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Original Research Article

BIOSYNTHESIS, PARTIAL PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR L-ASPARAGINASE FROM NOVEL *BACILLUS SUBTILIS SUBSP. SPIZIZENII* TU-B-10 ISOLATED FROM GCU GARDEN SOIL MICROFLORA

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Abstract *This study focused on L-asparaginase (LA), which breaks down L-asparagine into ammonia and aspartic acid and is known for its anti-carcinogenic properties. A bacterial strain from GCU garden soil was isolated, characterized, and screened for LA production using various assays. Physical and nutritional parameters for maximum LA biosynthesis were optimized by employing one factor at one time (OFAT) optimization strategy. Partial purification of the enzyme was followed by the determination of its various biochemical properties. The phylogenetic analysis has confirmed the close relation of soil isolate SHF-11 to Bacillus subtilis subsp. spizizenii TU-B-10. A twofold increase in enzyme activity with 23.05 IU/ml corresponding to 100 IU/mg of protein was achieved using 2% inoculum size, initial pH 7, agitation at 200 rpm, incubation time, and temperature of 72 hrs and 37 °C, respectively under submerged fermentation consuming 0.1% sucrose as carbon source and 1.5% asparagine as an inducer in the presence of 0.5% tryptone and 0.25% yeast extract as nitrogen source. SDS-PAGE analysis showed that the enzyme exhibited a protein band of almost 40 kDa. The highest activity of LA from Bacillus subtilis subsp. spizizenii TU-B-10 (BsLA) was observed at pH 8 and 37 ºC. EDTA was found to be a strong inhibitor of BsLA activity at the final concentration of 0.1 M, while Mg+2 ions were found to be a strong activator of BsLA. By optimizing purification parameters, more potential and specific preparations of LA are likely to come up that can meet industrial and biomedical standards.*

Keywords: L-asparaginase, *Bacillus subtilis subsp. spizizenii* TU-B-10, Submerged fermentation, Biochemical properties

Introduction

Biotechnology is making use of a broad range of enzymes produced on a commercial scale utilizing purposely screened microbes; called microbial enzymes. Microbial enzymes are ideal and preferred because of their consistency, high yields, economic feasibility, ease of optimization and modification of product, rapid microbial growth on low-cost media, stability, regular supply due to lack of seasonal variations and more excellent catalytic activity. These microbial enzymes have established their value in bio-industries like food, leather and textiles, animal feed, bioremediations, and bio-conversions (Niyonzima *et al.*, 2020). One of the most demanding microbial enzymes in the industrial and biomedical fields is L-asparaginase (LA). LA is a biodegradable, non-toxic amino-hydrolase (E.C no. 3.5.1.1) enzyme that has made a radical influence in the area of healthcare because of its universal use in the treatment of various lymphoproliferative disorders and lymphomas (Vimal & Kumar, 2022).

[Citation Fatima, H., Hussain, Z., Saleem, R., Shaikh, Z.A., Siyal, S., Jokhio, S.A., Unar, A.A., Unar, K., Shaikh, S.A., Shaikh, A.I., Siyal, F.J. (2023). Biosynthesis, partial purification and characterization of extracellular l-asparaginase from novel bacillus subtilis *subsp. spizizenii* tu-b-10 isolated from GCU garden soil microflora. *Biol. Clin. Sci. Res. J.*, **2023***:* 447. doi: https://doi.org/10.54112/bcsrj.v2023i1.447]

LA works by eliminating the serum circulatory asparagines by hydrolyzing it into aspartic acid and ammonia thus stopping protein synthesis in cancer cells, leading to the arrest of G1 cell-cycle. This causes massive destruction of tumor cells but does not harm the normal healthy cells because they can synthesize L-asparagine using L-asparagine synthetase present in them in sufficient capacity (Shrivastava *et al.,* 2015). LA has also been used broadly as a food processing aid by inhibiting acrylamide formation throughout food processing **(**Jia *et al*., 2021**).**

High levels of LA production by *Erwinia species*, *Escherichia coli, Bacillus subtilis* and *Streptomyces* species are helpful as bacterial substitutes for microbial sources producing LA for cancer therapy. Latter two species are superior for asparaginase production as these have considerable stability towards harsh condition (Ghasemian *et al*., 2019). *Bacillus sp*ecies are predominant in soil microbial flora (Wafula *et al.,* 2014) thus considered as ideal source for the isolation and screening of various *Bacillus* species. The *Bacillus subtilis* species complex is comprised of closely related species and is considered a heterogeneous group. Many isolates, which have now been categorized as *Bacillus mojavensis (*Roberts *et al.,* 1994), *Bacillus vallismortis* (Roberts, *et al.,* 1996), and *Bacillus atrophaeus (*Nakamura, 1989), were previously classified as *Bacillus subtilis* in general. The Bacillus subtilis was partitioned into three subspecies named *B. subtilis* subsp. *Inaquosorum*, *Bacillus subtilis* subsp. *subtilis*, *B. subtilis* subsp. *spizizenii* and (Nakamura *et al.,* 1999; Rooney *et al.,* 2009). *B. subtilis* subsp. *spizizenii* strain TU-B-10 is a strain for *Bacillus subtilis subspecies spizizenii*, and its close relatedness with newly identified lab strain W23 has been proved. It was identified as gram positive, spore producer, resistant to adverse environmental conditions, and eco-friendly, with the potential to biosynthesize various enzymes (Earl *et al.,* 2012).

Submerged fermentation is usually employed when bacterial cultivation for LA production is desired because (i) control of fermentation parameters (temperature, pH, moisture etc.) is simple and easy (ii) substrate pre-treatment is not required (iii) product purification is more straightforward, (iv) exploitation of genetically modified organism is supported greatly (Vimal & Kumar, 2017). As organisms have their physico-chemical condition to give optimal levels of enzyme production, no defined medium is established for getting the highest possible yield of LA from diverse microbes. Screening optimal concentrations of medium nutrients is vital to control the cost of the production process globally. Enzyme purification is critical to endorse its characterization plus therapeutic usage with fewer adversarial effects.

Therefore, the objective of this study is production, optimization, partial purification followed by characterization of extracellular LA from novel *B. subtilis* subsp. *spizizenii* strain TU-B-10 (*Bs*LA) isolated from GCU garden soil microflora.

Material and methods

Chemicals and Reagents

All chemicals and solvents employed in this study were purchased from Acros (Belgium), Merck (Germany), Fluka (Switzerland) and Sigma (USA).

Collection of Soil Samples

Soil samples were collected from four locations in Pakistan (Jhelum River bank, Sialkot agriculture field, Govt. college University (GCU) Lahore, and Islamabad industrial site) during early summer to isolate LA-producing bacteria. Physicochemical properties (pH, temperature, color, and odor) were recorded. The samples were labeled, transported to the laboratory, sieved to remove debris, heat treated at 120 °C for 15 minutes, and stored in polythene bags at room temperature.

