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# BIOACTIVE METABOLITE PRODUCTION BY *TRITIRACHIUM EGENUM* ISOLATED FROM SOUTHEAST COAST OF ANDHRA PRADESH, INDIA

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**Keywords:**

*Tritirachium egenum*, Antimicrobial metabolites, Marine fungi

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**ABSTRACT:** A study has been undertaken to isolate potent fungi from the South-east coastal zone of Andhra Pradesh. Among the 11 fungal strains isolated and screened, one potent strain with broad-spectrum antagonistic activity was found. Based on the morphological, cultural, and molecular characteristics, the potent strain was identified as *Tritirachium egenum*. Production of bioactive metabolites by the strain was high in malt extract broth compared to other media tested. The strain utilized mannitol and ammonium sulphate as good carbon and nitrogen sources for the elaboration of antimicrobial metabolites. The optimum pH and temperature for bioactive metabolite production by strain were recorded at 4.0 and 30°C, respectively. The secondary metabolites produced by the strain grown under optimal conditions exhibited high antagonistic activity against Gram-positive and Gram-negative bacteria and fungi. This is the first report of antimicrobial metabolites produced by *Tritirachium egenum* isolated from the south-east coast of Andhra Pradesh.

**INTRODUCTION:** The need for novel substances to treat several human diseases like cancer supports the intensive exploration of new substances from marine organisms [1]. Oceans are sources of a large group of structurally unique natural products mainly accumulated in marine organisms. Several of these secondary metabolites possess pronounced pharmacological activities [2]. Microorganisms such as fungi and bacteria are truly prolific producers of bioactive molecules [3–5]. The marine ecosystem is characterized by very special conditions that differ from other habitats. Marine microorganisms often produce structurally unique bioactive secondary metabolites that are not found in terrestrial organisms [6].

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Focus on marine microbes is a highly sustainable approach aiming to conserve natural habitats since only a small quantity of samples is required. These microbes are considered an excellent source of secondary metabolites, presumably evolved for specific functions like protecting the host against diseases. From a biotechnological point of view, many of these compounds have pharmaceutical properties, often antibiotic or cytotoxic, that may be useful as lead structures for developing new drugs [7]. Therefore, using marine fungi, prolific producers of bioactive compounds provides a solution for the supply issue.

Marine fungi are known to produce natural products for various ecological purposes. Fungal natural products are often produced in response to many environmental cues, which might also be small molecules [8]. Despite their potential for secondary metabolite production, marine fungi are still poorly characterized and under-utilized for biotechnological applications [9]. A literature survey covering more than 23,000 bioactive microbial

products, *i.e.*, antifungal, antibacterial, antiviral, cytotoxic, and immune-suppressive agents, shows that the producers are mainly from fungal kingdom. Hence, fungi represent one of the most promising sources of bioactive compounds [10, 11]. Prominent examples of compounds isolated from marine fungi comprise ulocladol, halimide, avrainvillamide, pestalone and the halovirs A-E [12–14]. However, the number of available strains from marine sources is limited and the knowledge of marine fungi, in general, is scarce [15]. In the present work, an attempt has been made to isolate and identify potential fungus possessing antimicrobial activity from the samples collected from southeast coastal region of Andhra Pradesh. *Tritirachium egenum* isolated from the samples was maintained in Sabourauds dextrose agar (SDA) medium. Further studies are carried out to optimize the culture conditions of the potential isolate to enhance the production of biologically active compounds.

## MATERIALS AND METHODS:

**Collection of Soil Sample:** Soil samples collected from southeast coastal zone of Andhra Pradesh in sterile bags were transported to the research lab and preserved at 4°C.

**Isolation of Fungi:** Soil samples collected were shade dried and used for the isolation of fungi by serial dilution plate technique. Serial dilutions were prepared and 0.1ml of $10^{-3}$ and $10^{-4}$ dilutions were aseptically transferred to Petri plates containing SDA medium and incubated at 27±2°C for a week. The isolates were characterized morphologically using lactophenol cotton blue scotch tape technique [16]. The colonies were picked up and maintained as pure cultures on SDA slants and stored at 4°C for further study.

