EXPERIMENTAL
Chapter-3

1. INTRODUCTION

Important feature in food processing industry is the selection of appropriate machines, right ingredients, suitable process conditions and authentic quality test methods. In order to achieve the required parameters various study methods are discussed. Ground and steam conditioned soybean material was extruded in the extruder and process conditions monitored. Extruded soybean was subjected to quality tests to find out the levels of trypsin inhibitors and urease activity before utilizing in pet food formulations and it is detailed as follows.

I. Soybean Extrusion
II. Test Methods for Extruded soybean
III. Pet Food Formulations
IV. Shelf-life studies for Pet Food
V. Pet food Digestibility studies:

I. SOYBEAN EXTRUSION:

A. Pre extrusion process:

The pre-extrusion processing of the raw material involved 3 main steps: grinding, blending and moisturizing. The raw soybeans were ground through 4 mm screen in hammer mill with 20 HP. The ground material was transferred through a screw conveyor to the Twin screw steam conditioner for preconditioning of the material. Steam at 2 kg/cm² pressure was injected onto the soybean powder and the twin paddles in steam conditioner at 200 rpm helped in mixing of the material for 2 minutes. During preconditioning, the raw granular soybean powder was uniformly blended, moistened with live steam. The moisture and temperature of the soyabean powder was maintained at 12-14% and 60-70°C respectively through out the conditioning process.
i. Extruder:

Extruder Instapro 2000R was equipped with a single screw of 20mm pitch, grooved barrel of 150 mm ID and divided into three sets of barrels, feed, compression and die-set. Extruder barrels were not provided with any external heating facility.

ii. Soybean extrusion Process:

Preliminary extrusion trials were run to select the extruder conditions and reduce the number of variables. Extrusion conditions were maintained at constant screw speed of 500 rpm and feeder rate of 150 rpm and extruder motor load at 70-75 amps. After barrels were setup, the preconditioned soybean material was conveyed to the extruder and extruded at different temperatures in the range of 60°C –140°C. The material was extruded without injecting water into the extruder barrel. As the heat was generated by friction during the passage of the material through the barrels and screws, the built-in temperature was recorded. Temperature of the first, second and third barrel sections varied between 40-70, 70-100, 100-140°C respectively. The extruded samples were collected when the extrusion running conditions reached steady state as indicated by constant product temperature at the end barrel. After extrusion, total six extruded soybean samples were collected in polythene covers for each experimental design. Samples were collected directly from the ‘extruder cutter head’ at 60 - 70°C and 100 -140°C at 10 increments of temperature. Each collected sample was kept separately in polythene covers for cooling. After cooling, all samples were stored in cardboard boxes at ambient temperature for estimation of antinutritional factors.
II. TEST METHODS FOR EXTRUDED SOYBEAN:

Each extruded soybean sample was subjected for tests on antinutritional factors such as Trypsin Inhibitors and Urease activity. The inactivation of trypsin inhibitors and urease activity was evaluated at different processing conditions.

A. Urease Activity

Urease, an enzyme present in soybean hydrolyses urea to ammonia and other compounds, which are toxic to the animals. Some experiments were conducted to study the effects of extrusion temperature and moisture content on urease activity. The determination of urease activity of extruded soybean was primarily done to evaluate the cooking efficiency of such products. The term urease activity is defined as the quantity of ammonical nitrogen released in one minute under the specified operating conditions, being expressed as mg of nitrogen released per gram and referred to the product as it is. The raw and extruded soybean samples were subjected for urease activity estimation in order to study the extrusion conditions for inactivation of urease activity in extruded samples. The urease activity index values obtained by novel quick methods were compared with standard methods in order to understand the effect of extrusion on antinutritive factors.

i. ‘Novel quick test for Urease activity

a. Principle: The Urease present in the soybean sample releases ammonia from the urea (fig-01) when the urea is added to the soybean sample solution. The difference in pH after one minute between the control and treatment is measured as the Urease activity Index.

Figure-01:

Urease catalyzes the hydrolysis of urea:

$$(\text{NH}_2)_2\text{CO} + 3\text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{NH}_4\text{OH}$$
b. Materials:

i. Urea
ii. De-mineralised water
iii. A pH-meter [+ or- 0.02 pH]
iv. Magnetic stirrer
v. Glass beakers [100 ml capacity]

c. Procedure:

i. A known amount of sample (1 to 5g) was weighed and transferred into a beaker.
ii. Added 100 ml of water to the beaker stirred well to record the pH of the suspension.
iii. About 0.1 g of urea added into above solution and measured the pH.

d. Calculations:

i. Let the pH of the solution before adding urea = X.
ii. pH of the solution after adding urea at 1 min = Y.
iii. UREASE ACTIVITY INDEX = Y - X

ii. Urease activity by pH measurement using Phosphate Buffer.

a. Principle:

