Project code and title: ARP3522
Isolation, cloning and characterization of antibacterial protein(s) from silkworm, *Bombyx mori* L.
(A collaborative project with SBRL)

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Objectives:

- To isolate and characterize bacterial strains and study their pathogenecity [CSRTI]
- To isolate, purify and characterize antibacterial protein fractions elicited against bacterial strain [CSRTI]
- Molecular cloning and full length gene sequence using cDNA cloning for mass production of antibacterial protein [SBRL & CSRTI]

All the objectives were studied as per the time line and the methodology followed and results obtained with respect to achieving the same are depicted below under the respective heads.

Introduction:

Silkworm cocoon crops are extremely unpredictable considering the fact that the mulberry silkworm is susceptible to various diseases (Rahul *et al.*, 2018). The diseases in silkworm are broadly

classified into two major groups based on the causal agent - Microbial and amicrobial diseases. Viruses, bacteria, fungi and microsporidia are the major microbial pathogens that cause diseases in silkworm and such diseases are called microbial or infectious diseases. The respective diseases caused by these pathogenic agents are Virosis (Nuclear polyhedrosis, Cytoplasmic polyhedrosis, Viral flacherie and Densonucleosis); Bacteriosis (Bacterial flacherie, Bacterial diseases of digestive of digestive tract, Bacterial septicemia and Bacterial toxicosis); Mycosis (White, Green, Yellow, Red muscardine and Aspergillosis) and Microsporidiosis/Pebrine. The microbial pathogens usually invade the host, proliferate rapidly in them and extend the infection to other susceptible hosts.

Among the major diseases prevalent in the different sericultural regions of India, Bacteriosis caused by both Gram positive and Gram negative bacteria is most common and predominant during summer season. Sources of infection include infected/dead silkworms, their faecal matter, contaminated mulberry leaves, rearing appliances and cross contamination through pests of mulberry. Wide fluctuation in temperature and humidity with poor quality mulberry leaves are the major predisposing factors for flacherie.

In Insects like *B. mori*, innate immune mechanisms are the primary line of defence against invading pathogens. The innate immune system of *B. mori* is composed of cellular and humoral immunity. Cellular immunity involves phagocytosis, encapsulation and nodule formation where as the humoral immunity involves the activation of enzymes cascades like prophenol oxidase, induction of immune proteins such as lysozymes, antibacterial proteins, lectins and antifungal proteins. The antibacterial peptides are multi components of innate immune system in *B. mori*. Antibacterial proteins are induced mainly in the fat body and haemocytes upon bacterial infection. The various antimicrobial peptides include attacin, lebocin, moricin, defencin, cecropins, apolipophorin etc.

Insect lipoproteins, called lipophorins, are well studied complexes of multifunctional molecules which are non-covalent assemblies of lipids and proteins. They serve as lipid transport carriers and are involved in defense activities against pathogens. The protein moiety of lipophorin encompasses two glycosylated apolipoproteins, apolipophorin I (apoLp-I) and apolipophorin II (apoLp-II), and an exchangeable protein, apolipophorin III (apoLp-III). ApoLp-III is a copious protein occurring in the hemolymph in a lipid-free and bound state and plays an important role in lipid transport and insect innate immunity. In immune response apoLp-III serves as a pattern recognition molecule. ApoLp-III is reported to be involved in binding and detoxification processes of microbial cell wall components (lipopolysaccharide, lipoteichoic acid, and β-1,3-glucan), activation

and expression of antimicrobial peptides and proteins, regulation of the phenoloxidase system and in hemolymph clotting. In addition, the protein is also involved in cellular immune response, influencing hemocyte adhesion, phagocytosis and nodule formation, and in gut immunity. In the present study, apolipophorin-III of silkworm which was elicited against bacterial infection was successfully cloned and expressed in *Pichia pastoris*.

Methodology

- **Isolation of bacterial strains responsible for causing bacteriosis in silkworm:**

Surface sterilization of the silkworm larval samples exhibiting symptoms of bacterial flacherie was done using 0.5% sodium hypochlorite solution.The oral and anal ends of the diseased silkworm were tied with sterilized cotton thread and the long end of the thread was retained to handle the insect. The insect handled with a thread was dipped into sterile saline (0.85% NaCl) in a sterilized tube. This process was repeated thrice. The insect was kept on a clean tissue paper to dry. Then the diseased insect was transferred into 0.5% sodium hypochlorite solution and was left for 5min with intermittent shaking. Then it was transferred to sodium thiosulphate (10%) solution and rinsed thoroughly for 1-2 minutes after which it was thoroughly rinsed with sterile distilled water to wash off the germicide.

The surface sterilized diseased insect sample was dissected and the gut contents were crushed in sterile NaCl solution using mortar and pestle and a few microliters were spread onto nutrient agar (g/l: peptone, 5; beef extract, 1.5; yeast extract, 1.5; NaCl, 5 and agar 15) plates. The bacterial isolates were purified by repeatedly streaking on nutrient agar plates. Single colonies were sub-cultured onto fresh agar plates till identical colonies were observed on two successive plates. Contamination from other bacteria was checked by observing the characters of the culture like color of the culture, colony morphology and by examining microscopically. Pathogenicity of the isolated strains was confirmed by employing Koch postulates. The isolated bacterial pathogens were identified by 16S rRNA gene sequencing. For 16S rRNA gene sequencing, DNA was extracted and purified from 5 ml of well grown liquid cultures by the modified Marmur (1961) method. The presence and quality of the extracted DNA was checked by electrophoresing 10 µl of genomic DNA and 10 µl of standard genomic DNA (as marker) on an horizontal agarose gel (0.8 %, w/v) in TAE buffer at 15 V cm^{-1}. The gel was stained in 0.5 µg ml^{-1} ethidium bromide and visualized in an UV gel documentation system (BIORAD). Amplification is performed using a DNA thermal cycler (MJ Mini Personal Thermal

Cycler – BIO-RAD) in 50 µl volumes taken in 0.2 ml microfuge tubes. All plastic ware were sterilized by autoclaving and ultraviolet irradiation. The primers used for the amplification of the 16S rRNA gene were Eub27F (5'GAGTTTGATCCTGGCTCAG-3') and Univ1492R (5' GGTTACCTTGTTACGACTT-3'). The concentration and volume of the reaction mixture are Primers: 2 µl of each primer (10 pmol μl^{-1}); Template: 5 µl of DNA template (25 ng μl^{-1}); DNase free Water: 19 µl and Master mix: 25 µl. The cycling parameters were given in Table 1. Quality of the amplified DNA was checked by horizontal agarose gelelectrophoresis [2 % (w/v) in TAE buffer at 15 V cm^{-1}] of 5 µl of amplicon and 5 µl of 1 kb DNA marker (O'GeneRuler™ 1 kb DNA Ladder, 250-10000 bp, Catalog No. SM1163). The gel was stained in ethidium bromide solution (0.5 µg ml^{-1}) and visualized on Gel Doc (BIORAD).

The almost complete length of the 16S rRNA gene sequence (> 1400 bp) was obtained by sequencing the PCR amplicon with 2 primers viz., Eub27F (5'-GAGTTTGATCCTGGCTCAG-3'), 2 and Univ1492R (5'-GGTTACCTTGTTACGACTT-3'). The 16S rRNA gene amplicon was sequenced at MTCC and Xcelris labs, India. The sequences obtained as *.scf format were assembled using "SeqMan" software in the DNA STAR Lasergene 7 package. To know the nearest phylogenetic relative, the single contig sequence of approximately 1350 to 1450 bp length was submitted to the NCBI-BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). EzTaxon server (http://www.ezbiocloud.net/eztaxon) was more useful for comparison of 16S rRNA gene sequences with type strain sequences.

- **Isolation, purification and characterization of antibacterial protein fractions elicited upon infecting the healthy silkworm larvae with the pathogenic strains**

Healthy silkworm larvae were infected per oral with the isolated pathogenic strains at a concentration of 1 x 10^6 CFU/ml. Haemolymph was collected at regular intervals i.e after 24h, 48h and 72h by puncturing with a micro syringe (Hamilton) at the base of 1st prolegs. To prevent clotting, PTU & Protease inhibitor (Himedia) was added to the haemolymph and was stored with at -20°C till further use. Haemolymph was tested for antibacterial activity by Zone of inhibition assay against isolated bacteria. Total protein was extracted from haemolymph samples showing a positive zone of inhibition assay. The protein fraction was run on a 15% SDS. Proteins that were elicited upon infection were sent for mass spectrometric analysis for identification which was outsourced to CCAMP, Bangalore.

➢ **Partial cloning of anti bacterial gene**

Cloning was carried out by reverse transcription method as expressed RNA will be up-regulated in infected silkworm when compared to the control insect. For partial cloning, total RNA was extracted from fat body of both control and infected silkworm as per manufacturer's instruction (RNAeasy kit-Takara). 1st Strand cDNA was synthesized from the extracted RNA by employing Primescript cDNA synthesis kit (Takara), following manufacturer's instructions. Target antibacterial genes were PCR amplified by using 1st strand cDNA as template and gene specific designed primers. Designed primers were outsourced for synthesis from Eurofins, Bangalore.

PCR products were purified and cloned into sequencing pJET plasmid (Blunt end vector). Recombinant plasmids were transformed into *E. coli* Dh5α cells. Recombinant colonies were selected and insert was confirmed by colony PCR. Purified recombinant plasmids were sequenced by outsourcing (Eurofins, Bangalore).

➢ **Full length cloning of anti bacterial gene**

Full length cloning of antibacterial gene was done using RACE kit, Takara following manufacturer's protocol. Both 5' and 3' primers were designed, RACE products were cloned into sequencing plasmid (pJET blunt end cloning), recombinant colonies were selected and insert was confirmed by colony PCR. Purified recombinant plasmids were sequenced by outsourcing (Eurofins, Bangalore). Sequence analysis was done by tools in NCBI.

➢ **Expression of cloned antibacterial gene**

Recombinant gene was expressed in *Pichia pastoris* expression system (Invitrogen). Forward and reverse primers were designed and synthesized by outsourcing (Eurofins, Bangalore).To clone in *Pichia*pink HC (High copy number) plasmid, restriction sites were added and additional 6-Histidine patch was added to the 3' end of the plasmid. Gene was amplified from the 1st strand cDNA isolated from fatbody of silkworm. PCR product and the vector was restricted and successfully ligated. Recombinant plasmids were incorporated in *P. pastoris* chemically competent cells and were grown at 30 °C for 48 hrs in PAD plates. White colonies were selected (non recombinant cells grow in pink colour) and confirmed by colony PCR.

The expression of cloned gene was done as per manufacturer's instruction (Invitrogen-*Pichia* expression kit). Transformed clones were transferred to BMGY medium for successive growth under ambient temperature. After 24 hours, cells were induced with methanol (BMMY medium). Cells and supernatant were collected by centrifugation. As there was no secretion signal added to the plasmid, recombinant protein was accumulated in cells. Both cells and supernatant were collected at the regular intervals of 24 hrs, 48hrs and 72hrs. *Pichia* cells transformed with *Pichia* HC plasmid (without insert) served as a control. Proteins were extracted from cells by breaking them with acid washed glass beads (0.1mm). Extracted protein was subjected for 15% SDS to check recombinant target protein. Presence of expressed recombinant ApoLPIII protein was confirmed by Western blot by antihistidine antibody and mass spectrometry analysis.

- **Purification of recombinanat protein and *in vitro* assay of antibacterial activity of purified recombinant protein by disc diffusion method**

Recombinant protein having Histidine patch was purified by His patch-Ni2+ purification kit (Takara). Purified protein was checked in 15% SDS-PAGE. Protease inhibitor was added to the purified protein to prevent degradation. In vitro assay of the recombinant protein was checked by disc diffusion method.

Observations / Results:

A total of five bacterial strains were isolated in pure form of which three strains were found to cause bacteriosis in silkworm. The three strains were identified as belonging to the genera *Staphylococccus, Escherichia and Providencia.* Haemolymph collected from silkworms upon infecting with *Staphylococccus* sp., *Escherichia* sp. and *Providencia* sp. was subjected to zone of inhibition assay. It was observed that the haemolymph fraction that was collected upon 24 hrs of infection exhibited a good zone of inhibition against the bacterial pathogen (Fig 1).

SDS PAGE profile of protein that was isolated from the haemolymph of the silkworm upon 24 and 48 hrs of infection with *Staphylococcus* sp. is depicted in (Fig 2). From the gel profile, it is observed that bands pertaining to molecular mass 50, 45, 40, 24, 15 and 10 kDa were detected in the protein sample that was isolated from haemolymph of silkworm upon 24 hrs of infection with *Staphylococcus* sp. and the same could not be detected in protein collected from the haemplymph of 48 hrs sample nor the control sample.

SDS PAGE profile of protein that was isolated from the haemolymph of the silkworm upon 24 and 48 hrs of infection with *Escherichia and Providencia* sp. is depicted in Fig 3. From the gel profile, it is observed that bands pertaining to 15 and 26 kDa were detected in the protein sample that was isolated from haemolymph of silkworm upon 24 hrs of infection with *Providencia* sp. and the same could not be detected in protein collected from the haemplymph of 48 hrs sample nor the control sample. Also it was observed that the band pertaining to 28 and 72 kDa was intense in the protein isolated from haemolymph of silkworm upon 24 and 48 hrs of infection with *Providencia* sp. Bands of 15, 28, 72 kDa were also detected from the protein isolated from haemolymph of silkworm upon 24 and 48 hrs of infection with *Escherichia* sp. Based on the SDS profile, bands pertaining to molecular mass of 72, 50, 45, 40, 28, 26, 24, 15 and 10 kDa were sent for peptide analysis by mass spectrometry. (Table 2) depicts the similarity index of the sequenced samples to the known antibacterial peptides.

Based on analyzed mass spectrometric data, genes pertaining to Uncharacterized protein (~12kDa), Gloverin 4-like protein (~14kDa), Lysozyme (~7kDa) and Lipoprotein (~20kDa) were selected for partial cloning based on the available literature of their potential as antibacterial peptides and also, to know any sequence variations in the above reported antibacterial proteins.

1. Uncharaterized protein

(Forward primer): 5'-GATATTCACGACTTTGTCACTTGG-3'

(Reverse primer): 5'-TTACCACTCGTGAGTAATCTGGCCTG-3'

2. Gloverin 4-like protein

(Forward primer): 5'-GTCTTCGGCACGCTGGGCCAAAATG-3'

(Reverse primer): 5'-GTCATTCCAGATCTGCCTCCGATCTG-3'

3. Lysozyme

(Forward primer): 5'-GACTACGGATTCACCAGAT-3'

(Reverse primer): 5'-CAGTGATTCTTCCATCCGTACCA-3'

4. Lipoprotein

(Forward primer):5'-ATGGCCGCCAAGTTCGTAG-3'

(Reverse primer): 5'-TTACTGCTTGGCGTTGGCGGCCT-3'

All the four genes pertaining to the above mentioned peptides were partially cloned in pJET vector which was confirmed by colony PCR. The PCR amplified products of ~363 bp, ~400 bp, ~200 bp and ~565 bp pertaining to Uncharaterized protein, Gloverin 4-like protein, Lysozyme and Lipoprotein respectively is depicted in (Fig 4). NCBI BLAST analysis of partially cloned sequences of Uncharacterized protein, Gloverin 4 like protein, Lysozyme and Lipoprotein exhibited similarities of 98% with Gloverin-1, 99% with Gloverin-4 like protein, 99% with Lysozyme and 94% with *Bombyx mori* apolipophorin III (*B. mori* apoLp-III) respectively. Based on the results of partial cloning and sequence analysis, it was proposed to clone and express the gene pertaining to *Bombyx mori* apolipophorin III. It was the only antimicrobial protein less characterized in *B. mori.*

5' UTR and 3' UTR of apolipophorin III gene was amplified by RACE employing designed primers 5' RACE PRIMER: 5'-GTCGGGAGCGTCACGTCGCACCA-3' and 3' RACE PRIMER: 5'-CAGGAGGTGTTCAAGAAGATCCAG-3' and Adapter primers from Takara RACE Kit. The gene-specific primers were designed on the sequence obtained from the partial cloning of apoLp-III gene. Amplified product was cloned in pJET blunt end cloning vector and was confirmed by colony PCR. The PCR amplified products of ~965 bp pertaining to *B. mori* Apolipophorin III gene is depicted in (Fig.5). The sequence pertaining to the partial and full length cloning of *B. mori* Apolipophorin III gene is depicted in (Fig. 6). NCBI BLAST analysis of fully cloned sequences of *B. mori* Apolipophorin III exhibited similarities of 97% with *Bombyx mori* Apolipophorin III. Though nucleotide variations were present in the full cDNA sequence, there was no amino acid change. Further, Apolipophorin III gene was cloned in *Pichia* plasmid to express the recombinant protein (Fig 7). Transformed *Pichia* cells were grown as white colored colonies (Fig. 8). Transformation was confirmed by colony PCR [desired product size of ~965 bp. (Fig. 9)].Transformed cells were cultured in BMGY media and induced with BMMY media. Recombinant protein was extracted and the presence of the same was checked on a 15% SDS PAGE. The gel profile indicated the presence of 2 particular proteins of ~20kDa and ~33kDa. Both the protein bands were absent in the control lane (Fig 10). Further confirmation of the expressed recombinant protein was done by western blot using antihistidine antibody (Fig 11). Both the bands were sent for mass spectroscopic analysis and the results indicated that both were exhibiting highest similarity with *B. mori* ApoLpIII (Table 3).

Recombinant protein was purified by Ni^{2+} column. The final concentration of the recombinant protein obtained was 10 ng/ul and the same was tested for antibacterial activity against pathogenic Gram +ve *Staphylococcus sp.* (Fig 12) and Gram –ve *E.coli* (Fig 13). A good zone of inhibition

against the pathogens was observed in both the cases indicating the potential antibacterial activity of the recombinant protein.

Discussion:

Silkworm *Bombyx mori* is an economically important domesticated lepidopteran insect. During the process of its incessant domestication, the silkworm has lost some of its capabilities which include resistance to infection caused by pathogenic microbes. Silkworm *Bombyx mori* is known to have both humoral and cellular immune system which together forms a potent barrier against invading microorganisms. Cellular immunity involves phagocytosis, encapsulation and nodule formation where as humoral immune mechanisms involve the activation of enzymes cascades like prophenol oxidase, induction of immune proteins such as lysozymes, antibacterial proteins, lectins and antifungal proteins.

Bacterial diseases in silkworm are generally classified as bacterial flacherie, bacterimia, septicemia and toxicosis. Different species of bacteria belonging to the genera *Aeromonas*, *Alcaligenes*, *Bacillus*, *Escherichia*, *Micrococcus*, *Pseudomonas*, *Proteus*, *Streptococcus*, *Staphylococcus* and *Serratia* are reported to cause bacterial disease in silkworm (Sakthivel *et al.*, 2012, Zhang *et al.*, 2013). Upon bacterial infection, antibacterial proteins are induced mainly in the fatbody and haemocytes of the silkworm to combat the bacterial infection. In silkworm antibacterial genes are transcribed in fatbody and expressed in haemolymph, where it functions as effective immune barrier. Most anti microbial peptides are synthesized as inactive precursor proteins or pro-proteins, and active peptides are generated by limited proteolysis during immune components upregulation upon infection (Hui-Yu Yi *et al.*, 2014). The antibacterial peptides are multi components of innate immune system in *B. mori*. The various antimicrobial peptides include attacin, lebocin, moricin, defencin, cecropins, apolipophorin etc. (Cheng *et al.*, 2006, Gandhe *et al.*, 2007)

In the present study, a total of five bacterial strains were isolated in pure form of which three strains were found to cause bacteriosis in silkworm. The pathogenic strains were identified as belonging to the genera *Staphylococccus, Escherichia and Providencia* where as both the non pathogenic strains isolated belonged to the genera *Bacillus.* Several researchers have reported that strains belonging to the above mentioned genera to be pathogenic to silkworm. Though members of *Bacillus* were also reported to be pathogenic to silkworm, the strains isolated in this study were not

inflicting any symptoms of bacteriosis in silkworm. This may be attributed to the fact that pathogenic potential is strain-specific in many species. Strain-level variation based on the genetic make up of a microbial species is crucial in determining the pathogenic potential. Factors like degree of host susceptibility/resistance, environmental factors favouring/disfavouring the disease infliction also come into play in determining the microbial species to be pathogenic or otherwise.

Upon infecting the silkworm with the above pathogenic strains, haemolymph samples were collected as regular intervals. The same were screened for the presence of low molecular weight peptides considering the fact that most of antimicrobial peptides reported till date from insect sources belong to this category. SDS PAGE profile indicated that the bands pertaining to molecular mass 72, 50, 45, 40, 28, 26, 24, 15 and 10 kDa were detected in the protein samples that were isolated from haemolymph of silkworm upon infecting with pathogenic bacterial strains. A zone of inhibition assay against pathogenic bacteria was also performed. SDS PAGE profiling and Zone of inhibition assay indicated that most of the antibacterial compounds were synthesized in the haemolymph of the silkworm within 24 hrs of infection and the concentration of the same declined with increase in time. Yang *et al.*, 1999 reported that Northern blotting analyses revealed that the gene expression of cecropin D (antimicrobial protein) is detectable by 4 hrs after the bacterial injection and reaches the maximal level at 24 h. That high level is maintained up to 48 hrs post-immunization.

