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Screening and Purification of L-asparaginase Production by *Aspergillus quadrilineatus* Using Agro Wastes and Vegetable Peels

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Abstract

Enzymes are the name for the biocatalysts that are created by living cells. These are intricate protein molecules that start chemical reactions essential to life. They are colloidal, thermolabile, and have a particular action. L-asparaginase (also called L-asparagine amido hydrolase, E.C.3.5.1.1) is an extracellular enzyme that has attracted a lot of attention since it is used as an anticancer drug. To assess the growth capacity of the selected fungus *Aspergillus quadrilineatus*, a preliminary experiment was carried out using vegetable peels and agricultural waste as substrate. The by-products that were utilized included wheat bran, cornmeal, gram flour, wheat flour, rice flour, rice husk, and peels from green peas, onions, carrots, and papayas. On the other hand, papaya peel medium worked well as a source of media for improving growth in *Aspergillus species*. This work focuses on the purification, mass-scale production, screening, and characterization of the Fusarium proliferative-derived L-asparaginase enzyme. The crude enzyme was removed, followed by precipitation with ammonium sulphate, filtration over a Sephadex column, and ion exchange chromatography to further purify the L-Asparaginase. The reaction of the enzyme to different temperatures, pH values, substrate concentrations, and incubation times was evaluated in order to define it. Because it can catalyze the conversion of l-asparagine to l-aspartic acid and ammonia, l-asparaginase is one such crucial enzyme that has found commercial usage in the food and pharmaceutical industries. Although l-asparaginase may be produced by a variety of bacterial and fungal sources, researchers are particularly interested in how these organisms might generate the enzyme commercially utilizing less expensive substrates.

Keywords: Fungi, Vegetables, L-asparaginase, *Aspergillus quadrilineatus*, papaya peels.

Introduction

India, a global leader in agriculture with the greatest irrigated crop area, is a resource hub for producing agricultural wastes; according to reports, the country produces 1500 lakh tons of agricultural trash annually (1) (shakambari *et al.*,2017). These agricultural wastes typically consist of bagasse, straw, fruit and vegetable peels, husks, and other agricultural materials produced during processing that have been shown to be used in the creation of numerous value-added goods. By using fermentation technology to transform these nutrient-rich byproducts into valuable bioproducts, the method can reduce environmental pollution risks while simultaneously minimizing production costs(2) (Pallem *et al.*, 2018). A portion of the organic wastes have been utilized to make garbage enzyme, which is used to treat household, municipal, and industrial wastes as well as create antibacterial agents. Various wastes, including those from

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the sugar industry, municipal solid wastes, and even agricultural wastes, have been employed as an alternate substrate to increase the synthesis of several of these crucial enzymes. L-asparaginase is one such significant enzyme that is used in the food and pharmaceutical industries due to its ability to catalyze the conversion of L-asparagine to L-aspartic acid and ammonia. For their growth, acute lymphoblastic leukemia (ALL) and tumor-suspected cells depend on the circulating asparagine that is produced by normal cells. Asparagine is a non-essential amino acid. The leukemic cells are deprived of circulating asparagine, which results in cell death, due to asparaginase's catalysis of the conversion of L-asparagine to aspartic acid and ammonia. According to Hosamani and Kaliwal *et al*., (2011)(3), L-aspartate functions as a precursor molecule for ornithine in the urea cycle and creates oxaloacetate in the transamination reaction in the gluconeogenic pathway, which leads to the synthesis of glucose in humans. L-asparaginase activity was originally reported by Lang in 1904 (4); and Clementi in 1922 (6). Kidd reported in 1953(7) that guinea pig serum may produce transplanted lymphomas in vivo in mouse and rat cells. This was eventually linked to the serum's L-asparaginase activity (Dolowy *et al*., 1966)(8). However, it was challenging to produce and purify this enzyme on a large scale from guinea pigs for therapeutic use, which prompted researchers to look for other sources with comparable anti-leukemic properties. Microorganisms, animals, and plants all naturally contain asparaginase. In order to address these financial concerns, we must discover a different source for microbe cultivation. The solution to replace synthetic culture medium is to use massive amounts of agricultural waste products for the cultivation of microorganisms, especially fungus. In order to maximize enzyme production during submerged fermentation, the current work was designed to use vegetable peels and agricultural waste products such as wheat bran, rice husk, corn flour, gram flour, rice flour, onion peels, garlic peels, carrot peels, papaya peels, green pea peels, and corn peels as substrate. For this investigation, papaya peels with the highest level of enzyme activity were selected. There are several reports on *Aspergillus quadrilineatus* producing L-asparaginase, but there are none on the full purification and characterisation of the free and immobilized enzyme. Thus, we report on the purification of fermentation conditions for *Aspergillus quadrilineatus* L-asparaginase synthesis in this study.

