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Analysis of alkaline phosphatase and its relationship with commercial characters of silkworm *Bombyx mori* L.

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ABSTRACT

Four pure mulberry silkworm breeds viz., Pure Mysore, Nistari, NB_4D_2 & CSR_2 and two hybrid (Pure Mysore x CSR_2 and Nistari x NB_4D_2) silkworms were selected for the present study. The specific activity of alkaline phosphatase (ALKP) in midgut and fat body tissues were estimated. The qualitative analysis of ALKP was carried out by Native-PAGE. The commercial characters viz., fecundity, larval weight, larval duration, cocoon weight, shell weight, shell ratio, filament length, denier and renditta were selected. The activity levels of ALKP of midgut and fat body tissue showed statistically significant ($P<0.001$) changes in their activity levels during fifth instar. The results of quantitative analysis were subjected for regression analysis against selected commercial characters to know the correlation coefficient between them. The results of regression analysis clearly showed that midgut ALKP has positive correlation with fecundity, larval duration and renditta only. The activity levels of fat body ALKP showed positive correlation with fecundity, larval weight and renditta only. The zymograms of ALKP in midgut and fat body also exhibited variation among the selected silkworm varieties.

Keywords: *Bombyx mori*, midgut, fat body, alkaline phosphatase, commercial characters.

INTRODUCTION

Recent advances in plant and animal breeding have highlighted the prospects of using linked molecular markers (Isozyme/DNA) for improvement of desirable traits. Therefore, identification of suitable markers, holds the key to successful implementation of marker assisted selection (MAS) which is gaining ground fast in other fields of breeding [1]. In addition, biochemical markers are useful for screening germplasm with minimum cost in time and labour [2]. Alkaline phosphatase (Phosphoric monoester hydrolases; E. C. 3.1.3.1.) are metalloenzymes, nonspecifc, phosphomonoesterases [3], which exists in various organisms from bacteria to mammals [4,5,6], involving as mediators in the energy transfer wherever ADP and ATP are involved in metabolic pathways. The activity level during various stages of ontogeny reflects the generation and utilization of energy. In insects, alkaline phosphatases are involved in several biological processes and responds to stress, pathogenesis or infection [7]. Hegde and Krishnamurthy [8] and Raje Urs [9] analyzed phosphatases in some silkworm races. Umakanth [10] studied stage-specific, sex-specific, pupal- specific and moth- specific bands of phasphatases. The analysis of enzymes like amylase, succinate dehydrogenase [11,12,13,14], alkaline phosphatase and alkaline protease [15] may help in silkworm breeding programme for cocoon characters and disease resistance. However, correlation studies combining alkaline phosphatase with commercial characters of silkworm *Bombyx mori* are rather scarce. Hence, the present investigation was undertaken.

MATERIALS AND METHODS

Four pure mulberry silkworm breeds *viz*., Pure Mysore, Nistari, NB_4D_2 & CSR_2 and two hybrid (Pure Mysore x CSR_2 and Nistari x NB_4D_2) silkworms were selected for the present investigation. The silkworm rearing was conducted in the laboratory following the method described by Krishnaswamy [16,17]. All experimental batches were maintained in triplicate. In each replication 500 larvae were kept after third moult. The economic traits selected for present study included weight of fifth instar larva, larval duration, cocoon weight, shell weight, shell ratio, filament length, denier, renditta and fecundity.

The midgut and fat body tissues were obtained from five silkworms of fifth instar by dissecting the larvae in ice cold water and the gut contents were removed. The tissues were thoroughly washed in distilled water. A 10 % (w/v) homogenate of the tissues were prepared in pre cooled distilled water using mortar and pestle. The homogenate was centrifuged at 3000 rpm for 10 minutes in a cooling centrifuge at 5°C. The clear supernatant was used for the enzyme analysis.

The total soluble protein present in the haemolymph and midgut tissue was estimated by following the method of Lowry *et al*. [18]. Bovine Serum Albumin was used as standard protein.

Quantitative analysis of alkaline phosphatase was done in haemolymph, midgut and fatbody tissues following the method. The reaction mixture contained 1ml of 0.1M sodium carbonate buffer (pH 10) containing 50 mM paranitrophenol phosphate was incubated at 37ºC for 5 min. After this pre incubation, appropriately (1:10) diluted 10 µl haemolymph for haemolymph alkaline phosphatase assay and 10 µl tissue (0.5 %) extract for midgut and fat body alkaline phosphatase assay respectively. Incubation of this mixture was carried out for 30 min at 37°C in a water bath. After 30 min, 2 ml of 0.1 N NaOH was added and the contents were mixed thoroughly. Then the volume was made up to 4 ml with buffer. The contents were shaken vigorously and the optical density was measured at 540 nm setting the spectrophotometer to zero with blank consisted of incubation mixture to which enzyme sample was added after termination of the reaction. The activity of the enzyme was expressed as µmoles of paranitrophenol released /mg protein/min at 37ºC. Paranitrophenol was used as standard.

The experimental data were statistically analyzed through SPSS by one way ANOVA [19], Scheffe's post hoc [20] and linear regression analysis [21] wherever they were applicable.

The qualitative analysis of alkaline phosphatase isozymes was carried out in Native Poly Acrylamide Gel Electrophoresis (PAGE) with the discontinuous buffer system containing 5% stacking and 8% separating gel. The vertical slab gel apparatus was used. The gels, soon after the removal, washed in running distilled water followed by the incubation in 100 ml of Tris-HCl buffer (50mM pH 8.5) containing polyvinyl pyrolidone 500 mg, fast blue RR salt 100 mg, sodium alpha napthyl phosphate 100mg, magnesium chloride 60 mg, manganese chloride 60 mg and sodium chloride 2 g at 37ºC in a rotary shaker in dark for 1 h or until the bands appeared. Then the gels were scanned, analyzed and photographed in a gel scanner (Vilber Laurmat Bioprofil image analysis system).

RESULTS

The summary of the studied commercial characters are presented in the table1. From the table it is clear that the two bivoltine races are superior for productivity traits whereas multivoltines are superior for viability traits. The hybrids showed average values of their parents. The results of one way ANOVA revealed that the variation in all commercial characters among the experimental batches are all significant at 0.1 % ($P<0.001$). The specific activity of alkaline phosphatase in haemolymph was nil. The specific activity of alkaline phosphatase in midgut and fat body tissue samples is shown in the tables 2 and 3 respectively. The activity of alkaline phosphatase in midgut and fat body tissues samples showed significant changes in their activity levels at every 24 hours till the end of fifth instar. Almost similar trend was observed in both the tissues of all the experimental batches. The results of one way ANOVA revealed that the variation among the experimental sets is found to be significant at 0.1% ($P<0.001$). In the case of midgut tissue, the highest alkaline phosphatase activity was observed in Nistari (9.38 µM/mg/min at 37°C was the average during fifth instar) followed by Pure Mysore (8.41 µM/mg/min at 37°C), NB_4D_2 (8.38 µM/ mg/ min at 37°C), CSR_2 (8.15 µM/mg/min at 37°C), Pure Mysore x CSR_2 (7.17 µM/ mg/ min at 37°C) and Nistari x NB_4D_2 (6.79 µM/mg/min at 37°C). In the case of fat body tissue, the highest activity was observed in Nistari x NB_4D_2 (6.72 µM/mg/min at 37°C) followed by NB_4D_2 (6.47 µM/mg/min at 37°C), Pure Mysore (6.24 µM/mg/min at 37°C), Nistari (6.20 µM/ mg/min at 37°C), CSR_2 (6.00 µM/mg/min at 37°C) and Pure Mysore x CSR_2 (5.82 µM/ mg/ min at 37°C). The results of quantitative analysis were subjected for regression analysis against selected commercial characters to know the level of correlation coefficient between them.

