pathway has evolved from various different primary and secondary biosynthesis pathways, Spiering et al., (2005).

Fungal endophytes of the genera *Epichloe* and *Neotyphodium* form symbioses with grasses of the subfamily Pooideae, in which they can synthesize an array of bioprotective alkaloids. Some strains produce the ergopeptine alkaloid ergovaline which is implicated in livestock toxicosis caused by ingestion of endophyte infected grasses. Cloning and analysis of a nonribosomal peptide synthetase (NRPS) gene from *N. lolii* revealed a putative gene cluster for ergovaline biosynthesis. All genes in the cluster were highly expressed in planta but expression was very low or undetectable in mycelia from axenic culture. This work provides a genetic foundation for elucidating biochemical steps in the ergovaline pathway, the ecological role of individual ergot alkaloid compounds and the regulation of their synthesis in planta, Fleetwood et al., (2007).

**MATERIALS AND METHODS**

1. **COLLECTION OF PLANT SAMPLES**

   Selection of host plants from appropriate sites and obtaining fresh plant material is important for studying the occurrence and distribution of fungal endophytes.

1.1 **Host and site collection**

   Two medicinal plant samples were chosen and collected from the Irula tribe women’s welfare society (ITWSS), Thandarai (12°39′32″–12.6°39′32″N:79.6°2′45″- 80°2′45″E), with an average annual temperature 29°C, Uthiramerur taluk, Kancheepuram district near Chengalpet which is located in South Chennai. The samples were collected every 3 months during a year (June – Aug, Sep – Nov, Dec – Feb, Mar - May) and the collection was repeated for two years.

1.2 **Collection of Plant samples**
The two plants were chosen on the rationale of potent medicinal property, growing among the tropics and clade.

The two medicinal plants were:

I. *Asparagus racemosus* (Shatawari)

II. *Hemidesmus indicus* (Nannari)

1.2.1 *Asparagus racemosus*

*Asparagus racemosus* Wild. belongs to the lily family, Liliaceae, Clade - Monocots. Some of the other important medicinal plant species belonging to this genus are: *Asparagus adscendens* Roxb., *A. filicinus* Lam., *A. gonoclados* Baker, *A. officinalis* Linn. and *A. sarmentosus* Wild.

It is a much-branched, spinous woody climber growing to 1-2 m length. The stem is scandent, woody, triquetrous, striate, terete and climbing. Young stems are very delicate, brittle and smooth. Leaves are reduced to minute chaffy scales and spines, cladodes triquetrous and curved in tufts of 2-6. The needle-like pinnate leaves were small and uniform. Flowers were white fragrant in simple or branched racemes on the naked nodes of the main shoots or in the axils of the thorns. Fruits were globular or obscurely 3-lobed, pulpy berries, purplish black when ripe, seeds with hard and brittle testa. The tuberous succulent roots were 30 cm to 100 cm or more in length, fascicled at the stem base, smooth tapering at both ends and are finger-like and clustered (Fig: 1) (Chawla et al., 2011; Joy et al., 1998).

1.2.2 *Hemidesmus indicus*

*Hemidesmus indicus* (Linn.) *R. Br. syn. Periploca indica* Linn. belongs to the family Asclepiadaceae, Clade - Eudicots.

It is a perennial, slender, laticiferous, twining or prostrate, wiry shrub with woody rootstock and numerous slender, terete stems having thickened nodes. The thin creeper vine trails on the ground and climbs by means of tendrils growing in pairs from the petioles of the alternate, orbicular to ovate, evergreen slender leaves closely resembling blades of grass, and they maintain a uniform shiny dark green color throughout the year. Leaves are simple, opposite, very variable from elliptic-oblong to linear-lanceolate, variegated with white above and silvery white and pubescent beneath. Flowers are greenish purple crowded in sub-sessile cymes in the opposite leaf-axils. Fruits are slender follicles, cylindrical, 10 cm long, tapering to a point at the apex. Seeds are
flattened, black, ovate-oblong and silvery white. The vine emerges from a long, tuberous rootstock, and can reach up to 1-3 m. The tuberous root is dark-brown, silvery white, tortuous with transversely cracked and are known to be very aromatic, emitting a sweet scent reminiscent of a combination of vanilla, cinnamon and almonds. The Ayurvedic texts mention two varieties, viz. a krsna or black variety and a sveta or white variety which together constitute the pair, Saribadvayam (Fig: 2),(Aiyer et al., 1957; Joy et al., 1998).

