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Isolation, Optimization and Molecular characterization of Bio potential halotolerant deep-sea fungus *Aspergillus* isolated from Agatti Island

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ABSTRACT
The Agatti Island is at a distance of 459 km from Kochi is located to the west of Kavaratti Island. It lies between 10º 48′ and 10º 53′ N latitude and 72º 09′ and 7º 13′ E longitude, having an area of 3.84 sq. km, with a maximum length of 10 km and width of 10km. It has a north-east, south-west trend with a long tail on the south. Agatti Island is rich for its microbial diversity especially fungi. Out of 100 different fungal organisms isolated from the deep-sea sediment of Agatti Island, 80% of the major dominated species belonged to the genus *Aspergillus*. Further studies of molecular characterization helped us to understand the diversity up to their species level. An attempt was made to isolate such a diverse fungi which makes an extraordinarily contribution in managing disease in humans and other animals. The deep-sea fungi that inhabit the sediment at depths of over 800 m to 1000m below the surface, have become an important source of various compounds (industrial, pharmaceutical and agricultural) based on their diversity. Reports say that there are more than 180 bioactive secondary metabolites derived from deep-sea fungi and include compounds with antimicrobial, antihelminthic, anti-cancerous, and antiviral activities. Such bioactive compounds produced by these deep-sea fungi have the potential treatments for human diseases. A halotolerant fungus *Aspergillus* isolated from a depth of 1429m at the Agatti was found to have antimicrobial and anticancer activity. Based on the internal transcribed spacer, 18S rRNA, and additional β tubulin and calmodulin sequencing the isolate was identified as *Aspergillus terreus*. Optimized production of secondary metabolites (SM) was determined at 7.0 pH, 30ºC temperature, and 10% salinity, sucrose as carbon and tyrosine and ammonium sulfate as the nitrogen source.

Keywords: *Aspergillus terreus*, ITS, Calmodulin, Deep sea fungus, secondary metabolites.

1. INTRODUCTION

Isolation of Fungi

A small portion of the sediment was removed using an alcohol sterilized spatula from the middle of the sub sample that had not been in contact with the walls of the cylinder and was used for the isolation of the fungi [2]. 1mg of the sample from the core was taken and diluted in test tube containing 5ml of saline. The test tube was vortexed well for 5 minutes and serially diluted using sterilized saline water for 8 dilutions ranging from 10-2 to 10-8. A volume of 100µL of the diluents was spread on Sterile Media plates using spread plate method. The plates were kept for incubation at room temperature (27ºC-30 ºC) for 40 days and the isolation of different fungi was carried out from 41st day onwards. The isolated culture showing different morphological features were selected for purification. The cultures were 27 purified using streaking method. The purified isolates were then cultured on Potato dextrose slants and stored at -40ºC.

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Macro Morphological Identification of *Aspergillus*

The isolated species on PDA plates after 7 days of incubation at 28ºC showed brown colored powdery colonies with white border having a flat elevation and a rhizoid margin (Figure 1 A) The reverse mat showed pale yellow color. Globose to sub globose conidial morphology was observed under the light microscope. The isolate was cultured for 7 days in PDB to accumulate SMs in the culture medium at 28ºC (Figure 1 B)

(A) (B)

Figure 1. (A) *Aspergillus* (B) n-butanol extract of SMs of *Aspergillus terreus*

Micromorphological Identification

For SEM studies, the bioactive fungi were cultured on the media and after the growth the colonies were gently inoculated in a broth with a sterile loop and the resultant conidia were fixed with 3% glutaraldehyde in phosphate buffer (0.1%) at room temperature. Conidial samples placed on top of a 13mm glass coverslip was incubated in a chamber for final dehydration with ethanol vapours. The glass cover slip was later mounted on an aluminium stub with silver paint and gold sputtering was done. The samples were finally observed with FEI QUANTA 200F.

The fungal isolate *Aspergillus sps* morphological features observed under the scanning electron microscope showed the conidial heads bearing conidia in compact columnar arrangement at 5000X .Conidia bearing structures were clearly visible in *Aspergillus.* Conidia were very small and smooth walled and were produced directly on the hypae . Conidia were connected to each other. As in *Aspergillus* the morphology especially the shape of the conidial head, number of branching structures between vesicles and phialides forms a major role in identification of the *sps [4]*. The hyphae were long without any branching. Vesicles were young and immature without any branching. Spores were globular in shape with depression at sides were visible at 14000X (Fig .2)

Molecular Characterisation

The isolate was grown in 1/4th concentration of Potato Dextrose Broth (PBD-Difco, USA) prepared in 100% filtered sea water and incubated at 28ºC. The DNA was isolated and **1** mL of the purified DNA was added to 20 mL of PCR mixture. The reactions were carried out in

triplicates. The 18SrRNA was amplified using 42F: forward 5'CTCAARGAYTAAGCCATGCA3' and 1492R:reverse3' ACCTTGTTACGRCTT5'. The ITS region was amplified using ITS1-ITS4 primers (forward 5'TCCGTAGGTGAACCTGCGG 3' and reverse 3'TCCTCCGCTTATTGATATGC 5') using Veriti 96 well thermocycler. Because of the constraints related to ITS as a species marker a secondary barcode or identification marker is commonly required for identification at the species level. In addition to add a taxonomic value of the organism another marker calmodulin (CaM) and βtubulin was amplified. The primers used for amplification is listed in Table1.The PCR amplification was carried out using the following conditions. The amplified PCR products were purified using the Quigen® PCR kit.