Table 1: Mean values ± SD of nine commercial characters in six breeds of silkworm, *Bombyx mori*

SILKWORM BRREDS	FECUNDITY	LARVAL WEIGHT (g)	LARVAL DURATION (h)	COCOON WEIGHT (g)	SHELL WEIGHT (g)	SHELL RATIO (%)	FILAMENT LENGTH (m)	DENIER	RENDITTA
Pure Mysore	467.22±10.96	2.01±0.06	660±10.39	1.02±0.75	0.12±0.01	12.57±0.49	426.44±19.83	1.77±0.09	11.77±0.82
Nistari	485.11±5.30	2.83±0.06	564.88±10.01	1.14±0.71	0.15±0.01	13.41±0.87	435.66±17.21	1.78±0.07	13.26±0.24
CSR_2	509.10±16.58	4.07±0.05	578.88±6.45	1.81±0.47	0.43±0.01	24.02±0.18	1011.99±12.34	2.93±0.22	5.78±0.23
NB_4D_2	520.55±16.65	4.16±0.05	576.67±11.08	1.76±0.30	0.35±0.01	20.27±0.15	1020±29.96	2.48±0.06	8.34±0.47
Pure Mysore x CSR_2	466.66±11.52	2.68±0.07	610±11.10	1.67±0.23	0.28±0.01	17.29±0.21	910±18.74	2.75±0.06	7.64±0.12
Nistari x NB_4D_2	490.77±6.81	3.46±0.04	557±10.21	1.47±0.22	0.23±0.01	16.06±0.85	805.99±12.36	1.83±0.02	9.22±0.85
F	89.775	4210.79	853.92	3570.99	3898.36	1484.63	65.17	311.48	28230.47

Values are the mean± SD of Pre monsoon, Monsoon and post monsoon observations.
The variation between the races is statistically significant at 0.1 % (P<0.001).

Table 2: Alkaline phosphatase activity levels (µ moles of product released/mg protein/min at 37°C) in midgut tissue

SILKWORM BREEDS	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day	8th day	AVERAGE
Pure Mysore	6.88	8.33 (+21.07)	9.01 (+8.16)	8.92 (+0.99)	8.95 (+0.33)	8.90 (-0.55)	8.54 (-4.04)	7.78 (-8.89)	8.41
Nistari	5.89	8.03 (+36.33)	10.30 (+28.26)	11.69 (+13.49)	10.99 (-5.98)	9.40 (-14.46)	-	-	9.38
CSR_2	6.01	7.38 (+22.79)	8.09 (+9.62)	9.16 (+13.22)	9.96 (+8.73)	8.33 (-16.36)	-	-	8.15
NB_4D_2	6.13	7.66 (+24.95)	8.55 (+11.61)	9.33 (+9.12)	9.69 (+3.85)	8.94 (-7.73)	-	-	8.38
Pure Mysore x CSR_2	5.97	6.49 (+8.71)	8.18 (+26.04)	7.57 (-7.45)	7.10 (-6.20)	7.41 (+4.36)	7.52 (+1.48)	-	7.17
Nistari x NB_4D_2	5.55	5.76 (+3.78)	6.44 (+11.80)	6.93 (+7.60)	8.20 (+18.32)	7.88 (-3.90)	-	-	6.79

The variation between the races is statistically significant at 0.1 % (P<0.001).
Values within parentheses represent per cent change over previous day.

Table 3: Alkaline phosphatase activity levels (µ moles of product released/mg protein/min at 37°C) in fat body

SILKWORM BREEDS	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day	8th day	AVERAGE
Pure Mysore	4.61	5.20 (+12.79)	5.61 (+7.88)	7.36 (+31.19)	7.22 (-1.90)	6.80 (-5.81)	6.56 (-3.52)	6.63 (+1.06)	6.24
Nistari	4.00	4.19 (+4.75)	6.59 (+57.27)	8.00 (+21.39)	7.40 (-7.50)	7.06 (-4.59)	-	-	6.20
CSR_2	4.61	4.79 (+3.90)	6.03 (+25.88)	7.14 (+18.40)	7.17 (+0.42)	6.27 (-12.55)	-	-	6.00
NB_4D_2	4.19	5.64 (+34.60)	6.90 (+22.34)	7.35 (+6.52)	7.41 (+0.81)	7.35 (-0.80)	-	-	6.47
Pure Mysore x CSR_2	4.32	5.37 (+24.30)	5.61 (+4.46)	6.02 (+7.30)	6.89 (+14.45)	6.08 (-11.75)	6.48 (+6.57)	-	5.82
Nistari x NB_4D_2	4.38	7.16 (+63.47)	6.86 (-4.18)	7.41 (+8.01)	7.00 (-5.53)	7.51 (+7.28)	-	-	6.72

The variation between the races is statistically significant at 0.1 % (P<0.001).
Values within parentheses represent per cent change over previous day.

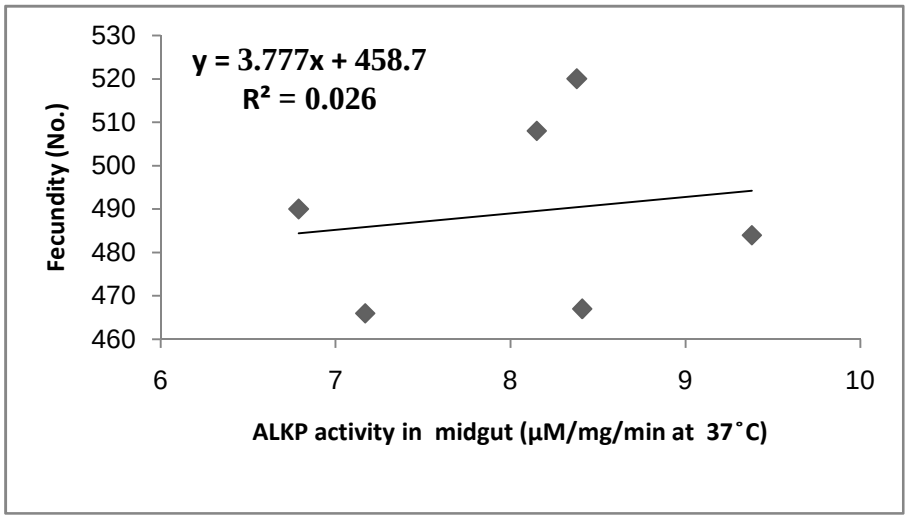

Figure 1: Correlation between midgut alkaline phosphatase activity level and fecundity

