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BIOCHEMICAL AND MOLECULAR IDENTIFICATION OF *ASPERGILLUS FLAVUS STRAIN MARUTY* C_1 FROM MARINE SEDIMENTS

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ABSTRACT

L-asparaginase is a potential anti-carcinogenic enzyme. Microbial system plays the important role in the production of L-asparaginase enzyme. Collected marine samples were screened for potential producers of L-asparaginase using modified Czapek dox's agar containing L-asparagine and phenol red indicator. The selected strain has been characterized by biochemical tests was found to be *Aspergllus* species. Based on the molecular characterization, 16SrDNA sequence was identified, strain have been closely related to *Aspergillus flavus* subsp CJ-B4 (100% similarity). As the organism was varied with substantial differences in some of the polyphasic and biochemical characteristics it was proposed as a strain variety of *Aspergillus flavus* and designated as *Aspergillus flavus Maruty* C_1.

KEY WORDS: L-asparaginase, 16SrDNA sequence analysis, phenotypic, *Aspergillus flavus.*

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INTRODUCTION

Enzyme therapy involves taking enzyme supplements as an alternative form of cancer treatment. Enzymes are natural proteins that stimulate and accelerate many biological reactions in the body. Enzymes are sometimes used in mainstream medicine and also, approved chemotherapy drug asparaginase is an enzyme. The L-asparaginase has been attracting a great attention as an amino acid degrading enzyme exhibiting antineoplastic activity [1,2,3]. Cancer cells (Neoplastic cells) are differentiated from normal cells based on their expression levels of L-asparaginase (4,5), for this reason, cells are not able to produce L-asparagine, and mainly depend on the L-asparagine from the circulating plasma pools [4].The production of L-asparaginase using microbial systems has been concerned great attention, due to the profitable and eco-friendly in nature. There are many microorganisms such as filamentous fungi, yeasts, and a bacterium has been proved to be beneficial sources of this enzyme [6, 7, 8, 9, 10]. L-asparaginase is using as a chemotherapeutic agent for over 30 years, mainly two bacterial strains of *E. coli* and *Erwinia chrysanthemi*are play the important role in world wide in cancer patients[10, 11, 12] but the bacterial L-asparaginases cause side effects like allergic reactions and anaphylaxis etc.[13, 14]. The search for other asparaginase sources, like eukaryotic microorganisms, can lead to an enzyme with less adverse effects. It has been observed that some eukaryotic microbes like yeast and filamentous fungi have a potential for L- asparaginase production [15, 16,17].Previous studies reported that the mitosporic fungi genera such as *Fusarium, Penicillium*, and *Aspergillus* are commonly produce asparaginase [18, 19]. Therefore, the present investigation, an attempt has been made to isolate and identify the fungi as a better source of L-asparaginase from marine sediments.

MATERIALS AND METHODS

COLLECTION OF SAMPLE

Seven marine sediment samples collected from different regions of Visakhapatnam coast of Bay of Bengal at a depth of 30 to 40 cm and were carried in Zip lock covers to research lab for further analysis.

ISOLATION OF FUNGI

A total of 48 fungal strains were isolated from seven marine sediment samples collected from different places of Visakhaptnam regions and three fungal isolates C_1, B_4, D_2 shown the L-asparaginase activity potential of which isolate C_1 from marine sediments shown high L-asparaginase activity is maintained on PDA and subcultured monthly. L-asparaginase producing microorganisms namely *Fusarium oxysporum* NCIM 1008, *Aspergillus wentii* NCIM 941, *Aspergillus terreus* MTCC 1782 were procured from MTCC, Chandigarh and NCIM, Pune, India for comparing the best L-asparaginase producing isolate.

SCREENING OF FUNGI BY PLATE ASSAY

A novel and semi quantitative rapid plate assay for screening of L-asparaginase producers was adopted [20].

L-ASPARAGINASE ENZYME ASSAY

L-Asparaginase catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia. L-asparaginase activity was measured by using Nesslerization which is based on the determination of ammonia liberated from L-asparagine by enzyme in the Nessler's reaction. One international unit of L-Asparaginase activity is defined as that amount of enzyme which catalyses the formation of 1 µ mole of ammonia per minute under the conditions of the assay [21].

BIOCHEMICAL IDENTIFICATION

Of the 48 isolates from seven marine sediment samples only 3 isolates *(B_4, D_2, C_1)* shown the L-asparaginase activity potential and when compared with the procured microorganisms the isolate C_1 exhibited maximum L-asparaginase activity. This promising isolate was subjected to polyphasic technique to observe morphological characteristics which includes a drop of lacto phenol blue on a clean slide with a cover glass placed on it. This

biochemical test includes the macro morphology like the color, texture and the micro morphology like shape, size, arrangement of conidial heads, length, vesicle shape, wall character using light and scanning electron microscope.