The bands pertaining to above molecular mass were sent for identification of the same by mass spectrometry. Based on analyzed mass spectrometric data, it was observed that the peptides were showing similarity to a wide range of antimicrobial peptides which include attacin, arylphorin, lysozyme, gloverin, lipoproteins and uncharacterized proteins. Uncharacterized protein (~12kDa), Gloverin 4-like protein (~14kDa), Lysozyme(~7kDa) and Lipoprotein (~20kDa) were selected for partial cloning based on the available literature of their potential as antibacterial peptides. NCBI BLAST analysis of partially cloned sequences of Uncharacterized protein, Gloverin 4 like protein, Lysozyme and Lipoprotein exhibited similarities of 98% with Gloverin-1, 99% with Gloverin-4 like protein, 99% with Lysozyme and 94% with *Bombyx mori* Apolipophorin III (*B. mori* ApoLpIII) respectively. Based on the results of partial cloning, it is proposed to clone and express the gene pertaining to *Bombyx mori* Apolipophorin III considering its vast potential as an antimicrobial peptide. ApoLp-III is a copious protein occurring in the hemolymph in a lipid-free and bound state and plays an important role in lipid transport and insect innate immunity. In immune response, apoLp-III serves as a pattern recognition molecule. ApoLp-III is reported to be involved in binding

and detoxification processes of microbial cell wall components (lipopolysaccharide, lipoteichoic acid, and β-1,3-glucan), activation and expression of antimicrobial peptides and proteins, regulation of the phenoloxidase system and in hemolymph clotting. In addition, the protein is also involved in cellular immune response, influencing hemocyte adhesion, phagocytosis and nodule formation, and in gut immunity.

A few researchers have performed studies related to cloning and expression of antibacterial proteins from silkworm. *Bombyx mori* Cecropin B was cloned (Kato *et al.,* 1993) and expressed in silkworm larvae using *Bm*NPV/Bac-to-Bac Expression System (Yamano *et al.*, 1994). *B. mori* lebocin was expressed in *E. coli* expression system (Lü *et al.*, 2017). cDNA and Deduced Amino Acid Sequences of Apolipophorin-IIIs from *Bombyx mori* has been characterized (Yamauchi *et al.*, 2000), however there is no report of its large scale expression studies in *Pichia pastoris* expression system.

In the present study, apolipophorin-III of silkworm which was elicited against bacterial infection was successfully cloned and expressed in *Pichia pastoris*. An alternate to the prokaryotic expression system i.e eukaryotic expression system *Pichia pastoris* was selected in the present study as it can tolerate the stress generated by the antibacterial gene while expressing it. In this study SDS-PAGE of expressed recombinant ApoLpIII exhibited two corresponding bands at ~20kDa and ~33kDa, which is due to glycosylation property of the *Pichia* system that causes an increase in the molecular weight of the expressed protein (Yokoyama *et al.*, 2013). The final concentration of the recombinant protein obtained was 10 ng/ul and the same was exhibiting good antibacterial activity against pathogenic Gram +ve *Staphylococcus sp.* and Gram –ve *E. coli* indicating its potential as an antibacterial peptide. Many researchers have reported the potential of apoLp-III in insects in combating microbial infections as it is involved in the molecular pattern recognition, synergistic action with antimicrobial peptides and lysozyme (Andrä *et al.*, 2001), activation of hemocytes during non-self recognition, and detoxification of bacterial cell wall components (Kang *et al.*, 2003). Moreover, it has opsonizing activity and its hemagglutinating as well as anti-oxidant properties were also described (Chung and Ourth, 2002). However, recombinant protein production has to be upscaled by fermentation techniques (Shen *et al.*, 2007) inorder to perform further studies to ascertain and establish the role ApoLPIII as an antibacterial peptide in combating bacterial infections in silkworm.

Inference / Recommendations:

An antimicrobial protein Apolipophorin III was successfully cloned and expressed in *Pichia pastoris*. From 250 ml of recombinant-ApoLpIII *Pichia* culture, ~80ul of recombinant protein was purified with a concentration of 10 ng/ul. The recombinant protein was exhibiting antibacterial activity against both Gram positive and negative bacteria responsible for causing flacherie, indicating its broad spectrum potential. However, further studies indicated have to be performed to explore the feasibility of utilizing the outcome.

Studies have to be performed to scale up the expression (using fermentor) of Apolipophorin III in *Pichia* as the concentration of the protein being obtained is too low. Bioassay studies have to be performed by fortification of the mulberry leaves or artificial diet with the recombinant protein to ascertain the role of the same in combating bacterial infection *invivo*. Studies related to direct application of the recombinant protein on silkworm which will make its way through the cuticle and can provide necessary immunity against bacterial infection have to be performed.

Applications made for patenting / commercialization if any: - No

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Papers Published:-

Summary:

Three bacterial strains responsible for causing flacherie in silkworm were isolated in pure cultures and were identified as belonging to the genera *Staphylococccus*, *Escherichia* and *Providencia*. Haemolymph samples were collected at regular intervals from silkworms upon infecting

with the pathogenic strains mentioned above. Protein was extracted from the haemolymph samples and an SDS PAGE profiling was performed. Based on the SDS profile, bands pertaining to molecular mass 72, 50, 45, 40, 28, 26, 24, 15 and 10 kDa that were detected in the haemolymph samples post infection and not in control were sent for peptide analysis by mass spectrometry. Based on analyzed mass spectrometric data, genes pertaining to Uncharacterized protein (~12kDa), Gloverin 4-like protein (~14kDa), Lysozyme (~7kDa) and Lipoprotein (~20kDa) were selected for partial cloning based on the available literature indicating their potential as antibacterial peptides. All the four genes pertaining to the above mentioned peptides were partially cloned in pJETvector and confirmed by colony PCR. NCBI BLAST analysis of partially cloned sequences of Uncharacterized protein, Gloverin 4 like protein, Lysozyme and Lipoprotein exhibited similarities of 98% with Gloverin-1, 99% with Gloverin-4 like protein, 99% with Lysozyme and 94% with *Bombyx mori* Apolipophorin III (*B. mori* ApoLPIII) respectively. Based on the results of partial cloning and sequence analysis, it was proposed to clone and express the gene pertaining to *B. mori* Apolipophorin III considering it is the less characterized protein and its antibacterial potential is not known in *B. mori*. 5' UTR and 3' UTR of Apolipophorin III gene was amplified by RACE employing designed primers. Amplified product was cloned in pJET blunt end cloning vector and was confirmed by colony PCR. Full length gene sequence of Apolipophorin III was obtained and it was expressed successfully in *Pichia pastoris* expression system which was confirmed by Western blot analysis using antihistidine antibody and mass-spectrometry analysis. From 250 ml of recombinant-ApolpIII *Pichia* culture, ~80ul of recombinant protein was purified with a concentration of 10 ng/ul. The recombinant protein was exhibiting antibacterial activity against both Gram positive and negative bacteria responsible for causing flacherie, indicating its broad spectrum potential.

