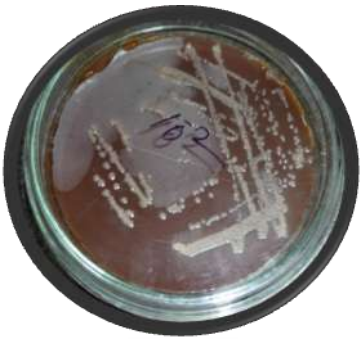
A collaborative research project

ARP3522: Isolation, Cloning and Characterization of Antibacterial Protein (s) from Silkworm, *Bombyx mori*

Period: 3 years
(May, 2015 – Apr, 2018)



Submitted to -

Central Silk Board
Ministry of Textiles: Government of India
Bangalore

Total cost of the project Rs: 52.80 lakhs
(CSR&TI –37.8 lakh & SBRL – 15.0 lakh)

By

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PROFORMA – I

PROFORMA FOR SUBMISSION OF PROJECT PROPOSALS ON RESEARCH AND DEVELOPMENT, PROGRAMME SUPPORT

(To be filled by the applicant)

PART I: GENERAL INFORMATION

1.	Name of the Institute/ University/ Organisation submitting the Project Proposal	:	Central Sericultural Research & Training Institute, Central Silk Board, Ministry of Textiles, Govt. of India, Berhampore, Murshidabad, W.B.-742101, India.
2.	Status of the Institute(s)	:	Govt.
3.	Name and designation of the Executive Authority of the Institute/University forwarding the application	:	**Dr.S.Nirmal Kumar, Director**
4.	Project Title	:	Isolation, cloning and characterization of antibacterial protein (s) from silkworm, *Bombyx mori*
5.	Category of the Project	:	Research and Development
6.	Specific area	:	Silkworm Pathology
7.	Duration	:	03 years
8.	Total cost	:	TOTAL: 52.80 Lakh (CSR&TI – 37.80 lakh and SBRL – 15.0 lakh)
9.	Is the project Single Institutional or Multiple-Institutional (S/M):	:	Multi - Institutional
10.	If the project is multi-institutional, please furnish the following: Name of Project Co-ordinator Affiliation & Address	:	Dr. (Mrs) Kanika Trivedi, Director, Co-Ordinatoor, Seri-biotech Research Laboratory, Kodathi, Bangalore.
11 (a)	Project Summary	:	Bacterial diseases affecting silkworm are collectively known as **'flacherie'** due to the flaccid conditions of the diseased larvae and it is caused primarily by bacteria mainly by *Coccus* and *Bacillus* sp. The biological / chemical control measures and development of disease tolerant silkworm breeds are found to be insufficient to control the bacterial diseases as it is caused by varities of pathogens. Presently, studies are focussed on resistance and immunity as promising alternative to control bacterial disease as innate immunity in multicellular organism is the first line of defense against invading bacteria. In insects, two immune systems exist - cellular and humoral. Silkworms are attacked frequently by pathogens due to failure of cellular defense system as total haemocytes count (THC) remains almost same for a particular breed while multiplication of bacteria is comparatively higher. Alternately, humoral immune system has efficient self-defence mechanism against bacterial infection through induction of **antibacterial proteins by its detergent**

			properties *(mode of action)*. In insects, a large number of antibacterial proteins have been reported and secreted into the haemolymph to attack against the bacteria**. One of the promising alternatives to control bacterial disease is the antibacterial protein from insect source, which can kill their specific targets by damaging the bacterial cell membrane. The detergent properties of these antibacterial proteins disrupt the cell membranes of the invading bacteria leading death of bacteria; thereby control the disease *(Mode of action)*.** Recently, we identified one new bacterial strain ***Staphylococcus vitulinus strain*** **ATCC51145T** identified from MTCC,Chandigarh based on phenotypic and genotypic characterization ***(Reports are enclosed herewith as ANNEX-I)*** isolated from **'flacherie'** infected silkworm, *Bombyx mori*, prevalent in Eastern and NE region***.*** **The bacteria strain is totally different and never been reported as causal agent for 'flacherie' disease in *B. mori*. The strain is highly virulent and cent percent larval mortality was observed due to its infection. Through study of review of literature, it is stated that the bacteria strain have not been used before for induction of antibacterial protein from silkworm, *B.mori*.** Moreover, we have completed two pilot studies on immunization of silkworm at this Institute and out come of the studies indicate i) the advantage of humoral immunity derived components as well as their application in the field to control the bacterial diseases in silkworm and ii) all varities of pathogenic bacteria causing **'flacherie'** diseases in silkworm are not controlled by immunogen(s) used. Hence, there is a need to look for antibacterial protein(s) as an effective alternative for control the bacterial diseases in silkworm. Hence, the present study aims at searching antibacterial protein(s) from *B.mori* using *Staphylococcus vitulinus strain* ATCC51145T, a virulent strain (s) of pathogenic bacteria prevalent in Eastern and NE region; as inducer as it has not been used earlier to explore antibacterial protein. In the present proposal, isolation and identification of antibacterial protein(s) will be done and it will be tested in silkworm system followed by full length gene will be characterised using cDNA cloning. Later on, such characterized antibacterial gene (s) to be functionally expressed in suitable system for mass production of the same. Identification and functional characterization of novel antibacterial protein (s) are needed to control the resistance of bacteria against known antimicrobials, *Staphylococcus vitulinus strain* ATCC51145T. The resultant recombinant protein (s) will be used to control the bacterial diseases of the silkworm which will be a boon for the sericulture industry.

11 (b)	Aims and objectives	:	1) Isolation and characterization of bacterial strain(s) and their pathogenicity studies. 2) Isolation, purification and characterization of novel immunogenic antibacterial protein(s) fractions elicited against, *Staphylococcus vitulinus* strain ATCC51145T strain-specific pathogenic bacteria. 3) Molecular cloning and full length of gene sequence using cDNA cloning for mass production of antibacterial protein.

