Materials & Methods
3.1 Isolation and screening of actinomycete strains from laterite soil sample

Laterite soil sample collected for the isolation of potent actinomycete strains was initially analysed for physico-chemical properties such as moisture content (%), pH, organic carbon (%) and total nitrogen content (%).

3.1.1 Soil Characteristics

3.1.1.1 Moisture content

To determine the moisture content, 10 g of soil sample was dried in a hot air oven at 105°C until a constant weight is obtained. The difference between the weights of pre-drying and post-drying was taken as the moisture content of the initial soil sample.

3.1.1.2 Soil pH

The pH of the collected soil sample was determined with the help of digital pH meter of model Di-707 (Jackson, 1973). 20 g of soil sample was taken in a 100 ml beaker and 40 ml of distilled water was added. The suspension was mixed thoroughly and allowed to settle the suspended clay particles from the suspension for about 1h before recording the pH.

3.1.1.3 Organic carbon

Organic carbon content of the soil sample was estimated by following Walkey and Black method (1934). 10 g of soil sample was taken into 500 ml Erlenmeyer flask. To this 10 ml of 1N \( \text{K}_2\text{Cr}_2\text{O}_7 \) was added and shaken gently to disperse the soil into the solution. 20 ml of Conc. \( \text{H}_2\text{SO}_4 \) was added followed by vigorous agitation for 1min. 200 ml of distilled water was added and filtered. Few drops of \( \text{O} - \) phenanthroline indicator was added to the filtered solution and titrated against 0.5N \( \text{FeSO}_4 \cdot 7\text{H}_2\text{O} \). Sample without soil served as blank. The amount of organic carbon in the soil sample was calculated by using the following formula.
(milliequivalents of K$_2$Cr$_2$O$_7$ – milliequivalents of FeSO$_4$. 7H$_2$O)x 0.003x$100$
Organic carbon (%) = \frac{\text{Soil weight (g)}}{f}
\quad f = \text{correlation factor} = 1.33

3.1.1.D Total nitrogen

Total nitrogen in the soil sample was determined by Micro - Kjeldahl method.

Reagents

- Conc. H$_2$SO$_4$, mercuric oxide, K$_2$SO$_4$, sodium hydroxide – sodium thiosulphate solution (60% NaOH and 5%Na$_2$SO$_3$. 5H$_2$O in distilled water), 0.02N standard HCl , 4% boric acid solution , mixed indicator solution (10ml of 0.2% methyl red in ethanol mixed with 50ml of 0.2% bromocresol green in ethanol)

Procedure

To the finely sieved soil sample (100 g) taken into a digestion flask, 2 g of K$_2$SO$_4$, 90 mg of mercuric oxide and 2 ml of Conc. H$_2$SO$_4$ were added , mixed thoroughly and kept for digestion on heater. Before carrying out of distillation process,limited addition of distilled water (5 ml) to the flask was followed by the addition of Sodium hydroxide – sodium thiosulphate solution was added. Ammonia was collected in boric acid. The distillate (20ml) collected was titrated against 0.02N HCl. Appearance of violet colour is the end point. Blank was maintained using equal volume of distilled water instead of distillate. Total nitrogen present in the soil sample was calculated by using the formula

\[
\text{Total nitrogen} = \frac{[\{\text{HCl (ml) in sample} - \text{HCl (ml) in blank}\} \times \text{normality of acid} \times 14.01 \times 100]}{\text{Soil weight (mg)}}
\]
3.1.2 Isolation of actinomycete strains

Laterite soil sample collected at a depth of 6 – 10 cm was pretreated with calcium carbonate (1:1 w/w) and dried at 45°C for 1 h in order to reduce the incidence of bacteria and molds (El-Nakeeb and Lechhevalier, 1963). Yeast extract malt extract dextrose agar (YMD) and starch casein salts agar media were prepared, sterilized at 15 lbs pressure (120°C) for 15 min and poured into Petri plates under aseptic conditions. Both streptomycin (100 µg/ml) and nystatin (50 µg/ml) were added to the media just before pouring into Petri plate. Soil dilution plate technique was employed for isolation of actinomycete strains (Williams and Cross, 1971). The pretreated soil (1 g) sample was suspended in 100 ml of sterile distilled water. Serial dilutions were prepared and 0.1ml of $10^{-3}$ and $10^{-4}$ dilutions were plated on media with the help of a spreader. The inoculated plates were incubated at 30°C for 10 days. After incubation, actinomycete colonies were isolated from soil.

Streak plate method was used to purify the cultures of actinomycete strains. The colonies were picked either by straight wire or loop according to the condition. The picked up specks of the colonies were streaked over YMD agar medium followed by incubation at 30°C for 7 days. Further, pure cultures were maintained on YMD agar slants and stored at 4°C for further study (Williams and Cross, 1971).