Budget Utilized: Rs. 22,30,183 (Details attached as Annexure-I)

ANNEXURE - I

		Allotted	Expenditure incurred
A. Non-Recurring (e.g. equipments, accessories, etc.)	1. Laminar Flow Hood (CSR&TI)	1,50,000	59,430
	2. BOD incubator with Shaker (CSR&TI)	2,50,000	2,05,939
	3. Deep Freezer -20° C (CSR&TI)	2,00,000	1,63,735
	4. High speed refrigerated centrifuge with rotor head (CSR&TI)	4,40,000	3,46,029
	5. Mini Vertical Electrophoresis(CSR&TI)	60,000	-
	6. PCR machine(CSR&TI)	4,00,000	3,99,840
	7. Air Conditioner 1.5 Ton (CSR&TI)	50,000	-
	8. Trinocular phase contrast microscope with image analysis software, digital live camera with PC and out put device (CSR&TI)	5,00,000	-
	9. Refrigerator double door 300 L (CSR&TI)	60,000	-
	10. Semi-Dry Blotter (SBRL)	3,50,000	-
	11. Deep Freezer - 40° C (SBRL)	2,00,000	-
	Total	**26,60,000**	**11,74,973**
B. Manpower: **JRF/SRF – 01(CSR&TI)**	May' 2015-June'2017(JRF)	5,20,000	3,30,000
	July'2017- June,2018(SRF)		1,84,800
	Total	**5,20,000**	**5,14,800**
C. Consumables	**Total**	**11,00,000**	**2,59,184**
D. Others 1. Travel : (CSR&TI,Berhampore & SBRL, Kodathi)	**Total**	**7,00,000**	**72,944**
2. Contingency : (CSR&TI,Berhampore SBRL,Kodathi)	1. Plasticware & Glassware		65,398
	2. Proteomic Analysis		1,11,735
	3. pET Vector		21,546
	4. Protein purification filter		9,603
	Total	**3,00,000**	**2,08,282**
	GRAND TOTAL	**52,80,000**	**22,30,183**

Figures and Tables

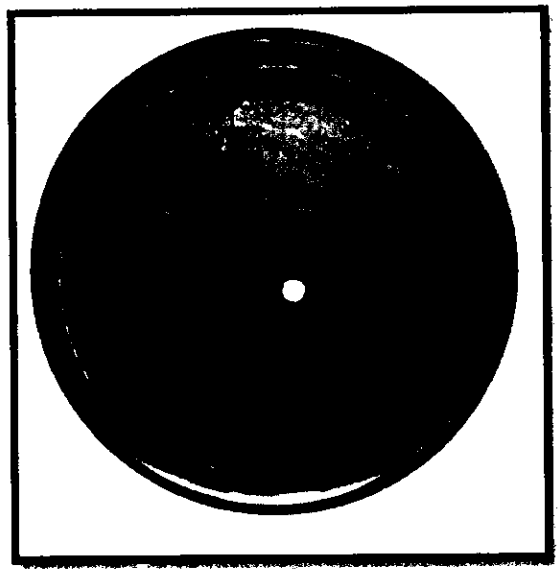


Fig 1: Disc diffusion assay of silkworm haemolymph against *Staphylococcus* sp. collected at regular intervals upon infection with the same bacterium [T1: 24 hours post inoculation (p.i.); T2: 48 hours p.i.; T3: 72 hours p.i.]

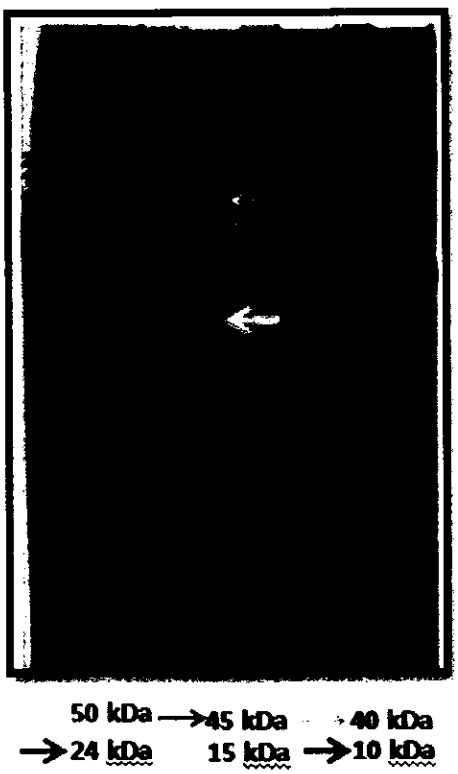


Fig 2: SDS PAGE of silkworm haemolymph protein upon *Staphylococcus sp.* infection

Lane 1-4: Marker, 24 h p.i., 48 h p.i., 24 h control
12 µl of protein sample was loaded in each lane.

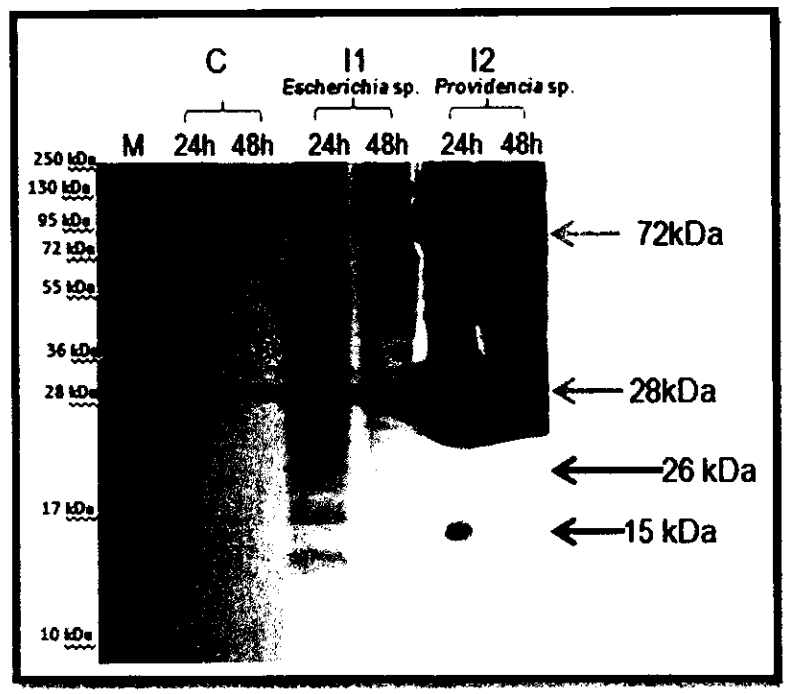


Fig 3: SDS PAGE of silkworm haemolymph protein upon *Escherichia sp.* and *Providencia sp.* infection

Lane1: Marker; Lane 2-3: 24 h & 48 h control
Lane 4-5: 24 h & 48 h p.i. (*Escherichia sp.*)
Lane 5-6: 24 h & 48 h p.i. (*Providencia sp.*)
12 µl of protein sample was loaded in each lane

