Production and anti-cancer Activity of L-Asparaginase from Bacillus sp.

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Abstract

Microbial L-asparaginase (L-asnase) is a widely used anti-tumor enzyme that catalyzes the process of converting L-asparagine to aspartate and ammonia, successfully starving cancer cells of an essential nutrient. L-asnase does occur in a variety of other organisms, including plants and fungi, but it is relatively little perennial for bacteria. The aim of this study was to isolate, screen and characterize soil-borne bacterial strains for their capability to produce L-asparaginase, and evaluate the anticancer activity of this enzyme on the Pan-I cell line. Herein, eight bacterial isolates were recovered from soil samples collected from forested areas in Tehran, Iran. These specimens were cultured in vitro and screened for their capable of producing extracellular and intracellular L-asparaginase. Following isolation, the samples were cultured in M9 medium which allowed for identification of colonies producing the enzyme with a change in color from yellow to pink. L-asnase was confirmed for its presence by spectrophotometry, and its molecular mass was analyzed by SDS-PAGE on heterogeneous purified samples. The extracted L-asparaginase was then tested for its anticancer activity was assessed against the Pan-I cell line using the MTT assay. An L-asnase- was able to isolate Bacillus subtilis strain excited from the soil samples which exhibited positive anticancer activity as shown by decreased cell viability of treated Pan-I cells.

Keywords: L-asparaginase; *Bacillus*; MTT; Pancreatic cancer cell.

Introduction

Pancreatic cancer is among the most aggressive malignancies with a highly progressive disease and poor prognosis. In fact, about 80% of cases have metastasized by the time of diagnosis, and this is partly why its reputation as an extremely aggressive cancer type (which it is) is well-deserved. Pancreatic cancer is the fourthleading cause of cancer-related mortality in both men and women after lung (1, 3) and colorectal (4) and prostate (5) for men and after breast (1, 6), colorectal (7), and lung cancers (8) in women according to a 2014 European epidemiological study. Despite progress in diagnostic and therapeutic technologies, pancreatic cancer is still associated with a heightened five-year survival rate, estimated to be somewhere around 4% (3).

Undoubtedly, L-asparaginase is one of the treatments that aroused enthusiasm in cancer therapy in general and in the treatment of pancreatic cancer. For the last 40-plus years, this enzyme has been the focus of research and debate over whether it has any usefulness or not. L-asparaginase is an anti-cancer enzyme that is used to treat a variety of hematologic malignancies, such as acute lymphoblastic leukemia (ALL), reticulosarcoma, Hodgkin's disease, acute myeloid leukemia, acute myelomonocytic leukemia, and chronic lymphocytic leukemia (CLL). It is generally prescribed with other therapies. This enzyme has been universally approved by Food and Drug Administration (FDA) and World Health Organization (WHO) for a wide range of clinical promise (4).

Mechanistically, L-asparaginase acts through the hydrolysis of L-asparagine into L-aspartic acid and ammonia resulting in a depletion of circulating asparagine levels (5). Such depletion perturbs the normal assembly of cancer cells in their proliferation cycle, especially during the G1 phase of the cycle which results in decreased protein and nucleic acid synthesis and further promotes cell death (6). Significantly, L-asparaginase additionally exhibits co-activity as a glutaminase, an attributeu of note if L-asparaginase is to prove effective in targeting pancreatic cancer cells, which often demonstrate increased reliance upon glutamine metabolism for survival (7). Mutations in the KRAS gene, prevalent in pancreatic cancer, further augment glutamine utilization, which allows tumor growth and proliferation(8). L-asparaginase-induced glutamine depletion has been shown to inhibit the growth of human hepatocellular carcinoma

xenografts (9), underscoring the important role of glutamine metabolism in the survival of cancer cells.

L-asparagiase can be obtained from different organisms, including plants, animals, and microorganisms, and displays antiproliferative activity against multiple cancer types, such as breast cancer (4). The demand for L-asparaginase will rise significantly due to its clinical potential and its applications in food processing. Microbial production, in particular using bacteria and fungi, has various advantages: rapid enzymes production (24–48 h), inexpensive and easy screening of different species (10). The use of pH indicators allows direct visualization that permits fast identification of active cultures(11).

