IJBPAS, July, 2013, 2(7): 1488-1500

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF A LIPOLYTIC MARINE FUNGAL ISOLATE *ASPERGILLUS SYDOWII* STRAIN BTSS1005

PANCHAGNULA B[1*] AND TERLI R[2]

1: Department of Biotechnology, Andhra University, Visakhapatnam-530003, Andhra Pradesh, India

2: School of Life Sciences, GITAM University, Visakhapatnam-530045, Andhra Pradesh, India

***Corresponding Author: E Mail: bindiya.au@gmail.com; Mob.: +91-9966176065**

ABSTRACT

Lipolytic fungal isolates were screened from forty four marine sediment samples, collected from South East Coast of Bay of Bengal, from 8 field stations by tributyrin agar clearing method and submerged fermentation. Out of 66 strains a marine fungus isolated from Divipoint location was observed to produce higher extracellular lipase and was subjected to characterization. Phenotypic and genotypic methods were used to identify the isolate. Scanning electron micrograph indicated echinuate spore morphology. Phylogenetic analysis of 18s rRNA gene highlighted it as *Aspergillus sydowii*. Fatty acid methyl esters were analyzed by gas chromatography and major cellular lipids were found to be linoleic acid (46.43%), oleic acid (19.90%) and palmitic acid (19.67%).

Keywords: Lipolytic Fungi, *Aspergillus*, Characterization, Fatty Acid Composition

INTRODUCTION

Filamentous fungi were used for production of a range of valuable products. *Aspergillus* is an important genus that has been used for basic genetic research **[1-3]**. The wide diversity of bioactive fungal metabolites identified is a strong motivation to develop *Aspergillus* for more exploration into secondary metabolite engineering. The occurrence of fungi in deep sea sediments has been poorly studied and documented **[4-8]**. Using selective media, 66 lipolytic fungal strains were isolated from 44 marine

sediments. On the basis of morphological and molecular analysis the most promising isolate BTSS-1005 was characterized. Data on fatty acid composition is used for physiological, chemotaxonomic and intrageneric differentiation studies of many organisms. Fatty acids make up a relatively constant proportion of the cell biomass and signature fatty acids exist that can differentiate major taxonomic groups within a community **[9-10]**. FAME analysis provides information on the microbial community composition based on groupings of fatty acids **[11-12]**. Hence fatty acid methyl esters were analyzed by gas chromatography (GC) using the Sherlock Microbial Identification System (MIDI Inc.). We report here the occurrence of fungi in deep-sea sediments and characterization of most promising lipolytic fungal isolate, identified as *Aspergillus sydowii*, based on morphological and molecular traits.

MATERIAL AND METHODS

Strain and Cultural Conditions

A filamentous fungi was isolated from a marine sediment sample collected from Bay of Bengal at Divipoint location (Latitude 15°59.813N and Longitude 81°29.045E) at a depth of 191 meters. The purified strain is picked up and maintained on PDA slants. Biomass for characterization studies was obtained by cultivation in shaker flasks on YEME broth.

Lipase Activity

Primary Screening for Lipolytic Isolates

Detection of lipolytic fungi is done by tributyrin agar diffusion method **[13]**. Twenty ml of tributyrin agar medium was inoculated with a loopful of isolate and incubated at 28ºC for five days. The composition of tributyrin agar medium is (g/l): $(NH_4)_2SO_4$, 5; Na_2HPO_4, 6; KH_2PO_4, 2; $MgSO_4$, 3; $CaCl_2$, 3; agar 20 and tributyrin, 10ml with pH 6.0. Lipolytic zone of the isolates was measured and these isolates were subjected to secondary screening.

Secondary Screening for Lipase Production

The selected isolates were cultivated in a synthetic medium containing olive oil (source of natural triglyceride, triolein) as the sole carbon source under submerged fermentation conditions and assayed for the lipolytic activity of the culture filtrates. 45ml of production medium is taken in 250ml Erlenmeyer flask and inoculated with a loopful culture of each isolate. The flasks were incubated at 28ºC for 4 days on a rotary shaker (120 rpm). The culture broth was filtered and the clear filtrate was used as the source of crude enzyme. The composition of the production medium is (g/l): Olive oil, 10;

$(NH_4)_2$ SO_4, 5; Na_2HPO_4, 6; KH_2PO_4, 2; $MgSO_4$, 3; $CaCl_2$, 3 with pH 6.0.

