Microbiology Research International
Vol. 12(1), pp. 1-9, February 2024
DOI: 10.30918/MRI.121.23.024
ISSN: 2354-2128
Full Length Research Paper

Isolation and characterization of antibacterial phylloplane bacteria *Bacillus subtilis* isolated from *Terminalia arjuna* leaves

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Accepted 12 June, 2023

ABSTRACT

Bacterial diseases are becoming a major constraint during the rearing of tropical tasar silkworms. Because of the devastating nature of the disease, crop loss is severe; it is necessary to develop an eco-friendly biocontrol strategy that can be utilized in the tasar rearing plots. In the present study, the phylloplane bacteria were isolated from *Terminalia arjuna* and *Terminalia tomentosa* and were screened for antimicrobial potential against the tasar silkworm infecting pathogenic bacterial isolates. The promising bacteria was identified as *Bacillus subtilis* using biochemical as well molecular techniques (16s Ribosomal gene amplification). The treatment of *Bacillus subtilis* showed more survival as well as improved cocoon characteristics in both outdoor and indoor rearing conditions. Further, the treatment of *Bacillus subtilis* along with bacterial pathogens showed induction of protein, carbohydrate, and peroxidase activity and reduction in catalase activity which helps the tasar silkworm build resistance against bacterial pathogens. Hence, treatment of *Bacillus subtilis* during the rearing of tasar silkworm will reduce the bacterial infection which leads to enhanced tasar silk production.

Keywords: Phylloplane bacteria, *Terminalia arjuna*, *Bacillus subtilis*, molecular characterization.

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INTRODUCTION

Sericulture has been one of the main branches of agriculture in Asian countries for centuries. Silkworm disease is considered one of the major technical problems. Tropical Tasar silkworms (*Antheraea mylitta* Drury) are infected by different kinds of pathogens viz., microsporidia (pebrine), bacteria (bacteriosis), fungi (mycosis), and virus (virosis). These pathogens cause considerable yield loss of up to 40% (Sahay et al., 2000). Among all the diseases, bacterial diseases are most prevalent during the rearing season causing considerable yield loss. Three types of bacteria symptoms can be noticed in tropical tasar silkworms viz., Sealing of Anal Lips, Rectal Protrusion, and Chain Type Excreta caused by different bacterial pathogens.

Bacteria that colonize the aerial plant parts such as the phylloplane are exposed to higher temperature and moisture fluctuations with limited nutrient availability and such bacteria have a better chance of survivability and reproduction in a nutritionally enriched rhizosphere soil. The phylloplane provides a diversity of beneficial bacteria because of the frequent drift in the microbial communities. Phylloplane bacteria have been identified as biocontrol agents (Andrews, 1992).

Earlier, some work has been carried out to isolate the phylloplane bacteria for the management of bacterial diseases of tropical tasar silkworms (Sahay et al., 2000). The present work also aims to evaluate the *Bacillus subtilis* on the rearing performance of tropical tasar

silkworm (*Antheraea mylitta* D.) and study the biochemical changes induced by biocontrol agents in silkworm larvae against pathogens.

MATERIALS AND METHODS

Maintenance of pathogenic bacterial isolates

The pre-maintained pathogenic bacteria species *Staphylococcus* sp. (CTE), *Micrococcus* sp. (SAL), and *Serratia* sp. (RP) were inoculated to test tubes and flasks. The inoculated test tubes and flasks were kept at room temperature (25^{o}C) for 48hrs and observed for bacterial growth using turbidimetric determination.

Collection of leaves

Four months old leaves (10 per plant species) were collected from *Terminalia tomentosa* and *Terminalia arjuna* in the plantation area of Central tasar research and training institute, Ranchi. The leaves were picked with sterile forceps and placed in sterile polyethylene bags, which were kept in a refrigerator (4^{0}C) till further processing (for a period that usually did not exceed 4−5 hours).

Phylloplane bacteria isolation

Phylloplane bacteria were isolated from leaf washings. Leaves from each plant species were pooled in 10 g lots and washed by mechanical shaking in 200 ml of sterilized ultra-pure water at room temperature **(25^{o}C)** for 1 hour and the water suspension was decanted. The water suspension containing bacteria was used for the serial dilution method. The bacteria cultured on the nutrient agar plate were isolated individually and used for further studies.