The urease present in the sample releases ammonia when urea phosphate buffer solution is added to the sample mixture. The difference in pH for one minute of sample and test is measured as the urease activity Index. The urease activity is determined by the quantity of ammonical nitrogen released by 1g of the product in 1min at a temp of 30°C from a urea buffer solution.

b. Materials:

i. 0.05M Phosphate Buffer solution: Dissolve 4.45 gram of di-potassium hydrogen phosphate and 3.403 gram of Potassium dihydrogen phosphate separately in 100 ml of DM water, mix and make up to 1000 ml with water. Adjust the pH of the solution to 7.0 by using either strong base or acid.
ii. Urea Phosphate buffer solution: Dissolve 15 gram urea in 500 ml of phosphate buffer (refer: 6.221) and adjust the pH to 7.0.

iii. Water bath capable of maintaining the temperature at 30 ± 0.5 degree centigrade.

iv. A pH Meter. [+ or- 0.02 pH]
v. Test tubes: 20 mm x 150 mm, fitted with glass stoppers.

c. Preparation of the Sample:

i. Sample was ground without raising the temperature and mixed well.

ii. Sieved through mesh No 40 U.S sieve and ensured that at least 60% of the sample was passed through the mesh.

d. Procedure:

i. To 0.200 gram of a test sample in a glass-stoppered test tube, added 10 ml of urea phosphate buffer, mixed and placed in the water bath at 30°C.

ii. Prepared a blank by taking 0.2 gram of the sample into a test tube and added 10 ml of phosphate buffer, mixed and placed on a water bath at 30°C.

iii. Allowed 5 min interval between the preparation of test and blank solutions.

iv. Agitated the contents of the tubes regularly at 5 min. intervals.

v. Removed the test and blank from the water bath after 30 minutes.

vi. Decanted the contents of the tubes into 10ml beakers and determined the pH at 5 minutes interval after removing from the water bath.

e. Calculations:

Urease activity = ‘X’ - ‘Y’

Where, X = pH of test sample

Y = pH of the blank sample
B. Measurement Of Antitryptic Activity\textsuperscript{1,2}:

i. Principle:

The enzymatic activity of trypsin results in smaller peptides, but this catalytic action gets reduced in presence of trypsin inhibitors. Trypsin inhibitors, if present in soybean sample interfere with the catalysis and slow down the enzymatic reactions of trypsin leading to reduction in the release of end products such as peptides thereby difference in wavelength.

ii. Procedure:

The casein at 2\%w/v solution was used as the substrate and the substrate concentration was kept constant for all the experimental trials. The soybean samples are extracted for protein and the concentrated protein sample was used in the experimental calculations. Trypsin inhibitory activity in soybean samples at different concentration was obtained from the trypsin standard curve.

iii. Expression of activity:

One trypsin unit (TU) is subjectively defined as an increase of 0.01 absorbance units at 280 \textit{nm} in 20 min. per 10 ml of reaction mixture under the set conditions. Trypsin inhibitor activity is defined as the number of trypsin units inhibited (TUI).

C. Determination of Lipase activity:

i. Principle:

Lipase (Triacylglycerol acylhydrolase, EC 3.1.1.3) hydrolyses emulsified triglycerides\textsuperscript{9} of the long-chain fatty acids. The site of the action of lipase is the interface between the oil drops and aqueous phase. Lipase is present in Soybean seeds but when the soybean powder is exposed to high temperature and pressure during extrusion, the lipase will get inactivated. When the lipase is inactivated it cannot involve in the catalytic reactions, there by not releasing free fatty acids and hence the change in pH is less or nil. One fatty acid is liberated by the catalytical action of lipase in each step.
The pH decreases as the fatty acid is liberated in each step. The difference in pH before and after the extrusion, gives an indication of activity of lipase in the test sample.

**ii. Procedure:**

i. Extruded soybean was ground through 1 mm screen and sieved the sample through #20 mesh.

ii. 5 gm of the sample was weighed and suspended in DM water.

iii. Stirred the suspension for 30 min.

iv. Measured the initial pH of the sample and allowed to stand for 24 hr at 37°C.

v. Measured the final pH and noted down the difference in pH from initial value.

vi. Same procedure was followed for raw soybean powder to use as control.

**Inference:**

The difference in pH is an indication of activity of lipase in the sample.

If the difference in pH is less, it indicates that enzyme lipase is not active.

**D. Determination of Acid value**

a. **Principle:**

The free fatty acids are liberated from the catalytic action of lipase, which is present in Soybean samples. Lipase gets inactivated by extrusion, and does not participate in catalytic action and hence does not liberate free fatty acids, which are measured as acid value. Acid value is the number, which expresses in milligrams, the amount of Potassium hydroxide necessary to neutralize the free acids present in the 1 gram of substance.
The acid value was measured in raw soybean samples and in the samples extruded at different temperature conditions ranging from 60°C to 140°C.