The molecular characteristics including 18S rRNA and ribosomal intergenic spacer region (ITS) confirmed that the isolate belong to the genus *Aspergillus and* is most closely related to *Aspergillus terreus* showing 100 % homology similarity. The ITS sequences were deposited in gene bank through NCBI portal and accession number MF037880 was obtained. Mega 7.0 with bootstrap values calculated from 1000 replicates were used to generate phylogenetic tree.

Phylogenetic markers Beta tubulin (Bt2) and calmodulin (CaM) were amplified to confirm *Aspergillus* to their species level. β tubulin and calmodulin is easy to amplify and distinguishes *among* all the *Aspergilli*(Visagie *et al.* 2014).The isolate showed close similarities with *Aspergillus terreus. Both* βtubulin and calmodulin (CaM) were deposited in the NCBI through the portal and the accession number MH357352 and MH357353 respectively were obtained.

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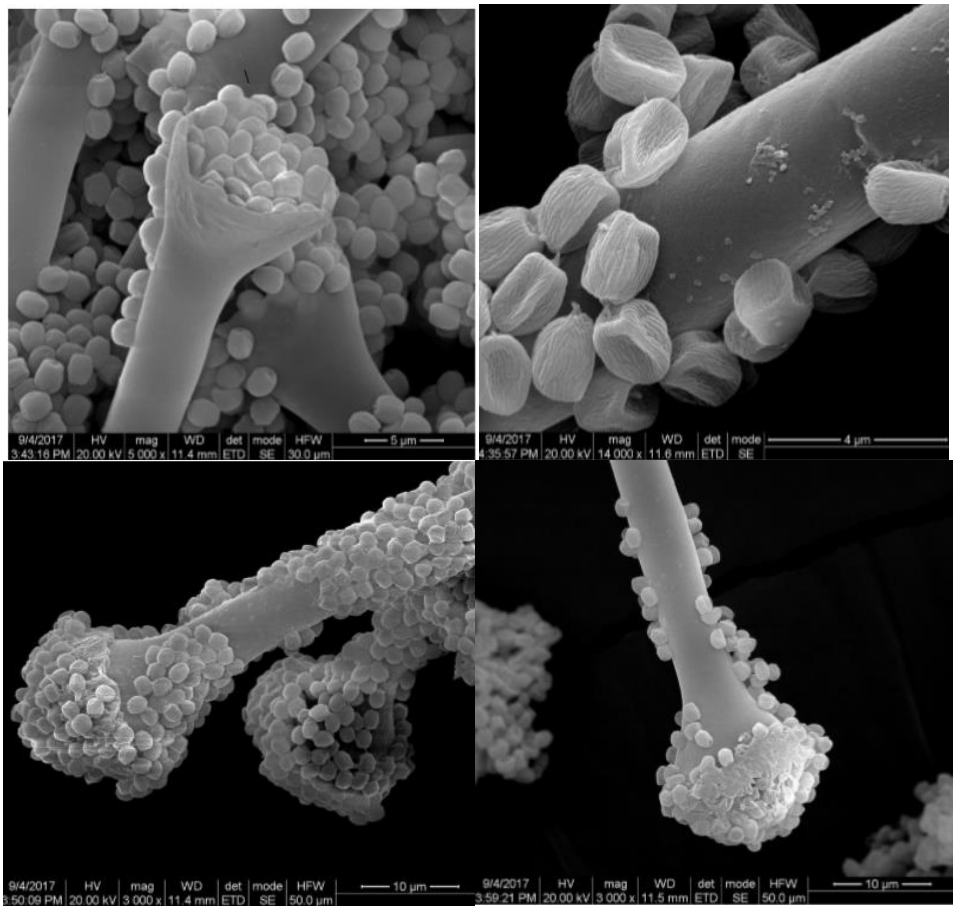

Fig 2. Scanning Electron Microscopy Observation

Phylogenetic Tree

The obtained DNA sequences were compared with sequences within the NCBI gene database using NCBI blast. This tool was used to identify the organism even at the species level. MEGA tool was used to align the sequences. The phylogenetic tree was generated using NJ method along with evolutionary distance. 1000 boot strap replicates was used to evaluate the robustness of the inferred tree.