Materials and Methods

Screening of different basal media for L-asparaginase production

Twelve numbers of basal media (Wheat bran, Corn flour, Gram flour, Wheat flour, Rice flour, Rice Husk, Corn peels, Onion peels, Carrot peels, Papaya peels and Green peas peels, garlic peels) were used for present study. All the vegetable peels were collected, air dried and then oven dried at 50˚C for 48 hours. For studying the effect of L-asparagine, 250 ml of each basal medium were prepared. The organism *Aspergillus quadrilineatus* was inoculated by punching out 0.6 mm of the agar-plate culture and transferred broth medium. Cultures were incubated at 30°C, for 6 days of incubation period. The culture filtrate produced in each treatment was tested for L-asparaginase enzyme production.

Estimation method

The ammonia measurement procedure was carried out by conducting an enzyme assay using Nesslerization method as described by Imada(8). To make the enzyme test mixture, 0.5ml of 0.04M L-asparagine was mixed with 0.5ml of Tris HCl buffer (pH 8.6). Then 0.5ml of enzyme and 0.5ml of distilled water were added. Subsequently, the combination was subjected to incubation for 30 minutes at a temperature of 37^0C degrees Celsius. Following the incubation period, the reaction was halted by introducing 0.5ml of 30% TCA. To estimate the enzyme activity, 7ml of distilled water was combined with 0.5ml of enzyme mixture followed by the addition of 1ml of Nesseler's Reagent. Next, the op-

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tical density was measured at a wavelength of 480nm using a spectrophotometer to estimate the enzyme activity. The quantity of ammonia emitted by the test sample was evaluated by comparing it to the reference graph. The measurement of the enzymatic activity of L-asparaginase was conducted using International Units (IU). An International Unit (IU) of L-asparaginase activity is the amount of enzyme required to produce one micromole of ammonia per milliliter per minute at a temperature of 37°C and a pH of 8.6.

Purification studies

Mass production, extraction and purification

The organism was introduced into the selected broth media using carrot peels and kept in a stationary environment at a temperature of 30ºC for 6 days. The samples were collected following the appropriate incubation period. The samples then subjected to treatment with a 0.05 M Tris-HCL buffer at a pH of 8.5. The sample was centrifuged at a speed of 600 rpm for 20 minutes at a temperature of 4^0C which led to the formation of a liquid. The statement described above was considered to be the basic composition of the enzyme. The enzyme activity and protein content were quantified and thereafter, the sample underwent the initial purification phase using ammonium sulfate precipitation.

Ammonium sulphate precipitation

The initial solution underwent precipitation by adding finely powdered ammonium sulfate until it reached a saturation level of 80%. The temperature was kept constant at 4ºC. The material underwent centrifugation at a speed of 6000 rpm for 10 minutes, continuing overnight. Afterward, the pellet that was retrieved was dissolved in a Tris-HCl buffer solution with a pH of 8.5, which had a concentration of 0.05 M (Suchita *et al*.,2010) (9).

Sephadex G-100 gel filtration

The samples that underwent precipitation with ammonium sulfate were assessed for their enzymatic activity and protein concentration. Afterward, they were subjected to gel filtration using Sephadex G-100 (Patro and Gupta,2012) (10).

Gel-column preparation and sample application

A glass tubing chromatography column measuring 60 cm in height and 2.2 cm in diameter was utilized. The eluent was 0.05 M Tris-HCL buffer with a pH of 8.5. To inhibit the formation of bubbles within the gel bed. Then the eluent was transferred from securely sealed brown bottles into containers containing the same temperature. The gel slurry was prepared by dissolving 5 grams of Sephadex in 200 ml of 0.05 M Tris-HCL buffer and allowing it to swell at room temperature for twenty-four hours. The column was secured and closed using Sephadex. Ammonium sulphate was used to precipitate the samples which were then continuously poured onto the column. The resulting fractions were collected in containers with a volume of 5ml. A random assortment of the gathered fractions underwent protein and enzyme activity analysis, and the fractions demonstrating exceptional enzyme activity were combined.