E. Determination of alpha amino acid residues

Amino acid analysis can be used to quantify protein and peptides, to determine the identity of proteins or peptides based on their amino acid composition, to support protein and peptide structure analysis, to evaluate fragmentation strategies for peptide mapping, and to detect atypical amino acids that might be present in a protein or peptide which are macromolecules consisting of covalently bonded amino acid residues organized as a linear polymer.

a. Materials and methods

i. Reagent A-Ninhydrin reagent
   - Disodium hydrogen orthophosphate-6.5g
   - Potassium dihydrogen phosphate-6.0g
   - fructose–0.3g
   - ninhydrin-0.5g

ii. Reagent B-Diluent:
   2 gram of Potassium iodate dissolved in 600ml of distilled water and then 400ml of ethanol is added. Store in a refrigerator

iii. Glycine stock solution (1mg/ml): Dissolve 107.2mg Glycine in 100ml distilled water. Store in a refrigerator.

iv. Glycine standard solution:
   1 ml of glycine stock solution is dissolved in 100mL of distilled water.

b. Procedure

i. Filtered the incubation mixture through whatmann no. 1 filter paper.

ii. Diluted the filtrate in 1:100 ratio with distilled water.

iii. 2 ml of distilled water was taken as blank.

iv. Pipetted out 2 ml of the diluted filtrate and 2 ml of glycine standard solution into separate test tubes in duplicates.
v. 1 ml of Ninhydrin reagent was added, stoppered the test tubes to prevent evaporation. Boiled in a water bath for 16 minutes

vi. Cooled at 20°C for 20 minutes and add 5 ml of diluent, incubate at room temperature for 30 minutes.

vii. Read the absorbance at 570 nm using distilled water as a blank.

c. Calculation:

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\text{AAN (mg/L)} = \frac{\text{Net absorbance of the sample} \times 2 \times \text{dilution factor}}{\text{Net absorbance of the glycine std.}}
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F. Proximate analysis for Pet food:

The fat coated pellets were subjected to proximate analysis such as moisture, crude protein, crude fat, crude fibre, calcium and phosphorous. Proximate analysis was planned to study the changes in nutritional profile, during extrusion in some of the major nutrients present in the ‘Pet food with extruded soyabean’. This analysis was done for the samples of ‘Pet food with extruded soyabean’ kept at accelerated conditions and for the samples kept at ambient temperature. The analysis included the pre-extruded samples, 45th day samples kept at accelerated condition and for the samples kept at ambient temperature for 365 days.
III. PET FOOD FORMULATIONS

Preparation of a formulation mixture included pulverizing the ingredients through 1mm screen, followed by sieving to remove larger particles. These process conditions helped the ingredients in maintaining closely identical particle size thus ensuring proper mixing and providing uniformity in pellet shape and colour during extrusion.

Different pet food formulations were done with extruded soybean and as well as with raw soyabean. The Formula with ‘extruded soybean’ was considered as ‘Test’ and the formula with the ‘raw soybean’ as ‘Control’. The formulation details are discussed in chapter –05.

A. Pet Food extrusion:

The blend of Pet foods containing ‘extruded soyabean’ and ‘raw soyabean’ were conveyed to the steam conditioner separately. Steam conditioned material was extruded as pellets. Extrusion was carried out at 100-120°C temperature and the extruded samples were collected directly into polythene covers and cooled to room temperature. After cooling, the pellets of pre-extrusion and post extrusion samples were subjected to moisture analysis in IR moisture balance at 60-70°C. Extruded material was conveyed to the drier through the conveyor and drying was done for the extruded pellets at 120-140°C to reduce the moisture levels to 10%. Pre drying and post drying operations were carried out at hygienic conditions. After drying, the pellets were cooled and coated with fat.

IV. SHELF-LIFE STUDIES FOR PET FOOD

‘Pet food with extruded soybean’ and ‘pet food with raw soybean’ were subjected to product shelf life studies. Neatly and tightly packed samples in polythene covers were kept in the stability chamber and at ambient temperature for stability studies. Shelf life studies were planned for determining the stability of the pet foods kept at atmospheric conditions 25 to 35°C temperature and 55 to 65% RH and at accelerated conditions ie., 40°C ± 1°C temperature and 60%± 1% RH. Analysis was carried out for Peroxide value, which was used as the criteria to find out the activity of lipase for determining the shelf life of the pet foods.
V. PET FOOD DIGESTIBILITY STUDIES

The digestibility study\textsuperscript{14} for ‘pet food with extruded soyabean’ was conducted on ten dogs of different breeds and aged approximately 30 months for a period of ten days, comprising of acclimatization period of 5 days, collection period of five days. During the period of collection, the fecal output was weighed and approximately 50 grams of sample was collected and refrigerated for further analysis of crude protein, crude fibre, ether extract (crude fat), ash, nitrogen free extract and moisture by proximate method of analysis\textsuperscript{11} of feed ingredients. The metabolizable energy was calculated according to AAFCO\textsuperscript{11} standards.
References: