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**Seetharamulu J**
Silkworm Breeding Division, Andhra Pradesh State Sericulture Research and Development Institute, Kirikera, Hindupur, Andhra Pradesh, India

**Anitha Mamillapalli**
Department of Biotechnology GITAM Deemed to be University Visakhapatnam, Andhra Pradesh, India

**Jayashree**
Silkworm Breeding Division, Andhra Pradesh State Sericulture Research and Development Institute, Kirikera, Hindupur, Andhra Pradesh, India

**Alekhya Rani**
Department of Biotechnology GITAM Deemed to be University Visakhapatnam, Andhra Pradesh, India

**Madhavi K**
Silkworm Breeding Division, Andhra Pradesh State Sericulture Research and Development Institute, Kirikera, Hindupur, Andhra Pradesh, India

**PJ Raju**
Silkworm Breeding Division, Andhra Pradesh State Sericulture Research and Development Institute, Kirikera, Hindupur, Andhra Pradesh, India

**Corresponding Author:**
**Seetharamulu J**
Silkworm Breeding Division, Andhra Pradesh State Sericulture Research and Development Institute, Kirikera, Hindupur, Andhra Pradesh, India

# Studies on the amylase activity in the haemolymph of sex-limited silkworm *Bombyx mori* L. breeds for cocoon colour

**Seetharamulu J, Anitha Mamillapalli, Jayashree, Alekhya Rani, Madhavi K and PJ Raju**

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**Abstract**
Enzymes play a very significant role in growth and physiology of silkworm (*Bombyx mori* L.). The growth of the silkworm during larval stage is enormous, increase in growth by size and weight necessitates involvement of various enzymes. Amylase is a hydrolytic enzyme involved in digestion and metabolism of carbohydrates present in the mulberry leaves in the form of starch. Amylase activity is determined by calculating the reducing component volume being released from the soluble starch. The haemolymph amylase was selected for the present study, since it is the only carrier for transport of every substance including amylase and other enzymes. Three different breeds *viz.*, Pure Mysore (Polyvoltine), APS45 (Bivoltine) and SLFC27 (Sex-limited breed for cocoon colour) were selected for estimation of amylase activity. The haemolymph of the selected strain at their 5$^{th}$ instar starting from first day to seventh day was considered to determine the amylase activity. The haemolymph of both the sexes was used for the assay. Increased trend of amylase activity was noted in the haemolymph of all the selected breeds from first day to fifth day which was from a low range of 127.35 µg/20 µL in SLFC27 on first day to a higher range of 427 µg/20 µL in APS45 on fourth day. Amylase activity of PM is highly significant on day 5 when compared to SLFC27 and Amylase activity of SLFC27 is significantly high on the 5$^{th}$ day when compared to APS45 (* $p$<0.05, ** $p$<0.01) indicating that there is a gradual decrease in the amylase activity on day six by 22-25% than fifth day in all the selected breeds as this may be due to maturation of silkworm larvae and leaf consumption getting reduced as they approach for spinning process. The study specifies that, all the breeds showed high enzyme activity during larval stage along with positive correlation with respect to cocoon parameters. The higher amylase activity was recorded during the larval stage of the silkworm and decreased gradually towards spinning stage.

**Keywords:** Amylase enzyme, haemolymph, cocoon colour sex-limited breeds, disease free laying (DFL) diapause and non-diapause strains, silkworm

## Introduction
The silkworm, *Bombyx mori* L. is a completely domesticated and an economically important insect, as it gives luxuriant silk thread through cocoon shells and the larvae fed on mulberry leaves in general. Mulberry leaf quality, genetic traits of silkworms, biotic and abiotic factors influence the growth and metamorphosis of silkworm and the quality of silk cocoon. Besides the commercial exploitation of silkworms, it has greater significance as a laboratory tool.

The growth of the silkworm during larval stage is enormous and increase in growth by size and weight depends on the various enzymes. Silkworm, *Bombyx mori* L. is a monophagous insect consuming mulberry leaves. The amylase activity directly effects the larvae's digestibleness. Amylase, the hydrolytic enzyme has its role in digestion and metabolism of carbohydrates present in the mulberry leaves in the form of starch. The growth, metamorphosis, immune levels against diseases, stresses and survival during several climatic situations are influenced by the enzyme activity (Chatterjee *et al*., 1992) [3]. The haemolymph and digestive fluid of many silkworm breeds contain different forms of amylase enzymes and the activity is seen in digestive juice and haemolymph too. However, the enzymes differ biochemically (Ito *et al*., 1959) [5] and genetically (Matsumura, 1934) [10]. Yokoyama (1959) [20] also has stated that there are two types of amylase activities like in haemolymph and in digestive fluid.

