



Comparative Evaluation of the Growth Parameters for Enhanced Extracellular L-Asparaginase Production by Locally Isolated *Rossellomorea marisflavi* Strain

Iqra Javed, Beenish Maqsood* and Muhammad Khurshid*

School of Biochemistry and Biotechnology, University of the Punjab, Lahore, 54590, Pakistan.

Iqra Javed and Beenish Maqsood first authorship.

ABSTRACT

L-asparaginase (EC 3.5.1.1) has gained prominence as an industrial enzyme in the recent decades. It is notable for its use as an anticancer drug and an acrylamide mitigator in the food industry. The screening of L-asparaginase from new microbial sources, with greater yield and fewer drawbacks is greatly appreciated. The present work attempts to screen and identify L-asparaginase producing bacteria from soil, as well as to optimize the bioprocess parameters for higher yield of extracellular L-asparaginase. For this purpose, fifteen isolates were screened by rapid plate assay. The two microbial colonies representing widest pink zone around them were identified as *Rossellomorea marisflavi* and *Bacillus cereus*, based on 16S ribosomal RNA gene sequencing results. *B. cereus* and *R. marisflavi* produced maximum L-asparaginase at neutral pH and pH 8 respectively, when cultured for 36 h using 1.5% inoculum size. For optimal L-asparaginase production, *R. marisflavi* required 40°C while *B. cereus* preferred 37°C temperature. Best liquid media was Czapek 2 for *R. marisflavi* that produced 583 U/mg enzyme and TSB for *B. cereus* that gave 250 U/mg L-asparaginase; while Corn cob as solid substrate facilitated L-asparaginase production maximally by both strains. The supplementation of metal ions altered L-asparaginase production differently. However, addition of Mg²⁺ under optimized fermentation conditions magnified L-asparaginase production to 882.35 U/mg by *R. marisflavi* and to 414.47 U/mg by *B. cereus*. Therefore, this study served to provide two isolates from Bacillaceae family that can utilize inexpensive substrates and Mg²⁺ to give enhanced L-asparaginase production under optimized fermentation conditions for future commercial applications.

Article Information

Received 04 August 2022

Revised 20 May 2023

Accepted 19 June 2023

Available online 09 September 2023
(early access)

Authors' Contribution

BM and MK conceived the study and drafted the manuscript. IJ and BM performed the experimental work. IJ, BM and MK analyzed the data.

Key words

Extracellular L-asparaginase, Bacillaceae family, Ribotyping, Optimization, Fermentation

INTRODUCTION

L-asparaginase (EC 3.5.1.1) is an amidohydrolase enzyme that catalyses the removal of the amino group from L-asparagine, resulting in the formation of L-aspartic acid and ammonia (Hill *et al.*, 1967). This enzyme in addition to being a cancer-fighting agent (Müller and Boos, 1998; Narta *et al.*, 2007) inhibits acrylamide formation

in processed foods as well (Gökmen and Palazogulu, 2008; Mottram *et al.*, 2002).

L-asparaginase is abundant in nature and can be found in microorganisms, higher plants (*Sphagnum fallax*), and animals (Borek *et al.*, 1999; Sinha *et al.*, 2013). It is an oncolytic enzyme which is used to treat acute myelocytic leukemia, chronic lymphocytic leukemia, Hodgkin's disease, melanomas and Lymphosarcomas (Kidd, 1953; Broome, 1961) with or without combination of drugs (Szymanska *et al.*, 2012). Even complete remission of acute lymphoblastic leukemia (ALL) has been reported following L-asparaginase therapy (Ashok *et al.*, 2019; Brumano *et al.*, 2019). This therapeutic protein catalyzes the breakdown of circulating L-asparagine in the blood stream (Kumar and Verma, 2012) and deprives the tumor cells of an essential nutrient and growth factor. Hence, RNA and protein synthesis stops and cell cycle arrests in G1 phase, leading to apoptosis of the tumor cells (Ali *et al.*, 2016; Rizzari *et al.*, 2000). Normal cells, on the other

* Corresponding author: beenish.ibb@pu.edu.pk, khurshid.ibb@pu.edu.pk
0030-9923/2023/0001-0001 \$ 9.00/0



Copyright 2023 by the authors. Licensee Zoological Society of Pakistan.

This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

hand survive due to the presence of the L-asparagine synthetase enzyme that resynthesizes L-asparagine from L-aspartic acid and L-glutamine (Zhang *et al.*, 2014). Some L-asparaginases have additional L-glutaminase activity and can catalyze L-glutamine as well (Chan *et al.*, 2014). However, the presence of this side activity has been reported to cause side effects in patients, such as bleeding problems, neurological disorders, hepatic dysfunction and pancreatic inflammation, among others, thereby leading to the discontinuation of L-asparaginase treatment (Stams *et al.*, 2003).

The ability of L-asparaginase to reduce acrylamide levels make it useful in the food industry. Acrylamide is a neurotoxin and a suspected carcinogen that is produced at higher temperatures as a result of the Maillard reaction between L-asparagine and reducing sugars, particularly in potato and cereal-based fried food products (Gökmen Palazuglu, 2008; Mottram *et al.*, 2002). L-asparaginases from different microbial sources have been used industrially to mitigate acrylamide levels in several food products efficiently (Cachumba *et al.*, 2016). L-asparaginases have recently been used in the development of biosensors that detect L-asparagine levels in food or in the blood of leukemic patients. Either whole cells of L-asparaginase producing microorganisms like *E. coli*, yeast, *Erwinia* sp. or the purified enzyme itself is co-immobilized with an indicator (phenol red) in an inert support (Tetra methyl ortho silicate sol-gel) at the tip of an electrode. When L-asparagine comes in its vicinity, it is degraded into ammonia and L-aspartate causing change in pH that alters the colour of the indicator to pinkish red. This initiates an electrical signal which is displayed on the screen showing the amount of L-asparagine in the samples (Verma *et al.*, 2012).

The isolation of intracellular and extracellular L-asparaginases has been reported from diverse sources and species showing tremendous variation in their properties (Sinha *et al.*, 2013). Bacterial L-asparaginases that are produced extracellularly are of greater choice amongst several other sources as they can be synthesized in bulk amount without much difficulty, lesser time and with easier recovery of the product at the end (Patro *et al.*, 2011; Amena *et al.*, 2010). To date, *Escherichia coli* and *Erwinia chrysanthemi* L-asparaginases have only been clinically approved to treat lymphoid system malignancies but they also possess side effects like shorter half-life, immunogenic reactions, lesser stability and short half-life etc. (Batool *et al.*, 2016). Therefore, to screen microbes with improved L-asparaginase properties, greater yield and minimal side effects are desirable. Large-scale production of L-asparaginases is typically accomplished through submerged and/or solid state fermentation, as

well as by varying physical and chemical parameters and then selecting the ones that result in increased production (Lopes *et al.*, 2017). Keeping in mind various biotechnological prospects of L-asparaginases, the current study presents screening and identification of extracellular L-asparaginase producing bacteria from local soil samples, along with the selection of most suitable physical and chemical parameters that could improve overall L-asparaginase yield.