**Extraction of Bioactive Metabolites:** Fungal isolates were inoculated into 250 ml Erlenmeyer flasks containing 100 ml Sabourauds dextrose broth (SDB) and incubated at room temperature for 21 days under stationary conditions. The contents were filtered to separate mycelium from the filtrate. The metabolites produced by strain were extracted twice with ethyl acetate and pooled solvent extracts were concentrated under a vacuum to yield a crude residue. The residue dissolved in ethyl acetate was used for testing antimicrobial activity.

**Screening Bioactive Properties of Fungal Metabolites:** Antibacterial activity of secondary metabolites extracted from fungi was tested against microorganisms such as *Staphylococcus aureus, Bacillus subtilis, Bacillus megaterium, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans* using agar well diffusion method. Wells were made with the help of a borer and extracts were transferred to separate wells. The zone of inhibition was detected after 24-48 h of incubation at 37°C. The presence of a zone of inhibition on plates was used as an indicator of bioactive nature of the strain. Based on the zone of inhibition, the potent metabolite-producing fungal culture is selected for further studies.

**Identification of Potent Fungal Strain:** The potent fungal strain is identified based on colony characteristics (colony size, colour, shape, appearance, pigment production) and micro-morphological (mycelium, conidiophores, and conidia) characteristics [17, 18, 19].

**Cultural and Morphological Characteristics of VJLB 1:** The strain was grown on several culture media such as Sabourauds dextrose agar (SDA), Czapek-Dox agar (CDA), potato dextrose agar (PDA), malt extract agar (MEA) and yeast extract malt extract dextrose agar (YMD) and nutrient agar for one week to study colony characteristics [16].

**Molecular Identification of Strain VJLB 1:** Molecular identification was done using 18SrRNA sequence analysis. These sequences were deposited in the gene bank (NCBI). Phylogenetic and molecular evolutionary analysis was conducted using Molecular Evolutionary Genetic Analysis (*MEGA*) version 5.0 [20].

**Growth Pattern of the Strain VJLB 1:** The strain was inoculated into SD broth and incubated at 30 ± 2°C on a rotary shaker at 180 rpm. At every 72 h interval up to 25 days, the flasks were harvested, and growth of the strain was measured in terms of the dry weight of biomass. The antimicrobial metabolite production was determined in terms of its antimicrobial activity. The culture filtrate extracted with ethylacetate was tested for antimicrobial activity by agar well diffusion method [21].

**Selection of Suitable Culture Medium for Enhanced Production of Bioactive Metabolites:** To select suitable growth medium, the isolate was grown on different culture media such as Czapek-Dox broth, Sabourauds broth, potato dextrose broth, malt extract broth, yeast extract malt extract broth and nutrient broth. The medium in which the isolate exhibited maximum bioactive metabolite production expressed in terms of zone of inhibition was notified as a suitable medium for further study. All the media were procured from HiMedia Laboratories, Mumbai, India.

**Effect of Temperature on Bioactive Metabolite Production:** The fungus was subjected to different temperature ranges (15 to 45°C) to record the optimum temperature required for bioactive metabolite yield. Under aseptic conditions, the medium was inoculated with the culture and incubated for 18 days. After incubation, the antimicrobial metabolite production was recorded.

**Effect of pH on Bioactive Metabolite Production:** The effect of pH on bioactive metabolite production by the isolate was tested at different pH levels (pH 4-9). The medium was adjusted to the desired pH by adding 0.1N NaOH or 0.1NHCl. Each flask was inoculated with mycelial discs (5mm) in sterile conditions. Inoculated flasks were incubated at 30 ± 2°C for 18 days and bioactive metabolite production was recorded.

**Effect of NaCl on Bioactive Metabolite Production:** The effect of salinity on bioactive metabolite production was carried out by growing the strain in a medium with different NaCl concentrations, ranging from 1-6%. The bioactive metabolite production at different sodium chloride concentrations was recorded.

