Production, characterization and anticancerous activity of L-asparaginase from Bacillus sp

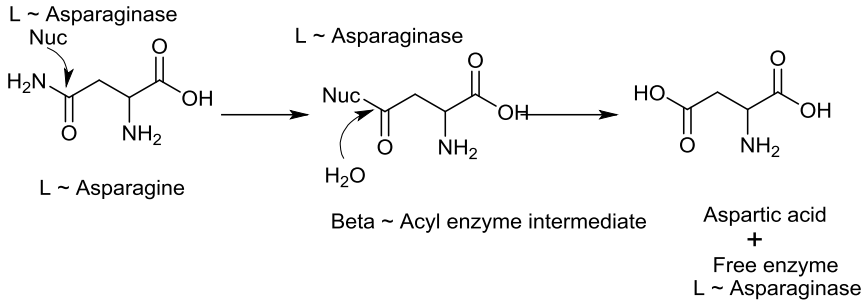
Keywords: L-Asparaginase, Acute Lymphoblastic Leukemia (ALL), Glutaminase-free L-Asparaginase, SDS-PAGE, MTT assay, Nessler's reagent

ABSTRACT

L-asparaginase, an enzyme that catalyzes the hydrolysis of L-asparagine to L-aspartate and ammonia, is widely used as an antineoplastic agent in the treatment of Acute Lymphoblastic Leukemia (ALL) and in the food industry. Microorganisms producing the enzyme were isolated from soil and cultured in Luria-Bertani (LB) media. The optimal conditions for enzyme production were determined to be 40°C, pH 6, and 48 hours of incubation. The addition of ammonium sulfate, sucrose, and cobalt chloride enhanced enzyme production. The enzyme was purified through salt dialysis, ion-exchange chromatography, and gel filtration. Gram staining and biochemical tests identified the producer as a *Bacillus* species. Characterization of the purified enzyme showed an activity of 0.27 U/mL at 30 minutes of incubation, 2.93 U/mL at 50 mM substrate concentration, 0.36 U/mL at pH 6, and 0.44 U/mL at room temperature. SDS-PAGE analysis revealed a molecular weight of 45 kDa. MTT assay using the 3T3 cell line demonstrated 62.37% inhibition, indicating significant anticancer potential. These findings highlight L-asparaginase as a promising candidate for therapeutic applications.

1. INTRODUCTION:

L-Asparagine, a key amino acid for protein and peptide synthesis, is produced within cells by the enzyme asparagine synthetase. This tetrameric protein deaminates asparagine and glutamine (Dhanam Jayam G and Kannan S, 2014)[1]. L-Asparaginase catalyzes the hydrolysis of L-Asparagine into aspartic acid and ammonia. While normal cells can regenerate L-Asparagine, cancer cells lack this ability and rely on blood serum for their supply. L-Asparaginase depletes L-Asparagine, disrupting protein synthesis, arresting the cell cycle in the G1 phase, and inducing apoptosis in cancer cells.



28

29 L-Asparaginase has gained significant attention in cancer therapy, particularly for treating
30 Acute Lymphoblastic Leukemia (ALL) and lymphomas. It is also widely used in the food
31 industry to reduce acrylamide formation, a carcinogenic byproduct of the Maillard reaction in
32 starchy foods during heating (Arastoo Badoei-Dalfard, 2014)[2].

33 Microorganisms serve as the primary source of L-Asparaginase, with *Escherichia coli* and
34 *Erwinia carotovora* being commonly used in pharmaceutical applications. However, these
35 sources have drawbacks, such as glutaminase activity, which can lead to side effects like
36 diabetes, allergic reactions, and coagulation disorders. To overcome these limitations,
37 alternative microbial sources, including fungi like *Aspergillus oryzae* and *Aspergillus niger*
38 (Jorge Javier Muso Cachumba et al., 2016) [3], and actinomycetes such as *Streptomyces*
39 *gulbargenis, S. olivalus* NEAE-119, *S. parvus* NEAE-95, and *S. brollosae* (Noura El-
40 Ahmady El-Naggar et al., 2016)[4], have been explored for enzyme production.

