MATERIALS AND METHODS

3.1 COLLECTION OF SOIL SAMPLES

The occurrence and distribution of soil mycoflora invariably depends on the soil environment that proper soil sampling should be done from different places at a well-defined depth of the collection site for effective isolation of soil saprophytes as well as for accurate soil analysis.

3.1.1. SITES CHOSEN FOR STUDY AND LOCATION:

The following sites (Fig. 3) variably polluted by distinguishing pollutants were chosen for the present study to mark the impact of stress induced upon the environment:

i) Plastic/garbage dumpsite at Pallikaranai

Pallikaranai Marsh Land (PML) (12°56’15.72”N: 80°12’55.08”E) is located 20 kms South of Chennai with an area of 50 sq. km. PML is surrounded by Old Mahabalipuram in the East, Tambaram-Velachery Road in its West, Pallikaranai-Perumbakkam road in the North, and Medavakkam-Karapakkam road in the South. PML has an average annual temperature 29°C, governed under Kancheepuram district in South Chennai. The site is highly dumped with plastic/garbage of nearly 4000 tonnes by the Chennai Corporation and Alandur Municipality.

ii) Chemical effluent site at Madurantakam

The site was at the Sathammai Village (12.5101°N 79.8849°E) Karunkhuzhi Post, located 77 km South West of Chennai, has an average temperature of 30°C. The site is extremely miserable due to the pharmaceutical industry discharging its untreated effluents contaminating the nearby water bodies, affecting the ground water and also the cultivable lands. It is governed by the Madurantakam Taluk in Kancheepuram district.
i) Plastic/garbage dumpsite at Pallikaranai

ii) Chemical effluent site at Madurantakam

Fig. 3 Sites chosen for the study
iii) Tannery affected site in Vellore

Ranipet (12.9275°N 79.3302°E), in Vellore is located 100 Km from Chennai, the fourth largest urban area in India. Ranipet has an average annual temperature 32°C, governed under Walajapet taluk, Vellore district. The site is highly polluted with tannery effluent discharges from the nearby industries resulted the land uncultivable.

iv) Garden soil (Control) from Chennai

Samples of garden soil were obtained from Bharati park in Selaiyur (12.93°N 80.14°E) located in Chennai, has an average annual temperature of 32°C, governed under Tambaram taluk, Kancheepuram district. The site is free from pollution and waste disposal, whose soil samples were employed as control.

3.1.2. METHOD FOR COLLECTION OF SOIL SAMPLE

Soil samples were collected according to the method of Saksena and Mehrotra (1952) for their studies. At each site an area of 3 m² was chosen for sampling. The surface deposits were removed to a depth of 15 cm and samples were collected up to a depth of 30 cm, each sample containing about 100 g of soil. Six soil samples were collected at different locations at random within 1.5 km radius in each site chosen. The samples were stored in sterile, thin-walled polythene bags and brought to the laboratory. All random soil samples of each site were put together to make a single composite sample for each site. Samples were stored in a refrigerator and analyzed within 48 h of collection for their physico – chemical parameters including heavy metals and chemicals. The garden soil samples obtained was also subjected for analysis that pertained as control.
iii) Tannery affected site in Vellore

iv) Garden soil (Control) from Chennai

Fig. 3 Sites chosen for the study
3.2 PHYSICO- CHEMICAL ANALYSIS OF SOIL SAMPLES

The pH values, electrical conductivity, soil moisture, organic carbon, nitrogen, phosphorous, potassium, iron, manganese, copper and zinc were analyzed. The macro nutrients such as Nitrogen (Alkali permanganate method), phosphorous (Olsen method), potassium (neutral normal ammonium acetate method), organic carbon (Walkley and Black method) and micro nutrients such as copper, iron, manganese and zinc were analyzed by DTPA extract method using atomic absorption spectrophotometer (Mishra et al., 2011).

3.3 ISOLATION OF SOIL MYCOFLORA

The spectrum of soil fungi isolated and the frequency of isolation is influenced by various factors like: Soil sampling procedures, transit and storage conditions etc. among which the isolation technique used is of major importance to enumerate most of the mycoflora.

3.3.1 Isolation of saprophytic fungi

Initially, all the required glasswares including petridish were sterilized in an autoclave at 121°C for 15 min. Analytical grade chemicals (Sigma – Aldrich, India) were used for the preparation of both liquid and solid media.

The soil micro fungi were enumerated by two methods: Soil dilution method (Waksman, 1927), and Soil plate method (Warcup, 1950).

3.3.1.1 Soil dilution method

A. sample of 10 g soil was placed in a graduated cylinder, added with sterilized distilled water to make a total of 100 ml. The suspension was stirred, poured into sterile 250 ml Erlenmeyer flask and shaken in orbital shaker thoroughly for 30 min. 1 ml of this suspension was pipetted aseptically and dispensed in dilution test tubes with 9 ml of sterilized distilled water. Series of soil dilutions of 1:10, 1:100, 1:1,000 and 1:10,000 were prepared. 1 ml of the desired dilution (10^{-3} and 10^{-4}) was transferred aseptically into sterile petridishes containing PDA amended with chloramphenicol (120 mgl^{-1}) to avoid bacterial contamination and spreaded with a glass spreader.
Composition of Potato Dextrose Agar (PDA)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
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<tbody>
<tr>
<td>Potato</td>
<td>200 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20 g</td>
</tr>
<tr>
<td>Agar</td>
<td>17 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>5.6 ± 0.2</td>
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</table>

The media was prepared by adding the potato infusion (boiled filtrate of macerated potatoes) along with dextrose and agar. pH was adjusted and the media was sterilized by autoclaving at 121°C, 15 lb pressure for 15 min. The media was then cooled and poured on to sterile petri dishes aseptically allowing for solidification.

Duplicates were maintained for each soil dilution that the inoculated plates were then incubated at room temperature of 27 ± 3°C for a period of 4 – 7 days and examined. After incubation, a small portion of mycelium from each fungal colony was transferred into PDA slants. Each colony that appeared on the plate was considered as one colony forming unit (CFU).