**Effect of Carbon Sources on Bioactive Metabolite Production:** Carbon sources such as glucose, starch, sucrose, fructose, lactose, mannitol, carboxy methyl cellulose and maltose are used to study their effect on metabolite production. Carbon source@1% was added to the MEB medium. The flasks were inoculated with 5 mm mycelial discs of seven-day-old fungal culture and incubated for 18 days. At the end of the incubation period, production of bioactive metabolites was recorded.

**Effect of Nitrogen Source on Bioactive Metabolite Production:** Nitrogen sources, including beef extract, yeast extract, peptone, ammonium sulphate, urea, malt extract and sodium nitrate were used to study their influence on bioactive metabolite production. Each nitrogen source @ 1% was added to the MEB medium and dextrose was used as the carbon source in all treatments. Flasks were inoculated with 5 mm mycelial discs of seven-day-old fungal culture under aseptic conditions and incubated for 18 days. The antimicrobial compound production was recorded at the end of the incubation period.

**Fermentation, Extraction and Antimicrobial Assay of Bioactive Compounds Produced by VJLB 1:** The strain was transferred aseptically into seed medium (ME broth). After 7 days of incubation, the seed culture at a rate of 10% was inoculated into a production medium of the same composition. Fermentation was carried out at 30 ± 2°C for 18 days under agitation at 180 rpm. Secondary metabolites produced by the strain were extracted twice with ethyl acetate and pooled solvent extracts were concentrated under a vacuum to yield a crude residue. The residue dissolved in ethyl acetate was used for testing antimicrobial activity [22].

**Antimicrobial Spectrum of *T. egenum* Grown on Optimized Medium:** The culture inoculated intooptimized medium was incubated at 30°C with shaking at 180 rpm for 18 days. The broth was then harvested and antimicrobial metabolite production was determined in terms of its antimicrobial activity.

**Statistical Analysis:** The results are statistically analyzed using AGRISTAT and MINITAB16 software.

## RESULTS AND DISCUSSION:

**Sample Collection, Isolation and Identification of Fungi:** A systematic study about fungal isolates of marine habitats of southeast coastal region of Andhra Pradesh was carried out to evaluate their capacity to produce bioactive compounds. A total of 11 fungal strains designated as VJLB 1, VJLB 2, VJLB 3, VJLB 8, VJLB 9, VJLB 10, VJLB 13, VJLB 14, VJLB 15, VJLB 18 and VJLB 20 were isolated from soil samples. All the fungal strains

were screened for bioactive metabolites. All 10 isolates showed antimicrobial activity **Fig. 1**. Among the 10 isolates, VJLB 1 was found potent against test bacteria and fungi. Hence an attempt was made to identify the VJLB 1 strain.

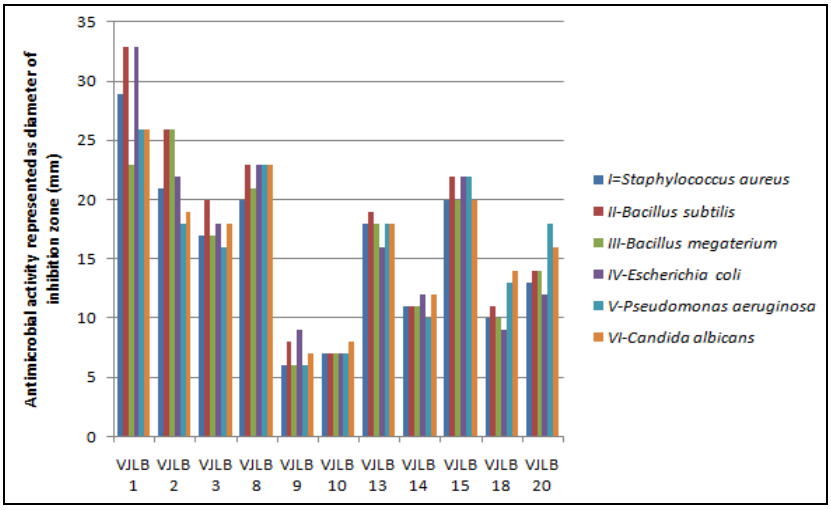


**FIG. 1: ANTIMICROBIAL METABOLITE PRODUCTION BY THE STRAINS VJLB 1 TO VJLB 20 AGAINST TEST MICROORGANISMS.** The results are analyzed statistically and found to be significant at 5% level.