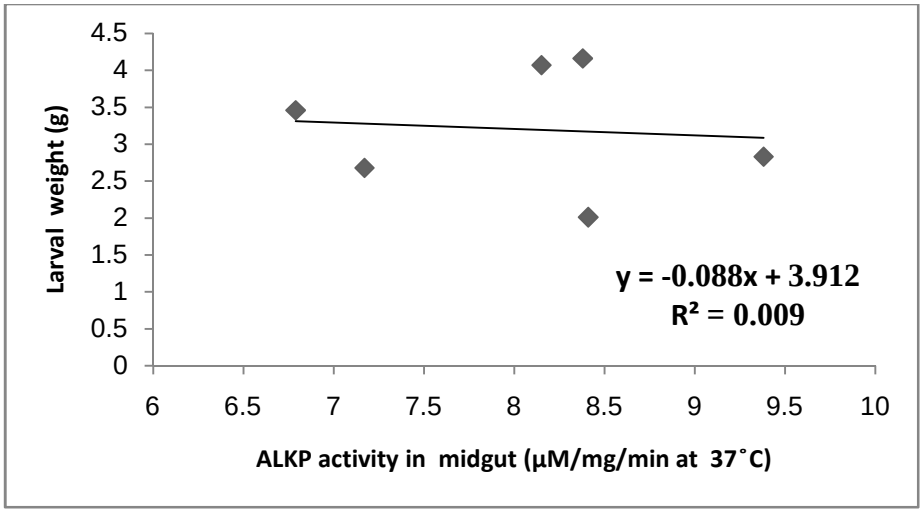

Figure 2: Correlation between midgut alkaline phosphatase activity level and larval weight

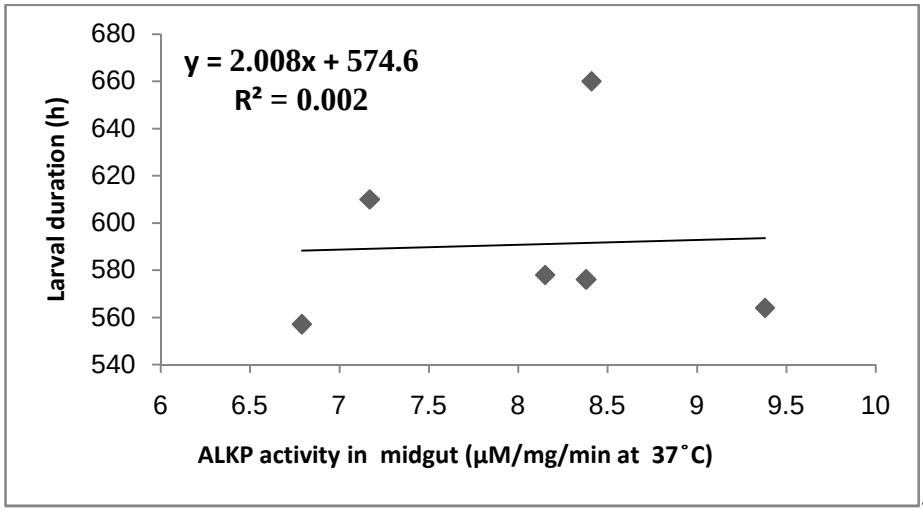

Figure 3: Correlation between midgut alkaline phosphatase activity level and larval duration

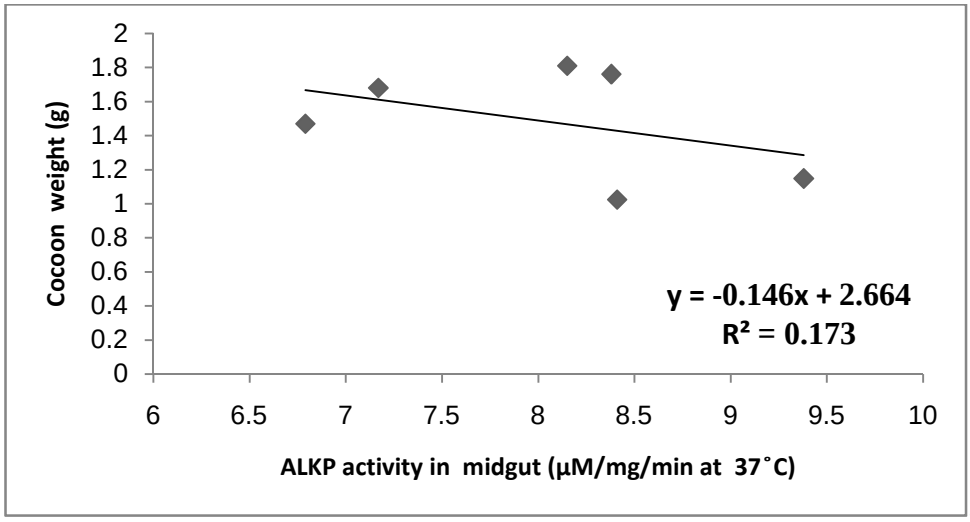

Figure 4: Correlation between midgut alkaline phosphatase activity level and cocoon weight

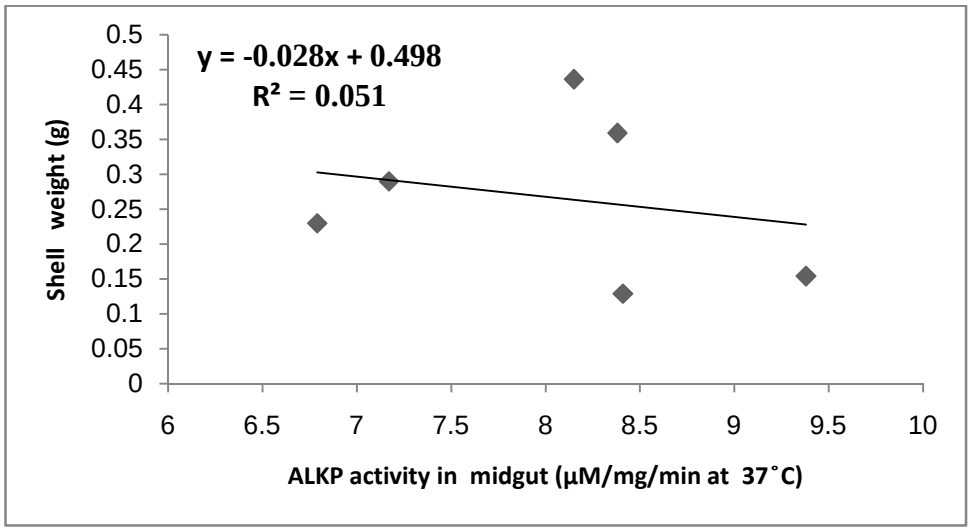

Figure 5: Correlation between midgut alkaline phosphatase activity level and shell weight

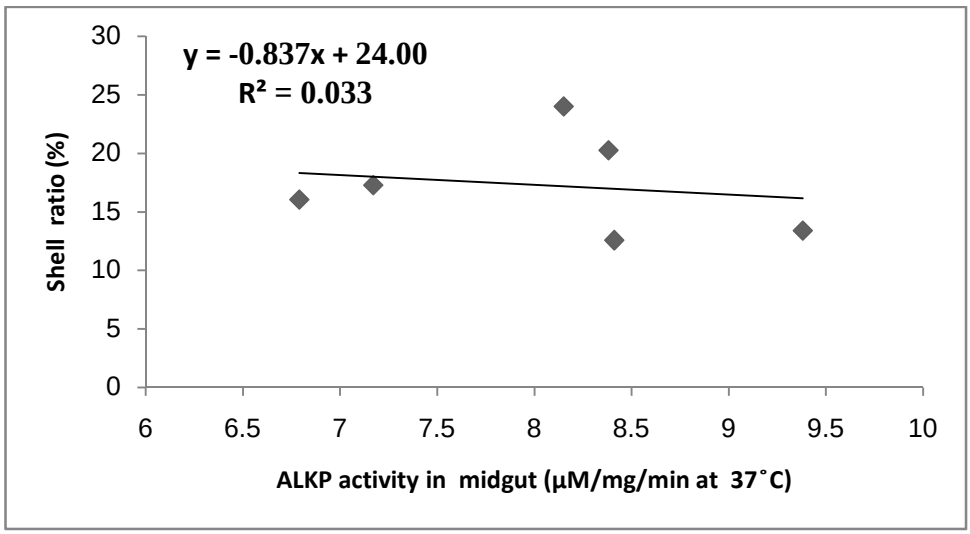

Figure 6: Correlation between midgut alkaline phosphatase activity level and shell ratio

