CHAPTER - II

MATERIALS AND METHODS
A. SURVEY

To determine the socio-economic conditions of the reelers engaged in muga reeling and to determine the quantum of availability of muga waste, the survey work is essential. As the present work is related to the utilization of waste muga pupae, which will indirectly benefit the muga reelers, it is necessary to have detailed information about socio-economic condition of these people, the amount of muga cocoon they use and the present utility, if any, of the waste muga pupae.

The present study was designed to conduct a sample survey at Sualkuchi during a phase of two years from 1991 to 1992. Sualkuchi is situated on the north bank of the river Brahmaputra, 40 km from Guwahati, and is the centre of muga silk industry of Assam. The area is constituted of 39 suburis (sub-locality) with a population of about 29,000. A total of 3429 families reside at Sualkuchi, out of which 319 families, selected at random, were directly interviewed and also asked to fill up a questionnaire supplied to them. The following information were gathered from them.

1. Number of families engaged in muga reeling.
2. Number of families, suburi wise, engaged in muga reeling.
3. Number of families from outside engaged in muga reeling.
4. Number of reelers, family wise, engaged in muga reeling.

5. Number of reelers undertaking reeling as (a) primary and (b) secondary occupation.

6. Number of muga cocoons purchased annually.

7. Number of muga cocoons reeled daily.

8. Total amount of pupae waste produced per day and its utilization, if any.

9. Total earning per family per year from muga reeling and from selling silk waste to spun silk mill.

The relevant data were then analyzed by applying standard statistical method (Mahajan, 1989).

Further, during the survey major traders of muga cocoons were interviewed to work out the total amount of muga cocoons used in Sualkuchi. It was then compared with the official figures of the State Sericulture Department.

B. BIOCHEMICAL ANALYSIS

For proper use of the muga waste it is essential to know its biochemical composition. Though waste muga silk worm pupae are available in large quantities in Assam (mainly at Sualkuchi), no information regarding its chemical composition is available, which is a prerequisite for its proper utilization. The present study is designed to evaluate the chemical composition of waste muga pupae.
Muga being a multivoltine species, produces six crops, namely, Chotia, Bohagi, Aherua, Bhodia, Jethua and Katia. Of these Jethua and Katia have reelability of 48% and 54% respectively (Sengupta and Ghose, 1991) and are considered as commercial crops. At Sualkuchi besides Jethua and Katia variety, Bohagi variety of corps are also reeled. During this study these three varieties were analyzed separately.

The pupae were collected from Sualkuchi, sun dried for three days and stored for analysis and utilization. Different constituents were analyzed by standard methods.

1. MOISTURE

The moisture of the dried pupae is estimated following the procedure described in AOAC (1984).

Process:

10 g of the pupae was accurately weighed in a crucible and dried in an oven at 100°C for 5 hours. The process of heating and cooling was repeated till a constant weight is achieved. The percentage of moisture (by mass) was calculated from the following relationship.

\[
\frac{\text{Initial mass} - \text{Final mass}}{\text{Mass of the sample}} \times 100
\]
2. ASH CONTENT

About 10 g of the sample was weighed accurately into a platinum crucible (which had previously been heated to 600°C and cooled). The crucible was placed on a clay pipe triangle and heated over a low flame till all the material was completely charred, followed by heating in a muffle furnace for about 5 hours at about 600°C. It was then cooled in a dessicator and weighed to ensure completion of ashing. The crucible was again heated in a muffle furnace for 30 minutes, cooled and weighed. This was continued till two consecutive weights were the same and the ash was almost white in colour (AOAC, 1984). The percentage of ash (by mass) was then calculated by applying the following relationship.

\[
\text{\% of Ash} = \frac{\text{Mass of ash}}{\text{Mass of the sample}} \times 100
\]

3. CRUDE FIBRE

Principle:

Crude fibre consists of mainly cellulose and lignin and some mineral matter. Crude fibre content is used as a measure of the nutritive value of poultry and livestock feeds. The estimation of crude fibre involves the treatment of fat free pupae first with boiling in sulphuric acid and then with boiling sodium hydroxide solution when
oxidative hydrolytic degradation of the native cellulose and lignin occurs. The residue obtained after final filtration is weighed, incinerated, cooled and weighed again. The loss in weight gives the crude fibre content. (Maynard, 1970).

**Reagents Required:**

1. **0.255 N Sulphuric acid**: 1.25 g of concentrated sulphuric acid (0.67 mL of concentrated sulphuric acid of specific gravity 1.86) was diluted to 100 mL with distilled water.

2. **0.313 N Sodium hydroxide solution**: 1.252 g of sodium hydroxide was dissolved in distilled water and the volume made up to 100 mL.