Isolation and Screening of Bacillus sp. Producing LA

Qualitative and Semi-quantitative Screening of Soil solates producing LA

Soil samples were diluted and plated on modified M9 medium ($Na_2HPO_4.2H_2O$, 6: L-asparagine, 5; NaCl, 0.5; KH_2PO_4, 3; 20% glucose stock solution, 10 mL, 0.1 mol/L $CaCl_2$ $2H_2O$, 1 mL; 1 mol/L $MgSO_4.7H_2O$, 2 mL; agar, 20, with 0.36 mL of 2.5% phenol red, set pH to 6.5) to isolate l-asparaginase (LA) producers qualitatively. Pink-red colonies were selected as potential LA producers and streaked on nutrient agar plates for pure culturing. Pure cultures were stored at 4 °C and revived every 14 days (Fatima *et al.,* 2019). Semi-quantitative analysis based on the hydrolysis zone diameter measurement was done to screen for the potent out of total LA producers on modified M9 agar plates. Microscopic and macroscopic characteristics, including staining and colony properties, were observed for morphological characterization (Gulati *et al.,* 1997; Mahajan *et al.,* 2013; Fatima et al., 2019)

Quantitative Screening

Submerged fermentation was conducted using 100 mL of sterile TGY-broth with 1% asparagine inoculated with a 24-hour-old bacterial culture (Fatima *et al.,* 2019). The flasks were incubated on a rotary shaker at 120 rpm and 37 °C for 24, 48, and 72 hours. Enzyme activity was assessed by nesslerization after centrifugation of the fermented broth at 10,000 ×g for 10 minutes (Shirfrin *et al.,* 1974).

Biochemical and Molecular Identification of Potent *Bacillus sp.*

[Citation Fatima, H., Hussain, Z., Saleem, R., Shaikh, Z.A., Siyal, S., Jokhio, S.A., Unar, A.A., Unar, K., Shaikh, S.A., Shaikh, A.I., Siyal, F.J. (2023). Biosynthesis, partial purification and characterization of extracellular l-asparaginase from novel bacillus subtilis *subsp. spizizenii* tu-b-10 isolated from GCU garden soil microflora. *Biol. Clin. Sci. Res. J.,* **2023***:* 447. doi: https://doi.org/10.54112/bcsrj.v2023i1.447]

Biochemical testing was performed on the most active LA-producing bacteria (Garrity *et al.,* 2004). The results were interpreted using the "Bergey's Manual of Determinative Bacteriology" (Holt, 1994). The soil isolates with the highest LA enzyme activity were sent for16S rRNA sequencing at Macrogen sequencing company Korea. BLAST analysis was conducted using the query sequence submitted to NCBI-BLAST, followed by multiple sequence alignment and neighbor-joining method to generate the evolutionary tree (Altschu *et al.,* 1997; Higgins *et al.,* 1988; Saitou & Nei, 1987).

Optimization of Physico-Nutritional parameters for BsLA Production under SmF

Process parameters were optimized using an OFAT strategy. Various fermentation media were tested, including TGY broth with 1% asparagine, TGY broth, M-9 media, and M-9 media with 1% asparagine. Cultures were incubated at 37°C and 200 rpm for 48 hours. Optimum incubation intervals were determined (24, 48, 72, 96, and 110 hours). Enzyme activity was measured in the collected supernatant.

The impact of different incubation temperatures (27°C, 37°C, 47°C, 57°C, and 67°C), initial pH values (6.5 to 8.5), inoculum sizes (1 mL, 2 mL, 3 mL, and 4 mL), carbon sources (lactose, maltose, fructose, and sucrose), glucose concentrations (0.1% to 0.5%), L-asparagine concentrations (0.5% to 2.5% w/v), yeast extract concentrations (0.25% to 2% w/v), and K2HPO4 concentrations (0.05% to 0.25% w/v) on BsLA production was assessed.

Partial Purification of BsLA and protein profiling by SDS-PAGE

Ammonium sulfate precipitation was performed to purify the crude enzyme extract partially. The supernatant was subjected to stepwise increases in salt concentration (20%, 30%, up to 80%) with agitation at 4°C for 1 hour. The mixture was then centrifuged, and the precipitate was suspended in a 50 mM Tris-HCl buffer (pH 8). The suspended precipitate was dialyzed against the same buffer at 4°C. Fractions were collected and assayed for protein and enzyme activity. Protein quantification was performed using the Bradford assay with bovine serum albumin (BSA) as a standard.

SDS-PAGE (12%) was prepared according to the modified technique proposed by Laemmli (1970) to determine the molecular weight of BsLA. Stacking gel (5%) and resolving gel (12%) were prepared. Protein samples mixed with gel loading buffer were loaded into the wells. The gel was operated at 80 volts until the bands were stacked and then increased to 100 volts for efficient resolution. The gel was stained and destained to visualize the protein bands.

Biochemical Characterization of *Bs*LA

The optimal incubation time for partial purification of LA was determined within 5-50 minutes. Enzyme activity assay was performed using direct nesslerization. Optimum pH was evaluated by assessing the catalytic activity of BsLA at 37 °C using various buffers (0.05 M) spanning different pH ranges: citrate–phosphate buffer (pH 3-6), phosphate buffer (pH 6-7), Tris-HCl buffer (pH 8-9), and glycine-NaOH buffer (pH 10-12). The optimal pH for BsLA enzyme activity was determined by assaying enzyme activity at temperatures ranging from 27-77 °C.

The impact of diverse metal ions (Mg2+, Cu2+, Ca2+, Mn2+, Ba2+, Ni2+, Co2+, Zn2+, and Cd2+) on partially purified LA was studied at a concentration of 0.1 mM. Residual activities were estimated and compared with the control (without metal ions), with the control activity set as 100%. Additionally, the effect of various concentrations of EDTA (0.0001, 0.001, 0.005, 0.01, 0.05, and 0.1 M) on partially purified BsLA was examined. Residual activities were measured and compared with the control (without EDTA addition), with the control activity considered as 100%.

Results

Properties of Soil

Analysis of the physico-chemical properties of all four soil samples demonstrated that the samples were brown, black, and red in color, comprising temperatures varied from 17 ± 1.2 to 27 ± 0.2 ºC, respectively. The samples were found to have pH varied from 6.4 ± 0.4 to 7.0 ± 0.2 with an earthy smell (Table 1).

Table 1: Physico-chemical properties of Soil

Sr. no.	Samples	Locations (GCU Departments)	Soil Properties			
			Ph	Temperature °C	Color	Smell
1.	I	Jhelum	6.4 ± 0.4	17 ± 1.2	Black	Earthy
2.	II	Sialkot	7.0 ± 0.2	27 ± 0.2	Black	Earthy
3.	III	Islamabad	6.5 ± 0.2	23 ± 0.6	Brick Red	Earthy
4.	IV	Lahore, GCU	6.7 ± 0.5	25 ± 1.0	Brown	Earthy

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Isolation and Screening

Qualitative and Semi-quantitative Screening of Soil isolates producing LA

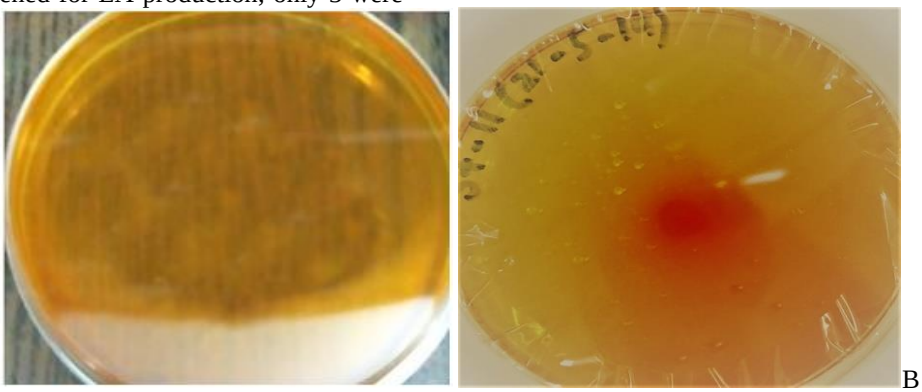
Primary soil isolates were screened for LA production by serial dilution technique on a modified M9 agar medium plate. Out of total 30 isolates obtained from various soil samples, there were only 11 isolates producing LA, labeled as (SHF-1, SHF-2, SHF-4,……,SHF-11), identified based on pink color that they produced around the colony (Table 2).