**Cultural and Morphological Characteristics of VJLB 1:** Cultural characteristics of **VJLB 1** were studied on six different media *viz*. SDA, NA, CDA, PDA, YMA and MEA. VJLB 1 grew luxuriantly on MEA followed by SDA and PDA. Morphological characteristics like morphology of mycelium, conidiophore, and conidia were assessed by using the slide culture technique. Colonies are velvety with a dark brown reverse. Mycelium was composed of branched, septate hyphae, at first thin and hyaline, but becoming slightly thicker and coloured pale brown with time. Conidiophores are well differentiated and erect, slightly finer at the base and tapering towards the tip, smooth and thick-walled, septate, brownish and verticillated branched bearing three to six whorls. Conidia are acropleurogenous, hyaline, ochre in mass, smooth thin-walled, globose measuring 2– 3µm in size [23] **Fig. 2.**

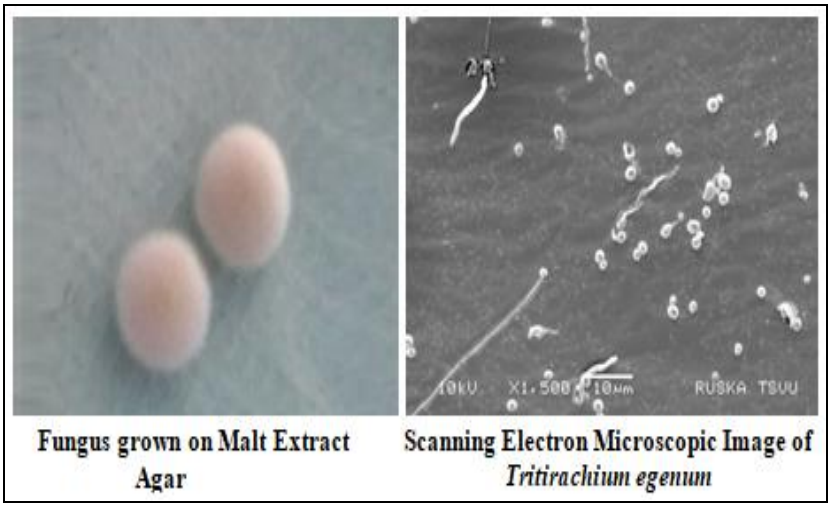


**FIG. 2: CULTURAL CHARACTERISTICS OF *TRITIRACHIUM EGENUM***

Identification of the strain based on molecular approach was also carried out based on 18S rRNA analysis. The partial sequence of the isolate was submitted to the GenBank database with accession number MH636844. The phylogenetic tree was constructed based on the Maximum Parsimony method **Fig. 3** and the strain was identified as *Tritirachium egenum.*