**Process:**

About 5 g of moisture and fat free (after soxhlet extraction) sample was accurately weighed and to it 200 mL of boiling 0.255 N sulphuric acid was added. The mixture was boiled for 30 minutes keeping the volume constant by adding water at frequent intervals. The mixture was filtered through a muslin cloth and residue was washed with hot water till free from acid. The residue was then treated with 200 mL of boiling 0.313 N sodium hydroxide solution for 30 minutes, keeping volume constant as before. The mixture was again filtered through a muslin cloth. The residue was washed with hot water till free from alkali, followed by washing with alcohol and ether. The residue was then taken into a crucible dried overnight.
80°C - 100°C and weighed \((W_1)\). The crucible was then heated for 2 hours at 600°C in a muffle furnace, cooled and weighed \((W_2)\). The mass percent of the crude fibre was then calculated using the following formula (Maynard, 1970).

\[
\% \text{ Crude fibre} = \frac{100 \times (W_1 - W_2)}{\text{Mass of moisture and fat free sample}}
\]

4. **PROTEIN**

*i) CRUDE PROTEIN* :

**Principle:**

Crude protein of muga waste pupae is evaluated by micro-kjeldahl method (Oser *et al.*, 1965). In the process the sample (about 100 mg) is digested with concentrated sulphuric acid to convert nitrogen in protein or any other organic matter to ammonium sulphate. The salt on steam distillation liberates ammonia which is collected in boric acid in the form of ammonium borate. It is finally tritrated against sulphuric acid to find out the percentage of nitrogen in the sample.
Reagents Required:

1. **Concentrated sulphuric acid**: Sulphuric acid of specific gravity 1.86.
2. **2% Boric acid solution**: 2 g boric acid dissolved in distilled water and the volume made up to 100 mL.
3. **0.01 N Sulphuric acid**: 0.49 g concentrated sulphuric acid (0.26 mL sulphuric acid of specific gravity 1.86) in 1 L solution.
4. **40% Sodium hydroxide solution**: 40 g of sodium hydroxide dissolved in water and volume made up to 100 mL.
5. **Bromocresol green indicator**: 0.1% solution in ethanol.

Process:

The accurately weighed sample (about 100 mg) was taken into micro-kjeldhal flask and digested on the micro-digestion unit after adding 2 mL concentrated sulphuric acid. The digestion was continued till the solution became clear. The digested sample was then subjected to steam distillation after adding about 8 mL 40% sodium hydroxide solution in a micro-kjeldhal apparatus and the distillate was collected in a 10 mL solution of 2% boric acid containing 2-3 drops of bromocresol green indicator. The distillation was continued till the receiving solution became about 30 mL.

The receiving solution was blue in colour. It was titrated against 0.01 N sulphuric acid till the original green colour was
obtained. A blank preparation of distilled water was also run similar to the sample.

The crude protein was calculated from the following relationship.

\[
\text{% Crude Protein} = \frac{1400 \times (\text{Titre} - \text{Blank})}{\text{Strength of H}_2\text{SO}_4 \times 6.25} \times \frac{1}{1000 \times \text{Amount taken}}
\]

* Since in most proteins, nitrogen constitutes 16% of the total makeup, the total nitrogen content of the sample is multiplied by a factor of 6.25 to arrive at the value of the crude protein.

ii) SOLUBLE PROTEIN:

Principle:

Soluble protein is estimated by the procedure of Lowry et al. (1951). A blue colour is developed by the reduction of Folin-Ciocalteau reagent by the amino acids of the protein and by the biuret reaction of the protein with the copper reagent. The amount of soluble protein is estimated by spectrophotometric method by measuring the colour intensity at 755 nm using uv-vis spectrophotometer 118 (Systronics).

Reagents required:

1. Alkaline copper reagent: A mixture of 50 mL of solution ‘A’ containing 2% sodium carbonate in 0.1 N sodium hydroxide and 1
mL of solution 'B' containing 0.5% copper sulphate (CuSO<sub>4</sub>·5H<sub>2</sub>O) in 1% sodium potassium tartrate.

2. **Folin—Ciocalteau reagent**: 1 volume of the reagent (Qualigens fine Chemicals) was diluted with 2 volumes of distilled water.

3. **1 N Sodium hydroxide solution**: 4 g of sodium hydroxide dissolved in distilled water and volume made up to 100 mL.

4. **0.1 N Sodium hydroxide solution**: Solution (3) was diluted 10 times.

5. **Trichloroacetic acid**: 1 g of trichloroacetic acid was dissolved in water and volume made up to 10 mL.

6. **Standard protein solution**: The standard solution is prepared with bovine serum albumin to have a concentration of 100 μg of protein per mL.

**Process**:

The accurately weighed sample (about 100 mg) was subjected to lipid extraction in soxhlet apparatus. The dried lipid extracted powder was soaked in 1.0 N NaOH (10 mL) and homogenized. The homogenate was incubated at 37°C for an hour and centrifuged. The centrifugate was diluted to make a solution of soluble protein in 0.1 N NaOH. To 2 mL of centrifugate 6 mL 10% trichloroacetic acid was added to precipitate the protein. The precipitated protein was collected by centrifugation and again dissolved in 1 mL of 1N NaOH solution. It was then diluted to 100 mL. To 1 mL of this solution 5 mL of
alkaline copper reagent was added, mixed well and allowed to stand. After 10 minutes 0.5 mL of Folin-Ciocalteau phenol reagent was added, mixed immediately by shaking and kept for 30 minutes. The blue colour was read against a 0.1 N NaOH blank at 755 nm and by comparing with standard calibration curve the amount of soluble protein was calculated.