MOLECULAR IDENTIFICATION BY GENOTYPIC CHARACTERISATION (16S rDNA analysis)

Genomic DNA was prepared by using Płaza et al [22, 23]. Fungal mycelium was collected from grown culture plates and 200-500mg of mycelium material (wet weight) was added to 1.5mL micro centrifuge tubes. Mycelium material from the fungi were suspended in 500 µl of a bead beating solution containing: 0.1M NaCl, 0.5M Tris-HCl, (pH8.0),and 5% sodium dodecyl sulfate. Approximately 0.2g of mixed diameter (1.0mm/0.5mm/0.1mm) glass beads for crushing of cell walls was also added. The tubes were then placed into a TurboMixTM adapter (Scientific Industries, INC.) attachment for a Vortex Genie 2 (FisherBioblock Scientific) and homogenized for 10 min at maximum speed. Then, the tubes were centrifuged for10 min at 5000rpm. After centrifugation, the supernatants were decanted into new tubes and the extraction procedure was repeated. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) (Amresco) was added to each sample; the samples were then vortexed briefly, and centrifuged for 5 min in a microcentrifuge. The aqueous layer was transferred to a new tube and extracted again with an equal volume of chloroform: isoamyl alcohol (24:1). The tubes were mixed vigorously and centrifuged for 5 min at 10,000 rpm. The supernatant was transferred to the new Eppendorf tubes, and 2.5 volumes of isopropanol were added for precipitation of DNA. The tubes were incubated in a refrigerator for1 hour, and centrifuged at 4°C for 10 min at 14,000 rpm. The pellets were washed twice with cold 70% ethanol, air-dried, and then resuspended in sterile double deionised water. The samples were treated with RNase. The subsequent DNA yields and quality have been analyzedby electrophoresistechnique using 1% (w/v) agarose gel. The amplified product was sequenced using forward primers NS1 (GTAGTCATATGCTTGTCTC) and reverse primer: ITS4 (TCCTCCGCTTATTGATATGC). The nucleotide sequences of the 16S rDNA obtained were compared with the sequences in Gene Bank using the basic local alignment search tool (BLAST) program then aligned with the related reference sequences retrieved from NCBI Gene Bank databases. Phylogenetic tree was constructed using the Molecular Evolution Genetics Analysis (MEGA) software version 5.0 [24].

RESULTS AND DISCUSSION

Isolation and microscopic studies of strain Maruty C_1

Microscopic observations showed that the conidia were close textured, velvety, with regular margins appearing in greenish yellow color. Conidial heads are typically radiate, later splitting to form loose columns, biseriate but having some heads with phialides borne directly on the vesicle. Conidiophores are hyaline and coarsely roughened, often more noticeable near the vesicle (30-75µm in dia) Figs 1& 2). Conidia are globose to subglobose, pale green and conspicuously echinulate. The isolate was morphologically identified as *Aspergillus sp*[25].

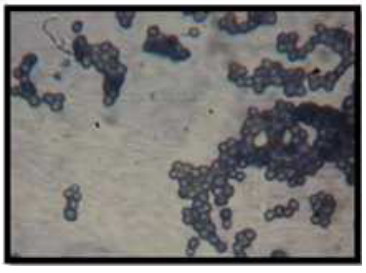

Figure 1
Aspergillus flavus C_1 cells observed under light microscope at 100X magnification.

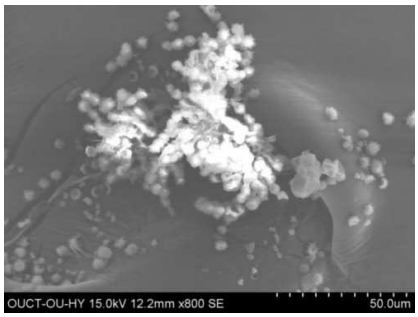

Figure 2
Aspergillus flavus C_1 cells observed under scanning electron microscope at 50X magnification.

