

Bioprospecting of Marine Fungi from Coastal Karnataka Region as Potential Source of Economically Important Enzyme L-Glutaminase and their Comparative Genomic Study

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Abstract

Marine fungi are important sources of new metabolites including certain enzymes of medical interest due to their enormous capacity to adapt themselves to extreme environments. Living in a highly competitive ecological niche, they produce certain unusual chemical moieties. Marine biological resources are green, abundant, renewable and aid in economic development. The present study investigates the production of L-Glutaminase which is of therapeutic and industrial importance, from marine fungi of coastal Karnataka. Primary screening on agar plates and submerged fermentation in broth was employed for enzyme production. Both marine yeasts (*Pichia* sp) and filamentous fungal strains (*Aspergillus*, *Penicillium*) were found to be efficient producers of L-Glutaminase. Of the 42 isolates, five potential strains were selected through primary screening and Thin Layer Chromatography was performed to confirm the production. Filamentous fungi were identified through morphological and molecular methods as *Penicillium* and *Aspergillus* strains with 99-100% similarity. *A. foveolatus* (MT667385) and *A. nidulans* (MT667422) were potential producers (1.58U/ml and 1.41IU/ml). The yeast identified was *Pichia kudriavzevii* (MT667428), which was a moderate producer of Glutaminase and first marine yeast reported for this enzyme production. *Neosartorya quadricincta* (MT667427) and *P. citrinum* (MT667426) are also moderate producers. After screening the marine fungi, the isolated strains' potential to produce L-Glutaminase was confirmed using SDS PAGE, FTIR and Mass analysis. This study emphasizes the necessity of marine fungal culturing and the scope of use of these fungi for further commercial production of L-Glutaminase which would uplift marine economy.

Keywords: Marine Fungi, Yeasts, L-Glutaminase, Molecular Identification, Submerged Fermentation, TLC, SDS-PAGE, FTIR, Mass Analysis, Marine Economy

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INTRODUCTION

L-Glutaminase is ubiquitous in distribution and is present in animal tissues, different plants, and also in microorganisms such as bacteria and fungi. It catalyses the deamination of L-Glutamine to form L-Glutamic acid and ammonia ions. It finds application in the food industry as a flavour enhancing agent,¹ as an antileukemic agent,^{2,3} and as an efficient anti-retroviral agent.⁴ *Trichoderma* and *Aspergillus* species are exploited on an industrial scale, for L-Glutaminase production.^{5,6,7} Dutt and his group (2010) studied the L-Glutaminase production from *Penicillium expansum*, an isolate from soil, and confirmed through TLC. The obligate need of salt tolerant L-Glutaminase in fine food industry has prompted the exploration of marine resources for screening of microorganisms. Research group from Tamilnadu reported good production of L-Glutaminase (352.4±0.23IU) from *Vibrio* species SFL-2 isolated from *Mallipallam lagoon* was previously reported (Jeyaprakash *et al.*, 2009). *Trichoderma koningii* was also shown to produce good amounts of L-Glutaminase when grown on Sesamum oil cake solid substrate.⁸

Report from National research centre, Cairo, Egypt, has shown the highest glutaminase activity from *Penicillium brevicompactum* NRC 829, when grown on modified CzapekDox's medium.⁹

The marine ecosystem is recognized as one of the untamed environments for unknown microorganisms to produce several secondary metabolites and enzymes. Marine fungi are known to produce a myriad of alkaloids, terpenes, peptides and enzymes of pharmacological importance.¹⁰ Microbial enzymes are drawing more attention than plant and animal enzymes due to their interesting characteristics such as specificity, stability and ease of production. They may have wide applications in the food industry, technical industries, leather industry, paper, household care, fine chemicals and pharmaceuticals.¹¹ Large numbers of microbial enzymes have played crucial roles in industrial bioprocesses, but there is still a need for more versatile/improved enzymes to develop economically-competitive and sustainable industrial processes. Study of microbial diversity and basic screening is still

used to identify potent strains.¹¹ The necessity of replacement of traditional chemical process by new enzyme technology in various industries especially in pharmaceuticals and food, has been driven by various factors like the safety of environment, depletion of natural resources, enhanced demand for industrial goods, need of cost reduction, nontoxic and low energy input in enzyme production *etc.*¹²

According to Singh *et al.*,¹³ the enzyme market is very significant with an estimated € 4.2 billion with about 6.5 to 10% annual growth (excluding pharmaceutical enzymes). Enzymes such as amylase and lipase were the first to be marketed in the 1980s. Though enzymes are present in all organisms and can be found in hundreds of different forms, only 25 enzymes have been industrialized and commercially produced, including amylase, cellulase, chymosin, lipase, lactase, glycomylase, glucose isomerase, protease, pullulanase, xylanase *etc.* The bioprocess technology provides an alternative method by providing unlimited and pure source of enzymes instead of various chemicals traditionally employed in industries to accelerate chemical reactions.¹³ Many fungi inhabit the deep sea (likely 10000 species) but only 600 have been reported emphasizing the need to explore more of them for industrially important enzymes.¹⁴

Marine filamentous fungi are unique in producing potential enzymes with novel physiological characteristics due to their ability to survive in harsh marine environments like salinity, variable temperature, nutrients, *etc.* Several such enzymes are biologically active and find applications in the food, textile, biofuel, agriculture and pharmaceutical industries.^{15,16} Various fermentation methods are employed for production using submerged fermentation and solid-state fermentation. Exploiting fungi in enzyme production is gaining importance as GRAS (Generally Referred as Safe) by the US FDA. *Aspergillus niger* is widely used to produce economically essential compounds, including citric acid and enzymes.¹⁷

Submerged fermentation using sugarcane bagasse as sole supporting substrate was employed to grow soil bacterium *Acinetobacter calcoaceticus* PJB1 to produce extracellular-L-

glutaminase.¹⁸ In Tamil Nadu, the production of enzyme L-glutaminase by *Aspergillus flavus* JK-79 isolated from soil sediments of the estuaries region of Parangipettai, Cuddalore District, was studied.¹⁹

Thus in the present investigation, marine resources were harvested for isolation of fungi. Their molecular identity and phylogeny were established and interested strains were studied for the production of industrially and medically important enzyme L-Glutaminase.

METHODOLOGY

Sampling

Marine water samples were collected from Someshwara, Hosabettu, Surathkal, Kaup, Murudeshwar, and Gokarna (Karnataka state, India). The sampling sites from Someshwara to Gokarna are shown in Figure 1. Sterile plastic bottles were used for collection of marine water samples laboratory, and stored in the laboratory at 4°C until use. The diluted water samples were inoculated onto Potato Dextrose Agar plates after serial dilution method. After 5-6 days of incubation in RT, the pure cultures were isolated and maintained in Potato Dextrose Agar slants at 4°C.

Qualitative assay of L-Glutaminase

For screening of L-Glutaminase producing fungal isolates, modified CzapekDox Agar (Glucose-

2g, Glutamine-10g, KH_2PO_4 -1.2G, KCl-0.52g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -0.52g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ -0.0019g, Agar-20g for 1Litre distilled water and 0.006%-Phenol Red) pH6.2 and modified PDA was prepared with 50% sea water. All the chemicals and media used are from Hi media. The pure cultures of the marine fungi were inoculated and incubated in RT for 3-5 days. The L-glutaminase activity was confirmed by the appearance of a pink zone around the fungal colony in a yellow medium. Measuring of the inner and outer diameter of the fungal colony and enzyme production is done to calculate the colony diameter and the zone diameter for all the test fungi.^{20,21} The zone index was calculated as the ratio of the outer to the inner diameter as shown in the equation below:

$$\text{Zone index} = \frac{\text{Zone diameter}}{\text{colony diameter}} \dots \dots \dots \text{Eq 1}$$

Rapid confirmation of enzyme by Thin Layer Chromatography

The culture extracts(10 days) of the selected fungi (Table 4) were used for confirmation by TLC.²² The standards L-Glutamine and L-Glutamic acid were prepared by adding 0.1g into 1ml of distilled water. The test extracts and standards were loaded onto the silica gel plates. The plates were then placed inside the chamber consisting of mobile phase, Butanol: Acetic acid: Water (5:1:4). After running the chromatogram, the plates were sprayed with 0.25% of ninhydrin in acetone



Figure 1. Fungal sampling sites used along the coast of Karnataka

Table 1. L-Glutaminase positive isolates

No.	Site of Collection (coast of Karnataka, India)	Source	Total Isolates	Fungal Strains Positive For L-Glutaminase
1.	Gokarna(Om beach, Kudle beach)-Uttara Kannada district	Marine water	6	2
2.	Murudeshwara, Uttara Kannada district	Marine water	6	2
3.	Kaup, Udupi district	Marine water	5	2
4.	Surathkal, Dakshina Kannada district.	Marine water	10	5
5.	Hosabettu, Dakshina Kannada district.	Marine water	5	2
6.	Someshwara Dakshina Kannada district.	Marine water	6	2
	Total		42	16

Table 2. Zone indices of selected marine fungi

Fungi	Diameter of the colony in cms	Diameter of colored zone in cms	Zone index
MF1	2.8	4.5	1.6
MF2	2.8	4.5	1.6
MF4	2.5	4.0	1.6
MF5	2.2	3.5	1.5
MF6	2.0	3.0	1.5
Std Glutamic acid	--		-

Table 3. Selected marine fungi and their glutaminase activity

No.	Fungi	Enzyme activity (Uml ⁻¹)
1.	MF1	1.58±0.0100
2.	MF2	1.41±0.0057
3.	MF4	1.41±0.0057
4.	MF5	1.4±0.0057
5.	MF6	1.39±0.0057

(values Mean±SD of triplicates)

and air-dried. The Rf values were calculated. TLC was performed to confirm the hydrolysis of L-Glutamine by Glutaminase produced from the selected species of *Aspergillus* and *Penicillium*. This was estimated by the redness of the spot developed by spraying the ninhydrin reagent.

