Isolation of asparaginase-producing microorganisms and evaluation of the enzymatic antitumor activity

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Background
L-asparaginase is an enzyme with very high biological activity owing to its activity on several tumor cells. It is mainly used to treat acute lymphoblastic leukemia. The complicated immunogenic adverse effects of present microbial sources present a need for switching to natural novel sources that have no immunogenic effect and better activity of L-asparaginase, so screening for other sources of L-asparaginase, like marine bacteria, may result in an enzyme having fewer adverse effects.
Objective
To screen and identify marine eco-friendly and potent L-asparaginase-producing bacteria, having a novel immunological property that possibly will avoid hypersensitivity.
Materials and methods
In the present study, bacterial strains were screened for extracellular L-asparaginase production from marine isolates, identified by 16 s rDNA technology, and L-asparaginase productivity was assessed using semiquantitative and quantitative enzymatic assays. The antiproliferative effect of the partially purified enzyme on different tumor human cell lines [HepG-2 (human hepatocellular carcinoma cell line), MCF-7 (breast cancer cell line), and PC-3 (prostate carcinoma cell line)] was assessed by the mitochondrial-dependent reduction of yellow MTT.
Results and conclusion
Bacillus safensis was established as the bacterial strain (Gene Bank accession number: MK541039). The extracellular enzyme-yielding capacity of the isolate *B. safensis* (518 IU/ml) was found to be 4.18 times higher than *Bacillus pumilus* (157.03 IU/ml) and higher than *Bacillus circulans* species (85 IU/ml).
The marine isolate is environmentally friendly and can be used to produce significant quantities of extracellular L-asparaginase for the treatment of a variety of tumors and preparation of acrylamide-free fry food.

Keywords:
asparaginase, cancer, culture optimization, extraction, isolation, marine

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Introduction

Asparaginase is nowadays being recognized as an enzyme of important medicinal value as it is widely found in multiple sources like animals, plants, and microorganisms.

L-asparaginase has been well documented to possess antilymphomic, anti-leukemic, and antineoplastic activities. The enzyme L-asparaginase is most used in the treatment of acute lymphoblastic leukemia, Hodgkin disease, chronic lymphocytic leukemia, and melanosarcoma [1]. L-asparaginase produced by microorganisms takes considerable attention, owing to its eco-friendly nature [2].

The recent commercially available asparaginase products produced from microorganisms can lead to hypersensitivity and toxicity during treatment, representing the need to detect new sources of these enzymes [3]. The complicated immunogenic adverse effects of present microbial sources present a need for switching to natural novel sources, like marine bacteria [4].

Presently, marine bacteria are considered a potential source of therapeutic enzymes, metabolites, and natural products, which possess novel properties [5].

The seas and oceans represent more than 70% of the Earth's surface and are considered a rich source of microorganisms and bioactive metabolites [6,7]. The

Red Sea is considered a valuable marine ecosystem with high salinity, water temperature, and microbial diversity in comparison with other tropical seas [8,9]. However, only a few studies have reported the isolation of marine microorganisms from the Red Sea water and sediments.

Marine isolates were proven to be an excellent source for production of L-asparaginase [10]. There are some reports on the production of L-asparaginase from marine sediments [11], as well as estuarine fish's gut [12,13]. Similarly, *Streptomyces noursei* MTCC 10469, isolated from the marine sponge *Callyspongia diffusa*, produces L-asparaginase [14].

In this regard, this study was conducted, and three marine isolates from the Red Sea were used to evaluate the productivity of the L-asparaginase enzyme and its activity on cancer cell lines.

Materials and method

Microorganism

Local marine isolates 12 Y, 12 OR, and 12 W from marine sponge *Phyllospongia lamellosa* were obtained from Culture Collection of the Pharmacognosy Department, Faculty of Pharmacy, Beni-Suef University, Egypt. The microorganisms were grown and maintained by weekly transfer on slants of modified malt yeast extract agar medium [15] adjusted to pH 7.0 and supplemented with 0.4% D-glucose as the only carbon source for growth. Malt yeast extract agar medium was used for bacterial growth (malt extract 1, glucose 0.4, K_2HPO_4 0.05, yeast 0.4, and agar 2), at concentration (wt./vol.) %.

Chemicals and buffers

Anhydrous L-asparagine was obtained from CARLO ERBA (Spain). Nessler's reagent chemicals were obtained from Fluka (USA). Sigma Chemical Company (St Louis, Missouri, USA) provided the trichloroacetic acid, bovine serum albumin, and electrophoresis reagents. Dialysis bag for partial purification was obtained from SERVA (Switzerland). Molecular weight markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were provided by Fermentas Company, the USA.

RPMI 1640 medium was purchased from Lonza Company, Belgium. MTT salt medium was purchased from Sigma Aldrich Company. All other chemicals were of the best analytical grade and high purity. Buffers were prepared according to Gomori [16].

Cell lines

Three cell lines were obtained from the VACCERA collection: HepG-2 (human hepatocellular carcinoma cell line), MCF-7 (breast cancer cell line), and PC-3 (prostate carcinoma cell line) (Egypt).

