Abasyn Journal of Life Sciences

Open Access

DOI: 10.34091/AJLS.4.1.2

Isolation and Identification of the Potential Novel L-asparaginase producing Bacillus Strains from Soil

Naushaba Nazli[1†], Rukhsar Masood[1†], Muhmmad Salman[1†], Bilal Nasir[1], Farah Shireen[1], Asif Mehmood[1], Sudhair Abbas Bangash[2], Farrukh Hussain[1] and Shabir Ahmad[1]*

[1]*Institute of Biological Sciences, Sarhad University of Science and Information Technology Peshawar, Pakistan*.
[2]*Department of Pharmacy, Sarhad University of Science and Information Technology Peshawar, Pakistan*
[†]These authors contributed equally to this work

Abstract

L-Asparaginase is a well know enzyme for its antineoplastic potential and is widely used to treat acute lymphoblastic leukemia and lymphosarcoma. The present work describes the isolation and characterization of novel L-asparaginase producing Bacillus strains from soil. Soil samples were collected from three different locations such as fruit garden, dairy farm and agricultural land in Peshawar Khyber Pakhtunkhwa, Pakistan. The isolates were screened to produce L-asparaginase in growth medium supplemented with 1% L-asparagine using a phenol red indicator. Among 30 bacterial isolates, only two strains initially coded as A5 and FG7 showed L-asparaginase activity. Based on biochemical and 16S rRNA sequencing analysis, the isolate A5 and FG7 were identified as *Bacillus amyloliquefaciens* and *Bacillus proteolyticus* respectively. Different factors like pH and time were optimized for maximum L-asparaginase activity. *Bacillus amyloliquefaciens* showed maximum asparaginase activity at pH 7 after 24 hours incubation at 30°C, while *Bacillus proteolyticus* showed optimum activity at pH 7 after 48 hours of incubation at 30°C. The present study first time reported the production of L-asparginase enzyme from *Bacillus amyloliquefaciens* and *Bacillus proteolyticus.*

Key Words: L-asparaginase, *Bacillusamyloliquefaciens*, *Bacillus proteolyticus*, 16sRNA.

Article Info:
Received:
May 23, 2021
Received Revised:
July 30, 2021
Accepted:
August 13, 2021
Available online:
September 11, 2021

**Corresponding Author:*
shabir.biotech@suit.edu.pk

1. INTRODUCTION

L-asparaginase is a well-known tetrameric enzyme that converts the amino acid L- asparagine to ammonia and L-aspartic acid via hydrolysis[1, 2]. The enzyme belongs to amidohydrolases family[3] and was discovered in 1922 in the serum of pig by Clementi[4]. L-asparginases, especially those isolated from microbes, are widely recognized due to their antineoplastic potential and uses in food industry[5]. It is the first enzyme that has been used in the treatment of cancer in human beings[6]. It has been actively used for 30 years to treat lymphoblastic leukemia, which mostly common in children[7, 8]. There are several advantages of this enzyme, such as, non-toxicity, biodegradability, and the ease of administration at local site[9]. Normal cell are able to synthesize L-aspargine, enough for their metabolic requirements via enzyme called asparagine synthase, however cancerous cells (malignant)lack this enzyme and they cannot synthesize L-asparagine and are therefore dependent upon the exogenous sources of L-asparaginase such as blood for growth[10]. After the administration of L-asapraginase, the concentration of the L-asapragine in plasma or serum is reduced, leading to the deprivation of malignant cells from large amount of L-asparagine important for growth[11, 12]. As the amount of L-asparagine reduces in the blood, it leads to the inhibition of DNA and RNA synthesis and ultimately results in neoplastic cell apoptosis[13]. L-asparaginase is also used in food industry as admixture for reduction of acrylamide produced by the high temperature in starchy foods and reduces the risk of cancer[12].