Table 2: Screening of LA producers out of total isolates

Sr. no.	Samples	Total bacteria	LA producers	Codes of LA producers	% of positive LA producers
1.	I	5	2	SHF-1 SHF-2	40
2.	II	7	3	SHF-3 SHF-4 SHF-5	42.8
3.	III	9	3	SHF-6 SHF-7 SHF-8	33.3
4.	IV	9	3	SHF-9 SHF-10 SHF-11	33.3

Screening of potent LA producers was done according to the method of Gulati *et al.* (1997). Based on the diameter of the hydrolysis zone, out of 11 isolates screened for LA production, only 3 were screened as potent producers of LA. Hydrolysis zone of soil isolate SHF-11 is shown in (Figure 1).

A



B

Fig 1: Modified M9 agar Medium plates [A] showing un-inoculated control plate [B] production of L-asparaginase by microorganisms as indicated by the pink zone formed around the colony.

From the semi-quantitative plate assay performed by measuring the hydrolysis zone diameter of each isolate on modified M9 agar plate, it was observed that out of all potent producers, the highest enzyme activity was exhibited by SHF-11 isolate of GCU Garden soil with 20 ± 3.2 of zone index followed by SHF-6 (14 ± 1.2) then SHF-5 (9 ± 0.5) after 72 hours of incubation (Table 3).

Sr. no.	Isolates	Semi quantitative Screening (Rapid plate assay) Zone index in mm			Quantitative Screening IU/ml/minute		
		1 DAY	2 DAY	3 DAY	1 DAY	2 DAY	3 DAY
1.	SHF- 5	6 ± 0.8	7 ± 1.4	9 ± 0.5	0.86 ± 0.13	2.16 ± 0.13	6.71 ± 0.01
2.	SHF- 6	9 ± 0.6	11 ± 2.2	14 ± 1.2	1.52 ± 0.01	3.81± 0.05	7.02 ± 0.03
3.	SHF- 11	12 ±1.3	16 ± 2.1	20 ± 3.2	2.56 ± 0.02	5.3 ± 0.01	10.9 ± 0.13

Table 3: Semi-Quantitative and Quantitative Screening of Potent Soil isolates producing LA

Quantitative Screening

Cell free extract was used for quantitaively enzyme assay by nesslerization. An enzyme unit is defined as a liberation of one micromole of ammonia per mL per minute. When the nessler's reagent reacts with ammonia released by LA-asparagine reaction, orange to brown precipitate forms. The intensity of color production is directly proportional to ammonia

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concentration (figure 2.a), and ammonia release is calculated using a standard curve of ammonium sulfate (Figure 2.b). It was observed that all three isolates exhibit maximum enzyme activities after 72 hours of incubation. Out of all potent producers, the highest enzyme activity was exhibited by SHF-11 isolate of GCU Garden soil with 10.9 ± 0.14 IU/mL followed by SHF-6 (7.02 ± 0.03) then SHF-5 (6.71 ± 0.01) after 72 hours of incubation (Table 3).

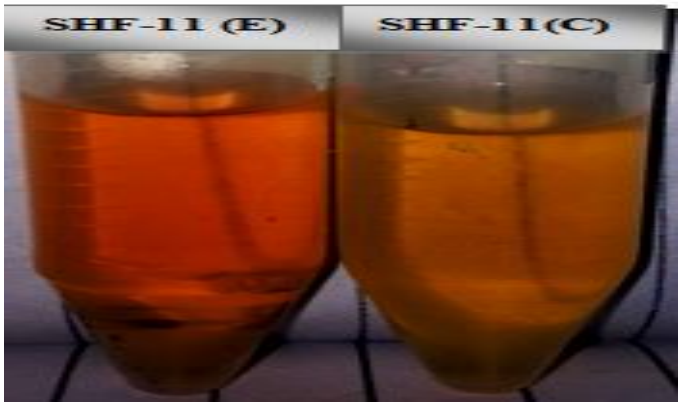

Fig. 2a: Enzyme Assay of extracellular fraction of SHF-11 by Nesslerization. E: In experimental tube Orange to brown precipitate denotes greater amount of ammonia release because of L-asparagine degradation by LA; (C): In control tube no orange to brown ppt because of TCA addition before enzyme addition, no asparaginase
-enzyme reaction, and no release of ammonia.

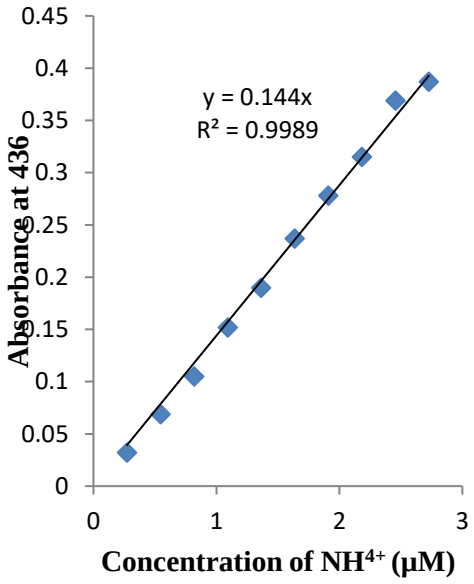

Fig. 2b: Standard curve of Ammonium Sulfate.

Biochemical and Molecular Identification

The morphological characters of a total 11 screened microbes were determined. On nutrient agar plates, their macroscopic characters like colony size, shape, texture, and margins were determined, and microscopic analysis, including gram staining and motility test by hanging drop method (Jain et al., 2020). Based on morphological characters, there were 7 gram positive; 5 were large rods, 2 short rods, and 4 were gram-negative with 3 cocci and 1 with short rods. In our research, SHF-5, SHF-6, and SHF-11, previously screened as potent producer were found to gram positive with large rods and were then biochemically characterized to identify them at least up to genus level, and results obtained were compared with Berge's Manual determinative of bacteriology. All of three isolates were positive for catalase, produced spore, hydrolyzed casein, gelatin, starch, and asparagine, illustrating that these are producers of gelatinase, protease, amylase, and asparaginase**.** Carbohydrate utilization test for various sugars denotes that these can ferment sugars like D- xylose, glucose, sucrose, arabinose, maltose, lactose, and fructose. SHF-11 and SHF-5 isolates were found negative for mannitol fermentation. Results obtained after biochemical testing of these isolates indicated that all of the 3 isolates belong to *Bacillus* genus (Table 4).