Lipase Activity Determination

The culture broth was filtered and the lipase activity in the culture filtrate was determined by titrimetry using olive oil substrate emulsion method **[14]**. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1µmole equivalent fatty acid / ml/ min at 30 ºC under the standard assay conditions. All the experiments were carried out in triplicate and the mean of the three values is presented.

Characterization

The best lipolytic fungal isolate, BTSS-1005 was grown on various types of media **[15]** for morphological studies. The colonies were observed after 7 days of cultivation at 28 ºC. The color names used in this study were taken from the Methuen Handbook of Colour **[15]** and taxonomic characterization was done **[16-17]**. Scanning Electron Micrograph was taken at Advanced Analytical Laboratory (using Scanning Electron Microscope JEOL; JSM-6610LV), Andhra University. Molecular and chemotaxonomical analysis was done by sequencing studies and by determining the fatty acid composition (using standard Microbial identification system) at MTCC, Chandigarh. For cellular fatty acid analysis, the strains were grown on PDA agar medium at 25°C for 48 hours and biomasses from which fatty acids were extracted were standardized for their physiological age at the point of harvest according to the protocol given by MIDI. Fatty acids were saponified, methylated and extracted using the standard protocol of MIDI (Sherlock Microbial Identification System, version 4.0). The fatty acids were analyzed by GC (Agilent 6890 Series) and identified using the TSBA50 database of the Microbial Identification System **[18]**.

Filamentous fungi were identified on the basis of their 18S rRNA gene and internal transcribed spacer regions of rDNA **[19, 20]**. The conditions for PCR amplification of both targets were 50 ng of DNA, 5 µL of 10X reaction buffer, 0.5 U of Taq DNA polymerase, 200 µM of the four deoxynucleotides (all components from Promega Italia s.r.l. Milan, Italy) and 0.2 µM of each primer was combined in a total volume of 50 µL. The PCR procedure for all primer sets used consisted of 30 cycles: initial denaturation at 95ºC for 5min,denaturation 95ºC for 30 s, annealing at 50 ºC for 30 s, and elongation at 72 ºC for 1:30 and final extension at 72 ºC for 10min .The primers used for PCR amplification of ITS-D1/D2 region: ITS1 (F) :TCC GTA GGT GAA CCT GCG G and NL4 (R) :GGT CCG TGT TTC

AAG ACG G. For Sequencing: For ITS region ITS1 (F) TCC GTA GGT GAA CCT GCG G ITS4 (R) TCC TCC GCT TAT TGA TAT G and for D1D2 region: NL1 (F) GCA TAT CAA TAA GCG GAG GAA AAG and NL4 (R) GGT CCG TGT TTC AAG ACG G. Sequencing was carried out and the results compared to sequences held in GenBank databases using the BLAST program **[21]**. Closely related or phylogenetically relevant sequences were obtained from the GenBank databases. Sequences were aligned using CLUSTAL W and the sequence alignment editor available in MEGA 5.05 **[22]**. Corrected pairwise distances were computed using the Jukes and Cantor correction **[23]**. Bootstrap analysis was based on 1000 re-samplings of the sequence alignment. The evolutionary tree was inferred by using Neighbour joining method with Mega 5.05 software.

Nucleotide Sequence Accession Number

The 18S rRNA gene sequence of strain (975nucleotides) has been deposited in GeneBank with the accession number JQ755254.

RESULTS AND DISCUSSION

Lipase Activity

Using the selective media, 66 lipolytic fungal strains were isolated. The samples of 7 locations are found suitable for the isolation of lipolytic fungi. Number of active strains found depends on many factors like the medium and methods of screening. The isolate BTSS-1005 of DIV 3 sediment of Divipoint location showed maximum lipolytic activity of 0.166U among all the isolates **(Table 1)**.

Characterization

Identification of the Most Promising Lipolytic Fungal Isolate

Morphological properties serve as the primary basis of characterization and the results indicate that BTSS 1005 was characterized by its bluish green colonies, moderate growth, reverse in shades of red, conidial heads radiate to nearly globose, conidiophores colourless, smooth, vesicles globose, sterigmata in two series, conidia globose to subglobose spores. On Potato dextrose agar (PDA) it was blue-green color, often with reddish exudate, reverse reddish and with extremely rough conidia. Colonies on malt extract agar grew more rapidly 4 to 5 cm. in 2 weeks, essentially plane, with crowded conidial structures arising from the sub-merged mycelium. Conidial heads typical of the species are produced in greater abundance and are characteristically more blue-green than on Czapek's agar; exudate lacking; reverse uncolored to pale reddish maroon or with the green color of the conidial heads

apparent through the substrate. The morphological and cultural features in all the media used for study coincided with the features of *A.sydowii* reported earlier by many researchers **(Table 2)** On the basis of morphological characteristics **(Figure 1)** strain BTSS-1005 was identified as *Aspergillus sydowii*. This report was confirmed after observing the scanning electron micrograph (Fig 2) and also sequencing studies on the isolate. While a few species of *Aspergillus*, including *A. sydowii*, have been isolated from the ocean before **[24-30]**, they are not considered normal inhabitants of the marine environment. This suggests that the marine sediments are also good sources for isolation of lipolytic *Aspergillus sydowii*.