Till date L-asparaginase has been isolated from various sources such as microbes (bacteria, fungi and actinomycetes), animals, algae and plants[14]. L-asparaginase obtained from microbes are preferred as microbes can be easily cultured and the enzyme can be extracted and purified from them easily[15]. However, *Escherichia coli* and *Erwinia chrysanthemi* are the only two species that are used to produce L-asparaginase at industrial level [11]. Other bacterial such as *Enterobacter aerogenes, Bacillus licheniformis, Pseudomonas aeruginosa*, *Staphylococcus* sp., *Zymomonas mobilis, Thermus aquaticus* and *Thermus thermophilus* have also been reported to be the potential producers of L-asparaginase[6]. Although bacterial based L-asparaginase are highly potent and beneficial but still some reports indicate that there are several drawbacks associated with the use of L-asparaginase obtained from microbes[14]. It has been shown that bacterial based L-asparaginase cause hypersensitivity in the long-term use, leading to allergic reactions and anaphylaxis[6]. Other side effects of L-asparaginases from bacteria include fatal hyperthermia, fever, abdominal cramps and chills[12]. Therefore, it is important to look for novel and efficient L-asparaginase producing bacterial sources[10]. The search for new bacterial strains therefore could lead to novel L-asparaginases that are efficient and have fewer side effects.

Therefore, while looking at the importance of bacteria as a source of L-asparaginase, the current study was aimed to screen the L-asparaginase activity of novel bacterial strains isolated from soil samples. The novel strains were identified through biochemical and 16SrRNA analysis. Different factors like PH and time were optimized for maximum L-asparaginase activity.

2. MATERIAL AND METHODS

2.1. Sample collection and processing

Three different soil samples were collected from different sites such as dairy farm, fruits garden and agriculture land of Peshawar region, Khyber Pakhtunkhwa, Pakistan. About 1gm of each soil sample was added into 3 different test tubes each contain 9ml distilled water and serially diluted to a level of 10^{-9}. About 0.1 ml was taken out from each tube and streaked on nutrient agar media. All the plates were incubated at 30°C for 24 hrs. Growing bacterial colonies were sub-cultured until pure growth was achieved.

2.2. Screening for L-asparaginase producing isolates

A total of 30 different bacterial isolates were screened for the production of L-asparaginase using nutrient agar media. The media was supplemented with 1% L-asparagine and 0.005% phenol red indicator. Sterile nutrient agar without L-asparagine and phenol red was used as control. The isolates were seed culture in 5 ml sterile nutrient broth for 24 hour at 30°C. About 30 ul of inoculum from seed culture was added into each well in nutrient agar plate. Plates were incubated at 30°C for 24 hrs. Isolates with pink zones around

the wells were considered L-asparaginase positive strains and were selected for further morphological and molecular characterization.

2.3. Morphological and molecular identification of selected isolates

2.3.1. Morphological Studies

Initially the selected isolates were identified based on morphological studies and gram staining.

2.3.2. Molecular characterization and phylogenetic analysis of the L-asparaginase strains

Overnight grown cultures of the selected isolates were processed for isolation of genomic DNA using DNA extraction kit (Qiagen,China). The extracted DNA was sent to Macrogen, South Korea for amplification and sequencing of 16S rRNA gene. Obtained Sequencing results were modeled via Finch TV (Version 1.4.0), and Codon code aligner (Version 8.0.2) softwares. The sequence homology of bacterial nucleotide sequence was checked with available sequences from the Gene Bank database using BLASTn program. A phylogenetic tree based on partial sequences of 16S rRNA was constructed using the Maximum–Likelihood Method using Mega X software version 10.0.4(GLEN STECHER). Finally, 16S rRNA sequences were submitted to NCBI for accession number.

2.4. Purification of L-asparaginase

The isolates were cultivated in 50 ml nutrient broth at 30°C for 72 hrs. on rotary shaker at 120 rpm. To obtain cell free supernatants, the culture broth from each flask was twice centrifuge at 10,000 rpm for 10 mints. The supernatant was filtered through Whatman's filter paper No.1. The filtrate was used as crude enzyme extract for measuring the enzyme activity.

