30 minutes. The samples were placed on acetone cleaned microscope slides and covered with a cover slip to prevent dehydration. The slides were then viewed under the microscope. The microscope was attached to a Wild MPS 05 system comprising a camera and an exposure meter set.
Table 1. Peak assignment for starch samples

<table>
<thead>
<tr>
<th>Wavenumbers cm(^{-1})</th>
<th>Assignments of peaks</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>426</td>
<td>Skeletal modes of carbohydrate rings (C-C-O)</td>
<td>Schuster \textit{et al.}, 2000</td>
</tr>
<tr>
<td>480</td>
<td>Skeletal modes</td>
<td>Schuster \textit{et al.}, 2000</td>
</tr>
<tr>
<td>Below 800</td>
<td>C-C and C-O deformations and skeletal breathing modes</td>
<td>Vandenabeele \textit{et al.}, 2000</td>
</tr>
<tr>
<td>947</td>
<td>α-1,4- glycosidic linkage</td>
<td>Schuster \textit{et al.}, 2000</td>
</tr>
<tr>
<td>950-800</td>
<td>C-O-C sugar ring vibration</td>
<td>Vandenabeele \textit{et al.}, 2000</td>
</tr>
<tr>
<td>1057</td>
<td>(C-C) + (C-O-H) modes</td>
<td>Schuster \textit{et al.}, 2000</td>
</tr>
<tr>
<td>1082</td>
<td>(C-O-H) modes</td>
<td>Schuster \textit{et al.}, 2000</td>
</tr>
<tr>
<td>1128</td>
<td>(C-O) and (C-O-H) modes</td>
<td>Schuster \textit{et al.}, 2000</td>
</tr>
<tr>
<td>1200-950</td>
<td>C-C and C-O symmetric stretching</td>
<td>Vandenabeele \textit{et al.}, 2000</td>
</tr>
<tr>
<td>1370-1380</td>
<td>(CH(_2)) scissoring</td>
<td>Schuster \textit{et al.}, 2000</td>
</tr>
<tr>
<td>1633</td>
<td>Water</td>
<td>Schuster \textit{et al.}, 2000</td>
</tr>
<tr>
<td>2908</td>
<td>(C-H)</td>
<td>Schuster \textit{et al.}, 2000</td>
</tr>
<tr>
<td>3213</td>
<td>(O-H)</td>
<td>Schuster \textit{et al.}, 2000</td>
</tr>
</tbody>
</table>
3.2.9. Rheological analysis

Small deformation test of flour/semolina solutions

Dynamic viscoelastic behaviour (DVB) of solutions (10% w/v) of rice flour, wheat semolina and ragi flour in the temperature range of 20°-90°C and cooling back to 20°C was measured using a Rheometrics Constant Stress 200 rheometer under oscillatory mode with a 4 cm parallel plate geometry. 10% solutions of the flour samples were used for DVB measurement and the sample was surrounded by silicone oil to prevent evaporation of solvent. The gap between measuring geometry and Peltier plate was adjusted to 0.3 mm. The applied stresses for rice flour, wheat semolina and ragi flour were 0.13 Pa, 1.8 Pa and 0.15 Pa respectively. Applied stress was compared with the resultant strain. The results of such measurement were expressed as the storage modulus (G') and loss modulus (G'’).

Protein-polysaccharide interaction

In order to study protein-polysaccharide interaction, Dynamic viscoelastic behaviour (DVB) of flour-ribbonfish mixture has been carried out as a function of time period at different temperatures (60°C, 70°C and 80°C) using a Carri Med Controlled Stress Rheometer (CSR-500, Carri-Med, Surrey, UK.) under oscillatory mode. The proteins from fresh ribbonfish meat were extracted using extraction buffer (EB) and the concentration of protein used for the study was 10 mg/ml. Flour samples (rice flour, wheat semolina and ragi flour) were mixed with protein solution and the concentration of flour used was 20% (w/v). The measuring geometry used was 2 cm parallel plate. The gap between measuring geometry and peltier plate was adjusted to 200 μm. The gap was set manually at 70°C using the micrometer provided with the system. Time sweep was carried out for 30 minutes at 60°C, 70°C and 80°C. The applied stress of 20 Pa was within the viscoelastic region. The linear viscoelastic region was determined by a torque sweep with a frequency of 1 Hz. Measurements were made by applying a small amplitude oscillation (0.0005 Rad) with a frequency of 1 Hz. Applied stress was compared with the resultant strain. The results of such measurement were expressed as the storage modulus (G’) and loss modulus (G’’). An average of three replicates was used for plotting the results.