Ion exchange chromatography

The column was constructed by dissolving the necessary quantity of DEAE-cellulose in Tris-HCl buffer overnight. After the packaging procedure, the column was rinsed with 5M KCl to produce the desired weak ion exchange material. This was followed by rinsing with 5M NaOH to remove ionic charges from the ion exchanger. Afterward, the material was washed with distilled water and a 0.05 M Tris-HCl buffer solution at a pH of 8.5. Following the utilization of Sephadex for fractionating the peak fractions, they were next transferred to the ion exchange column where 5 ml of the fractions were gathered. After assessing the protein and enzyme activity of the samples and the fractions comprising the most active enzymes were consolidated and stored in a deep freezer.

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Enzyme characterization based on partial purification

Substrate Specificity

The assay mixture was enriched with several substrates, such as L-arginine, L-phenylalanine, L-histidine, L-glutamine, and L-aspartic acid, to evaluate the enzyme's inclination towards a range of substrates. The substrates were present at a concentration of 0.04M, with L-asparagine serving as a control.

PH optima

The pH of the Tris-HCl buffer in the reaction mixture was methodically varied between 3 and 10 to identify the most favorable pH for enzyme activity. The enzymatic activity was measured at different pH values.

Temperature tolerance

The enzyme was maintained at temperatures ranging from 30 to 50ºCbefore its addition to the reaction mixer for the assay.

Incubation period

The enzyme was incubated at varying temperatures of 30, 37, and 50ºCbefore its addition to the reaction mixer for the assay.

Results and Discussion

Screening of substrates

The selection of a suitable substrate for submerged state fermentation is an important factor because it determines the production cost of the entire process. In this work, carrot peels have been selected for the production of asparaginase based on its nutritional composition, cost and availability. The results in (Table 1 and Fig.1) demonstrate that the highest level of L-asparaginase synthesis was observed i.e.3.0 IU/ml when carrot peels was used as the substrate followed by other substrates used as basal media. However, Varalakshmi and Raju,(2013) (11) reported that maximum L-asparaginase activity was achieved in a medium containing Bajra seed flour as the substrate whereas corn cob showed the lowest activity from *Aspergillus terrus.* whereas Abha Mishra *et al*., (2006) (12) reported production of L-asparaginase from *Aspergillus Niger* using agricultural substrates like bran of Cajanus Cajan, Phaseolus mungo and Glycine max.

Table 1. Screening of different substrates for L-asparaginase production by *Aspergillus quadrilineatus*

Samples	Enzyme activity (U ml^{-1})
Carrot peels	0.25±0.008
Papaya peels	2.99±0.011
Green pea peels	0.21±0.017
Corn peels	0.07±0.00
Onion peels	0.095±0.006
Garlic peels	0.11±0.00
Rice Husk	0.08±0.00
Rice flour	0.035±0.007
Gram flour	0.055±0.003
Corn flour	0.06±0.00
Wheat flour	0.022±0.003
Wheat bran	No Growth

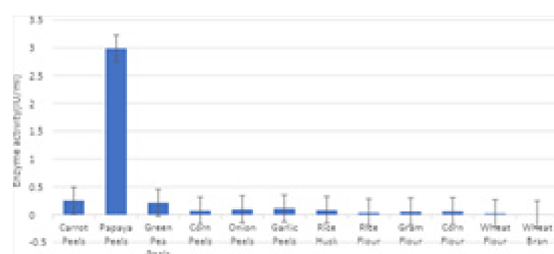

Fig-1: Screening of different substrates for L-asparaginase production by *Aspergillus quadrilineatus*

Purification studies

The enzyme L-asparaginase was purified from the culture filtrate of *Aspergillus quadrilineatus* using ammonium sulphate precipitation, ion exchange chromatography followed by gel filtration. The purification procedure summarized in (Table-2). The first step

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of purification by ammonium sulphate precipitation achieved 0.99fold purification with 106% enzyme recovery. The second purification step was performed by gel filtration using Sephadex G-100 column. This step showed 1.04-fold increase in enzyme activity with 126.66% enzyme recovery. The final step of purification was done by ion exchange chromatography using DEAE cellulose The fractions showing L-asparaginase activity in this step were collected and pooled. The final step of purification resulted in 1.05-fold increase in enzyme activity with yield of 136.60% enzyme recovery.