Molecular Characterization of the Isolate

The complete sequence of 18S r RNA gene of the strain of BTSS 1005 strain showed 100% similarity with *Aspergillus sydowii*. The sequence was deposited in the GenBank database (Accession No. JQ755254). This sequence was compared with the corresponding partial 18S r RNA gene sequence of representative members of the genus *Aspergillus* retrieved from the public database by using BLAST and the tree topology of 18S r RNA **(Figure 3)** confirms it as *Aspergillus sydowii* **[31-35]**.

Chemotaxonomy of the Isolate

The fatty acid methyl esters were identified on the basis of their retention times and quantified. The percentage contents of fatty acids vary from 0.08-46.43%. The compounds occurring in smaller quantities were C18:0 (4.65%) and C14:0 (1.85%). 21 compounds were present in concentrations less than 1%. The major cellular lipids were linoleic acid (46.43%), oleic acid (19.90%) and palmitic acid (19.67%) **(Figure 4)**. There are no reports available on the fatty acid profile of *Aspergillus sydowii*. Characteristic Fatty acid profiles are used to identify fungi taxonomically **[36-42]**.

Table 1: Location of the Promising Isolate and its Lipase Activity

Divipoint Sediment No.	Isolate No. BTSS	Depth (meters)	Latitude	Longitude	Lipolytic zone (R/r)	Lipase Activity (U/ml)
DIV3	1005	191	15°59.813N	81°29.045E	1.77	0.16600

Table 2: Morphological Characteristics of the Strain BTSS 1005

S. No.	Medium	Growth	Vegetative mycelium	Aerial mycelium	Spore Colour	Soluble pigment
1	Potato dextrose agar	Abundant	White	Yellow	Greenish	No pigment
2	Czapek	Abundant	White	Brown	Brown	Brown

	dox agar					
3	**Sabouraud dextrose agar**	**Abundant**	**White**	**Brown**	**Greenish**	**Purple**
4	**YEME Agar**	**Abundant**	**Reddish brown**	**Brown**	**Black**	**Brown**
5	**Oat meal agar**	**Good**	**White**	**Brown**	**Greenish black**	**No pigment**
6	**Inorganic salts starch agar**	**Good**	**White**	**Brown**	**Black**	**No pigment**
7	**Glycerol-Aspargine agar**	**Good**	**Reddish brown**	**Brown**	**Black**	**Brown**
8	**Peptone agar medium**	**Abundant**	**White**	**White brown**	**Dark brown**	**Orange red**
9	**Tryptone yeast glucose agar**	**Good**	**White**	**White**	**Dark brown**	**No pigment**
10	**Nutrient agar**	**Moderate**	**White**	**White**	**Brown**	**No pigment**