5. TOTAL FREE AMINO ACIDS

Principle:

Total free amino acid is estimated following the procedure described by Balasubramanium and Sadasivam (1987). Ninhydrin decarboxylates and oxidatively deaminates most alpha amino acids and its reduced product hydrindantin produces intense purple colour with ninhydrin, that is measured spectrophotometrically at 570 nm.

Reagents required:

1. **80% Methanol solution**: Methanol : water in 80 : 20 V/V.

2. **Ninhydrin solution**: 0.4 g of ninhydrin in 10 mL of 0.2 M citrate buffer solution.

3. **0.2 M Citrate buffer**: It was prepared by mixing 20.5 mL of 0.2 M citric acid (4.2 g in 100 mL) with 29.5 mL of 0.2 M sodium citrate (5.88 g in 100 mL) and diluted to 100 mL with distilled water.
4. **Diluent solvent**: Equal volumes of water and 1-propanol.

**Procedure**:

About 100 mg of pupae powder was homogenized in ice cooled 80% methanol in all glass micro-homogenizer. The homogenate was centrifuged and supernatant was collected. The residue was washed twice with 80% methanol and centrifuged. The pooled supernatant was evaporated to reduce the volume.

To 0.1 mL extract 1 mL of 4% ninhydrin solution in 0.2 M citrate buffer (pH = 5.0) was added, the volume was made to 2 mL with distilled water. The solution was then heated in a boiling water bath for 20 minute. The solution was cooled and 5 mL of a mixture of water and 1-propanol (1:1 V/V) was added. The intensity of the colour was measured at 570 nm. The reagent blank was prepared by taking 0.1 mL of 80% methanol instead of the extract.

A standard curve was prepared by taking a mixture of equal amount of alanine, aspartic acid, lysine, tryptophan, proline and leucine as standard.

6. **TOTAL GLYCOGEN**

**Principle**:

Total glycogen is determined by method of Carrol *et al.* (1956). In hot acidic medium glycogen is hydrolyzed to glucose,
which is dehydrated to hydroxymethylfurfural. The compound forms a green colour with anthrone reagent. The colour intensity was measured at 620 nm.

**Reagents required:**

1. **2.5 N Hydrochloric acid**: 20.8 mL of concentrated hydrochloric acid was diluted to 100 mL with distilled water.
2. **Anthrone reagent**: 200 mg of anthrone was dissolved in 100 mL of ice cold 95% sulphuric acid.
3. **30% Potassium hydroxide solution**: 3 g of potassium hydroxide dissolved in water and volume made up to 10 mL.

**Process:**

About 10 g of muga waste pupae was boiled with 4 mL 30% KOH solution for 1.5 hours with occasional stirring. The mixture was cooled in ice cold water and centrifuged. To the supernatant 2 volume of 95% ethanol was added, heated just to boiling and was kept overnight in cold and centrifuged. The precipitate was dissolved in 10 mL re-distilled water. The solution was hydrolyzed by keeping it in a boiling water bath for 3 hours with 5 mL of 2.5 N HCl and was cooled to room temperature. The solution was neutralized with NaOH using phenol red as indicator. The volume was then made up to 50 mL.
To 1 mL of the test solution freshly prepared 4 mL of anthrone reagent was added and heated for 10 minute in a boiling water bath. It was then rapidly cooled, read at 620 nm against a reagent blank.

The standard curve was prepared by taking glucose as standard. From the standard curve glucose content was determined. The value was multiplied by a factor 0.9 to arrive at glycogen content.

7. MINERALS

The ash of muga pupae taken in a platinum crucible was moistened with 1.0 mL of re-distilled water and 5.0 mL of concentrated HCl was added to it. The mixture was evaporated to dryness in boiling water bath. 5 mL of concentrated HCl was added again and evaporated to dryness as before. Finally 4 mL of concentrated HCl and 2.3 mL of redistilled water was added and mixture was warmed in a boiling water bath and filtered into a 100 mL volumetric flask. After cooling, the volume was made 100 mL and aliquots were used for the estimation of phosphorus, iron and calcium (Srikantia, 1976).

i) PHOSPHORUS

Principle:

Phosphorus in the form of phosphate reacts with ammonium molybdate to give rise to a blue coloured phosphomolybdate complex, which is measured spectrophotometrically at 660 nm. Amount of
phosphorus is determined from the standard curve prepared with potassium dihydrogen phosphate.

**Reagents required:**

1. **Hydroquinone solution:** 0.5 g of hydroquinone was dissolved in distilled water containing 4 drops of concentrated H$_2$SO$_4$ and the volume was made up to 100 mL.

2. **Sodium sulphite solution:** 5 g sodium sulphite dissolved in distilled water and volume made up to 100 mL.

3. **2.5% Ammonium molybdate solution:** 2.5 g of ammonium molybdate dissolved in distilled water and volume made up to 100 mL.

