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Characterization of Amylase Activity in the Midgut of Silkworm *Bombyx mori* (L.) by using Vermiwash Supplement

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Abstract

The silkworm *Bombyx mori* (L.) is a lepidopteran, monophagous insects that manily feed on mulberry leaves. The midgut is the major organ contains diversity of enzymes to digest the proteins and carbohydrates of mulberry leaves helps in the larval growth, development and silk production. Vermiwash is a watery extract produce from vermicompost contains variety of micro and macronutrients as well as many enzymes like amylase, protease, urease and phosphatase. However, the role of vermiwash on the midgut of silkworm enhances the protein content and regulation of amylase activity in limited. The main aim is Characterization of amylase activity in the midgut of silkworm *B. mori* (L.) by using vermiwash supplement. In the present study, we used vermiwash as supplement with different concentration such as 25 per cent (T1), 50% (T2), 75% (T3), 100% (T4) and (T0) Control. The IV and V instar bivoltine double hybrid (FC1×FC2) silkworm were fed with G4 mulberry leaves treated with different concentration of vermiwash. In this study we find out the protein content in midgut of silkworm and activity of amylase, specific activity of α-amylase, with respect to pH and temperature in midgut of silkworm. The results have shown that treated T4 showed the highest protein content in 3rd and 5th day of IV and V instar larvae (0.514 ± 0.16 mg of protein/gm tissue) and (0.513±0.25 mg of protein/gm tissue) which is significant (***P<0.01). In the midgut of IV and V instar larvae, activity of amylase was maximum in 3rd and 5th day (8.016 ± 0.36×10-6 µmol/min/ml), (9.504 ± 0.18×10-6µmol/min/ml) which is significant (***P<0.01) and specific activity of α-amylase was maximum in 3rd and 5th day of IV and V instar larvae (1.552 ± 0.39×10-7 µmol/ml/mg of protein) (***P<0.001) (2.128 ± 0.36 ×10-6µmol/ml/mg of protein) which is significant (***P<0.01). Further, maximum activity of amylase was observed at pH-9 and temperature 40ºC in both treated and control groups respectively. Hence, the present study would be of special importance to understand the activity of amylase with supplement of vermiwash.

Keywords : *Bombyx mori*, Vermiwash, Midgut, Enzymes, α-Amylase, G4 mulberry leaves

The mulberry silkworm, *B.mori* is a lepidopteran, monophagous insect, which feeds only on mulberry leaves. Insects need carbohydrate as a major component for their growth and development (Muniv *et al.,* 2011). The silkworm has ability to digest, proteins, carbohydrates and fats by digestive enzymes secreted in midgut. Silkworm has almost become an important model for several biochemical, physiological and genetic studies in insect group. The physiological and biochemical studies include general metabolism and morphogenesis in insects, digestion and digestive enzymes, protein synthesis and their metabolism (Yogananda Murthy, 2015). In silkworms, the digestive enzymes in the midgut breakdowns the complex form of nutrients present in the food in to simpler forms. These simpler forms are

easily absorbed in to the body through the semipermeable membrane of alimentary canal (Lokesh *et al.,* 2012). Enzymes play an important role in growth, development, metamorphosis and physiology of silkworm. So, the silkworm size, weight, duration of silkworm larvae depends on the enzyme activity. One such enzyme is an amylase which is a hydrolytic enzyme found in micro-organisms, plants and animals that helps in the digestion and carbohydrate metabolism in insects (Horie and Watanobe, 1980). α-amylase (α-1, 4-glucan 4-glucanohydrolase, E C 3.2.1.1) is one of the de-polymerases which breaks α-1.4-glucan bonds in starch and glycogen (Terra and Ferrira, 2012). The insects which feed on wood, concentration of α-amylase activity is low, where in phytophagous insects, α-amylase is abundant and its activity is high (Slansky, 1982 and Dow, 1984) α-amylase improves the digestive activity of insects leading to survival within different living conditions and increases their biological role and fitness (Kaur *et al*., 2014). The growth, metamorphosis, immune levels of silkworm against diseases, stress and survival during several climatic situations are influenced by the enzyme activity (Chatterjee *et al*., 1992).

Vermiwash is a liquid extract produced from vermicompost. It contains massive vitamins, different bioavaible minerals, hormones, enzymes and anti-microbial peptides (Kasahun *et al.*, 2021). Vermiwash was found to contain enzyme cocktail of proteases, amylase, urease and phosphatase (Purusothaman *et al.,* 2012). Vermiwash is the watery extract of vermicompost extracted in the presence of earthworm and contains several enzymes plant growth hormones, vitamins along with micro and macro nutrients which increase the resistance power of crops (Karuppasamy karthikairaj and Lourdu Isaiarasu 2013). Vermiwash of earthworm have growth stimulating agent and contain variety of micro and macronutrients as well as protein which play an important role in silk production and silk gland development (Dayal doss *et al.,* 2011). Thus, supplementations of nutrients play an important role in metabolic activity of mulberry silkworm (Simi and Asiya Nuzhat, 2022). The nutrients present in vermiwash are in water soluble form and intermediate requirement of a number of components that can be met from a single source (Udhaya Nandhini and Venmathi, 2017). Hence, vermiwash has been used as a supplementary nutrient to silkworm which has improved feeding, decline of diseases and increases the yield of cocoons (Priyadarshini and Yatheesh, 2008). The vermiwash can also be used as feed additives in silkworm rearing for higher performance of economic traits (Dayal doss *et al.,* 2011). So, in the present study, vermiwash is used as a supplement on mulberry leaves to document and to understand the effect of vermiwash on total protein content, total amylase activity, specific α-amylase activity, with respect to pH and temperature in the midgut of $FC_1 \times FC_2$ *B. mori* silkworm. Hence, the present study would be of special interest to the silkworm physiologist to improve the overall larval growth, better cocoon production and protein content by vermiwash supplement.

Material and Methods

To understand the effect and mechanism of vermiwash on enzyme activity in mulberry silkworm, the following standard procedures were employed.