In addition, in many silkworm breeding programmes, digestive amylase, because of its genetic divergence, its role in better digestibility and close association with survival, was used as an excellent marker. Kanekatsu (1972) [7] described that the activity of amylase is higher during active feeding stages of silkworm larvae and becomes low when larvae reach ecdysis stage.

In silkworms, amylase is mainly involved in the carbohydrate metabolism. The two forms of hydrolases namely α-amylase is for starch breakdown and α-glucosidase is for absorption. The alteration or inhibition of these enzymes show significant difference in the complete carbohydrate amounts of the silkworm larvae in haemolymph and gut (Ashutosh and Jha, 2011; Jeroh *et al*., 2011) [2, 6].

In tropical regions like India, Silkworm breeds lay non-diapausing eggs and can be reared throughout the year usually called Polyvoltine breeds which show more surviving capability with shorter larval durations and lesser silk fiber quality. While in temperate regions, like Japan, the Silkmoths lay diapausing eggs and undergo hibernation. These breeds called as bivoltine breeds will show two generations per year and secret long silk fibre. However, they have poor surviving capability under tropical conditions (Tazima, 1991; Murakami, 1989) [16, 21]. The biochemical differences between non-diapausing and diapausing breeds can be well studied for further understanding mechanisms for such differences (Gamo, 1983) [4]. Keeping this in view, the current research is on the differences in amylase enzymatic activities of diapause non-diapause and silkworm sex-limited breeds for cocoon colour.

## Material and Methods

The objective of the present study is to estimate the changes of the amylase activity in the haemolymph of the silkworm, *Bombyx mori* L. to assess the alteration in the amylase enzymatic activities of non-diapausing, diapausing strains and in sex-limited breeds (cocoon colour) of silkworms. The racial features and specific traits of the breeds (Fig 1 & table 1) selected for the study are as follows.

**Pure Mysore:** This is an indigenous polyvoltine silkworm and very popular among the farmers of South India for commercial usage. It has a glorious history of more than two centuries and it has been the foundation for sericulture development in India. At present 80% of the Raw silk produced in South India is by Pure Mysore x Bivoltine (Cross Breed) combination. It is a universal combiner, shows bimodal moth emergence, longer larval period, highly tolerant to vagaries of environmental conditions and its cocoon luster which is expressed in its hybrids also.

**APS45:** It is a productive disease tolerant oval bivoltine parent developed at Andhra Pradesh State Sericulture Research and Development Institute (APSSRDI), Hindupur, Andhra Praadesh. APS45 is a parent of authorized hybrid *i.e.* APS45 x APS12.

**SLFC27:** It is a sex-limited breed for cocoon colour developed at APSSRDI, Hindupur and also the male parent of the new Cross Breed (Seetharamulu *et al.,* 2017) [13]. The special trait of this breed is female silkworm larvae spins yellow colour cocoons and male larvae spins white colour cocoons. This is an added advantage at commercial silkworm egg production centers for separating both the sexes during cross breed eggs preparation which saves labour cost and silk waste.

The disease free layings (DFLs) of Pure Mysore (Polyvoltine), APS45 (Bivoltine) and SLFC27 (Sex-limited breed for cocoon colour) were reared at Andhra Pradesh State Sericulture Research and Development Institute under standard rearing techniques. The larvae were fed as per the rearing protocols with V1 variety mulberry leaf. Three replications were maintained with a count of 250 larvae in each replication. The racial features and the important economic traits of the silkworm *viz*., fecundity and hatching percentage, larval duration, cocoon yield, pupation rate, single cocoon weight, single shell weight, shell percentage were recorded (Table 1 & 2).

**Silkworm economic traits:** The economic traits of the silkworm *viz*., fecundity and hatching percentage, larval duration, cocoon yield, pupation rate, single cocoon weight, single shell weight, shell percentage are important for development/improvement of a race. The following are the formulae to calculate these parameters.