The leaf samples of both the plants were collected respectively and placed in separate self sealing plastic bags and returned to the laboratory on the same day and kept at 4°C until the next morning for the isolation of endophytic fungi.

2. ISOLATION OF ENDOPHYTES – SEASONAL RECURRENCE STUDIES

Fresh and healthy leaf parts of the plant are the critical requirements for isolation of endophytes as longer storage might result in desiccation and also a balanced adequate aeration is important to prevent the growth of secondary contaminating fungi/bacteria.
2.1 Surface sterilization of plant material

Preliminary screening was done by adopting ten different methods of surface sterilization procedures for evaluating the best method in yielding more number of endophytic isolates. Asymptomatic healthy plant samples were first thoroughly washed in running tap water for 10 min. Leaf segments measuring 5-8 mm² were then cut from the leaf samples using a sterile scalpel.

Following which surface sterilization procedure was carried out aseptically according to the procedure of Suryanarayan and Thennarasan (2004) and Schulz et al., (1993) with slight modifications. Different methods of sterilization as shown in Table 1 were adopted to kill the epiphytic fungal flora for the isolation of endophytes.

2.2 Preliminary sample inoculation

338 surface sterilized leaf segments per plant per method of sterilization were inoculated on to petri dishes containing PDA amended with chloramphenicol (120 mg l⁻¹).

Composition of Potato Dextrose Agar (PDA)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>200 g</td>
</tr>
</tbody>
</table>

Fig: 2 *Hemidesmus indicus*
Dextrose - 20 g  
Agar - 17 g  
Distilled water - 1000 ml  
pH - 5.6 ± 0.2

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. Five segments were placed in each petri dish using sterile scalpel under the laminar air hood and were kept for incubation in a light chamber with a light regimen of 12 h:12 h light:dark cycle for 21 days at 21±6°C (Suryanarayan and Thennarasan, 2004).

Table. 1 Different methods of treatment adopted for isolation of endophytes

<table>
<thead>
<tr>
<th>Method</th>
<th>Concentration Of Surface sterilizing agent</th>
<th>Time in solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.1% HgCl₂; 70% EtOH</td>
<td>1 min</td>
</tr>
<tr>
<td>II</td>
<td>0.2% HgCl₂; 70% EtOH</td>
<td>35 s in HgCl₂; 1 min in EtOH</td>
</tr>
<tr>
<td>III</td>
<td>0.3% HgCl₂; 70% EtOH</td>
<td>25 s in HgCl₂; 1 min in EtOH</td>
</tr>
<tr>
<td>IV</td>
<td>0.4% HgCl₂; 70% EtOH</td>
<td>15 s in HgCl₂; 1 min in EtOH</td>
</tr>
<tr>
<td>V</td>
<td>0.2% HgCl₂; 25% EtOH</td>
<td>30 s in HgCl₂; 2 min in EtOH</td>
</tr>
<tr>
<td>VI</td>
<td>0.2% HgCl₂; 50% EtOH</td>
<td>30 s in HgCl₂; 1.5 min in EtOH</td>
</tr>
</tbody>
</table>
Following preliminary inoculation procedures, number of endophytic isolates and their percentage in each method was determined respectively. The method yielding highest number of endophytic isolates was thus evaluated and chosen for further procedure.

2.3 Isolation of endophytes

Method VII (0.2% HgCl₂ for 30 s, 75% Ethanol for 1 min) yielding higher number of endophytic fungal isolates was chosen for surface sterilization of plant samples. 338 surface sterilized leaf segments per sampling per plant were inoculated on to petri dishes containing PDA amended with chloramphenicol (120 mg/l). Five segments were placed in each petridish using sterile scalpel under the laminar air hood and were kept for incubation in a light chamber with a light regimen of 12 h:12 h light:dark cycle for 21 days at 21±6°C.