Rearing of Silkworm

The Silkworm eggs of $FC_1 \times FC_2$ (Bivoltine double hybrid) were collected and used from chawkiworm rearing centre, Hollenhalli, Tumakuru, Karnataka, India. and were maintained in silkworm rearing room under laboratory condition (26±2 ºC and 78±2 % RH) by following standard protocol given by Krishnaswami 1978. The larvae were divided in to five experimental groups including the control; each group consists of 100 larvae. The larvae were reared in plastic trays measuring (20×15×5cm), covered with wax paper and placed in a metallic stand with ant wells. The treated and control groups were fed with G4 (*Morus multicaulis* × S-34) mulberry leaves. Feeding was given two times a day (*i.e.,* 9am and 4pm) and this was followed up to end of V instar larvae.

Plate 1 : V instars ($FC_1 \times FC_2$) silkworm larvae
a : Control, b : 25% vermiwash treated silkworms, c : 50% vermiwash treated silkworms,
d : 75% vermiwash treated silkworms e : 100% vermiwash treated silkworms

Preparation of Vermiwash Supplement

The earthworm (*Eudrilus eugeniae*) was used in vermicomposting unit, Durgadhalli, Tumakuru, Karnataka, India.

Mulberry Leaves Treated with Vermiwash

Different concentrations 25% (T_1), 50% (T_2), 75% (T_3) and 100% (T_4) were prepared, fresh G4 (*Morus multicaulis* × S-34) mulberry leaves were separately soaked in these concentrations for 10 min each and were air dried for 10 min and were fed to IV and V instar larvae.

Collection of Tissue and Preparation of Enzyme Extract

In each group, silkworms (n=10) were selected randomly daily from IV instar 1st day till end of V instar and dipped in distilled water for 2-5min and the silkworm were dissected and cleaned in Phosphate buffer saline (PBS) and midgut tissue was collected. The enzyme extract was prepared (Ishaaya and Swiriski, 1970) with slight modification as per lab conditions. The midgut tissue was then homogenized in 1ml of 0.2M Sodium phosphate buffer (pH-7). The homogenate was centrifuged at 8000 rpm for 30 min

TABLE 1
Treatment details

Treatment	Concentration of vermiwash + D/W
T_0	Control (D/W)
T_1	25 ml vermiwash+75ml D/W
T_2	50 ml vermiwash+50ml D/W
T_3	75 ml vermiwash+25ml D/W
T_4	100 ml vermiwash

Plate 3 : G4 mulberry plant

Plate 2: Different Concentrations of Vermiwash

at 4ºC and supernatant was collected and used as an enzyme extract.

Estimation of Protein in Midgut (Lowry *et al.*, 1951)

The concentration of protein present in the midgut, was estimated according to the Lowry's method (Lowry *et al.,* 1951) using Bovine serum albumin (BSA) as protein standard.

Activity Assay of Amylase

The total activity of amylase was performed using the 3, 5-dinitrosalicylic acid (DNSA), *i.e.,* DNS method (Bernfeld, 1955). A volume of 500µl of 1 per cent soluble starch (Substrate pH-6.8) was mixed with 300µl of enzyme extract and 200µl of 0.2M phosphate buffer was added to mixture. This sample preparation was pre-incubated at 37ºC incubated for 15min. In order to stop the reaction, 1000µl of DNS reagent (1gm of 3, 5-dinitrosalicylic acid was dissolved in 30ml of water, 30gm of sodium potassium tartrate was added along with 20ml of 2N NaOH added and made up to 100ml with distilled water) and finally the absorbance was measured at 540nm using UV-visible spectrophotometer. The whole experiment was carried out in triplicates.

Specific Activity Assay of α-Amylase

The specific activity of α-amylase was prepared by Lowry's method. 1ml of enzyme was mixed with 5 ml of Alkaline copper Reagent (Reagent-C) and incubated at room temperature (RT) for 10 min and then 0.6 ml of FC (Reagent-D) (1:2) was added. Incubated at RT for 30 min and absorbance was

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measured at 660nm by using UV-visible spectro photometer.

Effect of Temperature and pH on Amylase Activity

To determine the optimum temperature of amylase activity, the reaction mixture was incubated at different temperature ranges, *i.e.,* 4, 20, 30, 40, 50, 60, 70, 80, 90 and 100ºC for 30 min and also to determine the optimum pH amylase activity reaction mixture was treated by different pH of Phosphate buffer at 4, 5, 6, 7, 8, 9, 10 and 11 pH range for 30 min and readings was recorded by UV-Visible spectrophotometer.

Statistical Data and Analysis

The obtained data were analysed by one way ANOVA followed by Tukey's multiple comparison test for total protein content, total and specific activity by using Graph Pad Prism software (GPPS) 8.3.0. All values were represented as Mean ± SD values and were significant difference were found at (*P<0.05), (** P<0.01) and (*** P<0.001) respectively for correlation.

Results and Discussion

Total Protein Content in the Midgut

Protein content in midgut of IV and V instar silkworm was analysed and expressed in mg of protein/gm tissue. The protein content was observed on 1st day of IV instar larvae control (T_0) group that showed (0.066 ± 0.25 mg of protein/gm tissue), in treated groups T_1 (0.177 ± 0.68 mg of protein/gm tissue), T_2 (0.196 ± 0.45 mg of protein/gm tissue), T_3 (0.367 ± 0.14 mg of protein/gm tissue) and T_4 showed highest protein content (0.461 ± 0.28 mg of protein/gm tissue), which is significant (***P<0.01). A gradual increase in the protein content was observed in all treated groups of IV instar Table 2 and Fig. 1 but attain its maximum activity on 3rd day of IV instar T_0 (0.127 ± 0.12 mg of protein/gm tissue), T_1 (0.236 ± 0.96 mg of protein/gm tissue), T_2 (0.261 ± 0.69 mg of protein/gm tissue), T_3 (0.421 ± 0.87 mg of protein/gm tissue) and T_4 showed highest protein content (0.514 ± 0.16 mg of protein/gm tissue). Protein content in T_0 group of V instar larvae is summarised and presented in Table 3 and Fig. 2 on the 1st day (0.076 ± 0.15 mg of protein/gm tissue) was observed and in treated groups T_1 (0.233 ± 0.88 mg of protein/gm tissue), T_2 (0.264 ± 0.69 mg of protein/gm tissue), T_3 (0.398 ± 0.32 mg of protein/gm tissue) and T_4 (0.513 ± 0.25 mg of protein/gm tissue) which is significant (***P<0.01). A significant increases in the protein content was