MATERIALS AND METHODS

Analytical grade chemicals, purchased from local distributors of Thermo Fisher Scientific Inc. (Maryland, USA), Fluka™ and Sigma (Sigma-Aldrich Co. USA), were used in the present research. All steps involving the handling of bacterial isolates/cultures were performed aseptically.

Sample collection and pretreatment

Soil samples were collected separately in clean, zip-lock bags from five different garden plots around cafeterias in University of the Punjab (Quaid-e-Azam Campus), after digging to a depth of 15 cm with a sterile knife. The soil samples were spread evenly in trays and incubated at 37°C after physically eliminating unwanted stones, dried leaves, grass, branches etc. Dried and sieved soil (5 gm) was stirred in 50 ml of sterile distilled water and was incubated for 2 h in a shaker, adjusted to 37°C.

Isolation and screening of L-asparaginase producers

LB agar plates were maintained at 37°C overnight after streaking filtered, serially diluted supernatants. The rapid plate assay (Gulati *et al.*, 1997) for 15 randomly selected single colonies was performed, to screen extracellular L-asparaginase producing bacteria. The diameter of the pink zone that appeared around bacterial colonies after streaking them separately on M9 medium-agar plates was used to select best producers. (For 1 liter M9 medium (agar), pH 7.0: Na₂HPO₄·2H₂O 6g; KH₂PO₄ 3g; NaCl 0.5g; L-asparagine 10g; 20% glucose stock 10ml; 1molL⁻¹ MgSO₄·7H₂O 2ml; 0.1M solution of CaCl₂·2H₂O 1ml; 2 % agar and 3ml of 2.5% phenol red dye). Streaked plates were incubated overnight at 37°C.

Production of L-asparaginase

The quantification of extracellular L-asparaginase of selected microbial colonies was carried out. Freshly grown single colony of the bacterial strain/s from LB agar plate was inoculated in 10 ml Luria-Bertani (LB) broth (pH 7.0) and was incubated at 37 °C in a shaker. Then 1% of this inoculum was added to a 30 ml sterilized tryptic

soy broth (TSB) media (pH 7.0) in 250 ml conical flask and was cultured for 24 h in an orbital shaker (100 rpm) at 30°C. Following centrifugation (13000 rpm) at room temperature, the supernatant was saved as crude enzyme extract/s.

L-asparaginase assay

The crude enzyme supernatants were assayed for L-asparaginase activity by Nesslerization method (Campbell Mashbum, 1969). The absorbance was recorded at 425 nm after mixing the contents thoroughly. The enzyme activity was quantified using ammonium sulphate standard graph. One L-asparaginase unit was equivalent to the amount of enzyme liberating 1µmole of ammonia per minute at given temperature.

Protein assay

The total protein content in crude enzyme supernatants was estimated by Bradford assay (Bradford, 1976) at 595 nm. The amount of protein in the samples was quantified in µg/ml by comparing the absorbance values of standards in BSA standard graph.

Molecular identification of bacterial isolates

Ribotyping technique (Bouchet *et al.*, 2008) was used to identify the selected bacterial stains. The whole genomic DNA was isolated using kit (K0721; Thermo Scientific™). PCR was run to amplify 16S ribosomal RNA gene. Universal primers given below of 10 µM stock, template (1 µg whole genomic DNA) and DreamTaq green mix (K1081; Thermo Scientific™) were combined and placed on ice. 8F: 5' AGA GTT TGA TCC TGG CTC AG 3'; 1492R: 5' GGT TAC CTT GTT ACG ACT T 3'.

In Bio-Rad Thermocycler, initial denaturation at 95°C for 4 min followed by 30 amplification cycles at 95°C for 30 sec; 52°C for 30 sec; 72°C for 90 sec and then final extension at 72°C for 10 min was performed. The amplified gene product was resolved on 1% agarose gel along with DNA markers (SM0313; Thermo Scientific™). Bands exhibiting the desired size were excised and extracted from the gel using kit (K0692; Thermo Scientific™). The nucleotide sequence retrieved after sequencing was subjected to BLAST and the homology-based corresponding 16S rRNA sequences of different strains were aligned using Clustal W (online program) (Thompson *et al.*, 1994). The phylogenetic tree was designed using neighbor-joining method through MegAlign software (Clewley and Arnold, 1997).

Optimization of fermentation conditions

To achieve enhanced L-asparaginase production from selected bacterial isolates, fermentation process

was optimized by altering single parameter at a time. The enzyme activity and protein content in all crude enzyme extracts was assayed.

pH

The change in L-asparaginase production at different pH conditions was studied. 1% inoculum of the selected bacterial strain/s was added to 10 ml TSB media with different pH (4.0-10) and left for 24 h at 30°C in an orbital shaker. The cells were removed by centrifugation at 13,000 rpm at room temperature and the supernatant was saved as crude enzyme extract.

Temperature

L-asparaginase production by microbes under various temperatures was observed by adding 1% inoculum of the selected bacterial strains to 10 ml TSB media of optimal pH. The cultures were maintained at 30°C, 37°C, 40°C and 50°C temperatures separately for 24 h, with shaking. Centrifugation at 13000 rpm (room temperature) removed the cells from grown culture and the supernatant was saved as crude enzyme extract.

Incubation time

The impact of incubation time on extracellular L-asparaginase production was observed by adding 1% inoculum of selected bacterial strains to 10 ml TSB media of optimal pH for 12, 24, 36, 48, 60 and 72 h, respectively under optimal temperature conditions with shaking. The cells were removed by centrifugation at 13,000 rpm at room temperature and the supernatant was stocked as crude enzyme extract.

Inoculum size

Selected bacterial strains were cultured under optimal physical conditions to record changes in L-asparaginase production after adding different volumes of freshly grown inoculum (0.5, 1.0, 1.5, 2.0 and 2.5 %) to 10 ml TSB media. On completion of incubation, the cells were spinned down (13,000 rpm; room temperature) and the supernatant was saved as crude enzyme extract.

Growth media

Alteration in extracellular L-asparaginase concentration was studied for selected bacterial isolates by using 10 ml of different growth media (liquid and solid) under optimized conditions. The liquid media included Luria-Bertani broth (LB), tryptic soy broth (TSB), M9 media, L-asparagine broth media (ABM), modified Czapek-Dox's medium 1 and modified Czapek-Dox's medium 2. After completion of submerged fermentation process, the cells were removed at room temperature by centrifugation at 13,000 rpm and the supernatant was

saved as crude enzyme extract. For solid substrates corn cob, almond seed cake, coconut seed cake, sugar cane bagasse and wheat bran grounded to 1mm particle size (10 gm) and moistened with 40 ml L-asparagine broth medium, were tested. The enzyme was extracted in 0.1 M Tris HCl buffer (optimal pH) by shaking at 37°C for two h. Following filtration and centrifugation (13,000 rpm), the supernatant obtained was saved as crude enzyme extract.