2.4 Subculturing

The growing edges of colonies from the segments were transferred to a fresh PDA plates by hyphal tipping and subcultured. Pure cultures of the isolates were maintained.

2.5 Identification of fungal isolates
Pure cultures were examined periodically for sporulation and identified. Fungal 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. The identification of molds is based on the shape, method of production and arrangement of spores (conidial ontogeny). Also wet mount preparation by lactophenol cotton blue staining methods were 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>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton Blue</td>
<td>50 mg</td>
</tr>
<tr>
<td>Phenol</td>
<td>20 g</td>
</tr>
<tr>
<td>L(+)-Lactic Acid</td>
<td>20 ml</td>
</tr>
<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 (Philip Thomas et al., 1991).

### 2.6 Statistical analysis

Measurement of fungal occurrence was established by calculating colonization density, colonization rates and isolation rates. The density of colonization was calculated as the percentage of segments infected by one or more isolate(s) from the total number of segments of each tissue plated following the method of Petrini and Fisher (1988).

\[
\text{Colonization rate} = \frac{\text{Total no. of leaf segments in a sample yielding } \geq 1 \text{ isolates}}{\text{Total no. of leaf segments in that sample}}
\]

\[
\text{Total no. of isolates yielded by a given sample}
\]
Isolation rate = Total no. of leaf segments in that sample

The fungal endophytes were isolated and identified. Samples were collected every season and endophytes were isolated for a period of consecutive 24 months in duplicates.

One way ANOVA was performed to compare the isolation rates and colonization rates of fungal endophytes of each plant occurring from four different seasons (Kumar and Hyde, 2004). The final data was also comparatively studied for the consecutive period of 24 months for recurring or differing results significantly.

3. SECONDARY METABOLITE PRODUCTION OF A. STRICTUM

Among the several endophytic fungi isolated from the plant, A. strictum was chosen for further studies due to its common prevalence in both plants recurring mostly. Secondary metabolites are low molecular weight metabolites often have potent physiological activities produced by an organism that are not required for primary metabolic processes. There are so many factors influencing the growth in turn on the production of secondary metabolites and hence preliminary growth studies are necessitated.

3.1 Preliminary growth studies of Acremonium strictum

Two 500 ml Erlenmeyer flasks containing each 100 ml of Potato Dextrose Broth (PDB) amended with chloramphenicol (12 mg/100 ml) was autoclaved at 121°C for 15 min at 15 lb pressure. Each flask was inoculated with either 10 μl of a spore suspension of A. strictum (10^4 spore/ml) from 7 days old cultures of A. strictum on the solid PDA medium or 10 μl of sterile water (blank). 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 0th 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 (Meletiadis et al., 2003; Trinci, 1972).
3.2 Extraction and analysis of secondary metabolites

3.2.1 Preparation of fermentation media

500 ml Erlenmeyer flasks containing each 250 ml of PDB amended with chloramphenicol (12 mg/100 ml) was autoclaved at 121°C for 15 min at 15 lb pressure. Each flask was inoculated with cultures of *A. strictum* as previously described and were incubated for 18 days at 26°C.

3.2.2 Extract preparation

At the end of growth period, after 18 days of culture, inoculated flasks were collected and the culture fluid was passed through four layers of cheese cloth to remove solids and extracted with organic solvents (Strobel *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:

*Extraction of mycelia:*

The fresh mycelium of each fungus was washed three times with sterile distilled water to remove adherent filtrate and then plotted between folds of sterilized filter paper No 1. The plotted 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 three times, left in separating funnel for 15 min to precipitate. The crude extract was collected.

*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, 1999 and Lin et al., 2000).

3.2.3 Analysis of secondary metabolites

Physical properties of biologically active extract were subjected to the following analysis. UV spectra were obtained between 200 and 400 nm using UV-VIS spectrophotometer for all the extracts obtained from mycelia as well as filtrate. Similarly an Infra Red spectra (IR) was obtained on the Fourier Transform Infra Red spectrometer (Nicolet, model 670, USA) using pellets of KBr. The extracts were then subjected for qualitative analysis of various metabolites (Mabrouk et al., 2008).