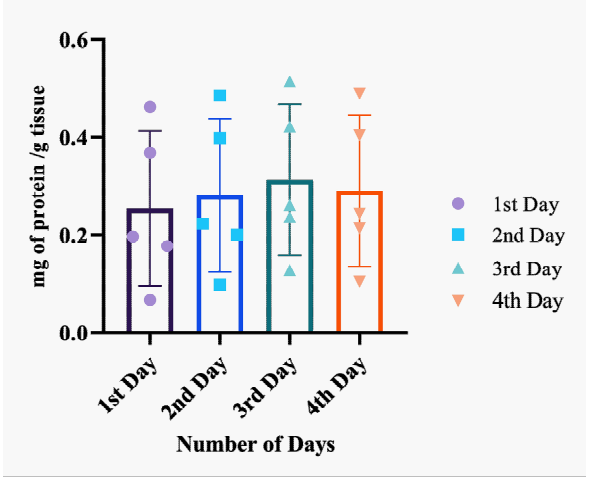

Fig. 1 : Effect of vermiwash on protein content in the midgut of IV instar silkworm

Table 2

Concentration of Protein in the Midgut of Silkworm, *B. mori* IV instars larvae

	1st Day	2nd Day	3rd Day	4th Day
Control (T_0)	0.066 ± 0.25	0.098 ± 0.86	0.127 ± 0.12	0.104 ± 0.28
25% (T_1)	0.177 ± 0.68	0.200 ± 0.58	0.236 ± 0.96	0.213 ± 0.73
50% (T_2)	0.196 ± 0.45	0.222 ± 0.98	0.261 ± 0.69	0.243 ± 0.47
75% (T_3)	0.367 ± 0.14	0.398 ± 0.95	0.421 ± 0.87	0.40 ± 0.69
100% (T_4)	0.461 ± 0.28	0.485 ± 0.34	0.514 ± 0.16	0.489 ± 0.66

TABLE 3

Concentration of Protein in the Midgut of Silkworm, *B. mori* V instar larvae

	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
Control (T_0)	0.076 ± 0.15	0.092 ± 0.66	0.118 ± 0.95	0.154 ± 0.12	0.172 ± 0.72	0.169 ± 0.21	0.157 ± 0.15
25% (T_1)	0.233 ± 0.88	0.252 ± 0.55	0.279 ± 0.52	0.300 ± 0.39	00.335 ± 0.58	0.324 ± 0.26	0.307 ± 0.22
50% (T_2)	0.264 ± 0.69	0.285 ± 0.41	0.315 ± 0.61	0.342 ± 0.54	0.354 ± 0.65	0.343 ± 0.44	0.325 ± 0.57
75% (T_3)	0.398 ± 0.32	0.438 ± 0.62	0.467 ± 0.13	0.491 ± 0.26	0.528 ± 0.33	0.517 ± 0.59	0.504 ± 0.36
100% (T_4)	0.513 ± 0.25	0.540 ± 0.99	0.569 ± 0.31	0.586 ± 0.35	0.633 ± 0.21	0.620 ± 0.67	0.598 ± 0.59

observed in control and all treated groups attained its maximum activity on 5th day V instar T_0 (0.172 ± 0.72 mg of protein/gm tissue), T_1 (0.335 ± 0.58 mg of protein/gm tissue), T_2 (0.354 ± 0.65 mg of protein/gm tissue), T_3 (0.528 ± 0.33 mg of protein/gm tissue) and T_4 (0.633 ± 0.21 mg of protein/gm tissue).

Total Activity of Amylase

The total activity of amylase was expressed in terms of amount of maltose released per µmol/min/ml. G4 mulberry leaves were treated with different concentration of vermiwash and were fed to the IV and V instar larvae. The total activity of amylase in midgut of IV instar larvae based on experiment, the increased amylase activity in the midgut of silkworm is summarized and presented in Table 4 and Fig. 3. A steady increase in the activity of amylase was observed both in control and treated groups. The activity of amylase was observed on 1st day of IV instar larvae was in T_0 (2.958 ± 0.07×10^{-6} µmol/min/ml), T_1 (4.067 ± 0.015×10^{-6} µmol/min/ml), (ns) T_2 (4.473

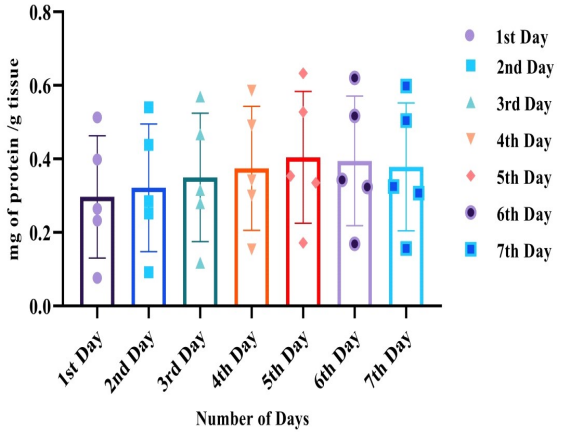

Fig. 2 : Effect of vermiwash on protein content in the midgut of V instar silkworm

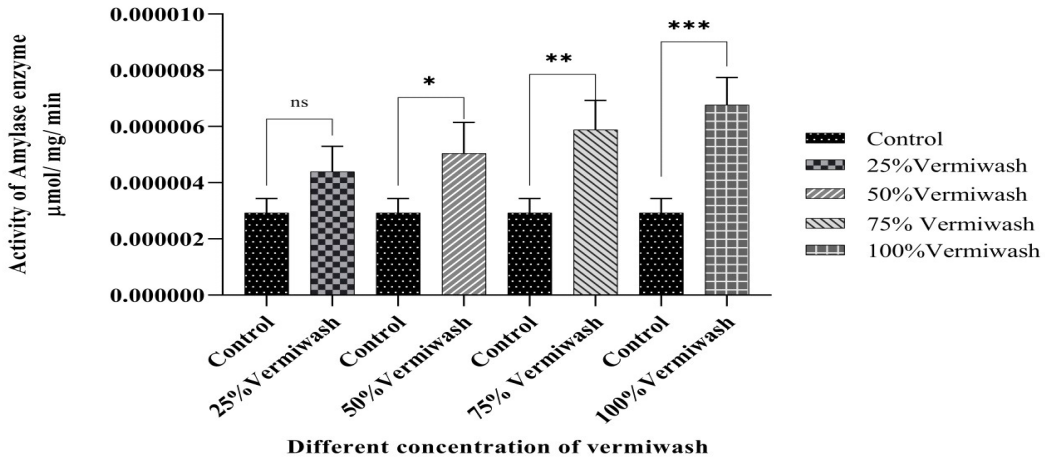

Fig. 3 : Effect of vermiwash on activity of amylase in the midgut of IV instar silkworm *B. mori*
NS-Non-significant,*P<0.05, **P<0.01, ***P<0.001