Table 4: Morphological and biochemical characterization of potent LA producers

TESTS	ISOLATES		
	SHF-5	**SHF-6**	**SHF-11**
Colony Size	Medium	Medium	Medium
Colony Margins	Entire	Lobate	Lobate
Colony Texture	Mucoid	Mucoid	Dry
Colony Color	White	White	White
Gram Stain	+	+	+
Shape of cells	Rod	Rod	Rod
Spore Production	+	+	+

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Motility	+	+	+
Catalase	+	+	+
MR test	-	-	-
Oxidase	-	-	-
Carbohydrates Fermentation **Tests**			
D-Xylose	+	+	+
D-Glucose	+	+	+
D-Sucrose	+	+	+
D-Arabinose	+	+	+
D-Maltose	+	+	+
D-Mannitol	-	+	-
D-Lactose	+	+	V
D-Fructose	+	+	+
Enzyme Hydrolysis Tests			
Starch Hydrolysis	+	+	+
Casein Hydrolysis	+	+	+
Gelatin Hydrolysis	+	+	+
Tyrosine Hydrolysis	-	-	-
L-asparagine	+	+	+
Urease	-	-	-
Probable identification	***Bacillus sp.***	***Bacillus sp.***	***Bacillus sp.***

Strain SHF-11 was identified as *Bacillus subtilis* through a BLAST search of the GenBank database using its query sequence (accession ID: CP002905.1). Using the neighbor-joining method, a phylogenetic tree (Figure 3) was constructed based on the 16S rRNA gene sequence (Saitou & Nei, 1987). The BLAST analysis and multiple sequence alignment confirmed the close relationship of SHF-11, a soil isolate, to Bacillus subtilis subsp. spizizenii TU-B-1-10.

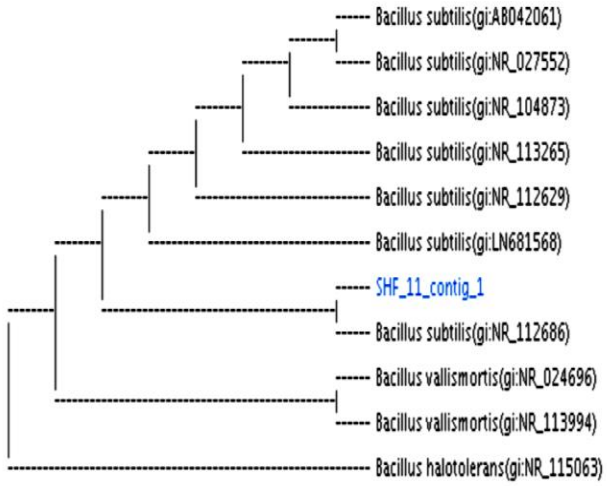

Fig 3: Phylogenetic tree showing the relationships of the soil isolate SHF-11 and their closest relatives according to the 16S rRNA gene.

Media optimization

Out of all media employed for enzyme production, TGY broth with 1% asparagine was observed as best medium for *Bs*LA production with 5.3 IU/mL enzyme activity after 48 hours of incubation followed by M-9 medium with 1% asparagine (3.2 IU/mL). Effect of medium on enzyme activity is shown in (figure 4). Hence, further optimization was conducted by using 1% TGY broth.

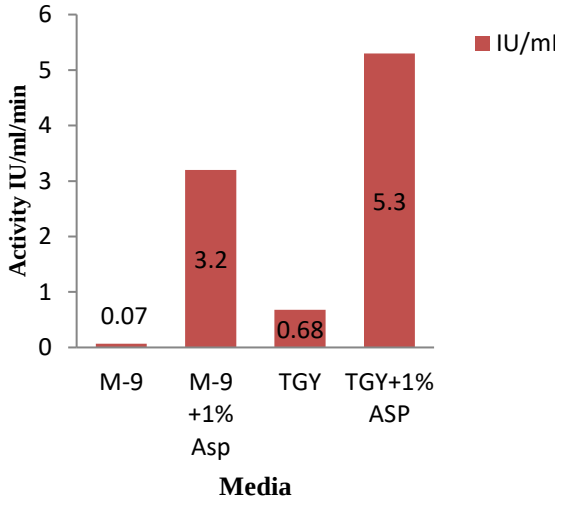

Fig.4: Media optimization

Effect of Incubation period on BsLA Production

To evaluate the highest possible enzyme production by desired strain, the incubation time was optimized. After 72 hours of incubation, the maximum level of *Bs*LA production with 10.5 IU/mL was achieved, followed by 9.9 IU/mL after 96 hours of incubation (figure 5). There was a tremendous decrease in enzyme activity with a further increase in incubation time.

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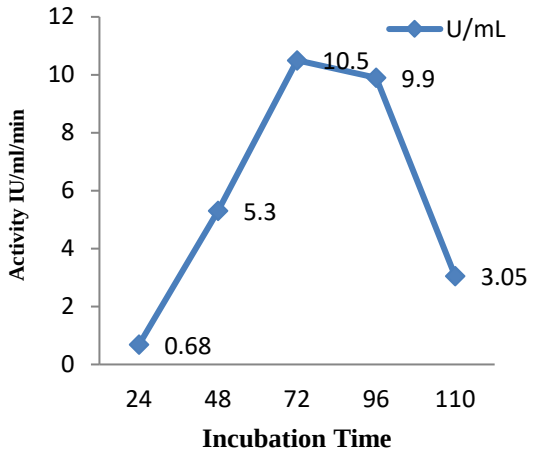

Fig 5: Effect of incubation time on LA production

Effect of Incubation Temperature on BsLA Production

Culture growth and enzyme production in production media is critically influenced by temperature as microbial growth depends on temperature. Maximum possible *Bs*LA production with highest enzyme activity of 13.75 IU/mL was achieved after 72 hours of incubation at 37 °C followed by 9.9 IU/mL at 47 °C. No enzyme productivity was observed at 67 °C (figure 6). This loss of productivity might be due to the microbial strain inactivation at higher temperatures caused by a lot of metabolic heat production.

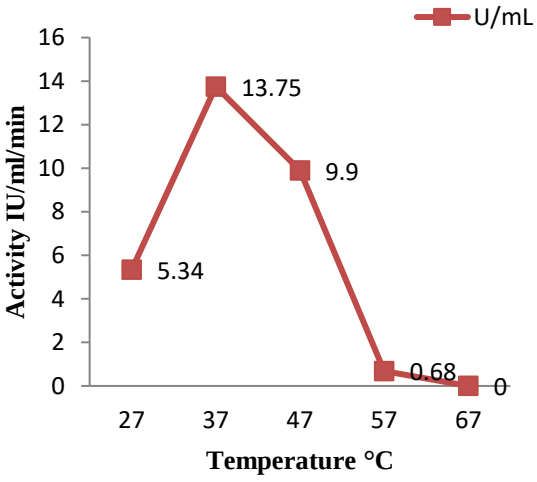

Fig.6: Effect of temperature on LA production

Effect of initial pH of medium of the Medium on BsLA Production

The effect of initial pH ranging from pH 6.0-8.5 on *Bs*LA production was analyzed (figure 7).

The effect of pH on *Bs*LA production was observed using TGY medium with 1% L-asparagine, and fermentation conditions was 200 rpm, 72 hours of incubation at 37 °C. Maximum enzyme yield with 13.9 IU/ml enzyme activity was observed at pH 7. Beyond this pH there was a gradual decrease in enzyme production. With increasing pH, enzyme production decreased with negligible production at pH 8.5 (1.52 IU/ml).