41 Soil, rich in microbial diversity, provides an excellent natural reservoir for L-Asparaginase-
42 producing organisms. Optimizing factors such as pH, temperature, incubation time, and
43 nutrient sources, including carbon, nitrogen, and trace elements, enhances enzyme yield. This
44 study focuses on the identification, isolation, and optimization of L-Asparaginase production
45 from various microorganisms with minimal glutaminase and urease activity. The enzyme was
46 further purified, determined its molecular weight and evaluated its anticancer potential.

47 **2. EXPERIMENTAL SECTION**

48 **2.1. Isolation of organism**

49 Soil samples were collected from different locations of Bangalore for isolation of organism
50 and was carried out by pour plate methodology. In this process, 1g of soil was dissolved in a
51 saline solution used as inoculum. Subsequently, 200µl of each soil solution was poured into a
52 sterile petriplates above that approximately 20mL modified in M9 media (Asep Awaludin
53 Prihanto, et.al. 2020)[19] was poured and allowed for salinification. After the process, the

plates were incubated in bacteriological incubator at 37℃. A bacterial identification was performed by a gram staining technique.

2.2. Screening for production

Approximately 20mL media was poured in sterile petriplates further streaked in a zig zag manner on the media and incubated for 24hrs at 37℃ and confirmed by the biological assay method. In this process, the reaction mixture contains 1mL of Tris HCl buffer, 0.1mL of 189mM of L-asparagine solution followed by incubation for 10min at 37℃ and centrifuged. 0.1mL of cell free media was added to the sample solution and incubated for 30min at 37℃ and stopped the reaction by adding 0.1mL of 1.5M trichloroacetic acid. Then, 0.2mL of sample solution was pipetted out, diluted with water and subsequently, 0.5mL of Nessler's reagent was added. The liberation of ammonia was analyzed by UV absorbance at 436nm against 6mM of ammonium sulphate standard solution to find 1 unit of enzyme activity which is defined as the amount of enzyme that catalyzes the reaction of 1µmol of substrate per minute.

2.3. Optimization of physical parameters

Physical parameters such as incubation time was carried out by the preparation of 50mL of media, inoculated the organism and incubated in shaker incubator and for every 24 hrs enzyme assay was carried out. After standardization of incubation time, it was subjected to optimization of pH. In this process, 50mL media was prepared in five different conical flasks adjusted to pH of 4 to 9 using NaOH solution and after 48 hours assay was performed. Thereafter, temperature was optimized with 50mL of media and adjusted to pH 6 and incubated at different temperatures such as 25℃, 30℃, 35℃ and 40℃. Enzyme assay was carried out to observe the optimum temperature.

2.4. Effect of nitrogen sources, carbon sources and trace elements

Five different types of nitrogen sources such as 1% tryptone, Peptone, Ammonium sulphate, Ammonium nitrate and Sodium nitrate were added into a media and adjusted to pH 6 and the organism was inoculated followed by the incubation for 48 hours at 40℃ .Assay was performed to determine a nitrogen source that has been utilized by an organism. Thereafter, a range of concentrations from 0.25% to 1% of selected nitrogen source were added to the broth and assay was carried out to determine the specific amount of nitrogen source used by the organism. Similarly, carbon sources such as 1% of cellulose, sucrose, starch, glucose and

maltose as well as trace elements includes 10mg $MnCl_2$, $MgSO_4$, $Fe(SO_4)_2$, $ZnCl_2$, $CoCl_2$ and $Cu(SO_4)_2$ were added into a l-asparagine broth and assays were performed for all the above chemical sources along with its different concentration.

2.5. Purification of L-asparaginase and protein estimation

The enzyme solution was centrifuged, and the cell free media obtained was subjected to purification which includes salt precipitation where 70% saturation was achieved by the addition of salt followed by dialysis. The dialysed sample was purified by ion exchange chromatography using a gradient elution. Diethylamino ether (DEAE) cellulose was added to a column as a matrix, and it served as a positively charged resin. An enzyme solution was then purified by gel filtration chromatography where, 75g sephadex gel was added to a column. Protein estimation was carried out against BSA standard at 660nm (Oliver H. Lowry et.al., 1951) [20] and assay was conducted for all the purified samples by modified Nesslerization method.

