**RPF - III**
**(PERFORMA FOR SUBMISSION OF FINAL REPORT OF RESEARCH PROJECTS)**

Part- I: General Information

**800 Project Code** :

8001 Institute Project Code No. : **Path XVIII (813)**

8002 ICAR Project Code No.

**801 Name of the Institute and Division**

8011 Name and address of Institute : Indian Institute of Spices Research, Calicut-673012

8012 Name of Division / Section : Crop Protection

8013 Location of the Project : IISR, Calicut

**802 Project Title** : **ISOLATION AND EVALUATION OF ANTIMICROBIAL COMPOUNDS FROM BACTERIAL ENDOPHYTES AGAINST MAJOR PATHOGENS OF SPICE CROPS**

**803 Priority Area** :

8031 Research Approach :

| Applied Research | Basic Research | Process/Technology development | Transfer of Technology |
|---|---|---|---|
| ✸✸ | ✸✸✸ | ✸ | ✸ |

804 Specific Area : **Biological Control**

**805 Duration of Project** :

8051 Date of start : April 2008

8052 Date of completion : March 2011

**806 Total cost /Expenditure Incurred**
(Give reasons for variation, if any from original estimated cost)

Total cost of the project : Rs. 106.40 lakhs
Expenditure incurred : Rs. 38.64 lakhs

(As the PI had gone abroad after the first year for his higher studies, many of the envisaged purchases were not made)

**807 Executive Summary**

Biosurfactants from different endophytic bacteria were extracted and the compounds were identified as massetolide A using RP-HPLC studies. Among the eight bacteria screened, the highest production of the surfactant was noticed in *P. aerugenosa* IISR GB9 followed by IISR BP 35. *In vitro* and *in vivo* studies using this cyclic lipopeptide showed biocidal activity against *Phytophthora capsici* and *Pythium myriotylum.* The biosurfactant, massetolide A, was tested against *Radopholus similis* and was found to possess no nematicidal properties.

Strains of plant associated *P. aeruginosa* isolated from plants such as *Piper nigrum* (IISR 6, IISR 13 & BP 35), *Zingiber officinale* (IISR 51 & GEB 9) and *Chromolaena odorata* (IISR 853) were characterized for the presence gene(s) coding for surfactant and phenazine antibiotics. All the six isolates of *P. aerugenosa* were compared by phenotypic and genotypic methods. Phenotypic characters such as antibiotic resistance, swarming motility and surfactant production were documented. Sequence analysis of gene encoding DNA repair protein (*recN*) indicated that the six isolates of *P. aeruginosa* isolated from plants formed two non-overlapping clusters. The cluster I consisted of IISR6, IISR13, & IISR51 and isolates IISR853, IISRBP35 & IISRGEB9 into another cluster. Allelic profiles of seven housekeeping genes (*acsA, aroE, guaA, mutL, nuoD, ppsA,* and *trpE*) were determined through PCR amplifications and sequencing and the isolates grouped into two lineages.

In an *in vitro* test, crude culture filtrates from six bacteria (*Bacillus amyloliquefaciens* GRB 35*, Serratia marcescens* GRB 68, *Enterobacter dissolvens* GRB 70, *Micrococcus* sp. BRB 3, unidentified BRB 13 and *Serratia* sp. BRB 49) were tested for their nematicidal activity. Culture filtrates of BRB 13 at 40 µl/ml caused cent per cent mortality of *R. similis* within 24 h. Protease enzyme from *P. aerugenosa* IISR 853, extracted and purified by RP-HPLC, possessed high nematicidal activity against *R. similis*.

608 **808 Key words** **:** *Bacillus,* biosurfactant, Massetolide, multi-locus sequence typing, Oomycetes, *Phytophthora*,

*Pseudomonas, Radopholus similis*

## Part-II : Investigator Profile

(Please identify clearly changes, if any in Project personnel)

**810 Principal Investigator :**

| | | |
|---|---|---|
| 8101 | Name | : **A. Kumar (2008-09)**<br>**Santhosh J. Eapen (2009-11)** |
| 8102 | Designation | : Senior Scientist |
| 8103 | Division/ Section | : Division of Crop Protection |
| 8104 | Location | : IISR, Calicut |
| 8105 | Institute Address | : P.B. No. 1701, Marikunnu Post<br>Calicut – 673 012, Kerala |