Screening of microorganism for high L-asparaginase production

The previous studies observed that L-asparaginase production is accompanied by an increase in pH of the culture filtrates. The plate assay was devised using this principle by incorporating the pH indicator phenol red in medium containing asparagine (sole nitrogen source). Phenol red at acidic pH is yellow and at alkaline pH turns pink, thus a pink zone is formed around microbial colonies producing L-asparaginase. The release of ammonia from asparagine in PDA plates led to increase in local pH and hence the pinkish appearance (due to the presence of phenol indicator) of plates harboring positive cultures. The plate assay is advantageous as the method is quick and L-asparaginase production can be visualized directly from the plates without performing time consuming assays [18, 26, 27].I this work a total of six cultures were screened for the production of L-asparaginase. Out of which three cultures were procured from NCIM, Pune and MTCC Chandigarh India, The isolated *Aspergillus flavus C_1* shows maximum zone of color change 19 mm, isolate B_4 15mm and isolateD_2 13mm (Fig 3&4 , Table1).

Table 1
Screening of microorganism for the Production of L-asparaginase

S No	Microorganisms	Medium	Zone of color change(mm)
1	*Fusarium oxysporum*	Modified CzapexDox's agar	9
2	*Aspergillus wentii*		12
3	*Aspergillus terreus*		14
4	*Isolate B_4*		15
5	*Isolate D_2*		13
6	***Aspergillus flavus Maruty C1*** **(Isolated)**		19

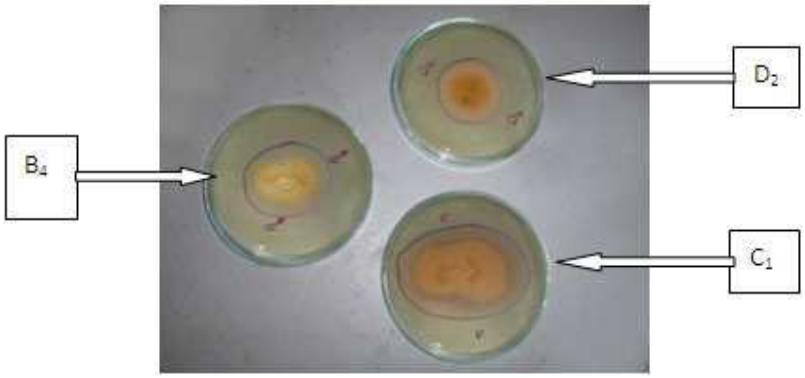

Figure 3
Aspergillus flavus C_1 showing L-asparaginase activity with a zone dia of 19mm, B_4 15mm and D_2 13mm.

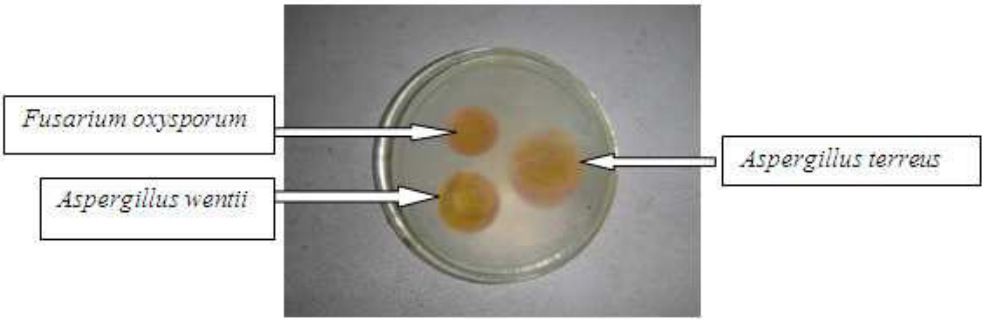

Figure 4
Rapid plate Assay for Screening of procured fungi for L-asparaginase production

MOLECULAR IDENTIFICATION OF STRAIN MARUTY C_1

The 16S rDNA sequence was aligned with other 16S rDNA fungal sequence obtained from Gen-Bank by basic local alignment search tool (BLAST) program [28]. The 16S rDNA gene sequence between the isolate *Maruty* C_1 and its near neighbor *Aspergillus flavus* subsp CJ-B4 was 100%similarity andthis value corresponds to 0 differences out of the 522 nucleotide positions compared (Table 2). An almost complete 16S rDNA sequence was determined for strain *Maruty* c_1 (522 nucleotides). Comparison of this with those of representation reference strains of the family fungi shows that the organism belongs to the genus *Aspergillus*. Based on the morphological, physiological, biochemical characteristics, phyogenetic tree constructionand molecular characterization by 16s rRNA sequencing, the strain has been identified as *Aspergillus flavus* strain *Maruty* C_1(Fig. 5 and 6).