2.6. Characterization and SDS page

The purified sample underwent further characterization such as, incubation time ranging from 5min to 30min and for substrate concentration, different concentrations of Tris HCl buffer (reagent A) were prepared, pH was adjusted to 4,5,6,7,8,9 and 10 using different buffers and incubated at different temperatures during the assay experiment and its activity was calculated for all the parameters. SDS page was performed to find a molecular weight (Sarina P. Khabade, et.al, 2024) [21].

2.7. Anti –Proliferating assay

The anticancer activity of L-asparaginase was evaluated using the 3TS cell line, a standard model for testing anticancer agents. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. Upon reaching 70%-80% confluency, they were trypsinized, counted, and seeded into 96-well plates at a density of 10,000 cells per well. After overnight adhesion, L-asparaginase was added in concentrations ranging from 0.1 to 10 U/mL in triplicate, while control wells contained only culture medium. The plates were incubated at 37°C with 5% CO_2 for 48 hours. Cell viability was assessed using an MTT assay. After incubation, 20 µL of MTT reagent (5 mg/mL in PBS) was added to each well and incubated for 4 hours. The media was then removed, and 150 µL of DMSO was added to

dissolve the formazan crystals formed by metabolically active cells. Absorbance was measured at 575 nm using a microplate reader.

3. RESULTS AND DISCUSSION

3.1. Confirmation of L-asparaginase activity

Soil was chosen as the source to isolate microbes producing L-asparaginase enzyme. Collected soil samples were dissolved in a saline solution (Fig 1) followed by its introduction into a petridish containing asparagine dextrose salts agar media (ADS). The presence of L-asparaginase enzyme was indicated by the formation of pink colour colonies due to the addition of phenol red indicator, thus there was a change in pH from acidic to alkaline (Fig 2a to 2e). The similar change was observed by Noha et.al.[14]. The gram staining technique identified the culture as belonging to Bacillus species.

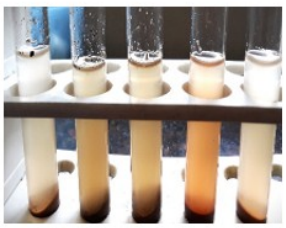


Fig 1

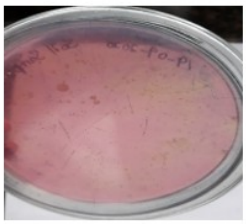


Fig 2a

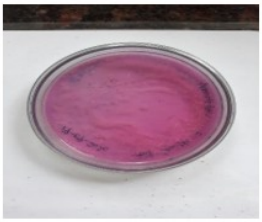


Fig 2b

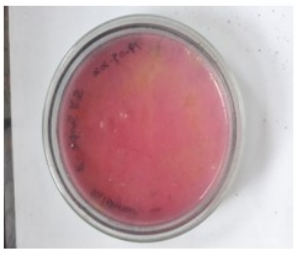


Fig 2c

Fig 2d

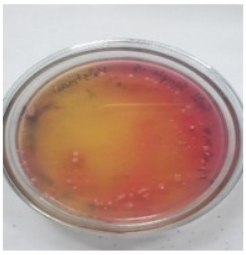


Fig 2e

Fig 1(soil sample dissolved in saline solution) Fig 2a to 2e (Sample showing pink colour colonies indicates the presence of L-asparaginase enzyme)