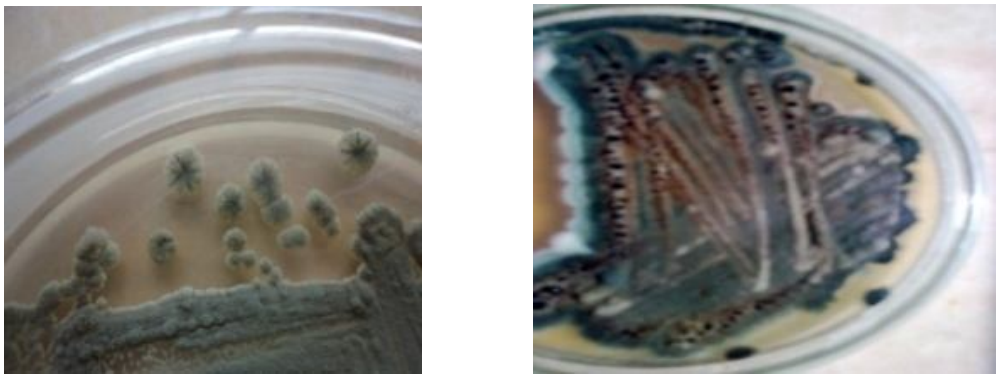

Figure 1: *Aspergillus sydowii* (Strain BTSS1005) on PDA Agar and YEME Agar

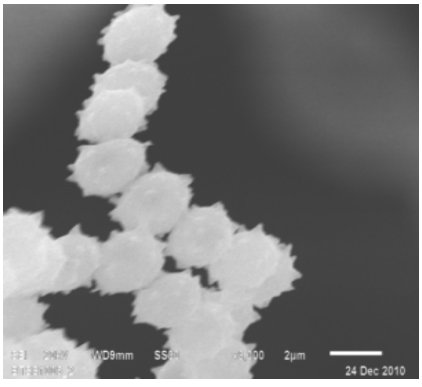

Figure 2: Scanning Electron Micrograph of Marine *Aspergillus sydowii* (Strain BTSS 1005) Grown on YEME Agar for 1 Week at 28ºC. Bar 2µm

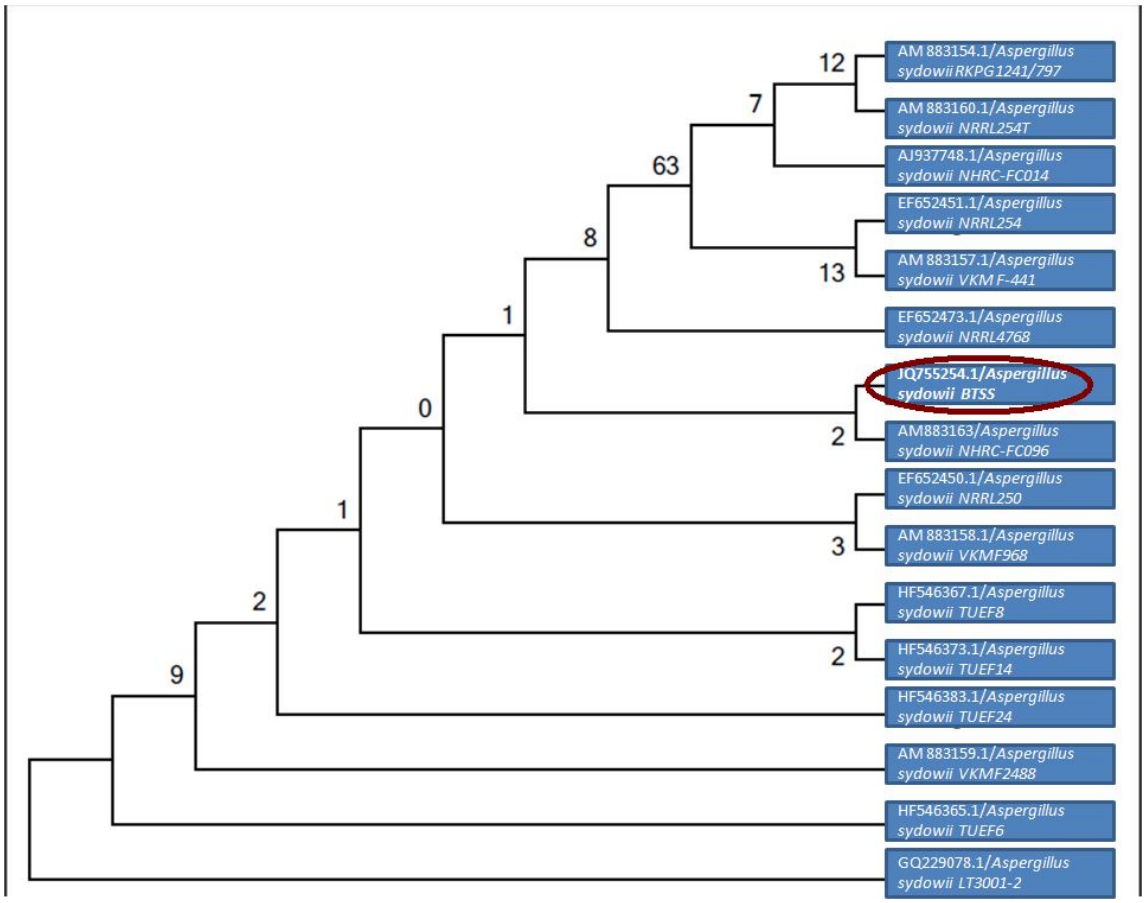

Figure 3: Phylogenetic Tree Based on 18S rRNA Gene Sequences Showing Relationship Between Marine Active Strain and Related Members of Genus *Aspergillus* by Neighbour Joining Method Using Mega 5.05 Version Software. Bootstrap Values (Expressed as Percentages of 1000 Replications) Greater Than 50% are Given at Nodes. Bar 0.01 Substitutions per Nucleotide Positions