Metal ions effect

To study the effect of metal ions on L-asparaginase production, 10 ml of optimal growth media were separately topped up with divalent ions like Zn²⁺, Mg²⁺, Fe²⁺, Ni²⁺, Co²⁺, Cu²⁺, Mn²⁺ and Ca²⁺ (1mM final concentration) and 5 mM EDTA. After completion of incubation under optimal fermentation parameters, the cells were excluded by centrifugation (13000 rpm; room temperature) and the supernatant was stored as crude enzyme extract.

RESULTS

Characteristics of isolated L-asparaginase producers

Fifteen randomly selected colonies were subjected to rapid plate assay. On the basis of the diameter of pink zone formed around the colonies, 9 bacterial isolates did not produce sufficient extracellular L-asparaginase, 4 bacterial isolates produced it moderately while two strains (Strain a and Strain b) were selected, as the pink zones around their colonies displayed 2.7 cm and 3.3 cm diameters (Fig. 1). The Strain_a and Strain_b were cultured and the clear supernatant after centrifugation gave specific activity of 125 Units/mg for Strain_a and 191 Units/mg for Strain b.

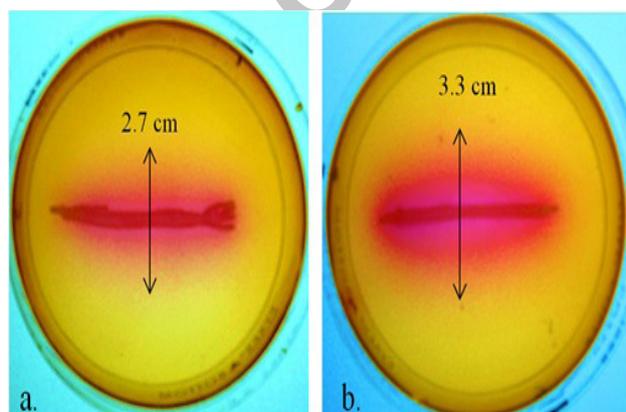


Fig. 1. Selected L-asparaginase producers on M9 agar plates. a, Strain_a showing 2.7 cm pink zone. b, Strain_b showing 3.3 cm pink zone.

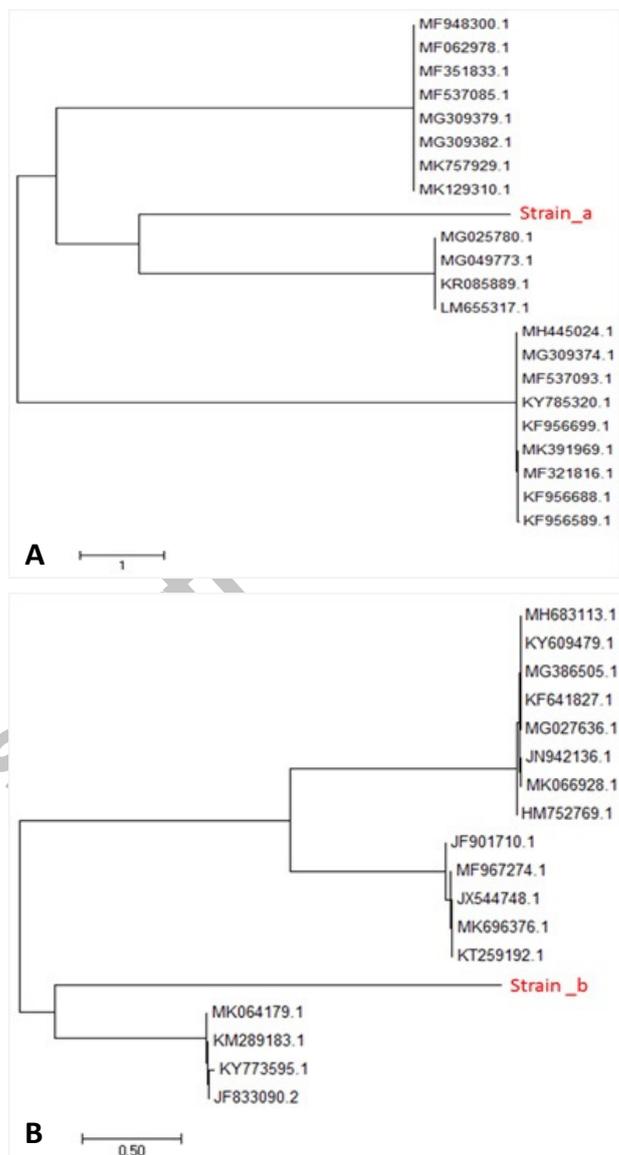


Fig. 2. Phylogenetic tree analysis. A. For Strain_a a phylogenetic tree was constructed through neighborjoining method using following closely related strains: MF948300.1 (*Bacillus* sp.), MF062978.1 (*Bacillus marisflavi*), MF351833.1 (*Bacillus marisflavi*), MF537093.1 (*Bacillus* sp.), MG309379.1 (*Bacillus* sp.), MG309382.1 (*Bacillus* sp.), MK757929.1 (*Bacillus* sp.), MK129310.1 (*Bacillus marisflavi*), MG025780.1 (*Bacillus marisflavi*), MG049773.1 (*Bacillus marisflavi*), KR085889.1 (*Bacillus marisflavi*), LM655317.1 (*Bacillus* sp.), MH445024.1 (Uncultured bacterium clone JSP_105), MH445024.1 (*Bacillus* sp.), MF537085.1 (*Bacillus* sp.), KY785320.1 (*Bacillus aquimaris*), KF956699.1 (*Bacillus* sp.), MK391969.1 (*Bacillus marisflavi*), MF321816.1 (*Bacillus marisflavi*), KF956688.1 (*Bacillus* sp.) and KF956589.1 (*Bacillus* sp.) Strain_a showed closest homology to

MG025780.1 strain and was identified as *Bacillus marisflavi*, preferably written as *Rossellomorea marisflavi*. B. For strain_b a phylogenetic tree was constructed through neighborjoining method using following closely related strains: MH683113.1 (*Bacillus* sp.), KY609479.1 (Uncultured bacterium clone), MG386505.1 (*Bacillus cereus*), KF641827.1 (*Bacillus cereus*), MG027636.1 (*Bacillus cereus*), JN942136.1 (*Bacillus* sp.), MK064179.1 (*Bacillus cereus*), HM752769.1 (*Bacillus cereus*), JF901710.1 (*Bacillus* sp.), MF967274.1 (*Bacillus* sp.), JX544748.1 (*Bacillus cereus*), MK696376.1 (*Bacillus* sp.), KT259192.1 (*Bacillus cereus*), MK064179.1 (*Bacillus cereus*), KM289183.1 (*Bacillus* Sp.), KY773595.1 (*Bacillus cereus*) and JF833090.2 (*Bacillus cereus*). Strain_b showed closest homology to MK064179.1 strain and was identified as *Bacillus cereus*.