2.5. Estimation of L-asparaginase activity

The method of Imada *et al.*[16] was followed for measuring the enzyme activity. Briefly, 0.5ml of cell free supernatant was mixed with 0.5ml of 40mM L-asparagine and 0.5ml of distilled water at pH 7 and incubated for 10 mints at 30°C in water bath. After incubation, 0.5 ml of 1.5 M trichloroacetic acid was added to the assay mixture for stopping the reaction. The ammonia liberation in the supernatants was measured to determine the enzyme activity. For this, 0.2ml Nessler's reagent was added to glass tubes containing 3.75 ml distilled water and 0.1 ml supernatant. The mixture was incubated at room temperature for 10 min. After incubation the absorbance of the supernatant was measured using spectrophotometer at wavelength of 450 nm. The activity of enzyme was measured in International unit (IU/U). One international unit of L-asparaginase activity is defined as; the amount of enzyme which liberates 1 µmol of ammonia, under the assay condition at PH 7 and 30°C [15]. The assay was carried out in triplicates and the average values were noted.

2.6. Optimization of L-asparaginase activity

2.6.1. Optimization of PH and Temperature condition

The optimum pH and temperature condition for enzyme activity was determined. The enzyme activity was checked at pH 5, 7, 9 and different incubation temperature such as 30, 40 and 50^{0}C.The activity of the enzyme at each pH and temperature was noted.

2.6.2. Effect of incubation time on L-asparaginase production

Different incubation periods of 24, 48 and 72 hours were employed to study the effect on L-asparaginase production. All experiments were carried out at pH 7 and 30°C. The optimum incubation time was noted.

2.6.3. Effect of carbon and nitrogen source on L-asparaginase production

The effect of different carbon and nitrogen sources on cell growth and L-asparaginase production was investigated. Different carbon (glucose, fructose and sucrose) and nitrogen sources (ammonium oxalate, ammonium citrate and sodium nitrate) at a concentration of 1% (w/v) and 0.2% (w/v) respectively were used in culturing media at 30^{0}C.

3. RESULTS AND DISCUSSION

3.1. Selection and identification of L-asparaginase producing bacteria among selected isolates

Among randomly selected 30 isolates only two isolates (A5 and F.G7) showed L-asparaginase activity when inoculated on nutrient agar plate supplemented with L-asparagine as substrate and 1% phenol red indicator and were further subjected to morphological and molecular identification. The isolates were identified as Bacillus species. The results of morphological analysis are given in Table 1.

Table 1: Morphological characteristics of the selected strains.

S.No	Bacterial strain	Media used	Macroscopic Characteristics	Microscopic Characteristics	Bacteria
1	*Bacillus amyloliquefaciens* A5	Nutrient agar	Opaque, Mucoid, Uniform growth	Gram positive, Purple color, Rod-shaped, Appearance	*Bacillus spp.*
2	*Bacillus proteolyticus* FG7	Nutrient Agar	Opaque, Mucoid, Uniform growth	Gram positive, Purple color, Rod-shaped, Appearance	*Bacillus spp.*

3.2. Phylogenetic analysis of the strains

To further confirm the identity of selected isolates, 16S rRNA sequencing analysis was performed. The blast and phylogenetic analysis of 16S rRNA gene sequences of strain FG7 and A5 showed 99.9% sequence similarity with *Bacillus proteolyticus* and *Bacillus amyloliquefaciens* with Gene Bank Accession as MN833036 and MN833030 respectively. A phylogenetic tree was constructed based on 16S rRNA gene sequence obtained from Macrogen, South Korea. The outcomes are depicted in (Fig 1(a) and Fig 1(b)).

Bacillus species are the most important and common source of different types of important enzyme as most of the Bacillus spp. are non-pathogenic and are widely distributed[17]. Previously, the production of L-asparaginase from different Bacillus species has been reported by Kamble*et al.*[18], Audipudi*et al.*[19] and Mahmoud *et al*[4].