While being an efficient drug, the glutaminase activity of L-asparaginase gave way to its side effects on normal cells. Continued usage of common substances such as E. coli and D. chrysanthemi may induce immune responses that lead to allergic reactions, anaphylaxis, and possible resistance in tumoral cells (12). 2, L-asparaginases isolated from microbes are divided into two types according to their substrate affinity, where the Km values of Type II L-asparaginases (micromolar range) are lower than that of Type I L-asparaginases (millimolar range) (13). The higher target affinity of Type II enzymes is beneficial for therapeutic use, in which it is essential that asparagine is successfully degraded. Recently, a strain of Bacillus subtilis isolated from Red Sea sponges has been found to have anticancer activities with a low adverse effect (14).

L-asparaginase sources from Bacillus species would be a good option only if those obtained from soil as the soil ecosystem is the richest with diverse microorganisms(15).

Hence, this study seeks to assess the role of therapeutic potential of L-asparaginase targeting pancreatic cancer and its effect on tumor growth and metabolic dependencies. Our exploration of the glutaminase activity, substrate affinity, and possible side effects of L-asparaginase is helping us evaluate its potential as an effective and safe therapeutic agent against pancreatic cancer. We also intend to explore new sources of L-asparaginase having improved therapeutic properties, especially from soil originated Bacillus species in order to extend its clinical use with very high property against side effects.

Material and Methods

Collection and Preparation of Soil Sample

Soil samples were collected from various locations within a forest park in Tehran, Iran, at a depth of 10 cm and stored at 4 °C in plastic containers. Each sample was serially diluted in sterile normal saline, followed by centrifugation at 1000 rpm for 3 minutes. From the 10^{-4} and 10^{-6} dilutions, 25 µL of supernatant was cultured on 8 cm LB agar plates under sterile conditions. The cultures were spread evenly across the plates with a sterile L-shaped Pasteur pipette and incubated at 37 °C for 48 hours. Bacterial colonies were then purified, and biochemical tests were performed to identify and isolate Bacillus strains.

M9 medium

Table1 shows the properties of M9 medium used in this study.Additionally, it contained 1.5 % agar with phenol red (0.04-0.36 ml of a solution containing 2.5 %) to maintain a pH of approximately.

Table 1: Ingredients of the culture medium M9 for 1 Liter

material	amount
MgSO 4. 7 H 2 O	0/4 ml
CaCl 2. 2 H 2 O	0/2 ml
Glucose	2 ml
Phenol red	6 ml
Agar	4gr

Na 2HPO 4. 2 H 2 O	3gr
KH 2PO 4	1/5 gr
NaCl	0/25
L -asparagine	2/5

Colony selection

Morphologically different colonies were streaked onto sterile plates with modified M9 medium with the addition of Phenol Red as pH indicator. Control plates contained un-inoculated medium. Plates were incubated at 37 ± 2°C for 24–72 h, and L-asparaginase activity was characterized by the color development of the medium from yellow to pink due to pH change (Fig. 1). The positive colonies were further streaked on the nutrient agar plates to purify the L-asparaginase producer. The purified cultures were stored at 4°C for further study.

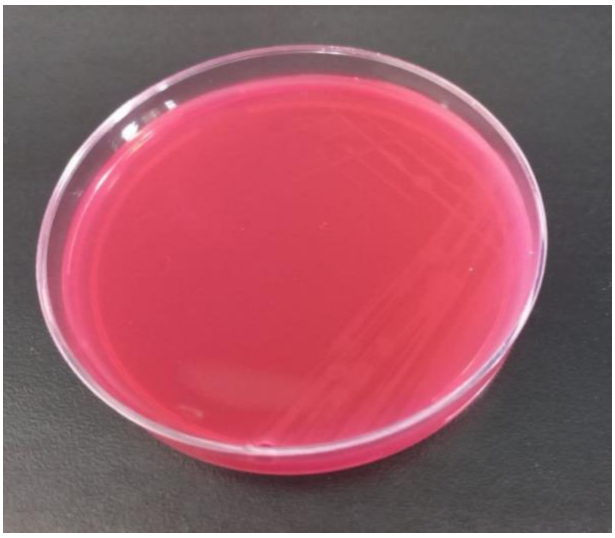

Fig 1. Detection of L-ASNase- producting isolates in the modified M9 medium.