Mass screening of Phylloplane bacterial species against pathogenic bacterial species (In vitro Inhibition Assay)

Each Pathogenic and antagonistic bacterial suspension *(B. subtilis)* was prepared in sterilized water and the initial concentration of bacteria was adjusted, approximately to 10^{8} colony-forming units (CFU)/ml (by reading absorbance at 700nm in spectrophotometer). Each phylloplane bacteria was screened against the pathogenic bacterial pathogens by using the gel diffusion method (the test agar plate is swabbed with a standardized concentration of the test organism, and then paper disks containing a defined biocontrol agent suspension were placed on the lawn of bacteria) and the promising bacterium was used for further studies. The Ampicillin was used as the standard.

Characterization of the promising biocontrol agent

The promising biocontrol agent was characterized by using biochemical and molecular methods. The bacteria were characterized biochemically by Gram Staining, Starch Hydrolysis, Catalase activity, Lipase activity, Casein hydrolysis, and KOH solubility test.

Molecular characterization of the promising biocontrol agent

Antagonistic bacterial isolates were grown in Nutrient broth at 25°C for 2 days. The bacterial suspension was centrifuged at 10,000 rpm for 10 minutes at 4^{o}C. The bacterial cells were scraped from tubes with a sterile scalpel, collected into microtubes, and were lysed with lysis buffer (50 mMTris, 250mM NaCl, 50 mM EDTA, 0.3% SDS (w/v), pH 8,0) mixed with glass beads (425−600 µm). After centrifugation, the supernatant was transferred to clean tubes with Tris-EDTA (10 mM Tris, 1 mM EDTA) and RNAse (Sigma, USA) (60 µg/mL) and incubated for 30 min, at 37°C. Chloroform/isoamyl alcohol (24:1) was then added for extraction. The supernatant was transferred to a new tube and DNA was precipitated with cold ethanol. The resulting pellet was air-dried and resuspended in Tris-EDTA. PCR reactions were carried out in a 50 µl mixture containing 1µl of genomic DNA, 2.5 units of Taq DNA polymerase, 125 µM of each dNTP, and 0.2 µM of primer (1µl) (Sigma, USA) of the target gene. The cycle numbers were optimized to ensure that the amplification of the gene of interest remained within the exponential amplification range. Amplification was carried out according to the following temperature profile: 94^{o}C for 2 min for denaturation, followed by 30 cycles for 94^{o}C for 30 s, 58^{o}C for 30 s, and 72^{o}C for 1 min, with a final elongation at 72^{o}C for 10 min. The obtained PCR product was separated by ethidium bromide-stained 1.4% TAE-agarose gels and documented (Infinitti, VilberLourmat, France). The PCR product (50 µl) was also purified using a Minielute™ Gel extraction kit (Qiagen, France) according to the manufacturer`s instructions for sequencing. The PCR product was sequenced by the dideoxy nucleotide method using the Big Dye Terminator ver. 3.0 Kit (Applied Biosystems) from both strands and multiple sequence alignment was performed using Multalin software (France) and deposited to NCBI Gene bank (Accession number JX006081).

The following set of Primers was used in the study:

27F (Forward) AGAGTTTGATCMTGGCTCAG

1492R (Reverse) ACCTTGTTACGACTT (Lane et al., 1991).

In vivo evaluation of newly isolated biocontrol agents (outdoor rearing)

Disease-free larvae (500 numbers) for each treatment were brushed on *Terminalia arjuna* plants. After 24hrs of brushing, the bushes containing silkworm larvae were spray treated with the *Bacillus subtilis* suspension with a concentration of 10^8 colony-forming units (CFU)/ml. The untreated batches of larvae were used as a control. The final yield was recorded in terms of the number of cocoons harvested. The Shell Ratio (SR%) was recorded from the harvested cocoons to analyze the impact of *B. subtilis* on the cocoon characters.

In vitro evaluation of biocontrol agents (indoor rearing)

Disease-free larvae (50 larvae) before the 4^{th} moult, which were reared in outdoor conditions transferred to indoor conditions. Twenty-four hours after 4^{th} Moult, the larvae were inoculated with pathogenic bacteria (*Staphylococcus* sp.) along with un-inoculated control. After 24h of inoculation with pathogenic bacteria, biocontrol agents were treated to larvae. Survival % was recorded.

Biochemical changes induced by *Bacillus subtilis* in tasar silkworm larvae under pathogen inoculated condition

Collection, Preparation of Samples for biochemical studies

Fifty (50) larvae were brushed on *Terminalia arjuna* (arjun) plants in indoor conditions. Twenty-four hours after the 4^{th} moult, the larvae were inoculated with different pathogenic bacteria along with un-inoculated control. After 24h of inoculation with pathogenic bacteria, biocontrol agents were treated to larvae. The collected larvae were stored at -20^{o}C until further use.