Production of L-Glutaminase by submerged fermentation

Primarily screened strains (*Aspergillus*, *Penicillium* and *Pichia*) positive for L-Glutaminase production, were used for submerged fermentation. About 100ml of the modified Czapek Dox broth (pH 6.0) supplemented with glutamine and phenol red was taken in 250ml Erlenmeyer flask and inoculated with the cultures. The flasks were incubated at 28-30°C in an orbital shaker at 100rpm for 96-120 days. The samples were observed every 24 hrs for colour change.²³

Assay of L-Glutaminase

About 150ml of CzapekDox broth was taken in 250ml Erlenmeyer flask. Spore

suspensions of selected fungi were inoculated into different flasks. The flasks with inoculated fungi were incubated at 120rpm in a rotary shaker for 4 days. After this, they were incubated at room temperature for 3 days (static culture). Then, the mycelium was removed by centrifugation at 8000rpm for 10min. The clear supernatant was used for the L-Glutaminase assay. The samples were prepared in triplicate for each fungus.^{21,24}

Nesslerization method was employed to detect L-Glutaminase activity by measuring ammonia released during the enzyme reaction.²⁵ The enzyme activity was evaluated by spectrophotometric analysis from the proportional release of NH⁴⁺ ions after adding Nessler's reagent to the sample.

Briefly, 0.5ml of glutamine (0.2M) was added to 0.5ml of the culture filtrate. After 30 min of incubation, 1ml of 10% TCA was added. To 0.1ml of the above mixture, 3.9 ml of distilled water and 0.2ml of Nessler's reagent was added. After 15 min, the enzyme activity was calculated from the absorbance spectra using the spectrophotometer

Table 4. Analysis of variance results for comparing enzyme production using R program

	Df	Sum Sq	Mean Sq	F
Ind	40.0729	0.0182	390.6	6.21e-11***
Residuals	10	0.0004	0.000047	

Table 5. Tukey's test results

MF2-MF1	1.6666	0.0000
MF4-MF1	1.6666	0.0000
MF5-MF1	1.7333	0.0000
MF6-MF1	1.8333	0.0000
MF4-MF2	2.2204	1.0000
MF5-MF2	6.6666	0.7542
MF6-MF2	2.0000	0.0316
MF5-MF4	6.6666	0.7542
MF6-MF4	2.0000	0.0316
MF6-MF5	1.3333	0.1949

(Systronics) at 450nm. The samples were prepared in triplicate. A blank was prepared with distilled water and Nessler's reagent. To determine the enzyme activity a standard curve was plotted using ammonium sulphate with respect to the ammonia liberated during the reaction. The enzyme activity was calculated by the amount of ammonia released using a standard graph and the following equation:

Table 6. Molecular identification of potential L-Glutaminase producing marine fungi

No.	Fungi selected	Fungi identified as	NCBI Accession numbers
1.	MF1	<i>Aspergillus foveolatus</i>	MT 667385
2.	MF2	<i>Aspergillus nidulans</i>	MT667422
3.	MF4	<i>Penicillium citrinum</i>	MT667426
4.	MF5	<i>Neosartorya quadricincta</i>	MT667427
5.	MF6	<i>Pichia kudriavzevii</i>	MT667428

Enzyme activity(Uml⁻¹)=amount of NH⁴⁺ liberated/incubation time x ml of enzyme taken.....Eq2.

Purification of L-glutaminase and molecular weight determination

Culture broth of potential five fungal strains were filtered. The concentrated enzyme was treated with ammonium sulphate fractionation with a concentration of 80% according to the method of Orabi *et al.*²⁶ 0.2 M phosphate buffer (pH 6.0) was used to dissolve the precipitate of crude enzyme and dialyzed over night at 4°C in a dialysis bag against the same buffer. From ammonium sulphate precipitation (80%) the L-glutaminase was collected. An equal volume of cold ethanol

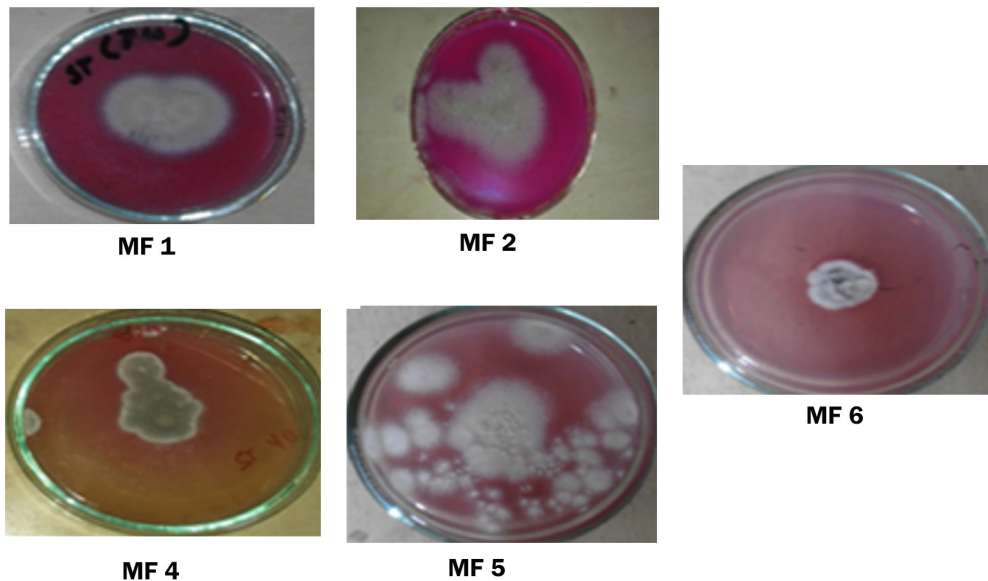


Figure 2. Isolates of marine fungi on glutamine supplemented medium positive for L-Glutaminase

added gently to a crude enzyme with gentle stirring for 15 min at 4°C and the precipitated protein was obtained by centrifugation 10,000 rpm for 30 min. the precipitated protein collected and dried. The molecular weight of the purified L-glutaminase was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using standard molecular weight markers (Bio-Rad, Hercules, CA) as described by Orabi *et al.*,²⁶ The gel was stained by Coomassie brilliant blue stain.

Morphological and Molecular identification with Phylogenetic Analysis of most promising cultures of fungi

The fungi were identified microscopically for conidial heads, fruiting bodies, and degree of sporulation by optical light microscope (Olympus, 40X) using lactophenol cotton blue staining.²⁷

The DNA identification of the six potential fungi was done employing universal primers of ITS.

The genomic DNA was isolated using 100mg of fungal mycelium. PCR amplification was carried out in a PCR thermal cycler and gene AMP PCR system 9700 (Applied Biosystems) using forward (ITS-IF) and reverse(ITS 4R) primers.

ITS-IF(sequence 5'→3'TCCGTAGGTGAACCTGCGG) ITS-4R(sequence 5'→3'TCCTCCGCTTATTGATATGC)

For PCR, a total volume of 20µl with following substrates were combined including 1Xphire PCR buffer(containing 1.5mM MgCl₂), 0.2mM each dNTPs (dATPs, dGTPs, dCTPs, and dTTPs), 1µl DNA, 0.2µl Phiro HOTSPOT II DNA polymerase enzyme, 0.1mg/ml BSA and DMSO, 0.5M Betaine, and 5pmol (0.5µl) each of the forward and reverse primers. This step was performed on ice. The automated thermocycler was used to incubate complete reaction mixture. After PCR the products were run in 1.2% Agarose gels using 0.5X TBE buffer containing 0.5µl/ml EtBr.

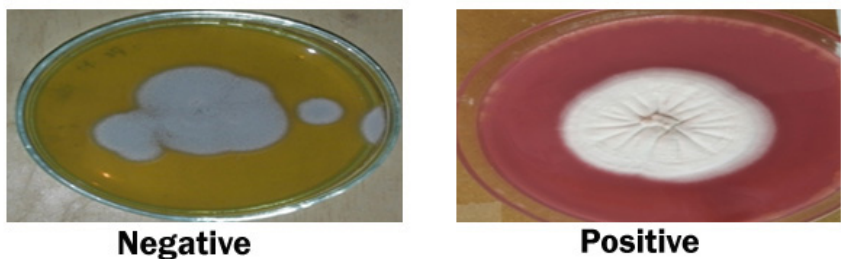


Figure 3. Plates showing negative(yellow) and positive(pink) results on phenol red supplemented medium

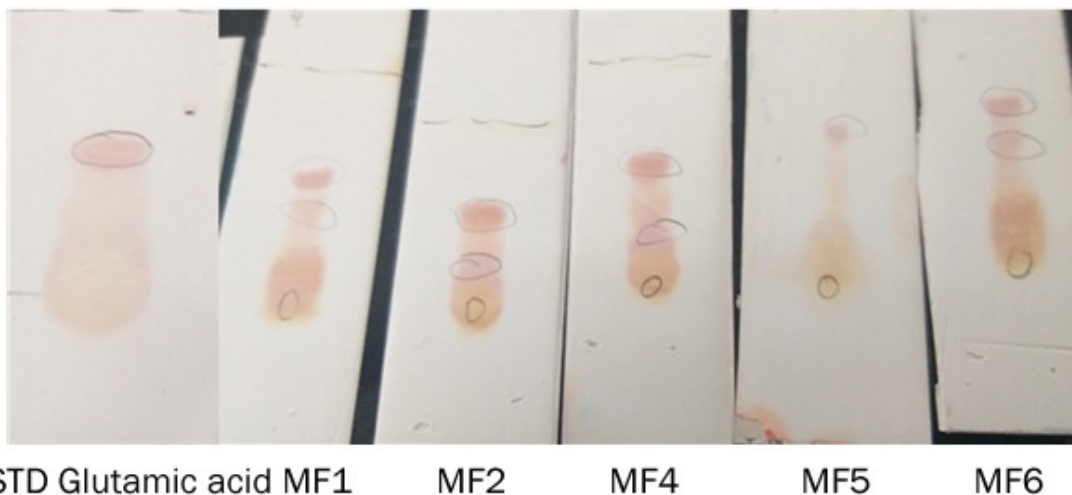


Figure 4. TLC plates for cultures showing similar Rf values for isolates of fungi as standard

Gel elution was done to purify the PCR products, and the purified products were sequenced by cycle sequencing with dideoxy mediated chain termination in a PCR thermal cycler (Gene Amp PCR system 9700, Applied Biosystems) using the Big Dye Terminator V3.1 cycle sequencing kit (Applied Biosystems, USA) following the manufacturer's protocol. The PCR mix consisting of 10-20ng PCR product, 3-2pM primer, 0.28µl sequencing mix, and 1.86µl 5X reaction buffer was made up to 10 µl with distilled water.