The phylogenetic tree was constructed using NJ Method for ITS (Figure 3), 18S rRna (Figure 4.), β tubulin (Figure 5) and calmodulin (Figure 6) and processed using MEGA 7.0.The tree was evaluated with 1000 bootstrap replicates

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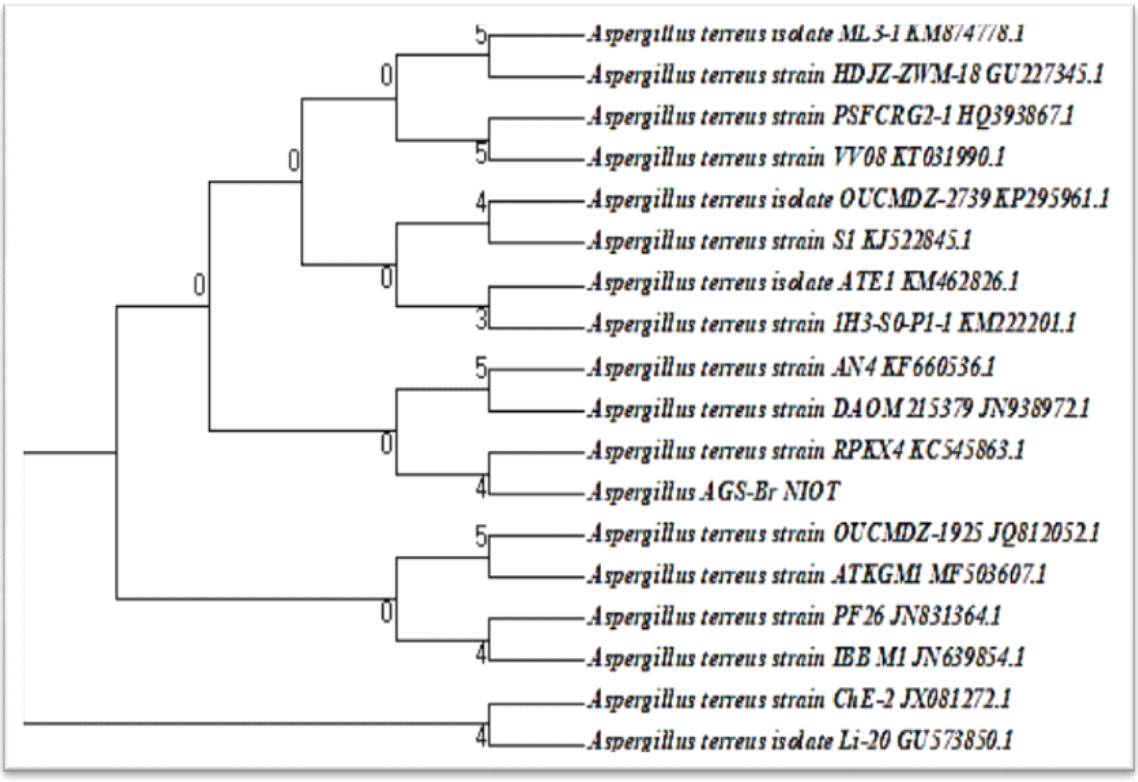

Figure.3.Phylogenetic tree of Deep-Sea derived *Aspergillus terreus* using the NJ method obtained from ITS

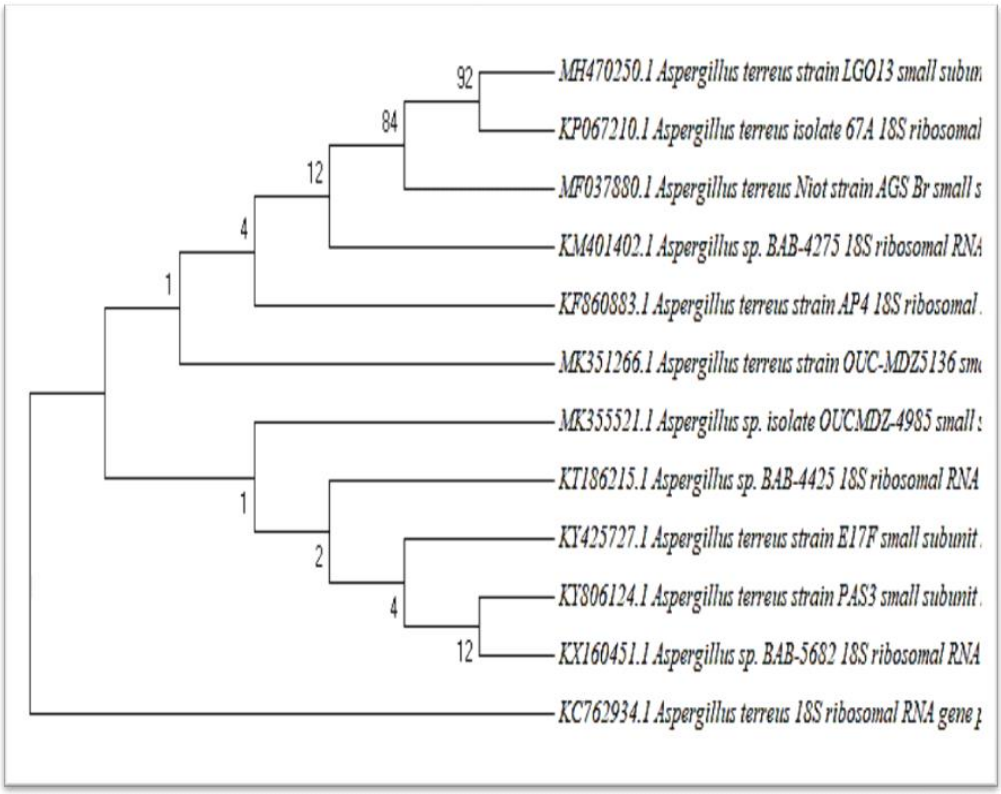

Figure.4.Phylogenetic tree of Deep-Sea derived *Aspergillus terreus* using the NJ method obtained from 18S

