International Journal of Current Advanced Research
ISSN: O: 2319-6475, ISSN: P: 2319-6505, Impact Factor: 6.614
Available Online at www.journalijcar.org
Volume 7; Issue 12(E); December 2018; Page No. 16712-16717
DOI: http://dx.doi.org/10.24327/ijcar.2018.16717.3099

Research Article

ISOLATION AND IDENTIFICATION OF L-GLUTAMINASE PRODUCING FUNGI FROM AGRICULTURAL SOIL

Hemalatha V[1]., Kalyani P[1]., Chandana Vineela K[1] and Hemalatha K.P.J*[2]

[1]Department of Microbiology, Andhra University, Visakhapatnam
[2]Department of Biochemistry, Andhra University, Visakhapatnam

A R T I C L E I N F O

Article History:
Received 06th September, 2018
Received in revised form 14th October, 2018
Accepted 23rd November, 2018
Published online 28th December, 2018

Key words:
L-glutaminase, Serial dilution method, Gram staining, Biochemical characterization of microorganisms, Production of L-glutaminase.

A B S T R A C T

In the present study, L-glutaminase producing fungi were isolated from agricultural soil collected from the area of Andhra University, Visakhapatnam. L-glutamiase from selected five bacterial isolates were produced by submerged fermentation. The potential soil fungal isolate was identified as *Aspergillus flavus* strain S4 based on its morphological, cultural, biochemical and physiological characteristics.

INTRODUCTION

Enzymes are biocatalysts produced by the living cells to bring about specific biochemical reactions generally forming part of the metabolic processes of the cells. Enzymes are highly specific in their action on substrates and often many different enzymes are required to bring about, by concerted action, the sequence of metabolic reactions performed by the living cell (Nagwa *et al*., 2013). In recent years, biomedical sciences accentuate the involvement of the enzyme L-glutaminase and other amino acid depleting enzymes as therapeutic agents for the treatment of tumor (Holcenber, 1982). L-glutaminase is an extracellular enzyme that deaminates L-glutamine. It also acts as an endopeptidase, which hydrolyses the peptide bond present in the interior of the protein molecules. Glutaminases are ubiquitous in the biological world (Ohshima *et al*., 1976; Iyer and Singhal, 2010). A variety of microorganisms, including bacteria, yeast, molds and filamentous fungi have been reported to produce L-glutaminase (Kashyap *et al*., 2002; Weingand- Ziade *et al*., 2003; Iyer and Singhal 2008), of which most potent producers are fungi (Balagurunathan *et al*., 2010). Microbial glutaminases are supposed to be more stable than corresponding enzymes derived from plant and animal counterparts (Nathiya *et al*., 2011). The microbial production of metabolites and enzymes mainly depend on the genetic nature of the organism, fermentation medium components and their concentration, physiological growth conditions and interactive influence of all the above factors. Hence optimization of the above conditions is vital in order to get higher yield and to develop effective bioprocess system for industrial application. Many authors reported that the increased enzyme yield depends upon optimization of bioprocess conditions using different fermentation strategies (Sathish and Prakasham, 2010; Sathish and Hymavathi, 2010; Mahalaxmi *et al*., 2009). On an industrial scale, glutaminases are produced mainly by *Aspergillus* sp. and *Trichoderma* sp. (Tomita *et al*., 1988; Masuoa *et al*., 2004; El-Sayed, 2009; Palem *et al*., 2010).

The enzyme L-glutaminase (L-glutaminase amidohydrolase E.C.3.5.1.2) is an amodohydrolase which catalyses the hydrolysis of L-glutamine that results in the production of L-glutamic acid and ammonia. L-glutaminase has substantial contributory role in cellular nitrogen metabolism in all living cells and it has a central role in mammalian tissues. Interest on the amidohydrolases started with the discovery of their antitumor properties and a lot of efforts have gone into extensive studies on microbial L-glutaminases with the intention of developing them as antitumor agents.

MATERIALS AND METHODS

Collection of soil sample

Soil sample was collected from Garden of Horticulture, Andhra University, Visakhapatnam, Andhra Pradesh, India.