The sequences of the ITS rRNA of the isolates were first analyzed using the advanced BLAST search programme at the National Centre for Biotechnology Information (NCBI) website <http://www.ncbi.nlm.nih.gov> BLAST to assess the degree of similarity. Pure cultures of five potential producers were deposited in NCBI Gene data bank and accession numbers obtained. The results are depicted in Table 6. Phylogenetic tree was constructed using MEGA X software for determining relatedness of fungi isolated through

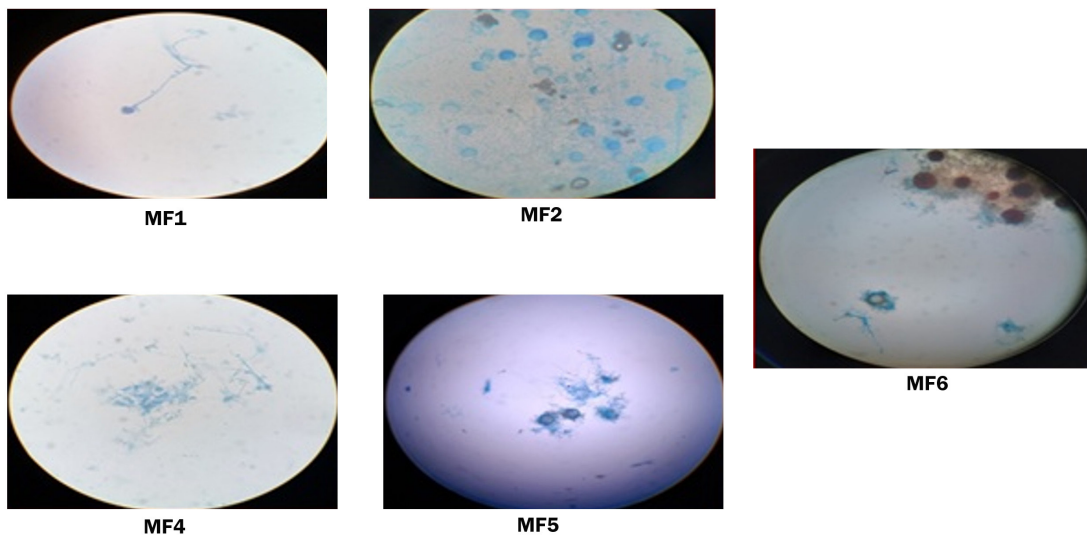


Figure 5. Microscopic images of selected isolates of marine fungi (40x)

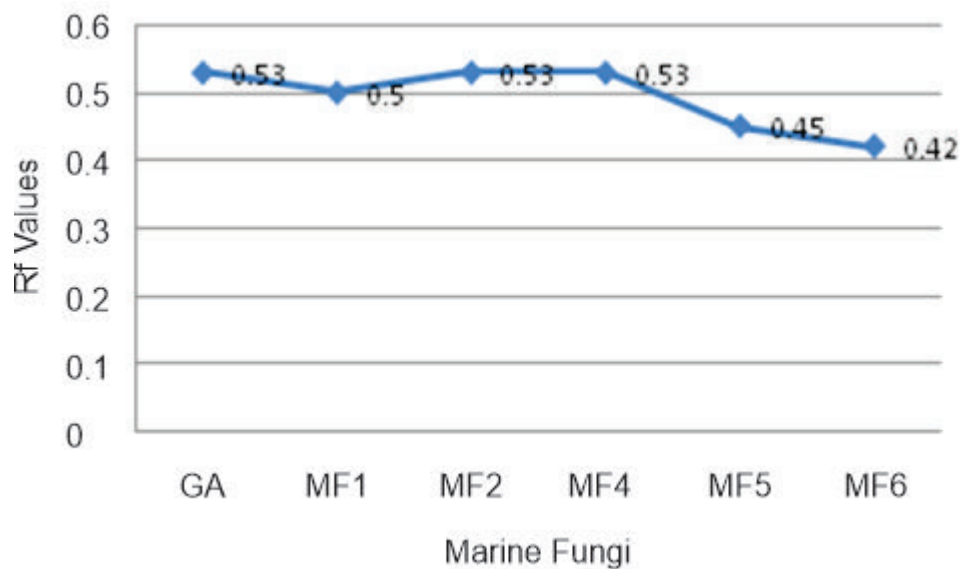


Figure 6. TLC-Rf values of standard glutamic acid and selected marine fungi extracts

the neighbor-joining tree method. (Figure 8). Next we examined the evolutionary relationships of each isolated organism based on a phylogeny derived from 14 sequences from NCBI databases.

FTIR Analysis

Ethyl acetate extracts of 5 potential fungal cultures were prepared. Fourier transform

infrared (FTIR) spectrophotometer (IR Prestige-21, Shimadzu) was employed for spectral analysis. Dried samples were scanned at 4.0cm^{-1} resolution in a special range of $4000\text{-}500\text{cm}^{-1}$. Spectral data analysis, baseline correction, normalization and band area were performed using Perkin Elmer Applications spectrum software. FTIR peaks were identified with the help of reports and databases available.^{28,29}

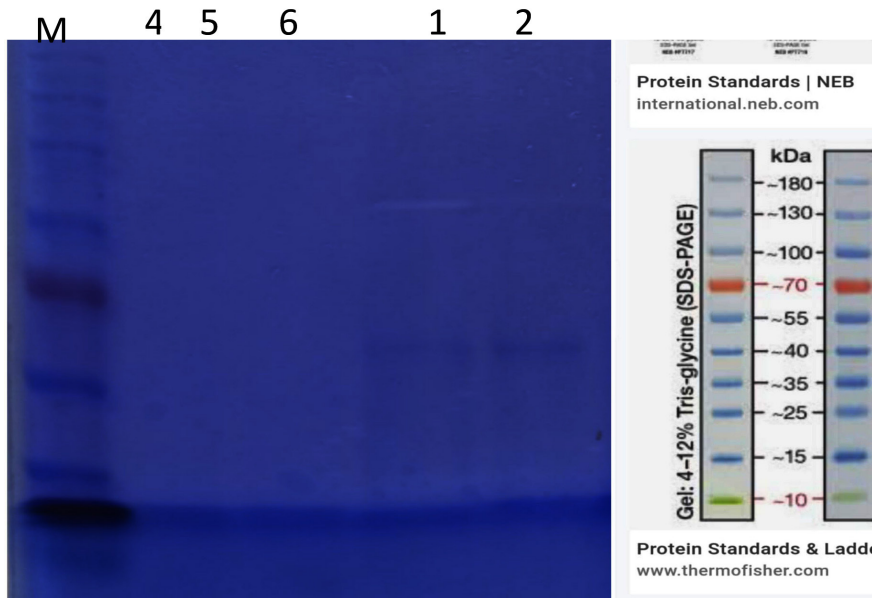


Figure 7. SDS- PAGE to determine the molecular weight of the purified enzyme.(Lane1-MF1, Lane 2-MF2, Lane 4-MF 4. Lane 5-MF 5, Lane 5-MF5, Lane 6-MF 6)



Figure 8. Dendrogram showing relatedness of fungi isolated from marine waters of Karnataka coast

LCMS

Ethyl acetate extracts of five potential fungi were employed further and tandem mass spectrometry (LCMS/MS) was used to elucidate

the molecular mass of chemical constituents in them.³⁰ Standard glutaminase was employed for comparison of mass spectrum obtained with fungal extracts. Mobile phase consisted (A)