3.2. Determination of physical parameters by L-asparaginase assay

The physical parameters were optimized with respect to pH, incubation time and temperature and thereby, enzyme activity was determined by l-asparaginase assay. Ammonium sulpahate was used as a standard .The highest enzyme activity was observed in the first organism at 48hrs incubation time (Fig 3a). Further, it remained stable as compared to other organisms where, the enzyme activity differed. The selected organism was further analysed for pH and

temperature in which the maximum enzyme activity was observed at 40°C and pH 8 (Fig 3b and 3c). Pallavi et.al.[11] reported the maximium enzyme activity at 37°C and pH 9.6 and Khabade et.al.[21] observed the similar results for temperature at 37°C and pH 7. By optimizing these parameters production of the enzyme can be increased. Biochemical tests were performed on the bacterial culture in order to understand the correlation of the compounds associated with the enzyme which will be helpful in the preparation of growth

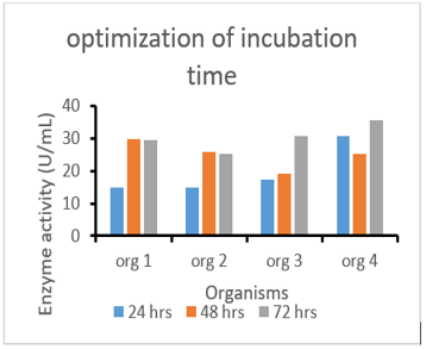


Fig 3a

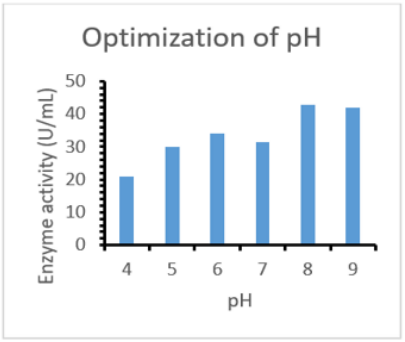


Fig 3b

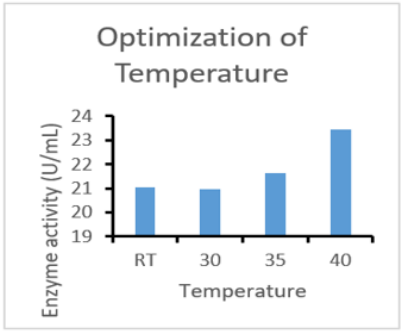


Fig 3c

media as well as in clinical aspects. Nineteen biochemical tests were conducted and among them some of them showed positive results as represented in table 1.

Fig 3 – optimization of physical parameters a) incubation time b) pH c) Temperature

Table 1 – Biochemical tests of a bacterial culture

	Positive	**Negative**
Carbohydrate fermentation test		
Glucose	Positive	-
Maltose	-	Negative
Lactose	-	Negative
Sucrose	Positive	-
Indole	-	Negative
Methyl red	-	Negative
Voger Proskauer	Positive	-
Citrate	-	Negative
Gelatin	-	Negative
H_2S	-	Negative

Nitrate reduction	Positive	-
Catalase	Positive	-
Oxidase	-	Negative
TSi	Positive	-
Urease	Positive	-
Lipid hydrolysis	-	Negative
Starch hydrolysis	-	Negative
Cellulose degradation	Positive	-
Casein hydrolysis	Positive	-

3.3. Effect of different chemical sources

Among five nitrogen sources, the more enzyme activity was found with 1.25% ammonium sulphate, (Fig 4a and 4b). In carbon sources, the greatest activity was observed in 1. 5% of sucrose (Fig 4c and 4d) and in trace elements 5mg of cobalt chloride showed the highest activity (Fig 4e and 4f). Similar results were observed by Narendra et.al. [22] Whereas, Ali et.al.[13] showed the maximum enzyme activity result in glucose and the least activity in sucrose. Noura et.al. [23] obtained the maximum values for manganese and cobalt metal ions such as 145.15% and 143.04% respectively. The addition of these chemical sources will enhance the enzyme activity.