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TABLE 4

Total activity of Amylase using DNS Method in IV Instar Larva (Day 1 to Day 4)

	1st day		2nd day		3rd day		4th day	
	OD/ Concentration	Activity	OD/ Concentration	Activity	OD/ Concentration	Activity	OD/ Concentration	Activity
Control (T_0)	0.139/273.5	2.958 ± 0.07×10 $^{-6}$	0.145/288.5	3.121 ± 0.12×10 $^{-6}$	0.156/316	3.418 ± 0.23 ×10 $^{-6}$	0.112/206	2.228 ± 0.06×10 $^{-6}$
25% (T_1)	0.180/376	4.067 ± 0.15×10 $^{-6}$	0.198/421	4.554 ± 0.32×10 $^{-6}$	0.235/513.5	5.555 ± 0.36 ×10 $^{-6}$	0.156/316	3.418 ± 0.09×10 $^{-6}$
50% (T_2)	0.195/413.5	4.473 ± 0.201×10 $^{-6}$	0.225/488.5	5.285 ± 0.12 ×10 $^{-6}$	0.269/598.5	6.475 ± 0.42 ×10 $^{-6}$	0.176/366	3.959 ± 0.06 ×10^{-6}
75% (T_3)	0.235/513.5	5.555 ± 0.514×10 $^{-6}$	0.256/566	6.123 ± 0.22 ×10 $^{-6}$	0.295/663.5	7.178 ± 0.53 ×10 $^{-6}$	0.203/433.5	4.690 ± 0.02 ×10^{-6}
100% (T_4)	0.262/581	6.285 ± 0.041×10 $^{-6}$	0.289/648.5	7.016 ± 0.02 ×10 $^{-6}$	0.326/741	8.016 ± 0.36 ×10 $^{-6}$	0.243/533.5	5.771 ± 0.01 ×10^{-6}

OD-Optical Density @540nm, Concentration -µmol of maltose released, Activity-µmol of maltose/mg of tissue/min, Each value is represented as Mean ±SD, n=10

TABLE 5

Total activity of Amylase using DNS Method in V Instar Larva (Day 1 to Day 7)

	1st day		2nd day		3rd day		4th day		5th day		6th day		7th day	
	OD/ Concentration	Activity	OD/ Concentration	Activity	OD/ Concentration	Activity	OD/ Concentration	Activity	OD/ Concentration	Activity	OD/ Concentration	Activity	OD/ Concentration	Activity
Control (T_0)	0.123/ 233.5	2.526 ± 0.12 ×10^{-6}	0.165/ 338.5	3.662 ± 0.56 ×10^{-6}	0.182/ 381	4.122 ±0.32 ×10^{-6}	0.199/ 423.5	4.581 ± 0.33 ×10^{-6}	0.256/ 566	6.123 ± 0.32 ×10^{-6}	0.243/ 533.5	5.771 ± 0.17 ×10^{-6}	0.221/ 478.5	5.176 ± 0.09 ×10^{-6}
25% (T_1)	0.169/ 348.5	3.770 ± 0.26 ×10^{-6}	0.194/ 411	4.446 ± 0.23 ×10^{-6}	0.240/ 526	5.609 ±0.65 ×10^{-6}	0.268/ 596	6.448 ± 0.21 ×10^{-6}	0.293/ 658.5	7.124 ± 0.24 ×10^{-6}	0.276/ 616	6.664 ± 0.20 ×10^{-6}	0.241/ 528.5	5.717 ± 0.33 ×10^{-6}
50% (T_2)	0.186/ 391	4.230 ± 0.21 ×10^{-6}	0.218/ 471	5.095 ± 0.13 ×10^{-6}	0.273/ 608.5	6.583 ±0.10 ×10^{-6}	0.295/ 663.5	7.178 ± 0.19 ×10^{-6}	0.332/ 756	8.179 ± 0.36 ×10^{-6}	0.316/ 716	7.746 ± 0.32 ×10^{-6}	0.292/ 656	7.097 ± 0.30 ×10^{-6}
75% (T_3)	0.224/ 486	5.258 ± 0.03 ×10^{-6}	0.264/ 586	6.339 ± 0.11 ×10^{-6}	0.299/ 673.5	7.286 ±0.26 ×10^{-6}	0.342/ 781	8.449 ± 0.28 ×10^{-6}	0.363/ 833.5	9..017 ± 0.23 ×10^{-6}	0.344/ 786	8.503 ± 0.39 ×10^{-6}	0.312/ 706	7.638 ± 0.31 ×10^{-6}
100% (T_4)	0.257/ 568.5	6.150 ± 0.36 ×10^{-6}	0.296/ 666	7.205 ± 0.07 ×10^{-6}	0.333/ 758.5	8.206 ±0.12 ×10^{-6}	0.359/ 823.5	8.909 ± 0.15 ×10^{-6}	0.381/ 878.5	9.504 ± 0.18 ×10^{-6}	0.369/ 848.5	9.179 ± 0.37 ×10^{-6}	0.340/ 776	8.395 ± 0.29 ×10^{-6}

D-Optical Density @540nm, Concentration -µmol of maltose released, Activity-µmol of maltose/mg of tissue/min, Each is value represented as Mean ±SD, n=10

