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Analysis of digestive enzymes in different breeds of *Bombyx mori* in response to *Beauveria bassiana* infection

M Sheeba Praveena, G Savithri
Department of Biosciences and Sericulture, Sri Padmavati Mahila Visvavidyalayam (Women's University) Tirupati, Andhra Pradesh, India

Abstract
The study has been focused to examine the digestive enzymes such as amylase, trerhalase and lipase in the blood of the three breeds viz., bivoltine double hybrid(CSR2 X CSR27) X (CSR6 X CSR 26), crossbreed (PM x CSR2) and bivoltine single hybrid (CSR2 × CSR4) of fifth instar silkworm *Bombyx mori* during the development of fungal pathogen *Beauveria bassiana*. The enzymes play a significant role in breaking down the complex food material into simpler micro-biomolecules for absorption and assimilation, to provide energy and necessary metabolites for appropriate growth and development of an organism. Varied levels enzymes activity was recorded in the selected silkworm breeds under the fungal pathogen induced stress conditions in the haemolymph of silkworm. Steady drop in amylase and trehalaseenzymes activity was noticed throughout the 5th instar larvae with *Beauveria bassiana* induced stress conditions in all the breeds selected for the investigation, whereas the lipase activity was declined in the early stages of infection then the enzyme activity was elevated in diseased silkworms. The results of thecurrent investigationindicated that the activity of the digestive enzymes such as amylase, trehalase and lipase was higher in bivoltine double hybrid followed by crossbreed and bivoltine single hybrid in healthy and disease induced larvaethat indicates higher positive correlation with higher resistance.

Keywords: *Bombyx mori*, *Beauveria bassiana*, haemolymph, amylase, trehalase, lipase

Introduction
The silkworm, *Bombyx mori* L. is one of the important productive commercially significant sericigenous insects cultured under controlled microclimatic conditions and exploited for production of tradable queen of textile "Silk". Silkworm *Bombyx mori* is delicate and susceptible to different pathogenic micro-organisms, due to domestication for centuries which leads to cocoon crop loss and loss occurs particularly in final instars after substantial energy and money have been spent. *Beauveria bassiana*is one of the highly infectious and most destructive fungal pathogens, that causes heavy loss to cocoon crops which influences the quality of the silk. Enzymes are important biomolecules involved in thousands of biological inter conversions such as growth and development, healing diseases, breathing, digestion, reproduction, and many more biological activities that sustain life. Enzymes potentially support the most challenging health disorders in any host system by providing energy and eliminating the free radical damage. The most important digestive enzymes in the midgut of insects consist of amylases, lipases and proteases that catalyses macromolecules into substances that the digestive tract can absorb to provide energy source for the host system. During deficiency of digestive enzymes, the organism may take enzymes from the immune system to operate, in turn weakening immune function. Digestive enzymes are the energy providers necessary for metabolic responses essential to immune health and stimulate the natural defences. The enzymes are beneficial to relieve stress and strengthen the defence mechanism that can manage the healing process (Rajitha and Savithri 2014) [16].
Investigation on enzymatic studies during the development of the fungal pathogen *Beauveria bassiana*in the host organism i.e., in silkworm *Bombyx mori* may be helpful in understanding the interaction between the host system and the fungal pathogen as a part of a survival approach. As the studies on these lines is very scanty; an attempt has been made to examine the activity of the digestive enzymes viz., amylase, trehalase and lipase in the haemolymph of three breeds of silkworm *Bombyx mori* viz., bivoltine double hybrid (CSR2 X CSR27) X (CSR6 X CSR 26), crossbreed (PM x CSR2) and bivoltine single hybrid (CSR2 × CSR4) in *Beauveria bassiana* inoculated silkworms.

Material and methods
Maintenance of Silkworm Stock
Three silkworm breeds i.e., Bivoltinedouble hybrid (CSR2 X CSR27) X (CSR6 X CSR 26), crossbreed (PM x CSR2) and bivoltine single hybrid (CSR2 × CSR4)selected for the study were culture daccording to Dandin*et al*(2003) under required optimum conditions for appropriate growth and development of silkworms for the experimentation.

Culturing of Fungal Pathogen and Determination of LD50
Six days after death of *Beauveria bassiana* infected silkworms were collected and placed in a sterile container, then white powdery fungal mycelial sporeswere collected gently with the help of scalpel into a sterile petri plate. These fungal conidial spores were transferred onto the petri plates consists of Potato Dextrose Agar (PDA) media in a laminar flow and maintained 240 C ± 1^0 C for 4-7 days. Then conidia from a single colony of the *Beauveria bassiana* were transferred to sterile Potato Dextrose Agar (PDA) slants and the pure culture of the fungal pathogen was maintained by repeated transfers for every 15 days.