Soil sample was collected and taken to the laboratory in sterile polythene bags to avoid contamination.

Growth medium

The organisms were grown on slightly modified Potato Dextrose Agar (PDA) medium containing (g/l distilled water) L-glutamine- 20.0; agar-20.0; phenol red- 2.5%; pH 7.

Isolation of fungi

The serial dilution of collected sample was carried out by taking 1 gm of soil sample in 10 ml of sterile distilled water. It was mixed thoroughly to get soil suspension. From this soil suspension, serial dilution was performed and from that 10^{-4} and 10^{-5} dilutions were further spreaded on Potato Dextrose Agar Media for getting the single isolated colonies. The plates were incubated at 28^{o}C for 7 days. Then colonies were isolated from each plate and subcultured on PDA medium until a pure isolate was obtained.

Pure cultures were maintained on potato dextrose agar. The single isolated colonies were further screened by streaking on potato dextrose agar medium for screening of L-glutaminase production.

Screening of L-glutaminase producers by using PDA

PDA medium contains L-glutamine as sole carbon and nitrogen source and phenol red acts as a pH indicator. The color change of the medium from yellow to pink is an indication of the extracellular L-glutaminase production by the fungal isolate. This color change is due to the change in the pH of the medium, as the L-glutaminase causes the breakdown of amide bond in L-glutamine and liberates ammonia. The four fungal isolates collected were inoculated on PDA individually. After 4 days of incubation at room temperature the plates were turned to pink color. It indicates that the selected isolates have the ability to produce L-glutaminase. These fungal isolates were used for further analysis.

Extraction of crude enzyme

The crude enzyme was extracted from 2 days of old PDB culture. The culture was taken and centrifuged at 5,000 rpm for 20 min. The supernatant was collected and used for enzyme assay by using Imada *et al*., (1973) method.

Estimation of L-glutaminase activity

The L-glutaminase activity was measured by estimating the amount of ammonia liberated from L-glutamine. Enzyme extract (0.5 ml) was added to 0.5 ml of 0.04 M L-glutamine and 0.5 ml of distilled water. To this 0.5 ml of 0.1 M phosphate buffer, pH 7 was added. The reaction mixture was mixed thoroughly and incubated at 37ºC for 30 min. The reaction was stopped by adding 0.5 ml of 1.5 M trichloroacetic acid (TCA). The reaction mixture was centrifuged at 5,000 rpm for 20 min. The blank was prepared similarly without adding enzyme preparation. To 0.1 ml of the supernatant, 3.7 ml of distilled water and 0.2 ml of Nessler's reagent were added. The absorbance was measured at 480nm using spectrophotometer. Then standard graph was constructed by treating 1 ml of various concentrations (10mM, 20mM, 30mM, 40mM etc.) of ammonium chloride with phosphate buffer (pH 7), TCA and Nessler's reagent.

One unit of glutaminase was defined as amount of enzyme that liberates one micromole of ammonia under optimum conditions. The enzyme yield was expressed as units/ml (U/ml).

Five fungal isolates exhibited L-glutaminase activity during the screening process. Those are isolate S1, isolate S2, isolate S3, isolate S4 and isolate S5. Among these isolates, isolate S4 has promising L-glutaminase activity and it was subjected to molecular characterization and optimization studies.

Estimation of protein

Protein concentration was measured according to Lowry *et al*., (1951) method by using bovine serum albumin as a standard. The protein concentration was expressed as mg/ml of crude enzyme.

Characterization of fungal isolates

Microscopic characterization

Identification of fungal isolates was preformed as per the manuals of Domsch *et al.,* (1980) and Barnett and Hunter, (1972). After the isolation of fungal isolates they were subcultured on the PDA plates and were primarily subjected to the Lacto phenol cotton blue staining, and then analyzed the morphology by Scanning Electron Microscope (JSM-6610 instrument model, JEOL/EO format, Japan) to observe morphology of mycelium and spore structure.