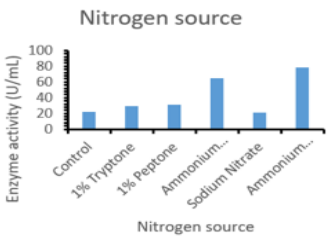


Fig 4a

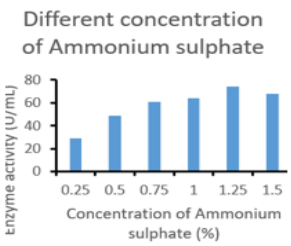


Fig 4b

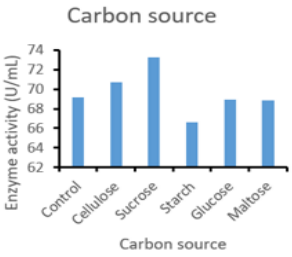


Fig 4c

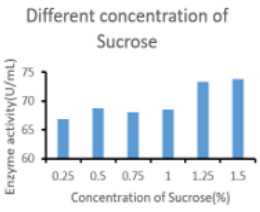


Fig 4d

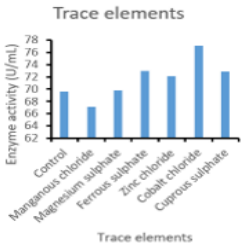


Fig 4e

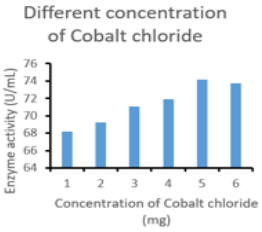


Fig 4f

Fig 4- different chemical parameters a) Nitrogen source b) Concentartion of ammonium sulphate c) Carbon source d) Concentration of Sucrose e) Trace elements f) Concentration of Cobalt chloride

3.4. Purification and protein estimation

The optimized sample was further purified by salt precipitation, dialysis and ion exchange chromatography. Protein estimation was performed at 660nm for all the purified samples (Fig 5) against a BSA standard. Enzyme activity was found to be 75.31 units/mL in salt precipitate whereas, the activity decreased gradually to 5.42 units/mL for ion exchange sample. The activity was even more decreased when subjected to gel filteration chromatography. Similar results were reported by Narendra et.al.[22] and P.Devaghi et.al.[24].

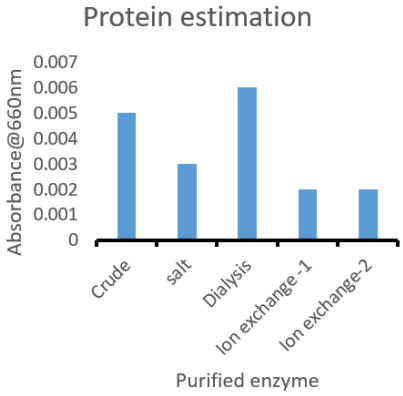


Fig 5- Protein estimation of all the purified samples

3.5. Characterization of purified enzyme and SDS page for molecular determination

The purified enzyme was characterized based on pH, temperature, incubation time, and substrate concentration. Enzyme activity showed a sharp increase, reaching 0.22 U/mL at 20 minutes of incubation before declining at 30 minutes (Fig 6a). The highest activity was observed at pH 6 (0.36 U/mL), with a decline in alkaline conditions (Fig 6b). Maximum activity was recorded at a 100 mM substrate concentration, remaining stable up to 150 mM (Fig 6c). The optimal temperature for enzyme activity was 35°C (Fig 6d). Similar findings were reported by Estefania et al. [16], who observed peak activity at pH 6 and 37.5°C, while Narendra et al. [22] found maximum activity at pH 9, 40 minutes of incubation, and 40°C. The molecular weight of the enzyme was determined to be 45 kDa (Fig 7), aligning with

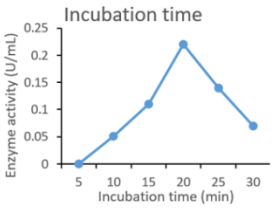


Fig 6a

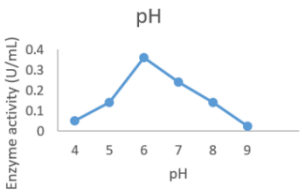


Fig 6b

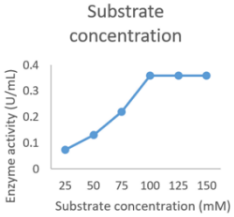


Fig 6c

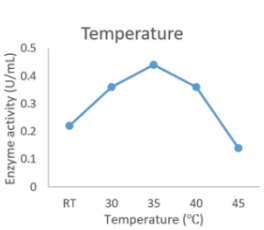


Fig 6d

previous studies, where Noura et al. [12] reported 64 kDa and Estefania et al. [16] found 37 kDa.