3.3.1.2 Soil plate method

About 0.05 g of soil was scattered on the bottom of a sterile petri dish to which molten, cooled (40-45°C) sterile potato dextrose agar medium was added aseptically and then rotated gently to disperse the soil particles in the medium. Duplicates were maintained for each sample that the inoculated plates were then incubated at 27 ± 3°C for a period of 4 – 7 days and examined.

3.3.2 Subculturing

From day 2, observations were made each day for the fast growing flocculent types such as *Rhizopus, Mucor* and *Trichoderma*, etc., has grown excessively to interfere with observations of other species. Therefore the slow growing organisms were subcultured first in separate media (PDA) to avoid being overrun by the more aggressive types and allowing their further growth enabling for identification (Saravanakumar and Kaviyarasan, 2010). The growing edges of fungal colonies in the soil dilution plates were then transferred to a fresh PDA plates by hyphal tipping and subcultured. Pure cultures of the isolates were maintained on PDA agar slants.
3.3.3 Identification of fungal isolates

Identification of fungal cultures involves both macroscopic and microscopic studies revealing the morphological features of the fungi. Pure cultures of the fungi obtained were periodically examined for sporulation and identified accordingly. These identification methods were based on the morphology of the fungal culture, the mechanism of spore production and characteristics of the spore by following the standard mycological manuals. Also, the shape, method of spore production and the arrangement of spores (conidial ontogeny) is examined. Macroscopically, colony features (Colour and Texture) were also noted.

Microscopic examination using wet mount preparation by lactophenol cotton blue staining method was employed (Ellis, 1971; Sutton, 1980). Lactophenol Blue Solution is a mounting medium and staining agent used in the preparation of slides for microscopic examination of fungi. Fungal elements are stained intensely blue.

### Composition of Lactophenol Blue

<table>
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<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>Phenol</td>
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</tr>
<tr>
<td>L (+)-Lactic Acid</td>
<td>20 ml</td>
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<tr>
<td>Glycerol</td>
<td>40 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>20 ml</td>
</tr>
</tbody>
</table>

A drop of lactophenol blue solution was placed on a slide. Fungal culture was carefully teased using an inoculating needle. A coverslip was placed on the slide without any air bubble. After 5 min, slide was observed under a microscope with low power for screening in low intensity. The edges of the coverslip can be sealed with nail polish or permount to preserve the mount (Thomas et al., 1991).

3.3.4 Statistical analysis

Following the isolation and identification of fungi, the following data were calculated for statistical analysis to evaluate the diversity of fungi present in different soil samples notoriously contaminated by distinguishing pollutants.
3.3.4.1 Frequency and Relative Density

The prominence of a species in the fungal community can be assessed quantitatively both as frequency (F) and as Relative density (R.D).

a) **Frequency:** It is the percentage of sites at which the species occurred.

\[
\text{Frequency (F)} = \frac{\text{No. of soil samples in which a particular fungus occurred}}{\text{Total No. of soil samples examined}} \times 100
\]

b) **Relative density:** It is the percentage of total isolates contributed by the ‘i’th species.

\[
\text{Relative Density (R.D)} = \frac{\text{No. of isolates of the ‘i’ th species}}{\text{Total No. of isolates}} \times 100
\]

3.3.4.2 Variation

To determine the total variation, expressed as sum of variation due to different ecological types of soils and due to chance causes analysis of variance (ANOVA) was performed (Paterson 2008).

3.3.4.3 Species diversity

The diversity of species was studied in terms of species richness and relative dominance of the species.

a) **Species richness (S):** It represents the total number of different species in a particular area (Harrison 2004).

b) **Relative dominance (d):** It was measured by calculating the Berger- Parker dominance (Harrison 2004).

\[
d = \frac{n}{N}
\]

Where \( n = \text{no of individuals in a species.} \)

\( N = S = \text{total no of individual.} \)
3.3.4.4 Simpsons Diversity index:

This is a simple mathematical measure that characterizes species diversity in a community. It is calculated using the following formula:

\[
D = \frac{\sum_{i=1}^{R} n_i(n_i - 1)}{N(N - 1)}
\]

Where, \( n \) = No. of individuals in each species.
\( N \) = Total no. of individuals.

The D assumes value between 0 and 1. \( D = 0 \) indicates maximum diversity while \( D = 1 \) represents the least diversity (http://en.wikipedia.org/wiki/Diversity_index).

3.4. SECONDARY METABOLITE PRODUCTION OF ASPERGILLUS TERREUS

Although different mycoflora were present in the distinguishing soil samples, commonly prevalent fungal species i.e., four strains of A. terreus, each isolated from three distinguishingly polluted sites and the garden soil were chosen for further studies. These strains of A. terreus chosen hereafter will be designated as: AT - PAL (Plastic dump site isolate), AT - TAN (Tannery isolate), AT - CHM (Chemical effluent isolate) and Garden soil isolate as Control strain.

Each organism produces distinct metabolites. These low molecular secondary metabolites often have potent physiological activities whose production is influenced by various factors depending on their growth and hence preliminary growth studies are necessitated.

3.4.1. Preliminary growth studies of A. terreus

Four sets of two 500 ml Erlenmeyer flasks each, containing 100 ml of Potato Dextrose Broth amended with chloramphenicol (12 mg/100 ml) were autoclaved at 121°C for 15 min at 15 lb pressure. One flask in each set was inoculated with 10 μl of a spore suspension of different strain of A. terreus chosen(10⁴ spore/ml) from 7 days old cultures of A. terreus on the solid PDA medium while the other flasks were inoculated with 10 μl of sterile water (blank). All the inoculated flasks were incubated on a rotary shaker at 65 rpm, for 20 days at 26°C.
Culture turbidity was evaluated as parameter for mould growth. Optical density was recorded following inoculation at 0\textsuperscript{th} hour at 405 nm using UV-VIS spectrophotometer. For every 24 h sequential measurements were recorded until the decline phase was reached. Growth curves were then plotted using optical density values against time taken. The time obtained to attain maximum growth was hence determined from the growth curve (Trinci, 1972).

### 3.4.2 Extraction and analysis of secondary metabolites

#### 3.4.2.1 Preparation of fermentation media

250 ml of Potato Dextrose Broth amended with chloramphenicol (12 mg/100 ml) was prepared in 500 ml Erlenmeyer flasks and autoclaved at 121°C for 15 min at 15 lb pressure. Each flask was then inoculated with different strain of *A. terreus* chosen, as previously described and were incubated for 10 days at 26°C.

