



Analysis of gut juice biochemical profiles in different silkworm (*Bombyx mori*) breeds and their correlation with economic traits

MOHD YOUNUS WANI^{1*}, NISAR AHMAD GANIE¹, MUSHTAQ RASOOL MIR¹, ASIF RAFIQ¹ and KHURSHEED AHMAD SAHAF¹

College of Temperate Sericulture, Sher-e-Kashmir University of Agricultural Sciences and Technology-Kashmir, Mirgund, Baramulla, Jammu and Kashmir 193 121, India

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ABSTRACT

The lack of strong silkworm (*Bombyx mori* L.) breeds in Jammu and Kashmir is a major obstacle to achieve optimal cocoon yields. This study introduces standardised methodology for biochemical assessment of digestive enzymes in bivoltine silkworms by employing a chloroform-induced gut juice collection protocol with a defined effective exposure window. The collected gut fluid was normalised by protein content to ensure comparability across 10 distinct breeds. Temporal profiling of key digestive enzymes - amylase, protease, alkaline phosphatase and acid phosphatase throughout the 5th instar was performed and integrated with economic traits using correlation and cluster analyses. Significant differences were found between breeds, with SK1 showing the highest protein concentration at 42.76 mg/mL, along with the highest enzyme activities followed by other breeds. The temporal analysis indicated peak amylase and protein levels on the 5th day, whereas protease and phosphatase levels peaked on the 7th day. This integrative biochemical-economic framework provides specific reference for identifying enzyme-based markers linked to larval vigour and cocoon productivity in Kashmir. The study identifies enzymatic activity as possible biochemical markers for undertaking breeding programmes to evolve new breeds that are resilient with high cocoon yields.

Keywords: Biochemical, Climate, Cocoon, Sericulture, Temporal

The silkworm, (*Bombyx mori* L.), is an economically important sericigenous insect, which feeds solely on mulberry leaves and transforms their proteins into silk (Babu *et al.* 2009). Most factors determining the production of silk are polygenic in character (Konate *et al.* 2011). The larval digestive system is a straight tube made up of the foregut, midgut and hindgut, each with different functions (Wu *et al.* 2010). The foregut contributes to the intake of food, grinding and short-term storage, whereas the hindgut reabsorbs salts and amino acids to achieve ionic balance (Baggio *et al.* 2014). The midgut, which makes up approximately 78% of the digestive system, is the main location of digestion and absorption and is covered by a peritrophic membrane (Vallet-Gely *et al.* 2008).

Digestive enzymes released by cylindrical and cup-shaped midgut cells break down complex nutrients into simpler forms that can be absorbed (Hu *et al.* 2015). Amylase activity serves as a valuable biochemical marker

in conjunction with conventional phenotypic selection to enhance the survival potential of high-yielding silkworm breeds (Devamani and Umakanth 2023). In silkworm, positive correlation between various enzyme activities and cocoon quality was reported by Miao (2002). Similarly, acid phosphatase has been recognised as an effective biochemical indicator for assessing genetic variability among silkworm strains (Staykova *et al.* 2010).

India is the 2nd largest producer of silk in the world, with most of its production coming from multivoltine races with low-quality silk. The Kashmir region remains the major univoltine-bivoltine region of the country, producing high-quality mulberry silk owing to its suitable climatic conditions. Nevertheless, the unavailability of high-yielding bivoltine breeds that have been locally adapted reduces cocoon productivity. Given the central role of biochemistry in silkworm physiology, this research explores the activity of digestive enzymes in gut juice across various days of the 5th instar and analyses their association with economically significant characters to determine potential biochemical markers for improvement in breeding. Most breeding programmes still rely on phenotypic traits, with very little integration of enzyme activity or other biochemical markers for early and precise selection.