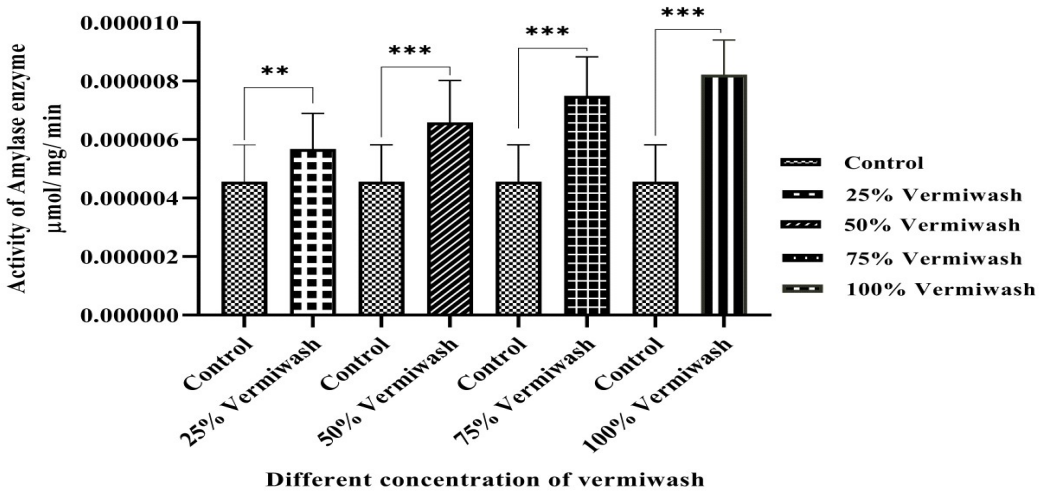

Fig. 4 : Effect of vermiwash on amylase activity in the midgut of V instar silkworm *B. mori*
NS-Non-significant,*P<0.05, **P<0.01, ***P<0.001

± 0.20×10^{-6} µmol/min/ml) (*p<0.05), T_3 (5.555 ± 0.514×10^{-6} µmol/min/ml) (**p<0.01) and T_4 (6.285 ± 0.041×10^{-6} µmol/min/ml) it was found to be significantly highest activity in T_4 at (***P<0.001) which were gradually increase in the amylase activity. However in 2nd day also there was increase in the activity of amylase in both control and all treated groups, on the 3rd day of IV instar maximum activity was seen in T_0 (3.148 ± 0.023 $\times10^{-6}$ µmol/min/ml), T_1(5.555 ± 0.36 × 10^{-6}µmol/min/ml), T_2(6.475 ± 0.42 × 10^{-6}µmol/min/ml), T_3(7.178 ± 0.53 × 10^{-6} µmol/min/ml) and T_4 (8.016 ± 0.36 × 10^{-6} µmol/min/ml) on 4th day of IV instar there was decrease in the amylase activity in all control and treated groups which might be due to stoppage of feeding to undergo moult. Beside vermiwash was also responsible for increase in the activity of amylase in the midgut during IV instar. The total activity of amylase in midgut of V instar silkworm was analysed. In general irrespective of the silkworm fed with different concentrations of vermiwash, the 1st day of V instar larvae activity of amylase was less in T_0 (2.526 ± 0.12×10^{-6} µmol/min/ml), and more in T_1 (3.770 ± 0.26×10^{-6} µmol/min/ml) (**p<0.01), T_2 (4.230 ± 0.21×10^{-6} µmol/min/ml) (***P<0.001), T_3 (5.258 ± 0.03×10^{-6} µmol/min/ml) (***P<0.001) and T_4 (6.150 ± 0.36×10^{-6} µmol/min/ml) (***P<0.001) Table 5 and Fig. 4. However it was observed that on 5th day of V instar showed maximum activity of amylase. The 100 per cent vermiwash treated T_4 (9.504 ± 0.18×10^{-6} µmol/min/ml), silkworm showed higher amylase activity which was on par with T_3 (9.017 ± 0.23×10^{-6} µmol/min/ml) followed by T_2 (8.179 ± 0.36×10^{-6} µmol/min/ml), T_1 (7.124 ± 0.24×10^{-6} µmol/min/ml) and lowest activity of amylase was observed in control and the gradually decrease in the activity was due to less feeding stage of silkworms and readiness for cocoon formation.

Specific Activity of α-Amylase

The specific activity of α-amylase was analysed in the midgut of IV and V instar larvae and it was expressed in µmol/ml/mg of protein released. In general irrespective of the silkworm fed with different concentrations of vermiwash. The α-amylase activity was less in the T_0 (0.2246 ± 0.31×10^{-7} µmol/ml/mg of protein) on 1st day of IV instar and gradually increased in all treated groups T_1(0.5960 ± 0.26×10^{-7} µmol/ml/mg of protein) (***P<0.001), T_2(0.6612 ± 0.03×10^{-7} µmol/ml/mg of protein) (***P<0.001), T_3 (1.237 ± 0.85×10^{-7} µmol/ml/mg of protein) (***P<0.001) and T_4 (1.552 ± 0.39×10^{-7} µmol/ml/mg of protein) (***P<0.001). The activity of α-Amylase attained maximum at 3rd day of IV instar (Table 6 and Fig. 5). V instar larvae also showed gradually increase in

α-amylase activity on the 1st day in control and all treated groups. T_4 (1.724 ± 0.91×10^{-6} µmol/ml/mg of protein) (***P<0.001) showed highest activity of α-amylase followed by T_3 (1.33 ± 0.95×10^{-6} µmol/ml/mg of protein) (***P<0.001), T_2 (8.886 ± 0.65 × 10^{-7} µmol/ml/mg of protein) (***P<0.001), T_1 (7.82 ± 0.46 ×10^{-7} µmol/ml/mg of protein) (***P<0.001), and low enzyme activity was observed in T_0 (2.569 ± 0.23×10^{-7} µmol/ml/mg of protein) (***P<0.001) Table 7 and Fig. 6.

However, it was observed that on 5th day of V instar showed maximum activity of á-amylase, in T_4 (2.128 ± 0.36 × 10^{-6} µmol/ml/mg of protein), (***P<0.001), which was on part with T_3 (1.775 ± 0.55×10^{-6}µmol/ml/mg of protein) (***P<0.001), followed by T_2 (1.144 ± 0.2×10^{-6} µmol/ml/mg of protein) (***P<0.001), T_1 (1.125 ± 0.24×10^{-6}µmol/ml/mg of protein) (***P <0.001), Lowest enzyme activity observed in T_0 (5.775 ± 0.65 ×10^{-7} µmol/ml/mg of protein).