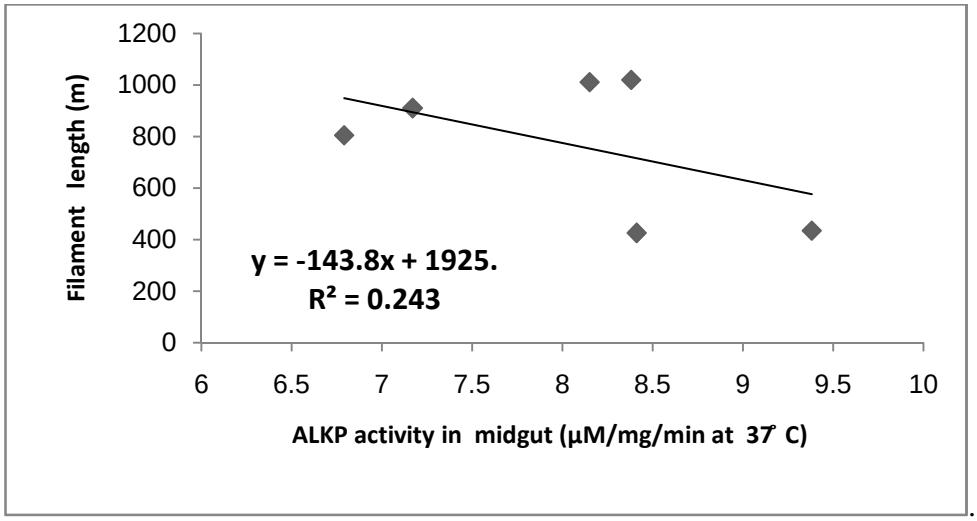

Figure 7: Correlation between midgut alkaline phosphatase activity level and filament length

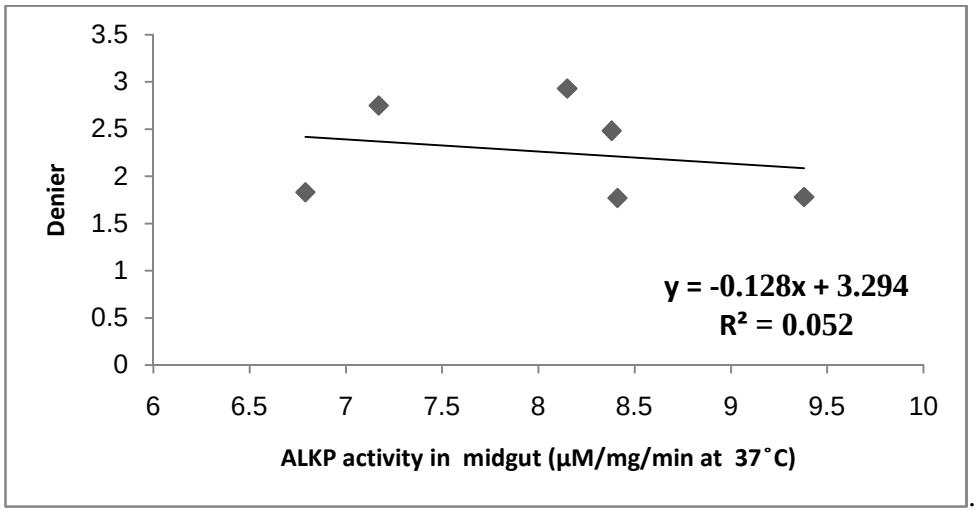

Figure 8: Correlation between midgut alkaline phosphatase activity level and denier

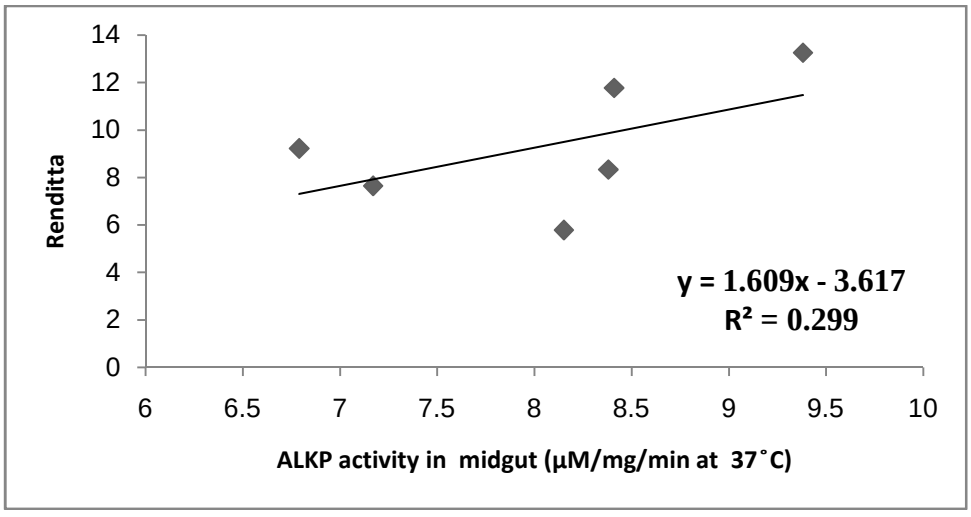

Figure 9: Correlation between midgut alkaline phosphatase activity level and renditta

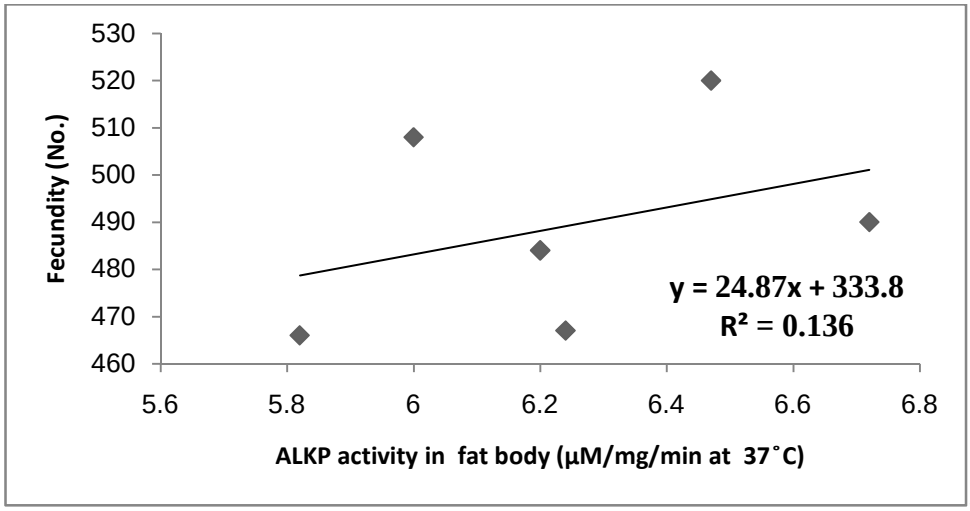

Figure 10: Correlation between fat body alkaline phosphatase activity level and fecundity