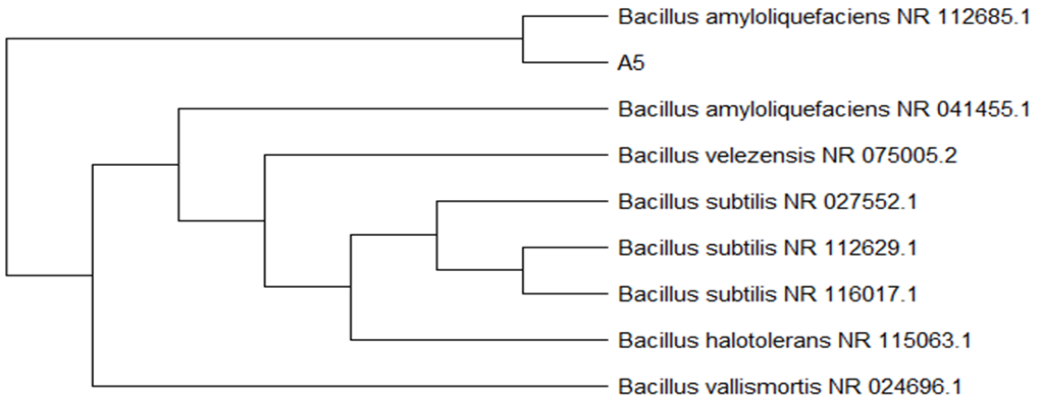

Figure 1a: Phylogenetic analysis of strain A5, showing 99.9% sequence similarity with *Bacillus amyloliquefaciens.*

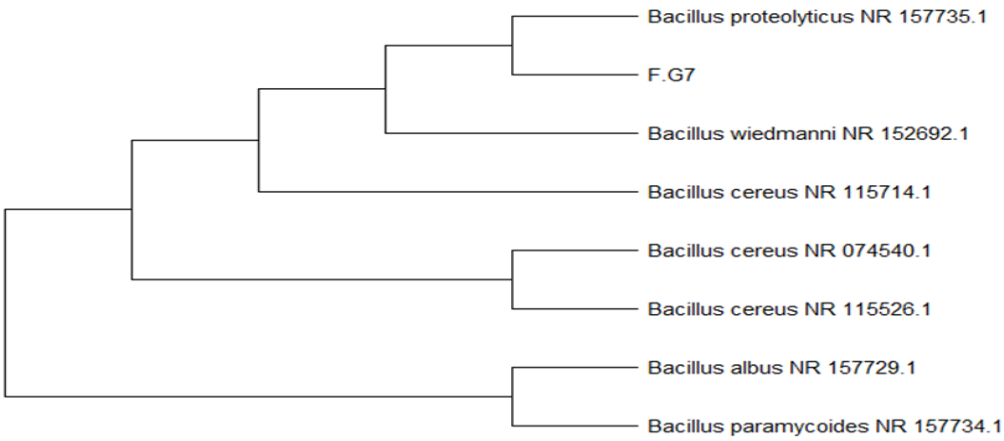

Figure2b: Phylogenetic analysis of strain FG7, showing 99.9% sequence similarity with *Bacillus proteolyticus.*

3.3. Optimum pH for L-asparaginase activity

The effect of pH on growth and enzyme production was observed by inoculating the culture of *Bacillus proteolyticus* FG7 and *Bacillus amyloliquefaciens* A5 in broth medium having different pH values i.e., 5, 7 and 9. The results showed that the maximum L-asparaginase activity was observed at pH 7 for both the strains. The L-asperginase activity of strain *Bacillus proteolyticus* FG7 at pH 7 was 2.587±0.01 U/ml, while for *Bacillus amyloliquefaciens* A5, the maximum L-asparaginase activity at pH 7 reported was 0.452±0.03 U/ml (Table 2). Our findings are supported by the previous study of Rahimzadeh *M etal*[20], where optimum activity of L-asparaginase from *Bacillus PG04* was reported at pH 7.

