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Effect of Endophytic Bacteria *Burkholderia cepacia* on Growth, Cocoon Characters and Enzyme Activity of Silkworm, *Bombyx mori* L.

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Authors' contributions

This work was carried out in collaboration between both authors. Author VG designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author AS managed the analyses of the study and the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Microorganisms have always been of scientific prominence and their indulgence in industrial and research facets cannot be denied. Several microorganisms have been employed as research tool to amend various parameters of industrial prominence. The current research article is from the context of significance emphasizing on the silk industry. The research article has focused on the impact of *Burkholderia cepacia* which is an endophytic bacterium on the silk worm and the affirmative aspects were recorded. The research work included the inoculation of the bacterium with the silk worm which has resulted in enhanced production of silk from the inoculated lot. In addition, the research has also demonstrated higher activity of protease and amylase in inoculated lot when compared to control population. The results obtained have substantiated the practical aspect of the research.

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Keywords: Burkholderia cepacia; Bombyx mori; endophytic bacteria; microorgan; silk worm.

1. INTRODUCTION

SILK-The queen of textiles, is the natural fiber, spells luxury, elegance, class and comfort, which is secreted by silkworm. India is the second largest consumer of silk in the world. India is the unique country in the world to produce all the four types of commercial silk and stands second in the production of mulberry silk (Murugesh et al. 2004). Silk worm has various advantages as experimental animal such as the low cost for rearing and fewer ethical issues [1]. Generally, at the first larvae development high temperaure prolong life span and determine cocoon character. However, the fluctuation and wide range temperature inhibit larvae development [2]. Silkworm (*B. mori L.)* is well known Lepidopteron (Family: Bombycidae), the larvae instars of which feed on the leaves of mulberry used for silk production. Indian silk industry is based largely on the mulberry silkworm. As the insect for silk production, silk worm has very economicvalue because silk is a very good textile material and is utilized widely [3]. Economics of silk production depends on the quality of cocoons produced by the worm [4], which in turn is depended upon the nutritional demands of silkworm. The silkworm is considered a central model species for lepidopteran genomics and genetics and it is second only to the fruit fly (*Drosophila melanogaster*) as an insect model for biological studies [5]. Mulberry silkworm, *B. mori* susceptible to a number of diseases and also to the attack of pests and parasites. There is no silkworm race at present, which can be deemed as totally resistant to diseases or pest [6]. The fungi, bacteria, nematode and viral diseases persist throughout the year. Though most of these diseases appear and cause maximum damage during rainy and winter season, there are also few diseases that appear during summer and cause reduction in plant growth Aleksey [7]. Silkworm is poikilotherm; it cannot regulate its body temperature and is susceptible to several diseases [8]. Diseases in silkworm and mulberry plants caused by pathogens reduce the quality and quantity of silk production which in turn affects normal economy. Attempts have been made in sericulture with nutrient such as protein, vitamin, carbohydrates, amino acids, hormones and antibiotic etc for better performance of good quality of cocoons [9].

Mulberry (*Morus alba* L.) leaves, being the only source of nourishment is certainly imperative that the supply of good quality leaf is most important for getting good quality cocoons [10]. However, disease resistance and other improved traits which can augment productivity and quality are needed to be inoculated to enhance the economic benefits to the sericulture farmers [6]. Mulberry harbors a large number of microbes which include bacteria like *B. cepacia, Bacillus subtilis, Pseudomonas aeruginosa* etc and fungi, actinomycetes. Among which *B. cepacia, B. subtilis* and *P. aeruginosa*have been reported to be endophytic bacteria of mulberry which improves plant growth and control of foliar and soil borne fungal and bacterial pathogens of mulberry. Mulberry is infected by a number of root diseases among them root rot disease of mulberry caused by *Rhizoctonia bataticola* poses a serious threat in all mulberry growing areas throughout the year leading to the death of plants within a short period (Philip et al., 1997, Chowdary et al., 2003). Mulberry *(M. alba* L.) the only food crop for silkworm is widely cultivated throughout subtropical, tropical and temperate regions in the world. In India, mulberry is cultivated in 2 lakh hectares under different agro climatic conditions. Integrated methods used for control of diseases especially root rot is still posing threats to mulberry cultivation. Besides pathogens, it also contains a group of beneficial microbe viz nitrogen fixers, phosphate solubilizers, potassium solubilizers and antagonistic bacteria/fungi. Among them few endophytic bacteria were proved to be effective incontrol of root rot of mulberry (Gunasekar et al. 2011) and also improve plant growth [11]. Recent approaches in this direction include the application of VAM fungi and bacterial biofertilizers to improve the mulberry leaves quality and thereby the cocoon characters [12]. The quality of feed is determined by its major components such as water, carbohydrates, proteins, minerals, elements,fats, amino acids and vitamins [13].