¹College of Temperate Sericulture, Sher-e-Kashmir University of Agricultural Sciences and Technology-Kashmir, Mirgund, Baramulla, Jammu and Kashmir. *Corresponding author email: younus.wani692@gmail.com

MATERIALS AND METHODS

Bivoltine silkworm breeds were procured from the Division of Sericulture Crop Improvement, College of Temperate Sericulture, Sher-e-Kashmir University of Agricultural Sciences and Technology (34°21'N, 74°23'E; at an elevation of 1,590 m amsl), Mirgund, Baramulla, Kashmir. The selected breeds included SK-1, SK-6, SK-22, SK-28, SK-33, CSR4, CSR2, NB4D2, DUN6 and APS4. Four disease-free layings (DFLs) of each breed were reared following standard rearing protocols (SKUAST-K 2003). The experiment was laid out in a completely randomised design (CRD) with three replications, using 200 larvae/breed in each replication.

Gut juice collection: Since the 5th instar represents the most active stage of larval growth and silk synthesis, digestive juice samples were collected from larvae at this stage. From each replication, 10 larvae were randomly selected and placed in sterilised Petri plates (100 mm × 15 mm). Larvae were briefly exposed to chloroform vapours to induce gut juice secretion. To calculate optimal exposure period for inducing gut juice secretion while minimising larval mortality, the larvae were exposed to chloroform vapours for varying durations. Exposure durations were categorised into three groups based on the larval response: sub effective (20–30 sec), effective (30–40 sec) and lethal (>40 sec) (Ganie *et al.* 2018).

The expelled digestive juice was collected using sterilised syringes into pre-cooled 2 mL eppendorf tubes. Samples were centrifuged at 10,000 rpm for 10 min and the resulting supernatant was transferred to fresh tubes and stored at -20 °C for subsequent enzymatic analyses.

For enzyme activity estimation, equal volumes of digestive juice with equivalent protein concentrations were used for each breed.

Standardisation of gut juice for estimation of different enzymes: The volume of digestive juice used for estimation of enzyme activities included APS4 (50.26 µL), Dun6 (34.84 µL), CSR2 (32.49 µL), CSR4 (30.98 µL), SK33 (28.95 µL), NB4D2 (28.87 µL), SK28 (26.63 µL), SK22 (26.42 µL), SK6 (26.19 µL) and SK1 (23.52 µL).

The following parameters were taken during the study.

Total protein: The quantitative estimation of protein in digestive juice was carried out using crystalline bovine serum albumin (BSA) as standard and expressed in mg of protein/mL (Lowry *et al.* 1951).

Amylase activity: The digestive juice amylase activity of silkworm was estimated by the procedure suggested by Chatterjee *et al.* (1993) and Ashwath *et al.* (2010) and amylase activity was expressed as U/mL.

Protease activity: Protease activity in the digestive juice was assayed according to Eguchi and Iwamoto (1982) by using tyrosine as standard. The protease activity was expressed as U/mL.

Acid phosphatase activity: Acid phosphatase activity in digestive juice was carried out according to the method described by Linhardt and Walter (1965) by using para nitrophenol as standard. The activity was expressed as U/mL.

Alkaline phosphatase (ALP) activity: The digestive juice alkaline phosphatase activity was estimated by the method of Kind and King (1954) by using pure crystalline phenol as standard. The activity was expressed as U/mL.

Correlation: The nature and magnitude of correlation of economic parameters (weight of 10 mature larvae (g) on 5th day, 5th instar larval duration (days), total larval duration (days), Cocoon yield/10,000 larvae (by number), cocoon yield/10,000 larvae by weight (kg), pupation rate (%), single cocoon weight (g), single shell weight (g), shell ratio (%), average filament length (m), raw silk recovery (%), denier, silk productivity (cg/day) and fecundity) with digestive juice protein, amylase activity, protease activity, alkaline phosphatase activity, and acid phosphatase activity were worked out. The data collected was compiled and analysed using CPCS standard statistical software.

