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ISOLATION, IDENTIFICATION AND OPTIMIZATION STUDIES FOR L-ASPARAGINASE PRODUCTION FROM FUNGAL ISOLATES OF MARINE SEDIMENTS

RASHMI HOSAMANI[1*], KALIWAL B B[2] ,AND SHREERANGEGOWDA THIPPESWAMY[1]

[1]Department of Microbiology,University College of Science, Tumkur University, Tumkur-572103
[2]Vice-Chancellor, Davanagere University, Shivgangotri, Davangere-577002

ABSTRACT

Marine microflora are a potential source of anticancer compounds and are the least explored, an attempt has been made to isolate the fungi from marine sediments of different coasts and screened for potential L-asparaginase production. Since malignant cells are dependent on L-asparagine for its survival. Out of eighteen isolates from five different coasts, three of isolates have exhibited significant L-asparaginase production on modified Czapek-Dox medium incorporated with 1% L-asparaginase and 0.009% phenol red indicator. Initial screening for CD21 & MD22 for asparaginase activity showed an activity of 0.14 and 0.07 IU/ml, respectively and were considered potential L-asparaginase producers. The molecular identification revealed that CD21 showed 99% similarity to *Cladosporium tenuissimum* and MD22 shows 94% similarity to *Ramichloridium apiculatum.* Optimization studies were carried out under submerged fermentation conditions. *Cladosporium tenuissimum* has shown maximum L-asparaginase production at 28°C, 1.5% glucose and 2% NaCl concentration. Similarly, *Ramichloridium apiculatum* exhibited the maximum activity at 28°C, 2% glucose and 2% NaCl concentration. The present study revealed that the two marine fungi are capable of producing bioactive enzyme L-asparaginase and can be further purified and characterized. This is the first report on L-asparaginase production by *Cladosporium tenuissimum and Ramichloridium apiculatum.*

KEYWORDS: **L-asparaginase, marine sediments, *Cladosporium tenuissimum, Ramichloridium apiculatum,* submerged fermentation.**

RASHMI HOSAMANI*
Department of Microbiology,University College of Science,
Tumkur University, Tumkur-572103

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INTRODUCTION

Nature continues to be the most prolific source of biologically active and diverse chemo types and it is becoming increasingly evident that associated microbes may often be the sources of biologically active compounds originally isolated from host microorganisms. Marine ecosystems have a high diversity of living organisms compared to terrestrial ecosystems providing numerous resources for human nutrition and health.[1] Rawat *et al*.[2] have reported that over the past few decades, during which the study of marine natural products has begun in earnest, approximately 16,000 novel marine natural products have been discovered. Marine microorganisms have unique properties since they have to adapt to extreme marine environmental conditions such as high or low temperature, alkaline or acidic water, high pressure and limited substrate in the deep-sea water. These distinctive characteristics have attracted many researchers to explore in depth since there is a potential for marine microorganisms to be used in industry.[3] Marine microorganisms have acquired special importance as the most potent source of antibiotics, enzymes and other bioactive secondary metabolites.[4] The secondary metabolites derived from a number of marine organisms possess antibiotic, anti-parasitic, antiviral and anti-cancer activities.[5-6] Therefore research into marine microorganisms and their metabolites has become a major task in the search for novel pharmaceuticals.[7] Among the various sources of anticancer drugs, fungal sources are more advantageous with respect their potential in producing diverse compounds since they have multiple metabolic pathways, can generate a substantial new structure, activity and diverse secondary metabolites. Many recent reports state that a huge number of naturally-occurring compounds are unexplored as therapeutic/chemical agents in pharmaceutical, food and agricultural industries due to the lack of scientific and systematic investigations, particularly systematic extraction and bioactivity screening, isolation, identification and characterization of bioactive compounds from marine fungi. Marine fungi are rich profile of biologically active metabolites, especially from genera *Penicillium, Aspergillus* and *Fusarium* have been used aiming the development of novel therapies for treating cancer. Whereas the marine fungi are least studied than terrestrial fungi due to their production of new metabolites which are not found in terrestrial fungi.[8] In clinical research enzymes have obtained great importance in recent years. L-asparaginase is one of them which are widely present in nature.[9] The discovery of L-asparaginase (L-asparaginase aminohydrolase, E.C.3.5.1.1),a medicinal agent for the treatment of malignant tumors, was made in 1922.[10] Figure 1 depicts that L-asparaginase (E.C 3.5.1.1) is a tetrameric protein that hydrolyzes free L-asparagine to give aspartic acid and ammonia.[11-12] The malignant cells depend on an exogenous source of L-asparagine for survival, where as the normal cells are able to synthesize L-asparagine[13-14] and are thus less affected by its rapid depletion on treatment with the enzyme L-asparaginase. This enzyme is the drug of choice used in combination therapy for treating acute lymphoblastic leukemia in children.[12,15-17] A great deal of interest has emerged in studying the possibilities of harnessing potential microorganisms that produce this enzyme. The enzyme is produced by a large number of bacteria including *Pseudomonas aeruginosa*[18] and *Citrobacter* sps.[19]. However, the administration of such an enzyme protein for a long duration, in general, produces the corresponding antibody in the tissues, resulting in anaphylactic shock or neutralization of drug effect. Therefore, the use of new serologically different L-asparaginase with a similar therapeutic effect is highly desirable.[20] Eukaryotic microorganisms such as yeast and filamentous fungi genera such as *Aspergillus*, *Penicillum* and *Fusarium* are commonly reported in scientific literature to produce L-asparaginase with less adverse effects.[21-22] Marine sediment may be a good source for active asparaginase producing fungi, because marine environment, particularly seawater, which is saline in nature and chemically closer to human blood plasma, it is expected that marine fungi could provide L-asparaginase with lesser side effects to human.[23] In spite of the above reports on asparaginase production by fungi from marine sediments, limited literatures are available on enzyme producing marine fungi. Therefore, the aim of the study is to isolate and identify filamentous fungi from marine sediments and to investigate on production and exploration of bioactive L-asparaginase from such isolates.