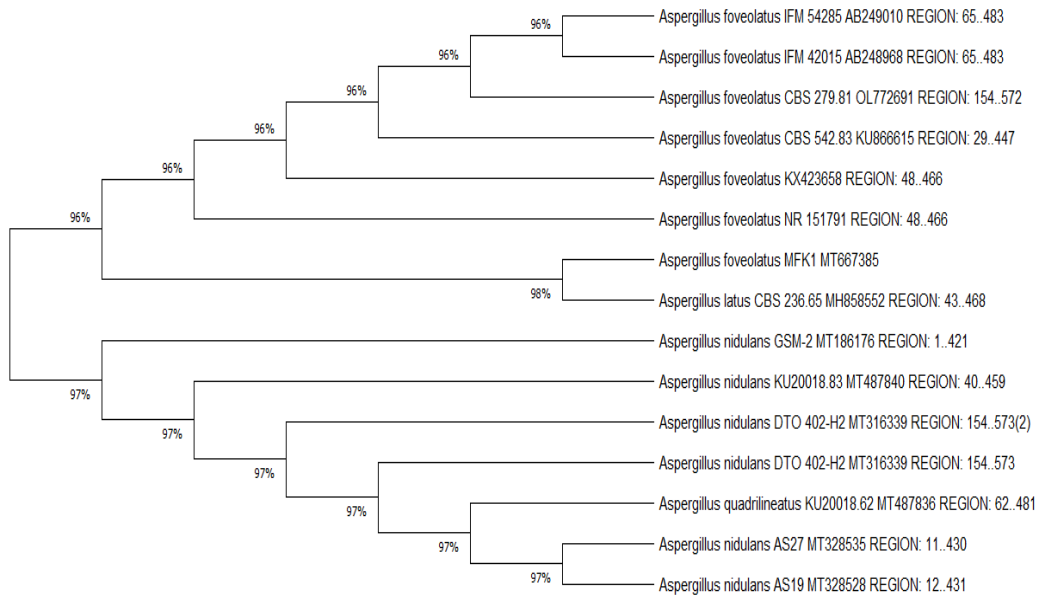


Figure 9. Dendrogram using the hierarchical cluster analysis of relatedness among the strains of *Aspergillus foveolatus*



Figure 10. Dendrogram using the hierarchical cluster analysis of relatedness among the strains of *Aspergillus nidulans*

0.1% formic acid and (B) ACN. Mass analysis was performed on an SHIMADZU LCMS 8030 equipped with a jet stream ESI source. MRM was performed in the positive and negative ion mode. Other MS parameters included are: Interface temperature at 350°C, DL temperature at 250°C, nebulizing gas

flow at 3L/min, drying gas flow at 15L/min and interface voltage at 4.50Kv.

Statistical analysis

For experimental results, the samples were taken in triplicate for the assay of

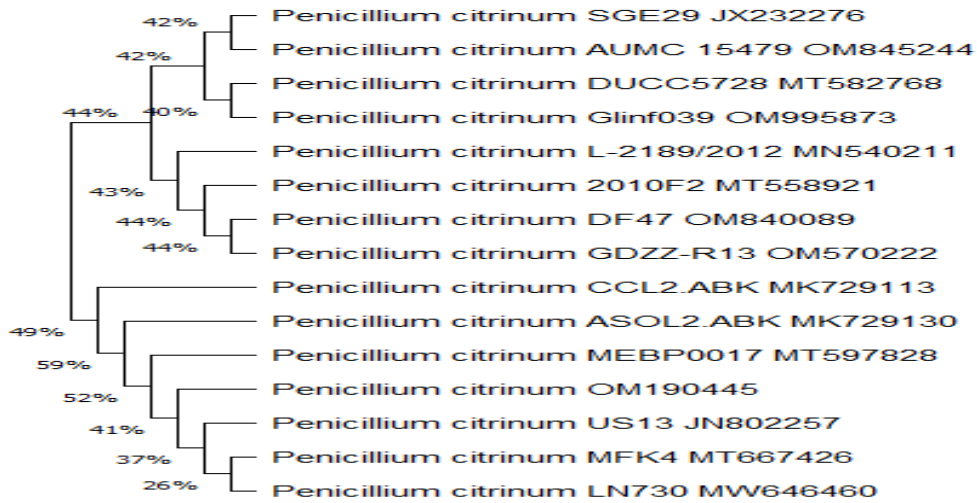


Figure 11. Dendrogram using the hierarchical cluster analysis of relatedness among the strains of *Penicillium citrinum*

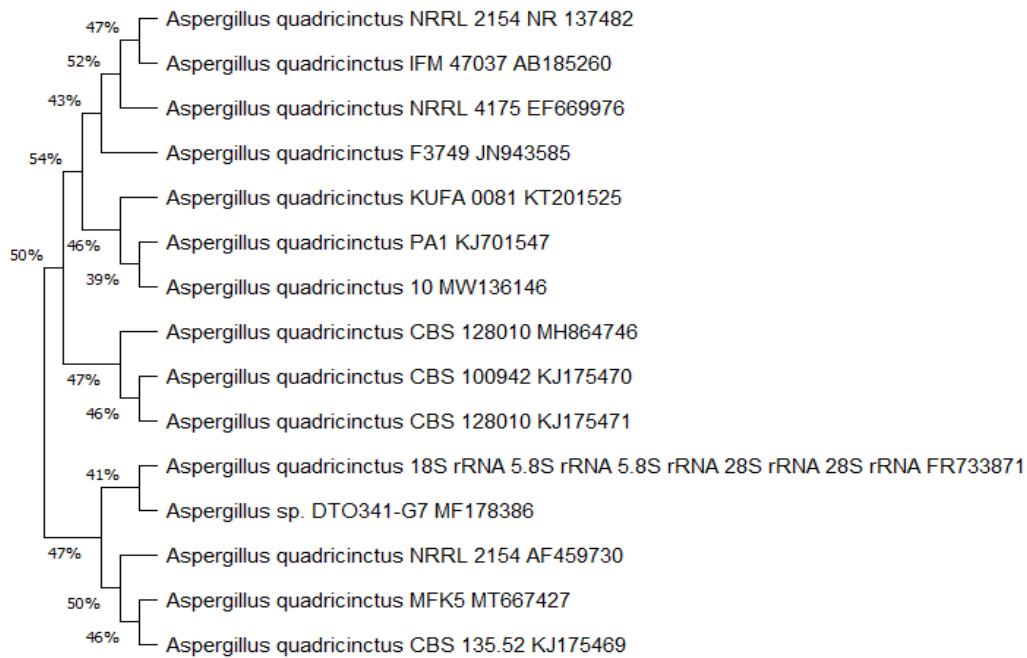


Figure 12. Dendrogram using the hierarchical cluster analysis of relatedness among the strains of *Aspergillus quadricinctus*

L-glutaminase. The results are presented as MEAN±STDEV. Analysis was carried out using R version 4.1.3. Statistical testing was carried out to examine differences in enzyme production

of marine fungi employed. Analysis of variance was carried out to verify the difference in glutaminase production among marine fungi employed. Since there is significant difference

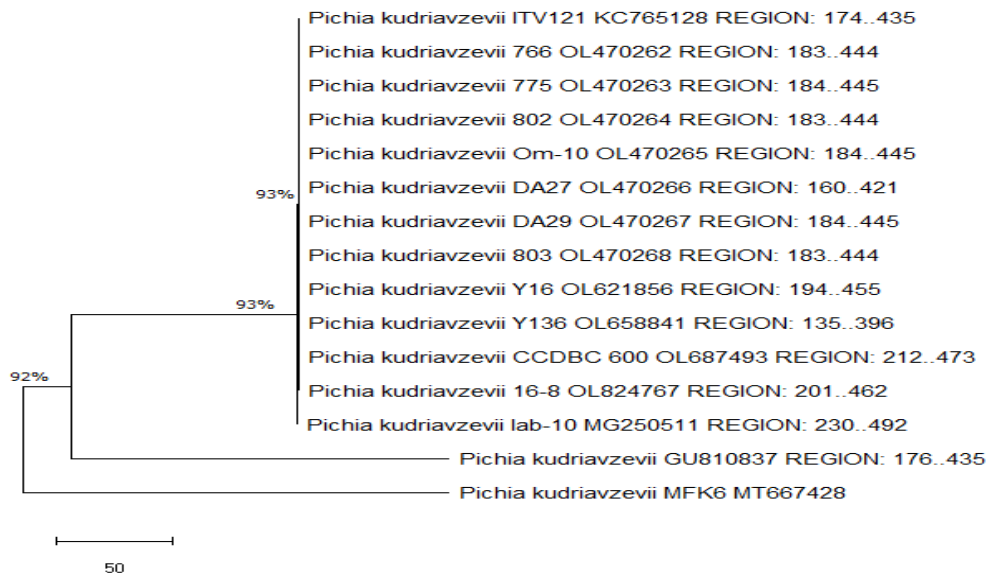


Figure 13. Dendrogram using the hierarchical cluster analysis of relatedness among the strains of *Pichia kudriavzevii*

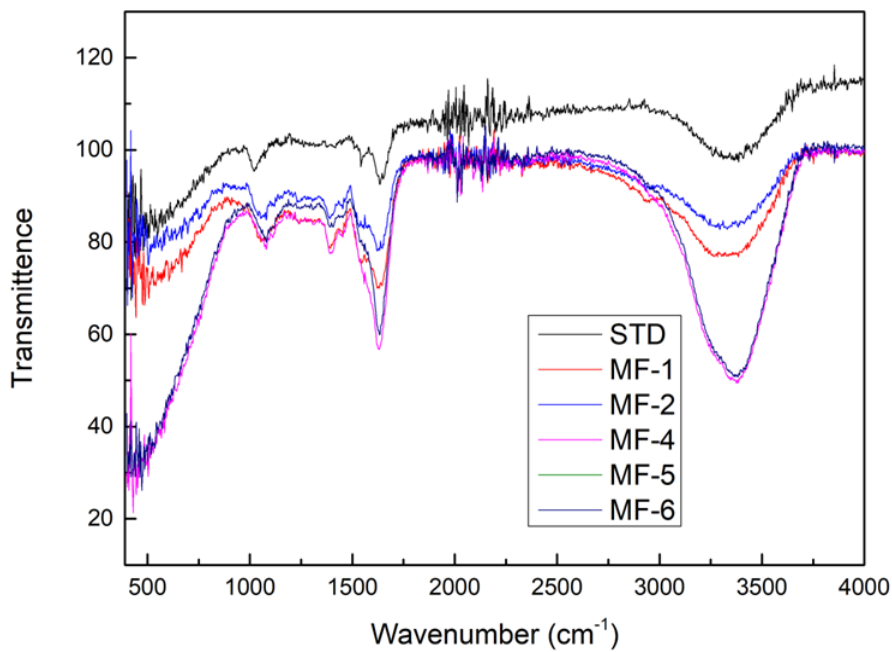


Figure 14. FTIR spectra of selected fungal extracts and reference standard

among marine fungi employed, as found in ANOVA, we examined pairwise comparison of marine fungi for glutaminase production in the given conditions.