Sherlock Sample Report E12201555A Page 1

Volume: DATA File: E122015.55A Samp Ctr: 3 ID Number: 1000
Type: Samp Bottle: 2 Method: FUNGI6
Created: 2/1/2012 2:05:55 PM
Sample ID: BTSS 1005

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
0.060	373	0.037	----	3.699		----	< min rt	
1.730	4.674E+8	0.029	----	7.004	SOLVENT PEAK	----	< min rt	
1.927	5415	0.021	----	7.392		----	< min rt	
1.970	16754	0.023	----	7.477		----	< min rt	
2.002	10781	0.026	----	7.541		----	< min rt	
2.080	9948	0.023	----	7.694		----	< min rt	
2.116	1974	0.027	----	7.766		----	< min rt	
2.339	407	0.024	----	8.205		----	< min rt	
2.743	425	0.028	1.230	9.003	9:0	0.22	ECL deviates 0.003	Reference 0.002
3.033	213	0.015	----	9.576		----	< min ar/ht	
3.246	394	0.022	1.155	9.998	10:0	0.19	ECL deviates -0.002	Reference -0.003
3.282	72	0.011	----	10.049		----	< min ar/ht	
3.596	391	0.043	----	10.500		----		
3.945	434	0.037	1.095	11.000	11:0	0.20	ECL deviates 0.000	Reference 0.000
4.325	630	0.061	----	11.412		----		
4.632	495	0.027	----	11.745		----		
4.864	1258	0.033	1.047	11.996	12:0	0.55	ECL deviates -0.004	Reference -0.004
5.079	1189	0.032	----	12.183		----		
5.592	792	0.033	----	12.628		----		
5.934	433	0.033	1.012	12.923	13:1 AT 12-13	0.18	ECL deviates -0.008	
6.023	195	0.022	1.010	13.001	13:0	0.08	ECL deviates 0.001	Reference 0.000
7.252	808	0.035	0.985	13.896	Sum In Feature 1	0.33	ECL deviates 0.002	14:1 TRANS 9/CIS 9
7.391	4506	0.038	0.983	13.997	14:0	1.85	ECL deviates -0.003	Reference -0.003
7.879	550	0.029	0.976	14.313	2-Me-14:0	0.22	ECL deviates -0.003	
8.359	715	0.039	0.970	14.625	15:0 ISO	0.29	ECL deviates 0.004	Reference 0.003
8.499	889	0.035	0.968	14.715	15:0 ANTEISO	0.36	ECL deviates 0.004	Reference 0.004
8.937	1535	0.038	0.963	14.999	15:0	0.62	ECL deviates -0.001	Reference -0.002
9.555	801	0.039	----	15.369		----		
9.858	539	0.036	0.955	15.551	C16 N Alcohol	0.22	ECL deviates 0.002	
10.302	1146	0.030	0.952	15.817	16:1 Cis 9 (w 7)	0.46	ECL deviates 0.000	
10.490	1174	0.044	0.950	15.930	16:1 Cis 13 (w 3)	0.47	ECL deviates -0.009	
10.605	49576	0.043	0.949	15.998	16:0	19.67	ECL deviates -0.002	Reference -0.003
11.370	683	0.036	0.945	16.441	Iso 17:1 G (w 11)	0.27	ECL deviates 0.007	
11.975	2199	0.050	0.942	16.790	17:1 Cis 9 (w 8)	0.87	ECL deviates -0.002	
12.342	1646	0.047	0.940	17.002	17:0	0.65	ECL deviates 0.002	Reference 0.000
13.156	608	0.039	0.936	17.464	18:1 ISO H	0.24	ECL deviates 0.004	
13.616	118889	0.046	0.935	17.725	18:2 CIS 9,12/18:0a	46.43	ECL deviates 0.005	
13.701	50974	0.059	0.934	17.773	Sum In Feature 8	19.90	ECL deviates 0.000	18:1 CIS 9 (w 9)
14.099	11937	0.045	0.933	17.999	18:0	4.65	ECL deviates -0.001	Reference -0.003
14.377	351	0.032	0.932	18.157	17:0 ISO 3OH	0.14	ECL deviates -0.007	
15.089	816	0.045	----	18.564		----		
15.854	565	0.037	0.926	19.001	19:0	0.22	ECL deviates 0.001	Reference -0.002
16.347	423	0.037	----	19.285		----		
17.053	1143	0.047	----	19.694		----		
17.581	1913	0.044	0.919	19.999	20:0	0.73	ECL deviates -0.001	Reference -0.005
18.248	1942	0.043	----	20.385		----	> max rt	
----	808	---	----	----	Summed Feature 1	0.33	14:1 TRANS 9/CIS 9	14:1 CIS 9/TRANS 9
----	50974	---	----	----	Summed Feature 8	19.90	18:1 CIS 9 (w 9)	18:1 (w 8)