3.1.3 Screening of predominant actinomycete strain for bioactive metabolites

Out of 10 actinomycete strains isolated from two different media, one predominant strain designated as MSL was chosen for bioactive metabolite production. The secondary metabolites produced by actinomycete strain were extracted by the method of Elliah et al. (2005). The pure culture of the strain was transferred aseptically into the seed medium (ISP-2 broth). After 24 h of incubation, the seed culture at a rate of 10% was inoculated into the production medium of the
same composition. Fermentation was carried out at 30°C for 1 week under agitation at 180 rpm. At every 24 h interval, the flasks were harvested and the biomass was separated from the broth. The dry weight of the biomass was recorded and expressed as g/100ml. The culture filtrate was extracted twice with ethyl acetate and the pooled solvent extracts were concentrated under vacuum to yield a crude residue. The residue dissolved in methanol was used for testing antimicrobial activity.

3.1.3. A Antimicrobial assay

Bacteria employed for testing include *Bacillus cereus*, *B. megaterium*, *B. subtilis*, *Escherichia coli*, *Klebsiella* sp., *Pseudomonas aeruginosa*, *P. solanacearum*, *Salmonella typhi*, *Shigella flexneri*, *Staphylococcus aureus*, *Vibrio cholerae* and *Xanthomonas campestris* while *Alternaria* sp., *Asperigillus niger*, *Botrytis cinerea*, *Candida albicans*, *Fusarium solani*, *F. oxysporum* and *Verticillium alboatrum* were used as test fungi.

The antimicrobial activity of metabolites produced by the strain was determined by agar well diffusion method (Cappuccino and Sherman, 2004). Nutrient agar (NA) and Czapek-Dox (CD) agar media were used for culturing the test bacteria and fungi respectively. NA medium (100 ml) was sterilized at 15 lbs pressure (121°C) for 15 min, cooled and inoculated with 0.1 ml of test bacterial suspension. After thorough mixing, the inoculated medium was poured into Petri plates under aseptic conditions. After solidification of agar medium, wells of about 6 mm diameter were punched with sterilized cork borer. In case of antifungal assay, test fungus (10^5 spores/ml) was plated onto the solidified CD agar plate. Methanolic extract (50 ppm) was added to each well while the addition of only methanol served as control. The inoculated plates were incubated at 30°C and the diameter of the inhibition zone was measured after 24 h of incubation for bacteria and 24-72 h for yeast and filamentous fungi.
As the strain MSL exhibited high antimicrobial activity against bacteria and fungi tested, attempts were made for its identification.

3.2 Taxonomic studies of the actinomycete strain MSL

Cultural, morphological and physiological characteristics together with genomic (16s rDNA gene sequencing) analysis of the strain were studied.

3.2.1 Cultural characterization

The growth characteristics of the strain was studied on seven ISP media such as ISP-1 (Tryptone-yeast extract agar), ISP-2 (Yeast extract malt extract dextrose agar), ISP-3 (Oat meal agar), ISP-4 (Inorganic salts starch agar), ISP-5 (Glycerol-asparagine salts agar), ISP-6 (Peptone yeast extract iron agar medium) and ISP-7 (Tyrosine agar) as well as on five non ISP media like Nutrient agar (NA), Czapek Dox (CD) agar, Bennett’s agar, Glucose Tryptone (GT) agar and Starch casein (SC) agar with initial pH 7.2 maintained at 30°C (Dietz and Thayer, 1980). The composition of the culture media is presented in table 2. Cultural characters such as growth, color of the aerial and substrate mycelia and pigment production were observed (Shirling and Gottlieb, 1966).