4. **Standard phosphorus solution:** 0.088 g of KH$_2$PO$_4$ was dissolved in double distilled water along with 0.7 mL of concentrated H$_2$SO$_4$ and volume made up to 250 mL.

**Process:**

To 0.1 mL of mineral solution, 1 mL of ammonium molybdate, 1 mL of hydroquinone and 1 mL of Na$_2$SO$_3$ was added one after another with constant shaking. The volume of the mixture was made up to 15 mL with double distilled water. The mixture was allowed to stand and after half an hour the colour was measured at 660 nm against a re-distilled water reagent blank.
The standard curve of phosphorus was prepared by taking KH₂PO₄ solution as standard and from the standard curve the amount of phosphorus was determined.

ii) IRON

Principle:

Ferric iron gives a blood red colour with potassium thiocyanate solution which is spectrophotometrically measured at 540 nm. Amount of iron is determined from the standard curve prepared with ferrous ammonium sulphate.

Reagents required:

1. **30% Sulphuric acid**
2. **7% Potassium persulphate solution**: 7 g of potassium persulphate dissolved in distilled water and volume made up to 100 mL.
3. **40% Potassium thiocyanate(KCNS) solution**: 40 g of KCNS dissolved in 90 mL of double distilled water, 4 mL of acetone was added and volume made up to 100 mL.
4. **Standard iron solution**: 702.2 mg ferrous ammonium sulphate was dissolved in 100 mL of double distilled water and after addition of 5 mL of 1:1 HCl, the volume was made up to 1L. A solution containing 10 μg per mL was prepared by diluting this solution 10 fold.
Process:

3 mL of mineral solution was mixed with 3.5 mL of distilled water and 1 mL 30% H₂SO₄. To the solution 1 mL 7% potassium persulphate and 1.5 mL 40% potassium thiocyanate were added. After each addition the solution was shaken well. The red colour, that developed, was measured within 20 minute at 540 nm taking redistilled water as blank.

The standard curve of iron was prepared by taking ferrous ammonium sulphate solution as standard solution and from the standard curve the amount of iron in the sample was determined.

iii) CALCIUM

Principle:

Calcium in waste muga pupae is determined by the method of Oser et al. (1957). Calcium is precipitated as calcium oxalate in ammoniacal medium. The precipitate is dissolved in H₂SO₄ and titrated against standard potassium permanganate solution.

Reagents required:

1. 4% Ammonium oxalate solution: 4 g ammonium oxalate dissolved in water and volume made up to 100 mL.
2. Dilute ammonia solution: 2 mL liquor ammonia + 98 mL water.
3. 1 N Sulphuric acid: 2.8 mL of concentrated sulphuric acid diluted to 100 mL.
4. **0.01 N Potassium permanganate solution**: 0.316 g of potassium permanganate dissolved in distilled water and volume made up to 1 L.

5. **0.01 N Standard oxalic acid solution**: 0.63 g of oxalic acid dissolved in distilled water and volume made up to 1 L.

**Process**:

25 mL of the mineral solution was diluted to 150 mL with double distilled water and to it 6-8 drops of methyl red indicator was added when the solution became pink. To this solution concentrated ammonia solution was added drop wise till the colour became yellow. It was then heated in a boiling water bath with 10 mL ammonium oxalate solution for 8-10 minutes. To this solution glacial acetic acid was added drop wise till the colour again became pink.

The solution was then allowed to stand for an hour, filtered and the precipitate was washed 7-8 times with warm double distilled water to wash away the oxalate ion.

The precipitate was transferred to a beaker with 50 mL 2 N 
\( \text{H}_2\text{SO}_4 \) and the solution was heated at 70°C. It was cooled and titrated against standard 0.01N potassium permanganate solution standardized against standard 0.01 N oxalic acid solution. Amount of calcium was calculated from the following relationship.

\[
1 \text{ mL of 0.01 N KMnO}_4 = 0.2004 \text{ mg of } \text{Ca}^{2+} \text{ ion.}
\]
8. QUALITATIVE DETERMINATION OF FREE AMINO ACIDS

Qualitative determination of free amino acids was carried out by two dimensional paper chromatographic procedure using butanol : acetic acid : water and phenol : water as solvents.

100 mg pupae powder was homogenized in ice cold 80% methanol, the homogenate was centrifuged and the supernatant was collected. The residue was washed twice with 80% methanol and centrifuged. The pooled supernatant was reduced by evaporation. The reduced supernatant was desalted and 100 μL was spotted on Whatman- 41 chromatographic paper. Then it was irrigated with butanol : acetic acid : water (4:1:5). The paper was air dried and placed in the chromatographic chamber, previously saturated with phenol : water (3:1), and was run with the solvent. The paper was then air dried, sprayed with ninhydrin solution and heated at 50°C for 1 hour and left overnight in the dark. The paper was finally sprayed with copper nitrate solution and the intensity of spots were determined visually and expressed as + (traces) to ++++ (abundant).