ECL Deviation: 0.004 Reference ECL Shift: 0.003 Number Reference Peaks: 14
Total Response: 261303 Total Named: 254339
Percent Named: 97.34% Total Amount: 239316

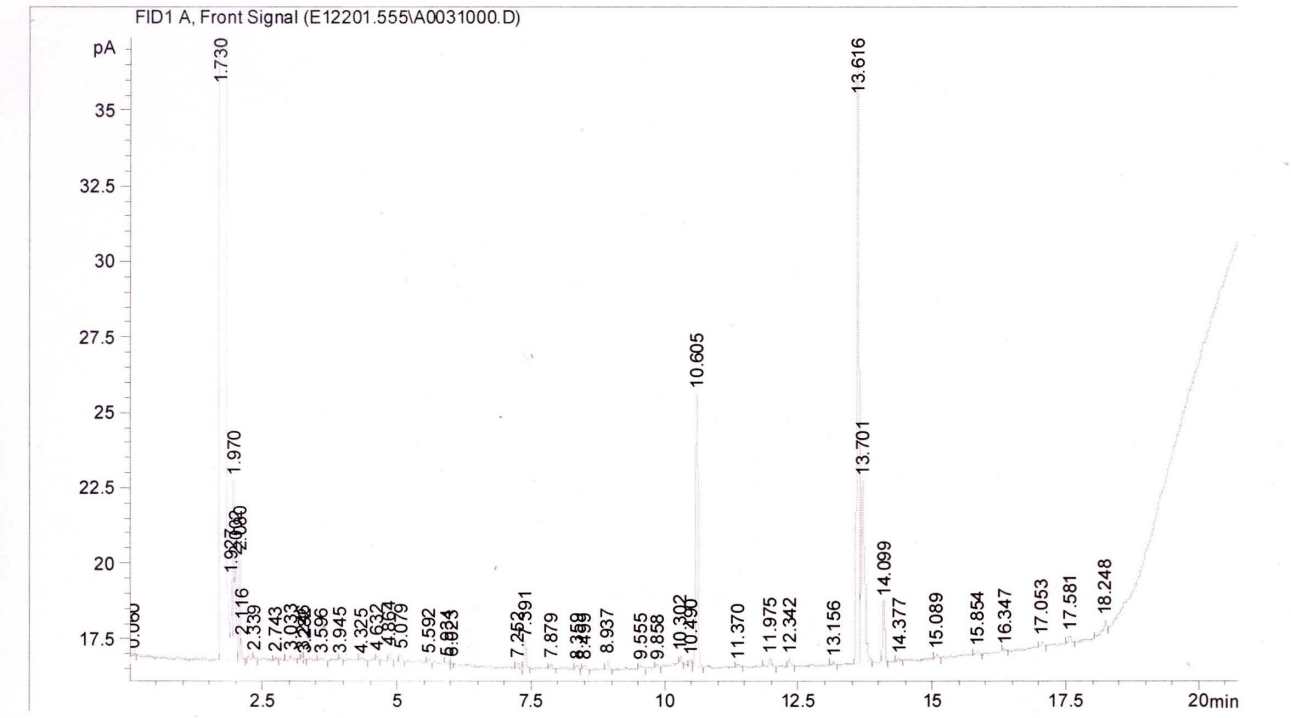

Figure 4: Fatty Acid Profile of *Aspergillus sydowii* (Strain BTSS 1005)

CONCLUSION

Filamentous fungi are recognized as the best lipase producers and are currently the preferred sources; since they produce extracellular lipases their extraction from fermentation media is easy. It is the first report on *Aspergillus sydowii* from marine sediment producing lipase. In the present study the morphological and molecular characterization has been carried out to identify the filamentous fungi Aspergillus sydowii. Phylogenetic analysis largely confirms the morphological identification, specifying accurately the taxonomic position and their possible molecular traceability. Considering the high lipase activity, it is further proposed that the marine *Aspergillus sydowii* holds enormous scope for commercial exploitation of lipase production and there is abundant scope for research on marine fungi for production of bioactive compounds.