#### 3.4.2.2 Preparation of fungal extracts

The inoculated flasks were collected at the end of growth period of 10 days of culture, whose culture fluid was passed through four layers of cheese cloth to remove the solids. Extraction was carried out with different organic solvents of varying polarity (Strobel \textit{et al.}, 1996). 500 ml of culture filtrate was processed for each organic solvent extraction. Both mycelia and filtrate were separately subjected to solvent extraction as follows:

#### 3.4.2.3 Extraction of mycelia:

Using sterile distilled water, the fresh mycelium of each fungus was washed thrice to remove adherent filtrate and then blotted between folds of sterilized filter paper No 1. The blotted mycelium was then dried in hot air oven at 60°C, crushed in mortar, extracted with the solvent to obtain intracellular metabolites. Both crushing and extraction were repeated thrice, left in separating funnel for 15 min to precipitate. The crude extract was collected.

#### 3.4.2.4 Extraction of the filtrate:

The filtrate of fungus was extracted several times with solvent (v/v) in a separating funnel.
The crude extracts of mycelia and filtrate were collected and the solvent phase was then removed by evaporation under reduced pressure at 35°C using rotary vacuum evaporator. The dry solid residue was re-dissolved in respective solvents and subjected for subsequent studies (Belofsky et al., 1998; Holler et al., 2000 and Lin et al., 2000).

3.4.2.5 Analysis of secondary metabolites

Physical properties of the bioactive crude extracts can be analysed via UV and IR spectroscopy. UV spectra were obtained between 200 and 400 nm for all the extracts obtained from mycelia as well as filtrate using UV-VIS spectrophotometer. Similarly an Infra Red spectra (IR) was obtained using pellets of KBr on the Fourier Transform Infra Red spectrometer (Nicolet, model 670, USA).

The extracts were then analyzed qualitatively for the presence of various metabolites (Mabrouk et al., 2008).

The following reagents were prepared for the different chemical groups test.

**Mayer’s reagent**: 1.36 g mercuric iodide in 60 ml of water was mixed with a solution containing 5 g of potassium iodide in 20 ml of water.

**Fehling’s solution A**: 34.64 g copper sulphate was dissolved in a mixture of 0.50 ml of sulfuric acid and sufficient water to produce 500 ml.

**Fehling’s solution B**: 176 g of sodium potassium tartarate and 77 g of sodium hydroxide were dissolved in sufficient water to produce 500 ml equal volume of the above solution was mixed at the time of use.

**Liebermann-Burchard Reagent**: 5 ml acetic anhydride was carefully mixed under cooling with 5 ml Conc. sulfuric acid. This mixture was added cautiously to 50 ml absolute ethanol with cooling.
The extracts were then tested for different chemical groups as follows:

**Test for Alkaloids (Mayer’s test)**

To 3 ml of extract, 1 ml of 1% HCl followed by 2 drops of Mayer’s reagent was added to give a creamy white precipitate.

**Test for Flavanoids (Shinoda test)**

To 3 ml of extract, 1 g of Magnesium Sulphate followed by 2 drops of conc. HCl was added to give red colouration.

**Test for Saponins (Frothing test)**

2 ml of the extract was vigorously shook and observed for stable frothing.

**Test for Carbohydrates (Fehling's test)**

Equal volume of Fehling's A (Copper Sulfate in distilled water) and Fehling's B (Potassium Tartarate and Sodium Hydroxide in distilled water) reagents were mixed and a few drops of extract is added and boiled. A brick red precipitate of cuprous oxide was observed, hence indicating the presence of reducing sugars.

**Test for Tannins (Ferric chloride test)**

To 1 ml of extract, 2 drops of 5% Ferric Chloride were added and observed for a dirty green precipitate.

**Test for Steroids (Liebermann-Burchard test)**

To 1 ml of extract, 2 ml of Liebermann-Burchard reagent was added and observed for reddish purple colour.

The results of different solvent extracts indicating the presence of different chemical group of metabolites were tabulated and studied comparatively
3.5 BETA CAROTENE AND FATTY ACID PRODUCTION FROM ASPERGILLUS TERREUS

Lipids are produced by microorganisms for essential functioning of cell membranes and other membranous structures. However optimization of process parameters such as media variables like different carbon and nitrogen sources, pH, temperature etc. affects the growth and the lipid accumulation, hence media standardization for strain improvement is necessary.

3.5.1 Inoculum preparation

The different strains of *A. terreus* chosen *i.e.*, AT - PAL, AT - TAN, AT - CHM and Control were maintained on PDA plates at 4°C. For conidial production, *A. terreus* strains were grown on PDA at 20 – 25 °C for 7 days. The spores were then harvested and suspended in 10 ml of sterile distilled water containing 0.05% (v/v) Tween 80. Using a Neubauer chamber, the concentration of spore suspension was determined and adjusted with sterile distilled water to 1×10^4 spores/ml. Conidia of different strains of *A. terreus* were thus obtained from 7 day old PDA cultures grown at 20 – 25 °C. The concentration was also adjusted to 1×10^4 spores/ml (Calvo *et al.*, 2007).