RESULTS AND DISCUSSION

Response of silkworm larvae to chloroform exposure: At sub-effective exposure (20–30 sec), larvae did not vomit but displayed mild irritability and signs of nausea, particularly at around 23 sec in some breeds, suggesting partial stimulation of the neuro-physiological response in the insect's nervous system (Ganie *et al.* 2018). Prolonged exposure for 30–40

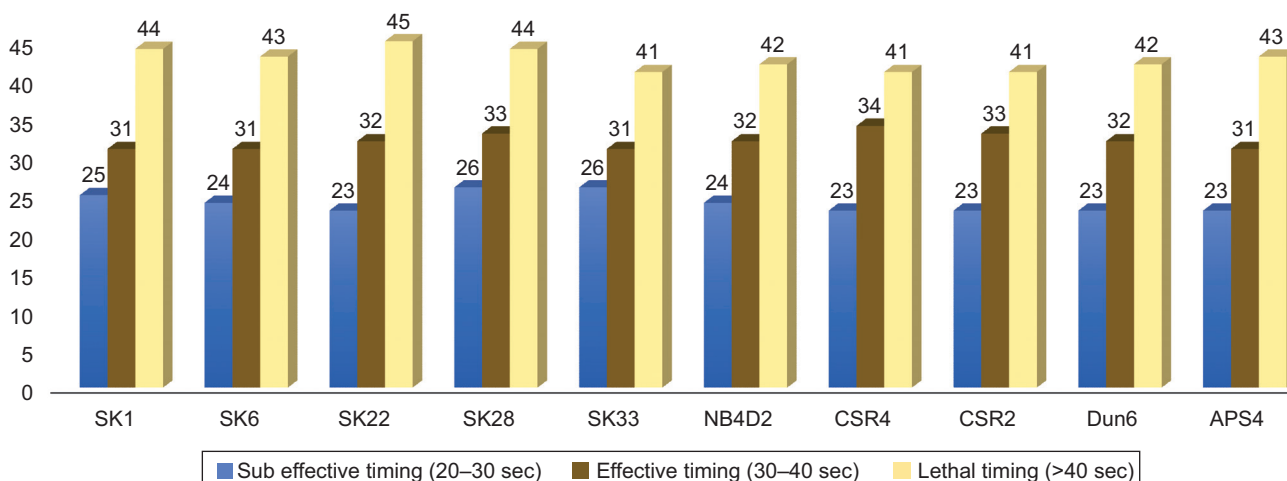


Fig. 1 Duration of response of various silkworm breeds to chloroform exposure.

sec effectively induced gut juice expulsion, attributed to complete activation of the vomiting centre. However, exposure exceeding 40 sec led to larval mortality, likely due to central nervous system depression and dehydration induced by chloroform toxicity (Vennart and McKee 1955).

Notably, variation in response times among the tested breeds indicated differential tolerance to chloroform vapours. This observation may reflect breed-specific physiological sensitivity and metabolic adaptability. No prior reports exist on the use of chloroform-induced gut juice collection in *Bombyx mori*, underscoring the novelty and pioneering nature of this methodological approach. Nevertheless, similar physiological responses to chloroform have been documented in other animal models such as mice, dogs and pigs (Yang *et al.* 2010).

Variation in digestive juice protein content across bivoltine breeds and developmental stages: Pooled analysis of the two-year data (spring 2018 and 2019) revealed that highest digestive juice protein content of 42.76 mg/mL was recorded in the silkworm breed SK1 as compared to SK6 (38.36 mg/mL), SK22 (37.85 mg/mL), SK28 (36.27 mg/mL), SK33 (34.68 mg/mL), NB4D2 (34.67 mg/mL), CSR4 (32.37 mg/mL), CSR2 (30.81 mg/mL) and Dun6 (28.73 mg/mL). The lowest peak of digestive juice protein content of 19.97 mg/mL was recorded in the silkworm breed APS4 (Fig. 2).