Fig 6 – Characterization of purified enzyme a) Incubation time b)pH c)Substrate concentration d)Temperature.

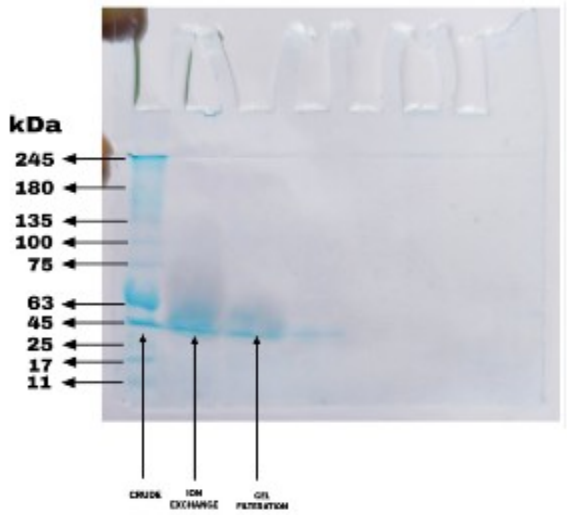


Fig 7– SDS page showing the molecular weight of the protein

3.6. Effect of enzyme volume on cell inhibition

This study has evaluated and confirmed the anti-cancerous properties of the enzyme extracted from a soil-isolated microorganism. An assay performed using the 3T3 cell line demonstrated a gradual increase in the percentage of inhibition with the incremental addition of the sample. At a sample volume of 10 µl, the inhibition percentage was recorded at 13.67%, whereas at 50 µl, it increased to 40.39% (Table 2) and IC-50 was calculated from the graph (Fig 8b), and it was found to be 62.37%. This indicates that the lower sample volume resulted in minimal reduction in cell viability, while the higher volume produced a more significant effect. The comparative analysis with Manish Bhat et al. [25] revealed a 98% inhibition upon the addition of 0.019 IU/mL of the enzyme sample, whereas Islam Husain et al. [26] reported a 31.79% inhibition after adding 10 IU/mL of the sample.

Table 2 – Effect of enzyme volume on cell inhibition

Volume of enzyme	OD at 575nm	Percentage of Inhibition
Control	0.9765	-
10µl	0.843	13.67127 %
20µl	0.764	21.76139 %
30µl	0.688	29.54429 %

40µl	0.6285	35.63748 %
50µl	0.582	40.39939 %

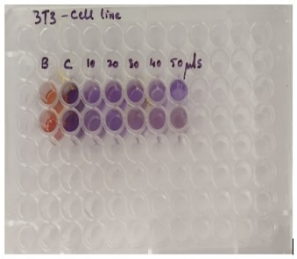


Fig 8a

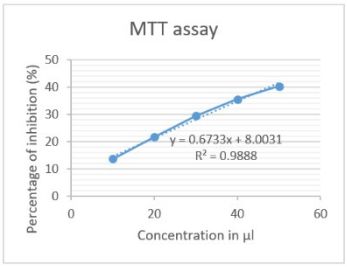


Fig 8b

Fig 8 a) – Various concentration of control and enzyme solutions, Fig b) – Percentage of inhibition for different volumes of enzyme

4. CONCLUSION

This study successfully optimized and characterized L-asparaginase from soil-isolated *Bacillus* species. Enhanced enzyme production was achieved through optimized physical and chemical conditions, followed by purification using salt precipitation, dialysis, ion-exchange, and gel filtration chromatography. Characterization confirmed its activity across various parameters, with SDS-PAGE revealing a molecular weight of 45 kDa. MTT assay using the 3T3 cell line showed 62.37% inhibition, demonstrating significant anticancer potential. These findings reinforce the therapeutic relevance of L-asparaginase, particularly in Acute Lymphoblastic Leukemia treatment, and highlight its broader industrial applications.

