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Research Article

Enhanced production of glutaminase-free L-asparaginase by marine *Bacillus velezensis* and cytotoxic activity against breast cancer cell lines

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ABSTRACT

Background: The increasing rate of breast cancer globally requires extraordinary efforts to discover new effective sources of chemotherapy with fewer side effects. Glutaminase-free L-asparaginase is a vital chemotherapeutic agent for various tumor malignancies. Microorganisms from extreme sources, such as marine bacteria, might have high L-asparaginase productivity and efficiency with exceptional antitumor action toward breast cancer cell lines.

Results: L-Asparaginase-producing bacteria, *Bacillus velezensis* isolated from marine sediments, were identified by 16S rRNA sequencing. L-Asparaginase production by immobilized cells was 61.04% higher than that by free cells fermentation. The significant productivity of enzyme occurred at 72 h, pH 6.5, 37°C, 100 rpm. Optimum carbon and nitrogen sources for enzyme production were glucose and NH_4Cl, respectively. L-Asparaginase was free from glutaminase activity, which was crucial medically in terms of their severe side effects. The molecular weight of the purified enzyme is 39.7 KDa by SDS-PAGE analysis and was ideally active at pH 7.5 and 37°C. Notwithstanding, the highest stability of the enzyme was found at pH 8.5 and 70°C for 1 h. The enzyme kinetic parameters displayed V_{max} at 41.49 μmol/mL/min and a K_m of 3.6×10^{-5} M, which serve as a proof of the affinity to its substrate. The anticancer activity of the enzyme against breast adenocarcinoma cell lines demonstrated significant activity toward MDA-MB-231 cells when compared with MCF-7 cells with IC_{50} values of 12.6 ± 1.2 μg/mL and 17.3 ± 2.8 μg/mL, respectively.

Conclusion: This study provides the first potential of glutaminase-free L-asparaginase production from the marine bacterium *Bacillus velezensis* as a prospect anticancer pharmaceutical agent for two different breast cancer cell lines.

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1. Introduction

Globally, breast cancer stands out among other cancers [1], causing high mortality among Third World Women and has a prevalence rate of 21.8% in Saudi Arabia [2,3]. L-Asparaginase is a favorable chemotherapeutic agent for acute lymphoblastic leukemia and strong tumor malignancies [4,5]. Cancer cells are incapable of producing asparagine synthase; therefore, a high amount of asparagine from serum and cerebrospinal fluid is required [6]. L-Asparaginase can hydrolyze asparagine into aspartate and ammonia, thereby causing apoptosis of cancer cells [7]. The formation of secondary tumors through metastasis of breast cancer cells causes most cases of mortality. The change in asparagine accessibility substantially affects the regulation of metastatic headway [6]. Commercial L-asparaginase as a potential chemotherapeutic agent for malignancy is derived from *Erwinia chrysanthemi* and *Escherichia coli* [8,9]. It exhibits glutaminase activity with severe symptoms such as liver diseases, kidney problems, pancreatitis, leukopenia, neurological seizures, and coagulation anomalies [5]. Thus, discovering new microbial sources to produce large amounts of glutaminase-free L-asparaginase with high

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therapeutic activity is highly significant [10]. Recently, marine microorganisms have been considered as producers of L-asparaginase because of their interesting properties and fewer side effects [11,12]. The Red Sea is an extraordinary marine ecosystem occupied by an incredible variety of microbial communities with novel properties for the biopharmaceutical industry; therefore, this is distinct enough to warrant further investigation [13]. There are numerous reports about the potential biological applications of marine *B. velezensis* because it is capable of detoxifying zearalenone in feeds [14], producing biosurfactants, antimicrobial agents [15], and exopolysaccharides that induce apoptosis of malignant breast cells [16]. Submerged and solid state fermentation were utilized for microbial L-asparaginase production [17]. Cell immobilization is a valuable process for upgrading the fermentation industry with more advantages for the facilitating continuous operations, potential reuse of immobilized cells, and reducing the expense of modern procedures through high productivity and proficiency [10,17]. L-Asparaginase purification aims to promote these characteristics and improve its therapeutic uses [18]. The crude enzyme was precipitated by ammonium sulfate, dialysis, and chromatography [19,20,21]. Glutaminase-free L-asparaginase was investigated in terms of the potential applications of L-asparaginase and the requirement for new economic and effective sources of chemotherapy, production, purification, characterization, and anti-breast cancer activity of *B. velezensis*.

2. Materials and methods

2.1. Isolation and screening of bacterial strains

L-Asparaginase-producing bacteria were isolated from sediment samples of the Red Sea, Saudi Arabia, by the Millipore membrane filter method and using asparagine agar medium containing (%) beef extract (0.6), peptone (1), KH_3PO_4 (0.33), L-asparagine (0.1), and agar (20), supplemented with phenol red (0.03%) as an indicator (pH 6.5). The appearance of a pink zone around the colonies after 3 d of incubation at 37°C and 250 rpm was measured [22].

2.2. Molecular identification of the bacterial isolate

For DNA extraction, the isolate was incubated for 24 h under moderate shaking at 200 rpm and then centrifuged for 15 min at 6708 × *g*. Genomic DNA extraction was carried out using the QIAamp DNA Mini kit (Qiagen Inc., Valencia, CA) [23]. The amplification and sequencing of the 16S rRNA gene using universal primers were accomplished. The primers 27 F (5' CCA GCA GCC GCG GTA ATA CG 3′) and 1492 R (5' ATC GG(C/T) TAC CTT GTT ACG ACT TC 3′) were applied to amplify the specified gene using the PCR Master Mix kit (TAKARA, Japan). The 50-μL PCR product was mixed with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, each dNTP at 0.2 mM, 1.25 IU of Taq polymerase, each primer at 2 μM, and 2 μL of DNA template, with the final volume brought up to 50 μL with water. The PCR program involved a denaturation step (94°C for 10 min), followed by 1 min of denaturation at 94°C for 35 cycles, 1 min of annealing at 55°C, 2 min of extension at 72°C, and 10 min at 72°C for the last extension step. The formed PCR product was separated through a 1.5% agarose gel in 1× TBE running buffer for 30 min using electrophoresis unit. The migrated bands of the PCR product were observed under UV light and photographed using a gel documentation system (BIORAD, USA). The remaining PCR product were subsequently submitted to sequencing (Macrogen, Korea). The acquired sequence was aligned and compared with the gene sequences deposited in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences of the amplified 16S rRNA gene of the strain reported in this study have been deposited in the GenBank nucleotide sequence database. The relationship between the acquired sequence and other related sequences was obtained through the construction of a phylogenetic tree using MEGA version 5.0.