Rapid plate assay for preliminary screening of L-asparaginase producers

The three marine isolates were tested for L-asparaginase production using a modified M9 medium (composition for 1 l: 6.0 g $Na_2HPO_4{\cdot}2H_2O$; 3.0 g KH_2PO_4; 0.5 g NaCl; 5.0 g L-asparagine; 0.5 g $MgSO_4.7H_2O$; 0.014 g $CaCl_2.2H_2O$; 2.0% w/v glucose; and 20.0 g agar) incorporated with a pH indicator (phenol red) [17]. L-asparaginase activity was identified by a pink zone formed around colonies.

Secondary screening for L-asparaginase producers

The microorganisms chosen based on preliminary screening were further tested. To check for L-asparaginase producers, the two active marine isolates (12 Y and 12 OR) were screened on Modified asparagine glucose salt (AGS) agar, pH 6.8. The composition of the AGS agar medium was L-asparagine 1, glucose 0.1, $MgSO_4.7H_2O$ 0.05, K_2HPO_4 0.05, NaCl 0.05, yeast 0.2, phenol red dye 100 µl (0.009 in methanol), and agar 2, at concentration (wt./vol.) %.

AGS medium was prepared by mixing all the components well, except agar, and pH was adjusted with 1 N HCl to 6.8, and then agar was mixed and medium was autoclaved at 121°C, 15 psi for 15 min. After that medium was allowed to cool down. Plating was done, and a 5-mm plug of 48-h.-old culture was inoculated on AGS agar plates. The plates were then kept at 28±2°C for 2–4 days and were monitored for formation of the pink zone after every 24 h for 4 days. Plates without asparagine and others without the dye were used to control color change.

Study of growth curve and effect of different medium composition on isolates showing maximum L-asparaginase productivity

To study the effect of different media composition on isolates showing maximum L-asparaginase productivity, two different medium compositions were prepared as follows:

Medium 1: L-asparagine 0.05, glucose 1, $MgSO_4.7H_2O$ 0.05, K_2HPO_4 0.05, yeast 0.2, pH 7, at concentration (wt./vol.) %.

Medium 2: L-asparagine 0.1, glucose 1, $MgSO_4.7H_2O$ 0.05, K_2HPO_4 0.05, yeast 0.2, pH 6.8, at concentration (wt./vol.) %.

Overall, 100 ml of medium 1 and medium 2 was prepared and autoclaved. Then, 1 ml of inoculum (24 h old in malt yeast broth) was added and incubated in a shaker. Every 24 h, samples were withdrawn for measuring culture density indicated by optical density at 600 nm and enzyme activity indicated by I.U. as calculated at 436 nm.

Crude enzyme production by submerged fermentation on the chosen medium

For the preparation of fermentation, an inoculum of 24-h-old medium was inoculated in 15-ml malt yeast medium composition without agar.

The fermentation was carried out in a 1-l Erlenmeyer flask. Overall, 400 ml of production medium (chosen medium) was poured into the flask, and the flask was sterilized in the autoclave at 121°C and 15 lbs pressure and then cooled at room temperature. One milliliter of bacterial inoculum (24 h old) was transferred to a flask. The flask was then placed in the rotary incubator shaker oscillating at 150 rpm at 30°C for 96 h. At the end of the fermentation period, the crude enzyme was prepared by centrifugation at 10 000 rpm for 20 min. The crude enzyme was the cell-free supernatant [18].

Enzymatic assay (determination of enzyme activity)

The activity of L-asparaginase was determined using the method described by Imada *et al.* [19]. The reaction was started by mixing 0.1 ml of the crude extract with 0.1 ml of 0.04 M L-asparagine and 1 ml of 0.05 M tris (hydroxymethyl) aminomethane (tris-HCl) buffer, pH 8.6, and incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.1-ml 1.5 M trichloroacetic acid.

The ammonia released is measured by adding 0.5 ml of Nessler's reagent to tubes containing 0.2-ml supernatant and 4.3-ml distilled water, incubated at room temperature for 10 min, and then the absorbance of the supernatant was read with a ultraviolet-spectrophotometer at 436 nm.

The liberated ammonia content was determined, and one unit of asparaginase is the amount of enzyme required to catalyze the formation of 1 μmol ammonia per minute at 37°C.

Partial purification of L-asparaginase

The purification was carried out using crude enzyme extract [20]. The partial purification protocol includes two steps: ammonium sulfate precipitation and dialysis at 0–4°C unless otherwise mentioned.

Ammonium sulfate precipitation (step I)

A trial was run to determine the optimal concentration required for the enzyme precipitation with various concentrations of ammonium sulfate. For this purpose, the supernatant obtained after centrifugation was subjected to ammonium sulfate precipitation. Ammonium sulfate was added in various concentrations ranging from 20 to 80% w/v saturation. The obtained precipitate was suspended in Tris buffer solution (2 ml) and tested for L-asparaginase activity and total protein content. The salting-out concentration of crude enzyme was established based on enzyme activity. To obtain complete precipitation of the crude enzyme, the remaining harvested fluid was subjected to ammonium sulfate precipitation at 80% w/v saturation.

Dialysis (step II)

The formed precipitate was separated by centrifugation at 10 000 rpm at 4°C for 15 min, resuspended in cold 50 mM Tris-HCl buffer (100 ml), and dialyzed overnight against 1 l of 50 mM Tris-HCl buffer of pH 8.6 with three changes every 8 h using a dialysis membrane of 12–14 kD cut off-limit. Following dialysis, the solution was centrifuged, and the supernatant was lyophilized and stored at 4°C for future use.