ACKNOWLEDGEMENTS

The authors thank the Department of Biotechnology and Advanced Analytical Laboratory, Andhra University for providing the necessary facilities. The authors wish to thank MTCC, Chandigarh for providing

services for gene sequencing and GC FAME analysis.

REFERENCES

[1] Carvalho PO, Contesini FJ, Bizaco R, Macedo GA, Food Biotechnol., 19, 2005, 183-192.

[2] Maia MMD, Heasley A, Camargo De Morais MM, Melo EHM, Morais Jr MA, Ledingham WM *et al*., Effect of culture conditions on lipase production by *Fusarium solani* in batch fermentation, Bioresource Technol., 76, 2001, 23-27.

[3] Mahadik ND, Puntambekar US, Bastawde KB, Khire JM, Gokhale DV, Production of acidic lipase by *Aspergillus niger* in solid state fermentation, Process Biochem., 38, 2001, 715-21.

[4] Raghukumar C, Raghukumar S, Sharma S and Chandramohan D, Endolithic fungi from deep-sea calcareous substrata: Isolation and laboratory studies, In: Desai, B.N. (Ed.) Oceanography of the Indian Ocean, Oxford IBH Publication, New Delhi, 1992.

[5] Lorenz R and Molitoris HP, High-pressure cultivation of marine fungi: cultivation experiments, In: Balny C, Hayashi R, Masson, P. (Eds.) High Pressure and Biotechnology John Libbey & Co. London, 1992, 315-319.

[6] Takami H, Isolation and characterization of microorganisms from deep-sea mud, In: Horikoshi K, Tsujii K, (Eds.) Extremophiles in Deep-Sea Environments, Springer Tokyo 1999, 3-26.

[7] Takami H, Inoue A, Fuji F and Horikoshi K, Microbial flora in the deepest sea mud of the Mariana Trench, FEMS Microbiol. Letters, 152, 1997, 279-285.

[8] Zaunstock B, Molitoris HP, Germination of fungal spores under deep-sea conditions, Abstr., VI International Marine Mycology Symposium, Portsmouth, 1995.

[9] Graham JH, Hodge NC and Morton JB, Fatty acid methyl ester profiles for characterization of Glomalean fungi and their endomycorrhizae, Appl. Environ. Microbiol., 61, 1995, 58-64.

[10] Siciliano SD, Germida JJ, Biolog. analysis and fatty acid methyl ester profiles indicate that Pseudomonad inoculants that promote phytoremediation alter the root-associated microbial community of *Bromus biebersteinii*., Soil Biol. Biochem., 30, 1998, 1717-1723.

[11] Ibekwe AM and Kennedy AC, Phospholipid fatty acid profiles and carbon utilization patterns for analysis of microbial community structure under field and greenhouse conditions, FEMS Microbiol. Ecol., 1998, 26, 151-163.

[12] Zelles L, Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review, Biol. Fertil. Soils, 29, 1999, 111-129.

[13] Jani TR, Patei RB, Sharma G, Shah DA and Dave SR, Screening of lipase using oil seed industry wastes, J. Sci. Ind. Res., 57, 1998, 785-789.

[14] Musantra A, Use of lipase in the resolution of racemic ibuprofen, Appl. Microbiol. Biotechnol., 38, 1992, 61-66.

[15] Kornerup A and Wanscher JH, Methuen Handbook of colour, 3rd Ed., Methuen, London, 1978.

[16] Alexopolus CJ and Mims CW, Introductory Mycology, 3rd Ed., Wiley Eastern Ltd. Publishers, New Delhi, India, 1979.

[17] Arx J Von, The Genera of Fungi Sporulating in Pure Cultures, 3rd Ed., J. Cramer, Germany, 1981.

[18] Sasser M, Identification of bacteria by gas chromatography of cellular fatty acids, MIDI Technical note 101, Newark, DE: MIDI Inc., 1990.

[19] White TJ, Bruns T, Lee S and Taylor J, Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, In M. A. Innis, D. H. Gelfand, 1990.

[20] Sninsky JJ and White TJ (Eds.), PCR protocols, A guide to methods and applications (pp. 315e322), San Diego, CA, USA: Academic Press.