Screening for L-asparaginase producing *Bacillus*

Soil samples were analyzed to screen for L-asparaginase (L-ASNase)-producing isolates by determining colonies with a color change (pink or reddish color) for the presence of the enzymatic activity. L-ASNase activity from bacteria grown on modified M9 medium was determined by measuring ammonia secretion by the Nesslerization method (16). Enzyme activity was determined by measuring ammonia levels against an ammonium sulfate standard. Furthermore, the yellow brown coloration of Nessler's reagent symbolizing presence of ammonia enabled one the ability to detect minute amounts of ammonia, which yielded activity of L-ASNase that converts asparagine into ammonia and aspartate.

$$Unit\ mL^{-1}Ensyme = \frac{Micromoles\ of\ ammonia\ liberated\ \times X}{V \times T \times Y}$$

X = initial volume (mL) of enzyme mixture of the first step

Y = volume of enzyme mixture (mL) (taken from first step) used in the second step

T = incubation time (min)

V = volume of enzyme taken as crude enzyme (mL).

Partial purification of L-asparaginase

L-asparaginase was partially purified from the crude enzyme extract by ammonium sulfate (85%) precipitation. The precipitate enzyme was resuspended in a minimal volume of 1 M Tris HCl (pH 7.5) and dialyzed overnight through a semi-permeable membrane against the same buffer at 4°C.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

We used SDS PAGE for screening the purity of asparaginase enzyme. To this, the enzyme fractions were resuspended in 200 µL of lysis buffer (62.5 mM Tris -HCl, pH 6.8), SDS (2%), glycerol (15%), 2-mercaptoethanol (5%) and Bromophenol Blue 0.001% (tracking dye). The samples were boiled for 5 min at 100° C and loaded onto a 15% SDS polyacrylamide gel and run at 100 V for 7–8 hours. After electrophoresis, the gel was then stained with Coomassie

Brilliant Blue R-250, distained with 500 mL methanol, 100 mL acetic acid and distilled water to 1 L and visualized by Gel Documentation System. Determination of molecular weight of the partially purified L-asparaginase was performed by comparing with standard molecular weight markers

Cell toxicity assessment by MTT assay

Fig. 5: Cell viability was measured by the colorimetric MTT assay using [3-(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]-based reduction into a purple formazane product by metabolically active cells. The Panc-1 cells, purchased from Pasteur Institute of Iran, were cultured in DMEM with penicillin and streptomycin to prevent contamination. To obtain a final concentration of 10% of fetal bovine serum (FBS), this was supplemented to a complete culture medium necessary for good cell growth. Cell viability was confirmed with trypan blue dye exclusion, followed by counting with a hemocytometer and seeding in 96-well plates at a density of 10,000 cells per well.

104 human existed in each well of the 96-well plates, Panc-1cell line. After incubation for 24 hours (5% CO2 pressure, 98% humidity, 37 degrees), culture medium was drained. Next, L-ASNase at concentrations between 20 and 100 mg/ml was added to the cells in the culture medium in the 96 wells plate. Complete culture medium served as a 100% non-toxic control in three control wells.