Midgut, hemolymph, and fat bodies of both control and infected were collected separately for the biochemical studies. Hemolymph was collected by cutting the pro leg of silkworm larvae in a pre-cooled micro-centrifuge tube containing a pinch of phenylthiourea as an anticoagulant and centrifuged at 5,000 rpm for 5 min at 4°C. The supernatant was collected and stored at -20^{o}C until further use. Dissection was carried out for the tissues like the midgut and fat bodies. Homogenization of tissues was done in a homogenizer using a phosphate buffer of pH 7. The homogenate was transferred to a clean centrifuge tube and centrifuged at 5000rpm for 5 min in cooling conditions.

The supernatant was collected for biochemical constituent analysis. The total protein (Bradford, 1976) and reducing sugar concentration (Miller, 1959) were estimated by using standard protocols.

Determination of peroxidase activity

The tissue sample (0.1 g) was extracted with phosphate buffer (pH 7.0, 0.1M) in a pre-chilled pestle and mortar. The extracted sample was centrifuged at 4°C at 10,000 rpm for 10 minutes. The protein content in the supernatant was estimated by using the dye-binding method (Bradford, 1976). The reaction mixture, 3 ml of phosphate buffer along with Guaiacol, was taken in the spectrophotometer sample cuvette along with 40 µl crude extract sample and 40 µl of substrate H_2O_2 (10mM). The reaction was measured spectrophotometrically at 470nm (Hammerschmidt et al., 1982). Peroxidase activity was expressed as a change in units mg^{-1} $protein^{-1}min^{-1}$.

Determination of catalase activity

By using phosphate buffer (0.1 M, pH 7.0), a tissue sample (0.1g) was extracted using a pre-chilled pestle and mortar. The extracted sample was centrifuged at 4°C at 10,000 rpm for 10 minutes. The protein content in the supernatant was estimated by using the dye-binding method (Bradford, 1976). The reaction mixture containing 3ml of phosphate buffer along with 40 µl crude extract was taken in the spectrophotometer sample cuvette and 40 µl of H_2O_2 substrate was added. The reaction was measured spectrophotometrically at 240 nm. Catalase activity was expressed as a change in units mg^{-1} $protein^{-1}min^{-1}$ (Havir and McHale, 1987).

Data Analysis

For data analysis, the statistical computer application package SPSS 23.0 was employed. The data generated were an average of three independent experiments. Data were subjected to analysis of variance (ANOVA) and the means were compared for significance using Duncan's Multiple Range Test (DMRT; P = 0.05).

RESULTS

Inhibition of pathogenic bacteria by antagonistic phylloplane bacterium (In vitro inhibition assay)

All the bacteria isolated from the leaf of *T. arjuna* and *T. tomentosa* showed a zone of inhibition against all the

pathogenic bacterial species. The bacterium TT103 showed activity against all three bacterial pathogens tested in the study whereas other bacterial species showed inhibitory activity against one or two bacteria. Hence TT103 bacterium was used for further studies (Table 1). The TT103 bacterium showed maximum activity against *Staphylococcus* sp. (Chain type excreta) (1.90±0.47) in comparison with *Micrococcus* sp. (Sealing of Anal Lips) (1.20±0.45) and *Serratia* sp. (Rectal protrusion) (1.50±0.34) (Table 1 and Figure 1).

Table 1. Inhibition of different pathogenic bacteria by Phylloplane bacterium.

Bacteria isolated	*Staphylococcus* sp. (CTE) (in Cms)	*Micrococcus* sp. (SAL) (in Cms)	*Serratia* sp. (RP) (in Cms)
TT 101	0	0	0
TT 102	0	0	1.10±0.27^{c}
TT 103	**1.90±0.47^{a}**	**1.20±0.45^{b}**	**1.50±0.34^{b}**
TT 104	1.00±0.29^{c}	0	0.25±0.37^{g}
TT 105	0	0.80±0.35^{c}	0
TA 102	0	0.78±0.37^{c}	0
TA 103	0.30±0.67^{d}	0	0.40±0.25^{f}
TA 104	0	0	0.72±0.45^{d}
TA 10^{5}	0	0.12±0.72^{e}	0.25±0.37^{g}
TA $10^{1}2$	0	0.50±0.27^{d}	0.57±0.35^{e}
Positive control	1.74±0.75^{b}	1.74±0.25^{a}	1.72±0.25^{a}

The values represent the mean of three replications with standard error, values with different small alphabets are significantly different according to Duncan's Multiple Range Test (DMRT, (P≤0.05). (CTE= Chain type excreta, SAL=Sealing of Anal Lips, RP= Rectal protrusion).