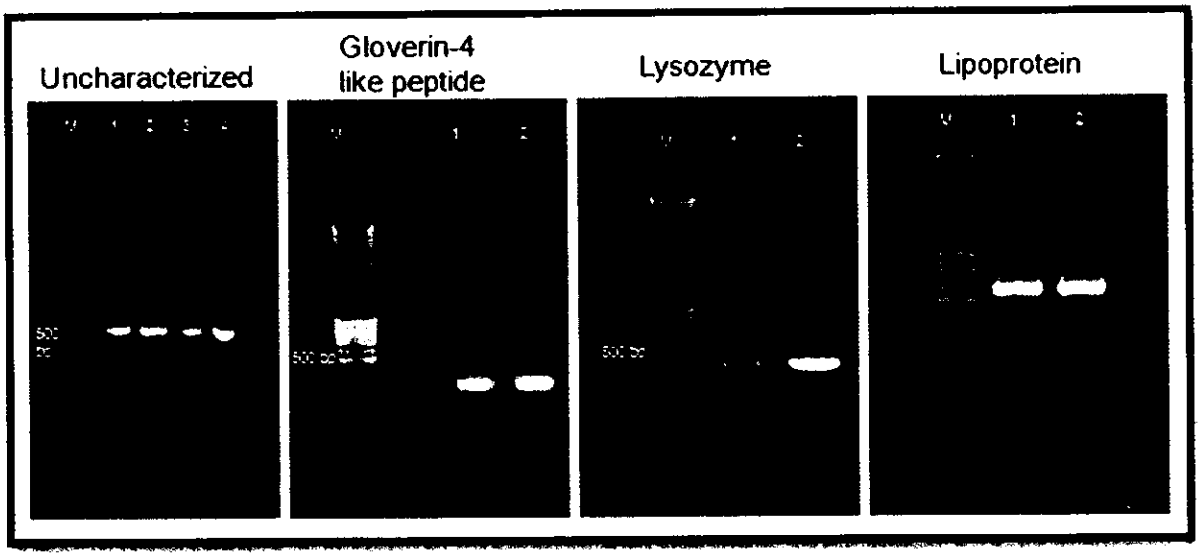


Fig 4: Partial cloning of the target antibacterial genes in pJET vector and confirmation of the same by colony PCR. Agarose gel electrophoresis image depicting the amplification of the genes pertaining to uncharacterized protein (~363 bp). Gloverin-4 like peptide (~400 bp). Lysozyme (~200 bp) and lipoprotein (~565 bp).

M-Marker: The other bands labelled in numericals in all the four images correspond to transformed colonies selected by amp selection from LB plate

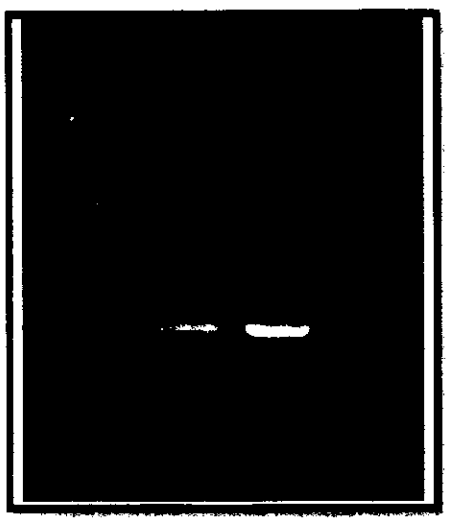


Fig 5: Full length cloning of the ApoLpIII gene in pJET vector and confirmation of the same by colony PCR. Agarose gel electrophoresis image depicting the amplification of the same (~965bp).

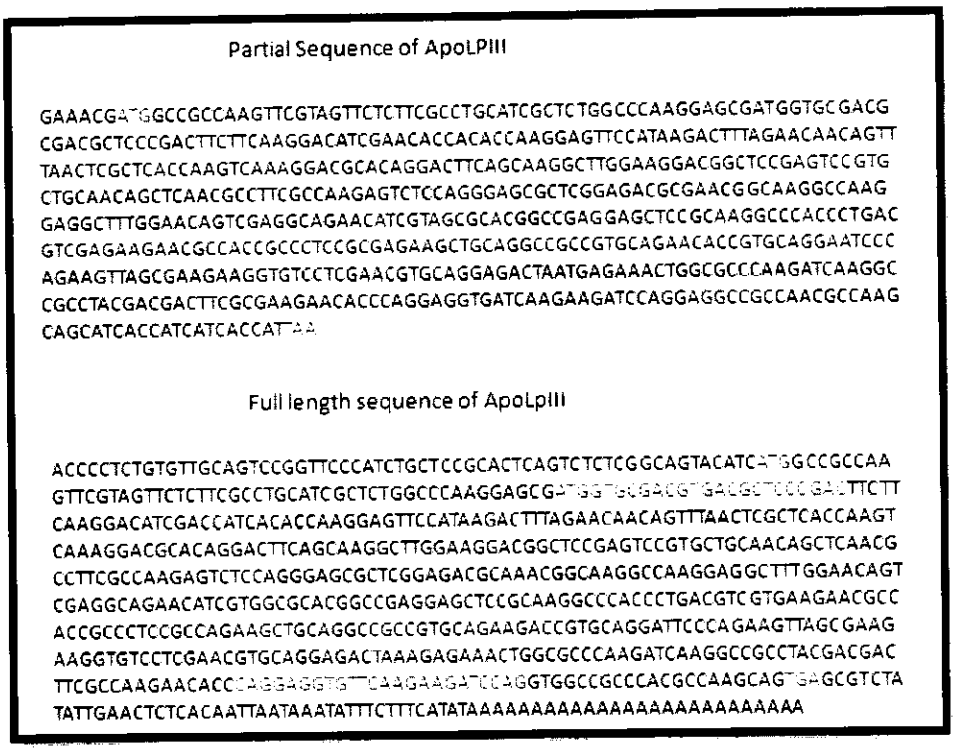


Fig 6: Partial and Full length (after RACE) cloned nucleotide sequences of *Bombyx* mori Apolipophorin III gene

