Genetic Variability in *Morus alba L* by Biochemical and Bioassay Methods for increased Silk Productivity

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ABSTRACT
The object of this study is to screen biochemical content of six varieties of mulberry leaves viz S13, S30, S36, ML, V1 and RFS-135 to find out nutritively richer one. Among six varieties V1 is the best one containing highest total sugar (16.72%), total protein (26.72%) and total phenol (4.56%) content compare to other five varieties, so V1 is the highly recommendable feed for silkworm (*Bombyx mori L)* to increase their silk productivity. Silkworms feeded with nutritively richer V1 variety were taken for the following biochemical and bioassay. Protein estimation in larval, pupal haemolymph and also in silkgland, enzymatic (amylase, protease) analysis of different (II to V) instars larvae. Likewise fibroin sericin ratios in cocoon were also done. The all the above parameters of silkworm (*Bombyx mori L)* feeded with V1 variety was found to be high and silk productivity was also high, which will fulfill existing market demand.

Key words: Silkworms, silk productivity, mulberry and protein.

INTRODUCTION

Silk is one of the nature's gifts to mankind produced by silkworm. Among silkworm the most commercially exploited one are mulberry silkworm *Bombyx mori L.*, silk is a natural fibre secreted for the protection of pupae in the process of completing their life cycle. The larvae of *Bombyx* mori *L,* is an elongated caterpillar commonly called as mulberry silkworm. Larvae are monophagous and feed only on mulberry plants. Mulberry leaves is the sole food plant for the silkworm *Bombyx mori L.* The fresh and nutritive quality of mulberry leaf plays an important role on the development of worm stabilizing the cocoon production and silk productivity.

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The various compositional factors of mulberry leaves are responsible for successful cocoon harvest and silk productivity, thus the mulberry leaf quality plays a predominant role in healthy growth of silkworm. Hence nutrition of silkworm, *Bombyx mori L*, is of primary importance as the cocoon production is directly influenced by the nutritive status of mulberry leaves. The quality of feed is determined by its major components such as water, carbohydrates, proteins, mineral, elements, fats, amino acids and vitamins.

MATERIALS AND METHODS

Mulberry leaf samples preparation: The six varieties (S13, S30, S36, ML, V1 and RFS-135) of mulberry leaves were collected from mulberry plants (*Morus alba L*) in G.K.V.K, Bangalore. The leaves were dried in oven and then made

into fine powder using mortar and pestle. The powdered samples were preserved in butter paper/polythene cover and stored for further use.

Estimation of total protein: For the estimation of total protein, leaf samples were extracted with the following chemicals: Pre chilled acetone (-20 C), 0.2M phosphate buffer (pH 7.0), 0.056 M beta mercapto-ethanol and the mixture was centrifuged at 12 K for 10 minutes at 4°C.The supernatant was used to estimate total soluble protein as per the standard method [1]. This leaf samples were mixed with 5 ml of Lowry's reagent and allowed to stand for 30 minutes and color development was read at 660 nm against water blank

***Preparation of mulberry leaf sample for the estimation of total soluble sugar and total phenol*:** Six different mulberry leaf samples were extracted with 10ml of 80% warm ethanol and centrifuged at 10 K for 10 minutes, collected the Supernatant and evaporated to dryness in hot water bath and the residue was dissolved in 5 ml of distilled water and used for total sugars, reducing sugar and total phenol estimation.

Estimation of total soluble sugar: The total soluble sugar content of leaf samples were estimated as per the standard method [2]. For this leaf samples were mixed with 0.5 ml of phenol, 5ml of sulphuric acid and placed in a water bath at 30° C for 20 minutes and color development was read at 490 nm against water blank

Estimation of total phenol: The total phenol content of leaf samples were estimated as per the method described [3]. For this leaf extract samples were mixed with 0.5 ml of Folin ciocalteau reagent (FCR) followed by the addition of 2 ml of 20% sodium carbonate and placed the tubes in boiling water bath for 10 minutes cool downed the tubes and the absorbance was read at 650 nm against water blank.

Biochemical and enzymatic analysis of mulberry silkworm: Qualitative and quantitative analysis of enzymes (protease, amylase),estimation of protein content in silk gland and haemolymph of fifth instar larvae were analyzed after feeding the silkworm only with V1 variety of mulberry leaves likewise pupal haemolymph protein and glycerol, glycogen content of eggs, fibroin and sericin ratio in cocoon were also analyzed.