Flow behaviour of gelatin from bull’s eye skin (Shear Stress Sweep)

The flow properties of gelatin were measured as a function of temperature using Controlled Stress Rheometer (CSR Carri-Med model CSL 500, Dorking, Surrey, UK) with
flow software. Gelatin solutions of different concentrations were analyzed as a function of temperature. Shear stress sweep of gelatin solution (10 mg/ml) has been carried out at 28°C, 40°C and 50°C. Gelatin solutions (15 mg/ml and 30 mg/ml) were analyzed at 25°C, 40°C, 50°C and 60°C. At a concentration of 30 mg/ml shear stress sweep has been carried out at 10°C. The sample was equilibrated for 5 min before the shearing experiment was started. The measuring geometry used was 4 cm cone and plate with truncation of 59 μm. The range of stress applied varied between 2 to 5 Pa depending on the angular velocity in the pre-shear experiment. The ascent and descent time were 2 min each. Shear stress sweep of the gelatin solutions were performed in triplicate and average values was taken for plotting. A flow curve was obtained by plotting log viscosity and log shear rate.

3.2.10. Expansion ratio of extruded products

Expansion ratio of the extruded products was determined as the ratio of the diameter of the extruded product to die diameter and expressed as percentage.

\[ \text{Expansion ratio} = \frac{\text{Diameter of the extruded sample}}{\text{Die diameter}} \times 100 \]

3.2.11. Water absorption capacity (WAC) of extruded products

Water absorption capacity of extruded products was determined by the method of Sousulski (1962). About 0.3 g of extruded sample was taken in a pre-weighed dried centrifugation tube. After weighing the sample with tube, 5 ml of water was added and mixed by using vortex mixer. The sample was kept for 30 minutes for the absorption of water and then centrifuged at 7000 x g for 10 minutes, so that excess water can get released from sample. The released water was decanted by inverting the tubes at an angle of 45° for 30 minutes at 50°C. The tubes were weighed again. The water absorption capacity was expressed as gram of water absorbed per gram of dried material. The average of three values was reported as WAC of dried material.

3.2.12. Color analysis

Color of the extruded products was determined using a Hunter Lab (Hunter Lab, Mini Scan XE Plus, Sunset Hills Road, Reston, Virginia, USA), color-measuring instrument. Extruded products were powdered using a mixer. The powder was then sieved through a sieve having mesh size of 250 μm. The particles passed through this mesh size
were collected and allowed to sieve through another sieve with a mesh size of 150 μm. The particles, which are retained in this second sieve, are collected for color measurement. A uniform thick layer of the powder is taken in the sample holder of the colorimeter and values corresponding to L*, a* and b* were measured. Measurement has been carried out in triplicate and average value was taken for plotting.

3.2.13. Texture analysis

Crispness

Texture analysis of extruded products from different flours (rice, wheat and ragi) and fish mince (ribbonfish/Bull’s eye fish) has been carried out using the Stable Microsystems TA-XT2 texture analyzer (Stable Microsystems, Godalming, UK). After a trigger force of 10g was attained the probe then proceeded to penetrate into the extruded product to a depth of 15mm. At this depth the maximum force reading (the resistance to penetration) was obtained and translated as the fracturibility of the extruded product.

Stable Microsystems TA XT2 texture analyzer setting.

- Mode : Measure Force in Compression
- Option : Return to start
- Pre-test speed : 1.0 mm/s
- Test speed : 2.0 mm/s
- Post test speed : 10.0 mm/s
- Distance : 15 mm
- Trigger type : Auto – 10g
- Data Acquisition rate : 500pps

5 mm diameter cylinder stainless probe (P5), Using 25Kg load cell

Breaking strength

The breaking strength of the extruded products was measured using Lloyd texture analyzer (Ametek, Lloyd instruments Ltd., Ametek Inc., Model LRX Plus, Hampshire, UK) attached with a Warner-Bratzler shear attachment. Instrument was set up to ‘Compression to limit’ mode, with a pre-load of 2 N and test speed of 500 mm/minute. The stiffness of the force required to break the product was measured. The measurement was carried out 10 times for each sample to get consistent results. The measurements were
recorded in a computer with NEXYGEN, Ver. 4.1, software and breaking strength was expressed as Newton.