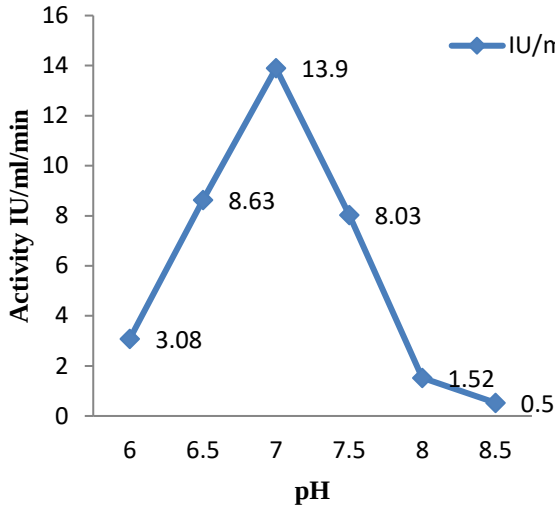

Fig.7: Effect of pH on LA production

Effect of Inoculum level on BsLA Production

The inoculum size was optimized for the maximum possible enzyme production by fermentation culture. Initially, there was an increase in enzyme productivity from 12.02 IU/mL at 1% inoculum size to the peak value of 14.0 IU/mL at 2% inoculum size. Beyond this inoculum size, there was a decrease in enzyme production with almost negligible production (0.53 IU/mL), when the fermentation medium was inoculated with 4% inoculum size. The effect of inoculum on *Bs*LA production is shown in (figure 8).

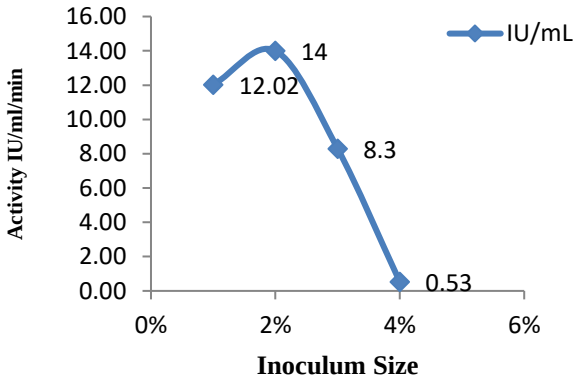

Fig.8: Effect of Inoculum size on LA production

[Citation Fatima, H., Hussain, Z., Saleem, R., Shaikh, Z.A., Siyal, S., Jokhio, S.A., Unar, A.A., Unar, K., Shaikh, S.A., Shaikh, A.I., Siyal, F.J. (2023). Biosynthesis, partial purification and characterization of extracellular l-asparaginase from novel bacillus subtilis *subsp. spizizenii* tu-b-10 isolated from GCU garden soil microflora. *Biol. Clin. Sci. Res. J.*, **2023***:* 447. doi: https://doi.org/10.54112/bcsrj.v2023i1.447]

3.4.6. Effect of various carbon sources on BsLA Production

The influence of various carbon sources i.e., glucose, sucrose, fructose, lactose, and maltose was studied for *Bs*LA production under optimized physical parameters. Highest enzyme activity was observed by using 0.1% sucrose followed by glucose, fructose, lactose, and maltose with 17.01 IU/ml, 13.65 IU/ml and 11.03 IU/ml, 6.1 IU/ml and 1.52 IU/ml, respectively (figure 9). Among various concentrations of glucose maximum level of the enzyme was produced by fermentation culture in the presence of 0.4% glucose with 15.53 IU/mL (figure 10). But the overall production of enzyme in the presence of 0.4% glucose was lower than in the presence of 0.1 % sucrose. It is evident from various literature reports that microbial synthesis of LA is under catabolic repression and entails a smaller amount of carbon source, so 0.1 % sucrose was opted for further optimization studies.

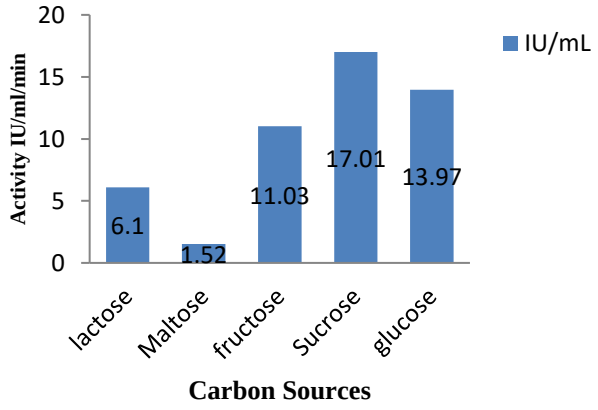

Fig. 9: Effect of Various Carbon sources

Effect of Salt concentrations BsLA Production

The effect of various concentrations of K_2HPO_4 ranging from 0.05%, 0.1%, 0.15 %, 0.2%, and 1.25% on *Bs*LA production was studied using TSY medium with 1% L-asparagine. The fermentation was performed at optimized conditions. In fermented broth, Maximum LA production with the highest enzyme activity of 17.02 IU/mL was analyzed in 0.1% K_2HPO_4. The effect of various concentrations of salts on LA production is shown in (figure 11).