RESULTS

Qualitative assay of L-Glutaminase

From the marine water of 8 sampling sites 42 fungi were isolated on different fungal media supplemented with glutamine. Of the 42 isolates, 16 were positive for the production of L-Glutaminase.

The results of the Glutaminase active plate assay are presented in Figure 2 and 3. All the isolates were subjected to plate assay using

the CDA medium supplemented with glutamine and phenol red. The rapid plate assay revealed a distinct zone of hydrolysis. The results obtained showed that among the 42 isolates, 16 strains exhibited L-Glutaminase activity. The results are produced in Table 1. Among the sixteen, five promising strains including MF1, MF2, and MF4 were seen to have high zone indices after 5 days of the incubation period. MF5 and MF 6 are with moderate zones (Table 2). Enzyme activity was calculated by relative ratio of zone diameter to colony diameter. Comparison of zone indices of all selected fungi was done using phenol red. Direct visualisation and activity of enzyme can be measured by this rapid plate assay.

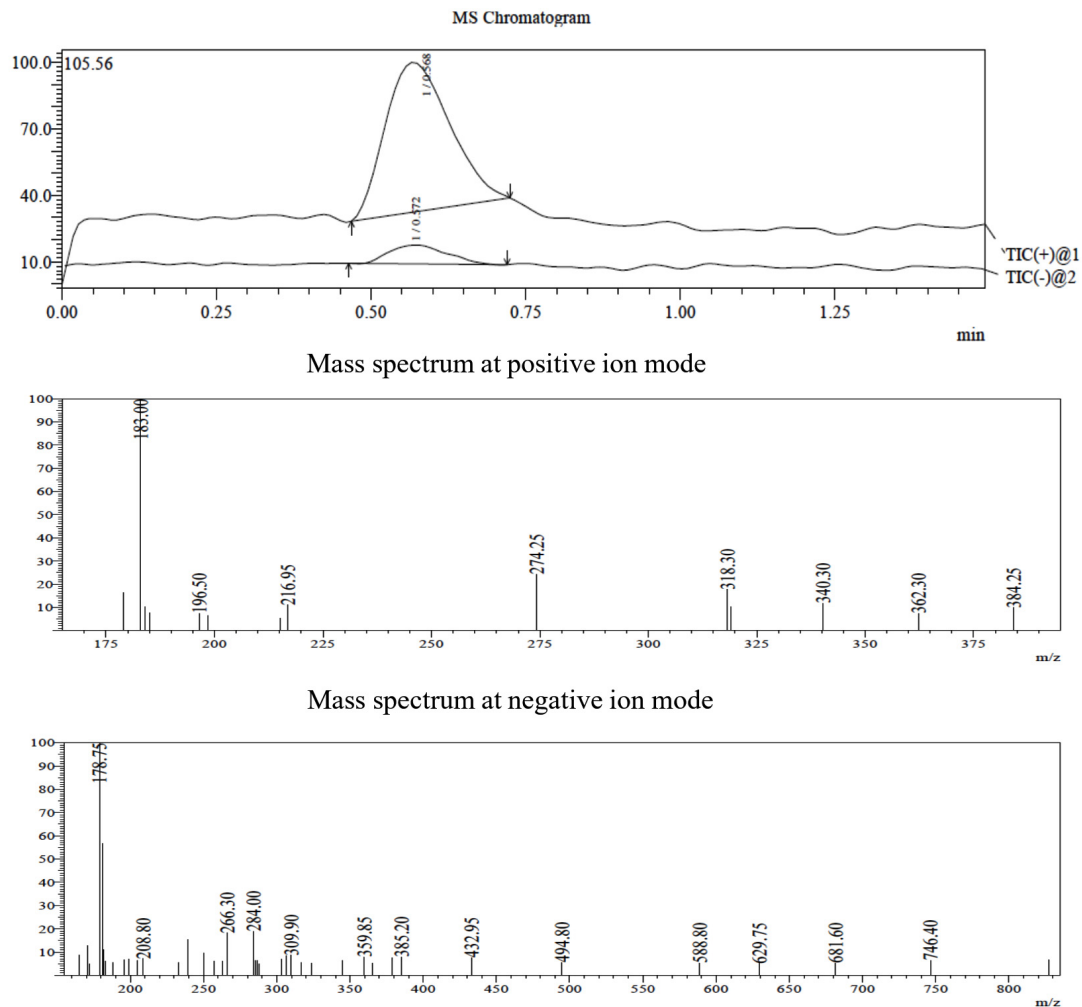


Figure 15. MS chromatogram and MS spectrum of reference standard

Rapid confirmation of enzyme by TLC

The appearance of two separated spots indicates that Glutamine was acted upon by L-Glutaminase and hydrolysed to Glutamic acid (Figure 4). The results of the confirmation of L-Glutaminase production from the potential isolates are presented in Figure 6. All the test fungi extracts exhibited Rf values (0.42-0.53) nearing to that of Glutamic acid (0.53) with the employed solvent mixture.

Production of L-Glutaminase by submerged fermentation

After 96 hrs of fermentation in a rotary shaker, the selected five strains of fungi grown in broth, changed the colour of the medium from yellow to pink showing that the fungi produce extracellular L-Glutaminase. The present study used a broth supplemented with Glutamine and Phenol red as pH indicator. The intensity of the pink colour increased after 120hrs of incubation.

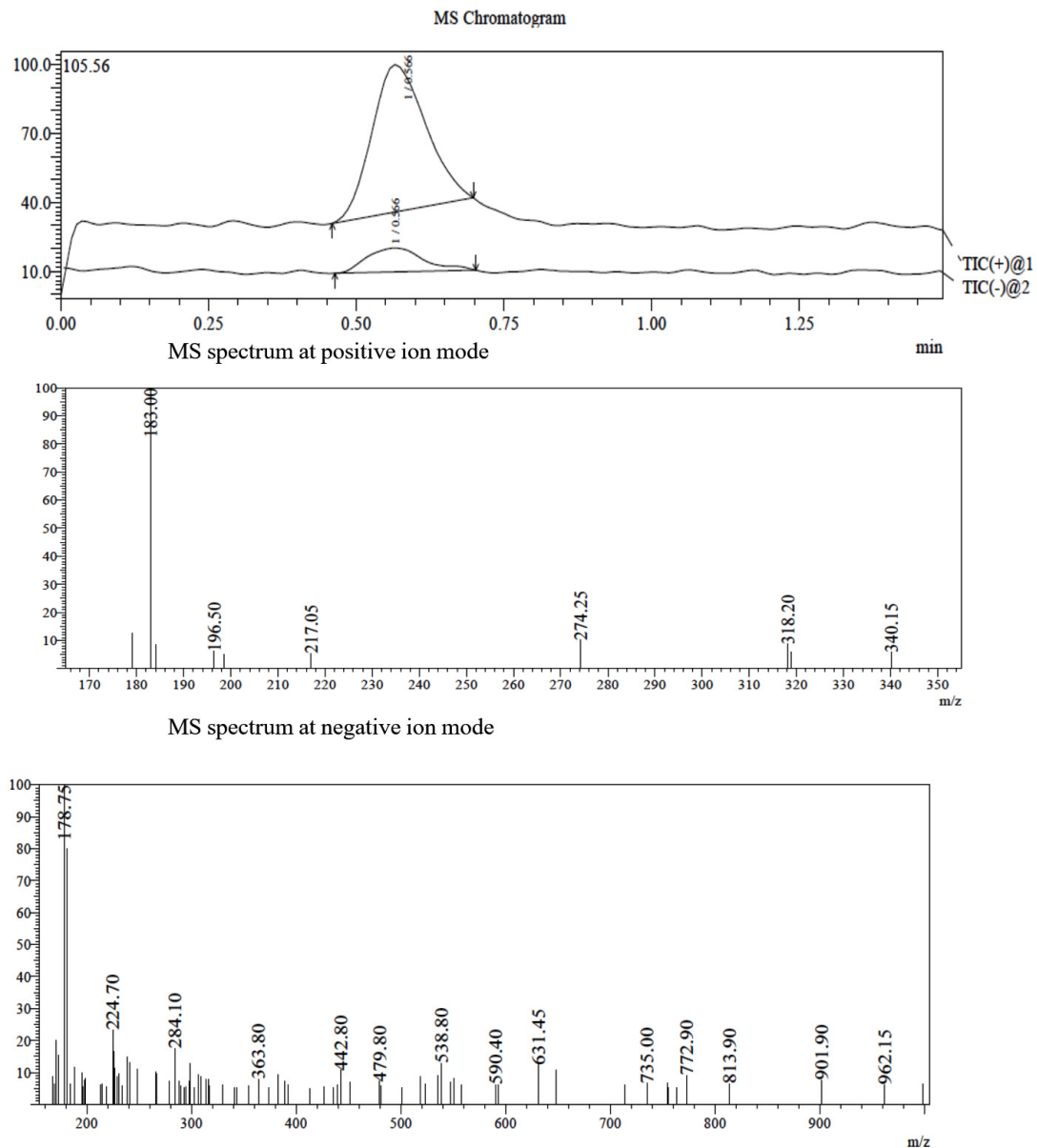


Figure 16. MS chromatogram and MS spectrum of MF1

Assay of L-Glutaminase

Among the isolates, the test fungi which showed positive results during qualitative tests were used for submerged fermentation. Five potential strains inoculated in the modified Czapek Dox broth for L-Glutaminase activity showed that the strains MF1 (1.58 ± 0.01), MF2 (1.41 ± 0.05), and MF4 (1.41 ± 0.05) exhibited greater activity. The results are shown in Table 3. Results obtained revealed that equivalent relation existed between zone index and enzyme activity measured from broth.