**811 Co- Investigator :**

| | | |
|---|---|---|
| 8111 | Name | : **Santhosh J. Eapen (2008-09)** |
| 8102 | Designation | : Senior Scientist |
| 8103 | Division/ Section | : Division of Crop Protection |
| 8104 | Location | : IISR, Calicut |
| 8105 | Institute Address | : P.B. No. 1701, Marikunnu Post<br>Calicut – 673 012, Kerala |

## Par-III: Technical Details

**820 Introduction and objectives**

8201 Project Objectives :

*Immediate objectives*

1. To select strains harboring known antimicrobial gene(s) e.g. DAPG/PCA etc using PCR based (marker assisted) selection
2. To isolate cyclic lipopeptides (CLPs) from bacterial endophytes
3. To evaluate the activities of cyclic lipopeptides on different growth stages of *Phytophthora capsici, Pythium myriotylum, Ralstonia solanacearum Radopholus similis, Meloidogyne incognita in vitro*
4. To evaluate the metabolites *in vivo* for disease suppression and growth promotion of host plants
5. To purify/identify the potential metabolites using chromatography (HPLC/TLC/GC-

MS)

*Long term objectives*

To manage fungal, bacterial and nematode diseases of spice crops using biosurfactant producing bacterial antagonists

8202 Background information and importance of the projects

Microbes are source for several novel molecules that can be used in crop protection. Recent experience showed that the endophytic bacteria produce molecules that can be exploited for disease management. Existing method of biological control of plant diseases depends on the use of live cells in the formulation for field application. This method of application necessitates generation of safety guidelines (biosafety data) while releasing the biological control agents. Though several useful bacteria have been isolated during the past years, bioprospecting of microbes have not been seriously attempted. This is one of the lacunae in the exploitation of microbial wealth of our country.

Bacterial antagonists are bacteria that negatively affect the growth of other organisms. Many antagonists inhibit the growth of fungi by various mechanisms, e.g., secretion of lytic enzymes, siderophore and antibiotics. Such inhibition of fungal growth may indirectly support plant growth. Metabolites from bacterial biocontrol agents offer novel compounds for management of plant diseases. Among the metabolites, surfactants produced by microorganisms have attracted the attention in the recent times owing to its versatile functions. A variety of microorganisms, including bacteria, fungi, and yeasts, have been reported to produce biosurfactants. Several of these biosurfactants are well described chemically and categorized into high- and low-molecular-mass compounds. The low-molecular-mass biosurfactants include glycolipids and lipopeptides, such as rhamnolipids and surfactin. The high-molecular-mass compounds include proteins and lipoproteins, or complex mixtures of these polymers. Although biosurfactants are structurally diverse, they all have an amphiphilic nature, i.e., they contain both hydrophobic and hydrophilic groups. The hydrophobic moieties are usually saturated, unsaturated, or hydroxylated fatty acids or apolar amino acids, like leucin and isoleucin. The hydrophilic moieties consist of mono-, di, or polysaccharides, carboxylic acids, polar amino acids, or peptides. Among them bacterial biosurfactants produced by *Pseudomonas* spp. have been shown to present several interesting biological activities, restricting the growth of bacteria and showing zoosporicidal activity on zoosporic phytopathogens. It has been suggested that the interaction with the membrane could ultimately be responsible for these actions.

Several bacterial endophytes having anti-oomycete as well as nematicidal properties have been isolated and identified in an earlier externally funded project. Out of these, one of the

isolate *Ps. aeruginosa* BP 35, was found to secrete a biosurfactant belonging to cyclic lipopeptide in Kings B and *Pseudomonas* Agar medium that showed biocidal activity against zoospores and mycelium of *Phytophthora capsici in vitro.*

The proposed project consists of two tasks, the first of which focuses on biochemical analyses of biosurfactant production in *Pseudomonas* and *Bacillus* as well as on the activity of the identified compounds against plant pathogens. The second task focuses on synergism between biosurfactants and other metabolites of bacteria. The ultimate goal of the proposed project is to design new approaches to control Oomycetes and other plant pathogens by treatment of planting materials of spice crops and substrates with antagonistic microorganisms or their bio-active compounds.

## 821 Project Technical Profile

8211 Technical programme
(Indicate briefly plan of procedure, techniques, instruments and special materials, organisms, special environments etc.)

1. Isolation and extraction of CLPs during different growth stages of bacteria (BP35, BP25, BP17, GEB19, GEB18, GEB17, GEB13 and GEB9)

   Bacterial cultures will be obtained from the Bacterial Repository of Indian Institute of Spice Research, Calicut. Bacteria will be screened for gene(s) encoding antifungal proteins using standard protocols. The biosurfactants will be extracted as described previously by de Souza *et al*., 2003.