Fecundity: Number of eggs laid by the single moth.

$$\text{Hatching Percentage} = \frac{\text{No. of hatched eggs in the DFL}}{\text{Total number of eggs in the DFL}} \text{ X } 100$$

ERR = Effective Rate of Rearing

$$\text{ERR by weight} = \frac{\text{Wt. of good cocoons harvested (kg)}}{\text{Total no. of larvae retained after } 3^{rd} \text{ moult}} \text{ X } 10000$$

$$\text{Pupation Rate (\%)} = \frac{\text{Total no. of live cocoons (with live Pupae) harvested}}{\text{Total no. of larvae retained after } 3^{rd} \text{ moult}} \text{ X } 100$$

$$\text{Weight of single cocoon (gm)} = \frac{\text{Wt. of 10 male cocoons + Weight of 10 female cocoons}}{\text{Total No. of cocoons (20)}}$$

$$\text{Weight of single shell (gm)} = \frac{\text{Wt. of 10 male cocoon shells + weight of 10 female cocoon shells}}{\text{Total No. of cocoon shells (20)}}$$

$$\text{Shell ratio (\%)} = \frac{\text{Single shell weight (gm)}}{\text{Single cocoon weight (gm)}} \text{ X } 100$$

From the 1st day of the 5th instar to 7th day of the 5th instar larvae, the haemolymph was collected by cutting the abdominal legs of the silkworm larvae in pre-chilled Eppendorf tubes containing the crystals of phenylthiourea to prevent oxidation. After proper dilutions, the samples were centrifuged at 3000 rpm for 10-15 min. the supernatant was collected for further amylase activity estimation.

Considering three replication batches, three test tubes one for each replication was taken having 0.5 ml of hemolymph (20-fold diluted solution in 0.01M phosphate buffer, pH 6.8) and 2 ml of soluble starch solution (2 mg/ml starch; 0.05M NaCl; 0.01 M phosphate buffer pH 6.8). The mixture was incubated for 30 min at 37 ℃ followed by keeping the test tubes in boiling water around 50-60 °C in a water bath for 3min to terminate the reaction. The protein gets coagulated and was subjected to centrifugation. The supernatant-0.5 ml was mixed with 0.5 ml of 3, 5-dinitrosalicylic acid reagent

and the optical density values were taken (Smith and Stocker, 1949) [14]. Breed wise values of the end product maltose were noted using Spectrophotometer at 525 nm (expressed as µgm per 20 µL of haemolymph in 30 min).

**Results and Discussion**

The amylase activity in the haemolymph of selected strains Pure Mysore (Polyvoltine breed), APS45 (Bivoltine breed) and SLFC27 (Sex-limited breed for cocoon colour) was determined from the first day of fifth instar larval up to the seventh day. The haemolymph of mixed sex was used for the assay. Activity levels of amylase enzyme in the haemolymph of PM, APS45 and SLFC27 are presented in Graph 1 and 2. Increased trend of amylase activity was recorded in the haemolymph of all the selected breeds from first day to fifth day from low range of 127.35 µg/20 µL of haemolymph in SLFC27 on first day to high range of 427 µg/20 µL of haemolymph in APS45 on fourth day. The enzyme activity in silkworm haemolymph of PM ranges between 165.58 µg/20 µL (1st day) to 411.36 µg/20 µL (5th day). In case of APS45 the amylase activity was least on day 1 with 149.94 µg/20 µL of haemolymph and highest on day 4 *i.e.*, 427 µg/20 µL. Similarly, in SLFC27 lowest amylase activity was recorded on day 1 of 127.35 µg/20 µL of haemolymph and highest on day 4 was 409.62 µg/20 µL of haemolymph. Since, PM is a polyvoltine breed and have longer larval duration hence highest amylase activity was recorded in the 5th day where as in bivoltine breeds *viz.*, APS45 and SLFC27 the highest amylase activity was noticed on the 4th day of 5th instar, which is very active feeding stage silkworm lifecycle. Amylase activity of PM is highly significant on day 5 when compared to SLFC27 and Amylase activity of SLFC27 is significantly high on day 5 when compared to APS 45 (* $p<0.05$, ** $p<0.01$).

By 22-25% decrease in the amylase activity was recorded during the 6th day than fifth day in all the selected breeds as this may be due to maturation of larvae and leaf consumption get reduced as they approach spinning process. Enzyme activity was observed in all the breeds during larval development. Especially in bivoltine breed (APS45), wherein high amount of enzyme activity was recorded throughout the larval development except on first and second day.

The current study revealed that there is a tremendous increase in the amylase enzymatic activity among all selected silkworm breeds from first day to fifth day of the fifth instar larvae and started decreasing from the next day of the same instar. Other research findings also indicate that, gradual increase in amylase activity was noticed during all the feeding stages of entire larval stage of *Bombyx mori* L (Umakanth and Devamani 2016) [17]. The increased levels of amylase may be due to ingestion and digestion of mulberry leaves consumed by the silkworm larvae which facilitates a suitable environment for absorption and utilization during the growth and developmental process. Wyatt (1967) [19] addressed that, degradation of glycogen in the haemolymph involves haemolymph amylase participation that is released during histolysis. The existence of amylase in profusion during larval stages of both non-diapausing and diapausing breeds infer that amylase has a keen role in carbohydrate metabolism and physiological role in digestion of the polysachharide starch present in mulberry leaves.