2.3. Cell immobilization

The free inoculum (37°C, 250 rpm, 24 h) was centrifuged (10,000 × *g*/20 min), and the separated cells were blended with 3% (w/v) sodium alginate. The mixture was extruded from a pipette (2 mL) into $CaCl_2$ (1.5% w/v). The capsules framed were allowed to solidify for 1.0 h, washed with 0.09% NaCl, and safeguarded in $CaCl_2$ in a cooler at 4°C [10].

2.4. L-Asparaginase production

Enzyme production was carried out in 250 mL Erlenmeyer flasks containing 50 mL of asparagine medium at 37°C/250 rpm in shaking incubator (Shel Lab, SSI 5, USA). The cell suspension of 24 h grown cells in 50 mL of the medium was used as the inoculum for freely suspended cultures (10% v/v). For the immobilized cell cultures, the medium was inoculated with bacterial cells equivalent to those used in the free cultures by 5% (w/v) of the alginate capsules. Thereafter, the centrifuged fermentation broth (10,000 × *g*/20 min) was used as the crude enzyme. Bacterial growth was monitored as the cell dry weight (mg/mL). Conditions of incubation period (12–96 h), pH (5.0–8.0), temperature (25–50°C), agitation (0.0–300 rpm), carbon sources (glycerol, fructose, glucose, maltose, sucrose, dextrin, and starch), and nitrogen sources (asparagine, yeast extract, urea, casein, ammonium sulfate, ammonium chloride, sodium nitrate, and potassium nitrate) were optimized. All experiments were performed in three replicates.

2.5. Enzyme assay

The activity of L-asparaginase was determined by the Nesslerization procedure [24]. The absorbance of mixtures was estimated with Nessler's reagent at 500 nm. Further, L-glutaminase was assayed by the same method using L-glutamine as the substrate. One unit of enzyme activity was defined as the measure of the enzyme that freed 1 μmol of ammonia under assay conditions.

2.6. Protein assay

The protein content of the enzyme was measured according to the Lowry method using bovine serum albumin (BSA) as the standard [25].

2.7. Purification of L-asparaginase

The purification procedure began by adding ammonium sulfate to the crude enzyme at 70%. After centrifugation (4000 × *g* for 20 min), the precipitated mixture was resolved in Tris–HCl buffer (50 mM) and then dialyzed at 4°C. Afterwards, it was stacked onto a 45 × 1.5 cm Sephadex G-100 column and eluted with 0.05 M Tris–HCl buffer and 0.1 M KCl. The protein content and enzyme activity were measured for the eluted fractions [20]. The main fraction was lyophilized and stocked at 4°C.

2.8. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE analysis was performed to determine the molecular weight of the purified enzyme. The samples were suspended in 200 μL of lysis Tris/HCl buffer, SDS (2%), glycerol (15%), 2-mercaptoethanol (5%), and bromophenol blue (0.001%) and stacked onto an SDS polyacrylamide gel (15%). The lysates were boiled, stacked onto a 15% SDS polyacrylamide gel, and kept running at 100 V before staining with Coomassie Blue, and noticeable blue bands were imaged.

Standard protein markers were used to determine the molecular weight of the enzyme protein [21].

2.9. Kinetic properties

The ideal activity of the enzyme was assessed at various pH values (5 to 10) and temperatures (30 to 100°C). In terms of the enzyme's pH stability and thermal stability, the enzyme was incubated at similar pH and temperature values for 1 h and the relative activity was measured. The kinetic parameters K_m and V_{max} were assessed using the Lineweaver-Burk plot [4].

2.10. Anti-breast cancer assay

Breast cancer cells, namely, MCF-7 and MDA-MB-231, were acquired from VACSERA (Egypt). MCF-7 cells were kept in the RPMI-1640 medium, while MDA-MB-231 cells were preserved in DMEM supplemented with antibiotics [Penicillin-G (100 units/mL) and streptomycin sulfate (100 μg/mL)] and 10% (v/v) bovine serum. The viability of the breast cancer cell lines was assessed by the sulforhodamine B (SRB) assay [26]. A 150-μL cell suspension was seeded (2×10^3 cells/well) in 96-well plates, and the plates were incubated at 37°C for 24 h in a CO_2 incubator. The cancer cells were treated with another 150 μL of media containing an enzyme (0.01 to 100 μg) for 72 h. The cells were fixed by supplanting the media with 100 μL of 10% TCA and incubated (at 4°C for 1 h). Further, 0.4% (w/v) SRB solution was added, and the cells were incubated in a dim spot for 15 min. Thereafter, 1% acetic acid was used for washing the plates, which were then air-dried. At this point, 50 μL of Tris–HCl (10 mM) was added, and the absorbance was estimated at 570 nm.

2.11. Statistical analysis

One-way ANOVA was performed, and the significant differences at $P \leq 0.05$ were assayed using Minitab (version 15). The error bars represent the standard error of the mean for $n = 3$. Sigma Plot was used to calculate the IC_{50} values.