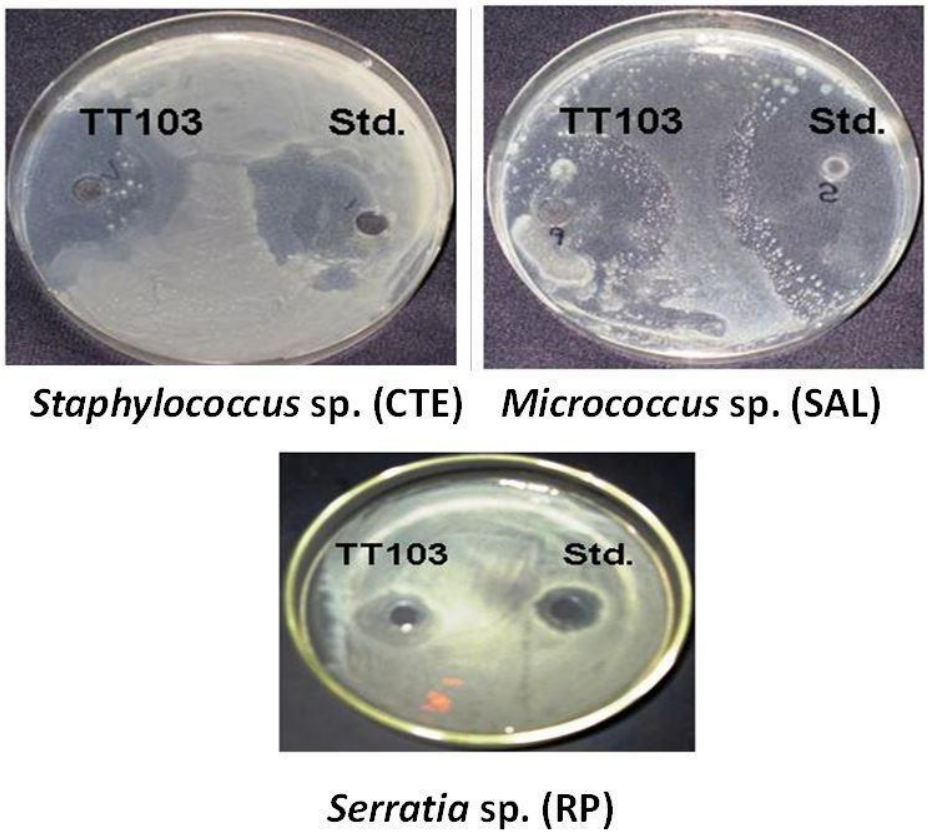

Figure 1. Photographs showing the antagonism of TT103 bacterium against different pathogenic bacteria.

Biochemical Characterization

The isolated antagonistic phylloplane bacteria (TT 103) showed positive results towards gram staining, catalase activity, lipase activity, casein hydrolysis, and KOH hydrolysis whereas negative results were noticed in starch hydrolysis (Table 2).

Bacterial identification by using 16s rRNA gene

The amplification of bacterial genomic DNA by using primers produced the 628bp product (Figure 2). The obtained PCR product was separated by agarose gel, purified, and sequenced. The obtained sequence was analyzed using the BLAST server of NCBI. The results revealed that the obtained sequence was *Bacillus subtilis*. The obtained sequence was deposited to NCBI Gene bank (Accession number JX006081).

Evaluation of biocontrol agents on rearing performance (Outdoor/field condition)

Bacillus subtilis showed maximum silkworm survivability compared to the control. The *Bacillus subtilis* showed 92% survival in comparison with the control (79%) (Table 3). Little improvement in SR% was noticed in cocoons harvested from the lot which were treated with the *Bacillus subtilis* (Table 4).

Table 2. Biochemical Characterization of antagonistic phylloplane bacteria.

Tests conducted	Results
Gram Staining	+ve
Starch Hydrolysis	-ve
Catalase activity	+ve
Lipase activity	+ve
Casein hydrolysis	+ve
KOH solubility test	+ve