Few endophytic bacteria were proved to be effective in control of root rot of mulberry (Gunasekhar et al. 2011) and endophyticin nature, when applied to soil it reaches to leaf within short period. The endophytic bacteria (*B. subtilis, B. cepacia* and *P. aeruginosa*) are reported to produce plant growth hormones, solubilize phosphates, fix nitrogen and produce siderophores in plants. For silkworm growth and development, amylase and protease activity in the gut region play much role for digestion and

growth of larvae. In the present study, an attempt has been made to know the probiotic/deleterious effect of *B. cepacia* on silkworm growth and development and the effect on amylase and protease activity in silkworm in midgut tissue and midgut juice. This is informative to know the probiotic activity of bacteria in silkworm which improves larval growth.

Gut micro flora is regarded as valuable metabolic resources for the insect on suboptimal diets, but apart from this, most relationship and their micro biota remain undefined. Microbial transformation of plant secondary compounds in an insect gut and adoption by the host to use resulting common metabolites are unique to insects [14]. Some of the gut micro flora of silkworm includes *B. cereus, B. subtilis, Lactococcus lactis, Staphylococcus lactis, Enterobacter aerogenes* etc [15].

Probiotics are the live microbial food supplements beneficially affecting host by improving the microbial balance and enhanced rapid cellular growth and development (Fuller et al. 1993). The gut probiotics are involved in the digestive utilization of feeds and detoxification of metabolite. Stimulation of non specific immune system. They also promote the production of vitamins and increase host resistance and compete with pathogenic bacteria by producing organic and antibiotic substances. The *Lactobacillus plantarum* is a probiotic which improves the cocoon production of mulberry silkworm *B. mori* [16]. Certain probiotic bacteria inhibit the growth of microbes.

*S. nourse*iare probiotic microbes which prove the antibacterial activity and good ecofriendly management of silkworm diseases [17]. Impact of robotics (*Lactobacillus, Saccharomyces cereviciae* and effective microorganisms) treatment on mulberry leaves to modulate the economic parameters of 5th instars larvae of *B. mori* were studied (Jeyapaul et al. 2004) Amala et al. 2011 had stated that *S. cereviciae* serves as an immune modulating agent in silkworm *B. mori.* When the probiotic *S. cereviciae* was used for the treatment there was a considerable increase on the energy budget and the commercial characteristics of *B. mori* and also there was an increase in the level of protein content in treated worms. Yeast improves the protein content and commercial production. The leaves of mulberry are the sole source of food for larval instars of silkworm *B. mori,* biochemically constituted with proteins, lipids, carbohydrates and minerals. Therefore, corresponding diversity of enzymes capable of hydrolyzing the bio compounds of mulberry is exhibited by mid gut of larval instars of silkworm, *B. mori*.

Horie et al., (2010) that, molecular proteins are hydrolyzed into peptides by digestive fluid content and into amino acids with peptidase in the mid gut tissue likewise, the polysaccharides, are digested in the insect gut lumen by digestive fluid and disaccharides and/or trisaccharides get hydrolyzed into their constituent monosaccharide sugars mainly in the gut tissue.

The digestibility of silkworm larva depends upon the activity of an enzyme called amylase. Amylase is one of the most important enzyme which helps in digestion of starch in silkworm. It is the key enzyme involved in digestion and carbohydrate metabolism in insect. Of the various enzymes analyzed amylase is well worked out because of its close association with the economic parameters of silkworm [18]. The literature viz Sengupta et al. [19]; Mathavan et al., 1984; Jeyapaul et al., 2003a and 2003b and Sheeba et al., 2007 reveals that the biochemical formulations promote the levels of enzyme activity which ultimately enhances the quality of the traits.

Strain Lu10-1 of *B. cetacean* (Gene bank EF 546394) is an antagonistic entophyte originally isolated from mulberry (*Morusalba*) leaves. *B. cepacia* strain Lu10-1 is an endophyte that can multiply and spread in mulberry seedlings rapidly and efficiently. The strain is antagonistic to *C. dematium* and act as an efficient plant growth promoting agent on mulberry seedlings and is therefore a promising candidate as a biocontrol and growth promoting agent [11]. In the present study, an attempt has been made to know the effect of endophytic bacteria *B. cepacia* on growth and development of silkworm as well as the activity of digestive enzymes i.e., amylase and protease are estimated by standard procedures.