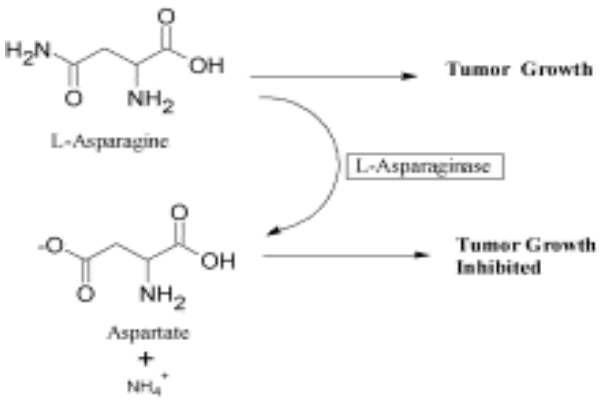

Figure 1
Schematic illustration of the reaction mechanism of L-asparaginase.

MATERIALS AND METHODS

Sample Collection

Sediment samples are collected by a core sampler at different coasts of Karnataka such as Gokarna, Devbagh, Murdeshwar and Karwar as well as from Cancona coast of Goa. The sediment cores are fractionated and transferred to sterile falcon tubes along with sea water and store at 4°C for until shipping to the laboratory, then stored at -20°C.[3]

Isolation of fungi

The isolation was carried out on Marine agar (Hi-media) by employing dilution plate-method. The isolated organisms were maintained on modified Czapek Dox medium containing (g/L of distilled water): glucose (2.0g), L-asparagine (10.0g), K_2HPO_4 (1.52g), KCl (0.52g), $FeSO_4 \cdot 7H_2O$ (0.03g), $ZnSO_4 \cdot 7H_2O$ (0.05g), $NaNO_3$ (0.3g) and Agar (18.0g) at pH 6.2.[8]

Screening of L-asparaginase producing fungi

The fungal isolates were subjected to rapid screening for L-asparaginase production by plate assay following the procedure of Gulati *et al.*[24] Modified Czapek Dox's (mCD) medium, pH 6.2, used for fungi contained 0.2% (w/v) glucose, 1% (w/v) L-asparagine, 0.152% (w/v) K_2PO_4, 0.052% (w/v) KCl, 0.052% (w/v) $MgSO_4.7H_2O$, 0.003% (w/v) $CuNO_3$. $3H_2O$, 0.005% (w/v) $ZnSO_4.7H_2O$, 0.003% (w/v) $FeSO_4.7H_2O$, 1.8% (w/v) agar, initial pH 6.2 was supplemented with 0.009% (v/v) phenol red as indicator. The media was sterilized; the plates were inoculated and incubated at 28±2°C for 96 h. Control plates contained modified Czapek-Dox's medium without dye and L-asparaginase. The zone of L-asperaginase production and colony diameter were measured after 48 h. Colonies with pink zones were considered as positive L-asparaginase producers. Isolates with maximum zone diameter were selected for further study.