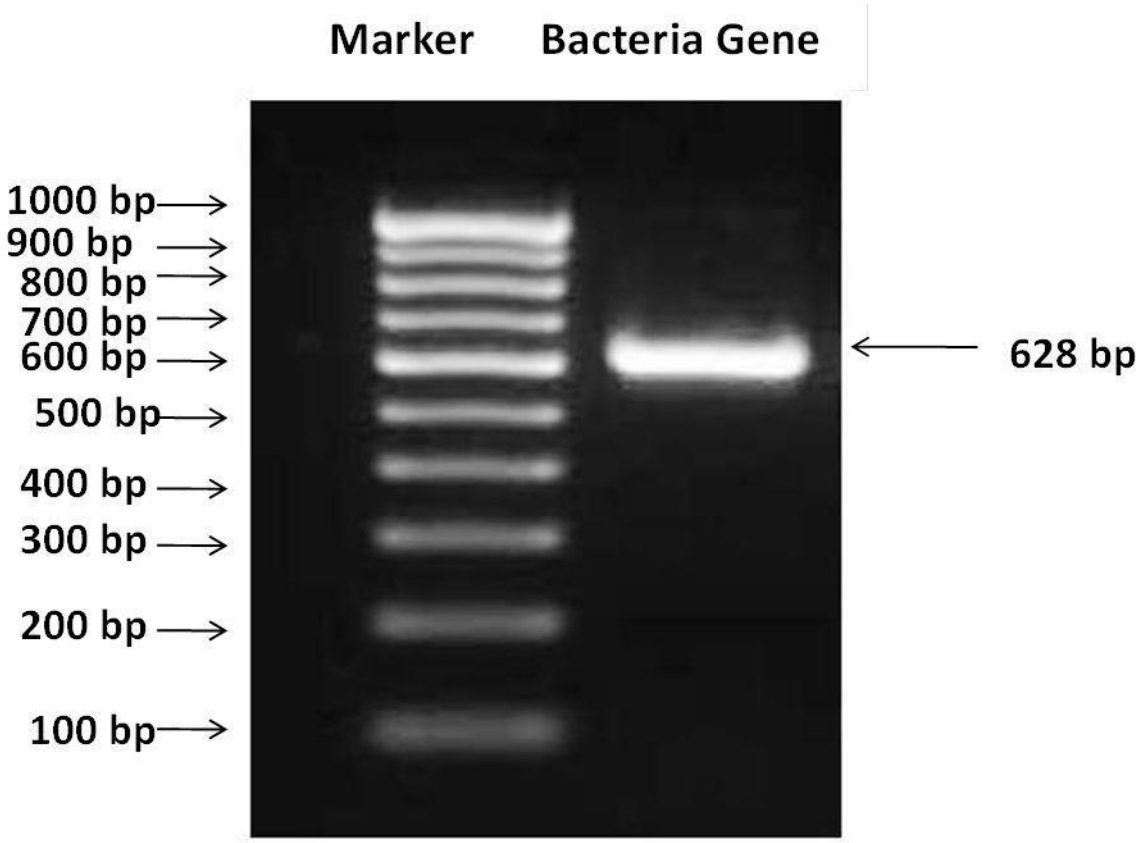

Figure 2. The 16s Ribosomal RNA gene amplification of TT103 bacterium.

Table 3. The impact of *B. subtilis* on the survival % of tasar silkworm in outdoor rearing conditions.

Treatments	No. of Cocoons harvested	Survival %
Control	390	78
Bacillus subtilis	460	**92**

*The number of larvae brushed was 500 for both control and *B. subtilis* treatment.

Table 4. The impact of *B. subtilis* on the cocoon characters.

Treatments	Male			Female		
	Cocoon wt	Single shell wt	SR%	Cocoon wt	Single shell wt	SR%
Control	10.19 ± 0.27^{b}	1.44 ± 0.33^{b}	14.09	15.55 ± 0.37^{a}	2.10 ± 0.33^{a}	13.48
Bacillus subtilis	10.49 ± 0.43^{a}	1.54 ± 0.54^{a}	14.72	14.19 ± 0.43^{b}	2.05 ± 0.57^{b}	14.45

The values represent the mean of three replications with standard error, values with different small alphabets are significantly different according to Duncan's Multiple Range Test (DMRT), P≤00.05.

Effect of biocontrol agents on the survivability of bacteria inoculated tropical tasar silkworm (Indoor condition)

In indoor conditions also, *Bacillus subtilis* showed maximum survivability in comparison with the control. *Bacillus subtilis* (92%) showed more survivability than control (Table 5).

Temporal changes in Protein and Carbohydrates concentration

After 48hrs treatments with biocontrol agents to larvae that were control inoculated with pathogenic bacteria, a significant increase in protein and carbohydrate concentration was noticed in comparison with control and only pathogen inoculated larvae. The maximum level of protein and carbohydrate concentration was noticed in midgut tissue at the constitutive level. In all three-tissue examined, an almost similar trend of increased protein and carbohydrate concentration was noticed (Figure 3 and 4).