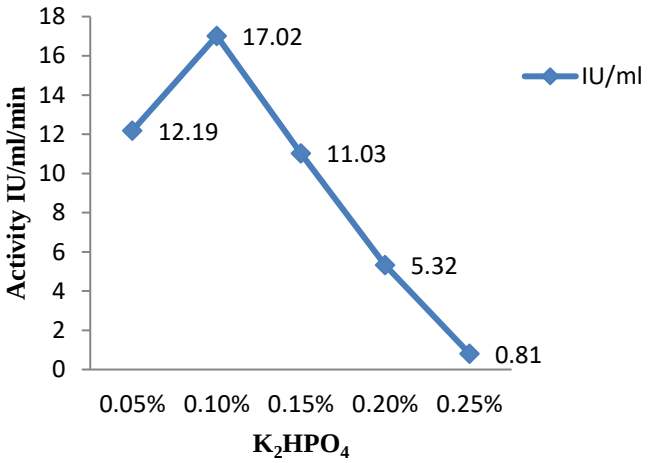

Fig. 11: Effect of Various concentration of K_2HPO_4

3.4.8. Influence of various concentrations of inducer on BsLA Production

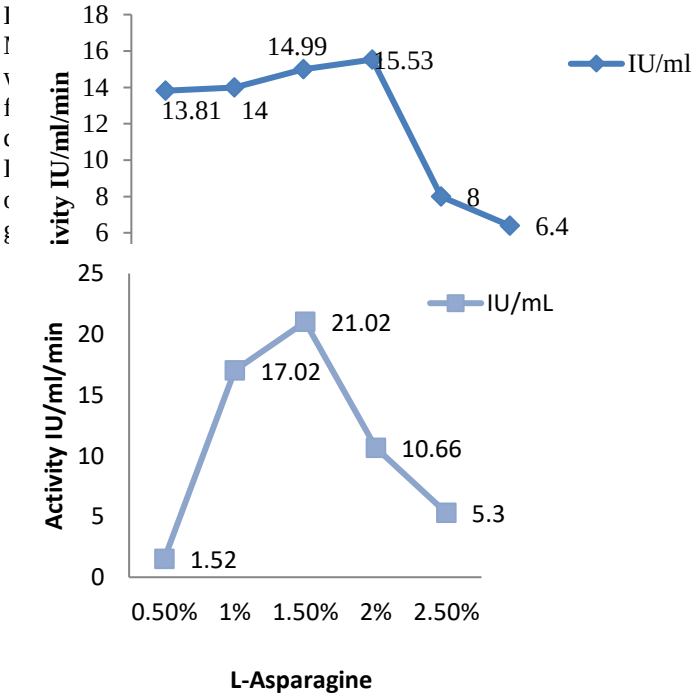

Fig. 12: Effect of various conc. of L-asparagine

Effect of various concentrations of Yeast Extract on BsLA Production

A nitrogen source is a limiting nutrient and plays a key role in *Bs*LA production. The effect of various concentrations of yeast extract ranging from 0.25%, 0.5%, 1%, and 1.5% on *Bs*LA production was studied using TSY medium with pH 7, inoculated with 2% inoculum and was then incubated at 37 °C,

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for 72 hours at shaking conditions. With 1.5% L-asparagine and 0.5% tryptone, maximum enzyme production was achieved at 0.25 % of yeast extract with 23.05 IU/ml (figure 13).

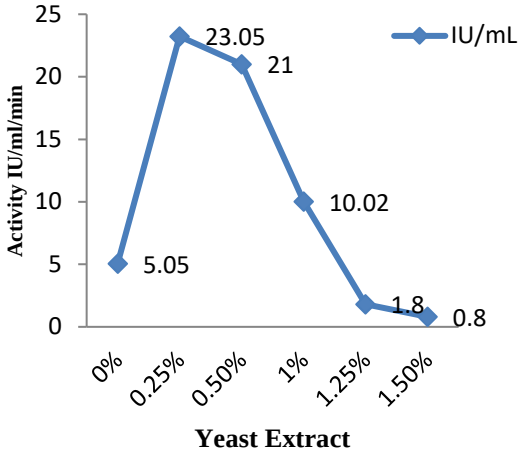

Fig. 13: Effect of various conc. of Yeast Extract

Partial Purification of BsLA

Supernatant obtained from the centrifugation of the fermented broth was subjected to ammonium sulfate precipitation followed by dialysis to purify BsLA partially. The precipitation range of BsLA was associated with the 30-80% fractions, with the highest enzyme activity observed at 80%. The ammonium sulfate precipitation was followed by dialysis, which resulted in a further increase in specific activity.

The stepwise purification of BsLA is summarized in Table 5. This purification process was effective in achieving partial purification of LA. With each purification step, there was a decrease in activity, protein content, and yield, while there was an increase in specific activity and fold purification. After dialysis, the partially purified *Bs*LA had a final protein content of 0.072 mg, approximately 2-fold purity, and a yield of 61%. After dialysis, the total activity of the partially purified BsLA was 14 IU/mL, with a specific activity of 100.22 IU/mg of protein.

Table 5: Purification Profile of *Bs*LA

Sr. no.	Purification Steps	Activity (IU/mL)	Protein (mg)	Specific Activity (IU/mg)	Yield (%)	Fold purity
1.	Crude extract	23.05	0.230	100.22	100	0
2.	ASP*	19.99	0.126	142.7	87	1.42
3.	Dialysis	14.0	0.072	199.4	61	1.99

Molecular weight of partially purified *Bs*LA was determined by performing 12% SDS-PAGE and a protein band of approximately 40 kDa was observed (figure 14).

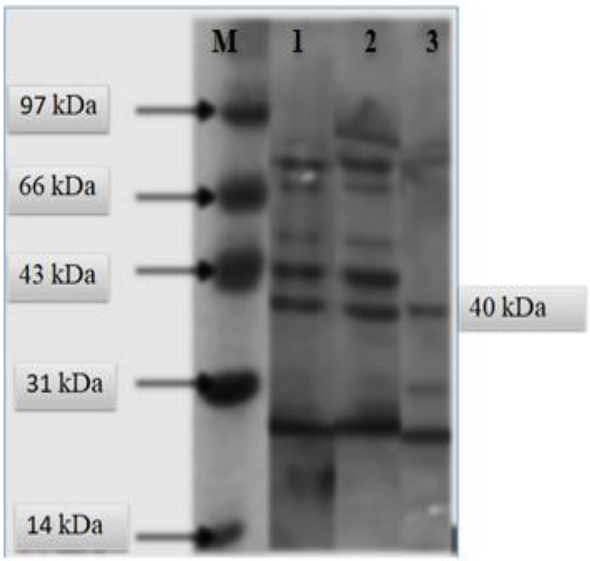

Figure 14: Lane M, Marker; Lane 1 & 2, Crude enzyme fractions; Lane 3, Dialyzed Enzyme sample

CHARACTERIZATION

Effect of Incubation Time on BsLA BsLA activity was investigated at various time intervals ranges from 5-50 minute. There was maximum activity at 10 minutes of incubation. Above or below this time of incubation, there was gradual decrease in enzyme activity (figure 15K)

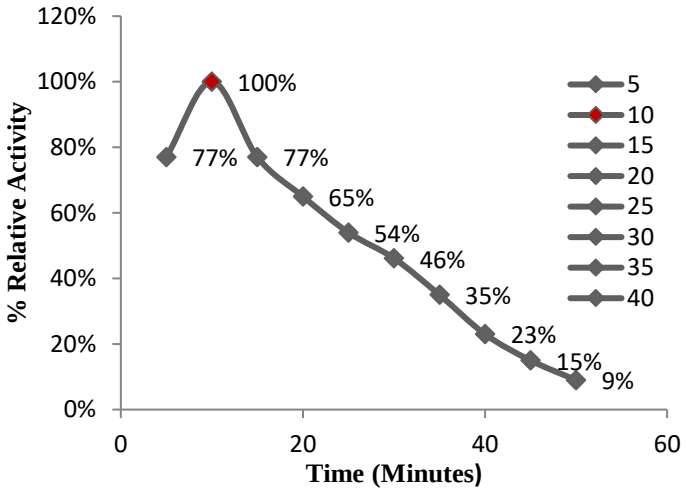

Fig. 15: Effect of incubation time on *Bs*LA activity

[Citation Fatima, H., Hussain, Z., Saleem, R., Shaikh, Z.A., Siyal, S., Jokhio, S.A., Unar, A.A., Unar, K., Shaikh, S.A., Shaikh, A.I., Siyal, F.J. (2023). Biosynthesis, partial purification and characterization of extracellular l-asparaginase from novel bacillus subtilis *subsp. spizizenii* tu-b-10 isolated from GCU garden soil microflora. *Biol. Clin. Sci. Res. J.*, **2023***:* 447. doi: https://doi.org/10.54112/bcsrj.v2023i1.447]