3.5.2 Media standardization

250 ml of Potato dextrose broth cultures amended with chloramphenicol (12 mg/100 ml) were prepared in 500 ml Erlenmeyer flasks and subjected to conditions of varying pH ranging from 3 to 9, varying temperature at 21°C, 26°C, 37°C and 12:12 h of Light & Dark conditions (Higashiyama *et al.*, 2002). Also cultures containing 250 ml of different types of media were employed such as:

**Potato Dextrose Broth**

<p>| | | |</p>
<table>
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</thead>
<tbody>
<tr>
<td>Potato</td>
<td>-</td>
<td>200 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>-</td>
<td>20 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>-</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>-</td>
<td>5.6 ± 0.2</td>
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**Potato Sucrose Broth**

<table>
<thead>
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<th>Quantity</th>
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<tbody>
<tr>
<td>Potato</td>
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</tr>
<tr>
<td>Sucrose</td>
<td>20 g</td>
</tr>
<tr>
<td>Distilled water</td>
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</tr>
<tr>
<td>pH</td>
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**Richards’s Broth**

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<td>Magnesium sulphate</td>
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<tr>
<td>Potassium dihydrogen phosphate</td>
<td>5 g</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>10 g</td>
</tr>
<tr>
<td>Potato starch</td>
<td>10 g</td>
</tr>
<tr>
<td>Sucrose</td>
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<td>Distilled water</td>
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**Czapek Dox Broth**

<table>
<thead>
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<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>30</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>3</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>1</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
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</tr>
<tr>
<td>Potassium chloride</td>
<td>0.5</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
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<tr>
<td>pH</td>
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</table>

Potato dextrose broth cultures adjusted to pH 5.6 served as the mother culture. Optical density was recorded following inoculation at 0th hour at 405 nm using UV-VIS spectrophotometer to check the growth phase. For every 24 h sequential measurements were recorded periodically until the decline phase was reached. Growth curves were then plotted using optical density values against time taken. The time and the conditions required obtained to attain maximum growth was hence determined from the growth curve (Trinci, 1972).
3.5.3 Biomass production

Following the end of growth period the mycelial biomass was collected from the inoculated flasks via separation. After 10 days of culture incubation, the inoculated flasks were collected and the culture was passed through Whatmann filter paper No.1 to separate the mycelia biomass. Sterile distilled water was used to wash the filtered mycelia twice and then it was dried in hot air oven at 60°C (Yongmanitchai and Ward, 1991). The biomass of the dried cultures was weighed and the results were represented for comparative studies of cultural conditions influencing the production of highest biomass.

3.5.4 Analysis of β - carotene

3.5.4.1 Extraction of carotenoid

The extraction was performed by adding acetone to 500 mg mycelial powder obtained by the disruption of the mycelium with pestle and mortar followed by vortexing. This step was repeated until the pellet was found to be colourless. After combining the extracts, they were partitioned with an equal volume of 10 % diethyl ether in petroleum ether. To facilitate the separation and to remove dissolved acetone, 1 ml distilled water was added. Finally, petroleum ether fractions were combined, dried and redissolved in acetone. The absorbance of the extracts were scanned spectrophotometrically (Shimadzu-160A, Japan) between 300 and 600 nm against acetone blank (Papp et al., 2006) and λ max was recorded.

3.5.4.2 Estimation of β - carotene

Carotenoids in solution obey the Beer–Lambert law, that is, their absorbance is directly proportional to the concentration. Hence using the optical density values, carotenoids can be quantified spectrophotometrically according to method of Davis (1976) and Britton (1995) with slight modifications. 1 ml of the sample (fungal extract) was taken in a 50 ml volumetric flask and diluted up to the volume using cyclohexane. Following which, the absorbance was measured spectrophotometrically at 450 nm against cyclohexane as blank. Reference standards of pure β - carotene (≥95% HPLC grade) was obtained from Sigma-Aldrich.
Conc. Of β - carotene (µg/ml) = \( \frac{A \times V_1}{A^{1\%}} \times C^{1\%} \)

Where A is the absorbance reading of the diluted sample, V 1 is the dilution factor (50×), A 1% is the absorbance of a 1% solution i.e., the extinction coefficient; 2592 AU), and C 1% is the concentration of a 1% solution (10 mg/ml) (US Pharmacopeial convention, 2012).

3.5.4.3 TLC profiling of β - carotene

Following the spectral analysis, the extracts were subjected to TLC (Thin Layer Chromatography) profiling to identify the presence of β - carotene qualitatively in TLC plates. Precoated silica gel 60 F\(_{254}\) TLC plates (E. Merck, 0.2 mm thickness) and the reference standard of 500 µg/ml of β - carotene (≥95% HPLC grade) were employed. 5 µl of samples were applied on the plate using a capillary tube. The plate was then transferred to a rectangular glass chamber saturated with the solvent system of the mobile phase consisting petroleum ether: diethyl ether: acetone (75:15:10, v/v/v). Care was taken to keep the level of solvent slightly below the level of the spots. Plates were kept slanting on the walls of the chamber and closed tightly with the lid. The solvent was allowed to run up to 2/3\(^{rd}\) portion of the plate after which it was taken out and allowed to dry in air. The chamber was kept undisturbed till the run was complete and the spots were identified using potassium permanganate. R\(_f\) values were calculated and the TLC profiles were compared (Cano and Ancos, 1994).

3.5.5 Analysis of fatty acids

3.5.5.1 Extraction of fatty acid

An economical way of extraction of lipids is using the Bligh and Dyer method, a simple adaptation of the Folch procedure. Total extraction of cellular lipids was done by disrupting the cells using a solvent mixture of chloroform, methanol and water to perform the extraction and the solvent ratios were balanced such that a single phase is formed with the sample water (Bligh and Dyer, 1959). The lipids were suspended in the chloroform phase. The fat content was determined in an aliquot of the chloroform phase by weighing the lipids after evaporation of the solvent. Modifications of this method have been made by using hexane and acetone instead of
chloroform and methanol. The dried biomass was weighed (30 mg) and powdered using mortar and pestle to disrupt the cells, extracted with 5 ml of acetone followed by 5 ml of hexane and the fractions were collected. The tube containing the fraction was centrifuged and the supernatant was collected. The remaining biomass was re-extracted twice by the addition of solvents that a total volume of 20 ml was then concentrated and diluted with same solvent mixture for further analysis.

3.5.5.2 TLC profiling

The extracts obtained were then subjected for TLC (Thin Layer Chromatography) profiling on precoated silica gel 60 F254 TLC plate (E. Merck, 0.2 mm thickness) using palm oil as standard. 5 µl of samples were applied on the plate using a capillary tube. The plate was then transferred to a rectangular glass chamber saturated with the solvent system of diethyl ether: hexane: acetic acid (4:13:0.4). Care was taken to keep the level of solvent slightly below the level of the spots. Plates were kept slanting on the walls of the chamber and closed tightly with the lid. The solvent was allowed to run up to 2/3rd portion of the plate after which it was taken out and allowed to dry in air. The chamber was kept undisturbed till the run was complete and the spots were identified using potassium permanganate. Rf values were calculated and the TLC profiles were compared (Christie and Han, 2010).