PART II: PARTICULARS OF INVESTIGATORS

(*One or more co-investigators are preferred in every project. Inclusion of co-investigator(s) is mandatory for investigators retiring before completion of the project*)

Principal Investigator - I

12.1 Name: Dr.S.Chakrabarty
Date of Birth: 09-12-1964; Sex : Male
Designation: Scientist-C
Department: Silkworm Pathology Laboratory, Central Sericultural Research & Training Institute
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Number of research projects being handled at present: 1

Principal Investigator - II

12.2 Name: Dr.G.Ravikumar
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Number of research projects being handled at present: 01

Co-ordinator - I

12.3. Name: Dr.(Mrs) Kanika Trivedi
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Department: Seri-Biotech Research Laboratory
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Number of research projects being handled at present: Several

Co-ordinator - II

12.4. Name: Dr.A.K.Saha
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Executive Authority

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Name: Dr. S.Nirmal Kumar
Sex: Male
Designation: Director
Department: Central Sericultural Research & Training Institute
Institute/University: Central Silk Board, Ministry of Textiles, Govt. of India.
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Number of Research projects being handled at present: Several

13. No. of Projects being handled by each investigator at present: 01 each by PI

14. Research Fellow: One

JRF participation will be the main scientific support to carry out the research work. The proposed work is voluminous and no other scientists are available as Co-Investigators in both the Institutes.

Bacterial cultures, silkworm rearing, infections to silkworms, a number of purification steps of antibacterial proteins using sephadex and HPLC, and antibacterial assays their infections to silkworms, electrophoresis are involved.

Isolation of RNA, 1st strand syntheisis, PCR, cDNA cloning which involes cutting out and ligating of PCR fragments, transformation, and plasmid purification are required. Vector constructions for recombinant expression, optimization, purification of recombinant proteins, Western blotting, antibacterial assays, sequence analysis are to be performed.

In addition, JRF working in this project will be imparted basic as well as advanced training in insect molecular biology, which will form an effective human resource development for the country.

PART III: TECHNICAL DETAILS OF PROJECT

15. **Introduction**

In India, 'flacherie' is one of the serious diseases of silkworm. Prevalence of bacterial 'flacherie' is more and cause substantial crop loss in the eastern and NE region other than viral / bacterial diseases.

- The disease incidence is prevalent during hot and humid seasons.
- Poor disinfection, accumulation of pathogenic load in the rearing trays, feeding of contaminated mulberry leaves and improper use of bactericide leads to large scale crop loss due to bacterial diseases.

The biological / chemical control measures and development of disease tolerant silkworm breeds are found to be insufficient to control the bacterial diseases as it is caused by varities of pathogens. Presently, studies are focussed on resistance and immunity as promising alternative to control bacterial disease as innate immunity in multicellular organism is the first line of defense against invading bacteria. In insects, two immune systems exist - cellular and humoral. Silkworms are attacked frequently by pathogens due to failure of cellular defense system as total haemocytes count (THC) remains almost same for a particular breed while multiplication of bacteria is comparatively higher *(Annual Report: CSR&TI, Berhampore, W.B., 2001-02)*. Humoral immune system has efficient self-defence mechanism against bacterial infection through induction of antibacterial proteins by its detergent properties *(mode of action).* (Dunn, 1986 and Stanley-Samuelson *et al*., 1991).

In insects, a large number of antibacterial proteins have been reported (Hultmark *et al*, 1980) which are synthesized mainly in fat body of silkworm (equivalent to liver in mammals) and secreted into the haemolymph to attack enzymatically against the bacteria by hydrolysing their peptidoglycan cell walls (Dunn, 1986; Russel and Dunn, 1996; Brey, *et al.,* 1993). **The detergent properties of these antibacterial proteins disrupt the cell membranes of the invading bacteria. Both cellular**

and humoral immune system may occur simultaneously or discretely, depending on the degree of infection and the nature of pathogen (Krishnan *et al*., 2000).

Role of Antibacterial proteins

- A group of immune proteins that protect the host from infection.
- Antibacterial proteins are important factors which can eliminate infectious bacteria using the innate immune system.
- In insects, a large number of antibacterial proteins have been isolated
- Antibacterial proteins have emerged as novel antimicrobial agents for use in therapeutics, animal drug and food preservatives.
- To make antibacterial proteins more economically viable, researchers have sought to mass production of antibacterial proteins using recombinant means such as insects, baculovirus, yeast and *E. coli* based systems

Salient findings of previous works on Antibacterial proteins of silkworm

- **Cecropins** exhibit a broad spectrum antibacterial activity most effectively against gram negative bacteria but also against gram positive bacteria on interaction with bacterial membranes resulting in the formation of ion channels. It also resisting fungi.
- **Lysozyme** directly attacks bacteria by hydrolysing their peptidoglycan layer of the cell wall.
- **Glycin-rich-Attacin** act against gram negative bacteria and was shown to inhibit the synthesis outer membrane protein of bacteria.
- **Glycin-rich-Gloverin** inhibits the formation of outer membrane leads to increase permeability of the bacterial membrane.
- **Proline-rich Lebocin** has weak antibacterial activity on bacterial membrane but its antibacterial activity becomes much higher when it exists together (synergistic effect) with Cecropin-D.
- **Moricin** active against gram negative bacteria and also gram positive bacteria by increasing permeability bacterial membrane and form ion channels.

Mode of action of antibacterial proteins

- Usually when insects are infected by bacteria, antibacterial proteins are induced rapidly and these play an important role in eliminating different types of invading bacteria.
- Antibacterial proteins are synthesized mainly in fat body of silkworm and secreted into the haemolymph to enzymatically attack the bacteria by hydrolysing their peptidoglycan cell walls.
- The detergent properties of these antibacterial proteins disrupt the cell membranes of the invading bacteria.
- Both cellular and humoral immune systems may occur simultaneously or discretely and form a potent defence against invading bacteria, depending on the 'degree of infection' and the 'nature of pathogen'

This Institute had already conducted a research project on cellular defense mechanism of silkworms and screened the available multivoltine and bivoltine breeds (Krishnan *et al*., 2000). Besides, two pilot studies have been completed on immunization of silkworm. The out come of all these studies indicates advantage of humoral immunity derived components as well as their application in the field to control the bacterial disease. However, not all bacteria were controlled by the immunogen(s) used. This Institute also isolated *Staphylococcus vitulinus strain* ATCC51145T**,** a totally different strain of virulent bacteria isolated from 'flacherie' infected silkworm larvae. Hence, there is a need to look for an effective antibacterial protein for control the bacterial diseases. Hence, the present study aims at searching antibacterial protein(s) from silkworm, *B.mori* using *Staphylococcus vitulinus* strain ATCC51145T, the highly virulent strain of pathogenic bacteria prevalent in eastern and NE region, as inducer and it has not been used earlier to explore antibacterial protein. In the present proposal, isolation and identification of antibacterial protein(s) will be done and it will be tested in silkworm system followed by full length gene will be characterised using cDNA cloning. Later on, such characterized antibacterial gene(s) to be functionally expressed in suitable system for mass production of the same.