Enzyme extraction and qualitative analysis of enzymes: Silkworm were dissected and taken its three different gut (fore, mid and hind) regions and it was homogenized well with 5 ml of distilled water in separate test tubes and this homogenate were used for qualitative analysis of enzymes.

Invertase: Few drops of gut homogenate samples were mixed with 1ml of 5% sucrose solution and incubated at room temperature for one hour. Addition of few drops of Fehling's reagent to the above reaction mixture. Red precipitate formation indicates the presence of invertase.

Amylase: Few drops of gut homogenate samples were mixed with 1ml of starch solution and incubated at room temperature for one hour. A drop of iodine was added to the above reaction mixture. Formation of reddish brown or violet color indicates the presence of amylase.

Lipase: Few drops of gut homogenate samples were mixed with 1ml of methylsalicyclate, 5 ml of methyl red and incubated at room temperature for one hour. Formation of orange color indicates the presence of lipase.

Protease: Few drops of gut homogenate samples were added to the bits of exposed film and kept them in refrigerator for 1-2 days. If film turned white/transparent indicates the presence of protease.

Quantitative analysis of amylase: Different instars (II to V) mulberry silkworm larval midgut samples were collected by dissecting the larvae. Midgut samples subjected to homogenization were centrifuged and collected the digestive juice supernatant and used for quantitative analysis. Amylase activity of the different instars midgut samples were analyzed [4, 5]. For this 2 ml of different instars midgut samples were mixed with 2ml of starch solution (2mg/ml) and incubated at 37°C for 30 minutes. Then the reaction was stopped by adding 2 ml of 3-5, DNSA.The color was developed by keeping the tubes in boiling water bath and the color development was read at 525 nm.

Quantative analysis of protease: *Protease* activity of the different instars midgut samples were analyzed [6]. For this following chemicals were used; 1% casein, 0.1 M borate buffer (ph 11), 10% TCA.The mixture was centrifuged at 3K for10 minutes and to the supernatant 2 ml of 0.5 N sodium hydroxide, 0.5 ml of FCR were mixed and allowed this mixture to stand at room temperature for 30 minutes and absorbance was read at 660nm against water blank.

Estimation of protein in silk gland and haemolymph: Fifth instar *Bombyx mori L*., larval was dissected inorder to collect silk gland and haemolymph, likewise pupal haemolymph was also collected by dissecting the pupae. Haemolymph samples were diluted with distilled water and 1g of silk gland was homogenized well with distilled water. The protein content of haemolymph and silk gland samples were estimated [1] and color development was read at 660nm against water blank.

Analysis of fibroin and sericin protein ratios in cocoon: The dry weight of the three different colored cocoon were recorded before treating with 2% potassium hydroxide at 70-80°C with constant stirring for few minutes. The chemically treated cocoons become fluffy and it was washed thoroughly in tap water before treating with acetic acid solution. Chemically treated cocoons wet weight and dry weights were recorded for estimating fibroin and sericin ratio in cocoon. The percentage of fibroin and sericin in silk shell (cocoon) was calculated using the following formula

$$\text{Fibroin \%} = \frac{\text{Weight of fibroin (g)}}{\text{Weight of shell (g)}} \times 100$$

Sericin % = 100- fibroin %

RESULTS

Biochemical analyses of six different (S13, S30, S36, ML, V1 and RFS-135) varieties of mulberry leaves were presented in table I.

Table I: Biochemical analysis of six different mulberries (*Morus alba L*)

S.No.	Mulberry Varieties	Total protein %	Total sugar %	Total phenol%
1	S-13	23.42	15.50	3.22
2	S-30	24.14	15.74	4.42
3	S-36	23.67	15.94	3.92
4	MYSORE LOCAL	22.40	14.30	3.30
5	V-1	26.72	16.72	4.56
6	RFS-135	21.30	14.64	3.42

Table II: Qualitative analysis of enzymes in gut regions of mulberry silkworm *(Bombyx mori L)* larvae

S.No.	Enzymes tested	Foregut	Midgut	Hindgut
1	Amylase	++	+++	++
2	Invertase	+	++	+
3	Protease	++	++	++
4	Lipase	-	-	-