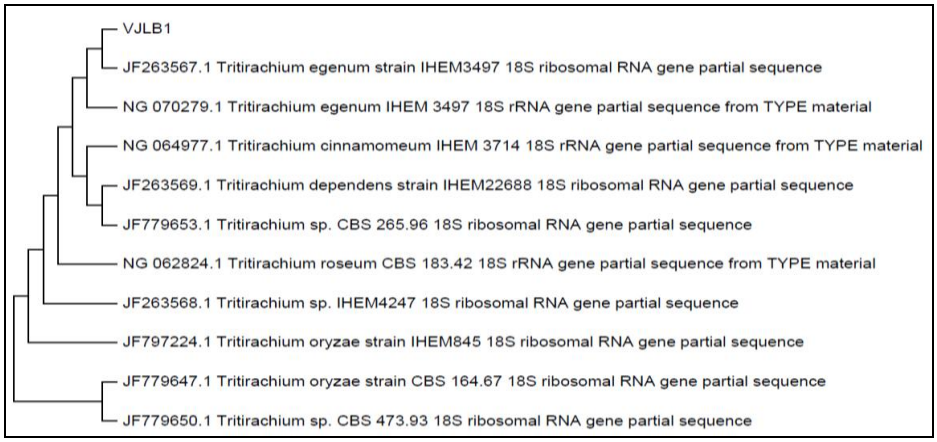


**FIG. 3: MAXIMUM PARSIMONY TREE BASED ON 18S RRNA GENE SEQUENCE SHOWING RELATIONSHIP BETWEEN ISOLATE VJLB 1 AND RELATED MEMBERS OF THE GENUS *TRITIRACHIUM EGENUM***

**Growth Pattern of VJLB 1:** Analysis of the growth pattern revealed that the culture entered into log phase on 6$^{th}$ day of incubation which extended up to 14$^{th}$ day followed by a stationary phase from 15-18 days and finally entered into the decline phase **Fig. 4.**

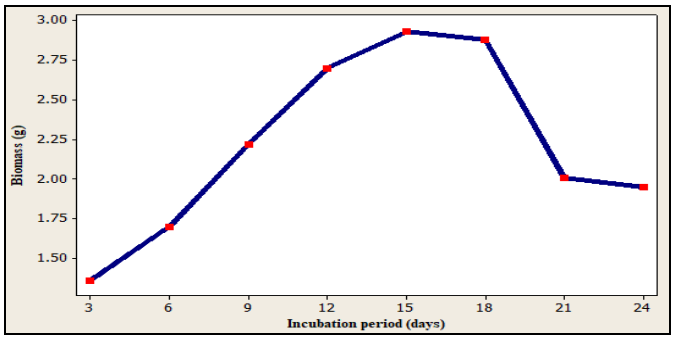


**FIG. 4: GROWTH PATTERN OF *T. EGENUM***

**Effect of Culture Media on Antimicrobial Metabolite Production by *T. egenum:*** Among the six culture media tested, ME broth was found to support antimicrobial metabolite production, followed by YMB, PDB **Fig. 5.**

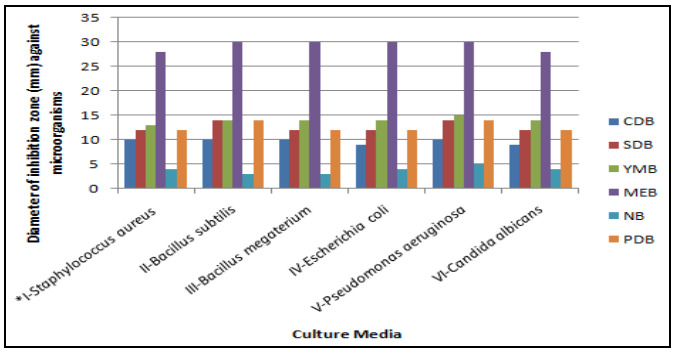


**FIG. 5: INFLUENCE OF CULTURE MEDIA ON ANTIMICROBIAL METABOLITE PRODUCTION BY *T. EGENUM* AGAINST TEST MICROORGANISMS.** The results are analyzed statistically and found to be significant at 5% level.

**Effect of pH on Antimicrobial Metabolite Production by *T. egenum*:** The influence of pH on bioactive metabolite production was determined by adjusting the pH of MEB from 4.0 to 9.0. Maximum antimicrobial metabolite production was observed at pH 4.0 **Fig. 6.**

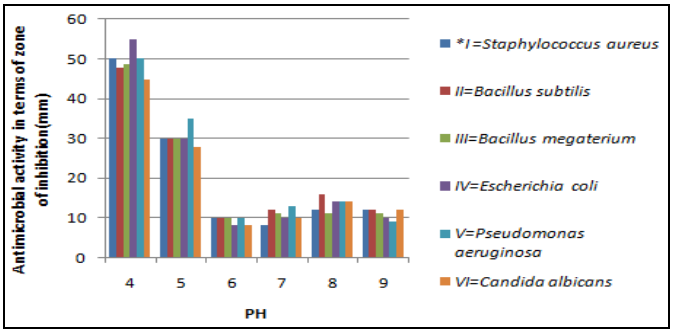


**FIG. 6: EFFECT OF PH ON ANTIMICROBIAL METABOLITE PRODUCTION BY *TRITIRACHIUM EGENUM*.** The results are analyzed statistically and found to be significant at 5% level.