List of gut micro flora of silkworm *(B. mori*):

Bacteria	References
Bacillus cereus	Sekar et al. [15] George John study on the establishment of bacterial
Bacillus subtilis	micro the gut of silkworm *Bombyx mori*. Internation of current research.

Bacteria	References
Lactococcus lactis *Staphylococcus lactis* *Enterobacter aerogenes*	
Pseudomonas aeruginosa *Bacillus circulans* *Proteus vulgaris* *Klebsiella pneumonia* *Escherichia coli* *Citrobacter freundii* *Serratia liquefaciens* *Enterobacter* sp. *Pseudomonas fluorescens* *Aeromonas* sp. *Erwinia* sp.	Vitthalrao [20] Khyadeand Rjendra M. Mara diversity of bacterial flora in the midget of fi larvae of silkworm, *Bombyx mori* (Race: PM×CSR2). G.J.B.B.
Streptomyces noursei *Bacillus subtilis*	Mohanraj and Subramanian [21]. Ant activity of gut flora isolates from mulberry *Bombyx mori*. An International Quarterly J. of Environmental Sciences.

2. MATERIALS AND METHODS

To study the effect of endophytic bacteria, *Burkholderia cepacia* (Rifampicine resistant bacterial strain) was collected from the stock culture of Agronomy section, CSRTI, Mysuru. Silkworm weight and other statistical data were collected from CSRTI rearing section, Mysuru, Karnataka, India.

Materials required: Starch solution (1%), DNS reagent, Phosphate buffer (pH 6.8), Casein solution (1%), Ninhydrin reagent and Sucrose solution 0.25 M.

Inoculum preparation: *B. cepacia* culture was multiplied on Luria Bertani agar media. Streaked bacteria on LB agar plate were incubated at 30+-2°C for 24 hours. A loopful of 24 hour bacterial culture, *B. cepacia* was inoculated to LB broth. The inoculated cultures were incubated at 30+-2°C at 100 rpm in an orbital shaking incubator (Paragon RPM-0249). 48 hours old bacterial culture was then centrifuged at 12000 rpm for 30 minute (Refrigerated centrifuge REMI CH 12). The supernatant was discarded and the pellets obtained were dissolved in 100 ml physiological saline water. Bacterial cell concentration was adjusted to 10^8 CFU/ml (by adding physiological saline water) with the help of UV or visible spectrophotometer (ELICO SL 171 mini spec) at 660nm (Optical density for 10^8 CFU/ml at 0.1). From 10^8 CFU/ml concentrations, 10^6 CFU/ml suspensions were prepared using serial dilution method.

Bioassay: A popular silkworm double hybrid (CSR50×CSR52) × (CSR51×CSR53) was used for the bioassay experiments. The layings were obtained from silkworm seed production centre, Mysore and the experiments were conducted at silkworm physiology laboratory, CSRTI, Mysore. The hatched larvae are reared in plastic trays as per standard procedures. After fourth moult, the larvae were used for experimentation. 100 healthy larvae of 5th stage (Before 1st feeding) were selected and kept in plastic trays. For each treatment, 3 replicates were maintained.

Inoculation of bacteria to silkworm: For 1st feeding of 5th stage larvae*, B. cepacia* suspensions were injected orally by feeding through mulberry leaves. Two concentrations of bacteria 10^6 and 10^8 CFU/ml was prepared as described earlier. Healthy mulberry leaves were cut into 10cm discs, 5 such discs were fed to 100 larvae of silkworm. Before feeding, 1 ml of inoculums was evenly spread on the dorsal side of the leaf disc with sterile plastic spreader. 2 treatments 10^6 and 10^8 CFU/ml were tested. For control 5 such discs were treated with 1 ml of physiological saline water. 2nd feeding onwards normal leaves were fed up to the spinning. For each treatment 3 replicates were maintained.

Data on larval growth: During 5th stage of larvae, before first feeding 10 larval weights were recorded. 3 replicates were maintained for each treatment and control. During 5th stage of larvae, the data on larval weight was recorded at 24 hours interval up to 6 days i.e., up to the maturity of worms for spinning.

Amylase and Protease activity: Amylase and protease activity in silkworm gut juice and tissue was estimated on 5th day larvae of 5th instar. Mid

gut tissue and mid gut juice was collected for the estimation of amylase and protease activity.