Color intensity scoring for enzymatic activity:

\+ = Enzymatic activity - color intensity is low
++ = Enzymatic activity - color intensity is medium
+++ = Enzymatic activity - color intensity is high
\- = Absence of enzymatic activity

Table III: Quantitative analysis of enzymes (amylase and protease) in different instars larval midgut samples of mulberry silkworm (Bombyx *mori L)*

S.No.	Midgut samples from different Instars of mulberry silkworm	Amylase activity (mg of maltose released/minutes/ml of midgut sample)	Protease activity (µg of tyrosine released/minutes/ml of midgut sample)
1	II	0.164	1.064
2	III	0.192	1.124
3	IV	0.226	1.178
4	V	0.260	1.242

Table IV: Protein profiles in fifth in star larval silk gland, haemolymph and in pupal haemolymph of mulberry silkworm (*Bombyx mori L)*

S.No.	Silkworm samples for protein estimation	Fifth instar larval silkglandprotein (mg/g)	Fifth instar larval haemolymph protein (mg/ml)	Pupal haemolymph protein (mg/ml)
1	I	93.80	66.72	54.72
2	II	95.50	65.50	55.34
3	III	96.20	69.32	52.42
4	IV	98.50	67.40	56.14
5	Mean value	96.00	67.23	54.65

Table V: Analysis of fibroin and sericin content in different colored cocoons of mulberry silkworm *(Bombyx mori L)*.

S.No.	Cocoon color	Fibroin %	Sericin %
1	White	75.30	24.70
2	Pale yellow	74.60	25.40
3	Dark yellow	73.80	26.20

Total protein: Total protein values ranged from 21.30-26.72%.In that maximum value was observed in V1 (26.72%) followed by S30 (24.14%).The least value was observed in RFS-135 (21.30%).The values obtained from the six leaf varieties were tabulated against standard BSA values.

Total sugar: Total sugar values ranged from 14.30-16.72%.In that maximum value was observed in V1 (16.72%) followed by S36 (15.94%).The least value was observed in Mysore Local (14.30%).The values obtained from the six leaf varieties were tabulated against standard glucose values.

Total phenol: Total phenol values ranged from 3.22-4.56%.In that maximum value was observed in V1 (4.56%) followed by S30 (4.42%).The least value was observed in S13 (3.22%).The values obtained from the six leaf varieties were tabulated against standard catechol values.

Qualitative analysis of enzymes: Amylase, protease, invertase and lipase enzymes has been qualitatively analyzed in three different (fore, mid and hind) gut samples of larvae and the results were presented in table II. Qualitative analysis of enzymes in gut region were revealed that amylase, protease and

invertase enzymes were present in all three gut regions and lipase enzyme was absent in all three gut regions.

Quantitative analysis of amylase enzymes: Four different instars (II to V) larval midgut samples were quantitatively analyzed for amylase activity and the results were presented in table III. Amylase activity of midgut sample ranges from 0.164 to 0.260mg of maltose released/minute/ml of midgut sample. In that maximum amylase activity was found in V instar midgut sample (0.260mg of maltose released/minute/ml of midgut sample). The least amylase activity was found in II instar midgut sample (0.164 mg of maltose released /minute /ml of midgut sample). The values obtained from the test were tabulated against standard maltose values.

Quantitative analysis of protease enzymes: Four different instars (II to V) larval midgut samples were quantitatively analyzed for protease activity and the results were presented in table III. Protease activity of midgut sample ranges from 1.064 to 1.242mg of tyrosine released/minute/ml of midgut sample. In that maximum amylase activity was found in V instar midgut sample (1.242mg of tyrosine released/minute/ml of midgut sample).The least amylase activity was found in II instar midgut sample (1.064 mg of tyrosine released /minute /ml of midgut sample).The values obtained from the tests were tabulated against standard values.

***Estimation of protein in silkgland*:** Fifth instar larval silkgland protein content was analyzed and the results were presented in table IV, which has the ranges from 93.80 to 98.50mg/g of silkgland and the mean value of 96mg/g.In that maximum protein content was observed in sample IV (98.50mg/g) followed by sample III (96.20mg/g).The least protein content was observed in sample I (93.80mg/g). The values obtained from test were tabulated against standard BSA values.