Nine serial dilutions of spore suspension were prepared from the pure culture and four replications were used and cumulative percentages of mortality of the different treatments were recorded. Then LD50 value was determined for different breeds selected for the study (2.5X 10^4 conidia/ml in crossbreed and Bivoltine single hybrid and 2.5 X 10^3 conidia/ml in Bivoltine double hybrid) in 5th instar silkworms) by using the probit analysis (Leora software, 1987).

Inoculation of *Beauveria bassiana* Spore suspension:

After completion of 4th instar, a set of 100 silkworms were selected per replication from the stock of all the breeds and fungal pathogen was inoculated in the sub-lethal concentration (2.15 X 10^6conidia/ml in crossbreed and Bivoltine single hybrid and 2.15 X 10^5 conidia/ml in Bivoltine Double hybrid) of ***Beauveria bassiana***spore suspension @ 50 ml/100 worms for 45 seconds. Four replications were maintained for control and treated silkworms. Then haemolymph of the control and inoculated larvae were collected every day from 1st day to 6th day of the 5th instar silkworm larvae, by clipping the third pair of prolegs. The haemolymph was drained into pre-chilled centrifuge tubes with a pinch of phenylthiourea from all the replications and then the haemolymph was used for the analysis three enzymes viz., amylase, trahalase and lipase by following standard procedure and protocols as mentioned below

- Amylase -Noetling and Bernfeld (1948) [15] Baker (1991) [2]
- Trehalase-Ishaaya and Swirsiki (1976).
- Lipase-Bier (1957) [3]

The data recorded was subjected to statistical analysis in terms of mean, standard deviation (SD), test of significance and per cent changes were calculated by using three-way ANOVA by using SPSS 3.1 software.

Results and discussion

Amylase

The results of amylase activity were showed in Table-1 and Graph-1. The raise of amylase activity was observed from 1st to 6th day in bivoltine double hybrid (151.8 IU/L to 169.1 IU/L). In crossbreed (147.6 IU/L to 158.2 IU/L) and bivoltine single hybrid (139.6 IU/L to 154.6 IU/L) elevation of amylase activity was noticed up to 3rd day then reduction of the enzyme activity was noticed on 4th day of both races viz., crossbreed (142.7 IU/L) and bivoltine single hybrid(140.2 IU/L) then again elevation of amylase activity was noticed in the rest of instar of both the races i.e., crossbreed (148.3 IU/L and 151.2 IU/L) and bivoltine single hybrid (144.5 IU/L and 148.3 IU/L) in healthy silkworms.

In *Beauveria bassiana*inoculated silkworms there is gradual reduction of amylase enzyme activity noticed from 1st day to 6th day of the 5th instar in all the breeds selected for the study i.e., bivoltine double hybrid (148.89 IU/L to 73.73 IU/L), crossbreed (141.53 IU/L to 67.36 IU/L) and in bivoltine single hybrid (136.2 IU/L to 60.4 IU/L) in compare to control. In bivoltine double hybrid higher levels of amylase activity were noticed followed by crossbreed and bivoltine single hybrid in both treated and untreated larvae.