Temporal changes in Peroxidase and Catalase activity

The maximum peroxidase activity was noticed in the *Bacillus subtilis* treatment in comparison with control from 24hrs post-treatment (Figure 5) whereas a decrease in catalase activity was noticed after 24hrs in new biocontrol agents in comparison with control (Figure 6). This trend was noticed in all three tissues used in our study.

Table 5. The impact of *B. subtilis* on the survival % of tasar silkworm under indoor rearing condition.

Treatments	No. of Larvae used	Mortality (bacterial infection)	No. of cocoons harvested	Survival %
Pathogen inoculated	50	36	14	28
Pathogen inoculated + *Bacillus subtilis*	50	04	46	92

DISCUSSION

Tasar silkworm is affected by different pathogens which are causing considerable yield loss Among them, bacterial diseases are becoming a major constraint for the production of tropical tasar silkworms. Some work has been done to isolate the phylloplane bacteria to treat bacterial diseases of tropical tasar silkworms (Sahay et al., 2000).

Phylloplane bacteria and fungi are major sources of antimicrobial agents (Maji et al., 2003). Not much information is available about the use of biocontrol agents isolated from plants against animal diseases. Even though some scanty reports are available about the Leaf surface microbes to control diseases of tropical tasar silkworms. In the present study, *Bacillus subtilis*

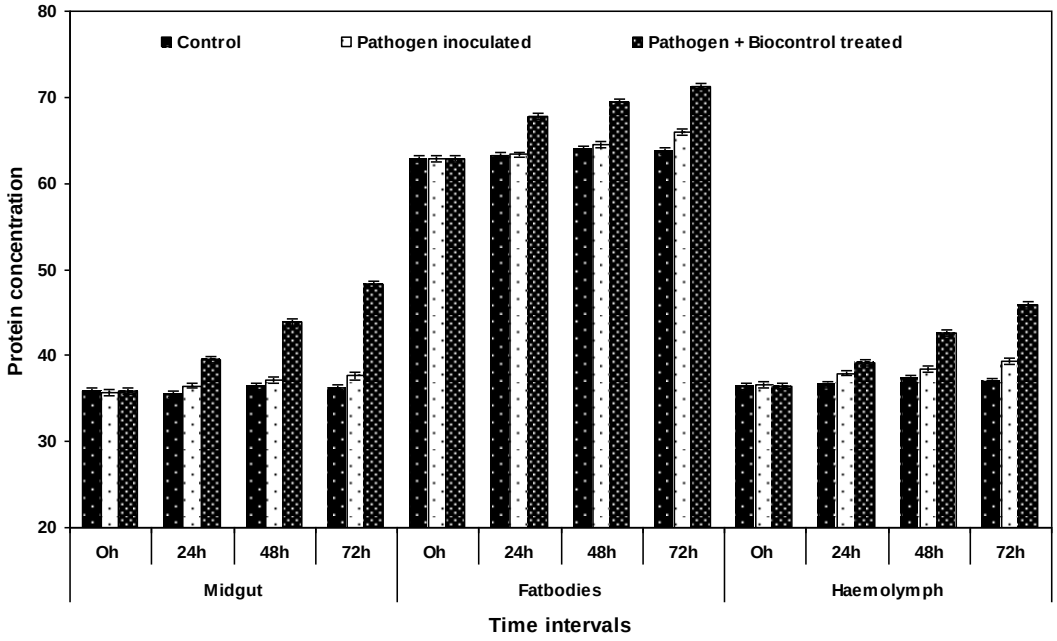

Figure 3. Comparative temporal changes in total protein concentration in uninoculated control, biocontrol agent treated, and biocontrol agent with the pathogen.