15.1 Definition of the Problem

(a) Origin of the project

In India, **'flacherie'** is one of the serious diseases of silkworm. Prevalence of bacterial 'flacherie' is more and cause substantial crop loss in the eastern region other than viral / bacterial diseases. Biological / chemical control measures and development of disease tolerant silkworm breeds are insufficient to contain the bacterial diseases. Recently, this Institute have identified one new bacteria strain *Staphylococcus vitulinus* strain ATCC51145T isolated from 'flacherie' infected silkworm, *Bombyx mori*, based on phenotypic and genotypic characterization**.** The bacteria strain is totally different causing 'flacherie' disease in *B. mori*. The strain is highly virulent and cent percent larval mortality was observed due to 'flacherie' disease. Through study of review of literature, it is stated that the bacteria strain have not been used before for induction of antibacterial protein from silkworm.

Hence, there is a need a new antibacterial protein(s) for combating the bacterial disease. Hence the present study aims at searching antibacterial protein(s) from silkworm, *B.mori* using above virulent strain (s) of pathogenic bacteria prevalent in eastern region. In the present proposal isolation and identification of antibacterial protein(s) will be done and it will be tested in silkworm system followed by full length gene will be characterised using cDNA cloning for mass production of antibacterial protein..

b) Expected outcome

We hope to get new antibacterial protein (s) in the present proposal. Hence the present study aims at searching antibacterial protein(s) from *B.mori* using *Staphylococcus vitulinus* strain ATCC51145T, the highly virulent strain of pathogenic bacteria prevalent in eastern and NE region. In the present proposal isolation and identification of antibacterial protein(s) will be done and it will be tested in silkworm system followed by full length gene will be characterised using cDNA cloning for mass production of antibacterial protein.In addition, the new protein(s) may be patented and also we expect a good scientific publication out of this project.

15.2 Origin of the Proposal / Rationale of the Study

Bacterial diseases of silkworms are very much prevalent in the eastern and NE region and recurring crop loss is attributed in this region. Chemical / botanical are used in the field to control the disease as well as the silkworm breeds are screened on the basis of haemocyte counts to control the disease. But none of the methods provide complete control of the disease. Cellular defense response involves direct interactions between circulating haemocytes and bacteria through phagocytes, nodulation and encapsulation (Gupta, 1986 and 1991). Humoral reactions involve inducted synthesis of antibacterial proteins (Cociancich *et al*., 1994). In insects, a large number of antibacterial proteins have been isolated and these antibacterial proteins are classified in five major groups (Hultmark *et al*,, 1980). Cecropins, lysozyme and prolin-rich antibacterial proteins have been reported from immunized haemolymph of silkworm, *B.mori* (Chadwick and Aston, 1991 and Hara and Yamakawa, 1995). The detergent properties of these antibacterial proteins disrupt the cell membranes of the invading bacteria and enzymatically attack bacteria by hydrolysing their peptidoglycan cell walls (Dunn, 1986 and Russel and Dunn, 1996). All three processes may occur simultaneously or discretely, depending on the degree of infection and the nature of pathogen (Krishnan *et al*., 2000). Antibacterial proteins play an important role in eliminating invaders. Antibacterial proteins are amphiphillic, positively charged molecules that protect the host from infection (Dunn, 1986; Boman and Hultmark, 1981; Boman *et al*,, 1974, 1998; Hoffman *et al*, 1981).

The strain *Staphylococcus vitulinus* ATCC51145T isolated from 'flacherie' infected *Bombyx mori*, based on phenotypic and genotypic characterization by MTCC, Chandigarh. The bacteria strain is totally different causing 'flacherie' disease in *B. mori*. The strain is highly virulent for causing 'Flacherie' disease. Through study of review of literature, it is observed that the bacteria strain have not been used earlier by any workers for induction of antibacterial protein from silkworm.

The present proposal aims identifying and isolating immunogenic antibacterial proteins produced in *Bombyx mori* in response to infection by the above strain causing bacterial disease or

'flacherie'. In addition, it is proposed to test these antibacterial protein(s) in silkworm and also clone the cDNA (s) encoding the induced antibacterial proteins.

15.3 Relevance to the current issues and expected outcome

We have identified one new bacteria strain *Staphylococcus vitulinus* strain ATCC51145T from flacherie infected *B.mori* by MTCC, Chandigarh based on genotypic and phenotypic characterization *(Reports are enclosed herewithas ANNEX-I)***.** The bacteria strain is totally different causing 'flacherie' disease in *Bombyx mori* L. The strain is highly virulent and cent percent larval mortality was observed due to causing'Flacherie' disease in *B.mori* L.The strain have not been used for induction of antibacterial strain.

We hope to get new antibacterial protein(s) in the present proposal. Hence the present study aims at searching antibacterial protein(s) from silkworm, *B.mori* using the most virulent strain (s) of pathogenic bacteria prevalent in eastern region. In the present proposal isolation and identification of antibacterial protein(s) will be done and it will be tested in silkworm system followed by full length gene will be characterised using cDNA cloning for mass production of antibacterial protein.In addition, the new protein (s) may be patented and also we expect a good scientific publication out of this project.

The novelties of the project are-

- Novel virulent strain specific antibacterial protein (s) to be isolated and identified to control the bacterial disease.
- Such identified & purified antibacterial protein (s) will be sequenced and will be tested in silkworm system.
- Full length of gene will be characterised using cDNA cloning for mass production of antibacterial protein.

15.4 Objectives

i. Isolation and characterization of bacterial strains and their pathogenicity studies
ii. Isolation, purification and characterization of novel immunogenic antibacterial protein(s) fractions elicited against *Staphylococcus vitulinus* strain ATCC51145T , strain-specific pathogenic bacteria
iii. Molecular cloning and full length of gene sequence using cDNA cloning for mass production of antibacterial protein.