Protein estimation in crude and purified enzyme

Total protein content was estimated by using bicinchoninic acid (BCA) assay [21]. First, the standard curve of bovine serum albumin (BSA) was made. Different dilutions of BSA ranging from 0.02 to 1 mg/ml were prepared by adding an adequate volume of BSA stock solution (1 mg/ml) and double-distilled water in test tubes. BCA Working Reagent (WR) was made by calculating the total volume of WR required. The WR was made by combining 50 parts BCA reagent A and one part BCA reagent B (50 : 1, reagent A : B) (the mixture appears to be a clear and green solution).

Each sample requires 2.0 ml of the WR for measurement in a test tube method. The sample-to-WR ratio was set to 1 : 20. Tubes were incubated at 37°C for 30 min and before being measured were kept at room temperature for 10 min The absorbance was measured at 562 nm. All of the readings were taken in triplicate. The absorbance versus concentration graph was used to deduce the protein content of the unknown protein sample.

Molecular weight determination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The molecular weight of the extracted enzyme was determined by performing SDS-PAGE according to the method of Laemmli [22], with a separating acrylamide gel of 10% and stacking gel of 5% containing 0.1% SDS. After the 2-h electrophoresis, the gel was stained in 0.025 Coomassie brilliant blue R-250 for 2 h and then destained several times for 2 h with a 4 : 1 : 5 solution of methanol, acetic acid, and water. The molecular weight of the purified L-asparaginase was determined in comparison with standard molecular weight markers (molecular mass range: 14-20-44-67-97-116-200 kD).

Antitumor activity

The antiproliferative effect of the partially purified enzyme on different tumor human cell lines [HepG-2 (human hepatocellular carcinoma cell line), MCF-7 (breast cancer cell line), and PC-3 (prostate carcinoma cell line)] was assessed by the mitochondrial-dependent reduction of yellow MTT (3-(4, 5-dimethylthiazol-2- yl)-2, 5-diphenyl tetrazolium bromide) to purple formazan [23].

All procedures were carried out in a sterile environment using a biosafety class II Laminar flow cabinet (Thermo Fisher Scientific Inc., USA). Each of the three cell lines was seeded in 96-well microtiter plastic plates with fresh complete growth medium at a concentration of 104 cells/well for 24 h at 37°C. under 5% CO_2 using a water-jacketed carbon dioxide incubator (Thermo Fisher Scientific Inc.). For background absorption, some wells were remained cell-free, that is, as blank control. The medium was aspirated, fresh medium (without serum) was added, and cells were incubated either alone (negative control) or with different concentrations of partially purified L-asparaginase dissolved in DMSO to give a final concentration of 620-310-155-77.5-38.75-19.38-9.69-4.84-2.42-1.21-0.61 IU/ml. The cells were suspended in RPMI 1640 medium containing 1% antibiotic-antimycotic mixture (10 000 U/ml potassium penicillin, 10 000 g/ml streptomycin sulfate, and 25 g/ml amphotericin B) and 1% L-glutamine in a 96-well flat-bottom microplate at 37°C under 5% CO_2. After 48 h of incubation, the medium was aspirated; 40 µl of MTT salt (2.5 µg/ml) was added to each well and incubated for another 4 h at 37°C with 5% CO_2. To stop the reaction and dissolve the formed crystals, 200 µl of 10% SDS in deionized water was added to each well and incubated overnight at 37°C.

Positive control of adriamycin (doxorubicin) (Mw=579.99) in concentrations of 100 µg/ml was used as a known cytotoxic natural agent with 100% lethality under the same conditions [24].

The absorbance was measured using a microplate multiwell reader (Berthold Technologies, Germany) at 595 nm and a reference wavelength of 620 nm. The percentage of viable cells was calculated from the formula: survival fraction=optical density of treatment cell/optical density of control cells. The IC50 was calculated by fitting the survival curve using GraphPad Prism software [25].

Identification of 12 Y marine isolate by 16SrRNA sequencing

Bacterial DNA isolation and purification were performed using a bacterial genomic DNA isolation kit. The isolated DNA was then amplified by PCR. The PCR was carried out using the PCR Master Mix Kit. The size and purity of the genomic DNA were analyzed by agarose gel electrophoresis. The PCR product was directly sequenced using the genetic analyzer. RNA sequences were compared with the already submitted sequence in the NCBI database using BLAST software and a phylogenetic tree was viewed using MEGA7 software to analyze evolutionary relationships among sequences of isolated microorganisms and nearest neighbors.

Results and discussion

To obtain pure cultures, the isolates were subcultured on Malt Yeast extract agar and stored at 4°C for future use. The isolates released characteristic color on colonies, and on this basis, they were named 12 Y (Yellow), 12 W (White), and 12 OR (Orange).

Screening for L-asparaginase production

The three isolates were preliminarily tested for asparaginase productivity by inoculating them on a modified M9 medium in which phenol red was incorporated as an indicator for pH change in 24-h intervals. The yellow color background turns pink in color after 48 h, which implies the test was positive.