Quantitative estimations of enzyme activities were carried out using modified Czapek-Dox broth medium. Erlenmeyer flasks (250 ml) containing 50 ml of the modified Czapek-Dox medium were inoculated with each of the test organisms. The flasks were incubated at 28±2°C at 250 rpm for 48 h, in a controlled environment using rotary shaking incubator. Uninoculated medium served as control. The flask contents were centrifuged at 4000 rpm for 15 min. Quantitative estimation was carried out following Nessler's reagent according to the method of Imada *et al*.[25] and the enzyme activity is expressed as IU. One international unit of L-asparaginase activity is defined as 1 µmol of ammonia per ml per min under the conditions of the assay.

Identification of the strain

The isolates CD21 and MD22 which have produced maximum zone diameter by plate assay method were cultivated in Erlenmeyer flask containing modified Czapek Dox's broth medium in controlled conditions using rotary shaking incubator at 250 rpm. After 48 hours of incubation pellets were collected by centrifugation and suspended in PBS buffer and sent to Bioserve Biotechnologies (India) Pvt. Ltd, Hyderabad for further molecular identification by gene sequencing.

Standardization of growth parameters for potential isolates

Submerged fermentation was carried out for the fungal isolates using 250 ml capacity Erlenmeyer flasks. The optimum physiological growth parameters of potential fungal isolates were determined under submerged conditions varying each parameter at a time. Erlenmeyer flasks, containing 100 ml of modified Czapek-Dox's medium (production medium) with 1ml culture suspension (three days old) were incubated at 28±2°C for five days on a rotary shaking incubator at 250 rpm. All experiments were conducted in three replicates and the mean values were presented.

Effect of incubation temperature

The effect of initial temperature was studied by cultivating CD21 and MD22 at different temperature levels. The selected experimental temperatures in the medium were 4 °C, 28 °C, 37 °C and 50 °C for every 72, 96 and 120 hrs of incubation and enzyme assay was carried out as described by Imada *et al.*[25] Among the four temperature levels, the optimum temperature for the maximum enzyme activity was taken into consideration for further studies.

Effect of glucose concentration

To investigate the effect of glucose concentration, the production medium was supplemented with 0.5%, 1.0%, 1.5% and 2.0% (w/v) glucose respectively. The isolates CD21 and MD22 were cultivated at 28±2°C and the enzyme assay was carried out after 72, 96 and 120 h.

Effect of salt concentration

To study the effect of salt concentration the production medium was supplemented with 0.5%, 1.0%, 1.5% and 2.0% (w/v) sodium chloride respectively and the enzyme activity was measured spectrophotometrically by recording optical density (ELICO SL-210, India).

RESULTS

Isolation and screening of L-asparaginase producers by plate assay method

The results revealed that out of eighteen isolates from five different coasts, eight strains were positive for L-asparaginase with different ranges of zone diameters. Among the 8 different strains, only 3 strains showed highest zone (>40%) viz., CD21 has highest colony diameter of 16 mm with 9 mm zone diameter compared to MD22 which has colony diameter of 16 mm with 8 mm zone diameter, whereas CK21 had colony diameter of 24 mm and zone diameter of 11 mm (Table-1). Hence, these potential isolates were selected for further studies and also subjected to molecular identification.The broth studies of enzyme activity revealed that CD21 has highest activity 0.14 IU/ml followed by MD22 (0.07 IU/ml) and CK21 (0.04 IU/ml) (Table-2). Out of these three positive isolates, CD21 and MD22 showed maximum activity and were considered as the potential strains for further studies.

Identification of the strain

DNA was isolated from the different fungal cultures, The DNA was subjected to PCR to amplify the ITS region using ITS 1 and ITS 4 primers. The 400 - 900 bp amplicon was gel eluted and the product was sequenced by Sanger's method of DNA sequencing. The sequencing results were assembled and compared with NCBI data base.The molecular analysis for sample CD21 showed 99% identity to the sequence of *Cladosporium tenuissimum* the sample is most probably *Cladosporium tenuissimum* (Figures 2A). The molecular analysis for sample MD22 showed 94% identity to the sequence *of Ramichloridium apiculatum ,* hence the sample is most probably *Ramichloridium apiculatum* (Figures 3A). Further, fermentation studies were carried out with the same strains for the production of L-asparaginase under submerged fermentation.