It was also revealed that highest digestive juice protein content of 35.72 mg/mL was recorded on 5th day of 5th instar which was statistically higher than rest of the days. The lowest peak of digestive juice protein content of 31.48 mg/mL was recorded on 4th day of the 5th instar (Fig. 3). The results were similar to the findings of Wani *et al.* (2021; 2023a; 2026).

Amylase activity: Pooled analysis of the two-year data (spring 2018 and 2019) revealed that highest digestive juice

amylase activity of 4.19 U/mL was recorded in the silkworm breed SK1. The digestive juice amylase activities in other breeds include: SK6 (3.29 U/mL), SK33 (3.28 U/mL), SK22 (3.17 U/mL), SK28 (3.15 U/mL), NB4D2 (3.13 U/mL), CSR4 (3.10 U/mL), CSR2 (3.05 U/mL) and Dun6 (2.70 U/mL). The least value for digestive juice amylase activity of 2.19 U/mL was recorded in the silkworm breed APS4 (Fig. 2). During the study, day wise digestive juice amylase activities was also recorded and it was found that highest peak of digestive juice amylase activity of 3.21 U/mL was recorded on 5th day of 5th instar. The lowest peak of digestive juice amylase activity of 3.01 U/mL was recorded on 7th day of the 5th instar (Fig. 3). Similar results were reported by Ganie *et al.* (2017a) and Wani *et al.* (2023b).

Protease activity: Pooled analysis of the two-year data (spring 2018 and 2019) revealed that highest digestive juice protease activity of 2.45 U/mL was recorded in silkworm breed SK1. The protease activities on other breeds includes: SK6 (2.21 U/mL), SK22 (2.19 U/mL), SK28 (2.16 U/mL), SK33 (2.14 U/mL), NB4D2 (2.10 U/mL), CSR4 (2.07 U/mL), CSR2 (1.23 U/mL) and Dun6 (0.84 U/mL). The least value of digestive juice protease activity of 0.58 U/mL was recorded in the silkworm breed APS4 (Fig. 2). Day wise digestive juice protease activity revealed that highest digestive juice protease activity of 1.85 U/mL was recorded on 7th day of 5th instar. The least digestive juice protease activity of 1.75 U/mL was recorded on 4th day of 5th instar (Fig. 3). Similar findings have also been reported by Kumar *et al.* (2011) and Abudabos (2012).

Alkaline phosphatase activity: The digestive juice alkaline phosphatase activity was estimated in different breeds of silkworm. Pooled analysis of the two-year data (spring 2018 and 2019) revealed that highest digestive juice alkaline phosphatase activity of 12.79 U/mL was recorded in the silkworm breed SK1. The digestive juice alkaline