Isolation of midgut tissue and midgut juice: 0.5 ml of mid gut juice was drawn from the anterior end of silkworm 5th day of 5th instar larvae in an eppendroff's tube rinsed with an anticoagulant Thiourea. Similarly mid gut tissue was excised by cutting larval skin dorsally in a dissection tray containing ice cold ringer solution with TrisHCl buffer (pH 7). Mid gut tissue was collected by separating anterior and posterior part of the gut and transferred to a pre cooled plastic vials.

Enzyme assay: 0.1 gram of mid gut tissue was collected and ground with 5 ml of 0.25 M sucrose solution in a mortar and pestle. 0.5 ml of mid gut juice and 5 ml of sucrose was mixed. Then the suspensions were centrifuged at 4000 rpm for 30 minutes. 0.5 ml of supernatant from both tissue and juice samples were collected separately in respectively labeled test tubes. 2 ml of phosphate buffer pH (6.8) was added to each test tubes including control. Then 1 ml of 1% starch solution was added to test tubes meant for amylase activity and 1% casein solution was added to test tubes meant for protease activity. The test tubes were incubated at room temperature for 15 minutes. Then 2 ml of DNS reagent and Ninhydrin reagents were added to amylase and protease test tubes respectively. The test tubes were kept for water bath for 30 minutes. After cooling, enzyme activity was measured at 540nm spectrophotometrically.

Amylase activity = (Concentration of product formed × 2) / (Molecular weight of glucose × time of incubation)

Protease activity = (Concentration of product formed × 2) / (Molecular weight of tyrosine × time of incubation)

Composition of Luria Bertani (LB) media:

Ingredients: Gms / Litre
Casein enzymic hydrolysate: 10.000
Yeast extract 5.000 Sodium chloride: 10.000
Final pH: (at 25°C) 7.5±0.2

3. RESULTS AND DISCUSSION

The results obtained were very transparent and have substantiated our research work. The research work has indeed considered several facets ranging from weight to enzyme activity and larval gut microbial profile. Table 1 depicts the outcome of *B. cepacia* on the larval growth which has confirmed an upsurge in larval weight.

The larval weight was kept under constant observation and the weight was recorded from the 1st day of 5th in-star at intervals of 24 hours for 5 days. The product of the research work has demonstrated an increase in the weight in succession since day 1. The research has also substantiated the relation between the larvae culture and the extent of bacterial load. It was found that the larvae weight enhancement was directly proportional to the extent of bacterial culture. Higher concentrations of bacterial cultures have indeed had an affirmative impact and have positively contributed to larvae weight.

The increase in the larval weight was in correspondence to amylase and protease activities measured on 5th day larvae and have been depicted in Table 2. Control was used in accordance to the treated sample in order to decipher the outcome for productive interpretation. Similarly the amylase activity of mid gut juice was recorded as 0.0272 µmoles/min/ml, 0.0278 µmoles/min/ml and 0.0279 µmoles/min/ml in control, T1 and T2 respectively. These results validated the fundamental shift in microbial profile in silkworm larval gut which is beneficial to the host and in turn may significantly contribute to increase silk production. Food has also been a vital criteria in deciding the amount of silk production and has been regulated by its physical nature and presence of phago stimulants in the food [22]. Silkworm *B. mori* (L) reared on mulberry leaves supplemented with minerals, oral extracts, plant growth hormones [23] are reported to have beneficial effects on economic parameters.

The increase in the larval and cocoon weight was in correspondence of protease activity measured on 5th day larvae. Protease activity in tissue was observed to be 0.042 µmoles/min/ml of sample in control and 0.070 µmoles/min/ml and 0.082 µmoles/min/ml in T1 and T2 respectively. Similarly the protease activity in mid gut juice was observed to be 0.178 µmoles/min/ml in control, 0.296 µmoles/min/ml in T1 and 0.334 µmoles/min/ml in T2 respectively. *B. cepacia* was fed to silkworm orally through mulberry leaf, it reaches to mid gut and survive for life time and increases the enzyme activity of silkworm and improves its digestivity.