Molecular identification and phylogenetic analysis

The 16S rRNA gene of 1500 base pairs (bp) size, was successfully amplified by using PCR technique, from both selected strains (Fig. 2). Its sequence was subjected to BLAST and the nucleotide sequences showing greatest homology to the amplified gene were aligned using ClustalW software. A phylogenetic tree was designed by using neighbor-joining method through MegAlign software. Strain_a was 98.16% similar to *Bacillus marisflavi* strain LQ1 (GenBank: MG025780.1) (Fig. 2A), preferably written as *Rossellomorea marisflavi* (Gupta *et al.*, 2020) while Strain_b showed 99% similarity to *Bacillus cereus* strain R2 (GenBank: MK064179.1) (Fig. 2B). The 16S rRNA nucleotide sequences have been received by GenBank and given accession numbers: SUB11884202 Strain_a OP117229 and SUB11884202 Strain_b OP117230, respectively.

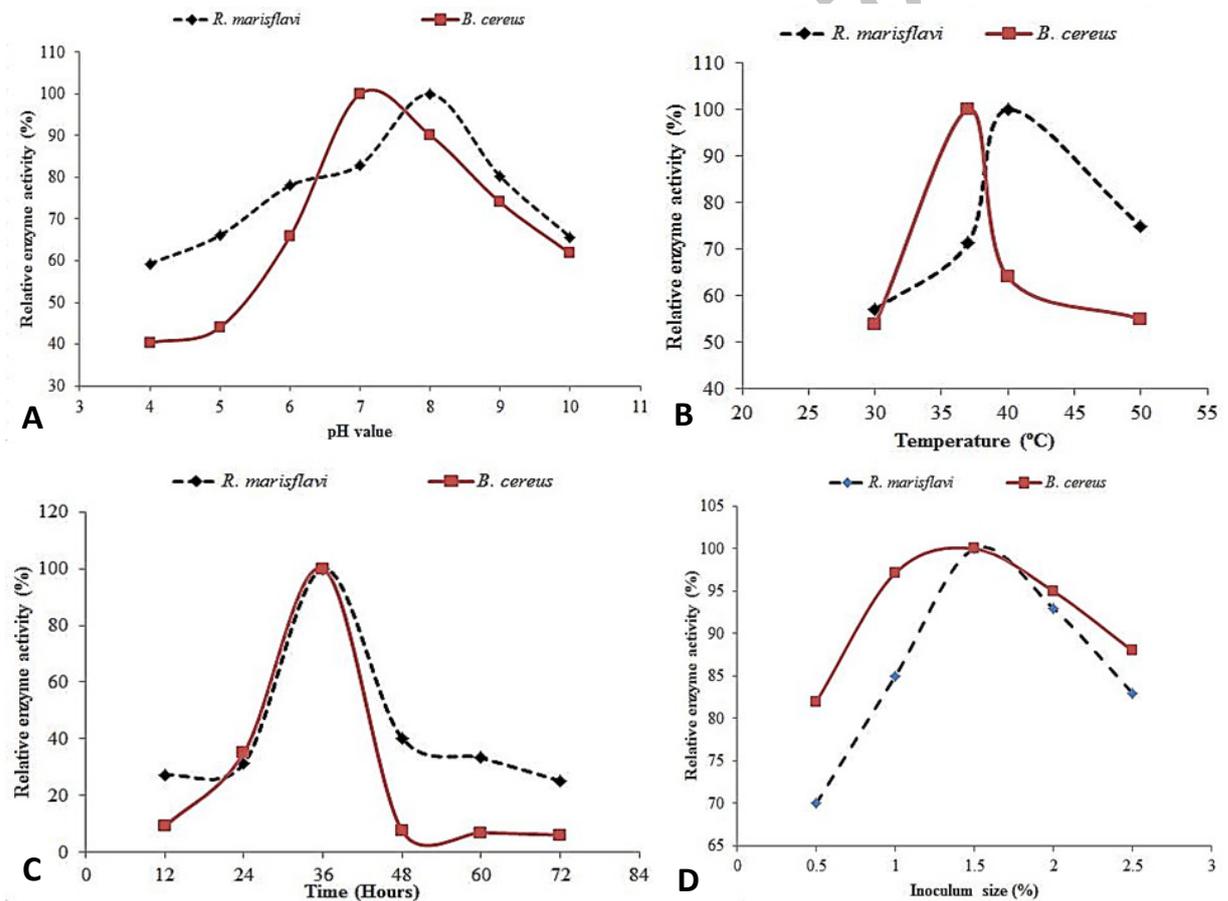


Fig. 3. L-asparaginase production. **A**, At different pH conditions. L-asparaginase production by *Bacillus cereus* and *Rossellomorea marisflavi* was observed to be optimal at neutral pH and pH 8 respectively under suitable temperature conditions. **B**, At different temperatures (°C). *R. marisflavi* optimally produced L-asparaginase at 40°C while *B. cereus* produced it maximally at 37°C under optimal pH conditions. **C**, Under different incubation times (h). L-asparaginase activity was highest in both strains on the completion of 36 h, under optimal temperature and pH conditions. **D**, Using different inoculum sizes (%). L-asparaginase production was greatest with 1.5% inoculum size on the completion of 36 h, under optimal by both strains.

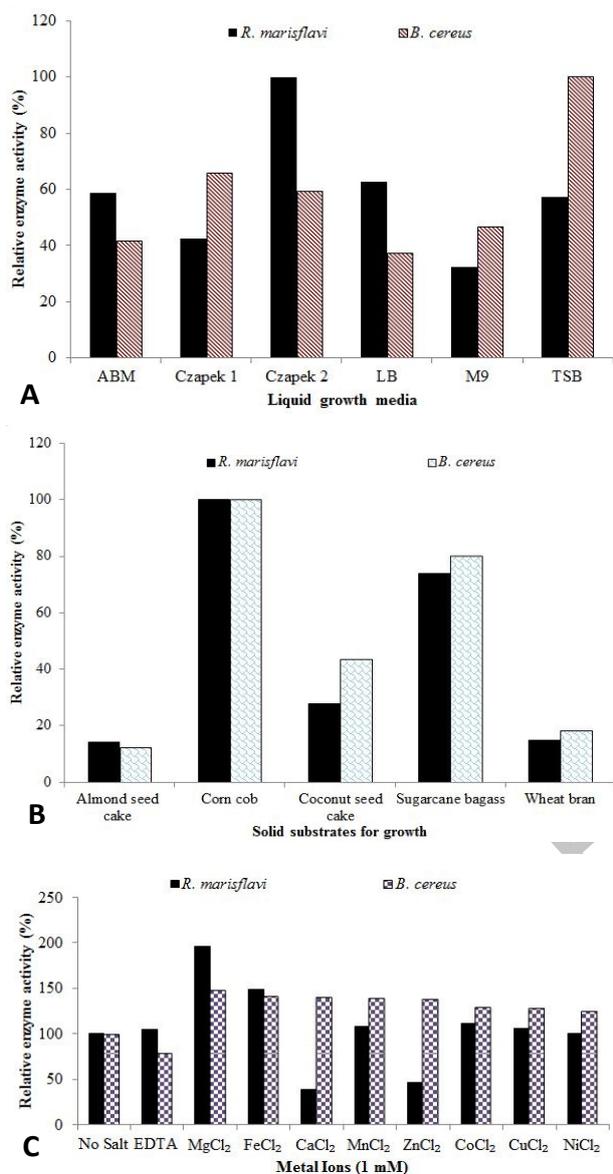


Fig. 4. Change in L-asparaginase production in the presence of different liquid growth media (A), solid substrate (B) and metal ions (C). A, *R. marisflavi* maximally produced L-asparaginase when grown in modified Czapek-Dox's medium 2, while TSB proved to be the most suitable media for L-asparaginase production by *B. cereus*. Experiment was performed under optimal conditions. B, strains gave highest L-asparaginase activity when grown on corn cob under optimal conditions. C, the effect of metal ions (0.1 mM final concentration) and EDTA (5 mM final concentration) on L-asparaginase production by both strains showed variable results. Mg²⁺ was the best L-asparaginase inducer that enhanced the activity to 195.8% (882.35 U/mg) in *R. marisflavi* and to 148.02% (414.47 U/mg) in *B. cereus* in contrast to No-salt control (100% L-asparaginase activity).