Of the supplied three marine isolates, two showed in preliminary screening (qualitative) on M9 active enzyme productivity. L-asparaginase activity was detected by the formation of red color around the colony, as shown in Fig. 1. Similarly, Kamble *et al.* screened efficient L-asparaginase-producing bacteria [26]. Similar screening of L-asparaginase production

by rapid plate assay was reported in bacterial strains such as *Bacillus circulans* [27], *Streptomyces sp.* PDK7 [28], and *Streptomyces sp.* [29].

For more screening, the two active strains were further grown on modified AGS medium with and without asparagine and with and without dye for 24-h intervals. It was found that the two active strains were 12 Y and 12 OR, as shown in Fig. 2.

Diameter of zone of activity of 12 Y and 12 OR in centimeter

The zone of activity was calculated by measuring the colored zone, and it was noticed that 12 Y is the most active one as shown in Fig. 3a and b.

Study of growth parameters

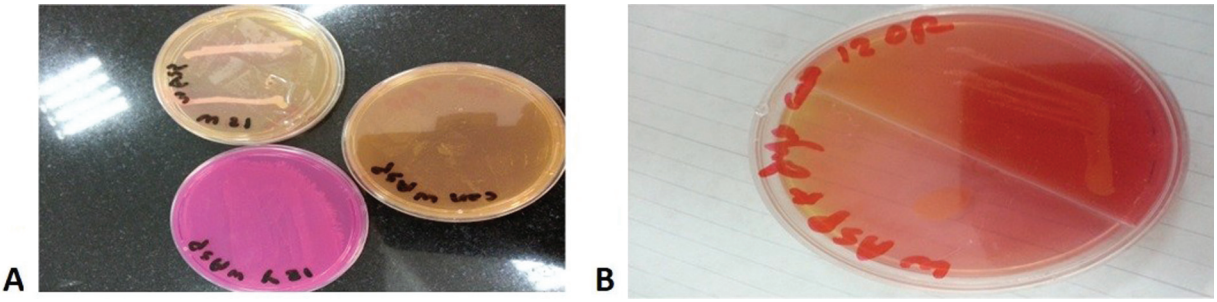
Each organism has its requirements for maximum enzyme production. As a result, specific medium optimization was performed to maintain a balance between the various medium components, thereby minimizing component utilization at the end of fermentation [30]. An important factor to be monitored while developing a production medium is the cost-effectiveness of the medium.

The incubation period is one of the most important parameters in submerged fermentation conditions. In general, most of the enzymes are synthesized and secreted as proteinaceous molecules. They are active in the range of 24–96 h. Only after a certain incubation period could maximum enzyme production be obtained, allowing the culture to grow at a steady state [31]. Enzyme secretion of each strain relies on the specific growth rate of the strain.

Growth rate and enzyme synthesis of the culture are the two key features that are mostly influenced by incubation time [32].

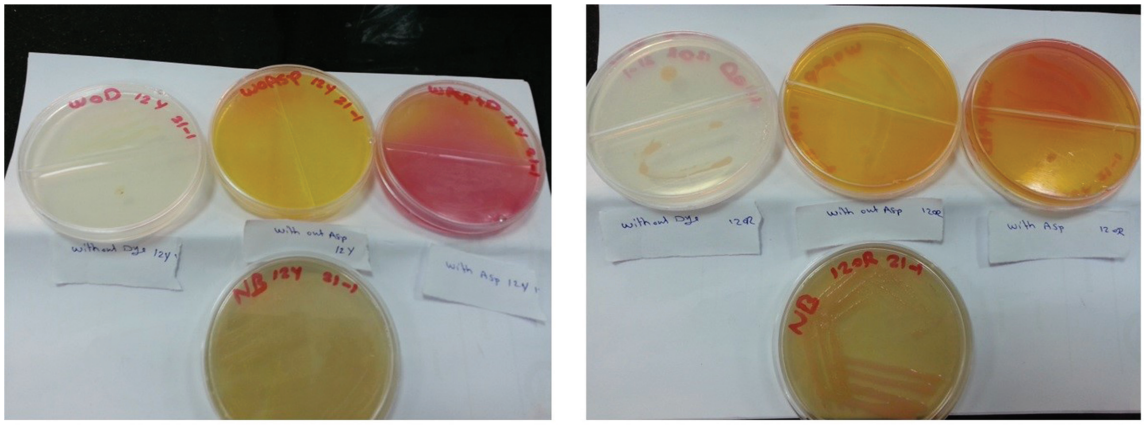
In the present study, the growth curve of the two active strains was studied on two media (identified as medium 1 and medium 2) by taking its absorbance reading at 600 nm at 24-h interval and along which

Figure 1



Activity of asparaginase on M9 media (A) show positive 12 Y(pink color) and 12 W and control (yellow) (B) show 12 OR positive (pink color).