**Effect of Temperature on Antimicrobial Metabolite Production by *T. egenum:*** Temperature profoundly affects bioactive metabolite production.

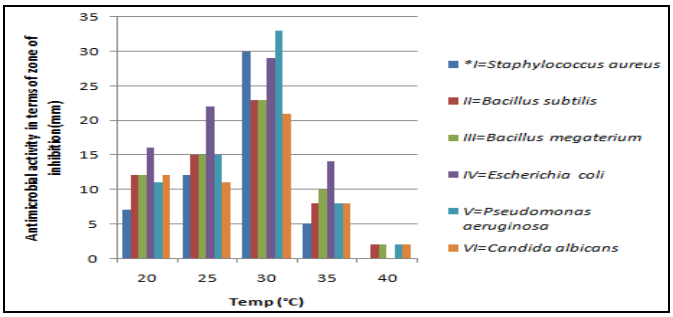


**FIG. 7: EFFECT OF TEMPERATURE ON ANTIMICROBIAL METABOLITE PRODUCTION BY *T. EGENUM*.** The results are statistically analyzed and found to be significant at 5%.

The yield of bioactive metabolites was recorded when grown at temperatures ranging from 20 to 40°C, the optimum being 30°C indicating the mesophilic nature of the strain **Fig. 7.**

**Effect of NaCl on Antimicrobial Metabolite Productionby *T. egenum*:** The influence of NaCl on bioactive metabolite production was determined by adjusting the NaCl of MEB from 1% to 6%. Antimicrobial metabolite production was high at 5% **Fig. 8.**

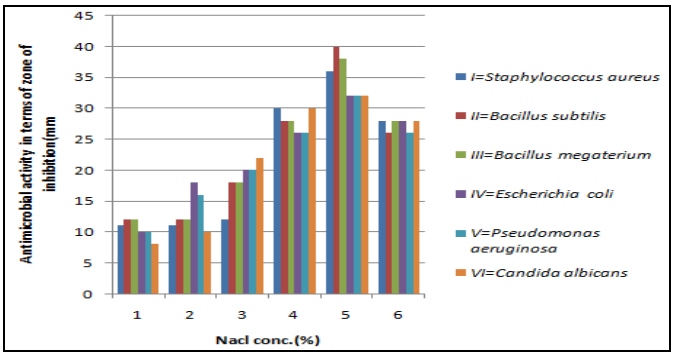


**FIG. 8: EFFECT OF NACL ON ANTIMICROBIAL METABOLITE PRODUCTION BY *T. EGENUM*.** The results are analysed statistically and found to be significant at 5% level.

**Effect of Carbon Sources on Antimicrobial Metabolite Production by *Tritirachium egenum*:** The strain exhibited good antimicrobial activity in the medium supplemented with mannitol followed by starch and carboxy methyl cellulose as a carbon source while it was moderate with fructose, maltose, lactose compared to dextrose and sucrose **Fig. 9.**

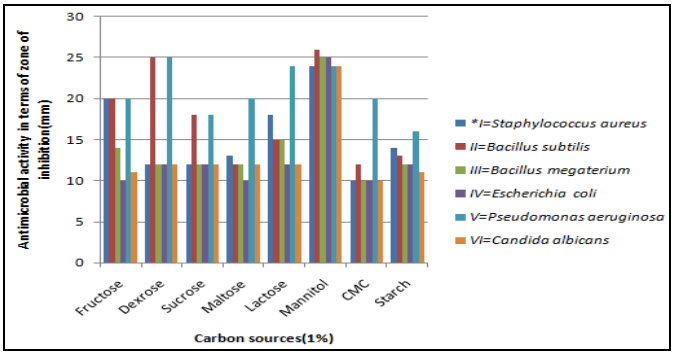


**FIG. 9 EFFECT OF CARBON SOURCESON ANTIMICROBIAL METABOLITE PRODUCTION BY *TRITIRACHIUM EGENUM*.** The results are analysed statistically and found to be significant at 5% level.