Optimum fermentation conditions

pH

The effect of pH (4.0-10) on L-asparaginase production by both *R. marisflavi* and *B. cereus* was observed (Fig. 3A). The results suggested that *R. marisflavi* seemed to give greater specific activity of L-asparaginase over a wide range of pH (6.0-10) as compared to *B. cereus*, giving maximum value of 406.7 U/mg compared to 214.3 U/mg L-asparaginase produced by *B. cereus*.

Temperature

The change in extracellular L-asparaginase production by both strains was observed at different temperatures. An increase in temperature from 30°C also increased the enzyme production that started to decline after 40°C (Fig. 3B). However, *R. marisflavi* L-asparaginase seemed to be more heat tolerant in contrast to *B. cereus* L-asparaginase.

Incubation time

The L-asparaginase synthesis by both strains spiked after 24 h. It was greatest on the completion of 36 h under optimal temperature and pH conditions (Fig. 3C). *R. marisflavi* produced 444.4 U/mg whereas *B. cereus* gave 244 U/mg L-asparaginase concentration in crude extract. However, after 36 h the enzyme relative activity started to reduce. It was decreased from 40.2% to 25.2% in *R. marisflavi* between 48 to 72 h and in case of *B. cereus*, only 6.91% L-asparaginase relative activity was recorded after 72 h.

Inoculum size

Freshly prepared inoculum (0.5-2.5%) was added to 10 ml TSB media and cultivated under optimal physical conditions to monitor changes in L-asparaginase production. With an increase in inoculum size, extracellular L-asparaginase production was enhanced by both strains. Addition of 1.5 % inoculum proved to be optimal for highest L-asparaginase synthesis by both *R. marisflavi* and *B. cereus* strains, giving 446.67 U/mg and 279.17 U/mg L-asparaginase specific activities, respectively (Fig. 3D). Interestingly, *B. cereus* produced significant amount of L-asparaginase i.e., ~87% even at lowest inoculum size (0.5%) in contrast to 52.49% L-asparaginase that was produced by *R. marisflavi* under same conditions.

Growth media

L-asparaginase production by both selected strains was analyzed in liquid media as well as with solid substrates. The results showed that *R. marisflavi* maximally produced L-asparaginase when it was grown in modified Czapek-Dox's medium 2 giving 583 U/mg enzyme, respectively. On the other hand, TSB was the best media for L-asparaginase

production by *B. cereus*, giving 250 U/mg L-asparaginase (Fig. 3). Interestingly, both *B. cereus* and *R. marisflavi* gave highest L-asparaginase activity when cultured on corn cob under optimal fermentation conditions producing 138 U/mg and 121.87 U/mg L-asparaginase in crude extract (Fig. 4A). Comparatively, overall production of extracellular L-asparaginase using solid substrates by both strains was relatively lower as compared to the liquid growth media. Hence, Czapek-Dox's medium 2 for *R. marisflavi* and TSB medium for *B. cereus* were selected for cultivation using submerged fermentation.

Effect of metal ions

The effect of metal ions (0.1 mM final concentration) and EDTA (5 mM final concentration) on L-asparaginase production by both strains showed variable results under optimal physical and chemical conditions (Fig. 4B). The change in L-asparaginase production was monitored by taking "No-salt" crude enzyme extract as control (100 % L-asparaginase activity). In case of *R. marisflavi*, EDTA slightly enhanced the L-asparaginase activity to 104% while it was inhibited to 78.9% in *B. cereus*. *R. marisflavi*'s L-asparaginase activity remained unchanged on the addition of Ni^{+2} but it was inhibited in the presence of Zn^{2+} and Ca^{+2} to 46.2% and 38.83% while Fe^{+2} , Co^{+2} , Cu^{+2} and Mn^{+2} increased the enzyme activity. Mg^{2+} was the best L-asparaginase inducer, spiking the activity to 195.8% (882.35 U/mg) in *R. marisflavi* and to 148.02% (414.47 U/mg) in *B. cereus*. The L-asparaginase synthesis by *B. cereus* was not inhibited by metal ions rather Fe^{+2} , Ni^{+2} , Co^{+2} , Cu^{+2} , Mn^{+2} , Zn^{2+} and Ca^{+2} acted as L-asparaginase activity boosters.

DISCUSSION

In an ambition to isolate extracellular L-asparaginase producing bacteria from soil and to gain greatest L-asparaginase production from them, current study was undertaken. Microbial colonies were randomly chosen after rapid plate assay technique which is being practiced even today (Gulati *et al.*, 1997), owing to its usefulness (Nour *et al.*, 2019; Fatima *et al.*, 2019). The two microbial isolates, Strain_a and Strain_b with largest pink zones were identified as *Rossellomorea marisflavi* and *Bacillus cereus* by ribotyping (Bouchet *et al.*, 2008). Similar strategy for the screening and identification of L-asparaginase producing *Bacillus* strains from soil have been previously practiced (Wakil *et al.*, 2015). Earlier, the term *Bacillus marisflavi* was used for *Rossellomorea marisflavi* however presently *Bacillus* species only encompass species of *Bacillus cereus* and *Bacillus subtilis* (Gupta *et al.*, 2020).

Various fermentation parameters such as pH,

temperature, incubation time, inoculum size, growth media, solid substrates and metal ions concentration were optimized for maximum extracellular L-asparaginase production by both strains. *R. marisflavi* was more active over a wide range of pH (6.0-10) as compared to *B. cereus*. There was a gradual increase in L-asparaginase activity from pH 4.0-7.0 that got maximum at pH 8.0 in *R. marisflavi*. This finding is similar to the optimal pH for L-asparaginase production by *Citrobacter* sp. (Shah *et al.*, 2010). However, *B. cereus* showed greatest L-asparaginase activity at pH 7.0 which coincided to the optimal pH for L-asparaginase production by *Streptomyces tendae* (Kavitha *et al.*, 2010). On the contrary, in previous reports L-asparaginase production was highest at pH 6 by *Aspergillus terreus* (Farag *et al.*, 2015) and at pH 8.6 by *Bacillus licheniformis* (Abdelrazek *et al.*, 2019).