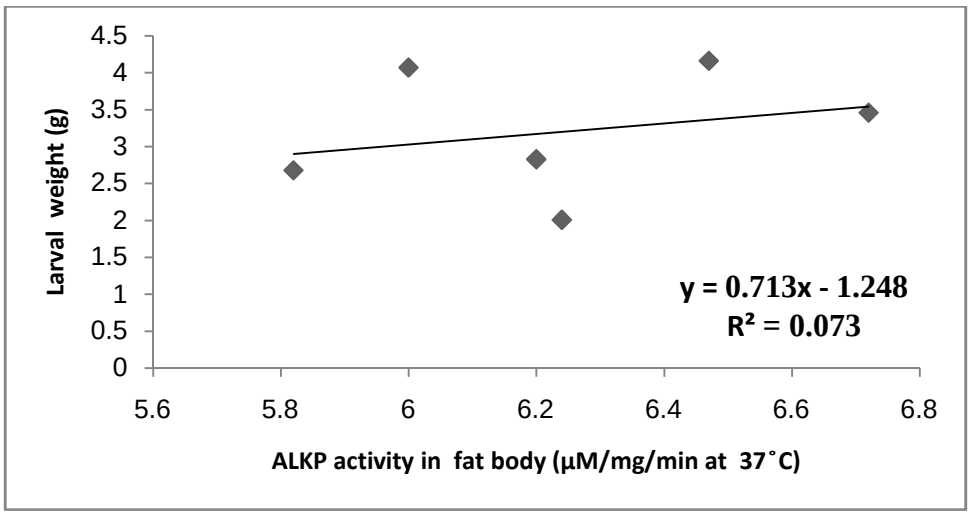

Figure 11: Correlation between fat body alkaline phosphatase activity level and larval weight

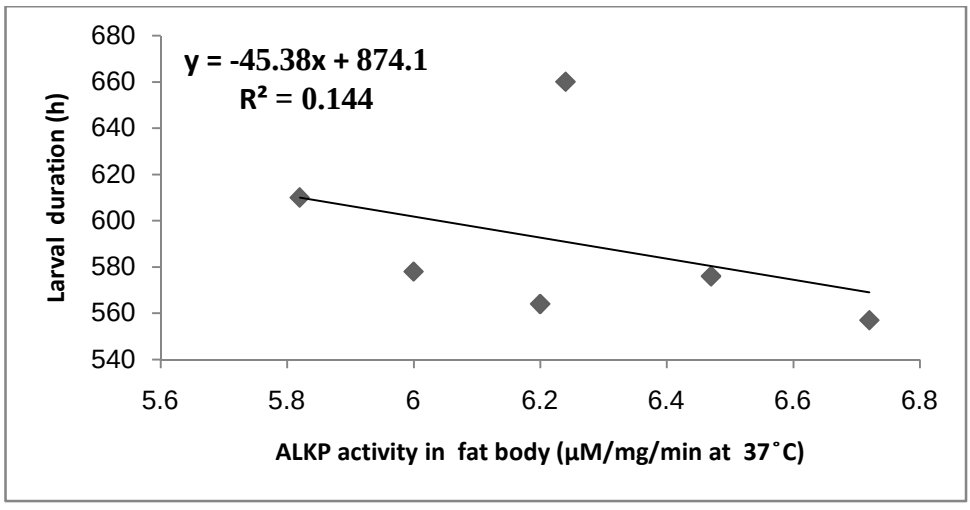

Figure 12: Correlation between fat body alkaline phosphatase activity level and larval duration

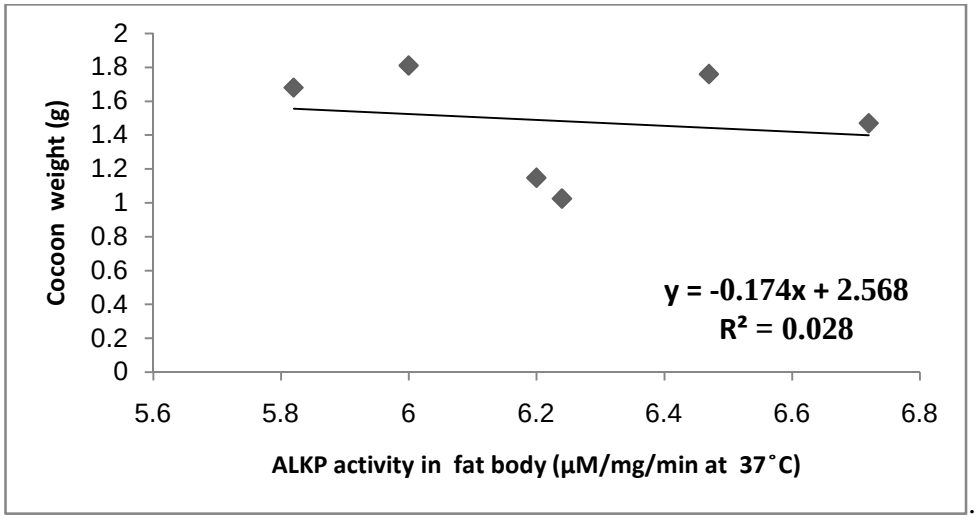

Figure 13: Correlation between fat body alkaline phosphatase activity level and cocoon weight

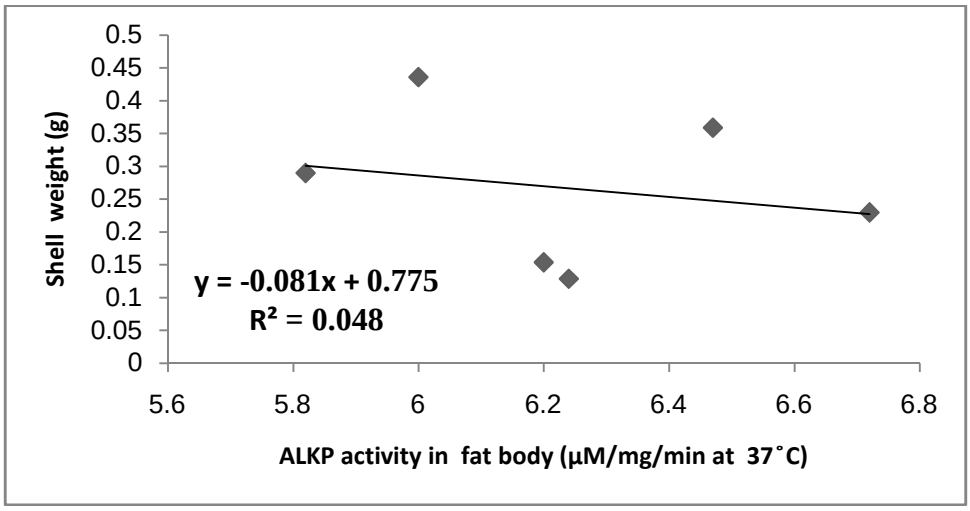

Figure 14: Correlation between fat body alkaline phosphatase activity level and shell weight