Figure 2

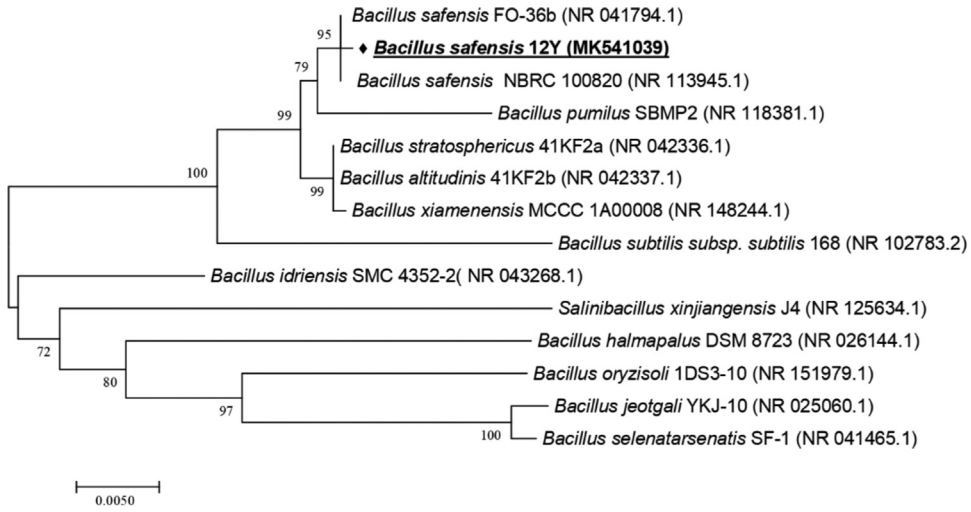


Asparagine Glucose salts (AGS) media of 12 Y without dye(left), without asparagine (center) and with asparagine and dye right. below NB of bacteria.

Asparagine Glucose salts (AGS) media of 12 OR without dye(left), without asparagine (center) and with asparagine and dye right. below NB of bacteria.

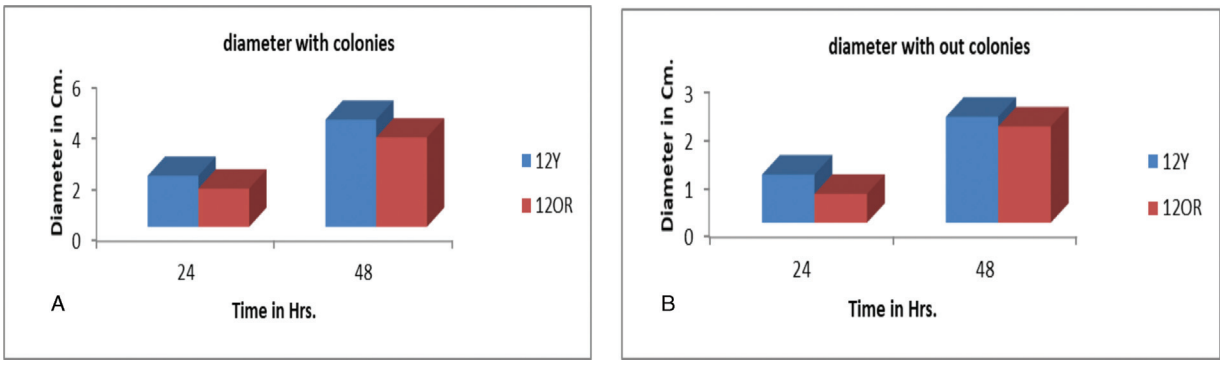
Asparagine Glucose salts (AGS) media.

Figure 3



Evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length=0.17099107 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1355 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Figure 4



The zone of activity for 12 Y and 12 OR asparaginase was calculated by measuring the colored zone. (a) diameter with colonies and (b) diameter without colonies.

the activity was assayed, as done by Deshpande *et al.* [33]. The stationary phase of growth was observed between third and fourth day, as shown in Fig. 4a and b.

The maximum enzyme production by 12 Y and 12 OR was obtained at 96 h (540 and 232 U/ml, respectively, in medium 1 and 635 and 459 U/ml, respectively, in medium 2). In the same way, asparaginase production by bacterial strains with optimum yield was detected by *Streptomyces marcescens* and *Bacillus methylotrophicus* at 96 h [34], for *Bacillus sp.* R36 at 24 h [35], for *Bacillus subtilis* at 36 h [36], and for *Lactobacillus salivarius* at 120 h [37].

Similarly, a previous study [38] has stated that maximum activity was obtained at 120 h of incubation from the fungal endophytes of seaweeds. Comparing the activity, 12 Y on medium 2 was the best to produce the enzyme and with optimum growth.

16 S rRNA gene sequence comparisons and phylogenetic analysis of 12 Y marine isolate

The 16 S rRNA gene sequence (1361 bp) was determined for strain 12 Y. A BLAST search of the GenBank database using this sequence revealed similarities to many species of the genus *Bacillus sp.*

Using MEGA7, a phylogenetic tree based on 16 S rRNA gene sequences of *Bacillus sp.* members was constructed using the neighbor-joining method of Saitou and Nei [39,40] as shown in Fig. 5.

