Chapter 3

Materials and Methods

SECTION I: *MICROBIOLOGICAL METHODS*

3.1 Isolation of microorganisms

3.1.1 Source material and collection of samples

The microbes were isolated from water, soil and biological fluids. Water and soil samples were collected from different ecological locations for isolation of bacteria and fungi whereas biological fluid used was human urine from urinary tract infection (UTI) patients for isolation of bacteria. Due care was taken to ensure that the sample collection points were widely varying with regard to geographical distribution. All samples were collected in sterile screw capped bottles. A total of 69 bacterial samples, including soil, water and human urine, and 12 fungi samples from water and soil have been collected.

3.1.2 Isolation of microorganisms

Serial dilutions of samples were prepared separately with sterile saline. Suitably diluted samples were pour plated using SDA for fungi and LB agar for bacteria. Upon incubation for 2-3 days, the fungal colonies which had grown were isolated, sub-cultured and preserved in Czapek dox agar slants. Similarly bacterial cultures were maintained on LB agar. Pure isolated cultures were preserved at -80^0C in 10% glycerol for fungus and 15% glycerol for bacteria.

3.2 Screening of microbes for production of HMGR inhibitors:

3.2.1 Preparation of inoculum

Fungal spore suspensions using 5 mL sterile water containing 0.1% Triton X-100 were prepared from fully sporulated slants. The count of spores was adjusted to 5×10^6

spores/mL by suitable dilution. Spore count was performed with cell counting haemocytometer. 10% v/v spore suspension was added in Erlenmeyer's flask containing Czapek Dox broth. The flasks were kept in rotary shaker and agitated at 150 rpm for 48 h at 28ºC. After incubation, the cell mass was collected by centrifugation at 1500 rpm for 15 min, washed twice with sterile water and suspended in 10 mL of water.

Discrete bacterial colonies were isolated from the master plates; and revived in 10 ml autoclaved LB medium. Bacterial cell count was done by reading OD at 600nm.

3.2.2 Submerged fermentation process for production of HMGR inhibitors

Fungus *Aspergillus sp* (AC) identified from unexploited coastal region has been used to check for the production of statins as their secondary metabolites in fermentation reaction. The glycerol stocks which are available for above strains were plated on Sabaroud Dextrose Agar (SDA) plates and kept in the incubator for 3 days at 28°C. After the incubation, the spores which are formed on the SDA plates are removed using a 0.5% tween 80 solution and collected in 15 ml centrifuge tube. Then the spores were preserved using 60% glycerol and stored at -20°C.

Collected spores were inoculated in 100 ml of production medium (Czapek dox medium, pH 7.0) (Hi-Media) and kept in the shaking incubator for 7 days at 180 rpm at 28°C.

Biotransformation reaction for bacterial culture:

10 ml of bacterial culture with O.D > 0.5 was taken and pellet down at 4000 rpm for 10 min. 2 ml of fresh LB media was added along with MJ (Monacolin J) 133ul (15mM) and DMB 17ul (25mM). The reaction mixture was kept for shaking incubation at 37°C for 24 hours.

3.2.3 Downstream processing of Statins using solvent-solvent extraction method

For fungal culture:

At the end of 7th day, the fermentation broth was acidified to pH 3.0 with 10% HCl. Then equal volume of ethyl acetate was added and kept for shaking in incubator at 28°c for 2 hours. Wattman paper (10 mm to 500 mm diameter circles and 460 mm x 570 mm sheets) was used to separate out the spores from the reaction suspension. The culture supernatant was subsequently centrifuged at 3000 x g for 15 min. The organic phase was collected in a fresh tube and allowed to dry and concentrated using a rotary evaporator. The residue obtained was dissolved in methanol and used for estimation and analysis of statin molecules.