Table 2: L-asparaginase activity from *Bacillus amyloliquefaciens* A5 and *Bacillus proteolyticus* FG7 strains at different PH values. The values are expressed in IU/ml with standard deviation.

S. No	Culture Code	pH value	Enzyme activity (U)
1	*Bacillus amyloliquefaciens* A5	5 7 9	0.439±0.04 0.452±0.06 0.407±0.01
2	*Bacillus proteolyticus* FG7	5 7 9	2.560±0.07 2.587±0.02 2.531±0.02

Enzyme activity of L-asparaginase is expressed as Mean ±SD of Asparaginase produced SD= standard deviation

3.4. Optimum temperature for L-asparaginase activity

Optimum temperatures for L-asparaginase activity from both the strains are given in table 3. *Bacillus proteolyticus*FG7 showed optimum activity at 50°C, while L-asparaginase from *Bacillus amyloliquefaciens* A5 was maximally active at 40°C. Our results are in accordance with that of Rahimzadeh M. *et al.*[20] and Komathi *et al.*[21], where optimum temperature for L-asparaginase activities were 40 and 50°C respectively.

Table 3: Enzymatic activities of L-asparaginase at different temperatures isolated from *Bacillus amyloliquefaciens* A5 and *Bacillus proteolyticus* FG7**.**

S. No	Culture Code	Temperature (C)	Enzyme activity (U)
1	*Bacillus amyloliquefaciens* A5	30 40 50	0.253±0.03 0.577±0.06 0.451±0.03
2	*Bacillus proteolyticus* FG7	30 40 50	0.013±0.04 0.569±0.02 0.668±0.03

Enzyme activity of L-asparaginase is expressed as Mean ±SD of Asparaginase produced SD =standard deviation

3.5. Optimum incubation time for L-asparaginase production

The optimum time for production of enzyme was determined by incubating the broth culture at 30^{0}C for different time intervals such as 24, 48 and 72 hours. Spectrophotometric analysis showed that strain *Bacillus proteolyticus* FG7 showed maximum activity (2.540±0.04U/ml) after 48 hours of incubation while *Bacillus amyloliquefaciens* A5 showed maximum activity (0.422±0.01U/ml) after 24 hours of incubation (Table 4).

Our results are in agreement with the study of Hosamani and Kaliwal[22] and Tallur*iet al.*[15], who reported maximum asparaginase production after 48 and 24 hours incubation from *Fusarium equiseti* and *Myroides* species respectively. However, our findings are not in line with results obtained by Alrumman*et al.*[23], where maximum L-asparaginase activity from *Bacillus licheniformis* was reported after 72 hrs of incubation. Therefore, it is important to note that the culturing period plays an essential role in L-asparaginase productivity by bacterial strains.

Table 4: L-asparaginase activity of both the strains after different incubation time period.

S. No	Culture Code	Incubation Time	Enzyme activity (U)
1.	*Bacillus amyloliquefaciens* A5	24 hrs. 48 hrs. 72 hrs.	0.422±0.01 0.214±0.05 0.326±0.02
2.	*Bacillus proteolyticus* FG7	24hrs 48 hrs. 72 hrs.	0.532±0.02 2.540±0.04 2.527±0.01

Enzyme activity of L-asparaginase is expressed as Mean ±SD of Asparaginase produced SD= standard deviation

3.6. Effect of carbon and nitrogen sources

Results showed that all the carbon (1%w/v) have remarkable effect on L-asparaginase activity. However, compared to other carbon sources the highest L-asparaginase activity was observed when glucose was used as a carbon source (Table 5). Deshpande N[7]*et al* also reported maximum L-asparaginase production using glucose as carbon source.

Varied L-asparaginase activities were observed with different nitrogen sources tested. Amongst different nitrogen sources used, Sodium nitrate favored maximum enzyme production by both the strains (Table 6).