3.2.2 Morphological studies

ISP-2 grown culture was used to observe the detailed micromorphology of the strain through Scanning Electron Microscopy (SEM) as per the procedure described by the Bozzola and Russella (1999). The culture was fixed in 2.5% gluteraldehyde prepared in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4°C followed by the post fixation step in 2% aqueous osmium tetroxide for 4 h in the same buffer. The sample was then dehydrated in ethanol and then dried up to critical with the help of Electron Microscopy science CPD unit (Ruska Labs, Acharya NG
<table>
<thead>
<tr>
<th>Medium code</th>
<th>Name of the medium</th>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ISP media</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISP-1</td>
<td>Tryptone - yeast extract agar</td>
<td>Tryptone: 1.0; yeast extract: 0.3; agar: 2</td>
</tr>
<tr>
<td>ISP-2</td>
<td>Yeast extract malt extract dextrose agar</td>
<td>Yeast extract: 0.4; malt extract: 1.0; Dextrose: 0.4; CaCO₃: 0.2; agar: 2</td>
</tr>
<tr>
<td>ISP-3</td>
<td>Oat meal agar</td>
<td>Oat meal: 2; trace salt solution (FeSO₄·7H₂O: 0.01, MnCl₂·4H₂O: 0.01, ZnSO₄·7H₂O: 0.01); agar: 2</td>
</tr>
<tr>
<td>ISP-4</td>
<td>Inorganic salts starch agar</td>
<td>Soluble starch: 1; K₂HPO₄: 0.1; MgSO₄·7H₂O: 0.1; NaCl: 0.1; (NH₄)₂SO₄: 0.2; CaCO₃: 0.2; trace salt solution (FeSO₄·7H₂O: 0.01, MnCl₂·4H₂O: 0.01, ZnSO₄·7H₂O: 0.01); agar: 2</td>
</tr>
<tr>
<td>ISP-5</td>
<td>Glycerol - asparagine salts agar</td>
<td>L-asparagine: 0.1; glycerol: 1; K₂HPO₄: 0.1; trace salt solution (FeSO₄·7H₂O: 0.01, MnCl₂·4H₂O: 0.01, ZnSO₄·7H₂O: 0.01); agar: 2</td>
</tr>
<tr>
<td>ISP-6</td>
<td>Peptone yeast extract iron agar medium</td>
<td>Peptone: 1; yeast extract: 0.4; FeSO₄·7H₂O: 1.0; agar: 2</td>
</tr>
<tr>
<td>ISP-7</td>
<td>Tyrosine agar</td>
<td>L-tyrosine: 0.1; agar: 2</td>
</tr>
<tr>
<td><strong>Non-ISP media</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>Nutrient agar</td>
<td>Peptone: 0.5; Beef extract: 0.3; NaCl: 0.5; agar: 2</td>
</tr>
<tr>
<td>CD agar</td>
<td>Czapek Dox agar</td>
<td>Sucrose: 3; sodium nitrate: 0.2; K₂HPO₄: 0.1; MgSO₄·7H₂O: 0.05; KCl: 0.05; FeSO₄·7H₂O: 0.01; agar: 2</td>
</tr>
<tr>
<td>Bennett’s agar</td>
<td></td>
<td>Glucose: 2; yeast extract: 0.1; K₂HPO₄: 0.1; MgSO₄·7H₂O: 0.05; KCl: 0.05; FeSO₄·7H₂O: 0.01; agar: 2</td>
</tr>
<tr>
<td>GT agar</td>
<td>Glucose tryptone agar</td>
<td>Glucose: 1; tryptone: 0.5; K₂HPO₄: 0.1; FeSO₄·7H₂O: 0.01; agar: 2</td>
</tr>
<tr>
<td>SC agar</td>
<td>Starch casein agar</td>
<td>Starch: 0.1, casein: 0.2; K₂HPO₄: 0.1; MgSO₄·7H₂O: 0.1; FeSO₄·7H₂O: 0.01; agar: 2</td>
</tr>
</tbody>
</table>
Ranga University, Hyderabad, India). The dried sample was mounted on aluminum stubs covered with double-sided carbon tape. A thin layer of gold coating was applied over the sample by using automated sputter coaster for 3 min (JEOL JFC -1600, Japan). Finally, the samples were examined under SEM at various magnifications (Model: JOEL-JSM 5600, Japan).

3.2.3 Physiological and biochemical tests of the predominant strain MSL

3.2.3.1 Utilization of carbon compounds

The utilization of carbon sources by the strains was carried out according to the method described by Gottlieb (1961). The minimal medium employed for carbon utilization test consists of KH$_2$PO$_4$ - 0.2%, K$_2$HPO$_4$ - 0.5%, MgSO$_4$·7H$_2$O - 0.1%, CuSO$_4$·3H$_2$O - 0.1%, FeSO$_4$·7H$_2$O - 0.1%, MnCl$_2$·4H$_2$O - 0.1%, ZnSO$_4$·7H$_2$O - 0.02%, (NH$_4$)$_2$SO$_4$ - 0.2% and agar - 2% with pH adjusted to 7.0. Carbon sources such as dextrose, fructose, galactose, glycerol, lactose, maltose, mannitol, sucrose and xylose were added separately at the rate of 1% to the molten carbohydrate free minimal medium and poured into sterilized Petri plates. After solidification, the plates were inoculated with the strain and incubated at 30°C for 7 days. Growth of the organism on different carbon sources was rated as good, moderate and poor.

3.2.3.2 Sodium chloride tolerance test

Sodium chloride (NaCl) tolerance of the strain was determined by the method suggested by Tresner et al. (1968). ISP-2 medium was prepared and amended with different concentrations of NaCl (0-7%) and autoclaved at 15 lbs pressure (121°C) for 15 min. Sterilized medium was poured into Petri plates and allowed to solidify. Growth of the strain at different concentrations of NaCl was recorded by the inoculating the plates with the strain and incubated at 30°C for 7 days.
3.2.3.3 Indole test

This test facilitates to determine the ability of the strain for the production of indole from tryptophan. Tryptone broth was prepared and sterilized in test tubes. A loopful culture of the strain was inoculated and incubated at 28-30°C for 48-72 h. After incubation, 10 drops of Kovac’s reagent was added and the test tubes were left undisturbed for a few min. The tubes were observed for the development of cherry red color (Holding and Collee, 1971).