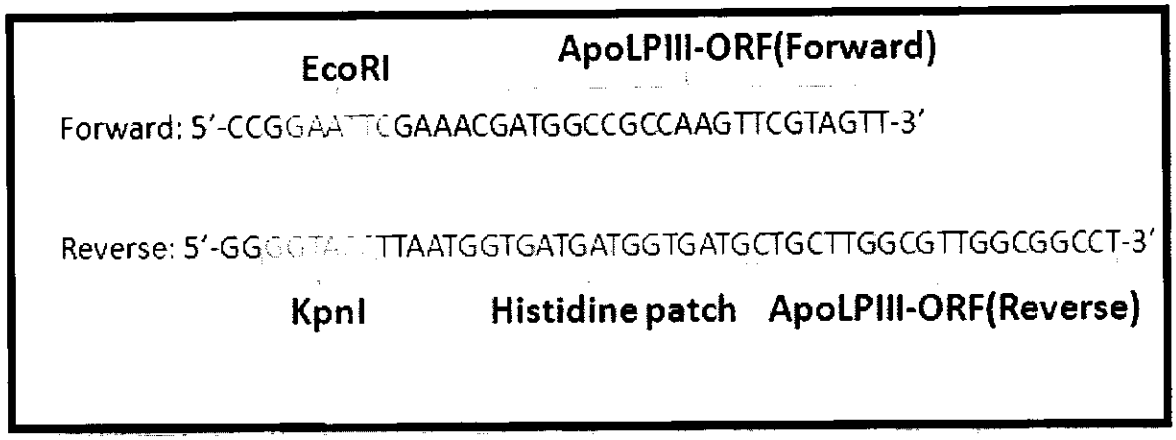


Fig 7: Vector construction for ApoLpIII gene expression in *Pichia pastoris*

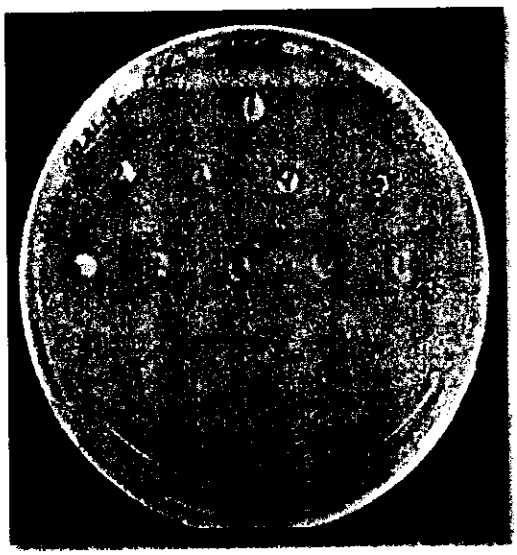


Fig 8: Recombinant *Pichia*-ApoLpIII growing as white coloured colonies

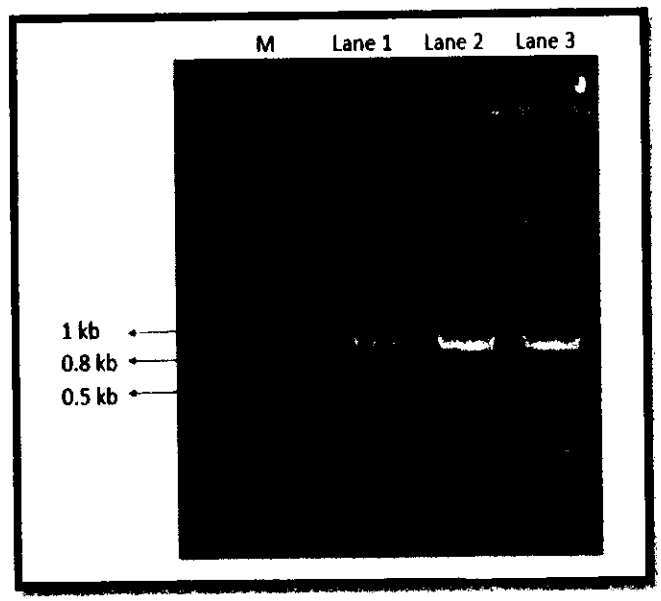


Fig 9: Agarose gel electrophoresis image depicting the presence of insert (~965bp)

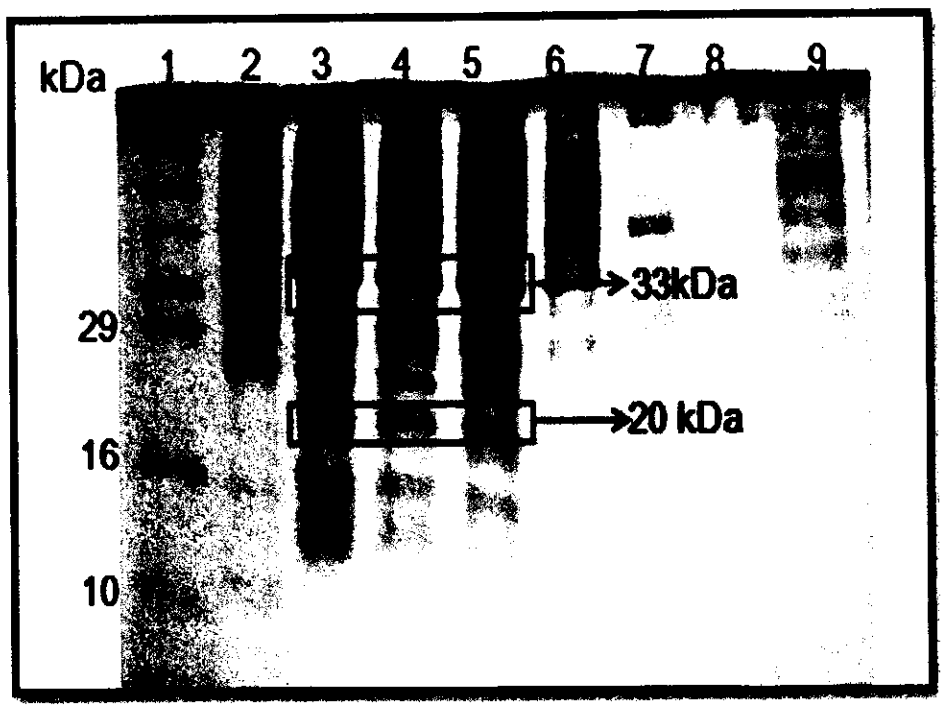


Fig 10: SDS PAGE profile of proteins extracted from *Pichia* cells and supernatant upon expression. Bands pertaining to 33kDa and 20kDa protein are exclusively present in recombinant expression

Lane1: Marker

Lane 2: Control (with out insert)

Lane 3-5: Detection of *Pichia*-ApoLpIII recombinant protein from the pellet harvested after 24h, 48h and 72h

Lane 6-9: *Pichia*-ApoLpIII recombinant was not detected from the supernatant harvested after 24h, 48h and 72h

12 µl of protein sample was loaded in each lane

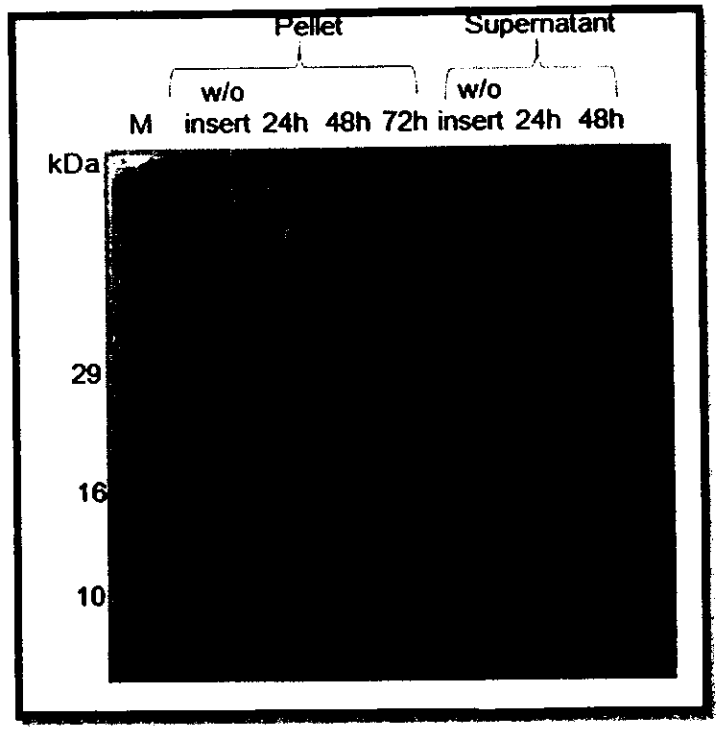
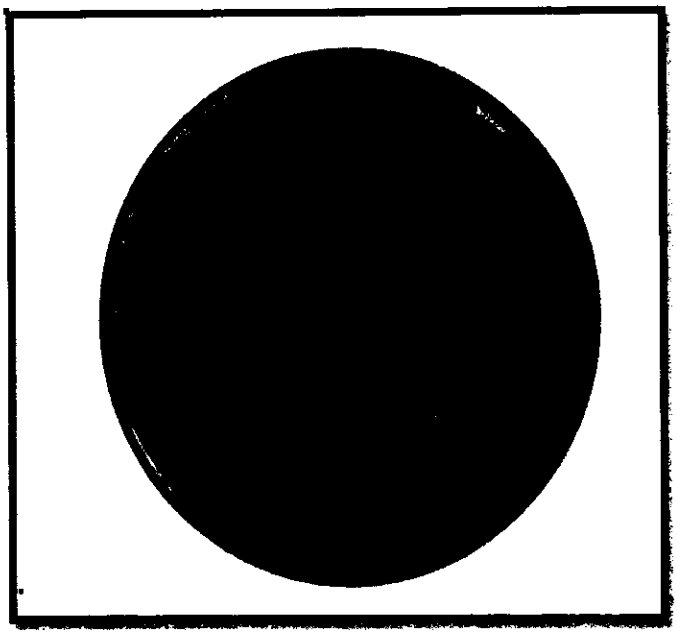
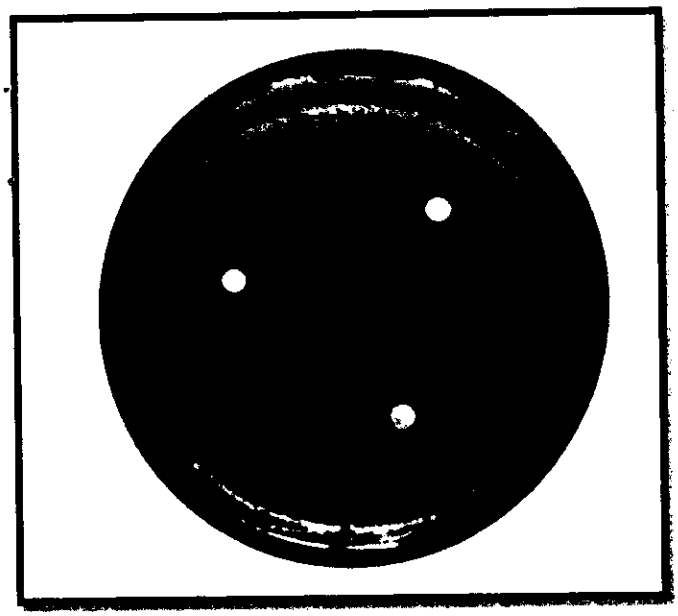