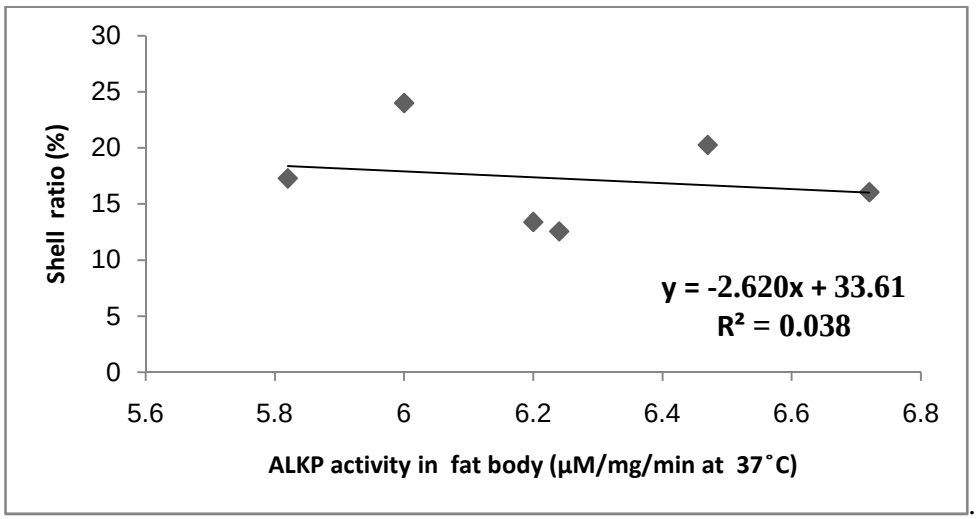

Figure 15: Correlation between fat body alkaline phosphatase activity level and shell ratio

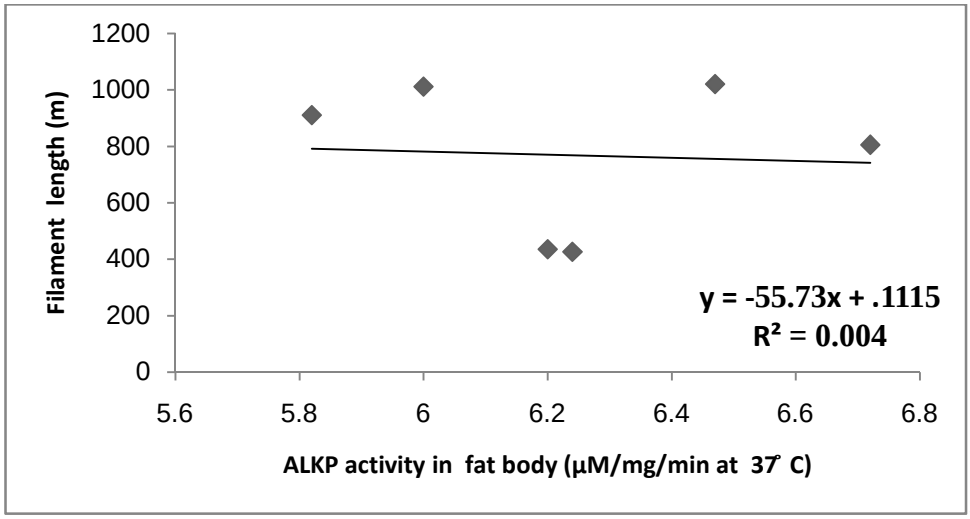

Figure 16: Correlation between fat body alkaline phosphatase activity level and filament length

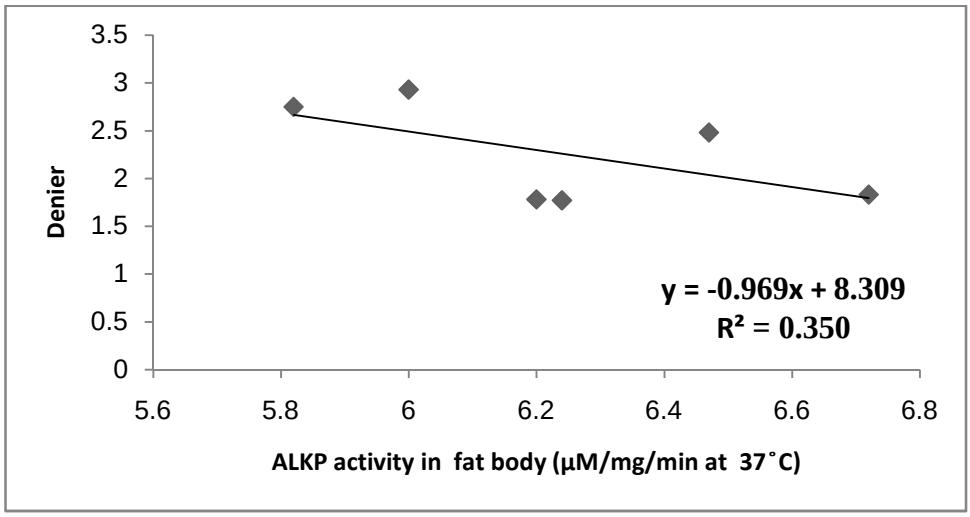

Figure 17: Correlation between fat body alkaline phosphatase activity level and denier

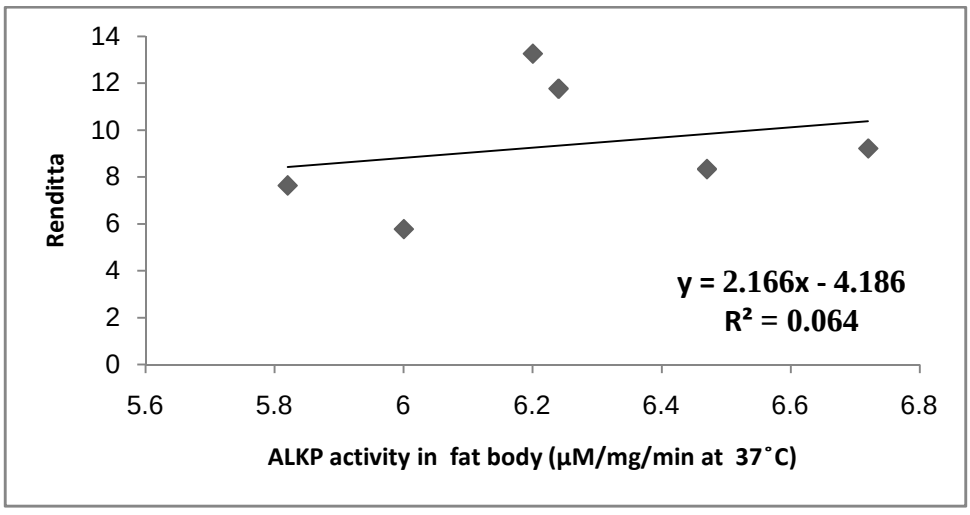

Figure 18: Correlation between fat body alkaline phosphatase activity level and renditta

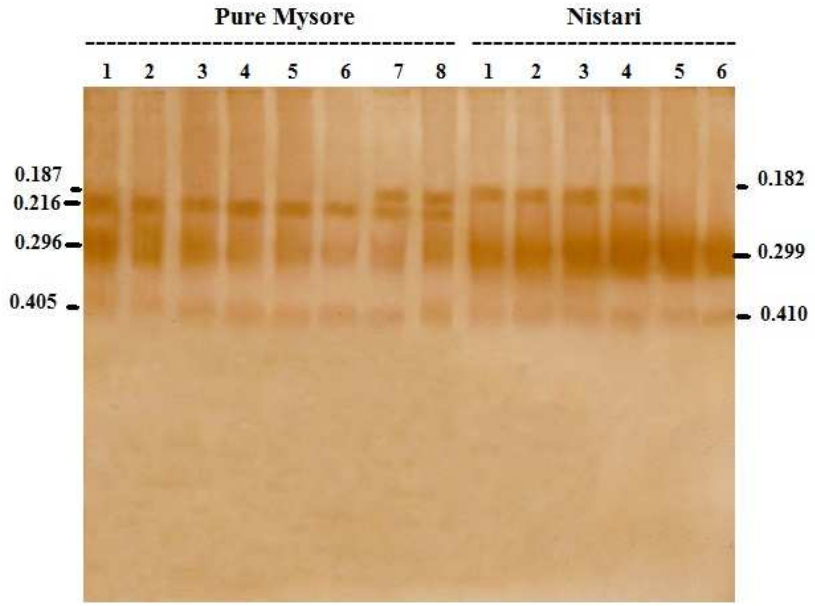

Figure 19: Native PAGE analysis of midgut alkaline phosphatase of Pure Mysore and Nistari silkworms. Lanes: 1-8 days in fifth instar.