REFERENCES

1. Dhanam Jayam,G and Kannan, S, " The various sources of L-asparaginase", *International journal of recent scientific research*, Vol 5(2), 342-346, Feb 2014.
2. Arastoo Badoei-Dalfard, "L-asparaginase production in the pseudomonas pseudoalcaligenes strain JHS-71 isolated from jooshan hot-spring", *Molecular biology research communications*, Vol 5(1), 1-10, 2016.
3. Jorge Javier Muso Cachumba, Felipe Antonio Fernandes Antunes, Guilherme Fernando Dias Peres, Larissa Pereira Brumano, Julio Cesar Dos santos, Silvio Silverio Da Silva, "Current applications and different approaches for microbial L-asparaginase production", *Brazilian journal of microbiology*, 77-85, Oct 2016.

4. Noura El-Ahmady El-Naggar, Sahar F. Deraz, Hoda M.Soliman, Nehal M. El-Deeb and Sara M. El-Ewasy, “Purification, characterization, cytotoxicity and anticancer activities of L-asparaginase, anti-colon cancer protein from the newly isolated alkaliphilic Streptomyces fradiae NEAE-82", *Scientific reports*, Sep 2016”.
5. Vishal P. Oza, Pritesh P. Parmar, Sushil Kumar, R.B. Subramanian, “Anticancer properties of highly purified L-asparaginase from *Withenia somnifera* L. against acute lymphoblastic leukemia”, *Applied biochemistry and biotechnology*, Vol 160, 1833-1840, 2010.
6. Sahar W.M. Hassan, Aida M. Farag and Ehab A. Beltagy, “Purification, characterization and anticancer activity of L-asparaginase produced by marine Aspergillus terreus”, *Journal of pure and applied microbiology*, Vol 12(4), 1845-1854, Dec 2018.
7. Anup Ashok, Kruthi Doriya, Jyothi Vithal Rao, Asif Qureshi, Anoop Kumar Tiwari and Devarai Santhosh Kumar, “Microbes producing L-asparaginase free of glutaminase and urease isolated from extreme locations of Antarctic soil and moss”, *Scientific reports*, Vol 9, 1423, Feb 2019.
8. Jahanvi Vachhani and Binita Desai, “Isolation, identification and production of L-asparaginase producing bacteria from soil”, *BMR Microbiology*, Vol 4(1), 1-6, Apr 2018.
9. M. R. Bhat, J. S. Nair and T. Marar, “Isolation and identification of L-asparaginase producing salinicoccus sp. M. KJ997975 from soil microbial flora”, *International journal of pharmaceutical sciences and research*, Vol 6(8), 3599-3605, 2015.
10. S. A. Alrumman, Y. S. Mostafa, Kholood A. Al-izran, M. Y. Alfaifi, T. H. Taha and S. E. Elbehairi, “Production and anticancer activity of an L-asparaginase from Bacillus licheniformis isolated from the red sea, Saudi arabia”, *Scientific reports*, Vol 9, 3756, 2019.
11. Pallavi Rudrapati and Amrutha V. Audipudi, “Production and purification of anticancer enzyme L-asparaginase from Bacillus firmus AVP 18 of mangrove sample through submerged fermentation”, *International journal of current microbiology and applied sciences*, Vol 5, 1-18, 2017.
12. Noura El-Ahmady El-Naggar and Nancy M. El-Shweihy, “Bioprocess development for L-asparaginase production by Streptomyces rochei, purification and in-vitro efficacy against various human carcinoma cell lines”, *Scientific reports*, Vol 10, 7942, 2020.
13. Ali Mohamed Elshafei and Dina Helmy El-Ghonemy, “Screening and media optimizaiton for enhancing L-asparaginase production, an anticancer agent from different filamentous fungi in solid state fermentation”, *British biotechnology journal*, Vol 9(3), 1-15,2015.