The potent strain was known as *Bacillus safensis*, and it was close to the member of the genus *Bacillus sp.* and showed 99.78% similarity in sequence and homology

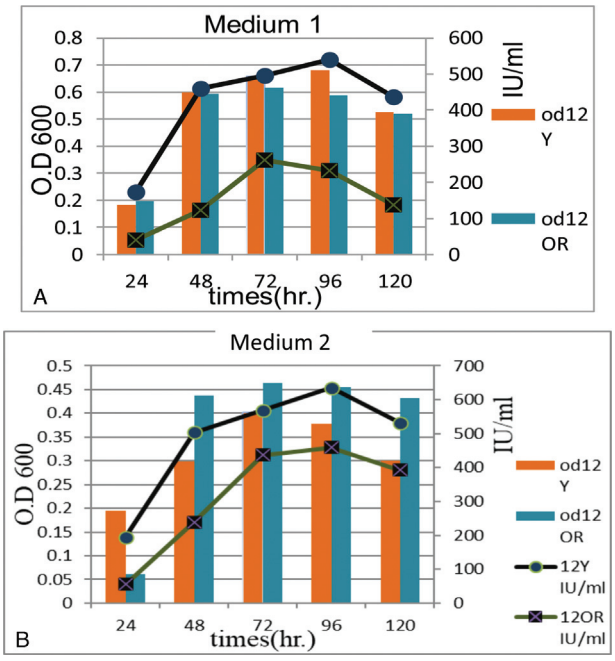
with *B. safensis* strain FO-36b (NR 041794.1) and *B. safensis* strain NBRC 100820 (NR 113945.1). The sequences were submitted to Gene Bank and were assigned a Gene Bank accession number as MK541039.

B. safensis belongs to the phylum Firmicutes. Firmicutes are one of the main bacterial phyla in the Red Sea like Proteobacteria, Fusobacteria, Bacteroidetes, and Spirochetes [11]. Asparaginase has been recognized in some marine bacteria while the most copious bacterial isolates were *Bacillus sp.* and *Pseudomonas sp.* [41]. The copiousness of *Bacillus sp.* throughout marine habitats is related to its ability to produce spores, which display high resistance to environmental stresses [42].

Production of L-asparaginase from *Bacillus safensis* MK541039

Using screening data, the culture with maximum activity was used to produce L-asparaginase enzyme using shake flask fermentation as done by Priya *et al.* [43]. Production of L-asparaginase by submerged fermentation yielded crude enzyme with an activity of 657 IU/ml. This was found to be 4.18 times higher than *Bacillus pumilus* (157.03 IU/ml) and higher than *B. circulans* species (85 IU/ml), *Bacillus licheniformis* MTCC 429 (71.11 IU/ml), *B. subtilis* strain hswx88 (23.8 IU/mL), *Bacillus sp.* R36 (20.15 U/ml), and *Bacillus sp.* WF67 (11.82 U/ml).

Figure 5



Growth curve of the two active strains was studied on two media, identified as medium 1 (a) and medium 2 (b), by taking its absorbance reading at24-h interval and along which the activity was assayed.

Crude L-asparaginase production from marine *Streptomyces sp.* PDK7 with 374.6 IU total activity and 489.5 mg total protein was reported by Dhevagi and Poorani [28]. Lower production of crude L-asparaginase from marine *Streptomyces sp.* (S3, S4, and K8) with total activity of 61.53 IU and total protein of 0.16 mg was also stated by Basha *et al.* [29]. Varying amounts of L-asparaginase were testified in many genera of marine microorganisms [44].

During the process of production and partial purification, activity and protein content were assayed; the results are shown in Table 1.

Purification

Purification of *B. safensis* MK541039 L-asparaginase was achieved by using 80% ammonium sulfate saturation and dialyzed using 50 mM Tris-HCl buffer of pH 8.6 to remove excess salt. The specific activity of an enzyme (units/mg) is an indicator for purity, as its value increases as the purity of the enzyme increases as the amount of protein contaminants characteristically declines.

The enzyme was purified 4.6 times while its specific activity increased from 41.2 to 189.3 IU/mg for the crude extract and the final dialysis, respectively. This specific activity of the partially purified enzyme was around 5.24 times of *B. licheniformis* (36.08 U/mg), whereas it is significantly higher as compared with L-asparaginase from *Streptomyces fradiae* NEAE-82 (30.636 U/mg) [45].

Electrophoretic separation of the partially purified enzyme

Using a standard wide-range molecular weight marker (14–200 kD), the approximate molecular weight of

Table 1 Purification profile of L-asparaginase from *Bacillus safensis* MK541039

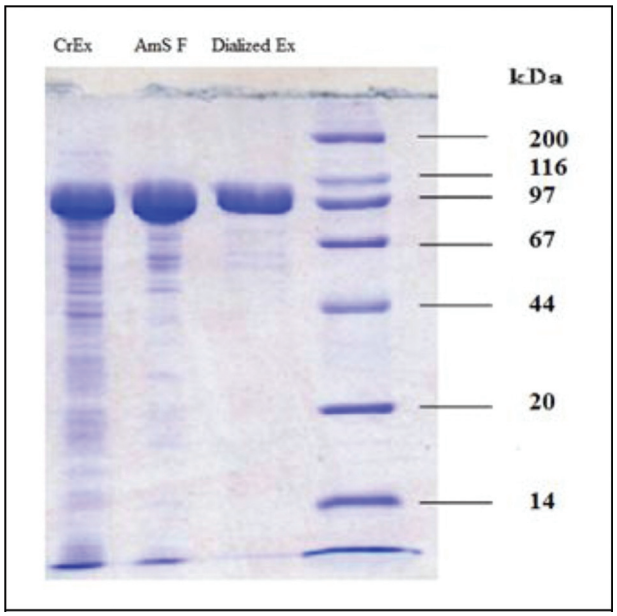
Steps	Collected volume (ml)	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification (fold)	Yield (%)
Crude extract	300	155 400	3768	41.2	0.00	100
(NH_4SO_4) precipitation	200	112 400	1480	75.9	1.84	72.3
Dialysis	100	65 700	347	189.3	4.6	42.3

Yield=total activity of step/total activity of crude.
Purification fold= specific activity of step/specific activity of crude.