Reference standard were also simultaneously maintained for the identification of spots.
9. (i) LIPID EXTRACT

Accurately weighed pupae powder was extracted by using different solvents with soxhlet extractor. The extraction was carried out for about 6 hours when around 8 washings occur. The lipid solution in solvent was concentrated by distillation when most of the solvent was recovered. Finally concentrated solution of lipid was transferred quantitatively to a volumetric flask and estimated gravimetrically. The different solvents used were

i) Petroleum ether (40°C – 60°C)
ii) n-Hexane
iii) Chloroform-methanol mixture (2:1 V/V)

The extraction was also tried by refluxing with petroleum ether as solvent using magnetic stirrer.

(ii) TOTAL LIPID

Extraction of total lipid was done by using modified Folch method (Folch et al., 1957).

a) 10 g of sample was taken in a conical flask and properly labeled.
b) To the flask 100 mL of chloroform : methanol (2:1 V/V) mixture was added and kept inside a refrigerator overnight.
c) Next day it was filtered and residue was again put in 100 mL chloroform : methanol (2:1 V/V) mixture and kept overnight inside a refrigerator. Filtrate was also kept overnight.
d) Next day it was again filtered and the filtrate of both i.e., the earlier one and later one were mixed.
e) The filtrate mixture was then evaporated to dryness in a rotary evaporator and then again dissolved in 100 mL chloroform : methanol (2:1 V/V) mixture and transferred into the original conical flask.
f) After that, 20 mL of 1% saline solution was added to the extract, mixed by vigorous shaking and allowed to stand overnight inside a refrigerator.
g) The lower layer was taken out next day in a flask with the help of a pipette and then evaporated to dryness in a rotary evaporator.
h) It was then mixed with 50 mL of chloroform and evaporated to dryness again in a rotary evaporator.
i) The process was repeated.
j) It was then transferred to a volumetric flask and the volume made up to a known value with chloroform : methanol (2:1 V/V) mixture by repeated rinsing of the flask which contained the lipid until the yellow colour of the lipid disappeared completely.

The volumetric flask containing the lipid was properly labeled and stored inside a refrigerator. Amount of lipid extracted was determined by gravimetric process.
10. LIPID PARAMETERS

For characterizing the lipid of waste muga pupae a few parameters, viz., iodine value, saponification value and acid value were determined by standard methods (Sadasivam et al., 1996).

i) IODINE VALUE

Principle:

Iodine value is the gram of iodine absorbed by 100 g of the oil or fat. Iodine value is a measure of the degree of unsaturation in an oil or fat. It is a useful parameter for studying oxidative rancidity of oil or fat because higher is the unsaturation greater is the possibility of it to get rancid.

Reagents required:

1. Iodine monochloride (ICl) solution: 5 mL of ICl was dissolved in 500 mL of glacial acetic acid.
2. 0.1 N Sodium thiosulphate solution: 24.8 g sodium thiosulphate dissolved in distilled water and volume made up to 1L.
3. 10% Potassium iodide solution
4. Starch solution

Process:

0.2 g of lipid was taken in a dry iodine flask and dissolved by adding 10 mL of chloroform. 25 mL of ICl solution was then added.
The flask was stoppered with previously moistened stopper in KI solution and the mixture was kept in dark for 30 minutes at a temperature 15° C – 25° C. A blank was set up simultaneously with all the reagents except the lipid. After the stipulated period the stopper and the flask was rinsed with 50 mL of re-distilled water followed by the addition of 15 mL of KI solution. The iodine content was then titrated with sodium thiosulphate solution till the solution became pale yellow. To this solution 1 mL of starch solution was added and the titration was continued until the blue colour disappeared.

If ‘a’ be the volume of sodium thiosulphate solution required in test solution, ‘b’ be the volume of sodium thiosulphate solution required in blank solution and N be the normality of sodium thiosulphate solution then,

\[
\text{Iodine value} = \frac{(b - a) \times 0.01269 \times 100 \times N}{\text{Mass of sample in g} \times 0.1}
\]

ii) SAPONIFICATION VALUE

Principle:

Saponification value is the amount of potassium hydroxide (KOH) in mg required to hydrolyze 1 g of lipid. This value gives an idea about fatty acid chain length in oil or fat. A known quantity of fat is boiled with excess of alcoholic KOH solution. After complete
saponification, the remaining KOH is estimated by titrating against a standard acid.

**Reagents required:**

1. **Alcoholic KOH solution (0.5N):** 7 g KOH was dissolved in 20 mL of water and the volume made up to 250 mL with 95% ethanol and allowed to stand overnight.
2. **Ethanol – ether mixture:** Equal volume of 95% ethanol and ether were mixed.
3. **HCl (0.5 N):** Standardized with NaHCO₃ using methyl orange as indicator.
4. **Phenolphthalein indicator:** 1% solution in 95% ethanol.