2. Evaluation of CLPs *in vitro* and *in vivo* against *Phytophthora capsici*, *Pythium myriotylum Radopholus similis, Meloidogyne incognita* and *Ralstonia solanacearum*

   Black pepper stem cuttings and rooted cuttings of suitable variety and ginger rhizomes will be collected from Peruvannamuzhi Farm and will be maintained in greenhouse of Indian Institute of Spices Research. *In vitro, in vivo* and *in planta* bioassays for pathogen suppression will be done by adopting appropriate methodology

3. Chemical characterization of CLPs and other metabolites

   RP–HPLC analysis will be performed to identify the biosurfactant extracted. RP-HPLC analysis will be done as described by de Souza *et al*. (2003) and de Bruijn *et al*. (2007).

8212 Total man months involvement of component project workers

| | | |
|---|---|---|
| a) | Scientific | 36 |
| b) | Technical | 18 |
| c) | Supporting | 18 |

## 822 Final Report on the Project

Detailed report containing all relevant data with a summary of results (not exceeding 2-5 pages)

8221 Achievements in terms of targets fixed for each activity

***1. Isolation of primary and secondary metabolites from endophytes***

Biosurfactants from eight endophytic bacteria viz. *Pseudomonas aeruginosa* IISR-BP35, *Pseudomonas putida* IISR-BP25, *Bacillus megaterium* IISR-BP17, *Pseudomonas aeruginosa* IISR-GEB 9, *Stenotrophomonas maltophila* IISR-GEB 13, *Enterobacter* sp IISR-GEB 17, *Klebsiella* sp IISR-GEB 18 and *Acinetobacter calcoaceticus* IISR-GEB19 were extracted. Among the eight bacteria screened, the highest production of the surfactant was noticed in *P. aerugenosa* IISR GEB9 while GEB 19 did not produce any biosurfactant. Maximum production of biosurfactant was observed in 48-72 h old culture. *P. aerugenosa* IISR BP35 produced 88-90µg of biosurfactant per ml which was inhibitory to *P. capsici*. The biosurfactants from different bacteria were identified as massetolide A using RP-HPLC studies.

### 2. PCR based screening for production of antimicrobial metabolites

Strains of plant associated *P. aeruginosa* isolated from plants such as *Piper nigrum* (IISR 6, IISR 13 & BP 35), *Zingiber officinale* (IISR 51 & GEB 9) and *Chromolaena odorata* (IISR 853) were characterized for the presence gene(s) coding for surfactant and phenazine antibiotics. Rhamnolipid production in *P. aeruginosa* has been reported to require both the *rhl* system and *rhlA* and *rhlC*. *rhlA* (1100bp) and *rhlC* (1200bp) could be detected in all six strains as well as in the reference strain, PAO1. Similarly, specific amplification of *rhlI* (377bp) and *rhlR* (266bp) gene fragments, the regulatory genes for rhamnolipid biosynthesis in *P. aeruginosa*, was obtained in a PCR assay in all the six strains as well as the reference strain, PAO1.

Role of phenazines was also confirmed by the amplification of the gene encoding phenazine biosynthesis enzyme (*phzF*) using specific primers (Ps-up1 5-ATC TTC ACC CCG GTC AAC G-3, Ps-low1 5-CCR TAG GCC GGT GAG AAC-3).

### 3. In vitro and in vivo evaluation of aqueous and organic fraction against fungal, bacterial and nematode pathogens

*In vitro* studies using this cyclic lipopeptide showed biocidal activity against zoospores and mycelium of *P. capsici*. Zoospores of *P. capsici* disintegrated on exposure to the biosurfactant. The mycelial growth and their density was also inhibited by this compound at a concentration of 50-500µg per ml. It also inhibited mycelial growth of *Pythium myriotylum* also.