The key role of digestive enzymes in the body of the insect is converting of complex food material into simpler micro-molecules essential for growth, development and other imperative functions (Wyatt and Kalf 1957) [24]. In silkworm *Bombyx mori* which is considered one of the major economically important insectsworldwide, amylase has been found as one of the key enzymes engaged in digestion and carbohydrate metabolism. Besides having significance in the dietary efficiency in *Bombyx mori* amylases have been found to possess close relations with the survival of the insect as well (Chatterjee *et al*1993) [6]. From histolytic tissues, amylase accrues in the haemolymph then it moves to the histo-genetic region where energy is required. It was then hydrolysis to α-D 1, 4- glycan linkage in starch and related carbohydrates by the catalytic action of amylase enzyme (Strobl *et al* 1998) [19]. In insect's body amylases have been differentiated from different origins (Baker 1991) [2]. In silkworm during their development and metamorphosis amylase converts starch to maltose, hydrolysis of maltose results in glucose by α-glycosidases to meet the energy demands. Christopher *et al* (1985) [7] suggested that in lepidopteron larvae rational food consumption was directly correlated with the activity of amylase. The larva which consumes 100% of food are found will have highest amylase activity. During the progress of *Beauveria bassiana* infection larva ceases food results in lower levels of amylase activity. Jeroh*et al* (2011) reported that inhibition of digestive enzymes that involved in digestion food and absorption and assimilation of carbohydrates can substantially alter the total carbohydrate content in the midgut and haemolymph resulting in the poor survival of the silkworm. Chatterjee *et al* (1973) [4] reported the importance of digestive amylase activity for the survival of the silkworm by way of disease resistance. The importance of amylase in silkworm concerning genetic variability, for better digestion andhigher survival has also been well documented (Chatterjee *et al*1993 and Aswath*et al* 2010) [7]. The amylase activity has been found to vary from breed to breed (Chatterjee *et al* 1988). Venugopal *et al* (1987) observed high degree of amylase activity in multivoltines than bivoltine. The result of the study was also supported by the investigations of Rajitha and Savithri (2014) [16] and the researchers reported that the decreased amylase activity in the haemolymph of fungal infected silkworm with reference to the control is due to less intake of food.

Table 1: Dynamics of Amylase enzyme activity (IU/L) in the haemolymph of silkworm *Bombyx mori* L. treated with fungal pathogen *Beauveria bassiana* (Bals.) Vuill.in three breeds selected for the study during fifth instar compared to control.

S.NO	Days	Amylaseenzyme					
		(CSR 2 x CSR 27) x (CSR 6 x CSR 26)		(PM X CSR2)		(CSR2 X CSR 4)	
		Control	Inoculated	Control	Inoculated	Control	Inoculated
1	Day 1	151.83	148.89	147.6	141.53	139.6	136.2
2	Day 2	156.7	131.6	152.3	125.5	146.4	120.4
3	Day 3	161.6	126.3	158.2	114.66	154.6	113.48
4	Day 4	165.6	113.3	142.7	103.53	140.2	98.6

5	Day 5	168.4	93.53	148.3	88.5	144.5	84.29
6	Day 6	169.1	73.73	151.2	67.36	148.3	60.4
Mean		162.20	114.566	150.05	106.85	145.60	102.22
Std. Deviation		6.339	25.692	7.485	25.126	8.245	25.54

Tests of Between-Subjects Effects					
Dependent Variable: AMYLASEENZYME					
Source	Type III Sum of Squares	df	Mean Square	F	P value	SIG
Breeds	4180.640	2	2090.320	210.210	0.000	**
Treatments	49120.005	1	49120.005	4939.673	0.000	**
Days	18612.953	5	3722.591	374.356	0.000	**
Breeds*Treatments	161.586	2	80.793	8.125	0.001	**
Breeds* Days	576.431	10	57.643	5.797	0.000	**
Treatments * Days	15350.764	5	3070.153	308.745	0.000	**
Breeds *Treatments * Days	589.977	10	58.998	5.933	0.000	**
Error	715.966	72	9.944			
Corrected Total	89308.323	107				
a R Squared =.992 (Adjusted R Squared =.988)						

p<0.05 Significant at 0.05 levelp<0.01 significant at 0.01 levelp>0.05 Not significant