3. Results

3.1. Isolation of L-asparaginase-producing bacteria

Twelve L-asparaginase-producing marine bacteria were isolated from sediments of the Red Sea, Saudi Arabia. Only two isolates, namely, KB-9 and KB-11, showed a pink area around the colony with sizes of 2.5 cm and 1.8 cm, respectively, indicating the highest ability to produce L-asparaginase that hydrolyzes asparagine to aspartic acid and ammonia, where the indicator turns from yellow to pink under alkaline conditions.

3.2. Molecular identification of the marine bacterial strain

Identification of the promising bacterial isolate KB-9 principally relies on the amplification and sequencing of the 16S rRNA gene. The selected primers (27 F and 1492 R) were used to amplify the required gene with a total length of 1450 bp. Alignment of the 16S rRNA gene sequences of the isolate along with sequences obtained using BLAST search and MACROGEN results revealed that this strain demonstrated 99% similarity to *Bacillus velezensis*. Various sequences taken from the GenBank database were used to build the phylogenetic tree (Fig. 1). The phylogenetic tree indicated that each isolate was clustered together as one, with more similar strains found in GenBank. The sequence was subsequently deposited in GenBank (GenBank accession no. MG580925).

3.3. L-Asparaginase production

3.3.1. Effect of time course

The difference in the kinetics of L-asparaginase synthesis using free and immobilized cells of *B. velezensis* was investigated (Fig. 2). The development of immobilized bacterial cells expanded progressively until 72 h of cultivation, although, with free cells, gradual growth was noted up to 48 h in the stationary growth stage. An inoculum of immobilized cells was more noteworthy than that of the freely suspended cells by 61.04%. Extension of incubation after an ideal period reduced enzyme production drastically. L-Glutaminase activity of the enzyme preparation was not detected.

3.3.2. Effect of initial pH

The most favorable initial pH value for L-asparaginase production and microbial growth production was 6.5 (Fig. 3). Enzyme secretion at pH 7.0 demonstrated a small decrease of 9.37%, although, at pH 6, a 34.79% drop in the production of the enzyme occurred.

3.3.3. Effect of incubation temperature

The outcomes depicted a critical connection between L-asparaginase production and incubation temperature of up to 37°C, achieving a yield of 7.73 U/mL (Fig. 4). The decrease in enzyme productivity was seen beyond the ideal temperature, with a critical drop occurring at 42°C, achieving 38.80%, which expanded to 64.68% at the highest tested temperature.

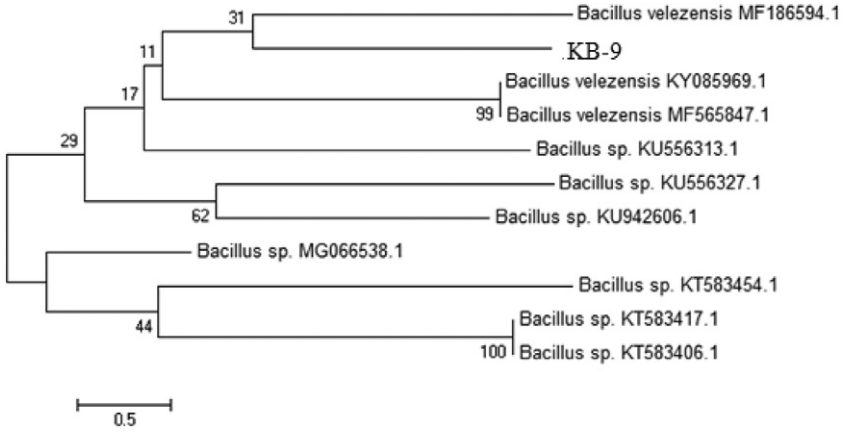


Fig. 1. Phylogenetic relationship between the bacterial isolates; *Bacillus velezensis* (a) and *Pseudomonas pachastrellae* (b) with other 16S rRNA gene sequences of published strains generated by the Neighbor-Joining method using MEGA 5.0 software.

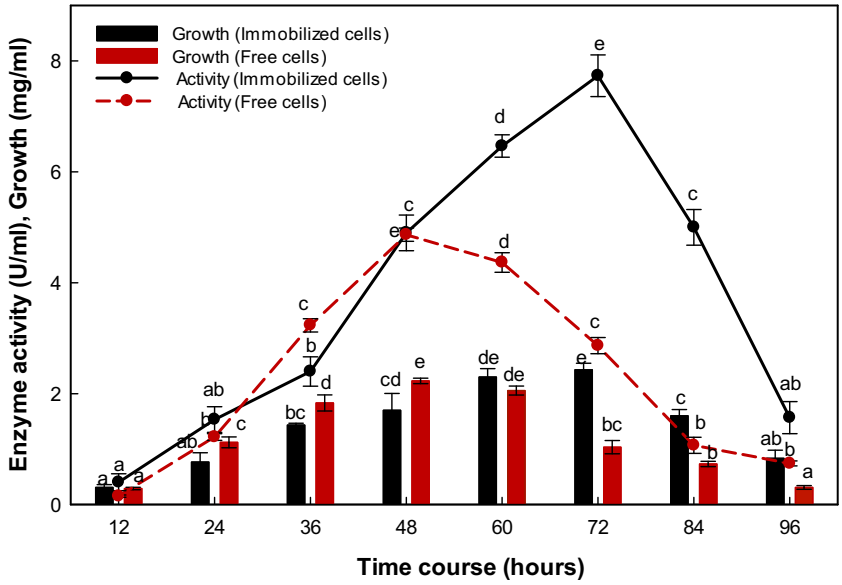


Fig. 2. Effect of time course on bacterial growth and L-asparaginase production using free and immobilized cells.

3.3.4. Effect of agitation rate

The greatest enzyme production occurred at 100 rpm (Fig. 5). Under static condition, enzyme production diminished, achieving only 33.86%. In addition, increase in the agitation rate above the ideal value reduced L-asparaginase productivity.