TABLE 6

Specific activity of á-Amylase using Lowry's Method in IV Instar Larva (Day1 to Day4)

	1st day		2nd day		3rd day		4th day	
	OD/ Concentration	Activity	OD/ Concentration	Activity	OD/ Concentration	Activity	OD/ Concentration	Activity
Control (T_0)	0.055/ 66.8	0.2246 ± 0.31 × 10^{-7}	0.078/ 98.5	3.313 ± 0.26 ×10^{-7}	0.099/ 127.5	4.288 ± 0.59 ×10^{-7}	0.082/ 104.1	3.501 ± 0.42 ×10^{-7}
25% (T_1)	0.135/ 180	0.5960 ± 0.26 ×10^{-7}	0.152/ 200.7	6.690 ± 0.28 ×10^{-7}	0.178/ 236.6	7.958 ± 0.39 ×10^{-7}	0.161/ 213.1	7.167 ± 0.83 ×10^{-7}
50% (T_2)	0.149/ 196.6	0.6612 ± 0.03 ×10^{-7}	0.168/ 222.8	7.494 ± 0.16 ×10^{-7}	0.196/ 261.4	8.713 ± 0.36 ×10^{-7}	0.183/ 243.5	8.190 ± 0.38 ×10^{-7}
75% (T_3)	0.273/ 367.8	1.237 ± 0.85 ×10^{-7}	0.295/ 398.1	1.339 ± 0.20 ×10^{-7}	0.312/ 421.6	1.418 ± 0.49 ×10^{-7}	0.299/ 403.7	1.357 ± 0.18 ×10^{-7}
100% (T4)	0.341/ 461.6	1.552 ± 0.39 ×10^{-7}	0.358/ 485.1	1.631 ± 0.69 ×10^{-7}	0.379/ 514.1	1.729 ± 0.32 ×10^{-7}	0.361/ 489.3	1.645 ± 0.14 ×10^{-7}

OD-Optical Density @660nm, Concentration -µmol of protein released, Activity-µmol/ml/mg of protein ,
Each value is represented as Mean ± SD, n=10

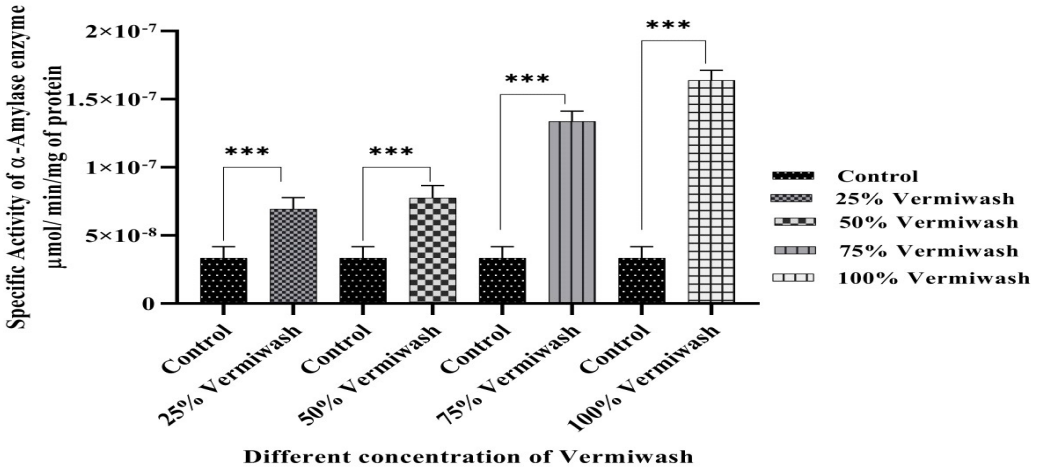

Fig. 5 : Effect of vermiwash on specific activity α- amylase in the midgut of IV instars Silkworm *B. mori*
NS-Non-significant,*P<0.05, **P<0.01, ***P<0.001

TABLE 7

Specific activity of á-Amylase using Lowry's Method in V Instar Larva (Day1 to Day7)

	1st day		2nd day		3rd day		4th day		5th day		6th day		7th day	
	OD/ Concentration	Activity	OD/ Concentration	Activity	OD/ Concentration	Activity	OD/ Concentration	Activity	OD/ Concentration	Activity	OD/ Concentration	Activity	OD/ Concentration	Activity
Control (T0)	0.062/ 76.4	2.569 ± 0.23 $\times10^{-7}$	0.073/ 91.6	3.081 ± 0.29 $\times10^{-7}$	0.092/ 117.9	3.965 ± 0.32 $\times10^{-7}$	0.118/ 153.8	5.173 ± 0.16 $\times10^{-7}$	0.131/ 171.7	5.775 ± 0.65 $\times10^{-7}$	0.129/ 168.9	5.681 ± 0.15 $\times10^{-7}$	0.120/ 156.5	5.264 ± 0.69 $\times10^{-7}$
25% (T1)	0.175/ 232.5	7.82 ± 0.46 $\times10^{-7}$	0.189/ 251.8	8.469 ± 0.12 $\times10^{-7}$	0.209/ 279.4	9.398 ± 0.95 $\times10^{-7}$	0.224/ 300.1	1.009 ± 0.37 $\times10^{-6}$	0.249/ 334.6	1.125 ± 0.24 $\times10^{-6}$	0.241/ 323.6	1.088 ± 0.27 $\times10^{-6}$	0.229/ 307.0	1.032 ± 0.25 $\times10^{-6}$
50% (T2)	0.198/ 264.2	8.886 ± 0.65 $\times10^{-7}$	0.213/ 284.9	9.583 ± 0.57 $\times10^{-7}$	0.235/ 315.3	1.060 ± 0.17 $\times10^{-6}$	0.254/ 341.5	1.148 ± 0.22 $\times10^{-6}$	0.263/ 353.9	1.190 ± 0.13 $\times10^{-6}$	0.255/ 342.9	1.153 ± 0.46 $\times10^{-6}$	0.242/ 325.0	1.093 ± 0.85 $\times10^{-6}$
75% (T3)	0.295/ 398.1	1.33 ± 0.95 $\times10^{-6}$	0.324/ 438.2	1.473 ± 0.32 $\times10^{-6}$	0.345/ 467.2	1.571 ± 0.73 $\times10^{-6}$	0.362/ 490.6	1.650 ± 0.65 $\times10^{-6}$	0.389/ 527.9	1.77 5 ± 0.55 $\times10^{-6}$	0.381/ 516.9	1.738 ± 0.66 $\times10^{-6}$	0.372/ 504.4	1.696 ± 0.48 $\times10^{-6}$
100% (T4)	0.378/ 512.7	1.724 ± 0.91 $\times10^{-6}$	0.398/ 540.3	1.817 ± 0.27 $\times10^{-6}$	0.419/ 569.3	1.914 ± 0.29 $\times10^{-6}$	0.431/ 585.9	1.970 ± 0.33 $\times10^{-6}$	0.465/ 632.8	2.128 ± 0.36 $\times10^{-6}$	0.456/ 620.4	2.08 6± 0.22 $\times10^{-6}$	0.440/ 598.3	2.012 ± 0.67 $\times10^{-6}$