L-asparaginase was determined. The extracted enzyme molecular weight was determined using the Laemmli [22] method of SDS-PAGE, with a separating acrylamide gel of 10% and stacking gel 5% containing 0.1% SDS. In comparison with standard molecular weight markers, the molecular weight of the partially purified L-asparaginase was determined and it was found to be nearly 97 kD, as shown in Fig. 6.

The molecular weight of L-asparaginase was found to vary depending on the source of enzyme, such as 37 kD in *B. licheniformis*, 80 kD in *Corynebacterium glutamicum*, 140 kD in *Streptomyces sp.* PDK223, and 116 kD in *S*treptomyces *albidoflavus* [46]. SDS-PAGE analysis of purified L-asparaginase from *Streptomyces tendae* reveals a protein band around 97.4 kD. *E*scherichia *coli* functional L-asparaginase is a homotetramer with a molecular weight of about 142 kD [47].

Figure 6



Molecular weight and purification steps of L-asparaginase on SDS gel from the left side: CrEX resembles crude extract of the enzyme, AmSF resembles ammonium sulfate purification fraction, dialized Ex resembles the dialyzed fraction, and finally, the biomarker proteins. All fractions show a band to be nearly 97 kD. SDS, sodium dodecyl sulfate.

Enzyme activity

After each step of production and partial purification, the enzyme activity was assayed using nesslerization method and evaluated as shown in Fig. 7.

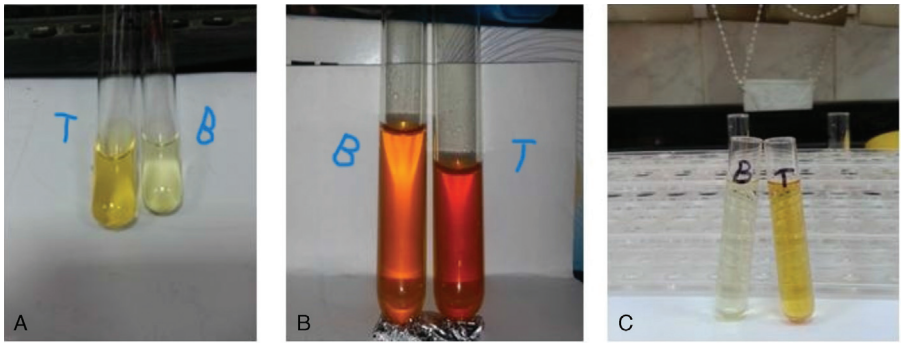
Cytotoxic activity of the partially purified extracts

Tumor rates are increasing worldwide; in Egypt, liver cancer ranked first among distributed cancers followed by breast cancer [48]. Prostate cancer is a growing concern in Egypt and currently ranks as the fourth most common cancer in the country [49]. The antitumor action of the asparaginase produced by *B. safensis* in this study was estimated for tumor cell lines.

Results showed that L-asparaginase has a higher effect in inhibition against HepG-2 and PC-3 but a lower effect against MCF-7 cancer cell line (Fig. 8). The effect of L-asparaginase on HepG-2 and PC-3 cancer cell line in vitro revealed that L-asparaginase is capable of inhibiting the cell proliferation of human HepG-2 and PC-3.

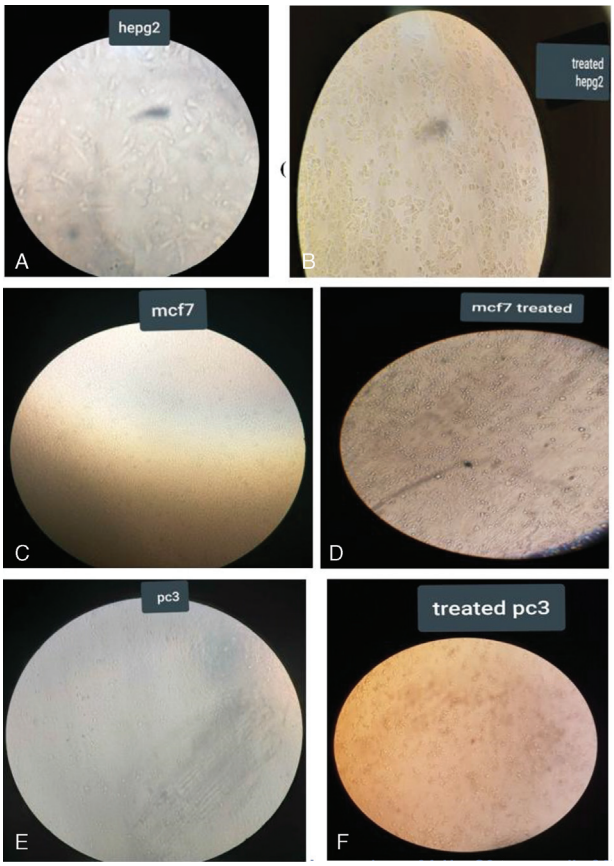
Therefore, (Using MTT assay) cytotoxic effect on the cell lines was further evaluated using a partially purified enzyme using doxorubicin as a positive control. Doxorubicin is known to induce apoptosis in several cancer cells [24]. Different concentrations of the partially purified extracts (620, 310, 155, 77.5, 38.75, 19.38, 9.69, 4.84, 2.42, 1.21, and 0.61 IU/ml) were incubated with the cells for 24 h. The partially