3.3.5. Effect of carbon sources

The efficiency of *B. velezensis* to utilize different carbon sources for L-asparaginase production was investigated. It was demonstrated that glucose significantly influenced enzyme secretion by expanding production by 9.3% (Fig. 6). Other carbon sources used did not improve the microbial growth and enzyme productivity. The greatest reduction in L-asparaginase was seen with starch and dextrin: enzyme productivity diminished to 29.26% and 34.69%, respectively.

3.3.6. Effect of nitrogen sources

The effect of providing nitrogen sources for the production of L-asparaginase demonstrated that ammonium chloride is the ideal nitrogen source, realizing an increase in enzyme production of up to 23.28% (Fig. 7). Non-noteworthy changes in enzyme productivity were observed using organic sources such as yeast extract, urea, and casein. On the other hand, $NaNO_3$ and KNO_3 decreased enzyme production by 71.30% and 73.62%, respectively.

3.4. Purification of L-asparaginase

Ammonium sulfate precipitation, dialysis, and a Sephadex G-100 column were used for the purification of the enzyme extracted from *B. velezensis* (Table 1). In the obtained 17 fractions, one portion showed enzyme activity. The specific activity and the purity of the enzyme expanded with each progression of purification, although total protein, total activity, and yield diminished relatively. The

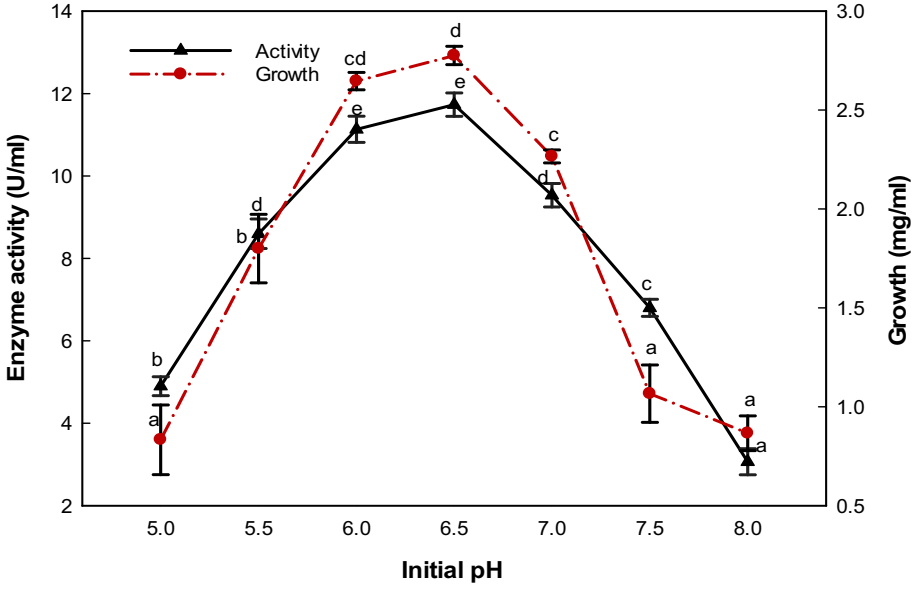


Fig. 3. Effect of initial pH on bacterial growth and L-asparaginase production.

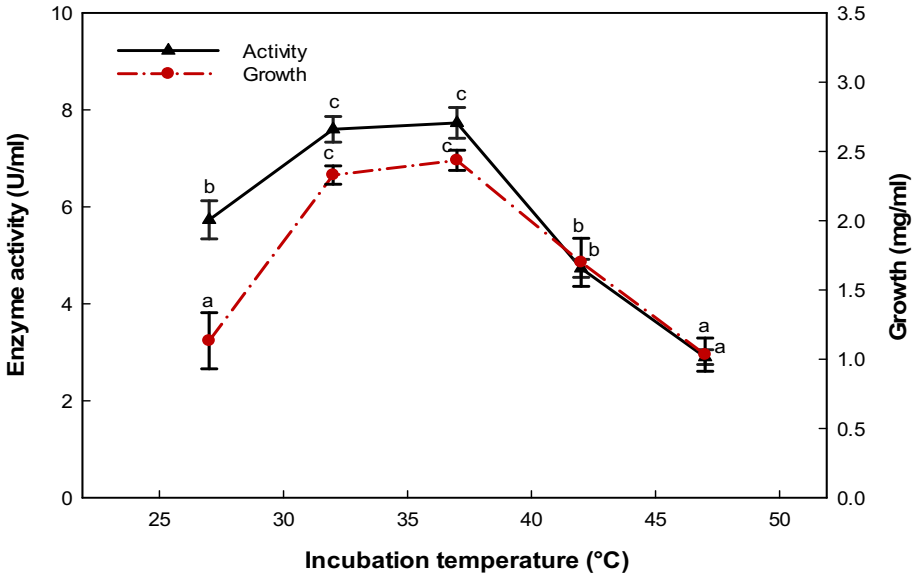


Fig. 4. Effect of incubation temperature on bacterial growth and L-asparaginase production.

purification process yielded 8.04 mg of protein and 255.5 U of enzyme activity with specific activity at 31.77 U/mg, which yielded approximately 4.98-fold purification with 36.41% yield of the enzyme.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to estimate the molecular weight of the purified enzyme. The single particular protein band was seen with a molecular weight of 39.7 kDa, which exhibited the purity of the enzyme (Fig. 8).

3.5. Kinetic properties

L-Asparaginase activity and stability were investigated as a function of pH and temperature. The increase in enzyme activity notably progressed up to pH 7.0–7.5 and then decreased sharply, losing 12.03% of its activity at pH 9.0 and 9.53% at pH 5.0. The enzyme remained stable at a pH range of 7.0–9.0. The rapid fall from its initial activity was seen at pH 10.0, reaching 42.76% (Fig. 9a). Optimum enzyme activity was recorded at 37°C, and thermostability was in the range of 30–70°C (Fig. 9b). The kinetic values of the enzyme were 3.6 $\times$ 10^{-5} M and 41.49 μmol/mL/min for K_m and V_{max}, respectively, as obtained in the Lineweaver Burk plot (Fig. 9c).