OD-Optical Density @660nm, Concentration -µmol of protein released, Activity µmol/ml/mg of protein,
Each value is represented as Mean ±SD, n=10

Effect of Temperature and pH on Amylase Activity

The optimum temperature was recorded at 40ºC in both treated and control groups. The enzyme activity gradually increased and attained maximum activity at 40ºC and decreased with increase in temperature (Fig. 7). The activity of amylase was maximum at pH-9 in all treated and control groups (Fig. 8).

Vermiwash is a liquid used as a foliar spary and contains major nutrients like Mg, Cl, Ca, organic carbon, Nitrate, P and carbon (Jaikishun *et al.,* 2018) and also biochemical components like protein, carbohydrates, lipids, amino acids and other nutrients are also found in vermiwash (Ansari & Jaikishun 2010 and Ansari, 2012). The vermiwash as a supplement is rich is micro and macro nutrients, enzymes and proteins which play crucial role in growth, development and physiological activities in mulberry silkworm. In the present study, we have used the vermiwash as a supplement to see its effect on overall metabolism of silkworm. In this paper, we found out the effect of vermiwash on protein content in midgut of IV and V instar silkworm which showed significant increase in 25, 50, 75 and 100 per cent groups followed by control group respectively. The maximum protein content in the midgut of silkworm was due to supplement of vermiwash in IV and V instar larvae. 80-85 per cent of leaves were consumed during 3rd day of IV instar and 5th day in V instar larvae. Ananda kumar and Ann Sandhya Michael 2012, observed that the increase in the protein content in midgut of silkworm that fed on 1 per cent foliar applicant treated mulberry leaves. Purusothaman *et al*., 2012, reported that biochemical composition of mulberry leaves enriched with vermiwash and control, the levels of carbohydrates, protein and lipid is greater in vermiwash enriched leaves. The level of enzymes such as amylase, alkaline phosphatase and acid phosphatase in fifth instar larvae showed significant increase when they were fed with vermiwash enriched leaves. Yogananda murthy, 2015 observed active absorption of food constituents by the midgut and increase in assimilation and conversion rates during V instar larval development.

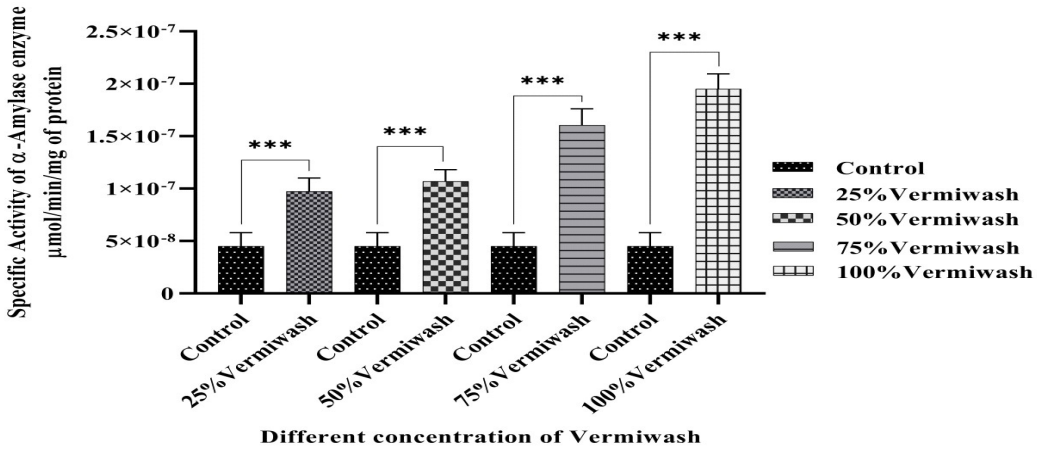

Fig. 6 : Effect of vermiwash on specific activity α- amylase in the midgut of V instar Silkworm *B. mori*
NS-Non-significant,*P<0.05, **P<0.01, ***P<0.001

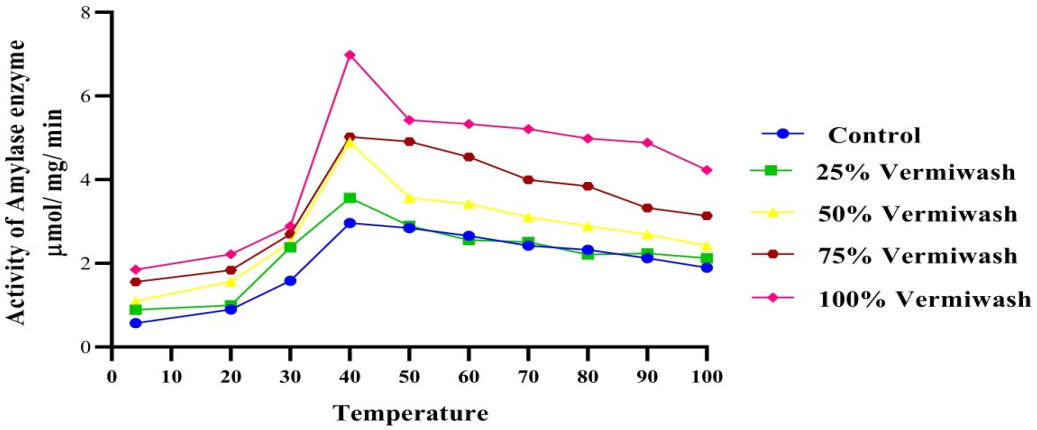

Fig. 7 : Effect of Temperature on activity of amylase in midgut of silkworm *B. mori* in IV and V instar larvae