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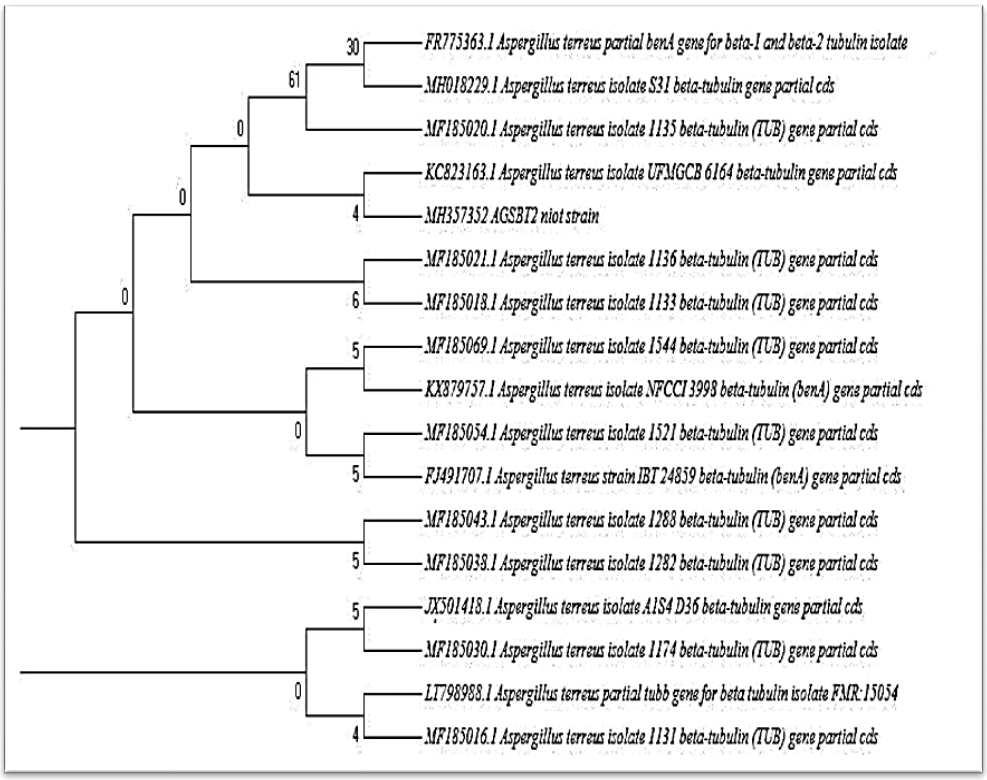

Figure: 5.Phylogenetic tree of a marker gene beta tubulin confirming *Aspergillus terreus*

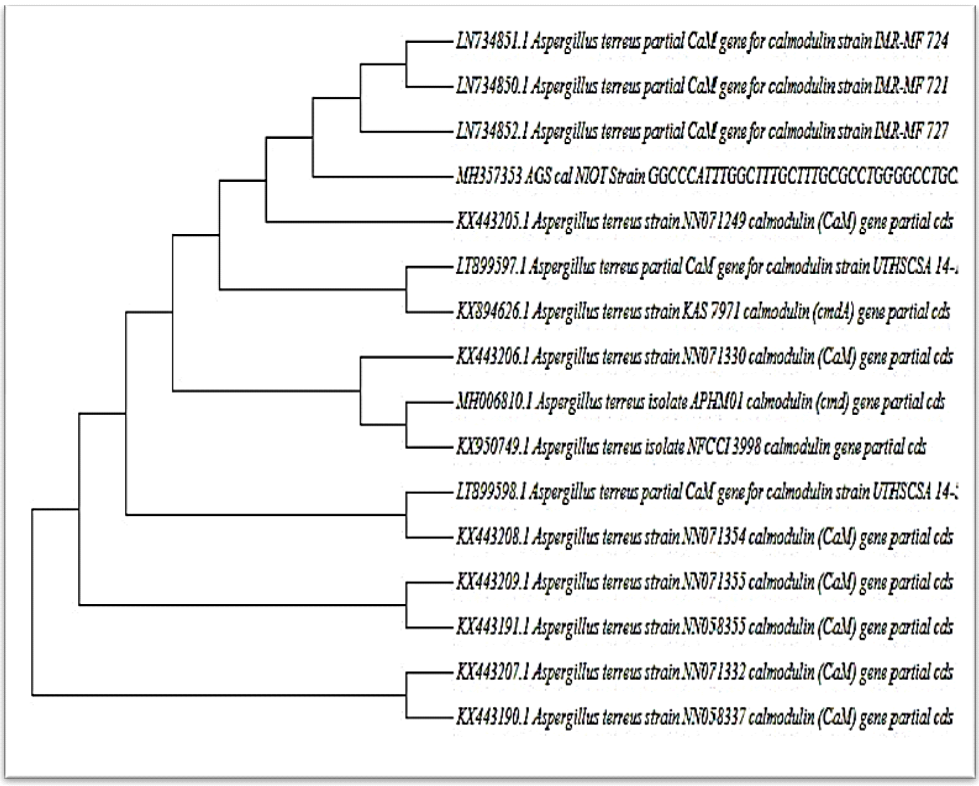

Figure: 6. Phylogenetic tree of a marker gene Calmodulin confirming *Aspergillus terreus*

2. OPTIMISATION

Minimal salt medium with filter sterilized, trace salt solution was used as the basal medium. To select the suitable culture media the fungal isolates were grown in 5 different culture media like Potato Dextrose broth, Sarbourod Dextrose Broth, CzapekDox broth, Malt extract broth and Corn Meal Agar broth prepared in 100% sea water. The medium in which the fungal isolates produced maximum secondary metabolite was optimized for further study.