The following reagents were used 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 concentrated 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 Flavonoids (Shinoda test)
To 3 ml of extract, 1 g of Magnesium Sulphate followed by 2 drops of concentrated HCl was added to give red colouration.

Test for Saponins (Frothing test)
2 ml of the extract was vigorously shaken 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 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.

4. **FATTY ACIDS PRODUCTION FROM ACREMONIUM STRICTUM**

   Microorganisms produce lipids 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.

4.1 Microorganism and inoculum preparation
A. strictum, the chosen endophytic strain was maintained on PDA plates at 4°C. The standard inoculum used was in order of $10^5$ spores/ml (final concentration) harvested from 7 day-old plates. For conidial production, A. strictum 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 $1\times10^5$ spores/ml. Conidia of A. strictum were thus obtained from 7 day old PDA cultures grown at 20–25°C. The concentration was also adjusted to $1\times10^5$ spores/ml (Calvo et al., 2007).

4.2 Standardization of media for strain improvement

250 ml of Potato dextrose broth cultures amended with chloramphenicol (12mg/100 ml) were prepared in 500 ml Erlenmeyer flasks and subjected to conditions of varying pH ranging from 3 to 8, 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**

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

**Potato Sucrose Broth**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>200 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>5.6 ± 0.2</td>
</tr>
</tbody>
</table>

**Richards’s Broth**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium sulphate</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>5 g</td>
</tr>
</tbody>
</table>
Potassium nitrate - 10 g
Potato starch - 10 g
Sucrose - 50 g
Distilled water - 1000 ml
pH - 5.4

**Oat Meal Broth**
Rolled oats - 30 g
Distilled water - 1000 ml
pH - 7.2

**Corn Meal Broth**
Corn - 50 g
Distilled water - 1000 ml
pH - 6.5

**Potato Dextrose Broth with Yeast Extract (PDB-YE)**
Potato - 20 g
Dextrose - 2 g
Ammonium tartarate - 0.1 g
Potassium dihydrogen phosphate - 0.7 g
Magnesium sulphate - 0.15 g
Disodium hydrogen phosphate - 0.2 g
Yeast extract - 0.15 g
Calcium chloride - 0.01 g
Cobalt nitrate - 0.00001 g
Distilled water - 100 ml
pH - 6

Culture of potato dextrose broth adjusted to pH 5.6 served as the mother culture. The growth phase was periodically checked to attain the maximum growth by taking optical density at 405 nm using UV-VIS spectrophotometer (Meletiadis et al., 2003; Trinci, 1972). 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 and the conditions required to obtain maximum growth was hence determined from the growth curve.

4.3 Biomass production

At the end of growth period, after 18 days of culture, the inoculated flasks were collected and the culture was passed through Whatmann filter paper No.1 to separate the mycelia biomass. The filtered mycelia was then washed twice with sterile distilled water and dried in hot air oven at 60°C (Yongnanitichai 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.

4.4 Extraction of fatty acid

The Bligh and Dyer method is a simple adaptation of the Folch procedure and was developed merely as an economical means (in terms of solvent volumes) of extracting lipids. 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.

4.5 TLC profiling

The extracts obtained were then subjected for TLC (Thin Layer Chromatography) profiling on precoated silica gel 60 F$_{254}$ 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).

4.6 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:

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

4.7 Preparation of FAMEs

Owing to increased acid value, the fatty acid extracted from the culture grown in PDB-YE was chosen for FAMEs preparation to be analyzed 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-MS (Nelson, 2010).

4.8 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 250ºC respectively. The oven was programmed for 2 min at 160ºC then increased to 180ºC at 6ºC/min, maintained for 2 min at 180ºC, increased further to 230ºC at 4ºC/min and finally maintained for 10 min at 230ºC. 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).