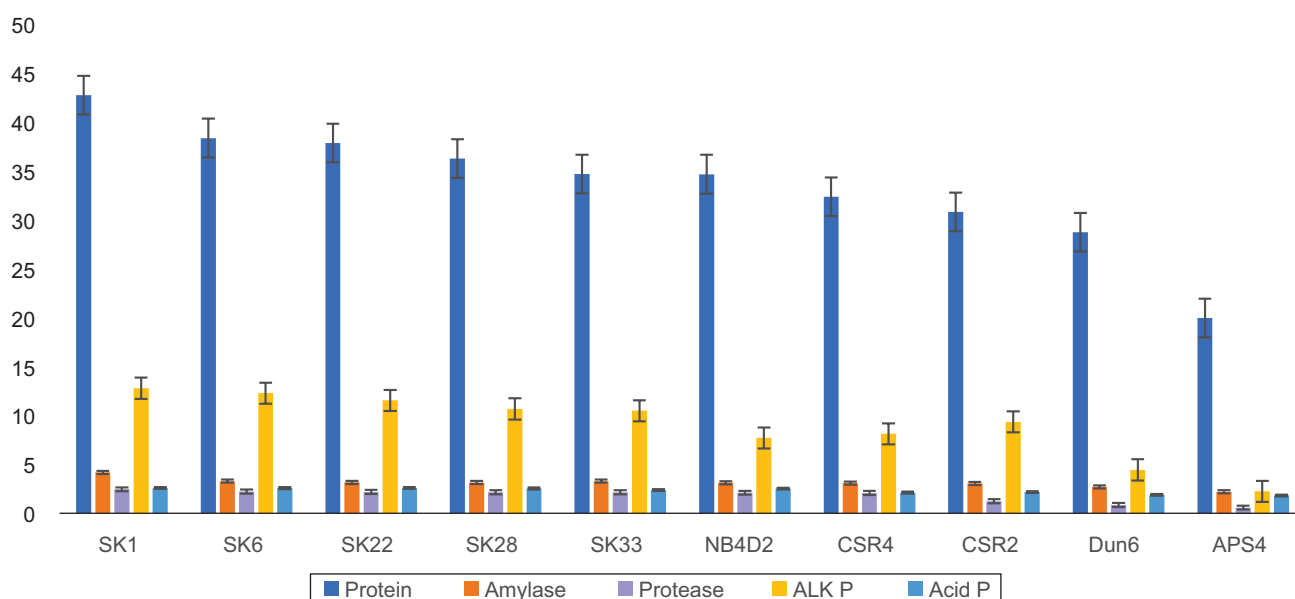


Fig. 2 Enzyme dynamics in different breeds.

ALK P, Alkaline phosphatase activity; Acid, Acid phosphatase activity.

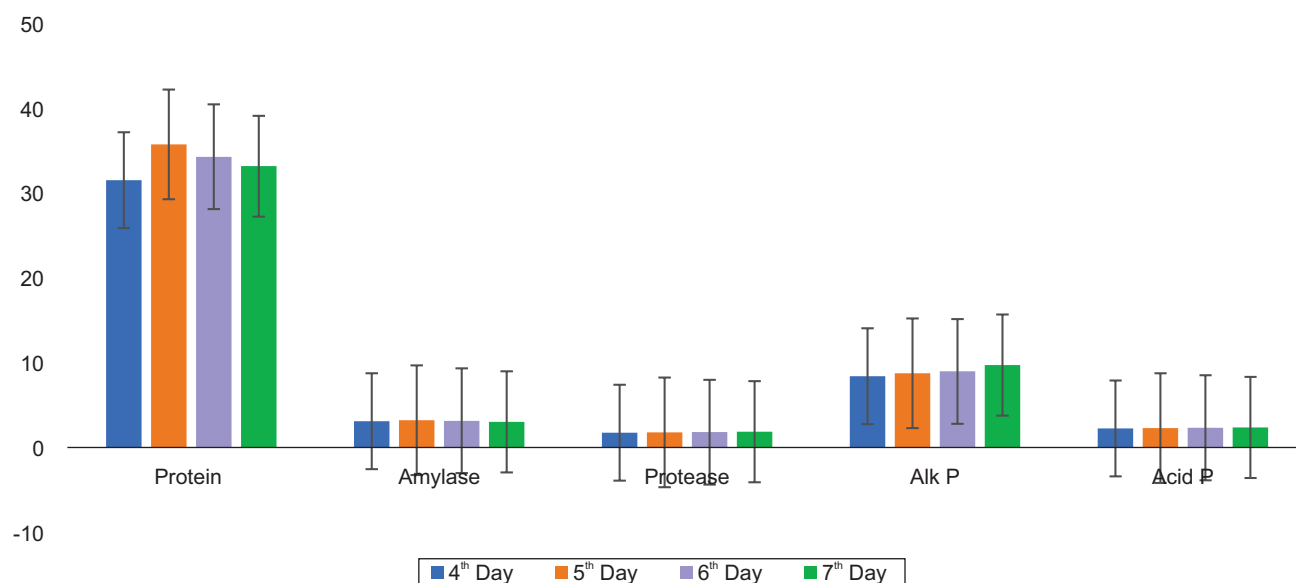


Fig. 3 Enzyme activities at different days of 5th instar.
ALK P, Alkaline phosphatase activity; Acid, Acid phosphatase activity.