Carbon Source

Dextrose, sucrose, starch, cellulose and maltose were the different carbon sources used for the study. 1% of the carbon source was added to the MSM medium individually. Each flask with different carbon source was inoculated with 1mL of the fungal inoculum and incubated under shaking conditions. The fermentation broth was collected every 24h up to 144h. The broth was spun at 12,000 rpm for 20 min, and the residual biomass was removed by filtering it in whatmann filter (No. 2). The dry weight of the mycelial biomass was quantified by filtering in the filter paper and drying in a hot air oven at 80ºC for 4 h.

The growth of the isolate was initially slow; there was an increase in biomass with increase in SMs production. Maximum SMs was recorded at 120th hour. The different sugar type used had an evident effect on the growth of the fungal culture and in the current results saccharides were very effective. The Growth rate of *Aspergillus terreus* was significantly increased when sucrose was used as carbon source for *Aspergillus terreus* and it supports maximum growth (1.074. g/L) as well as SMs synthesis (0.094 g/L) when compared with dextrose (0.632g/L), starch (0.181 g/L) and cellulose (0.4188 g/L).The starch was the least used carbon source (Figure 7).Hence sucrose was selected as the carbon source for further studies.

Nitrogen Source

Ammonium dihydrogen phosphate, ammonium sulphate, ammonium nitrate, peptone, beef extract, tryptophan, phenyl alanine and leucine were the different nitrogen source used for the study. 0.1% of the nitrogen source and sucrose (carbon source) was added to the conical flask containing MSM media under shaking conditions. The fermentation broth was collected every 24h up to 144h. The broth was spun at 12,000 rpm for 20 min, and the residual biomass was removed by filtering it in whatmann filter (No. 2). The dry weight of the mycelial biomass was quantified by filtering using the filter paper and drying in a hot air oven at 80ºC for 4 h. Nitrogen which is a main ingredient for increasing the fungal growth but, also the SMs biosynthesis. Various nitrogen sources (inorganic and organic) such as ammonium nitrate, ammonium sulphate, ammonium dihydrogen phosphate, yeast extract, beef extract, peptone, leucine, tryptophan, tyrosine and phenyl alanine on fungus growth and SMs production were analysed (Figure 8). Different nitrogen sources stimulated the growth of the *Aspergillus sp.* and some sources increased the production of SMs. Based on the inorganic nitrogen sources tested, the ammonium nitrate was the best nitrogen source and its supplementation significantly increased the mycelia biomass (6.308 gL-1) and SMs (0.308 gL-1) production significantly. In addition, among the organic nitrogen sources tested, peptone influenced the biomass (7.205 gL-1) and SMs (0.355 gL-1). Interestingly, the addition of aromatic amino acid tyrosine and phenyl alanine stimulated both the mycelial

biomass (10.249.2 gL-1), (5.762 gL-1) and SMs (0.309 gL-1), (0.317 gL-1) biosynthesis respectively on day 5 of the culture medium.

Effect of Temperature and pH

The various temperature for optimization for *A.terreus* ranged from 10 to 50ºC at pH 7.0 and incubated. Maximum growth was observed between 20 to 30ºC while medium growth was observed at 40 and no growth was observed at 50ºC.Mycelial biomass (2.5g/L) and SMs (0.195 g/L) was found to be high at temperature of 30ºC. The growth of fungus and production of SMs was tested at different pH ranging from (5.0-10.0) in PDB. This isolate produced high mycelial biomass (2.5 g/L) and SMs (0.195 g/L) at initial pH value of 7.0. Our results indicate that the maximum growth of the bioactive *A.terreus* grew in the range of pH 7, pH6, pH 5, pH 8 and pH 9. Thus the pH was maintained at neutral pH throughout the subsequent experiments.

Effect of Salinity

The effect of the salinity on biomass and the metabolite production ranged from 0-20% for *Aspergillus* with 1% carbon and 0.1% nitrogen source. The flasks were inoculated and incubated at 28ºC and placing it in the shaker. The fermentation broth was collected every 24h up to 144h. The broth was spun at 12,000 rpm for 20 min, and the residual biomass was removed by filtering it in whatmann filter (No. 2). The dry weight of the mycelial biomass was quantified by filtering in the filter paper and drying in a hot air oven at 80ºC for 4 h.The biomass and the SMs production for each saline concentration was estimated and recorded.

All the above experiments were performed in triplicates.