3.5.5.3 Estimation of free fatty acids

Free fatty acid content is known as the acid number/acid value. The fatty acids were estimated by titrating them against the methanolic KOH in the presence of phenolphthalein indicator. 0.5 g of fatty acid sample was dissolved in 50 ml of fat solvent (1:1 ratio of methanol: diethyl ether) and titrated against 0.1 N methanolic KOH using 1% phenolphthalein as indicator (Cox and Pearson, 1962). Appearance of pale pink colour is the end point and the titre values were obtained. Calculations were done using the following formula:

\[
\text{Acid value} = \frac{\text{Titre value} \times \text{Normality of KOH} \times 56.1}{\text{Weight of the sample (g)}}
\]
3.5.5.4 Preparation of FAMEs

Owing to increased acid value, the fatty acid extracted from the fungal cultures grown in PDB, at 26°C of pH – 6 were chosen for FAMEs preparation to be analysed by gas chromatography. Fatty acid components need to be converted into fatty acid methyl esters (FAME) in order to improve their volatility and thus ensuring better gas chromatographic peak shape. The preparation of FAME involves extraction of the lipid molecules from the sample matrix breaking of the ester bonds and formation of methyl esters. The two last steps may be combined by trans-esterifying the lipids directly with acid or base in methanolic solution. The lipid sample (50 mg) obtained previously was dissolved in toluene (1 ml) in a test tube fitted with a condenser and 1% sulfuric acid in methanol (2 ml) was added before the mixture was refluxed for 2 h (or alternatively the mixture be left overnight in a stoppered tube at 50°C). Water (5 ml) containing sodium chloride (5 %) was added and the required esters were extracted with hexane (2 × 5 ml) using Pasteur pipette to separate the layers. The hexane layer was washed with water (4 ml) containing potassium bicarbonate (2 %) and dried over anhydrous sodium sulfate and then subjected to GC-FID analysis (Nelson, 2010).

3.5.5.5 Gas Chromatographic conditions

Capillary columns and flame ionization detector (FID) are most commonly used to characterize the fatty acid profile in biological materials. Fused silica columns can easily achieve high resolution, their temperature can be programmed or the flow velocity of the carrier gas can be raised to optimize resolution and reduce the analysis time. The FAMEs obtained were analyzed using Gas Chromatography equipped with Flame Ionization Detection (GC - FID). This common method is simple and rapid and is thus advantageous for analysis. Individual fatty acid methyl ester standards (all greater than 95% purity) including methyl myristate (C14:0), methyl pentadecanoate (C15:0), methyl palmitate (C16:0), and methyl docosahexaenoate (C22:6) standards were obtained from Sigma Aldrich and used for Gas Chromatography calibrations. The FAME standards were prepared in a hexane solution at concentrations of 0.2 mg/ml and used in GC analysis to determine the unknown fatty acid concentrations (Nelson, 2010).

GC was performed on Agilent 6890 series Gas Chromatograph equipped with ALS 7673 auto injector, FID and the capillary column VF- 23MS (30 m x 0.25 mm x 0.25 µ). Injector and detector temperatures were maintained at 230 and 250°C, respectively. The oven was
programmed for 2 min at 160°C then increased to 180°C at 6°C/min, maintained for 2 min at 180°C, increased further to 230°C at 4°C/min and finally maintained for 10 min at 230°C. The carrier gas nitrogen was used at a flow rate of 1.5 ml/min. The injection volume was 1 ml with a split ratio of 50:1 (Ahmed et al., 2006). Data from the chromatographic analysis were sent to the computer system where fatty acids were identified on the basis of their retention times relative to known standards and quantified relatively to other fatty acids in the sample on the basis of peak width and area data.

Thereby fatty acids extracted from various fungal cultures being optimized for various parameters was subjected for acid value estimation of fatty acids and quantified. The strains grown from the culture media corresponding to increased biomass and fatty acid production optimized for its own parameters was further employed for development of mutants.

3.6 GENERATION OF MUTANTS OF ASPERGILLUS TERREUS

Mutagenesis is an effective way to attain genetic modification in an organism improving the stain for production in industrial inputs. A eukaryotic system generally has a natural tendency of reverting back to its wild form that a second mutation is often necessary for development of a highly mutated and stable strain.

In the present investigations, two steps of mutagenesis were followed:

1. The chosen different strains of A. terreus were mutated by exposing to chemical mutagen – Ethidium bromide at various time intervals and various concentrations.
2. The best mutant developed from each different strain of A. terreus of first step mutation were again exposed to two different mutagens – Ethidium bromide, Mosquito Coil (powdered) at various time intervals and various concentrations and double mutant strains were obtained.

3.6.1 Primary mutants

The four different chosen strains of A. terreus were grown on potato dextrose agar (PDA) slants for 7 days until sporulation. Following which, the spores were scraped off into sterile
phosphate buffer (0.02 M and pH 7.0) containing Tween - 80 (1:5000) to give a uniform suspension. The suspension was transferred into a sterile conical flask and thoroughly shaken for 30 min on a rotary shaker to break the spore chains. The spore suspension was then filtered through a thin sterile cotton wad into a sterile tube, to remove vegetative mycelium from the suspension. This spore suspension was then used for EtBr treatment.

10 ml of spore suspension was added to 10 ml of EtBr solution with a range of concentration of (200 – 800 μg/ml) and the reaction was allowed to proceed. 2 ml of this solution was taken after every 15, 30, 45, 60 min and centrifuged immediately for 10 min at 5000 rpm. The supernatant solution was decanted and the pelleted cells were washed thrice with sterile water to remove traces of mutagen and resuspended in 10ml of sterile phosphate buffer. These samples were then inoculated on to Potato dextrose broth amended with chloramphenicol (12 mg/100 ml) and incubated for 10 days at 26°C. The cultures following incubation were harvested and analysed for β - carotene and fatty acid production as described in section 3.7 below. (Dhawan et al., 2003, Akbar et al., 2012).