Effect of pH on BsLA Activity

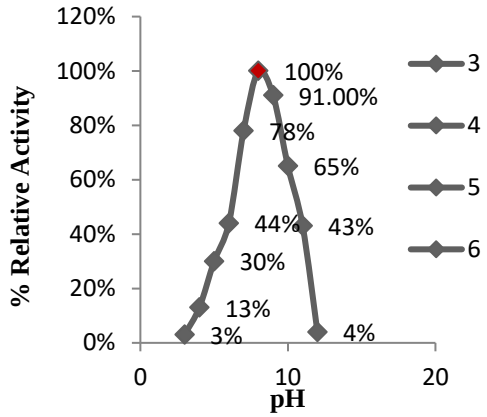

Fig 16: effect of Ph on BsLA activity

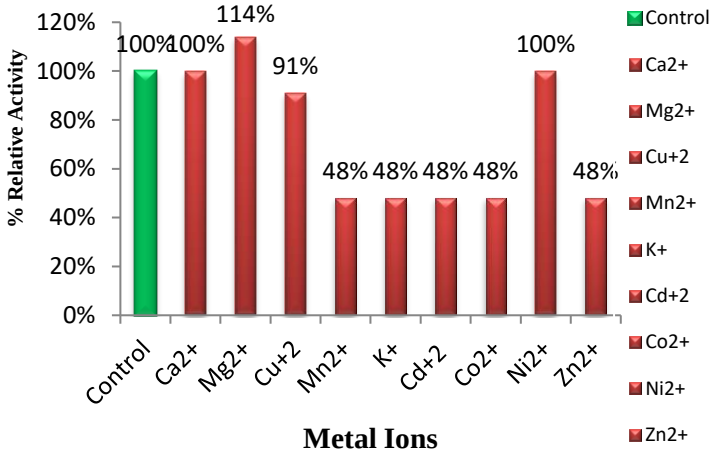

Fig. 18: Effect of various metal ions on *Bs*LA activity

Effect of Temperature on BsLA Activity

Characterization of *Bs*LA was done for various parameters. Each enzyme works its best at a specific pH called its optimum pH. LA exhibited maximum activity (100%) at pH 8 (figure 16). Optimum pH for this enzyme was signified as pH 8.0 as above or below this pH there was a decrease in its catalytic activity. Each enzyme works its best at a specific temperature called its optimum temperature. LA activity assay was performed at temperatures ranges from 27-77 °C. The maximum activity of recombinant LA was observed at 37 °C temperature. Optimum temperature for this enzyme was recorded 37 °C, as above and below this temperature, there was a decrease in its catalytic activity (figure 17).

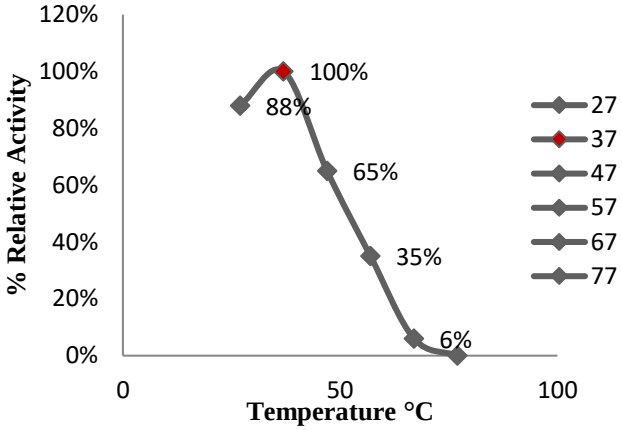

Fig. 17: Effect of temperature on *Bs*LA activity

Effect of Metal ions on BsLA activity

LA activity was investigated in metal ions like Cd^{2+}, Co^{2+}, Ni^{2+}, K^{+}, $Cu^{2+,}$ $Mg^{2+,}$ Ca^{2+}, Zn^{2+} and Mn^{2+}. Mg^{2+} was found to increase asparaginase activity by 14%, while Cd^{2+}, Zn^{2+}, K^{+}, Mn^{2+}, and Co^{2+} were found to decrease the enzyme activity by 52%. Enzyme activity retained 100% in the presence of Ca^{2+} and Ni^{2+}. Residual activity of the enzyme was 90% in Cu^{2+} presence, causing slight inhibition of enzyme activity by 10% (figure 18).

Effect of EDTA on BsLA activity

Effect of various concentrations of EDTA was checked on the *Bs*LA catalytic activity. It was considered a strong inhibitor of BsLA activity as enzyme activity diminishes in the presence of 0.1 M EDTA. There was almost negligible residual enzyme activity (2% and 23%) in the presence of 0.05 and 0.01 M EDTA. There was 5% suppression in enzyme activity in the presence of 0.0001 M EDTA with residual activity of 95%. The residual activity was less than 50% in the presence of 0.005 M EDTA and greater than 50% in the presence of 0.001 M EDTA (figure 19).

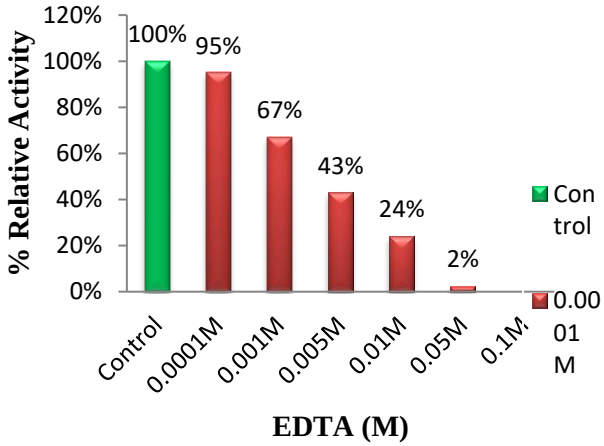

Fig. 19: Effect of EDTA on *Bs*LA activity

[Citation Fatima, H., Hussain, Z., Saleem, R., Shaikh, Z.A., Siyal, S., Jokhio, S.A., Unar, A.A., Unar, K., Shaikh, S.A., Shaikh, A.I., Siyal, F.J. (2023). Biosynthesis, partial purification and characterization of extracellular l-asparaginase from novel bacillus subtilis *subsp. spizizenii* tu-b-10 isolated from GCU garden soil microflora. *Biol. Clin. Sci. Res. J.*, **2023***:* 447. doi: https://doi.org/10.54112/bcsrj.v2023i1.447]

Discussion

Following previous research, this study found that soil microorganisms could produce L-asparaginase. A rapid plate assay, semi-quantitative and quantitative screening, was employed to identify 11 isolates out of 30 isolates with L-asparaginase production potential, and three highly potent isolates were obtained, with SHF-11 exhibiting the highest enzyme activity 20 ± 3.2 of zone index and 10.9 ± 0.14 IU/mL. These screening strategies have been widely employed in the search for potential L-asparaginase-producing microorganisms, as reported by previous studies (*Kumar et al.,* 20119; Darnel *et al.,* 2023; El-Naggar & El-Shweihy, 2020; Alzaeemi *et al.,* 2023; Bakeer *et al.,* 2022). Nazli and colleagues (2021) screened soil microbes for LA production using similar strategies and found that soil samples are best for bacterial isolation producing LA. Soil bacteria are a promising source for anticancer LAs through submerged fermentation. In addition, Nour El-Dein and colleagues (2019) found that extracellular LA activity was highest in submerged fermentation using tryptone glucose yeast (TGY) with 1% asparagine medium. Furthermore, the study characterized the biochemistry and morphology of the isolates, classifying them under the Bacillus genus. Using BLAST analysis and multiple sequence alignment, the study found that SHF-11 was related to *Bacillus subtilis* subsp. *spizizenii* TU-B-1-10. This further strengthens previous studies in LA production and identification, emphasizing the importance of soil sampling and identification techniques when searching for new potential isolates (Darnel *et al.,* 2023; Nazli *et al.,* 2021).