**Process:**

1 g of accurately weighed lipid was dissolved in 5 mL of ethanol - ether solvent. The content was quantitatively transferred to 250 mL conical flask by rinsing the beaker three times with further amount of solvent. 25 mL of 0.5 N alcoholic KOH solution was then added to the mixture. By using air condenser the contents were refluxed in a boiling water bath for 60 minutes. The contents of flask were frequently stirred to mix. Simultaneously, a blank was prepared by refluxing a similar mixture not containing the lipid. The contents were cooled to room temperature and titrated with 0.5N HCl using phenolphthalein as indicator.
If ‘a’ mL and ‘b’ mL are the titre values of test and blank solutions then,

\[
\frac{(b-a) \times 0.02805 \times 1000}{\text{Mass in g of the fat}}
\]

iii) ACID VALUE

**Principle:**

The acid value of a fat is the number of mg of KOH required to neutralize the free acid in 1 g of lipid. It is estimated by titrating against a standard KOH solution.

**Reagents required:**

1. A mixture of equal volume of ethanol and ether.
2. 1% Phenolphthalein in 95% ethanol.
3. 0.1N KOH solution: Standardized with 0.1N oxalic acid using phenolphthalein as indicator.

**Process:**

About 2 g of accurately weighed fat was dissolved in 30 mL of alcohol-ether mixture and slightly warmed. The mixture was titrated with standard 0.1 N KOH solution using phenolphthalein as indicator. If the titre value was ‘a’ mL then,

\[
\frac{a \times 0.00561 \times 100}{\text{Mass in g of the fat} \times 0.1}
\]
C. UTILIZATION OF WASTE MUGA SILK WORM PUPAE AS INGREDIENT OF BROILER RATION

In Assam broiler rearing is a very fast growing industry. The broiler ration should contain a definite amount of animal protein for proper growth of the bird. There is dearth of good quality animal protein in this region and presently most of the animal protein in the form of fish meal (FM) is imported from coastal areas of the country. Quality of these fish meal is often found to be inferior. As such a substitute for fish meal (FM) in poultry ration for this region is desirable. As the analysis of waste muga pupae show the presence of high percentage of protein along with a number of essential amino acids it was thought worthwhile to try waste muga pupae as substitute for fish meal in the broiler ration.

The present study intends to go into the details of the utility of waste muga pupae as animal protein ingredient of broiler ration. Three sets of experiments were conducted for this purpose.

SET-I

Fish meal in conventional broiler ration was 100% substituted by (i) raw waste muga pupae meal and (ii) de-oiled waste muga pupae meal.

SET-II
Fish meal in conventional broiler ration was 100% and 50% substituted by de-oiled waste muga pupae meal.

**SET-III**

Stimulating effect of antibiotic and probiotic on broiler performance fed on de-oiled MSWPM as animal protein ingredient.

**SET-I**: *Fish meal is 100% substituted by (i) Raw waste muga pupae meal and (ii) De-oiled waste muga pupae meal*

Waste muga pupae were collected, sun dried, powdered, de-oiled and stored in airtight containers. The pupae powder, both raw and de-oiled, were tested four monthly for quality change and putrefaction, if any.

Feeding trials were conducted on 120 one day chicks for 45 days. They were divided into 12 groups at random, each containing 10 birds. Three sets of diet, viz., Control (C) and Experimental–1 ($E_1$) and Experimental–2 ($E_2$) were prepared incorporating fish meal, raw waste muga pupae meal and de-oiled waste muga pupae meal respectively on iso-nitrogenous basis as animal protein. Four replicate groups were allotted randomly to each set.

The whole experiment was repeated three times during 1993-94 to confirm the findings of the present study.
Control diet (C) was prepared with the conventional ingredients along with 10% fish meal. The conventional ingredients were yellow maize, groundnut cake, til oil cake, soya bean meal, rice polish, wheat bran along with minerals, salt, vitamins and medicines. Different ingredients were mixed in the proportion given in the standard procedure keeping the percentage of protein in the recommended level. Two sets of diets, viz., starter and finisher, were prepared where percentage of protein were kept at 22.3% and 19.9% respectively (Table 2.1).

Experimental -1 (E₁) diet was prepared by using raw waste muga pupae meal as animal protein ingredient. In the starter ration 7.3% pupae and in the finisher 5.8% pupae were used to keep the animal protein percentage and crude protein percentage at the same level as that of control ration.

Experimental -2 (E₂) diet was prepared by using de-oiled waste muga pupae meal as animal protein ingredient. In the starter ration 6% and in the finisher ration 4.8% de-oiled pupae were used to keep the animal protein percentage and crude protein percentage same as those of controlled diet. In both E₁ and E₂ percentage of some of the common ingredients were also varied from the control ration.