Lactophenol cotton blue staining

A drop of the lactophenol cotton blue stain was placed in the center of clean glass slide. A fragment of the fungus colony was taken with a sterile needle and placed it on the drop of lacto phenol cotton blue stain. Gently the colony was teased and applied a cover slip. It should not be pushed down or tapped as this may dislodge conidia from the conidiophores. Then it was observed under compound microscope with required magnification.

Scanning electron microscopic (SEM) analysis

To observe the morphology of the selected fungal isolate under the SEM, the isolates were primarily fixed by 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.0) for 24 h at 4^{o}C and post fixed in 2% Osmium tetroxide for 4 h in the phosphate buffer. Then these samples were washed and dehydrated with different concentrations of ethyl alcohol (30 to 80% for 20 min). The samples were dried and mounted over the tube with double-sided carbon tape. A thin layer of gold coat was applied over the samples using an automated sputter coated by gold shadowing technique for 3 min, later the samples were scanned under SEM at desired magnification.

Molecular Characterization of Fungal Isolates

ITS/5.8S rRNA gene sequence analysis

Molecular characterization of selected fungal isolate was done by intergenic transcribed spacer (ITS)/5.8S rRNA gene sequence analysis. The intergenic transcribed spacer (ITS) regions and the 5.8S rRNA gene sequence were used to analyze the interspecific relationships among the species. Taxonomic relationship among *Aspergillus* species is based on ITS sequence which explains the comparison with morphological and physiological features.

Phylogenetic tree

Construction of phylogenetic tree was based on evolutionary relationship of taxa based on ITS sequence data by Neighbor-

Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 100 replicates, which were taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The evolutionary distances were computed using Kimura2-parameter method (Kimura, 1980) and evolutionary analysis was conducted in MEGA6 (Tamura *et al.,* 2011).

RESULTS AND DISCUSSION

Isolation and screening of L-glutaminase producing fungi

On Potato Dextrose Agar plates, colonies with powdery consistency, in different colors were observed. Pink color zone was observed around the colonies and such colonies were further subcultured and purified by streaking on PDA media containing L-glutamine and pH indicator, Phenol red. From this, five morphologically different colonies were selected. Based on the cultural characteristics and microscopic appearance all strains were identified as fungi. These five colonies were able to produce glutaminase enzyme. The yield of five isolated colonies were given in Table 1. In most of the microbial screening programs for enzyme production, the microorganisms are first isolated from different environments by isolation procedures and then screened for enzymatic activity by methods like zone of color change or zone of clearance on agar medium supplemented with suitable substrate (Ranjker *et al*., 2002). Rajeswari and Shome, (2001) reported microbial asparaginase from mangrove sediments of Andaman Islands. They have screened 200 bacterial isolates by plate method by using asparagine as substrate and found that 108 (54%) were asparaginase producers.

Isolation and screening was done in a single step process which reduced the cost and time needed for primary screening. Pure cultures of fungi were identified by comparing the characteristic features of fungi described in the manual of soil fungi. The isolated fungal strains were identified at genus level on the basis of macroscopic characteristics like color, colony, morphology, shape, texture, diameter, and appearance of colony and by microscopic characteristics like mycelium, presence of specific reproductive structures, structure and shape of conidia.