3.6. Anti-breast cancer activity of L-asparaginase

Two different breast cancer cell lines (MCF-7 and MDA-MB-231) were exposed to different concentrations of *B. velezensis* L-asparaginase (0.01–100 μg) to assess their antiproliferative effects. The dose–response curve and IC_{50} values were determined using a Sigma Plot as shown in Fig. 10. The toxicity outcome of the enzyme toward malignant breast cells showed a piecemeal rise in the potency of the enzyme. The enzyme displayed antiproliferative activity toward MDA-MB-231 cells relative to MCF-7 cells with an IC_{50} value of 12.6 $\pm$ 1.2 μg/mL and 17.3 $\pm$ 2.8 μg/mL, respectively. After treatment with

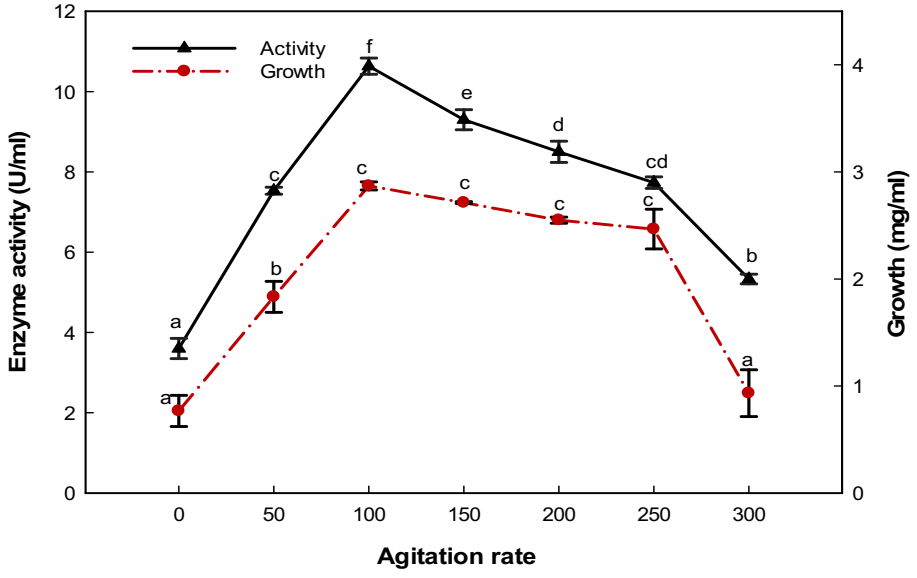


Fig. 5. Effect of the agitation rate on bacterial growth and L-asparaginase production.

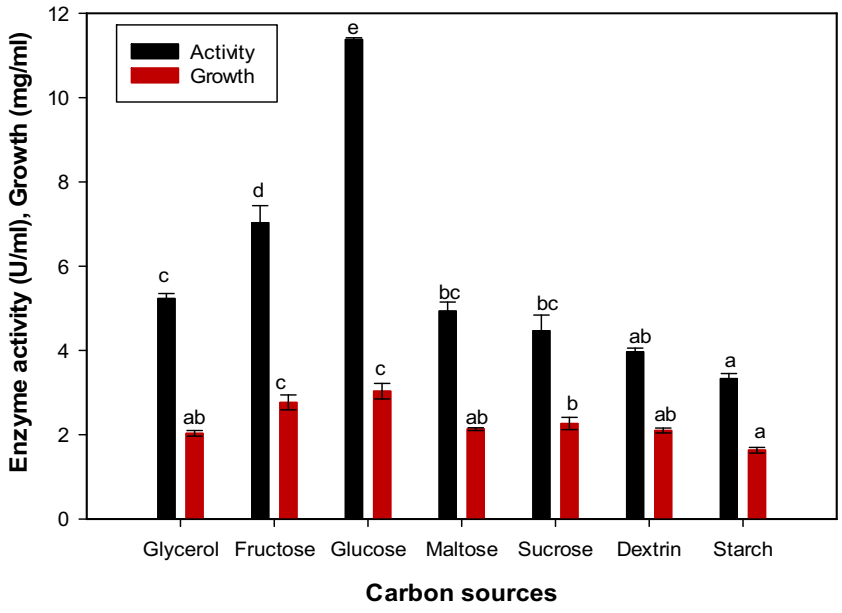


Fig. 6. Effect of carbon sources on bacterial growth and L-asparaginase production.

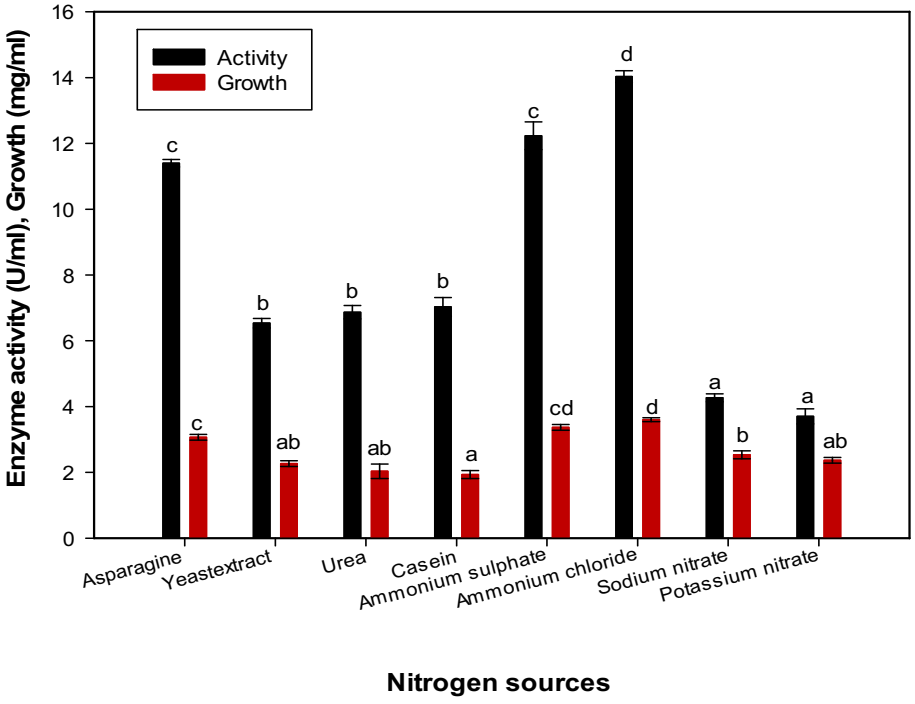


Fig. 7. Effect of nitrogen sources on bacterial growth and L-asparaginase production.