phosphatase activities in other breeds was recorded as SK6 (12.28 U/mL), SK22 (11.54 U/mL), SK28 (10.67 U/mL), SK33 (10.48 U/mL), CSR2 (9.35 U/mL), CSR4 (8.12 U/mL), NB4D2 (7.70 U/mL) and Dun6 (4.44 U/mL). The least value of digestive juice alkaline phosphatase activity of 2.24 U/mL was recorded in the silkworm breed APS4 (Fig. 2). The digestive juice alkaline phosphatase activity was also recorded on different days of 5th instar and it was found that highest peak of digestive juice alkaline phosphatase activity of 9.71 U/mL was recorded on 7th day of 5th instar and lowest peak of digestive juice alkaline phosphatase activity of 8.41 U/mL was recorded on 4th day of 5th instar (Fig. 3). The results are supported by Mahesha *et al.* (2015) and Manjula *et al.* (2010a).

Acid phosphatase activity: Pooled analysis of the two-year data (spring 2018 and 2019) revealed that highest digestive juice acid phosphatase activity of 2.60 U/mL was recorded in the silkworm breed SK1. The digestive juice alkaline phosphatase activities in other breeds included: SK22 (2.59 U/mL), SK6 (2.57 U/mL), SK28 (2.54 U/mL), NB4D2 (2.51 U/mL), SK33 (2.37 U/mL), CSR2 (2.17 U/mL), CSR4 (2.12 U/mL) and Dun6 (1.88 U/mL). The least value of digestive juice acid phosphatase activity of 1.82 μ moles was recorded in the silkworm breed APS4 (Fig. 2). During the study, day wise digestive juice acid phosphatase activities were also measured which revealed that highest peak of digestive juice acid phosphatase activity of 2.37 U/mL was recorded on 7th day of 5th instar and lowest peak of digestive juice acid phosphatase activity of 2.26 U/mL was recorded on 5th day of 5th instar (Fig. 3). The results were supported by findings of Sumithra *et al.* (2010).

Clustering: The 10 silkworm breeds were grouped in different clusters with different enzyme activities, revealing diversity between the silkworm breeds that can be used for parental selection in silkworm breeding programmes (Fig. 4)

(Aurade *et al.* 2024).

Correlation studies: Analysis of correlation studies between digestive juice protein and some enzyme activities with economic parameters of different bivoltine breeds of silkworm during spring 2018 and 2019 revealed that, the highly significant ($p \leq 0.01$) positive correlations were obtained between digestive juice protein and pupation rate ($r = +0.928$). The moderately significant ($p \leq 0.05$) positive correlations were obtained between digestive juice protein and weight of 10 mature larvae ($r = +0.725$), digestive juice protein and single cocoon weight ($r = +0.628$), digestive juice protein and single shell weight ($r = +0.713$), digestive juice protein and shell ratio ($r = +0.622$), digestive juice protein and silk productivity ($r = +0.715$), digestive juice protein and cocoon yield/10,000 larvae by weight ($r = +0.629$), digestive juice protein and fecundity ($r = +0.621$) and digestive juice protein and raw silk (%) ($r = +0.626$) (Table 1).

Highly significant ($p \leq 0.01$) positive correlations were obtained between digestive juice amylase activity and pupation rate ($r = +0.925$). The moderately significant ($p \leq 0.05$) negative correlations of digestive juice amylase activity with weight of 10 mature larvae on 5th day ($r = -0.721$), single shell weight ($r = -0.716$), single cocoon weight ($r = -0.656$), shell ratio ($r = -0.642$), filament length ($r = -0.629$), raw silk (%) ($r = -0.736$) and the moderately significant ($p \leq 0.05$) positive correlations of digestive juice amylase activity with fecundity ($r = +0.621$), filament size ($r = +0.623$), 5th instar larval duration ($r = +0.645$), silk productivity ($r = +0.658$) and with cocoon yield/10,000 larvae by weight ($r = +0.629$) were also recorded (Table 1).