Table 5: Effect of different carbon sources on the production of L-asparaginase from *Bacillus amyloliquefaciens* A5 and *Bacillus proteolyticus* FG7 strains.

S. No	Strain code	Carbon sources	Enzyme activity (U)
1	*Bacillus amyloliquefaciens* A5	Glucose Fructose Sucrose	1.091±0.01 0.988±0.02 0.717±0.25
2	*Bacillus proteolyticus* FG7	Glucose Fructose Sucrose	2.926±0.12 2.715±0.01 2.122±0.05

Enzyme activity of L-asparaginase is expressed as Mean ±SD of Asparaginase produced

Table 6: Effect of different carbon sources on the production of L-asparaginase from *Bacillus amyloliquefaciens* A5 *and Bacillus proteolyticus* FG7 strains.

S. No	Strain code	Nitrogen sources	Enzyme activity (U)
1	*Bacillus amyloliquefaciens* A5	Ammonium oxalate Ammonium citrate Sodium nitrate	-0.301±0.15 -0.147±0.15 1.633±0.01
2	*Bacillus proteolyticus FG7*	Ammonium oxalate Ammonium citrate Sodium nitrate	-0.551±0.26 -0.301±0.02 0.121±0.01

Enzyme activity of L-asparaginase is expressed as Mean ±SD of Asparaginase produced.

4. CONCLUSION

The current study for the first time reported two L-asparaginase producing *bacillus* strains from soil. It was observed that the L-asparaginase obtained from *Bacillus proteolyticus* FG7 showed maximum activity at pH 7 at 50 °C, while the optimum pH and temperature reported for L-asparaginase from *Bacillus amyloliquefaciens* A5 were pH 7 and 40°C respectively. Other parameters such as incubation period, carbon and nitrogen sources have shown remarkable effect on the production of L-asparaginase. However, further studies are required to characterize the L-asparaginase enzyme from these two novel strains at molecular level. The enzyme from these strains might be used for pharmaceutical and commercial purposes in the future.

ACKNOWLEDGMENT

The authors are thankful to the Institute of Biological Sciences, Sarhad University, Peshawar for financial assistance

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Badoei-Dalfard A. L-asparaginase production in the strain JHS-71 isolated from Jooshan Hot-spring. Molecular Biology Research Communications. 2016; 5(1): 1-10.
2. Shukla S, Mandal SK. Production optimization of extracellular L-asparaginase through solid-state fermentation by isolated *Bacillus subtilis*. International Journal of Applied Biology and Pharmaceutical Technology.2013; 4(1): 219-226.
3. Tomar RS, Sharma N, Kaushik S. Isolation and characterization of L-asparaginase extracellular enzyme producing bacteria from industrial soil samples. International Journal of Pharmaceutical Sciences and Research.2019; 10(11): 4937-4941.