3.6.2 Secondary mutants

Four different mutants each representing from the parent strains i.e., AT ET - 49, AT ET - 34, AT ET - 2, AT ET - 17, yielding high amount of fatty acid and β - carotene were chosen for the second mutation using EtBr and mosquito coil mutagens.

The chosen mutant strains of A. terreus were grown on potato dextrose agar (PDA) slants for 7 days until sporulation. Following which, the spores were scraped off into sterile phosphate buffer (0.02 M and pH 7.0) containing Tween- 80 (1:5000) to give a uniform suspension. The suspension was transferred into a sterile conical flask and thoroughly shaken for 30 min on a rotary shaker to break the spore chains. The spore suspension was then filtered through a thin sterile cotton wad into a sterile tube, to remove vegetative mycelium from the suspension. This spore suspension was then used for mutagen treatment.

10 ml of spore suspension was added to 10 ml of EtBr solution and 10 ml of mosquito coil solution with a range of concentration of (200 – 800 μg/ml) and the reaction was allowed to proceed. 2 ml of this solution was taken after every 15 and 30 min and centrifuged immediately for 10 min at 5000 rpm. The supernatant solution was decanted and the pelleted cells were
washed thrice with sterile water to remove traces of mutagen and resuspended in 10 ml of sterile phosphate buffer. These samples were then inoculated on to Potato dextrose broth amended with chloramphenicol (12 mg/100 ml) and incubated for 10 days at 26°C. The cultures following incubation were harvested and analysed for β-carotene and fatty acid production as described in section 3.7 below. (Dhawan et al., 2003, Akbar et al., 2012).

3.7 ANALYSIS OF BETA CAROTENE AND FATTY ACID PRODUCTION FROM MUTANTS OF ASPERGILLUS TERRIEUS

3.7.1 Biomass production

The cultures treated with mutagen were harvested following incubation period of 10 days. The mycelial biomass was collected from the inoculated flasks via separation by passing through Whatmann filter paper No.1 that the filtered mycelia was washed twice using sterile distilled water and then it was dried in hot air oven at 60°C (Yongnanitchai and Ward, 1991). The biomass of the dried cultures was weighed and the results were represented for comparative studies of mutation influencing the production of highest biomass.

3.7.2 Analysis of β-carotene

The carotenoids were extracted from the mycelia of the mutant cultures, estimated using UV spectrophotometry and subjected for TLC profiling as described in Sec. 3.5.4

3.7.3 Analysis of fatty acids

Total lipids were extracted from dried biomass of mutant cultures, subjected for TLC profiling and the free fatty acids were estimated as described in Sec 3.5.5.1, 3.5.5.2 and 3.5.5.3. Owing to increased acid value and high β-carotene content, the fatty acid extracted from the mutated strains AT SMC – 123 and AT SET – 83 were chosen for FAMEs preparation to be analysed by gas chromatography. FAMEs were prepared and analysed using GC – FID as described in Sec 3.5.5.4 and 3.5.5.5.
3.8 PURIFICATION OF BETA CAROTENE USING HPLC ANALYSIS

Purification was carried out using Shimadzu LC 10 ATVP High Performance Liquid Chromatograph fitted with a Luna 5µ column and a SPD - 20AV UV-Vis Detector set at 452 nm. The analytical column was a phenomenex C-18 110A column of dimension 250 × 4.6 mm with particle size 5 µm. The developed HPLC analytical method for separation of β-carotene was achieved isocratically with the C18 column kept under constant temperature (22.9°C set at 21°C) inside a condenser and a flow rate set at 2.0 ml/min. The mobile phase used was a mixture of Acetonitrile: Dichloromethane: Methanol (70: 20: 10). The detection of β-carotene was obtained at 452 nm. 1 mg of the extract was dissolved in 1 ml of the mobile phase and 20 µl of this aliquot was injected into the column. Data of peak height and peak area of each spot was recorded. The calibration curve was prepared by plotting concentration (mg/spot) versus peak area corresponding to each spot (Norshazila et al., 2012).

3.9 ANTICANCER ACTIVITY OF BETA CAROTENE AGAINST MCF-7 AND HEP-G2 CELL LINES

3.9.1 Cell line and culture
Cancer cell lines MCF-7 (Breast cancer cell line), Hep-G2 (Liver cancer cell line) and Vero cell lines were purchased from National Centre for Cell Sciences Pune (NCCS). The cells were thawed in a water bath at 37°C for approximately one to two min and added to 10 ml of pre-warmed (37°C) antibiotic free RPMI 1640 (Sigma) growth medium containing 10% Foetal Calf Serum (FCS) (Sigma) in order to dilute out the DMSO. The cells were centrifuged (500 × g, 5 min, 25°C) in order to obtain a cell pellet and the supernatant discarded. The pellet was resuspended in 10 ml pre-warmed total growth medium and transferred to a cell culture dish. The cells were incubated in a humidified CO₂ incubator (5% CO₂) at 37°C until the monolayer was subconfluent (Rose and Connolly, 1990).

3.9.2 Subculture of Cells
The cultures were viewed using an inverted phase contrast microscope to assess the degree of confluency and confirmed the absence of bacterial and fungal contaminants. Standard
trypsinization procedures were performed (trypsin obtained from Roche) and a cell count was obtained by using 20 μl of the cell suspension and counting with an improved Neubauer haemocytometer. The required numbers of cells were transferred to new labeled cell culture dishes containing pre-warmed antibiotic free RPMI 1640 with 10% FCS and these dishes were incubated as before (Rose and Connolly, 1990).

3.9.3 Cell Quantification

Under sterile conditions 20 μl of cell suspension was transferred to an eppendorf tube and an equal volume of 0.4% trypan blue in PBSA was added and mixed by gentle pipetting. An improved Neubauer haemocytometer was used to perform the count and the number of viable (colourless) and non-viable cells (blue) were counted and the percentage of viable cells was calculated (Mascotti et al., 2000).

3.9.4 In vitro assay for Cytotoxicity activity (MTT assay).

The reduction of tetrazolium salts is now widely accepted as a reliable way to determine cell proliferation and viability referred to as MTT (3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay (Mossman, 1983). The yellow tetrazolium salt MTT (3 (4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells in part by the action of dehydrogenase enzymes. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The MTT cell proliferation assay measures the cell proliferation rate and conversely when metabolic events lead to cell death, the reduction in cell viability. The MTT reagent yields low background absorbance values in the absence of cells.