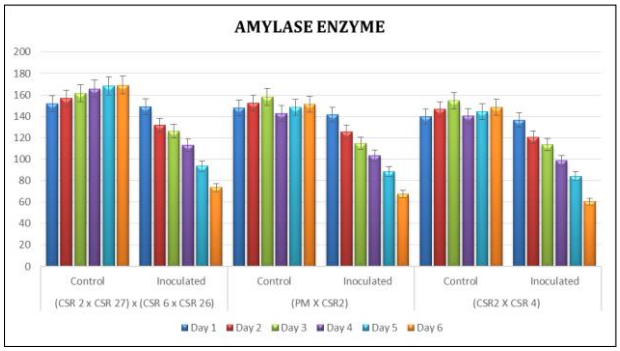

Fig 1

Trehalase

Results of trehalaseenzyme activity were presented in Table-2 and Graph-2. Increase of trehalase, enzyme activity was noticed from 1st to 6th day in bivoltine double hybrid (38.45 IU/L to 45.29 IU/L) followed by crossbreed (34.41 IU/L to 46.41 IU/L) and bivoltine single hybrid (32.01IU/L to 43.62 IU/L) in healthy silkworms. In bivoltine double hybrid a gradual decrease of the trehalase activity was recorded infected with fungal pathogen *Beauveria bassiana* up to 4th day (38.45 IU/L to 34.48 IU/L) and then the slightly increasing trend of trehalase activity was noticed (35.39 IU/L and 37.55 IU/L) in remaining days of the instar. In crossbreed (32.49 IU/L to 23.88 IU/L) and bivoltine single hybrid (23.61 IU/L to 20.33 IU/L) gradual decrease of trehalase activity was recorded from 1st day to 3rd day of the instar and then a gradual increase of trehalase enzyme activity was observed in the later days of the instar i.e., from 4th to 6th day in crossbreed (24.25 IU/L to 29.49 IU/L) and bivoltine single hybrid (21.48 IU/L to 28.76 IU/L). Compare to control, lower level of trehalase enzyme activity was recorded in fungal infected silkworms of all the breeds selected for the study throughout the instar. Maximum levels of trehalase enzyme activity were noticed in bivoltine double hybrid followed by crossbreed and bivoltine single hybrid in both treated and untreated silkworms.

Trehalose the chief blood sugar in insects differs from glucose in mammals. The enzyme trehalase catalyses trehalose into glucose in the organelle membrane or the cytoplasm and also the enzyme integrate into tissue cells. Soluble and membrane-bound trehalase proteins have been isolated from insects (Saito 1963) [17]. Tang *et al* (2008) [20] reported thattrehalase is one of the main factors that regulate trehalose levels in insect haemolymph. Xia, Glarkson and Charnley (2002) [25] stated that trehalose and trehalase activity play an important role in the regulation of insect development and growth. Trehalose of silkworm body fluid rapidly disappeared on starvation suggesting that it might be used as an energy source. The changes in trehalase activity levels are closely related to alterations in physiological conditions and developmental events thus trehalase is effectively involved both in regulating homeostasis in the body and to supply energy for development by hydrolyzing the reserve trehalose in the metabolically active tissues such as fat body and haemolymph (Terra and Ferreira 1994; Silva *et al*2009) [21, 18]. Under severe stress conditions and high energy demand trehalase activity and trehalose levels are inversely related, in *Beauveria bassiana* inoculated larvae trehalase activity diminishes due to decline of hydrolysis of trehalose to release glucose molecules (Hasegawa and Yamashita 1970) [10]. Gururaj *et al* (1999) [9] observed no significant variation in trehalase activity in the silkworm blood infected with *Bombyx mori* nuclear polyhedrosis virus (BmNPV) and in untreated silkworm up to 96 hours then the enzyme activity was elevated in the remaining days of the instar. He suggested that the enrichment of the enzyme activity is correlated with diminishing levels of trehalose. Yaginuma *et al* (1990) [26] recorded elevation of trehalase enzyme activity in the midgut of silkworm during the mid-phase of cytoplasmic polyhedrosis viral infection.

Table 2: Dynamics of trehalase enzyme activity (IU/L) in the haemolymph of silkworm *Bombyx mori* L. treated with fungal pathogen *Beauveria bassiana* (Bals.) Vuill.in three breeds selected for the study during fifth instar compared to control.

TREHALASEENZYME							
S.NO	Days	(CSR 2 x CSR 27) x (CSR 6 x CSR 26)		(PM X CSR2)		(CSR2 X CSR 4)	
		Control	Inoculated	Control	Inoculated	Control	Inoculated
1	Day 1	38.45	38.45	34.41	32.49	32.01	23.61
2	Day 2	39.03	37.51	34.51	29.49	35.6	21.46
3	Day 3	39.15	36.4	35.55	23.88	34.42	20.33

4	Day 4	43.21	34.48	38.53	24.25	37.11	21.48
5	Day 5	44.55	35.39	44.34	25.39	40.66	24.28
6	Day 6	45.29	37.55	46.41	29.49	43.62	28.76
	Mean	41.613	32.132	38.962	22.33	37.421	20.49
	Std. Deviation	3.104	6.984	4.964	2.298	4.177	1.881

Tests of Between-Subjects Effects

Dependent Variable: Trehalseenzyme

Source	Type III Sum of Squares	df	Mean Square	F	P value	SIG
Breeds	1252.167	2	626.084	402.308	0.000	**
Treatments	5480.408	1	5480.408	3521.595	0.000	**
Days	8.306	5	1.661	1.068	0.386	NS
Breeds*Treatments	322.270	2	161.135	103.542	0.000	**
Breeds* Days	348.934	10	34.893	22.422	0.000	**
Treatments * Days	1288.915	5	257.783	165.646	0.000	**
Breeds *Treatments * Days	100.598	10	10.060	6.464	0.000	**
Error	112.048	72	1.556			
Corrected Total	8913.647	107				

a R Squared =.987 (Adjusted R Squared =.981)