The effect of salinity ranging from 0 to 20% on the growth of the fungus which was studied (Figure 9) fungal growths were observed in wide range of salinity from 0 to 20%. Immature colonies were white in colour. Mature colonies were brown in colour with white border and grew bigger in size. Since it also grows in 0% salinity (Distilled water) it may not be a halophilic but could be a halo tolerant organism. However, metabolites biosynthesis (0.195 g/L) in fungus reached peak at 8% salinity, while a slow decline in the metabolite production was observed from 10% salinity (Figure 10)

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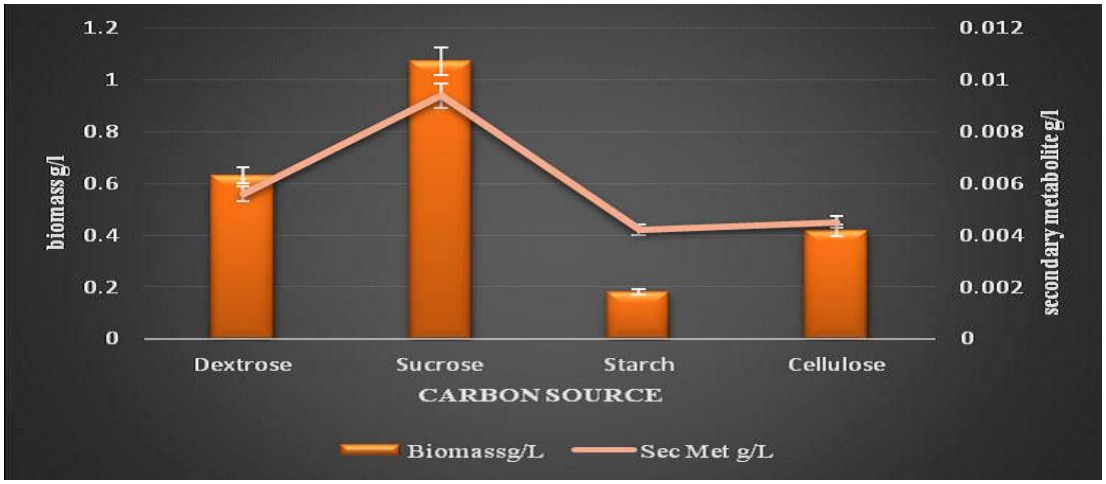

Figure: 7. Effect of Different Carbon source on biomass and secondary metabolite production

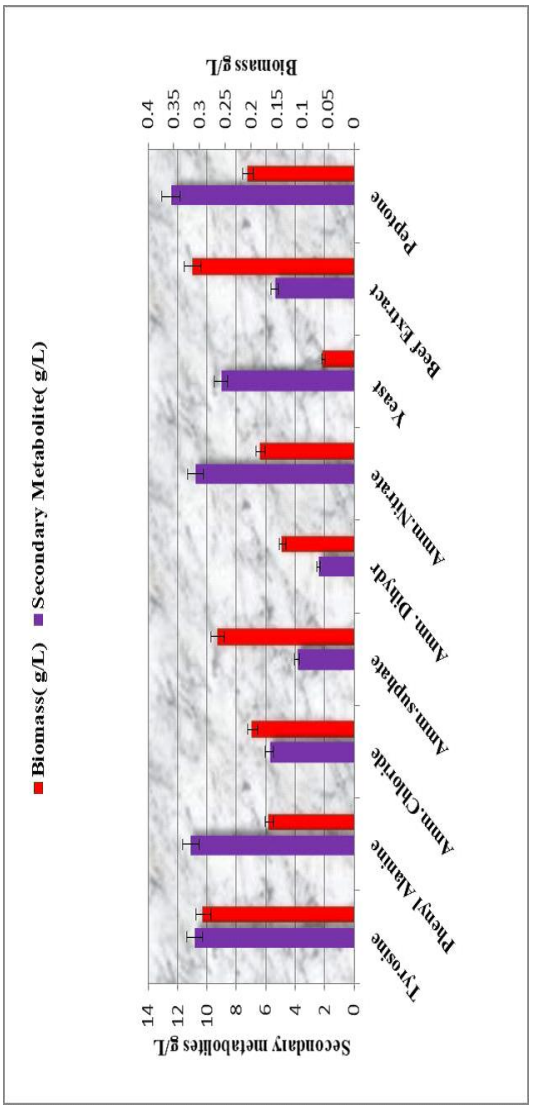

Figure: 8. Effect of nitrogen sources and secondary metabolite production

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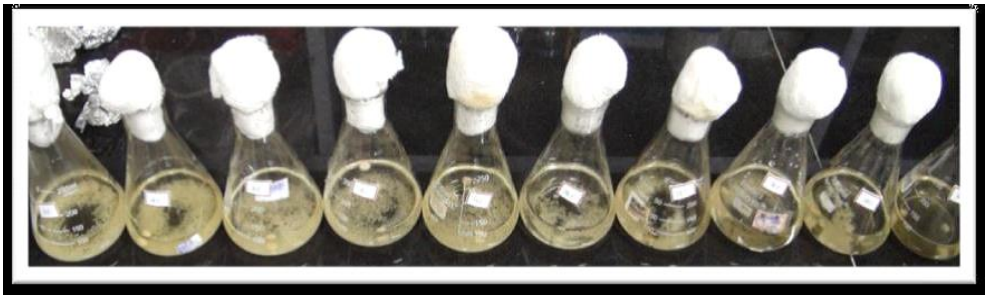