Table 1
Purification procedures of the cell-free extract of *Bacillus velezensis*.

Steps	Enzyme activity (U)	Protein (mg)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude extract	701.5	110	6.37	1.0	100
Ammonium sulfate	586	70.85	8.27	1.29	83.53
Sephadex G-100 column	255.5	8.04	31.77	4.98	36.41

bacterial L-asparaginase, the morphological changes in the cancer cell lines were assessed using an inverted microscope. The cells appeared to be separated from the wells, which indicated cell demise. Likewise, shrinkage, cell rounding, and membrane blabbing of the cells indicated the attributes of cell death.

4. Discussion

The current use of medicinal bacterial L-asparaginase leads to extreme touchiness and poisonous effects through chemotherapy.

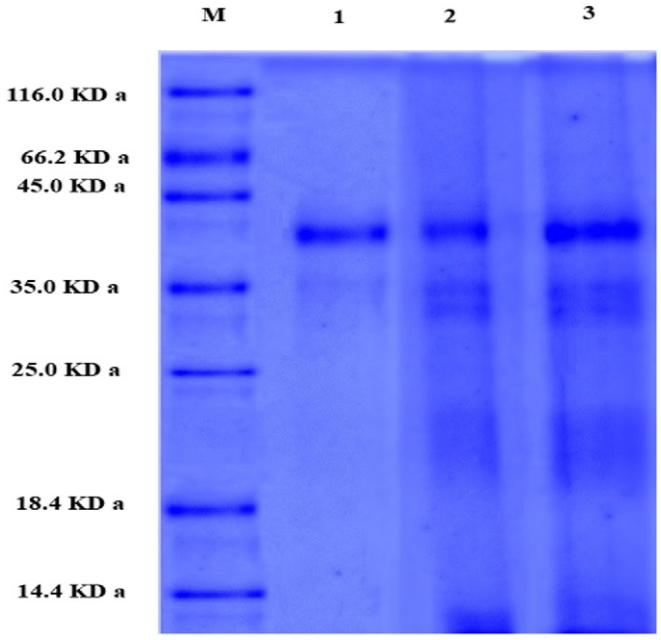


Fig. 8. SDS-polyacrylamide gel electrophoresis of the purified L-asparaginase. Lane 1 (M): Protein marker; Lanes 1–3: Sephadex G-100 (purified enzyme); Ammonium sulfate fractions; Crude enzyme.

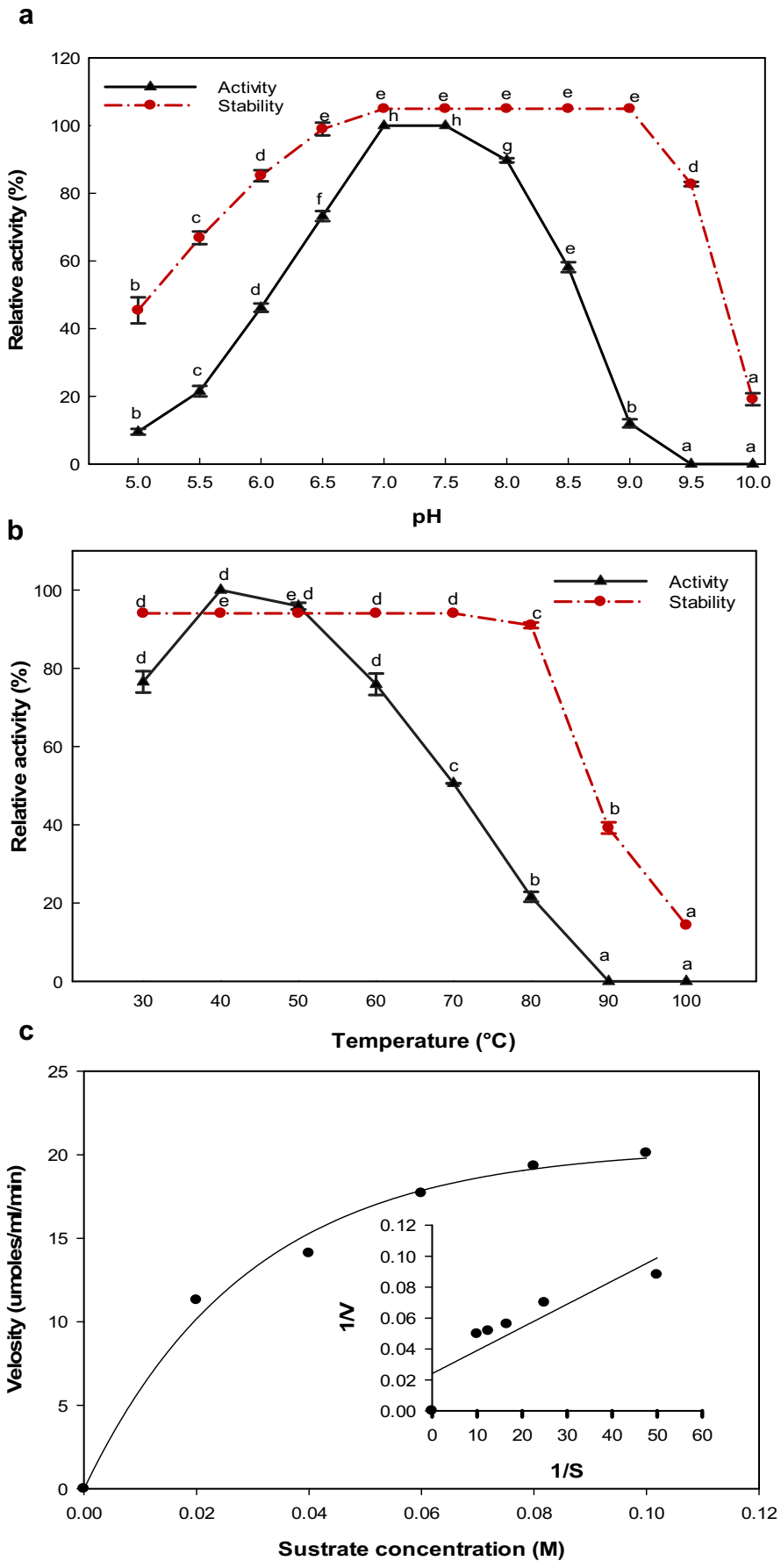
Therefore, there is a pressing need to discover new sources of enzyme production with more effective therapeutic properties [27]. The Red Sea ecosystem is characterized by unique physiochemical and biota properties [10]. These features provide an opportunity to yield unique microbial diversity with unrivaled marine metabolites [12]. The bacterial strains isolated from marine sources represent a propitious source of L-asparaginase production, offering unique therapeutic properties [28]. The promising L-asparaginase-producing marine bacteria *B. velezensis* isolated from the sediments of the Red Sea belong to phylum Firmicutes, which are predominantly found in the Red Sea [29]. The spore-forming bacteria *Bacillus* sp. are prevalent in marine ecosystems because of their high resilience to environmental stresses [30,31].