The L-asparaginase production was tested between 30-50°C temperatures. An increase in temperature from 30°C also increased the enzyme production. At 40°C maximum production was shown by *R. marisflavi* while *B. cereus* optimally produced L-asparaginase at 37°C. Hence, thermophilic profile of *R. marisflavi* L-asparaginase was better than *B. cereus* and could be tested for its potential in acrylamide reduction in food materials in future. L-asparaginase was previously observed to be optimally produced by *Bacillus licheniformis* at 40°C (Abdelrazek *et al.*, 2019) and at 37°C by *Citrobacter* sp. (Shah *et al.*, 2010) which is in harmony with our findings but opposing to *Streptomyces tendae* that showed highest L-asparaginase activity at 30°C (Kavitha *et al.*, 2010) and *Bacillus firmus* and *Bacillus circulans* that optimally produced L-asparaginase at 45°C (Wakil and Adeegan, 2015), respectively.

The study displayed that 36 h were required for maximal L-asparaginase production by both strains and led to decrease in enzyme activity after 72 h, probably owing to the consumption of essential nutrients and accumulation of waste products in the medium. This finding is not in accordance with previously reported *Bacillus halotolerans* L-asparaginase that was optimally produced within 28 h (Orabi *et al.*, 2018) and to *Bacillus* Sp. BCCS 034 whose optimal incubation time was 48 h (Ebrahiminezhad *et al.*, 2011). Since, optimization of inoculum size is important for greater enzyme yield so different volumes of freshly grown inoculum were tested and addition of 1.5% inoculum proved to be optimal for highest L-asparaginase production by both *R. marisflavi* and *B. cereus*, respectively. This finding was in contrast to the already reported 1% inoculum size that was sufficient for highest L-asparaginase production by *Aspergillus terreus* (Baskar and Sahadevan, 2012).

Media and substrates are a source of essential

carbon and nitrogen nutrients that are vital for microbial cellular activities. Hence, various liquid media and solid substrates were tested in this study for optimal L-asparaginase production. A report showed that maximum L-asparaginase activity in *Streptomyces* spp. was achieved by modified Czapek Dox's media (El-Hadi *et al.*, 2019) which is in accordance with our findings for *R. marisflavi* L-asparaginase. Similarly, TSB media was previously utilized to produce L-asparaginase extracellularly from *Corynebacterium glutamicum* (Mesas *et al.*, 1990) which is the optimal growth media for *B. cereus* in our study. Results proposed Corn cob to be the most suitable solid substrate for both strains which is contrary to the earlier findings from *Bacillus halotolerans* that utilized sugarcane bagasse for best L-asparaginase production and corn bran as second choice (Orabi *et al.*, 2018).

Variable changes in L-asparaginase production were observed when EDTA and metal ions were supplemented in the growth media. Only slight increase in enzyme production was induced by EDTA in *R. marisflavi*, comparable to the findings that reported 105% increase in *Nocardia levis* L-asparaginase activity in the presence of EDTA (Kavitha and Vijayalakshmi, 2012). However, L-asparaginase activity was inhibited by EDTA in *B. cereus* which is in accordance with a previous report showing inhibitory effects of EDTA on L-asparaginase production by *Bacillus* sp. (Moorthy *et al.*, 2010). While studying the effect of metal ions, L-asparaginase synthesis remained unchanged by *R. marisflavi*'s on the addition of Ni^{+2} which is analogous to the behaviour of previously documented *Bacillus* sp. (Mohapatra *et al.*, 1995). However, inhibition of enzyme production by *R. marisflavi* was observed upon the addition of Zn^{2+} and Ca^{+2} , similar to the former findings that displayed the inhibitory effects of Zn^{2+} and Ca^{+2} on L-asparaginase activity in *Bacillus* sp. GH5 (Gholamian *et al.*, 2013). Moreover, present study supports retardation in L-asparaginase synthesis with Ni^{+2} , Zn^{2+} and Ca^{+2} ions by *B. cereus*. Yet, in another study accelerated effects of Fe^{+2} , Co^{+2} , Cu^{+2} and Mn^{+2} on L-asparaginase activity in *Erwinia* sp. were reported (Borkotaky and Bezbaruah, 2002) that are comparable to our results reported for both *R. marisflavi* and *B. cereus*. Mg^{2+} was the best activator amongst all metal ions in both strains, similar to its previously reported enhanced effects on the *Aspergillus terreus* (Farag *et al.*, 2015) and *Bacillus* sp. L-asparaginases (Moorthy *et al.*, 2010). Nonetheless Mg^{2+} supplementation reduced L-asparaginase activity in *Fusarium equiseti* in a previous study (Hosamani and Kaliwal, 2011).

The present study was found to be useful in designing the optimal fermentation conditions for enhanced extracellular L-asparaginase production by both *R. marisflavi* and *B. cereus*. The specific activity of enzyme

enhanced to 583 U/mg from 125 U/mg in *R. marisflavi* and to 250 U/mg from 191 U/mg in *B. cereus*, respectively (without supplementation of Mg^{2+} ions). Increase in L-asparaginase production after optimization studies has been previously reported in *Fusarium equiseti* (Hosamani and Kaliwal, 2011) and *Aspergillus terreus* (Varalakshmi and Raju, 2013; Farag *et al.*, 2015) among others.

CONCLUSION

Soil is home to many important microbes that can synthesize metabolites and byproducts of commercial importance. The present study was conducted to isolate extracellular L-asparaginase producing bacterial strains from the soil that were identified as *Rosellomorea marisflavi* and *Bacillus cereus* by ribotyping. They displayed significant L-asparaginase activity at physiological pH and temperature. They exhibited marked increase in enzyme production when growth media was supplemented with Mg^{+2} . Moreover, greater L-asparaginase quantities with less inoculum size and simpler fermentation conditions were achieved. Hence, it is proposed that application of L-asparaginases from locally screened *R. marisflavi* and *B. cereus* strains as therapeutic agents and as acrylamide reducers in food industry shall be explored.

ACKNOWLEDGEMENT

Authors are thankful to the School of Biochemistry and Biotechnology, University of the Punjab, Lahore for provision of research facilities.

Funding

No organization funded the research work.

IRB approval

.....?