Purification of L-glutaminase and molecular weight determination

The molecular weight of the purified L-glutaminase was detected by using standard molecular weight markers (Bio-Rad, Hercules, CA). L-glutaminase showed a band with a molecular weight of 45 kDa (Figure 7).

Morphological and Molecular identification with Phylogenetic Analysis of most promising cultures of fungi.

The identification of the five most promising cultures of marine fungi through

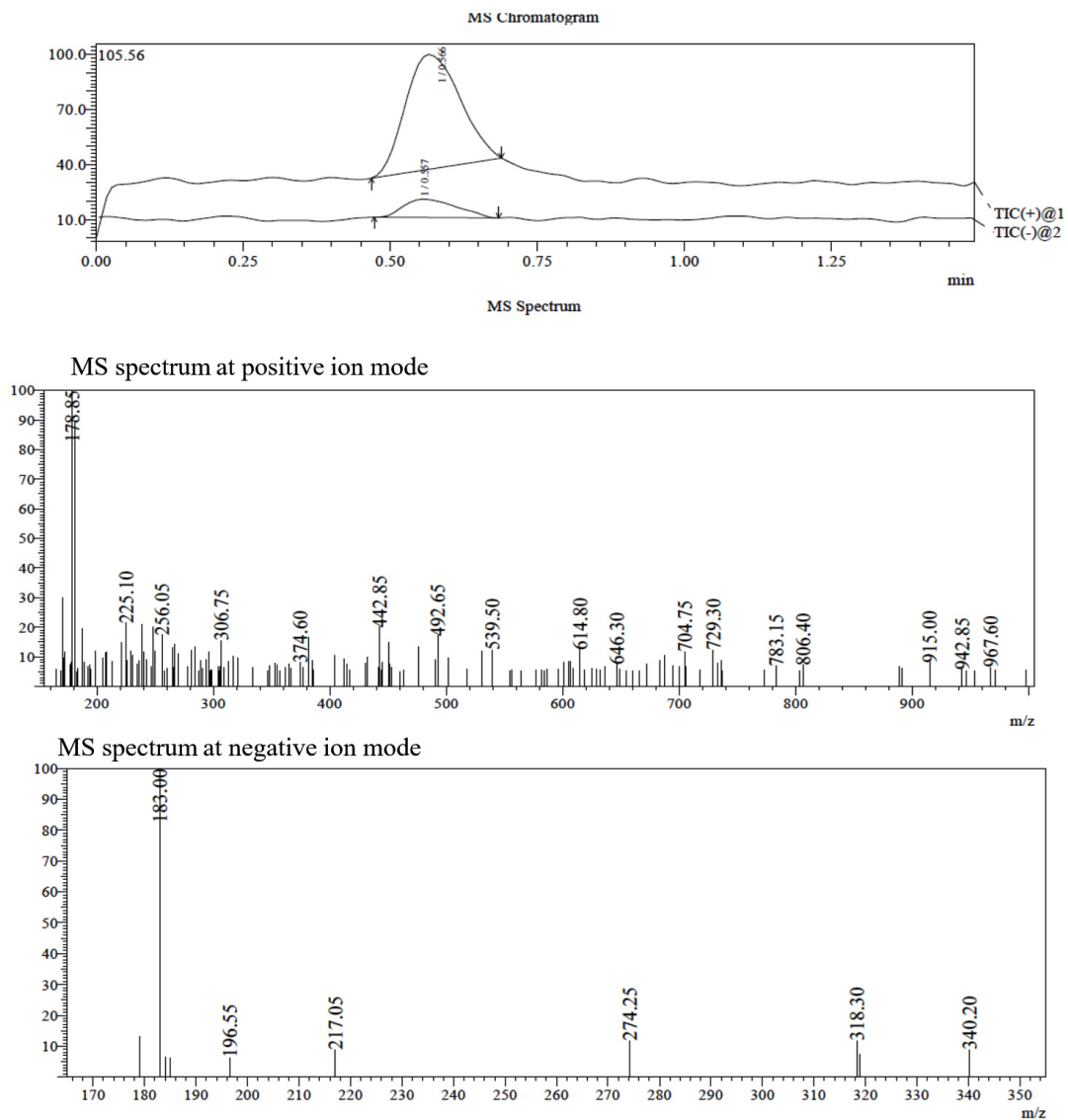


Figure 17. MS chromatogram and MS spectrum of MF2

microscopic images(40x) revealed that they are filamentous fungi (class Ascomycetes) with hyphae and conidiophores, except one, which is a yeast species of the class Ascomycetes. Their images are shown in Figure 5.

Further confirmation was done through gene sequencing. The nucleotide sequences of the gene in elected fungi have been compared with similar ITS rRNA sequences in GenBank.

The sequences of the isolates confirmed MF1/MFK1 as *Aspergillus foveolatus*, MF2/MFK2

as *Aspergillus nidulans*, MF4/MFK4 as *Penicillium citrinum*, MF5/MFK5 as *Neosartorya quadricincta* and MF6/MFK6 as *Pichia kudriavzevii*. MF3 was not identified so not mentioned in the text. MF1 was identified as *Aspergillus foveolatus* with 100% similarity. MF5 was confirmed as *Neosartorya quadri cincta* with 100% similarity, which was identified as a teleomorph of *Aspergillus fumigatus*. The others were identified with 99% similarity. Phylogenetic analysis reveals that *Aspergillus foveolatus* (MFK1) and *Penicillium citrinum* (MFK4)

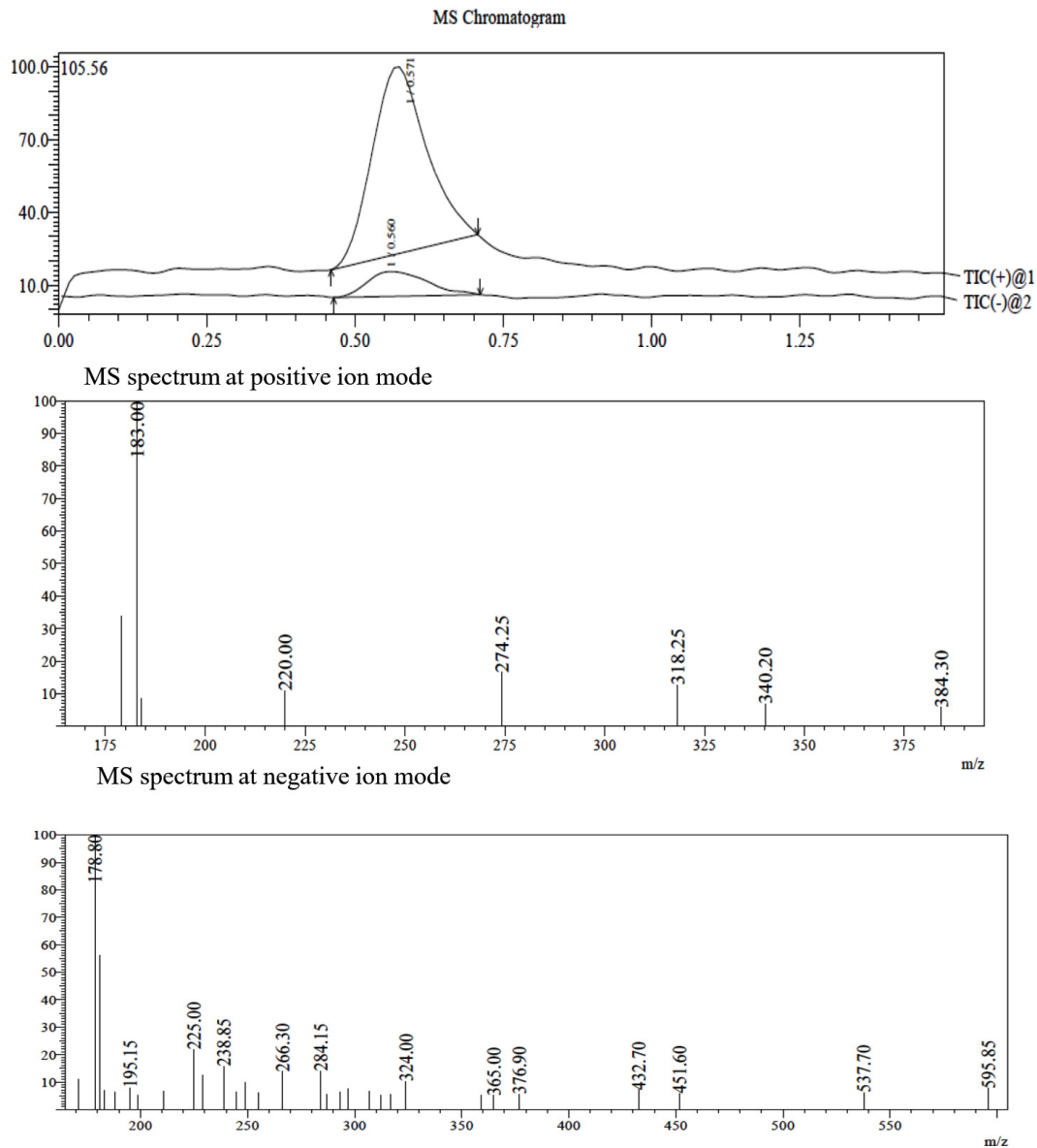


Figure 18. MS chromatogram and MS spectrum of MF4

are more closely related and in sister clades, and distant from the other three. while *Aspergillus nidulans* (MFK2) and *Pichia kudriavzevii* (MFK6) are more closely related. *Neosartorya quadricincta* (MFK5) or *Aspergillus quadricincta* is an out group and more related to MFK2 and MFK6 as shown in the dendrogram (Figure 8).