Cells were grown in RPMI-1640 medium at 37°C incubated for 6-7 h 5% CO₂ in a humified incubator. Cells were harvested, counted (3 × 10⁴ cells/ml) and transferred into a 24 well plate (Costar Corning, Rochester, NY) and incubated for 48 h. Vero cell lines were employed as control. Prior to the addition of β - carotene sample, serial dilutions were prepared by dissolving compounds in DMSO followed by dilution with RPMI-1640 medium to give final concentration at 10, 5.0, 2.5, 1.25, 0.625, 0.3125 and 0.156 mg/ml. Sample at 10 μl and cell lines at 90 μl were incubated for 72 h. MTT solution at 5 mg/ml was dissolved in 1 ml of Phosphate Buffer Solution (PBS) (pH 7.4) and 100 μl of it was added to each of the 24 wells. The wells
were wrapped with aluminium foil and incubated at 37°C for 4 h. The solution in each well containing media, unband MTT and dead cells that were removed by suction and 150 μl of DMSO was added to each well. Then the plates were shaken and optical density was recorded using a microplate reader (spectrophotometer) at 570 nm using DMSO as a blank. Control and samples were assayed and replicated for each concentration and replicated three times for each cell line. After 24 h incubation of the mononuclear cells with β - carotene fraction the cytotoxicity on the cancer cell lines was evaluated using MTT assay (Hagopian et al., 1999; Huq et al., 2004). The cytotoxicity was obtained by comparing the absorbance between the samples and control. The values were then used to calculate the concentration of β - carotene required to cause a 50% reduction (IC₅₀) in growth (cell number) for each cell lines.

\[ \% \text{ cell viability} = \frac{A_{570} \text{ of treated cells}}{A_{570} \text{ of control cells}} \times 100\% . \]

### 3.10 ANTIOXIDANT ACTIVITY OF BETA CAROTENE USING DPPH ASSAY

The radical scavenging activity of β - carotene fraction was determined using DPPH (2, 2 ′ -diphenyl-1-picrylhydrazyl stable radicals) assay according to Nenadis and Tsimidou (2002), with little modification. The decrease of the absorption at 517 nm of the DPPH solution after the addition of the antioxidant (β - carotene) was measured in a cuvette containing 2.96 ml of 0.1 mM ethanolic DPPH solution that was mixed with 40 µl of 20-200 μg/ml of β - carotene fraction. Blank contained 0.1 mM ethanolic DPPH solution without β - carotene and vortexed thoroughly. The setup was left in the dark at room temperature. The absorption was monitored after 20 min. Butylated Hydroxy Toluene (BHT) was used as reference standard. The ability to scavenge DPPH radical was calculated by the following equation. % of DPPH radical scavenging activity (% RSA) = \[ \frac{(A_{\text{control}} - A_{\text{test}})}{A_{\text{control}}} \times 100 \]. \( A_{\text{control}} \) is the absorbance of DPPH radical + ethanol; \( A_{\text{sample}} \) is the absorbance of DPPH radical + β - carotene.

Measurements were performed in triplicates. Absorbance values were corrected for radical decay using blank solutions. The IC₅₀ (concentration providing 50% inhibition) was calculated graphically using a calibration curve plotted with scavenging percentage against essential β - carotene concentration.
3.11 APOPTOTIC DNA FRAGMENTATION ASSAY

0.5 ml of cell suspension was suspended and centrifuged at 2000 × g at 4°C for 10 min. 0.5 ml of TTE (10 mM Tris (pH 7.4), 5 mM EDTA, 0.2% Triton solution was added to pellet and vortexed vigorously. This procedure allows the release of fragmented chromatin from nuclei after cell lysis (due to the presence of Triton X-100 in the TTE solution) and disruption of the nuclear structure (following Mg²⁺ chelation by EDTA in the TTE Solution). To separate fragmented DNA from intact chromatin, tubes were centrifuged at 20,000 × g for 10 min at 4°C. Carefully supernatant was transferred in new tubes and 0.5 ml volume of ice-cold 5 M NaCl was added and vortexed again vigorously. The addition of the salt should be able to remove histones from DNA. 0.7 ml of ice-cold isopropanol added again and vortexed vigorously precipitation was allowed to proceed overnight at -20°C. This step can be shortened by putting samples in a bath of ethanol/dry ice for 1 h. After precipitation DNA was recovered by pelleting for 10 min at 20,000 × g at 4°C. Supernatant was discarded by aspiration or by rapidly inverting tubes and drops of fluid were carefully removed which remains adherent to the wall of the tubes with a paper towel corner. This can be a critical step because the pellet could be lost or hard to be seen. Pellets were rinsed by adding 0.5-0.7 ml ice-cold ethanol and centrifuged at 20,000 × g for 10 min at 4°C. Again supernatant was discarded by aspiration or by rapidly inverting tubes. Drops adherent to the wall were carefully removed by inverting tubes over an absorbent paper towel for 30 min. The tubes were air dried in upright position for at least 3 h before proceeding. DNA was dissolved by adding to each tube 20-50 µl of TE (10 mM Tris–HCl (pH – 7.5) and 1 mM EDTA) solution. Samples of DNA were mixed with loading buffer by adding 10X loading buffer to a final concentration of 1X. The addition of loading buffer to samples allows to load gel wells more easily and to monitor the run of samples. Electrophoresis was run in standard 1X TBE buffer (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA) after setting the voltage to the desired level. During electrophoresis it is possible to monitor the migration of samples by following the migration of bromophenol blue dye contained in the loading dye. Electrophoresis was stopped when the dye reaches about 3 cm from the end of the gel. DNA bands were visualized by placing the gel on a UV Transilluminator (Kotamraju et al., 2000).
3.12 MOLECULAR STUDY USING PCR

Molecular approaches have led to the isolation of a number of genes involved in carotenogenesis, in particular the genes coding for bifunctional enzymes like phytoene synthase. Owing to higher production of β-carotene in the AT SMC - 123, initial molecular studies were done to isolate the phytoene synthase gene mediating β-carotene production using PCR amplification technique. The carotenoid pathway begins with the condensation, by phytoene synthase, of two molecules of geranylgeranyl pyrophosphate (GGPP), to give rise to phytoene. Phytoene dehydrogenase then introduces four dehydrogenations into phytoene to produce lycopene. The next step in carotenoid biosynthesis is the cyclization of lycopene, giving rise to β-carotene via the intermediate γ-carotene, which is carried out by lycopene cyclase (Velayos et al., 2000).