For bacterial culture:

After 24 hours of incubation, the fermentation broth was acidified to pH 3.0 with 10% HCl. Then equal volume of ethyl acetate was added and kept for shaking in incubator at 37°C for 2 hours. The extract from the bacterial samples were subsequently centrifuged at 3000 x g for 15 min and the organic phase was collected in a fresh tube. The solvent portion collected is allowed to dry and concentrated in vacuum evaporator and residue obtained was dissolved in methanol. Extract collected was used for estimation and analysis of statin molecules.

3.3 Biological methods for characterization of statins

3.3.1 Yeast growth inhibition bio-assay agar plate method

Revival of yeast (*Saccharomyces cerevisiae*): To 0.25gm weigh yeast powder 4ml of luke warm water was added and kept in dark place for fermentation at 28°C for 15 min.

YEPD media preparation:

Components	Quantity/100ml
Agar-agar	1.5gm
Yeast extract	0.5gm
Dextrose	2gm
Peptic Extract from animal tissue	1gm

Yeast growth inhibition bioassays were performed by seeding 20ml of YEPD (Yeast Extract Peptone Dextrose) medium with 0.25 ml of yeast (*Saccharomyces cerevisiae*) suspension. Yeast inoculated medium was poured into a 15 cm diameter glass petri dish. After solidifying, 6 mm diameter wells were made with the aid of a sterile cork borer. 30µl of each of the ten-fold concentrated fungal extract samples were added to these wells. Ethyl acetate and standard solution of lovastatin were used as negative and positive controls respectively. Plates were incubated at 26°C for 42-48 hours and afterwards the inhibition zones were scored.

3.3.2 Yeast growth inhibition bio-assay- effect on growth rate

For testing growth in liquid cultures, inocula of *S. cerevisiae* were added to liquid minimal media supplemented with one of the statins/HMGR inhibitors or buffer as a negative control. Cultures were grown with shaking at 30°C and OD600 was measured every two hours. Each culture was assayed in triplicate and the results were averaged. Based on OD600 values growth curves of each culture were plotted.

3.3.3 Triglyceride Release Assay:

Cells (1×10^5) were plated in 35 mm culture dish and treated with SK-02 and SV for 48

hours. Cells were lysed in PBS containing 2% Triton X-100 for 10 min. After centrifugation (12,000 rpm, 15 min), resulting supernatant was used for triglyceride estimation. Triglyceride was estimated by using kit with sensitivity range up to 800 mg/dL purchased from Accurex as per the manufacturer's instructions.

3.3.4 Cytotoxicity assay- MTT (methylthioazole tetrazolium) assay

MTT assay measures the cell viability and proliferation and forms the basis of numerous in vitro assays of a cell population's response to external factors. MTT [3- (4,5-dimethyl thiazol-2-yl)-2,5,-diphenyl tetrazolium bromide] is based on the mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium of the pale yellow MTT and form a dark blue formazan crystals which are largely impermeable to cell membranes, thus resulting in its accumulation in healthy cells. Solublization of the cells by the addition of detergent results in the liberation solublized crystals. The number of surviving cells is directly proportional to the level of formazan product formed. The colour is quantified using simple colorimetric assay. Protocol followed for MTT assay is briefly described below:

- After 48 hours of incubation of cells, the media was removed.
- Fresh media (100 µl) which contained 20 µl MTT (5mg/ml) was added and incubated for 4 hours.
- The crystals formed were dissolved by adding 100 µl of DMSO
- The clear supernatant obtained was read at 570nm.

3.4 Identification of Microbes of Interest

The isolates were used to grow new colonies on the same media to ensure purity of isolated bacterial/fungal strains. Bacterial inoculums were then prepared by suspending the freshly-grown bacterial colonies in 10 ml sterile LB and incubated at 37°C, which were then used for Gram staining as well as inoculated in both HI chrome UTI agar and MacConkey agar plates followed by incubation at 37°C for 24-48 hours for bacterial identification based on specific metabolism of chromogenic substrates. Himedia rapid biochemical identification kits were used in the present study as the comprehensive identification system. Kit was opened aseptically and sealing tape was peeled off. Each well was inoculated with 50µl of the above inoculum by surface inoculation method and kept for inoculation at 35-37°C for 18-24 hours. At the end of the incubation period, a series of reagents were added in designated wells as per manufacturer's specifications to carry out different biochemical tests. Fungal inoculums were prepared by suspending the freshly-grown fungi in 10 ml sterile potato dextrose media and incubated at 28°C, which were then used for lactose cotton blue staining for fungal identification based on specific staining. Further the cultures were sent to ARI, Pune for complete identification.