The metabolizable energy (in kcal / kg) of different rations were calculated following Mallete et al., (1968).
### Table 2.1
Composition of rations - Control (C), Experimental-1 (E1) and Experimental-2 (E2)
(All the values are expressed as mass percent i.e., g per 100 g)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Starter</th>
<th>Finisher</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>E₁</td>
</tr>
<tr>
<td>Yellow maize</td>
<td>46.5</td>
<td>50.0</td>
</tr>
<tr>
<td>Ground nut cake</td>
<td>15.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Til oil cake</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Soya bean meal</td>
<td>6.0</td>
<td>5.2</td>
</tr>
<tr>
<td>Rice polish</td>
<td>5.0</td>
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<tr>
<td>Wheat bran</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Fish meal</td>
<td>10.0</td>
<td>-</td>
</tr>
<tr>
<td>MSWPM (Raw)</td>
<td>-</td>
<td>7.3</td>
</tr>
<tr>
<td>MSWPM (De-oiled)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>* Mineral mixture</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Salt</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>** Minovit</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Coccidiostat</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C.P.(%)</td>
<td>22.3</td>
<td>22.3</td>
</tr>
<tr>
<td>M.E. kcal/kg (Calculated)</td>
<td>3279</td>
<td>3494</td>
</tr>
</tbody>
</table>

* Minerals present: Ca - 6.3 g, P - 1.35 g, I₂ - 3.5 mg, Fe - 30 mg, Mn - 95.5 mg, (Per kg of the food) Cu - 5.5 mg, Co - 9 mg, Zn - 69 mg, F - 4.5 mg.

** Vitamins present: Vitamin A - 10,000 I.U., Vitamin D₃ - 2,000 I.U., Vitamin B₂ - 4 mg, (Per kg of the food) Vitamin B₁₂ - 0.01 mg, Vitamin K - 2 mg, Vitamin E - 1.51 I.U., Calcium pentothenate - 5 mg, Nicotinamide - 20 mg.

Supplemented amino acids: L - Lysine - 40 mg, DL - Methionine - 0.40 mg.
The chicks were fed *ad libitum* with starter ration from 0-5 weeks and finisher ration from 6\textsuperscript{th} week till the end of the experiment. Broiler chicks were kept within the recommended space and at proper temperature range at different stages of their growth.

During the experiment, feed consumption and weight gain were recorded. For the study of the body weight gain initial body weight of each chick was recorded and wing banded. During the feeding trial the following operations were performed.

i) Daily weighed quantity of feed was supplied and the residue left was recorded.
ii) Daily feed consumption and faeces voided were recorded.
iii) Weekly feed consumption per bird, group wise and for the whole set were noted.
iv) Gain in live weight per bird per week and mortality were carefully recorded.

Chicks were vaccinated for the protection against different diseases and during the progress of the work they were given different medicines when necessary.

Feed efficiency ratio was calculated from the average body weight gain and average feed consumption. Similarly protein efficiency was calculated from the average consumption of protein.
and body weight gain. Food economy was computed from different relevant data.

A metabolism trial on three birds from each set on three days collection was performed to find out the nitrogen retention per bird.

At the end of the study period two male and two female birds were sacrificed from each replicate to determine the percentage of dressing, edible carcass, giblet yield, heart yield, gizzard yield and liver yield. All the data were statistically analyzed (Mahajan, 1989).

**SET- II : Fish meal is 100% and 50% substituted by De- oiled waste muga pupae meal**

The waste muga pupae were collected, sun dried, ground to powder and the lipid fraction extracted in soxhlet using petroleum ether. The de-oiled muga pupae was dried and stored in airtight container to be used for preparing ration.

A feeding trial was conducted on 60 one day old chicks for 45 days. The chicks were randomly divided into 6 groups, each containing 10 birds. Experiments were conducted using three sets of diets, viz., Control (C), Experimental ration -1 (E₁) and Experimental ration -2 (E₂). Two replicate groups were allotted to each set.

The control diet was prepared with conventional ingredients including fish meal (FM) as animal protein. In E₁ ration FM was
### Table 2.2

**Composition of rations - Control (C), Experimental -1 (E₁) and Experimental -2 (E₂)**

(All values are expressed as mass percent i.e. g per 100 g)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Starter</th>
<th>Finisher</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>E₁</td>
</tr>
<tr>
<td>Yellow maize</td>
<td>46.5</td>
<td>51.5</td>
</tr>
<tr>
<td>Ground nut cake</td>
<td>15.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Til oil cake</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Soya bean meal</td>
<td>6.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Rice polish</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Fish Meal</td>
<td>10.0</td>
<td>−</td>
</tr>
<tr>
<td>MSWPM</td>
<td>−</td>
<td>6.0</td>
</tr>
<tr>
<td>*Mineral mixture</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Salt</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Minovit</strong></td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Coccidiostat</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C.P. (%)</td>
<td>22.4</td>
<td>22.4</td>
</tr>
<tr>
<td>M.E.(kcal/kg) (Calculated)</td>
<td>3279</td>
<td>3425</td>
</tr>
</tbody>
</table>

* Minerals present: Ca – 31.5%, P – 6.75% of the mineral mixture added.

**Vitamins present: Vitamin A – 10,000 I.U., Vitamin D₃ - 2,000 I.U., Vitamin B₂ – 4 mg, Vitamin B₁₂ – 0.12 mg, Vitamin K – 2 mg, Vitamin E – 1.5 mg, Calcium pantothenate – 5 mg, Nicotinamide – 20 mg, Choline chloride 33 mg.