Table 2. Purification profile of L-asparaginase from *Aspergillus quadrilineatus.*

Steps	Collected volume(ml)	Total activity (IU)	Total protein(mg)	Specific activity (IU/mg)	purification fold	Yield (%)
culture filtrate	3200	300	125	2.4	0	100
Ammonium sulfate precipitation	150	320	134	2.38	0.99	106.66
Gel filtration	120	380	151	2.51	1.04	126.66
Ion exchange chromatography	100	410	162	2.53	1.05	136.60

L-asparaginase from various vegetable peels and agro waste substrates have been purified and characterized and reported earlier. Similar findings were made by Aparna & Raju (2015) (13), who discovered that the ratios of these substrates showed a synergistic relationship and that corn ear and cauliflower stem greatly enhanced l-asparaginase synthesis by *A. terreus* MTCC 1782. According to Dias *et al*., (2015) (14), *A. niger* LBA02 produced a maximum L-asparaginase production of 89.22U/g when a ternary mixture containing equal amounts of soybean meal, cottonseed meal, and wheat bran was used. After 96 hours of fermentation, yields increased by 13.53, 13.53, and 71.5-fold, respectively, in comparison to the individual feedstocks. Furthermore, employing Passion fruit peel flour as a substrate, *A. niger* LBA02 l-asparaginase activity reached 2380.11U/gds (de Cunha *et al.,* 2018) (15). Maximum l-asparaginase yield for *A. niger* was supported using the bran of Glycine max (39.9±3.92U/gds), *Phaseolus mungo* (30.7±3.69U/gds) and *Cajanus cajan* (26.14±3.67U/gds) (Mishra, 2006) (16). L-asparaginase production by *Pseudomonas plecoglossicida* RS1 was increased twofold by onion peel extract and garlic peel extract supplemented with (0.3% w/w) L-asparagine. Several studies examined the asparagine content of wastes used as substrates. Further, pomegranate peel powder contains asparate in a ratio of 0.3g/100g (Rowayshed *et al*., 2013) (17).

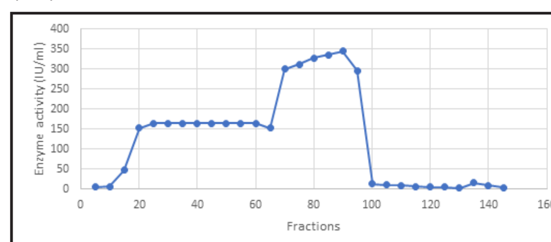

Fig-2: purification of L-asparaginase from the *Aspergillus quadrilineatus* gel filtration column chromatography (Sephadex G-100) of the fractions collected from ammonium sulphate precipitation fractions.

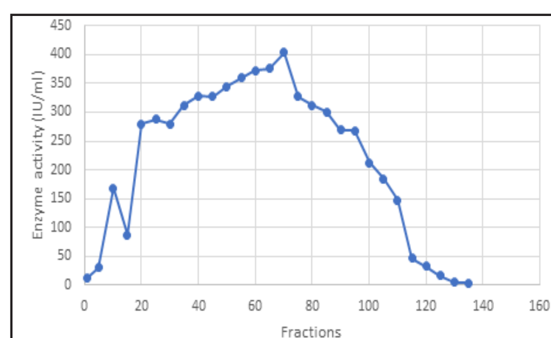

Fig-3: Purification of L-asparaginase from *Aspergillus quadrilineatus* ion exchange column chromatography fractions.