Estimation of protein in larval and pupal haemolymph: Fifth instar larval and pupal haemolymph protein content was analyzed and the results were presented in table IV, larval haemolymph protein has the ranges from 65.50 to 69.32mg/ml and the mean value of 67.23mg/ml. In that maximum protein content was observed in sample III (69.32mg/ml) followed by sample IV (67.40mg/ml). The least protein content was observed in sample II (65.50mg/ml). Pupal haemolymph protein has the range from 52.42 to 56.14 mg/ml and the mean value of 54.65mg/ml. In that maximum protein content was observed in sample IV (56.14mg/ml) followed by sample II (55.34mg/ml). The least protein content was observed in sample III (52.42mg/ml).The values obtained from test were tabulated against standard BSA values.

***Analysis of fibroin and sericin protein ratios in cocoon*:** Fibroin and sericin content of three colored cocoons were analyzed and the results were presented in table V. White colored cocoon has fibroin: sericin ratio of 75.30%: 24.70%. Pale yellow colored cocoon has fibroin: sericin ratio of 74.60%: 25.40%. Dark yellow colored cocoon has fibroin: sericin ratio of 73.80%: 26.20% respectively.

DISCUSSION

Biochemical analysis of six different varieties of mulberry leaves were analyzed among the six varieties V1 contain highest total sugar (16.72%),total protein (26.72%) and total phenol (4.56%).so V1 mulberry leaves is highly recommendable for mulberry silkworm for their increased silk productivity.

Protease and amylase activity of the mulberry silkworm (*Bombyx mori L*) fifth instar larvae feeded with V1 mulberry leaves found to contain high protease, amylase activity and haemolymph, silkgland protein content was also high. Amylase, protease activity of fifth instar larvae mainly for the digestion and absorption of sugar and protein content of the mulberry leaves consequently which will increase haemolymph and silk gland protein content ultimately which will increase silk productivity of the silkworm.

From the above observation we found that V1 mulberry leaves recommended for mulberry silkworm for their increased silk productivity. Weight of the cocoon produced by the silkworm feeded with V1 variety of mulberry leaves was found to be high compare to normal/other cocoon weight and also glycerol content of egg were rapidly increased day by day and glycogen content of the egg were rapidly decreased day by day compared to other eggs this is the better indication of conversion of embryo form to larval form of the silkworm.

Sericulture in India is an important cottage industry based on agro forestry earning foreign exchange worth about Rs.1500 crores per annum. Presently, sericulture is practised in more than 60,000 villages providing employment to 60 lakh people who hail from the weaker section of the society and are in rural areas. Silk production has reached over 15,000 tones and India is the second largest silk producer (18% of world production) after China (69%). Even so, our present production falls very much short of the domestic demand. Nearly 90% of our silk is mori silk or mulberry silk produced by the silkworm *Bombyx mori*. Feeding with V1 variety of mulberry leaves inorder to increase the silk production in the country which decreases our domestic silk demand and silk importing from China.

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REFERENCES

[1] Lowry O.H, Rosebrough N., Farr A. and Randall R.1951.Protein measurement with the folin phenol reagent. *Journal of biological chemistry*.193: 265-275.

[2] Dubios M,Yilles K A.,Hamilton J K.,Robers PA and Smith F.1951.Colorimetric method for determination of sugars and related substances. *Anal Chem.* 28(3):350-353.

[3] Malik C P and Singh M B.1980. *Plant enzymology and histoenzymology*, A test

manual.kalyani publishers, New Delhi .286.

[4] Nagaraju J, Abraham EG**.** 1995. Purification and characterization of digestive amylase from the tasar silkworm, *Antheraea mylitta* (Lepidoptera: Saturniidae). *Comparative Biochemistry and Physiology* 1: 201-209.

[5] Ishimoto M, Yamada T, Kaga A.1999. Insecticidal activity of an α-amylase inhibitor-like protein resembling a putative precursor of α-amylase inhibitor in the common bean, *Phaseolus vulgaris* L. *Biochimical Biophysica Acta* 1432: 104-112.

[6] Eguchi M and Iwamoto A. 1976. Alkaline proteases in the midgut tissue and digestive fluid of the silkworm *Bombyx mori L.* Insect Biochem 6: 491 - 496.