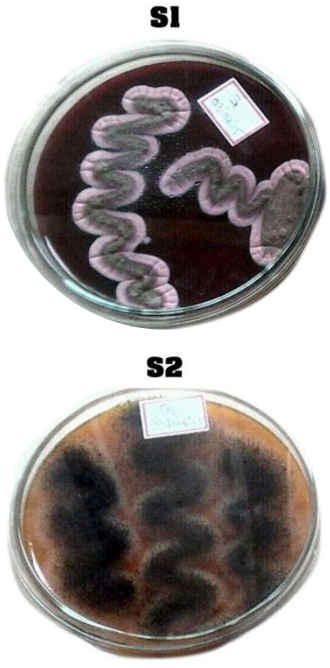


Fig 1 Screening of L-glutaminase producing fungi on PDA medium

Enzyme activity of screened isolates

In the present study isolates S1, S2, S3, S4, S5 were used to check L-glutaminase activity. Isolate S4 exhibited maximum L-glutaminase activity of 195.30 U/ml among all five isolated strains. The results showing the enzyme (glutaminase) yield of five isolated strains were given in Table 1. The activity of L-glutaminase was also studied by Prasanth Kumar *et al*., (2009). They have reported 30 marine bacterial strains for L-glutaminase production. Among these isolates LG24 exhibited maximum L-glutaminase activity of 22.68 U/ml. Thadikamala Sathish *et al*., (2012) reported 196 U/ml of L-glutaminase

production by *Bacillus subtilis* under optimum conditions. Chanakya *et al*., (2010) studied L-glutaminase production by *Trichoderma koningii* under solid state fermentation. They reported maximum yield of 19.41 U/gds of enzyme under optimum conditions. Tullimilli Anusri, (2014) reported maximum yield of 692.30 U/ml of enzyme under optimum conditions.

Table 1 Enzyme activity of screened isolates

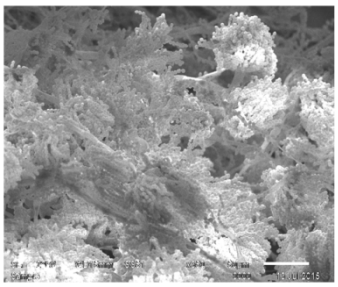
S.No.	Fungal isolate	Colony color	L-glutaminase detection (Pink zone (mm)	Enzyme activity (U/ml)	Specific activity (U/mg protein)
1	S1	Grey	12	94.46	3.14
2	S2	Black	14	121.76	2.70
3	S3	Dark green with white back ground	18	110.08	1.84
4	S4	Light green with white puffy back ground	20	195.30	6.28
5	S5	Yellow	16	99.80	1.99

Isolate S4 has maximum L-glutaminase activity. This strain was used for further studies. The amount of protein produced from isolate S4 was observed as 20 mg/ml.

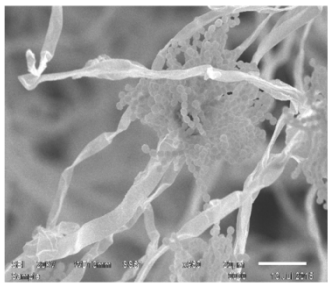
Morphological characteristics of selected fungi (S4)

The morphological identification needs sufficient growth in order to evaluate the colony characteristics and microscopic features. Hence, PDA was used to accelerate the growth rate and the production of conidia, as reported by Diba *et al.,* (2007). The colony of S4 on PDA grew white initially and then turned to pink (Fig. 2a). The microscopic appearance of S4 showed radiate to loosely columnar, spherical to ovoidal conidia, smooth walled, septate branched hyphae and greenish to grey color were observed under scanning electron microscopic analysis (Figs. 2b and 2c). The isolate (S4) was characterized morphologically by lactophenol cotton blue staining (Fig. 3) and scanning electron microscopic analysis and the details were presented in Table 2. According to morphological characteristics isolated fungus (S4) belongs to *Aspergillus* species.

Fig 2 a) Isolate S4 on Potato dextrose agar



b) SEM image of isolate S4 at x350 magnification



c) SEM image of isolate S4 at x950 magnification

Fig. 2 Macroscopic ((a) Potato dextrose agar) Microscopic ((b) and (c) Scanning electron micrograph SEM images)

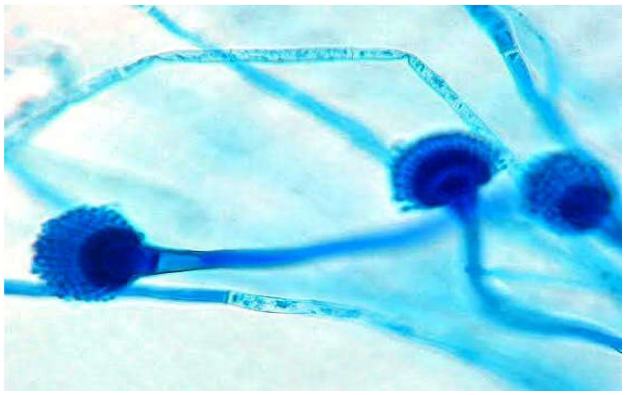


Fig 3 Lactophenol cotton blue staining image of Isolate S4 at x400 magnification

Table 2 Cultural characteristics of selected isolate (S4)