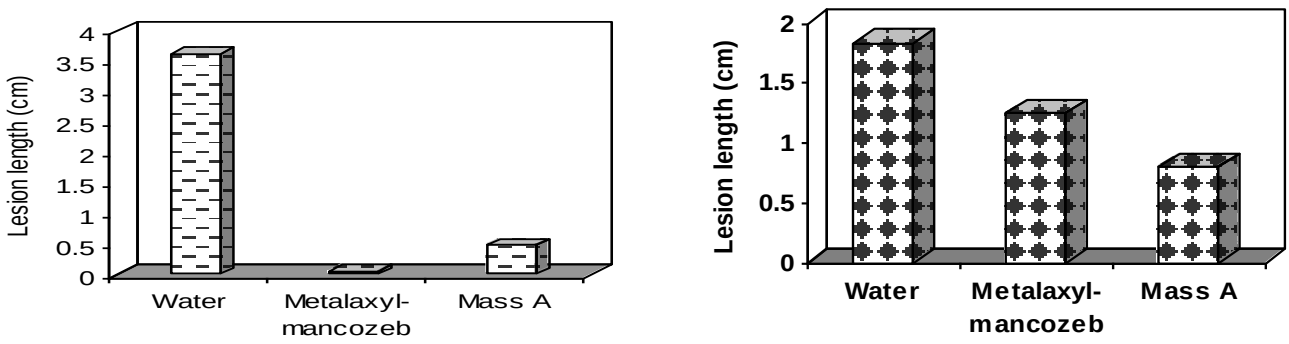


**Fig. 1.** Effect of treatment of black pepper stem cuttings with biosurfactants for protection against *Phytophthora* infection. Left – Prophylactic treatment (LSD $_{0.05}$ - 1.614) Right – Curative action

*In vivo* studies using stem cuttings of black pepper treated with the surfactant also proved its bioefficacy against *P. capsici.* Biosurfactant tested for lesion suppression on cut shoot revealed that the surfactant significantly inhibited the lesion development on stem cutting when compared to chemical (Metalaxyl- mancozeb). It was found that the prophylactic treatment of biosurfactant for 30 min showed protection against infection (Fig. 1). On dipping the *P. capsici* infected single noded black pepper cuttings for 30 min, arrested the spread of infection. Curative effect of biosurfactant was found to be superior over water and chemical control (Metalaxyl- mancozeb).

Massetolide A, a biosurfactant produced by endophytic bacteria BP 35, was tested against burrowing nematode, *R. similis*, in an in vitro bioassay. The biosurfactant was dissolved in sterile water at three concentrations viz. 0, 100 and 200 µg/ml. Two hundred microlitres of this suspension was added to 24 well microtitre plates and each treatment was replicated thrice. About 50 µl nematode suspension (containing ~10-15 *R. similis*) was added to each well. Wells containing sterile distilled water served as control. The plates were incubated at 27°C and the number of live and dead nematodes was counted after 72 h under a stereomicroscope by adding 20 µl of 1N NaOH (Chen & Dickson, 2000). The percentage mortality of nematodes observed indicated that massetolide does not possess any nematicidal properties at the concentrations tested.

Seventeen compounds produced by *Bacillus megaterium* were docked against GHF5 endo 1, 4 beta glucanase of *R. similis* out of which eight compounds showed promising results. They are 2-nonanone, 2-pentylfuran,

2-undecacone, 2, 6, 10-trimethyl-dodecane, benzene acetaldehyde, benzene ethanol, decanal and hexadecane.

Culture filtrates of six bacteria viz. GRB 35 (*Bacillus amyloliquefaciens*), GRB 68 (*Serratia marcescens*), GRB 70 (*Enterobacter dissolvens*), BRB 3 (*Micrococcus* sp.), BRB 13 (Unidentified) and BRB 49 (*Serratia* sp.) were tested for their efficacy to kill *R. similis*. Except BRB 13, none of the isolates showed any nematicidal activity.

Protease enzyme present in crude extracts of *P. aerugenosa* IISR 853 was purified by RP-HPLC and tested against *R. similis* for its nematicidal activity. Fraction 2 was found to contain maximum enzyme and nematicidal activities.

### *4. Genotypic characterization Pseudomonas aerugenosa*

All the six isolates of *P. aerugenosa* were compared by phenotypic and genotypic methods. Phenotypic characters such as antibiotic resistance, swarming motility and surfactant production were documented. All the six plant associated *P. aeruginosa* exhibited a characteristic round, 'moving away from middle' like growth pattern with remarkable accumulation of biomass within 12-16 hour of incubation at 37°C on soft KMB agar (0.6%). Besides they showed intrinsic antibiotic resistance against kanamycin.

PCR amplification with primers specific for gene encoding DNA repair protein (*recN*) in *P. aeruginosa* yielded partial *recN* gene sequences (937bp) from all the six strains which had 99% similarity with three of the fully sequenced strains such as PAO1, PA14 and LESB58. Based on the multiple alignments of sequences, a phylogenetic tree was constructed by UPGMA which was subjected to 1000 bootstrap trials. The analysis indicated that *P. aeruginosa* isolated from plants formed two non-overlapping clusters. The cluster I consist of IISR6, IISR13, & IISR51 and isolates IISR853, IISRBP35 & IISRGEB9 into the other cluster.