Table 1. Represents average weight of 10 larvae of 5th instar from 1st to 5th day

Treatment	Grams				
	1st day	2nd day	3rd day	4th day	5th day
C	14.079	20.458	31.474	40.909	44.403
T1	14.391	23.328	33.324	43.79	45.478
T2	16.381	25.013	34.095	44.838	46.459

Table 2. Represents amylase and protease activity of 5th day larvae of 5th instar

Treatment	µmoles/min/ml of sample for amylase activity		µmoles/min/ml of sample for protease activity	
	Mid gut tissue	Mid gut juice	Mid gut tissue	Mid gut juice
C	0.0346	0.0272	0.042	0.178
T1	0.0359	0.0278	0.070	0.296
T2	0.0376	0.0279	0.082	0.334

Table 3. Represents results on cocoon characters

	SCW	SSW	SR%
C	2.096	0.446	21.27
T1	2.121	0.459	21.64
T2	2.051	0.467	22.76

SCW - Single cocoon weight, SSW - Single shell weight and SR% - Shell ratio

Experimental results on the isolation of *B. cepacia* fed to the silkworm from the fecal matter after ingestion to the larvae revealed that the bacteria survived in the digestive tract. Similarly as amylase and protease activity also represented in Table 2, the results indicated that the amylase and protease activity of 5th in star larvae mainly for the digestion and absorption of sugar and protein content of the mulberry leaves consequently which increases haemolymph and silk gland protein content ultimately increases silk productivity of the silkworm Thirumalaisamy et al. [13]. Glycogen being a storage polysaccharide was found to be high in the experimental groups of silkworm *B. mori*. It is significant to correlate to the availability of increased sugars, which may undergo glycogenesis resulting in more amount of glycogen.

Amylase catalyses the specific hydrolysis of the glycosidic bonds in specific hydrolysis of the glycosidic bonds in glycogen [24]. Hence the increased amount of glycogen may bring about the increased secretion of digestive enzyme amylase. Increase in protease activity may be attributed to the increased concentration of silk protein for silk production. The digestive tissue may be tuned to synthesize more of protease enzyme since the protein content increased significantly over control category on UV ray treatment at 280-400 nm [25]. The results on cocoon characters were presented in Table 3. Single cocoon weight, Single shell weight and SR% was increased in both treatments. The SR% in control was 21.27 and T1 and T2 was 21.64 and 22.76 respectively. The SR% may be in correspondence with amylase and protease activity of silkworm larvae treated with 10^6 (T1) and 10^8 (T2) concentrations of *B. cepacia.*

Fig. 1. Dorsal side of mulberry leaves spread with inoculum were fed to 1st day of 5th instar larvae

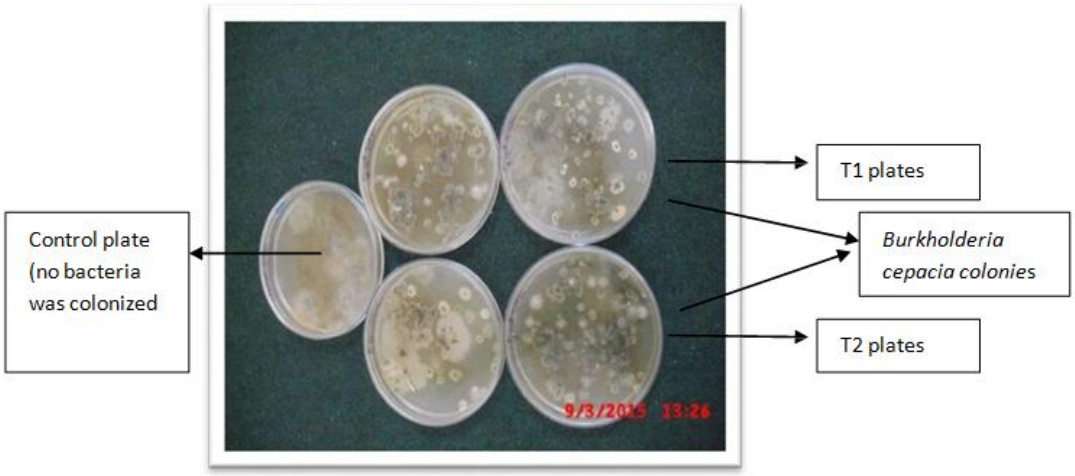

Fig. 2. Isolation of *B. cepacia* from the fecal matter of silkworm fed with bacterial inoculum

4. CONCLUSION

The study of endophytic bacteria, *B. cepacia* on silkworm (*B. mori*) growth and enzyme activity gives a conclusion that the endophytic bacteria increases the growth of the silkworm compared to that of the normal values. There was an increase in the weight of the cocoon, which in turn increase the silk yield. The increase in the larval and cocoon weight was in correspondence of protease activity. The increased amount of glycogen may bring about the increased secretion of digestive enzyme amylase. Increase in protease activity may be attributed to the increased concentration of silk protein for silk.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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