In insects, the compound food materials are processed by the action of different digestive juices in the gut to simpler food particles followed by their utilization for various biological processes. Hence, the enzyme system in silkworm too has pivotal role in transforming the organic complex compounds of leaves to the useful small biomolecules. In general, the consumption of mulberry leaf by a silkworm in its fifth instar is more and constitutes to 80% during its larval stage. Simultaneously, the food consumed at this stage effectively supports for the formation of silk proteins (Lokesh *et al*., 2006) [9].

Both haemolymph and midgut digestive enzymes show increased activity in the mid of the fifth instar larval stage. Similar observations were also reported by Tanaka and Kuzhano, 1979 [15]. In the similar manner, the current study also revealed that there is a tremendous increase in the amylase enzymatic activity among all selected silkworm breeds from first day to fifth day of the fifth instar larvae and started decreasing from the next day of the same instar. This might be due to the maximum leaf consumption and presence of more food in the gut part that is stimulating the release of digestive enzymes (Sarangi, 1985; Kumar *et al*., 2011) [12, 8]. The study reveals that, irrespective of the breed, the haemolymph amylase activity was increased throughout feeding stage of the 5th instar and gradually decreased towards spinning as leaf consumption get reduced.

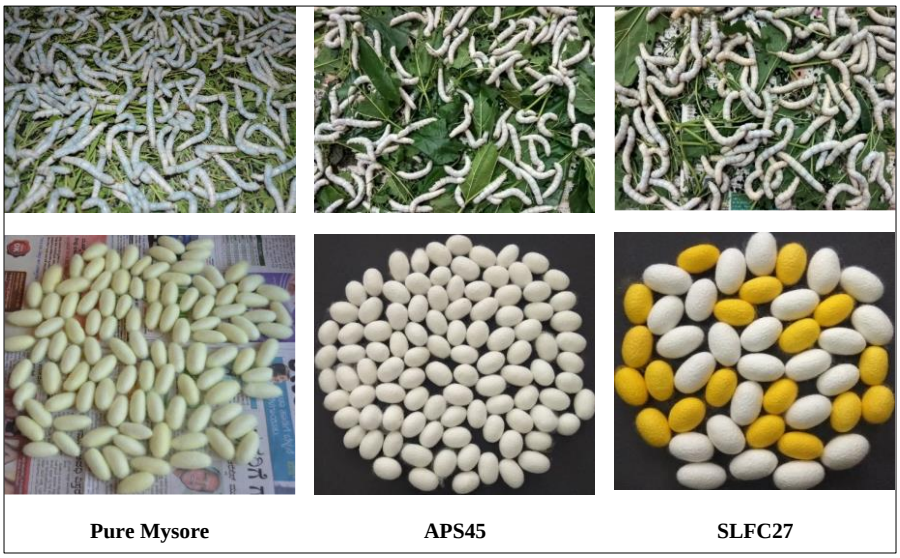