PCR amplification and sequencing of seven housekeeping genes (*acsA, aroE, guaA, mutL, nuoD, ppsA,* and *trpE*) was performed and compared to the data available in the MLST database for *P. aeruginosa* (http://pubmlst.org/paeruginosa). The allelic profiles served an input data to analyze the strain relatedness using eBurst, a novel clustering algorithm designed for MLST data. The P. aeruginosa isolates obtained from plants were grouped into two lineages as in the previous analysis. Allelic profiles of

the strains Pa-IISR6, Pa-IISR13 and Pa-IISR51 matched with sequence type 760, a strain documented from human sputum in China. The strain Pa-BP35, Pa-IISR853, Pa-GEB9 are single locus variant of ST575, it does not have any double locus variants and has 15 other three locus variants in the population. Sequence Type 575 is a strain documented from disease (human) habitat from Utrecht Medical Center, Utrecht, The Netherlands.

8222 Questions- Answered

1. Do spice associated bacteria secrete biosurfactants?
   Yes
2. What is the chemical nature of the biosurfactant?
   A cyclic lipopeptide, massetolide A
3. Whether they suppress the growth of the pathogens of spice crops?
   In vitro and in vivo studies proved their efficacy against oomycetes.
4. Do they work against nematodes?
   No. In vitro studies showed that they are not effective against nematodes. However, crude metabolites some of these bacteria showed nematicidal properties.
5. Do they work against bacterial plant pathogens?

   No. More studies required.

8223 Process/ Product/ Technology/ Developed

Massetolide A, the cyclic lipopeptide, secreted by endophytic bacteria can be developed as an effective chemical for controlling *P. capsici*

8224 Practical Utility
(not more than 150 words)

Several bacteria (*Pseudomonas aerugenosa*, *Acinetobacter calcoaceticus* etc.) having antimicrobial activity could be isolated and made available. Most of the *P. aerugenosa* strains produce Massetolide A, a cyclic pepetide, having very high anti-oomycete activity. Biocontrol technology based on biosurfactant producing bacteria can be evolved against *Phytophthora.* Bacterial metabolites having very strong nematicidal activity were also identified through this study.

8225 Constraints, if any

Nil

**823 Publications and Material Development**

(One copy each to be supplied with this proforma.)

8231 *Research papers*

a. Aravind, R., Kumar, A., Eapen, S.J. and Ramana, K.V. 2009. Endophytic bacterial flora in root and stem tissues of black pepper (*Piper nigrum* L.) genotype: isolation, identification and evaluation against *Phytophthora capsici*. *Letters in Applied Microbiology* 48: 58–64.

b. Aravind, R., Eapen, S.J., Kumar, A., Dinu A. and Ramana, K.V. 2010.Screening of endophytic bacteria and evaluation of selected isolates for suppression of burrowing nematode (*Radopholus similis* Thorne) using three varieties of black pepper (*Piper nigrum* L.). *Crop Protection* 29: 318–324.

*Papers presented in Symposia/Seminars*

a. Kumar A. Aravind R, Rajina A S, Joseph T, Adithya V, Eapen S J and Vipina V 2008 Endogenous movement of *Pseudomonas aeruginosa* IISR BP35 in *Piper nigrum* L. *vis-vis* its density and duration dependent protection against *Phytophthora capsici* infection. National Seminar on Piperaceae, 21-22 November 2008, IISR, Calicut.

b. Kumar A. Aravind R, Priyanka A, Eapen S J and Vinod V 2008 Massetolide A: A zoosporicidal biosurfactant produced by black pepper associated endophytic bacterium *Pseudomonas aeruginosa* IIISR BP35. National Seminar on Piperaceae, 21-22 November 2008, IISR, Calicut.

c. Dinu Antony, S. Balaji, K.V. Asha, Joyal Joseph, R. Aravind and S.J. Eapen 2010. Molecular characterization of burrowing nematode (*Radopholus similis*) population from India using ITS-PCR. National Conference on Innovations in Nematological Research for Agricultural Sustainability – Challenges and A Roadmap Ahead, 23-25 February 2010, TNAU, Coimbatore.

d. Kumar A., Raaijmakers J.M. and Eapen S.J. (2010). Multilocus Sequence Typing of tropical plant-associated *Pseudomonas aeruginosa*: a genotypic tool for selection of biocontrol strains. In Symposium on Changing Plant Disease Scenario in Relation to Climate Change. 22-23 October 2010, Indian Phytopathological Society (Southern Zone), IISR, Calicut.