L-Asparaginase production by immobilized microbial cells represent a key procedure to improve its yield because of the expanded growth of cells in the gel matrix, thus maintaining enzyme activity for an extended period, easy harvesting of products, reuse of the beads, and lower processing costs [10,32,33]. However, nutritional and environmental conditions affect the optimization of L-asparaginase production [34]. Ideal enzyme production was achieved at the stationary stage of bacterial growth at 72 h of incubation. The same outcome was noted for L-asparaginase production by *Bacillus licheniformis* [35] and *Streptomyces albidoflavus* [36]. Enzyme production was optimal at pH 6.5, as aforesaid for *B. subtilis* [37]. The initial pH affected the permeability of nutrients and metabolic components through the cell membrane and is dependent on genetic characteristics [38]. L-Asparaginase production was optimal at 37°C, which is close to human physiological conditions [39]. Similar outcomes have been noted for many *Bacillus* species [37,38,39]. Maximum enzyme production was mentioned at an agitation rate of 100 rpm. The ideal agitation rate for enzyme productivity differs among bacterial strains [28,37]. The oxidation–reduction process is based on microbial growth and the biosynthesis of the enzyme in the submerged fermentation [38,40]. The synthesis of L-asparaginase is affected by carbon sources, where the simple sugar glucose is ideal for bacterial growth and enzyme production, and it supports bacterial metabolism when compared with other sources [35,41]. Ammonium chloride provides an optimum nitrogen source for enzyme production as reported by Baskar and Renganathan [42]. L-Asparaginase production is a nitrogen-regulated operation, and it is more substantial for the organism's growth and enzyme production by affecting the synthesis of nucleic acids and proteins [43]. In summary, under optimized conditions, 14.03 U/mL of L-asparaginase was produced. This result was comparable with the results of other researchers, where it was higher than the L-asparaginase produced by *Bacillus polymyxa*, *Bacillus firmus*, *Streptococcus* spp. D2, and *Paenibacillus validus,* which showed optimum enzyme production of 7.037 U/mL, 5.368 U/mL, 6.006 U/mL, and 4.754 U/mL, respectively [44]. Moreover, the highest L-asparaginase productivity was measured in the marine bacterium *Pseudomonas* sp. PG-Ol, with a yield of 1.6 U/mL [37]. On the other hand, L-asparaginase production by *Bacillus licheniformis* (RAM-8) and *Streptomyces gulbargensis* was approximately 32.26 U/mL and 30 U/mL under optimized conditions [38,45]. Moreover, the production of L-asparaginase from marine *B. licheniformis* reached to 17.4 U/mL [42].

The specific activity and the purity of the enzyme increased step by step with the purification process as mentioned in the findings of El-Naggar et al. [18,21]. The SDS-PAGE analysis showed that the molecular weights of *B. velezensis* L-asparaginase (39.7 kDa) varied slightly according to the source of the enzyme among the *Bacillus* species [46,47]. No L-glutaminase activity was detected in L-asparaginase preparations as reported for various *Bacillus* species [46,47,48]. These outcomes are more advantageous medically because the current enzymatic preparations containing L-glutaminase activity cause many severe symptoms in the liver and pancreas, neurological crisis, and anomalous coagulation [49,50]. The activity and stability of the enzyme in relation to pH and temperature prove to be highly effective under conditions analogous to the human body [19,21]. The ideal pH for L-asparaginase activity and stability was observed to be 7.0 and 9.0, and the ideal temperature for activity and stability was at 37°C and 70°C. These properties are alluring for enzymes utilized as therapeutics [3]. L-Asparaginase is an amidase that is usually active and stable at pH values of 6.0–10.0 [21,46]. The increase in the enzyme's thermal stability can be due to the destabilization of the enzyme protein by alteration of the amino acid charges [47]. The enzyme has a high affinity to the substrate, with a lower K_m value of 3.6×10^{-5} M in addition to a V_{max} value of 41.49 μmol/mL/min as shown by the Lineweaver Burk plot [20]. Our results can be contrasted effectively with K_m and V_{max} values for L-asparaginase from *B. licheniformis* [38] and *Streptomyces fradiae* NEAE-82 [21].