Figure: 9. Growth of *Aspergillus* in different salinity ranges

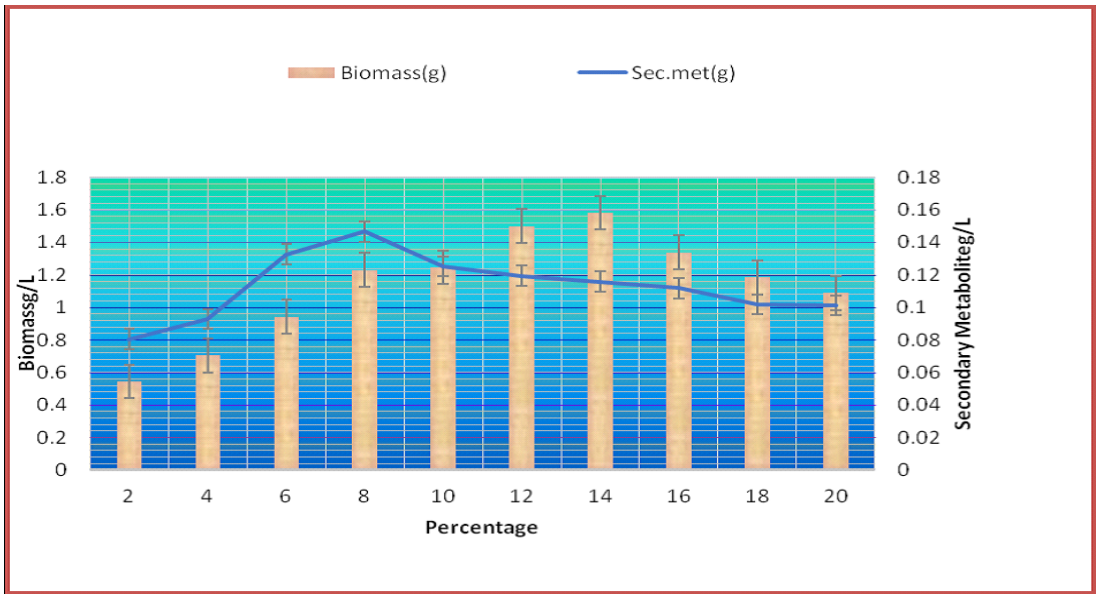

Figure: 10. Effect of salinity on Biomass and SMs production

3. DISCUSSION

The marine environment is an extreme environment which has low temperature, high salt content, low pH and excess radiation. Marine microrganisms living in such an environment are called extremotolerants as they live in such extreme environments [11]. Fungi derived from Marine environment have been found to be a potent source of novel metabolites [14]. *Aspergillus* is a halotolerant fungi which can survive in such extreme environment. *Aspergillus* belong to the division Ascomycota, class Eurotiomycetes, Order Eurotiales and family Trichocomaceae. *Aspergillus* is a diverse fungi with a very high social and economic impact. It is found worldwide and they are known in the production of metabolites such as antibiotics, medicines and enzymes. It is one of the source of the production of Lovastatin, (a drug mainly used for lowering the cholesterol). *Aspergillus terreus* is also called as *A.terrestris* is a (mold) fungus found worldwide in the soil. Production of bioactive SMs, organic acids and enzyme inhibitor, insecticides, anti-tumour metabolites, and vitamin production is usually reported from the members of *Aspergillus [3]* An attempt was made to isolate, characterise and purify the bioactive secondary metabolite from *Aspergillus* obtained from the subsurface sediment of Deep-sea. *Aspergillus terreus* one of the easily available halophilic fungi among all fungi. It requires atleast 10% NaCl and grows under a variety of salinity ranging from 0 to 20%.

The colony on PDA grown at 28ºC were 50mm in diameter brown colored mat with white border, reverse mat was seen with yellowish cream colour with wrinkled appearance.

Aspergillus terreus is brownish in colour and becomes darker with white border as it becomes mature. Our results were in concurrence with the study of Zain *et al*., 2009 which states that the surface and the reverse mat and the 121 number of spore formation depends on the type of medium used. The top mat of *Aspergillus* grown on PDA was brown in colour and the reverse mat showed a lemon colour. Based on the morphological characteristics the isolate was classified as *Aspergillus*. In submerged culture, *A.terreus* grows in different morphological forms from dispersed mycelia to tightly packed pellets, the condition of the culture and the method of inoculation determine the morphological form [6]. Conidia were very small and smooth walled produced directly on hyphae. The hyphae were long without any branching