Effect of incubation temperature

The isolates were incubated at various temperature ranges of 4 ^{o}C, 28 ^{o}C, 37 ^{o}C and 50 ^{o}C and the optimum activity was measured at standard assay conditions. Other media conditions remained constant as stated above. The results are presented in the Graph 1. The maximum L-asparaginase activity of 0.45 IU/ml was obtained for MD22 at 28°C where as CD21 has shown maximum activity of 0.23 IU/ml at 28°C.

Effect of glucose concentration

The effect of glucose as the carbon source was investigated at concentration of 0.5%, 1%, 1.5% and 2% (w/v) (Graph 2). The maximum activity of 0.69 IU/ml was observed for CD21 at a concentration of 1.5 % where as MD22 has shown the maximum activity of 0.73 IU/ml at a concentration of 2%.

Effect of salt concentration

The results of initial salt concentration of the isolates CD21 and MD22 are presented in Graph 3.The results revealed that CD21 has shown optimum activity of 0.64 IU/ml at 2% NaCl concentration after 96 and 120 hrs of incubation. Similar observation has been done for MD22 at 2% NaCl concentration has shown the optimum activity of 0.53 IU/ml after 96 and 120 hrs. The present investigation confirms that the growth of these fungal isolates as well as L-asparaginase production was dependent upon physiological parameters.

Table 1
Colony diameter and zone diameter of different marine fungal isolates after 72 hrs of incubation

Sl. No.	Isolate No.	Colony diameter (mm)	Zone Diameter (mm)	% activity
1	MG21	12	2	16.0
2	MG31	10	0	0.0
3	MD21	20	3	15.0
4	**MD22**	**16**	**8**	**50.0**
5	MD31	18	0	0.0
6	MC21	22	0	0.0
7	MC30	14	0	0.0
8	MC31	16	3	18.7
9	MC32	15	0	0.0
10	MC33	15	0	0.0
11	MK21	19	4	21.0
12	MK 22	4	0	0.0
13	MK23	5	0	0.0
14	**CD21**	**16**	**9**	**62.5**
15	CD31	18	2	11.1
16	CC21	16	0	0.0
17	**CK21**	**24**	**11**	**45.8**
18	CK22	17	0	0.0

Table 2
L-asparaginase activity of the culture filtrate

Sl. No.	Isolate No.	Enzyme Activity (IU/ml)
1	CD21	0.14
2	MD22	0.07
3	CK21	0.04

Figure 2A
Sequence of CD21 isolate

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  1 gtagggtgaa cctgcggagg gatcattaca agtgaccccg gtctaaccac cgggatgttc
 61 ataacccttt gttgtccgac tctgttgcct ccggggcgac cctgccttcg ggcgggggct
121 ccgggtggac acttcaaact cttgcgtaac tttgcagtct gagtaaactt aattaataaa
181 ttaaaacttt taacaacgga tctcttggtt ctggcatcga tgaagaacgc agcgaaatgc
241 gataagtaat gtgaattgca gaattcagtg aatcatcgaa tctttgaacg cacattgcgc
301 cccctggtat tccgggggggc atgcctgttc gagcgtcatt tcaccactca agcctcgctt
361 ggtattgggc aacgcggtcc gccgcgtgcc tcaaatcgac cggctgggtc ttctgtcccc
421 taagcgttgt ggaaactatt cgctaaaggg tgctcgggag gctacgccgt aaaacaaacc
481 catttctaag gttgacctcg gatcaggtag ggatacccgc t
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Figure 3A
Sequence of MD22 strain