SECTION II: *ANALYTICAL METHODS*

3.5 Analytical methods for characterization of statins:

3.5.1 UV Spectrophotometric analysis

Prepared sample were analyzed for the presence of statin at different wavelength (200 nm-300 nm) in triplicate. Absorbance of samples with different dilution was calculated along with standard (lovastatin). Dilutions were prepared in methanol and methanol was also taken as blank for subsequent readings. Absorbance reading was taken with scanning

wavelength spectrophotometer in the range of 200-300nm UV/Visible spectrophotometer (DU® 730 Beckman Coulter).

3.5.2 TLC (Thin layer chromatographic)

Thin layer chromatographic plates pre-coated with silica gel-G (Merck & Co.), 200 mesh and 1 mm thickness were used. A quantity of 10µl of the diluted crude extract in methanol was spotted along with standard. The plates were developed in a solvent system of dichloromethane:ethyl acetate, 70:30 v/v (Hi-Media). For Each plate took around 40 minutes for completing the run. Individual spots on the TLC plates were marked as observed under UV light and Rf values were calculated..

3.5.3 HPLC (High-performance liquid chromatography)

HPLC is a chromatographic technique used to separate a mixture of compounds in analytical chemistry and biochemistry with the purpose of identifying, quantifying or purifying the individual components of a mixture.

Chromatography can be described as a mass transfer process involving adsorption. HPLC relies on pumps to pass a pressurized liquid and a sample mixture through a column filled with a sorbent, leading to the separation of the sample components. The active component of the column, the sorbent, is typically a granular material made of solid particles (e.g. silica, polymers, etc.), 2-50 micrometers in size. The components of the sample mixture are separated from each other due to their different degrees of interaction with the sorbent particles. The pressurized liquid is typically a mixture of solvents (e.g. water, acetonitrile and/or methanol) and is referred to as "mobile phase". Its composition and temperature plays a major role in the separation process by influencing the

interactions taking place between sample components and sorbent. Components and conditions on Shimdzu instrument used are described below:

Table 3.1: Conditions for High performance liquid chromatography

Components	Conditions
Column	150X4.6 mm, 5µ
Flow rate	1ml/min
Injection Volume	20 ul
Mobile Phase	ACN : H_2O (70:30)
Column temperature	35°C
Wavelength	238nm

3.5.4 Infrared Spectroscopy (IR)

Weighed amounts of sample, approx 2 mg, and KBr powder, approx. 300 mg was transferred into an agate mortar. The KBr powder used wasof spectroscopic grade purity and spectroscopically dry. The powders were ground together, with an agate pestle, until the sample was well dispersed and the mixture developed a consistency of afine flour. The die was assembled, with the lower pellet polished face up. The ground mixture was transferred into the cylinder bore so that it was evenly distributed across the polished face of the lower pellet. A flat, even surface was achieved by gently inserting the plunger and lightly swiveling. The second pellet was inserted, polished face towards the mixture, into the bore followed by the plunger. The die assembly was placed into a hydraulic press, between the ram and the piston ensuring that the die was firmly held in the press. A vacuum tube was connected and the High Vacuum pump switched on. The die assembly was left under vacuum for approximately 2 min for removing air from the disk. The pressure was increased in the press to 10-15 tons (as per the manufacturer's instructions

for max pressure for a given die). After approximately 1 minute, the pressure was slowly released followed by careful release of the vacuum and removal of the die from the press. After dismantling the die, the KBr disk was transferred to a spectrometer disk holder without touching the faces of the disk. It was ensured that the disk is translucent and that the sample is homogeneously distributed in the disk. The disk was mounted on holder in the spectrometer to obtain the spectra.