The phylogenetic analysis of the obtained sequence and related sequences in GenBank revealed that all the selected strains share the same ancestor followed by multiple internal nodes and branches sharing the same genus and species identification.

The phylogenetic tree for *Aspergillus foveolatus* was constructed with sequences retrieved from Genbank in NCBI with their accession numbers using NJ method in MEGA X programme. All *Aspergillus foveolatus* species were placed into 2 groups, 1st with 96% similarity and 2nd with 97% similarity. MFK1 is closely related to CBS236.65 with 98% similarity and share the same ancestor. MFK1 is more related to 1st group than the 2nd group (Figure 9).

Figure 10 shows *Aspergillus nidulans* (MFK2) is closely related to FO25 with 45%

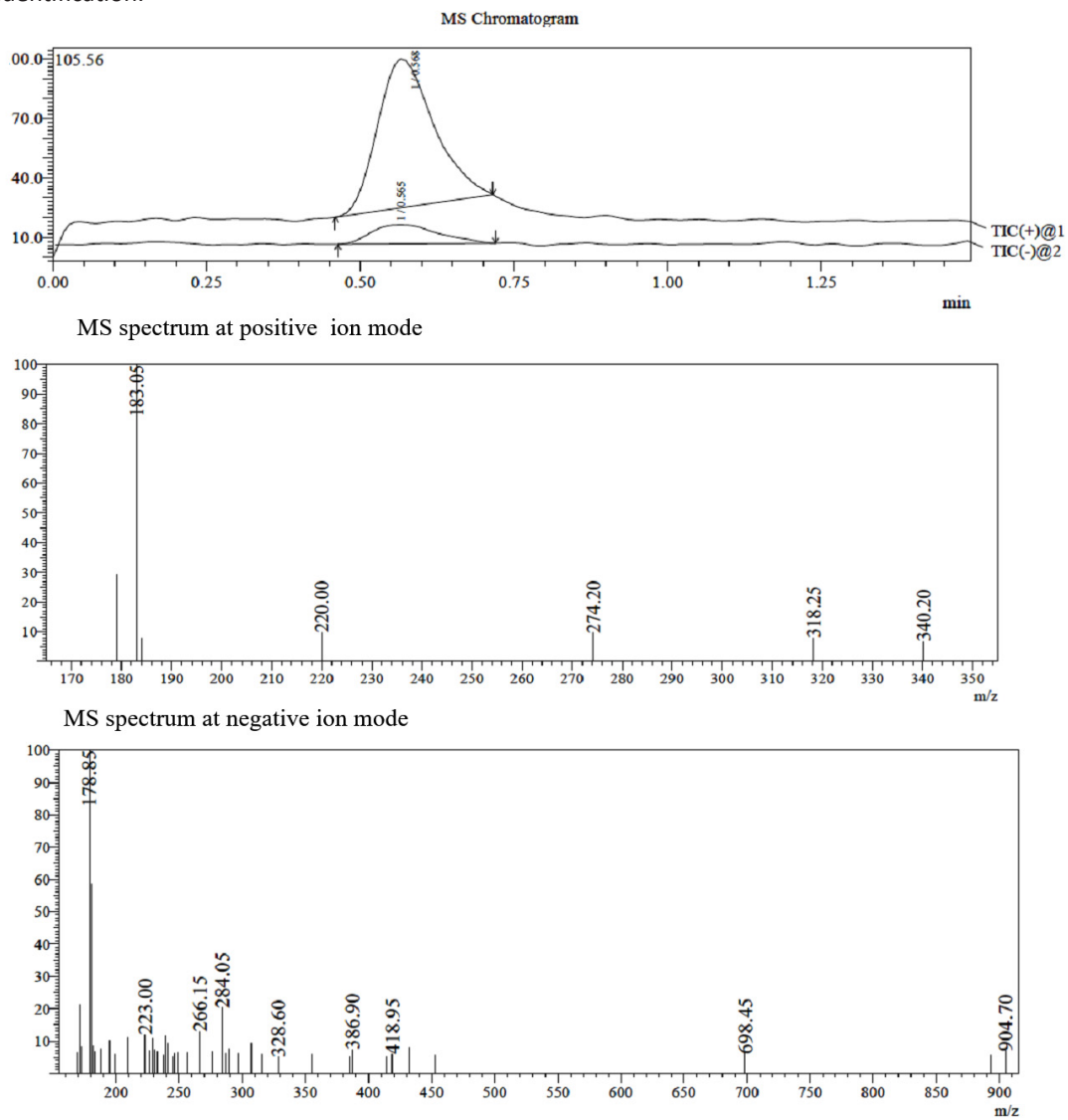


Figure 19. MS chromatogram and MS spectrum of MF5

similarity and is in the first group. Whereas 2nd group has four other species (AP3, A1, AS27 and AS 19) with greater similarity than MFK2.

In Figure 11, *Penicillium citrinum*(MFK4) is in 2nd group and more closely related to LN730 with 26% similarity. 1st group has organisms with around 40% similarity.

Figure 12 shows close relatedness of *Neosartorya quadricincta* (MFK5) with CBS 135.52 with 52% similarity. Tree has two major branches, 1st with further small branches with 50-47% similarity. MFK5 belongs to 2nd branch.

Figure 13 explains the association of *Pichia kudriavzevii* (MFK6) with other species. It is an out group but shares 93% similarity with GU8108 and share the same ancestor. While all others species are in other group/clade.

FTIR Analysis

The FTIR spectra of selected fungal extracts and reference standard is shown in Figure 14. The IR spectrum for each selected fungal extract obtained was compared with that of the

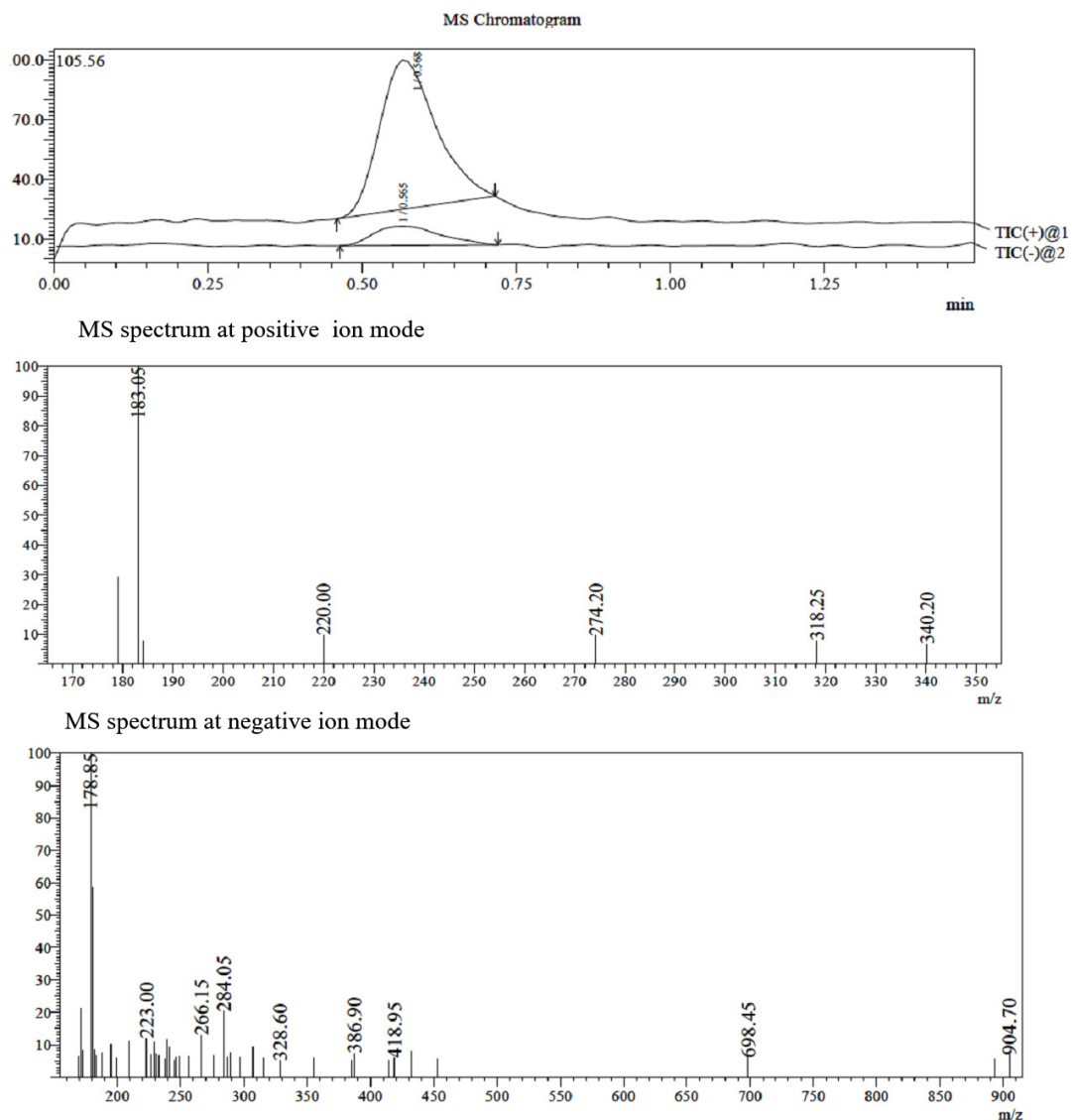


Figure 20. MS chromatogram and MS spectrum of MF6

standard (*Glutaminase enzyme*). Two regions in the IR spectrum of the fungal extract are dominated.