16. Review of status of Research and Development on the subject.

16.1. International Status

In insects two broad categories of immune systems exists - cellular and humoral. Humoral immune system does not involve antigen-antibody reactions and have efficient self-defence mechanism against bacterial infection through induction of antibacterial peptides (Dunn, 1986 and Stanley-Samuelson *et al*., 1991). Usually when insects are infected with bacteria, antibacterial proteins are induced rapidly and simultaneously the network of antibacterial proteins plays an important role in eliminating different types of invading bacteria. To understand the role of such a network, it is important to identify individual peptides. But structurally and functionally these peptides are different from antibodies produced in the higher vertebrates. To date, many antibacterial peptides have been isolated from different species of insects and can be classified into five major groups, cecropins, insect defensins, attacin-like proteins, prolin-rich peptides and lysozymes. Certain peptides, phenoloxydases, lectins, haemolin, attacins etc. of low molecular weight have also role to improve humoral defence in insects. Cecropins, lysozyme and prolin-rich peptide have been reported as antibacterial peptides from immunized haemolymph of silkworm, *B.mori* L. (Chadwick and Aston, 1991 and Hara and Yamakawa, 1995). Cecropin, a small family of lytic peptide was found in giant silk moth, *Hyalophora cecropia*. These proteins are small and strongly basic,and comprise three major forms,A,B and D. Cecropins exhibit a broad spectrum antibacterial activity against both Gram-

negative and positive bacteria by adopting α - helical structure on interaction with bacterial membranes resulting in the formation of ion channels (Christensen *et al*.,1988). Attacin and Lysozyme were isolated and characterized from immunized larval haemolymph of the wild eri silk moth *Samia cynthia ricini* (Kishimoto *et al.*, 2002; Fujimoto *et al*., 2001). Kato *et al*., (1993) studied on expression and characterization of cDNAs for cecropin B of *B. mori*.

This study will generate basic information on the humoral defense mechanism in silkworm *Bombyx mori* L. that can be used for immunization of the insect against pathogenic bacterial strains. Induction of humoral defence can also be done with non-pathogenic or heat killed / irradiated microorganism. Healthy larvae of insects when immunized after specific time gap, the survival percentage of larvae was higher than that of control (Sharma *et al*., 2005, Raz and Fierer, 2006). Antimicrobial defence mechanism in animals was performed using insect model (Metchnikoff, 1884). In insects, morphologically distinct hemocyte types have been shown to play different roles in the host defence reaction i.e., cell mediated immunity (Price and Ratcliffe, 1974). Defence peptides play a crucial role in insect immunity against invading pathogens (Cytrynska *et al.,* 2006). Non- pathogenic or heat-killed bacteria can acquire resistance to subsequent challenge by bacterial pathogen and those silkworms are used for induction of immunity because of high volume of protein-rich haemolymph (Sharma, *et.al*., 2005). Cuboni and Garbini (1980) isolated *Bacillus cubonianus* and reported them to be the cause of bacterial flacherie. Later Bacillus thuringiensis sotto (Ishiwata, 1902), *Streptococcus bombycis* (Steinhaus, 1949), *Serratia marcescens* (Metalnikov and Chorine, 1928), *Streptococcus faecalis, S.facium* were also reported to be pathogenic to silkworm causing flacherie (Vago, 1963).

Usually when insects are infected with bacteria, antibacterial proteins are induced rapidly and simultaneously the network of antibacterial proteins plays an important role in eliminating different types of invading bacteria. To understand the role of such a network, it is important to identify more antibacterial proteins. Live or formalin treated *Escheria coli* K-12 induced antibacterial activity in the hemolymph of silkworm, *B.mori* L. (Ichimori *et al.*, 1992 and Sumida *et al.*, 1992). Oligodeoxynucleotides (ODN) are known to stimulate immune response in silkworm, *B.mori* L. (Hsu *et al.,* 1999 and Kim, *et al*., 2002). Induction of humoral defence can also be done with non-pathogenic or heat killed / irradiated microorganism.

Silkworm have developed an efficient host defense against invading micro-organisms, which involves three major components such as phagocytosis,cellular encapsulation, prophenol oxidate cascade and synthesis of antibacterial proteins,where as the interrelationships among immune mechanisms and other mechanisms is yet to be investigated in *B.mori*. Due to the enormous use of antibiotics, most of the clinical pathogens are becoming resistant to those antibiotics. Promising alternative antibiotics are the anti bacterial peptide antibiotics from insect source, which can kill their specific targets by damaging the bacterial cell membrane. In India the indigenous tropical polyvoltine races showed more resistance to diseases than temperate bivoltine races.In order to understand the differential response at molecular level, it may be an ideal approach in compare the expression level of antibacterial genes in hardy polyvoltine races like Pure Mysore and Nistari with temperate races (Ponnuvel and Yamakawa, 2002).

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16.2. National Status

Preliminary work done so far

This Institute has already conducted a research project entitled '*Hemocyte immunity study of silkworm, Bombyx mori* on cellular defense mechanism of silkworms and screened the available multivoltine and bivoltine breeds based on haemocytic study including encapsulation and nodulation in response to highly, medium and non-pathogenic bacteria (Annual Report: CSR &TI, Berhampore,

West Bengal, 2001-02, Choudhry *et al.*, 2002 ; Krishnan *et al*., 1998, 2000a, 2000b, 2002a, 2002b, 2002c; Mitra,2008). Through this study the breeds have been screened on the basis of total and differential hemocyte counts (THC and DHC) for further use to select the breeds as parents in future breeding programme. Besides, a pilot study entitled '*Immunization of silkworm Bombyx mori L. against bacterial disease'* has just been completed on immunization of silkworm through the feed supplement of immunogens along with mulberry leaf (Pilot Study, Central Sericultural Research & Training Institute, Berhampore, West Bengal, 2009 - 10). This study screened the available immunogens to improve the humoral system in silkworm *B. mori.* The out come of both the studies indicates advantage of humoral immunity as well as its application in the field to control the bacterial disease. Both of these studies gave a lead to pursue a molecular based approach to elucidate candidate gene(s) responsible for silkworm immunity against virulent pathogenic bacterial strains/species found predominately in the eastern and NE region of India. In addition, bacterial resistance to some antimicrobials is also a concern. This study should lead to find better and more effective potential to control the flacherie disease of silkworm as an alternative of the classical approach to use chemicals/ botanicals. We have identified one new bacteria strain *Staphylococcus vitulinus strain* ATCC51145T based on genotypic and phenotypic characterization *(Reports are enclosed herewith)* through MTCC, Chandigarh and MTCC, Chandigarh from 'flacherie' infected *B.mori.* The bacteria strain is totally different causing 'flacherie' disease in *Bombyx mori* L. The strain is highly virulent and cent percent larval mortality moratality was observed due to causing'Flacherie' disease in *B.mori* L.The strain have not been reported from Silkworm and not been used for induction of antibacterial strain.