3.5.5 Mass Spectroscopy (MS)

Mass spectra were obtained using Shimadzu LC/MS-MS mass spectrometer in positive ion ionization mode.

SECTION III: *MOLECULAR BIOLOGY METHODS*

3.6 Screening for the presence of acyltransferase coding genes

3.6.1 Fungal DNA Isolation

Solutions and Reagents

- CTAB
- Tris pH 8.0
- EDTA pH 8.0
- SDS
- Proteinase K
- Sodium Chloride (NaCl)
- Sodium Acetate
- Isopropanol
- Chloroform–isoamyl alcohol (24:1, v/v)

Extraction buffer: 100mM Tris, 10mM EDTA and 2% SDS

Procedure:

1. 50 milligram of lyophilized mycelium was placed in a clean cold mortar and liquid nitrogen was added to keep the material frozen and brittle.
2. The material was then ground to a very fine powder with a pestle and later the contents were transferred to a 50 ml polypropylene centrifuge tube.
3. 500 µl of pre warmed (at 60^{o}C) extraction buffer was added to the tube followed by addition of 50 µg proteinase K and shaken vigorously.
4. The tube was incubated at 60^{o}C for one hour.
5. Afterwards, the tube was cooled to room temperature, and 140 µl of 5 M NaCl, one tenth volume of 10% w/v of CTAB were added sequentially and well mixed after each addition.
6. The tube was incubated for 10 min at 65^{o}C, and then equal volume of CA was added.
7. The tube was centrifuged at 12,000g for 10 min at 4^{o}C.
8. The supernatant was transferred to another new tube and 0.6-fold volumes of iso propyl alcohol was added and mixed thoroughly for precipitating the DNA overnight at -20^{o}C.
9. Subsequently the DNA was pelleted at 12,000g for 30 min at 4^{o}C, washed with 70% ethanol twice, air dried in a vacuum, re-dissolved in 100 µl double distilled autoclaved water, and stored at -80^{o}C for further use.

3.6.2 Bacterial DNA isolation

Solutions and Reagents

- SET buffer
- Tris saturated phenol

- EDTA pH 8.0
- 10% SDS solution
- Proteinase K
- Lysozyme
- 5 M Sodium Chloride (NaCl)
- Sodium Acetate
- Isopropanol
- Chloroform–isoamyl alcohol (24:1, v/v)

SET buffer: 50mM Sucrose, 10mM EDTA and 25 mM Tris-Cl

Procedure:

1. Incubate culture with shaking overnight.
2. Take 3 ml culture in 1.5 ml eppendorf tube (in two rounds of 1.5ml each) and harvest cells by centrifugation at 6000 rpm for 10 min.
3. Wash in SET buffer and resuspend cells by gentle tapping followed by centrifugation at 6000 rpm for 10 min.
4. Carefully decant the supernatant and resuspend the pellet in 500 µl of SET buffer.
5. Add lysozyme at a final concentration of 1 mg/ml. Incubate at 37^{o}C for 30 min.
6. Add one tenth volume of 10% w/v of SDS solution and proteinase K at the concentration of 100 µg/ml.
7. Incubate at 55^{0}C for 90-120 min with occasional inversion at regular interval of 20 min.
8. Add 1/3 vol of 5 M NaCl and equal volume phenol:chloroform:iso amyl alcohol (25:24:1).
9. Mix properly by inversion, incubate at room temperature for 30 minute with

frequent inversion.

10. The tube was centrifuged at 10,000g for 10 min at 4^{o}C.
11. The supernatant was transferred to another new tube, 2-fold volume of absolute ethanol, added and mixed thoroughly for precipitating the DNA overnight at -20^{o}C.
12. Subsequently the DNA was pelleted at 10,000g for 15 min at 4^{o}C, washed in 70% ethanol twice, air dried under vacuum, re-dissolved in 100 µl double distilled autoclaved water and stored at -80^{o}C for further use.