Highly significant positive correlations ($p \leq 0.01$) were obtained between digestive juice protease activity and cocoon yield/10,000 larvae by weight ($r = +0.948$), digestive juice protease activity and cocoon yield/10,000 larvae by

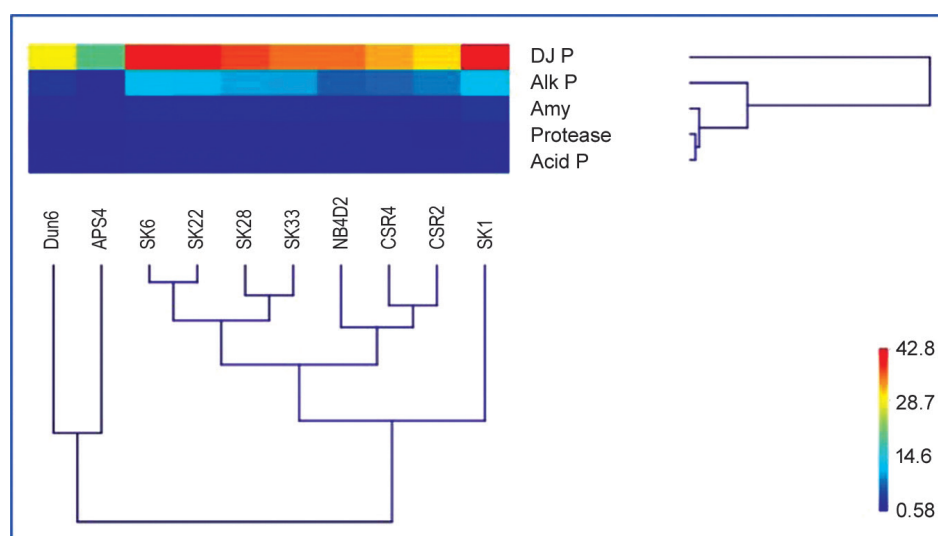


Fig. 4 Cluster analysis of different bivoltine silkworm breeds.

DJP, Digestive juice protein; ALK P, Alkaline phosphatase activity; Amy, Amylase activity; Acid P, Acid phosphatase activity.

number ($r = +0.935$) and digestive juice protease activity and fecundity ($r = 0.975$). The moderately ($p \leq 0.05$) significant positive correlations were obtained between digestive juice protease activity and weight of 10 mature larvae on 5th day ($r = 0.625$), digestive juice protease activity and single shell weight ($r = 0.708$), digestive juice protease activity and single cocoon weight ($r = 0.780$), digestive juice protease activity and shell ratio ($r = 0.759$) and digestive juice protease activity and silk productivity ($r = +0.724$) (Table 1).

Highly significant positive correlation ($p \leq 0.01$) was

obtained between digestive juice alkaline phosphatase activity and cocoon yield/10,000 larvae by weight ($r = +0.921$). The moderately significant positive correlations ($p \leq 0.05$) were obtained between digestive juice alkaline phosphatase activity and 5th instar larval duration ($r = +0.628$), digestive juice alkaline phosphatase activity and total larval duration ($r = +0.622$), digestive juice alkaline phosphatase activity and single shell weight ($r = +0.712$), digestive juice alkaline phosphatase activity and single cocoon weight ($r = +0.723$), digestive juice alkaline phosphatase activity and shell ratio ($r = +0.629$),

digestive juice alkaline phosphatase activity and silk productivity ($r = +0.632$), digestive juice alkaline phosphatase activity and cocoon yield/10000 larvae by number ($r = +0.634$), digestive juice alkaline phosphatase activity and pupation rate ($r = +0.624$) and digestive juice alkaline phosphatase activity and fecundity ($r = +0.621$) (Table 1).