**Fig 1:** Silkworm larvae and cocoons of the three selected breeds

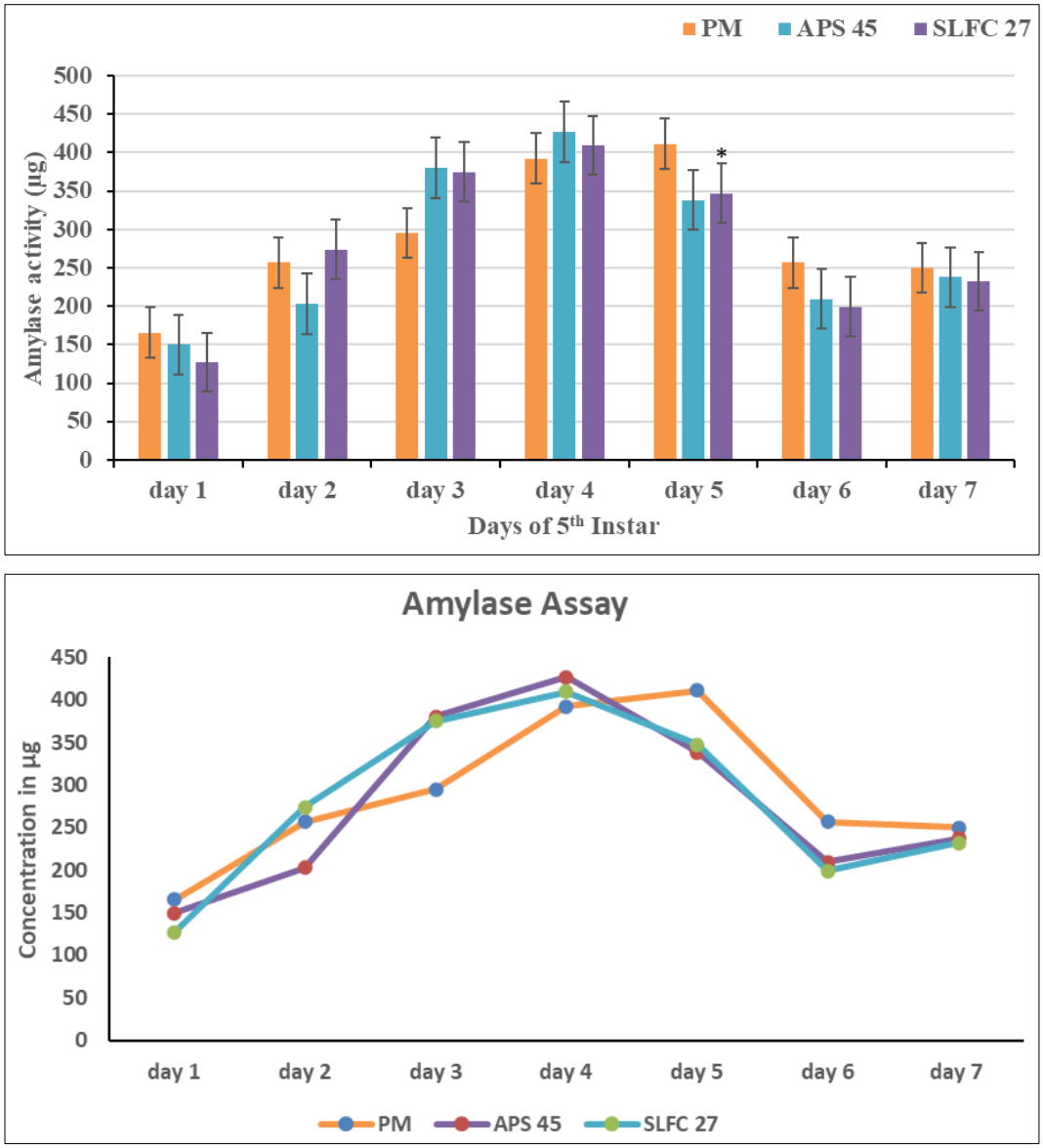


**Fig 2:** Changes in haemolymph amylase activity in various breeds of *Bombyx mori* L. throughout the 5th instar larval stage (day 1-day7). Amylase activity is measured as µg of maltose released per 20 µL of haemolymph in 30 min.

**Table 1:** Racial features silkworm breeds

| Breed | Voltinism | Silkworm Larval Markings | Cocoon characters | | | |
|---|---|---|---|---|---|---|
| | | | shape | Colour | Size | Grains |
| Pure Mysore | Multivoltine | Plain | Spindle | Greenish yellow | Small | Flossy cocoons |
| APS45 | Bivoltine | Plain | Oval shape | White | Medium | Medium |
| SLFC27 | Bivoltine | Plain | Oval shape | Sex-limited breed for cocoon colour (Male larvae spins white & Female larvae spins yellow colour cocoons | Medium | Medium |

**Table 2:** Rearing performance of the selected silkworm breeds

| Breed | Fecundity | Hatching% | Silkworm larval duration (Days: Hours) | Yield/10000 larvae by Wt. (kg) | Pupation rate (%) | Single cocoon wt. (g) | Single shell wt. (g) | Shell ratio (%) |
|---|---|---|---|---|---|---|---|---|
| Pure Mysore | 524±10 | 98.4±1.1 | 26.00±2.5 | 9.74±0.24 | 95.17±2.3 | 1.02±0.03 | 0.157±0.007 | 15.39±0.12 |
| APS45 | 541±20 | 98.0±1.9 | 23.00±1.5 | 16.14±1.1 | 93.67±3.2 | 1.718±0.06 | 0.345±0.006 | 20.08±0.23 |
| SLFC 27 | 528±17 | 97.5±2.2 | 22.12±1.0 | 15.44±0.61 | 97.17±2.4 | 1.570±0.08 | 0.342±0.004 | 21.78±0.16 |

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