Thereby fatty acids extracted from various seed cultures being optimized for various parameters was subjected for acid value estimation of fatty acids and quantified. The culture media corresponding to increased biomass and fatty acid production optimized for its own parameters was further employed for strain improvement studies.

5. ENHANCEMENT OF FATTY ACID PRODUCTION USING ELICITORS

5.1 Microorganism and inoculum preparation
A. strictum, the chosen endophytic strain was maintained on PDA plates at 4°C. The standard inoculum used was in order of $10^5$ spores/ml (final concentration) harvested from 7 day-old plates. For conidial production, A. strictum 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 $1\times10^5$ spores/ml. Conidia of A. strictum were thus obtained from 7 day old PDA cultures grown at 20–25°C. The concentration was also adjusted to $1\times10^5$ spores/ml (Calvo et al., 2007).

5.2 Preparation of culture media and conditions

PDB-YE (optimized by standardization) was prepared according to the following composition amended with following different metal ion salt concentrations as elicitors ranging from 1 µg to 500 mg of Magnesium sulphate ($\text{MgSO}_4\cdot7\text{H}_2\text{O}$), Manganese sulphate ($\text{MnSO}_4\cdot5\text{H}_2\text{O}$), Copper sulphate ($\text{CuSO}_4\cdot5\text{H}_2\text{O}$), Zinc sulphate ($\text{ZnSO}_4\cdot7\text{H}_2\text{O}$) and Ferric chloride ($\text{FeCl}_3\cdot6\text{H}_2\text{O}$).

**Composition of PDB-YE**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>20 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>2 g</td>
</tr>
<tr>
<td>Ammonium tartarate</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>0.7 g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.15 g</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.15 g</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.01 g</td>
</tr>
<tr>
<td>Cobalt nitrate</td>
<td>0.00001 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
<tr>
<td>pH (optimized by standardization)</td>
<td>6</td>
</tr>
</tbody>
</table>

Different stock solutions of metallic salts were prepared according to Table 2. To 50 ml of the culture media required amount of stock solution was added according to Table 3 and made up to 100 ml, chloramphenicol (12 mg/100 ml) was added followed by autoclaving at 121°C at 15 lb pressure for 15 min.
Culture of PDB-YE served as the control. The cultures following inoculation were maintained at room temperature with 12 h cycle of light and dark conditions. The cultures were harvested after 18 days of inoculation (Muhid et al., 2008).

5.3 Biomass production

At the end of growth period, after 18 days of culture, inoculated flasks were collected and the culture was passed through Whatmann filter paper No.1 to separate the mycelia biomass. The filtered mycelia was then washed twice with sterile distilled water and 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 cultural conditions influencing the production of highest biomass.

5.4 Extraction of fatty acid

The Bligh and Dyer method is a simple adaptation of the Folch procedure and was developed merely as an economical means (in terms of solvent volumes) of extracting lipids. 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 chloroform phase. The fat content was usually 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 30 mg dried biomass was weighed, powdered by 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.

5.5 Thin Layer Chromatography (TLC) profiling

The extracts obtained were then subjected for TLC profiling on pre-coated silica gel 60 F\textsubscript{254} TLC plate (E. Merck 0.2 mm thick) using palm oil as standard. 5 μl of samples were applied on the plate using a capillary tube in a horizontal line. 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 2/3\textsuperscript{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 (Christie and Han, 2010).

Table: 2 Stock solution preparation of metal salts

<table>
<thead>
<tr>
<th>Metal salt</th>
<th>Stock 1 (mg/ml)</th>
<th>Stock 2 (mg/ml)</th>
<th>Stock 3 (mg/ml)</th>
<th>Stock 4 (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO(_4).7H(_2)O</td>
<td>10</td>
<td>50</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>MnSO(_4).5H(_2)O</td>
<td>10</td>
<td>50</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>CuSO(_4).5H(_2)O</td>
<td>10</td>
<td>50</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>ZnSO(_4).7H(_2)O</td>
<td>10</td>
<td>50</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>FeCl(_3).6H(_2)O</td>
<td>10</td>
<td>50</td>
<td>100</td>
<td>200</td>
</tr>
</tbody>
</table>