S.No	Parameter	Characteristics of Isolate (S4)
1	Colony color	Light green
2	Colony diameter	30 mm
3	Hyphae	Septate branched
4	Spore arrangement	Bunch of spores with single chain
5	Spore shape	Oval

Molecular characterization of S4

Among the five isolates, S4 showed maximum L-glutaminase activity and hence the S4 isolate was subjected to molecular characterization. Molecular characterization of the isolate (S4) was performed by ITS/5.8 rRNA gene sequence analysis and the isolate was confirmed as *Aspergillus flavus*. ITS/5.8 rRNA gene sequence and phylogenetic Evolutionary relationship of taxa based on ITS sequence were shown in Figs. 4 and 5. The gene sequence was submitted to NCBI Gene Bank database with accession number MK138677. Park *et al.,* (2007) and Litaker *et al.,* (2007) reported that intergenic transcribed spacer (ITS) region was found to be the most promising for identification of the *Aspergillus* species. Witiak *et al.,* (2007) reported that the Bootstrap support was generally used to find relationship among species within sections and subgenera. The ITS regions have been used as targets for phylogenetic analysis because they generally display sequence variation between species, but only minor variation within strains of the same species (Lee *et al.,* 1998). Gaskell *et al.*, (1997) investigated sequence variation in ITS regions to distinguish *Aspergillus* from other allergenic molds. They found little variation between *Aspergillus* and *Penicillium* within the ITS 2 region but concluded that the ITS 1 region may be sufficient for identification.

CCGAGTGTAGGGTTCCTAGCGAGCCCAACCTCCCACC
CGTGTTTACTGTACCTTTTGCTTCGGCGGGCCCGCCAT
TCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCG

CCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTG
AAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTT
TCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGA
ACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGA
ATTCCGTGAATCATCGAGTCTTTGAACGCACATTGCG
CCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGT
CATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTC
GTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGC
AGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGG
GCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTG
CCGAACGCAAATCAATCTTTTTCCAGGTTGACCTCGG
ATCAGGTAGGGATACCCGCTGAACTTAAG

Fig 4 *Aspergillus flavus*, Strain "(S4)", ITS/5.8S rRNA gene sequence data

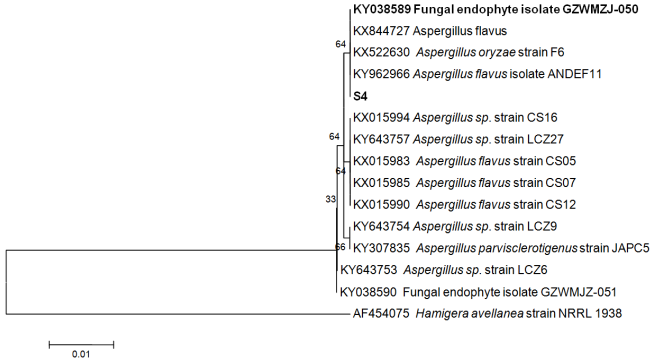


Fig 5 *Aspergillus flavus*, Strain "(S4)", Phylogenitic Evolutionary relationship of taxa based on ITS sequence

The evolutionary history was inferred using the Neighbor-Joining method (Saitou *et al*., 1987). The optimal tree with the sum of branch length = 0.10819617 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein *et al*., 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The analysis involved 15 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 527 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

References

Abdallah, N.A., Amer, S.K. and Habeeb, M.K., 2013. Production, purification and characterization of L-glutaminase enzyme from *Streptomyces avermitilis*. *African Journal of Microbiology Research*, 7(14), pp.1184-1190.