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Characterization of partially purified enzyme

Another crucial element that needs to be adjusted and regulated is temperature. It is unclear how temperature affects the synthesis of enzymes (Chaloupka, 1985) (18). The current investigation examined the impact of incubation temperature on A. terreus's ability to produce L-asparaginase. As a result, the synthesis of L-asparaginase was shown to be highest at 37°C (4.11 IU/ml) and minimal at 50°C (1.60 IU/ml). According to Sarqius *et al*., (2004) (19), 30°C is ideal for submerged fermentation employing *A. tamarii* and *A. terreus* to produce L-asparaginase. According to Manna et al. (1995) (20), 37°C is the ideal temperature for *Pseudomonas stutzeri* to be at its most active. For the synthesis of L-asparaginase to be successful, the substrate's initial pH level is crucial. According to earlier research (Sivaramakrishnan *et al*., 2006) (21), pH plays a significant role in controlling development, metabolism, and the synthesis of enzymes. Since each creature has a unique pH optimal range, any changes to these ranges may cause an organism's enzyme function to decline (Adinarayana and Ellaiah, 2002) (22). The current investigation found that this fungus produced the most enzyme at pH 8 (4.28 IU/ml), and that pH values over 10 caused the fungus to produce less L-asparaginase. At pH 10, the least amount of enzyme was produced (0.43IU/ml). Similar findings indicating that pH 7.0 is ideal for L-asparaginase synthesis during submerged fermentation have been reported by De Angeli *et al*., (1970) (23). Koshy *et al*., (1997) (24) discovered that *Streptomyces plicatus* produces the most L-asparaginase at a pH of 8.0. The analysis of substrate specificity demonstrated that the enzyme acts as a catalyst, particularly using L-asparagine as its substrate. When enzyme production was examined under various reaction times, a significant amount of variance was seen, with the highest output being detected at 10 minutes.

Table 3. Effect of pH, Temperature, and substrate on partially purified L-asparaginase.

Parameters	Enzyme activity (U ml^{-1})
Temperature Effect	
30	3.57
37	4.11
50	1.6
Incubation Period Effect	
10 min	4.22
20 min	3.85
30 min	3.02
40 min	2.2
Effect of PH	
3	3.09
4	3.11
5	3.25
6	3.69
7	3.75
8	4.28
9	1.2
10	0.43
Effect of Substrate Specificity	
L-arginine	2.52
L-phenylalanine	2.24
L-Histidine	2.06
L-Glutamine	2.33
L-aspartic	3.10
L-asparagine	4.54

Conclusion

The observations obtained in this study found that *Fusarium proliferative* has the capability to produce substantial amount of L-asparaginase enzyme. For the submerged state fermentation process, carrot peel is found to be the best substrate and it clearly enhances the

practicality of this work by the way of economically feasible for the large-scale production of L-asparaginase. The L-Asparaginase was further purified using gel filtration chromatography with a Sephadex G-100 column. The final overall yield recovery was 121.21%, and the purification resulted in a fold increase of 0.95. Research on the physical factors affecting the effectiveness of pure L-Asparaginase has shown that it remains functional throughout a wide pH range of 8 and at a temperature of 37ºC. The enzyme demonstrates a high level of selectivity towards its natural substrate, L-asparagine and demonstrates a high level of stability at a pH of 8.

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References

1. Shakambari, G., Kumar, R.S., Ashokkumar, B.,Varalakshmi, P. Agro wastes utilization for cost-effective production of L-asparaginase by Pseudomonas plecoglossicida RS1 with anticancer and acrylamide mitigation potential. *Acs Omega*.,2017;2,8108-8117. Doi.10.1021/acsomega.7b01429.

2. Pallem, C., Solid-state fermentation of corn husk for the synthesis of Asparaginase by Fusarium oxysporum. *Asian J Pharm Pharmacol*.,2019; *5*(4),2007678-681.

3. Hosamani, R. and Kaliwal, B.B.,L-asparaginase an anti-tumor agent production by Fusarium equiseti using solid state fermentation. *Int J Drug Discov*.,2011; *3*(2),88-99.

4. Lang, S., Über desamidierung im Tierkörper. *Beitr chem Physiol Pathol*.,1904; *5*,321-345.

5. Clementi, A.,La désamidation enzymatique de l’asparagine chez les différentes espéces animales et la signification physio logique de sa presence dans l’organisme. *Archives Internationales de Physiologie.*, 1922;*19*(4), pp.369-398.