$p<0.05$ Significant at 0.05 level $p<0.01$ significant at 0.01 levelp>0.05 Not significant

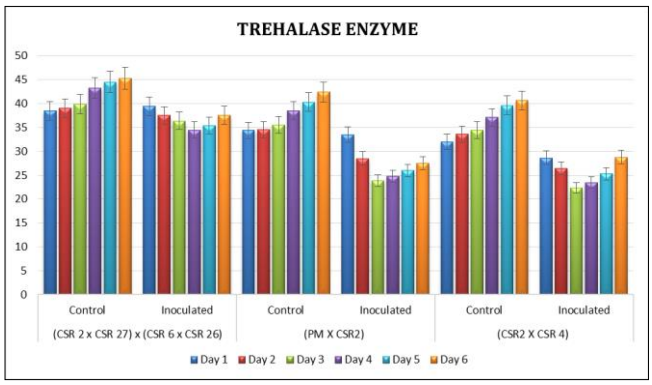

Fig 2

Lipase

The results of lipase enzyme activity are presented in Table-3 and Graph-3. Gradual decline of lipase enzyme activity was noted from 1st to 6th day of 5th instar silkworm larvae in three breeds selected for the study i.e., bivoltine double hybrid (62.71 IU/L to 36.50 IU/L), crossbreed (57.01 IU/L to 36.50 IU/L) and bivoltine single hybrid (53.25 IU/L to 33.75 IU/L) in control silkworm. Gradual decrease of the lipase activity was noticed from 1st day to 4th day in bivoltine double hybrid (53.25 IU/L to 43.12 IU/L) and then drastic increase of the enzyme activity was observed (46.25 IU/L and 47.36 IU/L) in remaining days of the instar in *Beauveria bassiana* inoculated silkworms whereas in crossbreed (50.99 IU/L to 41.85 IU/L) decreased trend of lipase activity was observed up to 3rd day and in bivoltine single hybrid (49.41 IU/L and 43.65 IU/L) decreased trend of lipase activity was noticed up to 2nd day, then increase of the lipase activity was noticed in remaining days of the instar in both crossbreed(42.25 IU/L to 32.31 IU/L) and bivoltine single hybrid (44.85 IU/L to 50.28 IU/L) of the instar. In contrast with healthy worms high levels of lipase activity was noticed throughout the instar i.e., 1st day to end of the instar in bivoltine double hybrid but in case of crossbreed higher lipase activity was observed only on 4th and 5th day and in bivoltine single hybrid higher level of lipase activity was noticed from 3rd day to till the end of the instar. Higher level of lipase activity was noticed in i.e. bivoltine double hybrid followed by crossbreed and bivoltine single hybrid in both inoculated and control batches. Lipase is an enzyme that catalyzes the hydrolysis the fats (lipids), which is subclass of the esterases. In most of living organism's lipases perform a crucial role in digestion, transportation and processing of dietary lipids (e.g. triglycerides, fats, oils). They hydrolyze lipids to form fatty acids and glycerol. The insect fat body plays vital role in the life of insects, the fat body tissue is dynamic that involved in several metabolic functions such as storage and release of energy in response to the energy demands of the insect. During energy requirement the organism access triglyceride stores by the action of lipases (lipolysis). Kangayam (2003) [14] observed that at the initial site of viral infection against Bm NPV insect lipase enzyme acts as a physiological barrier. Horne *et al* (2009) [11] observed that in storing, utilizing and transmitting lipids lipase enzyme plays a key role and also, they are essential for basic physiological processes such as reproduction, development, defending against pathogens and oxidative stress, and pheromone signalling in insects. According to Terra and Ferreira (2012) [12] in insects' lipases are divided into triacylglycerol lipases (TAG-lipases) and alkaline and acid phosphatases in addition to phospholipases. Besides Lipases have a crucial role physiological active; the catabolism of triacylglycerols (TAGs) stored as fat depots and those from dietary lipids.