Fig. 11: Detection of recombinant ApoLPIII protein by antihistidine antibody. Both 20kDa and 33kDa pertaining to recombinant ApoLPIII protein was detected from *Pichia* pellet (24h, 48h and 72h) and the same were not detected in *Pichia* supernatant and control sample.



Sl.No	Mol. Wt (kDa)	Concentration (ng/µl)
1.	20 (Elution 1)	10
2.	33 (Elution 1)	12
3.	20 (Elution 2)	8
4.	33 (Elution 2)	10
5.	20 (Elution 1)	20
6.	33 (Elution 1)	18

Fig 12: Antibacterial activity of purified recombinant Apolipophorin III against *Staphylococcus* sp. by disc diffusion assay



Sl.No	Mol. Wt (kDa)	Concentration (ng/µl)
1.	20 (Elution 1)	20
2.	18 (Elution 1)	12

Fig 13: Antibacterial activity of recombinant Apolipophorin III against *Escherichia* sp. by disc diffusion assay

No. of cycles	**Denaturation**		**Annealing**		**Elongation**	
	Temp (°C)	**Time**	**Temp (°C)**	**Time**	**Temp (°C)**	**Time**
1	94	2 min				
34	92	40 s	52	40 s	72	1 min
1			50	1 min	72	5 min

Table 1: PCR cycling parameters for the amplification of 16S rRNA gene for the identification of bacteria

	Low molecular 30 kDa lipoprotein	30K protein	Uncharacterized protein	Arylphonn	Attacin	Lysozyme	Heat shock protein	Gloverin	Anti trypsin	Antibacterial peptide
A_72	Y	Y	Y	Y	N	N	Y	Y	N	Y
H_50	Y	Y	N	Y	N	N	N	N	Y	Y
I_45	Y	Y	N	Y	N	N	N	N	Y	N
J_40	Y	Y	N	Y	N	N	N	N	Y	N
D_28	Y	Y	Y	N	N	Y	N	N	N	N
K_24	Y	Y	N	Y	Y	N	Y	N	Y	N
E_26	Y	Y	Y	Y	Y	N	N	N	N	N
F_15	Y	Y	Y	Y	N	Y	N	Y	N	N
M_10	Y	Y	N	Y	Y	Y	N	Y	Y	N

Table 2: Data analysis of mass spectroscopic results depicting the similarity of sequenced protein bands to antibacterial proteins

Y-Present; N-Absent

Mass: Score: Expect: Matches:

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide

Table 3: NCBI BLAST analysis of recombinant protein sequence obtained by mass spectroscopy analysis showing homology with *B. mori* Lipoprotein.

Certificate

Certified that the study has been carried out and financial expenditure incurred for executing the study are in accordance with the declaration/certification submitted at the time of submission of the proposal and sanction obtained from time to time thereafter as per the revisions made.

(K. Rahul)
Scientist-B
Principal Investigator
CSR&TI, Berhampore
Date: 10/10/2018

के. राहुल/K. RAHUL
वैज्ञानिक-बी/Scientist-B
के.रे.उ.अ.व प्र.सं./C.S.R.&T.I.
केन्द्रीय रेशम बोर्ड/Central Silk Board
भारत सरकार/Govt. of India
बहरमपुर-742101/Berhampore-742101
मुर्शिदाबाद(प.बं.)/Murshidabad (W.B.)

(Dr.G.Ravikumar)
Scientist-D
Principal Investigator
SBRL, Kodathi
Date: 10/10/2018

Director
Chief Executive Authority
Date:.....

निदेशक / Director (I/c)
केन्द्रीय रेशम उत्पादन अनुसंधान संस्थान
Central Sericultural Research & Training Institute
केंद्रीय रेशम बोर्ड/Central Silk Board
बहरमपुर-742101/Berhampore-742101
मुर्शिदाबाद(प.बं.)/Murshidabad (W.B.)

Directors Comment::

The project has been concluded without any deviation of milestones and the results obtained as per the objectives proposed. The project has also been extended for a period of 2 months from the actual date of conclusion (May 2015 to April 2018). The project resulted in the isolation of three bacterial pathogens in pure cultures, partial cloning of four antibacterial genes and cloning and expression of an antimicrobial protein Apolipophorin III in *Pichia pastoris*.

Three pathogenic bacterial strains that were isolated in the present study must be sub-cultured periodically and the pure cultures must be maintained for further research activities. 16S rRNA gene sequences pertaining to the same may be deposited with public databases like NCBI indicating that these strains were pathogenic to silkworm and were isolated from flacherie infected silkworms.

Based on the results, it was observed that the antimicrobial protein (Apolipophorin III) cloned is exhibiting a very good antibacterial activity against the silkworm bacterial pathogens. However, the protein that was expressed is too low in quantity. It is understood that the output cannot be extrapolated to the field level at this juncture. Further studies are necessarily required. The PI may concentrate on taking up further studies in relation to scaling up the same through fermentation or any other modern methods available. The infrastructural and technical support from CSB/other institutes may be sought in this regard. The PI may take up a new project in order to explore the efficacy of the antimicrobial protein *in vivo*.

DIRECTOR
CSR&TI, BERHAMPORE

निदेशक / Director
केन्द्रीय रेशम उत्पादन अनुसंधान एवं प्रशिक्षण संस्थान
Central Sericultural Research & Training Institute
केन्द्रीय रेशम बोर्ड/Central Silk Board
बरहमपुर-742101/Berhampore-742101
मुर्शिदाबाद (प.बं.)/Murshidabad (W.B.)

Comments of the 48th RAC of CSR&TI Berhampore:

Observations of RAC	Action taken
The project has been concluded as per the milestones. However, explore the utility of the outcome of the study for future plan of work.	New project will be proposed in order to scale up the antimicrobial protein and explore its efficacy *in vivo*.