3.2.3.4 Methly red and Voges – Proskaeur (MR-VP) test

MR-VP broth contains glucose, peptone, and a phosphate buffer. Organisms that perform mixed-acid fermentation produce enough acid to overcome the buffering capacity of the broth, so a decrease in pH results. Organisms that perform other kinds of fermentation cannot overcome the buffering capacity of the broth. After incubation, the pH indicator Methyl Red is added to the broth. Methyl Red is red at pH below 4.4 (this would be a positive result) and yellow at pH above 6.0. The strain MSL was inoculated in MR – VP broth, incubated at 30°C for 48 – 72 h and observed for the color change.

3.2.3.5 Citrate utilization test

Citrate utilization test was performed to assess the ability of the strain to utilize sodium citrate. A loopful culture of the strain was inoculated into the test tubes slants containing sterilized Simmons citrate agar medium. After incubation of the test tubes at 28-30°C for 48-72 h, the color change of the medium from green color to blue was observed (Holding and Collee, 1971).

3.2.3.6 Melanin Test

The ability of the strain to produce melanin pigment was tested on ISP-7 medium. The strain was inoculated on tyrosine agar plates and kept for incubation at 30°C for 5-7 days. After
incubation, the plates were examined for brown or black color around the colonies that indicates the melanin test as positive (Baumann et.al., 1976).

3.2.3.7 Hydrogen sulphide test

Sulphide Indole Motility (SIM) agar medium was used to test the ability of the strain for the production of H₂S. The medium was prepared as per the composition and the strain was inoculated by Stab technique. Control was maintained without inoculation of the strain. The tubes were incubated at 28°C for 48-72 h and observed for the color change along the stab (Kuster and Williams, 1984).

3.2.3.8 Enzymatic profile of the strain

3.2.3.8.A Amylase

Starch agar medium was used to test the strain for the production of amylase. The culture was inoculated in the Petri plate containing solidified starch agar medium. The Petri dish was incubated at 28-30°C for 48 h. After incubation the plate was flooded with Gram’s iodine solution (2:1 of potassium iodide and iodine in 300 ml of distilled water). A clear zone around the colony against the blue background was taken as positive (Holding and colle, 1971).

3.2.3.8.B Cellulase

To detect the cellulolytic activity of the strains, modified ISP-4 medium with cellulose (1%) in place of starch was employed. Inoculated plates were incubated at 30°C for 7 days. Hydrolysis of cellulose was seen as clear yellow zone within a reddish brown background after flooding the plates with a mixture of hydrochloric acid and iodine solution, prepared by mixing 1 ml of 0.1 N HCl with 5ml of 2% KI (Yeoh et al., 1985).
3.2.3.8.C L-asparaginase

To determine the ability of the strain to secrete L-asparaginase that catalyzes the hydrolysis of L-asparaginase to L-aspartic acid and ammonium ion. Modified ISP-5 medium with L-asparagine (1%) and phenol red (0.03%) with initial pH 7.0 was prepared. Sterilized medium was poured into Petri plates and allowed to solidify. The plates were inoculated with the strain and incubated at 30°C for 48-72 h. Change of dye color around the actinomycete colony from yellow to pink indicates the positive reaction while no color change interprets the test as negative (Gulati et al., 1997).

3.2.3.8.D Catalase

Sterilized trypticase soy agar slants inoculated with the strain were incubated at 30°C for 48-72 h. After incubation, 3-4 drops of hydrogen peroxide was allowed to flow over the culture. The tubes were observed for the appearance or absence of air bubbles (Holding and Collee, 1971).

3.2.3.8.E Chitinase

Colloidal chitin was prepared according to the method of Avelizapa et al. (1999). Chitin powder (5 mg) was added slowly to 90 ml of concentrated HCl under vigorous stirring for 2 h, followed by the addition of 1L distilled water. Thus obtained fine white precipitate was collected at 4°C by centrifugation (6,000 rpm) for 10 min. It was then washed repeatedly with distilled water until the colloidal chitin became neutral (pH 7) followed by drying at 50°C and used for further studies. Colloidal chitin (0.5%) was added to the rest of the components of Chitin Yeast extract Salts (CYS) broth. The plates were inoculated with the strain and observed for a zone of clearance around the actinomycete colony after incubation at 30°C for 7 days.
3.2.3.8.F Deoxyribonuclease (DNase)

Production of the enzyme capable of degrading deoxyribonucleic acid (DNA) was tested by inoculating the strain on DNA agar plates. After 3-5 days of incubation, plates were flooded with 1N HCl and the appearance of clear zone around the actinomycete colony infers the degradation of DNA in the medium (Hankin and Anagnostakis, 1975).

3.2.3.8.G Nitrate reductase

Sterilized trypticase nitrate broth inoculated with the strain was incubated at 30°C for 6 days. After incubation, 5 drops of reagent A (8 g of sulphanilic acid in 1000 ml of 5 N acetic acid) and 5 drops of solution B (10 g of α-naphthalamine in 1000 ml of 5 N acetic acid) were added to the culture tubes and observed for color change. Appearance of cherry red color indicates the ability of the strain to produce nitrate reductase (Holding and Collee, 1971).