### 3.2.14. Determination of bloom strength of gelatin gels

Bloom value was determined using a TA-XT2 Texture analyzer (Stable Microsystems, Godalming, UK) according to the method described by Stable Micro System. Gelatin (5 g) was placed in cold water to make 5% w/v solution, stirred with a glass rod, covered and allowed to stand at room temperature (not more than 22°C) for 3 hrs. After this time, the mixture was heated in a water bath at 60°C (but not exceeding) and stirred on a magnetic stirrer for 15 min to dissolve the gelatin completely. Gelatin solution (150 ml) was immediately poured into standard bloom jar (SCHOTTGLAS. Mainz. Bloom test vessel. Product No. 2112501) over which a cover was placed. After 2 min bloom jars were kept in the cold room (4°C) overnight (17 hrs), and immediately tested using Stable Microsystems TA-XT2 texture analyzer. The bloom jar was placed centrally under the standard probe and the penetration test was commenced. After a trigger force of 4 g was attained the probe proceeded to penetrate into the gel to a depth of 4mm. At this depth, the maximum force reading (the resistance to penetration) was obtained and translated as the Bloom strength (g) of the gel.

**TA-XT2 Setting**

- **Mode**: Measure force in compression
- **Option**: Return to start
- **Pre-test speed**: 0.5 mm/s
- **Test speed**: 0.5 mm/s
- **Post test speed**: 0.5 mm/s
- **Distance**: 4 mm
- **Trigger type**: Auto-4g
- **Data Acquisition rate**: 200 pps
- **0.5 Radius cylinder (P/0.5R) using 5 Kg load cell.**

The method described corresponds to the British standard method for sampling and testing gelatins (BSI 757, 1975).
3.2.15. Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) of purified gelatin was carried out under reduced condition according to the method as described by Laemmli (1970). Electrophoresis was carried out using polyacrylamide gel slabs of 10x8 cm (length x width) in a vertical slab gel electrophoresis apparatus (Model SE 260; Hoefer Pharmacia Biotech Inc., USA). A discontinuous gel of acrylamide concentration T% = 10 and C% = 25 was used. For polymerization of the gel, TEMED (N, N', N', N'-tetramethylene diamine), used as the initiator and APS (ammonium persulphate) as catalyst. The gels were cast in a duel gel caster and thickness of the gel was 0.75 mm and the number of wells in each slab was 10.

One gram of gelatin was mixed with 3 ml of treatment buffer (Tris-HCl, pH 7.0, 10% SDS, 20% glycerol, 0.2% 2-mercaptoethanol and 0.02% bromophenol blue) and macerated. Then the samples were heated on a boiling water bath for two minutes, cooled and centrifuged at 8000 rpm for five minutes to get a clear supernatant. Supernatant was stored in vials and kept at −20°C till electrophoresis was carried out.

Two microlitre of the clear solution was loaded into the wells of the gel. Run was carried out on a constant current mode using an electrophoresis power pack (Model PS-3000; Hoefer Pharmacia Biotech Inc., USA). A constant current of two milliampere per well of the gel was applied during the run. Run was terminated when the dye touched the bottom of gel. A standard sigma marker of wide range molecular weight was loaded into separate wells of the gel. After completing the run, gel was stained in comassie brilliant blue R-250 (0.025% in 40% methanol and 7% acetic acid) overnight. Gels were destained using acetic acid- methanol mixture (7% acetic acid and 2% methanol) repeatedly till protein bands were clearly visible. Molecular weight of the protein bands obtained in the sample was approximated by measuring the relative mobility of the standard molecular weight markers.

3.2.16. Setting index of gelatin

Different concentration of gelatin viz., 10, 20, 30 and 40 mg/ml was prepared in test tube and kept at 5°C for different durations. At periodic intervals visual observation was made for solidification process. The solidification process was given index depending on the extent of solidification. A maximum index of ‘100’ was taken as complete solid and ‘0’ as complete liquid. A plot of time in minutes and setting index was obtained.
3.2.17. Statistical analysis

The results obtained are the mean of three trials and the standard deviation has been found out. Two way analysis of variance technique was used in order to ascertain, whether there is any significant difference in the quality attributes of extruded products prepared with and without fish meat or between the samples prepared at different extrusion variables like barrel temperature, screw speed and die diameter.