The MTT solution was added to each well with one-tenth of the total supernatant volume of the cells added, and the resulting 2 with each added cell-culture media were removed from the incubator after 24 h. Followed by placing the plate in the incubator for 4 hours. Then, 200 µl of DMSO dimethyl sulfoxide solution was added to the formazan crystals, and the solution optical absorption was read with an ELISA reader at a 560 nm wavelength. MTT converted to the insoluble formazan salt was used to determine relative cell viability. The data are expressed as the mean percentage of viable cells relative to the respective control cultures treated with the solve

Results

Screening of bacteria isolates

Eight soil samples were collected from various environments, including forested park areas, to investigate the presence of Bacillus species capable of producing the enzyme L-asparaginase (L-ASNase). Results revealed that four bacterial isolates were able to produce ammonia, evidenced by a color change to pink in the culture medium due to pH changes associated with asparagine breakdown. Of these isolates, four displayed a prominent pink halo around their colonies, indicating higher enzyme activity. The soil samples collected near the forest park had the greatest concentration of positive isolates, accompanied by the most intense pink coloration surrounding the bacterial growth.

Biochemical tests

Biochemical tests were performed on enzyme-producing isolates, which included assessments of colony morphology, gram staining, catalase, oxidase, indole production, citrate decomposition, Methyl Red (MR), Voges-Proskauer (VP), and Hydrogen Sulfide (H2S) production. The results indicated that, from the four bacilli bacterial isolates tested, three were gram-positive while one was gram-negative. Table 2 presents the outcomes of the additional biochemical tests categorized by the type of reaction for each bacterial isolate.

Table2: biochemical tests

Bacteria code	gr	catalase	oxidase	indole	citrate	MR	VP	Urease
BAC1	+	+	-	+	+	+	+	-
BAC2	-	-	-	+	+	+	+	-
BAC3	+	+	+	-	+	-	+	-
BAC4	+	+	-	+	+	+	+	-

Bacteria number 1, 3 and 4 were selected for the next tests

Estimation of L-asparaginase production

The absorbance rate of the test and standard samples was measured using a spectrophotometer and calculated based on the Beer-Lambert law. The results are presented in Table 3. By plotting absorbance changes in different concentrations of standard samples, a standard absorbance

chart (Figure 2) was obtained, and unknown sample concentrations were calculated using the line slope formula (Table 4).

Table3: Absorption of standard samples

Absorption rate (OD) 480 nm	**Standard ascending concentrations Cs (µm/ml)**
300	0.24
400	0.32
500	0.4
600	0.45
700	0.52
800	0.53

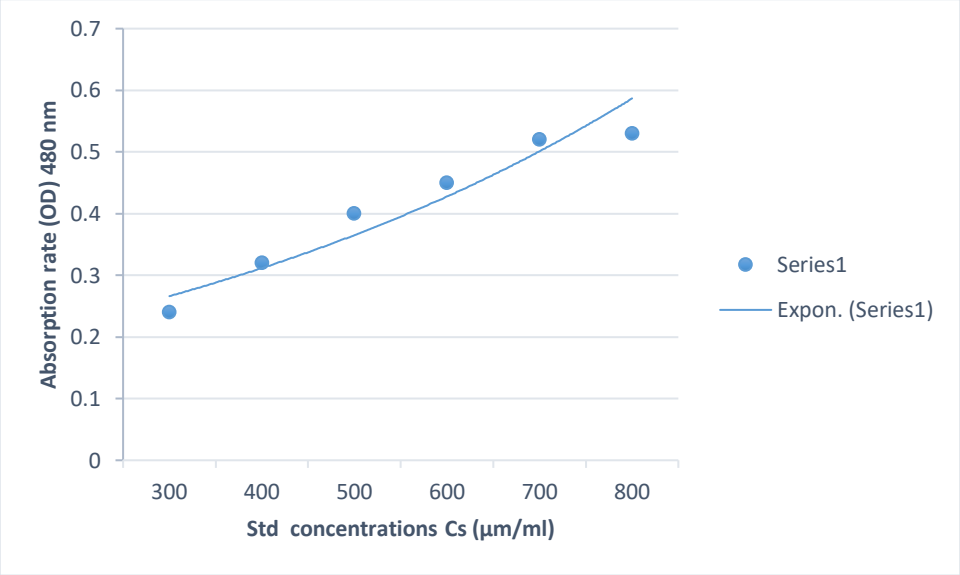

Fig 2. Standard absorption diagram of different investigated concentrations;

Table4: Absorption and unit of test samples (bacterial enzymes)

Bacteria Code	**Absorbance 480 nm**	**Calculated concentrations Cs (µm/ml)**	**Enzyme Units/ml**
BAC1	0.46	720.3	72.03
BAC3	0.42	365.1	65.31
BAC4	0.67	1403.7	104.37

L-asparaginase activities of three Bacteria were recorded to range of 65.31-104.37 unit/mL−1. The isolates of BAC4, exhibited a maximum enzyme activity with 104.37unit/mL−1. Other results can be seen in the table 4.