Figure 7



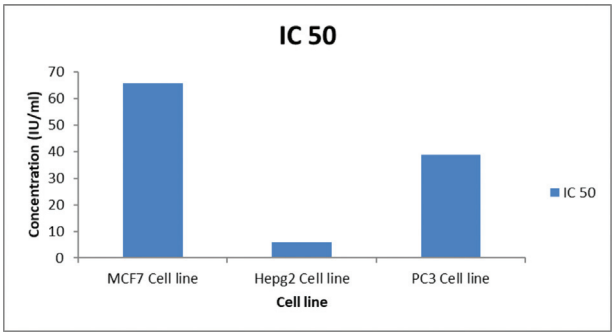
The activity obtained from nesslerization B (blank) and T (tested enzyme) (a) Crude enzyme, (b) ammonium precipitation fraction, and (c) dialyzed fraction.

Figure 8



Control and treated tumor cells with L-asparaginase 9A HepG-2 tumor cells and 9B treated HepG-2 tumor cells, 9C MCF-7 tumor cells and 9D treated MCF-7 tumor cells, and 9E PC-3 tumor cells and 9F treated PC-3 tumor cells.

Figure 9



IC50 curve representing that the highest cytotoxic activity for *Bacillus safensis* asparaginase was found on the Hep-G2 cell line, as it has lowered IC50 values of compared with the IC50 values of PC-3 and MCF-7.

purified enzyme revealed a significant concentration-dependent decrease in the cell viability of the three cancer cell lines. The highest cytotoxic activity was found on the Hep-G2 cell line, as it has lowered IC50 values of 5.718±0.03 IU/ml compared with the IC50 values of 38.75 and 65.53 of PC-3 and MCF-7, respectively (Fig. 9).

These findings were consistent with previous research, which found that asparaginase from the *B. licheniformis* strain had the highest cytotoxic activity against the cancer cell lines Jurkat clone E6-1, MCF-7, and K-562, with IC50 values of 0.22, 0.78, and 0.153 IU, respectively [50]. In addition, asparaginase isolated from *Bacillus sp.* R36 inhibited the growth of two human cell lines (HCT-116 and HepG-2), with IC50 values of 218. and 112.19 µg/ml, respectively [35]. Furthermore, Shafei *et al.* [51] described asparaginase that inhibited the growth of three human cell lines, with IC50 values of 14, 12.5, and 37 µg/ml against hepatocellular, breast, and prostate carcinoma, respectively. Asparaginase has been shown to inhibit glycosylation of a variety of newly synthesized proteins [52].

In this connection, the cytotoxicity of L-asparaginase from *Aspergillus flavus* KUFS20 toward the MCF-7 cell line by the MTT assay (IC50 120.875 µg/ml) was informed by Rani *et al.* [53]. Sirisha and Haritha [54] mentioned the highest antitumor activity was recorded toward MCF-7 (82.3%), and also purified L-asparaginase from *B. licheniformis* RAM-8 was found to be highly effective against MCF-7, with IC50 values as low as 1 IU/ml [50]. Elshafei *et al.* [55] reported IC50 109.9 µg/ml toward Hep-G2 cell line from the partially purified L-asparaginase of *Penicillium brevicompactum* NRC 829. The LD50 of ECAR LANS 5 and 7.5 IU/ml for Hep-G2 and MCF-7, respectively, is mentioned by Abakumova *et al.* [56].

However, the cytotoxicity of L-asparaginase from *Aspergillus oryzae* CCT 3940 toward PC-3 cell line by the MTT assay (total growth inhibition 3.2±4.1 IU/ml) was informed by Dias *et al.* [57].

Asparagine is involved in the Krebs cycle inducing cell metabolism after its conversion into oxaloacetic acid [58]. In addition, extracellular serine uptake is enhanced by intracellular asparagine, which is then utilized to synthesize nucleic acids [59]. Leukemic cell lines and other tumor cells need extreme amounts of asparagine to produce energy and produce biomolecules, so declines in this amino acid supply prevent the growth of these cells and multiplication [60].

Muñoz-Pinedo *et al.* [61] describes how exposure to anticancer enzymes leads cancer cells to undergo starvation conditions, by catalyzing the transformation of a specific amino acid into a form that cancer cells cannot use. In addition, owing to the absence of L-asparagine synthetase gene on tumor cells

leads to failure to produce L-asparagine, causing cell cycle disruption and apoptosis to occur [62].

Conclusion

The results of this study demonstrate that the marine environment of the Red Sea is a powerful source for bacterial strains and discovery of potent anticancer drugs. Asparaginase-producing strains with better yield should be identified paying attention to improve the cost-effectiveness to produce this enzyme. The anticancer activity of the partially purified enzyme explains the excellent possibility for using it as a powerful anticancer agent. However, more comprehensive studies are needed to estimate that, including immunogenicity and shelf-life determination analyses, as well as pharmacokinetic and pharmacodynamic profiling in model animals and human clinical trials.

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Conflicts of interest

There are no conflicts of interest.

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