3.2.3.8.H Protease

Proteolytic activity of the strain was determined in terms of caseinolysis. Starch casein agar medium were prepared, autoclaved and poured into sterilized Petri plates. After solidification, the strain was inoculated and incubated at 30°C. After 3 days of incubation, appearance of clear zone around the actinomycete colony indicates the test as positive (Holding and Collee, 1971).

3.2.3.8.I Ribonuclease (RNase):

The culture medium described by Jeffries et al., (1957) was used to detect the ability of the strains to degrade ribonucleic acid used as a test substrate. Test medium was prepared with a pH of 7.0. The RNA (2 mg/ml) was dissolved carefully in 1N NaOH and added to the liquefied basal medium. Plates were poured as soon as the medium cooled to 50°C. After solidification, the plates were inoculated with the strain and incubated at 30°C for 5 days. Degradation of RNA
in the test medium was observed in the form of clear zone around the actinomycete colony after flooding the plates with 1N HCl (Hankin and Anagnostakis, 1975).

3.2.3.8. Urease:

To detect the urease activity of the strain, Christensen agar medium was prepared, sterilized and poured into sterile Petri plates. The strain was inoculated at the center of the agar medium and the plates were incubated at 30°C for 48 h and observed for color change from yellow to pink around the colonies indicating positive reaction (Holding and Collee, 1971).

3.2.4 Molecular identification of the strain based on 16s rDNA sequence analysis

The strain was grown in YMD broth for 3 days and was centrifuged at 10,000 rpm for 20 min and the pellet was used for the extraction of DNA (Mehling et al., 1995). PCR mixture consisted of 2.5 μl of 10× buffer, 3.5 μl of MgCl₂ (25 mM), 2 μl of dNTP (0.4 mM), 1 μl of 16S rDNA actino specific primer - forward (10 pmol/μl), 1 μl of 16S rDNA actino specific primer- reverse (10 pmol/μl), Taq polymerase (2 U/μl) and 2 μl template DNA. PCR amplification was carried out as follows: Initial denaturation step at 94 °C for 3 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 1 min, with a further 5 min extension at 72°C. The PCR product was purified with Agarose Gel DNA Purification Kit (SoluteReady® Genomic DNA purification kit, PCR Master Mix, Agarose gel electrophoresis consumables and Primers from HELINI Biomolecules, Chennai, India). The 750 bp 16S rDNA sequence was determined with 16S rDNA actino specific forward and reverse primers. The deduced 16s rDNA sequence was compared with the sequences in GenBank (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST) then aligned with the related reference sequences retrieved from NCBI GenBank databases using the
Clustal W method. Phylogenetic and molecular evolutionary analyses were conducted using Molecular Evolutionary Genetic analysis (MEGA) version 4.0 (Tamura et al. 2007).

3.3 Optimization of physiological and cultural parameters for the enhanced production of secondary metabolites by the strain

3.3.1 Effect of Incubation time on growth and metabolite production

The strain was inoculated into 500 ml flasks containing 200 ml YMD broth and incubated at 30°C on a rotary shaker at 180 rpm. At every 24 h interval up to 7 days, the flasks were harvested, the growth of the strain was measured in terms of dry weight of biomass while antimicrobial metabolite production was determined in terms of its antimicrobial spectrum. The culture filtrate extracted with ethyl acetate was tested for antimicrobial activity by agar well diffusion method (Cappuccino and Sherman, 2004).

3.3.2 Influence of pH and temperature on growth and antimicrobial metabolite production

The strain was inoculated into sterilized YMD broth with pH ranging from 5-9 adjusted using HCl (1M) and NaOH (1M). For temperature study, YMD broth with optimized pH was inoculated and incubated at different temperatures ranging from 20-45°C. Both the growth and bioactive metabolite production by the strain was measured and recorded.

3.3.3 Effect of carbon and nitrogen sources on growth and production of bioactive metabolites

To determine the effect of carbon sources on growth and bioactive metabolite production, different carbon sources such as D-fructose, lactose, galactose, sucrose, maltose, arabinose, mannitol, xylose and starch each at a concentration of 0.4% were added to the YMD medium replacing dextrose. Influence of various levels of the best carbon source (0.2 - 5%) among the carbon sources tested on growth and bioactive metabolite production was also examined.
The influence of various nitrogen sources such as sodium nitrate, ammonium nitrite, ammonium sulphate, ammonium chloride, peptone, beef extract, tryptone, casein, L-asparagine, tyrosine and urea was studied by adding nitrogen source (0.4%) to the medium with optimized carbon source. Further, the optimal levels of the best nitrogen source (0.2 - 5%) among the nitrogen sources tested supporting optimal yields of bioactive metabolites was also recorded. The optimal concentration of carbon and nitrogen sources supporting the maximum production of metabolite was selected for the further studies.