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1 tccgttggtg aacctgcgga gggatcatta ccagaagccg cgtcggccgt gaggtcggcg
61 ccttcgccca acccttttgtg aacgatattg attgcctcgg ggggacccgc gcccacacgg
121 gcgtggcccc ccggtggccc cccataactc ttgttgtatc tgccstctga gtcgcawatg
181 caaatgaaac aaaactttca acaacggrwy tmttggttct ggcwtcratk aasaacrcag
241 craawtgcga taagtaatgt gaattgcaga attcagtgaa tcatcgaatc tttgaacgca
301 cattgcgccc cctggtattc cgggggggcat gcctgttcga gcgtcattac aaccaatcca
361 gcccggctgg gtgttgggcg tykcrgcctk ccrcscscct cwrmktcctc kksggaagcc
421 gcccgttcct ctgcgtgatg atccatcgcc gc
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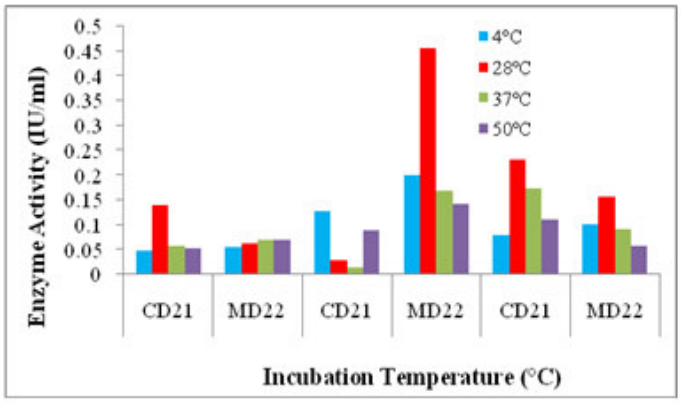
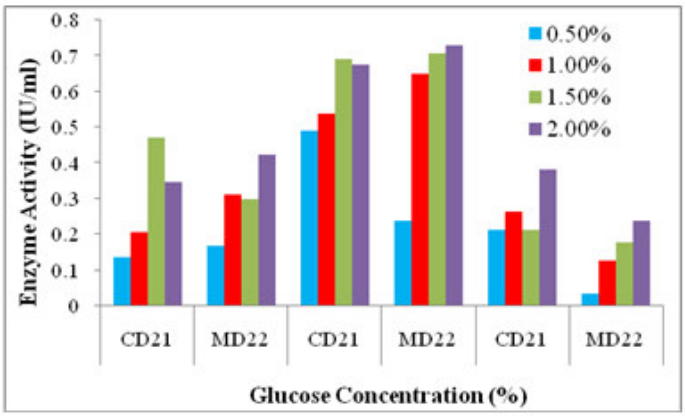
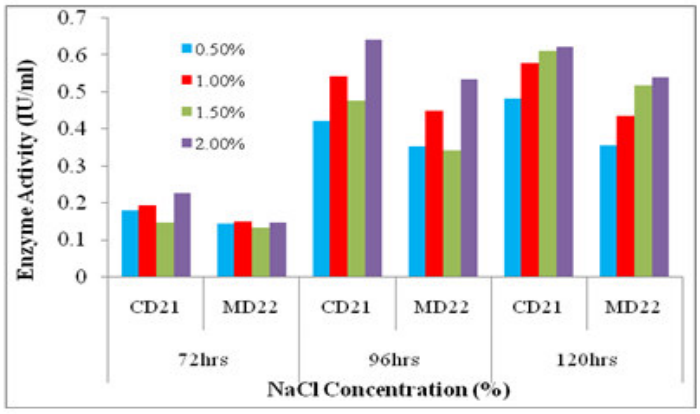
Graph 1
Effect of Temperature on the production of L-asparaginase



Graph 2
Effect of Glucose concentration on the production of L-asparaginase



Graph 3
Effect of Sodium chloride on the production of L-asparaginase



DISCUSSION

Isolation and screening of L-asparaginase producers by plate assay method

A total of eighteen isolates from five different coasts were screened for L-asparaginase production, out of which eight strains were positive for L-asparaginase with different zone diameters. The two among these showed highest zone (>40%) viz., CD21 and MD22 respectively. The broth studies of enzyme activity revealed that CD21 has highest activity 0.14/ml IU followed by MD22 which has shown the activity of 0.07 IU/ml. As the isolates CD21 and MD22 exhibited more activity when compared to the other organisms, hence they were considered as potential strains for further studies. The molecular analysis was carried out as described by Bruns *et al.*[26] for the isolates CD21 and MD22. CD21 has shown 99% identity to the sequence of *Cladosporium tenuissimum* (Fig 2A) and the isolate MD22 showed 94% identity to *Ramichloridium apiculatum* (Fig 3A). Lapmak *et al.*[27] have reported the identification based on internal transcribed spacer (ITS) regions of ribosomal DNA and morphological study showed that isolate BR438 from brown rice was *Bipolaris* sp. and was closely related to *Bipolaris australiensis* and *B. ovariicola.*Nutritional requirements for optimal synthesis of L-asparaginase vary from one microorganism to the other. Indeed, the rate of synthesis varies in the same organism as a function of culture conditions. In this study, fermentation parameters have been optimized which has shown the enzymatic activity at lowest values in the log phase and increasing in the exponential phase it reached to the maximum values and continued in decreasing at late stationary phase.