Table: 3 Amount of Stock to be added for the preparation of culture media

<table>
<thead>
<tr>
<th>Metal salt concentration</th>
<th>Amount of stock to be added</th>
<th>Stock solution used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µg</td>
<td>0.1 µl</td>
<td>Stock 1</td>
</tr>
<tr>
<td>5 µg</td>
<td>0.1 µl</td>
<td>Stock 2</td>
</tr>
<tr>
<td>10 µg</td>
<td>1 µl</td>
<td>Stock 1</td>
</tr>
<tr>
<td>25 µg</td>
<td>0.5 µl</td>
<td>Stock 2</td>
</tr>
<tr>
<td>Concentration (µg)</td>
<td>Volume (µl)</td>
<td>Stock</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------</td>
<td>-------</td>
</tr>
<tr>
<td>50 µg</td>
<td>1 µl</td>
<td>Stock 2</td>
</tr>
<tr>
<td>75 µg</td>
<td>1.5 µl</td>
<td>Stock 2</td>
</tr>
<tr>
<td>100 µg</td>
<td>10 µl</td>
<td>Stock 1</td>
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<tr>
<td>500 µg</td>
<td>10 µl</td>
<td>Stock 2</td>
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<tr>
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<tr>
<td>5 mg</td>
<td>50 µl</td>
<td>Stock 3</td>
</tr>
<tr>
<td>10 mg</td>
<td>0.1 ml</td>
<td>Stock 3</td>
</tr>
<tr>
<td>15 mg</td>
<td>75 µl</td>
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</tr>
<tr>
<td>20 mg</td>
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<td>3 ml</td>
<td>Stock 3</td>
</tr>
<tr>
<td>400 mg</td>
<td>4 ml</td>
<td>Stock 3</td>
</tr>
<tr>
<td>500 mg</td>
<td>5 ml</td>
<td>Stock 3</td>
</tr>
</tbody>
</table>
5.6 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 formulae:

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

Thereby fatty acids extracted were subjected for acid value estimation and quantification.

5.7 Preparation of FAMEs

Owing to increased acid value, the strain grown in PDB-YE amended with 500 µg salt concentration, hereafter referred to as ASIS01 was chosen for FAME preparation. 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 50 mg lipid sample obtained previously
were dissolved in 1 ml toluene in a test tube fitted with a condenser and 2 ml of 1% sulfuric acid in methanol was added before the mixture was refluxed for 2 h (or alternatively the mixture can be left overnight in a stoppered tube at 50°C. 5 ml water 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 4 ml water containing potassium bicarbonate (2%) and dried over anhydrous sodium sulfate and then subjected to GC – FID (Nelson, 2010).

5.8 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).

Gas Chromatography was performed on Agilent 6890 series GC set 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.

The improved strain ASIS01 yielding higher biomass and fatty acid production was chosen for further studies.
6. **PURIFICATION OF OLEIC ACID**

The extracted fatty acid methyl esters were then subjected for High Performance Liquid Chromatography (HPLC). The analytical conditions were as follows: pump, LC-5A (Shimadzu); column, Inertsil ODS-2 (4.6 × 250 mm; GL Science, Tokyo, Japan); detector, SPD-2A (Shimadzu); wavelength, 205 nm; mobile phase, acetonitrile/water (95:5, vol/vol); flow rate, 1 ml/min; and column temperature, 30°C. Fraction containing Oleic acid ester was eluted, concentrated and subjected for GC-FID analysis under the same conditions described above comparing with the standard. Chromatogram was obtained. The purified oleic acid was further checked for its potent biological activity (Shirasaka et al., 1998).

7. **ANTICANCER STUDY OF OLEIC ACID AGAINST MCF-7 AND HEP-G2 CELL LINES**

7.1 **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 minutes 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).

7.2 **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).

7.3 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).

7.4 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 Oleic acid sample, serial dilutions were prepared by dissolving compounds in DMSO followed by dilution with RPMI-1640 medium to give final concentration at 10, 50, 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 Oleic acid 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 Oleic acid required to cause a 50% reduction (IC$_{50}$) in growth (cell number) for each cell lines.