Holcenberg, J.S., 1982. Enzyme therapy: problems and solutions. Annual review of biochemistry, 51(1), pp.795-812.

Ohshima, M., Yamamoto, T. and Soda, K., 1976. Effect of glutamine analogs on glutaminase formation in *Pseudomonas aeruginosa*.

Iyer, P.V. and Singhal, R.S., 2010. Glutaminase production using *Zygosaccharomyces rouxii* NRRL-Y 2547: Effect of aeration, agitation regimes and feeding strategies. Chemical Engineering and Technology: Industrial Chemistry Plant Equipment Process Engineering Biotechnology, 33(1), pp.52-62.

Kashyap, P., Sabu, A., Pandey, A., Szakacs, G. and Soccol, C.R., 2002. Extra-cellular L-glutaminase production by *Zygosaccharomyces rouxii* under solid-state fermentation. Process Biochemistry, 38(3), pp.307-312.

Weingand-Ziade, A., Gerber-Decombaz, C. and Affolter, M., 2003. Functional characterization of a salt and thermotolerant glutaminase from *Lactobacillus rhamnosus*. Enzyme and Microbial Technology, 32(7), pp.862-867.

Iyer, P. and Singhal, R.S., 2008. Production of glutaminase (EC 3.2. 1.5) from *Zygosaccharomyces rouxii*: Statistical optimization using response surface methodology. Bioresource technology, 99(10), pp.4300-4307.

Balagurunathan, R., Radhakrishnan, M. and Somasundaram, S.T., 2010. L-glutaminase producing actinomycetes from marine sediments–selective isolation, semi quantitative assay and characterization of potential strain. Aust J Basic Appl Sci, 4(5), pp.698-705.

Nathiya, K., Nath, S.S., Angayarkanni, J. and Palaniswamy, M., 2011. Optimised production of L-glutaminase: A tumour inhibitor from *Aspergillus flavus* cultured on agroindustrial residues. African Journal of Biotechnology, 10(63), pp.13887-13894.

Sathish, T. and Prakasham, R.S., 2010. Enrichment of glutaminase production by *Bacillus subtilis* RSP-GLU in submerged cultivation based on neural network genetic algorithm approach. Journal of Chemical Technology and Biotechnology, 85(1), pp.50-58.

Hymavathi, M., Sathish, T., Brahmaiah, P. and Prakasham, R.S., 2010. Impact of carbon and nitrogen sources on L-asparaginase production by isolated *Bacillus circulans* (MTCC 8574): Application of saturated Plackett-Burman design. Chemical and biochemical engineering quarterly, 24(4), pp.473-480.

Mahalaxmi, Y., Sathish, T. and Prakasham, R.S., 2009. Development of balanced medium composition for improved rifamycin B production by isolated *Amycolatopsis* sp., RSP-3. Letters in applied microbiology, 49(5), pp.533-538.

Tomita, K., Yano, T., Kumagai, H. and Tochikura, T., 1988. Formation of γ-glutamylglycyglycine by extracellular glutaminase of *Aspergillus oryzae*. Journal of fermentation technology, 66(3), pp.299-304.

El-Sayed, A.S., 2009. L-glutaminase production by *Trichoderma koningii* under solid-state fermentation. Indian journal of microbiology, 49(3), pp.243-250.

Masuo, N., Ito, K., Yoshimune, K., Hoshino, M., Matsushima, K., Koyama, Y. and Moriguchi, M., 2004. Molecular cloning, overexpression, and purification of *Micrococcus luteus* K-3-type glutaminase from *Aspergillus oryzae* RIB40. Protein expression and purification, 38(2), pp.272-278.

Pallem, C., Manipati, S. and Somalanka, S.R., 2010. Process optimization of L-glutaminase production by *Trichoderma koningii* under solid state fermentation (SSF). Int. J. Appl. Biol. Pharm. Technol, 1, pp.1168-1174.