3.6.3 Assessment of DNA Quality and Quantity:

DNA concentration and purity were measured using a NanoDrop Spectrophotometer (Biolab ND-8000). The concentration of purified DNA was determined by measuring the absorbance at 260 nm and 280 nm in a spectrophotometer. The reading at 260 nm allows for the calculation of the nucleic acids concentration in the sample. The ratio of the absorbance readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have OD_{260}/OD_{280} of 1.8 and 2.0 respectively. A ratio of the readings at 260 nm and 230 nm (OD_{260}/OD_{230}) can aid in evaluating the level of solvent carryover in the purified nucleic acid. As a guideline, the OD_{260}/OD_{230} is best if it is greater than 1.5.

3.6.3.1 Materials Used

1.5 ml microcentrifuge tubes, tips and Milli Q water for dissolution, Kim wipes

Equipment: Nanodrop -8000 (UV spectroscopy).

3.6.3.2 Procedure

1. 2 µl of purified genomic DNA sample was diluted with 8 µl of water (1/5 dilution).
2. Each receptor (8) of Nano drop was first cleaned and wiped with Kim wipes.
3. The system was then set blank with autoclaved Milli Q water by loading 2µl of water on to each receptor.
4. 2µl of each of the diluted genomic DNA sample was loaded with the help of 2-20µl multichannel micropipette on to the receptor platform of Nano drop.
5. The absorbance of the diluted sample was then measured at 260, 280, 230 nm

3.6.4 Molecular genetic analysis of acyltransferase coding gene

3.6.4.1 Sequence of the acyltransferase gene and primer synthesis

Sequences for acyltransferase coding LovD gene was obtained from databases such as NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unis). Primer sequences were synthesized by Sigma-Aldrich and procured from Hychem, India. The primer sequences used are:

Table 3.2: Primer sequences for lovD gene amplification

Primer Code	Primer sequence-5'-3'	PCR amplicon
LV-400	LV-F: TCTGTCTTAGATGGATGAATGG LV-1R: GGGATGGATGGAAAAGATTC	399 bp
LV-1000	LV-2F: AGGAACAAAGTCTCATTCTCTTG LV-R:ACAGATATCATTTACAACACATGTG	998 bp

3.6.4.2 Amplification by polymerase chain reaction (PCR)

Amplification was carried out on eppendorf thermal cycler in a final volume a 10 µl

reaction. Reaction cocktail for each sample contained a total of 2.5 µl of 4X cocktail mix (4X-CT), 2.0 µl (0.5-1.0µg/ul) of genomic DNA, 0.15 µl (5U/µl) of Sigma Taq DNA Polymerase, 6.7% DMSO and the final volume was made up to 10 µl using ultra-pure water. The basic composition of the 4X-CT for each mix is listed in Table 4.4. PCR cycling conditions are amplification of markers in duplex is summarized in Table 4.5. The PCR tubes were maintained at 4ºC during dispensing of the master mix.

Table 3.3: Composition of basic 4X cocktail for each mix for 100 reactions

Components (for 100 reactions)	**4X CT**
10X PCR Buffer (Sigma)	31.3µl
10mM dNTPS	20 µl
Forward Primer	1 µl
Reverse Primer	1 µl
Ultrapure Water	197 µl
Total	250 µl

The final concentration of salts or reagents in the 10 µl reaction mixture was 1.5mM $MgCl_2$, 200µM of dNTP's mix, 1 picomol/ µl each of forward and reverse primer.

Table 3.4: PCR amplification conditions are:

Steps	**Conditions**
Initial Denaturation	95ºC-5'
Denaturation	94ºC-1'
Annealing	66ºC-1.5'
Extension	72ºC-5'
Cycles	40 cycles
Final extension	72ºC-8'

3.6.5 Agarose gel electrophoresis

PCR products were checked for product by electrophoresis on 1.5% agarose gel to confirm the amplification and its quality.