SDS PAGE and determination of monomeric molecular of the protein

All the bands related to the enzymes of all the samples were revealed approximately in the range of 45 kDa. As expected, the negative control samples lacked the enzyme band

According to the obtained electrophoresis pattern, the extraction of L-asparaginase is confirmed (Figure 3).

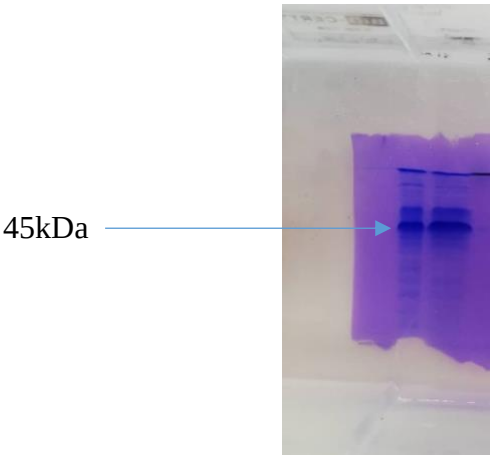

Fig 3.SDS-polyacrylamide gel electrophoresis of the purified L-asparaginase produced by Lane 1: Protein marker; Lane 2: Purified L-asparaginase;

Anti-cancer activity

Using MTT assay, the in vitro cytotoxicity effect of Bacillus sp asparaginase enzyme on the growth of pan-1 cell lines was studied. percentage of viable cells were calculated As shown in (Figure 4, table5).

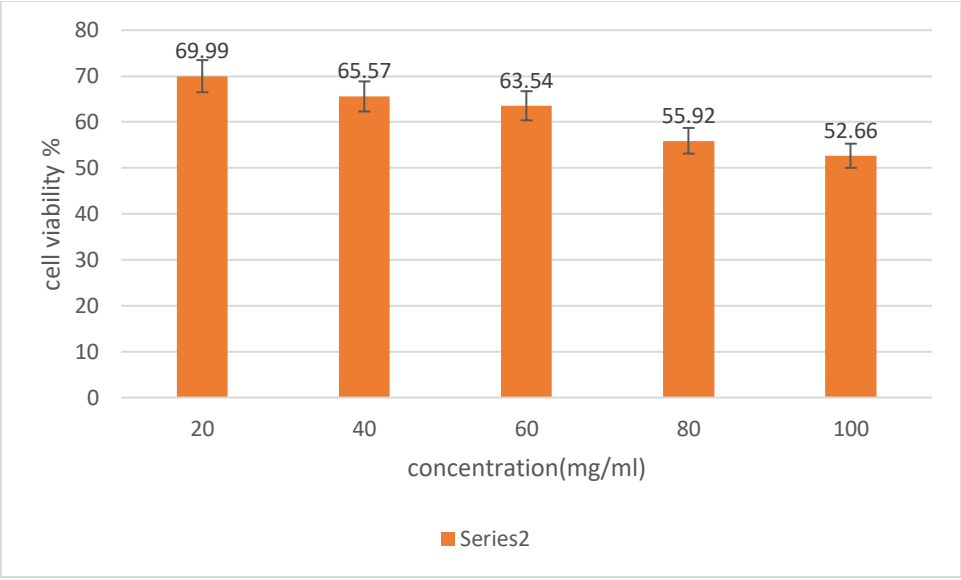

Fig 4. Cytotoxic effect of L-ASNase's conccentrations on pancreatic cancer cell line. The percentage of cell viability was measured by MTT assay at 24 h.