3.3.4 Impact of sodium chloride (NaCl) on growth and antimicrobial activity of the strain

Salt concentration has a profound effect on the production of antibiotic from microorganism due to its effect on the osmotic pressure to the medium (Pelczar et al., 1993). NaCl (0-5%) was added to the medium containing optimized carbon and nitrogen sources. Both the growth and antimicrobial activity was tested at each concentration of NaCl and the results were recorded.

3.3.5 Antimicrobial spectrum of the strain in the optimized medium

The culture inoculated into the optimized medium (100 ml) was incubated in 250 ml flasks with shaking at 180 rpm for 120 h at 30°C. The broth was then harvested and growth of the strain was measured in terms of dry weight of biomass and antimicrobial metabolite production was determined in terms of its antimicrobial spectrum.

3.4 Extraction, purification and structural confirmation of bioactive compounds produced by the strain

3.4.1 Production of bioactive metabolites

For the large scale production of bioactive compounds from the strain, 10 % of seed broth was inoculated into the optimized production medium (galactose @ 6g, yeast extract @ 10g, malt extract @ 10g, sodium chloride @ 10g dissolved in one liter distilled water and adjusted to
pH 7.0) for the enhanced secondary metabolite production. The fermentation was carried out in 1L Roux bottles for 120 h at 30 °C.

3.4.2 Isolation, purification and identification of bioactive compounds

The bioactive compounds from the fermented broth was harvested by filtration of biomass through Whatman filter paper no. 42 (Merck, Mumbai, India). The culture filtrate (25L) was extracted twice with an equal volume of ethyl acetate, pooled and the organic layer was concentrated in a Rotavac. The deep brown semi solid compound (3.21g) obtained was applied to a silica gel G column (80x2.5cm, Silica gel, Merck, Mumbai, India). The separation of the crude extract was conducted via gradient elution with Hexane: Ethyl acetate. The eluent was run over the column and small volumes of eluent collected in test tubes were analyzed via thin-layer chromatography (TLC) using silica gel plates (Silica gel, Merck, Mumbai, India) with Hexane: Ethyl acetate solvent system. Compounds with identical retention factors ($R_f$) were combined and assayed for antimicrobial activities. The crude eluent was recuperated in 5-10 ml of ethyl acetate, and was further purified.

Among the 14 main fractions eluted, 10 fractions were polar and 4 were non polar residues. Antimicrobial activity was tested for all the fractions obtained. Among the 10 polar fractions eluted from the crude extract 4 fractions exhibited high antimicrobial activity. B1 (90-10v/v), B2 (70-30v/v), B3 (60-40v/v) and B4 (50-50v/v) were the fractions collected at different eluent Hexane:Ethyl acetate solvent system. B2 and B3 compounds exhibiting the highest antimicrobial activity were rechromatographed using different gradient eluent systems for final elucidation of compounds. The fractions B1 and B4 on further purification yielded two compounds each in pure form. The structure of these active fractions was analyzed on the basis of Fourier Transform Infrared (FTIR); model: Thermo Nicolet Nexus 670 spectrophotometer.
with NaCl optics, Electron Ionization Mass/Electron Spray Ionization Mass Spectrophotometry (EIMS/ESIMS); model: Micromass VG – 7070H, 70eV spectrophotometer, Nuclear Magnetic Resonance (\(^1\)H NMR and \(^13\)C NMR) model: Varian Gemini 200 and samples were made in CDCl\(_3\) with Trimethyl silane as standard.

3.4.3 Minimum Inhibitory Concentration (MIC) of bioactive compounds

Antibacterial activity of compounds was done by micro-dilution method in 96 well microplates. The bioactive fractions were dissolved in MeOH to prepare the required concentrations of the compounds ranging from 0 to 500 µg/ml. Each well was inoculated with 10 µl of suspension containing \(10^5\) CFU/ml of test bacteria. Antibiotic tetracycline was used as the positive control. The plates were incubated at 37°C for 24 h. After incubation 10 µl of 2, 3, 5-triphenyltetrazolium chloride was added to each well. Appearance of red color indicates bacterial growth, while the absence of color indicates the inhibition of bacterial growth by the bioactive compounds. Similarly to determine the MIC of the compound against test fungi, \(10^5\) spores/ml were dispensed into the 24 well microtitre plates. Increasing concentrations of the compounds (0 to 500µg/ml) was added to the wells and incubated at 28±2°C. The concentration of the compounds that completely inhibited the growth of fungi was recorded after 3-4 days.