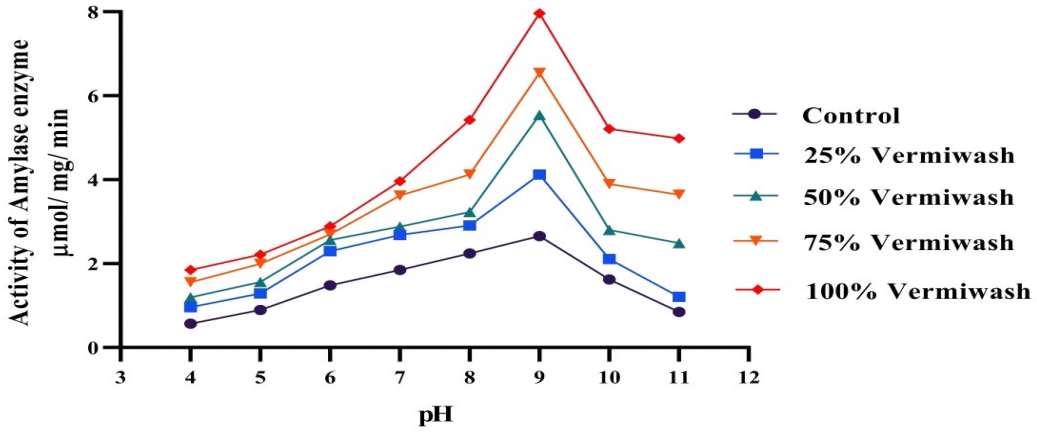

Fig. 8: Effect of pH on activity of amylase in midgut of silkworm *B. mori* in IV and V instar larvae

We investigated the effect of vermiwsah on activity of amylase in midgut of silkworm *B. mori* and as per our results obtained there was a gradual increase in activity of amylase and attained its maximum range at 3rd day of IV instar larva and on 5th day of V instar larvae respectively. The results are in unanimity with other studies, though their supplement was different. In the earlier studies, digestive amylase activity attained its peak on the 5th day of V instar larvae in diapausing and non diapausing strains of silkworm (Ganie *et al*., 2017). The effect of 7.5 per cent *Vigna unguiculata* supplementation with mulberry on the midgut of Urease, Amylase, Sucrose, Protease and Trehalose activities of silkworm breed LNB_4, D2 showed significant increases when compared with control group (Manjula *et al.,* 2011). Gradual increase in amylase activity was noticed during all the feeding stages of entire larval stages of *B. mori*. (Umakanth & Devamani 2022 and Seetharamula *et al.,* 2022). The midgut amylase activity in larvae [(CSR6×CSR26) × (CSR2×CSR27)] fed with leaves of M5 variety treated with aqueous solution of Eurhodin powder with 25.0ppm (6.869 ± 1.2174), 50.0ppm (7.549 ± 2.320) 75.00ppm (7.719 ± 3.31) and 100ppm (8.539 ± 3.78) had shown significant improvement in the activities of amylase in midgut (Samiksha *et al*., 2019). In silkworm during their development and metamorphosis, amylase converts starch to maltose that results in glucose by α-glycosidase to meet the energy demands (Sheeba praveena and Savithri 2021).

In our study, the activity of midgut amylase was highest in 100 per cent vermiwash treated leaves fed by silkworm (FC_1× FC_2) which could explain that vermiwash contains different enzymes like protease, amylase, urease and phosphatase needed for the growth and development of silkworm. (Sumit Sow and Shivani Ranjan, 2021).Vermiwash contains various enzyme mixtures of protease, amylase, urease and phosphatase, similar finding was observed by (Zambare *et al*., 2008), vermiwash contain various enzymes such as protease, amylase, urease and phosphatase. Similar result was reported on plant crops which resulted in increased crop yield and showed diseased resistance by applying vermiwash in different concentrations (Purusothaman *et al.,* 2012). Even vermiwash contain growth stimulating agent and a variety of polypeptides, micro and macro nutrients and proteins, which play an important role in silk production and silk gland development. The activity of amylase pH range from 4-11 but in the present study the optimum pH was 9 (Alkaline) in all treated groups along with control, whereas Nijagal *et al.,* 2017 also reported different pH in treated groups (Corn, soya, Horse gram flour) that showed decreased in activity of amylase when compared with control group. Amylase activity of dipausing and non-diapausing strain is pH-9.2 which falls under alkaline range (Abraham *et al*., 1972). In other lepidopteron, insects *Chilo supressalis* (Zibaee *et al.,* 2008) *Glyphoder pyloalis* (Yezdani *et al*., 2010), *Cameraria ohridells* (Stygal *et al.,* 2010) amylase activity falls in alkaline range. However, in lepidopteron insects, digestive tract shows higher activity of amylase in alkaline pH and it would be because of presence of alkaline RNQ (Arg, Asn and Gln) (Terra and Ferreria 2012). It may be inferred that optimum pH for amylase activity in midgut of silkworm is alkaline. In case of optimum temperature, maximum α-amylase activity was on *Balatella aresmanica* with 50ºC (Applebaum, 1985) (Kanekatysa, 1978) and 37ºC in *Glyphoder pyloalis* (Yezdani *et al.,* 2010), In Nistari and Kolar gold optimum temperature was 60ºC (Muniv *et al.,* 2011). In the present study the optimum temperature of α-amylase activity was 40ºC for all treated and untreated in the experiment and it is presumed that carbohydrate metabolism occurs in optimum temperature.

The present study was carried out to assess the supplement of vermiwash which increased protein content as well as amylase enzyme activity in the midgut of FC_1× FC_2 (Bivoltine double hybrid). Vermiwash is a watery extract of earthworm which contains many beneficial biochemical components like proteins, carbohydrates, amino acids and also it contain many enzymes like amylase, protease, urease and phosphatase.These enzymes which were responsible for converting the mulberry leaf protein and carbohydrates as essentials nutrients for the

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growth and developments of silkworm. Result revealed that 100 per cent vermiwash treated group of silkworm has shown promising results of increased protein content and activity of amylase enzyme. In spite of this, control group had low level of protein content and amylase activity, Hence, it is concluded that supplement of vermiwash with 100 per cent concentration to silkworm will have beneficial effects in growth, development, metamorphosis and disease resistance. Further, intensifying the extension activities helps in improving the knowledge level of enzymes activity correlated with larval length, larval weight, larval duration, cocoon weight and silk filament length. Being an economical crop, silkworm helps to increase farmer returns for producing good quality cocoons under different nutritional supplements.

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