Conidia were globular in shape connected to each other. As in *Aspergillus* the morphology especially the shape of the conidial head, number of branching structures between vesicles and phialides forms a major role in identification of the *sps* [19]. Vesicles were young and immature without any branching. DNA Sequences are essential for robust identification of the genus *Aspergillus*. The molecular characterisation including 18S rRNA and ribosomal intergenic spacer region (ITS) confirmed that the isolate belong to the genus *Aspergillus and* is most closely related to *Aspergillus terreus* showing 100 % homology similarity. Also phylogenetic markers Beta tubulin (Bt2) and calmodulin (CaM) were amplified to confirm their species level The sequences were deposited in NCBI through the portal, BankIt .Mega 7.0 with bootstrap values calculated from 1000 replicates were used to generate phylogenetic tree. The genus currently contains 354 accepted species [19]. To add to the taxonomic value of the List, accession numbers of ITS, 18S, β tubulin and Calmodulin sequences, thereby supplying a verified set of sequences for the species of the genus. Our work is in concurrence with the work of Taylor *et al*.2000 [15] who detailed, by combining ITS, 18S, BenA and CaM will aid in deciding whether a species is new or not .The secondary marker namely Calmodulin, βtubulin or RNA polymerase II should be used for the identification or distinguish between species [12].This is in fact common practise most studies for describing and characterising *Aspergillus* species. Thus, the isolate was identified as *Aspergillus terreus* on the basis of both morphological and molecular studies.

The synthesis of SMs is usually regulated by a mechanism to prevent the excessive production, these regulatory mechanisms may sometimes become very 122 low. The yield of the SMs can be increased by optimization of physical factors like pH, salinity and temperature and the chemical factors like the media composition [7,10,13,16,21]. Though the SMs are chemically diverse and exhibiting wide range of activities, all are produced by a few common biosynthetic pathways, often in conjunction with morphological development [5]. The growth of the Deep-Sea isolate was initially slow; there was an increase in Biomass with increase in SMs production. Maximum SMs was recorded as 120th hour.

All sugar type used had an apparent effect on the growth of the fungal culture but some saccharides were remarkably effective. The Growth rate of *Aspergillus terreus* was significantly increased when sucrose was used as carbon source for *Aspergillus terreus* and it supports maximum growth as well as SMs synthesis g/L) when compared with dextrose,

starch and cellulose. The starch was the least used carbon source (Figure 4.46). Our study was in agreement with the study of Bhattacharyya and Jha (2011) which stated that the sucrose was the best carbon source for the production of mycelial biomass along with the SMs production and starch was the least utilized carbon source [1].

Nitrogen is an important factor influencing not only fungal growth and differentiation, but also on the SMs biosynthesis. Effects of different nitrogen sources (inorganic and organic) such as ammonium nitrate, ammonium sulphate, ammonium dihydrogen phosphate, yeast extract, beef extract, peptone, leucine, tryptophan, tyrosine and phenyl alanine on fungus growth and SMs production were analysed. Various nitrogen sources stimulated the growth of the *Aspergillus sp.* and some sources increased the production of SMs. Based on the inorganic nitrogen sources tested, the ammonium nitrate was the best nitrogen source and it supplementation significantly increased the mycelia biomass and SMs production significantly when compared to the other nitrogen sources used on day 5 of the culture medium. In contrarory to our study the biomass production and the SMs production was increased when yeast was added to the culture medium [16]. The growth of fungus and production of SMs was tested at different pH ranging from (5.010.0) in PDB. This isolate produced high mycelial biomass and SMs at initial pH value of 7.0. Our results indicate that the maximum growth of the bioactive *A.terreus* grew in the range of pH 7, pH6, pH 5, pH 8 and pH 9. Thus the pH was maintained at neutral pH throughout the subsequent experiments. Most of the microbes have the property to produce anti-microbial metabolite with pH values ranging from pH5.5 to pH8.5 [17,18].

The yield of the SMs was more when grown in a culture medium with pH value at 5.5. The various temperature for optimization for *A.terreus* ranged from 10 to 50ºC at pH 7.0 and incubated. Maximum growth was observed between 20 to 30ºC while medium growth was observed at 40 and no growth was observed at 50ºC.Mycelial biomass and SMs was found to be high at temperature of 30ºC. Our results were in good agreement with Bhattacharyya and Jha, (2011) which stated the yield of the SMs increased at 30ºC [1]. The effect of salinity ranging from 0 to 20% on the growth of the fungus which was studied. Fungal growths were observed in wide range of salinity from 0 to 20%. Since it was also grows in 0% salinity (Distilled water) it may not be a halophilic but could be a halo tolerant organism. However, metabolites biosynthesis in *Aspergillus* reached a peak at 8% salinity, while a sharp decline in the metabolite production was observed from 10% salinity Halotolerant fungi belong to extremophiles and using such halotolerant fungi to produce a secondary metabolite is rarely studied [8,9,20].The salinity concentration was found to possess profound effect on growth and metabolites biosynthesis in fungi. Though our isolate was found to grow at a faster rate at even at 15% NaCl concentration but the production of secondary metabolite started decreasing at 10% salinity. Salt-tolerant fungi belong to extremophiles which can survive under the conditions of zero to high salinity. However, using salt-tolerant fungi to produce SMs at high salinity was rarely reported.

Thus to conclude the understanding of the Deep-Sea ecosystem as reported by Tyler, 2003 is interlinked with the scientific explorations and development technologies and the microbes in

such an environment have become an important resource for the discovery of many antibiotics. Also they show a wide diversity in chemical structure. Further the bioactivity of the fraction obtained from Deep-Sea microbes is comparable with the earlier available antibiotics which can cure many infectious disease. These marine fungi supply a numerous number of variety of SMs which is especially used for the commercial development

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