Supplemented amino acids: L-Lysine- 40 mg, DL- Methionine- 0.40 mg.
replaced on iso-nitrogenous basis totally (100%) by de-oiled muga silk worm pupae meal (MSWPM) and in E\textsubscript{2} ration the FM was replaced partially (50%) by de-oiled MSWPM. The composition of three sets of diets is presented in table 2.2.

The chicks were fed \textit{ad libitum} with starter ration from 0-35 days and with finisher ration for the remaining period of the experiment. In the experiment the feed efficiency ratio and food economy were worked out. A metabolic trial on three birds from each set on three days collection was performed to find the nitrogen retention per bird. At the end of the study period two male and two female birds were sacrificed from each replicate to determine the percentage of dressing, the edible carcass, giblet yield, heart yield, gizzard yield and liver yield. Lastly all the data were statistically analyzed (Mahajan, 1989).

\textit{SET – III : Stimulating effect of Antibiotic and Probiotic supplemented broiler ration incorporated with De-oiled MSWPM}

In order to study the growth stimulating effect of probiotic and antibiotic supplemented broiler ration another set of experiment was carried out.

Study was conducted on 75 one day old commercial broiler chicks for 7 weeks. The chicks were randomly divided in 9 groups, each containing 8-9 birds. Three sets of diets, viz., Control (C),
Experimental ration-1 (E₁) and Experimental ration-2 (E₂). Two replicate groups were allotted to each set of experiment. In each set de-oiled MSWPM was used as animal protein supplement. Control diet (C) did not contain either antibiotic or probiotic. E₁ contained antibiotic, teracyline hydrochloride (0.02 %) in addition to the normal ingredients, while E₂ contained probiotic, G-Pro (0.05 %).

Two sets of rations were prepared – the starter ration for 0-5 week old chicks and finisher ration for 6-7 week old chicks. Starter ration contained 6.0% and finisher ration contained 4.8% of de-oiled MSWPM. The starter ration contained 22.4% crude protein while the finisher ration contained 19.9% crude protein. (Table - 2.3).

Feed efficiency, protein efficiency, body weight gain and economy were determined for each set of birds by applying standard methods.

At the end of the study period two male and two female birds were sacrificed from each set and dressing percentage, giblet yield percentage, liver yield percentage, heart yield percentage and gizzard yield percentage were determined. Lastly the data were statistically analyzed.
Table 2.3
Composition of rations – Control (C), Experimental – 1 (E₁) and Experimental – 2 (E₂)
(All values are expressed as mass percent i.e. g per 100 g)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Starter</th>
<th>Finisher</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>E₁</td>
</tr>
<tr>
<td>Yellow maize</td>
<td>40.00</td>
<td>39.95</td>
</tr>
<tr>
<td>Ground nut cake</td>
<td>14.92</td>
<td>14.95</td>
</tr>
<tr>
<td>Til oil cake</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Soya bean meal</td>
<td>14.80</td>
<td>14.80</td>
</tr>
<tr>
<td>Rice polish</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>MSWPM</td>
<td>7.70</td>
<td>7.70</td>
</tr>
<tr>
<td>*Mineral mixture</td>
<td>1.50</td>
<td>1.50</td>
</tr>
<tr>
<td>Salt</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>**Minovit</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Furazolidone</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Amprolium</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Probiotic</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>--</td>
<td>0.02</td>
</tr>
<tr>
<td>CP (%) [Calculated]</td>
<td>23.11</td>
<td>23.19</td>
</tr>
<tr>
<td>CP (%) [Experimental]</td>
<td>22.29</td>
<td>22.89</td>
</tr>
</tbody>
</table>

* Minerals present: Ca – 31.5%, P – 6.75% of the mineral mixture added.

**Vitamins present: Vitamin A – 10,000 I.U., Vitamin D₃ – 2,000 I.U., Vitamin
(Per kg of the food) B₂ – 4 mg, Vitamin B₁₂ – 0.12 mg, Vitamin K – 2 mg,
Vitamin E – 1.5 mg, Calcium pantothenate – 5 mg,
Nicotinamide – 20 mg, Choline chloride 33 mg.
Supplemented amino acids: L- Lysine – 40 mg, DL- Methionine- 0.40 mg.
D. STATISTICAL METHOD

Analysis of Variance:

This test involves comparison of sample variance and is also called as F test. At first sum squares, between the classes and within the classes are calculated. The sum squares within the classes is found by subtracting sum of squares between the classes from the total sum of squares of entire sample. The F ratio is calculated as per the following table (Mahajan, 1984).

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Sum of squares</th>
<th>Mean sum of squares</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Between the classes</td>
<td></td>
<td></td>
<td>MSS (3-2)</td>
<td></td>
</tr>
<tr>
<td>B. Within the classes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The significance of F is found by referring to F table. F table gives variance ratio values at different level of significance at df \( (n_1 - 1) \) given horizontally and \( (n_2-2) \) given vertically. If calculated value is greater than table value, \( H_0 \) (Null hypothesis) is accepted, if calculated value is greater than the table value, \( H_0 \) is rejected and \( H_1 \) (Alternate hypothesis) of significant variation between the means is accepted.