3.5 L-asparaginase production by Streptomyces sp. MSL

To screen the strain for the production of L-asparaginase, culture was inoculated into modified ISP-5 agar containing glycerol (1%), L-asparagine (1%), K\(_2\)HPO\(_4\) (0.1%), trace salt solution (Fe\(\text{SO}_4\).7\(\text{H}_2\)\(\text{O}\) - 0.01%, Mn\(\text{Cl}_2\).7\(\text{H}_2\)\(\text{O}\) - 0.01%, Zn\(\text{SO}_4\).7\(\text{H}_2\)\(\text{O}\) - 0.01%) - 0.1% with initial pH 7.0. Phenol red (0.1%) was added to the Petri dish before inoculation. The inoculated Petri plates were incubated at 35°C for 4 days and observed for the formation of red color around the colony.
3.5.1 L-asparaginase assay

The enzyme assay was performed as described by Pertson and Ciegler (1969) with minor modifications. Biomass was separated by centrifuging the culture broth at 10,000 rpm for 15 min and was mixed with Tris-HCl buffer. It was again centrifuged and the cell free extract (0.2 ml) obtained was mixed with 0.8 ml of 0.05M L-asparagine. After incubating the reaction mixture for 15 min at 37°C in a waterbath shaker, the reaction was terminated by the addition of 0.5 ml of trichloroacetic acid (15% w/v). Precipitated proteins were removed by centrifugation and the liberated ammonia was determined spectrophotometrically at 500 nm by Nesslerization. Tubes kept at zero time incubation served as control. Enzyme production was determined on the basis of liberation of ammonia calculated with reference to a standard curve of ammonium sulfate. One L-asparaginase unit (U) equals to the amount of enzyme which releases 1µM of ammonia per minute at 37°C. Growth of the strain was also recorded by measuring the dry weight of the biomass obtained during centrifugation step.

3.5.2 Optimization of L-asparaginase production

Influence of various parameters including pH, temperature, carbon and nitrogen sources on the production of L-asparaginase was determined by growing the strain in modified ISP-5 broth for 96 h.

3.5.2.A Impact of pH on growth and L-asparaginase production by the strain

Impact of pH on L-asparaginase production was examined by culturing the strain in modified ISP – 5 broth adjusted to pH levels ranging from 4 to 10. Both the growth of the strain and enzyme activity was determined. The optimal pH achieved at this step was used for further study.
3.5.2.B Influence of temperature on growth and enzyme production by the strain

To determine the optimal temperature for growth and L-asparaginase production, the strain inoculated in modified ISP-5 broth with optimal pH was incubated at different temperatures viz., 20ºC to 40ºC for 96 h.

3.5.2.C Effect of carbon sources on growth and enzyme production by the strain

To investigate the effect of carbon sources on L-asparaginase production by the strain, modified ISP-5 broth was supplemented with different carbon sources such as arabinose, fructose, galactose, glucose, lactose, maltose, starch and sucrose each at a concentration of 1% (w/v). The carbon source which supports good yields of L-asparaginase production by the strain was chosen for further study. Impact of different concentrations of the best carbon source (0.2-5%) among the other carbon sources tested on L-asparaginase production by the strain was studied and the optimal level achieved was fixed for further studies.

3.5.2.D Effect of nitrogen sources on growth and enzyme production by the strain

The effect of nitrogen sources was determined by amending different inorganic and organic nitrogen sources viz., beef extract, peptone, tryptone, yeast extract, ammonium nitrate, sodium nitrate and urea at the rate of 1% (w/v) to modified ISP-5 broth. The nitrogen source found to be good for optimal L-asparaginase productivity was selected for further study. Besides, the optimal concentration of nitrogen source (0.2-5% w/v) supporting high yields of L-asparaginase production by the strain was determined by maintaining all other conditions at optimal level.
3.6 Chitinase production by *Streptomyces* sp. MSL

For screening the strain for production of chitinase, the strain was inoculated in colloidal chitin agar medium and incubated at 28-30°C for about 4 days. Clear zone around the colony indicates the utilization of colloidal chitin by the production of chitinase enzyme.

3.6.1 Chitinase assay:

Chitinolytic activity in the medium was determined by using colloidal chitin as a substrate (Thamthiankul et al., 2001) by measuring the release of reducing sugars (GlcNAc) with dinitrosalicylic acid method (DNS) (Miller, 1959). One milliliter of reaction mixture contained 1ml of substrate (1% w/v) aqueous suspension of the colloidal chitin), 0.5 ml of 0.05 M citrate phosphate buffer (pH 6.0) and 1ml of the enzyme sample (culture supernatant). Reaction mixture was incubated in a water bath at 35°C for 60 min. Reaction was stopped by adding 3 ml of dinitrosalicylic acid followed by boiling in a water bath for 10 min. The reaction mixture was diluted to obtain a total volume of 10 ml with distilled water. The OD values were recorded 540 nm using UV Spectrophotometer. A standard curve for N-acetyl glucosamine was carried out in parallel to measure reducing sugar released. A unit of enzyme activity was defined as the amount of enzyme required to release 1µM of NAG h⁻¹ at 37°C.

3.6.2 Optimization of Nutritional and Physiological conditions for chitinase production:

3.6.2.A Effect of Incubation period on Growth and Enzyme activity:

The strain was inoculated into 500 ml conical flasks containing 100 ml CC broth. They were incubated at 30°C for optimum yields on a rotary shaker at 180 rpm. At every 24 h interval, the flasks were harvested and chitinase enzyme production was estimated in terms of IU/5mL and the growth was measured in terms of dry weight of biomass.
3.6.2.B Study on effect of chitin concentration on chitinase activity:

Chitin concentration was studied by adding different concentrations (0.5%, 1%, 1.5%, 2%, 3% and 5%) of colloidal chitin to the broth. The effect of chitin concentration on growth and enzyme production was measured. The experiment was performed in triplicate.