Chitra *et al*. (1973) isolated *Aerobacter cloacae, Achromobacter delmarvae* and *A.superficalis* from haemolymph and *Achromobacter cloacae, Pseudomonas boreopolis, P.ovalis, Escheria freundii and Staphylococcus albus* from the gut of 'flaccid' silkworm. However, the species of *Streptococci, Staphylococci, Bacillus* and *Serratia* are the most common bacteria in silkworm, *Bombyx mori* L. in India (Anitha *et al*., 1994 and Patil, 1994). Abraham *et al.* (1995) examined the nature of antibacterial response in *B.mori* and the major protein involved in the antibacterial response of silkworm was of the lysozyme type. Choudhury *et al.* (2004) and Sharma *et al.,* (2005) isolated antibacterial protein(s) from non-mulberry silkworm against flacherie causing *Pseudomonas aeruginosa* AC-3.

In this context, this Institute has already conducted a research project entitled '*Hemocyte immunity study of silkworm, Bombyx mori* L.' (Annual Report: CSR&TI, Berhampore, W.B, 2001-02) on cellular defense mechanism of silkworms and screened the available multivoltine and bivoltine breeds based on haemocytic study including nodule formation in response to pathothgenic and non-pathogenic bacteria (Mitra, 2008). Detail study on encapsulation and nodulation responses were coducted by Choudhry *et al.* (2002) and Krishnan *et al*., (1998, 2000a, 2000b, 2002a, 2002b, 2002c) at this Institute.

Besides, a pilot study entitled '*Immunization of silkworm Bombyx mori L. against bacterial disease'* has just been completed on immunization of silkworm through the feed supplement of immunogens along with mulberry leaf (Pilot study, 2010).

The out come of both the studies indicate advantage of humoral immunity as well as application of antibacterial protein in the field to control the bacterial disease.

The CSR&TI, Berhampore had already conducted a research project on cellular defense mechanism of silkworms and screened the available multivoltine and bivoltine breeds (Krishnan *et al*., 2000). Besides, two pilot studies have been completed on immunization of silkworm. The out come of all these studies indicates advantage of humoral immunity derived components as well as their application in the field to control the bacterial disease. However, all bacteria are not controlled by the immunogen (s) used. This Institute also isolated *Staphylococcus vitulinus strain* ATCC51145T**,** a totally different strain of virulent bacteria from 'flacherie' infected silkworm larvae. Hence, there is a need to look for an effective antibacterial protein for control bacterial disease. Hence, the present study aims at searching antibacterial protein(s) from silkworm, *B.mori* using *Staphylococcus vitulinus strain* ATCC51145T, the most virulent strain (s) of pathogenic bacteria prevalent in eastern and NE region. In this project, isolation, and characterization of antibacterial protein (s) and full length of gene will be characterised and will be tested in silkworm system.

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16.3 Importance of the proposed project in the context of current status:

We have identified one new bacteria strain *Staphylococcus vitulinus strain* ATCC51145T based on genotypic and phenotypic characterization *(Reports are enclosed herewith)* through MTCC, Chandigarh and MTCC Chandigarh from 'flacherie' infected *B.mori.* The bacteria strain is totally different causing 'flacherie' disease in *Bombyx mori* L. The strain is highly virulent and 100 % larval mortality moratality was observed due to causing'Flacherie' disease in *B.mori* L.The strain have not been used for induction of antibacterial strain.

We hope to get new antibacterial protein (s) in the present proposal. Hence the present study aims at searching antibacterial protein(s) from silkworm, *B.mori* using the most virulent strain (s) of pathogenic bacteria prevalent in eastern region. In the present proposal isolation and identification of antibacterial protein(s) will be done and it will be tested in silkworm system followed by full length gene will be characterised using cDNA cloning for mass production of antibacterial protein.

16.4 Anticipated Products, processes / Technology, Packages / Information or other outcome from the project and their expected utility:

Antibacterial proteins have been isolated from insects as novel antimicrobial agents for the use in therapeutics, animal drug and food preservatives. To make antibacterial proteins more economically viable, researchers have sought to produce antibacterial proteins. In the present proposal isolation and identification of antibacterial protein(s) will be done and it will be tested in silkworm system followed by full length gene will be characterised using cDNA cloning for mass production of antibacterial protein.

16.5. Expertise available with proposed investigation group/institution on the subject of the project:

Name of the Scientists	Desig.	Experience
Dr.S.Chakrabarty	Scientist C	He is having doctoral degree in Zoology with specialization in Parasitology (Immunology) and have working experience in silkworm pathology since 1997. He has completed 2 pilot studies based on '*Immunization of silkworm Bombyx mori L.' against bacterial diseases*. He has working experience in immunological methods and assays. He undergone/ participated training/ workshop at SBRL,Kodathi, Bangalore on silkworm molecular pathology / biology viz.,detection of diseases using PCR, utilization of RAPD & ISSR markers for molecular phylogenetic analysis;extraction of cuticle protein, HPLC purification & 2-D analysis of cuticle protein;SDS PAGE analysis of haemolymph protein; cloning of PCR products,plasmid DNA isolation; cloning of SSU-rRNA sequence *etc.* He concluded two pilot projects as PI entitled, '*Immunization of silkworm Bombyx mori L. against bacterial disease*" followed by *'Testing of immunogen for prevention of silkworm diseases in Bombyx mori L."* using amino acids, vitamin etc. He is one of the co-inventor of one product 'Sericillin' a silkworm bed disinfectant and it is under patenting with NRDC, New Delhi. Two Entrepreneurs have been developed for commercialization of the nproduct. Recently he has identified one bacteria *Staphylococcus vitulinus strain* ATCC51145T a different strain from 'Flecherie' infected silkworm. He formulated 'Ghar Sodhon' a fumigant cost-effective room disinfectant for labour saving and drudgery reduction especially useful in eastern and NE region. He published more than 38 papers (including international 6) and attended 16 symposiums (including international-1).
Dr. G.Ravikumar	Scientist D	Recently he came back from Japan after completing nearly seven years of research at the National Institute of Agrobiological Sciences (NIAS), formerly called as National Institute of Sericulture and Entomological Sciences (NISES) Tsukuba, Japan. During this period, he had the opportunity to work in frontier areas of molecular biology using insect as experimental model. His work was focused on the cloning and expression of novel genes from insects. He has cloned a carboxylesterase from a parasitic wasp, Dinocampus coccinellae, cloned and expressed a lipophorin receptor from the silkworm, and expressed a termite cellulase in yeast. Apart from this, he had been actively associated with the Bombyx mori genome project with Dr. Kazuei Mita (Head, insect Genome Lab, NIAS) and was co-author of the paper on silkworm genome. During the last 6 years at Seri-biotech Research Lab, he has developed a multiplex PCR based detection system for the identification of three main diseases, pebrine, grasserie and flacherie of the silkworm. This is patent pending with NRDC, New Delhi and is the first patent from the Seri-biotech Research Lab. In addition, he is investigating the functional characterization of the brain-specific lipophorin receptor variant using frontier areas of molecular biology (RNAi and protein protein interactions using yeast two-hybrid system). He has all the necessary expertise in basic as well as frontier areas of molecular biology research and am capable of independently planning and carrying out research projects. In addition, he has close association with leading scientists in the field from Japan and other countries. He is confident of doing cutting edge molecular biology research and he would like to use his expertise for the advancement of insect molecular biology in general and Indian sericulture in particular.