The bonds between 3500cm^{-1} and 300cm^{-1} corresponding to fatty acids and the signals between 1750cm^{-1} to 1500cm^{-1} corresponding to proteins. 1690cm^{-1} to 1650cm^{-1} corresponding to amides are also observed, which are also present in reference standard IR spectrum.

LCMS

MS chromatogram and mass spectra obtained for the reference standard (Glutaminase) and the Selected fungal strains (Figures 15-20) are matching with each other. They exhibit similar chromatogram and peaks, which reveals the presence of Glutaminase in the culture media of selected fungi.

Analysis of the data

One of the analyses is to compare the marine fungi; this is done statistically through the Analysis of variance. The result of Analysis of Variance for the experiment was that there was a significant difference among marine fungi in glutaminase production at 0.1% level of significance (Table 4). Now the quest is which marine fungi are significantly different. Statistically, Tukey's test is used for pairwise comparison. This test result is shown in Table 5. MF1 was highly significant with all other fungi. Whereas MF6 showed significant differences with MF2 and MF4.

DISCUSSION

L-Glutaminase is present in plants, animals, and microorganisms. The advantage of employing microorganisms for extracellular L-Glutaminase is that we can go for large-scale production using simple substrates in a short period. It is also easy to manipulate microorganisms genetically to obtain greater amounts of enzymes. *Beauveria* Sp and *Beauveria bassiana* were isolated from marine sediments of Cochin, Kerala, were seen to produce L-Glutaminase.³¹ There are very few studies on L-Glutaminase production as it is difficult to obtain sufficient quantities of enzyme from marine fungi.³² Thus, we screened

marine water for fungi producing L-Glutaminase. There are several bacteria, filamentous fungi and yeasts studied for this enzyme production. Krishna Kumar *et al.*,³³ from Chennai reported extracellular production with optimizing conditions from an actinomycete (marine alkaliphilic *Streptomyces* species-SBU1) isolated at the Cape Comorin coast. Several bacteria like *E coli*, *Pseudomonas*, *Klebsiella* and *Aerobacter* and fungi like *Aspergillus*, *Beauveria*, *Verticillium* and *Trichoderma* sps have been reported to produce L-Glutaminase. Marine fungi produce diverse and bioactive monoterpenes, diterpenes, triterpenes, tetraterpenes, sesquiterpenes, and steroids. Studies on the production of these from *Penicillium* and *Aspergillus* have been reported by Kim.³⁴ Yeasts such as *Rhodotorula*, *Hansenula*, *Candida*, *Cryptococcus* and *Torulopsis* have also been studied for L-Glutaminase production.³⁵ Marine fungi were isolated for the production of bioactive metabolites and their antibacterial activity against *Staphylococcus* and other pathogens. These were identified as *Aspergillus* and *Curvularia* species.³⁶ The marine biosphere is the richest habitat for microorganisms, especially fungi and halotolerant enzymes as they provide an important alternative for therapeutic purposes, since they are capable of functioning under extreme conditions that leads to precipitation/denaturation of most proteins from other fungi. Another advantage is that seawater and its salinity, which is closer to human blood plasma, can cause these fungi to produce biomolecules, especially enzymes that can cause lesser side effects during therapeutics³⁷. Unissa *et al.*³⁸ in their review listed out the various applications of L-glutaminase and the manufacture of thianine from L-glutaminase, which is an important amino acid infused in to tea to enhance its taste. They also observed that commercial production of L-glutaminase from *Bacillus* sp is commercialized in Japan and UK, though the production is done using yeasts and *Aspergillus*.

The search for a novel L-glutaminase producing fungi from rare sources and the selection of isolates with high enzyme production for commercial and industrial applications is of great interest to scientists. Marine fungi are one of the richest sources of structurally novel and biologically active metabolites. Many marine fungi are also being explored for the production

of extracellular enzymes. Aquatic fungi are better choices for the production of secondary metabolites against their terrestrial counterparts, as they are more resistant to physical conditions. Marine fungi are tolerant to harsh environments such as increased temperature, salinity, pH, and other extremities. Thus, their products can be exploited in a plethora of industries including food, textile, medicine, environmental, cleaning, and pharmaceuticals.¹⁴ Mervat Morsyet *al.*,³⁹ isolated marine endophytic fungus *Aspergillus* species ALAA-2000 as potential L-glutaminase producer and studied the optimization of conditions using low cost substrates for enzyme production. *Aspergillus fumigatus* and its teleomorph *Neosartorya* are known to produce important bioactive secondary metabolites. Several pentaketides some benzofuran-1-one derivatives, some benzoic acid derivatives and benzoxepine derivatives, have been isolated from *Neosartorya quadricincta* KUFA-4-one, which is a marine sponge associated fungus.⁴⁰ Halophilic and halotolerant bacterial strains producing L-glutaminases were isolated from Urmia salt lake in Iran.⁴¹

In the present study, a total of 42 marine fungi were collected and tested for their potential for L-glutaminase production on solid and broth media. Of the 42 isolates, 16 isolates exhibited positive result in rapid plate assay. L-glutaminase activity by fungi is usually determined by Nessler's method using L-glutamine as substrate. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 μ l of ammonia under optical assay conditions.⁴² Greater enzyme production was recorded for *Aspergillus foveolatus* after 8 days of incubation at room temperature in a rotary shaker. All the four filamentous fungi identified through DNA sequencing were capable of production. In our study, fungal enzyme index varied from 1.5-1.6. For *Aspergillus* it is 1.06, which is in line with study conducted by Doriya and Santhoshkumar.⁴³

Tandem mass spectrometry (LC-MS/MS) was used to detect the phenolic compounds in antifungal extracts of stem bark and wood of *Terminalia brownie*.⁴⁴ Azzollini *et al.*,⁴⁵ employed GC-MS and LC-HRMS for detection of metabolites from fungal cultures. In this investigation we employed mass spectrometry to compare the

fungal cultures components with that of standard glutaminase, to confirm the production of enzyme by our cultures.

Many species of *Penicillium* such as *P.citrinum*, *P. chrysogenum*, *P.solium*, *P.hirsutum*, etc. have been isolated from saline habitats and their phylogenetic relationships have been studied.⁴⁶ In this study, we studied and constructed phylogenetic tree of our marine fungus MFK4 with other species whose sequences are drawn from NCBI. All marine fungi obtained were compared with others by constructing phylogenetic tree, which revealed their similarities and distant associations.

Identifying fungi based on morphology alone can be challenging as there are a fewer morphological characters that can be checked for their identification. The three nuclear ribosomal genes used in fungal identification and the potential advantages and limitations of the ITS region, which is the official DNA barcoding marker for species-level identification of Fungi.⁴⁶ The yeast identified was *Pichia kudriavzevii*, which was studied as a teleomorph of *Candida krusei*, and exhibited L-Glutaminase activity as reported for the first time in the present study. *P. kudriavzevii* has several genetically modified strains, which are employed in large scale production of glycerol and succinate and are also made use of in food industry.⁴⁷ Though phenotype remains as integrative part of fungal taxonomy, in the molecular level ITS acts as universal fungal barcode to identify phylogenetic lineages. It helps to differentiate various species from different habitats.⁴⁸ Glutaminase enzyme preparation obtained from *Aspergillus niger* strain GT 147 is studied and study concluded that this can be used safely in food industry.⁴⁹ Gutaminase produced by the bacterial strain *A. xylosoxidans* RSHG1 was known to be stable in a wide range of pH and temperature conditions, having a high affinity for its substrate. Glutaminase enzyme activity was enhanced by a number of metal ions, such as sodium chloride and thus can be used in food industry.⁵⁰ These studies show the importance of marine filamentous fungi in production of glutaminase enzyme. Several bacteria also were shown to produce Glutaminase enzyme. *Bacillus subtilis* NRRL 1315 was the most potent producer

for L-glutaminase enzyme and, the crude enzyme exhibited considerable DPPH radical scavenging activity.⁵¹

The dry rot fungus *Serpula lacrymans* and its intra species characterization was done using FTIR analysis, and study explores the applicability of FTIR in identification and discrimination of different strains of the species.⁵² We employed FTIR studies to understand the presence of our enzyme glutaminase in selected fungal culture broths, by analysing the presence of spectral data obtained. Investigation of 15 halophilic bacterial strains isolated from the marine environment that produced extracellular L-glutaminase was done. *Bacillus sp.* DV2-37 was selected as the most potent strain, showed potent cytotoxic activity and antitumor effect against human breast (MCF-7), hepatocellular (HepG-2), and colon (HCT-116) carcinoma cell lines.⁵³

CONCLUSION

It can be concluded that the screened marine fungi exhibited different phylogenetic associations with the fungi from NCBI and are potent in producing glutaminase. Microbial L-glutaminases with improved properties like universal presence, thermo-resistance and salt resilience discovers applications in nourishment industry just as in malignant growth treatment. Marine fungi isolated and identified in this study include a yeast species producing glutaminase is a novel work reported.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

KBS and MS designed the experimental protocols. TPMP analysed the data. SR wrote the

manuscript. All authors read and approved the final manuscript for publication.

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None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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