3.6.2.C Effect of pH on chitinase activity:

Impact of pH on chitinase production was examined by culturing the strain in CC broth adjusted to pH levels ranging from 4 to 10. Growth of the strain as well as enzyme production by the strain were determined. The optimal pH achieved at this step was used for further study.

3.6.2.D Effect of temperature on chitinase activity:

To determine the optimal temperature for growth and chitinase production, the strain inoculated in CC broth with optimal pH was incubated at varied temperatures viz., 20ºC to 40ºC for 96 h.

3.6.2.E Effect of supplementation of Carbon and Nitrogen sources on growth and enzyme activity of the strain

The carbon sources (1%) used were glucose, fructose, galactose, arabinose, raffinose, starch and Nitrogen sources (0.1%) tested were peptone, yeast extract, tryptone, L-asparagine, ammonium nitrate, potassium nitrite and Urea. Control was maintained for each set.

3.6.2.F Effect of different concentrations of optimized carbon and nitrogen sources

Once the carbon and nitrogen sources have been optimized attempts were made to determine the effect of various levels of optimized carbon and nitrogen source. Increasing concentrations of carbon sources such as 0.25, 0.5, 1.0, 1.5 and 2% while 0.05, 0.1, 0.15, 0.2, 0.5, 1 and 2% of nitrogen sources were added in addition to chitin in the medium. Triplicates were maintained for each set of experiment.
3.7 Screening of the strain for the production of IAA

To determine the production of IAA, culture suspension of the strain was inoculated in glucose tryptone broth supplemented with 0.5% L-Tryptophan (L-Trp) adjusted to initial pH 7.0 and incubated at 30°C. After incubation, culture broth was centrifuged at 10,000 rpm for 20 min. The supernatant (5 ml) was mixed with 1ml of 1N HCl and 4 ml of Salkowski reagent and observed for the development of pink colour. Optical density was read at 530 nm in a spectrophotometer (Glickmann and Dessaux, 1995). Standard Graph was constructed using increasing concentration of IAA (Himedia,Mumbai, India).

3.7.2 Extraction of IAA from culture broth

A loopful culture of the strain was inoculated into the GT broth containing 0.5% L-tryptophan and incubated at 30°C and incubated for 7 days. The broth was harvested every 24 h and tested for the production of IAA in the culture broth. The culture filtrate was collected and adjusted to pH 3.5 with 1N HCl. The acidified culture filtrate when extracted with ethyl acetate and vacuum dried at 40°C appeared as a brown semisolid compound. The crude extract was partially purified using silica gel column chromatography and fractions were collected during ethyl acetate : hexane (20:80 v/v). Each fraction was tested on TLC with the solvent system and then developed with the Salkowski reagent (0.01M FeCl2 in 35% HClO4) (Ehmann, 1977). The indole containing fraction was further purified in preparative high-performance liquid chromatography (HPLC, normal phase, silica column, 10x250 mm, 5 µl using hexane/ 2-propanol, 8:2) and the pure compound obtained was structurally identified by (1H NMR), 13C NMR and EIMS.
3.7.3 Determination of optimal nutritional and physiological parameters for improved growth and production of IAA

3.7.3.A Effect of Precursor (L-Tryptophan) concentration, pH and temperature on growth and IAA production of the strain

Precursor concentration was studied by adding different concentrations (0.5%, 1%, 1.5%, 2%, 3% and 5%) of L-Tryptophan to GT broth. A range of pH (5, 6, 7, 8 and 9) was adjusted to the GT broth amended with optimized precursor concentration. The optimal temperature of the strain for the growth and IAA production was determined by incubating the inoculated culture broth maintained with optimized initial pH in a range of temperatures (20, 30, 40 and 50).

3.7.3.B Impact of carbon and nitrogen sources on growth and IAA production of the strain

Impact of various carbon sources on growth and IAA production was determined by the addition of lactose, fructose, galactose, arabinose, raffinose and starch (1%) to the GT broth replacing glucose. For the investigation of suitable nitrogen source, beef extract, peptone, sodium nitrate, potassium nitrate, urea and L-asparagine (0.5%) were supplemented to the culture broth containing optimized L-Tryptophan concentration maintaining already optimized physiological conditions.

3.7.3.C Influence of various levels of optimized carbon and nitrogen sources on growth and IAA production

Once the carbon and nitrogen sources effecting the growth and IAA production of the strain was determined, investigations were carried out to verify the levels of optimized carbon and nitrogen sources. To achieve this, increasing concentrations of carbon and nitrogen sources (0.5%, 1%, 1.5%, 02%, 3% and 5%) were supplemented to the production medium. The growth of the strain was determined in terms of weight of biomass (mg/100ml) and IAA production in terms of µg/ml.