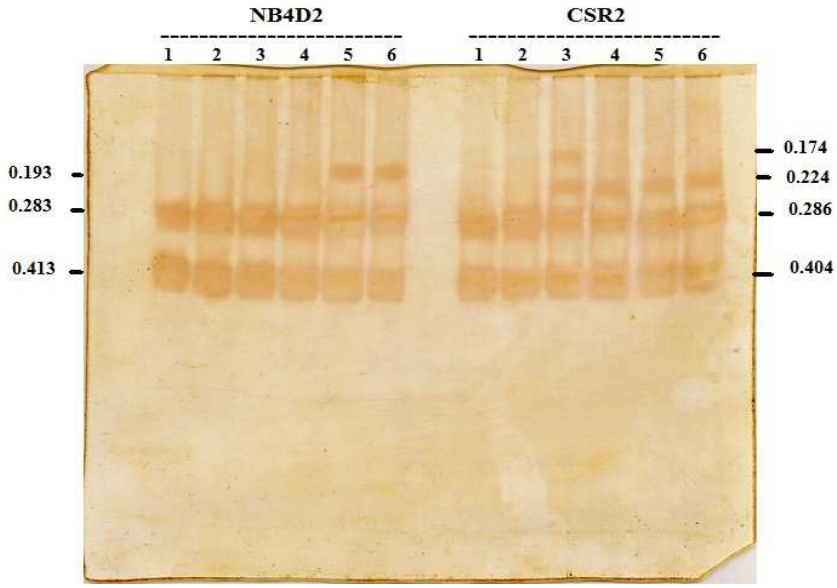

Figure 20: Native PAGE analysis of midgut alkaline phosphatase of NB_4D_2 and CSR_2 silkworms. Lanes: 1-6 days in fifth instar.

The results of regression analysis between the midgut alkaline phosphatase activity levels and commercial characters are presented in figures 1-9. The results of statistical analysis clearly showed that the midgut alkaline phosphatase activity levels exhibited moderately positive correlation with renditta (R^2=0.299), fecundity (R^2 = 0.026) and larval duration (R^2=0.002) only. Also, in the case of fat body tissue, the results of regression analysis are presented in figures 10-18. The results of statistical analysis clearly indicated that the fat body alkaline phosphatase activity levels exhibited positive correlation with fecundity (R^2 = 0.136), larval weight (R^2 = 0.073) and renditta (R^2=0.064) only.

The zymograms of alkaline phosphatase also exhibited variation among the selected silkworm varieties. A number of quantitative and qualitative variations were observed in the zymograms (figures 19-24). In the case of multivoltines, the midgut alkaline phosphatase isozymes in Pure Mysore exhibited entirely different pattern of banding when compared to Nistari silkworms. In the case of Pure Mysore larvae, an isozyme fraction with R.F. 0.187 was observed only in 7th and 8th day. Also, another band with R.F. 0.405 was prominent on 8th day only. In the case of Nistari silkworms, a band with R.F. 0.182 was present only from 1st to 4th day. Among the bivoltines, in the case of NB_4D_2, one band with R.F. 0.193 was prominent only on 5th and 6th day. In the case of CSR_2 silkworms,

an isozyme fraction with 0.174 was clear only on third day. Of the hybrids, Pure Mysore x CSR_2 silkworms, two bands with R.F. 0.290 and 0.338 were prominent on 1st and 2nd day. In the case of Nistari x NB_4D_2 silkworms, an isozyme fraction with R.F. 0.335 was prominent from 3rd to 6th day.

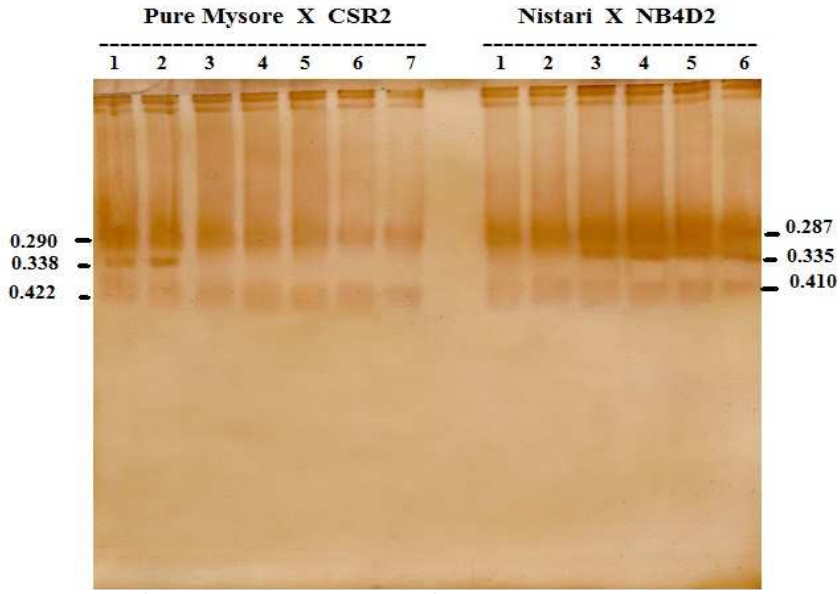

Figure 21: Native PAGE analysis of midgut alkaline phosphatase of Nistari x NB_4D_2 and Pure Mysore x CSR_2 silkworms. Lanes: 1-6 days in fifth instar.

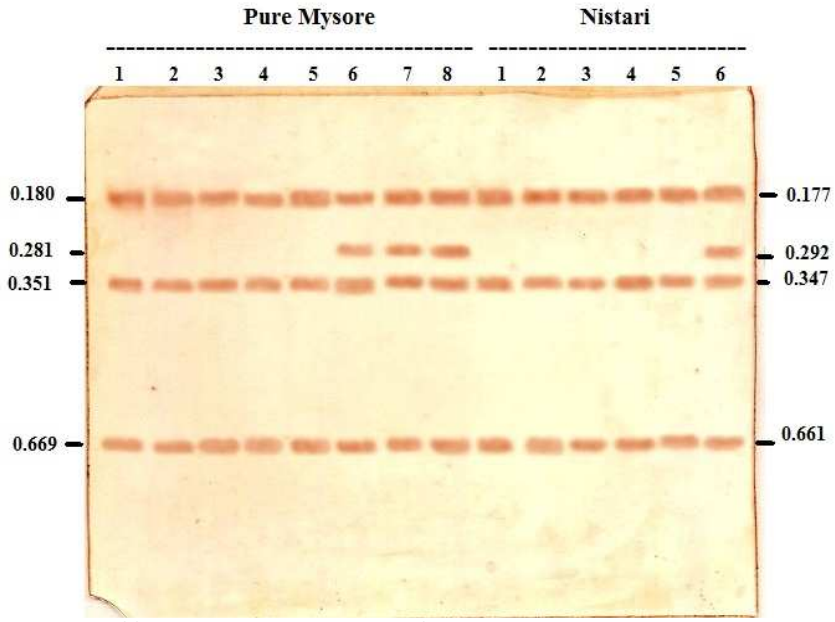

Figure 22: Native PAGE analysis of fat body alkaline phosphatase of Pure Mysore and Nistari silkworms. Lanes: 1-8 days in fifth instar.