Effect of incubation temperature

The effect of different initial temperature levels on the production of L-asparaginase was studied using the isolated *Cladosporium tenuissimum* and *Ramichloridium apiculatum.* The maximum activity of 0.45 IU/ml was obtained for *Ramichloridium apiculatum* at 28°C where as the lowest activity of 0.14 IU/ml at 50°C. Similarly, *Cladosporium tenuissimum* has shown maximum activity of 0.23 IU/ml at 28°C and minimum activity of 0.08 IU/ml at 4°C. The data shows that any temperature beyond the optimum range is found to have some adverse effect on the metabolic activities of the microorganisms. Gurunathan and Sahadevan[28] have reported the maximum enzyme activity of 23.57 IU/ml at temperature of 35°C using *Aspergillus terreus* MTCC 1782. Swathi *et al.*[29] have reported the fungal strain *Beauveria bassiana* (MSS18/41) isolated from marine sediments of Visakhapatnam has shown the maximum enzyme production of (66.5 U/gds) at 26°C.

Effect of glucose concentration

The ameliorate production of L-asparaginase by the addition of glucose may be attributed to the positive influence of additional carbon sources on enhanced biosynthesis. The L-asparaginase production of 0.69 IU/ml was recorded at 1.5 % (w/v) glucose concentration by *Cladosporium tenuissimum*. Likewise 0.73 IU/ml at 2% glucose concentration was observed for *Ramichloridium apiculatum.* The maximum production of L-asparaginase (8.14 IU) was observed at the concentration of 0.3 %.[30] The results of Aida *et al.*[31] revealed that dextrose has brought highest L-asparaginase production (8.26 U/mg protein) compared to other carbon sources using *A. terreus* culture.

Effect salt concentration

Salinity is one of the important factor influencing the marine fungi. Therefore, optimization of initial salt concentration is essential for L-asparaginase production. The results of initial salt concentration of *Cladosporium tenuissimum* was 0.64 IU/ml at 2% NaCl concentration. Likewise 0.53 IU/ml at 2% NaCl concentration was observed for *Ramichloridium apiculatum.*. Similar observation has been made for L-asparaginase from *Bacillus* sp. isolated from an intertidal marine alga (*Sargassum* sp.) The maximum L-asparaginase activity was found to be 2% NaCl concentration.[32] Aparna and Raju[33] have reported 1% (w/v) NaCl for the highest yield of L-asparaginase from *Aspergillus terreus* MTCC 1782.

CONCLUSION

Marine ecosystems are least unexplored, and may provide a rich source of the microorganisms producing

novel and efficient bioactive compounds. As on date, only limited research on fungal microflora from marine sediments have been reported. No studies have been reported from the coasts of Gokarna, Devbagh, Murdeshwar & Karwar of Karnataka and Cancona coasts of Goa. Hence, this is the first report on *Cladosporium tenuissimum and Ramichloridium apiculatum* for L-asparaginase production from *the* marine sediments. In the present study, isolation, screening, identification and optimization of fermentation parameters were carried out. A total of 18 fungal isolates were screened for novel L-asparaginase production from five different coasts, eight strains showed positive for L-asparaginase with different ranges of zone diameters. Out of which the two CD21 and MD22 have shown highest zone (>40%) i.e., CD21 has highest colony diameter of 16 mm with 9 mm of zone diameter compared to MD22 which has colony diameter of 16 mm with 8 mm of zone diameter, and the enzyme activities of 0.14 IU/ml and 0.07 IU/ml at 28°C, respectively. The molecular identification revealed that CD21 showed 99% identity to the sequence *Cladosporium tenuissimum* and MD22 showed 94% identity to the sequence of *Ramichloridium apiculatum*. The maximum values of L-asparaginase activity obtained after the optimization of the fermentation parameters was found to be 28°C, 96hrs, 1.5% glucose and 2% NaCl concentration for *Cladosporium tenuissimum* and 28°C, 96hrs, 2% glucose and 2% NaCl concentration exhibited by *Ramichloridium apiculatum.* Hence, these marine fungi are a promising source for novel bioactive molecules such as L-asparaginase. There is scope for further studies with regard to production, purification and characterization of this enzyme.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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Reviewers of this article

Dr Ponnamma S.LI M.Sc.,M.Phil.,Ph.D

Associate Professor
Dept of Bio-technology,Microbiology
Presidency college
Hebbal ,Bangalore

Prof.Dr.Prapurna Chandra Rao

Assistant Proffessor, KLE University,
Belgaum, Karnataka

Prof.Dr.K.Suriaprabha

Asst. Editor , International Journal
of Pharma and Bio sciences.

Prof.P.Muthuprasanna

Managing Editor , International
Journal of Pharma and Bio sciences.

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