14. Noha E Abdel-Razik, Khaled Z el-Baghdady, Einas H El-Shatoury, Nahla G Mohamed, "Isolation, optimization and antitumor activity of L-asparaginase extracted from pectobacterium carotovorum and serratia marcescenson human breast adenocarcinoma and human hepatocellular carcinoma cancer cell lines", *Asian journal of pharmaceutical and clinical research*, Vol 12(2), 332-337, 2019.
15. Noura El-Ahmady El-Naggar, Hassan Moawad, Nayera A. M. Abdelwahed, "Optimization of fermentation conditions for enhancing extracellular production of L-asparaginase, an anti-leukemic agent, by newly isolated Streptomyces brollosae NEAE-115 using solid state fermentation", *Ann microbial*, Vol 67, 1-15, 2017.
16. Estefania Arevalo-Tristancho, Luis E. Diaz, Jorge E. Cortazar and Manuel F. Valero, "Production and characterization of L-asparaginases of Streptomyces isolated from the Arauca riverbank (Colombia)", *The open microbiology journal*, Vol 13, 2019.
17. N Saleem Basha, R Rekha, M Komala and S Ruby, "Production of extracellular anti-leukaemic enzyme L-asparaginase from marine actinomycetes by solid –state and submerged fermentation: purification and characterization", *Tropical journal of pharmaceutical research*, Vol 8(4), 353-360, Aug 2009.
18. Maria P. MacWilliams and Min-Ken Liao, "Luria Broth (LB) Luria Agar (LA) Media and their uses protocol", *American society for microbiology*,
19. Asep Awaludin Prihanto, Indah Yanti, Mohammad Achsanil Murtazam and Yoga Dwi Jatmiko, "Optimization of glutaminase-free L-asparaginase production using mangrove endophytic *Lysinibacillus fusiformis* B27", 8, May 2020.
20. Oliver H. Lowry, Nira J. Rosebrough, A. Lewis Farr and Rose J. Randnol "Protein measurement with the folin phenol reagent", May 28, 1951.
21. Sarina P. Khabade, Divijendra Natha Reddy SIrigiri and Anshu Beulah Ram, "L-asparaginase from Solanum lycopersicum as a nutraceutical for acute lymphoblastic leukemia", *ACS Omega*, Vol 9, 3616-3624, May 2024.
22. Narendra Kumar S, Mohammed Haseeb Nawaz, Shyam Shankar Mishra, Satya Suman, Lingayya Hiremath, Praveen Kumar Gupta, Ajeet Kumar Shrivastava and Mahesh M, " Anti-cancer enzyme(L-asparaginase) production, purification and characterization from a soil isolate of Pseudomonas sp", *International journal of advanced research*, Vol 7(12), 753-761, Dec 2019.
23. Noura El-Ahmady El Naggar, Sahar F. Deraz, Sara M. El-Ewasy and Ghada M. Suddek, "Purification, characterization, immunogenicity assessment of glutaminase free L-

asparaginase from Streptomyces brollosae NEAE-115", *BMC pharmacology and Toxicology*, 2018.

24. P. Dhevagi and E. Poorani, "L-Asparaginase from Marine Actinomycetes of Thoothukudi Coastal Ecosystem", *International Journal of Current Microbiology and Applied Sciences*, Vol 5(7), 295-306, July 2016.
25. Manish Bhat and Thankamani Marar, "Cytotoxic Effect of Purified L-asparaginase from *Salinicoccus sp*. MKJ997975", *International Journal of Current Microbiology and Applied Sciences*, Vol 4(4), 701-712, July 2015.
26. Islam Husain, Anjana Sharma, Suresh Kumar and Fayaz Malik, "Purification and Characterization of Glutaminase free asparaginase from *Enterobacter cloacae*: In –vitro evaluation of Cytotoxic Potential against Human Myeloid Leukemia HL-60 Cells", *PLoS One*, Vol 11(2), Feb 2016.