[21] Altschul SF, Madden TL, Schaffer AA, Zhang Z, Miller W and Lipman DJ, Gapped BLAST and PSI-BLAST: a new generation of protein databases search programs, Nucleic Acids Res., 25, 1997, e 3389-3402.

[22] Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S, MEGA 5, Mol. Biol. and Evolution, 28, 2011, 2731-2739.

[23] Jukes TH and Cantor CR, Evolution of protein molecules, In H. N. Munro (Ed.), Mammalian protein metabolism, (21e132), New York, NY, USA: Academic Press, 1969.

[24] Roth FJ, Orpurt PA and Ahearn DG, Occurrence and distribution of fungi

in a subtropical marine environment, Can. J. Bot., 42, 1964, 375-383.

[25] Sweeney JC, Migaki G, Vainik PM and Conklin RH, Systemic mycoses in marine mammals, J. Am. Vet. Med. Assoc., 169, 1976, 946-948.

[26] Kendrick B, Risk M, Michaelides J and Bergman K, Amphibious microborers: bioeroding fungi isolated from live corals, Bull. Mar. Sci., 32, 1982, 862-867.

[27] Abrell LM, Borgeson B and Crews P, Chloro polyketides from the cultured fungus (Aspergillus) separated from a marine sponge, Tet. Let., 1996, 37, 2331-2333.

[28] Belofsky G, Jensen PR, Renner MK, Fenical W, New cytotoxic sesquiterpenoid nitrobenzoyl esters from a marine strain of the fungus *Aspergillus versicolor* Tetrahedron 54, 1998, 1715-1724.

[29] Raghukumar C, Raghukumar S. Barotolerance of fungi isolated from deep-sea sediments of the Indian Ocean. Aquat. microbiol. Ecol. 1998; 15:153–163.

[30] Toske SG, Jensen PR, Kauffman CA and Fenical W, Aspergill amides A and B: modified cytotoxic tripeptides produced by a marine fungus of the genus *Aspergillus*, Tetrahed, 1998, 54, 13459-13466.

[31] Zuluaga-Montero A, Ramrez-Camejo L, Rauscher J and Bayman P, Marine isolates of *Aspergillus flavus*: denizens of the deep or lost at sea? Fungal Ecol., 2010, 3, e 386-391.

[32] White TJ, Bruns TD, Lee SB and Taylor JW, Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis N, Gelfand D, Sninsky J, White TJ (eds), PCR Protocols and Applications e a laboratory manual, Academic, New York, 1990, e 315-322.

[33] Thompson JD, Higgins DG and Gibson TJ, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice, Nucleic Acids Res., 22, 1994, e 4673-4680.

[34] Singh P, Raghukumar C, Verma P and Shouche Y, Assessment of fungal diversity in deep-sea sediments by multiple primer approach, World J. Microbiol. and Biotechnol., 2011, doi:10.1007/s11274-011-0859-3.

[35] Anderson IC, Campbell CD and Prosser JI, Potential bias of fungal 18S rDNA and internal transcribed spacer polymerase chain reaction primers for estimating fungal biodiversity in soil, Environmen. Microbiol., 5, 2003, e 36-47.

[36] Menyawi El I, Ogerbauer WM, Sigmund H, Burgmann H and Graninger W, Identification of yeast species by fatty acid profiling as measured by gas-liquid chromatography, J. Chromatography B: Biomedical Sciences and Applications, 2000, 742, 13e.

[37] Madan R, Pankhurst C, Hawke B and Smith S, Use of fatty acids for identification of AM fungi and estimation of the biomass of AM spores in soil, Soil Biol. and Biochem., 2002, 34, e 125-128.

[38] Schutter ME and Dick RP, Comparison of Fatty Acid Methyl Ester (FAME) methods for characterizing microbial communities, Soil Science Society of America J., 2000, 64, e 1659-1668.

[39] Welch DF, Applications of cellular fatty acid analysis, Clinical Microbiol., 1991, 4, e 422-438.

[40] Brondz I, Hiland K and Ekeberg D, Multivariate analysis of fatty acids in spores of higher basidiomycetes: a new method for chemotaxonomical classification of fungi, J. Chromatography B., 2004, 800, 303-307.

[41] Cavigelli AM, Robertson GP and Klug MJ, Fatty acid methyl ester (FAME) profiles as measures of microbial community structure, Plant and Soil, 170, 1995, 99-113.

[42] Kock JLF and Botha A, Fatty acids in fungal taxonomy, In: Frisva J, Bridge PD, Arora DK, (Eds.), Chemical Fungal Taxonomy, Marcel Dekker, New York, 1998, 219-246.