8232 *Popular articles*

a. Pervez, R. and Eapen, S. J. 2011. Kalimirch mein sutrkirmiyoon ki samsya avam samadhan. Phal aur Phool, 21-23. (In Hindi)

b. Pervez, R., Eapen, S.J. and Devasahayam, S. 2010. Keetnashak sutrakrimi: saflta ki kunzi. Malabar Jyoti, pp. 37-38. (In Hindi)

8233 *Reports*

a. Santhosh J. Eapen 2008. Endophytic Bacteria for the Biological System Management of *Radopholus similis*, the Key Nematode Pest of Black Pepper (*Piper nigrum* L.) - Final Report of the Project. Indian Institute of Spices Research, Calicut, India. 93 pp.

*M. Sc. Project Report*

a. Anju Philip 2008. Inhibition of *Radopholus similis* by extracellular proteases from nematode antagonistic bacteria. School of Biotechnology, VIT University, Vellore, T.N. 61 pp.

*Books*

a. Santhosh J. Eapen, Kumar A. and Anandaraj M. (Eds.) 2008. *Plant Pathogens and Their Biocontrol Agents – Diagnostics and Characterization*. Indian Institute of Spices Research, Calicut, Kerala. 262 pp.

8234 Seminars, conferences and workshops (relevant to the project) in which the scientists have participated. (List abstracts forwarded)

1. National Seminar on Piperaceae, 21-22 November 2008, IISR, Calicut.
2. National Conference on Innovations in Nematological Research for Agricultural Sustainability – Challenges and A Roadmap Ahead, 23-25 February 2010, TNAU, Coimbatore.
3. Symposium on Changing Plant Disease Scenario in Relation to Climate Change. 22-23 October 2010, Indian Phytopathological Society (Southern Zone), IISR, Calicut.

**824 Infrastructural facilities developed**

(Details of field, laboratory, note books and final material and their location)

- BIOLOG Microbial Identification System

825 Comments / Suggestions of Project Leader regarding possible future line of work that may be taken up arising out of this Project.

The project will be continued as an externally aided project to investigate further on the biochemical profiles of these promising bacteria. As *Pseudomonas aerugenosa* is a known human pathogen, we need to shift our focus to the metabolites produced by these highly effective microorganisms than organisms

*per se*. Structural and functional analysis of genes/gene clusters of these potential metabolites needs to be undertaken with active collaboration with other groups having expertise in the relevant subject domains. The leads obtained can be further validated and scaled up through such a multi-institutional project.

**Part-IV: Project Expenditure**
(Summary)
Year 2008-2011

**830 Total Recurring Expenditure (Rs. In lakhs)**

| | | Estimated | Actual |
|---|---|---|---|
| 8301 | Salaries: (Designation with pay scale) | | |
| | i) Scientific | 6.00 | 7.15 |
| | ii) Technical | 1.50 | 0.80 |
| | iii) Supporting | 0.75 | 0.00 |
| | iv) Wages | 0.30 | 0.00 |
| Sub-Total | | 8.55 | 7.95 |
| 8302 | Consumables | | |
| | i) Chemicals & Glasswares | 1.50 | 1.10 |
| | ii) Others | 0.75 | 0.30 |
| Sub-Total | | 2.25 | 1.40 |
| 8303 | Travel | 0.30 | 0.00 |
| 8304 | Miscellaneous (other costs) | 0.30 | 0.00 |
| 8305 | Sub-Total (Recurring) | 11.40 | 9.35 |
| **831** | **Total Non – Recurring** Expenditure (Equipments and works) | | |
| | i) High capacity flash evaporator | 3.00 | - |
| | ii) High capacity vacuum concentrator | 5.00 | - |
| | iii) Transilluminator with epiUV | 2.00 | - |
| | iv) Biolog | 20.00 | 29.29 |
| | v) Other equipments | 65.00 | - |
| **823** | **Total** (830 and 831) | 106.40 | 38.64 |

## Part-V : Declaration

This is to certify that the final report of the Project has been submitted in full consultation with the Project workers as per the approved objectives and technical programme and the relevant records, note-books, materials are available for the same.

Signature of the Project Investigator:

Co-Investigators: Nil

Signature & Comments of the Head
Of the Division/ Section

Signature & Comments of the
Joint Director (Research)

Signature & Comments of the
Director