17. **Work Plan**

17.1 **Methodology**

1. Isolation of bacteria

Staphylococcus vitulinus strain ATCC51145T, bacteria from stock solution at the Silkworm Pathology Laboratory of this Institute and fresh bacteria will be isolated from 'flacherie infected' silkworm from the farmer's house and our laboratory will be used for the study.

2. Purification of bacteria

The infected silkworms will be homogenized aseptically. Homogenate will be examined under phase contrast microscope for the presence of bacteria. An aliquot of the homogenate to be streaked on NB plates (beef extract 0.5%,peptone 0.3%,NaCl 0.2% and agar 2.0% , pH 7.0) and incubate overnight at 36 ± 2ºC and relative humidity of 60 ± 5ºC. The growth of individual colonies shall be observed and an individual colony to be separated. Pure culture shall be maintained for the isolate on nutrient agar slant. Bacterial isolate shall be sent to the Institute of Microbial Technology, Chandigarh, India for identification.

3. Insect

Laboratories colonies of the silkworm *Bombyx mori* (Strain- Nistari) shall be reared at room temperature on fresh mulberry leaves *ad lib* under ambient conditions as per the standard rearing method (Krishnaswami, 1978). Leaf surface shall to be sterilized prior to feeding to silkworms. Short rinses in 75% ethanol follwed by distill water rinse and air dry is sufficient to eliminate the influence of pathogens in leaves (Sheeba Rajakumari *et al*., 2007). Care shall be taken to ensure that all insects to be healthy and robust at the time of experiment.

4. Pathogenicity

For each of the different bacterial strain (s), three replicates of 100 third instar larvae will be maintained. Three bacterial doses of three replicates of 10^7, 10^6 and 10^5 cfu / ml will be used as infective doses for the treatment. A piece of mulberry leaf *(Morus* sp. Variety S1635) measuring 5 cm^2 will be smeared with the bacterial suspension of 0.2 ml and inoculated *per os* to 5 healthy larvae on the first day of the third instar and ensure that the entire leaf should be consumed by the larvae. The infected larvae will be subsequently reared with fresh leaves following the standard procedures. Then the dead larvae shall be counted from the second day of inoculation and smear of dead larvae were collected and examined under phase contrast microscope to confirm the mortality of the larvae due to bacterial disease. Then the Lethal Concentration (LC) 50 / LC90 shall be measured following standard procedure (Woolf, 1968). Most virulent pathogen shall be determined through the detection of lethal concentration and shall be taken for the next step of experiment.

5. Immunization or Inoculation

Most virulent strain of bacteria prevalent in the region shall be injected to the silkworm larvae for studying induction kinetics of antibacterial activity. LC50 dose of most virulent strain of bacteria will be determined and with suitable adjuvant shall be injected for immunization. The 3-day-old V stage larvae to be injected with 10-µl suspension of bacteria log phased bacteria washed and suspended in saline (0.3M NaCl, 0.005M KCl at 10^7 cfu / ml.).

6. Collection of haemolymph

Haemolymph samples shall be collected from 3-day old fifth instar larvae at different intervals of post injection into pre-cooled at below 4^oC eppendorf tubes containing few crystals of phenylthiourea to prevent oxidation of haemolymph and centrifuged subsequently at 6000 rpm for 5 min to remove haemocytes and other tissue debris (pellete) and supernatant shall be stored at - 20^oC until use. Haemolymph from uninfected samples shall be used as control.

7. Purification of antibacterial protein

Collected haemolymph shall be applied to a CM-Sephadex C-50 (20X1cm) column equilibrated in 0.3M ammonium acetate, pH 7.0, at a flow rate of 10 ml/hr. The column will be washed with the same buffer and the bound proteins will be eluted. Protein peaks will be detected by monitoring absorbance at 280 nm using a UV-Visible Spectrophotometer. Fractions shall be separately pooled, concentrated by lyophilization and kept at 4^{o}C. All the peak fractions shall be separately used to check for antibacterial activity in the next process.

8. Antibacterial Assay

Antibacterial activity shall be assayed by measuring the zone of bacterial growth inhibition in thin agar plates with isolated and purified bacterial pathogens. The zone of bacterial growth inhibition shall be measured and compared to that of control haemolymph. Serially diluted immunized and control peak fraction samples shall be applied into the wells on a thin agar plate seeded with bacteria (Hultmark *et al*, 1983).

9. Reverse Phase – High Pressure Liqued Chromatography (RP-HPLC)

The peak fractions with marked activity against bacterial pathogens will be further (final) purified by RP- HPLC using C4 column (250 x 4.6 mm) at a flow rate of 1 ml / min @ 25ºC (Shimadzu, Japan). Peaks will be detected by using λ absorbance detector at 280 nm. The protein concentration in each active fraction will be measured (Bradford,1976).

10. Electrophoresis of purified proteins

HPLC purified proteins will be confirmed by SDS and native PAGE. For SDS-PAGE, electrophoresis under reducing/non-reducing conditions (presence or absence of β-mercaptoethanol) will be performed according to the protocol of Laemmli (1970). Standard molecular weight markers will be used for comparing the molecular weight of the proteins separated from the samples.

11. Matrix-assisted laser desorption / ionization time-of-flight mass spectrometry (**MALDI – TOF MS**)

The purified active protein(s) will be digested with enzymes and subjected to MALDI -TOF MS analysis. The peptide sequence data generated from MALDI -TOF will be blasted against the protein databases for homology search.

12. Cloning of cDNA

Total RNA to be isolated from haemolymph/ fat bodies of fifth instar 3^{rd} day larvae after 10 hr of immunization with most virulent pathogenic bacteria in the region and the mRNA will be purified using a quick preparation mRNA purification kit (Pharmecia). The mRNA will be subjected to reverse transcription and the first strand cDNA will be utilized for PCR (with sense and antisense degenerative primers, designed from the amino acid sequence of the purified protein) to get partial sequence by TA cloning method. This will be followed 5' and 3' Rapid Amplification of cDNA ends (RACE)-PCR using adapter and gene specific primers and the sequence will be determined as above to get the full length sequence.