Breast cancer rates are increasing worldwide with a high mortality rates [1,2]. Breast cancer is an intricate and heterogeneous disease, and breast cancer cell lines elicit heterogeneous responses to targeted therapeutic agents [51]. Therefore, two cell lines were used in this study, namely, MDA-MB-231 and MCF-7. L-Asparaginase was more toxic toward the MDA-MB-231 cell line than toward the MCF-7 cell line. The MDA-MB-231 cell line represents a more offensive, invasive, triple-negative breast cancer cell line used in the mediator pathways of metastatic to the bones, the brain, and the lungs, while the MCF-7 cell line is considered hormone sensitive [52]. Asparagine is a key amino acid for breast cancer metastasis, and the spread of malignant cells to different parts of the body can be halted by limiting this nutrient [6]. L-Asparaginase from marine *Bacillus* strains possesses the highest activity against human cell lines: E6-1, MCF-7, and K-562 [38,47]. Moreover, Sirisha and Haritha [51] showed that L-asparaginase from *Streptomyces enissocaesilis* was reported to be a potent therapeutic agent for the breast malignant cells MCF-7 and

Fig. 9. Effect of pH (a) and temperature (b) on the activity and stability of L-asparaginase. The plot of the reaction velocities (V) *vs.* substrate concentration fitted to the Michaelis–Menten equation and determination of the K_m and V_{max} values of the purified enzyme by the Lineweaver-Burk plot (c).


a
Activity
Stability
Relative activity (%)
pH
b
Activity
Stability
Relative activity (%)
Temperature (°C)
c
Velosity (umoles/ml/min)
Sustrate concentration (M)
1/V
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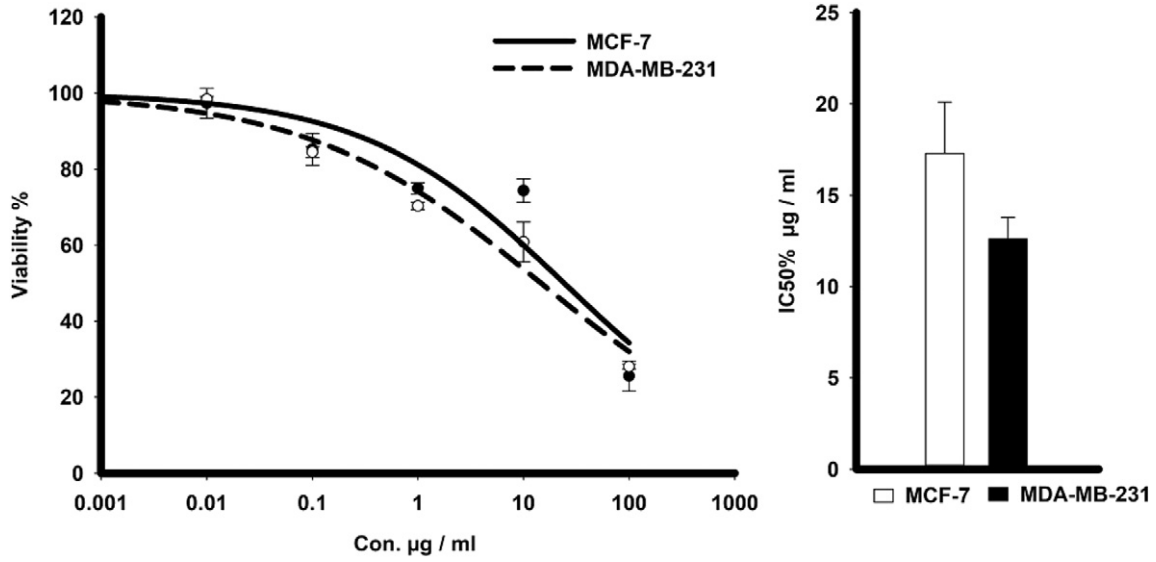

Fig. 10. Dose–response curve of the cytotoxicity of L-asparaginase toward MCF-7 and MDA-MB-231 cell lines. The cells were exposed to enzyme at different concentrations for 72 h. Cell viability was determined by SRB stain (A) and the IC50 values of MCF-7 and MDA-MB-231 cells (B).

MDA-MB435S. The enzyme had a distinct effect on expanding the number of apoptotic cells by hydrolysis of asparagine alongside arrested asparagine-dependent protein synthesis, which caused hindrance in cell growth and diminished the multiplication of malignant cells [4,44]. Furthermore, intracellular asparagine plays a vital role in obtaining the extracellular serine used for the formation of nucleic acids [45]. It also affects cell metabolism by its conversion to oxaloacetic acid involved in the Krebs cycle [53]. Moreover, the proliferation of cancer cells is inhibited by the L-asparaginase which leads to cell apoptosis with the dissipation of mitochondrial membrane potential [26].

5. Conclusion

The marine bacterium *B. velezensis* isolated from the Red Sea is a valuable potential producer of L-asparaginase. Enzyme production by immobilized cells was 61.04% higher than that by free cells under optimized conditions: 72 h, pH 6.5, 37°C, 100 rpm, with glucose and NH_4Cl as carbon and nitrogen sources, respectively. The kinetic characteristics of the purified enzyme appear to have high activity and stability over a wide range of temperatures and pH with parameters of V_{max} and K_m (41.49 μmol/mL/min and 3.6×10^{-5} M), which established high substrate specificity. The profound anti-breast cancer effect of the enzyme against MDA-MB-231 cell line (IC_{50} 12.6 ± 1.2 μg/mL) makes it a potent anticancer agent. This is the main report on L-asparaginase production from the marine bacterium *B. velezensis*, its purification, and its anti-breast cancer activity. Nevertheless, more assessment of its industrial production and *in vivo* adequacy as an anticancer agent in animal and human clinical trials is required.

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Conflict of interest

The authors declare no competing interests.

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