6. Kidd, J.G.,.Regression of transplanted lymphomas induced in vivo by means of normal guinea pig serum: I. course of transplanted cancers of various kinds in mice and rats given guinea pig serum, horse serum, or rabbit serum. *The Journal of experimental medicine*.,1953; *98*(6), 565-582.

7. Dolowy, W.C., Henson, D., Cornet, J. and Sellin, H., Toxic and antineoplastic effects of l-asparaginase: Study of mice with lymphoma and normal monkeys and report on a child with leukemia. *Cancer*,1966; *19*(12),1813-1819.

8. Imada A, Igarasi S, Nakahama K, Isono M. Asparaginase and glutaminase activities of microorganisms. *J. Gen. Microbial*.,1973; 76(1): 85- 99.

9. Suchita C, Warangkar and Chadrahas N Khobragade. Purification, characterization, and effect of Thiol compounds on Activity of the Erwinia carotovora L-asparaginase. *Enzyme research*,2010.

10. Patro, K.R., Basak, U.C., Mohapatra, A.K. and Gupta, N.,Development of new medium composition for en d production of L-asparaginase by Aspergillus f. *Journal of environmental biology*.,2012; *35*, 295-300.

11. Varalakshmi, V. and Raju, K.J., Optimization of l-asparaginase production by Aspergillus terreus mtcc 1782 using bajra seed flour under solid state fermentation. *Int J Res Eng Technol*.,2013;121-129.

12. Aparna, C., Raju Jaya, K.Optimization of process parameters for L-asparaginase

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DOI: 10.5530/ctbp.2025.2.19

production by Aspergillus terreus MTCC 1782 under solid state fermentation using mixed substrate. *International Journal of Research in Engineering and Technology*.,2015; 4(5), 354-360.

13. Dias, F.F.G., de Castro, R.J.S., Ohara, A., Nishide,T.G., Bagagli, M.P., Sato, H.H.Simplex centroid mixture design to improve L-asparaginase production in solid-state fermentation using agroindustrial wastess. *Biocatalysis and Agricultural Biotechnology*., 2015;4(4), 528-534.

14. da Cunha, M.C., Silva, L.C., Sato, H.H., de Castro, R.J.S. Using response surface methodology to improve the L-asparaginase production by Aspergillus niger under solid-state fermentation. *Biocatalysis and Agricultural Biotechnology*.,2018; 16,31–36.

15. Mishra, A., Production of L-asparaginase, an anticancer agent, from Aspergillus niger using agricultural waste in solid state fermentation. *Applied biochemistry and biotechnology*.,2007;*135*,33-42.

16. Rowayshed, G., Salama, A., Abul-Fadl, M., AkilaHamza, S., Mohamed, E. A. Nutritional and chemical evaluation for pomegranate (Punica granatum L.) fruit peel and seeds powders by products. *Middle East Journal of Applied Sciences.,2013;*3, 169–179.

17. Chaloupka, J.,Regulation of the synthesis of extracellular proteinases in bacilli. In *Environmental Regulation of Microbial Metabolism.,1985;* 287-293.

18. Manna, S., Sinha A., Sadhukan R., Chakrabarty SL. Purification, characterization and antitumor activity of L-asparaginase isolated from Pseudomonas stutzeri MB-405. *Curr Microb*.,1995; 30: 291-298.

19. Sivaramakrishnan S, Gangadharan D, Nampoothiri KM, Soccol CR, Pandey A. αamylases from microbial sources-an overview on recent developments. *Food Tech Biotech*., 2006; 44: 173-184.

20. Adinarayana, K., Ellaiah P. Response surface optimization of the critical medium components for the production of the alkaline protease by a newly isolated Bacillus sp. *J Pharm Pharmaceu Sci*., 2002; 5: 272–278.

21. De-Angeli C., Pocciari F., Russi S., Tonolo A., Zurita VE., Ciaranf E., Perin A. Effect of L-asparaginase from Aspergillus terreus on ascites sarcoma in the rat. Nature, 1970; 225: 549-550.

22. Koshy, A., Dhevendaran, K., Georgekutty, M. I., & Natarajan, P. L-asparaginase in Streptomyces plicatus isolated from the alimentary canal of the fish, Gerres filamentosus (Cuvier). *Journal of Marine Biotechnology*,1997; *5*(2-3), 0181-0185.