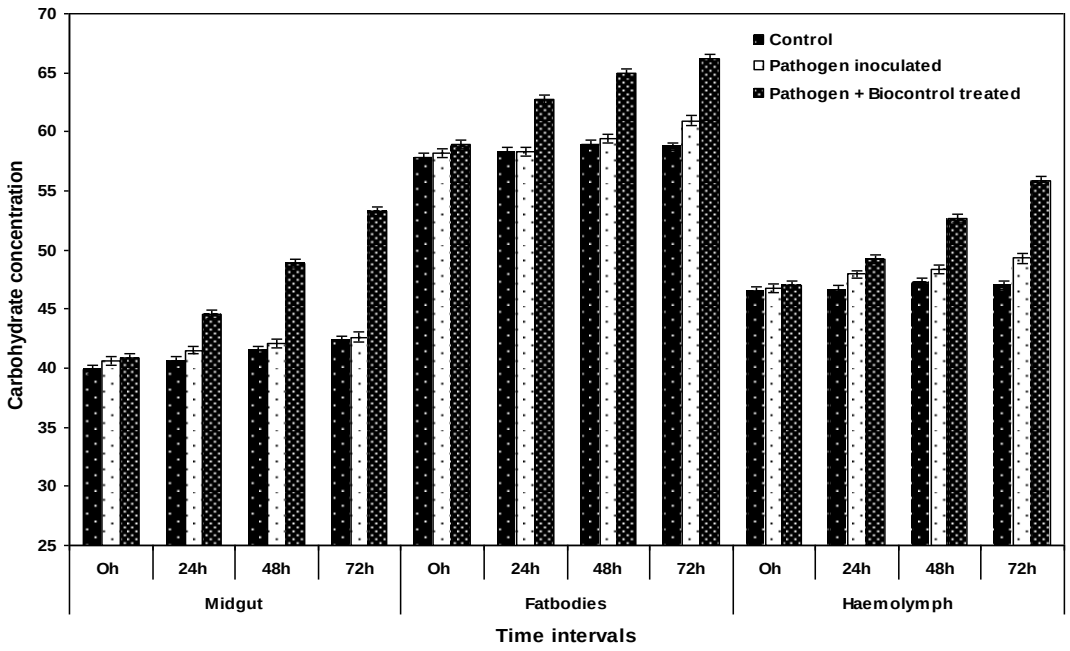

Figure 4. Comparative temporal changes in carbohydrate concentration in uninoculated control, biocontrol agent treated, and biocontrol agent with the pathogen.

showed a maximum survival % along with a slight improvement in cocoon characteristics in outdoor rearing conditions.

The studies on temporal changes in regulatory enzymes such as peroxidase and catalase revealed that an increase in peroxidase and their activity level was recorded in the hemolymph, midgut, and fat body of newly identified biocontrol agents treated along with LSM and control larvae. The results of our study confirm that there is upregulation of peroxidase activity and

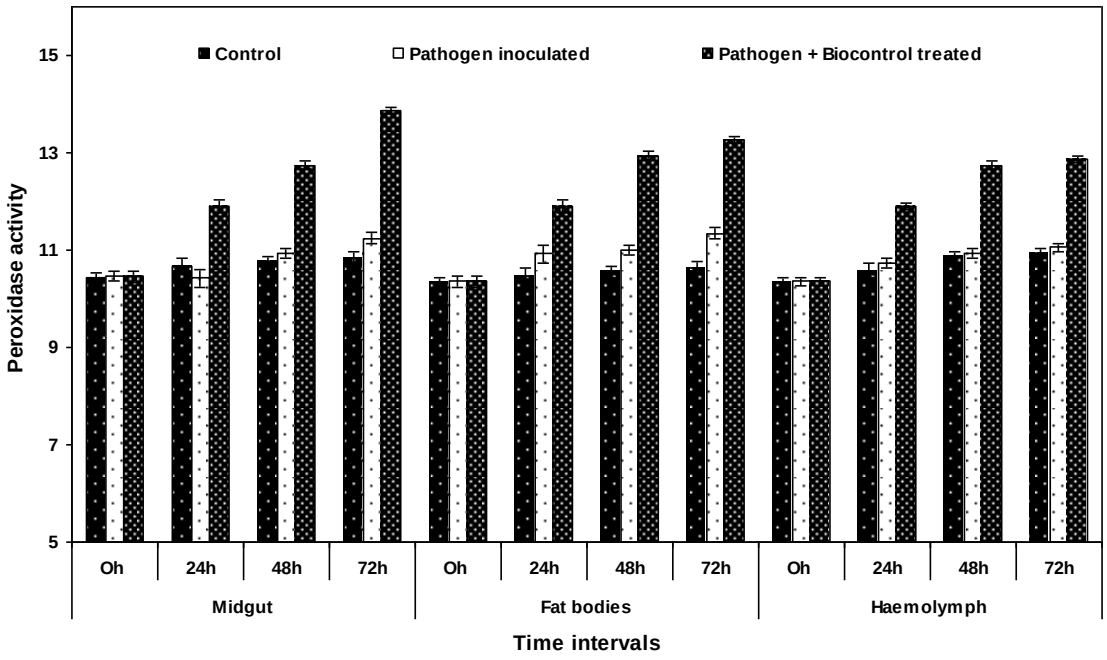

Fig. 5. Comparative temporal changes in Peroxidase activity in uninoculated control, biocontrol agent treated, biocontrol agent with the pathogen.