Imada, A., Igarasi, S., Nakahama, K. and Isono, M., 1973. Asparaginase and glutaminase activities of microorganisms. Microbiology, 76(1), pp.85-99.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., Grynkiewicz, G., Poenie, M., Tsien, R.Y., Folch, J., Lees, M., Stanley, G.S. and Alessi, D.R., 1951. 26. The colorimetric determination of phosphorus. J. biol. Chem, 193, p.265.

Domsch, K.H., Gams, W. and Anderson, T.H., 1980. Compendium of soil fungi. Volume 1. Academic Press (London) Ltd..

Barnett, H.L. and Hunter, B.B., 1972. Illustrated genera of imperfect fungi. Illustrated genera of imperfect fungi., (3rd ed).

Saitou, N. and Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular biology and evolution, 4(4), pp.406-425.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution, 39(4), pp.783-791.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of molecular evolution, 16(2), pp.111-120.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution, 28(10), pp.2731-2739.

Ranjekar, M.K. and Sridhar, K.R., 2002. occurrence and extracellular enzyme potential of actinomycetes of a thermal spring, Southern India. Asian Journal of Microbiology Biotechnology and Environmental Sciences, 4(1), pp.59-64.

Shome, R. and Shome, B.R., 2001. Microbial L-asparaginase from mangroves of Andaman Islands.

Prasanth Kumar, K., Prabhakar, T., Satish, T., Girija Sankar, G., Moges, F., Swarajya Lakhmi, G., Ramana, H., 2009. J. Pharmacy. Chemistry, 3(1), pp. 4-7.

Thadikamala Sathish and Reddy Shetty Prakasham., 2012. Influence of Fermentation Process Parameters on L-glutaminase production by *Bacillus subtilis* RSP-GLU. International journal of pharmaceutical and chemical sciences, 1(3), pp.625-631.

Chanankya, P., Manipati, S. and Somlanka, S.R., 2010. Process optimisation of L-glutaminase production by. *Trichoderma koningi*, pp.1168-1174.

Anusri, T., Jagarlamudi, S., Bai, B.P. and Rao, C.R., studies on isolation, screening and molecular characterisation of L-glutaminase producing marine isolates.

Diba, K., Kordbacheh, P., Mirhendi, S.H., Rezaie, S. and Mahmoudi, M., 2007. Identification of *Aspergillus* species using morphological characteristics. Pakistan Journal of Medical Sciences, 23(6), p.867.

Park, K.C., Kwon, S.J., Kim, P.H., Bureau, T. and Kim, N.S., 2007. Gene structure dynamics and divergence of the polygalacturonase gene family of plants and fungus. Genome, 51(1), pp.30-40.

Wayne Litaker, R., Vandersea, M.W., Kibler, S.R., Reece, K.S., Stokes, N.A., Lutzoni, F.M., Yonish, B.A., West, M.A., Black, M.N. and Tester, P.A., 2007. Recognizing dinoflagellate species using its rDNA sequences 1. Journal of phycology, 43(2), pp.344-355.

Witiak, S.M., Samson, R.A., Varga, J., Rokas, A. and Geiser, D.M., 2007. Phylogenetic markers for the genus *Aspergillus* developed from complete genome sequences. In 24th Fungal Genetics Conference, Asilomar, Abstract (No. 130).

Gaskell, G.J., Carter, D.A., Britton, W.J., Tovey, E.R., Benyon, F.H. and Lovborg, U., 1997. Analysis of the internal transcribed spacer regions of ribosomal DNA in common airborne allergenic fungi. Electrophoresis, 18(9), pp.1567-1569.

Saitou, N. and Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular biology and evolution, 4(4), pp.406-425.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution, 39(4), pp.783-791.

Tamura, K., Nei, M. and Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences, 101(30), pp.11030-11035.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular biology and evolution, 30(12), pp.2725-2729.

How to cite this article:

Hemalatha V *et al* (2018) 'Status of Post Traumatic Stress in Children in Armed Conflict', *International Journal of Current Advanced Research,* 07(12), pp. 16712-16717. DOI: http://dx.doi.org/10.24327/ijcar.2018.16717.3099

****