4. Nour El-Dein M, Mousa M, Metwally N. Isolation and survey of L-asparaginase producing bacteria from soil. Journal of Egyptian Academic Society for Environmental Development. D, Environmental Studies.2019; 20(1): 19-29.
5. Shukla D, Shrivastav VK, Jana AM, Shrivastav A. Exploration of the potential L-asparaginase producing bacteria from the soil of Gwalior (India). International Journal of Current Microbiology and Applied Sciences. 2014; 3(5): 665-672.
6. El-Sabbagh SM, El-Batanony NH, Salem TA. L-Asparaginase produced by *Streptomyces* strain isolated from Egyptian soil: Purification, characterization and evaluation of its anti-tumor. African Journal of Microbiology Research.2013; 7(50): 5677-5686.
7. Deshpande N,Choubey P, Agashe M. Studies on optimization of growth parameters for L-asparaginase production by *Streptomyces ginsengisoli*. The Scientific World Journal. 2014.
8. Ashok A, Doriya K, Rao JV, Qureshi A, Tiwari AK, Kumar DS. Microbes Producing L-asparaginase free of glutaminase and urease isolated from extreme locations of Antarctic soil and moss. Scientific reports. 2019; 9(1):1-10.
9. Sherifah M, Wakil, Adesewa,Adelegan A. Screening, Production and optimization of L-Asparaginase from soil bacteria isolated in Ibadan, South-western Nigeria. Journal of Basic and Applied Sciences.2015;11: 39-51
10. Bhat R, Nair JS, Marar T. Isolation and identification of L-asparaginase producing *Salinicoccus sp*. M KJ997975 from soil microbial flora. International Journal of Pharmaceutical Sciences and Research. 2015; 6 (8): 3599-3605.
11. Pourmolaei S, Asghar Tobi S. Isolation and Identification of L-Asparaginase producing bacteria and From Forest Soil of Western Mazandaran. International Journal of Molecular and Clinical Microbiology. 2020; 10(2): 1386-1391.
12. Hatamzadeh S, Rahnama K, Nasrollahnejad S, Fotouhifar KB, Hemmati K, White JF, Taliei F. Isolation and identification of L-asparaginase producing endophytic fungi from the Asteraceae family plant species of Iran. Peer J. 2020; 8: e8309.
13. Piatkowska-Jakubas B, Krawczyk‐Kulis M, Giebel S, Adamczyk‐Cioch M, Czyz A, Lech-Maranda E, Paluszewska M, Pałynyczko G, Piszcz J, Holowiecki J. Use of L‐asparaginase in acute lymphoblastic leukemia: recommendations of the Polish Adult Leukemia Group. PolskieArchiwumMedycynyWewnetrznejPolish Archives of Internal Medicine. 2008; 118(11):664-668.
14. Ameen F, Alshehri WA, Al-Enazi NM, Almansob A. L-Asparaginase activity analysis, ansZ gene identification and anticancer activity of a new *Bacillus subtilis* isolated from sponges of the Red Sea. Bioscience, Biotechnology and Biochemistry. 2020; 84(12): 2576-2584.
15. Prasad Talluri VS SL, Bhavana M, Rajagopa SV. Isolation and screening of L-asparaginase producing bacteria from Visakhapatnam soil samples. International journal of pharmaceutical, chemical and biological sciences. 2013; 3(4): 1121-1125.
16. Imada A, Igarasi S, Nakahama K, Isono M. Asparaginase and glutaminase activities of micro-organisms. Microbiology.1973; 76(1): 85-99.
17. Danilova I, Sharipova M. The practical potential of *Bacilli* and their enzymes for industrial production. Frontiers in Microbiology.2020; 11.
18. Kamble KD, Bidwe PR, Muley VY, Kamble LH, Bhadange DG, Musaddiq M. Characterization of L-asparaginase producing bacteria from water, farm and saline soil. Bioscience discovery. 2012; 3(1): 116-119.
19. Audipudi AV, Pallavi R, Supriya, G. Characterization of L-asparaginase producing bacteria from mangrove soil. International Journal of ChemTech Research.2013; 5 (1): 109-112.
20. Rahimzadeh M, Poodat M, Javadpour S, Qeshmi FI, Shamsipour F. Purification, characterization and comparison between two new L-asparaginases from *Bacillus* PG03 and *Bacillus* PG04. The open biochemistry journal.2016; 10: 35-45.
21. Komathi S, Rajalakshmi G, Savetha S. Balaji, S. Rajalakshmi G. Isolation, production and partial purification of l-asparaginase from *Pseudomonas aeruginosa* by solid state fermentation.Sch. Acad. J. Pharm., 2013; 2(2):55-59

22. Hosamani R, Kaliwal BB. L-asparaginase an anti-tumor agent production by *Fusarium equiseti* using solid state fermentation. International journal of drug discovery. 2011; 3(2): 88-99.
23. Alrumman SA, Mostafa YS, Al-Izran KA, Alfaifi MY, Taha TH, Elbehairi SE. Production and anticancer activity of an L-asparaginase from *Bacillus licheniformis* isolated from the Red Sea, Saudi Arabia. Scientific reports. 2019; 9(1): 1-14.