3.6.5.1 Materials Used

250 ml conical flask, Tips and Milli Q water for dissolution, Tris base (Trizma, Sigma-Aldrich, USA), Boric acid (Himedia), EDTA, Ethidium bromide (EtBr), SRL, India, Agarose; Sigma-Aldrich, USA

3.6.5.2 Reagents and working solutions

5X TBE: 0.09M tris-borate, 0.002M EDTA

50X TAE: 0.04M tris-acetate, 0.001M EDTA

6X loading dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% v/v glycerol

Ethidium Bromide: 10mg/ml

3.6.5.3 Agarose gels for resolving PCR products and quality check of genomic DNA/purified PCR products

Agarose gels (1.5- 2.0% w/v agarose; Sigma-Aldrich, USA) were prepared by dissolving the required quantity of agarose in 1X TAE/0.5X TBE electrophoresis buffer by heating in a microwave oven until the solution became transparent, followed by addition of 0.5 µg/ml ethidium bromide. The agarose solution was poured into a gel casting tray containing a comb (8 well or 15 well as per requirement) and was allowed to cool for solidification. Following this the tray was placed in an electrophoresis tank and submerged in 1X TAE/0.5X TBE buffer. DNA samples were mixed with 6X loading buffer and loaded on the gel along with DNA marker mix (MBI Fermentas, Lithuania). Horizontal electrophoresis was carried out at approximately 80-100V for ~2 hrs. DNA fragments were detected by visualizing in a UV trans-illuminator (Alphaimager, Alpha Innotech Corporation, USA).

3.7 Effect of HMGR inhibitors on the mevalonic acid pathway in recombinant yeast

strains expressing human HMGR

The MB10-1D yeast strain (carrying the human HMGR gene) was grown at 28^{o}C for 24 hours in 10-ml liquid minimal medium supplemented with histidine, uracil, lysine and the appropriate volume of each inhibitor. The corresponding volume of methanol was used as a control. SK-03 and SK-04 were tested in duplicates. Optical density at 600 nm was measured after 24 hours of growth. Total RNA was isolated from yeast cells using the MagNA Pure Compact RNA Isolation Kit (Roche). Reverse transcription (RT) was performed using the QuantiTect Reverse Transcription Kit (QIAGEN). RT-qPCR amplification was performed using a LightCycler 1.5 and LightCycler FastStart DNA Master SYBR Green I (Roche). Data normalization was carried out in relation to the transcript of the housekeeping 35S rRNA gene. The sequences of primers used and the qPCR parameters are detailed in table below:

Table 3.5: PCR conditions for HMGR gene expression in recombinant yeast

Gene	Primer Sequence	Annealing Temp.	Product Size
HMGR	F: 5′-ATGCTCACAGTCGCTGGATA-3′ R: 5′-ACAGCCAGAAGGAGAGCTAA-3′	61^{o}C	199bp

SECTION IV: *BIOINFORMATIC APPROACHES*

In-silico tools have been used for analyzing sequence-structure-function dependencies to better discern the function of genes and proteins under study. Since the structure of a protein determines what function it can perform, knowledge of structure is useful in predicting protein function. To find out the fungal analog in bacterial genome, we translated the whole bacterial genome using the translate tool of EXPASY tools. The bacterial proteome obtained after translation was searched for the presence of amino acid

sequence similar to fungal lovD protein. The sequence thus obtained was used in BLAST for validating the presence of a particular protein in bacteria of interest. Based on the BLAST results the structure was retrieved from PDB database, PDB Id: 3CJM-beta-lactamase. Similarly structure for fungal lovD was taken from PDB database, PDB Id: 3HLD. To understand the structural relationship between fungal and bacterial protein, structural alignment was performed using SALIGN. The RMSD between the aligned structures was calculated using CHIMERA online tool. The RMSD of two aligned structures indicates their divergence from one another. A correlation was observed between functional similarity and RMSD between the pairs of proteins studied. Further phylogenetic tree for bacterial protein and these two fungal proteins was generated using MEGA 6 phylogenetic analysis software to find evolutionary relationship among them.