Highly significant positive correlation ($p \leq 0.01$) was obtained between digestive juice acid phosphatase activity and fecundity ($r = +0.978$). The moderately significant

Table 1 Correlation matrix showing relationships between economic traits and digestive enzyme activities in silkworm breeds

	DJ Protein	DJ Amylase	DJ Protease	DJ ALK P	DJ ACP
5 th LDD	-0.338	0.645*	0.129	0.628*	-0.712*
TLDD	-0.256	0.286	0.572	0.622*	-0.625*
W5 th D	0.725*	-0.721*	0.625*	-0.051	0.652*
SSW	0.713*	-0.716*	0.708*	0.712*	0.623*
SCW	0.628*	-0.656*	0.780*	0.723*	0.631*
SR	0.622*	-0.642*	0.759*	0.629*	0.633*
SP	0.715*	0.658*	0.724*	0.632*	0.634*
CYN	0.217	-0.042	0.935**	0.634*	0.633*
CYW	0.629*	0.629*	0.948**	0.921**	0.628*
PR	0.928**	0.925**	-0.255	0.624*	0.721*
Fecundity	0.621*	0.621*	0.975**	0.621*	0.978**
FL	0.123	-0.629*	0.225	-0.021	-0.025
FS	0.026	0.623*	-0.131	-0.103	-0.034
RSP	0.626*	-0.736*	-0.466	-0.082	-0.012

DJ, Digestive juice; SSW, Single shell weight; SCW, Single cocoon weight; SR, Shell ratio; SP, Silk productivity; CYN, Cocoon yield/10,000 larvae by number; CYW, Cocoon yield/10,000 larvae by weight; PR, Pupation rate; FL, Filament length; FS, Filament size; RSP, Raw silk (%); 5th LDD, 5th instar larval duration; TLDD, Total larval duration; W5thD, Weight of 10 mature larvae on 5th day; ALK P, Alkaline phosphatase activity; ACP, Acid phosphatase activity.

positive correlations ($p \leq 0.05$) were obtained between digestive juice acid phosphatase activity and weight of 10 mature larvae on 5th day ($r = +0.652$), digestive juice acid phosphatase activity and single shell weight ($r = +0.623$), digestive juice acid phosphatase activity and single cocoon weight ($r = +0.631$), digestive juice acid phosphatase activity and shell ratio ($r = +0.633$), digestive juice acid phosphatase activity and silk productivity ($r = +0.634$), digestive juice acid phosphatase activity and cocoon yield/10,000 larvae by weight ($r = +0.628$), digestive juice acid phosphatase activity and cocoon yield/10000 larvae by number ($r = +0.633$) and digestive juice acid phosphatase activity and pupation rate ($r = +0.721$). The moderately significant negative correlations ($p \leq 0.05$) were obtained between digestive juice acid phosphatase activity and 5th instar larval duration ($r = -0.712$) and digestive juice acid phosphatase activity and total larval duration ($r = -0.625$) (Table 1).

The study identified several bivoltine breeds, SK1, SK6, SK22, SK28, SK33, CSR2 and CSR4 that show high levels of protein, amylase, protease and both acid and alkaline phosphatase activities. It was also observed that an effective timing of 30–40 sec results in larvae expelling gut juice. Most economic traits positively correlate with these breeds/enzymes, making them promising candidates. These breeds could potentially be used in breeding programmes aimed at developing silkworm breeds/hybrids that are well-suited to the Kashmir valley, with resistance to local conditions, seasons and diseases. The findings indicate that biochemicals may serve as potential supplementary indicators for early selection but are not sufficient as standalone markers. Future research should focus on validating these enzyme-trait relationships and integrating biochemical markers with molecular tools to improve selection precision.

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