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

8. **ANTIOXIDANT ACTIVITY OF OLEIC ACID USING DPPH ASSAY**

The radical scavenging activity of Oleic acid 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 (Oleic acid) 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 Oleic acid fraction. Blank contained 0.1 mM ethanolic DPPH solution without Oleic acid and vortexed thoroughly. The setup was left at 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 + Oleic acid.

Measurements were performed in triplicates. Absorbance values were corrected for radical decay using blank solutions. The IC$_{50}$ (concentration providing 50% inhibition) was calculated graphically using a calibration curve plotted with scavenging percentage against essential Oleic acid concentration.

9. **APOPTOTIC DNA FRAGMENTATION ASSAY**

0.5 ml of cell suspension was suspended and centrifuged at 200 x 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 Mg<sup>2+</sup> 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 was 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 and transparent 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).

10. MOLECULAR STUDY USING PCR

Owing to higher production of Oleic acid in the ASIS01, initial molecular studies were done to isolate the δ-9 Fatty Acid Desaturase gene mediating Oleic acid production using PCR amplification technique. The pathway for fatty acid desaturation and elongation from Stearic acid (18:0) to MUFAs (Monounsaturated fatty acids) and long-chain polyunsaturated fatty acids (LCPUFAs) has been elucidated both by biochemical means and by studying mutant strains that
multiple fatty acid δ-9 desaturases encoded by distinct genes have been found so far in fungi, rat, mouse, carp, Drosophila, Caenorhabditis and Arabidopsis.

10.1 Microorganism and Inoculum preparation

A. strictum, the chosen endophytic strain was maintained on PDA plates at 4°C. The standard inoculum used was in order of 10^5 spores/ml (final concentration) harvested from 7 day-old plates. For conidial production, A. strictum 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. Conidia of A. strictum were thus obtained from 7 day old PDA cultures grown at 20–25°C. The concentration was also adjusted to 1×10^5 spores/ml (Calvo et al., 2007).

10.2 Preparation of fungal cultures

Culture of A. strictum was grown in PDB, adjusted to pH 5.6. The strain ASIS01 was grown in the PDB-YE culture media standardized with 500 μg of salt concentrations (MgSO_4·7H_2O, MnSO_4·5H_2O, CuSO_4·5H_2O, ZnSO_4·7H_2O and FeCl_3·6H_2O) for higher Oleic acid production. 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 18 days of inoculation.

10.3 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.

10.4 Gel Electrophoresis

Genomic DNA extracted was determined using 0.8% agarose gel on Tris-acetate-EDTA (TAE) buffer. Ethidium bromide 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).

10.5 Generation of PCR primers for δ-9 fatty acid desaturase (D-9, FAS)

The δ-9 Fatty Acid Desaturase is the key enzyme involved in the initial D9-desaturation of Stearic acid 18:0 (stearoyl-CoA) to Oleic acid (18:1) in fatty acid biosynthesis degenerate oligonucleotides were designed for use as PCR primers. Two sets of primers were designed for the δ -9 fatty acid desaturase gene responsible for Oleic acid production (Table 4) (Kowalchuk et al., 2002).

10.6 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 95°C 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 ethidium bromide staining (Kowalchuk et al., 2002).

10.7 Gel Electrophoresis

DNA quality and PCR products were determined using 1% agarose gel on Tris-acetate-EDTA (TAE) buffer. Ethidium bromide 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).
Table: 4 Primers for δ -9 fatty acid desaturase

<table>
<thead>
<tr>
<th>PRIMER NAME</th>
<th>SEQUENCE(5’-3’)</th>
</tr>
</thead>
</table>
| δ -9 , FAS Primer 1 (469 bp) | F 5’TACCACRWMTTCCRCCACSMKT3’  
R 5’CGCTTCCAAAYCTCMACCTCRC3’ |
| δ -9 , FAS Primer 2 (483 bp) | F 5’CCCTHGGHAGGGHTACCAYAA3’  
R 5’CCGAGCTGCTCCAGATCCAGA3’ |