Ethical statement

This article does not contain any studies with human or animal subjects.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

- Abdelrazek, N.A., Elkhatib, W.F., Raafat, M.M. and Aboulwafa, M.M., 2019. Experimental and bioinformatics study for production of L-asparaginase from *Bacillus licheniformis*: A promising enzyme for medical application. *AMB*

- Expr.*, **9**: 39. <https://doi.org/10.1186/s13568-019-0751-3>
- Ali, U., Naveed, M., Ullah, A., Ali, K., Shah, S.A., Fahad, S. and Mumtaz, A.S., 2016. L-asparaginase as a critical component to combat acute lymphoblastic leukaemia (ALL): A novel approach to target aLL. *Eur. J. Pharmacol.*, **771**: 199-210. <https://doi.org/10.1016/j.ejphar.2015.12.023>
- Amena, S., Vishalakshi, N., Prabhakar, M., Dayanand, A. and Lingappa, K., 2010. Production, purification and characterization of L-asparaginase from *Streptomyces gulbargensis*. *Braz. J. Microbiol.*, **41**: 173-178. <https://doi.org/10.1590/S1517-83822010000100025>
- Ashok, A., Doriya, K., Rao, J.V., Qureshi, A., Tiwari, A.K. and Kumar, D.S., 2019. Microbes producing L-asparaginase free of glutaminase and urease isolated from extreme locations of Antarctic soil and moss. *Sci. Rep.*, **9**: 1-10. <https://doi.org/10.1038/s41598-018-38094-1>
- Baskar, G. and Sahadevan, R., 2012. Optimization of culture conditions and bench-scale production of L-Asparaginase by submerged fermentation of *Aspergillus terreus* MTCC 1782. *J. Microbiol. Biotechnol.*, **22**: 923-929. <https://doi.org/10.4014/jmb.1112.12002>
- Batool, T., Makky, E.A., Jalal, M. and Yusoff, M.M., 2016. A comprehensive review on L-asparaginase and its applications. *Appl. Biochem. Biotechnol.*, **178**: 900-923. <https://doi.org/10.1007/s12010-015-1917-3>
- Borek, D., Podkowinski, J., Kisiel, A. and Jaskolski, M., 1999. Isolation and characterization of cDNA encoding L-asparaginase from *Lupinus luteus*. *Pl. Physiol.*, **119**: 1568-1570.
- Borkotaky, B. and Bezbaruah, R.L., 2002. Production and properties of asparaginase from a new *Erwinia* sp. *Folia Microbiol.*, **47**: 473-476. <https://doi.org/10.1007/BF02818783>
- Bouchet, V., Huot, H. and Goldstein, R., 2008. Molecular genetic basis of ribotyping. *Clin. Microbiol. Rev.*, **21**: 262-273. <https://doi.org/10.1128/CMR.00026-07>
- Bradford, N.A., 1976. A rapid and sensitive method for the quantitation microgram quantities of a protein isolated from red cell membranes. *Anal. Biochem.*, **72**: 248-254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3)
- Broome, J.D., 1961. Evidence that the L-asparaginase activity of guinea pig serum is responsible for its antilymphoma effects. *Nature*, **191**: 1114-1115. <https://doi.org/10.1038/1911114a0>
- Brumano, L.P., da Silva, F.V.S., Costa-Silva, T.A., Apolinário, A.C., Santos, J.H.P.M., Kleingesinds, E.K. and Junior, A.P., 2019. Development of L-Asparaginase biobetters: Current research status and review of the desirable quality profiles. *Front. Bioeng. Biotechnol.*, **6**: 212. <https://doi.org/10.3389/fbioe.2018.00212>
- Cachumba, J.J.M., Antunes, F.A.F., Peres, G.F.D., Brumano, L.P., Dos Santos, J.C. and Da Silva, S.S., 2016. Current applications and different approaches for microbial l-asparaginase production. *Braz. J. Microbiol.*, **47**: 77-85. <https://doi.org/10.1016/j.bjm.2016.10.004>
- Campbell, H.A. and Mashburn, L.T., 1969. L-Asparaginase EC-2 from *Escherichia coli*. Some substrate specificity characteristics. *Biochemistry*, **8**: 3768-3775. <https://doi.org/10.1021/bi00837a042>
- Chan, W.K., Lorenzi, P.L., Anishkin, A., Purwaha, P., Rogers, D.M., Sukharev, S. and Weinstein, J.N., 2014. The glutaminase activity of L-asparaginase is not required for anticancer activity against ASNS-negative cells. *Blood*, **123**: 3596-3606. <https://doi.org/10.1182/blood-2013-10-535112>
- Clewley, J.P. and Arnold, C., 1997. MEGALIGN. The multiple alignment module of LASERGENE. *Methods mol. Biol.* (Clifton, N.J.), **70**: 119-129. <https://doi.org/10.1385/0-89603-358-9:119>
- Ebrahiminezhad, A., Rasoul-Amini, S. and Ghasemi, Y., 2011. l-Asparaginase production by moderate halophilic bacteria isolated from Maharloo Salt Lake. *Indian J. Microbiol.*, **51**: 307-311. <https://doi.org/10.1007/s12088-011-0158-6>
- El-Hadi, A.A., Ahmed, H.M. and Hamzawy, R.A., 2019. Optimization and characterization of l-asparaginase production by a novel isolated *Streptomyces* spp. strain. *Egypt. Pharm. J.*, **18**: 111.
- Farag, A.M., Hassan, S.W., Beltagy, E.A. and El-Shenawy, M.A., 2015. Optimization of production of anti-tumor l-asparaginase by free and immobilized marine *Aspergillus terreus*. *Egypt. J. aquat. Res.*, **41**: 295-302. <https://doi.org/10.1016/j.ejar.2015.10.002>
- Fatima, N., Khan, M.M. and Khan, I.A., 2019. L-asparaginase produced from soil isolates of *Pseudomonas aeruginosa* shows potent anti-cancer activity on HeLa cells. *Saudi J. Biol. Sci.*, **26**: 1146-1153. <https://doi.org/10.1016/j.sjbs.2019.05.001>
- Gholamian, S., Gholamian, S., Nazemi, A. and Nargesi, M.M., 2013. Optimization of culture media for L-asparaginase production by newly isolated bacteria, *Bacillus* sp. GH5. *Microbiology*, **82**: 856-863. <https://doi.org/10.1134/S0026261714010032>