Alternate strategies

We will be using standard and proved techniques to isolate, clone and characterise the proteins. In the case of any short coming, which is unlikely, differential gene expression of silkworm races (resistant versus sucesptible) against bacteria will be done to identify the proteins upregulated in the resistant silkworm race.

17.2 Organization of Work Elements

Name of Scientists	Desig.	Time	Organization of work elements
Dr. S.Chakrabarty	Sci-C	50%	Isolation, purification and characterization of the common bacterial strain/s causing 'flacherie' disease in silkworm. Purification and characterization of the common bacterial strain (s) Pathogenicity studies and immunization. Partial purification by gel filtration followed by *in vitro* and *in vivo* assay. Complete purification of antibacterial proteins by RP-HPLC, followed by *in vitro* and *in vivo* assay. Antibacterial activity assay, Final purification to homogeneity. Testing of antibacterial protein in silkworm. Electrophoresis of proteins with simultaneous MALDI-TOF sequence determination. Analysis and compilation of final report.
Dr. G.Ravikumar	Sci-D	50%	mRNA isolation and cDNA cloning and full length gene sequence determination. Vector construction for recombinant protein expression. Recombinant protein production of antibacterial protein(s). Analysis and compilation of final report.
Shri Gourab Roy , M.Sc	JRF	100%	Bacterial cultures, silkworm rearing, infections to silkworms, a number of purification steps of antibacterial proteins using sephadex and HPLC, and antibacterial assays their infections to silkworms, electrophoresis are involved.Isolation of RNA, 1^{st} strand syntheisis, PCR, cDNA cloning which involes cutting out and ligating of PCR fragments, transformation, and plasmid purification. Vector constructions for recombinant expression, optimization, and purification of recombinant proteins, Western blotting, antibacterial assays, and sequence analysis.

17.3 **Proprietary / Patented items, if any, expected to be used for this Project**: No.

17.4 **Suggested plan of action for utilization of the expected outcome from the project:**

Antibacterial proteins are being used to combat bacterial diseases in insects including silkworms. However, not all are fully effective in controlling the flacherie disease as they have shown mixed responses. There is a possibility that new and novel antibacterial proteins are present in the silkworm genome that is yet to be identified. By challenging with *Staphylococcus vitulinus strain* ATCC51145T, the virulent bacterial strain; it may be possible to isolate such antibacterial protein(s) from the silkworm. Such a possibility will be explored in the present proposal.

17.5 **Time Schedule of activities giving milestones**

#	Organization of work/Milestone /Activity	Period of study	
		Starting	**Completion**
1	E01: Procurement of chemicals / equipments and initiation of bacterial strain (s) isolation (CSR&TI).	May , 2015	Jul, 2015
2	E02: Purification and characterization of the common bacterial strain (s). Pathogenicity studies and immunization (CSR&TI)	Aug,2015	Jan,2016
3	E03: Partial purification by gel filtration followed by *in vitro* and *in vivo* assay (CSR&TI)	Feb,2016	Apr,2016
4	E04: Complete purification of antibacterial proteins by RP-HPLC, (Out source) followed by *in vitro* and *in vivo* assay (CSR&TI)	May,2016	Jul,2016
5	E05: Electrophoresis of proteins with simultaneous MALDI-TOF sequence determination (Out source). cDNA cloning start (SBRL)	Aug,2016	Oct,2016
6	E06: Full length cloning and gene expression studies (SBRL)	Nov,2016	Apr,2017
7	E07: Vector construction for recombinant protein expression.Recombinant protein production of antibacterial protein(s) (SBRL)	May,2017	Oct,2017
8	E08: Characterization of functionally expressed recombinant protein(s) (SBRL) and their testing for antibacterial activity (CSR&TI & SBRL)	Nov,2017	Jan,2018
9	E09: Characterization of functionally expressed recombinant protein(s) (SBRL) and their testing for antibacterial activity. Submission of final report (CSR&TI & SBRL)	Feb,2018	Apr,2018

17.6 **Project Implementing Agency / Agencies**

Name of the agency	Address of the agency	Proposed Research Aspects	Proposed Amount	Cost Sharing %
Central Silk Board, Bangalore	BTM Layout Madiwala Bangalore	Silkworm Crop protection	52.80 lakh	100 %

PART-IV: BUDGET PARTICULARS

18. BUDGET (in Lakhs): [In case of multi-institutional projects, the budget details should be provided separately for each of the Institute]

TOTAL: 52.80 Lakh (CSR&TI –37.8 lakh and SBRL – 15.0 lakh)

A. Non-Recurring (e.g. equipments, accessories, etc.)
[Total 26.60 (CSR&TI- 21.1 and SBRL-5.5)]

#	Item	Year 1	Year 2	Year 3	Total (Rs in lakh)
1.	Laminar Flow Hood (CSR&TI)	1.50	-	-	1.50
2.	BOD incubator with Shaker (CSR&TI)	2.50	-	-	2.50
3.	Semi-Dry Blotter (SBRL)	3.50	-	-	3.50
4	Deep Freezer - 40º C (SBRL)	2.00	-	-	2.00
5	Deep Freezer -20º C (CSR&TI)	2.00	-	-	2.00
6	Mini Vertical Electrophoresis (CSR&TI)	0.60	-	-	0.60
7	High speed refrigerated centrifuge with rotar head (CSRTI)	4.40			4.40
8	Trinocular phase contrast microscope with image analysis software, digital live camera with PC and out put device (CSR&TI)	5.00	-	-	5.00
9	Refrigerator double door 300 L (CSR&TI)	0.60	-	-	0.60
10	Air Conditioner 1.5 Ton (CSR&TI)	0.50	-	-	0.50
11	PCR machine (CSR&TI)	4.00			4.00
	Sub total A	**26.60**	-	-	**26.60**

B) Recurring

B.1. Manpower: JRF – 01 (CSR&TI - 3.5 lakh)

#	Position No.	Consolidated Emolument	Year 1	Year 2	Year 3	Total (Rs)
1	JRF 1 No	8000+10% HRA (I & II year) 9000+10% HRA (III year)	1.60	1.60	2.00	5.20
					Total	**5.20**

B.2 Consumables (CSR&TI- 4.0 and SBRL-7.0)

#	Item	Qty	Year 1	Year 2	Year 3	Total (Rs)
1.	Molecular Biology Reagents (CSR&TI,Berhampore)		2.50	1.00	0.50	4.00
2.	Molecular Biology Reagents (SBRL, Kodathi)			3.00	4.00	7.00
Sub total						**11.0**

Molecular biology reagents are expensive. The cost will also cover sequencing, synthesis of oligos, HPLC work, MALDI-TOF sequence determination etc. that will be done through outside agencies. Indicated amount will be the minimum requirements.