Two most important groups of lipases are Liposomal (intracellular) and digestive lipases. Decreased levels of lipase enzyme activity in the haemolymph of ***Beauveria bassiana*** infected larvae during the early stage of infection may be due to a higher rate of lipolysis to cope up with the stress induced by the fungal pathogen. Decrease of lipase activity indicates the increased lipid levels in haemolymph which is very clearly obvious from the study. The enzyme may also be released by the pathogen to degrade structural lipids which may lead to distraction of structural organs of the host, by this, the pathogen could be nurture and developed strongly. Hanan Sadawy *et al* (2009) noticed that in *Parasarcophagaaegyptiaca* and *Argas (persicargas) persicus* higher levels of lipase enzyme activity and reduction of total lipids in parasitised hosts.

Table 3: Dynamics of Lipase enzyme activity (IU/L) in the haemolymph of silkworm *Bombyx mori* L. treated with fungal pathogen *Beauveria bassiana* (Bals.) Vuill.in three breeds selected for the study during fifth instar compared to control.

		LIPASEENZYME					
S.NO	**Days**	**(CSR 2 x CSR 27) x (CSR 6 x CSR 26)**		**(PM X CSR2)**		**(CSR2 X CSR 4)**	
		Control	**Inoculated**	**Control**	**Inoculated**	**Control**	**Inoculated**
1	Day 1	52.71	53.25	57.01	50.99	53.25	49.41
2	Day 2	48.89	49.27	48.49	46.8	47.83	43.65
3	Day 3	45.4	45.02	45.4	41.85	43.12	44.85
4	Day 4	43.74	43.12	43.74	42.25	40.23	45.47
5	Day 5	38.41	46.25	38.41	43.35	39.11	48.55
6	Day 6	36.5	47.36	36.5	45.91	33.75	50.28
Mean		45.872	47.373	44.153	45.191	42.9	47.035
Std. Deviation		9.406	14.7621	7.548	12.937	6.47	12.694

Tests of Between-Subjects Effects Dependent Variable: LIPASE Enzyme						
Source	Type III Sum of Squares	df	Mean Square	F	P value	SIG
Breeds	329.585	2	164.792	45.538	0.000	**
Treatments	3412.239	1	3412.239	942.922	0.000	**
Days	10983.926	5	2196.785	607.049	0.000	**
Breeds*Treatments	30.416	2	15.208	4.203	0.019	**
Breeds* days	153.740	10	15.374	4.248	0.000	**
Treatments * Days	1057.841	5	211.568	58.464	0.000	**
Breeds *Treatments * Days	18.091	10	1.809	0.500	0.885	NS
Error	260.553	72	3.619			
Corrected Total	16246.392	107				
a R Squared =.984 (Adjusted R Squared =.976)						

p<0.05 Significant at 0.05 levelp<0.01 significant at 0.01 levelp>0.05 Not significant

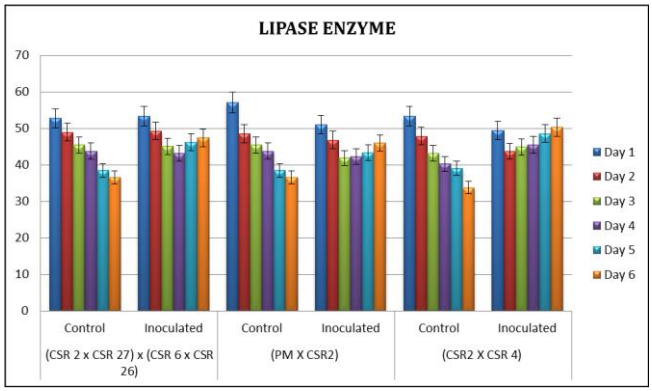

Fig 3

Conclusion

Dynamics of digestive enzyme in insect system signifies the level of health and immunity including infection. Enzymes play a defensive role against stress, any alterations in enzymatic parameters in infected silkworm would envisage the metabolic stress that the insect experience during the development of the pathogen.The ability to digest more food influences the healthy growth of any organism and resist diseases and physiological stress for better survival and in wide range of environmental conditions.The highest enzymes activity was observed in bivoltine double hybrid which indicates higher positive correlation with higher resistance under fungal infection followed by crossbreed and bivoltine single hybrid. Therefore, the degree of variations in enzymatic interactions in different breeds during the development of fungal pathogen *Beauveria bassiana* in silkworm *Bombyx mori* may provide a platform for the development of a diagnostic tool for the identification of the disease and in developing sustainable prophylactic strategies. The prevention of damage caused by diseases forms the foundation to harvest successful cocoon crops in order to provide quality silk to fetch better price.

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