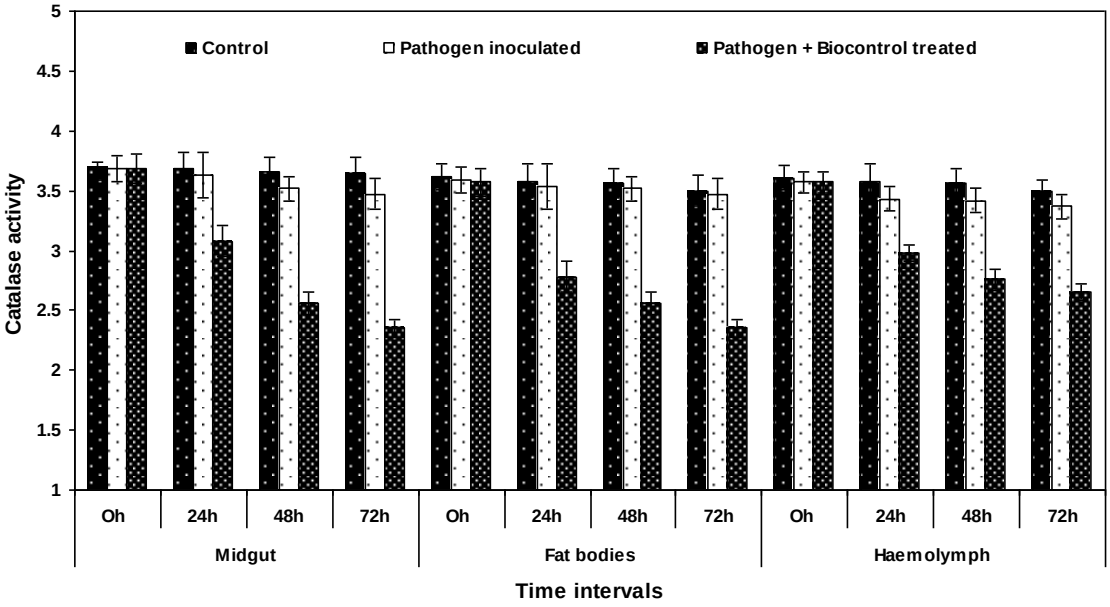

Figure 6. Comparative temporal changes in catalase activity in uninoculated control, biocontrol agent treated, biocontrol agent with the pathogen.

downregulation of amylase activity in biocontrol-treated larval samples used in the study.

Among the different phylloplane bacterial isolates screened, *Bacillus subtilis* showed more inhibition of bacterial pathogens in vitro and in vivo conditions. The *Bacillus subtilis* performed very well during both indoor and field conditions in comparison with control batches in terms of survival. Increased protein and carbohydrate concentration was noticed in *Bacillus subtilis* treated larvae. Upregulation of peroxidase and downregulation of catalase activity was noticed in the biocontrol agents treated larval tissues.

CONCLUSION

Based on the results of the present work, *Bacillus subtilis* can be utilized for the control of bacterial pathogens in the tasar silkworm rearing plots.

ACKNOWLEDGMENT

The authors wish to thank Central Tasar Research and Training Institute, Ranchi for providing laboratory facilities to conduct research work.

REFERENCES

Andrews JH, **1992**. Biological control in the phyllosphere. Ann Rev Phytopathol, 30(1): 603-635.

Bradford MM, **1976**. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 72(1-2): 248-254.

Hammerschmidt R, Nuckles EM, Kuć J, **1982**. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. Physiol Plant Pathol, 20(1): 73-82.

Havir EA, **McHale** NA, **1987**. Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. Plant Physiol, 84(2): 450-455.

Lane DJ, **1991**. 16S/23S rRNA Sequencing. In: Stackebrandt, E. and Goodfellow, M., Eds., Nucleic Acid Techniques in Bacterial Systematic, John Wiley and Sons, New York, 115-175.

Maji MD, Qadri SMH, Pal SC, **2003**. Evaluation of mulberry phylloplane microorganisms for biological control of bacterial leaf spot of mulberry caused by *Xanthomonas campestris* pv. *mori*. Indian J Agric Res, 37(4): 307-309.

Miller GL, **1959**. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem, 31(3): 426-428.

Sahay DN, Roy DK, Sahay A, **2000**. Diseases of tropical tasar silkworm, *Antharaea mylitta* D., symptoms and control measures. In: Lessons on tropical tasar. Ed. By K. Thangavelu, Central Tasar Research and Training Institute, Piska Nagri, Ranchi. Page: 104.

Citation: Madhusudhan KN, Tuboi L, Vinayarani G, Moorthy SM, Gandhi Doss S, 2024. Isolation and characterization of antibacterial phylloplane bacteria *Bacillus subtilis* isolated from *Terminalia arjuna* leaves. Microbiol Res Int, 12(1): 1-9