Optimizing various factors affecting LA production enhances the enzyme's productivity. One common optimization approach utilized in the study is the One-Factor-At-A-Time (OFAT) strategy. Other studies have employed similar methods to optimize different LA production variables, including carbon and nitrogen sources and concentration, incubation period, temperature, pH, and agitation rate. Yap *et al.* (2022) used OFAT and response surface methodology (RSM) to identify glucose and L-asparagine concentrations, incubation period, and temperature as the key factors affecting LA production by *C. gloeosporioides*. Our study found that the optimal culture incubation period for maximal LA production was 72 hours. *B. subtilis* reached the highest L-asparaginase production after 72 hours of incubation, consistent with other studies that found optimal incubation times between 48 and 96 hours for various *Bacillus strains*. Notably, the maximal productivity of LA occurs during the stationary phase of bacterial growth (Soliman *et al.,* 2019; Naggar and El-Shweihy, 2020; Niu *et al.,* 2022). Moreover, optimizing the pH is crucial as it affects numerous enzyme processes and the movement of different components across the microbial cell membrane. As demonstrated in previous studies, the optimal pH for LA production varies depending on the specific strain used. In our study, the maximum LA production was observed at pH 7, which is a consistent with the findings of Niu *et al.* (2022).

The incubation temperature also significantly affects LA production, affecting bacterial growth and enzyme production. In this study, incubating at 37°C was optimal for maximal enzyme productivity similar to the LA produced from other *B. subtilis* strains. (Ameen *et al.,* 2020). Producing L-asparaginase using *B. subtilis* at 37°C offers advantages due to its proximity to the human physiological temperature. This closeness helps minimize the potential risks of immunogenicity and toxicity associated with the enzyme when used as a therapeutic agent (Castro *et al.,* 2021).

Nutritional factors such as carbon and nitrogen sources and their concentration also impact LA production. In our study, yeast extract (0.25%) was the optimal nitrogen source. El-Naggar and El-Shweihy (2020) found yeast extract as the best nitrogen source at almost similar concentration for LA production by *Streptomyces rochei.* The role of glucose in LA synthesis is a subject of controversy. Higher glucose concentrations were typically considered to act as a catabolic repressor in bacterial LA synthesis, as observed in *Erwinia aeroideae and Escherichia coli.* This repression may occur through the inhibition of lactate transport components that are involved in stimulating LA synthesis. Sucrose (0.1%) was identified as the ideal sole carbon source. Concentration of salt, inducer, and inoculum size are other parameters that can significantly impact LA production. The optimum inoculum size of 2% was observed optimum for LA production (El-Naggar and El-Shweihy, 2020). Salt concentration is another factor that may affect LA production by *B. subtilis.* Soliman & colleagues (2019) reported the optimized condition for asparaginase production; 1.5% asparagine, 0.2% inoculum size, 0.1 % K_2HPO_4, and 37°C, which were following what we observed in our study.

Optimizing these factors is critical in enhancing LA production for different bacterial strains, potentially leading to novel cancer treatments. The maximal amount of LA enzyme activity achieved from the studied *Bacillus subtilis* strain was 23 IU/mL, which is comparable to the activity of 23.8 IU/mL observed in asparaginase from *Bacillus subtilis* strain hswx88 (Pradhan *et al.,* 2013). Partial purification by employing Ammonium sulfate precipitation followed by dialysis to remove excess salt has been a common

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and widely acceptable strategy. Using similar strategies of partial purification, Alzaeemi *et al.* (2023) enhanced the antitumor potential of LA from *Aspergillus arenarioides* EAN603 by achieving a 2.6-fold increase in its purity. The partially purified enzyme from *Bacillus subtilis* containing the specific asparaginase enzyme showed a specific activity of 680.9 IU/mg with a yield of 49.7% (Ameen *et al.*, 2020). Kavya and Madhu (2019) reported a 779.15 mg/mL activity for partially purified LA produced by endophytic bacteria from *Simarouba Gluaca*. The enzyme's yield and fold of purification vary depending on the source, even though similar sequential purification steps are used (Meghavarnam & Janakiraman., 2015).

The molecular weight of the enzyme was found to be strain-specific. SDS-PAGE analysis of the L-ASNase from *B. subtilis* showed a protein band of approximately 40 kDa, consistent with in silico modeling and previous studies (Agrawal *et al.*, 2021). As the molecular weight of the enzyme is strain-specific, other studies reported molecular weights for LA as 42 kDa for LA from Bacillus sp. (S8) (Poongothai *et al*., 2017), 39 kDa for recombinant enzyme produced by *B. subtilis* (Yano *et al.*, (2008) and 42 and 39.8 kDa for LA from *Erwinia carotovora* MTCC 1428 (Devi and Azmi, 2012) and 45 kDa for partially purified LA from a new strain of *B. subtilis* isolated from sponges of the Red Sea Ameen *et al.*, (2020).

The biochemical characterization of LA revealed its optimal conditions. *Bs*LA showed the highest activity after 10 minutes of incubation at pH 8 and 37°C. Mg2+ was found to be an activator, while EDTA strongly inhibited BsLA activity. Similarly, Abbas *et al*. (2015) reported the highest LA activity after 10 min of incubation at 37 °C, and observed the gradual decrease in enzyme activity by increasing the time to incubation beyond 10 min. Darwesh *et al., (*2022) reported optimum pH of 8 for enzyme action from *Burkholderia pseudomallei*. LA from *Bacillus licheniformis*, T. viride HK01 and *Pseudomonas sp*. PCH199 have similar optimum temperature of 37°C. **(**Alrumman *et al*., 2019; Luhana and Bariya, 2023; Darnel *et al.*, 2023). The enzyme activity decreased by Cu^{2+}, Co^{2+}, K ions Ba^{2+} and Hg^{2+} while Mn^{2+} and Mg^{2} increased the enzyme activity which is consistent with our study. The enzyme activity was inhibited by metal-enhanced the activity (Kabeer *et al.*, 2023; Darnel *et al.*, 2023). Moreover, EDTA acted as inhibitors of LA from *Streptomyces brollosae* NEAE-115 activity reducing its activity by 37.55 (El-Naggar et al., 2018). it was revealed that EDTA decreased the activity of *B. pseudomallei* LA by 60.7 and 41.2%, respectively, at concentrations of 1 mM and 5 mM (Darwesh *et al.*, 2022).

Conclusion

The current study involves the production of LA from novel *Bacillus subtilis subsp. spizizenii* TU-B-10 isolated from soil. Enzyme produced under optimized condition exhibited high levels of specific activity. The partially purified *Bs*LA was resistant to the wide range of pH values and temperature. However, it is also recommended to investigate the production of the desired enzyme in a bioreactor or on a large scale with subsequent purification by combining precipitation and chromatography steps.

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Declarations

Data Availability statement

All data generated or analyzed during the study are included in the manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

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Not applicable

Conflict of Interest

Regarding conflicts of interest, the authors state that their research was carried out independently without any affiliations or financial ties that could raise concerns about biases.

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