Table5: The percentage of cell viability

Concentrations	Average	Average control	viability
100 mg/ml	0. 51666667	0.981	52.66
80 mg/ml	0.548666667	0.981	55.92
60 mg/ml	0. 62333333	0.981	63.54
40 mg/ml	0.64333333	0.981	65.57
20 mg/ml	0.68666663	0.981	69.99

Discussion

Although L-asparaginase (L-ASNase) is typically more compatible with body tissues than classical chemotherapy drugs and new therapies (such as oligonucleotide therapies) (16), it has general side effects that can jeopardize patients (17). E. When sourced from bacteria, L-ASNase is known to induce toxicities and hypersensitivity reactions, making it unsuitable for certain patients. Hence, it is essential to find other sources for L-ASNase (18).

Soil environments with abundant vegetation, such as in forest parks, are a valuable means of exploring bacterial species with the potential for the generation of bioactive compounds.

Positive activity of L-ASNase was shown by four bacterial isolates in this study, which results in a color change of the medium when ammonia is generated upon the cleavage of L-asparagine (19). Bacteria have an incredible ability to adapt to different environmental circumstances, leading to the production of different enzymes (20) (both intracellular and extracellular) that help carry out essential biochemical reactions.

Biochemical testing of the isolates revealed that Bacillus subtilis showed the highest enzyme production; however, molecular assays are still necessary for further confirmation. According to its exotic properties, B. subtili is a Gram-positive bacterium which is usually present in the upper soil layers, took potential importance as a microbial cell factory, chiefly because of high fermentation, product yield, and low toxicien by-products such as of many cellular enzymes; therefore B. subtilis is a good micro-organism for anticancer L-ASNase production by using submerged fermentation (21).

Anti-cancer potential of bacterial L-ASNase Hence, bacterial L-ASNase was studied. For example, Moharam et al. Özdemir et al. [5] reported that L-ASNase from Bacillus sp. However, R36 was less effective against the Hep-G2 and HCT-116 cell lines, with IC50 values of 112.19 µg/mL and 218.7 µg/mL (22), respectively. Although L-ASNase upregulation could take some time, the enzyme used in the present study could still reduce Panc-1 cell viability in a dose-dependent manner (Figure 4); thus highlighting its anticancer effects.

Because each microorganism has a heterogeneous therapeutic efficacy and side effects as well (22), other natural sources are being investigated to find the most promising L-ASNases (23). For example, Alrumman et al. demonstrated that L-ASNase was produced by Bacillus licheniformis isolated from the Red Sea as a glutaminase-free enzyme, with a maximum production at 72 hours, pH 6.5, and 37 °C (24). Pradhan et al. attributed the extracellular enzymes production at the rate of 1.7 and 14.5 times lower than that of Bacillus sp. and Pectobacterium carotovorum to the use of Bacillus subtilis strain hswx88. This isolate also has potential such as large-scale production of thermophilic L-ASNase for pharmaceutical use and acrylamide-free food processing (25).

This study showed the generation of L-ASNase from soilderived Bacillus subtilis and its anticancer activity on Panc-1 cell line which is mediated through apoptosis as evidenced from MTT assay. These results highlight the feasibility of L-ASNase derived from B. subtilis and its potential as a potent and efficient therapeutic for cancer, providing the opportunity to improve

the current commercial preparations. The findings offer a strategy for the design of new anticancer therapeutics derived from bacterial L-ASNase.

References

1. Partyka O, Pajewska M, Kwaśniewska D, Czerw A, Deptała A, Budzik M, et al. Overview of pancreatic cancer epidemiology in europe and recommendations for screening in high-risk
Son J, Lyssiotis CA, Ying H, Wang X, Hua S, Ligorio M, et al. Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. Nature. 2013;496(7443):101-5.
9. Yang W-H, Qiu Y, Stamatatos O, Janowitz T, Lukey MJ. Enhancing the efficacy of glutamine metabolism inhibitors in cancer therapy. Trends in cancer. 2021;7(8):790-804.