Other items	**Year 1**	**Year 2**	**Year 3**	**Total (Rs)**
B.3 Contingency : SR&TI,Berhampore SBRL,Kodathi	0.75 0.50	0.75 0.50	0.50 0.00	2.00 1.00
Sub total B.3				**3.0**
B.4 Travel : CSR&TI,Berhampore SBRL, Kodathi	1.00 0.50	2.00 0.50	1.00 0.50	5.50 1.50
Sub total B.4				**7.0**
Sub-total of B (B.1+B.2+B.3+B.4)				**26.20**
Grand total (A+B)				**48.80**

Total cost of the project Rs: 52.80 lakhs
(CSR&TI – 37.80 lakh & SBRL – 15.0 lakh)

PART-V: EXISTING FACILITIES

19. Available equipment and accessories to be utilized for the project:

CSR&TI Berhampore

#	Name of the instruments	Make	Source	Year of Manufacturer
1	UV-160A UV-Visible Spectrophotometer	Shimadzu	CSB	1994
2	Vertical Gel Electrophoresis System	Bangalore GENEI,	CSB	2004
3	Water bath with orbital shaker	Digitech system	CSB	2011
4	Thermo controlled cooling system	Pharmacia	CSB	----------
5	Digital pH meter	Thermo-Orion	CSB	2005
6	Bench-top Centrifuge	Hermle	CSB	2002
7	Table-top Centrifuge	Remi	CSB	2006
8	Cooling Centrifuge C-24	Remi	CSB	2007
9	High speed Centrifuge	Sorvall	CSB	1992
10	Deep Freezer –80ºC	Thermo	CSB	----------
11	Gel Documentation unit	UVP	CSB	1997
12	Thermal Cycler	Eppendorf	CSB	2004
13	DNA Hybridization Chamber	Binder	CSB	2004
14	Steroscopic Binocular Compound Microscope	Wild Heerburgg M8, Make - Leitz	CSB	1993
15	Gel Rocker	Genei	CSB	2006
16	Satorious Water filter	Germany, 61316 RO and 611 DI	CSB	2007

SBRL, Kodathi

#.	Name of the equipments	Make	Model	Source	Year
1.	Thermal cycler (2 Nos.)	MJ Research	PTC 200	CSB	1998
2.	Thermal cycler (2 Nos.)	Eppendorf	Ep384, epgradient S	CSB	2004
3.	Gel Documentation unit	Syngene	Genegenius	CSB	2004
4.	Spectophotometer	Beckmen	DU530	CSB	2004
5.	Centrifuge	Eppendorf	5810R	CSB	2004
6.	Deep Frezer (-30°C)	Forma		CSB	1999
7.	Deep Frezer (-86°C)	Forma		CSB	
8.	Laminar flow	Microfilt		CSB	1990
9.	Tisue culture facilities, cold room, autoclave and other routine equipments				

PART VI: DECLARATION/CERTIFICATION

It is certified that

a) the research work proposed in the scheme/project does not in any way duplicate the work already done or being carried out elsewhere on the subject.
b) the same project proposal has not been submitted to any other agency for financial support.
c) the emoluments for the manpower proposed are those admissible to persons of corresponding status employed in the institute/university or as per the Ministry of Science & Technology guidelines (Annexure-III)
d) necessary provision for the scheme/project will be made in the Institute/University/State budget in anticipation of the sanction of the scheme/project.
e) if the project involves the utilisation of genetically engineered organisms, we agree to submit an application through our Institutional Biosafety Committee. We also declare that while conducting experiments, the Biosafety Guidelines of the Department of Biotechnology would be followed in toto.
f) if the project involves field trials/experiments/exchange of specimens, etc. we will ensure that ethical clearances would be taken from concerned ethical Committees/Competent authorities and the same would be conveyed to the Department of Biotechnology before implementing the project.
g) it is agreed that any research outcome or intellectual property right(s) on the invention(s) arising out of the project shall be taken in accordance with the instructions issued with the approval of the Ministry of Finance, Department of Expenditure, as contained in Annexure-V.
h) we agree to accept the terms and conditions as enclosed in Annexure-IV. The same is signed and enclosed.
i) the institute/university agrees that the equipment, other basic facilities and such other administrative facilities as per terms and conditions of the grant will be extended to investigator(s) throughout the duration of the project.
j) the Institute assumes to undertake the financial and other management responsibilities of the project.

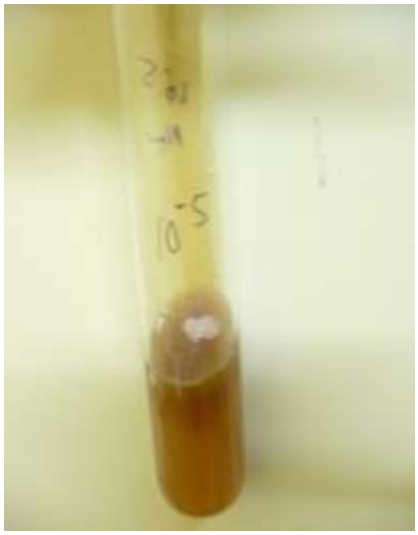
	(Dr S.Nirmal Kumar) Director Signature of Executive Authority of CSR&TI
(Dr. S.Chakrabarty) Signature of Principal Investigator-I CSR&TI,Berhampore(W.B.)	(Dr A K Saha) Scientist-D (Seri) Project Co- Ordinator - I CSR&TI, Berhampore
(Dr. G.Ravikumar) Signature of Principle Investigator – II SBRL, Kodathi	(Dr.Kanika Trivedi) Director Signature of Project Co ordinator - II SBRL Kodathi

'Flacherie' infected silkworm infected with *Staphylococcus vitulinus strain* ATCC51145T

***Staphylococcus vitulinus strain* ATCC51145T under light Microscope**

Plate contain *Staphylococcus vitulinus strain* ATCC51145T

'Flacheri' infected silkworm attacked by *Staphylococcus vitulinus strain* ATCC51145T collected from a farmer's house.



Slant contain *Staphylococcus vitulinus strain* ATCC51145T

Picture related with collection, subculture of bacteria, *Staphylococcus vitulinus strain* ATCC51145T