GTGCTGCTTTCTGGTATGTCTCAATGCCTTCGAGTTAGTATGCTTTGGACCA
AGGAACTCCTCAAAAGCATGATCTCGGATGTGTCCTGTTATATCTGCCACA
TGTTTGCTAACAACTTTGCAGGCAAACCATCTCTGGCGAGCACGGCCTTGA
CGGCTCCGGTGTGTAAGTACAGCCTGTATACACCTCGAACGAACGACGACC
ATATGGCATTAGAAGTTGGAATGGATCTGACGGCAAGGATAGTTACAATG
GCTCCTCCGATCTCCAGCTGGAGCGTATGAACGTCTACTTCAACGAGGTGC
GTACCTCAAAATTTCAGCATCTATGAAAACGCTTTGCAACTCCTGACCGCT
TCTCCAGGCCAGCGGAAACAAGTATGTCCCTCGTGCCGTCCTCGTTGATCT
TGAGCCTGGTACCATGGACGCCGTCCGTGCCGGTCCCTTCGGTCAGCTCTT
CCGTCCCGACAACTTCGTTTTCGGCCAGTCCGGTGCTGGTAACAACTGGGC
CAAGGGTCACTA

Figure 5
The DNA sequence for 16s rDNA gene of Aspergillus flavus strain Maruty C1.

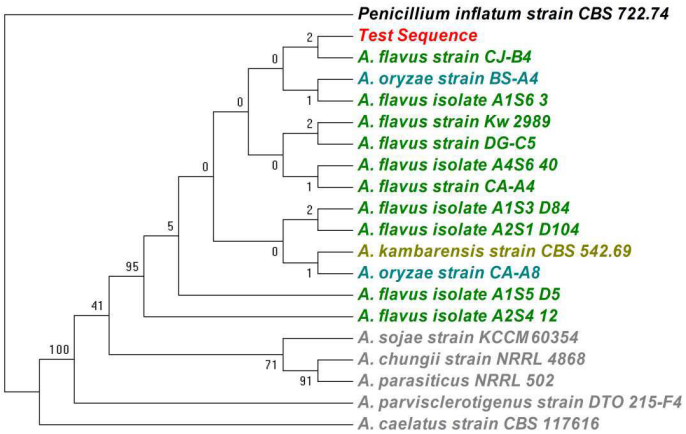

Figure6
Neighbour-joining tree based on almost complete 16S rDNA sequences showing relationshipsbetween strain Maruty C1 and the representatives of the family Aspergillus and related taxa.

Table 2
16S rDNA similarity values between strain Maruty C_1 and the representatives of the genus Aspergillus.

Rank	Name	Strain	Accession	Pair-wise Similarity	Identities	Gaps
1	*Aspergillus flavus*	CJ-B4	329458227	100%	522/522	0/522
2	*Aspergillus oryzae*	BS-A4	329458213	100%	522/522	0/522
3	*Aspergillus flavus*	A1S6_3	410178875	100%	522/522	0/522
4	*Aspergillus flavus*	Kw 2989	441414895	100%	522/522	0/522
5	*Aspergillus flavus*	DG-C5	329458232	100%	520/520	0/520
6	*Aspergillus flavus*	A4S6_40	410178907	100%	522/522	0/522
7	*Aspergillus flavus*	CA-A4	329458218	100%	522/522	0/522
8	*Aspergillus oryzae*	CA-A8	329458222	100%	522/522	0/522
9	*Aspergillus kambarensis*	CBS 542.69	133741567	100%	484/484	0/484
10	*Aspergillus flavus*	A2S1 D104	410178881	100%	522/522	0/522
11	*Aspergillus flavus*	A1S3 D84	410178871	100%	522/522	0/522
12	*Aspergillus flavus*	A1S5 D5	410178873	100%	522/522	0/522
13	*Aspergillus flavus*	A2S4 12	410178885	100%	522/522	0/522
14	*Aspergillus parasiticus*	NRRL 502	15147397	100%	484/484	0/484
15	*Aspergillus chungii*	NRRL 4868	359324389	98%	512/523	1/523
16	*Aspergillus sojae*	KCCM60354	329458364	98%	513/523	1/523
17	*Aspergillus parvisclerotigenus*	DTO 215-F4	459649526	98%	509/517	1/517
18	*Aspergillus caelatus*	CBS 117616	133741559	93%	453/487	3/487

CONCLUSION

From this study, it is clearly indicated that marine sediments can provide a rich source of L-asparaginase-producing fungi when compared to other soil sources. *Aspergillus flavus Maruty C_1* isolated from marine sediment samples has the ability to produce a significant amount of L-asparaginase enzyme. Then the efficient producer of marine fungal isolate was microscopically identified and genetic identification (16SrDNA) was carried out. However, more detail investigation is required to characterize this microbial enzyme, which may be effectively used in the large scale production for commercial and pharmaceutical purposes.

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