**Effect of Nitrogen Sources on Antimicrobial Metabolite Production by *T. egenum*:** Ammonium sulphate as nitrogen source supported good metabolite production **Fig. 10.**

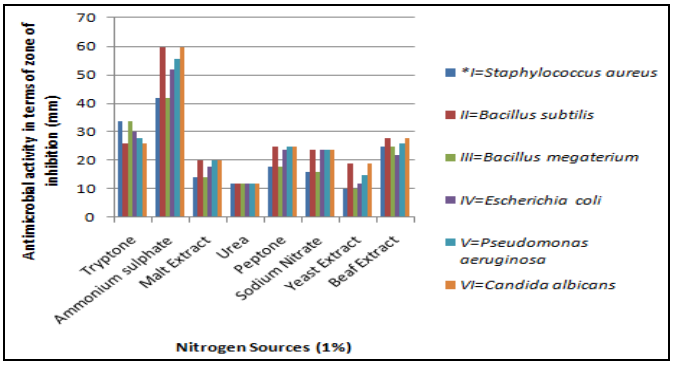


**FIG. 10: EFFECT OF NITROGEN SOURCES ON ANTIMICROBIAL METABOLITE PRODUCTION BY *T. EGENUM*.** The results are analyzed statistically and found to be significant at 5% level.

**Antimicrobial Spectrum of *T. egenum* Grown on Optimized Culture Medium:** *T. egenum* was cultured on optimized MEB (1% mannitol, 1% ammonium sulphate, 5% NaCl, temperature- 30°C, pH – 4.0) at optimal conditions for 18 days and the metabolite was harvested and tested for antimicrobial activity against test bacteria and fungi. High antimicrobial activity was recorded (Plate 1) when cultured under optimized conditions **Fig. 11**. Attempts are in progress for the characterization of bioactive metabolites produced by *Tritirachium egenum.*

**PLATE 1: ANTIMICROBIAL METABOLITE PRODUCTION AGAINST *B. MEGATERIUM***

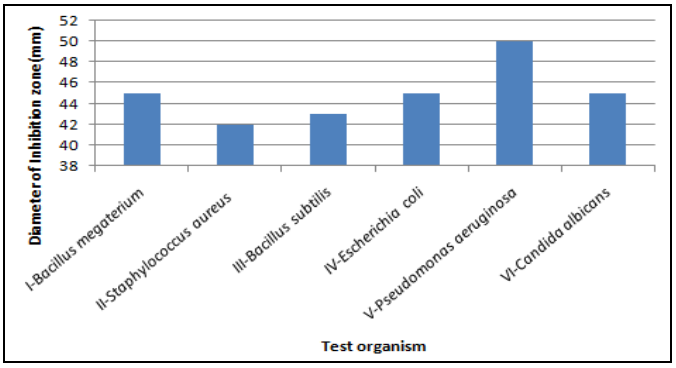


**FIG. 11: ANTIMICROBIAL ACTIVITY OF METABOLITES PRODUCED BY *TRITIRACHIUM EGENUM* ON OPTIMIZED MALT EXTRACT BROTH.** The results are analyzed statistically and found to be significant at 5%level.

**CONCLUSION:** This is the first report of *Tritirachium egenum* isolated from the marine habitats of southeast coast of Andhra Pradesh. In this study, *T. egenum* was cultured on CDA, PDA, NAM, SDA, MEA and YMA culture media. Optimized MEA promoted high metabolite yield reflected by high antimicrobial activity. Hence, *T. egenum* is the potent strain as it exhibited good antimicrobial activity. Attempts are in progress for the identification of bioactive metabolites produced by *Tritirachium egenum*.

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**CONFLICTS OF INTEREST:** Both authors confirm that the article content has no conflict of interest.

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