In the case of fat body the silkworms of Pure Mysore breed exhibited a band with R.F. 0.281 was clear during later ages of fifth instar *i.e.*, from 6th to 8th day. Similar pattern was also observed in the case of Nistari silkworms, wherein a band with R.F. 0.292 was present only on 6th day. Among the bivoltines, NB_4D_2 silkworms exhibited two fractions with R.F. 0.301 and 0.356 were present only during later stage of fifth instar *i.e.* on 5th and 6th day. In the case of CSR_2 larvae, two isozyme fractions with R.F. 0.294 and 0.353 were appeared as in case of NB_4D_2 silkworms. Of the hybrids, Pure Mysore x CSR_2 larvae showed a fraction with R.F. 0.480 was prominent from 1st to 3rd day. In the case of Nistari x NB_4D_2 breed, a band with R.F. 0.398 was present during early days of fifth instar.

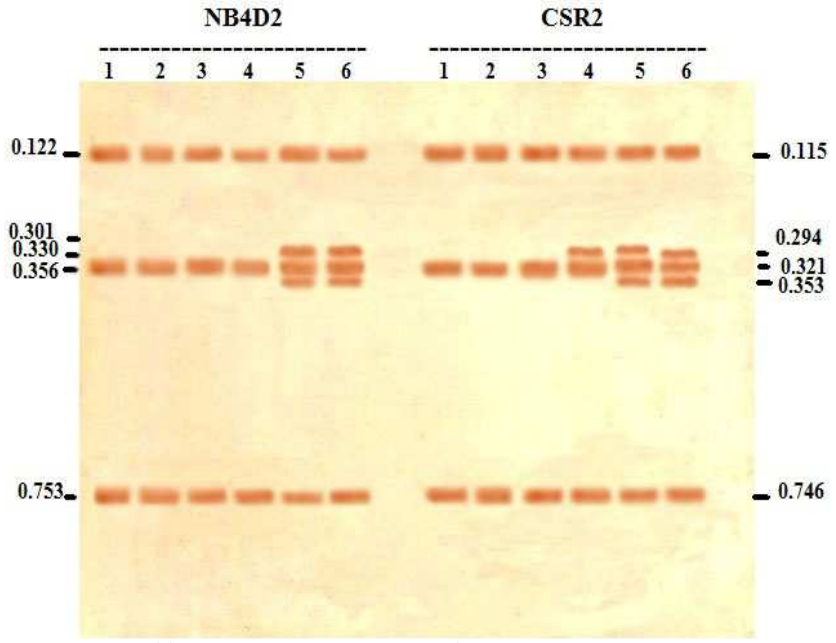

Figure 23: Native PAGE analysis of fat body alkaline phosphatase of NB_4D_2 and CSR_2 silkworms. Lanes: 1-6 days in fifth instar.

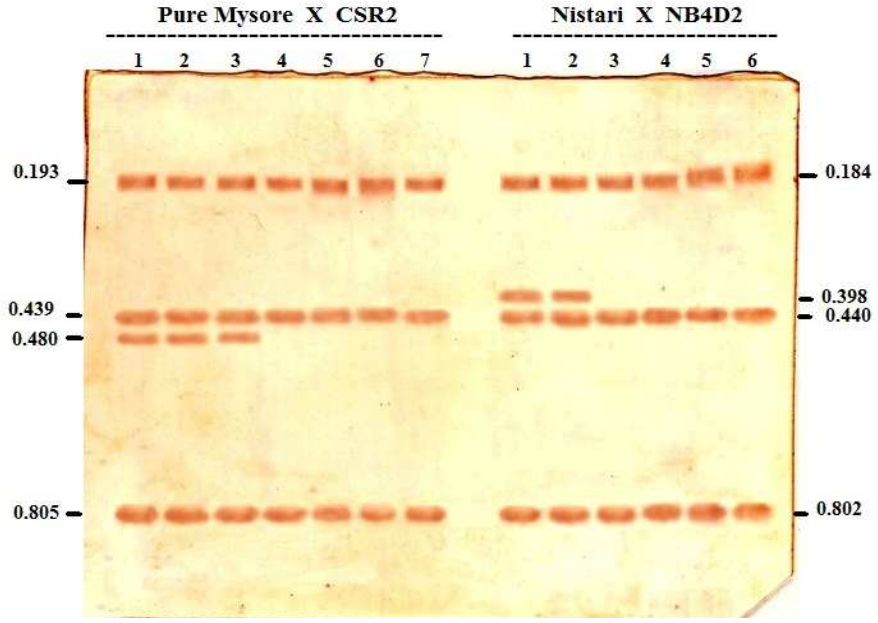

Figure 24: Native PAGE analysis of fat body alkaline phosphatase of Pure Mysore x CSR_2 and Nistari x NB_4D_2 silkworms. Lanes: 1-7 days in fifth instar.

DISCUSSION

Quantitative analysis of alkaline phosphatase activity levels clearly indicated two types of correlations *viz*., positive or negative correlation between activity levels with commercial characters. Alkaline phosphatase is a set of hydrolytic enzymes that hydrolyze phosphomonoesters under the alkaline condition. The activity of these enzymes is related to the physiological status of silkworms and reflects the absorption, digestion and positive transportation of nutrients in the midgut. Different stress and disease causes considerable decrease in the activity of ALKP [22]. Saravanan *et al.,* [23] reported that the increased ALKP activity levels indicates the better physiological situation and reflects the absorption, digestion and positive transportation of nutrients in the midgut. And also it indicates stress free and disease free condition of the silkworm. In the present study ALP level of activity increased

significantly indicates the better physiological situation and reflects the absorption, digestion and positive transportation of nutrients in the midgut. Several factors affect the activity of ALKP in the midgut of silkworm, such as viral and bacterial infection and administered chemicals [24].

Observation on the ALKP isozyme pattern has revealed that the banding pattern differs between pure races, between hybrids and between pure races and hybrids. The zymogram indicated the variation in R.F. and volume/intensity of the bands among the experimental silkworm breeds. The qualitative analysis of ALKP indicated six types of changes *i.e*., the intensity of the bands either more or less, besides, some of the bands either present or absent. Some of the bands increased or decreased in their intensity as the age advances in addition to altered R.F. value. Presence or absence of protein bands indicates either the non production or utilization or degradation of protein contents [25] when the studies are restricted within a particular race/breed. However, when the studies are concentrated between the races, it directly targets the genetic material as they are exactly determined by the genetic material of the organism. The variations in the activity levels and isozyme pattern clearly showed differences between the races. Therefore, by studying the silkworm ALKP with commercial characters, it is possible to have a clear picture about the kind and degree of correlation between them. An understanding of such correlations will help us to identify and exploit the marker molecule during breeding of new races of silkworm *Bombyx mori* with improved commercial characters.

CONCLUSSION

The present results clearly indicated that the alkaline phosphatase activity was nil in the haemolymph of silkworm *Bombyx mori* L. The midgut alkaline phosphatase activity levels showed moderately high positive correlation with renditta only. On the other hand fat body alkaline phosphatase activity level indicated slight positive correlation with fecundity only. In brief, alkaline phosphatase activity levels were not positively correlated with majority of the selected commercial traits. In view of the above, the alkaline phosphatase may be used as marker molecules during the evolution of new breeds of silkworm *Bombyx mori*.

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