10. Qeshmi FI, Homaei A, Fernandes P, Javadpour S. Marine microbial L-asparaginase: biochemistry, molecular approaches and applications in tumor therapy and in food industry. Microbiological Research. 2018;208:99-112.
11. Muneer F, Siddique MH, Azeem F, Rasul I, Muzammil S, Zubair M, et al. Microbial L-asparaginase: purification, characterization and applications. Archives of Microbiology. 2020;202:967-81.
12. Fonseca MHG, da Silva Fiúza T, de Morais SB, Trevizani R. Circumventing the side effects of L-asparaginase. Biomedicine & Pharmacotherapy. 2021;139:111616.
13. Batool T, Makky EA, Jalal M, Yusoff MM. A comprehensive review on L-asparaginase and its applications. Applied biochemistry and biotechnology. 2016;178:900-23.
14. Gholami N, Ebrahimipour GH, Yaghoubi Avini M. A Study of the Optimization of Anti-Tumor L-Asparaginase Production Using Bacillus subtilis Isolated from the Soil of East Azerbaijan Province. Journal of Microbial Biology. 2022;11(44):1-12.
15. Wakil SM, Adelegan AA. Screening, production and optimization of L-asparaginase from soil bacteria isolated in Ibadan, South-western Nigeria. Journal of Basic & Applied Sciences. 2015;11:39-51.
16. Bento HB, Paiva GB, Almeida MR, Silva CG, Carvalho PJ, Tavares AP, et al. A liivibrio fischeri L-Asparaginase production by engineered Bacillus subtilis: a potential new biopharmaceutical. Bioprocess and Biosystems Engineering. 2022;45(10):1635-44.
17. Ran T, Jiao L, Wang W, Chen J, Chi H, Lu Z, et al. Structures of L-asparaginase from Bacillus licheniformis reveal an essential residue for its substrate stereoselectivity. Journal of Agricultural and Food Chemistry. 2020;69(1):223-31.
18. Arredondo-Nuñez A, Monteiro G, Flores-Fernández CN, Antenucci L, Permi P, Zavaleta AI. Characterization of a Type II L-Asparaginase from the Halotolerant Bacillus subtilis CH11. Life. 2023;13(11):2145.
19. Niu J, Meng F, Zhou Y, Zhang C, Lu Z, Lu F, et al. Non-classical secretion of a type I L-asparaginase in Bacillus subtilis. International Journal of Biological Macromolecules. 2021;180:677-83.
20. Shafqat I, Shahzad S, Yasmin A, Chaudhry MT, Ahmed S, Javed A, et al. Characterization and applications of glutaminase free L-asparaginase from indigenous Bacillus halotolerans ASN9. Plos one. 2023;18(11):e0288620.
21. Aly N, El-Ahwany A, Ataya FS, Saeed H. Bacillus sonorensis L. asparaginase: Cloning, expression in E. coli and characterization. The Protein Journal. 2020;39:717-29.
22. Moharam M, Gamal-Eldeen A, El-Sayed S. Production, immobilization and anti-tumor activity of L-asparaginase of Bacillus sp R36. J Am Sci. 2010;6(8):131-40.
23. Chakravarty N, Singh J, Singh RP. A potential type-II L-asparaginase from marine isolate Bacillus australimaris NJB19: statistical optimization, in silico analysis and structural modeling. International Journal of Biological Macromolecules. 2021;174:527-39.
24. Alrumman S, Mostafa Y, Al-Izran KA, Alfaifi M, Taha T, Elbehairi S. Production and anticancer activity of an L-asparaginase from Bacillus licheniformis isolated from the Red Sea, Saudi Arabia. Scientific reports. 2019;9(1):3756.
25. Pradhan B, Dash SK, Sahoo S. Screening and characterization of extracelluar L-asparaginase producing Bacillus subtilis strain hswx88, isolated from Taptapani hotspring of Odisha, India. Asian Pacific journal of tropical biomedicine. 2013;3(12):936-41.