3.12.1 Microorganism and Inoculum preparation

Garden soil isolate of A. terreus and the mutant strain AT SMC - 123 were maintained on PDA plates at 4°C. The standard inoculum used was in the order of 10^5 spores/ml (final concentration) harvested from 7 day-old plates. For conidial production, these strains were grown on PDA at 20 – 25°C. After a week, spores were harvested and suspended in 10 ml of sterile distilled water containing 0.05% (v/v) Tween 80. The concentration of spore suspension was determined with a Neubauer chamber and adjusted with sterile distilled water to 1×10^5 spores/ml (Calvo et al., 2007).

3.12.2 Preparation of fungal cultures

Culture of A. terreus and the mutant AT SMC - 123 were grown in PDB, adjusted to pH 5.6. The cultures following inoculation were shaken at 200 rpm at room temperature maintained at room temperature with 12 h cycle of light and dark conditions. The cultures were harvested after 10 days of inoculation.

3.12.3 Isolation of Genomic DNA from fungi cultures

Fungal genomic DNA was extracted using the Shrimpex Microbial genomic DNA extraction kit (Shrimpex Biotech Services Ltd., Chennai, India). Fungal cultures obtained were transferred to 2 ml screw capped micro centrifuge tubes, centrifuged at 13000 rpm for 1 min and the supernatant was discarded. 1 ml of Lysis Buffer was added to harvested cells and vortexed
for 1 min with beads. Tubes were centrifuged at rate of 13,000 rpm for 3 min. 700 μl of the supernatant was transferred to another 1.5 ml tube and 700 μl of Binding buffer was also added, inverted five times to mix properly. 650 μl of lysate was loaded on to the spin columns and centrifuged at 10,000 rpm for 15 s. This process was carried out for entire volume (Sometimes we have to spin 2 to 3 times to process the entire volume, if processed volume is more). If the total volume of lysate is approximately 2 ml it will take up to 3 spins to process the entire volume. Avoid flow through lysate touching the bottom of the spin membranes. This is an optional step to improve the binding efficiency and to prevent loss of nucleic acids during subsequent wash steps. The volume was centrifuged once more at 10,000 rpm for 15 s to remove residual lysate left behind in the membranes. 600 μl of 70% ethanol was added to the spin columns and centrifuged at 10,000 rpm for 15 s and discarded the flow through. This wash step was repeated two more times to improve the quality of nucleic acid isolation. Flow through was discarded and spin column were placed into the new collection tube and again centrifuged for 3 min at 13,400 rpm to remove the residual ethanol. Column was transferred to a new collection tube and 50 to 100 μl of Elution Buffer was added on to the membrane and left for 1 min. It is optional to incubate the columns after adding the Elution Buffer for a period of 5 min to improve the elution efficiency. Tubes were centrifuged again at 13,000 rpm for 1 min for DNA elution and columns were discarded. DNA was ready for downstream applications.

3.12.4 Gel Electrophoresis

Genomic DNA extracted was determined using 0.8% agarose gel on Tris-acetate-EDTA (TAE) buffer. EtBr was added to the gel mix to a final concentration of 0.2 μg/ml and samples were mixed with 6X loading buffer (0.25 % w/v bromophenol blue and 40 % w/v sucrose). 10 μl of 1 kb DNA ladder were loaded onto the agarose gel and the electrophoresis was run at 100 watts for 45 min. Bands were visualized using a UV transilluminator and the gel images were captured using Gel documentation system (Gel Doc Fire Reader Documentation System GENi Bangalore, India) (Sambrook and Russel, 2001).
3.12.5 Generation of PCR primers for phytoene synthase

Phytoene synthase is the key enzyme involved in the initial production of phytoene, that also have a bifunctional activity of lycopene cyclase in producing β-carotene in the carotenoid pathway. Hence degenerated oligonucleotides were designed for use as PCR primers. Two sets of primers were designed for the phytoene synthase for PCR amplification (Table 1) (Velayos et al., 2000).

3.12.6 Amplification using PCR

PCR was done in 16 μl volume with 2 μl template DNA and 2 μl primer (0.5 μM) using the Taq PCR Master Mix system (Shrimpex Biotech, Chennai, India) with the manufacture’s recommended buffer, enzyme and nucleotide (1X PCR Master Mix: 0.05 U/µl of Taq DNA polymerase, Tris HCl (pH 9.0), reaction buffer, 2 mM MgCl₂ and 0.2 mM dNTPs mixture). Product was amplified in Eppendorf Realplex Fast Real Time Gradient PCR system using the following conditions: initial denaturation of 95ºC for 15 min; 35 cycles (denaturation of 95°C, 30 s; annealing at 55°C, 30 s; extension of 72°C, 1 min); final extension at 72°C, 6 min. PCR product was analyzed by agarose gel electrophoresis (1.0% (w/v) agarose; 100 V, 20 to 30 min) and EtBr staining (Velayos et al., 2000).

3.12.7 Gel Electrophoresis

DNA quality and PCR products were determined using 1% agarose gel on Tris-acetate-EDTA (TAE) buffer. EtBr was added to the gel mix to a final concentration of 0.2 μg/ml and samples were mixed with 6X loading buffer (0.25 % w/v bromophenol blue and 40 % w/v sucrose). 10 μl of 1 kb DNA ladder was loaded onto the agarose gel and the electrophoresis was run at 100 watts for 45 min. Bands were visualized using a UV transilluminator and the gel images were captured using Gel documentation system (Gel Doc Fire Reader Documentation System GENi Bangalore, India) (Sambrook and Russel, 2001).
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<td>R 5’ ATGCTCGAGCCGCAATCGGG 3’</td>
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