- Gökmen, V. and Palazoğlu, T.K., 2008. Acrylamide formation in foods during thermal processing with a focus on frying. *Fd. Bioproc. Tech.*, **1**: 35-42. <https://doi.org/10.1007/s11947-007-0005-2>
- Gulati, R., Saxena, R.K. and Gupta, R., 1997. A rapid plate assay for screening L-asparaginase producing micro-organisms. *Lett. appl. Microbiol.*, **24**: 23-26. <https://doi.org/10.1046/j.1472-765X.1997.00331.x>
- Gupta, R.S., Patel, S., Saini, N. and Chen, S., 2020. Robust demarcation of 17 distinct *Bacillus* species clades, proposed as novel *Bacillaceae* genera, by phylogenomics and comparative genomic analyses: Description of *Robertmurraya kyonggiensis* sp. nov. and proposal for an emended genus *Bacillus* limiting it only to the members of the *Subtilis* and *Cereus* clades of species. *Int. J. Syst. Evol. Microbiol.*, **70**: 5753-5798. <https://doi.org/10.1099/ijsem.0.004475>
- Hill, J.M., Roberts, J., Loeb, E., Khan, A., MacLellan, A. and Hill, R.W., 1967. L-asparaginase therapy for leukemia and other malignant neoplasms: Remission in human leukemia. *J. Am. med. Assoc.*, **202**: 882-888. <https://doi.org/10.1001/jama.202.9.882>
- Hosamani, R. and Kaliwal, B.B., 2011. Isolation, molecular identification and optimization of fermentation parameters for the production of L-asparaginase, an anticancer agent by *Fusarium equiseti*. *Int. J. Microbiol. Res.*, **3**: 108. <https://doi.org/10.9735/0975-5276.3.2.108-119>
- Kavitha, A. and Vijayalakshmi, M., 2012. A study on L-asparaginase of *Nocardia levis* MK-VL_113. *Sci. World J.*, 2012. <https://doi.org/10.1100/2012/160434>
- Kavitha, A. and Vijayalakshmi, M., 2010. Optimization and Purification of L-Asparaginase Produced by *Streptomyces tendae* TK-VL_333. *Z. Naturforsch C J. Biosci.*, **65**: 528-531. <https://doi.org/10.1515/znc-2010-7-817>
- Kidd, J.G., 1953. 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. *J. exp. Med.*, **98**: 565-582. <https://doi.org/10.1084/jem.98.6.565>
- Kumar, K. and Verma, N., 2012. The various sources and application of L-asparaginase. *Asian J. Biochem. Pharma. Res.*, **2**: 197-205.
- Lopes, A.M., Oliveira-Nascimento, L.D., Ribeiro, A., Tairum Jr, C.A., Breyer, C.A., Oliveira, M.A.D. and Cavaco-Paulo, A.M., 2017. Therapeutic L-asparaginase: Upstream, downstream and beyond. *Crit. Rev. Biotechnol.*, **37**: 82-99. <https://doi.org/10.3109/07388551.2015.1120705>
- Mesas, J.M., Gil, J.A. and Martín, J.F., 1990. Characterization and partial purification of L-asparaginase from *Corynebacterium glutamicum*. *Microbiology*, **136**: 515-519. <https://doi.org/10.1099/00221287-136-3-515>
- Mohapatra, B.R., Sani, R.K. and Banerjee, U.C., 1995. Characterization of L-asparaginase from *Bacillus* sp. isolated from an intertidal marine alga (*Sargassum* sp.). *Lett. appl. Microbiol.*, **21**: 380-383. <https://doi.org/10.1111/j.1472-765X.1995.tb01086.x>
- Moorthy, V., Ramalingam, A., Sumantha, A. and Shankaranaya, R.T., 2010. Production, purification and characterisation of extracellular L-asparaginase from a soil isolate of *Bacillus* sp. *Afr. J. Microbiol. Res.*, **4**: 1862-1867.
- Mottram, D.S., Wedzicha, B.L. and Dodson, A.T., 2002. Acrylamide is formed in the Maillard reaction. *Nature*, **419**: 448-449. <https://doi.org/10.1038/419448a>
- Müller, H.J. and Boos, J., 1998. Use of L-asparaginase in childhood all. *Crit. Rev. Oncol. Hematol.*, **28**(2): 97-113. [https://doi.org/10.1016/S1040-8428\(98\)00015-8](https://doi.org/10.1016/S1040-8428(98)00015-8)
- Narta, U.K., Kanwar, S.S. and Azmi, W., 2007. Pharmacological and clinical evaluation of L-asparaginase in the treatment of leukemia. *Crit. Rev. Oncol. Hematol.*, **61**: 208-221. <https://doi.org/10.1016/j.critrevonc.2006.07.009>
- Nour El-Dein, M., Mousa, M. and Metwally, N., 2019. Isolation and survey of L-asparaginase producing bacteria from soil. *J. Egypt. Acad. Soc. environ. Develop. Environ. Stud.*, **20**: 19-29. <https://doi.org/10.21608/jades.2019.67651>
- Orabi, H.M., El-Fakrany, E.M., Abdel Khalek, E.S., El-Kasaby, A.M. and Sidkey, N.M., 2018. Exteracellular L-asparaginase from local soil bacteria: Production and optimization of physiological condition. *Al-Azhar Bull. Sci.*, **29**: 1-7. <https://doi.org/10.21608/absb.2018.33806>
- Patro, K.K.R., Satpathy, S. and Gupta, N., 2011. Evaluation of some fungi for L- asparaginase production. *Indian J. Fundam. appl. Life Sci.*, **1**: 219-221.
- Rizzari, C., Zucchetti, M., Conter, V., Diomede, L., Bruno, A., Gavazzi, L. and Milani, M., 2000. L-asparagine depletion and L-asparaginase activity in children with acute lymphoblastic leukemia receiving im or iv *Erwinia C. or E. coli* L-asparaginase as first exposure. *Ann. Oncol.*, **11**: 189-193. <https://doi.org/10.1093/annonc/11.2.189>

- [org/10.1023/A:1008368916800](https://doi.org/10.1023/A:1008368916800)
- Shah, A.J., Karadi, R.V. and Parekh, P.P., 2010. Isolation, optimization and production of L-asparaginase from coliform bacteria. *Asian J. Biotechnol.*, **2**: 169-177. <https://doi.org/10.3923/ajbkr.2010.169.177>
- Sinha, R.A.T.I., Singh, H.R. and Jha, S.K., 2013. Microbial L-asparaginase: Present and future prospective. *Int. J. Innov. Res.*, **2**: 7031-7051.
- Stams, W.A., den Boer, M.L., Beverloo, H.B., Meijerink, J.P., Stigter, R.L., van Wering, E.R. and Pieters, R., 2003. Sensitivity to L-asparaginase is not associated with expression levels of asparagine synthetase in t (12; 21) + pediatric ALL. *Blood Am. J. Hematol.*, **101**: 2743-2747. <https://doi.org/10.1182/blood-2002-08-2446>
- Szymanska, B., Wilczynska-Kalak, U., Kang, M.H., Liem, N.L., Carol, H., Boehm, I. and Lock, R.B., 2012. Pharmacokinetic modeling of an induction regimen for *in vivo* combined testing of novel drugs against pediatric acute lymphoblastic leukemia -xenografts. *PLoS One*, **7**. <https://doi.org/10.1371/journal.pone.0033894>
- Thompson, J.D., Higgins, D.G. and Gibson, T.J., 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**: 4673-4680. <https://doi.org/10.1093/nar/22.22.4673>
- Varalakshmi, V. and Raju, K.J., 2013. Optimization of L-asparaginase production by *Aspergillus terreus* mtcc 1782 using bajra seed flour under solid state fermentation. *Int. J. Eng. Res. Technol.*, **2**: 121-129. <https://doi.org/10.15623/ijret.2013.0209020>
- Verma, N., Bansal, M. and Kumar, S., 2012. Whole cell based miniaturized fiber optic biosensor to monitor L-asparagine. *Adv. appl. Sci. Res.*, **3**: 809-814.
- Wakil, S.S. and Adelegan, A.A., 2015. Screening, production and optimization of L-asparaginase from soil bacteria isolated in Ibadan, South-western Nigeria. *J. Basic appl. Sci.*, **11**: 39-51. <https://doi.org/10.6000/1927-5129.2015.11.06>
- Zhang, J., Fan, J., Venneti, S., Cross, J.R., Takagi, T., Bhinder, B. and Thompson, C.B., 2014. Asparagine plays a critical role in regulating cellular adaptation to glutamine depletion. *Mol. Cell*, **56**: 205-218. <https://doi.org/10.1016/j.molcel.2014.08.018>